Human EGFR-2, EGFR and HDAC Triple-Inhibitor CUDC-101 Enhances Radiosensitivity of GBM Cells

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Radiotherapy remains the standard treatment for glioblastoma multiforme (GBM) following surgical resection. Given the aberrant expression of human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) which may play a role in therapeutic resistance to receptor tyrosine kinase inhibitors, and the emerging use of histone deacetylase (HDAC) inhibitors as radiosensitizers, we defined the effects of CUDC-101, a triple inhibitor of HER2, EGFR and HDAC on the radiosensitivity of GBM cells. Clonogenic survival was used to determine the in vitro radiosensitizing potential of CUDC-101 on GBM, breast cancer, and normal fibroblast cell lines. Inhibitory activity was defined using immunoblots and DNA double strand breaks were evaluated using γH2AX foci. Effects of CUDC-101 on cell cycle and radiation-induced cell kill were determined using flow cytometry and fluorescent microscopy. CUDC-101 inhibited HER2, EGFR and HDAC and enhanced in vitro radiosensitivity of both GBM and breast cancer cell lines, with no effect on normal fibroblasts. Retention of γH2AX foci was increased by CUDC-101 alone and in combination with irradiation for 24 h. Treatment with CUDC-101 increased the number of cells in G2 and M phase, with only increase in M phase statistically significant. An increase in mitotic catastrophe was seen in a time-dependent fashion with combination treatment. The results indicate the tumor specific CUDC-101 enhanced radiosensitization in GBM, and suggest that the effect involves inhibition of DNA repair.

INTRODUCTION

The development and application of molecularly targeted radiosensitizers is crucial for improving the efficacy of radiotherapy as a cancer treatment modality. Two main approaches exist regarding the incorporation of molecular inhibitors: incorporating multiple drugs known to target multiple distinct pathways, such as, in the treatment of hematological malignancies; or using a single agent to target a specific pathway, such as erlotinib, gefetinib or lapatinib which selectively inhibit receptor tyrosine kinases (RTKs). The latter has historically been the more popular approach in solid malignancies for the vast majority of newly investigated cancer treatment regimens, particularly when

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combined with radiotherapy. Single-target agents, despite their pharmacokinetic simplicity, lower cost and reduced risk of adverse effects, are often clinically limited due to the genetic heterogeneity and myriad dysregulated pathways that exist not only among different malignancies, but from cell to cell variations within the same histology (Lai et al., 2010).

Recently, single small molecular inhibitors have been designed to simultaneously target multiple critical cellular pathways to induce cell death. The drug, CUDC-101, was designed to target two members of the ERBB family, human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR), as well as function as a pan-histone deacetylase inhibitor (HDACi) (Lai et al., 2010). The overexpression of these two RTKs has been associated with tumorigenesis and aggressiveness in many cancers, including glioblastoma multiforme (GBM) (Cancer Genome Atlas Research, 2008; Kesavabhotla et al., 2012; Lal et al., 2002; Mittapalli et al., 2013; Pierga et al., 2013; Wang et al., 2013). Additionally, histone acetyltransferase inactivation has been associated with oncogenesis, yet it is the aberrant HDAC activity that is considered a potential target for cancer therapy. Depending on the experimental system, HDACi has been reported to induce tumor cell differentiation, apoptosis, and/or growth arrest, putatively via modulation of gene expression (Shabason et al., 2011).

CUDC-101 has been shown to be an effective agent as a monotherapy for treatment of various tumor cell lines in vitro including non-small cell lung cancer, pancreatic, breast, prostate, brain, and liver cancers (Lai et al., 2010), and is currently undergoing phase I and Ib clinical trials as a monotherapy or in combination with radiotherapy for multiple cancers. As an initial step in evaluating the potential of CUDC-101 as a clinically applicable radiosensitizer, we investigated the effects of CUDC-101 in a panel of cancer and normal cell lines. The data indicated that CUDC-101 selectively enhances tumor cell radiosensitivity in vitro. Moreover, the mechanism appears to involve inhibition of DNA double strand break (DSB) repair and modulation of the cell-cycle.

**MATERIALS AND METHODS**

**Cell lines and treatment**

The human GBM cell line U251, breast carcinoma cell line MDA-MB-231 and normal human lung fibroblast cell line MRC9 were obtained from American Type Culture Collection (ATCC). U251 and MDA-MB-231 cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen) and maintained at 37 °C, 5% CO₂. MRC9 cells were grown and maintained in minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS, non-essential...
amino acids (NEAA), and sodium pyruvate (Invitrogen). Lyophilized CUDC-101 (MW: 434.49) was purchased from Selleck chemicals and 3.43 mg of CUDC-101 was reconstituted in 1 mL of DMSO (solubility: 20 mg/mL) and stored at -20 ºC at a concentration of 10 mM. DMSO was not shown to have an effect and subsequent experiments were done with a media only control unless otherwise noted. Cultures were irradiated using the Pantak source at a dose of 2.27 Gy/min.

Clonogenic Assay
Cultures were dissociated with 0.25% trypsin EDTA (Invitrogen) to create a single cell suspension and a specified number of cells were seeded into each well of a six well tissue culture plate. After allowing cells time to adhere (24 h) cultures were treated with varying doses of radiation: 0, 2, 4, 6, and 8 Gy, followed by CUDC-101 (0.5 µM for U251 and 1.0 µM for MDA-MB-231). The drug was not removed for duration of the assay. Ten to fourteen days after seeding, colonies were stained with crystal violet, and the number of colonies (≥ 50 cells) were determined. Surviving fractions were calculated and survival curves generated by normalizing for the amount of CUDC-101-induced cell death. The data represents the mean ± standard error of mean (SEM) of minimum two independent experiments.

Cell cycle analysis
The evaluation of the cell cycle phase distribution was performed using the BD FACSCalibur. Treatment protocols were identical to the clonogenic treatment regimen and cells were seeded into 10 cm petri dishes. Samples were stained with propidium iodide (PI) and analyzed using flow cytometry. To determine the activation of the G2 cell cycle checkpoint, mitotic cells were distinguished from G2 cells as previously reported by Xu et al. (2002) utilizing the mouse monoclonal antibody (Cell Signaling) against phospho-H3 histone (S10) (6G3) followed by staining with a Alexa Fluor-488 F(ab’)2 fragment of goat anti-mouse conjugated secondary antibody (Invitrogen) (Xu and Kastan, 2004; Xu et al., 2002). In this assay the increase of M phase reflects the abrogation of the G2 checkpoint. The data represents the mean ± SEM of minimum of three independent experiments.

Apoptotic Cell Death
The BD Annexin V: FITC Apoptosis Detection Kit (Catalog Number: 556547) was performed as per the manufacturer’s instructions. In brief, cells were washed twice with cold PBS and resuspended in 1X binding buffer. Subsequently, 100 µL of cell suspension was incubated for 15 min. at room temperature (25 ºC) in the dark after adding 5 µL of FITC-Annexin V and PI. Samples were brought to a final volume of 500 µL and run on the FACSCalibur capturing 10,000 events.
**Immunofluorescent staining for γH2AX**
Immunofluorescent staining and counting of γH2AX nuclear foci was performed as previously described (Xu and Kastan, 2004; Xu et al., 2002). Cells were seeded on four well chamber slides and treated with 2 Gy irradiation followed by treatment with CUDC-101. Slides were examined on a Leica upright fluorescent microscope. Images were imported into ImageJ image software for analysis. For each treatment condition γH2AX foci were counted in 50 cells. The data represents the mean ± SEM of minimum three independent experiments.

**Mitotic Catastrophe**
The presence of fragmented nuclei was used to define cells undergoing mitotic catastrophe. Cells were seeded on cover slips and treated with 2 Gy irradiation followed by treatment with CUDC-101. To visualize nuclear fragmentation cells were fixed with a 10% neutral buffered formalin solution and incubated with α-tubulin followed by anti-mouse Alexa-555 and mounted with Prolong gold antifade reagent containing DAPI. Normal cells and cells undergoing mitotic catastrophe were manually counted as cells with nuclear fragmentation. For cells to be considered positive for mitotic catastrophe, cells with greater than 2 lobes were used as the limiting criteria. For each condition 100 cells were scored. The data represents the mean ± SEM of minimum three independent experiments.

**Immunoblotting and antibodies**
Cells were seeded onto 60-mm dishes, lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing phosphatase and protease inhibitors (Roche, Indianapolis, IN). Protein concentrations were quantified using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein was resolved on 4–20% Tris-Glycine gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories) and probed with the indicated antibodies. Primary antibodies were: actin, CD9 (Millipore, Germany), phospho-/total EGFR, and acetyl-/total histone H3 (Cell Signaling Technology, Danvers, MA) and phospho-/total HER2 (Upstate Cell Signaling Solutions, Lake Placid, NY). Blots with phosphorylated targets were visualized with Super-Signal West Femtoluminol substrate (Thermo Scientific, Rockfold IL) and ECL Prime luminol reagent (GE Health-care, Pittsburgh, PA) was used for total protein. Actin was used as a loading control.

**Statistical Analysis**
*In vitro* experiments were repeated minimum twice and Student's t-test was used for statistical analyses. Data are presented as mean ± SEM. A α value of $p < 0.05$ was considered significant. Analyses were done in GraphPad version 6 (Prism).
RESULTS

**CUDC-101 can effectively target intended ligands**

To assess the effects of CUDC-101 on the radiosensitivity of tumor cells, a cytotoxicity assay was performed initially to determine appropriate dosage with minimal toxic effects. For this study, cells were plated at clonogenic density, allowed to attach overnight, and treated with 4 Gy irradiation followed immediately by treatment with CUDC-101 using a dose range including the previously reported average IC₅₀ values of U251 and MDA-MB-231 cells. U251 and MDA-MB-231 cells were kept in drug containing media for duration of the assay (10–14 days). Cells were then stained with crystal violet and assessed for inhibition of colony formation. Dosing CUDC-101 at 0.5 μM and 1.0 μM immediately post-radiation for the duration of the entire assay was most effective in inhibiting colony formation while having minimal single agent toxic effect on U251 and MDA-MB-231 respectively (Data not shown). These concentrations were used to further investigate the radiosensitive effects of CUDC-101.

To confirm that CUDC-101 inhibited the molecule’s intended RTK targets, CUDC-101 was given alone or immediately following irradiation (4 Gy) to U251 cells and RTK inhibition was analyzed via immunoblot. Results confirmed that CUDC-101 targeted the intended ligands (Fig. 1). CUDC-101 treatment reduced the activation of phosphorylated HER2 (pHER2) dose dependently, irrespective to the addition of irradiation, while pEGFR levels were only modulated at 0.5 μM and no further decrease in ligands level with combination treatment was observed (Fig. 1A). Interestingly however, the levels of total EGFR decreased dose dependently in both single agent and combination treatment cohorts. Also, as expected of an HDACi, treatment with CUDC-101 increased levels of acetylated H3 in a dose dependent manner. The protein levels are quantitated and normalized against a loading control, actin (Fig. 1B).

**CUDC-101 treatment inhibits clonogenic survival tumor specifically**

The hallmark of radiosensitivity is reduced clonogenicity. In both the tumor cell lines, treatment with CUDC-101 following irradiation resulted in an increase in radiosensitivity as assessed by colony forming ability 10–14 days later. U251 cells treated with 0.5 μM CUDC-101 yielded a surviving fraction (SF) of 0.67 ± 0.12; while treatment of MDA-MB-231 cells with 1.0 μM gave a SF of 0.65 ± 0.07. These values indicate an approximate value for the degree of cytotoxicity for the evaluation of CUDC-101 in combination with radiation. As shown in Fig. 2A and B, a dose enhancement factor (DEF) of 1.42 in U251 and 1.37 in MDA-MB-231 was observed at a SF of 0.1.
Accordingly, the potential for tumor-specific actions of CUDC-101 on radiosensitivity was determined. The normal fibroblast cell line MRC9 was treated with both 0.5 μM and 1.0 μM using the same treatment schedule and colony formation was assessed as previously mentioned. CUDC-101 treatment reduced the MRC9 SF to 0.79 ± 0.15 and 0.87 ± 0.007 respectively; in contrast to the tumor cell lines, CUDC-101 had little effect on the radiosensitivity (1.13 at 0.5 μM; 1.25 at 1.0 μM) of MRC9 (Fig. 1C). Albeit some sensitivity was induced it was not statistically significant. Since the effect seen in GBM was significant compared to drug alone and the DEF was greater than that seen in the

![Image](https://example.com/image.png)

**Figure 1: CUDC-101 effectively targets the intended ligands.** The U251 was radiated (0 or 4 Gy) and incubated with 0.1 or 0.5 μM of CUDC-101 for 24 h. (A) Levels of phosphorylated HER2 and EGFR, and acetylated histone H3 were analyzed via immunoblot analysis. (B) Results show a decrease in phosphorylated HER2 and total EGFR in the presence or absence of radiation at 0.5 μM; however no effect was seen on pHER2 and pEGFR at 0.1 μM. Furthermore, acetyl-H3 is maintained and increased in the presence of CUDC-101 with/without radiation at both concentrations tested, supporting the effect seen by clonogenic survival.
breast cancer cell line, we focused on this histology. Further experiments to investigate the radiosensitization effects of CUDC-101 used U251 cells.

**CUDC-101 treatment increased retention of γH2AX foci**

The most lethal form of injury to cells is DNA damage. Thus drugs inhibiting or retarding DNA repair have the potential of effective radiosensitizers. Therefore, the rate of DNA repair via resolution of γH2AX foci was measured. The γ-variant of the H2A family was shown to be a biomarker of DNA double stranded breaks (DSBs) and delayed resolution of the foci indicate inhibition of DNA repair mechanisms (Kuo and Yang, 2008). CUDC-101 was added following irradiation (2 Gy) and at 1, 6 and 24 h and U251 nuclei were visualized via immunofluorescence for inhibition of γH2AX resolution. Representative images at 24 h are shown in Fig. 3A. At 1 h and 6 h a significant difference in the average number of foci per cell was not observed (Fig. 3B). However, there was a significant increase in foci between cells treated with drug only compared to no treatment at 24 h in U251 (6.33 ± 0.73; \( p = 0.003 \)) was observed. Furthermore, at 24 h there was a significant difference in the

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**Figure 2**: CUDC-101 radiosensitivity is tumor cell specific. Cells were radiated with increasing doses of radiation (closed circles) or post radiation incubated with various concentrations of CUDC-101 for 10 to 14 days (open circles). Colony-forming ability was assessed and survival curves generated after normalizing for the cytotoxicity of CUDC-101. (A) U251 cells were given 0.5 µM CUDC-101 and (B) MB231 cells were given 1.0 µM. (C) Normal fibroblast cell line MRC9 was irradiated with increasing doses of radiation (closed circles) or 0.5 µM CUDC-101 (open circles) or 1.0 µM CUDC-101 (closed triangles) following irradiation. Survival curves show that the radiosensitive activity of CUDC-101 is tumor cell specific, whereas MRC9 cells were not affected. Dose enhancement factors (DEFs) were assessed at surviving fractions (SFs) 0.1 and 0.01 where applicable. Data represents three-independent experiments (A–B) and two-independent experiments (C) with points representing mean, and error bars SEM.
average number of foci per cell in those cells treated with combination radiation and 0.5 μM CUDC-101 (14.40 ± 0.92; *p = 0.0003). As expected at 24 h almost complete resolution of DNA DSBs was seen in cells that were given irradiation only, as the average number of foci per cell returned to near baseline levels. The significant inhibition of γH2AX resolution at 24 h suggests that CUDC-101 inhibits repair of DNA DSBs.

**CUDC-101 post irradiation redistributes GBM cells within cell cycle**

Progress through the cell cycle depends on regulated set of checkpoints, which become activated in the event of DNA damage. It is known that radiation has an effect on inducing G2/M delay (Hall and Giaccia, 2006). Therefore, the distribution of tumor cells within the cell cycle was assessed by flow cytometry (Fig. 4A–B). As shown in Fig. 4C, at 24 h an increase of cells in G2 was observed, although the increase was not significant (25.8 ± 3.5% to 40.5 ± 7.0%; *p = 0.134). Additionally, at 24 h a significant increase in M phase from 2.30 ± 0.24% to 6.70 ± 1.1% was observed with CUDC-101 alone (*p = 0.02). The addition of radiation to CUDC-101, however, did not augment this increase in M phase. In fact, a slight decrease in M phase in cells treated with combination treatment (8.37 ± 1.1% to 6.86 ± 0.08%) was observed, however this difference was not statistically significant (*p = 0.247). Based on these results, it may be possible that treatment with CUDC-101 may push cells into M phase by having off-target effects in altering the activity of critical regulators of this checkpoint (e.g. ATM).
CUDC-101 increases mitotic catastrophe as mode of cell death

Lai et al. (2010) reported that treatment with CUDC-101 induced expression of proapoptotic and antiproliferative proteins in breast and colon cancer cells lines. To determine whether the increase in radiosensitivity resulting from CUDC-101 treatment was due to an enhancement of radiation-induced apoptosis, Annexin V staining 24 h after treatment was measured. As expected for a solid tumor cell line, radiation induced little apoptotic cell death; treatment with CUDC-101 yielded essentially identical levels of apoptosis, and the combination of irradiation (2 Gy) and CUDC-101 had no effect on the frequency of apoptotic cell death events, indicating that the CUDC-101-mediated radiosensitization of U251 glioma cells does not involve enhanced susceptibility to apoptosis (Data not shown).

The apparent inhibition of DSB repair, increase of cells in M phase, and no increase in radiation-induced apoptosis suggests that CUDC-101 induced radiosensitization involves an enhancement of mitotic catastrophe. Cells with nuclear fragmentation, and Chk2) regardless of the amount of DNA damage, and may partially account for CUDC-101-mediated enhancement in radiation-induced cell killing.

“Figure 4: CUDC-101 increases the percentage of cells in more sensitive phases of the cell cycle. U251 cells were seeded in 10-cm petri dishes, stained with PI and pH3 to differentiate mitotic cells and analyzed by flow cytometry. (A) Representative histogram of cell cycle distribution of U251 cells treated with drug or irradiation alone or combination treatment from an independent experiment. (B) Representative dot plots of cell cycle distribution of U251 cells from an independent experiment, gating and analysis was done using FloJo analysis software. (C) Treatment with 0.5 µM CUDC-101 in the absence of irradiation significantly increased the number of cells in M-phase; however this effect was not augmented by the addition of irradiation (2 Gy). Additionally an increase in the G2 phase was observed for both CUDC-101 alone and in combination with irradiation but was not statistically significant. Data represents three independent experiments. Columns represent the mean and error bars are the SEM. *p < 0.05; **p < 0.001.”
defined as the presence of 2 or more distinct nuclear lobes within a single cell, were classified as going through mitotic catastrophe. As shown in the representative fluorescent micrograph in Fig. 5A, cells undergoing mitotic catastrophe could be distinguished after treatment of irradiation (2 Gy), CUDC-101, and combination treatment. There was a time dependent increase in the number of cells undergoing mitotic catastrophe after the treatment with either radiation or CUDC-101 up to 72 h. In cells receiving the combination treatment, a significantly greater number of cells undergoing mitotic catastrophe was detected at 48 and 72 h, 61.6 ± 11.1% and 70.3 ± 6.2% respectively. Furthermore, this increase in mitotic catastrophe was greater than additive as compared to irradiation and CUDC-101 alone. These data suggest that the CUDC-101 mediated radiosensitization is achieved by an inhibition in DNA DSB repair resulting in an increase in cells undergoing mitotic catastrophe. Additionally, the data supports the observations seen in cell cycle analysis indicating that the increase in M phase seen with drug alone, maintained when combined with irradiation at 24 h, was indicative of an increase in mitotic catastrophe at later time-points.

**DISCUSSION**

GBM (WHO Grade IV) is the most common malignant central nervous system tumor with
an incidence of 0.4–2.8 per year per 100,000 persons, and typified by nuclear atypia, mitosis, endothelial proliferation and necrosis (Mineo et al., 2006). Despite aggressive therapies, prognoses for GBM remains poor, and average overall survival remains 12–16% (Stupp et al., 2009). A majority of these patients will at some point undergo radiotherapy typically combined with a radiosensitizing agent. Attempts to develop clinically relevant radiosensitzers have traditionally been limited to cytotoxic chemotherapeutic agents (Camphausen et al., 2005).

Recently, agents targeting HDAC have gained in popularity, as they are shown to enhance the radio response by relaxing the chromatin, leaving it more susceptible to DNA damage, among other mechanisms. Lai et al. (2010) showed the potent inhibition of multiple oncogenic pathways with CUDC-101 as a monotherapy, and are currently investigating the effects of this compound in a dose escalation study for various cancers as a single agent (NCT01171924), and in combination with radiation and cisplatin for locally advanced head and neck cancer (NCT01384799). We extended the investigation to assess the radiosensitizing potential of CUDC-101 in GBM.

In GBM, EGFR is genomically amplified in 40–50% of tumors, often followed by gene rearrangement resulting in a ligand-independent, constitutively phosphorylated and cell surface localized receptor tyrosine kinases (RTK) that enhances tumorigenicity (Heimberger et al., 2005; Lal et al., 2002; Lopez-Gines et al., 2010; Sugawa et al., 1990; Wikstrand et al., 1997). Furthermore, recent preclinical and clinical studies showed that EGFR may play a role in radioresistance through activation of downstream signaling cascades such as, PI3K/Akt/mTOR, and its involvement in regulating autophagy (Palumbo et al., 2014). Targeting HER2 is a well established target for breast cancer therapy and a negative prognostic factor for cancers of breast, lung and brain (Hiesiger et al., 1993; Tateishi et al., 1991). In regards to GBM, however, HER2 is not expressed in adult glial cells, but its expression has been shown to increase with the degree of astrocytoma degeneration (Mineo et al., 2006). Taken with the fact that the major action of HER2 is the heterodimerization of HER2 with other tyrosine kinase family members like EGFR; and this HER2 heterodimerization, produces a more potent RTK with higher ligand affinity and tyrosine kinase activity, and a lower internalization and degradation rate, inhibiting HER2 may not only decrease HER2 activity, but could also affect the activity of its dimerization partners (EGFR), thus helping to explain the effects observed here with CUDC-101. Limited evidence exists showing the role of HER2 in
radio response; however, a study by Duru et al. (2012) showed that a pro-survival network initiated by HER2 was responsible for radioresistance in breast cancer stem cells. Additionally, irradiation of breast cancer cell lines has been associated with increased expression of EGFR and HER2, which augments the response to HER2-targeted therapy with trastuzumab (Wattenberg et al., 2014). Interestingly, we also saw an increased expression of HER2 and EGFR in U251 cells treated with irradiation alone.

We show CUDC-101 inhibited HER2, EGFR and HDAC in U251 and MDA-MB-231 cell lines, which was accompanied by enhancement in radiosensitivity, while no significant radiation-induced cell kill was seen in a normal fibroblast cell line. Although 0.1µM CUDC-101 effectively maintained Ac-H3, it did not affect HER2 and EGFR deactivation or reduce colony formation. 0.5 µM CUDC-101 maintained or increased Ac-H3 levels, decreased levels of pHER2 and total EGFR, and achieved a significant DEF. Thus the question arises, if some combinatorial permutation of these targets is essential for inducing radiosensitization, or is it solely an HDACi effect? Albeit an important question, it was beyond the scope of this study, and needs further investigation.

The apparent mechanism of CUDC-101-induced radiosensitization appears to involve an inhibition of DNA DSB repair. γH2AX has been shown to correlate with amount of DNA DSBs (Kuo and Yang, 2008). When X-rays induce DNA damage γH2AX is recruited to the damage site and repair proteins such as ATM or DNA-PK phosphorylate γH2AX, and p53BP, MRN and BRCA1 are recruited to complete the repair complex (Mah et al., 2010). We have previously shown that the HDACi valproic acid induces retention in γH2AX foci on the sites of DNA DSBs yet, the sites of damage are being repaired as observed by comet assay (Camphausen et al., 2005). A similar effect may occur with CUDC-101. Additionally, based on the increase of retained foci at 24 h and cells in the M-phase of the cell cycle, CUDC-101 may be inducing DNA damage as a single agent, and therefore causing an abrogation of the G1/M checkpoint. Alternatively, CUDC-101 may show indirect effects on critical regulators of the checkpoint. Therefore, this conflict between the DNA repair complex remaining on the DNA damage sites after repair, and the cells still being pushed into M-phase, may explain the increase in mitotic catastrophe and the CUDC-101-induced radiosensitization.

In order for a putative radiosensitizing agent to be effective clinically, the effects seen in vitro must be replicated under in vivo tumor xenograft models. Aside from defining tumor radioresponsiveness, the ability of the agent to target its intended ligands is critical. The radiosensitizing ability seen in vitro by clonogenic survival with a DEF of 1.42 was not replicated in vivo (DEF 1.2) due to a high
degree of toxicity and substantial weight loss (data not shown). Various other agents have shown promise in vitro, but their effects were unable to be translated to in vivo models or clinically, limited by the toxicity (Camphausen et al., 2005). Much of the biological consequences and mechanisms of action explaining this phenomenon are unclear. Additionally, in the case of CUDC-101, whose targets are nuclear (HDAC) and surface receptors (EGFR and HER2), CUDC-101 may not effectively reach and inhibit the target(s) responsible for enhancing radiosensitization in sufficient number of tumor cells in vivo to achieve a significant response. It may be feasible to combine multiple drugs together as single agents (i.e. lapatinib + erlotinib + vorinostat) with radiotherapy, as reported in various haematologic cancers.

The evaluation of the radiosensitizing potential of CUDC-101, provides the basis for additional preclinical exploration of the radiosensitizing potential. Further investigation and understanding of the specific molecular mechanisms addressing the necessity to target all three ligands to achieve clinically applicable sensitization, is warranted.

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CONFLICT OF INTEREST
The authors claim no conflict of interest.

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