Research Article

Synthesis and Antitumor Activity of Novel Pyrazolo[3,4-D]Pyrimidine and their Tricyclic Derivatives

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ABSTRACT

Novel pyrazolo[3,4-*d*]pyrimidine derivatives carrying different groups at position 6 and varieties of tricyclic structures containing pyrazolopyrimidine core were synthesized aiming at having antitumor activity. Starting from 6-(chloromethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(1)**, several new compounds were synthesized. The newly synthesized compounds were tested *in vitro* against human adenocarcinoma cell line (MCF7). Some of the test compounds exhibited potent antitumor activity compared to the reference drug methotrexate, especially compounds **3b**, **3c** and **4a** which showed the highest activity (IC_{50}: 0.03µM) among the test compounds. Some azomethine derivatives and triazine ring enhanced the anticancer activity. While, diazine **6**, phenyl analogue of thiosemicarbazide **7b** and pyrazolone derivatives **9** decrease the activity.

Keywords: Pyrazolo[3,4-*d*]pyrimidine derivatives, Tricyclic structures, Antitumor activity, MCF7, Triazines, Diazines.

INTRODUCTION

Cancer still remains one of the most feared diseases in the world. When normal cells lose their regulatory mechanisms that control the growth and multiplication, cancer cells are formed. Cancer is caused by gene mutations or interfering with normal cell differentiation, initiated by chemicals, viruses, smoking or diet.[1]

Breast cancer is one of the most common cancer diseases in women worldwide.[2] Rapid increase in the incidence of breast cancer is attributed to the nature of the breast tissue and its complex with hormonal changes throughout the life.[3]

There are three traditional approaches for treatment of cancer: surgery, radiotherapy and chemotherapy. As combination therapy is more effective than using a single therapy, chemotherapy is usually used alongside surgery and radiotherapy.[4] Now, cancer chemotherapy is entering a new era by using molecular target therapeutics (highly selective agents which target specific molecular targets that are abnormal or over expressed in the cancer cells).[5]

It has been known that protein kinases (PKs) are enzymes that control most of aspect of cellular signal transmission under both physiological and pathological conditions. Phosphorylation of protein kinases resulted in the formation of the active form which catalyzes transferring of signals from cell surface receptors to inside the cell in order to elicit different cell functions. c-Src and EGFR –as protein kinases-are active in many solid tumors such as breast cancer. Compounds able to inhibit protein kinases play a critical role in cancer therapy.[6]

Recently, pyrazolo[3,4-*d*]pyrimidine derivatives have been evaluated *in vitro* as potent anticancer agents against several tumor cell lines especially breast cancer cell line (MCF7).[7]

The mechanism of action of most pyrazolo[3,4-*d*]pyrimidine derivatives is by binding the ATP-pocket of the protein kinases leading to have antiproliferative and apoptotic properties of many carcinomas.[8]

In 2011, He *et al*.[7] reported the broad-spectrum anticancer activity of 6-substituted pyrazolo[3,4-*d*]pyrimidine with a methylene spacer **I, II** and **III** (Fig. 1) against several tumor cell lines including A549, MCF7, HepG2 and PC-3.

In our previous work, 6-hydrazinocarbonyl methylpyrazolo [3,4-*d*] pyrimidine derivative **III** (Fig. 1) was synthesized and evaluated as antitcancer agent against MCF7.[9] Moreover, different moieties such as hydrazinyl[10] schiff's bases[9], pyrazole[11-13] and triazine [14] rings have been published and demonstrated as anticancer agents.

Based on the above findings, synthesis of novel 6-substituted pyrazolo[3,4-*d*]pyrimidines and their tricyclic derivatives is the aim of this study. Examination of their antitumor activity against breast cancer cell line (MCF7) is also one of the main objectives of this work.

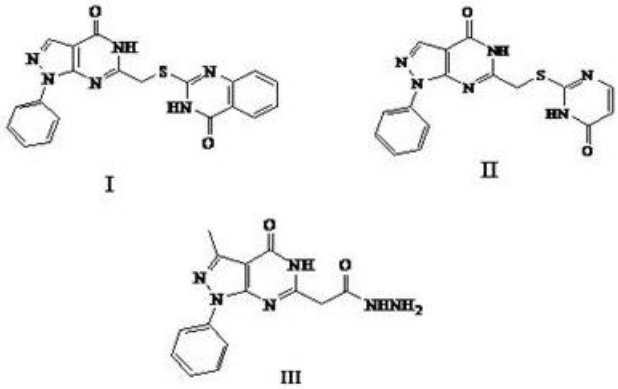


Figure 1: Some of the previously synthesized pyrazolo[3,4-*d*]pyrimidines.

MATERIALS AND METHODS

Chemistry

Melting points were determined using a Griffin apparatus and were uncorrected. IR spectra were recorded on Shimadzu IR 435 spectrophotometer and values were represented in cm^{-1}. ^1H NMR and ^{13}C NMR were carried out on Bruker 400 MHz spectrophotometer, Beni Suef University, Beni Suef, Egypt, using TMS as an internal standard and chemical shifts were recorded in ppm on δ scale. The electron impact (EI) mass spectra were recorded on Hewlett Packard 5988 spectrometer, Microanalytical center, Cairo University, Cairo, Egypt. Analytical thin layer chromatography (TLC) on silica gel plates containing UV indicator was employed routinely to follow the course of reactions and to check the purity of products. All reagents and solvents were purified and dried by standard techniques.

Synthesis of 6-(Hydrazinylmethyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (2)

6-(Chloromethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(1)** (5.48 g, 20 mmol) and hydrazine hydrate (99%, 4 mL, 80 mmol) were heated under reflux for 5 h. The solid formed on hot was cooled, washed with hot ethanol (95%, 20 mL), filtered, dried and crystallized from propanol. Yield: 75%; mp: 254-256^{0}C; IR (cm^{-1}): 3337, 3157 (2NH/NH_2), 2925, (CH aliphatic), 1712 (C=O); ^1H NMR ($CDCl_3$-d_6) δ ppm 2.65 (s, 3H, CH_3), 3.51 (s, 2H, CH_2), 6.40 (s, 2H, NH_2, D_2O exchangeable), 7.37 (t, 1H, J= 7.6 Hz, Ar-H), 7.43 (s, 1H, NH, D_2O exchangeable), 7.49 (t, 2H, J= 7.2 Hz, Ar-H), 8.04 (d, 2H, J= 7.6 Hz, Ar-H), 9.55 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR ($CDCl_3$-d_6): 13.63 (CH_3), 57.28 (CH_2), 122.27 , 125.85, 127.83, 129.16, 133.62, 149.44, 153.03, 164.56 (8C, aromatic carbon), 157.90 (Pyrimidine-C=O); MS *m/z*: 270 [$M^{+\cdot}$, 2.30%], 268 [(M - H_2)$^{+\cdot}$, 100%], 200 [($C_{11}H_{10}N_3O$)$^+$, 33.60%]. Anal. Calcd. For $C_{13}H_{14}N_6O$: C, 57.77; H, 5.22; N, 31.09. Found: C, 58.05; H, 5.50; N, 30.97.

General procedure for the synthesis of compounds (3a-c)

A mixture of 6-(Hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) and the appropriate aromatic aldehyde (1.5 mmol) in absolute ethanol (15 mL) were heated under reflux for 7 h. The separated solid was filtered, dried and crystallized from acetic acid 96%.

6-{[2-(4-Flurobenzylidene)hydrazinyl]methyl}-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (3a).

Yield: 79%; mp: 346-348^{0}C; IR (cm^{-1}): 3429 (br, 2NH), 2926, (CH aliphatic), 1680 (C=O); ^1H NMR (DMSO-d_6) δ ppm 2.67 (s, 3H, CH_3), 2.73 (s, 2H, CH_2), 7.17-8.33 (m, 9H, Ar-H), 8.35 (s, 1H, N=CH), 8.66 (s, 1H, NH, D_2O exchangeable), 9.97 (s, 1H, NH, D_2O exchangeable); MS *m/z*: 376 [$M^{+\cdot}$, 0.26%], 255 [(M - $4FC_6H_4CN$)$^{+\cdot}$, 0.32%], 80 [($C_4H_4N_2$)$^{+\cdot}$, 100%]. Anal. Calcd. For $C_{20}H_{17}FN_6O$: C, 63.82; H, 4.55; N, 22.33. Found: C, 64.20; H, 4.83; N, 22.40.

6-{[2-(4-Chlorobenzylidene)hydrazinyl]methyl}-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (3b).

Yield: 82%; mp: 269-271^{0}C; IR (cm^{-1}): 3426 (br, 2NH), 2979, (CH aliphatic), 1681 (C=O); ^1H NMR (DMSO-d_6) δ ppm 1.59 (s, 3H, CH_3), 2.73 (s, 2H, CH_2), 7.38-7.86 (m, 7H, Ar-H), 8.07 (d, 2H, J= 8 Hz, Ar-H), 8.34 (s, 1H, N=CH), 8.64 (s, 1H, NH, D_2O exchangeable), 9.93 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 13.62 (CH_3), 50.91 (CH_2), 122.25 , 127.29, 127.83, 128.03, 128.29, 129.17, 129.43, 129.77, 130.51, 131.40, 153.86, 164.56 (12C, aromatic carbon), 150.05 (N=CH), 161.09 (Pyrimidine-C=O); MS *m/z*: 393 [(M+2) $^{+\cdot}$,8.70%], 391 [(M) $^{+\cdot}$, 27.20%], 80 [($C_4H_4N_2$)$^{+\cdot}$, 100%]. Anal. Calcd. For $C_{20}H_{17}ClN_6O$: C, 61.15; H, 4.36; N, 21.39. Found: C, 60.70; H, 4.45; N, 21.03.

6-{[2-(2-Bromobenzylidene)hydrazinyl]methyl}-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (3c).

Yield: 82%; mp: 278-280^{0}C; IR (cm^{-1}): 3428 (br, 2NH), 2977, (CH aliphatic), 1693 (C=O); ^1H NMR (DMSO-d_6) δ ppm 1.68 (s, 3H, CH_3), 2.74 (s, 2H, CH_2), 7.32-7.70 (m, 6H, Ar-H), 8.08 (d, 2H, J= 7.2 Hz, Ar-H), 8.23 (t, 1H, J= 8.8 Hz, Ar-H), 8.34 (s, 1H, N=CH), 9.05 (s, 1H, NH, D_2O exchangeable), 10.40 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 13.63 (CH_3), 50.91 (CH_2), 122.27 , 125.85, 127.28, 127.67, 127.83, 128.76, 129.16, 129.29, 132.45, 133.38, 133.62, 150.03, 151.09 (13C, aromatic carbon), 150.05 (N=CH), 161.08 (Pyrimidine-C=O); MS *m/z*: 436 [(M) $^{+\cdot}$, 26.00%], 433 [(M-H_2) $^{+\cdot}$, 27.00%], 77 [C_6H_5)$^+$, 100%]. Anal. Calcd. For $C_{20}H_{17}BrN_6O$: C, 54.93; H, 3.92; N, 19.22. Found: C, 55.03; H, 4.28; N, 18.98.

General procedure for the synthesis of compounds (4a&b).

A mixture of 6-(Hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) and the appropriate aryl aldehyde (1.5 mmol) in absolute ethanol (15 mL), triethylamine (0.5 ml) was added. The mixture was heated under reflux for 9 h. The separated solid was filtered, dried and crystallized from benzene.

1-(Pyridin-3-yl)-[1,2,4]-triazino[3,4-d]pyrazolo[4,5-a]pyrimidin-6-one (4a).

Yield: 65%; mp: 260-262^{0}C; IR (cm^{-1}): 3137, 3058 (CH aromatic), 1673 (C=O); ^1H NMR (DMSO-d_6) δ ppm 2.57 (s, 3H, CH_3), 7.40 (m, 1H, Ar-H), 7.56 (m, 3H, Ar-H), 8.09 (d, 2H, J= 8 Hz, Ar-H), 8.72 (m, 3H, Ar-H), 9.05 (s, 1H, Ar-H); MS *m/z*: 355 [$M^{+\cdot}$, 16.33%], 354 [(M - H)$^+$, 20.06%], 68 [($C_3H_4N_2$)$^{+\cdot}$, 100%]. Anal. Calcd. For $C_{19}H_{13}N_7O$: C, 64.22; H, 3.69; N, 27.59. Found: C, 64.48; H, 3.83; N, 27.30.

1-(4-Chlorophenyl)-[1,2,4]-triazino[3,4-d]pyrazolo[4,5-a]pyrimidin-6-one (4b).

Yield: 58%; mp: 273-275^{0}C; IR (cm^{-1}): 3047 (CH aromatic), 2994-2939 (CH aliphatic), 1685 (C=O); ^1H NMR (DMSO-d_6) δ ppm 2.50 (s, 3H, CH_3), 7.53 (m, 1H, Ar-H), 7.89 (m, 4H, Ar-H), 8.04 (m, 2H, Ar-H), 8.38 (m, 2H, Ar-H), 8.72 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6): 13.78 (CH_3), 104.53, 121.68, 122.16, 126.85, 127.32, 128.79, 129.57, 129.71, 130.50,

131.61, 133.08, 136.50, 146.47 (13C, aromatic carbon), 161.06 (Pyrimidine-C=O); MS *m/z*: 388 [$M^{+\cdot}$, 11.28%], 275 [$(C_{14}H_7N_6O)^{+\cdot}$, 39.64%], 113 [$(C_6H_6Cl)^{+\cdot}$, 22.06%], 86 [$(C_7H_2)^{+}$, 100%]. Anal. Calcd. For $C_{20}H_{13}ClN_6O$: C, 61.78; H, 3.37; N, 21.61. Found: C, 61.64; H, 3.52; N, 21.65.

Synthesis of 1-oxo-[1,2,4]-triazino[3,4-d]pyrazolo[4,5-a]pyrimidin-6-one (5).

A solution of 6-(hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) in pyridine (15 mL), carbon disulfide (1 mL) was added. The mixture was heated under reflux for 24 h. The solution was evaporated to dryness, washed with ethanol 95%, filtered, dried and crystallized from benzene/ethyl acetate mixture.

Yield: 49%; mp: 250-252^{0}C; IR (cm^{-1}): 2361 (SH), 1692 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm 2.49 (s, 3H, CH_3), 7.30 (t, 1H, *J*= 4.8, Ar-H), 7.46 (m, 3H, Ar-H), 8.00 (m, 2H, Ar-H), 12.38 (s, 1H, SH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 13.76 (CH_3), 104.64, 121.63, 126.88, 129.51, 138.76, 146.31, 152.68, 157.48, 158.04 (9C, aromatic carbon), 158.76 (Pyrimidine-C=O), 158.85 (C-SH); MS *m/z*: 310 [$M^{+\cdot}$, 15.60%], 309 [(M - H)$^+$, 25.00%], 84 [$(C_2N_2S)^+$, 100%]. Anal. Calcd. For $C_{14}H_{10}N_6OS$: C, 54.18; H, 3.25; N, 27.08. Found: C, 53.99; H, 3.46; N, 27.32.

Synthesis of 3,9-dimethyl-1-phenyl-1H-5,7-dihydro-pyrazolo[4',3'-5,6]pyrimido[1,2-a]pyrazine-4-one (6).

A mixture of 6-(hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) and triethyl orthoacetate (15 mL) was heated under reflux for 4 h. The solid separated was filtered, dried and crystallized from ethanol 95%.

Yield: 43%; mp: 210-212^{0}C; IR (cm^{-1}): 3428, 3192 (2NH), 2964 (CH aliphatic), 1683 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm 2.34 (s, 3H, CH_3), 2.76 (s, 3H, CH_3), 7.38 (t, 1H, *J*= 7.6 Hz, Ar-H), 7.55 (t, 2H, *J*= 7.6 Hz, Ar-H), 7.67 (s, 1H, Ar-H), 8.00 (d, 2H, *J*= 8 Hz, Ar-H), 11.80 (s, 1H, NH, D_2O exchangeable), 12.19 (s, 1H, NH, D_2O exchangeable) ; ^{13}C NMR (DMSO-d_6): 13.78, 20.91 (2CH_3), 105.59, 121.74, 127.28, 129.66, 135.62, 138.59, 146.65, 152.62, 157.48, 168.43 (10C, aromatic carbon), 158.51 (Pyrimidine-C=O); MS *m/z*: 294 [$M^{+\cdot}$, 0.74%], 280 [$(C_{14}H_{12}N_6O)^{+\cdot}$, 1.00%], 77 [$(C_6H_5)^+$, 100%]. Anal. Calcd. For $C_{15}H_{14}N_6O$: C, 61.21; H, 4.79; N, 28.55. Found: C, 61.41; H, 4.52; N, 28.53.

General procedure for the synthesis of compounds (7a&b).

To a suspension of 6-(hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** in absolute ethanol (20 ml), the appropriate isothiocyanate derivative (1.5 mmol) was added. The mixture was heated under reflux for 10 h. The separated solid was filtered, dried and crystallized from dimethylformamide/ethanol 95%.

N-Ethyl-2-[(3-methyl-4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methyl]hydrazine carbothioamide (7a).

Yield: 53%; mp: 310-312^{0}C; IR (cm^{-1}): 3434 (br, 4NH), 2930 (CH aliphatic), 1679 (C=O), 1220 (C=S); ^{1}H NMR (DMSO-d_6) δ ppm 1.20 (t, 3H, *J*= 6.8 Hz, CH_2CH_3), 2.51 (s, 3H, CH_3), 3.34 (s, 2H, CH_2), 3.56 (q, 2H, *J*= 6.8 Hz, CH_2CH_3), 7.39 (t, 1H, *J*= 6.8 Hz, Ar-H), 7.56 (t, 2H, *J*= 6.4 Hz, Ar-H), 7.67 (s, 1H, NH, D_2O exchangeable), 7.98 (d, 2H, *J*= 7.6 Hz, Ar-H), 9.50, 12.09, 12.18 (3s, 3NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 13.78 (CH_3), 14.87 ($CH_2\underline{C}H_3$), 40.20 ($\underline{C}H_2CH_3$), 40.62 (CH_2), 105.55, 122.21, 127.40, 129.70, 133.40, 138.53, 146.69, 152.69, (8C, aromatic carbon), 158.66 (Pyrimidine-C=O), 177.54 (C=S); MS *m/z*: 357 [(M) $^{+\cdot}$, 9.15%], 310 [$(C_{15}H_{16}N_7O)^+$, 26.61%], 77 [$(C_6H_5)^+$, 100%]. Anal. Calcd. For $C_{16}H_{19}N_7OS$: C, 53.76; H, 5.36; N, 27.43. Found: C, 54.08; H, 5.03; N, 27.15.

2-[(3-Methyl-4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methyl]-N-phenyl hydrazine carbothioamide (7b).

Yield: 53%; mp: 276-278^{0}C; IR (cm^{-1}): 3432-3201 (4NH), 1677 (C=O), 1190 (C=S); ^{1}H NMR (DMSO-d_6) δ ppm 2.51 (s, 3H, CH_3), 3.35 (s, 2H, CH_2), 7.30 (t, 1H, *J*= 7.6 Hz, Ar-H), 7.41-7.47 (m, 5H, Ar-H), 7.56 (t, 2H, *J*= 7.6 Hz, Ar-H), 7.60 (s, 1H, NH, D_2O exchangeable), 7.99 (d, 2H, *J*= 7.6 Hz, Ar-H), 10.90, 12.36, 12.38 (3s, 3NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 13.78 (CH_3), 40.62 (CH_2), 105.65, 122.24, 126.77, 127.15, 127.44, 128.93, 129.72, 134.33, 138.53, 138.89, 146.72, 152.66 (12C, aromatic carbon), 158.63 (Pyrimidine-C=O), 177.41 (C=S); MS *m/z*: 405 [(M) $^{+\cdot}$, 0.27%], 310 [$(C_{14}H_{10}N_6OS)^{+\cdot}$, 100%], 281 [$(C_{14}H_9N_4OS)^+$, 40.31%]. Anal. Calcd. For $C_{20}H_{19}N_7OS$: C, 59.24; H, 4.72; N, 24.18. Found: C, 58.99; H, 4.54; N, 23.90.

Synthesis of 6-[(3,5-dimethyl-1H-pyrazol-1-yl)methyl]-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)one (8).

A mixture of 6-(hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) and acetyl acetone (0.15 g, 1.5 mmol) in absolute ethanol (15 mL) was heated under reflux for 8 h. After cooling, the separated solid was filtered, dried and crystallized from ethanol 95%. Yield: 62%; mp: 186-188^{0}C; IR (cm^{-1}): 3420 (NH), 2978-2925 (CH aliphatic), 1686 (C=O); ^{1}H NMR ($CDCl_3$-d_6) δ ppm 2.26 (s, 3H, CH_3), 2.86 (s, 3H, CH_3), 2.98 (s, 3H, CH_3), 3.64 (s, 2H, CH_2), 5.91 (s, 1H, NH, D_2O exchangeable), 6.15 (s, 1H, CH), 7.29-7.95 (m, 5H, Ar-H); ^{13}C NMR (DMSO-d_6): 11.11, 13.63, 14.68 (3CH_3), 50.76 (CH_2), 107.05 (pyrazole-CH), 105.59, 121.72, 121.96, 126.70, 128.90, 138.49, 140.76, 147.21, 150.13, 154.24 (10C, aromatic carbon), 157.84 (Pyrimidine-C=O); MS *m/z*: 334 [(M) $^{+\cdot}$, 46.8%], 255 [$(C_{13}H_{13}N_5O)^{+\cdot}$, 100%], 253 [$(C_{13}H_{11}N_5O)^{+\cdot}$, 23.58%]. Anal. Calcd. For $C_{18}H_{18}N_6O$: C, 64.66; H, 5.43; N, 25.13. Found: C, 64.47; H, 4.77; N, 25.18.

Synthesis of 1-[(3-methyl-4-oxo-2,5-dihydro-1H-pyrazolo [3,4-d]pyrimidin-6yl)methyl]pyrazoline-3,5-dione (9).

A mixture of 6-(Hydrazinylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(2)** (0.40 g, 1.5 mmol) and diethyl malonate (0.24 g, 1.5 mmol) in absolute ethanol (15 mL) was heated under reflux for 10 h. After cooling, the separated solid was filtered, dried and crystallized from ethanol 95%. Yield: 67%; mp: 319-321^{0}C; IR (cm^{-1}): 3339, 3157 (2NH), 2925 (CH aliphatic), 1711-1684 (3C=O); ^{1}H NMR (DMSO-d_6) δ ppm 2.51 (s, 3H, CH_3), 3.37 (s, 2H, CH_2), 4.13 (s, 2H, CH_2), 7.38 (m, 2H, Ar-H, and NH, D_2O exchangeable), 8.05 (m, 2H, Ar-H), 8.44 (m, 2H, Ar-H); ^{13}C NMR (DMSO-d_6): 13.80 (CH_3), 47.10 (CH_2), 50.60 (CH_2), 104.52, 121.65, 126.84, 127.24, 129.56, 138.96, 146.45, 153.52 (8C, aromatic carbon), 158.33 (Pyrimidine-C=O), 168.80, 169.90 (2 C=O); MS *m/z*: 338 [(M)$^{+\cdot}$, 7.33%], 230 [(M-C_6H_5N)$^{+\cdot}$, 27.00%], 87 [($C_3H_6N_2O$)$^+$, 100%]. Anal. Calcd. For $C_{16}H_{14}N_6O_3$: C, 56.80; H, 4.17; N, 24.84. Found: C, 56.50; H, 4.38; N, 24.60.

Biological testing

The human breast adenocarcinoma cell line (MCF7) was obtained as a gift from NCI, MD, USA. All chemicals and solvents were purchased from Sigma-Aldrich.

The cytotoxic activity of the newly synthesized compounds was examined *in vitro* on human breast adenocarcinoma cell line (MCF7) using Sulforhodamine-B stain (SRB) assay applying the method of Skehan *et al*. [15]

Table 1: Results of *in vitro* cytotoxic activity of the synthesized compounds on human breast adenocarcinoma cell line (MCF7).

Compound no	IC_{50} (µM)[a]	Compound no	IC_{50} (µM)[a]
3a	0.0888	**6**	0.1615
3b	0.0319	**7a**	0.0532
3c	0.0332	**7b**	0.1358
4a	0.0352	**8**	0.0706
4b	0.0739	**9**	0.1048
5	0.0558	**Methotrexate**	0.046

[a] The values given are means of three experiments.

Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the test compounds to allow attachment of the cells to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the test compound (5, 12.5, 25 and 50 mg/mL) were added to the cell monolayer. Triplicate wells prepared for each individual dose. Monolayer cells were incubated with the test compound for 48 h at 37 ^{o}C in atmosphere of 5% CO_2. After 48 h, cells were fixed with trichloroacetic acid, washed with water and stained for 30 min with 0.4% (wt/vol) Sulforhodamine-B stain dissolved with 1% acetic acid. Excess stain was removed by four washes with 1% acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in ELISA reader. The relation between surviving fraction and compound concentration was plotted and IC_{50} [the concentration required for 50% inhibition of cell viability] was calculated for each compound and results are given in Table 1 represented graphically in Chart 1.

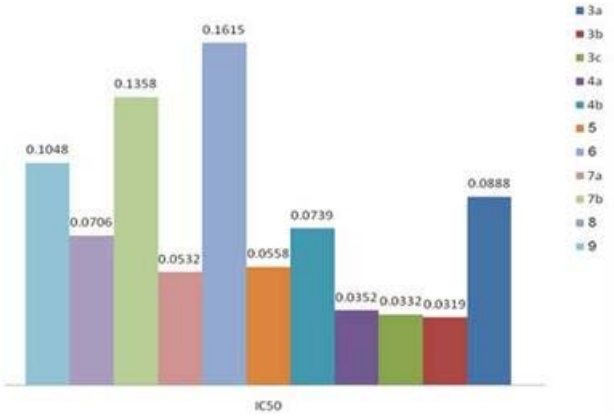


Chart 1: Represents IC_{50} of the newly synthesized compounds in µM on human adenocarcinoma cell line (MCF7).

RESULTS AND DISCUSSION

Chemistry

The target compounds depicted in Scheme 1 were synthesized from the reaction of 6-chloromethyl derivative **1**[9] and 4 molar equivalent of hydrazine hydrate (99%) to give 6-hydrazinylmethyl derivative **2** as the starting material.

Formation of compound **2** was confirmed on the basis of its correct elemental analysis and spectral data. IR spectrum of compound **2** showed the presence of bands for 2NH and NH_2 groups. The site of hydrazinolysis in compound **2** was supported by ^{1}HNMR spectrum which revealed D_2O exchangeable signals at δ 6.40, 7.43 and 9.55 ppm attributed to NH_2, NH and CONH, respectively. Moreover, the mass spectrum of compound **2** displayed molecular ion peak at *m/z* 270 (M$^{+\cdot}$).

The formation of different Schiff's bases **3a-c** was obtained by reacting **2** with the appropriate halogenated aromatic aldehyde in ethanol. Structures of compounds **3a-c** were based on analytical and spectral data. The ^{1}H NMR spectra of **3a-c** exhibited the characteristic signals for azomethine proton and 2NH protons at δ 8.34, 8.35; 8.64-9.05 and 9.93-10.40 ppm, sequentially. The ^{13}C NMR revealed the presence of (N=CH) at δ 150.05 ppm. In addition, the molecular ion peak of compound **3a** was found in the mass spectrum at *m/z* 376 (M$^{+\cdot}$).

On the other hand, the reaction of compound **2** and the corresponding aromatic or aryl aldehyde in ethanol in the presence of a basic catalyst such as triethyl amine afforded the tricyclic structure of compounds **4a&b**, through cyclization of the formed schiff's bases by the action of the basic catalyst.

Structures of compounds **4a&b** were established on the basis of their correct elemental analysis and spectral data. The IR spectra of compounds **4a&b** indicated the absence of the NH group. The ^{1}H NMR spectra of **4a&b** in (DMSO-d_6) showed the disappearance of CH_2 protons of the parent start and the appearance of =CH protons of triazine ring at δ 7.56 ppm. Moreover, the structure of **4a** was supported by its mass spectrum which displayed a molecular ion peak at m/z 355 ($M^{+\cdot}$).

A suggested mechanism for the formation of compounds **4a&b** was illustrated as in Fig. 2.

Also, triazinopyrazolopyrimidine derivative **5** was obtained from the hydrazinylmethyl derivative **2** and carbon disulfide in pyridine. The formation of **5** was ruled out on the basis of analytical and spectral data.

The IR spectrum of compound **5** showed the presence of (SH) group at 2361 cm^{-1}. Its ^{1}H NMR spectrum in (DMSO-d_6) revealed D_2O exchangeable singlet signal at δ 12.38 ppm attributed to (SH) proton. The ^{13}C NMR showed peak at δ 158.85 ppm for (C-SH). The mass spectrum of **5** exhibited a molecular ion peak at m/z 310 ($M^{+\cdot}$) with a base peak at 84.

In the present study, it was attempted to prepare ethoxyethylidene derivative **A** by reacting the starting material **2** with triethyl orthoacetate. Surprisingly, pyrazolopyrimidopyrazine derivative **6** was obtained instead. The structure of the unexpected compound **6** was confirmed on the basis of its IR, mass, ^{1}H NMR and ^{13}C NMR spectral data. The ^{1}H NMR spectrum indicated the absence of triplet and quartet peaks of ethyl moiety and CH_2 protons of the expected product **A**. Besides, the appearance of two CH_3 protons at δ 2.34 and 2.76 ppm. Also, =CH proton of the newly formed diazine ring in aromatic region confirmed the structure of compound **6**. Lacking of a peak for CH_2 and addition of a peak for =CH in aromatic part in ^{13}C NMR assigned for the formation of the unexpected product **6**. The mass spectrum of compound **6** showed the corresponding molecular ion peak at *m/z* 294 ($M^{+\cdot}$).

A possible mechanism for the formation of compound **6** was outlined in Fig. 3.

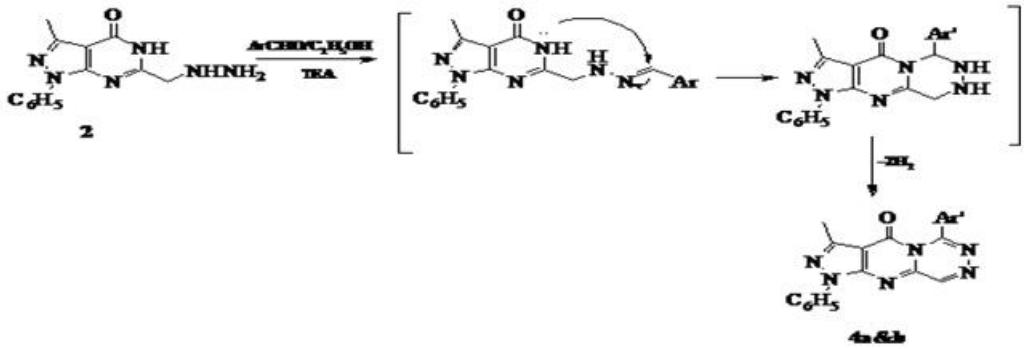


Figure 2: A suggested mechanism for the formation of compounds **4a&b**.

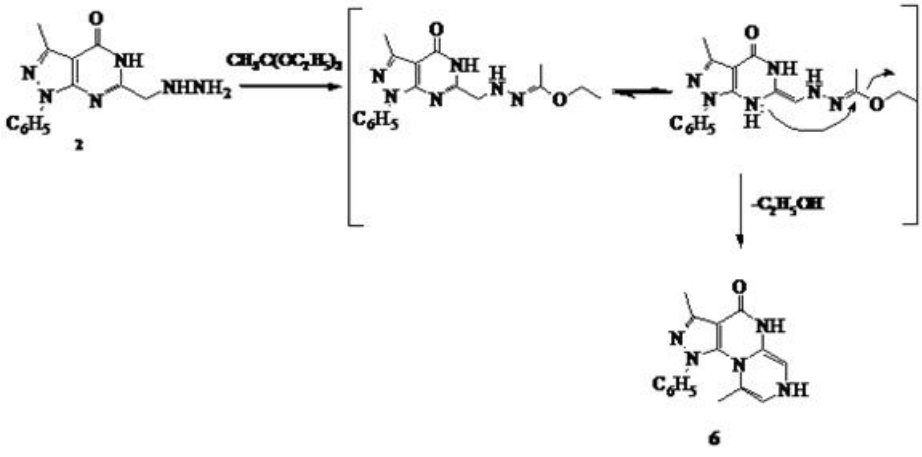


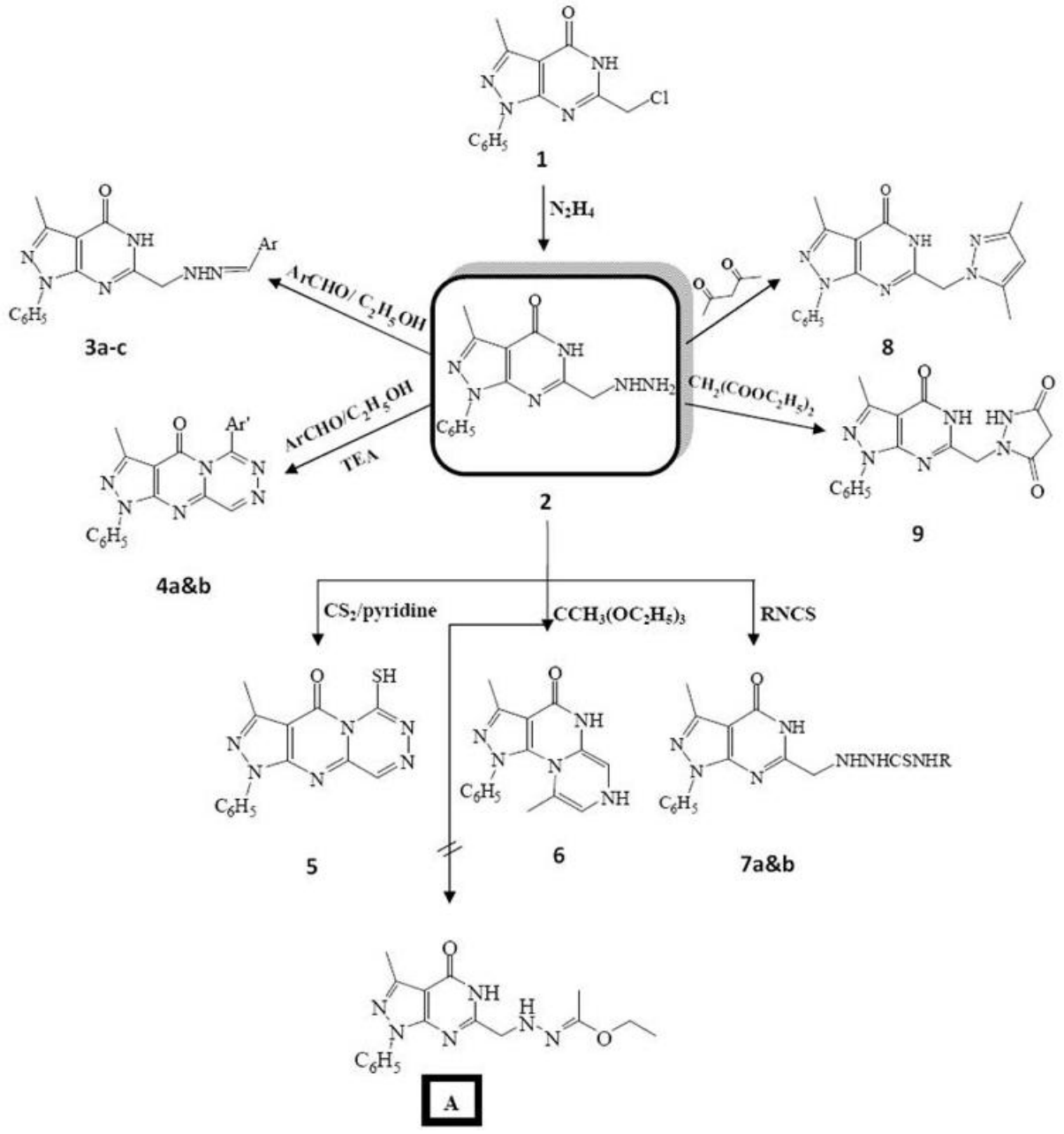
Figure 3: A possible mechanism for the formation of compound **6**.

Introducing thiosemicarbazide moiety in pyrazolo[3,4-*d*]pyrimidine ring at position 6 was achieved from reacting compound **2** with ethyl or phenyl isothiocyanate in absolute ethanol in a molar ratio (1:1) yielding compounds **7a&b**. Element analysis and spectral data are in agreement with the chemical structure of compounds **7a&b**. The IR spectra showed the presence of characteristic band for (C=S) group at 1249 and 1220 cm^{-1}. The ^{1}H NMR spectra indicated the presence of four D_2O exchangeable singlet signals assigned for four NH protons at δ 7.60, 7.67 – 9.50, 10.90 – 12.09, 12.36 and 12.18, 12.38 ppm. In addition, the molecular structure of compounds **7a&b** was established by ^{13}C NMR spectra

which recorded a signal at δ 177.41 and 177.54 ppm due to (C=S).

In this work, incorporating pyrazole and pyrazolone rings with pyrazolo[3,4-*d*]pyrimidine ring containing methylene spacer at position 6 was achieved *via* reacting of compound **2** either with acetyl acetone or diethyl malonate, respectively. Structures of the products **8** and **9** were substantiated on analytical and spectral data (See Experimental Section), Scheme 1.

The synthesized compounds were subjected to *in vitro* cytotoxic activity against human adenocarcinoma cell line (MCF7), using Sulforhodamine-B stain (SRB) assay[15]. IC_{50} [value corresponds to the concentration required for 50% inhibition of cell viability] was calculated from the relation between surviving fraction of MCF7 and drug concentration. The IC_{50} of the test compounds are shown in Table 1 and Chart 1.



R= C_2H_5, C_6H_5; Ar= 4-FC_6H_4, 4-ClC_6H_4, 2-BrC_6H_4; Ar'= 3-C_5H_3N, 4-ClC_6H_4

Scheme 1: Synthesis of the new compounds.

Structure-activity correlation of the test compounds exhibited that introducing different azomethine derivatives at 6-position of pyrazolo[3,4-*d*]pyrimidine nucleus afforded antitumor potency for **3b**> **3c**> **3a**. Moreover, triazine ring in **4a** showed almost equal antitumor activity with **3b** and **3c**. Meanwhile, conversion of hydrazine moiety of compound **2** into triazine in **4b** and **5** yielded marginal decrease in potency. Conversion of **2** to produce diazine derivative **6** decreases the antitumor activity. Conversion of the hydrazine part of **2** into the

corresponding thiosemicarbazides **7a** and **7b** increase the antitumor activity in **7a** and decrease the activity in phenyl analog **7b**. Cyclization of **2** into pyrazole derivative **8** showed good antitumor activity. While, pyrazolone analog **9** decreases the activity.

CONCLUSION

The aim of the present work was to synthesize novel pyrazolo[3,4-*d*] pyrimidines carrying different groups or rings at position 6. The newly synthesized compounds were tested *in vitro* on human breast adenocarcinoma cell line (MCF7). Some of the test compounds showed potent antitumor activity compared to the reference drug methotrexate, especially compound **3b** (4-Cl derivative), **3c** (2-bromo derivative) and **4a** (3-pyridyl derivative) which displayed the highest activity among the test compounds. The results of the anticancer screening suggested that some azomethine derivatives and triazine ring enhanced the anticancer activity. While, diazine **6**, phenyl analogue of thiosemicarbazide **7b** and pyrazolone **9** derivatives decrease the activity.

In conclusion, compounds 6-{[2-(4-chlorobenzylidene) hydrazinyl]methyl}-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(3b)**, 6-{[2-(2-bromobenzylidene) hydrazinyl]methyl}-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(3c)** and 1-(pyridin-3-yl)-[1,2,4]-triazino[3,4-*d*]pyrazolo[4,5-*a*]pyrimidin-6-one **(4a)** proved to be the most active compounds in the present study.

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