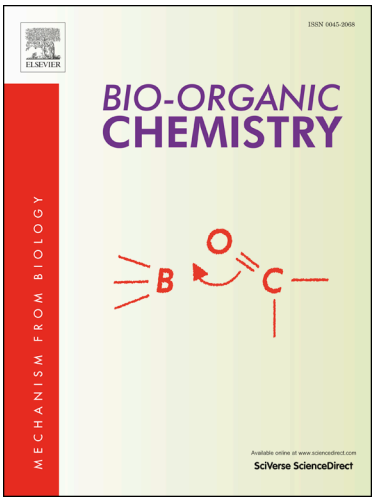

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Design and synthesis of novel imidazo[4,5-*b*]pyridine based compounds as potent anticancer agents with CDK9 inhibitory activity

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Abstract

New imidazo[4,5-*b*]pyridine derivatives were designed, synthesized and screened for their anticancer activity against breast (MCF-7) and colon (HCT116) cancer cell lines. Nine compounds (**I, II, IIIa, IIIb, IV, VI, VIIa, VIII, IX**) showed significant activity against MCF-7, while six compounds (**I, VIIc, VIIe, VIIf, VIII, IX)** elicited a remarkable activity against HCT116. Compounds showing significant anticancer activity revealed remarkable CDK9 inhibitory **potential** (IC_{50}= 0.63-1.32 μM) relative to **sorafenib (IC_{50}= 0.76μM)**. Moreover, a molecular docking study was performed to illustrate the binding mode of the most active compounds in the active site of CDK9 and revealed superior binding **affinity** relative to the natural ligand (T3C).

Key words: Imidazo[4,5-*b*]pyridines, anticancer, CDK9, HCT116, MCF-7.

1. Introduction

Cancer is considered as **the** second leading cause of death after cardiovascular diseases [1]. Targeted chemotherapeutic agents have advantages over the traditional ones due to their selectivity towards cancer cells and lower side effects [1].

Cyclin Dependent Kinases (CDKs) are a group of serine/threonine kinases. They are instrumental regulatory enzymes involved in cell cycle progression and cell proliferation. Their hyperactivity in breast, lung as well as colorectal tumors contributes to cancer cell proliferation, accordingly their inhibition is a valid targeted anticancer approach [2][3]**. Nevertheless, cells lacking CDK 2, 4 and 6 can proliferate normally** [4]**. Thus in many cancer cases specifically targeting those CDKs may not result in optimum activity. This may be due to cell compensation mechanisms and functional substitution. This drew the attention to targeting CDKs affecting transcription, namely CDK9/7** [5]**.**

CDK9 regulates the RNA transcription of short-lived anti-apoptotic proteins [6]. **Literature review revealed** that the scaffolds showing CDK9 inhibitory action include chromones, thiazole [7], pyrimidines [5,8,9], macrolides [10], imidazoles, purines, and aminopyrazole [7, 11].

It is worth pointing out that, the imidazoline derivative **A** is a potent CDK9 inhibitor with promising anticancer activity against HCT116 colon cancer cell line [12]. Furthermore, the imidazo[4,5-*b*]pyridine **B** was reported to have a potent anti-proliferative activity against MCF-7 **(IC_{50}: 0.58 – 78.66 µM)** and HCT116 **(IC^{50}: .49 – 87.33µM)** cancer cell lines **via selective inhibition of CDK9** [12,13] (Figure 1). Accordingly, the present study deals with the **design and** synthesis of new imidazo[4,5-*b*]pyridines as potential anticancer agents targeting CDK9 enzyme with improved activity.

The synthetic route adopted to prepare the new imidazo[4,5-*b*]pyridine derivatives was based on reaction of the key intermediate 4(1*H*)-imidazo[4,5-*b*]pyridine-2-yl)aniline **I** with either diethylethoxymethylenemalonate to obtain **II** followed by cyclization to the appropriate pyrazolidine **IIIa,b** and **IV,** pyrimidine **Va,b**, triazepine **VI** or via diazotization of the amino functionality of **I** followed by coupling with phenol, aniline or active methylene derivatives to

afford **VIIa-f**, **VIII** and **IX,** respectively. The biological evaluation of the synthesized compounds was performed in terms of anti-proliferative activity against MCF-7 and HCT 116 cancer cell lines and CDK9 enzyme inhibitory assay. Furthermore, a docking study of the synthesized imidazo[4,5-*b*]pyridine compounds and the native ligand T3C was carried out in the CDK9 active site in order to explore and compare their binding mode and validate their mechanism of action.

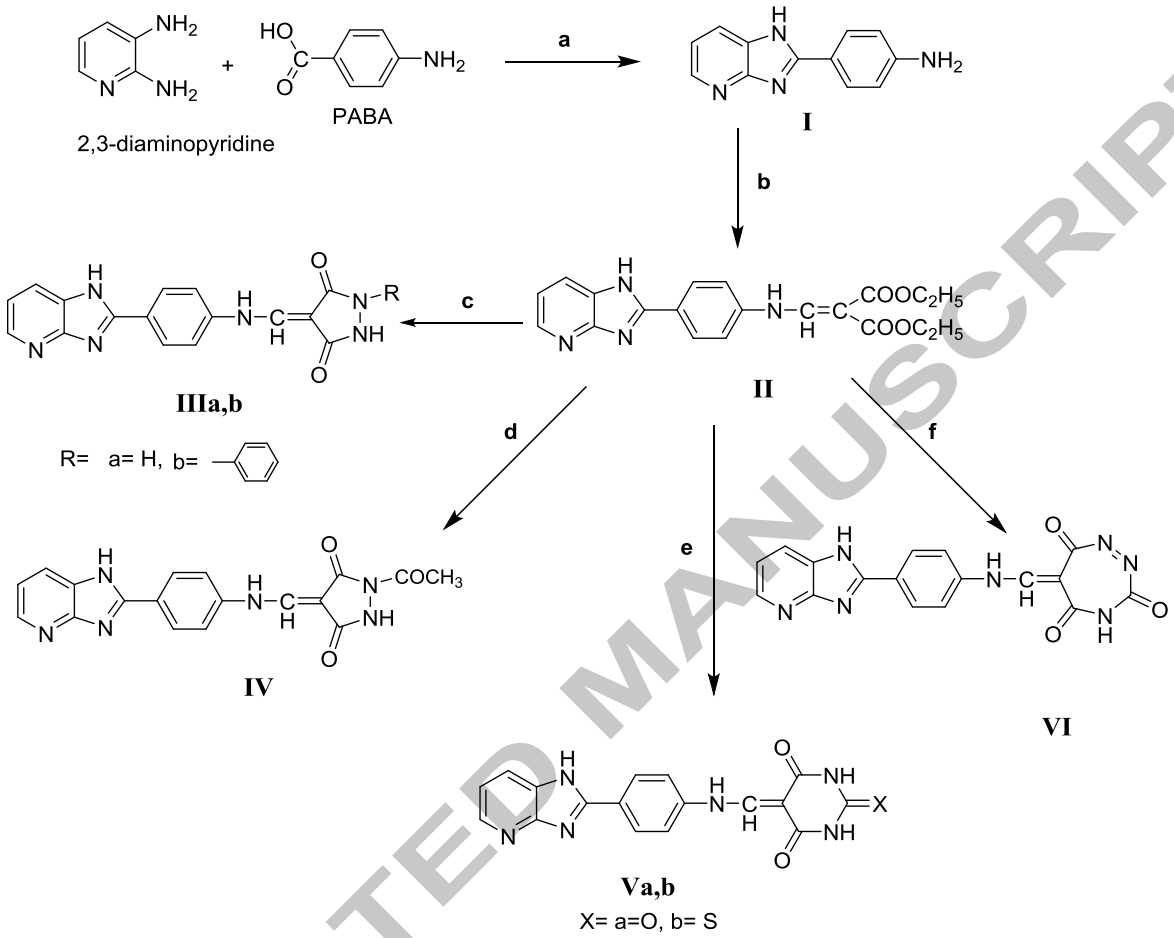
2. Results and discussion

2.1.Chemistry

The novel imidazo[4,5-*b*]pyridine derivatives were synthesized according to Schemes 1 and 2. **4-**(1*H*-imidazo[4,5-*b*]pyridin-2-yl)aniline **I** was prepared via condensation of 2,3-diaminopyridine with 4-aminobenzoic acid (PABA) in the presence of polyphosphoric acid (PPA). IR spectrum of **I** showed a forked band at 3344 and 3300 cm^{-1}assigned to NH_2 group, a broad band at 3128-3400 cm^{-1}attributed to NH group. ^{1}HNMR spectrum revealed two exchangeable singlet signals at 2.50 and 5.69 ppm attributed to NH and NH_2 protons respectively, in addition to doublet, doublet of doublet and doublet signals at 7.84, 7.90 and 8.20 ppm, respectively corresponding to pyridine protons. Reaction of **I** with diethyl ethoxymethylenemalonate in absolute ethanol resulted in the diethyl methylenemalonate derivative **II.** IR spectrum showed a strong band at 3402- 3325 cm^{-1} attributed to NH groups in addition to a band at 1708-1689 cm^{-1} assigned to C=O of ester.^{1}H NMR spectrum revealed the presence of the two geometrical isomers appearing as two adjacent singlet signals assigned to the olefinic proton at 5.92-5.94 ppm equal to 0.5 proton which does not disappear after D_2O, the triplet quartet pattern at 1.22 and 1.28 ppm and 4.11 and 4.22 ppm corresponding to $2C_2H_5$ protons, two exchangeable singlet signals at 2.51 and 9.05 ppm of 2NH protons. Furthermore, refluxing the diester **II** with hydrazine hydrate or phenyl hydrazine in the presence of Na ethoxide afforded **IIIa** or **IIIb**, respectively. Refluxing **II** with hydrazine hydrate in glacial acetic acid gave **IV**. IR spectra of **IIIa, IIIb** and **IV** showed a strong absorption band at 3194-3317 cm^{-1}corresponding to NH groups, a band at 3043-3100 cm^{-1} attributed to CH aromatic and a band at 1670-1708 cm^{-1} assigned for C=O groups. ^{1}H NMR spectra indicated the disappearance of aliphatic protons of the ethyl groups in addition to the presence of exchangeable singlet signals at 5.31-9.31 ppm corresponding to NH protons. Compound **IV** showed a singlet signal at 2.50 ppm attributed to CH_3 protons, whereas compound **IIIb** revealed

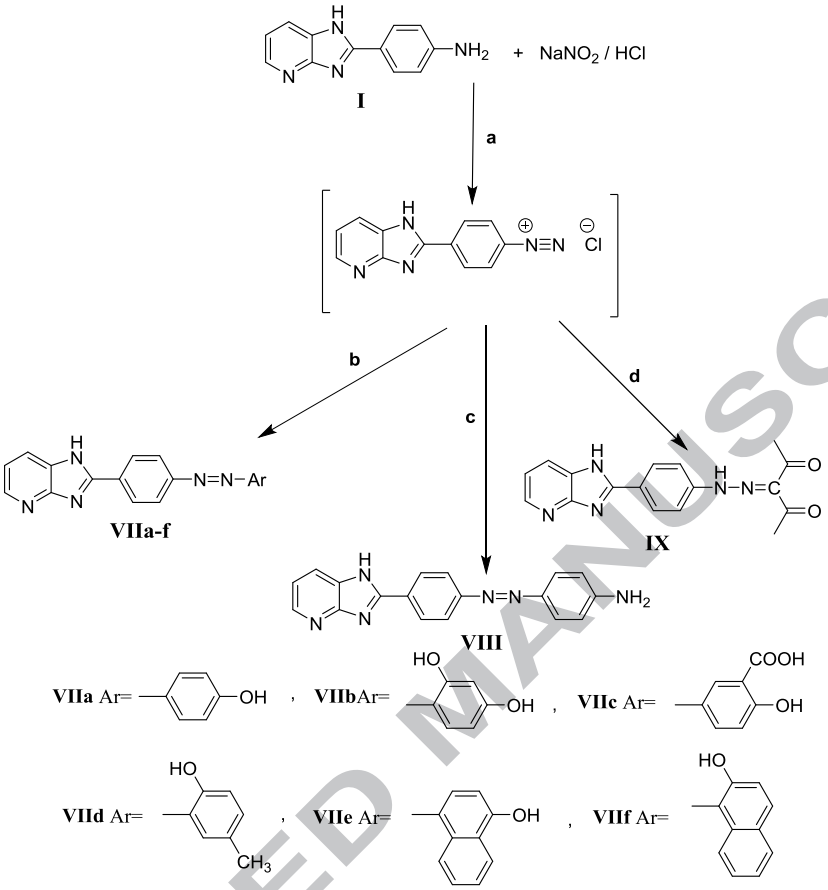
an increase in the integration of aromatic protons 6.63-8.11 ppm due to additional phenyl ring, moreover, the mass spectrum of compound **IV** displayed a molecular ion peak M^+ at of 396 m/z. Similarly, Pyrimidinetrione **Va** and thioxopyrimidinedione **Vb** were prepared via the condensation of diethylmalonate derivative **II** with urea or thiourea in the presence of sodium ethoxide. IR spectra of **Va,b** showed bands at 3275,3170 cm^{-1}corresponding to NH groups, and a band at 1650-1678 cm^{-1} assigned to C=O, while compound **Vb** showed additional band at 1261 cm^{-1} due to C=S. ^{1}H NMR spectra of compounds **Va,b** revealed an exchangeable singlet signal at 10.0 ppm corresponding to additional NH,a singlet signal at 5.42 ppm attributed to olefinic CH proton in addition to doublet, triplet and doublet signals of pyridine protons at 7.38, 8.16 and 8.26 ppm respectively. On the other hand, reaction of diethylmalonate ester **II** with semicarbazide in the presence of sodium ethoxide led to cyclization and formation of triazepine ring followed by the loss of hydrogen to form a more stable form of triazepinetrione derivative **VI**. IR spectrum showed a broad band at 3500- 3336 cm^{-1}assigned to NH groups in addition to a band at 1681cm^{-1} attributed to 3C=O. ^{1}H NMR spectra of **VI** displayed a singlet signal at 10.8 ppm exchanged with D_2O corresponding to additional NH proton and a singlet signal at 6.35 ppm attributed to olefinic CH proton (Scheme 1). **Proposed mechanisms are illustrated in figure 2.**

Scheme 1: Synthesis of 1*H*-imidazo[4,5-*b*]pyridine derivatives I-VI



Reagents and reaction conditions: a: PPA, 220 ^{o}C, 3h, **b:** diethylethoxymethylene malonate / ethanol, reflux 6 h, **c:** hydrazine hydrate/phenyl hydrazine, Na ethoxide in ethanol, reflux 6 h, **d:** hydrazine hydrate in glacial acetic acid, reflux 6 h, **e:** urea or thiourea / Na ethoxide in ethanol, reflux 6 h, **f:** semicarbazide / Na ethoxide in ethanol, reflux 6 h.

Scheme 2: Synthesis of substituted 1*H*-imidazo[4,5-*b*]pyridine derivatives VIIa-f, VIII and IX



Reagents and reaction conditions: a: 0-5 °C, **b**: Ar-OH/ NaOH,0-5 °C, **c**: Aniline, 0-5 °C, **d**: Acetyl acetone, NaOH, Ethanol.

Additionally, compounds **VIIa-f** were prepared by reacting the diazonium salt of **I** with the appropriate phenolic compounds namely, phenol, resorcinol, salicylic acid, *p*-cresol, α-naphthol and β-naphthol, in 10% sodium hydroxide at 0-5℃ (Scheme 2). IR spectra lacked the NH_2 band and showed the appearance of a broad band at 3394-3420 cm^{-1} assigned to OH group. Additionally, compound **VIIc** showed a band at 1680 cm^{-1}attributed to C=O group. ^{1}H NMR spectra displayed the disappearance of the singlet signal of NH_2 protons and indicated the presence of an exchangeable singlet signal at 4.59-7.12 ppm of the OH proton, while compound **VIIc** revealed additional exchangeable singlet signal at 16.09 ppm corresponding to COOH proton. Compound **VIId** showed a singlet signal at 2.30 ppm attributed to CH_3 protons. Moreover, compounds **VIIe**, **VIIf** elicited additional aromatic protons at 6.64-8.49 ppm corresponding to naphthalene ring. The mass spectrum of **VIIf** showed a molecular ion peak M^+

at 365 m/z. Similarly, **VIII** was obtained via reaction of the diazonium salt of **I** with aniline at 0-5ºC.IR spectrum showed the appearance of NH_2band at 3317 cm^{-1}in addition to NH band of imidazopyridine ring at 3197 cm^{-1}. ^{1}H NMR spectra revealed the appearance of a singlet signal of NH_2 protons at 4.05 ppmand a singlet signal at 4.37 ppm assigned to NH protons that were exchanged with D_2O. Finally, reaction of the diazonium salt of **I** with acetylacetone in ethanol in the presence of sodium hydroxide afforded compound **IX.** IR spectrum showed the appearance of NH band at 3402 $cm^{-1}$and the C=O band at 1670 cm^{-1}in addition to aliphatic CH band at 2924 cm^{-1}. ^{1}H NMR spectra indicated two singlet signals at 2.46 and 2.48 ppm corresponding to two CH_3protons in addition to two exchangeable signals at 4.46 and 13.70 assigned to 2NH protons.

2.2 *Anticancer activity*

2.2.1. In- vitro MTT assay

The anticancer activity of the newly synthesized compounds was performed against breast cancer cell line (MCF-7) and colon cancer cell line (HCT-116) using MTT assay according to the Mosmann's method [14,15]. Each experiment was performed in triplicate. The assay was performed in a concentration range of 0.01, 0.1, 1, 5, 10, 100 and 200 µM. **Cisplatin was used as an in-house internal standard while sorafinib was used as a positive control**. There was a good reproducibility between replicate wells with standard errors below 10 %. The results were expressed in terms of IC_{50} [µM] (**Table 1**).

Concerning MCF-7 breast cancer cell line, imidazopyridin-2-ylaniline derivative **I** and its diethylmethylenemalonateester **II** elicited potent anti-breast cancer activity (IC_{50} = 0.71 and 0.78µM), respectively, compared to cisplatin (IC_{50} = 2.84 µM) **and sorfinib (IC_{50}=4.16 µM)**. Cyclization of **II** to un/substituted pyrazolidinedione **IIIa, IIIb** and **IV** resulted in remarkable activity with IC_{50} of 1.59, 0.97 and 0.86 µM, respectively. Pyrimidine derivatives **Va, Vb** (IC_{50}= 4.80, 3.24 µM) were less active than the pyrazolidinedione (**IIIa and IIIb).** 2-Thioxopyrimidinedione **Vb** was slightly more active than its trione congener **Va**. In addition, further ring expansion to triazepine significantly increased the activity resulting in the most potent compound **VI** (IC_{50} =0.63 µM).

Coupling of diazonium salt with monohydroxy phenol resulted in compound **VIIa** with a promising activity (IC_{50} =1.23 µM), while dihydroxyphenol (resorcinol) **VIIb** greatly decreased the activity (IC_{50} = 8.90 µM). Additionally, the bioisosteric substitution of 4-OH with NH_2 **VIII**

elicited significant activity (IC_{50}= 2.11 μM) but less than that expressed by the 4-OH analogue. Moreover, Coupling of diazonium salt with phenol bearing an electron withdrawing group (COOH) **VIIc** showed moderate activity (IC_{50} = 5.60 μM), while electron donating group (*m*-CH_3) **VIId** markedly decreased the activity (IC_{50} = 27.04 μM). Coupling with 1-naphthol gave **VIIe** exhibiting moderate activity (IC_{50}= 5.77 μM), whereas 2-naphthol derivative **VII** revealed a sharp decrease in activity (IC_{50} = 12.70 μM). Also, substitution of diethylaminomethylenemalonate moiety of **II** by hydrazono pentane-2,4-dione produced **IX** showing a slight decrease in the anticancer activity (IC_{50}= 2.11 μM) relative to that expressed by **II** (0.78 μM), however, it was still more potent than cisplatin (IC_{50} = 2.84 μM) **and sorfinib (IC_{50}=4.16 μM)**.

Regarding HCT116 colon cancer cell line, six compounds **I**, **VIIc, VIIe, VIIf**, **VIII** and **IX** elicited promising anticancer activity as denoted by their IC_{50} values (1.69-3.06 μM) compared with cisplatin (IC_{50}= 2.82 μM) and **sorfinib (IC_{50}=6.12)**. The most active ones were hydrazonopentadione **IX**, diazenylbenzeneamine **VIII**, diazenyl naphthalen-2-ol **VIIe,** diazenylnaphthalen-1-ol **VIIf** and diazenyl 2-hydroxybenzoic acid **VIIc** with IC_{50} values 1.69, 1.83, 2.14, 2.31 and 2.52μM, respectively. **Figure 3 illustrates the structural activity relationship of the designed compounds.**

Finally, we can conclude that 9 out of 16 tested compounds exhibited promising anticancer activity against MCF-7. Five compounds **I**, **II**, **IIIb**, **IV**, **VI** elicited superior activity at a submicromolar level (IC_{50}= 0.63-0.97 μM) where compound **VI** is the most active one (IC_{50}=0.63 μM). **It's worth pointing out that each of these compounds was selectivity potent against one of the two tested cell lines. Nine compounds (I, II, IIIa, IIIb, IV, VI, VIIa, VIII, IX) showed significant activity against MCF-7, while six compounds (I, VIIc, VIIe, VIIf, VIII, IX) elicited a remarkable activity against HCT116 (Table 1).**

Table 1: *In vitro* growth inhibitory activity (IC_{50}μM) of compounds **I-IX** against MCF-7 breast cancer cell line and HCT-116 colon cancer cell line (MTT method):

Compound	Antiproliferation (IC_{50}, μM) MCF-7	Antiproliferation (IC_{50}, μM) HCT-116
Cisplatin	2.84	2.82
I	0.71	3.06

II	0.78	4.06
IIIa	1.59	4.65
IIIb	0.97	5.45
IV	0.86	4.03
Va	4.80	3.94
Vb	3.24	6.06
VI	0.63	5.30
VIIa	1.23	5.45
VIIb	8.90	6.43
VIIc	5.60	2.52
VIId	27.04	3.77
VIIe	5.77	2.14
VIIf	12.7	2.31
VIII	2.84	1.83
IX	2.11	1.69
Sorafinib	4.16	6.12

2.2.2. CDK9 Enzyme inhibition assay

Compounds (**I**, **II**, **IIIa**, **Vb**, **VI, VIIa** and **IX**) which elicited superior anticancer activity against one or both tested cancer cell lines were further screened for their CDK9 enzyme inhibitory activity in order to validate their proposed mechanism of action. **Sorafinib was used as a reference compound due to its kinase inhibitory potential.** Results were expressed in IC_{50} [µM] (**Table 2**). The tested compounds were active inhibitors of CDK9 (IC_{50} = 0.50-2.06 µM). The un-substituted aniline **I,** triazepine **VI** and hydrazonopentane-2,4-dione **IX** exhibited superior CDK9 inhibitory activity with IC_{50} ranging from 0.50 to 1.002 µM.

These results strongly agree with the *in-vitro* anti-proliferative assay where compounds **I** and **VI** showed a superior anticancer activity at a submicromolar level against MCF7 with (IC_{50}=0.71 and 0.63 µM), respectively. Also, compound **IX** was the most active against HCT116 cell line (IC_{50} = 1.69 µM).

Table 2. IC_{50} µM of the most active compounds as CDK9 enzyme inhibitors

Compound	IC_{50} (µM)
I	0.95
II	2.06

IIIa	1.32
VI	0.50
VIIa	1.10
IX	1.002
Sorafinib	**0.76**

2.3.Docking study

In order to validate the CDK9 inhibitory activity, docking study was performed and results were compared to those of the native ligand (T3C) **and the multi-kinase inhibitor sorafinib as a reference compound.** The data revealed that most of compounds had acceptable binding affinity as compared to T3C (**Table 3).**

The energy scores (Kcal/mol) of the native ligand binding affinity with the CDK9 was -21.96. This binding resulted from interaction between the amino group, phenyl ring, 1,4-diazepine, carbonitrile group, aminothiazole ring and pyrimidine moieties of the T3C native ligand and the ATP pocket of the CDK9. The main amino acid residues involved in the interaction were Cys 106, Ile 25, Val 33, Asp 109, Leu 156, Asp 167 and Ala 46.

Our designed compounds showed **comparable** affinity to CDK9 ranging from -16.84 to -26.72Kcal/mol. The interacting chemical groups of the proposed compounds and the corresponding amino acid residues of the CDK9 binding site are described in **table 3.**

Furthermore, docking study against CDK2/4 and 6 was performed in order to compare the selectivity of the test compounds against different CDKs. Results revealed that the compounds showed stronger selectivity towards CDK9 over other CDKs (Data are presented in supplementary materials tables 1 and 2).

Table 3. Docking Study of the native ligand, and the designed compounds:

Compound ID	Energy Score S (Kcal/mol)	Amino Acids Interaction	Interacting Groups	Hydrogen Bond Length (Å)
T3C Native ligand	-21.96	Cys 106	NH	2.05
		Ile 25	Phenyl ring	
		Val 33	Phenyl ring	
		Asp 109	1,4-diazepine	
		Leu 156	Carbonitrile	
		Asp 167	Aminothiazole ring	
		Ala 46	Pyrimidine ring	
I	-16.84	Asp 109	Phenyl ring	

		Ile 25	Phenyl ring	
		Val 33	Pyridine ring	
II	-23.81	Glu 107	NH imidazole	1.75
		Val 33	Phenyl ring	
		Gly 112	Pyridine ring	
		Asp 109	Pyridine ring	
		Leu 156	C=O	
		Ile 25	CH_3	
		Ala 153	Phenyl ring	
		Thr 29	Phenyl ring	
		His 108	Phenyl ring	
IIIa	-21.03	Lys 48	C=O	2.55
		Leu 156	C=CH	
		Phe 105	C=O	
		Ile 25	Phenyl ring, NH imidazole	
		Glu 107	Pyridine ring	
		Asp 167	Imidazole ring	
VI	-26.72	Lys 48	C=O	2.78
		Asp 109	Imidazopyridine ring	
		Leu 156	C=CH	
		Ile 25	Phenyl ring	
		Gly 112	Imidazopyridine ring	
		Asp 167	C=O	
VIIa	-24.73	Glu 107	NH Imidazole	1.88
		Asp 109	Pyridine ring	
		Ile 25	Phenyl ring	
		Leu 156	Phenyl ring	
IX	-21.44	Lys 48	N pyridine	2.85
		Ile 25	NH amino	1.75
		Asp 109	Diester group	
		Thr 29	Phenyl ring	
		Asp 167	Phenyl ring	
		Val 33	Phenyl ring	
Sorafinib	**-20.9152**	**Ile 25**	**Amide group**	
		Asp 109	**Pyridine ring**	
		Glu 107	**Phenyl ring**	
		His 108	**Methyl group**	
		Leu 156	**Methyl group**	
		Asp 167	**Trifluoromethyl group**	

2.4. Assessment of physicochemical properties and drug likeness

The Swiss-Adme tool [16] was used to determine physicochemical properties of the novel compounds. The molecular properties of the synthesized compounds, the theoretical lipophilicity and water solubility of the compounds are shown in Tables **3- 6** of supplementary materials.

Theoretical assessment of pharmacokinetics and cytochrome inhibition potential of the synthesized molecules is displayed in **table 7 of the supplementary materials**. The data show that the molecular attributes of the designed molecules have high potential to be absorbable from GIT except for compounds **IIIa**, **Vb** and **VIIc**. Apart from compound **I**, all the molecules cannot cross blood brain barrier. The designed compounds are not susceptible to Pgp efflux except **I**, **IIIa** and **IV**. Compounds **IV**, **VI** and **VIIc** are not expected to inhibit any cytochrome enzyme. Collectively compound **VI** is predicted to have best pharmacokinetic attributes.

Compound **I** show desirable drug-likeness attributes according to Lipinski, Ghose, Veber, Egan and Muegge rules. From medicinal chemistry perspective, the compound can be easily synthesized.

Similarly compound **II** had desirable drug-likeness however the compound molecular weight is more than 350 with more than 7 rotatable bonds and elevated lipophilicity. These characteristics slightly weaken its lead likeness.

Compound **IIIa** is compatible with Lipinski, Ghose, Veber and Muegge filters. It is considered as a lead like compound.

Compound **IIIb** has desirable drug likeness as per all medicinal chemistry filters. The lead likeness is compromised because its molecular weight (MW) is more than 350. Similarly was compound **IV.**

Compound **Va** attributes make it a drug-like compound. Additionally, the compound may act as a perfect lead like compound.

Compound **Vb** attributes make it a good drug like compound except for its elevated topological polar surface area which makes it incompatible with Veber and Egan rule. Its lead likeness may be compromised due to its larger molecular weight (>350).

Drug likeness of compound **VI** is similar to those of compound **Vb**. The large molecular weight (>350) is a manageable concern against its lead-likeness.

Compounds**VIIa-e** are perfect drug like compounds however their lead-likeness is compromised, while compound **VIIf** has an elevated LogP which hinders its compatibility with Muegge, Egan and Ghose as well as its drug-likeness.

Compounds **VIII** and **IX** have good drug-likeness where **IX** shows an additional good lead likeness.

Collectively the good predicted **pharmacokinetic profile** and drug-likeness attributes along with the improved docking profile of the proposed compounds strongly support their biological evaluation.

3. Conclusion

In this study, novel imidazo[4,5-*b*]pyridine derivatives were designed and synthesized. The synthesized derivatives had promising anticancer activity against either breast or colon cancer cell lines. Three compounds elicited remarkable activity against both cell lines **I**, **VIII**, **IX**. It is worth pointing out that, compound **VI** was the most potent one exhibiting a superior activity at a submicromolar level against breast cancer cell line (IC_{50}= 0.63 μM) and CDK9 enzyme assay (IC_{50}= 0.50 μM). Notably the molecular attributes of compound **VI** predict optimum pharmacokinetic profile and acceptable drug likeness. Finally, the promising anticancer activity of diazenyl benzamine **VIII** against MCF-7 and HCT116 cell lines (IC_{50}= 2.84 and 1.83 μM) and hydrazono pentane-2,4-dione **IX** (IC_{50} = 2.11 and 1.69 μM) along with its desirable drug-likeness attributes directed our attention to perform a future study by coupling diazonium salt of **I** with appropriate primary amines and methylene derivatives.

4. Experimental

4.1.Chemistry

2,3-Diaminopyridine and diethyl ethoxymethylenemalonate were purchased from Acros Organics. All reactions were monitored by TLC (aluminum sheet) using $CHCl_3/CH_3OH$ (9:1) and all spots were visualized at 254, 366 nm by UV Vilber Lourmat 77202 (Vilber, Marne La Vallee, France). All melting points were determined by open capillary tube method using Gallen Kamp melting point apparatus MFB-595-010M (Gallen Kamp, London, England) and were uncorrected. Elemental microanalysis was carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University. Infrared spectra (IR) were recorded as potassium bromide discs on Schimadzu FT-IR 8400S spectrophotometer (Shimadzu, Kyoto, Japan) and expressed in wave number (υ_{max}) cm^{-1}. ^{1}HNMR and ^{13}CNMR spectra were recorded on Varian Mercury VX-300 NMR spectrometer at 300 MHz and Bruker NMR spectrometer at 400 MHz. Chemical shifts are quoted in δ as parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. Mass spectra were recorded using Schimadzu Gas Chromatograph Mass spectrometer- QP 100 Ex (Schimadzu).

*4.1.1. 4-(1H-imidazo[4,5-b]pyridin-2-yl)aniline(**I**)*

2,3-Diaminopyridine (5.45 g, 0.05 mole), 4-aminobenzoic acid (6.85 g, 0.05 mole) in polyphosphoric acid (PPA) (85 g) were heated in an oil bath at 220 ºC for 3 h. The reaction mixture was cooled, and then poured onto ice-cooled 10% sodium carbonate solution (1 Liter). The formed solid product was filtered, washed with water. The crude product was crystallized from aqueous methanol. Yield (86%), m.p 328-330 ºC. IR υ_{max}, cm^{-1}: 3344, 3221 (NH_2), 3128 (NH), 3067 (CH Ar), 1630 (NH), 1604 (C=N), 1543, 1473 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.50 (s, 1H, NH exchanged with D_2O), 5.69 (s, 2H, NH_2, exchanged with D_2O), 6.67 (d, J = 8.64 Hz, 2H, H Ar), 7.12 (dd, J = 4.84, 7.84 Hz, 1H, H imidazopyridine), 7.84 (dd, J = 1.36, 7.88 Hz, 1H, H imidazopyridine), 7.90 (d, J = 7.52 Hz, 2H, H Ar), 8.20 (dd, J = 1.40, 4.80 Hz, 1H, H imidazopyridine). ^{13}CNMR (101 MHz, DMSO-d_6): δ 154.85, 152.02, 151.63, 142.80, 128.62, 122.75, 117.58, 117.20, 113.95, 111.96.MS m/z %:210 (M^+). Anal. calcd for $C_{12}H_{10}N_4$; C, 68.56; H, 4.79; N, 26.65; Found: C, 68.79; H, 4.88; N, 26.94.

*2-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}malonate (**II**).*

Equimolar amounts of compound **I** (4.2 g, 0.02 mole) and diethyl ethoxymethylenemalonate (4.32 g, 4.04 ml, 0.02 mole) in absolute ethanol (60 ml) were refluxed for 6 h. The solvent was evaporated under reduced pressure. The crude product was crystallized from ethanol. Yield (73%), m.p 151-152 ºC. IR υ_{max}, cm^{-1}: 3402, 3325 (2NH), 3063 (CH Ar), 2931 (CH Aliph), 1708 (2C=O), 1654 (NH), 1600 (C=N), 1593, 1481 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.22 (t, J = 7.08 Hz, 3H, CH_3), 1.28 (t, J = 7.24 Hz, 3H, CH_3), 4.11 (q, J = 7.04 Hz, 2H, CH_2), 4.22 (q, J = 7.08 Hz, 2H, CH_2), 5.92 (s, 1H, H olefinic, =C**H**), 6.66-6.69 (dd, J = 4.92, 7.68 Hz, 1H, H imidazopyridine), 7.48 (dd, J = 1.28, 7.68 Hz, 1H, H imidazopyridine), 7.86 (dd, J = 1.48, 4.88 Hz, 1H, H imidazopyridine), 8.169 (d, J = 13.36 Hz, 2H, H Ar), 9.04 (s, 1H, NH exchanged with D_2O), 9.07 (s, 1H, NH exchanged with D_2O), 10.18 (d, J = 13.2, 2H, H Ar). ^{13}CNMR (101 MHz, DMSO-d_6): δ 167.63, 165.33, 164.01, 152.77, 150.56, 144.09, 141.56, 128.62, 126.05, 123.84, 118.43, 118.14, 94.96, 60.29, 60.10, 14.70. MS m/z: 380 (M^+). Anal. calcd for $C_{20}H_{20}N_4O_4$; C, 63.15; H, 5.30; N, 14.73; Found: C, 63.39; H, 5.37; N, 14.59.

4.1.2. General procedure for preparation of **IIIa,b**

Compound **II** (3.80 g, 0.01mole) was added to sodium ethoxide solution prepared by dissolving Na metal (0.23g, 0.01 mole) in absolute ethanol (10 ml), followed by drop-wise addition of hydrazine hydrate or phenyl hydrazine (0.01 mole) while stirring. The mixture was refluxed for 6 h. The solvent was removed under reduced pressure. The crude product was crystallized from ethanol.

4.1.2.1.*4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}pyrazolidine-3,5-dione **(IIIa)***

Yield (78%), m.p. 168-169 ºC. IR υ_{max}, cm^{-1}: 3317, 3267, 3213, 3194 (4NH), 3070 (CH Ar), 1670 (2C=O), 1631 (NH), 1573 (C=N), 1523, 1473 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.50 (s, 1H, NH exchanged with D_2O), 5.31 (s, 2H, 2NH exchanged with D_2O), 6.34-6.37 (m, 3H, 2H imidazopyridine, 1H, =C**H**), 6.67 (d, J= 8.84 Hz, 2H, ArH), 7.26 (dd, J = 1.40, 4.88, 1H,

H imidazopyridine), 7.39 (s, 2H, Ar H), 8.40 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{16}H_{12}N_6O_2$; C, 60.00; H, 3.78; N, 26.24; Found: C, 59.78; H, 3.89; N, 26.17.

4.1.2.2.*4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}-1-phenylpyrazolidine-3,5-dione* ***(IIIb)***.

Yield (88%), m.p. 89-91 ºC. IR υ_{max}, cm^{-1}: 3255 (2NH), 3186 (NH), 3024 (CH Ar), 1685 (2C=O), 1597 (NH), 1543 (C=N), 1520, 1496 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.71 (s, 1H, NH exchanged with D_2O), 6.61 (s, 1H, H olefinic, =C**H**), 6.68-6.71 (m, 2H, Ar H), 6.85 (d, *J* = 7.84, 2H, Ar H), 6.99 (d, *J* = 7.84, 2H, Ar H), 7.17-7.72 (m, 2H, H imidazopyridine), 7.28 (d, *J* = 7.28, 2H, Ar H), 7.38 (t, 1H, Ar H), 8.11-8.16 (m, 1H, H imidazopyridine), 9.03 (s, 1H, NH exchanged with D_2O), 13.21 (s, 1H, NH exchanged with D_2O). MS m/z: 396 (M^+). Anal. calcd for $C_{22}H_{16}N_6O_2$; C, 66.66; H, 4.07; N, 21.20; Found: C, 66.38; H, 3.97; N, 21.43.

4.1.3. *4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}-1-acetylpyrazolidine-3,5-dione* **(IV).**

Compound **II** (3.80g, 0.01 mole) was dissolved in glacial acetic acid (10 ml) and then hydrazine hydrate (0.32 g, 0.316 ml, 0.01 mole) was added while stirring. The reaction mixture was heated under reflux for 6 h, then cooled and the formed precipitate was filtered, washed with ethanol (5 ml) and dried. The crude product was crystallized from ethanol. Yield (67%), m.p. 289-290 ºC. IR υ_{max}, cm^{-1}: 3332 (NH), 3224 (2NH), 3043 (CH Ar), 2893 (CH Aliph), 1697 (2C=O), 1660 (C=O), 1651 (NH), 1608 (C=N), 1558, 1504 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H, C**H$_3$**), 5.73 (s, 1H, NH, exchanged with D_2O), 5.90 (s, 1H, H olefinic , =C**H**), 6.67 (d, *J* = 8.24 Hz, 2H, H Ar), 7.20-7.24 (m, 1H, H imidazopyridine), 7.76 (d, *J* = 8.28 Hz, 2H, H Ar), 7.89 (d, *J* = 8.28 Hz, 1H, H imidazopyridine), 8.15 (d, *J* = 8.52 Hz, 1H, H imidazopyridine), 8.87 (s, 1H, NH exchanged with D_2O), 9.25 (s, 1H, NH exchanged with D_2O)**.** ^{13}CNMR (101 MHz, DMSO-d6) δ 174.63, 169.63, 168.59, 151.67, 150.63, 141.82, 128.63, 127.92, 124.64, 124.57, 119.41, 113.98, 59.34, 24.97. Anal. calcd for $C_{18}H_{14}N_6O_3$; C, 59.67; H, 3.89; N, 23.19; Found: C, 59.29; H, 3.95; N, 23.57.

4.1.4. General procedure for preparation of compounds**Va,b** and **VI**

Compound **II** (3.80g, 0.01 mole), urea/ thiourea or thiosemicarbazide (0.01 mole) were added to sodium ethoxide solution prepared by dissolving Na metal (0.23g, 0.01 mole) in absolute ethanol (10 ml) while stirring and the mixture was refluxed for 6 h. The solvent was removed under reduced pressure. The crude product was crystallized from ethanol.

4.1.4.1.*5-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}pyrimidine 2,4,6(1H,3H,5H)-trione* **(Va).**

Yield (58%), m.p. 213-214 ºC. IR υ_{max}, cm^{-1}: 3444 (2NH), 3348, 3209 (2NH), 3093 (CH Ar), 1678 (2C=O), 1620 (NH), 1608 (C=N), 1546, 1492 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.42 (s, 2H, 2NH exchanged with D_2O), 5.70 (s, 1H, NH exchanged with D_2O), 7.17-7.18 (m, 1H, H imidazopyridine), 7.22 (d, *J* = 8.72 Hz, 2H, H Ar), 7.89-7.93 (m, 2H, 1H imidazopyridine, 1H, olefinic, =C**H**), 8.15 (d, *J* = 8.72 Hz, 2H, H Ar), 8.26 (dd, *J* = 1.28,4.88 Hz , 1H, H imidazopyridine), 10.0 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{17}H_{12}N_6O_3$; C, 58.62; H, 3.47; N, 24.13; Found: C, 58.94; H, 3.61; N, 24.38.

4.1.4.2.*5-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}-2-thioxodihydro-pyrimidine-4,6(1H,5H)-dione* ***(Vb).***

Yield (74%), m.p. 350-352 ºC. IR υ_{max}, cm^{-1}: 3383 (2NH), 3275, 3170 (2NH), 3097 (CH Ar), 1650 (2C=O), 1610 (NH), 1604 (C=N), 1490, 1465 (C=C) 1261 (C=S). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.17 (s, 1H, H olefinic, =C**H**), 7.56(s, 1H, NH exchanged with D_2O), 8.07 (d, *J* = 7.36 Hz, 1H, H imidazopyridine), 8.17 (d, *J* = 8.48 Hz, 2H, H Ar), 8.32-8.34 (m, 2H , H Ar). 8.39-8.43 (m, 2H, H imidazopyridine), 8.92 (s, 2H, NH exchanged with D_2O), 13.59 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{17}H_{12}N_6O_2S$; C, 56.04; H, 3.32; N, 23.06; Found: C, 56.53; H, 3.62; N, 23.49.

4.1.4.3.*(Z)-6-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenylamino]methylene}-4H-1,2,4-triazepine-3,5,7(6H)-trione* ***(VI).***

Yield (58%), m.p. 223-224 ºC. IR υ_{max}, cm^{-1}: 3500, 3363, 3336 (NH), 3062 (CH Ar), 1681 (3C=O), 1640 (NH), 1604 (C=N), 1580, 1496 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 5.72 (s,1H, 1NH exchanged with D_2O), 6.35 (s, 1H, =C**H**), 6.67 (d, *J* = 8.64 Hz, 2H, H Ar), 7.11-7.22 (m, 1H, H imidazopyridine), 7.54 (d, *J* = 8.76, 2H, H Ar), 7.77 (d, *J* = 8.68 Hz, 1H, H imidazopyridine), 8.19-8.29 (m, 1H, H imidazopyridine), 10.20 (s, 1H, NH exchanged with D_2O), 10.80 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{17}H_{11}N_7O_3$; C, 56.51; H, 3.07; N, 27.14; Found: C, 56.81; H, 3.17; N, 27.45.

4.1.5. General procedure for preparation of Substituted 1-{4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl}diazene (Azocoupling products) **VIIa-f.**

A solution of sodium nitrite (0.89 g, 0.013 mole) in distilled water (5 ml) was added portion wise to a stirred ice-cooled solution of compound **I** (2.10g, 0.01 mole) in HCl (2.5 ml) and distilled water (5 ml). The cold diazonium solution was added portion wise to a solution of the appropriate phenolic compound (0.01 mole) in an aqueous solution of 10% sodium hydroxide (4 ml) while stirring and cooling. The reaction mixture was kept in ice for 2 h, filtered, dried. The crude product was crystallized from ethanol.

4.1.5.1.*4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}phenol* ***(VIIa).***

Yield (65%), m.p. 350-351 ºC. IR υ_{max}, cm^{-1}: 3400 (OH), 3363 (NH), 3062 (CH Ar), 1660 (NH), 1589 (C=N), 1480, 1454 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.77-6.80 (m, 3H, 2 Ar H, 1H, NH exchanged with D_2O), 7.12 (s, 1H , OH exchanged with D_2O), 7.18 (dd, *J* = 4.72, 7.88 Hz,1H, H imidazopyridine), 7.75 (d, *J* = 8.92 Hz, 2H, Ar H), 7.88 (d, *J* = 8.56 Hz, 2H, H Ar), 7.96 (dd, *J* = 7.92,1.44 Hz, 1H, H imidazopyridine), 8.30 (dd, *J* = 1.40,4.72 Hz, 1H, H imidazopyridine), 8.41 (d, *J* = 8.56 Hz, 2H, Ar H). MS m/z: 315 (M^+). Anal. calcd for $C_{18}H_{13}N_5O$; Calcd: C, 68.56; H, 4.16; N, 22.21; Found: C, 68.90; H, 4.27; N, 22.51.

4.1.5.2.*4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}benzene-1,3-diol* ***(VIIb).***

Yield (76%), m.p. 353-354 ºC. IR υ_{max}, cm^{-1}: 3394 (2OH), 3255 (NH), 3097 (CH Ar) 1680 (NH), 1610 (C=N), 1604, 1489 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.18-6.23 (m, 2H, Ar H), 6.47 (s, 1H, Ar H), 6.57 (d, J = 8.84 Hz, 2H, Ar H), 6.89 (t, J = 7.96 Hz , 1H, H imidazopyridine), 8.09 (d, J =8.24 Hz, 2H, Ar H), 8.40 (d, J =7.76 Hz, 1H, imiazopyridine H), 8.54 (d, J =8.24 Hz, 1H, imidazopyridine H), 9.23 (s, 1H, NH exchanged with D_2O), 11.00 (s, 1H, OH, exchanged with D_2O), 12.31 (s, 1H, NH exchanged with D_2O). MS m/z: 331 (M^+). Anal. calcd for $C_{18}H_{13}N_5O_2$; C, 65.25; H, 3.95; N, 21.14; Found: C, 65.43; H, 4.06; N, 21.31.

4.1.5.3.5-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}-2-hydroxybenzoic acid ***(VIIc).***

Yield (67%), m.p. 351-352 ºC. IR υ_{max}, cm^{-1}: Broad band 3417- 2500 OH, NH, CH Ar), 1680 (C=O), 1650 (NH), 1600 (C=N), 1581, 1485 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.58 (s, 1H, NH exchanged with D_2O), 6.60-6.64 (m, 4H, 3 Ar H + 1H imidazopyridine), 7.12 -7.17 (m, 3H, 2 Ar H + 1H imidazopyridine), 7.67-7.69 (m, 3H, 2 Ar H, 1H imidazopyridine), 16.09 (s, 2H, OH exchanged with D_2O). MS m/z: 359 (M^+). Anal. calcd for $C_{19}H_{13}N_5O_3$; C, 63.51; H, 3.65; N, 19.49; Found: C, 63.31; H, 3.78; N, 19.68.

4.1.5.4.*2-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}-4-methylphenol* ***(VIId).***

Yield (52%), m.p. 258-259 ºC. IR υ_{max}, cm^{-1}: 3400 (OH), 3383 (NH), 3097 (CH Ar), 2920 (CH Aliph), 1615 (NH), 1604 (C=N), 1530, 1496 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.30 (s, 3H, C**H$_3$**), 4.59 (s, 1H, OH exchanged with D_2O), 6.64 (d, J = 7.36 Hz, 1H, Ar H), 6.92-7.01 (m, 2H,1 Ar H, 1H imidazopyridine), 7.27 (d, J = 7.20 Hz , 1H, Ar H), 7.56 (s, 1H, Ar H), 8.20 (d, J = 7.52 Hz, 1H, H imidazopyridine), 8.22-8.24 (m , 2H, 1 Ar H, 1H imidazopyridine), 8.54 (d, J = 7.76 Hz, 2H, Ar H), 10.85 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{19}H_{15}N_5O$; C, 69.29; H, 4.59; N, 21.26; Found: C, 69.29; H, 4.72; N, 21.18.

4.1.5.5.*1-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}naphthalen-2-ol* ***(VIIe)***

Yield (91%), m.p. 308-309 ºC. IR υ_{max}, cm^{-1}: 3420 (OH), 3390 (NH), 3028 (CH Ar), 1627 (NH), 1593 (C=N), 1535, 1450 (C=C). ^{1}HNMR (400 MHz, DMSO-d_6): δ 5.19 (s, 1H, OH exchanged with D_2O), 6.64 (d, J = 10.44 Hz, 1H, Ar H), 7.03 (d, J = 10.08 Hz, 1H, Ar H), 7.11 (d, J = 7.00 Hz, 1H, Ar H), 7.48-7.53 (m, 3H, Ar H), 7.62-7.64 (m, 1H, H imidazopyridine), 7.90 (d, J = 8.84 Hz, 1H, H imidazopyridine), 7.95-8.02 (m, 2H, Ar H), 8.46 (d, J = 8.92 Hz, 2H, Ar H), 8.57 (d, J = 5.32 Hz, 1H, H imidazopyridine), 13.4 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{22}H_{15}N_5O$; C, 72.32; H, 4.14; N, 19.17; Found: C, 72.04; H, 4.19; N, 19.03.

4.1.5.6.*4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}naphthalen-1-ol* ***(VIIf).***

Yield (89%), m.p. 339-340 ºC. IR υ_{max}, cm^{-1}: 3410 (OH), 3390 (NH), 3024 (CH Ar), 1680 (NH), 1620 (C=N), 1600, 1496 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.10 (s, 1H, OH, exchanged with D_2O), 6.78 (d, J = 9.52 Hz, 1H , H Ar), 7.09-7.12 (m, 2H, 1H Ar, 1H imidazopyridine), 7.37 (t, J = 7.36 Hz, 1H, H Ar), 7.47 (t, J = 7.36 Hz 1H, H Ar), 7.61-7.67 (m, 3H, 2H Ar, 1H imidazopyridine), 7.71-7.76 (m, 2H, H Ar), 7.93 (d, J = 9.6 Hz, 1H, H Ar), 8.01 (d, J = 8.68 Hz, 1H, H Ar), 8.42 (d, J = 7.84 Hz, 1H, H Ar), 8.49 (dd, J = 4.08,8.60 Hz, 1H, H imidazopyridine), 15.87 (s, 1H, NH exchanged with D_2O). MS m/z: 365 (M^+). Anal. calcd for $C_{22}H_{15}N_5O$; C, 65.25; H, 3.95; N, 21.14; Found: C, 65.43; H, 4.06; N, 21.31.

4.1.6. *4-{[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]diazenyl}benzenamine* ***(VIII)***.

A solution of sodium nitrite (0.98g, 0.013 mole) in water (5ml) was added portion wise to a stirred ice-cooled solution of compound **I** (2.10 g, 0.01 mole) in HCl (2.5 ml) and cold distilled water (5 ml). Aniline (0.01 mole) was added dropwise to the cold diazonium solution while stirring and cooling. The reaction mixture was kept in ice for 2 h, filtered, dried and crystallized from ethanol. Yield (92%), m.p332-333 ºC. IR υ_{max}, cm^{-1}: 3317 (NH_2), 3197 (NH), 3097 (CH Ar), 1604 (NH), 1550 (C=N), 1520, 1496 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.05(s,2H, NH_2 exchanged with D_2O), 4.37 (s, 1H, NH exchanged with D_2O), 6.78 (d, J = 8.68 Hz, 2H, H Ar), 7.37 (d, J = 7.32 Hz, 2H, H Ar), 7.48-7.51 (m, 4H , H Ar), 7.68 (d, J = 8.72 Hz, 1H, H

imidazopyridine), 8.14 (d, *J* = 8.40 Hz, 1H, H imidazopyridine), 8.47 (d, *J* = 5.40 Hz, 1H, H imidazopyridine). Anal. calcd for $C_{18}H_{14}N_6$; C, 68.78; H, 4.49; N, 26.74; Found: C, 69.06; H, 4.58; N, 26.89.

4.1.7. *3-{2-[4-(1H-imidazo[4,5-b]pyridin-2-yl)phenyl]hydrazono}pentane-2,4-dione* ***(IX).***

The diazonium salt produced from compound **I** (4.20 g, 0.02 mole) was dissolved in absolute ethanol (15 ml) in presence of sodium hydroxide (2.40 g, 0.06 mole) and acetylacetone (3.00 g, 3.06 ml, 0.03mole) was added on cold while stirring. The reaction mixture was stirredat room temperature for 2 h. The obtained solid was filtered, dried and crystallized from ethanol. Yield (73%), m.p 295-296 ºC. IR υ_{max}, cm^{-1}: 3402 (2NH), 3089 (CH Ar), 2924 (CH Aliph), 1670 (2C=O), 1640 (NH), 1608 (C=N), 1504, 1465 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.46 (s, 3H, C**H**$_3$), 2.48 (s, 3H, C**H**$_3$), 4.46 (s, 1H, NH exchanged with D_2O), 7.54 (dd, *J* = 7.36, 5.6 Hz, 1H, H imidazopyridine), 7.78 (d, *J* = 8.64 Hz, 2H, ArH), 8.35 (d, *J* = 7.52 Hz, 1H, H imidazopyridine), 8.47 (d, *J* = 8.60 Hz, 2H, Ar H), 8.52 (d, *J* = 5 Hz, 1H, H imidazopyridine), 13.70 (s, 1H, NH exchanged with D_2O). Anal. calcd for $C_{17}H_{15}N_5O_2$; C, 63.54; H, 4.71; N, 21.79; Found: C, 63.19; H, 4.78; N, 22.05.

4.2. Anticancer activity

4.2.1. In-vitro MTT assay

Cell lines were provided by American Type Culture Collection (ATCC) and propagated in RPMI-1640. Cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone,), 10 µg/ml of insulin (Sigma), and 1% penicillin-streptomycin. All chemicals and reagents were from Sigma, or Invitrogen.

Cells suspended in medium (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100µl complete growth medium were seeded into a 96-well plate and incubated at 37°C in a CO_2 incubator overnight. After 24 h, the test sample (100 µl of the tested compound per well) was added to the cells in 96- well plates and cultured in fresh new medium at 37 ºC for 48 h. Media containing test compounds were discarded and the plate was washed with PBS. The cultured cells were mixed with 20µl of MTT dye solution and incubated for 4 h at 37 ºC. The supernatant was carefully removed from each well and 100 µl of DMSO were added to each well to dissolve

the formazan crystals which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by microplate reader using a test wavelength of 570 nm [12] 14]. The results were expressed in IC_{50}, which induces a 50 % inhibition of cell growth of treated cells when compared to the growth of control cells. Each experiment was performed at least 3 times in a concentration of 0.01, 0.1, 1, 5, 10, 100 and 200 μM. There was a good reproducibility between replicate wells with standard errors below 10 %.

4.2.2. CDK9 enzyme inhibition assay

The micro ELISA plate provided has been pre-coated with an antibody specific to CDK-9. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then the reactants become antibody-antigen-antibody-enzyme complex, after washing completely, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added and TMB substrate becomes blue color under HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of CDK9 in the samples is then determined by comparing the optical density (O.D) of the samples to the standard curve.

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Graphical abstract

Highlights

- New imidazo[4,5-*b*]pyridine derivatives were designed, synthesized and screened for their anticancer activity
- The new imidazo[4,5-*b*]pyridine derivatives had remarkable anti-proliferative activity against breast and colon cancer cell lines
- Synthesized compounds showed superior binding affinity relative to the natural ligand (T3C)
- Synthesized compounds had excellent physicochemical properties and drug-likeness attributes
- Synthesized compounds had excellent pharmacokinetic profile