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Optimization of 6,6-dimethyl pyrrolo[3,4-*c*]pyrazoles: Identification of PHA-793887, a potent CDK inhibitor suitable for intravenous dosing

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ABSTRACT

We have recently reported CDK inhibitors based on the 6-substituted pyrrolo[3,4-*c*]pyrazole core structure. Improvement of inhibitory potency against multiple CDKs, antiproliferative activity against cancer cell lines and optimization of the physico-chemical properties led to the identification of highly potent compounds. Compound **31** (PHA-793887) showed good efficacy in the human ovarian A2780, colon HCT-116 and pancreatic BX-PC3 carcinoma xenograft models and was well tolerated upon daily treatments by iv administration. It was identified as a drug candidate for clinical evaluation in patients with solid tumors.

1. Introduction

Cyclin dependent kinases (CDKs) are a family of serine/threonine kinases that, in concert with cyclins (positive regulators) and natural inhibitors (CDKI), control the cell cycle progression.[1] Deregulation of the activity of CDKs, mainly due to alterations of expression and/or genetic mutations of cyclins, CDKs, CDKIs and other components of the retinoblastoma protein (pRB) pathway, has been reported in more than 90% of human neoplasms. For example, cyclins E and A have been found over expressed in 50% of breast and lung cancer whereas decreased levels of the inhibitor p27 predict for a poor prognosis in breast, prostate, colon, gastric, lung and esophageal cancer.[2] The high frequency of alterations found in the core members of this pathway in human tumors led to the suggestion that its deregulation, leading to unscheduled proliferation, is an obligatory event for cancer development, because it results not only in proliferative advantages, but also in increased susceptibility to genetic alterations. Despite recent genetic studies in mice indicating that normal cells are not dependent on interphase CDKs (CDK4 and 2) for their growth, certain tumor cells, depending on their origin and their pathogenic spectrum of mutation, may be sensitive to the inhibition of CDKs.[1] Observations of functional redundancy within the CDK family have led to the belief that highly selective inhibitors of individual CDKs may not be therapeutically effective. The best combination of CDK activities that will lead to the greatest efficacy with minimal toxicity is still under debate. Several small molecules which inhibit the function of multiple CDKs such as Roscovitine/CYC-202,[3] BMS-387032/SNS-032[4] (both mainly CDK2 but also CDK7 and 9 inhibitors), PD0332991[5] (a selective CDK4 and 6 inhibitor) and recently R547[6] (CDK1, 2, 4 inhibitor), SCH-727965[7] (CDK1, 2, 5, 9 inhibitor), AT7519[8] (CDK1, 2, 4, 5 inhibitor) and AZD5597[9] (CDK1, 2 and 9 inhibitor) are in clinical development, confirming that agents which inhibit the function of multiple CDKs may be clinically more successful than very selective inhibitors of CDKs.[10]

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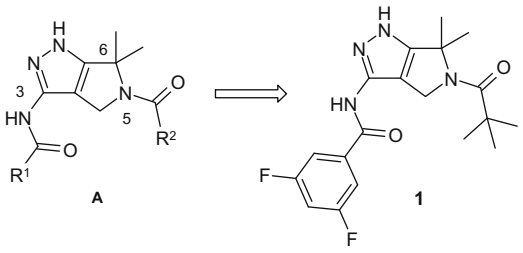

Figure 1. From 6,6-dimethyl pyrrolo[3,4-*c*]pyrazole core structure **A** to compound **1**.

We have recently reported CDK inhibitors based on the 6-substituted pyrrolo[3,4-*c*]pyrazole core structure **A**[11] (Fig. 1). This versatile adenino mimetic scaffold has been exploited for the conventional and polymer-assisted solution phase synthesis of a series of CDK2/cyclin A inhibitors. In this study, we identified compound **1** displaying nanomolar activity against CDK2/cyclin A and other CDKs, and selectivity against a panel of serine-threonine and tyrosine kinases (see Section 5, kinase assays). Within this class, selectivity was attained by the introduction of dimethyl group on position 6 of the bicyclic pyrrolo-pyrazole skeleton, exploiting the different size and shape of the buried region of CDK2 (defined by Ala31, Val64, Phe80, and Ala144) with respect to other kinases.[11–13]

Furthermore, the high binding affinity of compound **1** was also accompanied by inhibition of CDK2-mediated cell proliferation in vitro and by in vivo antitumor activity. These results prompted us to start a medicinal chemistry program with the aim to identify an analogue with a better profile with respect to potency and physico-chemical properties. Particular attention was focused on the solubility of the new compounds. In fact, notwithstanding the favorable pharmacodynamic profile of compound **1**, its low solubility in 5% dextrose solution (3.4 mg/mL) prevented its use by intravenous administration. In the present communication, we illustrate the synthesis and the biological evaluation of a set of compounds related to compound **1**.

2. Chemistry

The general synthetic approach that allowed the generation in a parallel fashion of a wide array of derivatives of general formula **A** is represented in Scheme 1. Doubly protected 3-amino-pyrrolo[3,4-*c*]pyrazole **B**, prepared as previously described,[11] was treated either with an acyl chloride in the presence of a tertiary amine or with an isocyanate to give, respectively, amides or ureas **C**. Removal of the *N*-Boc protection and treatment of the secondary amines **D** with either an acyl chloride or a carboxylic acid in the presence of a coupling agent or triphosgene followed by addition of an amine generated compounds of types **E**. Final deprotection of the ethyl carbamate group with triethylamine in methanol gave final compounds **1**–**36**.

3. Results and discussion

As previously reported,[11] optimization of 6,6-dimethyl pyrrolo[3,4-*c*]pyrazole core structure led to the identification of the *tert*-butyl moiety as potentially best R^2 group, although the most promising compounds (**1** and **2**) were characterized by poor solubility in neutral buffer.

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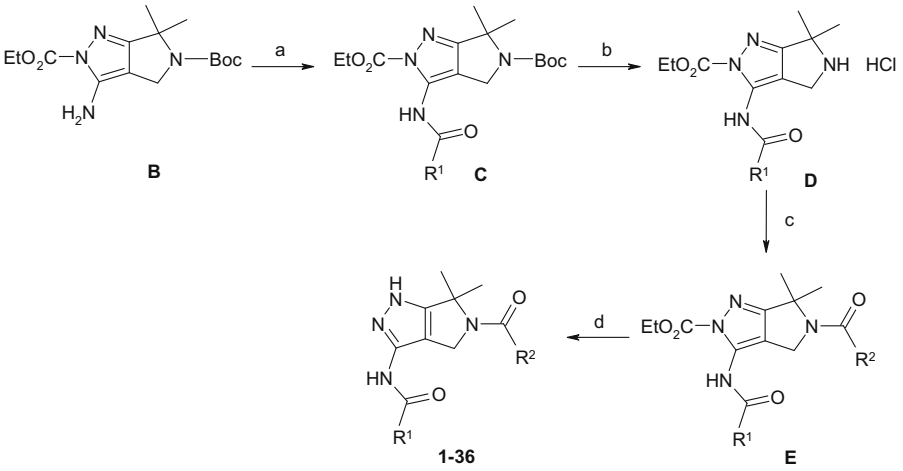
Therefore, in order to improve this property, we explored a series of amides at position 3 bearing an additional basic center (Table 1). 3-Pyridine and 4-pyridine carboxamide (**3** and **4**) maintained CDK2 inhibitory activity and potency in the cellular antiproliferative assay, in particular compound **4**, without a clear improvement in the solubility in neutral buffer that instead was achieved moving from aryl or heteroaryl moieties to heterocyclic or linear alkyl ones (**5**–**7**). Unfortunately, a small decrease in inhibitory activity against CDK2/cyclin A was also observed, except for compound **7**, which showed an interesting enzymatic activity. On the other hand, no significant improvement in A2780 cell proliferation assays was observed.

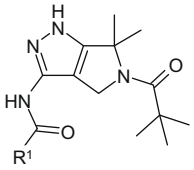
These results prompted us to introduce a basic moiety at position 5 (pointing towards the relatively spacious phosphate binding region) while keeping unvaried 4-fluoro benzamido group at position 3 (Table 2).

An initial set of piperidino amides **8**–**11** was synthesized and compared to compound **1**. No substitution (**8**) or insertion of small substituents such as methyl, ethyl, and cyclopropyl (**9**–**11**) provided compounds with good activity against CDK2/cyclin A. Interesting potency in the A2780 cell proliferation assay as well as good



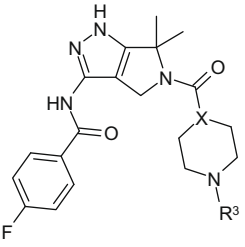
Scheme 1. Reagents and conditions: (a) R^1COCl, DIPEA, THF or R′NCO, THF; (b) HCl 4 N in dioxane; (c) R^2COCl, DIPEA, THF or R^2COOH, TBTU, DIPEA, DCM or triphosgene then NHR′R″; (d) TEA, MeOH.

Table 1
R^1 modification



Compd	R^1	CDK2/A IC_{50}, μM	A2780 IC_{50}, μM	Solubility μM, pH 7
1	3,5-Difluorophenyl	0.060	0.31	186
2	4-Fluorophenyl	0.030	0.27	235
3	3-Pyridinyl	0.050	0.69	207
4	4-Pyridinyl	0.049	0.11	205
5	4-Piperidinyl	0.169	0.65	>225
6	1-Methyl-piperidinyl	0.214	0.35	>225
7	Dimethylaminomethyl	0.077	0.58	>225

Table 2
R^3 modification



Compd	R^3	X	CDK2/A IC_{50}, μM	A2780 IC_{50}, μM	Solubility μM, pH 7
8	H	CH	0.030	0.65	204
9	Methyl	CH	0.023	0.12	>225
10	Ethyl	CH	0.030	0.29	>225
11	Cyclopropyl	CH	0.068	0.38	>225
12	Acetyl	CH	4.80	>10	204
13	Methyl	N	0.046	0.18	>225
14	Hydroxyethyl	N	0.060	>5	>225
15	Acetyl	N	0.54	>10	>225
16	Phenyl	N	3.5	nt	55

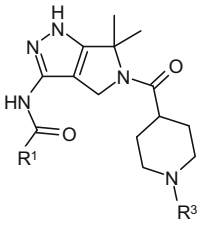
solubility in neutral buffer was observed, except for compound **8** which showed lower potency on cells with respect to compound **1**.

Acylation of the piperidino nitrogen (**12**) led to a drop in potency against CDK2/cyclin A and in cells. Moreover, the role of substituted piperazino ureas was explored. Compound **13** as well as hydroxyethyl derivative **14** displayed interesting enzymatic activity, whereas a bulkier residue such as phenyl (**16**) caused a marked decrease in activity against CDK2/cyclin A. Acylation of the piperazino nitrogen (**15**) led to a drop in potency in biological assay, even if less dramatic with respect to the corresponding acetyl piperidino **12**. No improvement in terms of cellular antiproliferative activity was achieved moving from methyl piperazino **13** to ureas **14**–**16**.

The interesting results obtained with *N*-substituted piperidino moiety (**9**–**11**) prompted us to expand this subclass. Thus, we prepared a set of 5-piperidino carboxamides to further explore the solvent accessible region of the ATP binding site of CDK2/cyclin A (Table 3).

A phenyl ring eventually bearing halogen substituents (**17**–**22**) gave compounds with strong activity against CDK2/cyclin A and in A2780 cell proliferation assays. In particular, the *p*-trifluoromethyl phenyl derivative **22** was more potent in the antiproliferative assay than the previous ones, but less soluble in neutral buffer. Also naphthyl derivatives (**23**–**24**) showed interesting potency

Table 3
R^1, R^3 modifications



Compd	R^1	R^3	CDK2/A IC_{50}, μM	A2780 IC_{50}, μM	Solubility μM, pH 7
17	Phenyl	Me	0.023	0.16	>225
18	3-Fluorophenyl	Me	0.014	0.20	>225
19	3,4-Difluorophenyl	Me	0.014	0.10	>225
20	3,5-Difluorophenyl	Me	0.016	0.13	>225
21	4-Chlorophenyl	Me	0.015	0.12	>225
22	4-Trifluoromethylphenyl	Me	0.026	0.06	191
23	2-Naphthyl	Me	0.027	0.27	219
24	1-Naphthyl	Me	0.016	0.06	200
25	4-Pyridinyl	Me	0.025	0.50	>225
26	2-Thienyl	Me	0.010	0.03	155
27	3-Thienyl	Me	0.005	0.14	>225
28	2-Furyl	Me	0.033	>5	>225
29	Cyclopropyl	Me	0.015	1.42	>225
30	Cyclobutyl	Me	0.009	0.14	>225
31	*i*-Bu	Me	0.008	0.09	>225
32	*i*-Bu	Et	0.014	0.41	>225
33	*i*-Bu	Cyclopropyl	0.024	0.32	>225
34	Benzylamino	Me	0.021	2.68	>225
35	Propylamino	Me	0.011	0.65	>225
36	3-Fluorophenylamino	Me	0.010	0.35	>225

against CDK2/cyclin A, in particular the naphthyl-1-carboxamide **24**, but without an improvement of solubility in neutral buffer.

Among the heteroaryl carboxamide derivatives (**25**–**28**), the 2-thienyl (**26**) was potent against CDK2/cyclin A and in antiproliferative assay, but less soluble in neutral buffer. The cyclopropyl and the cyclobutyl groups also gave potent CDK2/cyclin A inhibitors (**29**–**30**). Interestingly, the branched *iso*-butyl group gave the potent enzyme inhibitor **31** with an improvement of the antiproliferative activity in A2780 human ovarian carcinoma cells as well as solubility in neutral buffer, with respect to the parent compound **1**. Introduction of ethyl or cyclopropyl instead of methyl on the nitrogen of piperidine group (**32**–**33**) was compatible with the maintenance of the CDK2 inhibitory activity but potency in the cellular antiproliferative assay was diminished, with respect to analogue **1**. A small set of ureas (**34**–**36**) was tested against CDK2/cyclin A, showing interesting enzymatic activity, but a drop in the antiproliferative activity, in particular for benzylurea **34**.

The crystal structure of compound **31**, in complex with CDK2/cyclin A has been completely elucidated (Fig. 2).[14] The pyrrolo-pyrazole system occupies the adenine region of the ATP pocket, while the *iso*-butyl group points towards the solvent accessible region. This compound binds into the ATP pocket of CDK2/cyclin A by three hydrogen bonds with the protein backbone of the hinge region: the NH of the pyrazole binds to the carbonyl of Glu81, the nitrogen atom of the pyrazole core and the adjacent NH interact with the NH and the carbonyl oxygen of Leu83. The dimethyl group on position 6 occupies the buried region formed in CDK2 by Ala31, Val64, Phe80 and Ala144. In addition, the carbonyl group is within hydrogen bond distance of the side chain of Lys33 and the nitrogen atom of the piperidine binds to the side chain of Asp145. This additional hydrogen bond, not present in compound **1**, contributes to the improvement of activity against CDK2/cyclin A observed for compound **31** and, more in general, for compounds bearing an *N*-alkyl piperidin-4-yl moiety as R^2 group (Fig. 1).

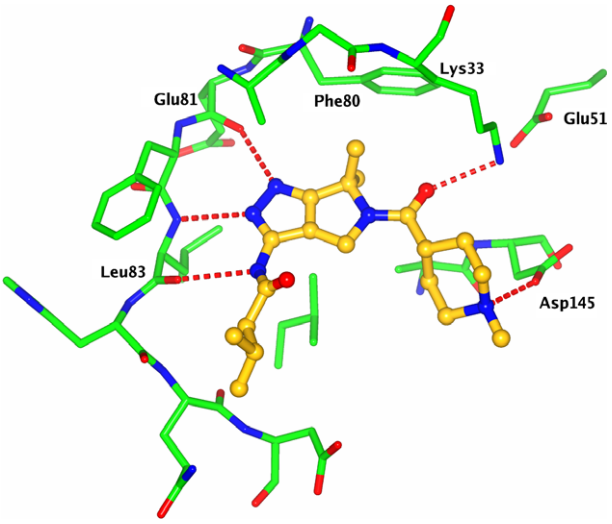


Figure 2. Crystal structure of compound **31** in complex with CDK2/cyclin A (PDB code 2WPA).

Based on the biochemical assay, cellular potency and preliminary physico-chemical properties compound **31** was selected for further assessment. Table 4 reports the selectivity profile of compounds **1** and **31** on a panel of 44 serine–threonine and tyrosine kinases.

Among the members of the CDK family that were assessed, compound **31** turned out to be a potent inhibitor of CDK2/cyclin A, CDK2/cyclin E, CDK5/p25 and CDK7/cyclin H at comparable levels, but also, albeit less potent, an inhibitor of CDK1/cyclin B and CDK4/cyclin D1 (ratio vs CDK2/cyclin A: 7.5 x). Among all the other enzymes in the panel, only GSK3 was inhibited (ratio vs CDK2/cyclin A: 10 x) by compound **31**, which generally demonstrated to be more potent than the parent compound **1**.

The effects of compound **31** on the cell cycle progression and DNA synthesis, measured as BrdU incorporation (Table 5), were analyzed using Flow cytometry and immunocytochemistry, respectively, on A2780 ovarian carcinoma cells in exponential growth in the presence or absence of compound, for 24 h, at the concentration of 3 μM and 1 μM. The cell cycle profile of the treated cells showed a mixed effect at 1 μM: a decrease in the S phase population and a subsequent increase of the G1 population, as expected for a CDK2/cyclin A inhibitor, and a slight accumulation of G2/M phase population compared to the control cells. At 3 μM only a strong G2/M increase was observed, which can be ascribed to inhibition of CDK1/cyclin B. The presence of cells with sub G1 DNA content (from 2.6% in control cells to 26.2% in cells treated with compound **31** at 3 μM) clearly implied cell death and apoptosis. A clear reduction in DNA synthesis was observed, compared to the control, as measured by BrdU incorporation. Also in these assays compound **31** showed to be more potent than the parent compound **1**.

Table 4
Selectivity profile of compounds **1** and **31** (IC_{50}, μM)

Protein Kinase	1	31
CDK2/cyclin A	0.060	0.008
CDK2/cyclin E	0.090	0.008
CDK5/p25	0.14	0.005
CDK7/cyclin H	Nd	0.010
CDK1/cyclin B	0.45	0.060
CDK4/cyclin D1	Nd	0.062
CDK9/cyclin T1	Nd	0.138
GSK3β	3.63	0.079
Other kinases[a]	>10	>10

[a] See Section 5.

Table 5
Effects on cell cycle progression and DNA synthesis of compounds **1** and **31** vs control cells

Compd	G0/G1%	S%	G2/M%	BrdU incorporation%
1[a]	+22	−52	+26	−96
31[a]	−11	−48	+263	−100
31[b]	+25	−76	+62	−85

[a] At 3 μM.
[b] At 1 μM.

In addition, the effect on the phosphorylation status of a known CDK substrate such as retinoblastoma protein (pRb) was analyzed in cells treated with compound **31** at concentrations of 1 and 3 μM. A clear reduction of the hyperphosphorylated form of pRb and an accumulation of the hypophosphorylated form of pRb was observed in the extracts of treated cells in comparison with the untreated cells, indicative of an effect on the activity of CDK (Fig. 3).

As far as preliminary in vitro and in vivo pharmacokinetic parameters are concerned, compound **31** displayed better solubility in vehicle (10 mg/mL in 5% dextrose solution as hydrochloride salt) with respect to compound **1** (3.4 mg/mL in 5% dextrose solution), stability to human cytochrome CYP4503A4 (91% remaining), and low permeability in the Caco-2 cell permeability assay (Table 6), which could explain the lack of oral bioavailability found in the pharmacokinetic experiment performed on healthy nude mice (F <1%). The plasma protein binding was relatively low. In vivo pharmacokinetic results in healthy nude mice (10 mg/kg by iv administration in 5% dextrose solution as hydrochloride salt) indicated a volume of distribution higher than the total body water suggesting tissue distribution, with a clearance in the range of 9% of the hepatic blood flow. The half-life is approximately 4.1 h. Furthermore, compound **31** demonstrated potent antiproliferative activity (Table 7) in several tumor cell lines, including sarcoma, melanoma, ovarian, colon, breast and prostate carcinoma cells. These results indicate a broad spectrum of activity of compound **31**.

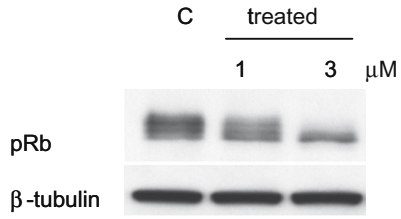


Figure 3. Compound **31** decreases the amount of pRB phosphorylation in treated cells in comparison with untreated cells (β-tubulin as control cellular protein).

Table 6
In vitro physico-chemical ADME properties and in vivo pharmacokinetic parameters of compound **31**

In vitro physico-chemical ADME properties	31
Solubility 5% dextrose (mg/mL)	10
CYP4503A4 (% remaining)	91
PPB (%)	54
$CaCO_2$ cell permeability	Low
In vivo pharmacokinetic parameters	**31**[a]
IV administration	10 mg/kg
C_{max} (μM)	36.5
AUC (μM·h)	19.2
CL (mL/min/kg)	23.9
$t_{1/2}$ (h)	4.1
V_{ss} (mL/Kg)	1360

[a] Dosed in 5% dextrose as hydrochloride salt.

Table 7
Inhibition of cell proliferation by compound **31**

Cell line	IC_{50}, μM
A2780 human ovarian carcinoma	0.088 ± 0.038
HCT-116 human colon carcinoma	0.163 ± 0.057
COLO-205 human colon carcinoma	0.188 ± 0.096
C-433 Ewing's sarcoma	0.285 ± 0.073
DU-145 human prostate carcinoma	0.303 ± 0.142
A375 human melanoma	0.396 ± 0.100
PC3 human prostate carcinoma	0.601 ± 0.324
MCF7 human breast carcinoma	1.287 ± 0.546
BX-PC3 human pancreatic carcinoma	3.444 ± 0.827

On the basis of these data compound **31** was evaluated for its in vivo antitumor activity in the human ovarian A2780 xenograft mouse model. The doses of 10, 20 and 30 mg/kg were selected and administered by iv route, once a day, for 10 consecutive days, on the basis of the plasma levels reached in the preliminary in vivo PK study. Compound **31** was dissolved in 5% dextrose solution and caused a dose-dependent inhibition of the A2780 tumor growth up to 76% at the dose of 30 mg/kg at the end of treatment (Fig. 4a). With the same schedule, compound **31** was tested at the doses of 10 and 20 mg/kg also on a model of human colon carcinoma (HCT-116) causing a dose-dependent inhibition of the tumor growth up to 81% at the dose of 20 mg/kg at the end of treatment (Fig. 4b) and on a model of human pancreatic carcinoma (BX-PC3) with a 84% of tumor growth inhibition at the end of treatment with 20 mg/kg (Fig. 4c). Compound **31** was well tolerated upon daily treatments by iv administration. In fact, in all experiments, body weight reduction was always marginal (<10% vs control mice) and no toxic effects were reported after gross autopsy.

In order to verify the mechanism of action of compound **31** also in vivo, A2780 xenograft tumors from mice treated at the dose of 30 mg/kg iv for 5 days were analyzed for BrdU incorporation and phosphorylation status of pRb by immunohistochemistry. Proliferation and CDK related markers were significantly reduced in treated tumors ($p = 0.0022$ and $p = 0.005$, respectively, for BrdU and phosho-pRb) compared to vehicles (Fig. 5).

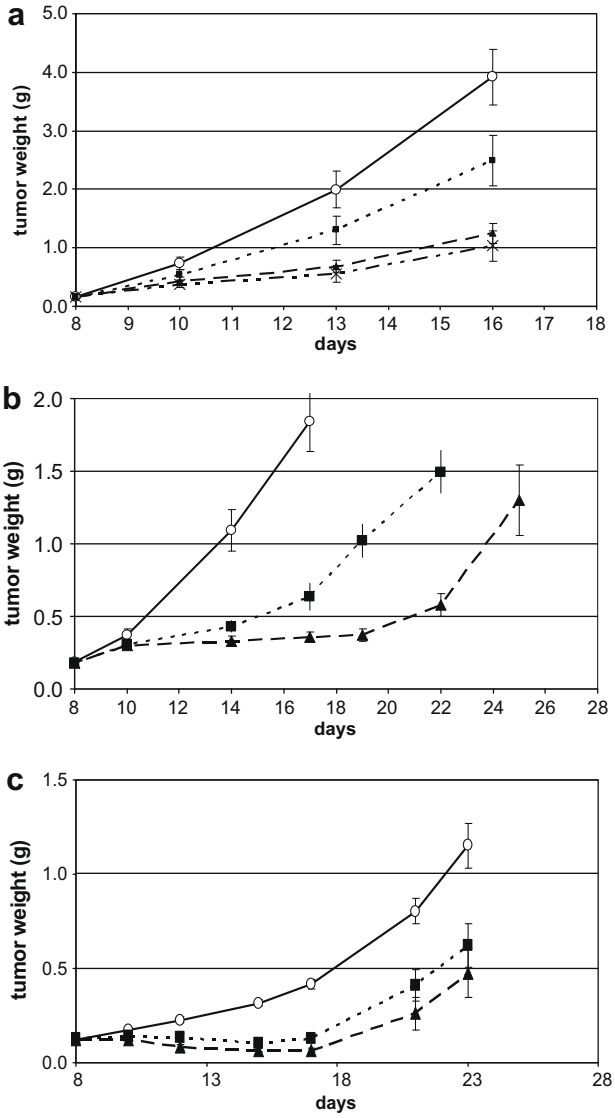


Figure 4. Compound **31** inhibits tumor growth of human ovarian A2780 (a), colon HCT-116 (b) and pancreatic BX-PC3 (c) carcinoma xenograft models. It was iv administered respectively at doses of 10 (square), 20 (triangle) and 30 (cross) mg/kg for 10 consecutive days starting from day 8. Vehicle-treated curve (control) is reported with open circle.

4. Conclusion

Starting from compound **1**, which showed good potency as CDK2/cyclin A inhibitor, a series of new analogues was synthesized with the aim of improving CDK inhibitor activity and antiproliferative activity against A2780 human ovarian carcinoma cells. Optimization of the physico-chemical properties led to the identification of compound **31** (PHA-793887), highly potent CDK inhibitor suitable for intravenous dosing that exhibited inhibition of tumor growth ranging from 76% to 84% in preclinical xenograft tumor models, well tolerated upon repeated daily treatments. The mechanism of action of compound **31** was demonstrated both in vitro and in vivo. PHA-793887 was selected for clinical evaluation as anticancer agent in patients with solid tumors. Further studies will be reported in the future.

5. Experimental

5.1. Chemistry

All solvents and reagents, unless otherwise stated, were commercially available, of the best grade and were used without further purification. All experiments dealing with moisture-sensitive compounds were carried out under dry nitrogen or argon atmosphere. Organic solutions were evaporated using a Heidolph WB 2001 rotary evaporator at 15–20 mmHg. Thin-layer chromatography was performed on Merck Silica Gel 60 F_{254} pre-coated plates. Column chromatography was conducted either under medium pressure on silica (Merck Silica Gel 40–63 μm) or on prepacked silica gel cartridges (Biotage). Components were visualized by UV light ($\lambda = 254$ nm) and by iodine vapor. ^{1}H NMR spectra were recorded in DMSO-d_6 at 28 °C on a Varian Inova 400 spectrometer operating at 400.50 MHz and equipped with a 5 mm ^{1}H{^{15}N–31P} z-axis-PFG indirect detection probe. DMSO-d_6 residual solvent signal was used as internal reference ($\delta = 2.50$ ppm for ^{1}H). Chemical shifts (δ) are reported in parts per million (ppm) and coupling constant (J) in Hz. The following abbreviations are used for multiplicities: s = singlet; br s = broad singlet; d = doublet; t = triplet; m = multiplet; dd = doublet of doublets. Electrospray (ESI) mass spectra were obtained on a Finnigan LCQ ion trap. HPLC–UV–MS analyses were carried out combining the ion trap MS instrument with HPLC system SSP4000 (Thermo Separation Products) equipped with an autosampler LC Pal (CTC Analytics) and UV6000LP diode array detector (UV detection 215–400 nm). Instrument control, data acquisition and processing were performed by using Xcalibur 1.2 software (Finnigan). HPLC

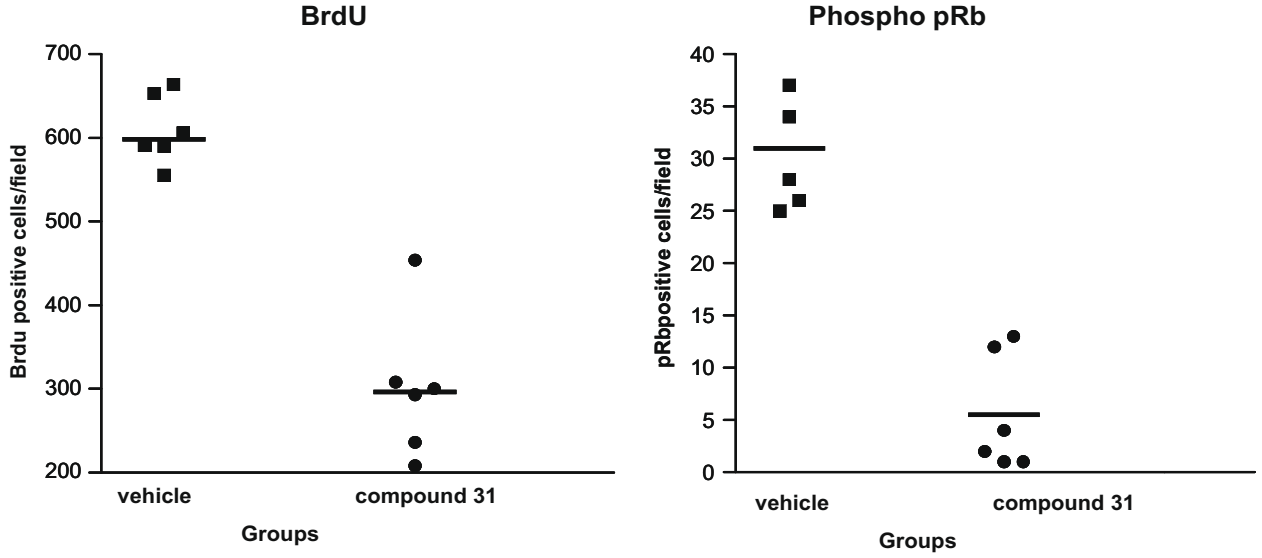


Figure 5. Compound **31** decreases the number of BrdU and phospho pRb positive cells in A2780 xenograft tumors as determined by immunohistochemistry.

chromatography was run at room temperature, and 1 mL/min flow rate, using a Waters X Terra RP 18 column (4.6 × 50 mm; 3.5 μm). Mobile phase A was ammonium acetate 5 mM buffer (pH 5.5 with acetic acid): acetonitrile 90:10, and mobile phase B was ammonium acetate 5 mM buffer (pH 5.5 with acetic acid): acetonitrile 10:90; the gradient was from 0% to 100% B in 7 min then hold 100% B for 2 min before requilibration. Mass are given as *m*/*z* ratio. ESI(+) high resolution mass spectra (HRMS) were obtained on a Waters Q-Tof Ultima directly connected with micro HPLC 1100 Agilent as previously described.[15] Elemental analyses were performed on a Carlo Erba 1110 instrument, and C, H, and N results were within ±0.4% of theoretical values unless specified.

5.1.1. General procedure A

General procedure for the preparation of compounds **1**–**7** is illustrated below for the preparation of **2.**

5.1.1.1. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]-4-fluorobenzamide (2).

5-*tert*-Butyl 2-ethyl 3-amino-6,6-dimethyl-4,6-dihydropyrrolo[3,4-*c*]pyrazole-2,5(4*H*,6*H*)-dicarboxylate (2.0 g, 6.16 mmol) was dissolved in THF (40 mL), treated with *N*,*N*-diisopropylethylamine (5.4 mL, 30.80 mmol) and then, at 0 °C dropwise, with 4-fluorobenzoyl chloride (0.80 mL, 6.77 mmol) dissolved in THF (8 mL). The reaction mixture was stirred at room temperature for 5 h, concentrated and dissolved in DCM, washed with saturated sodium hydrogen carbonate aqueous solution and with brine. The organic phase was dried over sodium sulfate, evaporated and purified by flash chromatography (hexane/EtOAc 80:20) to afford 5-*tert*-butyl 2-ethyl 3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethylpyrrolo[3,4-*c*]pyrazole-2,5(4*H*,6*H*)-dicarboxylate (2.50 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 1H), 7.97–8.03 (m, 2H), 7.41–7.48 (m, 2H), 4.52 and 4.54 (2 × s, 2H, conformers), 4.45 (q, *J* = 7.1 Hz, 2H), 1.61 and 1.63 (2 × s, 6H, conformers), 1.46 and 1.48 (2 × s, 9H, conformers), 1.36 (t, *J* = 7.1 Hz, 3H); LC–MS (ESI) *m*/*z* 447 [M+H]$^+$. This intermediate (2.49 g, 5.58 mmol) was dissolved in dioxane (50 mL) and treated with HCl 4 M in dioxane (28 mL, 20 equiv). After 2 h at 40 °C the reaction mixture was concentrated and the residue was treated with diethyl ether, filtered to afford ethyl 3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate hydrochloride (2.09 g, 98%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.90 (s, 1H), 9.88 (br s, 2H), 7.98–8.03 (m, 2H), 7.45–7.50 (m, 2H), 4.60 (br s, 2H), 4.48 (d, *J* = 7.1 Hz, 2H), 1.67 (s, 6H), 1.37 (t, *J* = 7.1 Hz, 3H); LC–MS (ESI) *m*/*z* 347 [M+H]$^+$. This intermediate (2.08 g, 5.33 mmol), in DCM (70 mL), was treated with *N*,*N*-diisopropylethylamine (1.6 mL, 9.2 mmol) and at 0 °C with pivaloyl chloride (0.78 mL, 6.3 mmol). Gradually the reaction was brought to room temperature and stirred overnight. The solution was washed with saturated sodium hydrogen carbonate aqueous solution and brine. The organic phase was dried over sodium sulfate, evaporated and purified by flash chromatography (DCM/EtOAc 90:10) to afford ethyl 5-(2,2-dimethylpropanoyl)-3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate (2.03 g, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.09–8.15 (m, 2H), 7.30–7.36 (m, 2H), 4.96 (s, 2H), 4.46 (q, *J* = 7.2 Hz, 2H), 1.68 (s, 6H), 1.36 (t, *J* = 7.2 Hz, 3H), 1.24 (s, 9H); LC–MS (ESI) *m*/*z* 431 [M+H]$^+$. This intermediate (2.0 g, 4.64 mmol) was dissolved in methanol (60 mL), treated with triethylamine (6.45 mL, 46.4 mmol) and stirred overnight at room temperature. After evaporation, the solid was treated with diethyl ether/hexane and filtered to afford the title compound **2** (1.43 g, 86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.44 (br s, 1H), 10.93 (s, 1H), 8.08 (dd, *J* = 5.5, 8.8 Hz, 2H), 7.33 (t, *J* = 8.8 Hz, 2H), 4.86 (br s, 2H), 1.66 (s, 6H), 1.23 (s, 9H); LC–MS (ESI) *m*/*z* 359 [M+H]$^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{23}FN_4O_2$+H$^+$ 359.1878, found 359.1889; Anal. Calcd for $C_{19}H_{23}FN_4O_2$: C, 63.67; H, 6.47; N, 15.63. Found: C, 63.63; H, 6.44; N, 15.55.

The following compounds **1**, **3**–**7** were prepared according to the general procedure A described above.

5.1.1.2. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]-3,5-difluorobenzamide (1).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.52 (br s, 1H), 11.10 (s, 1H), 7.68–7.76 (m, 2H), 7.45–7.55 (m, 1H), 4.87 (br s, 2H), 1.67 (s, 6H), 1.23 (s, 9H); LC–MS (ESI): *m*/*z* 377 [M+H]$^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{22}F_2N_4O_2$+H$^+$ 377.1784, found 377.1786; Anal. Calcd for $C_{19}H_{22}F_2N_4O_2$: C, 60.63; H, 5.89; N, 14.88. Found: C, 60.56; H, 5.91; N, 14.76.

5.1.1.3. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]pyridine-3-carboxamide (3).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.49 (br s, 1H), 11.15 (s, 1H), 9.12 (d, *J* = 2 Hz, 1H), 8.74 (d, *J* = 4.0 Hz, 1H), 8.32 (d, *J* = 7.9 Hz, 1H), 7.50–7.55 (m, 1H), 4.89 (br s, 2H), 1.67 (s, 6H), 1.23 (s, 9H); LC–MS (ESI): *m*/*z* 342 [M+H]$^+$; Anal. Calcd for $C_{18}H_{23}N_5O_2$: C, 63.32; H, 6.79; N, 20.51. Found: C, 62.95; H, 6.83; N, 20.32.

5.1.1.4. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]pyridine-4-carboxamide (4). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.53 (br s, 1H), 11.23 (s, 1H), 8.74 (d, J = 5 Hz, 2H), 7.90 (d, J = 5 Hz, 2H), 4.89 (s, 2H), 1.67 (s, 6H), 1.23 (s, 9H); LC–MS (ESI): m/z 342 $[M+H]^+$; Anal. Calcd for $C_{18}H_{23}N_5O_2$: C, 63.32; H, 6.79; N, 20.51. Found: C, 63.57; H, 6.89; N, 20.24.

5.1.1.5. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]piperidine-4-carboxamide (5). Compound **5** was obtained in 82% yield after deprotection of ethyl 5-(2,2-dimethylpropanoyl)-3-[({1-[(9*H*-fluoren-9-ylmethoxy)carbonyl]piperidin-4-yl}carbonyl)amino]-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate (prepared according the general procedure A) with 20% piperidine in methanol. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.26 (br s, 1H), 10.38 (br s, 1H), 4.80 (br s, 2H), 3.02–3.09 (m, 2H), 2.54–2.62 (m, 2H), 2.44–2.54 (m, 1H), 1.67–1.77 (m, 2H), 1.64 (s, 6H), 1.51–1.62 (m, 2H), 1.23 (s, 9H); LC–MS (ESI): m/z 348 $[M+H]^+$.

5.1.1.6. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]-1-methylpiperidine-4-carboxamide (6). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.26 (br s, 1H), 10.37 (s, 1H), 4.78 (s, 2H), 2.79–2.91 (m, 2H), 2.26–2.36 (m, 1H), 2.20 (br s, 3H), 1.85–2.03 (m, 2H), 1.55–1.79 (m, 4H), 1.62 (s, 6H), 1.21 (s, 9H); LC–MS (ESI): m/z 362 $[M+H]^+$; Anal. Calcd for $C_{19}H_{31}N_5O_2$: C, 63.13; H, 8.64; N, 19.37. Found: C, 62.84; H, 8.61; N, 19.12.

5.1.1.7. *N*-[5-(2,2-Dimethylpropanoyl)-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]-N^2,N^2-dimethylglycinamide (7). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.35 (br s, 1H), 10.06 (s, 1H), 4.80 (s, 2H), 3.13 (br s, 2H), 2.31 (s, 6H), 1.63 (s, 6H), 1.21 (s, 9H); LC–MS (ESI): m/z 322 $[M+H]^+$; Anal. Calcd for $C_{16}H_{27}N_5O_2$: C, 59.79; H, 8.47; N, 21.79. Found: C, 59.94; H, 8.51; N, 21.43.

5.1.2. General procedure B

General procedure for the preparation of compounds **8**–**12** and **17**–**33** is illustrated below for the preparation of **9**.

5.1.2.1. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (9). Ethyl 3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate hydrochloride (prepared according to the general method A) (0.5 g, 1.3 mmol) in DCM (25 mL), was treated with *N*,*N*-diisopropylethylamine (1.13 mL, 6.5 mmol) and TBTU (0.542 g, 1.69 mmol), at room temperature for 1 h, and then 1-methyl-piperidine-4-carboxylic acid hydrochloride (0.29 g, 1.61 mmol) was added. The reaction was stirred overnight. The solution was washed with saturated sodium hydrogen carbonate aqueous solution and brine, the organic phase was dried over sodium sulfate and concentrated. The residue was dissolved in methanol (16 mL), treated with triethylamine (2 mL, 14.3 mmol) and stirred overnight at room temperature. After evaporation, the solid was purified by flash chromatography (DCM/MeOH/NH_3 aq 90:10:2). The solid was treated with diisopropylether and filtered to afford the title compound **9** (0.36 g, 69%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (br s, 1H), 10.95 (s, 1H), 8.02–8.12 (m, 2H), 7.28–7.40 (m, 2H), 4.73 (br s, 2H), 2.80–2.94 (m, 2H), 2.30–2.43 (m, 1H), 2.22 (br s, 3H), 1.92–2.15 (m, 2H), 1.55–1.75 (m, 4H), 1.66 (s, 6H); LC–MS (ESI): m/z 400 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{21}H_{26}FN_5O_2$ + H^+ 400.2144, found 400.2149; Anal. Calcd for $C_{21}H_{26}FN_5O_2$: C, 63.14; H, 6.56; N, 17.53. Found: C, 62.78; H, 6.83; N, 17.32.

The following compounds **8**, **10**–**12** and **17**–**33** were prepared according to the general procedure B described above.

5.1.2.2. *N*-[6,6-Dimethyl-5-(piperidin-4-ylcarbonyl)-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl]-4-fluorobenzamide (8). Compound **8** was obtained in 77% yield after deprotection of ethyl 5-({1-[(9*H*-fluoren-9-ylmethoxy)carbonyl]piperidin-4-yl}-carbonyl)-3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate (prepared according the general procedure B) with 20% piperidine in methanol. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.43 (br s, 1H), 10.95 (br s, 1H), 8.07 (dd, J = 5.5, 8.9 Hz, 2H), 7.34 (t, J = 8.9 Hz, 2H), 4.72 (br s, 2H), 2.96–3.04 (m, 2H), 2.54–2.64 (m, 2H), 2.46–2.56 (m, 1H), 1.66 (m, 6H), 1.58–1.66 (m, 2H), 1.45–1.58 (m, 2H); LC–MS (ESI): m/z 386 $[M+H]^+$.

5.1.2.3. *N*-{5-[(1-Ethylpiperidin-4-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (10). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (br s, 1H), 10.95 (s, 1H), 8.01–8.13 (m, 2H), 7.28–7.40 (m, 2H), 4.74 (br s, 2H), 2.88–3.04 (m, 2H), 2.28–2.47 (m, 3H), 1.85–2.10 (m, 2H), 1.66 (s, 6H), 1.55–1.76 (m, 4H), 1.01 (t, J = 6.8 Hz, 3H); LC–MS (ESI): m/z 414 $[M+H]^+$; Anal. Calcd for $C_{22}H_{28}FN_5O_2$: C, 63.90; H, 6.83; N, 16.94. Found: C, 63.54; H, 6.98; N, 16.65.

5.1.2.4. *N*-{5-[(1-Cyclopropylpiperidin-4-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (11). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (br s, 1H), 10.95 (s, 1H), 8.03–8.11 (m, 2H), 7.28–7.39 (m, 2H), 4.74 (br s, 2H), 2.92–3.07 (m, 3H), 2.36–2.46 (m, 1H), 2.11–2.33 (m, 2H), 1.46–1.74 (m, 4H), 1.65 (s, 6H), 0.26–0.50 (m, 4H); LC–MS (ESI): m/z 426 $[M+H]^+$; Anal. Calcd for $C_{23}H_{28}FN_5O_2$: C, 64.92; H, 6.63; N, 16.46. Found: C, 64.95; H, 6.98; N, 16.65.

5.1.2.5. *N*-{5-[(1-Acetylpiperidin-4-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (12). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (br s, 1H), 10.96 (s, 1H), 8.04–8.11 (m, 2H), 7.28–7.38 (m, 2H), 4.77 (br s, 2H), 4.34–4.42 and 3.79–3.86 (2 × m, 2H), 3.06–3.16 and 2.56–2.66 (2 × m, 2H), 2.66–2.74 (m, 1H), 1.99 (s, 3H), 1.66 (s, 6H), 1.63–1.77 (m, 2H), 1.32–1.57 (2 × m, 2H); LC–MS (ESI): m/z 428 $[M+H]^+$; Anal. Calcd for $C_{22}H_{26}FN_5O_3$: C, 60.07; H, 6.10; N, 15.71. Found: C, 60.45; H, 6.13; N, 16.02.

5.1.2.6. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}benzamide (17). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (br s, 1H), 10.93 (br s, 1H), 7.96–8.01 (m, 2H), 7.56–7.62 (m, 1H), 7.48–7.54 (m, 2H), 4.73 (br s, 2H), 2.82–2.90 (m, 2H), 2.34–2.43 (m, 1H), 2.21 (s, 3H), 1.96–2.07 (m, 2H), 1.66 (s, 6H), 1.58–1.63 (m, 4H); LC–MS (ESI): m/z 382 $[M+H]^+$.

5.1.2.7. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-fluorobenzamide (18). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (br s, 1H), 11.07 (s, 1H), 7.85 (dt, J = 1.7, 8.0 Hz, 1H), 7.80 (ddd, J = 1.7, 2.4, 9.9 Hz, 1H), 7.57 (td, J = 5.8, 8.0 Hz, 1H), 7.42–7.50 (m, 1H), 4.79 (s, 2H), 2.75–2.83 (m, 2H), 2.23–2.34 (m, 1H), 2.14 (s, 3H), 1.83–1.94 (m, 2H), 1.67 (s, 6H), 1.59–1.68 (m, 4H); LC–MS (ESI): m/z 400 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{21}H_{26}FN_5O_2$+H^+ 400.2143, found 400.2142.

5.1.2.8. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3,4-difluorobenzamide (19). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.57 (br s, 1H), 11.09 (br s, 1H), 8.01–8.10 (m, 1H), 7.85–7.93 (m, 1H), 7.54–7.65 (m, 1H), 4.78 (br s, 2H), 2.75–2.84 (m, 2H), 2.25–2.35 (m, 1H), 2.15 (s, 3H), 1.82–1.93 (m, 2H), 1.62 (s, 6H), 1.54–1.65 (m, 4H); LC–MS (ESI): m/z 418 $[M+H]^+$.

5.1.2.9. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3,5-difluorobenzamide (20). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (br s, 1H), 11.17 (s, 1H), 7.67–7.75 (m, 2H), 7.48–7.55 (m, 1H), 4.78 (s, 2H), 2.74–2.84 (m, 2H), 2.26–2.34 (m, 1H), 2.16 (s, 3H), 1.82–1.93 (m, 2H), 1.62 (s, 6H), 1.56–1.67 (m, 4H); LC–MS (ESI): *m*/*z* 418 $[M+H]^+$; HRMS (ESI): *m*/*z* calcd for $C_{21}H_{25}F_2N_5O_2$+H^+ 418.2049, found 418.2055.

5.1.2.10. 4-Chloro-*N*-{6,6-dimethyl-5-[(1-methylpiperidin-4-yl)-carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}benzamide (21). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.53 (br s, 1H), 11.05 (br s, 1H), 8.01 (d, *J* = 8.4 Hz, 2H), 7.57 (d, *J* = 8.4 Hz, 2H), 4.79 (br s, 2H), 2.76–2.82 (m, 2H), 2.24–2.34 (m, 1H), 2.14 (s, 3H), 1.83–1.93 (m, 2H), 1.67 (s, 6H), 1.58–1.73 (m, 4H); LC–MS (ESI): *m*/*z* 416 $[M+H]^+$.

5.1.2.11. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-(trifluoromethyl)-benzamide (22). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.58 (br s, 1H), 11.23 (br s, 1H), 8.18 (d, *J* = 8.2 Hz, 2H), 7.89 (d, *J* = 8.2 Hz, 2H), 4.81 (br s, 2H), 2.73–2.84 (m, 2H), 2.25–2.34 (m, 1H), 2.14 (s, 3H), 1.67 (s, 6H), 1.54–1.69 (m, 4H); LC–MS (ESI): *m*/*z* 450 $[M+H]^+$.

5.1.2.12. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}naphthalene-2-carboxamide (23). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.49 (br s, 1H), 11.10 (br s, 1H), 8.65 (s, 1H), 7.97–8.09 (m, 4H), 7.58–7.69 (m, 2H), 4.78 (br s, 2H), 2.82–2.90 (m, 2H), 2.35–2.44 (m, 1H), 2.21 (s, 3H), 1.95–2.05 (m, 2H), 1.67 (s, 6H), 1.59–1.75 (m, 4H); LC–MS (ESI): *m*/*z* 432 $[M+H]^+$.

5.1.2.13. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}naphthalene-1-carboxamide (24). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.42 (br s, 1H), 11.11 (br s, 1H), 8.20–8.27 (m, 1H), 8.04–8.10 (m, 1H), 7.97–8.03 (m, 1H), 7.71–7.77 (m, 1H), 7.55–7.63 (m, 3H), 4.80 (br s, 2H), 2.80–2.87 (m, 2H), 2.34–2.42 (m, 1H), 2.17 (s, 3H), 1.90–2.00 (m, 2H), 1.68 (s, 6H), 1.59–1.74 (m, 4H); LC–MS (ESI): *m*/*z* 432 $[M+H]^+$.

5.1.2.14. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}pyridine-4-carboxamide (25). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.37 (s, 1H), 8.73–8.76 (m, 2H), 7.88–7.93 (m, 2H), 4.81 (br s, 2H), 2.78–2.85 (m, 2H), 2.28–2.37 (m, 1H), 2.17 (s, 3H), 1.90–2.01 (m, 2H), 1.68 (s, 6H), 1.58–1.74 (m, 4H); LC–MS (ESI): *m*/*z* 383 $[M+H]^+$.

5.1.2.15. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}thiophene-2-carboxamide (26). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.50 (br s, 1H), 11.06 (s, 1H), 8.10 (d, *J* = 3.8 Hz, 1H), 7.84 (dd, *J* = 1.0, 5.0 Hz, 1H), 7.20 (dd, *J* = 3.8, 5.0 Hz, 1H), 4.77 (s, 2H), 2.75–2.83 (m, 2H), 2.23–2.35 (m, 1H), 2.16 (s, 3H), 1.82–1.90 (m, 2H), 1.66 (s, 6H), 1.54–1.64 (m, 4H); LC–MS (ESI): *m*/*z* 388 $[M+H]^+$.

5.1.2.16. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}thiophene-3-carboxamide (27). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.45 (br s, 1H), 10.77 (s, 1H), 8.42 (br s, 1H), 7.61–7.70 (m, 2H), 4.73 (br s, 2H), 2.86–3.00 (m, 2H), 2.36–2.46 (m, 1H), 2.29 (br s, 3H), 2.07–2.22 (m, 2H), 1.59–1.77 (m, 4H), 1.66 (s, 6H); LC–MS (ESI): *m*/*z* 388 $[M+H]^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{25}N_5O_2S$+H^+ 388.1802, found 388.1806.

5.1.2.17. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}furan-2-carboxamide (28). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 7.91 (dd, *J* = 0.8, 1.7 Hz, 1H), 7.42 (dd, *J* = 0.8, 3.5 Hz, 1H), 6.68 (dd, *J* = 1.7, 3.5 Hz, 1H), 4.75 (s, 2H), 2.80–2.87 (m, 2H), 2.34–2.42 (m, 1H), 2.17 (s, 3H), 1.91–2.01 (m, 2H), 1.66 (s, 6H), 1.58–1.64 (m, 4H); LC–MS (ESI): *m*/*z* 372 $[M+H]^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{25}N_5O_3$+H^+ 372.2030, found 372.2013.

5.1.2.18. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}cyclopropanecarboxamide (29). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.28 (br s, 1H), 10.70 (br s, 1H), 4.61 (s, 2H), 2.81–2.90 (m, 2H), 2.27–2.36 (m, 1H), 2.23 (s, 3H), 1.97–2.07 (m, 2H), 1.83 (m, 1H), 1.61 (m, 6H), 1.56–1.68 (m, 4H), 0.72–0.80 (m, 4H); LC–MS (ESI): *m*/*z* 346 $[M+H]^+$.

5.1.2.19. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}cyclobutancarboxamide (30). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.25 (br s, 1H), 10.25 (br s, 1H), 4.67 (br s, 2H), 3.22 (m, 1H), 2.77–2.85 (m, 2H), 2.28–2.37 (m, 1H), 2.17 (s, 3H), 2.14–2.23 (m, 2H), 2.02–2.11 (m, 2H), 1.87–1.98 (m, 3H), 1.73–1.82 (m, 1H), 1.62 (s, 6H), 1.56–1.67 (m, 4H); LC–MS (ESI): m/z 360 $[M+H]^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{29}N_5O_2$ + H^+ 360.2394, found 360.2396; Anal. Calcd for $C_{19}H_{29}N_5O_2$: C, 63.48; H, 8.13; N, 19.48. Found: C, 63.25; H, 8.30; N, 19.12.

5.1.2.20. *N*-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-methylbutanamide (31). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.27 (br s, 1H), 10.34 (br s, 1H), 4.64 (br s, 2H), 2.82–2.89 (m, 2H), 2.30–2.37 (m, 1H), 2.21 (s, 3H), 2.12–2.18 (m, 2H), 2.15 (d, *J* = 7.1 Hz, 2H), 1.96–2.09 (m, 3H), 1.62 (s, 6H), 1.54–1.67 (m, 4H), 0.90 (d, *J* = 6.6 Hz, 6H); LC–MS (ESI): m/z 362 $[M+H]^+$; HRMS (ESI): *m*/*z* calcd for $C_{19}H_{31}N_5O_2$+H^+ 362.2551, found 362.2555; Anal. Calcd for $C_{19}H_{31}N_5O_2$: C, 63.13; H, 8.64; N, 19.37. Found: C, 63.01; H, 8.78; N, 19.25.

5.1.2.21. *N*-{5-[(1-Ethylpiperidin-4-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-methylbutanamide (32). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.32 (br s, 1H), 10.39 (s, 1H), 4.70 (s, 2H), 2.88–3.04 (m, 2H), 2.28–2.47 (m, 3H), 2.15 (d, *J* = 7.1 Hz, 2H), 1.98–2.08 (m, 1H), 1.85–2.10 (m, 2H), 1.63 (s, 6H), 1.55–1.76 (m, 4H), 1.01 (t, *J* = 6.8 Hz, 3H), 0.90 (d, *J* = 6.6 Hz, 6H); LC–MS (ESI): m/z 376 $[M+H]^+$.

5.1.2.22. *N*-{5-[(1-Cyclopropylpiperidin-4-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-methylbutanamide (33). ^{1}H NMR (400 MHz, DMSO–d_6) δ 12.33 (br s, 1H), 10.39 (s, 1H), 4.71 (s, 2H), 2.92–3.07 (m, 3H), 2.36–2.46 (m, 1H), 2.16 (d, *J* = 7.1 Hz), 2.11–2.33 (m, 2H), 1.97–2.08 (m, 1H), 1.46–1.74 (m, 4H), 1.63 (s, 6H), 0.90 (d, *J* = 6.6 Hz, 6H), 0.26–0.50 (m, 4H); LC–MS (ESI): m/z 388 $[M+H]^+$.

5.1.3. General procedure C

General procedure for the preparation of compounds **13**–**16** is illustrated below for the preparation of **13**.

5.1.3.1. *N*-{6,6-Dimethyl-5-[(4-methylpiperazin-1-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (13). Ethyl 3-{[(4-fluorophenyl)carbonyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate hydrochloride (prepared according to the general method A) (442 mg, 1.15 mmol) in DCM (30 mL) was added to a solution of triphosgene (195 mg, 0.65 mmol) in DCM (15 mL), followed by *N*,*N*-diisopropylethylamine (0.76 mL, 4.31 mmol). After 3 h, a solution of *N*-methylpiperazine (0.195 mL, 1.72 mmol) and diisopropylethylamine (0.30 mL, 1.72 mmol) in DCM (8 mL) was added. The

reaction was stirred overnight at room temperature. The solution was washed with brine, the organic phase was dried over sodium sulfate and concentrated. The residue was dissolved in methanol (16 mL), treated with triethylamine (1.6 mL, 11.5 mmol) and stirred overnight at room temperature. After evaporation, the solid was purified by flash chromatography (DCM/MeOH 90:10). The solid was treated with diisopropylether and filtered to afford the title compound **13** (0.294 g, 64%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (br s, 1H), 10.89 (s, 1H), 8.04 (dd, J = 5.5, 8.6 Hz, 2H), 7.33 (t, J = 8.6 Hz, 2H), 4.55 (br s, 2H), 2.99–3.12 (m, 4H), 2.32–2.48 (m, 4H), 2.24 (br s, 3H), 1.62 (s, 6H); LC–MS (ESI): m/z 401 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{20}H_{25}FN_6O_2$ + H$^+$ 401.2096, found 401.2091; Anal. Calcd for $C_{20}H_{25}FN_6O_2$: C, 59.99; H, 6.29; N, 20.99. Found: C, 59.70; H, 6.40; N, 20.66.

The following compounds **14**–**16** were prepared according to the general procedure C described above.

5.1.3.2. 4-Fluoro-*N*-(5-{[4-(2-hydroxyethyl)piperazin-1-yl]carbonyl}-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl)benzamide (14). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.41 (br s, 1H), 10.91 (s, 1H), 8.06 (dd, J = 5.5, 8.8 Hz, 2H), 7.33 (t, J = 8.8 Hz, 2H), 4.55 (s, 2H), 3.48–3.57 (m, 2H), 3.03–3.11 (m, 4H), 2.44–2.64 (m, 6H), 1.62 (s, 6H); LC–MS (ESI): m/z 431 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{21}H_{27}FN_6O_3$ + H$^+$ 431.2202, found 431.2200.

5.1.3.3. *N*-{5-[(4-Acetylpiperazin-1-yl)carbonyl]-6,6-dimethyl-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (15). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.45 (br s, 1H), 10.92 (s, 1H), 8.06 (dd, J = 5.5, 8.9 Hz, 2H), 7.33 (t, J = 8.9 Hz, 2H), 4.58 (br s, 2H), 3.44–3.49 (m, 4H), 3.05–3.10 (m, 2H), 2.99–3.04 (m, 2H), 2.00 (s, 3H), 1.63 (s, 6H); LC–MS (ESI): m/z 429 [M+H]$^+$.

5.1.3.4. *N*-{6,6-Dimethyl-5-[(4-phenylpiperazin-1-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-4-fluorobenzamide (16). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (br s, 1H), 10.92 (s, 1H), 8.06 (dd, J = 5.5, 8.8 Hz, 2 H), 7.32 (t, J = 8.8 Hz, 2 H), 7.19–7.26 (m, 2H), 6.93–6.98 (m, 2H), 6.77–6.82 (m, 1H), 4.62 (s, 2 H), 3.14–3.24 (m, 8H), 1.65 (s, 6 H); LC–MS (ESI): m/z 463 [M+H]$^+$.

5.1.4. General procedure D

General procedure for the preparation of compounds **34**–**36** is illustrated below for the preparation of **36**.

5.1.4.1. 1-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-(3-fluorophenyl)urea (36). 5-*tert*-Butyl 2-ethyl 3-amino-6,6-dimethyl-4,6-dihydropyrrolo[3,4-*c*]pyrazole-2,5(4*H*,6*H*)-dicarboxylate (3.0 g, 9.24 mmol) was dissolved in anhydrous THF (50 mL), treated at room temperature with 3-fluorophenyl-isocyanate (1.4 g, 10.21 mmol) and stirred overnight. Then the reaction mixture was evaporated, taken up with DCM and washed with brine. The organic phase was dried over sodium sulfate and evaporated to dryness. Purification by flash chromatography (DCM/MeOH 90:10) afforded 5-*tert*-butyl 2-ethyl 3-{[(3-fluorophenyl)carbamoyl]amino}-6,6-dimethyl-4,6-dihydropyrrolo[3,4-*c*]pyrazole-2,5-dicarboxylate (3.05 g, 71%). LC–MS (ESI): m/z 462 [M+H]$^+$. This intermediate (2.80 g, 6.06 mmol) was dissolved in dioxane (50 mL) and treated with HCl 4 M in dioxane (30 mL, 20 equiv). After 2 h at 40 °C the reaction mixture was concentrated and the residue was treated with diethyl ether, filtered to afford ethyl 3-{[(3-fluorophenyl)carbamoyl]amino}-6,6-dimethyl-5,6-dihydropyrrolo[3,4-*c*]pyrazole-2(4*H*)-carboxylate hydrochloride (2.30 g, 95%). LC–MS (ESI): m/z 362 [M+H]$^+$. This intermediate (2.30 g, 5.78 mmol), in DCM (100 mL), was treated with *N*,*N*-diisopropylethylamine (5.0 mL, 28.9 mmol) and TBTU (2.50 g, 7.78 mmol), at room temperature for 1 h, and then 1-methyl-piperidine-4-carboxylic acid hydrochloride (1.30 g, 7.24 mmol) was added. The reaction was stirred overnight. The solution was washed with saturated sodium hydrogen carbonate aqueous solution and brine, the organic phase was dried over sodium sulfate and concentrated. The residue was dissolved in methanol (72 mL), treated with triethylamine (8 mL, 14.3 mmol) and stirred overnight at room temperature. After evaporation, the solid was purified by flash chromatography (eluent: DCM/MeOH/NH$_3$ aq 90:10:2). The solid was treated with diisopropylether and filtered to afford the title compound **36** (1.80 g, 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.24 (br s, 1H), 9.03 (br s, 1H), 7.46 (dt, J = 2.2, 11.6 Hz, 1H), 7.28–7.34 (m, 1H), 7.10 (d, J = 9.5 Hz, 1H), 6.79 (ddd, J = 2.8, 8.0, 9.5 Hz, 1H), 4.66 (br s, 2H), 2.85–2.93 (m, 2H), 2.34–2.43 (m, 1H), 2.25 (br s, 3H), 2.03–2.15 (m, 2H), 1.63 (s, 6H), 1.57–1.73 (m, 4H); LC–MS (ESI): m/z 415 [M+H]$^+$.

The following compounds **34**–**35** were prepared according to the general procedure D described above.

5.1.4.2. 1-Benzyl-3-{6,6-dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}urea (34). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (br s, 1H), 8.83 (s, 1H), 7.22–7.36 (m, 5H), 6.89 (br s, 1H), 4.59 (br s, 2H), 4.31 (d, J = 5.9 Hz, 2H), 2.82–2.89 (m, 2H), 2.29–2.37 (m, 1H), 2.21 (s, 3H), 1.96–2.06 (m, 2H), 1.61 (s, 6H), 1.55–1.68 (m, 4H); LC–MS (ESI): m/z 411 [M+H]$^+$.

5.1.4.3. 1-{6,6-Dimethyl-5-[(1-methylpiperidin-4-yl)carbonyl]-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl}-3-propylurea (35). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (br s, 1H), 8.66 (s, 1H), 6.44 (br s, 1H), 4.58 (s, 2H), 3.00–3.07 (m, 2H), 2.79–2.86 (m, 2H), 2.26–2.35 (m, 1H), 2.18 (br s, 3H), 1.90–1.98 (m, 2H), 1.60 (s, 6H), 1.56–1.66 (m, 4H), 1.37–1.46 (m, 2H), 0.86 (t, J = 7.6 Hz, 3H); LC–MS (ESI): m/z 363 [M+H]$^+$.

5.2. Crystallographic methods

Expression, purification, crystallization and soaking procedures of the CDK2/cyclin A complex was carried out as previously described.[16] The final R-factor for the complex with compound **31** was 22.3% (Rfree 26.1%). The coordinates have been deposited in the Protein Data Bank with code 2WPA together with structure factors and detailed experimental conditions.

5.3. Kinase assays

Kinase assays were performed as previously described.[16] The panel includes: c-ABL, AKT1, ALK, Aur-A, Aur-B, CDC7, CDK2/A, CDK1/B, CDK2/E, CDK4/D1, CDK5/p25, CDK7/H, CDK9/T1, CHK1, CK2, EGFR, ERK2, FGFR1, FLT3, GSK3β, IGF1R, IKK2, IR, JAK2, C-KIT, LCK, MAPKAPK2, MET, NEK-6, NIM, PAK4, PDGFR, PDK1, PKAα, PKCα, PLK1, PLK2, P38α, RET, STLK2, SULU1, TRKA, VEGFR2, VEGFR3.

5.4. In vitro pharmacology. A2780 cells proliferation assay

Cells were seeded into 96- or 384-wells plates at final concentration ranging from 10000 to 30000 cells per cm^2 in appropriate medium plus 10% FCS. After 24 h cells were treated using serial dilution of compounds in two replicates. At 72 h after the treatment the amount of cells were evaluated using the Cell Titer_Glo assay (Promega). IC_{50} values were calculated using a sygmoidal fitting (Assay Explorer MDL). Experiments were replicated at least two times.

5.5. Flow cytometry analysis and BrdU incorporation

A2780 cells (human adenocarcinoma ovary, from ECACC), were seeded in T-75 tissue culture flasks, 25000 cells/cm^2 in RPMI 1640,

pH 7.4, 10% FBS, 2 mM L-glutamine, 1 × penicillin–streptomycin and maintained in 5% CO_2 at 37 °C with 96% relative humidity. After 24 h, cells were treated with compounds at 1 μM and 3 μM for 24 h. Cells in the supernatant and adherent cells were collected using 0.25% Trypsin, 0.02% EDTA. Cells were washed with PBS and were divided into three samples for flow cytometry analysis and for immunoblot and BrdU incorporation, as previously described.[16]

5.6. In vivo pharmacology

Evaluation of antitumor efficacy was performed as previously described.[16]

5.7. High-throughput solubility

Solubility at pH 7 was performed as previously described.[16]

5.8. Metabolic stability

Compounds were dissolved in DMSO at 10 μM concentration. Human cDNA expressed cytochrome P450 isoforms (supersomes) were purchased from Gentest (Woburn, MA). All chemicals used were of analytical grade and commercially available. The potential inhibitory effect was investigated against cDNA expressed human CYP4503A4 supersome using typical substrates incubated at their respective K_m concentration. The known inhibitor ketoconazole was included to check the inhibition response. Analysis of both substrate and metabolite was done by LC/MS/MS.

5.9. Plasma protein binding

Plasma protein binding was performed as previously described.[16]

5.10. In vivo pharmacokinetics

The pharmacokinetic profile of the compounds was investigated in overnight fasted male Nu/Nu mice following a single dose given intravenously (iv) or orally (po). The vehicle used was 5% dextrose solution. A total of six mice were treated (three for each leg). Blood samples of each mouse were collected from the saphenous vein at predose, 0.083, 0.5, 1, 6, and 24 h postdosing following iv dosing, and at predose, 0.25, 0.5, 1, 6 and 24 h following oral dosing. Samples were centrifuged at 10,000*g* for 3 min at 4 °C and the plasma was stored at −80 °C until analysis. Samples were analyzed by LC/MS/MS technique.

5.11. Immunohistochemistry

Immunohistochemistry was performed on serial sections of formalin fixed-paraffin embedded A2780 tumors. After dewaxing and heat-induced epitope retrival, sections were processed as previously described[17] for phosphor-pRb (pRb phosphorylated in Threonin 821 rabbit polyclonal antibody, Biosource cat 44–582). For BrdU staining, tumors, denaturated and pretreated with HCl 4 N and proteinase K, were incubated with Dako ARK kit following manufacturer's procedure. All samples were then counterstained with hematoxylin. Automated quantitative analysis was performed by two observers using a Image Pro Plus software. Five fields at 100× magnification were analyzed and results are reported as the mean of positive cells/field. Statistical analysis was performed by Mann–Withney test.

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