CHK1 and WEE1 inhibition combine synergistically to enhance therapeutic efficacy in acute myeloid leukemia *ex vivo*

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ABSTRACT

Novel combinations targeting new molecular vulnerabilities are needed to improve the outcome of patients with acute myeloid leukemia. We recently identified WEE1 kinase as a novel target in leukemias. To identify genes that are synthetically lethal with WEE1 inhibition, we performed a short interfering RNA screen directed against cell cycle and DNA repair genes during concurrent treatment with the WEE1 inhibitor MK1775. *CHK1* and *ATR*, genes encoding two replication checkpoint kinases, were among the genes whose silencing enhanced the effects of WEE1 inhibition most, whereas *CDK2* short interfering RNA antagonized MK1775 effects. Building on this observation, we examined the impact of combining MK1775 with selective small molecule inhibitors of CHK1, ATR and cyclin-dependent kinases. The CHK1 inhibitor MK8776 sensitized acute myeloid leukemia cell lines and primary leukemia specimens to MK1775 *ex vivo*, whereas smaller effects were observed with the MK1775/MK8776 combination in normal myeloid progenitors. The ATR inhibitor VE-821 likewise enhanced the antiproliferative effects of MK1775, whereas the cyclin-dependent kinase inhibitor roscovitine antagonized MK1775. Further studies showed that MK8776 enhanced MK1775-mediated activation of the ATR/CHK1 pathway in acute leukemia cell lines and *ex vivo*. These results indicate that combined cell cycle checkpoint interference with MK1775/MK8776 warrants further investigation as a potential treatment for acute myeloid leukemia.

Introduction

There is considerable interest in finding novel combination therapies to overcome resistance in acute myeloid leukemia (AML).[1] Checkpoint kinases are often aberrantly regulated in this disease and might, therefore, be an important new class of therapeutic targets in AML. Checkpoint pathways are evolutionarily conserved signaling cascades that are activated in response to DNA damage or replication errors.[2,3] Compared to normal cells, which have redundant checkpoints, AML cells are known to have an impaired G_1 checkpoint and, at the time of relapse, impaired p53 function, causing them to rely heavily on S- and G_2-checkpoints.[4-6] By exploiting these differences in checkpoint signaling between leukemic and normal cells, inhibition of checkpoint kinases could potentially achieve antileukemic activity.

We recently examined RNA interference (RNAi)-induced silencing of 572 kinases for effects on AraC (cytarabine) sensitivity.[7] This study identified *WEE1* and *CHK1* as important determinants of AraC activity in myeloid leukemia cells *in vitro* and *ex vivo*. Both genes are overexpressed in ~50-80% of myeloid leukemias as well as in B- and T-cell lymphoid leukemia samples compared to their levels in healthy bone marrow controls.[7]

WEE1 is a dual specificity kinase that regulates cell cycle progression by catalyzing inhibitory phosphorylation of Tyr-15 and Thr-14 on the cyclin-dependent kinases CDK2 and CDK1, thereby inhibiting progression in S and G_2 phases, respectively.[8] WEE1-deficient cells exhibit a decrease in replication fork speed with subsequent accumulation of cells in S phase,[9] and increased genomic instability.[10] Consistent with these observations, the potent and selective small molecule WEE1 inhibitor MK1775 has shown promise as a chemosensitizer in combination with carboplatin, cisplatin or gemcitabine in early clinical trials in solid tumors.[11,12]

CHK1, an essential serine/threonine kinase that is expressed during S and G_2 phases of the cell cycle[13-16] undergoes activating phosphorylation at Ser-345 and Ser-317 in response to DNA damage and replicative stress.[17] Once activated, CHK1 phosphorylates CDC25A and CDC25C[15,18] thereby failing to activate CDK2 and CDK1. As a result, cells arrest in the S and G_2 phases of the cell cycle.[13-15] CHK1 inhibitors alone or in combination with cytotoxic drugs have exhibited anti-tumor activity in hematologic and solid tumors.[19,20] For example, MK8776, a potent ATP-competitive inhibitor that is selective for Chk1 (IC_{50} = 3 nM) compared to CHK2 (IC_{50} = 1.5 μM) or CDK2 (IC_{50} = 160 nM),[19,21] exhibited promising clinical activity when combined with AraC in AML.[22]

In the past, checkpoint inhibitors were most commonly combined with conventional DNA damaging agents.[12,19,23,24] Given the crucial role of WEE1, we decided to investigate whether selective down-regulation of certain DNA damage

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pathway and checkpoint genes might sensitize to WEE1 inhibition as well. To identify the optimal targets for enhancing the effects of WEE1 pharmacological inhibition by MK1775, we used a customized short interfering RNA (siRNA) library against 41 siRNA from cell cycle checkpoint regulatory, DNA repair and ubiquitination processes. This approach identified ATR/CHK1 pathway inhibition as a potent sensitizer to MK1775 in AML, both in the siRNA screens and by combination with highly selective inhibitors of ATR (VE-821) and CHK1 (MK8776).

Methods

Cell culture and reagents

Primary patients' samples were collected according to Institutional Review Board-approved protocols, separated using Ficoll gradient centrifugation and cultured in RPMI-1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. MK1775 and MK8776 were kindly provided by Merck & Company Inc. (Upper Gwynedd, PA, USA). Roscovitine and VE-821 were obtained from Chemietek (Indianapolis, IN, USA).

High-throughput short interfering RNA screens

The high-throughput siRNA screens were performed using transfection conditions specifically adapted to myeloid suspension cells (see *Online Supplementary Methods*) as described previously.[7] Plates were assayed by CellTiter Glo, an ATP-based luminescent assay, to estimate cell survival and the effect of exposure for 48 h to MK1775 after siRNA-mediated gene silencing. Transfection efficiency in these assays was determined by a reduction in relative cell number after transfection of a custom-designed lethal siRNA (against ubiquitin, Qiagen) compared with the median relative cell number of all kinase siRNA.

Sensitization selection criteria

Sensitization was assessed through a two-step calculation. First, the relative light unit (RLU) for each target was normalized to its respective non-silencing siRNA for the given treatment (siRNA RLU/non-silencing RLU). In the second step, the decrement in cell viability was determined by subtracting the normalized RLU in the presence of MK1775 alone from the normalized RLU of cells treated with the same siRNA + MK1775.

Dual drug-response studies

Drug dose-response experiments with MK1775 were performed with seven concentrations in five cell lines with nine concentrations of MK8776.[7]

Cell cycle analysis

Following treatment with diluent, MK1775, MK8776 or their combination for 24 or 48 h as indicated, cells were sedimented at 140 x g for 5 min, resuspended in ice cold buffer consisting of 50 μg/mL propidium iodide and 0.1% (w/v) Triton X-100 in 0.1% (w/v) sodium citrate, incubated in the dark for a minimum of 4 h, and subjected to flow microfluorimetry on a Becton Dickinson FACSCanto II flow cytometer using a 488 nm laser and 530/30 filter. After 20,000 events had been collected, data were analyzed using ModFit software (Verity Software, Topsham, ME, USA).

Immunoblotting

For immunoblotting, cells were treated with MK1775 and/or MK8776 for 24 h as indicated in the figures, lysed in buffered guanidine hydrochloride, and prepared for sodium dodecylsulfate polyacrylamide gel electrophoresis followed by immunoblotting as recently described.[24]

Colony forming assays

To assess effects on colony formation, HL-60 or U937 cells plated in 0.3% agar in the medium of Pike and Robinson[25] were exposed to one or both drugs for 10-14 days, as indicated in the individual figures, and colonies containing ≥50 cells were counted. Likewise, aliquots containing $6x10^5$ freshly isolated marrow mononuclear cells from patients with AML were plated in Methocult medium (StemCell Technologies) containing one or both drugs (or diluent) and examined on day 14 for leukemic colonies according to established morphological criteria.[26]

Results

RNA interference screen and quality parameters

With the aim of identifying synthetic lethal interactions with WEE1 inhibition, a functional RNAi screen was employed to identify sensitizers to MK1775 in AML cell lines. A customized set of 41 genes was targeted by siRNA (with two different validated siRNA sequences per gene) in four myeloid cell lines, TF-1, THP-1, HEL and MDS-L, using transfection conditions that were optimized for each cell line as described previously.[7] For each run of the screen, cells were transfected with the library in 384-well plates (one siRNA/well). Negative controls included buffer and non-silencing siRNA, whereas a universally lethal siRNA (directed against ubiquitin) served as the positive control. Beginning 48 h after transfection, cells were left untreated (drug control) or treated with one of three doses of MK1775 (250, 750 and 1500 nM) for 48 h, then assayed for relative viability using CellTiter Glo.

Transfection efficiency, determined by the reduction in relative cell viability after transfection of a lethal siRNA in comparison to the median relative cell viability of all siRNA, was 96% and 97% for TF-1, 92% and 98% for THP-l, 92% and 90% for HEL in the two independent screens, and 70% for MDS-L (only one screen performed) (*Online Supplementary Figure S1A*). Non-specific toxicity, measured as the reduction in relative cell viability by non-silencing siRNA compared to transfection reagent only, was 12% and 7% for TF-1, 35% for THP-1 in both screens, 14% and 15% for HEL and 2% for MDS-L (*Online Supplementary Figure S1B*).

Because we custom-designed the siRNA library to target genes that are particularly relevant to WEE1 function, we anticipated a bias toward many positive constructs/hit. Accordingly, rather than defining a stringent criterion for selection of hits, we averaged the RLU signal from the two different siRNA sequences per gene and normalized this average to controls (non-silencing siRNA) first. Subsequently, we determined the magnitude of sensitization by measuring the delta or difference of MK1775 *versus* siRNA + MK1775, representing sensitization of all 41 genes continuously.

Using this parameter, CHK1 siRNA (adequate silencing characterized in *Online Supplementary Figure S2*) sensitized substantively with MK1775 in TF-1 and HEL in two independent screens (Figure 1A-C). In contrast, in THP-1, a complex MLL re-arranged cell line, and in MDS-L, CHK1 siRNA increased CellTiter Glo luminescence. This may indicate potential abrogation of MK1775-induced killing

(*Online Supplementary Figure S1C*). Given the biased gene set towards potential positive hits and the analytical approach, the siRNA gene hits represent a relative order rather than an absolute magnitude of sensitization to MK1775. Importantly, additional sensitizers were found both downstream and upstream of CHK1, including siRNA to NEK11 and Claspin (CLSPN), which are regulated either directly by CHK1[27] or within its pathway,[28,29] and siRNA to ATR, which is upstream of CHK1, was a mild sensitizer in TF-1, HEL and MDS-L at both 250 nM and 750 nM MK1775. In the parallel ATM/CHK2 pathway, CHK2 siRNA was a weak sensitizer to MK1775 in all of the lines except MDS-L and ATM siRNA was a mild sensitizer at 250 nM MK1775 (Figure 1A,B and *Online Supplementary Figure S1C*) but was antagonistic in three lines (THP-1, HEL, MDS-L) at 750 nM MK1775 (Figure 1B, C), indicating a dose-dependent effect. Interestingly, siRNA to WEE1 itself and PKMYT, a WEE1 family kinase,[8] were hits in TF-

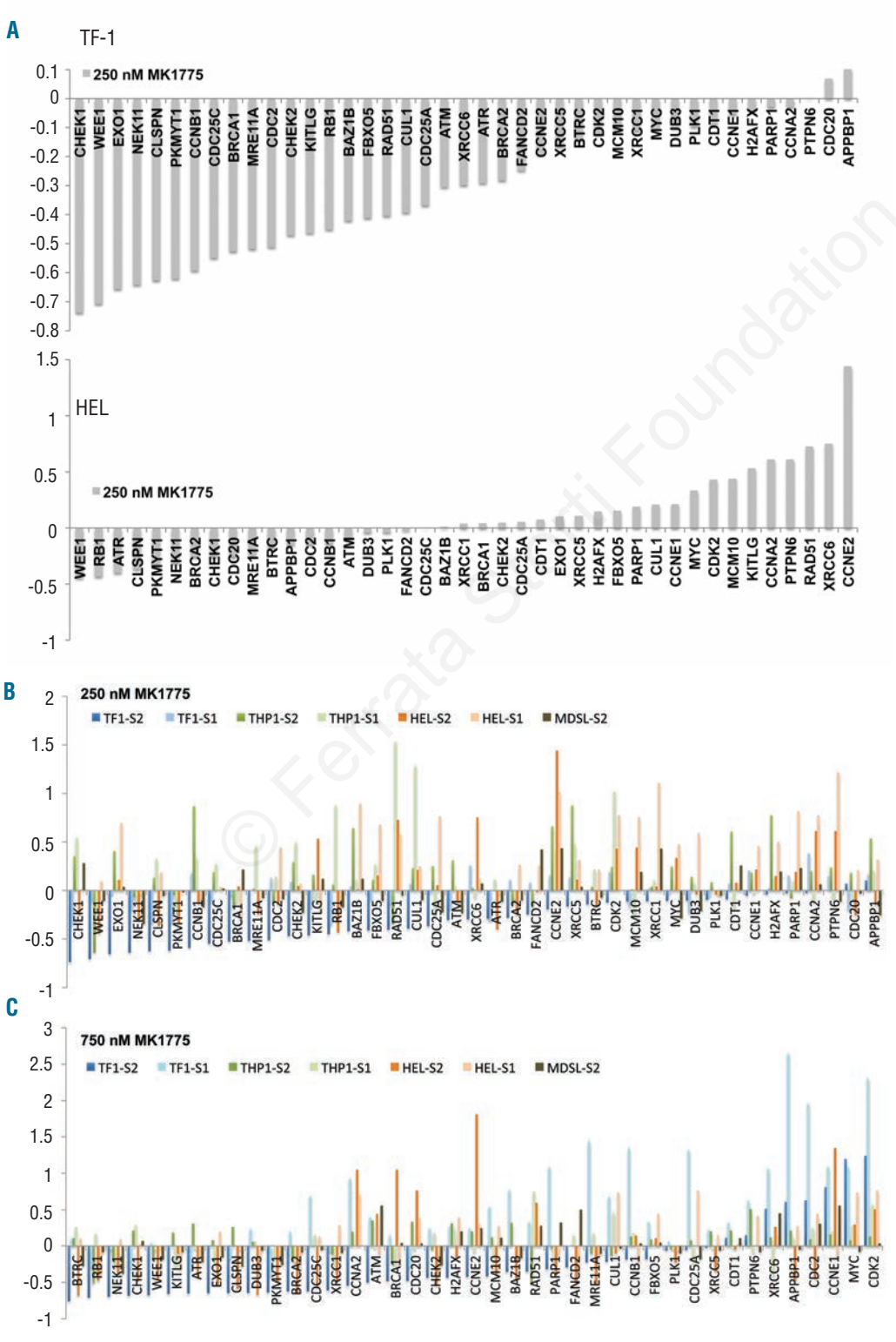

Figure 1. RNAi screen. (A) Graphical representation of the RNAi screen is shown for TF-1 and HEL cells treated with 250 nM MK1775. Data are plotted as the delta between the median of each (siRNA + 250 nM MK1775)/non-silencing siRNA (NS) and siRNA/NS. Each graph is representative of two screens. Graphical representation of the RNAi screen is shown for TF-1, THP-1, HEL and MDSL-2 for both the screens at (B) 250 nM MK1775 and (C) 750 nM MK1775. The RNAi screens were run in duplicate.

1, HEL and THP-1 (Figure 1A,B and *Online Supplementary Figure S1C*), suggesting that MK1775 itself at the concentrations utilized in these screens might have yielded incomplete inhibition of WEE1 kinase activity or that loss of the WEE1 protein has two effects, only one of which is produced by the kinase inhibitor (see *Discussion*).

CHK1, WEE1 and NEK11 siRNA were sensitizing hits at 250 and 750 nM MK1775. At 1500 nM MK1775, a concentration that has significant single agent activity, BRCA2, RAD51, PARP1 and FANCD2 siRNA became the most potent sensitizing hits, indicating that the homologous recombination pathway assumes a more important role in survival as WEE1 inhibition increases. In marked contrast, siRNA targeting species involved in cell cycle progression, such as CDK1 (CDC2), CDK2, cyclin E (CCNE1/2), cyclin A (CCNA2), CDC20 or MYC, became stronger antagonizing hits with increasing MK1775 concentrations (*data not shown*).

In summary, the siRNA results suggest that selective silencing of CHK1 is a potent way to sensitize to WEE1 pharmacological inhibition and ATR silencing shows some sensitization, whereas inhibition of cyclin-dependent kinases may antagonize WEE1 inhibition.

ATR inhibition sensitizes and cyclin-dependent kinase inhibition antagonizes WEE1 inhibition by MK1775

To follow up the siRNA observations, we next determined whether ATR inhibition would sensitize cells to MK1775 and whether CDK inhibition would be antagonistic to MK1775. To test these predictions, MK1775 was combined with VE-821, a selective small molecule inhibitor of ATR, or roscovitine, a selective small molecule inhibitor of CDK1, CDK2 and CDK5. VE-821 sensitized cells to MK1775 in a dose-dependent manner (Figure 2A, *Online Supplementary Table S1A*). In contrast, roscovitine antagonized the effects of MK1775 (Figure 2B, *Online Supplementary Table S1B*). These observations validate the results of the preceding siRNA screen and further support the idea that a functional RNAi approach in high-throughput fashion[7] or with a focused gene set, as demonstrated here, can yield important functional genomic information that can be extended to pharmacological inhibitors *in vitro*.

CHK1 inhibitor enhances the anti-leukemic activity of MK1775 in acute myeloid leukemia cell lines

Based on the observation that CHEK1 was one of the most significant sensitizing hits in our RNAi screen and on the availability of several clinically advanced small molecule inhibitors for CHK1, we focused additional studies around CHK1 inhibition. Specifically we examined MK8776, a highly selective CHK1 inhibitor with very little activity against CHK2.[19,21]

In dose-response studies of MK1775 with MK8776, MK8776 sensitized a panel of six AML and B-cell acute lymphoblastic leukemia cell lines to MK1775 from 2- to 26-fold at 96 h in a dose-dependent manner, as indicated by a shift in EC_{50} values (Figure 3). Importantly, sensitization was observed already at low MK1775 concentrations, starting at 40 and 150 nM, which were achievable plasma concentrations in clinical trials[30], and sensitization at the EC_{50} of MK1775 was potent (*Online Supplementary Table S2*).

Because diminished CellTiter Glo assay readout at the end of prolonged incubation can reflect an effect on cell cycle progression and/or induction of cell death, further experiments examined the impact of MK1775 and MK8776 on cell cycle distribution at 24 h. For these and subsequent studies we examined U937, HL-60 and ML-1 cells, a group of AML lines in which effects of MK8776 alone and in combination with AraC were established in previous work by our group,[24] permitting comparison to the AraC/MK8776 combination. In HL-60 and U937 cells (p53 deficient), MK1775 and MK8776 had little effect on cell cycle distribution as single agents and marginally changed the cell cycle distribution of cells when combined together (Figure 4A,B). Despite the absence of an obvious S phase

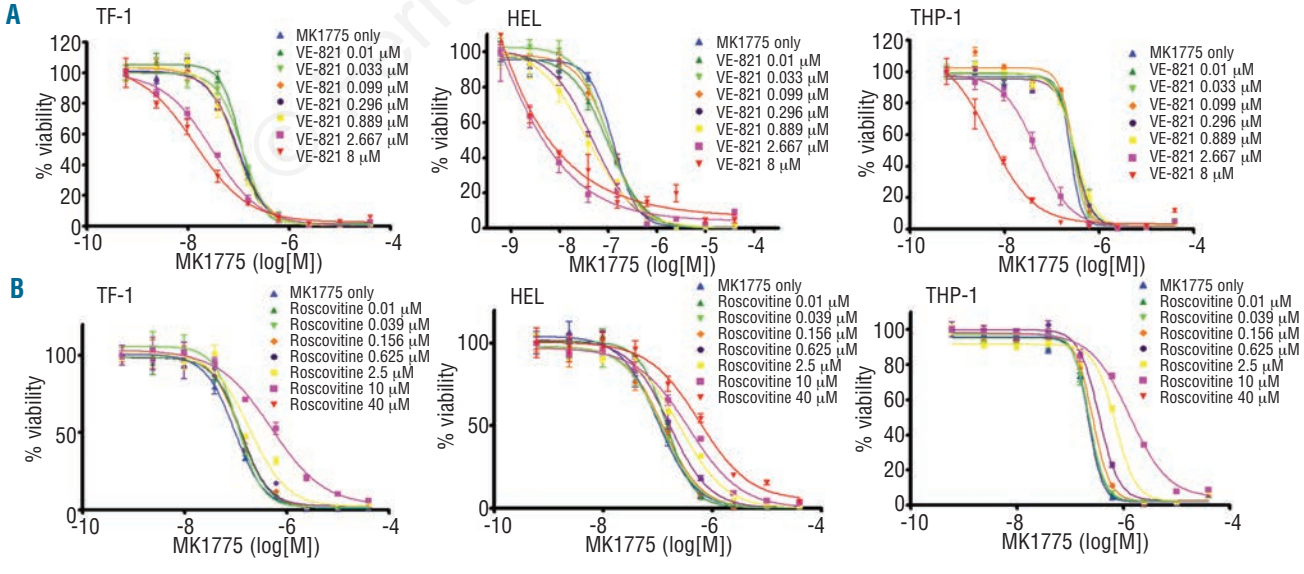

Figure 2. ATR inhibition sensitizes and CDK inhibition antagonizes WEE1 inhibition by MK1775. (A) TF-1, HEL and THP-1 cells were treated with nine doses of MK1775 alone and in combination with 0.011-8 μM VE-821, a selective small molecule inhibitor of ATR kinase. (B) TF-1, HEL and THP-1 cells were treated with nine doses of MK1775 alone and in combination with 0.01-40 μM roscovitine, a selective small molecule inhibitor of CDK1, CDK2 and CDK5. Viability was measured 96 h after drug treatment by CellTiter Glo. A representative result is shown from two independent experiments, each conducted in quadruplicate.

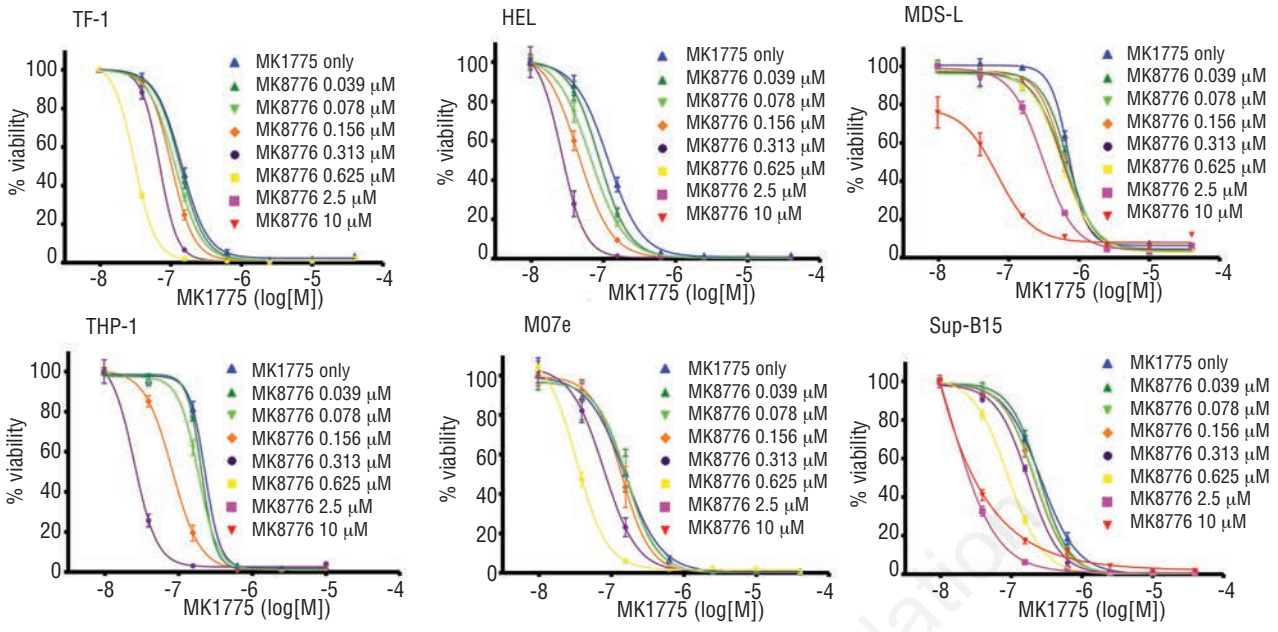

Figure 3. CHK1 kinase inhibition sensitizes to WEE1 kinase inhibition in AML *in vitro*. (A) Drug dose-response curves show a shift in MK1775 EC_{50} upon co-treatment with 0.010-10 μM MK8776, a selective small molecule inhibitor for CHK1 in four AML cell lines, a myelodysplastic syndrome and a B-acute lymphoblastic leukemia cell line. A representative result is shown from two independent experiments, each conducted in quadruplicate.

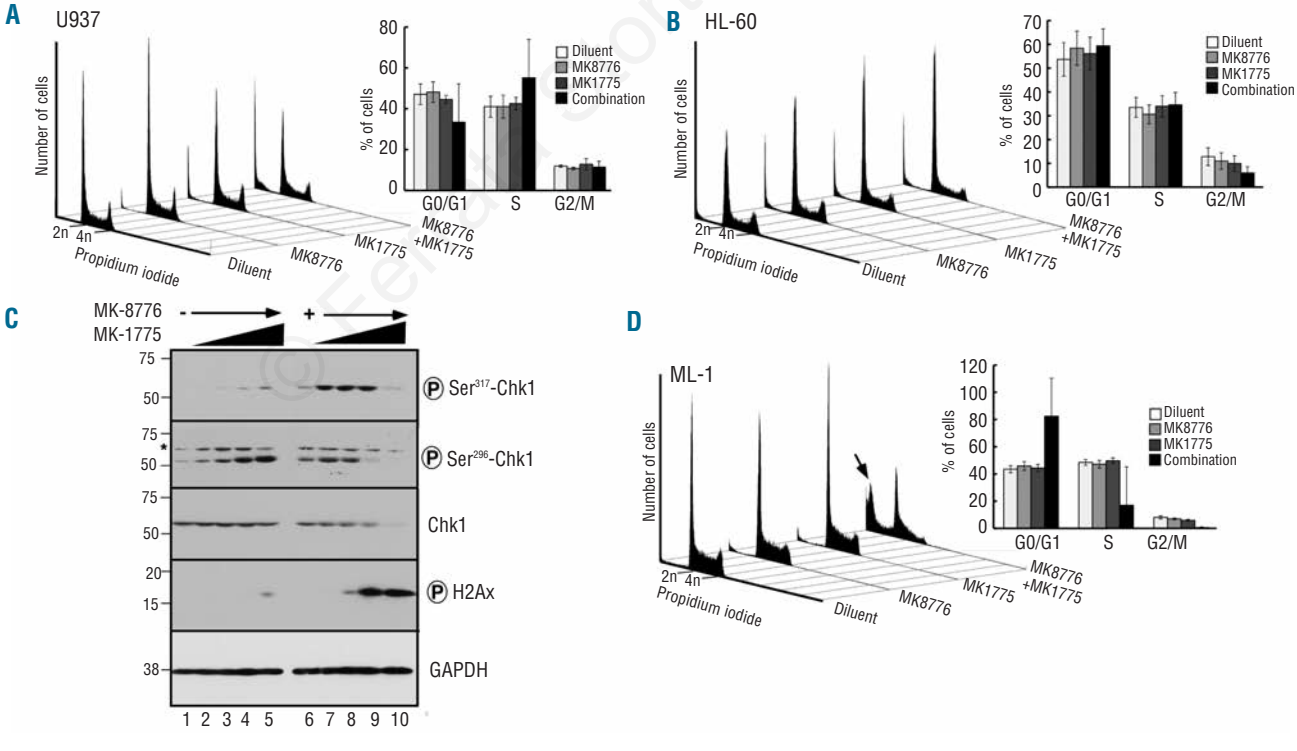

Figure 4. MK1775 activates the ATR/CHK1 pathway but does not induce cell cycle arrest. U937 (A), HL-60 (B) or ML-1 cells (D) were treated for 24 h with diluent, 100 nM MK8776, 100 nM MK1775, or the combination of 100 nM MK8776 + MK1775. At completion of the incubation, cells were stained with propidium iodide and subjected to flow microfluorimetry. Insets: cell cycle distribution as determined by CellQuest software after the indicated treatments. Arrow in (D), subdiploid cells suggesting apoptosis. (C) After U937 cells had been treated with MK1775 at 0, 25, 50, 100 and 200 nM in the absence of MK8776 (lanes 1-5) or presence of 100 nM MK8776 (lanes 6-10), whole cell lysates (50 μg of total cellular protein) were subjected to sodium dodecyl polyacrylamide gel electrophoresis followed by immunoblotting with antibodies that recognize the indicated antigen. Loss of the Chk1 signal in lanes 9 and 10 likely reflects previously described activation-induced degradation.

arrest, treatment with MK1775 induced dose-dependent phosphorylation of CHK1 on Ser-296, which is a CHK1 autophosphorylation site, and on Ser-317, suggesting that ATR has been activated (Figure 4C). Accordingly, it appears that MK1775 activates the ATR/CHK1 pathway (which is consistent with the siRNA data) but does not induce cell cycle arrest. Moreover, when MK8776 was added to MK1775, the MK-1775-induced phosphorylation of CHK1 on Ser-317 (an ATR-mediated event) and the downstream phosphorylation of H2AX were increased (Figure 4C), just as was observed when MK8776 was added to AraC.[24] In ML-1 cells, which harbor wild-type p53, we again saw no change in cell cycle distribution with MK1775 or MK8776 alone, although a trend toward decreased cells in the S and G_2/M phases of the cell cycle was observed with the combination (Figure 4D). In addition, ML-1 cells (Figure 4D, arrow) and, to a lesser extent, U937 cells (Figure 5A) showed evidence of DNA fragmentation, a hallmark of apoptosis, at 24 h with the combination.

Consistent with previous work showing that CHK1 inhibition increases the cytotoxicity of replication stress,[24] by 48 h we consistently observed that MK8776 increased the cytotoxicity of MK1775. Increased subdiploid cells, indicative of apoptosis, were observed in all three lines (Figure 5B-D). Additional assays measuring annexin V externalization confirmed increased apoptosis in cells treated with the combination compared to cells treated with either agent alone (*Online Supplementary Figure S3*).

MK8776 enhances effects of MK1775 on acute myeloid leukemia colony formation

In view of a recent study suggesting that MK8776 can accelerate the induction of apoptosis by the topoisomerase I poison SN-38 without altering the number of cells ultimately killed,[19] we examined the long-term effects of single-agent MK1775 or MK8776 and their combined effects in colony-forming assays. In the two cell lines that formed colonies in soft agar, HL-60 and U937, MK8776 up to concentrations of 200-400 nM had little effect on colony formation by itself (upper insets, Figure 6A,B) but nonetheless markedly enhanced the effects of MK1775 (main panel, Figure 6A,B). Formal analysis of these effects by the median effect method[31] indicated that the combination index values were <1, consistent with a synergistic interaction in both cell lines (lower insets, Figure 6A,B).

To determine whether similar effects would be observed in primary clinical samples from patients with myeloid malignancies, leukemic cells obtained from patients with AML were exposed to MK1775 ± MK8776 continuously for 14 days during colony formation in methylcellulose. Even though MK8776 had little effect on colony formation by itself, it shifted the dose-response curve of MK1775 to the left substantially. Similar effects were observed in four of six AML specimens examined (e.g., Figure 6C-E), whereas two were more resistant to MK1775 as a single agent and were not sensitized (e.g., *Online Supplementary Figure S4A*). Likewise, progenitor myeloid cells from normal volunteers were more resistant to single agent MK1775 and were not appreciably sensitized by MK8776 (*Online Supplementary Figure S4B-C*), suggesting the possibility of selectively affecting malignant myeloid *versus* normal myeloid progenitors.

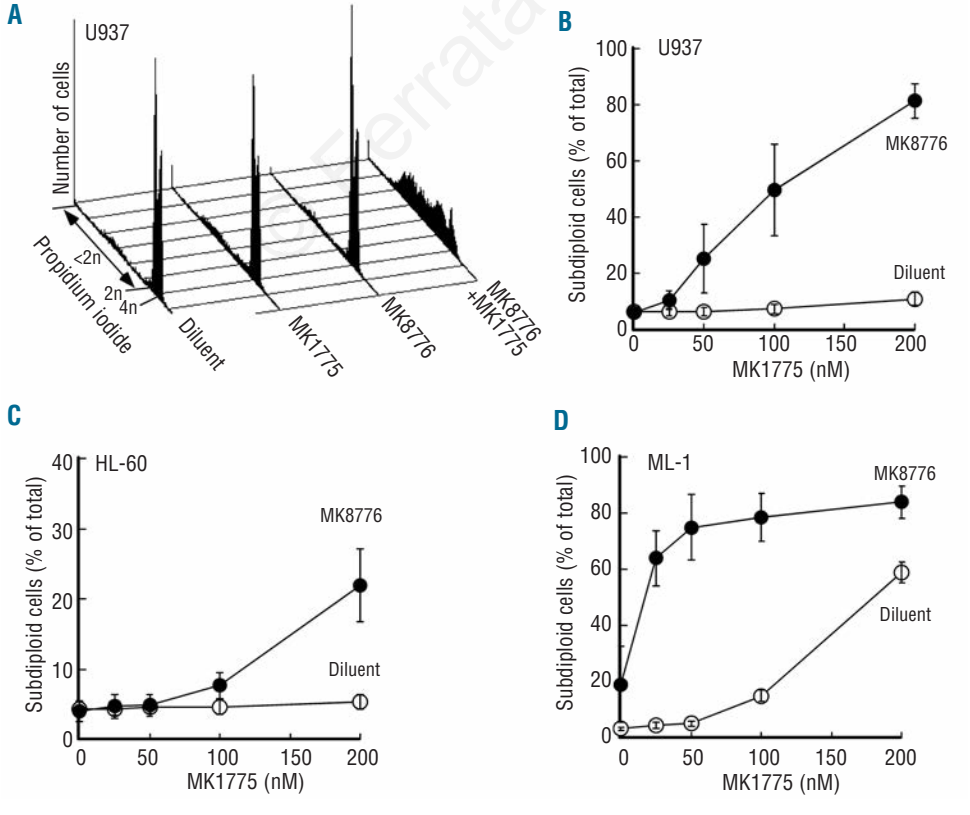

Figure 5. MK8776 enhances MK1775-induced apoptosis. (A) U937 cells were treated for 48 h with diluent, 100 nM MK8776, 100 nM MK1775, or the combination of 100 nM MK8776 + MK1775; stained with prodium iodide; and subjected to flow microfluorimetry. U937 (B), HL-60 (C) or ML-1 cells (D) were treated for 48 h with the indicated concentrations of MK1775 in the absence or presence of 100 nM MK 8776, stained with propidium iodide, subjected to flow microfluorimetry, and analyzed as illustrated in panel (A). Error bars, ± SEM from three or four independent experiments in each cell line. Experiments showing annexin V binding are presented in *Online Supplementary Figure S3*.

Discussion

Targeting DNA damage and cell cycle checkpoints has been proposed as a novel strategy for enhancing the efficacy of anticancer therapy. Toward this end, agents targeting DNA repair pathway components, including Chk1 and WEE1, are typically combined with DNA damaging agents such as AraC or cisplatin.[7,22,23,30] In the present study we report the first siRNA screen for pathways that sensitize to WEE1 inhibition and demonstrate for the first time the potential anti-leukemic activity of combined WEE1 and CHK1 inhibition in primary AML samples.

Our initial goal was to identify a molecular target that would sensitize AML cells to WEE1 inhibition. Because of the recently recognized role of WEE1 during S phase,[10] we focused on proteins and pathways related to CHK1, including proteins such as CHK1, ATR and CDK/cyclin complexes that could potentially be targeted with small molecule inhibitors. We assembled a customized gene list to identify genes that would sensitize leukemia cells to killing by the WEE1 inhibitor MK1775 when knocked down by siRNA. We identified that two independent sequences of siRNA to CHK1 strongly enhance the anti-proliferative effect of MK1775 compared to MK1775 alone in two of four leukemic cell lines tested. Building on this observation, we subsequently showed that pharmacological CHK1 inhibition synergistically enhanced MK1775 antiproliferative effects in AML cell lines and in primary AML samples.

For the most part the results of our siRNA screen and inhibitor studies are consistent with one another. However, the effects of mRNA down-regulation by siRNA and small molecule inhibitors are not always completely comparable.[32] This could be due to several factors including: (i) the ability to achieve greater inhibition of enzymatic signaling with small molecule inhibitors than with siRNA, and (ii) the non-enzymatic (scaffolding or dominant negative) effects of certain proteins, which can contribute to the effects of small molecule inhibitors but are lost when the protein is down-regulated by siRNA. Greater inhibition of CHK1 with a small molecule inhibitor might explain why MK8776 sensitizes to MK1775 more effectively than Chk1 siRNA in some of the cell lines (Figures 1 and 3). To search for alternative explanations, we also examined expression of WEE1 and CHK1 by immunoblotting but did not observe a clear correlation between protein expression levels and degree of sensitization when the two drugs were combined (*data not shown*).

Importantly, MK8776 had little impact on the effects of

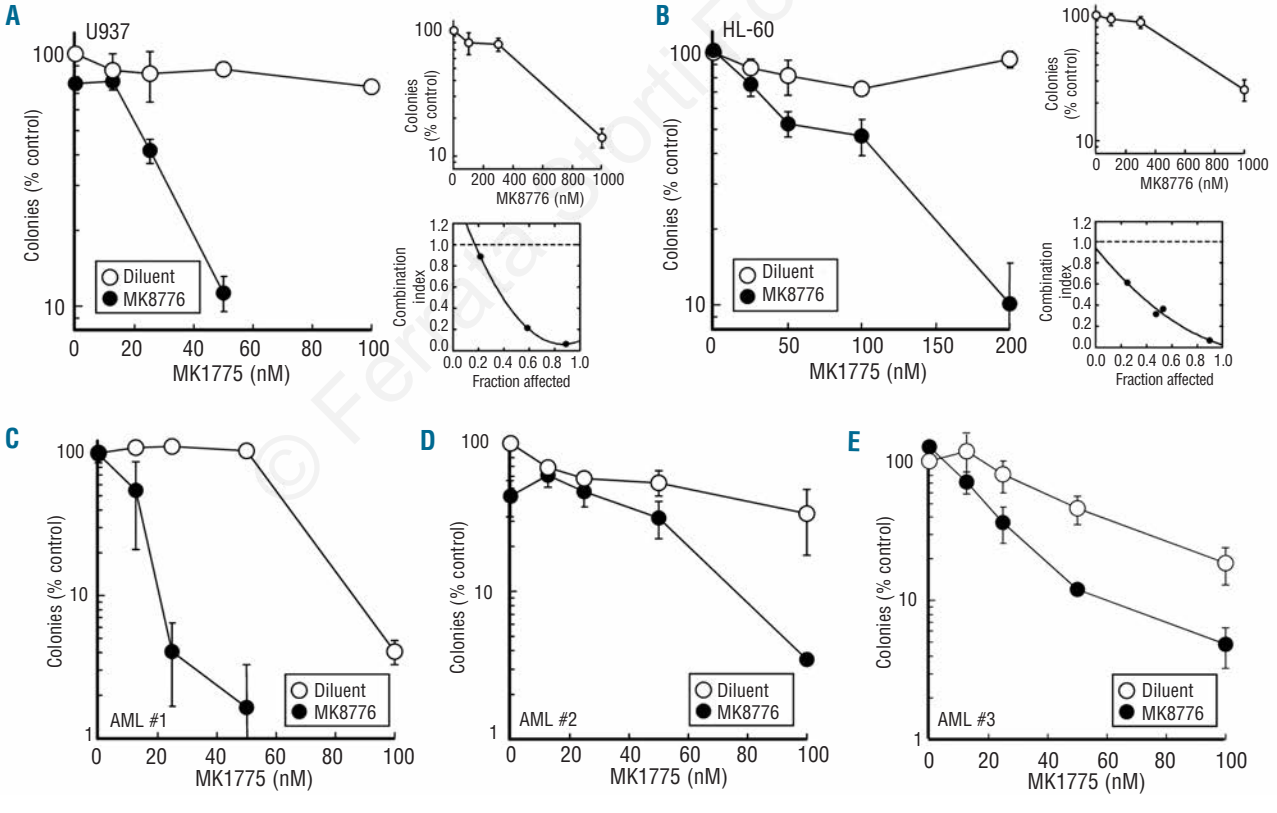

Figure 6. MK8776 enhances MK1775-induced antiproliferative effects in colony-forming assays of AML cell lines and primary AML specimens. U937 (A) and HL-60 (B) cells were plated in 0.3% agar containing the indicated concentrations of MK1775 in the absence or presence of 100 nM MK8776. Colonies were counted at 14 days and compared to those of samples containing diluent (0.2% DMSO). Upper insets in (A) and (B), the same cells were plated in 0.3% agar containing the indicated concentrations of MK8776. Lower insets in (A) and (B), combination index values calculated from data in the main panel and upper inset under the assumption that effects of the two agents are mutually exclusive. Note that combination values <1.0 indicate synergy. Error bars in (A) and (B): ± SEM from quadruplicate samples. (C-E). Three different AML samples were plated in Methocult® methycellulose containing the indicated concentrations of MK1775 in the absence or presence of 100 nM MK8776. Leukemic colonies were counted at 14 days and compared to those in samples containing diluent (0.2% DMSO). Error bars in (C-E): range of values from duplicate plates. As indicated in the text, four additional AML samples and three additional normal controls were examined.

MK1775 in normal myeloid progenitor cells (*Online Supplementary Figure S4*), just as MK8776 did not enhance the effects of AraC in normal progenitors,[24] pointing toward a possible therapeutic window in AML cells. The sensitization in primary AML leukemic cells was observed at concentrations of MK1775 and MK8776 that can be easily achieved in the clinic.[30]

In the siRNA screens, a number of other hits also converged around CHK1, including NEK11 and CLSPN. NEK11 plays an important role in regulation of the DNA damage-induced G_2/M checkpoint; NEK11 is activated by CHK1-mediated phosphorylation, which is important for CDC25A degradation.[27] CLSPN is a co-activator of CHK1 in response to DNA damage.[28] CHK1 has also been shown to interact with CLSPN to activate the ubiquitination of proliferating cell nuclear antigen (PCNA), thereby facilitating continued replication of stressed forks.[29] Interestingly, NEK11 is also downstream of cyclin-dependent kinases[33] as well as ATM and ATR[27] further solidifying the crucial role of CHK1 as an important downstream player and integrator of cell cycle checkpoint signaling. At lower (250 nM) or intermediate (750 nM) concentrations of MK1775, both WEE1 and PKMYT1 were also hits in the siRNA screens with MK1775. This led to the speculation that absence of WEE1 mRNA transcript/protein has effects that are different from and complementary to inhibiting WEE1 kinase activity.

Both CHK1 and WEE1 are regulators of the intra-S phase and G_2/M cell cycle checkpoints.[2,9,15,16,34] The enhanced activity of the MK1775/MK8776 combination relative to either agent alone highlights the notion that Chk1 and WEE1 have distinct but complementary functions. While MK1775 and MK8776 are chemosensitizers that enhance the antiproliferative effects of cytotoxic DNA damaging agents such as AraC and gemcitabine.[7,11,12,19,23,24,35] these agents also appear to cause DNA damage on their own. In particular, our results suggest that MK1775 activates the ATR/Chk1 pathway in AML lines, as indicated by Chk1 phosphorylation at Ser-317 and autophosphorylation at Ser-296 (Figure 4C, lane 5). This is accompanied by increased phosphorylation of H2AX, a marker of DNA damage (Figure 4C, lane 5). MK8776 has previously been reported to increase DNA damage-induced signaling due to incomplete replication fork stabilization and origin firing.[9] Just as MK8776 enhances the cytotoxicity of antimetabolites, such as AraC, which activate the S phase checkpoint,[19,24,36] it also appears to enhance the damaging effects of MK1775 after S phase checkpoint activation. Addition of MK8776 resulted in MK1775-induced phosphorylation of Chk1 on Ser-317 and H2AX phosphorylation on Ser-139 (Figure 4C, lanes 9 and 10) as well as evidence of increased DNA fragmentation, as observed by an increase in subdiploid cells (Figures 4A,D and 5). This enhanced toxicity is also manifest as a decrease in the ability of cells to form colonies (Figure 6) and an increase in the number of cells that bind annexin V, a hallmark of apoptosis (*Online Supplementary Figure S3*).

Interestingly, functional siRNA screens indicate that some of the pathways that sensitize to MK1775 appeared to do so in a manner that is MK1775 dose-dependent. At 1500 nM MK1775, WEE1 and PKMYT1 sensitized less, whereas genes of the homologous recombination pathway became more prominent sensitizers (*data not shown*). In contrast, CDK genes were antagonizing hits over a broad range of MK1775 concentrations, indicating a requirement for continued, at least partial, cell cycle progression mediated by cyclin-dependent kinases (Figure 1B,C). This conclusion is further supported by the antagonizing effect of adding roscovitine to MK1775 (Figure 2). Overall, these data suggest a model in which MK1775 alone induces limited DNA damage that is prominently enhanced by Chk1 inhibition. Continued cell cycle progression, mediated by cyclin-dependent kinases, is required at least to some degree to observe these effects. The inhibitory effect of combining the CDK inhibitor roscovitine with MK1775 (Figure 2) provides an important cautionary note that such combinations should probably be investigated in future clinical studies of WEE1 inhibitors.

Consistent with our results in AML, recent reports have shown the anti-proliferative effects of pharmacological Chk1 and WEE1 inhibition mainly in solid tumor malignancies.[37-40] Davies *et al.* performed a medium throughput screen to the Chk1 inhibitor AR458323 and identified WEE1 as their top hit in one lung cancer and two prostate cancer cell lines.[38] In a separate study by Carrassa *et al.*, high throughput siRNA screening identified WEE1 kinase to be synthetically lethal with the small molecule Chk1 inhibitor PF-00477736 in ovarian cancer cell lines.[39] Our study took the opposite approach and examined siRNA that sensitize to the WEE1 inhibitor MK1775. In contrast to the non-specific and broad effects of UCN-01, AR458323, PF-00477736 and AZD7762 used in previous studies, the third generation Chk1 inhibitor MK8776 selectively and preferentially inhibits Chk1 amongst the kinases examined, including cyclin-dependent kinases.[19,21] This is especially important in the context of antagonism between MK1775 and inhibition of cyclin-dependent kinases (by siRNA and roscovitine), at least in leukemia cells. Consequently, our study suggests that a more selective Chk1 inhibitor such as MK8776 should be combined with MK1775.

In conclusion, RNAi screening identified CHK1 as the top sensitizer to MK1775. *In vitro* and *ex-vivo* data indicate that combined treatment with a WEE1 inhibitor and a selective Chk1 inhibitor provides greater activity than either drug alone. While further investigation is needed to better define AML subsets that might be particularly susceptible to this combination, e.g., AML with enhanced basal levels of DNA damage that are more sensitive to single-agent Chk1 inhibition,[3] the present data provide a strong rationale for further preclinical and possible clinical investigation of combined WEE1 and Chk1 inhibitors in leukemias.

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