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Synergistic anti-leukemic interactions between panobinostat and MK-1775 in acute myeloid leukemia ex vivo

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MK-1775 is the first-in-class selective Wee1 inhibitor which has been demonstrated to synergize with CHK1 inhibitors in various malignancies. In this study, we report that the pan-histone deacetylase inhibitor (HDACI) panobinostat synergizes with MK-1775 in acute myeloid leukemia (AML), a malignancy which remains a clinical challenge and requires more effective therapies. Using both AML cell line models and primary patient samples, we demonstrated that panobinostat and MK-1775 synergistically induced proliferation arrest and cell death. We also demonstrated that panobinostat had equal anti-leukemic activities against primary AML blasts derived from patients either at initial diagnosis or at relapse. Interestingly, treatment with panobinostat alone or in combination with MK-1775 resulted in decreased Wee1 protein levels as well as downregulation of the CHK1 pathway. shRNA knockdown of CHK1 significantly sensitized AML cells to MK-1775 treatment, while knockdown of Wee1 significantly enhanced both MK-1775- and panobinostat-induced cell death. Our results demonstrate that panobinostat synergizes with MK-1775 in AML cells, at least in part through downregulation of CHK1 and/or Wee1, providing compelling evidence for the clinical development of the combination treatment in AML.

Introduction

Acute myeloid leukemia (AML) has a guarded prognosis, with overall survival rates of 25% and 65% in the adult and pediatric populations, respectively.[1,2] Standard treatment for AML consists primarily of a combination of cytarabine and an anthracycline.[3] These drugs are thought to act by targeting DNA in dividing cells, leading to DNA damage.[4,5] The DNA damage triggers activation of the cell cycle checkpoints, leading to cell cycle arrest and DNA damage repair. Therefore, activation of the cell cycle checkpoints remains a possible mechanism of drug resistance leading to treatment failure for AML.

Checkpoint kinase 1 (CHK1) plays an important role in replication initiation and fork stability, homologous recombination repair, progression of the cell cycle, and the S and G2/M cell cycle checkpoints.[6-9] In response to DNA damage or replication stress, CHK1 phosphorylates CDC25 phosphatases, inhibiting activation of CDK1/CDK2 and arresting cell cycle progression allowing for DNA repair and cell survival.[6,10-12] Another important checkpoint kinase is Wee1, whose primary function is inhibitory phosphorylation of CDK1 and CDK2 on Tyr-15 (Y15), which prevents cell cycle progression.[13,14] Thus, targeting these checkpoint kinases would interfere with the DNA damage response, allowing for accumulation of irreparable DNA damage and eventually leading to cell death. Therefore, inhibition of checkpoint kinases in combination with DNA damaging agents may become a therapeutic strategy for the treatment of various malignancies.[15-18]

Histone deacetylase (HDAC) inhibitors (HDACIs) have demonstrated anticancer activity in numerous malignancies including AML.[19-26] They have been demonstrated to induce cell cycle arrest, differentiation, and apoptosis in cancer cells, but less so in normal cells.[19-26] Though their single-agent efficacy in the clinic has been modest,[27-31] there are many clinical trials investigating combination therapies (NCT01242774, NCT01742793, NCT02061449, and NCT02145715, clinicaltrials.gov). Previously, we demonstrated that panobinostat, a pan-HDACI which was recently approved by the US FDA for the treatment of multiple myeloma, downregulates the CHK1 pathway in AML cells.[32,33] Also, we demonstrated that MK-1775, a potent and

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selective Wee1 inhibitor, synergizes with LY2603618, a CHK1 selective inhibitor, in AML cells.[34] Therefore, it is conceivable that the combination of panobinostat and MK-1775 would result in synergistic anti-leukemic activity in AML cells.

In this study, we demonstrate that panobinostat synergizes with MK-1775 to induce proliferation arrest and cell death in AML cell lines and primary patient samples. The combined panobinostat and MK-1775 treatment results in downregulation of Wee1 and CHK1. We also demonstrate that panobinostat treatment alone downregulates CHK1 and Wee1. Furthermore, shRNA knockdown of CHK1 enhances MK-1775-induced cell death, while shRNA knockdown of Wee1 enhances single drug panobinostat- and MK-1775-induced cell death. The results presented here support the clinical development of panobinostat in combination with MK-1775 for the treatment of AML.

Results

Panobinostat cooperates with MK-1775 in AML cells

To determine if panobinostat synergizes with MK-1775 in AML cells, we first assessed the effects of the 2 agents, alone or combined, on cell proliferation in U937 cells by MTT assays. The combined treatment resulted in synergistic anti-leukemic interactions, as determined by standard isobologram analyses, regardless of drug administration schedule (**Fig. 1A-C**). For ease of experimental setup, simultaneous treatment was used from here on out. We then confirmed the additive-to-synergistic anti-leukemic activities of the 2 agents in CTS, MOLM-13, and OCI-AML3 AML cell lines (**Fig. 1D-F**).

Next, we investigated panobinostat treatment in primary patient samples. Panobinostat IC_{50}s, as determined by MTT assays, for diagnostic AML blast samples obtained from patients at initial diagnosis ($n = 32$) or at relapse ($n = 7$) were similar (median IC_{50} 15.9 and 24.8 nM, respectively, $p = 0.129$, **Fig. 2A**). Then we determined *Wee1* transcript levels by real-time RT-PCR in the primary patient samples. Interestingly, panobinostat IC_{50}s positively correlated with *Wee1* transcript levels (**Fig. 2B**, $r = 0.37$, $p = 0.025$), suggesting that Wee1 may play an important role in panobinostat sensitivity in AML. We then tested the combined drug treatment in primary patient samples ($n = 11$), which had sufficient number of cells, by MTT assays. Consistent with the results obtained in the AML cell lines, MTT assays and CompuSyn software analyses revealed that the combination of panobinostat and MK-1775 resulted in additive-to-synergistic anti-leukemic interactions in all of the primary patient samples tested (**Table 1**). MK-1775 has been reported to reach maximal plasma concentrations of 400-500 nM when

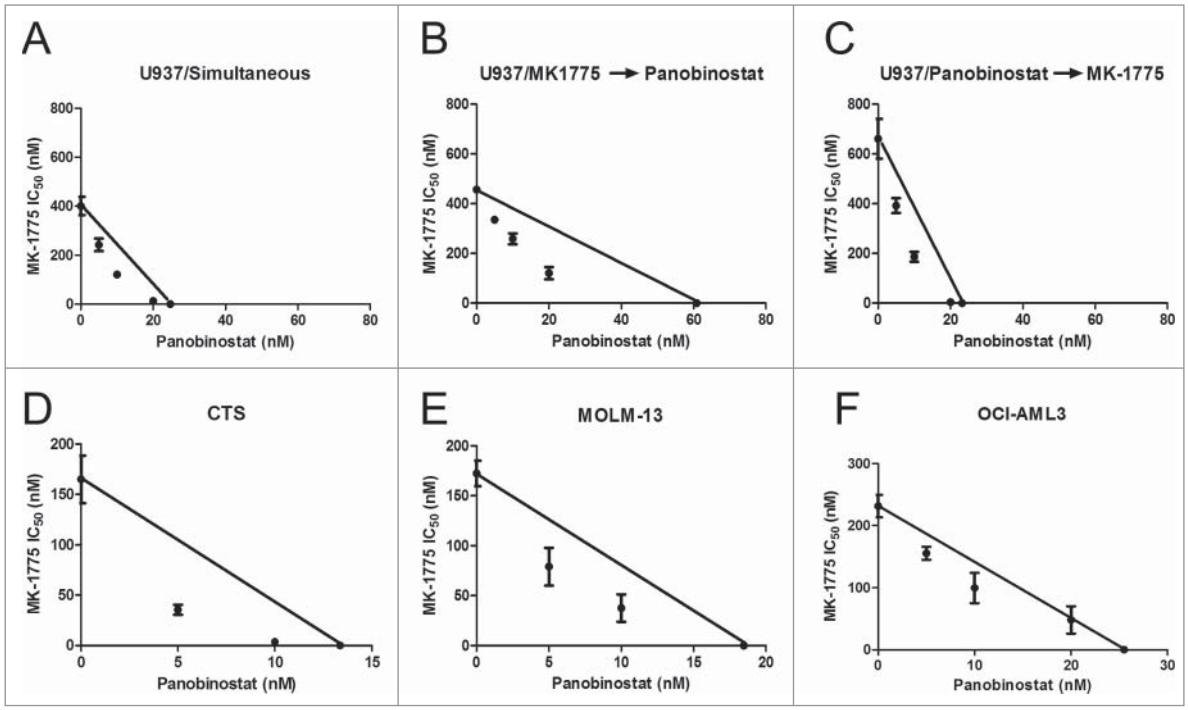

Figure 1. Panobinostat synergizes with MK-1775 to induce proliferation arrest in AML cells. Panels **A-C**: U937 cells were treated for 72 h simultaneously with MK-1775 -> Panobinostat (Pan), pretreated with MK-1775 for 24 h then simultaneous for 48 h (MK-1775 Panobinostat), or pretreated with panobinostat for 24 h then simultaneous for 48 h (Panobinostat -> MK-1775). Panels **D-F**: CTS (**Panel D**), MOLM-13 (**Panel E**), and OCI-AML3 (**Panel F**) cells were treated simultaneously with the 2 agents for 72 h. Viable cells post drug treatments were detected using MTT reagent. Anti-leukemic interactions between MK-1775 and panobinostat were determined by standard isobologram analyses. The IC_{50} values of each drug are plotted on the axes; the solid line represents the additive effect, while the points represent the concentrations of each drug resulting in 50% inhibition of proliferation. Points falling below the line indicate synergism whereas those above the line indicate antagonism. MTT assays were repeated at least 3 times and the data are presented as mean values ± s.e.m.

administered orally twice daily for 2.5 days[35-37] and the steady-state plasma concentrations of panobinostat range from 15-22 nM over 48 h (Novartis Investigator's brochure). Our MTT data shows ex vivo panobinostat IC_{50}s were clinically achievable or slightly higher than clinically achievable concentrations, while MK-1775 IC_{50}s varied widely, ranging from 233 nM to almost 6 μM and were at or significantly higher than maximal plasma concentrations. However, when clinically achievable concentrations of panobinostat were combined with MK-1775, the IC_{50}s for MK-1775 for all but one of the patient samples was at or below maximal MK-1775 plasma concentrations. Together, our results demonstrate global additive-to-synergistic anti-leukemic interactions between MK-1775 and panobinostat in AML.

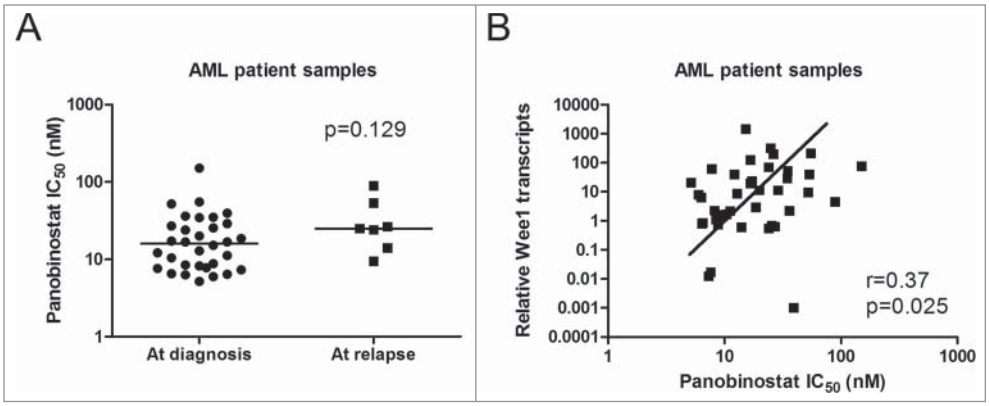

Figure 2. Wee1 transcript levels positively correlate with panobinostat IC_{50}s in *ex vivo* primary AML patient samples. Panel **A**: *Ex vivo* panobinostat sensitivity was determined by MTT assays in diagnostic AML blast samples. The horizontal lines indicate median panobinostat IC_{50} in each group of AML patient samples. Statistical significance between panobinostat IC_{50} for cases at initial diagnosis (n = 32) and cases at relapse (n = 7) was calculated using Mann-Whitney 2-sample U test. Panel **B**: Total RNAs were isolated from primary patient samples and *Wee1* transcript levels were quantified by Real-time RT-PCR. The relative *Wee1* transcript levels (normalized to *GAPDH*) were calculated using the comparative *Ct* method, and graphed versus the panobinostat IC_{50}s. The relationship between *Wee1* transcript levels and panobinostat IC_{50}s was determined by the nonparametric Spearman rank correlation coefficient.

Panobinostat synergistically enhances MK-1775-induced cell death in AML cells

We next assessed the effects of clinically achievable concentrations of panobinostat and MK-1775 treatments on cell death in 2 representative AML cell lines, U937 and CTS. The cells were treated with panobinostat and MK-1775, alone or in combination, for 24 h and then subjected to annexin V/propidium iodide (PI) staining and flow cytometry analyses. Panobinostat treatments significantly enhanced MK-1775-induced cell death in both U937 and CTS cells (**Fig. 3A-D**). The combination index (CI) values (determined using CompuSyn software) for the U937 cells treated with 10 nM panobinostat in combination with 250 nM or 500 nM MK-1775 were 0.95 and 0.73, while cells treated with 20 nM panobinostat in combination with 250 nM or 500 nM MK-1775 were 0.39 and 0.40, respectively. These results revealed that the drug combination did indeed result in additive to synergistic induction of cell death in U937 cells. Similar results were obtained in the CTS, though the CI values (10 nM panobinostat in combination with 125 nM or 250 nM MK-1775 were 0.6 and 0.6, while 20 nM panobinostat in combination with 125 nM or 250 nM MK-1775 were 0.5 and 0.5) indicated that all of the combinations tested were synergistic. Similar results were also obtained in 3 primary patient samples, though 2 of the data points for one patient sample (CI

Table 1. Effects of panobinostat (Pan) on MK-1775 anti-leukemic sensitivity in diagnostic AML blasts

		MK-1775 IC_{50} (nM) in the absence or presence of panobinostat			
AML patient samples	**Panobinostat IC_{50} (nM)**	**0 nM Pan**	**5 nM Pan**	**10 nM Pan**	**20 nM Pan**
AML#0	27.0	5996.5	2506.0 (0.61)	1408 (0.54)	157.1 (0.77)
AML#1	16.7	2448.5	684.7 (0.58)	200.7 (0.68)	ND
AML#2	34.9	3204.0	1682.0 (0.66)	1156.0 (0.65)	925.0 (0.86)
AML#3	55.2	461.0	383.6 (0.92)	307.4 (0.85)	236.4 (0.87)
AML#4	53.3	844.0	591.3 (0.79)	734.0 (0.70)	262.2 (0.69)
AML#5	14.0	948.3	449.2 (0.83)	130.1 (0.85)	ND
AML#6	34.6	233.7	168.3 (0.86)	139.8 (0.88)	113 (1.05)
AML#7	26.3	325.0	244.5 (0.94)	186.6 (0.95)	67.6 (0.97)
AML#8	36.1	887.9	651.6 (0.87)	528.3 (0.86)	302.7 (0.89)
AML#10	25.4	439.7	309.6 (0.90)	210.8 (0.87)	91.9 (1.00)
AML#13	17.0	3579.2	657.2 (0.47)	396.8 (0.70)	ND

Note: IC_{50}s are presented as mean of duplicates from one experiment. Numbers in the parentheses represent the combination index values. CI<0.9 indicates synergistic, 0.9<CI<1.1 indicates additive, and CI>1.1 indicates antagonistic anti-leukemic interactions. ND - not determined. AML#4, AML#5, and AML#7 were derived from patients at relapse. The rest of the primary AML patient samples were derived from patients at first diagnosis.

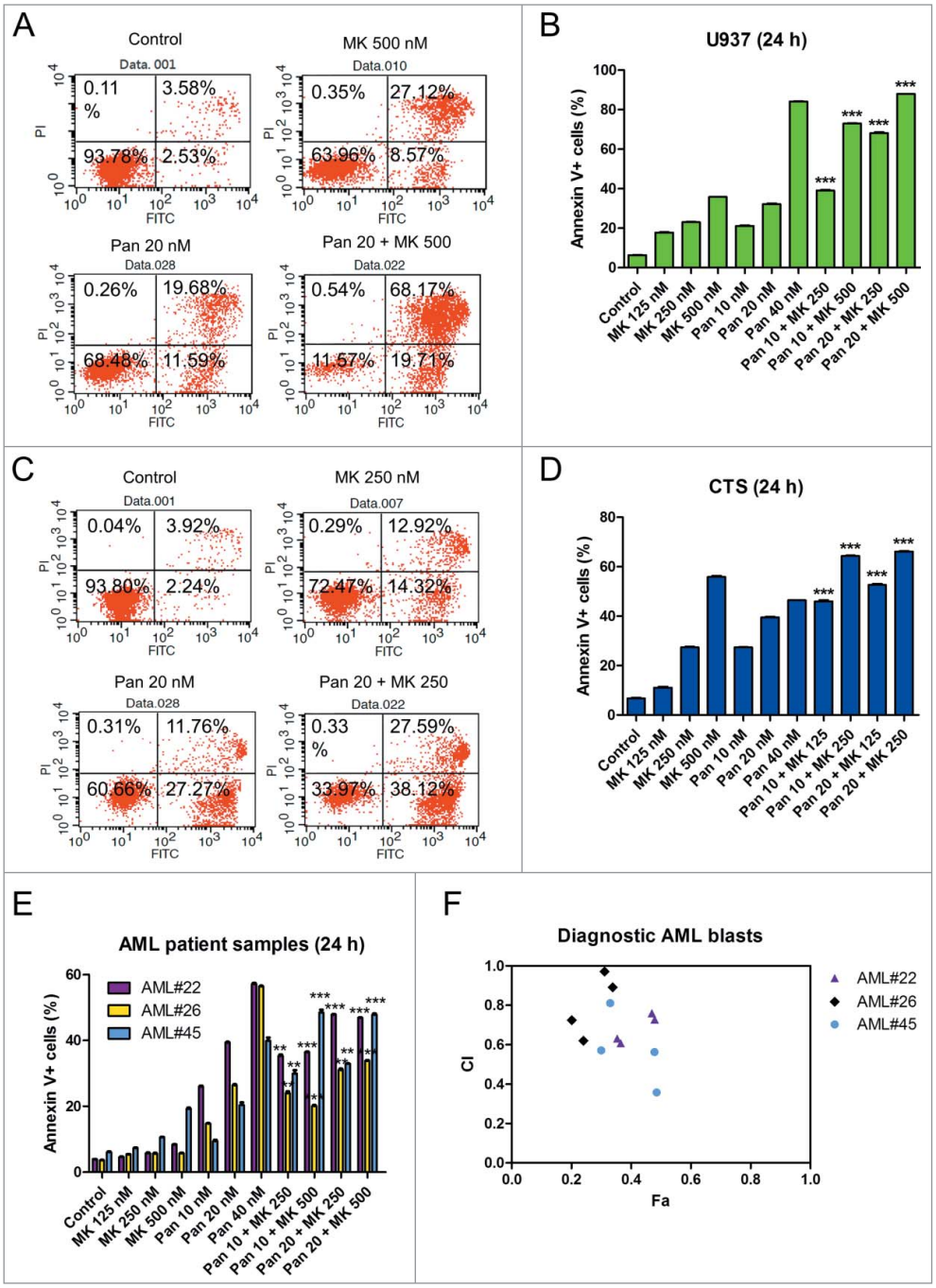

Figure 3. Panobinostat synergizes with MK-1775 to induce cell death in AML cells. Panels **A-D**: U937 (**Panels A and B**) and CTS (**Panels C and D**) cells were treated with the indicated drugs for 24 h. Cell death was determined by annexin V/PI staining and flow cytometry analyses. The experiment was performed at least 3 independent times. Representative annexinV/PI dot plots are shown in **Panels A and C**. The data are presented as means ± s.e.m of triplicates from one representative experiment (**Panels B and D**). Statistical significance was calculated using the pair-wise 2-sample t-test. ***indicates $p<0.0005$. Panel **E**: Primary patient samples were treated with the indicated drugs for 24 h. Cell death was determined by annexin V/PI staining and flow cytometry analyses. The data are presented as means of triplicates ± s.e.m. from one experiment due to limited sample. Panel **F**: CI vs. Fa plot (combination index vs. fraction affected) for the data presented in panel **E**. The CI values were calculated using CompuSyn software.

values of 1.0 and 0.9) indicated additivity, the majority (ranging from 0.4 to 0.8) indicated synergy (**Fig. 3E and F**, these samples were chosen based solely on the availability of sufficient number of cells for analysis). These results demonstrate that the combination of MK-1775 and panobinostat causes cell death in AML cells rather than merely causing proliferation arrest.

Panobinostat enhances MK-1775-induced cell death at least partially through downregulation of CHK1 and Wee1 in AML cells

Then we investigated the effects of panobinostat and MK-1775 on cell cycle progression in the CTS cell line. Panobinostat treatment resulted in S and G2 \M arrest, while MK-1775 treatment resulted in abrogation of the G2\M checkpoint (as indicated by the significant decrease of the percentage of G2\M phase cells, **Fig. 4**). The combined treatment resulted in abrogation of the G2\M cell cycle checkpoint and significantly increased cell death (indicated by the percent of cells with sub-G1 DNA content, **Fig. 4**). In the U937 cells, panobinostat treatment resulted in abrogation of the S and G2\M checkpoint, while MK-1775 resulted in decreased G0\G1. Similar to the CTS cells, U937 cells treated with the drug combination resulted in significant decrease of cells in G2\M.

As expected, increased levels of acetylated histone H4 were detected after panobinostat treatment of the CTS cells (**Fig. 5A**). In addition, treatment with MK-1775 or panobinostat alone resulted in significantly decreased levels of p-CDK1 and p-CDK2 compared to the no drug treatment control, which was not due to decrease of total CDK1 and CDK2 levels as the total levels remained largely unchanged or slightly increased ($p<0.05$, **Fig. 5A and B**). Compared to individual drug treatment, the combined drug treatment did result in further decrease of both p-CDK1 and p-CDK2 ($p<0.05$, **Fig. 5A and B**). Significantly, increased γH2AX was detected after individual drug treatments

and was further increased after combined drug treatment ($p<0.05$, **Fig. 5A and B**). Consistent with our previous studies,[38] CHK1 levels were decreased following panobinostat treatment and were accompanied by decreased levels of p-CDC25C. Surprisingly, MK-1775 treatment alone also caused significant decrease of CHK1, though it had no effect on CDC25C phosphorylation (**Fig. 5A and B**). CHK1 levels were further decreased in the combined drug treatment compared to individual drug treatment and p-CDC25C levels were decreased compared to MK-1775 treatment, but not to panobinostat treatment. Interestingly, panobinostat treatment caused downregulation of Wee1, which was further decreased in the combined drug treatment. In contrast, MK-1775 treatment had no effect on Wee1 level. Similar results were obtained in U937 cells, except panobinostat or MK-1775 treatment alone resulted in decreased CHK1, yet neither had an effect on CDC25C phosphorylation (**Fig. 5C and D**). These results provide evidence that cooperative downregulation of CHK1 and Wee1 by panobinostat and MK-1775 may represent a critical molecular mechanism underlying the synergistic anti-leukemic interactions between panobinostat and MK-1775 in AML cells.

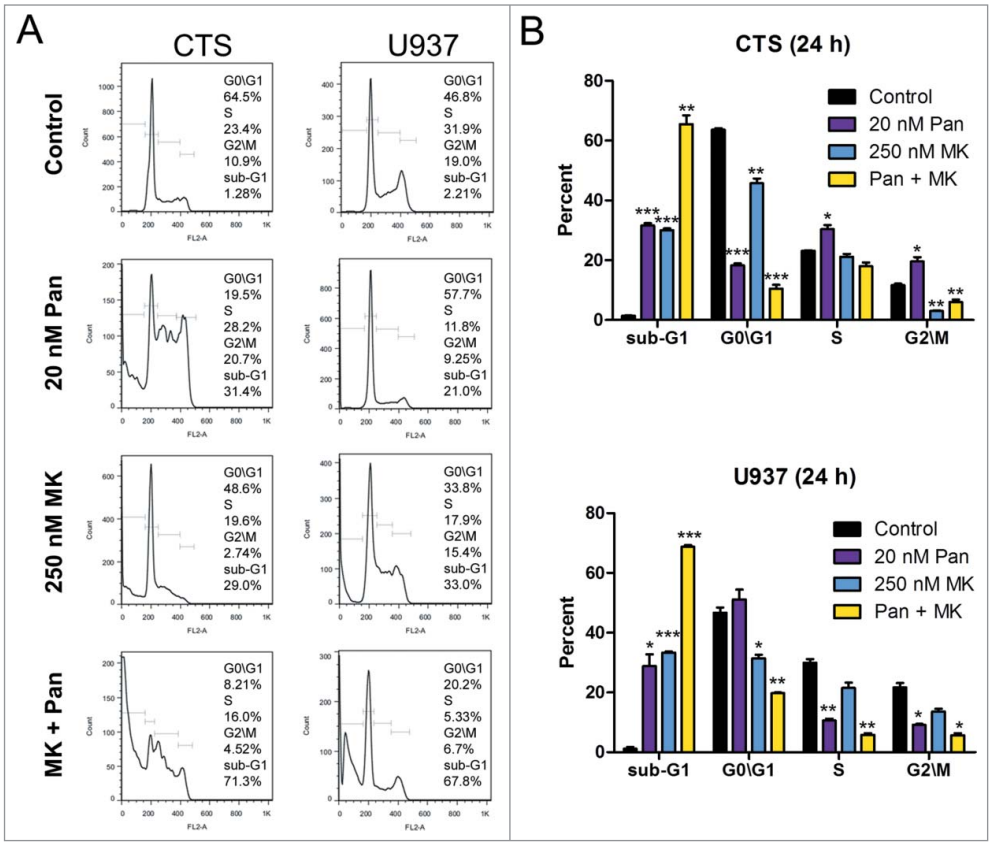

Figure 4. Effects of panobinostat and MK-1775, alone or in combination, on cell cycle progression in CTS and U937 cells. Panel **A**: CTS and U937 cells were treated with 20 nM panobinostat, 250 nM MK-1775, or in combination for 24 h. Then the cells were fixed with ethanol, stained with PI, and subjected to flow cytometry analyses. Panel **B**: The percentage of cells in each cell cycle phase after drug treatment are presented as means of triplicates ± s.e.m from one representative experiment. *indicates $p<0.05$, **indicates $p<0.005$, and ***indicates $p<0.0005$.

To provide evidence that CHK1 and Wee1 play critical roles in the anti-leukemic activities of MK-1775 and panobinostat, we used lentivirus shRNA to knockdown CHK1 and Wee1 in U937 cells (**Fig. 6**). Knockdown of CHK1 and Wee1 resulted in significantly increased MK-1775-induced cell death. Knockdown of Wee1 also significantly enhanced panobinostat-induced cell death, whereas CHK1 knockdown did not appear to have an effect on panobinostat-induced cell death (**Fig. 6B and D**).

Discussion

MK-1775 has demonstrated promising preclinical results in combination with DNA damaging agents.[16-18,39,40] Recent studies have investigated the combination of Wee1 and CHK1 inhibitors in preclinical models of different malignancies and have also found promising activity.[39,41,42] In addition, we have previously demonstrated that activation of CHK1 was a potential mechanism of resistance to MK-1775 which could be overcome by the addition of a CHK1 selective inhibitor.[34] HDACIs have shown promising results in combination with other chemotherapy agents and have been demonstrated to downregulate CHK1 in various malignancies, including AML;[38,43-45] therefore they could be used as a means to downregulate the CHK1 pathway. Many HDACIs are currently under clinical development for treating cancer and several of them have been FDA approved for the treatment of a number of malignancies,[46,47] which makes them attractive options for the clinical development of combination therapies with MK-1775. Of note, pracinostat has been granted Orphan Drug status by the FDA for the treatment of AML[48] and panobinostat has recently received FDA approval after being granted "priority review" status for the treatment of multiple myeloma.[32,33]

In this study, we demonstrated that combined panobinostat and MK-1775 treatment resulted in synergistic anti-leukemic activity in AML cell lines and *ex vivo* primary patient samples. In addition, we provide evidence to suggest that cooperative downregulation of CHK1 and Wee1 plays an important role in the synergistic anti-leukemic activity. While we were performing our studies, Zhou et al.

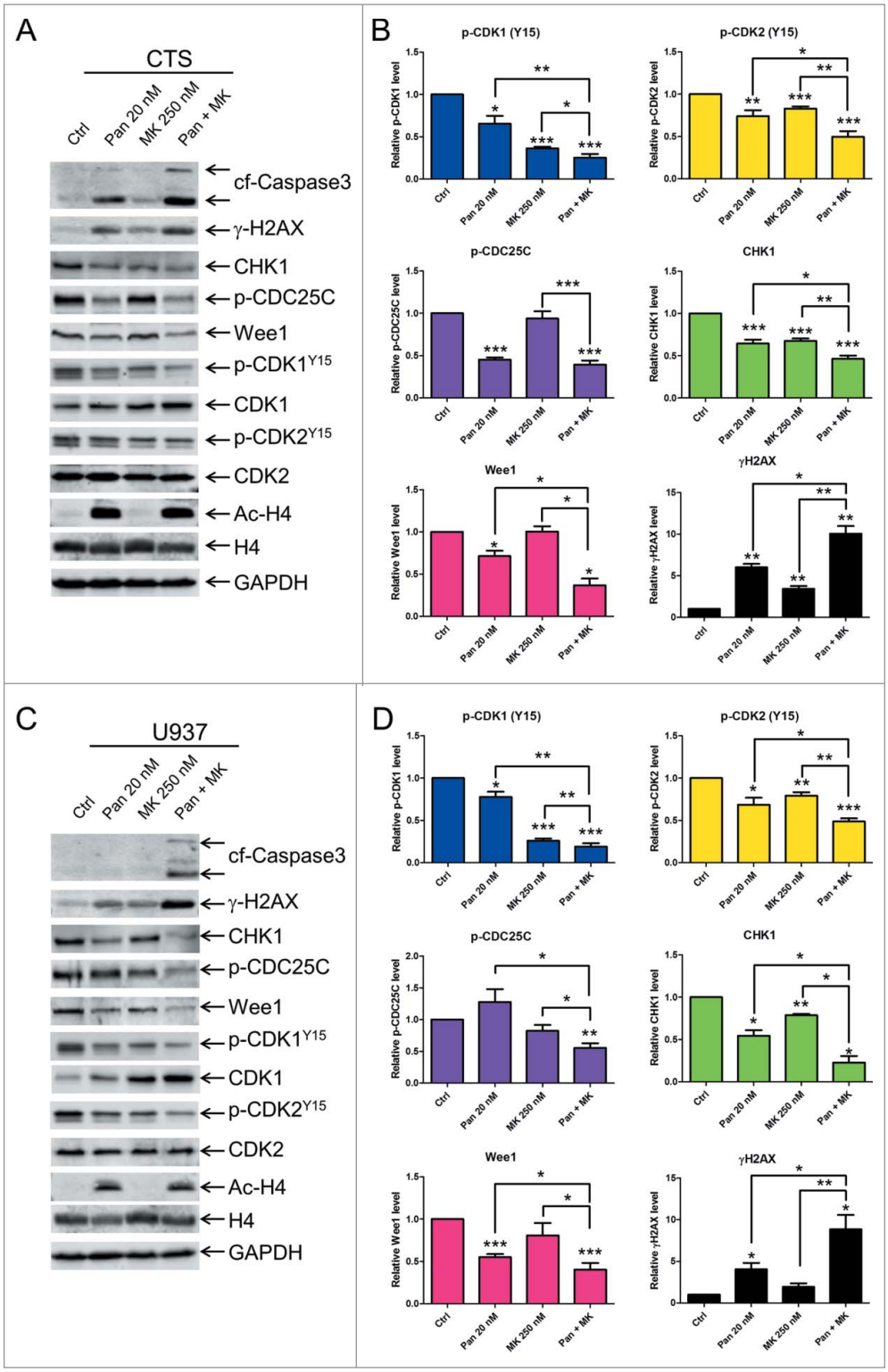

Figure 5. Cooperative downregulation of CHK1 and Wee1 by panobinostat and MK-1775 is common in different subtypes of AML. Panels **A** and **C**: CTS (Panel **A**) and U937 (Panel **C**) cells were treated with panobinostat, MK-1775, or in combination. Whole cell lysates were subjected to Western blotting and probed with anti-cleaved caspase-3 (cf-cleaved fragment), -p-CDK1, -CDK1, p-CDK2, -CDK2, -γH2AX, -CHK1, -p-CDC25c, -Wee1, -ac-H4, -H4, or -GAPDH antibody. Representative Western blots are shown. Panels **B** and **D**: Densitometry for protein expression, corresponding to experiments presented in Panels **A** and **C**, were measured using Oddyssey V3.0, normalized to GAPDH, and graphed as mean fold change (relative to no drug control) ± s.e.m from at least 3 independent experiments. For p-CDK1 and p-CDK2 the densitometry measurements were first normalized to the total CDK1 and CDK2 levels, respectively, then to GAPDH. *indicates $p<0.05$, **indicates $p<0.005$, and ***indicates $p<0.0005$.

published their study investigating the combination of Vorinostat, a pan-HDACI, with MK-1775 in AML.[45] In their study, the authors found diminished p-CDK1 following Vorinostat or MK-1775 treatment and further decrease after combined drug treatment,[45] which are similar to our results with panobinostat and MK-1775 treatment (**Fig. 5**). In contrast to Zhou et al., we found that panobinostat treatment alone resulted in decreased Wee1 protein levels. These differences may be due to the nature of the different HDACIs. Additionally, the HDACIs Vorinostat and valproic acid have been demonstrated to downregulate Wee1 expression in glioma cells[49] and panobinostat has been demonstrated to downregulate *Wee1* transcript levels in T-cell leukemia.[50] Our Wee1 knockdown demonstrated that Wee1 is important for panobinostat- and MK-1775-induced cell death. We also found that *Wee1* transcript levels positively correlated with panobinostat IC_{50}s in primary patient samples (**Fig. 2B**). Others have demonstrated that inhibition of CHK1 enhances MK-1775 activity[39,41,42] and we have previously demonstrated that CHK1 plays a role in MK-1775 resistance.[34] In line with those studies, our CHK1 knockdown further confirms that CHK1 plays a role in MK-1775-induced cell death. Based on all of these findings, the synergistic anti-leukemic interaction of panobinostat and MK-1775 is likely due to the cooperative downregulation of Wee1 and CHK1 in combination with inhibition of Wee1.

In addition to HDACIs, MK-1775 has been combined with other agents, such as cytarabine, in preclinical AML models.[36,40,51,52] The combined cytarabine and MK-1775 treatments resulted in synergistic inhibition of proliferation, regardless of p53 status.[40] It has been demonstrated that cytarabine-induced S-phase cell cycle arrest was overcome by the addition of MK-1775.[40,51,52] Tibes et al. used an RNAi screening approach to identify kinases involved in cytarabine sensitivity and found ATR, PKMYT1, and CHK1, among others, as kinases involved in cytarabine sensitivity.[36] In addition, they demonstrated synergistic anti-leukemic activity for combined MK-1775 and cytarabine treatment in cell line models. In contrast to MK-1775 combined with cytarabine, Van Linden et al. found that doxorubicin in combination with MK-1775 was largely antagonistic.[40]

In conclusion, the present findings indicate that panobinostat potentiates the anti-leukemic activity of MK-1775 in AML cell lines and primary patient samples at clinically achievable concentrations. Panobinostat treatment downregulates the CHK1 pathway and/or Wee1 expression levels in AML cells. As demonstrated by our shRNA knockdown studies in the U937 cells, the synergy between panobinostat and MK-1775 may be more likely due to panobinostat's ability to downregulate Wee1 than CHK1. However, panobinostat has been shown to regulate expression of many genes and acetylation of various proteins, which leads to cell death and tumor growth inhibition in various malignancies.[44,53-55] Therefore, we cannot rule out the possible involvement of other proteins in addition to CHK1 and Wee1.[38,53,56] The present findings provide support for the clinical development of panobinostat in combination with MK-1775 for the treatment of AML.

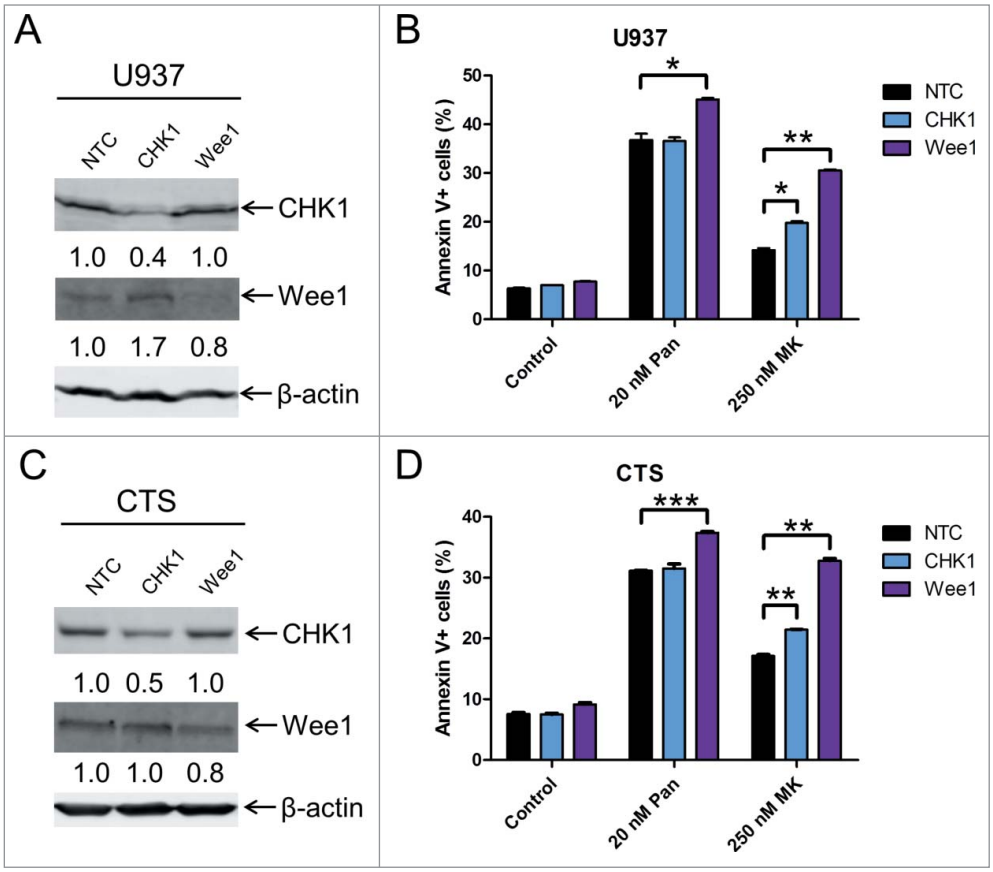

Figure 6. CHK1 and Wee1 play important roles in the anti-leukemic activity of MK-1775. Panel **A**: U937 cells were infected with CHK1, Wee1 or non-target control (NTC) shRNA lentivirus overnight, washed, and then incubated for 48 h. Whole cell lysates were subjected to Western blotting and probed with anti-CHK1 (Santa Cruz Biotechnology), -Wee1 (Cell Signaling Technology), or -β-actin antibody. Panel **B**: U937 cells were infected with CHK1, Wee1 or non-target control (NTC) shRNA lentivirus overnight, washed, incubated for 24 h. Then the cells were treated with panobinostat or MK-1775 for 24 h. Cell death was determined by annexin V/PI staining and flow cytometry analyses. Panel **C**: CTS cells were infected with CHK1, Wee1 or NTC shRNA lentivirus overnight, washed, and then incubated for 48 h. Whole cell lysates were subjected to Western blotting and probed with anti-CHK1, -Wee1, or -β-actin antibody. Panel **D**: CTS cells were infected with CHK1, Wee1 or NTC shRNA lentivirus overnight, washed, incubated for 24 h, and then the cells were treated with no drug control, panobinostat or MK-1775 for 24 h. Cell death was determined by annexin V/PI staining and flow cytometry analyses.

Methods

Drugs

MK-1775 (MK) and panobinostat (Pan) were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

The U937 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The OCI-AML3 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). MOLM-13 cells were purchased from AddexBio (San Diego, CA, USA). The CTS cell line was a gift from Dr. A Fuse from the National Institute of Infectious Diseases, Tokyo, Japan. The cell lines were cultured in RPMI 1640 (except OCI-AML3, which was cultured in alpha-MEM) with 10-15% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a 37°C humidified atmosphere containing 5% CO2/95% air.

Diagnostic AML blast samples derived from patients either at initial diagnosis or at relapse were purified by standard Ficoll-

Hypaque density centrifugation, then cultured in RPMI 1640 with 20% fetal bovine serum supplemented with ITS solution (Sigma-Aldrich, St. Louis, MO, USA) and 20% supernatant of the 5637 bladder cancer cell line (as a source of granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, interleukin-1 beta, macrophage colony-stimulating factor, and stem cell factor).[57-59]

Clinical samples

Diagnostic AML blast samples were obtained from the First Hospital of Jilin University. Written informed consent was provided according to the Declaration of Helsinki. This study was approved and carried out in accordance with the guidelines set forth by the Human Ethics Committee of the First Hospital of Jilin University. Clinical samples were screened for FLT3-ITD, NPM1, C-kit, CEBPA, IDH1, IDH2 and DNMT3A gene mutations. The samples were also screened for the following fusion genes by real-time RT-PCR: PML-RARα, BCR-ABL, AML1-MDS1, MLL-AF10, MLL-AF4, MLL-ELL, SET-CAN, TLS-ERG, NPM-RARα, E2A-PBX1, AML1-EAP, MLL-AF17, MLL-AF6, MLL-ENL, SIL-TAL1, HOX11, PLZF-RARα, TEL-AML1, DEK-CAN, MLL-AF1p, MLL-AF9, NPM-ALK, TEL-ABL, EIP1L1-PDGFRA, AML1-ETO, CBFB-MYH11, E2A-HLF, MLL-AF1q, MLL-AFX, NPM-MLF1, dupMLL, and TEL-PDGFB. Patient characteristics are presented in **Table S1**.

In vitro cytotoxicity assays

In vitro cytotoxicities of the AML cells were measured by using MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazoliumbromide, Sigma-Aldrich), as previously described.[60,61] Briefly, the cells were treated with variable concentrations of MK-1775, panobinostat, or in combination for 72 hours. MTT was added to a final concentration of 1 mM and cells were incubated for 4 hours at 37°C. The cells were lysed overnight using 10% SDS in 10 mM HCL and plates were read at 590 nm using a microplate reader. IC_{50} values were calculated as drug concentrations necessary to inhibit 50% growth compared to vehicle control treated cells. The IC_{50} values for the cell lines are presented as mean values ± standard errors from at least 3 independent experiments. The IC_{50} values for the patient samples are means of duplicates from one experiment, due to limited sample. Patient samples for the combined drug treatments were chosen based on sample availability. The combination index (CI) values were determined using CompuSyn software. $CI<0.9$ indicates synergistic, $0.9<CI<1.1$ indicates additive, and $CI>1.1$ indicates antagonistic anti-leukemic interactions.[62]

Quantification of gene expression by real-time RT-PCR

Total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA) and cDNAs were prepared from 2 μg total RNA using random hexamer primers and a RT-PCR kit (Life Technologies), and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) as previously described.[58,61,63] *Wee1* (Hs01119384_g1) transcripts were quantitated using TaqMan probes (Life Technologies) and a LightCycler® 480 real-time PCR machine (Roche Diagnostics, Indianapolis, IN, USA), based on the manufacturer's instructions. Real-time PCR data are presented as means of duplicates from one experiment, due to limited sample, and results were normalized to *GAPDH* (4333764) transcripts. Fold changes were calculated using the comparative C_t method.[64]

Western blot analysis

Cells were lysed in the presence of protease and phosphatase inhibitors (Roche Diagnostics). Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with anti-p-CDK2 (Y15) (ab76146) (Abcam, Hong Kong, China), p-CDK1 (Y15) (5757-1), -CDK1 (1484-1), -Wee1 (S2798), -p-CDC25C (S216) (1190-1) -CDK2 (1134-1) (Epitomics, Burlingame, CA, USA), -H4 (07-108), -ac-H4 (06-598) (Millipore, Billerica, MA, USA), -CHK1 (10362-1-AP), (Proteintech, Chicago, IL, USA), -cleaved caspase-3 (9661), -γH2AX (2577), -Wee1 (4936), -GAPDH (2118) (Cell Signaling Technology, Danvers, MA, USA), -CHK1 (sc-8408, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or -β-actin antibody (Sigma-Aldrich), as previously described.[65,66] Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer. Western blots were repeated at least 3 times and one representative blot is shown. Densitometry measurements were made using Odyssey software V3.0 (Li-Cor), normalized to GAPDH and graphed as fold change relative to the no drug control.

Annexin V/PI staining

AML cells were treated with MK-1775, panobinostat, or in combination and subjected to flow cytometry analysis to determine drug-induced cell death using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis Kit (Beckman Coulter; Brea, CA, USA), as previously described,[60,63] and a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Experiments with AML cell lines were performed 3 independent times in triplicates and data presented are from one representative experiment, while patient sample experiments were performed once in triplicates. Based on Galluzi et al.' s review, annexin V+ cells are referred to as dead cells.[67] Data are presented as mean percent of annexin V+ cells ± standard errors from one representative experiment. Due to limited sample, only 3 patient samples were evaluated for cell death induced by MK-1775 or panobinostat, alone or in combination, by flow cytometry.

Cell cycle progression

Cells were treated with the indicated drugs for 24 h. The cells were harvested and fixed with ice-cold 80% (v/v) ethanol for 24 h. The cells were pelleted, washed with PBS, and resuspended in PBS containing 50 μg/mL PI, 0.1% Triton X-100 (v/v), and 1 μg/mL DNase-free RNase. DNA content was determined by flow cytometry analysis using a FACS Calibur flow cytometer (Becton Dickinson), as previously described.[68] Cell cycle analysis was performed using ModFit LT 3.0 (Becton Dickinson).

Histograms were created using FlowJo v7.6.5 (Tree Star, Ashland, OR, USA).

Lentivirus production and shRNA knockdown of CHK1 and Wee1

The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. *CHK1*, *Wee1*, and non-target control shRNA lentiviral vectors were purchased from Sigma-Aldrich. Lentivirus production and transduction of U937 cells were carried out as previously described.[38]

Statistical analysis

Differences between treated (individually or combined) and untreated annexin V + cells were compared using the pair-wise 2-sample t-test. The *p* value for the differences between panobinostat IC_{50}s for the groups of patient samples was calculated using the Mann-Whitney 2-sample U test. The relationship between *Wee1* transcript levels and panobinostat IC_{50}s was determined by the nonparametric Spearman rank correlation coefficient. Statistical analyses were performed with GraphPad Prism 5.0. Error bars represent ± s.e.m. The level of significance was set at $p<0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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