Farnesyltransferase Inhibitors Induce DNA Damage via Reactive Oxygen Species in Human Cancer Cells

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Abstract

Farnesyltransferase inhibitors (FTIs) possess antitumor activity. Based on recent findings, we hypothesized that FTIs induce reactive oxygen species (ROS) that damage DNA, leading to DNA damage responses. To test this hypothesis, we investigated the effects of FTIs on the generation of ROS, DNA double-strand breaks (DSB), DNA damage responses, and RhoB, and the effects of quenching ROS on these FTI effects. We evaluated four FTIs in human cancer cell lines of different tissue origins. We found that FTIs induced ROS and DSBS. Suppressing expression of the β-subunit of farnesyltransferase with siRNA did not induce ROS, but slightly attenuated the ROS induced by FTIs. N-acetyl-t-cysteine (NAC), but not caspase inhibitors, blocked FTI-induced DSBS, suggesting that the DSBS were caused by ROS and did not result from apoptosis. The DSBS led to DNA damage responses. H2AX became phosphorylated and formed nuclear foci. The DNA-damage-sensing molecules involved were probably ataxia-telangiectasia mutated protein (ATM) and DNA-dependent protein kinase (DNA-PK) but not ATM- and Rad3-related protein (ATR). Key components of the homologous recombination and nonhomologous end joining repair pathways (DNA-PK, BRCA1, and NBS1) underwent phosphorylation and formed nuclear foci. RhoB, a mediator of the antineoplastic effect of FTIs, was increased by FTIs. This increase was blocked by NAC. We concluded that FTIs induced oxidative DNA damage by reducing ROS and initiated DNA damage responses, including RhoB induction, and there was a complex relationship among FTIs, farnesyltransferase, ROS, and RhoB. Our data also imply that inhibitors of DNA repair may accentuate the clinical efficacy of FTIs.

Introduction

Farnesyltransferase inhibitors (FTIs) possess antitumor activity against a variety of tumor cells, showing significant promise in preclinical studies; however, relatively poor clinical responses have been observed in clinical trials with FTIs as single agents (1, 2). FTIs were originally developed to inhibit the mitogenic function of ras oncogenes. However, the antineoplastic activity of FTIs also results, at least in part, from inhibition of the farnesylation of proteins other than Ras (3, 4). More than 10 uncharacterized isoprenylated proteins are affected by FTIs (5). Among the known farnesylated proteins other than Ras, lamins A and B, Rap2, RhoB and RhoE, and inositol triphosphate 5-phosphatase type I may be relevant in intracellular signaling and apoptosis. FTI treatment can decrease farnesylated RhoB and increase geranylgeranylated RhoB to inhibit cell cycling and reverse malignant transformation selectively in cancer cells (6). Geranylgeranylated RhoB is required for the antineoplastic action of FTIs (7). Cyclin B1 suppression may be an important step in FTI-induced apoptosis that is mediated by geranylgeranylated RhoB (8). Inhibition of the PI-3 kinase/Akt-mediated cell survival and adhesion pathway by FTIs has been shown to play a role in the induction of apoptosis (9). Heat shock protein 70 may also be a target for induction of apoptosis by FTIs as well (10, 11).

The FTI manumycin A is a natural product of Streptomyces parvulus (12), and a selective FTI (IC50 = 5 μmol/L) that inhibits geranylgeranyl transferase only at high concentrations (IC50 = 180 μmol/L). Manumycin inhibits farnesyltransferase through competition with the farnesyl PPI group (13), a mechanism of action different from peptide mimicry of the CAAX motif by FTIs, such as SCH66336 (lonafarnib), FTI-276, and L-744832 (14). We previously showed that the combination of manumycin and paclitaxel had a synergistic antineoplastic effect on anaplastic thyroid cancer, one of the most aggressive solid tumors (15). In the course of this investigation (15–17), we discovered that manumycin induced a paradoxical increase in [3H]thymidine incorporation (data not shown) similar to that induced by hydroxymethylglutaryl CoA-reductase inhibitors (18, 19). DNA repair was proposed as a possible explanation for the paradoxical increase in [3H]thymidine incorporation induced by lovastatin (18). Recently, FTIs were observed to induce reactive oxygen species (ROS) in chronic myelogenous leukemia cells (20).

On the basis of our findings and others, we hypothesized that FTIs induced ROS that damage DNA, leading to DNA damage responses. We tested this hypothesis by evaluating the effects of manumycin on generation of ROS, DNA damage, and DNA-damage-sensing kinases, the effect of quenching ROS on these responses to manumycin, and the relevance of ROS generation to the overall cytotoxicity of manumycin. The phenomena of ROS generation and DNA damage were also examined for four FTIs in a variety of human cancer cell lines of different tissue origins. Because RhoB is inducible after DNA damage (21) and required to mediate apoptosis in malignant cells after DNA damage (22), we also investigated whether FTIs indirectly increased RhoB via ROS-induced DNA damage.

Materials and Methods

Reagents. Sources of antibodies were as follows: histone H2A (H124), Santa Cruz Biotechnology (Santa Cruz, CA); NBS1, Novus Biologicals (Littleton, CO); phospho-NBS1 (Ser343), Cell Signaling Technology (Beverly,
trypsinization, pelleted by centrifugation, resuspended in PBS, and analyzed.

The cells were then collected by a kit purchased from BD Clontech (Palo Alto, CA) for 30 minutes and then measured using a membrane-permeable diaminofluorescein NO sensor dye experimental drug treatments (27).

A induced with 1 protein (ATR)-kinase dead-inducible cells, a gift from Dr. S.L. Schreiber with heat-treated bovine serum (20%). U2OS ATM- and Rad3-related M059J and M059K cells (26) were cultured in a medium supplemented with bovine serum (10%). AT22IJE-TpEBS7-YZ5 cells (ATM+/+, ATM−/−) were gifts from Dr. J.A. Fagin (University of Cincinnati, Cincinnati, OH) and 1 mmol/L phenylmethylsulfonyl fluoride. The DNA in the lysate was done using standard methods as described previously (15). The protein concentrations of samples were measured using a modified Lowry method (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein from each sample were loaded onto the SDS-polyacrylamide gel.

**Pulsed-field gel electrophoresis.** ARO cells treated with manumycin and control cells were washed and suspended with PBS after trypsin-EDTA treatment. Approximately 2 × 10⁶ ARO cells were washed and resuspended in cold PBS and quickly mixed with an equal volume of 1% low-melting point agarose prepared in PBS. The mixture was immediately poured into molds. The cells embedded in agarose were digested in lysis buffer containing 0.5 mol/L EDTA (pH 8.0), 10 mmol/L Tris, 1% (w/v) Sarkosyl, and 0.1 mg/mL proteinase K at 50°C for 24 hours, followed by washing with TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 0.2 mg/mL RNase A] for 3 hours with buffer changed each hour. The agarose plugs containing purified DNA were kept in a storage buffer [10 mmol/L Tris-HCl and 50 mmol/L EDTA (pH 8.0)] at 4°C. Then, the cellular DNA in the plugs was analyzed by pulsed-field gel electrophoresis (CHEF-DR II, Bio-Rad Laboratories) at 200 V with a switch time of 50 seconds for 16 hours at 7°C. The electrophoresis buffer contained 50 mmol/L Tris-borate (pH 8.2) and 1 mmol/L EDTA. After electrophoresis, gels were stained with ethidium bromide and then photographed on a UV transilluminator.

**Immunofluorescence staining.** Cells were seeded on coverslips in six-well plates and allowed to grow overnight. Cells were exposed to manumycin (54 μmol/L) for 1 hour and washed with PBS twice and then cells were fed with fresh medium. After another incubation for 2 hours, cells were fixed with 3% paraformaldehyde for 20 minutes. Then the cells were permeabilized in 1% Triton X-100 and 0.5% NP-40 for 20 minutes. Samples were blocked with 5% goat serum and then incubated with primary antibody for 2 hours and secondary (FITC) antibody for 1 hour. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI) to permit visualization of nuclear DNA. The coverslips were mounted onto glass slides with VectaShield antifade (Vector Laboratories, Burlingame, CA), and immunofluorescence was visualized by using a Leica DM LB fluorescence microscope. Images were captured with a Kodak digital imaging system.

**DNA damage detection.** The cell-free DNA damage detection assay (the 3D assay) was done as described by Barret et al. (31) with modifications. Purified genomic DNA (1 μg/mL, 50 μL) from KAT-4 cells was bound to poly-L-lysine–coated microplates. DNA was then treated for 3 hours with manumycin (54 μmol/L) or paclitaxel (22 μmol/L). UV light–damaged genomic DNA was used as a positive control. The treated DNA and control DNA were rinsed twice with PBS plus TWEEN 20 (PBST) and incubated for 3 hours at 37°C with 50 μL reaction mixture containing 30 μg Hela nuclear extract (Promega, Madison, WI), 70 mmol/L KCl, 40 mmol/L HEPES-KOH (pH 7.6), 5 mmol/L MgCl₂, 0.5 mmol/L DTT, 10 mmol/L phosphocreatine, 2.5 μg creatine phosphokinase, 2 mmol/L EDTA, 18 μg bovine serum albumin (BSA), 0.4 μmol/L dGTP, 0.4 μmol/L dCTP, 0.4 μmol/L dTTP, and 0.4 μmol/L biotin-14-dATP (Invitrogen). After being washed thrice with PBST plus 0.1% BSA, the DNA was incubated with streptavidin peroxidase and BSA diluted in PBST for 30 minutes. Chemiluminescence substrate (50 μL; Dura Substrate, Pierce Chemical Co., Rockford, IL) was added to each well after rinsing. The emitted light was measured with a Tanon Fluoroscan Plus plate reader in the luminescence mode.

**Small interfering RNA transfection.** SiGENOME SMARTpool duplexes against β-subunit of farnesyltransferase (FNTB) and small interfering RNA (siRNA) control duplex against Luciferase GL2 were purchased from Dharmacon RNA Tech. (Lafayette, CO). Transfection of the synthesized siRNA duplex of either anti-FNTB or the control into KAT-4 cells was done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Briefly, cells were grown in a six-well plate to 80% confluence and then incubated with a mixture of a 200 pmol siRNA duplex and 8 μL Lipofectamine 2000. After 4 hours, FBS was added to a final concentration of 10% (v/v). A second transfection was done 24 hours later. Experimental drug treatments were carried out 24 hours after the second transfection.

**Quantitative real-time reverse transcription-PCR.** DNA-free total RNA was prepared with RNeasy miRNA columns using the on-column DNase digestion step (Qiagen, Valencia, CA). One-step quantitative real-time
reverse transcription-PCR (RT-PCR) was done with ABI Prism 7900HT instrument (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's recommended protocol. The reaction was carried out in a 25-μl reaction volume in 96-well plates using the specific primers and probes, TaqMan Gene Expression Assays (FNTB Assay ID: Hs00157635_ml) and internal control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each sample was assayed in duplicate in each independent experiment. The comparative CT method (ΔΔCT) for relative quantitation of gene expression was used to analyze the mRNA expression. The mean cycle threshold (CT) value for FNTB and GAPDH across replicates of each sample was calculated. ΔCT (sample) = CT (target) − CT (GAPDH). ΔΔCT = ΔCT (sample) − the baseline’s ΔCT (mRNA control) and comparative expression level = 2−ΔΔCT. The results shown are representative of at least three independent experiments.

Results

Farnesyltransferase inhibitors induced reactive oxygen species formation. To determine whether manumycin induces ROS, we examined the effect of manumycin on levels of superoxide anion, a major ROS. We measured superoxide level with an established method involving oxidation of dihydroethidium (29). Four independent samples were measured for each data point. After exposure of cancer cells to 54 μmol/L manumycin for various durations up to 24 hours, superoxide levels in the cells (as measured by ethidium fluorescence after binding with mitochondrial DNA) were increased. The superoxide level peaked at ~6 hours of drug exposure and then decreased gradually. In KAT-4 cells, the average fluorescence values after 4, 6, and 8 hours of treatment were significantly higher than the fluorescence levels in control cells (P < 0.01, one-way ANOVA, post hoc comparisons, Tukey’s test; Fig. 1A). In all the figures in this paper, error bars represented the 95% confidence intervals. A similar time profile of superoxide induction was observed in ARO cells (data not shown). Manumycin (54 μmol/L, 6 hours) also induced superoxide production in ARO, MCF-7, and HCT116 cells (data not shown), suggesting this effect did not depend on the original tissue origins of the cell lines.

To find out whether the above observation is a class phenomenon for FTIs, we investigated the effects of 6-hour treatments of three other FTIs, SCH66336 (30 μmol/L), FTI-276 (40 μmol/L), and L-744832 (60 μmol/L). As shown in Fig. 1B, superoxide level was increased in various cancer cell lines after treatment with these FTIs. In contrast, SCH66336, FTI-276, and L-744832 did not increase ROS in human primary lung fibroblast CCD-37 cells as measured by the same method, but manumycin A increased ROS in these fibroblasts to only one fifth to one fourth of the levels seen in the malignant cell lines (data not shown). Taken together, these results suggested that various FTIs of diverse chemical structures induced ROS in a variety of cancer cells of different tissue origins.

We also investigated whether manumycin affected the generation of NO, another type of ROS. We used a proprietary membrane-permeable compound that fluoresces bright green upon binding NO to measure NO production in a fluorescence flow cytometry assay. Three independent experiments were done. Representative fluorescence distribution plots for the ARO and KAT-4 cell lines were shown in Fig. 1C. Manumycin (54 μmol/L) for 6 hours significantly increased the average green fluorescence (i.e., NO production; P < 0.01, Student’s t test) in both cell lines. When a cutoff value of fluorescence was defined such that 95% of the untreated control cells were below the cutoff value, the percentage of cells stained positive (higher fluorescence than the cutoff value) by the NO sensor dye was well above 50% in both cell lines.

We examined whether the ROS generation by FTIs was related to inhibition of farnesyltransferase. The two members of the prenyltransferase family, farnesyltransferase and geranylgeranyl transferase-1, are heterodimeric proteins that share an identical α-subunit but have different β-subunits (2). Therefore, we used siRNA targeting the FNTB to evaluate the effects of loss of farnesyltransferase on ROS generation. Transfection of KAT-4 cells with anti-FNTB siRNA decreased the target mRNA level by 90% when measured at 48 hours after the first transfection (Fig. 1D). However, suppressing the FNTB mRNA level by 90% was not sufficient to induce ROS (Fig. 1E). After a subsequent 6-hour exposure to FTIs (SCH66336, FTI-276, and L-744832), there were significant decreases in superoxide levels in cells transfected with anti-FNTB siRNA treated with an FTI compared with the respective cells transfected with control siRNA treated with the same FTI (P < 0.05, Student’s t test; Fig. 1E). However, the FTI-elicted superoxide generation was not attenuated proportionally like the 90% of inhibition of mRNA expression. These results suggested that inhibition of farnesyltransferase alone was not sufficient to generate ROS (in other words, the induction of ROS by FTIs was largely independent of farnesyltransferase) and that farnesyltransferase contributed partially to the generation of ROS by FTI in a yet unknown manner.

Farnesyltransferase inhibitors induced DNA double-strand breaks. ROS are well known to damage a variety of biomolecules: proteins, RNA, and DNA. To evaluate whether manumycin could lead to DNA damage, we looked for direct evidence of DSBs by pulsed-field gel electrophoresis (PFGE). ARO cells were exposed to manumycin (54 μmol/L) for 3 hours and harvested, and the degree of fragmentation in the genomic DNA (i.e., DSB) was assessed using PFGE. As shown in Fig. 2A, DSBs were evident in manumycin-treated cells but not in DMSO-treated control cells. In all the figures in this paper, the black dots indicated the presence of a drug or treatment. The presence of a pan-caspase inhibitor or a caspase-3 inhibitor did not interfere with DSB formation induced by manumycin (Fig. 2A), indicating that the formation of DSBs was not due to intermucleosomal DNA cleavage in the apoptotic process.

It is recently established that one of the early cellular responses to DSBs is the phosphorylation at Ser139 of H2AX (γ-H2AX), a subclass of eukaryotic histones that are part of the chromatin structure (32). Detection of γ-H2AX is probably the most specific and efficient technique identified to date for detecting DSBs in cells (33–35). Paclitaxel is an antitubulin antineoplastic agent that has activity against anaplastic thyroid cancer (24) and does not damage DNA. We compared paclitaxel and manumycin with respect to DNA breaks and damage secondary to apoptosis and cell death. We examined the formation of γ-H2AX in KAT-4 and ARO cells exposed to control medium (0.1% DMSO), manumycin alone, paclitaxel alone, or both drugs. After 3 and 6 hours of treatment, cells were harvested and cell lysates were analyzed by immunoblotting with the antibody specifically recognizing Ser139-phosphorylated H2AX. As shown in Fig. 2B, H2AX was phosphorylated in cells treated with manumycin or manumycin plus paclitaxel. The ratio of γ-H2AX to total histone 2A (H2A, as estimated by densitometric scanning of immunoblots) was 12 times as high in manumycin-treated cells as in control cells at 3 hours of treatment and 16 times as high in manumycin-treated cells as in control cells at 6 hours of treatment.

The addition of
paclitaxel, which enhanced apoptosis (15), did not enhance H2AX phosphorylation by manumycin, this again suggesting that the DSBs formed were not the result of apoptosis. The failure of paclitaxel to induce γ-H2AX was in agreement with its mechanism of antineoplastic action. To further investigate whether the DSBs were the result of apoptosis, we treated ARO cells with manumycin or manumycin plus paclitaxel in the presence of an inhibitor of caspase-3 and then assayed for DSBs by immunodetection of γ-H2AX. As shown in Fig. 2C, the phosphorylation of H2AX after manumycin or manumycin plus paclitaxel treatments were not dependent on caspase-3 activation. This result was consistent with the finding with PFGE (Fig. 2A) and indicated that the DSBs induced by manumycin did not result from apoptosis.

γ-H2AX organizes into discrete foci in the nucleus for DNA repair. Obvious γ-H2AX foci formation was observed in manumycin-treated ARO cells (Fig. 2D). The phosphorylation of H2AX and organization of γ-H2AX into nuclear foci not only indicated the existence of manumycin-induced DSBs but also revealed the activation of DSB detection mechanisms.

To ascertain whether manumycin led to DNA damage directly, we used the cell-free DNA damage detection assay (3D assay; ref. 36). Purified KAT-4 genomic DNA was incubated for 3 hours with control culture medium, manumycin, or paclitaxel. UV light–damaged genomic DNA was used as a positive control. The 3D assay did not detect significant damage in DNA treated directly with manumycin or paclitaxel, but did detect significant damage in UV-treated DNA (Fig. 2E). Therefore, direct incubation of DNA with manumycin was not sufficient to cause DNA damage detectable by the 3D assay.

To assess whether FTI-induced DSBs was a common phenomenon for different FTIs in different cell lines, additional FTIs were tested in two other different solid tumor cell lines. When treated with different FTIs, phosphorylation of H2AX was observed to increase significantly in HCT116 and MCF-7 cells (Fig. 2F). At the concentrations used, manumycin seemed to induce about twice as much phosphorylation of H2AX as the other FTIs. These data supported that DNA damage induced by FTIs might be a common phenomenon.
Manumycin induced DNA damage responses. DSBs pose a major threat to living cells and several mechanisms have evolved for repairing these lesions. Eukaryotes can repair DSBs by homologous recombination (HR) or nonhomologous end joining (NHEJ; ref. 37). HR requires interaction of the broken DNA molecule with an intact homologous copy and allows restoration of the original DNA sequence. NHEJ connects DNA ends irrespective of their nucleotide sequence; this mechanism of repair predominates in mitotic cells (38). Several proteins are involved in DSB repair: BRCA1 functions in HR, DNA-PK and Ku70 function in NHEJ (39), and the MRE11-NBS1-RAD50 complex functions in both HR and NHEJ. These proteins are activated by posttranslational modification (primarily phosphorylation). Therefore, we investigated the posttranslational modification of some of these key DNA damage response proteins after manumycin treatment.

The activation of BRCA1 by manumycin was evaluated by examining the relative mobility shift of the protein after posttranslational modification (phosphorylation) that activated the protein. In both KAT-4 and ARO cells, manumycin (54 μmol/L) induced a mobility shift of BRCA1 (Fig. 3A) characteristic of phosphorylated BRCA1. Mobility shift was not observed in the paclitaxel (22 μmol/L)-treated cells. The addition of paclitaxel did not affect the mobility shift of BRCA1 induced by manumycin.

The MRE11-NBS1-RAD50 complex is required for both HR and NHEJ, and NBS1 is phosphorylated at Ser234 in an ATM-dependent manner in vitro and in vivo after DNA damage (40). We examined the activation status (i.e., phosphorylation at Ser234) of NBS1 by immunoblotting with a phosphospecific antibody. A phospho-NBS1 (Ser234) band was observed in ARO cells treated with manumycin (Fig. 3A, right) but not in control or paclitaxel-treated ARO cells. The addition of paclitaxel did not affect manumycin-induced phosphorylation of NBS1. In KAT-4 cells, the phospho-NBS1 band was increased in cells treated with manumycin in comparison with the control- and paclitaxel-treated cells (Fig. 3A, left).

Figure 2. Manumycin induced DSBs indirectly in a caspase-independent manner. A, manumycin-induced DSBs. ARO cells were exposed to manumycin (54 μmol/L) in the absence or presence of caspase inhibitors (30 μmol/L pan-caspase inhibitor BOC-fmk or 100 μmol/L caspase-3 inhibitor Ac-DEVD-CHO) or control medium (0.1% v/v DMSO) for 3 hours and then harvested for analysis by PFGE. B, manumycin induced phosphorylation of H2AX. C, phosphorylation of H2AX was not dependent on caspase-3 activation. D, manumycin-induced γ-H2AX foci formation in ARO cells. E, no significant direct DNA damage by manumycin was detectable with the DNA damage assay (3D assay). *Statistically significant difference from the control (P < 0.05, Student’s t test). F, other FTIs increased phosphorylation of H2AX in additional cell lines.
NBS1 and BRCA1 nuclear foci were detectable by immunofluorescence microscopy in manumycin-treated ARO cells (Fig. 3B) and KAT-4 (data not shown). These findings of BRCA1 and NBS1 posttranslational modification and nuclear foci formation after exposure to manumycin, but not paclitaxel, suggested that manumycin induced DNA damage and that manumycin treatment affected key components of the HR repair pathway.

NHEJ differs from HR in that in NHEJ there is no requirement for an undamaged partner or for extensive homology between the two recombining ends. For NHEJ in mammalian cells, a key player in the initial recognition of the DNA lesion is DNA-PK (26), a member of the PIKK family of kinases. DNA-PK comprises a large catalytic subunit (DNA-PKcs) of ~450 kDa and two smaller Ku subunits (Ku70 and Ku80). As shown in Fig. 3A, we observed posttranslational modification manifesting as a mobility shift of DNA-PK in manumycin-treated cells but not paclitaxel-treated cells. This result suggested that this key component of the NHEJ pathway was affected by manumycin. It was previously reported that Ku-dependent autophosphorylation of DNA-PKcs at Tyr2689 was required for the repair of ionizing radiation–induced DSBs by NHEJ (41). Whether the DNA-PKcs phosphorylation caused by manumycin is autophosphorylation at this site remains to be tested. Nevertheless, these data together suggested that some key components of the HR and NHEJ pathways might be involved in the responses to DNA damage induced by manumycin.

ATM and DNA-dependent protein kinase, but not ATR, mediated the manumycin-induced DNA damage responses. Next, we explored which DNA damage sensors were responsible for initiating the DNA damage response upon manumycin treatment. Members of the PIKK family, including DNA-PK, ATM, and ATR, are central to the entire DNA damage response (26, 37, 42). Therefore, we investigated whether PIKKs were required for manumycin-induced H2AX phosphorylation. Pretreatment and continued presence of wortmannin, which inhibits PIKK family members, at concentrations as low as 1 μmol/L, reduced manumycin-induced phosphorylation of H2AX (Fig. 4A) by a large extent in both KAT-4 and ARO cells. The reduction of H2AX phosphorylation by wortmannin suggested that the DNA damage response to manumycin required PIKKs. It is known that wortmannin is a relatively potent inhibitor of DNA-PK at ~3.6 μmol/L, of ATM at ~5.8 μmol/L, and of ATR at >10 μmol/L (43), suggesting that ATR activity is significantly less sensitive than DNA-PK or ATM to wortmannin. ATM and ATR exhibit similar sensitivities to caffeine, a purine analogue, with 50% inhibition of kinase activity at concentrations (IC_{50}) of 0.2 and 1.1 mmol/L, respectively (44). In contrast, DNA-PK is relatively resistant to caffeine (IC_{50} 10 mmol/L; ref. 44). Our results (Fig. 4A) showed that caffeine slightly inhibited H2AX phosphorylation at 3 mmol/L and inhibited H2AX phosphorylation by >50% at 10 mmol/L. Our experimental results with wortmannin suggested that the functions of ATM

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**Figure 3.** BRCA1 and NBS1 were phosphorylated and formed nuclear foci in response to manumycin. A, ARO and KAT-4 cells were exposed to manumycin (54 μmol/L) and/or paclitaxel (22 μmol/L) for the indicated durations. Anti-phospho-NBS1, anti-NBS1, anti-DNA-PK (large catalytic subunit of DNA-PK), and anti-BRCA1 immunoblots are shown. B, manumycin induced BRCA1 and NBS1 nuclear foci in ARO cells. Fluorescent photomicrographs of DAPI-stained cells and antibody-stained cells are shown.
and DNA-PK, but not ATR, were important in the DNA damage responses after manumycin treatment.

Further investigation of the role of PIKK family members in manumycin-induced H2AX phosphorylation was carried out using ATM−/− versus ATM+/+ fibroblasts (25); DNA-PK mutant cells (MO59J) versus DNA-PK wild-type cells (MO59K; ref. 26); and U2OS cells with kinase-dead ATR inducible by doxycycline (27). In ATM−/− cells, H2AX expression was low but manumycin induced H2AX phosphorylation (Fig. 4B). In cells reconstituted with wild-type ATM, manumycin induced significant H2AX phosphorylation (Fig. 4B). Similarly, manumycin-induced H2AX phosphorylation was greatly reduced in DNA-PK mutant cells compared with DNA-PK wild-type cells (Fig. 4B). As shown in Fig. 4C, there was no difference between kinase-dead ATR cells with or without induction by doxycycline in response to manumycin, suggesting that ATR was not required for H2AX phosphorylation after exposure to manumycin.

Immunofluorescence microscopy showed that manumycin induced H2AX-containing nuclear foci in ATM+/+ cells and DNA-PK wild-type cells but not in ATM−/− cells and DNA-PK mutants (Fig. 4D). The presence of DSBs in ATM−/− and DNA-PK mutant cells (MO59J) after manumycin treatment was confirmed by neutral Comet assay (data not shown) using a standard protocol with a kit obtained from R&D Systems (Minneapolis, MN). The induction of kinase-dead ATR by doxycycline did not affect the formation of H2AX-containing nuclear foci after manumycin treatment in U2OS cells (Fig. 4D). Taken together, these results suggested that ATM and DNA-PK, but not ATR, were the sensors and/or mediators of the DNA damage response upon manumycin treatment.

Manumycin-induced DNA damage via reactive oxygen species generation. Given that manumycin induced ROS generation and that ROS could damage DNA, we evaluated the role of ROS in mediating manumycin-induced DNA damage by using the reducing agent N-acetyl-L-cysteine (NAC) to quench ROS. The thiol NAC is a source of cysteine for the synthesis of the endogenous antioxidant glutathione (GSH); thus, NAC is postulated to protect cells by increasing intracellular GSH levels and scavenging ROS (45). We measured the generation of NO using a flow cytometry method as described earlier. In both ARO and KAT-4 cells, manumycin (54 μmol/L) for 6 hours increased the average green fluorescence (i.e., NO production), but NAC coadministered with manumycin completely blocked the increase in NO production. When a cutoff value of fluorescence was defined such that 95% of the untreated control cells were below the cutoff value, the percentage of cells stained positive (higher fluorescence than the cutoff value) by the NO sensor dye was ~85% in the KAT-4 cells (Fig. 5A), and NAC blocked the increase in percentage of cells stained positive for NO. Similar results were observed with ARO cells (data not shown). Manumycin-induced increase in superoxide anion levels (as measured by ethidium fluorescence after binding with mitochondrial DNA; ref. 29) was also blocked by NAC in both ARO and KAT-4 cells (Fig. 5B).

Because NAC blocked manumycin-induced increase in ROS, NAC was expected to block manumycin-induced DNA DSBs and DNA

Figure 4. ATM and DNA-PK, but not ATR, mediated the manumycin-induced DNA damage responses. A and B, wortmannin and caffeine reduced the phosphorylation of H2AX induced by manumycin. ARO and KAT-4 cells were exposed to manumycin (54 μmol/L) for 3 hours in the presence of wortmannin or caffeine at the indicated concentrations. C and D, H2AX phosphorylation and foci formation depended on ATM and DNA-PK but not ATR. ATM−/−, ATM+/+, MO59J, and MO59K cells were exposed to manumycin (54 μmol/L) for 3 hours. The immunoblots are shown (C). U2OS cells with doxycycline-inducible kinase-dead ATR expression were induced with doxycycline for 48 hours and then exposed to manumycin (54 μmol/L) for 3 hours. The lysates of these cells and control cells not exposed to doxycycline were analyzed by immunoblotting (D). E, cells (as labeled) were treated and fluorescence analysis was done under the same condition as in Fig. 2D.
damage response if manumycin induced these changes via ROS. In fact, the formation of DSBs as detected by PFGE after manumycin treatment (54 μmol/L, 3 hours) was blocked by the presence of NAC (5 mmol/L; Fig. 5C). The phosphorylation of H2AX and H2AX nuclear foci formation were also blocked by coadministration of NAC with manumycin (Fig. 5D and E, respectively). Taken together, the blocking experiments with NAC suggested that manumycin caused DSBs and elicited DNA damage responses via ROS.

The role of NO in ROS-induced DNA DSBs was further investigated using deferoxamine. In the presence of deferoxamine (5 mmol/L), NO generation was selectively blocked after treatment with manumycin (Fig. 5F) without blocking generation of superoxide (data not shown). In the absence of deferoxamine, manumycin still induced phosphorylation of H2AX, but not as much as in the absence of deferoxamine. This suggested that NO played a partial role but was not absolutely required in the DNA damage after manumycin treatment.

Quenching reactive oxygen species antagonized the inhibitory effect of manumycin on protein biomass. To evaluate the overall importance of ROS generation by manumycin in the antineoplastic effect against anaplastic thyroid cancer cells, we measured to what degree NAC (5 mmol/L) could reverse the inhibitory effect of manumycin (54 μmol/L, 48 hours) on protein biomass in KAT-4 and ARO cells. Protein biomass (relative to control) was measured by sulforhodamine B staining. NAC blocked most of the manumycin-induced decrease in protein biomass in ARO cells and completely blocked the decrease in protein biomass in KAT-4 cells (Fig. 6). These findings indicated that the generation of ROS played an important role in the cytotoxic effect of manumycin.

Farnesyltransferase inhibitors increased RhoB via reactive oxygen species generation. Because of the importance of RhoB in FTI-induced apoptosis and the induction of RhoB by DNA damage (21, 22), we examined by immunoblotting whether FTIs induced

![Figure 5](image_url). NAC blocked the effects of manumycin on ROS generation, DNA DSB formation, and DNA damage responses. A, the generation of NO after manumycin treatment was blocked by NAC. NO generation was evaluated by flow cytometry. B, the generation of superoxide anions after manumycin treatment was blocked by NAC. C, formation of DSBs after manumycin treatment was blocked by NAC. ARO cells were exposed to manumycin (54 μmol/L) in the presence or absence of NAC (5 mmol/L) for 3 hours and then harvested for analysis by PFGE. Each lane represents an independent DNA sample. D, phosphorylation of H2AX after manumycin treatment was blocked by NAC as shown by immunoblotting. E, H2AX nuclear foci formation was blocked by NAC. F, deferoxamine blocked manumycin induction of NO in ARO cells. The NO induction was measured by flow cytometry of cells stained with NO sensor dye. G, deferoxamine slightly decreased manumycin-induced phosphorylation of H2AX as shown by immunoblotting.
RhoB protein level and whether the effect on RhoB was reversible by the ROS quencher NAC. We treated MCF-7 cells with FTI-276 (40 μmol/L) or L-744832 (60 μmol/L) in the presence or absence of NAC (5 mmol/L). Anti-RhoB immunoblots showed that the FTIs increased RhoB and that the increases were blocked by NAC (Fig. 7A). Similar results were observed in ARO and KAT-4 cells as well (data not shown). Based on our findings in this paper and studies of other investigators, we proposed a model (Fig. 7B) to weave together FTIs, ROS, DNA damage, DNA damage responses, and RhoB.

**Discussion**

In this report, we documented that a variety of FTIs induced DNA damage via generation of ROS in a variety of solid tumor cells. We identified that in the case of manumycin, the DNA-damage-sensing molecules involved are ATM and DNA-PK, but not ATR. RhoB was induced as part of the DNA damage responses. Our findings added a novel aspect to the spectrum of actions of FTIs. Our results are consistent with the observation of the ROS induction by FTIs in chronic leukemia cells by Selleri et al. (20). ROS generation induced by FTIs may be a common phenomenon because this has been observed for five different FTIs in eight solid tumor cell lines and three leukemia cell lines (20).

Inhibiting expression of farnesyltransferase by targeting FNTB transcription with siRNA was not sufficient to induce ROS production. Therefore, the ability of FTIs to induce ROS was probably not a direct result of farnesyltransferase inhibition. Although the amounts of ROS induced by different FTIs were attenuated in FNTB-silenced cells, this attenuation was small in magnitude when comparing with 90% transcriptional suppression in FNTB by siRNA. Moreover, the time course of changes documented in this report would precede the expected time course for a mechanism mediated through inhibition of protein farnesylation because for all but the farnesylated proteins with the shortest half-lives, 3 hours would be insufficient to produce significant effects on protein farnesylation. Taken together, ROS induction by FTIs is largely independent of farnesyltransferase inhibition. This complex relationship of farnesyltransferase and FTI-induced ROS production is yet to be defined.

Our results validated a link between the FTI-induced ROS and the manumycin-induced DNA damage. We assessed DSBs by PFGE and γ-H2AX. PFGE is a classic assay, whereas γ-H2AX is a recently established approach for assessing DSBs. Both PFGE and γ-H2AX (immunoblots and immunofluorescence results) provided clear evidence of DSBs following manumycin exposure (Fig. 2A–D). Quenching ROS with NAC significantly suppressed manumycin-mediated DSB formation (Fig. 5) and decreased protein biomass (Fig. 6). The involvement of ATM and DNA-PK in the response to manumycin (Fig. 4A–D) also supported the existence of DSBs. Although manumycin induced the generation of NO and superoxide, we identified that the selective blocking of NO generation by deferoxamine only slightly attenuated the H2AX phosphorylation.

**Figure 6.** NAC blocked the decrease in protein biomass caused by manumycin. Protein biomass (relative to control) was measured by sulforhodamine B staining.

**Figure 7.** FTIs induced RhoB via ROS. A, MCF-7 cells were exposed to FTIs (concentrations same as Fig. 1B) as indicated in the presence or absence of NAC (5 mmol/L). B, a model of the web of interaction among FTIs, ROS, DNA damage, DNA damage responses, RhoB, and apoptosis/growth inhibition. ↓ indicates an inhibitory effect. RhoB-F, farnesylated RhoB; RhoB-G, geranylgeranylated RhoB.

^5 Pooling our data in this paper, our unpublished data, and the data by Selleri et al.
phosphorylation by manumycin. This suggested that NO is not absolutely required for DNA DSBs but contributed to DSBs. It has been reported that peroxynitrite (ONOO⁻), formed by a fast reaction of NO with superoxide in cells simultaneously producing NO and superoxide, is a powerful agent of DNA damage (46). Thus, NO might enhance the DNA-damaging effects of superoxide after manumycin treatments.

Our results did not support direct damage of DNA by manumycin. Direct incubation of DNA with manumycin was not sufficient to cause DNA damage detectable by the 3D assay (Fig. 2E). Further, the blocking of caspases did not prevent manumycin-induced DSBs; in other words, DSB formation was not caused by caspase activation as a result of apoptosis (Fig. 2A and C). In contrast, NAC blocked manumycin-induced DSBs, indicating that DSB formation by manumycin depended on ROS.

Our data showed that ATM and DNA-PK, but not ATR, were involved in sensing the ROS-induced DNA damage. Two lines of evidence supported this. First, wortmannin pretreatment remarkably diminished the manumycin-induced DSBs (assayed by phosphorylation of H2AX; Fig. 4A) in a dose-dependent manner. ATM and DNA-PK are relatively sensitive to wortmannin, whereas ATR is relatively resistant to wortmannin. Second, induction of kinase-dead ATR cells with doxycycline did not affect manumycin-induced DSB formation (Fig. 4C and D), whereas inhibition of ATM and DNA-PK function significantly reduced the manumycin-induced DSB formation (Fig. 4B and D). However, it remains unclear why both ATM and DNA-PK are required. One possibility is that mutual interaction between ATM and DNA-PK contributes to the initiation of DNA damage response.

We also revealed that BRCA1 and NBS1 are involved in the DNA damage response to manumycin. Brca1 is a tumor suppressor gene that is mutated at a high frequency in hereditary breast and ovarian cancers (47). Following exposure to ionizing radiation, hydroxyurea, or UV irradiation, BRCA1 undergoes phosphorylation and forms nuclear foci (42). Association of BRCA1 with the DNA repair protein RAD51 further shows that BRCA1 participates in a pathway (or pathways) associated with HR. BRCA1 is the central part of a large multisubunit protein complex of tumor suppressors, DNA damage sensors, and signal transducers known as the BRCA1-associated genome surveillance complex (48). Among the DNA repair proteins in the complex are ATM, BLM, MSH2, MSH6, MLH1, the RAD50-MRE11-NBS1 complex, and the RFC1-RFC2-RFC4 complex. BRCA1, BLM, and the RAD50-MRE11-NBS1 complex colocalize to large nuclear foci after DNA damage. It has been suggested that the BRCA1-associated genome surveillance complex might serve as a sensor of abnormal DNA structures, a regulator of DNA repair, or both (48). Our data showed that BRCA1 phosphorylation and foci formation occurred after manumycin treatment (Fig. 3A and B). Similarly, NBS1 phosphorylation on Ser343 was also observed in manumycin-treated cells (Fig. 3A and B). BRCA1 has been reported to modulate response to genotoxic chemotherapy. Reconstitution of wild-type BRCA1 in BRCA1-mutant cells (HCC 1937) via transfection resulted in a reduced level of apoptosis after treatment with cisplatin, bleomycin, or etoposide (49). In addition, BRCA1-deficient mouse fibroblast cells were more sensitive to irinotecan, topotecan, etoposide, doxorubicin, carboplatin, or oxaliplatin than BRCA1-proficient cells (50). Therefore, our findings may imply that silencing the repair proteins BRCA1 and NBS1 may enhance the cytotoxicity of manumycin in tumor cells.

It has been shown that RhoB is one of the important mediators of FTI effect on apoptosis and proliferation inhibition (51). The essential role of RhoB has been validated by the knockout mouse studies (7). Geranylgeranylated RhoB may be a mediator of the effect of FTI on apoptosis and growth inhibition (6). Consistent with the other studies that DNA damage agents caused elevation of RhoB level (21, 22), we found that RhoB protein was increased after treatment with FTIs. Quenching ROS did prevent induction of RhoB by the FTIs, suggesting that RhoB was induced by FTI-ROS-mediated DNA damage. Linking FTI-induced DNA damage to RhoB added another section to the web of mechanism of action of FTIs.

In summary, FTIs induced ROS generation. ROS-mediated DNA damage led to formation of DSBs. DSB responses were then activated; ATM and DNA-PK but not ATR were the DNA damage sensors activated by manumycin. RhoB, an important mediator of FTI antineoplastic action, was induced as part of the DNA damage responses. This is the first report of DNA damage induced by FTIs via generation of ROS. Based on the findings in this paper and studies of other investigators, we proposed a model showing the relationship of FTI, ROS, DNA damage, DNA damage responses, and RhoB in the induction of growth inhibition and apoptosis of cancer cells (Fig. 7B). Future work will evaluate DNA repair mechanisms as potential therapeutic targets for combination therapy with manumycin. For instance, FTIs may be tested in combination with DNA repair inhibitors. The mechanism-based rational design of combination cancer chemotherapy might lead to improved survival of cancer patients.

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