RESEARCH ARTICLE

A convenient synthesis and molecular modeling study of novel pyrazolo[3,4-d]pyrimidine and pyrazole derivatives as anti-tumor agents

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Abstract

An efficient method to obtain ethyl 5-amino-1-tosyl-1H-pyrazole-4-carboxylate (**3**) was outlined using condensation reactions of 4-methylbenzenesulfonylhydrazide with (E)-ethyl 2-cyano-3-ethoxyacrylate. The cyclocondensation reaction of this substrate and its hydrazide derivative with urea, thiourea, formamide, formic acid, D-glucose, o-phenylenediamine, 4-dimethylaminobenzaldehyde, anthracene-9-carbaldehyde, thioglycolic acid and carbon disulphide then with hydrazine hydrate analogues furnished a series of pyrazolo[3,4-d]pyrimidine, pyrazolo[3,4-d]oxazin-4-one, pyrazole-4-glucoside, 4-benzo[d]imidazole, 1,3-thiazolidinone, 1,3,4-oxadiazol-2(3H)-thione and 1,2,4-triazol-5(4H)-thione derivatives respectively. The structure of the compound **3** was supported by X-Ray crystallographic data. Orally administrated, one of each of the series of pyrazoles showed significant effects in mouse tumor model cancer cell lines (EAC) and two human cancer cell lines of Colon cancer (HCT-29) and Breast cancer (MCF-7) with docking studies.

Introduction

The chemistry of 1H-pyrazole-containing compounds is particularly interesting because of their potential application in medicinal chemistry as analgesic[1,2], anti-inflammatory[3,4], anti-tumor[5,6], antimicrobial[7–11] and therapeutic agents[12], as well as based on their wide applications in agriculture as potent insecticides[13,14] and herbicides[15,16], although scarcely found in nature[17]. Due to many promising pharmacological, agrochemical, and analytical applications, a number of substituted pyrazoles are being used as inhibitors of heat-shock protein 90 (HSP90) and as therapeutics of cancer and therefore they have been the focus of many synthetic targets over the past decades[18]. Furthermore, it has also been found that 1H-pyrazole based heterocyclic structures have attracted synthetic interest for being an essential moieties in many chemotherapeutic agents with potential anti-parasitic[19,20], anti-malarial[21] and antiviral activities[22,23]. As far as the anticancer activity is concerned, literature citation revealed that a wide range of pyrazole derivatives were reported to contribute to a variety of anti-neoplastic potentials against a wide range of cancer cell lines[24–27]. Moreover, many pyrazole derivatives are associated with anti-fungal, anti-bacterial[28] and anti-pyretic[29] properties. Pyrazolopyrimidines and related fused heterocycles are of interest as potential bioactive molecules. They are known to exhibit pharmacological activities such as CNS-depressant[30], anticancer[31–33] and tuberculostatic[34] activities. Furthermore, some pyrazolo(3,4-d)pyrimidine derivatives

demonstrated significant antiviral activity[35,36]. There is no much difference in the basic structures of pyrazolopyrimidines and purines[37]. The above facts and our interest[38] in the synthesis of new biologically active isolated and fused substituted heterocycles prompted us to synthesize new substituted pyrazoles linked to five membered heterocycles and fused pyrazolopyrimidine and oxazine ring systems in order to increase its biological activity and evaluate its activity as anticancer agents.

Experimental

Chemistry

General procedures

All melting points were measured using a Reichert Thermovar apparatus (Depew, NY) and are uncorrected. Yields listed are of isolated compounds. The IR spectra were recorded on a perkin-Elmer model 1720 FTIR spectrometer (Waltham, MA) for KBr disc. NMR spectra were recorded on a varian Gemini 300 BB NMR Spectrometer (Buffalo, NY) at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts were reported in δ scale (ppm) relative to TMS as a reference standard and the coupling constants J values are given in Hz. Mass spectra were recorded on GC/MS Finnegan SSQ 7000 spectrophotometer and GC Ms-QP 1000 EX mass spectrometer at 70 eV (Waltham, MA). The progress of the reactions was monitored by TLC using aluminum silica gel plates 60 F245. Elemental analyses were performed at the Microanalytical Centre at Faculty of Science, Cairo University, Egypt.

Reaction of sulfonylhydrazide 1 with cyanoacrylate 2. To a solution of 4-methylbenzenesulfonylhydrazide (**1**)

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(1.86 g, 0.01 mol) in absolute ethanol (20 ml) was added (*E*)-ethyl 2-cyano-3-ethoxyacrylate (**2**) (1.69 g, 0.01 mol). The reaction mixture was heated at reflux temperature for 16 h, cooled to room temperature. The solvent was evaporated under reduced pressure. The resulting solid was recrystallized from ethanol to afford ethyl 5-amino-1-tosyl-1*H*-pyrazole-4-carboxylate (**3**) as a white solid, 2.01 g, (64.97%) m.p. 135–137 °C; dec. Diffraction-quality crystal was grown by slow diffusion of ethanol solution. IR (KBr) cm^{-1}: ν (NH$_2$) 3480, ν (C=O) 1693, ν (C=N) 1615; ^{1}H NMR (DMSO-d$_6$, 300 MHz): δ = 1.29 (t, 3H, J = 7.2, O–CH$_2$CH$_3$), 2.34 (s, 3H, CH$_3$), 4.18 (q, 2H, J = 2.7, O–CH$_2$CH$_3$), 4.22 (br.s, 2H, NH$_2$ exchangeable), 7.46 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.90 (s, 1H, pyrazole N=CH) ppm; ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 15.3 (CH$_3$), 21.2 (CH$_3$), 61.2 (CH$_2$), 106.5 (C-4), 128–140 (Ar. C + C-3), 148.5 (C-5), 160.5 (C=O) ppm; GC/MS: *m/z* (%) 309 $^{M+}$ (8.37), 245 (6.07), 199 (5.90), 155 (12.81), 91 (33.88), 63 (91.51), 44 (100). Anal. Calcd. for C$_{13}$H$_{15}$N$_3$O$_4$S (309.34): C, 50.47; H, 4.89; N, 13.58; Found: C, 50.39; H, 4.90; N, 13.45%.

Reaction of pyrazolocarboxylate 3 with formic acid. A suspension of **3** (3.09 g, 0.01 mol) in formic acid (85%; 20 ml) was heated at reflux temperature for 10 h. A solid product was obtained after cooling at room temperature was filtered off and recrystallized from ethanol to give 1-tosylpyrazolo(3,4-*d*1,3)oxazin-4(1*H*)-one (**4**) as a red powder 1.6 g (54.92%), m.p. 135–136 °C, IR (KBr) cm^{-1}: ν (C=O) 1644; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: δ = 2.34 (s, 3H, CH$_3$), 7.46 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.9 (s, 1H, pyrazole N=CH), 8.14 (s, 1H, oxazine N=CH); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 106.5 (C-3a), 128–140 (Ar. C + C-3), 147.0 (C-7a), 159.5 (C=O), 162 (C-6); Anal. Calcd. for C$_{12}$H$_9$N$_3$O$_4$S (291.28): C, 49.48, H, 3.11, N, 14.43; Found: C, 49.56, H, 3.0, N, 14.30.

Reaction of pyrazolocarboxylate 3 with formamide. A solution of **3** (3.09 g, 0.01 mol) in formamide (10 ml) was heated on oil bath at 180–190 °C for 4 h. The solution was then cooled to room temperature and diluted with 100 ml ice-cooled water. The precipitate that obtained was filtered off and recrystallized from ethanol to afford 1-tosyl-1*H*-pyrazolo(3,4-d)pyrimidin-4(5*H*)-one (**5**) as a yellow powder, 1.6 g (55.11%) m.p. 125–126 °C; IR (KBr) cm^{-1}: ν (NH) 3165, ν (C=O) 1700, ν (C=N) 1600; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: δ = 2.34 (s, 3H, CH$_3$), 7.46 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.9 (s, 1H, pyrazole N=CH), 8.0 (br.s, 1H, NH exchangeable), 9.0 (s, 1H, pyrimidine N=CH); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 105.2 (C-3a), 128–140 (Ar. C + C-3), 145.5 (C-6), 147 (C-7a), 157.65 (C=O); Anal. Calcd. for C$_{12}$H$_{10}$N$_4$O$_3$S (290.30): C, 49.65; H, 3.47; N, 19.30; Found: C, 49.60; H, 3.35; N, 19.35%.

General procedure for preparing compounds (6a,b). Ethyl 5-amino-1-tosyl-1*H*-pyrazole-4-carboxylate (**3**) (5 g, 1.61 mol) and urea (10 g, 16.66 mol) and/or thiourea (10 g, 13.15 mol) were heated together in an oil path at 150 °C for 30 min. The clear solution went mushy and heating was continued for ten minutes at 170 °C. The resulting solids were dissolved in dilute sodium hydroxide solution then acidified with acetic acid to obtain the crude products **6a** or **6b**.

1-Tosyl-1H-pyrazolo$^{3,4-d}$pyrimidin-4,6(5H,7H)-dione (6a). 2.72 g (54.88%) yellow powder, m.p. 290–291 °C, (ethyl acetate); IR (KBr) cm^{-1}: ν (NH) 3210, ν (C=O) 1690, ν (C=N) 1610; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: δ = 2.34 (s, 3H, CH$_3$), 7.46

(d, 2H, J = 8.1 Hz, Ar–H), 7.6 (s, 1H, -NH exchangeable), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.90 (s, 1H, N=CH pyrazole), 8.70 (s, 1H, NH exchangeable); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 127.2–140.7 (Ar. C + C-3), 148.5 (C-7a), 150.6 (C-3a), 154.3, 155.5 (2C=O); Anal. calcd. for C$_{12}$H$_{10}$N$_4$O$_4$S (306.30): C, 47.06; H, 3.29; N, 18.29; Found: C, 47.05; H, 3.25; N, 18.25.

6-Thioxo-1-tosyl-6,7-dihydro-1H-pyrazolo$^{3,4-d}$pyrimidin-4(5H)-one (6b). 2.63 g (52.6%) brown powder, m.p. 190–191 °C, (ethanol); IR (KBr) cm^{-1}: ν (NH) 3216, ν (C=O) 1670, ν (C=S) 1623, ν (C=N) 1610; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: δ = 2.34 (s, 3H, CH$_3$), 5.90 (br. s, 1H, NH exchangeable), 7.46 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.9 (s, 1H, N=CH pyrazole), 8.20 (br. s, 1H, NH exchangeable); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 127–140 (Ar. C + C-3), 148.6 (C-7a), 150.5 (C-3a), 155.23 (C=O), 172 (C=S); Anal. calcd. for C$_{12}$H$_{10}$N$_4$O$_3$S$_2$ (322.36): C, 44.71; H, 3.13; N, 17.38; Found: C, 44.65; H, 3.17; N, 17.46%.

Formation of 5-amino-1-tosyl-1H-pyrazol-4-carboxylic acid (7). A mixture of the pyrazolocarboxylate **3** (9.27 g, 0.03 mol) and sodium hydroxide (4 g, 0.1 mol) in 20 ml water was heated in an oil bath for 15 h. It was then cooled to 10 °C and acidified with HCl. The produced precipitate was filtered off, washed with water, and recrystallized from ethyl acetate to afford **7** as a red solid, 5.06 g (59.96%) m.p. 230–231 °C, IR (KBr) cm^{-1}: ν (OH) 3431, (NH) 3180, ν (C=O) 1640, ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: δ = 2.34 (s, 3H, CH$_3$), 4.50 (br. s, 2H, NH$_2$ exchangeable), 7.49 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 2H, J = 8.1 Hz, Ar–H), 7.9 (s, 1H, pyrazole N=CH), 11.00 (br. s, 1H, OH exchangeable), ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 106.2 (C-4), 128–140 (Ar. C + C-3), 146.6 (C-5), 165.0 (C=O); Anal. Calcd. for C$_{11}$H$_{11}$N$_3$O$_4$S (281.29): C, 46.97, H, 3.94, N, 14.94; Found: C, 46.84, H, 3.90, N, 14.84%.

Reaction of pyrazolocarboxylic acid 7 with Ac$_2$O. The pyrazolocarboxilic acid **7** (11.25 g, 0.04 mol) was heated with 30 ml acetic anhydride in an oil bath for 6 h. The mixture was cooled to room temperature, poured onto ice-cooled water, and stirred for 30 min. The solid obtained was filtered, washed with water, recrystallized from ethanol to afford 6-methyl-1-tosylpyrazolo(3,4-*d*1,3)oxazin-4(1*H*)-one (**8**) as a brown powder; 4.64 g (37.99%) m.p. 280–281 °C, IR (KBr) cm^{-1}: ν (C=O) 1696; ^{1}H NMR (DMSO-d$_6$) ppm: δ = 1.70 (s, 3H, CH$_3$), 2.34 (s, 3H, CH$_3$), 7.46 (d, J = 8.1, 2H, Ar–H), 7.70 (d, J = 8.1, 2H, Ar–H), 7.90 (s, 1H, pyrazole CH); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 21.2 (CH$_3$), 25.2 (CH$_3$), 106.2 (C-9), 128–140 (Ar. C + C-3), 147 (C-7a), 154.3 (C=O), 159.6 (C6); Anal. Calcd. for C$_{13}$H$_{11}$N$_3$O$_4$S (305.31): C, 51.14, H, 3.63, N, 13.76; Found: C, 51.0, H, 3.60, N, 13.80%.

Reaction of pyrazolocarboxylic acid 7 with o-phenylenediamine. A mixture of **7** (2.81 g, 0.01 mol) and *o*-phenylenediamine (1.08 g, 0.01 mol) in DMF (30 ml) was heated at reflux temperature for 6 h. The reaction mixture was allowed to cool at room temperature and poured onto cold water. The solid formed was filtered off, recrystallized from ethanol to afford 4-(1H-benzo^{d}imidazol-2-yl)-1-tosyl-1*H*-pyrazol-5-amine (**9**) as a brown solid; 2 g (56.59%) m.p. 170–172 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3307, ν (NH) 3190, ν (C=N) 1602; ^{1}H NMR (DMSO-d$_6$, 300 MHz): δ = 2.34 (s, 3H, CH$_3$), 4.22 (br.s, 2H, NH$_2$ exchangeable), 7.19 (m, 2H, Ar–H), 7.46 (d, 2H, J = 8.1 Hz, Ar–H), 7.6 (m, 2H, Ar–H), 7.70 (br. s,1H, NH exchangeable), 7.74 (d, 2H, J = 8.1 Hz, Ar–H), 7.9 (s, 1H,

pyrazole N=$\underline{CH}$); ^{13}C NMR (DMSO-d$_6$ 75 MHz): $\delta = 21.2$ (CH$_3$), 100.2 (C-4), 115.2–141.7 (Ar. C + C-3), 132.9 (C-5), 153.0 (C=N); Anal. calcd. for: C$_{17}$H$_{15}$N$_5$O$_2$S (353.40): C, 57.78; H, 4.28; N, 19.82. Found: C, 57.67; H, 4.39; N, 19.70%.

Formation of 5-amino-1-tosyl-1H-pyrazole-4-carbohydrazide (10). A solution of the pyrazolocarboxylate **3** (3.09 g, 0.01 mol) and hydrazine hydrate (0.15 mol) in ethanol (50 ml) was stirred at room temperature for 2 h. The solid formed after filtration was recrystallized from ethanol to yield **10** as a white solid; 1.33 g (45.03%) m.p. 140–142 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3457, ν (NH) 3219, ν (C=O) 1630; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: $\delta = 2.34$ (s,3H, CH$_3$), 4.22 (br. s, 2H, NH$_2$ exchangeable), 5.8 (br. s, 2H, NH$_2$ exchangeable), 7.46 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.87 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.9 (s, 1H, pyrazole N=$\underline{CH}$), 8.0 (br. s, 1H, NH exchangeable); ^{13}C NMR (DMSO-d$_6$,75 MHz): $\delta = 21.2$ (CH$_3$), 128–140 (Ar. C + C-3), 148.0 (C-5), 150.2 (C-4), 165.0 (C=O) ppm; Anal. Calcd. for C$_{11}$H$_{13}$N$_5$O$_3$S (295.32): C, 44.74; H, 4.44; N, 23.71; Found: C, 44.65; H, 4.36; N, 23.74%.

Reaction of carbohydrazide 10 with D-glucose. To a well stirred solution of the D-glucose (0.9 g, 5 mmol) in water (1 ml), and glacial acetic acid (1 ml); was added the carbohydrazide **10** (1.47 g, 5 mmol) in ethanol (15 ml). The mixture was heated under reflux for 3 h. and the resulting solution was concentrated and left to cool. The precipitate formed was filtered off, washed with water, then dried and recrystallized from ethanol to afford 5-amino-N′-(d-glucopyranosyl)-1-tosyl-1H-pyrazole-4-carbohydrazide (**11**) as a white powder, 1.37 g (59.9%) m.p. 143–144 °C; IR (KBr) cm^{-1}: ν (OH) 3460–3449, ν (NH) 3265,ν 1695 (C=O), ν (C=N) 1616; ^{1}H NMR (DMSO-d_6, 300 MHz): $\delta = 2.34$ (s, 3H, CH$_3$), 3.30–3.35 (m, 2H, H-6′,6″), 3.70 (m, 1H, H-5′), 4.12 (m, 1H, H-4′), 4.27 (t, $J = 7.4$ Hz, 1H, H-3′), 4.22 (br. s, 2H, NH$_2$ exchangeable), 4.34 (dd, $J = 7.4$ Hz, $J = 7.8$ Hz, 1H, H-2′), 4.44 (m, 1H, OH), 4.45 (d, $J = 6.4$ Hz, 1H, OH), 5.20 (m, 1H, OH), 5.60 (t, $J = 4.6$ Hz, 1H, OH), 5.79 (t, $J = 4.6$ Hz, 1H, OH), 7.0 (br. s, 1H, NH exchangeable), 7.47 (d, 2H, $J = 8.1$ Hz, Ph-H), 7.6 (s, 1H, N=$\underline{CH}$), 7.87 (d, 2H, $J = 8.4$ Hz, Ph-H), 7.89 (s, 1H, pyrazole N=$\underline{CH}$); ^{13}C NMR (DMSO-d$_6$, 75 MHz): $\delta = 21.2$ (CH$_3$), 62.1 (C-6′), 63.0 (C-5′), 69.2 (C-4′), 74.3 (C-3′), 75.7 (C-2′), 128–142 (Ar. C + C-3), 148.0 (C-5), 150.5 (C-4), 151.5 (C-1′), 164.2 (C=O) ppm, Anal. calcd. for: C$_{17}$H$_{23}$N$_5$O$_8$S (457.46): C, 44.63; H, 5.07; N, 15.31. Found: C, 44.56; H, 5.15; N, 15.37%.

5-(5-Amino-1-tosyl-1H-pyrazol-4-yl)-1,3,4-oxadiazole-2(3H)-thione (12). To a solution of **10** (3.37 g, 0.01 mol) in ethanol (20 ml) was added a potassium hydroxide solution (0.56 g, 0.01 mol) in water (2 ml) and carbon disulphide (4 ml). The mixture was heated under reflux for 10 h. The solvent was evaporated and the residue was dissolved in water, filtered, and the filtrate was acidified with dilute hydrochloric acid with continuous stirring. The precipitate that formed was filtered off, washed with water and recrystallized from ethanol to yield 2.7 g (80%) **12** as a yellow solid; m.p. 140–141 °C; IR (KBr) cm^{-1}: ν (NH$_2$) 3252, ν (NH) 3141, ν (C=S) 1670, ν (C=N) 1600; ^{1}H NMR (DMSO-d$_6$, 300 MHz): $\delta = 2.34$ (s,3H, CH$_3$), 4.20 (br. s, 2H, NH$_2$ exchangeable), 7.1 (br. s, 1H, NH exchangeable); 7.46 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.84 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.90 (s, 1H, pyrazole N=$\underline{CH}$),^{13}C NMR (DMSO-d$_6$, 75 MHz): $\delta = 21.2$ (CH$_3$), 98.2 (C-4), 128–142 (Ar. C + C-3), 148.0 (C-5), 156.5 (C=N imine), 190 (C=S) ppm; Anal. calcd. for: C$_{12}$H$_{11}$N$_5$O$_3$S$_2$ (337.38): C, 42.72; H, 3.29; N, 20.76; Found: C, 42.65; H, 3.35; N, 20.80%.

4-Amino-3-(5-amino-1-tosyl-1H-pyrazol-4-yl)-1H-1,2,4-triazole-5(4H)-thione (13). A solution of compound **12** (3.37 g, 001 mol) and hydrazine hydrate (0.15 mol) in ethanol (30 ml) was refluxed for 6 h. The reaction mixture was cooled at room temperature and the solvent was evaporated under reduced pressure. The solid that obtained was crystallized from ethanol to afford **13** as a white powder, 2.1 g (60%) m.p. 135–136 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3460, ν (NH) 3268, ν (C=S) 1696, ν (C=N) 1615; ^{1}H NMR (DMSO-d$_6$, 300 MHz): $\delta = 2.0$ (br. s, 2H, NH$_2$ exchangeable), 2.34 (s, 3H, CH$_3$), 4.22 (br. s, 2H, NH$_2$ exchangeable), 7.0 (br. s, 1H, NH exchangeable),7.46 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.84 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.90 (s, 1H, pyrazole N=$\underline{CH}$); ^{13}C NMR (75 MHz, DMSO): $\delta = 21.2$ (CH$_3$), 98.2 (C-4), 128–142 (Ar. C + C-3), 148.0 (C-5), 149.8 (C=N imine), 190.4 (C=S); Analysis Calcd. for C$_{12}$H$_{13}$N$_7$O$_2$S$_2$ (351.41): C, 41.01, H, 3.73, N, 27.90; Found C, 41.10, H, 3.65, N, 27.80%.

Generals procedure for preparation of the shiff's bases (15, 18). A solution of **10** (2.95 g, 0.01 mol) and the appropriate aldehydes, (4-(dimethylamino)-benzaldehyde **14** or anthracene-9-carbaldehyde **17** (0.01 mol) in ethanol (30 ml) and a few drops of acetic acid was heated at reflux temperature for 6 h. The reaction mixture was cooled at room temperature and the solvent was evaporated under reduced pressure. The resulting solid was crystallized from ethanol to obtain the shiff's bases **15** and/or **18**.

5-Amino-N′-(4-(dimethylamino)benzylidene)-1-tosyl-1H-pyrazole-4-carbohydrazide (15). This compound was obtained as a brown solid (benzene), 2.34 g (54.86%) m.p. 100–101 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3455, ν (NH) 3266, ν (C=O) 1666, ν (C=N) 1600; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: $\delta = 2.34$ (s, 3H, CH$_3$), 3.0 (s, 6H, 2CH$_3$), 4.22 (br. s, 2H, NH$_2$ exchangeable), 6.77 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.0 (br. s, 1H, NH exchangeable), 7.50 (d, 2H, $J = 8.2$ Hz, Ar–H), 7.70 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.86 (d, $J = 8.2$, 2H, Ar–H), 7.90 (s,1H, pyrazole N=$\underline{CH}$), 9.67 (s,1H, N=CH); ^{13}C NMR (DMSO-d$_6$, 75 MHz): $\delta = 21.2$ (CH$_3$), 41.0 (2CH$_3$), 128–142 (Ar. C + C-3), 148.0 (C-5), 149.3 (N=CH), 151 (C-4), 164.2 (C=O) ppm, Anal. Calcd. for C$_{20}$H$_{22}$N$_6$O$_3$S (426.49): C, 56.32, H, 5.20, N, 19.70; Found C, 56.36, H, 5. 30, N, 19.73%.

5-Amino-N′-(anthracen-9-ylmethylene)-1-tosyl-1H-pyrazole-4-carbohydrazide (18). This compound was obtained as a brown solid (benzene), 2.56 g (52.98%) m.p. 97–98 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3458, ν (NH) 3265, ν (C=O) 1666, ν (C=N) 1610; ^1H NMR (DMSO-d$_6$, 300 MHz) ppm: $\delta = 2.38$ (s, 3H, CH_3), 4.22 (br. s, 2H, NH_2 exchangeable), 7.0 (br. s, 1H, NH exchangeable), 7.46 (d, $J = 8.4$, 2H, Ar–H), 7.6 (m, 2H, Ar–H), 7.70 (m, 2H, Ar–H), 7.84 (d, $J = 8.2$, 2H, Ar–H), 7.90 (s, 1H, N=$\underline{CH}$ pyrazole), 8.20 (d, $J = 8.4$, 2H, Ar–H), 9.0 (d, $J = 9.3$, 2H, Ar–H), 9.05 (s, 1H, Ar–H), 11.48 (s, 1H, -N=$\underline{CH}$); ^{13}C NMR (DMSO-d$_6$,75 MHz): $\delta = 21.2$ (CH$_3$), 125.5–142.8 (Ar. C + C-3), 149.3 (CH=N), 148.0 (C-5), 151.6 (C-4), 164.2 (C=O), Anal. Calcd. for C$_{26}$H$_{21}$N$_5$O$_3$S (483.14): C, 64.58, H, 4.38, N, 14.48; Found C, 64.47, H, 4.25, N, 14.55%.

General procedure for preparing compounds (16, 19). A mixture of compounds **15** or **18** (0.01 mol) and thioglycolic acid (0.01 mol) in benzene (30 ml) was stirred at room temperature for 30 min, then refluxed for 10 h, cooled to room temperature. The solvent was removed under reduced pressure and the solid that obtained was recrystallized from the proper solvent to yield **16** and/or **19**.

5-Amino-N-(2-(4-(dimethylamino)phenyl)-4-oxothiazolidin-3-yl)-1-tosyl-1H-pyrazole-4-carboxamide (16). This compound was obtained as a yellow powder (ethyl acetate) 1.9 g (37.95%) m.p. 209–210 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3458, ν (NH) 3266,

ν (C=O) 1690, ν (C=N) 1610; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: $\delta = 2.39$ (s, 3H, CH$_3$), 3.0 (s, 6H, 2CH$_3$), 3.6 (s, 2H, CH$_2$), 4.22 (br. s, 2H, NH$_2$ exchangeable), 5.20 (s, 1H, thiazolidine CH), 6.90 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.0 (br. s, 1H, NH exchangeable), 7.2 (d, 2H, $J = 8.2$ Hz, Ar–H), 7.47 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.84 (d, $J = 8.2$, 2H, Ar–H), 7.9 (s,1H, pyrazole N=C<u>H</u>); ^{13}C NMR (DMSO-d$_6$ 75 MHz): $\delta = 21.2$ (CH$_3$), 39.4 (CH$_2$), 41.0 (2CH$_3$), 64.2 (CH), 128.0–145.0 (Ar. C + C-3), 148.0 (C-5), 151.5 (C-4), 165.0 (C=O), 169 (C=O); Anal. Calcd. for C$_{22}$H$_{24}$N$_6$O$_4$S$_2$ (500.59): C, 52.78, H, 4.83, N, 16.79; Found C, 52.69, H, 4.79, N, 16.85%.

*5-Amino-N-(2-(4-(anthracen-9-yl)-4-oxothiazolidin-3-yl)-1-tosyl-1H-pyrazole-4-carboxamide (**19**).*   This compound was obtained as a yellow powder (ethanol) 2.5 g (44.83%) m.p. 200–201 °C, IR (KBr) cm^{-1}: ν (NH$_2$) 3456, ν (NH) 3266, ν (C=O) 1670, (C=N) 1600; ^{1}H NMR (DMSO-d$_6$, 300 MHz) ppm: $\delta = 2.34$ (s, 3H, CH$_3$), 3.6 (s, 2H, CH$_2$), 4.22 (br. s, 2H, NH_2 exchangeable), 5.13 (s,1H, CH), 7.0 (br. s, 1H, NH exchangeable), 7.13 (d, $J = 7.8$, 2H, Ar–H), 7.33 (d, $J = 7.8$, 2H, Ar–H), 7.46 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.9 (s,1H, pyrazole N=CH), 8.20 (m, 2H, Ar–H), 8.40 (d, $J = 8.1$, 2H, Ar–H), 8.60 (s, 1H, Ar–H), 8.74 (d, $J = 8.2$, 2H, Ar–H); ^{13}C NMR (DMSO-d$_6$ 75 MHz): $\delta = 21.2$ (CH$_3$), 36.4 (CH$_2$), 64.2 (CH), 125.0–142.0 (Ar. C + C-3), 148.0 (C-5), 151.0 (C-4), 164.2 (C=O), 168.0 (C=O); Anal. Calcd. for C$_{28}$H$_{23}$N$_5$O$_4$S$_2$ (557.64): C, 60.31, H, 4.16, N, 12.56; Found C, 60.30, H, 4.10, N, 12.55.

Biology

Materials used in the study

EAC cell lines were supplied from National Cancer Institute, Cairo, Egypt. Colon cancer (HCT-29) and Breast cancer (MCF-7), cell lines were obtained from Centre of Mycology and Biotechnology, Al-Azhar University, DMSO, crystal violet and trypan blue dye were purchased from Sigma (St. Louis, MO). DMEM, RPMI-1640, Fatal bovine serum (FBS), HEPES buffer solution, L-glutamine, gentamycin and 0.25% trypsin-EDTA were purchased from (BioWhittaker@ Lonza, Belgium). Anticancer activity was performed at Centre of Mycology and Biotechnology, Al-Azhar University, Nasr city, Cairo, Egypt.

Evaluation of cytotoxic effects of the chemical compounds

Method of trypan blue exclusion cytotoxicity assay.   The *in vitro* cytotoxicity of the newly synthesized pyrazole derivatives were subjected to a preliminary screening for their anti-tumor activity in breast mouse tumor (EAC) using trypan blue exclusion method[39]. Stock solution of each of the tested samples was prepared of 100 µg of the tested material dissolved in 100 µL DMSO and 900 µL saline. From the stock solution, various dilutions of the tested materials were prepared. EAC cells were obtained by needle aspiration of Ascite under aseptic conditions. 10% of the tumor cell suspension (2.5 × 10^6) was prepared in saline (0.1 ml Ascite + 0.9 ml saline). From the stock solution, five concentrations of the tested compound (200, 100, 50, 30 and 10) µg/ml were prepared in five sterile test tubes, then 100 µL of 10% Ascite suspension was added, the volume was then completed to 1 ml by adding saline solution. The test tubes were incubated at 37 °C for 1 h. The supernatant was discarded from each tube, and one drop of trypan blue solution was added to the pellet. The number of viable cells were counted for trypan blue dye exclusion in a hemocytometer and examined by light microscopy. The viability assay for each compound was performed in triplicate.

Cell viability assay for human cell lines using crystal violet.   Crystal violet stain (1%) is composed of 5% (w/v) crystal

violet and 50% methanol then made up to volume with DD water and filtered through Whatmann filter paper. The Colon cancer cells (HCT) and Breast cancer cells (MCF-7) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fatal bovine serum, 1% glutamine, HEPES buffer and 50 µg/ml gentamycin. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO$_2$ and were subtracted two times a week.

X-ray crystallography

A single crystal of compound 3 was obtained by slow evaporation from a solution of ethanol. The crystal structure was solved and refined using data collection. The chemical structure and ring-labeling system is shown in Figure 1. Crystallographic data (excluding structure factors) for the structure in this paper have been published[40].

Results and discussion

Chemistry

Reaction of *p*-toluenesulfonylhydrazide (**1**) with (*E*)-ethyl 2-cyano-3-ethoxyacrylate (**2**) in absolute ethanol under reflux afforded the pyrazole derivative **3**.

The structure of compound **3** was evidenced from its spectral and analytical data. Its IR spectrum showed bands at 3483, 1693 and 1615 cm^{-1} corresponding to NH$_2$, C=O and C=N groups respectively, its ^{1}H NMR spectrum showed signals at δ 1.29 ppm as triplet (3H) and a quartet at δ 4.18 ppm (2H) indicating the presence of the ethyl ester group and also showed a singlet at δ 2.34 ppm (3H) for the tosylmethyl group, in addition to an exchangeable singlet for NH$_2$ at 4.22 ppm. The CH of the pyrazole at position 3 was appeared as a singlet at δ 7.90 ppm and the phenyl ring protons were appeared as two doublets at 7.46 and 7.84 ppm with J coupling 8.1 Hz. ^{13}C NMR of compound **3** supported its structure. The mass spectrum of **3** revealed the presence of the molecular ion peak at *m/z* 309 (M$^+$, 8.37%). Moreover, the X-ray diffraction[40] of **3** (Figure 1) added a clear evidence for its proposed structure following the cyclization pathway of the intermediate outlined in Scheme 1.

When compound **3** was refluxed with formic acid, the pyrazolo(3,4-*d*1,3)oxazinone derivative **4** was obtained. IR spectrum of **4** showed the band of C=O of the oxazinone ring at 1644 cm^{-1}. Its ^{1}H NMR spectrum displayed a singlet at 8.13 ppm of the CH of the oxazinone ring and ^{13}C NMR spectrum of **4** showed a signal at δ 154.0 ppm of the C=O group. However, refluxing compound **3** with formamide afforded the pyrazolo(*3,4-d*)pyrimidin-4(5*H*)-one derivative **5**. Its IR spectrum revealed bands at 3164, 1700 and 1590 cm^{-1} characteristic for NH, C=O and C=N groups respectively, while its ^{1}H NMR spectrum showed an exchangeable singlet signal of the NH proton at
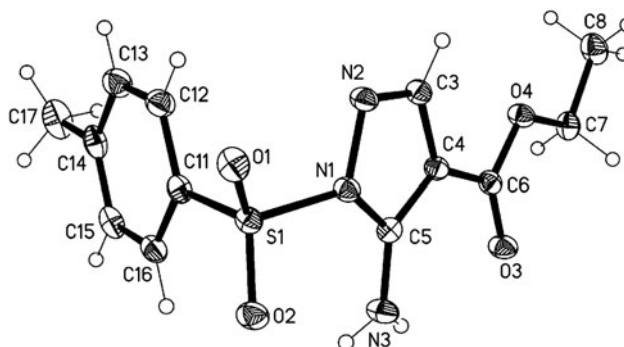

Figure 1. ORTEP Diagram of compound **3**.

δ 8.0 ppm in addition to a singlet at 9.0 ppm attributed to the CH of the pyrimidine ring. ^{13}C NMR spectrum of **5** showed a signal of the C=O group at 157.6 ppm in addition to the other signals for carbons of the fused rings.

On the other hand, fusion of **3** with urea and/or thiourea at 150 °C yielded the pyrazolo(3,4-*d*)pyrimidine-4,6-dione derivative **6a** and/or 6-thioxo-pyrazolo(3,4-*d*)pyrimidine-4(5*H*)-one

derivative **6b** respectively. The structures of compounds **6a**, **b** were confirmed by studying their IR, ^{1}H NMR, ^{13}C NMR spectra and elemental analyses which are in accord with the assigned structures cf. experimental. While refluxing **3** with NaOH (10% solution) followed by acidification with HCl afforded the acid derivative **7**, which upon heating with Ac$_2$O gave the pyrazolo(*3,4-d*1,3)oxazin-4(1*H*)-one derivative **8**. Furthermore,

Scheme 1. A proposed mechanism for the reaction of 4-methylbenzenesulfonylhydrazide (1) with (*E*)-ethyl 2-cyano-3-ethoxyacrylate (2) at refluxing temperature.
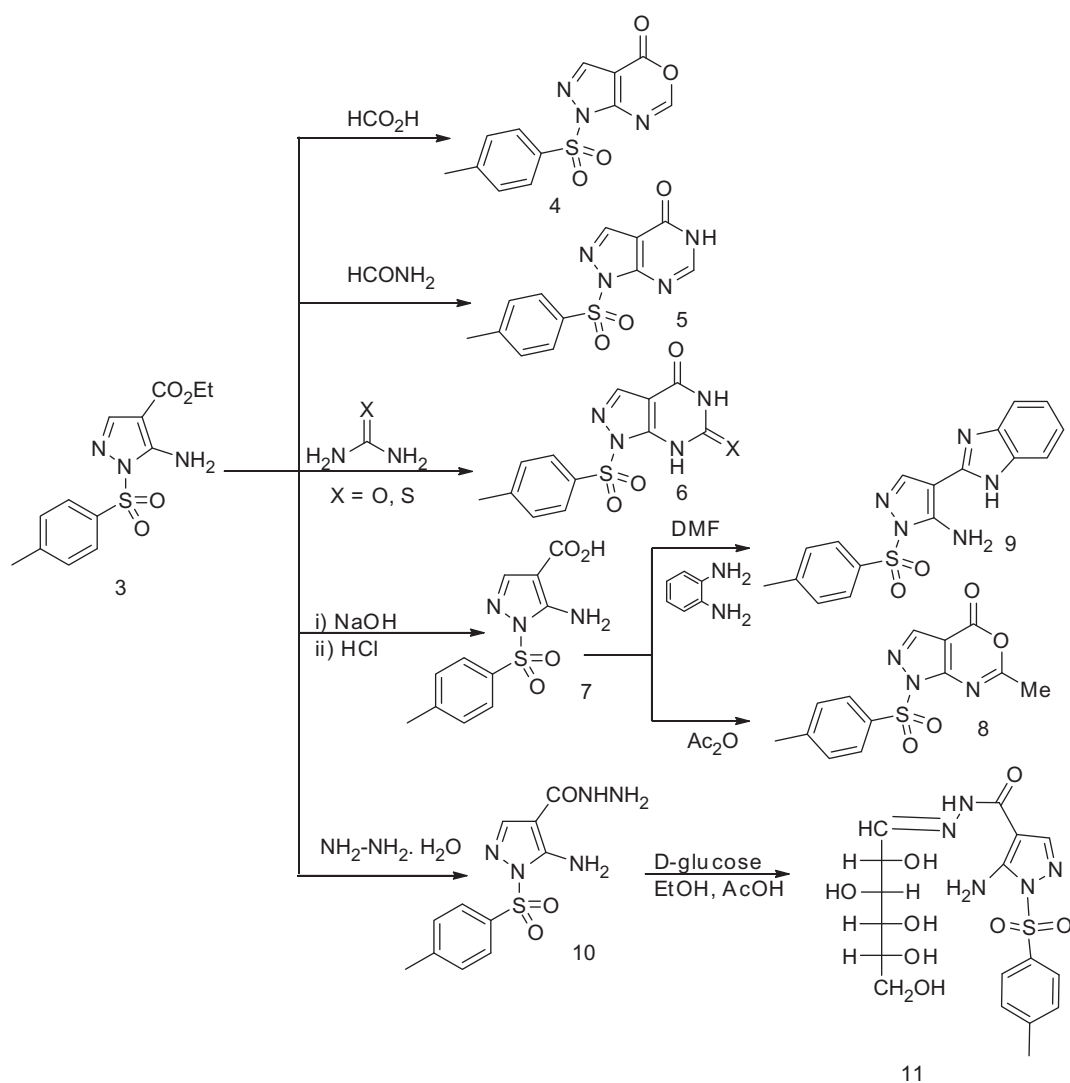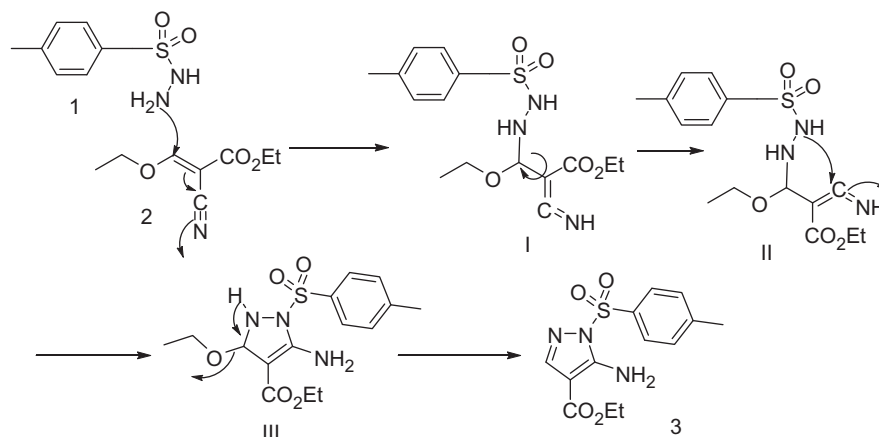
Scheme 2. Preparation pathways for compounds **4–11**.

reaction of **7** with *o*-phenylenediamine in DMF at reflux temperature afforded the benzo(*d*)imidazolopyrazole derivative **9**. The structures of compounds **7**, **8** and **9** were confirmed by IR, ^{1}H NMR, ^{13}C NMR spectra and elemental analyses cf. experimental.

Reaction of **3** with hydrazine hydrate in absolute ethanol at reflux temperature yielded the acid hydrazide derivative **10**. Its IR spectrum showed absorption bands of NH$_2$, NH and C=O groups respectively at 3457, 3219 and 1630 cm^{-1}. The ^{1}H NMR spectrum of **10** showed a singlet at δ 8.0 ppm corresponding to the NH group and a singlet at δ 5.8 ppm of the –NH$_2$ group of the hydrazide. The ^{13}C NMR spectrum of **10** showed a signals of the C=O group at 165.0 ppm.

The hydrazide **10** is used as a key starting material for the preparation of novel interesting heterocycles and sugar hydrazide. Thus, when compound **10** was allowed to react with D-glucose in an aqueous solution of ethanol and a catalytic amount of glacial acetic acid, the corresponding hydrazone **11** was obtained. The IR spectrum of **11** showed the bands of the OH groups of the glucose at 3460–3449 cm^{-1}. Its ^{1}H NMR spectrum displayed the presence of the CH$_2$, CH and OH groups of the sugar at their specific regions and so the ^{13}C NMR spectrum showed the presence of the sugar hydrazone carbons as outlined in the experimental section (Scheme 2).

On the other hand, refluxing the acid hydrazide **10** with carbon disulfide in alcoholic KOH afforded 1,3,4-oxadiazole-2(3*H*)-thione derivative **12**. Reaction of **12** with hydrazine hydrate in

ethanol gave the pyrazolothione derivative **13** in a good yield. The IR spectra of **12** and **13** revealed bands attributable to NH$_2$, NH and C=N groups, respectively. Further support for their assigned structures is gained from their ^{1}H NMR that revealed the presence of signals of NH$_2$, NH groups in **12** as well as one additional singlet signal for NH$_2$ group at 2.0 ppm for compound **13**. Their ^{13}C NMR spectra correspond very well with their assigned structures. Heating of the hydrazide derivative **10** with 4-dimethylaminobenzaldehyde **14** and/or anthracene-9-carbaldehyde **17** in ethanol and in the presence of a catalytic amount of acetic acid, the Shiff's bases **15**, **18** were afforded in good yields, which upon reaction with thioglycolic acid in benzene at reflux temperature afforded the thiazolidinone derivatives **16**, **19** in good yields (Scheme 3).

The structures of compounds **15–19** were evidenced from their IR spectra that revealed bands characteristic for NH$_2$, NH and CO groups. The ^{1}H NMR and ^{13}C NMR spectra correspond very well with their assigned structures.

Anticancer activity

The anticancer activity of the newly synthesized compounds were assessed in mouse tumor model cancer cell line (EAC) and two human cancer cell lines, Colon cancer (HCT-29), and Breast cancer (MCF-7) cell lines, using trypan blue exclusion assay and crystal violet for cytotoxicity assay. These assays are based on the alteration in the membrane integrity as determined by the uptake of the dye by dead cells, thereby giving a direct indication on cell
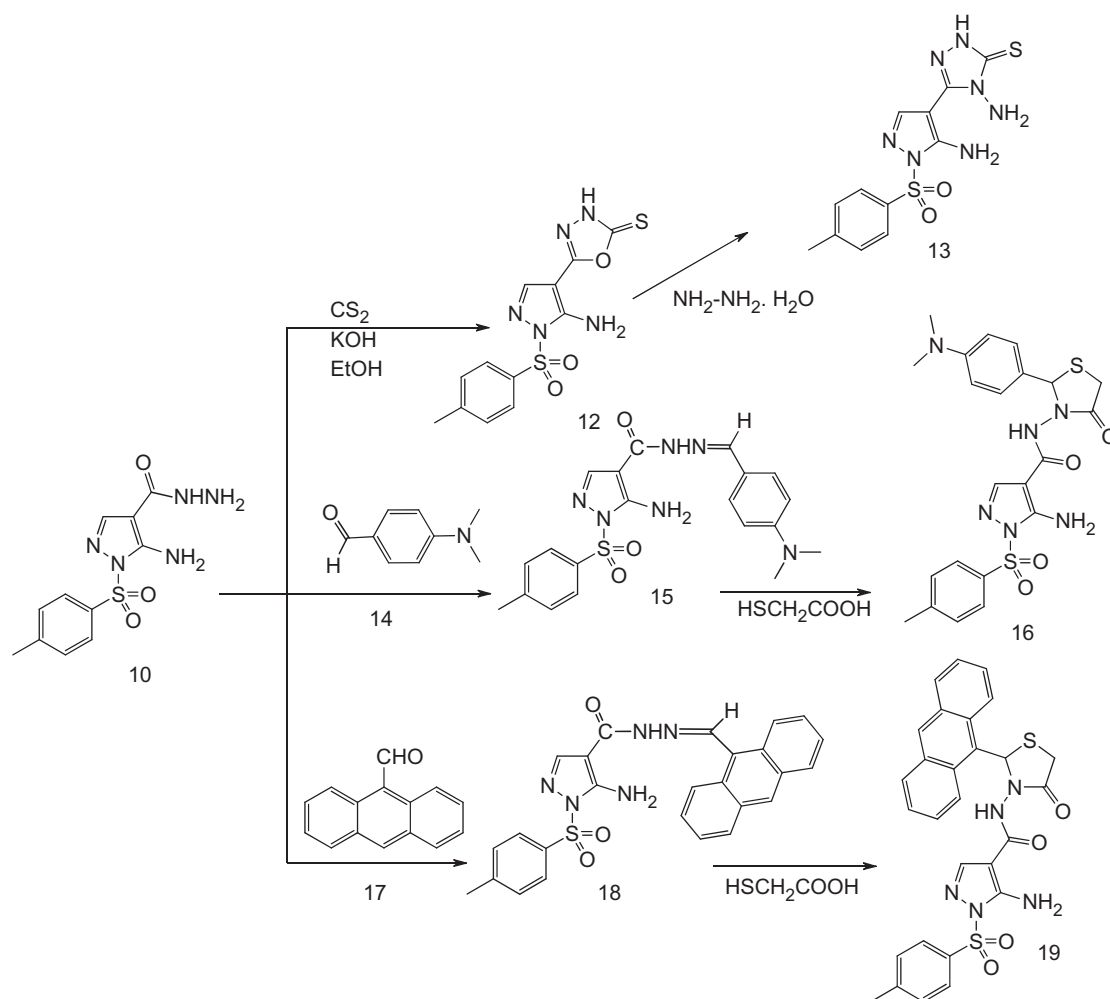
Scheme 3. Preparation pathways for compounds **12–19**.

viability. Cytotoxicity was considered as anticancer activity. Treatment of cell lines by chemical materials caused inhibition of the growth of cells.

Cytotoxicity evaluation

The cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100 μL of growth medium. Fresh medium containing different concentrations of the tested samples was added after 24 h of seeding. Serial two-folds dilutions of the tested chemical compounds were added to confluent cell monolayer dispensed into 96-well, flat-bottomed microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and/or without DMSO. (NB: The low DMSO percent used in the assay (maximal 0.1%) was found to be not effective). After incubation of cells for 24 h at 37 °C, various concentrations of samples (1.56, 3.125, 6.25, 12.5, 25, and 50) μg were added. Then the incubation continued for 48 h and the viable cells yield were determined by using trypan blue exclusion assay. All experiments were carried out in triplicate. The cytotoxicity of each compound was calculated, and the percentage of viable cells was plotted against the concentration. The preliminary investigation of the anticancer activity of the newly synthesized pyrazole derivatives was screened against EAC. The IC_{50} values of these compounds were determined compared to Doxorubicin.

Results and discussion for biology

The data expressed in (Table 1 and Figure 2) represents the cytotoxicity effect of the two compounds **6a** and **18** in colon cancer cells (HCT), the data showed that compound **18** exhibit the most potent antitumor activity ($IC_{50} = 10.24$ μ/ml) as compared to compound **6a** ($IC_{50} = 29.58$ μ/ml). The anticancer activity of the two compounds were also screened against breast cancer cell lines (MCF-7), in which both compounds exhibited anticancer activity with IC_{50} values (**18** $= 10.24$ μ/ml and **6a** $= 24.34$ μ/ml) respectively (Table 1, Figure 3).

From the data we concluded that the higher anticancer activity of compound **18** is attributed to the presence of the tosyl, C=N, and C=O groups in addition to NH_2 group that facilitates the H-Bonding with the active sites which increase its reactivity.

Molecular modeling

Ligand preparation. The structures of the newly synthesized compounds were drawn by using ChemDraw and converted to 3D structure with the help of 3D optimization tool. By using the LigPrep, the drawn ligand was geometry optimized by using the Optimized Potentials for Liquid Simulations-2005(OPLS-2005)

force field with the Steepest Descent followed by truncated Newton Conjugate gradient protocol. Partial atomic charges were computed using the OPLS-2005force field. The LigPrep is a utility in Schrodinger software suite that combines tools for generating3D structures from 1D (Smiles) and 2D (SDF) representation, searching for tautomers and steric isomers and geometry minimization of ligand. Finally, different poses had been prepared with different tautomeric and steric features for docking studies.

Protein preparation. The X-ray crystal structure of Cyclin Dependent Kinase2 (CDK2) was obtained from the RCSB protein data bank[41] (pdb: 3pj8). After evaluating numbers of entries, the best protein was selected by analyzing the protein with

Table 1. Docking score, binding energy and IC_{50} of the new compounds against mouse tumor model (EAC), HCT (colon) and MCF-7 (breast cancer cell lines).

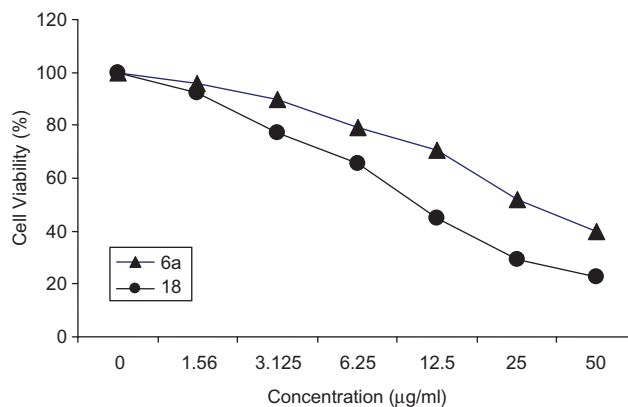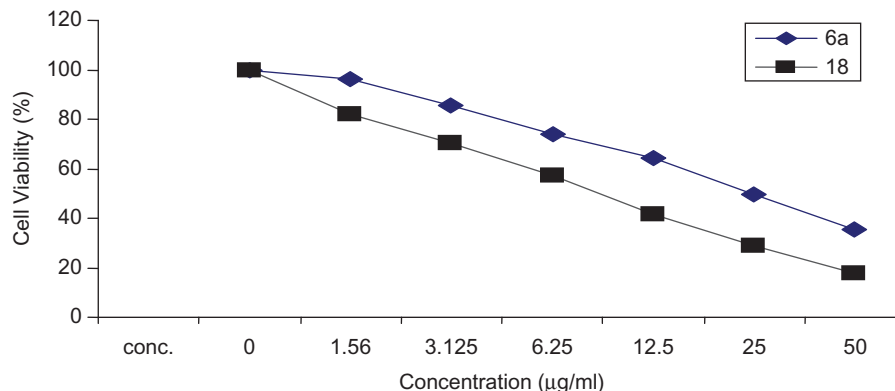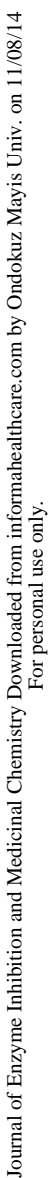| Tested compounds	IC_{50} (μg/ml) (EAC)	IC_{50} (μg/ml) (HCT)	IC_{50} (μg/ml) (MCF-7)	Docking score	Binding energy
4	200			−6.6	−51.125
5	200			−1.5	−54.981
6a	85	29.58	24.34	−8.3	−56.097
7	200			−6.5	−55.761
9	191			−5.0	−46.954
10	144			−5.48	−57.263
12	186			−5.6	−44.426
15	180			−4.6	−63.754
18	33	10.8	10.24	−5.9	−87.567
19	200			−4.6	−56.904

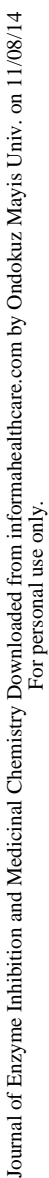Figure 2. *In vitro* cytotoxicity of the compounds **6a** and **18** on human cancer cell line (HCT).

Figure 3. *In vitro* cytotoxicity of the compounds **6a** and **18** on breast cancer cell line (MCF-7).

Figure 4. Electrostatic and hydrogen bond interactions of compound **18** with active site of CDK2.

Figure 5. Schematic interaction of compound **18** with amino acids in the active pocket of CDK2.

Ramachandran plot and ProCheck using SAVS server based on ligand and number of disallowed regions[42,43]. After selection, protein preparation wizard of Schrodinger suite[44] has been used to prepare protein. The protein was preprocessed separately by deleting the substrate cofactor as well as the crystallographic ally observed water molecules (water without *H* bonds), correcting the mistakes in PDB file, optimizing hydrogen bonds. After assigning charge and protonation state finally energy minimization with root mean square deviation (RMSD) value of 0.30 Å was done using OPLS2005 force field.

Docking studies.   The docking studies on compounds prepared through LigPrep were carried out in the active site of the protein. Receptor Vander Waals scaling for the non-polar atoms was set to 0.9 which makes the protein site ''roomier'' by moving back the surface of non-Polar Regions of the protein and ligand. This kind of adjustments emulate to some extent the effect of breathing motion to the protein site, it is a kind of giving breathing to the receptor, this approach softens the active site region of the receptor making it flexible[45]. The prepared protein and the ligand were employed to build energy grids
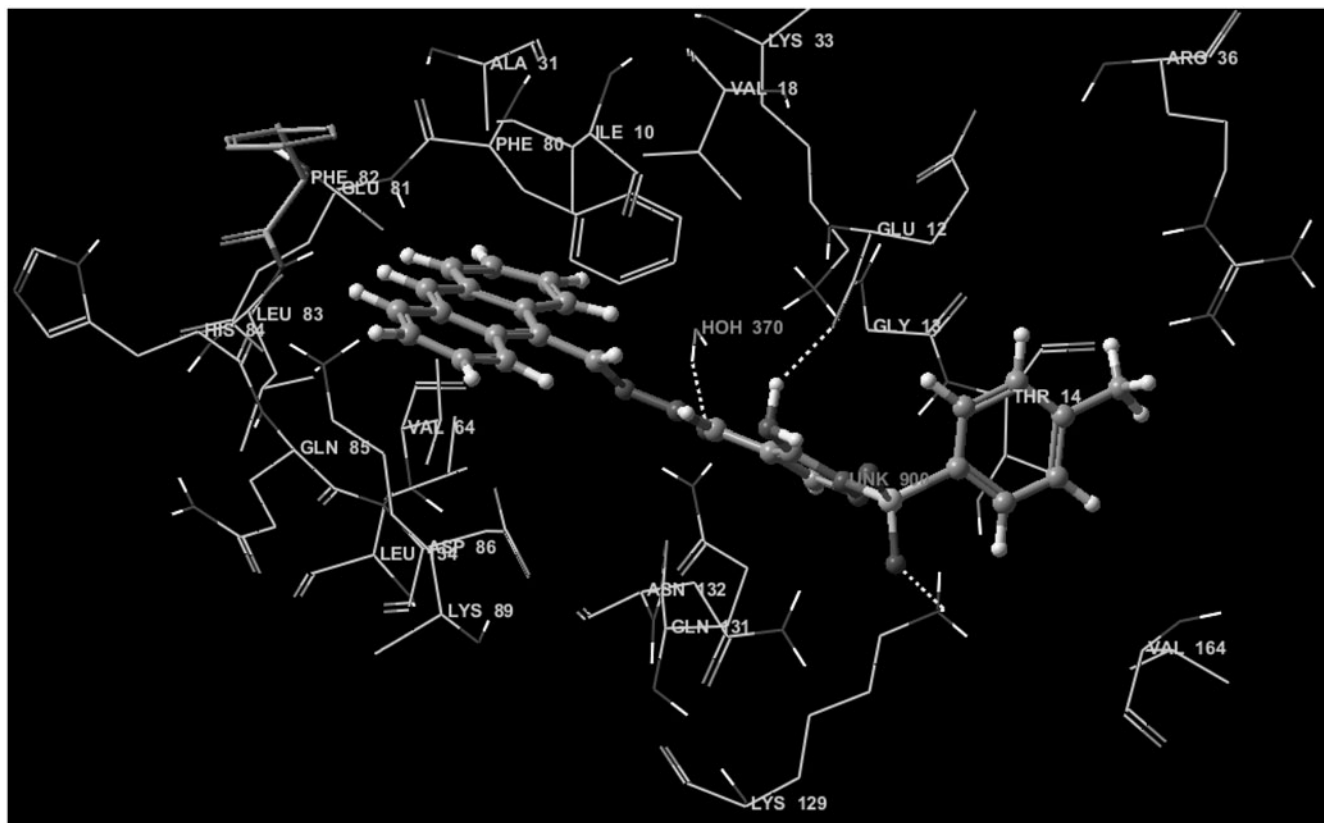
Figure 6. Hydrogen bond interactions of compound **18** with active site of CDK2.

using the default value of protein atom scaling (1.0 Å) within a cubic box, centered on the centroid of the X-ray ligand pose. After Grid generation, the ligand was docked with the protein by using Glide 5.5 module in extra precision mode (XP) which uses MCSA (Monte Carlo Based Simulated Algorithm) based minimization. The best docked pose (with lowest Glide Score value) obtained from Glide was analyzed[46,47]. The binding energy was calculated by Prim module. All the data are shown in Table (1). Figures 4–6 represents the Electrostatic and hydrogen bond interactions of compound **18** with active site of CDK2 and the interaction with amino acids in the active pocket of CDK2.

Conclusion

Ethyl 5-amino-1-tosyl-1H-pyrazole-4-carboxylate (**3**) was synthesized. The structure of the compound **3** was supported by X-Ray crystallographic data. Reactions of this substrate with urea, thiourea, formamide and formic acid furnished the bicyclic pyrazolooxazine and pyrazolo-pyrimidine ring systems. The hydrazide derivative was used as a key compound for synthesizing 1,3,4-oxadiazole, 1,2,4-triazole, thiazole and glucoside derivatives. Among the compounds screened, compound **18** showed the most potent anticancer activity and compound **6a** exhibited moderate anticancer activity towards (EAC) cancer cell line, while the rest of the other tested compounds exerted week anticancer activity. The two compounds **6a** and **18** were subjected to further evaluation of their antitumor activity in the two human cell lines (HCT-29 and MCF-7) using Crystal violet cytotoxicity assay and showed good to high activities. The results from docking study also represent that compound **18** had the more Electrostatic and hydrogen bond interactions with active site of CDK2.

Acknowledgements

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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