Natural product β-thujaplicin inhibits homologous recombination repair and sensitizes human osteosarcoma cells to radiation therapy

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Running title: β-thujaplicin functions as a DNA repair inhibitor and radiosensitizer

Abstract

Investigation of natural products is an attractive strategy to identify novel compounds for cancer prevention and treatment. Numerous studies have shown the efficacy and safety of natural products, and they have been widely used as alternative treatments for a wide range of illnesses, including cancers. However, it remains unknown whether natural products affect homologous recombination (HR)-mediated DNA repair and whether these compounds can be used as sensitizers with minimal toxicity to improve patients' responses to radiation therapy, a mainstay of treatment for many human cancers. In this study, in order to systematically identify natural products with an inhibitory effect on HR repair, we developed a high-throughput image-based HR repair screening assay and screened a chemical library containing natural products. Among the most interesting of the candidate compounds identified from the screen was β-thujaplicin, a bioactive compound isolated from the heart wood of plants in the Cupressaceae family, can significantly inhibit HR repair. We further demonstrated that β-thujaplicin inhibits HR repair by reducing the recruitment of a key HR repair protein, Rad51, to DNA double-strand breaks. More importantly, our results showed that β-thujaplicin can radiosensitize osteosarcoma cells, a cell type known to often be resistant to radiation therapy. Collectively, our findings for the first time identify natural compound β-thujaplicin, which has a good biosafety profile, as a novel HR repair inhibitor with great potential to be translated into clinical applications as a radiosensitizer. In addition, our study provides proof of the principle that our robust high-throughput functional HR repair assay can be used for a large-scale screening system to identify novel natural products that regulate DNA repair and cellular responses to DNA damage-inducing treatments such as radiation therapy.

Highlights

- Develop a functional high-throughput image-based HR repair assay
- Identify and validate β-thujaplicin as a novel chemical inhibitor of HR
- β-thujaplicin inhibits HR via regulating RAD51 recruitment
- β-thujaplicin sensitizes osteosarcoma cells to radiation

Key words: β-thujaplicin, Radiosensitizer, Homologous recombination, DNA repair, Osteosarcoma.

Introduction

Radiation therapy has been widely used in the treatment of solid tumors and is one of the most commonly used nonsurgical interventions in cancer treatment. However, response to radiation therapy is highly heterogeneous and can differ according to the type of radiation used and intrinsic tumor features, including genetic background, protein expression, the hypoxia status of tumor cells, and the efficiency of repair of DNA double-strand breaks (DSBs), a major form of DNA damage caused by radiation therapy [1].

Radioresistance causes radiation therapy failure and subsequent tumor relapse. The initial efforts to improve the clinical efficacy of radiation therapy resulted in combined-modality approaches, including radio-chemotherapy [2].In the past few decades, to improve the effectiveness of radiation therapy, extensive research has been conducted to search for radiosensitizers that can selectively increase the lethal effects of radiation in tumors. Among the potential radiosensitizers investigated are gold nanoparticles, hypoxia-specific cytotoxins, drugs involved in DNA repair, drugs involved in cell signaling, and growth factors [3]. Experimental and preclinical studies have demonstrated the efficacy of radiosensitizers in improving response to radiation therapy, and several inhibitors have been or are currently being tested as potential radiosensitizers in clinical trials, including poly(ADP-ribose) polymerase inhibitors [4-6], proteasome inhibitors [7-9], and epidermal growth factor receptor inhibitors [10,11]. Given the high cost of targeted therapeutic drugs as radisensitizers, further exploration of novel agents with promising radiosensitization effect, lower cost and fewer side effects is still clinically important. Therefore, identification of novel, affordable and safe radiosensitizers remains a critical clinical need in radiation therapy for solid tumors.

Ionizing radiation (IR) induces cell death through DNA damage. The major form of DNA damage caused by IR is DNA DSBs. Unrepaired DSBs directly contribute to the tumoricidal effect of radiation therapy, which mainly targets cells in the S/G2/M phases. To repair damaged DNA and survive, cells have evolved two major mechanisms to repair DSBs, nonhomologous end joining and HR [12].In contrast to nonhomologous end joining, HR-mediated DNA repair (HR repair) utilizes genetic information contained in the homologous sequences to repair DSBs. Thus, HR is considered an error-free, high-fidelity repair mechanism, which are predominates in the S/G2/M phases, when sister chromatids are available for homology searching [13, 14]. Given the fundamental role of HR repair in repairing DSBs in cells in the S/G2/M phases, HR repair deficiency not only predisposes to cancer development (e.g., breast and ovarian cancer risk is increased in people with mutations in *BRCA1* and *BRCA2*, which encode DNA repair proteins) but also sensitizes cancer cells to DNA damage-inducing therapy such as radiation therapy [15, 16]. More interestingly, chemical inhibitors that target kinases involved in HR

repair, such as ATM,ATR,CHK1, and CHK2 [12,17,18],have been shown to significantly enhance the sensitivity of cancer cells to DNA damage-inducing treatments, including radiation therapy and chemotherapy, in both experimental and clinical studies [19-22]. These findings suggest that novel agents targeting HR repair would be effective radiosensitizers. In order to identify chemical modulators of HR repair, we developed a high- throughput image-based assay as a functional assay of HR repair capacity.

Using this powerful and robust assay, we screened a chemical library containing natural products, which are produced from plants or chemically synthesized to be identical to products produced from plants. Accumulating studies have shown that natural products are effective and safe, particularly in the setting of cancer prevention, because natural products have lower cytotoxicity than chemotherapy agents and kinase inhibitors [23-25]. However, the potential clinical application of natural products as radiosensitizers has not been well studied. Many cancer patients receiving radiation therapy may also take supplementary natural products. How these supplementary compounds may have an impact on response to radiation therapy remains largely unknown. From our screening, we identified β-thujaplicin, a natural product also known as hinokitiol, as a HR repair inhibitor. We believe that our study is the first to show that β-thujaplicin has a strong inhibitory effect on HR repair. As expected, we found that β-thujaplicin treatment sensitized osteosarcoma cells to radiation therapy. Collectively, our findings provide proof of the principle that natural products with good safety profiles can be used as radiosensitizers.

Material and methods

1. Cell culture and irradiation

U2OS human osteosarcoma cells were purchased from the American Type Culture Collection. These cells were maintained in McCoy's 5A modified medium (Cellgro, 10-050-CV) supplemented with 10% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.Cells were irradiated with a RAD SOURCE RS-2000 X-ray irradiator (Suwanee, GA) at the indicated doses.

2. Compound library and antibodies

The compound library was obtained from the National Cancer Institute and was composed of 933 natural compounds and bioactive compounds (Natural Compounds Set III and 1mM Mechanistic Diversity Set II).Monoclonal antibodies for western blotting against phosphorylated RPA (p-RPA),RPA, and CTIP were purchased from Bethyl (Montgomery, TX);anti-p-CHK1 and anti-CHK1 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA);anti-BRCA1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX);and anti-tubulin antibody was purchased from Sigma (St. Louis, MO). Goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. Primary antibody for immunofluorescent staining against

Rad51 (SC-8349) was purchased from Santa Cruz Biotechnology, Inc.,anti-p-RPA32 (A300-245A)was purchased from Bethyl, and secondary antibody Alex Fluor-conjugated goat anti-rabbit IgG(A21206)was purchased from Life Technology(Waltham, MA).

3. Plasmids and transfection

The DR-GFP recombination substrate construct was a kind gift of Dr. Maria Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY). U2OS cells containing a single copy of the HR repair reporter substrate DR-GFP in a random locus were generated as previously described [26]. Positive clones have a single integrated copy of the reporter and exhibit two specific bands after digestion with *Hin*dIII and probing with the indicated Southern probe. FuGENE 6 (Promega, E2691) was used for all plasmid transfections following the manufacturer's protocols. Six hours after transfection, cells were dissociated in trypsin and re-suspended in fresh McCoy's 5A cell culture medium and ready to be used for compound screening.

4. High-throughput compound screening

For high-throughput compound screening, the plate and liquid handling were performed using a high-throughput screening platform composed of an EL406 washer dispenser (Biotek, Winooski, VT) and a JANUS automated liquid handling workstation (PerkinElmer, Billerica, MA).Cell seeding was performed in black 96-well imaging plates (Greiner Bio-One, Monroe, NC) at a density of 5×10^2 cells/well. On the same day, screening compounds were dispensed into assay plates at a final concentration of 10μM using the JANUS automated liquid handling workstation. These assay plates were incubated at 37°C for 3 days. On day 4, chemical treatment was withdrawn via discard medium. Cells were fixed with 0.4% PFA at 4°C. Then cells in assay plates were exposed to DAPI for 20 minutes. Images were acquired and staining intensity was quantified using an ImageXpress Micro XLS widefield high-content analysis system (Molecular Devices, Sunnyvale, CA).

5. Secondary validation assay

The positive hits identified from the primary screening were analyzed using a secondary validation assay. Briefly, cells were seeded in 96-well plates at a density of $5x10^2$ cells/well in a total volume of 100 μL in triplicate in each experiment, and nontransfected cells were used for control. On the same day, cells were treated with compounds identified as positive hits in the primary screening at indicated concentrations (0.1, 0.2, 0.5, 1, 2, 5, and 10μM). Seventy-two hours later, on day 4, chemical treatment was withdrawn via application of discard medium. Cells were fixed with 0.4% PFA at 4°C.Then cells in assay plates were exposed to DAPI for 20minutes.Images were acquired and staining intensity was quantified using an ImageXpress Micro XLS widefield high-content analysis system.

6. HR repair assay

In the HR repair assay system, the DR-GFP reporter substrate includes the SceGFP region, which contains an I-SceI endonuclease site within the coding region, and the iGFP region, which contains homologous sequence or the SceGFP region. Expression of I-SceI induces a single DSB in the genome. When this DSB is repaired by HR, the expression of green fluorescence protein (GFP) is restored and indicates the efficiency of HR repair. Six hours after transfection with an I-SceI transfection vector, cells were treated with β-thujaplicin at different concentrations (5, 10, 20 and 40 μM) or DMSO for 48 hours and then were analyzed to detect GFP-positive cells using a flow cytometer with CellQuest software (BD Biosciences, Franklin Lakes, NJ) at the Flow Cytometryand Cellular Imaging Facility at The University of Texas MD Anderson Cancer Center. Three independent experiments were performed, and mean values and their standard deviations (SDs) were calculated.

7. Colony formation assay

For colony formation assay, 250 U2OS cells were seeded in 6-well plates in triplicate and then treated the next day with β-thujaplicin at different concentrations (0.2, 0.5,1,2, and 5 μM) or not exposed to drugs. On the third day, cells were exposed to IR at a dose of 0.5, 1, or 1.5Gy or not exposed to IR. Cells were incubated for 2 weeks to allow colonies to form. The resulting colonies were detected by staining with 0.001% crystal violet. Colonies containing 50 or more cells were counted and analyzed.

8. Western blotting

Cells were washed in phosphate-buffered saline (PBS), and whole cellular extracts were incubated with urea buffer (8 M urea, 50 mMTris-HCl [pH 7.4], and 150 mM 2-mercaptoethanol) for 30 minutes on ice. Lysates were cleared by centrifugation, and proteins were separated by gel electrophoresis. Membranes were blocked in PBS–0.1% Tween-20 (PBST) with 5% (w/v) nonfat, dry milk for 1 hour at room temperature. Membranes were then incubated with 1:1000 dilutions of primary antibodies (anti-p-RPA, anti-RPA, anti-p-CHK1, anti-CHK1, anti-CTIP, anti-BRCA1, and anti-tubulin) overnight at 4°C. Subsequently, membranes were washed with PBST and incubated with secondary antibodies (1:1000) diluted in PBST with 5% nonfat, dry milk. Membranes were washed in PBST, and bound antibody was detected by enhanced chemiluminescence (GE Healthcare, Pittsburgh, PA).

9. Immunofluorescent staining

For detection of DNA damage–induced foci of p-RPA32 and Rad51, immunofluorescent staining was carried out essentially as described previously [26, 27]. Specifically, to detect Rad51 foci, U2OS cells grown on coverslips were treated with DMSO or 10μM β-thujaplicin for 16 hours and then exposed to IR (10 Gy).Eight hours after IR exposure, cells were exposed to cytoskeleton buffer, stripping buffer, and fixing buffer. Then cells were spread onto slides for staining.

Rabbit anti-Rad51 antibody (1:500) was used to detect Rad51 foci. Cells were incubated with primary antibodies overnight at 4°C, and cells were incubated with secondary antibody Alex Fluor-conjugated goat anti-rabbit IgG for 2 hours at room temperature. Slides were mounted in medium containing DAPI and analyzed under a fluorescence microscope. The number of foci per cell was scored for at least 50 cells per sample. To detect p-RPA32, β-thujaplicin at a concentration of 1, 2, 5, or 10μM was added into cells or DMSO was added, and 48 hours later, the same procedures as described above were performed.

10. Data analysis

All data were summarized as mean ± SD. Differences among groups were analyzed by using one-tailed Student's t-tests and two-way analysis of variance. Statistical significant was defined as $P < 0.05$. Calculations were performed with GraphPad Prism software (GraphPad Software Inc., LaJolla, CA).Protein levels were quantified using NIH Image J software (National Institutes of Health, Bethesda, MD).

Results

1. Development of a high-throughput image-based screening assay to identify chemical regulators of HR repair

We utilized DR-GFP cells to develop a high-throughput assay for functional identification of HR repair inhibitors. We previously established a DR-GFP cell line, which contains a DR-GFP HR reporter plasmid [26-28]. This plasmid was stably integrated into the cellular genome. It contains two mutant copies of GFP, which abolish GFP expression. The first mutant GFP contains a recognition site for I-*Sce*I. The second mutant GFP is truncated but contains a homologous sequence for the first GFP. When cells are transfected with I-*Sce*I plasmid, expression of I-*Sce*I enzyme results in a DSB in the first GFP. If this DSB is successfully repaired by HR repair utilizing the homologous sequence in the second GFP, GFP expression is restored. Basically, in this assay, the HR repair capacity is proportional to the percentage of GFP-positive cells (Figure 1A). We combined this DR-GFP cell-based functional HR repair assay with a high-throughput imaging system (Figure 1B). As shown in Figure 1C, we designed a plate layout to test each compound in triplicate. The negative control (DMSO) and positive control (ATMi) were arranged in separate columns, as were the non-transfected DR-GFP cells. As described previously [26], in this system, the efficiency of HR repair can be assessed by the expression of GFP. The nuclear (DAPI) and GFP fluorescence signals were acquired by high-content analysis system with different wavelengths (Figure 1D). After image acquisition and analysis, we presented the screening results in a histogram(Figure 1E).The screening results were also presented in a volcano plot(Figure 1F).Positive hits were defined as compounds that reduced the percentage of GFP-positive cells compared to the percentage in nontreated control cells and had p values less than 0.05 on statistical analysis (Figure 1F).

2. Validation of β-thujaplicin as an HR repair inhibitor by secondary screening

To validate β-thujaplicin as the positive hit in the primary screening, we performed a secondary validation assay to determine the dose-dependent effects of β-thujaplicin on HR repair. We treated DR-GFP cells with different doses of β-thujaplicin ranging from 0 μM to 10 μM. Cells were incubated with β-thujaplicin for 72 hours, the same amount of time as in the primary screening. We adapted four GFP signal-associated parameters to confirm β-thujaplicin's HR inhibitory function, including "the percentage of GFP-positive cells", "the number of GFP-positive cells", "all W2 mean stain area"(indicating the mean of GFP stained area of all fields in each well acquired by wavelength 2 in analysis system) and "all W2 mean stain integrity intensity"(indicating the mean of GFP stained integrity intensity of all fields in each well acquired by wavelength 2 in analysis system).Following treatment with β-thujaplicin for 72 hours, the percentage of GFP-positive cells (Figure 2A) and the number of GFP-positive cells(Figure 2B) decreased as β-thujaplicin concentration increased. At the doses of 2 μM, 5 μM and 10 μM, values of these two parameters in treated cells were significantly lower than values in the untreated control cells. The other two parameters we chose to measure GFP signal were "all W2 mean stain area" and "all W2 mean stain integrity intensity," which also decreased as β-thujaplicin concentration increased, with significant differences noted at the concentrations of 2 μM, 5 μM and 10 μM (Figure 2C and D). Notably, parameters chosen to represent all cell nuclei signal, "all nuclei mean area" (indicating the mean of DAPI stained area of all fields in each well in analysis system) and "all nuclei mean integrity intensity" (indicating the mean of DAPI stained integrity intensity of all fields in each well in analysis system), did not change significantly with increasing doses of β-thujaplicin (Figure 2E and F). These data strongly suggest that β-thujaplicin has a strong inhibitory effect on HR repair while it has undetectable inhibitory effects on overall cell growth and proliferation at the same concentration range. Collectively, our data validated the results from our primary screening and confirmed that β-thujaplicin is a potential potent HR repair inhibitor with minimal cytotoxic effects.

3. Confirmation of an inhibitory effect of β-thujaplicin on HR repair by classic HR repair assay

In our screening system, we used image-based screening to detect GFP expression restored by HR repair in DR-GFP cells. To confirm β-thujaplicin's role in HR repair inhibition, we analyzed the percentage of cells with I-*Sce*I-induced GFP expression using flow cytometry. Representative flow cytometry profiles are shown in Figure 3A. We observed a significant decrease in GFP-positive cells after cells were treated with different dosages of β-thujaplicin. The percentage of GFP-positive cells was 16.29% in control cells and 2.81% in cells treated with 40 μM β-thujaplicin, indicating defective HR repair after β-thujaplicin treatment. The quantitative summary of three independent experiments (Figure 3B) showed that the percentage of GFP-positive cells decreased in a dose-dependent manner after

β-thujaplicin treatment, with significant differences noted between control cells and treated cells at concentrations of at least 10 μM. In this assay, we observed that β-thujaplicin at 10 μM caused a 50% reduction of HR repair efficiency without any detectable inhibitory effect on cell survival. When we increased the dose of β-thujaplicin to 20 μM and 40 μM, we observed only minimal cell death due to a relatively short treatment time for HR repair assay. These data suggested that inhibition of HR repair by β-thujaplicin was not caused by cytotoxic effects. Our findings suggested that β-thujaplicin at 10 μM effectively inhibits HR repair without causing noticeable cytotoxic effects.

4.β-thujaplicin inhibits recruitment of Rad51 to DSBs

Recombinase Rad51 is a key protein in HR repair.Rad51 is recruited to DSBs and controls homology search and recombination, which permits cells to utilize genetic information contained in the sister chromatids to repair DNA damage in an error-free manner. Because I-*Sce*I-induced HR repair assays showed that β-thujaplicin inhibits HR repair, we explored the mechanism responsible for this effect. We first examined whether β-thujaplicin affects the recruitment of Rad51 to DSBs. We treated cells with β-thujaplicin and induced DSBs by exposure to IR. Rad51 was recruited to DSBs, forming discrete nuclear foci in control cells. In cells treated with β-thujaplicin, Rad51 foci formation was significantly reduced. These data suggest that β-thujaplicin inhibits HR through impaired recruitment of Rad51 to DSBs (Figure 4).

5.β-thujaplicin induces phosphorylation of RPA

When DSBs are repaired by the HR repair pathway, multiple nucleases, including CTIP, EXO1, BLM, and DNA2, are required to resect the DSB ends and generate a stretch of single-strand DNA (ssDNA). This ssDNA at the broken ends of DSBs serves as a platform to recruit ssDNA binding protein RPA. RPA is activated at DSB ends by phosphorylation, and activation of RPA then facilitates the recruitment of additional key repair proteins, such as Rad51.The recruitment of Rad51 replaces RPA and forms Rad51-coated ssDNA filaments, which initiate the homology search and recombination process of HR repair. Thus, defective Rad51 recruitment can result in persistent binding of RPA to ssDNA, which leads to RPA phosphorylation [29, 30]. As expected, when cells were treated with different concentrations of β-thujaplicin, we observed an increasing foci formation of p-RPA (Figure 5A and B). It is worthy of note that β-thujaplicin at a concentration of 10 μM induced p-RPA in the absence of exogenous DNA damage, as shown in Figure 5C and D. At a concentration of 10 μM, β-thujaplicin activated p-RPA protein expression and induced a significant seven fold increase in p-RPA protein level. The protein levels of other proteins, including RPA, p-CHK1, CHK1, CTIP, and BRCA1, exhibited no significant changes after β-thujaplicin treatment. We reasoned that β-thujaplicin may have perturbed HR repair, most likely due to the presence of endogenous DNA damage. Collectively, these data showed that the effect of β-thujaplicin on RPA activation was consistent with the reduced RAD51

foci formation observed in cells treated with β-thujaplicin.

6. β-thujaplicin sensitizes osteosarcoma cells to radiation

Patients with osteosarcoma often exhibit resistance to radiation therapy. As we observed an inhibitory effect of β-thujaplicin on HR repair, we next tested whether β-thujaplicin can sensitize osteosarcoma cells to radiation therapy. We used colony formation assay to determine whether β-thujaplicin reduced the survival of U2OS osteosarcoma cells after IR. To identify an optimal combination of β-thujaplicin concentration and IR doses, we treated cells with β-thujaplicin at a range of concentrations (0, 0.2, 0.5, 1, 2,5μM) in combination with IR at a range of doses (0, 0.5, 1, 1.5 Gy). Our results showed that at the IR dose of 1.5 Gy, β-thujaplicin radiosensitized cells in a β-thujaplicin dose-dependent manner. The colony number of U2OS cells decreased as the drug concentration increased (Figure 6A). Moreover, in the presence of β-thujaplicin at a concentration of 5μM, the colony number of U2OS cells decreased when the IR dosage was increased from 1Gy to 1.5Gy (Figure 6B).When β-thujaplicin was combined with IR treatment at different drug concentrations and IR doses, colony formation decreased significantly as the drug concentration and IR dose increased (Figure 6C). Two-way analysis of variance showed that both drug concentration (column factor) and IR dose (row factor) significantly affected cell proliferation. More importantly, the interaction of these two factors remarkably affected cell proliferation. These data strongly supported the concept that β-thujaplicin has a synergistic effect with IR and sensitizes osteosarcoma cells to radiation therapy (Figure 6D).

Discussion

In this study, we performed high-throughput image-based drug screening to identify natural compounds that act as HR repair inhibitors. From our screening, we identified β-thujaplicin as a natural compound with potent HR repair inhibitor activity. We also found that β-thujaplicin can inhibit the recruitment of a key HR repair protein, Rad51, to DNA damage sites, which can cause persistence of DNA damage as well as increased RPA phosphorylation. Finally, we found that β-thujaplicin can radiosensitize osteosarcoma cells, a cancer cell type normally insensitive to IR. Together, our results show that β-thujaplicin can be used as a novel natural compound–based radiosensitizer. Our data also provide proof of the principle that our high-throughput functional HR repair assay can be used as a robust chemical screening method to identify compounds with HR repair inhibitory activity.

Recently, essential oils have been reported to exhibit anticancer effects against different types of human tumors [31, 32]. β-thujaplicin (4-isopropyltropolone), isolated from the essential oil of the Japanese cypress, is a tropolone-based phenolic compound [33,34].This compound has a safety profile and has been approved for use in both clinical practice and health care products [35-38]. It exerts a wide variety of biologic activities, including antimicrobial, antioxidant, and

antitumor activities [39].Additionally, It has also been reported to regulate tumor growth and metastasis [37,40,41].β-thujaplicin's antitumor effects have been investigated in a variety of cancer models, including models of melanoma [41,42],breast cancer [40,43,44],lung cancer [45],colon cancer [37,40],prostate cancer [46], and oral cancer [47].Several studies have shown that β-thujaplicin can inhibit cancer cell proliferation by inducing the p53-independent DNA damage response [45],S or G1 cell cycle arrest [45,46]and apoptosis [37].These studies suggested a potential role of β-thujaplicin in cell cycle regulation and DNA damage response. However whether β-thujaplicin targets DNA damage response and repair pathways has not been studied intensively. In our study, because of β-thujaplicin's exceptional safety profile, widely clinical and health care usage and potential anti-tumor activity related to DNA damage response, it was identified as one of the most interesting candidates among all HR repair inhibitor hits in our screening for natural compounds. For the first time, our data show that β-thujaplicin inhibits the HR repair process. This inhibitory function leads to HR repair deficiency and an accumulation of DNA damage.

For decades, DNA damage–inducing agents have been the mainstay of anticancer therapy such as chemotherapy and radiation therapy. Inhibition of DNA repair has also been investigated experimentally and clinically as a sensitizing approach to improve responses to radiation therapy. On the basis of our study, β-thujaplicin, also known as hinokitiol, has great potential to be a novel radiosensitizer and should be further explored.β-thujaplicin is an antimicrobial agent with an established good safety profile, it is known to be of fairly low cytotoxicity and teratogenic effects [48], and has minimal side effects in animal models [49]. Thus, β-thujaplicin has been widely used as a preservative in toothpastes, skin lotions, body soaps and other health care products [39].Moreover, techniques have been established to improve the yield of β-thujaplicin production [50, 51].Therefore, we expect that β-thujaplicin and possibly similar natural products identified from our study will provide an important alternative approach to safely enhance the therapeutic effects of radiation by reducing DNA repair capacity in cancer cells. Furthermore, natural compounds cost less than targeted drugs that might be investigated for radiosensitizing effect, such as inhibitors of the DNA damage response kinases CHK1 and WEE1, and thus use of natural compounds could significantly reduce the economic burden for cancer patients [18-22, 52].

We chose osteosarcoma cells (U2OS) for our research reported here. One reason for this choice is that we could combine a DR-GFP cell-based HR repair assay and high-throughput screening to develop our novel functional screen of HR repair inhibitors. The other, more important, reason is that osteosarcoma is generally considered radioresistant although the mechanism remains unknown; in patients with osteosarcoma, chemotherapy and surgery in combination yield a 5-year survival rate of only 60% to 70% [53]. Radiation therapy is still an alternative combinatory therapy both in the initial treatment and for patients with locally

relapsed or recurrent disease [54, 55].Thus, there is increasing interest in treating the tumor by utilizing radiation therapy. In the future, the function of β-thujaplicin as a radiosensitizer will need to be explored in other cancer models. In fact, we plan to perform further in vivo and in vitro experiments to confirm the radiosensitization effects of β-thujaplicin in different cell models and animal models.

Conclusions

Natural compounds have been widely used as cancer prevention and treatment agents that function via targeting a variety of biological pathways. However, little is known about whether natural compounds can target DNA repair pathways and whether natural compounds can be used as radiosensitizers. Our findings from use of a functional DNA repair chemical screening assay indicate that the naturally occurring compound β-thujaplicin is a HR repair inhibitor that may serve as a novel radiosensitizer for osteosarcoma.

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

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Figure1

Performance of high-throughput image-based screening assay.

A: Schematic diagram of DR-GFP cell-based functional HR repair assay.
B: Schematic representation of the high-throughput screening (HTS).
C: Plate layout designation.
D: Automated imaging of U2OS cells engineered to permit assessment ofthe efficiency of HR repair. (Left)Cells stained with the nuclear dye DAPI (blue). (Right) Cells expressing green fluorescent protein (GFP) indicating successful HR repair.
E: Histogram of screening results.
F: Volcano plot of screening results.

Figure2

Results of secondary validation of β-thujaplicin as an HR repair inhibitor.

A-D: The percentage of GFP-positive cells(A), the number of GFP-positive cells (B), the W2 mean stain area (C), and the W2 mean stain integrity intensity (D) decreased with increasing β-thujaplicin concentration (0,0.1,0.2,0.5,1, 2,5, 10 μM).(W2 indicating wavelength 2 using by the analysis system to detect GFP signal by microscopy).
E-F: All nuclei mean area and all nuclei mean integrity intensity did not change significantly with increasing β-thujaplicin concentration.Error bars represent SD.*P<0.05, **P<0.01, ***P<0.001.

Figure3

β-thujaplicin decreases HR repair efficiency.

A: Representative flow cytometry profiles show a significant decrease of GFP-positive cells after treatment with different doses of β-thujaplicinas indicated by I-*Sce*1 HR reporter assay.
B: Quantitation of β-thujaplicin's effect on HR. Error bars represent SD.*P<0.05, **P<0.01, ***P<0.001.

Figure4

β-thujaplicin inhibits radiation-induced Rad51 foci formation.

A: Immunofluorescence staining of Rad51 foci (green) in U2OS cells following treatment with β-thujaplicin (10μM) followed 16 hours later by exposure to IR (10Gy). The nucleus is counterstained with DAPI (blue).
B: Quantitation of percentage of cells with Rad51-positivefoci number. Error bars represent SD from replicates.*P<0.05, **P<0.01.

Figure5

β-thujaplicin induces phosphorylation of RPA.

A: Immunofluorescence staining of p-RPA foci (green) in U2OS cells following treatment with DMSO or β-thujaplicin (1,2,5,or 10μM) for 48 hours. The nucleus is counterstained with DAPI (blue).

B: Quantitation of percentage of cells with Rad51-positive foci number. Error bars represent SD from replicates.*P<0.05, **P<0.01.
C: Western blot analysis of U2OS cells pretreated with DMSO or β-thujaplicin at the indicated concentrations.
D: Quantitation of P-RPA protein level Error bars represent SD from replicates (**P<0.01).

Figure6

Effect of β-thujaplicin on radiosensitivity of U2OS osteosarcoma cells.

A: Survival of U2OS cells treated with DMSO or with β-thujaplicinat the indicated doses and either not exposed to IR or exposed to IR at a dose of 1.5Gy.
B: Survival of U2OS cells exposed to IR at the indicated dosesand either not exposed to IR treated with β-thujaplicin at a dose of 5μM.
C: Survival of U2OS cells treated with DMSO or β-thujaplicinat the indicated doses and either not exposed to IR or exposed to IR at the indicated doses.
D: Statistical analysis of impact of interaction between β-thujaplicin concentration and IR dose on cell survival.*P<0.05, ****P<0.0001.

Figure 1

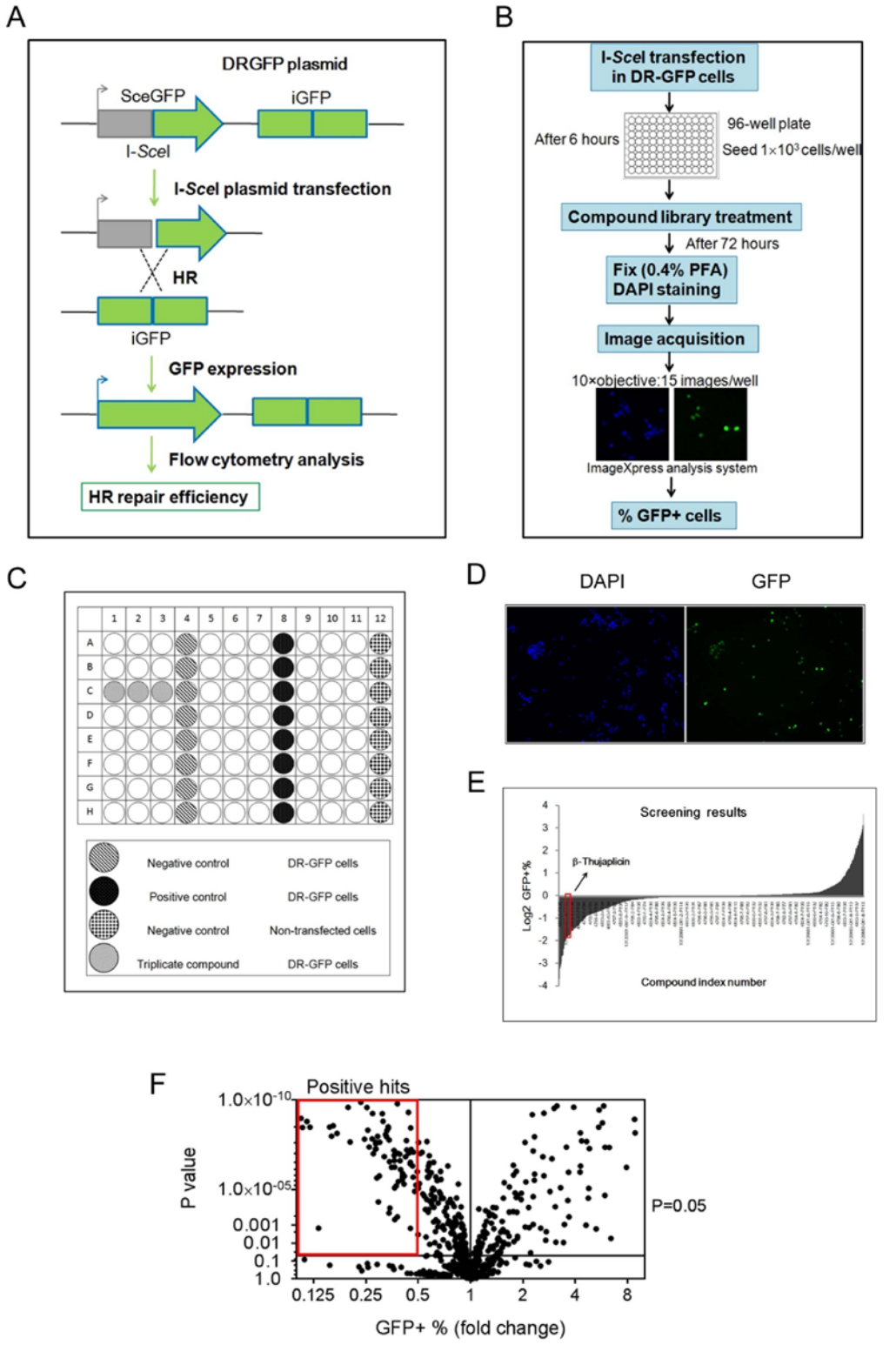

A
DRGFP plasmid
SceGFP
iGFP
I-SceI
I-SceI plasmid transfection
HR
iGFP
GFP expression
Flow cytometry analysis
HR repair efficiency
B
I-SceI transfection in DR-GFP cells
After 6 hours
96-well plate
Seed 1×10³ cells/well
Compound library treatment
After 72 hours
Fix (0.4% PFA) DAPI staining
Image acquisition
10×objective:15 images/well
ImageXpress analysis system
% GFP+ cells
C
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
Negative control DR-GFP cells
Positive control DR-GFP cells
Negative control Non-transfected cells
Triplicate compound DR-GFP cells
D
DAPI
GFP
E
Screening results
β-Thujaplicin
Log2 GFP+%
Compound index number
F
Positive hits
P value
1.0×10⁻¹⁰
1.0×10⁻⁰⁵
0.001
0.01
0.1
1.0
P=0.05
0.125 0.25 0.5 1 2 4 8
GFP+ % (fold change)

Figure 2

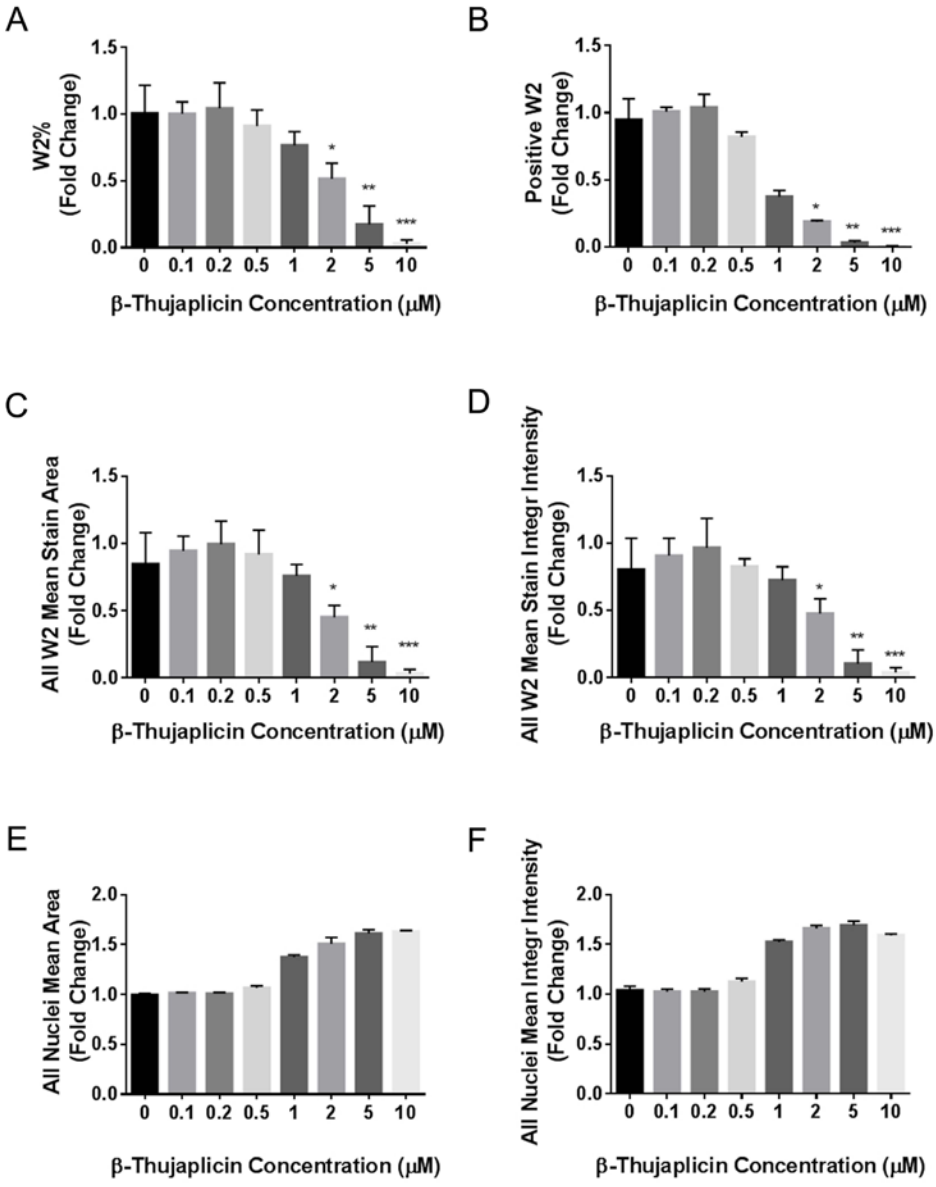

A
W2%
(Fold Change)
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)
B
Positive W2
(Fold Change)
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)
C
All W2 Mean Stain Area
(Fold Change)
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)
D
All W2 Mean Stain Integr Intensity
(Fold Change)
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)
E
All Nuclei Mean Area
(Fold Change)
2.0
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)
F
All Nuclei Mean Integr Intensity
(Fold Change)
2.0
1.5
1.0
0.5
0.0
0 0.1 0.2 0.5 1 2 5 10
β-Thujaplicin Concentration (μM)

Figure 3

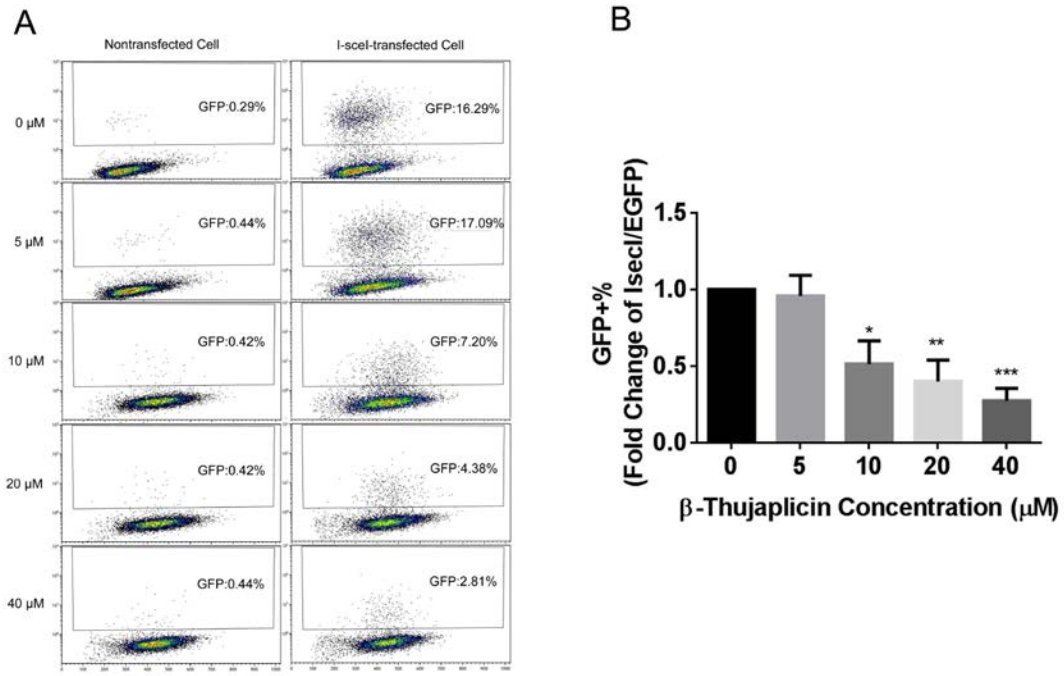

A
Nontransfected Cell
I-scel-transfected Cell
0 μM
5 μM
10 μM
20 μM
40 μM
GFP:0.29%
GFP:16.29%
GFP:0.44%
GFP:17.09%
GFP:0.42%
GFP:7.20%
GFP:0.42%
GFP:4.38%
GFP:0.44%
GFP:2.81%
B
GFP+%
(Fold Change of IsecI/EGFP)
1.5
1.0
0.5
0.0
*
**
***
0
5
10
20
40
β-Thujaplicin Concentration (μM)

Figure 4

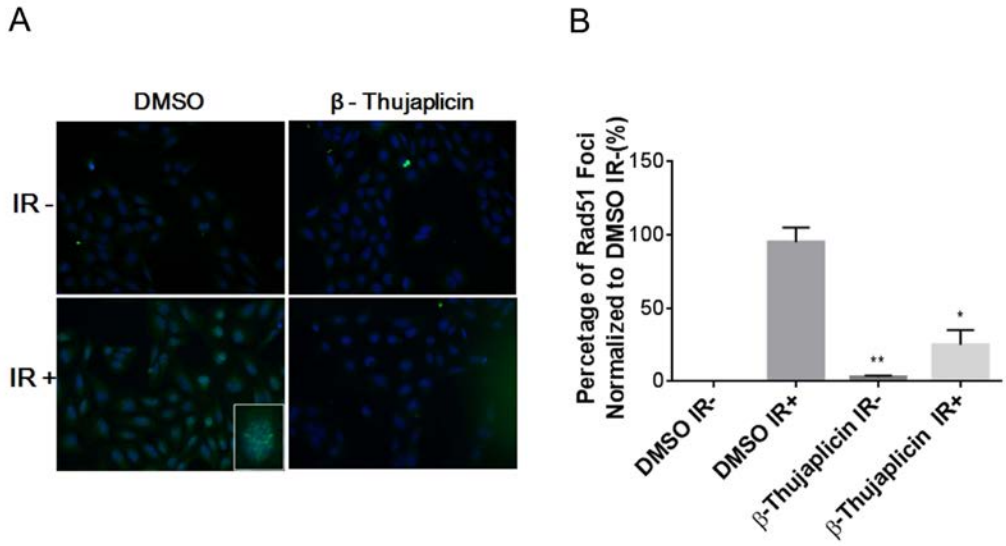
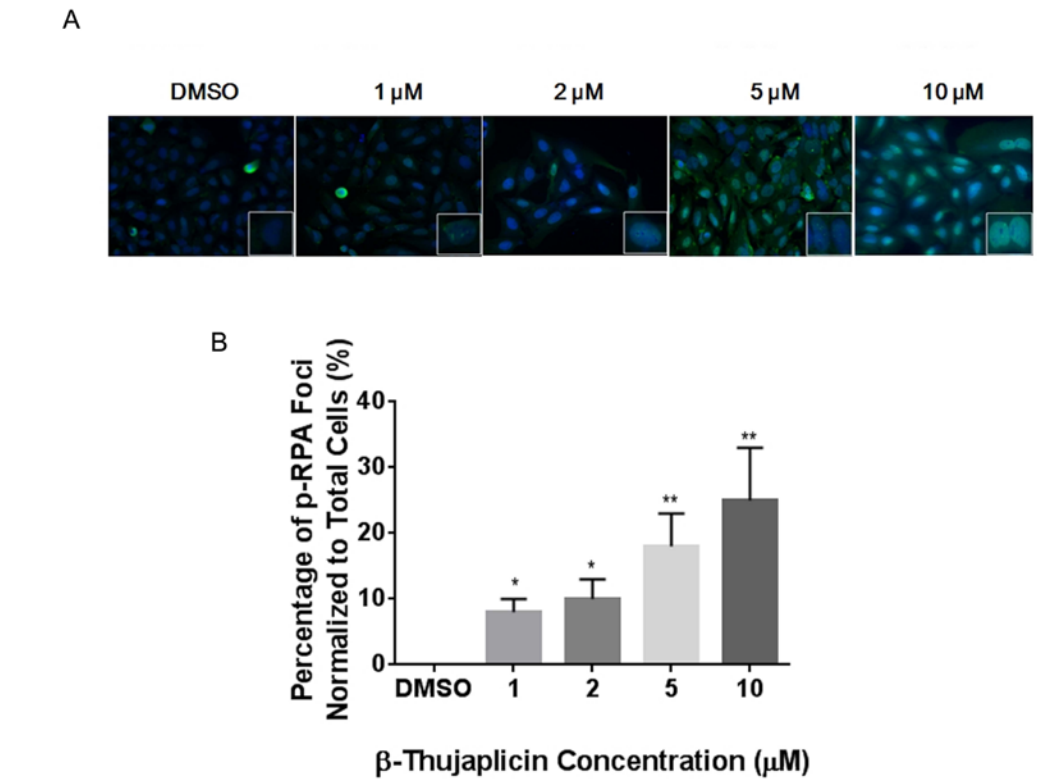

A
DMSO
β - Thujaplicin
IR -
IR +
B
Percetage of Rad51 Foci
Normalized to DMSO IR-(%)
150
100
50
0
**
*
DMSO IR-
DMSO IR+
β-Thujaplicin IR-
β-Thujaplicin IR+

Figure 5


A
DMSO
1 μM
2 μM
5 μM
10 μM
B
Percentage of p-RPA Foci Normalized to Total Cells (%)
0
10
20
30
40
*
*
**
**
DMSO
1
2
5
10
β-Thujaplicin Concentration (μM)

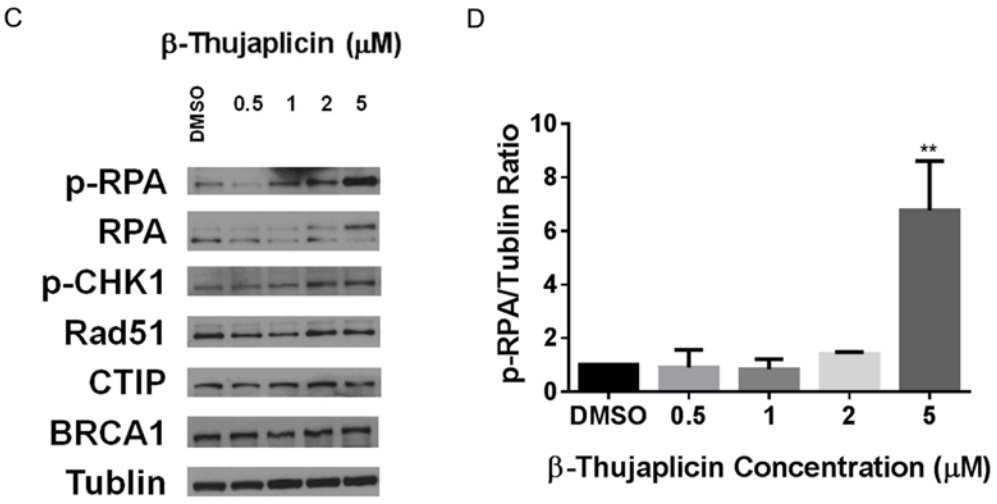
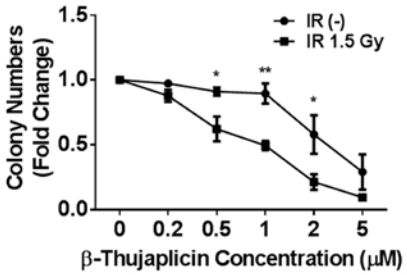
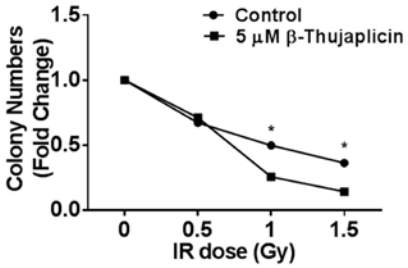

C
β-Thujaplicin (μM)
DMSO
0.5
1
2
5
p-RPA
RPA
p-CHK1
Rad51
CTIP
BRCA1
Tublin
D
p-RPA/Tublin Ratio
0
2
4
6
8
10
**
DMSO
0.5
1
2
5
β-Thujaplicin Concentration (μM)

Figure 6

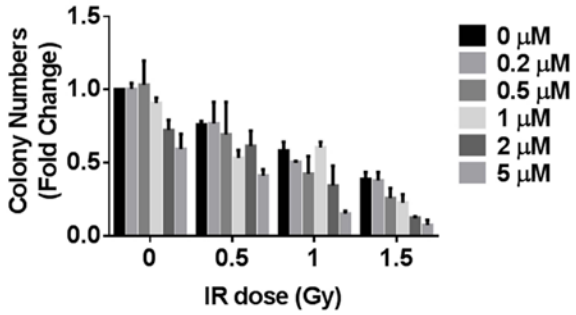
A



B



C



D

Source of Variation	% of total variation	P value	P value summary	Significant
Interaction	3.965	0.0352	*	Yes
Row Factor (IR Dose)	68.63	< 0.0001	****	Yes
Column Factor (Drug Concentration)	21.07	< 0.0001	****	Yes