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Desing of Novel 1,4-Naphthoquinone Derivatives: Evaluation of Apoptotic Effects and Anticancer Activity Against Triple Negative Breast Cancer Cells with Molecular Docking and Molecular Dynamics Studies

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Abstract

Worldwide, the number of deaths due to cancer is steadily increasing. Breast cancer is the most common type of cancer in the female population. Triple-negative breast cancer (TNBC) is one of the most aggressive subtypes of breast cancer and has a limited response to current treatments. Naphthoquinone compounds are a class of molecules with high anticancer potential, exhibit various biological activities such as antioxidant, anticancer, and antimicrobial effects.

In this study on quinone derivative compounds, *N,S,O*-substituted-1,4-naphthoquinone derivatives were synthesized as a result of reactions carried out between the compound 2,3-dichloro-1,4-naphthoquinone (**1**) and nucleophiles (**2, 3, 6, 7, 10**). All synthesized compounds were characterized by FT-IR, ^1H -/ ^{13}C -NMR and MS. It is our first study that the desining of *N,S,O*-substituted-1,4-Naphthoquinone derivatives and we evaluated of their apoptotic effects and anticancer activity against triple negative breast cancer cells with using molecular docking and molecular dynamics method. The anticancer activities of these compounds were determined in MDA-MB-231 triple-negative breast cancer (TNBC) cells using the CCK-8 assay, and their apoptotic activities were evaluated by Annexin V analysis and acridine orange/propidium iodide staining. All derivatives suppressed cell proliferation; however, compounds **4** and **13** showed the greatest effect against TNBC cells, with $\text{IC}_{50} = 2.7 \mu\text{g/mL}$ and $\text{IC}_{50} = 3 \mu\text{g/mL}$, respectively. Of the derivatives obtained, compounds **4** and **13** induced early apoptotic death, while compounds **11** and **12** led to late apoptotic death in TNBC cells compared to the control group. The AO/PI staining verified the apoptotic morphology of the cells and compounds **9, 11**, and **13** induced a greater formation of apoptotic bodies in the cells. The interactions of compound **4** and compound **13** with the MCL-1 protein responsible of apoptosis were analyzed using molecular docking followed by molecular dynamics simulations. A stronger docking score was observed for compound **13** (-8.4 kcal/mol) compared to compound **4** (-7.2 kcal/mol).

Furthermore, MD simulations were performed to shed light on the molecular dynamics behavior of both complexes. MCL1-4 and MCL1-13 both remained stable during simulations, with RMSD values below 3 Å. Higher positional stability was seen for compound **13**, while compound **4** showed slightly more flexibility within the binding pocket. The protein structure remained stable in both cases, and reduced residue fluctuations were observed around compound **4**, suggesting local stabilization. MM-GBSA results indicated a more favorable binding free energy for compound **4** (−52.18 kcal/mol) compared to compound **13** (−38.76 kcal/mol).

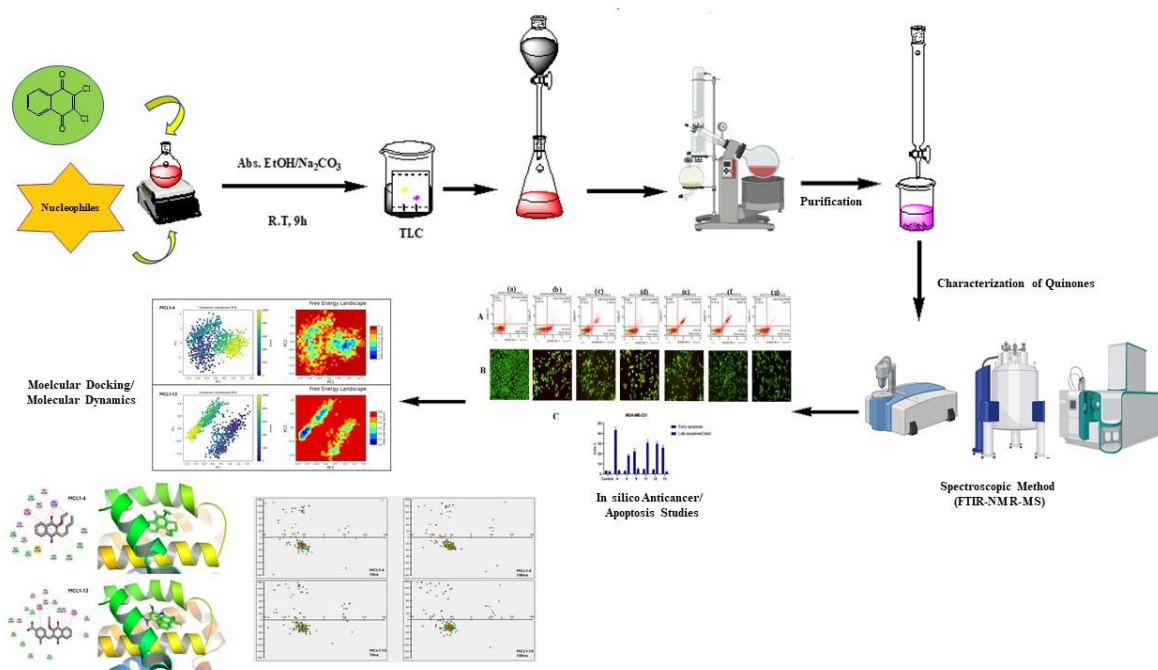
Keywords: 1,4-Naphthoquinones; Anticancer activity; Apoptosis effects; Molecular docking; Molecular dynamics simulations

Highlights

- *N,S,O*-substituted-1,4-naphthoquinone derivatives were synthesized via Michael addition mechanism.
- Structural characterization was confirmed by FT-IR, ¹H-NMR, ¹³C-NMR, MS.
- The anticancer activities of these compounds were determined in MDA-MB-231 triple-negative breast cancer (TNBC) cells using the CCK-8 assay.
- Their apoptotic activities were evaluated by Annexin V analysis and acridine orange/propidium iodide staining.
- The interactions of **4** and **13** with the MCL-1 protein responsible of apoptosis were analyzed using molecular docking and dynamics simulations.

Graphical Abstract

Desing of Novel 1,4-Naphthoquinone Derivatives: Evaluation of Apoptotic Effects and Anticancer Activity Against Triple Negative Breast Cancer Cells with Molecular Docking and Molecular Dynamics Studies



1. Introduction

Cancer is known as the uncontrolled proliferation of cells and is one of the leading causes of death worldwide. Due to increased life expectancy, environmental factors, and unhealthy lifestyle habits, a continuous rise has been observed in the number of new cases and deaths [1]. In 2020, as many as 19.3 million new cancer cases were recorded, and it is estimated that this number will rise significantly in the coming years [2]. Therefore, researchers are working to develop new drugs for cancer treatment [3, 4, 5].

One of the most prevalent cancerous conditions affecting women is breast cancer. Breast cancer, which remains a significant global public health problem, exhibits a highly heterogeneous structure in terms of its clinical and biological characteristics. This heterogeneity leads to a variety of treatment approaches and differences in disease course among patients [6]. Triple-negative breast cancer (TNBC) is characterized by lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2), and is a type of breast cancer that makes up a certain percentage of all breast cancers, with the highest recurrence rate and poor prognosis among breast cancer subtypes. [7, 8].

Apoptosis, a form of programmed cell death (PCD), is crucial for maintaining tissue balance and proper function. This process is controlled by genes and occurs in a regulated manner. During apoptosis, the cell's nucleus breaks down, its cytoplasm shrinks, and the cell disintegrates into small bodies, disappearing without harming the environment [9]. Escape from apoptosis plays a very important role in progression of TNBC. Apoptosis promotes the elimination of malignant cells through highly regulated signaling pathways. Disruptions in apoptotic processes cause tumor growth and progression, leading to the development of treatment resistance [10].

Quinones are secondary metabolites found in the kingdoms of fungi, bacteria, animals and plants. They are natural compounds with pharmacological properties that are involved in many important biological processes. Quinone compounds are derived from phenol oxidation and are known for containing two carbonyl groups conjugated with at least two double bonds. They are classified as benzoquinones, naphthoquinones, anthraquinones, and phenanthraquinones [11].

Bicyclic structures in which two carbonyl groups are located in the 1,4 or 1,2 positions form naphthoquinones (NQs). Studies on 1,4-naphthoquinone compounds are much more numerous than those on 1,2-naphthoquinones. 1,4-naphthoquinone compounds and their derivatives have an extensive range of biological activities such as antibacterial [12], antiviral [13], anticancer [14], antioxidant [15], antifungal [16], antiplasmodial [17], and anti-inflammatory activity [18-19]. The chemical and biological behavior of quinones varies according to the type, structure and position of the side groups (R) (such as hydroxyl, hydrogen, nitrogen, methyl, sulfur, halogen) attached to them [20]. In addition to natural compounds such as Lawsone, Juglone, Plumbagin and Lapachol, which have a 1,4-Naphthoquinone skeletal system; various synthetic derivatives such as Atovaquone, Menadion (Vitamin K₃) and Plasmodione (Figure 1) also show a wide range of biological activities and occupy an important place in pharmaceutical research [21-22].

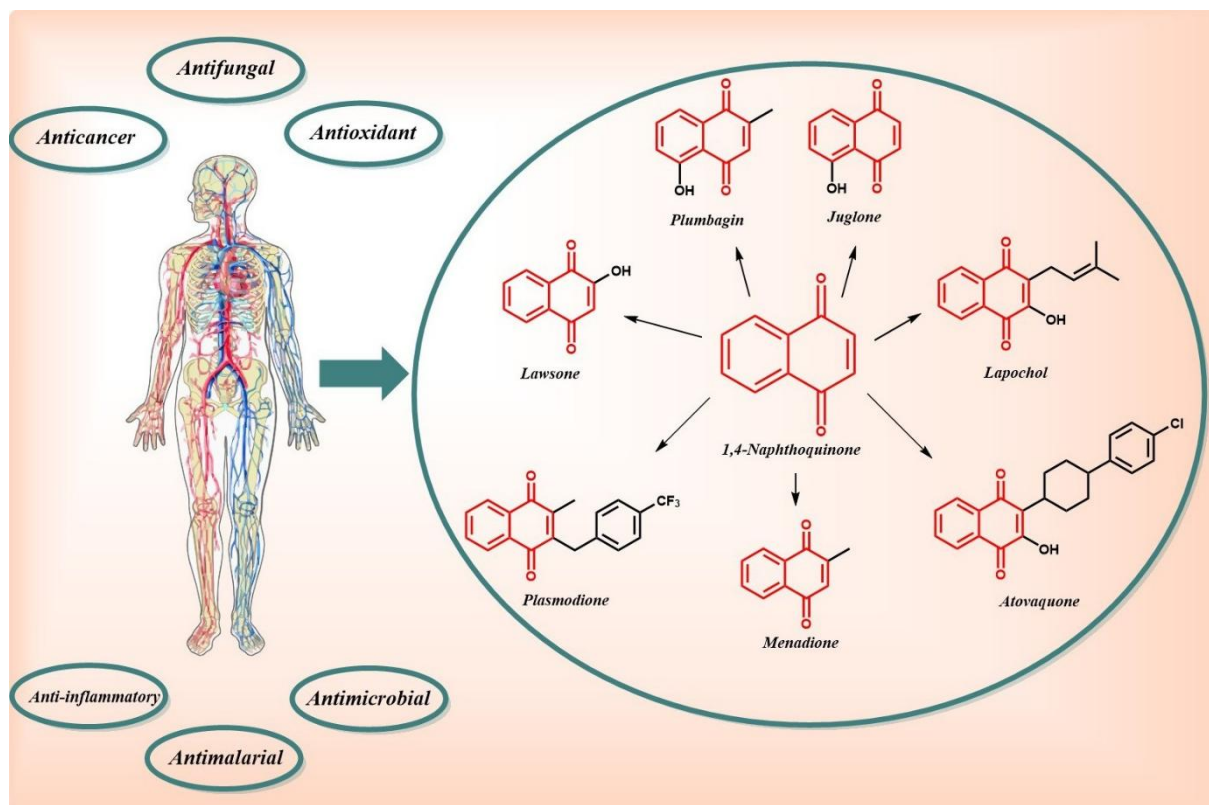


Figure 1. Biological activities of some natural and synthetic 1,4-naphthoquinone derivatives

This study aimed to synthesize seven 1,4-naphthoquinone derivatives using different amine derivatives, as shown in Scheme 1. To evaluate the anticancer activities of the synthesized naphthoquinone derivatives, a CCK-8 assay was performed on MDA-MB-231 triple-negative breast cancer (TNBC) cells. Annexin V analysis and acridine orange/propidium iodide staining were also performed to investigate the apoptosis effects of the compounds. It is our first study that the designing of *N,S,O*-substituted-1,4-Naphthoquinone derivatives and we evaluated of their apoptotic effects and anticancer activity against triple negative breast cancer cells with using molecular docking and molecular dynamics method.

The molecular docking and molecular dynamics approaches were simulation technique used to predict potential interactions in drug development processes. This method allows for the determination of interactions and binding affinities between drug candidate molecules and

their target receptors. Drug development efforts can benefit from generating a prediction profile by analyzing the interactions between disease-related macromolecules and potential therapeutic compounds, providing valuable insights to complement experimental studies. Given the anticancer properties of 1,4-NQ derivatives, molecular docking and molecular dynamics studies were conducted to explore the interactions between newly synthesized 1,4-Naphthoquinone substituted phenylpiperazine and thioether derivatives and various cancer-related targets in this study.

2. Material and methods

2.1. Experimental section

The melting points of all compounds were measured with Büchi B-540 melting point apparatus. FTIR spectra (cm^{-1}) were recorded as KBr pellets in nujol mulls on a Shimadzu IR Prestige 21 model Diamond spectrometer (ATR method). ^1H NMR and ^{13}C NMR spectra of the molecules were recorded on a Varian Unity Inova (500 MHz) spectrometer. TMS was used as the internal standard and deuterated chloroform (CDCl_3) was used as the solvent during the measurements. 60 Å silica gel (Merck 7734) was used as the stationary phase in column chromatography. Thin-layer chromatography (TLC) analyses were carried out using 60 Å silica gel on F254 aluminum plates (Merck 5554). Mass (MS) spectra were obtained using the Thermo Finnigan LCQ Advantage MAX LC/MS spectrometer according to the ESI probe. instrument. All chemicals were reagent grade and used without further purification. Moisture was excluded from the glass apparatus using CaCl_2 drying tubes. Solvents were of reagent grade unless otherwise stated and were distilled once before use.

2.2. Chemistry

All solvents used were dried and distilled before use. Column chromatography was performed on silica gel using various gradient eluent mixtures. All chemicals were reagent grade and used without further purification. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) (0 ppm) as a reference. Data are reported as: chemical shifts, multiplicity (s= singlet, d = doublet, t = triplet, q = quartet, m = multiplet).

2.3. Synthesis procedures

2.3.1. General procedure for the synthesis of compounds (4, 5, 8, 9, 11, 12, 13)

Compounds (4, 5, 8, 9, 11, 12, 13) formed by adding different aniline structures and thiol structures to the naphthoquinone skeleton were synthesized as shown in Scheme 1. 2,3-Dichloro-1,4-naphthoquinone compound was used as the naphthoquinone skeleton. Initially, sodium carbonate (1.52 g) dissolved in ethanol (45 mL) was added to 2,3-Dichloro-1,4-naphthoquinone (1) compound dissolved in ethanol. Different aniline and thiol derivatives (2, 3, 6, 7, 10) were dissolved in ethanol and added to the reaction medium. The solution was stirred at room temperature for 24 h and the mixture was terminated by TLC control. The reaction mixture was extracted with dichloromethane (10 mL x 3). The organic phase was dried over anhydrous Na₂SO₄ and an evaporator system was used to remove excess solvent. The crude dark solid obtained was purified by column chromatography and dried in a vacuum oven. The obtained *S,O*-, *N,O*-, *N*- and *N,S*- substituted naphthoquinone derivatives (4, 5, 8, 9, 11, 12, 13)

were elucidated by various spectroscopic methods such as FTIR, ^1H NMR, ^{13}C NMR, ^{13}C -APT NMR and MS.

2.3.2 Synthesis of 2-(butylthio)-3-ethoxynaphthalene-1,4-dione (**4**)

Compound **4** was synthesized from the reaction of **1** (2.0 g, 8.8 mmol) with **2** (0.795 g, 8.8 mmol) according to the synthesis procedure. Yield: 80%; Red oil; R_f = 0.40 (CHCl_3); FT-IR (KBr pellet, cm^{-1}): 3071 (CH_{arom} , 2959, 2929, 2872 (C-H), 1662 (C=O), 193 (C=C); ^1H NMR (499.74 MHz, CDCl_3 , ppm): δ = 0.95 (t, J = 8.34 Hz, 3H, CH_3), 1.21 (t, J = 7.32 Hz, 3H, CH_3), 1.44 (m, 2H, $-\text{CH}_2$), 1.66 (m, 2H, $-\text{CH}_2$), 3.13 (t, J = 6.75 Hz, 2H, S- CH_2), 4.22 (q, J = 6.21 Hz, 2H, O- CH_2), 7.60-8.25 (m, 4H, H_{arom}); ^{13}C NMR (125.66 MHz, CDCl_3): δ = 14.8, 15.0 (CH_3), 20.8, 28.6 (CH_2), 31.2 (S- CH_2), 68.8 ($-\text{OCH}_2$), 125.3, 125.5, 125.8, 125.9, 132.5, 132.5 (CH_{arom} , C_{arom}), 133.2 ($=\text{C-S}$), 156.7 ($=\text{CO}$), 177.9, 181.8 ppm (C=O); MS [+ESI]: m/z 291.0 $[\text{M}]^+$ for $\text{C}_{16}\text{H}_{18}\text{O}_3\text{S}$ (M_w , 290.38 g/mol).

2.3.3 Synthesis of 2-(butylthio)-3-(*m*-tolylamino)naphthalene-1,4-dione (**5**)

Compound **5** was synthesized from the reaction of **1** (2.0 g, 8.8 mmol) with **3** (0.9 g, 8.8 mmol) according to the synthesis procedure. Yield: 10%; Red oil; R_f = 0.26 (CHCl_3); FT-IR (KBr, cm^{-1}): 3327, (N-H), 3017 (CH_{arom}), 2961, 2925, 2853 (C-H), 1663 (C=O), 1592, 1551, 1512 (C=C), 760 ($\text{C}_{\text{arom-S}}$), 667 (C-S); ^1H NMR (499.74 MHz, CDCl_3 , ppm): δ = 0.95 (t, J = 7 Hz, 3H, CH_3), 1.18-1.32, 1.39-1.63 (m 4H, CH_2), 2.34 (s, 3H, $\text{C}_{\text{arom-CH}_3}$), 2.51-2.54 (t, 2H, J = 7.5 Hz, S- CH_2), 6.83 (s, 1H, NH), 6.93-8.12 (m, 8H, CH_{arom}); ^{13}C NMR (125.66 MHz, CDCl_3 , ppm): δ = 13.2 (CH_3), 21.7 ($\text{C}_{\text{arom-CH}_3}$), 22.1 ($-\text{CH}_2-$), 31.7 ($-\text{CH}_2-$), 33.7 (S- CH_2), 123.3 ($=\text{C-S}$), 138.6 ($=\text{C-NH}$), 102.5, 107.9, 119.7, 125.7, 126.9, 127.01, 128.5, 130.9, 132.9, 133.8, 134.7

(CH_{arom}, C_{arom}), 145.4 (C_{arom}-NH), 178.1, 179.2 (C=O); MS [+ESI]: m/z 352.2 [M]⁺ for C₂₁H₂₁NO₂S (M_w, 351.46 g/mol).

2.3.4 Synthesis of 2-chloro-3-((4-fluorophenyl)amino)naphthalene-1,4-dione (**8**)

Compound **8** was synthesized from the reaction of **1** (2.0 g, 8.8 mmol) with **6** (0.98 g, 8.8 mmol) according to the synthesis procedure. Yield: 68%; Dark red crystal solid; mp: 237.6-238.0°C, R_f = 0.67 (PET/CHCl₃ 1:1); FT-IR (ATR, cm⁻¹): 3423 (N-H), 3019 (CH_{arom}), 1671, 1635 (C=O), 1592, 1564 (C=C), 1215 (C-N); ¹H NMR (500 MHz, CDCl₃/DMSO-d₆, ppm): δ = 4.42 (s, 1H, N-H), 7.02-8.23 (m, 8H, H_{arom}); ¹³C NMR (125 MHz, CDCl₃, ppm): δ = 113.5, 125.3, 125.4, 125.9, 126.2, 128.7, 131.5, 131.9, 132.4, 134.1, 158.6, 160.6 (CH_{arom}, C_{arom}, C_{arom}-F), 114.4 (=C-Cl), 140.6 (=C-NH), 176.4, 179.4 (C=O); MS [-ESI]: m/z, 300.2 [M]⁻ for C₁₆H₉O₂NFCl (M_w, 301.70 g/mol).

2.3.5 Synthesis of 2-((4-fluorophenyl)amino)-3-((4-methyl-2-oxo-2H-chromen-6-yl)thio)naphthalene-1,4-dione (**9**)

Compound **9** was synthesized from the reaction of **1** (2.0 g, 8.8 mmol) with **6** (0.98 g, 8.8 mmol) and **7** (1.69 g, 8.8 mmol) according to the synthesis procedure. Yield: 25%; Dark red solid; mp: 181.7-182.5°C, R_f = 0.43 (PET/CHCl₃ 1:1); FT-IR (KBr, cm⁻¹): 3259 (N-H), 3061 (CH_{arom}), 2981 (C-H), 1717, 1673 (C=O), 1593, 1538 (C=C), 725 (C_{arom}-S); ¹H NMR (500 MHz, CDCl₃, ppm): δ = 2.32 (s, 3H, CH₃), 5.36 (s, 1H, N-H), 6.13 (s, 1H, =CH), 7.03-8.26 (m, 11H, H_{arom}); ¹³C NMR (125 MHz, CDCl₃, ppm): δ = 18.6 (CH₃), 114.8 (=CH), 108.2, 115.0, 117.4, 119.0, 122.0, 122.6, 125.2, 126.1, 127.1, 127.3, 130.1, 132.5, 133.3, 135.3, 159.7 (CH_{arom}, C_{arom}, C_{arom}-F), 140.5 (C_{arom}-NH), 145.4 (=C-NH), 124.3 (=C-S), 133.1 (C_{arom}-S),

152.0 (=C-CH₃), 153.5 (=C-O), 160.4 (C=O), 180.4, 180.4 (C=O); MS [-ESI]: m/z 456.2 [M]⁻ for C₂₆H₁₆FNO₄S (M_w, 457.48 g/mol).

2.3.6 Synthesis of 2-((2-amino-4-nitrophenyl)amino)-3-chloronaphthalene-1,4-dione (**11**)

Compound **11** was synthesized from the reaction of **1** (2.0 g, 8.8 mmol) with **10** (1.35 g, 8.8 mmol) according to the synthesis procedure. Yield: %24; Dark red solid; mp:254.5-255.0°C, R_f= 0.68 (PET/EtOAc 5:1); FT-IR (KBr, cm⁻¹): 3482, 3313 (NH₂), 3358 (N-H), 3019 (CH_{arom}), 1665, 1651 (C=O), 1598, 1570 (C=C), 1518 (NO₂); ¹H NMR (500 MHz, CDCl₃, ppm): δ= 4.15 (d, 2H, NH₂), 5.28 (s, 1H, NH), 6.71-8.27 (m, 7H, CH_{arom}); ¹³C NMR (125 MHz, CDCl₃, ppm): δ= 108.7, 120.6, 124.7, 126.0, 126.2, 128.7, 130.2, 131.2, 131.5 (CH_{arom}, C_{arom}), 117.4 (C-Cl), 132.0 (C_{arom}-NH), 132.1 (C_{arom}-NH₂), 134.1 (C_{arom}-NO₂), 141.7 (=C-NH), 176.2, 179.2 (C=O); MS [+ESI]: m/z 344.2 [M]⁺ for C₁₆H₁₀ClN₃O₄ (M_w, 343.72 g/mol).

2.3.7 Synthesis of 2,3-bis((2-amino-4-nitrophenyl)amino)naphthalene-1,4-dione (**12**)

Compound **12** was synthesized from the reaction of **2** (1.0 g, 8.8 mmol) with **10** (1.35 g, 8.8 mmol) according to the synthesis procedure. Yield: 54%; Brownish-yellow solid; mp:88.0-88.6°C, R_f= 0.32 (PET/EtOAc 5:1); FT-IR (KBr, cm⁻¹): 3424 (NH₂/N-H), 3019 (CH_{arom}), 1674 (C=O), 1596, 1570 (C=C), 1473 (NO₂); ¹H NMR (500 MHz, CDCl₃, ppm): δ= 4.54, 4.57 (m, 4H, NH₂), 5.32 (m, 2H, NH), 7.63-8.06 (m, 10H, CH_{arom}); ¹³C NMR (125 MHz, CDCl₃, ppm): δ=124.2, 124.9, 125.8, 125.9, 128.2, 129.7 (CH_{arom}, C_{arom}), 130.1 (=C-NH), 132.8 (C_{arom}-NH), 133.2 (C_{arom}-NH₂), 155.5 (C_{arom}-NO₂), 178.7, 177.6 (C=O); MS [+ESI]: m/z 461 [M]⁺ for C₂₂H₁₆N₆O₆ (M_w, 460.41 g/mol).

2.3.8 Synthesis of 2-((2-amino-4-nitrophenyl)amino)-3-ethoxynaphthalene-1,4-dione (**13**)

Compound **13** was synthesized from the reaction of **2** (2.0 g, 8.8 mmol) with **10** (1.35 g, 8.8 mmol) according to the synthesis procedure. Yield: 22%; Red solid; mp: 187.0-187.6°C, R_f = 0.56 (PET/EtOAc 5:1); FT-IR (KBr, cm^{-1}): 3440 (NH_2), 3280 (N-H), 3103, 3012 (CH_{arom}), 2955, 2922, 2850 (C-H), 1682, 1637 (C=O), 1600, 1568, 1508 (C=C), 1444 (NO_2); ^1H NMR (500 MHz, CDCl_3 , ppm): δ = 1.17 (t, J = 7.5 Hz, 3H, CH_3), 3.72 (q, J = 7 Hz, 2H, CH_2), 4.68 (m, 2H, $-\text{NH}_2$), 5.22 (d, J = 3 Hz, -NH), 6.11-6.28 (d, J = 1.5 Hz, 1H, H_{arom}), 6.68-8.12 (m, 7H, H_{arom}); ^{13}C NMR (125 MHz, CDCl_3 , ppm): δ = 17.6 (CH_3), 69.7 (CH_2), 113.0, 118.6, 126.1, 126.3, 129.1, 132.1, 134.3, 139.5, 140.4, (CH_{arom} , C_{arom}), 144.4 ($=\text{C}-\text{NH}$), 151.0 ($\text{C}_{\text{arom}}-\text{NH}$), 152.5 ($\text{C}_{\text{arom}}-\text{NH}_2$), 157.5 ($\text{C}_{\text{naphtha}}-\text{OC}_2\text{H}_5$), 160.6 ($\text{C}_{\text{arom}}-\text{NO}_2$), 182.0, 182.8 (C=O); MS [-ESI]: m/z 335.2 [$\text{M}-(\text{NH}_2+2\text{H})$] $^-$ for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$ (M_w , 353.33 g/mol).

2.4. Anticancer activity

2.4.1 CCK-8 proliferation assay

CCK-8 analysis was conducted to detect the cytotoxic effects of 1,4-naphthoquinone derivatives in MDA-MB-231 triple-negative breast cancer (TNBC) cells. MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified incubator.

For viability assay, 2×10^4 cells/well were cultured in 96-well plates and treated with 1, 2.5, 5 and 10 $\mu\text{g/mL}$ concentrations of the synthesized derivatives for 24 h. Then the cells were incubated with CCK-8 reagent for 45 min at 37°C. A microplate reader measured the absorbance at 450 nm. All tests in cell lines were accomplished in triplicate.

2.4.2 Annexin V analysis and Acridine orange/propidium iodide for apoptosis

For Annexin V analysis, the cells were seeded at 1×10^5 /well in a 6-well plate and incubated with 2.5 $\mu\text{g/mL}$ **4**, **8**, **9**, **11**, **12**, and **13** due to CCK-8 analysis. Following incubation, the cells were stained with Annexin V & Dead Cell Assay kit for 30 min after centrifugation. Stained cells were analyzed using Muse Cell Analyzer (Millipore, Germany) (n=3). To detect apoptotic morphological changes induced by the derivatives, MDA-MB-231 cells (5×10^5) were cultured in a six-well cell culture and incubated with **4**, **8**, **9**, **11**, **12**, and **13** for 24h. Then, the cells were fixed with 4% paraformaldehyde and then stained with acridine orange (AO)/ propidium iodide (PI) for 30 min and observed by the EVOS FL Cell Imaging System (Thermo Fisher Scientific, USA).

2.4.3 Statistical analysis

Statistical analysis was performed by GraphPad Prism. Multiple comparisons among the cells were analyzed by the one-way Analysis of Variance (ANOVA) using the Post-Hoc Tukey test. The $p < 0.05$ and $p < 0.01$ were regarded as statistically significant.

2.5 Computational Calculations

Multiple computational tools were utilized to study the interaction of the top hits compounds **4** and **13** within the biological environment. Furthermore, a literature review was performed to identify suitable therapeutic targets within the apoptosis pathway. This pathway was selected due to its frequent dysregulation in cancer and the well-known ability of cancer cells to evade apoptotic signals.

2.5.1 Molecular Docking

Myeloid cell leukemia 1 (MCL-1) is an anti-apoptotic member of the BCL-2 protein family that serves as a regulator of cell survival [33]. While its canonical function involves sequestering pro-apoptotic proteins at the mitochondrial membrane to prevent programmed cell death, MCL-1 is also implicated in non-canonical roles such as DNA replication and metabolic regulation[34]. The MCL1 gene is frequently amplified or overexpressed in a wide range of human malignancies, including triple-negative breast cancer and non-small cell lung cancer, where it drives tumorigenesis and confers resistance to conventional therapies. MCL-1 was selected as the target for this study because it emerged from the literature as a consistently amplified and clinically relevant survival factor, making it a high-priority candidate for therapeutic intervention [35]. To shed light on the interaction of MCL1 protein with the tested hits we used AutodockVina4 [36] to dock the structures inside the proteins catalytic site.

The crystal structure of the protein (PDB ID: 6OQD) was retrieved from the RCSB Protein Data Bank and prepared using AutoDock Tools 1.2.5 [36]. Preparation steps included the addition of polar hydrogens, calculation of Kollman partial charges, and the removal of water molecules and heteroatoms (co-ligands).

The preparation of the two top hits 4 and 13 was done by optimization of the three-dimensional structures built in GaussView 5.0 [37] using Gaussian09W software [38]. The final optimized structures were converted to pdbqt files before docking.

2.5.3 Molecular Dynamics:

Using the output of molecular docking simulations, the protein-ligand complexes pdb files were obtained using Biovia Discovery Studio [39]. For the Molecular Dynamics (MD)

simulations, we employed the Desmond software package [40]. A total of three independent simulations were performed: one involving the apo form of the protein, one with the protein in complex with compound **4**, and another with the protein in complex with compound **13**. Each system was placed in a cubic box measuring 100 x 100 x 100 Å and solvated using the TIP3P water model, ensuring a minimum distance of 10 Å between the protein and the box boundaries. All simulations were carried out under physiological conditions at pH 7.0 using an NPT ensemble, with temperature maintained at 303.15 K and pressure at 1 atm. The inclusion of periodic boundary conditions allowed for accurate modeling of solvation effects and ionization states throughout the simulation.

3. Results and Discussion

3.1. Chemistry

The synthesis pathway of heteroatom substituted 1,4-naphthoquinone derivatives as (**4**, **5**, **8**, **9**, **11**, **12**, **13**) was illustrated in Scheme 1. Compounds **5**, **9**, **12** and **13** are new compounds, while compounds **4** [23], **8** [24] and **11** [25] are known compounds that have been previously reported in the literature. Compounds **4** and **8** are that have been synthesized in our previous studies. Compound **11** was synthesized differently from the procedure in the literature.

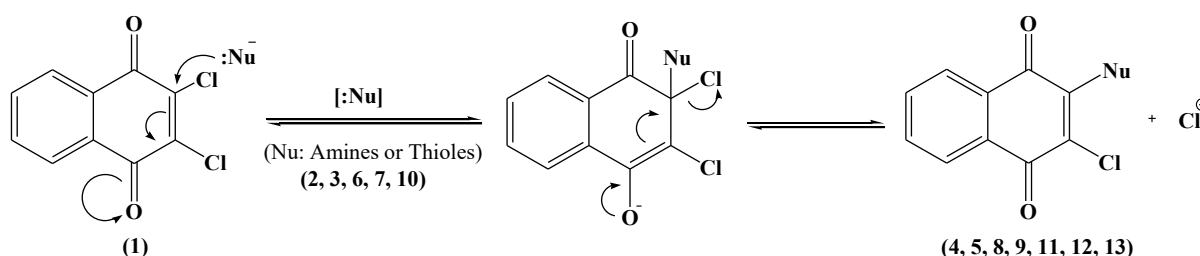
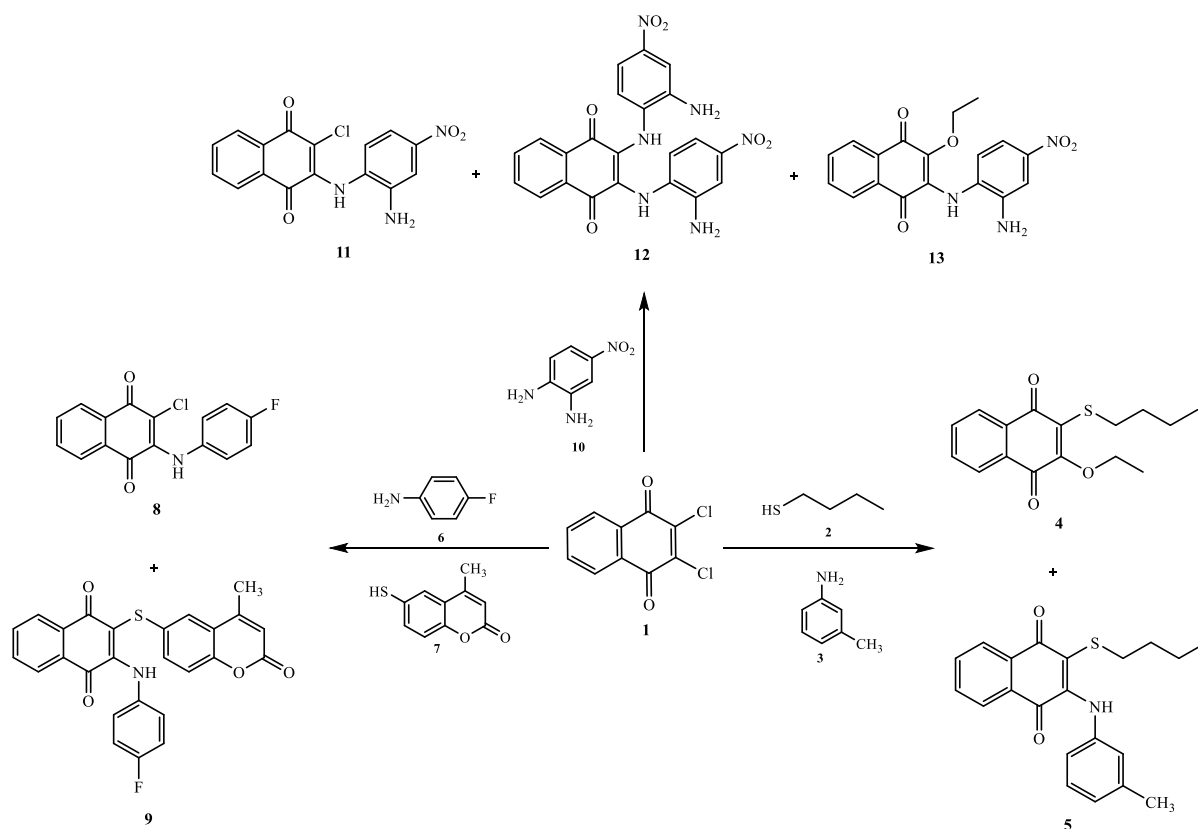


Figure 2. The proposed mechanism of synthesized compounds **4**, **5**, **8**, **9**, **11**, **12**, **13**.

The starting material, 2,3-dichloro-1,4-naphthoquinone (**1**), is an asymmetric Michael acceptor and exhibits a structure suitable for nucleophilic substitution reactions on the electrophilic sp^2 hybridized carbon atom. Therefore, by undergoing Michael-type addition reactions with various amines and thiols, it allows for the synthesis of the corresponding derivatives [26-28]. The compound 2,3-dichloro-1,4-naphthoquinone (**1**) was reacted with various amine and thiol derivatives (**2**, **3**, **6**, **7**, **10**) through the Michael 1,4-addition mechanism shown in Figure 2, resulting in the synthesis of compounds (**4**, **5**, **8**, **9**, **11**, **12**, **13**). The yields of the compounds we obtained as a result of the reactions vary between 10% and 80%.



Scheme 1. The reactions of 2,3-dichloro-1,4-naphthoquinone (**1**) with amines and thiols **2**, **3**, **6**, **7**, **10**

The synthesis of compounds **4**, **5**, **8**, **9**, **11**, **12** and **13** was carried out as shown in scheme 1. The obtained compounds were synthesized with high purity. When the FTIR spectra of the

compounds were examined, peaks belonging to the stretching bands of the two carbonyl groups (C=O) in the structures were observed in the range of 1633-1717 cm^{-1} . All of the synthesized compounds contain an aromatic ring, and the peaks corresponding to the aromatic C-H stretching bands were detected in the range of 3017–3165 cm^{-1} . Compounds **5**, **8**, **9**, **11**, **12** and **13** contain -NH and NH_2 groups in their structure. Peaks belonging to the -NH and NH_2 stretching bands were detected in the range of 3280-3482 cm^{-1} .

When the ^1H NMR spectra of the synthesized compounds were examined, the aromatic proton signals were recognized in the range of $\delta = 6.1\text{--}8.2$ ppm, as expected. The proton signals of the ethoxy group ($-\text{OCH}_2\text{CH}_3$) in the structure of compound **4** and compound **13** were recognized in the range of $\delta = 3.7\text{--}4.2$ ppm. The signals of -NH protons in the compounds were recognized in the range of $\delta = 4.4\text{--}6.8$ ppm and the signals of NH_2 protons were observed in the range of $\delta = 4.1\text{--}4.6$ ppm.

The ^{13}C NMR spectra showed that the carbonyl group signals of the compounds fell within the $\delta = 176.3\text{--}182.8$ ppm. Compound **9** has three carbonyl carbons in its structure. The third carbonyl carbon of compound **9** gave a signal at 160.46 ppm. The structure of compound **12** is a symmetric compound, and a single signal actually corresponds to two carbon atoms. The signals of the carbon atoms attached to the ($-\text{NH}$) group in compounds **5**, **8**, **9**, **11**, **12**, **13** were observed in the range of $\delta = 130.1\text{--}145.4$ ppm. The signals of the carbon atoms bonded to the sulfur atom in compounds **4**, **5** and **9** were given at 133.2, 123.3 and 124.3 ppm, respectively. The C-Cl peak in compounds **8** and **11** gave signals at 114.5 and 117.4 ppm, respectively.

The ion peaks obtained by the Mass Spectrum (MS) method of the synthesized compounds are as follows, respectively: m/z (%) $m/z = 291.0$ $[\text{M}]^+$ (Comp. **4**), 352.2 $[\text{M}]^+$ (Comp. **5**), 300.2 $[\text{M}]^-$ (Comp. **8**), 456.2 $[\text{M}]^-$ (Comp. **9**), 344.2 $[\text{M}]^+$ (Comp. **11**), 461.0 $[\text{M}]^+$ (Comp. **12**), 335.2 $[\text{M}-(\text{NH}_2+2\text{H})]^+$ (Compound **13**).

3.2 The assessment of cytotoxicity in TNBC cells

The CCK-8 assay was performed to evaluate the cytotoxicity of the derivatives in MDA-MB-231 cells. MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). According to our results (Figure 3), all derivatives significantly decreased the cell viability. Upon treatment with 1 µg/mL concentrations of **4**, **8**, **9**, **11**, **12**, and **13**, the inhibition rates of the cells were 31.3%, 37.1%, 39.9%, 32.1%, 42.8%, and 23.5%, respectively ($p < 0.05$). However, upon treatment with 2.5 µg/mL, cell viability was considerably reduced to 54.4%, 58.2%, 65.3%, 60.6%, 60.4%, and 57.5%, respectively ($p < 0.01$). Higher concentrations (5 and 10 µg/mL) were less effective than lower concentrations due to the aggressive behaviours of TNBC cells. Therefore, 2.5 µg/mL concentration was selected for further analysis because the inhibition rate was close to the IC₅₀ dose. The IC₅₀ concentration of **4**, **8**, **9**, **11**, **12**, and **13** was 2.7, 5.1, 50, 3.9, 14.9, and 3 µg/mL, respectively. Therefore, the most potent 1,4-naphthoquinone compounds were **4** and **13** due to lower IC₅₀ dose.

3.3 The assessment of apoptotic death in TNBC cells

To investigate the induction of apoptotic death by 1,4-naphthoquinone derivatives, Annexin V analysis and AO/PI staining were conducted. In Figure 4, 1,4-naphthoquinone derivatives induced early/late apoptosis in TNBC cells. Upon treatment with 2.5 µg/mL concentrations of **4**, **8**, **9**, **11**, **12** and **13**, the total apoptotic rate increased from 5.8% to 45.3%, 20.7%, 26.5%, 34.5%, 34.3% and 27.4%, respectively ($p < 0.05$). However, **4** and **13** induced more early apoptotic death, and **11** and **12** caused more late apoptotic death in TNBC cells compared with the control group.

To observe apoptotic morphology of the cells, AO/PI staining was performed as shown in Figure 4 1,4-naphthoquinone derivatives particularly induced nuclear damage and loss of cell interactions in TNBC cells. Furthermore, **9**, **11**, and **13** caused more apoptotic bodies in the cells. Our results showed that 1,4-naphthoquinone derivatives significantly inhibited the proliferation of TNBC cells and induced apoptotic death.

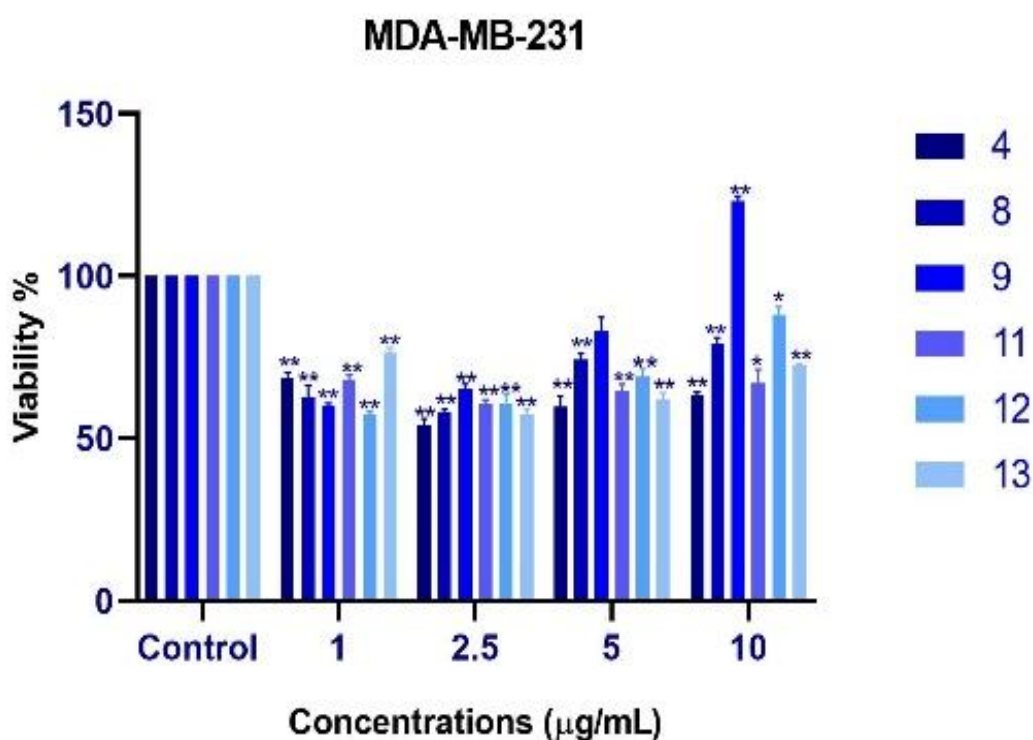


Figure 3. The cytotoxic effects of 1,4-naphthoquinone derivatives on MDA-MB-231 cells. (A) (a) The proliferation rate of the cells upon treatment with different concentrations (1-10 µg/mL) of compounds **4**, **8**, **9**, **11**, **12** and **13** for 24 h ($p < 0.05^*$, $p < 0.01^{**}$) by CCK-8 analysis.

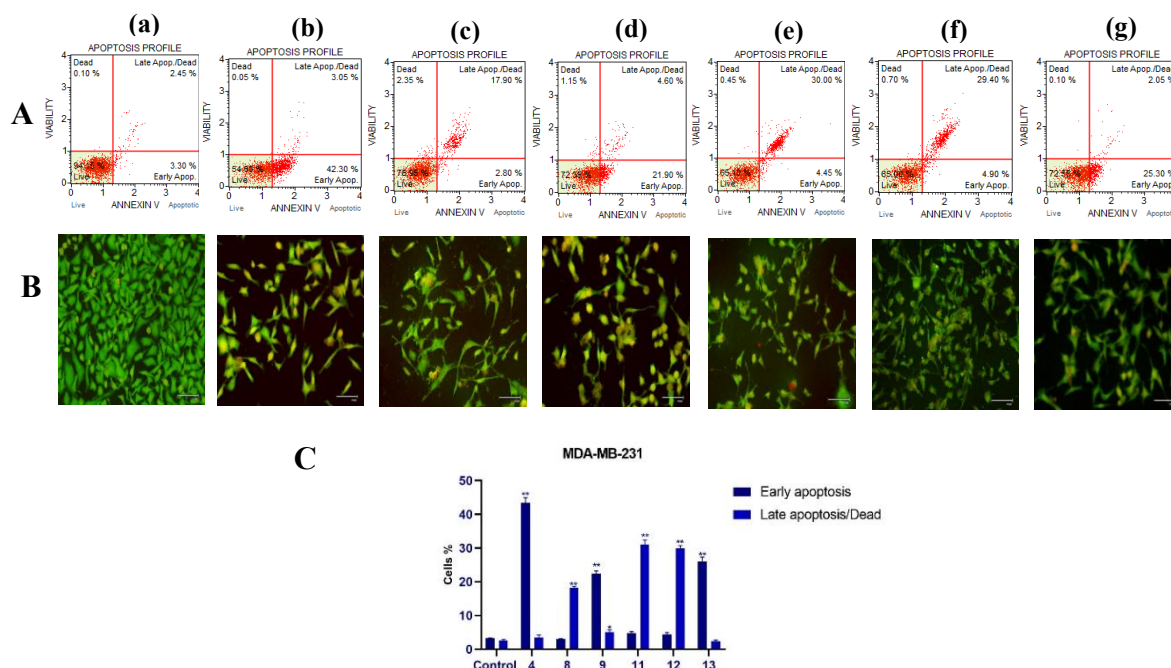


Figure 4. The apoptotic effects of 1,4-naphthoquinone derivatives on MDA-MB-231 cells. (A) Histograms of Annexin V analysis (a) Control, (b) Comp. 4, (c) Comp. 8, (d) Comp. 9, (e) Comp. 11, (f) Comp.12 and (g) Comp. 13. (B) Statistical comparison of early and late apoptotic cell death in the cells by Annexin V analysis ($p < 0.05^*$, $p < 0.01^{**}$). The cells treated with 2.5 $\mu\text{g/mL}$ for 24 h. (C) AO/PI staining results in TNBC cells.

In the study of Wang et al. (2019), two novel types of 1,4-naphthoquinone derivatives, including 2,3-dihydro-2,3-epoxy-2-propylsulfonyl-5,8-dimethoxy-1,4-naphthoquinone (EPDMNQ) and 2,3-dihydro-2,3-epoxy-2-nonylsulfonyl-5,8-dimethoxy-1,4-naphthoquinone (ENDMNQ), inhibit the proliferation of Hep3B cells and induce apoptosis through the MAPK and STA3 signaling pathways [29]. Kacmaz et al. (2019) state that N(H)-, S- and S,S-substituted-1,4-naphthoquinones reduce the viability of HeLa cervical cancer cells with lower IC₅₀ concentration, especially compound **3d** [30]. Gholampour et al. (2019) synthesized a novel series of aminonaphthoquinone compounds and determined the cytotoxicity of these

compounds in MCF-7 (breast cancer), HT-29 (colorectal cancer), and MOLT-4 (acute lymphoblastic leukemia). Among compounds, 56c exhibits especially potent anticancer activity in all cell types, with IC₅₀ concentrations of 10.4 μ M for MCF-7, 6.8 μ M for HT-29, and 8.4 μ M for MOLT-4 [31] Furthermore, a novel library of naphthoquinone derivatives (**3–5aa**) was synthesized, and especially compound **5v** exerted greater cytotoxicity in MCF-7 cells through G1/s arrest compared with doxorubicin [32]. In our study, 1,4-naphthoquinone compounds induced considerable cytotoxicity and apoptotic cell death in MDA-MB-231 cells. We mainly focused on the most aggressive subtype of breast cancer due to resistance to current treatment options and poor prognosis in patients. However, further studies should be conducted to elucidate the molecular mechanisms underlying 1,4-naphthoquinone-induced apoptotic cell death and to compare this mechanism with other cancer types.

4. Computational Analysis

The interactions of the top-ranked compounds, hits compounds **4** and **13**, with proteins involved in the apoptosis pathway were analyzed to better understand their potential therapeutic relevance. Because dysregulation of apoptosis is a hallmark of many cancers and allows tumor cells to evade programmed cell death, targeting this pathway represents a promising strategy for anticancer intervention. The docking and molecular dynamics results therefore provide insight into the binding behavior and stability of these compounds within the selected targets.

4.1.Molecular docking

To investigate their potential as MCL-1 inhibitors, both ligands were docked into the protein's hydrophobic binding groove as illustrated in Figure 5. The analysis predicted favorable binding energies for both molecules, with MCL1-4 achieving a score of $-7.2 \text{ kcal}\cdot\text{mol}^{-1}$ and the larger MCL1-13 exhibiting a stronger predicted affinity of $-8.4 \text{ kcal}\cdot\text{mol}^{-1}$. The structural basis for this activity becomes clear upon visual inspection of the predicted binding modes. The compact, fused-ring system of MCL1-4 allows it to settle snugly into a sub-pocket of the groove, where its planar aromatic core is ideally positioned for π - π stacking with PHE270. This central interaction is buttressed by an extensive network of hydrophobic contacts with a cluster of valine and leucine residues, including VAL249, VAL274, LEU246, LEU267, and others, effectively anchoring the ligand. Meanwhile, its carbonyl and thioether substituents project toward the solvent, where they engage in stabilizing hydrogen bonds with MET250 and LEU267. By contrast, the more extended and flexible scaffold of MCL1-13 allows it to stretch across a wider expanse of the binding pocket. Its elongated aromatic system maintains the critical π - π stack with PHE270, while its additional alkyl moieties form a broader network of hydrophobic interactions with residues like VAL253, LEU235, and LEU246. Furthermore, strategically placed polar groups on MCL1-13 reach out to form specific hydrogen bonds and electrostatic contacts with ARG263 and THR266, interactions that likely contribute to its enhanced predicted affinity. Collectively, these models demonstrate that the planarity, aromatic character, and precise placement of polar substituents in both ligands are key to achieving high complementarity with the MCL-1 pocket, providing a structural rationale for their favorable binding energies.

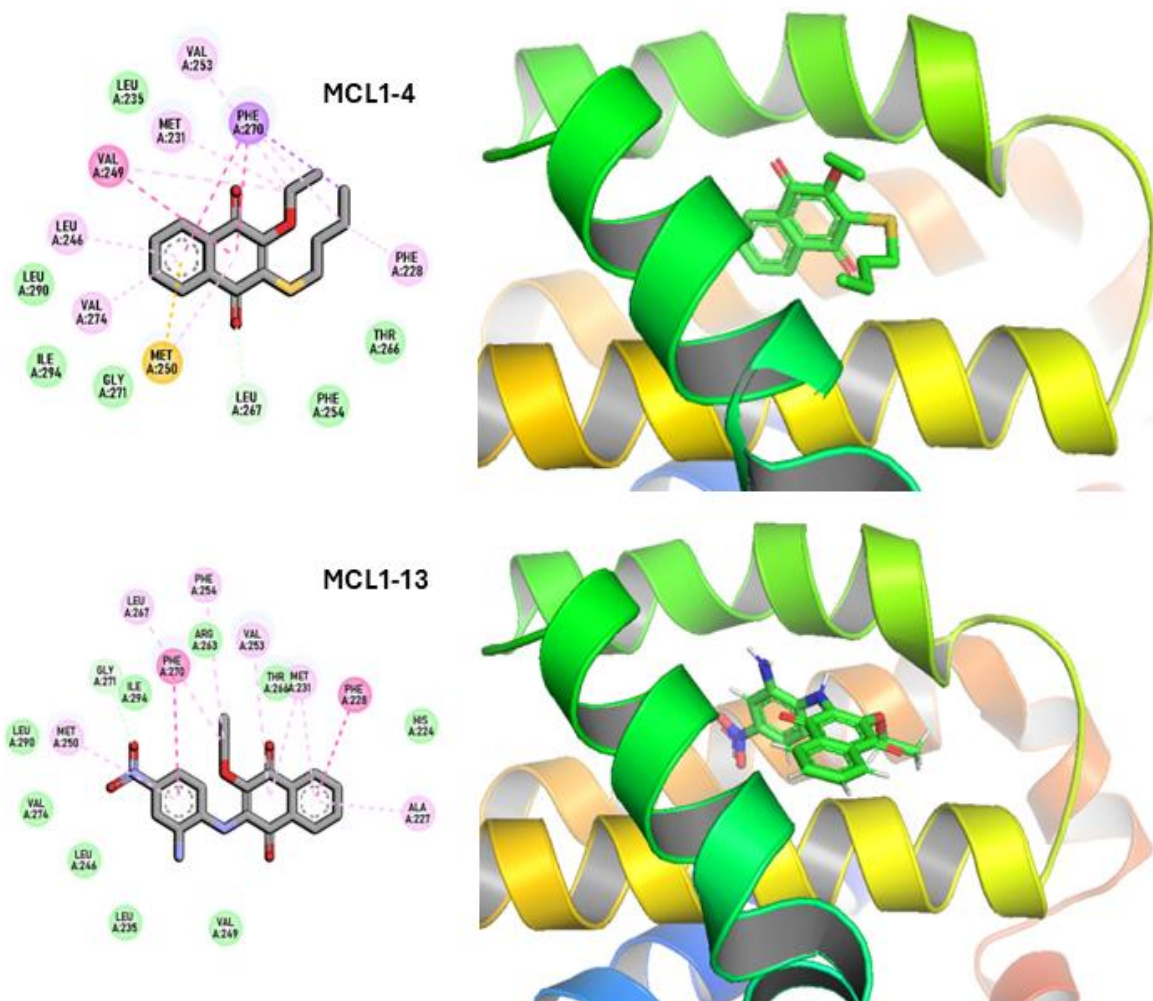


Figure 5. Molecular Docking 2D and 3D results of op hits **4** and **13** inside the catalytic site of MCL1.

5. Molecular Dynamics

The performed molecular dynamics simulations for both compounds **4** and **13** were analyzed using RMSD, RMSF, PCA, Ramachandran with SSE percentage plots and MMGBSA calculations. The two complexes simulations are visualized in Figure 6.

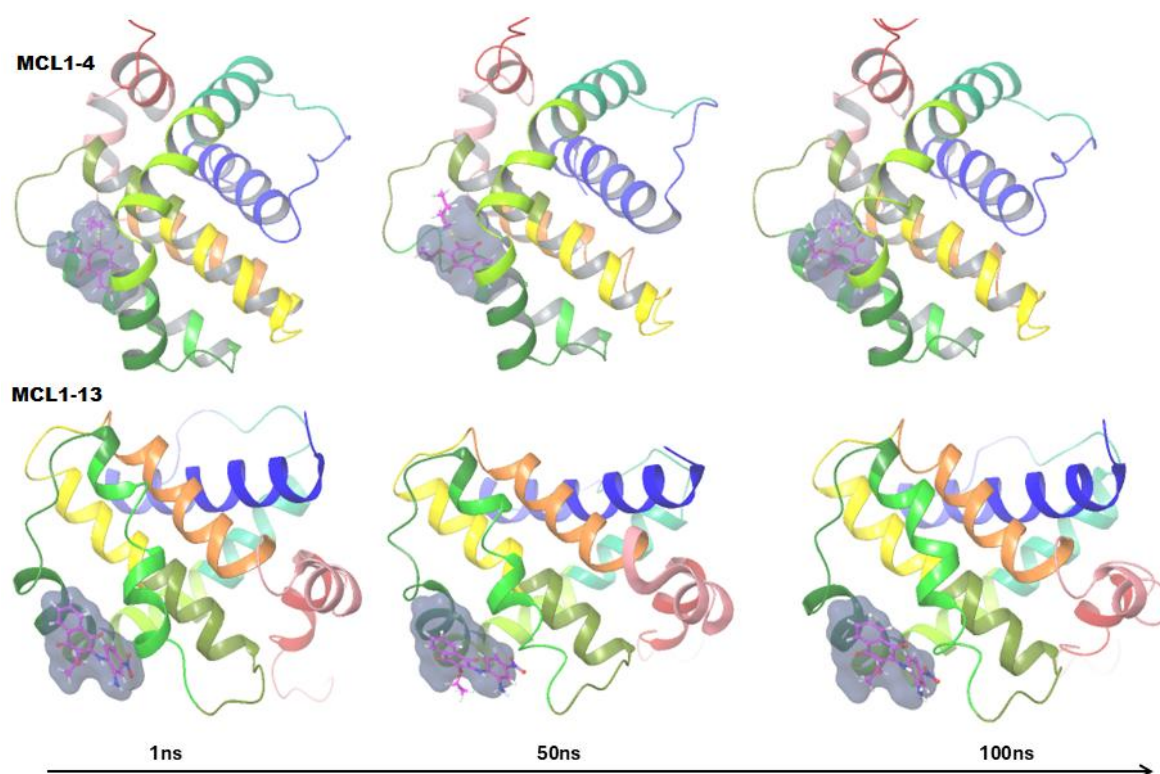


Figure 6. Vizualized simulated complexes named MCL1-4 and MCL1-13 through 100ns.

5.1.RMSD analysis

To evaluate the conformational stability of the simulated systems, the root-mean-square deviation (RMSD) of both the protein backbone and the ligand heavy atoms was calculated relative to their initial structures (Figure 7). The MCL-1 protein remained stable in both complexes, with average backbone RMSD values of 2.04 Å and 2.22 Å when bound to

compound **4** and compound **13**, respectively. As all RMSD values remained below 3 Å, these results indicate that the overall protein structure was well preserved throughout the simulations and that both complexes maintained stable binding conformations. Differences were nevertheless observed in ligand behavior. Compound **4** exhibited a moderate average RMSD of 1.86 Å, suggesting some degree of conformational flexibility or slight reorientation within the binding pocket. In contrast, compound **13** showed higher positional stability, with an average RMSD of only 0.63 Å. This difference suggests that compound **13** maintains a more consistent interaction pattern within the binding site, whereas compound **4** may explore a slightly broader range of conformations while remaining stably bound (Figure 8).

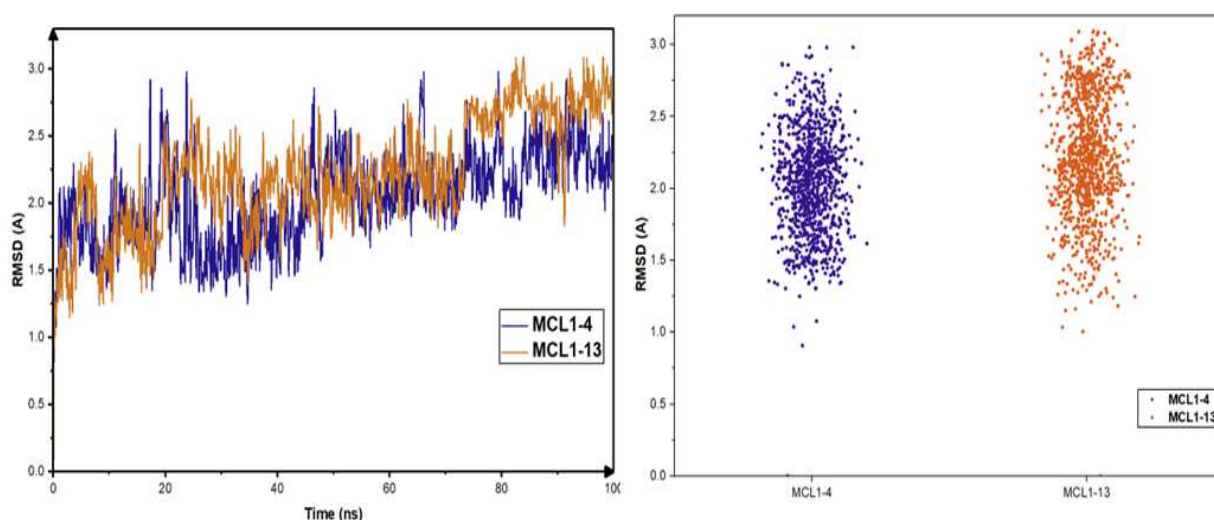


Figure 7. Backbone RMSD of MCL1 in complex with compounds **4** and **13** throughout the simulation trajectory.

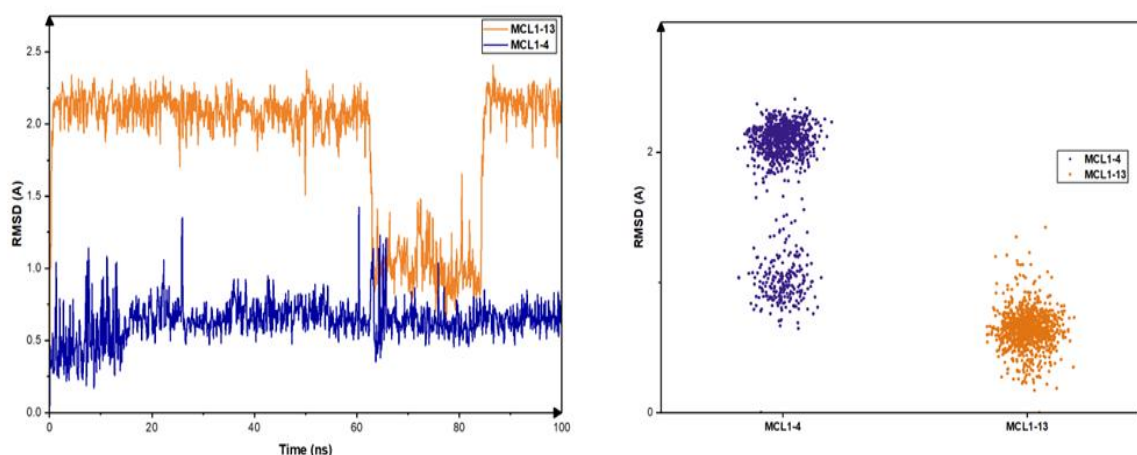


Figure 8. Heavy-atom RMSD of compounds 4 and 13 bound to MCL1 throughout the simulation trajectory.

5.2 RMSF Analysis:

To better understand the dynamic behavior of the protein at the residue level, the root-mean-square fluctuation (RMSF) of the MCL1 C α atoms was analyzed, with particular attention to residues that form the ligand-binding site (Figure 9). In the MCL1-4 complex, the average RMSF across all residues was 0.97 Å, whereas residues directly interacting with compound 4 displayed a lower average fluctuation of 0.83 Å. This decrease in mobility upon ligand binding suggests that compound 4 contributes to stabilizing the local protein environment. A different pattern was observed in the MCL-1/13 complex. In this system, the average RMSF for all residues was 0.94 Å, while residues in contact with compound **13** showed a slightly higher average fluctuation of 0.95 Å, a value comparable to the overall mobility of the protein. These results indicate that although compound **13** itself is highly rigid, as reflected by its low ligand RMSD, it does not induce a corresponding rigidification of the surrounding protein residues. Instead, the data point to a more dynamic binding interface in which the protein pocket maintains its intrinsic flexibility, potentially accommodating the stably bound ligand through

an adaptive rather than a rigidifying mechanism. This behavior contrasts with the MCL-1/4 complex, where ligand binding appears to dampen the motion of the interacting residues.

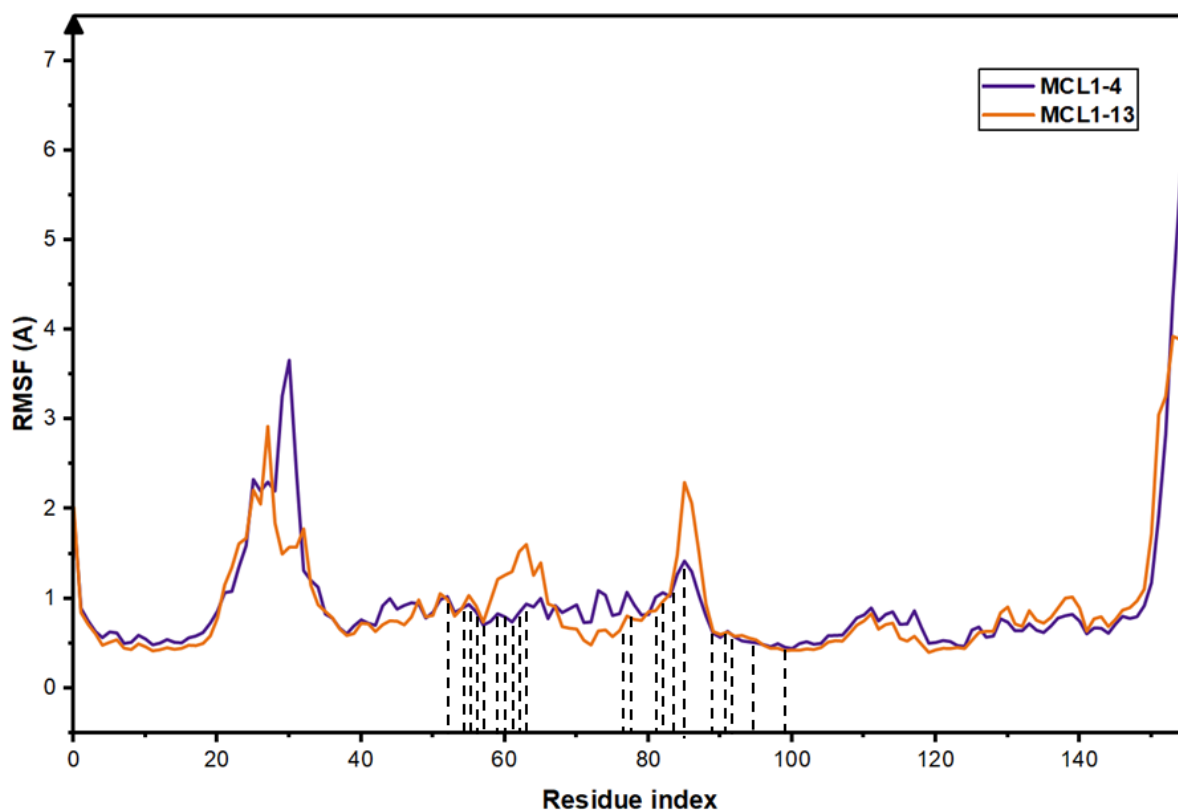


Figure 9. Residue-Level Root-Mean-Square Fluctuation (RMSF) of MCL-1 C α Atoms in Complexes with Compounds **4** and **13**

5.3. Principal Component Analysis

Principal component analysis (PCA) was carried out to examine the collective motions and conformational sampling of the MCL1 complexes bound to ligands 4 and 13 (Figure 10). In the PCA plots, each point corresponds to a simulation frame projected onto the first two principal components (PC1 and PC2), while the color gradient from purple to yellow represents the progression of simulation time from the initial to the final frames. For the MCL1–4 system,

the frames are broadly distributed across the PC1–PC2 space, indicating that the complex explores a wide conformational landscape during the simulation. The gradual color transition throughout the plot further suggests continuous sampling of multiple conformational states. This behavior is consistent with the corresponding free energy landscape, which displays several shallow minima spread across conformational space. In these maps, the color scale represents the Gibbs free energy, with blue indicating low-energy, highly stable conformations, green and yellow representing intermediate states, and red indicating higher-energy, less populated regions. In contrast, the PCA projection of the MCL1–13 complex shows a more compact and organized distribution of frames along a narrower pathway, suggesting more restricted conformational motion.

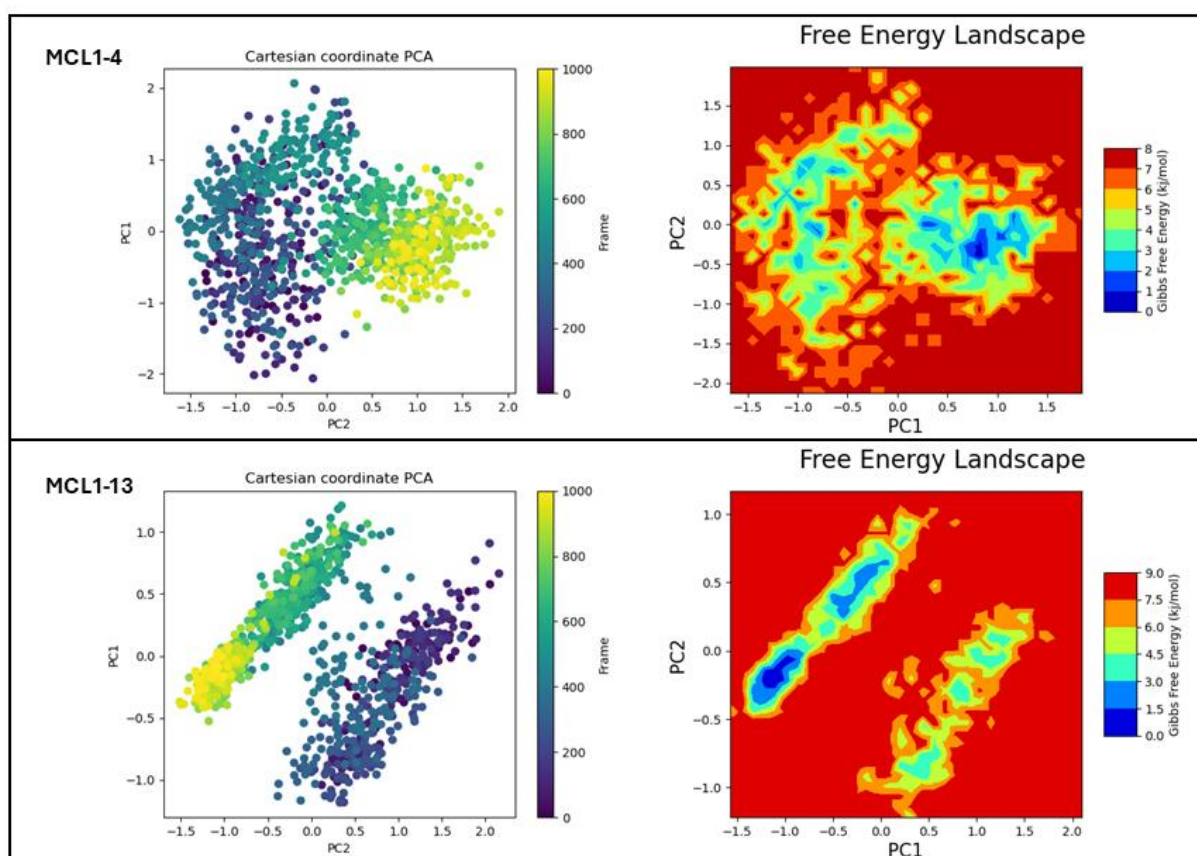


Figure 10. Principal component analysis (PCA) was carried out to examine the collective motions and conformational sampling of the MCL1 complexes bound to ligands **4** and **13**

5.4 MMGBSA Analysis

MM-GBSA calculations were performed to estimate the relative binding free energies of the two complexes (Table 1). The results indicate that the MCL-1/4 complex exhibits a more favorable binding free energy ($\Delta G_{\text{bind}} = -37.58$ kcal/mol) compared to the MCL-1/13 complex (-32.19 kcal/mol), suggesting a stronger predicted affinity for compound **4**. Energy decomposition analysis shows that van der Waals interactions serve as the primary driving force for binding in both systems, with compound **4** contributing significantly more (-52.18 kcal/mol) than compound **13** (-38.76 kcal/mol). This points to enhanced packing and improved shape complementarity of compound **4** within the binding pocket. Electrostatic contributions, while modest, are slightly more favorable for compound **13**. Notably, although previous RMSF analyses revealed increased rigidity of the binding site in the MCL-1/4 complex, the energetic data indicate that this rigidification is effectively offset by stronger hydrophobic interactions. (Figure 11)

Table 1. MM-GBSA Calculated Binding Energetics for the MCL-1 – compounds.

Parameter/ Complex	MMGBSA	Coulomb	HBond	Covalent	vDW
MCL1-4	-52.18284	-1.79199	1.40729	-0.00111	-32.19277
MCL1-13	-38.76418	-3.10498	1.05117	-0.2859	-37.5789

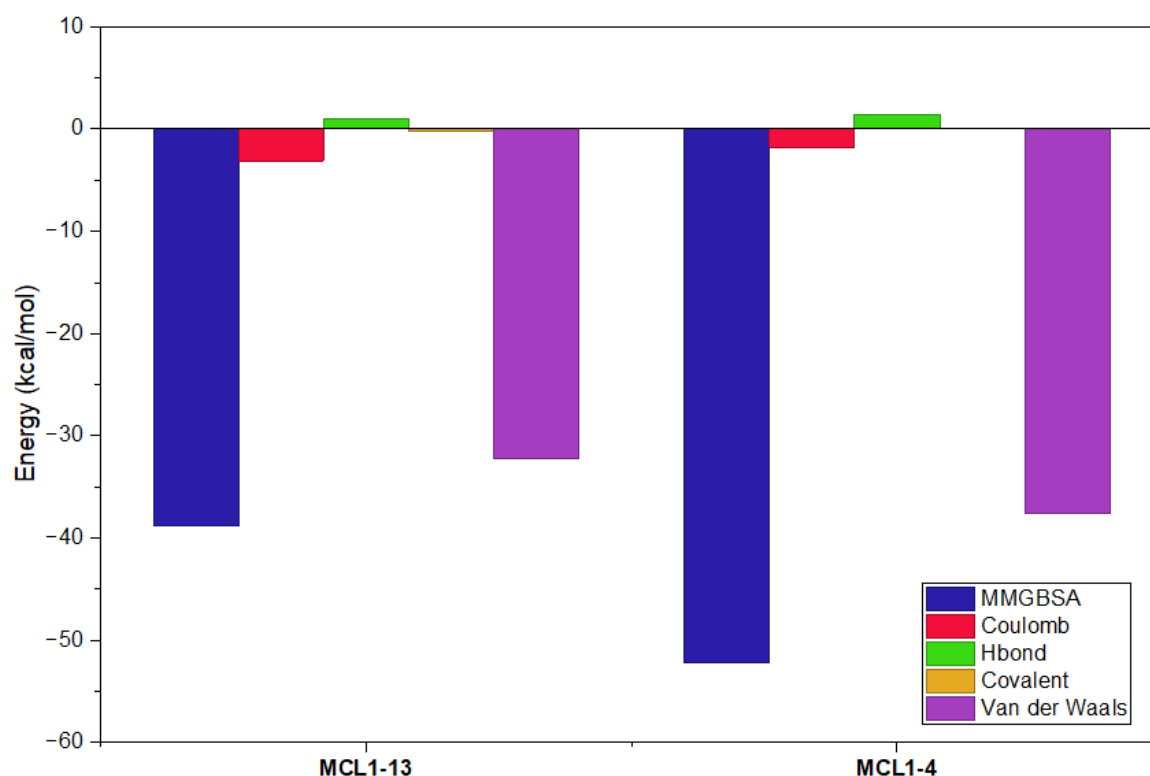


Figure 11. Graphical Representation of MM-GBSA Binding Free Energies and Key Intermolecular Interactions for the MCL-1 Complexes.

5.5. Protein Secondary Structure and Ramachandran plots analysis

Secondary structure and Ramachandran plot analyses were used to assess the structural integrity and stereochemical quality of the MCL-1 complexes during simulation. Secondary structure analysis describes the overall folding pattern and stability of structural elements, while Ramachandran plots evaluate backbone conformational geometry, confirming whether residues occupy energetically favorable conformations.

Both complexes remained predominantly α -helical, with essentially no β -strand content consistent with the known α -helical bundle architecture of MCL-1 within the Bcl-2 protein

family (Figure 12). The 4–MCL1 complex showed slightly higher helical content (70.51%) than the 13–MCL1 complex (64.76%).

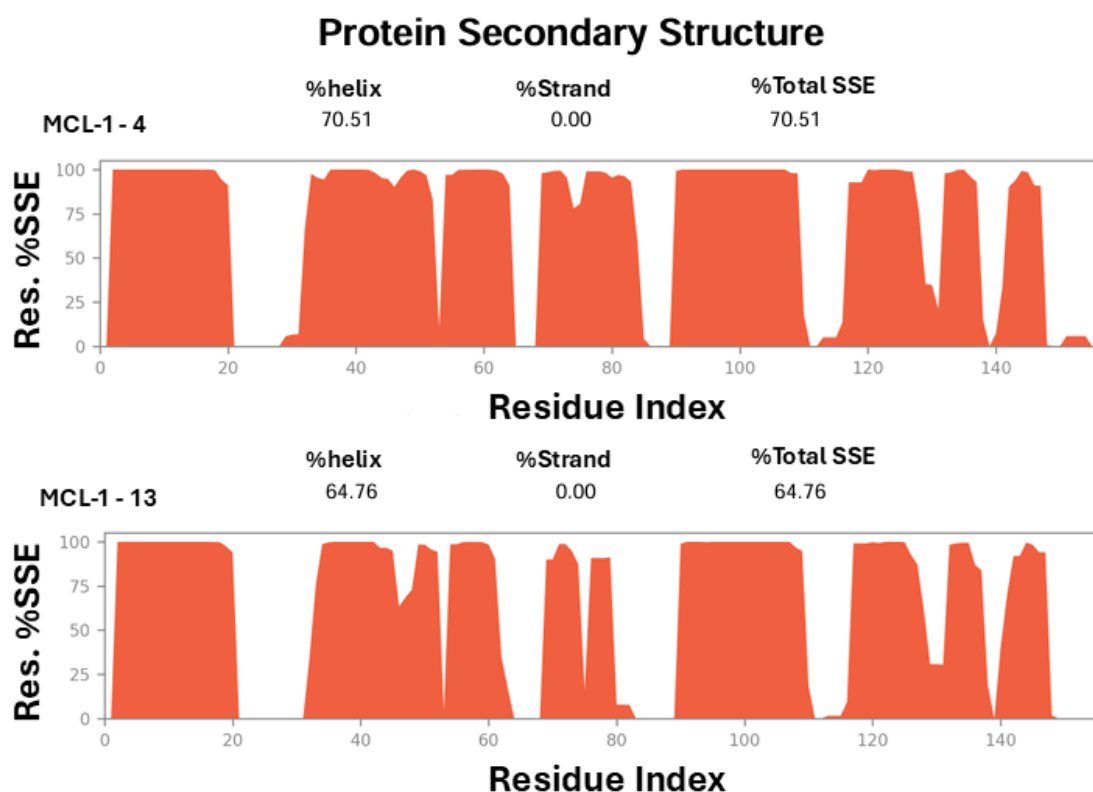


Figure 12. Percentage Distribution of Secondary Structure Elements in MCL-1 - 4 and MCL-1 -13 Complexes

To further assess structural quality, Ramachandran plots were examined before and after molecular dynamics (MD) simulation (Figure 13). Initially, the 13–MCL1 complex displayed better backbone geometry, with about 83% of residues in favored regions, whereas the 4–MCL1 complex had only ~75% favored residues and more outliers. After MD simulation, both systems improved markedly. The 13–MCL1 complex reached ~92% favored residues with only 2–3% outliers, while the 4–MCL1 complex improved to ~85% favored residues with 3–4% outliers, indicating a substantial reduction in disallowed conformations.

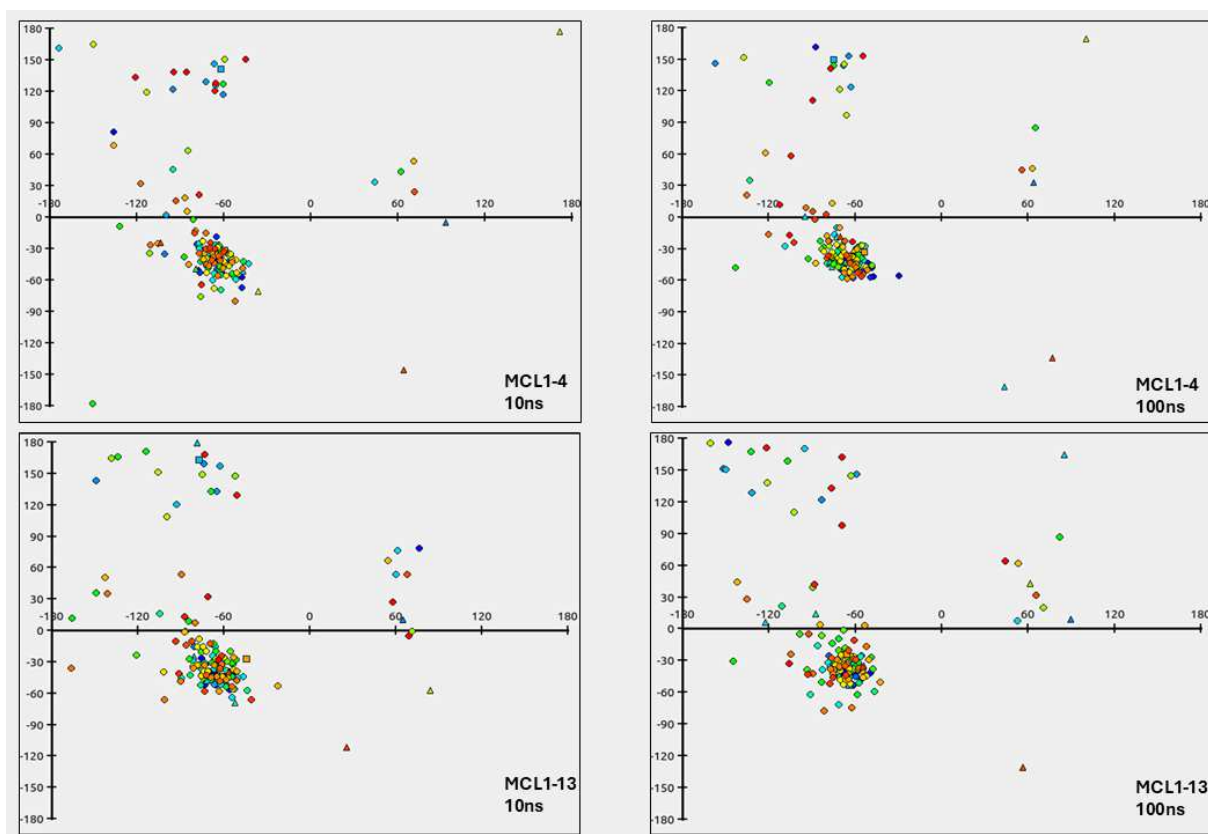


Figure 13. Ramachandran Plot Analysis of MCL-1 Complexes Before and After Molecular Dynamics Simulation

Conclusion

Quinone compounds, which can be of both natural and synthetic origin, have gained significant importance in the literature due to their differing biological activities. In this study, the compound 2,3-dichloro-1,4-naphthoquinone was reacted with different amine and thiol compounds within the framework of the 1,4-Michael addition mechanism. These reactions resulted in the synthesis of seven *S,O*-, *N,O*-, *N*- and *N,S*-substituted 1,4-naphthoquinone compounds (**4**, **5**, **8**, **9**, **11**, **12**, **13**). The structures of all the synthesized compounds were illuminated by ^{13}C NMR, ^1H NMR, FTIR and MS.

It is our first study that the desining of *N,S,O*-substituted-1,4-Naphthoquinone derivatives and we evaluated of their apoptotic effects and anticancer activity against triple negative breast

cancer cells with using molecular docking and molecular dynamics method. 1,4-naphthoquinone compounds significantly inhibited the proliferation of TNBC cells. However, particularly, compounds **4**, **11**, **12** and **13** induced more apoptotic cell death in MDA-MB-231 cells. Additionally, the apoptotic effects could be associated with the binding affinity of MCL-1 protein. Therefore, future studies could examine the molecular mechanisms underlying the apoptotic death induced by 1,4-naphthoquinone compounds. Further, molecular docking results showed that compound **13** exhibited a more favorable binding affinity compared to compound **4**, based on its lower docking score. This Monte Carlo simulation was followed by MD simulation to understand the behaviour of both molecules inside the protein catalytic site, results showed that both complexes remained stable throughout the simulation, with RMSD values below 3 Å. Higher positional stability was observed for compound **13**, while compound **4** displayed slightly more flexibility, suggesting some level of adaptation within the binding pocket. The MCL-1 protein structure was preserved in both systems, and reduced residue fluctuations were observed around compound **4**, indicating a stabilizing effect on the local protein environment. MM-GBSA calculations revealed that a more favorable binding free energy was associated with compound **4** compared to compound **13**, suggesting stronger binding despite its lower docking score. It can be concluded that while compound **13** showed better initial docking and stability, compound **4** demonstrated a more favorable binding profile when all parameters were taken into account.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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