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Short communication

Synthesis and cytotoxic activity of 2-methylimidazo[1,2-*a*]pyridine- and quinoline-substituted 2-aminopyrimidine derivatives

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ABSTRACT

A series of 2-methylimidazo[1,2-*a*]pyridine- and quinoline-substituted 2-aminopyrimidines derivatives were synthesized using a convenient synthetic route. We evaluate the isosteric replacement of methyl groups in 4-(2-methylimidazo[1,2-*a*]pyridin-3-yl)-*N*-*p*-tolylpyrimidin-2-amine (compound **1**) by trifluoromethyl groups and the isosteric substitution of the 2-methylimidazo[1,2-*a*]pyridin-3-yl scaffold by quinolin-4-yl or quinolin-3-yl moieties. The replacement of hydrogen by fluorine does not affect notably the cytotoxic activity and CDK inhibitor activity in this series. Quinolin-4-yl-substituted compound, **8**, presents cytotoxic activity and is most effective and selective against CDK1/CycA than against CDK2/CycB. Compound **11**, which has a quinolin-3-yl moiety is CDK inhibitor but presents null cytotoxic activity. Quinolin-4-yl-substituted compounds constitute a new lead of cytotoxic and CDK inhibitor compounds from which more compelling and selective inhibitors can be designed.

1. Introduction

Cancer is a leading cause of death. Approximately, one in four persons dies of cancer in the United States [1] and cancer is the third most common cause of dead worldwide [2]. The incidence of cancer has not dropped over the last decades; the most complicated cases are present in developing countries and it is expected that in the following years its incidence will be bigger than the incidence of cardiovascular diseases [2].

Regardless of the use of surgical treatment and irradiation, chemotherapy still remains an important option for the treatment of solid cancers. Despite extensive research efforts, the therapeutic treatment of tumor patients is still not satisfying. Chemotherapeutic drugs should preferentially target tumor cells without harming normal cells or tissues. Unfortunately, in most cases, chemotherapy remains a treatment modality with harsh side effects due to the toxicity of the drugs. Toxic effects on rapidly proliferating cells such as intestinal cells and bone marrow can cause life-threatening infections and other side effects, which seriously affect the quality of life of the patient. The indiscriminate nature of current cancer chemotherapy and drug resistance has initiated a search for more selective approaches.

Imidazo[1,2-*a*]pyridine scaffold has been extensively studied for their pharmacological properties, such as hypnotic [3], antiviral [4], antiparasitic [5] and proton pump inhibitor [6]. This moiety has revealed inhibitory properties on several kinases. Some examples are IRK-4 [7], GSK3 [8] and MAPK/ERK kinase [9]. Recently, 2-aminopyrimidines substituted with 2-methylimidazo[1,2-*a*]pyridine have showed excellent cytotoxic and inhibitory activity against cyclin-dependent kinase 2 (CDK2) [10,11]. Meridianines, indole-substituted 2-aminopyrimidines, are kinase inhibitors [12], showing that the replacement of the imidazo[1,2-*a*]pyridine is possible. As a part of our search for new antitumor compounds, a new series of imidazo[1,2-*a*]pyridine- and quinoline-substituted 2-aminopyrimidines were synthesized as potential antitumoral compounds. At the same time and with the same objective we explore the bioisosteric replacement in compound **1** (Table 1) of the hydrogen atoms in the methyl group of 2-methylimidazo[1,2-*a*]pyridine and in the tolyl part of the molecule by fluorine. In addition, since it is known that the bioisosteric substitution of the 2-methylimidazo[1,2-*a*]pyridine by a quinoline ring gave good antagonist compounds when applied to bradykinin B_2 receptor [13], we prepared compounds **7**–**12**. The synthesized compounds (Table 1) were tested against the following tumor human cell lines: U251 (glioma), PC-3 (prostate), K562 (Leukemia), HCT-15 (colon),

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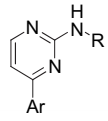
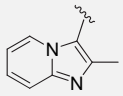
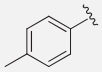
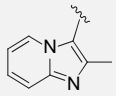
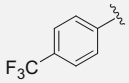
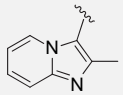
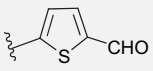
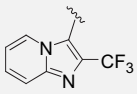
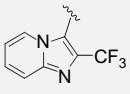
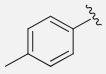
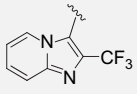
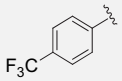
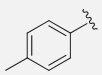
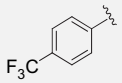
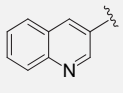
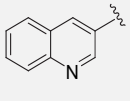
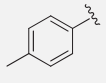
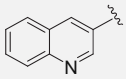
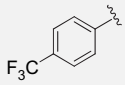
Table 1
In vitro susceptibility of SK-LU-1 and MCF7 cell lines of synthesized imidazo[1,2-*a*] pyridine and quinoline-substituted 2-aminopyrimidines.



Compound	Ar	R	IC_{50} (μM)[a]	
			SK-LU-1	MCF7
1			0.0197 ± 0.009	0.059 ± 0.007
2			0.034 ± 0.016	0.066 ± 0.002
3			1.540 ± 0.14	4.270 ± 0.36
4		H	56.510 ± 1.40	53.680 ± 21.62
5			0.175 ± 0.005	0.044 ± 0.02
6			0.745 ± 0.18	0.290 ± 0.24
7		H	>50	40.25 ± 0.25
8			1.400 ± 0.15	0.600 ± 0.04
9			1.720 ± 0.13	1.650 ± 0.06
10		H	>50.0	>50.0
11			NT	~50.0
12			NT	>50.0

NT = Not tested.
[a] The results show the concentration producing 50% of growth inhibition. Data are presented as mean ± SD of three independent experiments.

MCF7 (breast) and SK-LU-1 (lung). The effect on the cell cycle, cell death and inhibition of CDK2/CycA and CDK1/CycB of selected compounds was analyzed.

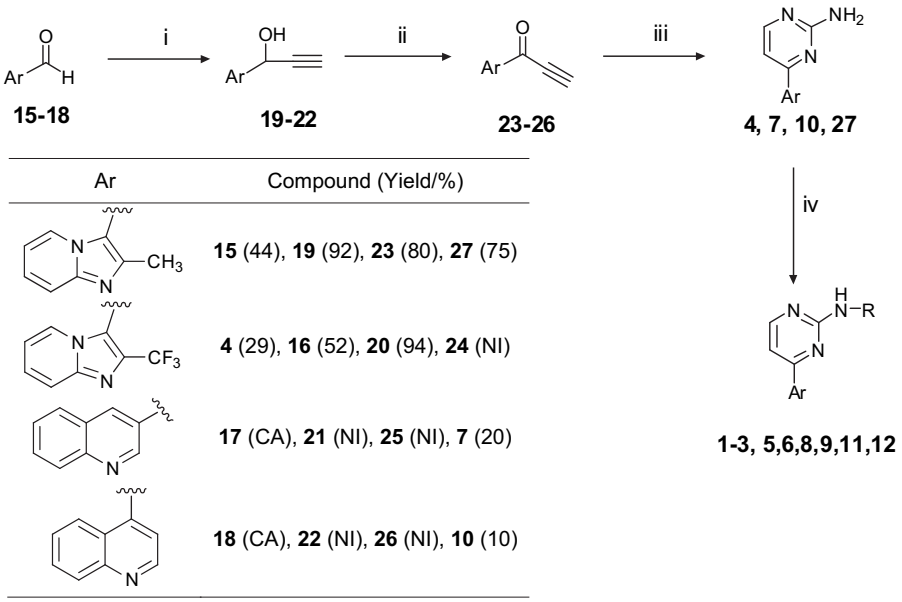
2. Chemistry

Synthesis of compounds **1**–**12** was achieved starting with the corresponding aldehyde (Scheme 1). 3- and 4-quinolinecarboxaldehyde are commercially available, 2-methyilimidazo[1,2-*a*]pyridine-3-carboxaldehyde (**15**) and 2-(trifluoromethyl)imidazo [1,2-*a*]pyridine-3-carboxaldehyde (**16**) were prepared through reaction of 2-aminopyridine with bromoacetone or 1,1,1-trifluoro-3-bromoacetone in ethanol-dioxane solution to afford compounds **13** and **14** (Scheme 2). Vilsmeier–Haack reaction of these with chloromethyliminium chloride yielded compounds **15** and **16** (Scheme 2).

Addition of ethynyl magnesium bromide 0.5 M in THF to the aromatic aldehydes **15**, **16**, **17** and **18** gave the propargylic alcohol derivatives **19**, **20**, **21** and **22**. These were oxidized with MnO_2 to afford the corresponding alkynyl ketone **23**, **25** and **26**. Oxidation of **20** with MnO_2 produce unsatisfactory results, but the use of calcium hypochlorite under phase-transfer catalyst condition produced **24**. Cyclocondensation of **23**, **24**, **25** and **26** with guanidine sulfate afforded imidazo[1,2-*a*]pyridines-substituted 2-aminopyrimidines **27** and **4** and quinoline-substituted 2-aminopyrimidines **7** and **10**. Then the desired products **1**, **2**, **3**, **5**, **6**, **8**, **9**, **11**, and **12** were obtained in excellent yields after the coupling between **27**, **4**, **7** and **10** and an aryl bromide or iodide under modified Buchwald–Hartig conditions [14]. All synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

3. Biological results and discussion

In this study we evaluated (1) the bioisosteric replacement in compound **1** of the methyl hydrogen atoms of 2-methyl-imidazo[1,2-*a*]pyridine by fluorine in compounds **4**, **5** and **6**, the substitution of methyl hydrogen atoms of the tolyl groups by fluorine in compound **2**, **6**, **9** and **12**. Fluorine presents the advantage of having a van der Waals radius comparable to that of hydrogen [15]. (2) The bioisosteric substitution of the complete 2-methylimidazo[1,2-*a*]pyridine scaffold by the isoelectronic quinoline-4-yl (compounds **7**, **8**, and **9**) and (3) the regioisomer quinoline-3-yl (compounds **10**, **11** and **12**). In compound **3** the entire toluene group was replaced with a thiazol ring. All compounds were evaluated for their cytotoxic activity in vitro against the tumor human cell lines U251 (glioma), PC-3 (prostate), K562 (leukemia), HCT-15 (colon), MCF7 (breast) and SK-LU-1 (lung). A primary screening at a fixed concentration of 50 μM showed cytotoxicity against the six human tumor cell lines (Table 2) as a positive control we used 5-fluorouracil at the same concentration. Although the type of cell lines tested differs among them in origin and genetic background some regularity could be observed. Compounds **1**–**9** showed better activity than 5-fluorouracil. In contrast, less activity or no activity was found for the

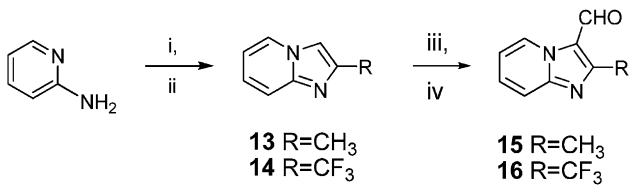


Scheme 1. Reagents: (i) Ethynyl magnesium bromide 0.5 M; (ii) MnO_2; for compound **20** $Ca(ClO)_2$, CH_2Cl_2, water, tetrabutylammonium bromide (catalytic); (iii) Guanidine sulfate, K_2CO_3, (iv) Xantphos (3%), $Pd(dba)_2$ (3%), Cs_2CO_3, toluene, aryl halide (1-iodo-4-methylbenzene, 1-iodo-4-(trifluoromethyl)benzene or 5-bromothiophene-2-carboxaldehyde). CA, commercially available; NI, not isolated.

compounds with the quinoline-3-yl substituent **10**, **11** and **12**. The most sensitive tumor human cell lines are MCF7 and SK-LU-1. In MCF7 cell line most of the synthesized compounds showed a 100 percent inhibition of cellular grown at 50 μM. We decided to determine the IC_{50} value of compounds **1**–**12** in the most sensitive cell lines MCF7 and SK-LU-1. Results are depicted in Table 1. The most potent compounds are **1** and **2** in both cell lines. The change of the tolyl group for a thiazolyl group reduces the biological activity of compound **3**. The bioisosteric replacement of the hydrogen atoms in the methyl group of the tolyl moiety do not affect significantly the biological activity of the compounds **2** and **9** comparing with **1** and **8** respectively. The substitution of the hydrogen atoms of the methyl group in position 2 of the imidazo[1,2-*a*]pyridine by fluorine, compound **5**, produces a nine fold decrement of potency in SK-LU-1 cells and a 0.74 fold decrement of potency in MCF7 cells than compound **1**. The replacement by fluorine in both methyl groups, tolyl and 2-methylimidazo[1,2-*a*]pyridin-3-yl moieties, produces a slight decrement in activity in MCF7 cell lines, and a moderate decrement in SK-LU-1 cells, this replacements cause an increase in the lipophilicity and an electron withdrawing effect which alter the biological properties of a molecule. Having a trifluoromethyl group in the molecule instead of methyl group increments the metabolic stability of the molecule. This could represent an advantage during in vivo studios.

Compounds **7** and **8** which enclose a quinolin-4-yl group instead of the imidazo[1,2-*a*]pyridin-3-yl scaffold, have modest cytotoxic activity in both SK-LU-1 and MCF7 cell lines. Comparing, compound **1** is 71 times more active in SK-LU-1 cell line and 10 times more active in MCF7 cell line than compound **8**; on the other hand, compound **2** is 50 times more active in SK-LU-1 cell line and 25 times more active than compound **9**. The cytotoxic activity exhibited by compounds **11** and **12** was not significant compared with compounds **8** and **9**. This last result indicates that quinolin-4-yl group is better than quinolin-3-yl group to replace the 2-methyl imidazo[1,2-*a*]pyridine-3-yl scaffold. Compounds **4** and **5** showed poor activity than compounds with and *N*-aryl moiety, e.g. **5** and **8**.

Next, in order to evaluate the effect of the selected compound (**1**, **3**, and **8**) on the grown and division of asynchronous SK-LU-1 and MCF-7 cell lines (Table 3, Fig. 1) we used flow cytometry, measuring the DNA content of each cell line after a 24 h treatment. As a positive control we used the CDK2 inhibitor Olomoucine and as a negative control DMSO alone at the highest quantity used in the experiments. All compounds were used at a concentration of approximately twice IC_{50} value in each cell line; except for compound **8** in SK-LU-1 cell line. The results show the proportion of cells emitting fluorescence proportional to the DNA content. In SK-LU-1 cells compound **1**, **3** and **8** significantly arrest cells in the G2/M phase of the cell cycle (Fig. 1) with a significant decrease in the proportion of cells in the G0/G1 phase in comparison with control



Scheme 2. Reagents: (i) Bromoacetone or 3-bromo-1,1,1-trifluoroacetone (ii) triethylamine; (iii) $POCl_3$, DMF; (iv) water.

Table 2
Inhibition of human tumor cells lines by compounds **1**–**12**.[a]

Compound	% of growth inhibition					
	U251	PC-3	K562	HCT-15	MCF7	SK-LU-1
1	100.0	94.2	89.4	95.2	88.5	100.0
2	85.6	64.5	84.8	88.6	83.4	100.0
3	57.8	49.5	88.1	85.4	75.9	100.0
4	92.2	74.0	56.3	274.0	100.0	71.0
5	100.0	100.0	100.0	100.0	100.0	100.0
6	100.0	92.0	94.2	94.4	100.0	95.0
7	100.0	77.4	9.4	NA	100.0	78.2
8	100.0	100.0	50.9	100.0	100.0	100.0
9	91.8	87.3	NA	100.0	100.0	100.0
10	38.5	31.9	NA	NA	100.0	59.3
11	87.7	59.5	NA	30.3	100.0	46.3
12	49.8	22.9	NA	24.0	80.9	35.4
5-fluorouracil	64.2	45.4	73.7	71.3	65.1	70.5

NA = Not active.
[a] 50 μM; mean of three experiments with three replicates each one.

Table 3
Cell cycle analysis of SK-LU-1 and MCF7 cell lines after treatment with **1**, **3**, and **8**. DMSO was used as vehicle control and Olomoucine, a CDK-2 inhibitor, as a positive control.

Compound	Cell cycle phase (%)[a]					
	SK-LU-1			MCF7		
	G1	S	G2/M	G1	S	G2/M
1	4.7 ± 0.4[b]	20.9 ± 10.7	77.5 ± 5.6 [b]	60.8 ± 1.8	25.3 ± 4.3	14.7 ± 7.0
3	42.5 ± 0.4[b]	13.9 ± 4.2	48.0 ± 8.5[b]	54.5 ± 6.3	15.7 ± 4.6	22.2 ± 1.6
8	8.1 ± 9.6[b]	22.0 ± 19.3	74.0 ± 11.4[b]	18.7 ± 5.6[b]	15.1 ± 6.7	64.7 ± 1.4[b]
DMSO	61.7 ± 2.9	18.2 ± 1.5	16.1 ± 0.9	57.0 ± 3.7	17.0 ± ± 1.2	23.2 ± 0.4
Olomoucine	44.2 ± 2.9	19.7 ± 8.6[b]	31.8 ± 3.7[b]	60.6 ± 3.1	15.1 ± 2.3	23.2 ± 0.4

[a] Results are expressed as means ± SD of three independent experiments. Cells were incubated with the compounds for 24 h.
[b] $P < 0.05$ as compared with solvent (DMSO) on 24 h treatment.

cells (DMSO, Fig. 1), S phase presents no difference with respect to control. Olomoucine has significant effect on S and G2/M phase of cell cycle of SK-LU-1 at the concentration used in these experiments (Table 3). MCF7 cell line shows a cell cycle pattern after treatment with compound **1** and **3** which possess a 2-methylimidazo[1,2-*a*]pyridine scaffold, similar to control cells, differences are statistically not significant. In contrast, compound **8** shows a strong arrest in G2/M phase of the cell cycle after a 24 h treatment in comparison with control cells (Table 3, Fig. 1) and a diminution in the G1/G0 phase of the cell cycle; S phase not presents significant differences in comparison with control cells. Olomoucine has no effect on cell cycle of MCF7 cells at the concentration used in these experiments (Table 3). The cell enlargement characteristic of a G2/M arrest was also evident as an enhancement in the FSC vs. SSC dot plots generated by all the tested compounds in SK-LU-1 cells and by compound **8** in MCF7.

Resistance to cell apoptosis is one of the mechanisms that is important in cancer drug resistance. To determine whether G2/M arrest was followed by induction of apoptosis, asynchronously growing SK-LU-1 cell line was exposed to compounds **1**, **3**, **5** and **8** for 24 and 48 h. Cell death was monitored by flow cytometry, using the binding of fluorescein isothiocyanate-labeled annexin V to phosphatidylserine (Annexin V-FITC). Apoptotic cells show positive fluorescence for annexin V-FITC; subsequently, an augment in the green fluorescence in the FLH-1 channel indicates an increase in apoptotic cell death. The upper left corner of the quadrant represents debris, lower left are live cells, upper right are late apoptotic or necrotic cells and lower right are apoptotic cells. After an incubation period of 24 h with the mentioned compounds, apoptosis was not significantly superior to control experiment. However, our data shows that compounds **1**, **3**, **5** and **8** are effective apoptotic inducers on SK-LU-1 cell line after a 48 h treatment (Fig. 2), showing a significant increase in apoptotic death cells comparing with DMSO treated cells. Not significant necrotic cells were found in cells treated with molecules **1**, **3**, **5** and **8**. If a molecule induces death via an apoptotic pathway, then this molecule will cause fewer toxic side reactions in vivo than if it induces death via necrosis.

It has been reported that substituted 2-aminopyrimidines are kinase inhibitors, especially of CDK2 [10–12]. We next analyzed the activity of compounds **1**, **2**, **3**, **8**, and **11** on recombinant CDK2/CycA and CDK1/CycB (Table 4). We used as positive control Olomoucine, it has been reported that the IC_{50} on CDK2/CycA of this compound is 7 μM [16]; for this reason we tested all compounds at the same concentration. In general all compounds tested are better than Olomoucine in both CDK2/CycA and CDK1/CycB. Compound **1**, **2** and **3** inhibit CDK1/CycB at the same extend at 7 μM. Interestingly, compound with a quinolinyl moiety present better inhibitory potency against CDK1/CycB, this observation could explain the G2/M arrest induced in MCF7 cell line when compounds **1** and **3** failed to exhibit this phenomena on this cell line (Fig. 1, Table 3). On CDK2/

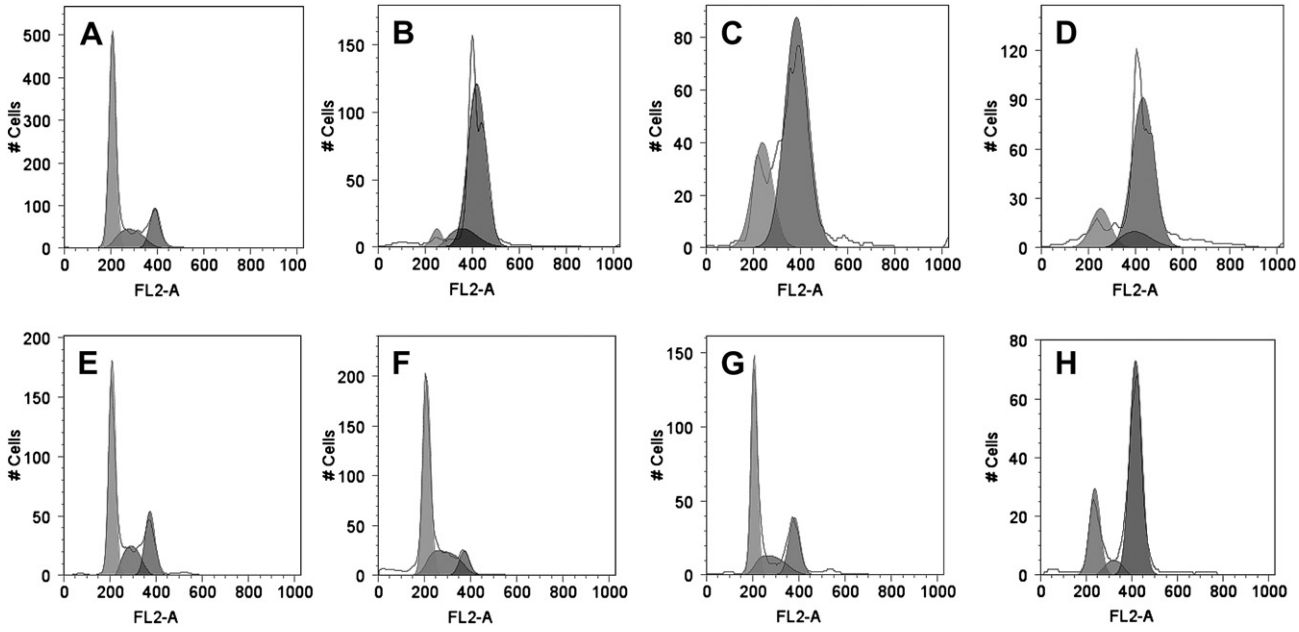


Fig. 1. Cell cycle phase effect of compound **1**, **3**, and **8** on SK-LU-1 and MCF7 cell lines. DMSO was used as solvent control (**A**, SK-LU-1; **E**, MCF7). Olomoucine was used as a positive control (not shown). Treatment with 0.04 μM compound **1** (**B**), 8.4 μM compound **3** (**C**), 1 μM compound **8** (**D**), and 70 μM Olomoucine were utilized on SK-LU-1 cells. 0.118 μM compound **1** (**F**), 8.4 μM compound **3** (**G**), 1 μM compound **8** (**H**), and 60 μM Olomoucine were used on MCF7 cells. Representative experiment out of three is shown.

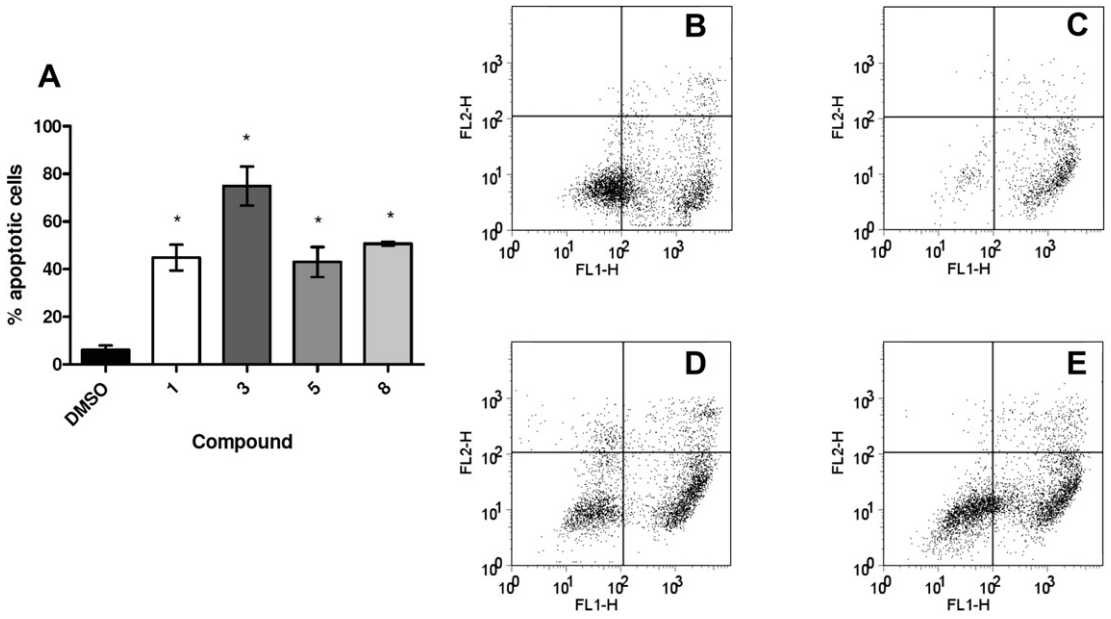


Fig. 2. Induction of apoptosis on SK-LU-1 after treatment with 0.04 μM compound **1**, 8.4 μM compound **3**, 0.4 μM compound **5** and 1 μM compound **8** for 48 h, DMSO was used as a negative control (**A**). Detection of apoptosis through annexin V and PI staining after treatment with **1** (**B**), **3** (**C**), **5** (**D**), and **8** (**E**) on SK-LU-1 cells. Representative experiment out of three is shown.

CycA most active compounds are **2** and **3**, albeit compound **3** does not exhibit the highest cytotoxic value compared with **1** and **2**. It is important to note that compound **11** was never significantly cytotoxic; however it shows inhibition on CDKs similar to **8** in both CDK1/CycB and CDK2/CycA (Table 1, Table 4). These differences in cytotoxic and CDKs inhibitory activity may be reflecting variations in cellular permeability, in intracellular distribution, and in metabolism.

In conclusion we have synthesized a series of 2-methylimidazo[1,2-*a*]pyridine- and quinoline-substituted 2-aminopyrimidines which inhibit CDK1/CycB and CDK2/CycA. Among the compounds containing a 2-methylimidazo[1,2-*a*]pyridine, hydrogen replacement by fluorine does not affect notably the cytotoxic activity. Quinoline-substituted compound, **8** and **11**, are most potent and selective against CDK1/CycB than against CDK2/CycA. Quinolin-4-yl-substituted compounds constitute a new family of cytotoxic and CDK inhibitor compounds from which more potent and selective inhibitors can be designed. The compounds synthesized broaden the knowledge of the activity of these kinds of derivatives.

4. Experimental protocols

Melting points were determined on a MEL-TEMP® capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Gemini 200 MHz, Varian Unity 300 MHz, and Varian Inova 500 MHz (^{1}H NMR and 125 MHz ^{13}C NMR). Chemical shifts are expressed as δ values relative to TMS as internal standard, J values are given in Hz; spectra were recorded in $CDCl_3$, or a mixture of $CDCl_3$ and DMSO-d_6 when problems of solubility were presented (compounds **2**, **3**, **7**, **10** and **12**). Mass spectra were recorded on a JEOL JMS-SX 10217 spectrometer by electron impact (EI). All reagents were acquired from Sigma-Aldrich unless otherwise stated. The solvents were distilled before being used. Tetrahydrofuran (THF) and toluene were dried with sodium using benzophenone as indicator. Bromoacetone was obtained from bromide and acetone and distilled under vacuum prior to use [17]. Silica gel (mesh 230–400) was purchased from Merck.

4.1. Chemistry

4.1.1. General method of synthesis of compounds *13* and *14*

2-Aminopyridine is dissolved in a 3:1 mixture of ethanol–dioxane and 1.1 equivalents of the appropriated bromoacetone are added slowly. The reaction mixture is vigorously mixed during 2 h, a solid is formed. Then three equivalents of triethylamine are added and the mixture is refluxed overnight. The volatiles are removed under vacuum and water is added to dissolve the formed salts.

*4.1.1.1. 2-Methylimidazo[1,2-a]pyridine (**13**).* The aqueous suspension is extracted with AcOEt. The organic phase is dried with anhydrous Na_2SO_4 and the solvent is evaporated in a Rotavapor evaporator. The oily residue is distillated under vacuum to give the title compound as yellow pale oil which solidifies and is stable at 4 °C. Yield 50%. B. p. 108–110 °C/6 mmHg. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 2.46 (d, 3H, $J = 0.8$ Hz), 6.71 (td, 1H, $J = 1.2$, 6.6 Hz), 7.10 (ddd, 1H, $J = 1.2$, 5.6, 9.0 Hz), 7.33 (s, 1H), 7.50 (dd, $J = 0.8$, 9.0 Hz), 8.02 (dt, 1H, $J = 1.2$, 6.8 Hz). $C_8H_8N_2$ calculated m. w.: 132.16. MS [EI+] m/z: 132 $[M]^+$ (100), 131 $[M-H]^+$ (80).

*4.1.1.2. 2-(Trifluoromethyl)imidazo[1,2-a]pyridine (**14**).* A solid is formed after adding water which is separated by suction. The solid is purified by column chromatography with EtOAc–Hexane 1:1. Recrystallization from hexane yields 75% of a white solid. M. p. 91–92 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.92 (td, 1H, $J = 1.2$, 6.9 Hz), 7.31 (ddd, 1H, $J = 1.2$, 6.9, 9.3 Hz), 7.89 (s, 1H), 8.15 (td, 1H, $J = 1.2$, 6.9 Hz). ^{13}C NMR ($CDCl_3$, 75.5 MHz), δ: 111.46, 113.96, 118.70,

Table 4
Percent of inhibition of human CDK/Cyc enzymes by compounds **1**, **2**, **3**, **8** and **11**.[a]

CDK/Cyc	Compound					
	Olomoucine	**1**	**2**	**3**	**8**	**11**
CDK1/CycB	48	59	60	52	75	73
CDK2/CycA	43	58	69	76	45	49

[a] 7 μM; mean of two independent experiments.

122.00 (q, $J = 267.5$ Hz), 126.25, 126.52, 135.96 (q, $J = 38$ Hz), 145.4. $C_8H_5F_3N_2$ calculated m. w.: 186.13 MS [EI+] m/z: 186 $[M]^+$ (100), 167 $[M - F]^+$ (15).

*4.1.2. General method of synthesis of compounds **15** and **16***

Over excess of anhydrous DMF at 0 °C are added slowly 2.5 equivalents of $POCl_3$ and the mixture is mixed during 15 min. Posterior, one equivalent of compound **13** or **14** is added and the reaction mixture is stirred vigorously by 1 h. Then the reaction is warmed at 60 °C by 24 h or until no starting material is detected by TLC. Chopped ice was added and the mixture is neutralized with ammonium hydroxide.

*4.1.2.1. 2-Methylimidazo[1,2-a]pyridine-3-carbaldehyde (**15**).* Oil was obtained which was solubilized in hot hexane, after cooling a solid was formed. Recrystallized from hexane to obtain a white solid. Yield 44% m. p. 119–120 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.73 (s, 3H), 7.07 (td, 1H, $J = 1.2$, 6.8 Hz), 7.52 (ddd, 1H, $J = 1.2$, 6.8, 9.0 Hz), 7.68 (td, 1H, $J = 1.2$, 9.0 Hz), 10.01 (s, 1H). ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ: 114.46, 114.83, 116.63, 121.19, 128.31, 130.01, 147.65, 157.08, 176.96. $C_9H_8N_2O$ calculated m. w.: 160.17 MS [EI+] m/z: 160 $[M]^+$ (100), 159 $[M - H]^+$ (98), 131 $[M - CHO]^+$ (25).

*4.1.2.2. 2-(Trifluoromethyl)imidazo[1,2-a]pyridine-3-carbaldehyde (**16**).* Purified by column chromatography (EtOAc–Hexane 1:9) and recrystallized from hexane to obtain 52% yield of white plaques. M. p. 116–118 °C. ^{1}H NMR ($CDCl_3$, 200 MHz) 7.28 (td, 1H, $J = 1.2$, 7.0 Hz), 7.68 (ddd, 1H, $J = 1.2$, 6.8, 9.0 Hz), 7.90 (dt, 1H, $J = 1.2$, 9.0 Hz), 9.65 (dt, 1H, $J = 1.2$, 7 Hz), 10.22 (s, 1H). ^{13}C ($CDCl_3$, 75.5 MHz) δ: 114.46, 114.83, 116.63, 122.00 (q, $J = 267.5$ Hz), 128.31, 130.01, 147.65, 157.08, 176.96. $C_9H_5F_3N_2O$ calculated m. w.: 214.14 MS [EI+] m/z: 214 $[M]^+$ (100), 195 $[M - F]^+$ (75), 185 $[M - CHO]^+$ (20).

*4.1.3. General method of synthesis of compounds **4**, **7**, **10** and **27***

The corresponding aldehyde **15**, **16**, **17** or **18** was reacted with 2 equivalents of ethynyl magnesium bromide 0.5 M in anhydrous THF at −70 °C under nitrogen atmosphere. The mixture was stirred by 30 min and allowed to reach room temperature. A saturated NH_4Cl solution was added and the aqueous layer was separated and extracted with THF; this reaction is quantitative.

The propargylic alcohol derivatives obtained **19**, **21** and **22** were oxidized with 35 equivalents of MnO_2 in reducing agents free acetone mixing for 30 min. After the completion of reaction, the MnO_2 was separated by suction filtration and washed with acetone. Concentration of solvent in a Rotavapor evaporator yielded compounds **23**, **25** or **26**. Compound **20** was oxidized employing 5 equivalents of $Ca(ClO)_2$ in CH_2Cl_2–water and using 7% of tetrabutylammonium hydrogen sulfate as phase-transfer catalyst, the mixture was stirred vigorously and heated to reflux for one hour, the reaction mixture is diluted with CH_2Cl_2 and extracted with brine. Concentration of the organic layer under vacuum afforded compound **24**. The alkynyl ketones **23**–**26** were used in the following reaction without further purification.

Compounds **23**–**26** were reacted with 2 equivalents of guanidine sulfate in presence of 3 equivalents of K_2CO_3 in *n*-butanol, reactions were heated in an oil bath to reflux overnight or until the reaction was over by TLC. All volatiles were removed under vacuum and the solid form was extracted with acetone. The suspended solid was removed by filtration and discarded. The solvent was removed under vacuum, and the resulting solid was purified. Following this procedure the following compounds were obtained.

*4.1.3.1. 4-(2-(Trifluoromethyl)imidazo[1,2-a]pyridin-3-yl)pyrimidin-2-amine (**4**).* Purified by column chromatography with EtOAc. Yield 29% of a yellow solid. M. p. 230–231 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.00 (d, 1H, $J = 5.4$ Hz), 7.02 (dd, 1H, $J = 1.2$, 9.2 Hz), 7.44 (td, 1H, $J = 1.4$, 9.2 Hz), 7.78 (dd, 1H, $J = 1.2$, 9.2 Hz), 8.40 (d, 1H, $J = 5.4$ Hz), 9.20 (dd, 1H, $J = 1.2$, 7.2 Hz). $C_{12}H_8F_3N_5$ calculated m. w.: 279.22 MS [EI+] m/z: 279 $[M]^+$ (100), 278 $[M - H]^+$ (55), 258 $[M - H_2F]^+$ (20).

*4.1.3.2. 4-(Quinolin-4-yl)pyrimidin-2-amine (**7**).* Purified by column chromatography with EtOAc–Hexane 9:1 to obtain 10% of the title compound. Yellow solid, m. p. 228–229 °C (desc.) ^{1}H NMR ($CDCl_3$ + DMSO-d_6, 500 MHz) δ: 6.30 (s, 2H); 6.78 (d, 1H, $J = 5.0$ Hz); 7.45 (d, 1H, $J = 4.5$ Hz); 7.50 (ddd, 1H, $J = 1.5$, 6.5, 8.5 Hz), 7.68 (ddd, 1H, $J = 1.4$, 6.8 Hz, 8.6 Hz); 8.04 (dd, 1H, $J = 0.5$ Hz, 8.0 Hz); 8.16 (dd, 1H, $J = 1.0$, 8.5 Hz); 8.35 (d, 1H, $J = 5.0$ Hz), 8.90 (d, 1H, $J = 4.5$ Hz). ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 125.7 MHz) δ: 110.35, 120.22, 124.64, 125.17, 126.45, 128.92, 129.14, 143.91, 148.05, 149.37, 158.45, 163.15, 164.06. $C_{13}H_{10}N_4$ calculated m. w.: 222.25 MS [EI+] m/z: 222 $[M]^+$ (70), 221 $[M - H]^+$ (100).

*4.1.3.3. 4-(Quinolin-3-yl)pyrimidin-2-amine (**10**).* Title compound was purified by column chromatography using EtOAc–Hexane 8:2 to yield 20% of a yellow solid, M. p. 252–253 °C (desc.), ^{1}H NMR ($CDCl_3$ + DMSO-d_6, 200 MHz) δ: 5.64 (ws, 2H), 7.21 (d, 1H, $J = 5.2$ Hz), 7.62 (td, 1H, $J = 1.2$, 7.0 Hz), 7.79 (td, 1H, $J = 1.2$, 6.8 Hz), 7.97 (dd, 1H, $J = 0.6$, 8.2 Hz), 8.14 (d, 1H, $J = 8.4$ Hz), 8.43 (d, 1H, $J = 5.2$ Hz, H-8), 8.81 (d, 1H, $J = 1.8$ Hz), 9.52 (d, 1H, $J = 2.4$ Hz). $C_{13}H_{10}N_4$ calculated m. w.: 222.25 MS [EI+] m/z: 222 $[M]^+$ (85), 221 $[M - H]^+$ (100).

*4.1.3.4. 4-(2-Methylimidazo[1,2-a]pyridin-3-yl)pyrimidin-2-amine (**27**).* Purified by column chromatography using EtOAc–acetone 1:1 to yield 75% of the yellow solid. M. p. 167 °C, ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.10 (s, 3H), 6.87 (d, 1H, $J = 5.4$ Hz), 6.88 (td, 1H, $J = 1.2$, 6.9 Hz), 7.30 (ddd, 1H, $J = 1.2$, 6.6, 9.0 Hz), 7.61 (dt, 1H, $J = 1.2$, 9.0 Hz), 9.62 (dt, 1H, $J = 1.2$, 9.0 Hz). ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ: 16.76, 108.95, 112.58, 116.64, 126.26, 127.85, 128.62, 146.22, 147.07, 158.01, 158.21, 162.62. $C_{12}H_{11}N_5$ calculated m. w.: 225.25 MS [EI+] m/z: 225 $[M]^+$ (85), 224 $[M - H]^+$ (100).

*4.1.4. General procedure to synthesize compounds **1**, **2**, **3**, **5**, **6**, **8**, **9**, **11**, and **12***

In an well oven-dried Schlenk flask were added 1 equivalent of **4**, **7**, **10** or **27**, 1.1 equivalents of the proper aryl halide, 3–5% Xantphos, 3–5% $Pd(dba)_2$ and 3 equivalents of anhydrous Cs_2CO_3. The flask was evacuated/backfilled with nitrogen at least three times and anhydrous toluene was added into the flask, the flask was evacuated/backfilled for others two times. The reaction mixture was warmed at 80 °C until the starting materials were completely consumed or the reaction did not proceed anymore (4–12 h). Then, the reaction was allowed to get room temperature and the solids were removed by vacuum filtration and rinsed plenty with EtOAc. All compounds were purified by column chromatography.

*4.1.4.1. 4-(2-Methylimidazo[1,2-a]pyridin-3-yl)-N-p-tolylpyrimidin-2-amine (**1**).* Eluted with EtOAc to obtain 80% of a pale green solid. M. p. 190 °C (desc.); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 2.36 (s, 3H), 2.74 (s, 3H), 6.78 (td, 1H, $J = 1.4$, 7.0 Hz), 6.95 (d, 1H, $J = 5.4$ Hz), 7.17 (d, 2H, $J = 8.2$ Hz), 7.29 (ddd, 1H, $J = 1.2$, 6.8, 9.0 Hz), 7.49 (d, 2H, $J = 8.4$ Hz), 7.61 (dt, 1H, $J = 1.2$, 9.0 Hz), 8.44 (d, 1 H, $J = 5.6$ Hz), 9.59 (dt, 1H, $J = 1.2$, 7.0 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ: 17.07, 20.85, 109.30, 112.55, 116.66, 120.92, 126.38, 128.28, 129.44, 132.85, 136.64, 146.26, 147.34, 157.62, 157.91, 160.08; $C_{19}H_{17}N_5$ calculated m. w.: 315.37 MS [EI+] m/z: 315 $[M]^+$ (95), 314 $[M - H]^+$ (100).

*4.1.4.2. 4-(2-Methylimidazo[1,2-a]pyridin-3-yl)-N-(4-(trifluoromethyl)-phenyl)-pyrimidin-2-amine (**2**).* Eluted with EtOAc to obtain 95% of the title compound as a yellow powder. M. p. 201–202 °C (desc.); ^{1}H

NMR ($CDCl_3$, 200 MHz) δ: 2.76 (s, 3H), 6.87 (td, 1H, $J = 1.2$, 6.8 Hz), 7.06 (d, 1H, $J = 5.4$ Hz), 7.35 (ddd, 1H, $J = 1.4$, 7.0, 9 Hz), 7.38 (ws, 1H), 7.59 (d, 2H, $J = 8.2$ Hz), 7.65 (dt, 1H, $J = 1.2$, 9.0 Hz), 7.79 (d, 2H, $J = 8.4$ Hz), 8.52 (d, 1H, $J = 5.4$ Hz), 9.55 (dt, 1H, $J = 1.2$, 7.0 Hz), ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 75.5 MHz) δ: 16.43, 109.64, 112.23, 116.05, 118.37, 119.85 (q, $J = 265.76$ Hz), 125.31 (q, $J = 4.53$ Hz), 126.09, 127.72, 143.03, 145.70, 146.73, 156.94, 157.28, 159.27. $C_{19}H_{14}F_3N_5$ calculated m. w.: 369.34 MS [EI+] m/z: 369 $[M]^+$ (100), 368 $[M - H]^+$ (92).

*4.1.4.3. 5-(4-(2-Methylimidazo[1,2-a]pyridin-3-yl)pyrimidin-2-yl-amino)thiophene-2-carbaldehyde (**3**).* Eluted with EtOAc–Hexane 6:4 obtaining 65% of dark green flakes. M. p. 235 °C (desc.); ^1H NMR ($CDCl_3$ + DMSO-d_6, 200 MHz) δ: 2.68 (s, 3H), 6.83 (d, 1H, $J = 4.2$ Hz), 6.92 (td, 1H, $J = 1.2$, 6.8 Hz), 7.02 (d, 1H, $J = 5.4$ Hz), 7.33 (ddd, 1H, $J = 1.0$ Hz, $J = 6.8$ Hz, $J = 8.8$ Hz), 7.55 (d, 1H, $J = 4.2$ Hz), 7.57 (d, 1H, $J = 8.8$ Hz), 8.53 (d, 1H, $J = 5.4$ Hz), 9.65 (s, 1H), 9.67 (d, 1H, $J = 8.6$ Hz), 11.05 (s, 1H); ^{13}C ($CDCl_3$ + DMSO-d_6, 75.5 MHz) δ: 16.18, 110.22, 110.93, 112.48, 115.80, 117.62, 126.62, 127.97, 131.70, 136.61, 145.51, 146.54, 152.01, 156.74, 156.86, 181.89. $C_{17}H_{13}N_5OS$ calculated m. w.: 335.38 MS [EI+] m/z: 335 $[M]^+$ (100), 209 $[M - C_5H_4ONS]^+$ (18).

*4.1.4.4. N-p-Tolyl-4-(2-(trifluoromethyl)imidazo[1,2-a]pyridin-3-yl)-pyrimidin-2-amine (**5**).* Eluted with EtOAc to yield 83% of a yellow solid. M. p. 133–134 °C; ^1H NMR ($CDCl_3$, 200 MHz) δ: 2.34 (s, 3H), 6.94 (td, 1H, $J = 7.0$, 1.4 Hz) 7.11 (dq, 1H, $J = 5.2$, 1.0 Hz), 7.15 (d, 2H, $J = 8.4$ Hz), 7.08 (d, 1H), 7.42 (ddd, 1H, $J = 1.4$, 6.4, 8.2 Hz), 7.49 (d, 2H, $J = 8.4$ Hz), 7.78 (ddd, 1H, $J = 1.0$, 6.2, 8.2 Hz), 8.54 (d,1H, $J = 5.2$ Hz), 9.21 (dd, 1H, $J = 1.2$, 7.2 Hz). $C_{19}H_{14}F_3N_5$ calculated m. w.: 369.34 MS [EI+] m/z: 329 $[M]^+$ (75), 328 $[M - H]^+$ (100).

*4.1.4.5. 4-(2-(Trifluoromethyl)imidazo[1,2-a]pyridin-3-yl)-N-(4-(trifluoromethyl)phenyl)-pyrimidin-2-amine (**6**).* Eluted with EtOAc–Hexane 9:1 to obtain 92% of a white solid. M. p. 217–218 °C; ^1H NMR ($CDCl_3$, 500 MHz) δ: 6.98 (td, 1H, $J = 1.5$, 7.0 Hz), 7.22 (dd, 1H, $J = 1.0$, 5.0 Hz), 7.46 (ddd, 1H, $J = 1.2$, 6.5, 9.0 Hz), 7.48 (ws, 1H), 7.58 (d, 2H, $J = 8.5$), 7.77 (d, 2H, $J = 8.5$ Hz), 7.81 (dt, 1H, $J = 1.0$, 9 Hz), 8.63 (d, 1H, $J = 5.5$ Hz), 9.14 (dt, 1H, $J = 1.5$, 7.5 Hz); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ: 113.63, 114.62, 118.64, 118.78, 118.89, 126.32, 126.94, 127.91, 142.19, 145.56, 155.40, 159.28, 159.37. $C_{19}H_{11}F_6N_5$ calculated m. w.: 423.31 MS [EI+] m/z: 423 $[M]^+$ (85), 422 $[M - H]^+$ (100).

*4.1.4.6. 4-(Quinolin-4-yl)-N-p-tolylpyrimidin-2-amine (**8**).* Eluted with EtOAc–Hexane 1:1 and recristallized from acetonitrile to obtain 75% of a pale yellow solid. M. p. 205–207 °C (desc.) ^1H NMR ($CDCl_3$, 200 MHz) δ: 2.32 (s, 3H), 7.00 (d, 1H, $J = 5.1$ Hz); 7.13 (d, 1H, $J = 8.4$ Hz), 7.36 (ws, 1H,), 7.53 (d, 1H, $J = 4.2$ Hz), 7.54 (d, 2H, $J = 8.4$ Hz), 7.58 (ddd, 1H, $J = 1.2$, 6.8, 8.4 Hz); 7.78 (ddd, 1H, $J = 1.4$, 6.8, 8.4 Hz); 7.8 (d, 2H, $J = 8.0$ Hz), 8.23 (dd, 1H, $J = 0.8$, 8.6 Hz); 8.24 (dd, 1H, $J = 1.0$, 8.4 Hz); 8.58 (d, 1H, $J = 5.0$ Hz); 9.02 (d, 1H, $J = 4.4$ Hz). ^{13}C NMR ($CDCl_3$, 200 MHz) δ: 20.79, 112.67, 119.66, 120.89, 125.35, 127.25, 129.49, 129.66, 130.06, 132.56, 136.51, 144.17, 148.86, 149.99, 158.73, 160.28, 164.97. $C_{20}H_{16}N_4$ calculated m. w.: 312.37 MS [EI+] m/z: 312 $[M]^+$ (95), 311 $[M - H]^+$ (100).

*4.1.4.7. 4-(Quinolin-4-yl)-N-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine (**9**).* Eluted with EtOAc–Hexane 1:1 to obtain the title compound (96%) as a white solid. M. p. 187–188 °C, ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.13 (d, 1 H, $J = 5.5$ Hz), 7.55 (d, 1H, $J = 4$ Hz), 7.56 (d, 2H, $J = 9.0$ Hz), 7.59 (ddd, 1H, $J = 1.2$, 6.5, 8.5 Hz), 7.70 (ws, 1H), 7.79 (ddd, 1H, $J = 1.2$, 7.0, 8.5 Hz), 7.81 (d, 2H, $J = 8.5$ Hz), 8.23 (dd, 1H, $J = 1.0$, 7.5 Hz), 8.24 (dd, 1H, $J = 1.0$, 8.0 Hz), 8.66 (d, 1H, $J = 5.0$ Hz), 9.05 (d, 1H, $J = 4.5$ Hz); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ: 113.83, 118.41, 120.92, 124.27 (q, $J = 33.18$), 124.33 (q, $J = 270.6$ Hz), 126.24 (q, $J = 3.8$ Hz), 127.40, 129.77, 130.21, 142.40, 143.78, 148.95, 158.78, 159.71, 165.21. $C_{20}H_{13}F_3N_4$ calculated m. w.: 366.34 MS [EI+] m/z: 366 $[M]^+$ (80), 365 $[M - H]^+$ (100).

*4.1.4.8. 4-(Quinolin-3-yl)-N-p-tolylpyrimidin-2-amine (**11**).* Eluted with EtOAc–Hexane 1:1 and recrystallized from acetonitrile to obtain 83% of a yellow solid. M.p. 209–210 °C; ^1H NMR ($CDCl_3$, 200 MHz) δ: 2.36 (s, 3H), 7.29 (d, 2H, $J = 8.4$ Hz), 7.28 (d, 1H, $J = 5.0$ Hz), 7.33 (ws, 1H), 7.60 (d, 2H, $J = 8.6$ Hz), 7.62 (td, 1H, $J = 1.2$, 8.0 Hz), 7.79 (ddd, 1H, $J = 1.4$, 7.0, 8.4 Hz), 7.95 (dd, 1H, $J = 1.2$, 8.2 Hz), 8.17 (d, 1H, $J = 8.2$), 8.53 (d, 1H, $J = 5.2$ Hz), 8.81 (d, 1H, $J = 1.8$ Hz), 9.61 (d, 1H, $J = 2.2$ Hz); ^{13}C ($CDCl_3$, 75.5 MHz) δ: 20.83, 108.15, 119.82, 127.26, 127.48, 128.74, 128.99, 129.37, 129.49, 129.74, 130.76, 132.48, 134.73, 136.68, 149.04, 159.02, 160.52, 162.50; $C_{20}H_{16}N_4$ calculated m. w.: 312.37 MS [EI+] m/z: 312 $[M]^+$ (85), 311 $[M - H]^+$ (100).

*4.1.4.9. 4-(Quinolin-3-yl)-N-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine (**12**).* Eluted with EtOAc–Hexane 1:1 to obtain 91% of a white solid. M. p. 258 °C (desc.) ^1H NMR ($CDCl_3$ + DMSO-d_6, 200 MHz) δ: 7.44 (d, 1H, $J = 5.4$ Hz), 7.59 (d, 2H, $J = 8.6$ Hz), 7.66 (ddd, 1H, $J = 1.2$, 6.8, 8.0 Hz), 7.83 (ddd, 1H, $J = 1.4$, 6.8, 8.4 Hz), 8.02 (d, 2H, $J = 8.6$ Hz), 8.02 (d, 1H, $J = 8.4$ Hz), 8.16 (d, 1H, $J = 8.6$ Hz), 8.62 (d, 1H, $J = 5.2$ Hz), 8.87 (d, 1H, $J = 2.2$ Hz), 9.31 (ws, 1H), 9.64 (d, 1H, $J = 2.2$ Hz). $C_{20}H_{13}F_3N_4$ calculated m. w.: 366.34 MS [EI+] m/z: 366 $[M]^{+\cdot}$ (5), 365 $[M - H]^+$ (10), 263 $[M - C_7H_5N]^+$(100).

4.2. Biology

4.2.1. Cell culture and assay for cytotoxic activity

The cytotoxic activity of **1**–**12** was assayed by the protein-binding method of sulphorhodamine B (SRB). Cell lines were cultured in RPMI-1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin sulfate, and 0.25 μg mL^{-1} amphotericin B. The cell line cultures were maintained in a 5% CO_2, 95% humidity atmosphere at 37 °C. In a 96-well plate were seeded 5–10 × 10^3 cells/well and incubated for 24 h. Then the cultured cell lines were grown in the presence of the putative cytotoxic compound for an additional 48 h. Test substances were dissolved in DMSO to create a stock solution, fluorouracil was used as a positive control. DMSO was added to control wells at the highest concentration used and no effect in cell grown was observed.

After the incubation with test compounds was over, adherent cell cultures were fixed in situ by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid and incubated at 4 °C for 1 h. The supernatant was discarded and the plates were washed with water and left dry to the air. The fixed cells were stained with 100 μL of 0.4% SRB solution. Protein-bonded dye was solubilized with 10 mM unbuffered Tris base and the optical density was read on a microplate reader (El_x 808; Bio-Tek Instruments, Inc., Winooski, VT, USA) with a test wavelength of 515 nm. A preliminary screening was made at 50 μM and the IC_{50} was determined in the most sensible cell lines. A dose-response curve was plotted for each compound, and the IC_{50} was estimated from non-linear regression using JMP software (version 3.2.1.; SAS Institute Inc. Cary, NC, USA).

4.2.2. Cell cycle analysis (Flow cytometry)

MCF7 and SK-LU-1 cells were subcultured to a final density of 1 × 10^6 cells in 100 × 20 mm culture Petri dishes. After a 24 h period of regular culture conditions, cell were cultured in the presence of compounds at approximately 2 times the IC_{50} (0.04 μM compound **1**, 8.4 μM compound **2** and 1 μM compound **8** were utilized for SK-LU-1 cells and 0.118 μM compound **1**, 8.4 μM compound **3** and 1 μM compound **10** were used with MCF7 cells)

for 24 h. After these treatment periods, cells were harvested and centrifuged for five minutes at 3000 rpm. The pellet was resuspended in PBS (pH = 7.4) and centrifuged again for five minutes at 3000 rpm. Cells were fixed with 70% ethanol at 4 °C for at least 12 h. Ethanol was eliminated by centrifugation and the pellet was washed with PBS. DNA was labeled with 5 μg mL^{-1} propidium iodide (PI) solution. Cell cycle analysis was made using a FACScan cytometer (BD Biosciences, San Jose, CA) and CELLQuest software (BD Biosciences). The cell cycle profile was obtained by analyzing 10,000 events using the FlowJo software Version 7.2.5 (Tree Star Inc, Ashland OR, USA).

4.2.3. Annexin V-FITC apoptosis assay

Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) was used to measure apoptosis. The kit contains annexin V labeled with a fluorophore that can identify apoptotic cells by binding phosphatidylserine exposed on the cytoplasmic surface of the cell membrane of apoptotic cells. In addition, the kit includes a red fluorescent propidium iodide (PI) nucleic acid binding dye that stains dead cells. Briefly, 2.5×10^5 SK-LU-1 cells in 2 mL medium were plated to each well of the six-well plate. After 24 h, cells were treated with either DMSO alone at the highest concentration used as control or with 0.04 μM compound **1**, 8.4 μM compound **3**, 0.4 μM compound **5** and 1 μM compound **8** for 48 h. At the end of the treatment, adherent and nonadherent cells were harvested and washed twice with PBS and then resuspended with 0.5 mL 1× annexin-binding buffer. Cells were incubated in the dark with 5 μL of annexin V and 10 μL of the PI solution for 10 minutes at room temperature. After incubation, the samples were analyzed with FACScan flow cytometry (BD Biosciences, San Jose, CA) using CellQuest Software (BD Biosciences). The percentage of apoptotic cells in the cell samples was obtained by analyzing 10,000 events using the FlowJo software Version 7.2.5 (Tree Star Inc, Ashland OR, USA).

4.2.4. CDK inhibition measurement

CDK/Cyclin activity was assayed by a protein kinase test using histone H1.2 as substrate. 220 ng of recombinant CDK1/CycB (Biaffin, Kassel, Germany) or 135 ng of recombinant Cdk2/CycA (Biaffin, Kassel, Germany) in 20 μL of kinase reaction buffer (pH = 7.4) Tris-HCl 50 mM, $MgCl_2$ 10 mM, NaF 50 mM, Na_3VO_4 500 μM, β-glicerofosfate 8 mM, DTT 1 mM, ATP 400 μM and $ATP^{\gamma 32}$ 4 μCi. (Perkin-Elmer, Wellesley, MA) is mixed with 0.5 μL 10 mg/ml recombinant Histone H1.2. (Calbiochem, San Diego, CA) and incubated 30 minutes at 30 °C. The CDK/Cyclin complexes were inhibited at 7 μM by compound **1**, **2**, **3**, **8**, and **11**. 7 μM Olomoucine was used as a positive control, vehicle solvent (DMSO) was used as a negative control at a 2.5% concentration or less.

5 μL of β-mercaptoethanol were added to the samples to stop the reaction. Samples were boiled 3 min in a screw-cap microcentrifuge tube to reduce the risk of vaporizing radioisotope. The samples were run on a 10% polyacrylamide gel. The amount of incorporated label was measured using a densitometry of the exposed film obtained with a Typhoon 9400 apparatus (Amersham Biosciences, Inc.) by using Kodak MI software (Carestream Health, Rochester, N.Y.).

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