Inhibition of NF-κB and DNA double-strand break repair by DMAPT sensitizes non-small-cell lung cancers to X-rays

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A B S T R A C T
We investigated the efficacy and mechanism of dimethylaminoparthenolide (DMAPT), an NF-κB inhibitor, to sensitize human lung cancer cells to X-ray killing in vitro and in vivo. We tested whether DMAPT increased the effectiveness of single and fractionated X-ray treatment through inhibition of NF-κB and/or DNA double-strand break (DSB) repair. Treatment with DMAPT decreased plating efficiency, inhibited constitutive and radiation-induced NF-κB binding activity, and enhanced radiation-induced cell killing by dose modification factors of 1.8 and 1.4 in vitro. X-ray fractionation demonstrated that DMAPT inhibited split-dose recovery/repair, and neutral DNA comet assays confirmed that DMAPT altered the fast and slow components of X-ray-induced DNA DSB repair. Knockdown of the NF-κB family member p65 by siRNA increased radiation sensitivity and completely inhibited split-dose recovery in a manner very similar to DMAPT treatment. The data suggest a link between inhibition of NF-κB and inhibition of DSB repair by DMAPT that leads to enhancement of X-ray-induced cell killing in vitro in non-small-cell lung cancer cells. Studies of A549 tumor xenografts in nude mice demonstrated that DMAPT enhanced X-ray-induced tumor growth delay in vivo.

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Introduction

Despite some recent advances in the treatment for non-small-cell lung cancer (NSCLC), the prognosis for many of the patients with this disease remains grim. In the U.S. population, lung cancer has the highest incidence of all cancer types, with over 222,520 cases projected for 2010, and is also the highest cause of mortality of all cancers, with over 157,300 projected in 2010 [1]. Not surprisingly, the prognosis for long-term survival of U.S. lung cancer patients ranks relatively low compared to other cancers, with a 5-year overall survival rate of 16% and 10-year overall survival of 11% [2].

As in most human cancers, the standard of treatment for NSCLC depends on the stage of the tumor. For a subset of patients with stage I and stage II pathology, surgical resection of the involved lobe gives the best prognosis for survival. For these patients, the 5-year survival has been reported to be in the range of 60 to 70% [3–5]. However, surgery is not always an option if these patients have existing comorbidities that make resection too risky to perform. For inoperable stage I and II NSCLC cases, stereotactic body radiation therapy (SBRT) has become a viable option [6–10].

Under optimal patient conditions, SBRT involves three large 18– to 20-Gy fractions of X-rays. This treatment regimen has been found to be as effective as surgical resection for patients with stage I disease [7–12]. But this treatment regimen is frequently not possible or has to be less aggressive if the tumor is staged T2 on the TNM scale, has broad thoracic wall contact, or is adjacent to the heart, hilus, or mediastinum [13,14]. These qualifiers used to help stage the disease for its size and location lead to a decrease in the overall subset of patients who can receive the standard SBRT treatment regimen. In fact, Timmerman and others have found that high-dose SBRT is appropriate only for peripherally located staged I disease and is contraindicated in central disease because of severe toxicity to centrally located structures of the thorax [13]. In addition, other investigators have found that although large-fraction radiation is effective in killing lung tumors, the surrounding healthy lung tissue can be at risk. Specifically, Wang et al. have shown that radiation-induced pneumonitis is the major dose-limiting factor in treating NSCLC with radiation therapy [15]. Finally, it has also been shown that when T2-staged NSCLC patients receive a less aggressive SBRT dosage regimen, those patients end up with lower disease-free survival (DFS) rates than the patients who receive the more aggressive T1-staged SBRT plan. Comparing the treatment and staging of these two groups, T1 patients who get three large-fraction doses have DFS at 1 year of 88% and at 2 years 85%. These T1 DFS rates are substantially better.


than the T2 patients who get smaller treatment doses and show DFS at 1 year of 76% and at 2 years only 54% [14].

Attempts to radiation-sensitize NSCLC by the addition of single agent or combinations of concurrent chemotherapy have had some success but overall survival remains relatively low [16,17]. Recently, attempts have been made to combine radiation with molecularly targeted agents in NSCLC treatment [18,19]. For example, NF-κB is a transcription factor that is constitutively activated in many types of cancer, including NSCLC, and has been associated with the ability to survive multiple cellular insults including chemotherapy and radiation treatment [20–26]. We and other investigators have shown that inhibition of NF-κB activity by the addition of parthenolide or other means can enhance radiation-induced cell killing in human tumor cells [21,26–28].

Parthenolide is the naturally occurring sesquiterpene lactone that is extracted from feverfew, a plant used in folk medicine for centuries because of its anti-inflammatory properties [29–31]. Parthenolide is an agent that is known to inhibit NF-κB activity by preventing its release into the cytoplasm from IκB [32,33]. Our lab and others have shown that parthenolide has radiosensitizing effects on tumor cell lines in vitro but low bioavailability may limit its clinical potential [27,28,34]. For this reason, Crooks et al. have modified parthenolide into dimethylaminoparthenolide (DMAPT), a form of the drug with a much greater bioavailability and, presumably, better clinical usefulness [35–37]. In fact, DMAPT has been shown in mouse and canine models to have a bioavailability of 70% [35].

In our work here, we assessed the radiosensitization effects of DMAPT with two radiation-resistant NSCLC cell lines, A549 and H1299. Both NSCLC lines have constitutive activation of NF-κB but A549 is p53 wild type and H1299 is p53 null. We found that DMAPT induced tumor cell death and radiosensitized these NSCLC lines by inhibiting NF-κB-mediated DNA double-strand break repair.

Materials and methods

Cell lines

A549 (p53 wild type) and H1299 (p53 null) non-small-cell lung cancer cell lines were obtained from Dr. John J. Turchi, Indiana University School of Medicine. Cells were grown in Dulbecco’s modification of Eagle’s medium X1 (DMEM X1; Mediatech, Herndon, VA, USA), 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Mediatech). Cells were cultured at 37 °C, 5% CO2, and 85% humidity. Subconfluent stock cultures plated at 2.4 × 104 cells were grown in T-75 tissue culture flasks 1 to 3 days before all experiments. A549 population doubling time was 17.2 ± 2.4 h and H1299 population doubling time was 17.0 ± 3.2 h. Irradiation studies were conducted using a 160-kVp Faxitron X-ray machine. Settings for the X-ray machine were as follows: 0.5 mm Cu filter, d = 33 cm, and dose rate of 62.8 cGy/min.

Assessment of DMAPT’s activity on constitutive NF-κB, plating efficiency, apoptosis, and cell growth in A549 and H1299 NSCLC cells

DMAPT, obtained from Christopher Sweeney, is a derivative of parthenolide, a sesquiterpene lactone, found in the feverfew plant [35–37]. A549 and H1299 cells were harvested from exponentially growing stock cultures and plated in T-25 flasks (Corning). Twenty-four and 48 h after plating appropriate volumes of 36 mM DMAPT drug stock diluted in DMEM were directly added to the cell culture flasks so that final concentrations were 0–15 μM for the A549 cells and 0–4 μM for the H1299 cells. Control and treated cells were harvested for NF-κB electrophoretic mobility-shift assay (EMSA)/gel shifts, cell counting/growth curve analysis, clonogenic potential, annexin V–phosphatidylserine/propidium iodide apoptosis analysis, and Western blot studies at 24 and 48 h after drug addition by our standard techniques [22,27,28]. The above experiments were performed with 15 μM DMAPT in A549 cells and 4 μM DMAPT in H1299 cells unless otherwise indicated.

EMSA

A549 cells were treated with 0, 2.5, 5, 7.5, 10, 15, or 20 μM DMAPT at 24 and 48 h as described above and also with 15 μM DMAPT plus 7 Gy irradiation and collected on a time course of 0, 2, 4, 6, and 8 h postirradiation. Likewise, H1299 cells were treated with 0, 2, or 4 μM DMAPT at 24 and 48 h as described above and also with 4 μM DMAPT plus 7 Gy irradiation and collected on a time course of 0, 2, 4, 6, and 8 h postirradiation. Cell extracts were harvested at the above time points by scraping flasks on ice for NF-κB EMSA/gel-shift analysis by our standard methods described previously [22,27,28]. The NF-κB oligonucleotide 5′-AGTTGACGGGAGCTTTCCCCAGG-3′ from Promega containing the NF-κB DNA binding consensus sequence was T4 kinase end-labeled with radioactive 32P (PerkinElmer, Waltham, MA, USA) and utilized for all studies.

Western blots

Twenty-four and 48 h after treatment with 0 or 15 μM DMAPT for A549 cells and 0 or 4 μM DMAPT for H1299 cells, the respective cell lines were analyzed for changes in protein expression by our standard Western analysis [27,28]. The primary antibodies utilized were anti-p53 (Ab-6, Calbiochem, and clone BP53-12, Upstate), anti-p21 (clone 1A12C1, Santa Cruz), anti-Bax (clone YTH-2D2, Trevigen), anti-Bcl-2 (clone 124, Dako), anti-Bid (BD Pharmingen), anti-MDM-2 (Upstate), anti-α-actin (clone AC-15, Sigma), and anti-α-tubulin (clone B-5-1-2, Sigma). The membranes were incubated with appropriate secondary antibodies conjugated with peroxidase (Pierce, Rockford, IL, USA) and the various protein levels were detected by chemiluminescence (Western Lighting Plus-ECL; PerkinElmer) and imaged with Kodak film (BioMax XAR; Kodak, Rochester, NY, USA).

Irradiation protocols with and without DMAPT single dose, and irradiation studies

A549 cells were treated with 0 or 15 μM DMAPT at 24 and 48 h and H1299 cells were treated with 0 or 4 μM DMAPT at 24 and 48 h. After treatment for 48 h the cells were then irradiated with 0–7 Gy of 160-kVp X-rays. After an additional 24 h for repair, the cells were trypsinized, and single-cell suspensions were prepared and counted. At each X-ray dose level appropriate numbers of cells were then plated into six T-25 flasks for standard colony-forming assays by our published methods to assess clonogenic capacity/plating efficiency and survival [27,28]. As previously stated, the surviving fractions (SF) after various X-ray doses and DMAPT treatments were always normalized for the radiosensitization effects of DMAPT alone. This was done to distinguish enhancement of radiation-induced cell killing from merely additive toxicity of the drug and radiation [27,28]. The mean ± standard deviation (SD) of the SFs of the radiation alone versus the mean ± SD of the SFs observed with DMAPT and radiation were compared using the Student t test for significance (p ≤ 0.05). Each experiment was performed three to five times and survival curves were constructed to calculate the dose modification factors for DMAPT acting on both cell lines. The survival curve parameters, D0, Dn, and n, were then calculated by fitting the data with the multitarget/single-hit model [RT version 2.0 software; Drs. Normand Albright and Marco Durante] [27,28].

Immediate plating and split-dose repair experiments

As previously described for these experiments, there were six experimental groups: control, DMAPT only (15 μM for A549 and 4 μM for H1299)
for H1299), 5 Gy with immediate plating, 5 Gy with DMAPT and immediate plating, 2×2.5 Gy split-dose group without DMAPT, and finally 2×2.5 Gy split-dose group with DMAPT. The split-dose radiation groups had a 4-h incubation period at 37 °C between the 2.5-Gy radiation doses, allowing for the cells to repair broken DNA strands [27,28,38,39]. For all groups in both lines, cells were irradiated on ice to prevent repair during irradiation [27,28].

**Comet assay protocol**

To study the role of DMAPT in DNA double-strand break (DSB) repair, neutral comet assays were performed using CometAssay kits (Trevigen 4252-040-K) [40–42]. A549 cells were treated with 0 or 15 μM DMAPT for 24 and 48 h and H1299 cells were treated with 0 or 4 μM DMAPT for 24 and 48 h. After the drug treatment, cells were irradiated at 20 Gy. To monitor DSB rejoining, cells were placed at 37 °C and trypsinized at a given 0-, 3-, 6-, 12-, or 24-h incubation period. After being washed with PBS, the cells were embedded in agarose, lysed, and subjected to neutral electrophoresis. Immediately before image analysis, the cells were treated with SYBR green. The cells were examined with a Leica CTR 5000 fluorescence microscope and comet images were captured using the SPOT RT software. The tail moments of comets were analyzed using the TriTekCometScore Freeware program (www.autocomet.com). Average tail moment values were estimated by examining at least 100 cells per sample [40–42].

**Protocol for in vivo A549 lung tumor xenograph studies in nude mice**

In vivo tumor studies in this experiment were carried out with female athymic nude mice purchased from Harlan. These studies followed our university-approved IACUC protocol. The mice were injected in the flank with 2 million A549 cells and the tumors were allowed to grow for 1 week. The mice with tumors were then placed in one of four groups: control, drugged only, irradiated only, and drugged plus irradiated. There were 10 mice/tumors per group. The control and irradiated-only groups were gavaged with 2.5% mannitol, and the drugged groups were orally gavaged at 100 mg/kg with DMAPT in 2.5% mannitol daily for 7 days. Irradiated groups received one 5-Gy treatment using a 250-kVp Precision X-ray machine with a collimator set at 2.0×2.0-cm field size to focus radiation treatment on the tumor. For the drug plus irradiation group, radiation treatment was done on the third day after drug treatment was started. The animals were followed for 74 days to monitor tumor progression. ANOVA statistical analyses of changes in tumor growth for each treatment group

![Fig. 1](image-url)

**Fig. 1.** (A) NF-κB EMSA gel shifts assayed for NF-κB DNA consensus sequence binding activity (left), cell population doubling times (middle), and plating efficiencies (right) of A549 human NSCLC cells treated with 0 or 15 μM DMAPT for 48 h. (B) NF-κB gel shifts, cell population doubling time, and plating efficiency of H1299 human NSCLC cells treated with 0 or 4 μM DMAPT for 48 h. The NF-κB gel shift for H1299 cells treated with 2 μM DMAPT for 48 h is also shown. We have previously demonstrated by antibody-based EMSA supershifts that the NF-κB consensus binding sequence we utilize under our EMSA conditions results in three shifted bands, with the p65/p50 heterodimer and the p50/p50 homodimer consistently resolving as the top two bands [22,27,28]. Changes in cell doubling time and plating efficiencies induced by DMAPT treatment in both A549 and H1299 cells were statistically significant (t test, *p*<0.05).
compared to the other three groups were performed using either relative tumor area versus time or relative tumor growth velocities at various days after treatment. Only $p$ values $\leq 0.05$ were reported as significant.

Results

**DMAPT decreases constitutive NF-$\kappa$B binding activity and inhibits cell proliferation in NSCLC cells**

EMSA were performed to investigate the effect of DMAPT on the binding activity of NF-$\kappa$B (p65/p50 heterodimer) to the NF-$\kappa$B DNA promoter consensus sequence in A549 and H1299 NSCLC cells. EMSA indicated that DMAPT reduces NF-$\kappa$B binding activity at 15 $\mu$M in A549 and 4 $\mu$M in H1299 (Figs. 1A and B, left). In addition to reducing constitutive NF-$\kappa$B binding, treatment with 15 $\mu$M DMAPT increased the cell population doubling time of A549 cells from 17.2 $\pm$ 2.4 to 40.3 $\pm$ 3.8 h (Fig. 1A, middle) ($t$ test, $p < 0.05$). Similarly we found that 4 $\mu$M DMAPT increased the doubling time in H1299 cells from 17.0 $\pm$ 3.2 to 32.6 $\pm$ 9.0 h (Fig. 1B, middle) ($t$ test, $p < 0.05$).

For both cell lines, clonogenic assays were then performed to determine if the increased doubling time was due to cell death. In A549 cells, we found that 48 h of exposure to 15 $\mu$M DMAPT significantly decreased the plating efficiency from 0.61 $\pm$ 0.18 to 0.26 $\pm$ 0.16 (Fig. 1A, right, $t$ test, $p < 0.05$). These data indicate that increased cell death clearly played a role in DMAPT growth inhibition. In Fig. 2A, we show that the increased cell death in A549 cells cannot be explained by the induction of apoptosis, because 15 $\mu$M DMAPT actually reduced the spontaneous apoptosis level in A549 cells from 10.0 $\pm$ 4.6% annexin V-positive cells to 4.4 $\pm$ 2.0% ($t$ test, $p = 0.13$). Western blot analysis confirmed that although exposure to DMAPT increased p53 and the p53-regulated cell cycle protein and the cyclin-dependent kinase inhibitor p21$\text{waf1/cip1}$, no discernible changes in the expression of the antiapoptotic Bcl-2 or proapoptotic Bax proteins were observed (Fig. 2A, right).

The increased population doubling time observed with H1299 DMAPT-treated cells also appears to be due to increased cell death, because 4 mM DMAPT significantly decreased plating efficiency from 0.61 $\pm$ 0.10 to 0.25 $\pm$ 0.13 ($t$ test, $p < 0.05$; Fig. 1B, right). However, once again, no significant induction of apoptosis was observed ($4.1 \pm 1.1\%$ annexin V-positive cells in controls versus $9.2 \pm 3.6\%$ in DMAPT-treated H1299 cells; Fig. 2B, left, $t$ test, $p = 0.08$). Western blots confirm no significant changes in pro- or antiapoptotic proteins at 48 h in the p53-null H1299 cells treated with DMAPT (Fig. 2B, right).

![Fig. 2.](image-url) (A) Percentage of apoptotic cells (determined by flow cytometry-based annexin V staining) and Western blots of protein extracts from A549 NSCLC cells treated with 0 or 15 $\mu$M DMAPT for 24 or 48 h and probed with the antibodies shown. (B) Percentage of apoptotic cells (determined by flow cytometry-based annexin V/PI staining) and Western blots of protein extracts from H1299 NSCLC cells treated with either 0 or 4 $\mu$M DMAPT for 24 or 48 h and probed with the antibodies shown. The changes in the percentage of apoptotic cells induced by DMAPT were not statistically significant in either A549 or H1299 cells ($t$ test, $p > 0.05$).
DMAPT inhibits radiation-induced NF-κB binding activity in NSCLC cells

To determine whether exposure to DMAPT would alter radiation-induced NF-κB activation in A549 and H1299 NSCLC cells, we irradiated exponentially growing cell cultures with 7 Gy of 250-kVp X-rays and collected cell extracts from each of them after incubation times of 0, 2, 4, 6, and 8 h. For 7-Gy-irradiated A549 and H1299 cells, we found that radiation-induced NF-κB binding activity reaches peak activation at 4 h after irradiation and then drops off (Figs. 3A and B, left). The presence of 15 μM DMAPT completely suppressed the radiation-induced increase in NF-κB binding activity for up to 8 h (Figs. 3A, right). By contrast, although DMAPT-induced suppression of radiation-induced NF-κB binding activity in H1299 cells was observed, it was relatively modest compared to irradiated A549 cells treated with DMAPT (Figs. 3A and B, right).

DMAPT enhances radiation-induced NSCLC cell killing

A549 and H1299 cells were irradiated at incremental doses from 0 to 6 Gy alone or after 48 h of incubation with 15 (A549) or 4 μM (H1299) DMAPT and clonogenic survival curves were constructed and compared (Fig. 4, left and right). We observed an enhancement of radiation-induced cell killing (significantly lower surviving fractions) for DMAPT-treated cells compared to irradiated-only controls across the spectrum of radiation doses for both A549 and H1299 NSCLC cells (t test, p < 0.05). For example, survival fraction at 2 Gy (SF2Gy) for A549 was 0.59 ± 0.06 for controls versus 0.35 ± 0.12 for DMAPT-treated cells (t test, p = 0.0039). SF2Gy for H1299 cells was 0.64 ± 0.12 versus 0.39 ± 0.07 for DMAPT-treated cells (t test, p = 0.0038). The surviving fractions of the DMAPT-treated cells were corrected for drug toxicity and therefore drug toxicity could not account for the enhanced X-ray-induced cell killing observed. The survival curves for both cell lines demonstrate that DMAPT reduced the size of the shoulder and increased the slope of the radiation survival curves. The survival curves were fit with a single-target multihit radiobiological model and the D0 (dose to reduce survival to 37%) and D10 (dose to reduce survival to 10%) were calculated. The dose modification factors (DMFs) for A549 cells treated with 15 μM DMAPT were 1.6 (5.8 Gy/3.8 Gy = 1.6) at the 10% survival dose and 1.8 (1.8 Gy/1.0 Gy) when calculated by the ratio of the D0 s. The DMF for H1299 cells treated with 4 μM DMAPT was 1.4 at the 10% survival dose (6.2 Gy/4.6 Gy = 1.4) or, when calculated by taking the ratio of the D0 s (2.2 Gy/1.6 Gy), 1.4.

DMAPT alters cell cycle distribution

We have demonstrated that incubation with DMAPT increases the radiation sensitivity of NSCLC cells in vitro. To investigate the mechanism of X-ray sensitization, we first performed flow cytometry to test whether altered cell cycle distribution could explain the DMAPT-induced radiosensitization of p53 wild-type A549 and p53-null H1299 lung cancer cells (Fig. 4B, left). Flow cytometry indicated that DMAPT significantly increased the number of A549 cells in the radiosensitive G2/M phase from 8.1 ± 3.5 to 29.4 ± 6.3% (t test, p = 0.007). The percentages of G1- and S-phase cells in A549 were reduced by DMAPT treatment, but not in a statistically significant manner. By contrast, H1299 cells treated with 4 μM DMAPT had no statistically significant alterations in the percentage of cells in any phases of the cell cycle (Fig. 4B, right). In summary, the increase in radiation sensitivity of A549 cells by DMAPT treatment could at least be partially explained by an increase in the percentage of X-ray-sensitive G2/M cells, but the H1299 X-ray sensitization does not seem to involve cell cycle alteration.

DMAPT inhibits sublethal split-dose repair and enhances fractionated X-ray-induced cell killing

To investigate whether DMAPT increased radiation-induced cell killing through inhibition of split-dose repair in both NSCLC cell lines, X-ray fractionation studies were performed (Figs. 5A and B).
Both A549 and H1299 received either a single 5-Gy-dose X-ray treatment with and without DMAPT present or two 2.5-Gy fractions of radiation with 4 h of recovery with or without DMAPT present. In A549 cells, we found that fractionating the radiation into two 2.5-Gy doses significantly increased survival to 17.4 ± 2.0% compared to a single fraction of 5 Gy, but incubation with 15 μM DMAPT completely inhibited the split-dose repair and resulted in survival of 0.7 ± 0.1% (t test, p < 0.0001). Therefore, the survival for A549 treated with the 2 × 2.5-Gy split dose plus DMAPT was nearly the same as the survival for these cells treated with a single dose of 5 Gy (Fig. 5A). For H1299 treated with 4 μM DMAPT, our results show that fractionating the radiation into two 2.5-Gy fractions and allowing for repair significantly increased survival to 25.6 ± 4.8%. As above, incubation with DMAPT completely inhibited split-dose recovery, resulting in survival of 2.5 ± 0.6% (t test, p = 0.0001). As we observed with A549 cells, the survival for the fractionated 2 × 2.5 Gy with DMAPT in H1299 was similar to the single 5-Gy-dose survival, suggesting that repair of radiation damage was significantly inhibited by DMAPT treatment (Fig. 5B).

Inhibition of p65 by siRNA knockdown inhibits sublethal split-dose repair

To test whether DMAPT’s inhibition of NF-κB was involved in the suppression of split-dose repair, we performed single and split doses with A549 and H1299 cells in which the NF-κB family member p65 was knocked down by treatment with siRNA against p65 mRNA. Survival was compared for single doses of 5 Gy and fractionated 2 × 2.5-Gy doses, with and without pretreatment with siRNA to knock down p65 protein expression. Western blots confirmed that the NF-κB protein p65 was knocked down by the siRNA treatment and that the expression of other proteins involved in cell cycle and apoptosis was not altered (Figs. 6A and B, left). Knockdown of p65 by siRNA treatment in both A549 and H1299 lines significantly reduced split-dose recovery and repair in these cells compared to those treated with scrambled control siRNA (Figs. 6A and B, right). In addition, it is important to note that treating A549 and H1299 cells with DMAPT after p65 knockdown by siRNA does not further enhance radiation-induced cell killing after single or split doses of X-rays (data not shown). This complements our published observations that the addition of parthenolide (DMAPT’s parental compound) after siRNA knockdown of p65 in prostate cancer cells did not further increase cell killing after single or split doses of X-rays (data not shown). Taken together, these results suggest that it is DMAPT’s inhibition of NF-κB activity that is at least partially responsible for the observed inhibition of split-dose recovery and repair in irradiated A549 and H1299 cells.

Comet assays for quantifying radiation-induced double-strand DNA breaks in DMAPT-treated cells versus untreated cells

Although the above data suggest that the inhibition of split-dose recovery by DMAPT could be due to inhibition of DNA double-strand break repair, a more direct test was required to demonstrate DMAPT altered DNA DSB repair. We performed neutral comet assays on both lines by standard methods to investigate this possibility [40–42]. Neutral comet assays have been shown to be an indication of the induction and repair of DNA DSBs in mammalian cells [40–42]. Groups of flasks with A549 and H1299 cells were irradiated on ice with 20 Gy of X-rays, processed, and then subjected to neutral comet assay to quantify tail moment length versus time after irradiation with and without DMAPT present. Tail moments were measured for each treatment condition and graphed in a time course for both cell lines (Fig. 7A, right and left). Our comet assay of DNA DSB repair
kinetics demonstrated the typical bimodal repair kinetics with fast and slow repair components, with the fast component of repair being completed during the first hour postirradiation [40–43]. The treatment of A549 cells with 15 μM DMAPT resulted in statistically significantly increased comet tail moment length at 3, 6, 14, and 24 h compared to control cells treated with 20 Gy of X-rays alone (t test, p < 0.05; Fig. 7A, left). In irradiated H1299 cell lines, DMAPT treatment also significantly slowed comet tail moment recovery at 3, 6, and 14 h compared to H1299 cells treated with 20 Gy alone (t test, p < 0.05; Fig. 7A, right, Supplementary Fig. 7S). The above data indicate that DMAPT seems to significantly alter the fast and slow components of DNA DSB repair as assessed by comet assay, which we and others have shown to correlate with increased cell death and lower survival after X-ray exposure of mammalian cells in vitro [40–43].

**DMAPT increases radiation-induced A549 tumor xenograft growth delay in vivo**

To test whether DMAPT can sensitize NSCLC cells to X-rays in human tumor xenographs in vivo, groups of nu/nu nude mice were each injected in the right flank with 2 million A549 NSCLC cells and the tumors were allowed to establish for 1 week. The tumor-bearing mice were then placed in four groups with 10 mice/tumors per treatment group. The treatment groups were control, DMAPT treated, irradiated (5 Gy), and DMAPT treated and irradiated (5 Gy). The control and irradiated-only groups were orally gavaged with 2.5% mannitol for 7 days, and the DMAPT-treated groups were orally gavaged with 100 mg/kg DMAPT in 2.5% mannitol daily for 7 days. Irradiated groups were given 5-Gy treatment using a 250-kVp Precision X-ray...
machine with a collimator set at 2.0 × 2.0-cm field size, which allowed us to focus the radiation treatment on the tumor and minimize normal tissue exposure. The tumors in each treatment group were measured with calipers and tumor area was calculated. The relative tumor area for each group was normalized to its tumor area on day 9 after subcutaneous injection. Relative tumor area versus days postinjection for control, DMAPT treated, 5-Gy irradiated, and 5-Gy irradiated with DMAPT is shown in Fig. 7B.

Daily treatment with 100 mg/kg DMAPT for 7 days slowed tumor growth compared to mannitol carrier controls and increased the time to attain 4× tumor area from 34 to 39 days, a growth delay of 5 days (Fig. 7B). Irradiation of tumors with 5 Gy compared to mannitol tumor controls also slowed tumor growth and increased the time to attain 4× tumor area from 34 to 44 days, a growth delay of 10 days. Treatment with DMAPT plus 5 Gy irradiation, however, slowed tumor growth and increased the time to attain 4× tumor area from 34 to 61 days, a growth delay of 27 days. Statistical analyses of relative tumor area versus time indicated that only A549 tumor xenographs treated with 100 mg/kg DMAPT for 7 days plus 5 Gy of X-rays were statistically different from the A549 tumor xenograph controls treated with the mannitol carrier for 7 days (ANOVA, *p = 0.026).

Discussion

Dimethylaminoparthenolide is a water-soluble derivative of the antineoplastic agent parthenolide with good bioavailability that is currently in phase 1 clinical trials [35–37]. In this study, we assessed the radiosensitization effects of DMAPT with two radiation-resistant NSCLC cell lines, A549 and H1299. Both these NSCLC lines have constitutive activation of NF-κB, but A549 and H1299 cells are p53 wild-type and null, respectively.

We found that treatment with DMAPT significantly reduced NF-κB constitutive binding activity, increased cell population doubling time, and decreased plating efficiency (Fig. 1). These studies are in general agreement with our recent findings with DMAPT [36,37]. Interestingly, we found that DMAPT did not induce significant apoptosis in the p53 wild-type A549 or p53-null H1299 cells (Fig. 2). We and others have observed apoptosis, necrosis, and an atypical form of apoptosis after parthenolide and/or DMAPT treatment [27,28,36,37,44]. The mode of cell death induced by parthenolide and DMAPT in various cancer cells seems to be both cell line and dose dependent [27,28,36,37,44].

Further investigation revealed that DMAPT treatment lowered radiation-induced NF-κB binding activity and enhanced X-ray-induced cell killing of both A549 and H1299 cells with single fractions of X-
rays, with dose modification factors of 1.6–1.8 for A549 and 1.4 for H1299 cells (Figs. 3 and 4A). Cell cycle analyses for A549 suggested that some of the radiosensitization might be due to an increase in the percentage of cells in the radiosensitive G2/M phase in A549 cells, but the shift into G2/M was not observed in the H1299 line (Fig. 4B). In fact, DMAPT treatment caused H1299 cells to shift slightly into the radiation-resistant S phase of the cell cycle.

DMAPT treatment reduced the shoulder of the single-dose radiation survival curves in both A549 and H1299 cells. Because a reduced survival curve shoulder has been correlated with repair inhibition, we investigated whether DMAPT would alter split-dose repair when radiation was fractionated (Fig. 5). Both A549 and H1299 were proficient at split-dose repair and recovery compared to the survival fractions of a 5-Gy single dose versus two 2.5-Gy fractions separated by 4 h for repair. Treatment with DMAPT completely inhibited the increase in survival fraction observed during split-dose repair/recovery in A549 and H1299 lung cancer cells (Fig. 5B).

NF-κB is a radiation-induced, prosurvival transcription factor. We hypothesized that it was DMAPT’s inhibition of NF-κB that is responsible for the inhibition of split-dose repair. We demonstrated by siRNA knockdown of p65, an active component of the NF-κB heterodimer in both A549 and H1299 cells, that split-dose repair/recovery was also inhibited (Figs. 6A and B). We then showed by neutral DNA comet assays that DMAPT’s inhibition of split-dose recovery was strongly correlated with inhibition of DNA double-strand break repair (Fig. 7A, right and left). Neutral comet assays have been shown to be a good indicator of the induction and repair of DNA DSBs in mammalian cells [40–42].

It is important to point out that as part of our preliminary studies, we performed DMAPT drug dose-escalation studies. These dose-escalation studies allowed us to find the optimal concentration of DMAPT that inhibited constitutive and radiation-induced NF-κB binding activity and increased cell killing in both A549 and H1299 cells. In addition, these preliminary experiments demonstrated that at drug concentrations at which DMAPT does not reduce constitutive and/or radiation-induced DMAPT binding activity, DMAPT does not increase radiation-induced cell killing of A549 or H1299 cells (data not shown). Therefore, at suboptimal DMAPT concentrations at which NF-κB activity remains elevated, we do not observe radiosensitization. The above in vitro observations further support our proposal that there is a strong relationship between inhibition of NF-κB activity by DMAPT and radiosensitization.

Both parthenolide and the more bioavailable derivative DMAPT have been shown both to induce oxidative stress (reactive oxygen species, ROS) and to inhibit NF-κB binding activity [22,36,37,45]. In addition, we have also shown that treatment with NAC can block DMAPT-mediated ROS generation [36,37]. However, the relationship between DMAPT’s actual mechanism of action, ROS generation, and NF-κB inhibition remains complex and unclear. Therefore, the potential role of altered cellular redox induced by DMAPT in our observed X-ray sensitization of lung cancer cells is unknown and will be part of our future studies.

Finally, our in vivo studies of A549 tumor xenografts injected subcutaneously into nude mice demonstrated that treatment with DMAPT for 7 days slowed tumor growth and significantly enhanced X-ray-induced tumor growth delay after 5 Gy from 10 to 27 days, indicating a strong enhancement of in vivo radiation-induced cell killing by DMAPT (Fig. 7B). This agrees with recent data by which we demonstrated in vivo tumor activity with DMAPT treatment alone in prostate and lung cancer mouse xenografts [36,37] and now demonstrates the potential of DMAPT to enhance the radiation response of NSCLC lung cancers. However, although we have shown here that the in vitro increase in radiation sensitivity induced by DMAPT is also observed when the NF-κB family member p65 is knocked down by siRNA treatment, it remains to be investigated whether the increase in radiation-induced growth delay of A549 lung tumor xenografts in vivo is due to alteration of radiation-induced NF-κB activity.

Conclusion

We demonstrate here that DMAPT inhibits radiation-induced NF-κB binding and DNA double-strand break repair in radiation-resistant NSCLC cells and enhances X-ray-induced cell killing in single and fractionated X-ray treatments both in vitro and in vivo. Guzman et al. have demonstrated that DMAPT has activity against leukemia stem cells but spares normal hematopoietic cells [35]. This potential therapeutic advantage, along with our observations here, supports that DMAPT has excellent potential for clinical translation for either standard or hypofractionated radiation treatments in NSCLC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2011.09.029.

References


