Inhibition of Wee1 Sensitizes Cancer Cells to Antimetabolite Chemotherapeutics In Vitro and In Vivo, Independent of p53 Functionality

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Abstract
Inhibition of Wee1 is emerging as a novel therapeutic strategy for cancer, and some data suggest that cells with dysfunctional p53 are more sensitive to Wee1 inhibition combined with conventional chemotherapy than those with functional p53. We and others found that Wee1 inhibition sensitizes leukemia cells to cytarabine. Thus, we sought to determine whether chemosensitization by Wee1 inhibition is dependent on p53 dysfunction and whether combining Wee1 inhibition is tolerable and effective in vivo. Synergistic inhibition of proliferation with a Wee1 inhibitor in clinical development, MK1775, and cytarabine was observed in all acute myelogenous leukemia (AML) cell lines tested, regardless of p53 functionality. Mechanistic studies indicate that inhibition of Wee1 abrogates the S-phase checkpoint and augments apoptosis induced by cytarabine. In AML and lung cancer cell lines, genetic disruption of p53 did not alter the cells’ enhanced sensitivity to antimetabolites with Wee1 inhibition. Finally, mice with AML were treated with cytarabine and/or MK1775. The combination of MK1775 and cytarabine was well tolerated in mice and enhanced the antileukemia effects of cytarabine, including survival. Thus, inhibition of Wee1 sensitizes hematologic and solid tumor cell lines to antimetabolite chemotherapeutics, whether p53 is functional or not, suggesting that the use of p53 mutation as a predictive biomarker for response to Wee1 inhibition may be restricted to certain cancers and/or chemotherapeutics. These data provide preclinical justification for testing MK1775 and cytarabine in patients with leukemia. Mol Cancer Ther; 12(12); 2675–84. ©2013 AACR.

Introduction
Cell-cycle checkpoint and DNA damage response proteins are critical mediators of successful DNA replication in the presence and absence of genotoxic stress. Cancer cells are particularly dependent on these processes, a phenomenon that could be exploited therapeutically (1, 2). For example, Chk1 has been studied extensively as an adjuvant therapeutic target in combination with anticancer therapy, including radiation and chemotherapy (3, 4). This strategy is expected to be particularly effective in tumors with disrupted p53 function, as they are highly dependent upon the G2–M checkpoint mediated by Chk1 (5).

Wee1 is a cell-cycle checkpoint protein downstream of Chk1 that is activated during the normal cell cycle, as well as in the context of DNA damage. The primary function of WEE1 is inhibitory phosphorylation of cyclin—dependent kinases (CDK) at tyrosine 15 (Y15), thereby inhibiting cell-cycle progression (6, 7). Most studies of Wee1 have focused on the phosphorylation of CDK1 in the context of DNA damage, which prevents progression through mitosis with levels of DNA damage that would result in mitotic catastrophe. Like Chk1, inhibition of Wee1 in combination with DNA-damaging agents has been explored as a therapeutic strategy for tumors with dysregulated p53. Indeed, in published reports, inhibition of Wee1 with small-molecule inhibitors in combination with DNA-damaging agents, including doxorubicin, has shown some specificity for TP53-mutated tumor models (8–11).

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Therefore, we sought to determine whether the function of p53 influences the sensitivity to Wee1 inhibition with chemotherapy in a broad panel of AML cell lines with various molecular abnormalities. In contrast with data from solid tumor models sensitized to DNA-damaging agents (8–11), we found that the functionality of p53 has no bearing on the chemosensitization of AML cells to cytarabine, as all of the cell lines tested were sensitized to cytarabine with Wee1 inhibition. Mechanistic studies indicate that inhibition of Wee1 abrogates the S-phase checkpoint and augments apoptosis induced by cytarabine. Furthermore, in isogenic models, in which wild-type p53 activity was impaired by RNA interference or dominant negative p53 constructs, we did not find enhanced chemosensitization with impaired p53. Also, in contrast with data from solid tumor models, we did not observe chemosensitization to doxorubicin with Wee1 inhibition in AML cells, even in cells with non-functional p53. In addition, we found that the chemosensitization to antimitobolite chemotherapeutics is not limited to leukemia, as lung cancer cells were equally sensitized to cytarabine and pemetrexed, whether p53 function was impaired or not. Finally, in mice with AML, we found that the combination of Wee1 inhibition with cytarabine slowed disease progression and prolonged survival better than cytarabine alone. These data support the development of clinical trials of antimitobolite chemotherapeutics and Wee1 inhibition for patients with cancers; however, distinct from DNA-damaging agents that induce the G2–M checkpoint, our data do not support the use of TP53 mutation as a biomarker to predict beneficial effects of Wee1 inhibition when combined with antimitobolites that induce the S-phase checkpoint.

Materials and Methods

Cell lines and tissue culture

Cell lines were generous gifts from the laboratories of Drs. Douglas Graham and James DeGregori (both at University of Colorado School of Medicine, Aurora, CO). Cell lines were DNA fingerprinted by multiplex PCR using the Profiler Plus or Identifier Kits (ABI) and confirmed to match published or internal databases as previously described (14). Cell lines and tissue culture were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics, except OCI-AML3 and Kasumi-1 which were cultured in RPMI supplemented with 20% heat-inactivated FBS. All AML cell lines were seeded at 1 to 2 x 10^5/mL before experimentation. A549 cells were plated at 1 to 2.5 x 10^5 cells/well the day before experimentation. Cells were counted by propidium iodide (Sigma) exclusion and flow cytometry (Guava EasyCyte Plus, Millipore). Apoptosis and cell cycle were measured with the Guava EasyCyte Plus using the Guava Nexin and Guava Cell Cycle reagents as per the manufacturer’s protocol (Millipore).

Vectors

MSCV-ires-GFP (MiG), MSCV-DDp53-GFP (DDp53), and MSCV-DNp53-GFP (DNp53) plasmids (provided by Dr. DeGregori) were packaged into viral particles and transduced into OCI-AML3 cells as previously described (15). Transduced cells were sorted for GFP using a MoFlow fluorescence-activated cell sorter (Dako Cytomation). Nonsilencing short hairpin RNA (shRNA) and shRNA-targeting p53 from the TRC collection (16) were purchased from the Functional Genomics Facility of the University of Colorado Cancer Center (Boulder, CO) and packaged as previously described (17). Transduced cells were selected in puromycin (Sigma-Alrdich).

Antibodies, chemicals, and reagents

Antibody directed against p21(CB1) was purchased from BD Biosciences; antibodies against phosphorylated CDK1 (Y15), CDK2 (Y15), and histone H3 (S10) were purchased from Cell Signaling Technology. Cytarabine and doxorubicin were purchased from Sigma-Aldrich and diluted in water. Nutlin-3 was purchased from Cayman Chemical and diluted in dimethyl sulfoxide (DMSO). Pemetrexed was purchased from Selleck. MK1775 was provided by the National Cancer Institute (Bethesda, MD) and Merck Sharp & Dohme Corporation.

Animal studies

Female C57BL/6J mice, 6 to 8 weeks old, were purchased from The Jackson Laboratory. All mice were housed in sterile micro-isolators in the Center for Comparative Medicine at the University of Colorado Denver (Denver, CO). Mice were treated with cytarabine 50 mg/kg/d by intraperitoneal injection and/or MK1775 40 mg/kg/d by oral gavage. One million luciferase expressing AML cells (18) were injected into unirradiated recipients to induce leukemia. Luciferase activity was measured 5 minutes after injection of luciferin using a Xenogen IVIS2000 imaging system. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Data analyses

Excel and GraphPad Prism 5 (GraphPad Software) were used for data sorting, analysis, and graphical depiction of data. Student t test was used to compare two samples; ANOVA and Bonferroni post-test were used to compare more than two samples. The Mantel–Cox (log-rank) test was used to test for significant differences in survival. Except as indicated in figure legends, figures demonstrate means of data collected from at least three independent experiments completed in duplicate or triplicate for each condition. Error bars in each figure depict the SEM, and may be obscured when narrow. For determination of synergistic effects, the extent of inhibition relative to control-treated cells was input into CalcuSyn (Biosoft) to calculate combination index (CI) values. CI values less than 1 are considered to represent synergistic inhibition of cellular proliferation (19). Chemiluminescence signals

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Results

We previously reported synergistic inhibition of proliferation in AML cells with cytarabine and MK1775, a small-molecule inhibitor of Wee1, in Molm13, MV4-11, and U937 cell lines (13). These data suggested that the combinatorial effect of cytarabine and Wee1 inhibition in AML cells is independent of p53 functionality, FLT3 mutation, and MLL rearrangement (Supplementary Table S1). We sought to confirm this in a broader panel of AML cells with wild-type and mutated TP53, as well as other AML-specific oncogenes (Supplementary Table S1). We first treated cells with the MK1775 as a single agent with a range of doses to determine the EC50 for each cell line. Consistent with a recent publication (21), we observed inhibition of proliferation of all cell lines with MK1775 as a single agent at clinically achievable concentrations, with a median EC50 value of 396.8 nmol/L (Supplementary Fig. S1 and Supplementary Table S1).

Despite single-agent activity, MK1775 would most likely be incorporated into clinical trials as a chemosensitizing agent in combination with conventional chemotherapeutics. We chose OCI-AML3 and HL60 for detailed study of the chemosensitizing effects of Wee1 inhibition, as they reportedly have wild-type and mutated TP53.
respectively (23, 24). To confirm the function of p53, we treated cells with nutlin-3a, a small molecule that interferes with the interaction of Mdm2 and p53, resulting in activation of p53 in cells in which it is not mutated (25). We found that OCI-AML3 cells were sensitive to treatment with nutlin-3a, whereas HL60 were not, consistent with the reported function of p53 in these cell lines (Fig. 1A). As a second test of p53 function, we performed Western blotting for p21 induction with DNA damage induced by doxorubicin (26). As expected, p21 was induced in OCI-AML3 cells, but not in HL60 (Fig. 1B). Despite discrepant p53 functionality, we found that in both cell lines, the concomitant treatment with MK1775 and cytarabine was synergistic (Fig. 1C and 1D and Supplementary Fig. S2). We observed synergistic inhibition in Nomo1 and Kasumi1 cells as well (Supplementary Fig. 3). Thus, three cell lines with mutated TP53 (HL60, Nomo1, and Kasumi1) and three cell lines with wild-type TP53 (OCI-AML3, Molm13, and MV4-11; ref. 13) are sensitized to cytarabine with MK1775, indicating that the beneficial effects of this combination are not dependent upon p53 dysfunction.

To better understand the mechanisms of chemosensitization to cytarabine with MK1775, we first examined inhibitory phosphorylation of tyrosine 15 of CDK2 by Western blot analysis, as a measure of S-phase checkpoint activation. Treatment with cytarabine alone led to enhanced phosphorylation of CDK2 (Y15), whereas the OCI-AML3 %Cells in S-phase

A+M

ARA-C

MK1775

DMSO

B

Figure 2. MK1775 abrogates the S-phase checkpoint and augments apoptosis induced by cytarabine. HL60 and OCI-AML3 cells were treated at the indicated doses of cytarabine and MK1775 (nmol/L). A, after 24 hours, cells were harvested and protein lysates subjected to Western blotting for P-CDK2 (Y) and actin. Relative phosphorylation as compared with DMSO-treated cells from at least two independent experiments was determined using ImageJ software and is depicted graphically. B, after 24 hours, cells were harvested, fixed and permeabilized, stained and analyzed by flow cytometry for cell-cycle distribution. Representative histograms are shown. The mean percentage of cells in S-phase from three independent experiments is depicted graphically (∗, P < 0.05; ANOVA with Bonferroni post-test). C, after 48 hours, cells were harvested, stained for Annexin V and with 7-aminoactinomycin D (7-AAD), and analyzed by flow cytometry. Cells that were Annexin V+/7-AAD− were considered in early apoptosis, and those Annexin V+/7-AAD+ in late apoptosis.
addition of MK1775 abrogated this modification, whether p53 was functional or not (Fig. 2A). Consistent with this, measurement of the cell cycle demonstrated S-phase arrest in cells treated with cytarabine that was diminished with the addition of MK1775 in both cell lines (Fig. 2B). Moreover, the addition of MK1775 enhanced the induction of apoptosis by cytarabine in both cell lines with discrepant p53 functionality (Fig. 2C). We also observed diminished phosphorylation of tyrosine 15 of CDK1 in cells treated with MK1775 (Supplementary Fig. S4A). However, in contrast with other reports in which MK1775 leads to unscheduled mitosis in chemotherapy-treated cells (8, 10, 21), we did not observe a dramatic increase in phosphorylated histone H3, (Supplementary Fig. S4B) in either cell line. These data indicate that the function of Wee1 in arresting cells in the S-phase of the cell cycle is critical to the survival of leukemia cells exposed to cytarabine, independent of p53 functionality.

Although cytarabine is one of the most active agents in AML, successful therapy also includes anthracyclines, such as doxorubicin, and MK1775 has been reported to sensitize a TP53-mutated colorectal cancer cell line (WiDr) to doxorubicin (27). Surprisingly, we found that the combination of MK1775 and doxorubicin did not have much synergistic effect in HL60 or OCI-AML3 (Fig. 3 and Supplementary Fig. S5), suggesting that dysfunctional p53 by itself is insufficient to sensitize cancer cells to this combination. In fact, the CI values at many of the dose combinations suggested an antagonistic effect of these two compounds, foreboding caution in the development of clinical trials incorporating MK1775 into induction regimens for AML.

Although these data suggest that the functionality of p53 has no bearing on the chemosensitization of AML cells to cytarabine, interpretation of data from genotypically diverse cell lines should be cautious. To more definitively test the relevance of p53 function in this context, we created isogenic cell lines with disrupted p53 activity by retroviral transduction of OCI-AML3 cells with a dominant negative form of p53 (DDp53) or the empty control vector (MiG). These cells were then treated with cytarabine or doxorubicin, with or without MK1775. Cells expressing DDp53 were less sensitive to nutlin-3a and had impaired p21 induction with doxorubicin treatment, as compared with cells expressing the empty vector, indicating impaired p53 activity (Fig. 4A). Cells expressing DDp53 were equally as sensitive to the combination of cytarabine and MK1775 as those cells expressing the empty vector (Fig. 4B). Experiments with isogenic cells in which p53 function has been disrupted also indicate that even in cells with dysfunctional p53, inhibition of Wee1 does not sensitize AML cells to doxorubicin (Fig. 4B). Similar observations were made with a second dominant negative form of p53 (DNp53), although the extent of p53 impairment was not as great as with DDp53 (Supplementary Fig. S6), and with shRNAs directed against p53 (Fig. 4C and Supplementary Fig. S6). These data confirm that the synergistic antileukemic activity of cytarabine and MK1775 is independent of p53 functionality.

Figure 3. Treatment with MK1775 does not sensitize AML cells to doxorubicin (Dox). A, cells were treated with doxorubicin and/or MK1775 at the indicated doses for 72 hours, then counted by flow cytometry and propidium iodide exclusion. The number of live cells relative to control cells is depicted. B, normalized isobolograms of CI values of data from A. Individual data points indicate different combinations of doxorubicin and MK1775. CI values are provided in Supplementary Fig. S4.
To determine whether our observations are specific to AML, we tested the combination of cytarabine and MK1775 in the lung cancer-derived A549 cell line. These cells have been described to have functional p53 (28), which we confirmed using nutlin-3a (Fig. 5A). Consistent with our data in AML cell lines, A549 cells were synergistically sensitized to cytarabine when treated with MK1775 (Fig. 5B and Supplementary Fig. S7). We next tested a more clinically relevant antimetabolite used for lung cancer treatment, pemetrexed, in combination with MK1775 and again found synergistic inhibition of proliferation (Fig. 5C and Supplementary Fig. S7). A549 cells were then transduced with MiG and DDp53 and the functionality of p53 probed with nutlin-3a (Fig. 5D). These cells were treated with pemetrexed and/or MK1775, and we again observed similar sensitivity to combination therapy (Fig. 5E), indicating that the beneficial effect of combining Wee1 inhibition with antimetabolite chemotherapeutics is not limited to AML and may be beneficial whether TP53 is mutated or not.

Finally, to determine whether the combination of antimetabolite chemotherapy plus Wee1 inhibition is tolerable and effective in vivo, we modeled therapy in mice. First, we treated mice without leukemia with cytarabine (50 mg/kg/d), with or without MK1775 (40 mg/kg/d) for 5, 7, or 10 days, and found that the longer courses of single or combination therapy were toxic, resulting in pancytopenia (not shown). In a second toxicity study, mice without leukemia were treated with cytarabine and/or MK1775 for 5 of 7 days for 3 consecutive weeks. The addition of MK1775 did not enhance the hematologic effects of cytarabine with this regimen (Fig. 6A). We then used an aggressive model of murine AML-expressing MLL-ENL, FLT3-ITD, and luciferase (18), to determine whether Wee1 inhibition would enhance the antileukemia effects of cytarabine. Consistent with the previous report (18), cytarabine alone slowed the progression of the leukemia, as measured by luciferase expression over time (Fig. 6B and Supplementary Fig. S8). The addition of MK1775 to cytarabine markedly enhanced the effects of

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Figure 4. Treatment of AML cells with cytarabine and MK1775 is synergistic independent of p53 function in an isogenic model. A, OCI-AML3 cells were transduced with a dominant negative form of p53 (DDp53) or the control vector (MiG) and sorted for GFP expression. Transduced cells were treated with nutlin-3a at the indicated doses for 48 hours then counted by flow cytometry and propidium iodide exclusion. The number of live cells, relative to DMSO-treated, is depicted. Cells were also treated with doxorubicin at the indicated doses, and the expression of p21 was assessed by Western blot analysis (insets). B, MiG and DD-p53 cells were treated with cytarabine or doxorubicin, with or without MK1775 for 72 hours and then counted by flow cytometry. The number of live cells relative to untreated is depicted. NS, P = nonsignificant (ANOVA). C, OCI-AML3 cells were transduced with vectors expressing nonsilencing (shNS) or p53-silencing (shp53) shRNA and selected in puromycin. Cells were then treated for 72 hours with cytarabine and/or MK1775 and counted. The number of live cells, relative to DMSO treated, is depicted. CI Values are provided in Supplementary Fig. S4.
cytarabine in slowing disease progression. Moreover, the addition of MK1775 to cytarabine significantly enhanced survival as compared with cytarabine alone (Fig. 6C).

Discussion

Targeting Wee1 to sensitize cancer cells to chemotherapy has emerged as a novel therapeutic strategy, and is currently being tested in early-phase clinical trials with the Wee1 inhibitor, MK1775. Although others have demonstrated that cell lines with TP53 mutation are particularly sensitive to the combination of MK1775 and DNA-damaging chemotherapy, we have shown here that the functionality of p53 does not influence sensitization to antimetabolite chemotherapeutics by MK1775 in AML cells and lung cancer cells. This conclusion is based on data not only from a panel of AML cell lines with discrepant molecular abnormalities, including TP53 mutation, but also isogenic cell line models of AML and lung cancer. We also demonstrate that inhibition of Wee1 does not sensitize AML cells to doxorubicin, even when p53 function is disrupted. Thus, the use of TP53 mutation as a biomarker to predict likely responders to chemosensitization with MK1775 must be cautious and considered in the context of the chemotherapy with which it will be paired, as well as the preclinical data supporting the combination. Moreover, we demonstrate for the first time that Wee1 inhibition can be effectively combined with cytarabine to slow leukemia progression in vivo.

As noted, tumors with dysfunctional p53 may be particularly susceptible to the combination of MK1775 and DNA-damaging agents (8–11), hypothetically due to the impaired G1 checkpoint and high dependence upon the G2–M checkpoint (5). The specificity of chemosensitization by MK1775 for TP53-mutated tumors was first reported in an ovarian cancer cell line (TOV21G) in which p53 had been knocked down by shRNA (8). In these experiments, cells with knockdown of p53 had greater sensitization to gemcitabine, carboplatin, and cisplatin as measured by sub-G1 DNA content, when treated with a single dose of MK1775 (8). Conversely, lung cancer cells with impaired p53 function (H1299) transduced with inducible p53 were sensitized to ionizing irradiation with MK1775 if p53 was not expressed, but not sensitized when p53 expression

Figure 5. MK1775 sensitizes lung cancer cells to cytarabine and pemetrexed independent of p53 function. A, A549 cells were treated with nutlin-3a for 72 hours, then counted by flow cytometry and propidium iodide exclusion. The number of live cells, relative to untreated cells is depicted. B, A549 cells were treated with cytarabine and/or MK1775 for 72 hours and counted by flow cytometry. The number of live cells relative to untreated is depicted. C, A549 cells were treated with pemetrexed and/or MK1775 for 72 hours and counted by flow cytometry, and the number of live cells relative to untreated is depicted. D, A549 cells were transduced with MiG or DDp53 and sorted for GFP. Cells were then treated with nutlin-3a at the indicated concentrations. The number of live cells, relative to DMSO-treated, is depicted. E, transduced A549 cells were treated with pemetrexed (PMTX) and/or MK1775 at the indicated concentration for 72 hours and counted by flow cytometry. The number of live cells relative to untreated is depicted. NS, P = nonsignificant (ANOVA).
was induced (9). Other reports of the specificity of chemosensitization in TP53-mutated cell lines rely on data from nonisogenic cell lines (10, 11). Notably, in a large panel of breast cell lines, although there was an association with mutated TP53 and synergy with gemcitabine and MK1775, many of the cell lines with mutated TP53 did not have CI values reflecting synergy, indicating that dysfunctional p53 does not always confer sensitivity to that particular combination (10). Our data do not directly contradict these reports, as we have looked specifically in the context of antimetabolite chemotherapeutics with distinct mechanisms of action. Rather, our data highlight the function of Wee1 in the S-phase checkpoint activated by antimetabolites (13), independent of p53, and broaden the potential clinical applicability of Wee1 inhibition. The fact that lung cancer cells were also sensitized to cytarabine and pemetrexed indicates that the beneficial effect of combining Wee1 inhibition with antimetabolite chemotherapeutics is not limited to AML and may be beneficial whether TP53 is mutated or not.

Importantly, we did not observe synergy with the anthracycline, doxorubicin, in combination with MK1775 in any of the AML cell lines tested. To the contrary, CI values suggested antagonism at some of the dose combinations tested. This information will be critical to consider in the design of clinical trials incorporating MK1775 into the induction regimens for AMLs that typically include both cytarabine and an anthracycline.

Not surprisingly, we observed hematopoietic toxicity in mice treated with cytarabine, with or without MK1775 when given daily for more than 5 days. Nonetheless, mice with leukemia survived longer due to...
enhanced disease control when treated with combination therapy as compared with cytarabine alone. Notably, we observed a decrease in disease burden with each course of combination therapy. Whether reducing the cytarabine dose and giving combined therapy continuously would be tolerable and more efficacious remains to be determined.

Taken together, these data suggest that the combination of Wee1 inhibition and cytarabine is a broadly applicable therapeutic strategy for AML, independent of several known molecular abnormalities, including mutation in TP53. Furthermore, the inhibition of Wee1 in combination with other clinically relevant antimetabolites should be tested, as this strategy may be applicable across a number of different cancer types including lung cancer. Although the use of TP53 mutation as a biomarker predictive of response for certain chemosensitization strategies may be appropriate, its use for combinations with antimetabolites seems to be limited.

Disclosure of Potential Conflicts of interest
No potential conflicts of interest were disclosed.

References

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