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**Bis-indolylmethane Derivatives as Anti-Apoptotic Bcl-2
Inhibitors: Synthesis, In Vitro Cytotoxicity, and MM-PBSA
Thermodynamic Validation**

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Abstract

Indole has shown remarkable anticancer activities, which resulted in the exploration of compounds containing the indole unit. In the present work, bis-indolylmethanes (**1-8**) bearing two indole molecules were synthesized in a one-pot reaction, and their antiproliferative activity against HL-60 cells was evaluated. Our findings suggest that all synthesized bis-indolylmethanes moderately inhibited the proliferation of HL-60 cells at a concentration of 50 μ M, except for ligands **4** and **7**, which have slightly higher activity and were selected as the best ligands for continuation of further biological and computational study. Ligands **4** bearing a 3,4,5-trimethoxy moiety and Ligand **7** substituted with a *para*-acetamido group, exhibited the highest inhibitory potency, yielding IC₅₀ values of 44.4 and 62.63 μ M, respectively, at a final concentration of 100 μ M. Morphological observation revealed numerous disintegrated nuclei, which signified apoptosis. In addition, UV-vis titration with ctDNA indicated the anticancer properties of bis-indolylmethanes, which have been ascribed to their capability in binding to DNA and leading to cancer cell death, with the binding constant value (K_b) for ligands **4** and **7** being $6.02 \times 10^4 \text{ M}^{-1}$ and $1.04 \times 10^4 \text{ M}^{-1}$ respectively. It was also found that both ligands (**4** and **7**) showed a non-carcinogenic behavior upon analysis using a DNA biosensor. Molecular docking study displayed that bis-indolylmethanes **4** and **7** stably bound to the anti-apoptotic protein (Bcl-2) binding hot spot (P2 and P4) via interactions formed with Arg143, Asp108, Phe101, Phe109, Met112, Glu149, Phe150, Leu134, Glu133, Ala146, and Val153. These initial binding poses were dynamically validated by 1000 ns molecular dynamics (MD) simulations and MM-PBSA calculations, which confirmed highly stable and thermodynamically favorable complexes. Collectively, our works highlight the potential of undiscovered bis-indolylmethane derivatives as antitumor agents.

1. Introduction

Apoptosis is a fundamental biological phenomenon in which cell death is genetically and biochemically regulated through the activation of specific signaling pathways, leading to an orderly cellular self-destruction program under physiological and pathological conditions (Mustafa et al., 2024). Since apoptosis is a healthy and normal process of cell death, cancer would develop if the balance between cell apoptosis and cell division goes astray. Bcl-2 family proteins are known for their regulation

of apoptosis, which consists of members (sub-families) that either promote or inhibit apoptosis (Atyabi, 2017). Anti-apoptotic BCL-2 family proteins, including BCL-2 and BCL-xL, promote cell survival by suppressing apoptosis. Their ectopic overexpression in human leukemia cells can increase resistance to diverse apoptosis-inducing agents and contribute to tumor progression and therapeutic resistance (Silva et al., 2026).

Indole nucleus is widely recognized as a privileged scaffold in medicinal chemistry because of its structural versatility and broad biological relevance, including promising anticancer activity. It is present in several endogenous and naturally occurring bioactive molecules, such as tryptophan, tryptamine, serotonin, and gramine (Drăgoi et al., 2026; Yakkala et al., 2026). The ubiquity of this heterocycle stems from its unique electronic and structural properties. Specifically, the indole ring features a planar, electron-rich heteroaromatic system capable of participating in robust π - π stacking and cation- π interactions within receptor binding pockets. Furthermore, the pyrrolic N-H moiety of the indole scaffold can act as a hydrogen-bond donor, facilitating important noncovalent interactions with target proteins (Drăgoi et al., 2026). Additionally, a number of indole derivatives that have been produced naturally or synthetically, endowed with varying structural complexities, have become the main subject and are being explored profusely (Janeiro & Marques, 2024). However, there are synthesized indoles that have not yet been studied with regard to their medicinal, pharmaceutical, or chemical behaviors. Consequently, exploiting the indole architecture provides a strategic rationale for designing high-affinity ligands, as these intrinsic physicochemical properties optimize biomolecular recognition and binding kinetics.

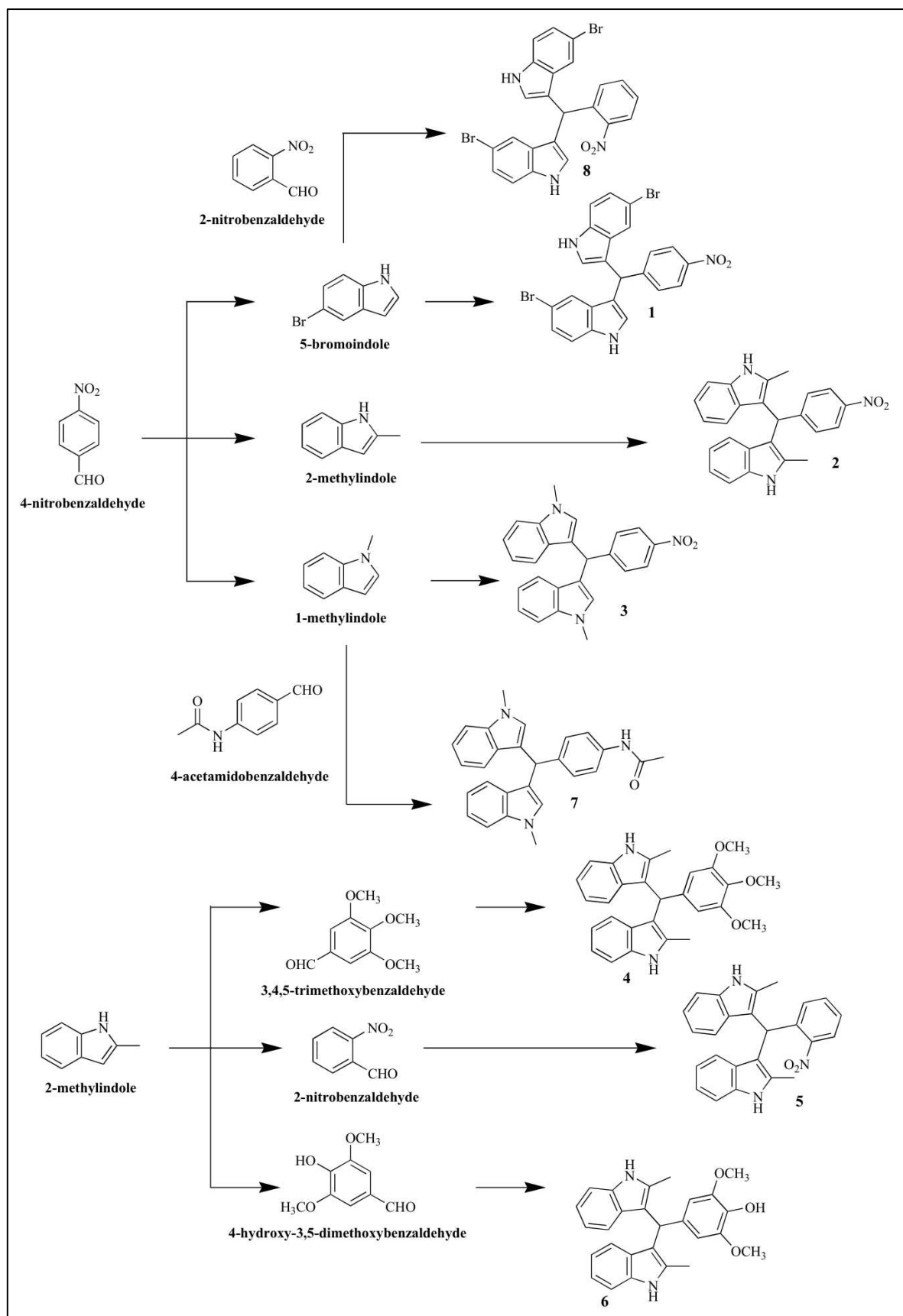
In this study, we synthesized eight bis-indole derivatives (bis-indolylmethanes) (1-8) whose activities have not been discovered as anticancer agents. Given the broad biological potential of indole-based scaffolds, the eight synthesized compounds may be promising candidates for further evaluation as BCL-2 inhibitors. All the bis-indolylmethanes were evaluated against the human leukemia HL-60 cells for their cytotoxic effect and apoptosis behavior. While *in vitro* assays provide crucial cytotoxicity

and mechanistic profiles, *in silico* approaches offer deep structural insights into receptor-ligand binding kinetics and stability. Therefore, molecular docking simulations were first conducted to identify the precise binding modes of the most potent synthesized bis-indolylmethanes within the Bcl-2 hydrophobic hotspots (P2 and P4). To rigorously validate the thermodynamic stability and conformational dynamics of these initial static poses, the docked complexes were subsequently subjected to 1000 ns (1 μ s) molecular dynamics (MD) simulations. Finally, Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) calculations were performed to quantify the binding free energies, ultimately providing a multidisciplinary framework to establish these novel indole derivatives as thermodynamically viable Bcl-2 inhibitors.

2. Results and discussion

2.1 Chemistry

The preparation of ligands is illustrated in Scheme 1. Two core structural elements required for synthesizing the target compounds **1-8** (Figure 1) are substituted benzaldehydes and substituted indoles. A convenient method involving one pot reaction was used as reported by (Fauzi et al., 2024; Misral et al., 2025) upon some modifications in order to achieve good percentage of yields.



Scheme 1 Synthesis of ligands **1–8** using a one-pot reaction. Condition and reagents: 18 h reflux, glacial acetic acid.

The commercially available material 4-nitrobenzaldehyde was used together with three different indole derivatives named 5-bromoindole, 2-methylindole, and 1-methylindole in order to yield compounds **1-3**. For production of ligands **4-6**, 2-methyl indole was reacted with 3,4,5-trimethoxy benzaldehyde, 2-nitrobenzaldehyde and 3,5-dimethoxy-4-hydroxy benzaldehyde correspondingly as shown in Scheme 1. Ligand **7** used 4-acetamidobenzaldehyde, which reacted with 1-methyl indole, while 2-nitrobenzaldehyde and 5-bromo indole were used to produce ligand **8**. All ligands were produced by acid-catalyzed, indole-aldehyde condensation reaction in a two-to-one equivalent ratio. These indoles experienced electrophilic substitution reaction with benzaldehydes in the presence of acetic acid for 18 hours of reflux to yield bis-indolylmethanes **1-8**. Acetic acid was added to the mixture in adequate amount for conjugation process to be happened.

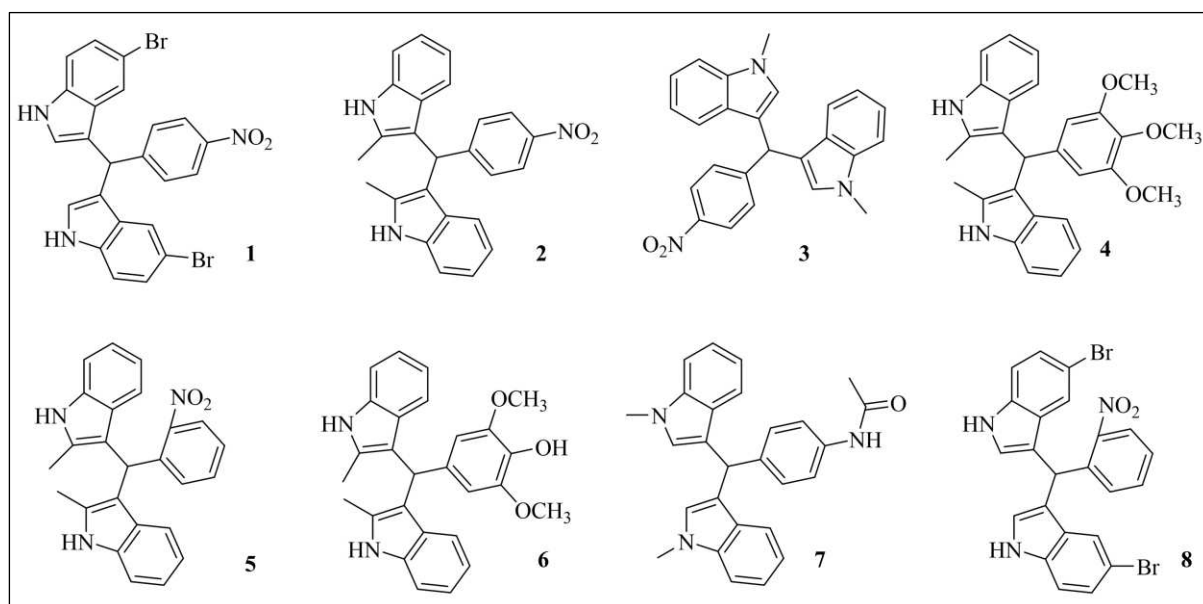
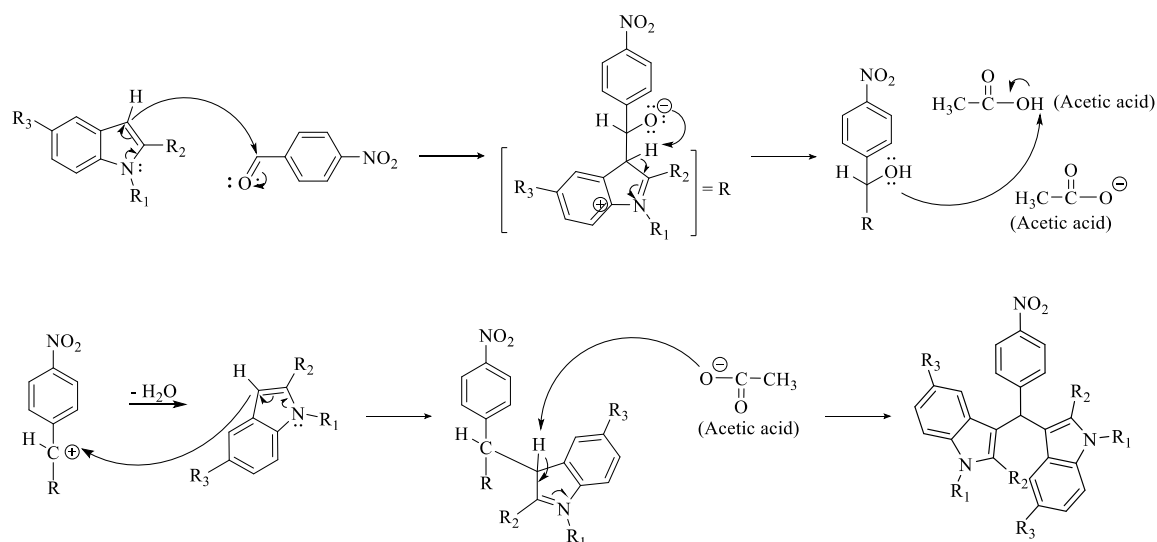


Figure 1 Structure of bis-indolylmethanes **1-8**.

All the synthesized compounds were purified by the flash chromatography technique and well characterized by spectroscopic techniques of NMR and FT-IR, which were in full accordance with the depicted structures. Generally, the ^1H NMR spectra of all compounds except for compound **3**

demonstrated the presence of secondary amine proton (N-H) as a singlet around 10-11 ppm. Both aromatic and pyrrole protons are resonated between 6.65 and 7.85 ppm. The indole proton located near bromine and nitrogen was determined to be slightly more deshielded compared to other aromatic protons on the indolic system with regard to the electron-withdrawing effect induced by both the amine and the bromine atom. For ligands that have substituents such as nitro and acetamido, the neighboring protons will be deshielded as influenced by their electronegativity, which depletes the electron density at the nucleus, deshielding the nucleus and hence resulting in a larger chemical shift. A singlet peak representing the CH group was observed around 6 ppm, which indirectly confirms the formation of the bis-indolylmethane compound. New singlets were detected in the spectrum of **4** and **6** around 3.53-3.65 ppm, with an integral of 3 protons, each of which belongs to the methoxy substituents. The furthest upfield peak belongs to the methyl group, which is located in the shielded area. The ^{13}C NMR spectra showed that the most downfield carbon is the one attached to electron-withdrawing groups such as bromine atom, nitro, and acetamido group, which the carbon peak exhibited between 146.14-168.57 ppm. The aromatic carbons appeared in the range of 110-140 ppm, and the methoxy substituent's carbon for ligands **4** and **6** showed an upfield shift at 56.24-60.63 ppm due to the shielding effect of the electron-donating methoxy groups. Almost similar patterns were elucidated in ^1H and ^{13}C NMR spectra for the rest of the compounds.

The FT-IR spectra exhibited a medium band at 3307-3423 cm^{-1} , representing the stretching frequencies of the secondary amine (NH) for all compounds except for compound **3**. Stretching of C=C in aromatic environment can be observed via a strong band around 1500 cm^{-1} . Both ligands **4** and **6** have a strong and distinct band at 1110-1117 cm^{-1} for the C-O stretch in addition to a broad OH band for ligand **6**. The FT-IR spectrum of ligand **7** has a strong band at 1668 cm^{-1} , which shows a typical stretching for the carbonyl of the acetamido group. For ligands having C-N and NO_2 groups, a strong band can be observed at 1070-1371 cm^{-1} .



Scheme 2 Proposed mechanism for bis-indolylmethane derivatives using ligand **1-3** as the example.

A plausible mechanism for the synthesis of bis-indolylmethanes is proposed in Scheme 2. The acid-catalyzed reaction occurs via condensation of substituted indoles and aldehyde derivatives. The initial step is commenced via nucleophilic attack from the first indole moiety onto the carbonyl group of the aldehyde, resulting in the formation of a negative formal charge of oxygen as an intermediate. In the next step, as acetic acid is a protic solvent that enables the proton donation, it is liable to be attacked by the lone pair on oxygen, which later undergoes elimination of water to form a carbocation with high electrophilicity. Then, the second indole molecule reacts with this carbocation, stabilized by acetic acid, resulting in aromatization and the formation of bis-indolylmethane derivatives. (Kalla et al., 2018).

2.2 Biological evaluation

2.2.1 Evaluation of antiproliferative activity

Extensive studies have been conducted on the antimetastatic activity of indole derivatives in various human cancer cell lines (Varlı et al., 2024). Here, the antileukemic activity of the identified bis-indolylmethanes was evaluated against the HL-60 cell line utilizing the CCK-8 assay (Sheryn et al., 2020). The results of the cell viability assays are presented in Figure 2 and Table 1, with noscapine

hydrochloride (Nos) acting as the positive control. In the first phase, all ligands **1-8** were observed and screened for dose-dependent cytotoxicity at concentrations of 25 and 50 μ M, respectively. At a concentration of 50 μ M, all compounds demonstrated cytotoxicity potency and inhibited the proliferative activity of the cell line (HL-60). The two best compounds, **4** and **7**, with cell viabilities of 23.1% and 29.2%, respectively, were selected for further analysis in phase two regarding their potential as anticancer agents.

Notably, bis-indolylmethane **4**, with three methoxy groups at the 3,4,5 positions on the benzene ring, showed the best antiproliferative activity among the tested compounds, including the positive control (noscapine). In a study by Liew et al., compounds with three methoxy groups attached to their side have marked greater cytotoxicity against the lung cancer cell line compared to the ones studied by previous researchers, named EF24 analogs (potent inhibitors) (Liew et al., 2020; Wu et al., 2017). In another study by Zhou and his team, compounds having a methoxy substituent at the position in the benzene ring contributed to higher activity in inhibiting HUVEC (Human Umbilical Vein Endothelial Cells) cells migration due to their electron-donating properties compared to those with electron-withdrawing groups such as nitro or chloro atom (Zhou et al., 2016). Together, the findings signify the importance of the methoxy group in the antiproliferative maneuver of the anticancer compounds. Furthermore, methyl substitution may enhance the antiproliferative activity of certain indole-based compounds. Al-Wahaibi et al. (Al-Wahaibi et al., 2024) reported that the introduction of an N-methyl group increased the antiproliferative potency of an indole derivative, which is consistent with the comparatively high activity observed for ligand **4**.

In contrast to bis-indolylmethane **6**, which possesses two methoxy groups at *meta* positions and a hydroxyl (OH) group at the *para* position, a low inhibition activity of 96% cell viability was perceived at 50 μ M concentration. This is due to the steric hindrance induced by the *para*-hydroxyl group, which depleted the compound's activity and influenced the less potent value of IC₅₀ reading recorded. Results for ligands **3** and **7**, which replace the nitro group with an acetamido group at the *para* position of the benzene ring, resulted in better activity in the cell line (HL-60). This finding suggests that the acetamido

moiety may contribute to the enhanced activity of ligand **7**. Recent literature indicates that acetamide-containing groups can facilitate favorable interactions with biological targets through hydrogen-bond donation and acceptance, potentially improving binding affinity and biological activity (Alshazly et al., 2025). Overall, ligands having a nitro group at *ortho* and *para* positions, such as bis-indolylmethanes **1-3**, **5**, and **8**, did not exhibit a superior effect on the antiproliferative activity.

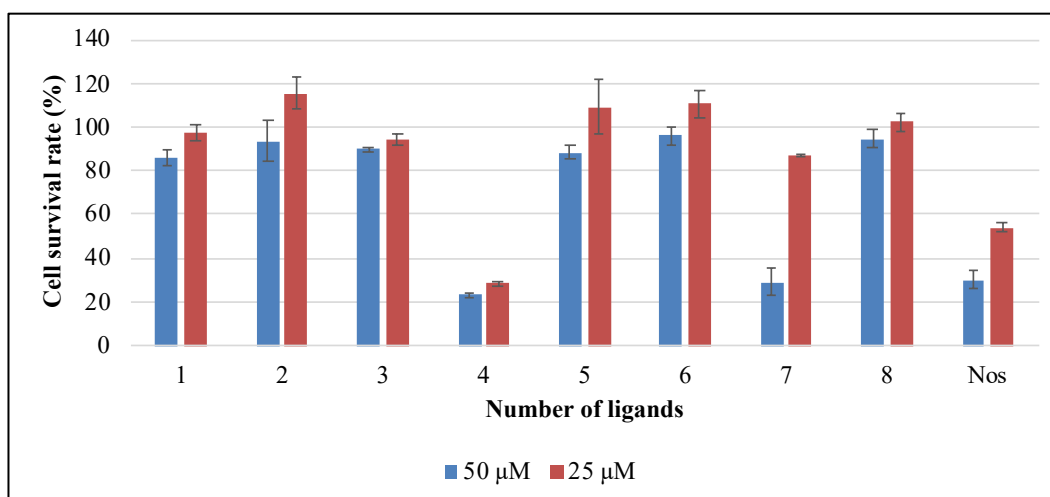


Figure 2 Antiproliferative effects of bis-indolylmethanes on HL-60 cells (means $\pm$ SEMs, $n=3$). Results are reported as a percentage of treated cells' viability.

Table 1 Cell viability of bis-indolylmethanes against HL-60 cells at a concentration of 50 μ M and their IC₅₀ values.

Ligand	HL-60 cells viability (% of control)	IC ₅₀ (μ M)
1	85.7 $\pm$ 3.9	>100
2	93.5 $\pm$ 9.5	99.23
3	89.8 $\pm$ 1.1	>100
4	23.1 $\pm$ 1.3	44.4
5	88.3 $\pm$ 3.2	95.44
6	96.0 $\pm$ 3.9	>100
7	29.2 $\pm$ 6.2	62.63
8	94.7 $\pm$ 3.8	>100
Noscapine	30.1 $\pm$ 4.4	28.97

In the second phase, two potent ligands of bis-indolylmethanes **4** and **7** were evaluated for two additional concentrations of the highest and lowest concentration at 100 μ M and 12.5 μ M, respectively, as shown in Figure 3. Both ligands exhibited excellent cytotoxicity activity at the final concentration of 100 μ M. Nuclear structural morphology studies were accomplished to ratify apoptosis induction of these two finalized ligands.

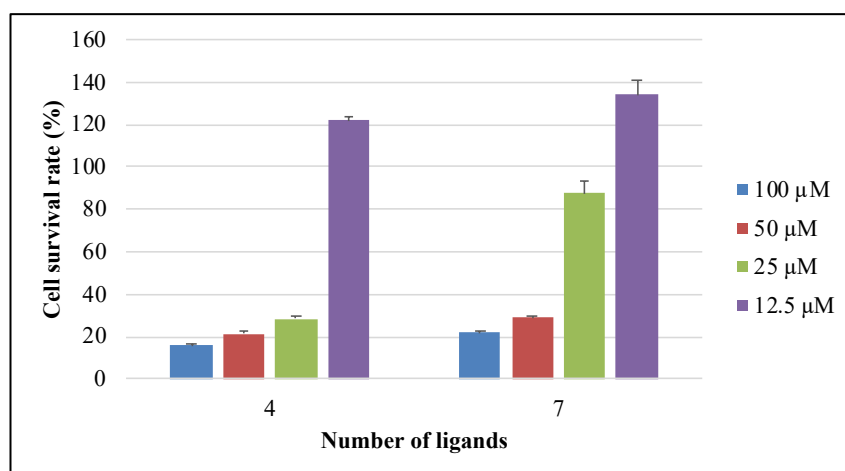


Figure 3 Antiproliferative effects of bis-indolylmethanes **4** and **7** on HL-60 cells (means $\pm$ SEMs, $n=3$). Results are reported as a percentage of treated cells viability.

Table 2 Cell viability of bis-indolylmethanes **4** and **7** against HL-60 cells at a final concentration of 100 μ M and their IC₅₀ values.

Ligand	HL-60 cells viability (% of control)	IC ₅₀ (μ M)
4	16.2 $\pm$ 0.3	44.4
7	22.7 $\pm$ 0.5	62.63

2.2.2 Apoptosis induction

Apoptosis of HL-60 cells instigated by the two particular ligands (**4** and **7**) with the best cytotoxicity activity was conducted using Hoechst 33258 staining and observed on a fluorescent microscope. The microscopic profile of the tested HL-60 cell line stained with Hoechst 33258 is

depicted in Figure 4. Treatment of HL-60 cells with compounds **6** and **7** (B and C) resulted in cell shrinkage, nuclear chromatin condensation, and membrane blebbing, which are characteristic morphological features of apoptosis (Sheryn et al., 2020). These apoptotic changes are associated with cytoskeletal reorganization and cell contraction, leading to the plasma membrane bulging and the formation of membrane blebs. Some membrane blebs may subsequently detach from the cell as apoptotic bodies containing portions of cytoplasm and other cellular components (Xiong et al., 2025). These results indicate the ability of the ligands to induce apoptosis in HL-60 cells, with obvious differences in cell structure compared to the untreated control group (A).

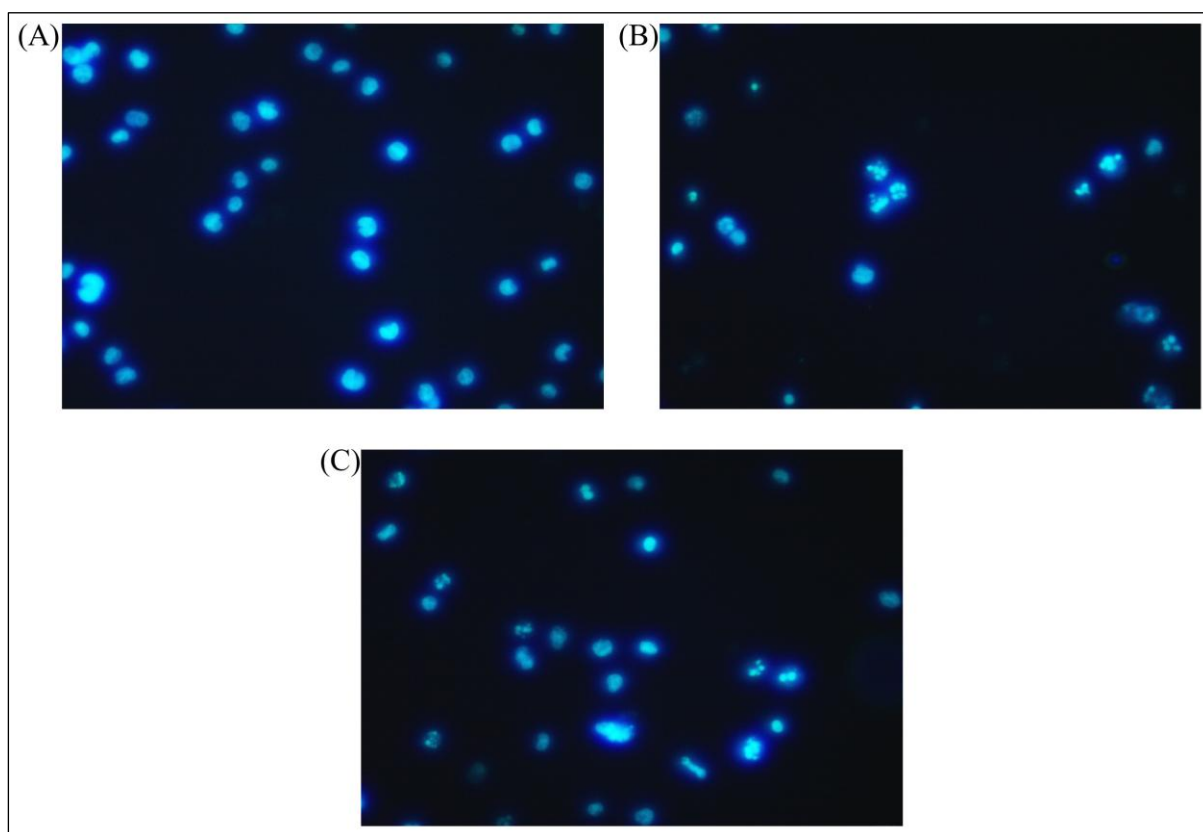


Figure 4 Morphological differences of HL-60 cells after being cured with 100 μ M (final concentration) of synthesized bis-indolylmethanes **4** and **7** for 48 h. (A) Controlled HL-60 (in individual DMSO) (B) Bis-indolylmethane **4** induced cell apoptosis in HL-60. (C) Bis-indolylmethane **7** induced cell apoptosis in HL-60.

2.2.3. *Ct-DNA binding properties*

The interaction between selected compounds (**4** and **7**) and ctDNA was studied using UV-vis spectroscopic titration. Electronic absorption spectroscopy was employed in this study in order to observe changes in the compounds' spectra with increasing amounts of DNA and to provide information on the interaction between double-stranded DNA and molecules. According to Kumar et al. (2025), the anticancer activity of certain indole-derived compounds may be attributed to their ability to interact with DNA through mechanisms such as DNA intercalation and topoisomerase inhibition, thereby disrupting DNA structure and DNA-dependent cellular processes and ultimately inducing apoptotic cell death. Therefore, to investigate the potential DNA-binding behavior of the synthesized bis-indolylmethanes, UV-Vis titrations of compounds **4** and **7** were performed in Tris-HCl buffer, and both of the compounds demonstrated strong absorption in the region between 220 and 320 nm and 450 to 550 nm, respectively.

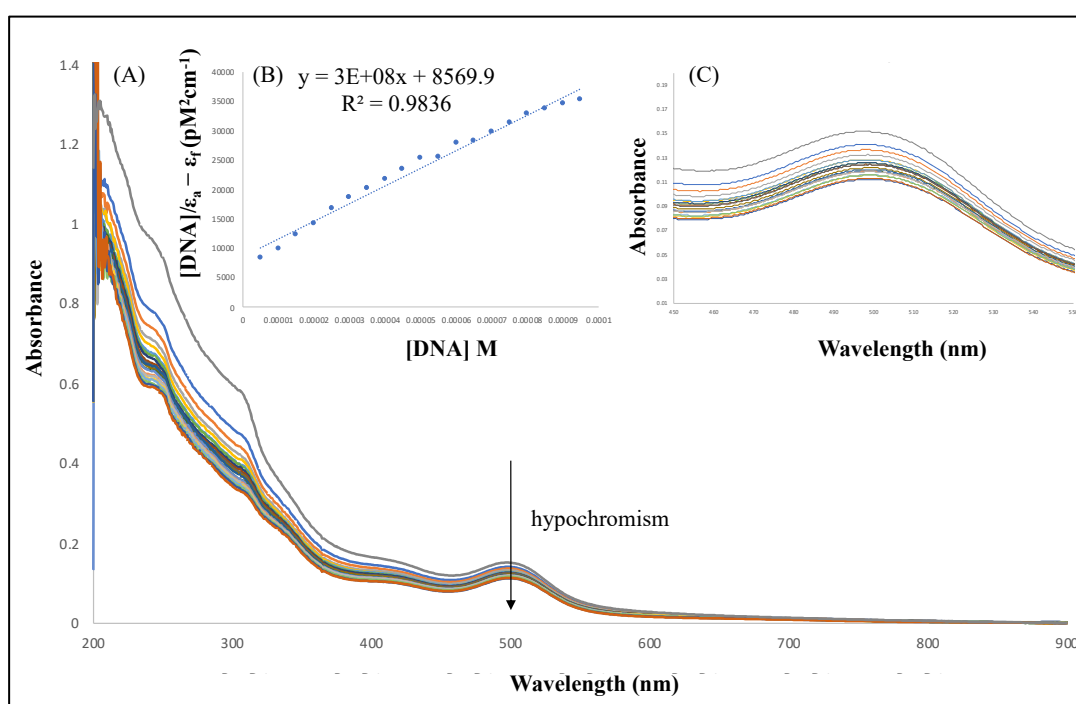


Figure 5 (A) Absorption spectral changes of bis-indolylmethane **7** (50 μM) with incremental concentration of ctDNA added. Arrow (↓) denotes the hypochromic effect. (B) Plot of $[DNA]/\epsilon_a - \epsilon_f$ (pM²cm⁻¹) as function of DNA concentration calculated from absorption spectral results. (C) Zoom version of (A) that focuses on the wavelength between 450 and 550 nm.

There are three types of spectral changes that can be observed in UV-vis titration, including hypochromism, hyperchromism, and bathochromism effects. In this study, both compounds (**4** and **7**) exhibited a hypochromic effect (Figures 5 and 6). Titration of increasing amounts of ctDNA into **4** and **7** solution steered to the degradation of UV-vis absorption (hypochromism effect). The observed hypochromic effect, reflected by a decrease in absorbance intensity upon the addition of DNA, indicates an interaction between the compound and DNA base pairs. Such interactions (e.g., between A-T and G-C base pairs), either through intercalation, electrostatic binding, or groove binding, may stabilize the double-stranded DNA helix through noncovalent forces and consequently produce a hypochromic effect (Naik et al., 2026). Hypochromism effect for bis-indolylmethane **7** at wavelength 506 nm is 29.67%, while for bis-indolylmethane **4**, hypochromism effect at wavelength 290 nm is 67.36%. Neither compound showed any red shift or blue shift. Generally, the simultaneous occurrence of hypochromism and a red shift in UV-Vis absorption spectra is commonly considered indicative of the intercalation of small molecules into DNA. This behavior is attributed to strong stacking interactions between the aromatic chromophore of the compound and DNA base pairs. Click or tap here to enter text.(Helal et al., 2026). On the other hand, according to Zhang et al. (2012) and Lafayette et al. (2017), if the DNA titration resulted in hypochromism without having a red or blue shift, it can be deduced that the groove binding interaction is the most susceptible mode of interaction. Thus, this phenomenon indirectly confirmed that the binding mode and interaction of both ligands **4** and **7** with ctDNA are groove binding. Additionally, the side chain associates with bis-indolylmethane derivatives such as N-H, C=O, and O-CH₃ give them additional hydrogen bond donor or acceptor atoms, which enable the molecule to remain in the minor groove with the assistance of van der Waals forces and hydrogen bonds. According to the Wolfe-Shimer equation, the intrinsic DNA-binding constant (K_b) was determined by plotting $[\text{DNA}]/(\epsilon_a - \epsilon_f)$ against $[\text{DNA}]$. The K_b value was subsequently calculated from the ratio of the slope to the intercept of the resulting linear plot (Li et al., 2026).

The DNA-binding constants (K_b) of compounds **7** and **4** were 1.04×10^4 and $6.02 \times 10^4 \text{ M}^{-1}$, respectively. These values are within the same order of magnitude as those reported for recent indole-

containing nitrogen heterocycles, which exhibited DNA-binding constants ranging from 10^4 to 10^5 M^{-1} (dos Santos et al., 2026).

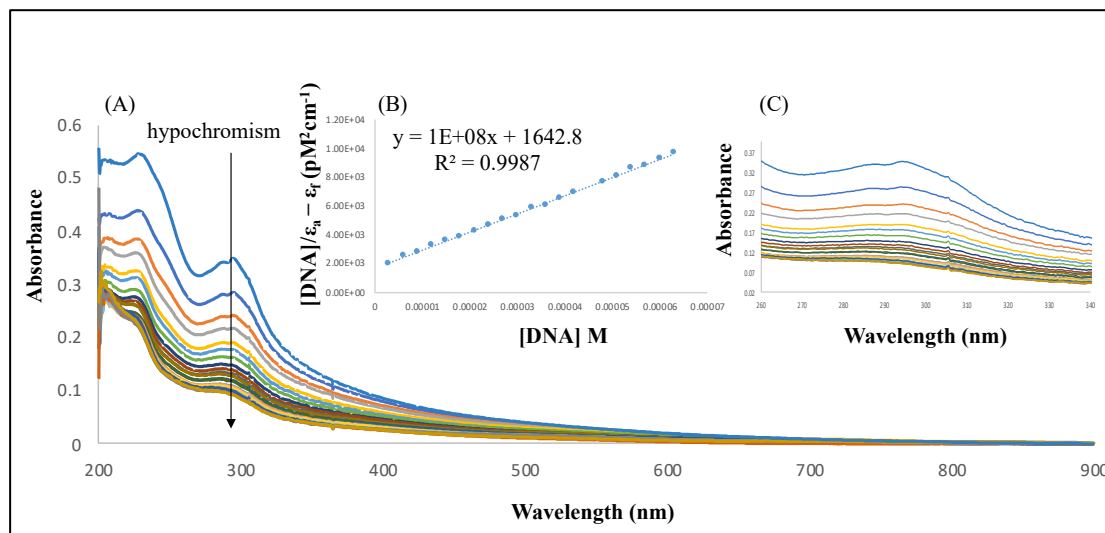


Figure 6 (A) Absorption spectral changes of bis-indolylmethane **4** (30 μM) with incremental concentration of ctDNA added. Arrow ($\downarrow$) denotes the hypochromic effect. (B) Plot of $[\text{DNA}]/\epsilon_a - \epsilon_f (\text{pM}^2\text{cm}^{-1})$ as a function of DNA concentration calculated from absorption spectral results (C). Zoom version of (A), focusing on the wavelength between 260 and 340 nm.

2.2.4 Carcinogenic test with DNA biosensor

The carcinogenicity of bis-indolylmethanes **4** and **7** were tested by employing the toxicity electrochemical DNA biosensor analysis. This toxicity biosensor comprises single-stranded DNA with a 24-base guanine-rich sequence and uses methylene blue (MB) as the redox indicator. Following the approach of Md. Sani et al. (2019), differential pulse voltammetry was employed in a DNA-based biosensor incorporating a 24-base guanine-rich single-stranded DNA probe to evaluate the DNA-binding potential of the tested compounds. This approach is relevant because DNA-reactive carcinogens can bind to and damage DNA, potentially leading to mutations. The guanine-rich DNA probe was selected because guanine-rich regions are susceptible to oxidative damage and may serve as important targets for DNA-reactive compounds (Cohen et al., 2025; Sani et al., 2019). Therefore, the finding suggests that the carcinogen's behavior is determined by its greater tendency to bind to DNA, which

has a high guanine content. Toxicity evaluation is determined by comparing the current signal recorded by the MB marker. The current values for both the carcinogen and the ligand tested were determined using the MB marker. Cadmium (II) chloride was utilized as the carcinogen control, in which it has been ascribed to yield a relatively low current signal, due to the binding that happens with the DNA, resulting in depletion of the current value observed, as a competition in binding to DNA was induced between the carcinogen and the MB marker. In this system, if a substance is classified as a carcinogen, the current signal of the MB indicator (MB + carcinogen) will be much lower than the free MB indicator (individual), indicating competition occurred in binding to DNA (rich guanine sequence).

Figure 7 shows a comparison of the MB marker with various concentrations of bisindolylmethanes **4** and **7** (0.1 to 0.0005 ppm) as well as hazardous heavy metal, cadmium(II) chloride. In this study, the current recorded by MB in the presence of both compounds is much lower than the free MB's (individual) spectrum, which is nearly the same as the spectrum of MB in the presence of carcinogen (cadmium). Generally, this phenomenon indicates competition between the ligands and MB for binding to DNA. Nevertheless, the current peaks recorded at -0.2 V demonstrated relatively large deviations (error bars), suggesting that the noise has become more significant (inset in Figure 7). According to Sani et al. (2019), as the concentration of the carcinogen increases, the current signal of the MB indicator recorded decreases, thus demonstrating a descending trend, which indicates a more stable binding scenario with the DNA. In contrast to the results for both compounds **4** and **7**, as the concentration of the ligands decreased from 0.1 to 0.0005 ppm (inset in 7(A) and 7(B)), no ascending trend in the current signal was observed, which signifies a non-uniformity. Hence, this occurrence suggested that there was no binding to the DNA (rich in guanine) and no binding competition with MB. Those compounds that have toxicity properties will possess lower errors compared to compounds with no toxicity, which indicates the binding with the DNA is happening, thus resulting in a stable current signal recorded (Sani et al., 2019).

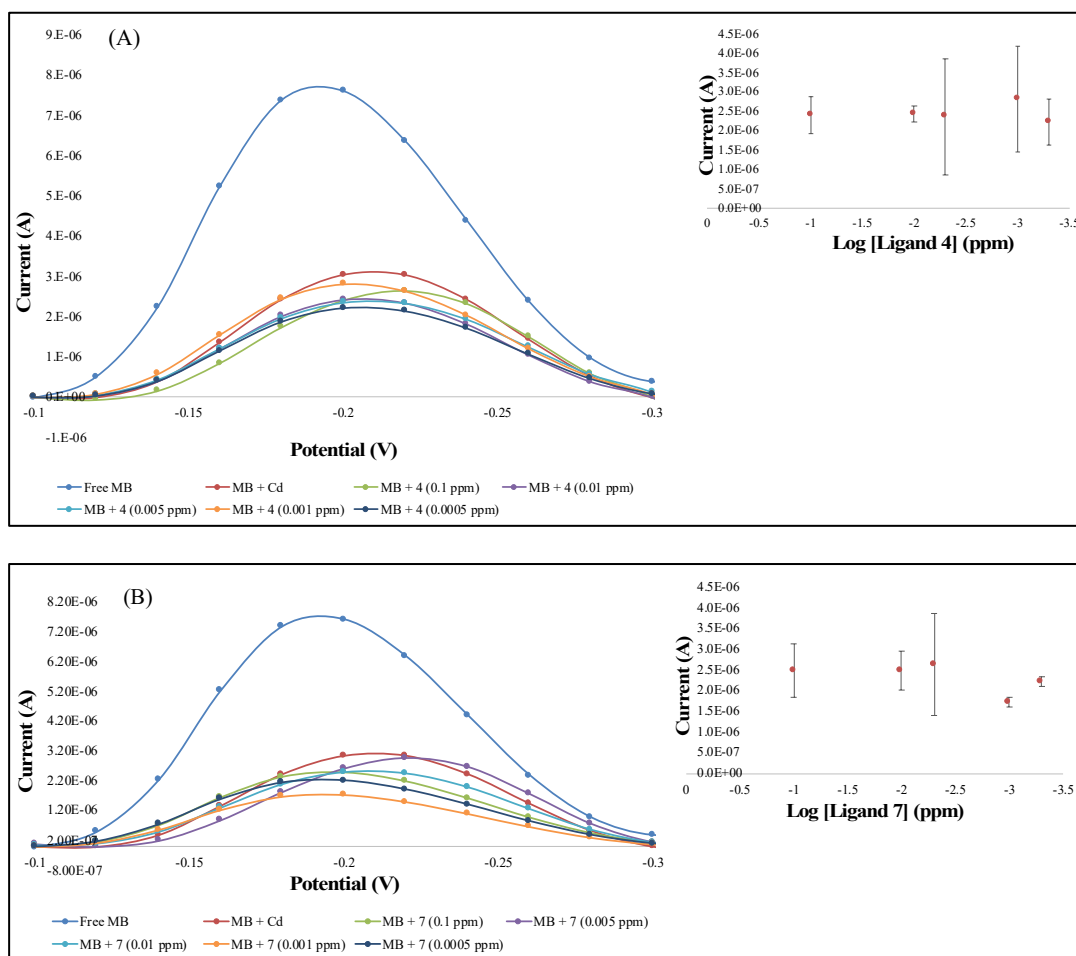


Figure 7 (A) Performance of DNA biosensor with ligand 4. (B) Performance of DNA biosensor with ligand 7. *

Large error bars (inset) display the non-carcinogenic behavior of the compounds.

2.3 Docking and modeling studies

2.3.1 Docking studies

Many cancers have a survival advantage because they are deprived of the apoptotic machinery, which involves the overexpression of the Bcl-2 family (Sheryn et al., 2020). Molecular docking simulations were conducted according to a previous study in order to observe the ligands' potential in suppressing the anti-apoptotic protein (Bcl-2) as well as discover possible receptor-ligand binding interactions. The two best-performing ligands, 4 and 7, were docked into the navitoclax-binding hotspots of BCL-2, specifically the P2 and P4 hydrophobic pockets. Navitoclax is an orally bioavailable BH3-mimetic

inhibitor that targets the anti-apoptotic BCL-2 family proteins, including BCL-2, BCL-xL, and BCL-w (Vogler et al., 2025). As the validation of the docking protocol, the co-crystallized ligand navitoclax was redocked and generated an RMSD value of 0.617 Å.

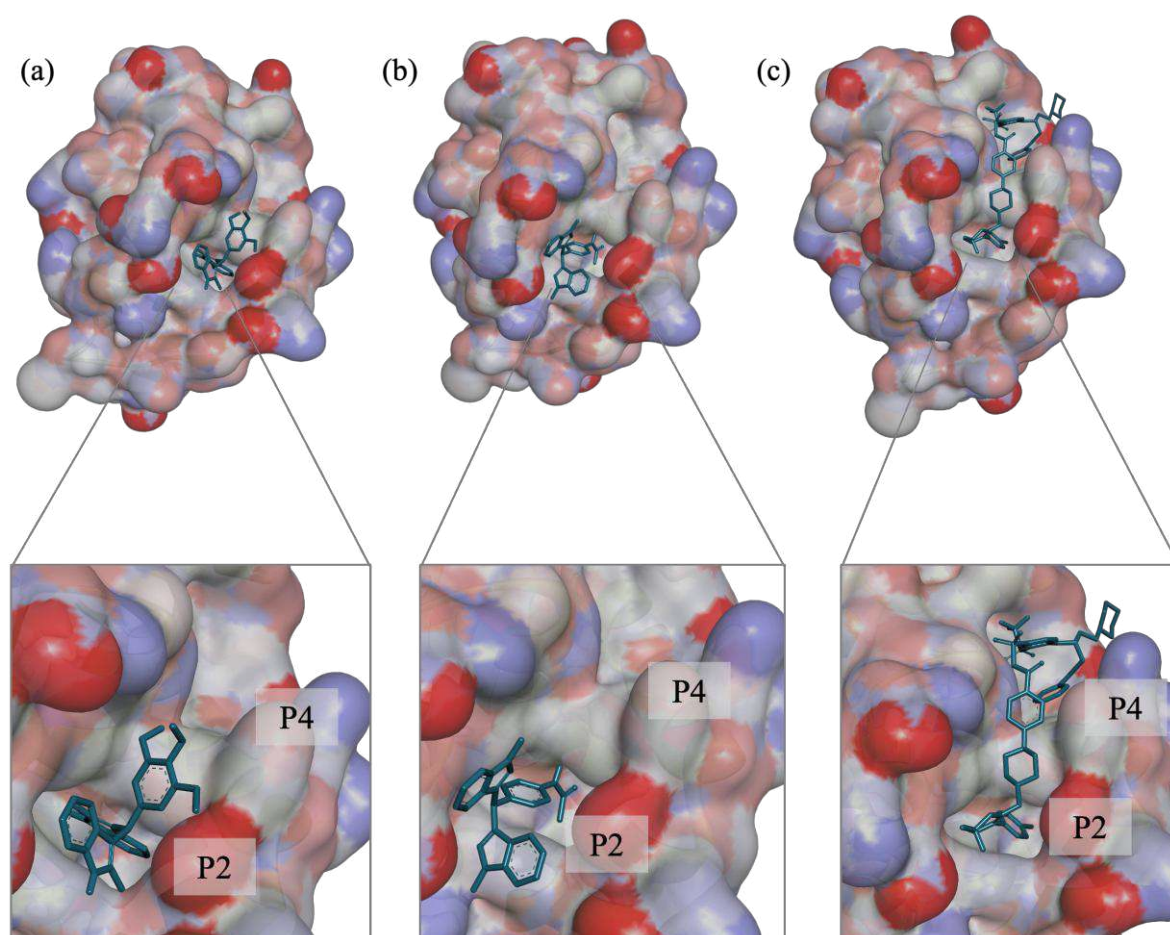


Figure 8 3D Bcl-2 molecular surface map displaying the docked poses of (A) **4**, (B) **7**, and (C) navitoclax into P2 and P4 binding hot spots.

The right conformation and sufficient energy, both congregate for the inhibition to come into existence, as they are the primary features in addressing successful inhibition activity (Imran et al., 2015). In this study which focuses on inhibition of Bcl-2 over expression, there are several factors being considered such as the position of ligands in the P2 and P4 hydrophobic pockets and formation of ligand-receptor interactions with important amino acid residues like Gly142, Arg104, Tyr199, Tyr 105,

Phe195, Leu198, Arg143, Asp108, Phe101, Phe109, Met112, Glu149, Phe150, Leu134, Glu133, Ala146, and Val153.

Generally, as illustrated in Figure 8, both docked ligands (**4** and **7**) exhibited a good fit with the receptor but occupied a smaller volume within the P2 hot spot than the established inhibitor, navitoclax, which occupied both P2 and P4 binding pockets. All simulated compounds attained the best configuration with binding free energies (DG) of -8.2 and -7.8 kcal/mol for ligands **4** and **7**, respectively, and for noscapine. Ligands **4** and **7** had the best docking scores, consistent with the experimental results compared with noscapine as the positive control. Both of them (**4** and **7**) incorporated with amino acid residues of Arg143, Asp108, Phe101, Phe109, Met112, Glu149, Phe150, Leu134, Glu133, Ala146, and Val153 at the P2 hot spot of the Bcl-2 protein. Among these amino acid residues, Ala146 forms a conventional H-bond with ligand **4** and a π -alkyl interaction with ligand **7** through one of the indole units. In addition, an interaction between the benzaldehyde unit for both compounds with Phe101 was observed through a π - π interaction, which led to the stability of ligand-receptor binding formation (Imran et al., 2015).

A carbon-hydrogen bond formation was spotted between the Asp108 residue and the methoxy group of **4**. On the other hand, the same interaction was observed in the indole unit of ligand **7**. Most amino acid residues interact with the ligands via Van Der Waals interactions, incorporating both the indole and benzaldehyde scaffolds. Despite not being able to occupy both P2 and P4 hot spots, it was observed that both scaffolds of indole and benzaldehyde in ligands **4** and **7** contributed to the binding affinity towards Bcl-2, thus suppressing its activity to prevent apoptosis in tumor cells.

2.3.2 Molecular Dynamics Simulations (1000 ns)

While molecular docking provides valuable static insights into receptor-ligand complementarity, it lacks the dynamic and solvent-inclusive environment characteristic of biological systems. To rigorously validate the stability of the docked complexes and further elucidate the conformational dynamics of

Bcl-2 upon binding, 1000 ns (1 μ s) molecular dynamics (MD) simulations were executed for both the Ligand **4** and Ligand **7** complexes. The dynamic behaviors were assessed utilizing root mean square deviation (RMSD), root mean square fluctuation (RMSF), hydrogen bond occupancy, and radius of gyration (Rg) (Figure 9).

Structural Stability (RMSD and Rg)

The structural stability of the protein-ligand complexes was first evaluated by monitoring the RMSD of the backbone atoms. As depicted in Figure 9A, both the Bcl-2/Ligand **4** and Bcl-2/Ligand **7** systems achieved rapid equilibration early in the simulation. Following this initial relaxation, the trajectories remained highly stable with minimal deviations, confirming that neither ligand induced structural unraveling or major conformational shifts within the Bcl-2 receptor over the 1 μ s timeframe. Further supporting this structural integrity, the radius of gyration (Rg), which measures the overall compactness of the protein core, remained remarkably steady (Figure 9D). Both systems maintained a consistent Rg profile, indicating that the receptor retained a tightly folded, stable conformation upon ligand accommodation.

Local Residue Flexibility (RMSF) and Hydrogen Bonding

To investigate the local flexibility of the receptor upon ligand binding, RMSF values were computed for all amino acid residues (Figure 9B). Both complexes exhibited similar fluctuation profiles. Notably, the residues lining the P2 hydrophobic pocket (including the key interacting residues identified during docking, such as Arg143, Asp108, and Ala146) displayed highly restricted movement. This signifies tight structural rigidification of the binding groove induced by the continuous presence of the ligands. The regions exhibiting higher fluctuations correspond primarily to the terminal ends and solvent-exposed loop regions, characteristic of globular proteins in an aqueous environment.

The persistence of intermolecular interactions, particularly hydrogen bonding, is a critical determinant of binding affinity. Throughout the 1 μ s simulation, both ligands maintained a dynamic but steady network of hydrogen bonds with the Bcl-2 active site (Figure 9C). These persistent H-bonds

serve to anchor the bis(indolyl)methane scaffold firmly within the anti-apoptotic binding groove, corroborating the specific interactions initially observed in the static docking models.

Trajectory Snapshots and Interaction Stability

To visually corroborate the quantitative stability metrics, structural snapshots of the Bcl-2/Ligand complexes were extracted at representative intervals across the 1000 ns trajectory (Figure 10). These snapshots clearly demonstrate that the bis(indolyl)methane core of both Ligands **4** and **7** remains deeply and securely anchored within the Bcl-2 P2 hydrophobic pocket throughout the entire 1 μ s simulation. The spatial orientation of the key functional groups of the trimethoxy moiety of Ligand **4** and the acetamido group of Ligand **7** exhibits minimal deviation from their initial docked conformations. The continuous retention of these poses visually confirms the persistence of the stabilizing intermolecular contacts, ensuring sustained blockade of the anti-apoptotic binding groove.

2.3.3 Conformational Dynamics (Principal Component Analysis)

To further interpret the dynamic stability and structural transitions of the Bcl-2 complexes, Principal Component Analysis (PCA) was performed. PCA isolates the large-scale, concerted collective motions of the protein backbone by projecting the high-dimensional MD trajectory onto a reduced 2D conformational landscape defined by the first two principal components (PC1 and PC2).

As shown in Figure 11, the conformational space sampled by both the Ligand **4** and Ligand **7** complexes is highly clustered into well-defined, singular conformational basins. The density contours (colored regions) confirm that the complexes rapidly locate and remain within a deep, stable structural well without undergoing chaotic scattering or massive structural unfolding over the 1 μ s simulation. Ligand **7** exhibits a slightly broader but equally dense projection compared to Ligand **4**, indicating a localized flexibility that correlates with its highly favorable van der Waals packing and superior binding free energy observed in the subsequent thermodynamic calculations. Overall, the PCA projections

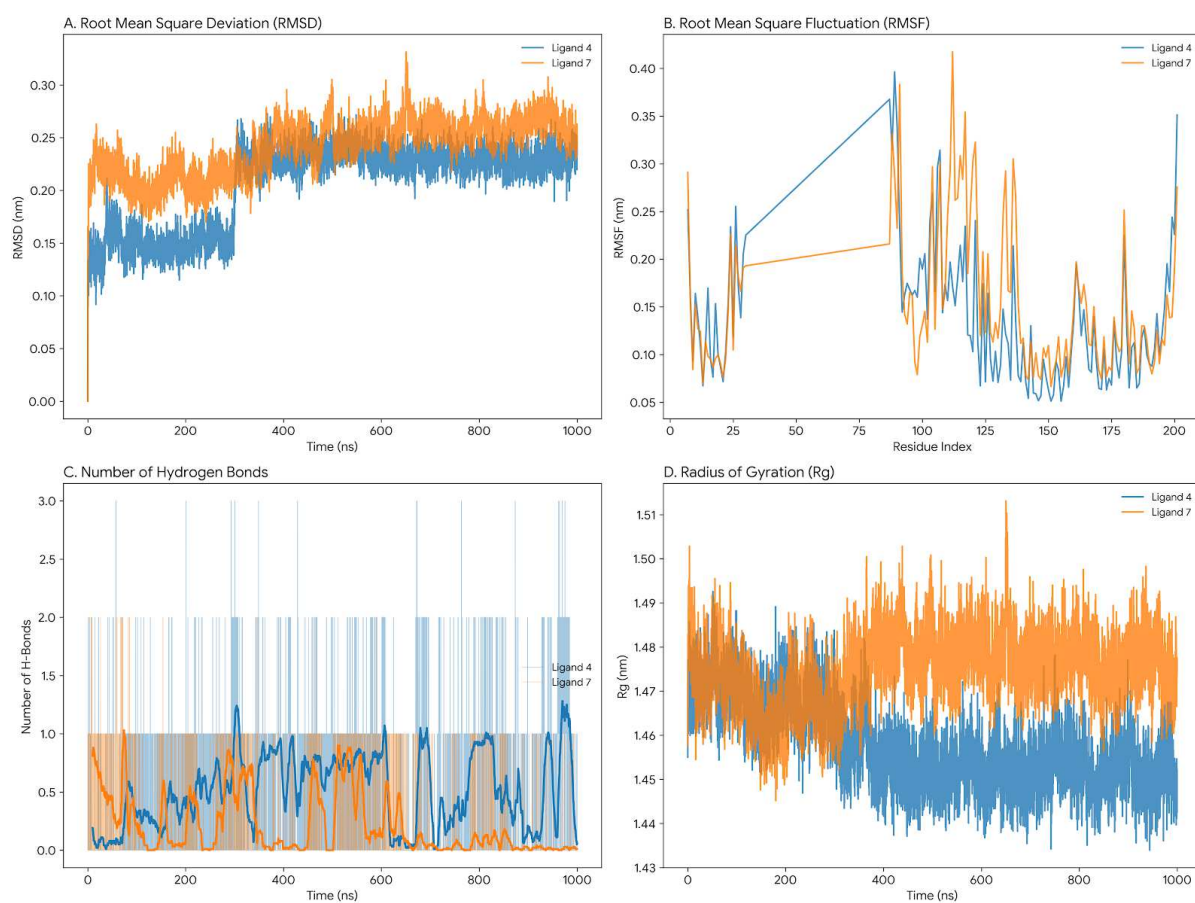
confirm that the bis(indolyl)methane scaffold effectively stabilizes the Bcl-2 apoptotic binding groove into a persistent, low-energy conformation.

2.3.4 Binding Free Energy Calculations (MM-PBSA)

To quantitatively validate the thermodynamic stability of the complexes observed during the MD simulations, Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) calculations were performed. The binding free energies (ΔG_{bind}) and their individual energy components were averaged over 10000 frames extracted from the 1000 ns trajectories (Table 3).

The calculations revealed that both bis(indolyl)methane derivatives possess highly favorable (negative) binding free energies, reinforcing their potential as Bcl-2 inhibitors. Ligand **7** exhibited the strongest binding affinity toward the Bcl-2 P2 pocket with a ΔG_{bind} of -36.83 ± 6.97 kcal/mol, while Ligand **4** recorded a ΔG_{bind} of -22.57 ± 11.61 kcal/mol. For both complexes, the binding is predominantly driven by massive favorable contributions from van der Waals interactions ($\Delta E_{\text{vdw}} = -48.66$ kcal/mol for Ligand **7** and -25.84 kcal/mol for Ligand **4**), which aligns perfectly with the highly hydrophobic nature of the Bcl-2 P2 binding groove. Electrostatic interactions (ΔE_{elec}) also contributed favorably to the complex stabilizations, whereas the polar solvation energies (ΔG_{PB}) acted as the primary opposing force to binding, a typical signature for deeply buried, hydrophobic ligand-receptor interactions.

Interestingly, while Ligand **7** demonstrated superior target-level affinity in silico, Ligand **4** exhibited slightly higher anti-proliferative potency in vitro against HL-60 cells ($\text{IC}_{50} = 44.4$ μM vs. 62.63 μM). This slight divergence between isolated thermodynamic affinity and whole-cell efficacy highlights the multifactorial nature of cellular cytotoxicity. The presence of three methoxy groups in Ligand **4** likely confers superior lipophilicity and membrane permeability compared to the polar acetamido group in Ligand **7**, enabling a higher intracellular concentration of Ligand **4** to reach Bcl-2 targets in vitro.



478

479 **Figure 9** Molecular dynamics trajectory analysis of the Bcl-2 receptor complexed with Ligand 4 (blue)
 480 and Ligand 7 (orange) over 1000 ns. (A) Root Mean Square Deviation (RMSD) of the protein-ligand
 481 complex backbone. (B) Root Mean Square Fluctuation (RMSF) of the protein residues, highlighting
 482 local amino acid flexibility. (C) Number of intermolecular hydrogen bonds formed between the ligands
 483 and the Bcl-2 active site. (D) Radius of gyration (R_g) indicating the structural compactness of the
 484 complexes.

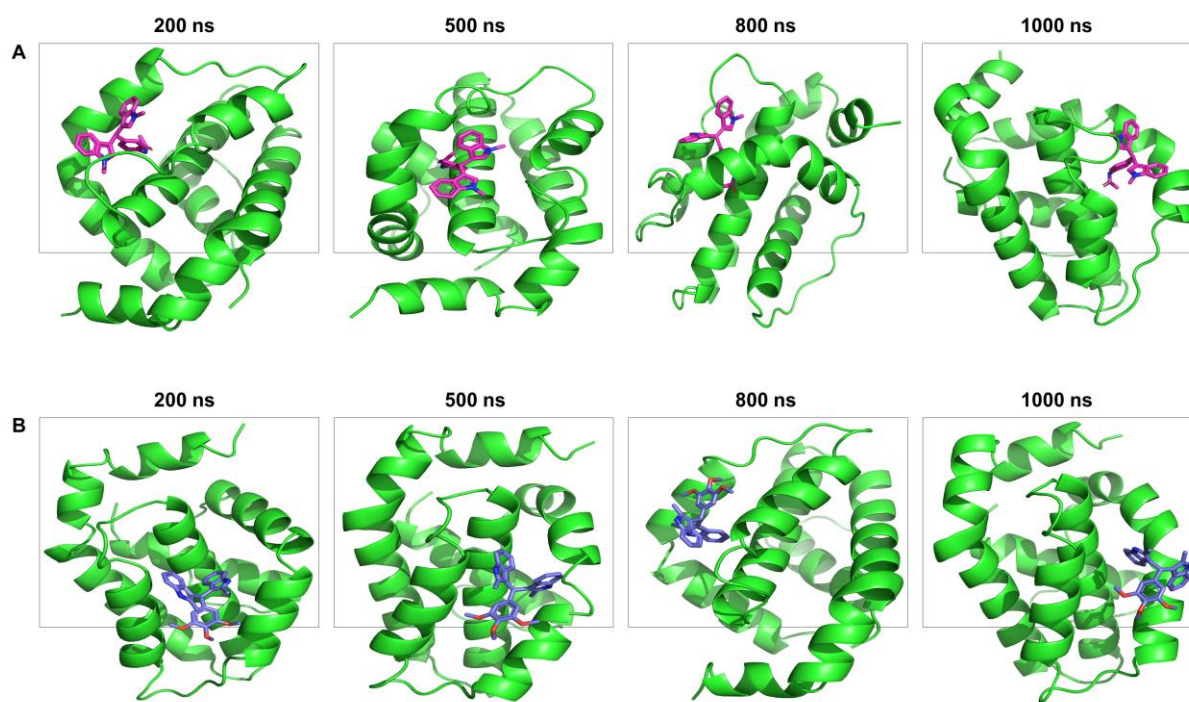


Figure 10 Structural snapshots of the Bcl-2 P2 active site complexed with (A) Ligand **4** and (B) Ligand **7**. The representative conformations were extracted at 0, 500, and 1000 ns intervals from the MD trajectories, visually illustrating the stable retention and persistent binding modes.

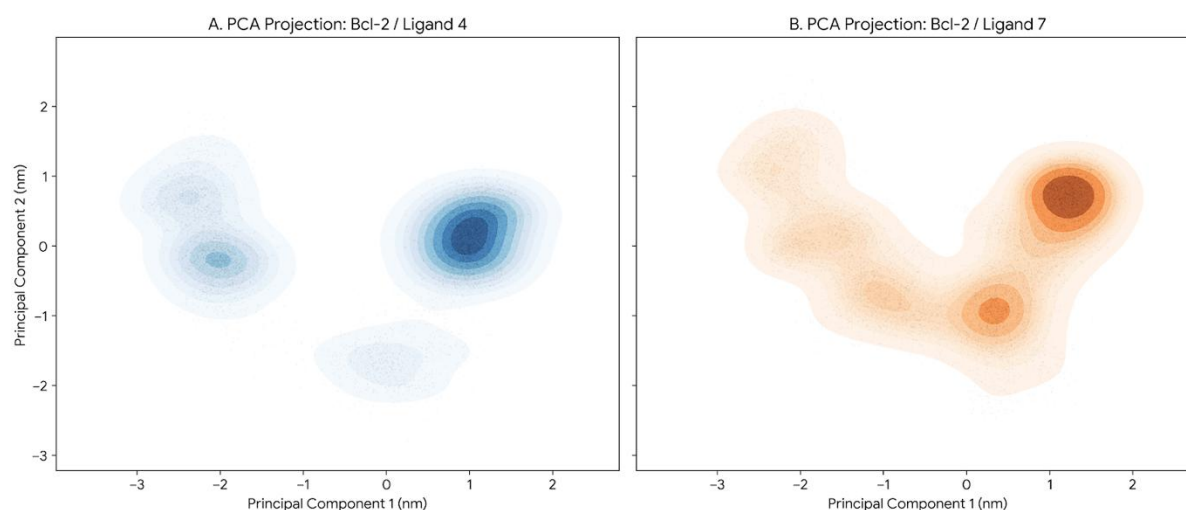


Figure 11 Principal Component Analysis (PCA) of the Bcl-2 receptor backbone dynamics. 2D projection plots of the first two principal components (PC1 vs. PC2) sampled over the 1000 ns MD trajectory for (A) Bcl-2/Ligand **4** and (B) Bcl-2/Ligand **7**. The concentrated density contours correspond to the highly populated, stable conformational basins continuously occupied by the complexes.

Table 3 Thermodynamic binding free energies (ΔG_{bind}) and individual energy components (kcal/mol) for the Bcl-2/Ligand complexes calculated using the MM-PBSA method, averaged over 1000 frames from the 1000 ns trajectories.

Ligand	ΔE_{vdw}	ΔE_{elec}	ΔG_{PB}	ΔG_{np}	ΔG_{bind} (Total)
Ligand 4	-25.84 ± 13.88	-12.51 ± 9.24	19.01 ± 12.26	-3.22 ± 1.59	-22.57 ± 11.61
Ligand 7	-48.66 ± 6.46	-13.75 ± 7.18	30.28 ± 9.16	-4.70 ± 0.48	-36.83 ± 6.97

(Note: vdw = van der Waals energy; elec = electrostatic energy; PB = polar solvation energy; np = non-polar solvation energy; Data presented as Average $\pm$ Standard Deviation).

3. Conclusion

In summary, a series of functionalized bis(indolyl)methane derivatives was successfully synthesized via an efficient acetic acid-catalyzed reaction and evaluated for their anti-leukemic potential. *In vitro* biological assays identified the trimethoxy (Ligand 4) and acetamido (Ligand 7) derivatives as the most promising candidates, demonstrating targeted cytotoxicity against the HL-60 cell line and effectively inducing apoptosis, as evidenced by morphological assessments. Crucially, electrochemical DNA biosensor analysis and UV-Vis spectroscopy confirmed that these active compounds exhibit a non-carcinogenic safety profile, distinguishing them from traditional DNA-damaging agents. To elucidate their specific molecular mechanism of action, comprehensive computational studies were conducted targeting the anti-apoptotic Bcl-2 protein. Molecular docking, rigorously validated by 1000 ns (1 μ s) molecular dynamics simulations and principal component analysis (PCA), revealed that both ligands are deeply and stably anchored within the P2 hydrophobic groove. Furthermore, MM-PBSA calculations confirmed highly favorable thermodynamic binding affinities. Collectively, this multidisciplinary study establishes the bis(indolyl)methane scaffold as a safe, highly stable, and

thermodynamically viable structural foundation for the future rational design of targeted anti-apoptotic therapeutics.

4. Experimental

4.1. General methodologies

All synthesis reactions were carried out under conventional reflux, and no preventive measures were taken to exclude air or moisture. All chemicals, solvents, and calf thymus DNA (ctDNA) were obtained and directly used from commercially available sources. Waters UPLC-MS system (Aquity UPLC XevoQTof) was utilized to obtain the MS spectra, while a Bruker Advance 400 MHz spectrometer at 400 MHz and 100 MHz was used in recording ^1H and ^{13}C NMR spectra, respectively, by employing d_6 -DMSO $_4$ as the product solvent. IR spectra were recorded on a Perkin Elmer Spectrum 400 FT-IR/FT-FIR spectrometer equipped with a PIKE GladiATR. UV-vis titration with ctDNA was conducted using a Shimadzu UV-1800 spectrophotometer. A DPV (differential pulse voltammetry) experiment was performed using an Autolab PGSTAT 12 potentiostat (Metrohm, Utrecht, The Netherlands). Thin-layer chromatography (TLC) spots on plates pre-coated with silica gel Merck 60 GF $_{254}$ (0.25mm) were identified with an ultraviolet (UV) lamp (254 nm).

4.2. Synthesis of bis-indolylmethane derivatives

The formation of bis-indolylmethane derivatives was conducted using the conventional method of reflux. The chemical structures of the synthesized ligands were elucidated and confirmed by spectroscopic studies. A mixture of substituted indole (6 mmol) and benzaldehyde derivatives (3 mmol) was stirred in a round-bottom flask containing an adequate amount of acetic acid (2.5 mL) and refluxed for 18 h. On the following day, the TLC method was utilized to monitor the reaction progress, and upon completion, deionized water was added drop by drop to initiate precipitation. Next, the mixture was displaced to a beaker filled with crushed ice to induce more precipitation. The crude precipitate formed

was filtered and washed using diethyl ether to afford pure products. The yielded pure product was dried at ambient conditions and followed by evaluation using spectroscopy studies.

4.2.1 3,3'-((4-nitrophenyl)methylene)bis(5-bromo-1H-indole) (**1**)

Compound **1** was synthesized from starting material 5-bromoindole and 4-nitrobenzaldehyde. This product was purified using diethyl ether to afford brown solid; Yield 80.0%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3421, 1508, 1210, 1341; ^1H NMR (400 MHz, DMSO) δ 11.19 (s, 2H, NH), 8.18 (d, $J = 8.8$ Hz, 2H, H-1, H-2), 7.60 (d, $J = 8.8$ Hz, 2H, H-3, H-4), 7.48 (d, $J = 1.9$ Hz, 2H, H-6, H-6'), 7.36 (d, $J = 8.6$ Hz, 2H, H-10, H-10'), 7.18 (dd, $J = 8.6, 1.9$ Hz, 2H, H-9, H-9'), 6.96 (d, $J = 2.2$ Hz, 2H, H-8, H-8'), 6.10 (s, 1H, H-5); ^{13}C NMR (101 MHz, DMSO) δ 152.92, 146.40, 135.68, 129.83, 128.61, 125.99, 124.13, 121.46, 116.84, 114.19, 111.61, 65.39, 15.63.

4.2.2 3,3'-((4-nitrophenyl)methylene)bis(2-methyl-1H-indole) (**2**)

Compound **2** was synthesized from the starting materials 2-methylindole and 4-nitrobenzaldehyde. This product was purified using diethyl ether to afford brown solid; Yield 90.0%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 1513, 1341, 1221; ^1H NMR (400 MHz, DMSO) δ 10.90 (s, 2H, NH), 8.26 – 8.05 (m, 2H, H-1, H-3), 7.43 (d, $J = 8.5$ Hz, 2H, H-2, H-4), 7.25 (d, $J = 8.0$ Hz, 2H, H-11, H-11'), 6.96 – 6.88 (m, 2H, H-9, H-9'), 6.81 (d, $J = 7.9$ Hz, 2H, H-8, H-8'), 6.75 – 6.66 (m, 2H, H-10-H-10'), 6.08 (s, 1H, H-5), 2.11 (s, 6H, H-6); ^{13}C NMR (101 MHz, DMSO) δ 153.37, 146.14, 135.56, 133.05, 130.29, 128.31, 123.72, 120.28, 118.71, 111.31, 111.02, 31.14, 12.37.

4.2.3 3,3'-((4-nitrophenyl)methylene)bis(1-methyl-1H-indole) (**3**)

Compound **3** was synthesized from the starting materials 1-methylindole and 4-nitrobenzaldehyde. This product was purified using diethyl ether to afford yellow solid; Yield 98.0%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 1550, 1339, 1153; ^1H NMR (400 MHz, DMSO) δ 8.16 (d, $J = 8.8$ Hz, 2H, H-1, H-2), 7.61 (d, $J = 8.7$ Hz, 2H, H-3, H-4), 7.40 (d, $J = 8.2$ Hz, 2H, H-8, H-8'), 7.32 (d, $J = 7.9$ Hz, 2H, H-11, H-11'), 7.17 – 7.10

(m, 2H, H-9, H-9'), 6.96 – 6.91 (m, 2H, H-10, H-10'), 6.90 (s, 2H, H-6, H-6'), 6.05 (s, 1H, H-7), 3.71 (s, 6H, H-7). ¹³C NMR (101 MHz, DMSO) δ 153.39, 146.26, 137.42, 129.87, 128.62, 127.08, 123.96, 121.78, 119.50, 119.08, 116.35, 110.24, 32.77, 31.14.

4.2.4 3,3'-((3,4,5-trimethoxyphenyl)methylene)bis(2-methyl-1H-indole) (**4**)

Compound **4** was synthesized from the starting materials 2-methylindole and 3,4,5-trimethoxybenzaldehyde. This product was purified using diethyl ether to afford maroon solid; Yield 80.5%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3330, 1502, 1117; ¹H NMR (400 MHz, DMSO) δ 10.72 (s, 2H, NH), 7.20 (d, $J = 8.5$ Hz, 2H, H-9, H-9'), 6.95 – 6.83 (m, 4H, H-10, H-10', H-12, H-12'), 6.71 (td, $J = 7.4, 0.9$ Hz, 2H, H-11, H-11'), 6.56 (s, 2H, H-4, H-5), 5.87 (s, 1H, H-6), 3.65 (s, 3H, H-2), 3.54 (s, 6H, H-1, H-3), 2.08 (s, 6H, H-7, H-7'); ¹³C NMR (101 MHz, DMSO) δ 152.99, 140.36, 136.32, 135.46, 132.39, 128.68, 120.04, 118.82, 118.39, 112.79, 110.77, 106.95, 60.63, 56.24, 12.43.

4.2.5 3,3'-((2-nitrophenyl)methylene)bis(2-methyl-1H-indole) (**5**)

Compound **5** was synthesized from the starting materials 2-methylindole and 2-nitrobenzaldehyde. This product was purified using diethyl ether to afford brown solid; Yield 81%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3391, 1527, 1371, 1301; ¹H NMR (400 MHz, DMSO) δ 10.87 (s, 2H, NH), 7.83 (dd, $J = 7.9, 1.4$ Hz, 1H, H-1), 7.57 (td, $J = 7.6, 1.4$ Hz, 1H, H-2), 7.50 (td, $J = 7.6, 1.3$ Hz, 1H, H-3), 7.26 (d, $J = 7.5$ Hz, 1H, H-4), 7.23 (d, $J = 8.1$ Hz, 2H, H-8, H-8'), 6.96 – 6.87 (m, 2H, H-9, H-9'), 6.76 – 6.66 (m, 4H, H-10, H-10'), 6.58 (s, 1H, H-5), 2.04 (s, 6H, H-6, H-6'); ¹³C NMR (101 MHz, DMSO) δ 150.54, 138.22, 135.49, 133.11, 132.84, 130.95, 128.53, 128.17, 124.99, 120.32, 118.82, 118.17, 111.02, 110.27, 34.36, 12.05.

4.2.6 4-(bis(2-methyl-1H-indol-3-yl)methyl)-2,6-dimethoxyphenol (**6**)

Compound **6** was synthesized from the starting materials 2-methylindole and 3,5-dimethoxy-4-hydroxybenzaldehyde. This product was purified using diethyl ether to afford brown solid; Yield

86.5%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3404, 3335, 1514, 1220, 1110; ^1H NMR (400 MHz, DMSO) δ 10.69 (s, 2H, NH), 8.20 (s, 1H, OH), 7.22 – 7.17 (m, 2H, H-10, H-10'), 6.92 – 6.85 (m, 4H, H-7, H-7', H-8, H-8'), 6.70 (td, $J = 7.4, 0.9$ Hz, 2H, H-9, H-9'), 6.49 (s, 2H, H-1, H-2), 5.82 (s, 1H, H-6), 3.53 (s, 6H, H-3, H-5), 2.07 (s, 6H, H-12); ^{13}C NMR (101 MHz, DMSO) δ 148.21, 135.43, 134.67, 134.24, 132.27, 128.75, 119.97, 118.90, 118.31, 113.16, 110.72, 107.27, 56.48, 12.42.

608

4.2.7 *N*-(4-(bis(1-methyl-1*H*-indol-3-yl)methyl)phenyl)acetamide (7)

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Compound **7** was synthesized from the starting materials 1-methylindole and 4-acetamidobenzaldehyde. This product was purified using diethyl ether to afford yellow solid; Yield 91.4%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3307, 1668, 1511, 1229; ^1H NMR (400 MHz, DMSO) δ 9.88 (s, 1H, NH), 7.46 (d, $J = 8.6$ Hz, 2H, H-3, H-5), 7.37 (d, $J = 8.2$ Hz, 2H, H-4, H-6), 7.27 (dd, $J = 13.6, 8.2$ Hz, 4H, H-10, H-10', H-13, 13'), 7.13 – 7.07 (m, 2H, H-11, H-11'), 6.93 – 6.87 (m, 2H, H-12, H-12'), 6.79 (s, 2H, H-8, H-8'), 5.77 (s, 1H, H-7), 3.69 (s, 6H, H-9, H-9'), 2.00 (s, 3H, H-1); ^{13}C NMR (101 MHz, DMSO) δ 168.57, 139.88, 137.61, 137.41, 128.82, 128.28, 127.31, 121.51, 119.69, 119.38, 118.77, 117.91, 110.06, 32.72, 24.35.

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4.2.8 3,3'-((2-nitrophenyl)methylene)bis(5-bromo-1*H*-indole) (8)

621

Compound **8** was synthesized from starting material 5-bromoindole and 2-nitrobenzaldehyde. This product was purified using diethyl ether to afford brown solid; Yield 90.0%; IR (ATR, $\nu_{\text{max}}/\text{cm}^{-1}$): 3423, 1519, 1339, 1070; ^1H NMR (400 MHz, DMSO) δ 11.18 (s, 2H, NH), 7.92 (dd, $J = 8.0, 1.3$ Hz, 1H, H-1), 7.60 (td, $J = 7.6, 1.3$ Hz, 1H, H-2), 7.50 (td, $J = 7.8, 1.4$ Hz, 1H, H-3), 7.37 (s, 2H, H-10, H-10'), 7.35 (d, $J = 1.3$ Hz, 2H, H-8, H-8'), 7.32 (dd, $J = 7.8, 1.3$ Hz, 1H, H-4), 7.18 (dd, $J = 8.7, 1.8$ Hz, 2H, H-7, H-7'), 6.86 (d, $J = 2.1$ Hz, 2H, H-6, H-6'), 6.37 (s, 1H, H-5). ^{13}C NMR (101 MHz, DMSO) δ 152.92, 146.40, 135.68, 129.83, 128.61, 125.99, 124.13, 121.46, 116.84, 114.19, 111.61, 65.39, 15.63.

629

4.3. Cell culture (HL-60)

631

632 HL-60 cell line was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and was cultured
633 in RPMI 1640 media (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cultivated media were
634 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics, and penicillin-
635 streptomycin (Gibco, Life Technologies, Thermo Fisher Scientific Inc., MA, USA). Cells were
636 preserved at 37 °C under a humidified atmosphere of 5% CO₂.

637

638 4.4. *In vitro* CCK-8 assay

639

640 A colorimetric assay using Cell Counting Kit-8 (CCK-8) was conducted as supplied by Dojindo
641 Molecular Technologies Inc. (Kumamoto, Japan), and noscapine hydrochloride hydrate, utilised as the
642 positive control, was acquired from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *In vitro*
643 cytotoxicity of the tested ligands was assessed against the human leukemia cell line HL-60 (2×10⁴
644 cells/mL, 100 µL) seeded in 96-well plates. Following a 24 h incubation period, solutions of the
645 evaluated compounds dissolved in DMSO were added to the cell culture, resulting in a final DMSO
646 concentration below 1%. CCK-8 solution (10 µL) was added after 48 h of incubation, and the plates
647 were incubated for an additional 3 h. A microplate reader (E_{max} Precision Microplate Reader, Molecular
648 Devices Japan, Tokyo, Japan) was used to record the absorbance at 436 nm.

649

650 4.5. *Hoechst 33342 staining*

651

652 For the morphological assessment of apoptosis, 100 µM of the compounds was added to the cultivated
653 HL-60 cells (2×10⁵ cells/mL) in 24-well plates, and the cells were incubated for 48 h. Next, individual
654 DMSO (10 µL) was added as the solvent control to another set of cells. The cultured medium was
655 stained with 5 µg/mL Hoechst 33342 and incubated for an additional 30 min. The excess dye was
656 discarded, and the cells obtained were washed with PBS after centrifugation before being analysed
657 under a fluorescence microscope (Axiovert 40, Carl Zeiss).

658

6594.6. UV-vis titration with ct-DNA

660

661 The stock solutions of both bis-indolylmethanes **4** and **7** were prepared at a concentration of 1 mM in
662 acetonitrile, from which the examined compounds' solutions were prepared by dilution with Tris-HCl
663 (5 mM, pH 7) buffer to achieve final concentrations of 30 μ M and 50 μ M, respectively. Calf thymus
664 DNA (ctDNA) was weighed at 0.0012 g and dissolved in 500 μ L of Tris-HCl (5 mM, pH 7), then stored
665 in a chiller overnight. The concentration of DNA was measured by the molar extinction coefficient
666 6600 M⁻¹ cm⁻¹ at 260 nm. The purity of DNA was evaluated by calculating the absorbance ratio
667 (A₂₆₀/A₂₈₀), and the DNA concentration was expressed as micromolar equivalents of the base pair.
668 The compounds were titrated with increasing concentrations of ctDNA. Each time before the analysis
669 (UV-vis) began, the system was swirled for 1 minute. The measurements were recorded using a
670 rectangular quartz cuvette of 1 cm path length. The intrinsic binding constant (K_b) was calculated by
671 substituting the obtained data into equation (1) (Li et al., 2026).

672

$$673 \quad [DNA]/(\epsilon_a - \epsilon_f) = [DNA]/(\epsilon_b - \epsilon_f) + 1 / K_b (\epsilon_b - \epsilon_f) \quad (1)$$

674

675 The symbols ϵ_a , ϵ_b , and ϵ_f denote the apparent, bound, and free extinction coefficients, respectively. Plot
676 fitting of $[DNA]/(\epsilon_a - \epsilon_f)$ vs. $[DNA]$ utilized K_b which was determined by dividing the slope ratio with
677 the Y intercept.

678

6794.7. Carcinogen behaviour (DNA Biosensor)

680

681 In order to prepare 1mM Methylene Blue (MB) solution, powdered MB was dissolved in deionized
682 water. 100 μ M of DNA stock solution was diluted to 10 μ M using potassium phosphate buffer (PBS)
683 at pH 7.0. The solution was stored at -5 °C. In this study, 24 bases of guanine-rich oligonucleotide with
684 CGG-CGG-CGG-CGG-CGG-CGG-CGG-CGG sequence were used. For the fabrication of the DNA
685 Biosensor, a screen-printed electrode (SPE) was modified with 15 μ L of colloidal gold nanoparticles
686 (0.0167 μ g/mL) and dried overnight. 10 μ L of suspended aminated SiNs (1.5 mg/mL) were dropped

evenly onto the AuNPs-modified SPE and dried for 10 to 15 min under ambient conditions. Next, SiNs-AuNPs-SPE was immersed in 10% of glutaraldehyde for 1 h, followed by an additional immersion in 10 μ M guanine-rich DNA solution (400 μ L) overnight to confirm that the full immobilization of the DNA onto the aminated SiNs had occurred. The next day, the modified SPE was rinsed with buffer solution to discard any unbound DNA, followed by immersion of the biosensor in a carcinogen or ligand solution for 10 min. The biosensor was then immersed in the MB indicator for 5 min. The biosensor was finally rinsed with buffer solution to remove any unbound MB and DNA, then scanned by DPV over the potential range from -0.4 to 0 V at a scan rate of 0.005 V/s in 0.05 M PBS (pH 7).

4.8. Docking studies

In docking simulations, AutoDock Vina was used to dock ligands into the protein active site. The Bcl-2 protein containing the native structure of navitoclax (PDB ID: 4LVT (Souers et al., 2013) was acquired from the Protein Data Bank (<http://www.rcsb.org>) of Research Collaboratory for Structural Bioinformatics and was utilised in executing the docking simulations. In the first step, the 3D structure of Bcl-2 was prepared by removing water molecules and adding hydrogens. The inhibitor binding site was then prepared for docking evaluation. Chem3D software was used to draw all compounds' structures, and their conformations were optimized via Gaussian09 software. The geometry optimization of ligands was conducted using the computational method of DFT at B3LYP/6-31G level. All docking simulations for each compound were carried out for 10 iterations by using AutoDock Vina v1.2.4 (Eberhardt et al. 2021; Trott & Olson 2010). Grid maps were arranged with $50 \times 62 \times 52$ points with a point spacing of 0.375 Å. The grid box was assigned at the centre of the protein using x,y,z coordinates of $6.021, -3.611, -5.598$, respectively. Protein-ligand interactions of the top-ranking pose of evaluated compounds towards the Bcl-2 protein were analysed and visualized using Discovery Studio version v4.5 (Dassault Systèmes, San Diego, CA).

4.9. Molecular Dynamics Simulation Protocol

To evaluate the dynamic stability of the receptor-ligand complexes, molecular dynamics (MD) simulations were performed using GROMACS 2025.2 (Abraham et al., 2015). The topologies for the Bcl-2 protein and the selected ligands (Ligands 4 and 7) were generated using the CHARMM36 force field (Huang et al., 2016). The complexes were solvated in a cubic box using the TIP3P water model and neutralized with the addition of counterions (Jorgensen et al., 1983). Prior to the production run, the systems underwent energy minimization using the steepest descent algorithm for 5,000 steps, converging when the maximum force fell below 1000.0 kJ/mol/nm. Position restraints were applied to the protein backbone (400.0 kJ/mol/nm²) and side chains (40.0 kJ/mol/nm²). Following minimization, the systems were equilibrated for 125 ps with a time step of 1 fs. The temperature was maintained at 303.15 K using the v-rescale thermostat with a coupling constant of 1.0 ps. The final production MD phase was executed for 1000 ns (1 μ s) utilizing a 2 fs time step. The system temperature was regulated at 303.15 K via the v-rescale thermostat, and the pressure was isotropically maintained at 1.0 bar using the C-rescale barostat with a compressibility of 4.5×10^{-5} bar⁻¹ and a relaxation time of 5.0 ps. Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) method, and van der Waals interactions were evaluated with a force-switch modifier and a cutoff radius of 1.2 nm. All hydrogen-containing bonds were constrained using the LINCS algorithm. To capture the large-scale collective motions of the complexes, Principal Component Analysis (PCA) was performed on the C α backbone atoms utilizing the built-in GROMACS trajectory analysis tools.

4.10. MM-PBSA Binding Free Energy Calculations

To quantify the thermodynamic binding affinity of the complexes, Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) calculations were performed. The binding free energy (ΔG_{bind}) was extracted from the 1000 ns MD trajectory using the gmx_MMPBSA tool (Valdés-Tresanco et al., 2021). A total of 1,000 structural frames were uniformly extracted across the entire 1 μ s trajectory (interval = 10) to ensure representative energetic sampling. The calculations were

executed utilizing a Poisson-Boltzmann (PB) model with a defined solvent ionic strength of 0.150 M.
Per-residue energy decomposition was also performed to evaluate individual energetic contributions.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Trial registration number: Not applicable. This study is not a clinical trial.

Availability of data and materials: The experimental data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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