G1 Phase Growth Arrest and Induction of p21Waf1/Cip1/Sdi1 in IB3-1 Cells Treated with 4-Sodium Phenylbutyrate

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Accepted for publication May 3, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

4-Sodium phenylbutyrate (4-PBA) has been used for many years in the treatment of urea cycle defects and has recently been studied as a chemotherapeutic agent for certain malignancies. 4-PBA has been shown to cause growth arrest, cellular differentiation, and apoptosis in certain malignant cells. Recently, it was shown that IB3-1 cells (a cystic fibrosis cell line, Δ508/W128X) treated with 4-PBA demonstrated a partial correction of the cystic fibrosis chloride channel defect. We were interested in evaluating the effect of 4-PBA on cell growth and cell cycle regulation in IB3-1 cells. We examined the effect of 4-PBA on cell growth and cell cycle regulation in IB3-1 cells treated with 2 to 10 mM concentrations. We found that cells treated with 2 mM concentrations of 4-PBA for 96 h underwent a significant decrease in cell growth (P < .007). Using flow cytometry, we were able to demonstrate that growth arrest occurred at the G1 phase of the cell cycle. This was detected as early as 24 h in IB3-1 cells treated with 5 mM 4-PBA (P < .03). Furthermore, the percentage of IB3-1 cells with less than a 2N DNA content increased with higher concentrations of 4-PBA, although this was not associated with an increase in apoptosis. Finally, p21Waf1/Cip1/Sdi1 protein levels were induced in IB3-1 cells receiving 2 and 5 mM concentrations of 4-PBA as early as 24 h of exposure, suggesting that G1 phase growth arrest in IB3-1 cells treated with 4-PBA is regulated through the p21Waf1/Cip1/Sdi1 pathway.

4-Sodium phenylbutyrate (4-PBA) has been used success-fully for many years in the treatment of urea cycle defects. 4-PBA binds glutamine and works by promoting an alterna-tive nitrogen excretion pathway in people with urea cycle defects. Recently, 4-PBA has been studied as a drug for the treatment of certain malignancies (Carducci et al., 1996; Gore et al., 1997; Melchior et al., 1999; DiGiuseppe et al., 1999; Yu et al., 1999). 4-PBA has been shown to cause growth arrest, cellular differentiation, and apoptosis in myeloid leukemic cells and prostatic cancers. Studies also have found that 4-PBA can act as a differentiating agent in colon carcinoma cells treated with fluorodeoxyuridine (Huang and Waxman, 1998). Recently, 4-PBA has been evaluated for use as a treatment modality in the disease cystic fibrosis (CF). Progressive lung disease and pancreatic insufficiency are the most common clinical manifestations of CF. CF is characterized by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Collins, 1992). The most common CF mutation is Δ508, a mutation that leads to a trafficking defect in which very little CFTR protein is released from the endoplasmic reticulum (ER) to the plasma membrane (Rubenstein and Zeitlin, 1998b). Recently, IB3-1 cells (a mutant CFTR lung epithelial cell line with the CFTR genotype of Δ508/W1282X) were treated with 2.5 mM concentrations of 4-PBA. IB3-1 cells have the characteristic trafficking defect found in the majority of people with CF. Single-channel recordings of these cells demonstrated that chloride currents consistent with CFTR channel activity were present. This is in contrast to control IB3-1 cells in which no CFTR activity was found (Rubenstein et al., 1997). These findings suggest that 4-PBA may be clinically useful in correcting the channel defect in people with CF. Although the mechanism of action of 4-PBA is not completely understood it is thought that 4-PBA promotes release of mutant CFTR from the ER. Rubenstein et al. (1997) suggested that 4-PBA might be increasing transcription of the Δ508 CFTR, resulting in more CFTR protein escaping ubiquination and being released from the ER to the plasma membrane, where it can act as a channel. We were interested in evaluating the effect of 4-PBA on cell growth and cell cycle regulation in IB3-1 cells. We examined the effect of 4-PBA on IB3-1 cells treated with 2 to 10 mM concentrations. We found that cells treated with 2 mM concentrations of 4-PBA for 96 h underwent a significant decrease in cell growth. Higher concentration of 4-PBA resulted in more pronounced growth inhibition at earlier time points. Using flow cytometry, we found a significant increase in the number of cells in the G1 phase by 24 h in IB3-1 cells treated with 5 mM 4-PBA. By

ABBREVIATIONS: 4-PBA, 4-sodium phenylbutyrate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; 2N, normal diploid.
72 h, a significant increase in the percentage of cells in G1 was present in cells receiving 2 mM concentrations of 4-PBA. The percentage of IB3-1 cells with less than a normal diploid (2N) DNA content (a marker of apoptosis) increased with higher concentrations of 4-PBA. A detectable increase in apoptosis by TUNEL assay, however, was not found. Finally, we found that p21<sup>Waf1/Cip1/Sdi1</sup> is involved in the G<sub>I</sub>/S and G<sub>S</sub>/M arrest in the cell cycle (O’Connor, 1997; Barboule et al., 1999), was induced at 24, 48, and 72 h in IB3-1 cells receiving 2 and 5 mM concentrations of 4-PBA. This suggests that p21<sup>Waf1/Cip1/Sdi1</sup> is involved in the G<sub>I</sub> phase growth arrest that occurred in IB3-1 cells treated with 4-PBA.

Materials and Methods

Cell Lines. IB3-1 cells (received from M. Egan, Department of Pediatrics, Yale University School of Medicine, New Haven, CT) were grown in LHC-8 media (Biofluids Inc., Rockville, MD), 5% fetal calf serum (Life Technologies, Gaithersburg, MD), penicillin/streptomycin (100 U/ml), fungizone (2.5 mg/ml), tobramycin (80 mg/ml), and imipenem (0.2 mg/ml) at 37°C in room air and 5% CO<sub>2</sub>. An equal number of cells was added to 25-cm<sup>2</sup> flasks. The following day cells were divided into four groups. 4-PBA (generous gift from S. Brusilow, Department of Pediatrics, Johns Hopkins Medical Center) was added at 2, 5, or 10 mM concentrations to cells in culture. Control cells received no 4-PBA. Filtered 4-PBA was added from a 1 M solution of 4-PBA dissolved in sterile water. Cell counting was performed at 24, 48, 72, and 96 h. IB3-1 cells were then grown for 72 h in the presence of either 2, 5, or 10 mM or no 4-PBA. Cells were trypsinized and 100,000 cells from each group were plated in medium without 4-PBA. Cells from each group were counted at 24, 72, and 120 h.

HCT116 cells (p21<sup>Waf1/Cip1/Sdi1</sup> and p21<sup>−/−</sup>) (generous gift from B. Vogelstein, Howard Hughes Medical Institute and Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine) were grown in McCoy’s 5A medium, 10% fetal calf serum, and penicillin/streptomycin (100 U/ml) and plated as described above. Cells were treated with 5 mM concentrations of 4-PBA for 24 h and analyzed by flow cytometry.

Detection of Apoptosis. IB3-1 cells were grown on vitrogen-coated glass coverslips. 4-PBA was added at 5 mM concentrations for 48 h. IB3-1 cells not treated with 4-PBA were used as controls. Apoptosis was determined by in situ labeling with terminal deoxynucleotidyl transferase (in situ cell death detection kit, fluorescein, no. 1 684 795; Boehringer Mannheim, Indianapolis, IN). Negative controls were performed with no enzyme. The 4-PBA-treated cells also were compared with IB3-1 cells not treated with 4-PBA.

Further evaluation for apoptosis was pursued with flow cytometry. Untreated and cells treated with 4-PBA were labeled with propidium iodide and annexin V-fluorescein isothiocyanate (FITC) and evaluated for induction of apoptosis (TACS annexin V-FITC, no. TA4638; R&D Systems, Minneapolis, MN). Assays for caspase 3 induction also were performed on IB3-1 cells receiving no 4-PBA, or 2, 5, or 10 mM 4-PBA for 48 h with ApoAlert caspase-3 calorimetric assay kit (no. K2027-1; Clontech, Palo Alto, CA) as per manufacturer’s instructions.

Western Blot Analysis. Whole cell lysates were solubilized in 2% SDS. Protein concentrations were determined with Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Lysates were loaded and run on a 12% SDS-polyacrylamide gel. Each protein gel contained equal amounts of protein per lane (either 15 or 25 µg/lane). Gels were then transferred to nitrocellulose and Western blot analysis was performed with either an anti-p21<sup>Waf1/Cip1/Sdi1</sup> monoclonal antibody (65961A) or an anti-p53 monoclonal antibody (14211A) (Pharmingen, San Diego, CA) at a concentration of 2 µg/ml as recommended in 5% Blotto/PBS and 0.05% Tween 20 overnight at 4°C. The blots were then washed three times in PBS-Tween, incubated with a mouse Ig, horseradish peroxidase-linked whole antibody (RPN 2108; Amersham, Arlington Heights, IL) for 1 h at a 1:500 dilution, and then washed and developed with enhanced chemiluminescence (RPN 2106; Amersham).

Flow Cytometry. IB3-1 and HCT116 cells were stained by incubation in PBS containing 0.025% Nonidet P-40, propidium iodide (100 µg/ml), and ribonuclease (10 µg/ml) on ice for 30 min. Cells were counted with an FACSScan (Becton Dickinson) and analyzed with the program Mod Fit LT (Verity, Topsham, ME). To evaluate checkpoint regulation, nocodazole, a mitotic inhibitor, was added (0.4 µg/ml) for 16 h to IB3-1 cells treated with 5 mM 4-PBA for 24 h.

Statistical Analysis. Statistical calculations were performed with the SPSS 8.0 statistical package for Windows (SPSS Inc., Chicago, IL). Differences in measured variables between experimental and control groups were made through comparison of the means by a one-way ANOVA. Statistical difference was accepted at P < .05.

Results

Cell Growth and Morphology in IB3-1 Cells Treated with 4-PBA. IB3-1 cells during exponential cell growth were exposed to 2, 5, and 10 mM concentrations of 4-PBA. 4-PBA had previously been shown to be cytostatic to certain malignant cells and we were interested in determining its effect on cell growth in IB3-1 cells. Treatment of IB3-1 cells with 5 and 10 mM concentrations of 4-PBA resulted in distinct changes in cell morphology as early as 24 h of treatment. The cells grew slowly with long spindle connections between adjacent cells (Fig. 1). These changes were notable at 24, 48, and 72 h of 4-PBA exposure. There was, however, little histological difference between control and IB3-1 cells exposed to 2 mM PBA at these time points. Cells were then counted. At 48 h, the number of cells in the 10 mM 4-PBA-treated group was significantly less than the number of cells in the 2 mM treated and control groups (P < .01 and P < .02, respectively). The IB3-1 cells in the 10 mM treated groups did not grow significantly after receiving 4-PBA. Furthermore, the number of viable cells in this group dropped progressively the longer the exposure to 4-PBA. Cells in the 5 mM treated group continued to grow, but did so at a markedly decreased rate compared with control IB3-1 cells. This difference was significant by 72 h (P < .03). By 96 h, the number of cells in the 2 mM treated group was significantly less than the number of cells in the control group (P < .007; Fig. 2). Cell survival after treatment with 4-PBA was then assessed. Cells were treated with 2, 5, and 10 mM 4-PBA for 72 h. Control cells received no 4-PBA. Cells were trypsinized and 100,000 cells/flask were seeded in 4-PBA-free medium. Cells were counted at 24, 72, and 120 h. At 72 h, a significant decrease in cell number was found in cells previously treated with 10 mM 4-PBA compared with control cells (Fig. 3). By 120 h, however, no significant difference in cell growth between the four groups was found, although there was borderline significance between the 10 mM treated group and the 5 mM treated group (P < .056). These findings suggest that IB3-1 cells are still viable after treatment with 4-PBA and are able to reenter the cell cycle when 4-PBA is removed.

G<sub>1</sub> Phase Growth Arrest in IB3-1 Cells Treated with 4-PBA. Flow cytometry was used to identify 4-PBA-induced changes in the cell cycle of IB3-1 cells (Fig. 4). At 24 h, cells treated with 5 mM concentrations of 4-PBA had a significantly greater percentage of cells in the G<sub>1</sub> phase (P < .03).
compared with control cells). The 5 mM treated group also had a significantly lower percentage of cells in G2/M phase (P < .01). These changes are consistent with growth arrest at the G1 phase of the cell cycle (Fig. 5). At 48 h, cells in the 10 mM 4-PBA-treated group had a significant increase in the G1 phase compared with control cells (P < .01). By 72 h, a significant increase in the percentage of cells in G1 phase was found in both the 2 and 5 mM 4-PBA-treated groups compared with control cells (P < .03 and P < .003, respectively). At 72 h, the 10 mM treated group had a significant drop in the percentage of cells in G1 phase, suggesting significant cell dysfunction from drug toxicity. Next, nocodazole, a mitotic inhibitor, was added to IB3-1 cells treated with 5 mM 4-PBA to help determine whether 4-PBA blocks cells at the G1/S checkpoint. In untreated IB3-1 cells, the cells underwent G2/M arrest when nocodazole was added. In cells treated with 5 mM 4-PBA, however, two populations of cells persisted, one population at G1/S and the other at G2/M (Fig. 6). This suggests that 5 mM 4-PBA is able to partially induce G1/S arrest in IB3-1 cells, however, not completely because a second population of cells is also present at G2/M.

**Apoptosis in IB3-1 Cells Receiving 4-PBA.** Next, we wanted to determine whether 4-PBA was an inducer of apoptosis in IB3-1 cells. 4-PBA had previously been shown to induce apoptosis in malignant cells obtained from the bone marrow of patients with myeloid leukemia, myeloid leukemia cells, and prostate cancer cells (Carducci et al., 1996; Gore et al., 1997; DiGiuseppe et al., 1999). We found by flow cytometry that the number of cells with less than a 2N DNA content was significantly increased in IB3-1 cells treated with 5 and 10 mM 4-PBA for 48 h (P < .007 and P < .001, respectively). TUNEL assays were then performed on control cells and IB3-1 cells treated with 5 mM 4-PBA for 48 h (n = 3). Differences in the number of cells undergoing apoptosis were not detectable between control and 5 mM 4-PBA-treated cells (Fig. 7). Furthermore only a slight increase in the percentage of cells undergoing apoptosis at 72 h was found in annexin V-FITC-labeled IB3-1 cells receiving 2, 5, or 10 mM 4-PBA (data not shown). Caspase 3 induction studies also were performed in control and 4-PBA-treated cells and no significant difference between control and treated cells was found at 48 h (data not shown). Therefore, our findings suggest that 4-PBA in concentrations up to 5 mM does not induce apoptosis in IB3-1 cells treated for up to 72 h, despite a significant increase in the percentage of cells with less than 2N DNA content by flow cytometry. This suggests that cells are undergoing a necrotic and not an apoptotic cell death.

**p21Waf1/Cip1/Sdi1 Induction in Growth-Arrested IB3-1 Cells Treated with 4-PBA.** Different environmental stresses such as genomic damage, hypoxia, infection, and hyperoxia can induce growth arrest in the cell (Jacks and Weinberg, 1996; McGrath, 1998). The tumor suppressor gene p53 has been shown to mediate cellular growth arrest during many of these stresses. p53 acts as a G1/S checkpoint in the cell cycle. It has a critical role in maintaining the integrity of the genome after cellular damage. p21Waf1/Cip1/Sdi1 has been shown to act downstream of p53 during certain conditions of stress that induce G1 phase growth arrest (el-Deiry et al., 1993). p21Waf1/Cip1/Sdi1 is a cyclin-dependent kinase inhibitor that can complex with a variety of cyclins, resulting in cell cycle arrest. We were interested in determining whether p53 and p21Waf1/Cip1/Sdi1 were involved in the G1 phase growth arrest that was induced in IB3-1 cells receiving 4-PBA. We found that p21Waf1/Cip1/Sdi1 protein levels were induced in cells treated for 24 h with 2 and 5 mM concentrations of 4-PBA. Protein levels of p21Waf1/Cip1/Sdi1 also were increased after 48 and 72 h of 4-PBA treatment (Fig. 8). We were, however, unable to detect changes in p53 protein levels between control, and 2 and 5 mM treated IB3-1 cells at 24, 48, and 72 h (data not shown).

Flow cytometry was then used to help elucidate the role of p21Waf1/Cip1/Sdi1 in G1/S checkpoint regulation in cells treated with 4-PBA. We used HCT116 cells (a human colon carcinoma cell line) for these studies. One cell line contained wild type (p21+/+) and the other null (p21−/−). Figure 9 shows the cell cycle profile of both cell lines when treated with 5 mM 4-PBA for 24 h. The cell cycle profile is different between the two groups of cells. HCT116 (p21−/−) cells have a larger subpopulation of cells at G1/M, whereas HCT116 (p21+/+) have a larger subpopulation of cells at G1/S. This demonstrates that p21Waf1/Cip1/Sdi1 null cells treated with 4-PBA do not undergo significant G1/S arrest. This suggests that p21Waf1/Cip1/Sdi1 does appear to influence G1/S checkpoint regulation in cells treated with 4-PBA.
Discussion

4-PBA is an aromatic fatty acid that has been used for many years as a scavenger of glutamine in urea cycle defects. Recently, 4-PBA has been shown to partially correct CFTR channel activity in IB3-1 cells through an undefined mechanism. 4-PBA also has been shown to induce growth arrest, apoptosis, and cellular differentiation in certain neoplastic cells. In this study we have investigated the effect of 4-PBA on cell growth and cell cycle regulation on IB3-1 cells, a lung epithelial cell line with a Δ508/W1282X CF mutation. We found that 4-PBA inhibited cell growth in IB3-1 cells receiving between 2 and 10 mM concentrations for up to 96 h and that growth inhibition was associated with an increase in the G1 phase of the cell cycle. We also found that growth arrest induced by 4-PBA may be through the p21Waf1/Cip1/Sdi1 pathway in IB3-1 cells.

IB3-1 cells treated with 2 mM concentrations of 4-PBA appeared histologically normal. Cell numbers by 96 h of continuous exposure, however, were significantly decreased compared with control cells. This suggests that long-term treatment with 4-PBA could potentially effect cell growth in vivo. This could be problematic particularly in organs of high tissue turnover such as the intestines. Our studies were performed in cells in culture and may not truly reflect what is happening in vivo. 4-PBA concentrations of 2 mM have been measured in the plasma of people taking 600 mg/kg/day 4-PBA for urea cycle defects, suggesting that if 4-PBA is having an effect on cell growth it may not be clinically significant (Brusilow and Maestri, 1996). Higher plasma levels of 4-PBA, however, may be necessary to achieve a therapeutic response in people with CF. Rubenstein et al. (1997) reported that IB3-1 cells had a significant increase in CFTR channel activity when exposed to 2.5 mM concentrations of 4-PBA (Rubenstein et al., 1997). In another study Rubenstein and Zeitlin (1998a) found that administration of 19 g/day 4-PBA over 1 week to people with CF resulted in a significant change in nasal potential differences toward a more normal potential difference pattern. Further clinical studies will be needed to determine the optimal dose of 4-PBA that is necessary to achieve a clinical response in people with CF. We found that the higher the concentration of 4-PBA used on IB3-1 cells, the more profound the effect on cell growth. The 10 mM concentrations of 4-PBA were very toxic to IB3-1 cells, resulting in essentially no cell growth and progressive cell loss with longer exposure times. We were able to show, how-
ever, that IB3-1 cells that survived treatment with 5 and 10 mM concentrations of 4-PBA for 72 h were able to recover and reenter the cell cycle.

The effect of 4-PBA on the cell cycle of IB3-1 cells appears to be primarily targeted at the G1 phase of the cell cycle. At 2 mM 4-PBA concentrations the percentage of cells in G1 was...
significantly increased after 72 h and the percentage of cells in G$_2$/M was significantly decreased. Although the increase in G$_1$ phase is modest in IB3-1 cells treated with 4-PBA, it is consistent with a G$_1$ phase growth arrest. This is not unique to IB3-1 cells. 4-PBA has previously been shown to cause G$_1$ phase arrest in leukemic cells (DiGiuseppe et al., 1999). 4-PBA-induced G$_1$ phase growth arrest may not entirely explain the striking decrease in cell growth found in cells treated with 5 and 10 mM 4-PBA. Our results suggest that many cells may be dying a necrotic cell death without undergoing a clear-cut G$_1$ arrest. We also found, by flow cytometry, an increase in the percentage of cells with less than a 2N DNA content in IB3-1 cells treated for longer time periods and with higher concentrations of 4-PBA. Cells with less than a 2N DNA content may reflect cells that are undergoing apoptosis. By TUNEL assay and caspase 3 assays, however, we did not find an increase in apoptosis in cells treated with 5 mM 4-PBA for 48 h compared with control cells. Other studies have reported significant increases in apoptosis in malignant cells treated with 4-PBA (Gore et al., 1997; DiGiuseppe et al., 1999). This may reflect an increase sensitivity of these particular malignant cells to 4-PBA. Alternatively, longer exposure periods to 4-PBA may be necessary before significant increases in apoptosis are found in IB3-1 cells. Furthermore, it is possible that IB3-1 cells treated with 4-PBA are dying through a nonapoptotic process.

We found that the cyclin-dependent kinase inhibitor p21$^{Waf/Cip1}$ was increased early in IB3-1 cells treated with 4-PBA, suggesting that the mechanism of growth arrest is mediated through a p21$^{Waf/Cip1}$ pathway. In 2 and 5 mM 4-PBA-treated cells, an increase in p21$^{Waf/Cip1}$ protein levels was found by Western blot analysis at 24 h. This
increase in protein levels occurred before the detection of G1 phase growth arrest by flow cytometry at 72 h and before the decrease in cell growth at 96 h. Our findings support the results of another study that reported an induction of p21<sup>Waf1/Cip1/Sdi1</sup> in myeloid leukemia cells treated with phenylbutyrate (DiGiuseppe et al., 1999). p21<sup>Waf1/Cip1/Sdi1</sup>-induced G1 phase growth arrest has previously been shown to be mediated through a p53 pathway (El-Deiry et al., 1993; Waldman et al., 1995; O'Connor, 1997). We were, however, unable to detect a change in p53 protein levels in cells treated with 4-PBA at 24, 48, and 72 h compared with control cells. This does not rule out the possibility that growth arrest induced by 4-PBA is p53 mediated because induction of p53 protein may be transient. Alternatively, we may have missed an earlier time point in which p53 protein was induced.

The mechanism by which 4-PBA acts on the cell is not entirely clear. Studies have shown that 4-PBA up-regulates the expression of many genes, including γ-globin, peroxisomal protein adrenoleukodystrophy protein, and genes that code for human leukocyte antigen class I (Dover, 1998; Kemp et al., 1998; Bar-Ner et al., 1999). 4-PBA can increase the production of fetal hemoglobin through the transcriptional activation of the γ-globin gene (Hudgins et al., 1996; Dover, 1998). 4-PBA, like butyrate, also may be a transcriptional regulator of CFTR in IB3-1 cells. As suggested by Rubenstein et al. (1997), partial correction of CFTR chloride function in CF cells treated with 4-PBA may be the result of increase transcription of CFTR, resulting in more mutated CFTR escaping ubiquination in the cytoplasm and being released to the plasma membrane. In this study we found that 4-PBA induces the protein levels of p21<sup>Waf1/Cip1/Sdi1</sup> in association with IB3-1 cell growth inhibition. The induction of p21<sup>Waf1/Cip1/Sdi1</sup> in IB3-1 cells treated with 4-PBA appears to be just one of many effects that 4-PBA has on cell function. The induction of p21<sup>Waf1/Cip1/Sdi1</sup> may be regulated through p53, however evidence of p53 induction was not found in this study.

In summary, we have shown that 4-PBA induces growth inhibition in IB3-1 cells at 2 mM concentrations and greater with evidence of growth arrest at the G1 phase of the cell cycle. Furthermore, the mechanism of growth arrest in IB3-1 cells treated with 4-PBA may be through p21<sup>Waf1/Cip1/Sdi1</sup> mediated inhibition of cyclin-dependent kinases.

**Acknowledgments**

We thank Drs. William Guggino, Marie Egan, Pamela Zeitlin, and Ron Rubenstein for useful discussions.

**References**


**Fig. 9.** Effect of 4-PBA on HCT116 (human colon carcinoma cell lines). Cells were treated with 5 mM 4-PBA for 24 h. p21 null cells (−/−) showed an increase in G2/M during treatment with 4-PBA compared with wild-type p21 cells.