Design, Synthesis and Biological Evaluation of dihydropyrimidine derivatives as potential anticancer agents

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Abstract:

CDK2, which plays a vital role as a regulator of cell growth, is one of the intensely studied TK targets of anticancer inhibitors. The present investigation dealt with design (docking and binding energy), which used to select the promising proposed compounds for the synthesis of novel, diverse 14 pyrido[2,3-d]pyrimidine derivatives as potential anticancer agents targeting CDK2. All the newly synthesized compounds were screened for their anticancer activity against MCF-7 and CaCO₂ and 14 compounds were found to be active. Compounds **6c** and **8d** showed significant activity with IC₅₀ values 7.4 and 5.5 on MCF-7 respectively. Most of the synthesized compounds were subjected to enzyme assay (CDK2 TK) for measuring their inhibitory activity. The preliminary results revealed that compound **8d**, which proves potent inhibitory activity toward tumor growth and potent activity on the CDK2 TK enzyme with 89% inhibition compared to ATP would be a potential anticancer agent.

Keywords:

pyrido[2,3-d] pyrimidines, CDK2 inhibitors, Docking study, Binding energy, Anti-proliferative activity

1. Introduction:

The cyclin-dependent kinases (Cdks) are a family of serine/threonine protein kinases whose members are small proteins (~34–40 kDa) composed of slightly more than the catalytic core joint by all protein kinases. By classification, all Cdks share the feature that their enzymatic initiation requires the binding of a regulatory cyclin subunit. In utmost cases, full initiation also needs phosphorylation of a threonine residue near the kinase active site. Gathered evidence displayed that overexpression of CDK2 would

cause the irregular regulation of cell-cycle, which would be straightly associated with hyperproliferation in cancer cells. Accordingly, CDK2 was observed as a possible therapeutic mark for cancer treatment. The finding of therapeutic agents, based upon the principle of inhibiting kinases, is not insignificant. Controlling the activity of protein kinases by low-molecular-weight compounds capable of interfering with either ligand binding (in the case of RTPKs) or with protein substrate binding has confirmed to be very tough [1-3].

Thus, targeting the catalytic site of kinases with ATP competitive inhibitors seems to be the most hopeful approach for drug intervention, while two major problems should be overcome: access to the intracellular targets and discernment of inhibition.

Uracil and its annulated derivatives like pyrido[2,3-*d*]pyrimidines and pyrimido[4,5-*d*]pyrimidines, characterize vital classes of heterocyclic compounds with biological significance, as they have similarity with pteridine system [4-6]. Numerous derivatives of these scaffolds show a wider range of pharmacological properties; e.g., Inhibitory activities against 5-phosphoribosyl-1-pyrophosphate synthetase [7], dihydrofolate reductases [8], and the tyrosine kinase of the epidermal growth factor receptor [9] have been completely confirmed. Recently, compounds such as RS-25344 and CP-77059 having the pyridopyrimidine moiety displayed excellent PDE-4 inhibitory activity [10].

As a part of our program devoted to developing highly expedient, selective and potent anticancer agents targeting CDK2, [11-15] we describe herein the design, an effective and quick method for the synthesis of novel pyrido[2,3-*d*]pyrimidines as CDK2 inhibitors.

2. Results and Discussion

2.1. Design:

2.1.1. Design of test molecules

The design of targeted compounds 2,5-disubstitutedpyrido[2,3-*d*]pyrimidine derivatives were derived from the structural optimization of the reference compound ATP which depends on the following two strategies: the reported SAR and molecular modeling studies. The bidentate hydrogen bonds of adenine with Hinge Region was replaced by N1 and N8 of pyrido[2,3-*d*]pyrimidine system, which could form the same hydrogen bonds. Adenine of ATP was bioisosterically replaced with pyrido[2,3-

d]pyrimidine to occupy the adenine region in the binding pocket. The phosphate binding region was replaced by substituted phenyl moiety. The sugar pocket was replaced by phenyl group at position 5 of pyrido[2,3-d]pyrimidine. The hydrophobic groups at position 2 of pyrido[2,3-d]pyrimidine was added to occupy the hydrophobic region II, which not occupied by ATP. These hydrophobic groups contain sulfonyl or hydroxyl groups to form H-bonds with the solvent exposure area (Fig.1).

Fig1: The proposed interaction features of pyrido[2,3-d]pyrimidines with ATP active site

2.1.2. Ligand Preparation

The three-dimensional structures of all proposed CDK2 inhibitors were prepared using the Ligand preparation module of Discovery Studio tools. The bond orders of these ligands were corrected and the ligands ‘cleaned’ through preparation specifying a pH of 6.5-8.5. There is a possibility that tautomeric forms interact differently with the binding site and one of the tautomers interacts more strongly relative to the other forms. Hence, most probable tautomers of the compounds were chosen based on their interactions with the proteins in the X-ray structures. In the final stage of Ligand preparation, the compounds were energy minimized with CHARMM forcefield.

2.1.3. Molecular docking and binding energy calculations

The CDK2 protein structure was gained from the RCSB Protein Data Bank (PDB; 2a4l) [16]. Protein Structure was organized using Discovery Studio (DS 2.0) software package [17]. The unacceptable or absent residues were added, and the structures were aligned via the protein structure alignment component. Hydrogen atoms were assigned, and the structure was minimized by means of CHARMM force field to relax

the backbone and to eliminate the clashes. For validation of the docking procedures, the native ligand Roscovitine was redocked in its respective CDK2/CyclinA2 active site. The docking results showed the same interaction features as Roscovitine in its native crystal structure and the RMSD was found to be 0.52. The proposed compounds with promising binding energy values were selected for synthesis. (Table 1).

Table 1: The newly synthesized compounds with their docking scores and binding energy compared with Roscovitine as a reference.

Code	Docking Score	Binding energy
Roscovitine	-10.31	-59.33
6a	-7.398	-38.522
6b	-5.436	-49.157
6c	-8.356	-52.78
6d	-7.709	-22.283
8a	-7.444	-41.393
8b	-7.922	-46.161
8c	-6.769	-47.779
8d	-8.112	-50.732
9a	-8.917	-48.936
9b	-8.825	-29.117
9c	-9.435	-50.569
9d	-9.317	-29.076
13a	-5.754	-32.70
13b	-6.224	-15.742

Docking studies of the proposed compounds in CDK2/CyclinA2 active site revealed that most of the proposed compounds conserved the coordination of C=O group of pyridone ring system in H-bond formation with LEU83 and Pyridone NH with GLU81(fig. 2).

Fig2: 3D and 2D interactions of compound **6c** with ATP active site of CDK2

Introduction of sulfa moiety in position 5 of pyrido[2,3-d]pyrimidine in compounds **8d** enhances the binding energy value, while introducing it in compound **6d** and **9d** had no effect because the SCH₃ group in compound **8d** occupied the hydrophobic pocket in ATP binding site, which pushes the sulfa moiety toward the sugar pocket and makes 2 hydrogen bonds with THR14 and LYS129(Fig. 3). Also, introducing a sulfanilamide moiety at position 2 enhance the binding energy due to the more favorable interactions with the active site (Fig4).

Fig3: 3D and 2D interactions of compound **8d** with ATP active site of CDK2

Fig4: 3D and 2D interactions of compound **9c** with ATP active site of CDK2

2.2. Chemistry:

The synthetic approach to obtain pyrido[2,3-d]pyrimidine derivatives were shown in Schemes 1, 3 and 4. The starting compound 6-amino-2-thioxo-2,3-dihydropyrimidin-4-one, **1**, was prepared by reaction of ethyl cyanoacetate with thiourea in presence of sodium ethoxide [18], which reacted with variously substituted arylidene malonic acids [18] to form a series of pyrido[2,3-d]pyrimidines.

Regarding to the options for its reactivity, electron deficient alkene could simply add on in a Michael way and could have been isolated or decarboxylated to yield uracil linked 5-alkene based carboxylic acids **3 or 4**. Here in our work, none of the three compounds **3-5** could occur but we obtained pyridines **6** as the only product (Scheme 1).

Scheme 1^a

^a) Reagent and condition: (i) DMF, Piperidine, RT, Reflux

The structure of the product is assigned as **6** on the basis of IR, ¹H NMR, and mass spectra. The ¹H NMR spectra recorded in DMSO-d₆ showed a multiplet at δ 7.34-6.89 for aromatic protons. The NH protons showed a singlet at δ 12.8, 11.8 and at 10.7, also a new CH proton of pyridine resonates as a singlet at δ 6.20. The IR spectra clearly showed absorption for NHs at 3420, 3330 and 3195 cm⁻¹. The two C=O bands were observed at 1690, 1660 cm⁻¹. In mass spectrum, a strong molecular ion peak was obtained at the corresponding molecular weight for compound **6**. The alternative

structure of product **3**, **4** and **5** (Scheme 1) is easily ruled out as no broad-band between 1710-1720 cm^{-1} was observed for the C=O function of acid. Final confirmation of structure came from mass where a strong molecular ion peak was observed at 287 $(\text{M}+\text{H})^+$ for **6a**. The benzalmalonic acid employed in this reaction was prepared by condensation of substituted benzaldehyde with malonic acid in THF using piperidine as a catalyst. ^1H NMR spectra of benzalmalonic acid showed a singlet at δ 7.52 for vinyl proton and phenyl protons appeared as a multiplet at δ 7.10-7.42.

Plausibly reaction must have proceeded *via* Michael addition it's difficult to confirm whether decarboxylation occurred before cyclization or after cyclization Scheme 2. To prove it we tried to react cinnamic acids **7** with 6-amino thiouracil neither there is Michael addition, nor any other product formation takes place and the starting materials were recovered. So evidently, arylidinemalonic acids **2a-g** undergoes Michael addition followed by cyclization then decarboxylation and oxidation to yield **6** (scheme 2). The plausible mechanism is as shown below:

Scheme 2

Another confirmation for that the cyclization occurred before decarboxylation, was obtained from quantum mechanics calculations. Compounds **3** and **4** were optimized (first the best tautomer was selected then the best conformer was chosen for optimization using semi-Empirical method) by DF theory with B3LYP method and 6-31G as basis set and the calculations showed that compound **3** is more stable than compound **4** (the difference in energy was 495.06 KJ) as shown in Fig5.

Fig5: Optimized structures of compound 3 and 4 by DFT(B3LYP/6-31G)

To simplify the synthetic work, we carried out the reaction in a multicomponent way by stirring the appropriate aldehyde with malonic acid in DMF in presence of Piperidine as a catalyst for 1h. then amino thiouracil was added and the reaction mixture was refluxed for 6-8 hrs. and we managed to obtain the target compounds in high yields.

In a trial to optimize the biological results, we prepared compound **9** by alkylation of compound **6** in basic medium then amination of compound **8** with sulfanilamide in refluxing DMF (Scheme3).

Scheme:3^a

a) Reagent and condition: (i) CH_3I , NaOH, stirring; (ii) Sulfanilamide, DMF, HCl, Reflux

To make another variation, we tried to prepare 7-aminopyrido[2,3-d]pyrimidine derivatives by reaction of 6-aminothiouracil **1** with various arylidene cyanoacetic acid [19] but the only product was 6-aminouracil-5-arylpropanenitriles (Scheme 4). Here are once more numerous possibilities, arylidene cyanoacetic acids may undertake Michael addition and then cyclize by an attack of the nucleophilic amino group on cyanide function or carboxyl may be tangled in cyclization to yield **11** and **12**

respectively (Scheme 4). We surprise that neither of the two possibilities was not achieved and compound **13** was obtained, which seems to be the result of initial Michael addition and fast decarboxylation therein prior to any cyclization and thus we successfully isolated a novel set of 6-aminouracil-5-aryl propanenitriles. The reaction is fairly general and product **13** was isolated in good yields.

Scheme 4^a

^a) Reagent and condition: (i) DMF, Piperidine, RT, Reflux

The structure of compound **13** was established by IR, which showed a new strong peak at 2220 cm^{-1} due to CN and ¹H NMR, which showed one triplet signal at δ 4.2 corresponding to CH and one doublet signal at δ 2.9-2.8 due to CH₂. Finally, the mass spectrum showed a strong molecular ion peak at the corresponding molecular weight of compound **13**.

2.3. Biology

Kinases inhibition and antitumor data of the newly synthesized compounds were reported in Table 2.

Table (2): Anticancer and CDK2 Activities of the newly synthesized compounds

Compds	IC ₅₀ (μM)		% inhibition CDK2	% inhibition VEGFR2
	MCF-7	CaCO2		
6a	37.5	41.6	56	8
6b	25.6	31.4	80	13
6c	7.4	18.0	85	17

8a	26.4	21.2	83	10
8b	36.8	21.1	88	21
8c	38.4	21.4	65	23
8d	5.5	4.2	89	18
9a	38.4	16.1	75	12
9b	21.7	14.9	90	15
9c	6.4	3.2	91	22
9d	61.3	38.8	23	11
13a	65.2	205.5	21	27
13b	55.6	36.6	41	17

The newly synthesized compounds exhibited effective CDK2 inhibitory activity with good to effective inhibition profile. Most of the newly synthesized compounds showed quite potent inhibitory efficiency against CDK2/cyclinA, with inhibition percent in the range of 56 to 90, except for compounds **13a** and **13b**. Compounds **6c**, **8d**, and **9c** showed the highest inhibitory activity and the highest antitumor activities against MCF-7 and CaCO₂ cell lines and could be used as lead compounds for further studies and optimization. The biological results were in good correlation with the modeling studies. In general, the derivatives with SCH₃ group were more active than the derivatives with C=S and the derivatives with sulfanilamide group were the most active one except for **9d**. All these results showed a good correlation between the modeling studies and the biological results. Compound **9d** may be inactive because of its geometrical shape of the two sulfanilamide groups, which prevent it from being buried well in the active site. All compounds were tested against VEGFR2 and none of them showed any significant inhibition, which showed some selectivity towards CDK2.

1. Conclusions

A series of novel pyrido[2,3-d]pyrimidine derivatives linked to various substitutions at positions 2 and 5 were docked into the active site of CDK2 and the binding energy was calculated and used for selection of the best pose. The promising compounds according to binding energy calculations were synthesized and evaluated as inhibitors of CDK2/CyclinA and as antitumor agents against MCF-7 and CaCO₂. The established compounds exhibited an actual upright inhibition of CDK2. Compounds **6c**, **8d**, and **9c** showed good selectivity towards CDK2 and a very good activity against breast and Colon cancer, so they can be used as a lead compound for further optimization. The biological results were in good correlation with the modeling

studies. In general, the derivatives with SCH₃ group were more active than the derivatives with C=S and the derivatives with sulfanilamide group were the most active as suggested by the modeling studies. All the newly produced compounds exhibited selectivity towards CDK2.

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3. Experiments:

3.1. Chemistry

All chemicals used were purchased from Sigma–Aldrich (USA) and SD-fine (India) and used without further purification. Separation of the compounds by column chromatography was carried out using silica gel 60 (200–300 mesh ASTM, E. Merck). The quantity of silica gel used was 50–100 times the weight charged on the column. Then, the reactions were monitored using TLC and visualized under UV light (254 nm). Melting points (uncorrected) were determined on an XT4 MP apparatus. Mass spectra were recorded on a Varian MAT 112 Spectrometer at Al-Azhar University. Analytical data (IR) were performed at the Micro-Analytical Data Center of Cairo University and National Research Center. Some of the ¹H NMR spectra were recorded on a Variant spectrometer 500 MHz, at the Micro-Analytical Data Center of Mansoura University and the main laboratories of the chemical war, while others were recorded at National Research Center, at 25 °C with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were performed on a CHNS-O-Rapid instrument in the Micro-Analytical Data Center at the main laboratories of the chemical war. Compounds were **1**¹⁸, **2**¹⁹ and **10**²⁰ synthesized according to reported methods.

Preparation of arylidene malonic acids (2**)¹⁷**

Malonic acid (5.2 g, 0.05 mole) and substituted benzaldehyde (0.05 mole) were taken in 20 mL of tetrahydrofuran (THF). To this stirred solution, added few drops of piperidine and continued stirring for further 1 hour. Evaporation of excess solvent under reduced pressure gave the colorless solid which was purified from ethanol to obtained pure product in 87 % yield.

5-substituted phenyl-2-thioxo-2,3-dihydropyrido[2,3-d]pyrimidine-4,7-dione Derivatives (6)

A mixture of arylidene malonic acids **2a-g** (10 mmol), uracil compound **1** (10 mmol) in 15ml nitrobenzene was stirred at 150°C for 3.0 to 4.5 h. After completion of reaction (monitored via TLC), the reaction mixture was cooled to room temperature and excess solvent was removed under vacuum. The solid thus obtained was recrystallized from methanol to afford pyrido[2,3-d]pyrimidines **6** in good yields.

5-(4-hydroxyphenyl)-2-thioxo-2,3-dihydropyrido[2,3-d]pyrimidine-4,7-dione 6a

Yellowish white powder. Yield = 80%. Mp = 216 - 219 °C. IR (cm⁻¹), 3421 (OH), 3335, 3269, 3189 (3NH), 1671 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.88 (s, 1H, NH exchangeable with D₂O), 11.87 (s, 1H, NH exchangeable with D₂O), 10.62 (s, 1H, NH exchangeable with D₂O), 8.67 (s, 1H, OH exchangeable with D₂O), 7.34 (d, *J* = 9.0 Hz, 2H), 6.80 (d, *J* = 9.0 Hz, 2H), 6.27 (s, 1H, H3 Pyridine). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 175.38, 164.67, 157.40, 156.29, 155.85, 153.03, 131.12, 130.17, 114.18, 110.58, 86.48; MS (m/z) = 287.29, [M⁺] = 288.21; Anal. Calcd for C₁₃H₉N₃O₃S: C, 54.35; H, 3.16; N, 14.63. Found: C, 53.87; H, 3.67; N, 15.17.

5-(4-hydroxy-3-methoxyphenyl)-2-thioxo-2,3-dihydropyrido[2,3-d]pyrimidine-4,7-dione 6b

colorless crystalline. Yield = 70%. Mp = 238-240 °C; IR (cm⁻¹), 3361 (OH), 3320, 3280, 3195 (3NH), 1660 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.65 (s, 1H, NH exchangeable with D₂O), 11.88 (s, 1H, NH exchangeable with D₂O), 10.73 (s, 1H, NH exchangeable with D₂O), 8.85 (s, 1H, OH exchangeable with D₂O), 7.23 – 7.14 (d, *J* = 8.5 Hz, 1H), 6.86 (d, *J* = 8.5 Hz, 1H), 6.26 (s, 1H, H3 Pyridine), 3.84 (s, 3H, OCH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 175.38, 164.66, 156.28, 155.77, 152.49, 147.97, 146.66, 130.85, 121.41, 113.44, 112.55, 110.49, 86.44, 56.02; MS (m/z) = 317; Anal. Calcd for C₁₄H₁₁N₃O₄S: C, 52.99; H, 3.49; N, 13.24. Found: C, 53.47; H, 3.17; N, 13.57.

4-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)phenyl benzoate 6c

Light brown powder. Yield = 74%. Mp = 191-194°C; IR (cm⁻¹), 3330, 3260, 3202 (3NH), 1710 (C=O ester), 1660 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.97 (s, 1H, NH exchangeable with D₂O), 11.92 (s, 1H, NH exchangeable with D₂O), 10.55 (s, 1H, NH exchangeable with D₂O), 8.13 (dd, *J* = 7.7, 1.5 Hz, 2H), 7.68 – 7.61 (m, 3H, ArH), 7.54 – 7.48 (d, 2H, *J* = 9.2 Hz, 2H), 7.31 – 7.25 (d, 2H, *J* = 9.2 Hz,

2H), 6.31 (s, 1H, H3 Pyridine). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 175.45, 165.38, 164.92, 156.29, 155.79, 153.04, 151.72, 133.77, 132.76, 130.32, 129.86 (d, $J = 10.5$ Hz), 128.96, 119.87, 110.46, 86.48; MS (m/z) = 391.40; Anal. Calcd for $\text{C}_{20}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$: C, 61.37; H, 3.35; N, 10.74. Found: C, 61.47; H, 3.13; N, 10.57.

4-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)phenyl benzenesulfonate 6d

Off white powder. Yield = 67%. Mp = 178-181°C; IR (cm^{-1}), 3410, 3302, 3250 (3NH), 1660 (C=O amide), 1350 (S=O); ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 13.29 (s, 1H, NH exchangeable with D_2O), 11.83 (s, 1H, NH exchangeable with D_2O), 10.27 (s, 1H, NH exchangeable with D_2O), 7.89 - 7.84 (dd, $J=7.9, 1.4\text{Hz}$, 2H), 7.78-7.74 (m, 3H, ArH), 7.41 (d, $J=8.9\text{Hz}$, 2H), 7.39 (d, $J=8.9\text{Hz}$, 2H), 6.29 (s, 1H, H3 Pyridine). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 175.38, 164.68, 156.29, 155.98, 153.03, 149.58, 136.86, 133.07 (d, $J = 11.7$ Hz), 129.22 (d, $J = 9.3$ Hz), 128.31, 119.51, 110.53, 86.48; MS (m/z) = 427.45; Anal. Calcd for $\text{C}_{19}\text{H}_{13}\text{N}_3\text{O}_5\text{S}_2$: C, 53.39; H, 3.07; N, 9.83. Found: C, 52.97; H, 3.23; N, 10.17.

2-(methylthio)-5-substituted phenylpyrido[2,3-d]pyrimidine-4,7-dione 8

To a solution of **7** (0.033 mol) in 0.83N NaOH (65 mL) was added CH_3I (5.0 g) and the reaction mixture was stirred at RT for 2h. The solution was treated with charcoal, filtered and the filtrate was acidified with acetic acid. The crude product was filtered and suspended in 100 mL of H_2O and the PH adjusted to **9** by ammonium hydroxide to dissolve any unreacted starting materials. The ammonical solution was filtered, washed with H_2O and collected to afford compound **8**.

5-(4-hydroxyphenyl)-2-(methylthio) pyrido[2,3-d]pyrimidine-4,7-dione 8a

Off white powder. Yield = 87%. Mp = 188-191 °C; IR (cm^{-1}), 3420 (OH), 3312, 3260 (2NH), 1670 (C=O amide); ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 11.81 (s, 1H, NH exchangeable with D_2O), 11.38 (s, 1H, NH exchangeable with D_2O), 8.67 (s, 1H, OH exchangeable with D_2O), 7.30 (d, $J = 9.0$ Hz, 2H), 6.80 (d, $J = 9.0$ Hz, 2H), 6.14 (s, 1H, H3 Pyridine), 2.53 (s, 3H, SCH_3). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 164.71, 159.79, 159.23 (d, $J = 16.9$ Hz), 157.97, 157.16, 130.97, 127.32, 114.12, 86.87, 12.81; MS (m/z) = 301.32; Anal. Calcd for $\text{C}_{14}\text{H}_{11}\text{N}_3\text{O}_3\text{S}$: C, 55.81; H, 3.68; N, 13.95. Found: C, 55.37; H, 3.33; N, 13.47.

5-(4-hydroxy-3-methoxyphenyl)-2-(methylthio)pyrido[2,3-d]pyrimidine-4,7-dione 8b

colorless crystalline. Yield = 90%. Mp = 210-213°C; IR (cm⁻¹), 3421 (OH), 3280, 3195 (2NH), 1690 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.09 (s, 1H, NH exchangeable with D₂O), 11.79 (s, 1H, NH exchangeable with D₂O), 8.84 (s, 1H, OH exchangeable with D₂O), 7.24 – 7.18 (d, *J* = 8.8 Hz, 1H), 7.21 (s, 1H, ArH), 6.89 (d, *J* = 8.8 Hz, 1H), 6.16 (s, 1H, H3 Pyridine), 3.84 (s, 3H, OCH₃), 2.51 (s, 3H, SCH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.64, 159.45 – 159.21 (m), 157.16, 147.99, 146.40, 127.56, 122.22, 113.77 (d, *J* = 9.5 Hz), 113.44, 86.90, 55.99, 12.78; MS (*m/z*) = 331.35; Anal. Calcd for C₁₅H₁₃N₃O₄S: C, 54.37; H, 3.95; N, 12.68. Found: C, 54.07; H, 3.83; N, 12.44.

4-(2-(methylthio)-4,7-dioxo-3,4,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-5-yl)phenyl benzoate 8c

brown powder. Yield = 82%. Mp = 170-174 °C; IR (cm⁻¹), 3310, 3240 (2NH), 1715 (C=O ester), 1670 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.91 (s, 1H, NH exchangeable with D₂O), 11.48 (s, 1H, NH exchangeable with D₂O), 8.12 (d, *J* = 6.3 Hz, 2H), 7.68-7.57 (m, 3H, ArH), 7.48 (d, *J* = 9.2 Hz, 2H), 7.31 (d, *J* = 9.1 Hz, 2H), 6.20 (s, 1H, H6 pyridine), 2.55 (s, 3H, SCH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.41, 164.69, 159.86, 159.31, 156.93, 151.72, 133.68, 131.04, 130.32, 129.82, 128.96, 119.84, 114.01, 87.08, 12.79; MS (*m/z*) = 405.43; Anal. Calcd for C₂₁H₁₅N₃O₄S: C, 62.21; H, 3.73; N, 10.36. Found: C, 62.07; H, 3.33; N, 10.13.

4-(2-(methylthio)-4,7-dioxo-3,4,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-5-yl)phenyl benzenesulfonate 8d

Colorless powder. Yield = 91%. Mp = 160-162°C; IR (cm⁻¹), 3320, 3230 (2NH), 1690 (C=O amide), 1330 (S=O); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.93 (s, 1H, NH exchangeable with D₂O), 11.42 (s, 1H, NH exchangeable with D₂O), 7.87 (dd, *J* = 8.0, 1.5 Hz, 2H), 7.79 - 7.67 (m, 3H, ArH), 7.39 (d, *J* = 8.8 Hz, 2H), 7.36 (d, *J* = 8.8 Hz, 2H), 6.16 (s, 1H, H6 Pyridine), 2.50 (s, 3H, SCH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.69, 159.86, 159.31, 156.77, 149.58, 136.88, 133.10, 130.44, 129.41, 129.20, 128.27, 119.51, 114.01, 87.08, 12.81; MS (*m/z*) = 441.48; Anal. Calcd for C₂₀H₁₅N₃O₅S₂: C, 54.41; H, 3.42; N, 9.52. Found: C, 54.27; H, 3.23; N, 9.23.

4-((5-(substituted phenyl)-4,7-dioxo-3,4,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-2-yl)amino)benzenesulfonamide 9

To a mixture of compound **8** (5 mmol) and sulfanilamide (10 mmol, 1.72 g) was added Conc. HCl (1 mL) and the reaction mixture was refluxed at 200 °C for 12 hrs.

After the reaction was completed, the product was collected by filtration and washed well with boiling H₂O and EtOH to afford the amino derivatives **9**

4-((5-(4-hydroxyphenyl)-4,7-dioxo-3,4,7,8-tetrahydropyrido[2,3-d]pyrimidin-2-yl) amino)benzenesulfonamide **9a**

Yellow powder. Yield = 80%. Mp = 236 - 239°C. IR (cm⁻¹), 3425 (OH), 3305, 3280, 3240, (2NH), 3189, 3151 (NH₂), 1681 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.58 (s, 1H, NH exchangeable with D₂O), 11.29 (s, 1H, NH exchangeable with D₂O), 10.60 (s, 1H, NH exchangeable with D₂O), 8.97 (s, 1H, OH exchangeable with D₂O), 7.72 (d, *J* = 8.7 Hz, 2H) 7.69 (d, *J* = 8.7 Hz, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.15 (s, 2H, NH₂ exchangeable with D₂O), 6.81 (d, *J* = 8.6 Hz, 2H), 6.22 (s, 1H, H6 Pyridine); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.20, 161.74, 158.96, 157.66, 153.78, 152.44, 143.75, 137.33, 130.96, 127.84, 127.21, 119.13, 114.53, 114.01, 91.16; MS (m/z) = 425.42; Anal. Calcd for C₁₉H₁₅N₅O₅S: C, 53.64; H, 3.55; N, 16.46. Found: C, 53.37; H, 3.33; N, 16.23.

4-((5-(4-hydroxy-3-methoxyphenyl)-4,7-dioxo-3,4,7,8-tetrahydropyrido[2,3-d]pyrimidin-2-yl)amino)benzenesulfonamide **9b**

Yellowish white crystals. Yield = 79%. Mp = 240-243 °C; IR (cm⁻¹), 3440 (OH), 3315, 3280, 3230, (2NH), 3190, 3161 (NH₂), 1675 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.48 (s, 1H, NH exchangeable with D₂O), 10.85 (s, 1H, NH exchangeable with D₂O), 10.30 (s, 1H, NH exchangeable with D₂O), 8.39 (s, 1H, OH exchangeable with D₂O), 7.71 (d, *J* = 8.8 Hz, 2H), 7.61 (d, *J* = 8.8 Hz, 2H), 7.23 (s, 1H, ArH), 7.01 (d, *J* = 8.4 Hz, 1H), 7.01 (s, 2H, NH₂), 6.90 (d, *J* = 8.4 Hz, 1H), 6.21 (s, 1H, H6 Pyridine), 3.81 (s, 3H, OCH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.14, 161.73, 158.45, 153.78, 152.36, 147.89, 146.79, 143.97, 137.12, 127.87, 127.53, 122.19, 119.13, 114.47, 113.74, 113.45, 90.65, 55.97; MS (m/z) = 455.45; Anal. Calcd for C₂₀H₁₇N₅O₆S: C, 52.74; H, 3.76; N, 15.38. Found: C, 52.47; H, 3.53; N, 15.26.

4-(4,7-dioxo-2-((4-sulfamoylphenyl)amino)-3,4,7,8-tetrahydropyrido[2,3-d]pyrimidin-5-yl)phenyl benzoate **9c**

Yellow powder. Yield = 69%. Mp = 195 - 197 °C. IR (cm⁻¹), 3325, 3270, 3220, (3NH), 3190, 3161 (NH₂), 1715 (C=O ester), 1681 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.56 (s, 1H, NH exchangeable with D₂O), 11.42 (s, 1H, NH

exchangeable with D₂O), 10.65 (s, 1H, NH exchangeable with D₂O), 8.13 (d, *J* = 9.0 Hz, 2H), 7.72 – 7.65 (m, 3H, ArH), 7.70 (d, *J* = 8.6 Hz, 2H), 7.68 (d, *J* = 8.6, 2H), 7.51 (d, *J* = 8.9 Hz, 2H), 7.32 (d, *J* = 8.8 Hz, 2H), 7.06 (s, 2H, NH₂), 6.26 (s, 1H, H6 Pyridine). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.40, 164.14, 161.74, 158.92, 153.75, 152.42, 152.13, 143.98, 137.06, 133.68, 131.08, 130.32, 129.82, 129.12, 128.93, 127.77, 119.91, 119.18, 114.06, 90.69; MS (*m/z*) = 529.53; Anal. Calcd for C₂₆H₁₉N₅O₆S: C, 58.97; H, 3.62; N, 13.23. Found: C, 58.46; H, 3.43; N, 13.35.

4-(4,7-dioxo-2-((4-sulfamoylphenyl)amino)-3,4,7,8-tetrahydropyrido[2,3-d]pyrimidin-5-yl)phenyl benzenesulfonate 9d

Yellow powder. Yield = 86%. Mp = 175-178 °C; IR (cm⁻¹), 3410, 3320, 3230 (2NH), 3199, 3176 (NH₂), 1690 (C=O amide), 1330 (S=O); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.56 (s, 1H, NH exchangeable with D₂O), 11.42 (s, 1H, NH exchangeable with D₂O), 10.65 (s, 1H, NH exchangeable with D₂O), 7.78 (d, *J* = 7.8 Hz, 2H), 7.70 (d, *J* = 8.6 Hz, 2H), 7.68 – 7.63 (m, 3H), 7.59 (d, *J* = 8.6 Hz, 2H), 7.50 (d, *J* = 8.5 Hz, 2H), 7.42 (d, *J* = 8.5 Hz, 2H), 7.21 (s, 2H, NH₂), 5.93 (s, 1H, H6 Pyridine). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.14, 161.74, 158.92, 153.75, 152.42, 149.75, 144.01, 137.05, 136.88, 132.94, 130.38, 129.41, 129.21, 128.32, 127.73, 119.49, 119.16, 114.06, 90.69; MS (*m/z*) = 565.58; Anal. Calcd for C₂₅H₁₉N₅O₇S₂: C, 53.09; H, 3.39; N, 12.38. Found: C, 53.26; H, 3.51; N, 12.28.

Preparation of arylidenecyanoacetic acids (10)¹⁸

Cyanoacetic acid (4.25 g, 0.05 mole) and substituted benzaldehyde (0.05 mole) was taken in 20 mL of tetrahydrofuran (THF). To this stirred solution, added few drops of piperidine and continued stirring for further 1 hour at 50 °C. Evaporation of the excess solvent under reduced pressure gave the solid which was purified from ethanol. Similarly, other arylidene cyanoacetic acids (**2a-e**) were prepared.

Preparation of 6-aminouracil-5-arylpropanenitriles (13):

A mixture of arylidene cyanoacetic acids **7** (10 mmol), uracil compound **1** (10 mmol) in 15 mL nitrobenzene was stirred at 150 °C for 2.5 to 4 h. After completion of reaction (monitored by TLC), the reaction mixture was cooled to room temperature and excess solvent was removed under vacuum. The solid thus obtained was recrystallized from ethyl acetate to afford 6-aminouracil-5-arylpropanenitriles **10** in good yields. The spectral data of products **10** are given below.

3-(6-amino-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-3-(4-hydroxyphenyl) propanenitrile 13a

Gray powder. Yield = 87%. Mp = 222 - 225°C. IR (cm⁻¹), 3411 (OH), 3335, 3280 (NH₂), 3269, 3189 (2NH), 2221 (CN), 1671 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.89 (s, 1H, NH exchangeable with D₂O), 11.18 (s, 1H, NH exchangeable with D₂O), 8.47 (s, 1H, OH exchangeable with D₂O), 7.19 (d, *J* = 8.8 Hz, 2H), 6.97 (s, 2H, NH₂), 6.72 (d, *J* = 8.8 Hz, 2H), 4.27 (t, *J* = 5.6 Hz, 1H, CH), 2.90 - 2.84 (t, 2H, CH₂). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 178.34, 157.53, 155.68, 151.99, 136.52, 129.28, 117.87, 115.70, 80.87, 38.51, 24.93; MS (m/z) = 288.33; Anal. Calcd for C₁₃H₁₂N₄O₂S: C, 54.16; H, 4.20; N, 19.43. Found: C, 54.36; H, 4.43; N, 19.28.

3-(6-amino-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-3-(4-hydroxy-3-methoxyphenyl)propanenitrile 13b

colorless crystalline. Yield = 79%. Mp = 208-210 °C; IR (cm⁻¹), 3341 (OH), 3320, 3290 (NH₂), 3250, 3175 (2NH), 2220 (CN), 1675 (C=O amide); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.88 (s, 1H, NH exchangeable with D₂O), 11.21 (s, 1H, NH exchangeable with D₂O), 8.69 (s, 1H, OH exchangeable with D₂O), 6.56 (s, 2H, NH₂ exchangeable with D₂O), 6.87 (s, 1H, ArH), 6.89 (d, *J* = 8.4 Hz, 1H), 6.76 (d, *J* = 8.4 Hz, 1H), 4.30 (t, *J* = 5.6 Hz, 1H), 3.83 (s, 3H, OCH₃), 2.92 - 2.87 (m, 2H, CH₂). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 178.34, 157.59, 152.00, 147.62, 145.86, 137.73, 121.50, 117.87, 115.48, 112.39, 80.73, 56.13, 38.70, 24.89; MS (m/z) = 318.35; Anal. Calcd for C₁₄H₁₄N₄O₃S: C, 52.82; H, 4.43; N, 17.60. Found: C, 52.39; H, 4.63; N, 17.82.

3.2. Biology

3.2.1. Cytotoxicity (MTT assay)

Human Breast (MCF-7) cancer cells were propagated 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 detached 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 × 10⁵ cells/ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 hours to achieve confluence. The medium was decanted and fresh medium containing various concentrations of bee venom and Lapatinib was

added for cytotoxicity determination using colorimetric MTT reduction assay. Dead cells were washed out using phosphate-buffered saline (PBS), and 50 µl of MTT stock solution (5 mg/ml) was added to each well. After 4 h incubation period, the supernatants were discarded and the formazan precipitates were solubilized by addition of 50 µl per well of dimethyl sulfoxide (DMSO) or 0.4% (v/v) acidified isopropanol. Plates were incubated in the dark for 30 min at a temperature of 37°C, and absorbance was determined at a wavelength of 570 nm using microplate reader (Biotek ELX -800, USA). The cell viability percentage was calculated using the following formula:

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

The cell viability (%) was plotted with the tested bee venom and Lapatinib concentrations. Statistical significance between treated and untreated cells was determined using one way ANOVA. Differences at P values less than 0.05 were considered significant. The IC₅₀ values of test bee venom and lapatinib were determined using Masterplex-2010 software program. The effect of bee venom and Lapatinib as an anticancer synergetic agent was examined by evaluating the cytotoxic potential of bee venom and Lapatinib in MCF-7 cells pretreated with 25 and 50 µg for 24 h. Morphological alterations of cells were analyzed using an inverted microscope (Nikon-Japan).

3.2.2. *in vitro* enzymatic assay (CDK2/CyclinA2)

3.2.2.1. Assay Conditions

The assay was performed using Kinase-Glo Plus luminescence kinase assay kit (Promega). It measures kinase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. The luminescent signal from the assay is correlated with the amount of ATP present and is inversely correlated with the amount of kinase activity.

The compounds were diluted in 10% DMSO and 5 µl of the dilution was added to a 50 µl reaction so that the final concentration of DMSO is 1% in all of the reactions.

All the enzymatic reactions were conducted at 30 °C for 40 minutes. The 50 µl reaction mixture contains 40 mM Tris, pH 7.4, 10 mM MgCl₂, 0.1 mg/ml BSA, 1 mM DTT, 10 µM ATP, Kinase substrate and the enzyme.

After the enzymatic reaction, 50 µl of Kinase-Glo Plus Luminescence kinase assay solution (Promega) was added to each reaction and incubate the plate for 5 minutes at room temperature. Luminescence signal was measured using a BioTek Synergy 2 microplate reader.

3.2.2.2. Data Analysis

Kinase activity assays were performed in duplicate at each concentration. The luminescence data were analyzed using the computer software, GraphPad Prism. The difference between luminescence intensities in the absence of Kinase (Lu_t) and in the presence of Kinase (Lu_c) was defined as 100 % activity ($Lu_t - Lu_c$). Using luminescence signal (Lu) in the presence of the compound, % activity was calculated as:

% activity = $\{(Lu_t - Lu)/(Lu_t - Lu_c)\} \times 100\%$, where Lu = the luminescence intensity in the presence of the compound (all percent activities below zero were shown zero in the table).

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