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Cyclin B1 is an efficacy-predicting biomarker for Chk1 inhibitors

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Abstract

Chk1 is the major mediator of cell-cycle checkpoints in response to various forms of genotoxic stress. Although it was previously speculated that checkpoint abrogation due to Chk1 inhibition may potentiate the efficacy of DNA-damaging agents through induction of mitotic catastrophe, there has not been direct evidence proving this process. Here, through both molecular marker and morphological analysis, we directly demonstrate that specific downregulation of Chk1 expression by Chk1 siRNA potentiates the cytotoxicities of topoisomerase inhibitors through the induction of premature chromosomal condensation and mitotic catastrophe. More importantly, we discovered that the cellular cyclin B1 level is the major determinant of the potentiation. We show that downregulation of cyclin B1 leads to impairment of the induction of mitotic catastrophe and correspondingly a reduction of the potentiation ability of either Chk1 siRNA or a small molecule Chk1 inhibitor. More significantly, we have extended the study by examining a panel of 10 cancer cell-lines with different tissue origins for their endogenous levels of cyclin B1 and the ability of a Chk1 inhibitor to sensitize the cells to DNA-damaging agents. The cellular levels of cyclin B1 positively correlate with the degrees of potentiation achieved. Of additional interest, we observed that the various colon cancer cell lines in general appear to express higher levels of cyclin B1 and also display higher sensitivity to Chk1 inhibitors, implying that Chk1 inhibitor may be more efficacious in treating colon cancers. In summary, we propose that cyclin B1 is a biomarker predictive of the efficacy of Chk1 inhibitors across different types of cancers. Unlike previously established efficacy-predictive biomarkers that are usually the direct targets of the therapeutic agents, cyclin B1 represents a non-drug-target biomarker that is based on the mechanism of action of the target inhibitor. This finding may be potentially very useful for the stratification of patients for Chk1 inhibitor clinical trials and hence, maximize its chance of success.

Keywords: *Biomarker, Chk1, cyclin B1, chemotherapeutics, siRNA, cell-cycle checkpoints, mitotic catastrophe*

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Introduction

Biomarkers are important tools for stratifying patients, establishing proper drug dose and schedule, and quantifying drug benefits in the clinical phases of drug development. The resulting information is critical for making informed decisions on drug

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safety and efficacy, which will help reduce the attrition rate in clinical studies, and thereby, decrease the overall cost of drug development. More importantly, biomarkers that are capable of predicting drug efficacy can be used to stratify patient populations and bring the new medicine to the right patients more rapidly than the conventional hit and miss approaches (Frank & Hargreaves 2003).

There are three main types of biomarker each serving different purposes: (1) cancer or disease progression related markers that can be used to evaluate drug efficacy; (2) mechanism-based markers that can predict the efficacy of the drug in different patient categories and hence help stratifying patients; (3) pharmacodynamic (PD) markers that can provide guidance in determining the most effective drug dose and schedule. Having a reliable PD marker constitutes a viable alternative to the conventional maximal tolerated dose (MTD), which may reduce undesirable adverse events. This marker is especially important for drugs that are devoid of any apparent toxicity, which makes it impossible or impractical to obtain a MTD.

We and others have demonstrated that Chk1 is the major mediator of cell-cycle checkpoints in response to various chemotherapeutics (Zhao et al. 2002, Chen et al. 2003, Zhou & Sausville 2003, Xiao et al. 2003, 2004, Zhou & Bartek 2004). Elimination of Chk1 will potentiate chemotherapeutics by abrogating these checkpoints leading to increased apoptosis and cell death (Eastman et al. 2002, Walworth 2000, Koniaris et al. 2001, Zhao et al. 2002, Chen et al. 2003, Xiao et al. 2003). It is clear now that Chk1 phosphorylates the family of Cdc25 phosphatases, which in turn inhibit CDK2 and Cdc2 activities and prevent their premature activation, thus maintaining the highly regulated temporal order of cell-cycle progression. In the event of cell-cycle alteration due to DNA damage, Chk1 is activated to phosphorylate Cdc25 leading to its degradation via the proteasomal pathway or sequestration to cytoplasm by binding to 14-3-3 protein (Bartek & Lucas 2003, Zhou & Sausville 2003). As a consequence, Cdc2 is inactivated and cells are arrested at checkpoints until damaged DNA is repaired. Under this circumstance, inhibition of Chk1 leads to the improper activation of Cdc2 resulting in checkpoint abrogation and presumably mitotic catastrophe. Therefore, we speculate that in the event of checkpoint abrogation, Cdc2 activity is a key factor to induce mitotic catastrophe and cell death.

It is well known that cyclin B1 is a cofactor required for Cdc2 activity. An increase in cyclin B1 expression in the late G2-phase is essential for Cdc2 activation leading to M phase entry (Johnson & Walker 1999). Consequently, cyclin B1 may also be responsible as a 'rate limiting factor' for the improper activation of Cdc2 activity resulting in premature mitosis and mitotic catastrophe incurred through the checkpoint abrogation. Here, through analysis of morphology and cell-cycle markers, we show that the abrogation of cell-cycle checkpoints by Chk1 siRNA results in the premature activation of Cdc2/cylin B1 leading to mitotic catastrophe. By revealing the underlying mechanism of the potentiation, we have discovered that the cellular cyclin B1 level is the major determinant for this potentiation. We further demonstrate that downregulation of cyclin B1 in HeLa cells leads to impairment of the induction of mitotic catastrophe as well as a decreased potentiation of topoisomerase inhibitors by either Chk1 siRNA or a small-molecule Chk1 inhibitor. Hence, these results validated our theory that the cyclin B1 level is a major factor determining the cellular efficacy of a Chk1 inhibitor in the potentiation of topoisomerase inhibitors leading to mitotic catastrophe and cell death. We additionally confirmed our theory by extending the study to an additional ten different cancer cell lines and comparing the endogenous

levels of cyclin B1 versus the ability of the Chk1 inhibitor to potentiate DNA-damaging agents in these cell lines. The cellular levels of cyclin B1 positively correlate with the degrees of potentiation achieved.

Experimental procedures

Cell culture

Human cervical cancer cell line HeLa and human lung cancer cell line H1299 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). H1299 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 μM sodium pyruvate, 1% penicillin–streptomycin and 0.45% glucose at 37°C in a 5% CO_2 incubator. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 1 μM sodium pyruvate, 1% penicillin–streptomycin and 0.45% glucose at 37°C in a 5% CO_2 incubator. Prior to transfection, the cells were switched to medium without any penicillin–streptomycin.

Antibodies and Chk1 inhibitors

Antibodies against Chk1, cyclin B and Cdc2 Y15P were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Chk1 (Chk1 S345P), caspase-8 and caspase-9 antibodies were from Cell Signaling Technology (Beverly, MA, USA). PARP antibody was from BD Biosciences (San Jose, CA, USA). Antibodies for phosphohistone H3 was obtained from Upstate Technology (Waltham, MA, USA). Tubulin antibody was obtained from Molecular Probes (Eugene, OR, USA). All the Chk1 inhibitors are made in house.

Transfection

Human Chk1 siRNA was as described previously (Xiao et al. 2003). Luciferase siRNA was used as control siRNA (5′ to 3′ sequence: AACACTTGTCACTACTTT CTC). Cyclin B siRNA was obtained from Dharmacon in the form of Smartpool siRNA (catalogue number: M-003206-01). Transfection protocols were also as described previously (Chen et al. 2003, Xiao et al. 2003). The final concentration of the siRNA is 50 nM. For 96-well transfection used in the MTS assay, 0.5 μl of 20 μM stock siRNAs was used in a final volume of 200 μl medium for each well, resulting in a final concentration of 50 nM.

Western blot analysis

Adherent cells in the tissue culture wells were rinsed with PBS and directly lysed in Laemmli sample buffer. Floating cells or cell fragments were also collected, lysed and combined with the above lysates. Samples were heated at 95°C for 5 min and resolved on the Novex mini-gel system (Invitrogen) under denaturing conditions and blotted to polyvinylidene difluoride membrane using a semi-dry transfer device (Amersham Biosciences). The membrane was blocked with 5% non-fat dry milk and probed with various antibodies. Enhanced chemiluminescent detection (ECL) was performed using ECL reagents according to the vendor's protocols (Santa Cruz Biotechnology).

Cell proliferation assay (MTS assay)

HeLa cells were seeded in 96-well plates and transfected with Chk1 siRNA or scrRNA with oligofectamine. The final siRNA concentration was maintained at 50 nM. Sixteen hours after transfection, the cells were treated with the indicated doses of 5-FU for 48 h. After treatment, MTS reagents that measure the amount of live cells (Promega, Madison, WI, USA) were added to the cells and allowed to develop for 20 min to 2 h. Colorimetric measurement was taken at 490 nm on Spectra MAX 190 from Molecular Devices (Sunnyvale, CA, USA).

Cell cycle analysis

HeLa cells after the indicated treatments were washed one time in PBS and fixed in 70% ethanol. The fixed cells were washed again twice with PBS and treated with RNase A at 37°C for 30 min. Finally, the cells were stained with propidium iodide and incubated in the dark for 60 min or overnight before analysis. The samples were analysed through flow cytometry using fluorescence-activated cell sorting (FACS) manufactured by BD Bioscience (San Jose, CA, USA) using the Cell-Quest program.

Immunofluorescence

HeLa cells were grown to 50% confluence on coverslips in six-well plates and transfected with Chk1 siRNA or control siRNA and treated as indicated. The cells were then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.15% Triton X-100 for 10 min, blocked with 5% normal goat serum (Invitrogen) for 30 min, and then stained with the appropriate dilution of the indicated antibody for 1 h at room temperature. After extensive washing, FITC-conjugated secondary antibody was added at a 1:500 dilution for 1 h (Molecular Probes). Before mounting, cells were stained with the nuclear stain Hoechst 33342 at 10 μg ml^{-1} for 10 min. Fluorescence signals were visualized with a Zeiss inverted microscope equipped for epifluorescence using a 488-nm excitation filter and a 522/535-nm emission filter. Images were recorded with a Hamamatsu Orca CCD camera and analysed with Axiovert software.

In vivo tumour xenograft studies

Animal studies were conducted following the guidelines of the internal Institutional Animal Care and Use Committee. Male *scid* mice were obtained from Charles River Laboratories (Wilmington, MA, USA) at 6–8 weeks of age. SW620 colon carcinoma cell-line were obtained from the ATCC and maintained in RPMI 1640 medium supplemented with 10% FBS. Then 1×10^6 cells in 50% matrigel (BD Biosciences, Bedford, MA, USA) were inoculated subcutaneously into the flank. Ten animals were assigned to each group, including controls, Chk1 inhibitor alone, irinotecan alone, and various combination regimens of both. Tumours were allowed to reach 200 mm^3 size and treatment was then commenced. Tumour size was evaluated by twice-weekly measurements with digital calipers. Tumour volume was estimated using the formula: $V = L \times W^2/2$. A-776574 was given intraperitoneally in a vehicle of 0.2% HPMC at a schedule of b.i.d. $\times$21-days.

Results

Downregulation of Chk1 abrogates the cell-cycle checkpoints induced by topoisomerase inhibitors and potentiates their cytotoxicities

Although some previous studies have shown that Chk1 inhibitors such as UCN-01 or caffeine can abrogate the DNA damage-induced cell-cycle checkpoint and potentiate apoptosis induction, these inhibitors are not highly specific for Chk1 and are known to hit many other cellular targets (Busby et al. 2000, Koniaras et al. 2001, Eastman et al. 2002, Zhou & Bartek 2004). Therefore, in order to demonstrate the potentiation effect resulting from the specific inhibition of Chk1, we used Chk1 siRNA to examine the impact of Chk1 downregulation on the cell-cycle checkpoints induced by camptothecin (CPT, a topoisomerase I inhibitor conferring S-phase arrest) and doxorubicin (Dox, a topoisomerase II inhibitor conferring G2/M arrest). HeLa cells were treated with 150 nM camptothecin (CPT, Figure 1A) or 200 nM of doxorubicin (Dox, Figure 1B) in the presence of either Chk1 siRNA or control siRNA for 24–48 h. After 24 h treatment with CPT and control siRNA, a majority of cells were arrested in S phase (Figure 1A, panel 2). However, Chk1 siRNA abrogates the S checkpoint and cells progressed into G2/M phase (panel 4). Correspondingly, the percentage of cells in sub-G0/G1 phase increased significantly reflecting the enhanced apoptosis induced by the checkpoint abrogation. At the 48-h time point, some of the control siRNA-treated cells have escaped the S phase arrest by CPT and migrated into the G2/M phase as evidenced by a hybrid S-G2/M peak (panel 3). This result indicates that 150 nM of CPT cannot achieve a permanent S-phase arrest in HeLa cells. Similarly, the cells treated with Dox and control siRNA for 24 h were arrested in a hybrid S-G2/M peak (Figure 1B, panel 2), whereas Chk1 siRNA abrogated this checkpoint and most of the cells accumulated in G2/M phase (panel 4). At the 48-h time point, Dox completely arrested control siRNA-transfected cells in G2/M checkpoint (panel 3), and Chk1 siRNA significantly enhanced the sub-G0/G1 population at the expense of the G2/M population, suggesting that the checkpoint abrogation leads to the onset of apoptosis (panel 6).

To further quantify the potentiation effect of Chk1 siRNA, we performed a MTS assay using HeLa cells treated with CPT and either Chk1 siRNA or control siRNA (Figure 2). We observed more than a 10-fold potentiation of CPT cytotoxicity by Chk1 siRNA. The EC_{50} value of CPT was decreased from 0.39 μM in the presence of control siRNA to 0.03 μM with Chk1 siRNA.

Abrogation of cell-cycle checkpoints by Chk1 siRNA results in a premature activation of Cdc2/cylin B1 leading to mitotic catastrophe

To ascertain the underlying mechanism of the potentiation, HeLa cells transfected with control siRNA or Chk1 siRNA were treated with CPT or Dox for various time points and the profiles of two major mitotic markers, Cdc2 Y15P and phosphohistone H3, were probed (Figure 3A, B). The result showed that both CPT and Dox induced time-dependent accumulation of Cdc2 Y15P (an inhibitory phosphorylation of the mitotic kinase Cdc2) in control siRNA-transfected cells, indicating the activation of cell-cycle checkpoints. This result is consistent with the observed cell-cycle arrest in the FACS analysis (Figure 1). In contrast, downregulation of Chk1 reversed this accumulation in the later time points (16 and 24 h), indicating reactivation of Cdc2,

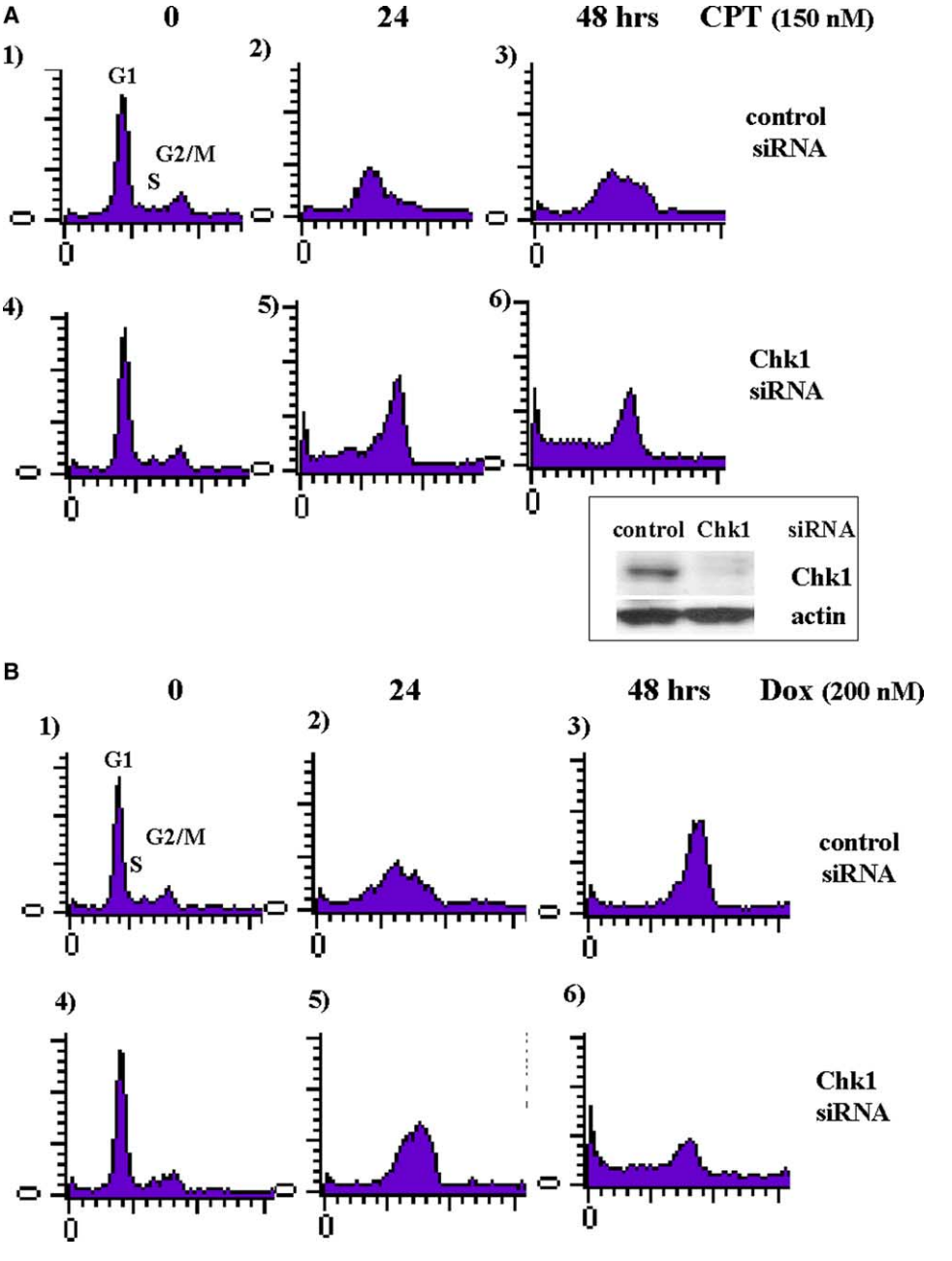

Figure 1. Chk1 siRNA abrogates DNA damage-induced cell-cycle checkpoints and confers apoptosis. HeLa cells were transfected with either GFP siRNA (as control) or Chk1 siRNA and then treated with (A) camptothecin (CPT) at 150 nM or (B) doxorubicin (Dox) at 200 nM for 24 or 48 h. Cells were processed for FACS analysis. Inset: Chk1 immunoblot demonstrates efficient elimination of Chk1 expression by the Chk1 siRNA.

which is consistent with the aforementioned abrogation of the checkpoints and migration of cell into M phase (Figure 1). It is known that Cdc2 activation leads to phosphorylation of histone H3 through aurora kinase and other mitotic kinases, and

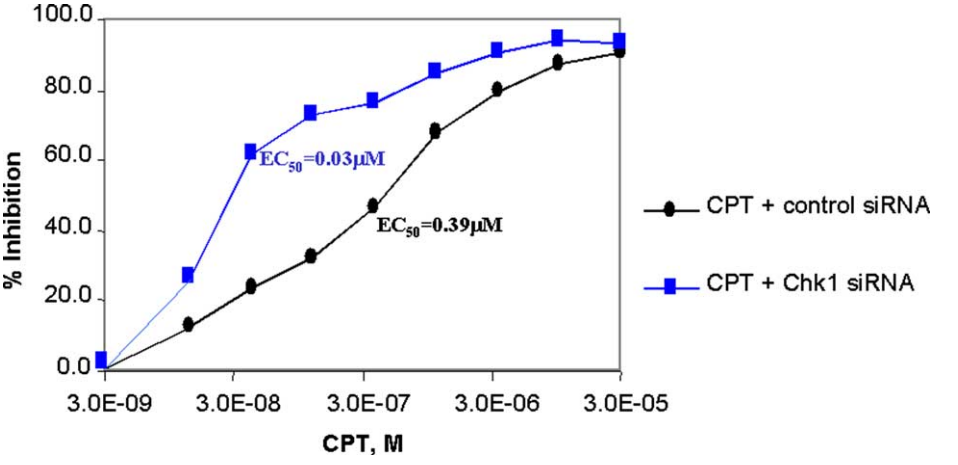

Figure 2. Chk1 downregulation potentiates the cytotoxicity of camptothecin (CPT) in HeLa cells. HeLa cells were transfected with either GFP siRNA or Chk1 siRNA and then treated with different doses of CPT for 48 h. The cells were then developed for cell-proliferation assay (MTS) and the IC_{50} values for inhibition of cell growth were calculated according to Prizm software.

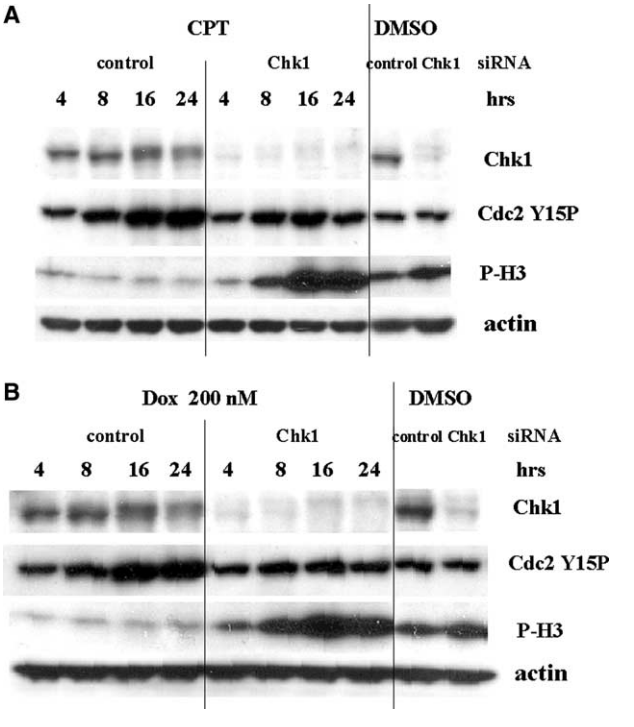

Figure 3. Chk1 downregulation abrogates DNA damage-induced cell-cycle arrest and confers excessive accumulation of the mitotic marker phosphohistone H3. HeLa cells were transfected with Chk1 siRNA or GFP siRNA, and then treated with 150 nM camptothecin (CPT) (A) or 200 nM doxorubicin (Dox) (B) for different time points (4, 8, 16 and 24 h). Cell lysates were harvested for each condition and immunoblotted for G2/M markers including Cdc2 Y15P and phosphohistone H3. Chk1 immunoblot shows efficient downregulation of Chk1 expression. Actin was probed as a loading control.

this is a prerequisite for chromosome condensation, and thus, a classic marker for mitosis (Pascreau et al. 2003, Prigent & Dimitrov 2003). As expected, in the control siRNA-transfected cells, phosphohistone H3 was depressed to a very low level with the treatment of CPT and Dox, indicating a depletion of M-phase cells due to the S- or G2-phase arrest. However, the combination treatment of DNA-damaging agents and Chk1 siRNA revealed an opposite profile. Phosphohistone H3 accumulated to extremely high levels, suggesting the abrogation of the checkpoints and extensive premature mitotic induction in the presence of DNA damage. Therefore, Chk1 siRNA abrogates checkpoints by causing dephosphorylation and activation of Cdc2, which induces the premature phosphorylation of histone H3 presumably leading to mitotic catastrophe.

A typical morphology for cells undergoing mitotic catastrophe is the appearance of multiple micronuclei (Hendry & West 1997, Roninson et al. 2001). To confirm further that Chk1 downregulation abrogates the checkpoints leading to mitotic catastrophe, we carried out the following cellular morphology study for HeLa cells undergoing checkpoint abrogation. HeLa cells were transfected with Chk1 siRNA or control siRNA and treated with CPT or Dox for 24 h. Cells were then fixed and stained for nuclei and β-tubulin to visualize the nuclear and microtubule profiles, respectively. Chk1 siRNA alone showed no effect on the nuclear and microtubule morphology (Figure 4A) since the staining profiles are indistinguishable from that of the control siRNA (data not shown). Under these experimental conditions, CPT or Dox alone did not induce a significant extent of apoptosis and hence failed to confer significant changes in the staining profiles either (Figure 4A, middle panels and Figure 4B, upper panels). However, cells transfected with Chk1 siRNA and treated with CPT or Dox showed a striking feature of frequent multimicronuclei formation (Figure 4A, B, bottom panels). The ring-shaped alignment of the multiple nuclei confers a distinctive horseshoe configuration (red arrows, bottom panel). Some of the multi-micronucleated cells contained supercondensed and abnormal nuclei (white arrows, bottom panel), indicating that the induced mitotic catastrophe eventually leads to apoptosis and cell death.

Mitotic catastrophe has been shown to either lead to apoptosis or, more frequently, to non-apoptotic events such as necrosis (Roninson et al. 2001). To ascertain whether the observed mitotic catastrophe led to apoptosis, we examined the effects of Chk1 inhibition on the major apoptotic pathways. Caspase-3 is the common downstream effector caspase for both the extrinsic/death receptor-induced apoptosis pathway involving caspase-8, and the intrinsic/mitochondria-triggered apoptotic pathway involving caspase-9 (Fesik 2000, Strasser et al. 2000). Activated caspase 3 will cleave its downstream substrates, for instance, PARP. Caspase-8 can activate caspase-3 either through direct cleavage or indirectly through the caspase-9 pathway by causing the cytosolic release of cytochrome *c* via tBid. The antibodies we used against pro-caspase-8 or pro-caspase-9 also recognize the active cleaved species. Under our experimental conditions, the pro-caspase-8 and pro-caspase-9 profiles remain fairly constant throughout the different time points of treatment by CPT or doxorubicin in control siRNA-transfected cells, and no cleavage of either pro-caspases was observed, indicating that no significant apoptosis occurred (Figure 5A, B, lanes 1–5). This is consistent with the previous FACS and morphological studies. In contrast, cells transfected with Chk1 siRNA and treated with CPT and Dox showed the cleavage of both pro-caspase-8 and pro-caspase-9 as well as the emergence of active/cleaved

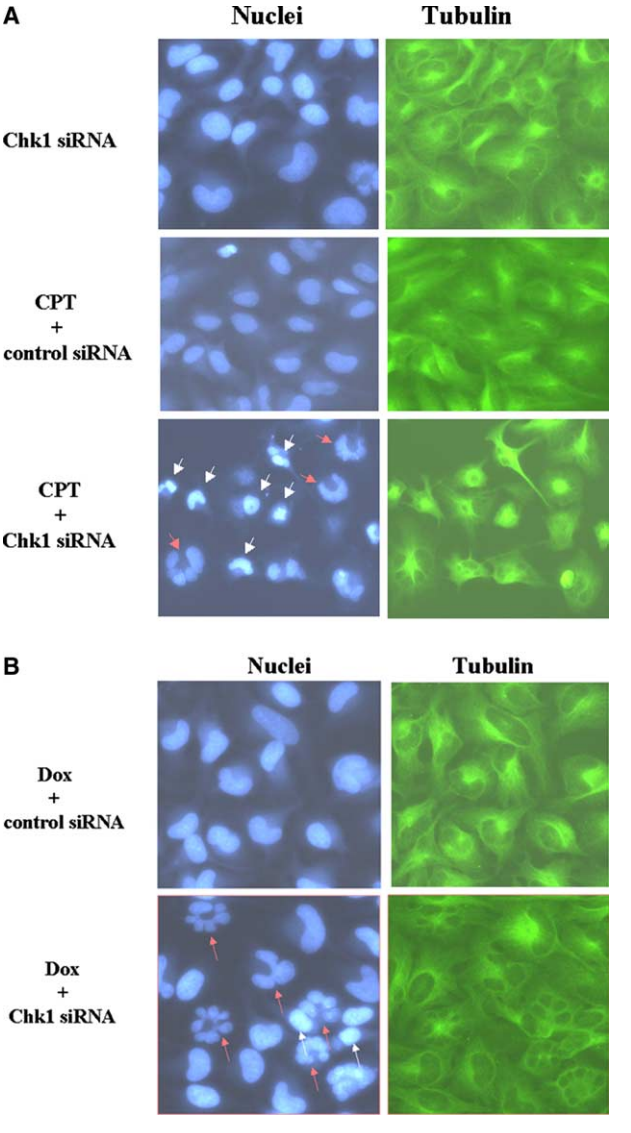

Figure 4. Chk1 downregulation in combination with topoisomerase inhibitors confers morphological features of mitotic catastrophe. HeLa cells were transfected with Chk1 siRNA or GFP siRNA, and then treated with 150 nM camptothecin (CPT) (A) or 200 nM doxorubicin (Dox) (B) for 18 h. Cells were fixed and permeabilized for immunocytochemical staining of tubulin as well as counterstained with DAPI to visualize the nuclear profiles. Red arrows indicate multimicronuclei (signs of mitotic catastrophe); white arrows denote condensed nuclei. The occurrence of both in the same field suggests that mitotic catastrophe eventually leads to apoptosis.

caspase-8 and caspase-9 in a time-dependent manner (Figure 5, lanes 6–10). Consequently, we observed cleavage of PARP at the same time points when caspase-8 and -9 were activated. This result indicates that Chk1 siRNA potentiates DNA-damaging agents through both the intrinsic/caspase-9 and extrinsic/caspase-8 pathways culminating in caspase-3 activation.

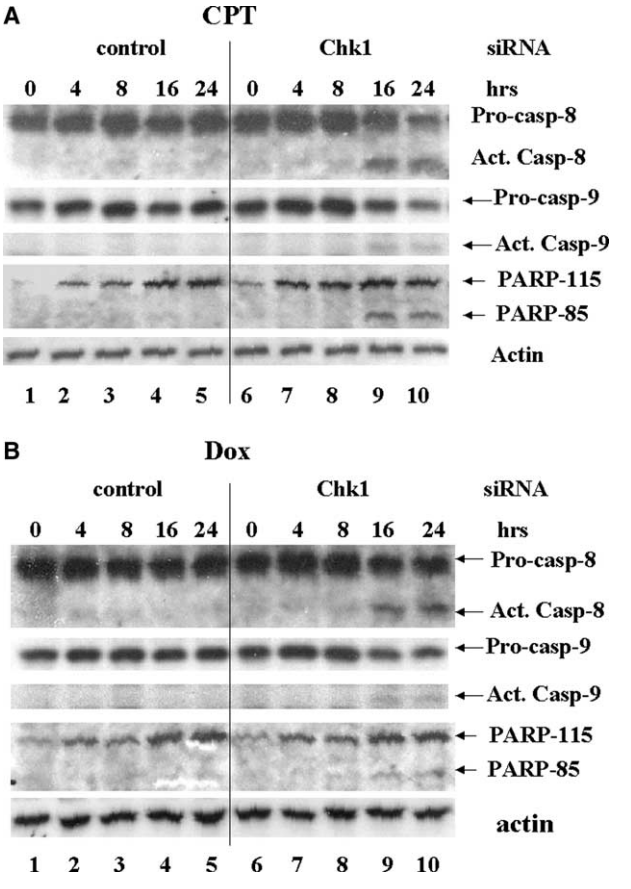

Figure 5. Chk1 siRNA potentiates topoisomerase inhibitors through both caspase-8 and caspase-9 pathways which eventually led to caspase-3 activation. HeLa cells were transfected with Chk1 siRNA or GFP siRNA, and then treated with 150 nM camptothecin (CPT) (A) or 200 nM doxorubicin (Dox) (B) for different time points (0, 4, 8, 16 and 24 h). Protein lysates were harvested for each condition and immunoblotted for caspase-8 and caspase-9 and PARP, a major downstream substrate for caspase-3. The antibodies used against caspase-8 and -9 can recognize both the inactive proform and the cleaved active species. Actin was also probed as a loading control.

Cyclin B downregulation prevents the Chk1 siRNA-induced accumulation of phosphohistone H3, reducing the induction of mitotic catastrophe and apoptosis

As previously noted, cyclin B1 is an important cofactor for Cdc2 activity, and we postulated that cyclin B1 could be the 'rate limiting factor' for the premature activation of Cdc2 during the checkpoint abrogation mediated by Chk1 siRNA. To verify this theory, we used cyclin B1 siRNA to specifically deplete cyclin B1 from HeLa cells and observed its effect on the induction of mitotic catastrophe and apoptosis in response to Chk1 siRNA and a DNA-damaging agent (CPT) (Figure 6A). Consistent with above results, in the cells transfected with control siRNA, the mitotic marker phosphohistone H3 (P-H3) was virtually eliminated by CPT, indicating a successful S arrest and a complete depletion of M-phase cells (lanes 1 and 2). In comparison, we observed the accumulation of P-H3 in the presence of

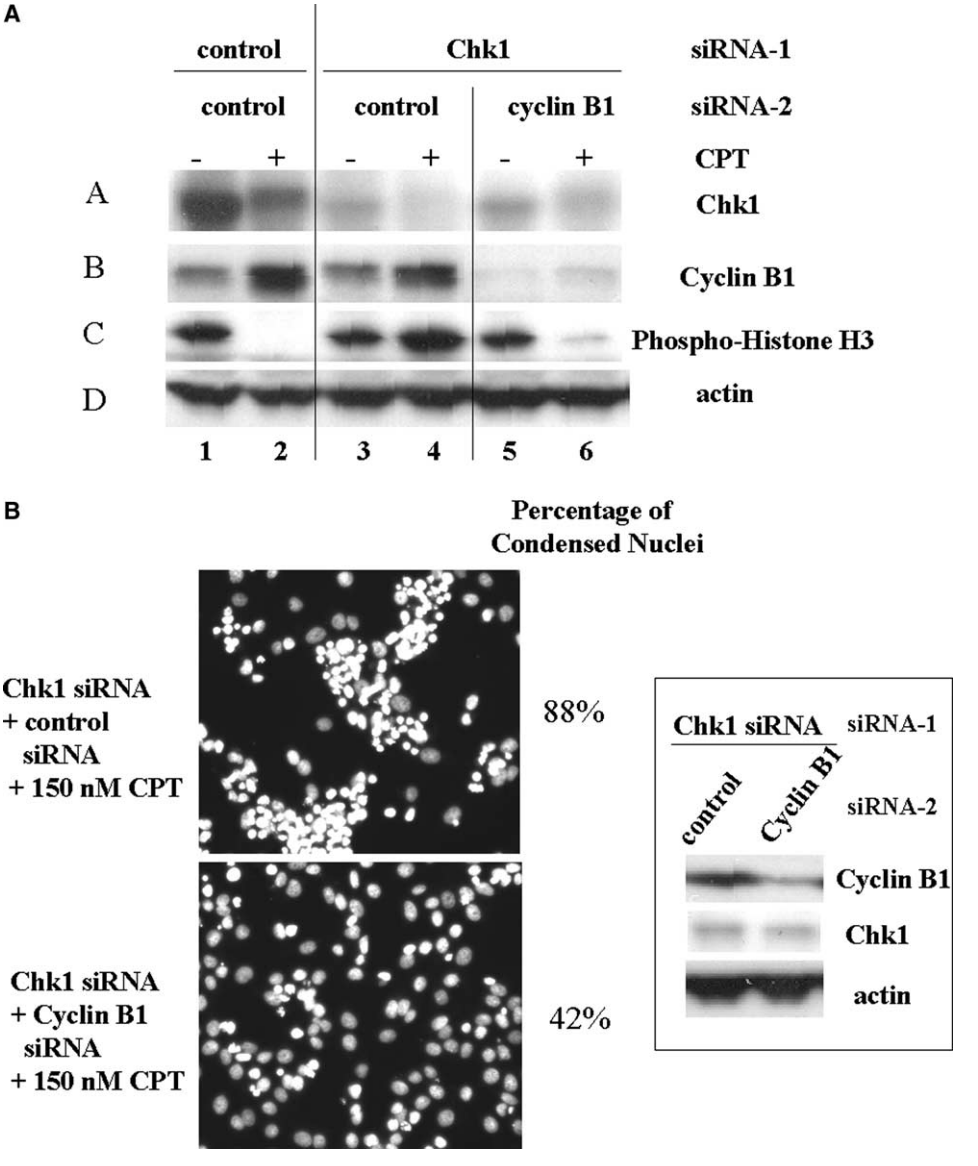

Figure 6. The induction of mitotic catastrophe by Chk1 siRNA in the presence of DNA damage is dependent on cyclin B. (A) HeLa cells were double-transfected with two siRNAs in the following scheme: control cells were transfected with GFP siRNA plus GFP siRNA (lanes 1 and 2). For Chk1 siRNA-transfected cells, they were additionally co-transfected with either GFP siRNA (lanes 3 and 4), or with cyclin B siRNA (lanes 5 and 6). Therefore lanes 3 and 4 represent cells with only Chk1 knockdown, where as lanes 5 and 6 represent cells with double Chk1 and cyclin B1 knockdown. The variously transfected cells were treated or not with 150 nM camptothecin (CPT) for 24 h. Cell lysates were collected and immunoblotted for Chk1, cyclin B and phosphohistone H3 levels. (B) HeLa cells were transfected with Chk1 siRNA in combination with GFP siRNA or cyclin B siRNA for 24 h. The cells were then treated with 150 nM CPT for 48 h. Nuclei were stained with DAPI and the percentages of cells with condensed nuclei were counted and calculated. Inset: the cyclin B and Chk1 immunoblot profiles of the treated samples.

CPT and Chk1 siRNA, suggesting abrogation of the S checkpoint and the onset of premature mitosis and mitotic catastrophe (lanes 3 and 4). Most importantly, the accumulation of P-H3 was mostly abolished in the cells transfected with cyclin B1 siRNA in addition to Chk1 siRNA (lane 6), suggesting that cyclin B1 is critically required for the premature mitotic entry and the induction of mitotic catastrophe. Corresponding nuclear staining profiles further demonstrated that downregulation of cyclin B1 not only abrogated the accumulation of P-H3, but also significantly decreased the formation of condensed nuclei, a sign of apoptosis (from 88% to 42%, Figure 6B). These observations were further confirmed by an MTS assay. Table I shows that cyclin B1 downregulation diminished the potentiation of CPT by Chk1 siRNA from 16-fold to 6-fold. We speculate that the remaining 6-fold potentiation may be due to incomplete cyclin B1 knockout or the presence of cyclin A to partially replace the mitotic functions of cyclin B1 (Table I). This was further corroborated by using our in-house specific small-molecule inhibitor of Chk1, A-690002 (Chen et al. 2006), to perform the potentiation assay in either luciferase siRNA-transfected cells or the cyclin B1 siRNA-transfected cells: A690002 produced a 21.2-fold potentiation of CPT in control luciferase-transfected cells, whereas it only achieved an 8-fold sensitization in cyclin B1 knockdown cells (Table I).

In conclusion, our results confirmed that cyclin B1 is required for the premature activation of Cdc2 and mitotic entry upon cell-cycle checkpoint abrogation. Therefore, its level may critically determine the potentiation effect of DNA-damaging agents and induction of mitotic catastrophe and cell death by the inhibition of Chk1.

Cyclin B1 is a mechanism-based biomarker predictive of the efficacy of Chk1 inhibitor in the potentiation of chemotherapeutics

To further substantiate and extend our observation that cyclin B1 is a major factor affecting the efficacy of the Chk1 inhibitor in potentiation of DNA-damaging agents, we examined other human cancer cell lines with different tissue origins to prove that cyclin B1 could broadly serve as an efficacy predicting biomarker (Figure 7). Again, we used A-690002 in this experiment as a potent and selective Chk1 inhibitor (Chen

Table I. Cyclin B1 knockdown attenuates the sensitivity of HeLa cells to Chk1 siRNA or Chk1 small-molecule inhibitor. HeLa cells transfected with luciferase siRNA (control) or cyclin B1 siRNA were further transfected with Chk1 siRNA (A), or treated with Chk1 small-molecule inhibitor A-690002 (B), in the presence of CPT in a potentiation assay. The EC_{50} values of CPT in the absence or the presence of Chk1 siRNA or Chk1 inhibitor were derived and the potentiation fold calculated as equal to the ratio of (CPT without Chk1 inhibition) versus (CPT with Chk1 inhibition).

	CPT	CPT+Chk1siRNA	Potentiation fold
A			
Control	348.6	21.6	16
Cyclin B1 KO	286.5	45.3	6
	CPT	CPT+A-690002	Potentiation fold
B			
Control	314	14.9	21.1
Cyclin B1 KO	163	20	8

et al. 2006). The IC_{50} value against Chk1 is 8 nM, which is from 125- to more than 6000-fold more selective against a broad panel of serine and threonine kinase tested. With this highly specific Chk1 inhibitor, we tested ten cancer cell lines with various tissue origins and compared their cyclin B1 levels versus the fold potentiation of CPT achieved in these cell lines in combination with the Chk1 inhibitor. Figure 7A shows the endogenous level of cyclin B1 in the cell lines grouped according to their respective tissue origins. We observed that the various colon cancer lines generally expressed higher levels of cyclin B1. In contrast, the selected leukaemia, prostate and renal cancer cell lines showed poor expression of cyclin B1. However, cyclin A, cyclin E and Cdc2 levels were fairly consistent across the cell lines and did not show such

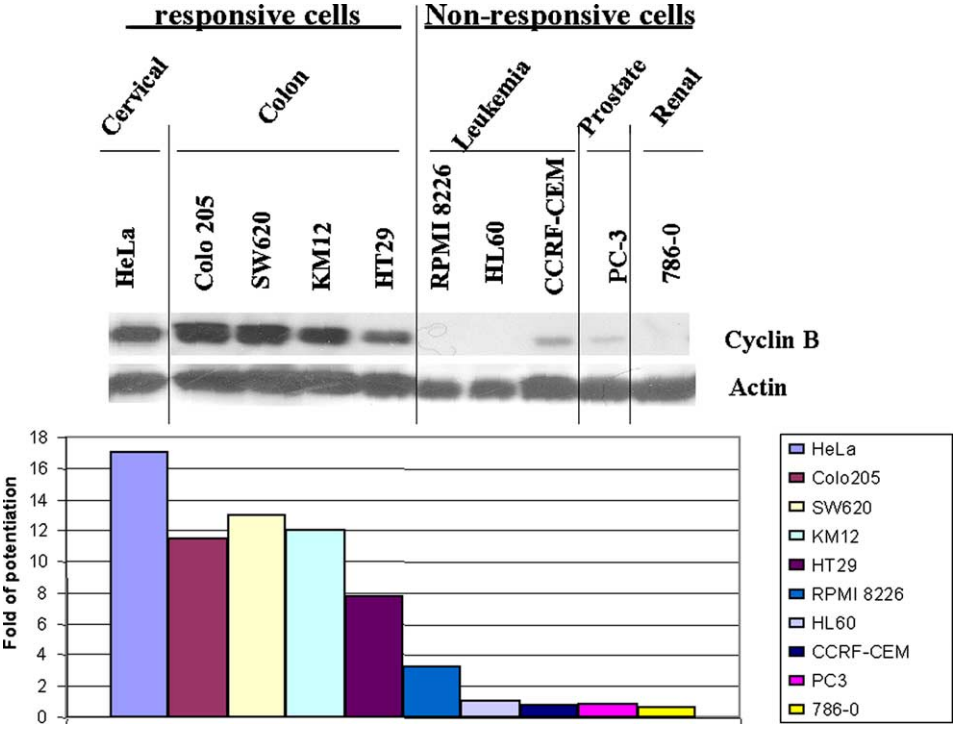

Figure 7. Cyclin B1 expression levels among different cancer cell lines from various tissue origins predict their responsiveness to Chk1 inhibitor in combination with topoisomerase inhibitors. (A) Eleven different cancer cell lines with different tissue origins such as colon, leukaemia, prostate and renal were harvested during log-phase growth when reaching 80–90% confluence. The cells were lysed and their protein samples were immunoblotted for cyclin B1. Actin was probed as a loading control. (B) Potentiation profile of a specific Chk1 inhibitor in the eleven cell lines. A-690002, a specific Chk1 inhibitor that displayed at least 200-fold selectivity against other serine/threonine kinases and >500-fold selectivity against tyrosine kinases (Chen et al. 2006), was used at 1.0 uM concentration in combination with the appropriate dose range of camptothecin (CPT) to treat each of the eleven cell lines. After 48 h, cells were developed for MTS assay to assess the inhibition of cell growth. IC_{50} values for CPT cytotoxicity in the absence or the presence of A-690002 were calculated and their ratio constitutes the fold of potentiation of CPT by Chk1 inhibitor in this cell line. The fold number of potentiation in each cell line was plotted as bar graph underneath the cyclin B1 immunoblot profile to detect if a significant match exists between the two profiles.

divergence (data not shown). Figure 7B shows the potentiation effect of CPT by the Chk1 inhibitor, A-690002, in the cell-proliferation assay with the same set of cell lines. The correlation between cyclin B1 expression and the scale of potentiation achieved is rather striking. Cell-lines with significant levels of cyclin B1 generally showed more than 7-fold potentiation; whereas cell-lines with low cyclin B1 expression generally displayed negligible potentiation. This indicates that cyclin B1 level can be used as an efficacy-predicting marker for Chk1 inhibitors.

Overall correlation of cyclinB1 expression levels in a wide range of cancer cell lines with their sensitivities to Chk1 inhibitor

To investigate the ability of cyclinB1 level as a predictive biomarker to predict cellular sensitivity to Chk1 inhibitor across a much wider range of cancer cell lines (50 cell lines spanning eight cancer categories), we used A-776574, another highly specific and potent Chk1 inhibitor (Wang et al. 2007), to carry out a potentiation assay in combination with camptothecin (Figure 8). The fold of potentiation produced by this inhibitor in each cell line was plotted against their respective cyclin B1 level according to groups of responders (showing >4-fold of potentiation) versuss non-responders (<2-fold of potentiation). As is obvious, we see a general correlation between responders with higher cyclinB1 levels, and non-responders with lower cyclinB1 levels.

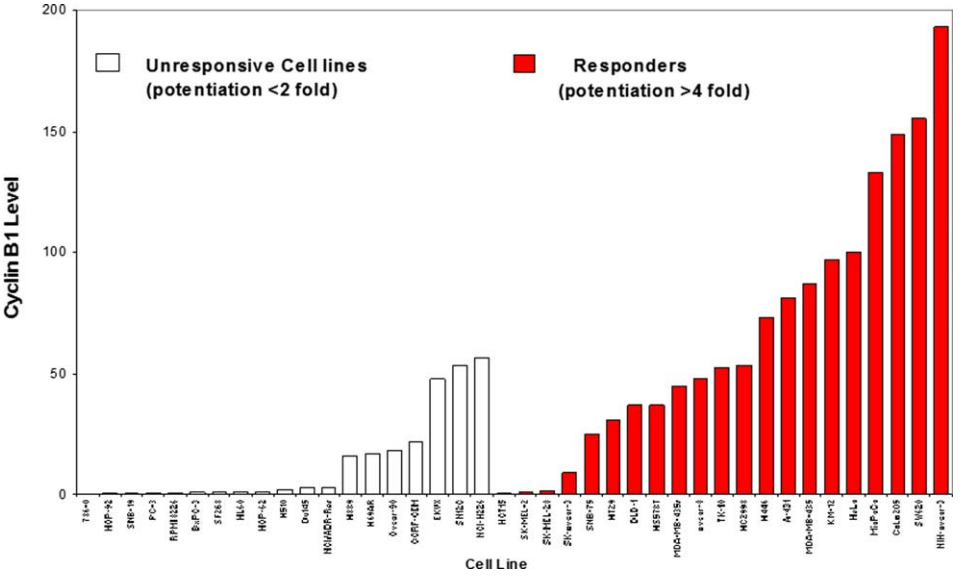

Figure 8. Cyclin B1 expression levels among 50 cancer cell lines from 11 tissue categories predict their responsiveness to Chk1 inhibitor in combination with camptothecin. Chk1 inhibitor A-776574 was used in combination with camptothecin across 50 most commonly used cancer cell lines spanning 11 cancer categories. Two-day cell growth assays were performed and fold of potentiation was calculated as described for Figure 7. Concomitantly, cell lysates were also prepared from the cancer cell lines and immunoblotted for cyclin B1 level in reference to HeLa cell level. The cell lines were then clustered into two groups according to their potentiation fold: those with >4-fold were grouped as responders, and those with <2-fold were grouped as unresponsive. They were plotted with their corresponding cyclin B1 levels.

In vivo efficacy of Chk1 inhibitor

To ascertain whether we could see translation of Chk1 inhibitor's *in vitro* efficacy into *in vivo* efficacy in tumour xenograft models, we carried out the SW620 tumour xenograft study and dosed the mice with Chk1 inhibitor A-776574 alone, irinotecan (a clinical analogue of camptothecin) at 1 mg kg^{-1} alone, or irinotecan in combination with A-776574 dosed IP at 10, 25 and 50 mg kg^{-1} level b.i.d. for 21 days. This colon cancer cell line contains very high cyclin B1 level and displayed great sensitivity to Chk1 inhibitor *in vitro* (>12-fold of potentiation of camptothecin, Figure 7). Tumour volumes were measured and calculated throughout the process (Figure 9). Chk1 inhibitor alone has minimal effect (blue curve); irinotecan alone at the used level only produced a very modest tumour growth delay (red curve); in contrast, combination of irinotecan with A-776574 showed a synergistic effect in tumour growth suppression, with 25 mg kg^{-1} of the inhibitor dose demonstrating the best efficacy (~59% reduced tumour load compared with vehicle control and ~46% reduced volume compared with irinotecan alone).

Discussion

It has been well established that Chk1 inhibition sensitizes tumour cells to IR and topoisomerase inhibitors through abrogation of the DNA-damage induced checkpoint. However, most of these studies were done with caffeine or UCN-01, both of which have multiple cellular targets besides Chk1. More importantly, the underlying mechanism of this sensitization has not been fully characterized. Specifically, it has

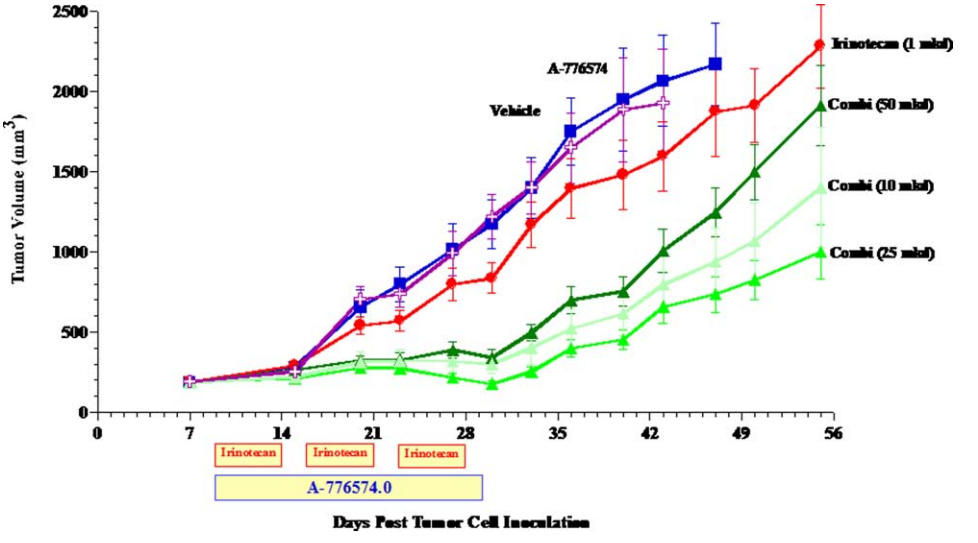

Figure 9. *In vivo* xenograft trial of Chk1 inhibitor. SW620 cells were inoculated into scid mice and tumours were allowed to grow to 200 mm^3 in size. The mice were dosed with Chk1 inhibitor A-776574 alone, irinotecan (a clinical analogue of camptothecin) at 1 mg kg^{-1} alone, or irinotecan in combination with A-776574 (combi) dosed intraperitoneally at 10, 25 and 50 mg kg^{-1} b.i.d. for 21 days. Ten animals were assigned to each group. Tumour size was evaluated by twice-weekly measurements with digital calipers. Tumour volume was estimated and plotted.

only been speculated, but not yet experimentally proven, that the potentiation effect is due to mitotic catastrophe. Using a Chk1 siRNA that confers specific downregulation of Chk1 expression, we have examined the underlying mechanism of this potentiation effect and demonstrated that premature mitosis and mitotic catastrophe constitute the major pathway for the sensitization effect. Most significantly, by revealing this mechanism, we discovered that the cyclin B1 level is able to predict the responsiveness of various cancer cell lines to the Chk1 inhibitor in combination therapy with DNA-damaging agents.

Clinical trials usually represent by far the most expensive and time-consuming stage of the drug development process. Additionally, the success rate of potential drugs (or new chemical entities (NCE)) to clear clinical trails is prohibitively low (around 20% on average) (Edmunds et al. 2001, Booth & Zemmel 2004). One of the main reasons for this high attrition rate is the inability to find the group of patients mostly likely to respond to the trial therapy due to the lack of effective efficacy-predictive biomarkers (Frank & Hargreaves 2003). Biomarkers are very important tools to stratify patients and quantify drug benefits in all clinical phases of drug development. Ultimately, they help to reduce the attrition rate in clinical studies and thereby, decrease the overall cost of drug development while bringing new medicines to the right patients much earlier than conventional approaches. Currently, all of the existing efficacy-predictive biomarkers are, in fact, the direct molecular targets of the therapeutic agents. In contrast, our present study revealed a novel type of efficacy-predictive biomarker, which is solely based upon the mechanism of action of the therapeutic agent, rather than the drug target. We found that the premature activation of Cdc2/cylin B1 is a key factor leading to mitotic catastrophe induced by Chk1 inhibitor, and cyclin B1 is a 'rate limiting factor' for the activity of Cdc2. This notion is validated by the fact that downregulation of cyclin B1 leads to impaired induction of mitotic catastrophe and decreased efficacy of the Chk1 inhibitor. We further substantiated and extended this notion by revealing a positive correlation between the cyclin B1 levels in various tumour cell lines and the potentiation ability of Chk1 inhibitor in these cell lines. In contrast, no such correlation was observed for cyclin A, cyclin E or Cdc2. Of additional interest, we observed that the various colon cancer cell lines in general appear to express higher level of cyclin B1 (Figure 7). Correspondingly, high levels of cyclin B1 were reported in colorectal cancer biopsies as well (Wang et al. 1997), implying that Chk1 inhibitor may be more efficacious in treating colon cancers.

Although cyclin B1 is the major mitotic cyclin, its mitotic function may be partially replaced to a limited extent by cyclin A, another cyclin also expressed during the G2/M transition. This may explain the fact that although cyclin B1 knockdown dramatically lowered the sensitivity of HeLa cells to Chk1 siRNA or inhibitor, Chk1 inhibition could still achieve 6–8-fold of potentiation in these cells. We are testing the possibility of double cyclin B1/cyclin A knockdown to validate this hypothesis. If true, this suggests that we could use cyclin B1 as the major biomarker while employing cyclin A as a secondary biomarker.

In summary, an efficacy predictive biomarker will significantly improve the overall response rate and in the process, reduce the frequency of resistance. It is especially important for drugs with novel or previously uncharacterized mechanisms of action since such a biomarker can be used to stratify patients and enhance their success rate in clinical trails. We believe that our discovery should facilitate the clinical trials for Chk1 inhibitors if they show sufficient therapeutic values.

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