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RAD51-IN-1 INDUCES DNA DAMAGE AND PROMOTES ROS-MEDIATED APOPTOSIS IN OVARIAN CANCER CELLS

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Aim. RAD51 protein is frequently overexpressed in ovarian cancer and plays a critical role in cancer cell development and survival. This study aims to elucidate, how RAD51-IN-1-induced DNA damage and apoptosis contribute to anti-cancer effects in ovarian cancer cell lines (MDAH-2774 and OVCAR-3).

Methods. This research explores the impact of RAD51-IN-1 on cell viability, colony formation, ROS levels, DNA damage, and apoptosis were assessed in MDAH-2774 and OVCAR cell lines through the application of the CVDK-8 viability kit, colony formation assays, DCFDA staining, Comet assays, and AO/ER double staining methods.

Results. Ovarian cancer cell lines were treated with varying doses of RAD51-IN-1, which resulted in a dose-dependent decline in both cell viability and colony formation, with the IC_{50} value for RAD51-IN-1 being determined. Furthermore, as shown by DCFDA staining, an increase in intracellular reactive oxygen species (ROS) and DNA damage as measured by the Comet assay was observed following RAD51-IN-1 treatment. RAD51-IN-1 was found to induce apoptosis by acridine orange/ethidium bromide staining.

Conclusions. This study demonstrates that RAD51-IN-1 effectively induces DNA damage and ROS-dependent apoptosis in ovarian cancer cell lines. Although RAD51-IN-1 requires further in vitro and in vivo evaluation as a treatment in ovarian cancer cells, these findings provide preliminary evidence of its potential efficacy.

Key words: RAD51-IN-1, Ovarian Cancer, DNA Damage, Apoptosis.

Deficiencies in DNA repair pathways are among the hallmarks of cancer. Cancer cells rely on these mechanisms to repair damage caused by replication stress and genotoxic agents. Impaired DNA repair leads to genomic instability, which contributes to tumor initiation, progression, and resistance to therapy [1]. In this context, DNA damage response (DDR) pathways have emerged as promising therapeutic targets, and numerous DDR pathway that target inhibitors have reached preclinical and clinical stages [2]. Homologous recombination (HR) is a central repair mechanism that addresses double-strand breaks (DSBs) in DNA caused by both endogenous stress and exogenous agents [3]. HR is initiated by the recruitment of the MRN complex (Mre11, Rad50, Nbs1) to the DSB site, followed by activation of ATM kinase. Activated ATM phosphorylates several downstream proteins such as γH2AX, 53BP1, and RAD50, thereby orchestrating the repair process [4]. Additionally, ATR and DNA-PK contribute to DDR by interacting with

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their respective co-activator complexes, RPA-ATRIP and XRCC6/XRCC5. RAD51, a homolog of *E. coli* RecA, is a key component of the HR pathway. It forms nucleoprotein filaments at the resected DNA ends, facilitating homologous sequence recognition and D-loop formation. This process is regulated by proteins such as BRCA2, RAD52, and the RAD51 paralogs (Holthausen, Wyman, and Kanaar, 2010). Overexpression of RAD51 has been observed in several human malignancies, including pancreatic, ovarian, and breast cancers [5–8]. RAD51 plays multifaceted roles in DNA repair, carcinogenesis, tumor progression, and therapeutic resistance [9]. It is implicated in drug resistance mechanisms associated with epithelial-to-mesenchymal transition (EMT), hypoxia, and cancer stem cells, and may also upregulate the expression of pro-metastatic genes. Functioning at the core of the HR pathway, RAD51 cooperates with regulatory proteins such as BRCA2, PALB2, and TOPBP1 [10]. However, its overexpression can result in aberrant recombination, leading to increased genomic instability, tumorigenesis, and drug tolerance. Recent studies have identified RAD51 as a potential biomarker for predicting HR repair capacity [11, 12].

RAD51 is highly expressed in many cancers, including ovarian cancer [4]. Elevated RAD51 levels are indicative of enhanced DNA repair capacity and are associated with therapy resistance and poor survival outcomes. RAD51 inhibitors, which target the protein to suppress the HR pathway, represent a promising class of anticancer [8, 13]. Under physiological conditions, RAD51 assembles into nucleoprotein filaments on single-stranded DNA, facilitating homology search and strand invasion. The inhibitors disrupt filament formation, thereby impairing DNA repair. This leads to the accumulation of DNA damage, inducing genomic instability and cell death, particularly in rapidly dividing cancer cells that heavily rely on HR repair [14, 15].

Furthermore, RAD51 inhibitors can enhance the cytotoxic effects of genotoxic therapies such as chemotherapy and radiotherapy, which primarily function by inducing DNA damage. Cancer cells capable of repairing such damage often survive and develop resistance. Inhibiting RAD51 impairs this repair capacity, leading to increased cancer cell death and a reduced likelihood of therapy resistance [13, 16, 17]. RAD51 inhibitors are particularly effective in cancers with high proliferation rates and in tumors harboring BRCA1/BRCA2 mutations, which disrupt HR and increase dependency on RAD51 for DNA repair. This makes such tumors more susceptible to RAD51-targeted therapies [18, 19]. The lack of a study in which the new generation small molecule inhibitor RAD51-IN-1 has previously targeted the DNA damage pathway in the ovarian cancer cell line MDAH-2774 leads us to question experimentally. This study aims to investigate the effect of RAD51-IN-1 on cell viability and colony formation, and the amount of ROS, DNA damage, and apoptosis.

Materials and Methods

Main reagents

RAD51 inhibitor RAD51-IN-1(Cat No: HY-122705) was purchased from MedChemExpress (Monmouth Junction, NJ 08852, USA). It was dissolved in sterile dimethyl sulfoxide (DMSO, sterile-filtered, suitable for cell culture) at a final concentration of 5 mM, and then stored in aliquots at 80 °C and working concentrations were diluted in culture medium. 2′,7′-Dichlorodihydrofluorescein diacetate(DCFH-DA)(CAS NO: 4091-99-0), Ethidium bromide solution(CAS NO:1239-45-8), Acridine Orange solution(CAS NO:65-61-2) was obtained from Sigma Aldrich company. Roswell Park Memorial Institute (RPMI) 1640 Medium, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from Gibco. CVDK-8 kit (Ecotech Biotechnology, Erzurum, T rkiye).

Cell Culture

This study utilised the ovarian cancer cell lines MDAH-2774 and OVCAR-3, which were provided by Bingol University Cancer Research Group (BUKAG). OVCAR-3 cells were cultured in RPMI medium supplemented with 20% fetal bovine serum (FBS), while MDAH-2774 cells were maintained in DMEM medium supplemented with 10% FBS 64 µg/mL penicillin, and 100 µg/mL streptomycin. The cell cultures were maintained in a humidified incubator at 37 °C with 5% CO_2.

NutriCulture Cell Viability Detection Kit-8(CVDK-8)

To determine the effective dose of RAD51-IN-1 molecule, cells were counted and seeded into 96-well plates with 2×10^3 cells each. Each well contained 100 µl of medium and drug mixture, and 0.5, 1, 2.5, 5, 10, 20, 40, 80, 100 µM of RAD51-IN-1 was applied to the cell

lines for 24 and 48 h. The DMSO concentration in the medium and drug mixture was adjusted to less than 0.5% to eliminate DMSO toxicity. The exact amounts of wells containing only DMSO were added for the untreated. According to the CVDK-8 (Cell Viability Detection Kit-8, NutriCulture) kit protocol, 10 µl of kit reagent was added, the 96-well plate was mixed orbitally for 2 minutes, and after 10 minutes of incubation, absorbance was measured at 450 nm wavelength in Microplate (Molecular devices LLC, USA). The result values were normalized and processed in the GraphPad Prism 10 program to determine IC_{50} (50% inhibitory concentration) values and graphs. The optimum dose was determined in the IC_{50} range for MDAH-2774 and OVCAR-3 cell lines, and then the experiments were continued according to these doses.

Colony Formation

Cell colony formation experiments will be performed as described in [20]. Cells at the density determined by preliminary studies were passaged into 6-well cell culture dishes. At the end of the RAD51-IN-1 agent treatments, the cells were washed 3 times with PBS. Cells were observed for colony formation in a humid 37 °C cell culture incubator containing 5% CO_2 for 7–14 days. The medium was refreshed every 3–4 days, and colony formations were examined under a microscope. The experiment was stopped at a particular time, considering the parameters that each colony should reach at least fifty cells, and the colonies should not contact each other. The cells were first washed with PBS, then fixed with Methanol-Acetic acid (3:1) solution for 5 min and treated with 0.5% crystal violet for 15 min. Observations with at least fifty cells were considered as colonies, and the results were evaluated statistically.

Determination of Intracellular Reactive Oxygen Species Level

Ovarian cancer cells were seeded in 6-well cell culture dishes with 2×10^5 cells/well. The next day, they were treated with the relevant agents and incubated in a CO_2 incubator for 24 h. After incubation, the cells were washed three times with PBS and then incubated with 5 µM DCFH-DA for 30 min in a carbon dioxide incubator. Then the cells were washed again with PBS 3 times and the images of the cells were recorded with Olympus CKX41 Inverted Microscope (Olympus, JAPAN) 20X objective [21]. Fluorometric analysis with DCFDA dye was performed as a different method to determine reactive oxygen species. Briefly, 25 µM DCFDA solution was added to the medium containing the cells after the treatments and kept in a 37 °C CO_2 incubator for 45 min. At the end of the incubation period, the medium was removed from the wells, and 1X wash solution was added to each well. The absorbance was then read at Ex 485 nm/Em 535 nm with a fluorescence spectrophotometer (Wu and Yotnda 2011).

DNA Damage Detection (Single Cell Gel Electrophoresis-Comet)

Single-cell gel electrophoresis method, also known as the Comet assay, was used to determine DNA damage after treatments with RAD51-IN-1 [22]. Briefly, 1% High Melting Agarose (HMA) in phosphate buffer will be dissolved in a microwave oven, and after spreading on rubbed slides, it will be kept at +4 degrees overnight. Cells were mixed with low-melting agarose (100µL) at 40–42 °C and spread on slides coated with HMA after the respective agents and combination treatments (1×10^4 cells). Immediately afterward, the slides were kept at +4 °C and in the dark for 5 min to solidify the agar. The slides were then placed in cold lysis solution and kept in the dark at +4 °C for 45 min. After the lysis time expired, the preparations were electrophoresed in the electrophoresis tank at 25 V for 20 min. After electrophoresis, the preparations were washed with cold neutralization buffer for 3×5 min and stained with 40 µL of 20 µg/mL Ethidium Bromide/Redsafe and observed under a fluorescence microscope. At least 50 cells from different photographs obtained from the preparations prepared for each group were analyzed using the ImageJ program (open comet) [23, 24].

Acridine Orange / Ethidium Bromide Staining

In 6-well plates, OVCAR-3 and MDAH-2774 (per well 1×10^5) cells were maintained in DMEM medium at 37 °C in a 5% CO_2 incubator. Cells were treated with concentrations of RAD51-IN-1 (0.5,10, 25 µM) for 48 h and then removed by trypsin-EDTA, followed by centrifugation. And then: 1 µl of AO/EB solution and 25 µl of cell suspension (2.0×10^6 cells/ml) were incubated. 10 µl of cell suspension was placed on a microscopic slide, covered with a glass coverslip, and the cells were examined in a fluorescence microscope using a fluorescent filter and a 20× objective.

Statistical Analysis

Statistical analysis was analyzed with GraphPad Prism 10.0 program according to multiple comparison Post Hoc Tests in the "One-way ANOVA" method. $P < 0.01$was considered significant in the analyses.

Results and Discussion

1. *RAD51-IN-1 reduced cell viability and colony formation in ovarian cancer cell lines*

CVDK-8 results showed that RAD51-IN-1 inhibited cell viability in a dose-time-dependent manner compared with untreated, and the 24and 48 h cell viability of MDAH-2774 and OVCAR-3 cell lines with RAD51-IN-1 are shown in Fig. 1, *A* and *B*. IC_{50} values for

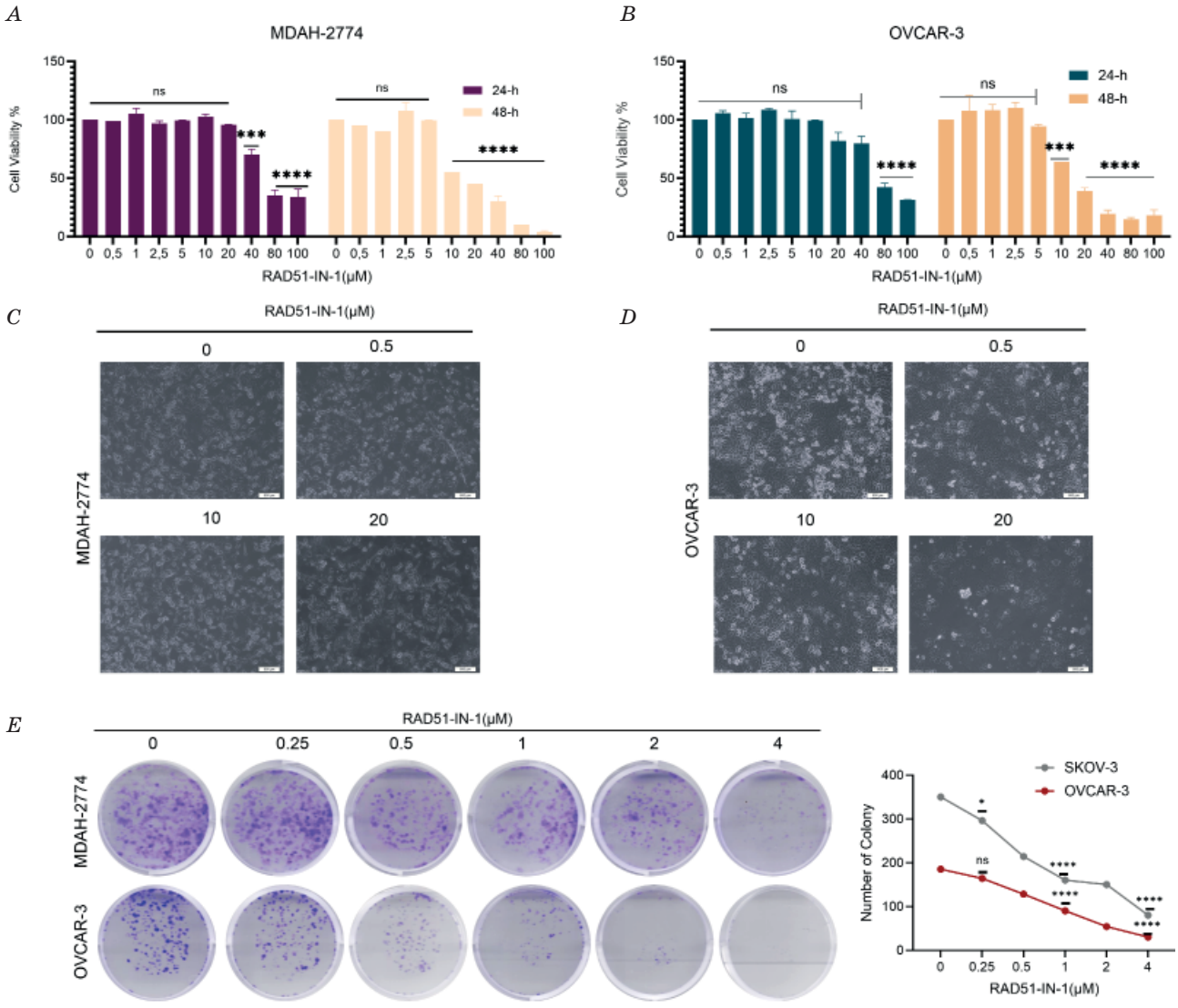

Fig. 1 **The effects of RAD51-IN-1 in MDAH-2774 and OVCAR-3 ovarian cancer lines viability and colony formation**

The cell lines MDAH-2774 (*A*) and OVCAR-3 (*B*) were exposed to various concentrations of RAD51-IN-1 (0.5, 1, 2.5, 5, 10, 20, 40, 80, 100 μM μM) for 24 and 48 h. MDAH-2774 (*A*) and OVCAR-3 (*B*), cell viability was suppressed in a concentration-dependent manner by RAD51-IN-1 treatment, as assessed by CVDK-8 assay. RAD51-IN-1 induced morphologic alteration 48 h as visualized by Olympus CKX41 inverted phase contrast fluorescence microscope (original magnification 10×) (*C* and *D*). B Colony formations are shown, and the number of colonies decreased in ovarian cancer cells at 48 h. (RAD51-IN-1: 0.25,0.5, 1, 2, and 4 μM). Cells were plated in 6-well plates, and 12–14 days later, after treatment, the cells were stained. The results were obtained by one-way ANOVA test ($^{*}P < 0.01$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$)

RAD51-IN-1 were calculated as MDAH-2774 (64.20 and 17.51 µM) and OVCAR-3 (68.88 and 17.18 µM) respectly, 24 and 48 h. However, a higher IC_{50} value was observed in the 24-h period, while a lower value was recorded in the 48-h period. In fact, in a study conducted by Ward and his teammates in Triple-negative breast cancer (TNBC) cell lines in 2017 [25], it was revealed that RAD51-IN-1 could reduce cell viability in a dose-dependent manner. Similarly, a dose-dependent decrease was also seen in the colony formation experiment, but OVCAR-3 cells showed a more sensitive effect than MDAH-2774 cells. In other sets of experiments, we determined the treatment dose and duration to be 48 h below the IC_{50} values.

2. RAD51-IN-1 treatment increases intracellular ROS levels

When MDAH-2774 and OVCAR-3 cells were treated with RAD51-IN-1, DCFDA fluorescence intensity in 10 and 20 µM groups was significantly increased compared to untreated groups and 0.5 µM groups; DCFDA fluorescence was almost negligible in untreated l groups and 0.5 µM groups (Fig. 2, *A*). At the same time, the results obtained from microscope images using DCFDA showed a significant change in the 20 µM group (Fig. 2, *B*, *C*). In this study, the highest concentration applied, 20 µM, led to significant increases in reactive oxygen species (ROS) across both cell lines. This evidence implies that RAD51-IN-1 can lead

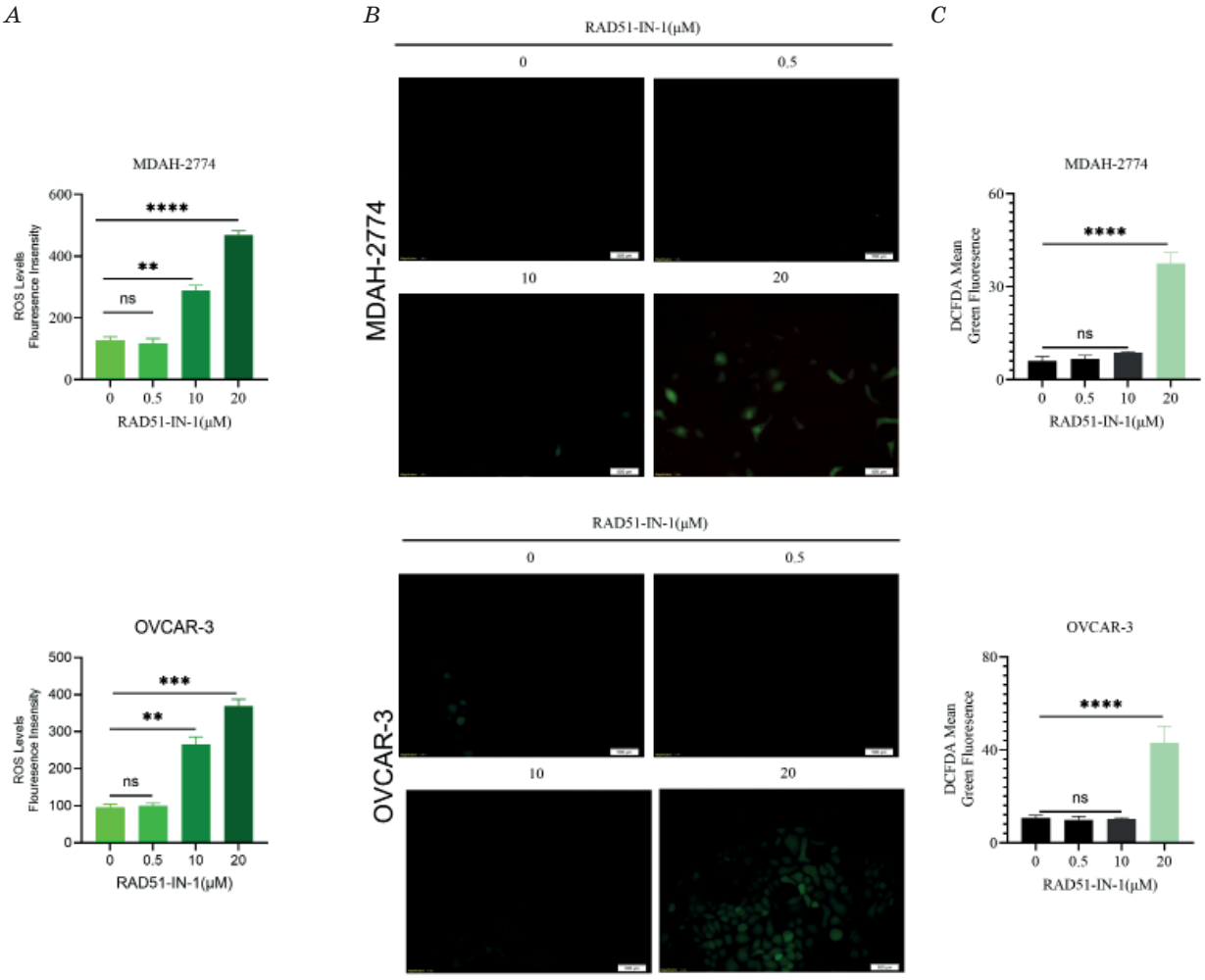

Fig. 2 **Intracellular ROS level induced by RAD51-IN-1 inhibitor**
(A)The cellular oxidative stress induced in the cells was measured using a fluorescent spectrophotometer(DCFDA probe), and the results were recorded and correlated to the levels of ROS produced. (B-C) Fluorescence microscopy(original magnification 20X). Image of treated cells and spectrophotometric measurement of fluorescence intensity(DCFDA), showing the increase of ROS in cells treated with RAD51-IN-1 alone at various concentrations for 48 h (A). The results were obtained by one-way ANOVA test (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$)

to intracellular ROS levels to a degree that triggers oxidative stress in cells, making cancer cells more prone to undergo apoptosis. In the study conducted on ovarian cancer cell lines, it was observed that the amount of ROS increased in A2780, HO8910, HEY, and SKOV3 cells as a result of RAD51 knockdown with siRNA [26] high level of ROS.

3. *RAD51-IN-1-induced enhanced DNA damage*

This novel inhibitor blocks the formation of RAD51 foci and sensitizes aggressive breast cancer cells to DNA damage [25]. Based on these findings, we investigated whether RAD51-IN-1 could similarly enhance DNA damage sensitivity in ovarian cancer cells. To evaluate this hypothesis, we conducted neutral comet assays in MDAH-2774 and OVCAR-3 cells to directly investigate whether it enhances RAD51-IN-1-induced DNA DSBs. The findings indicated that there was no notable difference in DNA tail moments between cells treated with the RAD51-IN-1 and those that were not. Nonetheless, treatment with RAD51-IN-1 alone significantly increased the count of DNA tails, signifying considerable DNA damage. The tail DNA and tail length shown in Fig. 3 were statistically significant in the 20 μM dose compared to the untreated group. Conversely, no notable difference was observed in either cell line within the 0.5 and 10 μM treatment, apart from the untreated group. Collectively, these findings suggest that inhibiting RAD51

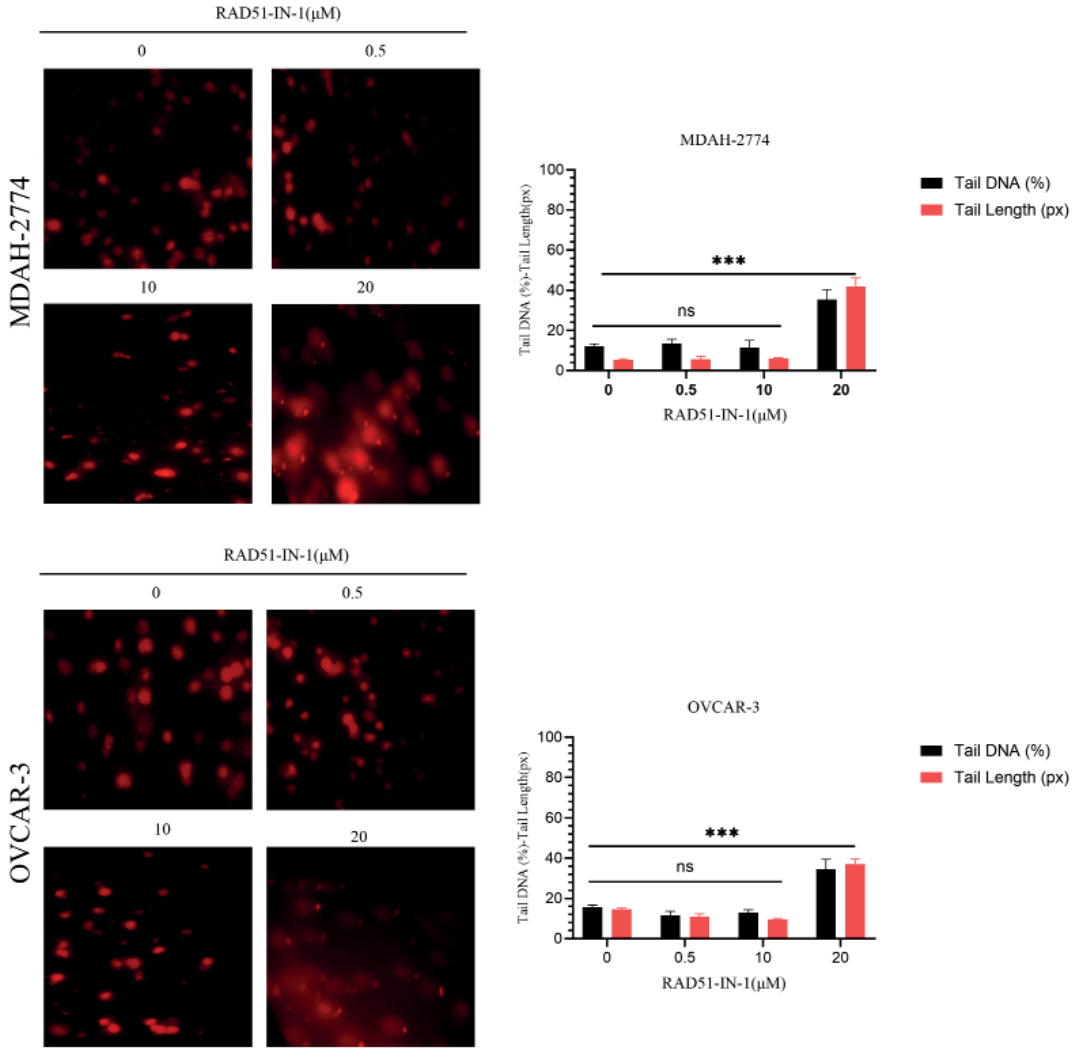

Fig. 3. **The comet assay evaluated the dsDNA breaks in MDAH-2774 and OVCAR-3 cells subjected to a treatment of RAD51-IN-1**

Images (original magnification 20X). Representing comets are displayed. Parameters of the comet assay encompass tail length and tail DNA% in MDAH-2774 and OVCAR-3 cells prompted by the treatment of RAD51-IN-1. The results were obtained by a One-way ANOVA test (***$P < 0.001$)

in ovarian cells results in an accumulation of DNA damage.

Formulating inhibitors that specifically target key aspects of DNA damage repair signaling is a critical method for improving the success rates of chemotherapy and radiotherapy in the treatment of cancer. The interaction between Nanog and Rad51 enhances the presence of γH2AX in mouse embryonic stem cells. Additionally, the comet assay demonstrated an increase in DNA damage [27].

4. RAD51-IN-1 Induced Apoptosis of MDAH-2774 and OVCAR-3 Cells

Our results demonstrated that increasing concentrations of RAD51-IN-1 resulted in a gradual rise in orange and red staining, alongside a decline in green staining of nuclei (Fig. 4, *A*), indicating cellular injury and apoptosis. The apoptotic rate was quantified by measuring the fluorescence intensities of red, orange, and green cells (Fig. 4). Following treatment with 20 µM RAD51 inhibitor for 48 h, a significant variation in the apoptotic rate was noted in the MDAH-2774 and OVCAR-3 cell lines compared to the untreated group. Thus, a high concentration of RAD51-IN-1 (20 µM) can lead to considerable damage in cells, suggesting that the apoptotic rate increases progressively with the concentration of RAD51-IN-1 and the duration of treatment. It was confirmed that approximately 20 µM of the inhibitor can induce apoptosis in the majority of cells after 48 h, aligning with the IC_{50} value results.

The small molecule IBR2, which has been newly identified, has been found to impede the multimerization of RAD51, promote the degradation of RAD51 protein via the proteasome, lessen the formation of RAD51 foci caused by ionizing radiation, disrupt homologous recombination processes, suppress cancer cell growth, and initiate

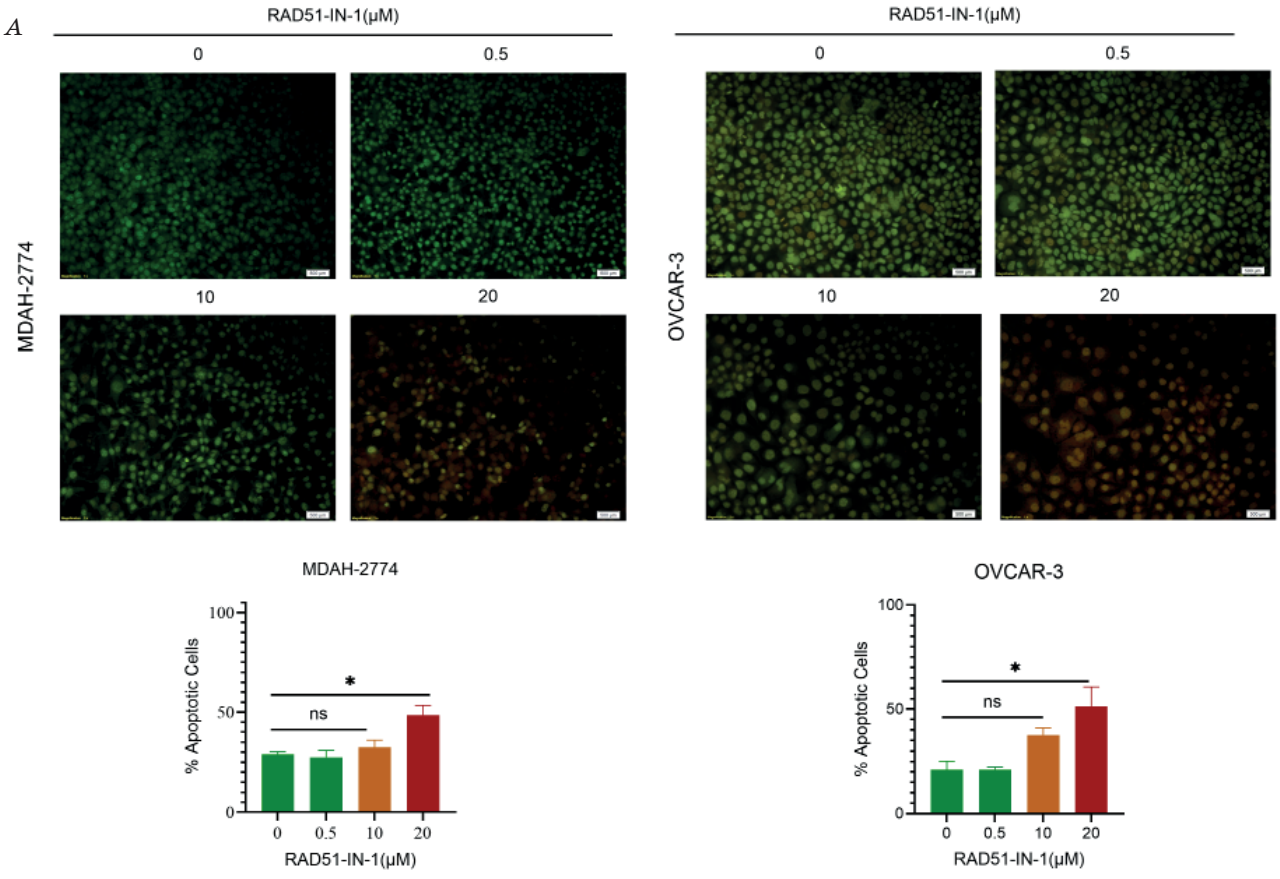

Fig. 4. **Apoptosis analysis by acridine orange/ethidium bromide**
Negative untreated group (normal cells); Cells showed yellow-green fluorescence by acridine orange staining (early apoptotic cells) and also showed orange fluorescence by ethidium bromide (late apoptotic cells), RAD51-IN-1 treatment. Quantification of apoptotic cells. Original magnification 20×. The results were obtained by a one-way ANOVA test (∗$P < 0.05$)

apoptosis [28]. A small molecule that inhibits the interaction between BRCA2 and RAD51 influences the assembly of RAD51 and has been shown to enhance cell death induced by DNA damage[18]. When B02 was used as a RAD51 inhibitor to target Rad51 as a strategy for the treatment of melanoma cells resistant to MAPK pathway inhibition, it was also found that apoptosis was activated [29]. RAD51 serves as a fundamental factor in the homologous recombination DNA repair pathway, playing a significant role in the maintenance of genomic stability. The overexpression of RAD51 is frequently noted in various malignancies, including ovarian [30], breast [31], and lung cancers [32]. It is associated with an unfavorable prognosis for the survival of cancer patients. The increased expression of RAD51 and other genes related to homologous recombination repair in tumor cells is hypothesized to improve DNA repair efficiency and increase resistance to DNA-damaging agents [33].

Conclusions

RAD51-IN-1 is a small molecule with preliminary effects for ovarian cancer; however, more *in vivo* studies are needed to confirm its efficacy and therapeutic significance.

Declaration of competing interest Authors have no conflict of interest.

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Deniz Özdemir and Can Ali Ağca are equal contributors to this work.

Notes

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RAD51-IN-1 ІНДУКУЄ ПОШКОДЖЕННЯ ДНК ТА СПРИЯЄ АПОПТОЗУ, ОПОСЕРЕДКОВАНОМУ ROS, У КЛІТИНАХ РАКУ ЯЄЧНИКІВ

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Мета. Протеїн RAD51 часто надмірно експресується при раку яєчників і відіграє вирішальну роль у розвитку та виживанні ракових клітин. Це дослідження має на меті вперше з'ясувати, як пошкодження ДНК та апоптоз, індуковані RAD51-IN-1, сприяють протиракової дії в лініях клітин раку яєчників (MDAH-2774 та OVCAR-3).

Методи. У роботі досліджували вплив RAD51-IN-1 на життєздатність клітин, формування колоній, рівні активних форм кисню, пошкодження ДНК та апоптоз, що оцінювали в клітинних лініях MDAH-2774 та OVCAR за допомогою набору для визначення життєздатності CVDK-8, аналізів формування колоній, фарбування DCFDA, аналізів Comet та методів подвійного фарбування AO/ER.

Результати. Лінії клітин раку яєчників обробляли різними дозами RAD51-IN-1, що призвело до дозозалежного зниження як життєздатності клітин, так і утворення колоній, при цьому було визначено значення IC50 для RAD51-IN-1. Крім того, як показано за допомогою фарбування DCFDA, після оброблення RAD51-IN-1 спостерігали збільшення внутрішньоклітинних активних форм кисню (ROS) та пошкодження ДНК, виміряного за допомогою аналізу Comet. Було виявлено, що RAD51-IN-1 індукує апоптоз за допомогою фарбування акридиновим оранжевим/бромідом етидію.

Висновок. Це дослідження демонструє, що RAD51-IN-1 ефективно індукує пошкодження ДНК та ROS-залежний апоптоз у лініях клітин раку яєчників. Хоча RAD51-IN-1 потребує подальшої оцінки *in vitro* та *in vivo* як методу лікування клітин раку яєчників, ці результати надають попередні докази його потенційної ефективності.

Ключові слова: RAD51-IN-1, рак яєчників, пошкодження ДНК, апоптоз.