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Presentation Abstract

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Abstract Body:	Polo-like kinase 1 (Plk1) is one of the emerging new generation of anti-mitotic targets. Plks are a family of serine-threonine kinases involved in the regulation of mitosis and maintenance of DNA integrity. Plk1 is required for almost every step of mitosis: promotes mitotic entry, contributes to centrosome maturation, regulates kinetochore assembly, contributes to spindle assemble checkpoint and initiates mitotic exit. Besides promoting proliferation, Plk1 overexpression contributes to oncogenesis by promoting chromosome instability and aneuploidy. Overexpression of Plk1 is associated with tumor development and many cancers have elevated Plk1 levels compared to surrounding normal tissues. Numerous studies have shown that Plk1 expression levels correlate with disease progression, invasiveness and poor patient prognosis. Furthermore, pre-clinical studies have demonstrated that cancer cell proliferation is blocked in vitro and in vivo via inhibition of Plk1 using siRNA or small molecule inhibitors. Collectively, these observations support the selection of Plk1 as an attractive target for cell cycle-directed cancer therapy and several small molecule inhibitors are currently being investigated in clinical trials. Using a de novo ligand design approach we have identified pyrimidodiazepinone small molecules, which are ATP-competitive, highly selective and potent Plk1 inhibitors. These molecules have good drug-like properties, oral bioavailability and efficacy in mouse xenograft models. To characterize the cell sensitivity toward the Plk1 inhibitors we have used washout and outgrowth assays in a panel of oesophageal, lung and colon cancer cell lines. The anti-proliferative activity of Plk1 inhibitors was correlated with the Plk1 expression level, p53 and Ras mutational status. Generally, we found that cell lines harbouring p53 mutations are more sensitive towards these inhibitors. Time-lapse microscopy and flow cytometry studies of RKO cell line (WT p53) treated with Plk1 inhibitors demonstrated that a proportion of cells arrested just before the G2-/-M transition and were able to resume normal proliferation upon compound removal. These results suggest that Plk1 inhibitors should have better therapeutic window when used for treatment of tumors with mutant p53, p53 mutational status could be used as a patient stratification marker, and tumor types with high frequency of p53 mutations, such as oesophageal cancer, could be suitable therapeutic indications for Plk1 inhibitors.

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Potent and selective small molecule inhibitors of Polo-like kinase 1: Biological characterization

Abstract #2814

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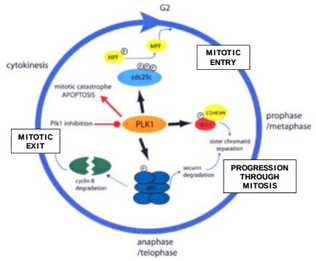
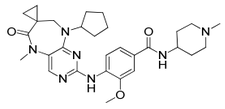
Introduction

Plk1, a serine / threonine kinase, is a key regulator of cell division controlling mitotic entry, bipolar spindle formation and mitotic exit.[1]



- Plk1 is frequently overexpressed in cancer[2]
- Level of expression correlates with aggressiveness and has prognostic value for predicting outcome[3-5]
- Cultured cells can be transformed by Plk1 overexpression and these cells can induce tumors in nude mice[6]
- Cancer cell proliferation is blocked *in vitro* and *in vivo* by small-molecule Plk1 inhibitors and Plk1 antisense / siRNA[7]. Plk1 inhibitors cause mitotic arrest and subsequent induction of apoptosis in cancer cells[8]
- Clinical Plk1 inhibitors have shown a small therapeutic window. Predictive selection marker will be critical for successful development of these agents
- Tumor sensitivity towards Plk1 inhibition has been related to TP53 mutations[9,10]
- Using *de novo* ligand design approach we have generated a pyrimidodiazepinone scaffold. Compound 1 is an early lead from this chemical series[11]. Compound 4 has been selected as development candidate following optimization for drug-like properties
- Here we present the biological characterization of Compound 4. Cellular phenotype was characterized in HeLa and RKO cell lines. Cellular sensitivity was studied in a panel of oesophageal (OE) cell lines with differing TP53 status
- Oesophageal cancer is one of the leading causes of death from cancer worldwide and a highly unmet medical need. 50 – 80% of OE tumors have mutant TP53, which is a major cause of chemo- and radioresistance. These mutant TP53 tumors may be sensitive to Plk1 inhibitors

Lead Compounds



Compound 1

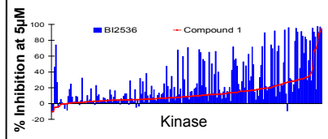
Compound 1 is an early lead from pyrimidodiazepinone chemical series. The related Compound 4 was selected as development candidate following optimization for drug-like properties

During lead optimization, physicochemical and ADME properties were tuned through variation in the solvent exposed regions of the inhibitor. Leads were optimized for solubility, cellular activity and pharmacokinetic profiles[11]. Values for selected compounds are given below.

	Cmpd 2	Cmpd 3	Cmpd 4
Mitotic induction activity ($PHH3\ EC_{50}$)	32 nM	15 nM	17 nM
Inhibition of 5 major CYP450 (IC_{50})	> 25μM	12-20 μM	>25 μM
hERG inhibition (IC_{50})	21 μM	6 μM	>30 μM
Human plasma protein binding	91%	77%	83%
Human microsomal half-life	34 min	65 min	49 min
PK: T½ mouse oral (λ_z)	1.1 h	3.9 h	2.5 h
Caco-2 (Efflux ratio B2A/A2B)	4.6	0.22	1.16
Interaction with MDR1	Pgp substrate	Possible potent Pgp inhibitor	No Pgp binding
Oral xenograft efficacy	100 mg/kg bid	40 mg/kg qd	40-67 mg/kg qd
Best activity – PO dosing	6% T/C (Regression)	9% T/C (Stabilization)	0% T/C (Cure)

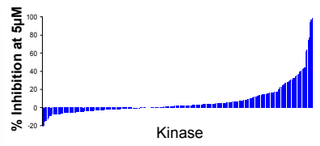
Compound 4 Characterization

Compound 1 selectivity was tested in 216 kinase panel at 5 μM. A high degree of selectivity for Plk1 was observed. Compound 1 was more selective for Plk1 inhibition than BI2536



Kinase	IC_{50} (μM) (Ratio vs. PLK1)	
	Compound 1	BI2536
Plk1	0.014 (1x)	0.007 (1x)
Plk2	0.231 (17x)	0.020 (3x)
Plk3	0.206 (15x)	0.011 (1.5x)

Compound 4 is a highly selective Plk1 inhibitor. Kinase selectivity was tested in a 283-kinase panel at 5 μM. IC_{50} values were determined against the kinases inhibited by more than 50%.

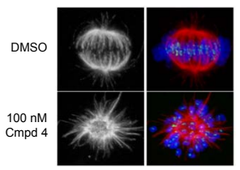


Kinase	Compound 4 IC_{50} (μM)	Ratio vs. PLK1
Plk1	0.003	1
Plk2	0.155	52
Plk3	0.297	99
EGFR(L858R)	1.32	440
CaMK2δ	1.63	543
EGFR(d746-750)	2.38	793
DAPK1	2.86	953

Low nM anti-proliferative activity of Compound 4, observed across a broad range of tumor cell lines in a standard 72-h cytotoxicity assay.

Cell line	Origin	IC_{50} (nM)	Cell line	Origin	IC_{50} (nM)
DU145	Prostate	39 ± 6	LU99A	Lung	33 ± 7
PC3	Prostate	48 ± 0	A2780	Ovary	7 ± 2
RKO	Colon	14 ± 9	MCF7	Breast	8 ± 7
Colo-205	Colon	2 ± 1	MesSa	Uterus	14 ± 2
A549	Lung	21 ± 7	HEL	Leukemia	34 ± 11
ABC-1	Lung	38 ± 9	HL60	Leukemia	53 ± 15
NCI-H23	Lung	34 ± 5	Jurkat	Leukemia	24 ±10
NCI-H1299	Lung	13 ± 3	K562	Leukemia	25 ± 14
NCI-H2122	Lung	34 ± 5	THP-1	Leukemia	49 ± 11

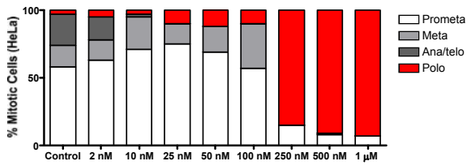
Treatment of proliferating cells with Compound 4 results in accumulation of cells in mitosis and increase in the proportion of mitotic cells with monopolar spindles, consistent with Plk1 inhibition.



HeLa cells were treated with compound for 24 h then fixed and stained with antibodies against α-tubulin (red) and the centromeric marker CREST (green). DNA was stained with DAPI (blue). Images were acquired by confocal microscopy at 100x.

	DMSO control	250 nM Cmpd 4
% Mitotic cells (n>250 cells/field)	2.4%	69.0%
% of mitotic cells with **monopolar spindles** (n=50 mitotic cells)	4%	74%

HeLa cells were treated for 2 h then fixed and stained with antibodies against S10-phospho-histone H3 as a mitotic marker and α-tubulin to determine spindle morphology. Mitotic cells with monopolar spindles (Polo) accumulate in a concentration dependent manner



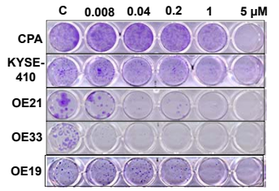
Cellular Sensitivity

Cellular sensitivity towards Compound 4 was studied in a panel of OE cell lines – benign Barrett's (CPA), oesophageal squamous cell carcinoma (ESCC; KYSE-410 and OE21) and Barrett's adenocarcinoma (BAC, OE33 and OE19). All four cancerous cell lines have mutant TP53 and two of them (OE33 and OE21) have non-functional p53[12].

Characteristic	CPA	KYSE-410	OE21	OE33	OE19
Type	Benign Barrett's	ESCC	ESCC	BAC	BAC
TP53 mutation	WT	c.1009C>T	c.269C>T c.270delC	c.404G>A	c.929dupA
p53 amino acid change	Nil	R337C	S90fs31X	C135Y	N310K Frame
p53 function	Yes	Yes	No	No	Yes

OE cell lines, seeded at 2.5 x 10^3 in 24-well plates, were treated for 24 h with Compound 4. Cells were then grown in drug-free media for additional 12 days, fixed and stained with 0.4% crystal violet solution.

- The ESCC and BAC cell lines are more sensitive towards the PLK1 inhibitor than the benign Barrett's cell line.
- The highest anti-proliferative potency of Compound 4 is detected in the cell lines lacking functional p53 i.e. OE33 and OE21



Cmpd4 IC_{50} (μM)

2.46 ± 2.33

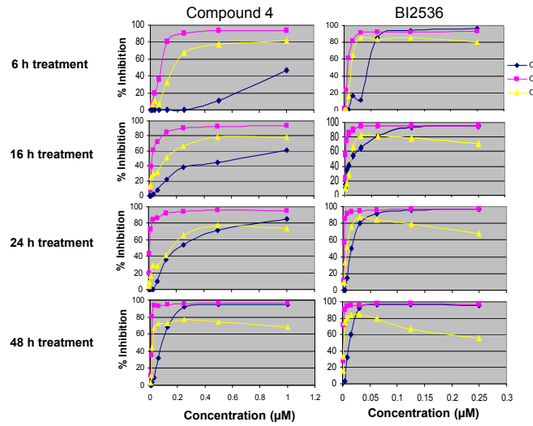
0.23 ± 0.21

0.010 ± 0.009

0.005 ± 0.003

0.061 ± 0.030

OE cell lines, seeded in 96-well plates, were treated for 6, 16, 24 or 48 h with Compound 4 or BI2536. Cells were then grown in drug-free media and the cell viability evaluated at 144 h using a resazurin assay.



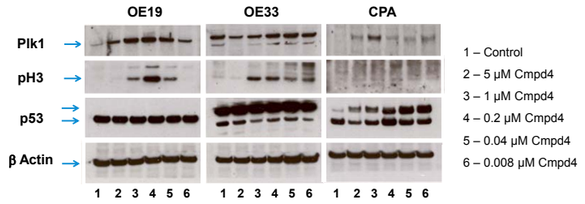
Treatment time	Cell line	IC50 (μM) Cmpd4	BI2536
6 h	CPA	>1	0.045
	OE33	0.069	0.007
	OE19	0.178	0.013
16 h	CPA	0.590	0.022
	OE33	0.020	0.002
	OE19	0.145	0.01
24 h	CPA	0.372	0.018
	OE33	0.009	0.002
	OE19	0.149	0.007
48 h	CPA	0.128	0.014
	OE33	0.009	0.001
	OE19	0.018	0.003

- Compound 4 inhibits more potently the proliferation of the two BAC cell lines (OE33 and OE19) than the benign OE cell line (CPA).
- 6 h treatment provides the best differentiation between cancerous and non-cancerous cells lines.
- BI2536 showed less differentiation between the BAC and the benign cell lines.
- Biphasic growth inhibition curve was observed for OE19 (mutant functional p53) with lower inhibition at high compound concentrations.

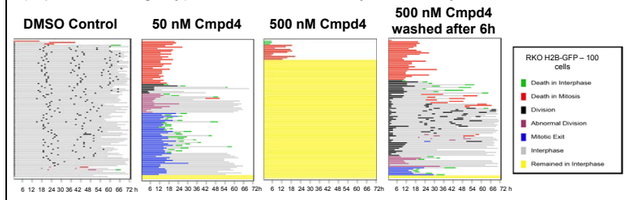
Cellular Mechanism of Action

OE cell lines were treated for 24 h with different concentrations of Compound 4. Cell lysates were subjected to SDS-PAGE, Western blotting and immunodetection.

- Highest level of Plk1 in OE33 cell line (non-functional p53).
- Compound 4-induced accumulation of Plk1 in OE19 and CPA (functional p53) but not in OE33.
- Plk1 levels do not strictly follow the change of mitotic index (phospho-Ser10 Histone H3 used as a marker).
- Biphasic induction of mitotic cell accumulation in OE19, which peaks at 0.2 μM. Higher concentrations have weaker effect.



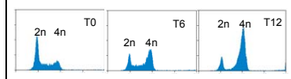
Concentration dependent cell cycle effects of Compound 4 were studied in RKO colon carcinoma cell line (WT p53) using time-lapse imaging and FACS analysis. Cells were blocked in thymidine for 16 h. Compound 4 was added 4 h after thymidine release. Imaging was performed using a Pathway Bioimager 855 (BD Biosciences) and a 20×/0.30 UPlan FLN objective. Images were collected every 5 min using a 0.1 s exposure, and image sequences were viewed using NIH ImageJ software (http://rsbweb.nih.gov/ij/). Cell behaviour was analysed manually.



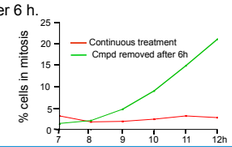
FACS analysis of RKO cells treated with 500 nM Compound 4. Cells stained with anti-phospho-histone H3 Ser10 antibody and propidium iodide.

- 50 nM Compound 4 causes mitotic arrest and rapid cell death in mitosis or shortly after mitotic exit.
- In contrast, large proportion of the cells treated with 500 nM Compound 4 were arrested in G2 and did not die during the experiment (72 h). Compound removal after 6 h allowed the cells to enter mitosis; ~ 25% of the cells recovered and resumed normal proliferation.

DNA content. Continuous treatment with 500 nM Compound 4.



Mitotic index. 500 nM Compound 4 washed after 6 h.



Summary

- **Potent and highly selective inhibitors of the mitotic kinase Plk1 have been identified. Compound 4 has good drug-like properties and has been selected for further development**
- **TP53 mutational and functional status correlate with sensitivity towards Compound 4. Cells with functional p53 are able to arrest in G2, accumulate Plk1 and partly recover from the treatment**
- **Oesophageal cell lines with deficient p53 are exquisitely sensitive towards Compound 4. Short treatment (6 h) provided the best therapeutic window. PK properties of Compound 4 will allow such treatment regimen**
- **Oesophageal cancer is a target therapeutic area for Compound 4 and p53 mutational and functional status could be used as a predictive biomarker**

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