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Synthesis, docking study and antitumor evaluation of certain newly synthesized pyrazolo[3,4-*d*]pyrimidine derivatives

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ABSTRACT

A series of new 4-substituted amino-1*H*-pyrazolo[3,4-*d*]pyrimidines **(6a-e)** and **(8a-e)** was synthesized using ethyl 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxylate **(1)** as the starting material. The cytotoxic activity of the newly synthesized compounds against human breast adenocarcinoma (MCF-7) cell line was investigatd. Most of the tested compounds showed potent to moderate growth inhibitory activity, especially 3,6-dimethyl-1-phenyl-4-[3-(1-acetyl-5—(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine **(8b)** exhibited the highest activity among the tested compounds with $IC_{50\%}$ equal to 28.157 µg/mL. Docking the synthesized compounds into the epidermal growth factor receptor (EGFR), which is highly expressed in breast cancer, was performed to explore the possible interactions of these compounds with the EGFR. The activity of the reported compounds supports its clinical promise as a component of therapeutic stratiges for cancer, for which high concentration of chemotherapeutic agents are always a major limitation.

KEYWORDS

Pyrazolo[3,4-*d*]pyrimidine;
Antitumor;
MCF-7;
Docking study;
EGFR.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (TK) of the ERB (HER) family[1]. In healthy tissues, activation of EGFR contribute to cell proliferation, differentiation, migration, adhesion, protection from apoptosis and angiogenesis[2]. Abnormal activtation of EGFR has been implicated in the pathogenesis and progression of many cancer, such as breast, ovarian, colon and prostate cancer[3]. Therefore, targeting EGFR represents a rational approach for the development of novel anticancer therapies[4]. Most EGFR inhibitors share common properties, low molecular weight (small molecules), hydrophobic heterocycles and act by competing with ATP for binding in ATP binding site[5].

A number of epidermal growth factor receptor (EGFR) kinase inhibitors had been evaluated in cancer clinical trials. For example, anilinoquinazoline-containing compounds erlotinib (Tarceva™)[6] and gefitinib

(Iressa™)[7] had been approved as chemotherapeutic agents for treatment of patients with advanced non small lung cancer. Also, lapatinib (Tykerb™)[8] was approved for treatment of HER2-positive advanced or metastatic breast cancer.

The pyrazolo[3,4-*d*]pyrimidine nucleus is considered as an isostere to purine nucleus and hence exhibits promising antitumor activity by acting as ATP competitive inhibitor for many kinase enzymes. Indeed, many pyrazolo[3,4-*d*]pyrimidines were reported to exhibit potent antitumor activity[9-14]. Their cytotoxic activities might be attributed to inhibition of several enzymes such as glycogen synthase kinase (GSK)[15], cyclin dependent kinase (CDK)[16], dual src/ Ab1kinase[17] and epidermal growth factor receptor (EGFR)[18].

Herein, we described the design and the synthesis of a new series of 4-substituted aminopyrazolo[3,4-*d*]pyrimidines as antitumor agents. The rational for the design of target compounds was based upon some structural modifications on the general features of anilinoquinazoline-containing compounds [Figure 1]. These modifications comprise a replacement of benzene moiety in quinazoline skeleton by a pyrazolo-moiety hence the pyrazolo-moiety is naturally occurring in

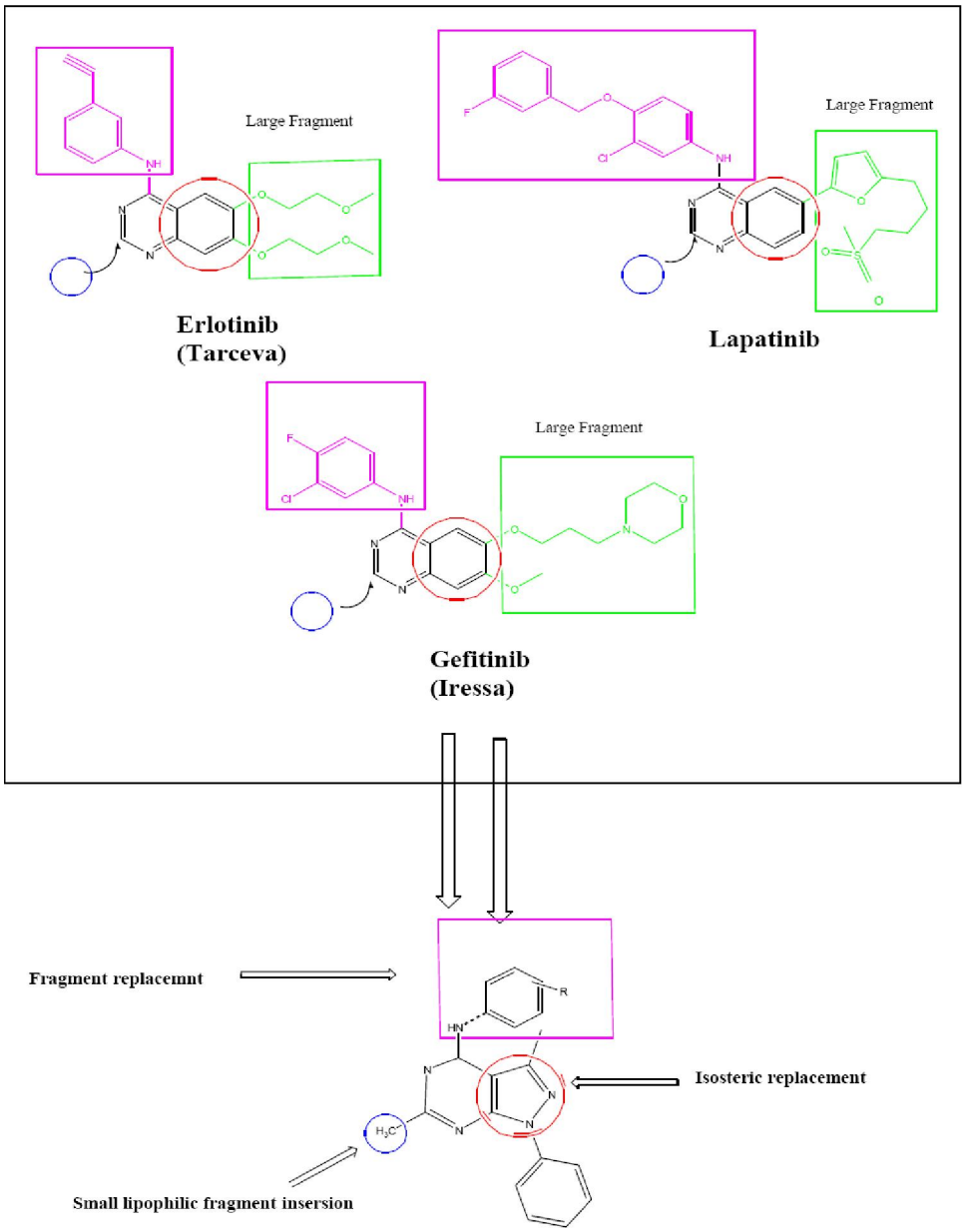


Figure 1 : Planned design of new pyrazolo[3,4-*d*]pyrimidine derivatives for cytotoxic activity

body purine bases and this expected to be more intensive for cytotoxic activity.

EXPERIMENTAL

Chemistry

Melting points were determined on a Griffin apparatus and are uncorrected. IR spectra were determined on Shimadzu IR 435 spectrophotometer and values were represented in cm^{-1}, at the Microanalytical center, Cairo University. ^1H NMR spectra were carried out on Varian Gemini 300 MHZ spectrometer, at nuclear magnetic resonance, Cairo University, using TMS as internal standard and chemical shifts were recorded in ppm on δ scale. The electron impact (EI) mass spectra were recorded on Shimadzu QP-2010 plus, at the Microanalytical center, Cairo University. Analytical thin layer chromatography (TLC) on silica gel plates containing UV indicator was employed routinely to follow the course of reactions and to check the purity of products. All reagents and solvents were purified and dried by standard techniques. Elemental microanalyses were carried out at the Microanalytical center, Cairo University.

Compound **(1)** was obtained according to the reported procedure[19].

5-Amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxylic acid (2)

A mixture of ethyl 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxylate **(1)** (12.25 gm, 0.05 mol) and sodium hydroxide (4.20 gm, 0.1 mol) in methanol (60 mL) was heated under reflux for 5h. After cooling, the reaction mixture was poured into ice-cold water, then adjustied pH of the mixture to 4 using concentrated hydrochloric acid. The obtained solid was filtered, dried and crystallized from ethanol/water.

Mp 156-157 °C; yield: 56.2%; IR (cm^{-1}): 3389.28-3204.15 (OH & NH_2); 3009.37 (CH aromatic); 2927.41 (CH aliphatic); 1651.73 (C=O); 1613.16 (C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.24 (s, 3H, CH_3); 6.30 (s, 2H, NH_2, D_2O exchangeable); 7.36-7.54 (m, 5H, ArH); 12.07 (s,1H, OH, D_2O exchangeable); MS *m/z*: 217 (M⁺, 27.65). Anal. Calcd for $C_{11}H_{11}N_3O_2$ (217.23): C, 60.82; H, 5.10; N, 19.34. Found: C, 60.69; H, 5.20; N, 19.67.

3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*][1,3]oxazin-4-one (3).

A mixture of 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxylic acid **(2)** (2.17 gm, 0.01 mol) and acetic anhydride (5 mL) was heated under reflux for 5 h. After cooling, the formed solid was filtered, dried and crystallized from methanol.

Mp 129-130 °C; yield: 49.79%; IR (cm^{-1}): 3078.80 (CH aromatic); 2923.56 (CH aliphatic); 1764.55 (C=O); 1599.66 (C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.46 (s, 3H, CH_3 of pyrazole); 2.49 (s, 3H, CH_3 of pyrimidine); 7.42 (t, *J*= 7.8 Hz, 1H, ArH); 7.56 (t, *J*= 7.8 Hz, 2H, ArH); 7.90 (d, *J*= 8.4 Hz, 2H, ArH); MS *m/z*: 241 (M, 100). Anal. Calcd for $C_{13}H_{11}N_3O_2$ (241.25):C, 64.72; H, 4.60; N, 17.42. Found: C, 65.01; H, 4.73; N, 17.19.

3,6-Dimethyl-1-phenyl-1,5-dihydropyrazolo[3,4-*d*]pyrimidin-4-one (4)

A mixture of the 3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*][1,3] oxazin-4-one **(3)** (2.41 gm, 0.01mol) and formamide (40 mL) was heated under reflux for 6 h. On cooling, the separated solid was filtered, washed with water and crystallized from ethanol.

Mp 298-300°C; yield: 60.19 %; IR (cm^{-1}): 3420.14 (NH); 3056.62 (CH aromatic); 2923.56 (CH aliphatic); 1676.80 (C=O); 1595.81 (C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.37 (s, 3H, CH_3 of pyrazole); 2.49 (s, 3H, CH_3 of pyrimidine); 7.34 (t, *J*= 7.2 Hz, 1H, ArH); 7.51 (t, *J*= 7.5 Hz, 2H, ArH); 8.02 (d, *J*= 8.7 Hz, 2H, ArH); 12.24 (s,1H, NH, D_2O exchangeable); MS m/z: 240 (M, 100). Anal. Calcd for $C_{13}H_{12}N_4O$ (240.27): C, 64.99; H, 5.03; N, 23.32. Found: C, 64.98; H, 4.93; N, 23.02.

4-Chloro-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (5)

A mixture of 3,6-dimethyl-1-phenyl-1,5-dihydropyrazolo[3,4-*d*] pyrimidin-4-one **(4)** (12 gm, 0.05 mol) and phosphorous oxychloride (300 mL) was heated under reflux for 5 h. Excess phosphorus oxychloride was distilled under reduced pressure and the residual syrup was poured onto crushed ice. The aqueous suspension was extracted with chloroform (3×300 mL). Drying the extract overnight over anhydrous so-

dium sulfate (10 gm) and chloroform was distilled to yield a slightly yellow coloured product. This crude product was crystallized from hexane.

Mp 100-102°C; yield: 60.19 %; IR (cm^{-1}): 3058.68 (CH aromatic); 2923.98 (CH aliphatic); 1601.55 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.25 (s, 3H, CH_3 of pyrazole); 2.47 (s, 3H, CH_3 of pyrimidine); 7.38 (t, *J*= 7.2 Hz, 1H, ArH); 7.55 (t, *J*= 7.2 Hz, 2H, ArH); 8.02 (d, *J*= 9 Hz, 2H, ArH); MS m/z: 258 (M, 75). Anal. Calcd for $C_{13}H_{11}ClN_4$ (258.71): C, 60.35; H, 4.29; N, 21.66. Found: C, 60.36; H, 4.19; N, 21.85.

General procedure for the synthesis of 3,6-Dimethyl-1-phenyl-4-substitutedamino-1*H*-pyrazolo[3,4-*d*]pyrimidines (6a-e)

A mixture of 4-chloro-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine **(5)** (2.58 gm, 0.01 mol), the appropriate aromatic amine (0.01 mol) and sodium iodide (0.2 gm) in isopropyl alcohol (20 mL) was heated under reflux for 4h. After cooling, the reaction mixture was neutralized with sodium carbonate solution (20%). The formed precipitate was collected by filtration, washed with water and crystallized from ethanol.

4-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenol (6a).

Mp 268-269 °C; yield: 60%; IR (cm^{-1}): 3367.10 (NH); 3204.15 (OH); 1517.70 (C=N);^{1}H NMR (DMSO-d_6) *δ* ppm: 2.42 (s, 3H, CH_3 of pyrazole); 2.65 (s, 3H, CH_3 of pyrimidine); 6.77 (d, *J*= 6.6 Hz, 2H, ArH); 7.28 (t, *J*= 7.2 Hz, 1H, ArH); 7.42 (d, *J*= 6.6 Hz, 2H, ArH); 7.50 (t, *J*= 7.2 Hz, 2H, ArH); 8.16 (d, *J*= 8.7 Hz, 2H, ArH); 8.47 (s, 1H, NH, D_2O exchangeable); 9.38 (s, 1H, OH, D_2O exchangeable).; MS *m/z*: 331 (M, 100); Anal. Calcd for $C_{19}H_{17}N_5O$ (331.38): C, 68.87; H, 5.17; N, 21.13. Found: C, 69.10; H, 4.88; N, 21.40.

4-(3-Acetylphenyl)amino-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (6b).

Mp 180-181°C; yield: 44.82 %; IR (cm^{-1}): 3435.56 (NH); 2920.66 (CH aliphatic); 1682.59 (C=O); 1564.95 (C=N); ^{1}H NMR ($CDCl_3$) δ ppm: 2.66 (s, 3H, CH_3 of pyrazole); 2.70 (s, 3H, CH_3 of pyrimidine); 2.79 (s, 3H, $COCH_3$); 7.31 (t, *J*= 6.9 Hz, 1H, ArH); 7.48-7.54 (m, 4H, 3ArH &1NH, D_2O exchangeable); 7.73 (d, *J*= 6.9 Hz, 1H, ArH); 8.14-8.22 (m, 3H, ArH); 8.34 (s, 1H, ArH); MS m/z: 357 (M, 100). Anal. Calcd for $C_{21}H_{19}N_5O$ (357.42): C, 70.57; H, 5.36; N, 19.59. Found: C, 70.31; H, 4.90; N, 20.01.

4-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)benzoic acid (6c)

Mp > 300°C; yield: 82%; IR (cm^{-1}): 3443.28 (NH); 2917.77 (OH); 1681.62 (C=O); 1603.52 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 22.51 (s, 3H, CH_3 of pyrazole); 2.57 (s, 3H, CH_3 of pyrimidine); 7.32 (t, *J*= 7.5 Hz, 1H, ArH); 7.54 (t, *J*= 5.7 Hz, 2H, ArH); 7.95-7.98 (m, 4H, ArH); 8.19 (d, *J*= 9 Hz, 2H, ArH); 8.89 (s, 1H, NH, D_2O exchangeable); 12.66 (s, 1H, OH, D_2O exchangeable); MS m/z: 359 (M, 100); Anal. Calcd for $C_{20}H_{17}N_5O_2$(359.39): C, 66.84; H, 4.77; N, 19.49. Found: C, 66.83; H, 4.67 ; N, 19.86.

2-(3,6-Dimethyl-1-phenyl-1H-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)benzoic acid (6d)

Mp 250-252°C; yield: 69%; IR (cm^{-1}): 3432.67 (NH); 3182.93 (OH); 1684.52 (C=O); 1605.45 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.53 (s, 3H, CH_3 of pyrazole); 2.71 (s, 3H, CH_3 of pyrimidine); 7.08-7.28 (m, 2H, ArH); 7.49-7.58 (m, 3H, ArH); 7.99-8.13 (m, 4H, ArH); 9.16 (s, 1H, NH, D_2O exchangeable); 11.52 (s, 1H, OH, D_2O exchangeable); MS m/z: 359 (M, 18.46); Anal. Calcd for $C_{20}H_{17}N_5O_2$(359.39): C, 66.84; H, 4.77; N, 19.49. Found: C, 66.45; H, 4.54; N,19.61.

Ethyl 4-(3,6-dimethyl-1-phenyl-1H-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)benzoate (6e).

Mp 229-231°C; yield: 83%; IR (cm^{-1}): 3310.21 (NH); 1710.55 (C=O); 1632.45 (C=N);^{1}H NMR ($CDCl_3$) δ ppm: 1.42 (t, *J*= 6.9 Hz, 3H, CH_2-C<u>H</u>$_3$); 2.70 (s, 3H, CH_3 of pyrazole); 2.73 (s, 3H, CH_3 of pyrimidine); 4.40 (q, *J*= 6.9 Hz, 2H, C<u>H</u>$_2$$CH_3$); 7.26-7.34 (m, 2H, 1ArH &1NH, D_2O exchangeable); 7.48-7.53 (m, 2H, ArH); 7.87 (d, *J*= 8.4 Hz, 2H, ArH); 8.11 (d, *J*= 8.4 Hz, 2H, ArH); 8.19 (d, *J*= 7.5 Hz, 2H, ArH); MS m/z: 387 (M, 100); Anal. Calcd for $C_{22}H_{21}N_5O_2$(387.44): C, 68.20; H, 5.46; N, 18.08. Found: C, 68.44; H, 5.53; N, 18.47.

General procedure for the synthesis of 1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]-3-(4-substitutedphenyl)prop-2-en-1-one (7a-e)

A mixture of compound 6b (0.36 gm, 0.001 mol) and an aqueous solution of sodium hydroxide (10%) (1 mL) was dissolved in ethanol (10 mL). After cooling in ice bath, the appropriate aromatic aldehyde (0.001 mol) was added while stirring and the temperature was not exceeded 20°C. The reaction mixture was stirred at room temperature for 12 h. The obtained solid was filtered, washed with water and crystallized from the appropriate solvent.

1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]-3-phenylprop-2-en-1-one (7a).

(Crystallized from acetic acid); m.p 180-181°C; yield: 44.82 %; IR (cm^{-1}): 3429.78 (NH); 3046.01 (CH aromatic); 2920.66 (CH aliphatic); 1655.59 (C=O); 1601.59 (C=N); ^{1}H NMR ($CDCl_3$) δ ppm: 2.72 (s, 3H, CH_3 of pyrazole); 2.80 (s, 3H, CH_3 of pyrimidine); 7.32-7.35 (m, 1H, ArH); 7.45-7.53 (m, 4H, 3ArH & =C<u>H</u>-Ar); 7.54-7.59 (m, 5H, 3ArH & O=C-CH= & NH, D_2O exchangeable); 7.61-7.70 (m, 3H, ArH); 7.87-8.17 (m, 3H, ArH); 8.19 (s, 1H, ArH); MS m/z: 357 (M, 100). Anal. Calcd for $C_{28}H_{23}N_5O$ (445.53): C, 75.49; H, 5.20; N, 15.72. Found: C, 75.51; H, 5.37; N, 15.87.

1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]-3-(4-fluorophenyl)prop-2-en-1-one (7b). (Crystallized from isopropyl alcohol); m.p 202-203° C; yield: 65%; IR (cm^{-1}): 3434.60 (NH); 3030.59 (CH aromatic); 2924.52 (CH aliphatic); 1681.62 (C=O); 1594.84 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.50 (s, 3H, CH_3 of pyrazole); 2.83 (s, 3H, CH_3 of pyrimidine); 7.33-7.35 (m, 1H, ArH); 7.52-7.88 (m, 4H, 2ArH & CH=CH); 8.03 (d, *J*= 7.5 Hz, 2H, ArH); 8.08-8.18 (m, 5H, ArH); 8.29 (d, *J*= 8.1 Hz, 2H, ArH); 8.47 (s, 1H, ArH); 9.03 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{28}H_{22}FN_5O$(463.52): C, 72.56; H, 4.78; N, 15.11. Found: C, 72.82; H, 4.75; N, 15.01.

1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]-3-(4-methoxyphenyl)prop-2-en-1-one (7c).

(Crystallized from acetic acid); m.p 255-256° C; yield: 55%; IR (cm^{-1}): 3433.64 (NH); 3051.80 (CH aromatic); 2924.52 (CH aliphatic); 1655.59 (C=O); 1602.56 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.48 (s, 3H, CH_3 of pyrazole); 2.79 (s, 3H, CH_3 of pyrimidine); 3.83 (s, 3H, OCH_3); 7.32-7.60 (m, 8H, 7ArH & =C<u>H</u>-Ar); 7.78-7.95 (m, 3H, ArH & O=C-CH=); 8.18-8.21 (m, 3H, ArH); 8.48 (s, 1H, ArH); 8.88 (s, 1H, NH, D_2O exchangeable); MS *m/z*: 475 (M, 88.81); Anal. Calcd for $C_{29}H_{25}N_5O_2$(475.55): C, 73.25; H, 5.30; N, 14.73. Found: C, 73.55; H, 5.40; N, 14.66.

3-(4-chlorophenyl)-1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]prop-2-en-1-one (7d).

(Crystallized from isopropyl alcohol); m.p 212-214° C; yield: 81%; IR (cm^{-1}): 3438.46 (NH); 3049.87 (CH aromatic); 2917.77 (CH aliphatic); 1657.52 (C=O); 1595.81 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.48 (s, 3H, CH_3 of pyrazole); 2.79 (s, 3H, CH_3 of pyrimidine); 7.32 (t, *J*= 7.2 Hz, 1H, ArH); 7.51-7.62 (m, 5H, ArH & =C<u>H</u>-Ar); 7.74-7.94 (m, 5H, ArH & O=C-CH=); 8.13-8.21 (m, 3H, ArH); 8.48 (s, 1H, ArH); 8.86 (s, 1H, NH, D_2O exchangeable); MS *m/z*: 479 (M, 100); Anal. Calcd for $C_{28}H_{22}ClN_5O$(479.97): C, 70.07; H, 4.62; N, 14.59. Found: C, 70.37; H, 4.90; N, 14.38.

1-[3-(3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)phenyl]-3-(4-nitrophenyl)prop-2-en-1-one (7e).

(Crystallized from isopropyl alcohol);m.p 220-221° C; yield: 80%; IR (cm^{-1}): 3423.03 (NH); 3073.01 (CH aromatic); 2922.59 (CH aliphatic); 1665.23 (C=O); 1598.70 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.72 (s, 3H, CH_3 of pyrazole); 2.82 (s, 3H, CH_3 of pyrimidine); 7.50-7.54 (m, 2H, ArH); 7.81-7.86 (m, 8H, ArH & CH=CH &NH, D_2O exchangeable); 8.20 (d, *J*= 7.6 Hz, 2H, ArH); 8.31 (d, *J*= 8 Hz, 3H, ArH); 8.49 (s, 1H, ArH); MS *m/z*: 490 (M, 100); Anal. Calcd for $C_{28}H_{22}N_6O_3$(490.53): C, 68.56; H, 4.52; N, 17.13. Found: C, 68.29; H, 4.70;N, 16.70.

General procedure for the synthesis of 3,6-Dimethyl-1-phenyl-4-[3-(1-acetyl-5-(4-substitutedphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidines (8a-e).

To a solution of hydrazine hydrate (99.9%, 0.1 mL,

0.002 mol) in glacial acetic acid (5 mL), the appropriate chalcone **(7a-e)** (0.001 mol) was added. The reaction mixture was heated under reflux for 5 h. After cooling, the solution was poured into ice-cold water. The obtained solid was collected by filteration, washed with water and crystallized from ethanol.

3,6-Dimethyl-1-phenyl-4-[3-(1-acetyl-5-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8a)

M.p > 300 °C; yield: 60%; IR (cm^{-1}): 3320.82 (NH); 3057.58 (CH aromatic); 2925.48 (CH aliphatic); 1690.30 (C=O); 1597.73 (C=N);^{1}H NMR (DMSO-d_6) δ ppm: 2.34 (s, 3H, CH_3 of pyrazole); 2.51 (s, 3H, CH_3 of pyrimidine); 2.76 (s, 3H, O=C-CH_3); 3.12 (dd, 1H, CH_2 of pyrazoline); 3.38 (s, 3H, O=C-CH_3); 3.88 (dd, 1H, CH_2 of pyrazoline); 5.56 (dd, 1H, CH of pyrazoline); 7.21-7.32 (m, 4H, ArH); 7.46-7.85 (m, 6H, ArH); 8.19-8.38 (m, 4H, ArH); 8.69 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{30}H_{27}N_7O$ (501.58): C, 71.84; H, 5.43; N, 19.55. Found: C, 71.69; H, 5.31; N, 19.37.

3,6-Dimethyl-1-phenyl-4-[3-(1-acetyl-5—(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8b).

M.p 160-161°C; yield: 68%; IR (cm^{-1}): 3417.24 (NH); 2923.56 (CH aliphatic); 1678.73 (C=O); 1542.77 (C=N);^{1}H NMR (DMSO-d_6) δ ppm: 2.37 (s, 3H, CH_3 of pyrazole); 2.50 (s, 3H, CH_3 of pyrimidine); 2.80 (s, 3H, O=C-CH_3); 3.03 (dd, 1H, CH_2 of pyrazoline); 4.01 (dd, 1H, CH_2 of pyrazoline); 5.74 (dd, 1H, CH of pyrazoline); 7.04 (d, *J*= 7.8 Hz, 2H, ArH); 7.24 (d, *J*= 7.8 Hz, 2H, ArH); 7.33-7.35 (m, 2H, ArH); 7.45-7.54 (m, 2H, ArH); 7.67 (d, *J*= 7.8 Hz, 1H, ArH); 7.89 (d, *J*= 7.8 Hz, 1H, ArH); 8.17 (d, *J*= 8.1 Hz, 2H, ArH); 8.44 (s, 1H, ArH); 8.91 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{30}H_{26}FN_7O$ (519.59): C, 69.35; H, 5.04; N, 18.87. Found: C, 69.08; H, 4.79; N, 18.50.

3,6-Dimethyl-1-phenyl-4-[3-(1-acetyl-5—(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8c).

M.p 270-271°C; yield: 50%; IR (cm^{-1}): 3433.64 (NH); 2929.34 (CH aliphatic); 1653.66 (C=O); 1563.99 (C=N);^{1}H NMR (DMSO-d_6) δ ppm: δ 2.48 (s, 3H, CH_3 of pyrazole); 2.70 (s, 6H, CH_3 of pyrimidine& O=C-CH_3); 3.20 (dd, 1H, CH_2 of pyrazoline); 3.74-3.86 (m, 4H, OCH_3 & CH of pyrazoline); 5.60 (dd, 1H, CH of pyrazoline); 6.81-6.85 (m, 2H, ArH); 7.25-7.31 (m, 3H, ArH); 7.46-7.51 (m, 5H, ArH & NH, D_2O exchangeable); 7.77 (s, 1H, ArH); 8.20 (d, *J*= 7.2 Hz, 2H, ArH); 8.37 (s, 1H, ArH); MS *m/z*: 531 (M, 84.43); Anal. Calcd for $C_{31}H_{29}N_7O_2$(531.62): C, 70.04; H, 5.50; N, 18.44. Found: C, 69.77; H, 5.20; N,18.00.

3,6-Dimethyl-1-phenyl-4-3-(1-acetyl-5—(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8d).

M.p 220-221°C; yield: 45%; IR (cm^{-1}): 3436.53 (NH); 3056.62 (CH aromatic); 2921.63 (CH aliphatic); 1657.52 (C=O); 1591.95 (C=N). ^{1}H NMR ($CDCl_3$) δ ppm: δ 2.33 (s, 3H, CH_3 of pyrazole); 2.50 (s, 3H, CH_3 of pyrimidine); 2.77 (s, 3H, O=C-CH_3); 3.11 (dd, 1H, CH_2 of pyrazoline); 3.88 (dd, 1H, CH_2 of pyrazoline); 5.56 (dd, 1H, CH of pyrazoline); 7.24 (d, *J*= 6.3 Hz, 2H, ArH); 7.25-7.34 (m, 1H, ArH); 7.39 (d, *J*= 6.6 Hz, 2H, ArH); 7.41-7.56 (m, 4H, ArH); 7.88 (d, *J*= 6.6 Hz, 1H, ArH); 8.19 (d, *J*= 9.6 Hz, 2H, ArH); 8.39 (s, 1H, ArH); 8.75 (s, 1H, NH, D_2O exchangeable); MS *m/z*: 535 (M, 40.28); Anal. Calcd for $C_{30}H_{26}ClN_7O$ (536.04): C, 67.22; H, 4.89; N, 18.29. Found: C, 66.85; H, 4.94; N, 18.00.

3,6-Dimethyl-1-phenyl-4-[3-(1-acetyl-5—(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8e).

M.p 220-221°C; yield: 45%; IR (cm^{-1}): 3438.46 (NH); 3055.66 (CH aromatic); 2926.45 (CH aliphatic); 1656.55 (C=O); 1590.99 (C=N). ^{1}H NMR ($CDCl_3$) δ ppm: δ 2.36 (s, 3H, CH_3 of pyrazole); 2.50 (s, 3H, CH_3 of pyrimidine); 2.77 (s, 3H, O=C-CH_3); 3.19 (dd, 1H, CH_2 of pyrazoline); 3.98 (dd, 1H, CH_2 of pyrazoline); 5.72 (dd, 1H, CH of pyrazoline); 7.30-7.34 (m, 1H, ArH); 7.48-7.56 (m, 6H, ArH); 8.15-8.22 (m, 5H, ArH); 8.41 (s, 1H, ArH); Anal. Calcd for $C_{30}H_{26}N_8O_3$ (546.59): C, 65.92; H, 4.79; N, 20.50. Found: C, 65.79; H, 4.90; N, 20.54.

***In vitro* antitumor activity measurement**

The cytotoxicity was carried out using sulphorhodamine-B (SRB) assay. Cells will be seeded

in 96 well microtiter plates at a concentration of 1000-2000 cells/well, 100 µl/well, After 24 hrs, cells will be incubated for 72 h with various concentrations of drugs (0, 0.01,0.1,1,10 and 100µg/ml). For each derivative concentration and doxorubicin, 3 wells were used. The plates were incubated for72 hours. The medium is discarded. The cells were fixed with 150 µl cold trichloroacetic acid 10% final concentration for 1 hour at 4 ^{0}C.

The plates were washed with distilled water using (automatic washer Tecan, Germany) and stained with 50 µl 0.4 % SRB dissolved in 1 % acetic acid for 30 minutes at room temperaturein dark. The plates were washed with 1 % acetic acid to remove unbound dye and air-dried [24 hrs.].

The dye was solubilized with 150 µl/well of 10 mMtris base (PH 7.4) for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well will be measured spectrophotometrically at 490 nm with an ELISA microplate reader.The mean background absorbance was automatically subtracted and mean values of each derivative and doxorubicin concentration was calculated. The experiment was repeated 3 times. The percentage of cell survival was calculated as follows:

Surviving fraction = O.D. (treated cells)/ O.D. (control cells).

Docking study

All molecular calculations and docking studies were performed using "Molecular Operating Environment" (MOE) version 2008.10 release of Chemical Computing Group's. The program operated under "Windows XP" operating system installed on an Intel Pentium IV PC with a 2.8 MHz processor and 512 RAM.

The target compounds were built using the MOE builder interface and subjected to energy minimization using the included MOPAC. The produced model was subjected to the Systematic Conformational Search where all items were set as default with RMS gradient of 0.01 kcal/mol and RMS distance of 0.1 A˚.

The X-ray crystallographic structure of the Tyrosine Kinase Domain from Epidermal Growth Factor Receptor EGFR TK complexed with erlotinib was obtained from the Protein Data Bank; code "1M17". The enzyme was prepared for docking studies as follows:

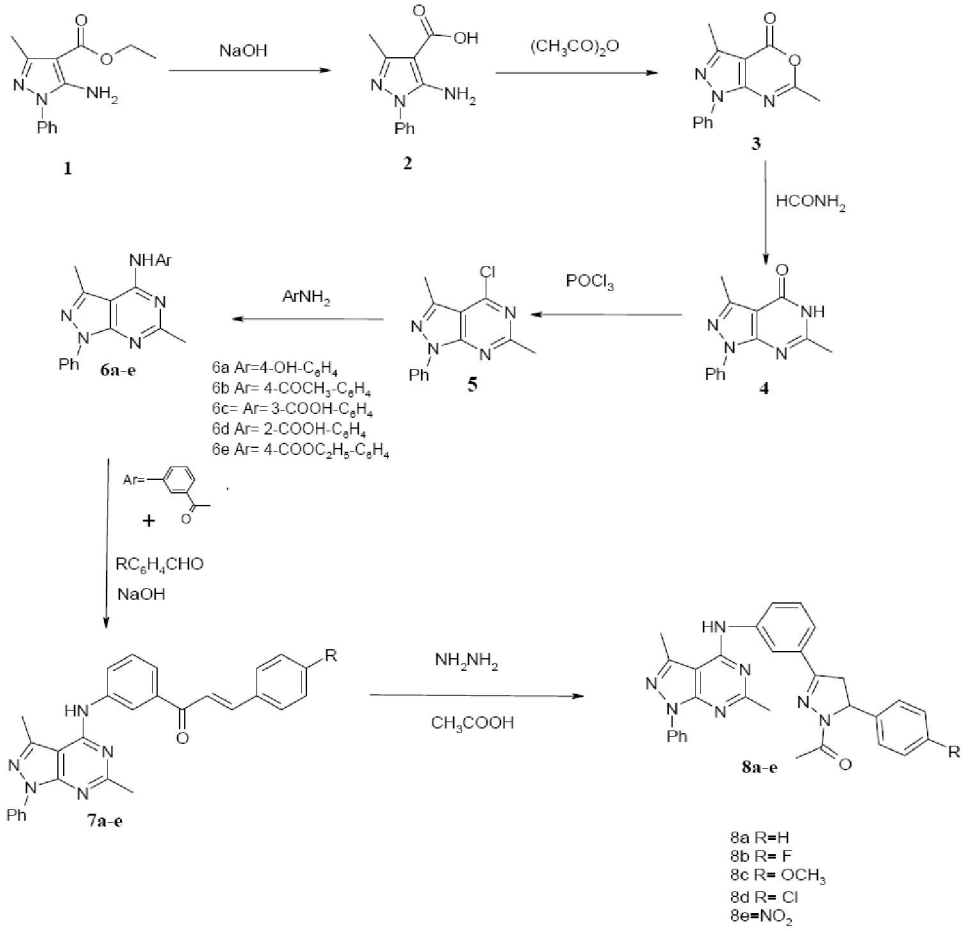
a) The ligand molecule was removed from the enzyme active site.
b) Hydrogen atoms were added to the the isolated target with their standard geometry.
c) A connect and type procedure was run for automatic completion of missed bonds during isolation and crystallization.
d) The target was fixed to be dealt as a rigid structure.
e) The active site was isolated by Alpha site finder tool using the binding amino acids as key elements in isolation.
f) Dummies were created around the active site.

RESULTS AND DISCUSSION

Chemistry

The compound 2 had been obtained through basic hydrolysis of ethyl 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxylate **(1)**[19] (Scheme 1). The compound 2 was confirmed by ^{1}H NMR that showed two exchangeable singlet signals at δ 6.30, 12.07 ppm corresponding to NH_2 and COOH, respectively. Heating compound 2 with acetic anhydride gave a cyclized product, 3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*][1,3]oxazin-4-one **(3)**. ^{1}H NMR spectrum of compound 3 revealed the appearance of a singlet signal at δ 2.49 ppm corresponding to CH_3 of pyrimidine protons disappearance of two D_2O exchangeable signals of NH_2 and COOH at δ 6.30 and at δ 12.07 ppm, respectively of compound **(2)**. The mass spectrum of compound **(3)** showed a molecular ion peak at *m/z* 241 which appeared as a base peak. Condensation of pyrazoloxazine derivative **(3)** with formamide afforded the pyrazolo[3,4-*d*]pyrimidin-4-one derivative **(4)**. The IR spectrum of 4 showed absorption band 3420.14 cm^{-1} due to NH group. The ^{1}H NMR showed one exchangeable singlet signal at δ 12.24 ppm corresponding to NH_2. Chlorination of pyrazolopyrimidinone derivative 4 with phosphorus oxychloride gave 4-chloro-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine **(5)**. Compounds 6a-e were synthesized by reacting compound 5 with the appropriate aromatic amines, the structure of compounds 6a-e were confirmed by ^{1}H NMR that revealed the appearance of exchangeable singlet signals at δ 7.26-9.16 ppm corresponding to NH proton.

Subsequent condensation of ketone 6b with vari-



Scheme 1

ous aldehydes in the presence of sodium hydroxide led to chalcones **(7a-e)**. The ^{1}H NMR spectra of chalcones **(7a-e)** showed signals atδ 7.32-7.95 ppm for CH=CH protons. Chalcones **(7a-e)** were cyclised with hydrazine hydrate in the presence of glacial acetic acid to form acetylpyrazoline 8a-e. The structure of compounds 8a-e were established and confirmed on the basis of elemental analysis and spectral data. The ^{1}H NMR spectra showed two protons of CH_2 pyrazoline protons as two doublet of doublet signals at δ 3.03-3.20 ppm and 3.86-3.98 ppm, in addition to the appearance of doublet of doublet signal at 5.56-5.74 ppm for CH pyrazoline proton.

In vitro anticancer screening

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast cell line (MCF7) using doxorubicin as the reference drug according to the method described as reported by Vichai and Kirtikara[20].Human breast cell line (MCF7) used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.) through the Tissue Culture Unit, the Egyptian Organization for Biological Products and Vaccines, Vacsera, 51 Wezaret EI Zeraa St., Agouza, Giza, Egypt. The tumor cell lines were maintained at Center for Genetic Engineering, Al-Azhar University, Cairo, Egypt by serial sub-culturing. The cytotoxicity was assessed at concentrations 0, 0.01, 0.1, 10 and 100 µg/mL. The relation between surviving fraction and drug concentration was plotted to obtain the survival curve of MCF7 tumor cell line after addition of the specified compound. The parameter used here is IC_{50}, which corresponds to the concentration required for 50% inhibition of cell viability. The IC_{50} of the synthesized compounds compared to the reference drug are shown in TABLE 1.

Docking study

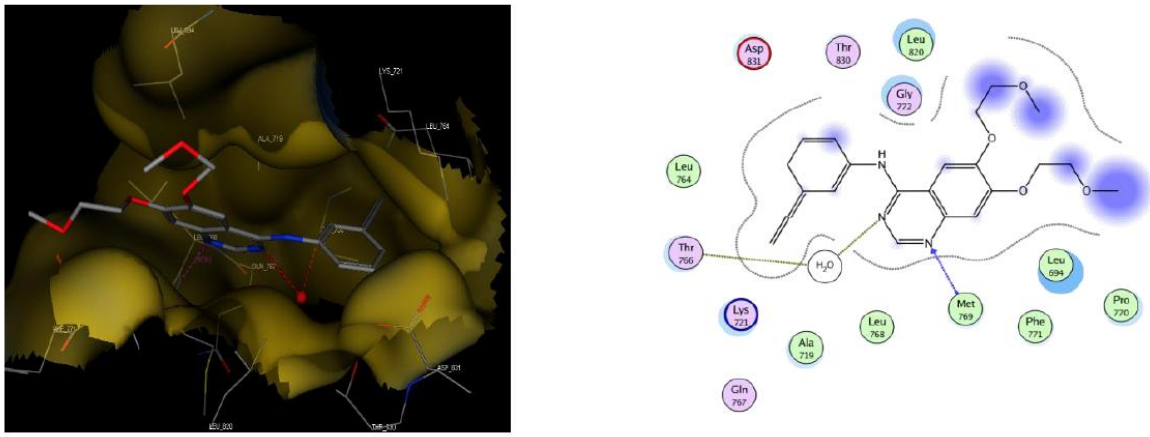
Docking study of designed compounds into EGFR tyrosine kinase was performed using "Molecular Op-

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

Compound no.	IC_{50} in µg/mL
Doxorubicin	43.254
6a	33.493
6b	71.432
6c	59.887
6d	53.952
6e	55.006
8a	49.557
8b	28.157
8c	51.523
8d	69.218
8e	58.075

erating Environment (MOE) version 2008.10 release of Chemical Computing Group's. The crystal structure of the enzyme with erlotinib was obtained from protein data bank; PDB code: 1M17.

To study the binding interactions of the reference ligand, redocking of erlotinib in the ATP binding region of the kinase binding site was carried out, revealing that two amino acids are involved in the interaction: Thr-766 and Met-769. N-1 of the quinazoline ring interacts with Met-769 via hydrogen bond (H-acceptor; bond length 2.70 A°), and similarly a water-mediated hydrogen bonding interaction was observed with N-3 of the quinazoline ring and Thr-766 (H-acceptor; bond length 2.78 A°) (Figure 2).



a b

Figure 2 : a) The interactions of erlotinib with EGFR (using MOE site finder program), the red dotted line represents H-bonding interaction between N-3 and Thr-766 through a water bridge (red sphere), and the violet dotted line represents H-bonding interaction between N-1 and Met-769. b) 2D interactions of erlotinib with Thr-766 and Met-769 acid residues.

The target compounds were modeled by positioning them in the erlotinib binding site. The result for the most active compound 8b showed two hydrogen bonding interactions with Thr-766 *via* a water molecule: one

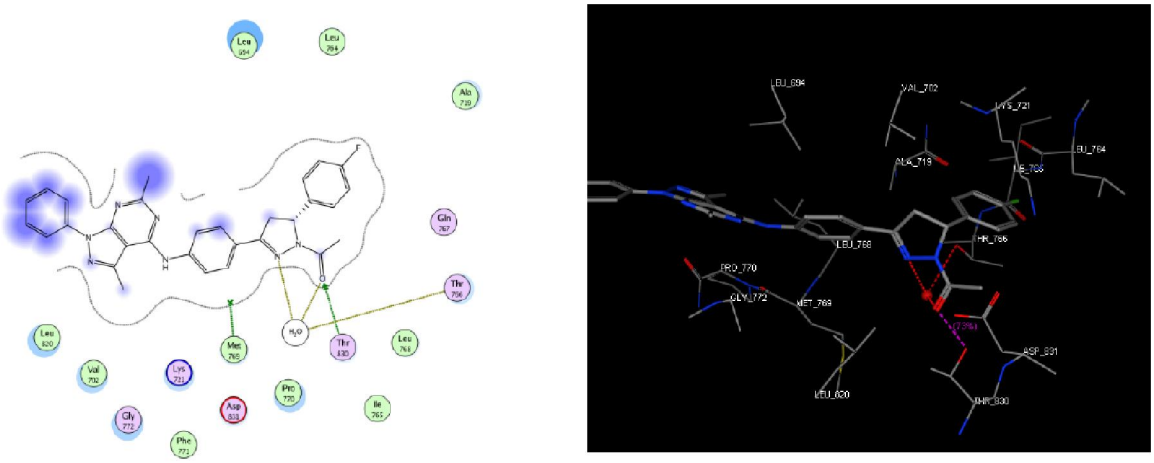


Figure 3 : Binding mode of compound 8b in the ATP binding site of EGFR

with N-2 of the pyrazole ring (H-acceptor; bond length 3.10 A°) and the other with C=O group of the same ring (H-acceptor; bond length 2.99 A°). Additional hydrogen bonding interaction of C=O group with Thr-830 (H-acceptor; bond length 2.11 A°) was observed (Figure 3).

The binding mode of compound 6a revealed the involvement of OH moiety by hydrogen bonding interaction with Thr-766 *via* a water molecule (H-donor; bond length 2.90 A°) (Figure 4).

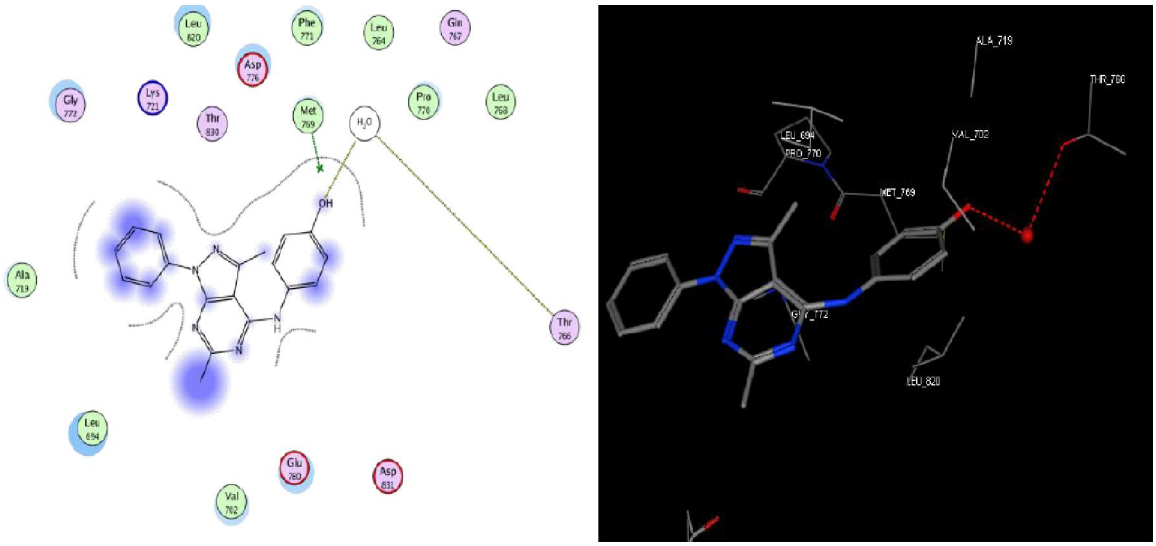


Figure 4 : Binding mode of compound 8b in the ATP binding site of EGFR

CONCLUSION

Novel derivatives of 4-substituted amino-1*H*-pyrazolo[3,4-*d*]pyrimidines 6a-e and 8a-e were designed and synthesized. All compounds were evaluated for their antiproliferative activity against human breast adenocarcinoma (MCF7) cell line. Most of the tested compounds displayed potent to moderate growth inhibitory activity, in particular compound 3,6-dimethyl-1-phenyl-4-[4-(1-acetyl-5—(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidine (8b) exhibited the highest activity among the tested compounds with $IC_{50\%}$ equal to 28.157 µg/mL. Docking study of the synthesized compounds in the ATP binding site of epidermal growth factor receptor (EGFR) tyrosine kinase may give a suggestion that the synthesized compounds may act as epidermal growth factor receptor (EGFR) inhibitors and this may contribute in part to their anticancer activity.

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