An Indolocarbazole Inhibitor of Human Checkpoint Kinase (Chk1) Abrogates Cell Cycle Arrest Caused by DNA Damage

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ABSTRACT

Many cancer therapies cause DNA damage to effectively kill proliferating tumor cells; however, a major limitation of current therapies is the emergence of resistant tumors following initial treatment. Cell cycle checkpoints are involved in the response to DNA damage and specifically prevent cell cycle progression to allow DNA repair. Tumor cells can take advantage of the G2 checkpoint to arrest following DNA damage and avoid immediate cell death. This can contribute to acquisition of drug resistance. By abrogating the G2 checkpoint arrest, it may be possible to synergistically augment tumor cell death induced by DNA damage and circumvent resistance. This requires an understanding of the molecules involved in regulating the checkpoints. Human Chk1 is a recently identified homologue of the Schizosaccharomyces pombe checkpoint kinase gene, which is required for G2 arrest in response to DNA damage. Chk1 phosphorylates the dual specificity phosphatase cdc25C on Ser-216, and this may be involved in preventing cdc25 from activating cdc2/cyclinB and initiating mitosis. To further study the role of Chk1 in G2 checkpoint control, we identified a potent and selective indolocarbazole inhibitor (SB-218078) of Chk1 kinase activity and used this compound to assess cell cycle checkpoint responses. Limited DNA damage induced by γ-irradiation or the topoisomerase I inhibitor topotecan was used to induce G2 arrest in HeLa cells. In the presence of the Chk1 inhibitor, the cells did not arrest following γ-irradiation or treatment with topotecan, but continued into mitosis. Abrogation of the damage-arrest checkpoint also enhanced the cytotoxicity of topoisomerase I inhibitors. These studies suggest that Chk1 activity is required for G2 arrest following DNA damage.

INTRODUCTION

The cellular response to DNA damage involves cell cycle delays, also known as checkpoints. The regulation of these cell cycle checkpoints is a critical determinant of the manner in which tumor cells respond to many chemotherapies and radiation (1, 2). Many effective cancer therapies work by causing DNA damage; however, resistance to these agents remains a significant limitation in the treatment of cancer. There are several mechanisms of drug resistance: an important one is attributed to checkpoint activation that causes cell cycle arrest, which provides both the opportunity and capacity for cells to repair DNA damage (1, 3, 4). The G2 checkpoint is of particular importance in cancer because many cancers have acquired defects in the G1 checkpoint, which provide the transformed cells with a growth advantage over normal cells (5). In contrast, there is no evidence that defective G2 checkpoints provide such a growth advantage. Rather, tumor cells are particularly dependent on the G2 DNA-damage checkpoint as their only means of confronting the consequences of DNA damage, repairing the effects, and recovering. Agents capable of overriding G2 arrest following DNA damage, such as methylxanthines and 7-hydroxystauarosporine (UCN-01; Refs. 6, 7), have been shown to enhance the cytotoxicity of DNA-damaging agents (8–10). Thus, it is likely that an appropriate checkpoint antagonist would have significant clinical utility in combination treatment with conventional therapies. It is essential, therefore, to elucidate the molecular mechanisms regulating checkpoint pathways and to identify critical points of the G2 checkpoint response. G2 checkpoints include regulation of cdc2 by inhibitory phosphorylations at Tyr-15 and Thr-14. At the G2-M transition, cdc2 usually is dephosphorylated at Tyr-15 and Thr-14 by the dual specificity phosphatase cdc25C, allowing cdc2 to phosphorylate its mitotic substrates (11). However, in response to DNA damage or inhibition of DNA replication, the inhibitory phosphorylation of cdc2 remains intact and the cell cycle arrests at the G2 phase. In S. pombe, the G2 checkpoint is regulated by the checkpoint kinase Chk1, which is essential for cell cycle arrest following DNA damage (12–14). Recently, Sanchez et al. (15) described the cloning and characterization of human checkpoint kinase (Chk1), a homologue of the yeast gene. The human Chk1 was shown to bind to and phosphorylate cdc25C on Ser-216, which results in the partial disruption of a G2 checkpoint and the inability of DNA damaged cells to arrest at the G2 phase. These results indicated that Ser-216 of cdc25C is a target of the DNA damage checkpoint. However, it is still important to determine whether human Chk1 is required for the G2 checkpoint response. In the present study, we identified and characterized a compound, SB-218078, that potently inhibited human Chk1. This compound prevented G2 arrest following DNA damage, suggesting that Chk1 activity is required for G2 checkpoint arrest. Consistent with the importance of the G2 checkpoint in the response to DNA damage, SB-218078 enhanced the cytotoxicity of DNA-damaging agents. These data are among the first to demonstrate a key regulatory role for human Chk1 in regulation of checkpoint controls and provide support that Chk1 inhibitors may be valuable adjuvant therapies.

MATERIALS AND METHODS

Compounds. SB-218078 [9,10,11,12-tetrahydro-9,12-epoxy-1H-diindolol[1,2,3-fg:3’,2’,1’-kl]pyrrolo[3,4-j][1,6]benzodiazocine-1,3(2H)-dione] was synthesized by Medicinal Chemistry, SmithKline Beecham, as described previously (17). Topotecan was also synthesized by Medicinal Chemistry, SmithKline Beecham (18), UCN-01 was kindly provided by NCI. Camptothecin was obtained from Biomol (Plymouth Meeting, PA).

Expression and Purification of GST-Chk1. A GST-Chk1 expression construct was constructed that has the GST2 gene fused to the NH2 terminus of full-length Chk1 kinase via a linker containing a thrombin cleavage site. This construct was cloned into the Baculovirus expression vector, pFASTBAC, which was used to make the viral stock for the subsequent infection. Spodoptera frugiperda cells (S9) were infected with the virus expressing the GST-Chk1, after which the cells were grown for 3 days, harvested, and then frozen until purification. To purify Chk1, an S9 cell pellet expressing GST-Chk1 was resuspended on ice in lysis buffer containing 50 mm Tris-HCl (pH 7.5), 250 mm NaCl, 1 mm DTT, 0.1% Brij, a protease inhibitor cocktail (2 μg/ml E-64, 1 mm AEBSF, and 1 μg/ml pepstatin A (Sigma)), and 1 mm sodium orthovanadate.

Received 7/19/99; accepted 11/29/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

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2 The abbreviations used are: GST, glutathione-S-transferase; FBS, fetal bovine serum; DAPI, 4′,6′-diamidino-2-phenylindole; PKC, protein kinase C.
date, and cells were lysed by sonication and centrifuged at 100,000 × g for 30 min. The supernatant was added to glutatione-Sepharose 4B (Pharmacia Biotech, Sweden), beads, equilibrated in wash buffer (20 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT, protease inhibitor cocktail, 1 mM sodium orthovanadate), and rocked for 30 min. The resin with the bound GST-Chk1 was centrifuged at 500 × g for 5 min and washed with 14 ml of wash buffer. The beads were spun as above and resuspended in another 14 ml of wash buffer. The suspension was transferred into a column and allowed to pack; the wash buffer was then allowed to flow through by gravity. The GST-Chk1 was eluted from the column with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0) in 500-μl fractions. Protein concentrations were determined on the fractions using the Bio-Rad protein assay kit according to the manufacturer’s instructions. Fractions containing the GST-Chk1 were pooled and dialyzed at 4°C in dialysis buffer (20 mM HEPES, pH 7.0, 1 mM MgCl₂, 100 mM NaCl, 0.05% Brij-35, 10% glycerol, 1 mM DTT, 1 mM sodium orthovanadate).

Expression and Purification of Human GST-cdc25C. The GST-cdc25C Escherichia coli expression construct was the generous gift of Dr. Laurent Meijer (Centre National de la Recherche Scientifique, Roscoff, France). The construct contained the GST gene fused to the N-terminus of full-length cdc25C by a linker containing a thrombin cleavage site. This was subcloned into the baculovirus expression vector, pFASTBAC, which was used to generate viral stock for subsequent infection. Sf9 cells were infected with virus and grown for 3 days prior to harvesting. Cell lysates were clarified by centrifugation at 28,000 × g for 4°C for 1 h. The supernatant was mixed with 10 ml of glutathione-Sepharose 4B and shaken gently at 4°C for 2 h. The resin was packed into a Pharmacia XK26/60 column, and unbound material was washed out with 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 12 mM MgCl₂, 1 mM DTT (wash buffer). Protein-bound to the resin were then eluted with 50 mM Tris-HCl (pH 8.0), 10 mM glutathione, and 0.05% Brij-35, and assayed and dialyzed as described above for Chk1.

Chk1 Kinase Assay. All of this well-96 Flashplate (Amersham, Arlington Heights, VA) was coated with 1 μg of the GST-cdc25C fusion protein diluted in PBS. Plates were incubated overnight at 4°C, and then washed twice in PBS and dried for 5–30 min at 37°C. DMSO vehicle or compounds are added as 2 μl/well prior to the addition of 5 μCi/well of [33P]-ATP, 10 μM cold ATP, and kinase reaction buffer containing 20 mM HEPES (pH 7.4), 50 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT. The reaction was initiated by the addition of GST-Chk1 (0.1 μg/well) and was allowed to proceed for a time predetermined to be linear on a time versus phosphorylation plot. Reactions were terminated with the addition of an equal volume (50 μl) of 50 mM EDTA. Plates are washed four times in PBS, dried for 30 min at 37°C, and quantitated by liquid scintillation counting.

Cell Cycle Studies. Cellular studies used HeLa S3 and HT-29 adenocarcinoma cells obtained from ATCC, HeLa and HT-29 cells were grown in DMEM and McCoy’s medium (Life Technologies, Inc., Gaithersburg, MD), respectively (all supplemented with 2 mM l-glutamine and 10% FBS) in a 37°C, 5% CO₂ environment. For cell cycle studies, cells were plated at a concentration sufficiently low such that 24 h later they were at 10–20% confluence (typically 2 × 10⁶ cells/15 cm²). Cells were then synchronized in S-phase by a recombination thymidine block. Briefly, cells were treated with 2 μM thymidine for 18 h, released for 8 h by three washes, and then treated again with thymidine. Following the second release from thymidine, 95% of cells were in S-phase. Synchronized cells were then returned to complete media containing 50 μM topotecan (a dosage we have found to be sufficient to arrest cells in early G₂ phase without inducing apoptosis) alone and in combination with SB-218078 for up to 18 h. Studies using γ-irradiation were carried out in a GAMMACELL 40 irradiator with a 137 Cs source. The exposure rate was 91 rad/h, and cells were exposed for 5.5 min to achieve a dose of 500 rads, or 5 Gy. Cell cycle profiles were performed cytometrically using a procedure for propidium iodide staining of nuclei (19).

Cytotoxicity Assays. The proliferation assay was performed with HeLa and HT-29 cells, and used a colorimetric change resulting from reduction of the tetrazolium reagent XTT into a formazan product by metabolically active cells (20). Cells were seeded in 100-μl aliquots into 96-well plates to roughly 10% confluence (HT-29, 2.75 × 10⁴ cells/well; HeLa, 5.5 × 10⁴ cells/well) and grown for 24 h. Compounds were then added with or without sufficient vehicle-containing medium to raise the cells to a 200-μl final volume containing chemical reagents in 0.2% DMSO. Cells received multiple concentrations of topotecan, SB-218078, and combination treatment at 37°C, 5% CO₂. Ninety-six h later, 50 μl of an XTT/phenozone methosulfate mixture were added to each well, and cells were left to incubate for 90 min. Plates were read at 450 nm, and antiproliferative effects were compared relative to maximal effect of 5 μM topotecan.

Clonogenic assays (10) were performed with HT-29 cells seeded at 5 × 10⁵ cells/ml into 12-well plates. Twenty-four h after plating cells, the medium was removed and various dilutions of camptothecin or vehicle were placed in the wells. After 7 h, the camptothecin medium was removed, and adherent cells were washed once with warm PBS and then dosed with dilutions of SB-218078, UCN-01, or vehicle. After 18 h of drug treatment, cells were harvested by trypsinization and reseded into 6-well plates at a consistent volume determined to give control cells a concentration of 3 × 10⁵ cells/well. Cells were grown for 10 days, and then fixed in 100% methanol for 5 min and stained with 0.04% methylene blue to permit counting.

Chromatin condensation studies (21) were performed with thymidine-synchronized HeLa cells. Drug or vehicle was introduced to cells 4 h after release from the final thymidine arrest. Thirty min later, cells were subjected to a mock dose or a 5-Gy dose of irradiation, sufficient to induce G₂ arrest. Cells were harvested beginning at 6 h post release and prepared for nuclear staining with DAPI (Calbiochem), according to the manufacturer’s protocol. Briefly, cells were swollen hypotonically for 5 min in 75 mM KCl, gently pelleted, and fixed in 0.5 ml of Carnoy’s fixative (3:1 methanol/glacial acetic acid, v/v) for 5 min. After a final wash and resuspension in fixative, cells were dropped on prewetted slides and allowed to air dry. Fixed cells were then stained in 1 μg/ml DAPI for 15 min, followed by one wash in methanol. Stained cells were visualized using an Olympus IX-70 digital camera with fluorescence filters (exciter filters, BP360-370 nm), and multiple cell field pictures were taken at random. One hundred fifty to 250 nuclei were then evaluated for condensed nuclei.

RESULTS

SB-218078 Inhibits Chk1 in Vitro. The current model of G₂ DNA-damage checkpoint regulation predicts that phosphorylation of cdc25C by Chk1 kinase is required to arrest cells in G₂ following DNA damage. We set out to identify inhibitors of Chk1 that would be useful to test this hypothesis. An in vitro kinase assay was established to monitor phosphorylation of recombinant human cdc25C by purified recombinant human Chk1. This enzyme was able to phosphorylate cdc25C in a time- and concentration-dependent manner when radiolabeled ATP was added to the kinase reaction (data not shown). We used this assay to test selected serine/threonine kinase inhibitors.

Staurosporine was identified as a potent inhibitor of Chk1, with an IC₅₀ of 8 nM (Fig. 1A). Staurosporine is known to inhibit many kinases, and the lack of selectivity (Table 1) limits its utility in cellular studies. Hoping to find a more selective Chk1 inhibitor, we examined the activities of other compounds related to staurosporine, and identified SB-218078 (Fig. 1B), which inhibited Chk1 phosphorylation of cdc25C with an IC₅₀ of 15 nM (Fig. 1A). In kinase selectivity assays, SB-218078 had IC₅₀ values for cdc2 and PKC of 250 nM and 1000 nM, respectively (Table 1). Selectivity over cdc2/cyclin B is of particular importance because Chk1 is predicted to have a role in negative regulation of cdc2/cyclin B. Thus, inhibition of Chk1 should allow activation of cdc2, and this would have only a cellular effect only if the inhibitor does not also inhibit cdc2. We also tested the related compound UCN-01 and determined that it had an IC₅₀ of 7 nm for Chk1. UCN-01 previously has been demonstrated to abrogate DNA-damage arrest (7), but the target of this effect had been unknown.

Cell Cycle Effects of DNA Damage and Chk1 Inhibition. In response to DNA damage, cells arrest in G₂ by a mechanism that involves prevention of cdc25C activation and thus maintenance of inhibitory phosphorylation on T14 and Y15 of cdc2. Chk1 kinase may be required for the negative regulation of cdc25C resulting in G₂
out the thymidine and were incubated for 1.5 h prior to treatment with 50 nm topotecan and 5 μM SB-218078. The cell cycle profile was then analyzed 16 h later. At the time of treatment, the cells had clearly progressed into S-phase (Fig. 2B, panel 2 compared with panel 1). Topotecan alone caused the majority of cells to arrest in G2, whereas SB-218078 caused the topotecan-treated cells to continue through the G1 and M phases and return to a normal cell cycle profile similar to untreated cells 18 h after release from the double-thymidine block (Fig. 2B, panels 3–5). Staurosporine, which also inhibits Chk1 but has no selectivity over cdc2, was also tested in this study, and we observed that 1 μM staurosporine alone caused the cells to arrest in G2. In addition, 1 μM staurosporine was unable to abrogate G2 arrest in the topotecan-treated cells (Fig. 2B, panels 7 and 8). We also evaluated a potent, selective PKC inhibitor, Ro 34-0432 (22), as a control because SB-218078 does have weak PKC inhibitory activity. Ro 34-0432 was used at 1 μM, which is more than sufficient to inhibit PKC in cells because cellular effects attributable to PKC inhibition have been reported to have IC50s in the 0.1 μM range (23). This PKC inhibitor was unable to prevent cells from arresting in G2 with topotecan (Fig. 2B, panel 6). A time course analysis of cell cycle progression in synchronized SB-218078-treated cells further demonstrated that this compound did not significantly perturb the cell cycle in the absence of DNA damage (Fig. 3). Control cells reached G2 6–8 h after release from a double-thymidine block, progressed through mitosis by 10 h, and had returned to a normal cell cycle profile by 20 h. Cells treated with 2.5 μM SB-218078 reached G2 with a slight delay, 8–10 h after release, progressed through mitosis by 12–16 h post release, and returned to a normal cell cycle profile by 20 h. The minor G2-M delay could be due to partial inhibition of cdc2/cyclin B at the concentration used. Additional studies demonstrated that concentrations of SB-218078 at 10 μM or higher caused significant G2 arrest (data not shown). Thus, the activity of SB-218078 as judged by complete abrogation of G2 arrest was limited to a range between 1.2 and 10 μM.

Chromatin condensation was followed by DAPI staining to discriminate between G2 and M phases in cells that have had the DNA-damage checkpoint perturbed by SB-218078. Synchronized control cells underwent an increase in chromatin condensation between 7 and 8 h after release from a double-thymidine block, indicating prophase entry (Fig. 4). This was followed by decondensation between 8 and 10 h as cells exited from mitosis. Cells that were γ-irradiated 4.5 h after release from double-thymidine block were at 50% condensation at 6 h. This increase over the control cells is likely due to some spontaneous premature chromatin condensation caused by the irradiation as reported previously (24). However, the irradiated cells did not have any significant progression in chromatin condensation between 6 and 10 h. In contrast, when irradiated cells were also treated with SB-218078, there was a transient peak in chromatin condensation ~1 h behind the control cells, which suggests only a slight delay in prophase entry, and normal progression through mitosis.

Chk1 Inhibitor Enhances Cytotoxicity of DNA Damage. Prevention of G2 arrest is predicted to decrease the ability to repair DNA damage. Therefore, it was of interest to determine whether a Chk1 inhibitor would increase the sensitivity of cells to DNA damage. Inhibition of Chk1 kinase activity by SB-218078. A, inhibition curves in a Flashplate-based Chk1 kinase assay. GST-cdc25 was coated in the wells of the plate, and GST-Chk1 was added in kinase buffer along with [33P]-γ-ATP as described in “Materials and Methods.” The percentage of control phosphorylation (% control phosphorylation) was calculated based on the signal from the kinase reaction with vehicle only (1% DMSO). Data were generated in triplicate and are shown as the average value; bars, SE.

Additional experiments were conducted to confirm that the cell cycle profile observed when cells were treated with SB-218078 and topotecan was due to abrogation of G2 arrest and not an alternative effect such as a G1 delay. HeLa cells were synchronized at the G1-S border by double-thymidine block. Cells were released by washing out the thymidine and were incubated for 1.5 h prior to treatment with 50 nm topotecan and 5 μM SB-218078. The cell cycle profile was then analyzed 16 h later. At the time of treatment, the cells had clearly progressed into S-phase (Fig. 2B, panel 2 compared with panel 1). Topotecan alone caused the majority of cells to arrest in G2, whereas SB-218078 caused the topotecan-treated cells to continue through the G1 and M phases and return to a normal cell cycle profile similar to untreated cells 18 h after release from the double-thymidine block (Fig. 2B, panels 3–5). Staurosporine, which also inhibits Chk1 but has no selectivity over cdc2, was also tested in this study, and we observed that 1 μM staurosporine alone caused the cells to arrest in G2. In addition, 1 μM staurosporine was unable to abrogate G2 arrest in the topotecan-treated cells (Fig. 2B, panels 7 and 8). We also evaluated a potent, selective PKC inhibitor, Ro 34-0432 (22), as a control because SB-218078 does have weak PKC inhibitory activity. Ro 34-0432 was used at 1 μM, which is more than sufficient to inhibit PKC in cells because cellular effects attributable to PKC inhibition have been reported to have IC50s in the 0.1 μM range (23). This PKC inhibitor was unable to prevent cells from arresting in G2 with topotecan (Fig. 2B, panel 6). A time course analysis of cell cycle progression in synchronized SB-218078-treated cells further demonstrated that this compound did not significantly perturb the cell cycle in the absence of DNA damage (Fig. 3). Control cells reached G2 6–8 h after release from a double-thymidine block, progressed through mitosis by 10 h, and had returned to a normal cell cycle profile by 20 h. Cells treated with 2.5 μM SB-218078 reached G2 with a slight delay, 8–10 h after release, progressed through mitosis by 12–16 h post release, and returned to a normal cell cycle profile by 20 h. The minor G2-M delay could be due to partial inhibition of cdc2/cyclin B at the concentration used. Additional studies demonstrated that concentrations of SB-218078 at 10 μM or higher caused significant G2 arrest (data not shown). Thus, the activity of SB-218078 as judged by complete abrogation of G2 arrest was limited to a range between 1.2 and 10 μM.

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damage and overcome the cytotoxic consequences. We examined the survival of cells treated with the combination of topotecan and SB-218078 in a 96-h cytotoxicity assay. On the basis of studies using a wide range of drug concentrations (data not shown), we chose concentrations of topotecan and SB-218078 that had little effect alone to test whether they would have a synergistic effect in combination. In HeLa cells, 12.5 nM topotecan or 625 nM SB-218078 alone had little or no cytotoxic effect (Fig. 5). However, when these treatments were combined, survival was decreased to 50%. Similar results were also seen with the HT-29 colon carcinoma cell line, suggesting that the Chk1 inhibitor can enhance the cytotoxicity of very low amounts of DNA damage.

Previous studies have demonstrated that abrogation of the G2 DNA-damage checkpoint with agents such as caffeine and UCN-01...
also increased the effectiveness of DNA-damaging drugs. The mechanism of these agents has been unclear, however. UCN-01 is structurally related to SB-218078 and also is a potent Chk1 inhibitor; thus, we compared the activity of these compounds side by side in a HT-29 clonogenic survival assay in which UCN-01 has been reported previously to enhance the cytotoxicity of camptothecin (10). SB-218078 significantly increased the cytotoxicity of low and relatively ineffective concentrations of camptothecin (Fig. 6). The effect was very similar to that of an equivalent concentration of UCN-01.

DISCUSSION

The G2 checkpoint is an important cellular response to DNA damage, and plays a key role in the sensitivity of tumor cells to many therapies (25–27). Agents that can abrogate the G2 checkpoint, such as methylxanthines, phosphatase inhibitors, or UCN-01, previously have been identified (6, 7, 9, 28), but the mechanism by which they affect checkpoint regulation is poorly understood. In S. pombe, Chk1 kinase is required for the G2 DNA-damage checkpoint (12), but the role of the newly identified human Chk1 is not clear. In the present study, we found that SB-218078 and UCN-01 are potent inhibitors of human Chk1 kinase and that SB-218078 abrogates G2 arrest caused by either γ-irradiation or a topoisomerase I inhibitor as reported previously for UCN-01 (7, 10). Consistent with the pharmacology of other agents that abrogate the G2 checkpoint, SB-218078 enhanced the cytotoxicity of both topotecan and camptothecin in either HeLa or HT-29 cell lines.

SB-218078 is related to UCN-01 and staurosporine, and we observed that these compounds are all potent inhibitors of Chk1 kinase (Table 1), although SB-218078 appears to be more selective versus PKC. Thus, it seems likely that the checkpoint abrogation of UCN-01 could be also attributed to Chk1 inhibition. Staurosporine has been reported to abrogate DNA-damage checkpoints (29) at very low concentrations, but it has also been reported to cause G2 arrest at slightly higher concentrations (30). This can be explained by the observation that staurosporine inhibits both Chk1 and cdc2 with similar potency, and illustrates why there are few reports of staurosporine overriding DNA-damage arrest. SB-218078 has significantly better selectivity versus cdc2, and is therefore a more useful pharmacological tool for checkpoint modulation. Another recent report identified isogranulatimide as a G2 checkpoint inhibitor by use of a specific cell-based screening assay (31). This compound is a substructural component of SB-218078 and also quite possibly is a Chk1 kinase inhibitor.

We observed that SB-218078 was able to significantly enhance the cytotoxicity of topotecan at concentrations where either agent alone had no significant effect (Fig. 5). The concentration of SB-218078 that caused this effect was 500–625 μM, which is 3–4-fold lower than that required for complete abrogation of G2 arrest by topotecan in a 1-day assay. Similar results have been reported for UCN-01, which required a concentration of at least 0.3 μM to completely abrogate G2 arrest yet was able to significantly enhance the lethal effects of...
γ-irradiation at a 3-fold lower concentration of 0.1 μM (7). This is an interesting observation and may reflect the difference in the duration of the assays. The survival assays are several days in length and are sensitive to lower concentrations of Chk1 inhibitor. Indeed, we also observed that higher concentrations of SB-218078, above 1 μM, did begin to inhibit cell proliferation in these longer-term assays. It is not clear if this was due to inhibition of Chk1 or another effect of the compound, for example, possible weak inhibition of cdc2. Nevertheless, we specifically chose subcytotoxic concentrations of SB-218078 and topotecan in these assays to maximally demonstrate their combined effects. These data suggest that it may not be necessary to completely abrogate G2 arrest to have a significant impact on the ability of cells to handle DNA damage and survive over several days.

In the clonogenic assay (Fig. 6), we sought to compare SB-218078 and UCN-01 directly, using conditions reported previously for UCN-01 (10). This study, which is 10–14 days in length, used a different treatment schedule in which the Chk1 inhibitor is added after transient treatment with camptothecin. Both SB-218078 and UCN-01, at 100 nM, enhanced the cytotoxicity of camptothecin, and the maximal enhancement was apparent at the lowest doses of camptothecin.

A second checkpoint kinase has been identified very recently. This kinase, known as Cds1 in S. pombe and Chk2 in humans, is both structurally and functionally distinct from Chk1 (32–36). Both Chk1 and Chk2/Cds1 phosphorylate cdc25C on Ser-216, and this phosphorylation has been proposed to be a critical event in preventing progression into mitosis (16). Nevertheless, it is unlikely that these two kinases are completely redundant. In S. pombe, both Cds1 and Chk1 must be mutated to override a replication block (32), but mutation of Chk1 alone is sufficient to override DNA-damage arrest (12). Moreover, these kinases may have different additional substrates other than cdc25C. For example, Cds1 can phosphorylate Wee1, whereas Chk1 does not (32). In humans, the extent of overlapping functions for Chk1 and Chk2 remains to be determined. We have demonstrated that the Chk1 inhibitor SB-218078 can inhibit the DNA-damage checkpoint, but the requirement for Chk2 activity in the checkpoint remains to be determined. On the basis of the structural divergence of Chk1 and Chk2, including the kinase domain, it is quite possible that SB-218078 would not be a potent inhibitor of Chk2, but this has not been tested.

The current model of checkpoint regulation mediated via phosphorylation of cdc25C on Ser-216 makes several predictions. For example, phosphorylation of Ser-216 and 14-3-3 binding should be inducible by DNA damage. If this were true, then one could test whether a Chk1 inhibitor such as SB-218078 could block Ser-216 phosphorylation in DNA-damaged cells. However, we and others have observed that Ser-216 on cdc25C is constitutively phosphorylated throughout interphase in both humans and yeast (data not shown; Refs. 16, 34, 37) and thus, DNA-damage-inducible phosphorylation on this residue cannot be observed during the S or G2 phases. It was interesting that the constitutive phosphorylation of Ser-216 was seen in a S. pombe strain lacking both Chkl and Cds1 (37), suggesting that additional kinase(s) also target this site. Indeed, there is a third human kinase, C-TAK1, that can phosphorylate cdc25C on Ser-216 (38). It is possible that cdc25C falls under the regulation of Chk1 or Chk2 only during mitosis, or that there are specific subcellular pools of cdc25C that are subject to Chk1 and/or Chk2 regulation. In any event, the regulation of DNA-damage and replication checkpoints by the Chk1 and Chk2 kinases is quite possibly more complicated than the simple model involving Ser-216 phosphorylation and 14-3-3 binding on cdc25C. Nevertheless, our data suggest a key role for Chk1 in regulating the DNA-damage checkpoint and suggest that Chk1 inhibitors may have therapeutic utility in cancer by enhancing the efficacy of some chemotherapeutics and preventing acquired resistance via repair of DNA damage.

**ACKNOWLEDGMENTS**

We thank Bin-Bing Zhou and Randall Johnson for advice and discussions during the course of these studies; Maureen Ho for providing the cdc2/cyclinB...
kinase data, Sean Flynn for the synthesis of SB-218078, Glenn Hofmann for the PKC data, and NCI for kindly providing UCN-01.

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