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Design, synthesis and cytotoxic activity of some novel compounds containing pyrazolo[3,4-*d*]pyrimidine nucleus

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ABSTRACT

*Novel pyrazolo[3,4-d]pyrimidines were designed and synthesized as anti tumor agents against human breast cancer adenoma (MCF-7). Molecular modeling and pharmacological screening were performed against breast cancer cell line and also certain synthetic pathways were developed in order to introduce functionality onto C6 and N5 positions of pyrimidine moiety. Surprisingly, all of the test compounds showed IC_{50} lower than that of the standard olomoucine **I**, especially compounds (**4b**, **8a**, **10b**, **11a&b**), which showed IC_{50} between (0.009-0.004 μM).*

Keywords: pyrazolo[3,4-*d*]pyrimidine derivatives; cytotoxic activity; MCF-7; cyclin-dependent kinase inhibitors (CDKI).

INTRODUCTION

Cyclin-dependent kinases (CDK) are enzymes of Ser/Thr kinase type. This family of enzymes plays an important role in cell cycle regulation and proliferation[1]. CDK enzymatic regulation requires the binding of catalytic subunits (CDK1-CDK8) with regulatory subunits (cyclin A- cyclin H) to give a fully active complex (CDK-cyclin), which is necessary for phosphorylation of key proteins that regulate the progression through the cell cycle[2].

Several types of CDK inhibitors (staurosporine, flavopiridole, butyrolactone-1 and purine derivatives) have so far been described[2].

Insight to different biological activities of pyrazolo[3,4-*d*]pyrimidines as antimetabolites in purine biochemical reactions, several mechanisms were described for their cytotoxic activities as EGFR inhibitors,[3] GSK-3 inhibitors,[4,5] xanthine oxidase inhibitors,[6] Mer receptor tyrosine kinase inhibitors,[7] tyrosine kinase c-Src inhibitors ,[8-11] adenosine receptor antagonist [12,13]and CDK inhibitors14,15].

CDKs are involved in the control of the cell cycle, but are over expressed or over active in many cancer cells. Since these enzymes are inactive in normal resting cells, drugs that target them should have fewer and less toxic side effects than conventional cytotoxic drugs, CDKs are activated by cyclins *via* binding of a cyclin with its associated kinase, which activates the enzyme and serves to move the cell from one phase of the cell cycle to another, and inhibited by CDKIs. So, particular attention has been focused on CDKIs that offer selective and tolerable treatment for cancer[16].

Moreover, pyrazolo [3, 4-*d*] pyrimidines was known to have broad spectrum Of biological activities including antimicrobial[17-19] antiviral[20,21] and antitumor activity[22-25].

Herein, we described the design, synthesis and pharmacological profile for a new class of compounds containing pyrazolo [3, 4-*d*] pyrimidine scaffold and having structure near from that of olomoucine **I**, the potent known CDK inhibitor[26].

MATERIALS AND METHODS

2.1 Docking studies

Molecular modeling was performed using the x-ray crystal structure of olomoucine **I** bound to CDK using "Molecular Operating Environment (MOE) version 2008.10".

Hydrogen atoms were added to the amino acids of the protein, free energy calculation was assigned. Ligand for docking was prepared, atom types, protonation states and formal charges were assigned using default settings. The root mean square deviation (RMSD) of the docked conformation of olomoucine **I** was 0.52^0A and the binding energy score was -19.41 Kcal/ mol. Test compounds were prepared as above and the top 10 poses were retained for each molecule from every docking run.

2.2 General

Melting points were determined on a Graffin apparatus and were uncorrected. Element analyses (C, H, and 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 spectra were carried out on a Bruker 300 MHz NMR Spectrophotometer in Cairo University, Egypt, using (Bruker, Munich, Germany) in DMSO-d_6 as a solvent, TMS as internal standard and chemical shifts were recorded in ppm on δ 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.

2.2.1. *6-Chloromethyl-3-methyl-1-phenyl-1, 5-dihydro-pyrazolo [3, 4-d] pyrimidin-4-one (**3**):* A mixture of compound **1** (1.06 g, 4.9 mmole) and chloroacetyl chloride (0.65 g, 5.8 mmole) was heated at 80°C for 6 hours. The mixture was cooled and neutralized with sodium carbonate solution (10%). The precipitated solid was collected and crystallized from acetone as white crystals (69% yield); m.p.: 246 - 248°C; IR (KBr, cm^{-1}): 3276, 2934, 2822, 1675; ^{1}H-NMR (DMSO-d_6) δ= 2.50 (s, 3H, CH_3), 4.59 (s, 2H, CH_2), 7.34-7.38 (t, 1H, C_4-H), 7.39-7.56 (t, 2H, C_3-H, C_5-H), 8.00-8.03 (d, 2H, C_2-H, C_6-H), and 12.61 (s, 1H, NH, D_2O exchangeable); MS (m/z): 274 (M^+, 85.80%), 77 (100%); Anal. Found: C, 56.84%; H, 4.04%; N, 20.40%; $C_{13}H_{11}ClN_4O$ Calcd C, 56.88; H, 4.10; N, 20.37%.

2.2.2 General procedure for the synthesis of 4 a-d

A mixture of compound **3** (0.52 g, 1.9 mmole), the appropriate primary aromatic amine (10 mmole) and absolute ethanol (20 ml) was heated under reflux for 8 hours. The solid that formed on hot, was filtered and crystallized from dioxane as white crystals.

*2.2.2.a 3-Methyl-1-Phenyl-6-phenylaminomethyl-1, 5-dihydro-pyrazolo [3, 4-d] pyrimidin-4-one (**4a**):* white colour solids; yield: 58%; m.p. 223-225^{0}C; IR (KBr, cm^{-1}): 3428, 2930, 2871, 1685, ^{1}H-NMR (DMSO-d_6) δ= 2.49 (s, 3H, CH_3), 4.30-4.32 (d, 2H, CH_2), 6.03-6.08 (t , 1H, NH, D_2O exchangeable), 6.57-6.59 (t, 1H, $C_{4'}$-H), 6.60-6.62 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 7.08-7.13 (t, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.29-7.32 (t, 1H, C_4-H), 7.40-7.45(t, 2H, C_3-H, C_5-H), 7.92-7.95 (d, 2H, C_2-H, C_6-H) , 12.15 (s, 1H, NH, D_2O exchangeable); MS (m/z): 331 (M^+ , 58.24%), 234 (100%); Anal. Found: C, 68.87 %; H, 5.01%; N, 21.09%; $C_{19}H_{17}N_5O$ Calcd C, 68.87; H, 5.17; N, 21.13%.

*2.2.2.b 3-Methyl-1-Phenyl-6-(p-tolylphenyl) aminomethyl-1, 5-dihydro-pyrazolo [3, 4-d] pyrimidin-4-one (**4b**):* white colour solids; yield: 63%; m.p. 217-219^{0}C; IR (KBr, cm^{-1}): 3427, 2999, 2924, 1684; ^{1}H-NMR (DMSO-d_6) δ= 2.13 (s, 3H, CH_3-ph), 2.49 (s, 3H, CH_3), 4.25-4.29 (d, 2H, CH_2), 5.84-5.86 (t, 1H, NH, D_2O exchangeable), 6.56-6.60 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.89-6.93 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.28-7.34 (t, 1H, C_4-H), 7.41-7.44 (t, 2H, C_3-H, C_5-H), 7.93-7.97(d, 2H, C_2-H, C_6-H), 12.10 (s, 1H, NH, D_2O exchangeable); MS (m/z): 345 (M^+ , 79.33%), 120 (100%); Anal. Found: C, 69.58%; H, 5.54 %; N, 20.32%; $C_{20}H_{19}N_5O$ Calcd C, 69.55; H, 5.54; N, 20.28%.

*2.2.2..c 3-Methyl-1-Phenyl-6-(p-hydroxyphenyl) aminomethyl-1, 5-dihydro-pyrazolo [3, 4-d] pyrimidin-4-one (**4c**):* white colour solids; yield: 60%; m.p. 298-300^{0}C; IR (KBr, cm^{-1}): 3439, 3249, 2979, 2924, 1675; ^{1}H-NMR (DMSO-d_6) δ= 2.48 (s, 3H, CH_3), 4.21-4.22 (d, 2H, CH_2), 5.49-5.51 (t, 1H, NH, D_2O exchangeable), 6.51-6.52 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.55-6.58 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.29-7.34 (t, 1H, C_4-H), 7.44-7.49(t, 2H, C_3-H, C_5-H), 7.95-7.98 (d, 2H, C_2-H, C_6-H), 8.48 (s, 1H, OH, D_2O exchangeable), 12.10 (s, 1H, NH, D_2O exchangeable); MS (m/z): 347 (M^+,

39.30%), 77 (100%); Anal. Found: C, 65.57%; H, 5.20%; N, 19.99%; $C_{19}H_{17}N_5O_2$ Calcd C, 65.69; H, 4.93; N, 20.16%.

*2.2.2..d 3-Methyl-1-Phenyl-6-(p-carboxyphenyl) aminomethyl-1, 5-dihydro-pyrazolo [3, 4-d] pyrimidin-4-one (**4d**):* white colour solids; yield: 66%; m.p. 281-283^{0}C; IR (KBr, cm^{-1}): 3404, 3263, 1664; ^{1}H-NMR (DMSO-d_6) δ= 2.49 (s, 3H, CH_3), 4.39-4.41 (d, 2H, CH_2), 6.70-6.82 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.81-6.83 (t, 1H, NH, D_2O exchangeable), 7.24-7.29 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.32-7.37 (t, 1H, C_4-H), 7.70-7.73 (t, 2H, C_3-H, C_5-H), 7.86-7.88 (d, 2H, C_2-H, C_6-H), 12.05 (s, 1H, NH, D_2O exchangeable), 12.27 (s, 1H, OH, D_2O exchangeable); MS (m/z): 375 (M^+ , 92.70%), 77 (100%); Anal. Found: C, 63.86%; H, 4.51%; N, 18.66%; $C_{20}H_{17}N_5O_3$ Calcd C, 63.99; H, 4.56; N, 18.66%.

2.2.3 General procedure for the synthesis of 5 a-d

To a solution of the appropriate compound **4a-d** (0.6 mmol) in propanol (10 ml), formaldehyde solution (3ml, 0.10 mole) was added while stirring during 10 min. The solution was stirred continuously for 2 hours. The formed precipitate was filtered and crystallized from propanol as white crystals

*2.2.3.a 1.3-Methyl-1-phenyl-7-phenyl-1, 4, 5, 6, 7,8hexahydropyrazolo[3, 4-d] imidazo [3, 4-a] pyrimidin-4-one (**5a**):* white colour solids; yield: 57%; m.p. 246-248^{0}C; IR (KBr, cm^{-1}): 2990-2857, 1687; ^{1}H-NMR (DMSO-d_6) δ= 2.54 (s, 3H, CH_3), 4.70 (s, 2H,C-CH_2-N), 5.37 (s, 2H, N-CH_2-N) and 6.75-6.79 (t, 1H, $C_{4'}$-H), 6.82-6.84 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 7.26-7.31 (t, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.34-7.40 (t, 1H, C_4-H), 7.52-7.57 (t, 2H, C_3-H, C_5-H), 7.99-8.03 (d, 2H, C_2-H, C_6-H); MS (m/z): 343 (M^+, 44.69%), 77 (100%); Anal. Found: C, 69.99%; H, 4.71%; N, 20.56%; $C_{20}H_{17}N_5O$ Calcd C, 69.96; H, 4.99; N, 20.40%.

*2.2.3.b 3-Methyl-1-phenyl-7-(p-tolylphenyl)-1, 4, 5, 6, 7,8hexahydropyrazolo[3, 4-d] imidazo [3, 4-a] pyrimidin-4-one (**5b**):* white colour csolids; yield: 56%; m.p. 284-286^{0}C; IR (KBr, cm^{-1}): 2921- 2858, 1704; ^{1}H-NMR (DMSO-d_6) δ= 2.22(s, 3H, CH_3-ph), 2.48(s, 3H, CH_3), 4.66 (s, 2H, C-CH_2-N), 5.34 (s, 2H, N-CH_2-N) and 6.67-6.70 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 7.08-7.11 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.30-7.37 (t, 1H, C_4-H), 7.52-7.58(t, 2H, C_3-H, C_5-H), 7.99-8.02 (d, 2H, C_2-H, C_6-H); MS (m/z): 357 (M^+, 63.96%), 240 (100%); Anal. Found: C, 70.40%; H, 5.49%; N, 19.41%; $C_{21}H_{19}N_5O$ Calcd C, 70.57; H, 5.36; N, 19.59%.

*2.2.3..c 3-Methyl-1-phenyl-7-(p-hydroxyphenyl)-1, 4,5,6,7, 8 hexahydropyrazolo[3,4-d]imidazo[3,4-a]pyrimidin-4-one (**5c**):* white colour solids; yield: 71%; m.p. 253-255^{0}C; IR (KBr, cm^{-1}): 3267, 2927- 2873, 1688; ^{1}H-NMR (DMSO-d_6) δ= 2.54 (s, 3H, CH_3), 4.60(s, 2H, C-CH_2-N), 5.29 (s, 2H, N-CH_2-N), 6.66-6.72 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.95-6.99 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.32-7.41 (t, 1H, C_4-H), 7.52-7.60 (t, 2H, C_3-H, C_5-H), 7.99-8.02 (d, 2H, C_2-H, C_6-H) , 8.80 (s, 1H, OH, D_2O exchangeable); MS (m/z): 359 (M^+, 0.27%), 240 (100%); Anal. Found: C, 66.79%; H, 4.59%; N, 19.39%; $C_{20}H_{17}N_5O_2$ Calcd C, 66.84; H, 4.77; N, 19.49%.

*2.2.3..d 3-Methyl-1-phenyl-7-(carboxyphenyl)-1, 4, 5, 6, 7,8hexahydropyrazolo[3, 4-d] imidazo [3, 4-a] pyrimidin-4-one (**5d**):* white colour solids; yield: 59%; m.p. 346-348^{0}C; IR (KBr, cm^{-1}): 3404, 1664; ^{1}H-NMR (DMSO-d_6) δ= 2.49 (s, 3H, CH_3), 4.39-4.41 (d, 2H, CH_2), 6.70-6.82 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.81-6.83 (t, 1H, NH, D_2O exchangeable), 7.24-7.29 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.32-7.37 (t, 1H, C_4-H), 7.70-7.73 (t, 2H, C_3-H, C_5-H), 7.86-7.88 (d, 2H, C_2-H, C_6-H), 12.05 (s, 1H, NH, D_2O exchangeable), 12.27 (s, 1H, OH, D_2O exchangeable); MS (m/z): 375 (M^+ , 92.70%),77 (100%); Anal. Found: C, 65.24%; H, 4.50%; N, 18.20%; $C_{21}H_{17}N_5O_3$ Calcd C, 65.11; H, 4.42; N, 18.08%.

2.2.4 General procedure for the synthesis of 6 a-c

A mixture of compound **3** (2.75 g, 0.01 mole) and the appropriate secondary amine (0.02 mole) in absolute ethanol (20 mL) was heated under reflux for 3 hours. The separated solid on hot, was filtered and crystallized from aqueous ethanol as white off crystals**.**

*2.2.4.a 6-Diethylaminomethyl-3-methyl-1-phenyl-1, 5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (**6a**):* white off colour crystals; yield: 47%; m.p. 158-160^{0}C; IR (KBr, cm^{-1}): 3424, 2966-2864, 1674; ^{1}H-NMR (DMSO-d_6) δ= 0.97-1.02 (t, 6H, *J*=7.2 Hz, 2 CH_2–$\underline{CH_3}$), 2.50 (s, 3H,CH_3), 2.58-2.65 (q, 4H, *J*=7.2 Hz, 2 CH_2-CH_3), 3.60 (s, 2H, CH_2), 7.31-7.36 (t, 1H, C_4-H), 7.45-7.54 (t, 2H, C_3-H, C_5-H), 8.02-8.06 (d, 2H, C_2-H, C_6-H), NH not visible; MS (m/z): 311 (M^+ , 0.34%),72 (100%); Anal. Found: C, 65.85%; H, 6.40%; N, 22.51%; $C_{17}H_{21}N_5O$ Calcd C, 65.57; H, 6.80; N, 22.49%.

*2.2.4..b 3-Methyl-1-phenyl-6-piperidin-1-ylmethyl-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (**6b**):* white off colour crystals; yield: 62%; m.p. 195-197^{0}C; IR (KBr, cm^{-1}): 3439, 2995-2852, 1673; ^{1}H-NMR (DMSO-d_6) δ= 1.59 (m, 4H, 2 CH_2), 1.83 (m, 2H, CH_2), 2.64 (s, 3H, CH_3), 2.86 (m, 4H, 2 N-CH_2), 7.32-7.35 (t, 1H, C_4-H), 7.48-7.59 (t,

2H, C_3-H, C_5-H), 7.99-8.01 (d, 2H, C_2-H, C_6-H), NH not visible; MS (m/z): 323 (M^+, 0.5%), 84 (100%); Anal. Found: C, 66.61%; H, 6.29%; N, 21.55%; $C_{18}H_{21}N_5O$ Calcd C, 66.85; H, 6.55; N, 21.66%.

*2.2.4.c 3-Methyl-6-morpholin-4-ylmethyl-1-phenyl-1, 5-dihydro-pyrazolo[3, 4-d] pyrimidin-4-one (**6c**):* white off colour crystals; yield: 62%**;** m.p. 219-221^{0}C; IR (KBr, cm^{-1}): 3467, 2990-2811, 1670; ^{1}H-NMR (DMSO-d_6) δ= 2.48-2.59 (m, 7H, CH_3 and 2CH_2-O), 3.50 (s, 2H, CH_2), 3.58-3.61 (m, 4H, 2CH_2-N), 7.32-7.34 (t, 1H, C_4-H), 7.49-7.56 (t, 2H, C_3-H, C_5-H), 8.01-8.05 (d, 2H, C_2-H, C_6-H); MS (m/z): 325 (M^+, 0.64%), 206 (100%); Anal. Found: C, 62.91%; H, 5.66%; N, 21.32%; $C_{17}H_{19}N_5O_2$ Calcd C, 62.75; H, 5.89; N, 21.52%.

2.2.5 General procedure for the synthesis of 7 a-c

A solution of compound **3** (0.82 g, 3 mmole) in sodium (methoxide, ethoxide and/or propoxide) solution (0.46g sodium in 30 ml absolute methanol, ethanol or propanol) was heated under reflux for 2 hours, the solvent was concentrated to half its volume and acidified with (0.01 N) hydrochloric acid until just acidic to litmus paper. The separated solid was filtered and crystallized from aqueous ethanol as white crystals**.**

*2.2.5.a 6-Methoxymethyl-3-methyl-1-phenyl-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (**7a**):* yellow colour solid; yield; 46%; m.p. 199-201^{0}C; IR (KBr, cm^{-1}): 3454, 2995-2928, 1676; ^{1}H-NMR (DMSO-d_6) δ= 2.47 (s, 3H, CH_3), 3.38 (s, 3H, OCH_3), 4.37 (s, 2H, CH_2), 7.33-7.38 (t, 1H, C_4-H), 7.51-7.56 (t, 2H, C_3-H, C_5-H), 8.01-8.05 (d, 2H, C_2-H, C_6-H), 12.20 (s, 1H, NH, D_2O exchangeable); MS (m/z): 270 (M^+ , 85.33%), 240 (100%); Anal. Found: C, 61.99%; H, 5.25%; N, 20.94%; $C_{14}H_{14}N_4O_2$ Calcd C, 62.21; H, 5.22; N, 20.73%.

*2.2.5.b 6-Ethoxymethyl-3-methyl-1-phenyl-1, 5-dihydro-pyrazolo[3, 4-d] pyrimidin-4-one (**7b**):* yellow colour solid; yield: 58%; m.p. 164-166^{0}C; IR (KBr, cm^{-1}): 3436, 2977-2929, 1677; ^{1}H-NMR (DMSO-d_6) δ= 1.14-1.19 (t, 3H, $CH_2\underline{CH_3}$),2.49 (s, 3H, CH_3), 3.55- 3.62 (q, 2H, $\underline{CH_2}CH_3$), 4.39 (s, 3H, CH_2), 7.33-7.36 (t, 1H, C_4-H), 7.49-7.56 (t, 2H, C_3-H, C_5-H), 8.04-8.06 (d, 2H, C_2-H, C_6-H) and NH not visible; MS (m/z): 284 (M^+ , 36.66%), 240 (100%); Anal. Found: C, 63.12%; H, 5.48%; N, 19.84%; $C_{15}H_{16}N_4O_2$ Calcd C, 63.37; H, 5.67; N, 19.71%.

*2.2.5.c 3-Methyl-1-phenyl-6-propoxymethyl-1, 5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (**7c**):* yellow colour solid; yield: 30%. m.p. 158-160^{0}C; IR (KBr, cm^{-1}): 3424, 2957-2871, 1686; ^{1}H-NMR (DMSO-d_6) δ= 0.97-1.02 (t, 3H, CH_2-$\underline{CH_3}$), 1.66-1.78 (m, 2H, CH_2-$\underline{CH_2}$-CH_3), 2.65 (s, 3H, CH_3), 3.57-3.62 (t, 2H, O-CH_2-), 4.53 (s, 2H, CH_2-O), 7.27-7.35 (t, 1H, C_4-H), 7.45-7.51 (t, 2H, C_3-H, C_5-H), 7.99-8.02 (d, 2H, C_2-H, C_6-H) , 9.46 (s, 1H, NH, D_2O exchangeable); MS (m/z): 298 (M^+ , 82.94%), 240 (100%); Anal. Found: C, 64.20%; H, 5.89%; N, 18.37%; $C_{16}H_{18}N_4O_2$ Calcd C, 64.41; H, 6.08; N, 18.78%.

2.2.6 General procedure for the synthesis of 8a&b

A mixture of compound **3** (2.75g, 0.01 mole), appropriate chloroacetanilide derivative (0.01 mole) and potassium hydroxide (1.12 g, 0.02 mole) in absolute ethanol (25 ml) was heated under reflux for 24 hours. The separated solid on hot, was filtered, washed with water and crystallized from dioxane as white off crystals.

*2.2.6.a 3-Methyl-1-phenyl-7oxo-8-phenyl-4, 5, 6, 7, 8, 9-hexahydro-pyrazolo[3, 4-d] pyrazino [4, 3-a] pyrimidin-4-one (**8a**):* white colour solid; yield: 18%; m.p. 245-247^{0}C; IR (KBr, cm^{-1}): 2916-2863, 1704 ; ^{1}H-NMR (DMSO-d_6) δ= 2.54 (s, 3H, CH_3), 4.77 (s, 2H, CH_2-N), 5.01 (s, 2H, CH_2-CO), 7.27-8.04 (m, 10H, Ar-H) ; MS (m/z): 371 (M^+ ,0.33%), 240 (100%); Anal. Found: C, 67.80%; H, 4.71%; N, 18.70%; $C_{21}H_{17}N_5O_2$ Calcd C, 67.91; H, 4.61; N, 18.86%.

*2.2.6..b 3-Methyl-1-phenyl-7oxo-8-(p-tolylphenyl)-4,5,6,7,8,9-hexahydro-pyrazolo[3,4-d]pyrazino[4,3-a]pyrimidin-4-one (**8b**):* white colour solid; yield: 15%; m.p. 252-254^{0}C; IR (KBr, cm^{-1}): 2924-2850, 1705, 1583; ^{1}H-NMR (DMSO-d_6) δ= 2.22 (s,3H, CH_3), 2.49 (s, 3H, CH_3), 4.66 (s, 2H, CH_2-N), 5.34 (s, 2H, CH_2-CO), 6.67-6.70 (d, 2H, $C_{2'}$-H, $C_{6'}$-H), 7.08-7.11 (d, 2H, $C_{3'}$-H, $C_{5'}$-H), 7.35-7.40 (t, 1H, C_4-H), 7.52-7.58 (t, 2H, C_3-H, C_5-H), 7.99-8.02 (d, 2H, C_2-H, C_6-H); MS (m/z): 385 (M^+ , 55.56%), 206 (100%); Anal. Found: C, 68.30%; H, 4.91%; N, 18.30%; $C_{22}H_{19}N_5O_2$ Calcd C, 68.56; H, 4.97; N, 18.17%.

2.2.7 6- Mercaptomethyl-3-methyl-1-phenyl-1H-pyrazolo [3, 4-d] pyrimidin-4-one

(9) : A mixture of compound **3** (1.58 g, 5.75 mmole) and thiourea (0.76 g, 0.01 mole) in absolute ethanol (25 ml) was heated under reflux for 4 hours. The product which was obtained on hot, was filtered, dissolved in sodium hydroxide (20 ml, 5%) and acidified with (0.01N) hydrochloric acid until just acidic. The solid separated was collected and crystallized from dimethyl formamide as yellow colour crystals; yield: 50%; m.p. 293-295^{0}C; IR (KBr, cm^{-1}): 3426, 2733, 1675; ^{1}H-NMR (DMSO-d_6) δ= 2.48 (s,3H, CH_3), 3.97 (s, 2H, CH_2), 7.28-7.31 (t, 1H, C_4-H), 7.42-7.47 (t, 3H, C_3-H, C_5-H and SH (D_2O exchangeable)), 7.99-8.01 (d, 2H, C_2-H, C_6-H), 12.35 (s, 1H, NH,

D_2O exchangeable); MS (m/z): 272 (M^+ , 45.30%), 198 (100%); Anal. Found: C, 57.11%; H, 4.55%; N, 20.60%; $C_{13}H_{12}N_4OS$ Calcd C, 57.34; H, 4.44; N, 20.57%.

2.2.8 General procedure for the synthesis of 10a&b

A mixture of compound **9** (1.04 g, 3.87 mmole), the appropriate halo compound (3.87 mmole) and sodium acetate (0.7 g, 8.50 mmole) in absolute ethanol was heated under reflux for 5 hours, and then allowed to cool. The separated solid was filtered, washed with water and crystallized from acetone as yellow crystals**.**

*2.2.8..a (3-Methyl-4-oxo-1-phenyl-4, 5-dihydro-1H-pyrazolo [3, 4-d] pyrimidin-6-ylmethylsulfanyl)-acetic acid ethyl ester (**10a**):* yellow colour solid; yield: 60%; m.p. 160-162^{0}C; IR (KBr, cm^{-1}): 3445, 2982-2851, 1734, 1674; ^{1}H-NMR (DMSO-d_6) δ=1.02-1.06 (t, 3H, $CH_2\underline{CH_3}$), 2.49 (s, 3H, CH_3), 3.54 (s, 2H, -CH_2- S), 3.73 (s, 2H, S-CH_2), 3.88-3.95 (q, 2H, $\underline{CH_2}CH_3$), 7.34-7.37 (t, 1H, C_4-H), 7.49-7.54 (t, 2H, C_3-H, C_5-H), 8.03-8.06 (d, 2H, C_2-H, C_6-H), 12.33 (s, 1H, NH, D_2O exchangeable); MS (m/z): 358 (M^+ , 17.90%), 240 (100%). Anal. Found: C, 57.19%; H, 5.25%; N, 15.89%; $C_{17}H_{18}N_4O_3S$ Calcd C, 56.97; H, 5.06; N, 15.63%.

*2.2.8.b 6-Benzylsulfanylmethyl-3-methyl-1-phenyl-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (**10b**):* yellow colour solid; yield: 65%; m.p. 217-219^{0}C; IR (KBr, cm^{-1}): 3439, 2990-2857, 1687; ^{1}H-NMR (DMSO-d6) δ= 2.38 (s, 3H, CH_3), 3.53 (s, 2H, -CH_2-S), 3.97 (s, 2H, S-CH_2-Ph), 7.18-8.09 (m, 10H, Ar-H) , 12.31 (s, 1H, NH, D_2O exchangeable); MS (m/z): 362 (M^+, 21.10%), 91 (100%). Anal. Found: C, 66.20%; H, 5.05%; N, 15.32%; $C_{20}H_{18}N_4OS$ Calcd C, 66.28; H, 5.01; N, 15.46%.

2.2.9 General procedure for the synthesis of 11a&b

A mixture of compound **9** (2.72 g, 0.01mole), the appropriate anilide compound (0.02 mole) and potassium carbonate (2.76 g, 0.02 mole) in acetone (20 ml), was heated under reflux for 10 hours, then allowed to cool. The separated solid was filtered and crystallized from dioxane as yellow crystals.

*2.2.9..a 2-(3-Methyl-4-oxo-1-phenyl-5-phenylcarbamoylmethyl-4, 5-dihydro-1H- pyrazolo [3, 4-d] pyrimidin-6-ylmethylsulfanyl)-N-phenyl-acetamide (**11a**):* yellow colour solid; yield: 58%; m.p. 280-282^{0}C; IR (KBr, cm^{-1}): 3437-3289, 2978.52-2926, 1691, 1662 ; ^{1}H-NMR (DMSO-d_6) δ= 2.49 (s, 3H, CH_3), 3.54 (s, 2H, -CH_2-S), 4.09 (s, 2H, S-CH_2), 5.08 (s, 2H, N-CH_2-CO), 6.99-8.03 (m, 15H, Ar-H), 9.94 (s, 1H, NH, D_2O exchangeable), 10.45 (s, 1H, NH, D_2O exchangeable); MS (m/z): 538 (M^+, 20.26%), 281 (100%); Anal. Found: C, 64.59%; H, 4.29%; N, 16.62%; $C_{29}H_{26}N_6O_3S$ Calcd C, 64.67; H, 4.87; N, 15.60%.

*2.2.9..b 2-[3-Methyl-4-oxo-1-phenyl-5-(p-tolylcarbamoyl-methyl)-4, 5-dihydro-1H-pyrazolo [3, 4-d] pyrimidin-6-ylmethylsulfanyl]-N-p-tolyl-acetamide (**11b**):* white colour solid; yield: 69%. m.p.; 289-291^{0}C; IR (KBr, cm^{-1}) 3437-3304, 3035-2921, 1671 (3C=O); ^{1}H-NMR (DMSO-d_6) δ= 2.19 (s, 6H, $2CH_3$), 2.49 (s, 3H, CH_3), 3.49 (s, 2H, -CH_2-S), 4.08 (s, 2H, S-CH_2), 5.05 (s, 2H, N-CH_2-CO), 6.97-8.15 (m, 13H, Ar-H), 9.86 (s, 1H, NH, D_2O exchangeable) and 10.36 (s, 1H, NH, D_2O exchangeable) ; MS (m/z): 566 (M^+, 10.40%), 55 (100%); Anal. Found: C, 65.44%; H, 5.56 %; N, 14.62 %; $C_{31}H_{30}N_6O_3S$ Calcd C, 65.70; H, 5.34; N, 14.83%.

Table 1. *In vitro* cytotoxic activity of some newly synthesized compounds and the standard olomoucin I.

Compound	Survival fraction (%) Concentration (µg/ mL)					
	50	25	12.5	5	IC_{50}[a] (µg/ml)	IC_{50} (µM)
4b	0.135	0.132	0.101	0.111	**2.7**	**0.008**
4d	0.163	0.105	0.473	0.918	11.6	0.030
5a	0.124	0.233	0.405	0.652	9.6	0.027
5c	0.289	0.201	0.271	0.472	8.84	0.013
5d	0.113	0.371	0.583	0.725	17.6	0.045
6a	0.188	0.181	0.297	0.523	5.55	0.017
6b	0.255	0.139	0.152	0.418	4.2	0.012
6c	0.173	0.391	0.581	0.762	17.7	0.054
7a	0.233	0.140	0.239	0.521	5.85	0.021
7b	0.214	0.297	0.392	0.754	10.2	0.035
7c	0.133	0.235	0.515	0.727	13.2	0.044
8a	0.201	0.113	0.186	0.307	3.45	**0.009**
8b	0.198	0.080	0.154	0.614	6.6	0.080
10a	0.234	0.161	0.377	0.599	8.4	0.023
10b	0.048	0.076	0.086	0.103	**2.85**	**0.007**
11a	0.123	0.077	0.080	0.404	4.2	**0.007**
11b	0.236	0.209	0.129	0.202	**2.85**	**0.004**
Olomoucine	-----	-----	-----	-----	-----	**7**

2.3 Antitumor bioassay

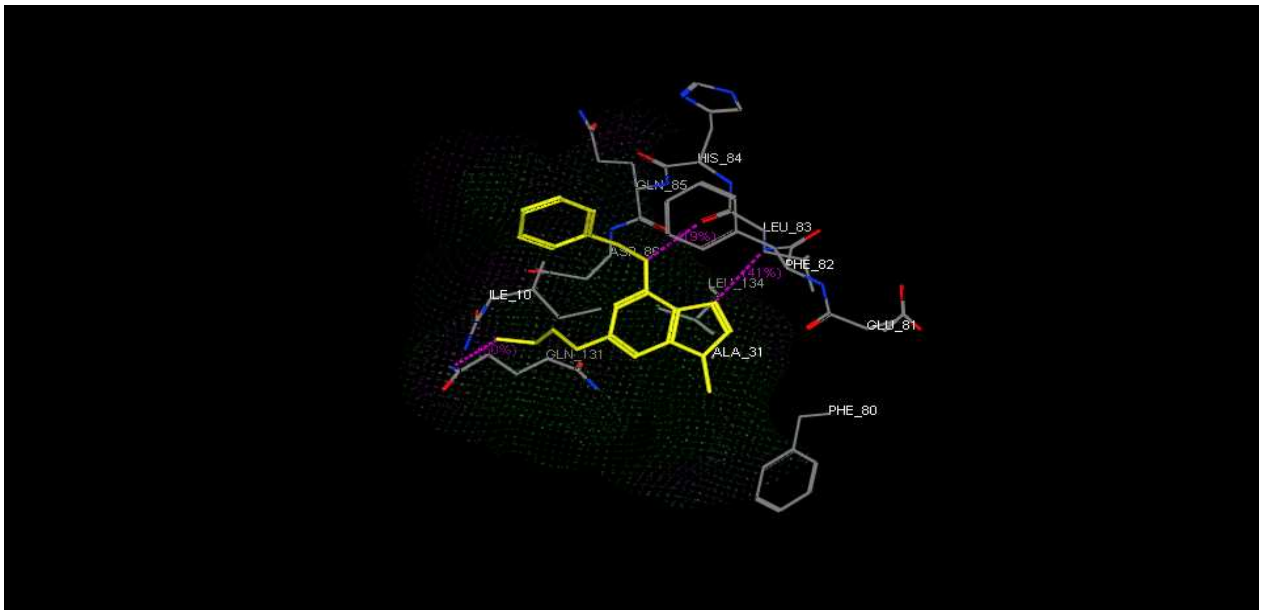
The effects of compounds on the growth of tumor cell lines (MCF-7), were evaluated according to the procedure adopted by the National Cancer Institute, Cairo, Egypt for the *in- vitro* anticancer drug screening that use the protein-binding dye sulforhodamine B (SRB) to assess growth inhibition[28]. Cell were routinely maintained as adherent cell cultures in RPMI- 1640 medium supplemented with 10% heat-inactived fetal bovine serum (FBS) and 1% penicillin/ streptomycin at 37^{o}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 ug/ml). Compounds were prepared in dimethylsulphoxide (DMSO), were freshly diluted with cell culture medium just prior the assays. Olomoucine **I** 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).

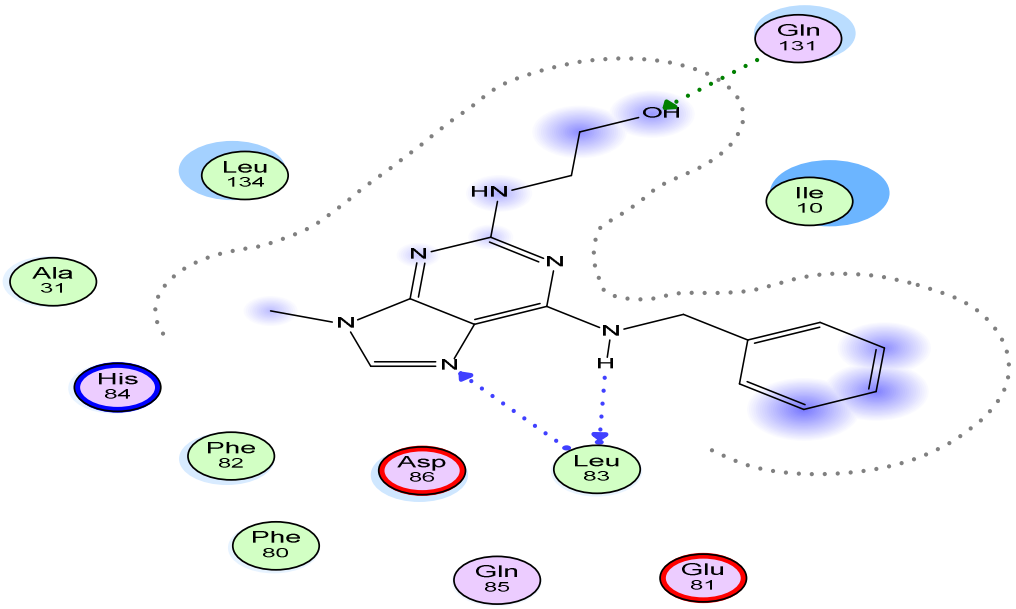
RESULTS AND DISCUSSION

3.1 Molecular modeling

The lead compound olomoucine **I** (PDB ID: 1W0X) ,[26] which was reported to have CDK inhibitory activity, was used to generate common feature hypothesis of CDK inhibitor as anti tumor agents by introducing pyrazole ring instead of imidazole ring on the purine analoguge. Both N7 and NH on C6 of imidazole ring of olomoucine **I** was found to make an H-bond with Leu 83 which is important for the activity. The C2 side chain is bound within the ATP ribose binding pocket, with the methyl group which interacting hydrophobically with glycine- rich loop and val 18. The hydroxyl group of the side chain can make an H- bond with the backbone carbonyl of Gln 131. The purine N9 of methyl group shows a strong hydrophobic interaction with the hydrophobic side chains of Val 18, Ala 31, Phe 80, Leu 134 and Ala 144 (Fig. 1).[1]



a



b

Figure 1. a) The proposed binding mode of olomoucine I inside the active site of CDK resulting from docking, the most important amino acids are shown together with their respective numbers. It forms two H-bonds with Leu 83 through its pyrimidine C6-NH and N7 of imidazol ring, and one H-bond with C2 side chain-OH group b) 2D interaction of olomoucine I with Leu 83 and Gln 131amino acid.

Searching in the common features of the lead compound **I**, we designed new pyrazolo[3,4-*d*]pyrimidine derivatives with the following modifications:

N1 Pyrazole nitrogen of pyrazolopyrimidine ring was substituted with phenyl group instead of the methyl group at N9 of the lead compound **I**. At the C6 side chain we added additional carbon before NH group to act as a spacer that showed good fitting to the receptor. We explored the usage of alkyl or aromatic side chain at C6 in order to increase the hydrophobic interaction toward the receptor. Additional modification was added at N5 of the pyrimidine ring to certain new compounds aiming to increase the hydrogen bonding interaction with the receptor binding site (Fig. 2).

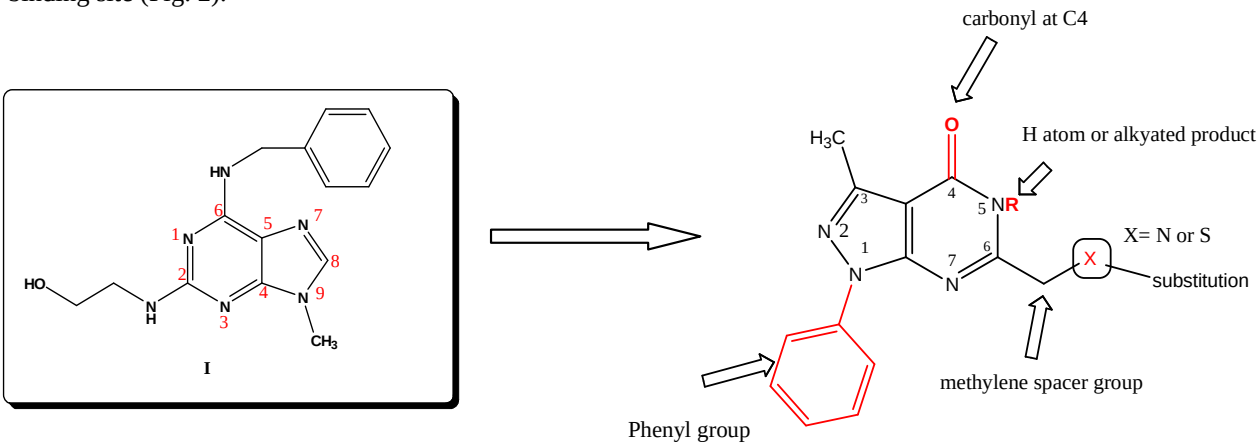


Figure 2. a) Structure of the lead compound olomoucine,
b) Common features for the newley synthesized compounds.

On standing on these modifications and docking of the new compounds with the CDK enzyme, a new common feature hypothesis for our compounds was flowering up (Fig. 2b). This hypothesis showed that carbonyl group at C4 and N5H make a hydrogen bond with Leu 83 and similar interaction also stated for the lead compound **I**. Alteration of methyl group in **I** with phenyl group in **II** resulted in better hydrophobic interaction, hence phenyl is more deeply

socked into ATP ribose binding pocket. By induction of different pharmacophores to C6 side chain, a variety of hydrogen bonding was estimated in certain compounds and this hydrogen bonding were with Gln 8, 131, His 84, Lys 89, 128, 129, Thr 14 and Asp 56, 145 amino acids (Fig. 3-5). However, most of our compounds interact with the receptor with a minimum binding energy scores as shown in (Table 2).

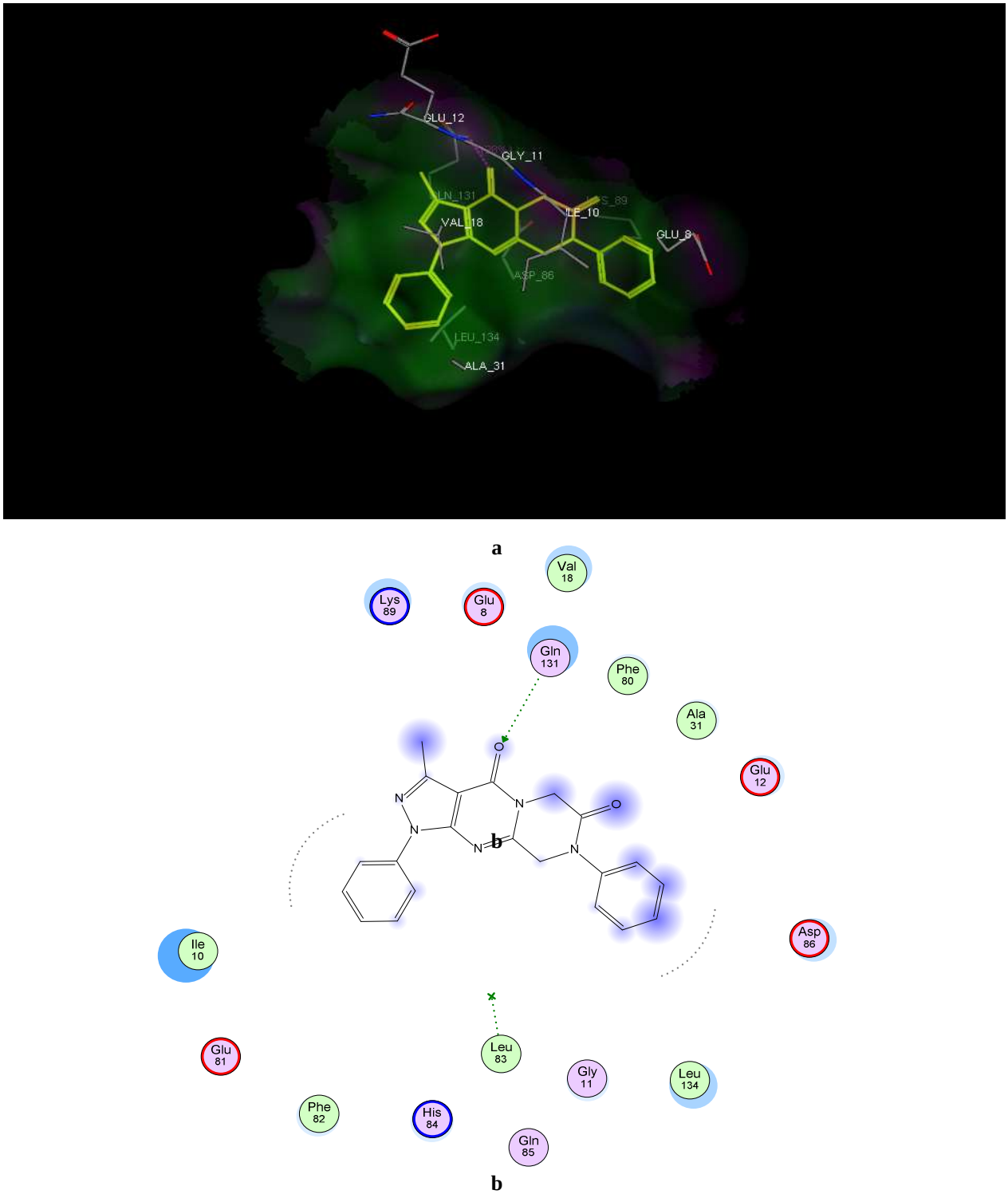
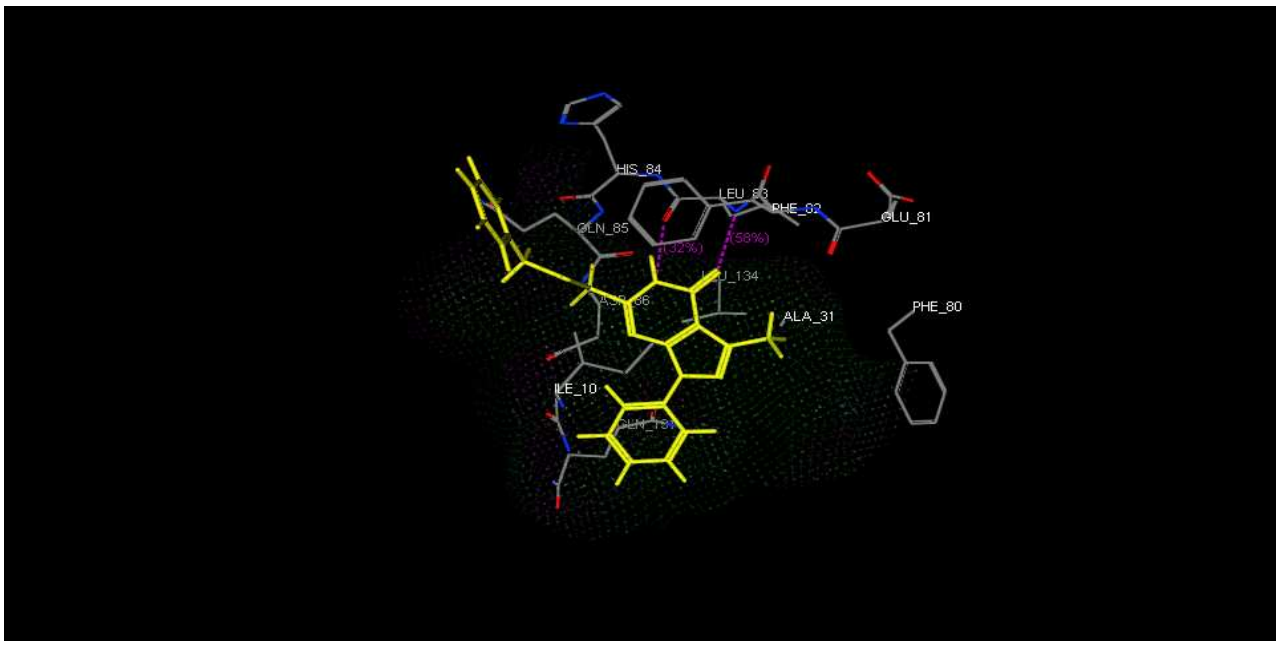


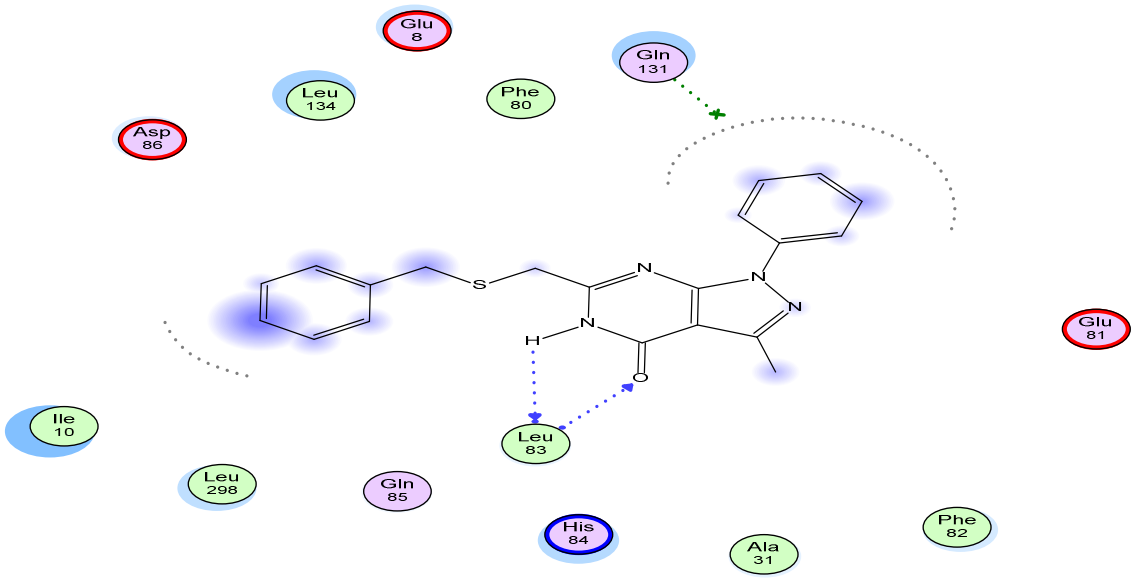
Figure 4. a) The proposed binding mode of compound 8a inside the active site of CDK resulting from docking, the most important amino acids are shown together with their respective numbers. Compound 8a forms one H-bonds with Gln 131 through its pyrimidine C=O, b) 2D interaction of compound 8a with Gln 131 amino acid.

Table 2. Docking study data and cytotoxic activity (IC_{50}) for olomoucine I and some newly synthesized compounds on CDK enzyme.

Compound No.	Number of H-bonds	Atoms of compound forming H-bonds	Amino acid residues forming H-bonds	Binding Energy score (Kcal/mol)
4b	2	C=O, NH	Leu 83 Leu 83	-23.1725
4d	3	C=O, NH, COO^-	Leu 83 Leu 83 His 84	-17.6897
5a	-	-	-	-23.8349
5c	1	OH	Lys 128	-18.2302
5d	2	C=O of (COOH), C-O^-	Thr 14 Lys 129	-11.7559
6a	1	NH	Asp 145	-19.1579
6b	3	C=O, NH, N piperidino group	Leu 83 Leu 83 Lys 89	-23.0477
6c	3	C=O, NH, N morpholino group	Leu 83 Leu 83 Lys 89	-23.2674
7a	1	C=O,	Gln 131	-18.1891
7b	2	C=O, NH	Leu 83 Leu 83	-21.2642
7c	2	C=O, NH	Leu 83 Leu 83	-20.1295
8a	1	C=O	Gln 131	-24.2298
8b	1	C=O	Gln 131	-25.0835
10a	2	C=O, NH	Leu 83 Leu 83	-21.0402
10b	2	C=O, NH	Leu 83 Leu 83	-24.4210
11a	1	C=O	Lys 89	-16.2786
11b	2	C=O , NH of (-SCH_2CONH-)	His 84 Asp 56	-21.6464
olomoucine	2	N6, NH	Leu 83, Leu 83	-19.4146

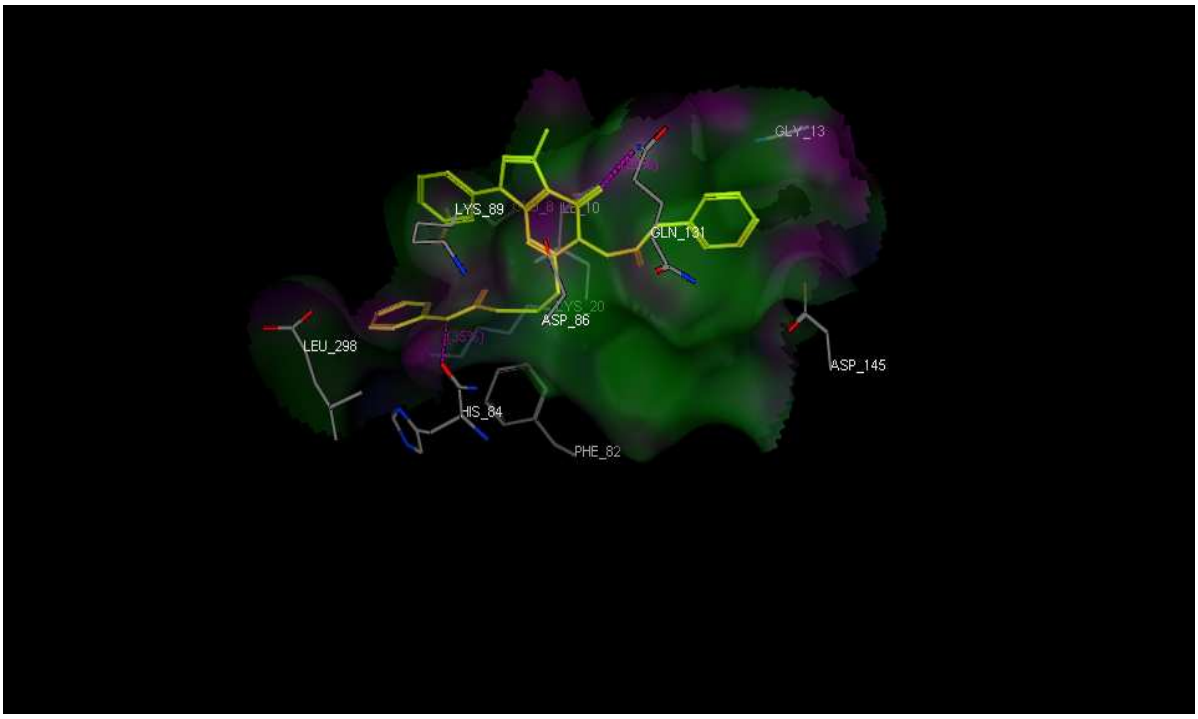


A

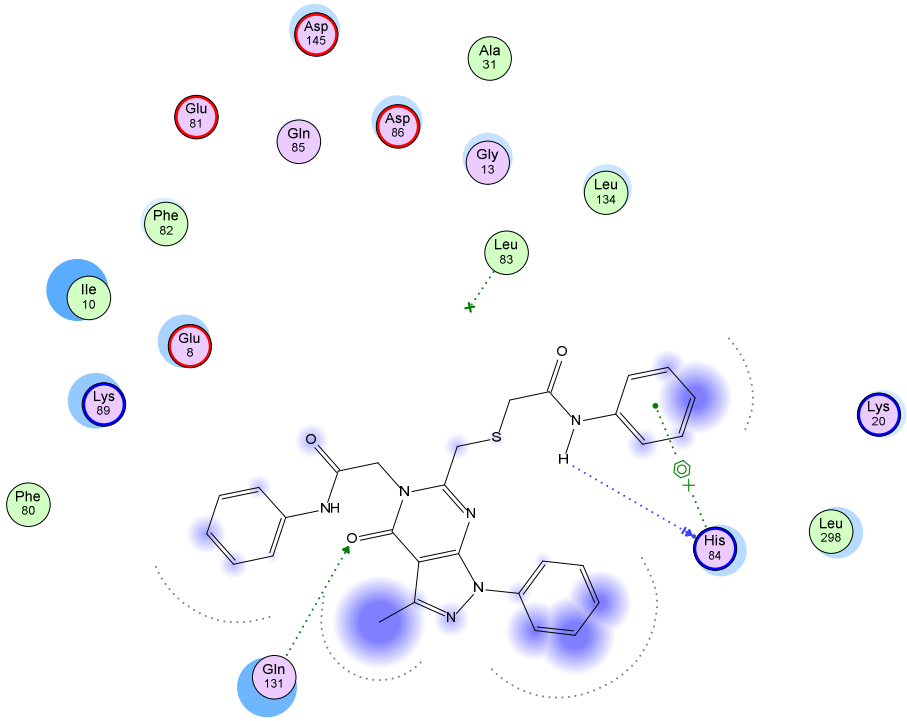


b)

Figure 5. a) The proposed binding mode of compound 10b inside the active site of CDK resulting from docking, the most important amino acids are shown together with their respective numbers. Compound 10b forms two H-bonds with Leu 83 through its pyrimidine NH and carbonyl group, b) 2D interaction of compound 13b with Leu 83 amino acid.

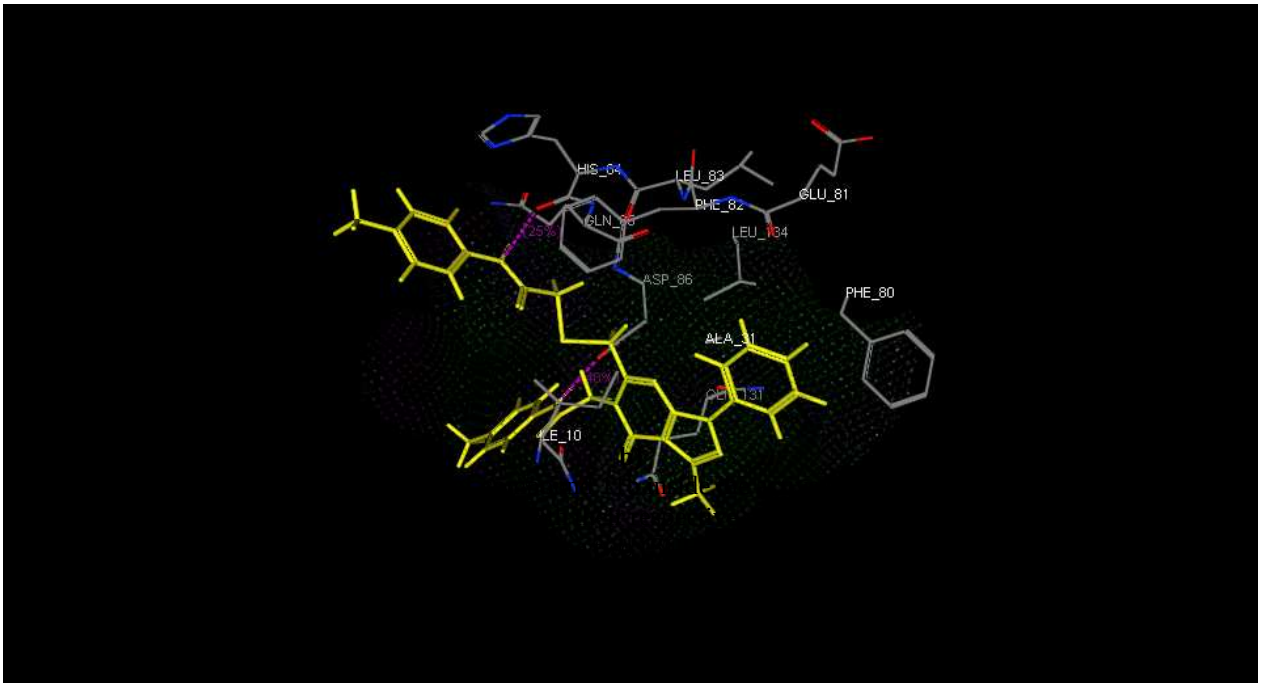


a)



b)

Figure 6. a) The proposed binding mode of compound 11a inside the active site of CDK resulting from docking, the most important amino acids are shown together with their respective numbers. Compound 11a forms two H-bonds with His 84 through its NH group and with Gln 131 through its pyrimidine C=O group, the other interaction is arene cation interaction with His 84 amino acid, b) 2D interaction of compound 11a with His 84 and Gln 131 amino acids.



a

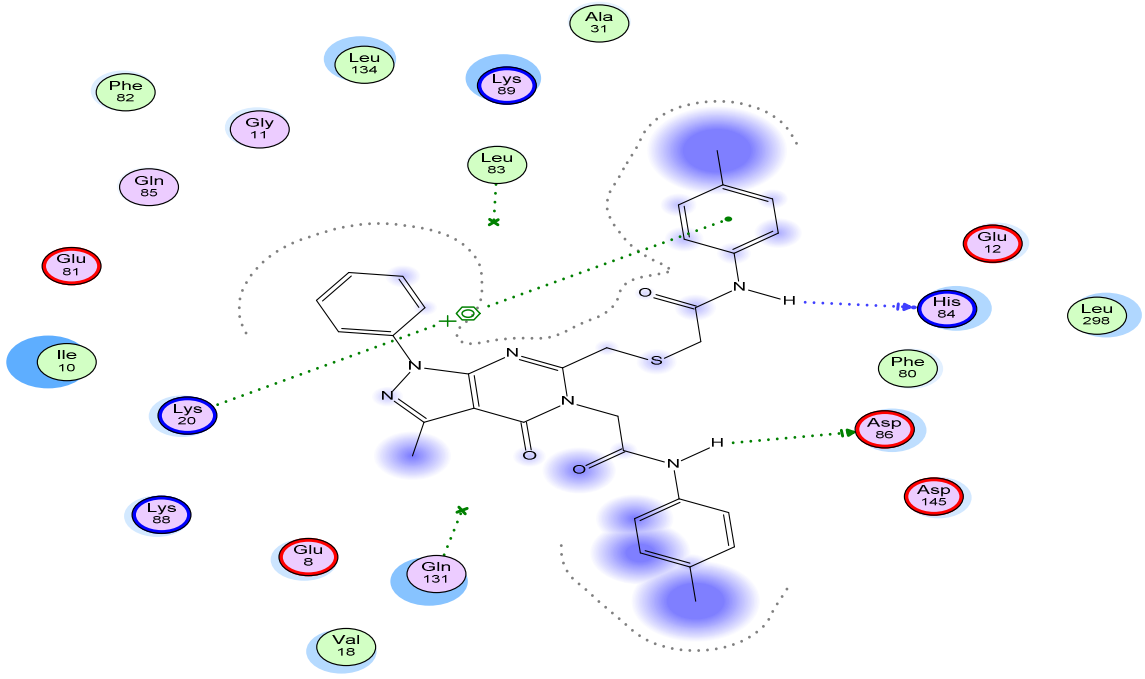


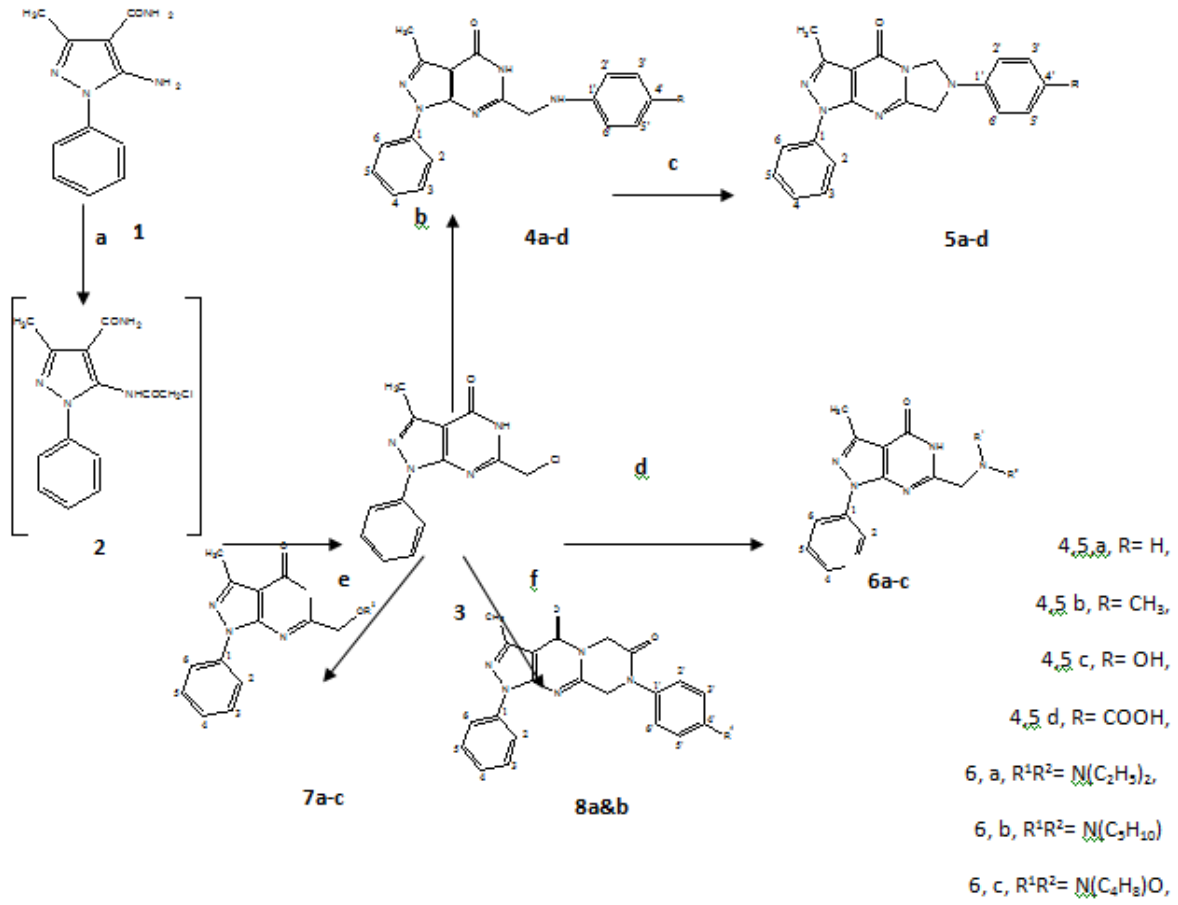
Figure 7. a) The proposed binding mode of compound 11b inside the active site of CDK resulting from docking, the most important amino acids are shown together with their respective numbers. Compound 11b forms two H-bonds with Asp 86 and His 84 through its 2NH groups of the two (SCH_2CONH) groups, the other interaction is arene cation interaction with Lys 20 amino acid, b) 2D interaction of compound 14b with Asp 86 and His 84 amino acids.

3.2 Experimental Section

Reacting of 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carboxamide **(1)**,[28] with Chloroacetyl chloride yielded 6-chloromethyl-3-methyl-1-phenyl pyrazolo [3, 4-*d*] pyrimidin-4-one (**3**) which was used as a starting material for the synthesis of novel pyrazolopyrimidines. Cyclization of **1** was preceded through chloroacetylation of amino group forming chloroacetyl aminopyrazole carboxamide **2** as non isolatable intermediate followed by dehydration to afford **3**. Structure of compound **3** was established on the basis of spectral analyses. IR spectrum showed absorption band at 3276 cm^{-1} and 1675 cm^{-1} attributed to the presence of (NH) and (C=O) groups, respectively. Moreover, the ^{1}H-NMR spectrum of compound **3** revealed a single signal at δ 4.45 assigned to the two protons of (CH_2-Cl) group, and a singlet at δ 12.61, D_2O exchangeable attributed to the (NH) proton. Also its mass spectrum exhibited a molecular ion peak at m/z 274 which agreement with the expected structure.

The key intermediate **3** was used as an alkylating agent to be reacted with different primary aliphatic amines, secondary aliphatic amines and some alkoxides giving series **4a-d**, **6a-c** and **7a-c,** respectively. All the prepared compounds were confirmed by element analysis and spectral data. Mannich reaction of arylaminomethylpyrazolopyrimidines **4a-d** with formalin (30%) solution was preceded *via* hydroxyl methylation of the NH group of aryl amino group which spontaneously underwent elimination of water to yield **5a-d**. The reaction of formaldehyde carbonyl group occurred at the amino NH rather than the pyrimidine NH which is present in tautomerism with the adjacent carbonyl group. The structure of **5a-d** was confirmed using spectral analyses. IR spectra of compounds **5a-d** showed the disappearance of bands characteristic of NH groups in the precursor compound. Also showed absorption bands at the range 1704- 1664 cm^{-1} for (C=O) group. Their ^{1}H-NMR showed the disappearance of D_2O exchangeable signals due to 2 NH protons and appearance of two singlet at δ 4.60-4.70 and at δ 5.29-5.37 characteristic of 2 CH_2 groups.

On our way to add alkyl pharmacophore to N5H of pyrimidine moiety, compound **3** was reacted with chloroacetanilide derivatives resulted in the tricyclic products **8a&b**. This reaction was suggested to be proceeded *via* double alkylation; one of them is of the amidic NH with methylene chloride group of pyrimidine **3** and the other for N5H of pyrimidine **3** and $–CH_2Cl$ of the anilide. The structure of compounds **8a&b** was confirmed by its IR, which revealed two (C=O) groups at the range of 1705-1704 cm^{-1} and disappearance of NH group. ^{1}H-NMR spectra showed two singlet signals at δ 4.66-4.77 and at δ 5.01-5.34 characteristic of 2 CH_2 groups. Also, the mass spectrum of compounds **8a&b** exhibited a molecular ion peak at m/z 371 and m/z 385, respectively (scheme I)

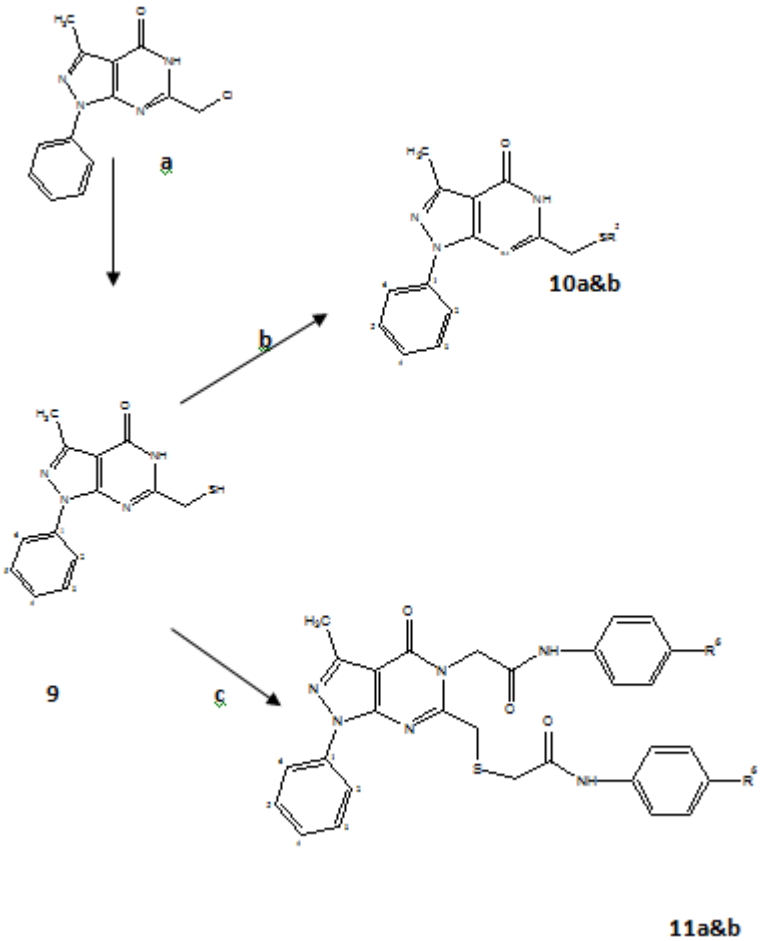


Scheme 1. Reagents: a, chloroacetyl chloride; b, primary aliphatic amines;
c, formaldehyde; d, secondary aliphatic amines; e, different alkoxides; f, chloro acetanilide derivatives

On the other hand, 6-chloromethyl-3-methyl-1-phenyl pyrazolo [3, 4-*d*] pyrimidin-4-one (**3)** was converted into the corresponding mercapto derivative **9** by refluxing with thiourea in ethanol resulted in the formation of the isothiouronium salt which then treated with sodium hydroxide followed by acidification with hydrochloric acid to give compound **9**. The structure of compound **9** was elucidated from its element and spectral analyses. IR spectrum showed absorption band at 2733 cm^{-1} for SH group. Its 1H-NMR showed a single signal at δ 3.97 for $–CH_2$. Mass spectrum of compound **9** revealed a peak at m/z 272 corresponding to molecular ion peak.

As an extension of this synthetic route, compound **9** was alkylated using halogenated compounds such as ethyl chloroacetate and/or benzyl chloride to give S-alkylated compounds **10a&b**. The structure of **10a&b** was elucidated using element and spectral analyses. IR spectra showed the disappearance of SH peak and the appearance of absorption bands in the range of 3445-3439 cm^{-1} for (NH) group, 1674-1687 cm^{-1} for (C=O), and 1734 cm^{-1} for (C=O) of ester moiety in compound **10a** . The ^{1}H-NMR showed the appearance of new signals characteristic for (-S-CH_2-) at δ 3.73, 3.97, also, mass spectra for compounds **10a&b** showed molecular ion peaks at m/z 358, 362 respectively which in agreement with the suggested structure.

In addition to S-alkylation, chloroacetanilide derivatives were used for S-alkylation, while the resulted structures **11a&b** were the doubly alkylated products on S- and N- atoms. This was attributed to the effect of (-CONH) group of the chloroacetanilide derivatives which increased the positive charge at (CH_2) and facilitate the acceptance of the lone pairs on (S and N) atoms leading to the alkylation on both (S and N). The structures were confirmed by micro analytical and spectral data, IR spectra showed absorption bands in the range 3437-3289 cm^{-1} for two amidic NH groups, 1691- 1662 cm^{-1} for (C=O) of pyrimidine ring and (2C=O) of amidic bond. ^{1}H- NMR showed the appearance of single signals characteristic for (-CH_2S-) at δ 3.49, 3.54, singlet signals at δ 4.08, 4.09 for (-S-CH_2-) , at δ 5.05- 5.08 for (-N-CH_2-CO-), also appearance of two D_2O exchangeable singlet signals at δ 9.86- 9.94 and 10.36- 10.45 referred to the two (NH) groups. Mass spectra for compounds **11a&b** showed molecular ion peak at m/z 538, 566, respectively which confirmed the suggested structure (Scheme 2).



10, a, R^5= $CH_2COOC_2H_5$,
10, b, R^5= $CH_2C_6H_5$
11, a, R^6= H,
Scheme 2. Reagents: a, thiourea; b, a) ethyl chloroacetate, b) benzyl chloride; c, a) anilide, b) *p*-tolyl anilide .

3.3 Antitumor activity

Olomoucine **I**, was used as the reference drug in this study. Some of the synthesized compounds were selected and screened for their anticancer activity. Each compound was tested at five different concentrations against human breast cancer cell line. The relationship between survival fraction and drug concentration was plotted to obtain the survival curve of human breast cancer cell line (MCF-7). The response parameters calculated was IC_{50} value, which corresponds to the compound concentration causing 50% mortality in net cells (Table 2).

CONCLUSION

The present data showed that, compounds (**4b**, **8a**, **10b**, **11a&b**) exhibited promising *in- vitro* cytotoxic activity against (MCF-7) giving the highest cytotoxic activity between (0.009-0.004 µM) when compared to the other test compounds and olomoucine **I** (7 µM) as a reference drug, all the other test compounds showed higher IC_{50} values than that of the reference drug, ranging from 0.012 to 0.080 µM. Docking studies confirmed this result as compounds (**4b**, **8a**, **10b**, **11a&b**) showed low binding energy scores from (-24.2298 to -16.2786 Kcal/mol), also showed binding mode similar to olomoucine **I** which was reported to have CDK inhibitory effects.IC_{50} values were plotted against binding energy scores which revealed that there was some sort of consistency between the docking studies and *in-vitro* screening, (Chart 1).

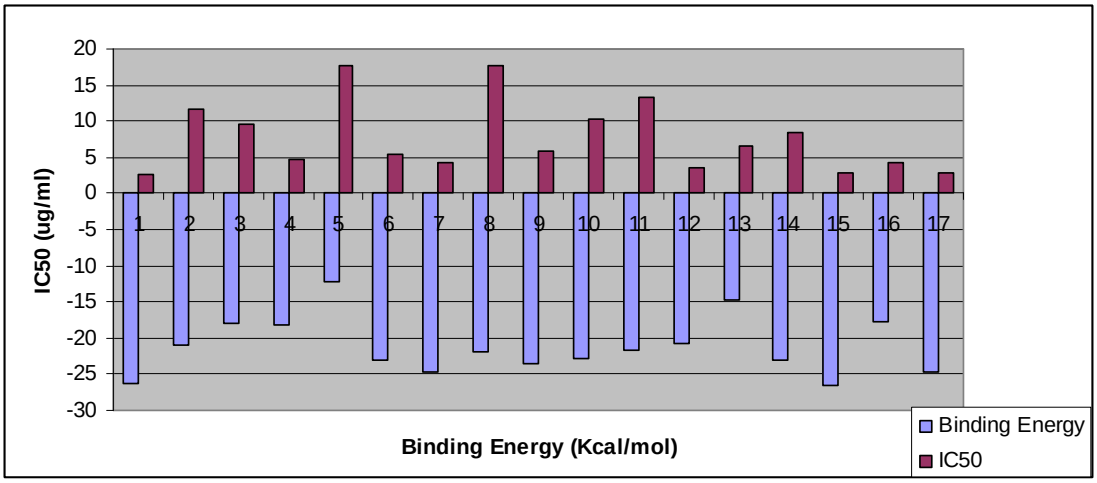


Chart 1. Docking scores against IC_{50}.

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