DNAPK Inhibition Preferentially Compromises the Repair of Radiation-induced DNA Double-strand Breaks in Chronically Hypoxic Tumor Cells in Xenograft Models

Yanyan Jiang¹, Elaine Willmore², Stephen R. Wedge², and Anderson J. Ryan¹

ABSTRACT

Radiation-induced DNA double-strand breaks (DSBs) can be repaired by homologous recombination (HR) and nonhomologous end joining (NHEJ). Recently, it has been found that chronic tumor hypoxia compromises HR repair of DNA DSBs but activates the NHEJ protein DNAPK. We therefore hypothesized that inhibition of DNAPK can preferentially potentiate the sensitivity of chronically hypoxic cancer cells to radiation through contextual synthetic lethality in vivo. In this study, we investigated the impact of DNAPK inhibition by a novel selective DNAPK inhibitor, NU5455, on the repair of radiation-induced DNA DSBs in chronically hypoxic and nonhypoxic cells across a range of xenograft models. We found that NU5455 inhibited DSB repair following radiation in both chronically hypoxic and nonhypoxic tumor cells. Most importantly, the inhibitory effect was more pronounced in chronically hypoxic tumor cells than in nonhypoxic tumor cells. This is the first in vivo study to indicate that DNAPK inhibition may preferentially sensitize chronically hypoxic tumor cells to radiotherapy, suggesting a broader therapeutic window for transient DNAPK inhibition combined with radiotherapy.

Introduction

Radiotherapy is a common treatment for solid tumors by inducing cell DNA damage, in which DNA double-strand breaks (DSBs) are the most lethal lesions (1). These DSBs can be repaired by homologous recombination (HR) or nonhomologous end joining (NHEJ). In mammalian cells, NHEJ is the predominant pathway for repairing radiation-induced DSBs (2, 3). In response to radiation, Ku70 and Ku80 bind to the DNA DSB sites, and the Ku70/Ku80 heterodimer then recruits the catalytic subunit DNAPK to form a DNAPK holoenzyme. The holoenzyme leads to DNAPK autophosphorylation and phosphorylation of downstream DNA repair proteins, and ultimately DSB ligation (4). Given the essential role of DNAPK in NHEJ, targeting DNAPK has been proposed as an appealing approach to augment the antitumor effect of radiotherapy. Pharmacologic inhibition of DNAPK has been shown to sensitize human cancer cells and xenografts to radiation (5–7).

Although the radiosensitizing effect of DNAPK inhibition under normoxia has been well studied (8, 9), few studies have addressed the effects of DNAPK inhibition on the radiosensitivity of hypoxic tumor cells. Recently, it has been reported that DNAPK inhibition preferentially sensitizes tumor cells to radiation under hypoxia in vitro (10, 11). However, to date, whether DNAPK inhibition sensitizes hypoxic tumor cells to radiation in vivo has not been reported.

Hypoxia is a characteristic feature of solid tumors, and it can be described as acute and chronic hypoxia. Acute hypoxia results from temporary change in blood perfusion, and chronic hypoxia results from imbalanced oxygen supply and demand (12, 13). Tumor hypoxia has long been recognized as a barrier to radiotherapy. It prevents fixation of radiation-induced DNA damage in tumor cells, causing radioresistance. However, it has recently been found that chronic hypoxia downregulates the HR protein Rad51 expression in tumor cells, resulting in less HR pathway repair (14–17). It has further been shown that chronic hypoxia can activate the NHEJ protein DNAPK in the absence of DNA damage (18, 19). Therefore, we hypothesized that DNAPK inhibition might preferentially potentiate the sensitivity of chronically hypoxic cancer cells to radiation through contextual synthetic lethality.

Our recent study has shown that transient DNAPK inhibition by a novel highly selective DNAPK inhibitor, NU5455, can favorably compromise the repair of radiation-induced DNA damage in tumor tissue versus normal tissues (7). However, the underlying mechanism for this preferential radiosensitization of tumors has not been investigated. We propose that tumor hypoxia might partially contribute to it.

In this study, we used NU5455 to evaluate the impact of DNAPK inhibition on the repair of radiation-induced DNA DSBs in both hypoxic and nonhypoxic tumor cells in various xenograft models. We show that transient DNAPK inhibition favorably compromises the repair of radiation-induced DNA DSBs in chronically hypoxic tumor cells in vivo.
Materials and Methods

Cell culture

Human non–small cell lung cancer (NSCLC) cell lines Calu-6 and A549 were purchased from the ATCC. HAP-1 DNA PK wild-type (WT) and knockout cell lines were purchased from Horizon Discovery. All cell lines were Mycoplasma negative as tested by PCR. Cells were cultured in advanced DMEM/F12 medium (GIBCO/Life Technologies), supplemented with 5% FBS, 2 mmol/L glutamine, and 50 μg/mL penicillin/streptomycin in a humidified atmosphere with 7.5% CO2.

Subcutaneous xenografts

All animal experiments were performed in accordance with the UK Home Office regulations and with the approval of the Oxford University Animal Welfare and Ethical Review Board. Subcutaneous xenografts were generated as described previously (16). In brief, 6- to 8-week-old female BALB/c nude mice (Harlan) were anesthetized with 2% isoflurane, and 5 × 10⁶ Calu-6, A549, HAP-1 DNA PK WT or knockout cells in 50% Matrigel (BD Biosciences) were subcutaneously injected into a single site on the back of the mouse respectively. Mouse weights and tumor volumes were measured three times a week (volume = 1/2 × length × width × depth).

Tumor irradiation and NU5455 treatment schedules

NU5455 was synthesized by the Medicinal Chemistry Department, Newcastle University (Newcastle upon Tyne, United Kingdom). Full synthesis details are provided in patent WO 2010/136778 (example 102; compound 143). The structure of NU5455 was reported previously (7). Tumor irradiation and NU5455 administration were conducted as described previously (7). In brief, when subcutaneous xenografts reached 200 mm³, mice carrying Calu-6 or A549 xenografts were divided into two groups (n = 4/group) to receive (i) vehicle (NMP: 30% Encapsin: PEG400 (1:6:3 v/v/v)) orally 30 minutes before 10-Gy tumor radiation; (ii) NU5455 (30 mg/kg, provided by the Medicinal Chemistry Department, Newcastle University, Newcastle upon Tyne, United Kingdom) orally 30 minutes before 10-Gy tumor radiation. Mice bearing HAP-1 DNA PK WT or knockout xenografts were divided into four groups (n = 4/group): (i) gavage fed with vehicle 30 minutes before mock radiation; (ii) gavage fed with NU5455 (30 mg/kg) 30 minutes before mock radiation; (iii) gavage fed with vehicle 30 minutes before 10-Gy tumor radiation; (iii) gavage fed with NU5455 (30 mg/kg) 30 minutes before 10-Gy tumor radiation.

Tumors were exposed to X-ray radiation delivered with a Gulmay medical RS320 irradiation system (Gulmay Medical Ltd) at a dose rate of 1.82 Gy/minute. The rest of the body was shielded by lead. To identify acute and chronic hypoxia, a hypoxia marker pimonidazole HCl (60 mg/kg, Hypoxyprobe) was intraperitoneally injected into mice immediately after radiation, and a second hypoxia marker EF5 (30 mg/kg, Hypoxy-Imaging) was injected at 24 hours postradiation as previously described (20). All mice were euthanized at 24 hours after radiation, and tumors were collected for H&E and immunofluorescence staining.

Hematoxylin & eosin (H&E) and immunofluorescence staining

Tumors were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Tumor sections (4 μm) were deparaffinized and rehydrated, and then stained with Harris’ Hematoxylin (VWR International Ltd) and Eosin (Merck Life Science) for necrosis assessment. The whole sections were scanned using the Aperio CS scanner (Aperio Technologies) and the necrotic fraction in each tumor section was determined using the ImageScope analysis software (Aperio Technologies). Necrosis was defined as areas that stained red with eosin but lacked blue hematoxylin stain.

For immunofluorescence staining, deparaffinized and rehydrated tumor sections (3 μm) were subjected to antigen retrieval by pressure heating in citrate buffer. The sections were blocked with 5% goat serum/2% BSA/0.5% Triton X-100 in PBS for 1 hour, and M.O.M (1:25 in PBS, Vector Laboratories) for 30 minutes. Sections were then incubated with mouse pimonidazole antibody (1:50, Hypoxyprobe catalog No. HPI–100) mixed with rabbit γH2AX antibody (1:160, Abcam catalog No. ab2893) or 53BP1 antibody (1:500, Bethyl Laboratories, catalog No. A300–272A) at 4°C overnight, followed by the mixture of goat-anti rabbit Alexa Fluor 488 (1:500, Life Technologies, catalog No. A11030) in dark for 1 hour at room temperature. Sections were then incubated with anti-EF5-Cy5 antibody (75 μg/mL, Hypoxia-Imaging) at 4°C overnight. Finally, sections were stained with Hoechst 33342 (1 μg/mL, Sigma-Aldrich) for 5 minutes before mounting. Images were acquired using a Leica DM IRBE microscope with a Hamamatsu C4742–95 camera.

The number of γH2AX or 53BP1 foci per nucleus was quantified from 100 nuclei across five randomly chosen fields of view per section using ImageJ software. Colocalization analysis was conducted using the ImageJ Coloc 2 plugin after deconvolution and background correction. Manders’ correlation coefficients M1 (pimonidazole: EF5) and M2 (EF5: pimonidazole) were used to determine the degree of overlap between pimonidazole and EF5 staining. Coefficient values ranging from 0.5 to 1.0 indicate a positive association (21).

Statistical analysis

Data were expressed as mean ± SEM, and analyzed by GraphPad Prism 8 software (GraphPad Software Inc). Statistical analysis was performed using Student t test (two groups comparison) or one-way ANOVA (more than two groups comparison) followed by Bonferroni correction. Statistical significance was defined as P < 0.05.

Results

DNA PK inhibition does not induce acute hypoxia or affect chronic hypoxia following radiation

Our previous study has shown that Calu-6 and A549 xenografts are highly hypoxic (16). To assess whether DNA PK inhibition affects tumor hypoxia following radiation, mice carrying Calu-6 or A549 subcutaneous xenografts were treated with vehicle or NU5455 prior to tumor radiation (10 Gy), and exogenous hypoxia markers pimonidazole and EF5 were sequentially administered (Fig. 1A). At 24 hours after radiation, tumors were collected for H&E and immunofluorescence staining. Acutely and chronically hypoxic cells were identified by spatial localization of the two hypoxia markers in tumors (pimonidazole /EF5: chronically hypoxic, pimonidazole /EF5: acutely hypoxic). Following radiation treatment with or without NU5455, Calu-6 and A549 tumors exhibited extensive plasma membrane staining for pimonidazole and EF5, mainly around necrosis areas (Fig. 1B and C). A high degree of colocalization between pimonidazole and EF5 staining was demonstrated in Calu-6 tumors (Fig. 1D) and A549 tumors (Fig. 1E) by Manders coefficient values (ranging from 0.85 to 0.91). In addition, there was no significant difference in M1 or M2 coefficients between radiation with NU5455 treatment and radiation without NU5455 treatment in Calu-6 tumors (M1: P = 0.76; M2: P = 0.74) and A549 tumors (M1: P = 0.48; M2: P = 0.86), suggesting that NU5455 does not induce acute hypoxia following radiation.
Tumor necrosis can be used as a morphologic marker of chronic hypoxia (22). To investigate whether NU5455 has an effect on chronic hypoxia after radiation, tumor necrosis was assessed using H&E staining. Necrotic areas were evident in both Calu-6 (Fig. 1F) and A549 tumors (Fig. 1G), and a good spatial correlation between H&E staining and pimonidazole/EF5 immunofluorescence staining was observed. There was no significant difference in necrotic fractions between radiation with NU5455 treatment and radiation without NU5455 treatment in Calu-6 tumors (P = 0.65; Fig. 1H) and A549 tumors (P = 0.81; Fig. 1I), suggesting that NU5455 does not affect chronic hypoxia following radiation.

DNAPK inhibition preferentially compromises the repair of radiation-induced DNA DSBs in chronically hypoxic cells in NSCLC xenografts

We first investigated the effect of DNAPK inhibition on the repair of radiation-induced DNA DSBs in chronically hypoxic and nonhypoxic tumor cells in NSCLC xenografts. Mice bearing Calu-6 or A549 xenografts were treated with 10-Gy of tumor radiation with or without NU5455, and the repair of radiation-induced DNA DSBs were assessed by quantification of γH2AX foci numbers in chronically hypoxic and nonhypoxic cells 24 hours after radiation (Fig. 2A). Because pimonidazole and EF5 staining were overlapped, we used pimonidazole and EF5 co-staining to analyze the repair of DNA DSBs in hypoxic and nonhypoxic cells.
cells to represent chronically hypoxic cells and pimonidazole− cells to represent nonhypoxic cells in this study to facilitate foci counting.

Resolution of the γH2AX foci was evident in Calu-6 tumors (Fig. 2B and C) and A549 tumors (Fig. 3B and C) 24 hours after radiation. As expected, radiation alone induced a greater number of unrepaired γH2AX foci in the nonhypoxic tumor cells compared with that in the chronically hypoxic cells (in Calu-6 tumors: 2.1-fold, Fig. 2D; in A549 tumors: 2.4-fold, Fig. 3D), suggesting that NU5455 inhibits the repair of radiation-induced DNA DSBs in both hypoxic and nonhypoxic cells. Moreover, the residual γH2AX foci ratio (NU5455 + 10 Gy vs. 10 Gy) in chronically hypoxic cells was significantly higher compared with that in nonhypoxic cells (P < 0.0001 in Calu-6 tumors, Fig. 2E; P < 0.001 in A549 tumors, Fig. 3E), suggesting a favorable inhibitory effect of NU5455 on the repair of radiation-induced DNA DSBs in chronically hypoxic cells.

DNAPK inhibition or deletion preferentially compromises the repair of radiation-induced DNA DSBs in chronically hypoxic cells of HAP-1 DNAPK isogenic xenografts

To confirm the inhibitory effect of DNAPK inhibition on the repair of radiation-induced DNA DSBs in both chronically hypoxic and nonhypoxic tumor cells, mice bearing HAP-1 DNAPK WT or isogenic knockout xenografts were treated with vehicle, NU5455, 10-Gy tumor irradiation with or without NU5455 (Figs. 4A and 5A). Residual DNA
DSBs in hypoxic and nonhypoxic tumor cells at 24 hours after radiation were assessed by γH2AX and 53BP1 foci quantification. NU5455 alone did not show an effect on the γH2AX or 53BP1 foci number in hypoxic and nonhypoxic tumor cells compared with the vehicle control in both DNAPK WT tumors (P > 0.99, Fig. 4B and C) and knockout tumors (P > 0.99, Fig. 5B and C; Supplementary Fig. S2A and S2B), indicating that NU5455 alone does not have a significant effect on DSB repair in HAP-1 xenografts. As expected, 24 hours postradiation, the number of unrepaired γH2AX or 53BP1 foci in the nonhypoxic cells was higher than that in the hypoxic cells in DNAPK WT tumors (γH2AX foci: 2.6-fold, Fig. 4B and C; 53BP1 foci: 2.1-fold; Supplementary Fig. S1A and S1B). Compared with radiation alone, the concurrent administration of NU5455 caused a significant increase in the number of unrepaired γH2AX foci (Supplementary Fig. S4B and S4C) or 53BP1 foci (Supplementary Fig. S1A and S1B) in both hypoxic cells (γH2AX or 53BP1 foci: P < 0.0001) and nonhypoxic cells (γH2AX foci: P < 0.05, 53BP1 foci: P < 0.01) in HAP-1 DNAPK WT tumors. Moreover, the residual γH2AX or 53BP1 foci ratio (NU5455 + 10 Gy vs. 10 Gy) in chronically hypoxic tumor cells was significantly greater than that in nonhypoxic tumor cells (γH2AX foci ratio: P < 0.001, Fig. 4D; 53BP1 foci ratio: P < 0.01, Supplementary Fig. S1C). This finding confirmed the observation in NSCLC xenografts that the inhibitory effect of NU5455 on radiation-induced DSB repair was greater in chronically hypoxic tumor cells than in nonhypoxic tumor cells.
In contrast, in DNAPK knockout xenografts, there was no significant difference in the number of γH2AX or 53BP1 foci per nucleus between the radiation group and the combination treatment group in both chronically hypoxic and nonhypoxic tumor cells (Fig. 5C; Supplementary Fig. 52B), and the γH2AX or 53BP1 foci ratio (NU5455 + 10 Gy vs. 10 Gy) in hypoxic and nonhypoxic cells was close to 1 (Fig. 5D; Supplementary Fig. 52C), suggesting that the effect of NU5455 is predominantly attributed to the inhibition of DNAPK activity. The γH2AX or 53BP1 foci ratio (DNAPK knockout vs. DNAPK WT) in chronically hypoxic cells was significantly higher than that in nonhypoxic cells 24 hours after radiation (γH2AX foci ratio: $P < 0.01$, Fig. 5D; 53BP1 foci ratio: $P < 0.05$, Supplementary Fig. 52C), suggesting that DNAPK deletion also favorably compromises the repair of radiation-induced DNA DSBs in chronically hypoxic cells.

**Discussion**

Although the radiosensitizing effect of DNAPK inhibition on oxygenated tumor cells is well established, the effect on hypoxic tumor cells is largely neglected. Currently, it remains unclear whether transient pharmacologic inhibition of DNAPK can overcome hypoxia-induced tumor resistance in vivo. Here, we show that the DNAPK inhibitor, NU5455 significantly inhibited DNA DSBs repair following irradiation in chronically hypoxic tumor cells.
radiation in both chronically hypoxic and nonhypoxic tumor cells across a range of xenograft models. Most importantly, this inhibitory effect was more pronounced in hypoxic cells than in nonhypoxic cells, suggesting that DNAPK inhibition may preferentially potentiate the sensitivity of chronically hypoxic cancer cells to radiation.

That DNAPK inhibition preferentially sensitizes hypoxic cancer cells to radiation in vitro has been reported by two groups. Klein and colleagues have found that the radiosensitizing effect of DNAPK inhibitor M3814 on A549 cells under hypoxia were significantly greater than under normoxia (10). He and colleagues have also observed that inhibition of DNAPK activity by an adenovirus-mediated DNKu70 gene transfer potentiates the radiosensitivity of U-87 MG cells and HCT-8 cells more greatly in hypoxia than in normoxia (11). Because survival of irradiated cells largely depends on the repair of radiation-induced DNA DSBs (23, 24), and radiosensitivity correlates with persistence of γH2AX (24, 25) and 53BP1 (26), we used residual γH2AX and 53BP1 foci as surrogates of cell radiosensitivity in this study. Our in vivo results here have further confirmed those reported in vitro findings.

Our previous study demonstrated that Calu-6 and A549 tumors contain extensive hypoxic regions that are associated with downregulation of the HR protein RAD51 expression (16), making the repair of radiation-induced DNA DSBs in hypoxic tumor cells rely more on the NHEJ pathway. Thus, it is plausible to observe that...
DNAPK inhibition or deletion compromises the repair of radiation-induced DNA DSBs in chronically hypoxic tumor cells to a greater extent than in nonhypoxic cells. NU5455 is a highly DNAPK selective inhibitor. We previously showed that NU5455 did not affect the response of HAP-1 DNAPK knockout cells to radiation, but markedly increased the radiosensitivity in HAP-1 DNAPK WT cells (7), suggesting that the radiosensitizing effect of NU5455 can be exclusively attributed to DNAPK inhibition. In this study, NU5455 significantly inhibited the repair of radiation-induced DNA DSBs in HAP-1 DNAPK WT tumor cells but not in the isogenic DNAPK knockout tumor cells, suggesting that NU5455 selectively inhibits DNAPK activity in vivo.

In the previous study, we also found that NU5455 increased the therapeutic index of radiotherapy in Calu-6 and A549 xenograft models by preferentially augmenting the cytotoxic effect of radiation on tumors without affecting DNA damage to surrounding normal tissues (7). Our findings here indicate that contextual synthetic lethality induced by tumor hypoxia and DNAPK inhibition could be the partial mechanism. It is of note that although NU5455 is a highly selective DNAPK inhibitor, it also has an inhibitory activity on the class III PI3 Kinase Vps34, an autophagy-related protein. The selectivity for DNAPK versus Vps34 is 8.7-fold. Because autophagy and apoptosis have been implicated in the effect of DNAPK inhibition and radiation (27, 28), it will be of interest to study further.

Currently, strategies for overcoming hypoxia-induced radioreistance include hypoxia-activated prodrugs (29), vascular remodeling to increase tumor oxygenation (30), and inhibition of cellular oxygen consumption (31). As hypoxia is a tumor-specific feature, our work here suggests that transient inhibition of DNAPK may be a promising strategy to selectively reverse radiosensitivity of hypoxic tumor cells and increase therapeutic gain.

At present, the use of DNAPK inhibitors in clinics is limited to trials in patients. Our data support further clinical evaluation of NU5455 as a selective radiosensitizer for treating tumors with high portion of hypoxia. This will be particularly useful in lung cancer treatment, as 25% of tumors from patients with NSCLC are highly hypoxic (32) and DNAPK expression is usually upregulated in NSCLC tumors (33). Our work also provides a strategy for the development of predictive biomarkers for the combination treatment.

In conclusion, our study suggests that DNAPK inhibition may preferentially enhance radiosensitivity in chronically hypoxic tumor cells in vivo, which could form the basis for clinical trials investigating the impact of DNAPK inhibitors in radioresistant hypoxic tumors.

**Authors’ Disclosures**

S.R. Wedge reports grants from Cancer Research UK during the conduct of the study, other support from AstraZeneca outside the submitted work, and is a member of the Cancer Research UK Drug Discovery Group, which originally discovered NU5455. No disclosures were reported by the other authors.

**Authors’ Contributions**

Y. Jiang: Conceptualization, formal analysis, investigation, methodology, writing—original draft. E. Willmore: Resources, validation, writing—review and editing. S.R. Wedge: Resources, validation, writing—review and editing. A.J. Ryan: Resources, supervision, funding acquisition, writing—review and editing.

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**References**


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