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Original article

# Design, synthesis and biological study of novel pyrido[2,3-d]pyrimidine as anti-proliferative CDK2 inhibitors

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ABSTRACT

The design and synthesis of a small library of 4-aminopyrido[2,3-d]pyrimidine derivatives is reported. The potential activity of these compounds as CDK2/Cyclin A, CDK4/Cyclin D, EGFR and anti-tumor was evaluated by cytotoxicity studies in A431a, SNU638b, HCT116 and inhibition of CDK2-Cyclin A, CDK4/Cyclin D and EGFR enzyme activity in vitro. The anti-proliferative and CDK2-Cyclin A inhibitory activity of compounds 4c and 11a was significantly more active than roscovotine with $IC_{50}$ 0.3 and 0.09 μM respectively. Molecular modeling study, including fitting to a 3D-pharmacophore model, docking into cyclin dependant kinase2 (CDK2) active site and binding energy calculations were carried out and these studies suggested the same binding orientation inside the CDK2 binding pocket for these analogs compared to ATP.



## 1. Introduction

Cyclin-dependent kinases (CDKs) are Ser/Thr protein kinases, which become active when they associate with their respective cyclin subunits. Cyclins are so called because of their characteristic pattern of appearance and disappearance during the cell cycle division [1]. To date, more than 13 CDKs and 25 cyclins have been discovered and their biological functions remain incompletely understood [2]. CDKs are key regulators of the cell cycle [3] and the proper regulation of CDK activity is crucial for the ordered execution of the phases of the cycle. A large number of human neoplasias show overexpression of positive regulators of CDKs and/or decrease in negative regulators [4]. Abnormal expression of CDK2/cyclin E has been detected in colorectal, ovarian, breast, and prostate cancers [5]. CDK inhibitors have been shown to induce apoptosis in different tumor cell lines [6]. Therefore, CDK inhibitors have the potential to enlarge the group of anti-cancer agents and a number of more or less selective CDK inhibitors have been described in the literature [7].

Pyrido[2,3-d]pyrimidine derivatives are of great interest due to their anti-tumor [8], antifolate [9], antibacterial [10], growth regulator [11], etc. The therapeutic importance of this nucleus is enthused us to develop selective procedures for synthesis in which substituents could be arranged in a pharmacophoric pattern to display high order pharmacological activities.

In continuation with our efforts in search of potential anti-proliferative CDK2 inhibitors [12–14], we carried out the chemical modification by the introduction of pyridine ring instead of imidazole ring on purine to give a specific binding mode as ATP or olomoucine analogs [15–18]. To this end, we designed and synthesized 2,5,7-trisubstituted pyrido[2,3-d]pyrimidines by introducing a variety of substituent groups at C-2, C-5 and C-7 of pyridopyrimidine ring for a potent and selective inhibitor of the CDK2 by competitive binding at the ATP binding site and evaluated for their CDK2, EGFR inhibitory activities and cell growth inhibitory activities.

## 2. Rationale and design

### 2.1. Structure preparation

The coordinate for the protein structure was obtained from the RCSB Protein Data Bank (PDB ID: 1AQ1). Protein Structure was prepared using Schrodinger Suite 2009 software package [19]. The invalid or missing residues were added and the structures were aligned using the protein structure alignment module. Hydrogen atoms were added and the structure was minimized to relax the

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backbone and to remove the clashes. The protein was inspected visually for accuracy in the X2 dihedral angle of Asn and His residues and the X3 angle of Gln, and rotated by 180° when needed to maximize hydrogen bonding. The proper His tautomer was also manually selected to maximize hydrogen bonding. The proposed compounds were optimized by semiempirical method (AM1) using Chem3D to eliminate bond length and bond angle biases and saved to be used in the pharmacophore mapping, which carried out by Accelrys DS 2.0 [20]. Finally, the proposed compounds with high fit-values were selected for docking and binding energy calculations, which carried out by Schrodinger Suite 2009.

### 2.2. Pharmacophore generation

Analysis of the many available crystal structures of kinase/inhibitor complexes reveals the existence of conserved interactions that appear to be determinant for the recognition of small heterocyclic molecules by the ATP (cofactor) binding site of this class of enzymes [21]. Prominent among these are hydrogen bond interactions that inhibitors make with the backbone of the amino acid stretch that connects the kinase N- and C-terminal domains, the so called 'hinge' loop. In particular, for CDK2, at least one hydrogen bond with hinge residue Leu 83 is observed in all reported crystal structures [22]. These hydrogen bonds position the inhibitors in an orientation that allow them to make multiple favorable contacts with the side chains of hydrophobic residues that normally form the environment of the adenine ring of ATP in the pocket. In the case of CDK2, these residues include Ile 10, Ala 31, Val 64, Phe 80, Leu 134 and Ala 144.

Our strategy to find new CDK2 inhibitors is based on the design of molecular scaffolds targeting ATP interactions so we used an X-ray crystal structure of CDK2 (PDB ID: 1HCK) to design pyrido [2,3-d]pyrimidine ligand scaffold. On the basis of the structural information from Adenosine Triphosphate (ATP), a set of features crucial for selectivity as CDK2 were considered to represent a pharmacophore hypotheses. The pharmacophore is expected to identify the common binding features and the hypothetical orientation of the active compounds interacting with CDK2 enzyme. This model was made up of a hydrophobic center, three Hydrogen bond acceptors (HBA) and three hydrogen bond donors associated to its protein acceptor site and acceptor atoms. HBD1 and HBA1 represent the bi-dentate interaction of adenine with GLU81A and LEU83A respectively and the hydrophobic center represents the aromatic region of ATP moiety. HBD2 and HBD3 represent the hydrogen bonds interaction of the 2 hydroxyl groups of sugar with GLN131A and ASP86A. Finally, HBA2 and HBA3 represent the hydrogen bond interactions of phosphate with TYR15A and LYS129A (Fig. 1).

### 2.3. Sterically refined pharmacophore

Although ligand-based pharmacophores serve as an excellent tools to probe ligand/macromolecule recognition and can serve as a useful 3D-QSAR models and 3D search queries, they suffer from a major drawback: They lack steric constrains necessary to define the size of the binding pocket. Therefore, we decided to complement our selected pharmacophore with exclusion spheres. Excluded volumes resemble sterically inaccessible regions within the binding site. All heavy atoms within 3 °A of the binding ligand (ATP) with CDK2 (PDB ID: 1HCK) were defined as excluded volumes. Fig. 2a, b shows the sterically-refined versions of our pharmacophore (34 added exclusion volumes) and how it maps one of the potent hits (11a). Finally, the proposed compounds with high fit-values were selected for docking step (Table 1).

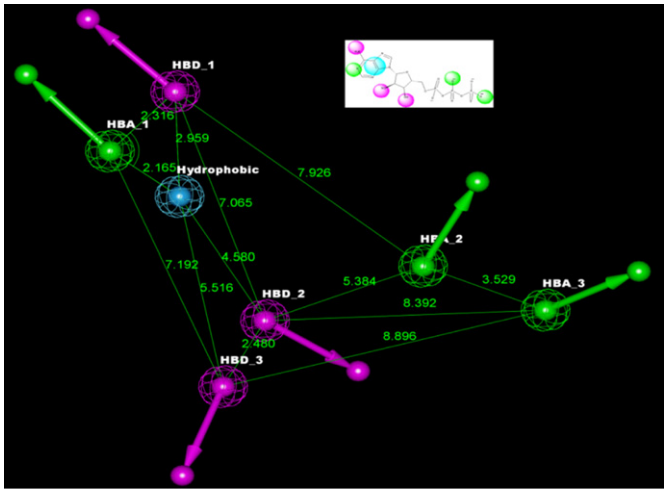



**Fig. 1.** Pharmacophore model, which derived from ATP and used in the pre-selection of the proposed compounds (Magenta HBD, Green HBA and Cyan hydrophobe). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

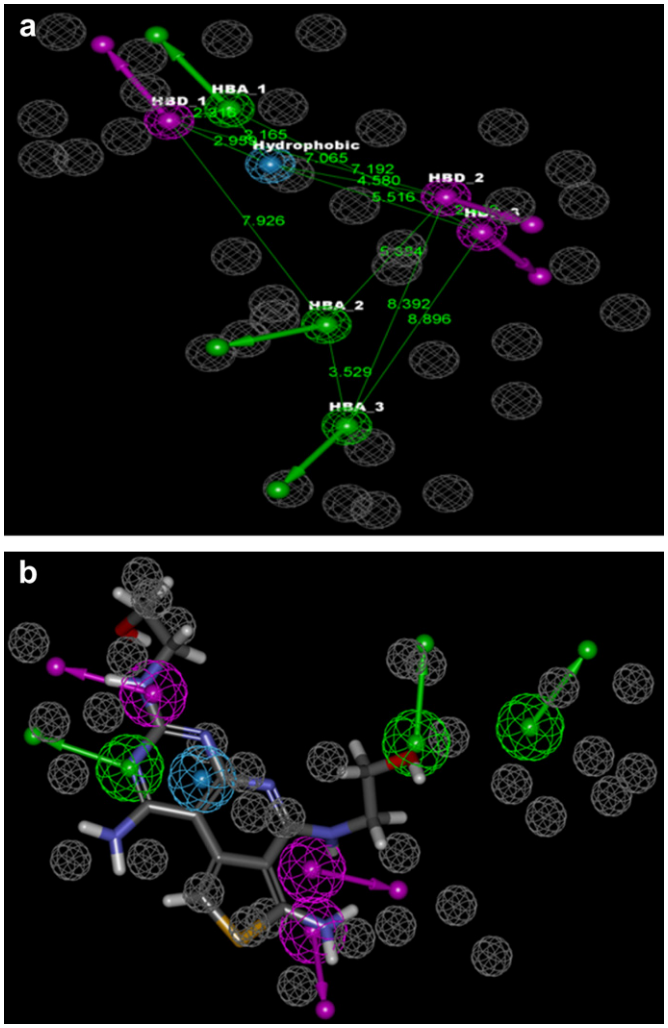
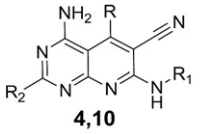
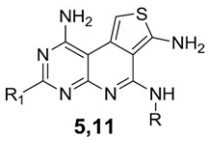



**Fig. 2.** a. Sterically-refined versions of our pharmacophore with 34 added exclusion volumes. b. Mapping of compound **11a** to the generated pharmacophore (FitValue = 3.31).

**Table 1**
Fit-Values, Docking Scores and Binding energies for the promising compounds.




| Compound | *R* | $R_1$ | $R_2$ | Fit-value | Docking Score (kcal/mol) | Binding Energy MMGBSA |
|---|---|---|---|---|---|---|
| **4a** | H | $C_2H_4OH$ | H | 3.00077 | −8.47 | −34.42 |
| **4b** | $CH_3$ | $C_2H_4OH$ | H | 3.03139 | −8.85 | −45.25 |
| **4c** | H | 4-$H_2NO_2SC_6H_4$ | H | 2.99167 | −9.59 | −83.4 |
| **4d** | $CH_3$ | 4-$H_2NO_2SC_6H_4$ | H | 2.96439 | −10.44 | −77.66 |
| **10a** | H | $C_2H_4OH$ | $HNC_2H_4OH$ | 3.13155 | −9.73 | −33.76 |
| **10b** | $CH_3$ | $C_2H_4OH$ | $HNC_2H_4OH$ | 3.28324 | −11.01 | −56.65 |
| **10c** | H | 4-$H_2NO_2SC_6H_4$ | $HNC_2H_4OH$ | 3.21189 | −12.9 | −47.72 |
| **10d** | $CH_3$ | 4-$H_2NO_2SC_6H_4$ | $HNC_2H_4OH$ | 3.1705 | −11.94 | −63.91 |
| **5a** | $C_2H_4OH$ | H | – | 2.86449 | −8.88 | −32.57 |
| **5b** | 4-$H_2NO_2SC_6H_4$ | H | – | 3.13155 | −9.73 | −33.76 |
| **11a** | $C_2H_4OH$ | $HNC_2H_4OH$ | – | 3.30766 | −10.26 | −185.32 |
| **11b** | 4-$H_2NO_2SC_6H_4$ | $HNC_2H_4OH$ | – | 3.62516 | −6.17 | −49.38 |

### *2.4. Docking and binding energy calculations*

However, since we were interested in finding CDK2 inhibitors representing novel chemotypes or at least chemotypes free of intellectual property (IP) constrains, we opted to keep our docking strategy unbiased. Grids for molecular docking with Glide [23] were calculated with hydrogen bond constraints to a backbone Glu 81 and LEU83 in the hinge region of CDK2. The newly proposed compounds which selected from mapping to the pharmacophore model were docked using Glide in extra-precision mode, with up to five poses saved per molecule. The docked poses were then minimized using the local optimization feature [24] in Prime, and the energies were calculated using the OPLS-AA force field [25] and the GBSA continuum model [26] in Maestro. We considered a binding mode to be reasonable if the key hydrogen bond was formed in any of the top five solutions (Fig. 3). Proposed compounds with the best score were selected for synthesis and for biological evaluation see Table 1.

## 3. Chemistry

The synthetic approach to obtain thieno[3′,4′:4,5]pyrido[2,3-d] pyrimidine derivatives 5 and 11 was shown in Scheme 1,2. 2-Amino-6-mercaptopyridin-3,5-dicarbonitrile derivatives 1a,b was prepared by reaction of 2-(ethoxymethylene)malononitrile or 2-(1-ethoxyethylidene)malononitrile with cyanothio-acetamide in sodium ethoxide solution under reflux. Cyclization of aminopyridine carbonitrile 1a,b was achieved by heating in formamide to afford pyrido[2,3-d] pyrimidine derivatives 2a,b which was alkylated withy methyl iodide in sodium hydroxide solution to afford thioether derivatives 3a,b. Amination of thioether derivatives 3a,b was achieved by refluxing with excess amine, which in turn reacted with sulfur under basic conditions to afford thieno[3′,4′:4,5]pyrido[2,3-d]pyrimidine derivatives 5a,b.

In attempt to use the hydrophobic channel, which was not occupied by ATP, we prepared compounds of type 10, 11 as depicted in Scheme 2. 2-Amino-6-mercaptopyridin-3,5-dicarbonitrile 1a,b was alkylated with methyl iodide to give thioether 6a,b, which reacted with different amines to afford amino derivatives 7. Triamino pyrido [2,3-d]pyrimidine derivatives 10 were obtained by heating amino derivatives 7 with excess thiourea, alkylation and amination in the same manner. Finally, thieno[3′,4′:4,5]pyrido[2,3-d]pyrimidine derivatives 11a,b were prepared by reaction of triamino derivatives 10 with sulfur under basic conditions.

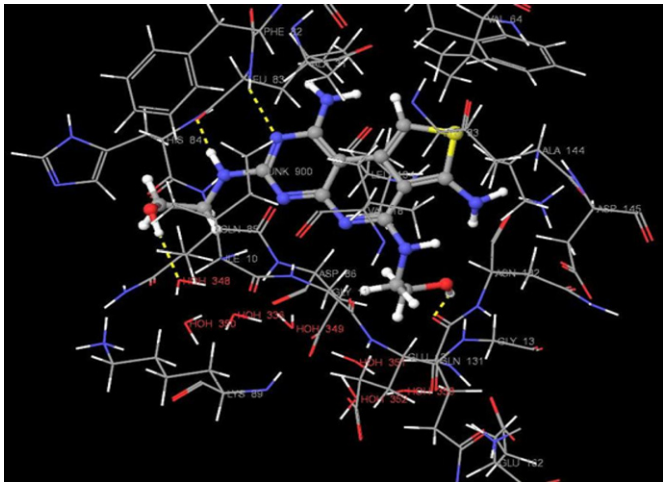
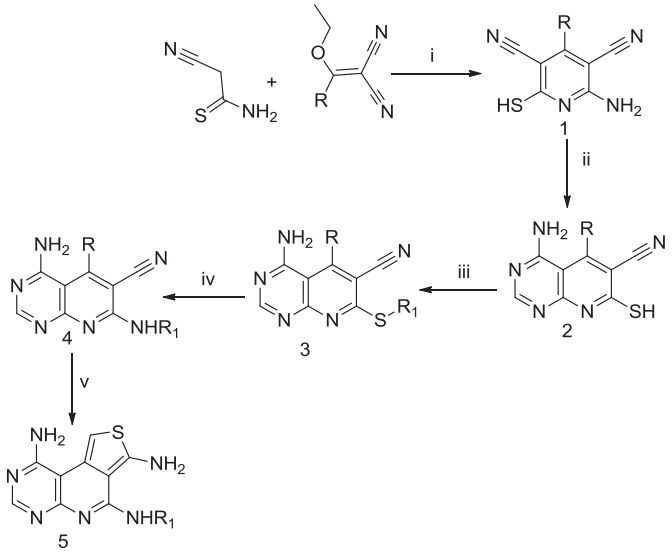
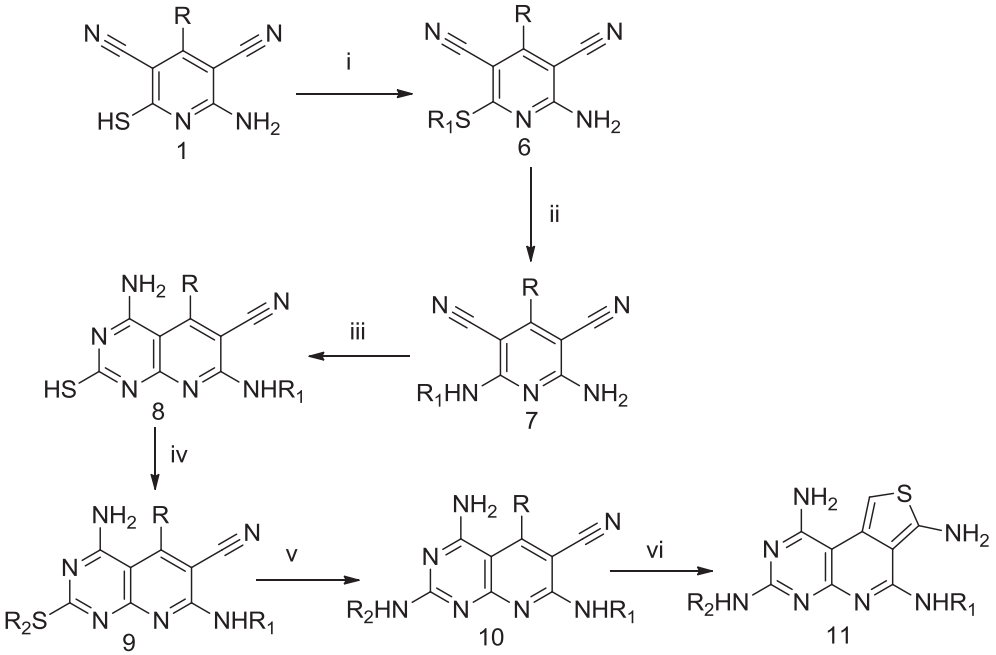


**Fig. 3.** Docking of compound **11a** into ATP binding site and its hydrogen bond interactions.




**Scheme 1.** Reagent and conditions: i) NaOEt, EtOH, Reflux, ii) $HCONH_2$, reflux, iii) RX, NaOH, iv) $R_1NH_2$, reflux, v) S, Morpholine, DMF.



**Scheme 2.** Reagent and conditions: i) RX, NaOH, ii) $R_1NH_2$, Reflux, iii) Thiourea, 180–200 °C, iv) RX, NaOH, v) $R_2NH_2$, reflux, vi) S, Morpholine, DMF.

## 4. Biology

CDK2, CDK4 and EGFR inhibitory activities of pyrido[2,3-d] pyrimidines, which prepared above were shown in Table 2, together with those of olomoucine, roscovitine and PYK2104 as reference compounds. Compounds were further evaluated for their cell division inhibitory activities together with doxorubicin against three human tumor cell lines. These tumor cells divided at least twice for 2-day period of the cell division inhibition test in the absence of inhibitory compounds and the kinase activity of CDK2/cyclin A and CDK4/Cyclin D are indispensable for the division of these cells. A measure of the inhibitory activity of compounds to its intended enzyme target is an important parameter for effective drug design. Therefore, pyrido[2,3-d]pyrimidines 4, 5, 10, 11 were tested for their CDK2, CDK4, EGFR and anti-proliferative properties against A431, SNU638, HCT116 and the data revealed that compounds 4c and 11a were more active than roscovotine with $IC_{50}$ 0.3 and 0.09 μM respectively. Compounds 4c, 5a, 10c, 10b and 10d were more active than olomoucine but less than roscovotine with $IC_{50}$, 0.3, 0.5, 0.9, 3.8 and 3.1 μM respectively. All other compounds had good to moderate activity against CDK2. On the other hand some of the tested compounds (4b–d, 5a and 10a,b) had very mild activities against CDK4 ranging from 10 to 36 μM and the rest of compounds had no significant activity. Finally, all the tested compounds had no significant activity against EGFR, which prove the selectivity of these compounds to CDK2. The effect of synthesized compounds on growth of A431, SNU638 and HCT116 cells were measured and showed good anti-proliferation activity. Compound 11a had the best activity with $GI_{50}$ 4.6, 2.57 and 11.4 μM on A431, SNU638 and HCT116 respectively. Compounds 4b, 4c, 4d and 5b showed strong activity with $IG_{50}$ ranging from 2.56 to 19.28 μM. The rest of tested compounds showed good to moderate inhibition. Compounds 10 and 11, which had substitutions at C2 were more active than the corresponding ones 4 and 5. This is prove the importance of the hydrophobic channel for activity and selectivity. All the biological data showed a good correlation between designing results, CDK2 and anti-proliferation activities.

**Table 2**
CDK2, CDK4, EGFR and cell division inhibitory activities of pyrido[2,3-d]pyrimidine derivatives.

| Compound | $IC_{50}$ (μM) | | | $GI_{50}$ (μM) | | |
|---|---|---|---|---|---|---|
| | CDK4/D | CDK2/A | EGFR | A431[a] | SNU638[b] | HCT116[c] |
| **4a** | >200 | 29.5 | 93.3 | 26.56 | 20.77 | 4.50 |
| **4b** | 36 | 29.5 | >100 | 5.06 | 2.58 | 4.10 |
| **4c** | 13 | 0.3 | >100 | 7.8 | 7.57 | 19.28 |
| **4d** | 16 | 10 | >100 | 2.94 | 6.20 | 16.61 |
| **5a** | 29 | 0.5 | >100 | 54.3 | 18.3 | 6.46 |
| **5b** | >120 | 36.3 | 29.5 | 3.94 | 2.56 | 8.89 |
| **10a** | 28 | 6.3 | >100 | 6.25 | 7.95 | 10.88 |
| **10b** | 10 | 3.8 | 47.9 | 31.86 | 18.16 | 20.07 |
| **10c** | 120 | 0.9 | >100 | 36.29 | 35.84 | 48.24 |
| **10d** | 95 | 3.1 | >100 | >100 | 17.07 | 38.19 |
| **11a** | >48 | 0.09 | 66.1 | 4.6 | 2.57 | 11.4 |
| **11b** | 44 | 8.1 | >100 | 21.88 | 26.11 | 47.68 |
| Olomoucine | 45 | 7.0 | – | 16.60 | 16.62 | 12.05 |
| Roscovotine | 15 | 0.5 | – | – | – | – |
| PYK2104 | | – | 0.0008 | – | – | – |
| Doxorubicine | | – | – | 0.18 | – | 0.13 |

[a] A431, human vulvar epidermoid carcinoma cell line.
[b] SNU638, human stomach epitherial carcinoma cell line.
[c] HCT116, human colon epitherial carcinoma cell line.

## 5. Structural activity relationship (SAR)

The structural activity relationship (SAR) of newly synthesized compounds 4a–d, 5a,b, 10a–d and 11a,b based on the observed results explored the importance of the planer bicyclic 4-aminopyrido[2,3-d]pyrimidine system in cycline-dependent kinase2 CDK2/cyclin A inhibition activity and to certain extent in the in vitro anti-tumor activity. The type of substituent attached at either position 2- or 7- is a controlling factor governing the total observed pharmacological properties. In most cases, the presence of a lipophilic function (LF) such as benzene sulfonamide group at the 7-amino group of a tested compound enhanced significantly the observed CDK2 inhibitory activity as exhibited in compounds 4c and 10c ($IC_{50}$ = 0.3 and 0.9 μM, respectively). Additionally, the fused thienyl function at position 5, 6 with tested analogs of pyrido

[2,3-d]pyrimidine gave enhanced cycline-dependent kinase2 (CDK2) inhibitory activity as shown in compounds 5a and 11a ($IC_{50}$ = 0.5 and 0.09 μM, respectively), together with appreciated anti-tumor activity against SNU638 HCT116 cell line. Furthermore, small (LF) with electron donating group (EDG) on position 2 of pyrido[2,3-d]pyrimidine moiety for Compounds 10a–d increase CDK2 inhibitory activity.

## 6. Conclusions

Using crystallographic data from the ATP/CDK2 complex, we rationally designed and synthesized a series of 4-aminopyrido[2,3-d] pyrimidine derivatives as potent CDK2 inhibitors. The results suggest that the nature of the side chain at position 2- and 7- plays an important role in the inhibitory activity against CDK2 and anti-proliferative. Compounds prepared in this study were potent inhibitors of CDK2 and there is a good correlation between the biological results and the molecular modeling studies. From this investigation we gained a better understanding of the structural requirements and limitations necessary for the preparation of selective CDK2 inhibitors. Compound 11a serves as a lead analog for rationale designed CDK2 inhibitory activity combined with anti-cancer inhibitory activity. Efforts are currently underway to improve the CDK2 inhibitory activity using 11a as the lead analog.

## 7. Experimental

### 7.1. General methods

Unless otherwise noted all reagents and solvents were used as supplied commercially (Merck, Sigma Aldrich, Fluka, Lancaster). All reactions were performed in oven-dried glassware. Melting points were determined on Electro-thermal capillary melting point apparatus and were uncorrected. $^1$H NMR spectra were obtained on a Bruker DRX- 400 NMR (400 MHz) spectrometer. Chemical shifts are expressed in ppm (δ) downfield from internal tetramethylsilane. Low-resolution mass spectra were determined on a Perkin–Elmer-SCIEX API 165 mass spectrometer using ES ionization modes (positive or negative).

#### 7.1.1. 2-amino-6-mercaptopyridine-3,5-dicarbonitrile derivatives **1a**,**b**

Cyanothioacetamide (22.3 mmol) was added to a solution of sodium ethoxide, prepared from Na (570 mg) in absolute ethyl alcohol (50 mL). To the resulting solution 2-(ethoxymethylene) malononitrile or 2-(1-ethoxyethylidene)malononitrile (23 mmol) was added and the reaction mixture was stirred for 30 min at room temperature then was heated at 90 °C for 4 h. The excess solvent was removed under reduced pressure then the resulting solid was triturated with cold water (100 mL), acidified with acetic acid and filtered off to give the desired compound (1), which purified by crystallization from alcohol.

*7.1.1.1. 2-amino-6-mercaptopyridine-3,5-dicarbonitrile* ***1a***. Yield 88%, mp 140–143 °C. IR: max/cm$^{-1}$ 3246, 3198, 2222, 2190, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.31 (s, 2H, $NH_2$), 8.19 (s, 1H, H4), 12.53 (s, 1H, SH or NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 94.1, 107.6, 115.8, 115.2, 145.6, 160.1, 180.2. MS (EI+): *m*/*z*: 176. Anal. Calcd for $C_7H_4N_4S$: C, 47.72; H, 2.29; N, 31.80; Found: C, 47. 3; H, 2.1; N, 31.3.

*7.1.1.2. 2-amino-6-mercapto-4-methylpyridine-3,5-dicarbonitrile* ***1b***. Yield 92%, mp 120–123 °C. IR: max/cm$^{-1}$ 3246, 3198, 2222, 2190, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.61 (s, 3H, $CH_3$), 7.51 (s, 2H, $NH_2$), 12.13 (s, 1H, SH or NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 19.5, 84.1, 85.6, 116.2, 117.3, 152.6, 161.1, 175.2. MS (EI+): *m*/*z*: 191 ($M^{+1}$). Anal. Calcd for $C_8H_6N_4S$: C, 50.51; H, 3.18; N, 29.45; Found: C, 49.8; H, 3.1; N, 28.8.

#### 7.1.2. 4-amino-7-mercaptopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives **2a**,**b**

A mixture of 2-amino-6-mercaptopyridine-3,5-dicarbonitrile derivatives 1a,b (0.01 mol), formamide (15 mL) and formic acid (2 mL) was heated under reflux for 8 h. The reaction mixture was allowed to stand overnight at R.T. The solid thus obtained was filtered, washed with cold ethyl alcohol, dried and crystallized from DMF-alcohol.

*7.1.2.1. 4-amino-7-mercaptopyrido[2,3-d]pyrimidine-6-carbonitrile* ***2a***. Yield 76%, mp 180–183 °C. IR: max/cm$^{-1}$ 3246, 3198, 2220, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.71 (s, 2H, $NH_2$), 8.1 (s, 1H, H7), 8.3 (s, 1H, H4), 11.8 (s, 1H, SH or NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 109.5, 111.7, 116.2, 132.3, 156.6, 163.1, 165.8, 173.2. MS (EI+): *m*/*z*: 203. Anal. Calcd for $C_8H_5N_5S$: C, 47.28; H, 2.48; N, 34.46; Found: C, 46.8; H, 3.1; N, 34.8.

*7.1.2.2. 4-amino-7-mercapto-5-methylpyrido[2,3-d]pyrimidine-6-carbonitrile* ***2b***. Yield 79%, mp 160–163 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 7.41 (s, 2H, $NH_2$), 7.9 (s, 1H, H7), 12.3 (s, 1H, SH or NH). MS (EI+): *m*/*z*: 217. Anal. Calcd for $C_9H_7N_5S$: C, 49.76; H, 3.25; N, 32.24; Found: C, 49.8; H, 3.1; N, 32.8.

#### 7.1.3. 4-amino-7-(methylthio)pyrido[2,3-d]pyrimidine-6-carbonitrile derivatives **3a**,**b**

4-amino-7-mercaptopyrido[2,3-d]pyrimidine-6-carbonitrile 2a,b (0.033 mol) was dissolved in NaOH (0.833 N, 65 mL). To this solution, methyl iodide (5.0 g) was added and the mixture was shaken at 20 °C for 45 min. The solution was treated with charcoal, filtered and the filtrate was acidified with acetic acid. The crude precipitate was filtered off and suspended in water (100 mL) and the PH was adjusted to 9 with ammonium hydroxide. The ammonical solution was filtered and the collected precipitate washed and recrystallized from dioxane to afford compound 3.

*7.1.3.1. 4-amino-7-(methylthio)pyrido[2,3-d]pyrimidine-6-carbonitrile* ***3a***. Yield 96%, mp 150–153 °C. IR: max/cm$^{-1}$ 3296, 3188, 2219, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.6 (s, 3H, $SCH_3$), 7.31 (s, 2H, $NH_2$), 7.8 (s, 1H, H4), 8.1 (s, 1H, H7). MS (EI+): *m*/*z*: 217. Anal. Calcd for $C_9H_7N_5S$: C, 49.76; H, 3.25; N, 32.24; Found: C, 49.4; H, 3.2; N, 31.9.

*7.1.3.2. 4-amino-5-methyl-7-(methylthio)pyrido[2,3-d]pyrimidine-6-carbonitrile* ***3b***. Yield 92%, mp 140–142 °C. IR: max/cm$^{-1}$ 3290, 3190, 2222, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.1 (s, 3H, $CH_3$), 2.6 (s, 3H, $SCH_3$), 7.61 (s, 2H, $NH_2$), 8.0 (s, 1H, H7). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 13.3, 19.5, 110.2, 115.7, 117.2, 144.3, 155.6, 159.1, 162.8, 166.3. MS (EI+): *m*/*z*: 231. Anal. Calcd for $C_{10}H_9N_5S$: C, 51.93; H, 3.92; N, 30.28; Found: C, 51.4; H, 3.6; N, 30.7.

#### 7.1.4. 4-amino-7-aminopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives **4a**–**d**

To a mixture of compound 3a,b (5 mmol) in dioxane (100 mL), the appropriate amine (8 mmol) was added and the reaction mixture was refluxed for 12 h. The excess solvent was removed under reduced pressure and the residual product was triturated with alcohol. The precipitate was collected by filtration and washed well with alcohol to give compound 4.

*7.1.4.1. 4-amino-7-((2-hydroxyethyl)amino)pyrido[2,3-d]pyrimidine-6-carbonitrile* ***4a***. Yield 66%, mp 170–173 °C. IR: max/cm$^{-1}$ 3360, 3196, 3120, 2215, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-

$d_6$): δ 3.2–3.3 (t, 2H, $CH_2$), 3.4–3.5 (t, 2H, $CH_2$), 3.9 (s, 1H, OH), 5.9 (s, 1H, NH), 7.6 (s, 2H, $NH_2$), 7.9 (s, 1H, H4), 8.2 (s, 1H, H7). MS (EI+): *m*/*z*: 230. Anal. Calcd for $C_{10}H_{10}N_6O$: C, 52.17; H, 4.38; N, 36.50; Found: C, 52.4; H, 4.2; N, 35.9.

*7.1.4.2. 4-amino-7-((2-hydroxyethyl)amino)-5-methylpyrido[2,3-d] pyrimidine-6-carbonitrile* ***4b***. Yield 70%, mp 180–183 °C. IR: max/cm$^{-1}$ 3450, 3236, 3140, 2222, 1240, 1215, 1180, 1109. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.6 (s, 3H, $CH_3$), 3.1–3.2 (t, 2H, $CH_2$), 3.5–3.6 (t, 2H, $CH_2$), 4.9 (s, 1H, OH), 5.8 (s, 1H, NH), 7.5 (s, 2H, $NH_2$), 8.1 (s, 1H, H7). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 18.6, 45.5, 61.1, 86.9, 106.7, 116.7, 150.2, 151.3, 157.6, 159.4, 165.3. MS (EI+): *m*/*z*: 244. Anal. Calcd for $C_{11}H_{12}N_6O$: C, 54.09; H, 4.95; N, 34.41; Found: C, 53.9; H, 4.5; N, 34.9.

*7.1.4.3. 4-((4-amino-6-cyanopyrido[2,3-d]pyrimidin-7-yl)amino) benzenesulfonamide* ***4c***. Yield 61%, mp 210–213 °C. IR: max/cm$^{-1}$ 3340, 3256, 3140, 2220, 1250, 1219, 1165, 1106. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 4.9 (s, 2H, $NH_2$), 7.2–7.5 (m, 4H, Ar–H), 7.9 (s, 2H, $NH_2$), 8.0 (s, 1H, H4), 8.3 (s, 1H, H7), 11.6 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 106.5, 110.4, 115.3, 117.9, 135.1, 136.6, 147.1, 151.2, 153.3, 156.4, 158.6, 161.5. MS (EI+): *m*/*z*: 341. Anal. Calcd for $C_{14}H_{11}N_7O_2S$: C, 49.26; H, 3.25; N, 28.72; Found: C, 48.9; H, 3.2; N, 28.5.

*7.1.4.4. 4-((4-amino-6-cyano-5-methylpyrido[2,3-d]pyrimidin-7-yl) amino)benzenesulfonamide* ***4d***. Yield 63%, mp 220–223 °C. IR: max/cm$^{-1}$ 3420, 3280, 3190, 2222, 1260, 1229, 1155, 1105. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 4.5 (s, 2H, $NH_2$), 7.1–7.4 (m, 4H, Ar–H), 7.7 (s, 2H, $NH_2$), 7.9 (s, 1H, H4), 8.1 (s, 1H, H7), 12.1 (s, 1H, NH). MS (EI+): *m*/*z*: 355. Anal. Calcd for $C_{15}H_{13}N_7O_2S$: C, 50.70; H, 3.69; N, 27.59; Found: C, 50.9; H, 3.4; N, 27.5.

*7.1.5. 1,7-diaminothieno[3′,4′:4,5]pyrido[2,3-d]pyrimidin-6-yl) amino derivatives* ***5a***,***b***

A mixture of 4-amino-5-methylpyrido[2,3-d]pyrimidine-6-carbonitrile derivatives 4b,d (0.01 mol) and sulfur (0.35 g, 0.01 mol) in 20 mL DMF in the presence of morpholine (3–4 drops) as catalyst was heated under reflux for 3 h. The reaction mixture was cooled to room temperature and diluted with cold water. The separated solid was filtered, washed with cold ethanol and dried. The crude product was recrystallized from DMF-ethanol to give pure 5.

*7.1.5.1. 2-((1,7-diaminothieno[3′,4′:4,5]pyrido[2,3-d]pyrimidin-6-yl) amino)ethanol* ***5a***. Yield 80%, mp 210–213 °C. IR: max/cm$^{-1}$ 3350, 3236, 3140, 2218, 1240, 1215, 1180, 1109. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.2–3.3 (t, 2H, $CH_2$), 3.4–3.5 (t, 2H, $CH_2$), 3.7 (s, 1H, OH), 6.6 (s, 1H, CH), 7.1 (s, 2H, $NH_2$), 7.6 (s, 1H, $NH_2$), 8.3 (s, 1H, H7), 11.2 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 44.5, 60.8, 86.9, 109.7, 121.7, 129.2, 140.3, 150.6, 155.9, 158.4, 160.3. MS (EI+): *m*/*z*: 276. Anal. Calcd for $C_{11}H_{12}N_6OS$: C, 47.81; H, 4.38; N, 30.41; Found: C, 47.9; H, 4.5; N, 30.3.

*7.1.5.2. 4-((1,7-diaminothieno[3′,4′:4,5]pyrido[2,3-d]pyrimidin-6-yl) amino)benzenesulfonamide* ***5b***. Yield 84%, mp 230–233 °C. IR: max/cm$^{-1}$ 3250, 3236, 3140, 2220, 1250, 1220, 1140, 1104. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 5.3 (s, 2H, $NH_2$), 6.5 (s, 1H, CH), 6.9 (s, 2H, $NH_2$), 7.1–7.3 (m, 4H, Ar–H), 7.8 (s, 1H, $NH_2$), 8.3 (s, 1H, H7), 12.2 (s, 1H, NH). MS (EI+): *m*/*z*: 387. Anal. Calcd for $C_{15}H_{13}N_7O_2S_2$: C, 46.50; H, 3.38; N, 25.31; Found: C, 46.9; H, 3.5; N, 25.3.

*7.1.6. 2-amino-6-(methylthio)pyridine-3,5-dicarbonitrile derivatives* ***6a***,***b***

Was prepared from **1a**,**b** with the same manner as compound **3**.

*7.1.6.1. 2-amino-6-(methylthio)pyridine-3,5-dicarbonitrile* ***6a***. Yield 95%, mp 125–128 °C. IR: max/cm$^{-1}$ 3230, 3180, 2220, 2202, 1246, 1217, 1175, 1103. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.4 (s, 3H, $SCH_3$), 7.9 (s, 2H, $NH_2$), 8.1 (s, 1H, H4). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 15.1, 98.1, 107.9, 117.8, 119.2, 148.6, 166.1, 168.2. MS (EI+): *m*/*z*: 190. Anal. Calcd for $C_8H_6N_4S$: C, 50.51; H, 3.18; N, 29.45; Found: C, 50.3; H, 3.1; N, 29.3.

*7.1.6.2. 2-amino-4-methyl-6-(methylthio)pyridine-3,5-dicarbonitrile* ***6b***. Yield 95%, mp 125–128 °C. IR: max/cm$^{-1}$ 3250, 3140, 2218, 2214, 1226, 1227, 1155, 1113. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 2.6 (s, 3H, $SCH_3$), 7.6 (s, 2H, $NH_2$). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 14.1, 20.1, 86.1, 102.9, 114.8, 118.2, 150.6, 161.1, 170.2. MS (EI+): *m*/*z*: 204. Anal. Calcd for $C_9H_8N_4S$: C, 52.92; H, 3.95; N, 27.43; Found: C, 52.3; H, 3.7; N, 27.3.

*7.1.7. 2-amino-6-((2-hydroxyethyl)amino)pyridine-3,5-dicarbonitrile derivatives* ***7a***,***b***

Was prepared from **6a**,**b** with the same manner as compound **4**.

*7.1.7.1. 2-amino-6-((2-hydroxyethyl)amino)pyridine-3,5-dicarbonitrile* ***7a***. Yield 55%, mp 145–148 °C. IR: max/cm$^{-1}$ 3450, 3350, 3210, 3150, 2219, 2215, 1236, 1227, 1165, 1113. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.1–3.2 (t, 2H, $CH_2$), 3.3–3.4 (t, 2H, $CH_2$), 3.6 (s, 1H, OH), 7.5 (s, 2H, $NH_2$), 8.0 (s, 1H, H4), 11.6 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 40.5, 60.6, 100.1, 115.9, 148.6, 162.1, 165.2. MS (EI+): *m*/*z*: 203. Anal. Calcd for $C_9H_9N_5O$: C, 53.20; H, 4.46; N, 34.47; Found: C, 53.3; H, 4.2; N, 34.3.

*7.1.7.2. 2-amino-6-((2-hydroxyethyl)amino)-4-methylpyridine-3,5-dicarbonitrile* ***7b***. Yield 62%, mp 151–153 °C. IR: max/cm$^{-1}$ 3430, 3310, 3190, 3110, 2220, 2216, 1256, 1217, 1175, 1106. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.1 (s, 3H, $CH_3$), 3.3–3.4 (t, 2H, $CH_2$), 3.5–3.6 (t, 2H, $CH_2$), 3.7 (s, 1H, OH), 7.9 (s, 2H, $NH_2$), 12.2 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 19.1, 43.5, 63.6, 80.1, 114.9, 155.6, 167.1, 170.2. MS (EI+): *m*/*z*: 217. Anal. Calcd for $C_{10}H_{11}N_5O$: C, 55.29; H, 5.10; N, 32.24; Found: C, 55.3; H, 5.2; N, 32.3.

*7.1.8. 4-amino-7-((2-hydroxyethyl)amino)-2-mercaptopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives* ***8a***,***b***

A mixture of Compound 7a,b (30 mmol) and thiourea (90 mmol, 7 g) was heated at 180 °C for 30 min until the clear solution became mushy. Heating was then continued at 200 °C for an additional 10 min. The cooled melt was dissolved in hot dilute sodium hydroxide. The hot solution was treated with charcoal and filtered and the boiling filtrate was carefully acidified with glacial acetic acid. The product was filtered from hot solution to yield light tan material.

*7.1.8.1. 4-amino-7-((2-hydroxyethyl)amino)-2-mercaptopyrido[2,3-d]pyrimidine-6-carbonitrile* ***8a***. Yield 75%, mp 245–248 °C. IR: max/cm$^{-1}$ 3450, 3320, 3220, 3120, 2229, 1256, 1237, 1175, 1105. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.0–3.1 (t, 2H, $CH_2$), 3.3–3.4 (t, 2H, $CH_2$), 3.5 (s, 1H, OH), 7.8 (s, 2H, $NH_2$), 8.1 (s, 1H, H4), 11.3 (s, 1H, NH), 11.9 (s, 1H, SH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 44.5, 63.6, 102.1, 111.9, 118.6, 140.6, 155.4, 160.1, 163.2, 175.4. MS (EI+): *m*/*z*: 262. Anal. Calcd for $C_{10}H_{10}N_6OS$: C, 45.79; H, 3.84; N, 32.04; Found: C, 45.3; H, 3.5; N, 32.3.

*7.1.8.2. 4-amino-7-((2-hydroxyethyl)amino)-2-mercapto-5-methylpyrido[2,3-d]pyrimidine-6-carbonitrile* ***8b***. Yield 72%, mp 265–268 °C. IR: max/cm$^{-1}$ 3410, 3300, 3240, 3160, 2220, 1236, 1227, 1155, 1115. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.1 (s, 3H, $CH_3$), 3.1–3.2 (t, 2H, $CH_2$), 3.3–3.4 (t, 2H, $CH_2$), 3.6 (s, 1H, OH), 7.7 (s, 2H, $NH_2$), 11.6 (s, 1H, NH), 12.2 (s, 1H, SH). MS (EI+): *m*/*z*: 276. Anal.

Calcd for $C_{11}H_{12}N_6OS$: C, 47.81; H, 4.38; N, 30.41; Found: C, 47.3; H, 4.5; N, 30.3.

*7.1.9. 4-amino-7-((2-hydroxyethyl)amino)-2-(methylthio)pyrido [2,3-d]pyrimidine-6-carbonitrile deriatives **9a**,**b***

Was prepared from **8a**,**b** with the same manner as compound **3**.

*7.1.9.1. 4-amino-7-((2-hydroxyethyl)amino)-2-(methylthio)pyrido [2,3-d]pyrimidine-6-carbonitrile **9a***. Yield 75%, mp 245–248 °C. IR: max/$cm^{-1}$ 3450, 3320, 3220, 3120, 2229, 1256, 1237, 1175, 1105. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.4 (s, 3H, $SCH_3$), 3.0–3.1 (t, 2H, $CH_2$), 3.3–3.4 (t, 2H, $CH_2$), 3.6 (s, 1H, OH), 7.9 (s, 2H, $NH_2$), 8.2 (s, 1H, H4), 11.9 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-d6): δ 13.2, 43.5, 60.6, 100.1, 114.9, 119.6, 141.6, 151.4, 158.1, 163.2, 170.4. MS (EI+): *m*/*z*: 276. Anal. Calcd for $C_{11}H_{12}N_6OS$: C, 47.81; H, 4.38; N, 30.41; Found: C, 47.5; H, 4.5; N, 30.3.

*7.1.9.2. 4-amino-7-((2-hydroxyethyl)amino)-5-methyl-2-(methylthio)pyrido[2,3-d]pyrimidine-6-carbonitrile **9b***. Yield 76%, mp 215–218 °C. IR: max/$cm^{-1}$ 3410, 3330, 3250, 3140, 2222, 1246, 1247, 1165, 1115. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 2.6 (s, 3H, $SCH_3$), 3.1–3.2 (t, 2H, $CH_2$), 3.3–3.5 (t, 2H, $CH_2$), 3.7 (s, 1H, OH), 7.6 (s, 2H, $NH_2$), 11.4 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-d6): δ 14.2, 18, 40.5, 63.6, 90.8, 105.1, 111.9, 150.6, 152.6, 155.4, 159.1, 168.2, 174.4. MS (EI+): *m*/*z*: 290. Anal. Calcd for $C_{12}H_{14}N_6OS$: C, 49.64; H, 4.86; N, 28.95; Found: C, 49.5; H, 4.5; N, 29.1.

*7.1.10. 4-amino-2,7-disubstituted aminopyrido[2,3-d]pyrimidine-6-carbonitrile **10a**–**d***

Was prepared from **9a**,**b** with the same manner as compound **4**.

*7.1.10.1. 4-amino-2,7-bis((2-hydroxyethyl)amino)pyrido[2,3-d] pyrimidine-6-carbonitrile **10a***. Yield 55%, mp 211–213 °C. IR: max/$cm^{-1}$ 3550, 3330, 3230, 3130, 2222, 1246, 1247, 1145, 1115. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.2–3.3 (m, 4H, $2CH_2$), 3.4–3.5 (m, 4H, $2CH_2$), 3.7 (s, 2H, OH), 7.6 (s, 2H, $NH_2$), 8.0 (s, 1H, H4), 11.3 (s, 2H, 2NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 48.5, 63.6, 95.1, 113.8, 118.9, 144.6, 156.4, 159.1, 161.2, 166.4. MS (EI+): *m*/*z*: 289. Anal. Calcd for $C_{12}H_{15}N_7O_2$: C, 49.82; H, 5.23; N, 33.89; Found: C, 49.5; H, 5.5; N, 33.3.

*7.1.10.2. 4-amino-2,7-bis((2-hydroxyethyl)amino)-5-methylpyrido [2,3-d]pyrimidine-6-carbonitrile **10b***. Yield 59%, mp 211–213 °C. IR: max/$cm^{-1}$ 3450, 3310, 3210, 3210, 2220, 1256, 1267, 1150, 1105. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 3.1–3.2 (m, 4H, $2CH_2$), 3.3–3.5 (m, 4H, $2CH_2$), 3.6 (s, 2H, OH), 7.9 (s, 2H, $NH_2$), 11.6 (s, 2H, 2NH). MS (EI+): *m*/*z*: 303. Anal. Calcd for $C_{13}H_{17}N_7O_2$: C, 51.48; H, 5.65; N, 32.32; Found: C, 51.5; H, 5.5; N, 32.1.

*7.1.10.3. 4-((4-amino-6-cyano-2-((2-hydroxyethyl)amino)pyrido [2,3-d]pyrimidin-7-yl)amino)benzenesul-fonamide **10c***. Yield 62%, mp 233–236 °C. IR: max/$cm^{-1}$ 3450, 3290, 3210, 3120, 2220, 1226, 1237, 1135, 1105. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.1 (t, 2H, $CH_2$), 3.3 (t, 2H, $CH_2$), 3.6 (s, 2H, OH), 7.2–7.5 (m, 4H, Ar–H), 7.9 (s, 2H, $NH_2$), 8.2 (s, 1H, H4), 9.2 (s, 2H, $NH_2$), 11.5 (s, 1H, NH), 11.9 (s, 1H, NH). $^{13}$C NMR (400 MHz, DMSO-d6): δ 44.1, 62.2, 95.1, 106.6, 112.8, 115.6, 130.3, 132.4, 143.4, 145.8, 153.6, 156.5, 160.1, 164.1. MS (EI+): *m*/*z*: 401. Anal. Calcd for $C_{16}H_{16}N_8O_3S$: C, 47.99; H, 4.03; N, 27.98; Found: C, 47.5; H, 4.2; N, 27.6.

*7.1.10.4. 4-((4-amino-6-cyano-2-((2-hydroxyethyl)amino)-5-methylpyrido[2,3-d]pyrimidin-7-yl)amino)benzene-sulfonamide **10d***. Yield 64%, mp 253–255 °C. IR: max/$cm^{-1}$ 3430, 3280, 3220, 3110, 2222, 1236, 1247, 1145, 1115. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.2 (s, 3H, $CH_3$), 3.3 (t, 2H, $CH_2$), 3.4 (t, 2H, $CH_2$), 3.8 (s, 2H, OH), 7.2–7.4 (m, 4H, Ar–H), 7.8 (s, 2H, $NH_2$), 9.0 (s, 2H, $NH_2$), 11.2 (s, 1H, NH), 12.1 (s, 1H, NH). MS (EI+): *m*/*z*: 401. Anal. Calcd for $C_{17}H_{18}N_8O_3S$: C, 49.27; H, 4.38; N, 27.04; Found: C, 49.5; H, 4.2; N, 27.3.

*7.1.11. 1,7-diaminothieno[3′,4′:4,5]pyrido[2,3-d]pyrimidine **11a**,**b***

Was prepared from **10b**,**d** with the same manner as compound **5**.

*7.1.11.1. 2,2′–((1,7-diaminothieno[3′,4′:4,5]pyrido[2,3-d]pyrimidine-3,6-diyl)bis(azanediyl))diethanol **11a***. Yield 77%, mp 244–246 °C. IR: max/$cm^{-1}$ 3450, 3286, 31s70, 1250, 1255, 1150, 1151. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.2–3.4 (m, 4H, $2CH_2$), 3.5–3.6 (m, 4H, $2CH_2$), 3.8 (s, 1H, OH), 6.8 (s, 1H, CH), 7.8 (s, 2H, $NH_2$), 8.3 (s, 1H, $NH_2$), 11.2 (s, 2H, 2NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 46.5, 62.8, 96.9, 121.7, 131.7, 136.2, 152.5, 156.6, 165.9, 168.4. MS (EI+): *m*/*z*: 335. Anal. Calcd for $C_{13}H_{17}N_7O_2S$: C, 46.56; H, 5.11; N, 29.23; Found: C, 46.7; H, 5.4; N, 29.3.

*7.1.11.2. 4-((1,7-diamino-3-((2-hydroxyethyl)amino)thieno[3′,4′:4,5] pyrido[2,3-d]pyrimidin-6-yl)amino)benzenesulfonamide **11b***. Yield 80%, mp 266–268 °C. IR: max/$cm^{-1}$ 3440, 3266, 3160, 1260, 1266, 1160, 1101. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 3.1–3.2 (t, 2H, $CH_2$), 3.3–3.4 (t, 2H, $CH_2$), 3.6 (s, 1H, OH), 6.6 (s, 1H, CH), 7.0 (s, 2H, $NH_2$), 7.2–7.4 (m, 4H, Ar–H), 7.7 (s, 2H, $NH_2$), 8.9 (s, 1H, $NH_2$), 11.1 (s, 2H, 2NH), 11.5 (s, 2H, 2NH). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 44.2, 60.1, 99.1, 113.2, 125.5, 130.6, 132.2, 140.3, 146.6, 150.4, 160.5, 162.2, 165.5. MS (EI+): *m*/*z*: 335. Anal. Calcd for $C_{17}H_{18}N_8O_3S_2$: C, 45.73; H, 4.06; N, 25.10; Found: C, 45.5; H, 4.4; N, 25.3.

## 8. Microbiology methods

### 8.1. Enzymatic activity inhibition assay

The inhibition studies of cell cycle dependent kinase2 and 4 were performed for the synthesized compounds along with olomoucine and roscovitine as reference compounds. CDK2/cyclin A enzyme was purified from infected sf21 insect cells. For baculoviral overexpression of proteins, human CDK2 c-DNA tagged by hexahistidine was subcloned on its N-terminal and human cyclin A c-DNA into pBacPak 8 expression vector, respectively, and baculovirus which carries each gene was generated using baculovirus generating kit. CDK2/cyclin A enzyme was purified using $Ni^{2+}$ affinity resin from sf21 insect cell culture into which CDK2 and cyclin A carrying baculoviruses were cotransfected. Enzyme assays were done in 20 μL of 50 mM Tris–HCl containing 10 μM ATP, 0.2 μCi of gamma-$P^{32}$ ATP, 10 mM $MgCl_2$, 5 mM DTT and 4 μg of histone H1 was used as a substrate. The reaction was continued for 10 min in the presence of inhibitors and stopped by adding 10 μL of 30% phosphoric acid. The stopped mixtures were spotted onto P81 paper and were washed with 10 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl for five times. The radioactivity of each spot was quantified with BAS imager. The inhibition studies of human EGFR tyrosine kinase activities were done using C-terminal human EGFR tyrosine kinase domain and PYK2104 was used as a reference compound [27]. The concentration of inhibitor that gives 50% inhibition was designated as $IC_{50}$ value.

### 8.2. Cell growth inhibition assay

Human cancer cell lines, A431 (cervical cancer cell line), SNU638 (stomach cancer cell line), and HCT116 (colon cancer cell line) were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C and 5% $CO_2$. For cell division inhibition assay, 1000 cells were plated on 96 well plate and at the next day, the tested compounds as well as olomoucine and doxorubicin as reference compounds

were added to the cells at various concentrations. Cells were allowed to grow further for two days in the presence of the compounds, then fixed by adding equal volumes of 4% formalin for 30 min. Fixed cells were washed with tap water for five times and stained in 0.1% sulforhodamine B for 30 min. Subsequently cells were washed with 1% acetic acid for four times and the dyes attached to cells were eluted by adding 100 μL of 0.1 M Tris/HCl (pH 8.0) and shaking for 10 min. The absorbance was measured at 520 nm wavelength using microplate reader. The absorbance is proportional to cell number in each well. Measurements were done triplicate and averaged. The absorbance from cells at the time of compound treatment was designated as 0% and the absorbance from cells after two days growth with no compound treatment was assigned as 100%. The $GI_{50}$ value was defined as the inhibitor concentration which gives 50% cell growth inhibition during 2 days period of compound treatment.

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