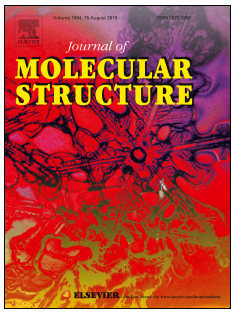

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Credit Author Statement

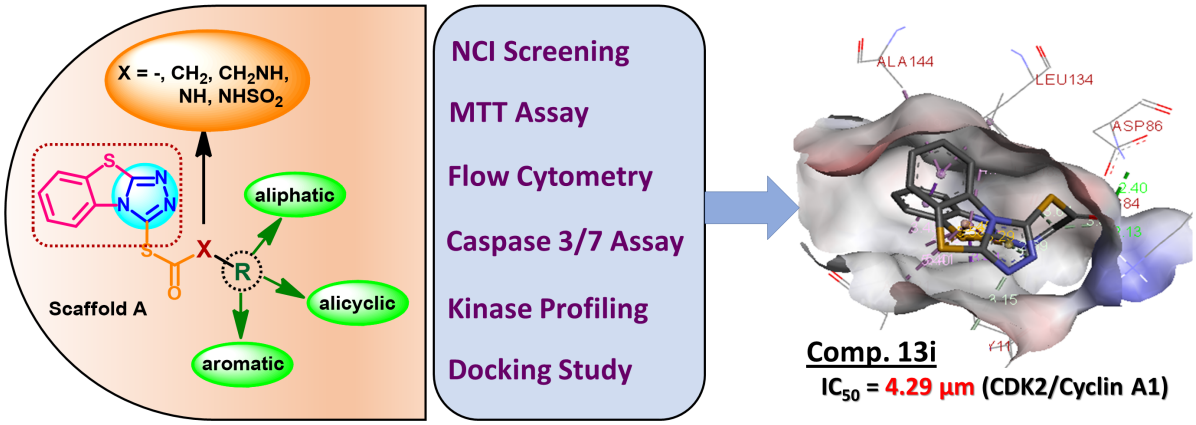
Ahmed H. Abdelazeem: Conceptualization, Conducting a research (chemical synthesis and analysis part) and Writing-Original draft preparation and Funding acquisition.

Alaa Alqahtani: Project administration, Funding acquisition, Supervision and Contributed to the design and implementation of the research.

Hany A. Omar: Methodology and Conducting a research (Biological part)

Syed Nasir Abbas: Writing - Review & Editing and Investigation

Ahmed M. Gouda: Writing - Review & Editing, Software, Investigation and Contributed to the design and implementation of the research.



Synthesis, biological evaluation and kinase profiling of novel *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole derivatives as cytotoxic agents with apoptosis-inducing activity

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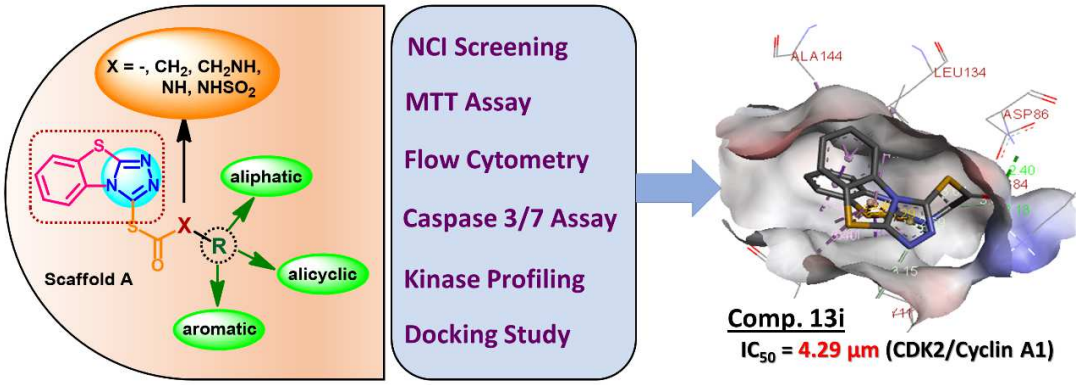
Graphical Abstract:



Highlights

1- A novel set of *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazoles was synthesized.

2- Compounds **13f**, **13g**, and **13i** showed the highest cytotoxic activity.

3- Compound **13i** activated caspase-3/7 and induced apoptosis.

4- Compound **13i** exhibited the highest inhibitory activity against CDK2/Cyclin A1.

Abstract

A novel set of *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazoles was synthesized. Cytotoxicity of these compounds was evaluated against three cancer cell lines of different origins, Hep3B, A549, and MCF-7. Three of these compounds were screened by NCI for growth inhibitory activities against 60 cancer cell lines. The results revealed significant cytotoxic activities for compounds **13a-i**. Among these derivatives, compounds **13c** and **13f-13i** exhibited the highest cytotoxicity against the selected cancer cell lines with IC_{50} values between 3.17-14.18 μM. The structure-activity relationship of compounds **13a-i** indicated favorable cytotoxic results on the expansion of the cyclic amine and the substitution with aminothiazole moiety. A mechanistic study revealed the activation of caspase-3/7 in A549 cells on treatment with compounds **13f-i** at 5-20 μM. Moreover, the results of flow cytometric analysis suggested that compound **13i** efficiently induced apoptosis in a dose-dependent manner. Compounds **13f**,**g**,**i** also exhibited a weak to moderate inhibition of multiple kinases where compound **13i** was the most active in inhibiting the activity of CDK2/Cyclin A1 (IC_{50} = 4.65 μM). The current work provided a novel set of compounds with cytotoxic, kinase inhibition, and apoptosis-inducing activities, which can serve as a lead for further optimization.

Keywords: *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole; anticancer; Apoptosis; caspase-3/7, flow cytometry; kinase profiling; CDK2/Cyclin A1.

1. Introduction

Cancer is one of the leading causes of death in the world [1]. In high-income countries, the number of deaths caused by cancers surpasses deaths caused by cardiovascular diseases [2]. Currently, combination therapy and multi-targeted anticancer agents have proved high efficacy in the treatment of cancers [3,4]. However, their high cost, side effects, drug interaction, and resistance problems encouraged the research to find cheap and effective alternatives [5].

Identification of molecular targets involved in cancer biology became an essential process for the rational design of potent anticancer agents [6-8]. Of these promising targets, protein kinases (PKs) attracted much attention due to their pivotal role in the regulation of cell survival and proliferation [9]. In the last two decades, oncogenic kinases was emerged as potential targets in cancer treatment [10,11]. Obviously, several kinase inhibitors succeeded to reach the drug market exhibiting high response rate in treatment of lung cancer [12]. Among these kinases, cyclin-dependent kinases (CDKs) which have a crucial role in the control of the cell proliferation and transcription [13]. However, acquired resistance was reported to several kinase inhibitors [14].

Recently, several triazoles derivatives were reported with high anticancer activities [15-17]. Lin *et al*. have reported a series of 1,2,4-triazole derivatives with potent anticancer activity against a panel of six cancer cell lines [18]. Among these derivatives, compound **1** displayed IC_{50} values in the range of 0.12-0.75 µM against several cancer cell lines. This activity was mediated by potent inhibition of multiple kinases including CDK1/cyclin B, GSK-3, and VEGF-R2, **Fig. 1**. Moreover, compound **2** displayed inhibitory activity against Src kinase with IC_{50} value of 33.9 µM [19].

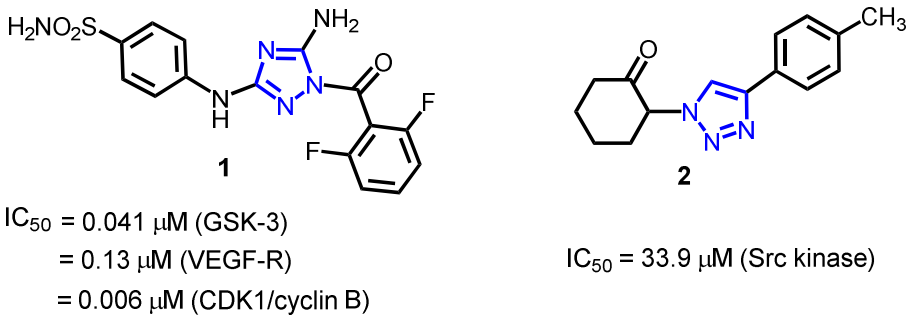


Fig. 1. Triazole-based multi-kinase inhibitors

In addition, several 2-aminobenzothiazole derivatives (**Fig. 2**) displayed broad spectra of anticancer potential against different types of cancer cell lines [18,20]. These activities were mediated by the induction of apoptosis [21,22]. Remarkably, compound **3** was developed as an anti-leukemic agent with potent inhibitory activity against both wild-type BCR-ABL (BaF3/WT) and T315I-mutated BCR-ABL (BaF3/T315I) cells. The activity of compound **3** was mediated by cell cycle arrest at the G0/G1 phase and induction of apoptosis [23-25]. Moreover, aminobenzothiazole **4** displayed potent antiproliferative activity against the M-CSF-dependent murine leukemia cell line MNFS60 (EC_{50} = 67 nM) [26]. The mechanism of action of compound **4** was mediated by inhibiting colony-stimulating factor 1 receptor (CSF-1R) [27].

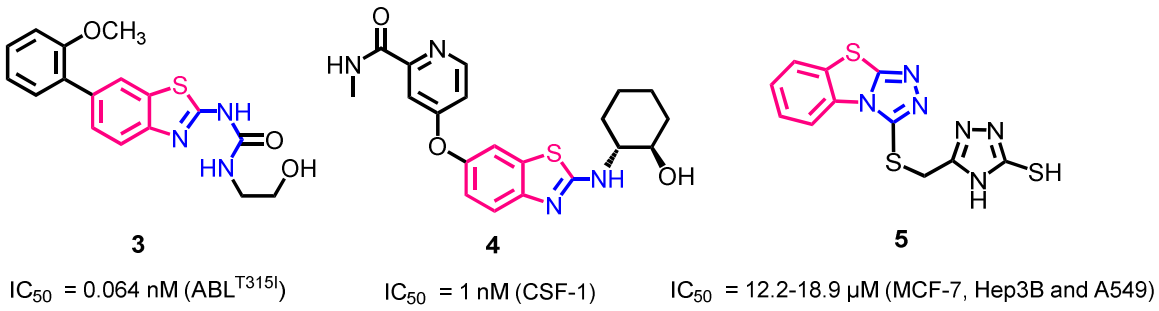


Fig. 2. Benzothiazole derivatives with anticancer/kinase inhibitory activities

More recently, our research group has reported the anticancer activity, for the first time, of a novel series incorporating *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole scaffold that is composed of

two fused triazole and aminobenzothiazole heterocyclic rings [28]. Of these derivatives, compound **5** exhibited anticancer activity against three (MCF-7, Hep3B, and A549) cancer cell lines, **Fig. 2**. Kinase profiling results of compound **5** revealed weak to moderate inhibitory activities against ten of the known oncogenic kinases. However, CDK2/Cyclin A1 was the most sensitive kinase to the action of compound **5**.

Rational Design

Based on these findings, we have designed the benzothiazolotriazole scaffold (A) incorporating 1,2,4-triazole with benzothiazole moieties. The structure activity relationship (SAR) of the novel scaffold was investigated through structural modifications in two points. Firstly, several derivatives were designed through variation of the substitution groups (R) using different aliphatic, heterocyclic, and aromatic groups **Fig. 3**. Secondly, the impact of the spacer group (X) on the anticancer activity of the new compounds was studied by alteration of the spacer length/type, **Fig. 3**.

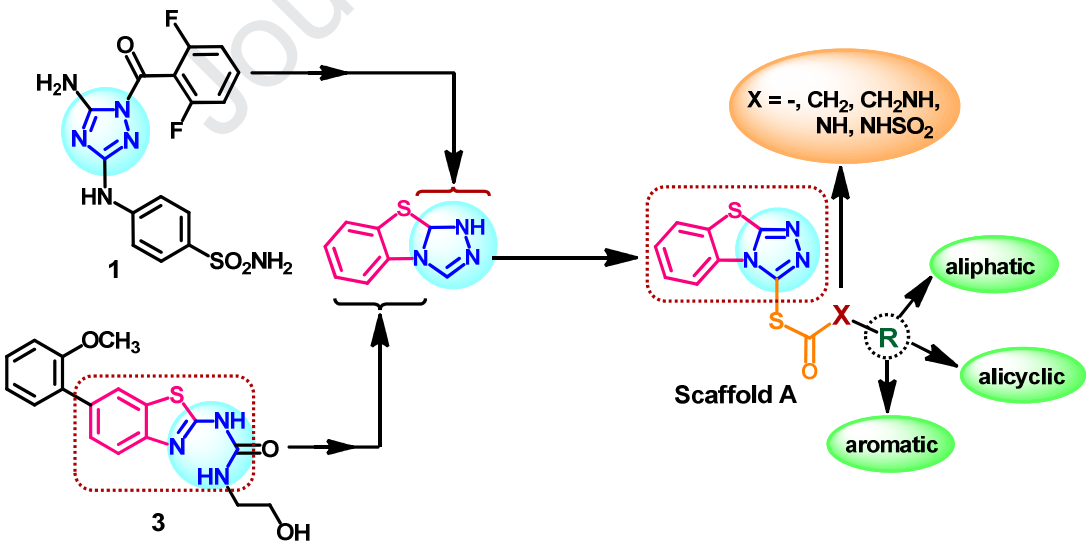


Fig. 3. Rational design and structural modifications of scaffold A.

2. Materials & Methods

2.1. Chemistry

The chemical reagents were purchased from commercial sources and used without purification. Solvents were purchased from commercial sources and dried by standard methods when necessary. ^{1}H-NMR and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively. these spectra were obtained on a Bruker APX400 at 100 MHz at Faculty of Pharmacy, Beni-Suef University. Both mass spectra (record on Finnigan MAT, SSQ 7000) and quantitative CHN analysis were performed in the microanalytical center, Faculty of Science, Cairo University. Melting points (uncorrected) were determined by the digital melting point apparatus (IA 9100MK series). The preparation of compound **8** was achieved according to the previous reports [28,29].

2.1.1. General procedure (A) for the preparation of compounds 9, 10, and 11a-d

A mixture of benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole-3-thiol (0.5 g, 2.41 mmol), the appropriate isocyanate derivative (2.41 mmol) and triethylamine (0.3 g, 3.1 mmol) was refluxed in 30 ml ethanol for 6 hr. the reaction was monitored by TLC until completion. The reaction mixture was left to cool, and the precipitate was filtered and washed with cold ethanol. Recrystallization was carried out using ethanol to furnish the final targeted compounds.

2.1.1.1. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-tosylcarbamothioate (9).

The title compound was prepared using the general procedure **A**. Compound **9** was obtained as off-white solid, m.p. 182-185 °C, yield 58%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.88 (s, 1H, N<u>H</u>), 8.09 (d, J = 7.9 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.65 (d, J = 7.7 Hz, 2H), 7.23-7.54 (m, 4H), 2.34 (s, 3H, C<u>H</u>$_3$).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 174.4 (C=O), 155.3 (C), 148.8 (C), 133.2 (C), 131.6 (C), 130.6 (C), 128.9 (C), 127.3 (CH), 127.1 (CH), 125.7 (CH),

125.4 (CH), 122.5 (CH), 114.8 (CH), 21.4 (CH_3). MS (EI) *m/z* 405.00 (M^++1). Anal. calcd. for: $C_{16}H_{12}N_4O_3S_3$: C, 47.51; H, 2.99; N, 13.85. Found: C, 47.33; H, 3.24; N, 14.01.

2.1.1.1. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-(1R,3S,5R,7R)-adamantan-2-yl-carbamothioate (10).

The title compound was prepared using the general procedure A. Compound **10** was obtained as orange solid, m.p. 176-179 °C, yield 62%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.89 (s, 1H, NH), 8.09 (d, *J* = 8.4 Hz, 1H), 7.93 (d, *J* = 7.9 Hz, 1H), 7.54 (t, *J* = 7.3 Hz, 1H), 7.45 (t, *J* = 7.9 Hz, 1H), 1.99 (s, 2H), 1.84 (s, 4H), 1.65-1.52 (m, 4H), 1.42-1.14 (m, 5H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 171.5 (C=O), 154.7 (C), 138.7 (C), 133.4 (C), 130.5 (C), 127.0 (CH), 125.7 (CH), 122.3 (CH), 115.0 (CH), 50.1 (C), 41.6 ($3CH_2$), 36.5 ($3CH_2$), 29.3 (3CH). MS (EI) *m/z* 385.00 (M^++1). Anal. calcd. for: $C_{19}H_{20}N_4OS_2$: C, 59.35; H, 5.24; N, 14.57. Found: C, 59.68; H, 5.14; N, 14.71.

2.1.1.3. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-*p*-tolylcarbamothioate (11a).

The title compound was prepared using the general procedure A. Compound **11a** was obtained as yellow solid, m.p. 200-203 °C, yield 68%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.50 (s, 1H, NH), 8.67 (d, *J* = 7.8 Hz, 1H), 8.12 (d, *J* = 7.9 Hz, 1H), 7.78 (d, *J* = 7.8 Hz, 2H), 7.06-7.45 (m, 4H), 2.22 (s, 3H, CH_3).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 163.9 (C=O), 161.3 (C), 154.0 (C), 152.3 (C), 137.2 (C), 131.5 (C), 129.5 (C), 127.1 (CH), 127.0 (CH), 125.1 (CH), 118.7 (CH), 115.6 (CH), 114.8 (CH), 20.7 (CH_3). MS (EI) *m/z* 340.00 (M^+). Anal. calcd. for: $C_{16}H_{12}N_4OS_2$: C, 56.45; H, 3.55; N, 16.46. Found: C, 56.12; H, 367; N, 16.94.

2.1.1.4. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-(4-chlorophenyl)carbamothioate (11b).

The title compound was prepared using the general procedure A. Compound **11b** was obtained as yellow solid, m.p. 205-208 °C, yield 66%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.65 (s, 1H, NH), 8.18 (d, J = 8.0 Hz, 1H), 8.09 (d, J = 8.3 Hz, 1H), 7.76 (d, J = 7.7 Hz, 2H), 7.36-7.63 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 165.3 (C=O), 160.7 (C), 153.3 (C), 152.5 (C), 135.5 (C), 131.7 (C), 131.0 (C), 127.1 (CH), 126.9 (CH), 125.7 (CH), 115.8 (CH), 115.4 (CH), 113.7 (CH). MS (EI) *m/z* 360.00 (M^+). Anal. calcd. for: $C_{15}H_9ClN_4OS_2$: C, 49.93; H, 2.51; N, 15.53. Found: C, 49.51; H, 2.73; N, 15.09.

2.1.1.5. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-(4-fluoroophenyl)carbamothioate (11c).

The title compound was prepared using the general procedure A. Compound **11c** was obtained as yellow solid, m.p. 220-223 °C, yield 56%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.64 (s, 1H, NH), 8.86 (d, J = 8.0 Hz, 1H), 8.01 (d, J = 7.9 Hz, 1H), 7.77 (d, J = 7.8 Hz, 2H), 7.11-7.55 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 164.2 (C=O), 161.8 (C), 154.1 (C), 152.2 (C), 136.0 (C), 131.5 (C), 130.5 (C), 127.0 (CH), 127.0 (CH), 125.3 (CH), 115.8 (CH), 115.6 (CH), 114.8 (CH). MS (EI) *m/z* 344.00 (M^+). Anal. calcd. for: $C_{15}H_9FN_4OS_2$: C, 52.32; H, 2.63; N, 16.27. Found: C, 52.54; H, 2.61; N, 16.11.

2.1.1.6. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-(4-nitrophenyl)carbamothioate (11d).

The title compound was prepared using the general procedure A. Compound **11d** was obtained as yellow solid, m.p. 193-197 °C, yield 64%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.99 (s, 1H, NH), 8.59 (d, J = 7.9 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 7.7 Hz, 2H), 7.16-7.41 (m, 4H).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 164.4 (C=O), 160.0 (C), 154.2 (C), 152.5 (C), 137.3 (C), 131.5 (C), 129.0 (C), 127.0 (CH), 126.7 (CH), 125.5 (CH), 118.6 (CH), 115.1 (CH), 114.0 (CH). MS (EI) *m/z* 371.00 (M^+). Anal. calcd. for: $C_{15}H_9N_5O_3S_2$: C, 48.51; H, 2.44; N, 18.86. Found: C, 48.76; H, 2.54; N, 18.51.

2.1.1.7. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-chloroethanethioate (12).

Chloroacetyl chloride (3.5 gm, 31.6 mmol) was added slowly to a cold solution of benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole-3-thiol **8** (5 gm, 24.3 mmol) and triethylamine (7.3 g, 72.81 mmol) in CH_2Cl_2. The reaction mixture was stirred for 1 h at 0 ^oC then for 5 h at room temperature. The organic layer was then separated and evaporated in vacuo. The residue obtained was used directly for the next coupling step without purification. MS (EI) *m/z* 283.00 (M^+).

2.1.2. General procedure (B) for the preparation of compounds 13a-i.

The appropriate amine (3.3 mmol) and K_2CO_3 (1.4 g, 9.9 mmol) were added, under mechanical stirring, to a solution of *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl 2-chloroethanethioate **12** (0.9 g, 3.3 mmol) in 30 mL DMF. The reaction mixture was heated at 60 ^oC for 4 h. After cooling, the mixture was poured onto 100 mL H_2O, extracted with ethyl acetate (3 x 50 mL), washed with brine and dried. The solvent was removed in vacuo, and the residue was chromatographed on a silica gel column using methylene chloride/methanol (9.5:0.5) as eluent to afford the final compounds **13a-i** in good yields.

2.1.2.1. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(pyrrolidin-1-yl)ethanethioate (13a).

The title compound was prepared using the general procedure B. Compound **13a** was obtained as yellow oil. ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.16 (d, J = 7.9 Hz, 1H), 8.08 (d, J = 8.2 Hz, 1H), 7.62 (t, J = 7.8 Hz, 1H), 7.52 (t, J = 8.0 Hz, 1H), 4.23 (s, 2H, COC$\underline{H_2}$), 2.11-1.65 (m, 8H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 165.1 (C=O), 156.5 (C), 143.3 (C), 131.9 (C), 130.0 (C), 127.5 (CH), 126.9 (CH), 126.0 (CH), 114.9 (CH), 52.9 (CH_2), 46.3 (CH_2), 25.7 (CH_2). MS (EI) *m/z* 318.00 (M^+). Anal. calcd. for: $C_{14}H_{14}N_4OS_2$: C, 52.81; H, 4.43; N, 17.60. Found: C, 52.69; H, 4.34; N, 17.48.

2.1.2.2. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(piperidin-1-yl)ethanethioate (13b).

The title compound was prepared using the general procedure B. Compound **13b** was obtained as off-white solid, m.p. 195-198 °C, yield 62%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.16 (d, J = 8.1 Hz, 1H), 7.52-8.08 (m, 3H), 4.35 (s, 1H, COC$\underline{H}_2$), 1.62-1.36 (m, 10H).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 165.2 (C=O), 156.6 (C), 143.2 (C), 131.9 (C), 130.0 (C), 127.6 (CH), 126.9 (CH), 126.0 (CH), 114.8 (CH), 46.9 (CH_2), 42.9 (CH_2), 26.3 (CH_2), 24.2 (CH_2). MS (EI) *m/z* 332.00 (M^+). Anal. calcd. for: $C_{15}H_{16}N_4OS_2$: C, 54.19; H, 4.85; N, 16.85. Found: C, 54.61; H, 4.57; N, 17.13.

2.1.2.3. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(azepan-1-yl)ethanethioate (13c).

The title compound was prepared using the general procedure B. Compound **13c** was obtained as orange solid, m.p. 186-189 °C, yield 66%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.16 (d, J = 8.0 Hz, 1H), 8.09 (d, J = 8.0 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.53 (t, J = 7.8 Hz, 1H), 4.36 (s, 1H, COC$\underline{H}_2$), 1.74-1.49 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.5 (C=O), 153.2 (C), 143.4 (C), 131.9 (C), 130.0 (C), 127.6 (CH), 126.9 (CH), 126.0 (CH), 114.8 (CH), 48.0 (CH_2), 45.6 (CH_2), 28.7 (CH_2), 24.9 (CH_2). MS (EI) *m/z* 346.00 (M^+). Anal. calcd. for: $C_{16}H_{18}N_4OS_2$: C, 55.47; H, 5.24; N, 16.17. Found: C, 55.15; H, 5.37; N, 15.94.

2.1.2.4. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(cyclohexylamino)ethanethioate (13d).

The title compound was prepared using the general procedure B. Compound **13d** was obtained as light brown solid, m.p. 194-197 °C, yield 57%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.21 (d, J = 8.3 Hz, 1H), 8.03-7.51 (m, 3H), 7.10 (s, 1H, N$\underline{H}$), 3.83 (s, 2H, COC$\underline{H}_2$), 3.02-2.94 (m, 1H), 1.94-1.47 (m, 4H), 1.41-0.91 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.0 (C=O), 158.8 (C), 149.0 (C), 131.9 (C), 127.4 (C), 126.9 (CH), 125.9 (CH), 115.0 (CH), 113.2 (CH), 48.3 (CH_2), 47.5 (CH), 32.8 (CH_2), 25.0 (CH_2), 24.2 (CH_2). MS (EI) *m/z* 346.00 (M^+). Anal. calcd. for: $C_{16}H_{18}N_4OS_2$: C, 55.47; H, 5.24; N, 16.17. Found: C, 55.19; H, 5.64; N, 15.87.

2.1.2.5. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-morpholinoethanethioate (13e).

The title compound was prepared using the general procedure B. Compound **13e** was obtained as brown solid, m.p. 187-190 °C, yield 61%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.18 (d, J = 8.3 Hz, 1H), 8.10-7.54 (m, 3H), 4.35 (s, 2H, COC$\underline{H}_2$), 4.09 (t, J = 8.0 Hz, 4H), 3.09 (t, J = 8.0 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.0 (C=O), 155.8 (C), 143.0 (C), 139.8 (C), 131.9 (C), 129.7 (CH), 127.6 (CH), 119.6 (CH), 114.9 (CH), 66.7 (CH_2), 59.6 ($2CH_2$), 46.2 ($2CH_2$). MS (EI) *m/z* 334.00 (M^+). Anal. calcd. for: $C_{14}H_{14}N_4O_2S_2$: C, 50.28; H, 4.22; N, 16.75. Found: C, 50.63; H, 3.99; N, 16.35.

2.1.2.6. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(4-methylpiperazin-1-yl)ethanethioate (13f).

The title compound was prepared using the general procedure B. Compound **13f** was obtained as brown oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.20 (d, J = 8.1 Hz, 1H), 8.11-7.50 (m, 3H), 4.15 (s, 2H, COC$\underline{H}_2$), 2.97 (t, J = 8.0 Hz, 4H), 2.72 (t, J = 7.8 Hz, 4H), 2.31 (s, 3H, C$\underline{H}_3$). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 167.5 (C=O), 153.9 (C), 143.4 (C), 138.7 (C), 131.9 (C), 129.1 (CH), 126.4 (CH), 120.0 (CH), 114.9 (CH), 66.7 (CH_2), 60.8 ($2CH_2$), 59.6 ($2CH_2$), 46.2 (CH_3). MS (EI) *m/z* 347.00 (M^+). Anal. calcd. for: $C_{15}H_{17}N_5OS_2$: C, 51.85; H, 4.93; N, 20.16. Found: C, 51.71; H, 5.18; N, 20.39.

2.1.2.7. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(hexylamino)ethanethioate (13g).

The title compound was prepared using the general procedure B. Compound **13g** was obtained as light brown solid, m.p. 234-237 °C, yield 52%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.21 (d, J = 8.2 Hz, 1H), 8.09-7.53 (m, 3H), 3.87 (s, 2H, COC$\underline{H}_2$), 3.12 (s, 1H, N$\underline{H}$), 2.93 (t, J = 8.0 Hz, 2H), 1.30-1.10 (m, 5H), 0.86-0.81 (m, 5H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.9 (C=O), 156.7 (C), 142.9 (C), 131.9 (C), 130.0 (C), 127.4 (CH), 126.9 (CH), 126.0 (CH), 115.0 (CH), 56.4 (CH_2), 38.2 (CH_2), 31.4 (CH_2), 29.2 (CH_2), 26.4 (CH_2), 22.5 (CH_2), 14.4 (CH-

$_2$). MS (EI) *m/z* 348.00 (M^+). Anal. calcd. for: $C_{16}H_{20}N_4OS_2$: C, 55.15; H, 5.79; N, 16.08. Found: C, 55.34; H, 6.12; N, 15.87.

2.1.2.8. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-((1*R*,2*S*,5*S*)-adamantan-2-ylamino)ethanethioate (13h).

The title compound was prepared using the general procedure B. Compound **13h** was obtained as white solid, m.p. 210-213 °C, yield 59%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.13 (d, *J* = 8.1 Hz, 1H), 8.09 (d, *J* = 8.1 Hz, 1H), 7.63 (t, *J* = 7.8 Hz, 1H), 7.53 (t, *J* = 7.6 Hz, 1H), 4.31 (s, 2H, COC<u>H</u>$_2$), 4.18 (s, 1H, N<u>H</u>), 2.02-1.58 (m, 15H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 169.1 (C=O), 156.8 (C), 142.6 (C), 131.9 (C), 129.8 (C), 127.6 (CH), 127.0 (CH), 126.1 (CH), 114.8 (CH), 57.1 (CH_2), 53.0 (C), 37.3 ($3CH_2$), 36.0 ($3CH_2$), 27.2 (3CH). MS (EI) *m/z* 398.00 (M^+). Anal. calcd. for: $C_{20}H_{22}N_4OS_2$: C, 60.27; H, 5.56; N, 14.06. Found: C, 59.98; H, 5.37; N, 13.89.

2.1.2.8. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-2-(benzo[*d*]thiazol-2-ylamino)ethanethioate (13i).

The title compound was prepared using the general procedure B. Compound **13i** was obtained as light brown solid, m.p. 189-191 °C, yield 56%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.64 (d, *J* = 7.8 Hz, 1H), 7.60-7.41 (m, 4H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.20 (t, *J* = 7.6 Hz, 1H), 7.00 (t, *J* = 7.5 Hz, 1H), 5.11 (s, 1H, N<u>H</u>), 3.55 (s, 2H, COC<u>H</u>$_2$).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 167.0 (C=O), 162.8 (C), 153.1 (C), 151.2 (C), 149.3 (C), 148.4 (C), 142.3 (C), 141.0 (C), 139.0 (CH), 131.3 (CH), 130.3 (CH), 125.9 (CH), 121.4 (CH), 119.3 (CH), 118.2 (CH), 114.5 (CH), 58.6 (CH_2). MS (EI) *m/z* 397.00 (M^+). Anal. calcd. for: $C_{17}H_{11}N_5OS_3$: C, 51.37; H, 2.79; N, 17.62. Found: C, 51.26; H, 3.01; N, 17.95.

2.1.3. General procedure (C) for the preparation of compounds 14a-d.

A solution of an appropriate acid chloride (2.41 mmol) in 10 mL methylene chloride or tetrahydrofuran was added dropwise to a mixture of benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole-3-thiol **8** (0.5 g, 2.41 mmol) and triethylamine (0.3 g, 3.1 mmol) in 20 mL methylene chloride or tetrahydrofuran. The reaction mixture was heated for 5 h while monitoring by TLC until completion. The reaction mixture was then evaporated, and 50 mL of H_2O was added followed by extraction with ethyl acetate (3 x 20 mL). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and the residue was purified by flash column chromatography using ethyl acetate/hexane (2:8) as eluent to give the final compounds **14a-d**.

2.1.3.1. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-benzothioate (14a).

The title compound was prepared using the general procedure C. Compound **14a** was obtained as yellow solid, m.p. 211-214 °C, yield 67%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.51-7.97 (m, 4H), 7.89-7.44 (m, 5H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.4 (C=O), 163.3 (C), 153.4 (C), 134.2 (C), 132.2 (C), 131.2 (C), 130.5 (CH), 129.7 (CH), 128.9 (CH), 127.8 (CH), 127.2 (CH), 125.5 (CH), 115.1 (CH). MS (EI) *m/z* 311.00 (M^+). Anal. calcd. for: $C_{15}H_9N_3OS_2$: C, 57.86; H, 2.91; N, 13.49. Found: C, 58.23; H, 3.11; N, 13.67.

2.1.3.2. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-3,4,5-trimethoxybenzothioate (14b).

The title compound was prepared using the general procedure C. Compound **14b** was obtained as yellow solid, m.p. 237-239 °C, yield 59%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.20-7.79 (m, 2H), 7.63-7.45 (m, 2H), 7.23 (s, 2H), 3.82 (s, 9H, 3OC$\underline{H_3}$). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 167.4 (C=O), 165.8 (C), 153.1 (C), 130.6 (C), 127.8 (C), 127.2 (C), 126.4 (C), 125.5 (C), 115.1 (CH), 114.4 (CH), 109.4 (CH), 108.2 (CH), 107.0 (CH), 60.9 ($2CH_3$), 56.5 (CH_3). MS (EI) *m/z* 401.00 (M^+). Anal. calcd. for: $C_{18}H_{15}N_3O_4S_2$: C, 53.85; H, 3.77; N, 10.47. Found: C, 53.91; H, 3.57; N, 10.81.

2.1.3.3. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-3,5-dinitrobenzothioate (14c).

The title compound was prepared using the general procedure C. Compound **14c** was obtained as yellow solid, m.p. 246-249 °C, yield 73%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.99 (s, 1H), 8.87 (s, 2H), 8.13-7.86 (m, 2H), 7.64-7.33 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 164.37 (C=O), 161.7 (C), 152.1 (C), 148.7 (C), 134.5 (C), 131.5 (C), 130.6 (C), 129.3 (CH), 127.1 (CH), 127.0 (CH), 125.3 (CH), 122.5 (CH), 114.7 (CH). MS (EI) *m/z* 401.00 (M^+). Anal. calcd. for: $C_{15}H_7N_5O_5S_2$: C, 44.89; H, 1.76; N, 17.45. Found: C, 45.11; H, 2.15; N, 17.67.

2.1.3.4. *S*-Benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazol-3-yl-adamantane-1-carbothioate (14d).

The title compound was prepared using the general procedure C. Compound **14d** was obtained as white solid, m.p. 187-190 °C, yield 61%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.06 (d, J = 6.3 Hz, 1H), 7.87-7.40 (m, 3H), 1.95 (s, 3H), 1.80-1.73 (m, 6H), 1.71-1.59 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 166.8 (C=O), 161.8 (C), 158.5 (C), 131.6 (C), 129.0 (C), 127.2 (CH), 116.2 (CH), 114.8 (CH), 113.1 (CH), 43.5 (C), 39.5 ($3CH_2$), 36.5 ($3CH_2$), 27.8 (3CH). MS (EI) *m/z* 369.00 (M^+). Anal. calcd. for: $C_{19}H_{19}N_3OS_2$: C, 61.76; H, 5.18; N, 11.37. Found: C, 61.89; H, 5.36; N, 11.31.

2.2. Pharmacological Screening

2.2.1. Anticancer activity

2.2.1.1. Evaluation of growth inhibitory activity by NCI

Three of the new compounds (**11b**, **13b**, and **13h**) were evaluated for their growth inhibitory activity against 60 cancer cell lines in the drug development program (DDP) by the NCI-USA. The test was performed using the SRB assay [30,31]. The most relevant results were presented in **Table 1**.

2.2.1.2. Cell Viability Assay

Three of the American Type human (MCF-7, A549, and Hep3B) cancer cell lines (ATCC, Manassas, VA) were used in the cell viability (MTT) assay. The cancer cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F-12) in humidified incubator containing 5% CO_2 at 37 °C according to the previous report [32]. The MTT was used to evaluate the effect of the new compounds **9-14** on the viability of the three cancer cell lines according to the previous reports [33-35]. After seeding the cancer cells in 96-well plates, the latter were incubated and incubated overnight then treated with the test compounds for 72 h. Following this treatment, cells were treated with the MTT for 2 h then the reduced MTT was dissolved in DMSO and its absorbance was determined using Varioskan™ Flash Multimode Reader (Thermo Scientific, USA), **Table 2**.

2.2.2. Flow Cytometric analysis

Flow cytometry was used for the determination of cell cycle perturbations and the ability of test compounds to induce apoptosis as mentioned before [36]. The A549 cells were exposed to compound **13i** at two concentrations, 5 and 10 μM for 48 h then the cells were stained with propidium iodide, and flow cytometry was performed. Data analysis was performed using BD FACSDiva 6.0 software (BD Pharmingen, San Diego, CA, USA). The results were presented in **Fig. 5**.

2.2.3. Caspase-3/7 Assay

The ability of the novel compounds to induce apoptosis was tested using the Caspase-Glo 3/7 assay kit (Promega, Madison, WI) according to the manufacturer's instructions and as mentioned before [37,38]. Following the incubation process, the luminescence was measured using

Varioskan™ Flash Multimode Reader (Thermo Scientific, USA). The results were presented in **Fig. 6**.

2.2.4. Kinase profiling assay protocol

Kinase profiling test was done at Reaction Biology Corp., PA, USA, using their kinases analysis services [39]. Kinases activity is expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions, **Tables 3** and **4**.

2.3. Molecular Docking Study

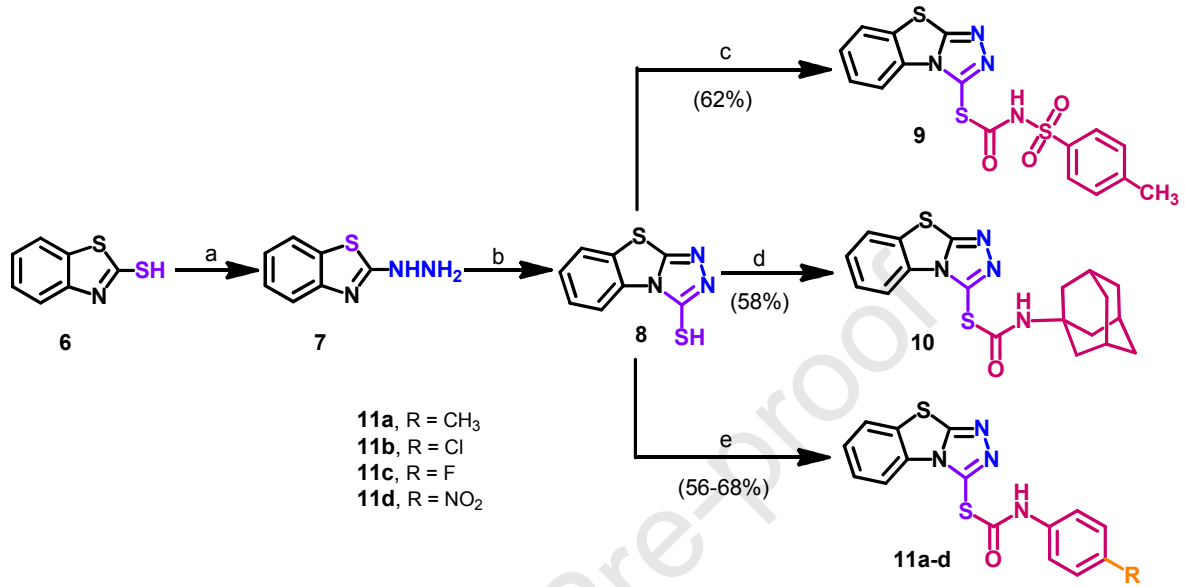
AutoDock 4.2 [40] was used to perform the docking study of the new compounds into CDK2 kinase. The crystal structures of CDK-2 [41] was obtained the Protein Data Bank (https://www.rcsb.org/, PDB code: 3TNW). This study was carried out using the previously used docking protocol [33,42]. The PDB format of the test compounds were prepared according to the previous reports [33,42]. During the preparation of the protein structure, water molecules and bound ligands were removed to avoid interference with the study. The 2/3D binding modes of the new compounds were generated using Discovery studio visualizer (DSV, v16.1.0.15350) [43]. The results were presented in **Table 5** and **Figs. 7-9**.

3. Results and Discussion

3.1 Chemistry

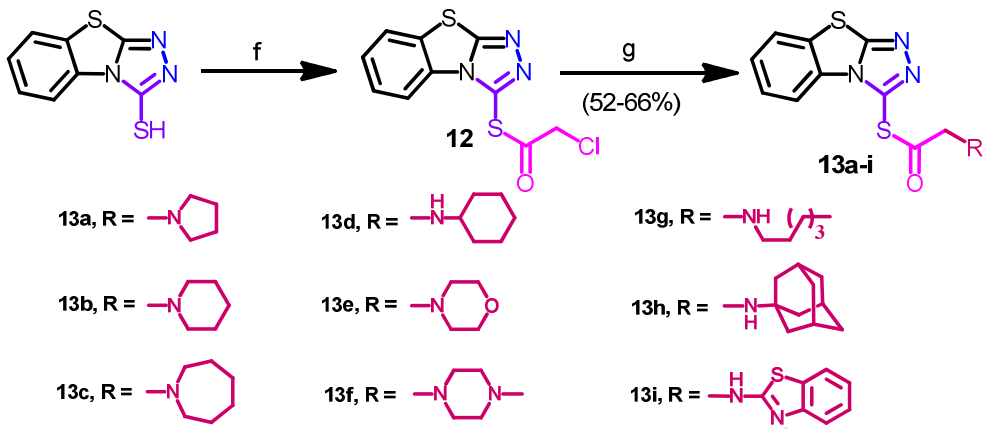
Synthesis of the starting material benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole-3-thiol derivative **8** was described in **Schemes 1**. It was achieved through the cyclization of 2-hydrazinobenzothiazole **7**, obtained from refluxing 2-mercaptobenzothiazole **6** with hydrazine hydrate in ethanol, with carbon disulfide in alcoholic potassium hydroxide as previously reported [28,29]. Subsequently, the reaction of thiol intermediate **8** with various isocyanate derivatives in

the presence of trimethylamine afforded the final *S*-thiocarbamate **(9, 10, 11a-d)** derivatives in good yield (56-68%).



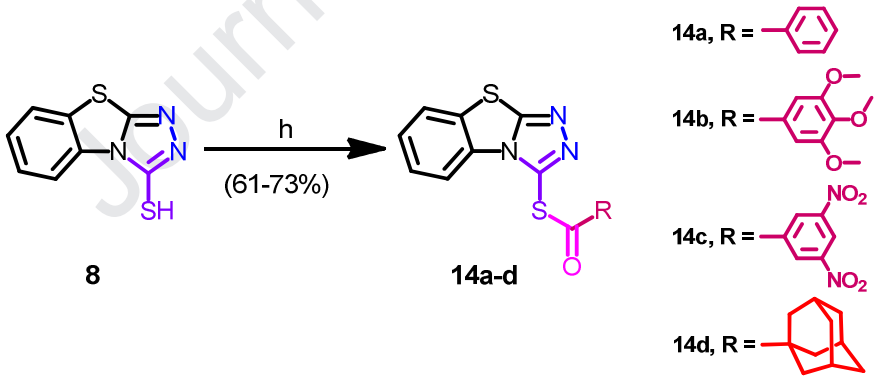
Scheme 1. Reagents and reaction conditions: a) $N_2H_4.H_2O$, reflux, EtOH, 20 h; b) i. CS_2, KOH, EtOH, reflux; ii. Conc. HCl; c) *p*-toluenesulfonyl isocyanate, DCM, TEA, reflux, 12 h; d) 1-Adamantyl isocyanate, DCM, TEA, reflux, 12 h; e) appropriate substituted-phenyl isocyanate, DCM, TEA, reflux, 12 h.

In addition, compound **12** was prepared from the *S*-alkylation of thiol intermediate **8** with chloroacetyl chloride. Compounds **13a-i** were obtained from the reaction of primary/secondary amines with compound **12**, **Scheme 2**. This reaction takes place through nucleophilic substitution with elimination of hydrochloride acid. The anhydrous potassium carbonate was used as acid removing agent.



Scheme 2. Reagents and reaction conditions: f) $ClCH_2COCl$, TEA, DCM, 0 °C, 1 h, rt, 5 h; g) appropriate amine, K_2CO_3, DMF, 60 °C, 3 h.

Similarly, compounds **14a-d** were prepared by acylation of the thiol group in compound **8** with different acid chlorides including benzoyl, 3,4,5-trimethoxybenzoyl, 3,5-dinitrobenzoyl and adamantly chloride, **Scheme 3**. The reaction was performed in DCM in the presence of TEA as a catalyst. Quantitative elemental and spectral (mass, ^{1}H-NMR, and ^{13}C-NMR) analyses were used to confirm the chemical structures of all new compounds.



Scheme 3. Reagents and reaction conditions: h) Appropriate acyl chloride, TEA, DCM, 2 h.

3.2. Pharmacological screening

3.2.1. Anticancer activity

3.2.1.1. Structure activity relationship (SAR)

The relationship between chemical structure and cytotoxicity of the new compounds (SAR) against the selected cancer lines was presented in **Fig. 4**. Among the carbamate derivatives **9-11**, compound **11d** bearing 4-nitophenyl substituent was the most active as cytotoxic agent. In addition, replacement of 4-nitrophenyl in compound **11d** moiety with 4-fluoro or 4-methyl groups resulted in a noticeable decrease in the cytotoxic activity. SAR study of compounds **13a-i** revealed the importance of the size of the cyclic amine on cytotoxic activity. A significant increase in cytotoxic activity on the increase in ring size from five-membered to seven-membered ring. Compounds **13c** bearing the bulky azepine ring exhibited higher cytotoxicity compared to compounds **13a** and **13b,** , **Fig. 4**.

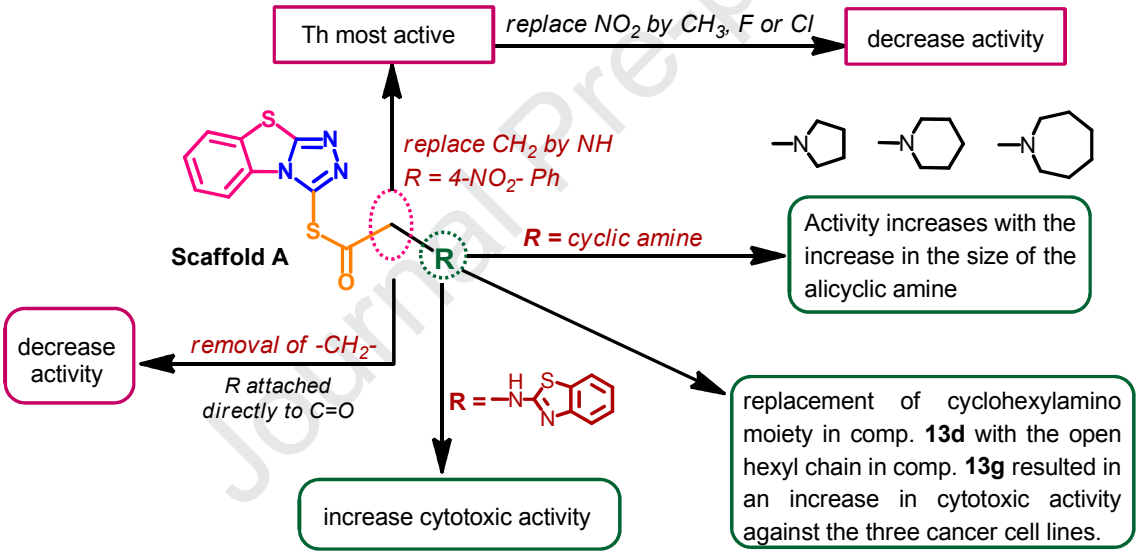


Fig. 4. SAR of cytotoxic activity of compounds **9-14**.

Moreover, it was found that the replacement of the cyclohexylamino moiety in compound **13d** with the open hexyl chain in compound **13g** resulted in increase in cytotoxicity. The high activity of compound **13i** might be explained by the well-known anticancer activity of the attached 2-aminobenzothiazole moiety [20]. Finally, the thioesters **14a-d** were also less active than compounds **13a-i**. Among the four derivatives, compound **14c** bearing the 3,5-dinitrophenyl moiety was the most active as cytotoxic agent. Cytotoxic activity of compounds 14 decreased on

removal/replacement of the dinitro groups with 3,4,5-trimethoxy or adamantly groups, **Fig. 4**. Obviously, these results were in concordance to some extent with the results of NCI screening assay, where compound **13h** exhibited high cytotoxic activity (IC_{50} in the range of 4.83-10.68 μM), while compounds **11b** and **13b** showed weaker or no cytotoxic activity (IC_{50} in the range of 9.23-31.55 μM).

2.2.1.2. NCI Anticancer Screening

The one-stage screening process started with the selection of three of the obtained compounds (i.e. **11b**, **13b** and **13h**) by the Drug Evaluation Branch of National Cancer Institute (NCI-USA). The selected compounds were subjected to a primary *in vitro* evaluation against 57 cell lines at a single dose of 10 μM (it is referred to as one-dose assay). The 57 cell panel is derived from nine different cancer strains: Leukemia, Lung, Melanoma, Colon, CNS, Ovary, Renal, Breast, and Prostate cancers [30,31]. The output from the single-dose screening was reported as a mean graph available for analysis by the COMPARE program, **(Supplementary data, Table S1)**.

Interestingly, the three compounds (**11b**, **13b**, and **13h**) exhibited nearly similar antiproliferative activity against SNB-75 cell line with growth inhibition in the range of 23.05-25.41% (i.e. 74.59-76.95% growth). In addition, compounds **11b** and **13h** showed moderate growth inhibitory activities towards several breast (MCF-7, MDA-MB-231/ATCC, T-47D) and prostate (PC-3) cancer cell lines. On the other hand, the tested compounds (**11b**, **13b**, and **13h**) displayed no or very low growth inhibitory activities against leukemia, colon cancer, and melanoma cell lines.

Moreover, the results of the one-dose assays revealed that compound **13h** has the highest antiproliferative activity against HOP-92 (Non-Small Cell Lung Cancer) cells with growth inhibition of 53.76% (i.e. 46.24% growth), **Table S1**. Moreover, compound **13h** caused 33.48% inhibition **(**i.e. 76.52% growth**)** in the growth of SNB-75 (CNS cancer) cell line, **Table 1**.

Table 1. Selected NCI anticancer screening data at single dose assay (10 μM) as percentage cell growth against selected human cancer cell line.

Cell lines	Percentage cell growth			Cell lines	Percentage cell growth		
	11b	**13b**	**13h**		**11b**	**13b**	**13h**
Non-Small Cell Lung Cancer				**Renal Cancer**			
HOP-62	99.65	89.87	104.06	CAKI-1	80.01	86.23	76.41
NCI-H226	87.8	84.92	91.31	UO-31	74.83	68.29	78.30
Leukemia				**Prostate Cancer**			
CCRF-CEM	99.80	103.54	86.05	PC-3	81.39	92.97	77.45
CNS Cancer				**Breast Cancer**			
SNB-75	76.95	74.59	76.52	MCF-7	88.02	92.86	88.72
Ovarian Cancer				HS 578T	93.68	109.77	89.46
OVCAR-4	98.6	104.73	87.62	T-47D	87.84	92.22	87.22
OVCAR-5	89.08	144.13	100.03	MDA-MB-468	87.06	97.09	101.97

Bold values represent growth% < 90.00%.

Compound **11b** exhibited the highest growth inhibitory activity against UO-31 (renal cancer)cell line with growth inhibition of 25.17 (i.e. 74.83% growth)**,** while compound **13b** showed the highest effect against UO-31 (renal cancer) cell line with growth inhibition of 31.71% (i.e. 68.29% growth).

3.2.1.3. MTT assay

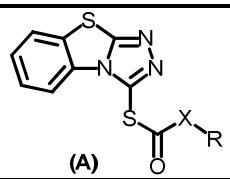
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to evaluate the anti-proliferative potential of the new compounds **9-14**. The MTT assay was performed against three (Hep3B, A549, and MCF-7) cancer cell lines according to the previous reports [33-35]. The cytotoxic activity of the new compounds expressed as IC_{50} values (the concentration that cause inhibition of 50%) compared to doxorubicin was presented in **Table 2**.

The carbamates **9-11** exhibited cytotoxic activity against Hep3B cells with IC_{50} in the range of 5.05-35.25 μM. Among the six derivatives, compound **11d** was the most active against the three cancer cell lines. On the other hand, compounds **9**, **10**, and **11b** were inactive against A549 cells.

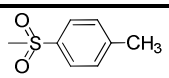
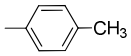
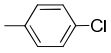
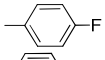
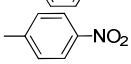
The results revealed higher anticancer potential for the compounds **13a-i** than both the thioesters **14a-d** the thiocarbamates **9, 10**, and **11a-d**. Among these derivatives, compounds **13c** and **13f-13i** exhibited the highest cytotoxic activity (IC_{50} in the range of 3.17-14.18 μM). Compound **13c** was more active than compounds **13a** and **13b**. In addition, the compound **13f** was more active than the morpholino **13e** and hexylamino **13g** analogs. Among the nine derivatives, compound **13i** showed the highest cytotoxicity against A549 and MCF-7 cells, while compound **13f** exhibited the highest growth inhibitory activity against the Hpe3B cells.

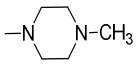
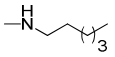
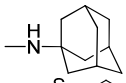
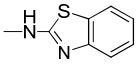
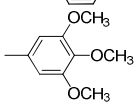
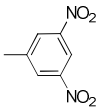
The thioesters **14a-d** showed weaker cytotoxic activity than compounds **13a-i**. However, compounds **14a-d** displayed the highest cytotoxic activity against Hep3B cells (IC_{50} values in the range of 6.95-1885 μM). Compound **14c** was the most active among the four derivatives against the three cancer cell lines.

Table 2. Anticancer activity of compound **9-14** against A549, MCF-7 and Hep3B cancer cell lines



(A)

Comp. No	X	R	IC_{50}[a]		
			A549	**MCF-7**	**Hep3B**
9	NH		>60	20.33±1.84	16.25±1.32
10	NH		>60	>60	35.25±2.27
11a	NH		34.56±2.19	9.28±1.03	14.23±1.93
11b	NH		>60	31.55±2.76	22.72±2.23
11c	NH		17.42±1.72	9.83±1.18	11.42±1.12
11d	NH		7.88±0.66	5.84±0.41	5.05±0.62

13a	CH_2		43.56±4.17	26.82±2.39	28.75±3.29
13b	CH_2		11.25±1.11	9.23±1.06	15.69±1.76
13c	CH_2		4.62±0.85	5.65±1.02	4.98±0.73
13d	CH_2		38.56±3.41	19.23±1.23	16.25±1.56
13e	CH_2		24.57±2.52	16.61±1.88	20.27±1.93
13f	CH_2		6.05±1.03	7.55±0.96	3.37±0.36
13g	CH_2		14.18±1.33	8.28±0.61	13.82±1.43
13h	CH_2		10.68±1.28	4.83±0.56	8.23±1.16
13i	CH_2		3.55±0.37	3.17±0.92	4.32±0.66
14a	-		37.50±3.19	9.74±0.42	18.51±1.79
14b	-		14.05±1.22	13.08±1.26	11.38±1.10
14c	-		13.81±1.37	8.13±0.92	6.95±0.74
14d	-		>60	36.12±3.31	18.85±1.52
Doxorubicin	-	-	2.3 ± 0.15	2.4 ± 0.17	1.7 ± 0.31

Results were presented as mean ±S.D. (n = 3), after 72 h treatment with test compounds or vehicle (control).

3.2.2. Flow Cytometric analysis

In identify the potential mechanism of the newly benzothiazolotriazole derivatives and to determine whether the inhibition of cancer cell growth was related to cell cycle arrest, the most potent compound **13i** was selected to be examined using flow cytometry to analyze the effect on the cell cycle in A549 cell line [36]. A549 cells were treated with compound **13i** at two concentrations of 5 and 10 μM for 48 h. While there was no significant change in the cell cycle distribution, the results revealed the ability of **13i** to induce apoptosis in A549 cells as indicated by the increase of the fraction of apoptotic cells to 8.23% and 14.9% at 5 and 10 μM concentrations, respectively compared to control (2.34%), **Fig. 5**.

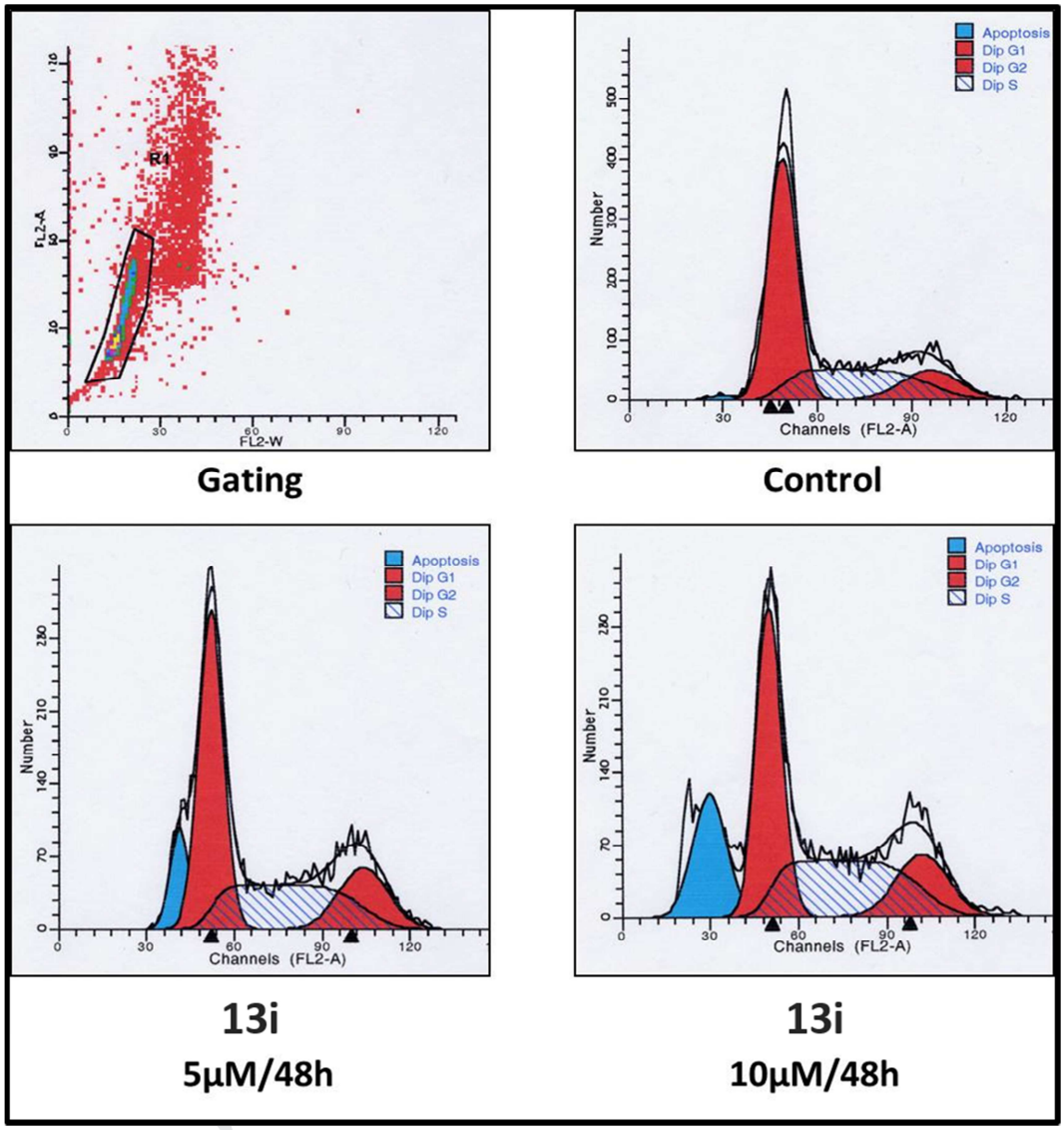


Fig. 5. Cell cycle analysis. Cells were treated with test compound or vehicle for 48 h, stained with propidium iodide, and analyzed by flow cytometry.

We did not observe a significant cell cycle arrest at that time point which could be due to the extensive cell death at that late stage. These findings suggested that compound **13i** can efficiently induce A549 cells apoptosis in a dose-dependent manner.

3.2.3. Caspase 3/7 Assay

It is well known that caspases, a family of cysteine proteases play a pivotal role in the induction of apoptosis especially, caspase-3/7. To further evidence the apoptotic inducing mechanism, the

activity of caspase-3/7 was determined for some of the most potent compounds (**13f-i)** in cell viability analysis. After treatment with the test compounds **13f-i**, the activation of caspase-3/7 in A549 cells was determined according to the previous reports [37,38]. The results revealed induction of caspase-3/7 activity in A549 cells after treatment with 5-20 µM of compounds **13f**,**g** and **13i**, **Fig. 5**. The three compounds increase the activity caspase-3/7 by nearly 3- to 4-fold. On the other hand, compound **13h** exhibited only weak induction in the activity of caspase-3/7. Interestingly, compound **13i** was the most active compound which confirms the consistency of these results with that of the cell viability analysis and flow cytometry as well. These results indicated that the tested compounds have the ability to induce apoptosis through the activation of effector caspase-3/7 family.

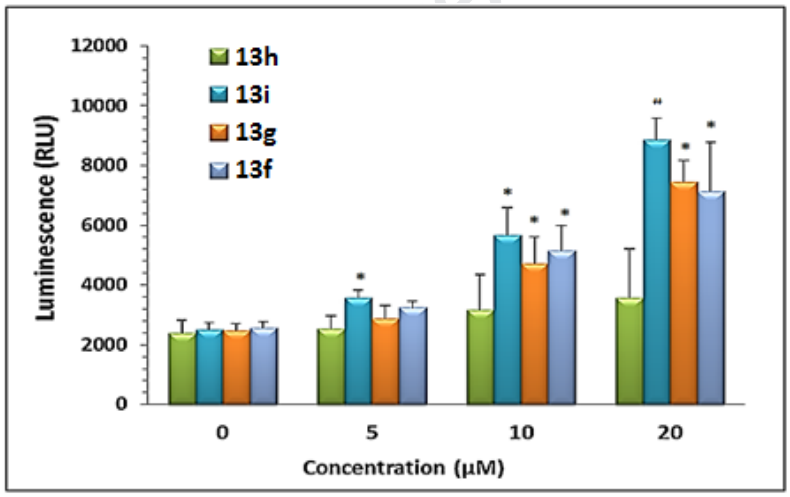


Fig. 6. *The effect of test compounds* ***13h-g*** *and* ***13f*** *on caspases 3/7 activity in A549 cells. Caspase-3/7activities were measured using Caspase-Glo Assay Kit after the indicated treatment for 48 h. All data are depicted as mean ± S.D (n=4). Significant difference from untreated control condition at *p < 0.05, #p<0.01.*

3.2.4. Kinase Profiling Assay

To identify the possible molecular anticancer targets which could mediate the anticancer potential of the new *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazoles, kinase profiling assay was performed. **13f**, **13g** and **13i** were selected for their high cytotoxic activity, **Table 2**. The

profiling assay was performed against ten different kinases of different families and types. The kinase profiling assays were performed at Reaction Biology Corp., PA, USA using the radiolabeled ATP determination method as reported [39]. The compounds were tested in single dose duplicate mode at a conc. of 10 μM using staurosporine, GW5074 and SB202190 as positive controls.

The results revealed that the three tested compounds **13f**, **13g** and **13i** exhibited weak to moderate inhibition against ten kinases with inhibition percentages up to 29.67%, **Table 3**. Among the tested kinases, CDK2/Cyclin A1 was the most sensitive kinase to the three selected compounds with enzyme activity percentages of 70.33 to 74.91%. Compound **13g** displayed the highest inhibitory activity against CDK2/Cyclin A1. Moreover, compounds **13g** and **13i** showed good activity against ABL1 kinase as well with enzyme activity of 79.78 and 76.24%, respectively.

Table 3. Kinase profiling results (enzyme activity percentages) of compounds **13f**, **13g** and **13i** against ten different kinases relative to DMSO control.

Kinase\Compd.	**Kinase activity %**			**Positive Control**	**IC_{50} (nM)**
	13i	**13g**	**13f**		
ABL1	76.24	79.78	95.06	Staurosporine	17.1
AKT1	93.22	82.37	80.97	Staurosporine	2.58
B-RAF	87.79	86.24	89.33	GW5074	21.9
c-Src	104.92	87.44	88.48	Staurosporine	1.51
CDK2/Cyclin A1	**74.91**	**70.33**	**73.41**	Staurosporine	1.31
EGFR	90.01	87.13	84.63	Staurosporine	30.4
FGFR1	96.46	94.66	93.29	Staurosporine	1.76
KDR/VEGFR2	89.33	94.56	83.81	Staurosporine	2.28
LCK	93.64	84.83	85.28	Staurosporine	1.51

P38a/MAPK14	205.02	180.26	163.96	SB202190	18.6

Compounds were tested in single dose duplicate mode at a conc. of 10 μM; Staurosporine was tested in 10-dose IC_{50} mode with 4-fold serial dilution starting at 20 μM; Alternate control compounds (GW5074 and SB202190) were tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 20 μM; Reactions were carried out at 1 μM ATP.

Based on the kinase profiling results, the three compounds **13f**, **13g**, and **13i** were tested in five-dose IC_{50} mode with a 10-fold serial dilution starting at 100 μM against two kinases that have been significantly inhibited by the action of these compounds including ABL1 and CDK2/Cyclin A1. The inhibitory activities of the tested compounds were expressed in terms of IC_{50} values and depicted in **Table 4**. The results revealed that compound **13i** showed potent inhibitory activity against CDK2/Cyclin A1 with IC_{50} value of 4.65 μM while, compound **13f** showed very weak activity with IC_{50} value of 100 μM. However, compound **13g** displayed no activity against the same kinase. Meanwhile, the three compounds **13f**, **13g**, and **13i** were tested against ABL1 kinase where **13f** showed no activity while compounds **13g** and **13i** exhibited weak to moderate activity with IC_{50} values of 69.1 and 30.7 μM, respectively. These results indicate that the class of compounds may exert their anticancer activity via inhibition of CDK2/Cyclin A1 kinase as another possible molecular mechanism that should be further investigated.

Table 4. Inhibitory activity of compounds **13f**, **13g** and **13i** against ABL1 and CDK2/cyclin A1.

Compd.	**Kinase Inhibition IC_{50}**	
	ABL1	**CDK2/Cyclin A1**
13f	NA	100 μM
13g	69.1 μM	NA
13i	30.7 μM	4.29 μM
Staurosporine	20.1 nM	1.43 nM

Compounds were tested in 5-dose IC_{50} mode with a 10-fold serial dilution starting at 100 μM; Staurosporine (positive control) was tested in 10-dose IC_{50} mode with 4-fold serial dilution starting at 20 μM. Reactions were carried out at 1 μM ATP; NA: Means no activity or compound activity that could not be fit to an IC_{50} curve.

3.3. Molecular Docking Study

A molecular docking study of compounds **9**-**14** into CDK2/Cyclin A1 (PDB code: 3TNW) [41] was performed to evaluate their binding mode/affinities into the active site of this kinase. The protein kinase [41] was downloaded from the protein data bank (https://www.rcsb.org/). The study was performed by AutoDock 4.2 [40]. Ligand, protein, and docking parameters were prepared according to the previously reports [33,42]. The results were handled by discovery studio visualizer [43]. The native ligand CAN508 was redocked into the active site of the kinase to validate the docking procedures. The results revealed binding free energy (ΔG_b) of -6.47 kcal/mol and inhibition constant (K_i) of 18.20 µM. The redocked CAN508 exhibited similar binding interactions with those of the native ligand [41]. The redocked CAN508 superimposed over the native ligand into CDK2 with RMSD of 1.11 Å, **Fig. 7**.

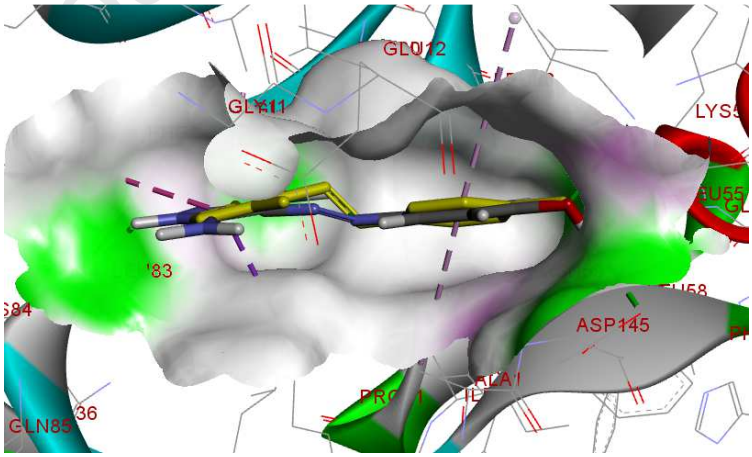


Fig. 7. The superimposition of redocked CAN508 (colored in yellow) over the position of the native ligand (colored by element) into the active site of CDK2/Cyclin A kinase (PDB code: 3TNW) with RMSD of 1.11 Å. The 3D protein was represented by solvent hydrogen bond donor/acceptor Surface. Hydrogen atoms were omitted from the redocked CAN508 for clarity.

The docking study of compounds **9-14** was performed following the previous report [33,42]. The results of the docking study were represented in **Table 5**. The results revealed a higher affinity than CAN508 for CDK2. The new compounds showed binding free energies in the range of -7.07 to -9.86 kcal/mol and inhibition constants in the range of 58.83 nM to 6.59 μM. Compound **9** showed the highest affinity toward CDK2, while compound **13g** displayed the lowest affinity. The thiocarbamate derivatives **9** and **10** displayed higher binding affinities to CDK2 the carbamate derivatives **11a-d**. In addition, compounds **11a-c** showed similar interactions pattern with CDK2. They all displayed three hydrogen bonds with bond length in the range of 1.91-2.71 Å with the amino acids in CDK2. These bonds were formed between carbonyl oxygen, thiazolyl sulfur, and amide hydrogen with LEU83, LYS89, and GLU81, respectively.

Table 5. Docking results of compounds **9-14** and CAN508 into CDK2 kinases.

Comp. No	ΔG_b [a]	K_i [b]	HBs [c]	Atoms in H-bonding		Length [d] (Å)
				In ligand	In protein	
9	-9.68	80.47 nM	2	SO_2	NH of ASP145	2.02
				SO_2	NH_2 of LYS33	2.19
10	-9.86	58.83 nM	2	C=O	NH of LEU83	1.80
				NH	C=O of LEU83	2.11
11a	-8.91	292.62 nM	3	C=O	NH of LEU83	1.91
				Thiazole S	NH_2 of LYS89	2.38
				NH	C=O of GLU81	2.69
11b	-9.08	220.17 nM	3	C=O	NH of LEU83	1.94
				Thiazole S	NH_2 of LYS89	2.36
				NH	C=O of GLU81	2.71
11c	-8.36	748.14nM	3	C=O	NH of LEU83	1.93
				Thiazole S	NH_2 of LYS89	2.32
				NH	C=O of GLU81	2.67
11d	-9.59	93.28 nM	2	NO_2	NH of ASP86	2.06
				C=O	NH of LEU83	2.19

13a	-7.41	3.72 μM	2	C=O	NH of LEU83	1.96
				Thiazole S	NH_2 of LYS89	2.34
13b	-7.93	1.53 μM	2	C=O	NH of LEU83	1.96
				Thiazole S	NH_2 of LYS89	2.34
13c	-8.45	634.94 nM	2	C=O	NH of LEU83	2.03
				Thiazole S	NH_2 of LYS89	2.37
13d	-8.58	516.64 nM	2	NH	NH of ASP145	1.95
				C=O	NH of LYS33	1.96
13e	-7.62	2.61 μM	1	C=O	NH of LEU83	1.74
13f	-8.0	1.37 μM	2	C=O	NH of ASP145	2.15
				Thiazole S	NH_2 of LEU83	2.47
13g	-7.07	6.59 μM	2	NH	C=O of LEU83	2.08
				C=O	NH of LEU83	2.20
13h	-9.25	167.06 nM	3	NH	C=O of ASP86	1.83
				Triazole N^1	NH of LEU83	2.35
				C=O	NH_2 of LYS89	2.54
13i	-8.73	399.02 nM	2	C=O	NH of LYS89	2.13
				C=O	NH of ASP86	2.40
14a	-8.08	1.2 μM	- [e]	-	-	-
14b	-8.96	272.19 nM	4	3-CH_3O	NH_2 of LYS89	2.13
				4-CH_3O	NH_2 of LYS89	2.48
				Triazole N^1	NH of LEU83	2.80
				C=O	NH of LEU83	2.87
14c	-9.73	73.16 nM	4	NO_2	NH of ASP86	1.88
				NO_2	NH_2 of LYS89	2.83
				Triazole N^1	NH of LEU83	2.53
				C=O	NH of LEU83	2.93
14d	-9.66	83.46 nM	1	Triazole N^1	NH of LEU83	2.43
CAN508 [f]	-6.47	18.20 μM	2	NH_2	C=O of GLU81	2.06
				OH	C=O of GLU51	1.88
				pyrazole NH	C=O of LEU83	2.71

[a] Binding free energy (kcal/mol); [b] Inhibition constant; [c]HBs, number of conventional hydrogen bonds; [d] length in angstrom (Å); [e] No hydrogen bond detected; [f]CAN508, (E)-4-((3,5-diamino-1H-pyrazol-4-yl)diazenyl)phenol.

On the other hand, a sharp decrease in binding affinity was observed on replacement of the carbamate NH in compounds **11a-c** with methylene group in compounds **13**.

Moreover, the docking results revealed the ability of compound **13i** to bind nicely to the active site of the CDK2/Cyclin A1 with binding free energy (ΔG_b) of -8.73 kcal/mol, **Table 5**. Compound **13i** displayed also an inhibition constant of 399.02 nM which was about 45 times lower than that of CAN508, indicating higher potential activity. The 2D/3D binding mode of compound **13i** showed two hydrogen bonds with LYS89 and ASP86_amino acids with bond lengths of 2.13 and 2.40 Å, respectively. Additionally, it was found that compound **13i** was involved in several hydrophobic interactions with the amino acids in CDK2/Cyclin A1, **Fig. 8**.

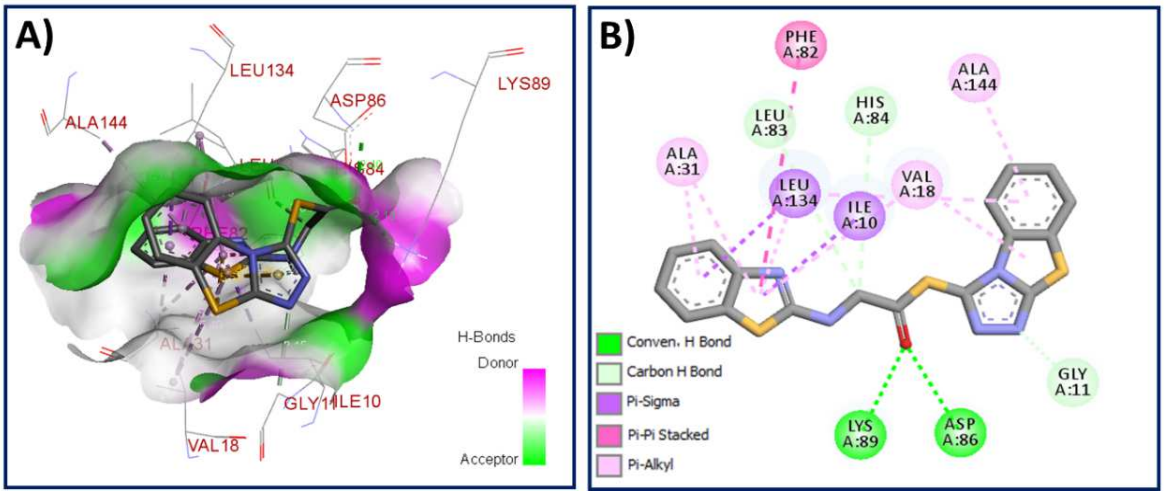


Fig. 8. Binding modes of compound **13i** into CDK2/Cyclin A kinase (PDB code; 3TNW): (A) 3D binding mode of compound **13i** into the active site of CDK2/Cyclin A kinase showing two H-bonds with Leu83 and Lys89 amino acid residues; (B) 2D binding mode of compound **13i** into the active site of CDK2/Cyclin A kinase showing two H-bonds and several hydrophobic interactions. H-bonds were represented as dashed green lines.

Among the three derivatives tested against CDK2 (**Table 4**), compound **13g** displayed the lowest affinity to CDK2 (ΔG_b = of -7.07 kcal/mol) which was matched with the results of kinase

inhibitory activity. Moreover, compound **13f** displayed binding free energy of (ΔG_b -8.0 kcal/mol) less than that of compound 13i. These results was also in concordance with the results of CDK2 inhibitory activity, **Table 4**.

To visualize the binding interactions of the other two series with CDK2, the 3D binding modes of compounds **11d** and **14c** which showed the highest affinity were presented in **Fig. 9**. Compound **11d** displayed two conventional hydrogen bonds with LEU83 and ASP86 and eleven hydrophobic interactions with amino acids in the active site of CDK2. On the other hand, compound **14c** exhibited 4 conventional hydrogen bonds with LEU83, ASP86, and LYS89 amino acids with bond length in the range of 1.88-2.93 Å. One pi-sulfur interaction and thirteen hydrophobic interactions were also observed with amino acids in the active site of CDK2.

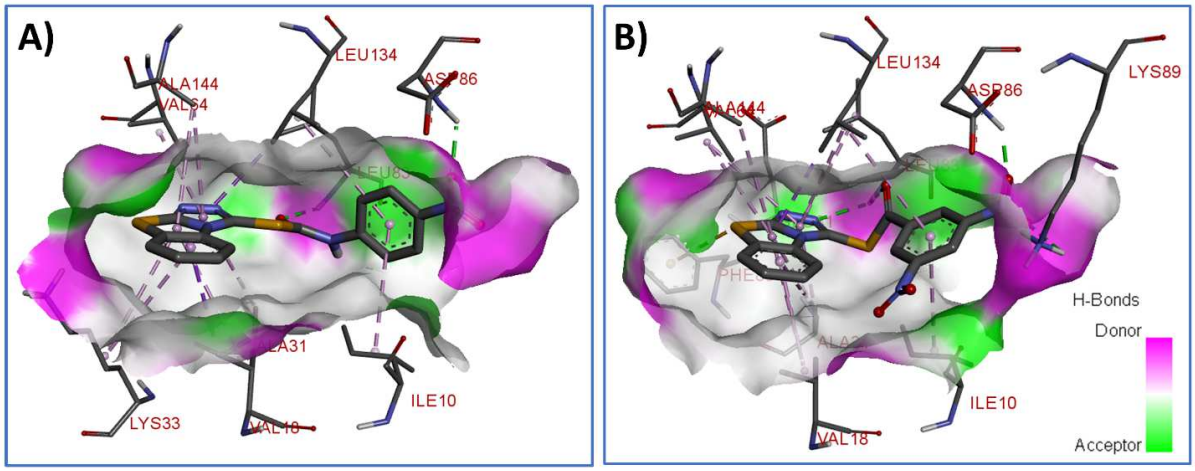


Fig. 9. Binding modes of compound **11d** into CDK2/Cyclin A kinase (PDB code; 3TNW): (A) 3D binding mode of compound **11d** into the active site of CDK2/Cyclin A kinase (PDB code; 3TNW) showing two conventional H-bonds with LEU83 and ASP86 amino acid residues; (B) 3D binding mode of comp **14c** into the active site of CDK2/Cyclin A kinase showing four conventional H-bonds and several hydrophobic interactions. H-bonds were represented as dashed green lines

4. Conclusions

In summary, nineteen *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole-based derivatives were synthesized and evaluated for their cytotoxic activity against three cancer cell lines (A-549, MCF-7, and Hep3B). Among this series, compounds **13c** and **13f-i** showed the most promising anticancer activity with IC_{50} values in the range of 3.17-14.18 μM. Flow cytometric analysis was employed to investigate the possible mechanism of the anticancer activity exerted by compound **13i**. The results suggested that compound **13i** has significant apoptosis-inducing activity in A549 cells. The induction of apoptosis was mediated by the activation of the effector caspase-3/7 family. Kinase profiling assay for the most active compounds **13f**, **13g,** and **13i** revealed a weak to moderate inhibitory activity against ten kinases. Compound **13i** inhibited the activity of CDK2/Cyclin A1 with an IC_{50} value of 4.65 μM. Therefore, it could be deduced that *S*-benzo[4,5]thiazolo[2,3-*c*][1,2,4]triazole as a novel anticancer scaffold with promising antiproliferative, CDK2/Cyclin A1 kinase and apoptosis-inducing activities deserves to be taken up as a lead for further structural optimization.

Statistical analysis

For statistical significance, the results were analyzed using one-way ANOVA followed by the Neuman‑Keuls test for multiple comparisons SPSS software (SPSS, Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

Acknowledgments

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some of our potent compounds against ten different kinases in a kinase profiling assay followed by determining IC_{50} values for best of them.

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Highlights

1) A novel set of S-benzo[4,5]thiazolo[2,3-c][1,2,4]triazoles was synthesized.
2) Compounds 13f, 13g, and 13i showed the highest cytotoxic activity.
3) Compound 13i activated caspase-3/7 and induced apoptosis.
4) Compound 13i exhibited the highest inhibitory activity against CDK2/Cyclin A1.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: