

RESEARCH PAPER



Diosmetin enhances the sensitivity of radiotherapy by suppressing homologous recombination in endometrial cancer

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ABSTRACT

Radiotherapy is an essential treatment for endometrial cancer (EC), especially in advanced, metastatic, and recurrent cases. Combining radiotherapy, which mainly causes DNA double-strand breaks (DSBs), with small molecules targeting aberrantly activated homologous recombination (HR) repair pathways holds great potential for treating ECs in advanced stages. Here, we demonstrate that diosmetin (DIO), a natural flavonoid, suppresses HR, therefore inhibiting cell proliferation and enhancing the sensitivity of EC to radiotherapy. Clonogenic experiments revealed that combining DIO and X-ray significantly inhibited the viability of EC cells compared to cells treated with diosmetin or X-ray alone. The survival fraction of EC cells decreased to 40% when combining 0.4 Gy X-ray and 4 μ M DIO; however, each treatment alone only caused death in approximately 15% and 22% of cancer cells, respectively. Further mechanistic studies showed that diosmetin inhibited the recruitment of RPA2 and RAD51, two critical factors involved in the HR repair pathway, upon the occurrence of DSBs. Thus, we propose that a combination of diosmetin and irradiation is a promising therapeutic strategy for treating endometrial cancer.

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Introduction

Endometrial cancer is the fourth most common gynecologic malignancy and the sixth leading cause of cancer death in women, as estimated by the American Cancer Society, in the United States [1], with 76,000 deaths among women every year worldwide [2]. The incidence and death rates of endometrial cancer are still rapidly rising. Contemporary treatment of endometrial cancer mainly includes surgery, which is often followed by radiotherapy, chemotherapy, or both, depending on risk factors including grade, lymph node metastasis, and muscle layer and vascular invasion [3]. Radiotherapy, which involves the production of excessive DNA damage to induce cell death, has been widely used for decades to treat more than 50% of cancer patients, and postoperative radiotherapy significantly reduces the risk of local recurrence in EC patients [4]. DNA double-strand breaks

(DSBs), the most lethal type of DNA damage to eukaryotic cells, are mainly repaired by homologous recombination (HR) and non-homologous end joining (NHEJ) in higher eukaryotes. NHEJ can be activated throughout the cell cycle, and HR is an accurate repair pathway that occurs during S and G2 phases, when sister chromatids are present [5]. As malignant endometrial cells rapidly divide [6], they may experience high replication stress. HR is the major pathway for relieving such stress [7], thereby promoting cell survival. Indeed, several studies have indicated that HR is upregulated in a number of tumor types in comparison to normal adjacent tissues [8–10]. On the other hand, upregulated DSB repair by HR in cancer cells might confer radioresistance. Therefore, suppressing DNA repair pathways, especially HR, might constitute an efficacious strategy to increase the sensitivity of EC cells to DNA damage inducers such as irradiation.

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Diosmetin (DIO) is an O-methylated flavone (3,5,7-trihydroxy-4-methoxyflavone) and the aglycone component of the flavonoid glycoside diosmin. Diosmetin can be extracted from citrus fruit and olive leaves [11,12]. It is also the active compound of *Galium verum* L, a traditional Chinese herb that has been used for decades to promote blood circulation and expel miasma [13]. Previous studies have shown that diosmetin possesses anti-inflammatory [14], antioxidant [15] and antitumor properties in different biological contexts. As reported, diosmetin induces cell cycle arrest as well as apoptosis in liver cancer [16], prostate cancer [17], breast cancer [18] and chronic myeloid leukemia [19] by targeting different pathways. Diosmetin has also been shown to downregulate expression of MMP-2 and MMP-9 to inhibit metastasis of hepatocellular carcinoma cells [20]. In addition, diosmetin has been demonstrated to act as a natural dietary agonist of AhR that is capable of inhibiting CYP1A1 enzyme activity to suppress carcinogen activation in breast cancer [21,22]. Nevertheless, its effect on endometrial cancer is not yet clear. Here, we corroborate that diosmetin destabilizes genomes by suppressing DSB repair, especially HR, in a cell cycle-independent manner. Diosmetin inhibits the recruitment of both RPA2 and RAD51 upon the occurrence of DSBs induced by X-ray. Further clonogenic studies indicate that combining diosmetin with X-ray synergistically suppresses the survival of endometrial cancer cells, suggesting that it has potential in future clinical applications.

Materials and methods

Cell culture

Hec-1B, ISK and AN3CA cells were cultured in DME/F-12 (SH30023.01, HyClone, Jiangsu, China) supplemented with 10% FBS (10270-106, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (15140-122, Gibco, Grand Island, NY, USA). HCA2-hTERT cells were cultured in Minimum Essential Medium Eagle (M4655, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (10270-106, Gibco, Grand Island, NY, USA), 1% MEM NEAA (11140-050, Gibco, Grand Island,

NY, USA) and 1% penicillin/streptomycin (15140-122, Gibco, Grand Island, NY, USA).

All cell lines were incubated in a 5% CO₂ humidified incubator (Thermo Fisher Heracell 240i, Thermo Fisher, Waltham, MA, USA) at 37°C. Mycoplasma testing was conducted regularly.

Cell viability assay

Hec-1B, ISK and AN3CA cells were seeded in 6-well plates at a density of 20,000 cells per well, followed by treatment with DMSO or diosmetin at the indicated concentrations at 24 hours after passaging. The cells were then collected, and cell numbers were calculated after 24, 48, and 72 hours of incubation.

Clonogenic assay

Hec-1B, ISK and AN3CA cells were seeded in 6-well plates at a density of 200 cells per well. At 24 hours postseeding, the cells were treated with DMSO or diosmetin at the indicated concentrations, followed by a 72-hour incubation. Afterward, the medium was replaced with fresh medium with no supplemented drugs.

Hec-1B cells were seeded in 6-well plates at a density of 200 cells per well. On day 1 postseeding, the cells were incubated with DMSO or diosmetin at the indicated concentrations for 24 hours. Then, the cells were X-ray irradiated at increasing dosages. The irradiated cells were cultured in the presence of diosmetin for 2 days, after which the medium was replaced with fresh medium.

The cells were incubated for 14 days before staining with Coomassie reagent (methanol: acetic acid: Coomassie: H₂O = 50: 10: 0.25: 40) for a time period of 4 hours. Afterward, the cells were washed with distilled water three times. Colonies with at least 50 cells were counted.

Comet assay

Hec-1B cells were seeded in 6-well plates at a density of 20,000 cells per well. After 24 hours, the cells were incubated with DMSO or the indicated concentration of diosmetin for 72 hours. The

cells were collected, washed with PBS once and diluted to 300,000 cells per milliliter. Genomic stability was analyzed with a Trevigen alkaline comet assay kit (Cat. number: 4250-050-K, Trevigen, Gaithersburg, MD, USA) according to the instructions. Pictures were taken and analyzed with Cometscore software (Sumerduck, VA, USA). At least 50 cells were quantified for each sample.

EdU incorporation assay

Hec-1B cells were seeded in 6-well plates at a density of 20,000 cells per well. At 24 hours postseeding, the cells were treated with DMSO or diosmetin at the indicated concentrations, followed by a 72-hour incubation. The cells were then harvested, incubated with 10 μ M EdU at 37°C for 2 hours, collected and treated with a Click-iT EdU Assay Kit (Invitrogen, Waltham, MA, USA, C10634) according to the manufacturer's instructions. EdU-positive cells were quantified using a FACSVerse (BD Biosciences, San Jose, CA, USA). At least 10,000 cells for each sample were analyzed.

Analysis of HR/NHEJ repair efficiency

Hec-1B cells were seeded in 10-cm dishes at a density of 1,000,000 cells per dish. At 24 hours postseeding, the cells were pretreated with DMSO or diosmetin at the indicated concentration for 24 hours, followed by transfection using a Lonza 4D machine. The transfected cells were immediately incubated with the above reagents for 72 hours before analysis using a FACSVerse (BD Biosciences, San Jose, CA, USA). At least 20,000 cells for each sample were analyzed.

Immunofluorescence assay

HCA2-hTERT cells were seeded on coverslips in 12-well plates at a density of 10,000 cells per well. After 24 hours, the cells were pretreated with DMSO or diosmetin (10 μ M) for 24 hours and then irradiated with X-ray (2 Gy) in the presence of diosmetin. At the indicated time points post-IR, the medium was removed, and the cells were

washed twice with PBS, followed by fixation with 4% paraformaldehyde for 15 min and permeabilization with 0.25% Triton X-100 (Sigma, Cat. #X100) for 10 min. The fixed and permeabilized cells were blocked with 2% bovine serum albumin for 1 hour and subsequently incubated with the primary antibody at 4°C overnight, followed by incubation with the specific fluorescent secondary antibody for 1 hour in the dark. Pictures were taken with a laser scanning confocal microscope (TCS SP8; Leica, Wetzlar, Germany), and for each sample, the number of foci for at least 50 cells was counted.

Results

Diosmetin impairs the genomic stability of EC cells and inhibits cell proliferation

The structure of diosmetin used in this study is shown in Figure S1A. We first examined whether diosmetin affects EC cell proliferation. Three different EC cell lines, ISK, Hec-1B and AN3CA, were treated with diosmetin at increasing concentrations. We observed a dose-dependent decline in cell proliferation rates in all three cell lines (Figure 1a), indicating a toxic effect of diosmetin on EC cells. Next, we examined whether diosmetin is able to impact genome integrity, therefore causing toxicity in EC cells. By using an alkaline comet assay, which is a sensitive method to measure genomic stability at the single-cell level, we observed that diosmetin destabilized the genomes of Hec-1B cells. Indeed, the tail moments of 5 μ M diosmetin-treated Hec-1B cells were 2-fold higher than those of DMSO-treated cells (Figure 1b–c), demonstrating that diosmetin impaired the genomic stability of Hec-1B cells.

Diosmetin negatively regulates HR repair in EC cells

Given that diosmetin influences genomic stability and that DNA repair plays an indispensable role in the maintenance of genomic stability [23], we hypothesized that diosmetin might have an impact on DNA repair. We compared the efficiency of HR and NHEJ, two major DSB repair pathways,

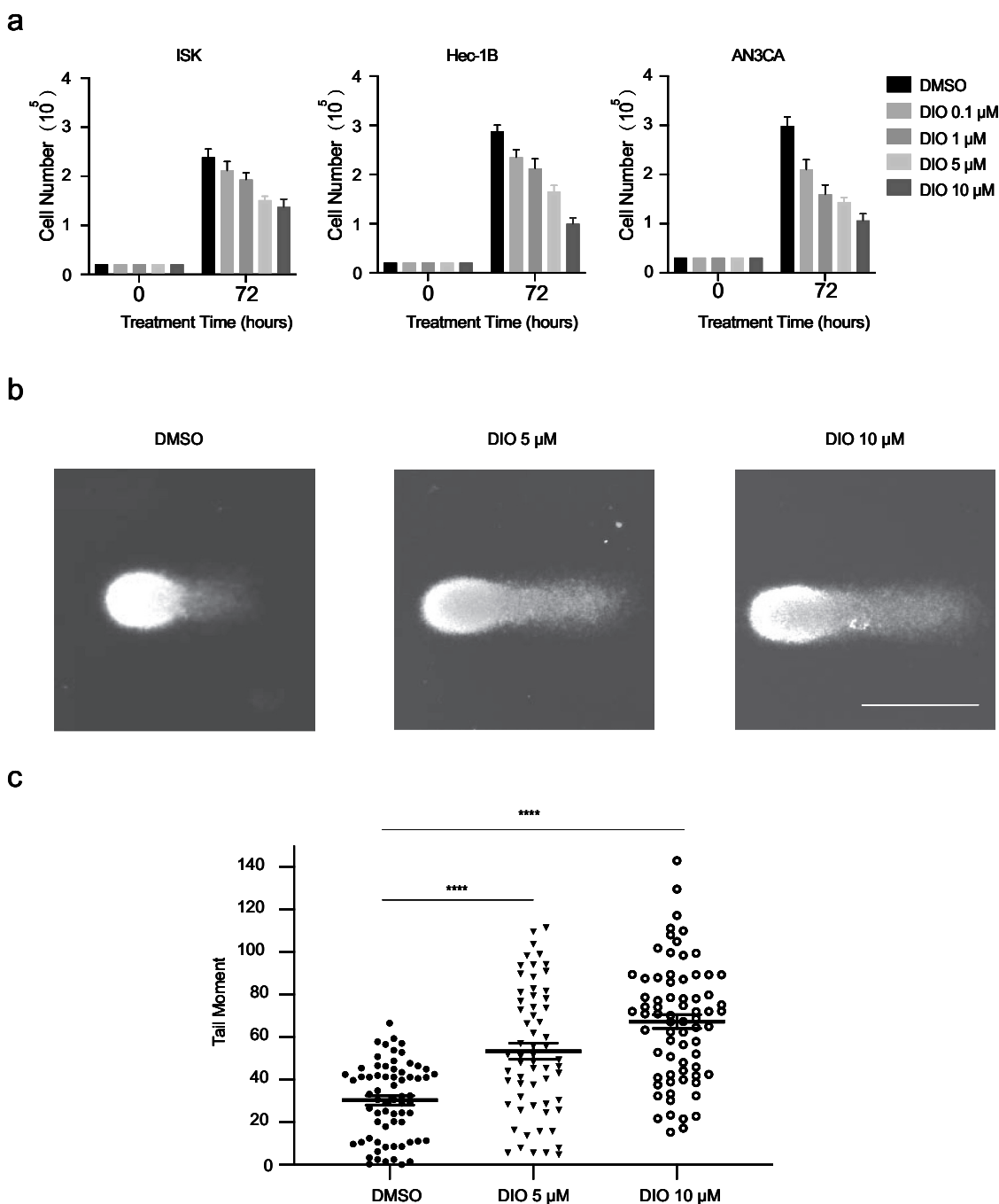


Figure 1. Diosmetin impairs the genomic stability of EC cells and inhibits the cell proliferation.

(a): Effects of diosmetin on the proliferation of ISK, Hec-1B, AN3CA cells. Cells were seeded in 6-well plates at 2×10^5 cells (ISK, Hec-1B) or 3×10^5 cells (AN3CA) per well and treated by DMSO or diosmetin in indicated concentration for 72 hours, followed by cell number counting. Error bars represent SD. (b): Representative pictures of alkaline comet assay. Hec-1B cells were incubated with DMSO or diosmetin in indicated concentration for 72 hours and subjected to the comet assay. Scale bar, 100 μM . (c): Statistical analysis of alkaline comet assay. The tail moments of at least 50 cells were quantified using the Cometscore software (Sumerduck, VA, USA). Results were presented as mean \pm SEM. Mann-Whitney U Test was used to compare the difference. ****: $P < 0.0001$.

between diosmetin-treated and DMSO-treated Hec-1B cells. A previously described [24–26] well-established DSB repair reporter system was used to analyze the HR and NHEJ efficiency of Hec-1B cells in a quantitative manner (Figure 2a,b). For

this assay, functional GFP genes were reconstructed and expressed only as an outcome of successful HR or NHEJ repair events. We transfected HR or NHEJ reporters digested by I-SceI enzymes *in vitro*, along with DsRed plasmids for

normalization of transfection efficiency, into Hec-1B cells in the presence of diosmetin or DMSO, and the numbers of GFP⁺ and DsRed⁺ cells were quantified by flow cytometry at 72 hours post transfection. We observed a remarkable reduction in the HR efficiency of diosmetin-treated Hec-1B cells in a dose-dependent fashion (Figure 2c) and a slight decrease in NHEJ efficiency (Figure 2d). The HR efficiency of Hec-1B cells decreased by 50% after treatment with diosmetin at a concentration of 10 μ M, which might explain the genomic instability caused by diosmetin. Moreover, we did not observe a significant change in the number of cells in S phase after diosmetin treatment for a 24-hour period (Figure 2e,f), indicating that the observed decrease in HR was not a secondary effect of cell cycle arrest.

Diosmetin reduces recruitment of RPA2

To reveal the underlying mechanisms involved, we first examined expression of major proteins involved in HR and NHEJ [27], though we did not observe any significant difference in DSB repair-associated proteins in Hec-1B cells after 72 hours of diosmetin treatment (Figure S2A-B). ATM, a member of the phosphoinositide 3-kinase (PI3K)-related kinase (PIKK) family, is the major factor sensing DNA damage and initiating signal transduction, especially in response to IR-induced DSBs [28]. In response to irradiation, ATM autophosphorylates serine residue S1981, which is crucial for its kinase activity [29]. However, we did not observe any change in the level of ATM protein or ATM S1981 phosphorylation with or without diosmetin incubation after IR treatment (Figure S2C). Consequently, we did not detect any significant difference between the control and experimental groups with regard to the level of CHK2, the target of ATM, or CHK2-pT68, the ATM phosphorylated product [30] (Figure S2C). Moreover, the level of H2AX phosphorylation at S139, which is also activated by ATM, did not decrease in the presence of diosmetin at 30 min post-IR (Figure S2C). All these data indicate that diosmetin suppresses DNA repair by HR in an ATM-independent manner, and the regulation of

HR by diosmetin probably occurs at steps after activation of ATM and phosphorylation of H2AX.

Next, we examined the recruitment kinetics of phosphorylated H2AX (γ H2AX), a well-recognized DSB damage marker, in response to the occurrence of DSBs upon X-ray treatment at different time points. We found that diosmetin treatment did not affect early foci formation of γ H2AX, consistent with our above findings. Nonetheless, diosmetin treatment significantly delayed the clearance of γ H2AX foci (Figure 3a). For instance, at 24 hours after X-ray treatment, the average number of γ H2AX foci remained over 30% of the peak number in the presence of diosmetin; in control cells, the average number of γ H2AX foci dropped to the basal level (Figure 3a). These data are in accordance with the above-mentioned results that diosmetin impairs HR repair, destabilizing the genomes of EC cells.

Then, we set out to identify the step at which diosmetin affects HR repair. We examined the kinetics of the recruitment of RPA2, a single-strand-binding protein involved in the step of end resection in the process of HR. We found that diosmetin strongly inhibited the recruitment of RPA2 to sites of DNA damage (Figure 3b), indicating that diosmetin disrupted the end resection step of HR repair. As a consequence, subsequent recruitment of RAD51, the major recombinase involved in HR repair [23], was also abrogated in EC cells by diosmetin supplementation (Figure 3c). In contrast, we did not observe any significant change in the recruitment of the critical NHEJ factor 53BP1, which is in agreement with the result that the presence of diosmetin did not affect NHEJ efficiency (Figure S3A).

Diosmetin sensitizes EC cells to ionizing radiation

As radiotherapy is one of the most critical therapeutic methods in curing EC, we then examined whether diosmetin sensitizes EC cells to X-ray using a clonogenic assay. We found that in Hec-1B cells, the IC₅₀ of X-ray or diosmetin alone was 0.98 Gy and 6.22 μ M, respectively (Figure 4a,b). Intriguingly, combining the two treatments greatly reduced the dosages of X-ray and diosmetin

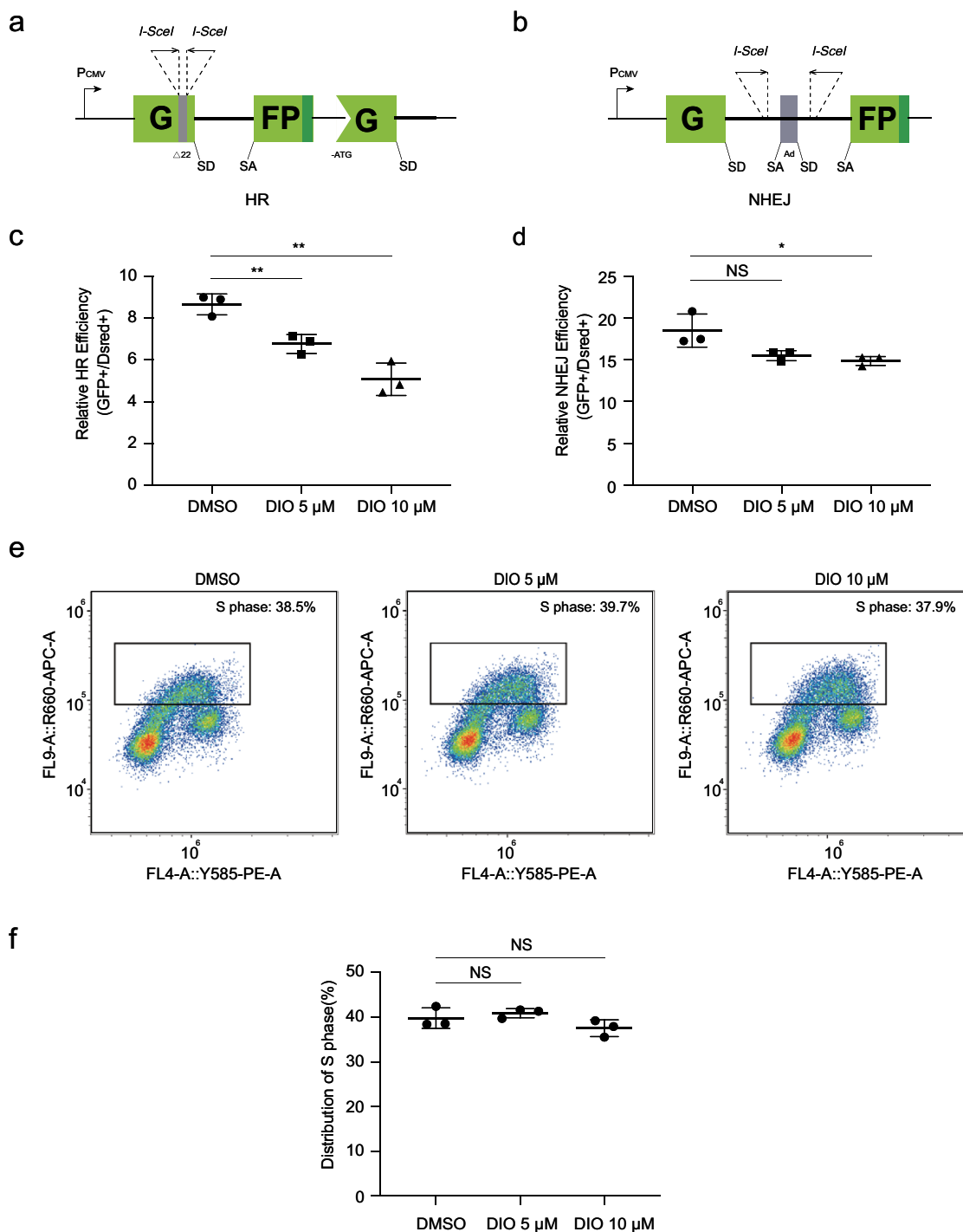


Figure 2. Diosmetin negatively regulates HR repair in EC cells.

(a,b): Reporter constructs for DSB repair analysis. HR (a) and NHEJ (b) reporters are constructed and function as previously described [10,26]. (c,d): Analysis of relative HR (c) and NHEJ (d) efficiencies in cells treated by DMSO or diosmetin in indicated concentration. Hec-1B cells were pretreated with DMSO or diosmetin for 24 hours, followed by being transfected with I-SceI digested reporters along with DsRed plasmids. Transfected Hec-1B cells were continued to be treated with the above reagents until 72 hours in the incubator. Cells were collected and examined on the flow cytometry. The ratio of GFP+ versus DsRed+ cells was measured as relative HR or NHEJ efficiency. Student's t test was used to compare the difference, *: $P < 0.05$, **: $P < 0.01$, NS: no significance. Error bars represent SD. (e): Representative pictures of EdU incorporation assay. Hec-1B cells were incubated with DMSO or diosmetin in indicated concentration for 72 hours and subjected to the EdU incorporation assay and then examined on flow cytometry. (f): Statistical analysis of EdU incorporation assay. The distribution of S phase was quantified on the FLOWJO software. At least 10,000 cells were examined. Student's t test was used to compare the difference. NS: no significance. Error bars represent SD.

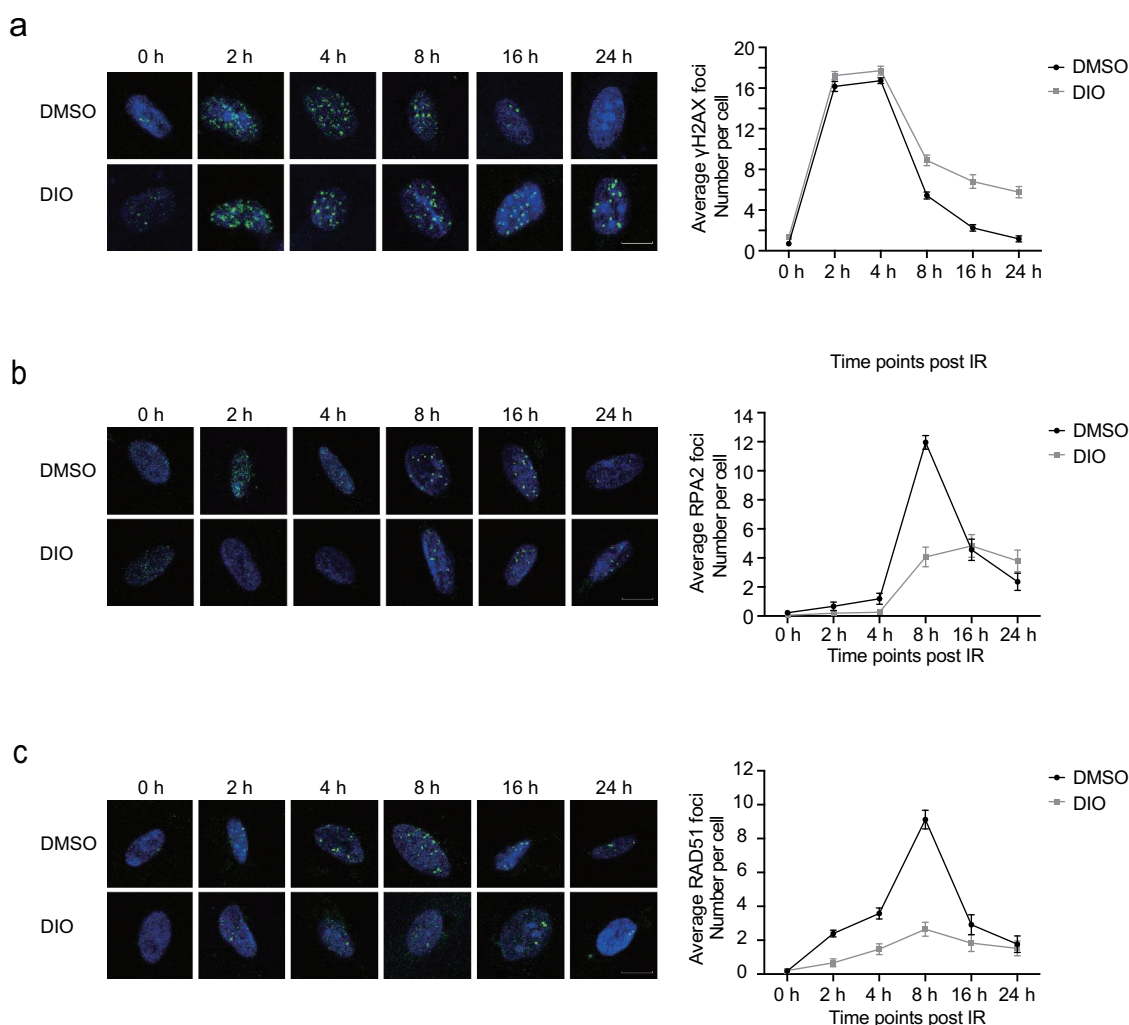


Figure 3. Diosmetin reduces the recruitment of RPA2.

(a–c): Representative images of γ H2AX (a), RPA2 (b), RAD51 (c) foci at different time points post IR (left) and statistical quantifications of the foci number (right). HCA2-hTERT cells were incubated with DMSO or diosmetin for 24 hours, then treated with X-ray (2 Gy). Cells were harvested immediately (0 h) or cultured for 2 h, 4 h, 16 h, 24 h before being harvested for immunofluorescence assay. Images were taken with a laser scanning confocal microscope (TCS SP8; Leica, Wetzlar, Germany). Scale bar, 10 μ M. For each time point, at least 50 cells were counted. Error bars represent SEM.

required, with IC₅₀s of 0.31 Gy for X-ray and 3.08 μ M for diosmetin (Figure 4a,b). These data strongly indicate that the combination of X-ray and diosmetin synergistically suppresses the survival of EC cells, hinting that diosmetin has potential for treating EC as a sensitizer of radiotherapy.

Discussion

Diosmetin is a natural flavonoid abundant in citrus fruit, olive leaves, and some other kind of teas and dry herbs [12], and it has been featured as an antitumor agent in various kinds of cancers. For instance, diosmetin induces apoptosis in

prostate cancer by inhibiting X-linked inhibitor of apoptosis (XIAP) [17]. In acute myeloid leukemia, diosmetin is reported to induce apoptosis through estrogen receptor β [31]. In addition, diosmetin has been shown to inhibit cell proliferation and induce apoptosis by regulating autophagy [32] and by upregulating p53 via the TGF- β signaling pathway [33] in hepatocellular carcinoma HepG2 cells. Diosmetin also induces ROS accumulation by downregulating the PI3K/Akt/GSK-3 β /Nrf2 pathway, resulting in apoptosis in NSCLC cells [34]. In the present study, we found that diosmetin enhanced the effect of radiotherapy in endometrial cancer by suppressing DSB repair

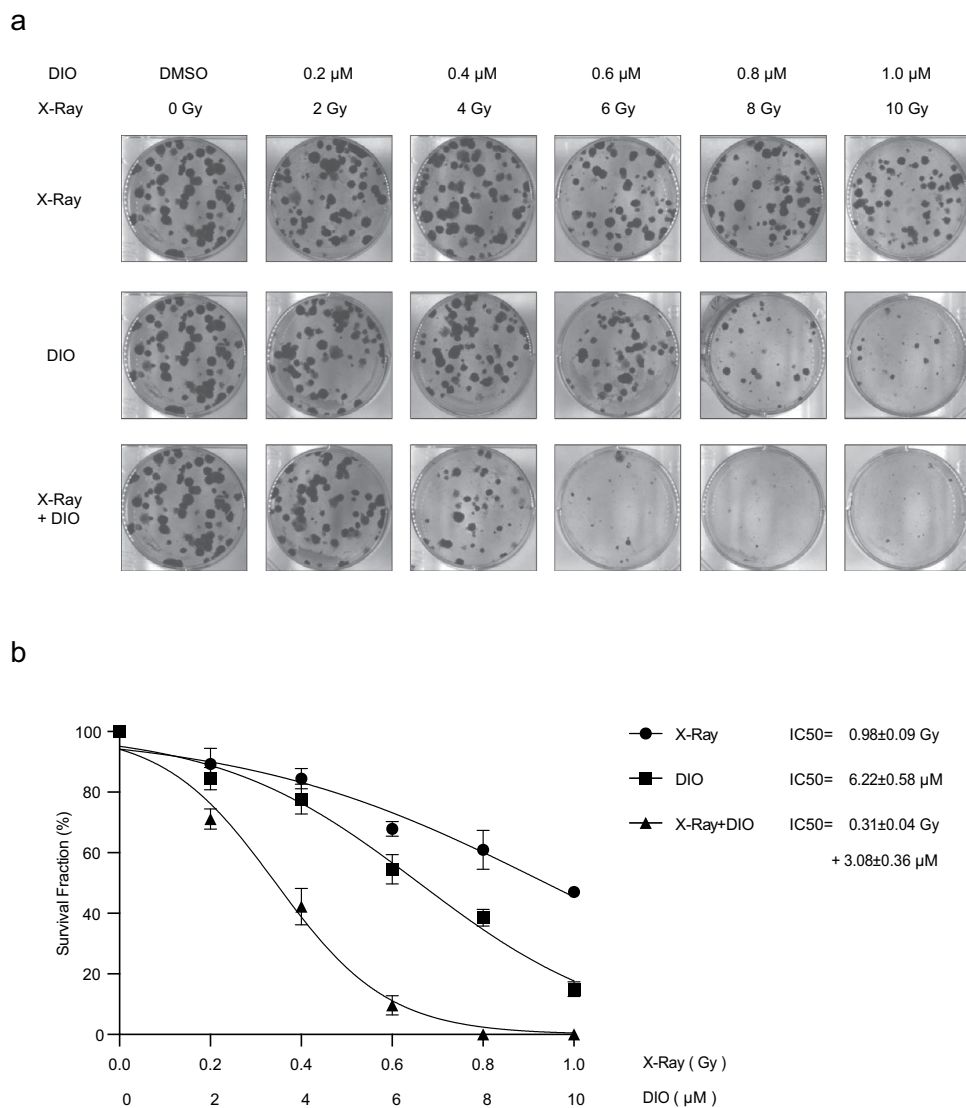


Figure 4. Diosmetin sensitizes EC cells to ionizing radiation.

(a): Representative pictures of clonogenic assay manifesting viability of Hec-1B cells after diosmetin or IR treatment or both. (b): Statistical analysis of clonogenic assay. Hec-1B cells were pretreated with DMSO or diosmetin in indicated concentration for 24 hours, followed by irradiation in indicated dose. Then cells were continued to be treated with DMSO or diosmetin for 72 hours and incubated in the incubator until 2 weeks. Colonies with at least 50 cells were qualified. Analysis of nonlinear regression was used. The IC50 values were analyzed using a dose-effect analysis software. Error bars represent SD.

by negatively regulating DNA end resection (Figure S4A). This finding extends the antitumor spectrum of diosmetin and identifies a novel role of this drug.

Notably, in some previous studies, the antitumor function of diosmetin has been largely attributed to its effect on the cell cycle. For example, diosmetin caused S-phase arrest at concentrations of 5 μ M, 10 μ M, 20 μ M, and 40 μ M in PC-3 cells [17]. However, we did not observe any significant discrepancy in S-phase distribution between the diosmetin-treated group and DMSO-treated

group of Hec-1B cells (Figure 2e,f), which suggested that diosmetin at concentrations of 5 μ M and 10 μ M might not have an S-phase arrest effect in these cells. In addition, Ma [16] and colleagues demonstrated that diosmetin induces Chk2-dependent G2/M cell cycle arrest at concentrations of 10 μ g/ml (33.3 μ M) and 15 μ g/ml (50 μ M); in our experiments, we did not observe obvious alterations in the protein levels of Chk2 and p-Chk2 after diosmetin treatment at a concentration of 10 μ M, which is nearly as low as one-fifth of the dosage used by Ma et al.

Therefore, we assume that diosmetin exerts its cell cycle arrest function only at high dosages, while at low concentrations, it can sensitize cancer cells to radiotherapy by targeting HR repair.

Previous studies have indicated that diosmetin might play distinct roles in different types of cancers, and the underlying mechanisms could be tissue type dependent. In skin cancer, diosmetin has been reported to impair tumor angiogenesis [35]. Moreover, it blocks tumor metastasis by inhibiting the epithelial-mesenchymal transition in human bronchial epithelial cells [36], by activating E-cadherin expression and inhibiting the TGF- β signaling pathway in glioma cells [37] and by downregulating expression of MMP-2 and MMP-9 in liver cancer cells [20]. To date, no research has focused on diosmetin's effect on endometrial cancer, and our study showed that diosmetin is able to inhibit cell proliferation and enhance sensitivity to radiotherapy by suppressing homologous recombination in endometrial cancer, providing additional mechanisms of diosmetin's antitumor effect. Although we demonstrated that diosmetin modulates HR by suppressing recruitment of RPA2 as well as RAD51, how diosmetin affects RPA2 recruitment remains to be elucidated. Chen and colleagues [38] showed that diosmetin can reduce the stability of Nrf2, which is reported to be capable of regulating many factors in HR repair and that inhibiting Nrf2 using all-trans retinoic acid (ATRA) or Nrf2 knockdown caused significant downregulation of HR repair. Whether diosmetin impairs the recruitment of RPA2 and RAD51 through Nrf2 needs further investigation. Xu and colleagues [39] observed that diosmetin elevates levels of γ H2AX at 1 hour post-IR in lung cancer cells, which is in agreement with our results in EC cells. They also found that the Akt signaling pathway was downregulated and speculated that Akt signaling may be involved in the diosmetin-mediated DNA damage response. Activated Akt is reported to suppress HR and activate NHEJ via different mechanisms [40], which may partially explain why NHEJ efficiency declines slightly in diosmetin-treated Hec-1B cells.

Radiotherapy has been widely used for decades either alone or in combination with surgery,

chemotherapy and targeted medicines to treat more than 50% of cancer patients; however, the response to irradiation treatment differs due to the complexity of a variety of factors [41] and is limited by acquired resistance, which can result in relapse and metastasis [42]. Additionally, a sufficient dose of pelvic radiotherapy is often associated with serious toxicities, such as urinary incontinence and fecal leakage, which will have a long-lasting influence on the quality of life of patients [43]. Attempts have been made to improve radiotherapy efficacy and to reduce the effective dose. It is believed that DNA repair inhibitors targeting several crucial molecules in DNA repair pathways can effectively sensitize cancer cells to irradiation treatment and, more importantly, reduce the effective dose of irradiation [23]. For example, AZD1390, an ATM inhibitor, is proven to be an effective radiosensitizer in central nervous system malignancies in early clinical development [44]. The PARP inhibitor talazoparib is reported to enhance the efficacy of radiotherapy in small-cell lung cancer both *in vivo* and *in vitro* [45], and RI-1 and B02, small molecules targeting RAD51, cause significant radiosensitization in glioblastoma [46].

Whether patients can benefit from such DNA repair inhibitors as radiosensitizers is partially dependent on the correlation of DNA repair and cancer type or subtype [41]. Previous research has demonstrated that the development of endometrial cancer is tightly associated with DSB repair; for example, 27% of high-risk ECs are associated with loss of nuclear RAD51 [47]. Decreased expression of Ku70 is also found in endometrioid endometrial cancer [48]. Our study showed that diosmetin suppresses DSB repair by negatively regulating the DNA end resection step. As a result, diosmetin not only inhibits proliferation but also sensitizes endometrial cancers to radiotherapy. Altogether, we propose that the combination of diosmetin and irradiation is a promising therapeutic strategy for endometrial cancer.

Disclosure statement

All authors declare no competing interests.

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