p53 Suppression of Arsenite-Induced Mitotic Catastrophe Is Mediated by p21\textsuperscript{CIP1/WAF1}

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ABSTRACT

Arsenic trioxide, an acute promyelocytic leukemia chemotherapeutic, may be an efficacious treatment for other cancers. Understanding the mechanism as well as genetic and molecular characteristics associated with sensitivity to arsenite-induced cell death is key to providing effective chemotherapeutic usage of arsenite. Arsenite sensitivity correlates with deficient p53 pathways in multiple cell lines. The role of p53 in preventing arsenite-induced mitotic arrest-associated apoptosis (MAAA), a form of mitotic catastrophe, was examined in TR9-7 cells, a model cell line with p53 exogenously regulated in a tetracycline-off expression system. Arsenite activated G\textsubscript{1} and G\textsubscript{2} cell cycle checkpoints independently of p53, but mitotic catastrophe occurred preferentially in p53\textsuperscript{(+)} cells. Cyclin B/CDC2(CDK1) stabilization and caspase-3 activation persisted in arsenite-treated p53\textsuperscript{(+)} cells consistent with MAAA/mitotic catastrophe. N-Benzylxycarboxyl-Val-Ala-Asp-fluoromethyl keitone, a pan-caspase inhibitor, completely abolished arsenite-induced MAAA/mitotic catastrophe and greatly increased the mitotic index. WEE1 and p21\textsuperscript{CIP1/WAF1} inhibit cyclin B/CDC2 by CDC2 tyrosine-15 phosphorylation and direct binding, respectively. CDC2-Y15-P was transiently elevated in arsenite-treated p53\textsuperscript{(+)} cells but persisted in p53\textsuperscript{(-)} cells. Arsenite induced p53-S15-P and p21\textsuperscript{CIP1/WAF1} only in p53\textsuperscript{(+)} cells. P21\textsuperscript{CIP1/WAF1}-siRNA-treated p53\textsuperscript{(+)} cells were similar to p53\textsuperscript{(-)} cells in mitotic index and cell cycle protein levels. p53-inducible proteins GADD45\textalpha and 14-3-3\textalpha are capable of inhibiting cyclin B/CDC2 but did not play a p53-dependent role in mitotic escape in TR9-7 cells. The data indicate that p53 mediates cyclin B/CDC2 inactivation and mitotic release directly via p21\textsuperscript{CIP1/WAF1} induction.
Chemotherapeutic concentrations of arsenite induce cell death in a variety of cancer cell lines and virally immortalized cell lines. In NB4 cells, an acute promyelogenous leukemia cell line, arsenic induces apoptosis and down-regulates Bcl-2 expression at concentrations of 2 to 4 μM and causes arrest in early M phase and induction of apoptosis (Cai et al., 2003). In cells lacking functional p53, such as SV40-transformed human fibroblasts (States et al., 2002) and in HeLa, KB (Huang and Lee, 1998), or U937 cells (McCabe et al., 2000; McCollum et al., 2005) arsenic induces mitotic arrest and apoptosis. In contrast, these moderate concentrations slow the growth of diploid human fibroblasts and telomerase-immortalized human fibroblasts (S. C. McNeely and J. C. States, unpublished data) but do not induce cell death through mitotic catastrophe (Yih et al., 1997; States et al., 2002). However, arsenite delays normal mitotic progression and induces aneuploidy in human diploid fibroblasts (Yih et al., 1997) and peripheral blood lymphocytes (Vega et al., 1995). Thus, arsenite is a mitotic disruptor, and understanding its differential cytotoxicity between normal and tumor cells may be pertinent to understanding how other mitotic disruptors may selectively kill tumor cells.

A common feature of the cell lines in which arsenite induces apoptosis is a p53-deficient phenotype. Jurkat (human T cell leukemia) cells and human peripheral blood lymphocytes transfected with mutant p53 show increased sensitivity to arsenite treatment compared with cells transfected with wild-type p53, indicating induction of functional p53 confers resistance to arsenite (Salazar et al., 1997). Arsenite has been shown to induce apoptosis in a p53-dependent manner in multiple myeloma cell lines with varying p53 status (Liu et al., 2003). U266, RPMI 8226, and ARH-77 cell lines expressing mutated p53 and ARP-1 cells (p53 null) are sensitive to low pharmacological doses of arsenite (4–8 μM) and undergo G2/M arrest and apoptosis. In contrast, p53 wild-type cell lines, IM9, MC-CAR, and HS-Sultan, are relatively resistant to arsenite-induced apoptosis (Liu et al., 2003). p53 regulates cell cycle progression and apoptosis in response to genetic damage. The molecular mechanisms by which p53 arrests cell cycle progression in G1 and G2 in response to DNA damage are reasonably well understood (Jin and Levine, 2001); hence, a role for p53 in preventing apoptosis in response to mitotic disruptors may appear paradoxical. p53 is activated in response to mitotic disruption by arsenic (Yih and Lee, 2000). Therefore, we hypothesize that p53 protects against arsenite-induced mitotic disruption and subsequent apoptosis. However, the signals for p53 activation and the role that p53 plays in the cellular response to mitotic disruption are not yet understood. Therefore, we have investigated the effect of p53 expression on arsenite-induced mitotic arrest and apoptosis in a model cell line expressing p53 under exogenous control in a tetracycline-off system (TR9-7 cells). We show here that p53-dependent p21<sup>CIP1/WAF1</sup> induction is involved in allowing escape from arsenite-induced mitotic arrest and mitotic arrest-associated apoptosis (MABA), a form of mitotic catastrophe (Castedo et al., 2004).

**Materials and Methods**

**Cell Culture and Specialty Chemicals.** TR9-7 cells were the kind gift of Dr. Michael A. Tainsky (Wayne State University, Detroit, MI). Cells were cultured as described by Agarwal et al. (1995). p53 expression was suppressed in half the cultures by direct addition of tetracycline (1 mg/ml) to the media to a final concentration of 2.0 μg/ml. To allow moderate p53 expression, tetracycline (10 μg/ml) was added directly to the media to get a final concentration of 15 mg/ml. Working aqueous solutions of NaAsO2 (Sigma Chemical Co., St. Louis, MO) were prepared freshly on the day of treatment and filter sterilized prior to use.

**Viability Assays.** Cells were seeded in 96-well plates for measuring cell growth during arsenite treatment using the AlamarBlue fluorescence assay (BioSource International, Inc., Camarillo, CA). AlamarBlue (resazurin) is a soluble, nontoxic dye used to monitor the functional electron transport chain of a viable cell by reduction of the dye from its oxidized, nonfluorescent (blue) state to the reduced, fluorescent (pink) state (resorufin). AlamarBlue measurement of cell viability was performed as described by (States et al., 2002). Data are reported as arbitrary fluorescence units above the blank versus time (days). Viability was also determined using trypan blue exclusion assay (Sigma). Three independent experiments were analyzed.

**Flow Cytometry.** For cell cycle/DNA synthesis analysis, TR9-7 cells were pulsed for 1 h with 10 μM 5-bromo-2′-deoxyuridine (BrDU) (Sigma) before cells were harvested by trypsinization and fixed in 70% ethanol overnight. Fixed cells were collected by centrifugation (480g) and then resuspended in denaturing buffer (PBS, 9.1% HCl, 0.02% pepsin) for 10 min at 37°C. Following denaturation, cells were washed three times in dilution buffer (PBS, 0.5% Tween 20, 0.5% BSA) and then probed with FITC-conjugated mouse α-bromodeoxyuridine (BD Biosciences Pharmingen, San Diego, CA) in the dark overnight at 4°C and washed in dilution buffer. Cells were then stained with 10 μg/ml propidium iodide in the presence of RNase A (100 μM final) for at least 30 min at room temperature. Cell cycle/mitotic index analysis was performed as described previously (McCollum et al., 2005). Cell cycle analysis (bivariate plots of BrDU incorporation (FITC) and DNA content or TG-3 (allophycocyanin) staining and DNA content) was performed on a FACSCalibur (BD Biosciences). A minimum of 20,000 cells/sample was analyzed. Data were collected and analyzed using CellQuest software. BrdU analysis consisted of three independent experiments, and a representative sample is shown. TG-3 analysis was performed as duplicate independent experiments.

**Mitotic Index and Mitotic Catastrophe Index Determination.** Cells were harvested for mitotic index as described previously (States et al., 2002). Slides were examined under a microscope, and at least 900 cells were counted on each slide for determination of mitotic index and mitotic catastrophe index. Only cells with distinct interphase nuclei, metaphase spreads, or mitotic catastrophe appearance were counted. Three independent experiments were analyzed. DAPI stain in mounting media (Molecular Probes, Invitrogen, Eugene, OR) added directly to slides was used to identify DNA in interphase nuclei, mitotic spreads, and mitotic catastrophe by fluorescence microscopy.

**Western Blot Analyses.** Cells were washed with PBS, and total cellular lysates were prepared by directly lysing the cells in the plates with 200 μl/10 cm dish of lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM Na2-EDTA, 0.1% SDS, and 180 μg/ml phenylmethylsulfonyl fluoride). Protein concentration in the lysates was determined by Bradford assay (Bio-Rad, Hercules, CA). Proteins were resolved by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gels and transferred to supported nitrocellulose membranes by electroblotting. For p53 analyses, 5 μg of protein/lane was analyzed, whereas all other western blot analysis contained 15 μg protein/lane. Proteins were visualized by probing membranes with mouse monoclonal antibodies for p53 DO-1 (LabVision), 14-3-3ζ (Upstate Cell Signaling Solutions, Chicago, IL), PARP (EMD Biosciences, Inc., San Diego, CA), cyclin B1 (BD Biosciences Pharmingen), and β-actin (Sigma), or rabbit polyclonal antibodies for p53-S15-P, p53-S20-P, CDC2-Y15-P, caspase-3 (Cell Signaling Technology, Inc., Beverly, MA), p21<sup>CIP1/WAF1</sup> (H-164), GADD45α (H-165) (Santa Cruz Biotechnologies, Santa Cruz, CA), WEE1,
CDC2 (CDK1), and CDC25c (Upstate Cell Signaling Solutions). Binding of secondary antibodies, rabbit anti-mouse antibody and goat anti-rabbit antibody (Zymed, San Francisco, CA), both conjugated to horseradish peroxidase, was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). β-Actin was used as a loading control. Western blot experiments were performed in three independent experiments, and blot images were quantified using ImageQuant software (GE Healthcare, Piscataway, NJ). Statistical analysis was performed using two-tailed Student’s t test in SlideWrite (Advanced Graphics Software, Inc., Encinitas, CA). In addition, all blots were stained with Ponceau S prior to antibody probing. Ponceau S staining was used to check for equal loading, quality transfer, and normalization during quantification analysis.

**P21cip1/waf1 siRNA.** Cells exposed either to high (2000 ng/ml) or low (15 ng/ml) tetracycline were transfected with 30 nM CIP1 (p21cip1/waf1) Smartpool siRNA (Dharmacon, Lafayette, CO), nonspecific control (NSC) siRNA (Dharmacon), or mock-transfected with Lipofectamine 2000 (Invitrogen) alone, then exposed to 0 or 5 μM NaAsO2 for up to 3 days. Samples were taken before arsenite exposure and after 24-, 48-, and 72-h treatment and analyzed by western blot. Twenty-four-hour treatment samples were also analyzed by mitotic index analysis. Three independent experiments were analyzed.

**Statistical Analyses.** Comparison of mitotic indices of arsenite-treated p53+/− and p53−/− cells was performed by two-tailed Student’s t test using SlideWrite software and confirmed by ANOVA.

## Results

### Modulated p53 Expression in TR9-7 Cells.

We tested the hypothesis that p53 protects against mitotic arrest and subsequent apoptosis/mitotic catastrophe using TR9-7 cells, a spontaneously immortalized Li-Fraumeni fibroblast cell line that is stably transfected with a tetracycline-regulated p53 expression plasmid (Agarwal et al., 1995). TR9-7 cells cultured in the absence of tetracycline express high levels of p53 and arrest in G1 and G2 phases (Agarwal et al., 1995). We have observed previously that noncycling cells are resistant to arsenite toxicity (States et al., 2002). Our hypothesis requires cycling cells to analyze the effect of arsenite on cells moving into and/or out of mitosis. TR9-7 cells were incubated with 0 to 1500 ng/ml tetracycline. Total cellular protein extracts were resolved by SDS-PAGE and electroblotted to nitrocellulose for probing with DO-1 antibody to p53 and detection by chemiluminescence. The bands on the lumigram were quantified by image analysis, and the data were plotted using SlideWrite software. B, TR9-7 cells were treated with tetracycline at 15 or 1500 ng/ml. AlamarBlue assays were performed in separate cultures after 4, 24, 48, 72, and 144 h in culture as indicated, and AlamarBlue fluorescence was recorded. Means ± S.D. of triplicate cultures are plotted.

### Flow-Cytometric Analysis of Cell Cycle and Cell Viability.

Flow-cytometric analysis of DNA content in arsenite-treated SV40 transformed cells (States et al., 2002) suggested that cells arrested in mitosis were degenerating, losing DNA and artifically appearing in the S phase compartment. We examined the effect of arsenite treatment on cell cycle in TR9-7 cells expressing and not expressing p53 and observed a similar increase in “S phase” (by DNA content) cells in p53−/− cells (data not shown). Therefore, DNA synthesis was monitored by pulsing the cells with a halogenated thymidine (dT) analog, BrdU, 1 h before harvesting. BrdU incorporation and DNA staining were then quantified by flow cytometry (Fig. 2A). The PI versus BrdU contour plots of p53−/− and p53+/− cells not treated with arsenite show a pattern of progression indicating gradual accumulation of cells in G2/G0 (bottom left quadrant) as expected in a culture undergoing contact inhibition. Thus, both p53−/− and p53+/− cells not exposed to arsenite have equal distributions at the end of 3 days in culture.

Exposure to arsenite caused marked changes in the cell cycle distributions of p53−/− and p53+/− TR9-7 cells (Fig. 2A). Within the first 24 h following arsenite exposure, there was an accumulation of cells in G2/M (i.e., bottom right quadrant; high DNA content, low BrdU-incorporating cells) and diminution of cells in the other three quadrants. In cultures exposed to arsenite for 24 h, BrdU incorporation was dramatically reduced and restricted to cells in late S phase. There were essentially no cells in early S (top left quadrants) treated with arsenite for 24 h. The fraction of cells accumulating in G2/M within the first 24 h following arsenite exposure was greater for p53−/− cells than it was for p53+/− cells (70.8 versus 55.8%). This increase was likely attributable to the increased number of mitotic cells in arsenite-treated p53−/− cells (see below).

The fate of cells accumulating in G2/M following arsenite treatment differed between p53−/− and p53+/− cells. Within 48 h of arsenite treatment, the fraction of p53−/− cells accumulated in G2/M had decreased substantially in comparison with the 24-h time point. At 48 h, a large number of p53−/− cells had an intermediate or S phase DNA content with the peak of the contour plot in the middle of the plot between G2/M and G1. However, the fact that such S phase DNA content cells were not incorporating BrdU suggested that these cells were actually degenerated G2/M cells or sub-G2 cells. It is also noteworthy to describe the shape of the contour plots in the p53−/− cells at 72 h. p53+/− cells treated with arsenite exhibit clear peaks in one or both of the two lower
quadrants, showing a distinct shift over time from a peak in G2/M at 24 h to a peak in G1/G0 at 48 and 72 h. In contrast, p53<sup>-/-</sup> cells never reach a G1/G0 peak and exhibit a plateau in a pseudo-S phase (“sub-G2”) area extending from G2/M quadrant to the G1 quadrant with no defined peaks. The p53<sup>-/-</sup> cultures also contained cellular debris after 72 h of arsenite exposure, indicating cell fragmentation had occurred. In contrast to the p53<sup>-/-</sup> cells, cell cycle transit through G2 and M phase was delayed in arsenite-treated p53<sup>+/+</sup> cells, but by 72 h, cells originally in G2/M (55.8 and 48.3% at 24 and 48 h, respectively) had survived and emerged as G1 cells (73.5%, lower left quadrant). Unfortunately, the sub-G2 cells are not quantitatively distinguished from true G1, G2, or M cells, nor are M phase cells distinguished from G2 cells in the G2/M quadrant. To address these problems, PI versus TG-3 bivariate analysis was performed (Fig. 2B). The TG-3 antibody recognizes a phosphoepitope on nucleolin that is specific to mitosis (Vincent et al., 1998), allowing us to distinguish mitotic cells from G2 cells. Thus, we were able to obtain the percentage of cells in G1, S, G2, and M phases. Because we have shown that treatment with arsenite results in little or no true S phase cells via PI versus BrdU incorporation analysis, we can assume the majority of cells identified as S phase by DNA content in arsenite-treated samples are not truly in S phase but are sub-G2 cells potentially undergoing mitotic catastrophe. The data show that at 72 h, the G1 population in arsenite-treated p53<sup>+/+</sup> cells is higher than in arsenite-treated p53<sup>-/-</sup> cells (47% versus 27%) and approaching control levels. The fraction of cells with S phase DNA content in both p53<sup>+/+</sup> and p53<sup>-/-</sup> cells treated with arsenite drops significantly at 24 h of treatment below their respective untreated controls. p53<sup>-/-</sup> cells have an apparent increase in

Fig. 2. Cell cycle analysis and cell viability. Replicate cultures of TR9-7 cells were incubated with tetracycline at 15 [p53<sup>+/+</sup>] or 1500 [p53<sup>-/-</sup>] ng/ml and with and without 5 μM Na3AsO<sub>3</sub> (As) for 3 days. After 24, 48, and 72 h, cells were harvested from each group and analyzed for: BrdU incorporation and DNA content (PI stain) (A) or TG-3 mitotic marker and DNA content (PI stain) (B) by flow cytometry. In A, contour plots with cells incorporating BrdU above the horizontal midlines and low DNA content on the left (G1) of the vertical midline are shown. The mean percentages within each quadrant are indicated in the corners of each plot. In B, percent G1, S, G2, and M phase is plotted as means ± S.D. C, cell viability assessed by trypan blue exclusion after 72 h. †, p < 0.05; ††, p < 0.01; †††, p < 0.001, arsenite-treated p53<sup>+/+</sup> or p53<sup>-/-</sup> cells are significantly different from respective untreated controls only; †, p < 0.05; ††, p < 0.01, arsenite-treated p53<sup>-/-</sup> cells are significantly different from arsenite-treated p53<sup>+/+</sup> cells. A minimum of 20,000 events were counted per sample. Representative results of biological triplicates (A) and combined results of biological duplicates (B) are shown. Combined results of biological triplicates from trypan blue exclusion assay are shown (C).
percent S phase DNA content cells rising above untreated cells at 48 and 72 h. In contrast, the fraction of arsenite-treated p53<sup>−/−</sup> cells with S phase DNA content increases only slightly to the level of untreated cells by 72 h. Both p53<sup>−/−</sup> and p53<sup>−/−</sup> cells treated with arsenite have a significant increase in percent G<sub>2</sub> above respective untreated controls at 24 and 48 h, whereas at 72 h, the arsenite-treated p53<sup>−/−</sup> cells remain somewhat higher. The percent M phase increases significantly above respective untreated controls in both arsenite-treated p53<sup>+/+</sup> and p53<sup>−/−</sup> cells at 24 h. Importantly, the percent M phase was significantly higher in treated p53<sup>−/−</sup> cells than in treated p53<sup>+</sup> cells at 24 h and remained significantly higher in p53<sup>−/−</sup> cells out to 72 h as percent M phase in p53<sup>+</sup> cells decreased to control levels by 72 h. The significant rise in apparent S phase DNA content cells after a prominent spike in M phase at 24 h in arsenite-treated p53<sup>−/−</sup> cells, combined with the data from the PI versus BrdU analysis showing a plateau of cells emanating from G<sub>2</sub>/M phase cell population, suggests that p53<sup>−/−</sup> cells preferentially undergo mitotic arrest and cell death as they attempt to exit from arsenite-induced mitotic arrest resulting in a sub-G<sub>2</sub> population at 72 h.

To determine the effect of arsenite on cell viability, we performed a trypan blue exclusion assay on p53<sup>+</sup> and p53<sup>−/−</sup> TR9-7 cells treated with and without 5 μM arsenite for 72 h (Fig. 2C). Trypan blue is excluded from cells with intact membranes. Thus, trypan blue exclusion does not distinguish early apoptotic cells with intact membranes from viable cells. Dead and necrotic cells have permeable membranes and stain readily. However, cells undergoing apoptosis in cell culture rapidly progress to a secondary necrosis in the late stages of apoptosis, and the cell membrane becomes permeable (Cejna et al., 1994). Therefore, trypan blue exclusion assay may be used to measure viability, but other studies are needed to distinguish apoptotic cell death from necrotic cell death. The results (Fig. 2C) show that arsenite treatment has a greater impact on viability of p53<sup>−/−</sup> compared with p53<sup>+</sup> TR9-7 cells.

**Mitotic Arrest-Associated Apoptosis/Mitotic Catastrophe.** The mitotic index appeared low in light of the large fraction of cells in G<sub>2</sub>/M determined by flow cytometry and the large number of rounded cells lifting off the surface in the arsenite-treated cultures. We observed indistinct structures on the mitotic index slides that had no nuclear envelope and no obvious chromosomes present. These structures were neither interphase nuclei nor chromosomal spreads (Fig. 3A). However, most of the structures resembled the general shape and size of a mitotic spread. The intensity of Giemsa stain varied from nearly as dark as chromosomes in a mitotic spread to very faint and almost invisible (Fig. 3A, I–L, respectively). It was noted that these were only present in high frequency in arsenite-treated cells and were more frequent in p53<sup>−/−</sup> cells than in p53<sup>+</sup> cells (Fig. 3A, A–H). These structures (Fig. 3A, P) stained positively with DAPI (Fig. 3A, T), indicating that they contained DNA. We speculated that these were mitotic cells undergoing apoptosis, which would explain the sub-G<sub>2</sub> population in the BrdU-propidium iodide flow cytometry data. These structures likely represent mitotic arrest-associated apoptosis or mitotic catastrophe, which has been shown to occur in a caspase-3-dependent manner in cells lacking cell cycle checkpoints such as loss of p53 (Castedo et al., 2004). Some cells dying in arsenite-treated cultures exhibited typical morphology of fragmented, condensed nuclei (Fig. 3A, N and R), normally characteristic of apoptosis in non M-phase parts of the cell cycle. However, these cells were rare.

**Arsenite Induces Mitotic Arrest Preferentially in p53<sup>−/−</sup> Cells.** The effect of moderate p53 expression on arsenite induction of mitotic arrest and mitotic catastrophe was examined (Fig. 3B). Cells were incubated with and without 5 μM arsenite for 24 and 48 h. Both p53<sup>−/−</sup> and p53<sup>−/−</sup> cells had a low mitotic index (2–3%) at 0 h that showed a modest increase to 3 and 5%, respectively, after 24-h incubation without arsenite. Exposure to 5 μM NaAsO<sub>2</sub> for 24 h more than doubled the mitotic index in p53<sup>−/−</sup> cells to ~12% but had only a modest effect on p53<sup>−/−</sup> cells (5–6%). At 48 h,
mitotic indices decreased in both p53(knockout) and p53(wild-type) cells; however, the mitotic index in p53(knockout) cells remained much higher (6–7%) relative to p53(wild-type) cells (1–2%). At 24 h, p53(knockout) cells had significantly higher mitotic catastrophe index (~19%) than p53(wild-type) cells (~10%). Analysis at 48 h revealed that as mitotic index decreased in p53(knockout) cells, the mitotic catastrophe index increased to ~26%. In contrast, p53(wild-type) cells displayed decreased mitotic catastrophe index from ~10% at 24 h to ~5% at 48 h as mitotic index also decreased from ~5 to ~1%. Decreases in mitotic index and mitotic catastrophe index in p53(wild-type) cells were likely a consequence of the successful completion of cytokinesis and progression into G1 (see Fig. 2), causing the denominator in mitotic index quantification to increase. This observation suggests that p53 expression played a pivotal role in preventing mitotic catastrophe induced by arsenite.

**Protein Markers of Mitotic Arrest and Apoptosis.**

Cells exposed either to high (2000 ng/ml) or low (15 ng/ml) tetracycline were treated with 0 or 5 μM NaAsO₂ for up to 3 days. Samples were taken before arsenite exposure and after 24, 48, and 72 h continuous exposure and analyzed by western blot for protein markers of mitotic arrest and apoptosis (Fig. 4). p53 regulation was confirmed by presence of p53 in cells exposed to 15 ng/ml tetracycline and absence in cells exposed to 2000 ng/ml tetracycline (Fig. 4). Probing with antibodies specific for phosphorylation of p53 on serine-15 and -20 (p53-S15-P, p53-S20-P) revealed phosphorylation on serine-15 but not serine-20 in any experimental condition. Serine-15 phosphorylation was observed only in p53(wild-type) cells without arsenite exposure and decreased with time. Arsenite exposure increased serine-15 phosphorylation at all three time points (significant changes of 2.3- and 4.1-fold at 48 and 72 h) in p53(wild-type) cells (Fig. 4). Cyclin-dependent kinase inhibitor p21<sub>CIP1/WAF1</sub> expression appears to be induced by arsenite, correlating with p53 expression and p53-S15-P, and in cells treated with agents capable of inducing necrotic or lytic cell death (Fig. 4).

**Fig. 4.** Western blot analysis of protein markers of mitotic arrest and apoptosis. TR9-7 cells were treated with 0 or 5 μM NaAsO₂ (As) and 15 [p53<sup>−/−</sup>] and 2000 [p53<sup>−/−</sup>] ng/ml tetracycline (Tet) as indicated. Total cellular lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed for p53, p53-S15-P, p53-S20-P, 14-3-3, GADD45α, p21<sub>CIP1/WAF1</sub>, cyclin B, CDC2, CDC2-Y15-P, WEE1, CDC25c, caspase-3, PARP cleavage, and β-actin as indicated from top to bottom, respectively. UV-treated 293 cell lysate was used as a positive control for p53-S20-P antibody, and epidermal growth factor-stimulated A431 cell lysate was used as a positive control for 14-3-3 protein. Representative results of biological triplicates are shown.

Prevention of Mitotic Catastrophe Using Z-VAD-FMK. To determine whether cell death was caspase-dependent mitotic catastrophe, we performed mitotic shake
treatments on both untreated and treated cells followed by mitotic index analysis of the nonadherent cells and adherent cells. We discovered that the mitotic cells and mitotic catastrophe cells were only present in the shaken portion of treated cells (data not shown). The presence of apparent mitotic catastrophe in the...
shaken fraction does not prove mitotic origin because apoptotic cells from other cell cycle phases will lift off of the plate. Therefore, we used Z-VAD-FMK, a broad-spectrum caspase inhibitor, to determine caspase dependence. Z-VAD-FMK treatment near completely removed the appearance of mitotic catastrophe figures (Fig. 5A). We also observed an increase in mitotic index in arsenite-treated cultures treated with Z-VAD-FMK such that the mitotic index approximately equals the mitotic index plus the mitotic catastrophe index in arsenite-treated cultures lacking Z-VAD-FMK (Fig. 5A). The mitotic index of p53(-/-) cells remained significantly higher than in p53(+/+) cells in the presence of both arsenite and Z-VAD-FMK. Western blot analysis (Fig. 5B) showed consistent p53 regulation, p53-S15-P induction by arsenite treated p53(+/+) cells raised the mitotic index to that of arsenite-treated p53(-/-) cells transfected with p21CIP1/WAF1-specific siRNA, NSC siRNA, or mock transfected with Lipofectamine 2000 and treated with arsenite. Western blots of mock-transfected cells appeared identical to western blots results from untransfected cells (see Fig. 4) and are not shown. Tetracycline regulation of p53 was confirmed again. P21CIP1/WAF1 was expressed in p53(-/-) cells transfected with NSC siRNA and was induced strongly by arsenite. P21CIP1/WAF1 knockdown in arsenite-treated p53(-/-) cells transfected with p21CIP1/WAF1-specific siRNA, indicating efficient suppression. Cyclin B was very low in all p53(-/-) cells. Cyclin B was stabilized in arsenite-treated p53(+/+) cells with p21CIP1/WAF1 suppressed by p21CIP1/WAF1-specific siRNA transfection but not in arsenite-treated NSC siRNA-transfected p53(-/-) cells. Cyclin B was stabilized in arsenite-treated p53(-/-) cells regardless of specific siRNA (Fig. 6A).

In general, p21CIP1/WAF1 knockdown increased CDC2 and CDC2-Y15-P. Much like in nontransfected p53(-/-) cells (Fig. 4), arsenite consistently induced prolonged stabilization of CDC2 and CDC2-Y15-P in p53(+/+) cells transfected with p21CIP1/WAF1-specific siRNA (Fig. 6A; 7.4- and 15.5-fold for CDC2 and 6.9- and 4.1-fold for CDC2-Y15-P at 48 and 72 h relative to NSC-siRNA-transfected cells). Like in nontransfected p53(-/-) cells (Fig. 4), arsenite induced CDC2 and CDC2-Y15-P only at 24 h in NSC-siRNA-transfected p53(+/+) cells (Fig. 6A). The effects of siRNA transfection on cyclin B levels were less pronounced but nonetheless similar.

The effect of p21CIP1/WAF1 knockdown by siRNA transfection on arsenite induction of mitotic arrest and mitotic catastrophe was examined (Fig. 6B). P21CIP1/WAF1 knockdown in untreated p53(-/-) cells slightly increased the mitotic index to the level of untreated p53(-/-) cells with no p21CIP1/WAF1 knockdown. P21CIP1/WAF1 knockdown in arsenite-treated p53(-/-) cells raised the mitotic index to that of arsenite-treated p53(-/-) cells. P21CIP1/WAF1 knockdown had no effect on the mitotic index of arsenite-treated p53(-/-) cells. Similar to mitotic index observations, p21CIP1/WAF1 knockdown in arsenite-treated p53(-/-) cells caused an increase in mitotic catastrophe index to the level of mitotic catastrophe in p53(-/-) cells during arsenite treatment. P21CIP1/WAF1 knockdown had no effect on the mitotic catastrophe index in arsenite-treated p53(-/-) cells. Thus, p21CIP1/WAF1 knockdown sensitized cells to arsenite-induced mitotic catastrophe and conferred a p53-null phenotype in p53(-/-) cells as measured by mitotic index and mitotic catastrophe index.

**Discussion**

Treatment of patients based on biological markers of their cancers, rather than type of cancer, will become increasingly common. Thus, understanding molecular mechanisms of sensitivity to cytotoxic drug action is important. Regarding arsenite, understanding p53’s role in arsenite sensitivity may lead to treatments for solid tumors using arsenic trioxide.
Our data show that arsenite induces accumulation of cells in mitosis and that these arrested cells undergo apoptotic death. These events are p53-dependent in that p53-deficient cells are more susceptible to MAAA (a form of mitotic catastrophe; Broker et al., 2005) than are cells expressing p53. We identified and quantified cells undergoing mitotic catastrophe and showed that this cell death is caspase-dependent. Our observation that the mitotic index rises, whereas mitotic catastrophe decreases when caspases are inhibited, indicates that “exit” from arsenite-induced mitotic arrest is via apoptosis.

p53 expression resulted in a significant reduction of apoptotic biochemical markers such as caspase-3 and PARP cleavage in response to arsenite (Fig. 4). Western blot data supported early microscopic observations and viability data (Fig. 2C), indicating that p53−/− TR9-7 cells were sensitive to arsenite-induced apoptosis. Arsenite treatment of TR9-7 cells caused mitotic catastrophe index decrease and an equal mitotic index increase (Fig. 5, A and B). This direct correlation indicated that the mitotic catastrophe figures were mitotic cells undergoing apoptosis, explaining the sub-G2 population in flow cytometry analysis (Fig. 2).

TG-3 is an antibody that recognizes phosphonucleolin, a marker of early mitosis (Vincent et al., 1998). TG-3 staining combined with mitotic index and mitotic catastrophe index determination demonstrated increases in mitotically arrested cells undergoing apoptotic death preferentially in p53−/− cells (Figs. 2B and 3B). These results suggest that escape from arsenite-induced MAAA/mitotic catastrophe is p53-dependent. Other studies in our laboratory following progression from G2 syncy to G2/M, indicated that the p53−/− independent delayed G2 exit but p53-dependent mitotic exit (S. C. McNeely, B. F. Taylor, and J. C. States, unpublished data).

Arsenite activates G2 checkpoint signaling in TR9-7 cells (Fig. 4) as in BEAS-2B (SV40-immortalized human bronchial epithelial) cells (Chen et al., 2002). Checkpoint activation in arsenite-treated U937 cells (McCabe et al., 2000; McCollum et al., 2005) and SV40-immortalized fibroblasts (States et al., 2002) may be the reason these cells accumulate in G2/M as determined by cell cycle distribution analysis using propidium iodide staining. We have shown that TR9-7 cells, like U937 cells (McCabe et al., 2000; McCollum et al., 2005), may suffer delayed G2 transit but arrest in mitosis. In addition to the increase in G2/M cells, an increase in TR9-7 cells with S phase DNA content was observed during prolonged arsenite exposure. It is unlikely that these cells rapidly progressed through G1 into mid-S and then ceased DNA synthesis. More likely, they degenerated from M phase arrest and appeared “exit” from arsenite-induced MAAA, indicating that p53−/− MG cells have been arrested in mitosis. In addition to the increase in G2/M cells, an increase in TR9-7 cells with S phase DNA content was observed during prolonged arsenite exposure. It is unlikely that these cells rapidly progressed through G1 into mid-S and then ceased DNA synthesis. More likely, they degenerated from M phase arrest and appeared as a sub-G2 population.
GADD45

Like others (Chen et al., 2001), we observed p53-independent activating cyclin B/CDC2 complex (Taylor and Stark, 2001). Mitotic cells lack nuclear membranes; thus, p53-dependent loss of CDC2 activity was due to neither 14-3-3 (Fig. 4). Thus, p53-dependent loss of CDC2 activity was due to 14-3-3 binding the CDC2 promoter (Taylor and Stark, 2001). Thus, p53 activation induces GADD45α, 14-3-3σ, and p21CIP1/WAF1 (Taylor and Stark, 2001) to effect G2 arrest by cyclin B/CDC2 inactivation (Fig. 7). GADD45α transcription can be induced by both p53-dependent and -independent mechanisms. GADD45α contributes to G2 arrest by dissociating and inactivating cyclin B/CDC2 complex (Taylor and Stark, 2001). Like others (Chen et al., 2001), we observed p53-independent GADD45α induction by arsenite (Fig. 4). Thus, GADD45α induction is not a p53-dependent mechanism of escape from arsenite-induced mitotic arrest but likely contributes to a p53-independent delay in G2.

14-3-3σ sequesters cyclin B/CDC2 in the cytoplasm of G2 cells, where it is unable to promote entry into mitosis (Taylor and Stark, 2001). Mitotic cells lack nuclear membranes; thus, 14-3-3σ could not sequester cyclin B1/CDC2 but could bind to and activate WEE1. WEE1 phosphorylates CDC2 on threonine-15 and inactivates cyclin B/CDC2 causing G2 arrest (Rothblum-Ovitt et al., 2001). 14-3-3σ was neither detected in TR9-7 cells nor induced by p53 expression or arsenite treatment (Fig. 4). Absence of 14-3-3σ after p53 stimulation by DNA damage was also demonstrated in TR9-7 cells released from mimosine (Taylor et al., 1999). Despite the increase in CDC2 and corresponding CDC2-Y15-P in arsenite-treated cells, our results surprisingly show that WEE1 expression is suppressed by arsenite, independent of p53 (Fig. 4). Thus, p53-dependent loss of CDC2 activity was due to neither 14-3-3σ induction nor increased WEE1-dependent CDC2-Y15 phosphorylation. Arsenite has been shown to suppress CDC25c expression, associated with p53-independent G2 arrest, by enhancing its ubiquitination (Chen et al., 2002). Thus, arsenite-induced CDC2-Y15-phosphorylation may result from decreased CDC25c phosphatase (Fig. 4), although other kinases such as MYT1 could also play a role. Furthermore, CDC2 and CDC2-Y15-P levels were reduced in p53−/− cells relative to p53+− cells in response to arsenite treatment, demonstrating that whatever the mechanism of CDC2 phosphorylation, it was neither dependent on p53 expression nor sufficient to allow mitotic exit.

P21CIP1/WAF1 can inhibit cyclin B/CDC2 by directly binding to p53 (Taylor and Stark, 2001) and/or inhibiting threonine-161 phosphorylation (Taylor and Stark, 2001), required for activity. P21CIP1/WAF1 expression is dependent on p53 expression in TR9-7 cells. P21CIP1/WAF1 levels appeared induced by arsenite and correlated with p53-S15 phosphorylation. siRNA knockdown of p21CIP1/WAF1 in p53−/− cells induced a p53−/− phenotype in response to arsenite as indicated by increased mitotic index, increased mitotic catastrophe (Figs. 3B and 6B), and cyclin B/CDC2 stabilization (Figs. 4 and 6A). These results suggest p21CIP1/WAF1 mediates p53-dependent escape from arsenite-induced mitotic arrest and cell survival through cyclin B/CDC2 inhibition (Fig. 7). Similarly, when arsenite arrested mitotic HeLa cells are treated with staurosporine and 2-amino purine, potent CDK1 (CDC2) and CDK2 inhibitors, rapid degradation of cyclin B occurred, whereas apoptosis was abrogated (Huang et al., 2000). P21CIP1/WAF1 expression also promotes exit from paclitaxel-induced mitotic arrest in MCF-7 cells (Barboule et al., 1997). Again, 2-amino purine allowed escape from paclitaxel-induced mitotic arrest (Chadebec et al., 2000). Other reports noted that retarded mitotic progression (associated with mitotic catastrophe) following G2 DNA damage checkpoint abrogation occurs in p53-null cells lacking p21CIP1/WAF1 expression (Minemoto et al., 2003). Addition of butyrolactone, a potent CDK1 (CDC2) and CDK2 inhibitor, to p53-null cells promoted degradation of cyclin B and prevented mitotic catastrophe as observed in p53−/− cells expressing p21CIP1/WAF1 (Minemoto et al., 2003). Thus, p21CIP1/WAF1 expression or adding cyclin-dependent kinase inhibitors to substitute for p21CIP1/WAF1 activity, releases cells from mitotic arrest, suggesting p21CIP1/WAF1 is a strong p53-dependent candidate for blocking mitotic catastrophe and allowing cells to survive arsenite exposure.

Our siRNA data provide strong evidence for the role of p21CIP1/WAF1 in preventing arsenite-induced MAAA. CDC2 stabilization by p21CIP1/WAF1 knockdown was much more pronounced than effects on cyclin B. Both p53 and p21CIP1/WAF1 inhibit CDC2 transcription, whereas only p53 inhibits cyclin B transcription (Taylor et al., 1999; Taylor and Stark, 2001). However, because there is no p53 consensus binding element in the CDC2 promoter, it is thought that unlike for cyclin B suppression, p53 does not directly bind the CDC2 promoter (Taylor et al., 1999). P21CIP1/WAF1 inhibits CDC2 transcription by interacting with the promoter sequence and factors binding the CDC2 promoter (Taylor and Stark, 2001). Thus, loss of p53 or p21CIP1/WAF1 knockdown results in deregulation of cyclin B and CDC2.

In some systems, arsenite-induced apoptosis and caspase-3 cleavage are dependent on caspase-9 activation in the mitochondrial pathway via oxidative stress (Huang et al., 2000; Chen et al., 2003; Liu et al., 2003). However, activation of this pathway should not limit apoptosis to mitosis. We have not observed caspase-9 or -8 cleavage in TR9-7 cells following arsenite treatment (data not shown). However, the pan-caspase inhibitor, Z-VAD-FMK, eliminated caspase-3 cleavage, suggesting upstream initiator caspase activity is necessary for caspase-3 cleavage and activation.
cleavage occurs in mitotic but not interphase Xenopus embryonic extracts, suggesting a correlation between CDC2 activity and caspase-3 activation (Gu et al., 2003). Ectopic CDC2 kinase expression in mammalian cells also activates caspase-3 and induces apoptosis (Gu et al., 2003). Similarly, we observed correlation between elevated CDC2 and caspase-3 activation in arsenite-treated TR9-7 cells (Fig. 4).

In p53-TR9-7 cells, disappearance of caspase-3 cleavage occurs with disappearance of CDC2 after 24-h arsenite treatment. We interpret the ability of p53-expressing cells to escape mitotic arrest as a result of p53-mediated p21<sup>CIP1/WAF1</sup> induction. P21CIP1/WAF1 inhibition of CDC2 allows not only for mitotic escape but also for reduction in caspase-3 cleavage and increased cell survival. A mechanistic link between CDC2 activity and mitotic caspase-3 cleavage remains to be elucidated. Our results suggest p53 plays an important role in prevention of arsenite-induced mitotic arrest and subsequent mitotic catastrophe. Understanding the link between p53 and prevention of arsenite-induced mitotic catastrophe will provide essential information for the effective use of arsenite and other mitotic disruptors in cancer therapy.

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References


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