RNA Silencing of Checkpoint Regulators Sensitizes p53-Defective Prostate Cancer Cells to Chemotherapy while Sparing Normal Cells

Utpal K. Mukhopadhyay,1 Adrian M. Senderowicz,2 and Gerardo Ferbeyre1

1Département de Biochimie, Université de Montréal, Montreal, Quebec, Canada and 2Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland

Abstract
p53 is frequently mutated in patients with prostate cancer, especially in those with advanced disease. Therefore, the selective elimination of p53 mutant cells will likely have an impact in the treatment of prostate cancer. Because p53 has important roles in cell cycle checkpoints, it has been anticipated that modulation of checkpoint pathways should sensitize p53-defective cells to chemotherapy while sparing normal cells. To test this idea, we knocked down ataxia telangiectasia mutated (ATM) gene by RNA interference in prostate cancer cell lines and in normal human diploid fibroblasts IMR90. ATM knockdown in p53-defective PC3 prostate cancer cells accelerated their cell cycle transition, increased both E2F activity and proliferating cell nuclear antigen expression, and compromised cell cycle checkpoints, which are normally induced by DNA damage. Consequently, PC3 cells were sensitized to the killing effects of the DNA-damaging drug doxorubicin. Combining ATM knockdown with the Chk1 inhibitor UCN-01 further increased doxorubicin sensitivity in these cells. In contrast, the same strategy did not sensitize either IMR90 or LNCaP prostate cancer cells, both of which have normal p53. However, IMR90 and LNCaP cells became more sensitive to doxorubicin or doxorubicin plus UCN-01 when both p53 and ATM functions were suppressed. In addition, knockdown of the G2 checkpoint regulators ATR and Chk1 also sensitized PC3 cells to doxorubicin and increased the expression of the E2F target gene PCNA. Together, our data support the concept of selective elimination of p53 mutant cells by combining DNA damage with checkpoint inhibitors and suggest a novel mechanistic insight into how such treatment may selectively kill tumor cells.

(Cancer Res 2005; 65(7): 2872-81)

Introduction
Cell cycle checkpoints are signaling pathways that sense the state of the cell and the progression of cell cycle events to orchestrate a cellular response to damage (1). The outcome of activating checkpoints depends on the extent of the damage. Moderate injury generally triggers a cell cycle arrest to allow time for repair, dissipation of stressors, or accumulation of essential factors for cell cycle progression. On the other hand, significant damage induces programmed cell death to protect the organism from the expansion of cells with extensive genomic alterations that could eventually lead to neoplastic transformation (2). Failure to activate these checkpoint pathways may cause the development of cancer and other diseases (2). In agreement, cancer cells have altered checkpoint mechanisms that help them to maintain cell proliferation in the presence of oncogenic stress. Therefore, a major issue in cancer therapeutics is to find targets whose modulation will exploit these checkpoint defects in tumor cells to make them more sensitive to chemotherapy (3).

One of the most common genetic alterations in cancer patients involves the p53 tumor suppressor gene. In prostate cancer, altered p53 expression has been correlated with a higher Gleason score and worse prognosis (4–9). p53 mutations occur early in the process of prostate tumorigenesis inasmuch as they have been found in prostatic intraepithelial neoplasia and benign prostatic hyperplasia (10–14). Significantly, cells with p53 mutations found in primary prostate tumors seemed clonally expanded in their metastases (15, 16). Collectively, these studies indicate that the p53 tumor suppressor pathway is often disrupted in prostate neoplasms.

As a rule, cells with defective p53 fail to undergo apoptosis in response to a variety of proapoptotic stimuli and generally have a growth advantage over cells that have intact p53 (17). However, p53 is also involved in DNA repair and is an important component of cell cycle checkpoints. Consequently, p53 null cells have increased sensitivity to certain DNA-damaging stress (18, 19). For instance, caffeine, which inhibits the G2 checkpoint, sensitizes p53 null cells to radiation (20, 21). One of the most important mediators of the checkpoint pathways is the caffeine target ataxia telangiectasia mutated (ATM). ATM is a member of the phosphatidylinositol 3-kinase family of proteins that also includes ATM and ATR, DNA-PK, and mTor. ATM activates DNA repair and checkpoint pathways by phosphorylating multiple targets, including p53, Mdm2, BRCA1, Chk2, and Nbs1 (22). It has been shown that an antisense RNA directed against ATM renders prostate cancer cells more sensitive to radiation (23, 24), suggesting that disabling ATM might be part of an approach aimed to kill tumor cells.

Recently, Collis and colleagues used RNA interference (RNAi) to knock down ATM, DNA-PK, or ATR in prostate cancer cell lines. Knocking down ATM or DNA-PK sensitized the cells to radiation whereas the small interfering RNA against ATR sensitized the cells to the DNA-damaging agent methyl methanesulfonate (25). This study was done with p53-disabled prostate cancer cell lines that are also known for containing other genetic changes. Therefore, it was not possible to conclude that the p53 status determined the chemosensitizing effect of knocking down checkpoint proteins. In addition, the effects of silencing checkpoint regulators in normal cells were not investigated. This is relevant, because in theory modulation of checkpoints would be more effective in cells deficient for p53 (3, 18, 19, 26).

To address these issues we developed a retroviral vector system for stable expression of small hairpin RNAs (shRNA) in prostate cancer cells and normal human diploid fibroblasts. We achieved a...
stable and almost complete inhibition of ATM, ATR, and Chk1 levels in the p53 null PC3 prostate cancer cells, rendering them more sensitive to doxorubicin. Treatment with the G2 checkpoint inhibitor UCN-01 cooperated with the anti-ATM shRNA to promote drug sensitivity. We also found that ATM knockdown increased the mitotic index of PC3 prostate cancer cells, augmented E2F activity and proliferating cell nuclear antigen (PCNA) expression, and inhibited G2 arrest in response to DNA damage. Significantly, RNAs against ATM did not increase the chemosensitivity of p53-proficient normal fibroblasts or the prostate cancer cell line LNCaP. However, upon p53 inhibition, those cells became sensitized to doxorubicin by the anti-ATM shRNA as observed in the p53 mutant PC3 cells. Finally, we discuss a new model proposing that chemosensitivity after ATM knockdown in p53 mutant cells possibly results from the combined effect of checkpoint defects, low levels of p21, high E2F activity, and increased PCNA expression.

Materials and Methods

Cell lines and culturing. Prostate cancer cell lines PC3 (American Type Culture Collection, Manassas, VA) and LNCaP and their derivatives were grown in RPMI (Invitrogen, San Diego, CA). Human diploid fibroblast cells IMR90, their derivatives, and packaging cells Bing and Phoenix were grown in DMEM (Invitrogen). Both media were supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), 2 mmol/L l-glutamine (Life Technologies), and 1% streptomycin-penicillin (Life Technologies). All cell lines were grown in 5% CO2 at 37°C in an incubator.

Transfection of PC3 and LNCaP cells were carried out using Metafectene (Biontex, Munich, Germany) according to manufacturer instructions. For monitoring transfection, an enhanced green fluorescent protein (EGFP) expression plasmid (pLPC-GFP) was cotransfected with shRNA-expressing PCR cassettes.

Retroviral infections were done as described (27). However, to increase infection efficiency in PC3 cells, retroviruses were pseudotyped with VSV-G by cotransfecting a VSV-G-expressing plasmid with the retroviral plasmid into the packaging cell line. 

Infected PC3 and IMR90-derived cell lines were selected with 2 μg/mL of puromycin (Sigma, St. Louis, MO). Infected LNCaP-derived cell lines were selected with 5 μg/mL of puromycin for 2 days wherever applicable. IMR90 and LNCaP expressing dominant-negative p53 were grown in the presence of 50 (IMR90) or 100 μg/mL hygromycin (LNCaP).

Construction of retroviral vectors expressing anti-ATM small hairpin RNAs and hammerhead ribozymes. Plasmid pMSCV-puro was obtained from Clontech (Palo Alto, CA). pLPC-GFP and pWZL dominant-negative p53 were described in refs. (27, 28). Two shRNAs, each against human ATM, ATR, and Chk1 sequences (GenBank accession nos. NM-000051, NM-001184, and AF016882, respectively), were designed with the "RNAi oligo retriever" software (http://www.cshl.org/public/SCIENCE/hannon.html). The corresponding nucleotide positions targeted (with respect to "A" of AUG as +1) were 1-29 and 9062-9091 for ATM; 1-29 and 9062-9091 for ATR; and 48-76 and 92-112 for Chk1. The short hairpin activated gene silencing–PCR technique (29) was used to synthesize an expression cassette containing U6 promoter, target-specific hairpin sequence, and PotIII terminator using pGEMU6 (N. Hernandez, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as template. Two restriction endonuclease sites (BglII and EcoRI) were also incorporated through primers for convenient directional cloning (Supplementary Fig. 1A). Resulting 600-bp PCR products were either cloned between BglII and EcoRI sites of retrovector pMSCV-puro (Clontech) or used directly for transfection. To control the shRNA experiments we used a retrovector expressing a hairpin that does not recognize any human protein (control hairpin). Ribozyme constructs are described in Supplementary Fig. 1B.

Fluorescence-activated cell sorting and cell cycle analysis. Cells were cotransfected with equimolar quantities of pLPC-GFP plasmid along with shRNA/hammerhead ribozyme expression cassette. Twelve to 24 hours after transfection, cells were trypsinized, washed with PBS, and resuspended in PBS at a concentration of 2 × 10^5 cells/mL. EGFP+ cells were sorted using FACS Vantage SE (Becton Dickinson, San Jose, CA) flow cytometer with a laser excitation wavelength of 488 nm and a band-pass filter at 530 ± 50 nm. Data were analyzed by the software CellQuest (version 3.3).

For cell cycle analysis, 3 × 10^5 cells were plated onto 6-cm plates and grown overnight. Doxorubicin (20 ng/mL) was added 24 hours after plating and 12 hours before harvesting. UCN-01 treatment was started 30 minutes before the addition of doxorubicin. Trypsinized cells were washed and resuspended in PBS. Then they were fixed with an equal volume of ethanol, washed twice with PBS, and treated with RNase (100 μg/mL in 1.12% sodium citrate) for 60 minutes at 37°C. Finally, DNA was stained with the addition of 100 μL of 50 μg/mL propidium iodide solution in 1.12% sodium citrate for 60 minutes at room temperature. Propidium iodide–stained cells were analyzed using FACS Calibur (Becton Dickinson) flow cytometer with a band-pass filter at 585 ± 42 nm. Data were analyzed by ModFit (version 3.1) software.

Analysis of mitotic index. Two sets of 2.5 × 10^5 cells per condition were plated onto 6-cm plates. To one set, we added 100 ng/mL nocodazole (Sigma) 14 hours after plating. To the other set, along with nocodazole, we also added 100 ng/mL doxorubicin. Cells were harvested 2, 4, and 6 hours post treatment. Subsequent steps of fixation, permeabilization, and staining with Alexa Fluor conjugated phosphohistone H3 (Ser10) antibody (Cell Signalling Technology, Beverly, MA), were carried out per manufacturer's instructions. Antibody-stained cells were analyzed using FACS Calibur (Becton Dickinson) flow cytometer. For each sample, 10,000 cells were analyzed with an excitation wavelength of 633 nm (red diode) and emission at 660 nm (FL4). Data acquisition and analysis were carried out by CellQuest (version 3.3 software).

Cell death and DNA ladder assay. For the cell death assay, we plated 2 × 10^5 cells per well in 12-well plates in triplicate. Drugs (20 ng/mL of doxorubicin and 100 μmol/L UCN-01) were added 12 hours after plating and the cells were grown for 4 days. At the end of incubation, both attached and floating cells were harvested by trypsinization and centrifugation. The resulting cell pellet was resuspended in PBS plus an equal volume of 0.4% trypan blue staining solution and allowed to stand at room temperature for 30 minutes. Stained and nonstained cells were counted under light microscope. To characterize apoptosis, cells were incubated for 2 days in the absence or presence of drugs (100 ng/mL of doxorubicin and 100 μmol/L UCN-01). DNA fragmentation was detected as described (30).

Cell growth assay. Growth of p53-proficient/deficient human fibroblast cell lines IMR90/IMR90dmp53 derivatives was monitored by crystal violet dye retention assay (31). A total of 2 × 10^4 cells per well in triplicate were plated onto 12-well plates. Twelve hours after plating, one set of plates was fixed with 0.5% glutaraldehyde and stored at -4°C to be used as 0-day control, whereas the other set was allowed to grow for further 6 days and then fixed as before. Fixed cells were stained with 0.5% crystal violet and extensively washed with water. The retained dye was extracted in 0.5 to 2 mL of 10% (v/v) acetic acid solution. An aliquot (0.1-0.2 mL) was placed into a 96-well plate and absorbance was read at 595 nm in a microplate reader.

Clonogenic cell survival assay. Five hundred cells per well were plated onto 6-well plates. Colonies appeared ~ 8 to 12 days after plating. They were fixed with 0.5% glutaraldehyde, stained with 0.5% crystal violet, and counted. Data are displayed as mean ± SD of at least three independent experiments.

Western blotting. Whole-cell lysates equivalent to 25 to 100 μg total protein were resolved through 6% to 10% SDS-PAGE. Resolved proteins were transferred onto polyvinylidene difluoride membranes and probed with specific antibodies. The following antibodies were used: anti-ATM (from M.B. Kastan, St. Jude Children's Research Hospital, TN) anti-p21 (C-19, 1:250, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (PC10, 1:2000, from B. Stillman, Cold Spring Harbor, NY), anti-phospho-Thr^34 Chk2 (2661, 1:1000, Cell Signaling), anti-tubulin (B-5-1-2, 1:5000, Sigma), anti-ATR (N-19, 1:500, Santa Cruz Biotechnology), and anti-Chk1 (F-746, 1:500, Santa Cruz Biotechnology). The proteins were visualized using respective species-specific, horseradish peroxidase–conjugated secondary antibodies (Sigma) and Lumilight Plus (Amersham, Uppsala, Sweden) chemiluminescence detection kit.
E2F luciferase reporter assay. E2F activity was measured using the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. We used the plasmid HsOrc1-luc as E2F reporter (32) and the Renilla luciferase pRL plasmid as internal control.

Results

Hammerhead ribozymes and small hairpin RNAs against ATM provide reagents for a graded inhibition of ATM gene expression. To inhibit the expression of ATM, we designed two RNA hairpins with complementarity to sequences in the NH2- and COOH-terminal ends of the protein coding sequence. We also designed two hammerhead ribozymes targeted against approximately the same regions of the mRNA. We included polyadenyllic acid sequences in the 3’ end of the ribozyme to increase their activity (33). shRNA expression cassettes (Supplementary Fig. 1A) and ribozymes encoding sequences (Supplementary Fig. 1B), were subcloned into the pMSCV retroviral vector and stably introduced into PC3 prostate cancer cells by retroviral infections. Hairpins were more efficient than ribozymes in inhibiting ATM expression. ATM-Hp2 was the most efficient, followed by ATM-Hp1 and then Rz1. Rz2 showed no effect (Fig. 1A). These gene knockdown effects were sustained for at least 10 passages after retroviral infection (Fig. 1A).

Neither the hairpins nor the ribozymes reduced PC3 cells growth or their clonogenic ability (not shown). The ability of the most efficient shRNA to interfere with the ATM pathway was also verified by measuring the phosphorylation of the ATM target Chk2 at Thr2874 (Fig. 1B). Both basal and drug-induced phosphorylation of Chk2 were significantly inhibited by expression of the anti–ATM-Hp2.

Chemosensitivity in PC3 cells expressing anti-ATM small hairpin RNAs or anti-ATM ribozymes. To study whether expression of RNA hairpins and ribozymes enhance the sensitivity of PC3 prostate cancer cells to the DNA-damaging drug doxorubicin, we generated stable cell populations expressing the two hairpins and the two ribozymes described above. We treated these cell populations with doxorubicin or a combination of doxorubicin and UCN-01. The latter is known for inhibiting the G2 checkpoint control (34). The concentration of doxorubicin used (20 ng/mL) was selected after pilot experiments to determine the maximum amount of drug that revealed survival differences between PC3 and its derivatives expressing anti-ATM shRNAs. The concentration of UCN-01 (100 nmol/L) did not significantly affect the viability of PC3 (Supplementary Fig. 1C). We found that both anti-ATM shRNAs and Rz1 sensitized the cells to doxorubicin (Fig. 1C). The degree of sensitization was dependent on the degree of inhibition of ATM expression (Fig. 1A and C). Of note, the combination of doxorubicin and the Chk1 inhibitor UCN-01 promoted an even more potent clonogenic cell death (Fig. 1C). Retrovirally derived ATM-Hp2 increased drug sensitivity by a factor of 3.1 and ATM-Hp1 by a factor of 1.9 (Fig. 1D). Increased sensitivity to doxorubicin was seen in a dose response experiment as well, wherein anti-ATM-Hp2 reduced both the IC50 and the IC90 for the drug (Fig. 1E). Furthermore, the time required for complete clonogenic cell death after treatment with a combination of doxorubicin (40 ng/mL) and UCN-01 (100 nmol/L) was reduced upon expression of ATM-Hp2. Four days were sufficient to attain 100% killing in cells expressing ATM-Hp2, whereas a significant fraction of cells with a vector control survived even 5 days of treatment (Supplementary Fig. 1D).

Next we evaluated the ability of shRNAs and ribozymes to enhance chemosensitivity in transient transfection assays. To this end, cells were cotransfected with EGFP-expressing vector and EGFP-positive cells were sorted by fluorescence-activated cell sorting and treated with doxorubicin or a combination of doxorubicin and UCN-01. The results are presented in Supplementary Fig. 2. Chemosensitivity was enhanced by a factor of 1.8 for ATM-Hp2, 1.4 for ATM-Hp1, and very slightly for ATM-Rz1. Therefore, although the efficiency of killing was higher when the anti-ATM hairpins were stably expressed it was also possible to sensitize PC3 cells to doxorubicin in transient assays.

It has been proposed that inhibiting ATM expression in p53 null cells increases drug sensitivity because p53 mutant cells use G2 checkpoint pathways controlled by ATM in response to DNA damage (20, 23, 24). Consistent with that proposal we observed a reduction in the number of cells in G2 after ATM knockdown by RNAi in PC3 cells (Fig. 1F). This reduction was further increased by UCN-01 and was more notorious after treatment with low doses of doxorubicin (Fig. 1F). Notably, this decrease in G2 cells was not accompanied by a preferential accumulation of cells into G1 or S phases (not shown) suggesting that ATM-disabled PC3 cells were not activating checkpoints in G1 and S. This result is consistent with the p53 status of PC3 cells.

Effects of ATM knockdown on the cell cycle and the G2 checkpoint in PC3 cells. Next, we wanted to confirm that ATM knockdown in p53-deficient cells impaired the G2 checkpoint, increasing cell cycle progression into mitosis. To this end, cells
were treated with doxorubicin to induce DNA damage and nocodazol to stop the cell cycle at mitosis. Mitotic cells were detected using an anti-phosphohistone H3 (Ser\(^{10}\)) antibody (Fig. 2A). As expected, the number of cells entering mitosis in PC3 cells bearing a vector control was reduced after treatment with doxorubicin. ATM knockdown in PC3 cells also reduced the number of cells getting into mitosis, perhaps a reflection of the activation of compensatory G2 checkpoint proteins such as ATR. However, in contrast to PC3 cells bearing the vector control, a larger fraction of ATM-disabled cells still entered mitosis in the presence of doxorubicin (Fig. 2A). Even more remarkable, ATM-disabled cells displayed an increase in mitotic cells (mitotic index) in the absence of drug, suggesting that ATM slows down the cell cycle in the absence of DNA-damaging drugs as well. In agreement with this idea, ATM knockdown was accompanied by a dramatic increase in PCNA (Fig. 2B), a protein associated with high proliferation rates and DNA synthesis (35). PCNA and many other genes involved in DNA replication are regulated by the E2F family of transcription factors (36). Because E2F1 has been associated with drug response and drug sensitivity in p53-defective cells (37, 38), we evaluated total E2F activity in PC3 cells expressing a vector control or anti–ATM-Hp2. To do this, we did reporter assays using a well-known E2F-responsive promoter (32). We found that ATM-Hp2 increased E2F activity in PC3 cells (Fig. 2C) and this effect was even more pronounced after treatment with doxorubicin. Hence, suppressing ATM gene expression in PC3 cells accelerated cell cycle progression, increased E2F activity and PCNA expression, and impaired the G2 checkpoint. Consequently, these cells entered mitosis in the presence of DNA-damaging drugs.

**Figure 2.** Effect of ATM knockdown on mitotic index, PCNA expression, and E2F activity in PC3 cells. **A,** percent of mitotic cells labeled with Alexa-conjugated anti-phosphohistone H3 (Ser\(^{10}\)) antibody in PC3 cells with Vector Control or anti-ATM shRNA (ATM-Hp2). Left, no drug; right, 100 ng/mL doxorubicin. **B,** PCNA expression in PC3 cells expressing a vector control or anti–ATM-Hp2. The intensity of the bands was quantified by densitometry. Levels were normalized to tubulin and expressed relative to the value of the top lane. **C,** ATM knockdown increases E2F activity. PC3 cells cotransfected with the luciferase reporter construct HsOrc1-luc, which contains E2F sites, and a vector control or its derivative expressing anti–ATM-Hp2. Cells were then treated with doxorubicin or vehicle. Columns, mean of three independent measurements; bars, SD.
Inhibition of Chk1 and ATR also increased PCNA expression in PC3 cells. Next, we wanted to investigate the effects of inhibiting the ATR/Chk1 checkpoint pathway in p53-deficient PC3 cells. To do the experiments we first tested several shRNAs against ATR and Chk1 and found one for each gene that efficiently suppressed their expression (Fig. 3A). Knocking down either ATR or Chk1 after transient transfection of PC3 cells with these shRNAs increased their sensitivity to doxorubicin to a similar extent as with the anti-ATM hairpin (Fig. 3B and C). In addition, knocking down ATR or Chk1 also increased the expression of the E2F target and proliferation marker PCNA (Fig. 3D). Taken together, our results suggest that the chemosensitizing effects of G2 checkpoint inhibitors in p53-deficient cells are not only associated with checkpoint defects but may also involve the effects of PCNA and other E2F target genes.

Effect of ATM suppression on normal human diploid fibroblasts. To evaluate whether suppressing ATM specifically sensitized p53-deficient cells we used the normal human diploid fibroblast line IMR90. These normal cells are often used as p53 wild-type control cells and may allow us to discern whether the effects of ATM small interfering RNAs and DNA-damaging agents may spare normal cells. One limitation of using primary cells is their inability to form colonies in a clonogenic assay. Therefore, we used a growth assay on the whole cell population to evaluate the effects of drugs and ATM inhibition in these cells.

After infection of IMR90 with the retroviral vectors expressing anti-ATM hairpins and ribozymes, the degree of ATM inhibition was similar to that obtained in PC3 cells (Fig. 4A). Again, the shRNA was more effective than ribozymes in reducing ATM gene expression. Neither the hairpins nor the ribozymes significantly inhibited cell growth in IMR90 cells (Supplementary Fig. 3A). Treatment with doxorubicin, at the concentration used in PC3 cells, led to inhibition of cell growth by a factor of 1.6. Notably, reducing ATM gene expression with RNA hairpins or ATM-Rz1 made these cells more resistant to the toxic effects of doxorubicin (Fig. 4B). This is consistent with a role of ATM in sensing DNA damage and activating p53-dependent cell death (22). Finally, treating IMR90 cells with doxorubicin plus UCN-01 was more effective in inhibiting cell growth, but reducing ATM levels with shRNA did not sensitize these cells to such treatment (Fig. 4B). Taken together and in contrast to the p53-defective PC3 cell line, normal IMR90 fibroblasts were not sensitized to DNA-damaging drugs by ATM knockdown.

The enhancement in chemosensitivity seen in PC3 cells but not in IMR90 cells after ATM inhibition could be due, at least in part, to the p53 status of these cells or to any other of the multiple mutations present in PC3 cells and not in IMR90 cells. We reasoned that if ATM inhibition sensitizes p53-defective cells to chemotherapy, then blocking p53 functions in IMR90 cells should sensitize these cells to chemotherapy after ATM knockdown. To verify this hypothesis we used a stable cell line derived from IMR90 cells expressing a dominant-negative p53 (IMR90-dnp53; ref. 39). IMR90-dnp53 cells were resistant to cellular senescence induced by p14ARF, a well-known p53 activator (39) and did not induce p21 upon expression of oncogenic ras (data not shown). First, we depleted
ATM in IMR90-dnp53 using ATM-Hp1 or ATM-Hp2 (Fig. 4A). ATM inhibition did not affect the growth of these cells (Supplementary Fig. 3B) but sensitized them to doxorubicin and UCN-01, as observed previously in PC3 cells (Fig. 4D). A comparison of the sensitivity to doxorubicin and UCN-01 of IMR90 and IMR90-dnp53 over three different experiments is presented in Fig. 4E. Both anti-ATM shRNAs increased the sensitivity of IMR90-dnp53 to doxorubicin or doxorubicin plus UCN-01, whereas they have a moderate protective effect against doxorubicin in IMR90 cells.

Effects of ATM suppression on p53-proficient LNCaP prostate cancer cells. The experiments described above have two caveats. First, we have used different assays to measure drug response in normal IMR90 (growth curve) and PC3 tumor cell line (clonogenic assay). Second, normal fibroblasts might have a different organization of the checkpoint pathways in comparison with prostate cells. To address these concerns we used the p53-proficient and androgen-sensitive prostate cell line LNCaP and its derivative, wherein we introduced dominant-negative p53 (LNCaP-dnp53). We introduced ATM-Hp2 and a control hairpin in LNCaP and LNCaP-dnp53 cells by retroviral gene transfer and observed a similar inhibition of ATM expression in both cell lines (Fig. 5A). To determine whether dominant-negative p53 disabled the p53 pathway in LNCaP cells we tested the expression of p21, a known p53 downstream protein. As expected, dnp53 decreased the basal levels of p21 in this cell line, p21 levels in doxorubicin-treated cells were also significantly inhibited by dnp53 (Fig. 5A, bottom). Interestingly, the anti-ATM shRNA also reduced the basal levels of p21 in LNCaP cells. This is consistent with an ATM requirement for some but not all p53 functions (40). This reduction was not observed in cells treated with doxorubicin.

Next we treated LNCaP cells and LNCaP-dnp53 cells expressing the anti-ATM hairpin or a hairpin control with doxorubicin, UCN-01, or a combination of doxorubicin and UCN-01. It is worth mentioning that although 100 nmol/L UCN-01 did not significantly affect PC3 cells, it did reduce growth and clonogenicity of LNCaP cells (Supplementary Fig. 1). Regardless of this difference, p53-proficient LNCaP cells behaved like IMR90 cells; that is, reduction of ATM expression did not increase their sensitivity to the chemotherapeutic drugs used (Fig. 5B). In contrast, p53-disabled LNCaP-dnp53 cells were more sensitive to doxorubicin, UCN-01, or doxorubicin plus UCN-01 after inhibition of ATM expression (Fig. 5C; Supplementary Fig. 1). As seen in PC3 cells, anti-ATM shRNA increased the sensitivity of LNCaP-dnp53 by a factor of 3.4 (Fig. 5D), and had no effect or even brought slight protection to p53 wild-type LNCaP (Fig. 5D). Cell cycle analysis in LNCaP cells showed that ATM-Hp2 moderately decreased the percentage of cells in G2-M in untreated cells but not in cells treated with doxorubicin (Fig. 5E). Thus, the G2 checkpoint in LNCaP cells is less dependent on ATM than in PC3 cells.
cells, because ATM-depleted PC3 cells exposed to doxorubicin showed a clear decrease in the G2 phase. Addition of UCN-01 decreased the number of cells in G2-M in both cells not treated and treated with doxorubicin. Because UCN-01 blocks additional G2 checkpoint regulators (i.e., Chk1) this result is consistent with the idea that the ATR-Chk1 pathway, targeted by UCN-01, plays an important role in the control of the G2 checkpoint in LNCaP cells.

In LNCaP-dnp53 cells, expression of the shRNA against ATM actually increased the number of cells arrested in G2-M (Fig. 5E). Hence, blocking p53 and ATM may trigger a compensatory prolongation of G2-M in LNCaP cells. UCN-01 reduced the number of cells arrested in G2-M after treatment with doxorubicin in LNCaP-dnp53 as well (Fig. 5E). The data suggest that in LNCaP-dnp53 cells, abrogation of the G2 checkpoint cannot explain the increased sensitivity to doxorubicin after ATM depletion. However, abrogation of this checkpoint with UCN-01 improved the killing efficiency of doxorubicin.

We then investigated whether an increase in E2F activity was associated with ATM depletion in LNCaP cells. To do the experiment we did similar reporter assays as described with PC3 cells. We found that ATM depletion moderately increased E2F activity in LNCaP-dnp53 but not in LNCaP wild-type cells. More importantly, upon treatment with doxorubicin E2F activity significantly increased only in LNCaP-dnp53 ATM-depleted cells (Fig. 5F). Taken together, the data suggest that in LNCaP cells the chemosensitizing effects of ATM knockdown require a disabled p53 and is linked to induction of E2F activity by doxorubicin.

Suppressing ATM gene expression in p53 null cells increase cell death in response to DNA-damaging drugs. We have shown that reducing ATM levels by shRNA sensitizes PC3, LNCaP-dnp53, and IMR90-dnp53 to doxorubicin or a combination of doxorubicin and UCN-01. We have measured this effect in clonogenic survival and growth assays. These assays score growth inhibition without distinguishing cell cycle arrest from cell death. To gain further insight into the mechanisms of cell growth inhibition after treatment with doxorubicin and UCN-01 we measured cell death by a trypan blue staining assay. We found a good correlation between the percentage of cell death as

![Figure 5.](http://example.com/image.png)

*Figure 5.* Chemosensitivity of LNCaP and LNCaP-dnp53 cells after knockdown of ATM. A, Western blot detection of ATM, p53, and p21 from stably transduced LNCaP and LNCaP-dnp53 treated or not treated with doxorubicin. B and C, clonogenic survival assay of LNCaP and LNCaP-dnp53 cells expressing a control vector, anti–ATM shRNA, or a control shRNA. Cells were treated with doxorubicin or doxorubicin + UCN-01 and survival was expressed as percentage of respective untreated controls (100%). D, fold increase in sensitivity to the drug mixture (i.e., doxorubicin + UCN01) for ATM-suppressed LNCaP and LNCaP-dnp53 cells were calculated from the results presented in B and C by dividing the percent survival in cells expressing vector control with the percent survival in cells expressing anti–ATM-Hp2. E, effect of shRNA against ATM, and a control shRNA on the relative abundance of cells in G2-M stage of the cell cycle in cells treated or not treated with doxorubicin or UCN-01. F, ATM knockdown increases E2F activity only in LNCaP-dnp53 cells. LNCaP and LNCaP-dnp53 cells were cotransfected with the luciferase reporter construct HsOrc1-luc, which contains ERF sites and a vector control or its derivative expressing anti–ATM-Hp2. Cells were then treated with doxorubicin or vehicle. Columns, average of three means; bars, SD.
measured by trypan blue staining and the degree of growth inhibition reported previously. For example, drugs induced much more cell death in PC3 cells expressing an anti-ATM hairpin than in control PC3 cells (Fig. 6A). This cell death had features of apoptosis such as fragmented DNA into multimers of nucleosomal-sized (180 bp) fragments (Fig. 6B; ref. 30). In contrast, depletion of ATM in p53-proficient IMR90 or LNCaP cells did not lead to an increase of cell death over the value of control cells with wild-type levels of ATM. On the contrary, ATM knockdown protected these cells from the killing effects of the drugs (Fig. 6C and D). It is remarkable that the drug combination UCN-01 + doxorubicin killed ~15% of PC3 and around 40% of IMR90 cells after 4 days in culture. In contrast, upon ATM knockdown the sensitivity of PC3 cells increased whereas normal IMR90 fibroblasts became more resistant to the treatment. Taken together, our results show that PC3 cells were three times more resistant to drug treatment than normal fibroblasts (Fig. 6A and C). However, ATM knockdown exquisitely sensitized PC3 cells while conferring protection to normal cells. In agreement with the results presented above, the ability of the anti-ATM shRNA to sensitize cells to doxorubicin-induced cell death was dependent on the p53 status. Blocking p53 functions in IMR90 and LNCaP cells changed their response to ATM inhibition, making them more sensitive to drug-induced cell death (Fig. 6C and D). Therefore, p53-deficient cells are more sensitive to DNA-damaging agents when ATM is depleted. Moreover, reduction of ATM levels not only sensitized p53 mutant tumor cells to chemotherapy but also protected normal cells from DNA-damaging agents.

Discussion

We have shown that silencing ATM expression with shRNAs increased the effect of chemotherapy in p53-deficient cells but not in p53-proficient cells. Because p53 is inactivated in at least 50% of human cancers, drugs targeting ATM and, perhaps, other checkpoint regulators should be highly valuable in treating patients suffering from neoplastic diseases. Previous studies in PC3 prostate cancer cells reached a similar conclusion (23–25). However, other mutations present in those cells could be responsible for their chemosensitivity. Here we directly examined the role of p53 status in chemosensitivity by disabling p53 in normal human diploid fibroblasts and in the p53-proficient LNCaP cells by stable expression of dominant-negative p53. Because IMR90 cells do not possess oncogenic mutations and LNCaP cells are poorly tumorigenic their increased chemosensitivity after disabling both p53 and ATM gives substantial support to the concept of selective killing of p53-deficient cells at any stage of tumor formation.

Figure 6. Cell death as a result of ATM deprivation in normal or prostate cancer cells with or without p53. A, cell death (Trypan blue dye exclusion) assay for PC3 cells. B, DNA fragmentation (DNA ladders) in PC3 cells after treatment with doxorubicin. C, cell death assay for p53-proficient/deficient IMR90. D, cell death assay for LNCaP and LNCaP-dnp53. For the cell death assay, we counted the percentages of blue cells in a total of 500 cells for each condition in three independent experiments. Columns, average of three means; bars, SD.
It might seem paradoxical that p53-deficient cells can be sensitized to DNA-damaging drugs inasmuch as p53 is a major mediator of cell death in response to DNA damage (17). We propose that different effects of p53 and ATM inhibition cooperate to increase chemosensitivity (Fig. 7). We agree with previous reports that reducing ATM levels and inhibiting the S-G2 checkpoints should be more deleterious in p53-deficient cells, in which the G1 checkpoint is already compromised (22). This idea is consistent with models proposing that p53 seems to be more prominent in the control of DNA repair and the G1 checkpoint (19), whereas ATM is a major regulator of the S and G2 checkpoints (41). Consistent with this explanation, the Chk1 inhibitor UCN-01 cooperated with the anti-ATM shRNA to increase chemosensitivity. However, our data also indicate that checkpoint inhibition is not the only mechanism explaining the chemosensitivity of cells lacking both p53 and ATM. We have found that cells with defective p53 and ATM possess a unique biochemical signature that may explain their increased sensitivity to DNA-damaging drugs. These molecular changes include a dramatic increase in mitotic index, augmented PCNA expression and E2F activity, and a poor expression of p21. These changes may act in separate pathways but they may also promote synergistic interactions. For example, p21 is a major PCNA inhibitor (42); thus, cells disabled for both p53 and the ATM/ATR checkpoint pathways may have a synergistic increase in PCNA activity. 

PCNA may increase drug sensitivity by accelerating DNA replication (35), inducing p53-independent apoptosis via the candidate tumor suppressor ING1b (43, 44), or promoting translesion DNA synthesis by faulty DNA polymerases (45–47). For its part, E2F activity may increase drug sensitivity by inducing proapoptotic genes such as p73, Apaf1, caspase 3, and caspase 7 (36–38). Consistent with the apoptotic mechanism we detected nucleosomal-sized DNA fragments in PC3 cells expressing anti-ATM shRNA after treatment with doxorubicin. It remains to be investigated how ATM knockdown increases E2F activity in p53-deficient cell but not in p53 wild-type cells. It has been reported that active Cdk2-cyclin E complexes phosphorylate E2F5, increasing its transcriptional activity and cell cycle progression (48). The activity of Cdk2-cyclin A/E complex may be higher in cells lacking p53 and ATM due to the simultaneous defects in p21, a CDK inhibitor, and Chk2, which inhibits Cdc25, a CDK activator. Hence, the simultaneous inhibition of p53 and ATM creates a new cellular condition of high sensitivity to DNA damage that may result from the combined effects of checkpoint deficiencies and high E2F, PCNA, and perhaps, CDK activity.

Intriguingly, blocking ATM expression reduced the number of cells in G2-M in PC3 cells but not in LNCaP cells. This result suggests that compensatory mechanisms restore the G2 checkpoint in LNCaP cells. The G2 checkpoint is controlled by the kinases ATM and ATR that receive DNA-damage signals activating downstream effectors such as the checkpoint kinases Chk1 and Chk2 (41, 49, 50). Chk1 and Chk2 phosphorylate and inactivate the Cdc25 family of phosphatases, which are required to dephosphorylate and activate Cdk1, an enzyme essential for cell cycle passage from G2 to M (41). Thus, in the absence of ATM the ATR/Chk1 pathway can compensate for the G2 checkpoint defects as has been observed in fibroblasts from patients with ataxia telangiectasia (51). In this sense, LNCaP cells are similar to murine fibroblasts wherein loss of ATM sensitized p53 null cells to anticancer agents without interfering with the G2 checkpoint (52). Together, these data support our model (Fig. 7) proposing that the chemosensitizing effect of the ATM knockdown is not only the result of checkpoint defects.

Because in our experiments we have disabled the ATM/Chk2 or the ATR/Chk1 pathway, a critical question is how the cells that survived the treatment handle DNA damage. As mentioned before, the ATR/Chk1 pathway can compensate for ATM/Chk2 defects and vice versa (51). Microarray analysis of p53 null and ATM null cells could suggest potential compensatory pathways. For example, B-cell lymphocytic leukemia cells with disabled ATM and p53 express high levels of the Rad51-like protein XRCC2, a protein that mediates homologous recombination (53). Several studies point to a causal role for the homologous recombination repair pathway in resistance to DNA-damaging drugs (54). Hence, combining G2 checkpoint blockers with inhibitors of homologous recombination might enhance the efficacy of chemotherapy (53, 55). An additional important finding of our study is that inhibiting ATM functions by shRNA did not sensitize normal human fibroblasts to doxorubicin. This observation contrasts with the well-known susceptibility of ATM null cells to agents causing DNA-strand breaks. This difference might simply result from the short duration of our growth test (1-2 weeks) or the low concentration of doxorubicin used in our experiments. It is also possible that the low levels of ATM not inhibited by RNAi can still carry out some essential functions. This is a very important issue and a good reason to use RNAi instead of complete knockouts to validate whether a particular gene is a good target to develop drugs against cancer.

In conclusion, we have described a strategy that moderately increase the sensitivity of tumor cells to chemotherapy and at the same time protects normal cells. Because chemotherapy works on a very narrow therapeutic window, knocking down ATM, and perhaps other G2 checkpoint regulators, may significantly improve current cancer therapeutics. In particular, prostate tumors, known for their low proliferation rates, are resistant to chemotherapeutic agents (56). Interfering with ATM functions may be a useful strategy to force slow-growing prostate tumor cells into rapidly cycling cells more susceptible to chemotherapy.
Chemosensitization of p53-Defective Prostate Cancer Cells

Acknowledgments

Received 7/15/2004; revised 12/5/2004; accepted 1/19/2005.

Grant support: Canadian Prostate Cancer Research Initiative and the Prostate Cancer Research Foundation of Canada (G. Ferbeyre). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Moulay Alaoui-Jamali, V. Bourdeau, and A. Mukhopadhyay and members of the Ferbeyre laboratory for reviewing the manuscript, Dr. J. De Gregory for EZF repressor construct, Dr. M. Kastan for anti-ATM antibody, and Dr. Mario Chevreau for LNCaP cells.

References