MK1775, a Selective Wee1 Inhibitor, Shows Single-Agent Antitumor Activity against Sarcoma Cells

Jenny M. Kreahling1, Jennifer Y. Gemmer1, Damon Reed1,2, Douglas Letson1,2, Marilyn Bui1,2, and Soner Altiok1

Abstract

Wee1 is a critical component of the G₂–M cell-cycle checkpoint control and mediates cell-cycle arrest by regulating the phosphorylation of CDC2. Inhibition of Wee1 by a selective small molecule inhibitor MK1775 can abrogate G₂–M checkpoint, resulting in premature mitotic entry and cell death. MK1775 has recently been tested in preclinical and clinical studies of human carcinoma to enhance the cytotoxic effect of DNA-damaging agents. However, its role in mesenchymal tumors, especially as a single agent, has not been explored. Here, we studied the cytotoxic effect of MK1775 in various sarcoma cell lines and patient-derived tumor explants ex vivo. Our data show that MK1775 treatment at clinically relevant concentrations leads to unscheduled entry into mitosis and initiation of apoptotic cell death in all sarcomas tested. In MK1775-treated cells, CDC2 activity was enhanced, as determined by decreased inhibitory phosphorylation of tyrosine-15 residue and increased expression of phosphorylated histone H3, a marker of mitotic entry. The cytotoxic effect of Wee1 inhibition on sarcoma cells seems to be independent of p53 status as all sarcoma cell lines with different p53 mutation were highly sensitive to MK1775 treatment. Finally, in patient-derived sarcoma samples, we showed that MK1775 as a single agent causes significant apoptotic cell death, suggesting that Wee1 inhibition may represent a novel approach in the treatment of sarcomas. Mol Cancer Ther; 11(1); 174–82. ©2011 AACR.

Introduction

Sarcomas are rare (1) and heterogeneous forms of cancer with more than 70 recognized histologies (2). While conventional, cytotoxic chemotherapy clearly plays a role in the treatment of some patients with sarcoma, it is unlikely that future escalations of conventional chemotherapy will be tolerable or improve survival and new approaches to therapy are required (3, 4). Toxicity with these agents is high, response rates remain modest, and improvement in overall survival, especially in the metastatic disease setting, remains negligible. Accordingly, new agents are needed for the treatment of this heterogeneous group of diseases (3, 4).

The characterization of important signaling pathways in cancer has led to the development of molecularly targeted therapies that interfere with pathways critical to cancer cell survival (3, 4). Targeted agents are aimed at essential components of cancer cell viability, sparing normal cells and resulting in fewer side effects (5). Inhibition of the pathways critical to tumor cell survival by molecularly targeted therapy represents an opportunity to reverse the biologic basis of tumor formation. Imatinib treatment for gastrointestinal stromal tumor (GIST) provided the proof of principle that a kinase-directed agent could be effective in a specific sarcoma subtype (6, 7).

The DNA-damage pathway plays an important role in survival of tumor cells upon treatment with DNA-damaging agents. Thus, the regulation of the G₂–M transition in eukaryotes through activation of the CDC2–cyclin B complex offers an attractive molecular target in tumor therapy. The proper regulation of CDC2 requires an activating phosphorylation on threonine-161 and inhibitory phosphorylations on threonine-14 and tyrosine-15 (Tyr15; refs. 8, 9). The inhibitory phosphorylation on Tyr15 maintains the CDC2–cyclin B complex in an inactive state if there is incompletely replicated DNA or damaged DNA in the cell (10). CDC2 activation through removal of its inhibitory phosphorylation allows cells to enter the mitotic phase of the cell cycle (8–10). A critical component of this DNA checkpoint is Wee1, a tyrosine kinase that inactivates CDC2 through selective phosphorylation of its Tyr15 residue. In response to DNA damage, Wee1 causes inactivation of CDC2 and consequently leads to G₂ arrest that gives tumor cells survival advantage by allowing time to repair their damaged DNA (11–13). Inhibition of Wee1 has been shown to abrogate the G₂–M checkpoint, forcing cancer cells with DNA damage to enter into unscheduled mitosis to undergo cell death, often referred to as mitotic catastrophe (14). Furthermore,
Wee1 has also been shown to play important roles in spindle formation (15).

Several studies showed that pharmacologic inhibition of Wee1 by a small molecule kinase inhibitor MK1775 exerts antitumorigenic effects in non–small cell lung carcinoma (16), breast (17), prostate (18), pancreatic (19), ovarian (20), and colon (21) cancer when combined with cytotoxic agents. These studies showed that MK1775 is particularly effective in p53-defective cancer cells (11, 16–21).

Here, we show that Wee1 inhibition by MK1775 results in activation of CDC2, as assessed by decreased Tyr15 phosphorylation, unscheduled mitotic entry, and significant cell death in sarcoma cell lines as well as in patient-derived sarcoma tumors. Our data show that MK1775 shows its cytotoxic effect in sarcoma cells as a single agent in a p53-independent manner and represents a promising therapeutic agent in treatment of sarcoma.

Materials and Methods

Cell culture and experimental treatments

MG63 (ATCC CRL-1427), SW-872 (ATCC HTB-92), SK-UT-1 (ATCC HTB-114), A673 (ATCC CRL-1598), SK-ES-1 (ATCC HTB-86), MNNG (ATCC CRL-1547), U2OS (ATCC HTB-96), and HT-1080 (ATCC CCL-121) cells (American Type Culture Collection) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine at 37°C in a 5% CO2 incubator. Stock solutions of the Wee1 inhibitor MK1775 (Selleck Chemicals) was dissolved in dimethyl sulfoxide (DMSO) and added to the media at the indicated concentrations. Control cells were treated with vehicle alone. Commericially obtained cells were not authenticated by the authors. (Fig. 1).

Cell growth and viability assays

Cells were seeded into 384-well plates and treated with various increasing doses of MK1775 for 72 hours. Cell viability was measured by the CT-Blue assay (Promega). The half maximal effective concentration (EC50) was determined for each cell line using a method developed by Chou and Talalay (22). This method involves plotting dose–effect curves for each agent and their combination, using a median-effect equation: \( f_a/f_b = (D_m/D_a)m \), where \( D \) is dose of drug, \( D_m \) is dose required for a 50% effect (equivalent to IC50 value), \( f_a \) and \( f_b \) are affected and unaffected fractions, respectively (\( f_a = 1 − f_b \)), and \( m \) is the exponent signifying the sigmoidicity of the dose–effect curve. The computer software Xlfit version 4.3.1 (ID Business Solutions) was used to calculate the values of \( D_m \) and \( m \). The combination index (CI) used for the analysis of the drug combinations was determined by the isobologram-equation for mutually nonexclusive drugs that have different modes of action: \( CI = (D_1/D_m1) + (D_2/D_m2) + [(D_1D_2)/(D_1D_2)] \), where \( D_1 \) and \( D_2 \) are relative concentrations of drugs 1 and 2 and \( x \) is the percentage of inhibition.

Western blot analysis

Both adherent and detached cells in tissue culture wells were collected in 15-mL conical tubes and centrifuged at 4°C for 5 minutes at 1,000 rpm in an Eppendorf 5810 R centrifuge. The supernatant was removed, and the cell pellet was rinsed with ice-cold PBS, after which ice-cold Universal Cell lysis buffer (Millipore) was added. Samples were sonicated, vortexed on ice every 10 minutes for 30 minutes, and then transferred to 1.5-mL microcentrifuge tubes and centrifuged for 10 minutes at 13,000 rpm at 4°C in an Eppendorf 5417R microcentrifuge. We used the Pierce BCA assay kit to determine protein concentrations, following manufacturer’s protocol (Thermo Fisher Scientific). Samples were heated to 95°C for 10 minutes before resolving on an SDS-PAGE with a 4% to 20% gradient gel (BioRad Industries) and transferred to a polyvinylidene difluoride membrane (Millipore) with a semi-dry transfer device (BioRad Industries). The membrane was blocked for 1 hour at room temperature in Pierce Superblock (Thermo Fisher Scientific) and probed for various antibodies. Enhanced chemiluminescence was carried out following manufacturer’s protocols (Thermo Fisher Scientific).

Antibodies

Rabbit γH2AX, total CDC2, CDC2 Y15P, and total PARP (IPARP) antibodies were purchased from Cell Signaling Technology. Mouse p53 antibody was purchased from Calbiochem, EMD Chemicals. Mouse β-actin antibody was purchased from Sigma Aldrich Corporation.

Determination of Annexin V–positive cells by flow cytometry

U2OS, MG63, A673, or HT1080 cells were seeded in 6-well plates at a density of (1 × 105) cells per well. Cells were treated the next day with 500 nmol/L MK1775 and collected 24 hours later for analysis by the BD Annexin APC kit for Flow Cytometry Kit (BD Bioscience) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich Corporation) per manufacturers’ protocols. Cells were detached with AccuMax (Innovative
Cell Technologies, Inc.), combined with floating cells, and centrifuged for 5 minutes at 1,000 rpm at 4°C in an Eppendorf Model 5417R centrifuge. Cell pellets were then rinsed 1 x with ice-cold 1 x Dulbecco’s Phosphate-Buffered Saline (DPBS) and centrifuged again for 5 minutes at 1,000 rpm and 4°C. Cells were then resuspended in 1 x Annexin V Binding Buffer at a concentration of 1 x 10⁶ cells per mL. An aliquot of 100 μL of this cell suspension was then stained by addition of 5 μL Annexin V/APC solution and 5 μL of DAPI (70 ng/mL) solution and allowed to incubate for 15 minutes on ice in the dark. Positive control cells were prepared by heating an aliquot of cells to 85°C for 2 minutes. Separate aliquots of cells were prepared for Annexin V/APC-only controls and DAPI-only controls. Aliquots of healthy, untreated cells were added to these controls postheating to obtain a representative profile of healthy and unhealthy populations for gating. After the 15-minute incubation was completed, 400 μL of Annexin V Binding Buffer was added to each sample and mixed. Samples were analyzed within 30 minutes on a BD FACScan instrument with Flowjo (Tree Star, Inc.) software to determine the percentage of Annexin V–positive cells.

**Determination of phosphorylated histone 3–positive cells and cell-cycle analysis by flow cytometry**

U2OS, MG63, A673, or HT1080 cells were seeded in 100-cm² plates at a density of (1 x 10⁶) cells per plate. Cells were treated the next day with 500 nmol/L MK1775 and collected 24 hours later for analysis by BD Alexa Fluor 647 Rat anti-Histone H3 (pS28; BD Bioscience) and counterstained with DAPI for cell-cycle analysis (Sigma Aldrich Corporation) per manufacturers’ protocols. Cells were detached with Accumax (Innovative Cell Technologies, Inc.) and centrifuged for 5 minutes at 1,000 rpm at 4°C in an Eppendorf Model 5417R centrifuge. Cell pellets were then rinsed 1 x with ice-cold 1 x DPBS and centrifuged again for 5 minutes at 1,000 rpm and 4°C. Cells were then fixed by resuspending at 1 x 10⁶ cells per mL in 100 μL BD CytofixTM fixation buffer (BD Bioscience) and incubated for 10 minutes at room temperature. The fixative was removed, and the cells were washed 3 times with 100 μL PBS. PBS was removed, and cells were blocked by adding 100 μL of BD Phosphoantigenic Antibody Stain Buffer (FBS; BD Bioscience) and incubated for 30 minutes at room temperature. The FBS was removed and 100 μL of a 1:50 dilution of the antibody conjugates in PBS was added to each well and incubated for 1 hour at room temperature. The diluted antibody conjugates were removed, and the cells were washed 3 times with 100 μL of 1 x PBS. The nuclei were counterstained with 100 μL ProLong Gold antifade reagent with DAPI (Invitrogen), then viewed with a Zeiss-inverted microscope and analyzed with Axiosview software.

For multinucleation, the cells were fixed and permeabilized as earlier and nuclei stained with 100 μL ProLong Gold antifade reagent with DAPI (Invitrogen), then viewed with a Zeiss-inverted microscope and analyzed with Axiosview software.

**Patient-derived ex vivo studies**

The ex vivo assays were conducted as previously described (23). Briefly, tumor explants were exposed to MK1775 at 500 nmol/L concentrations for 18 hours, and vehicles as well as MK1775-treated tissue fragments were collected for Western blotting and morphologic studies. All experimental protocols were approved by the Animal Care and Use Committee and Institutional Review Board at the University of South Florida (Tampa, FL).

**Results**

**Sarcoma cells are highly sensitive to the cytotoxic effect of MK1775**

To determine the cytotoxic efficacy of MK1775 on different sarcoma cell types, asynchronously growing MG63,
MK1775 induces apoptotic cell death in sarcoma cell lines

To better understand the molecular mechanisms of cell death in the presence of MK1775, we conducted Western blot analysis. As illustrated in Fig. 2B, all 8 sarcoma cell lines showed a dramatic decrease in CDC2 Tyr15 phosphorylation and a significant increase in cleaved PARP (cPARP) whereas total CDC2 levels remained the same, this shows that MK1775 treatment induces apoptotic cell death in these cell lines at clinically relevant doses (26). In addition, MK1775 treatment led to increased serine 139 phosphorylation of γ-H2AX indicating that MK1775 may also cause DNA damage and lead to mitotic catastrophe by allowing cells with DNA damage to enter into mitosis.

Next, we conducted FACS analysis to evaluate MK1775-mediated cell death and its relationship to p53 mutation in sarcoma cells by using Annexin V and DAPI staining. For this purpose, we chose 4 representative sarcoma cell lines with varying p53 status including U2OS (osteosarcoma—p53 wild-type), MG63 (osteosarcoma—p53 null), A673 (Ewing sarcoma—p53 mutant), and HT1080 (fibrosarcoma—p53 wild-type). Apoptotic cell death was assessed by increased percentage of Annexin V–bound cells (Fig. 3A). The bottom right quadrant of plots in Fig. 3A shows the number of cells in early apoptosis, whereas the top right quadrant shows cells in late cell death. Our results show that all 4 cell lines underwent apoptotic cell death in response to MK1775 independent of their p53 status. MG63 cells had a nearly 15-fold increase in Annexin V positivity, whereas HT1080, A673, and U2OS cells showed 10.2-, 6.6-, and 3.4-fold increase in the Annexin V–positive populations, respectively, compared with vehicle-treated control cells (Fig. 3B). To further determine whether

Table 1. Eight cell lines summarizing type of sarcoma, p53 mutational status, and IC50 doses for MK1775 in each of the 9 cell lines calculated from the cell viability assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53</th>
<th>Tumor type</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG63</td>
<td>Null</td>
<td>Osteosarcoma</td>
<td>300.2-E9</td>
</tr>
<tr>
<td>U2OS</td>
<td>WT</td>
<td>Osteosarcoma</td>
<td>546.2-E9</td>
</tr>
<tr>
<td>SW872</td>
<td>Mut</td>
<td>Liposarcoma</td>
<td>321.8-E9</td>
</tr>
<tr>
<td>A673</td>
<td>Mut</td>
<td>Ewing sarcoma</td>
<td>517.7-E9</td>
</tr>
<tr>
<td>SKUT</td>
<td>Mut</td>
<td>Leiomyosarcoma</td>
<td>430.5-E9</td>
</tr>
<tr>
<td>MNNG</td>
<td>Null</td>
<td>Osteosarcoma</td>
<td>338.1-E9</td>
</tr>
<tr>
<td>HT1080</td>
<td>WT</td>
<td>Fibrosarcoma</td>
<td>168.7-E9</td>
</tr>
<tr>
<td>SKES</td>
<td>Mut</td>
<td>Ewing sarcoma</td>
<td>112.2-E9</td>
</tr>
</tbody>
</table>

NOTE: Data shown as mean ± SD n = 3.
Abbreviations: Mut, mutant; WT, wild-type.
MK1775 treatment leads to unscheduled mitotic entry, we first analyzed the microscopic features of sarcoma cells after treatment with MK1775. Cells were treated with MK1775 at indicated concentrations for 24 hours followed by nuclear staining with DAPI. As illustrated in Fig. 3A, bottom, MK1775 treatment caused marked nuclear abnormalities, including multinucleation in all cell lines indicating abnormal mitosis with failed cytokinesis (27, 28). Interestingly, in the same multinucleated cells some of the nuclei show morphologic features of apoptotic cell death consistent with mitotic catastrophe (29). Taken together, these results provide the first evidence that MK1775 monotherapy effectively induces cell death in sarcoma cells in a p53-independent manner.

MK1775 induces unscheduled mitotic entry and cell death in sarcomas

To provide further evidence that inhibition of Wee1 by MK1775 leads to mitotic cell death in sarcoma cells, we next conducted immunofluorescence studies to colocalize mitosis and cell death. Mitotic entry was evaluated by pH3 (30, 31), whereas apoptotic cell death was assessed by increased expression of cleaved Caspase-3. As illustrated in Fig. 4A, microscopic examination of MG63, U2OS, A673, and HT1080 cells treated with MK1775 produced micro- and multinucleation with significantly higher pH3 staining. Furthermore, MK1775-treated cells also often showed double staining with pH3 and cleaved caspase-3 compared with vehicle-treated
control cells. To further quantify this increase, we conducted FACS analysis using pH3 antibody (Fig. 4B). Cells were counterstained with DAPI to assess DNA fragmentation (data not shown). In each of the cell lines we tested, there was a significant increase in the total amount of pH3 staining upon MK1775 treatment. Taken together, these data show that MK1775 treatment causes cell death associated with abnormal mitosis and failed cytokinesis, which is suggestive of mitotic catastrophe, a type of cell death that occurs during mitosis.

**MK1775 treatment results in apoptotic cell death in patient-derived tumor explants ex vivo**

To evaluate MK1775 treatment in clinically relevant tumor samples, we tested the effect of MK1775 on patient-derived tumors including undifferentiated high-grade sarcoma (UHGS), malignant peripheral nerve sheath tumor (MPNST), and pleomorphic spindle cell tumor (PMSS; Fig. 5A, top). Tumor explants were treated with MK1775 ex vivo at a clinically relevant concentration, as previously described (23). After drug treatment for 24 hours, tumor explants were collected for microscopic evaluation and Western blot analysis to assess target inhibition and cell death (Fig. 5). As illustrated in Fig. 5A, bottom, ex vivo treatment of cells with MK1775 (500 nmol/L) caused increased cell death and activation of CDC2 in all samples, as analyzed by PARP cleavage and CDC2 Tyr15 inhibition, whereas total CDC2 levels remained the same. The histologic analysis of tumor explants confirmed that MK1775 treatment caused dramatic cell death, hematoxylin and eosin (H&E)-stained paraffin section of vehicle and MK1775-treated UHGS, PMSS, and MPNST explants are shown in Fig. 5B.
These results further support the findings presented earlier with sarcoma cell lines and strongly suggest that MK1775 could be used as a single agent in the treatment of patients with sarcoma in future clinical studies.

Discussion

Sarcomas are heterogeneous mesenchymal tumors affecting both pediatric and adult populations (4). Approximately 10% of children with cancer are diagnosed with sarcomas, compared with 8% for young adults and 1% of adults (>40 years old) diagnosed with cancer. Ewing sarcoma, osteosarcoma, and rhabdomyosarcoma constitute about 3 quarters of all children with sarcomas, whereas liposarcoma, leiomyosarcoma, undifferentiated sarcomas, synovial sarcoma, and MPNST represent some of the more common histologies in the adult population (32, 33). Localized sarcomas are frequently treated with multimodal therapy including surgery, radiation therapy, and anthracycline-based chemotherapy with curative intent. However, disappointingly, cure rates have only been very modestly improved for recurrent and metastatic sarcomas. Multiple attempts at increasing the doses of these conventional chemotherapeutic agents have increased toxicity without improved efficacy (34, 35). Thus, there is a pressing need to develop novel therapies to improve outcomes in patients with sarcoma. Here, we showed that various soft tissue and bone sarcomas including osteosarcoma, Ewing sarcoma, and MPNST are highly sensitive to the cytotoxic effect of MK1775 when used as a single agent at clinically achievable concentrations.

The G2–M DNA damage checkpoint is an important cell-cycle checkpoint in eukaryotic organisms ensuring that cells do not initiate mitosis before they repair damaged DNA after replication. Wee1 is a tyrosine kinase with a key role as an inhibitory regulator of the G2–M checkpoint that precedes entry into mitosis (9). There is compelling evidence that abrogation of this checkpoint through Wee1 inhibition may result in increased antitumor activity of agents that cause DNA damage such as radiation therapy or some cytotoxic agents (14).

Our data presented here show that MK1775 treatment leads to unscheduled CDC2\textsuperscript{Y15} activation and consequently premature mitotic entry and cell death of sarcoma cells. MK1775 treatment led to increased serine 139 phosphorylation. A, Ewing sarcoma, osteosarcoma, and rhabdomyosarcoma constitute about 3 quarters of all children with sarcomas, whereas liposarcoma, leiomyosarcoma, undifferentiated sarcomas, synovial sarcoma, and MPNST represent some of the more common histologies in the adult population (32, 33). Localized sarcomas are frequently treated with multimodal therapy including surgery, radiation therapy, and anthracycline-based chemotherapy with curative intent. However, disappointingly, cure rates have only been very modestly improved for recurrent and metastatic sarcomas. Multiple attempts at increasing the doses of these conventional chemotherapeutic agents have increased toxicity without improved efficacy (34, 35). Thus, there is a pressing need to develop novel therapies to improve outcomes in patients with sarcoma. Here, we showed that various soft tissue and bone sarcomas including osteosarcoma, Ewing sarcoma, and MPNST are highly sensitive to the cytotoxic effect of MK1775 when used as a single agent at clinically achievable concentrations.

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Previous reports using MK1775 and other Wee1 inhibitors in combination with DNA-damaging agents or radiotherapy showed that p53-deficient cancer cells are more sensitive to Wee1 inhibition (14, 16–20, 37). These studies indicated that defective p53 pathway allows cells to pass G1–S checkpoint making them largely dependent on G2–M arrest to repair damage by cytotoxic agents. Therefore, p53 mutation has been proposed as a predictor of tumor response to Wee1 inhibitors in clinical trials (14, 38). Our data show that p53 status is not predictive of MK1775 response in sarcoma cells. We showed that MK1775 is effective as monotherapy in sarcoma cells independent of their...
p53 status, as we observed a similar degree of cell death in sarcoma cells with wild-type p53, mutant p53, and null p53. However, the role of p53 in response of sarcoma cells to combination treatment with MK1775 and DNA-damaging agents remains to be elucidated.

Recent studies showed that MK1775 is a well tolerated drug that has not shown a clear dose-limiting toxicity (39). The data presented here together with the high safety profile of this drug strongly suggest that MK1775 can be used as a potential therapeutic agent in the treatment of both adult as well as pediatric patients with sarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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