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New 1,3,4-thiadiazoles as potential anticancer agents: pro-apoptotic, cell cycle arrest, molecular modelling, and ADMET profile†

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A series of novel 1,3,4-thiadiazoles was synthesized via the reaction of N-(5-(2-cyanoacetamido)-1,3,4thiadiazol-2-yl)benzamide (3) with different carbon electrophiles and evaluated as potential anticancer agents. The chemical structures of these derivatives were fully elucidated using various spectral and elemental analyses. Out of 24 new thiadiazoles, derivatives 4, 6b, 7a, 7d, and 19 have significant antiproliferative activity. However, derivatives 4, 7a, and 7d were toxic to the normal fibroblasts, and therefore were excluded from further investigations. Derivatives 6b and 19 with IC₅₀ at less than $10~\mu M$ and with high selectivity were selected for further studies in breast cells (MCF-7). Derivative 19 arrested the breast cells at G2/M probably through inhibition of CDK1, while 6b significantly increased the sub-G1 percent of cells probably through induction of necrosis. These results were confirmed by the annexin V-PI assay where 6b did not induce apoptosis and increased the necrotic cells to 12.5%, and compound 19 significantly increased the early apoptosis to 15% and increased the necrotic cells to 15%. Molecular docking showed that compound 19 was like FB8, an inhibitor of CDK1, in binding the CDK1 pocket. Therefore, compound 19 could be a potential CDK1 inhibitor. Derivatives 6b and 19 did not violate Lipinski's rule of five. In silico studies showed that these derivatives have a low blood-brain barrier penetration capability and high intestinal absorption. Taken together, derivatives 6b and 19 could serve as potential anticancer agents and merit further investigations.

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1. Introduction

Heterocyclic compounds are key structural moieties in several anticancer drugs. According to the FDA, most novel molecular anticancer agents contain heterocyclic rings within their structures.¹ Due to their adaptability, heterocycle-based drugs can easily target various metabolic pathways and cellular processes in cancer pathology.² Various heterocyclic compounds have been synthesized and reported for their potential applications as therapeutic agents. Among these compounds, the five-membered heterocyclic scaffolds, oxadiazoles, triazoles, and thiadiazoles are the most significant compounds. Thiadiazoles have been reported to exhibit several biological activities. Among the thiadiazole isomers, 1,3,4-derivatives showed preferable metabolic stability and availability.³ This is the most promising moiety with different

The interesting pharmacological properties of 1,3,4-thiadiazoles is attributed to their high aromaticity, *in vivo* stability, and lack of toxicity. Moreover, the capability of the hydrogen binding domain allows the use of 1,3,4-thiadiazole as one of the potential agents in many FDA-approved¹⁰ drugs such as desaglybuzole, acetazolamide, sulfamethizole, litronesib, filanesib, and methazolamide¹¹ (Fig. 1).

The present study aimed at synthesizing new thiadiazole derivatives and investigating the antiproliferative effect on various human cell lines as well as investigating the possible mechanism(s) of action through assessing the effect of these derivatives on cell cycle and apoptosis by flow cytometry. Different *in silico* and docking studies were performed to predict some of the pharmacological characteristics of the new thiadiazoles.

2. Experimental

2.1 Chemistry

All melting points were determined using uncorrected Griffin and George melting-point equipment (Griffin & George Ltd.,

therapeutic activities such as anticancer,⁴ antiviral,⁵ antiinflammatory,⁶ antidiabetic activity,⁷ and antidepressant activity.⁸

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H₃C CH₃
H₃C Desaglybuzole
(Anti-diabetic)

Methazolamide
(Glaucoma)

NH₂

Methazolamide
(Glaucoma)

NH₃C NH₄

Methazolamide
(Glaucoma)

NH₃C NH₄

Methazolamide
(Glaucoma)

NH₄

NH₅

NH₆

NH₇

Filanesib
(Cancer)

Sulfamethizole
(Urinary tract infection)

Litronesib (leukaemia)

Fig. 1 FDA-approved drugs of 1,3,4-thiadiazole derivatives showing their common pharmacophoric features.

Wembley, Middlesex, UK). The quality of the produced compounds was controlled using aluminum sheet silica gel $\rm F_{254}$ (Merck) thin layer chromatography (TLC). The KBr wafer approach was used to record IR-spectra using a Pye Unicam SP1200 spectrophotometer (Pye Unicam Ltd., Cambridge, UK). $^{\rm 1}$ H-NMR spectra were obtained on a Bruker Avance (III) utilizing a Varian-Gemini 400 MHz and an internal standard of tetramethyl silane (chemical shifts in scale $\delta_{\rm ppm}$), whereas $^{\rm 13}$ C-NMR spectra were obtained at 100 MHz. In deuterated dimethyl sulfoxide, TMS was used as an internal standard (DMSO- d_6). Chemical changes were described as elemental studies were performed using a PerkinElmer 2400 CHN elemental analyzer at the Microanalytical Unit, Faculty of Science, Ain Shams University, and satisfactory analytical data (± 0.4) were obtained for all derivatives.

2.1.1 *N*-(5-Amino-1,3,4-thiadiazol-2-yl)benzamide (2). Method (I): a solution of *N*-(2-carbamothioyl)ydrazine-1-carbonothioyl)benzamide (1) (2.5 g, 10 mmol) in acetic acid (15 mL) was refluxed for three hours. The deposited solid while

heating was filtered off and then recrystallized from acetic acid affording the pure compound 2 as yellow crystals; (2.1 g, 91%); m.p. 278–280 °C; IR (KBr) (ν_{max} , cm⁻¹): 3448, 3446 (NH₂), 3164 (NH), 3060 (CH aromatic), 1672, 1638 (C=O), 1601 (C=N). Anal. calcd for C₉H₈N₄OS (220.25) C, 49.08; H, 3.66; N, 25.44; found: C, 49.18; H, 3.63; N, 25.60.

Method (II): a mixture of N-(2-carbamothioylhydrazine-1-carbonothioyl)benzamide (1) (2.5 g, 10 mmol) and sulfuric acid 98% (10 mL) was stirred at room temperature for 1.5 h, kept in refrigerator overnight, and then poured onto cold water. The precipitated solid was separated by filtration, dried, and recrystallized from N,N-dimethyl formamide affording the pure compound 2 as yellow crystals; (1.1 g, 50%) (identity m.p., TLC, and IR).

2.1.2 N-(5-(2-Cyanoacetamido)-1,3,4-thiadiazol-2-yl)

benzamide (3). A mixture of compound 2 (2.2 g, 10 mmol) and ethyl cyanoacetate (1.13 mL, 10 mmol) with a catalytic amount of TEA was stirred in 10 mL of absolute ethanol for three hours. The reaction mixture was acidified by cold dilute HCl, the

deposited solid was filtered off and recrystallized from EtOH/dioxane [1:3] giving the pure compound 3 as yellow crystals; (2.1 g, 75%); m.p. 204–206 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3213 (NH), 3080 (CH aromatic), 2918, 2894 (CH aliphatic), 2257 (C≡N), 1691, 1673 (C=O), 1612 (C=N). ¹H-NMR (300 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 3.88 (s, 2H, CH₂), 7.46–7.95 (m, 5H, ArH), 11.69 (br s, 1H, CH₂CONH, exchangeable with D₂O), 12.59 (br s, 1H, PhCONH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 24.06, 115.46, 115.60, 128.53, 128.84, 131.83, 133.30, 160.11, 166.58, 167.40, 168.10, 178.14. Anal. calcd for C₁₂H₉N₅O₂S (287.30) C, 50.17; H, 3.16; N, 24.38; found: C, 50.27; H, 3.01; N, 24.30.

2.1.3 N-(5-Benzamido-1,3,4-thiadiazol-2-yl)-2-imino-2Hchromene-3-carboxamide (4). A mixture of compound 3 (2.8 g, 10 mmol) and salicylaldehyde (1.2 mL, 10 mmol) with a catalytic amount of piperidine in absolute ethanol 10 mL was heated under reflux for three hours. The deposited solid while heating was collected by filtration, dried, and recrystallized from dioxane producing 4 as orange crystals; (3.5 g, 84%); m.p. 240-242 °C; IR (KBr) ($\nu_{\rm max}$, cm $^{-1}$): 3409, 3158 (NH), 3060, 3044 (CH aromatic), 2971 (CH aliphatic), 1679 (C=O), 1636 (C=N). 1H-NMR (300 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 7.28–8.00 (m, 9H, ArH), 8.58 (s, 1H, C=CH olefinic), 11.76 (br s, 1H, C = NH, exchangeable with D₂O), 13.98 (br s, 2H, 2NH CO, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm} : 115.41, 118.12, 118.50, 124.71, 128.58 (2C), 128.70, 128.86, 129.40, 130.40, 131.94, 133.01, 133.27, 133.78, 142.42, 153.24, 156.25, 156.80, 168.39. Anal. calcd for C₁₉H₁₃N₅O₃S (391.41) %: C, 58.31; H, 3.35; N, 17.89; found: C, 58.28; H, 3.38; N, 17.70.

The filtrate was acidified by cold dilute HCl. The deposited solid was filtered out, dried, and recrystallized from EtOH/dioxane [3:1] affording compound 5.

2.1.4 *N*-(5-Benzamido-1,3,4-thiadiazol-2-yl)-2-oxo-2*H*-chromene-3-carboxamide (5). Yellow crystals; (3 g, 78%); m.p. > 300 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3221, 3179 (NH), 3038 (CH aromatic), 1713 (C=O_{lactone}), 1679, 1655 (C=O_{amide}), 1608 (C=N). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm}: 7.48–7.57 (m, 5H, ArH), 7.66 (d, J=7.24 Hz, 1H, ArH), 7.79 (d, J=7.76 Hz, 1H, ArH), 8.00–8.08 (m, 2H, ArH), 9.04 (s, 1H, C=CH olefinic), 12.20 (br s, 1H, NH, exchangeable with D₂O), 13.74 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm}: 104.51, 116.56, 117.96, 120.23, 123.32, 123.76, 126.28 (2C), 128.58, 130, 131.67, 134.63, 140.30 (2C), 152.43 (2C), 166.99 (2C), 169.65. Anal. calcd for C₁₉H₁₂N₄O₄S (392.39) %: C, 58.16; H, 3.08; N, 14.28; found: C, 58.20; H, 3.10; N, 14.25.

2.1.5 *N*-(5-(6-amino-4-argio-3,5-dicyano-2-oxopyridin-1(2*H*)-yl)-1,3,4-thiadiazol-2-yl)benzamide (6a). A mixture of compound 3 (2.8 g, 10 mmol) and 2-(4-methoxy-benzylidene) malononitrile "arylidene" (1.8 g, 10 mmol) with a catalytic amount of piperidine was refluxed in absolute ethanol 10 mL for 4.5 h. The precipitated crystals were filtered off and washed with boiling ethanol affording compound 6a as gray crystals; (3.3 g, 73%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm⁻¹): 3455, 3394 (NH₂), 3263 (NH), 3099 (CH aromatic), 2944, 2844 (CH aliphatic), 2219 (C≡N), 1660, 1641 (C=O). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 3.88 (s, 3H, OCH₃), 5.59 (s, 2H, NH₂, exchangeable with D₂O), 7.18 (d, J = 8.80 Hz, 2H, ArH), 7.54 (t, J

= 7.76 Hz, 2H, ArH), 7.67 (t, J = 7.4 Hz, 1H, ArH), 7.97–8.22 (m, 4H, ArH), 12.42 (br s, 1H, NH CO, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 66.13, 71.23, 116.39, 117.57, 121.90, 123.72, 124.02, 124.39, 126.28, 127.08 (2C), 127.74, 129.80, 130.83, 132.22, 135.78, 147.67, 152.57, 153.56, 153.81, 162.56, 170.13, 170.20. Anal. calcd for C₂₃H₁₅N₇O₃S (469.48) %: C, 58.84; H, 3.22; N, 20.88; found: C, 58.80; H, 3.28; N, 20.82.

The reaction filtrate was poured onto ice cold dilute HCl with stirring. The crude material was filtered off and recrystallized from dioxane giving **6b**.

2.1.6 *N*-(5-(2-Cyano-3-(4-methoxyphenyl)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (6b). Orange crystals; (2.6 g, 65%); m.p. 252–254 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3233 (NH), 3066, 3020 (CH aromatic), 2984, 2935 (CH aliphatic), 2207 (C \equiv N), 1681 (C \equiv O). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 3.88 (s, 3H, OCH₃), 7.18 (d, J = 8.80 Hz, 2H, ArH), 7.54 (t, J = 7.76 Hz, 2H, ArH), 7.67 (t, J = 7.4 Hz, 1H, ArH), 7.97–8.04 (m, 4H, ArH), 8.22 (s, 1H, \equiv CH _{olefinic}), 11.11 (br s, 1H, NH, exchangeable with D₂O), 11.81 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 20.58, 55.62, 112.29 (2C), 114.63 (2C), 118.80, 127.50 (2C), 128.99 (2C), 129.53 (2C), 137.06 (2C), 146.06 (2C), 159.78, 170.44, 178.66. Anal. calcd for C₂₀H₁₅N₅O₃S (405.43) %: C, 59.25; H, 3.73; N, 17.27; found: C, 59.30; H, 3.70; N, 17.30.

2.1.7 General procedure for the synthesis of compounds (7a-f). A mixture of compound 3 (2.8 g, 10 mmol) and the appropriate aldehyde derivatives namely; 2,4-dichlorobenzaldehyde (1.7 g, 10 mmol), p-chlorobenzaldehyde (1.4 g, 10 mmol), furan-2-carbaldehyde (0.96 mL, 10 mmol), thiophene-2-carbaldehyde (1.2 mL, 10 mmol), p-nitrobenzaldehyde (1.5)g, 10 mmol), or 4-hydroxy-3methoxybenzaldehyde (1.5 g, 10 mmol) in absolute ethanol (10 mL) in the presence of a catalytic amount of piperidine was refluxed for three hours. The precipitated crystals were filtered off and recrystallized from ethanol affording compounds 7a-f.

2.1.8 *N*-(5-(2-Cyano-3-(2,4-dichlorophenyl)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7a). Yellow crystals; (3.1 g, 72%); m.p. > 300 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3197, 3154 (NH), 3095, 3069, 3019 (CH aromatic), 2921 (CH aliphatic), 2229 (C \equiv N), 1660 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 7.57–8.17 (m, 8H, ArH), 8.29 (s, 1H, \equiv CH_{olefinic}), 12.72 (br s, 1H, NH, exchangeable with D₂O), 13.47 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 103.93, 112.94, 115.85, 117.93, 129.74 (2C), 131.93 (2C), 132.04, 136.85 (2C), 146.12, 146.39, 148.22, 156.91, 159.10, 160.49, 170.08, 170.69. Anal. calcd for C₁₉H₁₁Cl₂N₅O₂S (444.29) %: C, 51.36; H, 2.50; Cl, 15.96; N, 15.76; found: C, 51.30; H, 2.43; Cl, 15.89; N, 15.80.

2.1.9 N-(5-(3-(4-Chlorophenyl)-2-cyanoacrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7b). Orange crystals; (3.2 g, 80%); m.p. 258–260 °C; IR (KBr) ($v_{\rm max}$, cm $^{-1}$): 3237 (NH_{br}), 3056 (CH aromatic), 2970 (CH aliphatic), 2212 (C \equiv N), 1686, 1665 (C \equiv O_{amide}). 1 H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 7.53–8.02 (m, 9H, ArH), 8.29 (s, 1H, \equiv CH_{olefinic}), 11.28 (br s, 1H, \equiv NH CO, exchangeable with D₂O), 12.40 (br s, 1H, CONH, exchangeable with D₂O). 13 C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 68.28, 70.01, 120.90, 123.02, 123.13, 125.59, 126.03, 126.71, 127, 128.11, 128.46, 128.55, 129.80, 129.89, 135.19, 146.74, 167.69, 170.62, 170.77. Anal. calcd for C₁₉H₁₂ClN₅O₂S (409.85) %: C, 55.68; H,

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2.95; Cl, 8.65; N, 17.09; found: C, 55.70; H, 2.98; Cl, 8.72; N, 17.15.

2.1.10 *N*-(5-(2-Cyano-3-(furan-2-yl)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7c). White crystals; (3.4 g, 95%); m.p. 276–278 °C; IR (KBr) (ν_{max} , cm⁻¹): 3291, 3238 (NH), 3067, 3040 (CH aromatic), 2215 (C \equiv N), 1684, 1664 (C \equiv O_{amide}), 1614 (C \equiv N). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 6.87–6.88 (m, 1H, ArH), 7.48–7.56 (m, 4H, ArH), 7.67 (t, J=7.36 Hz, 2H, ArH), 7.97–8.08 (m, 1H, ArH), 8.20 (s, 1H, \equiv CH_{olefinic}), 11.82 (br s, 1H, \equiv NHCO, exchangeable with D₂O), 12.43 (br s, 1H, CONH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm} : 102.91, 117.04, 118.04, 121.51, 124.32, 124.76, 127.11 (2C), 129.58 (2C), 131.00, 132.67, 135.63, 141.39, 153.43, 167.98, 170.65. Anal. calcd for C₁₇H₁₁N₅O₃S (365.37) %: C, 55.89; H, 3.03; N, 19.17; found: C, 55.93; H, 3.10; N, 19.15.

2.1.11 *N*-(5-(2-Cyano-3-(thiophen-2-yl)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7d). Gray crystals; (3.2 g, 85%); m.p. 268–270 °C; IR (KBr) ($\nu_{\rm max}$, cm $^{-1}$): 3244 (NH), 3098, 3018 (CH aromatic), 2924 (CH aliphatic), 2209 (C \equiv N), 1687, 1667 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 7.36 (t, J=8.76 Hz, 1H, ArH), 7.54 (t, J=7.84 Hz, 2H, ArH), 7.67 (t, J=7.4 Hz, 1H, ArH), 7.97–7.99 (m, 3H, ArH), 8.18 (d, J=4.96 Hz, 1H, ArH), 8.52 (s, 1H, \equiv CH_{olefinic}), 11.82 (br s, 1H, \equiv MHCO, exchangeable with D₂O), 12.41 (br s, 1H, CONH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 109.22, 119.18 (2C), 120.67, 123.35, 126.23, 126.67, 129.11, 131.85, 133.27, 134.76, 137.48, 143.22, 155.37 (2C), 169.99, 172.42. Anal. calcd for C₁₇H₁₁N₅O₂S₂ (381.43) %: C, 53.53; H, 2.91; N, 18.36; found: C, 53.58; H, 2.80; N, 18.25.

2.1.12 *N*-(5-(2-Cyano-3-(4-nitrophenyl)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7e). Yellow crystals; (3.0 g, 73%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm⁻¹): 3203, 3154 (NH), 3068, 3053 (CH aromatic), 2936 (CH aliphatic), 2233 (C \equiv N), 1669 (C \equiv O). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 7.59 (t, J=7.32 Hz, 2H, ArH), 7.69 (t, J=7.2 Hz, 1H, ArH), 8.15 (d, J=7.48 Hz, 2H, ArH), 8.25 (d, J=8.16 Hz, 2H, ArH), 8.40–8.45 (m, 2H, ArH + 1H, \equiv CH_{olefinic}), 12.55 (br s, 1H, NH, exchangeable with D₂O), 13.45 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm} : 112.94, 113.29, 116.98, 117.83, 129.28, 129.52, 130.28 (2C), 131.05, 131.25, 134.62, 135.78, 148.16, 154.35, 160.65, 161.47, 170.82, 174.14, 174.73. Anal. calcd for C₁₉H₁₂N₆O₄S (420.40) %: C, 54.28; H, 2.88; N, 19.99; found: C, 54.30; H, 2.85; N, 19.90.

2.1.13 *N*-(5-(2-Cyano-3-(4-hydroxy-3-methoxyphenyl) acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (7f). Pale yellow crystals; (3.7 g, 90%); m.p. 240–242 °C; IR (KBr) ($v_{\rm max}$, cm⁻¹): 3259 (OH), 3181, 3106 (NH), 3006 (CH aromatic), 2922, 2839 (CH aliphatic), 2219 (C \equiv N), 1667 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 3.85 (s, 3H, OCH₃), 6.96 (d, J=8.32 Hz, 1H, ArH), 7.56–7.78 (m, 5H, ArH), 8.11–8.15 (m, 2H, ArH + 1H, \equiv CH_{Olefinic}), 10.24 (br s, 1H, OH, exchangeable with D₂O), 12.07 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 65.73, 66.81, 68.92, 116.04, 117.41, 120.44, 123.33, 123.69, 126.08, 128.56, 129.98, 131.66, 134.43, 140.38, 152.45, 158.87, 159.51, 166.93, 168.85, 169.66. Anal. calcd for C₂₀H₁₅N₅O₄S

(421.43) %: C, 57.00; H, 3.59; N, 16.62; found: C, 57.09; H, 3.62; N, 16.58.

2.1.14 General procedure for the synthesis of compounds (9 and 10). A mixture of compound 3 (2.8 g, 10 mmol) and the appropriate diketone derivatives namely acetylacetone (1.00 mL, 10 mmol) or benzoyl acetone (1.6 mL, 10 mmol) in absolute ethanol (10 mL) with a catalytic base of fused sodium acetate was refluxed for three hours. The separated solid was filtered off, dried, and recrystallized from ethanol affording compounds 9 and 10, respectively.

2.1.15 *N*-(5-(3-Cyano-4,6-dimethyl-2-oxopyridin-1(2*H*)-yl)-1,3,4-thiadiazol-2-yl)benzamide (9). White crystals; (2.2 g, 64%); m.p. 298–300 °C; IR (KBr) ($\nu_{\rm max}$, cm $^{-1}$): 3224 (NH), 3054, 3021 (CH aromatic), 2964, 2922 (CH aliphatic), 2215 (C=N), 1648 (C=O). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 3.96 (s, 3H, CH₃), 3.99 (s, 3H, CH₃), 7.54–8.11 (m, 6H, ArH), 12.87 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 21.01, 21.13, 115.85, 116.66, 120.90, 123.13, 125.59, 126.71, 127.77, 129.80, 135.19, 152.70, 161.70, 167.67, 170.77, 180.28, 182.01. Anal. calcd for C₁₇H₁₃N₅O₂S (351.38) %: C, 58.11; H, 3.73; N, 19.93; found: C, 58.20; H, 3.65; N, 19.88.

2.1.16 *N*-(5-(3-Cyano-4-methyl-2-oxo-6-phenylpyridin-1(2*H*)-yl)-1,3,4-thiadiazol-2-yl)benzamide (10). White crystals; (2.4 g, 60%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm⁻¹): 3258, 3174 (NH), 3067 (CH aromatic), 2970, 2934 (CH aliphatic), 2218 (C \equiv N), 1684 (C \equiv O), 1649 (C \equiv N). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 2.15 (s, 3H, CH₃), 7.57–8.30 (m, 11H, ArH), 13.37 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm} : 22.83, 68.92, 116.04 (2C), 117.41, 120.44, 123.33, 123.69, 126.08 (2C), 128.56, 129.98, 131.66, 134.43, 140.38, 152.45, 158.87, 159.51, 166.93, 168.85 (2C), 169.66. Anal. calcd for C₂₂H₁₅N₅O₂S (413.46) %: C, 63.91; H, 3.66; N, 16.94; found: C, 63.88; H, 3.71; N, 16.81.

2.1.17 General procedure for the synthesis of compounds (11–16). 1,3,4-Thiadiazole derivative 3 (2.8 g, 10 mmol) was added to a cold suspension of finely divided potassium hydroxide (0.56 g, 10 mmol) in DMF (15 mL) with stirring for 30 minutes. Phenyl isothiocyanate (1.35 mL, 10 mmol) was added dropwise and then the mixture was allowed to stand overnight. Ethyl chloroacetate (1.22 mL, 10 mmol), chloro acetylchloride (1.12 mL, 10 mmol), diethyl malonate (1.6 mL, 10 mmol), 1,2-dibromoethane (1.87 mL, 10 mmol), methyl iodide (1.41 mL, 10 mmol), or HCl was added dropwise, stirred at room temperature for three hours and then allowed to stand overnight. The reaction mixture was placed into ice cold dilute HCl. The separated solid was filtered, washed with water, dried, and recrystallized from the suitable solvent producing 11–16, respectively.

2.1.18 Ethyl (*E,Z*)-2-((3-((5-benzamido-1,3,4-thiadiazol-2-yl) amino)-2-cyano-3-oxo-1-(phenylamino) prop-1-en-1-yl)thio) acetate (11). Brown crystals; (2.7 g, 55%); m.p. 288–290 °C; IR (KBr) (ν_{max} , cm⁻¹): 3148 (NH), 3064 (CH aromatic), 2982 (CH aliphatic), 2195 (C \equiv N), 1734 (C=O_{ester}), br. 1673 (C=O_{amide}), 1637 (C=N). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 1.19 (t, 3H, CH₂CH₃), 4.06 (q, 2H, CH₂CH₃), 4.59 (s, 2H, SCH₂CO), 6.75–6.77 (m, 5H, ArH), 7.66–8.07 (m, 5H, ArH), 11.96 (br s, 1H, NH Ph, exchangeable with D₂O), 12.85–13.05 (br s, 2H, CO<u>NH</u>,

exchangeable with D_2O). Anal. calcd for $C_{23}H_{20}N_6O_4S_2$ (508.57) %: C, 54.32; H, 3.96; N, 16.53; found: C, 54.25; H, 3.87; N, 16.59.

2.1.19 *N*-(5-(2-Cyano-2-(5-oxo-3-phenylthiazolidin-2-ylidene)acetamido)-1,3,4-thiadiazol-2-yl)benzamide (12). Red crystals; (3.0 g, 64%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm $^{-1}$): 3210, 3154 (NH), 3061 (CH aromatic), 2922 (CH aliphatic), 2204 (C \equiv N), 1741 (C \equiv O_{Ketone}), br. 1670 (C \equiv O_{amide}). 1 H-NMR (400 MHz, DMSO- d_6) $δ_{ppm}$: 4.10 (s, 2H, CH_2 CO), 6.72–6.77 (m, 5H, ArH), 7.64–8.05 (m, 5H, ArH), 12.95 (br s, 1H, NH, exchangeable with D₂O), 14.69 (br s, H, NH, exchangeable with D₂O). Anal. calcd for C₂₁H₁₄N₆O₃S₂ (462.50) %: C, 54.54; H, 3.05; N, 18.17; found: C, 54.51; H, 3.13; N, 18.11.

2.1.20 *N*-(5-(2-Cyano-2-(4,6-dioxo-1-phenylpiperidin-2-ylidene)acetamido)-1,3,4-thiadiazol-2-yl)benzamide (13). Reddish brown crystals; (2.7 g, 58%); m.p. > 300 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3150 (NH), 3064 (CH aromatic), 2981 (CH aliphatic), 2206 (C≡N), 1740 (C=O_{Ketone}), 1672 (C=O_{amide}), 1638 (C=N). 1 H-NMR (400 MHz, DMSO- $d_{\rm 6}$) $\delta_{\rm ppm}$: 4.62 (s, 2H, COCH₂ CO), 7.49–7.59 (m, 5H, ArH), 7.84–8.04 (m, 5H, ArH), 11.89 (br s, 1H, NH, exchangeable with D₂O), 12.50 (br s, 1H, NH, exchangeable with D₂O). Anal. calcd for C₂₂H₁₄N₆O₄S₂ (490.51) %: C, 53.87; H, 2.88; N, 17.13; found: C, 53.96; H, 2.94; N, 17.09.

2.1.21 *N*-(5-(2-Cyano-2-(3-phenylthiazolidin-2-ylidene) acetamido)-1,3,4-thiadiazol-2-yl)benzamide (14). Reddish brown crystals; (2.9 g, 68%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm $^{-1}$): 3157 (NH), 3055 (CH aromatic), 2962, 2921 (CH aliphatic), 2174 (C=N), 1673 (C=O_{amide}). 1 H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 3.75–3.85 (m, 4H, 2CH2), 6.72–6.77 (m, 5H, ArH), 7.55–8.05 (m, 5H, ArH), 9.21 (br s, 1H, NH, exchangeable with D₂O), 13.01 (br s, 1H, NH, exchangeable with D₂O). Anal. calcd for C₂₁H₁₆N₆O₂S₂ (448.52) %: C, 56.24; H, 3.60; N, 18.74; found: C, 56.29; H, 3.55; N, 18.64.

2.1.22 *N*-(6-Cyano-7-oxo-5-(phenylamino)-7*H*-[1,3,4]thia-diazolo[3,2-*a*]pyrimidin-2-yl)benzamide (15). Brown crystals; (2.7 g, 73%); m.p. > 300 °C; IR (KBr) ($\nu_{\rm max}$, cm⁻¹): 3418 (NH), 3060 (CH aromatic), 2923 (CH aliphatic), 2192 (C \equiv N), 1688 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 7.05–7.59 (m, 10H, ArH), 12.47 (br s, 1H, NH Ph, exchangeable with D₂O), 13.74 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 68, 116, 117, 120, 123.32, 123.76, 126, 128, 130, 131, 134, 140, 152, 166, 169, 180. Anal. calcd for C₁₉H₁₂N₆O₂S (388.41) %: C, 58.76; H, 3.11; N, 21.64; found: C, 58.65; H, 3.14; N, 21.59.

thioxopropanamido)-1,3,4-thiadiazol-2-yl)benzamide (16). Brown crystals; (2.2 g, 54%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm⁻¹): 3283, 3211 (NH), 3063 (CH aromatic), 2923 (CH aliphatic), 2205 (C \equiv N), 1669 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 3.5 (s, 1H, CHCN), 7.46–7.67 (m, 5H, ArH), 8.12–8.13 (m, 5H, ArH), 0.244 (br.s. 1H, NH, evaluation of the sum o

2.1.23 N-(5-(2-Cyano-3-(phenylamino)-3-

3.5 (s, 1H, <u>CH</u>CN), 7.46–7.67 (m, 5H, ArH), 8.12–8.13 (m, 5H, ArH), 9.24 (br s, 1H, NH, exchangeable with D_2O), 13.10 (br s, 1H, NH, exchangeable with D_2O), 13.15 (br s, 1H, <u>NH</u>Ph, exchangeable with D_2O). Anal. calcd for $C_{19}H_{14}N_6O_2S_2$ (422.48) %: C, 54.02; H, 3.34; N, 19.89; found: C, 54.05; H, 3.36; N, 19.85.

2.1.24 *N*-(5-(2-Cyano-3,3-bis(methylthio)acrylamido)-1,3,4-thiadiazol-2-yl)benzamide (17). 1,3,4-Thiadiazole derivative 3 (2.8 g, 10 mmol) was added to a cold suspension of finely

divided potassium hydroxide (0.56 g, 10 mmol) in DMF (15 mL) with stirring for 30 minutes. Carbon disulfide (2 mL) was added dropwise and then, the mixture was allowed to stand overnight. Methyl iodide (1.4 mL, 10 mmol) was added dropwise, stirred at room temperature for three hours and then, allowed to stand overnight. With stirring, the reaction mixture was placed into ice-cold dilute HCl. The separated solid was filtered, washed with water, dried, and crystallized from dioxane producing 17 as yellow crystals; (2.3 g, 60%); m.p. 250-252 °C; IR (KBr) (v_{max}) cm⁻¹): 3160 (NH), 3002 (CH aromatic), 2932 (CH aliphatic), 2213 (C \equiv N), 1664 (C=O_{amide}). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm} : 2.67 (s, 3H, SCH₃), 2.70 (s, 3H, SCH₃), 7.51-8.26 (m, 5H, ArH), 13.25 (br s, 1H, NHCO, exchangeable with D_2O), 14.74 (br s, 1H, CONH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) δ_{ppm} : 20.98 (2C), 69.90, 117.06, 118.04, 121.52, 127.10, 129.60, 131.00, 132.68, 133.65, 141.38, 153.43, 167.99, 170.64. Anal. calcd for C₁₅H₁₃N₅O₂S₃ (391.48) %: C, 46.02; H, 3.35; N, 17.89; found: C, 46.15; H, 3.42; N, 17.93.

2.1.25 General procedure for the synthesis of compounds (18–20). A mixture of compound 17 (1.9 g, 5 mmol) and the appropriate bidentate derivatives namely, *o*-phenylenediamine (0.9 g, 5 mmol), *o*-aminophenol (0.5 g, 5 mmol), or 2,3-dihydroxypyridine (0.5 g, 5 mmol) in dioxane (10 mL) with a catalytic amount of triethylamine was refluxed for four hours. The precipitated solid was filtered off, dried, and crystallized from ethanol/dioxane [1:3] affording compounds 18–20, respectively.

2.1.26 *N*-(5-(2-Cyano-2-(1,3-dihydro-2*H*-benzo[*d*]imidazole-2-ylidene)acetamido)-1,3,4-thiadiazol-2-yl)benzamide (18). Pale yellow crystals; (2.7 g, 68%); m.p. > 300 °C; IR (KBr) (ν_{max} , cm⁻¹): br. 3191 (NH), 3054 (CH aromatic), 2979 (CH aliphatic), 2171 (C \equiv N), br. 1688 (C \equiv O_{amide}). ¹H-NMR (400 MHz, DMSO-*d*₆) δ_{ppm} : 7.18–7.20 (m, 2H, ArH), 7.36–7.68 (m, 5H, ArH), 8.12 (d, *J* = 7.52 Hz, 2H, ArH), 11.24 (br s, 1H, NH CO, exchangeable with D₂O), 12.5 (br s, 2H, 2NH Ph, exchangeable with D₂O), 14.52 (br s, 1H, CONH, exchangeable with D₂O). Anal. calcd for C₁₉H₁₃N₇O₂S (403.42) %: C, 56.57; H, 3.25; N, 24.30; found: C, 56.47; H, 3.32; N, 24.41.

2.1.27 *N*-(5-(2-(Benzo[*d*]oxazol-2(3*H*)-ylidene)-2-cyanoacetamido)-1,3,4-thiadiazol-2-yl)benzamide (19). Pale yellow crystals; (3.4 g, 81%); m.p. 200–202 °C; IR (KBr) (v_{max} -cm⁻¹): 3187, 3134 (NH), 3063 (CH aromatic), 2209 (C \equiv N), br. 1665 (C \equiv O_{amide}), 1634 (C \equiv N). ¹H-NMR (400 MHz, DMSO-*d*₆) δ_{ppm} : 6.73 (t, *J* = 7.24 Hz 1H, ArH), 6.96 (d, *J* = 8.72 Hz, 2H, ArH), 7.07 (d, *J* = 7.72 Hz, 1H, ArH), 7.21 (t, *J* = 8.24 Hz, 4H, ArH), 7.60 (d, *J* = 8.72 Hz, 1H, ArH), 10.15 (br s, 1H, NH Ph, exchangeable with D₂O), 12.87 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO-*d*₆) δ_{ppm} : 55.62, 112.29 (2C), 114.63 (2C), 118.80, 127.50, 128.99 (2C), 129.53 (2C), 137.06 (2C), 146.06, 154.68, 155.99 (2C), 159.78, 185.65. Anal. calcd for C₁₉H₁₂N₆O₃S (404.40) %: C, 56.43; H, 2.99; N, 20.78; found: C, 56.39; H, 2.85; N, 20.81.

2.1.28 *N*-(5-(2-([1,3]dioxolo[4,5-*b*]pyridin-2-ylidene)-2-cyanoacetamido)-1,3,4-thiadiazol-2-yl)benzamide (20). Brown crystals; (2.1 g, 54%); m.p. 226–228 °C; IR (KBr) (ν_{max} , cm⁻¹): 3190, 3147 (NH), 3064 (CH aromatic), 2993 (CH aliphatic), 2209 (C≡N), br. 1665 (C=O_{amide}), 1633 (C=N). ¹H-NMR (400 MHz, DMSO- d_6) δ_{ppm}: 7.21 (d, J = 9.12 Hz, 1H, ArH), 7.36–7.39 (m, 2H,

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ArH), 7.59–7.63 (m, 2H, ArH), 8.01 (t, J=6.8 Hz, 1H, ArH), 8.63 (d, J=8.3 Hz, 2H, ArH), 11.9 (br s, 1H, NH, exchangeable with D₂O), 12.34 (br s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (100 MHz, DMSO- d_6) $\delta_{\rm ppm}$: 66.81, 116.04, 117.41, 120.44, 123.33, 123.69, 126.08, 128.56, 129.98, 131.66, 134.43, 158.87, 159.51, 166.93, 168.85, 169.66, 185.16, 192.83. Anal. calcd for C₁₈H₁₀N₆O₄S (406.38) %: C, 53.20; H, 2.48; N, 20.68; found: C, 53.18H, 2.55; N, 20.74.

2.2. Biological evaluation

- 2.2.1 Antiproliferative activity. All cell lines and media were obtained from the ATCC (Virginia, USA). The cells were cultured using the media and conditions recommended by the ATCC. The cell viability was determined using MTT as described elsewhere. 12-14 Briefly, the cells were seeded at a density of 2 \times 10⁶ per well in 48-well plate. The cells were treated with the thiadiazole derivatives (0.5–100 μ M) and incubated for 48 h. For comparison, untreated control cells and cells treated with doxorubicin were run under the same conditions. The data were expressed as mean \pm SEM for three independent experiments. The study was approved by the Research Ethics Committee at Ain shams University and was performed in accordance with the guidelines of the National Institute of Health (NIH).
- 2.2.2 Cell cycle analysis. MCF-7 cells were seeded in 6-well plate (2 mL per well) overnight at 37 °C and 5% CO₂. The cells were incubated with serum-free medium for 24 hours, then cells were treated with the IC₅₀ concentration of **6b** or **19** for 48 h. After incubation, cells were washed twice with ice-cold $1 \times$ PBS and collected after trypsinization. The cells were centrifuged at $500 \times g$ for five minutes at 4 °C. The cell pellet was resuspended in ice-cold $1 \times$ PBS and fixed with absolute ethanol and incubated at -20 °C for two hours. The fixed cells were then washed with ice-cold $1 \times$ PBS. Cell pellet was resuspended in propidium (PI)/RNase (BD Biosciences, BDB550825, NJ, USA) and incubated for 30 min at room temperature in dark, then DNA content in each phase was measured using BD FACSCanto IITM Flow Cytometer. More details could be reviewed elsewhere. ¹⁵
- 2.2.3 Apoptosis assay by annexin V. MCF-7 cells were seeded in a 6-well plate (1 \times 10⁵ cells per mL) and incubated overnight at 37 °C and 5% CO₂. To a fresh medium, **DMSO**, **6b**, or **19** was added at the IC₅₀ concentration and incubated for 48 hours. The cells were trypsinized, harvested, and washed twice with 1× PBS. The cells were stained with 5 μ L annexin-V/FITC and 5 μ L propidium iodide (Elabscience Biotechnology Inc, E-CK-A211) in the binding buffer for 15 minutes. The stained cells were analyzed by BD FACSCanto IITM flow cytometer.
- **2.2.4 Statistical analysis of data.** The data distribution was examined by the Kolmogorov–Smirnov test. Results were expressed as mean \pm SEM. The statistical analyses were performed using one-way analysis of variance followed by the Tukey-HSD test for multiple comparisons. Differences were considered significant at p < 0.05.

2.3. In silico studies

2.3.1 Docking studies. Using the MOE 2019 suite, the newly designed and synthesized compounds (**6b** and **19**) were

proposed as CDK1.¹⁶ As a result, two separate docking processes were performed for the investigated derivatives towards CDK1, with the co-crystallized inhibitor (**FB8**) serving as a reference standard.

- 2.3.2 Preparation of the newly designed and synthesized derivatives. As previously mentioned,¹⁷ all of the synthesized derivatives were prepared for docking by adding partial charges and minimizing energy after being sketched using the Chem-Draw professional program and added to the MOE program window. With the extracted co-crystallized FB8 in the case of CDK1 docking process.
- 2.3.3 CDK-1 pockets preparation and docking. The protein data bank was used to obtain the X-ray structures of the CDK-1 proteins (PDB codes ID: 6GU7). In each case, the target proteins were 3D protonated, error corrected, and energy minimized before being docked, as previously described in detail. According to each instance's default methodology, the two docking processes were carried out in accordance with the general docking protocol. For additional research, the docked poses with the most acceptable binding scores, RMSD values, and binding modes 6 were chosen.
- **2.3.4 Pharmacokinetic profiling.** Discovery studio 2.5 was used to investigate the pharmacokinetic profile of the tested derivatives and **FB8** as previously described.
- 2.3.5 ADMET predictions. Using Discovery Studio 2.5, the tested candidates' ADMET descriptors-namely, absorption, distribution, metabolism, excretion, and toxicity-were assessed. According to the default protocol, the target derivatives and FB8 were prepared and minimized. ¹⁹ After that, ADMET studies were conducted. ²⁰

Results and discussion

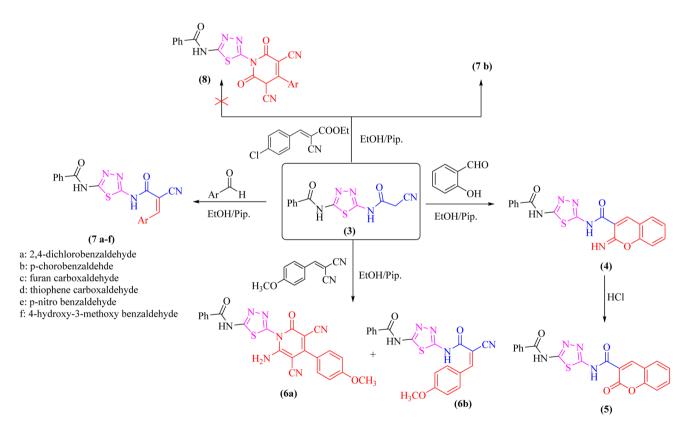
3.1 Chemistry

As a part of our ongoing research that aims at developing simple and efficient synthetic techniques for the synthesis of novel heterocyclic compounds with potential anticancer applications,21-31 the present study reported the synthesis of numerous new substituted 1,3,4-thiadiazole derivatives incorporating benzene moiety. N-(5-Amino-1,3,4-thiadiazol-2-yl) benzamide (2) was synthesized as a sole product through reaction of benzoylisothiocyanate with thiosemicarbzide in dry acetonitrile and then underwent cyclization according to the previously published methodology32-36 (Scheme 1). Treatment of compound 2 with ethyl cyanoacetate in the presence of a catalytic amount of triethylamine afforded N-(5-(2-cyanoacetamido)-1,3,4-thiadiazol-2-yl)benzamide (3) in a high yield, which was used as a key starting material (Scheme 1). The structures of compounds 2 and 3 were deduced from their elemental and spectral analyses.

The nucleophilic reactivity of the intermediate 3 towards various carbon electrophiles has been investigated. Refluxing compound 3 with salicylaldehyde in an ethanolic solution containing a catalytic amount of piperidine yielded 2-iminocoumarin derivative 4 (Scheme 2). The presence of absorption bands for –NH groups at 3409 and 3158 cm⁻¹ along with the absence of absorption band for the nitrile group confirmed the assigned

(3)

Scheme 1 Synthesis of 1,3,4-thiadiazole derivative 3.



Scheme 2 Reaction of the β -ketonitrile 3 with some aromatic aldehydes and arylidene derivatives.

structure 4. In addition, acidification of the filtrate gave the expected coumarin derivative 5 (Scheme 2). Ample evidence for the assigned structure 5 was forthcoming from the correct elemental analysis and spectral data. The presence of a high frequency C=O stretching at 1713 cm⁻¹ was attributed to the coumarin

oxo-group in the IR spectrum as well as the absence of NH proton of imino group at 11.76 ppm in the ¹H-NMR spectrum of compound 5 agrees well with the proposed structure.

Subjecting compound 3 to react with 2-(4-methox-ybenzylidene)malononitrile gave a grey crystalline product

upon reflux which was identified as 2-oxopyridine thiadiazole derivative 6a (major product, Scheme 2), while acidification of the reaction filtrate with cold dilute HCl furnished an orange precipitate that was detected as the arylidene derivative 6b (minor product). Furthermore, the Knoevenagel condensation of 1,3,4-thiadiazole derivative 3 with aromatic aldehydes namely: 2,4-dichlorobenzaldehde, p-chlorobenzaldehyde, furan-2-carbaldehyde, thiophene-2-carbaldehyde, p-nitro benzaldehyde, or 4-hydroxy-3-methoxy benzaldehyde for three hours in refluxing ethanol containing a catalytic amount of piperidine afforded the corresponding arylidene derivatives 7a-f, respectively (Scheme 2). Moreover, the reaction of compound 3 with ethyl 3-(4-chlorophenyl)-2-cyanoacrylate under the same reaction conditions afforded the arylidene derivative 7b rather than 2-oxopyridin derivative 8.

The proclivity of thiadiazole derivative 3 towards β -diketones was also investigated in this study. Thus, 2-pyridone derivatives 9 and 10 were obtained from the reaction of 3 with acetyl acetone and benzoyl acetone, respectively in ethanolic solution containing catalytic amount of fused sodium acetate (Scheme 3). 1 H-NMR spectrum of compound 9 showed signals at 3.96 and 3.99 ppm which were attributed to protons of CH₃ group, as well as the absence of a singlet signal for protons of CH₂CN group which confirmed the proposed structure 9. Furthermore, the 13 C-NMR spectrum revealed the presence of two methyl groups at δ 21.01 and 21.12 ppm (Scheme 3).

At room temperature, the active methylene moiety in the cyanoacetamide derivative 3 readily adds to one carbon donor such as phenyl isothiocyanate in DMF-containing potassium hydroxide forming a non-isolable intermediate potassium thiocarbamate salt (A), which *in situ* reacted with ethyl chloroacetate, chloroacetyl chloride, diethylmalonate, 1,2-dibromo ethane, methyliodide, or hydrochloric acid giving the corresponding ethyl thioacetate, 1,3-thiazolone, 4,6-dioxo-1,3-thiazinan, 1,3-thiazole, thiadiazolo[3,2-a]pyrimidin, and thioxopropanamide derivatives 11–16, respectively (Scheme 4). The structures of derivatives 11–16 were clarified using elemental

analysis and spectral data (*c.f.* experimental). For instance, the appearance of strong absorption bands in the IR spectrum at 2195 and 1734 cm⁻¹ for C \equiv N and C \equiv O_{ester} groups, respectively was completely consistent with the suggested structure **11** and ruled out the cyclized compound **12**.

Likewise, addition of 1,3,4-thiadiazole derivative 3 to carbon disulfide in DMF-containing potassium hydroxide at room temperature gave the non-isolable dipotassium dithiocarbazate salt (B), which *in situ* reacted with methyl iodide affording the ketene *S,S*-dithioacetal 17 (Scheme 5). The elemental analysis and spectral data were used to determine the structure of compound 17.

Further, the reactivity of **17** towards some nitrogen and oxygen nucleophiles was investigated. Thus, compound **17** reacted under reflux with bidentate nucleophiles such as *o*-phenylenediamine, *o*-aminophenol, or 2,3-dihydroxy pyridine giving the corresponding benzimidazole derivative **18**, benzox-azole derivative **19**, and dioxolo[4,5-*b*]pyridine derivative **20**, respectively (Scheme 5). The structures of compounds **18–20** were consistent with the ¹H-NMR and ¹³C-NMR spectra which revealed the absence of two SCH₃ groups.

3.2 Biological evaluation

3.2.1 Antiproliferative activity. According to the National Cancer Institute (NCI) criteria, only novel moieties with antiproliferative activity at 10 μ M or less against cancer cell lines are considered for further studies.³⁷ Based on this criterion, only derivatives **4**, **6b**, **7a**, **7d**, and **19** are to be considered (Table 1). However, derivatives **4** and **7d** were only effective against the breast cells and with very low selectivity. Although derivative **7a** was effective against all cell lines investigated, it has a low selectivity index (SI = 1.5). In other words, derivatives **4**, **7a**, and **7d** were as toxic to the cancer cells as to the normal fibroblasts, and therefore, these derivatives were excluded from further investigations (Table 1). Derivatives **6b** and **19** with IC₅₀ at less than 10 μ M against the breast, colon, and prostate cell lines and with high selectivity (SI = \sim 5 and 6 folds, respectively), were

Scheme 3 Synthesis of some new 2-pyridone derivatives (9 and 10) from the cyanoacetamide 3.

Scheme 4 Reaction of potassium thiocarbamate (A) with some electrophiles.

Scheme 5 Treatment of the ketene S,S-dithioacetal 17 with some bidentate nucleophiles

selected for further studies. It is noteworthy that other than 7a, no other derivative exhibited a significant antiproliferative activity against the liver HepG2 cells. It is also noteworthy that

18 compounds out of 24 were toxic (not selective) to the normal fibroblasts. Doxorubicin had antiproliferative activity against all cell lines investigated with no selectivity ($\mathrm{SI}=1.2$). The breast

Table 1 The antiproliferative activity (IC₅₀ in μ M) of the new thiadiazole derivatives against human breast (MCF-7), colon (HCT-116), prostate (PC-3), and liver (HepG2) cell lines, and normal fibroblasts (WI-38) and their selectivity index (SI)^a

Cpds	MCF-7	HCT-116	PC-3	HepG2	WI-38	SI ^c
DOX	4.17 ± 0.2^b	5.23 ± 0.3^b	8.87 ± 0.6^{b}	4.50 ± 0.2^b	6.72 ± 0.5^b	1.2
2	12.36 ± 3.0	15.26 ± 2.8	30.01 ± 3.1	14.11 ± 1.5	17.56 ± 4.1	1.0
3	23.81 ± 1.9	25.72 ± 1.9	39.23 ± 2.8	31.26 ± 5.1	37.41 ± 2.5	1.2
4	8.25 ± 3.5^b	15.84 ± 3.2	20.97 ± 3.3	48.51 ± 2.9	29.35 ± 2.2	1.3
5	64.12 ± 3.8	47.22 ± 3.5	66.53 ± 3.9	63.93 ± 8.9	91.23 ± 4.6	1.5
6a	41.57 ± 2.7	29.19 ± 2.3	35.76 ± 2.6	54.62 ± 6.8	18.64 ± 1.4	0.5
6b	9.48 ± 0.8^b	8.15 ± 0.7^b	7.41 ± 0.6^{b}	15.88 ± 1.3	50.39 ± 0.9	4.9
7a	5.51 ± 0.4^b	3.97 ± 0.2^{b}	9.10 ± 0.8^b	6.34 ± 0.9^{b}	9.18 ± 3.6^{b}	1.5
7 b	17.73 ± 0.6	16.18 ± 0.4	21.86 ± 1.1	14.68 ± 2.1	42.06 ± 2.7	2.4
7 c	28.13 ± 3.5	52.63 ± 3.7	13.30 ± 3.6	16.34 ± 1.8	22.57 ± 3.0	0.8
7 d	8.02 ± 1.4^b	34.14 ± 2.6	11.12 ± 4.7	66.10 ± 3.5	12.28 ± 1.2	0.4
7 e	45.11 ± 4.0	51.39 ± 4.1	75.22 ± 4.7	56.33 ± 7.8	23.39 ± 3.6	0.4
7 f	13.59 ± 1.1	10.92 ± 0.9	18.35 ± 1.5	21.26 ± 3.7	14.54 ± 0.3	0.9
9	14.13 ± 1.2	12.78 ± 1.1	15.38 ± 1.3	39.07 ± 2.7	50.39 ± 0.9	2.5
10	72.41 ± 4.1	68.01 ± 3.9	86.47 ± 4.8	19.40 ± 1.5	>100	1.7
11	69.70 ± 3.9	63.12 ± 3.8	71.94 ± 4.0	52.87 ± 4.6	22.65 ± 3.8	0.4
12	84.81 ± 4.4	91.27 ± 4.6	>100	69.54 ± 6.5	>100	1.2
13	37.95 ± 3.2	46.14 ± 3.7	73.56 ± 4.3	45.21 ± 6.3	23.58 ± 3.2	0.5
14	36.23 ± 2.4	40.18 ± 3.1	92.28 ± 3.2	>100	53.75 ± 3.4	0.8
15	12.74 ± 2.1	17.03 ± 1.4	10.49 ± 1.9	16.84 ± 1.7	19.38 ± 1.5	1.4
16	19.58 ± 1.6	21.62 ± 1.7	28.80 ± 2.3	14.68 ± 2.3	85.91 ± 1.9	4.1
17	15.10 ± 1.3	14.67 ± 0.9	12.17 ± 0.8	12.47 ± 1.0	81.20 ± 4.1	6.0
18	84.65 ± 4.6	56.85 ± 3.6	81.82 ± 4.5	47.33 ± 8.2	>100	1.5
19	7.70 ± 0.5^{b}	5.98 ± 0.3^{b}	6.35 ± 0.5^{b}	19.40 ± 1.5	60.28 ± 3.7	6.1
20	36.78 ± 2.7	18.32 ± 1.7	31.39 ± 2.2	29.64 ± 3.1	42.74 ± 2.7	1.5

^a The IC₅₀ was calculated from a dose–response curve. Doxorubicin (Dox) was used as a positive control. The data are expressed as mean \pm SEM from three independent experiments. ^b Significant (less than 10 μM) antiproliferative activity as compared to the untreated cells. ^c SI: selectivity index = WI-38 IC₅₀/IC₅₀ against cancer cells.

cells (MCF-7) were the most responsive to the thiadiazole derivatives. Therefore, these cells were selected for further investigations on compounds **6b** and **19**.

3.2.2 Cell cycle and apoptosis analysis. In the cell cycle analysis assay, the treated cells are stained with propidium iodide (DNA binding stain) and analyzed by flow cytometry. Treatment of MCF7 cells with 19 led to G2/M arrest with 23.3% of cells arrested at this stage compared to 4.6% in the cells treated with DMSO (Fig. 2). The arrested cells usually undergo apoptosis. Compound 6b increased the sub-G1 percent of cells from 7.4% in the DMSO-treated cells to 53.6%. The sub-G1 population is characterized by low DNA level, and it is usually comprised of cell fragments, often because of apoptosis or sometimes necrosis. Since the sub-G1 peak appears very close to the G0/G1 peak, we believe it is mainly due to necrosis rather than to apoptosis. To exclude the contribution of any mechanical stress to the cells from excessive pipetting, vortex, or harsh centrifugation that usually results in elevation of the sub-G1 cell population, the experiment was repeated thrice with much care and restricted gating strategy.

The arrest of cells at the G2/M indicates a severe DNA damage that is beyond repair.³⁸ The crucial event in cell-cycle progression through the G2/M checkpoint is the upregulation of expression of Chk2 protein and increase in the phosphorylation of Chk2 and CDC25C, leading to inhibition of the formation CDK1/cyclin B complex and cell cycle arrest at the G2/M phase.^{39,40} Many proteins such as p53, p21, and p38/MAPK

could contribute to the arrest at the G2/M phase.⁴¹ p53 induces the transcription of p21 which inhibits CDK resulting in the arrest of cells at the G2/M.^{42,43} In the current study, we have not investigated these different pathways.

We have also measured apoptosis by annexin V-PI assay. The level of apoptotic cells either in the early stage (lower-right quadrant, LR) or late apoptotic stage (upper-right quadrant, UR), in addition to the necrotic cells in the upper left (UL) quadrant were all quantified by flow cytometry (Fig. 3). It is evident that compound 6b did not induce apoptosis and increased the necrotic cells by 12.5% compared to 0.1% when the cells were treated with **DMSO**. This confirms the data we have obtained from the cell cycle analysis. As expected, compound 19 significantly increased the early apoptosis to 15% and increased the necrotic cells to 15% (Fig. 3). Compound 19 evidently arrested the cells at the G2/M forcing them to apoptosis possibly through induction of p53 and different caspases.44-46 Derivatives 6b and 19 induced necrosis in addition to the apoptosis caused by derivative 19. Although the importance of necrosis in the cancer therapy is not as fully understood as apoptosis, recent studies suggest that necrosis or necroptosis have potential applications as anticancer target. 47,48

3.3. In silico studies

3.3.1 Molecular docking study. First, two validation procedures were completed to verify the MOE program's

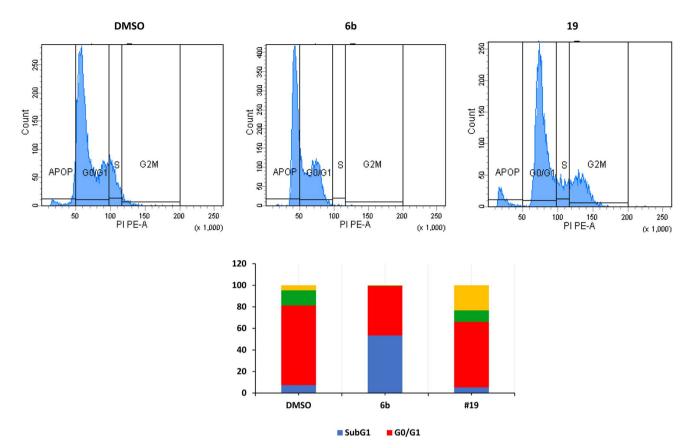


Fig. 2 Cell cycle analysis of MCF7 after treatment with derivatives **6b** and **19**. Data are expressed as means \pm SEM (n=3). The top panel represents one sample, and the bottom figure represents the average of 3 independent experiments expressed as percentage of cells in every stage.

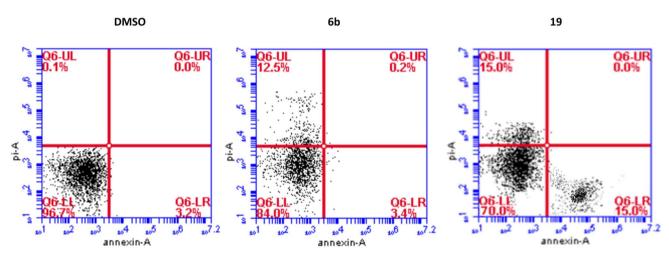


Fig. 3 Detection of apoptosis by derivatives 6b and 19 in MCF7 cells by flow cytometry.

reliability in producing accurate docking results. This was proven by redocking the co-crystallized inhibitor of the CDK-1.49 The successful execution of the docking protocol was guaranteed by the low values of RMSD = 1.043 Å that were obtained between the native and redocked poses of the co-crystallized inhibitor. 50

It was shown that **FB8**, a native co-crystallized inhibitor of the CDK-1, forms four H-bonds with the amino acids Glu81, Leu83, Leu83 and Lys89, one ionic bond with Asp146, and one pi-bond with Phe80.⁴⁹ More thorough research and comparisons with the docked **FB8** of the CDK-1 binding pocket was conducted on the two most promising biologically active newly synthesized thiadiazol derivatives (**6b** and **19**). After looking at

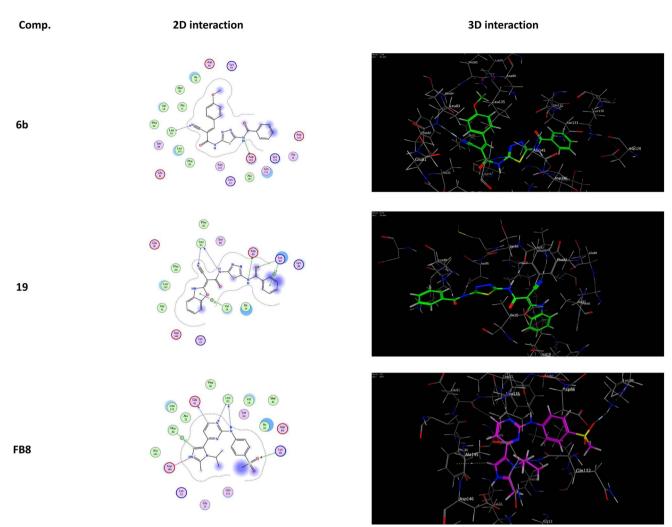


Fig. 4 The interactions of the most promising derivatives (**6b** and **19**) at CDK-1 binding pocket, alongside the docked co-crystallized inhibitor (FB8), are depicted in 2D and 3D interactions. H-bonds (dashed blue lines), ionic bond (red dashed lines) and H-pi bonds (green dashed lines).

the binding modes of the aforementioned compounds (Fig. 4), we concluded that:

- (a) Compound **6b** formed a H-bond donor with the Asp146 amino acids at 2.97 Å and a H-bond acceptor with the Leu83 amino acid at 3.17 Å when positioned inside the largest binding pocket of the CDK-1 (S=-7.220 kcal mol⁻¹ and RMSD = 1.713). It is not a good inhibitor of the enzyme.
- (b) Compound **19** formed two H-bonds donor with Leu83 and Asp86 amino acids at 3.53 and 2.93 Å, respectively. Furthermore, it formed two H-bonds acceptor with Lys89 and Leu83 amino acid at 3.03 and 3.18 Å, respectively, as well as two pi–H bonds with Val18 and Lys89 amino acids at 3.78 and 3.66 Å, respectively inside the binding site of the CDK-1 (S = -6.671 kcal mol⁻¹ and RMSD = 1.067). Therefore, compound **19** is a good CDK1 inhibitor candidate.
- (c) Finally, it was shown that the docked co-crystallized inhibitor of the CDK-1 (FB8, S=-6.787 kcal mol^{-1} and RMSD = 1.124) formed two H-bonds donor with Glu81 and Leu83 amino acid at 3.39 and 2.99 Å, respectively. Furthermore, it formed two H-bonds acceptor with Leu83 and Lys89 amino

acid at 3.42 and 3.13 Å, respectively. Also, formed one pi–H bond with Phe80 amino acids at 4.42 Å. In addition to one ionic bond with Asp146 amino acid at 4.42 Å.

3.3.2 Pharmacokinetics profiling study. In the current study, FB8 was used as a reference compound to assess the pharmacokinetics and physicochemical properties of all the designed compounds *in silico*. ^{51,52} According to Lipinski, a molecule is predicted to be absorbed more effectively if it complies with at least three of the following four principles: (i) molecular weight <500; (ii) H-bond donors [HBD] (OH, NH, and SH) \leq 5; (iii) H-bond acceptors [HBA] (N, O, and S atoms) \leq 10; (iv) $\log P < 5$. More than one of these requirements must be met for a compound to have a high level of bioavailability. Additionally, it was found that the number of rotatable bonds and the reduction in molecular flexibility were important indicators of excellent oral bioavailability. ⁵³ For substances with ten or fewer rotatable bonds and a molecular polar surface area of 140 Å or less, oral bioavailability is likely to be high. ⁵⁴

Except for compound 11, which violates more than one of the Lipinski's rule of five, all tested, and reference compound

Table 2 Physicochemical characteristics of the new thiadiazole derivatives and the reference compound

	Lipinski's rule of five					Verber's rule	
Comp	$\log P$	Mol. wt	HBD	НВА	Violation of Lipinski's rule	Number of rotatable bonds	TPSA
2	1.13	220.25	2	3	0	3	109.14
3	0.95	287.30	2	5	0	6	136.01
4	2.66	391.40	3	6	0	6	149.21
5	2.57	392.39	2	6	0	6	142.43
6a	2.42	469.48	2	7	0	6	187.95
6 b	2.58	405.43	2	6	0	8	145.24
7a	3.59	444.29	2	5	0	7	136.01
7 b	3.07	409.85	2	5	0	7	136.01
7 c	1.86	365.37	2	6	0	7	149.15
7 d	2.53	381.43	2	5	0	7	164.25
7e	1.91	420.40	2	7	0	8	181.83
7 f	2.25	421.43	3	7	0	8	165.47
9	2.39	351.38	1	5	0	4	128.91
10	3.30	413.45	1	5	0	5	128.91
11	3.00	508.57	3	7	2	13	199.64
12	2.32	462.50	2	6	0	7	181.62
13	1.88	490.51	2	7	0	7	198.69
14	2.62	448.52	2	5	0	7	164.55
15	2.57	388.40	2	5	0	5	140.42
16	2.45	422.48	3	5	0	9	180.13
17	2.16	391.49	2	5	0	8	186.61
18	1.78	403.42	4	5	0	6	167.59
19	2.11	404.40	3	6	0	6	164.94
20	1.60	406.37	2	8	0	6	167.36
FB8	2.51	371.46	1	5	0	5	98.15

(FB8) listed in Table 2 displayed drug-like molecular (DLM) nature, according to the study. Only one of Lipinski's rule of five was violated and only by compound 11. All other investigated molecules had $\log P$, molecular weight, number of H-bond donors, and number of H-bond acceptors less than 5, 500, 5, and 10, respectively. Except for compound 11, which showed 13 rotatable bonds, all the examined molecules' topology polar surface areas (TPSA) and numbers of ratable bonds fall within the acceptable ranges of less than 10 and 140 Å, respectively.

3.3.3 ADMET analysis. All the compounds examined were subjected to ADMET analyses using Discovery Studio 2.5, with the reference compound **FB8**. Many descriptors were used in ADMET evaluations, including hepatotoxicity, intestinal absorption, aqueous solubility, plasma protein binding, and molecular blood-brain barrier (BBB) penetration, ⁵⁵ Table 3.

The results showed that BBB penetration levels varied from none (derivatives 2, 4, 9, 11, 18, and 20, in addition to FBB) to very high (derivatives 5, 7a–f, 13, and 14). Derivatives 3, 6a, 6b, 10, 12, 15–17, and 19 showed low penetration ability to the BBB. Most important that derivatives 6b and 19 are safe to the CNS.

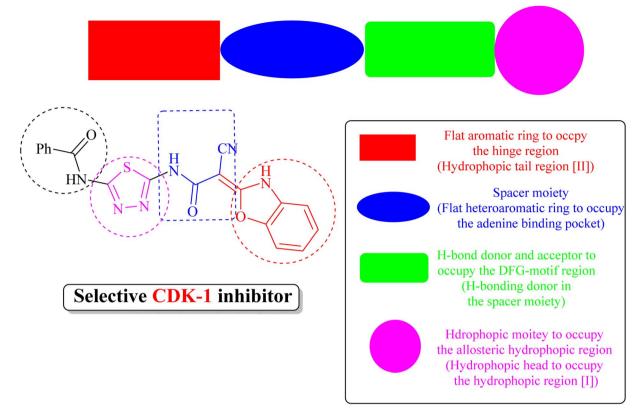
Clinical trials for a number of pharmaceutical candidates have been documented as being unsuccessful because of absorption issues. ⁵⁶ Intestinal absorption is the proportion of a drug that is taken up by the intestinal mucosa. A drug is well-absorbed if at least 90% of it is absorbed into the bloodstream. ⁵⁷ All the tested compounds, except for 5, 7a–f, 13 and 14, which showed moderate to poor levels of absorption, have good absorption properties, according to the findings of these studies. All the novel compounds as well as the reference

Table 3 Predicted ADMET descriptions for the thiadiazoles and FB8

Comp	BBB level ^a	Absorption level ^b	Hepatotoxicity probability c	PPB^d
2	0	0	0.857	0
3	1	0	0.901	0
4	0	0	0.834	2
5	4	2	0.518	2
6a	1	0	0.909	2
6b	1	0	0.878	2
7a	4	1	0.89	2
7 b	4	1	0.530	2
7 c	4	1	0.887	2
7 d	4	2	0.87	2
7e	4	2	0.516	2
7 f	4	2	0.894	2
9	0	0	0.86	2
10	1	0	0.974	1
11	0	0	0.907	2
12	2	0	0.859	2
13	4	2	0.905	2
14	4	2	0.955	2
15	1	0	0.905	2
16	1	0	0.865	2
17	1	0	0.794	2
18	0	0	0.92	2
19	1	0	0.973	1
20	0	0	0.912	2
FB8	0	2	0.517	1

 ^a BBB level, blood-brain barrier level, 0 = very high, 1 = high, 2 = medium, 3 = low, 4 = very low.
 ^b Absorption level, 0 = good, 1 = moderate, 2 = poor, 3 = very poor.
 ^c Hepatotoxicity probability, value > 0.5 means toxic, value < 0.5 means non-toxic.
 ^d PPB, plasma protein binding, 0 means less than 90%, 1 means more than 90%, 2 means more than 95%.

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Derivative 19 as a potential inhibitor of CDK1.

compound could have potential hepatotoxic effects according to predictions. All tested members and reference compound can bind plasma protein greater than 90% (highly bound), except for compounds 2 and 3, which showed plasma protein binding less than 90%.

Structure activity relationship (SAR) study

The SAR of the newly synthesized derivatives of the two series of thiadiazole and benzoxazole-based yields very promising results.

- (i) Concerning the antiproliferative activity of thiadiazolebased derivatives, compound 6b was found to be superior to compound 7a and then compound 7d on the four cell lines studied. This indicates that the 4-CH₃ substituent on the side phenyl moiety is superior to the 2,4-diCl substituent, which is also superior to the thiophene moiety.
- (ii) In contrast, we can conclude about the benzoxazole-based candidate compound 19 with benzo [d] oxazole ring together with the thiadiazole moiety achieved the best antiproliferative actions. The cyanoacetamido moiety in position-2 of the benzoxazole ring and benzamide side chain, demonstrated strong antiproliferative effects on all four cancer cell lines.
- (iii) Derivative 19 shares some of the common characteristics of the known CDK1 inhibitors (Fig. 5). These include a hydrophobic head to occupy the hydrophobic region I, an H-bond donor in the spacer moiety, a flat heteroaromatic ring to occupy the ATP-binding pocket, and a hydrophobic tail to occupy the

hydrophobic region I. In addition, derivative 19 has various Hbond donor and/or acceptor moieties such as cyanoacetamide. The presence of a linker (thiourea or cyanoacetamide) or rigidified to heteroaromatic ring facilitates the occupation to the hydrophobic region of the enzyme. A substituted phenyl ring and heteroaromatic ring with various electron-donating or withdrawing groups added to the aforementioned core probably improved the inhibiting potential of derivative 19.

4. Conclusion

In this study, a group of 24 annulated 1,3,4-thiadiazole compounds were synthesized and investigated as anticancer agents. Only five derivatives had a significant antiproliferative activity against a panel of four cancer cell lines. However, three of these were not selective and caused toxicity to the normal cells. Two derivatives 6b and 19 were effective and selective. Compound 19 arrested the MCF-7 cells at the G2/M phase probably through inhibition of CDK1 and elicited apoptosis in these cells confirmed by the annexin V-PI assay. Compound 6b acted through a different pathway and significantly increased the sub-G1 percent of cells probably through induction of necrosis. Molecular docking showed that compound 19 is a potential CDK1 inhibitor candidate. Derivatives 6b and 19 did not violate Lipinski's rule of five and therefore, they are considered hits for further drug discovery research. In silico studies showed that these derivatives also have a low bloodbrain barrier penetration capability and high intestinal absorption. Taken together, derivatives **6b** and **19** could serve as potential anticancer agents and merit further investigations.

Author contributions

Data curation: PSF and WME. Formal analysis: PSF and WME. Investigation: MHH, PSF, and WME. Writing – original draft: MHH, PSF, and WME. Conceptualization: MHH, MMH, WME. Supervision: MMH. Resources: AAE, AIH, WME. Software: PSF. Methodology: MHH, PSF, and WME. Validation: MHH, WME. Writing – review & editing: PSF, and WME. All authors read and approved the final version of the manuscript.

Conflicts of interest

Authors declare that they have no conflict of interest.

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