Synthesis and Cytotoxicity Evaluation of Novel Indole Derivatives as Potential Anti-Cancer Agents

Mona M. Kamel, Mohamed K. Abdel-hameid, Hala B. El-Nassan, Eman A. El-Khouly

Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt

Address: 33 Kasr El-Aini street, Cairo, Egypt
Tel: 00202-23632245
Fax: 00202-23635140
*E-mail: hala_bakr@hotmail.com

Abstract

A series of bioisosteres of marine indole alkaloids (meridianins) was synthesized and the compounds were tested for their *in vitro* anti-proliferative activity against HCT-116 cell-line. In the design of the targeted analogues, the 2-aminopyrimidine ring of merdianins was replaced with 5-aminopyrazoles, pyrazolo[1,5-*a*]pyrimidines and pyrazolo[3,4-*b*]pyridines. The cytotoxic screening of the synthesized compounds revealed that pyrazolo[1,5-*a*]pyrimidines (compounds **9c** and **11a**) had the most potent cytotoxic activity with IC_{50} = **0.31 µM** and **0.34 µM** respectively. Compounds **9c** and **11a** were further investigated for their kinase inhibitory potencies toward six kinases (CDK5/p25, CK1ð/ε, GSK-3α/β, Dyrk1A, Erk2, and CLK1). The results showed that both compounds were highly potent inhibitors of GSK-3α/β and Erk2 enzymes.

Keywords: Indole, meridianins, cytotoxic activity, Erk2

Introduction

Cancer is the second leading cause of death across the globe which affect billions of people worldwide [1]. Recently, a number of essential antineoplastic agents that are in preclinical and clinical evaluation have been isolated from marine flora and fauna [2,3]. Marine invertebrates are considered as a potent source of different biologically active alkaloids [4].

The marine alkaloids meridianins A-G (Figure 1) are isolated from the south Atlantic tunicate *Aplidium meridianum*. Meridianins are indole derivatives substituted at position 3 with 2-aminopyrimidine ring. They inhibit many protein kinases such as cyclin dependent kinases, glycogen synthase kinase-3 and casein kinase-1 and display potent antitumor activity related to their enzyme inhibitory activity [5].

Meridianin B and meridianin E are the most potent kinase inhibitors and cytotoxic agents. The SAR reveals that shifting the 2-aminopyrimidine ring from position 3 to position 2 on the indole ring abolished the enzyme inhibitory activity. It is hypothesized that meridianins bind at the ATP kinase binding site *via* two H-bonds between the pyrimidine *N*-1 and the 2-amino group of the pyrimidine and both fix the molecule in the hinge region while the indole ring lays in the hydrophobic pocket [6–8].

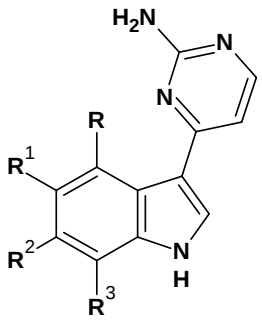
With such a wealth of biological activity, meridianins represent promising lead structures to prepare more potent and selective kinase inhibitors and many analogues have been synthesized during the last two decades[5,9]. Most of the published work focused on changing the substitution on the pyrimidine ring or the indole nitrogen. Thus, the pyrimidine ring was substituted at position 5 by aryl group or iodine [8,10]. However, the 5-arylpyrimidinyl substitution (for example compound **I**) was detrimental to the cytotoxic activity and enzyme inhibition of CDK5/p25, CK1ð/ε, GSK3α/β and Erk2, although their inhibitory activity of Dyrk1A was high. Dyrk1A enzyme is involved in Alzheimer's disease and Down syndrome.

On the other hand, the modifications reported on the indole ring involved substitution by alkyl group (*N*-methyl derivatives) [10], sulphonyl moieties [11], or acyl groups [7]. Both sulphonation and acylation of the indole nitrogen and the presence of 5-iodopyrimidine ring enhanced the inhibitory activity against Dyrk1A [4, 5, 6].

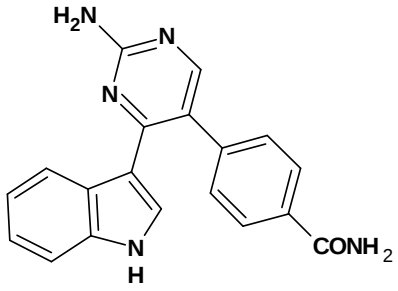
Other modifications of the meridianins structure included the isosteric replacement of the 2-aminopyrimidine ring by uracil[12] or pyridine [6] and the synthesis of bisindolylpyridines[13], bisindolylpyrimidines (Compound **II**) or bisindolylpyridazines[14,15].

However, no previous publications discussed the replacement of the pyrimidine ring by five membered ring or fused five and six membered rings.

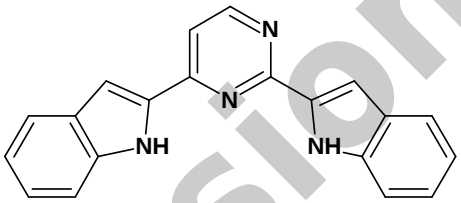
In this work, indole analogues of meridianins were synthesized in which the pyrimidine ring was replaced by 5-aminopyrazole, pyrazolo[3,4-*b*]pyridine and pyrazolo[1,5-*a*]pyrimidine (Figure 2). Upon designing the compounds, the 7-amino group on the pyrazolo[1,5-*a*]pyrimidines **4-9** was added to mimic the binding of the 2-aminopyrimidine ring in meridianins. Different substitutions were added at position 5 (H, methyl, aryl) to examine the effect of different substitutions on the cytotoxic activity. Both 5,7-bisphenylpyrazolo[1,5-*a*]pyrimidines **11a,b** and pyrazolo[3,4-*b*]pyridines **12,13** lack the amino group and only can act as H-bond acceptor. Besides, the latter compounds have large aromatic rings and can make hydrophobic interactions with the kinase binding site. The synthesized compounds were tested for their *in vitro* anti-proliferative activities toward HCT-116 cell-line. The kinase inhibitory potencies of the most potent compounds were examined toward six kinases (CDK5/p25, CK1ð/ε, GSK3α/β, Dyrk1A, CLK1 and Erk2).



		R	R^1	R^2	R^3
Merdianin	A	OH	H	H	H
	B	OH	H	Br	H
	C	H	Br	H	H
	D	H	H	Br	H
	E	OH	H	H	Br
	F	H	Br	Br	H
	G	H	H	H	H



I



II

Fig. 1: Merdianins and their reported analogues

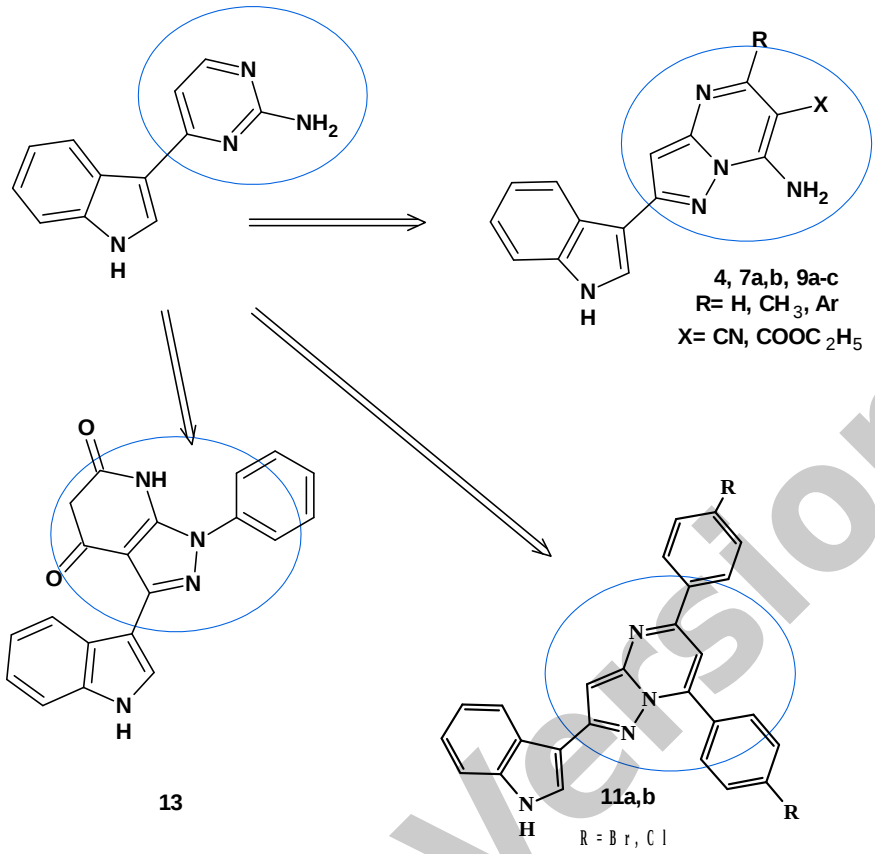


Fig. 2: Design of Merdianin analogues

Results and discussion

Chemistry

Schemes 1-3 outline the synthesis of the target compounds. The key precursor, 3-cyanoacetylindole (**1**) was prepared in excellent yield (91%) by heating a mixture of cyanoacetic acid and indole in acetic anhydride at 85 °C for 10 mins [16]. In the present study, attempts to prepare compound **2a** via refluxing one molar equivalent of compound **1** with three molar equivalent of hydrazine hydrate in ethanol as reported by Chiara *et al* [17] were unsuccessful and the starting compound was obtained unreacted. Trials to prepare compound **2b** using phenyl hydrazine in ethanol and refluxing up to 13 hrs were also fruitless.

Nevertheless the targeted aminopyrazole derivatives were obtained by fusion of compound **1** either with 8 molar equivalents of hydrazine hydrate or 1 molar equivalent of phenyl hydrazine. (**Scheme 1**). The IR spectra of compounds **2a,b** indicated the appearance of NH_2 absorption bands at 3417-3325 cm^{-1} and the disappearance of C≡N and C=O absorption band. Moreover, the ^1H NMR spectrum of compound **2a** displayed two exchangeable singlet signals at δ 4.59 ppm and δ 11.25 ppm corresponding to NH_2 protons and NH proton of the pyrazole ring, respectively. While, the ^1H NMR spectrum of compound **2b** showed an exchangeable singlet signal at δ 5.36 ppm corresponding to NH_2 protons. Their ^{13}C NMR spectra revealed the disappearance of aliphatic signals and the appearance of aromatic carbons at δ 88.0-154.2 ppm.

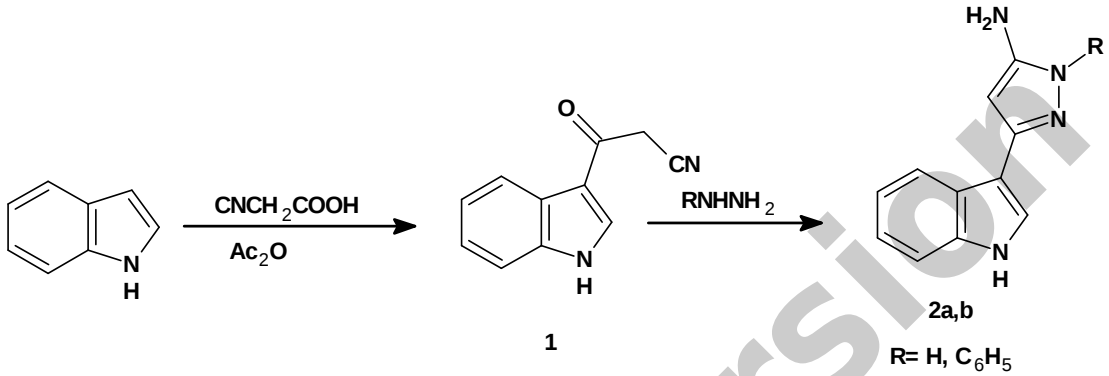
Compound **2a** was reacted with ethyl ethoxymethylenecyanoacetate **3a** [18] in glacial acetic acid to afford the 7-aminopyrazolo[1,5-a]pyrimidine **4**. Whilst, the 7-oxo derivative **5** was unexpectedly afforded upon reacting compound **2a** with ethoxymethylenecyanoacetate **3b**. This reaction proceeded via initial nucleophilic substitution of the ethoxy group of compound **3** by the exocyclic amino group of pyrazole, followed by intramolecular cyclization through nucleophilic addition of the pyrazole NH on either the cyano group to give compound **4** or the ester group to give compound **5**. [**Fig. 3**] Elemental analyses and spectral data were in favor of the proposed structures **4** and **5**. Thus, the IR spectrum of compound **4** indicated the presence of ester C=O absorption band at 1678 cm^{-1}. While that of compound **5** showed cyano band at 2214 cm^{-1} and C=O group at 1662 cm^{-1}. The ^1H NMR spectrum of compound **4** showed an exchangeable singlet signal at δ 8.61-8.84 ppm corresponding to NH_2 protons. Furthermore, triplet and quartet signals appeared at δ 1.34-1.37

ppm and δ 4.32-4.37 ppm assigned to ethyl protons. Both evidence proved that the cyclization occurred on the cyano group. The ^{13}C NMR spectrum of compound **4** revealed the presence of aliphatic carbons at δ 60.9 ppm and δ 14.6 ppm, in addition to C=O carbon at δ 166.6 ppm. On the other hand, the ^{1}H NMR spectrum of compound **5** revealed the presence of a singlet signal at δ 2.51 ppm assigned to CH_3 protons and, an exchangeable singlet signal at δ 13.37 ppm assigned to NH of the pyrimidine ring. Its ^{13}C NMR spectrum showed the methyl carbon at δ 19.2 ppm, the C≡N carbon at δ 116.3 ppm and C=O carbon at δ 158.1 ppm. Based on these findings, the cyclization was assumed to occur on the ester group.

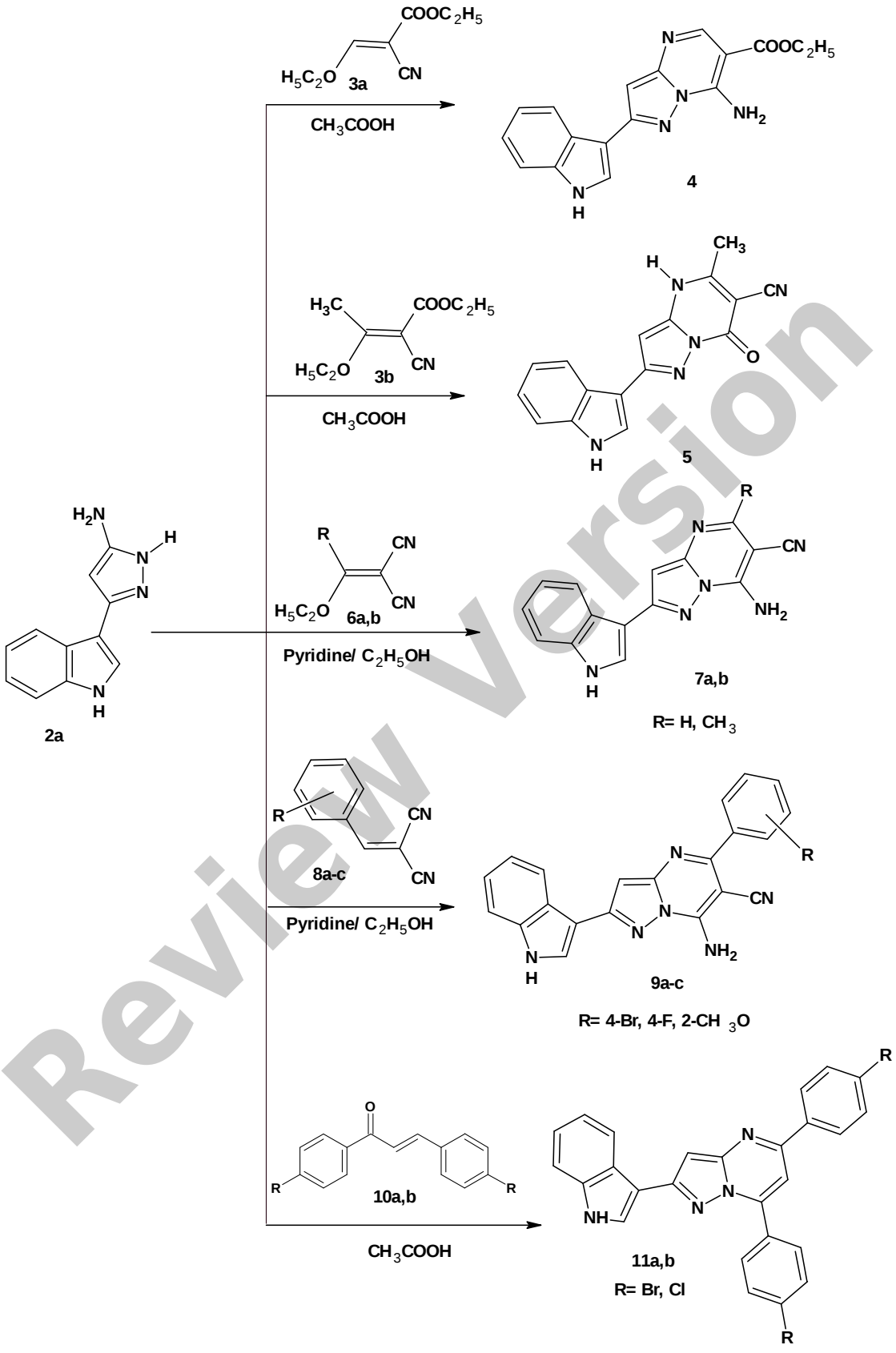
Using similar approach, ethoxymethylenemalononitriles **6a,b** [20,21] and arylidenemalononitriles **8a-c** [22–24] were heated under reflux with compound **2a** in ethanol/pyridine mixture to give the pyrazolo[1,5-*a*]pyrimidines **7a,b** and **9a-c**, respectively (**Scheme 2**). The cyclocondensation reaction of 5-aminopyrazole **2a** chalcones **10a,b** in acidic medium afforded 5,7-bisphenyl derivatives **11a,b**.

Compound **2b** was reacted with acetylacetone or diethyl malonate in acetic acid to afford pyrazolo[3, 4-*b*]pyridines **12** and **13**, respectively (**Scheme 3**). Elemental analysis and spectral data were in favor of the proposed structures. The IR spectra indicated the disappearance of the amino group. The IR spectrum of compound **13** indicated the appearance of two C=O absorption bands at 1636 and 1635 $cm^{-1.}$ Its ^{1}H NMR spectrum showed a singlet signal at δ 2.03 ppm assigned to -CO-$\underline{CH_2}$-CO- and an exchangeable singlet signal appeared at δ 9.98 ppm corresponding to NH proton of the pyridine ring. While, the ^{13}C NMR

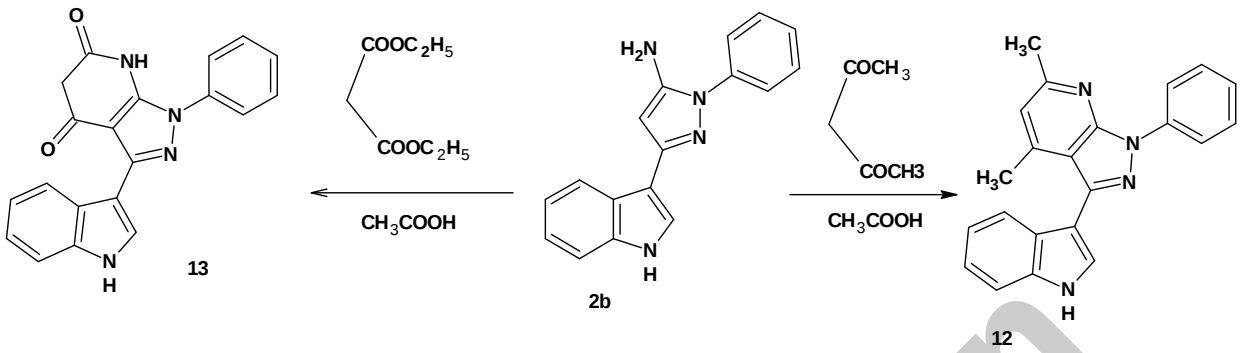
spectrum of compound **13** revealed the appearance of aliphatic protons at δ 24.9 ppm and two C=O carbons at δ 169.5 and 170.8 ppm.



Scheme 1: Synthesis of indol-3-yl pyrazoles



Scheme 2: Synthesis of pyrazolo[1,5-*a*]pyrimidines



Scheme 3: Synthesis of pyrazolo[3,4-*a*]pyridines

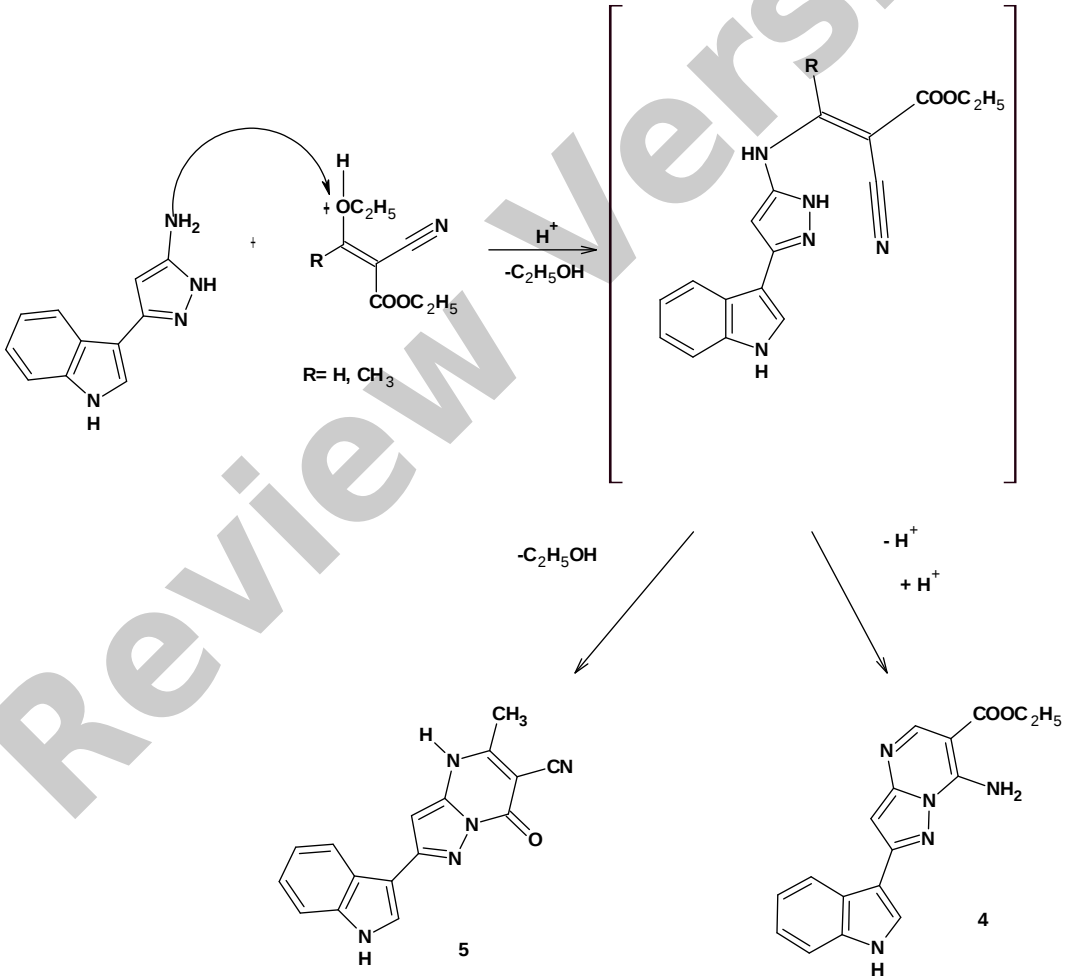


Fig. 3: Proposed mechanism for the synthesis of compounds **4** and **5**

***In vitro* cytotoxic activity**

All the prepared compounds were tested for their *in vitro* cytotoxic activity on HCT-116 cell-line using MTT- cytotoxicity assay [25–30]. Staurosporine was used as a reference compound and the results expressed as IC_{50} in µM are displayed in **Table 1** and represented graphically in **Fig. 4**.

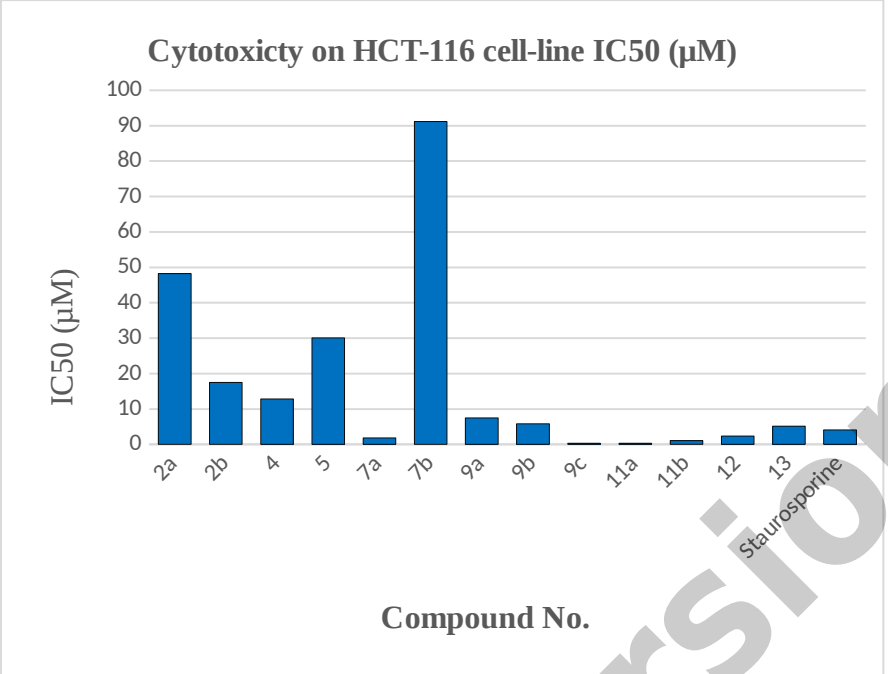
The results indicated that some of the compounds showed cytotoxic activity comparable to Staurosporine. SAR study of the derivatives indicated that the 5-aminopyrazoles **2a,b** showed poor cytotoxic activity IC_{50} = 48.25 and 17.42 µM, respectively. This was expected since the 5-aminopyrazole ring cannot form efficient H-bond with the kinase enzyme as the 2-aminopyrimidine ring. Better activity was observed in case of pyrazolo[1,5-*a*]pyrimidine and pyrazolo[3,4-*b*]pyridine due to their larger size.

The most potent compounds were **9c** (IC_{50}= 0.31 µM) and **11a** (IC_{50}= 0.34 µM).

Table 1: In-*vitro* cytotoxicity against the HCT cell-line.

Compound No.	IC_{50} (µM)
2a	48.25 ± 1.2
2b	17.47 ± 0.59
4	12.85 ± 0.4
5	30.06 ± 1.2
7a	1.80 ± 0.03
7b	91.12 ± 3.4
9a	7.45 ± 0.18
9b	5.80 ± 0.21
9c	0.31 ± 0.01
11a	0.34 ± 0.01
11b	1.06 ± 0.03
12	2.34 ± 0.07
13	5.13 ± 0.18
Staurosporine	4.1 ± 0.08

Fig. 4: In-*vitro* cytotoxicity against the HCT-116 cell-line.



Kinase inhibitory activity

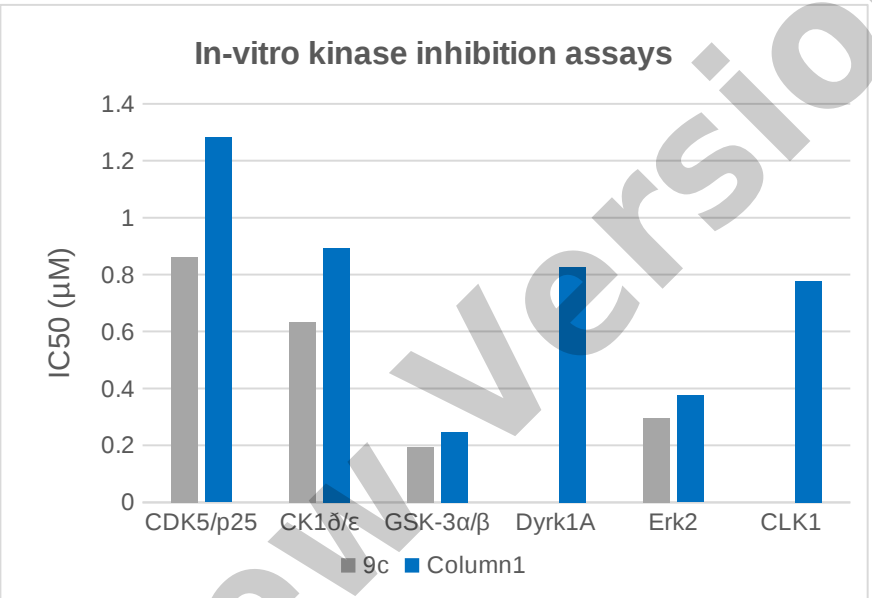
Compounds **9c** and **11a** which had the most potent cytotoxic activity were subjected to further *In vitro* Kinase inhibition assays. Their kinases inhibitory potencies were tested toward six kinases (CDK5/p25, CK1ð/ε, GSK-3α/β, Dyrk1A, Erk2, CLK1) and the results in terms of IC_{50} were displayed in **Table 2** and represented graphically in **Figure 5**.

The results indicated that compounds **9c** and **11a** displayed sub-micromolar inhibition potencies toward most of the kinases tested but the significant inhibitory activity was against GSK-3α/β and Erk2 enzymes [GSK-3α/β (IC_{50} = **0.196 µM** and **0.246 µM,** respectively**)** and Erk2 (IC_{50} = **0.295 µM** and **0.376 µM,** respectively)]. Both GSK-3α/β and Erk2 known to be involved in intestinal cell differentiation so had an effect on colon cancer cells [31–33].

Table 2: Results of *in-vitro* kinase inhibition assays

Compound No.	Kinase inhibition-IC_{50} in µM					
	CDK5/p25	**CK1ð/ε**	**GSK-3α/β**	**Dyrk1A**	**Erk2**	**CLK1**
9c	0.863	0.633	0.196	52.36	0.295	12.76
11a	1.281	0.893	0.246	0.827	0.376	0.777

Fig. 5: Results of *in-vitro* kinase inhibition assays



Conclusion

In summary, series of novel indolylpyrazoles, indolylpyrazolo[1,5-*a*]pyrimidines and indolylpyrazolo[3,4-*b*]pyridines were synthesized that revealed potent cytotoxic effect on HCT-116 cell-line. Compounds **9c** and **11a** showed the most potent cytotoxic activity with IC_{50} = **0.31 µM** and **0.34 µM**, respectively. Besides, they exhibited effective inhibition of GSK-3α/β (IC_{50} = **0.196 µM** and **0.246 µM,** respectively**)** and Erk2 (IC_{50} = **0.295 µM** and **0.376 µM,** respectively). Further work on meridianins scaffold is still needed to obtain more potent and selective kinase inhibitors.

Experimental

All the melting points were determined on Stuart apparatus and the values given are uncorrected. The IR spectra were determined using KBr discs on Shimadzu IR 435 spectrophotometer, microanalytical unit, Faculty of pharmacy, Cairo University and the values are represented in cm^{-1}.The ^{1}H NMR and ^{13}C NMR spectra were carried out using a 400 MHz Bruker spectrophotometer, microanalytical unit, Faculty of pharmacy, Cairo University, Egypt, using TMS as internal standard. Chemical shift values are recorded in ppm on δ scale. The elemental analyses were carried out at the Regional center for Mycology and Biotechnology, Azher University, Egypt. The progress of the reactions was monitored by TLC using TLC sheets pre-coated with UV fluorescent silica gel Merck 60 F 254 and the sheets were visualized using UV lamp. Reagents purchased commercially were used without further purification. Solvents were dried using standard procedures.

General procedure for the synthesis of 3-(1*H*-indol-3-yl)pyrazol-5-amines 2a,b.

A mixture of compound **1** (3.68 g, 0.02 mol), hydrazine hydrate (99%, 5.00 g, 5 mL, 0.10 mol) or phenyl hydrazine (2.16 g, 2 mL, 0.02 mol) was fused for 2 hrs. The reaction mixture was cooled. In case of compound **2a**, the fused product was triturated with water where greyish brown solid was formed**.** Compound **2b** was triturated with ethanol where yellow solid was formed. The solid was filtered, dried, washed with methanol (25 mL) and crystallized from ethanol.

3-(1*H*-Indol-3-yl)-1*H*-pyrazol-5-amine (2a)

Yield (71%), mp (219-221°C), IR (KBr) cm^{-1}: 3414, 3332 (NH_2), 3167, 3113 (NH), ^1H NMR (DMSO-d_6, ppm) δ: 4.59 (s, 2H, NH_2, D_2O exchangeable), 5.74 (s, 1H, pyrazole Ar-H), 7.06-7.84 (m, 5H, Indole Ar-H), 11.25 (s, 1H, NH, D_2O exchangeable), 11.52 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 88.3 (CH-C(NH_2)-N-), 107.8, 112.2, 119.9, 120.2, 121.9, 123.1, 125.9, 136.7, 140.8, 154.2 (Ar-C), Anal. calcd for $C_{11}H_{10}N_4$: C,66.65, H, 5.08, N, 28.26. Found: C, 66.91, H, 5.17, N, 28.49.

3-(1*H*-Indol-3-yl)-1-phenyl-pyrazol-5-amine (2b)

Yield (67%), mp (209-211°C), IR (KBr) cm^{-1}: 3417, 3325 (NH_2), 3221 (NH), ^1H NMR (DMSO-d_6, ppm) δ: 5.36 (s, 2H, NH_2, D_2O exchangeable), 5.85 (s, 1H, pyrazole Ar-H), 7.04-8.19 (m, 10H, Ar-H), 11.15 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 88.0 (CH-C(NH_2)-N-N(C_6H_5)), 110.1, 111.9, 119.7, 121.6, 121.8, 122.6, 123.9, 125.5, 125.9, 129.5, 136.9, 140.21, 147.7, 148.0 (Ar-C), Anal. calcd for $C_{17}H_{14}N_4$: C, 74.43, H, 5.14, N, 20.42. Found: C, 74.70, H, 5.28, N, 20.13.

Ethyl 7-amino-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidine-6-carboxylate (4).

A mixture of compound **2a** (0.5 g, 0.0025 mol) and compound **3a** (0.43 g, 0.0025 mol) in glacial acetic acid (20 mL) was heated under reflux for 8 hrs. The reaction was cooled and the separated solid was filtered, dried and crystallized from acetone.

Yield (51%), mp (233-235°C), IR (KBr) cm^{-1}: 3441, 3332 (NH_2), 3209 (NH), 2920, 2850 (CH-aliphatic), 1678 (C=O), ^1H NMR (DMSO-d_6, ppm) δ: 1.33-1.37 (t, 3H, CH_3CH_2O-, J=7.08 Hz), 4.31-4.36 (q, 2H, CH_3CH_2O-, J=7.08 Hz), 6.93 (s, 1H, pyrazole Ar-H), 7.15-8.59 (m, 6H,

Ar-H), 8.61 (s, 2H, NH_2, D_2O exchangeable), 11.56 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 14.6 ($\underline{CH_3}CH_2O$-), 60.9 ($CH_3\underline{CH_2}O$-), 89.9, 93.8 (pyrazole CH), 108.6, 112.2, 120.7, 122.2, 122.5, 125.1, 127.0, 137.0, 149.0, 149.5, 150.8, 154.5 (Ar-C), 166.6 (C=O). Anal. Calcd for $C_{17}H_{15}N_5O_2$, C, 63.54, H, 4.71, N, 21.79. Found: C, 63.67, H, 4.94, N, 22.12.

2-(1*H*-Indol-3-yl)-5-methyl-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (5).

A mixture of compound **2a** (0.5 g, 0.0025 mol) and compound **3b** (0.46 g, 0.0025 mol) in glacial acetic acid (20 mL) was heated under reflux for 15 hrs. Compound **5** gave a precipitate while hot. The reaction mixture was concentrated under reduced pressure and the separated solid was filtered, dried and crystallized from ethanol.

Yield (30%), mp (˃300°C), IR (KBr) cm^{-1}: 3410, 3309 (2 NH), 2920, 2850 (CH-aliphatic) 2214 (CN), 1662 (C=O), ^1H NMR (DMSO-d_6, ppm) δ: 2.51(s, 3H, CH_3), 6.67 (s, 1H, pyrazole Ar-H), 7.14-8.32 (m, 5H, Ar-H), 11.56 (s, 1H, NH, D_2O exchangeable), 13.38 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 19.2 (CH_3), 83.1, 88.5 (pyrazole CH), 116.3 (CN), 108.2, 112.2, 120.5, 121.7, 122.4, 125.2, 127.2, 137.0, 140.9, 152.6, 154.4, (Ar-C), 158.1 (C=O), Anal. Calcd for $C_{16}H_{11}N_5O$: C, 66.43, H, 3.83, N, 24.21. Found: C, 66.70, H, 4.05, N, 24.13.

General procedure for the synthesis of 7-amino-2-(1*H*-indol-3-yl)-5-substitutedpyrazolo[1,5-*a*]pyrimidine-6-carbonitriles 7a, b.

A mixture of compound **2a** (0.5 g, 0.0025 mol) and compound **6a** or compound **6b** (0.0025 mol) in ethanol (20 mL) was heated under reflux for 12-15 hrs in the presence of pyridine (1 mL) as a catalyst. Solid was precipitated on hot, the mixture was concentrated under reduced pressure. The separated solid was filtered, dried and crystallized from ethanol.

7-Amino-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (7a)

Yield (58%), mp (˃300°C), IR (KBr) cm^{-1}: 3433, 3394 (NH_2), 3325 (NH), 2222 (CN), ^{1}H NMR (DMSO-d_6, ppm) δ: 6.96 (s, 1H, pyrazole Ar-H), 7.13-8.69 (m, 6H, Ar-H), 8.81 (s, 2H, NH_2, D_2O exchangeable), 11.55 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 72.8, 94.2 (pyrazole C), 117.0 (CN), 108.6, 112.0, 120.5, 122.4, 122.9, 125.2, 127.2, 137.1, 149.3, 149.7, 151.5, 154.5 (Ar-C), Anal. Calcd for $C_{15}H_{10}N_6$: C, 65.68, H, 3.67, N, 30.64. Found: C, 65.37, H, 3.88, N, 30.91.

7-Amino-2-(1*H*-indol-3-yl)-5-methylpyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (7b)

Yield (76%), mp (˃300°C), IR (KBr) cm^{-1}: 3394, 3294 (NH_2), 3143 (NH), 2916 (CH-aliphatic), 2214 (CN), ^{1}H NMR (DMSO-d_6, ppm) δ: 2.49 (s, 3H, CH_3), 6.81 (s, 1H, pyrazole Ar-H), 7.12-8.65 (m, 5H, Ar-H), 8.67 (s, 2H, NH_2, D_2O exchangeable), 11.53 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 24.1 (CH_3), 72.7, 93.2 (pyrazole CH), 117.1 (CN), 108.7, 112.0, 120.4, 122.3, 122.9, 125.2, 127.1, 137.1, 148.6, 149.7, 154.4, 159.5 (Ar-C), Anal. Calcd for $C_{16}H_{12}N_6$: C, 66.66, H, 4.20, N, 29.15. Found: C, 66.43, H, 4.39, N, 29.41.

General procedure for the synthesis of 7-amino-2-(1*H*-indol-3-yl)-5-substitutedphenylpyrazolo[1,5-*a*]pyrimidine-6-carbonitriles 9a-c.

A mixture of compound **2a** (0.5 g, 0.0025 mol) and compound **8a-c** (0.0025 mol) in absolute ethanol (20 mL) was heated under reflux in presence of pyridine (1 mL) as a catalyst. Solid was precipitated on hot, except for compound **9c** (precipitated after cooling). The mixture was concentrated under reduced pressure, the separated solid was filtered, dried and crystallized from ethanol.

7-Amino-5-(4-bromophenyl)-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (9a)

Reflux for 28 hrs, Yield (82%), mp (284-286°C), IR (KBr) cm^{-1}: 3437, 3402 (NH_2), 3294 (NH), 2202 (CN), ^1H NMR (DMSO-d_6, ppm) δ: 7.00 (s, 1H, pyrazole Ar-H), 7.14-8.72 (m, 9H, Ar-H), 8.84 (s, 2H, NH_2, D_2O exchangeable), 11.58 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 71.5, 94.5 (pyrazole CH), 117.3 (CN), 108.6, 112.0, 120.5, 122.4, 122.9, 124.0, 125.2, 127.3, 131.1, 131.7, 137.15, 148.5, 150.7, 155.0, 157.8, 162.7 (Ar-C), Anal. Calcd for $C_{21}H_{13}BrN_6$: C, 58.76, H, 3.05, N, 19.58. Found: C, 58.90, H, 3.21, N, 19.63.

7-Amino-5-(4-fluorophenyl)-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (9b)

Reflux for 16 hrs, Yield (50%), mp (294-296°C), IR (KBr) cm^{-1}: 3444, 3387 (NH_2), 3309 (NH), 2206 (CN), ^1H NMR (DMSO-d_6, ppm) δ: 6.99 (s, 1H, pyrazole Ar-H), 7.14-8.72 (m, 9H, Ar-H), 8.82 (s, 2H, NH_2, D_2O exchangeable), 11.58 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 71.6, 94.4 (pyrazole CH), 117.4 (CN), 108.6, 112.0, 115.6, 120.5, 122.9, 125.2, 127.3, 131.3, 134.5, 137.1, 148.5, 150.7, 155.0, 157.9, 162.3, 164.7 (Ar-C), Anal. Calcd for $C_{21}H_{13}FN_6$: C, 68.47, H, 3.56, N, 22.81. Found: C, 68.90, H, 3.67, N, 22.72.

7-Amino-2-(1*H*-indol-3-yl)-5-(2-methoxyphenyl)pyrazolo[1,5-*a*]pyrimidine-6-carbonitrile (9c)

Reflux for 36 hrs, Yield (35%), mp (˃300°C), IR (KBr) cm^{-1}: 3437, 3294, (NH_2), 3228 (NH), 2916, 2850 (C-H, aliphatic), 2229 (CN), ^{1}H NMR (DMSO-d_6, ppm) δ: 3.84 (s, 3H, OCH_3), 6.94 (s, 1H, pyrazole Ar-H), 7.07-8.69 (m, 9H, Ar-H), 8.71 (s, 2H, NH_2, D_2O exchangeable), 11.57 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 55.9 (-OCH_3), 74.4, 94.2 (pyrazole CH), 116.8 (CN), 108.6, 112.0, 120.5, 120.8, 122.4, 122.8, 124.4, 125.2, 127.2, 127.7, 130.4, 131.5, 137.1, 148.7, 149.7, 154.6, 156.9, 158.5 (Ar-C), Anal. Calcd for $C_{22}H_{16}N_6O$: C, 69.46, H, 4.24, N, 22.09. Found: C, 69.72, H, 4.38, N, 22.37.

General procedure for the synthesis of 5,7-bis(4-halophenyl)-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidines 11a, b.

A mixture of compound **2a** (0.5 g, 0.0025 mol), and compound **10a** or compound **10b** (0.0025 mol) in glacial acetic acid (20 mL) was heated under reflux for 30 hrs. The mixture was cooled, the separated solid was filtered, dried and crystallized from ethanol.

5,7-Bis(4-bromophenyl)-2-(1*H*-Indol-3-yl)pyrazolo[1,5-*a*]pyrimidine (11a)

Yield (42%), mp (˃ 300°C), IR (KBr) cm^{-1}: 3402 (NH), ^{1}HNMR (DMSO-d_6, ppm) δ: 6.38 (s, 1H, Pyrazole Ar-H), 6.40-8.23 (m, 14H, Ar-H), 11.11 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 108.4, 109.7, 111.7, 114.8, 119.7, 120.8, 121.8, 122.6, 123.5, 126.4, 126.6, 129.7, 131.0, 131.7, 132.2, 136.1, 136.9, 138.27, 140.0, 145.1, 153.8, 154.4 (Ar-C), Anal. calcd for $C_{26}H_{16}Br_2N_4$: C, 57.38, H, 2.96, N, 10.29. Found: C, 57.43, H, 3.12, N, 10.43.

5,7-Bis(4-chlorophenyl)-2-(1*H*-indol-3-yl)pyrazolo[1,5-*a*]pyrimidine (11b)

Yield (55%), mp (˃ 300°C), IR (KBr) cm^{-1}: 3398 (NH), ^{1}HNMR (DMSO-d_6, ppm) δ: 6.96 (s, 1H, Pyrazole Ar-H), 6.99-8.33 (m, 14H, Ar-H), 11.10 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 108.4, 109.8, 111.7, 115.0, 119.7, 120.8, 121.8, 126.4, 126.6, 128.1, 129.3, 129.5, 131.5, 133.9, 134.8, 136.0, 136.6, 137.92, 140.0, 145.1, 153.7, 154.3 (Ar-C), Anal. calcd for $C_{26}H_{16}Cl_2N_4$: C, 68.58, H, 3.54, N, 12.30. Found: C, 68.23, H, 3.62, N, 12.49.

3-(1*H*-Indol-3-yl)-4,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine (12).

A mixture of compound **2b** (0.69 g, 0.0025 mol) and acetylacetone (0.25 g, 0.0025 mol) in glacial acetic acid (20 mL) was heated under reflux for 16 hr. The reaction was concentrated under reduced pressure, then few drops of water was added, the obtained emulsion was left to settle in the fridge overnight. The separated solid was filtered, dried and crystallized from ethanol.

Yield (43%), mp (198-200°C), IR (KBr) cm^{-1}: 3398 (NH), 2954, 2850 (CH-aliphatic), ^{1}H NMR (DMSO-d_6, ppm) δ: 2.51 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 7.05-8.40 (m, 11H, Ar-H), 11.53 (s, 1H, NH, D2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 19.8, 24.9 (CH_3), 108.1, 112.2, 114.6, 120.0, 120.2, 120.4, 120.9, 122.2, 125.8, 127.1, 127.4, 129.5, 136.5, 140.0, 141.1, 143.8, 151.2, 158.7 (Ar-C). Anal. calcd for $C_{22}H_{18}N_4$: C, 78.08, H, 5.36, N 16.56. Found: C, 78.35, H, 5.48, N, 16.67.

3-(1*H*-Indol-3-yl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-4,6(5*H*,7*H*)-dione (13).

A mixture of compound **2b** (0.69 g, 0.0025 mol) and diethyl malonate (0.40 g, 0.0025 mol) in glacial acetic acid (20 mL) was heated under reflux for 18 hr. The reaction was concentrated under reduced pressure, then few drops of water was added, the obtained emulsion was left to settle in the fridge overnight. The separated solid was filtered, dried and crystallized from ethanol. Yield (47%), mp (207-209°C), IR (KBr) cm^{-1}: 3221, 3190 (NH), 2920, 2850 (CH-aliphatic), 1634, 1635 (2 C=O), ^{1}H NMR (DMSO-d_6, ppm) δ: 2.03 (s, 2H, CO-$\underline{CH_2}$-CO), 6.75-8.21 (m, 10H, Ar-H), 9.98 (s, 1H, NH, D_2O exchangeable), 11.31 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR ($CDCl_3$, ppm): 24.9 (CH_2), 79.6, 100.3, 112.0, 115.8, 118.0, 119.9, 121.4, 122.0, 123.5, 129.6, 136.8, 139.5, 149.2, 155.7, 157.6, 169.5 (C=O), 170.8 (C=O), Anal. Calcd for $C_{20}H_{14}N_4O_2$: C, 70.17, H, 4.12, N, 16.37. Found: C, 70.35, H, 4.29, N, 16.70.

In vitro cytotoxic screening

The synthesized merdianin analogues were evaluated for their cytotoxic activity using MTT- cytotoxicity assay on HCT-116 (colon carcinoma cell-line). The test compounds were dissolved in DMSO, and Staurosporine was used as a reference.

Cell Line cells were obtained from American Type Culture Collection, cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. All other chemicals and reagents were obtained from Sigma-Aldrich, or Invitrogen.

Cell culture protocol. The culture medium was removed to a centrifuge tube, and the cell layer was briefly rinsed with 0.25% (w/v) Trypsin 0.53 mM EDTA solution, to remove all traces of serum which contains Trypsin inhibitor. Then 2-3 L of the Trypsin-EDTA solution was added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed. After that, 6-8 L of the complete growth medium was added, and cells were aspirated gently by gently pipetting. The cell suspension was transferred to the centrifuge tube with the medium and cells from the first step, and it was centrifuged at 125 xg for 5 to 10 min, and the supernatant was discarded. The cell pellet was re-suspended in a fresh growth medium, and the appropriate aliquots of cell suspension were added to new culture vessels. Finally, the cultures were incubated at 37 for 24 h.

MTT – Cytotoxicity assay protocol:

Plate cells (cell density 1.2-1.810,000 cells/well) in a volume of 100 L complete growth medium 100 L of the tested compound per well in a 96-well plate were incubated for 24 h before the MTT assay. A series of concentrations (0.01, 0.1, 1, 10, 100M) of the panel of tested compounds were added to cells. After 24 h incubation, MTT stain (10L per 100L of the culture medium) was added, and the cells were incubated for additional 4h. The formed formazan crystals were dissolved in MTT Solubilization Solution [Sigma catalogue no. M-8910], and gently mixed in a gyratory shaker. The cultures were pipette up and down to completely dissolve the MTT formazan crystals. The absorbance of each cell was measured at a wavelength of 450 nm by BIOLINE ElIZA reader. The relation between surviving fraction and compound concentration was plotted and IC_{50} values expressed in M as the mean values of triplicate

wells from at least three experiments were calculated and reported as the mean standard error.

Kinase inhibitory activity

The kinase inhibitory activity of the synthesized compounds was determined using Human proto-oncogene serine/threonine-protein kinase **GSK-3α/β** ELISA Kit (catalog #V9361), **Dyrk1A** (catalog #D09-10G), **CK1ð/ε** (catalog #CS0400)**, CLK1** (catalog #V4057), **Erk2** (catalog #V9291) and **CDK5/p25** (catalog #C33-10G-10). All the compounds were tested for their inhibitory activity against kinases at 0.2, 1, 5 and 25 µM. The results were displayed in terms of IC_{50}. **Table 2** and **Figure 5** show the obtained results.

References

(1) Melaku, Y. A.; Appleton, S. L.; Gill, T. K.; Ogbo, F. A.; Buckley, E.; Shi, Z.; Driscoll, T.; Adams, R.; Cowie, B. C.; Fitzmaurice, C. Incidence, Prevalence, Mortality, Disability-Adjusted Life Years and Risk Factors of Cancer in Australia and Comparison with OECD Countries, 1990–2015: Findings from the Global Burden of Disease Study 2015. *Cancer Epidemiol.* **2018**, *52*, 43–54.

(2) Munro, M. H. G.; Blunt, J. W.; Dumdei, E. J.; Hickford, S. J. H.; Lill, R. E.; Li, S.; Battershill, C. N.; Duckworth, A. R. The Discovery and Development of Marine Compounds with Pharmaceutical Potential. *J. Biotechnol.* **1999**, *70* (1–3), 15–25.

(3) B Bharate, S.; R Yadav, R.; Battula, S.; A Vishwakarma, R. Meridianins: Marine-Derived Potent Kinase Inhibitors. *Mini Rev. Med. Chem.* **2012**, *12* (7), 618–631.

(4) Faulkner, D. J. Marine Natural Products. *Nat. Prod. Rep.* **2001**, *18* (1), 1–49.

(5) Bharate, S. B.; Yadav, R. R.; Battula, S.; Vishwakarma, R. A.; Reviews, M.; Chemistry, M.; Medicine, I. Meridianins : Marine-Derived Potent Kinase Inhibitors. **2012**, No. July 2017, 1–14. https://doi.org/10.2174/138955712800626728.

(6) Shaw, S. J.; Goff, D. A.; Lin, N.; Singh, R.; Li, W.; McLaughlin, J.; Baltgalvis, K. A.; Payan, D. G.; Kinsella, T. M. Developing DYRK Inhibitors Derived from the Meridianins as a Means of Increasing Levels of NFAT in the Nucleus. *Bioorganic Med. Chem. Lett.* **2017**, *27* (11), 2617–2621. https://doi.org/10.1016/j.bmcl.2017.03.037.

(7) Yadav, R. R.; Sharma, S.; Joshi, P.; Wani, A.; Vishwakarma, R. A.; Kumar, A.; Bharate, S. B. Meridianin Derivatives as Potent Dyrk1A Inhibitors and Neuroprotective Agents. *Bioorganic Med. Chem. Lett.* **2015**, *25* (15), 2948–2952. https://doi.org/10.1016/j.bmcl.2015.05.034.

(8) Giraud, F.; Alves, G.; Debiton, E.; Nauton, L.; Th, V.; Durieu, E.; Ferandin, Y.; Lozach, O.; Meijer, L.; Anizon, F.; et al. Synthesis , Protein Kinase Inhibitory Potencies , and in Vitro Antiproliferative Activities of Meridianin Derivatives. **2011**, 4474–4489.

(9) Radwan, M. A. A.; El-Sherbiny, M. Synthesis and Antitumor Activity of Indolylpyrimidines: Marine Natural Product Meridianin D Analogues. *Bioorg. Med. Chem.* **2007**, *15* (3), 1206–1211.

(10) Akue-Gedu, R.; Debiton, E.; Ferandin, Y.; Meijer, L.; Prudhomme, M.; Anizon, F.; Moreau, P. Synthesis and Biological Activities of Aminopyrimidyl-Indoles Structurally Related to Meridianins. *Bioorganic Med. Chem.* **2009**, *17* (13), 4420–4424. https://doi.org/10.1016/j.bmc.2009.05.017.

(11) Yadav, R. R.; Khan, S. I.; Singh, S.; Khan, I. A.; Vishwakarma, R. A.; Bharate, S. B. Synthesis, Antimalarial and Antitubercular Activities of Meridianin Derivatives. *Eur. J. Med. Chem.* **2015**, *98*, 160–169. https://doi.org/10.1016/j.ejmech.2015.05.020.

(12) Časar, Z.; Bevk, D.; Svete, J.; Stanovnik, B. Reaction of Methyl (2E)-3-Dimethylamino-2-(1H-Indol-3-Yl)-Propenoate with Ureas: Facile Entry into the Polycyclic Meridianin Analogues with Uracil Structural Unit. *Tetrahedron* **2005**, *61* (31), 7508–7519. https://doi.org/10.1016/j.tet.2005.05.075.

(13) Jiang, B.; Xiong, X.-N.; Yang, C.-G. Synthesis and Antitumor Evaluation of Novel Monoindolyl-4-Trifluoromethylpyridines and Bisindolyl-4-Trifluoromethylpyridines. *Bioorg. Med. Chem. Lett.* **2001**, *11* (4), 475–477.

(14) Jiang, B.; Yang, C.-G.; Xiong, W.-N.; Wang, J. Synthesis and Cytotoxicity Evaluation of Novel Indolylpyrimidines and Indolylpyrazines as Potential Antitumor Agents. *Bioorg. Med. Chem.* **2001**, *9* (5), 1149–1154.

(15) Xiong, W.; Yang, C.; Jiang, B. Synthesis of Novel Analogues of Marine Indole Alkaloids : Mono (Indolyl) -4-Trifluoromethylpyridines and Bis (Indolyl) -4-Trifluoromethylpyridines as Potential Anticancer Agents. *Bioorg. Med. Chem.* **2001**, *9*, 1773–1780.

(16) Slaett, J.; Romero, I.; Bergman, J. Cyanoacetylation of Indoles, Pyrroles and Aromatic Amines with the Combination Cyanoacetic Acid and Acetic Anhydride. *Synth.* **2004**, No. 16, 2760–2765.

(17) Chiara, Ghiron; Arianna, Nencini; Iolanda, Micco; Riccardo, Zanaletti; Laura, Maccari; Hendrick, Bothmann; Simon, Haydar; Maurizio, Varrone; Carmela, Pratelli; Boyd, H. No Title Azoles as Nicotinic Acetylcholine Receptor Modulators and Their Preparation, Pharmaceutical Compositions and Use in the Treatment of Diseases, 2008.

(18) Borisova, I. A.; Zubarev, A. A.; Rodinovskaya, L. A.; Shestopalov, A. M. Synthesis and Physicochemical Properties of Merocyanine Dyes Based on Dihydropyridine and Fragments of Cyanoacetic Acid Derivatives. *Arkivoc* **2017**, No. part iii, 73–86.

(19) Huppatz, J. L. Systemic Fungicides. The Synthesis of Certain Pyrazole Analogues of Carboxin. *Aust. J. Chem.* **1983**, *36* (1), 135–147.

(20) Jones, R. G. Reactions of Orthoesters with Active Methylene Compounds. *J. Am. Chem. Soc.* **1952**, *74* (19), 4889–4891.

(21) McCall, M. A. Reactions of Substituted Methylenemalononitriles and Their Derivatives1. *J. Org. Chem.* **1962**, *27* (7), 2433–2439.

(22) Gazit, A.; Yaish, P.; Gilon, C.; Levitzki, A. Tyrphostins I: Synthesis and Biological Activity of Protein Tyrosine Kinase Inhibitors. *J. Med. Chem.* **1989**, *32* (10), 2344–2352.

(23) Chen, X.; Chen, X.; Zhao, Z.; Lue, P.; Wang, Y. 2, 4-Dicyano-3-

diethylamino-9, 9-diethylfluorene Based Blue Light-emitting Star-shaped Compounds: Synthesis and Properties. *Chinese J. Chem.* **2009**, *27* (5), 971–977.

(24) Angeletti, E.; Canepa, C.; Martinetti, G.; Venturello, P. Amino Groups Immobilized on Silica Gel: An Efficient and Reusable Heterogeneous Catalyst for the Knoevenagel Condensation. *J. Chem. Soc. Perkin Trans. 1* **1989**, No. 1, 105–107. https://doi.org/10.1039/P19890000105.

(25) Denizot, F.; Lang, R. Rapid Colorimetric Assay for Cell Growth and Survival: Modifications to the Tetrazolium Dye Procedure Giving Improved Sensitivity and Reliability. *J. Immunol. Methods* **1986**, *89* (2), 271–277.

(26) Takeuchi, H.; Baba, M.; Shigeta, S. An Application of Tetrazolium (MTT) Colorimetric Assay for the Screening of Anti-Herpes Simplex Virus Compounds. *J. Virol. Methods* **1991**, *33* (1–2), 61–71.

(27) Vistica, D. T.; Skehan, P.; Scudiero, D.; Monks, A.; Pittman, A.; Boyd, M. R. Tetrazolium-Based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production. *Cancer Res.* **1991**, *51* (10), 2515–2520.

(28) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a Tetrazolium-Based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity Testing. *Cancer Res.* **1987**, *47* (4), 936–942.

(29) Edmondson, J. M.; Armstrong, L. S.; Martinez, A. O. A Rapid and Simple MTT-Based Spectrophotometric Assay for Determining Drug Sensitivity in Monolayer Cultures. *J. Tissue Cult. Methods* **1988**, *11* (1), 15–17.

(30) Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and

Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65* (1–2), 55–63.

(31) Wang, Q.; Zhou, Y.; Wang, X.; Evers, B. M. Glycogen Synthase Kinase-3 Is a Negative Regulator of Extracellular Signal-Regulated Kinase. *Oncogene* **2006**, *25* (1), 43.

(32) Wang, Q.; Wang, X.; Hernandez, A.; Kim, S.; Evers, B. M. Inhibition of the Phosphatidylinositol 3-Kinase Pathway Contributes to HT29 and Caco-2 Intestinal Cell Differentiation. *Gastroenterology* **2001**, *120* (6), 1381–1392.

(33) Ding, Q.; Wang, Q.; Evers, B. M. Alterations of MAPK Activities Associated with Intestinal Cell Differentiation. *Biochem. Biophys. Res. Commun.* **2001**, *284* (2), 282–288.