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T0070907 inhibits repair of radiation-induced DNA damage by targeting RAD51

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ABSTRACT

T0070907 (T007), a PPARγ inhibitor, can reduce α and β tubulin proteins in some cancer cell lines. Thus, T007 has been suggested as an antimicrotubule drug. We previously reported that T007 increased radiosensitivity by inducing mitotic catastrophe in cervical cancer cells. In this study, we investigated the underlying mechanisms of the T007-mediated increase in radiosensitivity. T007 pre-treatment attenuated RAD51 protein levels and ionising radiation (IR)-induced nuclear foci formation, resulting in more frequent centrosome amplification and multipolar mitotic spindle formation in cervical cancer cells. Furthermore, T007 pre-treatment delayed the clearance of IR-induced γ-H2AX and increased radiosensitivity in cervical cancer cells. In contrast, none of these changes were observed in normal cells. Our data demonstrate for the first time that T007 impairs the repair of IR-induced DNA double-strand breaks by inhibiting RAD51, a key protein in homologous recombination repair, increases IR-induced mitotic catastrophe, and leads to increased death of IR-treated cells. These findings support T007 as a potential RAD51 inhibitor to increase tumour response to radiation therapy.

1. Introduction

DNA double-strand breaks (DSBs) are potentially the most lethal damage to DNA and can be induced by endogenous processes, ionising radiation (IR), or chemicals. If they are not repaired, DSBs can lead to chromosomal breakage, genomic instability, and a complicated cascade of cellular reactions (Mladenov et al., 2013). Phosphorylated histone H2AX (γ-H2AX) is known as an early and reliable surrogate for DSBs and is used to measure the induction and repair of DSBs (Banath et al., 2010). DSBs are primarily repaired by two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is carried out by DNA-dependent protein kinase (DNA-PK), consisting of the heterodimer KU70/KU80 and the DNA-PK catalytic subunit (DNA-PKcs), and rejoins DNA ends without requiring sequence homologies. Thus, NHEJ is an efficient but error-prone repair mechanism that is active throughout the cell cycle. In contrast, HR requires a sister chromatid as a template and is therefore, performed in the late S and G2/M phases of the cell cycle. HR is an error-free repair mechanism that plays an essential role in maintaining genome integrity (Chernikova et al., 2012; Mladenov et al., 2013). RAD51 is a key protein in HR and its nuclei foci provide convenient surrogate markers for monitoring the presence of DSBs or the recruitment of HR repair proteins (Willers et al., 2015).

T0070907 (T007), a peroxisome proliferator-activated receptor gamma (PPARγ) inhibitor, can reduce α and β tubulin protein levels in colon cancer cell lines (Schaefer et al., 2007). Therefore, T007 was suggested as a novel antimicrotubule drug, but its therapeutic targets have not yet been exploited (Schaefer, 2008). We previously reported that T007 decreased α and β tubulin protein levels in some cervical cancer cell lines and increased radiosensitivity by inducing mitotic catastrophe (An et al., 2014), which is a type of cell death caused by aneuploidy, micronuclei or multinucleated giant cells. DNA damage-induced centrosome amplification is a key step in aberrant cytokinesis followed by aneuploidy and mitotic catastrophe (Eriksson and Stigbrand, 2010; Loffler et al., 2013; Pannu et al., 2012). The DSBs repair proteins are correlated with DNA damage induced-centrosome amplification and aneuploidy occurrence (Dodson et al., 2004; Rodrigue et al., 2013). Here, we

Abbreviations: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; FBS, foetal bovine serum; HR, homologous recombination; IR, ionising radiation; NHEJ, non-homologous end joining; PPARγ, peroxisome proliferator-activated receptor gamma; RT, radiation therapy; T007, T0070907; TBST, Tris-buffered saline and Tween 20.

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investigated new mechanisms of increased radiosensitivity by T007. Notably, we found that T007 significantly reduced the RAD51 protein levels in cervical cancer cells, resulting in prolongation of IR-induced DNA damage. Furthermore, the maintenance of unrepaired DNA damage leads to centrosome amplification, resulting in the formation of multipolar mitotic spindles, aneuploidy and cell death.

2. Materials and methods

2.1. Cell culture and treatments

For this study, three cervical cell lines (HeLa [HPV 18], ME-180 [HPV 68], and SiHa [HPV 16]) and human normal embryonic kidney 293 (293) cells were used. Although normal cervical cells would be more appropriate as a normal cell model, 293 cells are very easy to grow, well characterized, and widely used in cell biology research as a model of normal cells; therefore, we used 293 cells herein. The cell lines were obtained from the Korea Cell Line Bank (Seoul, Korea); cultured in DMEM or RPMI medium (WelGENE, Daegu, Korea) with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% foetal bovine serum (FBS); and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. T007 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMSO. Cells were treated without or with 50 μM T007 in 0.1% FBS media for the indicated times and then irradiated using a 6-MV linear accelerator (Mevatron M6700, Siemens Healthcare, Concord, CA, USA) with a dose rate of 3 Gy/min at room temperature. Dosimetry was carried out using a waterproof 0.6 cm^3 Farmer chamber (30013, PTW, Freiburg, Germany) connected to an electrometer system (UNIDOS E Universal Dosimeter, PTW) that is accredited by Korea Laboratory Accreditation. Cell culture dishes were placed on an acryl table and irradiated posteriorly by rotating the gantry to 180°. Except for irradiation, the non-irradiated cells were subjected to the same experimental conditions. After irradiation, the medium was changed to include 10% FBS, and no T007, and the cells were incubated at 37 °C for the indicated periods. In our previous study (An et al., 2014), we found that treatment with 50 μM T007 for 24 h was sufficient to reduce α and β tubulin protein levels and induce G2-M arrest, and the radiosensitising effect combined with T007 was observed well with 4 Gy in clonogenic survival assay when compared with other doses. Therefore, we used this dose test for changes in protein expression and radiosensitivity in this study.

2.2. Western blot analysis

After treatment, cells were washed with ice-cold PBS and lysed using cell lysis buffer (Cell Signaling, Beverly, CA, USA) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany) as previously described (An et al., 2012). Cellular debris was cleared by centrifugation at 12,000 × *g* for 15 min at 4 °C. The protein concentration in each sample was determined by Bradford assay using BioRad reagent (Bio-Rad, Hercules, CA, USA). An equal amount of protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, CA, USA). The membrane was then blocked using blocking buffer and washed in 0.2% Tris-buffered saline and Tween 20 (TBST) for 5 min. The membrane was incubated overnight with the appropriate primary antibodies: anti-γ-H2AX (EMD Millipore, MA, USA), anti-p-DNA-PKcs (Abcam, Cambridge, UK), anti-DNA-PKcs, anti-RAD51, anti-p-ATM, anti-p-ATR, anti-p-Chk1, anti-p-Chk2 and anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the membrane was washed in 0.2% TBST for 30 min, followed by incubation with the corresponding secondary antibody. The proteins were visualised using enhanced chemiluminescence (West-Zol plus, iNtRON Biotechnology, Seongnam, Korea). The density values of the band were measured by Multi Gauge V3.1 software (Fujifilm, Tokyo, Japan), and ratios were compared with the ratios in control samples set at 1.

2.3. Confocal microscopy

Cells were grown on cover slips placed in 35-mm dishes, were pretreated with or without T007, and irradiated (4 Gy) as described before (An et al., 2014). After incubation for the indicated periods, cells were fixed with 4% paraformaldehyde in PBS, permeabilised with 0.1% Triton X-100, blocked with 10% FBS for 30 min, and incubated overnight with primary antibodies (anti-γ-H2AX [EMD Millipore], anti-RAD51, α-tubulin, γ-tubulin [Santa Cruz]) at a dilution of 1:100 in 2% FBS/PBS at 4 °C. Cells were further incubated with Alexa-488 and Alexa-594-conjugated secondary antibodies (Invitrogen, CA, USA) for 1 h. Nuclei were stained with DAPI (1 μg/mL, Sigma). Cover slips were mounted on slides and images were captured and analysed using a confocal microscope (Leica DM-IRB, Mannheim, Germany).

2.4. Clonogenic assay

Cells in the growing phase were treated either with vehicle (DMSO) or 50 μM T007 for 15 h and then irradiated. Varying numbers (100 − 1000) of cells, to allow optimal colony counting, were seeded in 60-mm dishes; samples represented the presence or absence of T007 and radiation. After 7–14 days, the cells were stained with 0.1% crystal violet solution and colonies composed of at least 50 cells were counted. The surviving fraction was calculated by dividing the plating efficiency of treated cells by that of the control.

2.5. Statistical analysis

All data are represented as mean ± SEM. Differences between groups were calculated using Student's *t*-test. Results reaching $P < 0.05$ were considered statistically significant.

3. Results

3.1. T007 pre-treatment prolongs IR- induced DSBs

First, we conjectured that the radiosensitising effect of T007 on cancer cells might be caused by impairment in the repair of DSBs. We therefore determined the levels of DSBs by immunofluorescence staining of γ-H2AX foci at different time points post exposure to X-rays. In both ME-180 and SiHa cells, most γ-H2AX foci were cleared at 24 h after exposure to 4 Gy X-rays, and the number of γ-H2AX positive cells (cells with ≥10 foci) subsided to near basal level (Fig. 1A, B). In contrast, when pretreated with T007 for 24 h more γ-H2AX positive cells persisted up to 24 h in both cell lines (Fig. 1A, B). This was also confirmed from the protein level by western blot. Both cell lines showed delayed clearance of the IR-induced γ-H2AX when pretreated with T007 (Fig. 1C). This result indicates that T007 pre-treatment impairs repair of IR-induced DNA DSBs.

To identify the effect of T007 on the proteins related to repair of DSBs, IR-induced p-ATM, p-ATR and p-Chk1/2 levels without or with T007 pre-treatment were checked by Western blot. Pre-treatment with T007 did not cause significant changes in the phosphorylated forms of any of the four proteins (Fig. 1C).

3.2. T007 pre-treatment attenuates the radiation-induced upregulation of RAD51

We next determined whether the levels of DNA-PKcs and RAD51 proteins were changed by treatment with T007 (0–50 μM) for various incubation periods (12–48 h). Treatment with T007 significantly decreased the levels of DNA-PKcs and RAD51 proteins in ME-180 and SiHa cells at all the time points but not in HeLa and 293 cells (Fig. 2A). Next, the combined effect of T007 pre-treatment and radiation on the expression of DNA-PKcs and RAD51 proteins was determined using Western blot in ME-180 and SiHa cells. IR-induced p-DNA-PKcs was