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

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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. Hence, conventional DNA-damaging radiotherapy (RT) remains the standard treatment, providing transient neurological improvement without improving probability of overall survival. During RT, 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 as compared to 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 demonstrate 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 RT 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 two-year survival rate less than 10% (1,2). Its hallmarks are the specific anatomical 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 (RT), which provides transient neurological 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 effectivity of RT in DIPG are urgently needed.

We have previously shown that inhibition of WEE1 kinase, one of the main gatekeepers of the G_2 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 to cancer cells, which often have a deficient G_1 arrest due to aberrant p53 signaling and hence heavily rely on the G_2 checkpoint to repair DNA damage caused by irradiation (IR) (10). Abrogation of the G_2 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 G_1 checkpoint, including TP53, MDM2, CDKN2A, ATM (12-19), suggesting a dysfunctional G_1 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 pyrazolo pyrimidine 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 utilizing xenograft animal models (6,9,26-30). Moreover, preliminary results of a phase I 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 performed 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. 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 here described research 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 (17) (n=27; GSE26576) was compared to post-mortem normal brain regions (33) (n=172; GSE11882), consisting of hippocampus, entorhinal cortex, superior frontal gyrus and postcentral gyrus, and 2 samples of post-mortem normal brainstem from the DIPG dataset (17) (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 < 0.000005 was considered significant. To compare WEE1 mRNA expression between groups within DIPG dataset and normal brain tissue datasets, one sided analysis of variance (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 Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands) (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 DMEM medium (PAA, Cölbe, Germany) containing 10% FBS, and antibiotics. DIPG tissue and control non-neoplastic brain tissue were obtained post-mortem from five DIPG patients (VUMC-
DIPG-1, 2, 3, 4, and 5) (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, Groningen, The Netherlands) was resuspended in DMSO to a concentration of 100 mM 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 blotting (WB) in both tissue samples and cell lines as described elsewhere (6). In brief, after cell lysis (for CDC2-pY15 a phospho-lysis buffer was used), 30 μg protein was transferred to a PVDF membrane and incubated with the primary antibodies: mouse anti-WEE1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-actin (1:10,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-CDC2-pY15 (1:2,000) (Abcam, Cambridge, UK), and subsequently incubated with HRP-labeled goat-anti-mouse or HRP-labeled goat-anti-rabbit immunoglobulins (DAKO, Aachen, Germany). Protein detection and visualization was performed using ECL+ Western Blotting Detection Reagents (Pierce, Rockford, IL, USA).

**Immunohistochemistry**

Paraffin-embedded DIPG tissue samples and matched non-neoplastic brain samples were deparaffinized and rehydrated. Endogenous peroxidase was inhibited by 30 min 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 min, followed by three times washing in phosphate-buffered saline (PBS). Slides were incubated with mouse anti-WEE1 (1:50) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Slides were washed three times in PBS and incubated with the secondary antibody, Envision+ Poly-HRP immunohistochemistry (IHC) Kit (Immunologic, Duiven, The Netherlands) for 30 min 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 hrs after treatment cells were washed twice with PBS containing 1% FBS and subsequently fixed in 70% ethanol for 24 hrs. Next, the cells were washed once in PBS containing 1% FBS followed by RNAse A treatment (0.15 mg/ml) for 20 min and subsequent DNA staining with 50 µg/µl propidium iodide (PI) for 30 min. Cell cycle distribution assessment was performed using a FacsCalibur Flow Cytometer and CellQuest Pro software (Becton-Dickinson, Breda, The Netherlands). Subsequently, data were analyzed using ModfitLT software (Verity Software House, Topsham, ME, USA).

**Immunofluorescence staining**

Cells were fixed in 3% paraformaldehyde at different timepoints post-irradiation (15 min, 30 min, 1, 24, 48, 72 hrs). Prior to staining, the cells were rinsed in PBS and permeabilized in PBS containing 0.1% Trition X-100 for 30 min at room temperature and blocked in PBS containing 5% FCS. Cover slips were incubated with both mouse-anti-γ-histone-H2AX (1:100) (Millipore) and rabbit-anti-53BP1 (1:100) (Novus...
Biologicals, Littleton, CO, USA) 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/FITC immunoglobulins (1:100) (DAKO) both in PBS containing 5% FCS for 30 min at room temperature in the dark. Slips were rinsed in PBS three times and nuclei were stained with 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, Burlingame, CA, USA) and analyzed with a Carl Zeiss Axioskop 20 microscope at 100x objective.

Cell counts

At four 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, Royston, UK).

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

Female athymic nude mice (age six–eight 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 elsewhere (32). In short, tumors were generated via stereotactic injection of 0.5x10^6 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 seven after intracranial injection, mice were stratified on the basis of BLI signal intensities into four treatment groups (n=12) with
comparable mean Fluc activity and 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 six injections per mouse. At day eight, mice received head-only IR with a single dose of 2 Gy using Clinac D/E (Varian Medical Systems, Palo Alto, CA, USA), as described elsewhere (32). Mice were sacrificed once humane endpoints were reached via sedation (1.5 L O₂/minute 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 +/- 2xSD range were excluded from further analysis. Next, BLI signals were log₁₀-transformed and unpaired Student’s t-test was performed. 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 to non-malignant brain tissue (33,36), including two tissue samples of normal brainstem (17), to identify the top up-regulated kinases for this malignancy. To compare the differential kinase gene expression of cancer versus normal samples, the fold-change to non-malignant brain tissue was determined for all kinases within the dataset. A false discovery rate (FDR) corrected moderated t-test was used to compare means of kinase gene expression levels between the datasets and a p-value <0.000005 was considered significant. Analysis of the kinase expression data
demonstrated 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, non-malignant brain regions and normal cerebellum (Fig. 1A). Using one-way analysis of variance (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 as compared to 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 WB in five 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 as compared to 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 WB in a primary DIPG cell line and in E98-FM glioma cells (32), again demonstrating 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 1I-K left panels) as compared to 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 blood vessels (Fig. 1F). Nuclei in the leptomeningeal tumor component also revealed WEE1 nuclear staining (Fig. 1G). Of note, WEE1 was found to be overexpressed in tumor cells invading brain areas beyond the brainstem (Fig. 1H and 1I-K right panels). 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*. Since WEE1 phosphorylates CDC2 upon IR (37-39), we performed WB analysis for phosphorylated CDC2 (Y15) 16 hrs after treatment with 0.1 µM MK-1775 and IR (Fig. 2B). IR at 6 Gy resulted in increased CDC2-pY15 levels, which were reduced upon 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 hrs, indicated by an accumulation of cells with doubled DNA content and indicative for a dysfunctional G1 arrest, which was reduced upon treatment with 0.1 µM 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 γH2AX and 53BP1 at 15 min, 30 min, and 1, 24, 48, and 72 hrs after treatment with IR at 4 Gy in the presence or absence of 0.1 µM MK-1775. As shown in Figure 2D γH2AX and 53BP1 colocalize. These results indicate DNA damage in both cell lines after IR treatment as compared to untreated cells. Cells treated with IR showed fast onset of DSBs (as early as 15 min after IR), however, a decreased 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). Of note, 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 as compared to cells treated with IR at 4 Gy alone (Fig. 2D and E, E98-FM and VUMC-DIPG-A p<0.001). These results demonstrate that MK-1775 is capable of functional WEE1 inhibition and, thereby, of attenuating the IR-induced G2 arrest in G2-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 G2 arrest by MK-1775 reduces viability of these cells *in vitro*. A clonogenic survival assay is the gold standard for this purpose (40) but since 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 µM MK-1775. After four days, cells were stained with DAPI and cell numbers were counted. Treatment of the E98-FM cells with 0.1 µM MK-1775 or IR
monotherapy resulted in a significant but moderate decrease in cell viability compared to 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 µM MK-1775 in combination with IR at 4 Gy on the cell viability of primary VUMC-DIPG-A cells. Both 0.1 µM MK-1775 and IR monotherapy demonstrate only a limited effect on VUMC-DIPG-A cell viability. However, combined treatment with MK-1775 and IR resulted in additional diminished cell viability, as compared to IR alone (p<0.05) (Fig. 3C and D).

**MK-1775 enhances the radiation response in the orthotopic E98-FM DIPG mouse model**

We employed 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 BLI of the photon activity of firefly luciferase encoded by the E98-FM cells. Seven days after intracranial injection of the cells, mice were randomized in four groups (n=12 per group) based on BLI intensity. Two groups of mice received MK-1775 intraperitoneally (i.p.) (90 mg/kg), and two 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 eight after injection of the cells. BLI revealed significant tumor progression in both non-irradiated vehicle-treated and MK-1775-treated mice, whereas tumor growth was significantly delayed in irradiated and irradiated MK-1775-treated mice compared to the control group (week 2 and 3, and week 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 to 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), whereas the groups receiving only MK-1775 or IR showed no significant effect on survival as compared to the untreated mice (Fig. 4C). These results indicate that pharmacological targeting of WEE1 with MK-1775 in combination with IR delays the growth of E98-FM DIPG tumors \textit{in vivo}, although the effects observed under these conditions were modest.

**Discussion**

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 \textit{in vitro} and \textit{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 \textit{in vitro} and the orthotopic E98-FM DIPG model (32) to demonstrate the radiation enhancing effects of WEE1 inhibition \textit{in vivo}.

Kinase expression data analysis showed WEE1 kinase to be in the top-10 overexpressed kinases in a dataset of DIPG samples compared to normal brain tissues. Although the other overexpressed kinases could also be of value for investigating therapeutic targeting in DIPG, we analyzed WEE1 kinase, since it was previously demonstrated 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 since 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 chosen given its proven selective action upon 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 demonstrated 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, though 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 hrs for VUMC-DIPG-A versus 24 hrs for E98-FM) (30).

We also investigated the radiation enhancing effects of MK-1775 in the E98-FM DIPG mouse model in vivo. Once injected into the mouse pons the human E98-
FM glioma cells give 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 to 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 non-treated control group, while IR alone did not yield such a significant survival advantage. This indicates that combining IR with MK-1775 improves the anti-tumor effect of IR alone. Finally, while treatment with MK-1775 alone showed lower cell counts in vitro, mono-treatment with MK-1775 in vivo did not result in a significant anti-tumor 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, though 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, since MK-1775 blood level was found to be undetectable by 10 hrs (30). Finally, previous studies have employed subcutaneous tumors to assess the effects of MK-1775 in vivo (9,27-30). Hence, the limited radiation
enhancing effect observed on DIPG \textit{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 that its inhibition \textit{in vitro} and \textit{in vivo} resulted in additional anti-tumor effects when combined with IR. Ultimately, DIPG is a very aggressive and heterogeneous tumor and the development of a clinical multi-target approach will be necessary. In this context, WEE1 inhibition in conjunction with RT may be one of the therapeutic strategies employed in the treatment armamentarium needed to treat this now still fatal disease.

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**References**


Table 1. Top 20 up-regulated kinases in DIPG.

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<th>Rank</th>
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<td>ROR2 receptor tyrosine kinase-like orphan receptor 2</td>
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<td>1.54</td>
<td>3.62E-06</td>
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<td>18</td>
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<td>205479_at</td>
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<td>5.41</td>
<td>1.54</td>
<td>2.29E-14</td>
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<td>DYRK3 dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3</td>
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Top 20 up-regulated kinases in DIPG tumor samples (n=27) (17) sorted on log2 fold increase as compared to non-malignant brain tissues (n=174) (33) including two samples of normal brain stem tissue from the DIPG dataset (17). Wee1 homolog (WEE1) is identified as a highly differentially overexpressed kinase in DIPG.
Figure legends

Figure 1. WEE1 is overexpressed in DIPG tissues and cells.

(A) WEE1 mRNA level in 27 DIPG samples, 6 brainstem low grade glioma (LGG) samples, 2 normal brainstem samples, 172 non-malignant brain region and 9 normal cerebellum samples. (B) WB analysis of WEE1 expression in DIPG tissues and matching controls. (C) WB analysis of WEE1 expression in E98-FM cells and primary DIPG cells. Non-malignant brain tissue collected from a pediatric patient that underwent surgery due to refractory epilepsy was used as a negative control. (D-M) IHC of WEE1 expression in five DIPG patients, VUMC-DIPG-1 (D-H), VUMC-DIPG-2 (I), VUMC-DIPG-3 (J), VUMC-DIPG-4 and -5 (K left and right panel, respectively). (D-G and I-K, left panels), (H, I and J, right panels) and (K, right panel) show DIPG at the level of the pons, cerebellum and insula, respectively. (H) Arrows indicate DIPG cells subpial accumulation. (L) Matched control tissue collected from the cerebellum and the pons for VUMC-DIPG-2 and VUMC-DIPG-1 patients, respectively. (M) IHC of WEE1 expression in the E98-FM tumor tissue. The triangle indicates absence of WEE1 nuclear staining in murine pons tissue microscopically free of disease. Size bar=20 µm (E-G,L, K), 40 µm (D,H-J and M, right panel), 80 µm (M left panel).

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) WB analysis of phosphorylated CDC2 16 hrs after treatment of E98-FM and VUMC-DIPG-A cells with 6 Gy and/or 0.1 µM MK-1775. Numbers represent relative phosphorylated CDC2 expression after normalization against control performed using imageJ software. (C) PI cell cycle
analysis of E98-FM and VUMC-DIPG-A cells 16 hrs 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 µM 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 min, 30 min, 1, 24, 48, and 72 hrs 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's t-test). At least 20 nuclei per time point were analyzed for this quantification.

**Figure 3. MK-1775 enhances the radiation response in E98-FM and DIPG cells.**

(A-B) Analysis of cell counts of E98-FM cells after treatment with IR at 4 Gy and/or 0.1 µM MK-1775. Relative percentages of cell counts was 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-D) Similar analysis as in (A-B) using primary VUMC-DIPG-A cells. Results are depicted as averages of an experiment performed in triplicate, error bars indicate standard error of the mean (*p<0.05, ***p<0.001, Student’s t-test). Size bar=20 µm.

**Figure 4. In vivo analysis of MK-1775 using the orthotopic E98-FM DIPG model.**

(A) Log10-transformed BLI values of individual mice per treatment group (n=12 per group) at different time points after injection of the E98-FM cells in the pons, and (B) representative bioluminescence images. The Fluc signal activity is calculated as photons/second/cm². The horizontal bar indicates the median and error bars indicate range (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test). (C) Kaplan-Meier survival
curves of the different treatment groups. Only mice treated with IR and MK-1775 showed a significantly longer survival as compared to untreated mice, in contrast to mice treated with MK-1775 or IR alone (log-rank, p<0.05).
Figure 2

A

MK-1775

B

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<tr>
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<th>IR+MK-1775</th>
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C

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<td>G2/M: 23%</td>
<td>G2/M: 19%</td>
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D

4 Gy

γH2AX  S3BP1  DAPI  merge

No IR

15 min

30 min

1 hr

24 hrs

48 hrs

72 hrs

4 Gy + MK-1775

γH2AX  S3BP1  DAPI  merge

E

<table>
<thead>
<tr>
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<th>CTRL</th>
<th>MK-1775</th>
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<tr>
<td>E98-FM</td>
<td>Avg number of γH2AX foci/nucleus</td>
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<tr>
<td>VUMC-DIPG-A</td>
<td>Avg number of γH2AX foci/nucleus</td>
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**Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.**
Figure 3

A

Relative % cell viability (log scale)

CTRL  MK-1775  IR  IR+MK-1775

B

CTRL  MK-1775

IR  IR+MK-1775

C

Relative % cell viability (log scale)

CTRL  MK-1775  IR  IR+MK-1775

D

CTRL  MK-1775

IR  IR+MK-1775
Figure 4

A

Log10 fluq activity

WEEK 1
CTRL MK-1775 IR IR+MK-1775

WEEK 2
CTRL MK-1775 IR IR+MK-1775

WEEK 3
CTRL MK-1775 IR IR+MK-1775

WEEK 4
CTRL MK-1775 IR IR+MK-1775

B

1 2 3 4
CTRL MK-1775 IR IR+MK-1775

C

Survival

Days after surgery

CTRL vs IR+MK-1775 p<0.05