

REV1 inhibition enhances radioresistance and autophagy

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Abstract

Cancer therapy resistance is a persistent clinical challenge. Recently, inhibition of the mutagenic translesion synthesis (TLS) protein REV1 was shown to enhance tumor cell response to chemotherapy by triggering senescence hallmarks. These observations suggest REV1's important role in determining cancer cell response to chemotherapy. Whether REV1 inhibition would similarly sensitize cancer cells to radiation treatment is unknown. This study reports a lack of radiosensitization in response to REV1 inhibition by small molecule inhibitors in ionizing radiation-exposed cancer cells. Instead, REV1 inhibition unexpectedly triggers autophagy, which is a known biomarker of radioresistance. Collectively, we report a possible role of REV1 TLS protein in determining cancer treatment outcomes depending upon the type of DNA damage inflicted. *Furthermore, we discover REV1 inhibition directly triggers autophagy, an uncharacterized REV1 phenotype, with significant bearing on cancer treatment regimens.*

Introduction

Intrinsic and acquired resistance to DNA-damaging cancer therapy is a persistent clinical challenge that ultimately limits successful clinical outcomes in patients ¹. Recent evidence suggests that a possible strategy to sensitize tumors and reduce chemotherapy resistance is to inhibit the mutagenic translesion-synthesis (TLS) pathway by targeting REV1 TLS polymerase ²⁻⁵. Translesion synthesis is a DNA-damage bypass process involving a set of specialized DNA polymerases that collectively tolerate DNA damage and cause mutations ¹. REV1 plays a central role in this process by engaging in protein-protein interactions via two distinct interfaces at its C-terminus domain (CTD) ^{6, 7}. Small molecule inhibitors targeting these interfaces can effectively inhibit the mutagenic translesion synthesis and suppress tumor growth ³⁻⁵. Remarkably, during a chemotherapy regimen, REV1 inhibition also switches the biology of cisplatin-dependent cell death response from apoptosis to senescence and triggers an immune response in treated cells ⁸. It is unknown how exactly REV1-inhibition triggers senescence.

Radiotherapy is one of the mainstay treatment modules for roughly half of all cancer patients, typically involving high energy X-rays between 1-2 Gy dose per treatment fraction ⁹. Despite the promising initial success with radiotherapy in inhibiting tumor growth, relapse of the incumbent tumor and the subsequent requirement for a higher dose of radiation results in a patient fatality. Several studies in the past few years have explored adjuvant therapies that might facilitate continued sensitivity of the tumor to radiotherapy, but the underlying complex heterogeneity of the tumor itself has not allowed appreciable success. Mechanisms conducive to therapeutic resistance to radiation range from

enhanced DNA double-strand break repair (DSBR), to altered expression of DNA damage signaling, in addition to the hypoxia-dependent protective effects from surrounding tissue¹⁰. Furthermore, radioresistance biomarkers have continued to be discovered within the clusters of transcriptional regulation of DNA metabolic processes, inhibition of apoptosis, response to hypoxia, and DNA repair. Because REV1 functionally links DSBR with enhanced mutations, and REV1 inhibitors suppress apoptosis and trigger senescence to sensitize cancer cells to chemotherapy^{8, 11, 12}, it is unknown whether REV1 may similarly sensitize cancer cells to radiation therapy and prevent radioresistance.

This study tested whether REV1 inhibition via CTD-specific small molecule inhibitors sensitizes cancer cells to radiation treatment. In contrast to their chemosensitization effects^{2-5, 8}, REV1 inhibition failed to sensitize cancer cells to ionizing radiation. We confirmed the lack of radiosensitization by using five different REV1 inhibitors, varying doses of REV1 inhibitors, testing physiologically relevant increasing ionizing radiation doses, and examining radioresistant cell lines. Unexpectedly, we discovered that REV1 inhibition by small molecule inhibitors triggered autophagy, which is known to cause therapy resistance in cancer cells under certain conditions. Further, we found a narrow range within which an autophagy inhibitor might aid in sensitizing IR and REV1 inhibitor-exposed cancer cells^{18, 21}. These results highlight an unexpected new function of REV1, which is beyond translesion synthesis, in determining therapy resistance with critical cancer therapy outcomes.

Results

REV1 inhibition does not sensitize cancer cells to IR

REV1 protein plays a crucial role in the DNA damage bypass process by functioning as a scaffolding molecule that facilitates protein-protein interactions with other TLS polymerases via its CTD ¹. *Previously, REV1 inhibitors that targeted specific interfaces of this CTD were shown to sensitize cancer cells to chemotherapy treatment, which suggested that the REV1-dependent DNA damage bypass of chemotherapy-induced damage was the cause of chemoresistance* ^{2-5, 8}. Whether REV1 inhibition would similarly sensitize cancer cells to radiation treatment is currently unknown. Because REV1 plays a role in the DSBR pathway and suppresses apoptosis—processes known to be involved in radioresistance—we hypothesized that REV1 inhibition might sensitize cancer cells to radiation treatment ²¹. To test this hypothesis, we exposed MEF (mouse embryonic fibroblasts), HT1080 (fibrosarcoma), HCT116 (colorectal), and REV1 KO (knockout MEF) cells to physiologically relevant increasing doses of IR (1 Gy and 4 Gy) and tested five different REV1 inhibitors that target different interfaces at 1 μ M dose. *These drugs were: 4 (7922759), 5 (7587885), and 6 (7127492) which target the RIR (REV1-interacting region) interface; and drugs JH-1 (JH-RE-06) and JH-2 (JH-RE06.NaOH) which target the REV7 interface of REV1 (Supplementary Fig. 1)* ^{3,4}. *The RIR-specific drugs successfully inhibit REV1's ability to interact with TLS polymerases - POL η , POL ι , and POL κ . JH-RE-06 specifically induces dimerization of the REV1 CTD that precludes REV7 from its binding pocket. The REV1/REV7 interface is also considered to be more mutagenic than the RIR interface as the RIR polymerases function redundantly. All these drugs have previously been reported to suppress chemoresistance in cancer cells, where the sensitization effects of the REV7 inhibitor were stronger than the RIR inhibitors* ^{3,4}.

Additionally, the drug JH-RE-06.NaOH is a newer more stable version of JH-RE-06 with an additional NaOH moiety. Using colony survival assays, our results showed no increased sensitization of these IR-exposed cancer cells to REV1 inhibitors (**Fig. 1A; Supplementary Fig. 2 and 3**). Colony survival assays measure the relative potential of exposed cells to proliferate and form colonies. Similarly, we tested the ability of REV1 inhibitors to sensitize the cancer cells to increasing doses of IR in the cytotoxicity assays and observed no synergy in cytotoxicity (**Supplementary Fig. 3**). *Additionally, the MEF REV1 KO cells were not sensitized to 1 Gy or 4 Gy in colony survival and cytotoxicity assays unlike the cisplatin (chemotherapy) sensitization as observed in REV1 KO cells (Fig 1C; Supplementary Fig. 3).* In fact, we consistently observed an increased proliferation potential post-treatment with REV1 inhibitors in all our assays, suggesting REV1 inhibition played a cytoprotective role in IR-exposed cells. *The relative lack of toxicity observed here is in line with the previously published trends in these specific cell lines* ²⁵⁻²⁸. We also tested higher doses of IR – 10 Gy and 100 Gy – on HT1080 cells but were unable to note synergy due to higher toxicity of the radiation (**Fig. 1B**). In order to test whether there was any discernible indication of double strand breaks from IR, which is the physiological response in cells and observed induction of γ H2AX in HCT116 cells as shown in **Fig. 1D**.

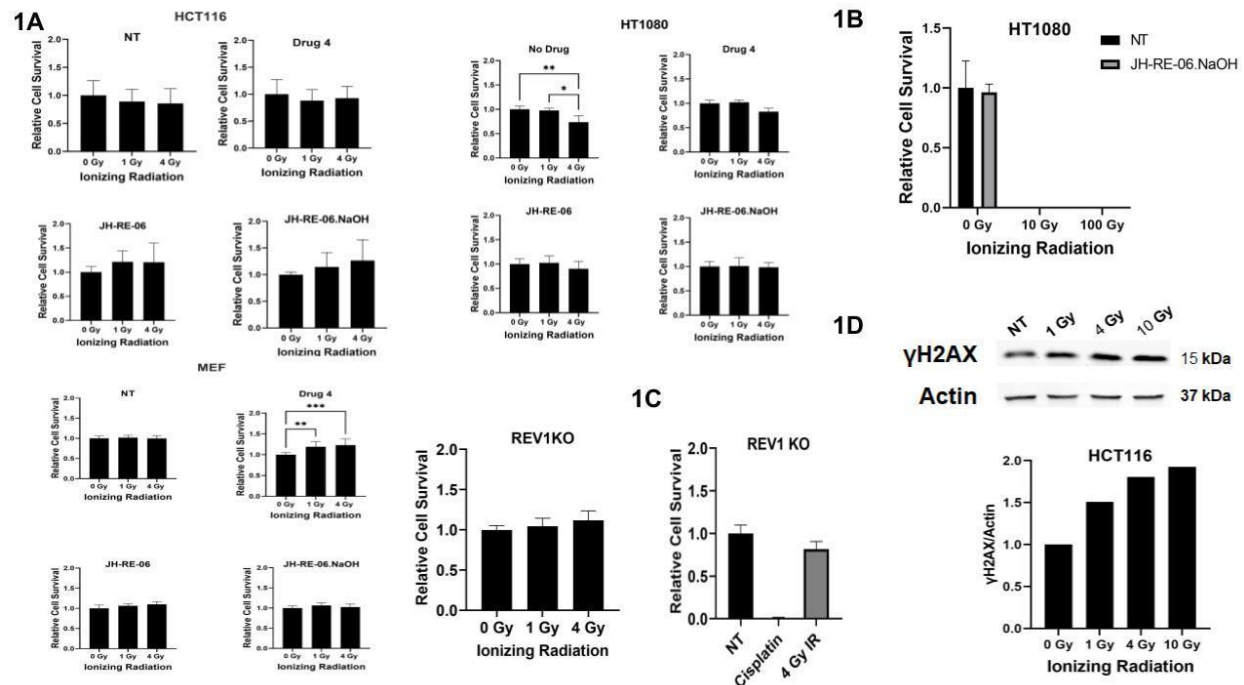


Figure 1: REV1 inhibition does not sensitize cancer cells to ionizing radiations.

1A. Relative cell survival of HCT116 (colorectal), HT1080 (fibrosarcoma), MEF (mouse embryonic fibroblasts), and REV1 KO (knockout; MEFs) in response to increasing doses of ionizing radiation of 1 Gy and 4 Gy, and REV1 inhibitor drugs at 1 μ M concentration: 4 (7922759), JH-1 (JH-RE-06), and JH-2 (JH-RE06.NaOH). **1B.** Relative cell survival of HT1080 cells with 0, 10 and 100 Gy. **1C.** Relative cell survival in REV1 KO cells after treatment with increasing doses of IR 0, 1 and 4 Gy (left graph) and with 10 μ M Cisplatin and 4 Gy of radiation (right graph). **1D.** Western blot show γ H2AX in HCT116 cells treated with 1 Gy, 4 Gy and 10 Gy of radiations. Graph shows relative quantification of the western blots. P values are * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. Error bars represent standard deviations. P values were calculated by two-way ANOVA. N=6 for all values.

To rule out the possibility that a longer time frame of REV1 inhibition was necessary to sensitize cells to IR, we exposed HT1080, HCT116, and MEF REV1 KO cells to increasing doses of 1 Gy and 4 Gy of IR and 1 μ M REV1 inhibitors 4 (7922759) and JH-RE-06 and measured relative differences in cytotoxicity at 24, 48, and 72 hours. We found no significant increase in synergy in cytotoxicity to increasing exposure times to REV1 inhibitors 4 and JH-RE-06 (**Fig. 2A and Supplementary Fig. 4**). The relative luminescence across different exposure times in treated cells was unchanged from the non-treated controls. These results suggested that increased exposure time to REV1 inhibitors did not sensitize cells to radiation treatment.

We also tested the alternative hypothesis that an increased concentration of the REV1 inhibitors, beyond the 1 μ M dose used above, would increase cytotoxicity with IR. To test this hypothesis, we treated IR-exposed HT1080 cells at 1 Gy to increasing concentrations of drug JH-RE-06 at 5 μ M, 15 μ M, and 30 μ M and measured relative increase in cytotoxicity. We observed that increasing the REV1 inhibitor's concentration conferred a cytoprotective effect, where IR-exposed cells treated with 5 μ M JH-RE-06 survived significantly better than the non-irradiated controls that received the drug alone (**Fig. 2B**). The cells treated with JH-RE-06 alone, in fact, lost 80% of their viability, which was rescued in the IR-exposed group treated with JH-RE-06, suggesting that REV1 inhibition specifically during radiation treatment has a cytoprotective effect. These data suggested that REV1 inhibition propels intrinsic resistance during radiation treatment, directly contrasting its role in sensitizing cancer cells to chemotherapy. *“Alternatively, it is also*

possible that this observed effect is unique to the JH-RE-06 drug, where it slows general cell proliferation and perhaps exhibits an opposite effect to being cytoprotective. Further work needs to be done to establish this effect more clearly.”

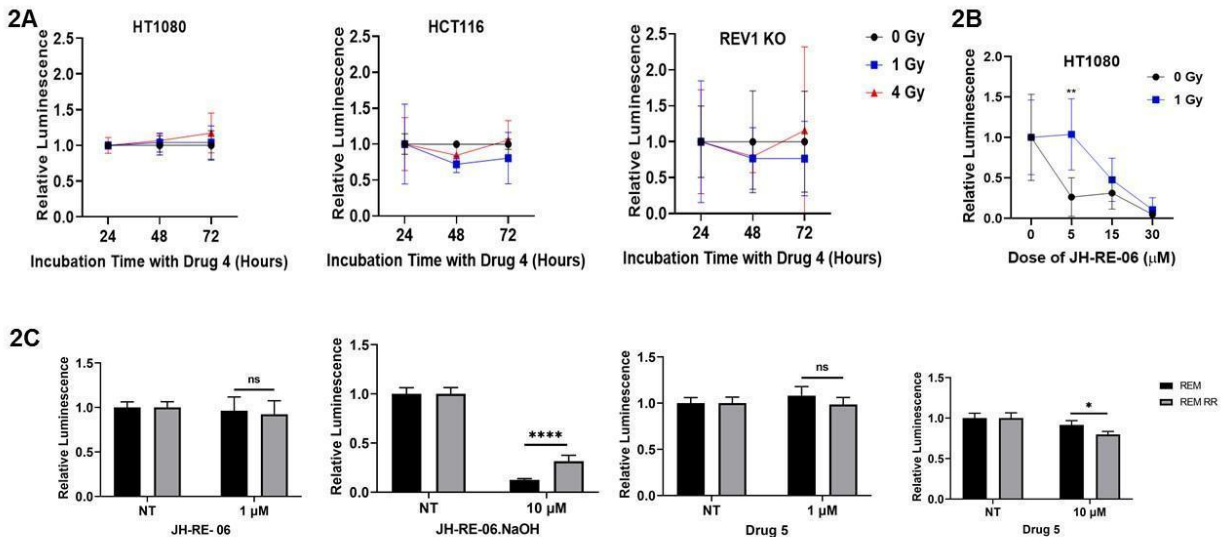


Figure 2. REV1 inhibition does not sensitize cancer cells to ionizing radiations.

2A. Graphs show relative luminescence with increasing incubation times (24, 48, and 72 hours) with drug 4 (7922759) and increasing doses of IR (0, 1, and 4 Gy) in the HT1080 and HCT116 cell lines. Also shown are relative luminescence intensities in MEF REV1 KO cells after exposure to 1 and 4 Gy ionizing radiations and incubated for 24, 48 and 72 hours. **2B.** Relative luminescence in HT1080 treated with 0, 5, 15 and 30 μ M of JH-RE-06 with 1 Gy of IR exposure. **2C.** Relative luminescence in REM and REM RR cells in response to treatment with JH-RE-06, JH-RE-06.NaOH and drug 5 (4053831). P values

are * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. Error bars represent standard deviations. P values were calculated by two-way ANOVA. $N=6$ for all values.

Because cancer cell lines over the course of cancer treatment tend to acquire resistance to therapy, we tested whether REV1 inhibition may sensitize acquired radioresistant cell lines to radiation treatment. To test this hypothesis, we treated isogenic pairs of cancer cells including human breast cancer cells (ZR751 and its radioresistant counterpart ZR-751 RR), and canine mammary cancer cells (REM and its radioresistant counterpart REM RR)^{13, 14} with increasing doses of drug 5 (7587885), JH-RE-06, and JH-RE-06.NaOH at 1 μM and 10 μM . Our results demonstrated no significant differences in cytotoxicity in the radioresistant cell lines compared to their isogenic parental controls (**Fig. 2C, and Supplementary Fig. 5**). The REM RR radioresistant cells upon addition of 10 μM REV1 inhibitor JH-RE-06.NaOH display a slight growth advantage compared to the parental cell line. These results suggest that REV1 inhibition might also provide a cytoprotective effect in cancer cells that have acquired radiation resistance over the course of treatment.

To further verify if the lack of cytotoxicity from REV1 inhibition during radiation treatment was simply an IR-specific effect, we exposed HT1080 cells to Etoposide (a cancer drug that functions similar to IR by inducing DNA strand-breakages and inducing cytotoxicity) and drugs 4 (7922759) and JH-RE-06. We did not observe any increase in synergy in cytotoxicity (**Supplementary Fig. 6**). Additionally, etoposide treatment did not sensitize

the MEF REV1 KO cells, suggesting that REV1 inhibition perhaps engages a different biological response that enables cancer resistance during strand breakages from IR or etoposide treatment, which contrasts to the reduction in mutagenesis and chemoresistance upon REV1 inhibition during cisplatin treatment or treatment with other drugs that make chemical modifications to DNA.

REV1 inhibition triggers autophagy, a radioresistance biomarker

During the course of our experiments, we observed an unusual phenotype of the MEF REV1 KO cells (**Supplementary Fig. 7**), where the cells appeared flattened under an inverted microscope as previously reported ⁸. However, we also observed a curious cellular morphology of enlarged cytoplasmic vesicles that prompted us to test whether the enlarged structures were lysosomes ¹⁵. Enlarged lysosomes are typically discernible with the DIC (differential interference contrast) brightfield microscopy. Next, we used the Cyto-ID green detection reagent (Enzo Life Sciences), a cationic dye with strong affinity for pre-autophagosomes, autophagosomes, and autophagolysosomes that are typically upregulated during autophagy. *More than 40% MEF REV1 KO cells exhibited marked staining suggestive of autophagy induction in the absence of REV1 (Fig. 3A). Because MEF REV1 KO cells grow slower than the MEF WT cells, we wanted to test whether growth dynamics influenced autophagy induction ⁸. To test this hypothesis, we used REV1 inhibitors, drugs 4 (7922759) and JH-RE-06 at 1 μ M concentration, to confirm the specificity of REV1's role in autophagy induction. We observed about 50% of the cells staining with the Cyto-ID green detection reagent (Fig. 3A and Supplementary Fig. 8).* The induction of autophagy from the two REV1 inhibitors in these immunofluorescence

assays was equivalent or stronger (more than 70% increase in lysosomal staining) than the positive control, chloroquine, and the MEF REV1 KO cells, suggesting that REV1 inhibition triggers autophagy (**Fig. 3A**). In addition to staining phagosomal- structures that mark induction of autophagy, increased LC3A/B protein ratios also successfully signal autophagy induction. LC3B is one of the three isoforms of the protein LC3. During autophagy, LC3A undergoes lipidation and converts to LC3B, which then associates with autophagosomes as part of the autophagy process ^{16, 17}. *Western blot analysis of the MEF REV1 KO cells shows an almost a 1.5-fold increased LC3B protein expression compared to their normal MEF cells, but we also observed a curious phenotype of an increased expression of LC3A exclusively in the MEF REV1 KO cells (Fig. 3B). The MEF REV1 KO cells also exhibited up to a 6-fold increase of p62 or SQSTM1 expression, which is another read out of the autophagy flux. The relative gene expression quantification from one representative image from two independent experiments is shown under the western blot images. Similarly, exposure of the HT1080 cells to REV1 inhibitor, JH-RE-06.NaOH, increased the LC3B levels by 10-fold, and the p62 levels by about 1.5-fold, compared to non-treated controls (Fig. 4B). Both these results indicate that REV1 inhibition triggers autophagy. It is interesting to note that the REV1 KO cell line upregulates LCA3, while the use of REV1 inhibitors increases the expression of LC3B. Future studies must address these unique differences in the autophagy induction by genetic perturbation of the key gene, versus the use of small molecule inhibitors to target gene function.*

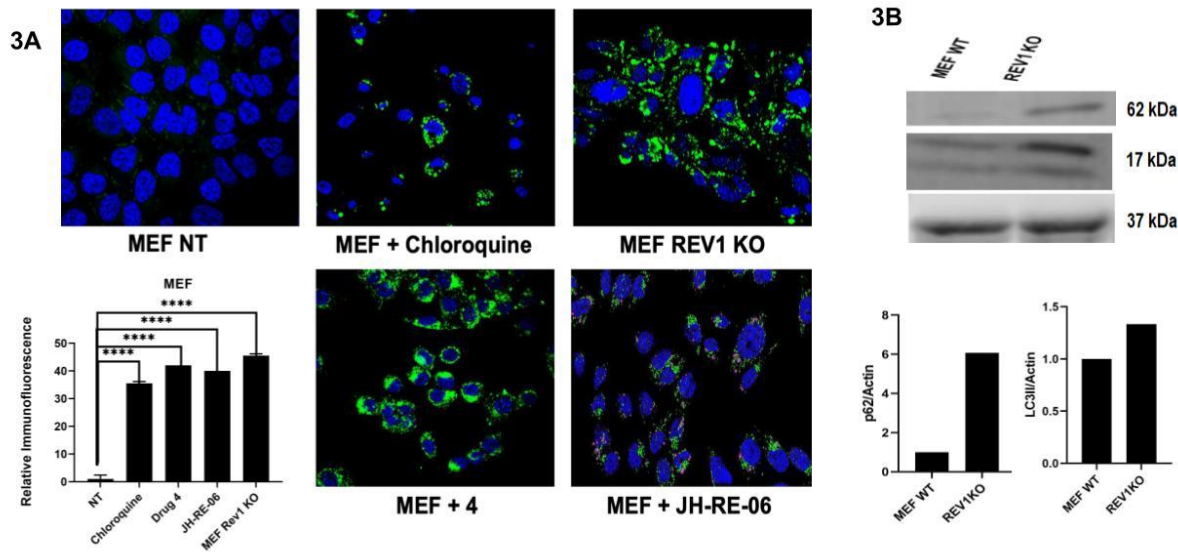


Figure 3. REV1 inhibition triggers autophagy.

3A. Immunofluorescence images show autophagy flux (green) in MEF WT cells treated with chloroquine (positive control), drug 4 (7922759) and JH-RE-06, and REV1KO cells as validation controls. Graph shows relative quantification of the cells expressing the green fluorescence signal. **3B** Representative image of a western blot showing expression of p62 and LC3a in MEF REV1 KO cells compared to the WT MEF. Graph shows relative quantification.

Autophagy is known to cause resistance to IR therapy in radioresistant cancer cells ¹⁸. The context within which REV1 inhibition plays into the induction or the maintenance of autophagy signals in cells is unknown. Similarly, whether REV1 inhibition-mediated radioresistance could be suppressed by inhibiting autophagy is not known. To test the latter hypothesis, we used an autophagy inhibitor Bafilomycin A1 (BFA) that targets the autophagosome-lysosome fusion to inhibit autophagy ¹⁹. We treated HT1080 cells with

increasing doses of BFA to establish the dose range at which it sensitizes JH-RE-06.NaOH-treated cells to ionizing radiation. Dose response curves suggest that the optimal dose to inhibit REV1-inhibition induced autophagy is 5 nM concentration of BFA in both IR exposed and controls (**Fig. 4A**). Higher doses of BFA triggered its own autophagy response as can be seen by the increase in toxicity in the dose response curves. To verify that higher concentrations of BFA was triggering autophagy and consequently toxicity in our dose response curves, we ran western blots of IR and REV1 inhibitor JH-RE06.NaOH-treated cells which were exposed to 50 nM of BFA, and observed an induction of both LC3B and p62 (**Fig. 4B**). These assays suggest that there is only a narrow range within which an autophagy inhibitor can potentially synergize cells to radiosensitization post-REV1 inhibition. Further studies are needed to systematically analyze the association of REV1 with autophagy and the utility of other autophagy inhibitors. However, because a functional reduction of REV1 was key in triggering the autophagy and the inhibition of REV1 failed to radiosensitize cells, REV1 expression dynamics may link cancer cell response to radiation treatment through potential induction of autophagy.

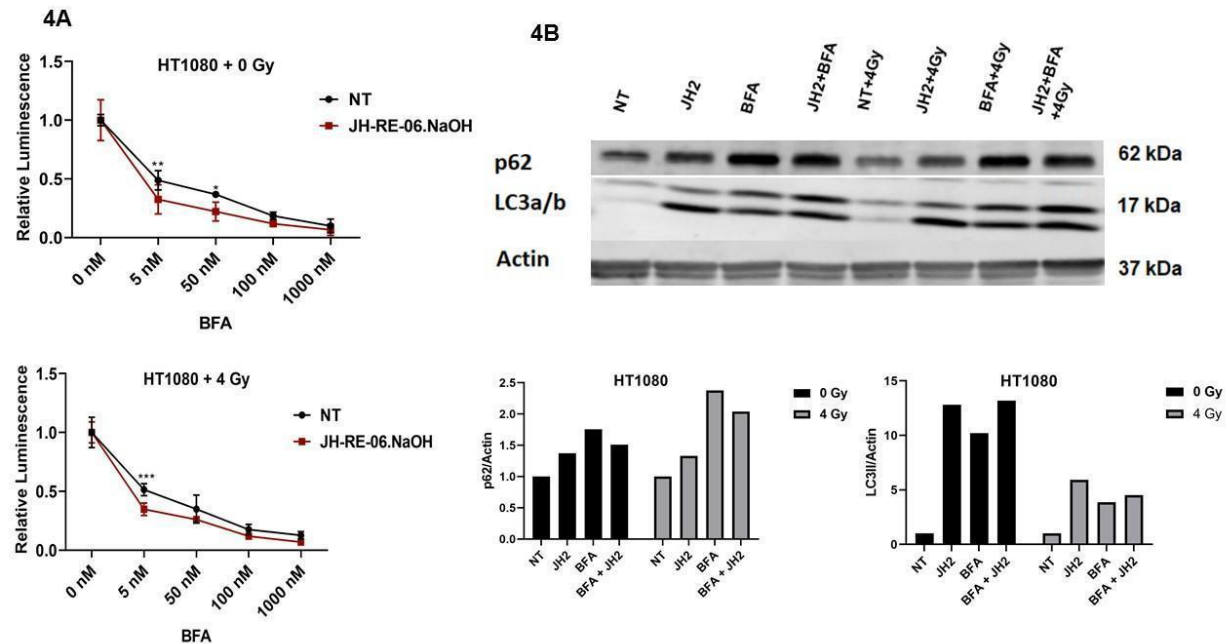


Figure 4: Autophagy inhibition has a narrow range to sensitize cancer cells treated with ionizing radiations and REV1 inhibitors

4A. Graphs show relative luminescence in HT1080 cells treated with 0 or 4 Gy ionizing radiation in the presence of increasing doses of autophagy inhibitor BFA and 1 μ M of JH-RE-06.NaOH. **4B.** Representative western blot images show expression patterns of p62 and LC3a/b in HT1080 cells treated with JH-RE-06.NaOH at 1 mM, BFA at 50 mM, and ionizing radiations at 0 Gy and 4 Gy respectively. Graphs show relative quantification of p62 and LC3a/b expression in HT1080 cells from the western blots above. P values are * $P < 0.05$, and *** $P < 0.001$. Error bars represent standard deviations. P values were calculated by two-way ANOVA. N=6 for all values

Discussion

In the last few years, the translesion synthesis pathway, especially the REV1 polymerase, has gained considerable traction in understanding how cancers acquire intrinsically and acquired resistance to therapy. The function of REV1, which was classically known to facilitate the formation of new mutations by functioning as a deoxycitidyl transferase, is also now known to allow protein-protein interactions with other TLS polymerases by folding into a scaffolding molecule ¹. These landmark observations provide a semblance as to why the cancer mutational spectrum continues to evolve and an understanding as to why there remains a continuity to clinical challenges in treating patients. Furthermore, the discovery of REV1 inhibitors provided a reliable platform for potential clinical adjuvant therapy ²⁻⁵. However, indications that REV1 inhibition can also switch the underlying biology of cancer cells, whereby it can suppress apoptosis in cisplatin-treated cells, and trigger senescence ⁸, suggested that REV1 may have a larger new role in cancer pathogenesis.

This study shows two unexpected observations that further our understanding of REV1 functional dynamics during cancer resistance and the consequences of targeting REV1 during different DNA damaging cancer treatments. *First, we found that REV1 inhibition during radiation therapy may not sensitize cancer cells to increased cell death as indicated from our in vitro data.* In colony survival assays we observed that REV1 inhibition, with five different REV1 inhibitors, did not sensitize cells to IR. Neither an increased exposure time to REV1 inhibitors nor increased concentration of REV1 inhibitors during IR showed a synergy in cytotoxicity. Additionally, acquired radioresistant

cancer cells REM and ZR751, developed by exposing the parental cells to repeat rounds of IR, survived similarly to non-treated controls when exposed to REV1 inhibitors. In summary, our data suggests that REV1 inhibition does not sensitize cancer cells with intrinsic and acquired resistance to radiation treatment. *This is in contrast to an earlier study in which siRNA depletion of REV1 in HeLa cells resulted in enhanced radiosensitivity²². However, it has also been shown that genetic heterogeneity of HeLa cells can lead to changes in protein expression and could impact interpretation of results gotten from this cell line^{23, 24}. The data presented from our research were obtained using 3 different cell lines and different REV1 inhibitors. Despite that, we did not see any enhanced radiosensitization. It is possible that these differences arise due to differences in cell lines or method of inhibiting REV1 function. It is also noted that small molecule inhibitors of REV1 that were used in this study were effective in chemosensitization as reported earlier, but not radiosensitization as observed in this study.*

Additionally, REV1 inhibition did not sensitize etoposide-treated cancer cells, suggesting that treatment modules that rely on DNA strand breaks to trigger cell death in cancer cells may not benefit from inhibiting REV1, unlike the significant potential of REV1 inhibition to sensitize cancer cells to chemotherapy treatment. That is, depending upon the type of DNA damaging cancer treatment chosen, levels of functional REV1 may either help enhance the efficacy of the said drug, such as would be for chemotherapy treatment that causes chemical modifications on DNA, where REV1 contributes to mutagenesis and consequent therapy resistance. In contrast, a similar reduction in functional REV1 during radiation treatment or drugs that cause strand breaks in cancer cells will actually propel

therapy resistance because REV1 inhibition evokes a newer function of induction of autophagy. This result further suggests that REV1 may be an essential biomarker for cancer treatment success, whereby its reduced levels during chemotherapy would be an indicator for good patient response to treatment. On the other hand, its increased levels would be the desired prognosis for patients undergoing radiotherapy. Further, the REV1 expression profile across 31 tumor samples and paired normal tissues shows that almost 50% of these tumor tissues exhibited reduced expression of REV1 (<http://gepia.cancer-pku.cn/detail.php?gene=REV1>).

We also show the first direct evidence of a REV1 inhibition-dependent activation of the autophagy flux, an uncharacterized biological consequence previously unknown to be associated with the REV1 translesion synthesis polymerase. We observed marked cytoplasmic vesicle formation in MEF REV1 KO cells that were stained as autophagosomes. Further, the induction of autophagosomes after REV1 inhibition in independent cell lines suggested that REV1 has an unanticipated role in regulating the autophagy stress response. Moreover, because we observed an upregulation of the LC3B isoform of LC3 in both the MEF REV1 KO and JH-RE-06-exposed HT1080 cells, REV1 may be an active modulator of autophagy.

Typically, autophagy is a potent mechanism triggered to combat the consequences of starvation stress, accumulation of damaged cellular components, and in certain instances promote cancer resistance. Besides, a dominant autophagic flux in certain circumstances can induce senescence in cancer cells ²⁰. Several questions remain unaddressed: how

REV1, a translesion synthesis polymerase, might fit within the complex interplay of autophagy induction; how REV1 serves to engage cellular responses such as autophagy versus regulating DNA repair versus senescence induction during IR-induced DNA strand damage? REV1 inhibition was previously shown to trigger senescence in chemotherapy-treated cells⁸. It is equally intriguing to evaluate whether REV1 might serve as a clinical biomarker for cancer cell response to other cancer treatments. For example, whether patients with higher REV1 expression levels respond better to radiation treatment, versus those with lower REV1 expression levels, where autophagy induction may result in poor response to radiation treatment.

This study collectively shows that REV1 inhibition confers a cytoprotective effect on the cancer therapies catered at inducing DNA strand breakages. We also observed that REV1 inhibition induces autophagy, a known biomarker of radioresistance. Further work needs to be done to determine a connection between REV1 inhibition, induction of autophagy and radioresistance.

Methods

Mammalian cell culturing

HT1080 cells (male, fibrosarcoma epithelial cells purchased from ATCC) were grown at 37 °C with 5% CO₂ in EMEM (ATCC) and RPMI (ATCC), 10% (v/v) FBS (Gibco), and 1% Penicillin-Streptomycin antibiotic (Gibco). HCT116 (male, colorectal epithelial cancer cells purchased from ATCC) were grown in McCoy's 5A (ATCC) 10% (v/v) FBS (Gibco), and 1% Penicillin-Streptomycin antibiotic (Gibco). MEF (Mouse embryonic fibroblasts) along with their REV1 knockout counterparts were grown at 37 °C with 5% CO₂ in DMEM (ATCC), 10% (v/v) FBS (Gibco), and 1% Penicillin-Streptomycin antibiotic (Gibco). REM (canine mammary cancer cells) and ZR751 (human breast cancer cell line) and their radioresistant counterparts, REM RR and ZR751 RR 10, were graciously donated by Mark Gray, University of Edinburgh. These cells were grown at 37 °C with 5% CO₂ in DMEM (ATCC), 10% (v/v) FBS (Gibco), and 1% Penicillin-Streptomycin antibiotic (Gibco). 0.25% trypsin was used for trypsinizing and splitting.

Drug inhibitors

REV1 inhibitors targeting the two interfaces of the C-terminus domain (CTD)—drugs 4 (7922759), 5 (4053831), 6 (7127492) targeting the RIR (REV1 interacting region) interface, and JH-1 (JH-RE-06), and JH-2 (JH-RE06.NaOH) targeting the REV7 interface—were used this study^{3,4,5}. BFA (Bafilomycin A1), a macrolide antibiotic that inhibits late phase autophagy was used an autophagy inhibitor (Sigma Cat # B1739).

Cytotoxicity assay

10,000 cells were plated into each well of a white-bottom 96-well plate (Corning). The cells were treated with varying concentrations (1 μ M, 5 μ M, 15 μ M, or 30 μ M) of REV1 inhibitor drugs (JH-RE-06, JH-RE-06.NaOH, Drug 4, Drug 5, Drug 6) with either 0, 1 or 4 Gy of ionizing radiation. After 24, 48, or 72 hours of incubation, the proportion of viable cells was evaluated by adding 100 μ L of the CellTiter-Glo Luminescence stain (Promega) was added into each well. The CellTiter Glo stain was prepared according to the manufacturer's recommendation. The endpoint luminescence was measured on the Synergy H1 Microplate Reader plate reader. Relative cell viability was determined by dividing treated sample luminescence measurements by their respective control samples without drugs and/or ionizing radiation.

Colony survival assay

Approximately 600 to 800 cells were plated in triplicate into each well of 6-well plates for 24 hours. The cells were treated with 1 μ M of various REV1 inhibitor drugs (JH-RE-06, JH-RE-06.NaOH, Drug 4, Drug 5, Drug 6) for 24 hours and exposed to varying levels of ionizing radiation (0, 1, or 4 Gy). The plates were incubated at 37°C for 24 hours, after which media was replaced with fresh media and plates were incubated at 37°C for 6-7 days. The media was aspirated, and the cells were fixed with 70% ethanol before staining with 1 mL of 0.1% crystal violet dye. Stained colonies containing at least 40 cells were counted and relative cell survival was quantified by dividing the average number of colonies from each condition by the average of their respective negative controls (no IR and/or no drugs).

Immunofluorescence detection of autophagy

MEF and HT1080 cells were plated in 35 mm dishes for 24 hours, after which they were treated with 1 μ M concentration of drug 4 and JH-RE-06 for another 24 hours. Cells were then fixed with 4% paraformaldehyde, and immunofluorescent detection of autophagy was assessed using the CYTO I.D. Autophagy Detection Kit 2.0 (Enzo Life Sciences; Catalog number: ENZ-KIT175-0050). The kit is optimized to detect autophagic vacuoles, as well as detect autophagic flux in lysosomal-inhibited live cells. A fluorescent green dye labels autophagic vacuole as they accumulate. Bright green fluorescence is indicative of autolysosomes. The stained samples were imaged at the UVM Cancer Center's Microscope Imaging Center (MIC).

Western blot analysis

Cells were lysed in RIPA lysis buffer (Pierce) with fresh protease inhibitors (Pierce), and the lysate was quantified using the Micro BCA Protein Assay Kit (Pierce). Samples were boiled for five minutes in 4x LDS Sample Buffer (Invitrogen) and separated by SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) for ninety minutes at 100V. The following antibodies were used in Blocking Buffer (Thermo Scientific): LC3A/LC3B (Invitrogen PA1-16931) at 1:500, SQSTM1 (Invitrogen PA1-27247) at 1:500, γ H2AX (Novus NB100-384) at 1:1000, and Actin (Invitrogen MA1-744) at 1:1000. The membrane was washed with dPBST (Corning, + 0.1% Tween) three times for fifteen minutes each and incubated with secondary antibody (LC3A/B/ γ H2AX/p62: IRDye 800CW, Goat Anti-Rabbit; Actin: IRDye 680RD, Goat Anti-Mouse) at a 1:20,000 dilution in blocking buffer

with 0.01% SDS and 0.1% Tween-20. Blots were visualized using a LiCOR imager and analyzed with Image Studio software.

Image studio was used to quantify relative expression of proteins in the western blots by dividing the A.U. for key genes with the corresponding actin control and were subsequently normalized to their respective experimental controls.

Statistical analysis

Statistical analysis was carried out by two-way analysis of variance (ANOVA). Each result was the sum of at least two biological replicates, with $n=6$ in most cases, unless otherwise noted. Standard deviations (S.D.) indicate the variance and is indicated as the mean \pm S.D., unless otherwise noted. Significance is noted as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, or $****P < 0.0001$.

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Author contributions

K.E.I. conducted the cytotoxicity experiments for increasing exposure time to REV1 inhibitors, autophagy inhibition, and tested the radioresistant cell lines. E.N.L. conducted the cytotoxicity and colony survival experiments with all the REV1 inhibitors. A.C. conducted all the immunofluorescence experiments and analysis. K.E.I., E.N.L., and A.C. helped analyze and plot the data. J.D., K.E.I., E.N.L. conducted the western blots. K.F. helped with the autophagy immunofluorescence experiments. M.G. and D.J.A. built the radioresistant cell lines. D.K. and K.H. synthesized drugs 4,5, and 6. P.Z., J.H. and W.Y.L. synthesized drugs JH-RE-06 and JH-RE-06.NaOH. N.C. designed and supervised the experiments. N.C. wrote the paper with the help of all authors.

Conflict of Interest Statement: The authors declare no potential conflicts of interest

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