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4,5-Dihydro-1*H*-pyrazolo[4,3-*h*]quinazolines as potent and selective Polo-like kinase 1 (PLK1) inhibitors

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ABSTRACT

A series of 4,5-dihydro-1*H*-pyrazolo[4,3-*h*]quinazoline derivatives was optimized as Polo-like kinase 1 inhibitors. Extensive SAR afforded a highly potent and selective PLK1 compound. The compound showed good antiproliferative activity when tested in a panel of tumor cell lines with PLK1 related mechanism of action and with good in vivo antitumor efficacy in two xenograft models after iv administration.

Polo-like kinases (PLKs) are a group of highly conserved serine/threonine kinases that regulate critical processes in the cell cycle.[1] Four mammalian PLK family members PLK1, PLK2 (SNK), PLK3 (FNK or PRK) and PLK4 (SAK) were identified.[2,3] Among them, PLK1 is the best characterized and is recognized to be a key component of the cell cycle control machinery with important roles in the mitotic entry, centrosome duplication, bipolar mitotic spindle formation, transition from metaphase to anaphase, cytokinesis and maintenance of genomic stability.[4,5] PLK1 is often over-expressed in many different tumor types including lung, colon, prostate, ovary, breast, head and neck squamous cell carcinoma, and its over-expression often correlates with poor prognosis.[6,7] Furthermore recent studies have identified a possible role of PLK1 in DNA damage recovery by phosphorylation and destabilization of Claspin, a known regulator of Chk1 activation.[8,9] PLK1 is not expressed in differentiated postmitotic cells like neurons where instead expression of PLK2 and PLK3 was reported indicating a potentially better safety profile for a PLK1 specific inhibitor.[10] Thus, PLK1 is thought to be a promising target for anti-cancer therapy and some PLK1 inhibitors are currently under evaluation in clinical trials.[11]

In order to identify PLK1 inhibitors a high-throughput screening (HTS) of the Nerviano Medical Sciences collection was conducted. Prior work in the 4,5-dihydro-1*H*-pyrazolo[4,3-*h*]quinazoline series was recently disclosed identifying the key scaffold positions to achieve selective and potent PLK1 inhibitors.[12] In order to develop a PLK1 specific inhibitor suitable for clinical trials, work on this class was continued and here we report the results obtained, starting from the hit compound **1**, by a focused expansion at the 5′- and 1-positions of the scaffold that led to the identification of the potent and selective compound **4** (Fig. 1).

Compounds modified at the 5′-position were prepared starting from the iodo amide **2**,[13] which was coupled under Buchwald–Hartwig conditions with the suitable anilines, $Pd(OAc)_2$ and (±)-BINAP as ligand to obtain compounds **1**, **3**–**5** (Scheme 1).[14] The bromo derivative **3** was in turn used as starting material to

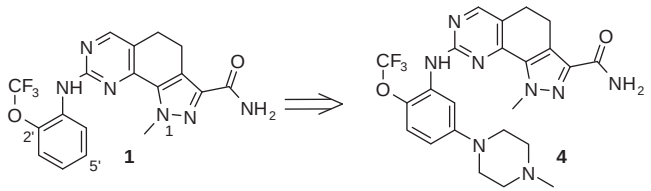

Figure 1. Evolution from the hit compounds **1**–**4**.

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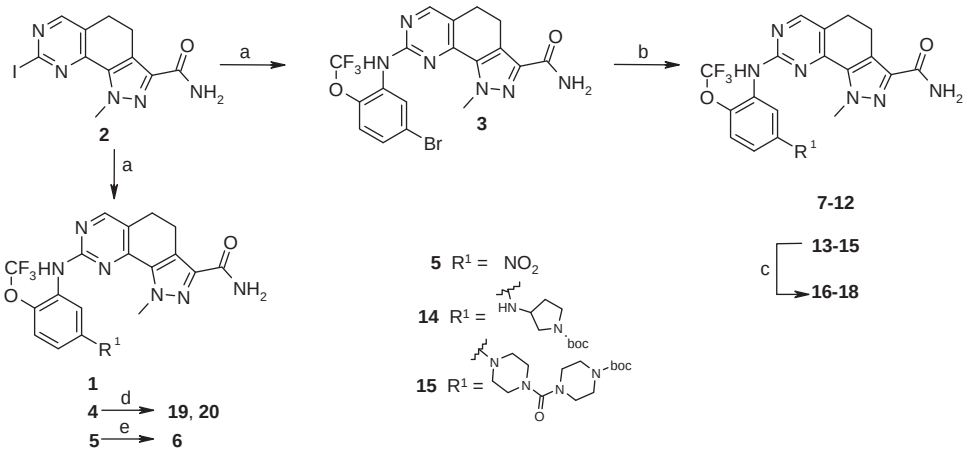
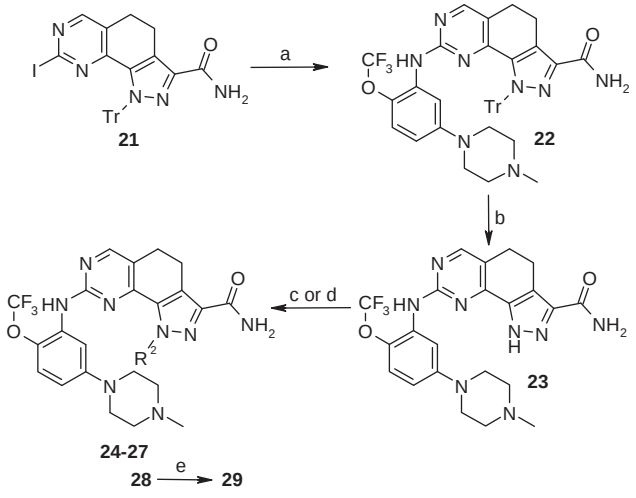


Scheme 1. Reagents and conditions: (a) $Pd(OAc)_2$, (±)-BINAP, K_2CO_3, anilines, DMF, 80 °C, 43–55%; (b) primary or secondary amines, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2′-(*N*,*N*-dimethylamino)-biphenyl, $LiN(TMS)_2$, THF, reflux, 5–70%; (c) 4 M HCl in dioxane, dioxane, rt, quant; (d) 0.1 M 3,3-dimethyl-dioxirane in acetone, DCM/acetone (1:1), rt, 40%; (e) Fe, NH_4Cl, $MeOH/H_2O$, reflux, quant.

access compounds **7–15**, by reaction with suitable amines, $Pd_2(dba)_3$ and 2-dicyclohexylphosphino-2′-(*N*,*N*-dimethylamino)-biphenyl. Removal of the *tert*-butoxycarbonyl protecting group from **13** to **15** under acid conditions afforded compounds **16**–**18** while, oxidation at the piperazine residue of **4** to yield compound **19** and **20** was performed with freshly prepared 3,3-dimethyl-dioxirane solution. Finally, compound **6** was prepared by reduction the nitro derivative **5** with iron and ammonium chloride.

Modifications at the 1-position of the pyrazole ring was performed reacting the advanced intermediate **23** with alcohols under Mitsunobu conditions using polimer-bound Ph_3P or, alternatively with alkyl halogens under basic conditions (Scheme 2).[15] The alkylation reaction proceeds in both cases regioselectively at the position 1 of the pyrazolo ring giving compounds **24**–**28**. Compound **23** was obtained from deprotection with hydrochloric acid of the trityl protected derivative **22** which was in turn prepared subjecting the iodo-1-trityl amide **21**[13] to Buchwald–Hartwig coupling conditions with 5-(4-methyl-piperazin-1-yl)-2-trifluoromethoxy-phenylamine, $Pd(OAc)_2$ and (±)-BINAP. Conversion of the chloro-ethyl derivative **28** to vinyl derivative **29** was performed in good yield with diaza(1,3)bicyclo[5.4.0]undecene (DBU).

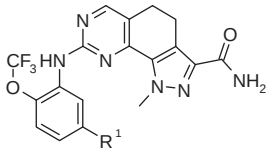


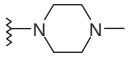
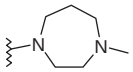
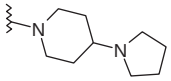
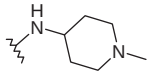
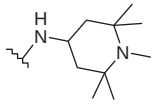
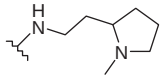
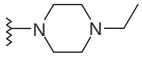
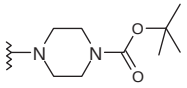
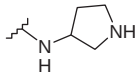
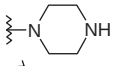
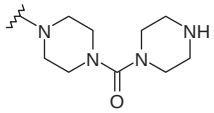
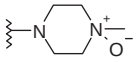
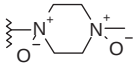
Scheme 2. Reagents and conditions: (a) $Pd(OAc)_2$, (±)-BINAP, K_2CO_3, amine, DMF, 80 °C, 30%; (b) TFA, DCM, rt, 71%; (c) DTBAD, Ph_3P polymer-bound, alcohol R_2-OH, THF; (d); Cs_2CO_3, R^2-Cl, DMF, rt; (e) DBU, 80 °C.

Table 1 summarizes the structure–activity relationship (SAR) of derivatives modified at the 5′-position of the hit compound **1**. Introduction at this position of simple heterocyclyl residues (**4**, **7**, **12** and **17**) resulted crucial to access good PLK1 activity, achieve selectivity against PLK2 and PLK3 and, improve selectivity vs CDK2/A with respect to the hit compound **1**. Among them, compounds **4** and **17** resulted to show the best compromise in terms of PLK1 activity, selectivity against PLK2, PLK3 and CDK2/A, cellular activity and acceptable solubility. Introduction of heterocyclyl-amino moieties (**9**–**11** and **16**) from one side contributed to increase compounds' solubility, from the other side determined a loss of PLK1 activity ranging from 38-fold (**16**) to more than 400-fold (**10**), a restored cross reactivity against PLK2, PLK3 and CDK2/A and a decreased cellular proliferation potency that in the best case (**10**) is 36-fold reduced with respect to the best inhibitor **17**. The worse biochemical profile shown by these compounds in comparison with analogs **4**, **7**, **12** and **17** bearing a piperazine or homopiperazine residue at the position 5′ of the aniline moiety is in agreement with the key role played by one of the piperazine nitrogens to pick-up a polar interaction with Glu 140 in the solvent accessible region of PLK1.[12] In case of compounds **9**–**11** and **16** the increased side chain freedom determined by the introduction of heterocyclyl-amino moieties impairs this interaction thus determining a decreased activity and selectivity with respect to piperazine analogs **4**, **12** and **17**. This hypothesis is further supported by the decreased PLK1 activity of **8** and **18** where the basic center of the 5′ residue was moved away with respect to the one of the piperazine moiety of compounds **4**, **12** and **17**. The same behavior was found with compound **6** lacking of the piperazino residue or, with compounds **13**, **19**, and **20** where the piperazine residue is unable to establish polar interaction with the acid residue Glu 140 and wherein the PLK1 activity is decreased from 20-fold (**6**) to around 1000-fold (**20**) with respect to compound **4**.

To verify if the methyl substituent at the pyrazolo ring of the compound **4** was the optimal group, a reduced number of derivatives modified at this position were tested (Table 2). Replacement of the methyl with trityl group (**22**) or 4-methoxy-benzyl moiety (**24**) gave a complete loss of activity. Compounds wherein the methyl group was removed (**23**) or replaced with small residues (**25**–**29**) exhibited a good antiproliferative activity (A2780 IC_{50} <100 nM), a maintained PLK1 activity (IC_{50} <20 nM) and for some of them (**25**, **26** and **28**) even an improved selectivity against PLK2 and PLK3 with respect to the compound **4**. These data confirmed the previous finding that small groups at the position 1 of the 4,5-dihydro-1*H*-pyrazolo[4,3-*h*]quinazoline template are

Table 1
SAR of 5′-position of the aniline residue



Compound	R^1	PLK1 IC_{50}[a] (μM)	PLK2 IC_{50}[a] (μM)	PLK3 IC_{50}[a] (μM)	CDK2/A IC_{50}[a] (μM)	A2780 IC_{50}[a] (μM)	Solubility pH 7 (μM)
1	H	0.117	0.206	0.032	1.762	6.412	<1
4		0.003	3.519	1.439	>10	0.021	72
6	NH_2	0.060	1.282	0.154	1.180	1.075	31
7		0.025	>10	>10	>10	0.941	95
8		0.033	>10	>10	>10	0.583	133
9		0.200	3.415	3.451	1.020	1.092	>225
10		0.964	>10	>10	3.019	1.754	147
11		0.123	0.429	0.485	0.969	2.556	157
12		0.008	3.387	1.215	>10	0.034	33
13		0.859	0.768	0.262	1.133	1.293	nd
16		0.077	0.762	0.239	0.414	2.217	171
17		0.002	>10	1.433	>10	0.03	114
18		0.035	0.190	0.083	0.622	0.538	85
19		0.127	>10	2.410	>10	0.782	>225
20		2.682	>10	>10	>10	>10	48

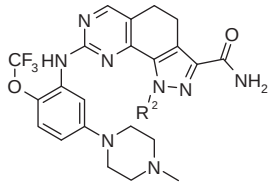
[a] Values are means of three experiments.

tolerated by PLK1.[12] A selection of the most promising compounds (**4**, **17**, **25** and **29**) was then profiled against a larger kinase panel (Table 3). All compounds showed good selectivity in a panel of more than 40 kinases, with a slightly more favorable profile for **4** in comparison with **17**, **25** and **29**.[16] Selected compounds **4**, **17**, **25** and **29** were further evaluated on the basis of solubility preformulation test and in vitro ADME properties (Table 4).[17,18] Among them compound **17** showed the worse profile with the lower solubility and the lower permeability in the PAMPA assay. Compounds **4** and **29** are both characterized by good solubility and by moderate metabolic stability in human liver microsomes with respect to **25**. In addition compound **4** compared to **29** exhibited a higher permeability value indicating a possible more favorable oral bioavailability.

In view of the ADME profile results, in vivo pharmacokinetic properties of **4**, **25** and **29** were evaluated in CD1 nu/nu mice following intravenous (iv) and oral (po) dosing (Table 5).[19] Compound **4** was considered to have the best pharmacokinetic balance with relatively high clearance (4.21 L/h/kg), a volume of distribution about five times the mouse total body water (3.39 L/kg), indicating a good tissue distribution, an half-life of 0.92 h and an AUC of 4.97 μM h. After po dosing, compound **4** showed a terminal half-life compatible to that found after iv administration and an acceptable oral bioavailability ($F = 21\%$).

On the basis of these favorable pharmacokinetic data, the acceptable solubility, the high selectivity and good antiproliferative activity, compound **4** was profiled against additional cancer cell lines in a 72 h proliferation assay (Table 6). The compound resulted active in cells derived from solid tumors as well as hematological malignancies showing a cytotoxicity ranging from 2 nM (KU812 chronic myeloid leukemia) to 195 nM (NCI-H1993 NSCLC). Interestingly compound **4**, when tested in A2780 ovary carcinoma

Table 2
Modifications at the 1 position of the pyrazole ring



Compound	R^2	PLK1 IC_{50}[a] (μM)	PLK2 IC_{50}[a] (μM)	PLK3 IC_{50}[a] (μM)	CDK2/A IC_{50}[a] (μM)	A2780 IC_{50}[a] (μM)	Solubility pH 7 (μM)
4	Methyl	0.003	3.519	1.439	>10	0.021	72
22	Trityl	>10	nd	nd	>10	2.105	<1
23	H	0.004	>10	>10	>10	0.093	12
24	4-Methoxy-benzyl	2.65	nd	nd	>10	3.624	<1
25	2-Fluoro-ethyl	0.004	>10	>10	>10	0.014	18
26	Ethyl	0.006	>10	>10	>10	0.011	21
27	2-Methoxy-ethyl	0.015	>10	>10	>10	0.075	136
28	2-Chloro-ethyl	0.007	>10	>10	2.01	0.031	9
29	Vinyl	0.006	0.335	0.182	>10	0.008	8

[a] Values are means of three experiments.

Table 3
Expanded kinase selectivity panel

Kinase	IC_{50}[a] (μM)			
	4	**17**	**25**	**29**
PLK1	0.003	0.002	0.004	0.006
CK2	0.140	0.059	0.134	0.069
FLT3	1.517	0.194	0.216	0.226
NEK6	1.664	0.595	>10	0.336
PDGFR	>10	>10	>10	1.461
ALK	>10	>10	>10	2.375
Others 37 kinases	>10	>10	>10	>10

[a] Values are means of three experiments.

Table 4
Solubility preformulation and in vitro ADME properties of selected compounds

Compd	In viro ADME properties		
	Solubility 10% Tween 80, (mg/mL)	PAMPA[a] (Papp 10^{-6} cm/s)	Cl_{int} (mL/min/kg) HLM[b] (1 μM)
4	>3.2	50.0	25.7
17	1.1	7.22	6..9
25	2.1	50.0	56.1
29	>3.2	19.6	28.8

[a] Parallel artificial membrane permeability.
[b] Human liver microsomes.

cell line, showed similar potency on parent cell line and in cells resistant to classical cytotoxic agents like doxorubicin (A2780 Adria), paclitaxel (A2780 PTX22) and cisplatin (A2780 CIS). In addition compound **4** was equally active on p53 proficient cells (A2780) or, defective for p53 (A2780 E6) thus indicating that the mechanism of action of the compound is not dependent from p53 status.

Table 6
Antiproliferative activity of compound **4**

Cell line	Tumor type	IC_{50} (μM)[a]
HT-29	Colon	0.008
LoVo	Colon	0.007
HCT-116	Colon	0.041
MIA-PaCa-2	Pancreas	0.052
PANC-1	Pancreas	0.126
A2780 PTX22	Ovarian	0.038
A2780 Adria	Ovarian	0.051
A2780 CIS	Ovarian	0.021
A2780-E6	Ovarian	0.005
NCI-H1993	NSCLC	0.195
HL-60	Acute myeloid leukemia	0.016
KU812	Chronic myeloid leukemia	0.002
KMS-11	Multiple myeloma	0.014

[a] IC_{50} values are reported as the mean of 2–3 experiments with a coefficient of variation below 35%.

Mechanism of action of compound **4** was first evaluated by array scan analysis (Cellomics) measuring different cell cycle markers in U2OS cells. Cells were treated for 24 h with different doses of compound and extent of mitotic block was established measuring the accumulation of cells positive for pSer10 Histone H3 and nuclear Cyclin B1 (Fig. 2, panels A and B). The specific PLK1 mechanism was confirmed evaluating the decrease of pSer46 TCTP signal in U2OS cells synchronized in mitosis by nocodazole and then, treated for 1 h with different doses of **4** (Fig. 2, panel C). Values of 260 and 320 nM, respectively, for pSer10 Histone H3 (20% positive cells) and Cyclin B1 (20% positive cells) (Fig. 2,

Table 5
In vivo pharmacokinetic parameters ± standard deviation of selected compounds in CD1 nu/nu mice[a]

Compound	PK data (iv), dose[b]: 10 mg/kg				PK data (po), dose[b]: 10 mg/kg			
	AUC_∞ (μM h)	CL (L/h/kg)	V_{ss} (L/kg)	$t_{1/2}$ (h)	C_{max} (μM)	AUC_∞ (μM h)	$t_{1/2}$ (h)	F^c (%)
4	4.97 ± 0.71	4.21 ± 0.45	3.39 ± 0.25	0.92 ± 0.12	0.29 ± 0.04	1.04 ± 0.10	1.44 ± 0.29	21
25	10.20 ± 5.97	2.42 ± 1.07	1.53 ± 1.04	0.74 ± 0.05	0.26 ± 0.08	0.192 ± 0.06	nd	2
29	5.37 ± 0.16	3.44 ± 0.15	2.58 ± 0.06	0.89 ± 0.02	0.61 ± 0.06	2.15 ± 0.18	2.58 ± 0.06	29

nd = not determined.
[a] $n = 3$ animals per study.
[b] Dosed as HCl in situ salt/glucosate.
[c] Bioavailability.

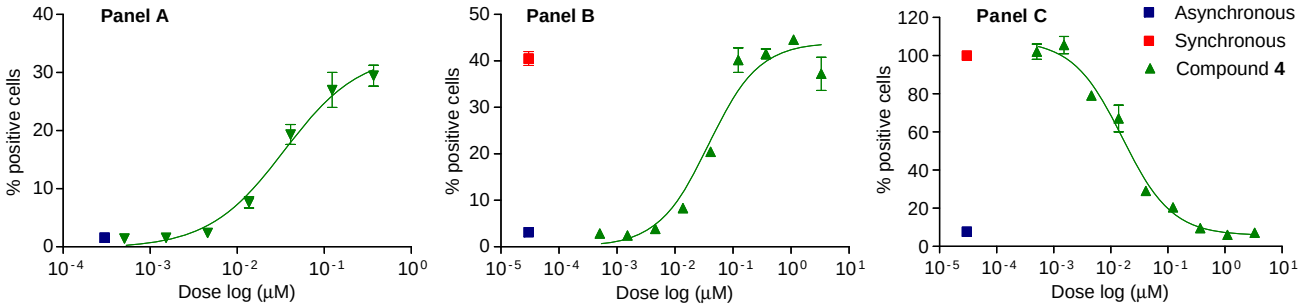


Figure 2. Modulation of phosphoSer10 Histone H3 (panel A), cyclin B1 (panel B) and phosphoSer46 TCTP (panel C) in U2OS cells treated with **4**.

panels A and B) and, IC_{50} of 95 nM for pSer46 TCTP signal inhibition were determined (Fig. 2, panel C).

A larger number of cell cycle and apoptosis markers were evaluated in A2780 and in HCT116 cell lines treated for 24 h with different doses of **4**. Clear modulation of markers was already appreciable at the dose of 50 nM with increase in PLK1 and cyclin B1 total levels, decrease in the phosphorylation of Tyrosine 15 on CDK1, increase in the phosphorylation of Histone H3 on Serine 10 and nucleophosmin on Threonine 199 indicating cells accumulating in the G2/M phase of the cell cycle. Apoptosis induction was observed in both cell lines as indicated by PARP cleavage signal (Fig. 3).

In vivo efficacy of compound **4** was examined in CD1 nu/nu mice xenografted with human ovarian A2780 or with HCT116 colon adenocarcinoma cells. The compound **4**, when administered intravenously at 30 mg/kg, bid on day 1, 2, 3 on A2780 xenograft mice model, showed a good tumor growth inhibition (TGI_{max} = 74%, day 12) with severe but reversible body weight loss (BWL_{max} 19%, day 12) indicating that this dose is potentially close to the maximal tolerated dose (Fig. 4). Even better efficacy was shown in the HCT116 xenograft model where, after iv treatment at 30 mg/kg, bid, given at day 1, 2 in a weekly scheduling × 3 cycles, the tumor growth inhibition reached 81% at day 32 with good tolerability (BWL_{max} = 14%) (Fig. 5). Antitumor efficacy of compound **4** was also evaluated orally in HCT116 xenograft model at 60 mg/kg, daily, given at day 1, 2, 3 × 2 cycles. In this case the compound exhibited moderate activity (TGI_{max} = 70%) with BWL_{max} = 13%.

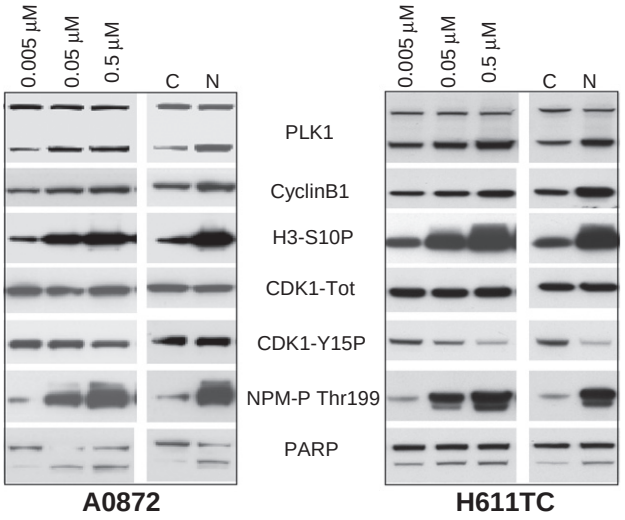


Figure 3. Western blot analysis of HCT116 and A2780 cells after 24 h treatment with three different doses of **4**.

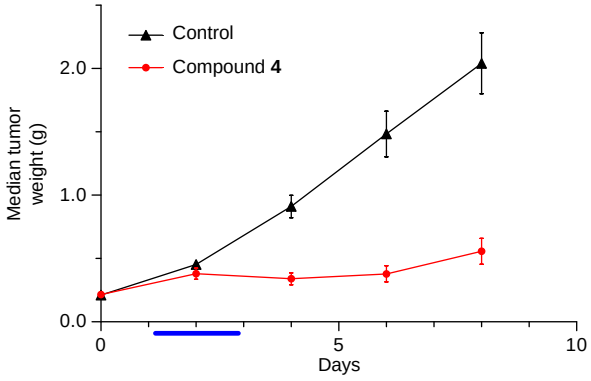


Figure 4. In vivo antitumor activity of **4** in A2780 xenograft model.

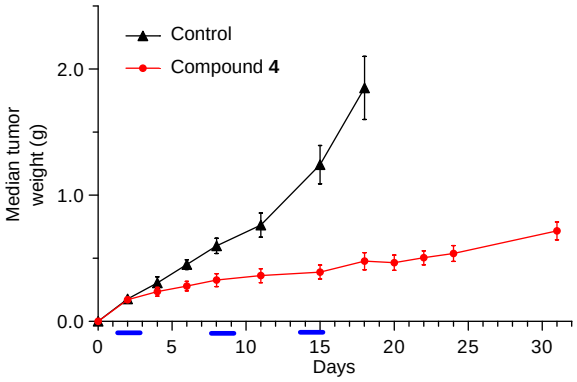


Figure 5. In vivo antitumor activity of **4** in HCT116 xenograft model.

In summary, we have reported the identification of a new PLK1 specific inhibitor with high potency on the target and demonstrated PLK1 mechanism of action in cells. Compound **4** showed good ADME properties suitable for moving it to in vivo efficacy experiments where it showed good activity in two different xenograft animal models with TGI_{max} ranging from 74% to 81% after iv administration. Furthermore, compound **4** exhibited a moderate activity also upon oral administration.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.060.

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16. The panel includes c-ABL, AKT1, Aur-A, Aur-B, CDC7, CDK1/B, CDK2/E, CDK4/D1, CDK5/P25, CHK1, EGFR1, ERK2, FGFR1, GSK3β IGF1R, IKK2, IR, JAK2, KIT, LCK, MAP-KAPK2, MET, MPS1, STK2, NIM, P38α, P38β, PAK4, PDK1, PKAα, PKCβ, RET, TAO3, TRKA, VEGFR2, VEGFR3 and, ZAP70.
17. Human liver microsomes was carried out as previously reported Ref. 12.
18. High-throughput solubility was carried out as previously reported Ref. 12.
19. The pharmacokinetic profiles of the compounds were investigated in overnight fasted male CD1 nu/nu mice following a single dose given intravenously (iv) or orally (po). Blood samples of each mouse were taken from saphenous vein at different time and after centrifugation plasma samples were analyzed by LC/MS/MS technique. For a more complete description see Ref. 12.