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Zebularine induces replication-dependent double-strand breaks which are preferentially repaired by Homologous Recombination.

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HIGHLIGHTS:

1. HR is the main pathway to repair DSB induced by zebularine.

2. Zebularine induces replicacion coupled-DSB that are repaired by HR through an exchange mechanism dependent on BRCA2.

3. XRCC1 deficiency is alleviated by an increase in HR to repair zebularine induced-lesions.

4. Our data offer the possibility that zebularine may have potential therapeutic value in the treatment of HR-deficient tumours.

ABSTRACT

Zebularine is a second-generation, highly stable hydrophilic inhibitor of DNA methylation with oral bioavailability that preferentially target cancer cells. It acts primarily as a trap for DNA methyl transferases (DNMTs) protein by forming covalent complexes between DNMT protein and zebularine-substrate DNA. It's well documented that replication-blocking DNA lesions can cause replication fork collapse and thereby to the formation of DNA double-strand breaks (DSB). DSB are dangerous lesions that can lead to potentially oncogenic genomic rearrangements or cell death. The two major pathways for repair of DSB are non-homologous end joining (NHEJ) and homologous recombination (HR). Recently, multiple functions for the HR machinery have been identified at arrested forks. Here we investigate in more detail the importance of the lesions induced by zebularine in terms of DNA damage and cytotoxicity as well as the role of HR in the repair of

these lesions. When we examined the contribution of NHEJ and HR in the repair of DSB induced by zebularine we found that these breaks were preferentially repaired by HR. Also we show that the production of DSB is dependent on active replication. To test this, we determined chromosome damage by zebularine while transiently inhibiting DNA synthesis. Here we report that cells deficient in single-strand break (SSB) repair are hypersensitive to zebularine. We have observed more DSB induced by zebularine in XRCC1 deficient cells, likely to be the result of conversion of SSB into toxic DSB when encountered by a replication fork. Furthermore we demonstrate that HR is required for the repair of these breaks. Overall, our data suggest that zebularine induces replication-dependent DSB which are preferentially repaired by HR.

Keywords: Zebularine, Homologous Recombination, Replication, DNA damage, BRCA2, XRCC1

1. Introduction

DNA methylation is controlled by a family of DNA methyltransferases (DNMT), enzymes that catalyze the transfer of a methyl moiety from S-adenosyl-l-methionine to the 5-position of cytosines in the CpG dinucleotide to silence gene expression [1]. Regulatory genes are often hypermethylated at their promoter 5' regions and silenced in cancer. Epigenetic therapy with DNA methylation inhibitors have been shown to result in the demethylation and reactivation of these genes and therefore, its downregulation by DNMT inhibitors could theoretically be advantageous in the treatment of cancer [2]. 5-Aza-cytidine (5-azaC) and 5-aza-2´-deoxycytidine (5-azadC) are two widely used DNMT inhibitors and recently have undergone several clinical trial for the treatment of patients with acute myeloid leukemia and myelodysplastic syndrome (MDS) [3]. Although the use of these agents may have clinical benefits, in the case of 5-azaC and 5-azadC toxicity and instability under physiological conditions may complicate their use in clinical setting.

Zebularine has recently been identified as an inhibitor of DNA methylation, which, in contrast to previously studied inhibitors, is stable in aqueous solution and has considerably less toxicity [4]. Moreover, zebularine has recently been shown to be preferentially incorporated into the DNA of tumor cells compared with normal cells in vitro [5]. Zebularine (1-[beta]-D-ribofuranosyl]-1,2-dihydropyrimidin-2-one) is similar in structure to azacytidine except that it lacks a 4′ amino group and a 5′ nitrogen. Besides inhibiting cytidine deaminase, zebularine was also found to form covalent complexes with DNA methyltransferases. As a result of the chemistry of the methyltrasferase reaction, the DNMT becomes covalently linked to DNA, in effect creating a protein–DNA cross-link [6]. This results in depletion of soluble DNMT protein levels, which induces replication-dependent global demethylation and gene reactivation [7].

Although there is a considerable amount of literature regarding the possible anti-cancer mode of action of DNMT inhibitors, the molecular mechanism of cell death still remains unclear. There are two non-exclusive models: one involves the reactivation of silenced genes involved in cell growth, which is accompanied by cell cycle arrest and/or apoptosis [8]. Another model emphasizes the importance of the formation of covalent DNMT–DNA adducts, which leads to DNA damage and cytotoxicity [2].

Adducts represent a physical barrier for enzymes sliding along the DNA molecule and when they are not efficiently removed produces collisions with the transcriptional and replicative DNA machinery and become highly cytotoxic lesions. The participation of different DNA repair pathway are necessary for remove these complex lesions. Cancer cells can develop resistance to these inhibitors by increasing the activities of DNA repair pathways, such as homologous recombination (HR) and non-homologous end joining (NHEJ). A better knowledge of the DNA repair pathways

activated by a specific chemotherapy agent could provide us insights about the induced lesions as well as indicate additional targets to improve the therapy. Recently we have demonstrated that cytotoxicity of the epigenetic drug 5-azadC can, at least in part, be explained by collapsed replication forks requiring HR for repair [9]. As a continuation of our previous work, in the current study we have evaluated the importance of the lesions induced by zebularine in terms of DNA damage and cytotoxicity as well as the role of HR in the repair of these lesions. Overall, our data suggest that zebularine induces replication-dependent double strand breaks (DSB) which are preferentially repaired by HR.

2. Materials and methods

2.1. Chemicals

Zebularine, 5-azadC and aphidicolin (APH), were purchased from Sigma (St Louis, MO, USA). Zebularine and 5-azadC were diluted in phosphate buffered saline (PBS) (10 mM sodium phosphate (pH 7.4), 140 mM NaCl and 3 mM KCl). APH was diluted in dimethyl sulfoxide. Both of them were aliquoted and stored at −80°C before use. Cytochalasin B (Sigma) was diluted in DMSO, aliquoted and stored at-20ºC.

2.2. Cell culture

The parental Chinese hamster ovary AA8 cell line and the repair deficient EM9 mutant were purchased from the American Type Culture Collection (ATCC), USA. EM9 cells were originally derived from AA8 cells and are known to be single strand break (SSB) repair deficient with a specific defect in XRCC1 [10,11]. The V3-3 DSB mutant cell line derived from AA8 known to be defective in DNA-PKcs (NHEJ) [12]. VC-8 cell line, derived from the V79 Chinese hamster lung fibroblast cell line defective in *BRCA2* gene resulting in impaired HR and VC-8B2 cell line (VC-8 cells complemented with human *BRCA2*) [13]. The SPD8 cell line derived from V79 carries a mutation in the *HPRT* gene and was used for determination of recombination frequencies [14]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (LONZA) containing 1000 mg/L glucose and 110 mg/L sodium pyruvate supplemented with 10% fetal bovine serum, 2 mM L-glutamine and the antibiotics penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were cultured at 37°C in an atmosphere containing 5% CO_2. Exponentially growing cultures were used for all experiments.

2.3. Clonogenic survival

Cell survival following zebularine treatment was measured by clonogenic assay. Cell lines were plated at low density onto 10 cm Petri dishes. After 4 h, cultures were incubated for 24 h (two rounds of DNA replication approximately) in the presence of different doses of zebularine (0.2, 0.4, 0.6 and 0.8 mM) or 5-azadC (ranging from 0.5-7.5 µM). For combined experiments (zebularine + APH), cells were treated for 12 h with zebularine. Then zebularine was discarded and cultures were allowed to recovery for 12h in the presence or absence of the DNA synthesis inhibitor APH (0.5 µM). After that, fresh media was added, and cells were allowed to grow from 7 to 10 days. Colonies were stained with methylene blue prepared in methanol (4 g/l). Surviving colonies made up of >50 cells per colony were counted. The data have been corrected according to cloning efficiencies of control cells.

2.4. Reversion assay and analysis of SCEs

For the reversion assay, SPD8 cells were grown in the presence of 5 µg/ml 6-thioguanine in order to reduce the frequency of spontaneous reversion prior to treatments. The protocol for the reversion assay with SPD8 cells began with the inoculation of flasks (75 cm^2) with 1.5×10^6 cells in DMEM 4 h prior to a 24 h treatment with different doses of zebularine (ranged from 0,1 to 0,4 mM) in a 5% CO_2 incubator. After treatment, the cells were rinsed three times with 10 ml of PBS, and 30 ml of DMEM was added to allow recovery for 48 h. The selection of revertants was performed by plating three dishes/group (3×10^5cells/dish) in the presence of hypoxanthine-L-azaserine-thymidine (HAsT; 50 mM hypoxanthine, 10 mM L-azaserine, 5 mM thymidine). The cells were grown for 12 days before fixation with methanol, and then stained with a 2% Giemsa solution [14,15].

For BrdU labelling, BRCA2-deficient VC-8 cells and their corresponding parental VC-8B2 cells were treated with different doses of zebularine (ranged from 20 to 80 µM) in the presence of 10 µM of 5-bromodeoxyuridine (BrdU) for 24 h (two cell cycle periods). After, zebularine was discarded, cells were washed in fresh medium and then allowed to recover in the presence of BrdU for another round of DNA replication (12 h) and pulsed with 0.1 µg of Colcemid per ml for the last 2,5 h. The flasks were shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 75 mM KCl for 2 min, fixed with methanol:acetic acid (3:1) and dropped onto clean glass microscope slides. Differential staining of BrdU-substituted sister chomatids was obtained by the fluorescence-plus-Giemsa (FPG) method of Perry and Wolf [16] modified by Morgan et al. [17]. A total of 100 metaphases were analysed from two independent experiments for each experimental point.

2.5. Analysis of chromosomal aberrations and micronuclei

Colcemid synchronized AA8 cells [18] were grown for 12 h in the presence of 20 and 40 mM of zebularine. After that, cells were incubated with fresh media for 12 h before mitotic arrest (colcemid 0.1 µg/ml). Then, metaphases were collected and analysed for chromosomal aberrations [9].

For micronuclei analysis, VC-8 and VC-8B2 cells were treated for 12 h with 0.4 mM of zebularine. After that, they were thoroughly washed and allowed to grow in the presence or absence of 0.5 µM of APH for a further 12 h. Then, APH was discarded, and cultures were allowed to recover in fresh media for 18 h in the presence of cytochalasin B at a final concentration of 3 µg/ml. At the end of the treatment, cells were trypsinized and then fixed in a methanol-acetic solution (3:1). Cytological preparations were made by dropping cells onto wet slides and staining with Giemsa. Two thousand binucleated cells was scored for micronucleus frequency in each experimental point.

2.6. Immunofluorescence labeling and microscopy (Foci detection)

VC-8 and VC-8B2 were cultured on coverslips and treated for 12 hours with 0.4 mM of zebularine. Then washed and allowed to grow with or without 0.5 µM of APH for a further 12 h. XRCC1-deficient EM9 cells and their corresponding parental AA8 cells were seeded on coverslips the day before being treated with zebularine (0.1, 0.2 and 0.4 mM) for 24 h.

At the end of the treatments, cells were washed with PBS and incubated for 30 s with cold 0.1% Triton X-100 in PBS to pre-extract soluble protein unlocated in foci. Afterwards, cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature. The antibodies used were a

rabbit polyclonal antibody α-RAD51 (H-92, Santa Cruz) or α-53BP1 (H-300, Santa Cruz) and a mouse monoclonal α-γH2AX (Upstate). Secondary fluorescent antibodies were an Alexa fluor 488-conjugated goat anti-mouse and an Alexa fluor 555-conjugated goat anti-rabbit (Invitrogen). DNA was stained with 100 nM of 4,6 diamidino-2-phenylindole for 15 min. Slides were mounted in anti-fade media (Vectashield). Immunofluorescence was observed using a Nikon eclipse 50i microscope with a 40-fold magnification objective. Cells with ≥10 or ≥20 foci were scored as positive. At least 200 nuclei were analyzed for each treatment.

2.7. Transfection procedures

All transient transfections were performed using JetPRIME® transfection reagent according to the manufacturer's protocol (PolyPlus). HeLa cells were transfected with XRCC1-RFP plasmid and 24 h later treated with zebularine (ranged from 0.4 to 1.6 mM) for 48 h. Cells were then fixed using 4% paraformaldehyde and mounted for microscopy. Cells containing more than 10 foci were scored as positive.

2.8. Statistical analysis

For the determination of significance of the difference between the means, Student's *t*-test was used (SPSS IBM software). The results come from at least two independent experiments and are presented as mean ± standard deviation (SD) of the mean. Differences were considered significant when $*P < 0.05$ or $**P < 0.01$.

3. Results

3.1. HR defective cells are preferentially sensitive to the cytotoxic effects of zebularine

Mammalian cells generally repair DSB either by NHEJ or by HR pathways. In order to study the importance of these two DNA repair pathways to zebularine, we used Chinese hamster cell lines with well characterized defects in NHEJ or HR [12,13]. Both parental and mutant rodent cells were plated at low density for clonogenic survival and then treated with increasing doses of zebularine. Survival data from Figure 1A shows that V3-3 cells were more sensitive to zebularine treatment, with a significant decrease in cell survival to all doses tested compared with its parental cell line AA8. The sensitization ranged from 1.3 to 2 times for the doses of 0.2 to 0.8 mM, respectively (Figure 1D). We could not observe any significative difference using the same model when cells were treated with 5-azadC (Figure 1B), a drug that also induces DNMT adducts. On the other hand, the VC-8 cell line defective in HR was more sensitive to zebularine as compared to VC-8B2 (Figure 1C). However in this case, the sensitization ranged from 1.7 to 4 times for the highest dose assayed (Figure 1C). We also wanted to see whether this sensitization could be observed in human cells deficient in HR. For that, control or BRCA2 siRNA depleted HeLa cells were seeded at low density and treated with zebularine for 48 hours. Then cells were allowed to form colonies in drug free media. Data presented in Supplementary Figure 1 show that BRCA2 silenced human cells are more sensitive to zebularine.

Overall these data suggest that both NHEJ and HR pathway prevent the accumulation of lethal DNA DSD induced by zebularine. Cell lines deficient in DNA DSB showed higher sensitivity to zebularine, with the HR defective cells VC-8 worst affected. This is consistent with a dominant role of HR in the repair of lesions induced by zebularine.

3.2. Zebularine promotes homologous recombination

Given that HR appears to be the predominat pathway choice to repair cytotoxic damage induced by zebularine, we wanted give further support this by employing the SPD8 reversion assay to detect functional HR by an exchange mechanism [14,15]. Here a partial duplication of exon 7 of the hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene that arose spontaneously in SPD8 cells, leads to expression of non-functional HPRT protein and reversion to wild type by homologous recombination can be selected for in HAsT media. Colonies formed following selection are therefore indicative of HR. We found a dose-dependent increase in HR in the *HPRT* gene when SPD8 cells were treated with zebularine (Figure 2A). In this system, following a 24 h treatment with 0.4 mM zebularine there was a nearly 5-fold induction in HR compared to spontaneous levels. As a second measure of HR, we explored the frequency of homologous recombination events evidenced as the exchange of large DNA regions between sister chromatids (SCEs) [16,17]. It is well know that HR between sister chromatid is the primary mechanism of SCEs [17]. They can be induced by various genotoxic treatments, and the analysis of SCEs in vertebrate cells provides a striking insight not only into the cellular response to potential carcinogens but also into the frequency of spontaneous lesions requiring HR-mediated repair during DNA replication and the accuracy with which the HR machinery repairs them [19]. On the other hand, the defective accumulation of SCEs indicates defects in HR activation at replication-associated DSB [20,21]. While the basal level of SCEs was the same, treatment with zebularine increased the frequency of SCEs in *BRCA2*-complemented VC-8B2 Chinese hamster fibroblasts but not in *BRCA2*- deficient cells (Figure 2B). This result suggests that zebularine-induced DSB are, at least in part, repaired by BRCA2- dependent sister chromatid exchange.

3.3. DNA damage induced by zebularine is dependent upon DNA replication

The prevailing model of how replication-dependent DSB and chromosomal aberrations arise after methylation damage involves replication fork stalling and collapse at methylated base adducts, similar to the mechanism of DSB formation after prolonged HU treatment [22].

To bring light about the nature of zebularine-induced chromosomal aberrations , we treated AA8 cells during one cell cycle with zebularine, then cells were allowed to recover in fresh media for another cell cycle before to be processed for chromosome aberrations analysis (Figure 3A).

Figure 3B shows that AA8 control metaphases showed only low percentages of abnormalities, whereas zebularine treatment induced an increase in chromosomal aberrations. Importantly, only a slight induction of isochromatid breaks was observed. This finding seems to indicate that zebularine behaves as a typical S-phase dependent agent.

We hypothesized that after zebularine treatment, complex DNA lesions such as DNA-protein adducts may represent obstacles to DNA replication that could be converted in one-ended double-strand ends generated at collapsed replication forks. The deficient resolution of replication-coupled DSB increases replication-derived chromatid aberrations, which are specifically generated in S phase [23].

While zebularine is not expected to directly cause DSB, DNA DSB could arise indirectly when the replication fork reaches a single strand break or lesion, and causes replication fork collapse. Replication inhibitors cause replication fork stalling and DSB does result from processing of stalled forks. During recovery from replication fork blocks, the HR factor RAD51 mediates fork restart and DSB repair [22]. HR therefore protects cells to replication inhibitors, with clear

implications for cancer therapy. Zebularine treatment induces the formation of protein covalent adducts. From this it could be argued that a reduction in DSB formation should be observed in the presence of the DNA synthesis inhibitor aphidicolin (APH) as long as damage induced by zebularine is related to their interaction with DNA replication fork progression. To test this, VC-8 and VC-8B2 cells were treated for 12 h, that is one cell cycle, with 0.4 mM zebularine, then cultures were washed and allowed to repair in fresh media or in media containing APH and DNA damage was measured as the percentage of γH2AX, 53BP1and RAD51 foci positive cells (Figure 4A). Since γH2AX foci can be formed in the absence of DSB [24], we evaluated other markers of DSB such as the phosphorylation of 53BP1 protein [25]. We saw that the percentage of cells with γH2AX foci did increase over the control values after zebularine treatment in both cell lines (Figure 4B). Similar results were obtained using 53BP1 DSB marker (Figure 4C). To investigate if HR is triggered by zebularine treatment, we monitored RAD51 foci formation. RAD51 is involved in the strand transfer reaction of HR and relocates into stable nuclear foci during HR repair [26]. We found that zebularine treatment increased the percentage of VC-8B2 cells containing RAD51 foci ~13 fold over spontaneous level. However, BRCA2 deficient cells displayed no RAD51 staining (Figure 4D).

Figure 4 also shows that treatment with APH alone induced DSB. However when APH was present during the second cell cycle, zebularine-induced γH2AX and 53BP1foci levels were specially reduced in BRCA2-deficient cells. More importantly, RAD51 foci were also disminished, which suggests that homologous recombination induction after zebularine treatment is linked to replication.

Micronuclei (MN) are formed when DSB are processed in a manner that excludes fragments of chromosomes from nuclei during/after karyokinesis and before cytokinesis [27]. Our data (Figure 4E) shows that BRCA2 deficient cells were markedly affected by zebularine treatment in line with data presented in Figures 1 and 2. When APH was present, we observed that MN frequencies were dramatically reduced in BRCA2 deficient cells.

Collectively, our observations imply that zebularine induces lesions that interfere with DNA replication where HR plays an essential role in the repair of these lesions.

3.4. Post-DNA damage replication inhibition rescues zebularine-induced toxicity

Cell survival was determined by clonogenic assay (Figure 5) using the same experimental schedule as for Figure 4. Survival data confirmed the increased sensitivity to zebularine of the mutant cell line defective in HR with a surviving fraction (SF) of 0.1 after 0.4 mM of zebularine. In contrast, zebularine treatment was well tolerated by the BRCA2 complemented cell line VC-8B2, showing high survival (SF of 0.8). As predicted, when APH was present during repair, the BRCA2 complemented cell line VC-8B2 was insensitive to changes in cell survival in contrast to the BRCA2-deficient cell line VC-8, which showed a 3-fold increase in cell survival. These results along with the data mentioned above (Figure 4) highlight the importance of HR in the processing of DSB that arise when replication forks are collapsed with zebularine lesions.

3.5. XRCC1 deficiency leads to induction of DSB that are substrates for Homologous Recombination after zebularine treatment.

As an anomalous base, it is possible that zebularine is recognized and repaired by an excision repair mechanism. After a DNA glycosilase excision and breakage of the sugar-phosphate backbond, downstream repair depends on XRCC1. XRCC1 is essential for both BER and SSB

repair and it has been shown that XRCC1 deficient mice are embryonic lethal [28]. Also XRCC1 deficient cells display increased levels of spontaneous γH2AX and RAD51 foci [29], and are hypersensitive to agents that induce SSB or base damage [30,31]. HeLa cells were transfected with XRCC1-RFP plasmid and the next day treated with different doses of zebularine for 48 h. The percentage of cells showing more than ten foci is depicted in Figure 6A. As can be seen, zebularine promotes XRCC1 recruitment at DNA damage sites which is important for repair.

To test whether XRCC1 plays an essential role in survival after a zebularine treatment, we used XRCC1 defective cells to determine a potential role of BER in repair of induced lesions. EM9 cells deficient for XRCC1 expression and its parental wild-type AA8 cells were plated at low density for clonogenic survival and then treated with increasing doses of zebularine. EM9 cells were shown to be significantly more sensitive to zebularine than XRCC1 WT AA8 cells (Figure 6B). It has been shown that SSB or unrepaired lesions can collapse replication forks and generate DSB [30]. These collapsed forks are known substrates for homologous recombination (HR) [29,31,32]. Therefore, we next wanted to determine whether the dramatic sensitization seen in EM9 cells was due to a lack in the repair of zebularine induced lesions. We show a steady increase in the levels of γH2AX/53BP1 and RAD51 foci in EM9 cells as compared with wild type cells (Figure 6C-E). Furthermore, γH2AX and RAD51 foci co-localized, showing that the induced DSB are indeed repaired by HR (Figures 6F and 7). Taken together, these data demonstrate that in the absence of XRCC1, zebularine induces lesions that result in higher levels of DSB and HR repair activation.

4. Discussion

Zebularine is a DNMT inhibitor with promising results as an antitumor agent because of its minimal toxicity, considerable stability, and selective effects toward cancer cells [33]. After its incorporation into DNA, it functions as a suicide substrate for DNMT enzymes. Trapped DNMT are thought to induce a DNA repair mechanism independently of its demethylation action [34].Therefore, the DNA-DNMT adduct lesions that results following zebularine treatment and the processes by which cells deal with them is predicted to play a major role in their antitumoral activity.

DSB repair occurs by homology-dependent and homology-independent recombination mechanisms. Homology-independent recombination relies on the direct joining of DNA ends and is referred to as NHEJ. This process is considered imprecise in many cases resulting in the deletion or insertion of a few nucleotides. Repair by HR relies on identical sequence stretches serving as templates and therefore usually restores the original sequence [35]. In this paper we have studied the response to zebularine of mutant cells defective in different DNA repair pathways. Our observations suggest that zebularine induces lesions that interfere with DNA replication where HR is the most important pathway in the repair of such lesions [32,36]. HR is required for repairing multiple types of DNA damage including single stranded DNA (ssDNA), DSB and DNA cross-links. In addition, HR is a critical mechanism for recovery of stalled or broken DNA replication forks. Furthermore, certain genetic alterations, such as *BRCA2* mutations, are associated with increased risk of malignancy and enhanced sensitivity to chemotherapeutic agents, including inhibitors of the enzyme poly-ADP ribose polymerase (PARP) [37]. Concerning this, we hypothesized that DNA-DNMT adducts induced by zebularine generate DNA replication blocking lesions either directly or due to repair intermediates formed during DNA repair. This could lead to collapse replication forks forming DSB, which can be the substrate for HR.

As replication fork associated DSB are repaired by HR and mainly produce SCE events, this model would explain the increase SCE levels observed in VC-8B2 cells treated with zebularine (Figure 2B). Further support for this is the evidence that DNA replication is required for zebularine-induced DSB. Concerning this, we found a significant reduction in zebularine-induced γH2AX and 53BP1 foci formation when VC-8 cells were cultured in the presence of the DNA polymerase inhibitor APH after zebularine incorporation. As expected, this protective effect of APH was also evidenced in cell survival and MN formation and was less evident in the BRCA2-proficient cell line (Figures 4 and 5). This observation suggests that zebularine may act by increasing the number of lesions that collapse replication forks during elongation [29].

Our results are in agreement with previous studies done in our laboratory showing that cytotoxicity of the epigenetic drug 5-azadC can, at least in part, be explained by the generation of collapsed replication forks requiring HR for repair after 5-azadC treatment [9]. Recombination-inducing replication intermediates cannot be repaired by pathways that require two free DNA ends, notably NHEJ and SSA. However, in our study, DNA-PK deficient cells (V3-3) resulted more sensitive to zebularine than wild type cells, so that NHEJ is needed to promote survival. We hypothesize that a minor induction of two-ended DSB could be generated and that NHEJ can contribute to a proper repair of zebularine lesions. Supporting this we report a small increase of isochromatid breaks when zebularine was administered during one cell cycle (Figure 3B).

However, cells deficient in NHEJ were not sensitized to 5-azadC. This means that in contrast to zebularine, lesions induced by 5-azadC do not appear to be repaired by NHEJ (Figure 1B). Relative to this, we think that there could be differences in the nature/processing of DNA damage induced by zebularine and 5-azadC, two cytidine analogs with apparent similar mechanism of action. Indeed, Champion et al [34] showed that 5-azadC forms irreversible complexes while those formed by zebularine appeared to be reversible. In agreement with this, we did not observe any induction of DNMT1 foci in DNMT1-GFP overexpressing cells treated with zebularine, as compared with 5-azaC or 5-azadC (data not shown).These data can be explained by the instability of zebularine-DNMT complexes and would be the reason for the different cytotoxicity and demethylating capacity showed by these two inhibitors. In a previous paper [38] we suggested that 5-azadC could induce clustered-DNA lesions in CpG islands. Clustered DNA lesions, i.e. those induced by ionizing radiation or oxidative stress, can be repaired via error-prone NHEJ leading to mutations [39]. Actually, 5-azadC induced mutations occur in CpG islands [40]. Given as Zebularine-DNMT complexes are less stable, it could be argued that clustered DNA lesions induced by Zebularine would be produced in a lower frequency and as a consequence, NHEJ could act in an error-free mode promoting survival.

On the other hand, our data agree with the findings of Liu et al. (2015) who observed in *Arabidopsis Thaliana* that zebularine-induced DNA damage is repaired by HR using mainly a synthesis-dependent strand annealing (SDSA) mechanism [41]. Concerning this, DSB can be repaired by several HR-mediated pathways, including double-strand break repair (DSBR) and SDSA [42]. In DSBR, double Holliday junctions are formed between the participating DNA molecules as recombination intermediates which have to be resolved. This resolution results in either crossovers visualized as SCEs or gene conversions (Figure 2). Alternatively, the reaction can proceed via SDSA, in this case, the repair product from SDSA is always non-crossover and does not generate SCEs. Therefore our data suggest DSBR as the main mechanism involved in the repair of DSB induced by zebularine in mammalian cells. In contrast SDSA and not the DSBR mechanism is suggested to be as the main used for the repair of DSB in somatic plant cells [41]. Pathway choice is determined by several factors including cell-cycle phase, time specificity, lesion structure, and organism [43]. HR is only one pathway of DSB repair and can collaborate with and compete with

other pathways to repair DSB. Therefore, the redundancy of multiple pathways plays a vital role in cell survival.

The XRCC1 protein does not have enzymatic activity, but acts as a scaffolding protein that interacts with multiple repair enzymes. The scaffolding allows these repair enzymes to then carry out their enzymatic steps in repairing DNA. XRCC1 is involved in single-strand break repair, base excision repair, nucleotide excision repair and alternative-NHEJ [44,45]. When we did evaluate the role that XRCC1 play in the repair of lesions induced by zebularine we found the following data supporting this notion: a) XRCC1 was recruited in foci in zebularine treated cells, b) XRCC1 deficient cells were more sensitive and displayed an increased level of DNA lesions, c) DNA lesions in XRCC1 defective cells were repaired preferentially by HR as γH2AX foci colocalizes with RAD51 (Figures 6 and 7). Overall our results show that SSB-deficient cells have increased levels of HR and zebularine contributes to increase replication fork collapse and thus the DSB observed.

DNA repair pathways can enable tumour cells to survive DNA damage that is induced by chemotherapeutic treatments; therefore, inhibitors of specific DNA repair pathways might prove efficacious when used in combination with DNA-damaging chemotherapeutic drugs. In addition, alterations in DNA repair pathways that arise during tumour development can make some cancer cells reliant on a reduced set of DNA repair pathways for survival. There is evidence that drugs that inhibit one of these pathways in such tumours could prove useful as single-agent therapies, with the potential advantage that this approach could be selective for tumour cells and have fewer side effects [46]. Considering the reduced survival of the BRCA2-deficient cell line after zebularine treatment, our results indicate that BRCA2 is involved in the repair of zebularine-induced DNA damage, and suggests that people carrying mutations in *BRCA2* (a gene whose mutation confers increased lifetime risk of developing breast and ovarian cancer), or sporadic cancer that have acquire this mutation, might be hypersensitive to the DNA-damaging and cytotoxic effects of zebularine. This offer the possibility that zebularine may have potential therapeutic value in the treatment of HR-deficient tumours. In closing, the data presented herein about the hypersensitivity of XRCC1-deficient cells to zebularine (presumably results from the accumulation of unrepaired SSB, which give rise to recombinogenic DSB), support a critical function for XRCC1 in DNA repair and also call attention to its potential as a therapeutic target.

In conclusion, our data point to a model to explain the effects of zebularine, where incorporated zebularine traps DNMT onto DNA, which becomes an obstacle to the second round of replication and results in a collapsed replication fork forming a DSB. Such replication-associated DSB is normally repaired in mammalian cells by RAD51-mediated HR, which results in an SCE.

Conflict of interest

The authors declare that there are no conflicts of interest

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FIGURE LEGENDS

Figure 1. Non Homologous End-Joining (NHEJ) and Homologous Recombination (HR) are required to promote survival after zebularine treatment. (**A, B**) Clonogenic survival of AA8 (wild type) and V3-3 (DNA-PKcs deficient) after zebularine and 5-azadC treatments respectively. (**C**) Survival of VC-8 (BRCA2 deficient) and VC-8B2 (VC-8 complemented with *BRCA2*) after zebularine treatments. For all survival experiments cells were seeded on Petri dishes and allowed to attach for 4h. Then, they were treated for 24 h with increasing doses of zebularine. After treatment, media was changed and they were allowed to form colonies. (**D**) Fold induction in cell death following zebularine treatment. Comparisons between NHEJ and HR deficient cell lines are depicted. Data show the mean ± SD from two to three independent experiments. Values marked with asterisks are statistically significant (*$p < 0.05$, **$p < 0.01$).

Figure 2. Zebularine promotes Homologous Recombination. (**A**) Recombination frequencies in SPD8 cells. Cells were treated for 24 h with increasing doses of zebularine. After treatment, media was changed and they were allowed to recover for 48h. $HPRT^+$ revertants were analysed seven days later. Data show the mean ± SD of two independent experiments. (*$p < 0.05$). **(B)** Zebularine induces sister chromatid exchanges in BRCA2 deficient cells. The data represent the increase in SCEs/cell after increasing doses of zebularine.

Figure 3. Chromosomal aberrations induced by zebularine.(**A**) Experimental schedule: G1 synchronized AA8 cells were cultured for 12 h (first round of DNA replication) in the presence of zebularine (20 and 40 μM), washed and allowed to recover for 12 h (second round of DNA replication) before mitotic arrest. (**B**) Two hundred metaphases were analyzed for chromosomal

aberrations in each experimental point. Breaks may occur during interphase, before and after replication. Only lesions produced after replication are visible in the following metaphase with only one chromatid affected. Data show that zebularine induce damage in a S-dependent manner. Data show the mean ± SD from two to three independent experiments. Values marked with asterisks are statistically significant (*$p < 0.05$).

Figure 4. Zebularine induces replication-dependent DSB. (**A**) Experimental schedule: VC-8 and VC-8B2 were treated with 0.4 mM of zebularine for 12 h, then cultures were washed and allowed to repair in fresh media or in media containing 0.5 μM of APH for an additional 12 h before analysis of nuclear γH2AX, 53BP1 and Rad51 foci by inmunofluorescence. Quantification of γH2AX (**B**), 53BP1 (**C**) and RAD51(**D**) foci was evaluated in 200 nuclei for each treatment. Cells with >10 foci were scored as positive. (**E**) Analysis of micronucleus induced by zebularine. As described in materials and methods, we used the cytokinesis-block micronucleus technique for measuring DNA damage. Two thousand binucleated cells were scored for micronucleus frequency in each experimental point. Data show the mean ± SD of two independent experiments. (*p < 0.05).

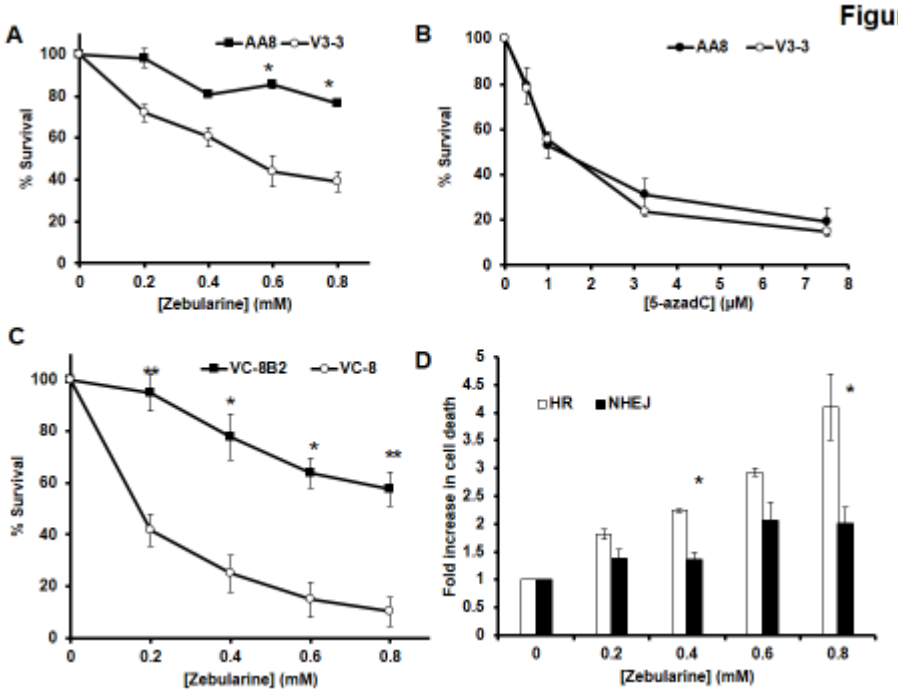
Figure 5. Cytotoxicity of zebularine is linked to DNA-replication damage. Cell survival of VC-8 and VC-8B2 treated for 12h with zebularine and co-treated for another 12h with and without 0.5 μM aphidicolin (APH), then left to grow in normal media for 10-14 days. The statistical significance compared with APH-treated cells was determined by Student`s *t*-test (*$p < 0.05$).

Figure 6. XRCC1 promotes survival after a treatment with zebularine. (**A**) HeLa cells expressing the XRCC1-RFP were seeded on coverslips and exposed to increasing doses of zebularine for 48 h. Then, cells were fixed and the percentage of XRCC1 positive cells was scored. (**B**) Clonogenic survival following exposure to zebularine for 24h was studied in parental AA8 cell line and XRCC1 deficient cell line EM9. The mean ± SD of three independent experiments is depicted. Quantification of γH2AX (**C**), 53BP1 (**D**) and RAD51 (**E**) foci in response to zebularine treatment. AA8 and EM9 cells were seeded on coverslips, treated for 24 h with increasing doses of zebularine, fixed and foci were inmunodetected and analysed. Two hundred nuclei for each experimental point was analysed. Cells with >10 foci were scored as positive. The mean ± SD of two independent experiments is depicted. The statistical significance was determined by Student`s *t*-test (*$p < 0.05$). (**F**) The number of RAD51 foci that colocalize with γH2AX per cell is depicted for AA8 and EM9 cells after 0.4 mM of zebularine for 24 hours. 30 cells from confocal microscope pictures were used to quantify colocalization (Image J).

Figure 7. BER deficiency results in higher levels of DSB and HR repair activation. Representative images of nuclei from control and zebularine treated (0.4mM, 24h) AA8 and EM9 cells are depicted. Panel shows nuclei stained for DAPI, γH2AX and RAD51 as well as colocalization of foci. RAD51 and γH2AX colocalization suggests that DSB formation recruits HR for the repair process.

Figure S1. BRCA2 depletion sensitizes HeLa cells to zebularine. **(A)** Depletion of BRCA2 after siRNA transfection. HeLa cells were transfected with control siRNA or BRCA2 siRNA for 72 hours. BRCA2 was monitored by SDS-PAGE **(B)** After transfection, cells were seeded at low density and treated with zebularine for 48 hours. Cells were allowed to form colonies in drug free media for 9 days. Values are relativized to vehicle control (100 %). The data represent the mean ± SE from two independent experiments. Values marked with asterisk are statistically significant (*$p < 0.05$)

Figure 1



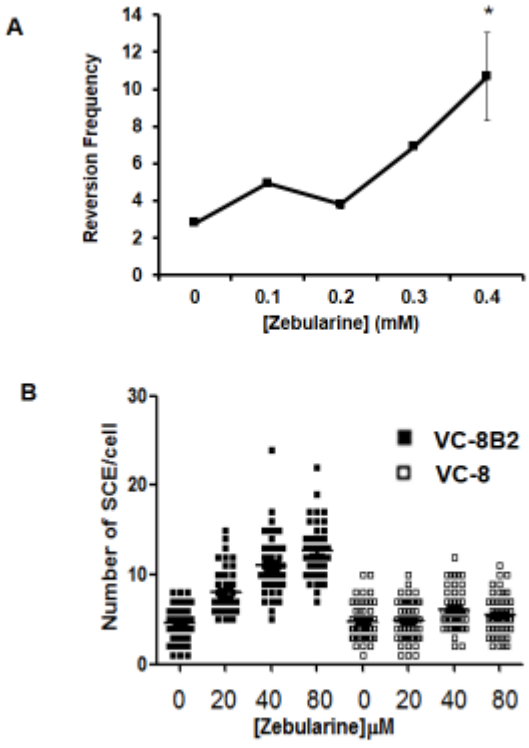


Figure 2

Figure 3

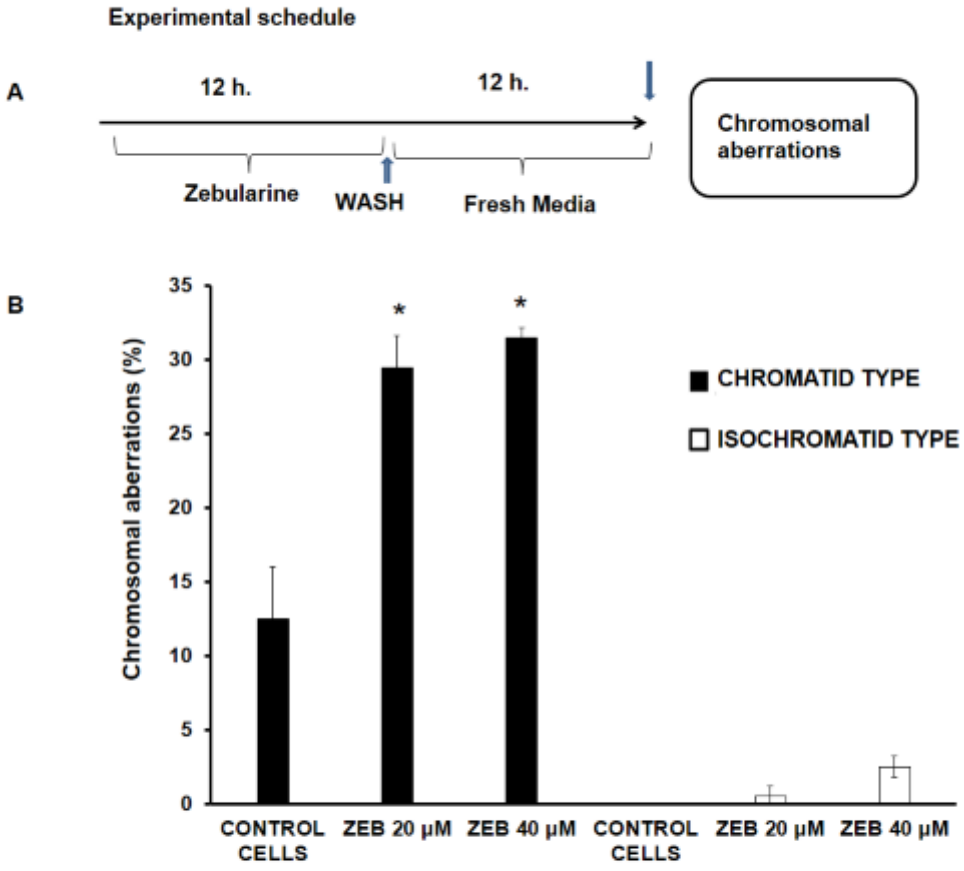


Figure 4

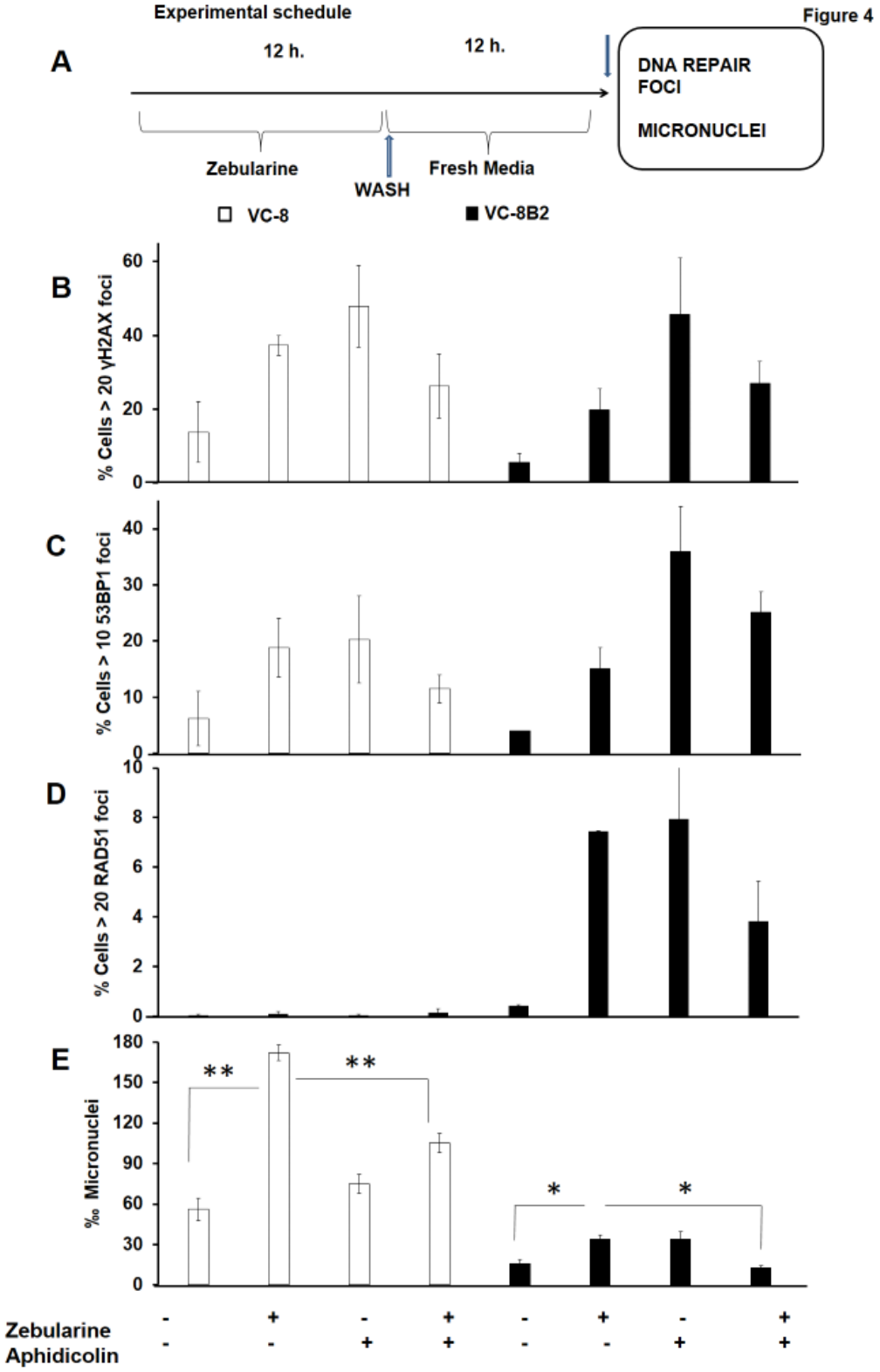


Figure 5

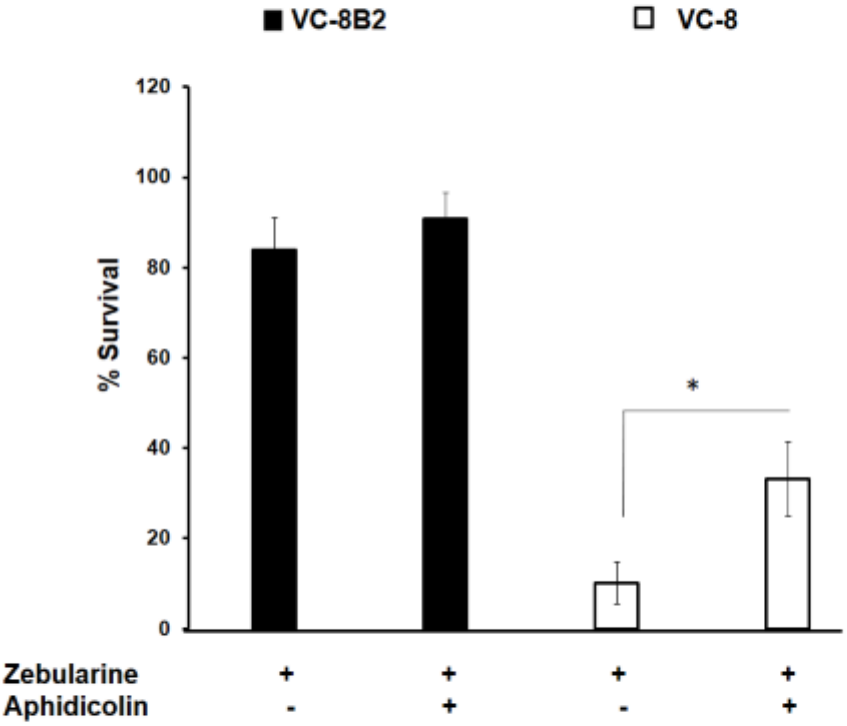


Figure 6

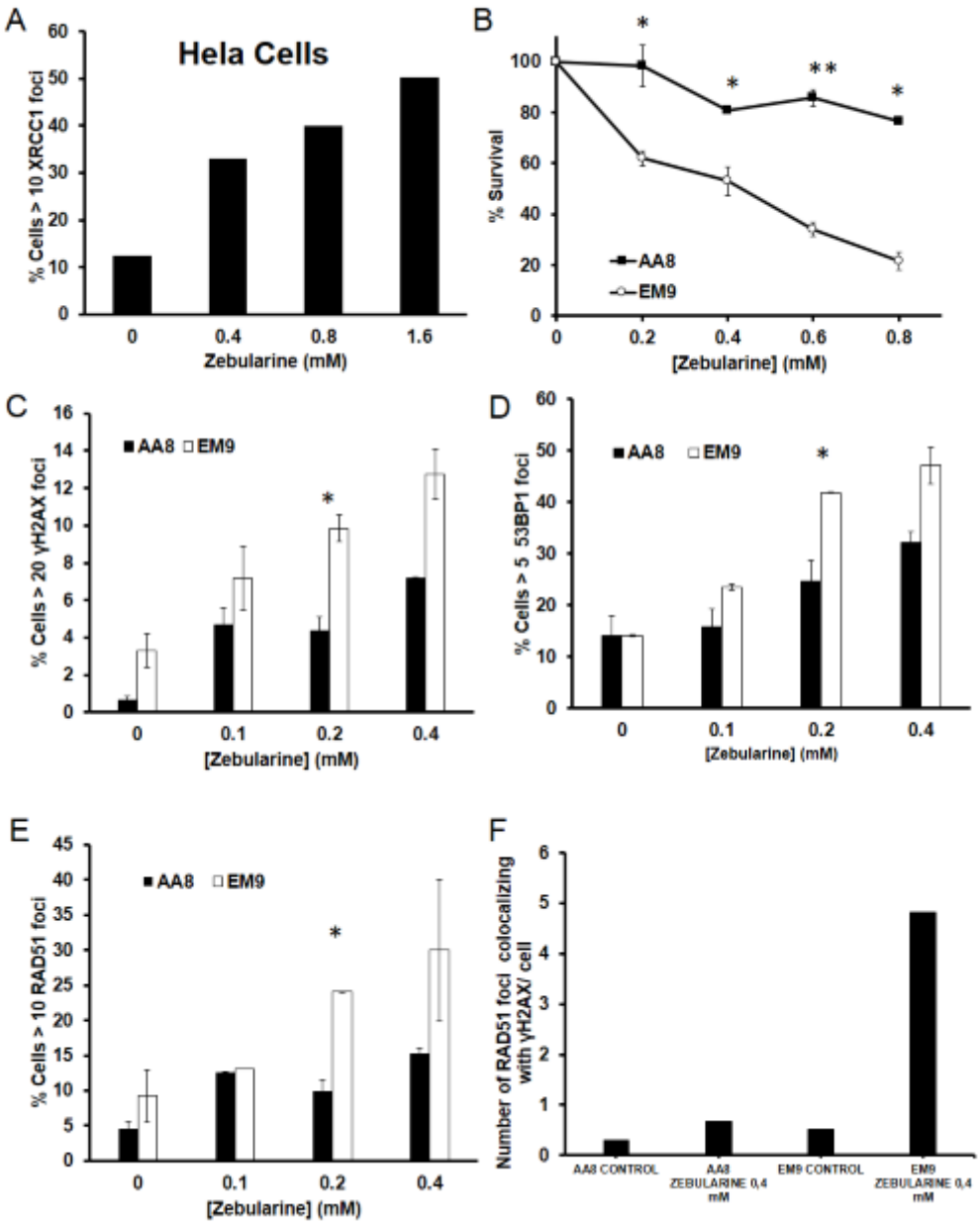


Figure 7