Replicative senescence is found in normal cells as a permanent, irreversible cell cycle arrest. Premature (or stress-induced) senescence is a major cellular response to chemotherapy in solid tumors and contributes to successful treatment. However, senescent tumor cells are resistant to apoptosis and may also reenter the cell cycle. We set out to find a means to specifically induce senescent tumor cells to undergo cell death and not to reenter the cell cycle that may have general application in cancer therapy.

Experimental Design: We investigated the mechanisms regulating cell survival in drug-induced senescent tumor cells. Using immunofluorescence and flow cytometry – based techniques, we established the status of the ataxia telangiectasia mutated (ATM) signaling pathway in these cells. We assayed the requirement of ATM signaling and p21CIP1 expression for survival in premature senescent tumor cells using pharmacologic inhibitors and antisense oligonucleotides.

Results: The ATM/ATR (ATM- and Rad3-related) signaling pathway was found to be constitutively active in drug-induced senescent tumor cells. We found that blocking ATM/ATR signaling with pharmacologic inhibitors, including the novel ATM inhibitors KU55933 and CGK733, induced senescent breast, lung, and colon carcinoma cells to undergo cell death. We show that the mechanism of action of this effect is directly via p21CIP1, which acts downstream of ATM. This is in contrast to the effects of ATM inhibitors on normal, untransformed senescent cells.

Conclusions: Blocking ATM and/or p21CIP1 following initial treatment with a low dose of senescence-inducing chemotherapy is a potentially less toxic and highly specific treatment for carcinomas.
may also reenter the cell cycle. However, if a treatment could be found to specifically induce senescent tumor cells to undergo cell death, then such a chemotherapeutic approach could be of great potential value in cancer therapy.

This prompted us to investigate the molecular pathways regulating cellular survival in tumor cells induced to undergo senescence by exposure to a chemotherapeutic drug. The sublethal concentrations of anticancer drugs that induce premature senescence in tumor cells activate the protein kinases ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and, possibly, DNA-dependent protein kinase (13) so that senescence can be regarded as a form of permanently maintained DNA damage response. We have found that blocking constitutive ATM/ATR signaling using multiple pharmacologic inhibitors induces senescent breast, lung, and colon carcinoma cells to undergo cell death. This cell death seems to be directly dependent on p21(CIP1), which acts downstream of ATM. Blocking ATM and/or p21(CIP1) following initial treatment with a low dose of senescence-inducing chemotherapy is a potentially less toxic and highly specific treatment for carcinomas.

Materials and Methods

Cell culture and drug treatment. A549 and HCT116 cells were obtained from American Type Culture Collection and cultured according to its instructions. MCF-7 cells were cultured in DMEM. All media were supplemented with 10% fetal bovine serum. The genetic background of the cell lines used is as follows:

- A549 (p53+; Rb+; Hdm2+; INK4a/ARF deleted).
- HCT116 (p53+; Rb+; Hdm2+; INK4a/ARF heterozygously mutated and methylated).
- MCF7 (p53+; Rb+; Hdm2+; INK4a/ARF deleted).

The cell culture media and reagents were purchased from Invitrogen. Doxorubicin (Calbiochem) was dissolved in sterile water. Caffeine (Sigma) was dissolved in DMEM (100 mmol/L stock solution). Camptothecin (Sigma) was dissolved in DMSO. A stabilized hydrogen peroxide solution, 30% (w/w), was purchased by Sigma. The DNA-dependent protein kinase inhibitor NIU7026 (Calbiochem) was dissolved in DMSO. The ATM inhibitors KI55933 and CGK733 (Sigma) were dissolved in DMSO. Z-Val-Ala-Asp-(OMe)-CH2F (z-VAD-fmk; Enzyme Systems Products) was dissolved in DMSO and used at concentration of 40 μmol/L.

Induction of premature senescence. Unless otherwise stated, A549 cells were treated with 200 nmol/L doxorubicin for 72 h. MCF-7 cells were treated with 100 nmol/L doxorubicin for 72 h. HCT116 cells were treated with 300 nmol/L doxorubicin for 72 h. Camptothecin (0.5 μmol/L) and hydrogen peroxide (100 μmol/L) were used for 72 h in MCF-7 cells and for 96 h in A549 cells. Cells were extensively washed and replated in drug-free medium. To allow the development of a fully senescent phenotype, cells were analyzed from 7 to 21 days after replating.

Cell viability and senescence-associated β-galactosidase activity. Staining for senescence-associated β-galactosidase was done as previously described (14). In proliferating HCT116 cells, the senescence-associated β-galactosidase staining solution was used at pH 6.2 to reduce background. Senescent cells were plated in triplicate in 6- or 12-well multidishes. Cell viability was determined either by counting or by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. For counting, adherent, senescence-associated β-galactosidase–positive cells were counted in three random fields under a bright-field microscope. For each independent determination, a minimum of 100 (MCF-7) or 50 (A549) senescent cells were counted.

For MTT assay, senescent cells were seeded in triplicate into either 24- or 12-well multidishes. A MTT stock solution was made in a phenol–red-free culture medium to a final concentration of 5 mg/ml. The MTT solution was added at 10% of the total culture volume and cells were incubated 4 h at 37°C. At the end of the incubation period, the medium was removed and the converted dye was solubilized with acidic isopropanol (0.1 N HCl in isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm on a Bio-Rad Absorbance Microplate Reader 680.

Immunofluorescence microscopy and 4′,6-diamidino-2-phenylindole staining. Cells were grown onto glass coverslips in six-well multidishes and allowed to adhere for 16 h. To detect γ-H2AX and p21(CIP1), cells were fixed with methanol (-20°C) and permeabilized with ice-cold acetone. To detect phosphorylated ATM or phosphorylated Chk2, cells were fixed in 2% formaldehyde and permeabilized with 0.5% NP40.

Cells were blocked with 10% fetal bovine serum in TBS and 0.1% Tween 20 (TBS-T) for 15 min. γ-H2AX was detected by incubating the cells with anti-γ-H2AX monoclonal antibody, in a 1:200 dilution, for 2 h. p21(CIP1) was detected by incubating the cells with anti-p21(CIP1) polyclonal antibody, in a 1:100 dilution, for 2 h. Phosphorylated ATM (Ser1981) was detected by incubating the cells with anti–phospho-ATM monoclonal antibody, in a 1:200 dilution, for 2 h. Phosphorylated Chk2 (Thr68) was detected by incubating the cells with anti–phospho-Chk2 polyclonal antibody, in a 1:200 dilution, for 2 h. Cells were washed with TBS-T and then incubated with 1:500 dilution of fluorescein-tagged secondary antibodies (Santa Cruz Biotechnology). After washes with TBS-T, cells were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 15 min. After washes with TBS-T, the coverslips were mounted on a microscope slide using a 90% solution of glycerol in TBS and analyzed with a Zeiss Axioplan microscope. γ-H2AX foci were counted visually in >50 cells by capturing images of randomly chosen fields.

Flow cytometry for γ-H2AX and phospho-ATM. To detect γ-H2AX, cells were fixed with 70% ethanol in PBS and routinely kept at -20°C overnight. Cells were washed twice with TBS and permeabilized with TBS, 4% fetal bovine serum, and 0.1% Triton X-100 for 10 min on ice. Cells were washed with TBS and incubated with anti-γ-H2AX monoclonal antibody, in a 1:200 dilution in TBS, 4% fetal bovine serum, for 2 h. Cells were washed twice with TBS-T and incubated with 1:200 dilution of fluorescein-tagged goat anti-mouse secondary antibody (Santa Cruz Biotechnology). After washes with TBS-T, cells were resuspended in TBS and analyzed using a CyAn ADP Flow Cytometer (DAKO) and Summit Software. Detection of phosphorylated ATM (Ser1981) was done as described by Kurose et al. (15).

Western blot analysis. Total cell protein preparations were obtained lysing cells in a lysis buffer containing 50 mmol/L Tris-Cl (pH 7.4), 2 mmol/L EDTA, 0.1% Triton X-100, 1% NP40, 100 mmol/L NaCl, 1 μg/mL aprotinin, 170 μg/mL phenylmethylsulfonyl fluoride, and phosphatase inhibitors (Sigma). Protein concentration was routinely measured by the Bio-Rad protein assay. Polyclonal antibodies (from 5% to 15%) were prepared essentially as described by Laemmli (16). Molecular weight standards were from New England Biolabs. Proteins separated on the polyacrylamide gels were blotted onto nitrocellulose filters (Hybond-C pure, GE Healthcare). Filters were washed and stained with specific primary antibodies and then with secondary antisera, conjugated with horseradish peroxidase diluted 1:2,000 (Bio-Rad). Filters were developed using the enhanced chemiluminescence Western blotting detection reagent (GE Healthcare). The anti-p21(CIP1) (C-9), p27(CIP1) (C-9), poly(ADP)ribose polymerase (PARP), Bcl-2, Bcl-X, Bax, and p53 antibodies were purchased from Santa Cruz Biotechnology; anti-pRB was from BD PharMingen; anti-γ-H2AX (JBW301) was from Upstate Biotechnology; anti–phospho-ATM (Ser1981), anti–phospho-Chk2 (Thr68), and anti–phospho-Chk1 (Ser345) were from Cell Signaling; and anti–α-tubulin antibodies from Serotec.
Cell cycle analysis. Cells were fixed with 70% ethanol in PBS and routinely kept at -20°C overnight. Cells were washed twice with PBS; resuspended in PBS, 40 μg/ml propidium iodide (Sigma), and 50 μg/ml RNase DNase-free (Roche); and incubated at room temperature for 20 min.

Evaluation of caspase activity. Caspase activity was estimated by using Caspase Fluorimetric Substrate Set PLUS assay system (Alexis Biochemicals) following the manufacturer’s protocol. Routinely, 50 μg protein lysates were incubated with AFC-conjugated caspase-specific substrates at 37°C for 2 h. Fluorescence was measured on a LS-50B Fluorescence Spectrometer (Perkin-Elmer).

Antisense oligonucleotides. Phosphorothioate antisense oligonucleotides and control oligonucleotides were synthesized by Sigma. The sequences of the p21CIP1 antisense oligonucleotides were as follows:

- a-p21: TGT CAT GCT GGT CTG CCC GC (17).
- a-p21.2: TCC CCA GCC GGT TGT GAC AT (19).

The control sense p21 oligonucleotide (ATG TCA GAA CCG GCT GGG GA) is complementary to the a-p21 oligonucleotide (19).

Subconfluent monolayers of senescent A549 and MCF-7 cells were transfected using Lipofectamine 2000 Reagent (Invitrogen), following the manufacturer’s protocol. A concentration of 0.8 μmol/L oligonucleotides was used.

Results

Constitutive activation of ATM/ATR DNA damage response in premature senescent tumor cells. The treatment of tumor cells with sublethal concentrations of several anticancer agents readily induces premature senescence (2, 20). We treated A549 (lung carcinoma), MCF-7 (breast carcinoma), and HCT116 (colon carcinoma) cells with the DNA topoisomerase II inhibitor, doxorubicin, widely used in cancer chemotherapy. Cellular responses to DNA-damaging agents range from cell death to senescence and to proliferation, with high levels of damage resulting in cell death and low levels resulting in repair and proliferation. In our experimental system, we calibrated both the duration of treatment and concentration of drug used so that we produced tissue culture dishes containing ~100% senescent cells. To allow the development of a fully senescent phenotype, cells were analyzed from 7 to 21 days after plating. The cells obtained were judged to be senescent by various criteria: increase in cell size and typical morphologic alterations (Supplementary Fig. S1A-C), permanent cell cycle arrest, and positive staining for senescence-associated β-galactosidase (Supplementary Fig. S1A-C). The senescent cells were maintained in culture for 3 months showing neither proliferation nor loss of viability. In line with previous observations (2), premature senescent cells were mainly arrested with a G2-M DNA content (Supplementary Fig. S1A-C). Senescent human cells are characterized by a constitutively active ATM/ATR–dependent DNA damage response (13, 21). To evaluate the activation of the ATM signaling pathway in our cell system, senescent A549 and MCF-7 cells were examined for the presence of DNA damage foci. Persistent phospho-ATM (Ser1981), phospho-Chk2 (Thr68), and γ-H2AX foci were detected in senescent A549 (Fig. 1A) and MCF-7 cells (data not shown). These observations confirm that a DNA damage response is constitutively active in premature senescent tumor cells (21). We also set out to confirm that senescent cells accumulate the cyclin-dependent kinase inhibitor p21CIP1 in their nuclei. To achieve this, a mixture of senescent cells and proliferating cells (as controls) were plated in the same dishes, and p21CIP1 was detected by immunofluorescence exclusively in the senescent cells (Fig. 1B). Nuclear localization of p21CIP1 was further confirmed by cellular fractionation (Fig. 1C) in premature senescent A549 cells.

Effects of ATM inhibition in premature senescent tumor cells. The maintenance of cell cycle arrest in replicative senescent cells depends on the continued activity of the DNA damage checkpoint apparatus (13). Inhibition of ATM signaling either by pharmacologic inhibitors (22) or by kinase-dead

![Fig. 1](DNA damage foci and p21CIP1 accumulation in doxorubicin-induced senescent carcinoma cells. A, senescent A549 cells were immunostained with anti-phospho-ATM (Ser1981), anti-phospho-Chk2 (Thr68), and anti-γ-H2AX antibodies followed by secondary fluorescein conjugate antibodies. Nuclei were stained with DAPI. B, selective p21CIP1 accumulation in the nuclei of senescent A549 cells compared with proliferating cells. Small arrows, small nuclei of normal, proliferating cells. C, senescent A549 cells were subjected to fractionation. Equal amounts of cytoplasmic (C) and nuclear (N) extracts (40 μg) were separated by SDS-PAGE and blotted with p21CIP1 antibodies. The same filters were stripped and reprobed with anti-α-tubulin antibody, as a cytoplasmic marker, and anti-PARP antibody as a nuclear marker.)
isoforms (13) induces the proliferation of normal senescent cells. Caffeine has been used to inhibit formation of telomere-associated, ATM-dependent, DNA damage foci (23). Hence, to assess the ability of caffeine to inhibit ATM signaling in senescent tumor cells, senescent A549 and MCF-7 cells were treated with 5 mmol/L caffeine for indicated times. Cells were immunostained with anti–phospho-ATM (Ser1981) antibodies followed by secondary fluorescein-conjugate antibodies. Nuclei were stained with DAPI. To senescent MCF-7 cells were treated with 5 mmol/L caffeine for indicated times. Cells were immunostained with anti–phospho-ATM (Ser1981) antibodies followed by secondary fluorescein-conjugate antibodies. Nuclei were stained with DAPI.

To assess the effects of ATM inhibition, we treated senescent MCF-7 and A549 cells with increasing concentrations of caffeine. As shown in Fig. 3A, caffeine-treated MCF-7 cells appeared detached and shrunken. Nuclear condensation and fragmentation, both known indicators of programmed cell death, were detected in DAPI-stained cells. The caffeine-dependent loss in viability was time and dose dependent (Fig. 3B). Extensive vacuolization was detected in caffeine-treated A549 cells (Fig. 3C). In addition, DAPI staining in A549 cells showed a distinct nuclear alteration, with some condensation although without visible fragmentation. Significant loss of viability in A549 cells was achieved by a 72-h treatment (Fig. 3D). Similarly, caffeine treatment led to decreased viability in HCT116 cells in a time- and dose-dependent manner (Fig. 3E). These results suggest a role for the ATM/ATR signaling pathway in modulating survival in senescent tumor cells.

Several anticancer agents have been shown to induce premature senescence (2, 24). Therefore, we established experimental conditions to induce senescence in MCF-7 and A549 cells by treatment with different agents (i.e., H2O2 or camptothecin), which induces topoisomerase I–mediated DNA damage. Caffeine induced a dose-dependent loss of viability in tumor cells induced to undergo senescence by either agent (data not shown). These data indicate that a caffeine-inhibitable prosurvival pathway is constitutively active in premature senescent tumor cells irrespective of the agent used to induce senescence.
To study the pathway of cell death activated by ATM inhibition, senescent MCF-7 cells were treated with caffeine in the presence of the pan-caspase inhibitor z-VAD-fmk. In line with previous results, a 24-hour treatment with 5 mmol/L caffeine reduced MCF-7 viability to <20% of the control (16% ± 3; Supplementary Fig. S2A). z-VAD-fmk effectively inhibited caffeine-induced death (76% ± 3 compared with control). Next, we analyzed caffeine-treated senescent MCF-7...
cells for molecular markers of apoptosis and found that caffeine treatment resulted in both PARP (25) and pRb cleavage (refs. 26, 27; Supplementary Fig. S2A), which suggest the activation of caspases in caffeine-treated senescent MCF-7 cells. Because MCF-7 cells fail to express procaspase-3 (28), we screened senescent MCF-7 for activation of several caspases. Caspase-2, caspase-7, and caspase-9 were significantly activated in caffeine-treated cells compared with untreated controls (Supplementary

Fig. 4. Effects of KU55933 and CGK733 on ATM activation and viability of senescent carcinoma cells. A, senescent A549 cells were treated with 20 μmol/L KU55933 for 72 h. Cells were immunostained with anti–phospho-ATM (Ser1981) antibodies followed by secondary fluorescein-conjugate antibodies. Nuclei were stained with DAPI. B, senescent A549 and HCT116 cells were treated with 20 and 40 μmol/L KU55933 (72 and 24 h, respectively). Phosphorylated ATM was detected by immunostaining with an anti–phospho-ATM (Ser1981) monoclonal antibody followed by secondary fluorescein-conjugate antibodies. Samples were analyzed by flow cytometry. C, dose- and time-dependent effect of KU55933 on viability of MCF-7 senescent cells. Columns, mean from three independent experiments; bars, SE. Statistical analysis by unpaired Student’s t test, *P < 0.001. D, dose- and time-dependent effect of KU55933 on viability of A549 senescent cells. Columns, mean from three independent experiments; bars, SE. Statistical analysis by unpaired Student’s t test, *P < 0.05. E, dose- and time-dependent effect of CGK733 on viability of MCF-7 senescent cells. Columns, mean from three independent experiments; bars, SE. Statistical analysis by unpaired Student’s t test, *P < 0.01.
ATM and p21CIP1 in Premature Senescence

Fig. S2B). Caffeine-treated senescent HCT116 cells were then analyzed for molecular markers of apoptosis. The caffeine treatment greatly enhanced PARP cleavage, which is highly indicative of caspase-mediated cell death (Supplementary Fig. S2C). These data indicate that inhibition of ATM induces apoptosis in senescent MCF-7 and HCT116 cells. We also examined caffeine-treated A549 cells for molecular markers of apoptosis. Neither PARP nor pRB were cleaved in senescent A549 cells after a 72-h incubation with caffeine (Supplementary Fig. S2D). In addition, z-VAD-fmk had no effect on caffeine-induced death in senescent A549 cells (Supplementary Fig. S2E). Hence, caffeine seems to induce a caspase-independent death pathway in senescent A549 cells.

**Specific inhibitors of ATM cause cell death in senescent tumor cells.** To confirm the crucial role of ATM in modulating the survival of premature senescent tumor cells, we investigated the effects of two novel, specific ATM inhibitors, KU55933 (29) and CGK733 (22).

Treating senescent cells with KU55933 resulted in disappearance of phospho-ATM foci (Fig. 4A and data not shown). Dose-dependent loss of ATM phosphorylation in senescent cells treated with KU55933 was also confirmed by flow cytometry (Fig. 4B). More importantly, inhibition of ATM activity by KU55933 determined a time- and dose-dependent loss of viability in senescent MCF-7, A549, and HCT116 cells (Fig. 4C and D and data not shown).

We also investigated the effects of CGK733 on viability of premature senescent tumor cells. A dose-dependent loss of viability was induced by a 24-hour treatment with CGK733 in senescent MCF-7 cells (Fig. 4E). In contrast, treatment of senescent tumor cells with the specific DNA-dependent protein kinase inhibitor NU7026 (ref. 30; 5-20 μmol/L) did not affect cell viability (data not shown).

These data confirm a critical role for ATM in preserving survival of premature senescent tumor cells.

**ATM inhibition results in p21CIP1 down-regulation in senescent tumor cells.** p21CIP1 acts as a positive regulator of premature senescence in tumor cells (31, 32) and has also been shown to modulate apoptosis (11, 12). Because p21CIP1 is also a downstream target of ATM, we examined p21CIP1 protein levels in senescent MCF-7, A549, and HCT116 cells, grown in the presence or in the absence of caffeine. Treatment with caffeine resulted in a time- and dose-dependent decrease of p21CIP1 in each cell line, as detected both by Western blot and immunofluorescence (Fig. 5A-C and data not shown). Expression of p53 (Fig. 5A) and the apoptotic regulatory molecules Bax, Bcl-2, and Bcl-XL (data not shown) was not affected by caffeine.

These data suggest that p21CIP1 might play a role downstream of ATM in determining the survival of premature senescent tumor cells. Therefore, we investigated the effect of KU55933 on p21CIP1 expression. As shown in Fig. 5D, KU55933 decreased p21CIP1 protein levels in a dose-dependent manner in all senescent cells analyzed. Similarly, treatment of senescent MCF-7 and HCT116 cells with CGK733 resulted in a dose-dependent decrease in p21CIP1 protein (Fig. 5E).

To understand whether down-regulation of p21CIP1 precedes caspase activation or if it is a downstream event in the apoptotic cascade induced by ATM inhibitors, the effect of the pan-caspase inhibitor z-VAD-fmk on p21CIP1 down-regulation in MCF-7 cells was investigated. Although z-VAD-fmk effectively inhibited caffeine-induced PARP cleavage, it did not affect p21CIP1 down-regulation (Fig. 5F). Furthermore, in time course experiments, decrease in p21CIP1 protein was found to precede cleavage of PARP (data not shown). Hence, down-regulation of p21CIP1 precedes caspase activation.

**Down-regulation of p21CIP1 in senescent carcinoma cells leads to cell death.** To confirm the critical role of p21CIP1 in modulating the survival of premature senescent tumor cells, antisense oligonucleotides were used to down-regulate p21CIP1 expression. Senescent MCF-7 cells were transfected with either p21CIP1 antisense oligonucleotides (a-p21, a-p21.1, and a-p21.2) or a sense control oligonucleotide, and cells were harvested for biochemical analysis or viability assays. As shown in Fig. 6A, exposure to each of the antisense oligonucleotides resulted in a decrease in p21CIP1 protein levels by 24 hour, a-p21.1 and a-p21.2 most effectively, whereas the control oligonucleotide had no effect. The down-regulation of p21CIP1 levels also resulted in PARP cleavage (Fig. 6B), which is highly indicative of the activation of apoptosis. Interestingly, the a-p21 oligonucleotide, which was less efficient in down-regulating p21CIP1 protein, was also less efficient in inducing PARP cleavage. In line with these data, exposure to antisense oligonucleotides resulted in a significant loss of viability in senescent MCF-7 cells (Fig. 6C). Next, we analyzed senescent A549 cells. Treatment with p21CIP1 antisense oligonucleotides for 72 or 96 hour also reduced both p21CIP1 protein levels (Fig. 6D) and cell viability (Fig. 6E). The levels of other common cyclin-dependent kinase inhibitor proteins, p27KIP1 and p57KIP2, were assayed as controls. The decrease in p27KIP1 levels detected at 96 hours appears to be secondary to extensive cell death as p27KIP1 was unaffected at 72 hours although cell viability was reduced to 40% and p57KIP2 levels remained unchanged.

These data show that inhibition of p21CIP1 protein expression in premature senescent tumor cells readily induces cell death even in the absence of additional apoptotic stimuli. **Inhibition of ATM signaling in senescent tumor cells does not induce reentry into the cell cycle.** The pharmacologic inhibition of ATM signaling in normal (i.e., non-tumor) human senescent cells induces cell cycle entry and proliferation (22). In line with these observations, direct interference with ATM downstream targets Chk2 and p21CIP1 in G1 arrested, senescent untransformed human fibroblasts reverts senescence and induces DNA synthesis (13, 33–35). These observations prompted us to assess if interfering with ATM signaling would also induce senescent tumor cells to enter the cell cycle. Because premature senescent tumor cells primarily accumulate in the G0-M phase of the cell cycle (Supplementary Fig. S1, middle and right), we first evaluated the ability of caffeine-treated senescent A549 and MCF-7 cells to enter mitosis. No entry of the cells into mitosis was observed by anti-phospho-histone H3 staining (Supplementary Fig. S3A and data not shown). In addition, caffeine did not induce DNA synthesis in either cell lines, as assessed by thymidine incorporation or by bromodeoxyuridine incorporation (data not shown), nor did caffeine-treated cells reexpress relevant cell cycle-related proteins, such as cyclin E, cyclin A, or Cdc2 (Supplementary Fig. S3B).

These data highlight a specific ability of ATM inhibitors to induce premature senescent tumor cells to undergo apoptosis and not to reenter cell cycle.
Discussion

We set out to investigate whether apoptosis-resistant, drug-induced senescent tumor cells could be induced to undergo cell death by interference with the constitutive DNA damage signaling pathway found in such senescent cells. The A549, lung adenocarcinoma; MCF-7, breast adenocarcinoma; and HCT116, colon carcinoma cell lines were specifically induced to undergo senescence by treatment with different DNA-damaging agents (doxorubicin, camptothecin, and hydrogen peroxide). These cells showed several markers of premature senescence (i.e., flat and enlarged morphology, expression of senescence-associated $\beta$-galactosidase, nuclear accumulation of $\alpha$-21CIP, cell cycle arrest, and permanent loss of proliferative

![Image of senescent carcinoma cells](image_url)

Fig. 5. Effect of ATM inhibitors on p21$^{\text{CIP1}}$ protein in senescent carcinoma cells. A. senescent cells were incubated with 5 mmol/L caffeine (24 h for MCF-7 and HCT116 cells; 72 h for A549 cells). Filters were stripped and reprobed with anti-p53 antibody and anti-\(\alpha\)-tubulin antibody as a loading control. Prol, proliferating. Sen, senescent. B. time-dependent effect of caffeine on p21$^{\text{CIP1}}$ protein expression in senescent A549 cells. Filters were stripped and reprobed with anti-\(\alpha\)-tubulin antibody as a loading control. C. the ability of caffeine to reduce the amount of p21$^{\text{CIP1}}$ protein in senescent cells was confirmed by immunofluorescence in senescent A549 cells. D. dose-dependent effect of KU55933 on p21$^{\text{CIP1}}$ protein expression in senescent tumor cells. Senescent cells were incubated with KU55933 (24 h for MCF-7 and HCT116 cells; 72 h for A549 cells). Filters were stripped and reprobed with anti-\(\alpha\)-tubulin antibody as a loading control. E. dose-dependent effect of CGK733 on p21$^{\text{CIP1}}$ protein expression in senescent MCF-7 and HCT116 cells. Senescent cells were incubated with CGK733 for 24 h. Filters were stripped and reprobed with anti-\(\alpha\)-tubulin antibody as a loading control. F. senescent MCF-7 cells were treated with 5 mmol/L caffeine in the presence of the pan-caspase inhibitor z-VAD-fmk. \(\alpha\)-Tubulin was used as loading control.
Fig. 6. Effects of p21\textsuperscript{CIP1} antisense oligonucleotides on viability of senescent MCF-7 and A549 cells. Senescent MCF-7 cells were treated for 24 h with either Lipofectamine 2000 alone (LF) or with anti p21\textsuperscript{CIP1} antisense oligonucleotides (a-p21, a-p21.1, and a-p21.2) or with p21\textsuperscript{CIP1} sense oligonucleotide. A, p21\textsuperscript{CIP1} protein levels were assessed by Western blot. p27\textsuperscript{KIP1} and \(\alpha\)-tubulin were used as controls. B, PARP cleavage was detected by Western blot. C, cell viability was assessed after 24 h by counting of three random fields. Columns, mean from three independent experiments; bars, SE. Statistical analysis by unpaired Student’s t test, \(P < 0.001\). Senescent A549 cells were treated for 72 and 96 h with either Lipofectamine 2000 alone or with anti p21\textsuperscript{CIP1} antisense oligonucleotides (a-p21, a-p21.1, and a-p21.2) or with p21\textsuperscript{CIP1} sense oligonucleotide. D, p21\textsuperscript{CIP1} protein levels were assessed by Western blot. p27\textsuperscript{KIP1}, p57\textsuperscript{KIP2}, and \(\alpha\)-tubulin were used as controls. E, cell viability was determined after 72 and 96 h by MTT assay. Columns, mean from three independent experiments; bars, SE. Statistical analysis by unpaired Student’s t test, \(P < 0.01\).
ability). Although replicative senescent cells arrest with a G1 DNA content, premature senescent tumor cells mainly arrest in the G2 phase of the cell cycle (2). Because senescence arrest reflects a DNA damage checkpoint response (13, 21), this difference is likely related to the finding that many cancers have defective G1 checkpoint and rely on the G2 checkpoint for DNA damage–induced arrest (36). Accordingly, persistent phospho-ATM (Ser1981), phospho-Chk2 (Thr68), and γ-H2AX foci were detected in these senescent cells. This clearly indicates that a DNA damage response is constitutively active in premature senescent tumor cells and is in line with the observation that senescing mammalian cells accumulate persistent DNA lesions that contain unreparable double-stranded DNA breaks (13, 21).

The effect of inhibiting ATM or p21CIP1 in normal senescent human cells has been previously investigated. Both pharmacologic inhibition of ATM signaling (22) and down-regulation of the ATM targets, Chk2 and p21CIP1, in normal human senescent cells reverses senescence and induces DNA synthesis (13, 33–35). Hence, blocking ATM function promotes normal untransformed senescent cells to reenter the cell cycle (13, 22). By contrast, inhibiting ATM in drug-induced senescent tumor cells, using both the broad spectrum ATM/ATR inhibitor (i.e., caffeine) and highly specific ATM inhibitors (i.e., KU55933 and CGK733) induced neither mitosis nor DNA synthesis and had the surprising effect of reducing viability leading to cell death in the absence of exogenous apoptotic stimuli. The cell death was dose dependent in each cell line. The same effect was observed in cells induced to undergo premature senescence by a variety of DNA-damaging agents such as doxorubicin, hydrogen peroxide, or the topoisomerase I inhibitor camptothecin. Therefore, ATM/ATR signaling has a crucial role in regulating the cell survival of tumor cells induced to undergo senescence by drug treatment.

The ability of replicative senescent cells to reenter the cell cycle upon abrogation of ATM signaling (13, 33–35) raises the question of whether nonmalignant cells induced to become senescent by exposure to chemotherapeutic drugs will also reenter the cell cycle and whether forced proliferation of these damaged cells might lead to malignant transformation. In contrast to tumor cells, the maintenance of senescence arrest in normal cells is mediated by two pathways: the p53-p21CIP1 pathway and the p16ink4a-pRb pathway (37). p16ink4a is increased during stress-induced senescence (37) and appears to play a critical role in cells that lack normal p53 function (38). Hence, the p16ink4a–pRb pathway may represent an additional barrier to proliferation of normal, drug-induced senescent cells following inhibition of ATM-p53-p21CIP1 signaling. Furthermore, cell cycle checkpoints and DNA repair mechanisms efficiently cooperate in preventing chromosome instability (39). Although a decline in some DNA repair functions has been reported (40), normal senescent cells appear to be generally proficient in DNA repair (41, 42). These observations suggest that therapeutic inhibition of ATM signaling is not likely to drive normal drug-induced senescent cells to malignant transformation. However, future investigations are required to accurately determine the probability of this occurring.

p21CIP1 is known to act downstream of ATM (43); therefore, we examined the levels of p21CIP1 in premature senescent tumor cells undergoing cell death in response to multiple inhibitors of ATM/ATR. p21CIP1 levels dramatically declined in these cells, whereas the levels of p53 and multiple Bcl-2 family members remained unchanged. The order of molecular events during MCF-7 apoptosis, following caffeine exposure, was shown to be p21CIP1 down-regulation followed by caspase activation, consistent with the known role of p21CIP1 in blocking cell death in cancer cells (11, 12). This indicates the primacy of interfering with ATM signaling in specifically inducing premature senescent tumor cells to lose viability. Because the direct down-regulation of p21CIP1 expression by antisense oligonucleotides results in cell death in the absence of any other apoptotic stimulus, premature senescent tumor cells must require p21CIP1 expression for survival.

We investigated the nature of the cell death found in senescent tumor cells following caffeine treatment. MCF-7 and HCT116 cells were clearly identified to undergo apoptosis as judged by several criteria, for example, PARP cleavage, caspase activation, and inhibition by the pan-caspase inhibitor zVAD. However, A549 cells did not undergo apoptosis but underwent a form of caspase-independent cell death instead (44).

Although the antiapoptotic activity of p21CIP1 is well documented, the mechanism by which p21CIP1 inhibits cell death is not known. It was shown that p21CIP1 can interact with procaspase-3 and suppress its activation by masking the proapoptotic activity site (45). Other work has suggested that the antiapoptotic function of p21CIP1 may be based on nuclear inhibition of cyclin-dependent kinases (46). More recently, p21CIP1 has been shown to act either upstream of the mitochondria to prevent cytochrome c release (11) or downstream of the mitochondria to inhibit cyclin-dependent kinase–mediated activation of caspase-9 (47). These variations suggest a cell type–specific effect of p21CIP1 or an ability of p21CIP1 to act at different levels of the death cascade.

Abrogation of the G2-M checkpoint has become a major therapeutic target in cancer therapy (36). Many cancers have a defective G1 checkpoint (e.g., due to Rb and p53 mutations), resulting in dependence on the G2 checkpoint during cell division; thus, abrogation of the G2 checkpoint can result in cell death. Our data suggest that the use of agents that disrupt the G2 checkpoint, such as caffeine, following initial treatment with DNA damage–based chemotherapy (or radiotherapy ref. 48) might also specifically target carcinoma cells that have developed the characteristics of senescence. This action may be due to premature senescent cells being primarily arrested in G2–M.

G2 checkpoint adaptation, that is, the ability of a cell to reenter cell cycle in the presence of damaged DNA following a sustained checkpoint arrest, has recently been described in human cells (49). Cyclin B–cyclin-dependent kinase 1 appears to play a central role in this process (49). In our cell system, no significant reentry of senescent cells in the cell cycle was observed either in the absence or in the presence of ATM inhibitors. However, a distinct feature of senescence is an increased heterochromatin formation that leads to silencing of genes involved in controls of proliferation (50). Hence, the lack of expression of relevant cell cycle–related proteins, such as cyclin A, cyclin B, and Cdc2, may hamper adaptation of senescent tumor cells. It is possible that the rare events of escape reported from therapy-induced accelerated senescence (6, 7) are related to G2 checkpoint adaptation.

These data have important implications for chemotherapy. Although the induction of senescence by chemotherapy
contributes to successful cancer therapy (4), the escape of tumor cells from senescence is highly likely to lead to relapse (6, 7). Therefore, successful chemotherapy to eradicate the disease could also take account of this and seek to kill off the senescent tumor cells while the tumor is in remission. Our data suggest that a two-hit approach, first inducing senescence with low concentrations of DNA damage–inducing drug, followed by a treatment that interferes with ATM signaling and/or p21 expression, may be a highly specific way to kill off carcinoma cells. As a basis for therapy, the advantage of this approach (apart from specificity) may be that a low dose of the primary drug could be sufficient to induce senescence or a senescent-like phenotype in carcinoma cells. This could be followed by a higher doses (or longer application) of a noncytotoxic drug that specifically interferes with the ATM/p21 pathway.

Acknowledgments

We thank Dr. Owen Williams (Institute of Child Health, University College London, London, United Kingdom) for comments on the manuscript.

References

27. Chen JH, Ozanne SE. Deep senescence human fibroblasts show diminished DNA damage foci but retain checkpoint capacity to oxidative stress. FEMS Lett 2006;350:689–73.
Ataxia Telangiectasia Mutated and p21\textsuperscript{Clp1} Modulate Cell Survival of Drug-Induced Senescent Tumor Cells: Implications for Chemotherapy

Elvira Crescenzi, Giuseppe Palumbo, Jasper de Boer, et al.

*Clin Cancer Res* 2008;14:1877-1887.

Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/6/1877

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2008/03/20/14.6.1877.DC1

Cited articles
This article cites 50 articles, 22 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/6/1877.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/6/1877.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.