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Synthesis and Anti-Breast Cancer Potency of Mono- and Bis-(pyrazolyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine) Derivatives as EGFR/CDK-2 Target Inhibitors

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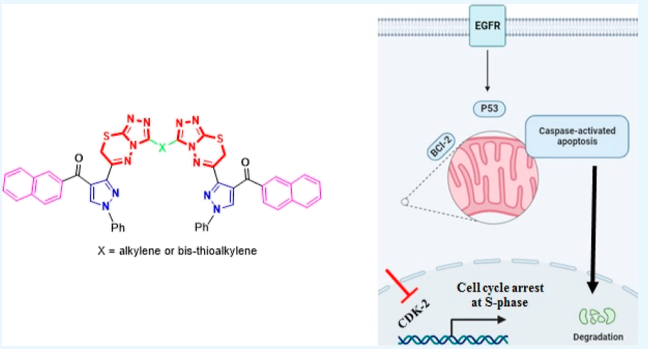
ABSTRACT: The target mono- and bis-(6-pyrazolyltriazolo-thiadiazine) derivatives **4a-c** and **6a-d** were synthesized using a straightforward protocol via reaction of 3-bromoacetylpyrazole **2** with 4-amino-*s*-triazole-3-thiols **3a-c** and bis(4-amino-5-mercapto-*s*-triazol-3-yl)alkanes **5a-d**, respectively. The bis(6-pyrazolyl-*s*-triazolo[3,4-*b*][1,3,4]thiadiazine) derivatives **8a,b** and **10** were also constructed by reaction of the triazolo[3,4-*b*][1,3,4]-thiadiazine-3-thiol **4c** with the proper dibromo compounds **7a,b** and **9**, respectively. Structures of the new substances were determined by spectroscopic and analytical data. Compounds **4b**, **4c**, and **6a** showed potent cytotoxicity against MCF-7 (IC_{50} = 3.16, 2.74, and 0.39 μM, respectively) and were safe against the MCF-10A cells. Compounds **4b**, **4c**, and **6a** also showed promising dual EGFR and CDK-2 inhibition activities, particularly **6a** was the most effective (IC_{50} = 19.6 and 87.9 nM, respectively), better than Erlotinib and Roscovitine. Compound **6a** treatment induced EGFR and CDK-2 enzyme inhibition by 97.18% and 94.11%, respectively, at 10 μM (the highest concentration). Compound **6a** notably induced cell apoptosis in MCF-7 cells, increasing the cell population by total apoptosis 43.3% compared to 1.29% for the untreated control group, increasing the cell population at the S-phase by 39.2% compared to 18.6% (control).



1. INTRODUCTION

Many potentially bioactive natural and synthetic organic molecules with promising anticancer activity were found to possess naphthalene ring.[1−7] Examples for such natural compounds are **Justicidin A** and **Furomollugin** (Figure 1) that demonstrate extraordinary anticancer activities.[8,9] Naphthalene moiety was also found in the commercially marketed anticancer drug; **Cinacalcet** as shown in Figure 1. Besides, pyrazoles are among the widest classes of nitrogen heterocycles that are available in numerous natural and synthetic organic hybrids that have significant anti-cancer activity.[10−12] For example, some pyrazole hybrids are commercially approved by FDA as anticancer drugs, such as **Ruxolitinib** and **Crizotinib**[13−15] (Figure 1). In addition, 1,2,4-triazoles are highly promising bioactive scaffolds and are integral parts in a number of commercial drugs for medical treatment of cancers, such as **vorozole**, **anastrozole**, **talazoparib**, **letrozole**, and **deferasirox** (Figure 1).[16,17]

The fusion of 1,2,4-triazole and 1,3,4-thiadiazine moieties produces the 1,2,4-triazolo- thiadiazine heterocycles that have a wide range of pharmacological properties. In particular, several publications reported the importance of triazolo[3,4-*b*]thiadiazine scaffolds as remarkable anticancer agents against many human cancer cell lines with minute evidence of toxicity.[18−28]

In addition, some naphthyl-pyrazole hybrids **A**−**C** showed fascinating anticancer performances versus MCF-7 (the human breast cancer cell line), and some of these derivatives had an extraordinary anticancer potency against MCF-7 with five-times more activity than the standard cisplatin drug (Figure 1).[29−32] Therefore, integrating two or more molecular fragments to build up a new structural hybrid is an essential path for designing new constructive therapeutic agents.[33,34]

Although the biological potency of 1,2,4-triazolo[3,4-*b*]-[1,3,4]thiadiazines has been widely documented, synthesis and biological evaluations of heterocyclic structures employing naphthalene, pyrazole, and triazolo-thiadiazine were not explored so far. The central point of our research concern is directed to the synthesis of diverse bioactive heterocyclic hybrids with wide biological importance, particularly the

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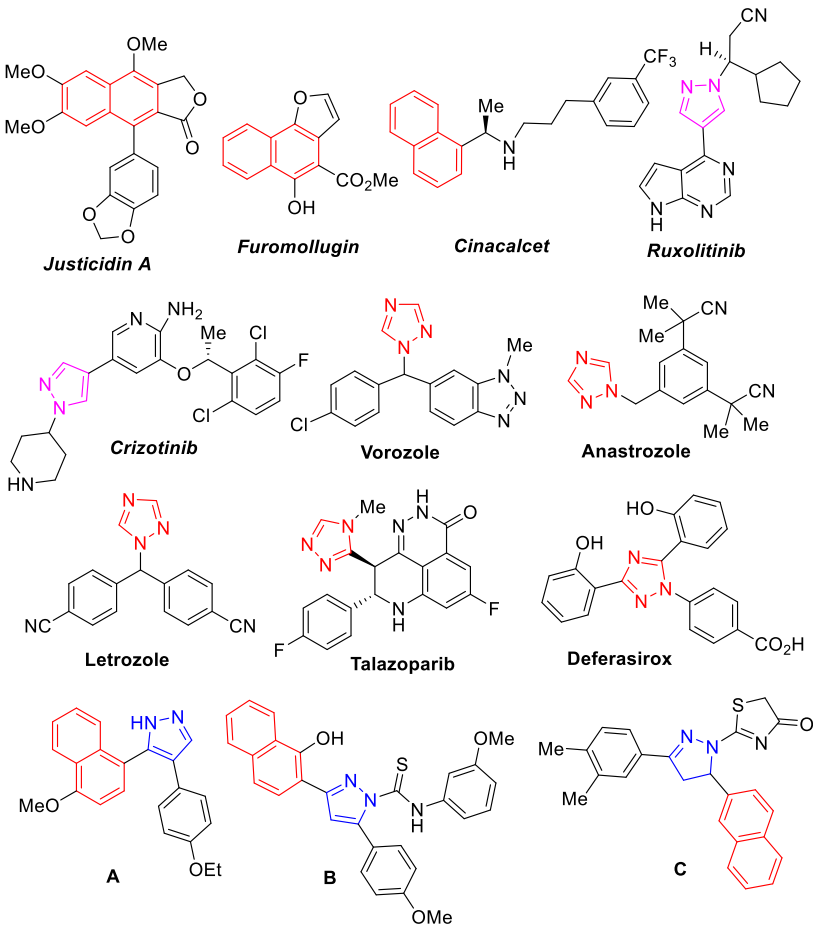

Figure 1. Examples of naphthalene-, pyrazole-, and 1,2,4-triazole-based anticancer drugs.

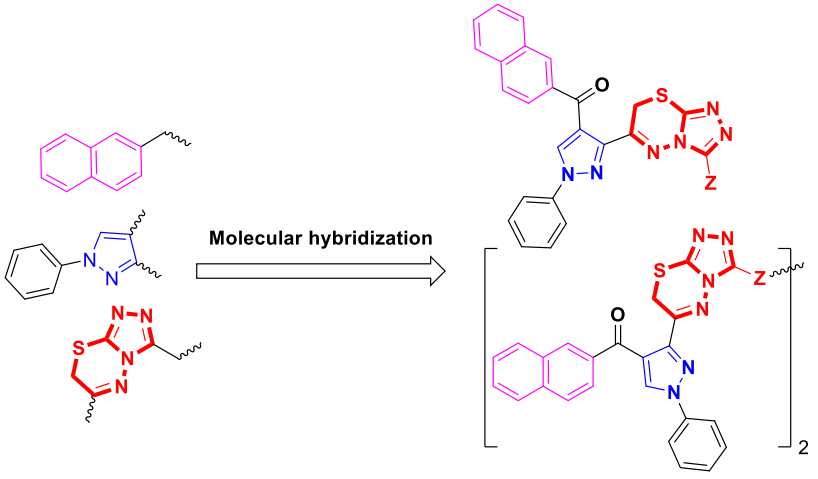

Figure 2. A rationale design for the new anticancer molecular hybrids.

anticancer potency.[35−47] Thus, we designed and constructed a group of new heterocyclic molecular hybrids integrating three organic fragments: naphthalene, pyrazole, and triazolothiadiazines in a mono- and symmetrical bis-structural forms targeting for the production of more therapeutically efficient anticancer agents (Figure 2).

2. RESULTS AND DISCUSSION

2.1. Chemistry. The starting substrate 3-bromoacetylpyrazole derivative **2** was built via acid catalyzed bromination of its precursor 3-acetylpyrazole derivative **1** in a good yield.[48] Then, the first new target compounds, pyrazolyl(triazolo[3,4-*b*]thiadiazines) **4a-c,** were produced in 76−85% from reaction of the 3-bromoacetylpyrazole derivative **2** with the respective 4-amino-1,2,4-triazole-3-thiol derivatives **3a-c** in refluxing ethanol using a few drops of the organic base catalyst; triethylamine as described in Scheme 1. Spectral data confirmed structures **4a-c**, for example, the IR spectrum of **4a** showed a distinctive carbonyl absorption peak at 1685 cm^{-1}, its ^1H NMR spectrum displayed four singlet signals at δ

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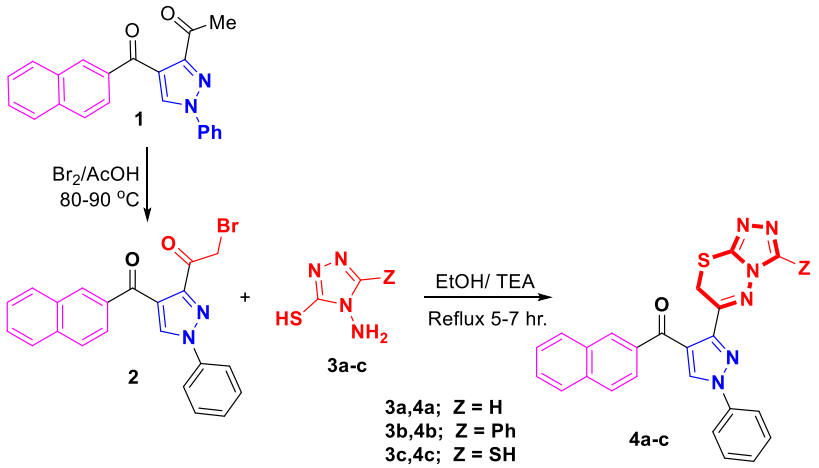
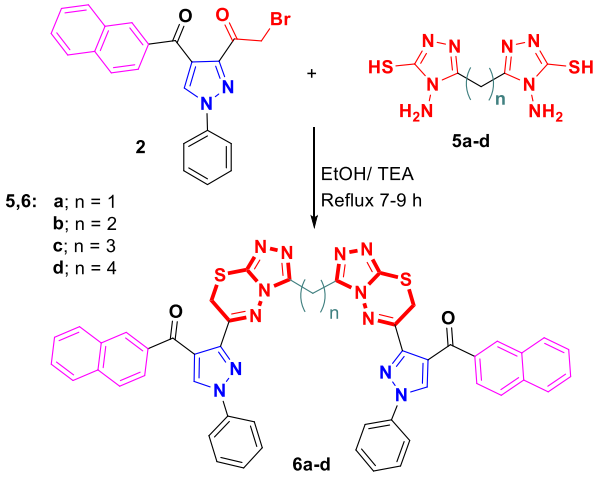
Scheme 1. Synthesis of the Pyrazolyl-triazolo[3,4-*b*]thiadiazine Derivatives 4a-c



4.50, 8.50, 8.64, and 9.23 assignable to the protons of thiadiazine-6-CH_2, naphthyl-1-CH, triazole-3-CH, and pyrazole-5-CH besides the aromatic multiplets at δ 7.44−8.12. The ^{13}C NMR of **4a** revealed one signal at δ 33 due to an sp^3 carbon and 21 signals due to sp^2 carbons including a characteristic C═O at δ 189.0.

Next is a straightforward and effective procedure for the construction of some newly reported bis-heterocyclic hybrids **6a-d**, in which two pyrazolyltriazolothiadiazine scaffolds are connected by alkylene spacers via the triazole moieties. To accomplish this target, the appropriate bis(4-amino-5-mercapto-1,2,4-triazol-3-yl)alkanes **5a-d** were first created as precursors for the desired bis-compounds **6a-d**. After being treated with a double ratio of **2** in ethyl alcohol at reflux condition catalyzed by triethylamine, these precursors gave rise to the corresponding bis(6-pyrazolyl triazolothiadiazines) **6a-d** in 71−77% yield, respectively (Scheme 2). The structures of

Scheme 2. Synthesis of Bis(naphthoylpyrazolyl-triazolothiadiazines) 6a-d



the purely isolated products were evidenced by elemental and spectroscopic studies. The IR spectra of the bis-fused heterocyclic derivatives **6a-d** were free of NH_2 stretching bands and showed, in each case, a carbonyl absorption around 1680 cm^{-1}. ^{1}H NMR spectrum of **6a** revealed the presence of two characteristic singlet signals due to spacer−CH_2 and thiadiazine-SCH_2 protons at δ 2.91 and 4.45, respectively, in addition to two other singlets due to naphthyl-1-CH and pyrazolyl-5-CH protons at δ 8.45 and 9.16, respectively. The ^{13}C NMR of **6a** displayed two signals at δ 19.2 and 22.9 due to two sp^3 carbons and 20 signals due to sp^2 carbons and a signal at δ 189.0 due to C═O carbon. In addition, the mass spectrum of compound **6a** exhibited a molecular ion peak at *m/z* 884.

Synthesis of the novel two-fold branched pyrazolyltriazolothiadiazines **8a,b** linked by an bis-thioalkylene spacer was carried out as outlined in Scheme 3. Thereby, when pyrazolyltriazolo[3,4-*b*][1,3,4]thiadiazine-3-thiol derivative **4c** was treated with the appropriate dibromoalkane **7a,b** (in 2:1 molar ratio) in refluxing ethyl alcohol containing potassium hydroxide as an inorganic base led to the construction of the bis-pyrazolyltriazolothiadiazines **8a,b** in 69 and 73% yields, respectively. The IR spectrum of **8a** showed an absorption at 1684 cm^{-1} (for C═O) and its ^{1}H NMR spectrum exhibited four characteristic singlet signals at δ 3.94, 4.56, 8.48, and 9.20 due to spacer−SCH_2, thiadiazine-SCH_2, naphthyl-1-CH, and pyrazolyl-5-CH protons, respectively. The ^{13}C NMR of **8a** revealed two signals at δ 27.9 and 30.2 due to two sp^3 carbons and 19 signals due to sp^2 carbons and a signal at δ 187.8 for C═O carbon. A molecular ion peak at *m/z* 962 appeared in the mass spectrum of compound **8a**.

Under the same reaction pathway, the bis(pyrazolyltriazolo-[3,4-*b*][1,3,4]thiadiazine) **10** linked to *ortho*-xylylene spacer via two thio-ether bonds was constructed. Thus, heating 1,2-bis(bromomethyl)benzene (**9**) with two equivalents of the triazolo[3,4-*b*][1,3,4]thiadiazine-3-thiol **4c** in ethanolic potassium hydroxide solution furnished the bis-pyrazolyltriazolothiadiazine derivative **10** in 65% yield (Scheme 3). Structure of compound **10** was elucidated according to its spectroscopic data (IR, ^{1}H- and ^{13}C NMR) along with its mass spectrometry and elemental analyses (as reported in the Experimental Section).

2.2. Biology. *2.2.1. Cytotoxicity.* Using the MTT bioassay, the newly constructed compounds were evaluated for their cytotoxicity against MCF-7 (breast cancer cells). As outlined in Table 1 and Figure 3, the bioassay results revealed that three molecular hybrids **4b, 4c,** and **6a** had a substantial cytotoxic activity against MCF-7 with IC_{50} values of 3.16, 2.74 and 0.39 μM, respectively, compared to Erlotinib (IC_{50} = 2.51 μM) and Roscovitine (IC_{50} = 1.9 μM). The tested compounds were also

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Scheme 3. Synthesis of Bis(naphthoylpyrazolyl-triazolothiadiazines) 8a,b and 10

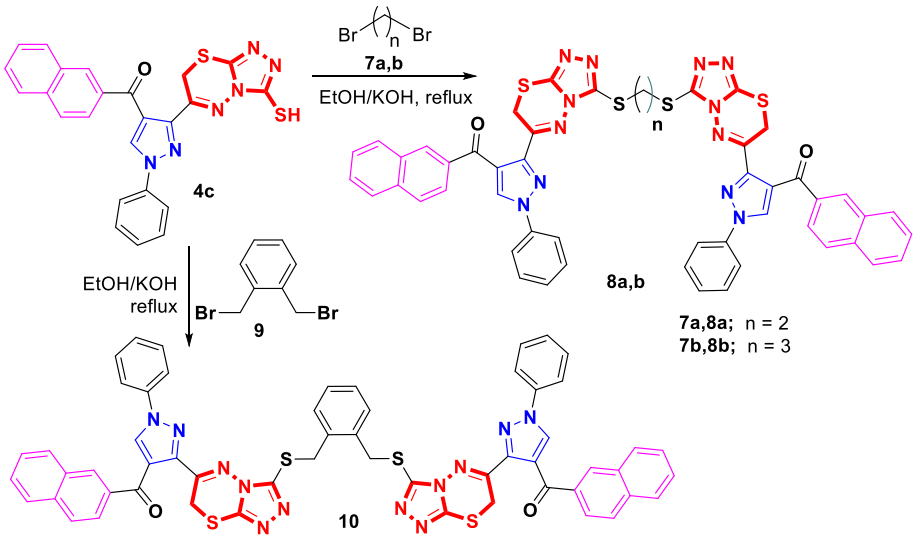

Table 1. Cytotoxic Activity of the Evaluated Substances Versus MCF-7 and MCF-10A Cell Lines

	$IC_{50} \pm SD^a$ (μM)	
compounds	MCF-7	MCF-10A
2	13.17 ± 0.75	37.5 ± 1.9
4a	6.24 ± 0.17	≥50
4b	3.16 ± 0.29	≥50
4c	2.74 ± 0.2	46.7 ± 1.7
6a	0.39 ± 0.1	≥50
6b	32.4 ± 0.75	41.2 ± 1.0
6c	9.15 ± 0.24	48.4 ± 1.8
6d	15.6 ± 0.27	31.4 ± 0.8
8a	7.69 ± 0.3	38.9 ± 0.89
8b	42.1 ± 0.96	29.6 ± 0.8
10	38.2 ± 0.75	31.2 ± 0.9
Erlotinib[b]	2.51 ± 0.11	≥50
Roscovitine[c]	1.91 ± 0.17	≥50

[a]Values are expressed as "mean ± SD of three independent triplets (n = 3)". [b]Standard cytotoxic with CDK-2 inhibition. [c]Standard cytotoxic with EGFR inhibition. NA: Non-Active.

non-cytotoxic versus the normal MCF-10A cells with high IC_{50} values. Furthermore, some other compounds **2, 4a, 6c,** and **8a** showed good cytotoxicity with IC_{50} values of 13.17, 6.24, 9.15 and 7.69 μM, respectively.

The effect of derivatization of the simple traiazolothiadiazine derivatives and their bis-hybrids is depicted in Figure 4. Based on their activities, the tested compounds were classified into three levels; level one composed the most active compounds as yellow-highlighted, level two for those with moderate activities as green-highlighted, and the third group were those with less active potency having IC_{50} > 15 μM. As shown in Figure 4, among the simple triazolothiadiazines **4a-c**, the order of activity was **4c** > **4b** > **4a**, indicating the greater effect of presence of the SH group (as hydrogen bond donor) in **4c**, and the phenyl group (for hydrophobic interaction) at the triazole moiety in **4b** than the unsubstituted triazole one in **4a**. All simple fused-triazoles **4a-c** showed better activity than their precursor **2**. For the bis-heterocyclic hybrids **6a-d**, compound **6a** (n = 1) having the shortest alkylene spacer showed the highest activity among the other bis-hybrids of this series having longer alkylene spacers (n = 2, 3, 4). Interestingly, compound **6a** presented almost 15-fold enhanced cytotoxicity compared with its mono-heterocyclic analogue **4a**. In addition, compound **6a** displayed substantial potency against MCF-7 cells compared with the other bis-molecular hybrids incorporating longer bis(thioalkylene) spacers (**8a,b** and **10**).

2.2.2. EGFR and CDK-2 Kinase Inhibitory Assay. Compounds **4b, 4c,** and **6a** with the promising cytotoxic activities against MCF-7 cells were tested against the EGFR and CDK-2 inhibition as molecular targets. The examined substances displayed interesting dual EGFR and CDK-2 inhibition activities, as seen in Table 2. Interestingly, compound **6a** had IC_{50} values of 19.6 and 87.9 nM, respectively, compared to Erlotinib (IC_{50} = 67.3 nM) and Roscovitine (IC_{50} = 67.3 nM). As seen in Figure 5, compound **6a** treatment induced EGFR and CDK-2 enzyme inhibition by 97.18 and 94.11%, respectively, at the highest concentration [10 μM]. Additionally, compound **4b** exhibited potent EGFR/CDK-2 inhibition with IC_{50} values of 71.7, 113.7 nM, respectively, while compound **4c** revealed good EGFR/CDK-2 inhibition with IC_{50} values of 89.6, 165.4 nM, respectively.

2.2.3. Apoptotic Investigation. 2.2.3.1. Annexin V/PI Staining with Cell Cycle Analysis. Apoptotic cell death in untreated and treated MCF-7 cells was analyzed by flow cytometric evaluation of Annexin V/PI staining to detect the apoptotic activity of compound **6a** (IC_{50} = 0.39 μM, 48h). Figure 6 showed that compound **6a** significantly induced the apoptotic cell death in MCF-7 cells, increasing the cell population by total apoptosis 43.3% (19.8% for early apoptosis and 23.5% for late apoptosis) compared to the untreated control group (1.29%). Additionally, compound **6a** treatment induced necrotic cell death by 8.6-fold, and increased the cell population by 7.35% compared to 0.85% in untreated control. Hence, dual apoptosis and necrosis cell death mechanisms were investigated.

Following cytotoxic treatment, DNA flow cytometry was utilized to quantify the proportion of cells in each phase of the cell cycle. As seen in Figure 7, treatment with compound **6a** dramatically boosted the percentage of cells in the S-phase by 39.2% compared to control (18.6%), while decreasing the percentage of cells in the G1 and G2/M phases. As a result,

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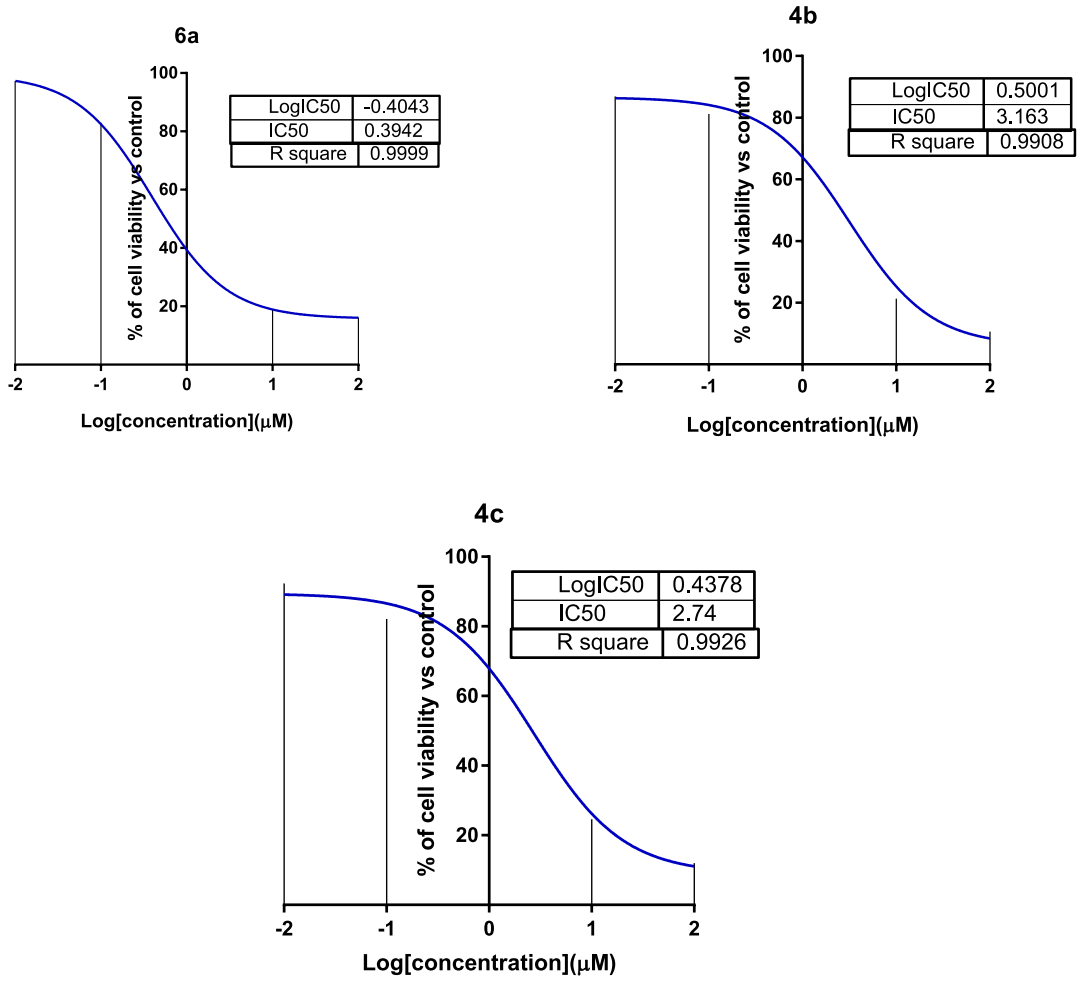

Figure 3. Dose−response nonlinear regression curve fitting the percentage of "cell viability vs log[con. μM]", $R^2 \approx 1$ using the GraphPad prism.

compound **6a** caused apoptosis in MCF-7 cells, which stopped cell division in the S-phase.

2.2.3.2. RT-PCR. RT-PCR was involved in analyzing gene expression level for the apoptosis-mediated genes of Bax, P53, Bcl-2, and caspase-3,-8,-9, in both treated and untreated MCF-7 cells, in addition to the flow cytometry data, to validate the apoptosis−induction activity in MCF-7 cells.

As shown in Figure 8, compound **6a** upregulated P53 by 12.3-fold, Bax by 7.6-fold, caspase-3,-8,-9 by 12.6, 6.5, and 10.6-fold, respectively, Bcl-2 expression was inhibited 0.17-fold. Thence, the obtained findings indicated the apoptosis-mediated cell death through both intrinsic and extrinsic pathways. Apoptosis-induction of **6a** was also investigated by the expression levels of apoptosis genes, P53, caspases-3,-8,-9, Bcl-2-associated X protein (Bax), B-cell lymphoma-2 (Bcl-2), which have been linked to cell signaling pathways that regulate cell survival and death, and this can facilitate to search for a new target and method of breast cancer therapy.

3. EXPERIMENTAL SECTION

3.1. Chemistry. Gallenkamp apparatus was used in measuring the melting points in open glass capillaries. Elemental analyses of the new structures were conducted at Cairo University Microanalytical Center. Potassium bromide disks were used for measuring the infrared spectra (IR), on a Pye-Unicam SP 3−300 and Shimaduz FTIR 8101 PC infrared spectrophotometer. Nuclear magnetic resonance spectra were carried out with a Varian Mercury (VXR-300 NMR spectrometer) at 75 MHz (^{13}C NMR) and at 300 MHz (^{1}H NMR). Mass spectra (EI) were measured at 70 eV using GC-MQP 1000 EX spectrometer from Shimadzu. Analytical TLC was carried out using Fluka precoated silica-gel 60,778 plates, and UV light (254 nm) was used to visualize the spots. Compounds 3-acetylpyrazole derivatives[48] **1** and **2,** 4-amino-3-mercapto-1,2,4-triazole derivatives[49,50] **3a,b**, 4-amino-4*H*-1,2,4-triazole-3,5-dithiol[51] (**3c**) and bis(4-amino-5-mercapto-1,2,4-triazol-3-yl)alkanes[52] **5a-d** were synthesized in our laboratory according to the previously reported methodology.

3.1.1. Synthesis of 6-(4-(2-Naphthoyl)-1-phenyl-1H-pyrazol-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazines ***4a-c.*** 3-Bromoacetyl-4-(2-naphthoyl)-1-phenyl-1*H*-pyrazole (**2**) (1 mmol) and 4-amino-1,2,4-triazole-3-thiol derivatives **3a-c** (1 mmol) were mixed together and dissolved in absolute ethyl alcohol (20 mL), then Et_3N (0.1 mL) was added, and the reaction mixture was left to reflux for 5−7 h then left to cool to room temperature. The precipitated solid product was collected by filtration then recrystallization from DMF/EtOH produced compounds **4a-c**.

3.1.2. 6-(4-(2-Naphthoyl)-1-phenyl-1H-pyrazol-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine ***(4a).*** Pale yellow solid, (76% yield), mp. 249−251 °C; IR (KBr) ν: 1685 (C═O), 1596 (C═N) cm^{-1}; ^{1}H NMR: δ 4.50 (s, 2H, thiadiazine-SCH_2), 7.44−8.12 (m, 11H, ArH), 8.50 (s, 1H, naphthyl-1-CH), 8.64 (s, 1H, triazolyl-3-CH), 9.23 (s, 1H,

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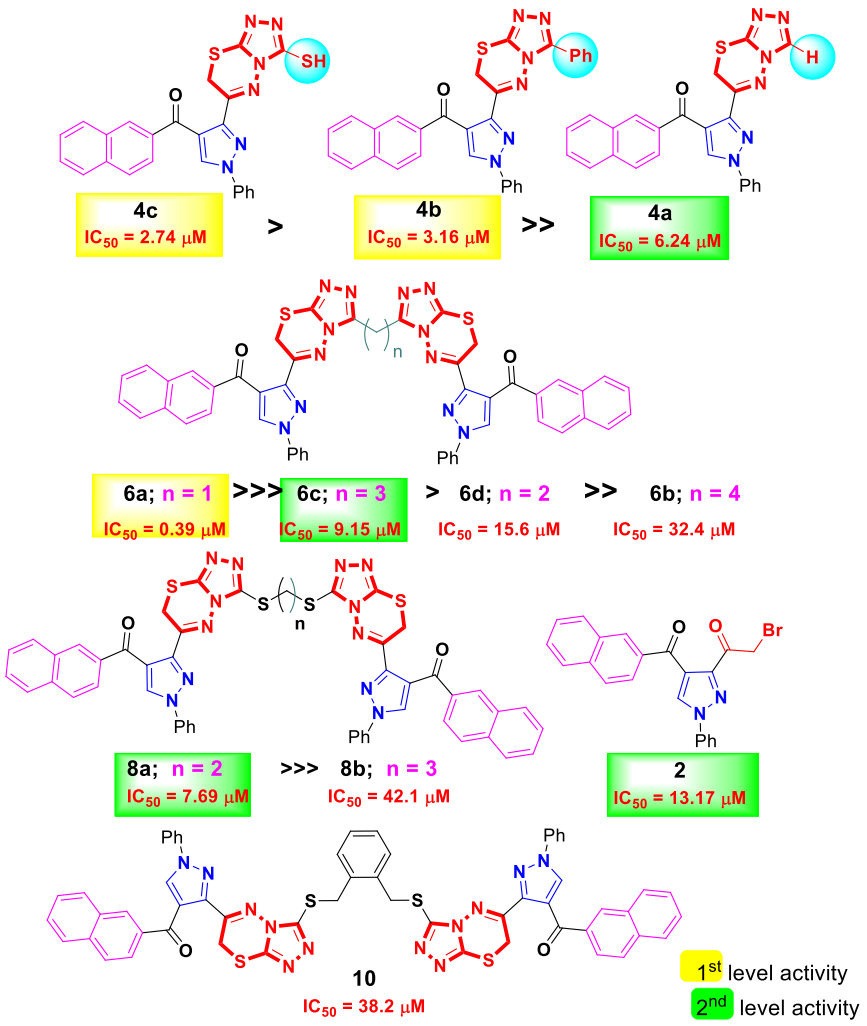

Figure 4. Derivatization effect on the simple- and bis-triazolothiadiazine hybrids on their cytotoxicities against MCF-7 cells.

Table 2. IC_{50} Values of EGFR and CDK-2 Kinase Inhibition Activities

	IC_{50} [nM][a]	
compound	EGFR kinase	CDK-2 kinase
4b	71.7 ± 2.1	113.7 ± 2.9
4c	89.6 ± 2.4	165.4 ± 3.14
6a	19.6 ± 0.64	87.9 ± 2.8
Erlotinib	67.3 ± 2.54	
Roscovitine		140 ± 3.6

[a]Values are expressed as "average of three independent replicates". "IC_{50} values were calculated using sigmoidal non-linear regression curve fit of percentage inhibition against five concentrations of each compound".

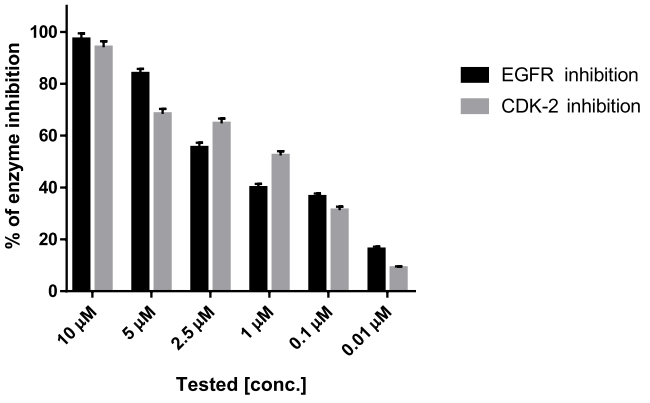

Figure 5. Dose−response curve for the activity of compound **6a** with percentage of kinase inhibition compared to concentrations range of [0.01−10 μM].

pyrazolyl-5-CH); ^{13}C NMR: δ 33.0, 119.0, 120.9, 124.7, 126.2, 126.8, 127.1, 127.6, 128.2, 128.5, 128.8, 129.6, 129.8, 131.6, 132.1, 134.3, 134.9, 135.7, 138.9, 147.9, 167.6, 189.0. MS: *m/z* 436 (M^+). Anal. Calcd for $C_{24}H_{16}N_6OS$: C, 66.04; H, 3.69; N, 19.25; S, 7.34. Found: C, 66.01; H, 3.67; N, 19.23; S, 7.30%.

3.1.3. 3-Phenyl-6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazine ***(4b)***. Off-white solid, (80% yield), mp. 258−260 °C; IR (KBr) ν: 1687 (C═O), 1597 (C═N) cm^{-1}; ^{1}H NMR: δ 4.58 (s, 2H, thiadiazine-SCH_2), 7.22−8.06 (m, 16H, ArH), 8.42 (s, 1H, naphthyl-1-CH), 9.19 (s, 1H, pyrazolyl-5-CH); MS: *m/z* 512 (M^+). Anal. Calcd for $C_{30}H_{20}N_6OS$: C, 70.30; H, 3.93; N, 16.40; S, 6.25. Found: C, 70.31; H, 3.90; N, 16.35; S, 6.27%.

3.1.4. 6-(4-(2-Naphthoyl)-1-phenyl-1H-pyrazol-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-3-thiol ***(4c)***. Yellow solid, (78% yield), mp 267−269 °C; IR (KBr) ν: 1683 (C═O), 1595 (C═N) cm^{-1}; ^{1}H NMR: δ 4.41 (s, 2H, thiadiazine-SCH_2), 7.44−8.11 (m, 11H, ArH), 8.49 (s, 1H, naphthyl-1-CH), 9.22 (s, 1H, pyrazolyl-5-CH), 13.77 (s, 1H, SH); MS:

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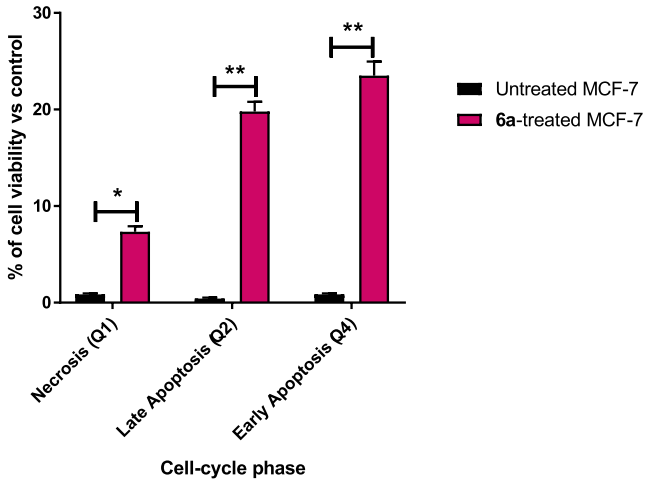

Figure 6. Apoptosis/necrosis assessment using "Annexin-V/Propidium Iodide staining" of untreated and **6a**-treated MCF-7 cells with the IC_{50} values, 48 h. "*($P \leq 0.05$) and **($P \leq 0.001$) significantly different between untreated and treated cells".

m/z 468 (M^+). Anal. Calcd for $C_{24}H_{16}N_6OS_2$: C, 61.52; H, 3.44; N, 17.94; S, 13.68. Found: C, 61.50; H, 3.43; N, 17.92; S, 13.67%.

*3.1.5. Synthesis of Bis(6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazin-3-ylalkanes **6a-d**.* A mixture the bis(4-amino-5-mercapto-1,2,4-triazole) derivatives **5a-d** (1 mmol) and two equivalents of 3-bromoacetylpyrazole derivative **2** (2 mmol) was dissolved in absolute ethyl alcohol (20 mL), then Et_3N (0.2 mL) was added portion-wise. After that, the mixed reaction components were heated at reflux temperature for 5−7 h then left to cool to ambient temperature. The solidified product was collected by filtration followed by recrystallization from DMF/EtOH to produce the bis-heterocyclic hybrids **6a-d**.

*3.1.6. Bis(6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-3-ylmethane **(6a)**.* Off-white solid, (72% yield), mp 262−264 °C; IR (KBr) ν: 1682 (C═O), 1572 (C═N) cm^{-1}; ^1H NMR: δ 2.91 (s, 2H, CH_2), 4.45 (s, 4H, thiadiazine-SCH_2), 7.44−8.06 (m, 22H, ArH), 8.45 (s, 2H, naphthyl-1-CH), 9.16 (s, 2H, pyrazolyl-5-CH); ^{13}C NMR: δ 19.2, 22.9, 119.3, 122.3, 124.1, 126.8, 127.4, 128.0, 128.3, 128.7, 129.6, 129.8, 131.8, 131.9, 132.4, 134.4, 134.9, 138.6, 140.3, 145.3, 147.5, 148.8, 189.0. MS: *m/z* 884 (M^+). Anal. Calcd for $C_{49}H_{32}N_{12}O_2S_2$: C, 66.50; H, 3.64; N, 18.99; S, 7.25. Found: C, 66.48; H, 3.63; N, 18.99; S, 7.22%.

*3.1.7. 1,2-Bis(6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylethane **(6b)**.* Brown solid, (71% yield), mp 243−245 °C; IR (KBr) ν: 1685 (C═O), 1595 (C═N) cm^{-1}; ^1H NMR: δ 3.05 (s, 4H, CH_2), 4.44 (s, 4H, thiadiazine-SCH_2), 7.44−8.07 (m, 22H, ArH), 8.36 (s, 2H, naphthyl-1-CH), 9.14 (s, 2H, pyrazolyl-5-CH); MS: *m/z* 898 (M^+). Anal. Calcd for $C_{50}H_{34}N_{12}O_2S_2$: C, 66.80; H, 3.81; N, 18.70; S, 7.13. Found: C, 66.78; H, 3.80; N, 18.71; S, 7.11%.

*3.1.8. 1,3-Bis(6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylpropane **(6c)**.* Brown solid, (77% yield), mp 276−278 °C; IR (KBr) ν: 1681 (C═O), 1592 (C═N) cm^{-1}; ^1H NMR: δ 1.80 (s, 2H, CH_2), 2.97 (t, 4H, J = 6.9, CH_2), 4.52 (s, 4H, thiadiazine-SCH_2), 7.46−8.07(m, 22H, ArH), 8.48 (s, 2H, naphthyl-1-CH), 9.18 (s, 2H, pyrazolyl-5-CH); MS: *m/z* 912 (M^+). Anal. Calcd for $C_{51}H_{36}N_{12}O_2S_2$: C, 67.09; H, 3.97; N, 18.41; S, 7.02. Found: C, 67.08; H, 3.98; N, 18.38; S, 7.01%.

*3.1.9. 1,4-Bis(6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylbutane **(6d)**.* Brown solid, (75% yield), mp 270−272 °C; IR (KBr) ν: 1680 (C═O), 1595 (C═N) cm^{-1}; ^1H NMR: δ 1.77 (s, 4H, CH_2), 2.72 (s, 4H, CH_2), 4.45 (s, 4H, thiadiazine-6-H), 7.45−8.06 (m, 22H, ArH), 8.47 (s, 2H, naphthalene-1-H), 9.16 (s, 2H, pyrazole-5-H); ^{13}C NMR: δ 17.6, 20.5, 31.3, 114.8, 119.8, 126.8, 127.0, 127.8, 128.3, 129.1, 129.4, 129.9, 132.7, 135.8, 141.2, 150.2, 153.1, 157.3, 159.6, 166.3, 168.3, 188.3. MS: *m/z* 926 (M^+). Anal. Calcd for $C_{52}H_{38}N_{12}O_2S_2$: C, 67.37; H, 4.13; N, 18.13; S, 6.92. Found: C, 67.35; H, 4.12; N, 18.10; S, 6.91%.

*3.1.10. Synthesis of Bis((6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazin-3-ylthio)alkanes **8a,b** and **10**.* The pyrazolyl[1,2,4]triazolo[3,4-*b*]thiadiazine-3-thiol derivative **4c** (2 mmol) was added portion-wise to a solution of KOH (0.112 g, 2 mmol) in absolute ethanol (20 mL) with stirring at ambient temperature. After complete addition, the bis-bromo derivatives **7a,b** or **9** (1 mmol) was added to the obtained component mixture, then

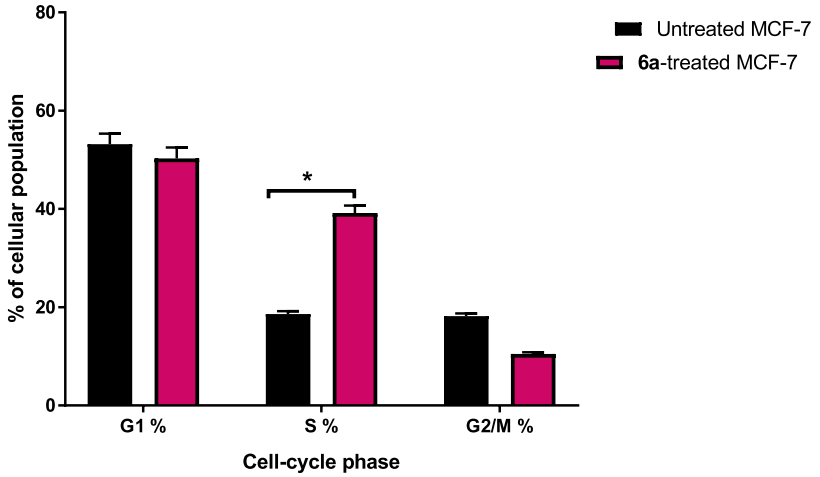

Figure 7. Cell population at each cell cycle G1, S, and G2/M in untreated and **6a**-treated MCF-7 cells with the IC_{50} values, 48 h using DNA content flow cytometry analysis. *($P \leq 0.05$) significantly different between untreated and treated cells.

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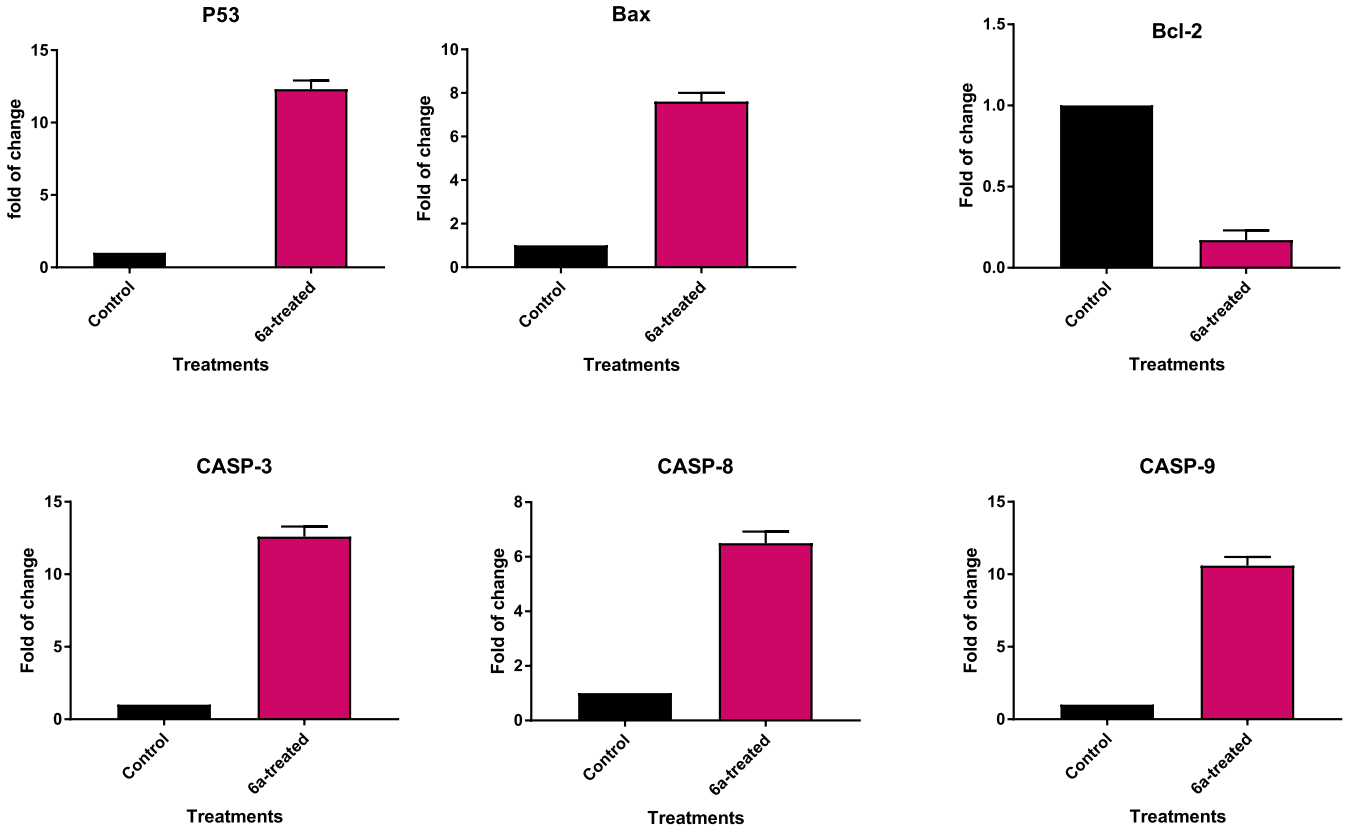

Figure 8. RT-PCR results analysis of the apoptosis-related genes; "P53, Bax, Caspases 3, 8, 9, and Bcl-2" in MCF-7 cells treated with **6a** with the IC_{50} values, 48 h. The data illustrated is the average of 3 independent experimental runs "mean ± SD".

the reaction flask was heated under conventional heating at reflux for 2−3 h then left to cool to an ambient temperature. The precipitated solid material was collected through filtration followed by recrystallization from DMF to yield the corresponding bis-heterocycles **8a,b** and **10,** respectively.

3.1.11. 1,2-Bis((6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylthio)-ethane ***(8a)***. Brown solid, (69% yield), mp 236−238 °C; IR (KBr) ν: 1684 (C═O), 1596 (C═N) cm^{-1}; ^{1}H NMR: δ 3.94 (s, 4H, CH_2S), 4.56 (s, 4H, thiadiazine-SCH_2), 7.44−8.06 (m, 22H, ArH), 8.48 (s, 2H, naphthyl-1-CH), 9.20 (s, 2H, pyrazolyl-5-CH); ^{13}C NMR: δ 27.9, 30.2, 114.8, 115.2, 121.3, 126.8, 127.1, 127.3, 127.4, 127.8, 128.3, 128.7, 137.1, 137.3, 141.1, 146.1, 151.0, 157.2, 159.2, 166.7, 168.3, 187.8. MS: *m/z* 962 (M^+). Anal. Calcd for $C_{50}H_{34}N_{12}O_2S_4$: C, 62.35; H, 3.56; N, 17.45; S, 13.31. Found: C, 62.32; H, 3.53; N, 17.44; S, 13.30%.

3.1.12. 1,3-Bis((6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylthio)-propane ***(8b)***. Brown powder, (73% yield), mp 249−251 °C; IR (KBr) 1684 (C═O), 1595 (C═N) cm^{-1}; ^{1}H NMR: δ 2.06 (s, 2H, CH_2), 4.41 (s, 4H, CH_2S), 4.50 (s, 4H, thiadiazine-SCH_2), 7.46−8.06(m, 22H, ArH), 8.50 (s, 2H, naphthyl-1-CH), 9.20 (s, 2H, pyrazolyl-5-CH); MS: *m/z* 976 (M^+). Anal. Calcd for $C_{51}H_{36}N_{12}O_2S_4$: C, 62.69; H, 3.71; N, 17.20; S, 13.12. Found: C, 62.67; H, 3.70; N, 17.21; S, 13.12%.

3.1.13. 1,2-Bis((6-(4-(2-naphthoyl)-1-phenyl-1H-pyrazol-3-yl))-7H-[1,2,4]triazolo[3,4-b][1,3,4]-thiadiazin-3-ylthiomethyl)benzene ***(10)***. Brown powder, (73% yield), mp 281−283 °C; IR (KBr) 1683 (C═O), 1595 (C═N) cm^{-1}; ^{1}H NMR: δ 4.13 (s, 4H, CH_2S), 4.53 (s, 4H, thiadiazine-SCH_2), 7.08−8.21 (m, 26H, ArH), 8.46 (s, 2H, naphthyl-1-CH), 9.18 (s, 2H, pyrazolyl-5-CH); ^{13}C NMR: δ 26.0, 31.3, 114.6, 121.3, 122.0, 126.6, 127.0, 127.2, 127.4, 127.9, 128.7, 131.2, 132.4, 137.0, 137.4, 146.4, 149.3, 149.9, 151.9, 158.4, 160.8, 166.6, 170.1, 191.8. MS: *m/z* 1038 (M^+). Anal. Calcd for $C_{56}H_{38}N_{12}O_2S_4$: C, 64.72; H, 3.69; N, 16.17; S, 12.34. Found: C, 64.71; H, 3.69; N, 16.16; S, 12.31%.

3.2. Biology. *3.2.1. Cytotoxicity.* Both MCF-7 (breast cancer) and MCF-10A (normal breast) cells were bought from National Research Institute, Egypt, and kept in RPMI-1640 medium L-glutamine (Lonza Verviers SPRL, Belgium, cat#12−604F). Both cell lines were provided with 10% fetal bovine serum (FBS, Sigma-Aldrich, MO, USA) and 1% penicillin−streptomycin (Lonza, Belgium). Cells were treated with the compounds at the (0.01, 0.1, 1, 10, and 100 μM) concentrations. Cell viability was assessed after 48 h using MTT solution (Promega, USA).[53,54]

3.2.2. EGFR and CDK-2 Kinase Inhibitory Assay. EGFR-TK assay kit (ADP-GloTM kinase assay, Cat no. V9261, Promega, USA) and CDK-2 luminescence kinase Assay kit (Catalog #79599, Kinase-Glo Plus, Promega, USA) were performed. Kinase inhibitory assays were carried out to evaluate the inhibitory efficacy of the most active substances **4b, 4c** and **6a** against the EGFR and CDK-2 following manufacturer instructions. The autophosphorylation % inhibition by compounds was counted employing the following equation: $100 - \left[\left(\frac{\text{A control}}{\text{A treated}} - \text{control}\right)\right]$ using the curves of % inhibition of five concentrations of each compound, IC_{50} was calculated using the GraphPad prism7 software.[55]

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3.2.3. Investigation of Apoptosis. 3.2.3.1. Annexin V/PI Staining and Cell Cycle Analysis. MCF-7 cells were seeded into 6-well culture plates (3−5 × 10^5 cells/well) and incubated overnight. Cells were then treated with compound **6a** at their IC_{50} values for 48 h. Next, media supernatants and cells were collected and rinsed with ice-cold PBS. The next step was suspending the cells in 100 μL of annexin binding buffer solution "25 mM CaCl2, 1.4 M NaCl, and 0.1 M Hepes/NaOH, pH 7.4" and incubation with "Annexin V-FITC solution (1:100) and propidium iodide (PI)" at a concentration that equals 10 μg/mL in the dark for 30 min. Stained cells were then acquired by Cytoflex FACS machine. Data were analyzed using cytExpert software.[56−58]

3.2.3.2. Real Time-Polymerase Chain Reaction for the Selected Genes. The expression of P53, Bax, Caspases-3,8,9 (proapoptotic genes), and Bcl-2 (antiapoptotic gene) was measured to learn more about the apoptotic process. MCF-7 cells were then treated with compound **6a** at their IC_{50} values for 48 h. After treatment, RT-PCR reaction was carried out following routine work. Then, the Ct values were gathered to determine the relative expression of genes in each sample, normalized to the -actin reference gene[56,59]

4. CONCLUSIONS

In this study, a series of naphthalene-based simple pyrazolyl-triazolo[3,4-*b*][1,3,4]thiadiazine derivatives **4a-c** and their bis-molecular hybrids with alkylene spacers **6a-d,** or with bis-thioalkylene spacers **8a,b** and **10,** were designed and synthesized using an easily applicable synthetic route. Structures of the constructed molecular hybrids were substantiated from their spectroscopic and analytical data. The cytotoxicity of the obtained molecular hybrids was evaluated against MCF-7 cell line and many compounds showed moderate to high potency. Compounds **4b, 4c,** and **6a** displayed potent cytotoxicity as well as promising dual EGFR and CDK-2 inhibition activities. Compounds **6a** had the most potent cytotoxicity against MCF-7 with IC_{50} = 0.39 μM and it was safe against the normal MCF-10A cells with high IC_{50} values. Compound **6a** was also the most active dual EGFR and CDK-2 inhibitor with IC_{50} values of 19.6 and 87.9 nM, respectively, better than Erlotinib and Roscovitine. Compound **6a** treatment induced EGFR and CDK-2 enzyme inhibition by 97.18 and 94.11%, respectively, at the highest concentration [10 μM]. For apoptosis activity, compound **6a** significantly activated apoptotic cell death in MCF-7 cells, increasing the cell population by total apoptosis 43.3% (19.8% for early apoptosis and 23.5% for late apoptosis) compared to the untreated control group (1.29%) increasing the cell population at the S-phase by 39.2% compared to control 18.6%. Therefore, compound **6a** induced a potent cytotoxicity against MCF-7 cells through EGFR/CDK-2 inhibition with apoptosis-induction, and it can be developed further as a chemo-therapeutic anti-breast cancer agent.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05309.

1H NMR spectrum of compound 4a; 13C NMR spectrum of compound 4a; 1H NMR spectrum of compound 4b; 1H NMR spectrum of compound 4c; 1H NMR spectrum of compound 6a; 13C NMR spectrum of compound 6a; 1H NMR spectrum of compound 6b; 1H NMR spectrum of compound 6c; 1H NMR spectrum of compound 6d; 13C NMR spectrum of compound 6d; 1H NMR spectrum of compound 8a; 13C NMR spectrum of compound 8a; 1H NMR spectrum of compound 8b; 1H NMR spectrum of compound 10; and 13C NMR spectrum of compound 10 (PDF)

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Notes

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