CHK1 Inhibition Radiosensitizes Head and Neck Cancers to Paclitaxel-Based Chemoradiotherapy

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a leading cause of cancer-related deaths, with increasingly more cases arising due to high-risk human papillomavirus (HPV) infection. Cisplatin-based chemoradiotherapy is a standard-of-care for locally advanced head and neck cancer but is frequently ineffective. Research into enhancing radiation responses as a means of improving treatment outcomes represents a high priority. Here, we evaluated a CHK1 inhibitor (CCT244747) as a radiosensitizer and investigated whether a mechanistically rational triple combination of radiation/paclitaxel/CHK1 inhibitor delivered according to an optimized schedule would provide added benefit. CCT244747 abrogated radiation-induced G₂ arrest in the p53-deficient HNSCC cell lines HN4 and HN5, causing cells to enter mitosis with unrepaired DNA damage. The addition of paclitaxel further increased cell kill and significantly reduced tumor growth in an HN5 xenograft model. Importantly, a lower dose of paclitaxel could be used when CCT244747 was included, therefore potentially limiting toxicity. Triple therapy reduced the expression of several markers of radioresistance. Moreover, the more radioresistant HN5 cell line exhibited greater radiation-mediated CHK1 activation and was more sensitive to triple therapy than HN4 cells. We analyzed CHK1 expression in a panel of head and neck tumors and observed that primary tumors from HPV⁻ patients, who went on to recur postradiotherapy, exhibited significantly stronger expression of total and activated CHK1. CHK1 may serve as a biomarker for identifying tumors likely to recur and, therefore, patients who may benefit from concomitant treatment with a CHK1 inhibitor and paclitaxel during radiotherapy. Clinical translation of this strategy is under development.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer worldwide (1), with smoking, alcohol consumption, and high-risk human papillomavirus (HPV) infection being the most common risk factors (2–⁴). Radiotherapy is a standard-of-care treatment for patients with HNSCC. Tumors of the same size and stage, however, may respond variably to radiation and new approaches to overcoming radioresistance and preventing tumor recurrence are continually being sought. Ionizing radiation induces a DNA damage response (DDR) that involves the cooperation of a complex network of proteins, which play roles in cell-cycle checkpoints and DNA repair (5). In normal cells, radiation-induced DNA damage predominantly induces a G₁-S cell-cycle arrest as a result of p53 activation. This G₁-S checkpoint is typically lost in cancer cells most often due to p53 loss, mutation, or inactivation (i.e., following HPV infection), or disruption of p53-regulated processes (6). These cells, therefore, depend heavily on the S or G₂-M cell-cycle checkpoints to repair DNA damage following ionizing radiation, a dependency that can be exploited therapeutically (⁷).

Ataxia telangiectasia and Rad3-related (ATR) and Ataxia telangiectasia mutated (ATM) are the initial DDR kinases activated by the sensors of DNA damage and, in turn, activate the downstream checkpoint effector kinases CHK1 and CHK2 (8). One consequence of CHK1 activation is inhibition of the phosphatase CDC25C and subsequent arrest of cells in the G₂-M phase of the cell cycle. CHK1 activity has also been shown to play a role in the G₁-S, intra-S, and mitotic spindle checkpoints, indicating that CHK1 is a fundamental component of the DDR and represents an ideal target for therapeutic intervention (⁹). We, and others, have shown that CHK1 inhibitors can enhance radiation cytotoxicity in cancer cells (reviewed in ref. ⁷). In the presence of CHK1 inhibitors, cells will attempt to undergo mitosis despite harboring radiation-induced DNA damage. This can lead to chromosomal missegregation or loss of chromatid fragments, resulting in abnormal cell division and cell death in a process called mitotic catastrophe (¹⁰).

Patients with locally advanced HNSCC typically receive cisplatin-based chemoradiotherapy (CCRT), but this is frequently ineffective and associated with severe acute and late toxicities (¹¹). Therefore, alternative approaches to CCRT for HNSCC need to be developed. Taxanes are potent radiosensitizers and have been shown to be tolerable and active in combination with...
radiotherapy in patients with HNSCC (12). Because they disrupt normal mitotic progression, they represent attractive agents to combine with radiation and radiosensitizers that target the G2–M phase of the cell cycle (e.g., CHK1 inhibitors).

CCT244747 is a selective and orally bioavailable CHK1 inhibitor, which has previously been shown to enhance the antitumor activity of several genotoxic agents (13). Here, we investigated whether including paclitaxel provided added benefit to CCT244747 and radiation combination therapy and defined the schedule-dependence of the combined therapy. We also investigated possible biomarkers of radiation resistance and assessed the potential for this triple combination therapy in overcoming radiation resistance in head and neck cancer patients.

**Materials and Methods**

**Cell lines**

HN4 and HN5 human HNSCC cell lines (LICR-LON-HN4 and LICR-LON-HN5) were kindly provided by Sue Eccles (The Institute of Cancer Research, Sutton, United Kingdom) and were authenticated by short tandem repeat analysis using the Puregene Cell (Qiagen) and GenePrint 10 (Promega) kits. Results were authenticated by short tandem repeat analysis using the Puregene Kit (Qiagen). SCC090 cells were obtained commercially from DMSZ (LICR-LON-HN5) were kindly provided by Sue Eccles (The Institute of Cancer Research, Sutton, United Kingdom). Irradiation was carried out using an AGO HS MP1 X-ray machine (AGO X-ray Ltd.) at 250 kV.

**Drug preparation and irradiation**

The CHK1 inhibitor CCT244747 was manufactured at the ICR. CCT244747 was dissolved in DMSO for in vitro experiments and in 10% DMSO, 5% Tween-80, 20% PEG 400, and 65% H2O for in vivo experiments. Paclitaxel was obtained from the Royal Marsden Hospital (London, United Kingdom). Irradiation was carried out using an AGO HS MP1 X-ray machine (AGO X-ray Ltd.) at 250 kV.

**MTT assays**

Cells were plated in 96-well plates, treated with increasing concentrations of CCT244747 or paclitaxel, and cell viability assessed 72 hours later using the MTT assay. Cell viability was calculated by comparing to vehicle-treated cells. Synergy was determined using the Bliss Independence Model, defined by the equation $E_{exp} = E_a + E_b - (E_a E_b)$, where $E_{exp}$ is the expected effect if the two drugs are additive and $E_a$ and $E_b$ are effects of the individual drugs (14). The equation $\Delta E = E_{obs} - E_{exp}$ is then used to ascertain synergy. If $\Delta E$ and the 95% CI values are all greater than 0, the two drugs demonstrate synergy.

**In vivo experiments**

HN5 cells were injected subcutaneously in female 6- to 8-week-old NOD scid gamma mice (NSG, JAX Mice). Once tumors had reached approximately 100 mm³, animals were divided into eight treatment groups (8–12 mice per group). Treatment was given three times on alternate days. Mice received CCT244747 (125 mg/kg) by oral gavage, paclitaxel (5 mg/kg) by intraperitoneal injection, and/or 2 Gy radiotherapy according to schedule 2. Tumor volume was obtained using the formula: $volume = width \times length \times depth \times 0.524 \ mm^3$. Mice were culled when tumors reached a maximum diameter of 15 mm in one dimension. All animal studies were conducted in accordance with National Cancer Research Institute (NCRI) guidelines (15). All animal research was reviewed and approved by the Institute of Cancer Research Ethics Committee. These experiments were performed under the authority given by UK Home Office Project License PPL 70/7947.

**Immunocytochemistry for DNA damage and nuclear morphology**

Cells were treated with CCT244747, paclitaxel, and/or 4 Gy radiation according to schedule 2. Cells were fixed with 4% paraformaldehyde, blocked with blocking buffer (1% BSA, 2% FCS), and incubated with rabbit anti-γ-H2AX (Cell Signaling Technology) and mouse anti-α-tubulin (Sigma-Aldrich) antibodies. Anti-rabbit 488 and anti-mouse 633 Alexa Fluor 647 (Cell Signaling Technology).
secondary antibodies were used (Invitrogen, Molecular Probes). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, Molecular Probes). Cells were imaged using an LSM 710 inverse laser scanning microscope (Zeiss) and captured with an LSM T-PMT detector (Zeiss). At least 275 cells from five fields of view and three independent experiments were counted. Cells with ≥5 γ-H2AX foci/nucleus were scored as positive.

**Long-term growth assays**

Cells were treated with CCT244747, paclitaxel, and/or 4 Gy radiation according to schedule 2. Cells were trypsinized at 96, 168, and 336 hours and counted. Before trypsinization at 336 hours, cells were imaged using an Eclipse TS100 microscope (Nikon) and images captured using a Digital Sight DS-L1 camera (Nikon).

**Apoptosis array**

Cells were treated with vehicle or triple therapy according to schedule 2 and lysed after 48 hours in RIPA buffer. Membranes from a Human Apoptosis Array Kit (R&D Systems) were incubated with prepared cell lysates and developed as per manufacturer’s instruction. Dot intensity was calculated using ImageJ software (NIH).

**Patient samples and IHC**

The PREDICTR-HNC study was approved by the Coventry Research Ethics Committee (reference number 10/H1210/9). Patient samples were collected at the Royal Marsden Hospital Research Ethics Committee (reference number 10/H1210/9). Patient samples and IHC were used in subsequent experiments unless otherwise stated. Western blot analysis of CCT drug on target activity was performed with antibodies specific for phospho-S345 CHK1 (Thermo Fisher Scientific), CHK1, phospho-Ser1981 ATM (Abcam), Survivin (Dako), and β-actin (Leica Biosystems). The percentage of HN4 and HN5 cells in mitosis up to 24 hours after irradiation was calculated for CCT244747 (1 μmol/L for HN4 cells and 0.4 μmol/L for HN5 cells) and were used in subsequent experiments unless otherwise stated (Supplementary Fig. S1A). As expected, radiation reduced the percentage of cells entering mitosis, suggesting that cells were arrested at the G2 phase of the cell cycle (Fig. 1A). CCT244747 administration significantly abrogated the radiation-mediated reduction of mitotic HN4 and HN5 cells at 4 and 8 hours after 4 Gy (Fig. 1A). We, and others, have previously shown that phospho-S345 CHK1 is the most consistent pharmacodynamic biomarker of CHK1 inhibition (16, 17). Western blot analysis confirmed CCT244747 drug-on-target activity as demonstrated by increased p-CHK1 expression (Fig. 1B). p-HH3 expression was reduced in cells following irradiation as before, whereas cells pretreated with CCT244747 had higher levels of p-HH3 expression at 4 and 8 hours after irradiation (Fig. 1B). Continued cell cycling after irradiation, due to CCT244747 treatment, resulted in the maintenance of DNA double-strand breaks (DSB) as shown by increased phosphorylation of histone H2AX at Ser139 (γ-H2AX) expression, most notably at 12, 24, and 48 hours after irradiation in both cell lines. Analysis of the sub-G1 population indicated that CCT244747 pretreatment significantly increased apoptosis at 48 hours in both cell lines, although levels of apoptosis overall were very low in HN5 cells (Supplementary Fig. S1B). Importantly, CCT244747 pretreatment led to significant radiosensitization of both HN4 and HN5 cells in clonogenic assays (Fig. 1C and Supplementary Fig. S1C).

**Results**

**CHK1 inhibition radiosensitizes HN4 and HN5 cells**

To test the effect of inhibiting CHK1 on radiation-mediated G2 arrest, cells were treated with the CHK1 inhibitor, CCT244747, 1 hour before irradiation and then collected at indicated time points. Mitotic cells were identified by phospho-Ser10 histone H3 (p-HH3) staining and flow cytometry. IC50 concentrations were calculated for CCT244747 (1 μmol/L for HN4 cells and 0.4 μmol/L for HN5 cells) and were used in subsequent experiments unless otherwise stated (Supplementary Fig. S1A). As expected, radiation reduced the percentage of cells entering mitosis, suggesting that cells were arrested at the G2 phase of the cell cycle (Fig. 1A). CCT244747 administration significantly abrogated the radiation-mediated reduction of mitotic HN4 and HN5 cells at 4 and 8 hours after 4 Gy (Fig. 1A). We, and others, have previously shown that phospho-S345 CHK1 is the most consistent pharmacodynamic biomarker of CHK1 inhibition (16, 17). Western blot analysis confirmed CCT244747 drug-on-target activity as demonstrated by increased p-CHK1 expression (Fig. 1B). p-HH3 expression was reduced in cells following irradiation as before, whereas cells pretreated with CCT244747 had higher levels of p-HH3 expression at 4 and 8 hours after irradiation (Fig. 1B). Continued cell cycling after irradiation, due to CCT244747 treatment, resulted in the maintenance of DNA double-strand breaks (DSB) as shown by increased phosphorylation of histone H2AX at Ser139 (γ-H2AX) expression, most notably at 12, 24, and 48 hours after irradiation in both cell lines. Analysis of the sub-G1 population indicated that CCT244747 pretreatment significantly increased apoptosis at 48 hours in both cell lines, although levels of apoptosis overall were very low in HN5 cells (Supplementary Fig. S1B). Importantly, CCT244747 pretreatment led to significant radiosensitization of both HN4 and HN5 cells in clonogenic assays (Fig. 1C and Supplementary Fig. S1C).

CHK1 inhibition reduces paclitaxel-mediated mitotic arrest, but the combination is not synergistic in HN4 and HN5 cells

To test the effect of combining CCT244747 with paclitaxel on the mitotic population, cells were treated with CCT244747 1 hour before paclitaxel treatment and then collected at indicated time points for quantification of p-HH3—expressing cells by flow cytometry. IC50 concentrations were determined for paclitaxel using MTT assays (8 nmol/L for HN4 cells and 4 nmol/L for HN5 cells) and were used in subsequent experiments unless otherwise stated (Supplementary Fig. S1A). Paclitaxel treatment increased the percentage of HN4 and HN5 cells in mitosis up to 24 hours (Fig. 1D). When cells were pretreated with CCT244747

Figure 1.

The CHK1 inhibitor CCT244747 overcomes radiation-mediated G2 arrest and reduces paclitaxel-mediated mitotic arrest in HN4 and HN5 cells. A, quantification of mitotic cells following 4 Gy of radiation (RT) with or without CCT244747 (CCT) pretreatment. Cells were fixed at indicated time points, stained with phosho-S10 Histone H3 (p-HH3), and analyzed by flow cytometry. Data expressed as fold changes (0 hour = 1). B, Western blot analysis of CCT drug on target activity (accumulation of phospho-S345 CHK1), mitotic cells (expression of p-HH3), and sustained DNA DSBs (expression of γ-H2AX) after 4 Gy of radiation with or without CCT pretreatment. Expression of β-actin provided a loading control. C, analysis of CCT244747 mediated radiosensitization using clonogenic assays. Cells were treated with 0.2 or 0.7 μmol/L CCT 1 hour before exposure to indicated doses of radiation and data normalized for drug effect. D, quantification of mitotic cells following exposure to paclitaxel (PTX) with or without CCT pretreatment, as described in A, E, Western blot analysis of CCT drug on target activity, mitotic cells, and sustained DNA DSBs after exposure to PTX with or without CCT pretreatment. Expression of β-actin provided a loading control. F, quantification of cell viability following CCT or PTX monotherapy, or combined treatment, at the indicated ratios of IC50 doses for CCT and PTX. Cell viability was quantified using MTT assays and calculated as a fraction of control cells. G, analysis of synergy using the Bliss Independence Model. The difference in observed and expected effects (ΔE = Eobs − Eexp) indicates synergy when ΔE and the 95% CI values are all greater than 0. **P < 0.001; *P < 0.01; *P < 0.05; †††P < 0.001; ††(* P < 0.0001; and ††††P < 0.00001.
accumulation in mitosis was initially unaffected; however, after 12 hours, the mitotic population was significantly reduced in both cell lines (P = 0.01 and 0.014 for HN4 and HN5 cells, respectively; Fig. 1D). Western blot analysis confirmed CCT244747 drug-on-target activity as demonstrated by increased p-CHK1 expression (Fig. 1E). p-HH3 expression was reduced slightly after 12 hours in cells pretreated with CCT244747 compared with those treated with paclitaxel alone; however, a p-HH3 reduction was most pronounced at 24 hours. Although the Western blotting and flow cytometry results vary slightly (possibly due to the sensitivities of the different antibodies used), both methods indicate that CHK1 inhibition enables cells to prematurely escape a paclitaxel-induced mitotic arrest. Moreover, CCT244747 pretreatment results in increased DNA DSBs, demonstrated by increased expression of γ-H2AX at 24 hours in both cell lines (Fig. 1E). Analysis of the sub-G1 population indicated that CCT244747 pretreatment did not increase apoptosis in HN4 or HN5 cells (Supplementary Fig. S1D). MTT assays were carried out to determine whether CCT244747 and paclitaxel acted synergistically. HN4 and HN5 cells were treated with varying ratios of IC_{50} concentrations of CCT244747 and paclitaxel independently or in combination and cell viability calculated relative to vehicle (DMSO) treated cells (Fig. 1F). Bliss analysis of the MTT results indicated that CCT244747 and paclitaxel only behaved synergistically in HN5 cells at a concentration of 0.5 × IC_{50} (Fig. 1G). Clonogenic assays showed a significant decrease in cell survival when cells were treated with CCT244747 and paclitaxel double therapy compared with monotherapy (Supplementary Fig. S1E).

A schedule for CCT244747, paclitaxel, and radiation triple therapy synergistically kills HN4 and HN5 cells

We hypothesized that cells prematurely entering mitosis with radiation-induced DNA damage, due to pretreatment with the CHK1 inhibitor, could be subsequently exposed to paclitaxel resulting in greater cell kill. Flow cytometry results suggested that treatment with paclitaxel 4 to 8 hours after CCT244747/radiation double therapy would affect the maximum number of damaged cells entering mitosis (Fig. 1A). In contrast, flow cytometry revealed that irradiation of cells 4 to 8 hours after CCT244747/paclitaxel double therapy would target the greatest number of cells arrested in mitosis, the most radiosensitive phase of the cell cycle (refs. 18–20; see Fig. 1D). Therefore, we tested two schedules where cells were treated with CCT244747 and then exposed to radiation before paclitaxel (Schedule 1) or vice versa (Schedule 2) over the same 6-hour therapeutic window (Fig. 2A). The short-term maintenance of DNA damage was determined by Western blotting for γ-H2AX expression. Triple therapy according to Schedule 2 resulted in a greater γ-H2AX signal at 24 hours in HN4 and HN5 cells compared with schedule 1 (Fig. 2B). The concurrent γ-H2AX and p-HH3 expression suggests that mitosis is occurring despite unrepaired DNA DSBs being present, therefore, cells are at significant risk of undergoing mitotic catastrophe. In long-term clonogenic assays, HN4 and HN5 cells were dosed with 0.5 μmol/L CCT244747, 3 nmol/L paclitaxel, and 2 Gy of radiation. The SF of cells treated with triple therapy was significantly lower than the SF of cells treated with CCT244747/radiation double therapy only in HN5 cells when using schedule 2 (P = 0.031; Fig. 2C). Normalizing SF values for CCT244747 and paclitaxel treatment again demonstrated a significant decrease in SF after triple therapy compared with CCT244747/radiation double therapy in HN5 cells when schedule 2 was used (P = 0.001; Fig. 2D). Clonogenic assays carried out with lower concentrations of 0.2 μmol/L CCT244747 and 1 nmol/L paclitaxel with 2 Gy of radiation resulted in a significantly lower SF following triple therapy only when compared with paclitaxel/radiation double therapy in HN5 cells using schedule 2 (P = 0.0002; Supplementary Fig. S2A). Normalizing for drug effect, a lower SF after triple therapy was only seen in HN5 cells using schedule 2 but this difference was not significant (Supplementary Fig. S2B). We used the Bliss Independence Model to determine whether paclitaxel synergized with CCT244747/radiation double therapy. Synergy was only observed for schedule 2 at the high drug concentrations in HN5 cells (Fig. 2E). Taking into account all of the results obtained for the two triple therapy schedules, schedule 2 was selected for further analysis.

Triple therapy reduces the growth rate of HN5 xenografts

To assess the efficacy of the triple therapy in vivo, HN5 cells were implanted subcutaneously in NSG mice and treated according to schedule 2 (Fig. 3A). Relatively low doses of all agents were used to ensure effects of the combination therapies would be apparent and to reduce toxicity in the combination groups. Radiotherapy was the only single agent to show a significant reduction in tumor growth compared with controls (P = 0.0343). Triple therapy significantly decreased tumor growth compared with all double therapy treatment groups (P < 0.0001 when comparing with the paclitaxel/radiotherapy combination; Fig. 3B). HN5 tumors appeared to be more sensitive to radiation than might have been expected from in vitro data, possibly due to the fractionated nature of the in vivo treatment regimen. Interestingly, combining CCT244747 or paclitaxel with radiation did not cause a significant reduction in tumor growth above what was seen for tumors treated with radiation alone. Three tumors from each group were collected 2 hours after the final treatment for pharmacodynamic analysis. Western blotting of lysates prepared from these tumors demonstrated that, in most cases, CCT244747 activity could be detected by expression of p-CHK1. Furthermore, CCT244747 caused an increase in the mitotic population within the tumors as assessed by p-HH3 staining (Fig. 3C).

Cells treated with CCT244747, paclitaxel, and radiation triple therapy undergo mitotic catastrophe

To assess the appearance of the nuclei in cells after triple therapy using schedule 2, treated cells were fixed at 24 and 48 hours and analyzed by confocal microscopy. Cells were stained with γ-H2AX to visualize DNA DSBs, and DAPI and α-tubulin to visualize nuclei and microtubules, respectively. Both HN4 and HN5 cells exhibited increased γ-H2AX foci after radiation, pan-H2AX staining (whole nuclear γ-H2AX staining) after CCT244747 treatment [as has been previously reported (21)], and abnormal nuclei after paclitaxel treatment (Fig. 4A and Supplementary Fig. S3A). Cells were quantified according to 6 categories: normal nuclear appearance, γ-H2AX foci, pan-H2AX staining, abnormal nuclear morphology (micronuclei and/or multinucleate), abnormal nuclear morphology with γ-H2AX foci, and abnormal nuclear morphology with pan-H2AX staining (Fig. 4B). Forty-eight hours after triple therapy, very few of the remaining cells had normal nuclear appearance. The most common characteristic in triple-treated cells was abnormal nuclear morphology, which was observed in 44.3% of HN4 cells and 67.5% of HN5 cells. HN4 cells exhibited...
Figure 2.
Scheduling of CCT244747, paclitaxel, and radiation triple therapy. A, schematic of the two schedules chosen for further analysis. B, Western blot analysis of CCT244747 (CCT) drug on target (accumulation of p-CHK1), mitotic cells (expression of p-HH3), and sustained DNA DSBs (expression of γ-H2AX) 24 hours after triple therapy according to schedule 1 or 2. Expression of β-actin provided a loading control. C, quantification of cell survival after triple therapy using clonogenic assays. Cells were treated with vehicle, 0.5 μmol/L CCT, 3 nmol/L paclitaxel (PTX), and/or 2 Gy radiation (RT) according to schedule 1 or 2. SFs were calculated by comparing treated wells with control wells. D, quantification of the SF when normalizing for drug effects. E, analysis of synergy using the Bliss Independence Model. The effect of triple therapy is compared with the effects of CCT and chemoradiotherapy (PTX+RT) independently. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.
significantly more micronuclei following triple therapy compared with HN5 cells (P = 0.001) and HN5 cells were significantly more likely to be multinuclear following triple therapy compared with HN4 cells (P = 0.044; Fig. 4C), indicating mitosis was aberrant in both cell lines. Pan-H2AX staining occurred in cells exposed to CCT244747 with 43.0% and 51.9% of HN4 and HN5 triple-treated cells exhibiting pan-H2AX staining 48 hours after triple therapy, respectively. Pan-H2AX staining has previously been linked to stalled replication (S-phase arrest), as well as being an indicator of inappropriate mitotic entry of cells harboring unrepaired DNA damage from replication stress or checkpoint inhibition (22, 23). We have previously observed by Western blotting that mitosis is occurring after triple therapy despite the presence of unresolved DNA DSBs (Fig. 2B). In addition, flow cytometry revealed that there was a 2.2- and 2.7-fold increase in the S-phase population of HN5 cells 24 and 48 hours after triple therapy, respectively (Supplementary Fig. S3B). Both of these findings could account for the increased pan-H2AX staining observed.

Triple therapy induces apoptosis and reduces clonogenicity in surviving cells

Pan-H2AX staining may also be an early indicator of cells destined for apoptosis (24). To assess whether HN4 and HN5 cells were undergoing apoptosis, sub-G1 analysis and Western blotting were carried out 48 hours after triple therapy. Flow cytometry revealed significantly more HN4 cells (19.9 ± 2.8%, compared with 6.1 ± 2.1% HN5 cells) were undergoing the late stages of apoptosis (as assessed by the percentage of cells in sub-G1) 48 hours after triple therapy (P = 0.016; Fig. 5A). Western blotting confirmed that HN4 cells were dying by apoptosis by virtue of decreased expression of MCL1 and the appearance of PARP and caspase-3 cleavage in lysates harvested from treated cells 48 hours after triple therapy (Fig. 5B). Decreased MCL1 expression could not be detected by Western blotting in HN5 cell lysates 48 hours after triple therapy, and only faint bands representing PARP and caspase-3 could be detected (Fig. 5B).
To assess whether HN5 cells were recovering at later time-points following triple therapy, as very few appeared to be undergoing apoptosis at 48 hours, long-term growth assays were carried out. Neither HN4 nor HN5 cells appeared to recover up to 2 weeks following treatment (Fig. 5C) and many of the cells that remained appeared large and flat, or small and apoptotic (Fig. 5D). Assays for senescence and autophagy were carried out with negative results (data not shown). The expression of apoptotic markers and cell-cycle proteins was assessed at later time points (96 and 120 hours) following triple therapy. Western blotting revealed that HN5 cells appeared to undergo delayed apoptosis because PARP and caspase-3...
Figure 5.
HN5 cells exhibit delayed apoptosis and loss of clonogenicity after triple therapy. A, quantification of cells in the sub-G₁ population following indicated treatments according to schedule 2. Cells were fixed at 24 and 48 hours, stained with PI and analyzed by flow cytometry. B, Western blot analysis of cell lysates 48 hours after indicated treatments according to schedule 2. Induction of apoptosis was analyzed by blotting for MCL1, PARP, and caspase-3. Expression of β-actin provided a loading control. C, long-term growth assays of treated cells. Drugs were washed off after 48 hours and cells counted at indicated time points. D, representative images of cells remaining at the end of the long-term growth assays described in A. Scale bar, 100 μm. E, Western blot analysis of HN4 and HN5 cell lysates 96 and 120 hours after indicated treatments. Apoptosis was analyzed by blotting for MCL1, PARP, and caspase-3. Cell-cycle progression was analyzed by blotting for Cyclin A2 and Cyclin B1. Expression of β-actin provided a loading control. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.
cleavage could be easily detected by 120 hours, despite constant expression of MCL1 (Fig. 5E). HN4 cells continued to display reduced expression of MCL1 following triple therapy, as well as sustained PARP and caspase-3 cleavage. Interestingly, CHK1 appeared to be reactivated 96 and 120 hours after triple therapy, more so in HN5 cells (demonstrated by p-CHK1 expression, as CCT244747 was washed off at 48 hours). Consequently, the expression of Cyclin A2 was reduced, most prominently at 120 hours, whereas Cyclin B1 expression remained constant (Fig. 5E), suggesting cells arrested in S-phase. These results imply that reactivation of CHK1 occurs in triple therapy–treated cells after drug wash-off, due to the presence of persistent DNA damage. When damage is too great, cells undergo apoptosis at this later stage, whereas surviving cells appear to express reduced levels of Cyclin A2 and lose their clonogenicity.

Triple therapy reduces the expression of markers of radiation resistance

To further study changes in protein expression in HN4 and HN5 cells following triple therapy, we used an apoptosis array. Cells were treated with triple therapy according to schedule 2 and lysates collected after 48 hours were used to probe an apoptosis array. The fold change in expression was calculated by comparing intensity of blots probed with lysate from treated cells with blots probed with lysate from vehicle-treated cells and converted to Log2 values (Supplementary Fig. S4A). The expression of a number of proteins was decreased by at least half (−1 on Log2 scale) in at least one cell line (Fig. 6A). The expression of cleaved caspase-3 was increased 3.1-fold and 1.6-fold in HN4 and HN5 cells, respectively, correlating with our previous Western blots (Fig. 5B). The expression of Claspin, a CHK1 adaptor protein, which undergoes proteasomal degradation during apoptosis, was decreased 9.0- and 4.0-fold in HN4 and HN5 cells, respectively, further confirming treatment efficacy. In addition to these findings, the expression of a number of proteins previously implicated in radiation resistance was reduced following triple therapy. Expression of the inhibitor of apoptosis proteins Survivin and XIAP was reduced 4.0- and 2.1-fold, respectively, in HN4 cells and 2.9- and 3.1-fold, respectively, in HN5 cells. We confirmed by Western blotting that downregulation of Survivin and XIAP persisted at least 120 hours after triple therapy (Fig. 6B).

**CHK1 expression and activity as markers of radiation resistance**

During this study, we noted that, despite HN5 cells being more resistant to radiation than HN4 cells, they appeared to be more sensitive to triple therapy. To investigate the DDR pathways activated in HN4 and HN5 cells in response to radiation, cells were treated with 10 Gy and collected at specific time points for analysis. DDR pathway activation was analyzed in prepared lysates by Western blotting for phospho-S1981 ATM (p-ATM), p-CHK1, and phospho-T68 CHK2 (p-CHK2). p-HH3 expression confirmed that radiation-mediated G2 arrest occurred within 2 hours and was maintained at least until 6 hours (Fig. 6C). HN4 cells exhibited strong activation of CHK2 (p-CHK2 expression), whereas HN5 cells had strong CHK1 activation (p-CHK1 expression; Fig. 6C). To determine whether CHK1 expression levels and activity could be a marker of resistance to radiotherapy, we analyzed a panel of HPV+ and HPV− head and neck tumors by IHC. Interestingly, high expression of total nuclear CHK1 protein and increased nuclear p-CHK1 expression significantly correlated with HPV+ tumors that recurred following radiotherapy (P = 0.005, Fig. 6D and E and Supplementary Fig. S5). We noted that high cytoplasmic expression of CHK1 and p-CHK1 also significantly correlated with recurring HPV+ tumors (P = 0.005 and 0.016, respectively; Supplementary Figs. S4B and S5). We also analyzed the expression of p-ATM, Ki67, and Survivin in these tumors. High expression of p-ATM was associated with recurring HPV+ tumors and low expression of Survivin was associated with nonrecurring HPV+ tumors; however, neither association was significant (P = 0.078 and 0.053, respectively; Supplementary Figs. S4C and S5). No correlation was observed between Ki67 expression levels and tumor recurrence.

Finally, we confirmed these findings in the SCC090 HNSCC cell line, which was isolated from a recurring HPV+ tumor. These cells were highly resistant to radiation in clonogenic assays (Supplementary Fig. S6A) and exhibited strong activation of CHK1 after exposure to 10 Gy radiation (Supplementary Fig. S6B). In accordance with these findings, SCC090 cells were very sensitive to CCT244747 (IC50 = 0.2 μmol/L; Supplementary Fig. S6C) and, consequently, triple therapy was highly effective in clonogenic and long-term MTT assays (Supplementary Fig. S6D and S6E).

**Discussion**

Detailed *in vitro* studies have been conducted to assess the efficacy of a range of CHK1 inhibitors in radiosensitizing cancer cells that rely on the G2-M checkpoint for DNA damage repair (7). However, CHK1 inhibitors have not yet been combined with radiotherapy in clinical trials, possibly due to inadequate selectivity and/or unexpected toxicities observed with earlier compounds (25, 26). The CHK1 inhibitor CCT244747 is a highly selective, potent, orally active ATP-competitive CHK1 inhibitor previously shown to enhance the activity of several genotoxic agents, including ionizing radiation (13). The activity of CCT244747 has not previously been investigated in HNSCC cell lines. Here we confirmed that CCT244747 could overcome radiation-mediated G2 arrest in the p53-deficient HN4 and HN5 cells, causing cells to enter mitosis with unrepaired DNA damage. This led to significant radiosensitization of both HN4 and HN5 cells in *vivo*.

We hypothesized that CCT244747 pretreated p53-deficient cells entering mitosis harboring radiation-mediated DNA damage could be further damaged with an antimitotic, such as paclitaxel. Indeed, greater cell kill was observed for triple therapy using this schedule (schedule 2). However, exploiting the ability of paclitaxel to arrest cells in mitosis, the most radiosensitive phase of the cell cycle, before exposing them to ionizing radiation proved to be more effective (schedule 2). These findings reinforce the critical importance of optimizing scheduling when combining different types of anticancer agents. Importantly, including CCT244747 in our triple therapy enabled a much lower dose of paclitaxel to be used, therefore potentially limiting toxicity. We hypothesize that tumors not harboring p53 mutations would also be sensitized to triple therapy as many still rely on the G2 checkpoint for DNA damage repair (7). However, normal tissue would be relatively protected due to harboring an intact G1 checkpoint, thus we would see an improvement in the therapeutic index when CCT244747 is present as part of a combination regimen. Finally, administration of the triple therapy according to schedule 2...
significantly reduced the growth rate of HN5 xenografts compared with all double combination therapies.

Cells receiving triple therapy exhibited many characteristics of mitotic catastrophe. The most common feature of cells exposed to triple therapy was the presence of nuclear abnormalities; cells often harbored micronuclei and/or were multinucleated. HN4 cells displayed more micronuclei, which could signal for them to be eliminated by apoptosis (27). Indeed, markers of apoptosis (namely an increased sub-G1 population, as well as caspase 3 and PARP cleavage) were increased in HN4 cells 48 hours after triple therapy. HN5 cells, however, were more likely to be multinucleated following triple therapy. Cell-cycle arrest was thought to be the primary response to multinucleation in HN5 cells, as the S-phase population more than doubled after triple therapy. S-phase arrest has also previously been associated with CHK1 activation and decreased expression of Cyclin A2 (28). We hypothesized that reactivation of CHK1 (after drug wash-off) due to persisting DNA damage could lead to cell-cycle arrest at later time points. If cells are unable to repair DNA damage at this later stage, they may undergo delayed apoptosis. Indeed, markers of apoptosis could

Figure 6.

Triple therapy downregulates markers of radiation resistance and high CHK1 expression and activity correlate with recurring HPV⁺ head and neck tumors. A, analysis of protein expression in HN4 and HN5 cells 48 hours after triple therapy according to schedule 2 using an apoptosis array. Proteins with greatest fold change (compared with untreated controls) shown. B, Western blot analysis of cell lysates 120 hours after indicated treatments according to schedule 2. Findings from the apoptosis array described in A were confirmed by blotting for Survivin and XIAP. Expression of β-actin provided a loading control. C, Western blot analysis of HN4 and HN5 cells following exposure to 10 Gy of radiation (RT). Checkpoint activity was determined by blotting for p-ATM, p-CHK1, and p-CHK2. The mitotic population was also analyzed by blotting for p-HH3. Expression of β-actin provided a loading control. D, quantification of CHK1 nuclear staining by IHC in HPV⁺ and HPV⁻ HNSCC samples obtained from patients in the PredictR-HNC trial. E, quantification of p-CHK1 nuclear staining in patient samples as described in D. **P < 0.01.
be detected 96 and 120 hours after triple therapy. Surviving cells appeared to have lost their clonogenicity and, as such, expressed decreased levels of the S-phase regulator Cyclin A2.

Finally, we found that triple therapy reduced the expression of several markers of radiation resistance, including Survivin and XIAP, in both HN4 and HN5 cells. High expression of these proteins has previously been linked to radiation resistance and worse prognosis (29–32). During this study, it was noted that despite HN5 cells being highly radioresistant they were more sensitive to CCT244747 monotherapy, as well as CCT244747 combined therapies, than HN4 cells. Consequently, HN5 cells exhibited greater activation of CHK1 following irradiation compared with HN4 cells. This correlates with findings from two recent studies carried out in non–small cell lung cancer where CHK1 levels and activity indicated sensitivity to CHK1-targeted therapy (33, 34). Likewise, we hypothesized that high CHK1 activity could be a marker of radiation resistance and may serve as a biomarker for tumors likely to relapse and, thus, identify the patients who would benefit the most from triple therapy. Analysis of a small panel of HPV+ and HPV− head and neck tumors revealed there was a significant correlation between high total CHK1 expression in the nucleus or cytoplasm with recurrence after radiotherapy in HPV+ tumors. High p-CHK1 expression in the nucleus or cytoplasm also significantly correlated with recurring HPV+ tumors. Although this is a small sample set, high CHK1 expression and/or activity has previously been correlated with therapy-resistant cancer cells (35, 36) and associated with high-grade, high-risk tumors often resistant to therapy and with a poor prognosis (36–40). Confirmation of our findings in a larger head and neck cancer dataset will be required.

We propose that cells with high CHK1 proficiency, which resist the first round of treatment with chemotheraphy or radiotherapy, will become dominant in a tumor and drive recurrence and relapse. Not only is it possible that CHK1 expression and activity will help identify those tumors associated with a worse prognosis, but also CHK1 is likely to continue being an important target for cancer treatment. The triple therapy we propose here; administration of a CHK1 inhibitor 1 hour prior to paclitaxel treatment and followed 6 hours later with radiotherapy, holds great promise for treating all tumors reliant on a G2 checkpoint for DNA damage repair but also for overcoming therapy resistance and hopefully preventing recurrence. The schedule that we have defined is highly compatible with current clinical practice in HNSCC. Biomarker-driven clinical studies in patients with locally advanced HNSCC represent a rapid and rational route to translating this strategy and offer a means of mitigating the severe toxicity associated with existing CCRT.

**Disclosure of Potential Conflicts of Interest**

Intelectual property from the research collaboration with Sareum Ltd. on Chk1 inhibitors was licensed from The Institute of Cancer Research to Sareum Ltd. The Institute of Cancer Research has benefited from this and requires its employees to declare this potential conflict of interest. This applies to H.E. Barker, R. Patel, M. McLaughlin, S. Zaidi, C. Nutting, K. Newbold, S. Bhude and K.J. Harrington. In addition, K. Newbold has received honoraria for speakers bureau and advisory boards for Eisai and AstraZeneca. U. Schick declared no potential conflicts of interest.

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**Acknowledgments**

We would like to thank Ian Collins, Michelle Garrett and Thomas Matthews for providing the CCT244747 compound; and Frances Daley, Anne Lowe, Margaret Smith and Jemma Shead in the Breakthrough Histopathology Facility at the Royal Marsden Hospital for carrying out the staining of the patient samples from the PredictR-HNC study.

**Grant Support**

This work was supported by grants from the Cancer Research UK Programme Grants C46/A10588 and C7224/A13407 (all authors), the NIHR Royal Marsden/Institute of Cancer Research Biomedical Research Centre (all authors), the Oracle Cancer Trust (to H.E. Barker) and Rosetrees Trust (to K.J. Harrington).

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Received December 23, 2015; revised June 6, 2016; accepted June 10, 2016; published OnlineFirst July 15, 2016.

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