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Anticancer activities of novel Mannich bases against prostate cancer cells

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Abstract

This study was designed to synthesize hybridizing molecules starting from compound of 6-(4-phenyl-piperazin-1-yl) pyridine-3-ylamine by enhancing its biological activity with other heterocycles and to determine anticancer activity of the resulting compounds. To this end, 6-(4-phenylpiperazin-1-yl)pyridin-3-ylamine (**4**) was used as the leading compound, which is known to exert anticancer activities. The synthesis of the leading compound was carried out using 1-(5-nitropyridin-2-yl)-4-phenylpiperazine (**3**) which was obtained by a novel method with the reaction of N-phenylpiperazine (**2**) and 2-chloro-5-nitropyridine. 6-(4-phenylpiperazin-1-yl)pyridin-3-ylamine (**4**) was converted to compound 5, an active intermediate compound, by substitution of one of the amine hydrogens with ethyl bromoacetate. The resulting ester product (**5**) followed by the hydrazidation (**6**) was added arylisocyanate to obtain the active intermediate (**8**). Then, by a series of substitution through cyclization and condensation reactions, thiazolidinone (**9**), 1,3,4-oxadiazole (**7**), and 1,2,4-triazole (**10**) were synthesized. Novel Mannich bases (**11a–11f** and **12a–12f**) were obtained using oxazole (**7**) and triazole (**10**) hetero rings with primer or secondary amine compounds. The characterization of the compounds was completed using FT IR, ^{1}H-NMR, ^{13}C-NMR, HRMS spectroscopic methods and elemental analysis technique. The chemicals, then, were tested for their anticancer activities against prostate cancer cell lines PC3 [ATCC, CRL-1435], LNCaP [ATCC, CRL-1740], and DU145 [ATCC, HTB-81]. The results revealed that the Mannich bases exhibited moderate cytotoxic activity against cancer cells tested.

Keywords Antitumor activity · triazole · oxadiazole · Mannich reaction

Introduction

Prostate cancer (PC) constitutes of nearly 29% of all newly diagnosed cancer incidents and is the most common cancer type among men. In 2019, 174,650 new cases are expected and ~31,620 will die due to PC in US (Siegel et al. 2019). For clinically localized cancer situations, radiotherapy and surgery are thought to be the main approaches. However, patients with advanced and metastatic cancer states are treated with androgen deprivation therapy if the cancer is hormone-sensitive. The patients with high Gleason scores generally acquires androgen-refractory phenotype in 1–2 years; hence, combinatorial strategy using surgical removal, radiotherapy, and chemotherapy is generally used as last optio (Oudard 2013; Jarvis et al. 2018). Inefficient radiotherapy, resistance to chemotherapies and significant cytotoxic effect of the chemicals used in the treatment bring the urgent necessity for safer, more efficient, and widely available approaches.

Since the beginning of cancer therapeutics, along with enhanced biological knowledge, synthetic chemistry has had different roles in the anticancer agent discovery (Neidle and Thurston 2005). In this manner, screening of anticancer activities of newly synthesized molecules holds great potential using ex vivo cell lines. One of the best examples of this potential is the discovery of temozolomide with good oral bioavailability, uptake, and distribution properties that is a well-established anticancer drug used to treat gliomas (Friedman et al. 2000). In the new drug active substance screening studies, homologous serial design, chain

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branching, chain-ring transformations, bioisosteric alterations are generally used to identify new analogs with an augmented therapeutic effect and index compared with a leader compound. The structural components of these leading molecules, which interact with the receptors and are, therefore, responsible for their biological activities, form their pharmacophores. Once it is defined, various variations on these pharmacophores are expected to lead more effective and less toxic analogs than the leading compound (Silverman et al. 1993).

The Mannich reactions occurs in a single reaction with three components have become one of the most prominently used methods in drug developments due to allowing the synthesis of organic molecules containing different functional groups in a single step. In recent years, screening for anticancer molecules with stronger activities among Mannich bases has become popular (Roman 2015). The structures of oxadiazole and triazole derivatives containing active –SH groups allow transferring functional groups to the leading compounds that increase the biological activity as a result of aminoalkylation by the Mannich reaction (Shivarama Holla et al. 2003; Li et al. 2009). Various types of Mannich bases have been presented in the literature to be highly reactive and possess various biological roles including antimicrobial (Idhayadhulla et al. 2014), anticancer (Shivarama Holla et al. 2003), analgesic and anti-inflammatory (Gökçe et al. 2005), anticonvulsant (Dimmock et al. 1992), and antimalarial activities (Lopes et al. 2004). Mannich bases have been evaluated for their cytotoxicity against lung cancer (Malhotra et al. 2012), leukemia (Hu et al. 2012), hepatocarcinoma, and breast cancer cell lines (Kumbhare et al. 2011).

In the present study, novel Mannich bases characterized by elemental analysis, IR, ^{1}H- and ^{13}C-NMR spectral data were synthesized and tested for their anticancer activities against PC cell lines; DU145, PC3, and LNCaP.

Materials and methods

Synthesis

All chemicals were purchased from Fluka Chemie AG Buchs (Switzerland). Melting points of the synthesized compounds were determined in open capillaries on a Buchi B-540 melting point apparatus and are uncorrected. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 aluminum sheets. FT IR spectra were recorded using a Mattson 1000 FT IR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Avance II 400 MHz NMR spectrometer (chemical shift in ppm downfield from TMS as an internal reference). The elemental analysis was performed on a Leco CHNS-932 (MI, USA) elemental analyzer. The mass spectra were obtained at a LC-MS TOF (1200/6210, Agilent) by electrospray ionization.

1-(5-Nitropyridin-2-yl)-4-phenylpiperazine (3)

2-Chloro-4-nitropyridine (**2**) (0.63 g, 4 mmol) was added dropwise to *N*-phenylpiperazine (**1**) (1.62 g, 10 mmol) at 0–5 °C over 20 min. The temperature was gradually raised to 135 °C and refluxed for 4 h. The solid obtained upon adding water into the mixture was filtered off. Crystallization with EtOAc-hexane (1:1) gave a pale brown product. Yield, 60% (1.68 g); mp, 155–156 °C; **^{1}H-NMR (400 MHz, $CDCl_3$):** δ 9.06 (d, 1H, H-3, *J*: 2.7 Hz), 8.23 (dd, 1H, H-5, *J*: 9.5, 2.7 Hz), 7.32–7.27 (m, 2H), 6.96–6.90 (m, 3H), 6.61 (d, 1H *J*:9.5 Hz), 3.94 (d, 4H, $2CH_2$ *J*:5.14 Hz), 3.42 (d, 4H, $2CH_2$ *J*:5.14 Hz). **APT (^{13}C-NMR) (100 MHz, DMSO-d_6):** δ 165.30, 155.80, 151.29, 139.59, 138.08, 134.23, 124.46, 120.86, 110.92, 53.44, 49.49; **FT IR (cm^{-1}):** 3072 (ar-CH), 1593–1290 (NO_2); **Elemental analysis for** $C_{15}H_{16}N_4O_2$ calculated; C, 63.37; H, 5.67; N, 19.71; found: C, 63.28; H, 5.85; N, 19.75; **HRMS (APCI):** *m/z* calculated: $C_{15}H_{16}N_4O_2$ ($M^+ + H$): 285.32; found: 285.13.

6-(4-Phenylpiperazin-1-yl)pyridin-3-amine (4)

The solution of compound **3** in (2.85 g, 10 mmol) n-butanol in a two necked flask was refluxed in the presence of Pd/C (0.53 g, 5 mmol) in an oil bath until refluxing started. Then, hydrazine hydrate (2.5 g, 4.04 mL, 50 mmol) was added dropwise over 20 min. The mixture was refluxed for 15 h. The hot reaction mixture was filtered through the celite. The solvent was removed by evaporation. Crystallization with EtOAc-hexane (1:1) gave a dark red product. Yield, 95% (2.41 g); mp,136–137 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.64 (d, 1H, *J*: 2.7 Hz), 7.23 (t, 2H, *J*: 7.8 Hz), 7.00–6.94 (m, 3H), 6.80 (t, 1H, *J*: 7.21 Hz), 6.70 (d, 1H, *J*: 8.76 Hz), 4.59 (s, 2H, NH_2), 3.36–3.49 (m, 4H, $2CH_2$), 3.26–3.16 (m, 4H, $2CH_2$); **APT (^{13}C-NMR) (100 MHz, DMSO-d_6):** δ 151.34, 134.92, 134.72, 129.16, 126.48, 120.00, 116.39, 116.37, 108.86, 49.29, 46.91; **FT IR (cm^{-1}):** 3309 and 3201 (NH_2), 3008 (ar-CH); **Elemental analysis for** $C_{17}H_{16}O_2$ calculated: C, 70.84; H, 7.13; N, 22.03, found: C, 70.49; H, 7.27; N, 21.99; **HRMS (APCI):** *m/z* calculated: $C_{15}H_{18}N_4$ ($M^+ + H$): 255.34; found: 255.15.

Ethyl (6-(4-phenylpiperazin-1-yl)pyridin-3-yl) glycinate (5)

To the solution of the corresponding compound **4** (2.54 g, 10 mmol) in tetrahydrofuran, triethylamine (1.4 ml, 1 g, 10 mmol), and ethyl bromoacetate (1.13 ml, 1.67 g, 10 mmol) were added at 0 °C and the mixture was stirred at

60 °C for 20 h. The precipitate was removed by filtration and the resulting solution was evaporated under reduced pressure to dryness. The solid obtained recrystallized from EtOH to give the pure compound. Light yellow solid. Yield, 90% (3.03 g); mp,137 °C; **^{1}H-NMR (400 MHz, $CDCl_3$):** δ 7.75 (s, 1H), 7.28 (t, 2H, *J*: 7.8 Hz), 7.00–6.97 (m, 3H), 6.88 (t, 1H, *J*: 7.3 Hz), 6.67 (d, 1H, *J*: 8.9 Hz), 4.24 (q, 2H, OCH_2, *J*: 7.1 Hz), 3.86 (s, 2H, CH_2), 3.55–3.52 (m, 4H, $2CH_2$), 3.32–3.31 (m, 4H, $2CH_2$), 1.29 (t, 3H, CH_3, *J*: 7.1 Hz); **APT (^{13}C-NMR)(100 MHz, $CDCl_3$):** δ 171,34, 154.54, 151.60, 136.59, 133.61, 129.36, 124.70, 120.16, 116.57, 109.04, 61.68, 49.49, 46.96, 45.19, 14.42; **FT IR (cm^{-1}):** 3386 (NH), 2977 (ar-CH), 1724 (C=O); **Elemental analysis for** $C_{19}H_{24}N_4O_2$ calculated: C, 67.04; H, 7.11; N, 16.46; found: C, 67.19; H, 7.07; N, 16.14; **HRMS (APCI):** *m/z* calculated $C_{19}H_{24}N_4O_2$ ($M^+ + H$): 341.43; found: 341.19.

2-((6-(4-Phenylpiperazin-1-yl)pyridin-3-yl)amino) acetohydrazide (6)

A solution of compound **5** (3.4 g, 10 mmol) in ethanol was refluxed with hydrazine hydrate (25 mmol, 1.21 ml, 1.25 g, 25 mmol l) for 13 h. The resulting solution was evaporated under reduced pressure to dryness. The solid recrystallized from EtOAc to give the pure compound. Light yellow solid. Yield, 88% (2.86 g); mp,154–155 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 9.11 (s, NH), 7.60 (d, 1H, *J*: 2.4 Hz), 7.23 (t, 2H, *J*: 7.7 Hz), 7.00–6.96 (m, 4H + NH), 6.79 (dd, 1H, *J*: 14.8, 8.0 Hz), 5.48 (s, 1H, NH_2), 3.60 (d, 2H, CH_2, *J*: 5.6 Hz), 3.44–3.39 (m, 4H, $2CH_2 + H_2O$), 3.22 (bs, 4H, $2CH_2$); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 169.90, 153.02, 151.59, 138.13, 132.34, 129.41, 123.71, 119.51, 116.14, 109.14, 54.39, 48.71, 46.86; **FT IR (cm^{-1}):** 3390, 3334, 3302 (NH_2–NH), 2834 (ar-CH), 1728 (C=O); **Elemental analysis for** $C_{17}H_{22}N_6O$ calculated: C, 62.56; H, 6.79; N, 25.75; found: C, 62.19; H, 6.68; N, 25.72; **HRMS (APCI):** *m/z* $C_{17}H_{22}N_6O$ calculated ($M^+ + H$): 327.40; found: 327.19.

5-(((6-(4-Phenylpiperazin-1-yl)pyridin-3-yl)amino) methyl)-1,3,4-oxadiazole-2(3*H*)-thione (7)

CS_2 (1.21 ml, 1.52 g, 20 mmol) was added to the solution of compound **6** (3.26 g, 10 mmol) in ethanol-water (1:1) and the mixture was refluxed in the presence of KOH (0.56 g, 10 mmol) for 12 h. Then, the resulting solution was cooled to room temperature and acidified to pH 4 with CH_3COOH. The solid precipitate was collected by filtration and recrystallized from EtOAc to give the pure compound. White solid. Yield, 66% (2.41 g); mp, 131–132 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 9.37 (s, 1H, NH), 7.69 (s, 1H), 7.23 (t, 2H, *J*: 7.5 Hz), 7.09 (d, 1H, *J*: 7.5 Hz), 6.99 (d, 2H, *J*: 8.0 Hz), 6.83–6.78 (m, 2H), 5.96 (s, 1H, NH), 4.39 (bs, 2H, CH_2), 3.42 (bs, 4H, $2CH_2$), 3.22 (bs, 4H, $2CH_2$); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 177.88, 162.24, 151.93, 150.91, 136.57, 136.38, 128.93, 125.13, 119.10, 115.66, 109.57, 48.08, 46.05, 38.38; **FT IR (cm^{-1}):** 3063 (ar-CH), 1228 (C=S); **Elemental analysis for** $C_{18}H_{20}N_6OS$ calculated: C, 58.68; H, 5.47; N, 22.81; S, 8.70 found: C, 58.63; H, 5.45; N, 22.84; S, 8.69; **HRMS (APCI):** *m/z* $C_{18}H_{20}N_6OS$ calculated ($M^+ + H$): 369.46; found: 369.15.

N-phenyl-2-((6-(4-phenylpiperazin-1-yl)pyridin-3-yl) glycyl)hydrazine-1-carbothioamide (8)

Phenylisothiocyanate (10 mmol) was added to the solution of compound **6** (10 mmol) in dried DCM dropwise and the mixture was refluxed for 8 h. The solid precipitate was collected by filtration and recrystallized from EtOAc to give the pure compound. Dark pink solid product. Yield 85% (2.35 g); mp, 197 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 10.11 (s, 1H, NH), 9.67 (bs, 1H, NH), 9.53 (bs, 1H, NH), 7.67 (s,1H), 7.44–7.42 (bs, 2H), 7.35 (t, 2H, *J*: 7.4 Hz), 7.26–7.18 (m, 3H), 6.99–7.06 (m, 3H), 6.80 (t, 2H, *J*: 9.7 Hz), 5.51 (s,1H, NH), 3.80 (bs, 2H, CH_2), 3.41 (bs, 4H, $2CH_2$), 3.23 (bs, 4H, $2CH_2$); **APT (^{13}C-NMR) (100 MHz, DMSO-d_6):** δ 160.43, 158.94, 153.24, 151.57, 139.21, 137.39, 132.62, 129.41, 123.91, 122.17, 119.52, 117.36, 116.15, 109.13, 48.71, 46.70, 38.81; **FT IR (cm^{-1}):** 3336, 3243 (4NH), 3083 (ar-CH), 1693 (C=O), 1230 (C=S); **Elemental analysis for** $C_{24}H_{27}N_7OS$ calculated: C, 62.45; H, 5.90; N, 21.24; S, 6.95 found: C, 62.39; H, 5.60; N, 21.29; S, 6.61; **HRMS (APCI):** *m/z* $C_{24}H_{27}N_7OS$ calculated ($M^+ + H$): 462.59; found: 462.20.

N′-(5-oxo-3-phenylthiazolidin-2-ylidene)-2-((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino) acetohydrazide (9)

A mixture of compound **8** and ethyl bromoacetate (10 mmol) in absolute ethanol was allowed to reflux in the presence of dried sodium acetate (50 mmol) for 15 h. The reaction mixture was then cooled to room temperature. After the solvent was removed under reduced pressure and added water, a solid appeared. This crude product was recrystallized from EtOAc to give the pure compound. Light Brown solid product. Yield 99% (4.95 g); mp, 79–80 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 10.83 (s, 1H, NH),7.70 (s, 1H), 7.37 (t, 2H, *J*: 7.6 Hz), 7.24 (t, 2H, *J*: 7.6 Hz), 7.14 (t, 1H, *J*: 7.2 Hz), 7.04 (dd, 1H, *J*: 8.8, 2.3 Hz), 6.99 (d, 2H, *J*: 8.4 Hz), 6.92 (d, 2H, *J*: 7.6 Hz), 6.80 (t, 1H, *J*: 6.0 Hz), 6.72 (d, 1H, *J*: 5.3 Hz), 4.15 (s, 2H, CH_2), 3.87 (d, 2H, CH_2, *J*: 6.0 Hz), 3.41–3.46 (m, 4H, $2CH_2 + H_2O$), 3.22–3.23 (m, 4H, $2CH_2$); **APT (^{13}C-NMR)(100**

MHz, DMSO-d_6): δ 169.85, 168.80, 153.13, 152.03, 151.58, 147.78, 137.97, 132.62, 129.78, 129.41, 124.95, 123.98, 121.29, 119.52, 116.15, 108.97, 56.51, 48.69, 46.82, 30.35; **FT IR (cm^{-1}):** 3278 (2NH), 2831 (ar-CH), 1759, 1733 (C=O), 1598 (N=C); **Elemental analysis for** $C_{26}H_{27}N_7O_2S$ calculated: C, 62.26; H, 5.43; N, 19.55; S, 6.39 found: C, 62.10; H, 5.75; N, 19.29; S, 6.23; **HRMS (APCI):** *m/z* calculated $C_{26}H_{27}N_7O_2S$ ($M^+ + H$): 502.61; found: 502.20.

4-Phenyl-5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl) amino)methyl)-4*H*-1,2,4-triazole-3-thiol (10)

The solution of the corresponding compound **8** (4.61 g, 10 mmol) in ethanol:water (1:1) was refluxed in the presence 100 mL EtOH:H_2O (1:1) of 2% NaOH for 3 h. Then the resulting solution was cooled to room temperature and acidified to pH 4 with 37% HCl (0.8 mL). The precipitate formed was filtered off, washed with water, and recrystallized from EtOH:H_2O (1:1) to afford the desired compound. White solid. Yield, 98% (3.32 g); mp, 155–156 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 13.85 (s, 1H, SH), 7.58–7.52 (m, 5H), 7.45 (d, 2H, *J*: 7.6 Hz), 7.31 (t, 1H NH, *J*: 7.6 Hz), 7.23 (t, 2H, *J*: 7.2 Hz), 6.99 (d, 2H, *J*: 8.4 Hz), 6.93 (d, 1H, *J*: 9.2 Hz), 6.80 (t, 1H, *J*:7.2 Hz), 6.74 (d, 1H, *J*: 8.8 Hz), 5.49 (s,1H, NH), 4.10 (s, 2H, CH_2), 3.39–3.40 (m, 4H, $2CH_2 + H_2O$), 3.21 (s, 4H, $2CH_2$); **^{13}C-NMR (100 MHz, DMSO-d_6):** δ 156.17, 151.45, 150.73, 141.68, 137.31, 133.95, 129.92, 129.82, 129.49, 128.58, 121.49, 119.59, 117.26, 116.16, 109.79, 48.58, 46.65, 39.69; **FT IR (cm^{-1}):** 3224 (NH), 3023 (ar-CH), 2102 (SH); **Elemental analysis for** $C_{24}H_{25}N_7S$ calculated: C, 64.99; H, 5.68; N, 22.10; S, 7.23 found: C, 64.75; H, 5.87; N, 22.10; S, 7.01; **HRMS (APCI):** *m/z* $C_{24}H_{25}N_7S$ calculated ($M^+ + H$): 444.57; found: 444.19.

General method for the synthesis of compounds 11a–11f, 12a–12f

To a solution of corresponding compound **7** (for **11a–11f**) and **10** (for **12a–12f**) (10 mmol) in dimethyl formamide containing $InCl_3$ (%10 mmol), 6-aminopenicilanic acid (6-apa) (10 mmol) (for **11a** and **12a**) (10 mmol), 7-aminocefalociporanic acid (7-aca) (for **11b** and **12b**) (10 mmol), ciprofloxacin (for **11c** and **12c**) (10 mmol), norfloxacin (for **11d** and **12d**) (10 mmol), 4-aminobenzylpiperidine (10 mmol) (for **11e** and **12e**), 1-Methylpiperazine (for **11f** and **12f**) (10 mmol) were added, and the mixture were stirred at room temperature in the presence of formaldehyde (%37, 30 mmol) for 24 h. The solid precipitate was collected by filtration, washed with water, and recrystallized from dimethylsulfoxide:water (1:1) to give the desired compound.

3,3-Dimethyl-7-oxo-6-(((5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-2-thioxo-1,3,4-oxadiazol-3(2*H*)-yl)methyl)amino)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (11a)

Light yellow solid. Yield, 50% (2.98 g); mp, 194–195°C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.91 (s, 1H, NH), 7.41 (s, 1H, NH), 7.23 (bs, 3H), 7.00–6.80 (m, ar-H, 5H), 5.51–5.46 (bs, 1H, CH), 5.29 (bs, 1H, CH), 4.40 (bs, 1H, CH), 4.22 (bs, 2H, CH_2), 3.68 (bs, 2H, CH_2), 3.54 (bs, 4H, $2CH_2$),3.23 (bs, 4H, $2CH_2$),1.57–1.45(m, 6H, $2CH_3$); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 175.30, 170.83, 162.58, 157.62, 155.81, 151.59, 137.95, 135.48, 129.71, 129.43, 119.60, 116.05, 108.69, 71.37, 64.21, 63.70, 59.80, 54.99, 48.63, 45.73, 44.98, 28.03, 27.58; **FT IR (cm^{-1}):** 3386 (NH + OH), 2973 (ar-CH), 1770 (2C=O); **Elemental analysis for** $C_{27}H_{32}N_8O_4S_2$ calculated: C, 54.35; H, 5.41; N, 18.78; S, 10.75; found: C, 54.37; H, 5.56; N, 18.74; S, 10.53; **HRMS (APCI):** *m/z* calculated $C_{27}H_{32}N_8O_4S_2$ (M^+): 595.73; found: 595.37.

3-(Acetoxymethyl)-8-oxo-7-(((5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-2-thioxo-1,3,4-oxadiazol-3(2H)-yl)methyl)amino)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (11b)

Light brown solid. Yield, 60% (3.91 g), mp, 198 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.73 (s, 1H, NH), 7.25–7.21 (m, 2H), 7.13 (d, 1H, NH *J*: 7.2 Hz), 7.01–6.92 (m, 4H), 6.79 (d, 2H, *J*: 8.4 Hz), 4.94 (s, 3H, $CH_2 + CH$), 4.67 (d, 1H, $J = 10.0$ Hz), 3.98 (s, 2H, CH_2), 3.65 (s, 2H, CH_2), 3.48 (s, 4H, $2CH_2$), 3.24 (s, 6H, $3CH_2$), 2.03 (s, 3H, CH_3); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 172.49, 170.69, 166.29, 157.95, 153.87, 151.53, 137.99, 135.60, 132.19, 128.89, 124.48, 123.55, 119.58, 116.39, 109.11, 63.41, 63.16, 62.83, 59.32, 50.86, 48.67, 46.52, 45.22, 21.50, 20.50; **FT IR (cm^{-1}):** 3386 (2NH + OH), 2973 (ar-CH), 1738 (2C=O); **Elemental analysis for** $C_{29}H_{32}N_8O_6S_2$ calculated: C, 53.36; H, 4.94; N, 17.17; S, 9.82; found: C, 53.67; H, 4.67; N, 17.40; S, 9.43; **HRMS (APCI):** *m/z* calculated $C_{29}H_{32}N_8O_6S_2$ (M^+): 652.75; found: 652.31.

Cyclopropyl-6-fluoro-4-oxo-7-(4-((5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-2-thioxo-1,3,4-oxadiazol-3(2*H*)-yl)methyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (11c)

Light yellow solid. Yield, 37% (2.63 g); mp, 238 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 8.65 (s, 1H, OH),7.72 (s, 1H), 7.23–7.21 (m, 4H), 7.12 (d, 1H, NH *J*: 8.4 Hz), 7.00–6.91 (m, 5H), 6.78–6.80 (m, 2H), 4.93 (s, 2H, CH_2), 3.97 (bs, 3H, $CH_2 + CH$), 3.46 (bs, 8H, $4CH_2$), 3.23 (bs,

8H, $4CH_2$), 2.88 (bs, 2H, CH_2), 1.34 (bs, 2H, CH_2); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 181.32, 176.68, 174.45, 166.59, 158.08, 153.40, 148.43, 145.59 and 151.51 (C–F *J*: 592 Hz), 139.50, 135.91, 135.44, 129.43, 124.14, 119.56, 116.19, 112.68, 111.62, 108.25, 107.30, 106.89, 69.58, 49.68, 48.56, 46.55, 45.70, 44.97, 30.89, 8.03; **FT IR (cm^{-1}):** 3326 (OH + NH), 3057 (ar-CH), 1689 (3C=O); **Elemental analysis for** $C_{36}H_{38}FN_9O_4S$ calculated: C, 60.75; H, 5.38; N, 17.71; S, 4.50 found: C, 60.72; H, 5.18; N, 17.42; S, 4.63; **HRMS (APCI):** *m/z* calculated $C_{36}H_{38}FN_9O_4S$ (M^+): 711.86; found ([$M^+ + H + Na$]): 735.27.

1-Ethyl-6-fluoro-4-oxo-7-(4-((5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-2-thioxo-1,3,4-oxadiazol-3(2H)-yl)methyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (11d)

Dark brown solid. Yield, 40% (2.80 g); mp, degraded at 178 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 8.93 (s, 1H, OH),7.71 (s, 1H), 7.22 (bs, 3H), 7.11 (d, 1H, NH *J*: 6.8 Hz), 6.99–6.90 (m, 5H), 6.80–6.78 (m, 2H), 4.93 (s, 2H, CH_2), 4.66–4.56 (m, 2H, CH_2), 3.96 (s, 2H, CH_2), 3.46 (bs, 8H, $4CH_2$), 3.22 (bs, 8H, $4CH_2$), 1.34–1.41 (m, 3H, CH_3); **APT (^{13}C-NMR)(100 MHz, $CDCl_3$):** δ 176.63, 166.54, 166.26, 153.87, 151.56, 149.58 and 145.81 (d *J*: 377 Hz), 148.91, 137.96, 137.67, 136.64, 135.81, 135.60, 131.98, 129.42, 123.29, 119.40, 116.04, 111.60, 108.89, 107.55, 106.27, 63.70, 50.87, 50.28, 50.13, 49.53, 48.54, 46.52, 14.81; **FT IR (cm^{-1}):** 3351 (OH + NH), 3054 (ar-CH), 1708 (3C=O); **Elemental analysis for** $C_{35}H_{38}FN_9O_4S$ calculated: C, 60.07; H, 5.47; N, 18.01; S, 4.58; found: C, 60.09; H, 5.50; N, 18.08; S, 4.58; **HRMS (APCI):** *m/z* calculated $C_{35}H_{38}FN_9O_4S$ ([$M^+ + H$]): 700.81; found: 700.27.

3-(((1-Benzylpiperidin-4-yl)amino)methyl)-5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-1,3,4-oxadiazole-2(3*H*)-thione (11e)

Recrystallized from EtOH. Yield, 35% (1.99 g); mp, 203 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.93 (d, 1H, *J*: 2.6 Hz), 7.42 (dd, ar-H + 2NH, *J*: 9.1, 2.6 Hz), 7.32–7.21 (m, 8H), 6.99 (d, 2H, *J*: 8.2 Hz), 6.89 (d, 1H, *J*: 9.2 Hz), 6.80 (t, 1H, *J*: 7.2 Hz), 5.25 (d, 2H, CH_2, *J*: 16.8 Hz), 4.14 (d, 2H, CH_2, *J*: 14.4 Hz), 3.53 (bs, 4H, $2CH_2$), 3.33 (s, 8H, $4CH_2$), 3.21 (d, 4H, $2CH_2$, *J*: 4.4 Hz), 2.86 (d, 2H, CH_2, *J*: 11.2 Hz); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 174.81, 162.84, 155.65, 151.51, 138.77, 137.91, 135.50, 129.76, 129.42, 129.27, 128.61, 128.38, 119.60, 116.19, 108.45, 63.60, 62.17, 53.61, 52.90, 52.42, 48.58, 45.76, 28.25; **FT IR (cm^{-1}):** 3100 (NH), 3046 (ar-CH), 1735 (C=O); **Elemental analysis for** $C_{31}H_{38}N_8OS$ calculated: C, 65.24; H, 6.71; N, 19.63; S, 5.62; found: C, 65.45; H, 6.82; N, 19.92; S, 5.42; **HRMS (APCI):** *m/z* calculated $C_{31}H_{38}N_8OS$ ($M^+ + H$): 571.76; found: 571.28.

3-((4-Methylpiperazin-1-yl)methyl)-5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-1,3,4-oxadiazole-2(3*H*)-thione (11f)

Recrystallized from EtOH. Brown solid. Yield, 99% (4.75 g); mp, 275 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.73–7.71 (m, 1H, NH), 7.23–7.22 (m, 3H), 6.98–6.93 (m, 3H), 6.80 (bs, 2H), 3.48 (bs, 4H, $2CH_2$), 3.40–3.36 (m, 10H, $5CH_2$), 3.23 (bs, 6H, $3CH_2$), 2.50 (s, CH_3); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 166.33, 153.84, 153.16, 151.56, 135.51, 131.98, 129.24, 123.18, 119.57, 116.14, 109.10, 63.56, 54.70, 50.88, 48.67, 46.70, 44.98, 14.56; **FT IR (cm^{-1}):** 3343 (NH), 3058 (ar-CH), 1772 (C=O); **Elemental analysis for** $C_{24}H_{32}N_8OS$ calculated: C, 59.98; H, 6.71; N, 23.31; S, 6.67 found: C, 59.85; H, 6.55; N, 23.51; S, 6.96; **HRMS (APCI):** *m/z* calculated $C_{24}H_{32}N_8OS$ ($M^+ + H$): 481.64; found: 481.25.

3,3-Dimethyl-7-oxo-6-(((4-phenyl-3-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-1-yl)methyl)amino)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (12a)

Light yellow solid. Yield, 81% (5.43 g); mp, 180 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 9.88 (bs, 1H, OH), 7.91 (bs, 1H, NH), 7.55–7.21 (m, 8H), 6.96–6.79 (m, 5H), 5.45–5.31 (m, 1H), 5.05 (bs, 1H), 4.73 (s, 1H), 4.32–4.26 (m, 2H, CH_2), 4.10 (s, 2H, CH_2), 3.39 (s, 4H, $2CH_2 + H_2O$), 3.20 (s, 4H, $2CH_2$), 1.53–1.41 (m, 6H, $2CH_3$); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 175.68, 172.09, 156.02, 151.57, 148.66, 141.77, 137.07, 134.13, 129.71, 128.97, 128.13, 126.99, 121.40, 119.57, 117.40, 116.14, 109.31, 107.74, 68.45, 67.26, 67.14, 63.34, 48.68, 46.74, 45.90, 27.08, 26.47; **FT IR (cm^{-1}):** 3343 (NH + OH), 2919 (ar-CH), 1762 (2C=O); **Elemental analysis for** $C_{33}H_{37}N_9O_3S_2$ calculated: C, 59.00; H, 5.55; N, 18.76; S, 9.54; found: C, 59.04; H, 5.62; N, 18.17; S, 9.64; **HRMS (APCI):** *m/z* calculated $C_{33}H_{37}N_9O_3S_2$ ($M^+ + H$): 672.84; found: 672.23.

3-(Acetoxymethyl)-8-oxo-7-(((4-phenyl-3-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-1-yl)methyl)amino)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (12b)

Light yellow solid. Yield, 72% (4.83 g); mp, 160 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 13.84 (s, 1H, OH), 9.85 (s, 1H, NH), 7.56 (d, 3H, *J*: 7.7 Hz), 7.46 (d, 2H, *J*: 4.9 Hz),

7.30 (t, 1H, NH, *J*: 7.0 Hz), 7.22 (d, 3H, *J*:6.4 Hz), 6.97 (d, 3H, *J*: 7.1 Hz), 6.73–6.80 (m, 2H), 5.45 (bs, 1H, CH), 2.03 (s, CH_3), 4.86–5.10 (m, 2H, CH_2), 4.69 (d, 2H, CH_2, *J*: 13.0 Hz), 4.11 (d, 2H, CH_2, *J*: 20.0 Hz), 3.94 (s, 1H), 3.39 (6H, $3CH_2 + H_2O$), 3.21 (s, 4H, $2CH_2$); **APT (^{13}C-NMR) (100 MHz, DMSO-d_6):** δ 170.60, 168.82, 163.46, 156.30, 153.16, 151.17, 150.94, 141.72, 137.28, 134.14, 130.11, 129.69, 128.34, 126.76, 124.12, 121.40, 119.57, 117.18, 116.04, 108.90, 68.42, 63.18, 58.34, 48.69, 46.70, 46.05, 31.17, 26.38, 21.04; **FT IR (cm^{-1}):** 3316 (OH + NH), 2919 (ar-CH), 1758 (C=O); **Elemental analysis for** $C_{35}H_{37}N_9O_5S_2$ calculated: C, 57.76; H, 5.12; N, 17.32; S, 8.81; found: C, 57.42; H, 5.08; N, 17.53; S, 8.71; **HRMS (APCI):** *m/z* calculated $C_{35}H_{37}N_9O_5S_2$ ($M^+ + H$): 728.86; found: 728.23.

1-Cyclopropyl-6-fluoro-4-oxo-7-(4-((4-phenyl-3-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-1-yl)methyl) piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (12c)

Light yellow solid. Yield, 97% (7.61 g); mp, 192–193 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 15.18 (s, 1H, OH), 9.83 (s, 1H, NH), 8.67–8.50 (m, 2H), 7.91–7.89 (m, 1H), 7.56–7.47 (m, 7H), 7.20–7.14 (m, 2H), 6.93 (bs, 1H), 6.79–6.64 (m, 3H), 5.19–5.23 (m, 4H, CH_2), 4.14–4.20 (m, 1H), 3.82 (bs, 2H, CH_2), 2.91–3.32 (m, $12H + 6CH_2$), 2.94 (d, 4H, $2CH_2$, *J*: 26.4 Hz), 1.10–1.32 (m, 4H, $2CH_2$); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 204.40, 176.84, 169.67, 166.33, 154.52, 153.17, 151.80 and 145.69 (d, C–F, *J*: 611 Hz), 151.36, 149.86, 148.23, 139.58, 137.08, 134.41, 133.26, 129.89, 129.42, 128.42, 121.48, 119.55, 117.25, 115.81, 111,52, 107.32, 106.91, 68.73, 51.10, 49.93, 48.47, 46.54, 45.89, 36.24, 8.04; **FT IR (cm^{-1}):** 3440 (OH + NH),3062 (ar-CH), 1716 (C=O); **Elemental analysis for** $C_{42}H_{43}FN_{10}O_3S$ calculated: C, 64.10; H, 5.51; N, 17.80; S, 4.07 found: C, 64.26; H, 5.38; N, 17.75; S, 4.05; **HRMS (APCI):** *m/z* calculated $C_{42}H_{43}FN_{10}O_3S$ ($M^+ + H$): 787.93; found: 787.31.

1-Ethyl-6-fluoro-4-oxo-7-(4-((4-phenyl-3-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-1-yl)methyl) piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (12d)

Light gray solid. Yield, 78% (6.03 g); mp, 220 °C; **^{1}H-NMR (400 MHz, CDMSO-d_6):** δ 8.90 (s, 1H, OH), 7.56–7.54 (m, 6H), 7.45–7.43 (m, 1H), 7.32–7.23 (m, 1H, NH), 7.23–7.13 (m, 4H), 6.99–6.90 (m, 2H), 6.78–6.63 (m, 3H), 5.18 (bs, 2H, CH_2), 4.56 (s, 2H, CH_2), 4.18 (s, 2H, CH_2), 3.34 (bs, 4H, $2CH_2 + H_2O$), 3.21 (s, 4H, $2CH_2$), 2.96–2.89 (m, 8H, $4CH_2$), 1.39 (t, 3H, CH_3, *J*: 6.8 Hz); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 176.65, 169.59, 167.13, 166.60, 154.61, 153.12, 152.34, 149.53 and 146.16 (d, C–F, *J*: 337 Hz), 141.66, 137.01, 134.41, 129.89, 129.43, 128.39, 123.90, 121.50, 119.77, 119.53, 117.39, 116.14, 115.71, 111,57, 108.58, 107.64, 106.34, 68.54, 50.00, 49.56, 48.68, 48.48, 46.58, 46.05, 14.82; **FT IR (cm^{-1}):** 3370 (OH + NH), 2977 (ar-CH), 1712 (C=O); **Elemental analysis for** $C_{41}H_{43}FN_{10}O_3S$ calculated: C, 63.55; H, 5.59; N, 18.08; S, 4.14; found: C, 63.13; H, 5.43; N, 18.02; S, 4.36. **HRMS (APCI):** *m/z* calculated $C_{41}H_{43}FN_{10}O_3S$ ($M^+ + H$): 775.32; found: 775.31.

2-(((1-Benzylpiperidin-4-yl)amino)methyl)-4-phenyl-5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino) methyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (12e)

Recrystallized from CH_2Cl_2:Hexane, provided a light purple solid product. Yield, 95% (6.13); mp, 137 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.55–7.22 (m,12H, ar-CH + NH), 6.95–6.80 (m, 6H), 5.44 (bs, 2H, CH_2), 4.14–4.11 (m, 2H, CH_2), 3.45 (s, 7H, $3CH_2 + CH$), 3.34 (s, 8H, $4CH_2$), 3.17 (s, 4H, $2CH_2$); **APT (^{13}C-NMR) (100 MHz, DMSO-d_6):** δ 168.71, 155.38, 153.20, 151.54, 147.78, 141.27, 139.06, 136.35, 131.50, 129.80, 129.41, 129.07, 128.47, 127.22, 124.17,119.59, 116.13, 108.68, 107.76, 62.36, 58.22,53.26, 51.92, 48.66, 46.63, 45.47, 31.22; **FT IR (cm^{-1}):** 3347 (NH), 3027 (ar-CH), 1230 (C=S); **Elemental analysis for** $C_{37}H_{43}N_9S$ calculated: C, 68.81; H, 6.71; N, 19.52; S, 4.96; found: C, 68.85; H, 6.74; N, 19.09; S, 4.90; **HRMS (APCI):** *m/z* calculated $C_{37}H_{43}N_9S$ (M^+): 645.87; found: ($M^+ + K + H_2O$): 702.36.

2-((4-Methylpiperazin-1-yl)methyl)-4-phenyl-5-(((6-(4-phenylpiperazin-1-yl)pyridin-3-yl)amino)methyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (12f)

Recrystallized from EtOAc, provided a light green solid product. Yield, 68% (3.77); mp, 94–95 °C; **^{1}H-NMR (400 MHz, DMSO-d_6):** δ 7.57–7.54 (m, 5H, ar-CH + NH), 7.45 (m, 2H), 7.23 (t, 2H, *J*: 7.6 Hz), 6.98 (d, 2H, *J*: 8.2), 6.89 (d, 1H, *J*: 8.8 Hz), 6.80 (t, 1H, *J*: 7.3 Hz), 6.71 (d, 1H, *J*: 8.9 Hz), 5.03 (s, 2H, CH_2), 4.14 (d, 2H, CH_2, *J*: 6.0 Hz), 3.36 (bs, 4H, $2CH_2 + H_2O$), 3.21 (s, 4H, $2CH_2$), 2.68 (s, 4CH, $2CH_2$), 2.8 (s, 4H, CH_2), 2.12 (s, 3H, CH_3); **APT (^{13}C-NMR)(100 MHz, DMSO-d_6):** δ 169.50, 153.25, 151.56, 149.35, 137.16, 134.43, 132.95, 130.03, 129.89, 129.42, 128.48, 123.99, 119.53, 116.14, 108.90, 69.04, 55.01, 51.34, 50.14, 48.69, 46.68, 46.25; **FT IR (cm^{-1}):** 3387 (NH), 3027 (ar-CH), 1226 (C=S); **Elemental analysis for** $C_{30}H_{37}N_9S$ calculated: C, 64.84; H, 6.71; N, 22.68; S, 5.77; found: C, 64.82; H, 6.14; N, 22.74; S, 5.38; **HRMS**

(APCI): *m/z* calculated $C_{30}H_{37}N_9S$ $(M^+ + H)$: 556.75; found: 556.29.

Anticancer activity

Cell lines and proliferation assay

Human PC cell lines, PC3 (CRL-1435), DU145 (HTB-81), and LNCaP (CRL-1740), were purchased from ATCC (Rockville, MD, USA). All cell lines were cultured in Dulbecco's Modified Eagle Media (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin-amphotericin (Invitrogen) in a humidified incubator at 37 °C and 5% CO_2. Anticancer activity of newly synthesized hybrid molecules against PC cell lines were analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and NCI60 response parameters was performed after 24 h incubation period. The results are given as % cell inhibition, and optic density for the cells treated with solvent only (DMSO) was accepted as 100%. Response parameters, IC50 (50% cell viability inhibition), GI50 (50% growth inhibition), TGI (total growth inhibition), and LC50 (50% cell death) were evaluated based on National Institutes of Health definitions using logarithmic functions (González-Vallinas et al. 2013).

Results and discussion

Chemistry

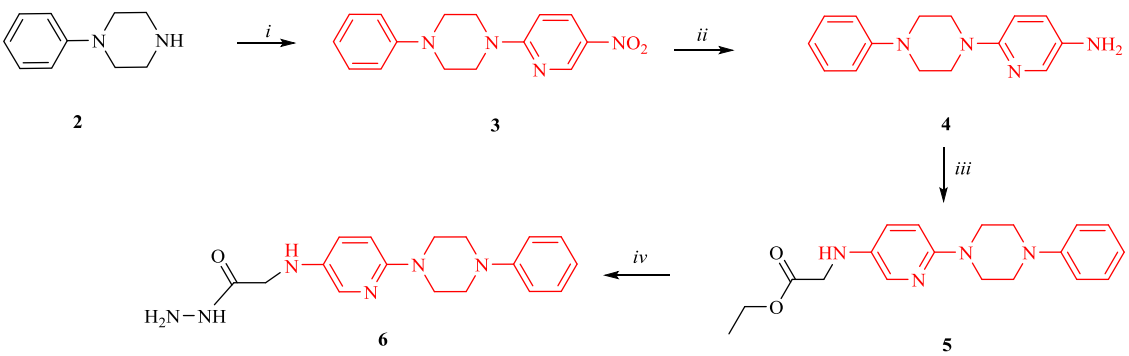
To obtain the commercially available 6-(4-phenyl-piperazin-1-yl)pyridine-3-ylamine (**4**) compound, 1-(5-nitropyridin-2-yl)-4-phenylpiperazine (**3**) was firstly synthesized. While the synthesis procedure for compound (**3**) was described in the literature, it was not possible to obtain the chemical by following the same protocol.

We showed that the most appropriate method to synthesize the compound (**3**) was in solvent-free environment in an oil bath. Then, synthesis of the leading compound (**4**) of the study was carried out by reduction of the compound (**3**) with hydrazine hydrate in the presence of catalytic Pd/C. While the molecule is known in the literature and commercially available, synthesis methodology, and spectroscopic data were presented in this work for the first time (Scheme 1).

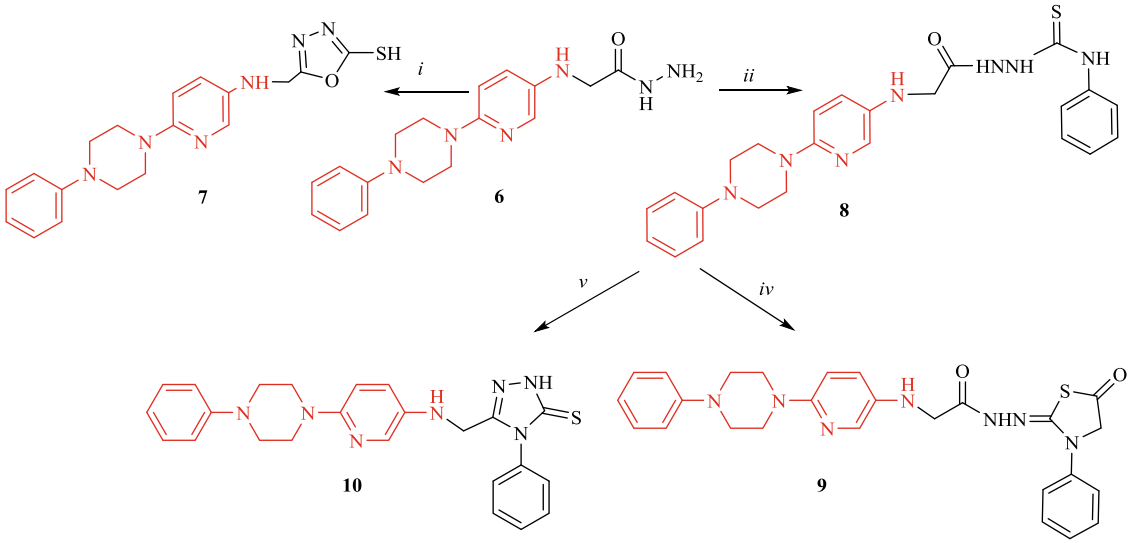
The synthesis of the hydrazide compound (**6**) was performed in two steps starting from the amine (**4**) compound. This compound (**6**) was then treated with CS_2 in the presence of KOH to be converted to 1,3,4-oxadiazole (**7**) derivative as a result of cyclo-condensation reaction (Scheme 2). Carbothioamide (**8**) was synthesized with the reaction of hydrazide compound (**6**) and isothiocyanate. The compound (**9**), which is a 4-oxo-1,3-thiazolidine derivative, was obtained from the treatment of the carbothioamide compound (**8**) with ethyl bromoacetate.

Elimination of signals in the compound (**9**) for –NH group that normally appears in 3336, 3243 cm^{-1} in FT IR spectra and 10.11–9.53 ppm in ^{1}H-NMR spectra of the compound (**8**), and observation of peaks for the C-2, C-4 and C-5 atoms of the 4-oxo-1,3-thiazolidine ring at the corresponding chemical shift values in ^{13}C-NMR are confirmations for the cyclo-condensation reaction. The chemical shift values for the compounds are in line with the previously published data (Küçükgüzel et al. 1999; Hubschwerlen et al. 2003; Skjemstad et al. 2004; Yolal et al. 2012). Resonance of the C-5 protons of the thiazolidinone ring at the aliphatic region in ^{1}H-NMR and ^{13}C-NMR as expected is a further proof for the reaction (Scheme 2).

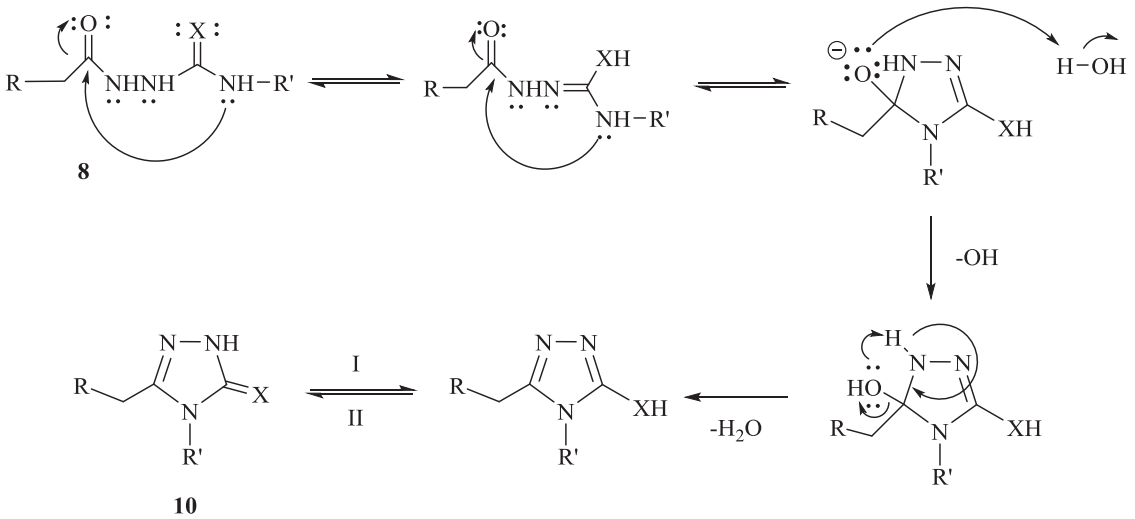
The 1,2,4-triazole compound (**10**) was obtained as a result of the intramolecular ring closure of the corresponding carbothioamide derivative (**8**) in the presence of base. As shown in Scheme 3, a tautomeric equilibrium is known to occur in the 1,2,4-triazole (**10**) compounds (Küçükgüzel



Scheme 1 Reaction and conditions for the synthesis of compounds 3–6. *(i)* 2-Chloro-4-nitropyridine in acetonitrile, 4 h, ruflux; *(ii)* $H_2NNH_2.H_2O$ in But-OH, Pd/C, 15 h, ruflux; *(iii)* $BrCH_2CO_2Et$ in THF, TEA, 60 °C, 20 h; *(iv)* $H_2NNH_2.H_2O$ in EtOH, reflux, 13 h



Scheme 2 *(i)* CS_2 in EtOH-H_2O, KOH, reflux, 12 h; *(ii)*: C_6H_5NCS, EtOH, reflux, 18 h; *(iv)* ethyl bromoacetate, dry CH_3COONa, absolute EtOH, ruflux, 18 h; *(v)*: NaOH, in EtOH:H_2O (1:1), reflux, 3 h



Scheme 3 Mechanism of 1,2,4-triazole

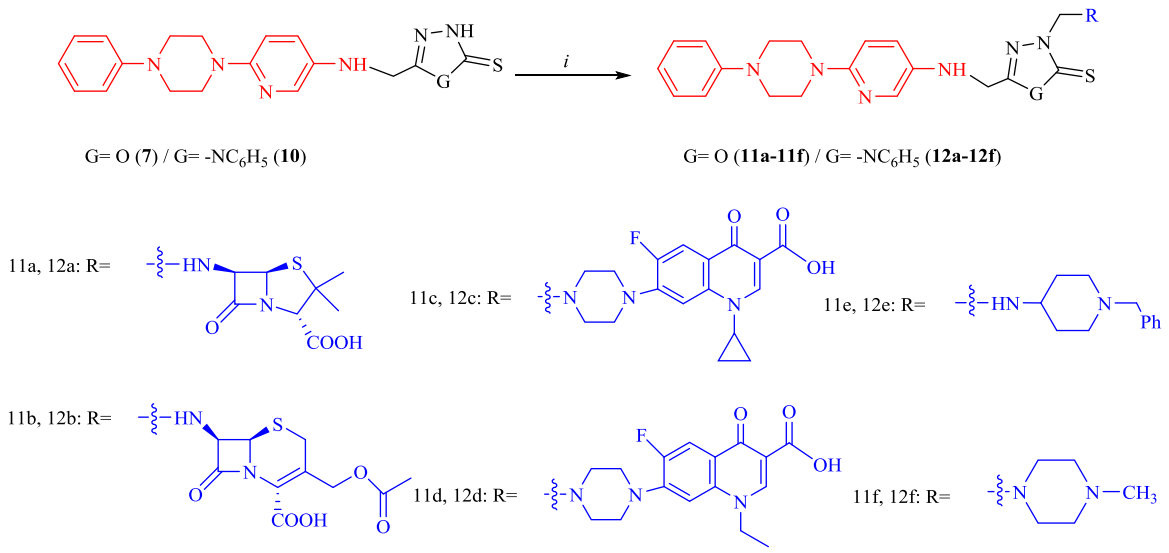
et al. 1999; Demirbas et al. 2004; Holla et al. 2005; Barbuceanu et al. 2009).

When X is the sulfur atom, the equilibrium is more favorable in the form of I, and when X is the oxygen atom, the equilibrium is shifted in favor of the II form. For the compound (**10**) synthesized in this study, the dominant tautomeric form is the mercapto form represented by I, as evidenced by FT IR and ^{1}H-NMR data. In the FT IR spectra of this compound (**10**), the signal from the voltage of the S–H bond is seen at 2102 cm^{-1}, while the –SH proton is resonant at 13.85 ppm in the ^{1}H-NMR spectra.

As in 1,2,4-triazole derivatives, it is also known that there is a tautomeric balance in 1,3,4-oxadiazole derivatives as indicated in I and II reactions. For the 1,3,4-oxadiazole compound (**7**), the spectroscopic data proposed that thiooxo form (II) was preferred as the dominant tautomeric form. In FT IR spectrum of this compound, the signal from –C=S bond was detected in 1228 cm^{-1}. In addition, the [M + 1] ionic peak of the molecule (**7**) matches with the appropriate *m/z* value and the results of the elemental analysis confirm the structure.

The Mannich reaction, a three-component reaction in a single step, that allows synthesis of organic molecules containing different functional groups in a single molecular structure has become one of the frequently used methods in drug design studies.

Three components involved in the Mannich reactions are a non-enolized carbonyl compound, enolizable carbonyl



Scheme 4 Amine, DMF, HCHO, $InCl_3$, 24 h, rt

compound, and a primary or secondary amine, in which alkyl (aryl) aminomethylated products can be synthesized (Roman 2015). In these reactions, any compound containing active hydrogen can be used instead of the enolable carbonyl compound. For example, Mannich bases exerting various biological activities have been obtained using 1,2,4-triazole derivatives as the active hydrogen component and methylpiperazine or morpholine as the amine component (Roman 2015).

In the current study, 1,3,4-oxadiazole compound (**7**) and 1,2,4-triazole compound (**10**) were used as the active hydrogen component in the synthesis of Mannich bases, **11a–11f** and **12a–12f**, respectively (Scheme 4). For the synthesis of Mannich bases, Lewis and Bronsted acid catalysts such as p-TSA, $FeCl_3$, $InCl_3$, and HCl are used in addition to polar solvents. In this study, $InCl_3$ catalyst was used for Mannich reactions (Mentese et al. 2016).

In the FT IR and NMR spectra of the Mannich bases, while there was not any peak confirming the presence of –SH or –NH proton of the compound (**7**) or (**10**), the signals of the amine used were recorded at the corresponding chemical shift values in the NMR spectra. These compounds also gave elemental analysis results and EI–MS spectra compatible with their structure.

Anticancer activity

Newly synthesized Mannich bases were evaluated for their anticancer activity against PC3, DU145, and LNCaP PC cell lines in ex vivo conditions. The results revealed that LC50 levels for all chemicals tested were higher than the highest concentration used (500 µM) for all cancer cell lines (Table 1). According to IC50 levels, LNCaP cells were more sensitive to Mannich bases compared with DU145 and PC3 cells. In addition, Mannich bases displayed strong growth inhibitions at relatively low concentrations (3.43–16.64 µM) to LNCaP cells which is comparable with control drug treatment (97.63 µM). DU145 cells were found to be the most resistant cell types against Mannich base treatment.

Evaluation of hybrid molecules according to NCI60 screening methodology

Although many anticancer drugs have been developed in modern chemotherapy, the development of a desirable level of therapeutic product has not yet been possible. In addition, due to the mechanisms of resistance against chemotherapeutic agents and their side effects, their therapeutic potency is decreasing day by day. Therefore, efforts are under way to develop new cancer drugs. In this study, an MTT test was performed to measure the effects of 21 newly synthesized hybrid molecules on cell proliferation (NCI60 scanning methodology) and IC50 values. According to this, none of the newly synthesized **3**, **5**, **11a**, and **11b** hybrid molecules, even at a high concentration of 125 µg/mL, have not shown antiproliferative activity on PC cell lines (Table 1). However, the fact that the anticancer activity of these compounds has not been able to demonstrate does not mean that they are pharmacologically worthless. A large scale analysis of these molecules, such as genome, transcriptome, and proteome, may be useful for the screening of different activities. In addition, when the G50 values of these molecules (2.5–59.5 µg/mL) are examined, it shows that there is potential for use if they are redesigned and increased antiproliferative effects. Interestingly, the

Table 1 Life parameters (GI50, TGI, and LC50) and IC50 values determined by MTT test of test molecules

Molecules (μg/mL)	DU145				PC3				LnCaP			
	GI50	TGI	LC50	IC50	GI50	TGI	LC50	IC50	GI50	TGI	LC50	IC50
3	4.9	>1000	>1000	>1000	21.3	>1000	>1000	>1000	22.5	>1000	>1000	>1000
4	2.4	44.5	>1000	44.6	4.7	40.8	>1000	40.8	4.6	>1000	>1000	>1000
5	2.5	>1000	>1000	>1000	6.0	>1000	>1000	>1000	4.3	>1000	>1000	>1000
6	7.9	>1000	>1000	>1000	6.2	122.5	>1000	70.9	1.2	11.5	>1000	8.9
7	>1000	>1000	>1000	>1000	12.6	>1000	>1000	>1000	4.2	40.5	>1000	18.5
8	>1000	>1000	>1000	>1000	10.5	>1000	>1000	>1000	4.9	211.3	>1000	47.2
9	10.9	>1000	>1000	>1000	5.2	402.7	>1000	129.8	5.6	>1000	>1000	>1000
10	16.3	>1000	>1000	>1000	2.3	626.7	>1000	113.8	3.1	82.3	>1000	14.5
11a	8.2	>1000	>1000	>1000	11.3	>1000	>1000	>1000	3.4	>1000	>1000	>1000
11b	59.5	>1000	>1000	>1000	3.6	>1000	>1000	>1000	5.5	>1000	>1000	>1000
11c	11.2	>1000	>1000	>1000	1.6	143.6	>1000	15.8	3.2	71.6	>1000	72.7
11d	129.0	>1000	>1000	>1000	6.2	>1000	>1000	>1000	2.6	349.5	>1000	368.2
11e	6.6	>1000	>1000	>1000	1.6	16.1	>1000	9.7	3.4	725.9	>1000	25.5
11f	22.7	>1000	>1000	>1000	43.4	>1000	>1000	>1000	8.0	532.3	>1000	61.7
12a	118.6	>1000	>1000	>1000	4.6	>1000	>1000	>1000	3.2	720.8	>1000	31.1
12b	12.7	>1000	>1000	>1000	148.3	>1000	>1000	>1000	6.5	805.6	>1000	113.4
12c	3.4	>1000	>1000	418.6	8.2	>1000	>1000	>1000	2.7	130.6	>1000	21.2
12d	179.6	>1000	>1000	>1000	16.6	>1000	>1000	>1000	3.1	115.5	>1000	20.7
12e	2.5	>1000	>1000	>1000	13.3	>1000	>1000	>1000	3.5	49.7	>1000	49.6
12f	2.1	420.3	>1000	88.7	3.9	>1000	>1000	>1000	2.3	13.8	>1000	13.8
5-FU	10.2	35.4	236.8	25.9	9.9	39.9	371.7	27.8	12.7	53.0	486.2	38.7

newly synthesized hybrid molecules **7, 8, 11d, 11f, 12a, 12b, 12d**, and **12e** showed strong antiproliferative properties only on LnCaP from the PC cell lines tested (GI50 values 2.6–8.0 μg/mL; TGI values 23.5–805.6 μg/mL, and IC50 values in the range of 18.5–368.2 μg/mL). However, the molecule **9** showed antiproliferative properties only on PC3 from PC cell lines tested (GI50 values was 5.2 μg/mL, TGI value was 402.7 μg/mL, and IC50 values were 129.8 μg/mL) (Table 1).

The emergence of such a result in different PC cell lines may have been effective in different molecular mechanisms of cells as well as newly synthesized hybrid molecules. When we examine the cells, these cells are susceptible to LnCaP hormone (androgen, AR+) and are able to secrete prostate-specific antigen (PSA).

These cells are also sensitive to 5-alpha-dihydrotestosterone, which is responsible for cell growth and modulation of acid phosphatase production. PC3 cells are hormone (androgen, AR-) insensitive and exhibit low acid phosphatase and testosterone-5-alpha-reductase activities. Molecules **6**, **10, 11c**, and **11e** showed antiproliferative properties similar to 5-fluorouracil (5-FU) which were used as positive control on both PC3 (G50 values 1.6–6.2 μg/mL, TGI values 16.1–626.7 μg/mL and IC50 values 9.7–113.8 μg/ml range) and LnCaP (G50 values 1.2–3.4 μg/mL, TGI values 11.5–725.9 μg/mL, and IC50 values 8.9–72.7 μg/mL) from the PC cell lines tested (Table 1).

While molecules **12c** and **12f** on the DU145 and LnCaP of the PC cell lines tested showed potent antiproliferative properties (DU145 cells, respectively, G50 value 3.4, 2.1 μg/mL and IC50 value 418.6, 88.7 μg/mL, GI50 2.7, 2.3 μg/mL and IC50 value 21.12, 13.8 μg/mL, respectively), only molecule **4** on both DU145 and PC3 of the PC cell lines showed antiproliferative (GI50 value 2.4 and 4.7 μg/mL, TGI value 44.5 and 40.8 μg/mL, respectively, IC50 value 44.6 and 40.8 μg/mL, respectively). Pharmacological activities of newly synthesized hybrid molecules on cells of the DU145 PC cell lines were understood to be remarkably few. This cell is independent of the hormone (androgen, AR−) and does not secrete the PSA but has a very weak acid phosphatase activity. We also have to point out that DU145 cells exhibit quite allogeneic properties.

Generally, when the IC50 data of the test results were examined, the molecules of the molecule **12c** (IC50 values 88.7 21 g/mL) on DU145 cells (Fig. 1), molecules **6, 11c, 11e** (IC50 values 70.9, 15.8, and 9.7 μg/mL, respectively) PC3 on the cells (Fig. 2), **6, 7, 8, 10, 11c, 11e, 11f, 12a, 12c, 12d, 12e, 12f** on LnCaP cells (IC50 value in the range of

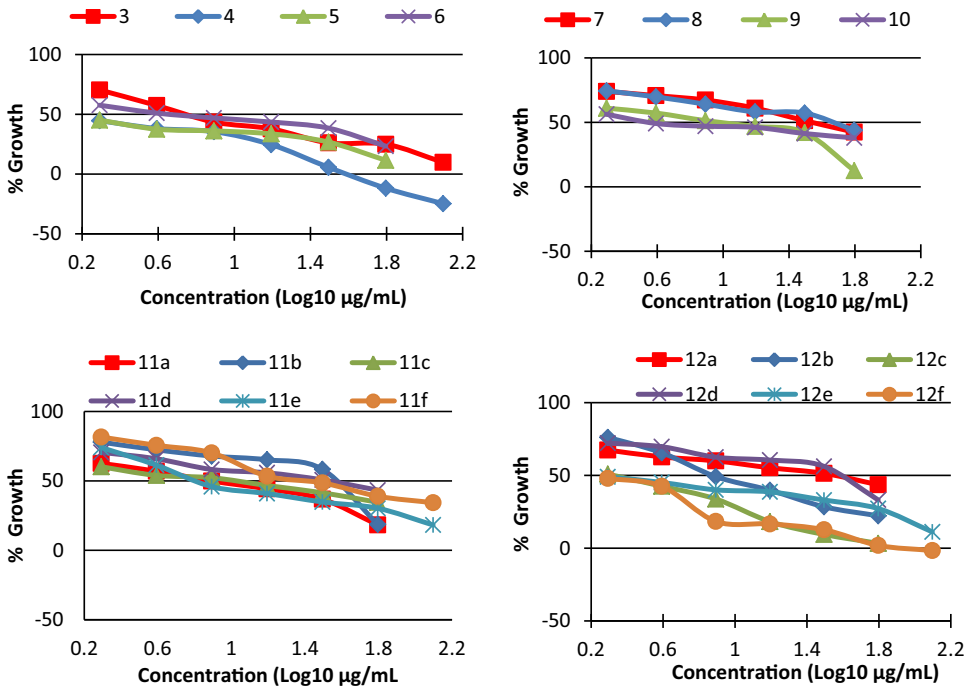


Fig. 1 Determination of antiproliferative effects of **3-12** test molecules on DU145 cell line by MTT test. Negative values on the graph indicate cell death, positive values indicate growth inhibition

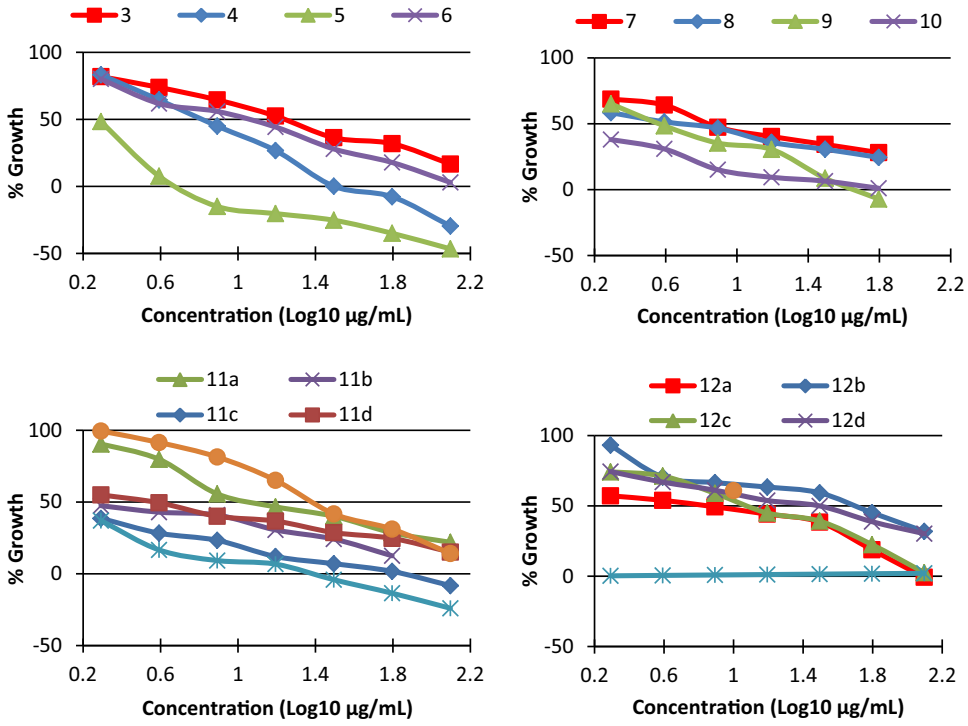


Fig. 2 Determination of antiproliferative effects of **3-12** test molecules on PC3 cell line by MTT test. Negative values on the graph indicate cell death, positive values indicate growth inhibition

8.9–72.7 µg/mL) (Figs. 3 and 4), inhibitory effected on total growth up to at least 5-FU (control anticancer molecule).

When the TGI values in Table 1 were examined, molecules **4** (TGI value 44.5 µg/ml) on DU145 cells (Fig. 1), molecules **4** and **11e** (TGI values were 40.8 and 16.1 µg/ml) on PC3 cells (Fig. 2), molecules **6, 7, 10, 11c, 12e, 12f** (TGI values respectively 11.5, 40.5, 82.3, 71.6, 49.7, and 13.8 µg/ml) LnCaP cells on (Fig. 3), inhibitory effected on total growth up to at least 5-FU.

The lower GI50, TGI, and IC50 values of the said molecules on cells similar to 5-FU are considered to be suitable for further clinical studies. Because all of molecules have very high concentration LC50 value (>1000 µg/mL), in terms of the concentration values of lethal (lethal,

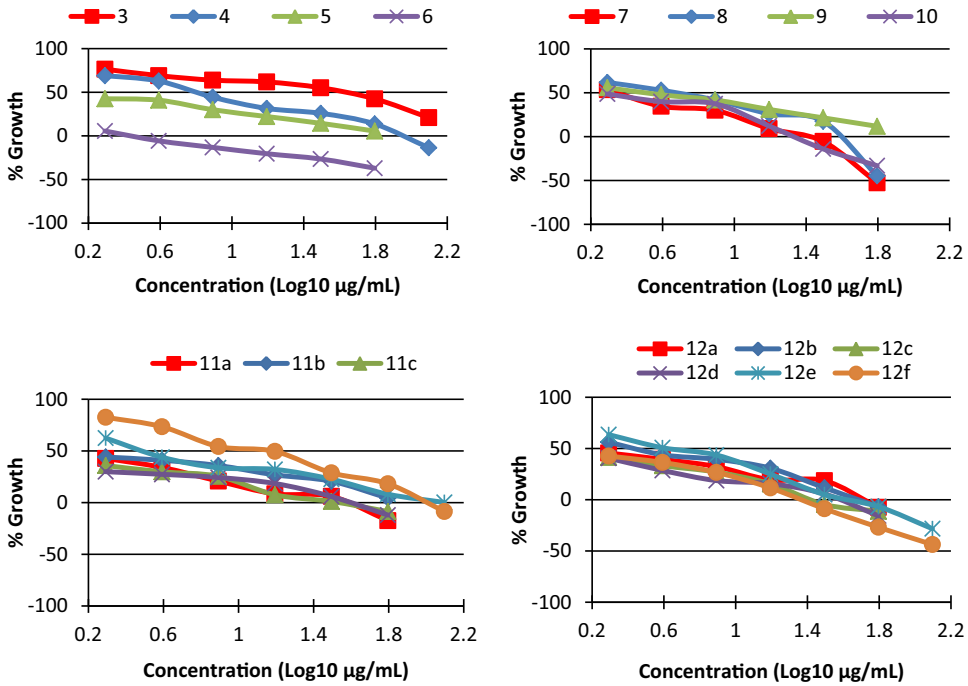


Fig. 3 Determination of MTT test of antiproliferative effects of test molecules **3-12** on the LnCaP cell line. Negative values on the graph indicate cell death, positive values indicate growth inhibition

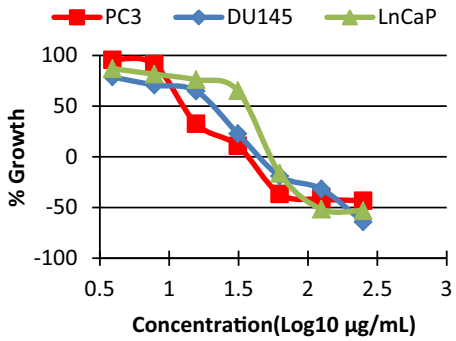


Fig. 4 Determination of antiproliferative effects of 5-FU control anticancer-acting molecules on PC3, DU145, and LnCaP cell line by the MTT assay

negative values on the graph), the new synthesized molecules not have a very high toxic effect on the cells and perform better than even positive control (Table 1).

Cancer is the second cause of death in the world, the amount spent for the treatment of cancer is rapidly increasing. Research and development studies for the development of national drugs in the fight against cancer have also gained speed. in this context, new hybrid molecules developed by us have been added to the science literature with anticancer properties. The aim of this article is to study in vitro and then in vivo, to determine the important pharmacological potentials of molecules, to study "phase 0" in order to see the availability in medicine and pharmacy. In addition, it is possible to develop new and more active molecules based on these molecules.

Fig. 5 DNA laddering test of newly synthesized molecule **4** and **12c**. The test log phase was performed using the DU145 cell line showing exponential growth and incubation time was limited to 24 h. K-Control

DNA ladder potentials of hybrid molecules

According to the DNA laddering test which determine the DNA degradation activity, which is an indicator of apoptosis, molecules **12c** and **4** of the new hybrid molecules tested on DU145 cells, DU145 cells have been shown to cause significant DNA breaks (Fig. 5).

According to the DNA laddering test, no DNA degradation was observed in the control well, whereas DNA fractures caused by Molecule **12c** did not cause very significant laddering (Fig. 5). The apopitosis mechanisms seem to be responsible for part of the anticancer activity of new hybrid molecules that cause partial breaks on DNA.

Morphological changes of molecules on cells

The effects of the new hybrid molecules used in two different concentrations (62.5 µg/mL and 125 µg/mL) on cell morphology were investigated and examined with the help of phase-contrast microscopy. Control cells show normal cell morphology, such as fibroblast or epithelial, and have been a reference for our evaluations. The first impression we get from phase-contrast microscope images is that cells begin to separate from the flask floor in a concentration-dependent manner. during the separation, respectively, cells begin to lose their normal forms such as fibroblast or epithelial, and then turn into rounded forms, and cytoplasmic bubble and spike formation, abnormal globular structures and apoptotic bodies appearing, and ultimately the cells are floating (cells are dead) and some occurs morphological changes. Findings from morphological analysis show that new hybrid molecules cause cell death by using apoptosis pathways, especially at high concentrations.

Conclusions

In the present study, the leading compound 6-(4-phenylpiperazin-1-yl)pyridine-3-ylamine was used to synthesize novel Mannich bases. These compounds were characterized using elemental analysis, IR, ^{1}H-, and ^{13}C-NMR spectral data and evaluated for their anticancer activities against PC cell lines. The results showed that the Mannich bases tested in the current study displayed moderate cytotoxic activities against PC cells. Further variations in the structure of the chemicals should be performed to screen more potent anticancer agent.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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