

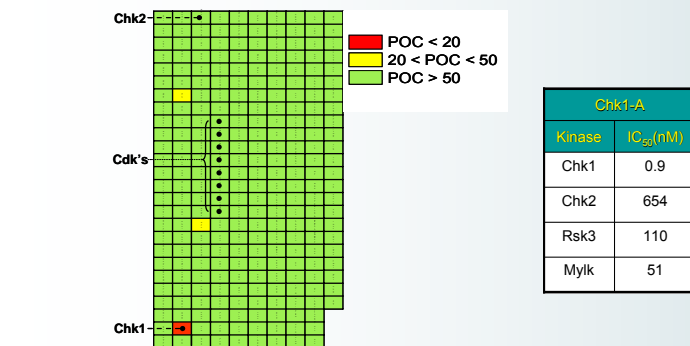
Introduction

Chk1 is a serine/threonine kinase that plays important roles in the cellular response to genotoxic stress. For this reason, there is a great deal of interest in using inhibitors of Chk1 to potentiate the effects of DNA-damaging chemotherapeutics. In addition, multiple studies have demonstrated that Chk1 activity is essential during an unperturbed cell cycle to ensure proper DNA replication and maintain genomic integrity. Therefore, it is plausible that a Chk1 inhibitor could also be efficacious as a single-agent therapeutic for human cancer.

Here we show that single-agent treatment with Chk1-A, a potent and selective small molecule inhibitor of Chk1, alone is anti-proliferative in human leukemia cell lines. Employing HEL92.1.7 cells, a line particularly sensitive to Chk1-A, we sought to understand the mechanisms by which Chk1 inhibition derives the anti-proliferative effect. Chk1-A treatment resulted in S-phase accumulation and induction of several biochemical markers of DNA damage and cell-cycle checkpoint activation. Furthermore, the anti-proliferative effect correlated with the induction of apoptosis but was not associated with pre-mature entry into mitosis. *In vivo*, we found that HEL92.1.7 tumor xenografts were sensitive to oral administration of Chk1-A at a dose that was well tolerated. Together, these studies suggest that inhibition of Chk1 results in DNA damage that induces apoptosis and that use of a Chk1 inhibitor as a single-agent could be an effective strategy to treat certain types of human cancers.

Results

Figure 1: Chk1-A is selective against a broad kinase panel



Selectivity Screen In a panel of 257 kinases, Chk1-A [0.1 μM] is highly selective for Chk1, with no significant Chk2 inhibition and no activity against other kinases involved in checkpoint control, cell cycle progression, or apoptosis.

Figure 2: Chk1-A exhibits low nanomolar cellular activity

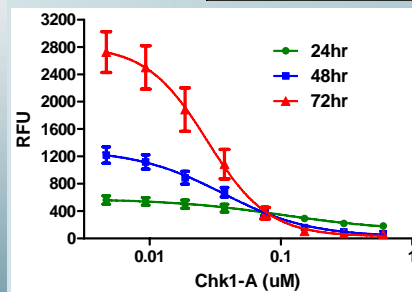
IC ₅₀ (nM)	
Auto-pChk1 (S296) inhibition	11
Checkpoint abrogation (pHH3)	25

Cellular Activity Assays For Chk1 serine-296 autophosphorylation inhibition assay, HT-29 colon carcinoma cells were treated for 30min with a dose range of Chk1-A and then treated with 100nM camptothecin for 2.5hr. Cells were then harvested and lysates were analyzed by Western blot. For checkpoint abrogation assay, HT-29 cells were treated with 100nM camptothecin for 16hr and then treated with a dose range of Chk1-A for 5hr. Induction of phosphorylation on serine-10 on histone H3 (mitotic marker) was then analyzed via an indirect immunofluorescence-based method using a LI-COR Aeries imager.

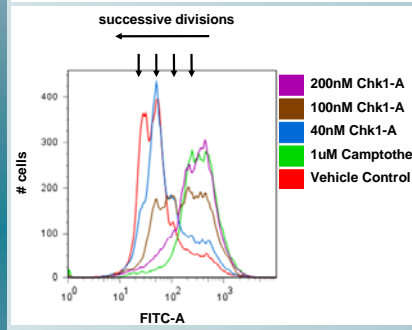
Results

Figure 3: Chk1-A is anti-proliferative as a single-agent in leukemia cells *in vitro*

Leukemia Cell Line	72hr Proliferation IC ₅₀ (nM)
HEL92.1.7	27
MV411	126
Molm13	39
K562	230

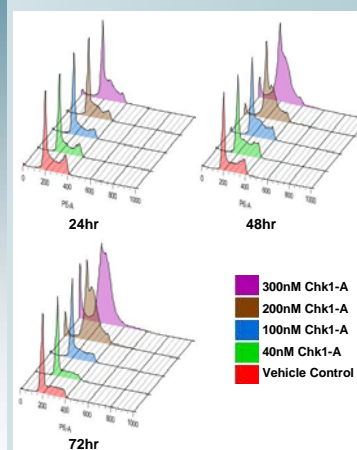


Proliferation Assay HEL92.1.7 cells were seeded at 4,000 cells/100 μL in 96-well plates and then immediately treated with a dose range of Chk1-A. Proliferation was determined at 24, 48, and 72hr using the CellTiter-Blue cellular viability assay (Promega). Values represent the mean ± S.E. (n=4 for 24 and 48hrs, n=5 for 72hrs).



CFSE Cell Division Assay HEL92.1.7 cells were synchronized with 300nM nocodazol for 16hr and then released and immediately stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen). Cells were then treated with a dose range of Chk1-A or 1uM camptothecin for 72hr, at which point they were analyzed by flow cytometry. Each successive peak on the histogram (from right to left) represents a division of the signal by 2 and thus 1 round of cell division.

Figure 4: Chk1-A induces S-phase accumulation and an eventual sub-G1 population in HEL92.1.7 cells

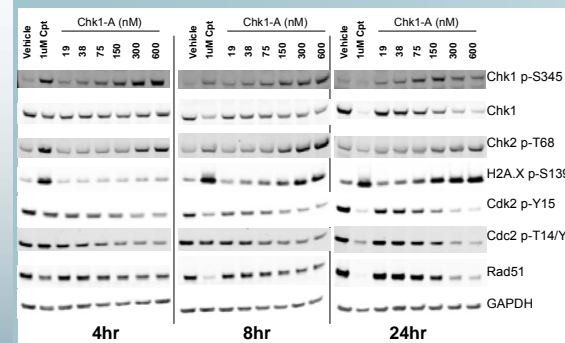


Cell Cycle Analysis HEL92.1.7 cells were treated with a dose range of Chk1-A for 24, 48, or 72hr then fixed with EtOH and stained with propidium iodide (BD Pharmingen). Cell cycle profile was determined by flow cytometry.

	24hr, 48hr, 72hr				
	%<G1	%G1	%S	%G2	%>G2
vehicle	3, 1, 3	51, 53, 57	26, 27, 29	20, 18, 10	1, 2, 2
40nM Chk1-A	3, 2, 2	54, 49, 62	26, 31, 25	15, 15, 8	2, 4, 4
100nM Chk1-A	2, 3, 3	57, 48, 54	27, 34, 29	12, 13, 11	3, 4, 4
200nM Chk1-A	4, 7, 10	50, 41, 32	31, 39, 51	12, 9, 7	3, 4, 4
300nM Chk1-A	7, 13, 15	46, 38, 28	36, 42, 53	9, 5, 2	3, 3, 3

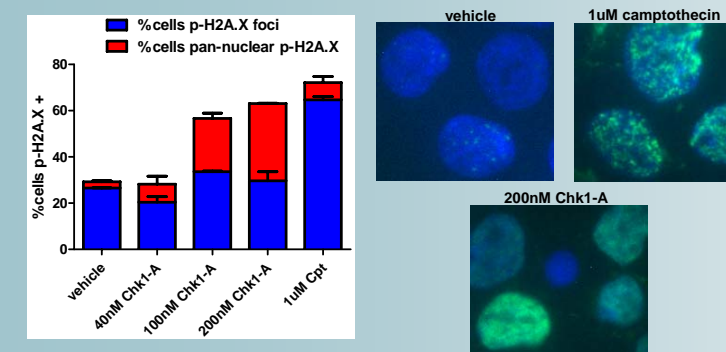
Results

Figure 5: Chk1-A induces activating phosphorylation of DNA damage response / cell-cycle checkpoint proteins and reduces inhibitory phosphorylation on cyclin-dependent kinases in HEL92.1.7 cells



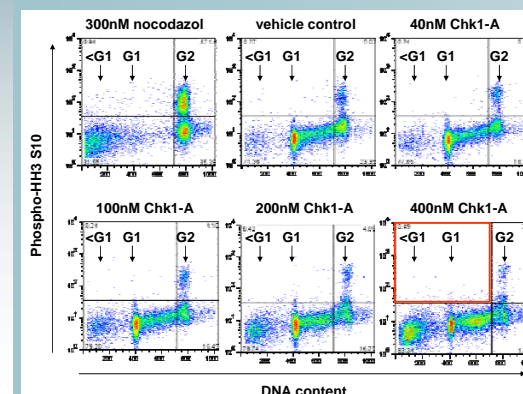
Western Analysis HEL92.1.7 cells were treated with a dose range of Chk1-A or 1uM camptothecin for 4, 8, or 24hr. Harvested cells were then lysed with RIPA buffer and cleared lysates were analyzed by Western blot using commercially available antibodies.

Figure 6: Chk1-A induces a primarily pan-nuclear distribution as opposed to nuclear foci of p-H2A.X in HEL92.1.7 cells



Immunofluorescent Analysis of H2A.X Phosphorylation HEL92.1.7 cells were synchronized with 300nM nocodazol for 16hr then released into media containing Chk1-A or 1uM camptothecin. Cells were harvested 14hr later (time corresponding to late S-phase), spun onto microscope slides with a Cytospin, and then fixed with 4% paraformaldehyde followed by MeOH. Cells were then stained with an antibody to H2A.X phospho-serine-139 (Millipore) and Hoechst 33342 DNA stain (Invitrogen). Images were acquired by fluorescent microscopy using a 60X objective. Experimenter was blinded to conditions during scoring (graph at left). For the representative images (right) p-H2A.X is green and DNA is blue.

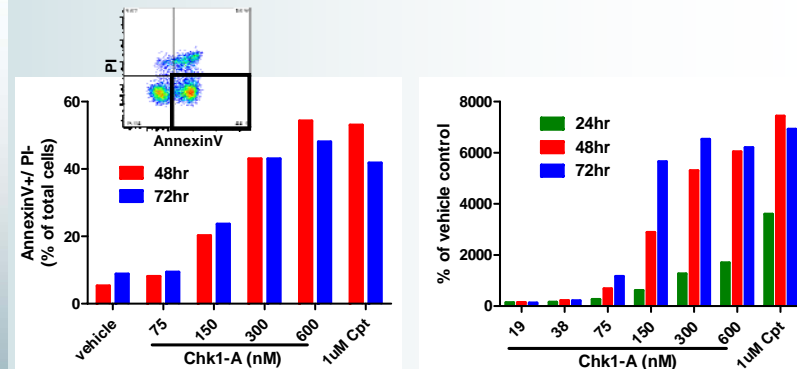
Figure 7: Chk1-A treatment does not lead to pre-mature mitosis in HEL92.1.7 cells



pHH3 and PI Staining HEL92.1.7 cells were treated with a dose range of Chk1-A or 300nM nocodazol (positive control for pHH3) for 24 hours and then fixed in EtOH. After fixation, cells were stained with an antibody to histone H3 phospho-serine-10 (Millipore) and then propidium iodide (BD Pharmingen). pHH3 positivity and DNA content were then determined by flow cytometry.

Results

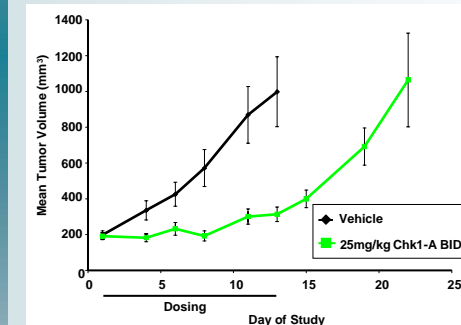
Figure 8: Chk1-A induces apoptosis in HEL92.1.7 cells



AnnexinV Apoptosis Assay HEL92.1.7 cells were treated with a dose range of Chk1-A or 1uM camptothecin for 48 or 72hr. Cells were then stained using Annexin-V-FLOUS kit (Roche). Stained cells were analyzed by flow cytometry. Graphed values represent the percentage of cells positive for AnnexinV staining but negative for propidium iodide staining (n=1). Inset is an example demonstrating the analyzed population.

Caspase 3/7 Assay HEL92.1.7 cells were seeded at 4,000 cells/100 μL in 96-well plates and then treated with a dose range of Chk1-A or 1uM camptothecin. Caspase 3/7 activity was determined at 24, 48, and 72hr using the Caspase-Glo 3/7 assay (Promega). Raw assay output was normalized to an approximation of cell number as determined by CellTiter-Blue cellular viability assay (Promega) (n=1).

Figure 9: Chk1-A inhibits growth of HEL92.1.7 tumor xenografts *in vivo*



In Vivo TGI Female SCID-Beige mice bearing growth-staged HEL92.1.7 tumors were administered Chk1-A (25 mg/kg PO, BID) for 13 days as indicated. This dosing regimen was well tolerated with minimal body weight loss over the course of the study.

Treatment	% Mean Regression	Max % Body Weight Loss	% TGI	Growth Delay (Days)
Vehicle	0	3.7, day 8	-	-
25 mg/kg Chk1-A	4.6, day 4	5.7, day 13	68.6	12.7

All *in vivo* studies were performed in accordance with IACUC guidelines and in harmony with the Guide for Laboratory Animal Care and Use.

Conclusions

- Chk1-A, a potent and selective inhibitor of Chk1, is anti-proliferative as a single-agent in leukemia cells *in vitro* and inhibits the growth of HEL92.1.7 tumor xenografts *in vivo*
- Inhibition of proliferation correlates with S-phase accumulation, induction of biochemical markers of DNA damage, and apoptosis - suggests that Chk1 inhibition produces lethal DNA lesions during replication
- Although Chk1-A treatment reduces inhibitory phosphorylation of Cdc2, it does not result in histone H3 phosphorylation in cells with <4N DNA content - suggests that apoptosis is not due to pre-mature mitotic entry
- Single-agent Chk1 inhibition may be an effective treatment strategy for certain cancers