TUMOR MICROENVIRONMENT AND THERAPY
Research Article • 10.2478/tumor-2013-0002 • TUMOR • 2012 • 37–45

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Impact of Wee1 inhibition on the hypoxia-induced DNA damage response

Abstract
Tumor hypoxia is a common feature associated with resistance to current anticancer therapies, such as radiotherapy and chemotherapy. Wee1 is a tyrosine kinase regarded as a gatekeeper of the G_2/M cell cycle transition. Wee1 has recently been highlighted as a therapeutic target in cells with a deficient G_1 checkpoint, since they are more reliant on the G_2 phase for repair of damage and survival. Here, we have assessed the impact of Wee1 inhibition on hypoxic cells and its therapeutic potential as a single agent under these conditions or in combination with radiation.
The p53 null non-small cell lung carcinoma cell line H1299 was used to assess Wee1 phosphorylation and signaling in a range of hypoxic conditions. Inhibition of Wee1 decreased the G_2 population in cells exposed to normoxia (21% O_2) or moderate hypoxia (1% O_2) but did not effect the cell cycle of cells exposed to levels of hypoxia associated with greatest radio-resistance (<0.1% O_2). In addition, DNA damage was induced in response to Wee1 inhibition although this was less significant in cells exposed to <0.1% O_2.
Wee1 inhibitors were then tested as single agents in a 2D and 3D model or in combination with radiation treatment, by clonogenic assay. Following continuous treatment, MK-1775 was shown to sensitize the H1299 cell line to different O_2 tensions. However, in combination with radiation, although MK-1775 had significant efficacy in normoxia, disappointing results were seen when these studies were carried out in hypoxia.

Keywords
Wee1 • DNA damage response • Hypoxia • MK-1775 • Radiation

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Received 01 February 2013
Accepted 18 April 2013

Introduction

Hypoxia occurs in the majority of tumors and is of significant clinical relevance due to its association with poor prognosis, increased metastasis and resistance to therapy [1]. Hypoxic cells have been shown to be chemo and radio-resistant for a variety of reasons including: inadequate tumor vasculature preventing efficient delivery and diffusion, poor proliferation in hypoxic environments, and reliance of radiotherapy on the production of reactive oxygen species to cause DNA damage [2-5]. It is therefore essential that the hypoxic fraction of a tumor be targeted during therapy.

The human genome is constantly exposed to damage either from endogenous or exogenous sources [6]. Since DNA has limited turnover and is central to all cell functions, organisms have developed a series of events that operate collectively and comprise the so-called DNA damage response (DDR). In order to maintain genome integrity when activated, the DDR can trigger a wide variety of cellular responses including cell cycle checkpoints, DNA repair, and in some cases apoptosis [7]. Severe levels of hypoxia (<0.1% O_2) have been shown to induce replication arrest and consequently activate the DDR, despite the absence of detectable DNA damage [8]. This hypoxia-induced DDR includes both ATR and ATM-mediated signaling [9]. Loss or inhibition of components of the DDR, such as ataxia telangiectasia- and rad3-related (ATR) or checkpoint kinase 1 (Chk1) has been reported to sensitize hypoxic cells [10]. For instance, we have previously demonstrated that inhibition or loss of the Chk1 or ATR increased sensitivity to hypoxia/reoxygenation [11-14]. We propose therefore that, additional studies focused on components of the hypoxia-induced DDR have the potential to establish new therapeutic targets [13].

To ensure cellular genomic integrity, a coordinated set of CDKs paired with the cyclin binding partners, regulate cell cycle progression and are crucial for cell survival. In particular, entry into mitosis is driven by CDK1, also known as CDC2, in complex with cyclin B. This complex is kept inactive following phosphorylation of CDK1 at the Tyr15 residue by Wee1 and Myt1 kinases [15]. Wee1 has been reported to be overexpressed in several tumor types and its depletion has been correlated with increased apoptosis [16-20]. Wee1 is a tyrosine kinase, which in response to DNA damage phosphorylates CDK1 during the G_2 phase of the cell cycle, and delays cell entry into mitosis. Wee1 is therefore regarded as a gatekeeper of the G_2-checkpoint making it an attractive target for therapeutic inhibition [16]. The principle surrounding the use of Wee1 inhibitors as anticancer agents

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relies on the loss of the G_1-checkpoint, which is often observed in tumors due to loss of function of the tumor suppressor protein p53 (approximately 50% of all solid tumors) [21]. Cancer cells with a deficient G_1-checkpoint accumulate DNA damage and consequently have a higher reliance on the G_2-checkpoint in comparison with normal cells [22]. Pharmacological inhibitors of Wee1 decrease the phosphorylation of CDK1 on tyrosine 15 (phospho-CDK1 (Tyr15)), allowing the cyclin B-CDK1 complex formation and cell entry into mitosis [23]. Cells with unrepaired damage then enter mitosis resulting in mitotic catastrophe and consequently cell death [16].

Wee1 inhibitors, PD0166285 and Wee1 inhibitor II have been shown to have anti-proliferative effects when used as single agents in melanoma and breast cancer [18,24]. In addition, studies with PD0166285 but also with the more selective Wee1 inhibitor (MK-1775) were shown to enhance the cytotoxic effects of several chemotherapeutics [25-27]. The Wee1 inhibitor MK-1775 is currently undergoing phase I and II clinical trials in combination with chemotherapeutics in ovarian and cervical cancer, and advanced solid tumors [16]. The potential of Wee1 inhibitors to enhance radiosensitivity of various tumor cell types including colon, lung, cervical, ovarian, glioblastoma and osteosarcoma, has also been assessed. In the majority of these studies, the increased radiosensitivity induced by Wee1 inhibitors was dependent on the loss of functional p53 [17,19,28-31]. However, to date no studies have been reported using these inhibitors in hypoxic conditions, which mimic the tumor microenvironment. Since we have previously reported that, inhibition of other key regulators of the G_2 checkpoint such as Chk1 and ATR showed efficacy in hypoxic cells we hypothesized that inhibition of Wee1 would also sensitize hypoxic cells [14,32]. Moreover, studies in *S. pombe* and *Xenopus* have reported Wee1 to be induced by Chk1 however this regulation has not been reported in mammalian cells [33,34].

In this study we have investigated the role of Wee1 in the hypoxia-induced DDR and the possibility of targeting hypoxic cells with Wee1 inhibitors as single agents. In addition, we have established whether Wee1 inhibitors may enhance the cytotoxic effects of radiotherapy under physiologically relevant oxygen tensions.

Materials and Methods

Compounds formulation

Stock solutions of PD0166285 (Pfizer), Wee1 inhibitor II (Calbiochem), MK-1775 (Merck) and Gö6976 (Calbiochem) were prepared in 100% dimethyl sulfoxide (DMSO: Sigma-Aldrich) at a concentration of 10 mM and stored at -20°C for further use. Stock solutions of Adriamycin (Pharmacia Company) were prepared in deionized H_2O and used at 2 μM.

Cell culture

H1299 (non-small cell lung carcinoma, p53 null) cells, obtained from the American Type Culture Collection (ATCC) were cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and Penicillin-Streptomycin. Cells were grown in standard culture conditions (in a humidified incubator at 37°C and 5% CO_2) and cell culture reagents were purchased from Sigma-Aldrich unless otherwise stated. Cells were routinely tested for *Mycoplasma* using a colorimetric assay (PlasmoTest™, Source BioScience Autogen).

Hypoxia treatments

Treatments in moderate hypoxic conditions (1% O_2) were carried out in an in vivo$_2$ 400 Ruskinn chamber (Biotrace Fred Baker) and treatments in severe hypoxic conditions (<0.1% O_2) were carried out in a Bactron II chamber (Shell Labs). Incubation periods under hypoxic and severe hypoxic conditions ranged from 2 - 24 h and are described in the figure legends, accordingly. In hypoxia studies, cells were plated with a minimum amount of media to reduce the time to eliminate O_2 from the medium and were harvested inside the chamber using equilibrated solutions.

Immunoblotting

Cells were lysed in UTB (9 M Urea, 75 mM Tris HCl (pH 7.5), 0.15 M β-mercaptoethanol), sonicated briefly and processed as described previously [35]. The antibodies used were anti-CDK1, anti-phospho CDK1 (Tyr15), anti-phospho Chk1 (Ser317), anti-phospho Wee1 (Ser642), anti-phospho H3 (Ser10), anti-H3 (Cell Signaling Technology), anti-phospho 53BP1 (ser25) (Bethyl), anti-Chk1, anti-β-actin, anti-Wee1 (Santa Cruz Biotechnology), anti-H2AX (Calbiochem), anti-γH2AX (Ser139) (Millipore), and HIF-1α (BD Transduction Labs). Proteins were detected using the LiCor Odyssey imaging system.

Quantitative PCR (qPCR)

RNA was firstly isolated from H1299 cells, using TRIzol® reagent (Life Technologies), following 0, 8, 16 and 24 h exposure to severe hypoxic (<0.1% O_2) conditions. cDNA was prepared from the RNA extracts using the Verso™ cDNA kit (Thermo Scientific), according to the manufacturer's instructions. The qPCR was carried out for *WEE1* and *GLUT1* in a 7500 Real-Time PCR system from Applied Biosystems. Primer sequences for *WEE1* and *GLUT1* are available upon request. All transcript levels are shown relative to 18s.

Alkaline Comet Assay

H1299 cells were seeded (6.5 x 10^4 cells) and allowed to adhere for 12 h before exposure to different O_2 tensions (21, 1 and <0.1% O_2) with or without 0.2 μM MK-1775, 0.5 μM PD0166285 or 10 μM Wee1 inhibitor II for 8 h. Following treatment cells were trypsinized, embedded in 1% low-melting agarose and allowed to set in cold pre-embedded agarose slides, in duplicate for each treatment condition. Slides were transferred to the lysis buffer (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris base, pH 10.5) for 1 h at room temperature in the dark under the different O_2 tensions. All solutions used up to this step were equilibrated at the required O_2 tension, to avoid additional O_2 accumulation. Following lysis, all slides were transferred to normoxic conditions, washed, and incubated with cold electrophoresis buffer (300 mM

NaOH, 1 mM EDTA and 1% DMSO, pH > 13) for 30 min and electrophoresis was carried out at 25 V, 300 mA for 25 min. After electrophoresis the slides were washed with neutralizing buffer (0.5 M Tris-HCl, pH 8.0) and stained with SYBR Gold (Invitrogen). At least 50 comets were analyzed per slide, using a Nikon 90i fluorescent microscope and Komet 5.5 image analysis software (Andor Technology).

Fluorescence-activated cell sorting (FACS)

H1299 cells were stained with propidium iodide (PI) for DNA content following treatment with or without 0.2 μM MK-1775, 0.5 μM PD0166285 or 10 μM Wee1 inhibitor II under different O_2 tensions (21, 1 and <0.1% O_2) for 6 h. FACS analysis was carried out as described previously using a Becton Dickinson FACSort [35], and CellQuest Pro and ModFit LT software were used for analysis.

2D colony formation assay and irradiation

In studies with MK-1775 as a single agent, H1299 cells were seeded at low densities (300 cells/6-well plate) for exposures up to 24 h and higher densities (1,000-100,000 cells/10-cm cm tissue culture dish) for continuous exposure throughout the assay. Cells were allowed to attach for 2 h, and treated with 0.2 μM MK-1775 under different O_2 tensions (21, 1, and <0.1% O_2) for the indicated times. Following exposure to variable O_2 tensions, MK-1775 treatment was replaced by fresh media, with the exception of the continuous treatment, and cells returned to standard culture conditions. In studies with MK-1775 in combination with irradiation, a Cs^{137} source (Gamma Service® GSR D1 irradiator) at a dose rate of 1.938 Gy/min was used. H1299 cells were seeded at low densities (500-1,500 cells/6-cm tissue culture dish), treated with 0.2 μM MK-1775 for 3 h under normoxic or severe hypoxic (<0.1 % O_2) conditions and irradiated with 0 - 8 Gy. Irradiation in hypoxic conditions was carried out as previously described [14]. Following irradiation, cells were returned to standard culture conditions for additional 21 h and MK-1775 treatment replaced with fresh media. Cells were incubated for 8 days to allow colony formation (> 50 cells), fixed and stained with crystal violet before counting. The platting efficiencies following treatment were calculated and normalized to the relevant control and the sensitizer enhancement ratio at 50% cell survival (SER_{50}) calculated.

3D colony formation assay

H1299 were seeded as single cells in 0.5 μg/μl of 3D laminin-rich extracellular matrix (lrECM: Matrigel, BD biosciences), as previously used [36] in complete DMEM media and allowed to grow for 24 h prior to treatment with or without 0.1 and 0.2 μM MK-1775 at different O_2 tensions (21, 1 and <0.1% O_2) for 24 h. Following exposure to the different O_2 tensions, cells were placed in standard culture conditions continuously throughout the assay and imaged on day 10 of the study. Cell colonies (>50 cells) were microscopically counted on a Nikon Eclipse TE2000-E microscope. Representative images were acquired with a Hamamatsu ORCA-ER camera and NIS-Elements advanced research software.

Results

Numerous components of the DNA damage response and DNA repair pathways are repressed in hypoxic conditions [37]. To date the levels of Wee1 have not been described in hypoxic conditions. We exposed H1299 cells to moderate hypoxia (1% O_2) and severe hypoxia (<0.1% O_2) and measured the levels of protein and mRNA of *WEE1*. In moderate hypoxia the Wee1 protein levels did not alter (Figure 1A), while at <0.1% O_2 the protein levels were initially induced and later reduced with prolonged exposure to hypoxia. Interestingly, the levels of Wee1 appear to mirror the levels of Chk1, which were also observed here, and previously to decrease over time in severe hypoxia [38]. In severe hypoxia (<0.1% O_2), the phosphorylation of Wee1 at serine 642 followed similar kinetics to Chk1 phosphorylation (Figure 1B). These data suggest that the hypoxia-induced DNA damage response might include Wee1. Additionally, the transcript levels of *WEE1* were measured (Figure 1C) together with the HIF-1 target, *GLUT1*, which was used as a hypoxia control. As expected, *GLUT1* mRNA levels increased in response to hypoxia (Supplementary Figure S1). In contrast, *WEE1* transcript levels decreased with time, which may in turn explain the reduction in protein levels, observed. Wee1 activity has been linked to Chk1, specifically; in *Xenopus* phosphorylated Wee1 at residue serine 642 has been reported to be a direct Chk1 target [33,39]. Therefore, we investigated this potential link further in severely hypoxic conditions. We exposed H1299 cells to severe hypoxia (<0.1% O_2) and to the Chk1 inhibitor Gö6976, and carried out western blot analysis for Wee1 and CDK1 (Figure 1D). Both total and phosphorylated forms of Wee1 decreased with time in severe hypoxia in the presence of Gö6976. The levels of CDK1-Tyr15 were also reduced in response to exposure to the Chk1 inhibitor suggesting that Wee1-mediated signaling had been compromised. Together, these data suggest that hypoxia-induced signaling to Wee1 could be affected by Chk1. Further investigation using more specific tools, for example siRNA to Wee1, are required to conclusively demonstrate direct Chk1 signaling to Wee1.

The induction of DNA damage and loss of the G_2 checkpoint by Wee1 inhibitors is oxygen dependent

Wee1 inhibition has been shown to induce DNA damage, which has been reported to be dependent on the Mus81-Eme1 complex [18,40,41]. The accumulation of DNA damage in response to Wee1 inhibition is independent of p53 status suggesting that this would not be restricted to tumor cells which, have lost the G_1 checkpoint (Supplementary Figure S2). Here, we have treated H1299 cells with MK-1775 and assessed the levels of DNA damage at relevant O_2 conditions by western blot for phosphorylated 53BP1 and γH2AX, as well as by comet assay. Following Wee1 inhibition the levels of phospho-CDK1 (Tyr15) were reduced indicating that the inhibitor effectively reduced Wee1 signaling. DNA damage was observed following treatment with MK-1775 in a time-dependent manner, and this was most significant under normoxic and moderate hypoxic

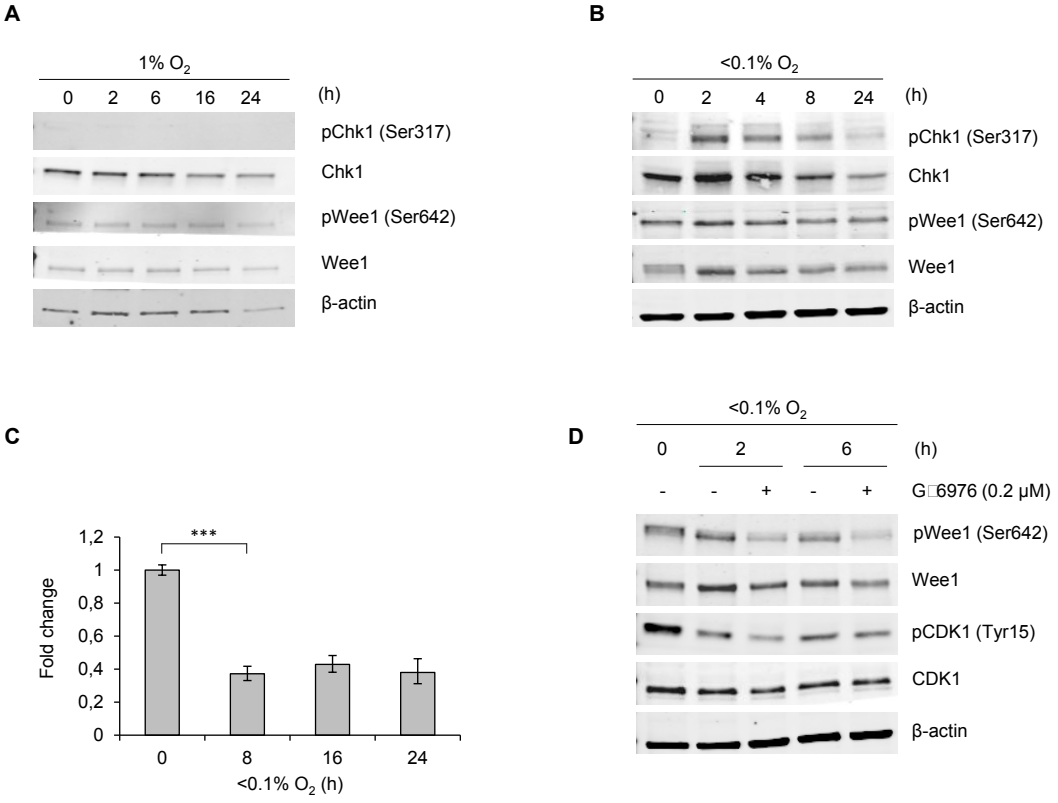

Figure 1. Wee1 is repressed in severely hypoxic conditions. In H1299 cells the protein levels of Chk1 and Wee1 were assessed under (A) hypoxia (1% O_2), and (B) severe hypoxia (<0.1% O_2) for the time periods indicated, by western blot. (C) Wee1 mRNA levels in H1299 cells exposed to severe hypoxia for the time periods indicated, by qPCR. (D) Wee1 is a potential Chk1 target in severely hypoxic conditions. The WEE1 downstream target (CDK1) activity was assessed in H1299 cells following treatment with or without Gö6976 (0.2 μM) in severe hypoxic conditions for the time periods indicated.

conditions (1% O_2) (Figure 2A). In cells treated with alternative Wee1 inhibitors, PD0166285 (Supplementary Figure S3) or Wee1 inhibitor II (Supplementary Figure S4), a similar induction of DNA damage was observed although this was somewhat delayed with the Wee1 inhibitor II treatment. Furthermore, comet assays confirmed a significant DNA damage induction following treatment with MK-1775 (Figure 2B), and with PD0166285 (Supplementary Figure S3B) with higher damage observed under normoxic and moderate hypoxic conditions.

Inhibition of Wee1 has been shown to lead to a reduction of the inhibitory phosphorylation on CDK1 promoting mitotic entry [42]. Under normoxic conditions all three Wee1 inhibitors led to an increase in phosphorylated histone 3 (phospho-H3 Ser10), in a time-dependent manner suggesting an increased mitotic population and premature exit from G_2 (Figure 2A, Supplementary Figure S3A and Supplementary Figure S4A). Under moderate hypoxic conditions only MK-1775 and PD0166285 were shown to promote mitosis, while Wee1 inhibitor II had no effect under these conditions or those of severe hypoxia. Cells treated with Wee1 inhibitors in severely hypoxic conditions (<0.1% O_2) showed no increase in the mitotic phase, suggesting that cells undergoing hypoxia-induced S-phase arrest do not undergo mitotic slippage [43]. Although PD0166285 promoted mitotic entry in severe O_2 conditions this was only observed up to 6 h treatment (Supplementary Figure S2A).

To confirm the effect of Wee1 inhibition on the cell cycle we tested MK-1775 in H1299 cells exposed to different O_2 tensions for 6 h (Figure 2C). MK-1775 treatment in both normoxic and moderate hypoxic (1% O_2) conditions caused a slight decrease in the G_2 phase cell population. In contrast, no effect was observed in severely hypoxic (<0.1% O_2) conditions. Treatment with PD0166285 showed a significant G_2 phase ablation (Supplementary Figure S5A) in both normoxia and moderate hypoxia, while treatment with the Wee1 inhibitor II showed little effect (Supplementary Figure S5B). Together, these data demonstrate that in hypoxic conditions severe enough to induce an S-phase arrest Wee1 inhibition does not have a significant effect on the cell cycle [35].

Effects of Wee1 inhibition on cell survival in hypoxic conditions

Wee1 inhibitors have primarily been tested in combination with either chemo or radiotherapy, although some reports have indicated single-agent activity [24,29]. We have observed that

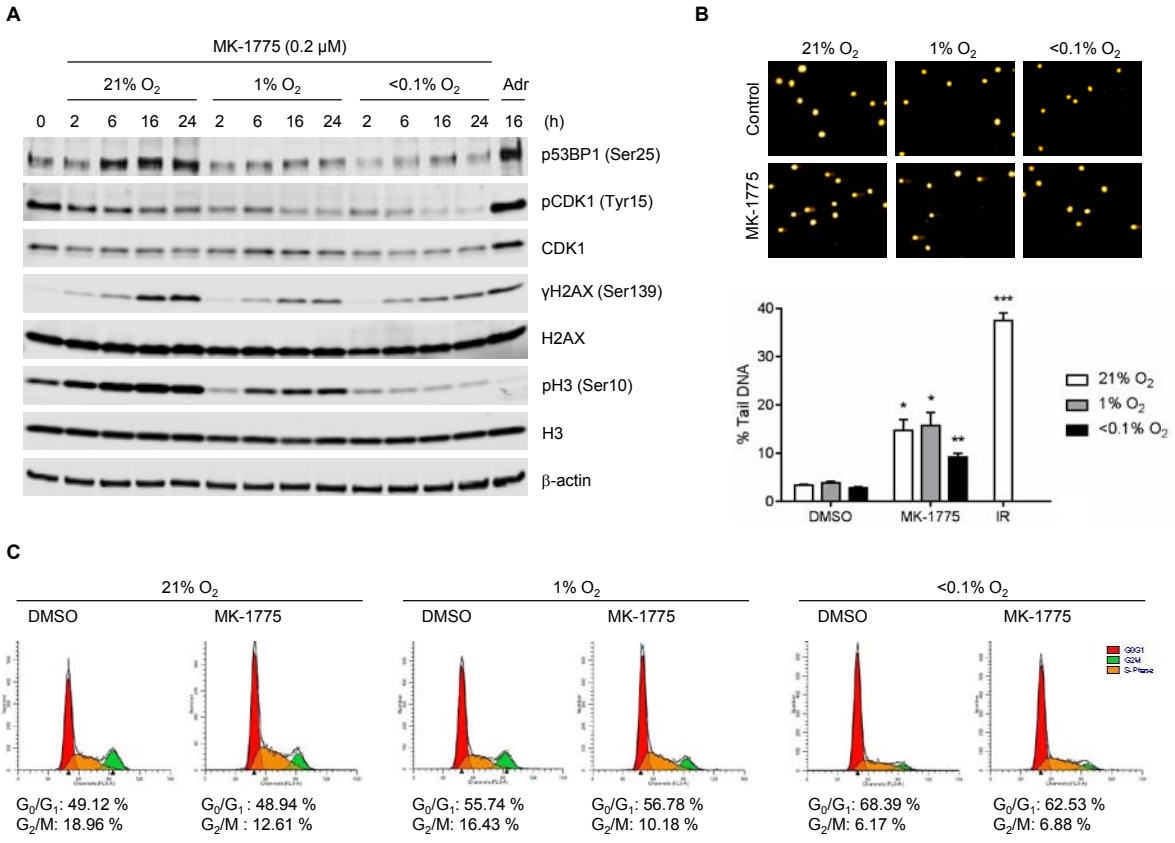

*Figure 2. Wee1 inhibition leads to induction of DNA damage and promotes premature mitotic entry. (A) H1299 cells were exposed to 0.2 μM MK-1775 for the time periods indicated in normoxia, and the CDK1 activity, 53BP1, γH2AX (Ser139) and phospho-H3 (Ser10) were analysed by western blot. Adriamycin at a concentration of 2 μM (Adr) was added for 16 h as a positive control for DNA damage. (B) H1299 cells were treated with or without 0.2 μM of MK-1775 for a period of 8 h in a range of O_2 tensions (21, 1 and < 0.1% O_2) and DNA damage was determined by alkaline comet assay. As a positive control for this assay H1299 cells irradiated with 6 Gy were used. The mean % tail DNA ± SEM (n=2) was plotted. *P<0.01, **P<0.001, and represents significant difference between treated and untreated cells. (C) Cell cycle profile of H1299 cells treated with 0.2 μM of MK-1775 for a period of 6 h in a range of O_2 tensions (21, 1, <0.1% O_2) analysed by FACS.*

Wee1 inhibition induces less DNA damage in severe hypoxia and therefore asked whether the single agent activity observed in normoxia is maintained in conditions of hypoxia. H1299 cells were exposed to 0.2 μM of MK-1775 under different O_2 tensions and the sensitivity was assessed using a 2D and 3D clonogenic survival assay. In the 2D model, the highest sensitivity was observed following exposure to MK-1775 for 24 h, which was most effective under normoxic conditions lowering the surviving fraction by approximately 30% (Figure 3A). When MK-1775 was used this way (24 h exposure) in conditions of mild and severe hypoxia the effect was less profound. However when 0.2 μM MK-1775 was used continuously throughout the experiment the surviving fraction was lowered to values near zero in all O_2 conditions, but was most significant in severe hypoxia (Figure 3B). In the 3D model, the H1299 cell line successfully formed colonies (Figure 3C). MK-1775 (0.2 μM), while slightly toxic in normoxic and moderate hypoxic conditions (20 - 30 % decrease in surviving fraction), was highly toxic in conditions of severe hypoxia where a 10-fold increase in sensitivity was observed (Figure 3D). Additionally, sensitivity to the other Wee1 inhibitors, PD0166285 and Wee1 inhibitor II, was tested (Supplementary Figure S6A). H1299 cells were highly sensitive to 0.1 μM PD0166285 under all O_2 tensions with more than 10-fold increase in sensitivity (Supplementary Figure S6B), while no pronounced effect was seen with the Wee1 inhibitor II across different O_2 tensions. Together, these data indicate that whilst severely hypoxic cells show increased sensitivity to continuous exposure to MK-1775 this is less apparent with either PD0166285 or Wee1 inhibitor II.

The radiosensitizing effect of MK-1775 is reduced under severe hypoxic conditions

One of the main resistance factors associated with radiotherapy is tumor hypoxia. Studies have reported that Wee1 inhibition improves the efficacy of radiation treatment however; so far its efficacy has not been demonstrated under hypoxic conditions [19,29]. Here we have determined the ability of MK-1775 to sensitize cells to radiation therapy in normoxic and severely hypoxic conditions. H1299 were exposed to DMSO or 0.2 μM MK-1775 at different O_2 tensions for 3 h, followed by irradiation

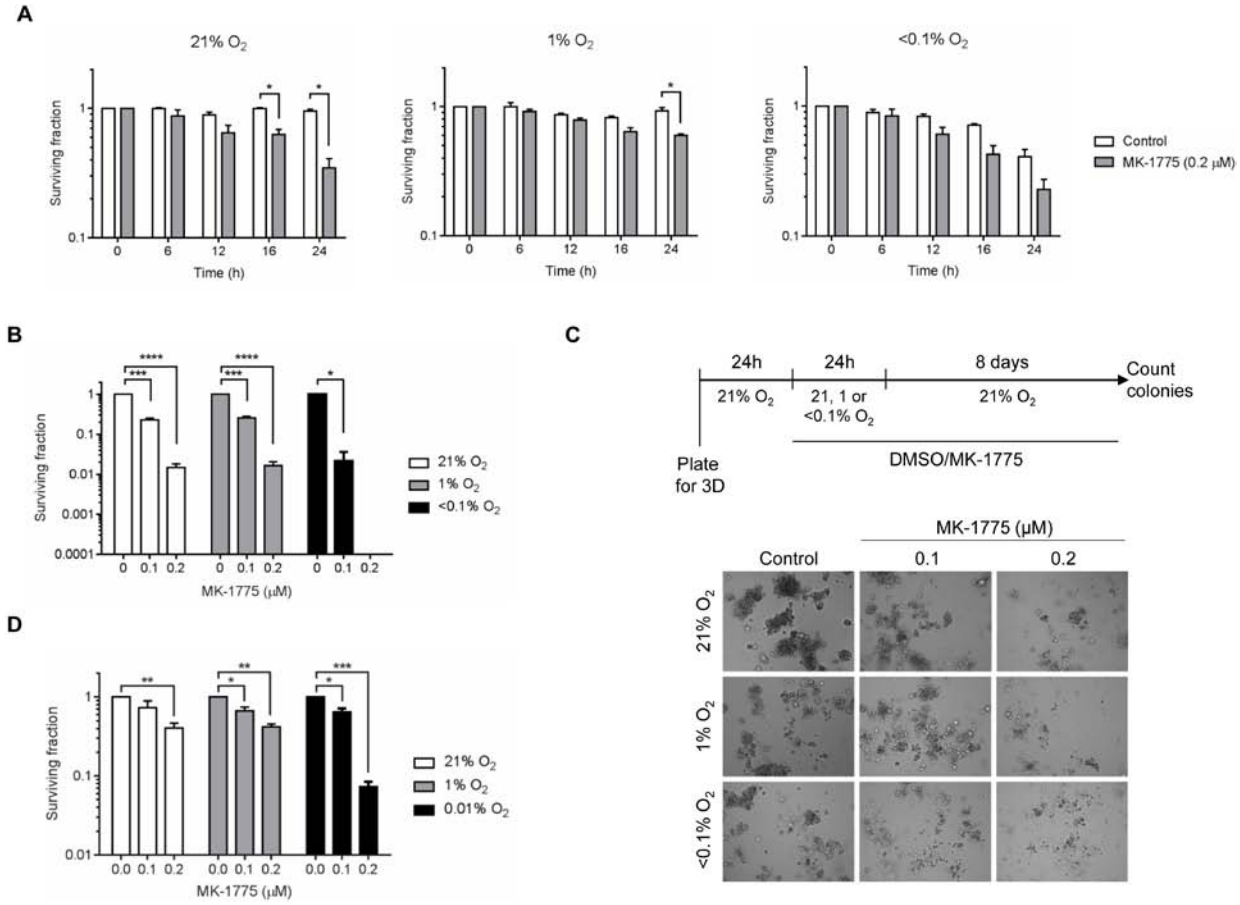

*Figure 3. MK-1775 effects in 2D and 3D clonogenic survival of H1299 cells in a range of O_2 tensions. (A) 2D surviving fraction of H1299 cells treated with or without 0.2 μM of MK-1775 for the time periods indicated at different O_2 tensions (21, 1, <0.1% O_2). (B) 2D H1299 cells surviving fraction following continuous treatment with or without 0.1 and 0.2 μM of MK-1775 at different O_2 tensions. (C) Schedule of treatment and representative images of the 3D colony formation of H1299 cells. H1299 cells were plated as single cells 24 h prior to treatment at different O_2 tensions (21, 1 and <0.1% O_2) for 24 h with or without 0.1 and 0.2 μM MK-1775 continuously throughout the assay. (D) 3D H1299 cells surviving fraction following continuous treatment with or without MK-1775 at different O_2 tensions. The mean surviving fraction ± SEM (n=3) was plotted. *P<0.01, **P<0.001, **P<0.0001 and represents significant difference between MK-1775 treated and untreated cells.*

and treatments removed 21 h later (Figure 4A). Under normoxic conditions, MK-1775 treatment potentiated the cytotoxicity of radiation treatment where the SER_{50} was 1.5, however in conditions of severe hypoxia the SER_{50} was reduced to 1.1 (Figure 4B).

Discussion

Studies in fission yeast (*S. pombe*) and *Xenopus* suggest that Wee1 is a direct target of Chk1 [33,34]. This raised the possibility that Wee1 signaling could be mediated by hypoxia-induced Chk1 activity. We have previously demonstrated that loss of ATR/Chk1 signaling is an effective way to increase sensitivity to hypoxia/reoxygenation [32,44]. Therefore, we asked if Wee1 inhibitors might be effective at sensitizing cells to hypoxia/reoxygenation and could also improve the radiation response of hypoxic cells. We observed an induction of DNA damage in response to Wee1 inhibition but found that this was less significant in severely hypoxic conditions. In addition, little or no effect on the cell cycle was observed in response to Wee1 inhibitors in these hypoxic conditions. These data suggest that cells, which are not replicating are somewhat resistant to Wee1 inhibitors. However, when we investigated the use of Wee1 inhibitors as a monotherapy for hypoxic cells we found that those cells at the most severe levels of hypoxia were generally more sensitive. This suggests that the loss of viability seen in response to Wee1 inhibition is not entirely mediated through the induction of DNA damage and the effect on the cell cycle. It is highly likely that in response to severe hypoxia the role Wee1 plays in replication is more significant. Cells exposed to severe hypoxia undergo replication re-start if oxygen is returned within an acute time period, and during this time it is likely that Wee1 activity is critical [38]. Recent studies from Davies *et al* and Dominguez-Kelly *et al*, have suggested Wee1 and Chk1 inhibition have additive effects on the delay of DNA replication and reduction of cell viability [41,45]. Our findings suggest that there may be direct signaling between Chk1 and Wee1 however these data are far from conclusive. Taken together with the potent effects we have demonstrated by inhibiting ATR/Chk1 in these conditions it seems likely that Chk1-mediated phosphorylation of Wee1 is not of major significance.

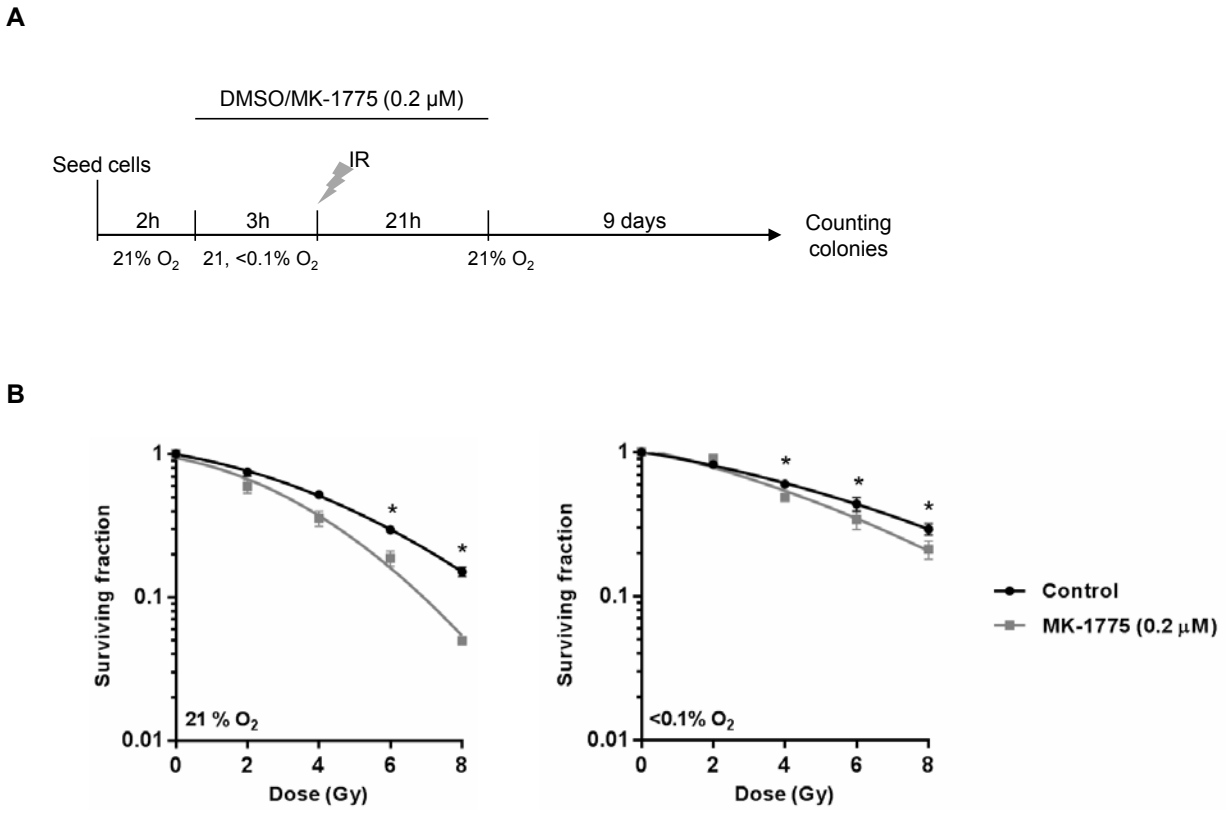

*Figure 4. Radiation survival curve of H1299 cells treated with MK-1775 under normoxic and severe hypoxic conditions. (A) Treatment schedule used for radiation studies using MK-1775. H1299 cells were treated with or without 0.2 μM MK-1775 for 3h at different O_2 tensions (21 and <0.1% O_2) followed by irradiation with 0, 2, 4, 6 and 8 Gy. Cells were returned to normoxic conditions and the MK-1775 treatment removed 21 h after irradiation and replaced with fresh media. The mean surviving fraction ± SEM (n=3) was plotted and the curve fitted according to the linear quadratic equation. *P<0.01, and represents significant difference between MK-1775 treated and untreated cells.*

In addition to its effect as a single agent in p53 null cells, Wee1 inhibition has been shown to sensitize cells to radiation therapy [19,29]. Here, although in normoxic conditions MK-1775 was shown to significantly sensitize cells to radiation therapy, this effect was less significant in conditions of severe hypoxia. In previous studies, MK-1775 has been shown to sensitize cells to different chemotherapeutics [25,26,46,47]. It remains to be established whether these effects will also be observed in conditions of hypoxia.

Here we have for the first time assessed the impact of Wee1 inhibition under hypoxic conditions. Most importantly, we have highlighted the need to carry out preclinical testing of potential cancer therapeutics in conditions, which resemble the tumor microenvironment as closely as possible. To this end we have made use of both physiologically relevant levels of oxygen and 3D culture. Although Wee1 inhibition remains an attractive target, caution should be taken regarding the clinical application of this novel compound, as the potential combination with other anticancer therapies might not be effective.

Acknowledgements

This work was supported by a Cancer Research UK grant awarded to EMH. SA is a Vertex scholar. We would like to thank Prof Nils Cordes and Dr Iris Eke at OncoRay (Dresden) for generously providing detailed methodology for the development of 3D cultures.

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