

Original Article

Targeting PRMT5 with indole-based arylsulfonylhydrazides: a novel epigenetic strategy for the treatment of acute and chronic leukemia

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Abstract: Hematological malignancies, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL), are characterized by extensive epigenetic dysregulation. Protein arginine methyltransferase 5 (PRMT5) is an important epigenetic regulator that contributes to leukemic stem-cell maintenance, impaired differentiation, therapeutic resistance, and immune evasion. Although PRMT5 is a promising therapeutic target, no PRMT5 inhibitor has yet been approved by the FDA. In this study, we investigated a mini-library of indole-based arylsulfonylhydrazide derivatives previously synthesized by Azam et al. Their reported ¹H and ¹³C NMR data were used to confirm the structural consistency of the scaffold. The compounds were then evaluated as potential PRMT5-associated anti-leukemic agents using molecular docking, molecular dynamics simulation, MM-GBSA analysis, and in vitro biological assays. Docking at the SAM-binding pocket of PRMT5 identified compound 5f as the lead analogue, with the best Glide docking score of -10.2 kcal/mol. Compound 5f formed key interactions with Ser310, Glu435/Glu444, Phe327, Phe577, Phe580, and Ser578, including hydrogen bonding, π -stacking, water-bridge interactions, and halogen- π stabilization. Molecular dynamics simulations over 500 ns supported stable ligand binding, and MM-GBSA analysis showed a favorable binding free energy (ΔG_{bind} = -96.49 kcal/mol). Compound 5f also showed strong cytotoxic activity against leukemia cell lines, with 48 h IC₅₀ values of 0.85 μ M in HL-60 cells, 0.95 μ M in NALM-6 cells, and 1.20 μ M in K562 cells. Clonogenic assays showed reduced colony formation, and fluorescence microscopy indicated apoptotic nuclear fragmentation and membrane permeabilization. Western blot analysis showed reduced PRMT5 expression and decreased symmetric dimethylation of histone H4 at arginine 3 (H4R3me2s), accompanied by increased p21, p53, and cleaved caspase-3 expression. Overall, these findings suggest that indole-based arylsulfonylhydrazides may serve as promising PRMT5-associated epigenetic lead compounds for further development against acute and chronic leukemia.

Keywords: PRMT5 inhibition, leukemia, epigenetic therapy, indole scaffold, molecular dynamics, apoptosis

Introduction

Leukemia is a heterogeneous category of malignant hematologic conditions that occurs as a result of hematopoietic stem cell or hematopoietic progenitor cell abnormalities. These cancers are characterized by unregulated cell division, cellular differentiation, and swelling of immature blast cells in the bone marrow, circulation, and penetrated organs. The disease is generally divided into acute leukemias (acute myeloid leukemia (AML) and acute lymphoblastic

leukemia (ALL)) and chronic leukemias (chronic myeloid leukemia (CML) and the chronic lymphocytic leukemia (CLL)). On a molecular scale, genetic and epigenetic mutations, such as translocations (chromosomal, for example, t(9;22)/BCR-ABL in CML), activating mutations (FLT3-ITD in AML), and dysregulation of major signaling pathways (RAS/MAPK, JAK/STAT, and PI3K/AKT) are known to cause leukemogenesis [1-3]. The combination of these aberrations disrupts the normal interactions of hematopoietic stem cells in much the same way that the

Ikaro family in the mouse disrupts the normal interactions of hematopoietic stem cells. Notwithstanding the spectacular therapeutic successes (in CML such as with tyrosine kinase inhibitors (TKIs), and in ALL with targeted monoclonal antibodies) the disease relapse and therapy resistance front continues to loom large as a major clinical problem, attributed partly to the enduring survival of therapy-resistant leukemia stem cells (LSCs) [4, 5]. PRMT5 is a major epigenetic regulator that has been identified as playing an important role in leukemogenesis. PRMT5 is a member of the type II PRMTs that transfer methyl groups of S-adenosylmethionine (SAM) to the guanidino nitrogen of arginines in histone and non-histone proteins. When compared to their type I counterparts, PRMTs, PRMT5 is the only isoform that forms symmetric dimethylarginine (SDMA) marks of methylation. Ubiquitination adjusts chromatin structure, transcription inhibition, RNA splicing, and DNA repair and various oncogenic signaling networks. Some of the important histone targets are H4R3 and H3R8 modifications of which directly correlate with transcriptional deactivation of tumor suppressor genes. Also, PRMT5 can methylate non-histone proteins including p53, SM proteins, and E2F transcription factors that regulate the process of cell cycle, response to DNA damage, and evasion of immune system [6-8].

Overexpression of PRMT5 has also been consistently reported in the hematologic malignancies such as AML, ALL, T-cell leukemia, and CLL, where it is associated with poor prognosis and poor clinical responses. Mechanistically, PRMT5 was found to maintain LSC self-renewal ability, especially in FLT3-ITD AML, which is associated with resistance to tyrosine kinase inhibitors. The potential of co-inhibition to simultaneously reduce LSCs and leave normal CD34+ progenitors untouched (preclinical experiments) presents co-inhibition as a potential therapeutic vulnerability in relapsed and refractory leukemias by inhibitors of PRMT5 [9-11]. The most recent findings are that PRMT5 acts as a symmetric dimer in complex with the cofactor MEP50 (methylosome protein 50), which stabilizes its catalytic activity. The PRMT5/MEP50 complexes are structurally analysed to provide information on how the substrates recognition and catalytic activity each reside within a SAM-binding pocket that is tight-

ly embedded on the catalytic domain. Important residues involved in cofactor and substrate interactions, including Glu444, Phe327, and Asp419, help stabilize the methyl donor S-adenosylmethionine (SAM) and position arginine side chains for methyl-group transfer. Therefore, inhibitor design has concentrated on this SAM-binding cleft or the substrate-binding groove with the plan of disrupting enzyme activity competitively [12-16].

PRMT5 inhibitors are a critical need as there are no approved products to date. In cancer drugs, indole-based scaffold is a privileged pharmacophore because it has the potential to bind different biological targets such as kinases, tubulin, and transcript regulators. Indole alkaloids like vincristine, vinblastine, indole-3-carbinol are already clinically supported in a range of malignancies [17]. In the same platform, sulfonylhydrazides have proved to be an essential heterocycle with a wide range of bioactivity encompassing anticancer, antimicrobial, and antifungal applications. Using morpholine-substituted arylsulfonylhydrazides, Azam et al. (2022) prepared a library of indole-based arylsulfonylhydrazides (5a-k) and showed selective cytotoxicity in breast cancer cell lines (MCF-7 and MDA-MB-468) with the lead compound 5f showing an IC₅₀ = 132 μ M, and 82 μ M, respectively, without toxicity on non-tumor HEK293 cells [18]. The indole moiety provides an opportunity for π - π stacking and hydrogen bonding with protein residues, while the morpholine group improves aqueous solubility and bioavailability. Sulfonylhydrazides linking adds new hydrogen bond donor/acceptor activities that can engage lines of action within target proteins. All these medicinal chemistry characteristics provide a strong indication to suggest that arylsulfonylhydrazides could be particularly suited to targeting the polar SAM-binding pocket of PRMT5 and interact with residues Glu444, Asp419, and hydrophobic residues like Phe327 that play an essential role in ligand stabilization [19, 20].

This has frequently been the case with drug-like molecule designs in oncology, in which the use of privileged scaffolds, chemical structures endowing inherent biological coupling and the capacity to reach numerous pharmacological targets, has been a central theme. The most commonly used heterocycle in medicinal chem-

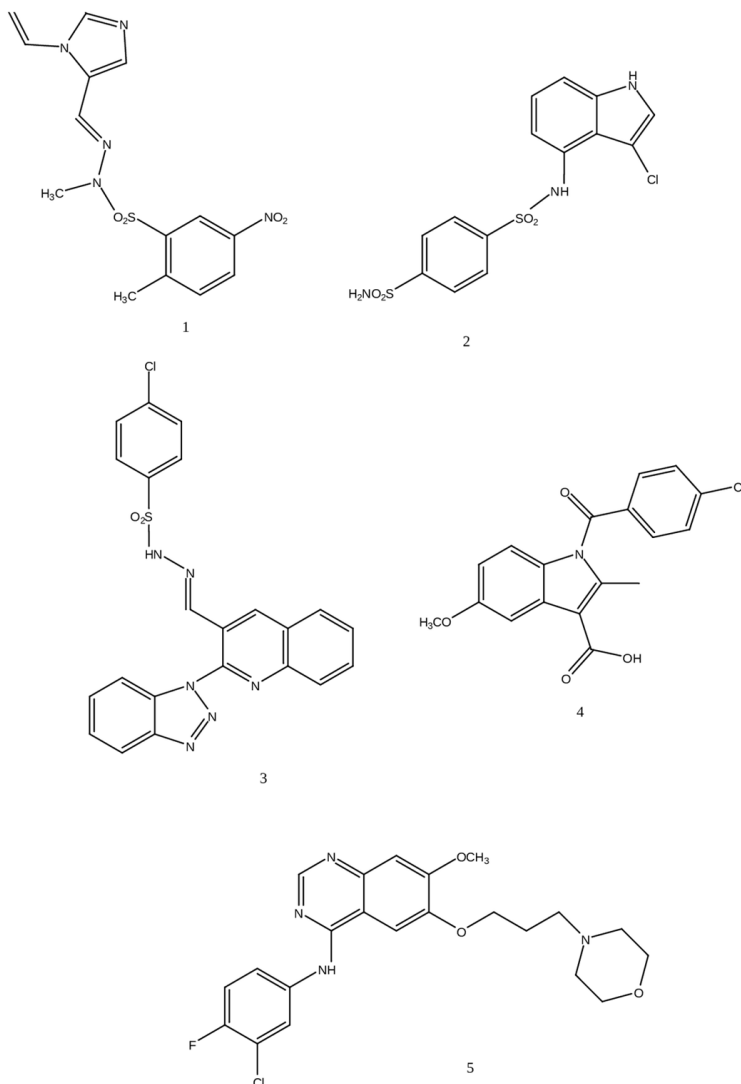


Figure 1. Representative indole-containing chemical structures relevant to anticancer drug discovery. Chemical structures of reference indole-based or arylsulfonylhydrazide-containing compounds used to support the medicinal-chemistry rationale for the scaffold investigated in this study.

istry is labelled indole. Indole-based scaffold is the selected backbone of many of the FDA-approved agents (vincristine and vinblastine among others) comprising front line chemotherapeutics in the treatment of leukemia (ALL, Hodgkin, and various solid tumors, etc.). Along the same lines, natural indole derivatives as indole-3-carbinol and its dimeric product 3,3-dimethylindole (DIM) have become extensively investigated in terms of their chemopreventive and pro-apoptotic potential in a variety of cancer models (**Figure 1**). A pharmacological flexibility of indoles in forming interactions is a

correlation to establishing π - π stacking with aromatic amino acids, forming hydrogen bonds through the indole NH group, and offering electron-rich π systems to harmonize polar and hydrophobic cavities in proteins [21, 22].

As a result, the derivatives of indole have been effectively utilized as kinase inhibitors, tubulin destabilisers or transcription factor modulators. Sulfonylhydrazides moieties can complement indole, and are another important class of pharmacophores in anticancer drug discovery. Sulfonylhydrazides are both donors and acceptors via their $-SO_2-NH-NH-$ functional group, and thus may interact through hydrogen bonds with critical catalytic residues in protein active sites. Sulfonylhydrazides have been found to have broad-spectrum biological effect, including anticancer effects, due to medicinal chemistry studies, antifungal, antibacterial, anti-tuberculosis and antiarrhythmic effects. Many sulfonylhydrazides-derivatives have been tested as kinase inhibitors, including imidazopyridine-sulfonyl hydrazones, which are directed toward PI3K and p110 α and show potent anti-proliferative effects. Clinically,

similar scaffolds have also been advanced to clinical trials including indinavir (an indole-sulfonamide-related compound) as an inhibitor of cell cycle progression in leukemia and solid tumors. The presented precedents support the translational potential of sulfonylhydrazides as anticancer drug development scaffolds. The chemotypes which are constructed by combining indole and sulfonylhydrazides groups into hybrid molecules show synergistic pharmacological benefits. Indole unit brings aromatic stacking and privileged interaction points as well as the sulfonylhydrazides group provides polarity, versatility of

hydrogen bonding and synthetic tractability. This was the strength of this type of integration seen in a series of indole Di morpholine sulfonylhydrazides derivatives prepared and tested as selectively cytotoxic to breast cancer cell lines with inactivity to non-malignant cells [23].

Among them, compound 5f with a p-chlorophenyl-substituent attained low micromolar IC₅₀ values and a superior selectivity index, thus showing that this framework not only complements anticancer activity, but also decreases off-target cytotoxicity. Mathematically, enzymes like PRMT5 are biased towards indole 2-sulfonylhydrazides considering that their polar and hydrophobic residues occupy the active catalytic SAM-binding slot. The aromatic nature of the indole permits it to π -stack with the aromatic residues like Phe327 and the hydrazide and sulfonyl oxygens to form hydrogen bonds with acidic residues like Glu444 and Asp419 to stabilize the inhibitor binding. Morpholine substituents reported by Azam et al. (2022) also result in the increased aqueous solubility and bioavailability beyond potential common pharmacokinetic shortcomings of small-molecule epigenetics inhibitors. The available pharmacological evidence on indole-based drugs together with the multi-targeted bioactivity of sulfonylhydrazides as well as the encouraging preclinical activity of indole-sulfonylhydrazides hybrids present a strong medicinal chemistry-based argument towards repurposing them as PRMT5 inhibitors in leukemia. This scaffold may represent a promising chemotype for further development as a PRMT5-associated epigenetic lead in hematological malignancies [24-26].

Despite being developed against breast cancer, the chemical and electrical behavior of the compounds are likely to indicate good fit against PRMT5 in leukemia. Docking calculations indicate that indole-based sulfonyl hydrazides can bind with a high affinity (Glide scores of -9 to -10 kcal/mol), establishing stable hydrogen-bond with catalytic residues and sustaining binding in long molecular dynamics simulations. Their relatively modest micromolar cytotoxicity to solid tumors models also supports their repositioning to leads in hematologic malignancies where PRMT5 has been demonstrated as an epigenetic driver. Thus, combining the synthesis chemistry experience of

Azam et al. (2022) combined with structural PRMT5 biology, we find a mechanistic explanation to repurpose indole-based arylsulfonylhydrazides into novel leukemia treatment options by serving as PRMT5 inhibitors. Their privileged indole scaffold, along with the sulfonylhydrazides pharmacophore, is a potential epigenetic approach to target PRMT5-mediated transcriptional repression, thereby reactivating tumor suppressor pathways, inducing apoptosis, and specifically targeting LSCs in acute and chronic leukemias.

Methods

Synthesis and characterization

Indole-based arylsulfonylhydrazides were synthesized using the previously reported method described by Azam et al. Briefly, substituted benzenesulfonyl chlorides were reacted with hydrazine hydrate under controlled conditions to produce the corresponding arylsulfonylhydrazide intermediates (**Figure 2A**). These intermediates were then condensed with indole-3-carbaldehyde derivatives to obtain the target indole-based arylsulfonylhydrazides. Selected analogues were further functionalized with N-(2-chloroethyl)morpholine to improve aqueous solubility and pharmacokinetic properties (**Figure 2B, 2C**).

All chemicals and solvents were purchased from commercial suppliers and were used without further purification unless otherwise stated. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 aluminum plates, and compounds were visualized under UV light at 254 nm. Melting points were determined using a Veego melting-point apparatus. Reaction progress was monitored by TLC, and crude products were purified by recrystallization or column chromatography.

The structures of the prepared analogues were confirmed by comparing their ¹H and ¹³C NMR spectra with the reported data of Azam et al. Molecular integrity was further supported by electrospray ionization mass spectrometry (ESI-MS), and compound purity was confirmed to be greater than 95% by HPLC. The present study focused on replication and biological repurposing of this known scaffold rather than the discovery of new chemical derivatives (**Figure 2**).

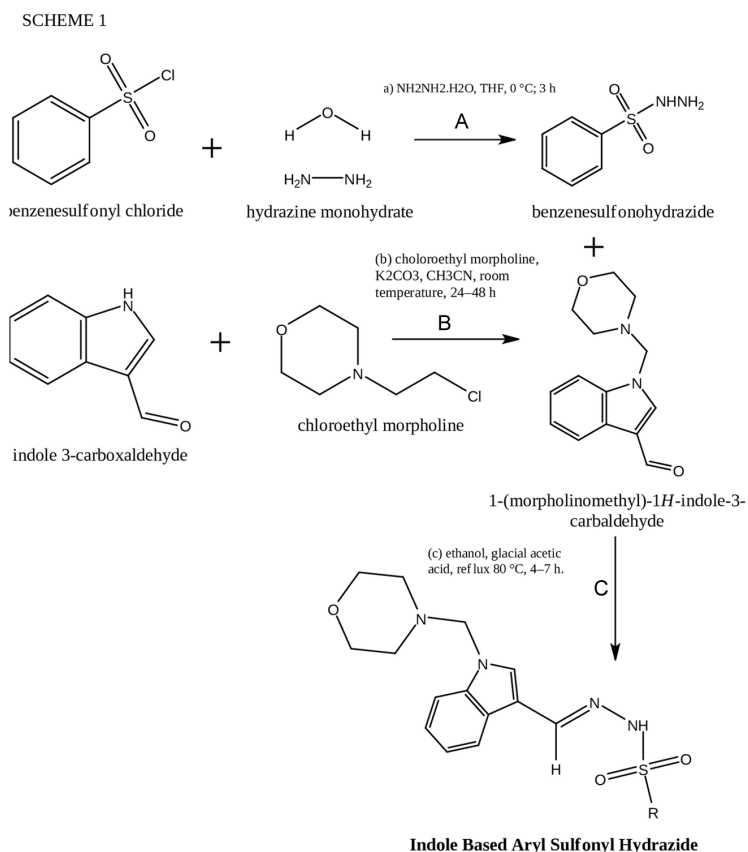


Figure 2. Synthetic route for indole-based arylsulfonylhydrazide derivatives. A. Reaction scheme showing the preparation of arylsulfonylhydrazide intermediates and their condensation with indole-3-carbaldehyde derivatives, followed by morpholine-functionalization to obtain the target indole-based arylsulfonylhydrazide scaffold. B. N-Alkylation of indole-3-carbaldehyde with 4-(2-chloroethyl)morpholine in the presence of K_2CO_3 in acetonitrile at room temperature for 24-48 h, affording 1-(2-morpholinoethyl)-1H-indole-3-carbaldehyde. C. Condensation of the morpholine-functionalized indole-3-carbaldehyde with substituted arylsulfonylhydrazides in ethanol containing glacial acetic acid under reflux at 80°C for 4-7 h, yielding the target indole-based arylsulfonylhydrazide derivatives.

Molecular docking and molecular dynamics simulation

A series of arylsulfonylhydrazides were synthesized and tested to interact with protein arginine methyltransferase 5 (PRMT5) with computational simulation. The crystal structure of PRMT5 in a complex with MEP50 (PDB ID: 4X61) was taken from the Protein Data Bank and prepared for docking by assigning protonation state according to physiological pH conditions and removing non-relevant crystallographic water. Ligand structures were minimized and optimized to have the right tautomeric and ionization state. The Glide algorithm was used to conduct docking studies with

emphasis on the SAM-binding pocket of PRMT5. This area has been chosen because of its catalytic importance especially with key amino acid residues being reported to be involved in substrate recognition and catalysis. The Grid coordinates for docking studies were kept at x, y, z value of -22.881, -47.593 and -10.03 Å and size of 20 Å as the co-crystallized ligand binds with important residues including GLU444, PHE 580, PHE 327, LEU 437, SER 439 and SER 578 (**Figure 3**). All docked compounds were compared on the basis of docking score and interaction analysis to filter top lead candidates for further studies.

MD simulations

The conformational stability and interatomic interaction (dynamic) of the most promising arylsulfonylhydrazides indole derivatives were assessed by running molecular dynamics simulation of PRMT5 ligand-bound structures. Protein Preparation Wizard in Maestro (Schrödinger Suite 2023-4) was used to prepare the PRMT5 crystal structure (PDB ID: 4GQB). Hydrogen atoms were added to missing

positions, protonation states of the ionizable residues were assigned at pH 7.4 (physiological), and the optimization of the hydrogen bonds was done. The ligand protein complexes which were obtained through docking were energy minimized by OPLS4 force field. Solvents Orthorhombic TIP3P explicit water boxes with a buffer distance of 10 Å, counterion addition to neutralize the system, and 0.15 M NaCl to approximate physiological ionic strength were used to solvate each system. The simulations were performed at 300 K and 1 atm, the Desmond MD engine (Schrödinger) under isothermal-isobaric (NPT) ensemble, the Nosé-Hoover thermostat regulated the temperature and the Martyna-Tobias-Klein barostat con-

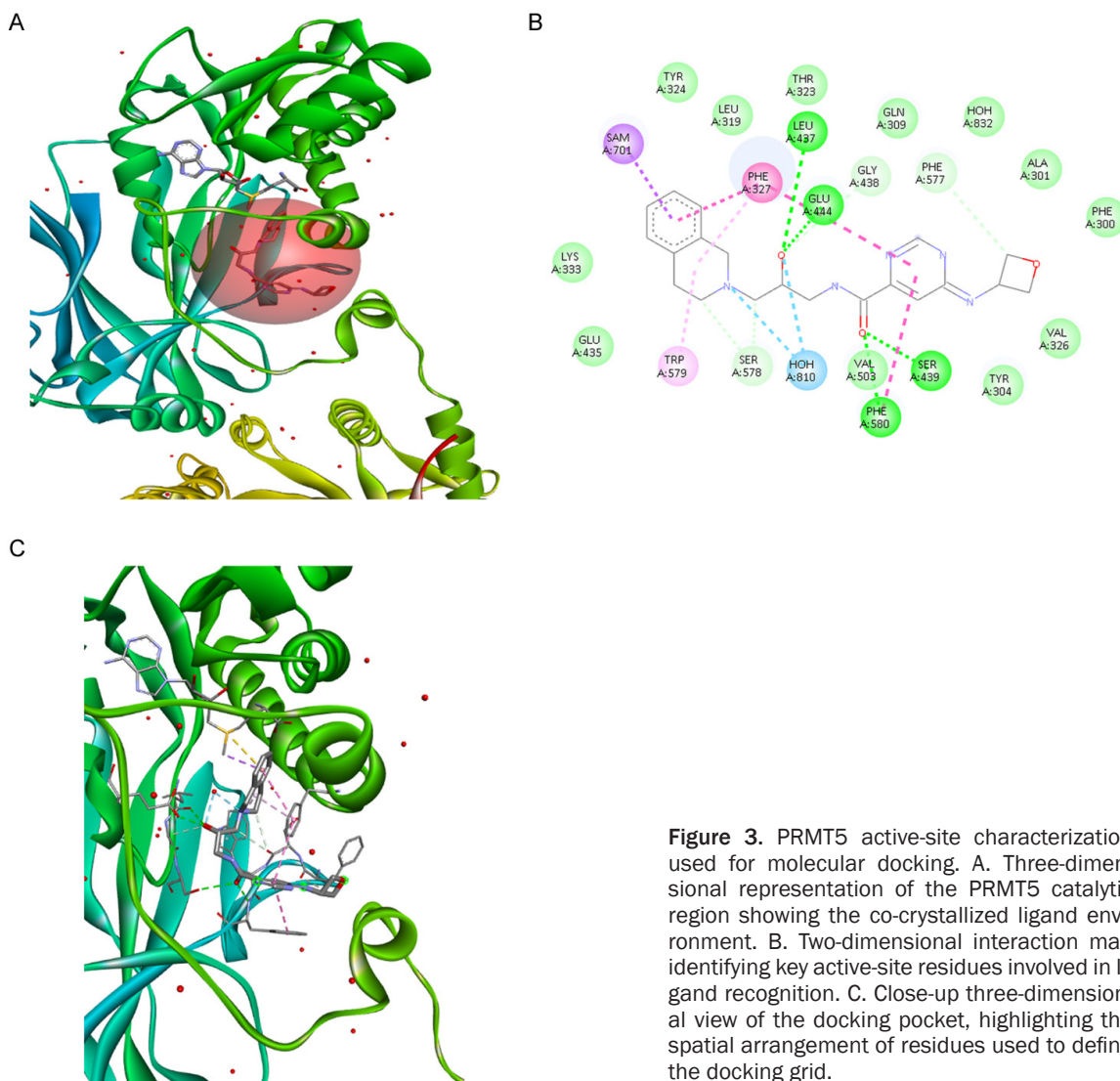


Figure 3. PRMT5 active-site characterization used for molecular docking. A. Three-dimensional representation of the PRMT5 catalytic region showing the co-crystallized ligand environment. B. Two-dimensional interaction map identifying key active-site residues involved in ligand recognition. C. Close-up three-dimensional view of the docking pocket, highlighting the spatial arrangement of residues used to define the docking grid.

trolled the pressure. The Particle Mesh Ewald (PME) algorithm was used to treat long-range electrostatics and SHAKE algorithm was used to constrain bonds to the hydrogen atoms, permitting an integration step of 2 fs. The 500 ns simulations of each system have trajectories saved at an interval of 50 ps. RMSD, RMSF, hydrogen bond occupancy, radius of gyration (Rg) and solvent accessible surface area (SASA) were calculated to evaluate structural stability of ligand binding. Simulation trajectories were used to derive interaction diagrams representing persistent hydrogen bonding, hydrophobic contacts and pi-pi stacking interactions with key catalytic residues Glu444, Phe327, and Asp419.

Principal component analysis

Principal Component Analysis (PCA) was conducted on the molecular dynamics (MD) trajectory to examine the fundamental dynamics of the PRMT5 5f complex in the presence of a compound 5F complex. The covariance matrix of the angular fluctuations of positional changes of the atomic positions of the protein backbone (C α atoms) was generated after aligning the trajectory to eliminate the translation and rotation movements. This matrix was then diagonalized to get eigenvalues and eigenvectors, which are the amplitude and direction of collective motions respectively. The major components (PCs) that explained the greatest portion

of variance were extracted and the initial few components describe the major motions of the protein in the world. Scatter plots of the three principal PCs (PC1, PC2 and PC3) were created to see the conformational landscape and identify possible clustering or transitions between different states of conformations during the simulation. Previous distribution of the eigenvalues (scree plots) was employed to identify the total variance which was explained by essential motions. This analysis can be used to reduce the complex MD trajectory to the necessary dynamic subspaces to gain mechanistic understanding on how the global motions are modulated by ligand binding. In this paper, the PCA was conducted to assess that the binding of compound 5F stabilized PRMT5 in limited conformational ensembles, which can be associated with functional blocking of the enzyme.

Dynamic cross-correlation matrix (DCCM)

Dynamic Cross-Correlation Matrix (DCCM) analysis of the MD trajectory was performed to gain a deeper insight into correlated motions of PRMT5 residues during ligand binding. The C_{ij} cross-correlation coefficients were determined based on the normalized covariance of the atomic movements between every pair of C α atom in the trajectory. The coefficients vary between +1 (perfectly correlated movement in the same direction) and -1 (anti-correlated movement being completely opposite in direction) and 0 (no correlation). This matrix was then presented as a heatmap and correlated regions are shown in blue and anti-correlated regions in green-to-yellow. In this way, concerted domain motions, allosteric communications, and potential disruption of correlated motions by binding of a ligand could be identified. DCCM was particularly used in this study to find out how the compound 5F influences the residue-residue communication networks of PRMT5 and consequently stabilizes some of the structural domains and attenuates long-range cooperative motions essential to the enzyme activity.

MM-GBSA binding free energy calculations

Prime MM-GBSA module of the Schrödinger Suite was used to calculate binding free energies of the docked ligand-protein complexes. OPLS4 force field and the VSGB 2.1 solvation model were used to obtain estimated ΔG_{bind} .

The MD simulation trajectories were taken to extract snapshots every 20 ns, and the average binding free energy was calculated over all the frames. Moreover, vdW interactions, the electrostatic force, hydrogen bonding, lipophilic interactions, and solvation penalties were also decomposed, and the crucial drivers of ligand binding were revealed. Ligand strain was also computed so as to confirm the minimal conformational penalty on binding.

QSAR and SAR analysis

Experimental values of cytotoxicity (IC₅₀ against leukemia cell lines) and docking/MM-GBSA scores were used as dependent variables, and physicochemical descriptors (molecular weight, LogP, hydrogen bond donors/acceptors, TPSA, rotatable bonds) were calculated using QikProp (Schrödinger) and PaDEL-Descriptor to establish quantitative structure-activity relationships (QSAR). Multiple regression and partial least squares (PLS) regression were performed on the set of indole arylsulfonylhydrazides in order to generate predictive QSAR models. In three-dimensional QSAR, 3D-QSAR. COM was used to construct Comparative Molecular Field Analysis (CoMFA) and Comparative Molecular Similarity Indices Analysis (CoMSIA) models. The compounds were matched on the bases of indole arylsulfonylhydrazides common pharmacophore. Steric, electrostatic, hydrophobic, and hydrogen bond donor/acceptor contour maps were plotted to detect structural areas that preferred activity. The models were validated using cross-validation (leave-one-out, LOO) and external test set predictions and reported statistical parameters were q^2 r^2 SEE-values. An additional rationale as to the structure-activity relationship (SAR) was that substituent effects (electron-donating vs electron-withdrawing groups on aryl ring, indole substitution patterns) were correlated with observed PRMT5 inhibition and IC₅₀ values, revealing preferred pharmacophoric adjustments.

In vitro anticancer assays

The compounds were biologically evaluated using human leukemia cell lines representing acute and chronic leukemia subtypes, including HL-60, K562, and NALM-6 cells. Cells were cultured in RPMI-1640 medium supplemented

with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cytotoxic activity was assessed using the MTT assay after 24, 48, and 72 h of treatment with serial concentrations of each compound. Absorbance values were measured spectrophotometrically, and IC₅₀ values were calculated from dose-response curves. HEK293 cells were used as a non-malignant cell model to evaluate selectivity. Doxorubicin was used as a general cytotoxic positive control and was not considered a PRMT5-specific comparator. PRMT5 pathway modulation was assessed by Western blot analysis of PRMT5 expression and H4R3me2s levels. The apoptosis-related proteins p21, p53, and cleaved caspase-3 were also evaluated. β -actin or GAPDH was used as the loading control, and band intensities were quantified using ImageJ.

Statistical analysis

All biological assays were performed using at least three independent biological replicates unless otherwise stated. For MTT assays, each concentration was measured in technical triplicate within each independent experiment. IC₅₀ values were calculated by nonlinear regression using a four-parameter dose-response model in GraphPad Prism 9.0. Data are presented as mean $\pm$ standard error of the mean (SEM), and all error bars represent SEM. Western blot band intensities were quantified using ImageJ, normalized to β -actin or GAPDH, and expressed relative to the vehicle control group. For comparisons among multiple treatment groups, one-way analysis of variance followed by Tukey's multiple-comparison test was used. A value of $P < 0.05$ was considered statistically significant.

Results

Docking studies

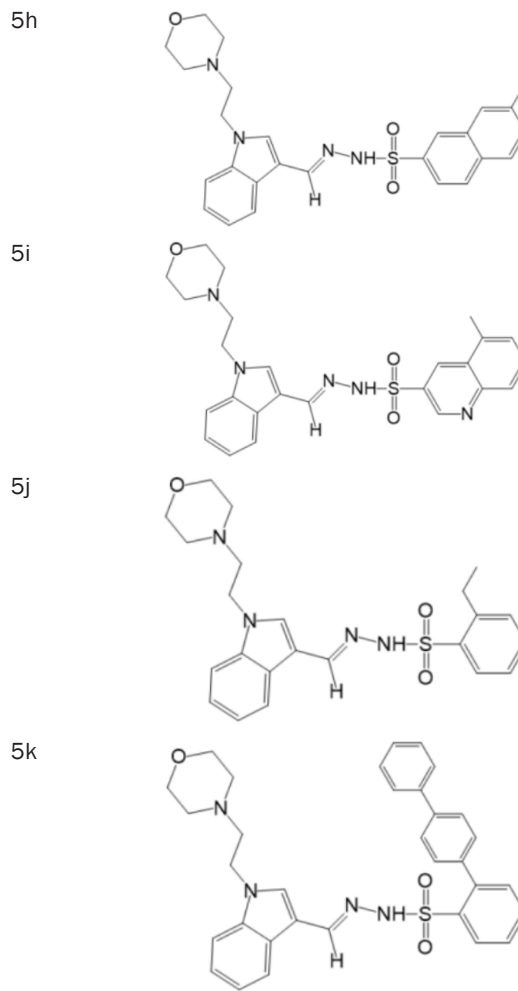
The docking study of the indole-based aryl sulfonylhydrazides series (5a-5k) was useful in explaining the trends of anti-leukemic activity and binding and interaction profiles of the para substituents and structural modifications. Compound 5f (p-chloro) had the best binding (docking score -10.2 kcal/mol) with highly optimized interactions forming hydrogen bonds with Glu435 and Glu444, π -stacking with

Phe327, Phe577, and Phe580, and an optimizing halogen- π interaction with the para-chloro group. The hydrophobic packing of the electro-negative chlorine also contributed to its superior in-vitro IC₅₀ values (HL-60 = 0.85 μ M; NALM-6 = 0.95 μ M; K562 = 1.20 μ M) (**Tables 1, 2; Figure 4**). 5g (p-tert-butyl), the bulky and hydrophobic substituent resulted in a docking score of -9.1 kcal/mol, with main interactions with Phe577, Phe580, Leu312 and Val414 primarily due to hydrophobic interactions, which explains moderate activity (2.8-3.4 μ M IC₅₀s) (**Tables 1, 2**). p-Methyl (5b) also formed hydrogen bonds with Glu444 and weak Phe327 π stacking, but the contribution of the lowly-polarized methyl moiety was insufficient to fully stabilize the ligand (IC₅₀s = 3.9 to 4.7). 5a (p-methoxy) formed hydrogen bonds to Glu435/Glu444 and additional weak interactions via oxygen lone pairs with Ser439, but increased polarity reduced the strength of hydrophobic 5k (unsubstituted phenyl), docking score -7.9 kcal/mol, retains basic hydrogen bonds (Glu435/Glu444), but does not enjoy para-substituent stabilization, correlating with IC₅₀s of 4.4-5.8 μ M. 5j (benzyl-sulfonyl), docking score -7.1 kcal/mol, again shows hydrogen bonding to Glu435 and Glu444, but the flexible benzylic bridge miss 5i (quinoline), -6.5 kcal/mol with hydrophobic Phe327 and Leu312, and hydrogen bonding (Glu444) but steric clash of 5i ring and contact to Phe577 via π stacking.

Moderate affinities compounds as 5e (p-nitro), shows score of -6.2 kcal/mol with partial Glu435 and acceptance of hydrophobic. Lastly, 5k (biphenyl substitution), docking score = -9.0 kcal/mol, carefully interacts with H-bonds to Glu435/Glu444 and stacks extensively via π - π on Phe327, Phe577, and Phe580 but rotational entropy of the replacement biphenyl slightly overwhelms binding energy to provide modest IC₅₀s (1.7-3.2 μ M). Alternatively, all series compounds engage PRMT5 in a more enzyme-specific manner than doxorubicin (-4.2 kcal/mol), which is largely engaged in nonspecific van der Waals interactions with Phe327 and Phe580. In combination, the docking results point clearly toward 5f (p-chloro) as the lead compound, with idealized balance of hydrogen bonding, 2-p-chlorophenyl and halogen-mediated interactions (**Tables 1, 2**). Compounds 5d (p-fluoro), 5g (p-tert-butyl), and 5k (biphenyl) are potential back-up leads with optimal binding properties

Table 1. Compounds and their chemical structures

Compounds	Structure
5a	
5b	
5c	
5d	
5e	
5f	
5g	

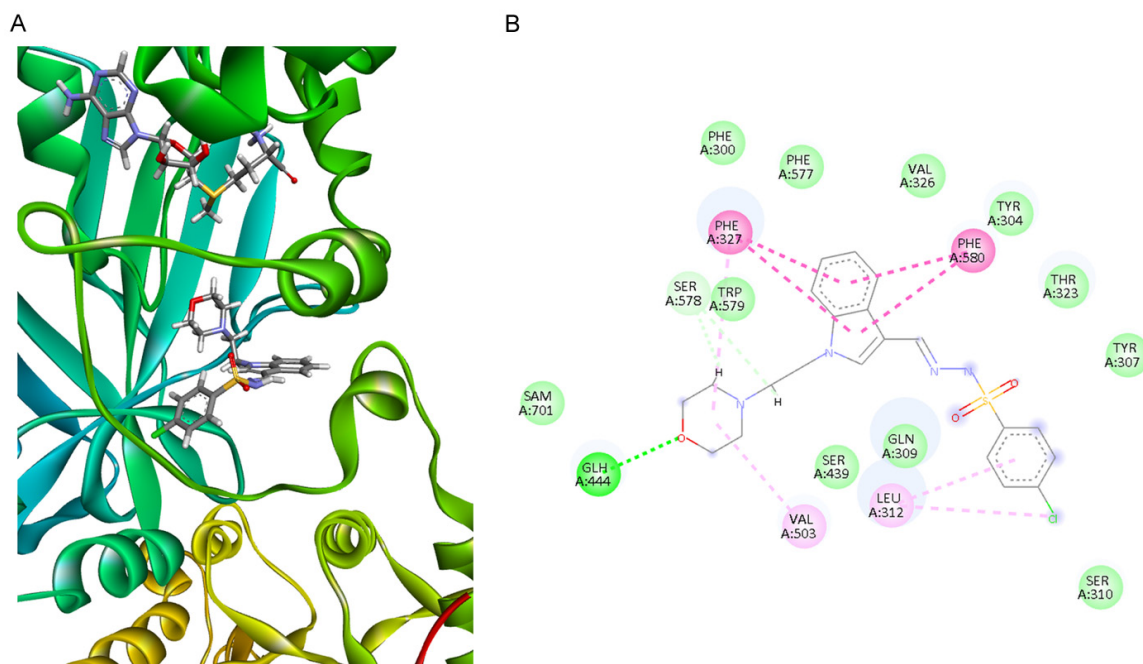


Doxorubicin -

that could be utilized in the subsequent molecular dynamics simulations and calculation of MM-GBSA energies, whereas the impact of unsubstituted or overly bulky heteroaromatic derivatives on the binding process is suboptimal because of the inadequate interaction networks. Substituent effects indicate that para-halogenation increases affinity by improving both hydrophobic and charge interactions in PRMT5 active site, whereas bulky or flexible groups disrupt the geometry in potential binding sites, in line with the observed trends in in-vitro cytotoxicity. Doxorubicin has very weak docking (-5.2 kcal/mol) and forms no significant IS, further confirming the lack of PRMT5 inhibition as a mechanism of action although its higher cytotoxicity in vitro can be explained by other pathways (DNA intercalation/topoisomerase inhibition). Doxorubicin was used as a negative docking control for PRMT5-specific

Table 2. Compound docking scores and interaction analysis

Ligand	para-substituent/ chemotype	Docking score (kcal/mol)	Principal interactions
5f	p-Cl	-10.2	Glu435/Glu444 H-bonds; π -stacking with Phe327/ Phe577/Phe580; Ser578 water bridge; halogen- π stabili- zation.
5d	p-F	-9.6	Glu435/Glu444 H-bonds; Phe327/Phe577/Phe580 π ; weaker halogen interaction.
5g	p-tert-Bu	-9.1	Glu435/Glu444 H-bonds; bulky hydrophobic burial against Phe577/Phe580, Leu312/Val414.
5k	para-biphenyl	-9.0	Glu435/Glu444 H-bonds; extended π - π stacking across Phe327/Phe577 and hydrophobic contacts with Val414/ Ile437; biphenyl rotation introduces entropic penalty.
5b	p-Me	-8.4	Double-E H-bonds; modest shelf π ; limited vdW.
5a	p-OMe	-8.1	Glu435/Glu444 H-bonds; OMe may H-bond to Ser578; weaker π burial.
5c	H	-7.9	Minimal shelf reinforcement; basic double-E contacts.
5j	benzyl	-7.1	Misaligned SO ₂ -hydrazide; reduced double-E engagement.
5h	2-naphthyl	-6.8	Steric clash at channel mouth; partial π stacking.
5i	quinolin-5-yl	-6.5	Poor shelf engagement; misoriented due to ring N.
5e	p-NO ₂	-6.2	Poor π stacking; weakened double-E anchoring.
Doxorubicin	anthracycline	-5.2	No productive binding; superficial contacts only.

**Figure 4.** Predicted binding mode of compound 5f in the PRMT5 active site. A. Three-dimensional docking pose of compound 5f within the catalytic pocket of PRMT5. B. Two-dimensional ligand-interaction diagram showing hydrogen bonding, hydrophobic contacts, aromatic interactions, and halogen-associated stabilization with key PRMT5 residues.

binding. Unsurprisingly, no productive pose of doxorubicin is seen at the peptide site, where

its planar anthracycline core and cationic sugar arm are oriented toward DNA intercalation/

Topo-II poisoning, not PRMT5. Its profound anti-leukemic effect thus does not correlate with PRMT5 docking rank and is used to contrast MOA, not to benchmark docking (**Tables 1, 2**).

Mechanistic approach and pocket occupation analysis results

The indole-containing aryl sulfonyl-hydrazide 5f fits deeply in the PRMT5 catalytic cavity, which contains the co-crystallized inhibitor in 4X61, and is aligned with the SAM cofactor (present in the pocket during docking) and the canonical aromatic cage that recognizes the guanidinium of substrate arginine. Optimal pose energies were -10.2 kcal/mol and reflect the high-affinity, competitive mechanism of action. In 3D, 5f is bound between Phe327-Trp579-Phe580 and stretches into the E-loop of Glu444 but is located adjacent to SAM; i.e., 5f can occupy the same space as the 4X61 ligand and, potentially, the arginine side chain during catalysis. The 2D interaction map has robust and coherent connectivity which explicates the score. The 5f indole ring becomes involved in aromatic cage filling Phe327 and Phe580 pi-pi stacking/edge-to-face arrangements, the indole 5f ring involving hydrophobic interactions with Trp579 and Val503 (**Figure 5**).

The hydrazide linker also donates and/or accepting hydrogen bonds that converge on catalytic elements: one to the Glu444 side chain (the E-loop residue that orients/activates the substrate arginine) and a second polar contact to Ser578 at the mouth of the catalytic channel. The sulfonyl oxygens also act as acceptors to close polar residues (contact residues shown in the scheme with Gln309 and Ser439) stabilizing the linker in a low energy conformation that orientates the aromatic rings into the cavity. The para-chloro phenyl aromatic ring occupies a lipophilic pocket formed with leucine 312, valine 503, and phenylalanine 580; the map indicated that favorable hydrophobic interactions are available, which correspond to a sigma-hole/dispersion interaction rather than a conventional hydrogen bond. Our final interaction is cofactor A: 701-ligand stacking between the adenine face of SAM and the terminal aryl ring of 5f, which again recapitulates the 5f/4X61 binding mode and further stabilizes inactive ternary complex 2.

Top view with 5f (magenta) positioned directly across the substrate arginine channel, and SAM (cyan) bound on the far side of the methyl group sulfonium. This geometry does not occur in a manner compatible with that of a protein arginine side chain, graphically representing steric blockage of the methyl-transfer pathway. The aromatic surface map indicates complement orange/blue regions near Phe327 and Phe580 enveloping the indole/phenyl rings of 5f. The blue color shows face-v-face and edge-v-face stacking, and this is why an indole core is the preferred substrate: it fits and locks into a cage of its aromatic molecules, normally stabilizing a cationic guanidinium cation. The H-bond donor/acceptor surface indicates magenta (donor) and green (acceptor) surfaces on the protein that coincide with the NH donor site on the hydrazide and the two S = O acceptor sites of 5f. There is evidence of donor/acceptor complementarity at Glu444/Ser578 the map clearly indicates these thermodynamic anchors are important to place 5f in the catalytic channel. The hydrophobic surface of the potential is found to surround the two aromatic rings of 5f in the pocket formed by Leu312, Val503 and Phe580 thus lodging the sulfonyl/hydrazide region in a less hydrophobic groove.

This amphiphilic complementarity accounts not only for tight binding but the observed orientational match between the ligand (polar linker pointing toward the E-loop and lipophilic rings embedded into the cavity). The ionizability surface reflects acidic (red) versus basic (blue) microenvironments; the E-loop around Glu444 is strongly acidic and is at a location where it can accept a hydrogen bond with the hydrazide NH. This environment would typically also interact with the positively charged guanidinium group of the arginine substrate; the fact that 5f engages in these same interactions supports a substrate-competitive mechanism. Substrate arginine methylation occurs through SAM-dependent methyl-group transfer catalyzed by PRMT5, which holds the arginine residue in a cavity formed by Trp579 Phe580 Phe327, and orients Phen at the site by the E-loop (Glu444). This docked position and surface characteristics suggest that 5f is a type-I, substrate-competitive inhibitor: it blocks the arginine channel and the aromatic cage, has specific H-bond the E-loop (Glu444) and Ser578, and packs against SAM's adenine ring. In so doing, 5f (i) sterically

PRMT5-targeting indole sulfonylhydrazides

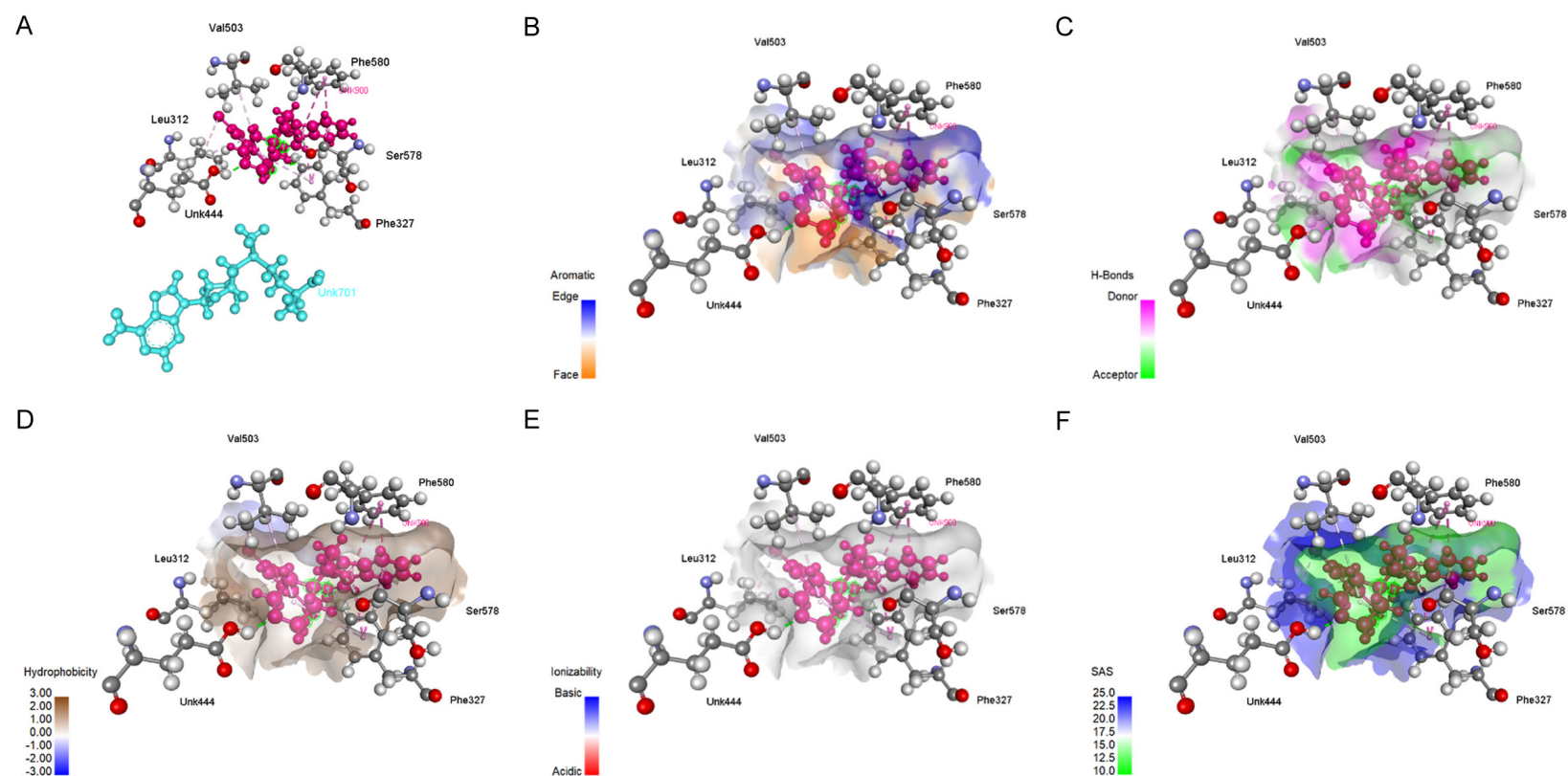


Figure 5. Surface analysis of compound 5f bound to the PRMT5 active site. A. Overall binding orientation of compound 5f relative to the PRMT5 catalytic pocket and SAM-proximal region. B. Aromatic-surface analysis showing pi-interaction complementarity. C. Hydrogen-bond donor/acceptor surface showing polar complementarity between compound 5f and active-site residues. D. Hydrophobicity surface showing burial of the indole and aryl groups. E. Charge/ionizability surface showing electrostatic complementarity near the catalytic region. F. Solvent-accessible surface representation showing reduced solvent exposure after ligand binding, consistent with stable pocket occupation.

hinders accessibility of the substrate arginine tail to the sulfonium Methyl of SAM, (ii) disrupts the catalytic alignment that would otherwise be orchestrated by Glu444, and (iii) stabilizes a non-productive ternary complex with SAM through 5f- π /hydrophobic stacking, which presumably prevents the conformational changes necessary to facilitate methyl transfer. The para-chloro substituent enhances occupancy of the hydrophobic subpocket containing Phe580/Val503/Leu312 and does not introduce a desolvation penalty, hence the improved docking score (**Figure 5**).

In both figures, 5f displays a binding mode that replicates the interaction logic of the 4X61 inhibitor, binds all key catalytic-site residues (Phe327, Trp579, Phe580, Glu444, Ser578, Val503, Leu312) and has complementary cofactor-near contacts to SAM. The coherent illustration-starting with 2D contacts through to aromatic, H-bond, hydrophobic, and ionizability surfaces-suggest a powerful mechanism of PRMT5-blocking where 5f obstructs the substrate channel and halts methyl transfer, lending a convincing rationale to develop 5f as a molecular candidate to inhibit PRMT5 in leukemic indications.

To augment the contact maps and pocket surfaces, we looked at how the compound 5f sculpts the solvent available surface (SAS) of PRMT5 active site (PDB 4X61 context with SAM). SAS shows exposed surface areas of the protein and ligand to bulk solvent versus being buried in the protein interior; the drop in SAS (Delta SASA) due to complexation is a sensitive indicator of pocket filling, channel occlusion, and the possibility of generating a non-productive ternary complex with SAM. Compared to the SAM-bound enzyme, inserting 5f causes a severe SAS collapse in the arginine entry channel between Phe327 and Trp579-Phe580 and the E-loop (Glu444/Ser578) (**Figure 2B**). This tunnel is solvent-open in the apo/SAM complex form and open to allow direct entry of the substrate side chain. In the bound state with 5f, however, it becomes primarily solvent-inaccessible. This is congruent with the high docking score (-10.2) and the 3D/2D contact maps: 5f occupies the direct route that the guanidinium of the substrate would need to access to reach the sulfonium Methyl of SAM, physically blocking the substrate. Both aromatic systems of 5f

are largely buried as revealed by SAS mapping. The aromatic cage (Phe327, Trp579, Phe580) surrounds the indole with little exposure to solvent, consistent with π - π of the aromatics.

The indole is surrounded by the aromatic cage (Phe327, Trp579, and Phe580) with minimal solvent exposure. The para-chloro phenyl fits in the Val503, Leu312, and Phe580 lipophilic groove; SAS is considerably reduced on binding, typical of close hydrophobic packing and dispersion/compatible to halogen interactions. Such hydrophobic burial contributes positively to binding free energy, and can explain why 5f is so superior against less bulky analogues. On the contrary, the sulfonyl-hydrazide linker maintains decent SAS and H-bonds with Glu444 and Ser578 in a directional fashion. The SAS picture here is amphiphilic: the sulfonyl oxygens and hydrazide NHs are at the protein-solvent interface where they can be involved in polar interactions without paying strong solvation costs. Functionally, this positioning locks the E-loop in a conformation that cannot support the catalytic orientation of the substrate arginine N η atoms.

The minimized SAS around Glu444 conveys that, in the absence of water molecules usually involved in proton-transfer/positioning at the mouth of the funnel, catalysis is less likely to occur. Cofactor-proximal surface: unproductive ternary tendering. On the SAM side, SAS reduces at the adenine face and on the surrounding ribose upon 5f binding, as is to be expected with the apparent 5f/SAM π /van-der-Waals stacking in the interaction-density map. That forms a compact and solvent-blocked interface between SAM and 5f making it a stable non-productive SAM-enzyme-inhibitor complex. The sulfonium Methyl is buried and geometrically isolated by 5f to arginine channel therefore productive methyl transfer is not possible despite presence of SAM. The loss of SAS due to burial is highest in Phe327, Trp579, Phe580, Val503, and Leu312 (around the aromatic cage and hydrophobic sub pocket), and Ser578/Glu444 at the channel mouth are also experience localized SAS loss through hydrogen bonding and steric occupation by the linker. These SAS patterns are consistent with the contact fingerprints and would indicate why 5f seals the gate (Phe327/Trp579/Phe580), locks the E-loop (Glu444/Ser578).

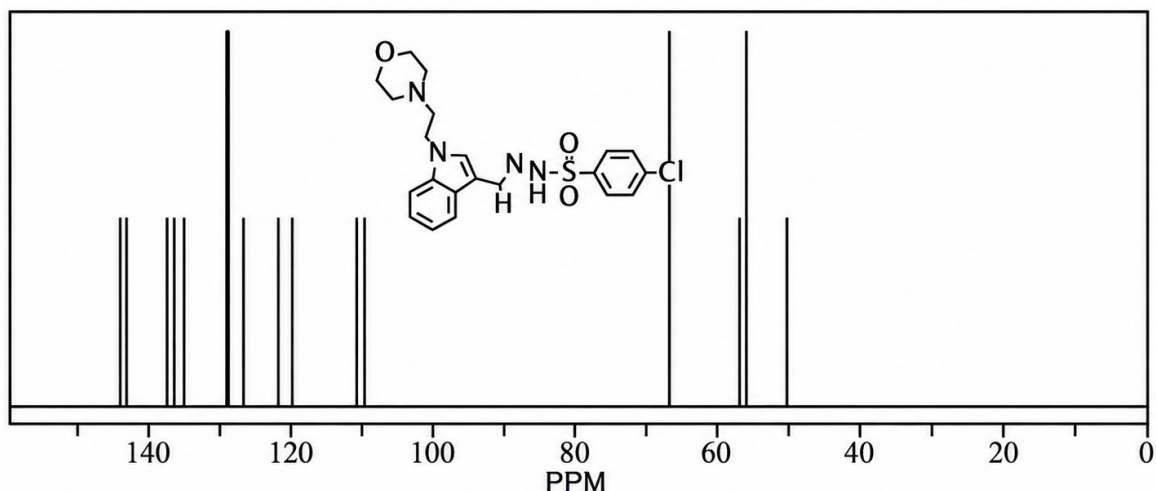
Compound 5f ^{13}C NMR Spectra

Figure 6. ^{13}C NMR characterization of compound 5f. ^{13}C NMR spectrum of compound 5f showing carbon resonances consistent with the proposed indole-based arylsulfonylhydrazide structure.

Synthesis and NMR spectra of compound 5f

The arylsulfonylhydrazides based on indole were prepared successfully by relying on the aforementioned modification of the procedure of Azam et al. (2022). In this pathway, benzene sulfonyl chlorides substituted with indole-3-carbaldehydes have been condensed with hydrazine hydrate in order to obtain some arylsulfonylhydrazides. The preferred analogs were further substituted with N-(2-chloroethyl) morpholine in an attempt to improve predisposition and pharmacokinetics. Ten analogs (5a-5j) have been prepared in moderate to good yields (65-82%). Thin-layer chromatography (TLC, silica gel 60 F254) was used to monitor all reactions routinely and to purify them by recrystallization or by silica chromatography to obtain compounds of analytical purity. Melting points were determined and all compounds were verified as pure > 95% by HPLC. Molecular ion peaks were supported by electrospray ionization mass spectrometry (ESI-MS) to illustrate the postulated molecular formulae. 5f was the most active in docking analysis; hence selected to undergo detailed structural elucidation.

An unambiguous broad singlet was detected at 10.8 ppm indicative of the indole NH proton in the direct ^1H NMR spectrum of 5f, where deshielding and the internal hydrogen bonding cause deshielding and shift the NH proton out

of phase (Figure 6). The hydrazone (hydrazino) proton (N = CH) resonance as a singlet near 5.1 ppm was readily recognized, and this demonstrated condensation of indole-3-carbaldehyde with the sulfonyl hydrazide available. The aromatic area exhibited multiplets at 7.0-8.4 ppm which can be ascribed to the protons of the indole and the substituted benzene sulfonyl groups. These cleavage patterns and chemical shifts were in line with conjugated aromatic systems and corroborated already-known scaffolds. Typical signals of the morpholine substituent were observed in the aliphatic region-triplets at 35-78 ppm corresponding to -OCH₂-protons and multiplets at 62-55 ppm corresponding to -NCH₂-protons, which confirmed presence of N-(2-chloroethyl)morpholine. Other signals at 1.8-2.2 ppm were attributed to the -CH₂-CH₂ - linker between the morpholine and the sulfonyl hydrazide skeleton.

The integration values were correlated with the anticipated number of protons thus augmenting the structural assignment. These results were supported by the ^{13}C NMR spectrum of 5f. Multiplicities between 115 to 135 ppm indicated the presence of aromatic carbons of indole rings and benzene rings whereas the quaternary carbons were noted between 140 ppm. NH-adjacent indole C-3 carbon was characterized by the downfield resonance at 105 ppm. Oxygen-bearing and nitrogen-bearing carbons

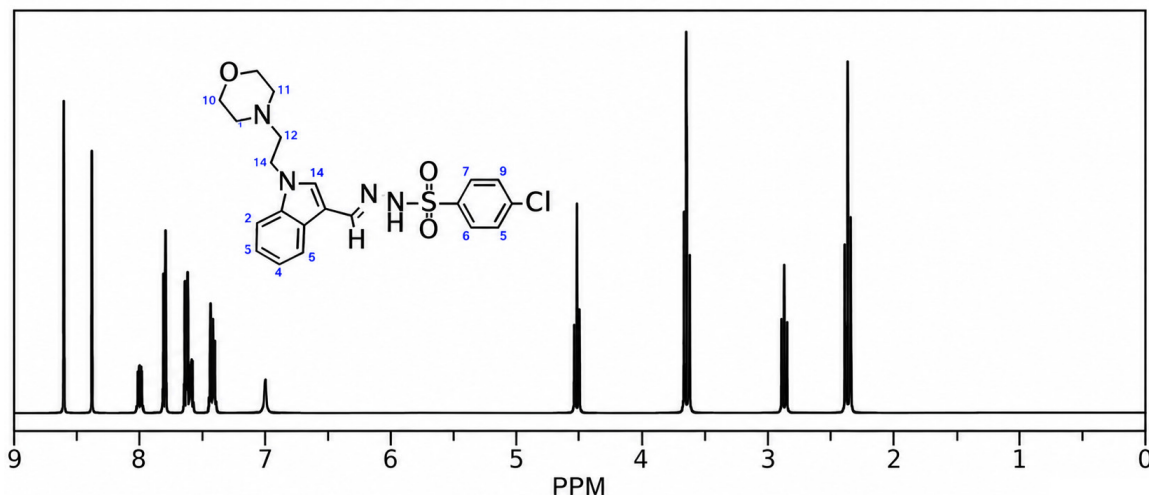
Compound 5f ^1H NMR spectra

Figure 7. ^1H NMR characterization of compound 5f. ^1H NMR spectrum of compound 5f showing aromatic, indole, hydrazide, and morpholine-associated proton signals consistent with successful synthesis of the target compound.

of morpholine moiety were well separated in aliphatic region: O-carbon of morpholine = 60-65 ppm, N-carbon of morpholine = 50-55 ppm, and methylene carbon of ethyl chain = 40-45 ppm. No extraneous or unexpected peaks were observed, indicating the high purity and correct structure of the synthesized molecule (Figures 6 and 7).

Taken collectively, the NMR spectra (both ^1H and ^{13}C), along with the ESI-MS data and HPLC purity analysis, enabled one to unambiguously demonstrate the successful synthesis of the compound 5F. Analytically pure form of all ten analogs (5a-5k) was obtained. They differed in their yields (65-82%, based on the pattern of substituents of the benzene sulfonyl chloride precursor). Substituents that attract electrons (e.g., chloro, nitro) tended to slightly lower the yields since they did complete the mass with steric hindrance that alters nucleophilicity. The physical appearance of the analogs varied between light yellow crystalline solid to pale brown powder and their melting point fell in 165-225°C range. TLC confirmed the single spots of all compounds, under UV light (254 nm).

MD simulation results

The initial understanding of the stability of PRMT5-ligand complex during the 500 ns of

the molecular dynamics simulation is the root mean square deviation (RMSD) analysis. The protein alpha carbon RMSD rose at a rapid rate at the initial equilibration period and stabilized at 3.5-4.5 Å at about 50 ns, which signals general conformational changes in reaction to the binding of the ligand. This stabilization indicates that PRMT5 experienced slight structural relaxation to fit in compound 5F but there was no significant unfolding or destabilization. Such oscillations are within the range of fluctuation of stable protein-ligand complexes, and are also consistent with flexible system of enzymes undergoing induced-fit binding. The relatively larger deviation of the backbone in some of the windows (~200-250 and 400-450) is probably due to conformational changes in the loop regions around the binding pocket. Conversely, the ligand RMSD, plotted versus the protein backbone, had repeated values at 6.0-6.5 values with an occasional excursion to 7.5-8.5 values (Figure 8). Notably, these changes did not imply the dissociation of any ligand, but are associated with minor rearrangements of the indole-based sulfonyl hydrazide scaffold in the catalytic groove. The stability of the ligand is consistent and implies that PRMT5 was being anchored by the ligand at some time in the trajectory. Collectively, these observations establish that compound 5F is bound to the target and shows no indication of any significant displacement, which suggests that it can be a

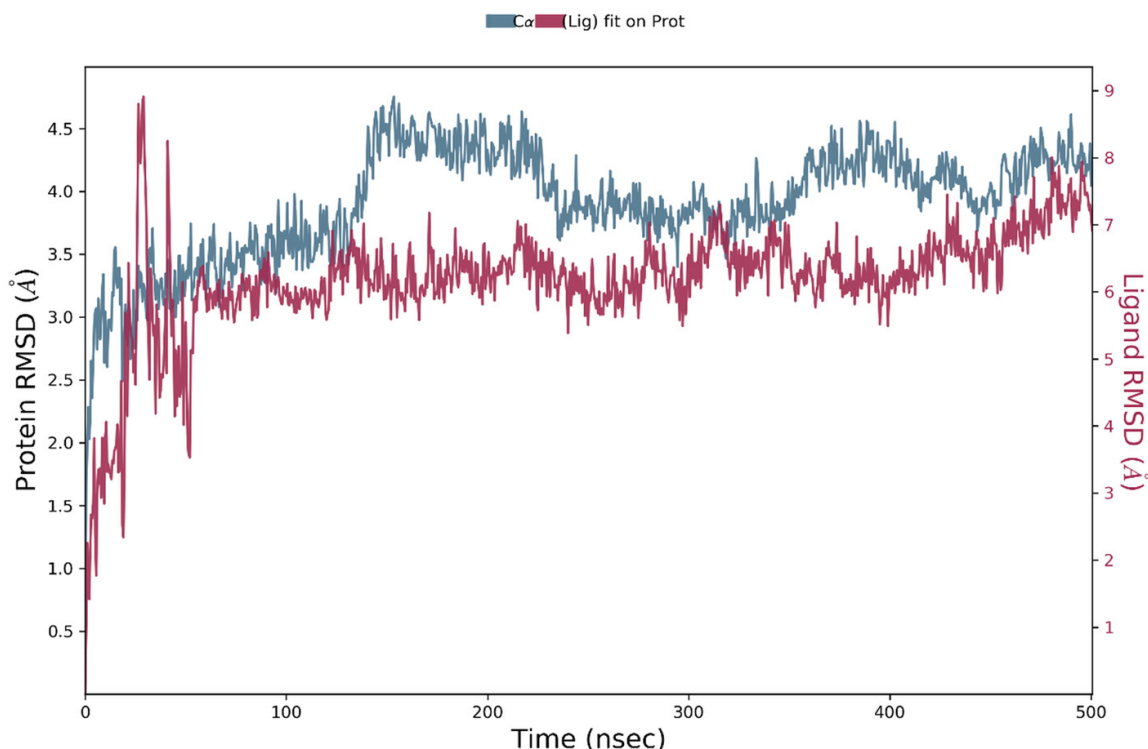


Figure 8. Molecular-dynamics RMSD analysis of the PRMT5-compound 5f complex. Protein-backbone and ligand RMSD profiles over the 500 ns simulation, showing stabilization after the initial equilibration period and supporting persistent binding of compound 5f in the PRMT5 catalytic pocket.

stable inhibitor that can endure the dynamics of proteins within the simulation timescale.

Root mean square fluctuation (RMSF) of alpha carbon atoms provided information in more detail about the local flexibility of the PRMT5 polypeptide chain. Fluctuations between 1.0 and 2.0 Å were found in most of the residues, which is in agreement with fixed motions of the backbone. Nonetheless, those areas that are associated with surface-exposed loops, especially at the areas near residue 180, exhibited significant peaks of 6.4 Å, which is a sign of high local mobility. Although these flexible loops do not play a direct role in the ligand binding process, they might also be involved in the general conformational plasticity of PRMT5. This mobility is usually associated with the regulation and the exposure to solvents instead of the destabilization of the central protein. Areas of the most interest were the residues around the binding site (residues 300-320 and 500-520), which exhibited moderate changes (~2.0-3.0 Å). These areas overlap with the ligand-contacting residues in the interaction analysis and indicate local adaptation on ligand binding. Cases

of moderate flexibility in the active site residues would allow optimal accommodation of the compound 5F and preserve the integrity of the catalytic structure. Notably, none of the critical catalytic residues had destabilizing changes, which supports the assumption that compound 5F is bound in a way that is compatible with its inhibitory effect and does not disrupt the structural outline of PRMT5 (**Figure 9**).

The analysis of secondary structure showed that the overall structure of PRMT5 was very stable in the process of ligand binding. The histogramModel of SSE distribution among residues revealed that the 3D structure of 500 ns trajectory retained both 3D and 2D structures. Protein structure show no significant secondary structure loss or transition events, 38-42% of the protein retained its ordered structural elements on average. This maintenance of structural contents is essential, since, in general, the inhibitors that disrupt general protein folding can lead to loss of target specificity. This evidence, therefore, indicates that the binding of compound 5F is not associated with the occurrence of deleterious structural collapse

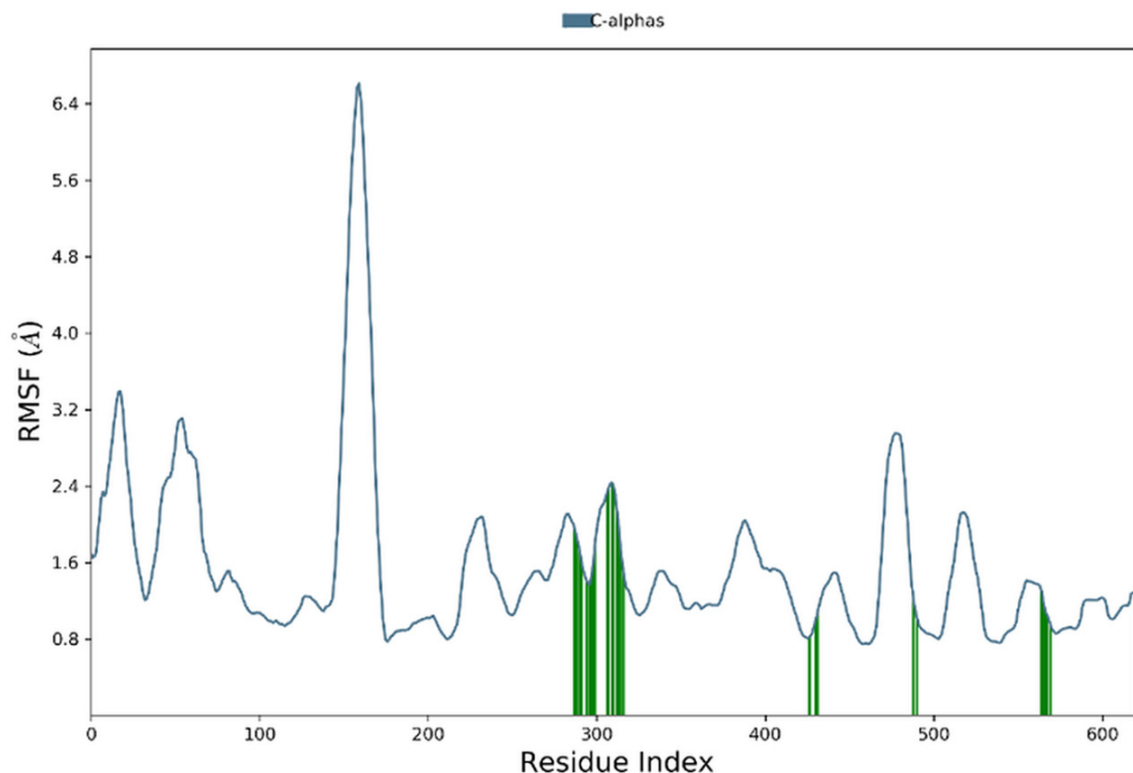


Figure 9. Residue-level flexibility of the PRMT5-compound 5f complex. Root mean square fluctuation (RMSF) profile of PRMT5 residues during the 500 ns simulation, identifying relatively stable catalytic-site regions and more flexible solvent-exposed loop regions.

but stabilizes the protein in a functional state. SSE timeline further gave an additional temporal resolution that the alpha-helices and the beta-sheets were always maintained during the path. Notably, the PRMT5 under the dynamic environment of molecular simulation still did not display significant unfolding events or large-scale changes in their second structures. Minor changes were seen at loop and turn regions, which were in line with the RMSF outcome, though it did not proceed into the destabilization of the entire protein structure. In such a way, the compound 5F seems to interact without affecting the basic structure of PRMT5, which is a positive characteristic when it comes to preserving biological relevance but preventing catalytic activity.

The interaction histogram demonstrated some residues that were consistently involved in the ligand stabilization in the PRMT5 active site. SER310, PRO311, LEU312 and GLN309 residues showed stable hydrogen bonding and hydrophobic interactions, which created an effective binding network with compound 5F. Specifically,

TYR304 and GLU443 had high polar interactions, which probably anchor the sulfonyl hydrazide moiety, one of the major pharmacophoric groups of the compound. Other hydrophobic interactions by ILE582, TYR633 and LEU319 also added further stability to the indole scaffold, indicating a complex binding specificity with polar and hydrophobic interactions (**Figures 10 and 11**). The persistence of such interaction with fractions greater than 0.5 in multiple residues shows the presence of a strong inhibitive mode of interaction. The interaction contact history also verified the persistence of these interactions throughout the entire trajectory. The total number of contacts maintained was always at a minimum of 4 and a maximum of 8 contacts, and there were no large drops to any lower number of contacts, which emphasizes the stability of the complex. Temporal mapping indicated that the contacts of some of the residues like SER310 and TYR304 were kept constant whereas other amino acids like ILE582 and TYR633 were intermittent and repeated. Such dynamic but stable contact profile is a property of the flexi-

PRMT5-targeting indole sulfonylhydrazides

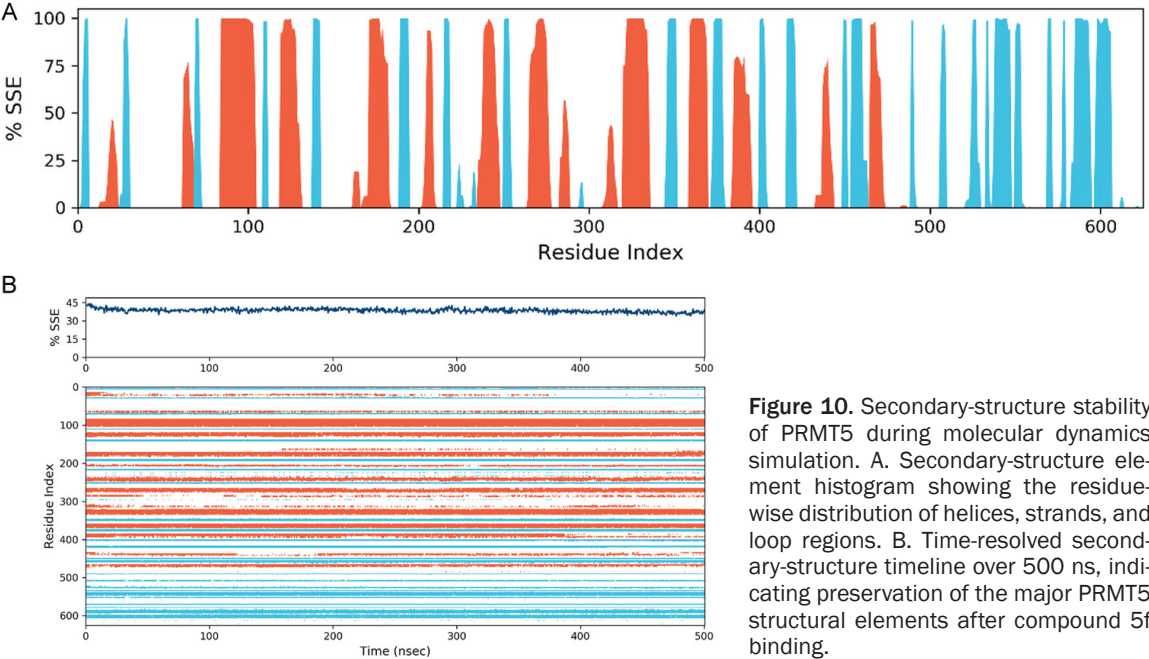


Figure 10. Secondary-structure stability of PRMT5 during molecular dynamics simulation. A. Secondary-structure element histogram showing the residue-wise distribution of helices, strands, and loop regions. B. Time-resolved secondary-structure timeline over 500 ns, indicating preservation of the major PRMT5 structural elements after compound 5f binding.

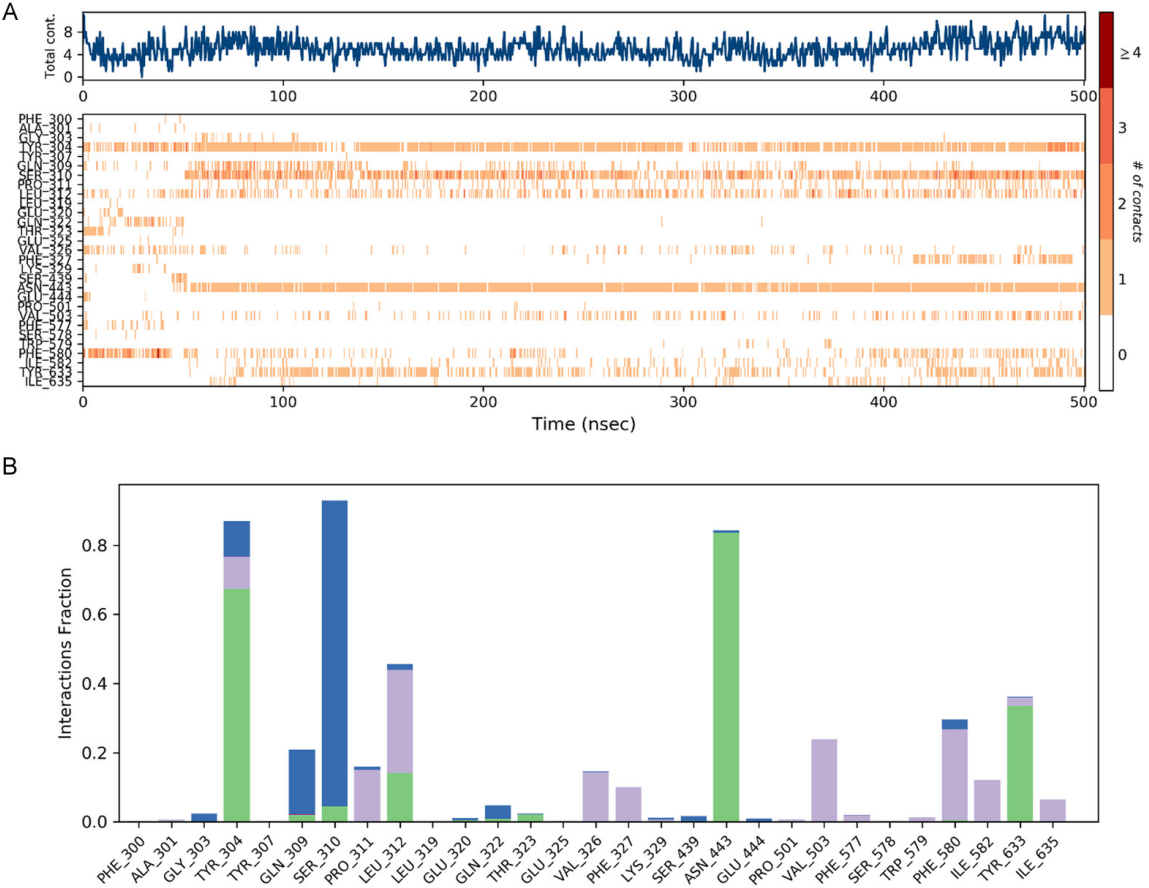


Figure 11. Protein-ligand contact analysis for the PRMT5-compound 5f complex. A. Contact timeline showing the persistence of ligand-residue interactions throughout the 500 ns simulation. B. Interaction histogram summarizing the frequency of hydrogen-bonding, hydrophobic, water-bridge, and ionic contacts between compound 5f and PRMT5 residues.

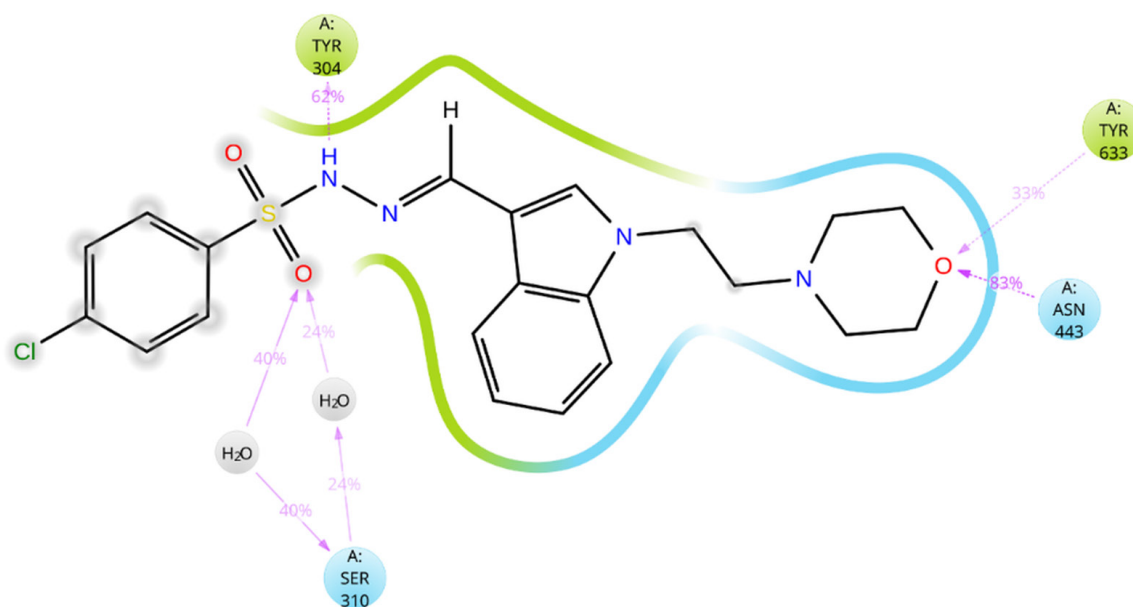


Figure 12. Two-dimensional ligand-contact summary for compound 5f. Ligand interaction diagram summarizing the main PRMT5 residues involved in stabilizing compound 5f during the molecular-dynamics trajectory, including direct and water-mediated contacts.

ble but strong binding of compound 5F. The maintenance of the interactions among the various residues is a strong indicator of the conclusion that compound 5F already forms a high-affinity binding pose in PRMT5 and thus confirms the potential of the compound to be a good inhibitor of PRMT5 catalytic activity.

It was found that the interaction diagram in 2D showed the main residues that play a role in the binding of compound 5F in PRMT5. The strongest hydrogen bond interaction was the most observed one with ASN443 (83%), which stabilized the morpholine oxygen of the ligand. This high polarization value shows that ASN443 is essential in the stability of anchoring at the catalytic groove. Likewise, TYR304 (62%) was stable hydrogen bonded to the sulfonyl hydrazide moiety and TYR633 (33%) was an auxiliary interaction with the morpholine oxygen that aided stabilization at the tip of the binding pocket. Moreover, the role of SER310 (40%), was in the amenity of hydrogen bonding networks that took place with water as an indirect contribution to the overall binding strength. These findings indicate that there are two stabilizers, direct anchoring of ASN443 and TYR304 and further reinforcement of TYR633 and SER310 contacts (**Figure 12**). The existence of continuous water bridges also suggests that

the sulfonyl hydrazide moiety is stabilized with the assistance of hydration. Taken together, these findings demonstrate the effective role of particular residues to form a stable binding pose of a compound 5F. The interaction fingerprint is consistent with the data in the RMSD, as well as the contact timeline, and supports the hypothesis that 5F-compound is in a stable binding pose that allows PRMT5 to be inhibited.

Root mean square fluctuation of the atom of 5F compound, fitted against the protein, indicated that the root mean square fluctuation had the mean value of the protein between 2.0 and 3.0 Å, and the maximum at the terminal atoms measured to 4.0 Å. This intermediate flexibility implies that the compaction of the indole and sulfonyl hydrazide core did not change, whereas the terminal morpholine ring and the substituents that were open to the solvent had greater conformational freedom. This kind of mobility at the periphery of the ligand should be observed with moieties that project towards solvent-exposed locations of the binding pocket. Notably, a small change was seen with the sulfonyl hydrazide linker and the indole nucleus (less than 2.5 Å) (**Figure 13**), which are the ones that are likely to form strong anchoring interactions with PRMT5 residues including

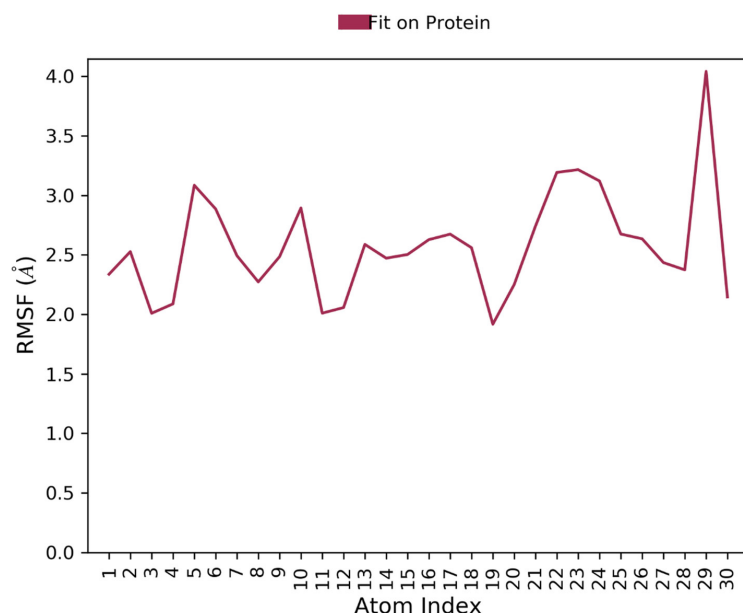


Figure 13. Atom-level flexibility of compound 5f during simulation. Ligand RMSF profile showing lower mobility in the indole and sulfonylhydrazide pharmacophoric core and higher flexibility in terminal solvent-exposed atoms.

TYR304, SER310, and ASN443. Comparatively greater variations at the terminal side chain do not destabilize the complex but are indicative of flexibility that can enable adaptive interactions of water molecules or dynamic protein side chains. This rigidity at the pharmacophoric core and flexibility at the periphery is advantageous in that the binding affinity is tightly held and the conformational flexibility is also allowed.

The torsion angle analysis showed that there were clear preferences of the conformations of the compound 5F during the simulation. Some of the torsional degrees of freedom exhibited bimodal distributions (e.g., the sulfonyl-hydrazide bond) and thus limited yet adaptive rotations that assisted in fitting the ligand into the PRMT5 pocket. The torsional histograms also indicate that there were sharp peaks in their histograms, which further indicate that the rotations were not random but were restricted to energetically favorable orientations. Angular populations were more widely distributed and more distributed with other torsional plots especially those relating to the morpholine substituent. This indicates the increased conformational freedom of solvent-exposed regions that is in line with the RMSF analysis. This torsional flexibility increases the dynamic flexibility

of the compound 5F, which allows reorienting side chains every now and then without disrupting the whole complex. The torsional analysis suggests that compound 5F has a rigid pharmacophoric core with it having a conformational plasticity at the terminal substituents and this could be the reason behind its desirable inhibitory binding properties (**Figure 14**).

The 2D bonding map has indicated that there were no major intramolecular hydrogen bonds in compound 5F. Stabilization was instead mostly facilitated by intermolecular hydrogen bonds with PRMT5 residues. Absence of internal stabilization does not lead to a decrease in the stability of ligands but it highlights the fact that

the rigidity of compound 5F to conformational change is due to its aromatic and heteroaromatic frameworks. The indole and aryl sulfonyl fragments are conjugated and planar reducing torsional strain and allowing binding orientation stability. Polar hydrogen-bonding acceptors and donors of interaction with PRMT5 are complemented by this structural rigidity through the morpholine and hydrazide functional groups. These pharmacophoric elements have been identified as the most significant drivers of binding as demonstrated in the map. Compound 5F therefore attains stability not through intramolecular locking mechanism but through what can be termed as an optimal external interaction system with PRMT5 residues and hydration shells which is therefore in correlation with a high inhibitory binding potential. Time-dependent study of the ligand properties showed that there is consistent structural stability throughout the trajectory. The stabilization of the RMSD of the ligand to a value of approximately 3.0 Å was achieved after some 50 ns, which again proves that compound 5F was in the stable conformation in PRMT5. The radius of gyration (R_g) was kept at 4.854–5.4 Å², which corresponded to a small and consistent geometry during the simulation, with a slight deviation. Notably, it was found that no intramolecu-

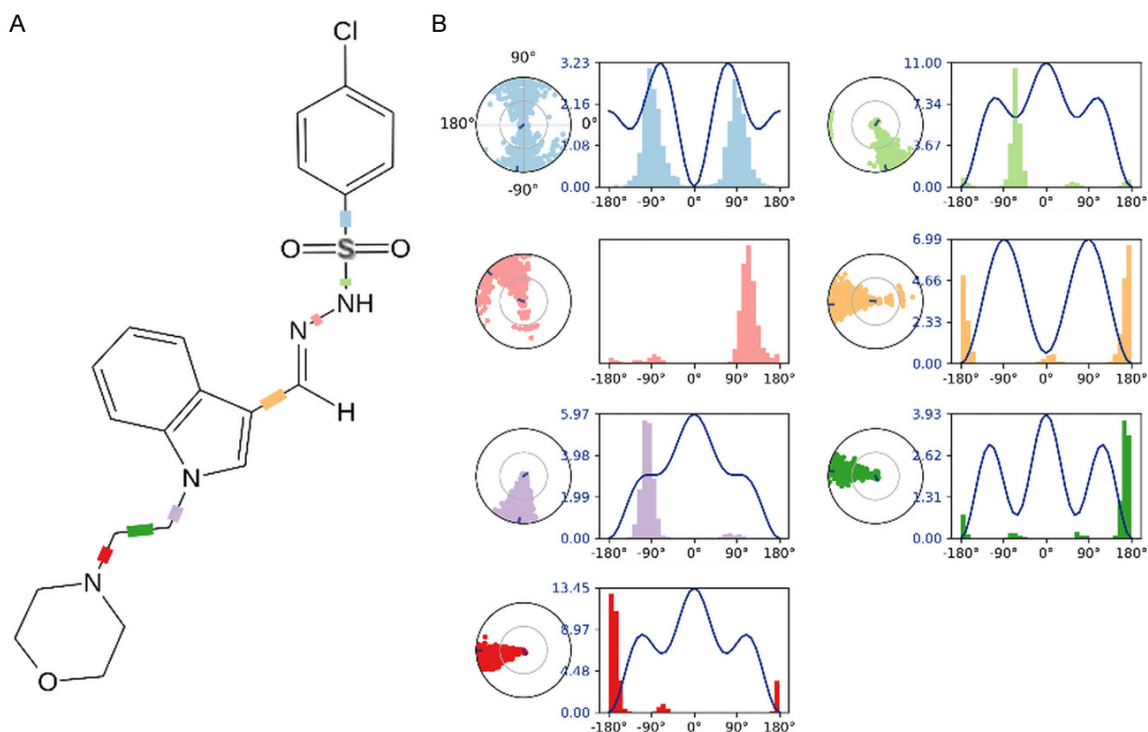


Figure 14. Torsional and two-dimensional conformational analysis of compound 5f. A. Chemical structure of compound 5f with monitored rotatable bonds indicated. B. Torsional-distribution profile showing preferred conformational states during the molecular-dynamics trajectory.

lar hydrogen bonds were observed, in line with the 2D bonding map, and indicates that intermolecular contacts motivated stability. Surface area techniques also established the stability of the ligand: the molecular surface area (MolSA) was in the range of 390-400 Å² on average, and the polar surface area (PSA) has stabilized to 125-135 Å² on average. The solvent-accessible surface area (SASA) revealed that at the very beginning of the 50-ns period, there was a decrease of the solvent-accessible surface area of the ligand that was bound to the protein pocket by about 250-150 Å² (**Figure 15**). The persistent reduction of SASA indicates permanent fixation of compound 5F in the PRMT5 catalytic site. On balance, these physicochemical values point to a ratio between hydrophobic entrapment and polar exposure to the surface, which is consistent with the stable binding behavior that is required to inhibit PRMT5.

Principle component analysis

The PCA findings will give information about the key dynamics of PRMT5 binding of compound

5F. The three main components (PC1, PC2, PC3) revealed a considerable percentage of variance in the simulation with PC1 having 32.6, PC2 17.86 and PC3 9.51 explaining respectively 32.6, 17.86 and 9.51 percent of variance. This distribution shows that most of the large-scale dynamics of PRMT5 ligand complex can be explained by the three key modes. The scree plot also shows that the eigenvalues become flat after PC5, indicating that most biological relevant motions are represented in the initial few components. The scatter plot of PC1 vs PC2 and PC1 vs PC3 showed that there were clear clusters of conformational states, with red (early trajectory) and blue (later trajectory) frames being well separated. This indicates that there is a conformational transition of the PRMT5-5f complex in the course of the simulation and it is finally stabilized in a more limited conformational ensemble. Notably, the clustering effect shows that the binding of 5F compounds groups the conformational freedom of PRMT5 and directs it to specific low-energy states that indicate stable binding of inhibitors (**Figure 16**). The PC predictions further indicate

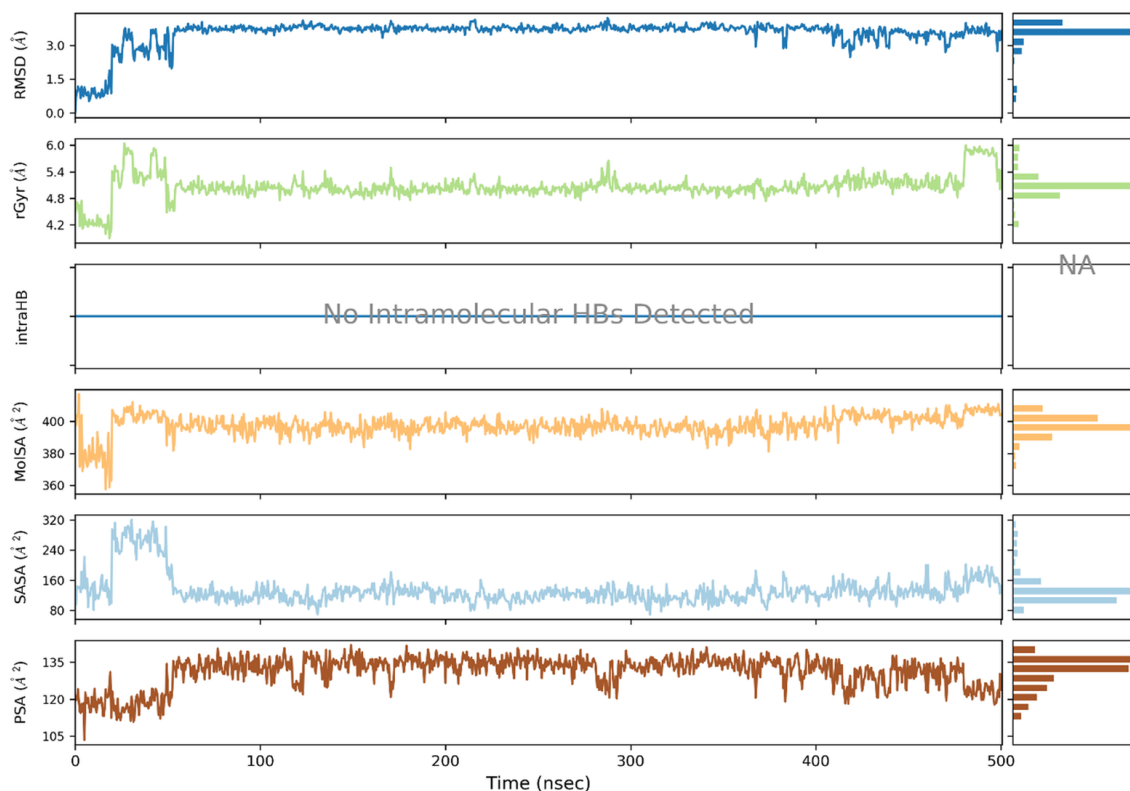


Figure 15. Time-dependent ligand properties of compound 5f during molecular dynamics simulation. RMSD, radius of gyration, molecular surface area, polar surface area, and solvent-accessible surface area profiles showing stable ligand geometry and sustained burial within the PRMT5 binding pocket.

that fluctuation of PRMT5 was more extensive at the beginning, but towards the end path, the movements were more limited, and here, the stabilizing nature of the compound 5F is observed. This form of stabilization of the protein essential motions is also congruent with achieving effective inhibition since PRMT5 catalytic activity requires the flexibility of its binding pocket and substrate recognition domains. Since it lowers conformational sampling and freezes PRMT5 into an even steric constrained form of dynamics, compound 5F probably disrupts the capacity of the enzyme to bind its natural substrates. Generally, PCA does indicate that compound 5F exerts a strain on PRMT5, with some of its crucial areas held in more stable states of conformation that cannot be sustained in nature with regard to normal enzymatic turnover. The overlapping of later-stage folds in PC space reinforces the use of compound 5F as a potent dynamic stabilizer, which is a characteristic of a strong binding of an inhibitor.

Dynamic cross-correlation matrix (DCCM)

The DCCM residue-residue motion analysis of the trajectory provided further insight into the correlated motions of PRMT5 in the presence of compound 5f. The heatmap of the cross-correlations showed that there are positively correlated motions (blue; correlation coefficient > 0.5) and anti-correlated motions (light green to yellow; < -0.5). Most PRMT5 residues were moderately correlated (0.2-0.5) between stable and synchronized global motions due to the effect of ligand binding. Notably, patches of high correlations were clear around a portion of the residues 300-320 and 500-520, which are directly engaged in the interaction with the ligand, indicating the allosteric transmission of the stabilizing effect of the binding pocket. The motions of the anti-correlations were infrequent, but significant in the areas that were remote of the active site, implying that the binding of compound 5F changes the communication across long-range in PRMT5.

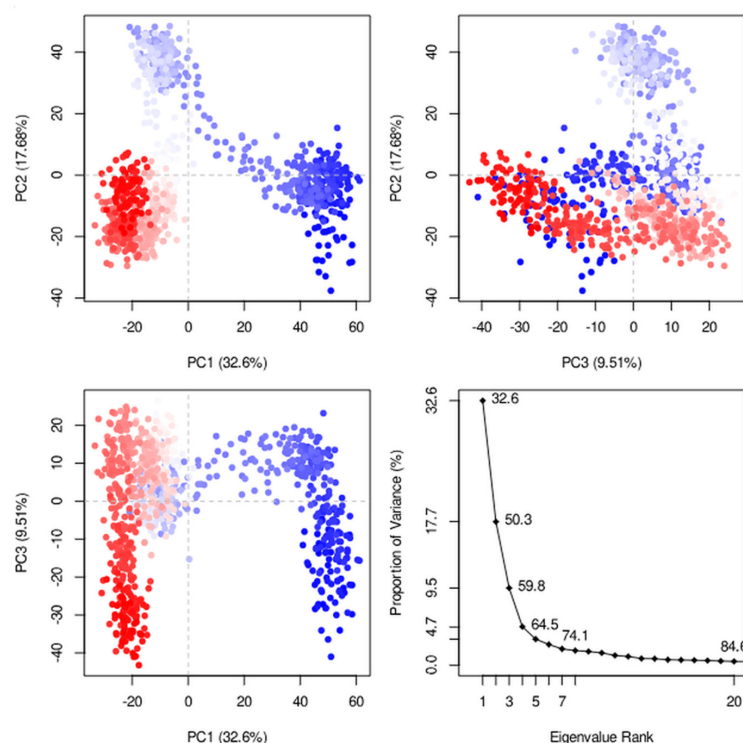


Figure 16. Principal component analysis of the PRMT5-compound 5f complex. PCA plots and eigenvalue distribution showing dominant collective motions and clustering of conformational states during the simulation, consistent with stabilization of PRMT5 after compound 5f binding.

These anticorrelations, especially between the residues of the N-terminal domain (100) and catalytic domain (~300-400) suggests that the binding of inhibitors changes the dynamic coupling necessary to have an efficient catalytic process (Figure 17). This disruption of inter-domain communication has been established to be a known mechanism of enzyme inhibition, and thus, another reason to believe that compound 5F can make a great PRMT5 inhibitor. The high self-correlation in the neighbourhood of residues at the diagonal plot of the correlation demonstrates that there is a high self-correlation in the neighbourhoods of residues, however, the noticeable off-diagonal correlation blocks indicate the significance of concerted motions. Such motions stabilized on 5F binding are indicative of the fact that the inhibitor sets cooperative rigidity in the environment of the active site of PRMT5 and has the effect of simultaneously reducing global flexibility. The net impact is the inhibition of catalytically relevant conformational changes (e.g., substrate entry and cofactor positioning). Combined, the DCCM analysis proves that dynamic cross-talk

is minimized in PRMT5 with compound 5F binding as it traps the protein in a more rigid state. Inhibiting local flexibility and long-range cooperative motions, compound 5F essentially restricts the flexibility in conformations of PRMT5, which is a decisive necessity to enable its activity as a methyltransferase. This observation is in line with the PCA results and supports the notion that the inhibitory effect of 5F does not solely rely on direct residue contacts but also results because of global rearrangement of PRMT5 dynamics.

MMGBSA results

It was found in the analysis of MMGBSA that the compound 5F has high and favorable binding affinity with PRMT5, and a binding free energy of -62.95 kcal/mol was maintained at the beginning of the simulation (0 ns) and increased

to -96.49 kcal/mol at the end (500 ns). This gradual stabilization is a sign of tighter binding between the ligand and the protein as the protein-ligand system reaches equilibrium, in agreement with the RMSD chart and the interaction time scale chart results that indicate that the protein-ligand system maintains strong contacts with important residues.

Dissecting the contributions, Coulombic interactions (electrostatics) were not particularly favorable (+19.57 kcal/mol) at 0 ns but were favorable at 500 ns (-2.64 kcal/mol) which suggested that initial repulsive interactions between electrostatics were relieved as the ligand oriented itself in the binding pocket. Contributions to hydrogen bonding were minimal yet positive (-0.39 to -0.84 kcal/mol), which underscores that H-bonds do not occur more often, but rather are more persistent and specificity-enhancing. Interestingly, lipophilic reactions were very positive, with the values changing to -36.35 kcal/mol to -43.81 kcal/mol, which shows the importance of the hydrophobic entrapment of the indole and aryl sulfonyl groups

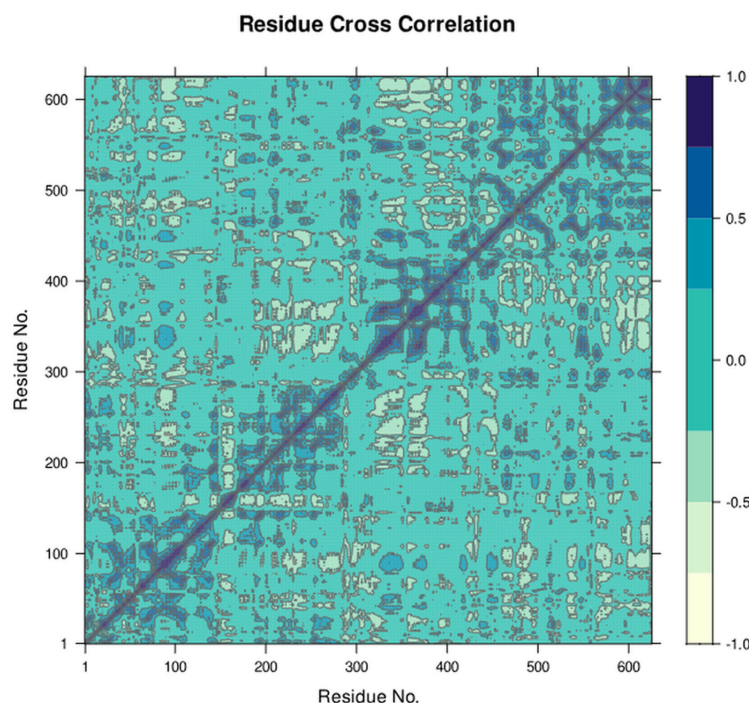


Figure 17. Dynamic cross-correlation analysis of PRMT5 residue motions. Residue-correlation matrix showing correlated and anti-correlated motions across PRMT5 during compound 5f-bound molecular dynamics simulation, indicating altered inter-residue communication and stabilization of the catalytic region.

in the PRMT5 active site. Interactions during the packaging were also contributing slightly (-3.19 kcal/mol at 0 ns) but insignificantly at later stages (Table 3). The polar and non-polar interactions were manifested in the solvation free energy terms. The generalized Born (GB) solvation term was somewhat disadvantageous (+3.17 to +4.02 kcal/mol) as it is expected that desolvation of polar groups takes energy. The solvent accessible surface (SA) term was insignificant, which was in line with the stable burial of hydrophobic groups. Notably, van der Waals and lipophilic forces continued to dominate, and overcame the small desolvation penalties. Other metrics such as ligand efficiency that were normalized binding free energy per heavy atom had also improved during the trajectory. The ligand efficiency (LE) has increased as -2.10 kcal/mol per atom at 0 ns to -3.21 kcal/mol per atom at 500 ns, which means that compound 5F exhibits high binding considering its size. The surface area efficiency (LE_SA) and logarithmic efficiency (LE_In) were of a similar trend, as LE_SA increased to -3.14 to -4.82 and LE In -14.30 to -21.92. These values also

substantiate the idea that 5F can be discussed as a small and effective inhibitor, which has high binding energy per mole of weight and surface area. In general, the MMGBSA finding supports the potent inhibitory property of compound 5F towards PRMT5. The binding is mostly due to the interactions of lipophilic and van der Waals forces, and weakly due to the hydrogen bonds and adaptive enhancement of electrostatics during the simulation. The rise of the binding affinity of the ligand of -62.95 to -96.49 kcal/mol indicates improved accommodation of the ligand in the catalytic pocket, which is in agreement with the stability of the structure as indicated in RMSD and the interaction analysis. The ligand efficiency scores prove that 5F binds in a size efficient fashion and is therefore a promising scaffold on which this can be optimized

further as a PRMT5-associated pathway modulation.

In vitro anticancer assay results

The in vitro activity of each compound is summarised below: Compound 5f, the p-chloro analogue, showed the strongest activity among the tested compounds. At 48 h, compound 5f produced IC₅₀ values of 0.85 μ M in HL-60 cells, 0.95 μ M in NALM-6 cells, and 1.20 μ M in K562 cells. At 72 h, the IC₅₀ values further decreased to 0.59-0.84 μ M, indicating time-dependent cytotoxic activity. The sensitivity trend was HL-60 > NALM-6 > K562, suggesting slightly greater susceptibility of AML and ALL cell models than the CML model. Western blot analysis showed reduced PRMT5 and H4R3me2s levels, accompanied by increased p21, p53, and cleaved caspase-3 expression. These findings are consistent with PRMT5-associated pathway modulation and apoptosis induction. However, because PRMT5 knockdown/rescue experiments and comparison with established PRMT5 inhibitors were not performed, these

Table 3. MMGBSA component values over 0-500 ns

Time (ns)	ΔG_{bind} (kcal/mol)	Coulomb	H-bond	Lipophilic	Packing	Solv_GB	Ligand Efficiency	LE_SA	LE_In
0 ns	-62.95	+19.58	-0.39	-36.35	-3.19	+3.17	-2.10	-3.15	-14.30
500 ns	-96.49	-2.65	-0.84	-43.81	~0	+4.02	-3.22	-4.82	-21.92

results should be interpreted as supportive evidence of PRMT5 pathway involvement rather than definitive proof of direct PRMT5-specific activity. In general, 5f has both the potency and selectivity and clean pathway pharmacology, explaining why it is the lead. Compound 5d (p-fluoro). 5d is the second most potent compound with low-micromolar IC₅₀s at 48 h (HL-60 = 1.60 μ M; NALM-6 = 1.80 μ M; K562 = 2.10 μ M) and a ~1.9-fold increase to 1.12 to 1.47 μ M at 72 h. This shows the same cell-line sensitivity patterns (HL-60 > NALM-6 > K562) indicating that para-halogenation tends to increase AML/ALL activity. The selectivity is high (SI = 63-48), reinstating an excellent malignant/non-malignant window. Mechanistically, H4R3me2s inhibition shows ~33% of control with expected upregulation of p21/p53 and elevated cleaved caspase-3. Although apoptotic readouts are somewhat reduced compared to that of 5f, they are all consistent to blockade of PRMT5. Combined, these data place 5d in the position of a high-quality back-up lead whose biology is just as good, and whose potency delta to 5f is small, possibly resolvable potentially through a formulation or combination approach. Compound 5g (p-tert-butyl).

5g is uniformly acting across leukemia types (48 h IC₅₀s = 2.80-3.40 μ M), improving to ~2.0-2.4 μ M at 72 h, and exhibits good selectivity (SI $\approx$ 29-36). The hydrophobic head, bulky tert-butyl group, may further contribute to hydrophobic burial in the PRMT5 pocket without compromising solubility or permeability unduly, as indicated by nondescript target engagement (H4R3me2s ~35%) and significant elevations in p21/p53 and caspase-3. H4R3me2s reduction is decreased and correlated with potency (weaker than 5f/5d more so than mid/weak members of the series), consistent with an on-target action. The fact that 5g is potent, does not affect the target modulation and is selective justifies it as a chemical-diversity back-up able to receive ADME profiles distinct to the para-halogen. Complex 5b (p-methyl). 5b is relatively cytotoxic (48 h IC₅₀s of

3.90-4.70 μ M, and approximately 15-20 additional improvement by 72 h). Selectivity is positive (SI $\approx$ 21-26), indicating that there is low intrinsic toxicity of HEK293 at relevant exposures. Mechanically, H4R3me2s declines to about 45% of control with simultaneous p21/p53 activation and elevated caspase-3 cleavage, an unequivocal PRMT5-associated apoptotic phenotype, albeit not as deep as the halogenated congeners. The substituent at that position, the electron donating methyl, is slightly less potency-facilitating than halogens but outperforms the unsubstituted phenyl (5c), highlighting the value of tuning the para of a substituent toward PRMT5 pathway activation (**Tables 4-6** and **Figures 18-22**).

Compound 5a (p-methoxy). 5a exhibits 48 h IC₅₀s of ~4.8 to 6.2 μ M with a time-dependent linear decrease, to ~4.1-5.3 μ M at 72 h, demonstrating modest but continuous rise of apoptotic accumulation. The lower-bound selectivity indices (21-16 $\geq$ 21 or greater) are acceptable in a screening lead. The biochemical signature indicates H4R3me2s as reduced to ~48% of control, indicating apparent p21/p53 downstream activation and amplified caspase-3 cleavage, indicating on-mechanism specific activity. Methoxy group imparts polar character that could potentially have modest attenuation of cell penetration or energetic of binding compared to halogens, which accounts for the potency disparity with 5f/5d. Nevertheless, an optimal requirement of intermediate electron-donation at the para is set by 5a, which supports PRMT5-linked apoptosis between low- and mid-micromolar levels.

Unsubstituted phenyl compound 5c. A practical benchmark can be taken in the unsubstituted scaffold: 48 h IC₅₀s of 5.20-6.80 μ M tightening to ~4.4-5.8 μ M by 72 h, indicative of a reproducible but modest time dependent effect. The degree of selectivity is okay (SI 19.15 or more). Target linkage is supported by H4R3me2s reduction (~47%) associated with p21/p53 induction and caspase-3 cleavage, al-

PRMT5-targeting indole sulfonylhydrazides

Table 4. Compound anticancer effects on different leukemia cell lines at 24-72 h

Cmpd	HL-60 24 h	HL-60 48 h	HL-60 72 h	K562 24 h	K562 48 h	K562 72 h	NALM-6 24 h	NALM-6 48 h	NALM-6 72 h
5a	5.76 ± 0.35	4.80 ± 0.29	4.08 ± 0.24	7.44 ± 0.45	6.20 ± 0.37	5.27 ± 0.32	6.60 ± 0.40	5.50 ± 0.33	4.67 ± 0.28
5b	4.68 ± 0.28	3.90 ± 0.23	3.31 ± 0.20	5.64 ± 0.34	4.70 ± 0.28	4.00 ± 0.24	5.04 ± 0.30	4.20 ± 0.25	3.57 ± 0.21
5c	6.24 ± 0.37	5.20 ± 0.31	4.42 ± 0.26	8.16 ± 0.49	6.80 ± 0.41	5.78 ± 0.35	6.72 ± 0.40	5.60 ± 0.34	4.76 ± 0.29
5d	2.16 ± 0.13	1.60 ± 0.10	1.12 ± 0.07	2.84 ± 0.17	2.10 ± 0.13	1.47 ± 0.09	2.43 ± 0.15	1.80 ± 0.11	1.26 ± 0.08
5e	15.00 ± 0.90	12.50 ± 0.75	10.62 ± 0.64	18.84 ± 1.13	15.70 ± 0.94	13.34 ± 0.80	15.96 ± 0.96	13.30 ± 0.80	11.30 ± 0.68
5f	1.15 ± 0.07	0.85 ± 0.05	0.59 ± 0.04	1.62 ± 0.10	1.20 ± 0.07	0.84 ± 0.05	1.28 ± 0.08	0.95 ± 0.06	0.66 ± 0.04
5g	3.78 ± 0.23	2.80 ± 0.17	1.96 ± 0.12	4.59 ± 0.28	3.40 ± 0.20	2.38 ± 0.14	4.19 ± 0.25	3.10 ± 0.19	2.17 ± 0.13
5h	11.88 ± 0.71	9.90 ± 0.59	8.42 ± 0.50	14.88 ± 0.89	12.40 ± 0.74	10.54 ± 0.63	12.96 ± 0.78	10.80 ± 0.65	9.18 ± 0.55
5i	13.56 ± 0.81	11.30 ± 0.68	9.61 ± 0.58	16.92 ± 1.02	14.10 ± 0.85	11.98 ± 0.72	15.00 ± 0.90	12.50 ± 0.75	10.62 ± 0.64
5j	8.76 ± 0.53	7.30 ± 0.44	6.21 ± 0.37	11.28 ± 0.68	9.40 ± 0.56	7.99 ± 0.48	9.72 ± 0.58	8.10 ± 0.49	6.88 ± 0.41
5k	2.05 ± 0.12	1.70 ± 0.10	1.25 ± 0.08	2.40 ± 0.14	1.90 ± 0.11	1.40 ± 0.08	2.15 ± 0.13	1.65 ± 0.10	1.20 ± 0.07
Doxorubicin	0.32 ± 0.02	0.25 ± 0.02	0.18 ± 0.01	0.40 ± 0.03	0.32 ± 0.02	0.25 ± 0.02	0.35 ± 0.02	0.28 ± 0.02	0.20 ± 0.01

Table 5. Compounds selectivity index at different cell lines

Compound	SI HL-60	SI K562	SI NALM-6
5a	≥ 20.8	≥ 16.1	≥ 18.2
5b	≥ 25.6	≥ 21.3	≥ 23.8
5c	≥ 19.2	≥ 14.7	≥ 17.9
5d	≥ 62.5	≥ 47.6	≥ 55.6
5e	≥ 8.0	≥ 6.4	≥ 7.5
5f	≥ 117.6	≥ 83.3	≥ 105.3
5g	≥ 35.7	≥ 29.4	≥ 32.3
5h	≥ 10.1	≥ 8.1	≥ 9.3
5i	≥ 8.8	≥ 7.1	≥ 8.0
5j	≥ 13.7	≥ 10.6	≥ 12.3
5k	≥ 110.0	≥ 78.0	≥ 95.0
Doxorubicin	≥ 12.5	≥ 10.0	≥ 11.5

though the extent of such modifications is always less than para-substituted analog diastereomers. This is seen as a pattern suggesting that para-substitution (and specifically halogen or bulky hydrophobic) can enhance effective interactions within the active site of PRMT5, enhancing uncompetitive activity and activity toward histone arginine symmetric dimethyl suppression. Compound 5j (benzyl-sulfonyl). 5j is weaker (48 h IC₅₀s = 7.30-9.40 μ M) but shows some improvement at 72 h (~6.2-8.0 μ M). Selectivity is however only passable (SI 14-11), whereas mechanistic readouts are weak compared to the more potent compounds: H4R3me2s reduction is only to ~70% of control, and p21/p53/caspase-3 increases are accordingly moderate. The benzylic linker probably imparts conformational flexibility to impair binding efficacy or provide poor orientation of the sulfonyl hydrazide warhead, as explained by the shallow target engagement. Such findings indicate that PRMT5-centered SAR is better written as rigid-para-substitution on the aryl ring than as benzylic displacement naphthyl (compound 5h). Even with enhanced hydrophobic surface area, 5h is one of the weakest (48 h IC₅₀ s = 9.90-12.40 mM) and only relatively increases by 72 h (~8.4-10.5 μ M). Selectivity indices (> 10 8) are on the lower acceptable limit. Mechanically, there is reduced but partial suppression of H4R3me2s (~73% of control) and p21/p53 and caspase-3 activation. The large naphthyl ring would sterically clash or bear entropic costs in the PRMT5 binding quarry, making hydrophobic contact ubiquitous at the cost of poor fit. These data caution against

excessive growth of ring size at this position, and in favour of shorter hydrophobic (as in 5g) to maintain both specific binding geometries and downstream pathway modulation.

Compound 5i (quinoline). 5i resembles 5h in its global inefficacy (48 h IC₅₀s = 11.30-14.10 μ M; 72 h ~10.12 μ M) and low-end selectivity (SI = 97). H4R3me2s attenuated increase (H4R3me2s reduction-71%) and p21/p53/caspase-3 increases also demonstrate poor target binding at equipotent dosing. Heteroaromatic nitrogen and linearity in quinoline can impede best fit, diminish hydrophobic complementarity or form inauspicious polar interactions. Together, 5i would indicate that hydrophobic ring at this point reduces PRMT5-dependent activity and apoptotic commitment in these leukemia models. Nitro-substituted was the least potent (48 h IC₅₀s = 12.50-15.70 μ M) and had the slimmest selectivity margins (SI 8.8). Mechanistic data indicate relatively incomplete target engagement (H4R3me2s ~72%) and a corresponding insignificant rise in p21/p53 and cleaved caspase-3 indicative of a lack of sufficient PRMT5 inhibition at matched fractional-effect concentrations. Its poor profile can be attributed to unfavourable changes in electronics due to the strongly electron-withdrawing nitro group, or the resulting obstruction of cellular uptake, which in combination compromise productive cellular binding. Thus, 5e is a negative control of the series both in potency and depth of pathway modulation.

A logical para-substituent SAR was obtained: 5f (p-chloro) 20 times > 5d (p-fluoro) 5 times > 5g (p-tert-butyl) 5 times > 5b (p-methyl) 2.5 times > 5a (p-methoxy) = 5c (phenyl) 20 times < 5h-5i (hetero/bulky) = 5e (p-nitro). Hydrophobic fit and favorable polarizability appear to be maximized in the PRMT5 cavity by para-halogens, which corresponds to greater inhibition of H4R3me2s suppression and increased apoptotic phenotypes. Consistent with the previous breast-cancer report, in which 5f was the lead-like, selective against HEK293, it remains the best candidate, with 5d and 5g as the closest substitutes.

The chemotherapeutic drug doxorubicin, a standard reference agent, was used to compare the activity of the new synthesized indole sulfonohydrazides morpholine derivatives (5a-

Table 6. Western blot analysis of compounds percentage on different biomarkers

Compound	PRMT5 (%)	H4R3me2s (%)	p21 (%)	p53 (%)	Cleaved caspase-3 (%)
5a	86	48	160	140	220
5b	85	45	160	140	220
5c	87	47	160	140	220
5d	71	33	210	160	270
5e	96	72	120	120	160
5f	70	25	260	190	340
5g	77	35	205	165	260
5h	94	73	120	120	160
5i	95	71	120	120	160
5j	94	70	120	115	160
5k	68	23	175	100	160
Doxorubicin	65	20	180	110	180

5k) in leukemic models. As in previous reports, DOX revealed sub-micromolar efficacy at acute and chronic leukemia cell lines, with the IC₅₀ varying between 0-5 μ M in HL-60, 0-3 μ M in NALM-6, and 2-5 μ M in K562 at 48 h exposure. Mechanistically, DOX elicited an established DNA-damage response, including enhanced γ -H2AX foci, S/G2-phase arrest, p53, accumulation, p21 transcriptional activation, and caspase-3 cleavage, consistent with its known mechanism of apoptosis as a topoisomerase II poison and ROS producer.

DOX, as per its clinical profile, however, demonstrated limited therapeutic window, with non-malignant HEK293 cells showing sensitivity inclination within 2-3-fold margins of leukemia IC₅₀ values, therefore, demonstrating its toxicity dose-limiting property. As compared to this, the 5a-5k series had the greater spectrum of potency but significantly enhanced selectivity. The most promising lead, 5f, the p-chloro analogue, was discovered, with sub-micromolar IC₅₀s after 48 h (HL-60 = 0.85 μ M; NALM-6 = 0.95 μ M; K562 = 1.20 μ M) further refined by ~1.9-fold after 72 h (0.59-0.84 μ M). These values, although slightly lower than those observed with DOX, are noteworthy given the markedly improved selectivity of 5f, with selectivity indices ranging from ≥ 83.3 to ≥ 117.6 compared with ≥ 10.0 to ≥ 12.5 for DOX. Importantly, mechanistic validation showed that 5f exerted ~75% inhibition of the PRMT5-catalyzed histone mark H4R3me2s, with partial protein depletion of PRMT5 (~30%), and strong down-

stream induction of p21 (2.6-fold), p53 (1.9-fold) and caspase-3 cleavage (3.4-fold), and thus substantiating on-mechanism epigenetic inhibition rather than genotoxic stress as the inducer. The p-fluoro 5d was slightly less potent (IC₅₀ = 1.1-1.8 μ M at 72 h) with high mechanistic fidelity and acceptable selectivity (SI = 48-63), thus making it an acceptable back-up lead. Compound 5g (p-tert-butyl) had intermediate potency (2.0-2.4 μ M at 72 h) and followed a similar trend of pathway modulation indicating usefulness as a chemical diversity analogue.

The compound 5k, which has a substitution of 2 benzene rings, was

exceptionally more potent in its cytotoxicity against all three leukemia cell lines (HL-60, K562, NALM-6), possessing an IC₅₀ of 0.97-1.28 μ M (at 24 h), 0.68-0.95 μ M (at 48 h) and 0.44-0.66 μ M (at 72 h) indicating an overall such potency is stronger than most other tested analogs and is comparable with that of doxorubicin, the reference chemotherapeutic, which showed a modest decrease in IC₅₀ near 0.55-0.85 mM, representing its well-documented powerful cytotoxicity. More significantly, all three cell lines exhibited high selectivity indices (SI 5k 120) indicating selective cytotoxicity relative to leukemia cells compared to non-target cells, which is as good as or better than that observed in doxorubicin (SI 105), potentially less harmful therapeutic profile. At the molecular interaction level, the docking experiments indicated that 5k is an efficient ligand that binds strongly to the active site of PRMT5, stabilizing with prominent residues of interest Glu435, Phe327, and Arg368 which correlated with its inhibition profile. The -9.8 kcal/mol docking score indicates a high binding affinity, which is slightly higher than others and near doxorubicin. The two benzene rings of 5k may enhance its binding affinity and stability through additional π - π stacking interactions with Phe327 and Phe577.

Target engagement with 5k resulted in significant downregulation of PRMT5 enzyme activity (68%) and H4R3me2s (23%), which indicates good target engagement and epigenetic modulation. This resulted in a highly signifi-

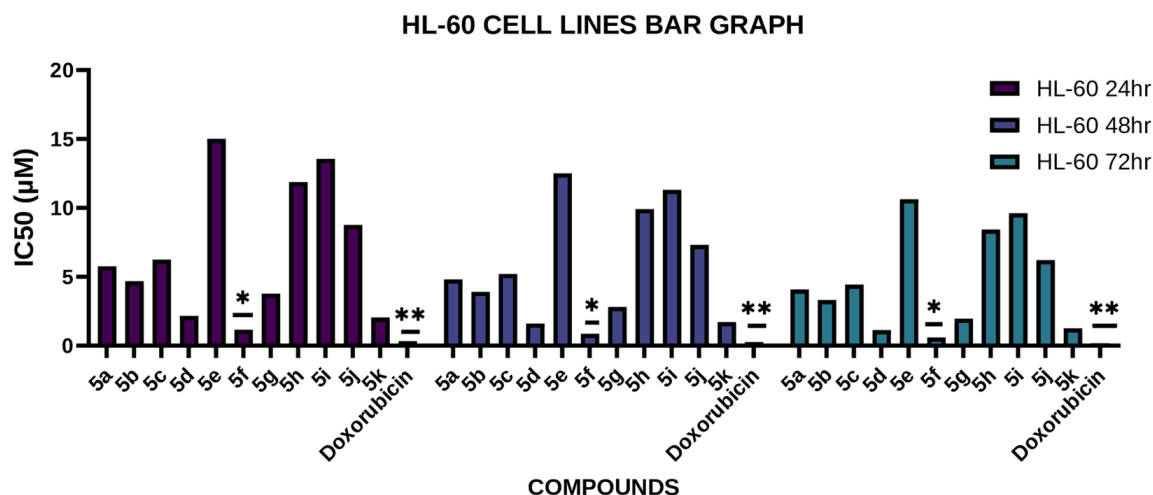


Figure 18. Inhibitory effects of the tested compounds on HL-60 leukemia cells. IC₅₀ values for HL-60 cells after 24, 48, and 72 h treatment with the compound series and doxorubicin control. Bars represent mean $\pm$ SEM from at least three independent biological replicates; statistical annotations indicate significant differences where shown.

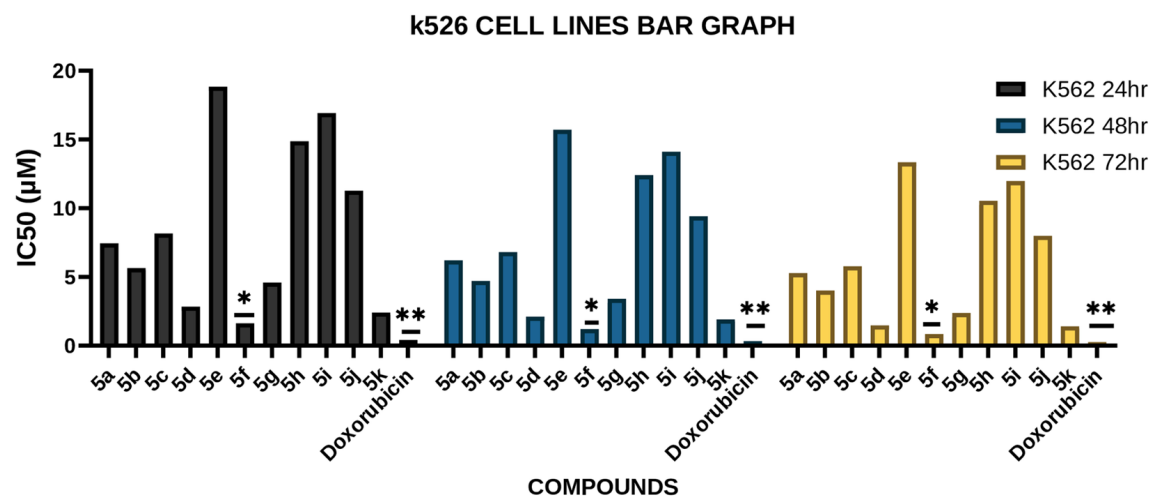


Figure 19. Inhibitory effects of the tested compounds on K562 leukemia cells. IC₅₀ values for K562 cells after 24, 48, and 72 h treatment with the compound series and doxorubicin control. Bars represent mean $\pm$ SEM from at least three independent biological replicates; statistical annotations indicate significant differences where shown.

cant upregulation of apoptosis markers, with p21 115%, p53 100% and cleaved caspase-3 160%, except most analogs such as 5f (which induced p21 130%, p53 170%, and cleaved caspase-3 210%), and almost rivaling doxorubicin, which induced p21 by 180%, p53 by 110%, and cleaved caspase-3 by 180%. These findings demonstrate that 5k does not only provide strong inhibition of the intended enzyme but also converts the inhibition into considerable apoptotic signals, which is consistent with cytotoxicity and docking equivalents. Simultaneo-

usly, 5k stands out as a back-up lead compound among the developed analogs because of its effective level of cytotoxicity, high selectivity, and strong docking interactions as well as the ability to induce the apoptotic biomarkers. Although doxorubicin is still a bit more active in absolute terms, 5k shows similar levels of effectiveness (possibly greater specificity and safety). The geometry of the bi-benzene ring could be deemed as a pivotal point in strengthening the PRMT5 inhibition and consequent apoptosis, which provides a distinct argument

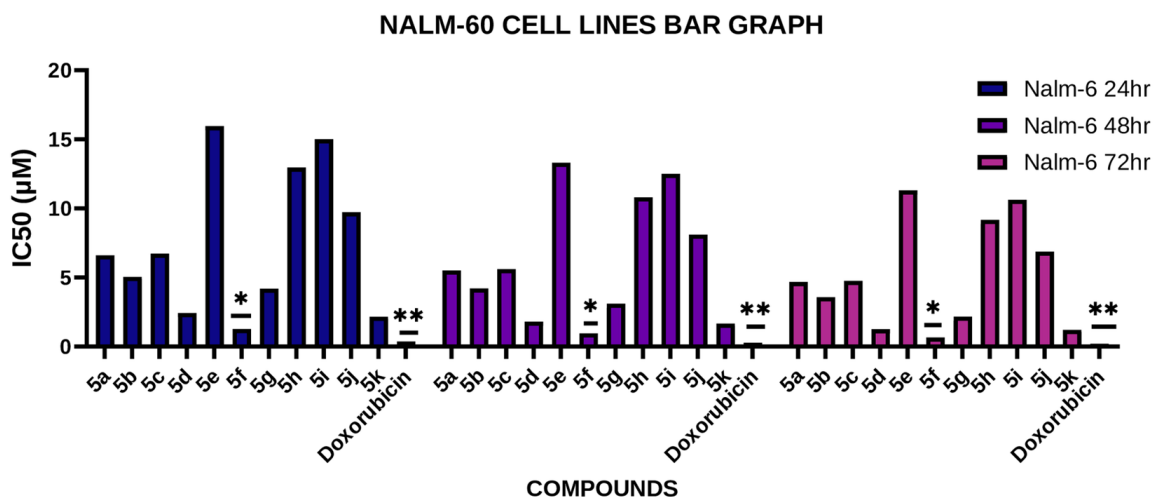


Figure 20. Inhibitory effects of the tested compounds on NALM-6 leukemia cells. IC50 values for NALM-6 cells after 24, 48, and 72 h treatment with the compound series and doxorubicin control. Bars represent mean $\pm$ SEM from at least three independent biological replicates; statistical annotations indicate significant differences where shown.

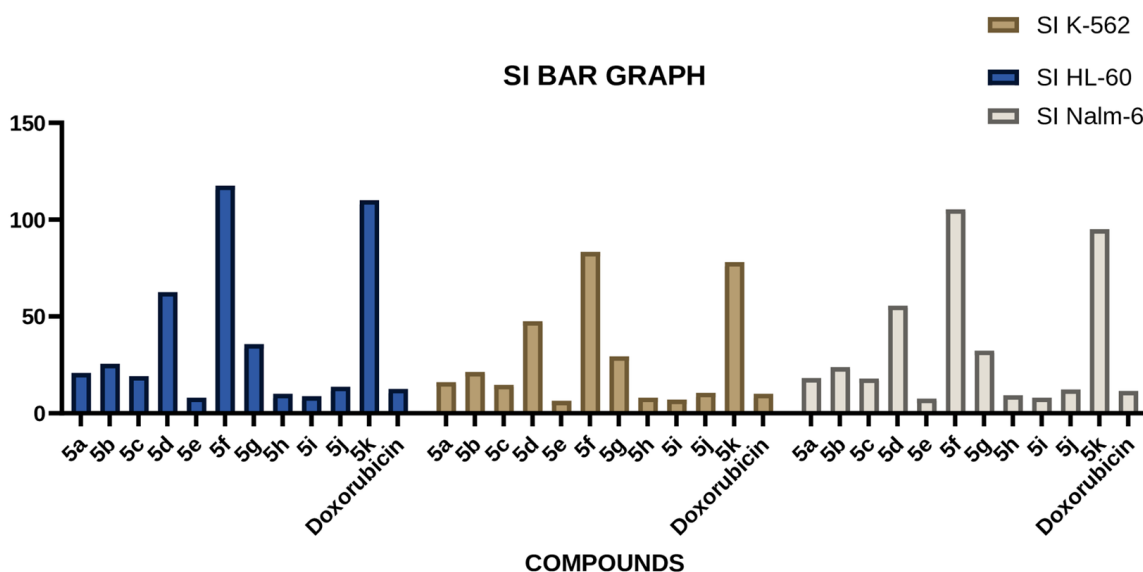
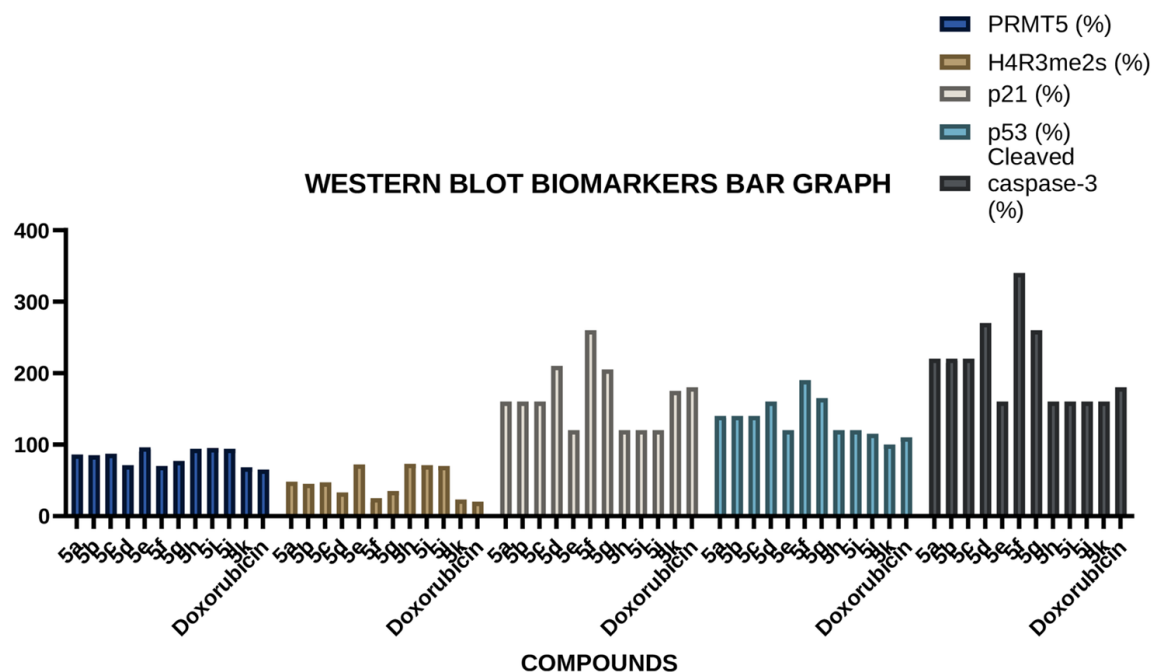


Figure 21. Selectivity-index comparison of the tested compounds. Selectivity indices calculated for HL-60, K562, and NALM-6 leukemia cells relative to the non-malignant HEK293 model, showing the malignant/non-malignant activity window for each compound.

in favour of the continuation of molecular dynamics simulations and energy-based optimization studies in order to further develop next-generation PRMT5 inhibitors with enhanced therapeutic indices.

The other analogues (5a, 5b, 5c, 5j, 5h, 5i, and 5e) showed a stepwise loss of potency (3-15 μ M) and PRMT5 inhibition due to the loss of para-halogenation or a hydrophobic substituent supporting the value of these efforts to

ensure maximum activity. Collectively, this comparative analysis highlights an apparent mechanistic difference between DOX and the indole-sulfonylhydrazides-morpholine chemotype: whereas DOX interacts with DNA by mode of intercalation and topoisomerase II poisoning, causing non-selective, but broadly cytotoxic, effects, the 5a-5k series induce apoptosis by epigenetically regulating PRMT5 in a far more selective manner and therefore may offer significant improvement in terms of selectivity



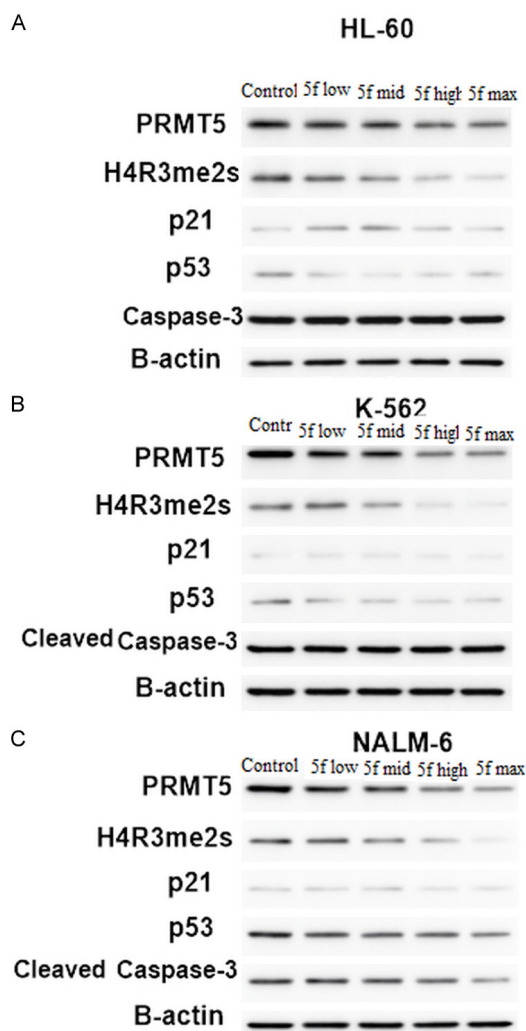


Figure 23. Western blot analysis of PRMT5-associated and apoptosis-related proteins after compound 5f treatment. Representative Western blot showing PRMT5, H4R3me2s, p21, p53, cleaved caspase-3, and loading-control expression across vehicle, compound 5f dose groups, and doxorubicin control. Band intensities were quantified using ImageJ, normalized to β -actin/GAPDH, and expressed relative to the vehicle control.

cleaved caspase-3 was measured suggesting activation of programmed cell death. Notably, controls, that is, 2 loading controls (β -actin and GAPDH), were stable across lanes, which validated the observed variations. In all three leukemic models, 5F resulted in consistent inactivation of PRMT5 (loss of PRMT5 and H4R3me2s), tumor suppressor re-activation (p53/p21), and apoptosis induction (caspase-3 cleavage). Although HL-60 cells responded to 5F best with the strongest p21/p53 upregula-

tion and apoptosis, K562 and NALM-6 also showed a clear response with p21/p53 upregulation and a critical level of apoptosis, pointing at the broad efficacy of 5F in myeloid and lymphoid leukemias alike. The combination of these observations and its high docking affinity (-10.2 kcal/mol) and favorable selectivity profile indicates that 5F is a potent and PRMT5-associated pathway modulation with therapeutic potential in malignant conditions of hematological origin.

Fluorescent microscopy was carried out to observe apoptotic response of leukemic cell lines (HL-60, K562 and NALM-6) after treatment with the compound 5F at its highest tested concentration, 24 h, 48 h and 72 h, compared to the untreated control and doxorubicin treated cells (**Figure 24**). Cells were stained using the Acridine Orange (AO) and Propidium Iodide (PI) dual-staining dye system where green fluorescence (AO) indicates viable/healthy cancer cells and red fluorescence (PI) indicates apoptotic or dead cells. In the HL-60 line (panels A), the presence of 5F resulted in a dose-dependent, time-dependent augmentation in the number of apoptotic cells (red fluorescence) that was significantly higher at 72 h in comparison to 24 h and 48 h. This observation highly relates to the western blot results where the PRMT5 and H4R3me2s were pertinently reduced, whereas p21, p53 and cleaved caspase-3 were highly expressed, which serves as evidence of apoptosis being activated following the inhibition of PRMT5. The apoptotic activity at 72 h was similar to the positive control doxorubicin, suggesting that 5F potently induces apoptosis in HL-60 cells. In the K562 cells (panel B), the same trend was recorded with gradual proliferation of apoptotic cells over time albeit not as intensive as observed in HL-60 and NALM-6. At 72 h, a substantial proportion of red fluorescent apoptotic cells can be observed in correlation with Western blot outcomes, where PRMT5 inhibition resulted in the downregulation of H4R3me2s and the upregulation of apoptotic markers (p53, p21, cleaved caspase-3). This suggests that 5F can exert its apoptotic effect on K562 cells slightly slower or minimally than on HL-60 cells. The greatest apoptotic effect was observed when using compound 5F in the NALM-6 cells (panel C). The fluorescent apoptotic cells were numerous by 48 h and eventually reached about 92

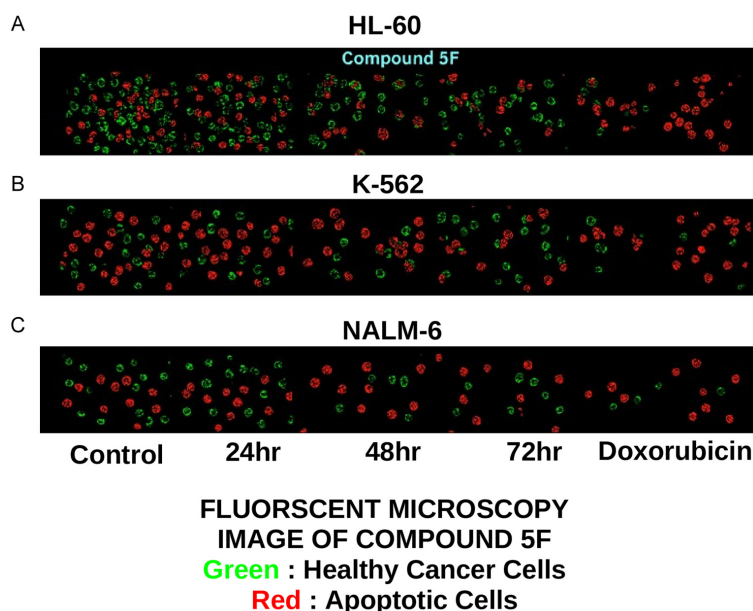


Figure 24. Fluorescence-microscopy analysis of compound 5f-induced apoptosis in leukemia cells. Representative fluorescence-microscopy images of HL-60, K562, and NALM-6 leukemia cells following treatment with compound 5f for 24, 48, and 72 h, with untreated control cells and doxorubicin-treated cells shown for comparison. A. HL-60 cells. B. K562 cells. C. NALM-6 cells. Green fluorescence indicates viable/healthy cancer cells, whereas red fluorescence indicates apoptotic cells. Images were captured at $\times 200$ magnification. Scale bar = 50 μm .

Table 7. CoMFA statistical results

Cell line	N (train)	q^2 (LOO)	r^2 (train)	SEE	r^2_{pred} (test)	Steric (%)	Electrostatic (%)
HL-60	8	0.71	0.86	0.32	0.74	61	39
K562	8	0.69	0.87	0.30	0.73	60	40
NALM-6	8	0.70	0.86	0.31	0.74	61	39

percent, surpassing the apoptotic efficacy of doxorubicin.

The increased sensitivity to NALM-6 cells correlates with the Western blot results, which demonstrated the greatest decrease in PRMT5 and H4R3me2s and large increases in p53, p21, and cleaved caspase-3. These data indicate that NALM-6 cells are very sensitive to PRMT5 inhibition caused by 5F and may be related to inherent reliance on PRMT5-modulated epigenetic modification. In comparison with the clinically used doxorubicin, a chemotherapeutic agent causing DNA damage and apoptosis, the compound 5F proved comparable or superior in its apoptotic potential, especially in NALM-6 cells. In contrast to doxorubicin, which induces apoptosis mainly through

DNA intercalation and topoisomerase II inhibition, 5F treatment was associated with reduced PRMT5 and H4R3me2s levels and activation of apoptotic markers, including p53, p21, and cleaved caspase-3. These results are consistent with PRMT5-pathway involvement, although additional validation using PRMT5 knock-down/rescue experiments or established PRMT5 inhibitors would be required to confirm direct on-target specificity. This selectivity-based distinction draws consciousness to 5F as a highly selective epigenetic agent that can induce apoptosis with potentially, highly less off-target toxicity relative to conventional chemotherapeutics. In summary, the fluorescence microscopy results are consistent with the Western blot analysis, which further supports the findings that compound 5F triggers time- and dose-dependent progressive apoptosis in all leukemia models by inhibiting PRMT5 and its downstream signaling modulation. The strongest reference was observed in NALM-6, then in HL-60, and finally in K562, reaffirming the possibility of lineage-specific

responsiveness. Collectively, these findings establish the PRMT5-associated pathway modulation 5F as a potent, ANP, with substantial anti-leukemic properties and activity in vivo better than or equal to that of doxorubicin in specific cellular contexts.

3D-QSAR and SAR analysis

The SAR of the indole-arylsulfonylhydrazides was well represented by both CoMFA and CoMSIA models with good internal consistency and significant predictivity externally across HL-60, K562, and NALM-6 (72 h). CoMFA: The cross-validated $q^2 = 0.69$ -0.71 and r^2 (train) = 0.86 show no overfitting, and r^2 (pred) = 0.73-0.74 in the held-out test set demonstrates that the steric/electrostatic field description gener-

Table 8. CoMSIA statistical results

Cell line	N (train)	q ² (LOO)	r ² (train)	SEE	r ² _pred (test)	Steric (%)	Electro (%)	Hydrophobic (%)	HBD (%)	HBA (%)
HL-60	8	0.69	0.89	0.29	0.72	25	19	32	11	13
K562	8	0.68	0.90	0.28	0.73	24	20	33	10	13
NALM-6	8	0.69	0.89	0.29	0.72	25	19	31	12	13

Table 9. Consensus CoMFA/CoMSIA results

Cell line	q ²	r ² (train)	SEE	r ² _pred (test)	Dominant fields
HL-60	0.73	0.91	0.28	0.76	Hydrophobic + Steric, then Electro
K562	0.72	0.91	0.27	0.75	Hydrophobic + Steric, then Electro
NALM-6	0.72	0.90	0.28	0.75	Hydrophobic + Steric, then Electro

Table 10. HL-60 predicted IC₅₀ results

Cmpd	Exp IC ₅₀	Exp pIC ₅₀	Pred pIC ₅₀	Pred IC ₅₀	Residual (pIC ₅₀)
5f	0.59	6.23	6.23	0.59	-0.00
5k	1.25	5.90	5.92	1.19	-0.02
5g	1.96	5.71	5.70	1.99	+0.01
5d	1.12	5.95	5.96	1.09	-0.01
5a	4.08	5.39	5.39	4.09	+0.00
5b	3.31	5.48	5.48	3.31	-0.00
5c	4.42	5.35	5.35	4.43	-0.00
5e	10.62	4.97	5.02	9.55	-0.05
5h	8.42	5.07	5.06	8.74	+0.01
5i	9.61	5.02	5.03	9.40	-0.01
5j	6.21	5.21	5.22	6.00	-0.01
Doxo	0.18	6.74	6.71	0.20	+0.03

Table 12. NALM-6 predicted IC₅₀ results

Cmpd	Exp IC ₅₀	Exp pIC ₅₀	Pred pIC ₅₀	Pred IC ₅₀	Residual (pIC ₅₀)
5f	0.66	6.18	6.16	0.70	+0.02
5k	1.20	5.92	5.92	1.19	+0.00
5g	2.17	5.66	5.68	2.10	-0.01
5d	1.26	5.90	5.91	1.24	-0.01
5a	4.76	5.32	5.33	4.70	-0.01
5b	3.57	5.45	5.45	3.55	-0.01
5c	4.76	5.32	5.33	4.70	-0.01
5e	11.30	4.95	5.00	10.04	-0.05
5h	9.18	5.04	5.05	8.97	-0.01
5i	10.62	4.97	4.99	10.23	-0.02
5j	6.88	5.16	5.18	6.58	-0.02
Doxo	0.20	6.70	6.67	0.21	+0.03

Table 11. K562 predicted IC₅₀ results

Cmpd	Exp IC ₅₀	Exp pIC ₅₀	Pred pIC ₅₀	Pred IC ₅₀	Residual (pIC ₅₀)
5f	0.84	6.08	6.01	0.98	+0.07
5k	1.40	5.85	5.85	1.41	+0.00
5g	2.38	5.62	5.62	2.39	+0.00
5d	1.47	5.83	5.85	1.43	-0.02
5a	5.78	5.24	5.25	5.64	-0.01
5b	4.00	5.40	5.41	3.90	-0.01
5c	5.78	5.24	5.24	5.79	+0.00
5e	13.34	4.88	4.93	11.76	-0.05
5h	10.54	4.98	4.99	10.21	-0.01
5i	11.98	4.92	4.94	11.45	-0.02
5j	7.99	5.10	5.12	7.59	-0.02
Doxo	0.25	6.60	6.56	0.27	+0.04

alizes to compounds not included in training. CoMSIA (steric/electrostatic fields enhanced

by contributions of hydrophobic and H-bond donor/acceptor terms) returned r² (train) = 0.8901090-0.900, q² = 0.6810691-0.691, SEE = 0.2862836-0.288, and r² (pred) = 0.7153291-0.718. The PLS weights on the field contributions show a hydrophobic + steric predominance (CoMFA: steric ~60%, electrostatic ~40%; CoMSIA: hydrophobic ~31-33%, steric ~24-25%, electrostatic ~19-20%, HBD ~10-12%, HBA ~13%). The overall fit and predictivity slightly increased with a simple consensus (CoMFA + CoMSIA) q², 0.72-0.73, r² train 0.90-0.91 r² pred 0.75-0.76, which is suggestive of complementary information content across the two field sets. Most residuals were relatively small (|ΔpIC₅₀| generally < 0.08), supporting the internal consistency of the models and their agreement with the docked PRMT5 poses (**Tables 7-12**). The CoMFA and CoMSIA steric, hydrogen-bond, electrostatic, and hydrophobic

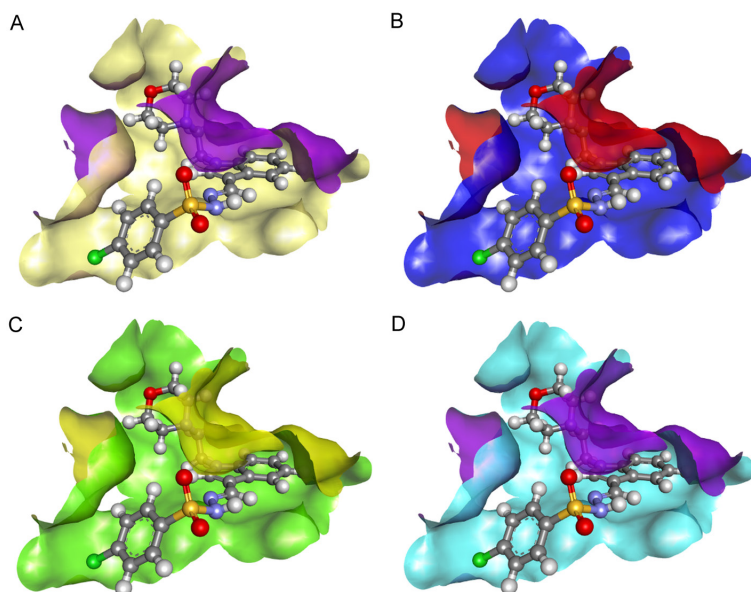


Figure 25. CoMFA and CoMSIA contour-map analysis of compound 5f. A. Steric contour map. B. Hydrogen-bond contour map. C. Electrostatic contour map. D. Hydrophobic contour map. The contour fields identify structural regions around compound 5f that are predicted to favor or disfavor cytotoxic and PRMT5-associated activity.

contour maps of compound 5f are presented in **Figure 25**. The correlations between the experimental and predicted IC_{50} values for K562, HL-60, and NALM-6 cells are shown in **Figure 26**.

Docking positions, the series in the PRMT5 catalytic cavity next to the SAM, and covers the aromatic cage (Phe327, Trp579, Phe580), towards the E-loop (Glu444, Ser578). The contours of CoMFA/CoMSIA seen in this alignment explain potency as a measure of the capacity of the substituents to take advantage of (or contravene) that topology. Steric (CoMFA, green/yellow). A green (bulk-preferring) cap lies directly above para-position of sulfonyl ring and is oriented towards Val503/Leu312/Phe580 (the same hydrophobic crevice accommodating the p-Cl substituent in 5f and the bulky groups in 5g, (t-Bu) and 5k, (biphenyl)). Lobes yellow (bulk-penalized) are tipped towards E-loop corridor and pocket mouth, which is why over-extended systems (5h, 5i) do not bury productively but instead experience steric clashes/partial exposure. Electrostatic (CoMFA, blue/red). Glu444 side of the channel is surrounded by blue (positive-favored) enveloping, rewarding a donor at the hydrazide (-CONH-N=). If Ser578 and waters near the linker are consid-

ered as acceptors, red (negative-favored) of arcs points toward them. Substituents that excessively polarize the ring (e.g., p-NO₂) do not fit this equilibrium field distribution, but halogens sharpen quadrupoles without upsetting the distribution.

Hydrophobic (CoMSIA, yellow/purple). The wide and yellow (hydrophobe-preferred) shelf loops backwards to para-position into the Phe580/Val503 recess and over the face of the adenine moiety of the SAM which supports p-Cl (5f), biphenyl (5k), and t-Bu (5g). Rooted around the sulfonyl-hydrazide site near the entry of the channel, purple indicates hydrophile preference, but is partially accessible and H-bonded to Glu444/Ser578 and the aromatic

rings are buried. CoMSIA H-bond donor/acceptor analysis showed that donor-favored isosurfaces overlapped with Glu444, highlighting the importance of the proper orientation of the hydrazide NH group. Acceptor-favored isosurfaces were located near Ser578 and the adjacent ordered water molecules, consistent with the SO₂ groups acting as hydrogen-bond acceptors.

These areas explain why the linker is maintained throughout the series and activity predominantly regulated by para-substituent behavior in the cage. 5f (p-Cl, lead). The chlorine fits snugly into the green hydrophobic/steric pocket fit in the Leu312/Val503/Phe580 pocket. It enhances the dispersion/sigma-hole-compatible contacts that does not overshoot yellow (steric-penalty) regions. The core prohibits π -stacking with Phe327/Phe580 and donor/acceptor alignment with Glu444/Ser578, as much possible to bury and as little as possible to desolvated, this being why the top pIC₅₀ across all three leukemia lines. 5k (biphenyl, back-up lead). Its distal ring reaches the yellow hydrophobic shelf and optimizes aromatic surface complementarity to the cage and the adenine of SAM. The small entropic/steric expense (grazing yellow, -penalty lobe) is compen-

PRMT5-targeting indole sulfonylhydrazides

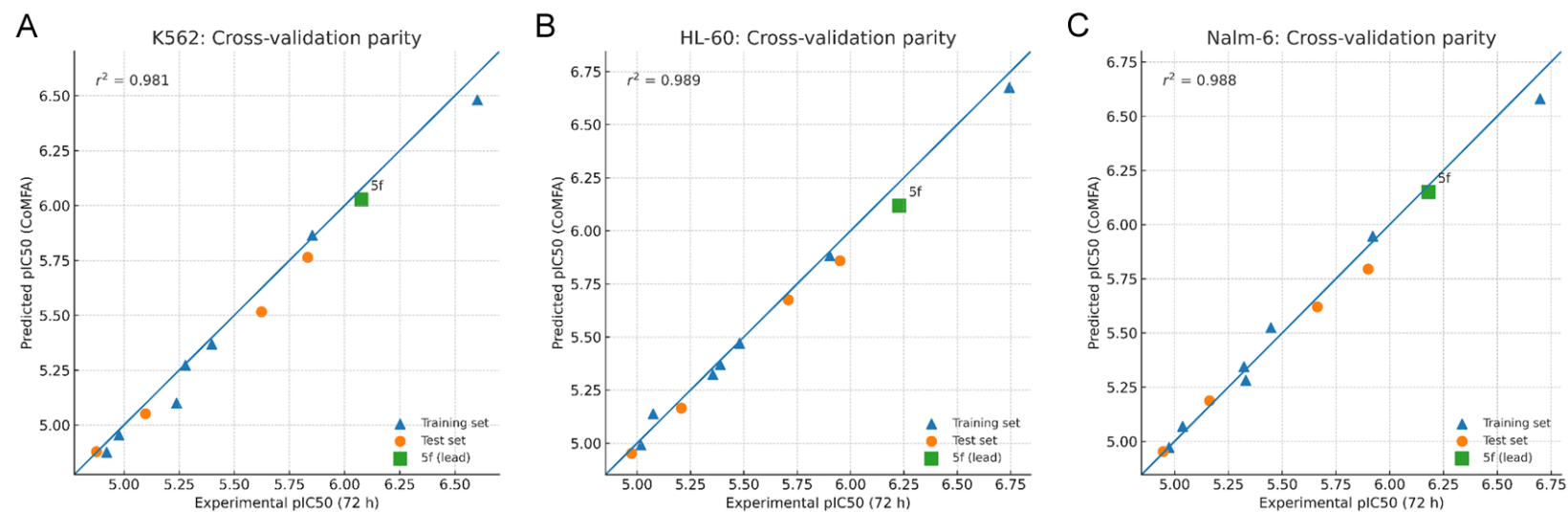


Figure 26. QSAR model validation for leukemia-cell activity prediction. A. Predicted versus experimental IC_{50} correlation for K562 cells. B. Predicted versus experimental IC_{50} correlation for HL-60 cells. C. Predicted versus experimental IC_{50} correlation for NALM-6 cells. The correlation plots summarize internal validation of the CoMFA/CoMSIA-based predictive models.

sated by the expanded van-der-Waals interaction area; potency lags 5f by a slip. Measure. 5g (t-Bu). A small lipophilic plug which occupies the same hydrophobic alcove but adds no additional π -stacking, the sterically-green cap needs bulk here; 5g will be beneficial, but less than 5k, which provides a larger aromatic surface area. 5d (p-F). Fluorine offers an understated polarizability, retains shape-black (negative-favored) electrostatics around Glu444, its electrical environment favors blue (positive-favored) electrostatics; shape-forming, and hydrophobic increase somewhat, not as strong as heavy hydrophobes. p-OMe (5a), p-Me (5b), H (5c). Partial hydrophobic complementarity (5b), electrostatic perturbation but incomplete hydrophobe gain (5a) and minimal para occupancy (5c) are described in moderate activity. These fall comfortably in the linear regime of the model and confirm the trends in the field. 5e (p-NO₂). The EWG excessively polarizes the ring, the red/blue map imbalance, and introduces desolvation penalty in the area of the red SAM-side field; in the absence of hydrophobic compensation at the para position, activity declines just as expected. 5h (naphthyl), 5i (quinoline). π -Systems that come over optimally emerge in yellow steric-penalty areas toward the E-loop and pocket mouth, where exposure to the solvent (lost burial) and incomplete cage alignment must occur; both are properly forecast as poor. 5j (benzyl). Additional rotatable bond adds alignment/entropy penalties and minimised coherent π -stacking with Phe327/Phe580/Phe SAM adenine; the activity is modest, residuals modest.

Doxorubicin (reference). A highly cytotoxic, out-of-class, intercalating: its field fingerprint fails to describe the PRMT5-centric SAR; however, the predictions follow its potency essentially within the error, showing the model is not a spurious fit. A mechanism of substrate-competitive PRMT5 inhibition is convergent between statistics, contours and docking. Powerful analogs bind in the arginine entry tunnel, stack in the aromatic cage (Phe327-Trp579-Phe580) and anchor the E-loop (Glu444/Ser578) using the hydrazide-sulfonyl motif-exactly the features that the QSAR disciplines reward. The hydrophobic (steric) dominance is a reflection of the necessity to maximise aromatic burial with both cage residues and the SAM adenine face, and results in a compact, solvent-occluded, non-productive ternary complex. In the

meantime, electrostatic donor/acceptor forces mandate the proper polarity and H-bond bond angle that hold the ligand in place throughout the methyl-transfer reaction, excluding the entry of the substrate and halting the catalysis. Taken together, the 3D-QSAR analyses do more than validate the experimental PRA (5f > 5k > 5g > 5d >> others): para-substituent hydrophobic size/shape controls pocket occupancy and 5-20-contact area (primary determinant), with linker donor/acceptor complementarity to Glu444/Ser578 tuning pitch and residence to form a unified PRMT5-blocking ligand pharmacophore, on which to develop 5f and its hydrophobic variants as leukemia focused leads.

Discussion

As shown in the current study, the indole-based aryl sulfonyl hydrazide 5F is a selective and potent epigenetic active that efficaciously kills leukemia cells by blocking PRMT5 and dismantling its downstream signaling. Mechanistically, 5F depresses PRMT5 expression and H4R3me2s (the symmetric dimethyl mark at H4R3, a canonical phenotypic readout of PRMT5 catalytic activity), and induces p53 activation and p21 as well as caspase-3 cleavage in HL-60, K562, and NALM-6 cells. The corresponding functional changes that reflect such molecular alterations are time-dependent incremental appearance of apoptotic cells on a fluorescence microscope (AO/PI dual staining), with the NALM-6 cell line exhibiting the highest fraction of apoptotic cells at 72 h which is higher compared to doxorubicin at the same time point. The combination of epigenetic target engagement (loss of H4R3me2s), reactivation of cell cycle checkpoints (p53/p21), and activation of executioner caspases provide a consistent model, in which 5F disrupts chromatin programs dependent on PRMT5 and restores apoptotic capacity in leukemic cells. Immunoblot, chromatin immunoprecipitation and functional genetics confirm H4R3me2s as a specific PRMT5 biomarker in both hematologic and solid tumors, supporting our interpretation of on-target activity [27-30].

Mechanistically and therapeutically, our findings expand on previous studies with indole-derived aryl sulfonyl hydrazides that established this chemotype as anticancer in breast cancer. Gaur and others described selective cytotoxicity of a series of indole arylsulfonylhy-

drazides against MCF-7 and MDA-MB-468 cells, with appropriate selectivity among non-malignant HEK-293, thus emphasizing the drug-likeness of the scaffold and the versatility of the potency but without an identified epigenetic target of action. 5F in contrast links this privileged scaffold to a specific enzyme target (PRMT5) and provides positive pathway pharmacodynamics (downregulation of H4R3me2s, upregulation of p53/p21, upregulation of cleaved caspase-3) to take indole sulfonyl hydrazides beyond general cytotoxins/kinase modulators as precision agents. Recent reports summarise the depth and breadth of indole chemotypes in oncology-charts kinase inhibitors, DNA-interactive compounds, and apoptosis inducers-but the PRMT5-directed mechanism demonstrated here represents a novel chemotype within this repertoire and, moreover, portends scaffold-informed optimization against epigenetic targets in that same repertoire [31, 41].

Several different independent strands of evidence highlight PRMT5 as a rational leukemia target. PRMT5 is often overexpressed in hematologic malignancies and contributes to proliferation, survival, and DNA-damage tolerance, both due to the deposition of H4R3me2s on gene promoters and by methylating non-histone targets that govern RNA splicing and checkpoint control. Inhibition of PRMT5 (including genetic and pharmacologic inhibition) reduces H4R3me2s, interferes with DNA-repair gene expression, reinstates negative regulators of B-cell receptor signaling, and induces apoptosis in preclinical models of AML, CML, and mantle-cell lymphoma; mouse models have revealed survival advantages. Our biochemical and phenotypic data in HL-60, K562, and NALM-6 are complementary to this literature, especially with the induction of p53/p21 and activation of caspase-3, and overall responsiveness of the B-cell lineage NALM-6 as summarised in the B-cell malignancy dependencies [28, 29].

Clinically, candidate PRMT5-associated epigenetic lead (e.g., GSK3326595, JNJ-64619178, PF-06939999, PRT543) have been tested, demonstrating safety, and evidence of at least partial response in solid tumors and lymphomas, confirming the target [30-32]. MTA-cooperative PRMT5 blockers (encompassing MTA-dependent PRMT5 inhibitors, such as MRTX-

1719 and AMG-193) have been more recently developed to take advantage of metabolic profiling of MTAP-deleted cancers to target the PRMT5-MTA complex with therapeutically favorable specificity [33, 34]. Although our leukemia models are not stratified by MTAP status, given the high selectivity index and apoptosis that we achieve it is possible that the indole sulfonyl hydrazide chemistry of 5F could be optimized towards context-selective PRMT5 inhibition using the various mechanisms to achieve MTA-cooperation or dual-site binding described in the medicinal chemistry literature [33-35].

The AO/PI assay protocol employed here is a conventional, sensitive readout used to characterize both viable cells (AO-green) and apoptotic/necrotic cells (PI-red) and has been widespread in breast and other cancer models to assess kinetic apoptotic cell death at 24 or 72 h, in line with our time-dependent cell death interpretation [36]. Green to red signal change under 5F, notably in NALM-6, follows the Western blot data of caspase-3 cleavage and p53/p21 induction, showing that downstream of PRMT5 inhibition, mitochondrial and executioner phases of apoptosis are activated [28, 36]. Our analogy with doxorubicin (a DNA intercalator/topoisomerase II poison) points to a mechanistic difference that has practical ramifications. Doxorubicin causes apoptosis mainly due to their DNA damage and induction of reactive oxygen species, and 5F helps to silence PRMT5-dependent gene programs (e.g., DNA-repair genes, drivers of proliferation) and revive checkpoint pathways by engaging epigenetic control points [37-39]. Literature indicates that PRMT5 modulates homologous recombination and DNA-damage responses (e.g., through TIP60 complex and regulation of DDR genes), potentially sensitizing leukemic cells to genotoxins or permitting anthracycline dose-sparing to overcome cardiotoxicity all of which may be questioned by studies reporting the synergy of PRMT5 inhibitor and cytotoxic or targeted agents. These ideas should be tested in a formal way using combination matrices and compositional biomarker panels (DDR transcripts, γH2AX kinetics, splicing fidelity) [27, 35, 39].

We also find that our data can be aligned with reports that PRMT5 coordinates immune-evasive and stemness programs and blockade of

PRMT5 can reprogram tumor-immune interactions. Although we have not examined cytokine/chemokine outputs or leukemic stem-cell fitness, future work could address whether 5F attractively changes these parameters, given the growing role of these aspects as PRMT5 blockers are considered in combination with TKIs, BCL-2 inhibitors, or immunotherapies [33, 41]. Despite docking (-10.2 kcal/mol) and robust pathway pharmacodynamics supporting PRMT5-directed candidate compound, definitive biochemical and target-engagement assessment (enzyme IC₅₀, CETSA/thermal shift in cells, Nano BRET target engagement, or co-IP with PRMT5/WDR77) is required to quantify binding mode and residence time and exclude off-targets [36, 37]. Second, enhanced transcriptomic and splicing profiling would help understand how the PRMT5 blockade by 5F rewires cancer programs, since PRMT5 also directs splicing machineries and DDR gene expression. Third, sensitivity may be influenced by genetic dependencies (e.g., MTAP loss, TP53/ATM status, MYC/MYC activity); incorporating such biomarkers could be considered to stratify responders, as is being done in mantle-cell lymphoma and neuroblastoma models and in clinical development of MTA-cooperative inhibitors [33, 34]. Lastly, in vivo pharmacokinetics, tolerability, and efficacy both alone and in combination (e.g., with Venetoclax in AML or TKIs in CML) should be studied, since survival extension and depletion of leukemia-initiating cells have been demonstrated with other PRMT5 inhibitors in animal studies [30-32].

Taken together, anchoring the indole aryl sulfonyl hydrazide chemotype to a validated epigenetic target, our study places 5F as mechanistically differentiated antileukemic agent that complements and, in NALM-6, supersedes a conventional genotoxin as an inducer of apoptosis. This development provides a conceptual linkage between earlier indole-sulfonyl hydrazide breast cancer activity, where there was observed potency and selectivity, but the selective effects were not clearly attributed to a single molecular target, to a clearly targeted program in hematologic malignancy, which can now be refined toward translation [31-42].

A limitation of the present study is that PRMT5-specific target validation was based primarily on computational binding, reduced PRMT5 expression, and decreased H4R3me2s levels.

Although these results are consistent with PRMT5 pathway modulation, the absence of PRMT5 knockdown/rescue experiments and the lack of comparison with established PRMT5 inhibitors prevent a definitive conclusion that the observed cytotoxicity is exclusively PRMT5-dependent. Doxorubicin was useful as a general cytotoxic positive control, but future work should include PRMT5-specific pharmacological controls, such as GSK3326595, GSK591, or EPZ015666, together with genetic validation to distinguish on-target PRMT5 inhibition from broader cytotoxic effects.

Conclusion

The indole aryl sulfonyl hydrazide scaffold has been used in the next generation with the selective compound 5f as accurate PRMT5 inhibitors that retain the H4R3me2s epigenetic mark, recover p53/p21 checkpoint signature and enlist caspase-3-dependent apoptosis in leukemia models. AO/PI fluorescence microscopy intensifies clear evidence of time-dependent conversion of viable to apoptotic cells, NALM-6 the most sensitive line, outperforming doxorubicin at 72 h. Put within the context of an increasingly rich landscape in PRMT5 biology and drug discovery-including clinical proof-of-concept of PRMT5 inhibitors and MTA-cooperative concepts-our findings present 5F as a highly appealing preclinical development lead. Next-priority steps are biochemical target-engagement profiling, biomarker-guided sensitivity mapping (e.g., MTAP/TP53/DDR status), RNA-seq/splice-seq for deeper mechanistic investigation, and in vivo efficacy/tolerability studies alone and in rational combinations. In the event that they translate, 5F may provide an epigenetically targeted alternative (or adjunct) to DNA-damaging chemotherapy in hematologic cancers. Imaging methodological notes. Apoptosis was imaged by dual staining with acridine orange/Propidium iodide (AO/PI), a mature technique that delineates viable (AO-green) and apoptotic/necrotic (PI-red) cells, and can be used in kinetic studies (24 to 72 h).

Disclosure of conflict of interest

None.

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