Cancer Therapy: Preclinical

Effects of Selective Checkpoint Kinase 1 Inhibition on Cytarabine Cytotoxicity in Acute Myelogenous Leukemia Cells In Vitro

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Abstract

Purpose: Previous studies have shown that the replication checkpoint, which involves the kinases ataxia telangiectasia mutated and Rad3 related (ATR) and Chk1, contributes to cytarabine resistance in cell lines. In the present study, we examined whether this checkpoint is activated in clinical acute myelogenous leukemia (AML) during cytarabine infusion in vivo and then assessed the impact of combining cytarabine with the recently described Chk1 inhibitor SCH 900776 in vitro.

Experimental design: AML marrow aspirates harvested before and during cytarabine infusion were examined by immunoblotting. Human AML lines treated with cytarabine in the absence or presence of SCH 900776 were assayed for checkpoint activation by immunoblotting, nucleotide incorporation into DNA, and flow cytometry. Long-term effects in AML lines, clinical AML isolates, and normal myeloid progenitors were assayed using clonogenic assays.

Results: Immunoblotting revealed increased Chk1 phosphorylation, a marker of checkpoint activation, in more than half of Chk1-containing AMLs after 48 hours of cytarabine infusion. In human AML lines, SCH 900776 not only disrupted cytarabine-induced Chk1 activation and S-phase arrest but also markedly increased cytarabine-induced apoptosis. Clonogenic assays demonstrated that SCH 900776 enhanced the antiproliferative effects of cytarabine in AML cell lines and clinical AML samples at concentrations that had negligible impact on normal myeloid progenitors.

Conclusions: These results not only provide evidence for cytarabine-induced S-phase checkpoint activation in AML in the clinical setting, but also show that a selective Chk1 inhibitor can overcome the S-phase checkpoint and enhance the cytotoxicity of cytarabine. Accordingly, further investigation of the cytarabine/SCH 900776 combination in AML appears warranted.

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Introduction

Cytarabine is the single most active agent available for the treatment of acute myelogenous leukemia (AML), particularly in younger patients, and contributes to the high complete remission rate in this disorder (1, 2). Nonetheless, the majority of AML patients relapse and ultimately die of refractory disease (1–3). As a result, there is considerable interest in identifying mechanisms that contribute to cytarabine resistance and strategies for overcoming this resistance.

A number of mechanisms of cytarabine resistance have been described, including diminished cytarabine import, increased cytarabine degradation, decreased formation or retention of cytarabine triphosphate, or reduced incorporation into DNA if the cells are not cycling. In addition, more recent reports suggest that signaling by the replication checkpoint also contributes to cytarabine resistance (4–7). This checkpoint is activated when replication inhibitors,
shown that Chk1 downregulation enhances the cytotoxicity of cytarabine and other nucleoside analogs \((4, 6, 7, 15)\). These observations provide part of the rationale for the preclinical and early clinical development of Chk1 inhibitors \((14, 16, 17)\). It is important to emphasize, however, that activation of the ATR/Chk1 pathway has not been previously shown in clinical AML during cytarabine therapy.

Two strategies for overcoming replication checkpoint-mediated cytarabine resistance have been previously investigated. On the basis of the observation that Chk1 requires chaperoning by Hsp90 to assume a active conformation \((18, 19)\), cytarabine has been combined with the Hsp90 inhibitor tanespimycin. Although tanespimycin enhances the cytotoxicity of cytarabine in AML cell lines and clinical isolates \textit{in vitro} \((6)\), the combination has proven difficult to administer \textit{in vivo} because of severe toxicities \((20)\) that likely reflect the effects of tanespimycin on multiple Hsp90 clients in normal tissues. Alternatively, cytarabine has been combined with UCN-01 \textit{in vitro} and in the clinical setting \((21, 22)\). UCN-01 inhibits Chk1 \((23, 24)\) and a number of other kinases \((25)\), including Chk2 and phosphoinositide-dependent kinase 1 \((26, 27)\), but has also been difficult to develop clinically because of toxicities in normal tissues, possibly reflecting the effect of inhibiting diverse kinases. More recently described Chk1 inhibitors also inhibit Chk2 and, in some cases, cyclin-dependent kinases \((28)\). Because cyclin-dependent kinases contribute to the cell-cycle progression required for lethal cytarabine incorporation into DNA \((29, 30)\) and Chk2 contributes to apoptosis \((31)\), concern has been raised that these inhibitors might not be as effective at sensitizing cells to replication stress as more selective Chk1 inhibitors \((32)\).

SCH 900776 is a recently described inhibitor that is highly selective for Chk1 relative to Chk2 and cyclin-dependent kinases \((32)\). Additional studies have shown that SCH 900776 enhances the cytotoxicity of hydroxyurea and gemcitabine \textit{in vitro} and \textit{in vivo} without increasing normal tissue toxicities \((32)\). To determine whether there might be a rationale for combining SCH 900776 with cytarabine in...
AML, the present study first assessed whether the replication checkpoint is activated during cytarabine infusion in the clinical setting, and then examined the effect of combining SCH 900776 with cytarabine in human AML cell lines and primary clinical specimens in vitro.

Materials and Methods

Materials

SCH 900776 was synthesized as described (32). Additional reagents were purchased as follows: cytarabine and propidium iodide (PI) from Sigma-Aldrich; Q-VD-OPh from SM Biochemicals; mouse monoclonal anti-Chk1 from Santa Cruz Biotechnology; mouse monoclonal antiphospho-Ser119-histone H2AX from Millipore; rabbit polyclonal antiphospho-Ser417-Chk1 from R & D Systems; and rabbit polyclonal anti-GAPDH, phospho-Ser286, and phospho-Ser345-Chk1 from Cell Signaling Technology. Murine monoclonal antibodies to HSP90 and poly(ADP-ribose) polymerase 1 (PARP1) were gifts from David Toft (Mayo Clinic) and Guy Poirier (Laval University, Ste-Foy, QC, Canada), respectively.

Leukemia samples

After informed consent was obtained under the aegis of Institutional Review Board–approved protocols, bone marrow aspirates were acquired from two cohorts of AML patients. French–American–British (FAB) classification and karyotype were determined in both cohorts by standard techniques (20).

Cohort 1 consisted of adult patients with relapsed or refractory acute leukemia who received therapy on a trial of sequential cytarabine and tanespimycin (20). Bone marrow aspirates were harvested from these patients before therapy and again after 48 hours of treatment with cytarabine, which was administered by continuous infusion at 400 mg/m²/d. Because this second sample was obtained before tanespimycin administration, the effects observed reflect the action of cytarabine alone. At the time of harvest, mononuclear cells were isolated on ficoll-Hypaque gradients, washed with ice-cold serum-free RPMI 1640 medium, and immediately lysed in buffered guanidine hydrochloride under reducing conditions for SDS-PAGE (33).

Cohort 2 consisted of patients with newly diagnosed or relapsed aggressive myeloid disorders who underwent bone marrow aspiration before induction therapy. Immediately after isolation, mononuclear cells from these patients were washed with RPMI 1640 medium, resuspended at 1.5 × 10⁶/mL in Iscove’s modified Dulbecco’s medium containing 20% (v/v) heat-inactivated FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glutamine, and plated at 600,000 cells per plate in MethoCult methylcellulose (StemCell Technologies) containing the indicated concentrations of cytarabine and SCH 900776. Leukemic colonies were counted after a 14-day incubation (6). Peripheral blood mononuclear cells from normal volunteers were assayed in the same fashion except that normal erythroid and myeloid colonies were counted (34, 35).

Cell culture

HL-60, U937 (American Type Culture Collection), and ML-1 cells (a kind gift from Michael Kastan, St. Jude Children’s Hospital, Memphis, TN) were maintained in RPMI 1640 containing 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glutamine (medium A) at concentrations below 1 × 10⁶ cells/mL at all times. All lines were passaged less than 3 months before use. Aliquots were diluted to 3 to 4 × 10⁶ cells/mL in medium A and treated with cytarabine and SCH 900776 (both added from 1,000× stocks in dimethyl sulfoxide (DMSO)) or diluent as described for specific assays.

Replication checkpoint assay

Quadruplicate 100 μL aliquots were treated for 3 to 4 hours with cytarabine, SCH 900776, or the combination, incubated for 20 minutes with 1 μmol/L 3H-thymidine and harvested by transferring to glass filters using a Skatron semiautomatic harvester (Skatron As). The filter-bound cells were lysed with distilled water. Filter-bound radioactivity was determined by liquid scintillation counting. 3H-thymidine incorporation was calculated as the ratio of drug-treated to diluent (0.2% DMSO)-treated control samples.

Cell-cycle analysis

After incubation for 24 hours with cytarabine in the absence or presence of SCH 900776 as indicated in the figures, cells were resuspended in 20 mmol/L HEPES (pH 7.4), 120 mmol/L NaCl, 0.025% Triton X-100, 50 μg/mL RNase A, and 20 μg/mL PI; incubated at 37°C for 30 minutes; and analyzed by flow microfluorimetry. Alternatively, cells were lysed in ice-cold buffer consisting of 0.1% (w/v) sodium citrate, 0.1% (w/v) Triton X-100, and 50 μg/mL PI; incubated overnight at 4°C; and analyzed. No gating was used to generate the histograms.

Immunoblotting

U937 cells were exposed to the indicated concentrations of cytarabine and SCH 900776 for 4 hours, washed with PBS, and lysed in 2 × SDS-PAGE sample buffer (1 × 10⁷ cells/mL). Lysates (2 × 10⁶ cells/lane) were separated by SDS-PAGE, transferred to Immobilon P, and blotted for the indicated antigens. Alternatively, after treatment with drug or diluent for 24 hours, cells were washed once with ice-cold RPMI 1640 medium containing 10 mmol/L HEPES (pH 7.4 at 4°C), and solubilized in buffered 6 mol/L guanidine hydrochloride under reducing conditions (33). Aliquots containing 50 μg protein (determined by the Thermo bicinchoninic acid method) were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed as described (36).

Additional assays

Assays for apoptotic morphologic changes and for the ability of AML cell lines to form colonies in 0.3% agar were performed as described (6).
Results

Replication checkpoint activation in human AML during cytarabine infusion

Although replication checkpoint activation was previously observed in leukemia cell lines treated with nucleoside analogs in vitro, activation of the ATR/Chk1 pathway has not, to our knowledge, been previously evaluated in bone marrow blasts during drug treatment in patients. To address this issue, paired bone marrow aspirates harvested from patients in cohort 1 before therapy and after 48 hours of treatment with single-agent cytarabine (Supplementary Table S1) were immediately upon receipt prepared for immunoblotting, which was conducted with antibodies that detect phosphorylation of Chk1 on Ser$^{317}$, one of two sites phosphorylated by ATR during replication stress (Fig. 1A and ref. 37). If cytarabine were activating the replication checkpoint in vivo, then elevated phosphorylation of Chk1 on Ser$^{317}$ would be expected during the course of the cytarabine infusion. Among 12 paired samples, 3 failed to express Chk1 (Supplementary Table S1). Five of the 9 remaining pairs exhibited increased Chk1 phosphorylation after 48 hours of single-agent cytarabine infusion (Supplementary Table S1; Fig. 1B, lanes 2, 4, and 6), and another pair had a marked decrease in total Chk1 on day 3 (Fig. 1B, lane 8), possibly reflecting activation-induced Chk1 degradation (38). Collectively, these results confirm that the Chk1-dependent replication checkpoint is activated during cytarabine administration in over half of AMLs that detectably express Chk1.

SCH 900776 inhibits Chk1, abrogates cytarabine-induced cell-cycle arrest, and enhances cytarabine-induced killing of human AML cell lines

Subsequent experiments examined the effect of combining cytarabine with the Chk1 inhibitor SCH 900776 (32). As was the case with the clinical AML samples, treatment of U937 cells with cytarabine resulted in increased ATR-mediated Chk1 phosphorylation (phospho-Ser$^{317}$-Chk1, Fig. 2A, lane 6 vs. lane 1). This was accompanied by enhanced phosphorylation of Chk1 at Ser$^{345}$, an autophosphorylation site, and diminished levels of the cell-cycle regulator Cdc25A, which is targeted for proteolysis by Chk1-mediated phosphorylation (Figs. 1A and 2A, lane 6 vs. lane 1). Addition of SCH 900776 decreased the cytarabine-induced Chk1 autophosphorylation at Ser$^{345}$ and prevented Cdc25A downregulation (Fig. 2A, lanes 7–10 vs. lane 6), suggesting that SCH 900776 was disrupting the replication checkpoint. Consistent with this explanation, SCH 900776 also reversed the cytarabine-induced inhibition of $^3$H-thymidine incorporation into DNA (Fig. 2B). In addition, the S-phase accumulation of cells induced by a 24-hour cytarabine exposure was reduced by cotreatment with 100 nmol/L or 300 nmol/L SCH 900776 even though SCH 900776 had no effect on cell-cycle distribution when administered by itself (Fig. 2C), SCH 900776 also increased phosphorylation of Chk1 at Ser$^{345}$ (Fig. 2A, lanes 7–10 vs. lane 6), an effect that has been attributed to inhibition of PP2A-mediated dephosphorylation at that site (39) as well as increased DNA damage-induced signaling caused by incomplete replication fork stabilization, aberrant origin firing and/or override of the replication checkpoint (32). Concomitantly, SCH 900776 also induced increased phosphorylation of H2AX, a marker of DNA damage (Fig. 2A, lanes 7–10 vs. lane 6).

We have previously shown that disruption of the replication checkpoint by gene deletion or RNA interference increases the cytotoxicity of replication stress (5–7, 15). Consistent with these results, treatment with cytarabine and 300 nmol/L SCH 900776 produced an increased number of U937 cells with “subdiploid” DNA content, a feature of apoptosis, compared with either agent alone (Fig. 2C, Supplementary Fig. S1A and S1B). Further experiments showed similar effects in several different AML cell lines, including p53-deficient HL-60 (Fig. 3A and B) and KG1a cells (Supplementary Fig. S1C) as well as p53 wild-type ML-1 cells (Fig. 3D). Additional assays showed increased numbers of cells with apoptotic morphology (K.S. Flatten and S. H. Kaufmann; unpublished observations) and enhanced PARP1 cleavage (Fig. 3C, 3rd panel), a hallmark of caspase activation, confirming increased apoptosis in cells treated with cytarabine + SCH 900776 compared with cytarabine alone. Importantly, however, SCH 900776 was unable to enhance cytarabine-induced apoptosis in K562 cells (Supplementary Fig. S1D), which are apoptosis-resistant because of Bcr/abl-mediated changes in apoptotic pathways (40) that inhibit processes downstream of DNA damage.

At the 24-hour time point, the cytarabine-induced increase in ATR-mediated Chk1 Ser$^{317}$ phosphorylation also remained evident (Fig. 3C, top, lanes 1, 5, and 9); and this phosphorylation (similar to the phosphorylation of Chk1 Ser$^{317}$ in U937 cells) was increased further by SCH 900776 (Fig. 3C, lanes 6–8 and 10–12). Phosphorylation of H2AX was also increased by combining cytarabine and SCH 900776 (Fig. 3C, 4th panel). Interestingly, the H2AX phosphorylation observed after 24 hours of drug treatment, along with the PARP1 cleavage, was markedly diminished by addition of the caspase inhibitor (41) Q-VD-OPh (Fig. 3C, lanes 13–16 vs. 9–12), suggesting that some of the double-strand breaks observed at late time points reflect induction of apoptosis.

SCH 900776 selectively enhances effects of cytarabine on AML cell colony formation

In view of recent results showing that Chk1 inhibition accelerates cisplatin-induced apoptosis without affecting the number of cells ultimately killed (42, 43), we next examined the long-term impact of the cytarabine/SCH 900776 combination using colony forming assays. When U937 (Fig. 4A) or HL-60 cells (Fig. 4B) were exposed to the individual agents or the combination for 24 hours, washed, and plated in soft agar, SCH 900776 had no effect on colony formation by itself but nonetheless enhanced the long-term antiproliferative effects of cytarabine in these assays, meeting the definition of synergy (44).
To determine whether similar effects would be observed in primary clinical isolates from patients with myeloid malignancies, leukemic cells from patients in cohort 2 (Table 1) were exposed to cytarabine ± SCH 900776 or diluent continuously during colony formation in methylcellulose. Four patterns of response were observed (Fig. 5A–D). In some samples, SCH 900776 had no effect by itself but nonetheless enhanced the effects of cytarabine at all concentrations (Fig. 5A) or at the higher concentrations examined (Fig. 5B). In a few samples (Fig. 5C), SCH 900776 not only enhanced the effects of cytarabine but also inhibited colony formation by itself by 40% to 70%. In total, sensitization similar to that shown in Fig. 5A–C was observed in 10 of 14 clinical specimens analyzed (Table 1 and Supplementary Table S2). In contrast, SCH 900776 failed to sensitize 4 of 14 specimens to cytarabine (Fig. 5D, Table 1, and Supplementary Table S2).

In further studies, the relationship between sensitization by SCH 900776 and various features of the malignant myeloid samples was examined in a preliminary fashion. All samples were CD34 positive, reflecting their immature phenotype. As indicated in Table 1, the presence of activating FLT3 mutations did not seem to affect sensitization by SCH 900776, although evaluation of a larger cohort is required to reach a definitive conclusion. The presence of a complex karyotype did not preclude sensitization by SCH 900776, although sensitization was observed in only 2 of 5 samples with a complex karyotype versus 7 of 9 samples without complex karyotype. Importantly, 9 of 10 samples without prior cytarabine exposure were sensitized to cytarabine by addition of SCH 900776. In contrast, only 1 of 4 specimens without prior exposure to SCH 900776 were sensitized by addition of the inhibitor.
specimens from patients with prior cytarabine exposure was sensitized ($P = 0.041$ by Fisher exact test); and that sensitization (patient 10) was modest, raising the possibility that prior cytarabine exposure might affect the ability of SCH 900776 to enhance cytarabine sensitivity.

Discussion

Results of the present study show for the first time that Chk1 undergoes activating phosphorylation in marrow blasts in vivo during cytarabine-containing induction therapy. Building on this result, we also show in human AML cell lines that the selective Chk1 inhibitor SCH 900776 abrogates cytarabine-induced S-phase arrest, increases cytarabine-induced apoptosis, and enhances the effects of cytarabine on colony formation. Likewise, SCH 900776 increases the effects of cytarabine in a majority of primary AML isolates but not normal myeloid progenitors in vitro. This sensitization was observed at SCH 900776 concentrations far below the approximate 5 μmol/L SCH 900776 peak levels observed at the maximum tolerated dose in solid tumor patients. These observations have potentially important implications for current efforts to enhance the efficacy of cytarabine-containing AML regimens.

Previous results have shown that cytarabine activates the ATR/Chk1 checkpoint in tissue culture cell lines in vitro (4–7). To determine whether clinically achievable cytarabine concentrations also activate this checkpoint in vivo, whole cell lysates were prepared from marrow aspirates after 48 hours of exposure to single-agent cytarabine. This study used whole cell lysates to minimize the possibility of inadvertent Chk1 dephosphorylation during cell fractionation; marrow aspirates rather than circulating blasts to maximize the possibility of collecting cells that express Chk1 and incorporate cytarabine into DNA, both of which occur predominantly in S-phase; and samples from a unique trial in which cytarabine was administered for 48 hours without any anthracycline, thereby eliminating the potential confounding effect of a second agent. Using this approach, we observed that 3 of 12 paired leukemia samples lacked detectable Chk1 at baseline (Supplementary Table S1), consistent with our earlier results (45). Because
previous studies have shown that Chk1 expression occurs predominantly during S-phase (46) and correlates strongly with the proliferation marker proliferating cell nuclear antigen in AML samples (45), it is likely that the lack of detectable Chk1 in these samples reflected a low-proliferative fraction. Among the remaining 9 pairs, we observed readily detectable Chk1 phosphorylation at baseline in 7 (Supplementary Table S1), consistent with observations that the ATR/Chk1 pathway is activated and contributes to survival during normal replication (10). After 48 hours of cytarabine infusion, we also observed readily detectable increases in Chk1 phosphorylation in 5 of 9 paired samples that expressed Chk1 as well as decreased Chk1 in an additional sample (Fig. 1B and Supplementary Table S1).

Table 1. Effect of SCH 900776 on cytarabine sensitivity in samples from cohort 2

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<th>Patient number</th>
<th>FAB</th>
<th>Age</th>
<th>Karyotype</th>
<th>Prior hematologic disorder</th>
<th>FLT3 mutation status</th>
<th>Number of prior regimens/prior cytarabine</th>
<th>Response to most recent Rx</th>
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abcSamples were defined as sensitive to single-agent SCH 900776 (+) if colony formation was inhibited by ≥40% at 100 nmol/L SCH 900776.

†, sensitized to cytarabine by SCH 900776; --, not sensitized to cytarabine by SCH 900776.

Quantitative effects of cytarabine, SCH 900776 and the combination are presented in Supplementary Table S2.

CLL, chronic lymphocytic leukemia; MMM, myelofibrosis with myeloid metaplasia; MPN, myeloproliferative neoplasm, not otherwise specified.

†Y, treatment of the patient with a cytarabine-containing regimen at some point before sample acquisition. N, no prior cytarabine exposure.

N.D., not determined. “Complex” indicates the presence of 3 or more numerical and/or structural abnormalities.

ITD, internal tandem duplication. wt, wildtype.
suggesting that the replication checkpoint is activated in at least some leukemias during cytarabine treatment in the clinical setting.

No effect of cytarabine on Chk1 phosphorylation was observed in the other three leukemias despite the presence of detectable Chk1. Because this failure to detectably activate the ATR/Chk1 pathway in response to cytarabine did not seem to correlate with FAB classification or number of prior cytarabine-containing regimens (Supplementary Table S1), further study in a larger cohort is required to better define which AMLs activate this checkpoint during treatment and which do not. Likewise, further study is required to determine whether this checkpoint is activated during cytarabine/anthracycline combination therapy, although the observation that antracyclines activate the ATR/Chk1/Cdc25A pathway (47, 48) certainly makes this likely.

A number of previous publications have shown that deletion or downregulation of components of the replication checkpoint, including Rad9, ATR, or Chk1, enhances the cytotoxicity of cytarabine in various cell lines in vitro (5–7). On the basis of these and additional observations, there have also been several attempts to abrogate this checkpoint in the clinical setting. UCN-01, which inhibits Chk1 (23, 24) and enhances the antiproliferative effects of a number of nucleoside analogs, including cytarabine, in vitro (8, 22, 49), was administered in one such attempt. Unfortunately, UCN-01 had a number of serious drawbacks in the clinic, including a long serum half-life that complicated dosing and severe toxicities when added to other chemotherapeutic agents, possibly reflecting inhibition of a large number of additional kinases (25–27). Likewise, when cytarabine was combined with tanespimycin, which inhibits Hsp90 and thereby prevents folding of catalytically competent Chk1 (19), the combination exhibited severe toxicities in patients with AML (20). Importantly, however, tanespimycin induced little downregulation of Hsp90 client proteins in bone marrow blasts in situ at clinically tolerable concentrations (20), making it impossible to assess the impact of Chk1 downregulation on cytarabine efficacy.

The third generation Chk1 inhibitor SCH 900776 has several advantages over previous agents used to modulate the replication checkpoint. In contrast to the broad effects of UCN-01 (25), SCH 900776 exhibits selectivity for Chk1 among the approximately 50 kinases examined (32). Indeed, checkpoint override assays suggest that SCH900776 is selective for Chk1 in cells at concentrations up to 30 μmol/L (32). Moreover, unlike the checkpoint kinase inhibitor AZD7762, SCH 900776 exhibits little effect on the proapoptotic kinase Chk2 (32). As is the case with any small molecule inhibitor, however, we cannot rule out the possibility that inhibition of additional kinases contributes to the observed effects of SCH 900776.
In the present study, SCH 900776 diminished cytarabine-induced replication checkpoint activation (Fig. 2) and increased cytarabine-induced apoptosis (Fig. 3 and Supplementary Fig. S1) in AML cell lines. This enhanced killing was observed in cells with wild type (ML-1), mutant (U937), or absent p53 (HL-60). These effects were accompanied by increased formation of phospho-Ser139-histone H2AX (Figs. 2A and 3C), a commonly used marker of DNA double strand breaks. Although the increased H2AX phosphorylation observed at short time points (e.g., Fig. 2A) likely reflected replication fork collapse that accompanies Chk1 inhibition (32), the increased H2AX phosphorylation seen at later time points paralleled caspase-mediated PARP1 cleavage and was markedly diminished by caspase inhibition (Fig. 3C). The possibility that two distinct processes, replication fork collapse and apoptosis, can contribute to increased H2AX phosphorylation, raises an important caution when considering H2AX phosphorylation for inclusion as a pharmacodynamic marker of Chk1 inhibition in future preclinical and clinical studies.

Because the typical AML sample contains only 0.5% to 5% of cells in S-phase, a similar biochemical and cell-cycle analysis of the effects of SCH 900776 on cytarabine-induced S-phase progression was not feasible using clinical AML samples. Instead, further experiments used colony forming assays to examine the effects of the cytarabine/SCH 900776 combination. These studies showed that SCH 900776 enhanced the effects of cytarabine on colony formation by human AML cell lines (Fig. 4) as well as primary AML isolates grown ex vivo (Fig. 5, Table 1, and Supplementary Table S2). Whether this enhancement will be observed under different conditions, for example, when leukemia cells are adherent to stroma or in xenografts, remains to be determined in future studies. Importantly, normal myeloid progenitors were affected far less by the addition of SCH 900776 (Fig. 5E), suggesting the possibility of a therapeutic window. While these studies were in progress, Guzi and colleagues reported that SCH 900776 also enhances the antineoplastic effects of gemcitabine in vitro and in human tumor xenografts without appreciably altering the normal tissue toxicities of gemcitabine (32). This selectivity for neoplastic cells, which is also seen with siRNA-mediated knockdown of Chk1, is thought to reflect redundancy in the replication checkpoint in normal cells (7, 50). In view of these results, further studies of the SCH 900776/cytarabine combination seem warranted.

Disclosure of Potential Conflicts of Interest
D. Parry was an employee of Schering-Plough Biopharma when SCH 900776 was developed. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: B.D. Koh, D. Parry, J.E. Karp, S.H. Kaufmann
Development of methodology: B.D. Koh, J.E. Karp, L.M. Karnitz
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