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Original Research Article

Design, synthesis and cytotoxic evaluation of 2-amino-4-aryl-6-substituted pyridine-3,5-dicarbonitrile derivatives

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Abstract

Purpose: *To synthesize novel pyridine derivatives and evaluate their efficiency as potent inhibitors of cyclin dependent kinase 2 (CDK2) enzyme for cancer therapy.*
Methods: *Pyridine scaffold were synthesized using one-pot multicomponent condensation reaction of arylidine with different primary amines. The cytotoxic potential of the new compounds was assessed using various cell lines. Furthermore, molecular docking studies based on the crystal structure of CDK2 was carried out to determine the possible binding modes that influence the anticancer activities.*
Results: *The results indicate that one-pot multicomponent reaction generated a series of functionalized pyridines with good yield. In vitro cytotoxicity study revealed superior cytotoxicity of the designed compounds against prostate and cervical cancer cell lines compared to 5-fluorouracil (standard anticancer compound) with half-maximal inhibitory concentration (IC_{50}) values of 0.1 – 0.85 and 1.2 – 74.1 µM, respectively. Finally, molecular modeling simulation of the newly synthesized compounds showed that they fit well and are stabilized into CDK2 active site via hydrogen bonding and hydrophobic interactions.*
Conclusion: *The results indicate that the newly synthesized pyridine can exert potent anticancer activity presumably via inhibition of CDK2. However, this will need to be confirmed in in vivo studies.*

Keywords: *5-Fluorouracil, Anticancer activity, Cyclin dependent kinase 2, Molecular docking, One-pot multicomponent reaction, Pyridine scaffold*

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INTRODUCTION

The development of Cyclin-dependent kinases (CDKs) inhibitors has sparked a lot of interest over the last two decades. This interest stemmed from the crucial roles of CDKs in regulating cell-cycle progression [1,2]. Cyclin-dependent kinase-2 (CDK2) is a member of protein kinase family

that regulates cell cycle progression [2,3]. Over-expression of CDK2 has been linked to poor prognosis in a variety of cancer cells [4-6]. Accordingly, CDK2 could be considered as a potential therapeutic target for cancer treatment.

Pyridine scaffold is gaining increased attention in modern pharmaceuticals with pyridine-containing compounds possessing multiple biological activities such as antimalarial [7], antiviral [8], anticholinesterase [9], antidiabetic [10] and antimicrobial activities [11]. Most interestingly, pyridine derivatives have been reported to exert potent cytotoxic effects against a variety of cancer cell lines, such as leukemia, colon, and ovarian cancer cell lines [9,12,13]. Byth *et al* [14] synthesized imidazo[1,2-a] pyridine derivatives as plausible CDK2 inhibitors and investigated their activities against breast cancer cell line (MCF-7). The synthesized pyridine derivatives showed potent CDK2 inhibitory activity as well as anti-carcinogenic activity even at lower concentrations ranging from 0.004 to 0.046 M.

Recently, a one-pot multicomponent reaction using readily accessible primary amines, malononitrile and aromatic aldehydes in the presence of different Lewis acids such as $FeCl_3$, $ZnCl_2$, and $AlCl_3$ has been adopted for the synthesis of substituted pyridines [15]. This multicomponent reaction generated a series of functionalized pyridines with good yield using catalyst and solvent-free conditions under the fusion condition. In the current study, we synthesized novel pyridine compounds through single-pot multi-component condensation reactions of arylidine and various primary amines. FTIR, ^{1}H NMR and mass spectroscopy were adopted for chemical characterization of the obtained pyridine derivatives. *In vitro* antitumor activity was also evaluated against a series of human cancer cells. Finally, virtual docking simulations were conducted to define the binding modes of the synthesized compound within the CDK2 active site. The results revealed that the synthesized pyridine derivatives fit well into CDK2 active site, and could represent novel CDK2 inhibitors with promising anti-proliferative activity against cancer cell lines; particularly, human prostate and cervical cancer cell lines.

EXPERIMENTAL

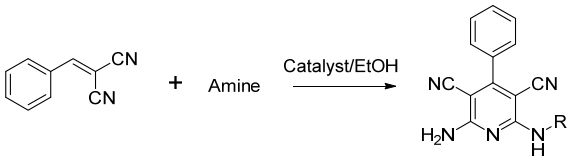
General

All reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were utilized without further purification unless mentioned otherwise.

Gallenkamp melting point apparatus (Gemini BV., Apeldoorn, Netherland) was used to measure the melting points. Pye-Unicam SP 3-300 spectrophotometer (Cambridge, UK) was utilized to record the FTIR spectra. A Varian Mercury VX-300 MHz spectrometer was employed to measure the ^{1}H-NMR spectra. The mass spectra were detailed on GCMS-QP 1000 EX Shimadzu mass spectrometer (Shimadzu Corporation, Kyoto, Japan) at 70 eV.

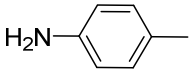
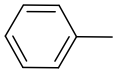
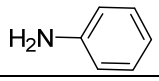
Synthesis

A one-pot multicomponent reactions were adopted for the synthesis of 2-Amino-4-Aryl-6-substituted pyridine-3,5-dicarbonitrile derivatives (S1–S4), as previously reported [15]. Briefly, a mixture of 0.06 mol arylidine, 0.069 mol of various primary amines (ethylamine, p-toluidine, aniline or a nucleophilic ammonium acetate) and anhydrous $ZnCl_2$ (0.09 mol) was agitated for 5 h at 80 ˚C in dry ethanol (20 ml) and then filtered. The residue from ethanol, then methanol, was recrystallized. The synthesis method of the target compounds is depicted in Scheme 1 and Table 1.



Scheme 1: Synthesis scheme for 2-amino-4-aryl-6-substituted pyridine-3,5-dicarbonitrile derivatives

Table 1: Chemical compounds (S1-S4)

Compound code	Amine	R	Chemical formula	Mol wt
S1	$CH_3CH_2NH_2$	CH_3CH_2	$C_{15}H_{13}N_5$	263
S2			$C_{20}H_{15}N_5$	325
S3	NH_4OAc	H	$C_{13}H_9N_5$	235
S4			$C_{19}H_{13}N_5$	311

Synthesis of 2-Amino-6-(ethylamino)-4-phenylpyridine-3,5-dicarbonitrile (S1)

A mixture of arylidine (0.06 mol), ethylamine as a nucleophile (0.069 mol) and anhydrous $ZnCl_2$ (0.09 mol) in dry ethanol (20 mL) then solution was stirred for 5 h at 80 ˚C. The formed precipitate was filtered and crystallized from ethanol, and then methanol with yield of 94%. Melting point: 226 ˚C, **^{1}H-NMR** (DMSO-d_6) δ (ppm): 7.43-7.52 (5H, m, Ar-H), 7.23 (4H, s, NH_2), **FT-IR** (cm^{-1}): 1623 (C=N), 2225 (C≡N), 2978 (C-H, aliphatic), 3108 (N-H), **MS**: m/z:263.3 calculated for $C_{15}H_{13}N_5$.

Synthesis of 2-Amino-4-phenyl-6-(p-tolylamino) pyridine-3,5-dicarbonitrile (S2)

A mixture of arylidine (0.06 mol), p-toluidine as a nucleophile (0.069 mol) and anhydrous $ZnCl_2$ (0.09 mol) in dry ethanol (20 mL) then solution was stirred for 5 h at 80 ˚C. The formed precipitate was filtered and crystallized from ethanol, and then methanol with yield of 91%. Melting point: 258 ˚C, **^{1}H-NMR** (DMSO-d_6) δ (ppm): 2.26 (3H, s, CH_3), 7.09-7.54 (9H, m, Ar-H), 7.49 (2H, s, NH_2), 9.02 (1H, s, NH), **FT-IR** (cm^{-1}): 1630 (C=N), 2208 (C≡N), 3108 (N-H), **MS**: m/z:325.1 calculated for $C_{20}H_{15}N_5$.

Synthesis of 2,6-Diamino-4-phenylpyridine-3,5-dicarbonitrile (S3)

A mixture of arylidine (0.06 mol), ammonium acetate as a nucleophile (0.069 mol) and anhydrous $ZnCl_2$ (0.09 mol) in dry ethanol (20 mL) then solution was stirred for 5 h at 80 ˚C. The formed precipitate was filtered and crystallized from ethanol, and then methanol with yield of 93%. Melting point: 292 ˚C, **^{1}H-NMR** (DMSO-d_6) δ (ppm): 7.23 (4H, s, NH_2), 7.43-7.52 (5H, m, Ar-H), **FT-IR** (cm^{-1}): 1623 (C=N), 2206 (C≡N), 3363-3424 (N-H), **MS**: m/z:235.3 calculated for $C_{13}H_9N_5$.

Synthesis of 2-Amino-4-phenyl-6-(phenyl-amino) pyridine-3,5-dicarbonitrile (S4)

A mixture of arylidine (0.06 mol), aniline as a nucleophile (0.069 mol) and anhydrous $ZnCl_2$ (0.09 mol) in dry ethanol was carried out without stirring at higher temperature. The formed precipitate was filtered and crystallized from ethanol, and then methanol with yield of 90%. Melting point: 251 ˚C, **^{1}H-NMR** (DMSO-d_6) δ (ppm): 7.64 (2H, s, NH_2), 7.05-7.56 (10H, m, Ar-H), 910 (1H, s, NH), **FT-IR** (cm^{-1}): 1630 (C=N), 2208 (C≡N), 3155 (N-H), **MS**: m/z:311.1 calculated for $C_{19}H_{13}N_5$.

***In vitro* cytotoxicity studies**

Tumor cell line

Human lung cancer cell line (A549) was kept in DMEM High Glucose (4.5 g/L), with stable L-Glutamine, and Sodium Pyruvate (Biowest, MO, USA). Human breast adenocarcinoma (MCF-7, and MDA-MB-231), hepatocellular carcinoma (HepG2), human prostate cancer (PC3) and Cervical cancer (Hela) were kept in RPMI-1640 medium with L-Glutamine (Lonza SPRL, Verviers, Belgium). Both media were supplemented with 10% FBS (Seralab, UK) and 1% antibiotic-antimycotic (Biowest, MO, USA). All cell lines were incubated under standard conditions (37 °C, 5 % CO_2).

Evaluation of cell proliferation

MTT tetrazolium assay was employed to assess the cytotoxic potential of the tested compounds on various cancer cell lines [16]. First, in a 96-well plate, 100 µL cell suspension corresponding to 1 x 10^4 cells/well were seeded and incubated for 24 h. Serial dilutions of tested compounds in dimethyl sulfoxide (DMSO) (ranging from 0.1 to 100 µM) were prepared. At 24 h post incubation, spent culture medium was replenished with a fresh medium containing the specified serial dilutions of tested compounds. The cells were further incubated for 48 h and cell viability was estimated by the MTT assay as described previously [16]. A microplate reader (TEKAN Japan, Kanagawa, Japan) was used to measure the absorbance of each well at 570 nm. The percent viable cells were plotted against tested compound concentration to determine the IC_{50} values summarized in Table 2.

Molecular docking

X-ray crystal structure of the molecular target along with its co-crystallized ligand was downloaded from the RCSB protein data bank (http://www.rcsb.org) with PDB code 1DI8. Chemsketch software was used to draw the molecular structures of the tested compounds and VEGAZZ software was used to calculate energy minimization of the tested compounds [17].

The molecular target was prepared for docking *via* Auto Dock Tools by removal of water molecules and heteroatoms followed by addition of hydrogen atoms and charge assignment. Search space for docking was defined by a grid box of appropriate dimensions placed around the co-crystallized ligand. Finally, docking was performed *via* AutoDock Vina [18] while

visualization of docking results was done *via* Chimera. The docking protocol was validated by redocking the co-crystallized ligand onto the molecular target and comparing the best generated binding pose with that of the co-crystallized ligand [19].

RESULTS

Chemistry

The new target 2-amino-4-aryl-6-substituted pyridine-3,5-dicarbonitrile derivatives (S1 - S4) were produced with good yield by one-pot multicomponent reactions as previously described [15]. Scheme 1 outlines the synthesis of the designed target compounds. The structure of the target compounds was completely identified by spectral data, as represented in the experimental section. In the IR spectra, characteristic bands at 3108, 2225 and 1623 cm^{-1} was observed refereing to N-H, C≡N and C=N groups, respectively. In the ^{1}H-NMR spectra, the presence of aromatic prorons at 7.1 - 7.55 ppm and NH_2 protons at 7.23 ppm were clearly observed. Molecular ion peaks in the mass spectra provided additional verification for the structure of the newly synthesized pyridine derivatives.

In vitro cell viability study

In order to assess the possible cytotoxic effect of the synthesized pyridine derivatives, the *in vitro* cytotoxicity of the compounds (S1-S4), over a concentration range of 0.1 - 100 µM, was validated against six human cancer cell lines (HepG2, A549, MDA-MB-231, MCF-7, PC3, HELA). The cytotoxicity of 5-flurouracil (5-FU), a standard anticancer compound, was also determined under the same experimental condition for the purpose of comparison. The IC_{50} values, expressed in µM, of the newly synthesized compounds along with that of 5-FU are represented in Table 2.

It was obvious that all the synthesized compounds exerted a dose-dependent cytotoxic effects against PC3, HepG2, MDA-MB-231 (except for S2), and HELA (except for S3) cancer cell lines. In particular, all the compounds under investigation showed a superior cytotoxic activity against PC3 cancer cell line, compared to the standard anticancer compound (5-FU). The IC_{50} values against PC3 cell line were 0.45, 0.85, 0.1 and 0.56 µM for S1, S2, S3 and S4, respectively. On the other hand, all the tested compounds showed inferior cytotoxic effects against breast adenocarcinoma (MDA-MB-231) compared to 5-FU. The IC_{50} values were 28.2, 69.2 and 81.3 µM for S1, S3 and S4 vs. 0.49 µM for 5-FU. Most importantly, 5-FU and all tested compounds exhibited poor or no cytotoxic activities against A549 or MCF-7 cell lines.

Molecular docking

Molecular docking was conducted to examine the possible binding interactions of the target compounds to their proposed CDK2 target. CDK2 was chosen as a target based on its potential role in controlling apoptosis and restricting cancer progression [1,3]. Consequently, targeting CDK2 might offer potential benefits for cancer therapy. Molecular docking was performed using AutoDock Vina, while visualization was done using Chimera.

Initially, the co-crystallized ligand was redocked into its molecular target, and the best proposed binding mode was compared to that of the co-crystallized ligand in order to validate our docking protocol (Figure 1). Results of the redocking showed that there was an RMSD value of 1.047Å (< 2Å) between the original co-crystal ligand position and the best generated docked pose, indicating the validity of our docking protocol [20].

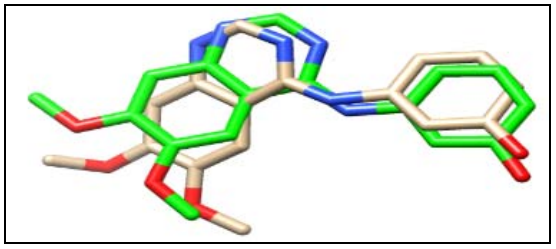


Figure 1: Docking validation by redocking the co-crystal ligands (Green color) to their corresponding receptors. Gray sticks represent the docked pose

Table 2: *In vitro* cytotoxicity activities of the tested compounds (S1-S4) against various cancer cell lines

Comp.	HepG2	MDA-MB-231	Hela	PC3	A549	MCF-7
S1	15.8	28.2	2.82	0.45	100	100
S2	0.2	100	1.2	0.85	100	100
S3	0.261	69.2	100	0.1	100	100
S4	0.242	81.3	74.1	0.56	100	100
5-FU	0.233	0.49	100	7.49	100	100

The binding modes of the synthesized pyridine derivative was analyzed to interpret their biological results and to gain further insight into the binding orientations and the activity of the new compounds (Figure 2). The X-ray crystallographic CDK2 enzyme substrate (PDB ID: 1DI8) being complexed with the reference ligand; 5-flourouracil, revealed the presence of two hydrogen bonds with LEU83 and LYS33. Interestingly, the presented docking study confirmed comparable binding modes between the designed compounds and the docked molecules. The binding energy and the binding mode of the biologically active synthesized compounds are summarized in Table 3.

Molecular docking analysis of the new compounds revealed that compounds S1, S2 and S4 retained at least one essential H-bonds with either LEU83 or LYS33, when compared with 5-fluorouracil. Furthermore, whereas compounds S1 and S3 forms only one H-bond with LEU83 or ASP145, respectively, and with different binding mode than that of the lead compound, they showed higher antitumor activity against PC3 cancer cell line.

Compound S2 showed -9.4 kJ mol^{-1} binding energy, forming two amino acid hydrogen bonds with LYS33 and GLN131 and exhibited superior antitumor efficacy against PC3 and Hela cancer cells when compared to the ligand reference. Similarly, Compound S4 shows -9.5 kJ mol^{-1} binding strength, forms four hydrogen bonds with amino acid and showed potent antitumor activities against PC3 and Hela cancer cells compared to the ligand reference.

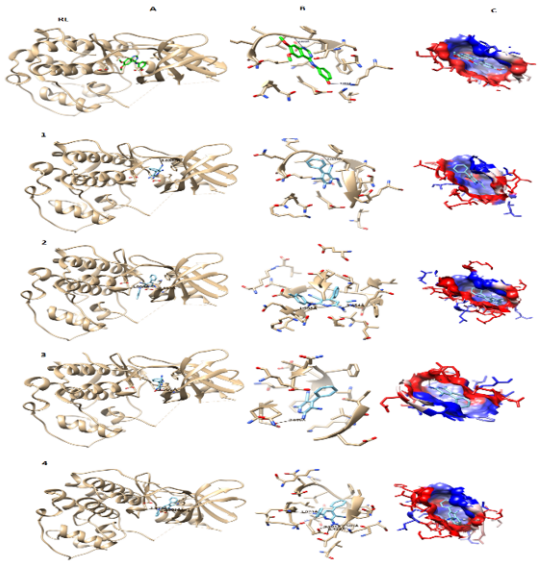


Figure 2: Interaction of the tested compounds and reference ligand (RL) with CDK2 protein. A) 3D interaction, B) H-bond formation, and C) hydrophobic interaction represented by blue color

DISCUSSION

Despite advances in diagnosis and care over the last 40 years, cancer remains a significant global public health problem. The search for new drug entities with anticancer activities is a complex and costly task, with only a few new compounds finding their way to the market, after being clinically tested. High Throughput Screening (HTS) is a critical method for detecting initial lead compounds for a particular target disease [21]. Unfortunately, HTS shows a high failure rate and, in many situations, fails to detect ideal viable drug leads.

Table 3: Results of the docking study of the test compounds against CDK2 binding pocket

Comp. no.	*CDK2*				Hydrophobic interaction
	Energy of free binding	H-Bond			
	ΔG_b [a]	*No.*	*Amino acid*	*Length Å*	
1	-8.6	1	LEU83	3.039	ILE10, LEU83, LEU134, LEU148, PHE82, VAL18, VAL64
2	-9.4	2	LYS33 GLN131	3.454 1.998	ILE10, LEU83, LEU133, LEU134, LEU148, LEU298, PHE82,VAL18, VAL30, VAL64
3	-8.3	1	ASP145	3.566	ILE10, VAL18, VAL30, VAL64
4	-9.5	4	LYS33 ASP145 ASP145 GLN131	3.503 2.429 2.501 1.973	ILE10, LEU83, LEU134, LEU148, PHE82, VAL18, VAL64
Reference ligand	-8.3	2	LYS33 LEU83	2.831 2.803	ILE10, LEU83, LEU133, LEU134, LEU148, LEU184, PHE82VAL18, VAL64

Molecular docking (an alternative HTS method) is a popular component of the drug discovery process and provides a straightforward way of evaluating possible binders from large chemical libraries with minimal costs [22].

Cyclin dependent kinase 2 (CDK2) is a well-known therapeutic target for conquering cancer [23]. In this study, we used a one-pot multicomponent condensation reaction to synthesize novel pyridine derivatives, and we investigated their efficiency as plausible inhibitors for CDK2 enzyme. All the compounds were efficiently synthesized with good yield. In addition, all the synthesized compounds showed remarkable cytotoxic activity against various tumor cell lines, particularly, prostate cancer (PC3) cell line compared to a reference compound, 5-fluorouracil (IC_{50} 7.49 µM). Furthermore, molecular docking studies in the CDK2 active site disclosed the efficient binding of the synthesized compounds to CDK2 active site, which was closely correlated with the cytotoxic potential of the newly synthesized compounds.

The MTT assay has been extensively used in cytotoxicity experiments to screen newly synthesized compounds for anticancer potential [16]. In this study, all the synthesized compounds demonstrated significant cytotoxic effect against various tumor cell lines, particularly, prostate cancer (PC3) cell line with an IC_{50} ranged from 0.1 to 0.85 µm, compared to a reference compound, 5-fluorouracil (IC_{50} 7.49 µm). The remarkable cytotoxic activities of the tested compounds when compared to 5-FU could be attributed to (i) the presence of an aryl hydrophobic group in position-4 of the pyridine ring which enhances binding energy via occupation of the unoccupied hydrophobic region binding pocket, and (ii) the presence of CN and NH groups at phenyl pyridine ring.

One of the most challenging computational chemistry tasks is to predict protein/ligand binding affinity. In this study, molecular docking revealed efficient binding interactions of the target compounds to their proposed CDK2 target (Figure 2). Furthermore, analysis of the binding modes of the synthesized pyridine derivative demonstrated that compounds S2 and S4 showed higher binding energies, compared to the ligand reference (Table 3). Collectively, these results suggest that the superior antitumor efficacy of compounds S2 and S4 against PC3 and Hela cancer cells, compared to the ligand reference, is closely correlated with the efficient binding of these compounds and the docked molecules.

CONCLUSION

Using a one-pot multicomponent condensation process, novel 2-amino-4-aryl-6-substituted pyridine-3,5-dicarbonitrile derivatives have been successfully synthesized. All the synthesized pyridine derivatives exhibit remarkable *in vitro* cytotoxic activities against various cancer cell lines. In addition, molecular docking results suggest that the synthesized pyridine derivatives might represent plausible CDK2 inhibitors for potential application in cancer therapy. Nevertheless, pharmacological studies are required to confirm their antitumor efficacy *in vivo*.

DECLARATIONS

Acknowledgement

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Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Amr Abu Lila, Marwa Abdallah, El-Sayed Khafagy and Shadeed Gad designed the study and supervised the data collection. Mohamed Omran and Kareem Younes analyzed and interpreted the data. Tamer Shehata and Mahmoud Soliman prepared and reviewed the manuscript for publication. All authors have read and approved the manuscript.

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