The Efficacy of CHK1 Inhibitors Is Not Altered by Hypoxia, but Is Enhanced after Reoxygenation

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Abstract

Inhibitors of CHK1 are in clinical trials for cancer treatment in combination with DNA-damaging agents. Importantly, it was previously suggested that hypoxic cancer cells may be particularly sensitive to CHK1 inhibition. However, this suggestion was based on studies in severe, toxic levels of hypoxia (anoxia). The influence of less severe hypoxia on the efficacy of CHK1 inhibitors, administered either as single agents or in combination with other treatments, remains to be investigated. Here, we have assayed the effects of the CHK1 inhibitors, AZD7762 and UCN-01, during various hypoxic conditions and after reoxygenation in the absence and presence of ionizing radiation. Treatment with CHK1 inhibitors during acute or prolonged hypoxia (< 0.03%, 0.2%, and 1% O2; 3 h or 20–24 h) gave similar effects on cell survival as treatment with these inhibitors during normoxia. CHK1 inhibitors combined with ionizing radiation showed similar radiosensitization in hypoxic and normoxic cells. However, when the inhibitors were administered after reoxygenation following prolonged hypoxia (< 0.03% and 0.2%; 20–24 h), we observed decreased cell survival and stronger induction of the DNA damage marker, γH2AX, in S-phase cells. This was accompanied by enhanced phosphorylation of the single-stranded DNA-binding replication protein A. These results suggest that the cytotoxic effects of CHK1 inhibitors are enhanced after reoxygenation following prolonged hypoxia, most likely due to the increased replication-associated DNA damage. Combining CHK1 inhibitors with other treatments that cause increased reoxygenation, such as fractionated radiotherapy, might therefore be beneficial. Mol Cancer Ther; 12(5); 705–16. ©2013 AACR.

Introduction

Most solid tumors contain regions of hypoxia, which can cause treatment resistance and tumor aggressiveness (1–3). Hypoxic cells are generally resistant to both radiotherapy and several chemotherapeutic drugs. The increased radioresistance is largely due to less induction of DNA double-strand breaks in the absence of oxygen (2, 4). In addition, hypoxia-induced changes in transcription and translation (5, 6), and/or altered DNA damage signaling (1, 7), may influence radio- and chemoresistance.

Although tumor hypoxia correlates with poor prognosis, hypoxic tissues also offer the advantage of being distinct from normal surrounding tissues. This difference between normal and cancer tissues might be exploited to achieve selective killing of cancer cells while sparing the surrounding normal tissue. One such distinction is that hypoxia can cause activation of DNA damage signaling (8, 9). Particularly, in response to extreme hypoxia, ATR-dependent phosphorylation of the checkpoint kinase CHK1 is observed in S-phase cells, an effect linked to replication stalling (10). It was proposed that hypoxic cells consequently may depend more on CHK1 for survival compared with normoxic cells, leading to the increased efficacy of CHK1 inhibitors in hypoxic tumors (7, 10). As CHK1 inhibitors are currently emerging as new anticancer agents (11, 12), this is an important hypothesis that could provide a rationale for further clinical testing of CHK1 inhibitors in hypoxic tumors. However, the experiments in support of this hypothesis were carried out at hypoxic conditions close to anoxia, which were toxic to the cells even in the absence of CHK1 inhibition (10, 13). It is therefore important to investigate the effects of CHK1 inhibitors under less severe hypoxic conditions.

CHK1 inhibitors are commonly used together with DNA-damaging agents (12, 14, 15). Therefore, another important issue is whether hypoxia alters the efficacy of such combination treatments. Combined treatment with CHK1 inhibitors and ionizing radio- or chemotherapeutic drugs lead to synergistic cell killing particularly in p53-negative cancer cells (12, 16). These effects have been attributed to abrogation of the G2 checkpoint and inhibition of homologous recombination (HR) repair (17).
Notably, it was shown that acutely hypoxic cells treated with ionizing radiation depend more on the homologous recombination repair pathway than ionizing radiation-treated normoxic cells (18). As CHK1 is required for HR repair (17, 19, 20), this could suggest that CHK1 inhibitors combined with ionizing radiation would cause greater radiosensitization of acutely hypoxic compared with normoxic cells.

To explore the potential of using CHK1 inhibitors to obtain selective killing of hypoxic cancer cells in the absence and presence of ionizing radiation, we have examined the effects of the CHK1 inhibitors, AZD7762 and UCN-01, during various hypoxic and normoxic conditions. Our results show that CHK1 inhibition leads to similar cytotoxic and radiosensitizing effects during normoxia and hypoxia. However, cancer cells display increased sensitivity to CHK1 inhibitors after reoxygenation following prolonged hypoxia. This effect is associated with enhanced DNA replication stress in response to CHK1 inhibitors after reoxygenation.

Materials and Methods

Cell culture, drugs, hypoxia treatment, and irradiation

Human osteosarcoma U2OS cells and colorectal HCT116<sup>+/−</sup> cells (21) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) with 10% FBS and 1% penicillin/streptomycin (P/S). U2OS-CHK1shRNA cells were maintained in DMEM containing puromycin (1 μg/mL) and blasticidin (5 μg/mL). The cell lines were authenticated by short tandem repeat profiling. AZD7762 was from Astra Zeneca and UCN-01 from R.J. Schulz (National Cancer Institute, Bethesda, MD). Nocodazole (Sigma) was used at 0.04 μg/mL. Hypoxia treatments were carried out in an INVIVO2 200 Hypoxic workstation supplied with a Gas mixer Q advanced gas mixing system and a De-tox sachet for optimum work-station environment (Ruskinn). Oxygen concentration setpoints used were 0.0% (referred to as 0.00%–0.03%), 0.2% (0.16%–0.24%), and 1% (0.9%–1.1%). Plastic tissue culture dishes and flasks were used throughout. Irradiation of cells was conducted with an X-ray generator (Faxitron CP160, 160kV, 6.3 mA) at a dose-rate of 1Gy/min. For ionizing radiation treatment of hypoxic cells, the cells were incubated in the hypoxic workstation for 3 hours in flasks with vented filter caps, which were replaced by sealed caps before the flasks were transferred to the X-ray generator.

Generation of U2OS cells with tetracycline-inducible CHK1 short hairpin RNA

The following oligos containing the CHK1 target sequence (indicated in bold below) were annealed and cloned into the p.Suprior.puro vector (from Oligogen):

**Forward oligo: 5’-GATCCCC**

GCAACAGATTTCCGATATA TTCAAGAGA TATACCGAAATCTGTTGC TTTA-3’

**Reverse oligo: 5’-ACCTAAAAAA**

GCAACAGATTTCCGATATA TTCTTGAAA TATACCGAAATCTGTTGC GGG-3’

For generation of cells with inducible expression of CHK1 short hairpin RNA (shRNA) upon addition of tetracycline, U2OS cells were cotransfected with the pSuperior.puro-CHK1shRNA and the pcDNA6-TR vector (Invitrogen). After 2 weeks of selection in medium containing puromycin (1 μg/mL) and blasticidin (5 μg/mL), separate surviving clones were isolated and tested for downregulation of CHK1 upon addition of tetracycline (2 μg/mL) to the growth medium. Results shown are from experiments with a single clone.

Immunoblotting

Cells were lysed in SDS boiling buffer (2% SDS, 10 mmol/L Tris-HCl pH 7.5, 100 μmol/L Na<sub>3</sub>VO<sub>4</sub>), and proteins were separated by gel electrophoresis (gels from Pierce/Thermo Scientific) and transferred to nitrocellulose membranes (Bio-Rad). Where indicated in the figure legends, cells were preextracted in detergent buffer (20 mmol/L HEPES, pH7.4; 50 mmol/L NaCl; 3 mmol/L MgCl<sub>2</sub>; 300 mmol/L Sucrose; 0.5% Triton-X-100) for 5 minutes on ice before cell lysis. The following antibodies were used for blotting: Anti-phospho-CHK1 (Ser296, Ser317, and Ser345) from Cell Signaling, anti-H4 from Santa Cruz, anti-HIF1α from Upstate, anti-CHK1 from Sigma, and anti-CHK1 from BD Transduction Laboratories, anti-α-tubulin from Sigma, and anti-CHK1 (DCS310; ref. 22).

Flow cytometry

Flow cytometry was conducted as described previously (23). For cell-cycle and DNA damage marker analysis, cells were stained with rabbit anti-phospho-H3 and mouse anti-phospho-H2AX (γH2AX) (Upstate, Millipore), anti-H4 from Sigma, and anti-CHK1 from BD Transduction Laboratories, anti-α-tubulin from Sigma, and anti-CHK1 from BD Transduction Laboratories, anti-α-tubulin from Sigma, and anti-CHK1 from BD Transduction Laboratories.

Immunofluorescence

Cells were fixed in 4% formaldehyde, permeabilized with 0.25% formaldehyde, permeabilized with 0.25% Triton X-100, and stained with antibodies to

### References


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cyclin A (Santa Cruz) and γH2AX [Upstate (Millipore)], followed by Alexa Fluor anti-rabbit 568 and anti-mouse 488 (Invitrogen). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector Laboratories). Images were obtained using a ×63 1.4 NA oil immersion objective on an Axio Imager Z1 microscope with ApoTome (Zeiss) using Axio Vision Release 4.8 software.

Clonogenic survival assay

Clonogenic survival assays were conducted as described previously (25). Cells were seeded to T25 flasks 16 to 20 hours before incubation in the hypoxia chamber for 3 to 24 hours. For experiments with CHK1shRNA expression, cells were seeded in medium containing 2μg/mL tetracycline. When indicated in the figure legends, treatment with UCN-01 (0–300 nmol/L), AZD7762 (0–100 nmol/L), and ionizing radiation (0–6 Gy) were added. For combined treatment with ionizing radiation and CHK1 inhibitors, CHK1 inhibitors were added to the culture medium for 10 to 15 minutes before ionizing radiation, and then washed off 24 hours later. For experiments with CHK1 inhibitors added after reoxygenation following prolonged hypoxia, cells were trypsinized and counted immediately after removal from the hypoxia chamber, irradiated in tubes when indicated, and plated out in 6 cm dishes with medium containing the indicated concentrations of AZD7762 or UCN-01. Cells were cultured for 13 to 14 days, fixed in 70% ethanol, and stained with methylene blue. Colonies containing more than 50 cells were counted as survivors. Survival fractions were calculated in each experiment as the average cloning efficiency (from 3 parallel dishes) after treatment with hypoxia, ionizing radiation, and/or the CHK1 inhibitors, divided by the average cloning efficiency for nontreated cells. All results shown are average of at least 3 independent experiments. The oxygen enhancement ratio (OER) was calculated as the ionizing radiation dose yielding a survival fraction of 10% for cells irradiated in hypoxia divided on the ionizing radiation dose yielding 10% survival for cells irradiated at normoxia.

Statistical analyses

Statistical significance was determined by a 2-tailed Student t test. P < 0.05 was considered significant. Error bars represent SEM for all plots (from at least 3 independent experiments).

Results

Exposure to < 0.03%, 0.2%, or 1% O2 does not reduce cell survival

To obtain acute and prolonged hypoxia treatments, we incubated cancer cells in the hypoxia chamber for 3 or 16 to 24 hours, respectively. Plastic, as opposed to glass, culture dishes were used to avoid anoxic conditions, as plastic binds oxygen and releases it gradually (26). Incubation of U2OS or HCT116 p^53−/− cells at < 0.03%, 0.2%, or 1% O2 for 20 to 24 hours did not significantly alter clonogenic cell survival (Fig. 1A). These hypoxia treatments were thus nontoxic to these cells. We used p53-negative HCTT16 cells in our experiments because CHK1-inhibitors are most often used for tumors that lack p53 function (12, 16), and HCTT16 parental cells are resistant to CHK1 inhibitors (25). U2OS cells contain wild-type p53, however, they lack a normal G1 checkpoint and are sensitive to CHK1 inhibitors independent of p53-status, consistent with deficient p53 function (25). Although acute exposure (3 hours) to either < 0.03%, 0.2%, or 1% O2 did not seem to alter cell-cycle progression, prolonged exposure (20 hours) to < 0.03% or 0.2% O2 resulted in a slight accumulation of cells in G1 (Fig. 1B and Supplementary Fig. S1A) with a concomitant reduction in the mitotic fraction (Fig. 1C). The effects were most pronounced following < 0.03% O2 (Fig. 1B). To verify that these cells were actively cycling, the microtubule inhibitor nocodazole was added for the last hour of hypoxia treatment. Addition of nocodazole resulted in an approximate doubling of the mitotic fraction, similar to what was observed for normoxic cells (Supplementary Fig. S1B). Also, cells could still incorporate the nucleotide analogue EdU during the last hour of hypoxia, showing ongoing replication (Supplementary Fig. S1C). These effects on cell-cycle progression and survival are consistent with an oxygen level in the growth medium between 0.01% and 0.1% (27) during exposure to < 0.03% O2 (in the gas phase) in our setup. In contrast, short exposure to severe hypoxia/anoxia (often referred to as < 0.02% O2 in previous publications) causes rapid, complete S-phase arrest, and massive cell death (10, 28).

Prolonged exposure to < 0.03% O2, but not 0.2% O2, leads to activation of DNA damage signaling

Previous studies have shown that severe, toxic levels of hypoxia can induce DNA damage signaling including phosphorylation of CHK1 (8, 10). To address whether our nontoxic hypoxic conditions lead to phosphorylation of CHK1, we conducted Western immunoblotting of lysates from cells exposed to varying durations and levels of hypoxia. We observed a robust phosphorylation of H2AX and CHK1 (Figs. 2A–D), activated at oxygen levels that do not cause a complete cell-cycle arrest (Fig. 1A). This finding was also confirmed by immunofluorescence analysis of γH2AX-positive cells in S-phase (Fig. 2C). This finding was also confirmed by immunofluorescence analysis of γH2AX after costaining with cyclin A to mark S-phase cells (Fig. 2D). Flow cytometric analysis revealed that the γH2AX-positive cells were in S-phase (Fig. 2C). This finding was also confirmed by immunofluorescence analysis of γH2AX after costaining with cyclin A to mark S-phase cells (Fig. 2D). Thus, our results show a hypoxia-induced replication stress response, including phosphorylation of H2AX and CHK1 (Figs. 2A–D), activated at oxygen levels that do not cause a complete cell-cycle arrest or reduction in cell survival (Fig. 1). We therefore reasoned that the replication stress response is most likely
Figure 1. Analysis of cell cycle and survival following treatment with hypoxia. A, clonogenic survival for U2OS and HCT116p53<sup>−−</sup> cells incubated at normoxia (21% O<sub>2</sub>) for 20 hours at <0.03% O<sub>2</sub> or 24 hours at 0.2% or 1% O<sub>2</sub>. B, cell-cycle profiles of U2OS cells following incubation at hypoxia (<0.03%, 0.2%, or 1% O<sub>2</sub>) for 0, 3, and 20 hours. Representative histograms of cell counts versus DNA content are shown. C, fraction of mitotic cells following incubation at normoxia (21% O<sub>2</sub>) or hypoxia (<0.03%, 0.2%, or 1% O<sub>2</sub> for 20 hours) as determined by gating of phospho-H3-positive cells from flow cytometric analysis.
reversible and will diminish following reoxygenation. Indeed, both γH2AX staining and CHK1 phosphorylation diminished rapidly following reoxygenation (Fig. 2A, lanes 3 and 9; Fig. 2B, lane 7; and Fig. 2E), consistent with a reversible response.

Cancer cells display unaltered sensitivity to CHK1 inhibitors UCN-01 or AZD7762 during hypoxic exposure

To explore whether cells are more sensitive to CHK1 inhibitors during hypoxia, we assayed clonogenic
Figure 3. Cell survival in response to CHK1-inhibitors is not altered by hypoxia. A, clonogenic survival of U2OS cells incubated in the hypoxic chamber at <0.03% O2 for 20 hours (left), at 0.2% O2 for 24 hours (middle), or at 1% O2 for 24 hours (right) in the presence of the indicated concentrations of AZD7762 (AZD) or UCN-01 (UCN). The inhibitors were washed off immediately after the hypoxic exposure. Survival fractions relative to nontreated cells are shown. In each graph, results for the normoxic (21% O2) cells are from the same experiments as the hypoxic cells; the normoxic and hypoxic cells were thus treated with identical stocks of the CHK1 inhibitors. (Different batches of the CHK1 inhibitors have varied slightly.) B, clonogenic survival of HCT116(p53−/−) cells treated with hypoxia and the CHK1 inhibitors as in A. C, analysis of CHK1 autophosphorylation (pCHK1-Ser 296) to monitor CHK1 activity during hypoxia (<0.03% O2) and normoxia. The hypoxic cells were incubated at <0.03% O2 for 3 hours before ionizing radiation (IR; 6Gy) and kept in the hypoxic environment until cell harvest. AZD7762 or UCN-01 (0, 25 or 100 nmol/L) was added 5 minutes before ionizing radiation, and cells were harvested 1 hour later. Ionizing radiation was included to induce detectable phosphorylation of CHK1-Ser296. D, structure of UCN-01 and AZD7762 (30, 31).
survival of U2OS or HCT116<sup>53-/-</sup> cells treated with either UCN-01 or AZD7762 during exposure to < 0.03% O<sub>2</sub>, 0.2%, or 1% O<sub>2</sub> with the inhibitors washed off immediately following reoxygenation. Remarkably, we observed no significant differences (P > 0.1) between normoxic and hypoxic cells in the effects of the CHK1 inhibitors on clonogenic survival (Fig. 3A and B). Thus, the efficacy of CHK1 inhibitors seems similar for normoxic and hypoxic cell populations, even though hypoxia induced CHK1 phosphorylation at < 0.03% O<sub>2</sub>. To confirm that AZD7762 and UCN-01 inhibited CHK1 to a similar extent under hypoxic and normoxic conditions, we monitored inhibition of CHK1 activity by examining CHK1 autophosphorylation on Ser296 in response to ionizing radiation (17, 29). Consistent with equal abrogation of CHK1 activity, we observed a similar decrease in Ser296 phosphorylation in ionizing radiation-treated cells that had been administered the drugs under either normoxia or hypoxia (Fig. 3C). The structure of the drugs are shown in Fig. 3D (30, 31).

**Cancer cells show increased sensitivity to inhibition of CHK1 after reoxygenation following prolonged hypoxia**

Although the CHK1 inhibitors, AZD7762 and UCN-01, showed similar cytotoxic effects during normoxia and hypoxia in experiments where the inhibitors were washed off immediately after reoxygenation (Fig. 3), the clonogenic survival following expression of CHK1 shRNA was significantly lower (P < 0.01) in hypoxic conditions (Fig. 4A). However, the level of CHK1 remained low for at least 24 hours after reoxygenation and removal of tetracycline from the medium (Fig. 4B). Thus, CHK1 was depleted both during hypoxia and after reoxygenation. Depletion of CHK1 by siRNA transfection also caused slightly lower survival in hypoxic conditions, although the magnitude of the effects varied between 2 different oligos (Supplementary Fig. S2). Taken together, these results led us to speculate that cells may be most sensitive to inhibition of CHK1 after reoxygenation.

To determine the cellular sensitivity to CHK1 inhibitors after reoxygenation, we seeded U2OS cells in the presence of AZD7762 and UCN-01 immediately after hypoxia treatment and assayed clonogenic survival. Cells pretreated in the hypoxia chamber at < 0.03% O<sub>2</sub> for 20 hours and exposed to 50 to 100 nmol/L AZD7762 or UCN-01 during the initial 24 hours after reoxygenation, showed a significantly lower survival (P < 0.01) than normoxic cells exposed to equal concentrations of the CHK1 inhibitors (Fig. 4C, left). Cells pretreated with 0.2% O<sub>2</sub> were also significantly (P < 0.05) more sensitive than normoxic cells to 100 nmol/L doses of UCN-01 and AZD7762, whereas no differences were observed for 50 nmol/L doses (Fig. 4C, middle). However, no significant differences were found for cells pretreated with 1% O<sub>2</sub> (Fig. 4C, right). Thus, U2OS cells show increased sensitivity to CHK1 inhibitors after reoxygenation following prolonged exposure to < 0.03% or 0.2% O<sub>2</sub>. HCT116<sup>53-/-</sup> cells are generally more resistant to the cytotoxic effects of CHK1-inhibitors than U2OS cells (Fig. 3A and B and ref. 25), and 50 nmol/L AZD7762 or 100 nmol/L UCN-01 therefore gave little reduction in clonogenic survival regardless of hypoxia or reoxygenation treatments. However, we observed a slightly decreased clonogenic survival with 100 nmol/L AZD7762 for HCT116<sup>53-/-</sup> cells pretreated at < 0.03% O<sub>2</sub> (results not shown), consistent with an increased sensitivity to CHK1 inhibitors after reoxygenation also in this cell line.

**CHK1 inhibition after reoxygenation leads to increased DNA damage in S-phase**

We previously have shown that CHK1 inhibition of normoxic S-phase cells leads to DNA damage in a process dependent on unscheduled replication initiation and subsequent replication stalling (32, 33). To explore whether CHK1 inhibition after reoxygenation may cause enhanced effects on such S-phase events, we first assayed γH2AX as a function of cell cycle by flow cytometric analysis. In the presence of UCN-01, the fraction of γH2AX-positive cells was elevated at 90 minutes and increased faster over the next 24 hours in the reoxygenated compared with normoxic cells (Fig. 5A and B, and Fig. 5C top histogram). Also, the fraction of cells displaying the strongest γH2AX staining (bottom gates Fig. 5A and B) was significantly higher in the reoxygenated cells at the later timepoints (24 hours; P < 0.05; ref. 5C; bottom histogram). These results suggest that the increased toxicity of CHK1 inhibitors after reoxygenation is associated with increased DNA damage in S-phase cells. Similar effects were found following UCN-01 treatment of reoxygenated HT29 and HCT116<sup>53-/-</sup> cells (Supplementary Fig. S3).

To address whether enhanced unscheduled initiation could be involved in causing the increased S-phase damage, we also monitored replication rates by uptake of the nucleotide analog EdU, and assayed loading of the replication initiation factor CDC45 (34, 35). Western blotting analysis of cells preextracted with detergent before lysis showed increased CDC45 chromatin loading after UCN-01 treatment of normoxic as well as reoxygenated cells (Fig. 5D). Notably, the effects of UCN-01 on CDC45 loading were not stronger in the reoxygenated compared with the normoxic cells, suggesting that the effects of UCN-01 on unscheduled initiation were not amplified after reoxygenation. Consistent with this finding, the total EdU uptake during the first hour after UCN-01 treatment was rather less increased after reoxygenation than in normoxia (Fig. 5E). On the other hand, we observed increased phosphorylation of replication protein A (RPA) on S4/S8 and S33 in reoxygenated compared with normoxic cells at 3 hours after treatment with UCN-01 (Fig. 5F and data not shown). As phosphorylation of RPA occurs at stalled replication forks (36, 37), this indicates increased...
Replication stalling. In agreement with this notion, the total cellular EdU uptake at 5 to 6 hours after UCN-01 treatment was decreased in reoxygenated compared with normoxic cells (Supplementary Fig. S4).

CHK1 inhibitors increase the radiosensitivity of acute hypoxic as well as normoxic cells

Next, we addressed whether acute hypoxic conditions could increase the synergistic effects between CHK1 inhibition and ionizing radiation. As CHK1 is required for homologous recombination repair (19, 20) and cells irradiated during acute hypoxia depend more on the homologous recombination repair pathway (18), we reasoned that inhibition of CHK1 potentially might lead to a greater radiosensitizing effect in cells irradiated under acute hypoxia than in normoxia. Clonogenic survival assays where U2OS cells were irradiated in the presence of UCN-01 during hypoxic versus normoxic conditions showed similar extent of radiosensitization for hypoxic and normoxic cells (Fig. 6A), and the same was observed for cells irradiated after CHK1 depletion by expression of CHK1 shRNA (Fig. 6B). Although there was a small tendency toward greater synergistic effects for cells irradiated during hypoxia (Fig. 6A and B), the differences in radiosensitization between hypoxic and normoxic cells were not significant. Furthermore, the radiosensitization for cells irradiated and treated with UCN-01 after reoxygenation following 24-hour pretreatment at 0.2% O2 seemed similar as for normoxic cells (although the radiosensitizing effects were small for both normoxic and hypoxic cells likely because UCN-01 was added after ionizing radiation; ref. Supplementary Fig. S5). At our hypoxic conditions, the extent of synergistic effects between CHK1-inhibition and ionizing radiation thus seems similar for normoxic and hypoxic cells.
Figure 5. Inhibition of CHK1 after reoxygenation leads to increased DNA damage in S-phase. A, flow cytometric analysis of γH2AX staining versus the DNA stain Hoechst for normoxic (21% O2) U2OS cells treated with 50 nmol/L UCN-01 for the indicated times. Gates indicate γH2AX-positive cells. The upper gate indicates cells with very strong γH2AX staining. B, similar analysis as in A for cells treated with 50 nmol/L UCN-01 for the indicated times after reoxygenation following 20 hours at <0.03% O2. C, quantification of the γH2AX-positive fractions from 3 independent experiments as in A and B. Top, displays the total fraction of γH2AX-positive cells; bottom, the fraction of cells with very strong γH2AX staining (top gates in A and B). D, CDC45 chromatin loading in U2OS cells after pre-extraction with detergent. Normoxic cells (21% O2) or cells reoxygenated after hypoxia (20 hours, <0.03% O2) were treated with UCN-01 (300 nmol/L) for 1 hour, pre-extracted with detergent for 5 minutes, and analyzed by Western blotting with the indicated antibodies. E, cellular EdU uptake within the first hour after UCN-01 treatment (300 nmol/L) of normoxic U2OS cells or following reoxygenation after hypoxia (20 hours, <0.03% O2). Cells were labeled with 1 μmol/L EdU and treated with UCN-01 immediately after reoxygenation and processed for the bar-coding flow cytometry technique 1 hour later. The average EdU median values from 3 independent experiments are shown. F, measurement of RPA S4/S8 phosphorylation by flow cytometry. Normoxic U2OS cells (21% O2) or U2OS cells reoxygenated following hypoxia (20 hours, <0.03% O2) were treated with UCN-01 (300 nmol/L) for 3 hours and processed for the flow cytometry bar-coding technique.
Tumor hypoxia is considered a major challenge for current cancer therapeutic approaches. On the other hand, hypoxia is a hallmark of solid tumors and might therefore be exploited to achieve tumor-specific treatments. Here, we have explored the hypothesis that CHK1 inhibitors would cause selective killing of hypoxic cancer cells. Our results suggest that the efficacy of CHK1 inhibitors will often be similar under hypoxic and normoxic conditions. We observed equivalent reductions in clonogenic survival by the CHK1 inhibitors in hypoxic and normoxic cells. Multiple concentrations of the CHK1 inhibitors were tested, some of which were toxic on their own, potentially eliminating cells that could otherwise have given rise to synergistic effects. However, we also observed no differences between hypoxic and normoxic cells in response to less toxic concentrations of the inhibitors (50 nmol/L UCN-01; Fig. 3). The synergistic effects following combined treatment with CHK1 inhibitors and ionizing radiation were also similar (Fig. 6). Only after reoxygenation following a prolonged hypoxia treatment (> 20 hours) did we observe an enhanced efficacy of the CHK1 inhibitors. This effect was most pronounced after the strongest level of hypoxia tested in our experiments (< 0.03% O₂), however, an effect was also found after prolonged treatment with 0.2% O₂ (Fig. 4). After reoxygenation following a short hypoxic exposure (3 hours), the reduction in clonogenic survival by CHK1 inhibitor was the same as for normoxic cells (data not shown). Provided successful delivery of such inhibitors to the tumors, CHK1 inhibitors would therefore most often be expected to work equally well in hypoxic as compared with well-oxygenated tumors. However, in cases where CHK1 inhibitors are administered to tumors undergoing periods of reoxygenation following prolonged hypoxia, the efficacy may be increased. In human tumors, reoxygenation following prolonged hypoxia can, for example, occur due to spontaneous changes in vascular networks (38).

CHK1 inhibitors are commonly used together with DNA-damaging agents (12, 25). On the basis of previous work showing that acutely hypoxic cells rely more on homologous recombination to repair ionizing radiation-induced DNA damage (18) and that CHK1 is required for homologous recombination repair (20), we initially hypothesized that the synergistic effects following combined treatment with CHK1 inhibitors and ionizing radiation may be increased in acutely hypoxic tumor cells. Although our results showed no significant differences in UCN-01–mediated radiosensitization between hypoxic and normoxic cells, there was a nonsignificant tendency toward greater radiosensitization following CHK1 inhibition in hypoxia (Fig. 6). In the publication by Sprong and colleagues, the OER for clonogenic survival of the hypoxic relative to normoxic human fibroblast cells was 1.8 to 2.2, whereas the OER in our experiments was about 1.5, consistent with more severe hypoxic conditions in the former study. We can therefore not exclude that a significantly increased radiosensitization by CHK1 inhibition might have been detected in our cells under more severe hypoxic/anoxic conditions. It is also noteworthy that hypoxia did not
cause a reduction of the radiosensitization effects. CHK1 inhibitors thus have the capability to radiosensitize cancer cells regardless of the hypoxic status. Taken together with the increased effects of CHK1 inhibitors after reoxygenation following prolonged hypoxia, the combination of CHK1 inhibitors with fractionated radiotherapy may potentially be beneficial.

During fractionated radiotherapy, ionizing radiation is delivered as daily fractions over several weeks, and reoxygenation may occur repeatedly in response to cell death (39–42).

With our hypoxia chamber at < 0.03% O₂, we observed phosphorylation of CHK1 and γH2AX staining consistent with hypoxia-induced replication stress. However, the cytotoxic effects of CHK1 inhibitors did not seem increased in these cells when the CHK1 inhibitors were washed off immediately after the hypoxic exposure. These findings are consistent with a previous publication where incubation with the CHK1 inhibitor G06976 after reoxygenation following severe hypoxia (anoxia) gave similar reduction in survival as incubation with G06976 during the periods of both hypoxia and after reoxygenation (13). As CHK1 is phosphorylated during hypoxia, and the phosphorylation of CHK1 decreases after reoxygenation (Fig. 2), it may seem counterintuitive that cells do not display the highest sensitivity to CHK1-inhibitors during hypoxia. A plausible explanation could be that hypoxia causes accumulation of cells in G₁ phase and thereby reduces the fraction of cells in the ‘CHK1-sensitive’ S and G₂ cell-cycle phases (Fig. 1). Furthermore, hypoxia-induced downregulation of CHK1 targets, such as CDC25A (43, 44), might potentially restrain the effects of CHK1 inhibition during hypoxia.

One intriguing issue is the mechanisms by which CHK1 inhibitors cause increased DNA damage in reoxygenated S-phase cells. Previous work, by us and others, suggests that such S-phase DNA damage can occur due to unscheduled replication initiation followed by replication fork stalling (33). Our analysis of CDC45 loading at 1 hour after addition of UCN-01 suggest that replication initiation is not more increased in the reoxygenated compared with the normoxic cells. However, increased RPA phosphorylation and decreased EdU uptake at 3 to 6 hours after UCN-01 indicate increased replication stalling in the reoxygenated cells (Fig. 5). When replication initiation is increased shortly after CHK1 inhibition, the extent of subsequent replication stalling may thus be enhanced, potentially due to deprivation of essential replication factors caused by the prolonged hypoxia treatment. In this context, it might be relevant that hypoxia can cause deprivation of the nucleotide pool (28). We recently found that WEE1 inhibition of normoxic cells causes DNA damage and replication stalling through increased initiation and subsequent nucleotide shortage (45). Although CHK1 may play different roles than WEE1 in this process (45), further studies may address whether nucleotide shortage contributes to replication stalling and DNA damage following CHK1 inhibition of reoxygenated cells. Importantly, stalled forks can act as substrates for endonucleases (46), which may cause excessive DNA damage if not controlled properly, and CHK1 may directly or indirectly be involved in suppression of such endonucleases (33, 47, 48).

In conclusion, we have shown that CHK1 inhibitors lead to similar reductions in cancer cell survival during hypoxia and normoxia, and the synergistic effects between CHK1 inhibitors and ionizing radiation are also similar. However, after reoxygenation following prolonged hypoxia, cells can display increased sensitivity to the cytotoxic effects of CHK1-inhibitors.

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

Authors’ Contributions
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