Synthesis and antitumor activity of novel pyrazolo[3,4-d]pyrimidines and related heterocycles

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ABSTRACT

The reaction between 5-amino-4-imino-3-methyl-1-phenyl-1,4-dihydro-pyrazolo[3,4-d]pyrimidine (2a) or (3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazine (3) and several available reactants afforded new heterocycles with pyrazolo[3,4-d]pyrimidine nucleus. Some of the newly synthesized compounds were screened against MCF-7 cell line, compounds 4b, 5a, 10c and 12c showed the highest activity among the tested compounds with IC₅₀ between 0.013 and 0.018 μM.

Key words: Pyrazolo[3,4-d]pyrimidine derivatives, Imino, MCF-7, Z and E Geometrical isomers.

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].

There are three traditional approaches for the treatment of cancer: surgery, radiotherapy and chemotherapy. As combination therapy is more effective than using a single drug, chemotherapy is usually used alongside surgery and radiotherapy [2]. 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) [3]. So, pharmaceutical attention has been focused on the more selective antineoplastic agents with the minimum side effects.

Several pyrazole derivatives received great attention due to their biological and pharmacological activities not only as potential inhibitors of HIV-1 [4], pesticides [5] fungicides [6], analgesic drugs [4], antihypertensive agents [7] and anticancer activity [8], but they are also important and useful as starting materials for the synthesis of other fused heterocyclic systems, among these, pyrazolo[3,4-d]pyrimidine derivatives [9], which have a considerable chemical and pharmacological importance as purine analogues [10-12]. Furthermore, compounds containing the triazolo[1,5-c]pyrimidine moiety were reported to exhibit remarkable adenosine receptor affinity [13]. Particularly, the 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazooline (1) was found to be a highly potent adenosine antagonist [14]. Also, the tricyclic heterocyclic pyrazolo[1,5-c]-1,2,4-triazolo[4,3-e]pyrimidines 2, 3 were planned as human A₂a and A₃ adenosine receptor subtype antagonists respectively [15,16].
Based on the above observation, a pyrazole nucleus was used as a starting material for the preparation of pyrazolo[3,4-d]pyrimidine with imino \( 2a \) or hydrazine \( 3 \) groups at position 4 of the pyrimidine ring. From these latter compounds, new targets - chemically related to adenosine antagonist - were synthesized, maintaining the pyrazolo[3,4-d]pyrimidine nucleus and replaced the 2-(2-furyl)-triazole moiety with five membered rings [pyrazole, triazole and tetrazole] or six membered ring fused to pyrazolo[3,4-d]pyrimidine ring system and functionalized by the introduction of several functions as ester, cyano and substituted amino groups. In addition, new pyrazolo[3,4-d]pyrimidine derivatives bearing either acyclic or heterocyclic pyrazole moiety at position 4 of the pyrimidine ring were also synthesized. Some of the newly synthesized compounds were tested against breast cancer cell line (MCF-7).

**MATERIALS AND METHODS**

**Experimental**

Melting points were determined on a Graffin apparatus and were uncorrected. Element analyses (C, H, N) were carried out on Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA) at the Micro analytical unit of Cairo University, Egypt. All compounds were within \( \pm 0.4\% \) of the theoretical values. IR spectra were determined as KBr discs on Shimadzu IR 435 Spectrophotometer and values were represented in cm\(^{-1}\); \(^1\)H NMR and \(^13\)C NMR spectra were carried out on Bruker 300 MHz NMR Spectrophotometer in Cairo University, Egypt, using (Bruker, Munich, Germany) in DMSO-\( d_6 \) or CDCl\(_3\)-\( d_6 \) as a solvent, TMS as internal standard and chemical shifts were recorded in ppm on \( \delta \) scale. Mass spectra were run on Hewlett Packard 5988 Spectrometer, Micro analytical center, Cairo University, Egypt. Progress of the reactions was monitored by TLC using TLC sheets precoated with UV fluorescent silica gel MERCK 60 F 254 that were visualized by UV lamp.

**General procedure for synthesis of compounds 2b&c.**

To a solution of compound \( 1 \) (2.54 g, 0.01 mol) in absolute ethanol (20 ml), the appropriate amine (0.01 mol) was added. The mixture was stirred at room temperature for 1 h. The formed precipitate was filtered, dried washed with ethanol to afford \( 2a \&b \). Spectroscopic data for all these compounds are given below.

3,5-Dimethyl-4-Imino-1-phenyl-5-methylamino-1H-4,5-dihydro-pyrazolo[3,4d]pyrimidines (2b). Isolated as white solid, m.p.: 145-147\(^0\)C (recrystallized from EtOH 95%); IR (KBr, cm\(^{-1}\)), 3433 (NH), 3042 (CH arom.), 2922 (CH aliph.), 1681 (C=N); \(^1\)H NMR (300 MHz, DMSO-\( d_6 \)) \( \delta \) ppm 2.53 (s, 3H, CH\(_3\)), 3.40 (s, 3H, N-CH\(_3\)), 7.30 (s, 1H, NH, D\(_2\)O exchangeable), 7.33 (t, 1H, J= 6.3 Hz, A-H), 7.50 (t, 2H, J= 6.3 Hz, Ar-H), 7.97 (d, 2H, J= 6.3 Hz, A-H) and 8.12 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 240 [M+\( \boxed{+}\), 17.12], 239 [M-\( \boxed{-}\), 100], 238 [M-H-\( \boxed{-}\), 37.50], 209 [C\(_11\)H\(_7\)N\(_5\) \( \boxed{-}\), 23.07], and 77 [C\(_6\)H\(_5\) \( \boxed{-}\), 21.50]; Anal. Calcd for C\(_{13}\)H\(_{13}\)N\(_5\): C, 65.25; H, 5.48; N, 29.27. Found: C, 65.51; H, 5.00; N, 29.84.

4-Imino-3-methyl-1-phenyl-5-phenylamino-1H-4,5-dihydro-pyrazolo[3,4d]pyrimidines (2c). Isolated as white solid, m.p.: 120-122\(^0\)C (recrystallized from EtOH 95%); IR (KBr, cm\(^{-1}\)), 3441, 3269 (2NH), 3049 (CH arom.), 2916 (CH aliph.), 1622 (C=N); \(^1\)H NMR (300 MHz, DMSO-\( d_6 \)) \( \delta \) ppm 2.62 (s, 3H, CH\(_3\)), 7.30 (s, 1H, NH, D\(_2\)O exchangeable), 7.33 (t, 1H, J= 6.3 Hz, A-H), 7.50 (t, 2H, J= 6.3 Hz, Ar-H), 7.97 (d, 2H, J= 6.3 Hz, A-H) and 8.12 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 240 [M+\( \boxed{+}\), 17.12], 239 [M-\( \boxed{-}\), 100], 238 [M-H-\( \boxed{-}\), 37.50], 209 [C\(_11\)H\(_7\)N\(_5\) \( \boxed{-}\), 23.07], and 77 [C\(_6\)H\(_5\) \( \boxed{-}\), 21.50]; Anal. Calcd for C\(_{14}\)H\(_{15}\)N\(_5\): C, 65.25; H, 5.48; N, 29.27. Found: C, 65.51; H, 5.00; N, 29.84.
3.27 (s, 1H, NH, D2O exchangeable), 6.74 (t, 1H, J = 7.2 Hz, Ar-H), 6.83 (d, 2H, J = 7.8 Hz, Ar-H), 7.17 (t, 2H, J = 7.2 Hz, Ar-H), 7.30 (t, 1H, J = 6.3 Hz, Ar-H), 7.52 (t, 2H, J = 8.4 Hz, Ar-H), 8.14 (d, 2H, J = 7.5 Hz, Ar-H), 8.33 (s, 1H, CH of pyrimidine) and 9.48 (s, 1H, NH, D2O exchangeable); EIMS (m/z) (relative abundance %), 317 [M+1 Δ+], 52.53; Anal. Calcd for C14H12N4: C, 60.16; H, 4.85; N, 34.60%.

General procedure for synthesis of 2-Ethylamino-9-methyl-7-phenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (4a)

A suspension of compound 2a (0.24 g, 0.001 mol) and the appropriate isothiocyanate derivative (0.001 mol) in absolute ethanol (10 ml) was heated under reflux for 1 h. The solid that formed on hot was filtered, to afford compounds 4a&b.

2-Ethylamino-9-methyl-7-phenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (4a) Isolated as white solid, Yield: 55%, mp: 165-167°C (recrystallized from DMSO/EtOH); IR (KBr, cm−1), 3415 (NH), 3069 (CH arom.), 2969, 2924, 2878 (CH aliph.), 1658 (C=N); 1H NMR (300 MHz, DMSO-d6) δ ppm 2.63 (s, 3H, CH3), 3.47 (m, 2H, NHCH2CH3), 7.03 (t, 1H, J = 5.7 Hz, NH, D2O exchangeable), 7.37 (t, 1H, J = 8.4 Hz, Ar-H), 7.56 (t, 2H, J = 6.6 Hz, Ar-H), 8.12 (d, 2H, J = 6.6 Hz, Ar-H) and 9.24 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 294 [M+1 Δ+], 52.53; Anal. Calcd for C14H12N4: C, 60.16; H, 4.85; N, 34.60%.

General procedure for synthesis of compounds 5a&b.

A mixture of 2a (0.24 g, 0.001 mol) and diethyl oxalate or diethyl malonate (5 ml) was heated under reflux for 10 h. The reaction mixture was concentrated and left to cool. The solid separated was filtered, to give compounds 5a&b.

Ethyl(3-methyl-1-phenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-yl)carboxylate (5a).

Isolated as yellow solid, Yield: 81%; mp: 197-199°C (recrystallized from dioxane); IR (KBr, cm−1), 3063 (CH arom.), 2986, 2928 (CH aliph.), 1739 (C=O), 1648 (C=N); 1H NMR (300 MHz, DMSO-d6) δ ppm 1.38 (t, 3H, J = 7.2 Hz, CH3CH2CH3), 2.77 (s, 3H, CH3), 4.46 (q, 2H, J = 7.2 Hz, CH2CH3), 7.44 (t, 1H, J = 7.2 Hz, Ar-H), 7.60 (t, 2H, J = 7.2 Hz, Ar-H), 8.08 (d, 2H, J = 8.7 Hz, Ar-H) and 9.78 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 323 [M+1 Δ+], 52.53; Anal. Calcd for C16H16N4O2: C, 59.62; H, 4.38; N, 26.07%. Found: C, 59.57; H, 4.21; N, 29.08%.

Ethyl(3-methyl-1-phenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-yl)methylcarboxylate (5b).

IR (KBr, cm−1), 3092 (CH arom.), 2979, 2929 (CH aliph.), 1735 (C=O), 1647 (C=N); 1H NMR (300 MHz, DMSO-d6) δ ppm 1.22 (t, 3H, J = 7.2 Hz, CH2CH3), 2.73 (s, 3H, CH3), 4.07 (s, 2H, CH2), 4.16 (q, 2H, J = 7.2 Hz, CH2CH3), 7.40 (t, 1H, J = 7.8 Hz, Ar-H), 7.59 (t, 2H, J = 7.8 Hz, Ar-H), 8.10 (d, 2H, J = 8.4 Hz, Ar-H) and 9.63 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 337 [M+1 Δ+], 52.53; Anal. Calcd for C16H16N4O2: C, 59.62; H, 4.38; N, 26.07%. Found: C, 59.57; H, 4.21; N, 29.08%.

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General procedure for synthesis of compounds 6a&b.
A mixture of 2a (2.4 g, 0.01 mol) and chloroacetyl chloride or ethyl cyanoacetate (1.12 g, 0.01 mol) was refluxed in dioxane (20 ml) for 6 h. The reaction mixture was filtered while hot and the filtrate was evaporated to dryness to give compounds 6a&b.

2-Chloromethyl-9-methyl-7-phenyl-2-(3-methoxyphenyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (6a)
Isolated as white solid. Yield: 63%. mp: 194-196°C (recrystallized from DMF/ethanol 95%); IR (KBr, cm⁻¹), 3091 (CH arom.), 2923 (CH aliph.), 1648 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 7.43 (t, 1H, J = 7.2 Hz, Ar-H), 7.60 (t, 2H, J = 8.1 Hz, Ar-H), 8.09 (d, 2H, J = 5.4 Hz, Ar-H) and 9.66 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 300 [M+2 ¹⁺], 16.34, 299 [M+1 ¹⁺], 12.50, 398 [M ²⁺], 51.94, 263 [C₁₉H₁₉N₆ ³⁻], 8.16 and 77 [C₆H₅ ⁴⁻, 100]; Anal. Calcd for C₁₉H₁₉N₆O₂: C, 60.71; H, 4.79; N, 24.99%. Found: C, 61.00; H, 4.89; N, 25.25%.

General procedure for synthesis of compounds 7a-d.
A mixture of compound 2a (2.4 g, 0.01 mol) and the appropriate aromatic aldehyde (0.01 mol) in absolute ethanol (30 ml), piperidine (0.5mL) was added and the mixture was heated under reflux for 3-5 h. The precipitate that formed on hot was filtered off and washed with ethanol to yield 7a-d.

9-Methyl-7-phenyl-2-(3-methoxyphenyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (7a).
Isolated as yellow solid. Yield: 53%. mp: 286-288°C (recrystallized from Acetone); IR (KBr, cm⁻¹), 2495 (OH), 1304 (CH arom.), 2927 (CH aliph.), 1649 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 7.41 (m, 2H, Ar-H), 7.61 (t, 2H, J = 7.5 Hz, Ar-H), 7.72 (m, 2H, Ar-H), 8.13 (d, 2H, J = 6.4 Hz, Ar-H), 9.72 (s, 1H, CH of pyrimidine) and 9.80 (s, 1H, OH, D₂O exchangeable); EIMS (m/z) (relative abundance %), 342 [M ²⁺, 3.66], 149 [C₁₃H₁₀N₅O ⁴⁻, 15.19], 95 [C₁₀H₅N₆ ⁵⁺, 28.35], 81 [C₁₄H₁₁N₄ ⁶⁻, 36.33] and 69 [C₁₅H₁₂N₇ ⁷⁺, 100]; Anal. Calcd for C₁₅H₁₂N₇O: C, 66.66; H, 4.12; N, 24.55%. Found: C, 66.42; H, 4.06; N, 24.38%.

9-Methyl-7-phenyl-2-(3-hydroxyphenyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (7b).
Isolated as yellow solid. Yield: 33%. mp: 232-234°C (recrystallized from dioxane); IR (KBr, cm⁻¹), 3072 (CH aromatic), 2932, 2835 (CH aliph.), 1652 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 8.12 (d, 2H, J = 7.8 Hz, Ar-H) and 9.69 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 300 [M+1 ¹⁺], 19.72, 289 [M ²⁺, 100], 288 [C₁₅H₁₄N₇ ³⁻, 30.38] and 77 [C₆H₅ ⁴⁻, 48.56]; Anal. Calcd for C₁₅H₁₄N₇: C, 62.28; H, 3.83; N, 33.89%. Found: C, 62.48; H, 3.80; N, 33.68 %.

9-Methyl-7-phenyl-2-(3-hydroxyphenyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (7c).
Isolated as orange solid. Yield: 35%. mp: 290-292°C (recrystallized from propanol); IR (KBr, cm⁻¹), 3067, 3002 (CH aromatic), 2932 (CH aliph.), 1646 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 7.42 (t, 1H, J = 7.2 Hz, Ar-H), 7.40 (m, 3H, Ar-H), 7.61 (t, 1H, J = 8.1 Hz, Ar-H), 7.72 (m, 2H, Ar-H), 7.76 (s, 1H, Ar-H), 7.68 (d, 1H, J = 7.8 Hz, Ar-H), 8.12 (d, 2H, J = 7.8 Hz, Ar-H) and 9.69 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 357 [M+1 ¹⁺, 23.53], 356 [M ²⁺, 100], 355 [M-1 ³⁺, 37.37], 326 [C₁₉H₁₄N₆ ⁴⁺, 15.31], and 77 [C₆H₅ ⁴⁻, 78.71]; Anal. Calcd for C₁₉H₁₄N₆O: C, 67.40; H, 4.53; N, 23.58%. Found: C, 67.44; H, 4.51; N, 23.83%.

9-Methyl-7-phenyl-2-(4-chlorophenyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (7d).
Isolated as white solid. Yield: 50%. mp: 243-245°C (recrystallized from ethanol): IR (KBr, cm⁻¹), 3067, 3002 (CH arom.), 2924 (CH aliph.), 1650 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 7.59 (t, 2H, J = 8.4 Hz, Ar-H), 8.08 (d, 2H, J = 7.2 Hz, A-H), 7.95 (t, 2H, J = 8.4 Hz, Ar-H), 8.38 (d, 2H, J = 8.7 Hz, Ar-H), 8.45 (d, 2H, J = 8.7 Hz, Ar-H) and 9.75 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 362 [M+2 ¹⁺, 30.57], 361 [M+1 ²⁻, 31.85], 360 [M ³⁻, 100], 118 [C₁₃H₁₀N₅Cl ⁴⁻, 8.11], 103 [C₁₀H₈N₄Cl ⁵⁻, 9.27] and 77 [C₆H₅ ⁶⁺, 33.40]; Anal. Calcd for C₁₀H₈ClN₆C: C, 63.25; H, 3.63; N, 23.29%. Found: C, 63.19 H, 3.80; N, 23.16%.
An ice-cold solution of sodium nitrite (0.21 g/5 ml H₂O, 0.003 mol) was added with stirring during five minutes to a cold solution of 2a (0.24 g, 0.001 mol) in acetic acid (10 ml). Stirring was then continued for 2 h. The reaction mixture was poured into water (≈30 ml). The separated solid was filtered off and washed with water to afford compound 8. Isolated as white solid, Yield: 50%, mp: 157-159°C (recrystallized from ethanol 95%); IR (KBr, cm⁻¹), 3187 (CH aliph.), 2925 (CH aliph.), 1649 (C≡N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.50 (s, 3H, CH₃), 7.66 (t, 1H, J = 8.4 Hz, Ar-H), 7.70 (d, 2H, J = 7.8 Hz, Ar-H) and 8.26 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 251 [M⁺⁻¹⁺, 0.28], 225 [C₁₁H₈N₆⁻¹⁺, 100], 224 [C₁₁H₈N₆⁻¹⁺, 28.50] and 77 [C₄H₆⁻¹⁺, 47.95]; Anal. Calcd for C₁₁H₁₀N₈O: C, 57.37; H, 3.61; N, 39.02%. Found: C, 57.21; H, 3.54; N, 38.89%.

General procedure for synthesis of 3-methyl-1-phenyl-1H,7H-5,6-dioxo-dihydro pyrazolo [3’4’:4,5] pyrimido[1,6-b][1,2,4]triazine (9).
A mixture of 2a (0.24 g, 0.001 mol) and oxalyl chloride (0.126 g, 0.001 mol) in dry benzene (10 ml) was heated under reflux for 8 h. The reaction mixture was evaporated to dryness, washed with 10% Na₂CO₃ solution (5 ml). The separated solid was filtered, dried to yield compound 9. Isolated as green solid, Yield: 76%, mp: 258-260°C (recrystallized from mixture of ethanol/benzene (1:1); IR (KBr, cm⁻¹), 3251 (NH), 3073 (CH aliph.), 2925 (CH aliph.), 1726, 1658 (C=O); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.50 (s, 3H, CH₃), 7.70 (t, 1H, J = 8.4 Hz, Ar-H), 7.50 (t, 2H, J = 8.4 Hz, Ar-H), 8.12 (d, 2H, J = 7.8 Hz, Ar-H) and 8.13 (s, 1H, CH of pyrimidine), and 12.31 (s, 1H, NH, D₂O exchangeable); EIMS (m/z) (relative abundance %), 295 [M⁺⁻¹⁺, 1.68], 294 [M⁺⁻¹⁺, 9.13], 252 [C₁₃H₁₈N₂O⁻¹⁺, 13.50], 251 [C₁₁H₈N₆⁻¹⁺, 100], 250 [C₁₁H₁₀N₈⁻¹⁺, 29.93], 236 [C₁₂H₁₀N₆⁻¹⁺, 13.22], 182 [C₁₀H₈N₄⁻¹⁺, 14.88] and 77 [C₄H₆⁻¹⁺, 37.18]; Anal. Calcd for C₁₃H₁₄N₈O: C, 57.14; H, 3.43; N, 38.56%. Found: C, 56.86; H, 3.40; N, 38.49%.

General procedure for synthesis of compounds 10a-d.
To a solution of 3 (2.40 g, 0.01 mol) in absolute ethanol (20 ml), the appropriate ethoxy derivative of malononitrile or cyanoacetate (0.01 mol) and TEA (0.5 ml) were added. The mixture was heated under reflux for 2-5 h. The mixture allowed to cool then poured into ice-cold water (≈30 ml). The solid separated was filtered off and washed with ethanol to give 10a-d.

5-Amino-1-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole-4-carbonitrile (10a).
Isolated as brown solid, Yield: 70%, mp: 245-247°C (recrystallized from EtOH 95%); IR (KBr, cm⁻¹), 3377, 3262 (NH₂), 2927 (CH aliph.), 2216 (C=C=N), 1625 (C≡N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.50 (s, 3H, CH₃), 7.40 (t, 1H, J = 8.4 Hz, Ar-H), 7.58 (t, 2H, J = 8.4 Hz, Ar-H), 8.12 (m, 3H, Ar-H and CH of pyrazole ring), 8.36 (s, 2H, NH₂, D₂O exchangeable) and 8.89 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 317 [M⁺⁻¹⁺, 21.71], 316 [M⁻¹⁺, 100], 315 [M⁻¹⁺, 10.96], 300 [C₁₀H₁₀N₇⁻¹⁺, 68.70], 288 [C₁₀H₉N₇⁻¹⁺, 13.94] and 77 [C₄H₆⁻¹⁺, 23.31]; Anal. Calcd for C₁₅H₁₂N₈O: C, 60.75; H, 3.82; N, 35.42%. Found: C, 60.74; H, 3.69; N, 35.56%.

5-amino-3-methyl-1-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole-4-carbonitrile (10b).
Isolated as brown solid, Yield: 82%, mp: 290-292°C (recrystallized from EtOH 95%); IR (KBr, cm⁻¹), 3366, 3270 (NH₂), 3057 (CH aliph.), 2993, 2924 9CH aliph.), 2213 (C≡N), 1641 (C≡N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.57 (s, 3H, CH₃), 2.79 (m, 3H, CH₂), 7.41 (t, 1H, J = 7.8 Hz, Ar-H), 7.57 (t, 2H, J = 7.8 Hz, Ar-H) and 8.12 (d, 2H, J = 7.8 Hz, Ar-H), 8.36 (2H, CH₂, NH₂, D₂O exchangeable) and 8.83 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 331 [M⁺⁻¹⁺, 9.43], 330 [M⁻¹⁺, 24.61], 209 [C₁₂H₁₀N₇⁻¹⁺, 28.73], 207 [C₁₁H₉N₇⁻¹⁺, 100] and 77 [C₄H₆⁻¹⁺, 90.80%; Anal. Calcd for C₁₇H₁₃N₇O: C, 61.81; H, 4.27; N, 33.92%. Found: C, 61.72; H, 4.42; N, 33.75%.

5-Amino-4-ethyl-1-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole-4-carboxylate (10c).
Isolated as white solid, Yield: 71%, mp: 143-145°C (recrystallized from EtOH 95%); IR (KBr, cm⁻¹), 3446, 3326 (NH₂), 3060 (CH aliph.), 2937 (CH aliph.), 1688 (C=O), 1608 (C≡N); ¹H NMR (300 MHz, CDCl₃-d₆) δ ppm 1.39 (t, 3H, J = 7.5 Hz, CH₃CH₂), 2.93 (s, 3H, CH₃), 4.34 (q, 2H, J = 7.5 Hz, CH₂CH₃), 7.35 (t, 1H, J = 8.1 Hz, Ar-H), 7.55 (t, 2H, J = 8.1 Hz, Ar-H), 7.60 (2H, NH₂, D₂O exchangeable), 7.91 (s, 1H, CH of pyrazole), 8.13 (d, 2H, J = 8.1 Hz, Ar-H).
Hz, Ar-H), and 8.78 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 364 [M+1 ▰, 25.55], 363 [M ▰, 100], 334 [C16H12N3O2 ▰, 17.67], 316 [C16H12N3O ▰, 29.90], 289 [C16H12N7 ▰, 23.73] and 226 [C16H12N6 ▰, 92.91]; Anal. Calcd for C16H12N3O2: C, 59.50; H, 4.72; N, 26.98%. Found: C, 59.19; H, 5.10; N, 26.92%.

5-Amino-4-ethyl-3-methyl-1-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole-4-carboxylate (10d).

Isolated as white off solid, Yield: 72%, mp: 151-153°C (recrystallized from EtOH 95%); IR (KBr, cm⁻¹), 3422, 3303 (NH2), 2929 (CH aliph.), 1678 (C=O), 1609 (C=N); 1H NMR (300 MHz, CDCl3-d6) δ ppm 1.40 (t, 3H, J= 14.4 Hz, CH2-CH3), 2.47 (s, 3H, CH3), 2.97 (s, 3H, CH3), 4.34 (q, 2H, J= 7.0 Hz, CH2-CH3), 7.36 (t, 1H, J= 7.2 Hz, Ar-H), 7.54 (t, 2H, J= 8.1 Hz, Ar-H), 7.68 (s, 2H, NH2, D2O exchangeable), 8.14 (d, 2H, J= 7.5 Hz, Ar-H), and 8.73 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 378 [M+1 ▰, 26.66], 377 [M+1 ▰, 100], 330 [C17H10N6O2 ▰, 23.98], 303 [C16H12N3O ▰, 24.08] and 209 [C17H12N5 ▰, 14.68]; Anal. Calcd for C17H10N6O2: C, 60.47; H, 5.07; N, 25.98%. Found: C, 60.70; H, 5.00; N, 25.58%.

General procedure for synthesis of 3,5-dimethyl-1-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole (11).

To a suspension of 3 (2.4 g, 0.01 mol) in absolute ethanol (20 ml), acetyl acetone (1 g, 0.01 mol) was added, the mixture was heated under reflux for 3 hrs. The solid separated on hot was filtered off and washed with ethanol to afford compound 11. Isolated as yellow solid, Yield: 42%, mp: 115-117°C (recrystallized from dioxane); IR (KBr, cm⁻¹), 3049 (CH aliph.), 2981, 2925 (CH aliph.), 1548 (C=O); 1H NMR (300 MHz, DMSO-d6) δ ppm 2.28 (s, 3H, CH3), 2.63 (s, 3H, CH3), 2.68 (s, 3H, CH3), 6.28 (s, 1H, CH), 7.38 (t, 1H, J= 11.1 Hz, Ar-H), 7.57 (t, 2H, J= 12 Hz, Ar-H), 8.15 (d, 2H, J= 11.4 Hz, Ar-H) and 8.88 (s, 1H, CH of pyrimidine); EIMS (m/z) (relative abundance %), 304 [M ▰, 100], 303 [C17H10N6 ▰, 17.90], 289 [C16H12N3O ▰, 100], 95 [C17H12N5 ▰, 35.70] and 77 [C16H2N2 ▰, 96.40]; Anal. Calcd for C17H10N6: C, 67.09; H, 5.30; N, 27.61%. Found: C, 66.93; H, 5.28; N, 27.40%.

General procedure for synthesis of 3-[(3-Methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazono]-(ZE)-butyric acid ethyl ester (12).

Method A:
To a suspension of 3 (2.4 g, 0.01 mol) in absolute ethanol (20 ml), ethyl acetoacetate (1.30 g, 0.01 mol) was added; the mixture was heated under reflux for 5 h. The solid separated on hot was filtered off and washed with water to afford compound 12.

Method B:
A mixture of 3 (2.40 g, 0.01 mol) and ethyl acetoacetate (1.30 g, 0.01 mol) was heated without solvent for 0.5 h. After cooling, absolute ethanol (30 ml) was added and the mixture was heated under reflux for additional 3 h. The solid separated on hot was filtered off and washed with water to yield 12. Isolated as yellow solid, Yield: 58%, mp: 142-144°C (recrystallized from chloroform /methanol (8:2)); IR (KBr, cm⁻¹), 3333 (NH), 3066 (CH aliph.), 2975, 2920 (CH aliph.), 1714 (C=O), 1654 (C=N); 1H NMR (300 MHz, DMSO-d6) δ ppm 1.17, 1.21 (2t, 3H, J= 14.1 Hz, -CH2-CH3), 2.12 (s, 3H, CH3), 2.53, 2.69 (s, 3H, CH3, (Z and E isomers)), 3.44, 3.60 (2s, 2H, CH2, (Z and E isomers)), 4.07, 4.16 (2q, 2H, J= 14.1 Hz, -CH2-CH3, (Z and E isomers)), 7.33 (t, 1H, J= 7.8 Hz, Ar-H), 7.53 (t, 2H, J= 7.8 Hz, Ar-H), 7.81 (s, 1H, CH of pyrimidine), 7.98 (m, 2H, Ar-H) and 11.66 (s, 1H, NH, D2O exchangeable); EIMS (m/z) (relative abundance %), 353 [M+1 ▰, 5.69], 352 [M ▰, 19.74], 266 [C14H10N5 ▰, 23.60], 265 [C14H11N6 ▰, 100] and 77 [C14H2N2 ▰, 42.32]; Anal. Calcd for C14H10N5O2: C, 61.35; H, 5.72; N, 23.85%. Found: C, 61.19; H, 5.68; N, 23.73%.

General procedure for synthesis of compounds 13a-d.

A mixture of compound 3 (2.40 g, 0.01 mol), the appropriate aromatic aldehyde (0.01 mol) and catalytic amount of piperidine (0.5 ml) was heated under reflux in absolute ethanol (30 ml) for 3-5 h. The precipitate that formed on hot was filtered off and washed with ethanol to yield 13a-d.

N-(Benzyldiene)-N'-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazine (13a). Isolated as yellow solid, Yield: 62%, mp: 170-172°C (recrystallized from dioxane); IR (KBr, cm⁻¹), 3438 (NH), 3127, 3051 (CH aliph.), 2982, 2927 (CH aliph.), 1576 (C=N); 1H NMR (300 MHz, DMSO-d6) δ ppm 2.56, 2.81 (2s, 3H, CH2, Z and E isomers), 7.44 (m, 6H, Ar-H), 7.50 (m, 4H, Ar-H), 7.95 (m, 5H, 4Ar-H and N=CH), 8.38, 8.45 (2s, 1H, CH of pyrimidine, Z and E isomers) and 11.81, 11.98 (2s, 1H, NH, D2O exchangeable, Z and E isomers); EIMS (m/z) (relative abundance %), 329 [M+1 ▰, 8.21], 328 [M ▰, 35.53], 327 [C15H13N6 ▰, 12.51], 251 [C13H11N2 ▰, 1709]

N-(3-Fluorobenzylidine)-N′-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazine (13b).
Isolated as yellow solid, Yield: 62%; mp: 170-172°C (recrystallized from dioxane); IR (KBr, cm⁻¹): 3206 (NH), 3059 (CH arom.), 2924 (CH aliph.), 1582 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.55, 2.79 (2s, 3H, CH₃ Z and E isomers), 7.32 (m, 3H, Ar-H), 7.52 (t, 2H, J = 8.4 Hz, Ar-H), 7.94 (s, 1H, N=CH), 8.06, 8.08 (2s, 1H, CH of pyrimidine, Z and E isomers) and 11.84, 12.00 (2s, 1H, NH, D₂O exchangeable, Z and E isomers); EIMS (m/z) (relative abundance %), 347 [M+1⁻, 15.05], 346 [M⁻, 60.59], 251 [C₁₃H₁₁N₆⁻, 37.49], 225 [C₁₃H₁₁N₆⁻, 100], 209 [C₁₂H₁₀N₄⁻, 25.02] and 77 [C₆H₅⁻, 77.56]; Anal. Calcd for C₁₉H₁₆N₆: C, 69.50; H, 4.91; N, 25.59%. Found: C, 69.69; H, 5.00; N, 25.60%.

N-(3-Methoxybenzylidine)-N′-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazine (13c).
Isolated as white solid, Yield: 49%; mp: 265-267°C (recrystallized from dioxane); IR (KBr, cm⁻¹): 3433 (NH), 3071 (CH arom.), 2986, 2922 (CH aliph.), 1587 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.55, 2.77 (2s, 3H, CH₃ Z and E isomers), 7.34 (t, 1H, J = 7.5 Hz, Ar-H), 7.52 (m, 4H, Ar-H), 7.94 (s, 1H, N=CH), 8.00 (m, 4H, Ar-H), 8.43 (s, 1H, CH of pyrimidine) and 11.77, 11.90 (2s, 1H, NH, D₂O exchangeable, Z and E isomers); EIMS (m/z) (relative abundance %), 388 [M⁻, 0.03], 251 [C₁₃H₁₁N₆⁻, 50.35], 225 [C₁₂H₁₀N₄⁻, 100] and 209 [C₁₂H₁₀N₄⁻, 30.11]; Anal. Calcd for C₂₀H₁₅NO: C, 67.02; H, 5.06; N, 23.45%. Found: C, 67.00; H, 4.99; N, 23.72%.

N-(ZE)-(4-Chlorobenzylidine)-N′-(3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-hydrazine (13d).
Isolated as white solid, Yield: 62%; mp: 170-172°C (recrystallized from dioxane); IR (KBr, cm⁻¹): 3433 (NH), 3071 (CH arom.), 2986, 2922 (CH aliph.), 1587 (C=N); ¹H NMR (300 MHz, DMSO-d₆) δ ppm 2.55, 2.77 (2s, 3H, CH₃ Z and E isomers), 7.34 (t, 1H, J = 7.5 Hz, Ar-H), 7.52 (m, 4H, Ar-H), 7.94 (s, 1H, N=CH), 8.00 (m, 4H, Ar-H), 8.43 (s, 1H, CH of pyrimidine) and 11.83, 12.02 (2s, 1H, NH, D₂O exchangeable, Z and E isomers); EIMS (m/z) (relative abundance %), 363 [M+1⁻, 20.00], 362 [M⁻, 24.07], 336 [C₁₃H₁₃ClN₆⁻, 23.33], 310 [C₁₉H₁₆N₆⁻, 20.00], 294 [C₁₈H₁₄N₆⁻, 26.30], 283 [C₁₂H₁₀N₄⁻, 28.89], 198 [C₁₁H₁₀N₄⁻, 23.33] and 80 [C₆H₅⁻, 100]; Anal. Calcd for C₁₉H₁₃ClN₆: C, 62.90; H, 4.17; N, 23.16%. Found: C, 63.20; H, 4.36; N, 23.17%.

RESULTS AND DISCUSSION

Chemistry
Compound 1 was synthesized as reported.¹⁷ Compound 2a as the starting material used in Scheme 1 was prepared from the reaction of formamic acid ethyl ester 1 and excess of hydrazine hydrate in methanol at 0°C [18]. Moreover, cyclization of formimidic acid ethyl ester 1 to the corresponding iminopyrimidine was occurred in ethanol using different basic reagents such as aliphatic amines (namely, methyl amine) or aromatic amines (namely, phenyl hydrazine) to afford 2b&c, respectively in good yield. The formation of compound 2b&c was confirmed by ¹H NMR which revealed the presence of =NH group at δ 7.30 and 3.20, sequentially.

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1710
Moreover, the mass spectrum of 2b&c wed that compound 3 was synthesized by Davoodnia et al. [19] from the reaction of 4-chloro-1H-pdisplayed molecular ion peak at m/z 239 and 316 (M⁺), respectively. Searching in literature shoyrazolo[3,4-d]pyrimidine A and hydrazine hydrate at room temperature for 10 h. In this work, a new method for the preparation of compound 3 was depicted from compound 1 depending on Dimorth rearrangement in basic conditions and under reflux in ethanol as in similar reactions [20-21]. The ¹HNMR spectrum of 3 showed the characteristic singlet signals at δ 4.74 ppm and at δ 8.79 ppm for the protons of NH₂ and NH, sequentially, which were disappeared upon addition of D₂O. Also, mass spectrum of compound 3 showed the corresponding molecular ion peak at m/z 240 (Scheme 1).

The 2-ethyl or phenyl amino derivatives 4a&b were obtained when 2a was treated with ethyl or phenyl isothiocyanate. ¹HNMR spectrum of 4a revealed the presence of the NH signal at δ 7.03 ppm as triplet. Besides, a multiplet signal appeared at δ 3.31 ppm corresponding to CH₂ protons. While CH₃ of the ester group appeared at δ 1.20 as triplet signal.

Heating compound 2a with diethyl oxalate or diethyl malonate under reflux conditions gave compounds 5a&b, sequentially. IR spectra of 5a&b showed absorption bands at 1739 and 1735 cm⁻¹, respectively, attributed to C=O of ester group. Also, the reaction of 2a with chloroacetyl chloride and ethyl cyanocetate in refluxing ethanol yielded the cyclized form of chloromethyl triazole 6a and methyl carbonitrile 6b. The IR spectrum of 6b showed the presence of cyano group at 2254 cm⁻¹. Furthermore, ¹HNMR spectrum for compound 6b revealed a singlet signal at δ 4.56 ppm attributed to two protons of CH₂CN. Reacting compound 2a with different aromatic aldehydes, the cyclized triazole derivatives were obtained 7a-d. The mass spectrum of compound 7a exhibited a molecular ion peak at m/z 342. Tetrazolo derivative 8 was obtained when compound 2a was subjected to the diazotization reaction conditions. Moreover, the reaction of 2a and oxalyl chloride afforded tricyclic structure 9 with the construction of six member ring system. IR spectrum of compound 9 showed two C=O groups at 1726, 1658 cm⁻¹. ¹HNMR spectrum of 9 revealed the presence of an exchangeable singlet peak at δ 12.31 ppm corresponding to NH proton (Scheme 2).
The second key compound 3 reacted with the appropriate ethoxymethylene or ethoxyethylidene malononitrile or cyanoacetate giving new pyrazole ring bearing ortho amino-cyano or amino ester groups 10a-d. The IR spectra of compounds 10a&b revealed the C≡N group at 2213 and 2216 cm\(^{-1}\), sequentially. \(^1\)HNMR showed a single signal at \(\delta 8.36\) ppm corresponding to two protons of NH\(_2\) which were D\(_2\)O exchangeable. The C=O group of the ester moiety in compounds 10c&d appeared in IR spectra at 1688 and 1678 cm\(^{-1}\), respectively. Besides, the NH\(_2\) group of 10c&d appeared at \(\delta 7.60\) and 7.68 ppm, sequentially. Reaction of 3 with acetyl acetone gave another pyrazole ring with two methyl groups 11. In contrast, when compound 3 was allowed to react with ethyl acetoacetate in refluxing ethanol, or fused with ethylacetoacetate then refluxed with ethanol, the pyrazole ring was not formed and we got an open chain product with IR spectrum has C=O group at 1714 cm\(^{-1}\). \(^1\)HNMR spectrum showed ethyl group of the ester moiety, two singlet peaks at \(\delta 1.17, 1.21\) ppm for CH\(_3\) (Z and E isomeric forms) and at \(\delta 3.44\) and 3.60 ppm for CH\(_2\) (Z and E isomeric forms). Also, the NH appeared as singlet signal at \(\delta 11.66\) ppm disappeared by D\(_2\)O. 

Mass spectrum of 11 showed a molecular ion peak at \(m/z\) 325 and a base peak at \(m/z\) 265. This confirmed the open acyclic structure of compound 11.

Different arylidene derivatives 12a-d were also obtained from compound 3 and different aromatic aldehydes. As arylidene-hydrazide structure was reported to exist in two geometrical isomers (E and Z) about –C=N double bond,\(^{22,23}\) the structure of compounds 12a-d was confirmed with IR, \(^1\)HNMR and mass spectra for their presence as Z and E geometrical isomers. \(^1\)HNMR of compound 12a showed two sets of signals at \(\delta 2.56\) and 2.81 ppm attributed to CH\(_3\) protons. Also, CH proton of pyrimidine ring appeared at \(\delta 8.38\) and 8.45 ppm. Moreover, Z and E isomers of NH proton appeared at \(\delta 11.81\) and 11.98 ppm. \(^1\)^CNMR of compound 12c revealed the presence of two peaks at \(\delta 14.07\) and 18.32 ppm corresponding to protons of CH\(_3\) group. Also –N=CH- appeared as two sets of peaks at \(\delta 147.66\) and 147.98 ppm (Scheme 3).
BIOLOGICAL TESTING

Materials and methods

The human breast tumor cell line (MCF-7) was obtained from NCI, Cairo, Egypt.

Measurement of potential cytotoxicity

The effects of compounds on the growth of tumor cell lines (MCF-7), were evaluated according to the procedure adopted by the National Cancer Institute, Cairo, Egypt for the *in vitro* anticancer drug screening that use the protein-binding dye sulforhodamine B (SRB) to assess growth inhibition.\(^2\) Cell were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in humidified atmosphere containing 5% CO\(_2\). The cell line was regularly subcultured to be maintained in the exponential growth phase. Cells were exposed for 48 h to five concentrations of compounds (0, 5, 12.5, 25 and 50 µg/ml). Compounds were prepared in dimethylsulphoxide (DMSO), were freshly diluted with cell
culture medium just prior the assays. Methotrexate was used as positive control. For each test compound and the cell line a dose-response curve was generated and the growth inhibition of 50% (IC$_{50}$), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined. The results of in vitro cytotoxic activity experiments are presented in (Table 1).

Table 1: Results of in vitro cytotoxic activity of some of the synthesized compounds on human breast adenocarcinoma cell line (MCF-7)

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>IC$_{50}$ in µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>0.057</td>
</tr>
<tr>
<td>4a</td>
<td>0.073</td>
</tr>
<tr>
<td>4b</td>
<td>0.013</td>
</tr>
<tr>
<td>5a</td>
<td>0.015</td>
</tr>
<tr>
<td>5b</td>
<td>0.049</td>
</tr>
<tr>
<td>6a</td>
<td>0.025</td>
</tr>
<tr>
<td>6b</td>
<td>0.051</td>
</tr>
<tr>
<td>7b</td>
<td>0.042</td>
</tr>
<tr>
<td>10c</td>
<td>0.018</td>
</tr>
<tr>
<td>12c</td>
<td>0.015</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.046</td>
</tr>
</tbody>
</table>

*The values given are means of three experiments.

RESULTS AND DISCUSSION

Six of the ten newly test compounds exhibited antitumor activity against MCF-7 with IC$_{50}$ between 0.013 to 0.042 µM. On the other hand, compound 4a gave the highest result than all the other compounds. Furthermore, the result obtained from compounds 5a, 6a, 7b, 10c and 12c showed good antitumor activity (IC$_{50}$ between 0.015 to 0.042 µM) than that of the other test compounds and the standard methotrexate.

CONCLUSION

New heterocycles were obtained from the reaction between compounds 2a and 3 with several commercially available reactants. All compounds obtained retain the pyrazolo [3,4-d] pyrimidine core. Some of the newly synthesized compounds were tested in vitro on human breast cancer cell line (MCF-7). Some of the test compounds showed potent antitumor activity, especially compound 4b with IC$_{50}$ equal to 0.013 µM. Compounds 5a, 6a, 7b, 10c and 12c showed also good antitumor activity with IC$_{50}$ between 0.015 and 0.042 µM. While, compounds 2a, 4a, 5b and 6b showed weak antitumor activity with IC$_{50}$ between 0.049 and 0.073 µM compared to methotrexate the positive control which has IC$_{50}$ equal to 0.046 µM. Thus, introducing substituted amino group or ester group at the triazole moiety or pyrazole ring resulted in higher cytotoxic activity.

REFERENCES