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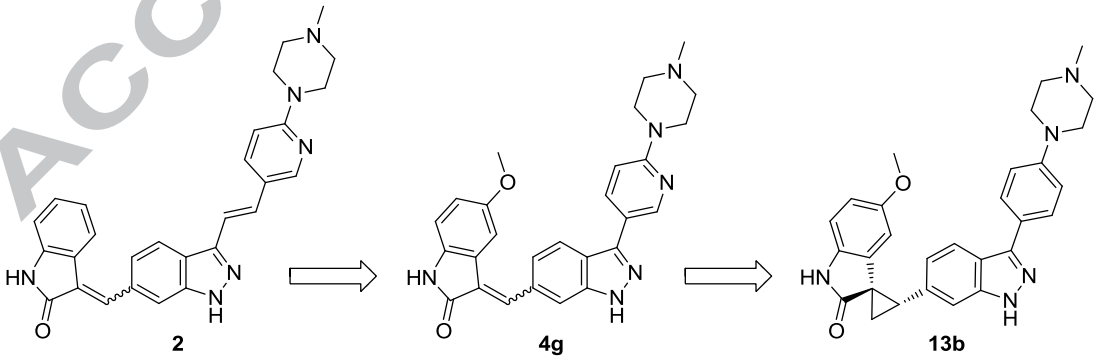
Keywords: Polo-Like kinase 4, PLK4 inhibitors, spiro[cyclopropane-1,3'-indolin]-2'-ones, (1H-indazol-6-yl)-methylene)indolin-2-ones, antitumor agent

Graphical abstract



Abstract

Previous efforts by our laboratory demonstrated that (*E*)-3-((3-(E)-vinylaryl)-1H-indazol-6-yl)methylene)-indolin-2-ones are potent PLK4 inhibitors with in vivo anticancer efficacy upon IP dosing. As part of a continued effort to develop selective and orally efficacious inhibitors, we examined variations on this theme wherein directly-linked aromatics, pendant from the indazole core, replace the arylvinyl moiety. Herein, we describe the design and optimization of this series which was ultimately superseded by (3-aryl-1H-indazol-6-yl)spiro[cyclopropane-1,3'-indolin]-2'-ones. The latter compounds are potent and selective inhibitors of PLK4 with oral exposure in rodents and in vivo anticancer activity. Compound **13b**, in particular, has a bioavailability of 22% and achieved a 96% tumor growth inhibition in an MDA-MB-468 xenograft study.

The family of Polo-like kinases (PLKs) comprises of five highly conserved serine/threonine protein kinases. They play a vital role in cell division and checkpoint regulation of mitosis.[1] Overexpression of PLKs found in human tumors coupled with their essential roles in cell division makes them potential anticancer targets.[2] In particular, PLK1 has been studied extensively with several inhibitors currently in clinical trials.[3] In contrast to PLKs 1–3, PLK4 is structurally divergent, containing only one polo-box domain instead of two.[4] PLK4 is essential in the centriole duplication pathway.[5,6,7] It has been shown that PLK4 is up-regulated in breast cancer, and overexpression of PLK4 is associated with a poor prognosis.[8,9,10] Hence, our laboratory is interested in the discovery of PLK4 inhibitors as novel therapeutic agents.[11]

Previously, we described PLK4 inhibitors based on an (*E*)-3-((1H-indazol-6-yl)methylene)-indolin-2-one core **1** (Figure 1); further elaboration culminated in highly potent

inhibitors such as **2.**[12] Subsequent optimization yielded a (1*R*,2*S*)-2-(1H-indazol-6-yl)spiro[cyclopropane-1,3'-indolin]-2'-one core[4] as that found in compound **3**.[13,14] Compounds **2** and **3** share a (*E*)-2-(6-(4-methylpiperazin-1-yl)pyridin-3-yl)vinyl moiety,[15,16] pendant from the indazole, that serves to fill a hydrophobic channel which terminates at the protein/solvent interface. Based on our PLK4 homology model (Figure 2),[17,18] we reasoned that "directly-linked" aromatics, such as the pyridin-3-yl moiety found in compound **4a**, would provide a viable alternative to populate the hydrophobic channel. Advantages to pursuing the directly-linked modification include reduced molecular weight and log P, and potentially enhanced selectivity profile and physicochemical properties. Specifically, we were interested in attenuating the off-target kinase activity which was observed in the early indazole based compounds[12] and improving the pharmacokinetic profile. Herein, we describe our efforts to develop potent and selective PLK4 inhibitors with optimized drug-like properties, starting from the (*E*)-3-((1H-indazol-6-yl)methylene)indolin-2-one core **1**.

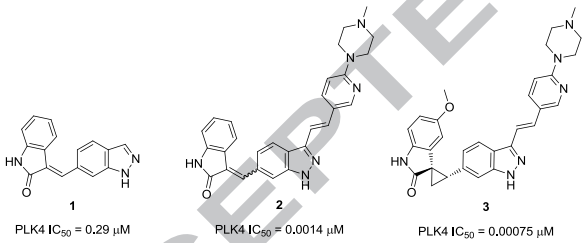


Figure 1. Structure of PLK4 Inhibitors.

Directly-linked analogs such as compound **4a** (*E*-isomer) were docked and scored in our previously described PLK4 homology model.[12] As was observed in previous docking experiments with the vinyl linked system, the Z-isomers scored poorly. For compound **4a** (*E*-isomer), hydrogen bonding interactions were observed between the indazole and the backbone carbonyl and NH of Glu90 and Cys92 respectively. Within the PLK4 active site, the indolinone heteroatoms are involved in hydrogen-bonding interactions to Lys41 and Asp154 (Figure 2A).

Leu89, the gatekeeper residue, is situated between the hinge region and the active site of PLK4. The pyridyl ring makes close contacts with the wall of the hydrophobic channel (Figure 2B).

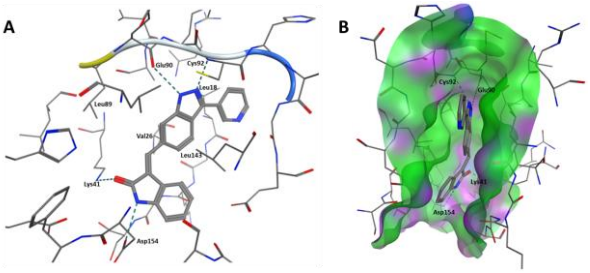
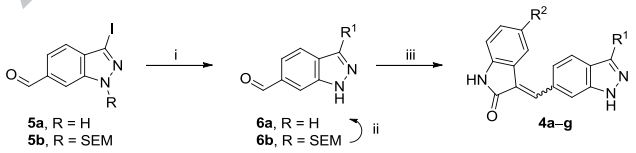


Figure 2. Binding mode of compound **4a** in the 3COK-based PLK4 homology model. Hydrogen bonds are shown as dashed lines. (A) H-bonds of the indazole moiety to Glu90 and Cys92 of the hinge region and H-bonds of the oxindole to Lys41 and Asp154. Leu89 is the gatekeeper residue of PLK4. (B) An orthogonal view of PLK4 active site surface with compound **4a**. The pyridyl moiety extends toward the enzyme/solvent interface, making hydrophobic interactions with the side chain of Leu18.

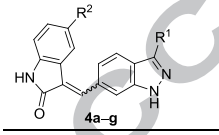
These observations prompted the synthesis of a number of directly-linked 3-((3-aryl-1H-indazol-6-yl)methylene)indolin-2-ones (Scheme 1). The compounds were prepared using precursors previously described in the preparation of arylvinyl compounds.[12] The heteroaryl moiety was installed via Suzuki-Miyaura cross coupling using either unprotected **5a** or SEM-protected 3-iodo-1H-indazole-6-carbaldehyde **5b**.[19] Removal of the SEM group, as necessary, with TBAF provided the aryl indazoles **6a**, which were subjected to Knoevenagel condensation with oxindoles to afford the desired PLK4 inhibitors **4a**–**g**.[20] Note that Knoevenagel condensation yielded a mixture of *E*- and *Z*-geometric isomers wherein the *E*-isomer was predominant.



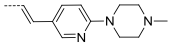
Scheme 1. Synthesis of 3-((3-aryl-1H-indazol-6-yl)methylene)indolin-2-one. Reagents and Conditions: (i) arylboronic acid pinacol ester, 10 mol% $PdCl_2(PPh_3)_2$, 7:1 DME/EtOH, 2 M aq. Na_2CO_3, 120 °C; (ii) TBAF, THF, 60 °C; (iii) oxindole, piperidine, MeOH, 60 °C.

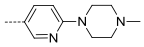
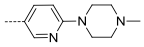
As shown in Table 1, the introduction of pyridin-3-yl group at the C3-position of indazole (**4a**) resulted in a 10-fold improvement in potency as compared to the unsubstituted lead compound **1**. The position of the nitrogen was important in that the 4-pyridyl analog **4b** was an order of magnitude less potent than the 3-pyridyl analog. Pyrrole substitution (**4c**) also enhanced potency vis a vis the core **1**, but indole **4d** and thiazole **4e** had reduced PLK4 potency, suggesting that size and electrostatic factors were important for optimal activity. Our binding model indicated that the increase in PLK4 activity was due to the hydrophobic interaction between the side chain of Leu18 and pyridyl ring (Figure 2).[12] The model, and results with the vinyl linked analogs, indicated that further extensions towards the solvent interface, e.g. polar and/or basic moieties, would be tolerated.[17,18] Accordingly, compound **4f**, with a (4-methylpiperazin-1-yl)pyridin-3-yl substituent, was prepared as a potent PLK4 inhibitor. In keeping with established SAR,[12] further increases in potency and selectivity were realized by incorporation of a methoxy group at the 5' position to give compound **4g**.

Table 1. In vitro activity and calculated physicochemical properties of 3-((3-aryl-1H-indazol-6-yl)methylene)indolin-2-one PLK4 inhibitors



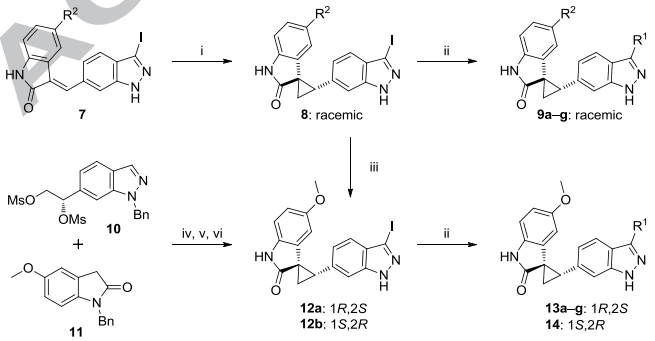
4a–g

Entry[a]	R^1	R^2	IC50 (μM)		GI50 (μM)
			PLK4	FLT3	MDA-MB-468
1	H	H	0.29	0.049	4.6
2		H	0.0014	0.013	0.022
4a		H	0.029	0.027	5.0
4b		H	0.22	0.12	9.9
4c		H	0.064	0.050	7.7

4d		H	0.54	ND	ND
4e[b]		H	0.49	0.072	ND
4f		H	0.013	0.017	1.3
4g		OMe	0.0024	0.028	1.3

[a]E/Z mixtures wherein E predominates; [b]E isomer.

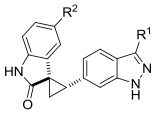
Analogs **4f** and **4g** exhibited good PLK4 potency while maintaining similar FLT3 selectivity as compound **2**; however, the enzyme inhibitory activity did not translate into cancer cell growth inhibitory activity. Both compounds showed modest inhibition of cell growth in human breast cancer cell lines. In addition, the inherent *E*/*Z*-isomerization of this class of inhibitors was a significant liability for future drug development.[12] Our approach to alleviate this problem was to replace the double bond linker with a bioisosteric cyclopropane ring.[13,14] Compounds were prepared in a convergent manner wherein the installation of the aryl moiety pendant from the indazole was the final step (Scheme 2). ((1H-Indazol-6-yl)-methylene)indolin-2-ones **7** were readily obtained via Knoevenagel condensation between oxindoles and 1H-indazole-6-carbaldehyde.[12,19,20] Subsequent cyclopropanation of **7** under Corey-Chaykovsky conditions[21] gave the desired major diastereomer **8**, which was readily separable from the minor diastereomer that did not possess inhibitory activity against PLK4.[13] Elaboration of the C3 position of the indazole was achieved by Suzuki-Miyaura cross coupling reaction to give the racemic **9** in moderate to good yields.



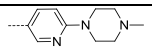
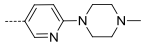
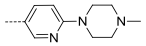
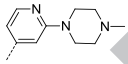
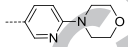
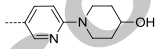
Scheme 2. Synthesis of racemic and optically active 2-(3-aryl-1H-indazol-6-yl)spiro[cyclopropane-1,3'-indolin]-2'-ones. Reagents and Conditions: (i) Me_3SOI, NaH, DMF, rt; (ii) arylboronic acid pinacol ester, 5 mol% $Pd(PPh_3)_4$, 2:1 $PhCH_3$/EtOH, 2 M aq. Na_2CO_3, 120 °C; (iii) chiral preparative SFC; (iv) NaH, THF, rt; (v) DMSO, *t*-BuOK, THF, O_2 (1 atm), rt; (vi) I_2, K_2CO_3, DMF, rt.

Application of the bioisostere strategy to one of the best compounds from Table 1, **4f** yielded compound **9a**. We were pleased to see that **9a** was a potent inhibitor of PLK4 with improved cancer cell growth inhibitory activity (Table 2). In addition, the replacement of the alkene with cyclopropane improved selectivity over FLT3. Compound **9a** constituted an excellent starting point for further optimization.

Table 2. In vitro activity and calculated physicochemical properties of racemic 2-(3-aryl-1H-indazol-6-yl)spiro[cyclopropane-1,3'-indolin]-2'-one PLK4 inhibitors



9a–g

Entry	R^1	R^2	IC50 (μM)		GI50 (μM)
			PLK4	FLT3	MDA-MB-468
9a		H	0.054	0.20	0.77
9b		F	0.0059	0.098	0.6
9c		OMe	0.0010	0.26	0.12
9d		OMe	0.0021	0.32	0.38
9e		OMe	0.0046	1.20	0.67
9f		OMe	0.0017	0.24	0.89

Previous studies showed that substitution of the oxindole ring in the 5'-position improved potency;[12] we explored the effect of this modification to the current series in more detail. As shown in Table 2, the replacement of 5'-hydrogen with fluorine and methoxy (**9b** and **9c** respectively) resulted in a marked improvement in potency against PLK4. Compound **9c**, the most effective, is a nanomolar inhibitor of PLK4 with over two orders of magnitude selectivity

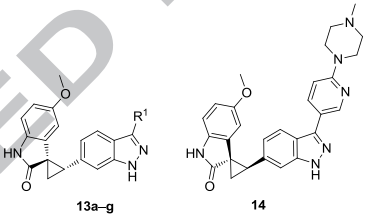
against FLT3 and potently attenuates the growth of MDA-MB-468 cells. Additional variations on this theme were explored; compounds **9d–f** were of similar potency and selectivity but none were superior to **9c** in the cell viability assay.

Our previous publication with styryl-1H-indazol-6-yl-spiro[cyclopropane-1,3'-indolin]-2'-ones has shown that the 1*R*,2*S* enantiomer is substantially more active against PLK4 than the 1*S*,2*R* enantiomer.[13,14] Hence, we decided to prepare enantiomerically pure adducts using the chemistry reported.[13,14] Enantiomerically pure spiro[cyclopropane-1,3'[3H]indol]-2'(1'H)-ones **12a** and **12b** were first isolated by preparative chiral SFC separations of racemic **8**, wherein R= OMe (Scheme 2). A stereoselective synthesis was subsequently developed.[14] The key reaction utilized a double S_N2 displacement of chiral dimesylate **10** with benzyl-protected oxindole **11** to give the protected spirocyclopropane with high enantiomeric excess (>98%). Subsequent oxidative debenzylation[22] followed by iodination gave the desired enantiomer **12a** in good yields. The final products (1*R*,2*S* stereochemistry) were obtained by Suzuki-Miyaura cross coupling reaction using conditions employed in the racemic synthesis. The 1*S*,2*R* enantiomer **14** was prepared similarly from **12b**.

Comparison of **13a** and **14** (Table 3) demonstrates a 40 fold increase in the PLK4 activity of the 1*R*,2*S* stereoisomer as compared to the much weaker 1*S*,2*R* stereoisomer. The former recapitulates the cell activity of the racemate **9c** whereas the latter showed much weaker inhibition of cancer cell proliferation. Subsequent analoging efforts were focused on the 1*R*,2*S* enantiomer. Although **13a** inhibited PLK4 potently it was not as effective as typical arylvinyl based analogs in the cell viability assay.[14] We postulated that the lower potency could be due to the moderate permeability of the compounds. To address this concern, we replaced the mildly basic and polar pyridine of **13a** with a phenyl moiety to yield **13b**. Both the lipophilicity (log D)

and polar surface area (PSA) of **13b** trended in the desired direction (Table 4); we were gratified to see that this replacement resulted not only in a marked improvement in cell potency, but also in a substantial enhancement in mouse oral exposure (Table 5). As anticipated, the replacement of pyridine with phenyl group had no adverse effect on CYP450 inhibition. To evaluate the kinase selectivity of these compounds as compared to their vinyl counterparts, PLK4 inhibitors **3**, **13a** and **13b** were profiled against a panel of 14 kinases at a screening concentration of 0.1 μM. We were pleased to see that replacement of vinyl aryl moiety with a "directly-linked" aryl group improved the kinase selectivity as illustrated by Figure 3. Compound **13b** was further evaluated against a large panel of kinases. Of the 274 kinases tested, only 15 were inhibited above 50% at a screening concentration of 0.1 μM.

Table 3. In vitro activity and calculated physicochemical properties of (1*S*,2*R*)- and (1*R*,2*S*)-5'-methoxy2-(3-aryl-1H-indazol-6-yl)spiro[cyclopropane-1,3'-indolin]-2'-one PLK4 inhibitors



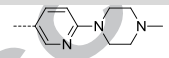
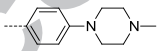
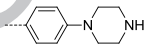
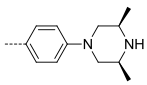
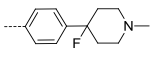
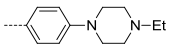
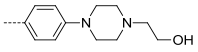
Entry	R^1	IC_{50} (nM)					GI_{50} (μM)		
		PLK4	FLT3	KDR	AURKA	AURKB	MDA-MB-468	MCF-7	HCC1954
4f		13	17	36	510	25	3.7	5.4	5.9
13a		0.69	97	14,000	>1,000	10	0.12	0.10	3.3
13b		0.94	45	9,400	>1,000	6.9	0.015	0.001	1.4
13c		0.45	44	5600	>1,000	6.9	<0.01	<0.01	3.6
13d		1.2	230	>5000	>1,000	13	0.0058	0.03	8.8
13e		1.5	200	ND	ND	ND	0.22	0.15	1.42
13f		1.6	62	ND	>1,000	8.5	0.031	0.0063	0.24
13g		1.2	77	ND	>1,000	7	0.068	1.7	3.0
14		30	6,500	>50,000	>1,000	2,100	3.7	5.4	5.9

Table 4. Calculated physicochemical data for selected PLK4 inhibitors[24]

	2	3	4g	13a	13b	13c	13d	13e	13f	13g
PSA	77	86	86	86	73	82	82	70	73	94
Log D at pH 7.4	4.5	4.2	3.5	3.3	4.0	2.6	3.4	4.0	4.8	4.0
LLE*	4.0	3.9	4.8	5.6	4.7	5.7	4.4	5.2	3.9	4.8

*LLE = pIC_{50} – clog P

Table 5. eADME and murine in vivo data for selected PLK4 inhibitors

Entry	CYP_{450} % inh at 1 μM	Microsomal $t_{1/2}$ (min)	Mouse PK (PO 25 mg/kg)		Rat PK (IV 1 mg/kg)			Rat PK (PO 5 mg/kg)	
	2C9/2C19/3A4	mouse/ human	C_{max} (μg/mL)	AUC (μg·h/mL)	CL (L/h·kg)	$t_{1/2}$ (h)	AUC (μg·h/mL)	AUC (μg·h/mL)	F%
2	67/83/55	4/24	< LOD	ND	6.4	5.1	0.17	< LOD	<1
3	73/93/76	9/16	ND	ND	3.1	2.4	0.33	0.25	15
4g	60/64/19	4/13	ND	ND	10.7	1.3	0.094	0.024	2
13a	34//30/6	8/20	0.071	0.14	3.8	1.6	0.27	0.14	10
13b	35/33/3	9/19	0.71	1.83	4.1	1.3	0.27	0.26	22
13c	ND	>60/59	< LOD	ND	ND	ND	ND	ND	ND
13d	44/54/-3	43/>60	0.11	0.17	ND	ND	ND	ND	ND
13f	37/60/6	15/>60	1.1	3.0	3.3	1.6	0.31	0.31	11
13g	41/28/11	28/>60	0.25	0.27	4.6	1.4	0.22	0.13	11

<LOD: below limit of detection; ND: not determined.

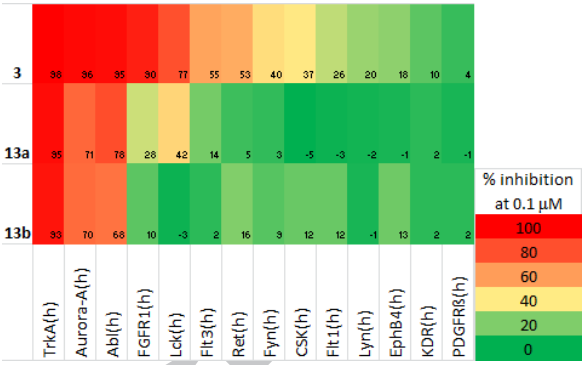


Figure 3. Heat map showing % inhibition of kinases at a screening concentration of 0.1 μM by PLK4 inhibitors **3**, **13a**, and **13b**.

Modifications of the solubilizing piperazine ring were undertaken with the view to scanning physiochemical properties in hopes of identifying a compound with improved ADME properties. We expected potency and selectivity would be maintained since modifications were limited to the solvent exposed region of the molecules. To assess selectivity these new PLK4 inhibitors were subjected to profiling against four other kinases, namely FLT3, AURKA,

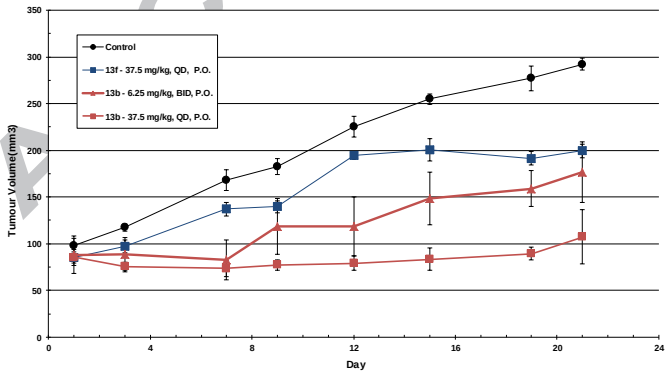
AURKB and KDR. Subsequently, compounds of interest were moved into rodent PK studies. Removing the *N*-methyl substituent attached to piperazine resulted in a potent PLK4 inhibitor **13c** with much improved microsomal stability (Table 5). The disubstituted piperazine **13d** with higher lipophilicity was prepared to address the potential loss in permeability of the desmethyl analogs (Table 4);[23] potency and selectivity against FLT3 and AURKB were maintained. It should be noted that the spiro[cyclopropane-1,3'[3H]indol]-2'(1'H)-ones (**13a**–**d**, **14**) all showed excellent selectivity against AURKA and KDR. In fact, the KDR selectivity appeared to be driven by the bioisosteric replacement of the alkene with the cyclopropane linker; consequently KDR was dropped from our routine selectivity panel. Replacing the piperazine with a 4-fluoro piperidine gave a potent and selective inhibitor **13e** with a mildly basic solubilizing element (pKa = 7.7).[24] However this compound had modest cancer cell growth inhibition. Replacing the terminal methyl with ethyl (**13f**) or hydroxyethyl (**13g**) resulted in compounds with similar PLK4 potency and somewhat attenuated kinase selectivity against FLT3 and AURKB. Nevertheless, both compounds showed improved human microsomal stability (Table 5) and were taken into mouse PK studies.

Our ultimate goal was to develop an orally efficacious PLK4 inhibitor. The beneficial effects of the alkene to cyclopropane transformation are made clear by comparison of compounds **4g** and **13a**. Aside from imparting configurational stability, **13a** exhibits improved ligand-lipophilicity efficiency, cell activity, KDR selectivity and reduced CYP inhibition (Tables 4 & 5). Moreover, a 5-fold increase in rat bioavailability was observed. Further improvements in exposure were seen upon conversion of the pyridyl to phenyl ring. Compound **13b** demonstrated an order of magnitude increase in mouse oral exposure vis a vis **13a**, although the increase in rat bioavailability was modest. The desmethyl analog **13c** had much improved microsomal stability

in mouse but poor oral exposure. The addition of flanking methyl groups only marginally improved the oral exposure (**13d**). The methyl and ethyl analogs (**13b** and **13f**) possessed the best combination of properties and hence were selected for in vivo efficacy evaluation.

The in vivo antitumor activity of compounds **13b** and **13f** was examined with mouse xenograft studies using the MDA-MB-468 human breast cancer cell line.[25,26] Two dosage regimens (6.25 mg/kg BID and 37.5 mg/kg QD) were chosen for the first xenograft study with compound **13b**. Compound **13b** were administered orally to mice for 21 days, and the observed tumor growth inhibition is summarized in Figure 4. A dose response was seen and compound **13b** showed the best response at 37.5 mg/kg QD with a 96% inhibition of tumor growth at day 21. Based on this encouraging result, a high dose of compound **13f** was used in a subsequent xenograft study. Compound **13f** was also orally efficacious, albeit to a lesser extent, with a 48% tumor growth inhibition. Both compounds were tolerated during the course of the experiment with less than 20% decrease in body weight during the course of the study. The promising results from in vivo studies provide a strong basis for further development of PLK4 inhibitors as antimitotic agents.

Figure 4. Tumor volume vs days of treatment with compounds **13b** and **13f** in an MDA-MB-468 mouse xenograft model (PO dosing)*



*Results are expressed as mean ± standard error (n = 8 per group); p < 0.06 compared to treatment with vehicle.

The use of "directly-linked" aromatic moieties was investigated in the quest of improving the drug-like properties of PLK4 inhibitors with the (E)-3-((1H-indazol-6-yl)methylene)indolin-2-one scaffold. Computational modelling suggested such modification should be well tolerated, and this work led to nanomolar PLK4 inhibitors. Cell growth inhibitory activity and kinase selectivity were further enhanced by replacing the alkene with a bioisosteric cyclopropane ring. Marked improvement in oral mouse exposure was achieved by substituting a pyridyl with a phenyl moiety, pendant from the indazole. Further optimization led to the discovery of compounds **13b** and **13f**. Both compounds are orally efficacious in inhibiting tumor growth in xenograft studies. Compound **13b**, for instance, resulted in a 96% inhibition of tumor growth in an MDA-MB-468 triple-negative breast cancer xenograft model. The results reported herein illustrate the potential of this class of compounds for cancer therapy.

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