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3-Amino-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazoles: A new class of CDK2 inhibitors

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Abstract—We have recently reported about a new class of Aurora-A inhibitors based on a bicyclic tetrahydropyrrolo[3,4-*c*]pyrazole scaffold. Here we describe the synthesis and early expansion of CDK2/cyclin A–E inhibitors belonging to the same chemical class. Synthesis of the compounds was accomplished using a solution-phase protocol amenable to rapid parallel expansion. Compounds with nanomolar activity in the biochemical assay and able to efficiently inhibit CDK2-mediated tumor cell proliferation have been obtained.

Uncontrolled cell growth and proliferation are hallmarks of all cancers and are directly linked to cell cycle dysregulation.[1] Cyclin-dependent kinase 2 (CDK2) in complex with cyclins E and/or A is a key cell cycle regulator and continues to be an attractive target for the discovery of new antitumor agents.[2] In particular, inhibitors of CDK2/cyclin A/E have already progressed into clinical trials with encouraging early results.[3] Recent findings in the biology of the CDKs show that CDK2 may be dispensable for tumor formation and maintenance[4] but compounds able to inhibit both CDK2 and CDK1 may still be regarded as potential anti-tumor agents.[5] We recently reported on a series of 3-aminopyrazoles as potent, orally available inhibitors of CDK2/cyclin A/E and CDK1/cyclin B, displaying activity in vivo in a murine tumor xenograft model.[6] A limitation associated with the 3-aminopyrazole scaffold is that high-throughput synthesis can be applied easily only for placing substituents at position 3, that is, toward the solvent-accessible region when the ligand is placed in the ATP-pocket of the kinase.[7] Positions 4- and 5-, which could be exploited to gain accessibility to the buried and phosphate binding regions to direct substituents are not synthetically amenable to rapid parallel medicinal chemistry.

We reasoned that by condensing a second heterocyclic ring onto the 3-aminopyrazole moiety we could gain access to the phosphate binding region of the ATP-kinase pocket. Thus, we would obtain a new chemical class that could be used to exploit an additional pocket in the ATP binding site (Fig. 1). The generation of this new chemical class, 1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazoles, was recently reported by us in a paper dealing with the characterization of potent Aurora-A inhibitors.[8] Here, we describe the solution-phase synthesis and early expansion of this class toward CDK2/cyclin A inhibitors. A general solution-phase synthetic methodology was needed to allow the generation of many derivatives using parallel medicinal chemistry methodologies. The synthesis of the scaffold 1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazole was accomplished as previously described.[8]

Scheme 1 reports a general solution-phase synthesis for this class of compounds that was applied to obtain

Abbreviations: CDK, cyclin-dependent kinase; ATP, adenosine triphosphate; FACS, fluorescence activated cell sorting; BrdU, bromodesoxyuridine; WB, Western blots; pRb, retinoblastoma protein; ADME, absorption-disposition-metabolism-excretion.

Keywords: CDK2; Cyclins; Kinase selectivity; Tumor cell proliferation inhibition.

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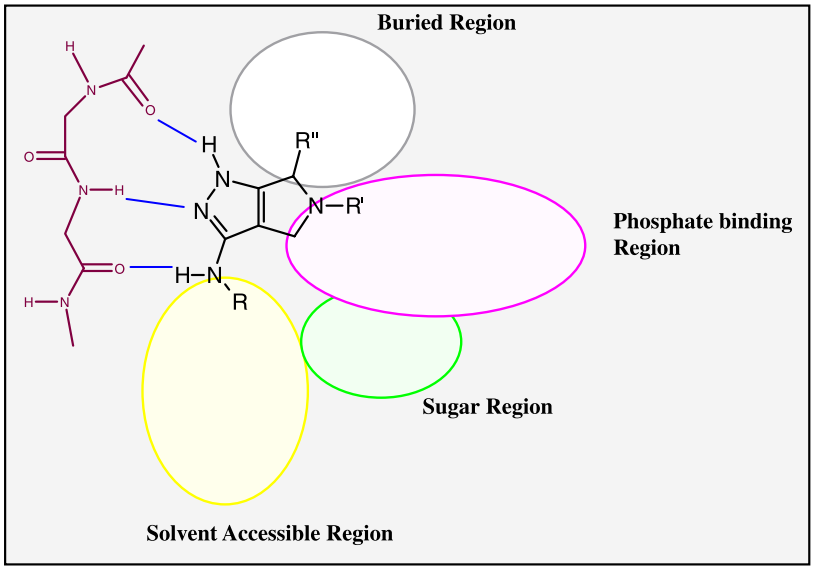
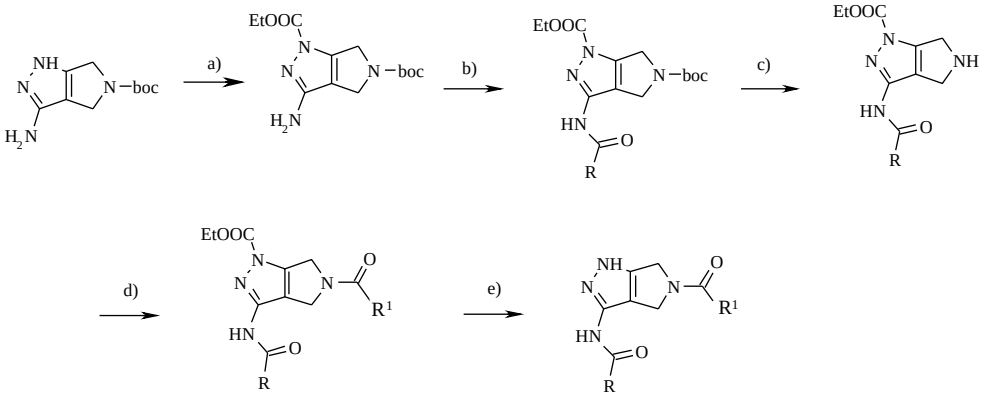


Figure 1. Schematic representation of the 1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazole scaffold in the kinase binding protein.



Scheme 1. Reagents and conditions: (a) EtOCOCl, DIEA, THF, 2 h, 0–5 °C; (b) RCOCl (1.2 equiv), DIEA (7.2 equiv), THF, 16 h, 22 °C; (c) TFA/DCM (1:1), 3 h, 22 °C; (d) R^1NCO or R^1COOH, TBTU, NMM, DCM or DMF, 24 h, 22 °C; (e) aq NaOH, MeOH, 72 h, 40 °C, then 35% HCl.

derivatives **1**–**18**. The 1,4,5,6-tetrahydropyrrolo[3,4-*c*]-pyrazole scaffold was selectively protected on the pyrazole ring using ethyl chloroformate. Acylation of the 3-position was accomplished using the corresponding acyl chlorides and di-isopropyl-ethylamine as a base. Removal of the N-Boc protection and treatment with a suitable acyl chloride, followed by a final deprotection of the ethylcarbamate group gave compounds **1**–**18**.[9]

Our strategy for class expansion was based on three main steps:

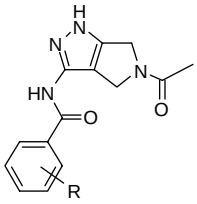
(a) Synthesize a limited number of inhibitors with moderate to good activity toward CDK2/cyclin A;
(b) Obtain X-ray structures of such inhibitors with CDK2/cyclin A;
(c) Use these structures to decide how to further expand this class.

In addition to testing compounds as CDK2/cyclin A inhibitor, all compounds were tested in parallel against Aurora-A, a mitotic serine-threonine kinase, since, at least in this compound series, inhibitors of CDK2/cyclin A often inhibited Aurora-A. Our primary goal at this stage was to dissociate CDK2/cyclin A from Aurora-A activity. While in principle a double specificity inhibitor of both serine-threonine kinases may be a superior tumor cell proliferation blocker, we wanted to get selective CDK2/cyclin A and selective Aurora-A inhibitors to possibly decoupling additive toxic effects and make an assessment of the advantages (or disadvantages) of a selective versus double specificity inhibitor.

A first series of 5-acetamido-3-benzamido-1,4,5,6-tetrahydro[3,4-*c*]pyrazoles was synthesized according to Scheme 1 and inhibitory activities are reported in Table 1.

All compounds **1**–**7** displayed moderate activity against both CDK2/cyclin A and Aurora-A. CDK2/cyclin A inhibition was less influenced by the nature of the substitution on the benzamido aromatic ring than was Aurora-A inhibition. The structure of CDK2/cyclin A was

Table 1. CDK2/cyclin A and Aurora-A inhibition of representative 5-acetyl-3-benzoylamino-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazoles



Compound	R	IC_{50} (μM)[6,13]	
		CDK2/cyclin A	Aurora-A
1	H	1.15	3.11
2	4-Me	1.08	2.14
3	4-F	2.54	>10
4	4-OMe	1.37	0.66
5	4-CN	1.59	8.5
6	3-F	2.11	4.85
7	3,4-Methylenedioxy	2.76	>10

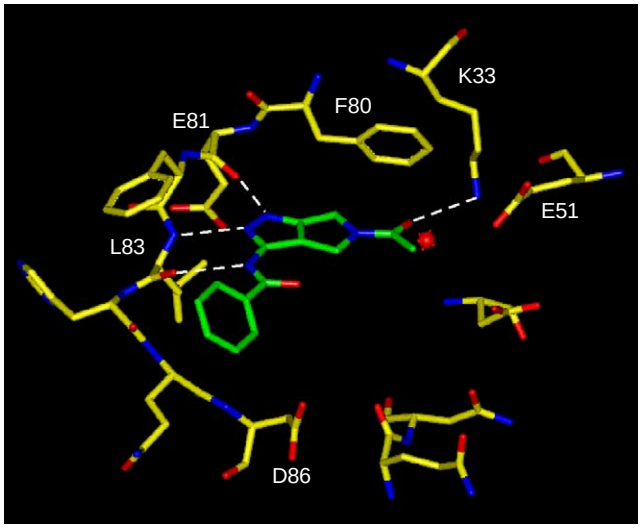


Figure 2. The crystal structure of CDK2/cyclin A in complex with compound **1** (PDB code 2C4G).

solved in complex with compound **1** according to known protocols.[6] Figure. 2 shows its mode of binding in the ATP-pocket of the kinase.

The 3-aminopyrazole moiety forms three hydrogen bonds with Glu81 and Leu83 at the hinge region of the CDK2 ATP binding site. The benzamido moiety occupies the solvent accessible region, and the tetrahydropyrrole moiety the hydrophobic pocket formed by Ala31, Val64, Phe80, and Ala144 (also known as buried region). In addition, the carbonyl oxygen of the amide at position 5 forms a hydrogen bond with Lys33. The hit compound was relatively simple with a low molecular weight and seemed suitable for further decoration to give improved activity, selectivity, and drug-likeness.

Libraries of tetrahydropyrrole[3,4-*c*]pyrazoles were made according to the method of Scheme 1 and three representative compounds are reported in Table 2.

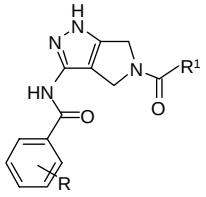
Amides or ureas built on the tetrahydropyrrole nitrogen (N-5) showed improved activity against the two reference kinases. In particular, ureas yielded activities in the high nanomolar range. On the other hand, the compounds did not show any selectivity for CDK2/cyclin A versus Aurora-A. (Table 2). Within the kinase superfamily the largest sequence variation is seen in the residues making up the solvent accessible region (see Fig. 1). In this region, Aurora-A differs from CDK2 due to the presence of an inserted glycine in Aurora-A (Gly216) that is absent in CDK2. This additional residue in Aurora-A causes a different protein conformation between the hinge region and the beginning of the C-terminal domain that tends to disfavor inhibitors that do not protrude out of the ATP pocket in a planar fashion. This region can be exploited both to improve selectivity as well as to modulate the ADME properties of the ligands (Fig. 3).

Based on this observation the hypothesis was made that 3-arylacetamido residues on the scaffold would generally be much more potent against CDK2/cyclin A, whilst 3-benzamido compounds would preferentially inhibit Aurora-A. 3-Benzamide containing compounds would make positive lipophilic interactions with Leu139 and Gly216 in Aurora-A, while 3-arylacetamido products could fit nicely into the lipophilic space delimited by Phe 82 and Ile10 in CDK2/cyclin A.

Indeed, when this substituent is varied the outcome is striking (Table 3). Compounds **11**–**14** are inhibitors of CDK2/cyclin A (down to nanomolar concentrations in the case of **13** and **14**) but are, at best, very weak inhibitors of Aurora-A.

The same observation can be made for the substituents pointing toward the solvent-accessible region of other classes of CDK2/cyclin A inhibitors and leads us to be-

Table 2. CDK2/cyclin A and Aurora-A inhibition of representative 3-benzoylamino-5-substituted-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazoles



Compound	R	R^1	IC_{50} (μM)[6,13]	
			CDK2/cyclin A	Aurora-A
8	4-Me		1.05	0.44
9	3,4-Methylenedioxy		0.43	0.16
10	3,4-Methylenedioxy		0.12	0.39

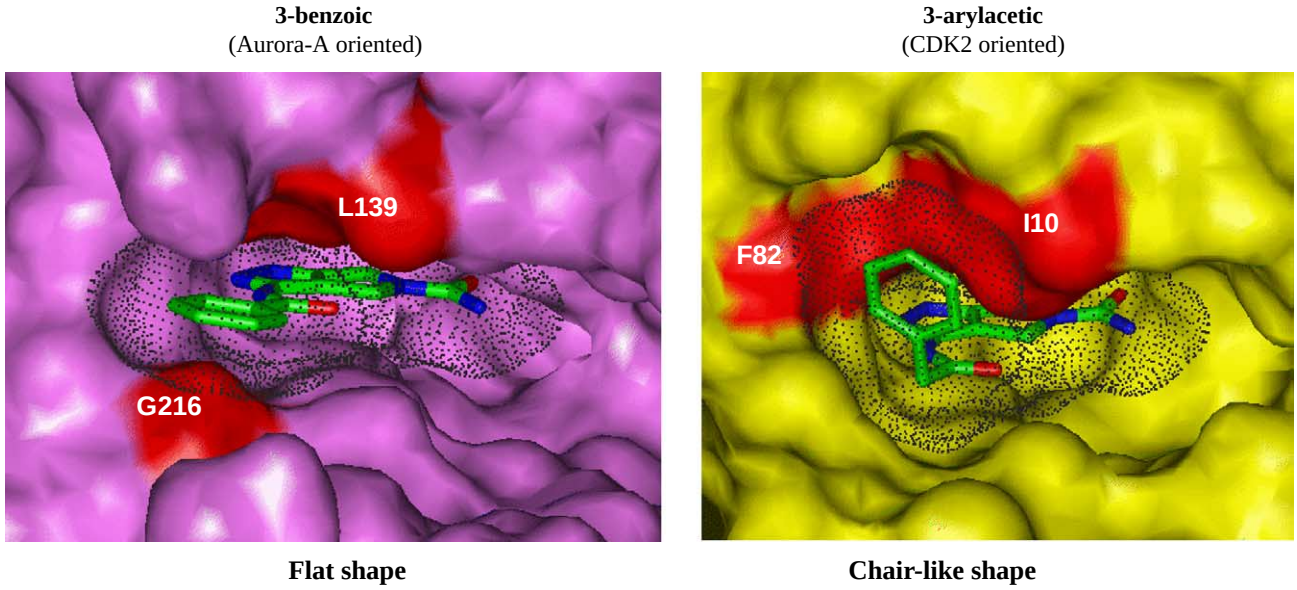
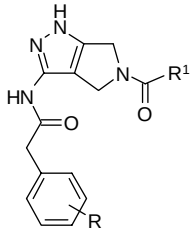


Figure 3. Compound **1** (flat-shape) and **11** (chair-like shape) docked in, respectively, Aurora-A and CDK2 crystal structures.

Table 3. CDK2/cyclin A and Aurora-A inhibition of representative 3-phenylacetylamino-5-substituted-1,4,5,6-tetrahydropyrrolo[4,3-*c*]pyrazoles



Compound	R	R^1	IC_{50} (μM)[6,13]	
			CDK2/cyclin A	Aurora-A
11	H	CH_3	0.44	2.31
12	H	NHPh	0.73	>10
13	H	NH2	0.030	>10
14	2-F	NH-*n*-Bu	0.051	>10

lieve that this simple rule is applicable across different chemical classes and across different kinases once the an inserted glycine is present among the aminoacids lining the solvent accessible region. We continued the expansion of this chemical class and we obtained several ureas (three of them, **15**, **16**, and **18** are reported in Table 4) with an interesting antiproliferative activity in cells (together with nanomolar inhibition of the target CDK2/cyclin A).

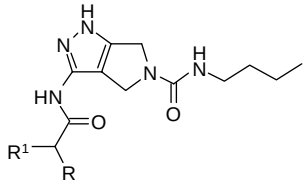
Interestingly, within this subclass of compounds there seems to be a correlation between lack of cellular activity and low C log *P* and Caco-2 cell permeability (entry **17**; Table 4).

Based on the biochemical assay and cellular potency, compounds **15** and **18** were selected for further assessment. Table 5 reports their activity against additional tumor cell lines, the selectivity profile on a panel of CDKs and two other cross-reacting protein kinases, GSK-3β and p42MAPK, and the preliminary in vitro parameters for predicting in vivo ADME.

Both compounds efficiently blocked at submicromolar concentrations the proliferation of an ovarian (A2780) and two colon (HCT116 and HT-29) tumor cell lines. The two compounds also displayed moderate buffer solubility, were sufficiently stable to the important human cytochrome CYP4503A4, showed moderate permeability in a Caco-2 permeability assay, and were seen to bind to plasma protein at high levels. When **15** and **18** were tested on a panel of CDKs, they were seen to inhibit CDK2/cyclin A and cyclin E at comparable levels, and CDK1/cyclin B and CDK5/p25 in the low micromolar range. On the other hand, CDK4/cyclin D1 was not significantly inhibited. Both compounds **15** and **18** did not significantly inhibit ($IC_{50} > 10$ μM) other protein kinases in a standard panel of 22 enzymes. Compound **18** was equipotent on CDK2/cyclin A and GSK-3β. It is frequently observed that CDK2 inhibition by small molecules is accompanied by inhibition of the structurally similar GSK-3β.[10] Whether this double inhibition may be advantageous (due to the recently reported activation of p53-dependent apoptosis by acute ablation of GSK-3β in colorectal cancer cells)[11] or detrimental due to the known link between proliferation and GSK-3β inhibition still remains to be established. However, that most of the cellular activity of these compounds is mediated through CDK2/cyclin A/E inhibition is further supported by an investigation into the mechanism of action as reported in Figure 4.

FACS analysis was performed on colon HT-29 tumor cells released from a nocodazole block. Untreated cells are not blocked in the G1/S phase of their cell cycle and the level of incorporation of BrdU is 62%. Conversely cells treated with the CDK inhibitor flavopiridol,

Table 4. CDK2/cyclin A, Aurora-A, A2780 tumor cell inhibition, C log *P* and Caco-2 permeability of representative 3-phenylacetylamino-5-ureidyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazoles



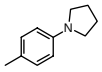
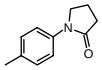
Compound	R	R^1	IC_{50} (μM)[6,13]			C log *P* (ACD Labs)	Caco-2 perm[6]
			CDK2/cyclin A	Aurora-A	A2780 cells		
15		H	0.033	4.2	0.94	1.4	Moderate
16		H	0.045	>10	0.54	2.4	Moderate
17		H	0.051	>10	8.27	0.4	Low
18		Me (*S*-confg)	0.036	>10	0.13	2.8	Moderate

Table 5. Kinase selectivity, tumor cell proliferation inhibition, and preliminary in vitro ADME parameters of compounds **15** and **18**

Compound	**15**	**18**
CDK2/cyclin A (IC_{50}; μM)[6,13]	0.033	0.036
CDK2/cyclin E (IC_{50}; μM)[6,13]	0.091	0.090
CDK1/cyclin B (IC_{50}; μM)[6,13]	0.40	0.75
CDK4/cyclin D1 (IC_{50}; μM)[6,13]	>10	>10
CDK5/p25 (IC_{50}; μM)[6,13]	0.28	0.15
GSK3-β (IC_{50}; μM)[6,13]	0.30	0.050
p42MAPK (IC_{50}; μM)[6,13]	0.28	0.38
HCT116 cells (IC_{50}; μM)[6]	0.90	0.12
HT-29 cells (IC_{50}; μM)[6]	0.20	0.07
Buffer solubility (μM)[6]	39	27
CYP4503A4 stability (% of remaining)[6]	Stable (100)	Stable (20)
Plasma protein binding[6]	95%	99%

used as a control, or compounds **15** or **18** showed a clear cell cycle block in G1/S and did not incorporate BrdU. This means that tumor cells are unable to synthesize the DNA in the S-phase.[12] Western blot (WB) analysis from the same cell cultures using specific antibodies labeling hypo- and hyper-phosphorylated forms of a CDK2/cyclin A target, pRb, showed that the compounds were able to block hyperphosphorylation of pRb, at concentrations of 1 and 3 μM, respectively.

We have described the early expansion of a new class of CDK2 inhibitors. 1,4,5,6-Tetrahydropyrrolo[3,4-*c*]pyrazole compounds were easily obtained from a versatile scaffold using a simple solution-phase chemistry amenable to rapid expansion.[8,9] Compounds with nanomolar

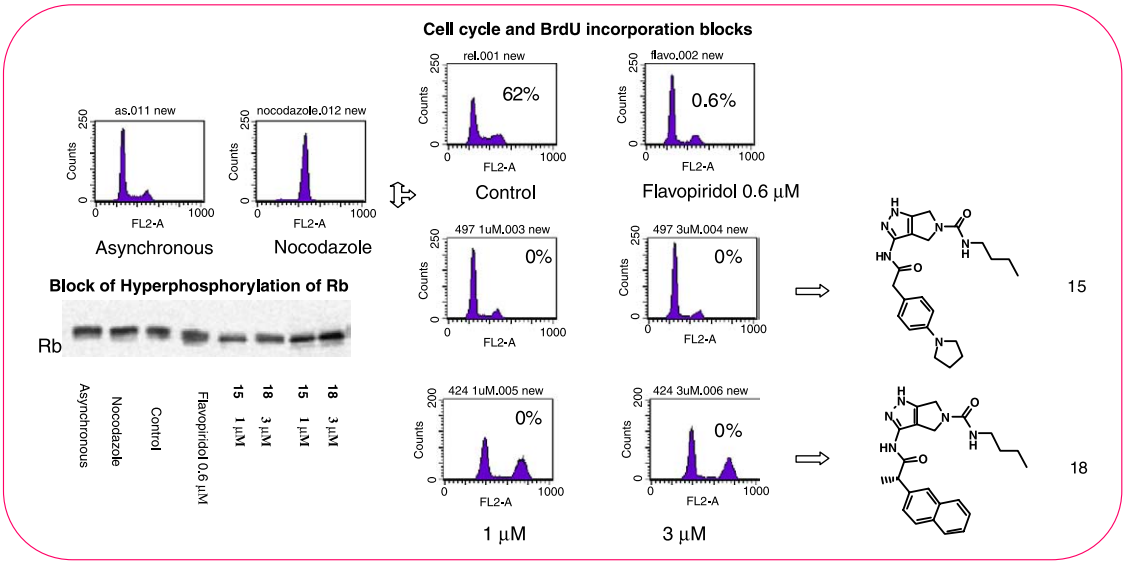


Figure 4. Molecular characterization of compounds **15** and **18** in HT-29 tumor cell line.

activity against CDK2/cyclin A/E were obtained which also show activity on cells at the submicromolar level. Analysis of the cell cycle profile and of the CDK2 substrate phosphorylation status points toward an antiproliferative effect that is mediated by CDK2 inhibition. From this study compounds **15** and **18** emerged as interesting leads. The unfavorable overall early ADME profile called for an optimization of these compounds in order to get inhibitors with drug-like in vivo pharmacokinetic profiles suitable for in vivo administration. This was the focus of further work around this class that will be reported in due course.

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9. Synthesis of **15**: A solution of ethyl chlorocarbonate (8.9 ml, 93 mmol) in THF (250 ml) was added slowly to a mixture of 3-amino-4,6-dihydro-1*H*-pyrrolo[3,4-*c*]pyrazole-5-carboxylic acid *tert*-butyl ester (20 g, 89 mmol) and DIEA (92 ml, 528 mmol) in THF (500 ml) at 0–5 °C. The reaction was kept at the same temperature for 2 h, then allowed to reach rt and stirred overnight. The reaction mixture was evaporated to dryness under vacuum. The resulting residue was taken up with AcOEt and water. The organic phase was separated, dried over sodium sulfate, and evaporated to dryness. The mixture was purified by flash chromatography (eluent: ethyl acetate/cyclohexane 4:6 to 7:3) to give 19 g (72% yield) of 3-amino-4,6-dihydro-pyrrolo[3,4-*c*]pyrazole-1,5-dicarboxylic acid 5-*tert*-butyl ester 1-ethyl ester. ESI MS: *m/z* 297 (MH^+); ^1H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ ppm 1.25 (t, $J = 7.1$ Hz, 3H), 1.42 (s, 9H), 4.12, 4.17 (m, 2H), 4.25 (q, $J = 7.1$ Hz, 2H), 4.42, 4.45 (m, 2H), 5.65 (br s, 2H). To a solution of (4-pyrrolidin-1-yl)-phenylacetic acid (2.67 g, 13.0 mmol) in DCM (60 ml) and DMF cat., $(COCl)_2$ (1.32 ml, 15.4 mmol) in DCM (15 ml) was added dropwise. The mixture was stirred at rt for 90 min and then concentrated under vacuum, taken up with toluene, and concentrated again. A solution of the so-obtained chloride in THF (60 ml) and DCM (40 ml) was added slowly to a mixture of 3-amino-4,6-dihydro-pyrrolo[3,4-*c*]pyrazole-1,5-dicarboxylic acid 5-*tert*-butyl ester 1-ethyl ester (3.5 g, 11.8 mmol) and DIEA (10.3 ml, 59.1 mmol) in THF (70 ml) at 0–5 °C. The reaction was allowed to reach rt and stirred at the same temperature overnight. The mixture was evaporated to dryness under vacuum. The resulting residue was taken up with DCM and the solution was washed with brine, dried over sodium sulfate, and evaporated to dryness. The crude product was purified by flash chromatography (eluent: ethyl acetate/cyclohexane 35:65 then 40:60) to give 2.4 g of 3-[2-(4-pyrrolidin-1-yl-phenyl)-acetylamino]-4,6-dihydro-pyrrolo[3,4-*c*]pyrazole-1,5-dicarboxylic acid 5-*tert*-butyl ester (42% yield). ESI MS: *m/z* 484 (MH^+); ^1H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ ppm 1.30 (t, $J = 7.0$ Hz, 3H), 1.41,1.42 (s, 9H), 1.92 (m, 4H), 3.16 (m, 4H), 3.43 (s, 2H), 4.34 (m, 4H), 4.50 (m, 2H), 6.46 (d, $J = 6.7$ Hz, 2H), 7.07 (d, $J = 6.7$ Hz, 2H), 11.10, 11.12 (s, 1H). 3-[2-(4-pyrrolidin-1-yl-phenyl)-acetylamino]-4,6-dihydro-pyrrolo[3,4-*c*]pyrazole-1,5-dicarboxylic acid 5-*tert*-butyl ester 2.4 g (5.0 mmol) in 25 ml DCM was treated with 16 ml of (1:1) TFA/DCM. The reaction mixture was stirred for 1 h and evaporated to dryness. The resulting residue was taken up with DCM, and treated with aq $NaHCO_3$. The precipitate was filtered, washed with water and DCM and then dried under vacuum to obtain 1.7 g (89%) of 3-[2-(4-pyrrolidin-1-yl-phenyl)-acetylamino]-5,6-dihydro-4*H*-pyrrolo[3,4-*c*]pyrazole-1-carboxylic acid ethyl ester. ESI MS: *m/z* 384 (MH^+); ^1H NMR (400 MHz, DMSO-d_6) δ ppm 1.28 (t, $J = 7.3$ Hz, 3H), 1.92 (m, 4H), 3.16 (m, 4H), 3.42 (s, 2H), 4.02 (s, 2H), 4.17 (s, 2H), 4.33 (q, $J = 7.3$ Hz, 2H), 6.45 (d, $J = 8.5$ Hz, 2H), 7.06 (d, $J = 8.5$ Hz, 2H), 11.0 (s, 1H). *n*-Butylisocyanate (530 μl, 4.8 mmol) was added to a suspension of 3-[2-(4-pyrrolidin-1-yl-phenyl)-acetylamino]-5,6-dihydro-4*H*-pyrrolo[3,4-*c*]pyrazole-1-carboxylic acid ethyl ester (1.4 g, 3.6 mmol) in THF (30 ml). The reaction mixture was stirred at room temperature for 18 h and then evaporated. The resulting residue was taken up with DCM and the solution was washed with brine, dried over sodium sulfate, and evaporated to dryness. The crude product was treated with 10% TEA in MeOH (36 ml) at 40 °C for 30 min and then evaporated. The solid was triturated with Et_2O, filtered, washed, and dried under vacuum to give 0.8 g of 3-[2-(4-pyrrolidin-1-yl-phenyl)-acetylamino]-4,6-dihydro-1*H*-pyrrolo[3,4-*c*]pyrazole-5-carboxylic acid butylamide (**15**) (53% yield). ESI MS: *m/z* 411 (MH^+); ^1H NMR (400 MHz, DMSO-d_6) δ ppm 0.85 (t, $J = 7.3$ Hz, 3H), 1.24 (m, 2H), 1.38 (m, 2 H), 1.92 (m, 4H), 3.01 (m, 2H), 3.17 (m, 4H), 3.42 (m, 2H), 4.29 (s, 4H), 6.18 (br s, 1H), 6.47 (d, $J = 7.7$ Hz, 2H), 7.08 (d, $J = 7.7$ Hz, 2H), 10.40 (s, 1H). HRMS: exact mass calcd: 411.2503; exact mass found: 411.2523. Combustion analysis: calcd: C (64.37); H (7.37); N (20.47); found: C (63.99); H (7.43); N (20.35).
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12. The apparent percentage of cells in the G2 phase in the FACS of **18** (Fig. 4) may be ascribed to a fraction of non-cycling cells unable to escape the nocodazole block,

rather than to an additional G2 block caused by the compound.
13. At least two independent experiments were performed for each compound in order to determine IC_{50} in replicates, and potency is expressed by the mean of IC_{50} values obtained by nonlinear regression analysis. Coefficient of variance /SD/mean) ranges from 10 to 24%.