Chem Biol Drug Des 2015; **85:** 608–622

Research Article

4-Substituted-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine Derivatives: Design, Synthesis, Antitumor and EGFR Tyrosine Kinase Inhibitory Activity

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Four series of some 4-substituted-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives 5a–f, 6a–f, 8a–f, and 9a–f were designed to be screened for their antitumor activity. All compounds were evaluated against breast (MCF-7) and lung (A-549) cell lines. Six compounds 5a, 5b, 6b, 6e, 9e, and 9f displaying activity against both cell lines were further estimated for their EGFR-TK inhibitory activity where they revealed 41–91% inhibition and compound 6b elicited the highest activity (91%). A docking study of these compounds into the ATP-binding site of EGFR-TK demonstrated their binding mode where H-bonding interaction with Met793 through N^1 of pyrimidine or N^2 of pyrazole was observed.

Key words: antitumor, EGFR-TK inhibition, molecular modeling, pyrazolo[3,4-d]pyrimidines, synthesis

Received 2 August 2014, revised 25 September 2014 and accepted for publication 4 October 2014

Protein tyrosine kinases (PTKs) are enzymes involved in many cellular processes such as cell proliferation, metabolism, and apoptosis. Several protein tyrosine kinases are activated in cancer cells resulting in tumor growth and progression (1). Inappropriate or uncontrolled activation of many of these kinases, by over-expression, constitutive activation or mutation, has shown to result in uncontrolled cell growth. PTKs can be broadly classified as receptor (RTK) or non-receptor kinases (2). The epidermal growth factor receptor (EGFR) protein belongs to the ErbB family of receptor tyrosine kinases (RTKs), which plays an important role in the regulation of cell growth, differentiation, and survival (3). EGFR is a glycoprotein that contains an extracellular ligand-binding domain, a transmembrane region, and an intracellular domain with kinase activity (4). Over-expression of these receptors was found in many cancers such as non-small cell lung cancer (NSCLC), breast, ovarian, colon, and prostate cancer and correlates with a poor prognosis in many cancer patients (5). Therefore, blocking of the kinase activity and its signal transduction pathway in cancer cells represents a rational approach to cancer therapy, and several drug discovery efforts have targeted this aberrant kinase activity in cancer (6,7). Inhibition of EGFR has been achieved through two main approaches: by blocking ligand binding to the extracellular domain with monoclonal antibodies or by using small-molecule inhibitors that interact at the ATP-binding site (8).

Pyrazolopyrimidine is an interesting bioactive core used for developing molecules of biological interest. Herein, through our ongoing efforts to develop highly potent antitumor agents, we focused on the pyrazolo[3,4-*d*]pyrimidines as a privileged lead molecule for scheming bioactive agents with promising antitumor activity. Screening the literature declared the antitumor activity of many 4-aralkylamino/anilino-pyrazolo[3,4-*d*]pyrimidines (9–18). Some of these derivatives exerted their antitumor activity via inhibition of EGFR tyrosine kinase as demonstrated by compounds **I**–**III** (19–21). Moreover, many derivatives with potent antitumor activity were found to possess 4-(un)substitutedphenylpiperidinyl **IV** (22) and 4-(un)substitutedphenyl-piperazinyl moieties **V** (23) at position 4 of the pyrazolopyrimidine nucleus (Chart 1). Accordingly, the 1-phenyl-pyrazolo[3,4-*d*]pyrimidine was selected as a pharmacophoric core for our target compounds. The first group of compounds **5a**–**f** was designed by incorporating phenylpiperidin-1-yl or (un) substitutedphenylpiperazin-1-yl moieties at position 4 of the pyrazolo[3,4-*d*]pyrimidine core.

Furthermore, regarding the pyrimidine template as an important component of nucleic acids and essential building block in many antitumor agents, several reports outlined the biological interest of compounds bearing the 2-mercapto-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile moiety as shown by compound **VI** which expressed antitumor activity against leukemia, lung and ovarian carcinoma (Chart 2) (24). Consequently, the design of the second and third groups of target compounds involved hybridization of the 1-phenylpyrazolopyrimidine nucleus to the 4-(un)substitutedphenyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile

 doi: 10.1111/cbdd.12451

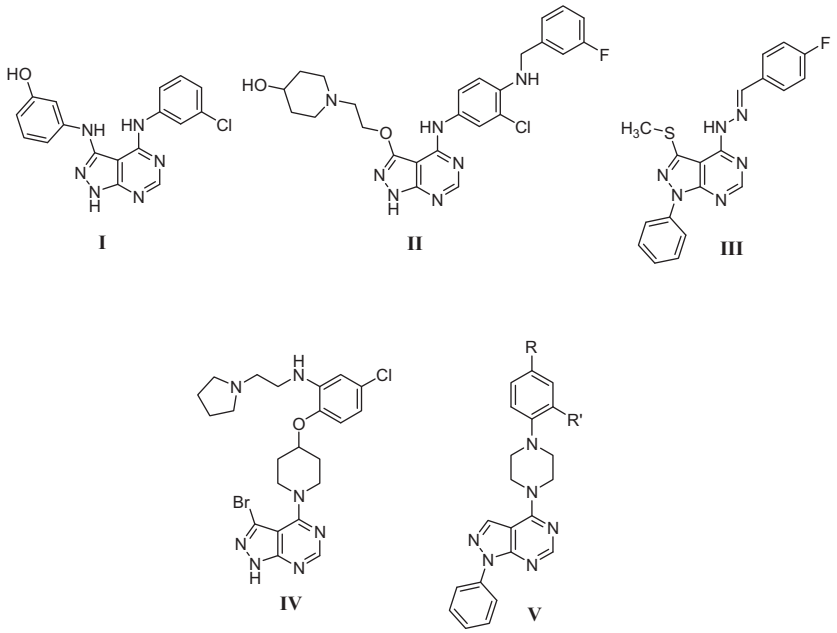


Chart 1: Examples of some pyrazolo[3,4-*d*] pyrimidines with antitumor activity.

group either through a sulfanyl or hydrazinyl spacer to produce compounds with the general formulae **6a**–**f** and **8a**–**f**, respectively.

In the same vein, and searching for another bioactive moiety of interest in the field of chemotherapy, many citations described the potent cytotoxic activity of imidazolone-based compounds as exemplified by the derivative **VII** (25). Also, it is worth mentioning that the imidazolone ring could be regarded as the ring contracted non-classical isostere of the pyrimidinone template used in compounds **6a**–**f** and **8a**–**f**. Motivated by these findings, it was thought worthwhile to carry out structural modification of compounds **6a**–**f** via substituting the pyrimidinone template by the ring contracted 2-phenyl-4-benzylidene-5-imidazolone ring along with isosteric substitution of the sulfanyl linker with the amino one to get the fourth group of target compounds of general formula **9a**–**f** (Chart 3). The substitution patterns of the designed compounds were selected to target additional regions in the ATP-binding site of the protein kinase domain to create differentially selective molecules. The designed compounds were tested for their antitumor activity against breast (MCF-7) and lung (A-549) cell lines. Compounds exhibiting activity against both cell lines were further subjected to *in vitro* EGFR tyrosine kinase activity to validate the mechanism of action of the compounds. In addition, flexible molecular docking study was performed to explore the binding mode of these compounds at the active site of the ATP-binding site of EGFR-TK.

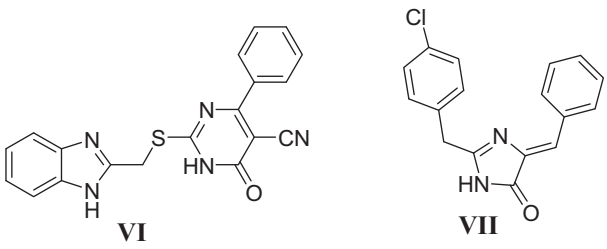


Chart 2: Pyrimidin-6-one and imidazol-5-one derivatives with potent cytotoxic activity.

Chemistry

The target compounds were synthesized as illustrated in Schemes 1 and 2. The key intermediate 4-chloro-1*H*-pyrazolo[3,4-*d*]pyrimidine **4** was prepared starting from ethyl 2-cyano-3-ethoxyacrylate **1** as depicted in Scheme 1. The latter was prepared according to the method of Guvigny and Normant from ethyl cyanoacetate and triethyl orthoformate in acetic anhydride (26). Reaction of the acrylate ester **1** with phenyl hydrazine in ethanol yielded the pyrazole derivative **2** (27) which upon condensation with formamide furnished 1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **3** (28). Chlorination of the pyrazolopyrimidinone **3** with phosphorous oxychloride gave the 4-chloro derivative **4** (29).

The first group of target compounds, 4-[(4-(un)substituted-phenyl)piperidin/piperazin-1-yl]-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidines **5a**–**f**, was synthesized from the corresponding 4-chloro intermediate **4** via reaction with 1-phenylpiperidine or the appropriate phenylpiperazine in dry dimethylformamide (DMF) in the presence of triethylamine (Scheme 2). The ^{1}H NMR spectrum of **5a** showed three multiplet signals at 1.62–2.21, 2.73–3.21, and 3.47–3.63 ppm assigned to the nine piperidine protons, in addition to multiplet signals in the aromatic region integrated

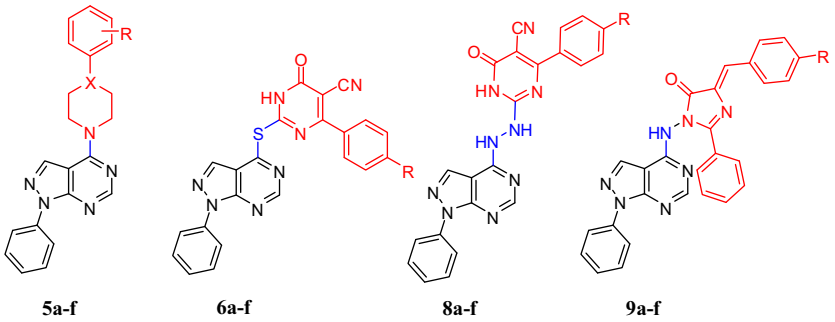
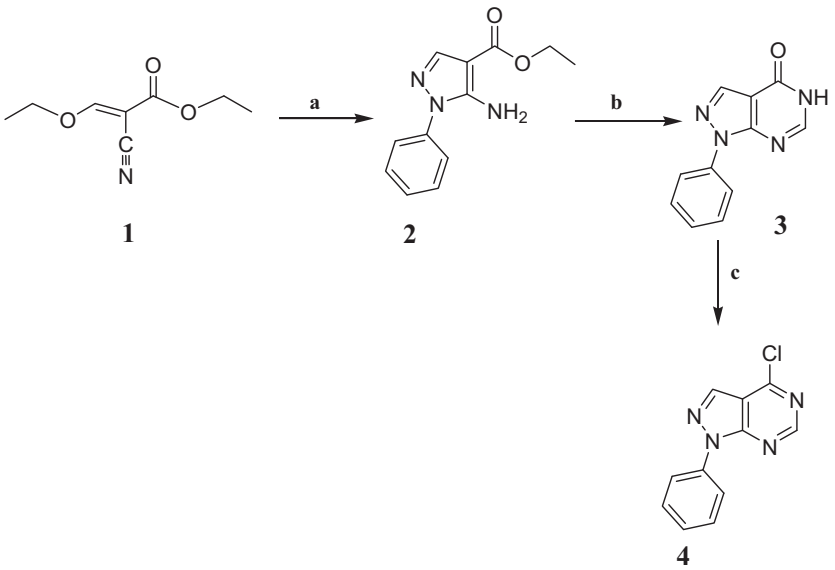


Chart 3: General structures of the target compounds.

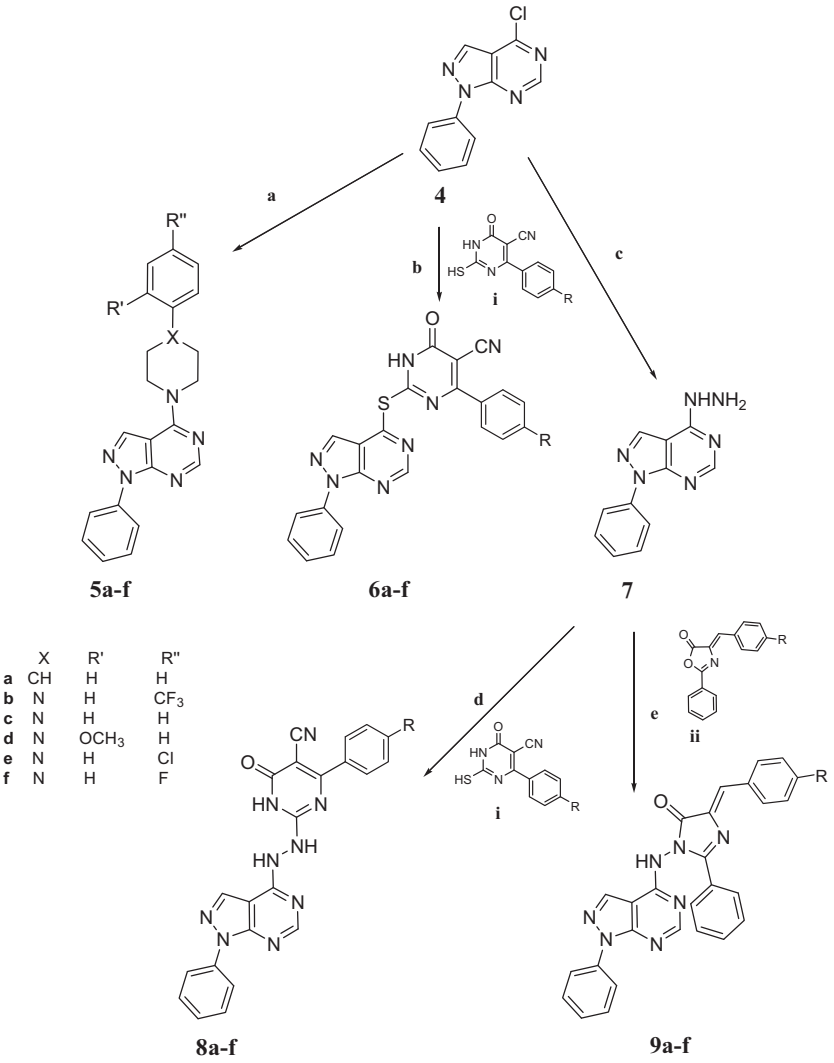


Scheme 1: Preparation of compound **4**. Reagents and reaction conditions. (a) $PhNHNH_2$, ethanol, reflux 15 h; (b) $HCONH_2$, 200–210 °C, 8 h; (c) $POCl_3$, reflux 1 h.

for the additional phenyl ring of the phenylpiperidine moiety. As for the piperazine derivatives **5b**–**e**, ^{1}H NMR spectra revealed the characteristic two triplets of the piperazine ring in the range of 3.20–3.49 and 3.75–4.23 ppm assigned for the 4 protons of the 2 methylene groups at N^4 and N^1 of the piperazine ring, respectively. In addition, other characteristic protons such as the methoxy protons of **5d** that appeared as singlet signal at 3.94 ppm were revealed.

The second group of compounds **6a**–**f** was synthesized by reacting the 4-chloro-pyrazolopyrimidine **4** and the appropriate 2-mercapto-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile derivative **i** (30) in absolute ethanol in the presence of potassium carbonate. The target compounds **6a**–**f** were characterized by IR spectra that showed the cyano and carbonyl stretching vibrations at 2229–2206 and 1714–1654 cm^{-1}, respectively. ^{1}H NMR spectra of **6a**–**f** revealed increase in the integration of aromatic protons attributed to the phenyl ring of the substituted pyrimidine moiety, in addition to the singlet signal of the NH proton appearing in the range of 10.20–12.42 ppm which disappeared upon deuteration. The 4-hydrazinyl derivative **7** was obtained according to the reported procedure by refluxing 4-chloropyrazolopyrimidine **4** with hydrazine hydrate in ethanol [29]. Reacting the 4-hydrazinyl derivative **7** with the appropriate pyrimidine-5-carbonitrile **i** in refluxing absolute ethanol afforded the third group of compounds **8a**–**f** (Scheme 2). The structure of compounds **8a**–**f** was confirmed by IR, ^{1}H NMR, and Mass spectroscopic analyses. ^{1}H NMR spectra of **8a**–**f** showed similar signals to those revealed with compounds **6a**–**f**, in addition to increase in the integration of broad exchangeable signals appearing in the range of 11.27–12.42 ppm assigned to 3 NH protons in all derivatives, except **8c** whose hydrazinyl protons appeared separately as a broad exchangeable signal at 6.60 ppm.

The fourth group including 4-(un)substitutedbenzylidene-2-phenyl-1[(1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-yl)amino]-1*H*-imidazol-5(4*H*)-ones **9a**–**f** was obtained via reaction of the 4-hydrazinyl derivative **7** with the appropriate oxazolones **ii** (31,32) in glacial acetic acid in the presence of freshly fused sodium acetate (Scheme 2). The structures assigned to compounds **9a**–**f** were confirmed from IR spectra that showed NH bands at 3422–3332 cm^{-1}, in addition to the carbonyl bands vibrating at 1705–1637 cm^{-1} (c.f. carbonyl bands of oxazolones **ii** appeared



	X	R'	R"
a	CH	H	H
b	N	H	CF_3
c	N	H	H
d	N	OCH_3	H
e	N	H	Cl
f	N	H	F

R, a = H, b = F, c = Cl, d = OCH_3, e = $N(CH_3)_2$, f = OH

Scheme 2: Synthesis of compounds **5a**–**f**, **6a**–**f**, **7**, **8a**–**f**, and **9a**–**f**. Reagents and reaction conditions. (a) Phenylpiperidine/ appropriate phenylpiperazine, dry DMF, triethylamine, reflux 15 h; (b) appropriate derivative **i**, absolute ethanol, anh. K_2CO_3, reflux 20 h; (c) $NH_2NH_2.H_2O$, ethanol, reflux, 2.5 h; (d) appropriate derivative **i**, dry DMF, TEA, reflux, 20 h; (e) appropriate derivative **ii**, gl. acetic acid, fused CH_3COONa, boiling water bath, 3 h.

at higher frequency, 1793–1762 cm^{-1}). ^1H NMR spectra revealed an increase in the integration of aromatic protons attributed to the 2 phenyl rings of the imidazolone moiety, in addition to the methine (-CH=) proton that appeared within the aromatic region and the singlet signal of the NH proton in the range of 9.90–13.40 ppm which disappeared upon deuteration. Other characteristic protons of some compounds appeared as singlet signals, namely the methoxy protons of compound **9d** at 3.86 ppm, the six protons of dimethylamino group of **9e** at 2.55 ppm, and the OH proton of **9f** at 5.80 ppm.

Experimental Section

General methods

Melting points were determined with Gallenkamp digital melting point Griffin apparatus 1901 and were uncorrected. FT-IR spectra were recorded on Bruker FT-IR 8400S spectrophotometer using KBr cell. Unless otherwise noted, ^1H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on Varian mercury 300BB at 300 MHz or Bruker Avance III 400 MHz FT-NMR at 400 MHz. ^{13}C NMR spectra were run at 75.46 or 100 MHz. Chemical Shifts were given in δ as parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. The electron impact (EI) mass spectra were recorded on Finnigan Mat SSQ 7000 (70 ev) mass spectrometer. Elemental microanalysis was performed at the Regional Center for Mycology and Biotechnology, Azhar University. TLC were monitored on Macherey-Nagel Alugram SIl G/UV_{254} silica gel plates (0.2 mm thickness) using chloroform/methanol (9.5:0.5) as eluent. The spots were visualized using Vilber Lourmet ultraviolet lamp at $\lambda = 254$ and 266 nm. All reagents and solvents were purified and dried by standard techniques.

Compounds **1** (26), **2** (27), **3** (28), **4** (29), **i** (30), **ii** (31,32), **5f** (23), and **7** (29) were prepared according to the reported procedures.

General procedure for the synthesis of compounds (5a–f)

To a solution of the 4-chloro derivative **4** (0.46 g, 2 mmol) in dry DMF (10 mL), the appropriate phenyl piperazine or 4-phenylpiperidine (2.1 mmol) and triethylamine (5 mL) were added and heated under reflux for 15 h. The solution was poured onto ice/water. The crude product was filtered off, dried, and crystallized from ethanol to give compounds **5a**–**f**.

1-Phenyl-4-[(4-phenylpiperidin)-1-yl]-1H-pyrazolo [3,4-d]pyrimidine (5a)

Creamy powder; m.p. 260–261 °C, yield, 0.46 g; 65%. IR υ_{max}/cm^{-1}: 3043 (CH aromatic); 2920, 2850 (CH aliphatic). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 1.62–2.21 (m, 4H, piperidine); 2.73–3.21 (m, 1H, piperidine); 3.47–3.63 (m, 4H, piperidine); 7.18–8.27 (m, 11H, Ar-Hs and pyrazole CH); 8.55 (s, 1H, pyrimidine CH). ^{13}C NMR (*CD*Cl$_3$, 100 MHz): δ 31.1 (C_3 + C_5 of piperidine); 42.0 (C_4 of piperidine); 46.3 (C_2 + C_6 of piperidine); 108.0, 122.2, 127.0, 127.6, 128.9, 129.6 (aromatic Cs); 136.4 (C_3 of pyrazolopyrimidine); 138.7 (C_1 of 1-phenyl ring); 149.3 (C_{7a} of pyrazolopyrimidine), 152.3 (C_6 of pyrazolopyrimidine); 157.7 (C_4 of pyrazolopyrimidine). MS m/z: 355.35 $[M]^+$. Anal. Calcd. for $C_{22}H_{21}N_5$ (355.44): C, 74.34; H, 5.96; N, 19.70. Found: C, 74.42 H, 6.02; N, 19.83.

1-Phenyl-4-[4-(4-trifluoromethylphenyl)piperazin-1-yl]-1H-pyrazolo[3,4-d]pyrimidine (5b)

Creamy powder; m.p. 285–287 °C, yield, 0.60 g; 70%. IR υ_{max}/cm^{-1}: 3109 (CH aromatic); 2958, 2843 (CH aliphatic). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 3.49 (t, 4H, J = 6, CH_2-2 and CH_2-6 piperazine); 4.23 (t, 4H, J = 6, CH_2-3 and CH_2-5 piperazine); 7.09–8.14 (m, 9H, Ar-Hs); 8.18 (s, 1H, pyrazole CH); 8.49 (s, 1H, pyrimidine CH). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 47.2 (piperazine Cs); 121.6, 122.6, 124.1, 126.3, 128.1, 129.6, 130.5 (aromatic Cs + CF_3); 135.5 (C_3 of pyrazolopyrimidine); 139.1 (C_1 of 1-phenyl ring); 149.0 (C_{7a} of pyrazolopyrimidine), 155.8 (C_1 of phenyl ring on piperazine); 156.9 (C_6 of pyrazolopyrimidine); 158.6 (C_4 of pyrazolopyrimidine). MS m/z: 425.35 $[M + 1]^+$.Anal. Calcd. for $C_{22}H_{19}F_3N_6$ (424.42): C, 62.26; H, 4.51; N, 19.80. Found: C, 62.49 H, 4.80; N, 19.93.

1-Phenyl-4-[(4-phenylpiperazin)-1-yl]-1H-pyrazolo [3,4-d]pyrimidine (5c)

Brown powder; m.p. 288–291 °C, yield, 0.42 g; 60%. IR υ_{max}/cm^{-1}: 3107 (CH aromatic); 2972, 2883 (CH aliphatic). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 3.20 (t, 4H, J = 6, CH_2-2 and CH_2-6 piperazine); 3.75 (t, 4H, J = 6, CH_2-3 and CH_2-5 piperazine); 7.16-8.06 (m, 10H, Ar-Hs), 8.14 (s, 1H, pyrazole CH); 8.29 (s, 1H, pyrimidine CH). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 48.2 (piperazine Cs); 121.8, 123.5, 124.2, 127.5, 127.9, 129.8 (aromatic Cs); 136.1 (C_3 of pyrazolopyrimidine); 139.1 (C_1 of 1-phenyl ring); 147.8 (C_{7a} of pyrazolopyrimidine), 153.0 (C_1 of phenyl ring on piperazine); 156.5 (C_6 of pyrazolopyrimidine); 160.4 (C_4 of pyrazolopyrimidine). MS m/z: 356.10 $[M]^+$. Anal. Calcd. for $C_{21}H_{20}N_6$ (356.42): C, 70.77; H, 5.66; N, 23.58. Found: C, 70.82; H, 5.64; N, 23.73.

4-[4-(2-Methoxyphenyl)piperazin-1-yl]-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (5d)

Creamy powder; m.p. 273–274 °C, yield, 0.60 g; 78%. IR υ_{max}/cm^{-1}: 3059 (CH aromatic); 2947, 2831 (CH aliphatic). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 3.26 (t, 4H, J = 5, CH_2-2 and CH_2-6 piperazine); 3.94 (s, 3H, OCH_3); 4.23 (t, 4H, J = 5, CH_2-3 and CH_2-5 piperazine); 6.94-8.14 (m, 9H, Ar-Hs); 8.19 (s, 1H, pyrazole CH); 8.49 (s, 1H, pyrimidine CH). ^{13}CNMR (CDCl$_3$, 75 MHz): δ 43.8 (piperazine Cs); 55.4 (OCH_3); 121.1, 122.1, 124.0, 125.9, 126.0, 127.2, 128.3, 129.8 (aromatic Cs); 136.0 (C_3 of pyrazolopyrimidine); 138.1 (C_1 of 1-phenyl ring); 144.5 (C_1 of phenyl piperazine); 146.5 (C_2 of phenyl on piperazine); 150.5 (C_{7a} of pyrazolopyrimidine), 157.8 (C_6 of pyrazolopyrimidne); 159.4 (C_4 of pyrazolopyrimidine). MS m/z: 386.15 $[M]^+$. Anal. Calcd. for $C_{22}H_{22}N_6O$ (386.45): C, 68.38; H, 5.74; N, 21.75. Found: C, 68.59; H, 5.40; N, 21.95.

4-[4-(4-Chlorophenyl)piperazin-1-yl]-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (5e)

Creamy powder; m.p. 284–285 °C, yield, 0.50 g; 65%. IR υ_{max}/cm^{-1}: 3116 (CH aromatic); 2974, 2885 (CH aliphatic); 779 (C-Cl). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 3.39 (t, 4H, J = 5, CH_2-2 and CH_2-6 piperazine), 4.21 (t, 4H, J = 5, CH_2-3 and CH_2-5 piperazine), 6.87–8.06 (m, 9H, Ar-Hs), 8.11 (s, 1H, pyrazole CH), 8.30 (s, 1H, pyrimidine CH). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 45.3 (piperazine Cs); 121.2, 122.5, 122.9, 125.0, 126.4, 128.3, 129.6, 129.8 (aromatic Cs); 135.6 (C_3 of pyrazolopyrimidine); 137.5 (C_1 of 1-phenyl ring); 148.7 (C_{7a} of pyrazolopyrimidine), 152.7 (C_1 of phenyl ring on piperazine); 160.0 (C_6 of pyrazolopyrimidine); 162.1 (C_4 of pyrazolopyrimidine). Anal. Calcd. for $C_{21}H_{19}ClN_6$ (390.87): C, 64.53; H, 4.90; N, 21.50. Found: C, 64.27; H, 4.79; N, 21.67.

4-[4-(4-Fluorophenyl)piperazin-1-yl]-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (5f)

Light creamy powder; m.p. 275–277 °C, yield, 0.52 g; 70%. IR υ_{max}/cm^{-1}: 3109 (CH aromatic); 2989 (CH aliphatic) (23). ^1H NMR (*CD*Cl$_3$, 300 MHz): δ 3.31 (t, 4H, J = 6, CH_2-2 and CH_2-6 piperazine), 4.20 (t, 4H, J = 6, CH_2-3 and CH_2-5 piperazine), 6.92-8.17 (m, 10H, Ar-Hs and pyrazole CH), 8.48 (s, 1H, pyrimidine CH). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 44.2 (piperazine Cs); 122.3, 122.6, 124.8, 126.3, 127.3, 129.3, 129.7, 131.2 (aromatic Cs); 135.6 (C_3 of pyrazolopyrimidine); 137.5 (C_1 of 1-phenyl ring); 148.7 (C_{7a} of pyrazolopyrimidine), 152.7

(C_1 of phenyl ring on piperazine); 160.0 (C_6 of pyrazolopyrimidine); 162.1 (C_4 of pyrazolopyrimidine). MS m/z: 374.40 $[M]^+$. Anal. Calcd. for $C_{21}H_{19}FN_6$ (374.42): C, 67.37; H, 5.12; N, 22.45. Found: C, 67.08; H, 5.27; N, 22.27.

General procedure for the synthesis of compounds (6a–f)

A mixture of the 4-chloro derivative **4** (2.30 g, 10 mmol), the appropriate 2-mercapto-dihydropyrimidine-5-carbonitrile derivative **i** (10 mmol) and anhydrous potassium carbonate (1.37 g, 10 mmol) was heated under reflux in absolute ethanol (20 mL) for 20 h. The reaction mixture was filtered while hot, and the residue was washed with hot ethanol. The filtrate and washings were concentrated, and the separated crude product was filtered off and crystallized from acetonitrile.

6-Oxo-4-phenyl-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6a)

Brown powder; m.p. 264–265 °C, yield, 3.20 g; 76%. IR υ_{max}/cm^{-1}: 3394 (NH); 3113 (CH aromatic); 2214 (C≡N); 1678 (C=O). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.27–8.06 (m, 10H, Ar-Hs); 8.06 (s, 1H, pyrazole CH); 8.30 (s, 1H, pyrimidine CH); 11.00 (s, 1H, NH exchanged with D_2O). MS m/z: 423.10 $[M]^+$. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 108.0 (C_5 of pyrimidinone); 122.2, 122.3, 127.6, 127.9, 128.7, 129.1, 129.6, 129.8, 132.5 (aromatic Cs + CN group); 136.4 (C_3 of pyrazolopyrimidine); 138.6 (C_1 of 1-phenyl ring); 147.9 (C_{7a} of pyrazolopyrimidine); 152.3 (C_6 of pyrazolopyrimidine); 160.7 (C_2 of pyrimidinone); 163.2 (C=O); 174.2 (C_4 of pyrimidinone); 179.1 (C_4 of pyrazolopyrimidine). Anal. Calcd. for $C_{22}H_{13}N_7OS$ (423.45): C, 62.40; H, 3.09; N, 23.15. Found: C, 62.10; H, 3.30; N, 23.47.

4-(4-Fluorophenyl)-6-oxo-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6b)

Dark creamy powder; m.p. 249–251 °C, yield, 3.50 g; 80%. IR υ_{max}/cm^{-1}: 3398 (NH); 3113 (CH aromatic); 2218 (C≡N); 1654 (C=O). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.44–8.16 (m, 9H, Ar-Hs); 8.28 (s, 1H, pyrazole CH); 8.47 (s, 1H, pyrimidine CH); 11.50 (s, 1H, NH exchanged with D_2O). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 108.0 (C_5 of pyrimidinone); 122.1, 122.4, 127.7, 128.2, 128.5, 128.7, 129.1, 129.3, 129.7, 130.0, 131.3 (aromatic Cs + CN group); 135.5 (C_3 of pyrazolopyrimidine); 138.7 (C_1 of 1-phenyl ring); 148.3 (C_{7a} of pyrazolopyrimidine); 150.9 (C_6 of pyrazolopyrimidine); 162.7 (C_2 of pyrimidinone); 165.2 (C=O); 173.8 (C_4 of pyrimidinone); 179.2 (C_4 of pyrazolopyrimidine). MS m/z: 442.20 $[M + 1]^+$. Anal. Calcd. for $C_{22}H_{12}FN_7OS$ (441.44): C, 59.86; H, 2.74; N, 22.21. Found: C, 59.93; H, 2.73; N, 22.00.

4-(4-Chlorophenyl)-6-oxo-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6c)

Brown powder; m.p. 288–291 °C, yield, 3.38 g; 74%. IR υ_{max}/cm^{-1}: 3421 (NH); 3118 (CH aromatic); 2198 (C≡N); 1651 (C=O). ^1H NMR (DMSO-d_6, 300 MHz): δ 7.21–8.13 (m, 9H, Ar-Hs); 8.18 (s, 1H, pyrazole CH); 8.53 (s, 1H, pyrimidine CH); 10.20 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 108.1 (C_5 of pyrimidinone); 122.2, 125.8, 126.6, 127.6, 128.2, 128.9, 129.6, 129.7 (aromatic Cs + CN group); 136.5 (C_3 of pyrazolopyrimidine); 138.7 (C_1 of 1-phenyl ring); 149.3 (C_{7a} of pyrazolopyrimidine); 152.3 (C_6 of pyrazolopyrimidine); 157.7 (C_4 of phenyl on pyrimidinone C-Cl); 161.4 (C_2 of pyrimidinone); 163.6 (C=O); 172.7 (C_4 of pyrimidinone); 176.3 (C_4 of pyrazolopyrimidine). Anal. Calcd. for $C_{22}H_{12}ClN_7OS$ (457.89): C, 57.71; H, 2.64; N, 21.41. Found: C, 57.60; H, 2.90; N, 21.31.

4-(4-Methoxyphenyl)-6-oxo-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6d)

Creamy powder; m.p. 296–297 °C, yield, 3.67 g; 81%. IR υ_{max}/cm^{-1}: 3340 (NH); 3116 (CH aromatic); 2927 (CH aliphatic); 2214 (C≡N); 1658 (C=O). ^1H NMR (DMSO-d_6, 300 MHz) δ 3.85 (s, 3H, OCH_3); 7.06–8.20 (m, 9H, Ar-Hs); 8.33 (s, 1H, pyrazole CH); 8.51 (s, 1H, pyrimidine CH); 12.40 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 55.5 (OCH_3); 107.5 (C_5 of pyrimidinone); 120.9, 121.6, 126.9, 127.2, 129.0, 129.1, 130.8 (aromatic Cs + CN group); 135.9 (C_3 of pyrazolopyrimidine); 138.2 (C_1 of 1-phenyl ring); 148.6 (C_{7a} of pyrazolopyrimidine); 157.1 (C_6 of pyrazolopyrimidine); 158.5 (C_4 of phenyl on pyrimidinone); 160.3 (C_2 of pyrimidinone); 162.3 (C=O); 176.1 (C_4 of pyrimidinone); 178.6 (C_4 of pyrazolopyrimidine). Anal. Calcd. for $C_{23}H_{15}N_7O_2S$ (453.48): C, 60.92; H, 3.33; N, 21.62. Found: C, 61.23; H, 3.61; N, 21.95.

4-[4-(*N*,*N*′-Dimethylamino)phenyl]-6-oxo-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6e)

Yellow powder; m.p. 220–222 °C, yield, 3.90 g; 85%. IR υ_{max}/cm^{-1}: 3298 (NH); 3110 (CH aromatic); 2920 (CH aliphatic); 2206 (C≡N); 1697 (C=O). ^1H NMR (DMSO-d_6, 300 MHz): δ 3.08 (s, 6H, $2CH_3$); 6.82-8.51 (m, 9H, Ar-Hs); 8.32 (s, 1H, pyrazole CH); 8.51 (s, 1H, pyrimidine CH); 12.40 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 62.3 (CH_3), 107.8 (C_5 of pyrimidinone); 116.1, 116.8, 122.3, 122.6, 127.1, 129.2, 129.6 (aromatic Cs + CN group), 134.4 (C_3 of pyrazolopyrimidine); 138.1 (C_1 of 1-phenyl ring); 150.1 (C_{7a} of pyrazolopyrimidine); 151.2 (C_6 of pyrazolopyrimidine); 157.5 (C_4 of phenyl on pyrimidinone); 158.6 (C_2 of pyrimidinone); 167.2 (C=O); 176.0 (C_4 of pyrimidinone); 178.8 (C_4 of pyrazolopyrimidine). MS m/z: 466.20 $[M]^+$. Anal. Calcd. for $C_{24}H_{18}N_8OS$ (466.52): C, 61.79; H, 3.89; N, 24.02. Found: C, 62.01; H, 4.09; N, 24.67.

4-(4-Hydroxyphenyl)-6-oxo-2-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)thio]-1,6-dihydropyrimidine-5-carbonitrile (6f)

Dark creamy powder; m.p. 262–263 °C, yield, 3.45 g; 79%. IR υ_{max}/cm^{-1}: 3288 (OH); 3161 (NH); 3082 (CH aromatic); 2229 (C≡N); 1714 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.14–8.10 (m, 9H, Ar-Hs); 8.19 (s, 1H, pyrazole CH); 8.32 (s, 1H, pyrimidine CH); 11.60 (s, 1H, OH, exchanged with D_2O), 12.42 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 108.1 (C_5 of pyrimidinone); 116.2, 116.9, 122.2, 122.8, 127.5, 129.6 (aromatic Cs + CN group), 134.5 (C_3 of pyrazolopyrimidine); 138.6 (C_1 of 1-phenyl ring); 149.2 (C_{7a} of pyrazolopyrimidine); 152.3 (C_6 of pyrazolopyrimidine); 155.1 (C_4 of phenyl on pyrimidinone); 157.7 (C_2 of pyrimidinone); 163.1 (C=O); 176.0 (C_4 of pyrimidinone); 179.0 (C_4 of pyrazolopyrimidine). MS m/z: 439.10 $[M]^+$. Anal. Calcd. for $C_{22}H_{13}N_7O_2S$ (439.45): C, 60.13; H, 2.98; N, 22.31. Found: C, 60.00; H, 3.21; N, 22.54.

General procedure for the synthesis of compounds (8a–f)

A mixture of the 4-hydrazinyl derivative **7** (0.45 g, 2 mmol), the appropriate 2-mercapto-dihydropyrimidine-5-carbonitrile derivative **i** (2 mmol) and triethylamine (3 mL) in DMF (5 mL) was heated under reflux for 20 h. The mixture was cooled and poured onto ice/water. The obtained precipitate was filtered off, washed with water, dried, and crystallized from DMF/water.

6-Oxo-4-phenyl-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8a)

Yellow powder; m.p. 260–262 °C, yield, 0.63 g; 75%. IR υ_{max}/cm^{-1}: 3435, 3415 (NHs); 3026 (CH aromatic); 2220 (C≡N); 1718 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.38–8.32 (m, 10H, Ar-Hs); 8.33 (s, 1H, pyrazole CH); 8.50 (s, 1H, pyrimidine CH); 12.42 (s, 3H, NHs exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 108.1 (C_5 of pyrimidinone); 114.8, 115.0, 115.4, 116.7, 121.3, 122.2, 124.4, 127.5, 129.1, 129.6, 130.4 (aromatic Cs + CN group); 133.9 (C_3 of pyrazolopyrimidine); 138.7 (C_1 of 1-phenyl); 149.2 (C_{7a} of pyrazolopyrimidine); 152.3 (C_6 of pyrazolopyrimidine); 157.6 (C_2 pyrimidinone); 162.8 (C=O); 164.0 (C_4 of pyrazolopyrimidine); 174.3 (C_4 of pyrimidinone). MS m/z: 421.30 $[M]^+$. Anal. Calcd. for $C_{22}H_{15}N_9O$ (421.41): C, 62.70; H, 3.59; N, 29.91. Found: C, 62.78; H, 3.62; N, 30.11.

4-(4-Fluorophenyl)-6-oxo-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8b)

Dark creamy powder; m.p. 218–219 °C, yield, 0.70 g; 80%. IR υ_{max}/cm^{-1}: 3414-3294 (NHs); 3055 (CH aromatic); 2220 (C≡N); 1674 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.00–7.95 (m, 9H, Ar-Hs); 8.23 (s, 1H, pyrazole CH); 8.45 (s, 1H, pyrimidine CH); 12.10 (s, 3H, NHs exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 108.0 (C_5 of pyrimidinone); 114.6, 115.5, 117.2, 122.5, 125.7, 127.1, 129.5, 129.7, 130.0 (aromatic Cs + CN group); 134.1 (C_3 of pyrazolopyrimidine); 138.6 (C_1 of 1-phenyl); 147.0 (C_{7a} of pyrazolopyrimidine); 154.2 (C_6 of pyrazolopyrimidine); 159.0 (C_2 pyrimidinone); 161.7 (C=O); 165.5 (C_4 of pyrazolopyrimidine); 171.3 (C_4 of pyrimidinone). MS m/z: 439.10 $[M]^+$. Anal. Calcd. for $C_{22}H_{14}FN_9O$ (439.40): C, 60.13; H, 3.21; N, 28.69. Found: C, 60.21; H, 3.28; N, 28.76.

4-(4-Chlorophenyl)-6-oxo-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8c)

Creamy powder; m.p. > 300 °C, yield, 0.75 g; 85%. IR υ_{max}/cm^{-1}: 3394 (NHs); 3050 (CH aromatic); 2209 (C≡N); 1663 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.60 (s, 2NHs exchanged with D_2O); 7.27–7.75 (m, 9H, Ar-Hs); 8.00 (s, 1H, pyrazole CH); 8.18 (s, 1H, pyrimidine CH); 11.27 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 108.0 (C_5 of pyrimidinone); 115.1, 116.5, 118.0, 123.3, 124.2, 126.6, 127.1, 129.5, 131.8 (aromatic Cs + CN group); 135.2 (C_3 of pyrazolopyrimidine); 140.6 (C_1 of 1-phenyl); 146.8 (C_{7a} of pyrazolopyrimidine); 155.2 (C_6 of pyrazolopyrimidine); 157.8 (C_2 pyrimidinone); 159.7 (C=O); 162.5 (C_4 of pyrazolopyrimidine); 173.9 (C_4 of pyrimidinone). MS m/z: 457.00, 455.00 $[M + 2, M]^+$. Anal. Calcd. for $C_{22}H_{14}ClN_9O$ (455.86): C, 57.96; H, 3.10; N, 27.65. Found: C, 57.94; H, 3.14; N, 27.78.

4-(4-Methoxyphenyl)-6-oxo-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8d)

Creamy powder; m.p. 282–283 °C, yield, 0.74 g; 82%. IR υ_{max}/cm^{-1}: 3448, 3298 (NHs); 3113 (CH aromatic); 2920 (CH aliphatic); 2206 (C≡N); 1697 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.87 (s, 3H, OCH_3); 7.13-8.20 (m, 9H, Ar-Hs); 8.31 (s, 1H, pyrazole CH); 8.33 (s, 1H, pyrimidine CH); 12.40 (s, 3H, NHs exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 55.3 (OCH_3); 108.1 (C_5 of pyrimidinone); 115.2, 115.6, 116.3, 118.2, 121.4, 122.6, 126.7, 128.5, 129.3 (aromatic Cs + CN group); 133.1 (C_3 of pyrazolopyrimidine); 137.9 (C_1 of 1-phenyl); 145.7 (C_{7a} of pyrazolopyrimidine); 153.8 (C_6 of pyrazolopyrimidine); 158.8 (C_2 pyrimidinone); 161.1 (C=O); 163.6 (C_4 of pyrazolopyrimidine); 174.0 (C_4 of pyrimidinone). MS m/z: 451.10 $[M]^+$. Anal. Calcd. for $C_{23}H_{17}N_9O_2$ (451.44): C, 61.19; H, 3.80; N, 27.92. Found: C, 61.22; H, 3.77; N, 28.04.

4-[4-(*N*,*N*′-Dimethylamino)phenyl]-6-oxo-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl) hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8e)

Yellow powder; m.p. 286–288 °C, yield, 0.74 g; 80%. IR υ_{max}/cm^{-1}: 3400 (NHs); 3045 (CH aromatic); 2951 (CH

aliphatic); 2208 (C≡N); 1705 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.08 (s, 6H, 2CH$_3$); 6.82-8.18 (m, 9H, Ar-Hs); 8.20 (s, 1H, pyrazole CH); 8.32 (s, 1H, pyrimidine CH); 12.40 (s, 3H, NHs exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 61.3 (CH$_3$); 107.6 (C$_5$ of pyrimidinone); 111.6–129.1 (aromatic Cs + CN group); 135.9 (C$_3$ of pyrazolopyrimidine); 138.2 (C$_1$ of 1-phenyl); 148.7 (C$_{7a}$ of pyrazolopyrimidine); 148.9 (C$_4$ of phenyl on pyrimidinone); 153.6 (C$_6$ of pyrazolopyrimidine); 157.1 (C$_2$ pyrimidinone); 163.4 (C=O); 163.6 (C$_4$ of pyrazolopyrimidine); 176.2 (C$_4$ of pyrimidinone). MS m/z: 464.10 [M]$^+$. Anal. Calcd. for $C_{24}H_{20}N_{10}O$ (464.48): C, 62.06; H, 4.34; N, 30.16. Found: C, 62.15; H, 4.41; N, 30.31.

4-(4-Hydroxyphenyl)-6-oxo-2-[2-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazinyl]-1,6-dihydropyrimidine-5-carbonitrile (8f)

Creamy powder; m.p. 256–258 °C, yield, 0.70 g; 79%. IR υ_{max}/cm^{-1}: 3286 (OH); 3159, 3113 (NHs); 3024 (CH aromatic); 2229 (C≡N); 1716 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.93–8.23 (m, 9H, Ar-Hs); 8.32 (s, 1H, pyrazole CH); 8.40 (s, 1H, pyrimidine CH); 10.79 (s, 1H, OH exchanged with D_2O); 12.42 (s, 3H, NHs exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 108.1 (C$_5$ of pyrimidinone); 116.9, 117.0, 122.2, 122.9, 127.6, 129.7 (aromatic Cs + CN group); 134.4 (C$_3$ of pyrazolopyrimidine); 138.7 (C$_1$ of 1-phenyl); 149.2 (C$_{7a}$ of pyrazolopyrimidine); 152.3 (C$_6$ of pyrazolopyrimidine); 155.1 (C$_4$ of phenyl on pyrimidinone); 157.6 (C$_2$ pyrimidinone); 163.1 (C=O); 163.4 (C$_4$ of pyrazolopyrimidine); 173.2 (C$_4$ of pyrimidinone). MS m/z: 437.10 [M]$^+$. Anal. Calcd. for $C_{22}H_{15}N_9O_2$ (437.13): C, 60.41; H, 3.46; N, 28.82. Found: C, 60.47; H, 3.49; N, 28.98.

General procedure for the synthesis of compounds (9a–f)

An equimolar mixture of the appropriate oxazolones **ii** (1 mmol) and the 4-hydrazinopyrazolo[3,4-*d*]pyrimidine derivative **7** (0.23 g, 1 mmol) in glacial acetic acid (5 mL) containing freshly fused sodium acetate (0.03 g, 0.36 mmol) was heated in a boiling water bath with constant stirring for 3 h. The solid product separated on cooling was filtered off, washed with water, and crystallized from ethanol.

4-Benzylidene-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]-1H-imidazol-5(4H)-one (9a)

Creamy powder; m.p. 260–262 °C, yield, 0.34 g; 75%. IR υ_{max}/cm^{-1}: 3421 (NH); 3058 (CH aromatic); 2976 (CH aliphatic); 1661 (C=O). ^{1}H NMR (C*D*Cl$_3$, 300 MHz): δ 7.25–8.20 (m, 16H, Ar-Hs + methine H); 8.22 (s, 1H, pyrazole CH); 8.26 (s, 1H, pyrimidine CH); 10.20 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 108.1 (methine C); 122.2, 124.1, 126.3, 127.4, 128.3, 129.3 (aromatic Cs); 136.4 (C$_3$ of pyrazolopyrimidine); 139.1 (C$_1$ of 1-phenyl ring); 149.3 (C$_{7a}$ of pyrazolopyrimidine), 152.6 (C$_2$ of pyrazolone); 158.4 (C$_6$ of pyrazolopyrimidine); 165.0 (C=O); 170.7 (C$_4$ of pyrazolopyrimidine). MS m/z: 457.00 [M]$^+$. Anal. Calcd. for $C_{27}H_{19}N_7O$ (457.50): C, 70.89; H, 4.19; N, 21.43. Found: C, 70.96; H, 4.22; N, 21.59.

4-(4-Fluorobenzylidene)-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]-1H-imidazol-5(4H)-one (9b)

White powder; m.p. 218–220 °C, yield, 0.38 g; 80%. IR υ_{max}/cm^{-1}: 3414 (NH); 3062 (CH aromatic); 2950 (CH aliphatic); 1693 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.38-8.19 (m, 15H, Ar-Hs + methine H); 8.32 (s, 1H, pyrazole CH); 8.38 (s, 1H, pyrimidine CH); 12.45 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 106.1 (methine C); 116.5, 116.8, 122.2, 125.5, 127.6, 128.4, 129.6, 129.8, 130.6 (aromatic Cs); 135.2 (C$_3$ of pyrazolopyrimidine); 138.7 (C$_1$ of 1-phenyl ring); 149.2 (C$_{7a}$ of pyrazolopyrimidine); 152.3 (C$_2$ of pyrazolone); 157.7 (C$_6$ of pyrazolopyrimidine); 162.7 (C$_4$ of phenyl C-F); 163.6 (C=O); 175.3 (C$_4$ of pyrazolopyrimidine). MS m/z: 475.00 [M]$^+$. Anal. Calcd. for $C_{28}H_{18}FN_7O$ (475.49): C, 68.20; H, 3.82; N, 20.62. Found: C, 68.29; H, 3.81; N, 20.71.

4-(4-Chlorobenzylidene)-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]-1H-imidazol-5(4H)-one (9c)

Yellow powder; m.p. > 300 °C, yield, 0.40 g; 85%. IR υ_{max}/cm^{-1}: 3338 (NH); 3057 (CH aromatic); 2941 (CH aliphatic); 1697 (C=O). ^{1}H NMR (C*D*Cl$_3$, 300 MHz): δ 7.21-8.22 (m, 15H, Ar-Hs + methine H); 8.24 (s, 1H, pyrazole CH); 8.51 (s, 1H, pyrimidine CH); 11.80 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (C*D*Cl$_3$, 100 MHz): δ 108.1 (methine C); 115.2, 122.2, 125.8, 126.7, 127.6, 128.2, 128.9, 129.7, 129.8, 131.8, 133.8 (aromatic Cs); 135.0 (C$_3$ of pyrazolopyrimidine); 138.7 (C$_1$ of 1-phenyl ring); 149.2 (C$_{7a}$ of pyrazolopyrimidine), 152.3 (C$_2$ of pyrazolone); 157.7 (C$_6$ of pyrazolopyrimidine); 162.4 (C$_4$ of phenyl C-Cl); 167.6 (C=O); 172.4 (C$_4$ of pyrazolopyrimidine). Anal. Calcd. for $C_{27}H_{18}ClN_7O$ (491.94): C, 65.92; H, 3.69; N, 19.93. Found: C, 65.98; H, 3.73; N, 20.04.

4-(4-Methoxybenzylidene)-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]-1H-imidazol-5(4H)-one (9d)

Creamy powder; m.p. 282–283 °C, yield, 0.40 g; 82%. IR υ_{max}/cm^{-1}: 3342 (NH); 3100 (CH aromatic); 2924 (CH aliphatic); 1637 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.86 (s, 3H, OCH$_3$); 7.09–8.29 (m, 15H, Ar-Hs + methine H); 8.32 (s, 1H, pyrazole CH); 8.33 (s, 1H, pyrimidine CH); 12.40 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (C*D*Cl$_3$, 100 MHz): δ 55.5 (OCH$_3$); 107.5 (methine C); 114.2, 122.3, 125.7, 127.4, 128.0, 128.9, 130.1, 131.8 (aromatic

Cs); 134.1 (C_3 of pyrazolopyrimidine); 137.9 (C_1 of 1-phenyl ring); 147.1 (C_{7a} of pyrazolopyrimidine); 150.3 (C_2 of pyrazolone); 156.2 (C_6 of pyrazolopyrimidine); 164.4 (C_4 of phenyl C-Cl); 166.9 (C=O); 171.9 (C_4 of pyrazolopyrimidine). MS m/z: 487.00 $[M]^+$. Anal. Calcd. for $C_{28}H_{21}N_7O_2$ (487.52): C, 68.98; H, 4.34; N, 20.11. Found: C, 68.95; H, 4.42; N, 20.19.

4-[4-(*N,N′*-Dimethylamino)benzylidene]-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl) amino]-1H-imidazol-5(4H)-one (9e)

Brown powder; m.p. 286–288 °C, yield, 0.40 g; 80%. IR υ_{max}/cm^{-1}: 3332 (NH); 3039 (CH aromatic); 2897 (CH aliphatic); 1693 (C=O). ^1H NMR (DMSO-d_6, 300 MHz): δ 2.55 (s, 6H, $2CH_3$); 7.38–8.19 (m, 15H, Ar-Hs + methine H); 8.30 (s, 1H, pyrazole); 8.33 (s, 1H, pyrimidine CH); 12.40 (s, 1H, NH exchanged with D_2O). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 62.5 (CH_3), 107.9 (methine C); 115.5, 117.9, 122.2, 124.8, 127.6, 128.7, 129.5, 130.4 (aromatic Cs); 133.6 (C_3 of pyrazolopyrimidine); 138.8 (C_1 of 1-phenyl ring); 145.2 (C_{7a} of pyrazolopyrimidine); 149.3 (C_2 of pyrazolone); 157.0 (C_6 of pyrazolopyrimidine); 165.4 (C_4 of phenyl C-Cl); 168.3 (C=O); 174.5 (C_4 of pyrazolopyrimidine). Anal. Calcd. for $C_{29}H_{24}N_8O$ (500.57): C, 69.59; H, 4.83; N, 22.39. Found: C, 69.62; H, 4.81; N, 22.48.

4-(4-Hydroxybenzylidene)-2-phenyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]-1H-imidazol-5(4H)-one (9f)

Brown powder; m.p. 256–257 °C, yield, 0.37 g; 79%. IR υ_{max}/cm^{-1}: 3410 (OH); 3230 (NH); 3062 (CH aromatic); 2929 (CH aliphatic); 1705 (C=O). ^1H NMR (DMSO-d_6, 300 MHz): δ 5.80 (s, 1H, OH exchanged with D_2O), 7.28–8.22 (m, 15H, Ar-Hs + methine H), 8.30 (s, 1H, pyrazole CH), 8.38 (s, 1H, pyrimidine CH), 12.40 (s, 1H, NH exchanged with D_2O). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 108.2 (methine C); 114.4, 116.3, 122.2, 124.4, 126.3, 127.2, 128.5, 131.3 (aromatic Cs); 134.3 (C_3 of pyrazolopyrimidine); 138.4 (C_1 of 1-phenyl ring); 145.2 (C_{7a} of pyrazolopyrimidine); 148.8 (C_2 of pyrazolone); 155.1 (C_6 of pyrazolopyrimidine); 165.4 (C_4 of phenyl C-Cl); 167.6 (C=O); 173.4 (C_4 of pyrazolopyrimidine). MS m/z: 473.10 $[M]^+$. Anal. Calcd. for $C_{27}H_{19}N_7O_2$ (473.50): C, 68.49; H, 4.04; N, 20.71. Found: C, 68.53; H, 4.08; N, 20.82.

In vitro *antitumor activity*

The cytotoxic activity was carried out at the Biotechnology Laboratory, Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University (33,34). The cytotoxic activity of the tested compounds was measured *in vitro* using the neutral red uptake assay method. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The amount of dye extracted from the cells is directly proportional to the cell mass. The cryovial containing the frozen cells was removed from liquid nitrogen storage and was immediately placed into a water bath; then, it was cultured in a flask containing complete media and incubated in an incubator at 37 °C. The cells were seeded in a plate, 55 000 per well for A-549 cell line and 35 000 per well for MCF-7 for 24 h at 37 °C. The tested compounds were added at different concentrations (0, 0.125, 0.25, 0.50, and 1 μmol/mL) in each well. Triplicate wells were prepared for each individual dose. The same procedure was applied for negative control and positive control drug (doxorubicin); then, all were incubated for 24 h. 100 μL of neutral red was added per well then incubated for 3 h. Excess unbound dye was removed using 150 μL of destain solution per well. The color intensity was measured in an ELISA reader at a wavelength of 490 nm. The percentage inhibition of growth of tumor cells was measured for all the tested compounds against breast MCF-7 and lung A-549 and is presented in Table 1.

In vitro *EGFR tyrosine kinase inhibition*

The kinase inhibitor compound profiling (KICP) was carried out at Kinexus Bioinformatics Corporation, Kinexus laboratory, Vancouver, B.C. Canada. The method was based on

Table 1: Percentage inhibition of breast MCF-7 and lung A-549 tumor cell lines caused by the synthesized compounds at a single dose of 1 μmol/mL and IC_{50} (μmol/mL) of compounds showing more than 50% inhibition

	Breast MCF-7		Lung A-549	
Compound	% inhibition at 1 μmol/mL	IC_{50} in μmol/mL	% inhibition at 1 μmol/mL	IC_{50} in μmol/mL
5a	83.36	0.72	76.33	0.28
5b	71.27	0.70	61.17	0.88
5c	NA	–	46.18	–
5d	30.14	–	67.05	0.16
5e	17.35	–	68.23	0.16
5f	59.68	0.85	NA	–
6a	17.60	–	48.09	–
6b	84.20	0.08	62.35	0.24
6c	44.20	–	45.29	–
6d	35.80	–	NA	–
6e	63.60	0.12	66.76	0.15
6f	21.60	–	67.64	0.94
8a	30.20	–	42.94	–
8b	NA	–	45.58	–
8c	34.60	–	61.17	0.18
8d	46.95	–	70.00	0.28
8e	17.60	–	NA	–
8f	88.39	0.10	29.41	–
9a	77.70	0.20	22.35	–
9b	84.06	0.10	47.64	–
9c	10.00	–	29.41	–
9d	NA	–	26.33	–
9e	78.30	0.10	52.67	0.32
9f	63.60	0.84	67.94	0.28

NA, no activity.

radioisotope assay in which direct quantification of radiolabeled phosphate from labeled ^{33}P-ATP onto a peptide or protein substrate of the target protein kinase was carried out. This assay provided a direct measure of the effect of a compound on the enzyme activity rather than a measure of the ability of a compound to bind near the active site of the kinase, Herein, profiling of the six compounds against EGFR (T790M/L858R mutant) at single concentration of 50 μM in duplicate was performed. Gefitinib was used as a positive control at dose of 10 μM.

Tested solutions were prepared to give final volume of 25 μL according to the following component ratios: Component 1: 5 μL of diluted active EGFR; Component 2: 5 μL of stock solution of peptide substrate; Component 3: 5 μL of kinase assay buffer; Component 4: 5 μL of compound (50 μM) or 10% DMSO (negative control) or 1 μM staurosporine (positive control), and Component 5: 5 μL of ^{33}P-ATP (250 μM stock, 0.8 μCi). The assay was initiated by the addition of ^{33}P-ATP, and the reaction mixture was incubated at room temperature for 20–30 min.

After the incubation period, the assay was terminated by spotting 10 μL of the reaction mixture onto multiscreen phosphocellulose P81 plate. The multiscreen phosphocellulose P81 plate was washed three times for approximately 15 min each in a 1% phosphoric acid solution. The radioactivity in the captured ^{33}P-labeled protein substrate on the P81 plate is quantified using a Trilux scintillation counter. Blank control was performed with equal volume of assay dilution buffer instead of the substrate.

The results were observed as % activity change compared to control (Table 2). Values of the results are significant if they cause >25% change in activity compared to the control.

Docking studies

The molecular docking study was carried out on Molecular Operating Environment (MOE, 10.2008) software provided by chemical computing group, Canada. The program operated on Windows XP operating system installed on Intel Pentium IV 2.80 MHz processor, 512 MB RAM. The target compounds were built using the MOE builder interface and subjected to energy minimization using the MMFF94X force field. The produced model was subjected to Systematic Conformational Search where all items were set as default with RMS gradient of 0.01 kcal/mol and RMS distance of 0.1 Å. The X-ray crystallographic structure of EGRF-TK co-crystallized with its ligand was downloaded from the protein data bank (PDB) code 3W2O. The enzyme was prepared for docking as followed:

- The ligand molecule was removed from the active site.
- H atoms were added to the structure with their standard geometry.
- MOE Alpha Site Finder was used for the active sites search in the enzyme structure, and dummy atoms were created from the obtained alpha spheres.
- Docking of the least energetic conformer for each compound in the active site of the ATP-binding site of EGFR-TK was performed.
- Docking protocol was verified by redocking of the co-crystallized ligand in the vicinity of the active site of the enzyme with energy score = 26.2922 kCal/mol and RMSD = 1.78.

Table 2: The EGFR tyrosine kinase assays of over EGFR tyrosine kinase at a single dose concentration of 50 μM

Compound	% Inhibition
5a	41
5b	51
6b	91
6e	71
9e	59
9f	77

Results and Discussion

In vitro *antitumor activity*

The synthesized compounds were screened for their antitumor activity against breast (MCF-7) and lung (A-549) cell lines at a single dose of 1 μmol/mL. Compounds eliciting more than 50% inhibition in the single-dose screening were screened at different dose levels to calculate their IC_{50} values (μmol/mL) (Table 1).

Concerning breast (MCF-7) cell lines: The results of the single-dose screening of the synthesized compounds against breast (MCF-7) cell line showed ten compounds achieving more than 50% inhibition. These compounds were further screened at different dose levels to calculate their IC_{50} values. Regarding the activity of the series of compounds **5a**–**f**, the 4-trifluoromethylphenylpiperazin-1-yl compound **5b** was the most active (IC_{50} = 0.70 μmol/mL) followed by the 4-phenylpiperidin-1-yl **5a** derivative (IC_{50} = 0.72 μmol/mL) then the 4-fluorophenyl piperazin-1-yl analogue **5f** (IC_{50} = 0.85 μmol/mL). On the other hand, out the derivatives bearing the 5-cyano-4-(4-substitutedphenyl)-6-oxo-pyrimidine template through a sulfanyl linker emerged two promising compounds, the 4-(4-fluorophenyl) derivative **6b** and its 4-(4-*N*,*N*'-dimethylamino)phenyl analogue **6e** which possessed IC_{50} = 0.08 and 0.12 μmol/mL, respectively. It is worth mentioning that compound **6b** was the most active among all synthesized compounds. Also, it was

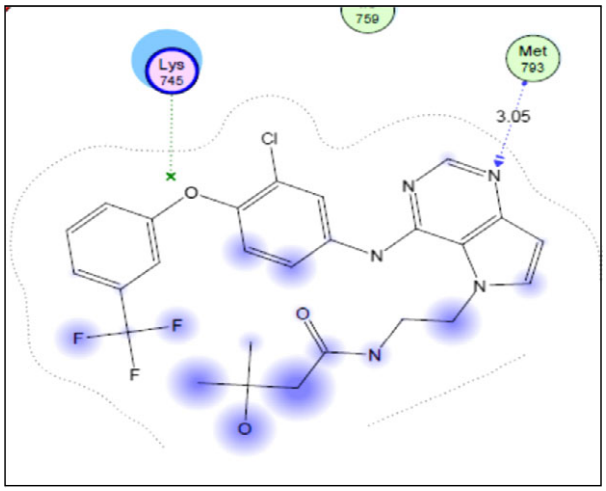


Chart 4: Co-crystallized ligand interaction with ATP-binding site of the EGFR tyrosine kinase enzyme (PDB code 3W2O).

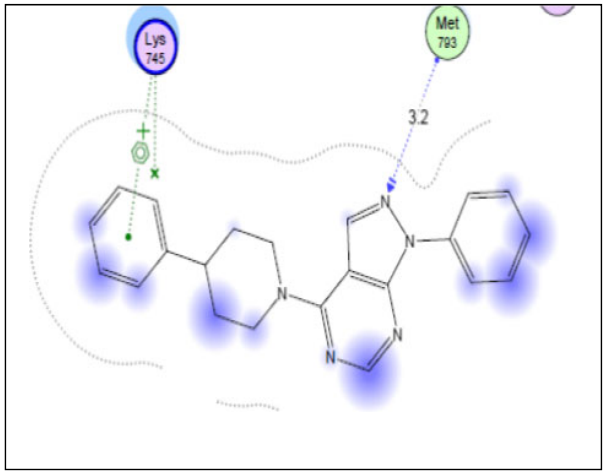


Chart 5: Docking of compound **5a** in the ATP-binding site of the EGFR tyrosine kinase enzyme.

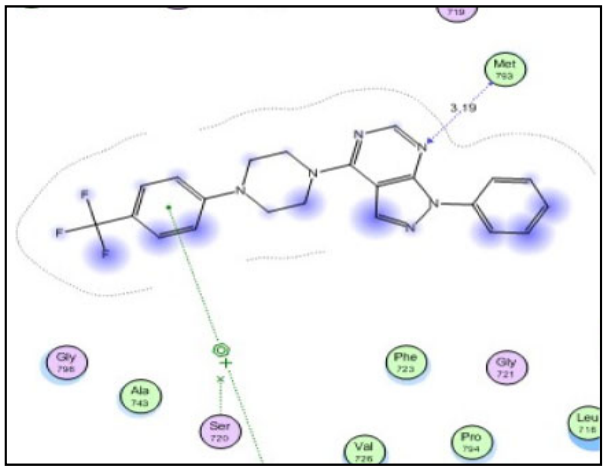


Chart 6: Docking of compound **5b** in the ATP-binding site of the EGFR tyrosine kinase enzyme.

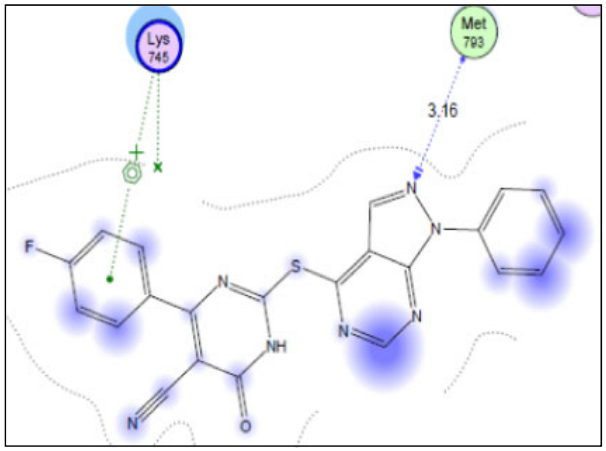


Chart 7: Docking of compound **6b** in the ATP-binding site of the EGFR tyrosine kinase enzyme.

observed that the series bearing the 6-oxopyrimidine moiety via a hydrazinyl spacer was generally of lower potency than that possessing the sulfanyl linker **6a**–**f** except for the 4-(4-hydroxyphenyl) derivative **8f** (IC_{50} = 0.10 μmol/mL). Finally, the compounds bearing the ring contracted 5-oxoimidazoline moiety **9a**–**f** were potentially more active than their 6-oxopyrimidine congeners. The 4-(4-fluorobenzylidene) derivative **9b** and the 4-(4- *N*,*N*'-dimethylaminobenzylidene) derivative **9e** were equipotent, and they were the most active in this series (IC_{50} = 0.10 μmol/mL). The 4-benzylidene compound **9a** possessed half the potency of **9b** and **9e** (IC_{50} = 0.20 μmol/mL). On the other hand, the 4-(4-hydroxybenzylidene) analogue **9f** was the least potent in this series (IC_{50} = 0.84 μmol/mL). Based on the antitumor activity of the tested compounds and their IC_{50} values, it could be concluded that derivatives bearing the substituted heterocyclic moieties 6-oxopyrimidine through a sulfanyl linker (**6a**–**f**) or the 5-oxoimidazoline via an amino spacer (**9a**–**f**) at position 4 of the pyrazolopyrimidine core were more active than those bearing either 6-oxopyrimidine moiety through a hydrazinyl bridge (**8a**–**f**) or hetero-alicyclic moiety (piperidine or piperazine) directly attached to the pyrazolopyrimidine core (**5a**–**f**).

Regarding lung (A-549) cell line: The single-dose screening of the synthesized compounds against lung A-549 cell line revealed that eleven compounds exhibited more than 50% inhibition, and accordingly, IC_{50} values for these compounds were calculated (Table 1). The results showed that compounds bearing the 4-phenylpiperidine/4-phenylpiperazine moieties were more effective against lung cell lines than breast cell lines as demonstrated by **5a**, **5b**, **5d**, and **5e** (IC_{50} = 0.28, 0.88, 0.16, and 0.16 μmol/mL, respectively). The 4-(2-methoxyphenyl)piperazin-1-yl derivative **5d** and its 4-(4-chlorophenyl)piperazin-1-yl analogue **5e** were

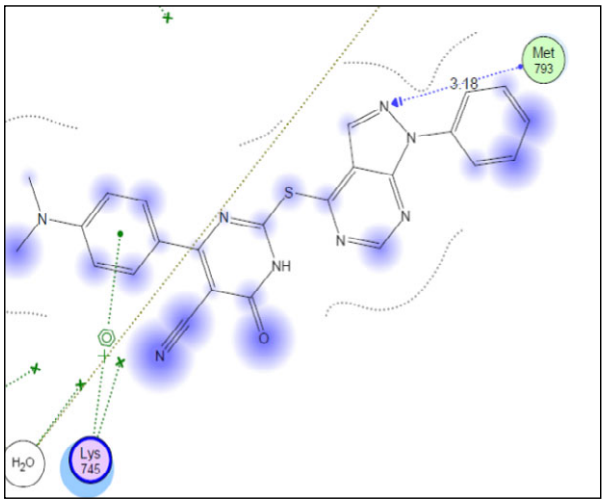


Chart 8: Docking of compound **6e** in the ATP-binding site of the EGFR tyrosine kinase enzyme.

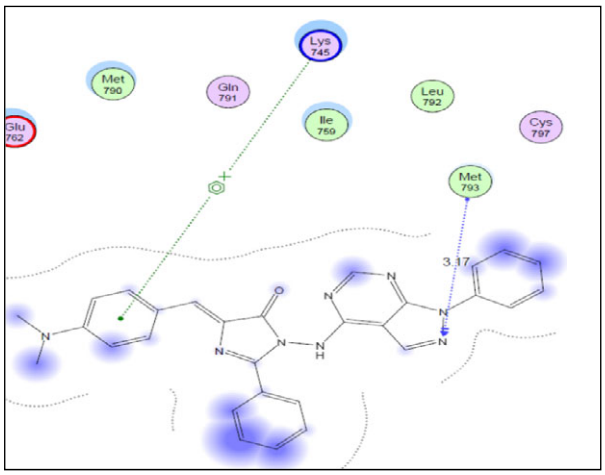


Chart 9: Docking of compound **9e** in the ATP-binding site of the EGFR tyrosine kinase enzyme.

equipotent, and they were the most active in this series. Compounds bearing the 6-oxopyrimidine moiety via the sulfanyl linker **6a**–**f** showed considerable activity where the 4-(4-*N*,*N*'-dimethylamino)phenyl analogue **6e** (IC_{50} = 0.15 μmol/mL) was the most active among this series followed by compounds **6b** and **6f** (IC_{50} = 0.24 and 0.94 μmol/mL, respectively). Alternatively, replacing the sulfanyl linker by a hydrazinyl one emerged two potentially active compounds, namely **8c** and **8d** with IC_{50} = 0.18 and 0.28 μmol/mL, respectively. On the other hand, the ring contracted 5-oxoimidazoline derivatives **9e** and **9f** exhibited good potencies (IC_{50} = 0.32 and 0.28 μmol/mL), respectively. By analyzing the results of the lung A-549 cell line, it could be claimed that the series eliciting considerable activity were the piperidin-/piperazin-1-yl derivatives **5a**–**f** and the 6-oxopyrimidine derivatives linked through the sulfanyl spacer **6a**–**f**.

In general, there was no consistent relation between the lipophilicity and/or electronic nature of the substituents (R) and the activity in any of the series.

Finally, it could be observed that some derivatives explored activity against both cell lines such as compounds **5a**, **5b**, **6b**, **6e**, **9e**, and **9f**. These compounds represent candidates for future optimization.

In vitro *EGFR tyrosine kinase inhibition*

Compounds exhibiting activity against both tested cell lines were subjected to EGFR tyrosine kinase inhibition testing. Gefitinib was used as positive control at dose of 10 μM where it exhibited 100% inhibition. The profiling data of the tested compounds **5a**, **5b**, **6b**, **6e**, **9e**, and **9f** against EGFR showed modest inhibition of EGFR target ranging from 41% to 91% as presented in Table 2. All compounds except **5a** showed inhibition percent more than 50%, and two compounds demonstrated more than 75% inhibition, namely compounds **6b** and **9f**. The 4-(4-phenyl)piperidin-1-yl derivative **5a**, the 4-(4-trifluoromethyl)phenyl-piperazin-1-yl derivative **5b**, and the 3-substituted-4-(4-*N*,*N*'-dimethylamino)benzylidene-2-phenyl-1*H*-imidazol-5(4*H*)-one **9e** showed moderate to good activity (41%, 51%, and 59% inhibition, respectively). Remarkable inhibitory activity was observed with compounds **6e** and **9f** (71% and 77% inhibition, respectively). The most potent compound was the 2-substituted-4-(4-fluorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile derivative **6b** (91% inhibition).

Docking studies

Compounds **5a**, **5b**, **6b**, **6e**, and **9e** eliciting activity against both tested cell lines and showing good to potent EGFR tyrosine kinase inhibition (41–91%) were docked into the active site of the ATP-binding site of EGRF-TK to visualize the orientation and binding mode of the active compounds. Molecular Operating Environment (MOE 10.2008) software provided by chemical computing group, Canada was used. The co-ordinates of the EGFR-TK structure were obtained from the crystal structure of EGFR with its co-crystallized inhibitor: *N*-(2-(4-[(3-chloro-4-(3-(trifluoromethyl)phenoxy)phenyl)amino]-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl)-3-hydroxy-3-methylbutanamide (PDB code 3W2O) which was chosen because of the structure similarity between the inhibitor and our target compounds. The energy minimization for the compounds was performed with MOE with MMFF94X force field, and the partial charges were automatically calculated. The root mean square difference (RMSD) between the top docking pose and original crystallographic geometry was 1.78. The main interaction between the co-crystallized ligand and the enzyme-active site is a H-bond involving N^1-pyrimidine and the amino acid Met793 with a bond length of 3.05 Å in addition to some hydrophobic interactions at different positions of the structure (Chart 4). Compound **5a** underwent H-bond interaction with Met793 (bond

length = 3.20 Å) through N^2-pyrazole, in addition to arene–cation interaction between Lys745 and the 4-phenylpiperidine ring (Chart 5). Compound **5b** showed similar H-bond interaction with Met793 through N^1-pyrimidine with a bond length 3.19 Å (Chart 6). Derivatives **6b** and **6e** revealed a H-bond interaction involving their N^2-pyrazole and Met793 with bond length = 3.16 and 3.18 Å, respectively. Arene–cation interaction with the phenyl ring on the 6-oxopyrimidine moiety and Lys745 was also observed for both compounds (Charts 7 and 8). The same binding mode was observed for compound **9e** that interacted with Met793 via a H-bond through its N^2-pyrazole (bond length = 3.17 Å) and arene–cation interaction between Lys745 and the benzylidene ring (Chart 9). The docking results suggested that the target compounds were successfully docked into the active site of the ATP-binding site of EGFR-TK and that the binding mode was similar to that of the docked ligand as demonstrated by the interactions with Met793. Most compounds showed additional interactions with Lys745 through the phenyl rings located on the substituents at position 4 of the pyrazolopyrimidine nucleus. Good correlation could be observed between the binding mode of the compounds in the ATP-binding site of EGFR-TK and their biological screening results. For instance, the closer interaction of compound **6b** with Met793 (H-bond = 3.16 Å) was coinciding with its high potency against breast cells (IC_{50} = 0.08 μmol/mL) and high *in vitro* inhibition percent of EGFR tyrosine inhibition (91%). On the other hand, compound **5a** which formed a H-bond of 3.20 Å with Met793 showed a lower potency (0.72 μmol/mL) against breast cells and a lower *in vitro* inhibition of EGFR tyrosine kinase (41%).

Conclusion

Four groups of compounds were designed involving the synthesis of some 4-substituted-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidines. The substitution pattern was selected to incorporate (un)substitutedphenyl azacyclic, substituted pyrimidine carbonitrile, or substituted imidazolinone moieties which were either directly attached to the pyrazolopyrimidine core or separated via different linkers, namely: sulfanyl, hydrazinyl, or amino. The first group of compounds was synthesized by reacting the 4-chloropyrazolopyrimidine **4** with the appropriate 4-(un)substitutedphenylpiperidine/piperazine derivatives to give compounds **5a**–**f**. The second and third groups comprised those compounds bearing 4-(4-(un)substitutedphenyl)-1,6-dihydro-6-oxo-pyrimidine-5-carbonitrile moiety either through a sulfanyl linker, **6a**–**f**, or a hydrazinyl spacer, **8a**–**f**, respectively. This was achieved by reacting the appropriate 2-thioxopyrimidine-5-carbonitrile **i** with either 4-chloro-pyrazolopyrimidine **4** to afford **6a**–**f** or with the 4-hydrazinyl intermediate **7** to obtain **8a**–**f**. Finally, the fourth group of compounds possessing the 4-(4-(un)substitutedbenzylidene)-2-phenyl-1*H*-imidazol-5(4*H*)-one moiety **9a**–**f** was synthesized by reacting the 4-hydrazinyl intermediate **7** with the appropriate oxazolones **ii**. All compounds were screened against breast (MCF-7) and lung (A-549) cell lines, where series **5a**–**f**, **6a**–**f**, and **9a**–**f** presented compounds that were the most effective against either or both cell lines. Six compounds displayed activity against both cell lines. EGRF-TK inhibitory activity of these compounds showed moderate activity. The most active compound was **6b** showing (91% enzyme inhibition). Docking of these compounds into the ATP-binding site of EGRF-TK demonstrated that the binding mode of these compounds involved H-bond interaction with Met793 through the N^1 of pyrimidine ring or the N^2 of pyrazole ring with a bond length ranging from 3.16 to 3.20 Å. Additional arene–cation interaction with Lys745 was revealed with some compounds. Good correlation between the docking study and EGFR-TK inhibition supported targeting of the EGFR-TK and presented compound **6b** as a promising and attractive antitumor agent that might be a promising lead for further studies.

Acknowledgments

The authors would like to thank Dr. Yassin M. Nissan, Molecular Modeling Laboratory of the Microanalytical Unit, Faculty of Pharmacy, Cairo University for his kind help in performing the molecular docking study.

Conflict of Interest

The authors have declared no conflict of interest.

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