A water soluble parthenolide analog suppresses in vivo tumor growth of two tobacco-associated cancers, lung and bladder cancer, by targeting NF-kB and generating reactive oxygen species

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Dimethylaminoparthenolide (DMAPT) is a water soluble parthenolide analog with preclinical activity in hematologic malignancies. Using non–small lung cancer (NSCLC) cell lines (A549 and H522) and an immortalized human bronchial epithelial cell line (BEAS2B) and TCC cell lines (UMUC-3, HT-1197 and HT-1376) and a bladder papilloma (RT-4), we aimed to characterize DMAPT’s anticancer activity in tobacco-associated neoplasms. Flow cytometric, electrophoretic mobility gel shift assays (EMSA), and Western blot studies measured generation of reactive oxygen species (ROS), inhibition of NFkB DNA binding, and changes in cell cycle distribution and apoptotic proteins. DMAPT generated ROS with subsequent JNK activation and also decreased NFkB DNA binding and antiapoptotic proteins, TRAF-2 and XIAP. DMAPT-induced apoptotic cell death and altered cell cycle distribution with upregulation of p21 and p73 levels in a cell type–dependent manner. DMAPT suppressed cyclin D1 in BEAS2B. DMAPT retained NFkB and cell cycle inhibitory activity in the presence of the tobacco carcinogen nitrosamine ketone, 4(methylnitrosamino)-1-(3–pyridyl)-1-butanone (NNK). Using a BrdU accumulation assay, 5–20 µM DMAPT was shown to inhibit cellular proliferation of all cell lines by more than 95%. Oral dosing of DMAPT suppressed in vivo A549 and UMUC-3 subcutaneous xenograft growth by 54% (p = 0.015) and 63% (p < 0.01), respectively, and A549 lung metastatic volume by 28% (p = 0.043). In total, this data demonstrates DMAPT’s novel anticancer properties in both early and late stage tobacco-associated neoplasms as well as its significant in vivo activity. The data provides support for the conduct of clinical trials in TCC and NSCLC.

Key words: dimethylaminoparthenolide, cell cycle, apoptosis, cancer

In the United States in 2008, over 68,000 individuals were diagnosed with bladder cancer, and more than 14,000 patients died from their disease. Transitional cell carcinoma (TCC) is the dominant histology in over 95% of cases.1 There were also more than 200,000 patients diagnosed with lung cancer with 160,390 deaths and non–small lung cancer (NSCLC) makes up more than three quarters of these cases.1 Tobacco smoking is a major modifiable risk factor for both bladder and NSCLCs and significant achievements in decreasing this risk factor have been made in some countries. Despite this progress, these cancers are still a major cause of the current death rate from cancer.2 Modest advances have recently been made in the treatment of NSCLC with epidermal growth factor receptor and angiogenesis inhibition.3,4 Therefore, novel strategies are needed to prevent the progression of epithelial cells which have entered the neoplastic...
process, prevent emergence of micrometastatic lesions after resection and treat established cancers.

Parthenolide, a sesquiterpene lactone obtained from the plant Tanacetum parthenium, concurrently promotes apoptosis in neoplastic cells by inducing oxidative stress while inhibiting the cancer promoting transcription factor, nuclear factor kappa B (NFkB). These unique properties are due to its α-methylene-γ-lactone ring and epoxide interacting with nucleophilic sites of biological molecules, which in turn generates reactive oxygen species (ROS) by depleting glutathione and other thiols and activates caspases and apoptosis. Parthenolide decreases NFkB DNA binding by both inhibiting IκB kinase and by directly preventing the p65 protein binding to DNA. Inhibition of NFkB signaling leads to reduced expression of many proteins including antiapoptotic proteins including Tumor necrosis factor receptor-associated factor (TRAF)-1 and TRAF-2. The downstream consequences of these effects include activation of p53 and caspases with consequent cell cycle arrest and promotion of cell death.

However, parthenolide has poor pharmaceutical properties and cannot be detected in plasma when humans have been given it as part of the herbal supplement, “feverfew.” We have also shown it has limited in vivo activity due to the poor bioavailability. Hence, the aminoanalogue, dimethylamnioparthenolide (DMAPT) was developed and entered phase 1 clinical trials after documenting 70% oral bioavailability. However, parthenolide has poor pharmaceutical properties and cannot be detected in plasma when humans have been given it as part of the herbal supplement, “feverfew.” We have also shown it has limited in vivo activity due to the poor bioavailability. Hence, the aminoanalogue, dimethylamnioparthenolide (DMAPT) was developed and entered phase 1 clinical trials after documenting 70% oral bioavailability, plasma concentrations in excess of 40 μM after oral administration and an acceptable toxicology profile in animal studies (unpublished data).

In this article, we describe the in vitro and in vivo activity of DMAPT in two smoking-related cancers, lung and bladder cancer as well as its ability to generate ROS, inhibit NFkB and both promote apoptosis and induce cell cycle arrest in a cell type-dependent manner. These findings are detailed for both early and late stage NSCLC and TCC and are shown to be both independent of p53 status and retained in the presence of the tobacco carcinogen, NNK. In doing so, this work adds to the data supporting the conduct of DMAPT clinical trials in hematological and solid tumor malignancies.

Material and Methods

Cell culture and treatment

DMAPT powder was produced from parthenolide sourced from Biomol (Plymouth Meeting, PA) and dissolved in sterile water. All cell lines were purchased from American Type Culture Collection (Manassas, VA) and kept in culture per specifications. Lung cancer cell lines: A549 [wild-type (wt) p53 and wt retinoblastoma (Rb)], H522 (mutant p53 and wt Rb) and BEAS2B (wt p53 and wt Rb) but immortalized with SV40 large T antigen effecting RB and p53 function. Bladder cell lines were: UMUC-3 (mutant p53 and wt Rb), HT1197 (mutant p53 and mutant Rb), HT1197 (mutant p53 and mutant Rb) and RT4 (wt p53 and mutant Rb). NNK was purchased from Toronto Research Chemicals (ON, Canada) and dissolved in water and added to the assays at the indicated time points.

Western blotting

Cell lines were treated with varying concentrations of DMAPT and after indicated durations, the medium was removed and the attached cells were washed with PBS. Whole cell proteins were extracted in protein extraction buffer (50 mM Tris pH 7.5, 0.25% sodium deoxycholate, 1% NP40, 150 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate, 1 mM sodium fluoride, 1 μM β-glycerophosphate, 0.5 mM PMSF, 2 μg/ml aprotinin, leupeptin and pepstatin). Protein concentrations were measured with Bio-Rad Protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein (50 μg) were loaded and run on 10% SDS-polyacrylamide gel with Tris-glycine running buffer and then transferred to a nitrocellulose filter. The filters were blocked with Tris-buffered saline containing 5% non-fat milk at 4°C overnight then probed. Antibodies against phospho-JNK, phospho-c-Jun, c-Jun, JNK and GAPDH were procured from Cell Signaling (Beverly, MA), p21, p65, from Santa Cruz Biotechnology (Santa Cruz, CA) and TRAF2, XIAP, Caspase 8 from B.D. Biosciences (San Diego, CA). Experiments were repeated two to four times with similar results.

Electrophoretic mobility gel shift assay

All cell lines were tested in exponential growth phase. DMAPT was added 3 hr before harvesting whole cell protein. Electrophoretic mobility gel shift assay (EMSA) was carried out as described earlier. To evaluate the effect of N-acetyl cysteine (NAC) on NFkB DNA binding, cells were exposed to NAC for 1 hr before DMAPT treatment. DNA binding activity of Oct 1 was measured as a control in untreated and DMAPT treated cellular extracts. DNA–protein complexes were separated by electrophoresis and visualized by autoradiography.

Determination of cellular ROS

ROS production was detected using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), a cell-permeable fluorescent probe (Invitrogen-Molecular Probes, Carlsbad, CA). Exponentially growing cells were loaded with 10 μmol/l H2DCFDA for 45 min before treatment at 37°C and were washed with PBS. The cells were allowed to recover for 15 min at 37°C in growth media and then treated with DMAPT alone or in presence of NAC. Following treatment, cells were washed in PBS and the green fluorescence intensity in cells was examined by FACS analysis.

Apoptosis analysis

For measurement of apoptosis, annexin V assay (Vybrant Apoptosis Kit# 3; Invitrogen, Carlsbad, CA) was performed according to the manufacturer’s instructions. Briefly, floating and adherent cells were harvested after exposure to DMAPT for 24 and 36 hr, washed twice with PBS, incubated with...
FITC-conjugated annexin V and propidium iodine (PI) for 15 min, and analyzed by FACScan (Becton Dickinson Bedford, MA). Experiments were performed with and without NAC pretreatment.

Cell cycle analysis
Cell cycle analysis by flow cytometry was undertaken of adherent cells only (2 × 10^5). The cells had been plated on a 60-mm plate, harvested by trypsinization then pelleted, and re-suspended in 1 ml of PBS. After spinning, cells were re-suspended in 125 μl of PBS with 2 mg/ml RNase A. Cells were further stained with 50 μg/ml PI solution (300 μl). Cell cycle analysis was carried out with a Becton Dickinson (Bedford, MA) FACScan flow cytometer. Data were analyzed with the Modfit LT software (Verity Software House, Topsham, ME).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was extracted from the cell lines using RNeasy kit (Qiagen, MD) according to manufacturer’s instructions. Total RNA (1 μg) was then reverse transcribed using the SuperScript First Strand-synthesis kit (Invitrogen Life Technologies). Five percent of the cDNA was used for each gene specific PCR. The reaction cycle was started by denaturation at 95°C for 5 min, followed by Taq73 specific condition 40 cycles at 94°C (30 sec), 56°C (40 sec) and 72°C (30 sec) using (sense, 5’-TCTCTGGAAACACAGCAC-3’) and (antisense, 5’-GGGATGTCGTTGAG-3’). P21 was amplified using p21 specific primers (sense, 5’-GCGACTGTGATGGCGTAATG-3’) and (antisense) 5’-AGAAGATCGACGC GGCGTTTG-3’ with 30 cycles 94°C (30 sec), 60°C (45 sec) and 72°C (45 sec). The Actin gene was chosen as an endogenous control expression sense (5’-GACCAACACCTTC TACAATGAGC-3’) and (antisense) 5’-GATAGACAGCC TGATAGACAC-3’) with 30 cycles 94°C (1 min), 55°C (1 min) and 72°C (1 min). Fifteen microliters of RT-PCR products were resolved in 2.0% agarose gels and were imaged and quantified with LabWorks analysis software (UVP Products, Cambridge, UK).

Small-interfering RNA treatment
Small-interfering RNA (siRNA) for luciferase (nonspecific control) p53 and p73 and the siRNA nontarget control were purchased from Dharmacon RNAi Technologies (Lafayette, CO). The UMUC-3 cells (4 × 10^5) were seeded into a 6-well dish and left for 24 hr. On the following day, a 2.5 μl aliquot of siRNA solution (20 pmol/μl) and 175 μl of Opti-MEM (Invitrogen) and 16 μl Opti-MEM + 3 μl of oligofectamine (Invitrogen) were each mixed separately. They were incubated for 20 min at room temperature after combining the two mixtures, and then added to the cells which had been seeded on the dish. The siRNA-transfected cells were used after 96 hr.

Proliferation assays
Cellular proliferation was measured using the Cell Proliferation ELISA, BrdU Kit (Roche, Indianapolis, IN) according to manufacturer’s instructions. Cells lines were plated in a 96-well plate in 100 μl of media and incubated in 5% CO₂ at 37°C for 24 hr. After 24 hr of plating, varying concentrations of DMAPT in 5 μl of media were added to each well and amount of cellular proliferation relative to untreated control was determined 48 hr after addition of DMAPT.

Trypan blue exclusion test for cell viability
A total of 0.5 million cells were placed in a 100-mm plate and drugs added at 24 hr when in exponential growth phase. Cells were harvested at 48 hr after drug exposure by trypsinization, made into a pellet and resuspended in PBS. Cells were diluted 1:1 ratio with Trypan Blue (0.4%, Sigma) and placed in a hemacytometer and counted for viable (opaque) and nonviable cells (stained blue).

In vivo tumor model
Male nude athymic mice aged 6–8 weeks were procured (Harlan Sprague Dawley Bioproducts for Science, Indianapolis, IN) and acclimatized for 1 week. A549 and UMUC-3 cells suspended in Matrigel (12–18 mg/ml) (Trevigen, Gaithersburg, MD) at a concentration of 1 × 10^5 cells per milliliter were injected into the subcutaneous tissue of the flank. The tumors were allowed to establish for 7 days before drug treatment and the mice were sorted with 10 mice per cohort for UMUC-3 and A549. Animals received either solvent control daily or the indicated doses of DMAPT either daily or twice daily by oral gavage. The mice were maintained in a pathogen-free environment with free access to food and water. Body weight and tumor volume (using the formula [sagittal dimension (mm) × cross-dimension^2 (mm)]/2) were measured twice weekly. Unpaired t tests were used to statistically compare the final tumor volumes. In the lung metastasis model, one million cells were injected into the tail vein of female athymic nude mice and DMAPT treatment commenced the following day and treated daily for 60 days.79 Mice were sacrificed and lungs removed and fixed in formalin and embedded in paraffin and stained with hematoxylin and eosin (H&E). The relative volume of lung metastasis was calculated by a technician blinded to treatment arm and using lung tissue stained with H&E. Normal tissue and adenocarcinoma areas were evaluated using a Bioquant Image Analyzing System (Version 1769, Bioquant Image Analysis, Nashville, TN). Stereological methods were used to calculate the relative volume of adenocarcinoma per lung.30 The sum of all adenocarcinomas per slide was divided by 1,000,000 for a total area in square millimeter; this value was divided by the total area (in square millimeter) of all lung tissue for each particular mouse and multiplied by 100 to obtain the relative volume (in cubic millimeter).
Immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded tissue sections. Serial sections (5-mm thick) of paraffin-embedded tissues were fixed on silane-coated glass slides, deparaffinized and rehydrated in tap water. Antibodies used were TRAF2 (clone 33A1293; Abcam, Cambridge, UK).
at dilution 1:200, and p21 (Calbiochem, Cat. OP64F) at 1:200. Antigen was retrieved using Target retrieval system (TRS, DAKO S1700) for 20 min at 95°C. Sections were blocked with Avidin-Biotin blocking system (DAKO) for 10 min and the endogenous peroxidase was quenched with 3% H2O2 for 15 min. Background was blocked using DAKO protein block for 30 min followed by primary antibody for 1 hr (2 mg/ml). Sections were exposed to secondary donkey antibody (30 min) and horseradish peroxidase- linked DAKO streptavidin for 30 min in 3,3-diaminobenzenyl tetrahydrochlide (DAB) as chromogen and counterstained with hematoxylin. The extent and intensity of staining in the cancer cells were evaluated by a single investigator blinded to the treatment arm. Microscopic fields were evaluated and those with the highest degree of immunoreactivity were pictured.

Results

**DMAPT generates ROS in both TCC and NSCLC cell lines with associated activation of JNK pathway**

We confirmed the ability of DMAPT (10 μM) to induce ROS63,1 in lung and urothelial neoplastic cell lines as measured by FACS (Fig. 1a) in UMUC-3 (upper panel) and A549 (lower panel). ROS generation was also observed in RT4 (data not shown) but to a lesser degree than the more advanced cancerous cell lines. The growth media of BEAS2B (BEGM, Lonza, Walkersville) contained phenol red and interfered with the FACS. Pretreatment with the antioxidant NAC blocked ROS generation in all cell lines due to its ability to elevate intracellular cysteine. This provides an abundance of intracellular glutathione to prevent the generation of ROS. It is of note that parthenolide activity is also blocked by other general antioxidants such as pyrrolidine dithiocarbamate (0.25 μM), and nordihydroguaiaretic acid (0.1 μM), and that inhibition of glutathione synthesis with buthionine sulfoximine, sensitizes the cells to parthenolide.6,31 Moreover, 0.5–1 mM of NAC is required to block DMAPT. This is 100 times in excess of the DMAPT concentration and one would expect inhibition of DMAPT's ability to generate ROS was associated with phosphorylation of c-JUN in A549, H522 and UMUC-3 cells and increased total JNK and phosphorylation of JNK in UMUC3 (Supporting Information Figure 1). This was blocked by NAC (Fig. 1b).

**DMAPT inhibits NFκB DNA binding and decreases proteins under its control in both urothelial and lung neoplastic cell lines**

EMSA confirmed the presence of constitutive NFκB DNA binding in the cell lines and DMAPT’s ability to inhibit NFκB DNA binding (cells exposed to DMAPT for 3 hr) in both the advanced (UMUC-3 and A549) and early (RT4 and BEAS2B) neoplastic cell lines (Figs. 1c and 1d). The inhibition was dose dependent with 10–20 μM causing a marked decrease in the cell lines tested. A kinetic analysis of NFκB DNA binding with time points of 0.5, 1, 2.0 and 3.0 hr (Supporting Information Figure 3) showed the decrease in NFκB DNA binding begins at 1 hr and is more pronounced at 2 and 3 hr. Proteins regulated by NFκB were decreased by DMAPT in a time, dose- and cell-dependent manner (Fig. 1e). The effective DMAPT doses were in the low micromolar range and corresponded with the doses which partially or completely inhibited NFκB DNA binding. It is of note a longer exposure (24 hr) to a lower dose (5 μM DMAPT) was more effective than 10 μM for 12 hr in decreasing TRAF-2 in UMUC-3 (left panel). In RT4 10 μM DMAPT was effective at 12 hr but not at 24 hr in decreasing XIAP (right panel). DMAPT (20 μM) also decreased NFκB regulated proteins of TRAF-2 in A549 from 6 to 24 hr (right panel) but no change was seen in TRAF-2 or XIAP in BEAS2B (data not shown). In total, the data indicates the dose, time and ability of DMAPT to decrease NFκB regulated proteins is cell dependent and does not always correspond to the amount of inhibition of NFκB DNA binding at 3 hr.

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DMAPT inhibits proliferation and decreases the viability of early and late lung and urothelial cell lines in a dose-dependent manner.

In general, the doses that caused the inhibition of proliferation, when measured using a BrdU accumulation assay, by greater than 95% (10 and 20 μM) corresponded with the doses that blocked NFκB DNA binding in UMUC-3 and A549 cells (Fig. 2a). This was also observed in HT1376 and HT1197 (EMSA data not shown). In contrast, the earlier neoplastic lesions RT4 (papilloma) and BEAS2B (immortalized human bronchial epithelial cell line) had greater than 80% inhibition of proliferation with 5 μM but required 3 hr of DMAPT exposure at 10 μM (BEAS2B) or 20 μM (RT4) before substantially inhibiting NFκB DNA binding. The trypan blue assay showed that after one dose the viability for each cell line at 48 hr was 26.2% for A549 with 20 μM, 20.9% for BEAS2B with 5 μM; 11.8% for UMUC-3 with 10 μM and 13.4% for RT4 with 10 μM. (b) Using a BrdU incorporation assay NAC was observed to decrease DMAPT’s in vitro antiproliferative activity in UMUC-3, A549 and BEAS2B in a dose-dependent manner. In all cell lines, 10 μM DMAPT suppressed proliferation by greater than 90% and this was almost completely abrogated by 1 mM NAC in UMUC-3 and A549 and DMAPT’s antiproliferative efficacy was limited to only 25% in BEAS2B cell line. (b, bottom right panel) Electrophoretic mobility gel shift assay demonstrating NAC abrogating DMAPT’s ability to inhibit NFκB DNA binding in a dose-dependent manner in UMUC-3.
NAC abrogated DMAPT’s ability to suppress proliferation of UMUC-3, A549 and BEAS2B in a dose-dependent manner (Fig. 1). Given this observation, we then sought to discern whether NAC’s effect was purely by blocking the oxidative stress and independent of DMAPT’s ability to block NFκB DNA binding. Using the EMSA for NFκB DNA binding in UMUC-3 cells, it was noted that NAC blocked DMAPT’s ability to inhibit NFκB DNA binding in a dose-dependent manner (Fig. 2, bottom right panel). These results suggest that DMAPT generation of ROS may be the initiating event.
and the subsequent reactions impact the NFκB DNA binding.

**DMAPT causes alteration of cell cycle in a cell type–dependent manner**

Because parthenolide alters the cell cycle by a number of possible mechanisms an evaluation of DMAPT’s ability to alter effectors of cell cycle (p73 and p21) was undertaken in advanced cancer cell lines with both functional p53 (A549) and mutant p53, (UMUC-3). In both cell lines DMAPT was able to increase p73 as measured by PCR (Fig. 3a, left panel and Fig. 3c, left panel) as well as p21 (Figs. 3b and 3c, right panel). Both siRNA inhibition of p73 and NAC exposure only partially blocked DMAPT’s ability to increase p21 expression in UMUC-3 (Fig. 3b). It is of note that the time course studies in A549 (Fig. 3c) of p21 and p73 transcription showed that DMAPT-mediated p21 increase occurred before the p73 increase and was at the time DMAPT started to decrease NFκB DNA binding at 1 hr (Supporting Information Figure 3). Altogether, this data indicates p73 has, at most, only a partial role in inducing p21 expression in UMUC-3 and appears not to be related to p21 induction in A549. The time course studies showing that the beginning of DMAPT-mediated inhibition of NFκB DNA binding (i.e., 1 hr) coincides with the p21 increase suggests a possible relationship between these two events in A549 cells.

FACS demonstrated DMAPT-induced accumulation of cells in S phase at 12 and 24 hr in UMUC-3 (p53 mutant, functional Rb) with 10 μM (Fig. 4d, bottom panel) and that this was blocked by NAC. In contrast, A549 cells with wt p53 and functional Rb undergo transient G2 accumulation upon treatment with 20 μM DMAPT (Fig. 4d, bottom panel). Specifically, NAC sensitive G2 accumulation was evident at 12 hr but not 24 hr after treatment. It is interesting to note that 20 μM of DMAPT was required for the cell cycle effects on A549 cells, which is consistent with less sensitivity of these cells to DMAPT in cell proliferation assays (Fig. 2d).

**DMAPT induces apoptosis**

In prior work, parthenolide has been shown to induce apoptosis as measured by flow cytometry with PI and annexin V staining. This was also confirmed with 10 μM DMAPT in
Figure 5. DMAPT inhibits NFκB and cancer cell cycle progression despite presence of tobacco-associated carcinogen, NNK, in early smoking-related cancers. (a) EMSA detailed NNK increased NFκB DNA binding in a dose-dependent manner in BEAS2B (left panel) and that DMAPT was able to block NNK-induced NFκB DNA binding in BEAS2B (right panel). (b) Western blotting detailed NNK increased the levels of cyclin D1 protein and that this was blocked by DMAPT (left panel). EMSA revealed NNK did not increase NFκB DNA binding in RT4 nor did it block the ability of DMAPT to inhibit NFκB DNA binding (right panel). In all EMSA figures, the first panel is p65 supershift demonstrating location of p65 in the p65-50 heterodimer in the other lanes. (c) Flow cytometry demonstrated DMAPT-induced S phase and G2 phase accumulation in RT4 and BEAS2B, respectively. These changes were present at 12 and 24 hr in both cell lines (data not shown for 12 hr time point) and were blocked by NAC. (d) NNK did not alter cell cycle distribution in RT4 but did increase the number of BEAS2B cells in G1 phase at 24 hr (37% in control vs. 55% with NNK treatment). The ability of DMAPT to induce S phase accumulation in RT4 was not abrogated by exposing the cells to NNK after DMAPT (top panel). In BEAS2B, whereas DMAPT alone induced G2 phase accumulation (c, lower panel), DMAPT treatment followed by NNK exposure caused cells to accumulate in G1 and S phases.
both early and late stage TCC and NSCLC [UMUC-3, RT 4, BEAS2B and A549] (Figs. 4a, 4c, 4d, and 4e). A549 cells treated with 10 μM DMAPT had a less robust increase of cells undergoing apoptosis at this concentration, consistent with the results of cell proliferation and cell cycle analysis, where 20 μM was more effective. In all cell lines that
underwent apoptosis, the type of apoptosis observed was both early and late apoptosis. In BEAS2B, necrosis was also evident. All types of DMAPT-induced cell death were inhibited by NAC. We further characterized apoptotic events in UMUC-3 cells. DMAPT decreased the levels of procaspase 8 and procaspase 3 and induced the apoptosis-specific cleavage of PARP in a time and dose-dependent manner (Fig. 4b). We were not able to detect active caspase 8 or 3 in these cells (presumably due to labile nature of active caspases).

**DMAPT blocks NNK-induced activation of NFκB and retains cell cycle inhibitory activity in the presence of NNK**

To explore the ability of DMAPT to negate molecular biological events that drive aberrant cellular proliferation of cells earlier in the neoplastic process, a urinary bladder papilloma (RT4) and an immortalized human bronchial epithelial cell line (BEAS2B) were studied. Both cell lines displayed modest levels of NFκB DNA binding activity (Figs. 5a and 5b, right panel). The tobacco carcinogen, NNK, increased NFκB DNA binding in BEAS2B in a dose-dependent manner but did not alter it in RT4. DMAPT efficiently reduced NFκB activation in both cell lines and this was not abrogated when NNK was given after DMAPT (Fig. 5a, right panel and Fig. 5b, right panel). The NNK induced increase in NFκB DNA binding in BEAS2B cells was associated with an increase in cyclin D1 and this was blocked when the cells were pretreated with DMAPT. Neither NNK alone nor DMAPT alone altered cyclin D1 expression in RT4 cells (data not shown) indicating the cell type–dependent effects from NNK exposure.

FACS evaluated whether DMAPT impacted the cell cycle in these “early” neoplastic cells either when given alone or in the presence of NAC. There were cell type specific differences in cell cycle stages. DMAPT treatment of RT4 cells lead to an accumulation in S phase (Fig. 5c, top panel), similar to the other bladder cancer cell line UMUC-3. In contrast, DMAPT treatment of BEAS2B, accumulated cells in G2 phase at both 12 and 24 hr after exposure (Fig. 5c, bottom panel). This was similar but more durable to the findings in the lung cancer cell line A549. These changes were blocked by NAC in both cell lines. We next examined the effect of DMAPT on cell cycle under NNK exposure (Fig. 5d).

NNK alone did not alter cell cycle distribution in RT4 (top panel). DMAPT in the presence of NNK maintained the ability to increase the number of RT4 cells in S phase. This suggests that DMAPT retains its ability to function in RT4 cells in the presence of tobacco carcinogens. In contrast, NNK alone did have an effect in BEAS2B and increased the percentage of cells in G1 phase in BEAS2B. DMAPT treatment followed by NNK increased the number of cells in G1 and S phases with a proportional decrease in G2 phase cells (unlike that seen with DMAPT alone in BEAS2B). These NNK specific effects in BEAS2B are consistent with NNK-induced cyclin D1 expression in this cell line. Specifically, cyclin D1 promotes the progression of the cell cycle from G1 to S phase. It is therefore consistent that DMAPT before NNK, by preventing an increase of cyclin D1, slowed progression of cells cycling from G1/S to G2 with a decrease of cells reaching the G2 phase (as was seen in Fig. 5d, bottom panel).

**DMAPT’s single agent in vivo anticancer activity in NSCLC and transitional cancer cell lines is associated with upregulation of p21 and decreased TRAF-2 expression**

The single agent in vivo anticancer activity of DMAPT was confirmed using subcutaneous xenografts of UMUC-3 and A549 in athymic nude mice. Daily oral DMAPT treatment slowed the growth of both A549 and UMUC-3 cell lines (Fig. 6a). At day 65, 100 mg/kg/day of oral DMAPT suppressed A549 subcutaneous xenograft tumor growth by 54% versus control (p = 0.016). In UMUC-3 there was a dose-dependent

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Figure 6. DMAPT has in vivo anticancer activity in both transitional cell and non–small lung cancers. (a) Established xenograft experiments with A549 (left panel) and UMUC-3 (middle panel) cells were conducted with the cell lines injected into the subcutaneous tissue of the flanks of athymic male nude mice. Treatment was commenced when tumors were palpable at day 7. DMAPT was given by daily oral gavage and was able to suppress tumor growth relative to untreated control in A549. In UMUC-3, DMAPT's ability to suppress tumor growth was observed to be dose dependent with 100 mg/kg twice per day (bid) being more effective than 50 mg/kg or 100 mg/kg daily (qd). The ability of orally administered DMAPT to inhibit the in vivo metastatic process (implantation of circulating cells) of A549 cell lines (right panel) was demonstrated by measuring the relative volume of lung involved with cancer 60 days after cells were injected by tail vein. Treatment was commenced on day 1 with daily oral DMAPT given by oral gavage for 48 days. (b) TRAF-2 immunohistochemical staining of tumors is depicted by the brown staining of cells. The tumors were extracted from the control and DMAPT single agent arms at the time of sacrifice of both A549 and UMUC-3 experiments. The photographs of the microscopic images demonstrate that compared with control, DMAPT decreased the expression of TRAF-2 in both A549 (top panel) and UMUC-3 (bottom panel) as evidenced by decreased intensity of brown staining of cytoplasm in both cell lines as well as in the nuclei of UMUC-3 cells. (c) p21 immunohistochemical staining of tumors is depicted by brown staining of cells. The tumors were extracted from the control and DMAPT single agent arms at the time of sacrifice of both A549 and UMUC-3 experiments. The photographs of the microscopic images demonstrate that compared with control, DMAPT increased the expression of p21 in both A549 (top panel) and UMUC-3 (bottom panel) as evidenced by increased amount of brown staining of the nuclei.
effect and 100 mg/kg oral twice per day suppressed tumor growth by 63% (<0.001). A lung metastasis model showed the ability of single agent DMAPT to decrease the metastatic burden of NSCLC cells (A549 cells injected via tail vein) in nude mice (Fig. 6a, right panel). With 40 mg/kg/day of DMAPT, 29% of the lung volume was replaced with cancer versus 39% with the solvent control (p = 0.043). As depicted by immunohistochemical staining of tumors at the time of sacrifice of both A549 and UMUC-3 subcutaneous xenograft experiments, DMAPT was able to upregulate p21 nuclear expression while decreasing TRAF-2 expression in both cell lines (Figs. 6b and 6c). This provided in vivo correlation of the in vitro findings of DMAPT treatment in these cell lines.

Discussion
The data presented details the antiproliferative activity of DMAPT, a bioavailable analog of parthenolide, in both early and late stage non-small lung and urothelial cells. Its activity is independent of p53. Moreover, single agent DMAPT is able to significantly decrease in vivo growth in both subcutaneous xenograft models tested as well as decrease the burden of metastatic disease from circulating tumor cells. The latter is presumably due to reducing circulating tumor cell implantation (one of the early phases of metastatic development)\(^{29}\) based on the fact therapy was started day 1 after tail vein injection. It is of note that the mice tolerated the therapy well. This preferential anticancer activity on neoplastic cells may be due to their dependence on NF\(_{\kappa}B^{14}\) and/or cancer cells having less capacity for dealing with ROS (eg less GST\(_{\pi}\) in cancer cells to generate glutathione).\(^{6,31}\) The ongoing phase 1 trial will determine the therapeutic window in humans.

The process by which DMAPT inhibits cancer cell proliferation is complex and cell type dependent. In this context, DMAPT resembles other natural compounds such as Curcumin.\(^{34}\) However, DMAPT’s good oral bioavailability and single agent in vivo activity is a distinguishing feature and is permitting clinical evaluation of DMAPT. The data presented indicates DMAPT both promotes apoptosis and slows progression through the cell cycle in a cell type–dependent manner. These changes occurred at a dose level (5–20 \(\mu\)M) achievable in the plasma after oral gavage. It is also proposed that the myriad of DMAPT effects seen at the 5–20 \(\mu\)M exposure level are not “off target” effects but downstream effects from the chemical reaction of DMAPT’s \(\alpha\)-methylene-\(\gamma\)-lactone ring and epoxide interacting with nucleophilic sites of biological molecules.\(^{7}\) This notion is supported by the fact the activity is consistently between 5 and 20 \(\mu\)M and this dose range is associated with inhibition of NF\(_{\kappa}B\) DNA binding and proteins under its control. DMAPT’s complex and multiple effects probably account for its broad activity in multiple cell types. This is in contrast to drugs with a specific target and a well-defined patient population (such as traztuzumab which is active only in HER-2 overexpressing cancers\(^{25}\)). The following discussion summarizes the molecular events that are thought to explain these complex and cell type–dependent events.

The oxidative stress from ROS generation and NF\(_{\kappa}B\) activation are inter-related. NF\(_{\kappa}B\) can be activated by oxidative stress which can protect the cells from JNK mediated apoptosis.\(^{36}\) However, previous work with parthenolide and DMAPT has shown that these agents induce oxidative stress as an early event and then inhibit NF\(_{\kappa}B^{14}\). It is also known that NF\(_{\kappa}B\) DNA binding is redox sensitive.\(^{37}\) It is therefore proposed that DMAPT inhibition of NF\(_{\kappa}B\) DNA binding is due to a combination of DMAPT’s alteration of redox state from ROS generation combined with a direct effect on NF\(_{\kappa}B\) DNA binding. The ability of NAC to overcome DMAPT-mediated inhibition of NF\(_{\kappa}B\) DNA binding supports at least some relationship with ROS generation. The concurrent blockade of NF\(_{\kappa}B\) (a cellular defense mechanism) in the presence of oxidative stress is a positive attribute and this dual activity probably accounts for its ability to overcome redundant survival pathways.

It is of note that we have recently shown that siJNK-2 in prostate cancer cell lines only partially blocks the anticancer efficacy of DMAPT.\(^{36}\) The anticancer activity in the absence of JNK-2 is probably due to other effects of oxidative stress and/or the inhibition of NF\(_{\kappa}B\). This includes free oxygen radical formation leading to DNA damage by breaking phosphodiester bonds of DNA helix which in turn activates ataxia telangiectasia mutated (ATM) [and ATR (ATM- and Rad3 related) and the signal transduction pathways which inhibit cell cycle progression.\(^{10,39}\) Furthermore, NF\(_{\kappa}B\)’s control of a number of genes results in its inhibition blocking many of the hallmarks of cancer including: invasion; propagation through the cell cycle; and evasion of apoptosis.\(^{40-46}\) Ongoing work using \(\kappa B\) super-repressor will determine the relative contribution of NF\(_{\kappa}B\) inhibition to DMAPT’s mechanism of action.

DMAPT’s effects are probably also dependent on the pathways activated in each unique cancer cell. To exemplify this fact, DMAPT caused cell cycle arrest in all cell types but the type of arrest is cell type dependent—brief G2 arrest in A549—(wt p53 and wt Rb),\(^{20,21}\) and prolonged S phase arrest in UMUC-3—(mutant p53 and wt Rb)\(^{28}\). The effects are independent of p53 and Rb status. Interestingly, DMAPT retained cell cycle inhibitory effects despite the presence of a tobacco carcinogen and displayed cell type–dependent effects on cell cycle regulatory proteins. DMAPT suppressed cyclin D1 expression in BEAS2B but not in RT4, UMUC-3 and A549. The lack of inhibition of cyclin D1 expression with DMAPT treatment despite NF\(_{\kappa}B\) inhibition in these cell types could be due to the redundancy of transcription factors such as AP-1, STATs and CREB:CREM:ATF-2, which regulate cyclin D1 expression.\(^{11}\) Induction of p21 was observed in all the cell lines we have examined so far. Similarly, TAp73 was induced in UMUC-3 and A549. The data presented suggests that TAp73 has a limited (if any) role in DMAPT-mediated induction of p21.
In summary, DMAPT is active as a single agent in two smoking-related tumors in both in vitro and in vivo models and in both early and late stage cancers. Work to date has also shown it has the positive attribute of concurrently generating oxidative stress and blocking the transcription factor NFκB. However, further work is required to determine the relevant contribution of each observation to DMAPT’s mechanism of action. In addition, the data in this article supports the conduct of clinical trials in bladder and non small lung cancers upon successful completion of the ongoing phase 1 clinical trial.

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7. Jehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, et al. Sesquiterpene lactones also show their positive attribute of concurrently generating oxidative stress and blocking the transcription factor NFκB. However, further work is required to determine the relevant contribution of each observation to DMAPT’s mechanism of action. In addition, the data in this article supports the conduct of clinical trials in bladder and non small lung cancers upon successful completion of the ongoing phase 1 clinical trial.

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**References**
Dimethylamnioparthenolide is an anticancer agent


