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Design and synthesis of some new tri-substituted pyrazole derivatives as anticancer agents

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Abstract A new series of tri-substituted pyrazole derivatives were designed as anti-cancer agents and synthesized, starting with the formylation of semicarbazone via the Vilsmeier–Haack reaction to give 3-(4-bromophenyl)-1*H*-pyrazole-4-carbaldehyde **I** which was the precursor of compounds **1**–**9**. The new chemical entities were screened for their anti-cancer activity on various human cancer cell lines, namely: hepatocellular carcinoma HepG2, breast cancer MCF-7, lung carcinoma A549 and prostatic cancer PC3. Most of the synthesized compounds showed remarkable activity on the tested cell lines, while compound **2** had the highest potency against the HepG2 cell line with an IC_{50} of 9.13 μM compared with doxorubicin (IC_{50} = 34.24 μM), the reference standard used in this study, and compound **7** was the most active on the rest of the three cell lines; MCF-7, A549 and PC3 (IC_{50} = 16.52, 6.52 and 9.13 μM, respectively) relative to IC_{50} = 20.85, 5.93 and 38.02 μM of the standard. Thus, some of the synthesized tri-substituted pyrazole derivatives, specially **2** and **7**, have the potential to be developed into potent anticancer agents.

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Introduction

Despite decades of research that have resulted in an enormous leap in cancer therapy, cancer continues to be one of the major causes of death all over the world and its incidence is rising in the developing as well as in the developed countries. The latest WHO cancer statistics reported that 8.2 million people worldwide died from cancer in 2012, and it is expected that annual cancer cases will rise from 14 million in 2012 to 22 million in the next two decades [1, 2]. It is worth mentioning that 60 % of world's total new annual cases occur in Africa, Asia and Central and South America [3].

Cancer is a compilation of changes in genetic expression leading to uncontrolled proliferation of somatic or germinal cells [4]. Normal cells lose control over their regulatory functions causing abnormal growth and division. There are currently more than 200 different types of cancer resulting from various cellular defects [5].

Meanwhile, pyrazole derivatives represent an important class of bio-active heterocycles attracting a progressive interest of many researchers due to their widely observed biological and pharmacological properties such as analgesic [6], anti-oxidative [7], antihypertensive [8], antiviral [9], anti-inflammatory [10], antiproliferative [11] and anticancer [12–15] activities. Ruxolitinib "(3R)-3-cyclopentyl-3-[4-(7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)pyrazol-1-yl]propanenitrile" and Crizotinib "3-[(1R)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl) pyridin-2-amine" are representative examples of commercially available anticancer drugs containing pyrazole scaffolds (Fig. 1) [16, 17].

On the basis of the afore-mentioned findings, the need for new anticancer agents is high and persistent.

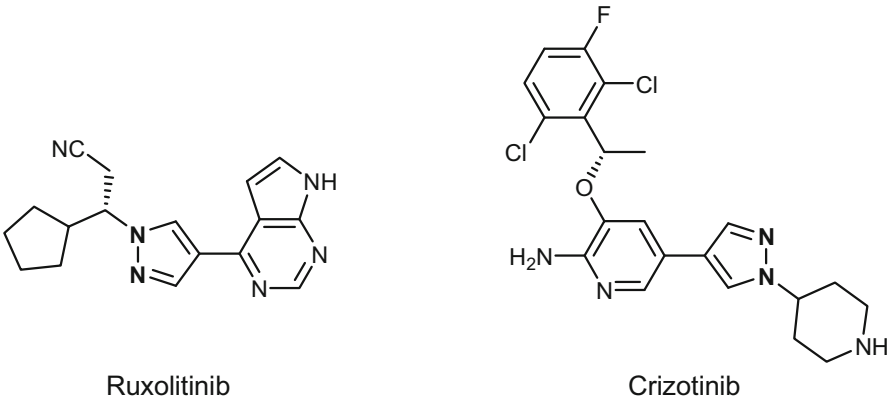


Fig. 1 Chemical structures of anticancer drugs containing pyrazole moiety available in the market

Thus, our current study is dealing with the rational design and synthesis of some new tri-substituted pyrazole-containing derivatives expecting potential activity with no or minimal side effects towards four aggressive cancer cell lines, namely: hepatocellular carcinoma HepG2, breast cancer MCF7, lung carcinoma A549 and prostatic cancer PC3.

Experimental

Chemistry

Melting points were recorded on a Stuart SMP30 melting point apparatus. IR spectra (KBr) were recorded on a JASCO 6100 spectrophotometer. NMR spectra were recorded on a JEOL AS 500 (DMSO-d_6, ^{1}H: 500 MHz, ^{13}C: 125 MHz), spectrometer. Chemical shifts (δ_H) are reported relative to TMS as the internal standard. All coupling constant (J) values are given in Hertz. Chemical shifts (δ_C) are reported relative to DMSO-d_6 as internal standards. Mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX (EI, 70 eV) spectrometer. Elemental microanalyses were recorded on a Vario El Elementar analyzer.

*Synthesis of 3-(4-bromophenyl)-1-ethyl-1H-pyrazole-4-carbaldehyde (**1**)*

A mixture of the carbaldehyde **I** (3 g, 11 mmol) and anhydrous potassium carbonate (2 g, 14 mmol) was stirred in DMF (10 mL) for 2 h. Iodoethane (0.9 mL, 11 mmol) was added dropwise, and stirring was continued overnight at room temperature, 25–30 °C. The reaction mixture was poured onto ice-cold water while stirring, the solid form was collected, washed with water, dried and crystallized from petroleum ether 60–80 to give **1**.

Colorless crystals; yield (70 %); mp 90–92 °C; IR: ν_{max}/cm^{-1}: 3027 (CH, aromatic), 2984, 2857 (CH, aliphatic), 1669 (C=O); ^{1}H NMR: δ 1.40 (*t*, J = 7.7 Hz, 3H), 4.21 (q, *J*= 7.7 Hz, 2H,), 7.63 (*d*, J = 8 Hz, 2H), 7.79 (*d*, J = 8 Hz, 2H), 8.58 (s, 1H), 9.82 (s, 1H); ^{13}C NMR: δ 15.5, 47.5, 115.9, 122.2, 129.4, 131.5, 132.3, 135.7, 150.7, 188.1; MS: m/z (%) 278 (M-1, 100); Anal. Calcd. For $C_{12}H_{11}BrN_2O$ (279): C, 51.63; H, 3.97; N, 10.04. Found: C, 51.85; H, 3.74; N, 10.15 %.

*Synthesis of (Z)-3-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-5-yl)-1-(4-chlorophenyl) prop-2-en-1-one (**2**)*

To a mixture of 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde (**1**) (2.8 g, 10 mmol) and 4-chloroacetophenone (0.8 mL, 10 mmol), sodium ethoxide (10 mmol, 0.23 g Na in EtOH absolute 20 mL) was added portionwise. The reaction mixture was stirred overnight at room temperature, 25–30 °C. The precipitate formed was filtered, dried and crystallized from ethanol to give **2**.

Yellow crystals; yield (58 %); mp 139–141 °C; IR: ν_{max}/cm^{-1} 3067 (CH, alkene), 1658 (C=O); ^{1}H NMR: δ 1.43 (*t*, J = 7.2 Hz, 3H), 4.20 (q, J = 7.2 Hz, 2H), 7.47 (*d*, J = 8.6 Hz, 2H), 7.60 (*d*, J = 8.6 Hz, 2H), 7.60 (*d*, J = 5 Hz, 1H),

7.62 (*d*, $J = 5$ Hz, 1H), 7.67 (*d*, $J = 7.7$ Hz, 2H), 8.05 (*d*, $J = 8.6$ Hz, 2H), 8.65 (s, 1H); ^{13}C NMR: $\delta\delta$ 15.5, 47.5, 115.9, 120.3, 122.2, 129.4, 130.6, 130.7, 131.5, 132.3, 135.8, 136.9, 138.4, 150.7, 188.1; MS: m/z (%) 416.09 (M + 1, 16); Anal. Calcd. For $C_{20}H_{16}BrClN_2O$ (415.7): C, 57.78; H, 3.88; N, 6.74. Found: C, 57.56; H, 3.65; N, 6.93 %.

*Synthesis of 2-amino-4-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-1,6-dihydro-6-oxopyrimidine-5-carbonitrile (**3**)*

To a mixture of 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde (**1**) (2.8 g, 10 mmol) and ethyl cyanoacetate (1 mL, 10 mmol), aqueous sodium hydroxide solution (40 %, 2 g NaOH in 5 mL H_2O) was added portionwise. The reaction mixture was stirred in ethanol (15 mL) at room temperature, 25–30 °C. After 15 min, guanidine sulfate (2.1 g, 10 mmol) was added to the reaction mixture and refluxed for an additional 11 h. The reaction mixture was poured onto ice-cold water while stirring, neutralized with dil. HCl, and the precipitate formed was filtered, washed with water, dried and crystallized from ethanol to give **3**.

Yellow powder; yield (72 %); mp 242–247 °C; IR: ν_{max}/cm^{-1} 3423, 3226 (NH_2, NH), 2217 (C≡N), 1646 (C=O); ^{1}H NMR: δ 1.41 (*t*, $J = 7.2$ Hz, 3H), 4.31 (q, $J = 7.2$ Hz, 2H), 7.47 (*d*, $J = 8.6$ Hz, 2H), 7.70 (*d*, $J = 7.6$ Hz, 2H), 8.74 (s, 1H), 13.69 (br. s, 1H); ^{13}C NMR: δ 15.7, 47.5, 99.8, 112.6, 117.2, 123.1, 131.0, 131.3, 132.5, 132.7, 145.8, 153.4, 164.2; MS: m/z (%) 385.22 (M + 2, 36.3); Anal. Calcd. For $C_{16}H_{13}BrN_6O$ (385.22): C, 49.89; H, 3.40; N, 21.82. Found: C, 49.64; H, 3.25; N, 21.96 %.

*General procedure for synthesis of (Z)-5-((3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)methylene)-3-aryl-2-thioxoimidazolidin-4-one (**4a–c**)*

A mixture of 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde (**1**) (1.9 g, 7 mmol), glycine (0.5 g, 7 mmol) and aryl isothiocyanate (7 mmol) was refluxed in glacial acetic acid (15 mL) for 10 h. The precipitate formed was filtered, washed with water, dried and crystallized from *n*-butanol to give ***4a–c***.

*(Z)-5-((3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)methylene)-3-phenyl-2-thioxoimidazolidin-4-one (**4a**)*

Yellow crystals; yield (70 %); mp 253–255 °C; IR: ν_{max}/cm^{-1} 3419 (NH), 3107 (CH, aromatic), 1649 (C=O), 1170 (C=S); ^{1}H NMR: δ 1.46 (*t*, $J = 7.2$ Hz, 3H), 4.21 (q, $J = 7.2$ Hz, 2H), 6.43 (s, 1H), 7.33 (*d*, J= 6.3 Hz, 2H), 7.44 (*d*, $J = 6.3$ Hz, 2H), 7.42-7.49 (m, 5H), 8.76 (s, 1H), 12.40 (br. s, 1H); ^{13}C NMR: δ 15.5, 47.5, 104.7, 111.6, 122.0, 125.0, 129.3, 129.4, 130.8, 131.9, 132.2, 132.4, 133.8, 150.8, 163.9, 177.8; MS: m/z (%) 454.3 (M + 1, 45); Anal. Calcd. For $C_{21}H_{17}BrN_4OS$ (453.35): C, 55.64; H, 3.78; N, 12.36; S, 7.07. Found: C, 55.89; H, 3.84; N, 12.44; S, 7.19 %.

*(Z)-5-((3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)methylene)-3-(4-fluorophenyl)-2-thioxoimidazolidin-4-one (**4b**)*

Yellow crystals; yield (59 %); mp 263–266 °C; IR: ν_{max}/cm^{-1} 3425 (NH), 3121 (CH, aromatic), 1720 (C=O), 1159 (C=S); ^{1}H NMR: δ 1.37 (*t*, $J = 7.2$ Hz, 3H), 4.19 (q, $J = 7.2$ Hz, 2H), 6.65 (s, 1H), 7.32-7.69 (m, 8H), 8.90 (s, 1H), 12.27 (br. s, 1H); ^{13}C NMR: δ 15.8, 47.5, 110.6, 112.1, 116.1, 116.3, 122.3, 126.0, 131.2, 131.6, 132.2, 132.8, 151.8, 161.0, 162.0, 163.0, 174.6; MS: m/z (%) 472.35 (M + 1, 100); Anal. Calcd. For $C_{21}H_{16}BrFN_4OS$ (471.35):C, 53.51; H, 3.42; N, 11.89; S, 6.8. Found: C, 53.78; H, 3.29; N, 11.65; S, 7.1 %.

*(Z)-5-((3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)methylene)-3-(4-methoxyphenyl)-2-thioxoimidazolidin-4-one (**4c**)*

Brown crystals; yield (56 %); mp 255–259 °C; IR: ν_{max}/cm^{-1} 3417 (NH), 3095 (CH, aromatic), 1716 (C=O), 1164 (C=S); ^{1}H NMR: δ 1.37 (*t*, $J = 7.1$ Hz, 3H), 3.77 (s, 3H), 4.20 (q, $J = 6.7$ Hz, 2H), 6.64 (s, 1H), 7.00 -7.70 (m, 8H), 8.90 (s, 1H), 12.20 (br. s, 1H); ^{13}C NMR: δ 15.5, 48.0, 55.9, 104.5, 110.4, 112.2, 114.4, 126.3, 130.4, 130.8, 131.2, 131.9, 132.2, 151.8, 159.7, 162.2, 164.1, 174.7; MS: m/z (%) 484.37 (M + 1, 100); Anal. Calcd. For $C_{22}H_{19}BrN_4O_2S$ (483.38): C, 54.66; H, 3.96; N, 11.59; S, 6.63. Found: C, 54.47; H, 3.72; N, 11.73; S, 6.71 %.

*Synthesis of 5-((3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (**5**)*

To a mixture of 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde (**1**) (1.9 g, 7 mmol) and barbituric acid (0.9 g, 7 mmol), few drops of piperidine were added. The reaction mixture was refluxed in ethanol (20 mL) for 5 h. The precipitate formed was filtered, dried and crystallized from glacial acetic acid to give **5**.

Dark yellow powder; yield (77 %); mp > 300 °C; IR: ν_{max}/cm^{-1}3441 (NH), 3185 (CH, aromatic), 1708 (2 C=O), 1650 (C=O); ^{1}H NMR: δ 1.41 (*t*, $J = 7.1$ Hz, 3H), 4.30 (q, $J = 5.7$ Hz, 2H), 7.44 (*d*, $J = 7.65$ Hz, 2H), 7.72 (*d*, $J = 7.65$ Hz, 2H), 8.04 (s, 1H), 9.30 (s, 1H), 11.24 (*d*, 2H); ^{13}C NMR: δ 15.6, 47.7, 113.2, 132.2, 131.0, 131.9, 132.3, 132.6, 133.0, 144.5, 150.8, 156.5, 163.2, 164.4; MS: m/z (%) 389.2 (M, 68.6); Anal. Calcd. For $C_{16}H_{13}BrN_4O_3$ (389.2): C 49.38; H 3.37; N 14.40. Found: C 49.68; H 3.11; N 14.15 %.

*General procedure for synthesis of 5-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-2H-1,2,4-triazols (**6a, b**)*

A mixture of 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde (**1**) (1.9 g, 7 mmol), semicarbazides (7 mmol) and anhydrous sodium acetate (0.8 g, 10 mmol) in ethanol (20 mL) was refluxed for 7 h. The reaction mixture was poured onto ice-cold water, and the solid formed was collected, washed with water, dried and crystallized from ethyl acetate affording **6a**, **b**.

*5-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-2H-1,2,4-triazol-3(4H)-one (**6a**)*

Faint yellow powder; yield (90 %); mp 150–152 °C; IR: ν_{max}/cm^{-1} 3471, 3235 (2 NH), 3165 (CH, aromatic), 1714 (C=O); ^{1}H NMR: δ 1.38 (*t*, $J = 7.2$ Hz, 3H), 4.13 (q, $J = 7.2$ Hz, 2H), 7.47 (*d*, $J = 8.6$ Hz, 2H), 7.61 (*d*, $J = 8.6$ Hz, 2H), 7.84 (br. s, 1H), 8.30 (s, 1H), 9.94 (br. s, 1H). ^{13}C NMR: δ 15.7, 47.5, 115.6, 121.7, 129.9, 130.4, 132.0, 133.1, 148.1, 157.2, 170.4; MS: m/z (%) 335.28 (M + 1, 30); Anal. Calcd. For $C_{13}H_{12}BrN_5O$ (334.17): C, 46.72; H, 3.62; N, 20.96. Found: C, 46.49; H, 3.82; N, 21.14 %.

*5-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-2H-1,2,4-triazole-3(4H)-thione (**6b**)*

Faint brown crystals; yield (97 %); mp 100–102 °C; IR: ν_{max}/cm^{-1} 3397, 3237 (2 NH), 1181 (C=S); ^{1}H NMR: δ 1.39 (*t*, $J = 7.2$ Hz, 3H), 4.13 (q, $J = 7.2$ Hz, 2H), 7.49 (*d*, $J =$ = 8.6 Hz, 2H), 7.62 (*d*, $J = 8.6$ Hz, 2H), 8.10 (s, 1H), 8.37 (s, 1H), 11.17 (s, 1H); ^{13}C NMR: δ 15.6, 47.5, 115.0, 121.9, 130.3, 130.4, 132.0, 132.4, 136.5, 148.9, 177.7; MS: m/z (%) 353.21 (M + 3, 13.49); Anal. Calcd. For $C_{13}H_{12}BrN_5S$ (350.24): C, 44.58; H, 3.45; N, 20.0; S, 9.16 Found: C, 44.39; H, 3.22; N, 20.18; S, 9.22 %.

*Synthesis of 4-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-6-(4-chlorophenyl)pyrimidin-2-amine (**7**)*

A mixture of (*E*)-3-(3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)-1-(4-chlorophenyl)prop-2-en-1-one (**2**) (1.8 g, 43 mmol) and guanidine sulfate (0.46 g, 43 mmol) was refluxed in ethanol (20 mL) for 2 h. then, aqueous NaOH (5 %, 6 mL) was added portionwise, refluxing was continued for 12 h., the reaction mixture was then poured onto ice-cold water and neutralized with dil. HCl. The solid formed was collected by filtration, washed with water, dried and crystallized from ethanol to give **7**.

Yellow crystals; yield (52 %), mp 146–148 °C; IR: ν_{max}/cm^{-1} 3420, 3310 (NH_2), 3195 (CH, aromatic); ^{1}H NMR: δ 1.42 (*tt*, $J = 7.6$ Hz, 3H), 4.20 (q, $J = 7.6$ Hz, 2H), 6.62 (s, 2H), 7.03 (s, 1H), 7.51 (*d*, $J = 8.6$ Hz, 2H), 7.55 (*d*, $J = 8.6$ Hz, 2H), 7.60 (*d*, $J = 8.6$ Hz, 2H), 7.94 (*d*, $J = 8.6$ Hz, 2H), 8.35 (s, 1H); ^{13}C NMR: δ 15.7, 47.5, 104.1, 119.1, 121.6, 128.8, 129.2, 131.3, 131.5, 132.8, 133.4, 135.6, 148.2, 161.8, 163.0, 164.3; MS: m/z (%) 454.17 (M, 100); Anal. Calcd. For $C_{21}H_{17}BrClN_5$ (454.17): C, 55.46; H, 3.77; N, 15.4. Found: C, 55.64; H, 3.87; N, 15.64 %.

*Synthesis of 4-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-6-(4-chlorophenyl)-1,2-dihydro-2-oxopyridine-3-carbonitrile (**8**)*

A mixture of (*E*)-3-(3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)-1-(4-chlorophenyl)prop-2-en-1-one (**2**) (1.2 g, 3 mmol), ethyl cyanoacetate (0.3 mL, 3 mmol) and anhydrous ammonium acetate (1.8 g, 24 mmol) was refluxed in absolute ethanol (15 mL) for 12 h. The reaction mixture was cooled and the precipitate formed was filtered, dried and crystallized from glacial acetic acid to give **8**.

Yellow crystals; yield (60 %); mp 345 °C; IR: ν_{max}/cm^{-1} 3436 (NH), 3114 (CH, aromatic), 2216 (C≡N), 1639 (C=O); ^{1}H NMR: δ 1.43 (*t*, $J = 13.4$ Hz, 3H), 4.23 (q, $J = 7.65$ Hz, 2H), 6.50 (s, 1H), 7.37-7.69 (m, 8H), 8.34 (s, 1H), 12.70 (br. s, 1H); ^{13}C NMR: δ 15.7, 47.5, 115.0, 121.8, 129.4, 129.9, 130.0, 132.1, 132.6, 132.7, 136.5, 148.0, 163.1, 172.9; MS: m/z (%) 480 (M + 1, 3); Anal. Calcd. For $C_{23}H_{16}BrClN_4O$ (479.76): C, 57.58; H, 3.36; N, 11.68. Found: C, 57.68; H, 3.62; N, 11.79 %.

*Synthesis of 1-(5-(3-(4-bromophenyl)-1-ethyl-1H-pyrazol-4-yl)-3-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl)ethanone (**9**)*

A mixture of (*E*)-3-(3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)-1-(4-chlorophenyl)prop-2-en-1-one (**2**) (2 g, 5 mmol) and hydrazine hydrate 99 % (0.4 mL, 8 mmol) was refluxed for 5 h. in glacial acetic acid (15 mL). The reaction mixture was poured onto ice-cold water, and the solid formed was collected, washed with water, dried and crystallized from ethanol to afford **9**.

Faint pink fluffy powder; yield (84 %); mp 158–160 °C; IR: ν_{max}/cm^{-1} 3124 (CH, aromatic), 1659 (C=O); ^{1}H NMR: δ 1.31 (*t*, $J = 7.2$ Hz, 3H), 2.24 (*t*, $J = 6.5$ Hz, 3H), 3.77 (q, $J = 6.5$ Hz, 2H), 4.03 (q, $J = 7.2$ Hz, 2H), 5.59-5.60 (m, 1H), 7.49 (*d*, $J = 7.7$ Hz, 2H), 7.57 (*d*, $J = 8.6$ Hz, 2H), 7.62 (*d*, $J = 7.7$ Hz, 2H), 7.72 (*d*, $J = 8.6$ Hz, 3H); ^{13}C NMR: δ 15.8, 22.3, 39.5, 43.5, 51.2, 120.8, 121.1, 128.8, 129, 129.3, 130.2, 131.9, 133.0, 134.0, 146.2, 153.6, 167.9; MS: m/z (%) 472.32 (M + 1, 100); Anal. Calcd. For $C_{22}H_{20}BrClN_4O$ (471.78): C, 56.01; H, 4.27; N,11.88. Found: C, 56.22; H, 4.34; N, 11.94 %.

In-vitro antitumor screening

Compounds **1–9** were screened against human cancer cell lines for anticancer activity using a cell-based approach [22–25]. Antitumor potency was tested on five human tumor cell lines obtained from the National Research Centre (Cairo, Egypt), namely: hepatocellular carcinoma HepG2, breast cancer MCF-7, lung carcinoma A549 and prostatic cancer PC3. Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] to purple Formosan [26, 27].

The in vitro antitumor screening was performed by adopting previously reported procedures [25–27]. Cells were suspended in RPMI-1640 medium for HepG2 and DMEM for MCF-7, A549 and PC3 in addition to 1 % antibiotic–antimycotic mixture (10,000 µg/ml potassium penicillin, 10,000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B) and 1 % L-glutamine at 37 °C, under 5 % CO_2 and 95 % humidity. Cells were seeded at a concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plates for 24 h. Media were aspirated, fresh medium (without serum) was added and cells were incubated with different concentrations of the sample to give a final concentration of (100, 50, 25 and 12.5 µM). An amount of 0.5 % DMSO was used as the negative control and doxorubicin was used as the positive control. A MTT assay was used for assessment of cytotoxicity [25–27]. After 72 h of incubation, the medium was aspirated, 40 µl

MTT salt (2.5 mg/ml) were added to each well and incubated for a further 4 h. To stop the reaction and dissolve the formed crystals, 200 µl of 10 % sodium dodecyl sulfate (SDS) in deionized water were added to each well and incubated overnight at 37 °C. The absorbance was then measured at 595 nm and a reference wave length of 620 nm. The cell surviving fraction was calculated as follows:

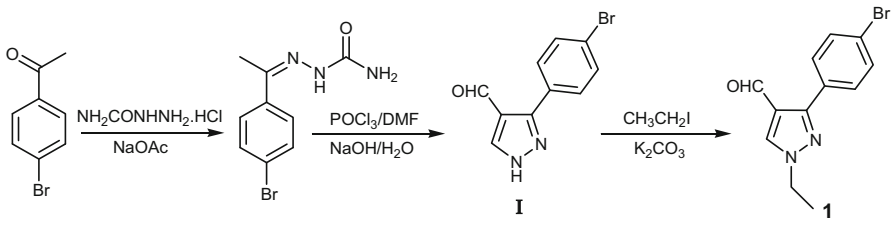
$$\text{Surviving fraction} = \text{Optical density (O.D.) of treated cells/O.D. of control cells.}$$

The IC_{50} (concentration required to produce 50 % inhibition of cell growth compared to the control experiment) was determined using Graph-Pad PRISM v.5 software. Statistical calculations for the determination of the mean and standard error values were determined by SPSS 11 software. IC_{50} results are mean values of three separate experiments using a probit analysis in the SPSS 11 program.

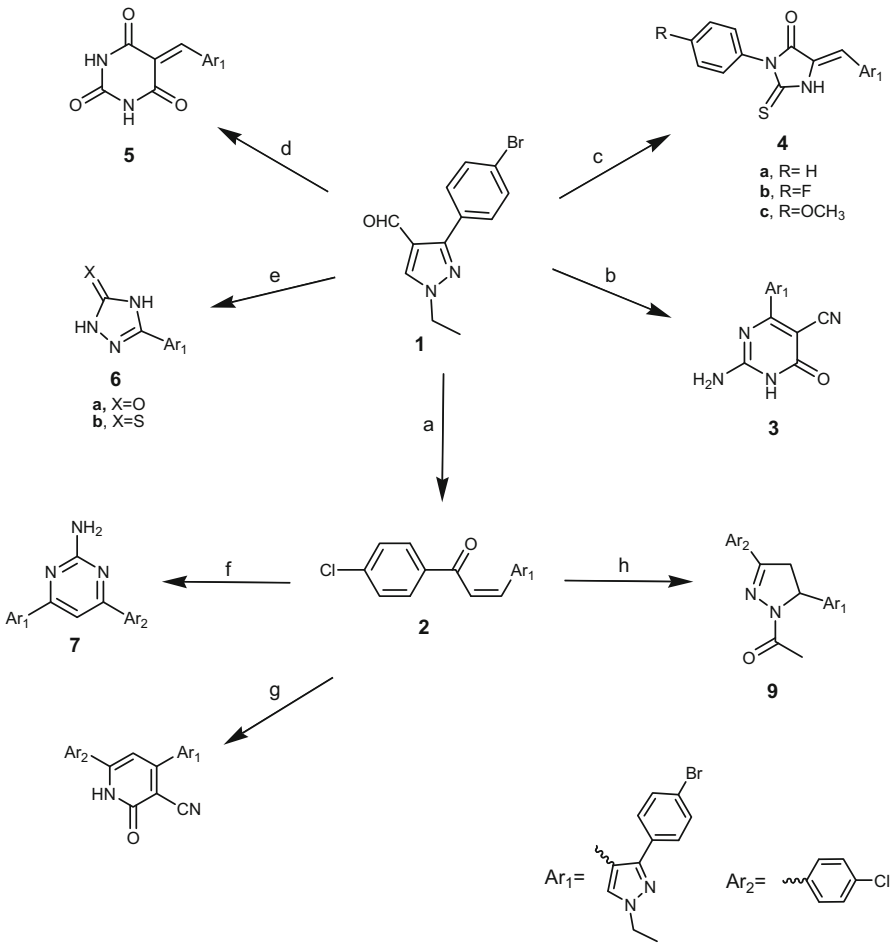
Results and discussion

Chemistry

The synthetic pathway employed to obtain some new tri-substituted pyrazole derivatives was started by the formylation of semicarbazone via Vilsmeier–Haack reaction to give 3-(4-bromophenyl)-1*H*-pyrazole-4-carbaldehyde **I** according to the earlier reported approach [18]. The formed carbaldehyde **I** was treated with iodoethane in DMF at room temperature in the presence of anhydrous potassium carbonate to give the corresponding N–alkylated analog 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde **1** (Scheme 1). The IR spectrum of **1** reveals the presence of a strong stretching carbonyl vibration band at $\nu = 1669$ cm^{-1} while, no assignable band due to NH stretching vibration was detected. ^{1}H NMR reveals the presence of a triplet signal at δ 1.40 ppm ($J = 7.7$ Hz) and a quartet signal at δ 4.21 ppm ($J = 7.7$ Hz) for CH_3 and CH_2, respectively, ^{13}C NMR reveals the presence of two peaks at δ 15.5 and 47.5 ppm representing CH_3 and CH_2, respectively, EI-MS spectrum reveals peak at m/z 278 (M-1). Compound **1**, the precursor of the target compounds **2–9** (Scheme 2), was treated via Claisen–Schmidt condensation with 4-chloroacetophenone in the presence of sodium ethoxide to give the corresponding chalcone (*E*)-3-(3-(4-bromophenyl)-1-ethyl-1*H*-



Scheme 1 Synthesis of the starting compound 3-(4-bromophenyl)-1-ethyl-1*H*-pyrazole-4-carbaldehyde **1**



Scheme 2 Synthesis route of compounds **2**–**9**. Reagents and conditions: (*a*) 4-Chloroacetophenone, EtONa, stirring rt; (*b*) ethylcyano acetate, guanidine sulfate, NaOH/H_2O, EtOH/reflux 11 h.; (*c*) glycine, arylisothiocyanates, gl. AcOH/reflux 10 h.; (*d*) barbituric acid, piperidine, EtOH/ reflux 5 h.; (*e*) semicarbazides, NaOAc, EtOH/reflux 7 h.; (*f*) guanidine sulfate, NaOH/H_2O, EtOH/reflux 12 h.; (*g*) ethyl cyanoacetate, amm. acetate, EtOH/reflux 12 h.; (*h*) hydrazine hydrate 99 %, gl. AcOH/reflux 5 h

pyrazol-4-yl)-1-(4-chlorophenyl)prop-2-en-1-one **2**. The IR spectrum of **2** revealed the presence of a strong stretching CH=CH vibration band at $v = 3067$ cm^{-1}. ^1H NMR spectrum shows a triplet signal at δ 1.43 ppm ($J = 7.2$ Hz) and a quartet signal at δ 4.20 ppm ($J = 7.2$ Hz) representing CH_3 and CH_2, respectively, as well as α, β-unsaturated ketone's protons, CH=CH, appeared as two doublets at 7.60 ppm ($J = 5$ Hz) and 7.62 ppm ($J = 5$ Hz). The ^{13}C NMR spectrum revealed the presence of peaks at δ 15.5, 47.5, 122.2, 138.4 and 188.1 ppm representing CH_3, CH_2, HC=CH and C=O, respectively. 2-amino-4-(3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)-1,6-dihydro-6-oxopyrimidine-5-carbonitrile **3** was obtained upon treatment of **1** with ethyl cyanoacetate and guanidine sulfate in 40 % aqueous

NaOH. In addition, compound **1** was heated under reflux in glacial acetic acid with aryl isothiocyanate derivatives for the proper time, and afforded the corresponding aryl thioxo imidazolidinone derivatives **4a–c**. The chemical shift of the hydantoin's CH proton of compounds **4a–c** are δ 6.43, 6.65 and 6.64 ppm, respectively, which indicate that the cis form is afforded [19–21]. Furthermore, the reaction of **1** and barbituric acid in ethanol with catalytic amount of piperidine gave the corresponding 5-((3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)methylene)pyrimidine-2,4,6(1*H*, 3*H*,5*H*)-trione (**5**). 5-(3-(4-bromophenyl)-1-ethyl-1*H*-pyrazol-4-yl)-2*H*-1,2,4-triazols **6a**,**b** were obtained via reaction of **1** with semicarbazide derivatives and anhydrous sodium acetate (Scheme 2). On the other hand, cyclocondensation of the chalcone **2** with guanidine sulfate in refluxing ethanol in the presence of 40 % aqueous NaOH solution gave 4-chlorophenyl)pyrimidin-2-amine **7**, while with ethyl cyanoacetate and anhydrous ammonium acetate in refluxing ethanol or hydrazine hydrate in glacial acetic acid afforded the corresponding 1,2-dihydro-2-oxopyridine-3-carbonitrile **8** and 4,5-dihydropyrazol-1-yl)ethanone **9** derivatives, respectively (Scheme 2) (cf. "Experimental" section, and Figs. 5–52 of Supplementary material).

Biological evaluations

In vitro antitumor screening

A cell-based approach was used for the in vitro antitumor screening [22–25] against 4 cancer cell lines, namely: hepatocellular carcinoma HepG2, breast cancer MCF-7, lung carcinoma A549 and prostatic cancer PC3. For cytotoxicity assessment, MTT assay was used [25–27], and DMSO and doxorubicin were used as negative and positive controls, respectively. Compounds **1–9** showed very good inhibitory effects on the four cell lines. IC_{50} values were calculated for compounds showing high antiproliferative activity using Graph-Pad PRISM v.5 software and the SPSS 11 program (Table 1), and three replicates were used for each tested concentration. The IC_{50} (potency) of the tested compounds were determined due to the attached dose response curves possessing standard error values for each data point (tested concentration) (Figs. 1–4 of Supplementary material). Most of our synthesized compounds; **3**, **4c**, **5**, **6b**, **7**, **8** and **9** showed higher activity than the standard reference doxorubicin (IC_{50} = 34.24 μM) on the HepG2 cell line with IC_{50} of 18.70, 21.74, 11.30, 24.57, 9.78, 10.44 and 11.74 μM, respectively, with compound **2** being the most potent with an IC_{50} of 9.13 μM, whereas compounds **4a** and **6a** showed low activity with IC_{50} values of 86.96 and 81.09 μM respectively. **4b** is the only inactive compound on the same cell line. As for the MCF-7 cell line, compound **7** is the most active (IC_{50} = 16.52 μM) in contrast to 20.85 μM for the standard on the same cell line. Compound **5** also showed higher activity (IC_{50} = 20 μM) than doxorubicin, while compounds **2**, **9**, **3c** and **6b** had moderate activities with IC_{50} values of 29.35, 52.17, 36.67 and 28.91 μM, respectively. The rest of the compounds showed minimal or no activity on MCF-7. Concerning the lung carcinoma cell line A549, compound **7** (IC_{50} = 6.52 μM) had an activity very

Table 1 Antiproliferative activity of compounds 1–9 four cell lines: hepatocellular carcinoma (*HepG2*), breast cancer (*MCF7*), lung carcinoma (*A549*) and prostate cancer (*PC3*) cell lines

Compound	IC_{50} μM			
	HepG2	MCF-7	A549	PC3
1	>100	>100	>100	>100
2	9.13	29.35	21.11	15.22
3	18.70	>100	>100	>100
4a	86.96	>100	>100	>100
4b	>100	91.78	22.17	>100
4c	21.74	36.67	>100	>100
5	11.30	20.00	>100	29.78
6a	81.09	99.11	>100	>100
6b	24.57	28.91	28.48	53.04
7	9.78	16.52	6.52	9.13
8	10.44	>100	12.39	>100
9	11.74	52.17	15.44	82.89
Doxorubicin	34.24	20.85	5.93	38.02

IC_{50} concentration which gives 50 % growth inhibition; N.B. IC_{50} values are the mean SD of three separate experiments

close to doxorubicin (IC_{50} = 5.93 μM), also compounds **2**, **4a**, **6b**, **8** and **9** had high activity of 21.11, 22.17, 28.48, 12.39 and 15.44 μM, respectively. Compounds **1**, **3**, **4a**, **4c**, **5** and **6a** had no activity on the A549 cell line. Finally, the prostatic cancer PC3 cell line which exhibited resistance towards most of our compounds, except for compounds **2**, **5** and **7** which had more potent activity (IC_{50} = 15.22, 29.78 and 9.13 μM) than our standard reference (IC_{50} = 38.02 μM). Compounds **6b** and **9** had low activity with IC_{50} values of 53.04 and 82.89 μM, respectively.

Conclusion

In conclusion, a series of new tri-substituted pyrazole derivatives **1–9** were designed, synthesized, and screened for their anticancer activity on various cancer human cell lines, namely; hepatocellular carcinoma HepG2, breast cancer MCF-7, lung carcinoma A549 and prostatic cancer PC3. Many of the newly synthesized compounds showed remarkable activity on the tested cell lines, while compounds **2**, **6b**, **7** and **9** showed antiproliferative activity on the four cell lines. Compound **2** had the highest activity on the HepG2 cell line (IC_{50} = 9.13 μM) compared to the reference standard doxorubicin (IC_{50} = 34.24 μM). Compound **7** was the most potent of the synthesized compounds, with IC_{50} values of 16.52, 6.52 and 9.13 μM, (doxorubicin IC_{50} = 20.85, 5.93 and 38.02 μM) on MCF-7, A549 and PC3 cell lines, respectively. Accordingly, the newly synthesized tri-substituted pyrazole derivatives can serve as a starting point for the development of potent anti-cancer agents.

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