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Synthesis and anticancer activities of 1,4-phenylene-bis-*N*-acetyl- and *N*-phenylpyrazoline derivatives

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Abstract Novel 1,4-phenylene-bis-*N*-acetyl- (**3a**–**h**) and bis-*N*-phenylpyrazoline derivatives (**4a**–**h**) were obtained by addition of hydrazine hydrate and phenylhydrazine to bis-chalcone derivatives (**1a**–**h**) in acetic acid and acetic acid/ethanol for 4 and 8 h in reflux conditions, respectively. The structures of the obtained bis-*N*-acetylpyrazoline and bis-*N*-phenylpyrazoline derivatives were characterized by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopic methods and elemental analysis. Compounds **3a**–**h** and **4a**–**h** were investigated to evaluate their anticancer activities against C6 (rat brain tumor cells) and HeLa (human uterus carcinoma) in vitro using a dose-dependent assay from 5 to 100 μM with 5-fluorouracil (5-FU) as standard anticancer drug. Compound **3a** showed higher cell-selective activity compared with 5-FU against HeLa cells. Compounds **3a**–**h** (except **3d**) were shown to have better activities than 5-FU against both cells, particularly at high concentration. Compound **4c** showed higher cell-selective activity compared with 5-FU against C6 cells. Compound **3a** may be particularly promising as an anticancer drug against HeLa cells.

Keywords Anticancer activity · Bis-*N*-acetylpyrazolines · Bis-*N*-biphenylpyrazolines · C6 · HeLa

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Introduction

Cancer has become one of the major health problems ravaging the world today, and one of the ways of treating cancer is known as chemotherapy [1–3]. Chemotherapy causes intense side-effects, due to its cytotoxic effect on normal cells [4]. For this reason, it is important that anticancer drugs show antiproliferative and cytotoxic activity against tumor cells without affecting normal tissues. Although numerous cytotoxic agents have been developed, there is a need to develop more potent and safer chemotherapeutic agents [5].

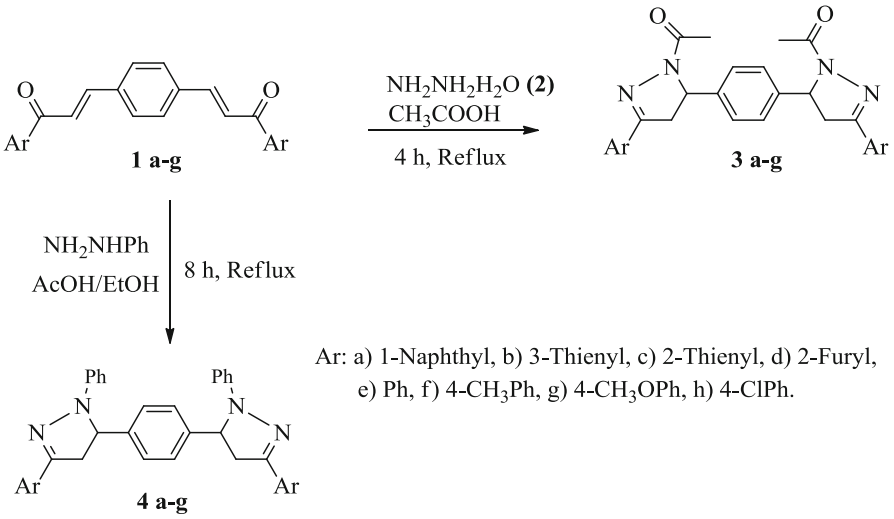
In this area, pyrazolines are of great interest due to their synthetic feasibility and biological importance [6–12]. Derivatives of pyrazolines are found to show good anticancer and radioprotective activity [13]. Pyrazoline is a prominent structural motif found in numerous antitumor agents [14]. Several 1,3,5-triaryl-4-alkylpyrazolines have been evaluated as breast cancer treatments with the goal of both reducing toxicity and increasing the response rate as an antitumor agent [15]. In addition, among anticancer pyrazolines, 1,3-diphenyl pyrazolines have been reported to be highly potent and efficient cytotoxic agents [16–20]. Also, a series of novel 3,5-diarylpyrazolines [21] and thiazolylpyrazoline derivatives were recently reported as potent anticancer agents [22].

Prompted by these results, we decided to investigate and report the synthesis and anticancer activity of a series of novel 1,4-phenylene-bis-*N*-acetylpyrazoline (**3a–h**) and 1,4-phenylene-bis-*N*-phenylpyrazoline derivatives (**4a–h**).

Results and discussion

Chemistry

The approach for synthesis of our target compounds is shown in Scheme 1. Initially, the known key 1,4-phenylene-bis-chalcones intermediates **1a–h** were resynthesized according to published procedure [23–26]. Bis-*N*-acetylpyrazoline derivatives **3a–h** were obtained in good yields (75–93 %) by refluxing 4 equivalents of hydrazine hydrate and the corresponding chalcones **1a–h** in acetic acid for 4 h (Scheme 1; Table 1). The compounds **3e–h** are known [27]; the others are novel, and they were synthesized by literature procedure [28]. The reaction involved nucleophilic attack of amino group on polarized carbonyl group, followed by intramolecular cyclization. Evidence for *N*-acetyl-4,5-dihydropyrazole ring formation was obtained from ^{1}H NMR spectra. The ^{1}H NMR spectra of the compounds displayed a typical ABX-type pattern of doublet of doublets due to three pyrazoline protons. Methine proton of pyrazoline was found at around 5.50 ppm, as a doublet of doublets with coupling constants of nearly 12 Hz and 4.0–4.8 Hz. Two methylene protons displayed two signals: a doublet of doublets at around 3.80–3.65 ppm with coupling constants of nearly 17 and 12 Hz, and a doublet of doublets at around 3.30–3.10 ppm. The ^{13}C NMR spectra of compounds **3a–h** confirmed their proposed structures, since C4 and C5 of pyrazoline ring resonated at around 40–45



Scheme 1 Synthesis of bis-*N*-acetyl- and bis-*N*-phenylpyrazoline derivatives **3** and **4**

Table 1 IC_{50} and IC_{75} values of **3a–h** and **4a–h** against C6 and HeLa cells (μM)

Compound	Ar	HeLa		C6	
		IC_{50}	IC_{75}	IC_{50}	IC_{75}
3a	1-Naphthyl	[a]	5.18	[a]	[a]
3b	3-Thienyl	9.79	43.90	[a]	12.58
3c	2-Thienyl	24.72	55.52	[a]	41.88
3d	2-Furyl	41.84	79.09	7.65	51.24
3e	Ph	49.74	73.60	29.43	55.24
3f	4-CH_3Ph	28.93	59.49	[a]	[a]
3g	4-CH_3OPh	[a]	34.33	[a]	[a]
3h	4-ClPh	43.23	64.11	[a]	36.68
4a	1-Naphthyl	63.64	82.10	[a]	20.85
4b	3-Thienyl	58.19	78.74	[a]	36.24
4c	2-Thienyl	63.03	91.24	23.88	56.92
4d	2-Furyl	49.70	72.34	[a]	35.76
4e	Ph	60.74	75.30	13.44	51.28
4f	4-CH_3Ph	68.14	86.19	[a]	35.76
4g	4-CH_3OPh	57.66	81.30	[a]	[a]
4h	4-ClPh	59.66	77.80	53.56	78.82
5-FU		16.32	[b]	5.8	[b]

[a] <5 μM, [b] no detection

and 55–60 ppm, respectively. Furthermore, in the ^{13}C NMR spectra, signals appearing at around 20–25 ppm indicated acetyl group connected to pyrazoline ring.

Similarly, heating at reflux of 4 equivalents of phenylhydrazine and the chalcones **1a–h** in acetic acid/ethanol mixture for 8 h afforded the corresponding bis-*N*-phenylpyrazoline derivatives **4a–h** in some good yields (76–93 %) (Scheme 1) [29]. Compound **4b** is novel; the others are known [30–32]. The structures of compounds **4a–h** were determined on the basis of spectral data. The spectral and microanalytical data of compounds **4a–h** were consistent with their structures.

Biological evaluation: anticancer activity

All compounds were tested for their potential growth inhibitory activity against rat brain tumor cells (C6) and human uterus carcinoma (HeLa) cell line using a proliferation BrdU enzyme-linked immunosorbent assay (ELISA). The tests were performed at concentrations of 5, 10, 20, 30, 40, 50, 75, and 100 μM with 5-fluorouracil (5-FU) as standard. The antiproliferative activities of the compounds increased with dose.

The activities of the *N*-acetylpyrazoline derivatives **3a–h** against C6 are shown in Fig. 1a and Table 1. All tested compounds **3a–h** showed anticancer activity with 50 % inhibitory concentration (IC_{50}) values ranging from <5 to 29.43 μM. In most cases, compounds **3a–h** were more active than 5-FU; in particular, compounds **3a**, **b**, **f–h** with IC_{50} values <5 μM exhibited significant activity at all concentrations.

The activities of the *N*-acetylpyrazoline derivatives **3a–h** against HeLa are shown in Fig. 1b and Table 1. As seen in Fig. 1b, the anticancer activities of all compounds **3a–h** (except **3d**, **e**) were better than 5-FU at high concentrations (75 and 100 μM). Moreover, compound **3a** exhibited very high activity with 95 % inhibition value at 100 to 20 μM concentrations. According to the IC_{50} values, the most active compounds were **3a**, **g** with IC_{50} value <5 μM, followed by **3b** (9.79 μM), **3c** (24.72 μM), and **3f** (28.93 μM).

The results for the anticancer activities of the *N*-phenylpyrazoline derivatives **4a–h** against C6 cells are shown in Fig. 2a and Table 1. According to Fig. 2a, the anticancer activity of **4c** was better than 5-FU at high concentrations (75 and 100 μM), and it showed significant activity at all concentrations. Compound **4d** showed almost the same activity as 5-FU. Compounds **4b**, **e–h** showed moderate anticancer effects. However, **4a** did not show significant activity compared with 5-FU. In addition, the compounds had very low IC_{50} values, except **4h**; while the IC_{50} values of **4a**, **b**, **d**, **f**, **g** were <5 μM, the others were 23.88 μM for **4a**, 13.44 μM for **4e**, and 53.56 μM for **4h** (Table 1).

The activities of **4a–h** against HeLa are shown in Fig. 2b and Table 1. According to these results, whereas compounds **4b–d**, **f** showed moderate activity at 100 μM concentration, the others were almost inactive compared with 5-FU.

According to these results, it was observed that compounds **3a–h** showed higher anticancer activities than compounds **4a–h** against both cell lines. Moreover, while compounds 3**a–h** exhibited significant activities against both cell lines, compounds **4a–h** demonstrated significant activities against the C6 cell line, particularly at high concentrations.

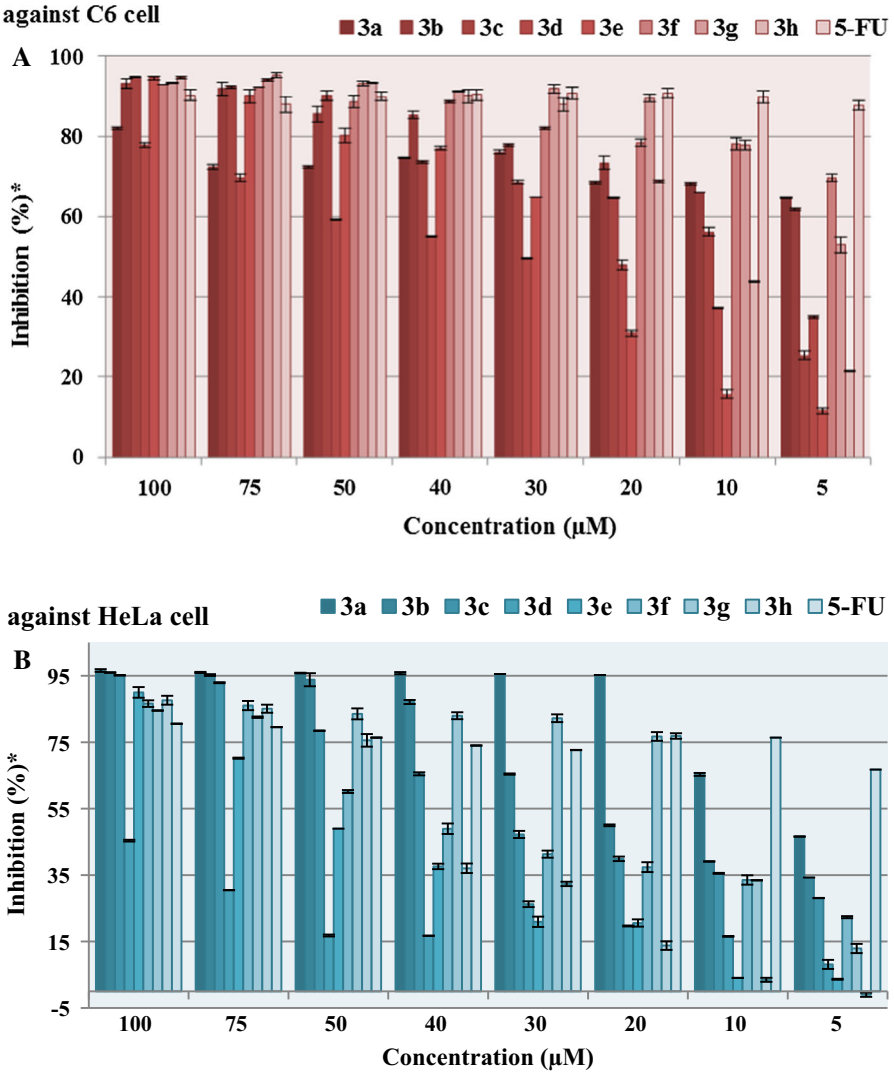


Fig. 1 Antiproliferative activities of **3a**–**h** against (**a**) C6 and (**b**) HeLa cells. *Each substance was tested twice in triplicates against cell lines. Data show averages of two individual experiments ($p < 0.01$)

Structure–activity relationship (SAR)

Comparing compounds **3a**–**h** with **4a**–**h**, it is obvious that the *N*-acetylpyrazolines **3a**–**h** showed higher growth inhibitory effect than the *N*-phenylpyrazolines **4a**–**h** against both cell lines. The higher cytotoxicity of **3a**–**h** is due to the acetyl (CH_3CO–) group attached to the pyrazoline ring, while in the case of compounds **4a**–**h**, the Ph group lowered the cytotoxicity.

Considering the *N*-acetylpyrazolines **3a**–**h**, all compounds showed high growth inhibitory effects against the C6 cell line at almost all concentrations. Compounds

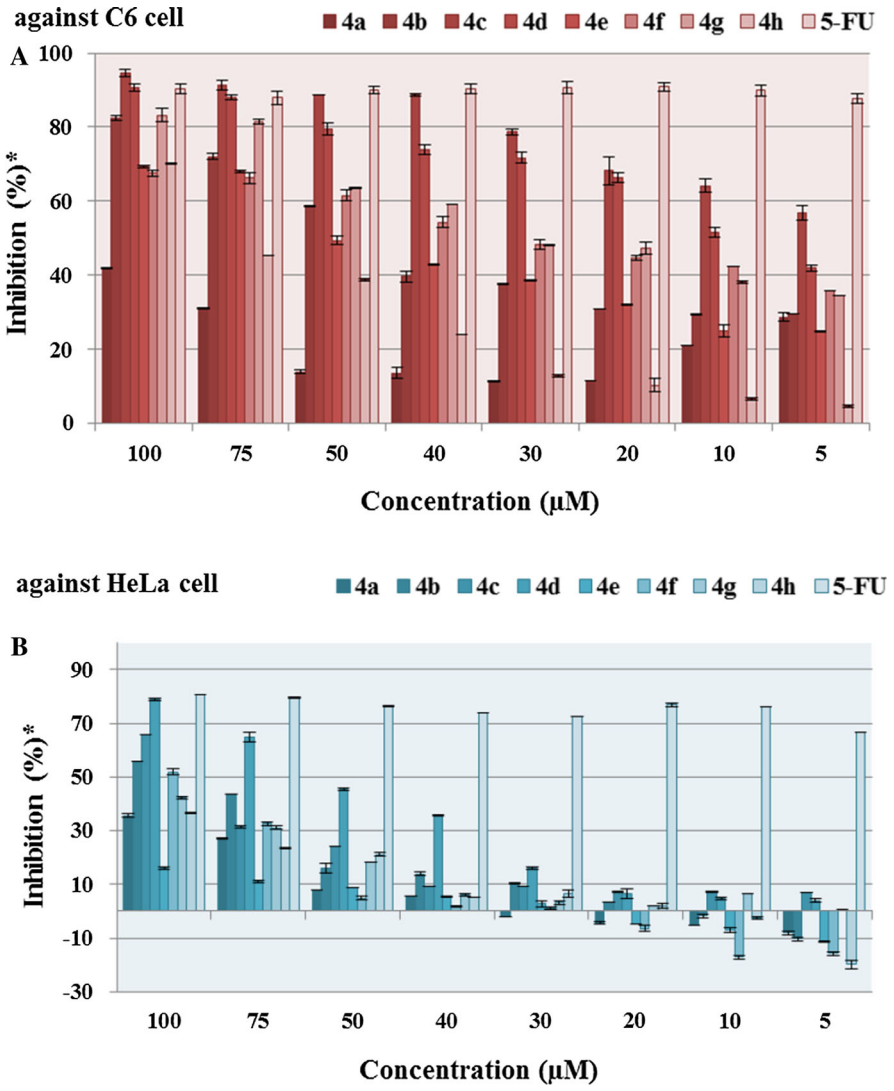


Fig. 2 Antiproliferative activities of **4a**–**h** against (**a**) C6 and (**b**) HeLa cells. *Asterisk* each substance was tested twice in triplicates against cell lines. Data show averages of two individual experiments ($p < 0.01$)

3a and **d** with 1-naphthyl and furyl substituents, respectively, showed lower activities, whereas the others exhibited activities higher than 5-FU against the C6 cell line at 100–50 μM concentrations.

The activities of all the compounds (except **3d** containing furyl ring) were better than 5-FU against HeLa cell lines at high concentrations. In addition, the activity of **3e** was low compared with 5-FU. However, compounds **3a** and **g** with 2-naphthyl and 4-methoxyphenyl substitution, respectively, exhibited activities higher than 5-FU against HeLa cell lines at 100 to 20 μM concentrations. This high cytotoxicity

of both compounds **3a** and **g** is attributed to the presence of naphthyl moieties and methoxide group, respectively.

Considering the *N*-phenylpyrazolines **4a**–**h**, all compounds **4a**–**h** showed selective growth inhibitory effects against the C6 cell line. All compounds (except **4a** containing naphthyl ring) exhibited remarkable activities, particularly at high concentrations. Among these compounds, the most active was **4c** (containing 2-thienyl ring), which showed significant activity at all concentrations. The remarkable activity of **4c** is due to the presence of the thiophene moiety.

All compounds showed very low activities except at 100 μM concentration against the HeLa cell line. The most active compound was **4d**, which is attributed to the presence of furan ring.

Conclusions

A series of 1,4-phenylene-bis-*N*-acetylpyrazoline (**3a**–**h**) and 1,4-phenylene-bis-*N*-phenylpyrazoline derivatives (**4a**–**h**) were synthesized in good yields by addition of hydrazine hydrate and phenyl hydrazine to 1,4-phenylene-bis-chalcones and evaluated for their anticancer activities against C6 (rat brain tumor) and HeLa (human uterus carcinoma) cell lines. Compounds **3a**–**h** showed potent anticancer activities against both cell lines. Most of them were higher in activity than 5-FU against the C6 and HeLa cell lines at high concentration. Compound **3a** exhibited very high activity against HeLa cell line cell selectively at 100 to 20 μM concentrations. Also, compounds **4a**–**h** (except **4a**) demonstrated significant activities against the C6 cell line, when compared with 5-FU at high concentrations. Moreover, only one compound (**4d**) showed remarkable activity against HeLa cell lines at 100 and 75 μM concentrations.

Experimental

The major chemicals were purchased from Sigma-Aldrich and Fluka. IR spectra (KCl disc) were recorded on a Jasco FT/IR-430 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-400 instrument. Tetramethylsilane (TMS) and $CDCl_3$ served as internal standard for ^{1}H and ^{13}C NMR spectroscopy with δ values of 0.00 and 77.0 ppm, respectively. *J* values are given in Hz. Signal multiplicities in the ^{1}H NMR spectra are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), and combinations thereof. Melting points were measured on Electrothermal 9100 apparatus. Elemental analyses were obtained using a LECO CHNS 932 elemental analyzer.

General method for synthesis of 1,4-phenylene-bis-*N*-acetylpyrazoline (3a–h) and 1,4-phenylene-bis-*N*-phenylpyrazoline derivatives (4a–h)

Bis-chalcone (**1a**–**h**) (1 mmol) and hydrazine hydrate (4 mmol) in acetic acid and/or phenylhydrazine (4 mmol) in acetic acid/ethanol mixture were refluxed for 4 and

8 h, respectively. The reaction mixture was poured into ice bath at the end of the reaction, and was checked with pH paper to ensure neutralization of the medium by addition of ammonia. It was observed that the collapse occurred when the mixture was allowed to stand overnight, and the resulting precipitate was filtered off and allowed to dry. The resulting material was obtained as pure.

*1,1′-(5,5′-(1,4-Phenylene)bis(3-(naphthalen-1-yl)-4,5-dihydro-1H-pyrazole-5,1-diyl)) diethanone (**3a**)* Yield: 86 %. M.p. 250–252 °C. IR (KCl) ν/cm^{-1}: 3046, 1648, 1508, 1419, 1394, 1268, 1143, 1014, 958, 804, 773, 557. ^{1}H NMR (400 MHz, DMSO, ppm): δ 9.24 (d, $J = 8.4$ Hz, 2H), 8.03–7.98 (m, 4H), 7.75 (d, $J = 6.8$ Hz, 2H), 7.68 (t, $J = 7.2$ Hz, 2H), 7.60 (t, $J = 7.2$ Hz, 2H), 7.55–7.52 (m, 2H), 7.24 (brs, 4H), 5.55–5.51 (dd, $J = 11.4$, 3.2 Hz, 2H), 4.10–4.03 (dd, $J = 17.6$, 12.4 Hz, 2H), 3.33–3.28 (m, 2H), 2.41 (s, 6H). ^{13}C NMR (100 MHz, DMSO, ppm): δ 168.4 (2C), 155.5 (2C), 141.6 (2C), 134.1 (2C), 131.5 (2C), 130.1 (2C), 129.5 (2C), 129.2 (4C), 128.3 (2C), 127.4 (2C), 126.8 (2C), 126.6 (2C), 126.3 (2C), 125.7 (2C), 58.5 (2C), 45.0 (2C), 22.2 (2C). Anal. Calcd. for $C_{36}H_{30}N_4O_2$: C, 78.52; H, 5.49; N, 10.17. Found: C, 78.48; H, 5.32; N, 10.11.

*1,1′-(5,5′-(1,4-Phenylene)bis(3-(thiophen-3-yl)-4,5-dihydro-1H-pyrazole-5,1-diyl)) diethanone (**3b**)* Yield: 77 %. M.p. 223–225 °C. IR (KCl) ν/cm^{-1}: 3023, 2924, 1644, 1438, 1409, 1338, 1102, 1037, 1018, 982, 813, 701, 635, 548. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.55–7.54 (m, 2H), 7.47–7.46 (m, 2H), 7.42–7.38 (m, 2H), 7.18–7.16 (m, 4H), 5.57–5.51 (dd, $J = 11.6$, 4.8 Hz, 2H), 3.73–3.65 (dd, $J = 17.6$, 11.6 Hz, 2H), 3.14–3.07 (dt, $J = 17.6$, 4.8 Hz, 2H), 2.43 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 168.8 (2C), 150.2 (2C), 141.0 (2C), 133.9 (2C), 126.9 (2C), 126.4 (4C), 126.1 (2C), 125.5 (2C), 59.5 (2C), 42.8 (2C), 21.9 (2C). Anal. Calcd. for $C_{24}H_{22}N_4O_2S_2$: C, 62.31; H, 4.79; N, 12.11. Found: C, 62.28; H, 4.67; N, 12.06.

*1,1′-(5,5′-(1,4-Phenylene)bis(3-(thiophen-2-yl)-4,5-dihydro-1H-pyrazole-5,1-diyl)) diethanone (**3c**)* Yield: 75 %. M.p. 306–308 °C. IR (KCl) ν/cm^{-1}: 3033, 2929, 1644, 1440, 1413, 1326, 1141, 1037, 1018, 952, 831, 761, 630, 555. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.45–7.41 (dd, $J = 5.2$, 0.8 Hz, 2H), 7.21–7.20 (m, 6H), 7.09–7.07 (dd, $J = 5.0$, 3.6 Hz, 2H), 5.58–5.55 (dd, $J = 11.6$, 4.4 Hz, 2H), 3.77–3.69 (dd, $J = 17.4$, 11.6 Hz, 2H), 3.19–3.13 (dd, $J = 17.2$, 4.4 Hz, 2H), 2.40 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 168.7 (2C), 149.6 (2C), 140.9 (2C), 134.8 (2C), 128.8 (2C), 128.7 (2C), 127.6 (2C), 126.1 (4C), 59.7 (2C), 42.8 (2C), 21.9 (2C). Anal. Calcd. for $C_{24}H_{22}N_4O_2S_2$: C, 62.31; H, 4.79; N, 12.11. Found: C, 62.25; H, 4.72; N, 12.09.

*1,1′-(5,5′-(1,4-Phenylene)bis(3-(furan-2-yl)-4,5-dihydro-1H-pyrazole-5,1-diyl))diethanone (**3d**)* Yield: 93 %. M.p. 251–253 °C. IR (KCl) ν/cm^{-1}: 3124, 1643, 1421, 1388, 1010, 983, 827, 769, 563. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.57 (s, 2H), 7.18 (s, 4H), 6.73 (d, $J = 2.8$ Hz, 2H), 6.54–6.52 (m, 2H), 5.58–5.54 (dd, $J = 12.0$, 4.8 Hz, 2H), 3.71–3.63 (dd, $J = 17.6$, 12.0 Hz, 2H), 3.12–3.06 (dt, $J = 17.6$, 3.6 Hz, 2H), 2.42 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 168.8 (2C), 146.7 (2C), 145.7 (2C), 144.8 (2C), 140.8 (2C), 126.1 (4C), 112.8 (2C), 111.9

(2C), 59.1 (2C), 41.9 (2C), 21.9 (2C). Anal. Calcd. for $C_{24}H_{22}N_4O_4$: C, 66.97; H, 5.15; N, 13.02. Found: C, 66.89; H, 5.09; N, 12.98.

1,1′-(5,5′-(1,4-Phenylene)bis(3-phenyl-4,5-dihydro-1H-pyrazole-5,1-diyl))diethanone ***(3e)*** Yield: 78 %. M.p. 296–298 °C. IR (KCl) ν/cm^{-1}: 3060, 1644, 1454, 1409, 1118, 958, 829, 771, 555. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.78 (d, $J = 4.0$ Hz, 4H), 7.46 (s, 6H), 7.14 (s, 4H), 5.54–5.51 (dd, $J = 11.8$, 4.0 Hz, 2H), 3.87–3.80 (dd, $J = 18.0$, 12.0 Hz, 2H), 3.15–3.10 (dd, $J = 18.0$, 4.0 Hz, 2H), 2.43 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 167.8 (2C), 154.6 (2C), 141.8 (2C), 131.5 (2C), 130.7 (2C), 129.2 (4C), 127.4 (2C), 127.0 (4C), 126.2 (2C), 59.5 (2C), 42.4 (2C), 22.1 (2C). Anal. Calcd. for $C_{28}H_{26}N_4O_2$: C, 74.65; H, 5.82; N, 12.44. Found: C, 74.59; H, 5.79; N, 12.38.

1,1′-(5,5′-(1,4-Phenylene)bis(3-(p-tolyl)-4,5-dihydro-1H-pyrazole-5,1-diyl))diethanone ***(3f)*** Yield: 78 %. M.p. 268–270 °C. IR (KCl) ν/cm^{-1}: 3027, 2921, 1662, 1417, 1326, 1143, 1027, 1010, 960, 867, 819, 543. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.63 (d, $J = 7.2$ Hz, 4H), 7.27 (d, $J = 7.6$ Hz, 4H), 7.18 (s, 4H), 5.59–5.53 (dd, $J = 12.0$, 4.8 Hz, 2H), 3.73–3.66 (dd, $J = 17.4$, 12.0 Hz, 2H), 3.19–3.12 (dt, $J = 17.4$, 4.4 Hz, 2H), 2.45 (s, 6H), 2.42 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 168.8 (2C), 154.2 (2C), 141.1 (2C), 140.7 (2C), 129.4 (4C), 128.4 (2C), 126.5 (4C), 126.1 (4C), 59.5 (2C), 42.2 (2C), 21.9 (2C), 21.5 (2C). Anal. Calcd. for $C_{30}H_{30}N_4O_2$: C, 75.29; H, 6.32; N, 11.71. Found: C, 75.21; H, 6.27; N, 11.63.

1,1′-(5,5′-(1,4-Phenylene)bis(3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole-5,1-diyl))diethanone ***(3g)*** Yield: 79 %. M.p. 248–250 °C. IR (KCl) ν/cm^{-1}: 3002, 2933, 2836, 1656, 1606, 1517, 1423, 1255, 1174, 1035, 960, 831, 547. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.71–7.66 (dd, $J = 8.8$, 2.8 Hz, 4H), 7.18 (s, 4H), 6.97–6.93 (dd, $J = 8.8$, 2.4 Hz, 4H), 5.58–5.54 (dd, $J = 12.0$, 4.8 Hz, 2H), 3.87 (s, 6H), 3.72–3.64 (dd, $J = 17.4$, 12.0 Hz, 2H), 3.17–3.12 (dt, $J = 17.4$, 4.4 Hz, 2H), 2.42 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 168.7 (2C), 161.3 (2C), 153.9 (2C), 141.1 (2C), 128.2 (4C), 126.2 (2C), 126.1 (2C), 123.9 (2C), 114.5 (4C), 59.5 (2C), 55.4 (2C), 42.2 (2C), 21.9 (2C). Anal. Calcd. for $C_{30}H_{30}N_4O_4$: C, 70.57; H, 5.92; N, 10.97. Found: C, 70.52; H, 5.83; N, 10.84.

1,1′-(5,5′-(1,4-Phenylene)bis(3-(4-chlorophenyl)-4,5-dihydro-1H-pyrazole-5,1-diyl))diethanone ***(3h)*** Yield: 81 %. M.p. 253–255 °C. IR (KCl) ν/cm^{-1}: 1666, 1592, 1417, 1398, 1322, 1145, 1091, 1010, 956, 827, 565, 538. ^{1}H NMR (400 MHz, $CDCl_3$ ppm): δ 7.70–7.65 (dd, $J = 8.8$, 2.8 Hz, 4H), 7.42–7.39 (dd, $J = 8.6$, 2.4 Hz, 4H), 7.19 (s, 4H), 5.61–5.55 (m, 2H), 3.73–3.62 (m, 2H), 3.17–3.10 (dt, $J = 17.6$, 4.4 Hz, 2H), 2.42 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 169.0 (2C), 153.0 (2C), 140.9 (2C), 136.3 (2C), 129.7 (2C), 129.0 (4C), 127.8 (4C), 126.1 (4C), 59.7 (2C), 42.0 (2C), 21.9 (2C). Anal. Calcd. for $C_{28}H_{24}Cl_2N_4O_2$: C, 64.74; H, 4.66; N, 10.79. Found: C, 64.66; H, 4.59; N, 10.72.

1,4-Bis(3-(naphthalen-1-yl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4a)*** Yield: 79 %. M.p. 271–273 °C. IR (KCl) ν/cm^{-1}: 3446, 3322, 1598, 1540, 1498, 1247, 1133, 997, 802, 779, 690. ^{1}H NMR (400 MHz, DMSO, ppm): δ 9.58 (d,

$J = 8.4$ Hz, 2H), 8.09–7.99 (m, 4H), 7.72 (t, $J = 7.2$ Hz, 2H), 7.68 (t, $J = 7.2$ Hz, 2H), 7.46–7.41 (m, 2H), 7.25–7.21 (m, 4H), 6.99–6.97 (m, 4H), 6.90–6.87 (m, 4H), 6.06–6.02 (m, 4H), 5.33–5.23 (m, 2H), 4.08–4.01 (dd, $J = 16.8$, 12.4 Hz, 2H), 3.39–3.33 (dd, $J = 16.8$, 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO, ppm): δ 152.1 (2C), 143.9 (2C), 143.3 (2C), 142.8 (2C), 137.0 (2C), 130.2 (4C), 129.6 (2C), 129.3 (4C), 128.2 (2C), 127.2 (4C), 127.1 (4C), 124.5 (4C), 120.0 (2C), 118.9 (2C), 113.2 (2C), 62.9 (2C), 40.5 (2C). Anal. Calcd. for $C_{44}H_{34}N_4$: C, 85.41; H, 5.54; N, 9.05. Found: C, 85.39; H, 5.35; N, 9.02.

1,4-Bis(1-phenyl-3-(thiophen-3-yl)-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4b)*** Yield: 85 %. M.p. 279–281 °C. IR (KCl) ν/cm^{-1}: 3315, 3097, 1596, 1498, 1361, 1103, 997, 746, 692. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.59–7.56 (dd, $J = 4.8$, 3.6 Hz, 2H), 7.30 (s, 4H), 7.24–7.22 (dd, $J = 3.6$, 1.2 Hz, 2H), 7.14 (t, $J = 8.0$ Hz, 6H), 6.90 (t, $J = 7.2$ Hz, 4H), 6.73 (t, $J = 7.2$ Hz, 2H), 5.39–5.35 (dd, $J = 12.4$, 6.4 Hz, 2H), 3.88–3.3.83 (dd, $J = 17.4$, 12.0 Hz, 2H), 3.10–3.02 (dd, $J = 17.2$, 6.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 153.0 (2C), 145.1 (2C), 144.8 (2C), 142.2 (2C), 129.3 (4C), 128.3 (2C), 128.1 (2C), 127.7 (4C), 126.8 (2C), 117.9 (2C), 110.8 (4C), 62.9 (2C), 40.5 (2C). Anal. Calcd. for $C_{32}H_{26}N_4S_2$: C, 72.42; H, 4.94; N, 10.56. Found: C, 72.39; H, 4.91; N, 10.48.

1,4-Bis(1-phenyl-3-(thiophen-2-yl)-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4c)*** Yield: 82 %. M.p. 231–233 °C. IR (KCl) ν/cm^{-1}: 3284, 3021, 1594, 1500, 1367, 1132, 998, 740, 690. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.58–7.56 (dd, $J = 4.8$, 3.6 Hz, 2H), 7.29 (s, 4H), 7.21 (t, $J = 3.6$ Hz, 2H), 7.13 (t, $J = 8.0$ Hz, 4H), 7.09–7.07 (m, 2H), 6.90 (d, $J = 7.2$ Hz, 4H), 6.71 (t, $J = 7.2$ Hz, 2H), 5.45–5.40 (dd, $J = 12.0$, 6.4 Hz, 2H), 3.90–3.87 (dd, $J = 17.2$, 12.0 Hz, 2H), 3.10–3.08 (dd, $J = 17.2$, 6.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 163.3 (2C), 144.5 (2C), 144.1 (2C), 142.0 (2C), 129.3 (4C), 128.2 (2C), 128.0 (2C), 127.9 (4C), 127.0 (2C), 119.1 (2C), 113.2 (4C), 63.3 (2C), 44.1 (2C). Anal. Calcd. for $C_{32}H_{26}N_4S_2$: C, 72.42; H, 4.94; N, 10.56. Found: C, 72.35; H, 4.89; N, 10.51.

1,4-Bis(3-(furan-2-yl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4d)*** Yield: 80 %. M.p. 246–248 °C. IR (KCl) ν/cm^{-1}: 3122, 2915, 1594, 1504, 1394, 1257, 1130, 1027, 829, 748, 690. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.52–7.51 (dd, $J = 3.2$, 1.6 Hz, 2H), 7.29 (s, 4H), 7.20–7.17 (m, 4H), 7.05–7.03 (m, 4H), 6.81 (t, $J = 6.8$ Hz, 2H), 6.57 (t, $J = 3.2$ Hz, 2H), 6.50–6.48 (m, 2H), 5.26–5.23 (dd, $J = 12.4$, 4.8 Hz, 2H), 3.82–3.74 (dd, $J = 16.8$, 2.4 Hz, 2H), 3.11–3.05 (dd, $J = 17.0$, 6.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 149.8 (2C), 139.6 (2C), 129.3 (4C), 129.1 (2C), 127.0 (2C), 119.1 (2C), 118.8 (2C), 113.3 (4C), 112.5 (4C), 112.4 (2C), 111.3 (2C), 62.6 (2C), 43.3 (2C). Anal. Calcd. for $C_{32}H_{26}N_4O_2$: C, 77.09; H, 5.26; N, 11.24. Found: C, 77.01; H, 5.19; N, 11.18.

1,4-Bis(1,3-diphenyl-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4e)*** Yield: 93 %. M.p. 224–226 °C. IR (KCl) ν/cm^{-1}: 3100, 1594, 1498, 1380, 1317, 1228, 1122, 1106, 827, 748, 709, 690. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.74–7.71 (dd, $J = 7.6$, 2.4 Hz, 4H), 7.43–7.39 (m, 6H), 7.29 (s, 4H), 7.14 (t, $J = 7.6$ Hz, 4H), 6.98 (d, $J = 7.6$ Hz, 4H), 6.73–6.69 (dd, $J = 12.2$, 6.8 Hz, 2H), 5.45–5.40 (dd, $J = 12.4$,

6.8 Hz, 2H), 3.93–3.85 (dd, $J = 17.4$ 12.4 Hz, 2H), 3.10–3.04 (dd, $J = 17.4$, 6.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 147.6 (2C), 144.5 (2C), 142.2 (2C), 132.6 (2C), 129.3 (4C), 129.1 (4C), 129.0 (4C), 127.0 (2C), 126.1 (4C), 119.1 (2C), 113.3 (4C), 63.3 (2C), 43.4 (2C). Anal. Calcd. for $C_{36}H_{30}N_4$: C, 83.37; H, 5.83; N, 10.80. Found: C, 83.29; H, 5.79; N, 10.78.

1,4-Bis(1-phenyl-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4f)*** Yield: 76 %. M.p. 278–280 °C. IR (KCl) ν/cm^{-1}: 3018, 2919, 1596, 1498, 1386, 1321, 1122, 817, 746, 690. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.64–7.63 (dd, $J = 7.4$, 4.4 Hz, 4H), 7.29 (s, 4H), 7.22–7.20 (m, 8H), 7.08 (d, $J = 7.6$ Hz, 4 H), 6.83–6.75 (m, 2H), 5.26–5.21 (dd, $J = 12.2$, 7.2 Hz, 2H), 3.85–3.77 (dd, $J = 15.0$, 2.0 Hz, 2H), 3.15–3.09 (dd, $J = 16.8$, 7.2 Hz, 2H), 2.40 (s, 6H). ^{13}C NMR (100 MHz, DMSO, ppm): δ 147.6 (2C), 143.5 (2C), 142.2 (2C), 137.1 (2C), 132.6 (2C), 129.9 (2C), 129.3 (4C), 129.0 (4C), 127.0 (2C), 126.1 (4C), 123.5 (2C), 113.3 (4C), 63.3 (2C), 43.4 (2C), 20.1 (2C). Anal. Calcd. for $C_{38}H_{34}N_4$: C, 83.48; H, 6.27; N, 10.25. Found: C, 83.37; H, 6.19; N, 10.15.

1,4-Bis(3-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4g)*** Yield: 79 %. M.p. 265–267 °C. IR (KCl) ν/cm^{-1}: 3007, 1733, 1716, 1698, 1683, 1558, 1540, 414. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.67–7.66 (m, 4H), 7.31 (s, 4H), 7.23 (t, $J = 7.6$ Hz, 4H), 7.04 (d, $J = 7.2$ Hz, 4H), 6.94–6.91 (m, 4H), 6.79 (t, $J = 7.2$ Hz, 2H), 5.23–5.18 (dd, $J = 12.2$, 7.2 Hz, 2H), 3.85–3.80 (m, 8H), 3.14–3.10 (dd, $J = 17.0$, 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO, ppm): δ 163.2 (2C), 147.7 (2C), 145.0 (2C), 142.2 (2C), 129.3 (4C), 127.7 (4C), 127.0 (2C), 125.2 (4C), 118.7 (2C), 114.5 (4C), 113.1 (4C), 63.2 (2C), 56.0 (2C), 43.6 (2C). Anal. Calcd. for $C_{38}H_{34}N_4O_2$: C, 78.87; H, 5.92; N, 9.68. Found: C, 78.81; H, 5.89; N, 9.59.

1,4-Bis(3-(4-chlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)benzene ***(4h)*** Yield: 84 %. M.p. 284–286 °C. IR (KCl) ν/cm^{-1}: 1594, 1502, 1488, 1386, 1321, 1132, 1087, 1010, 821, 742, 690. ^{1}H NMR (400 MHz, DMSO, ppm): δ 7.70 (d, $J = 8.0$ Hz, 4H), 7.41 (d, $J = 8.0$ Hz, 4H), 7.26 (s, 4H), 7.11 (t, $J = 7.2$ Hz, 4H), 6.96 (d, $J = 6.8$ Hz, 4H), 6.69 (t, $J = 6.8$ Hz, 2H), 5.44–5.40 (dd, $J = 12.0$, 6.4 Hz, 2H), 3.90–3.82 (dd, $J = 17.4$, 12.0 Hz, 2H), 3.08–3.02 (dd, $J = 17.4$, 6.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO, ppm): δ 150.7 (2C), 144.4 (2C), 140.0 (2C), 133.5 (2C), 133.0 (2C), 130.1 (2C), 129.9 (4C), 129.1 (4C), 128.6 (4C), 127.7 (4C), 113.2 (2C), 106.1 (2C), 63.4 (2C), 43.2 (2C). Anal. Calcd. for $C_{36}H_{28}Cl_2N_4$: C, 73.59; H, 4.80; N, 9.54. Found: C, 73.52; H, 4.79; N, 9.48.

Anticancer activity

Cell culture

Antiproliferative activities of the synthesized compounds were investigated on C6 (rat brain tumor cells) and HeLa (human uterus carcinoma) cells using a proliferation BrdU ELISA assay [33, 34]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10 % (v/v) fetal bovine

serum (Sigma, Germany) and PenStrep solution (Sigma, Germany) at 37 °C in 5 % CO_2 humidified atmosphere.

Cell proliferation assays

Cells were plated in 96-well culture plates (COSTAR, Corning, USA) at density of 3×10^4 cells per well. The activities of samples were investigated at eight concentrations. 5-FU was used as standard compound. The cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer's procedure. The amount of cell proliferation was determined as A450 nm by using a microplate reader (Awareness Chromate, USA). Results are reported as percentage inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100 % proliferation. Percentage of inhibition of cell proliferation was calculated as follows: $[1 - (A_{\text{treatment}}/A_{\text{vehicle control}})] \times 100$.

Stock solution of tested compounds was prepared in dimethyl sulfoxide (DMSO), and diluted with DMEM. The final DMSO concentration was below 0.1 % in all tests. IC_{50} and IC_{75} values were determined using ED50 plus v1.0. The results of the in vitro investigation are presented as mean ± standard deviation (SD) of six measurements. Differences between groups were tested with analysis of variance (ANOVA) with p values <0.01 considered significant, analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.).

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