DRUG SYNTHESIS

A CONVENIENT SYNTHESIS OF NOVEL PYRAZOLO[3,4-D] PYRIMIDINE AND PYRAZOLO[4,3-E][1,2,4]TRIAZOLO[1,5-C] PYRIMIDINE DERIVATIVES TARGETING CDK2

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Abstract: Cyclin-dependent kinase 2 (CDK2) is a critical protein kinase entangled in the cell cycle regulation. The irregular activity of CDK2 is correlating with cancer development and metastasis. Here structural resemblance of pyrazolopyrimidines to purines prompted the investigation of their chemical significance and their anticancer activity. Several new pyrazolopyrimidine compounds were obtained from 5-amino-3-(cyanomethyl)-1H-pyrazole-4-carbonitrile **1**, which was initially reacted with acetic anhydride at refluxed to form 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile **2** and N-(4-cyano-3-(cyano methyl)-1H-pyrazole-5-yl)acetamide **3**. The affinity of compound **2** towards nucleophiles was studied by its reaction with various primary aromatic amines to furnish the newly pyrazolo[3,4-d]pyrimidines **4a-c**. The reaction of 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile **2** with hydrazine hydrate gave pyrazolopyrimidine **5** which condensed with aromatic aldehydes to form pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine derivatives **6a-d**. All possible tautomers of 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl) acetonitrile **2** were studied by DFT (density functional theory) and the constancy of compounds **4** over compounds **3** was discussed and established by quantum mechanics.

Keywords: cyclin-dependent kinase 2, molecular docking, binding energy, pyrazolopyrimidines, Dimroth rearrangement, tautomeric structures, DFT

Protein kinases perform a vital role in catalyzing the phosphorylation of many substrate proteins that in turn regulate biological processes (1). In general, phosphorylation takes place on the specific amino acid residue, namely tyrosine, serine, and threonine, which generally control the cell cycle and cell development. Cyclin-dependent kinase 2 (CDK2) is one such protein kinase that is known to control the cell cycle by initiating the late G1 phase to enter the G1-S transition phase (2). Governance of the cell cycle to G2 from the S phase is promoted by cyclin A (3). Through DNA duplication, spindle fibers are designed with the help of centrioles and pro-centrioles principally depending on the proper functioning of CDK2 (4). Thus, the association of CDK2 with cyclin E and A specifically controls the regulation of the G1-S phase of the cell cycle.

The role of CDK2 in cell division is particularly important as it prohibits the dysfunction of the cell cycle. The overexpression of CDK2 gives rise to unusual regulation of the cell cycle that is straightly connected with the hyperproliferation of cancer cells. Numerous pieces of evidence are supporting

the idea of targeting CDK2 to slow down cancer progression (5). The dysregulation of CDK2 is noticed in the breast and other cancer, so making it a potential drug target for anticancer therapy (6, 7).

In recent years, organic chemists paid attention to pyrazolopyrimidine derivatives because of their prevalent potential biological and chemotherapeutic actions. Pyrazolopyrimidines and associated heterocycles are found to own comprehensive implementation in medicine and agriculture. Their structural likeness to purines encouraged biological investigations of their therapeutic significance (8-10). They showed widespread pharmacological activities like tuberculosis (11) antimicrobial (12), neuroleptic (13), antitumor (14), antihypertensive (15), and antileishmanial actions (16).

Starting with Robins's work (17) researchers have dedicated a considerable effort to investigate new approaches for the synthesis of pyrazolo[3,4-d]pyrimidine derivatives. There are two main strategies found in the literature to build up the pyrazolopyrimidines scaffold, the foremost starting from the pyrazole moiety and the other one starting from the pyrimidine moiety.

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Stimulated by the chemical and medical significance of pyrazolo[3,4-d]pyrimidines, and continuance to our work (18-20), the goal was to produce a new category of pyrazolo[3,4-d]pyrimidine and pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine analogs throughout the pyrazole moiety by inserting various groups at the pyrazolopyrimidine ring.

EXPERIMENTAL

Chemistry

All chemicals utilized in this study were acquired from Sigma-Aldrich (USA) and SD-Fine Chemicals (India) and were used without further purifications. Separation of the compounds by column chromatography was done utilizing silica gel 60 (200–300 mesh ASTM, E. Merck). The amount of silica gel employed was 75 times the weight of the sample charged in the column. The reactions were observed by TLC and displayed by UV light (254 nm). Melting points (uncorrected) were measured on an XT4 MP apparatus. Mass spectra were registered on a Varian MAT 112 spectrometer. Analytical data (FT-IR) were performed at Cairo University. ¹H NMR and ¹³CNMR spectra were listed on a Varian spectrometer (500 MHz). Elemental analyses were achieved on (SE Series Elemental Analyzer) for CHNS.

2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile **2**

A mixture of 5-amino-4-cyano-3-cyanomethylpyrazole **1** (7 mmol, 1.0 g) in acetic anhydride (10 mL) was boiled under reflux for 2 h. The reaction development was observed by TLC using ethyl acetate: n-hexane (1 : 1) as a mobile phase. The reaction mixture was cooled to deposit a shiny gray crystalline solid, which was filtered off and recrystallized from dioxane to produce compound **2**.

Shiny gray. Yield = 72%. Mp. = 195–197°C. IR (cm⁻¹), 3408, 3311 (2NH), 2231, 2222 (2CN), 1716 (C=O), 1649 (C=N); ¹H-NMR (500 MHz, DMSO-*d*₆) δ 12.32 (s, 1H, NH), 8.03 (s, 1H, NH), 4.10 (s, 2H, CH₂), 2.46 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 173.34 (C4), 155.57 (C6), 146.96 (C7a), 116.43 (CN), 113.12 (C3), 72.78 (C3a), 23.70 (CH₃), 16.99 (CH₂); MS (m/z) = 189.20; Analysis: calcd for C₈H₇N₅O: C, 50.79; H, 3.73; N, 37.02; found: C, 50.32; H, 4.13; N, 36.75.

N-(4-cyano-3-(cyanomethyl)-1H-pyrazol-5-yl)acetamide **2'**

Compound **2'** was obtained by dilution of the filtrate of compound **2** with ice-cold water to deposit a dark gray precipitate. Filtration, washing with water, and recrystallization from alcohol gave

compound **2'** as a pure sample (21). Yield 25% as gray crystals, Mp. = 212–214°C, reported (215°C).

2-(4-((substituted phenyl)amino)-6-methyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl) acetonitrile **4**

A mixture of 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile **2** (10 mmol, 1.9 g) and appropriate aromatic primary amine (11 mmol) refluxed in toluene (50 mL) with acetic acid (1 mL) for 24 h. The separated compound after cooling in an ice-bath was filtered, washed with diethyl ether, dried, and recrystallized from ethanol to give compounds **4**.

2-(4-((4-chlorophenyl)amino)-6-methyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)acetonitrile **4a**

Yield: 75% for light-yellow powder; Mp. = 233–235°C; IR: (ν max, cm⁻¹): 3335, 3221 (2NH), 2221 (CN), 1642 (amide C=N); ¹H-NMR (500 MHz, DMSO-*d*₆) δ 12.54 (s, 1H, NH), 10.54 (s, 1H, NH), 7.57 (d, *J* = 7.8 Hz, 2H), 6.82 (d, *J* = 7.8 Hz, 2H), 4.32 (s, 2H, CH₂), 2.35 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 161.60 (C6), 160.77 (C7a), 155.28 (C4), 153.98 (Ar-C1), 127.09 (C3), 113.35 (CN), 112.48 (Ar-C3,5), 110.70 (Ar-C4), 102.62 (Ar-C2,6), 97.01 (C3a), 25.05 (CH₃), 18.56 (CH₂); MS (m/z) = 300.75; Analysis: calcd for C₁₄H₁₁ClN₆: C, 56.29; H, 3.71; N, 28.13; found: C, 56.62; H, 4.03; N, 27.85.

2-(6-methyl-4-(p-tolylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)acetonitrile **4b**

Yield: 63% for white powder; Mp. = 221–223°C; IR: (ν max, cm⁻¹): 3325, 3181 (2NH), 2220 (CN), 1644 (amide C=N); ¹H-NMR (500 MHz, DMSO-*d*₆) δ 11.63 (s, 1H, NH), 9.09 (s, 1H, NH), 7.58 (d, *J* = 7.7 Hz, 2H), 7.47 (d, *J* = 7.7 Hz, 2H), 4.34 (s, 2H, CH₂), 3.26 (s, 3H, CH₃), 2.34 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.82 (C6), 157.38 (C7a), 144.95 (C4), 142.97 (Ar-C1), 130.08 (C3), 129.49 (Ar-C4), 117.62 (Ar-C3,5), 116.42 (CN), 115.1 (Ar-C2,6), 98.79 (C3a), 25.97 (CH₃), 16.74 (CH₂), 13.92 (CH₂); MS (m/z) = 278.8; Analysis: calcd for C₁₅H₁₄N₆: C, 64.73; H, 5.07; N, 30.20; found: C, 64.32; H, 4.73; N, 29.91.

4-((3-(cyanomethyl)-6-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino) benzenesulfonamide **4c**

Yield 62% for white powder; Mp. = 212–215°C; IR: (ν max, cm⁻¹): 3388 (NH₂), 3252, 3160 (2NH), 2219 (CN), 1640 (C=N); ¹H-NMR (500 MHz, DMSO-*d*₆) δ 11.81 (s, 1H, NH), 8.05 (s, 1H, NH), 7.74 (d, *J* = 9.6 Hz, 2H), 7.67 (d, *J* = 9.6 Hz, 2H), 7.61 (s, 2H, NH₂), 3.78 (s, 2H, CH₂), 2.20 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.01 (C6), 158.71

(C7a), 155.11 (C4), 150.97 (Ar-C1), 132.58 (C3), 130.19 (Ar-C4), 129.04 (Ar-C3,5), 127.90 (Ar-C2,6), 116.52 (CN), 93.82 (C3a), 31.27 (CH₃), 12.90 (CH₂); MS (m/z) = 344.1; Analysis: calcd for C₁₅H₁₃N₇O₂S: C, 48.97; H, 3.82; N, 28.56; found: C, 48.32; H, 4.33; N, 29.11.

2-(5-amino-4-imino-6-methyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-3-yl) acetonitrile 5

A suspension of 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile 2 (4.9 g, 25.9 mmol) in Hydrazine hydrate (1.3 mL, 25.9 mmol) was heated at 100°C in a water bath for 2 h with stirring. The reaction progress was followed by TLC using ethyl acetate: n-hexane (1: 1) as the mobile phase. The reaction mixture was diluted with ethanol/water, filtered, dried, and recrystallized from dioxane/ethyl alcohol to give the titled product 5.

Yield 74% for light brown powder; Mp. = 192-195°C; IR: (ν max, cm⁻¹): 3358 (NH₂), 3266, 3140 (2NH), 2222 (CN), 1645 (C=N); ¹H-NMR (500 MHz, DMSO-*d*₆) δ 11.9 (s, 1H, NH), 9.47 (s, 1H, NH), 6.08 (s, 2H, NH₂), 4.39 (s, 2H, CH₂), 2.4 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 161.62 (C4), 155.29 (C6), 154.03 (C7a), 127.08 (C3), 113.32 (CN), 102.63 (C3a), 22.35 (CH₃), 18.58 (CH₂); MS (m/z) = 204.1; Analysis: calcd for C₈H₉N₇: C, 47.29; H, 4.46; N, 48.25; found: C, 46.88; H, 4.13; N, 47.91.

2-(5-methyl-2-substitutedphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-9-yl)acetonitrile 6

Method 1

To a suspension of 2-(5-amino-4-imino-6-methyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-3-yl)acetonitrile 5 (1 g, 5 mmol) in acetonitrile (80 mL), appropriate benzaldehyde (5.2 mmol) was added with stirring and the reaction mixture was maintained stirring at r.t. Iodine (1.3 g, 5.1 mmol) was added over 20 min. and then the reaction mixture was heated at 125°C for 30 h. The reaction was monitored by TLC (Ethyl acetate: n-hexane 4: 1). The reaction mixture was added to a diluted solution of sodium thiosulfate to free the reaction from excess iodine then the light brown precipitate was filtered off, diluted with ethyl acetate, and recrystallized from ethyl alcohol.

Method 2

A mixture of hydrazide (4 mmol) and 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-d][1,3]oxazin-3-yl)acetonitrile 2 (0.82 g, 4 mmol) was heated at reflux for 24 h in toluene (10 mL) with acetic acid (0.5 mL). The product, which precipitates on cooling, was collected and recrystallized from ethanol to afford compound 6.

2-(5-methyl-2-phenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-9-yl)acetonitrile 6a

Yield: 69% for yellow powder, Mp. = 209-211°C; IR: (ν max, cm⁻¹): 3224 (NH), 2229 (CN), 1637(C=N); ¹H-NMR (300 MHz, DMSO-*d*₆) δ: 12.75 (s, 1H, NH), 7.45–7.25 (m, 2H), 7.20–6.83 (m, 3H), 4.37 (s, 2H, CH₂), 2.49 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 167.32 (C2), 154.17 (C6a), 136.47 (C5), 135.56 (C9b), 132.29 (C9), 130.98 (Ar-C1), 129.45 (Ar-C4), 128.11 (Ar-C3, 5), 124.27 (Ar-C2, 6), 113.38 (CN), 97.21 (C9a), 20.04 (CH₃), 13.74 (CH₂); MS (m/z) = 289.30; Analysis: calcd for C₁₅H₁₁N₇: C, 62.28; H, 3.83; N, 33.89; found: C, 62.78; H, 4.13; N, 33.91.

2-(2-(4-chlorophenyl)-5-methyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-9-yl)acetonitrile 6b

Yield: 71% for light-yellow powder, Mp. = 215-218°C; IR: (ν max, cm⁻¹): 3321 (NH), 2218 (CN), 1645 (C=N); ¹H-NMR (300 MHz, DMSO-*d*₆) δ: 12.61 (s, 1H, NH), 8.21 (d, *J* = 8.3 Hz, 2H), 7.64 (d, *J* = 8.3 Hz, 2H), 4.38 (s, 2H, CH₂), 2.41 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 157.98 (C2), 154.17 (C6a), 152.74 (C5), 144.44 (C9b), 140.00 (C9), 135.83 (Ar-C4), 131.85 (Ar-C1), 128.78 (Ar-C2, 6), 128.07 (Ar-C3, 5), 116.67 (CN), 97.43 (C9a), 19.97 (CH₃), 15.25 (CH₂); MS (m/z) = 324.1; Analysis: calcd for C₁₅H₁₀ClN₇: C, 55.65; H, 3.11; N, 30.29; found: C, 55.28; H, 3.63; N, 30.71.

2-(2-(4-(dimethylamino) phenyl)-5-methyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-9-yl)acetonitrile 6c

Yield: 58% for yellow powder, Mp. = 199-202°C; IR: (ν max, cm⁻¹): 3321 (NH), 2218 (CN), 1645 (C=N); ¹H-NMR (300 MHz, DMSO-*d*₆) δ: 11.91 (s, 1H, NH), 7.93 (d, *J* = 8.6 Hz, 2H), 7.53 (d, *J* = 8.2 Hz, 2H), 4.32 (s, 2H, CH₂), 2.94 (s, 6H, 2CH₃), 2.11 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 157.38 (C2), 154.64 (C6a), 144.94 (Ar-C4), 144.67 (C5), 142.97 (C9b), 142.05 (C9), 130.03 (Ar-C2, 6), 129.49 (Ar-C1), 117.16 (CN), 113.73 (Ar-C3, 5), 98.79 (C9a), 33.38 (2CH₃-N), 19.91 (CH₃), 16.74 (CH₂); MS (m/z) = 332.3; Analysis: calcd for C₁₇H₁₆N₈: C, 61.43; H, 4.85; N, 33.71; found: C, 60.98; H, 4.43; N, 33.91.

2-(2-(4-hydroxyphenyl)-5-methyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-9-yl)acetonitrile 6d

Yield: 69% for yellow powder, Mp. = 225-227°C; IR: (ν max, cm⁻¹): 3456 (OH), 3237 (NH), 2222 (CN), 1651 (C=N); ¹H-NMR (300 MHz, DMSO-*d*₆) δ: 11.89 (s, 1H, NH), 9.47 (s, 1H, OH), 7.02 (d, *J* = 8.9 Hz,

2H), 6.67 (d, $J = 8.9$ Hz, 2H), 4.39 (s, 2H, CH₂), 2.40 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 159.27 (Ar-C1), 157.79 (C2), 154.17 (C6a), 152.74 (C5), 144.44 (C9b), 140.00 (C9), 128.94 (Ar-C2, 6), 124.37 (Ar-C1), 116.67 (CN), 115.46 (Ar-C3, 5), 97.43 (C9a), 21.97 (CH₃), 19.25 (CH₂); MS: (m/z) = 305.41; Analysis: calcd for C₁₅H₁₁N₇O; C, 59.01; H, 3.63; N, 32.12; found: C, 59.33; H, 3.31; N, 31.86.

Computational methods

The geometrical optimization of compounds 2, 3, and 4 was carried out with the Gaussian 09 program (22), using B3LYP functional within the DFT level with the 6-311G (d, p) as basis sets in the gas phase. All geometries were taken as starting points using the AM1 method. These results were reoptimized at Becke's 3-parameter exact exchanges functional (B3) joint with gradient corrected correlation functional of Lee-Yang-Parr (LYP) method. For the optimized geometries, the frequencies were gained from the 2nd derivatives of the energy computed using analytically calculated first derivatives to roll out the compounds that exist in saddle points. All the optimized compounds were found to be at their stationary points congruent to local minima without imaginary frequency.

CDK2/cyclin A2 inhibition assay

The recently synthesized compounds have been inspected as ATP competitive inhibitors in contrast to CDK2 (at conc. 10 μ M) by BPS Bioscience Inc. Inhibition of CDK2/cyclinA2 was assayed by using the Kinase-Glo Plus luminescence kinase assay kit (Promega). It estimates kinase activity by calculating the amount of ATP residual in the solution following a kinase reaction, which is associated with the luminescent signal from the assay and is inversely correlated with the amount of kinase activity. The inhibition activity of the examined compound was revealed as the measurement of % inhibition of compounds on the enzyme and IC₅₀ μ M, which is defined as the concentration required for inhibiting the enzyme by 50%.

Cytotoxicity (MTT assay)

MCF-7 cancer cells were proliferated in 75 cm² cell culture flasks using RPMI-1640 medium (Gibco-USA) supplemented with 10% (v/v) fetal bovine serum (Gibco-USA) and incubated in 5% (v/v) CO₂ incubator at a temperature of 37°C. Confluent cells were separated using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylenediaminetetraacetic acid (Gibco-USA) for 5 min. Cells were plated at a concentration of 2 x 10⁵ cells/mL in 96-well cell culture

plates and incubated at a temperature of 37°C for 24 h to accomplish confluence. The medium was poured out and a fresh medium containing several concentrations of doxorubicin was added for cytotoxicity determination using colorimetric MTT reduction assay. Dead cells were washed with phosphate-buffered saline (PBS), and 50 μ L of MTT stock solution (5 mg/mL) was added to each well. After the 4 h incubation period, the supernatants were discarded and the formazan precipitates were solubilized by the addition of 50 μ L per well of dimethyl sulfoxide (DMSO). Plates were incubated in darkness for 30 min at 37°C, and absorbance was measured (at 570 nm) using a microplate reader (Biotek ELX -800, USA). To estimate the cell viability percentage, we applied the subsequent equation:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated wells} \times 100}{\text{OD of control wells}}$$

The IC₅₀ values of the test compounds were calculated by a Masterplex-2010 software program. An inverted microscope (Nikon-Japan) was applied to study and examine the morphological modifications of cells.

RESULTS AND DISCUSSION

Molecular modeling

Retrieval of CDK2 Inhibitor-like compounds

We retrieved a known inhibitor of CDK2 from the Drug Bank to identify our new proposed compounds having a close relation to them. The known inhibitors of CDK2, olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) (23) was used to screen our proposed compounds. The selection criteria for the known CDK2 inhibitors were structural similarity and its inhibitory potential. We retrieved all the proposed compounds having more than 56% structural similarity with olomoucine Figure 1.

Molecular Docking Approach

Molecular-docking for proposed compounds with similarity criteria was performed to detect suitable compounds that could strongly bind to CDK2. Subsequently, their bound conformations and binding affinities were estimated. The structure preparation of the receptor was initiated with the removal of co-crystallized ligands and water molecules before energy minimization using the CHARMM force field. Hydrogen atoms were added to the polar groups of the protein followed by adding the charges. The CDK2 coordinates were gained from the protein data bank (PDB 2fvd) and the structure was adjusted by

Figure 1. Chemical structures of pyrazolo[3,4-d]pyrimidines.

Figure 2. Hydrogen bond interactions of compound **4c** in the active site of CDK2 (ATP binding site). It forms 3 HB with Lys20, Asp86, and Glu 81. Figure 3. Hydrogen bond interactions of compound **6b** in the active site of CDK2 (ATP binding site). It forms 2 HB with Glu12, and Leu83, which is crucial for biological activity.

Accelrys Discovery Studio 2.5. The hydrogen atoms and the missing residues were added as well as the structure was relaxed to correct the protein errors. Finally, the binding site of CDK2 was distinct and all the suggested compounds that have high similarity values were nominated for docking and binding energy calculations. The investigated compounds were docked utilizing the default parameters of C-docker

protocol and the hopeful derivatives were elected for preparation (Figures 2, 3), (Table 1).

Any small molecule can stimulate a large conformational change in a protein after binding (24-27). Root-mean-square deviation (RMSD) is a significant approach to deduce the structural deviation and

Table 1. Docking energy, binding energy, and RMSD values of the proposed compounds

Compd.	-CDocker Energy	-CDocker interaction Energy	Binding Energy	Ligand Energy	Protein Energy	Complex Energy	Lig. RMSD to reference	No. of lig. conformations
4a	16.102	35.582	-1.355	-21.223	-14521.70	-14544.30	0.594	14
4b	20.033	38.667	-9.504	-21.829	-14511.90	-14543.20	0.443	16
4c	25.806	42.173	-20.228	-80.422	-14518.40	-14619.0	0.524	42
6a	15.20	34.563	-1.324	28.243	-14520.0	-14493.10	0.328	5
6b	12.371	38.901	-11.863	25.956	-14505.40	-14491.40	0.171	8
6c	11.152	40.993	-10.963	25.070	-14510.10	-14496.0	0.274	8
6d	10.718	39.014	-4.510	12.738	-14516.0	-14507.80	0.299	7

stability of a protein structure (28–31). The average RMSD for CDK2-proposed compounds complex was found to be in the range from 0.59 to 0.17 nm (Table 1), which suggests that the binding of the selected compound stabilizes the CDK2 structure and leads to a few conformational changes

Chemistry

The synthetic strategy to arise pyrazolo(3,4-*d*)pyrimidines was demonstrated in Schemes 1, 2 and 3. The starting compound 5-amino-3-(cyanomethyl)-1H-pyrazole-4-carbonitrile **1** was produced by the reaction of 2-aminoprop-1-ene-1,1,3-tricarbonitrile with hydrazine hydrate in boiling ethanol (21). Compound **1** was refluxed in acetic anhydride to produce 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-*d*][1,3]oxazin-3-yl)acetonitrile **2** and *N*-(4-cyano-3-(cyano methyl)-1H-pyrazole-5-yl)acetamide **2'**, the isolation of compound **2** from compound **2'** was achieved by fractional crystallization from acetic anhydride (Scheme 1). 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-*d*][1,3]oxazin-3-yl)acetonitrile **2** was utilized as precursors for the production of various pyrazolo[3,4-*d*]pyrimidines. The appropriate chemical structure of compound **2** was approved by spectroscopic data, which demonstrated a new peak at 1720 cm⁻¹ because the new oxazine ring and ¹H-NMR revealed a new singlet signal at 2.3 due to CH₃ and another one at 10.7 ppm consistent with the new NH group.

The stability of 2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-*d*][1,3]oxazin-3-yl) acetonitrile **2** tautomers were inspected by using the B3LYP functional (32, 33) within the density functional theory (DFT) (34) level using the 6-311G as a basis set. All geometries were engaged as initial points by AM1 geometry optimizations. These results were re-optimized at Becke's 3-parameter exact exchanges functional (B3) joint with gradient corrected correlation functional of Lee-Yang-Parr (LYP) method (35), Table 2.

The data in table 1 showed that tautomer **2A** is the most stable one while the HOMO-LUMO gaps showed that all tautomers are colorless (Figure 4). ¹H-NMR also helped us to roll out tautomer **2C** due to the existence of two signals equivalent to 2 NH.

The reaction of oxazine derivative **2** with different primary aromatic amines and hydrazine gave new pyrazolo[3,4-*d*]pyrimidines of possible important biological interest since such compounds are substituted analogs of the well-known drug allopurinol (36, 37).

2-(4-imino-6-methyl-1,4-dihydropyrazolo[3,4-*d*][1,3]oxazin-3-yl)acetonitrile **2** was reacted with various amines to yield the pyrazolopyrimidines **4a-c** in two steps. Primarily, the condensation of **2** with amines in toluene containing a catalytic amount of acetic acid formed the intermediate **3** by the nucleophilic attack of the NH₂ group on the oxazine ring. In the second step, the non-isolable pyrimidine

Scheme 1^a

^a) Reagents and conditions: i) EtOH, reflux; ii) AC₂O, reflux 2h.

Table 2. Different tautomeric structures of compound **2** with their energy, HOMO, LUMO, and HOMO-LUMO gaps.

Compd.	Structure	Energy (kJ/mol)	HOMO	LUMO	HOMO-LUMO gap
2A		-1728018.33	-654.09	-151.19	-502.9 (-5.21 eV)
2B		-1728009.95	-662.04	-117.06	-544.98 (-5.65 eV)
2C		-1727965.86	-632.68	-169.47	-463.21 (-4.80 eV)

Figure 4. HOMO and LUMO of the tautomeric structure **2A**.

3 was transformed into the novel pyrazolopyrimidines **4** via Dimroth rearrangement. The transformation of **3** to the thermodynamically stable isomer pyrazolopyrimidines derivative **4** (Scheme 2) seems to be established via acid /base-catalyzed in tandem of ring-opening followed by ring formation. This rearrangement coincided with that stated in some former reports (38, 39).

Again, the stabilities of compound **3** and compound **4** were studied with DFT with the above procedure and the data were compiled in Table 3.

The data showed that compounds **4a-c** were more stable than compounds **3a-c**, which proves the transformation of compound **3** to compound **4** as our study concluded.

The accurate structure of compound **4** was established by the IR spectrum, which showed the evanescence of a peak at 1720 cm^{-1} due to the

transformation of the oxazine ring to pyrimidine one. $^1\text{H-NMR}$ also displayed new aromatic protons at 7.1-7.6 ppm and finally, the mass spectrum manifested a proper ion peak, which is consistent with compound **4**.

It seemed interesting to study the analogous reactions of oxazine derivative **2** with hydrazine hydrate. Treatment of compound **2** with an equivalent amount of hydrazine gave 2-(5-amino-4-imino-6-methyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-3-yl)acetonitrile **5**, which reacted with different aromatic aldehydes to afford 2-(2-aryl,5-methyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine-9-yl)acetonitrile **6** in high yield. To omit the formation of compound **8**, compound **6** was prepared by another single step route by the reaction of oxazine derivative **2** with hydrazides in refluxing dioxane for 28 h.

Table 3. The calculated energy (kJ/mol) for compounds **3** and **4** derivatives.

Compd.	3D Structure	Energy (kJ/mol)
3a		-3489324.13
3b		-2357158.42
3c		-3828577.43
4a		-3489394.47
4b		-2357229.11
4c		-3828651.62

Scheme 2^a

^a) Reagents and conditions: i) Dioxane, AcOH, reflux 4 h.

Successive nucleophilic addition of the NH_2 group of hydrazides to the oxazine ring was observed to produce the intermediates amidopyrazolopyrimidines **9**. The formation of **9** was followed by cyclization via the removal of water to provide pyrazolotriazolopyrimidines **6** (Scheme 3).

The IR spectrum revealed the lack of a distinctive absorption band equivalent to the CO of the oxazine ring. $^1\text{H-NMR}$ exhibited new aromatic signals at 6.9-7.6 ppm and the mass spectrum established another prove for the accurate structure of derivative **6** by providing the correct ion peak.

Scheme 3^a

^a) Reagents and conditions: i) Dioxane, reflux 4 h.; ii) Dioxane, Et_3N , reflux 6 h.; iii) dioxane, reflux 24 h.

Biology results

Two evaluations of biological tests had been accomplished on the synthesized targets: CDK2/CyclinA assay and cytotoxicity assay to estimate the biological profile of the recently synthesized compounds towards CDK2.

In vitro CDK2/cyclin A2 inhibition activity

The established compounds were evaluated as CDK2/cyclinA and the results showed potent percent inhibition as outlined in Table 4 and for comparison, Staurosporine was used as positive controls.

By investigating the percentage inhibition data in Table 4, we found that seven compounds **4a-c** and **6a-d** had competitive percentage inhibitory changes values ranging from 40 to 90. Compounds **4c** and **6b** were found to be the most active derivatives with% inhibition values 90 and 82, respectively. On the other hand, two compounds **4a** and **6a** derivatives showed a low competitive inhibitory effect with percent activity changes values 45 and 40, respectively. The remaining compounds (**4b, c**, and **6a, c, d**) showed a moderate competitive inhibitory effect with percent activity changes values ranging from 66 to 75%.

The biological results showed a good correlation with docking and binding energy calculations. The most active compounds **4c** and **6b** showed the best binding energy calculations due to their good interactions with the CDK2 active site (Figures 3 and 4). Compound **4b**, for example, showed good hydrophobic interactions where its phenyl and its methyl groups were buried well in the active site. Also, it formed 3 hydrogen bonds with Lys20, Asp86, and Glu 81, which is decisive for biological activity.

Cytotoxicity assay

The cytotoxicity assay was done to measure the sensitivity scale and the selectivity of the tested compound against malignant and normal cells. The biological results of the synthesized compounds were measured as IC_{50} against the MCF-7 cancer cell line and 184B5 (non-malignant mammary epithelial cell) the data were given in Table 4. Some of the synthesized compounds displayed higher cytotoxicity against MCF-7 tumor cell lines but lower cytotoxicity against the 184B5 normal cell line. In the cytotoxicity assay with the MCF-7 cell line, the IC_{50} data declared that compound **4c** demonstrated the highest anticancer activity with an IC_{50} value of 0.81 μ M, which is more active than the reference. Since this compound demonstrated the uppermost IC_{50} values so it can be used as a primary hit. Compounds **6b** and **6c** presented very high activity versus the cell line reserved for the study with IC_{50} values 2.07 and 2.33 μ M, respectively. The other compounds demonstrated good anticancer activity concerning MCF-7 cancer cell line with IC_{50} values ranging from 7.39 to 3.55 μ M. Investigation of the anticancer activity of our produced compounds against the MCF-7 cell line revealed that compounds **4c** and **6b** exhibited the highest anticancer activity between the examined compounds expressed by their IC_{50} values 0.81 and 2.07 μ M, respectively related to doxorubicin $IC_{50} = 1.17 \mu$ M. The biological profile of these compounds could be enhanced and used as lead compounds by further optimization.

The results of the antitumor screening and CDK2/CyclinA assay revealed that compound **4c** had a high antitumor activity with IC_{50} 0.81 μ M and a high CDK2 percent inhibition of 90%, which confirmed that its antitumor action could be due to the suppression of CDK2 enzyme.

Table 4. Inhibitory effects of the newly synthesized compounds on CDK2/cyclin A2 Activity, cytotoxic activity against MCF-7 cell-line and non-cancer breast cell lines.

Compound	% Inhibition (on CDK2/cyclinA2) ^a	IC_{50} (μ M) ^b	
		MCF-7	184B5
4a	45	4.75	> 100
4b	66	3.73	> 100
4c	90	0.81	> 100
6a	40	7.39	> 100
6b	82	2.07	> 100
6c	72	2.33	> 100
6d	75	3.55	> 100
Staurosporine	100	---	---
Doxorubicin	---	1.21	---

Structure-activity relationship (SAR)

During the structure-activity relationship studies, we found that compounds containing the sulfonamide group at position 4 (**4a**) were more active against the MCF-7 cell line than those containing chloro (**6b**) and dimethylamino groups (**6c**). It was noted that the sulfonamide group had a remarkable effect on the antiproliferative activity against this cell line because it buried well in the active site and was directed to the solvent-exposed region. Compound **6b** and **6c** with the 2-phenyl triazole moiety that bearing a hydrophobic group showed more potent inhibitory effects than compounds with no substitution or with a hydrophilic group (OH) (**6a**, and **6d**).

Regarding the activity against the % inhibition of CDK2/cyclinA2, a similar structure-activity relationship was observed. Compound **4c** displayed the best inhibitory activity, indicating that the sulfonamide group was beneficial to the inhibitory activity when it was placed in position 4. This conclusion was further proved by compounds **6b** and **6c** which also showed good activities. The reason for the above compounds with potential activity might be a new hydrogen bond formation between the sulfonamide O atom serving as a hydrogen bond receptor and the enzyme serving as a hydrogen bond donor. For compounds **6b** and **6c**, another possible reason was the hydrophobic interaction of 2-phenyl moiety with the binding site.

CONCLUSION

Novel pyrazolopyrimidines were proposed and were selected according to their structural similarity with olomoucine (56% or more), docked into the ligand-binding site of CDK2 and subjected to binding energy calculation. According to the binding energy calculations the stimulated derivatives were chosen for preparation and estimation as inhibitors of CDK2. The obtained pyrazolopyrimidines showed very good inhibition for antitumor and CDK2 in the μM range. Compounds **4c** and **6b** exhibited the highest anticancer activity between the examined compounds expressed by their IC_{50} values 0.81 and 2.07 μM , respectively.

An unpretentious and adequate method for the synthesis of pyrazolo[3,4-d]pyrimidines was reported by cyclization followed by Dimroth rearrangement of oxazine **2**, which derived from 5-amino-3-(cyanomethyl)-1H-pyrazole-4-carbonitrile **1**, in the existence of primary amines. Also, the reaction of compound **2** with hydrazine was studied and gave compound **5**, which condensed with different

aldehydes yielded pyrazolo[4,3-d][1,2,4] triazolo[4,3-c]pyrimidines **6**. The structure of compound **6** was proved chemically by preparing it throughout another route by the reaction of compound **2** with some hydrazides derivatives in refluxing dioxane. Tautomeric structures of compound **2** were studied by DFT and the stability of compounds **4** over compounds **3** was established and approved by quantum mechanics calculations.

Conflicts of interest

The authors declare no conflict of interest.

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