WEE1 Kinase Inhibition Enhances the Radiation Response of Diffuse Intrinsic Pontine Gliomas

Viola Caretti1,2,4, Lotte Hiddingh1,2,4, Tonny Lagerweij1,2,4, Pepijn Schellen1,2,4, Phil W. Koken3, Esther Hulleman1,2,4, Dannis G. van Vuurden1,4, W. Peter Vandertop2, Gertjan J.L. Kaspers1, David P. Noske2,4, and Thomas Wurdinger2,4,5

Abstract

Diffuse intrinsic pontine glioma (DIPG) is a fatal pediatric disease. Thus far, no therapeutic agent has proven beneficial in the treatment of this malignancy. Therefore, conventional DNA-damaging radiotherapy remains the standard treatment, providing transient neurologic improvement without improving the probability of overall survival. During radiotherapy, WEE1 kinase controls the G2 cell-cycle checkpoint, allowing for repair of irradiation (IR)-induced DNA damage. Here, we show that WEE1 kinase is one of the highest overexpressed kinases in primary DIPG tissues compared with matching non-neoplastic brain tissues. Inhibition of WEE1 by MK-1775 treatment of DIPG cells inhibited the IR-induced WEE1-mediated phosphorylation of CDC2, resulting in reduced G2–M arrest and decreased cell viability. Finally, we show that MK-1775 enhances the radiation response of E98-Fluc-mCherry DIPG mouse xenografts. Altogether, these results show that inhibition of WEE1 kinase in conjunction with radiotherapy holds potential as a therapeutic approach for the treatment of DIPG.

Introduction

Diffuse intrinsic pontine glioma (DIPG) is an almost invariably fatal brain neoplasm affecting mainly children, with a 2-year survival rate less than 10% (1, 2). Its hallmarks are the specific anatomic location from which it originates, the pons (3), its diffuse phenotype, often spreading to the cerebellum and brain areas as far as the cerebral hemispheres (4), and its bleak prognosis (3). Despite various clinical trials, the standard treatment for DIPG patients remains conventional radiotherapy, which provides transient neurologic improvement, resulting in a better quality of life, but does not improve probability of overall survival (1, 2, 5). Therefore, novel treatment strategies to increase the efficacy of radiotherapy in DIPG are urgently needed.

We have previously shown that inhibition of WEE1 kinase, one of the main gatekeepers of the G2 cell-cycle checkpoint, is a potential therapeutic target for radiosensitization of adult gliomas (6) and of other type of cancers (7–9). Normal cells have functional cell-cycle checkpoints as compared with cancer cells, which often have a deficient G1 arrest due to aberrant p53 signaling and, therefore, heavily rely on the G2 checkpoint to repair DNA damage caused by irradiation (IR; ref. 10). Abrogation of the G2 checkpoint pushes glioma cells with unrepaired DNA damage into mitotic catastrophe, resulting in subsequent cell death (11). Interestingly, in DIPG, recent genomic studies have revealed aberrations in genes regulating the G1 checkpoint, including TP53, MDM2, CDKN2A, and ATM (12–19), suggesting a dysfunctional G1 arrest in DIPG cells. Therefore, inhibition of WEE1 could be a potential strategy to enhance the response to IR in DIPG cells.

A number of small-molecule compounds that inhibit WEE1 have been developed. These include PD0166285 (20, 21), PD0407824 (22, 23), WEE1 inhibitor II, and PHCD (23–25). The most promising WEE1 inhibitor may be MK-1775, a pyrazolopyrimidine derivative, because of its selectivity and potency to inhibit WEE1 kinase (26, 27).

In vivo, WEE1 inhibition has resulted in tumor growth reduction, increased survival, and absence of significant toxicity in several studies using xenograft animal models (6, 9, 26–30). Moreover, preliminary results of a phase 1 study of oral MK-1775 as monotherapy and in combination with gemcitabine, cisplatin, or carboplatin reported good tolerance and strong target engagement (31). We have previously shown that inhibition of WEE1 could function as a potential radiosensitizer of adult gliomas, both in vitro and in vivo. As an extension to these previous results, in this study, we investigated the
potential radiation-enhancing effects of a more potent and clinically relevant WEE1 inhibitor, MK-1775, in DIPG cells in culture and in vivo using the E98-Fluc-mCherry (E98-FM) DIPG mouse model, closely resembling the DIPG phenotype in humans (32).

Materials and Methods

Ethics statement

All animal experiments were conducted according to the guidelines established by the European community and following a protocol (NCH10-05) approved by the institutional ethical committee on animal experiments of the VU University (Amsterdam, the Netherlands). All patient samples, including the de novo cell line VUMC-DIPG-A, were used after appropriate written informed consent and under approval of the institutional medical ethical committee of the VU University Medical Center (Vumc). The research described here has been conducted according to the principles expressed in the Declaration of Helsinki.

In silico analysis of DIPG kinase expression

R2, a microarray analysis and visualization platform, provided by the Department of Oncogenomics of the Academic Medical Centre, Amsterdam, The Netherlands (http://r2.amc.nl), was used to obtain an overview of kinase mRNA expression in DIPG. A MAS5.0 normalized dataset of post-mortem DIPG tissues (ref. 17; n = 27; GSE GSE26576) was compared with post-mortem normal brain regions (ref. 33; n = 172; GSE11882), consisting of the hippocampus, entorhinal cortex, superior frontal gyrus, and postcentral gyrus, and 2 samples of post-mortem normal brainstem from the DIPG dataset (ref. 17; ref. GSE26576). To evaluate statistical significance, a false discovery rate (FDR)-corrected, moderated t-test (Linear Models for Microarray Data - Limma) was used to compare mean kinase mRNA expression levels between datasets. An FDR P value less than 0.000005 was considered significant. To compare WEE1 mRNA expression between groups within DIPG dataset and normal brain tissue datasets, 1-sided ANOVA was used.

Cells and tissue samples

The primary low-passage VUMC-DIPG-A cells were derived from tumor tissue surgically removed from a patient diagnosed with DIPG at the VUmc. E98 cells were obtained from the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands; ref. 34) and transduced with a lentiviral vector containing Fluc and mCherry at our institution (32). Cells used in this study were not authenticated. The primary VUMC-DIPG-A and E98-FM cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; PAA) containing 10% FBS and antibiotics. DIPG tissue and control non-neoplastic brain tissue were obtained post-mortem from 5 DIPG patients (VUMC-DIPG-1, 2, 3, 4, and 5; ref. 35), while VUMC-DIPG-A cells were isolated from surgical specimen after written informed consent.

WEE1 inhibitor and IR

The WEE1 inhibitor MK-1775 (Axon Medchem) was resuspended in dimethyl sulfoxide (DMSO) to a concentration of 100 mmol/L and diluted in PBS for the in vivo experiments and in medium for the in vitro experiments. Cells were irradiated in a Gammacell 220 Research Irradiator (MDS Nordion).

Western blotting

Expression levels of WEE1 were assessed by Western blot analysis in both tissue samples and cell lines as described previously (6). In brief, after cell lysis (for CDC2-p^Y15 a phosphoslysis buffer was used), 30 μg protein was transferred to a polyvinylidene difluoride (PVDF) membrane and incubated with the primary antibodies: mouse anti-WEE1 (1:1,000; Santa Cruz Biotechnology), mouse anti-β-actin (1:10,000; Santa Cruz Biotechnology), and rabbit anti-CDC2-p^Y15 (1:2,000; Abcam), and subsequently incubated with horseradish peroxidase (HRP)-labeled goat-anti-mouse or HRP-labeled goat-anti-rabbit immunoglobulins (DAKO). Protein detection and visualization was carried out using ECL+ Western Blotting Detection Reagents (Pierce).

Immunohistochemistry

Paraffin-embedded DIPG tissue samples and matched non-neoplastic brain samples were deparaffinized and rehydrated. Endogenous peroxidase was inhibited by 30-minute incubation in 0.3% H2O2, diluted in methanol. Antigens were retrieved by boiling in Tris/EDTA buffer (pH 9.0) in a microwave for 10 minutes, followed by washing 3 times in PBS. Slides were incubated with mouse anti-WEE1 (1:50; Cell Signaling Technology) overnight at 4°C. Slides were washed 3 times in PBS and incubated with the secondary antibody, Envision+ Poly-HRP immunohistochemistry (IHC) Kit (Immunologic) for 30 minutes at room temperature. Positive reactions were visualized by incubation with DAB chromogen solution. Slides were counterstained with hematoxylin, dehydrated, mounted, and analyzed by microscopy.

Flow cytometry

At 16 hours after treatment, cells were washed twice with PBS containing 1% FBS and subsequently fixed in 70% ethanol for 24 hours. Next, the cells were washed once in PBS containing 1% FBS followed by RNase A treatment (0.15 mg/mL) for 20 minutes and subsequent DNA staining with 50 μg/μL propidium iodide (PI) for 30 minutes. Cell-cycle distribution assessment was conducted using a FacsCalibur Flow Cytometer and CellQuest Pro software (Becton-Dickinson). Subsequently, data were analyzed using ModfitLT software (Verity Software House).

Immunofluorescence staining

Cells were fixed in 3% paraformaldehyde at different time points post irradiation (15 minutes, 30 minutes, and
1, 24, 48, and 72 hours). Before staining, the cells were rinsed in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 30 minutes at room temperature and blocked in PBS containing 5% FCS. Coverslips were incubated with both mouse-anti-γ-histone-H2AX (1:100; Millipore) and rabbit-anti-53BP1 (1:100; Novus Biologicals) in PBS containing 5% FCS overnight at 4°C. This was followed by secondary antibody incubation with goat-anti-rabbit/Alexa568 immunoglobulins (1:100; Invitrogen) and, subsequently, with rabbit-anti-mouse/fluorescein isothiocyanate (FITC) immunoglobulins (1:100; DAKO) both in PBS containing 5% FCS for 30 minutes at room temperature in the dark. Slips were rinsed in PBS 3 times and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000) in PBS at room temperature in the dark. This was followed by successive rinses in PBS and sterile water. The slips were then mounted on glass slides using Vectashield (Vector Laboratories) and visualized with a Carl Zeiss Axioskop 20 microscope at ×100 objective.

**Cell counts**

At 4 days after treatment, cells were fixed with 3.7% formaldehyde and DNA was stained with DAPI (0.3 μg/mL). Cell numbers were assessed by counting the number of DAPI-stained cells using the Acumen Ex3 laser scanning cytometer (TTP LabTech).

**In vivo analysis using the orthotopic E98-FM DIPG mouse model**

Female athymic nude mice (age 6–8 weeks; Harlan, Zeist, the Netherlands) were kept under specific pathogen-free conditions in air-filtered cages and received food and water ad libitum. E98-FM cells were injected as described previously (32). In short, tumors were generated via stereotactic injection of 0.5 × 10^6 E98-FM cells in a volume of 5 μL into the murine pons (coordinates from lambda: −1.0 mm X, −0.8 mm Y, 5.0 mm Z). Tumor growth was monitored twice-weekly by bioluminescence imaging (BLI), as previously reported (32). At day 7 after intracranial injection, mice were stratified on the basis of BLI signal intensities into 4 treatment groups (n = 12) with comparable mean Fluc activity and, then, received MK-1775 (90 mg/kg) or vehicle (PBS containing 18% DMSO) in a total volume of 200 μL via intraperitoneal injection every other day for a total of 6 injections per mouse. At day 8, mice received head-only IR with a single dose of 2 Gy using Clinac D/E (Varian Medical Systems), as described previously (32). Mice were sacrificed after humane endpoints were reached via sedation (1.5 L O2/min and 2.5% isoflurane), followed by cervical dislocation. To assess significant differences between treatment groups mice with BLI values within each treatment group outside the mean ± 2 × SD range were excluded from further analysis. Next, BLI signals were log_{10}-transformed and unpaired Student t test was carried out. A difference was considered significant when P < 0.05.

**Results**

**In silico analysis of kinase mRNA expression identifies WEE1 as a potential drug target in DIPG**

Using the publicly available microarray analysis and visualization platform R2 (http://r2.amc.nl), we analyzed a DIPG gene expression data set (17) and compared it with nonmalignant brain tissue (33, 36), including 2 tissue samples of normal brainstem (17), to identify the top upregulated kinase genes for this malignancy. To compare the differential kinase gene expression of cancer versus normal samples, the fold-change to nonmalignant brain tissue was determined for all kinases within the dataset. A FDR-corrected moderated t test was used to compare means of kinase gene expression levels between the data-sets and a P value less than 0.000005 was considered significant. Analysis of the kinase expression data showed that WEE1 ranked seventh among the overexpressed kinases (Table 1). Given our previous experience on the radiosensitizing effect of WEE1 inhibition in adult glioma and the existence of a selective inhibitor, MK-1775, currently being tested in clinical trials, we decided to further investigate WEE1 as a potential target to enhance the IR response in DIPG cells. Using the R2 platform, we specifically investigated WEE1 mRNA expression in DIPG, low-grade glioma (LGG) in the brainstem, normal brainstem, nonmalignant brain regions, and normal cerebellum (Fig. 1A). Using 1-way ANOVA, WEE1 mRNA levels were determined to be significantly higher in DIPG than in different regions of normal control brain. Interestingly, WEE1 mRNA levels were significantly higher in DIPG compared with LGG of the brainstem, as we previously observed in adult low- and high-grade gliomas (6).

**WEE1 protein is overexpressed in DIPG patient material and cell lines**

Next, we analyzed the protein expression of WEE1 kinase by Western blot analysis in 5 different post-mortem DIPG tissues (35) isolated from the pons and matched these to non-neoplastic brain tissues of the same patient (Fig. 1B). We observed significant overexpression of WEE1 protein in 4 out of 5 tumors compared with the non-neoplastic brain tissues. For patient VUMC-DIPG-1, we were not able to detect WEE1 protein overexpression in the tumor in the pons region, which may be due to the presence of tumor necrosis in the sample used. However, we detected significant WEE1 protein expression in VUMC-DIPG-1 tissue isolated from the frontal lobe of the same patient (Fig. 1B). In addition, we analyzed WEE1 protein expression by Western blot analysis in a primary DIPG cell line and in E98-FM glioma cells (32), again showing significant WEE1 protein levels (Fig. 1C). Furthermore, by IHC we found WEE1 protein overexpression, as shown by a clear nuclear staining, in DIPG tumors located in the pons (Fig. 1D–G and I–K), left as compared with matched non-neoplastic brain tissue (Fig. 1L). In particular, high levels of WEE1 were detected in the tumor bulk (Fig. 1D, asterisk), as well as in the infiltrative DIPG component (Fig. 1E), and in tumor cells surrounding the blood vessels (Fig. 1F). Nuclei in the
leptomeningeal tumor component also revealed WEE1 nuclear staining (Fig. 1G). Notably, WEE1 was found to be overexpressed in tumor cells invading brain areas beyond the brainstem (Fig. 1H and I–K, right). Finally, clear WEE1 nuclear staining was also detected in tumor tissue derived from the orthotopic E98-FM DIPG mouse model (Fig. 1M, arrow).

MK-1775 inhibits WEE1-regulated CDC2, IR-induced G2 arrest, and repair of IR-induced DNA damage in DIPG cells

E98-FM and VUMC-DIPG-A cells were used to study the effect of the WEE1 kinase inhibitor MK-1775 in vitro. Because WEE1 phosphorlates CDC2 on being subjected to IR (37–39), we conducted Western blot analysis for phosphorylated CDC2 (Y15) 16 hours after treatment with 0.1 μmol/L MK-1775 and IR (Fig. 2B). IR at 6 Gy resulted in increased CDC2-pY15 levels, which were reduced on treatment with MK-1775, indicating functional inhibition of WEE1 kinase activity in these cells. The effect of MK-1775-mediated WEE1 inhibition on the IR-induced G2 arrest was determined by cell-cycle analysis using flow cytometry (Fig. 2C). In both cell lines, IR at 6 Gy induced a G2–M arrest after 16 hours, indicated by an accumulation of cells with doubled DNA content and indicative for a dysfunctional G1 arrest, which was reduced on treatment with 0.1 μmol/L MK-1775. Subsequently, we examined the effect of WEE1 inhibition on the repair of IR-induced DNA damage within these cells (Fig. 2D and E). The IR-induced DNA damage was visualized using the DNA double-strand break (DSB) markers gH2AX and 53BP1 at 15 minutes, 30 minutes, and 1, 24, 48, and 72 hours after treatment with IR at 4 Gy in the presence or absence of 0.1 μmol/L MK-1775. As shown in Fig. 2D gH2AX and 53BP1 colocalize. These results indicate DNA damage in both cell lines after IR treatment as compared with untreated cells. Cells treated with IR showed fast onset of DSBs (as early as 15 minutes after IR); however, a decreased

Table 1. Top 20 upregulated kinases in DIPG

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>Name</th>
<th>Probeset</th>
<th>Expression normal brain (log2)</th>
<th>Expression DIPG (log2)</th>
<th>Fold increase (log2)</th>
<th>P value (FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TOP2A</td>
<td>Topoisomerase (DNA) II alpha</td>
<td>201292_at</td>
<td>1,05</td>
<td>7,85</td>
<td>7,45</td>
<td>3,30E-68</td>
</tr>
<tr>
<td>2</td>
<td>MELK</td>
<td>Maternal embryonic leucine</td>
<td>204825_at</td>
<td>1,61</td>
<td>7,49</td>
<td>4,66</td>
<td>6,64E-62</td>
</tr>
<tr>
<td>3</td>
<td>BUB1</td>
<td>Budding uninhibited by benzo-</td>
<td>209642_at</td>
<td>1,98</td>
<td>6,69</td>
<td>3,38</td>
<td>3,70E-44</td>
</tr>
<tr>
<td>4</td>
<td>TTK</td>
<td>TTK protein kinase</td>
<td>204822_at</td>
<td>2,12</td>
<td>6,83</td>
<td>3,23</td>
<td>2,78E-51</td>
</tr>
<tr>
<td>5</td>
<td>PBK</td>
<td>PDZ binding kinase</td>
<td>219148_at</td>
<td>2,46</td>
<td>7,61</td>
<td>3,10</td>
<td>5,90E-61</td>
</tr>
<tr>
<td>6</td>
<td>OSR1</td>
<td>Odd-skipped related 1 (Drosophila)</td>
<td>228399_at</td>
<td>1,87</td>
<td>5,66</td>
<td>3,03</td>
<td>7,03E-22</td>
</tr>
<tr>
<td>7</td>
<td>WEE1</td>
<td>WEE1 homolog</td>
<td>212533_at</td>
<td>3,60</td>
<td>8,81</td>
<td>2,45</td>
<td>2,60E-46</td>
</tr>
<tr>
<td>8</td>
<td>NEK2</td>
<td>NIMA (never in mitosis gene a)-related kinase 2</td>
<td>204641_at</td>
<td>3,44</td>
<td>6,74</td>
<td>1,96</td>
<td>8,43E-24</td>
</tr>
<tr>
<td>9</td>
<td>STK33</td>
<td>Serine/threonine kinase 33</td>
<td>228035_at</td>
<td>3,18</td>
<td>6,14</td>
<td>1,93</td>
<td>2,03E-19</td>
</tr>
<tr>
<td>10</td>
<td>TEX14</td>
<td>Testis expressed 14</td>
<td>221035_s_at</td>
<td>2,92</td>
<td>5,50</td>
<td>1,88</td>
<td>5,24E-14</td>
</tr>
<tr>
<td>11</td>
<td>CHEK1</td>
<td>CHK1 checkpoint homolog (S. pombe)</td>
<td>205394_at</td>
<td>2,90</td>
<td>5,37</td>
<td>1,85</td>
<td>2,57E-30</td>
</tr>
<tr>
<td>12</td>
<td>AURKB</td>
<td>Aurora kinase B</td>
<td>209464_at</td>
<td>3,05</td>
<td>5,32</td>
<td>1,74</td>
<td>2,45E-20</td>
</tr>
<tr>
<td>13</td>
<td>BUB1B</td>
<td>Budding uninhibited by benzo-</td>
<td>203755_at</td>
<td>3,98</td>
<td>6,84</td>
<td>1,72</td>
<td>4,08E-32</td>
</tr>
<tr>
<td>14</td>
<td>HK2</td>
<td>Hexokinase 2</td>
<td>202934_at</td>
<td>5,54</td>
<td>8,95</td>
<td>1,62</td>
<td>2,81E-32</td>
</tr>
<tr>
<td>15</td>
<td>CHEK2</td>
<td>CHK2 checkpoint homolog (S. pombe)</td>
<td>210416_s_at</td>
<td>3,51</td>
<td>5,43</td>
<td>1,55</td>
<td>3,41E-15</td>
</tr>
<tr>
<td>16</td>
<td>AURKA</td>
<td>Aurora kinase A</td>
<td>204092_s_at</td>
<td>4,26</td>
<td>6,57</td>
<td>1,54</td>
<td>4,37E-33</td>
</tr>
<tr>
<td>17</td>
<td>ROR2</td>
<td>Receptor tyrosine kinase-like</td>
<td>205578_at</td>
<td>2,41</td>
<td>3,71</td>
<td>1,54</td>
<td>3,62E-06</td>
</tr>
<tr>
<td>18</td>
<td>PLAU</td>
<td>Plasminogen activator, urokinase</td>
<td>205479_s_at</td>
<td>3,52</td>
<td>5,41</td>
<td>1,54</td>
<td>2,29E-14</td>
</tr>
<tr>
<td>19</td>
<td>DYRK3</td>
<td>Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3</td>
<td>210151_s_at</td>
<td>4,28</td>
<td>6,32</td>
<td>1,48</td>
<td>6,32E-15</td>
</tr>
<tr>
<td>20</td>
<td>MASTL</td>
<td>Microtubule-associated serine/threonine kinase-like</td>
<td>228468_at</td>
<td>4,21</td>
<td>6,13</td>
<td>1,46</td>
<td>3,76E-47</td>
</tr>
</tbody>
</table>

NOTE: Top 20 upregulated kinases in DIPG tumor samples (n = 27; ref. 17) sorted on log2 fold increase as compared with nonmalignant brain tissues (n = 174; ref. 33) including 2 samples of normal brain stem tissue from the DIPG dataset (17). WEE1 homolog (WEE1) is identified as a highly differentially overexpressed kinase in DIPG.
number of γH2AX and 53BP1 foci was observed over time in cells treated only with IR, indicating the occurrence of DNA damage repair within these cells (Fig. 2D and E). Notably, although no differences in the number of IR-induced DSB foci were observed at the early time points after IR, the cells treated with both IR and MK-1775 showed persistence of DNA damage over time, as indicated by a higher number of γH2AX and 53BP1 foci in these cells compared with cells treated with IR at 4 Gy alone (Fig. 2D and E; E98-FM and VUMC-DIPG-A, P < 0.001). These results show that MK-1775 is capable of functional WEE1 inhibition and, thereby, of attenuating the IR-induced G_{2} arrest in G_{2}-dependent E98-FM and VUMC-DIPG-A cells and, subsequently, of inhibiting the repair of IR-induced DNA damage in these cells.

**MK-1775 enhances the radiation response in cultured DIPG cells**

Next, we determined whether the attenuation of IR-induced G_{2} arrest by MK-1775 reduces viability of these cells *in vitro*. A clonogenic survival assay is the gold standard for this purpose (40), but because these cells do not form appreciable colonies, we monitored cell viability by counting DAPI-stained cells using a laser scanning cytometer. We treated E98-FM cells with IR at 4 Gy in the presence or absence of 0.1 μmol/L MK-1775. After 4 days, cells were stained with DAPI and cell numbers were counted. Treatment of the E98-FM cells with 0.1 μmol/L MK-1775 or IR monotherapy resulted in a significant but moderate decrease in cell viability compared with untreated cells. Treatment with MK-1775 in combination with IR resulted in radiosensitization of E98-FM cells (P < 0.001; Fig. 3A and B). Next, we analyzed the effect of 0.1 μmol/L MK-1775 in combination with IR at 4 Gy, on the cell viability of primary VUMC-DIPG-A cells. Both 0.1 μmol/L MK-1775 and IR monotherapy show only a limited effect on cell viability of VUMC-DIPG-A. However, combined treatment with MK-1775 and IR resulted in additional diminished cell viability, compared with IR alone (P < 0.05; Fig. 3C and D).
MK-1775 enhances the radiation response in the orthotopic E98-FM DIPG mouse model

We used the orthotopic E98-FM DIPG mouse model (32) to study the radiation-enhancing effects of MK-1775 in vivo. E98-FM glioma cells were stereotactically injected in the pons of nude mice. Tumor growth was monitored twice a week using the BLI intensity of the photon activity of firefly luciferase encoded by the E98-FM cells. Seven days after intracranial injection of the cells, mice were randomized into 4 groups (n = 12 per group) on the basis of BLI intensity. Two groups of mice received MK-1775 intraperitoneally (i.p.; 90 mg/kg) and 2 groups received vehicle (DMSO diluted in PBS) i.p, at 7, 9, 11, 13, 15, and 17 days after injection of the cells. One group of MK-1775 and vehicle-treated mice was irradiated with 2 Gy at day 8 after injection of the cells. BLI revealed significant tumor progression in both nonirradiated vehicle-treated and MK-1775-treated mice, while tumor growth was significantly delayed in irradiated and irradiated MK-1775-treated mice compared with the control group (weeks 2 and 3, and weeks 2, 3, and 4, respectively; Fig. 4A and B). Moreover, the mean BLI signal of the irradiated MK-1775-treated group was significantly decreased compared with the irradiated group at week 4 after injection of the cells (P < 0.05). In addition, survival analysis showed a significant advantage for combining IR with MK-1775 over the vehicle-treated control group (log-rank, P < 0.05), while the groups receiving only MK-1775 or IR showed no significant effect on survival as compared with the untreated mice (Fig. 4C). These results indicate that pharmacologic targeting of WEE1 with MK-1775 in combination with IR delays the growth of E98-FM DIPG tumors in vivo, although the effects observed under these conditions were modest.

Figure 2. MK-1775 inhibits WEE1-regulated CDC2 and IR-induced G2 arrest in E98-FM and DIPG cells. A, chemical structure of MK-1775. B, Western blot analysis of phosphorylated CDC2 16 hours after treatment of E98-FM and VUMC-DIPG-A cells with 6 Gy and/or 0.1 μmol/L MK-1775. Numbers represent relative phosphorylated CDC2 expression after normalization against control carried out using imageJ software. C, PI cell-cycle analysis of E98-FM and VUMC-DIPG-A cells 16 hours after treatment and treated as in B. The percentage of cells in the G2–M phase are indicated. D, analysis of DNA damage repair after treatment with IR at 4 Gy or IR and 0.1 μmol/L MK-1775, visualized by the DSB markers γH2AX and 53BP1. The panel shows representative images of γH2AX foci, 53BP1 foci, DAPI-stained nuclei, and merged images in VUMC-DIPG-A cells at 15 minutes, 30 minutes, and 1, 24, 48, and 72 hours after treatment. E, quantification of average number of γH2AX foci per nucleus in E98-FM and VUMC-DIPG-A cells at similar time points as in D ("", P < 0.05, "**, P < 0.01; *** P < 0.001 Student t test). At least 20 nuclei per time point were analyzed for this quantification.
DIPG is the most deadly pediatric malignancy. Radiotherapy remains the standard treatment, although this only causes temporary tumor regression. Despite numerous clinical trials, no therapeutic agent has, thus far, shown a survival benefit for DIPG patients (2). Nonetheless, the clinical use of these agents was not based on translational studies due to the lack of DIPG tissue and in vitro and in vivo models available for preclinical research. Here, we present a translational study with WEE1 target assessment on DIPG tissue, functional experiments using primary DIPG cells in vitro and the orthotopic E98-FM DIPG model (32) to show the radiation-enhancing effects of WEE1 inhibition in vivo.

Figure 3. MK-1775 enhances the radiation response in E98-FM and DIPG cells. A and B, analysis of cell counts of E98-FM cells after treatment with IR at 4 Gy and/or 0.1 μmol/L MK-1775. Relative percentages of cell counts were normalized to that of MK-1775-only treatment, as depicted in logarithmic scale. The panel in B shows representative images of DAPI-stained cells for the different treatment conditions. C and D, similar analysis as in A and B using primary VUMC-DIPG-A cells. Results are depicted as averages of an experiment conducted in triplicate; error bars indicate standard error of the mean (*, P < 0.05, ***, P < 0.001, Student t test). Size bar, 20 μm.

Kinase expression data analysis showed WEE1 kinase to be in the top-10 overexpressed kinases in a dataset of DIPG samples compared with normal brain tissues. Although the other overexpressed kinases could also be of value for investigating therapeutic targeting in DIPG, we analyzed WEE1 kinase, because it was previously shown to be a potential target for the radiosensitization of adult glioma cells (6), and because a clinically relevant WEE1 inhibitor was available (31). We found WEE1 to be highly overexpressed in the DIPG tissues analyzed. Interestingly, WEE1 was also overexpressed in tumor tissue invading brain areas beyond the brainstem (including cerebellum, insula, and frontal lobe), indicating that the infiltrative DIPG components can also be potentially targeted by WEE1 inhibitors. IHC confirmed WEE1...
overexpression in the bulk of the pontine tumor as well as in areas of the pons infiltrated by tumor cells, for example, in the invasive tumor front surrounding blood vessels. Finally, WEE1 expression level was found to be consistently lower in brain tissue not affected by the disease, indicating a therapeutic index and possible tumor-specific action of drugs inhibiting WEE1.

WEE1 inhibitors can function as enhancers of radiation responses because they abrogate the G2 checkpoint, thus preventing IR-induced DNA damage repair (41). To investigate the possible radiation-enhancing effects of WEE1 inhibition in DIPG, MK-1775 was selected, given its proven selective action on WEE1 and its clinical relevance (31, 41). In addition, a previous study reported that MK-1775 had no toxic effects on normal human astrocytes (30), and results from a clinical trial indicate that MK-1775 is tolerated by adult patients (31). Recently, preclinical studies have showed the radiosensitizing effects of MK-1775 in lung cancer (9), pancreatic cancer (29), and glioblastoma cell lines (30). Here, MK-1775 treatment resulted in an enhancement of the radiation response in primary DIPG cells, although less pronounced than on E98-FM glioma cells, which may be at least partly attributable to a difference in proliferation rate (doubling time of 72 hours for VUMC-DIPG-A vs. 24 hours for E98-FM; ref. 30).

We also investigated the radiation-enhancing effects of MK-1775 in the E98-FM DIPG mouse model in vivo. After injection into the mouse pons, the human E98-FM glioma cells gave rise to highly infiltrative tumors, accurately reproducing the invasive phenotype of DIPG, which represents one of the major challenges in the treatment of this disease (32). The mean BLI signal, indicative for E98-FM tumor size, was consistently lower in both the combined treatment and the irradiated treatment group as compared with the control group. This effect only persisted in the combination treatment group over time,
resulting in a significant difference in mean BLI between the irradiated and the combination treatment group at week 4 after injection of the cells. The group receiving both IR and MK-1775 showed a significant longer survival than the nontreated control group, while IR alone did not yield such a significant survival advantage. This indicates that combining IR with MK-1775 improves the antitumor effect of IR alone. Finally, while treatment with MK-1775 alone showed lower cell counts in vivo, monotherapy with MK-1775 in vivo did not result in a significant antitumor effect. In previous in vivo studies, MK-1775 monotherapy resulted in decreased tumor burden (9, 27–30). Thus, further studies using additional DIPG preclinical models and optimization of MK-1775 and IR dosing are needed. Moreover, supratentorial pediatric gliomas, although potentially representing a separate disease, may also respond to treatment with WEE1 inhibitors in combination with IR, and a comparison between infratentorial DIPG and supratentorial pediatric gliomas could be of importance in the context of drug delivery to these different regions of the brain. The route of administration may also influence the therapeutic outcome, and it would be of interest to compare i.p. versus oral administration of MK-1775 in combination with pharmacodynamic and pharmacokinetic analyses, because MK-1775 blood level was found to be undetectable by 10 hours (30). Finally, previous studies have used subcutaneous tumors to assess the effects of MK-1775 in vivo (9, 27–30). Therefore, the limited radiation-enhancing effect observed on DIPG in vivo may also be attributable to a possible inability of MK-1775 to cross the blood–brain barrier, which may be intact in large parts of the DIPG tumor (32).

In conclusion, in this study we showed that WEE1 is overexpressed in DIPG and its inhibition in vivo and in vitro resulted in additional antitumor effects when combined with IR. Ultimately, DIPG is a very aggressive and heterogeneous tumor and the development of a clinical multitarget approach will be necessary. In this context, WEE1 inhibition in conjunction with radiotherapy may be one of the therapeutic strategies used in the treatment armamentarium needed to treat this at present still fatal disease.

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

Authors’ Contributions

Conception and design: V. Caretti, L. Hiddinck, G.J.L. Kaspers, T. Wurdinger

Development of methodology: V. Caretti, L. Hiddinck, P.W. Koken, T. Wurdinger

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Caretti, L. Hiddinck, T. Lagerweij, P. Schellen, P.W. Koken, T. Wurdinger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Caretti, L. Hiddinck, E. Hullemann, D.G. van Vuurden, G.J.L. Kaspers, T. Wurdinger

Writing, review, and/or revision of the manuscript: V. Caretti, L. Hiddinck, P.W. Koken, E. Hullemann, D.G. van Vuurden, W.P. Vandertop, G.J.L. Kaspers, D.P. Noske, T. Wurdinger

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Wurdinger

Study supervision: G.J.L. Kaspers, D.P. Noske, T. Wurdinger

Acknowledgments

The authors thank Fred Buijs (Department of Nuclear Medicine & PET Research), Laurine Wedekind, and Petra van der Stoop (Neuro-oncology Research Group) for skilled technical support, all from the VU University Medical Center, Amsterdam, the Netherlands. The authors also kindly acknowledge Jan Koster (AMC, Amsterdam, the Netherlands) for the adaptations made in R2.

Grant Support

This research was financially supported by the Semmy foundation (G.J.L. Kaspers). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 17, 2012; revised November 19, 2012; accepted December 6, 2012; published OnlineFirst December 27, 2012.

References


www.aacrjournals.org

Mol Cancer Ther; 12(2) February 2013

OF9

Published OnlineFirst December 27, 2012; DOI: 10.1158/1535-7163.MCT-12-0735

Downloaded from mct.aacrjournals.org on June 21, 2017. © 2012 American Association for Cancer Research.


Molecular Cancer Therapeutics

WEE1 Kinase Inhibition Enhances the Radiation Response of Diffuse Intrinsic Pontine Gliomas

Viola Caretti, Lotte Hiddingh, Tonny Lagerweij, et al.

Mol Cancer Ther Published OnlineFirst December 27, 2012.

Updated version Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-0735

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.