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PRECLINICAL STUDIES

A phthalimidoalkanamide derived novel DNMT inhibitor enhanced radiosensitivity of A549 cells by inhibition of homologous recombination of DNA damage

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Summary

Purpose To elucidate the radiosensitizing effect and underlying mechanism of a new kind of DNA methyltransferase (DNMT) inhibitor with biological availability. *Methods* A novel non-nucleoside compound, designated as MA-17, was recently derived from a phthalimido alkanamide structure. DNMT expressions were confirmed in cultured human lung cancer (A549) and normal astrocyte (NHA) cells, radiosensitivity was measured using clonogenic assay, and assays of cell cycle alteration, apoptosis, DNA damage repair, and differential gene expression were undertaken. *Results* MA-17 significantly radiosensitized A549 cells with a mean dose enhancement ratio (DER) of 1.43 at the surviving fraction of 0.2 ($p < 0.05$ by one-tailed ratio paired t-test). MA-17 did not affect normal astrocytes (mean $DER_{0.2}$, 1.016; $p = 0.420$). MA-17 demonstrated a mean half-life of 1.0 h in vivo and a relatively even distribution in various tissues. Pretreatment with MA-17 increased sub-G1 fractions and inhibited the repair of DNA double-strand breaks, which are induced by irradiation. We found that MA-17 also down-regulated DNA homologous recombination and the Fanconi anemia pathway (*FANCA*, *BRCA1*, and *RAD51C*) in A549 cells. This bioinformatics finding was confirmed in validation Western blot to evaluate the expression of vital proteins. *Conclusions* A novel phthalimido alkanamide derivative, a DNMT inhibitor, possessed both biostability and favorable and substantial radiosensitizing effects by augmenting apoptosis or inhibiting DNA damage repair.

Keywords Radiosensitization · DNMT inhibitor · Epigenetics · Cancer

Introduction

The importance of epigenetic alterations in the initiation and progression of cancer has been established [1–4]. DNA methylation and histone code alteration are two principal mechanisms involved in epigenetic carcinogenesis and tumor progression. Treatments targeting this "non-coding" part of the biological process have been attempted. The successes observed in hematologic malignancies [5] have provoked preclinical and clinical research for clinical applications of epigenetic drugs in solid tumors [6].

It has been suggested that irradiation introduces epigenetic alterations [7]. Because epigenetic changes can potentially influence radiation response and the resultant clinical outcome, they may be considered as potential candidate targets of anticancer therapy that act as an enhancer of radiation response, a so-called radiation sensitizer. Several studies have focused on the clinical applicability of epigenetic drugs as

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radiosensitizers [8]. Psammaplin A (PsA), a non-nucleoside inhibitor, is an epigenetic modulator that sensitizes human cancer cells to radiation lethality. We have previously shown that PsA induces radiosensitivity in a lung cancer cell line [9]. However, a subsequent pharmacokinetic study reported that it is not stable across all biological matrices and that it tends to get rapidly eliminated in vivo [10]. To address this limitation, we synthesized a novel phthalimido alkylamide derivative, which has improved bioavailability and is capable of being used in vivo, named "MA-17".

We evaluated the radiosensitizing actions of MA-17 in a human lung cancer cell line. Currently, the molecular pathway, contributing to the underlying mechanism of DNMT inhibitors for enhancing the radiosensitivity of cancer cells, is not well understood. Although some studies suggest possible mechanisms of radiosensitization, including alteration of the cell cycle, apoptosis, and the DNA repair pathway [9, 11–13], a definitive mechanism at the gene expression level remains unknown. By identifying a method to assess differential gene expression, this method uses a radiation response modifying agent against cancer cells, to show genetic or epigenetic change. This study elucidates the mechanisms of the novel synthetic DNMT inhibitor, MA-17, using next-generation sequencing (NGS) of RNA and transcriptome analysis.

Materials and methods

DNMT inhibitors and irradiation

We synthesized radiosensitizers using PsA as the lead structure. A total of 26 novel compounds were synthesized and screened to identify compounds that enhanced radiation cell killing in human cancer cells and showed bio-stability in a mouse model. Finally, a phthalimido alkanamide derivative structure, MA-17, was selected as a potent radiosensitizer with enhanced bio-availability and a candidate for screening using an animal tumor model. For a comparative experiment, we used other DNMT inhibitors. 5-aza-2′-deoxycytidine (Dacogen) and PsA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

The 6 MX x-rays generated from a linear accelerator (Clinac 2100 C or Clinac 21EX, Varian Medical Systems, Palo Alto, CA, USA) were utilized. DNMT inhibitor, MA-17, was administered for 24 h, before the radiation treatment.

Cell culture

A549 cells, ascertained from a human non-small lung cancer cell (Korean Cell Line Bank, Korea), were used as cancer cells. NHA cells, derived from a normal human astrocyte cell, were used as normal control cells (Korean Cell Line Bank, Korea). A549 and NHA cells were cultured in RPMI and DMEM media, respectively, both supplemented with 10% fetal bovine serum and 12.5 μg/mL of gentamicin, at 37 °C in saturated air with 5% CO2.

Pharmacodynamics

The protocol to evaluate the pharmacokinetic properties of MA-17 was based on previous conventions established by Kim et al. [14]. In brief, male ICR mice were bred in plastic cages. The prepared MA-17 solution was injected via the penile vein at a dose of 10 mg/kg. Venous blood was collected at 5, 10, 15, 30, and 45 min by retro-orbital bleeding. Centrifugation of blood was done to harvest serum samples; these samples were stored at −80 °C. Concentrations of MA-17 in various tissues were measured. Sample preparation was performed after excision and homogenization in isotonic saline. All experiments were approved by the ethics committee for the treatment of laboratory animals at the Catholic University of Daegu (IACUC-2016-007). Animals were housed, and experimentation took place in the animal care facility at the Catholic University of Daegu.

Cell survival after irradiation or drug treatment

Cells were trypsinized, and the appropriate numbers of cells were seeded and incubated for 24 h before irradiation or drug treatment. The media was changed after treatment. After an incubation period of 14–21 days, the colonies were fixed with methanol and stained using 0.5% crystal violet. Colonies consisting of 50 cells or more were counted as survived cells, and the relative surviving fraction (SF) compared to the control plating efficiency was then calculated as the mean SF of three dishes at each radiation dose. When the cells were treated with DNMT inhibitors and irradiation, the SF was calculated relative to the SF after the DNMT inhibitor.

Flow cytometry, western blotting, and γH2AX assay

Flow cytometry was performed to identify the effects of drugs on the cell cycle. The expression of cleaved caspase-3, a marker of apoptosis, was examined using western blotting. Immunocytochemical analysis using anti-γH2AX antibodies, markers of DNA double-strand breaks (DSB), was performed to evaluate the effects of DNMT inhibitors on DNA repair of DSBs. The effects of DNMT inhibitors on DNMT expression were analyzed using western blotting for three DNMTs: DNMT1, 3A, and 3B.

A detailed protocol for flow cytometry, western blotting, and γH2AX assay has been previously described [9]. To summarize, the cells were treated with media or an IC_{50}s concentration (concentration resulting in 50% inhibition) of DNMT

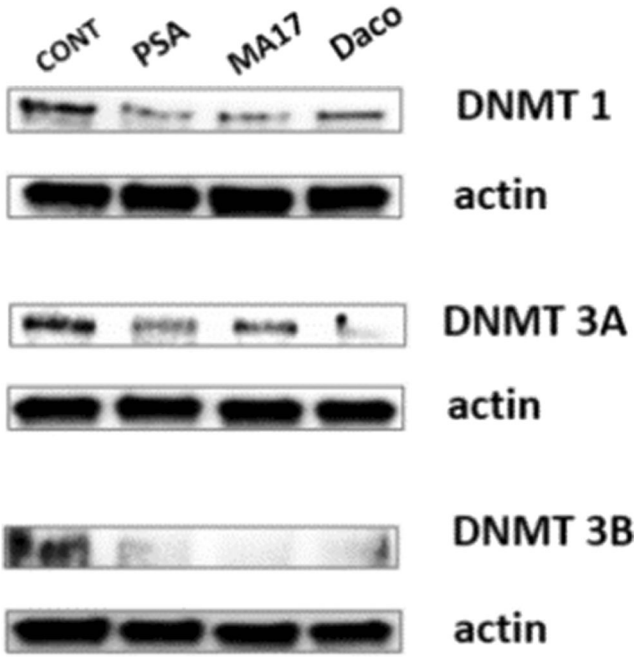

Fig. 1 DNMT expression after exposure to DNMT inhibitors was determined using Western blot analysis. The cells were treated with DNMT inhibitors for 24 h. Drastic depletion of DNMT1 and DNMT3A/3B by psammaplin A (PSA), 5-aza- 2'deoxycytidine (Dacogen), and MA-17 in A549 cell line was observed

inhibitors for 24 h, irradiated with 6 Gy, and then collected at 0, 2, 6, 24, and 48 h after radiation.

RNA isolation and sequencing

Quant-IT RiboGreen (Invitrogen) was used to calculate the total RNA concentration. Samples were run on TapeStation RNA ScreenTape (Agilent) to assess the integrity of the total RNA. Only high-quality RNA preparations (RNA integrity number > 7.0) were used for RNA library construction. A library was prepared with 1-μg of total RNA from each sample using the Illumina TruSeq mRNA Sample Prep kit (Illumina, Inc., San Diego, CA, USA). First, the poly (A)-containing mRNA molecules were purified using poly-T-attached magnetic beads. The mRNA was fragmented into small pieces with divalent cations at elevated temperatures. SuperScript II reverse transcriptase (Invitrogen) and random primers were used to copy the cleaved RNA fragments into first-strand cDNA. Second-strand cDNA synthesis using DNA polymerase I and RNase H were then performed. These cDNA fragments then underwent an end repair process, the addition of a single "A" base, and the ligation of indexing adapters. The products were purified and enriched with PCR to create the final cDNA library. The libraries were quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification Kits for Illumina sequencing platforms) and were then qualified using TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Indexed libraries were then sequenced with the HiSeq2500 platform (Illumina, San Diego, USA) by Macrogen Incorporated (Seoul, Korea).

Gene ontology (GO) and KEGG pathway analysis (KPA)

We performed gene set analysis for a functional study of the transcriptome data [15]. ToppGene was used to identify the enriched GO terms and pathways among the given list of genes that were differentially expressed in response to IR. Statistically overrepresented GO categories with Benjamini–Hochberg-adjusted p-values <0.05 were considered significant. KEGG Mapper [16–18] was used to map the expressed genes on KEGG pathways differentially. We used 320 overexpressed genes with a fold change of >1.5 or 433 downregulated genes with a fold change of <0.67 for this analysis.

Statistical analysis

Survival data were fitted using Kaleidagraph version 3.51 (Synergy Software, Reading, PA, USA) into a linear quadratic (LQ) model. Differences in mean values between groups were compared using the Student's t test. The clonogenic assay

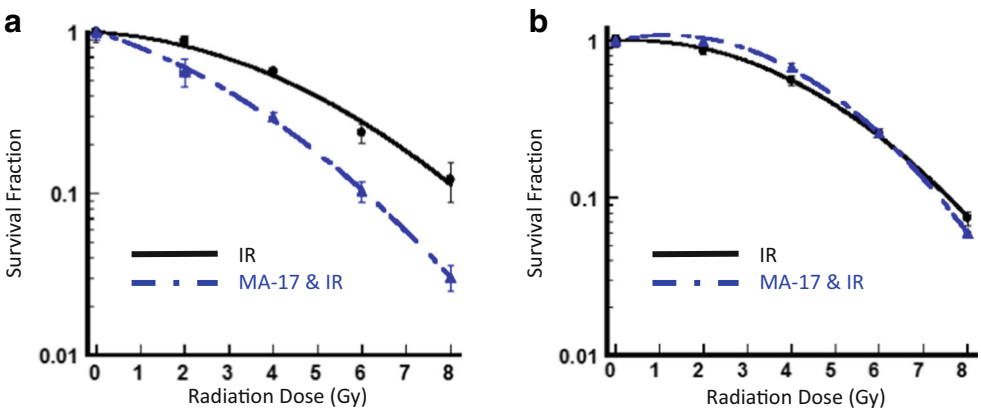

Fig. 2 The effects of MA-17 on A549 and NHA cell radiosensitivity. Survival curves of A549 (**a**) and NHA (**b**) cells treated with a combination of MA-17 pretreatment and radiation were compared with those of radiation treatment alone. Points, mean for three independent experiments; bars, SE

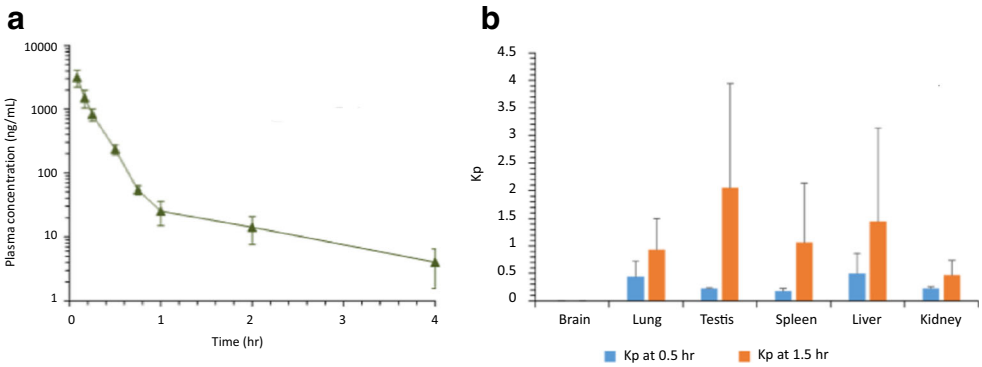

Fig. 3 Serum MA-17 concentration vs time curve (a) and tissue to serum partition coefficients (b). This curve was obtained after IV injection in mice at a dose of 10 mg/kg ($n = 5$). The tissue to serum partition coefficient was determined 30 and 90 min after IV injection

results were analyzed using repeated measures one-way ANOVA. A p value of <0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS 23.0 statistical software.

Result

IC50

The concentration of MA-17 used for the experiment was the concentration resulting in 50% inhibition of survival of cells, IC50 (inhibitory concentration of 50%). The IC50 of MA-17 was measured as 118.2 μM ± 33.9 μM in A549 cells and 301.9 μM ± 51.5 μM in NHA cells.

Efficacy of MA-17 as a DNMT inhibitor

The effects of DNMT inhibitors on the levels of DNMT expression are shown in Fig. 1. A drastic depletion of DNMT3A and DNMT3B by every DNMT inhibitor is demonstrated.

Enhancement of in vitro radiosensitivity

The combined treatment of MA-17 increased the cytotoxic effect of radiation in A549 cancer cells. In survival curve analysis, MA-17 enhanced the radiosensitivity of A549 cells. The ratio of the isoeffective radiation dose that is the dose enhancement ratio (DER) of MA-17 was 1.73, at an SF of 0.5 and 1.43 at an SF of 0.2 in A549 cells (Fig. 2a).

In contrast, the combined treatment of MA-17 did not enhance or weakly enhanced the radiosensitivity of the normal NHA cells. The DER of MA-17 was 1.02 at an SF of 0.5 and 1.11 at an SF of 0.2 in the NHA cells (Fig. 2b).

Pharmacodynamics

The MA-17 concentration obtained after a single IV injection is shown in Fig. 3a. The average terminal elimination half-life was 1.0 ± 0.3 h, and the systemic clearance was 184 ± 49.9 mL/min/kg. The tissue distribution of MA-17 was measured at 30 and 90 min after IV injection. The tissue concentrations and the tissue-to-serum partition coefficients (Kp) were obtained (Fig. 3b). The highest MA-17 concentration was observed in the testis. MA-17 concentrations were comparable with serum concentration in all tissues.

Cell cycle perturbation, apoptosis, and DNA damage repair

Flow cytometry showed the G2/M delay in A549 cells after radiation treatment alone. The effects of PsA pretreatment are shown in Fig. S1; the radiation-induced G2/M arrest was abrogated at 6-h. Conversely, delayed G2/M arrest at 24 h was

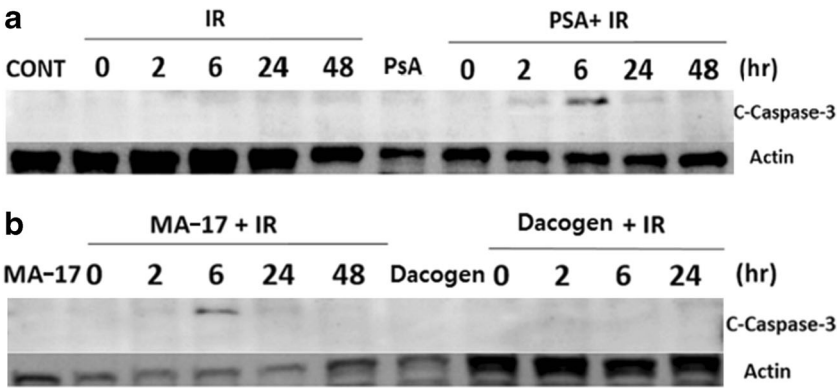

Fig. 4 Western blot analysis of cleaved caspase-3. The cells were treated with a combination of DNMT inhibitors and 6-Gy radiation. Increased cleaved caspase-3 protein levels were observed in cells treated with a combination of 6 Gy of radiation and psammaplin A or MA-17 in A549 cells

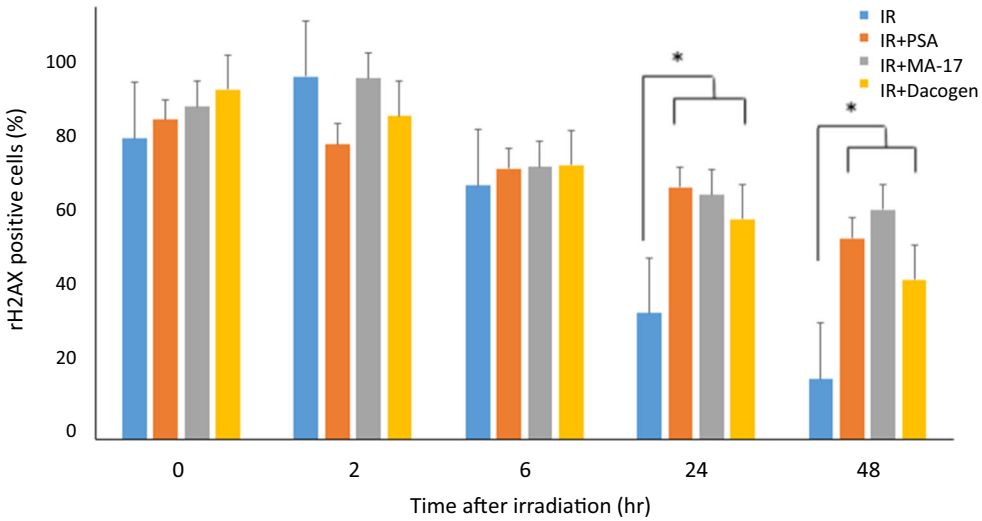

Fig. 5 Influence of DNMT inhibitors on radiation-induced γH2AX foci. A549 cells were fixed after pretreatment with DNMT inhibitors and radiation. Cells with more than five foci per nucleus were classified as positive for radiation-induced γH2AX. Foci were evaluated in 50 nuclei. Bars, SE. *$p < 0.01$ as determined by logistic regression compared with the group administered irradiation alone (6 Gy)

observed in cancer cells treated with MA-17 and radiation. Additionally, apoptotic rates were measured by the sub-G1 portion of flow cytometry; PsA and MA-17 pretreatments led to higher apoptotic rates than irradiation alone. An increased level of cleaved caspase-3 protein was observed by western blotting in cells treated with a combination of DNMT inhibitors and radiation (Fig. 4). The effects of combining Dacogen and radiation on the cell cycle and apoptosis were insignificant.

Immunocytochemistry using anti-γH2AX antibodies identified the effects of DNMT inhibitors on DNA damage repair. γH2AX foci could be visualized clearly after radiation treatment (Fig. S2), and the level of expression was shown to decrease over time. In cells treated with a combination of DNMT inhibitors and radiation, the level of γH2AX foci did not increase more at 1–6 h but declined less at 24–48 h (Fig. 5), compared to cells treated with radiation alone.

Transcriptome response modified by MA-17

The gene lists generated for irradiated A549 cancer cells with or without MA-17 pretreatment at 6-h after 6-Gy irradiation showed substantial differences in both the type and number of genes that were transcriptionally activated. Within 6 h of

Table 1 MA 17-associated pathways for radiosensitivity by Kegg pathway

Kegg pathway	q-value FDR B&H	Number of DEGs in pathway	Number of genes in pathway
Cell cycle	9.71E-18	28	124
DNA replication	1.78E-12	14	36
Homologous recombination	4.64E-08	11	41
Fanconi anemia pathway	1.12E-06	11	55
C5 isoprenoid biosynthesis, mevalonate pathway	2.29E-05	5	10
Cholesterol biosynthesis, squalene 2,3-epoxide = > cholesterol	3.86E-05	5	11
Steroid biosynthesis	6.41E-05	6	20
Oocyte meiosis	1.10E-04	13	124
Mismatch repair	1.49E-04	6	23
Terpenoid backbone biosynthesis	1.38E-03	5	22
Pyrimidine metabolism	2.07E-03	10	105
Progesterone-mediated oocyte maturation	4.31E-03	9	96
Nucleotide excision repair	6.94E-03	6	47
Base excision repair	8.18E-03	5	33
p53 signaling pathway	1.01E-02	7	69
HTLV-I infection	1.07E-02	15	256

FDR B&H, false discovery rate the Benjamini and Hochberg method; DEG, differentially expressed genes

Table 2 List of genes in DEG analysis relevant to apoptosis of the Kegg pathway

Gene	Log_2fold change
TNFRSF10D	0.868
JUN	0.836
BCL2A1	0.747
CTSL	0.700
BIRC3	0.664
MCL1	0.662

pretreatment, MA-17 appeared to significantly impact the transcriptional response in the A549 cell line, with regard to differentially regulated genes (using a 1.5-fold cut-off).

KEGG pathway analysis was performed for functional analysis of our gene list acquired from the RNA sequencing analyses. Interactions between significant differentially regulated genes were screened, and possible candidate hit-maps were selected. A total of 16 pathways were selected, which included several genes that were down-regulated more from combined MA-17 and radiation treatment than from irradiation alone (Table 1). Two pathways identified as relevant to DNA damage repair are shown in Fig. S3: homologous recombination and the Fanconi anemia pathway. Several genes have been demonstrated to have a fold change of >2 or < 0.5; these are highlighted in the blue boxes. These regulated genes include *FANCA*, *BRCA1*, and *RAD51C*. In gene set analysis to seek candidate genes related to the apoptotic pathway for studying radiosensitivity conferred by MA-17, we found several genes with paramount DEG when treated with MA-17 (Table 2).

Validation of protein expression relevant to radiosensitization

From GO and KEGG pathway analyses, we found that homologous recombination and the Fanconi anemia pathways are closely associated with the radiosensitizing effects of MA-17 on A549 cells. Based on this result, we chose the candidate genes for significant radiation-enhancing factors when combined with MA-17 (*RAD51C*, *BRCA1*, and *FANCA*). Expression levels following radiation and MA-17 pretreatment were evaluated using western blotting to validate our sequencing data. *RAD51C*, *BRCA1*, and *FANCA* expressions increased in A549 cells after radiation with a significant decrease in expression at 48 h. In contrast, decreased expression of all proteins was observed after administering a combination of MA-17 pretreatment and radiation treatment (Fig. 6).

Discussion

Radiation therapy is an established treatment modality for lung cancer. Dose-escalated radiation therapy can improve tumor control rates against radio-resistant cancer cells within a tumor, but it is limited by the threshold dose that can be tolerated by the surrounding, non-targeted normal tissues without severe sequelae. Any biological modulator that enhances the radiation response without dose escalation is advantageous in cancer treatment. Moreover, a radiosensitizer capable of facilitating tumor-specific effects can improve the therapeutic index of radiotherapy. Enhancement of radiation response strategies has been successful in the treatment of a variety of cancers [19–22].

In our study, radiosensitization of a lung cancer cell line by a novel synthetic DNMT inhibitor was demonstrated without affecting the normal cell line lethality from radiation. MA-17 significantly enhanced the radiation-induced apoptosis and DNA-damage repair inhibition of cancer cells. Also, MA-17 has demonstrated valuable clinical applicability due to its relatively high bioavailability with differential effects on cancer and normal cell lineages. MA-17 has a higher bioavailability than PsA: 1.0 ± 0.3 h in elimination half-life and 184 ± 49.9 mL/min/kg in systemic clearance in an animal study, and these properties are much better than those of PsA (0.16

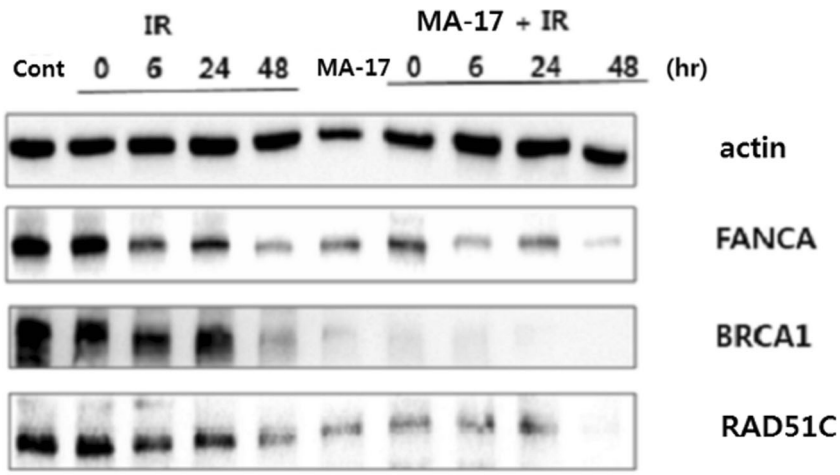

Fig. 6 Western blot analysis of *FANCA*, *BRCA1*, and *RAD51C*. Gene list was selected from candidate KEGG pathway analysis that supports in vitro results. The expression of three genes (*FANCA*, *BRCA1*, and *RAD51C*) was validated using Western blotting to compare between A549 cells treated with MA-17 before radiation and radiation versus those treated with radiation alone

± 0.02 h and 925 ± 570 mL/min/kg, in elimination half-life and systemic clearance, respectively). MA-17 showed a relatively even distribution in the current study [14]. Numerous candidate substances were validated to seek a bio-stable and effective DNMT inhibitor. MA-17 has been selected as an optimal drug. This work was conducted with interdisciplinary collaboration.

Typically, DNMT inhibitors have been known to get incorporated during the S-phase because of their nucleoside analog structure, thus inhibiting DNA synthesis [23]. DNMT inhibitors can inhibit the repair of radiation-induced DNA damage by reducing the number of tumor clonogens by conferring selective, preferential cytotoxicity to proliferative cells [8]. In our previous research, we demonstrated the radiosensitizing effects of PsA, Dacogen, and zebularine on lung cancer and glioblastoma cells by modulation of the impairment of the DNMA repair process. The expression of the DNA damage marker as determined by γH2AX was increased after co-treatment with DNMT inhibitors and radiation. This finding was confirmed in other in vitro [13] and in vivo [12] studies and reproduced in the present study. The cell cycle arrest abrogation is a possible mechanism for radiosensitization of DNMT though general inhibition of G2 checkpoint activation. Abrogation of the radiation-induced G2/M arrest was noted in the PsA-treated A549 cell line. However, this abrogation was not seen in the MA-17 pretreated cells. Finally, there is evidence suggesting that DNMT inhibitors trigger apoptosis. Qui et al. demonstrated that Dacogen increases the apoptotic rate of gastric cancer cells and results in enhanced radiosensitization [11]. De Schutter et al. also reported the role of Dacogen on head and neck cancer cells, inducing apoptosis when combined with radiation [24]. Similar results were noted in PsA- and MA-17-pretreated A549 cells in this study.

Although some results allude to the possible mechanisms of radiosensitization, no study has elucidated the precise mechanism. To our knowledge, this is the first study seeking possible mechanisms involved in the radiosensitizing effects of DNMT inhibitors using transcriptome data analysis. Using RNA sequencing, we identified that radiation-enhancing DNMT inhibitors induced transcriptome alteration in A549 cells. Comparing the RNA profiles altered by the MA-17 and radiation combination, we found that candidate genes had significant differential expression and were either up- or down-regulated by MA-17. Next, we performed GO and KEGG pathway analyses to search for significant and relevant molecular evidence. Several pathways were chosen for their statistical significance, and two pathways relevant to DNA damage repair that were identified supported our in vitro data.

The regulation of DNA repair and the expression of the Fanconi anemia-related genes (*RAD51C*, *BRCA1*, and *FANCA*) correlated with radiation sensitivity in A549 cells, and this was validated by western blotting. It is well known that ionizing radiation induces DNA damage [25, 26]; thus, it is not surprising that the down-regulation of DNA repair-related genes enhances the radiation response. These genes are of great importance in regulating the radiation response, and the finding that epigenetic modulation by MA-17 enhances the radiation response warrants further investigation. Also, *RAD51*, *BRCA1,* and another *FANC* family gene, *FANCG*, have previously been reported to be associated with treatment responses in various cancers [27–29]. However, no research investigating the association of radiosensitizer-induced down-regulation of these genes with radiation treatment efficacy against cancer cells exists. We cannot fully explain the mechanism of radiosensitization by the DNA repair pathway; however, our data suggests that the DNA repair pathway may be a possible therapeutic target for radiosensitization in NSCLC. Further studies are needed to support our conclusion.

Conclusion

In summary, our novel synthetic DNMT inhibitor has better bioavailability and enhances the radiosensitivity of A549 lung cancer cells in vitro through enhancement of apoptosis, inhibition of homologous recombination of DNA DSB, and probably modulation of the Fanconi anemia pathway. These findings suggest the potential of DNMT inhibiting agents for improved cancer radiotherapy.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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