

Aspirin inhibits camptothecin-induced p21^{CIP1} levels and potentiates apoptosis in human breast cancer cells

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Abstract. The ability of aspirin to trigger apoptosis in cancer cells is well known and is consistent with the clinical and epidemiological evidence on its chemopreventive effects in curtailing epithelial cancers, including breast cancer. We hypothesized that the anticancer effects of aspirin may involve acetylation of the tumor suppressor protein p53, a known regulator of apoptosis. In the present study, we determined if aspirin at the physiologically achievable concentration of 100 μ M acetylates p53 and modulates the expression of p21^{CIP1}, a protein involved in cell cycle arrest, and Bax, a pro-apoptotic protein. Using MDA-MB-231 human breast cancer cells, we demonstrate that aspirin at 100 μ M concentration markedly acetylated the p53 protein, which was primarily localized to the nucleus. Aspirin induced p21^{CIP1} protein levels in a transient fashion in contrast to the sustained induction of Bax. The induction of p21^{CIP1} protein levels began at 3 h and was maximal at 6-8 h; however, it decreased to control levels by 30 h. In contrast, the anticancer drug, camptothecin (CPT) induced a steady accumulation of p21^{CIP1} protein. Remarkably, when cells were co-treated with aspirin and CPT, p21^{CIP1} levels were drastically downregulated, and this phenomenon was observed in many cancer cell lines. Incubation of recombinant p21 with cytoplasmic extracts from aspirin-treated cells caused its degradation suggesting the involvement of proteases in the disappearance of p21^{CIP1}. Consistent with this data, aspirin decreased the survival of CPT-treated cells and greatly increased the extent of apoptosis. Our observation that aspirin has the ability to inhibit p21^{CIP1} after its initial induction has important implications in chemotherapy, and suggests its potential use to increase the efficacy of anticancer agents.

Introduction

Aspirin (acetylsalicylic acid) and other non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of minor injuries and headaches, degenerative joint diseases such as rheumatoid arthritis, and prophylaxis against cardiovascular diseases (1,2). NSAIDs inhibit the activity of cyclooxygenases (COX) leading to the inhibition of synthesis of prostaglandins (PGs) that cause inflammation, swelling, pain and fever (3,4). However, increasing evidence from human epidemiological studies, animal models, and *in vitro* experiments suggest that aspirin and other NSAIDs may prevent the occurrence of cancers of epithelial origin (5-9). Prolonged intake of NSAIDs has been shown to reduce the risk of cancer of the colon, breast, prostate, lung and skin (10-13). A comprehensive report compiled from 91 epidemiological studies shows a significant exponential decline in the risk for 7-10 malignancies with increasing intake of NSAIDs (12). Daily intake of NSAIDs, primarily aspirin, produced risk reductions of 63% for colon, 39% for breast, 36% for lung and 39% for prostate cancer. Significant risk reductions were also observed for esophageal (73%), stomach (62%) and ovarian cancer (47%) (12). These chemopreventive effects were apparent after 5 or more years of NSAID use and were stronger with longer duration. These observations have collectively initiated a wide variety of investigations to determine the mechanisms by which aspirin and other NSAIDs reduce the risk or progression of cancers.

The cellular pathways through which NSAIDs exert their anticancer effects are not well understood; however, they have been shown experimentally to induce apoptosis (6-9) and to inhibit angiogenesis (14,15), two important mechanisms that help to suppress tumor growth (5). Studies in literature suggest that in gastric, endometrial, and colorectal cancer cells, aspirin induces upregulation of mitochondrial outer membrane pro-apoptotic proteins such as Bax and Bak and downregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xl (16-19). In Jurkat, Raji, HL-60, HeLa, neuro 2 and B-chronic lymphocytic leukemia cells, aspirin increases mitochondrial membrane permeability and release of cytochrome c leading to the activation of caspases and cell apoptosis (20-24). One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduced PG synthesis through acetylation and inhibition of COX (3,4,25-27). On the contrary, NSAIDs

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have growth inhibitory effects against cancer cell lines that do not express COX-1 or -2 (28), and against mouse embryo fibroblasts that are null for both enzymes (29). These observations suggest that COX-independent pathways may also contribute to the anticancer effects of NSAIDs.

Aspirin causes inhibition of COX-1 and -2 by irreversible acetylation of serine residues (3,4,26,27); however, it is also known to acetylate lysine residues in serum proteins (30). Although the effects of aspirin on COX have been well-studied, little is known if it induces acetylation of cellular proteins, particularly those that regulate apoptosis, which may also contribute to its anticancer effects. We hypothesized that the ability of aspirin to induce apoptosis may involve acetylation of the tumor suppressor protein, p53, leading to modulation of its target genes, p21^{CIP1}, a protein involved in cell cycle arrest (31), and Bax, a mitochondrial proapoptotic protein (32-35).

Herein, we demonstrate that aspirin acetylates p53 in a dose- and time-dependent manner in MDA-MB-231 breast cancer cells. We also observed induction of the expression of p21^{CIP1} and Bax protein levels; however, induction of p21^{CIP1} was transient whereas that of Bax was sustained. Aspirin appears to exert a dual effect on p21^{CIP1} expression. This involves an initial induction phase which occurred within the first 8 h of treatment followed by an inhibitory phase that later decreased p21^{CIP1} protein levels. In contrast, the anticancer drug, camptothecin (CPT) caused a sustained increase in the p21^{CIP1} protein levels. Co-treatment of cells with aspirin and CPT caused a decrease in CPT-induced p21^{CIP1} protein levels after 8 h of exposure suggesting that aspirin activates an inhibitory pathway that causes degradation of p21^{CIP1}. Importantly, inhibition of p21^{CIP1} by aspirin potentiated the CPT-induced apoptosis. These results suggest that aspirin has the ability to enhance the action of the anticancer drug, CPT and therefore, it may prove beneficial in chemotherapy.

Materials and methods

Materials. Cell culture reagents were purchased from Invitrogen. Aspirin and salicylic acid were obtained from Sigma. Camptothecin was purchased from Calbiochem. Anti- β -actin antibody and anti-Bax antibodies were obtained from Cell Signaling Technology Inc. Anti-acetyl p53 antibody was purchased from Cell Signaling Technology Inc. or Biolegend Inc. Anti-p21 antibody was purchased from Santa Cruz Biotechnology; anti-p53 monoclonal antibody was obtained from Zymed Inc; goat anti-rabbit IgG and goat anti-mouse IgG were from Bio-Rad; protease inhibitor cocktail tablets were obtained from Roche; p21 full length cDNA was obtained from Origene Inc.; all other chemicals were either from Sigma or Fisher.

Cell culture. MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (ATCC) and maintained at 37°C in Leibovitz's L-15 medium containing 10% fetal bovine serum. Cells were grown for 12 to 24 h before the addition of aspirin or CPT for indicated times.

Cell fractionation and isolation of nuclei. All cell fractionation studies were carried out at 4°C as previously described (36).

Cells were harvested by scrapping into ice-cold phosphate-buffered saline (PBS), washed once with ice-cold PBS, resuspended in 0.8 ml of hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1X protease inhibitors (Roche), 1 mM sodium orthovanadate and 1 mM NaF] per cell pellet derived from two 100-mm petri plates. Using a loose fitting Dounce homogenizer, cells were gently broken into cytoplasmic and nuclear fractions as described earlier (36). Nuclei were removed from cell homogenate by low-speed centrifugation (1000 rpm for 4 min) in an Eppendorf centrifuge and extracted with high salt buffer (10 mM Tris-Cl, pH 7.4, 400 mM NaCl, 15% glycerol) for 30 min at 4°C and then centrifuged at 15,000 x g for 10 min. The supernatant represented nuclear extract, which was used for Western blot analysis and electrophoretic mobility shift assays. In some experiments protease inhibitors were omitted for the isolation of the cytoplasmic fraction.

Western blots. Cells were treated with various agents for the indicated times and washed with PBS. Cells were scraped in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton-X-100, 1 mM sodium orthovanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM NaF, protease inhibitor cocktail and 1 mM PMSF). Equal amounts of proteins were resolved by an 8 or 12% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose or PVDF membranes and incubated with their respective primary antibodies. Immunoreactive bands were visualized using chemiluminescence Western blotting system according to the manufacturer's instructions (Pierce). The densitometric intensity of each protein band on Western blots was quantified using the Bio-Rad Quantity One (version 4.5.2) software (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoretic mobility shift assay. Nuclear extracts were prepared from cells untreated or treated with aspirin or CPT or both. Electrophoretic mobility shift assays were performed as described previously (37,38), with slight modifications. Nuclear extract (5 μ g) were incubated with 1 μ g of poly(dI-dC)-poly(dI-dC), with or without competitor oligonucleotide in 20 μ l of 10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 10% glycerol for 20 min at 25°C. The samples were incubated with 1-2 fmol of ³²P-labeled p53 consensus DNA binding sequence (5'GGACATGCCCGGGCATGTCC-3') (~5000 cpm) for 10 min at 25°C. The DNA protein complex was analyzed on a 5% native polyacrylamide gel electrophoresis containing 0.5X Tris borate EDTA (TBE) buffer (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA). The gels were run at 200 V in a cold room (4-8°C) for 2 h in 0.5X TBE buffer, dried and exposed to X-ray film for 12-24 h.

Northern blots. Total RNA was prepared from cells untreated or treated with the drugs for the indicated times using TRIzol reagent (Invitrogen). Total RNA (20 μ g) was analyzed on 1.2% agarose-formaldehyde gels, transferred to nylon membranes and hybridized with ³²P-labeled p21 cDNA as previously described (39). The blots were washed with 0.2X SSC containing 0.1% SDS for 1 h at 65°C. The blots were dried and exposed to X-ray film.

Immunofluorescence studies. Cells were grown on glass coverslips (70% confluency) and left untreated or treated with various drugs. Following treatment for the indicated times, cells were fixed in 4% paraformaldehyde and incubated at 4°C with primary anti-acetyl p53 antibody (Biolegend Inc.) or anti-p21 antibody overnight, followed by a 1 h incubation with Alexa Fluor 488 secondary antibodies. Images of cells (x20) were acquired using a Zeiss Axiophot microscope (Oberkochen, Germany).

Expression of recombinant GST-tagged p21 protein. The full length cDNA of human p21^{CIP1} cloned in pGEX-2T expression vector (GST-p21) (40) was kindly provided by Dr Alan Tomkinson (University of Maryland, Baltimore, MD, USA). *Escherichia coli* BL21 (DE3, pLys) competent cells were transformed with the GST-p21 plasmid, cultured in L-broth supplemented with 60 µg/ml ampicillin, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 5 h at 37°C. The fusion protein present in the soluble fraction of the bacterial lysate was purified using GSH-agarose by standard procedures (41). The bound protein eluted with 10 mM GSH was largely homogeneous showing a major band of 43 kDa on SDS-PAGE.

Cell viability assay. Cytotoxicity of aspirin and CPT were measured by using MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl tetrazolium bromide) assay as previously described (42). Cells were plated onto 24-well plates in 400 µl of Leibovitz's L-15 medium containing 10% fetal bovine serum, at a density of 3.5x10⁴ cells per well. Twenty-four hours after seeding, cells were left untreated or treated with CPT alone (12.5, 25, 50, 75, 100, 125, 150 and 200 nM) in triplicate, or in combination with 100 µM aspirin. The total treatment time with the drugs was 72 h. The medium was aspirated from the wells, washed twice with PBS, 160 µl of MTT (2 mg/ml) added, and incubated for 4 h at 37°C. Thereafter, 800 µl dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals and the plates were incubated for 2 h at room temperature in a dark place. The absorbance was read at 570 nM using SpectraFlour Plus plate reader (Tecan, Inc., Santa Margarita, CA).

Flow cytometry. Cells were grown in 100-mm Petri dishes, left untreated or treated with aspirin alone, or CPT alone, or in combination with both drugs for 48 h. Cells were assayed for apoptosis using an annexin V-FITC apoptosis kit (Calbiochem) according to manufacturer's instructions. Analysis was conducted for 10,000 cells in a flow cytometer with CellQuest software (Becton-Dickinson, Rutherford, NJ) using FL1 and FL2 ranges for annexin V FITC and PI, respectively.

Clonogenic cell survival assay. Clonogenic assays were performed as previously described (43,44). Cells were grown in 100-mm plates up to 70% confluency, and left untreated or treated with aspirin alone, or CPT alone or aspirin and CPT for 72 h. The floating dead cells and the trypsinized adherent cells were collected and counted. The cells were then plated in triplicate into 100-mm plates at a density of 500 cells per plate to ensure the formation of colonies and allowed to grow for 15 days in normal media containing 10% FBS. The

cells were washed in PBS and fixed in 70% ethanol prepared in cold PBS, followed by staining for 30 min with 0.5% methylene blue prepared in 50% ethanol. The cells were washed in distilled water to remove the excess stain and the stained colonies were counted.

Statistical analysis. All experiments were performed 3-4 times independent of each other. Student's t-test or one-way ANOVA followed by Newman-Keuls multiple comparison test were adopted to compare group differences and significance was defined as P<0.05.

Results

Aspirin acetylates the p53 tumor suppressor protein. Acetylation of p53 has been shown to activate its sequence-specific DNA binding activity, and consequently increases the activation of its target genes (34,35,45). We determined if aspirin would induce its acetylation using the commercially available anti-acetyl p53 antibody, specific for the recognition of acetylation of lysine residue at position 382 (K-382). For our experiments, we used aspirin (acetylsalicylic acid) at a concentration of 100 µM, as this concentration has been demonstrated to be physiologically achievable on administration of an oral dose of aspirin (46). Therefore, MDA-MB-231 cells were left untreated or treated with aspirin for 24 h. As a negative control, cells were also treated with salicylic acid (100 µM) for the same time period. Cell lysates were prepared and immunoblotted with anti-acetyl-p53 antibody. Fig. 1A shows that as compared to the untreated control, aspirin significantly acetylated the p53 protein. However, the negative control, salicylic acid failed to induce this modification, suggesting that the acetyl group of aspirin is required for this reaction. To demonstrate equal loading, the samples representing Fig. 1A were immunoblotted with anti-p53 antibody (Fig. 1B). These samples contained similar levels of β-actin protein (Fig. 1C).

To establish if aspirin-induced acetylation of p53 was concentration-dependent, cells were treated with different concentrations of aspirin (25 µM to 2.5 mM), lysates prepared and immunoblotted with anti-acetyl p53 antibody. Fig. 1D demonstrates that aspirin induced acetylation of p53 at all concentrations tested with greater acetylation detected at higher concentrations. Interestingly, when the same blot was stripped and reprobed with anti-p53 antibody, we observed increased amount of p53 protein at lower doses up to 500 µM (Fig. 1E). However, at concentrations >500 µM, the amount of p53 detected was similar to the control levels or lower. This possibly reflects the stabilization of p53 reported to occur following acetylation of the protein (34). Additionally, we observed that the β-actin levels remained similar in all lanes (Fig. 1F) irrespective of the concentration of aspirin (loading control). This further suggests that acetylation of p53 at lower concentrations of aspirin (25-500 µM) stabilizes the protein.

To determine the time course of acetylation of p53, cells were left untreated or treated with 100 µM aspirin, lysates prepared at different time points and immunoblotted with anti-acetyl p53 (K-382) antibody. Fig. 1G demonstrates that acetylated p53 is detected as early as 3 h and increasing levels were observed at later time points such as 6-24 h.

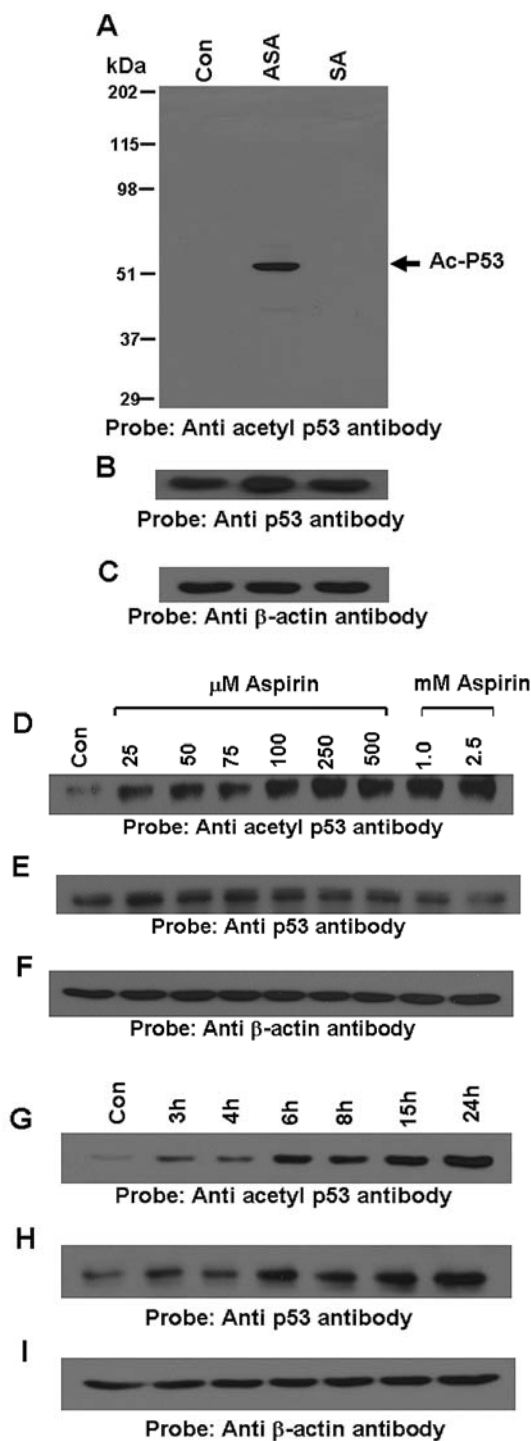


Figure 1. Aspirin acetylates p53 protein. A, shows that acetylated p53 is detected by anti-acetyl p53 (K382) antibody. Equal amount of protein from control, aspirin and salicylic acid-treated cells (100 μ M, 24 h) were run on an 8% SDS-PAGE and immunoblotted with anti-acetyl p53 antibody. B, the blot in A was stripped and reprobed with anti-p53 antibody. C, the samples of A were immunoblotted with anti- β -actin antibody. D, shows concentration-dependent p53 acetylation by aspirin. Cells were left untreated or treated for 8 h with different concentrations of aspirin as indicated, lysates prepared and immunoblotted with anti-acetyl p53 antibody. E, the blot in D was stripped and reprobed with anti-p53 antibody. F, the samples of D were immunoblotted with anti- β -actin antibody. G, shows time-dependent acetylation of p53 by aspirin. Cells were left untreated or treated with aspirin at 100 μ M for different time periods as indicated, lysates prepared and immunoblotted with anti-acetyl p53 antibody. H, the blot in G was stripped and reprobed with anti-p53 antibody. I, the samples of G were immunoblotted with anti- β -actin antibody. All blots are representative of 3 independent experiments. ASA, acetyl salicylic acid; SA, salicylic acid; Ac-p53, acetylated p53.

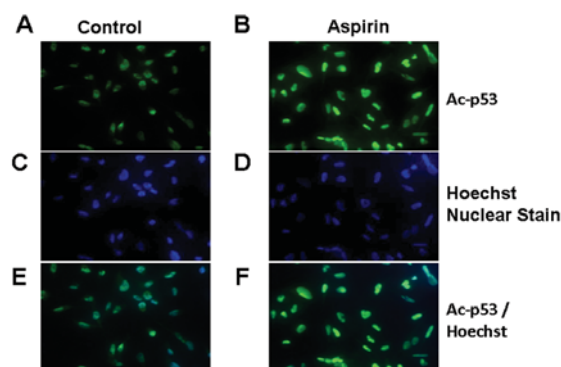


Figure 2. Acetylated p53 is localized mainly to the nucleus in aspirin-treated cells. For A and B, cells were left untreated or treated with aspirin (100 μ M, 24 h), fixed with paraformaldehyde and incubated with primary anti-acetyl p53 antibody followed by incubation with Alexa Fluor 488 secondary antibodies as described in Materials and methods. Images of cells (x20) were acquired using a Zeiss Axiophot microscope. C and D represent the Hoechst-stained nuclei of untreated and treated cells, respectively. E and F represent overlapping of both the images. These experiments were repeated 3 times.

Stripping and reprobing the blot of Fig. 1G showed increased amount of p53 protein particularly at later time points (6-24 h) (Fig. 1H), while β -actin in these samples were at similar levels (Fig. 1I). These results further confirm that aspirin-mediated acetylation of p53 stabilizes its levels.

Acetylated p53 is localized mainly to the nucleus in aspirin-treated cells. To further confirm the aspirin-induced acetylation of p53, we carried out immunofluorescence studies in cells untreated or treated with aspirin (100 μ M, 24 h) using anti-acetyl p53 antibody followed by detection with Alexa Fluor 488-labeled secondary antibody. Fig. 2 demonstrates that exposure of cells to aspirin induces higher levels of p53 acetylation (B) as compared to untreated control (A). Fig. 2C and D represent the Hoechst-stained nuclei of untreated and treated cells, respectively. Upon overlapping of the Alexa Fluor 488 and Hoechst-stained images, it is clear that almost all of the acetylated p53 was located in the nucleus (Fig. 2E and F).

Aspirin induces p53 DNA binding activity. Acetylation of p53 has been shown to increase its ability to bind to DNA (45). Since aspirin acetylated p53 at all time points examined (up to 30 h) and is localized to the nucleus, we determined using electrophoretic mobility shift assays, if it enhances p53 DNA binding activity in nuclear extracts. Fig. 3A shows that, compared to untreated control, aspirin exposure of cells enhanced the p53 DNA binding activity, and this was observed at all time points examined (3, 8, and 24 h). The DNA-protein complex was completely competed away by the addition of a 100-fold excess of unlabeled p53 binding DNA sequence, suggesting that the observed effect was the result of a specific DNA-protein interaction (data not shown).

Aspirin induces p21^{CIP1} protein and mRNA levels. p53 is known to be activated by posttranslational modifications involving acetylation/phosphorylation, leading to expression of its target genes (34,35,45). Since aspirin acetylates p53, and increases its DNA binding activity, we investigated if it

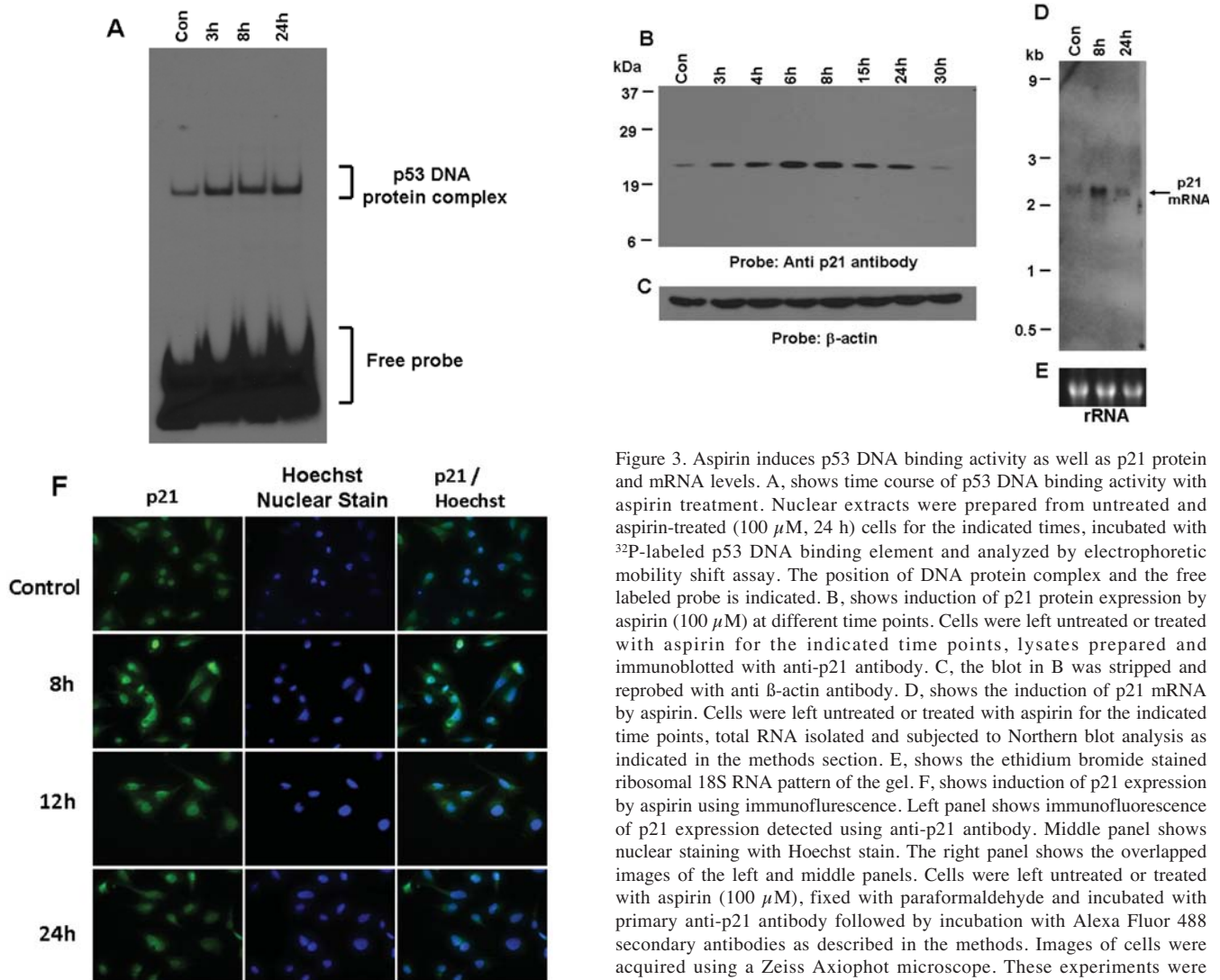


Figure 3. Aspirin induces p53 DNA binding activity as well as p21 protein and mRNA levels. A, shows time course of p53 DNA binding activity with aspirin treatment. Nuclear extracts were prepared from untreated and aspirin-treated (100 μ M, 24 h) cells for the indicated times, incubated with 32 P-labeled p53 DNA binding element and analyzed by electrophoretic mobility shift assay. The position of DNA protein complex and the free labeled probe is indicated. B, shows induction of p21 protein expression by aspirin (100 μ M) at different time points. Cells were left untreated or treated with aspirin for the indicated time points, lysates prepared and immunoblotted with anti-p21 antibody. C, the blot in B was stripped and reprobed with anti- β -actin antibody. D, shows the induction of p21 mRNA by aspirin. Cells were left untreated or treated with aspirin for the indicated time points, total RNA isolated and subjected to Northern blot analysis as indicated in the methods section. E, shows the ethidium bromide stained ribosomal 18S RNA pattern of the gel. F, shows induction of p21 expression by aspirin using immunofluorescence. Left panel shows immunofluorescence of p21 expression detected using anti-p21 antibody. Middle panel shows nuclear staining with Hoechst stain. The right panel shows the overlapped images of the left and middle panels. Cells were left untreated or treated with aspirin (100 μ M), fixed with paraformaldehyde and incubated with primary anti-p21 antibody followed by incubation with Alexa Fluor 488 secondary antibodies as described in the methods. Images of cells were acquired using a Zeiss Axiophot microscope. These experiments were repeated 3 times.

would induce the expression of p21^{CIP1}, a protein involved in cell cycle arrest (31). We exposed the cells to aspirin at 100 μ M for different time points from 3-30 h. Cell lysates were prepared and equal amount of proteins were analyzed by immunoblotting with anti-p21 antibody. Fig. 3B shows that aspirin induces p21^{CIP1} protein beginning at 3 h; however, maximal levels were detected ~6-8 h. Interestingly, the protein levels gradually decreased with longer time of aspirin exposure, and at 30 h the levels were similar to the untreated control. These samples contained similar amounts of β -actin (Fig. 3C), which shows that aspirin regulates the p21^{CIP1} protein levels in a transient fashion.

To determine if increased levels of p21^{CIP1} protein correlate with increased p21^{CIP1} mRNA synthesis, cells were left untreated or treated with aspirin for 8 and 24 h, total RNA was isolated and analyzed on Northern blots by probing with 32 P-labeled p21 cDNA. Fig. 3D demonstrates that aspirin clearly enhanced the p21^{CIP1} mRNA levels at 8 h; however, at 24 h its levels were significantly decreased. Fig. 3E shows the ethidium bromide-stained ribosomal 18S RNA pattern of the gel, confirming equal loading of RNA.

To further confirm the time-dependent activation of p21^{CIP1} by aspirin, immunofluorescence was carried out on cells

exposed to aspirin for different time periods (8, 12 and 24 h), and the protein levels were detected using anti-p21 antibody and Alexa Fluor 488-labeled secondary antibody. Fig. 3F, panel 1 confirms aspirin-induced expression of p21^{CIP1} at 8 h, and a decrease at 24 h, consistent with the Western blot experiments (Fig. 3B). These data clearly establish that aspirin regulates the expression of p21^{CIP1} protein by augmenting its transcription.

Comparison of p21^{CIP1} induction by aspirin and the anticancer drug camptothecin (CPT). To gain insight into the transient induction of p21^{CIP1} protein expression by aspirin, we compared its induction pattern with that of a known inducer of p21^{CIP1}, the anticancer drug, camptothecin (CPT). CPT causes DNA damage via inhibition of topoisomerase I (47) and this in turn upregulates of p21^{CIP1} (48). Cells were left untreated or treated with CPT (100 nM) for different time periods, lysates prepared and immunoblotted with anti-p21 antibody. Fig. 4A shows that CPT-induced p21^{CIP1} was detected as early as 3 h, however, the induction was sustained throughout the time points examined (up to 30 h). Fig. 4B demonstrates that these samples contained similar amounts of β -actin. Fig. 4C shows the comparison of the pattern of p21^{CIP1} induction for aspirin

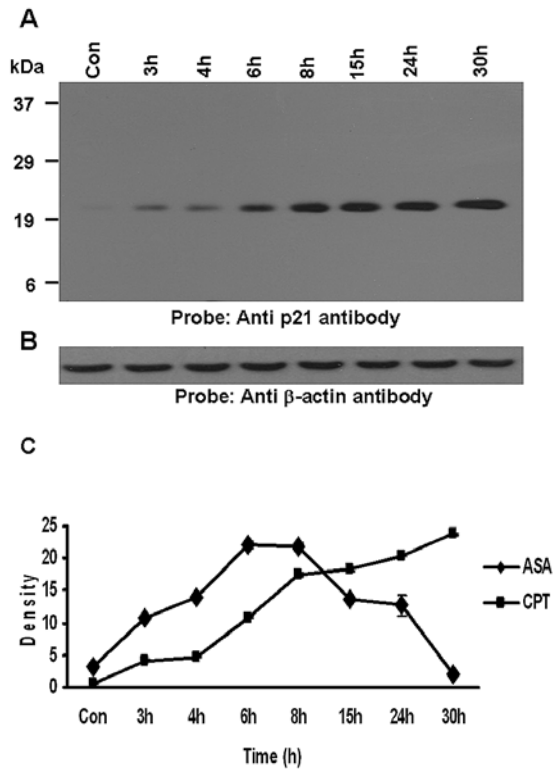


Figure 4. The anticancer drug CPT induces p21 in a sustained fashion. For A, cells were left untreated or treated with CPT (100 nM) for indicated time points, lysates prepared and immunoblotted with anti-p21 antibody. B, the blot in A was stripped and reprobed with anti- β -actin antibody. C, the p21 bands in Figs. 3B and 4A were quantified using densitometric scanning and graphically represented. These experiments were repeated 4 times.

in Fig. 3B and CPT in Fig. 4A. It is clear from Fig. 4C that the pattern of induction of p21^{CIP1} by aspirin was transient whereas that by CPT was more sustained. The results suggest that aspirin and CPT regulate p21^{CIP1} expression through distinct pathways.

Aspirin inhibits CPT-induced p21^{CIP1} expression. The results presented in Fig. 3B indicate that aspirin may regulate p21^{CIP1} protein levels in two distinct phases, a stimulatory phase which occurs in the first 1-8 h, followed by an inhibitory phase which starts after 8 h. In contrast, CPT appears to have only a sustained phase of p21^{CIP1} induction at all time points examined (up to 30 h) (Fig. 4A). To determine if aspirin-mediated downregulation of p21^{CIP1} protein at later time points (after 8 h) involves an inhibitory phase, we tested the ability of aspirin to inhibit CPT-induced p21^{CIP1} protein. Cells were left untreated or treated with aspirin (100 μ M) alone or CPT alone or co-treated with aspirin and CPT for 24 h. Cell lysates were prepared and immunoblotted with anti-p21 or anti-p53 or anti- β -actin or anti-acetyl p53 antibodies. Fig. 5A shows that in cells untreated or treated with aspirin alone, the p21^{CIP1} protein levels were not detected; however, CPT potently induced the p21^{CIP1} protein. The absence of p21^{CIP1} in aspirin-treated cells (5A) is consistent with the downregulation of p21^{CIP1} observed in 3B. Surprisingly, the co-treatment with aspirin significantly reduced CPT-induced p21^{CIP1} levels. Fig. 5B shows that acetylated p53 was present in cells treated

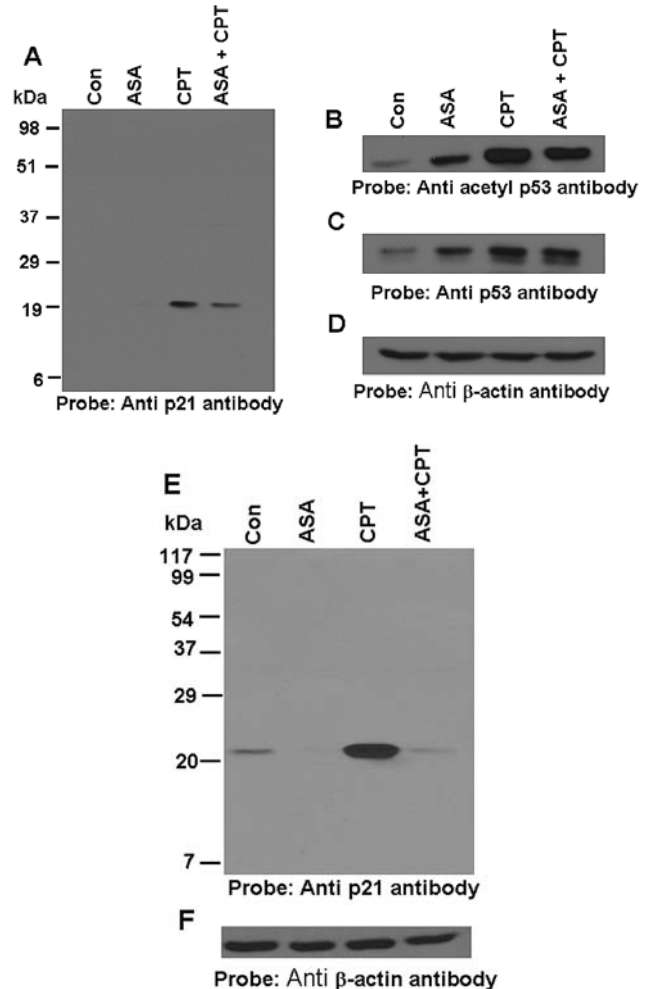


Figure 5. Aspirin inhibits CPT-induced p21 expression. For A, cells were left untreated or treated with aspirin alone (100 μ M), CPT alone (100 nM) or aspirin and CPT for 24 h, cell lysates prepared and immunoblotted with anti-p21 antibody. B, the blot in A was stripped and reprobed with anti-acetyl-p53 antibody. C, the samples of A were immunoblotted with anti-p53 antibody. D, the samples of A were immunoblotted with anti- β -actin antibody. For E, cells were left untreated or treated with aspirin alone (2.5 mM), CPT alone (100 nM) or aspirin and CPT for 24 h, cell lysates prepared and immunoblotted with anti-p21 antibody. F, the blot in E was stripped and reprobed with anti- β -actin antibody. These experiments were repeated 3 times.

with aspirin, CPT and aspirin + CPT combinations and Fig. 5C depicts that p53 was stabilized in these samples similar to the observations made in Fig. 1D and E. Fig. 5D shows that these samples contain similar amount of β -actin protein levels. When the same experiment was performed at 2.5 mM concentration of aspirin, we observed a more dramatic inhibition of CPT-induced p21^{CIP1} levels (Fig. 5E). Salicylic acid co-treatment with CPT did not inhibit p21^{CIP1} protein levels (data not shown) suggesting that the inhibitory action required the acetyl group. These findings demonstrate that aspirin has the ability to inhibit CPT-induced p21^{CIP1} protein levels and strongly suggest that downregulation of p21^{CIP1} protein levels may involve an inhibitory phase. Besides, MDA-MB-231 breast cancer cells, we observed that aspirin also inhibited CPT-induced p21^{CIP1} expression in other cancer cell lines such as HT-29 (colon), A549 (lung), SK-MEL-5 (skin) and Hep G2 (liver) (data not shown).

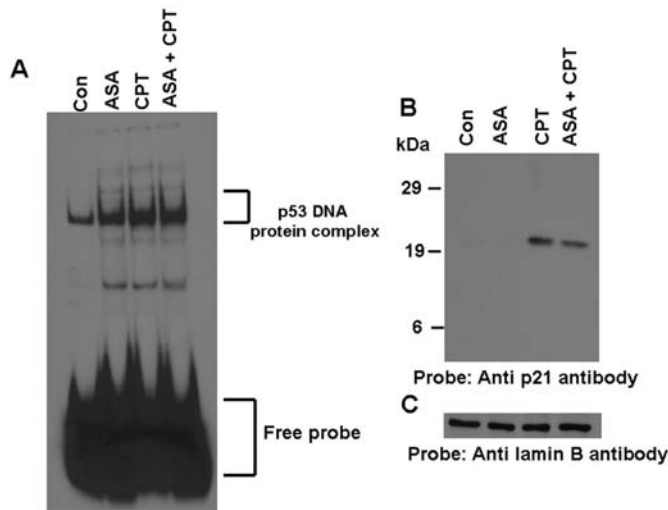


Figure 6. Aspirin does not inhibit CPT-induced p53 DNA binding activity. A, electrophoretic mobility shift assay. Cells were left untreated or treated with aspirin alone or CPT alone or co-treated with aspirin and CPT for 24 h, nuclear extracts prepared, and subjected to electrophoretic mobility shift assay using 32 P-labeled p53 DNA binding element. For B, equal amount of nuclear extracts used in A were immunoblotted with anti-p21 antibody. For C, the blot in B was stripped and reprobed with anti-lamin B antibody. These experiments were repeated 3 times.

Aspirin-mediated decrease in CPT-induced p21^{CIP1} protein levels is not due to a decrease in p53 DNA binding activity or p21^{CIP1} mRNA levels. To gain insight into the mechanism by which aspirin downregulates CPT-induced p21^{CIP1} protein levels, we determined if aspirin affects CPT-induced p53 DNA binding activity and p21^{CIP1} mRNA levels. Cells were left untreated or treated with aspirin alone or CPT alone or co-treated with aspirin and CPT for 24 h. Following treatment with the drugs, nuclear extracts or total RNA were prepared. The nuclear extracts were analyzed for the p53 DNA binding activity in an electrophoretic mobility shift assay, and total RNA were analyzed for specific p21^{CIP1} mRNA levels in Northern blots. Fig. 6A demonstrates that compared to control, higher levels of p53 DNA binding activity were observed in nuclear extracts prepared from cells treated with aspirin alone or CPT alone or with both drugs. In these nuclear extracts we also measured the p21^{CIP1} proteins levels to ensure the effectiveness of aspirin to inhibit CPT-induced p21^{CIP1} protein levels. We observed that aspirin inhibited CPT-induced p21^{CIP1} (Fig. 6B), consistent with the data described earlier in Fig. 5A and E with the total cell lysates. Fig. 6C demonstrates that all lanes contain similar levels of lamin B, a nuclear protein used as a control.

Fig. 7A shows the effect of aspirin on CPT-induced p21^{CIP1} mRNA levels. It is clear that low levels of p21^{CIP1} mRNA were detected in the untreated control. Aspirin treatment alone for 24 h showed a small decrease in p21^{CIP1} mRNA levels compared to control levels. In contrast, CPT induced higher levels of p21^{CIP1} mRNA; however, aspirin did not significantly inhibit the CPT-induced p21^{CIP1} mRNA levels. These results show that aspirin-mediated inhibition of CPT-induced p21^{CIP1} protein levels is neither due to decreased p53 DNA binding activity nor decreased synthesis of p21^{CIP1} mRNA.

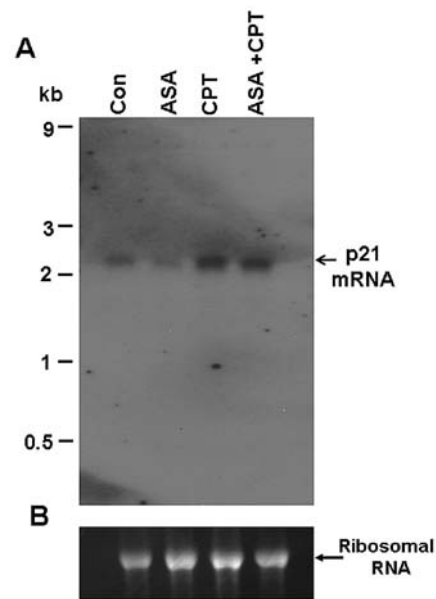


Figure 7. Aspirin does not inhibit CPT-induced p21 mRNA levels. For A, cells were left untreated or treated with aspirin alone or CPT alone or co-treated with aspirin and CPT for 24 h, RNA isolated and subjected to Northern blot analysis as indicated in Materials and methods. B, shows the ethidium bromide-stained ribosomal 18S RNA pattern of the gel. These experiments were repeated 3 times.

Cytoplasmic extract from aspirin-treated cells degrades recombinant GST-p21 protein. To explain the inhibitory effect of aspirin on CPT-induced p21^{CIP1} protein levels, we hypothesized that aspirin exposure to cells may activate proteases which specifically cause p21^{CIP1} protein degradation. To assess this possibility, cytoplasmic extracts (prepared without protease inhibitors) from untreated and aspirin-treated (100 μ M, 48 h) cells were incubated with GST-p21 recombinant protein. Fig. 8A demonstrates that incubation of exogenous p21 (recombinant) with cytoplasmic extracts from aspirin-treated cells resulted in its degradation; importantly, this degradation was prevented in the presence of protease inhibitor (PI) cocktail. This cocktail inhibits a broad spectrum of serine, cysteine and metalloproteases as well as calpains (Roche). However, cytoplasmic extract from untreated cells did not degrade the GST-p21 protein. These cytoplasmic extracts used contained similar amounts of β -actin protein (Fig. 8B). These results demonstrate that aspirin treated (48 h) cytoplasmic extracts contain proteolytic activity against p21^{CIP1}.

Aspirin induces Bax protein levels in a sustained pattern. We further investigated if aspirin induces the expression of the pro-apoptotic protein Bax. Lysates prepared from cells treated with aspirin at 100 μ M for different time points from 3 to 24 h were immunoblotted with anti-Bax antibody. Fig. 9A demonstrates that aspirin induces Bax beginning at 6 h, and its levels increased gradually with time. Fig. 9B shows similar levels of β -actin protein in all lanes. These data show that aspirin induces Bax protein levels.

Aspirin potentiates CPT-induced cell death. Previous studies have documented that elimination of p21^{CIP1} gene sensitizes the cells to apoptosis upon treatment with anticancer drugs

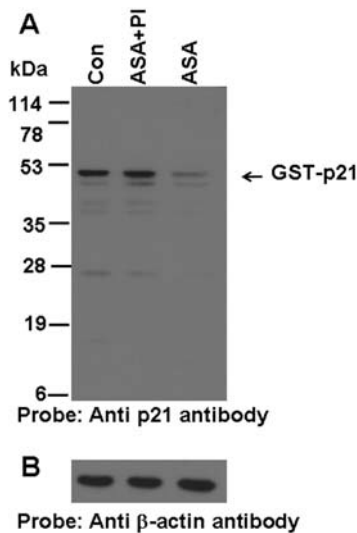


Figure 8. Lysate from aspirin-treated cells degrades recombinant GST-p21. For A, cytoplasmic extracts (prepared without protease inhibitors) from untreated cells were incubated with GST-p21. Separately, cytoplasmic extracts (prepared without protease inhibitors) from aspirin-treated (100 μ M, 48 h) cells were incubated with GST-p21 recombinant protein in the presence or absence of protease inhibitor (PI) cocktail (Roche). After 4 h, the samples were immunoblotted using anti-p21 antibody. B, the blot in A was stripped and re-probed with anti- β -actin antibody. These experiments were repeated 4 times. GST, glutathione-S transferase.

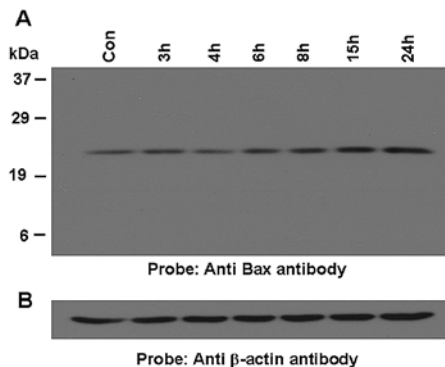


Figure 9. Aspirin induces Bax protein levels in a sustained fashion. For A, lysates were prepared from cells untreated or treated with 100 μ M aspirin for the time points indicated. Equal amount of protein was run on a 12% SDS-PAGE and immunoblotted with anti-Bax antibody. B, the blot in A, was stripped and re-probed with anti- β -actin antibody. These experiments were repeated 3 times.

(49-52). Since aspirin decreases CPT-induced p21^{CIP1} protein, we determined whether a co-treatment with both drugs would enhance the extent of cell death as compared to treatment with CPT alone. In initial experiments we determined the IC₅₀ (72 h) of CPT in MDA-MB-231 cells to be ~100 nM using the MTT assay. This is a colorimetric assay which measures the activity of the mitochondrial reductase enzymes and is often used to determine cell viability in response to cytotoxicity (42). Cells were treated with various concentrations of CPT from 0 to 200 nM alone or in combination with 100 μ M aspirin. The potentiating effects of aspirin on CPT-induced toxicity towards MDA-MB-231 cells were evident at 72 h after incubation (Fig. 10A). Enhanced cell killing was observed particularly from 12.5 to 100 nM CPT in combination with 100 μ M aspirin.

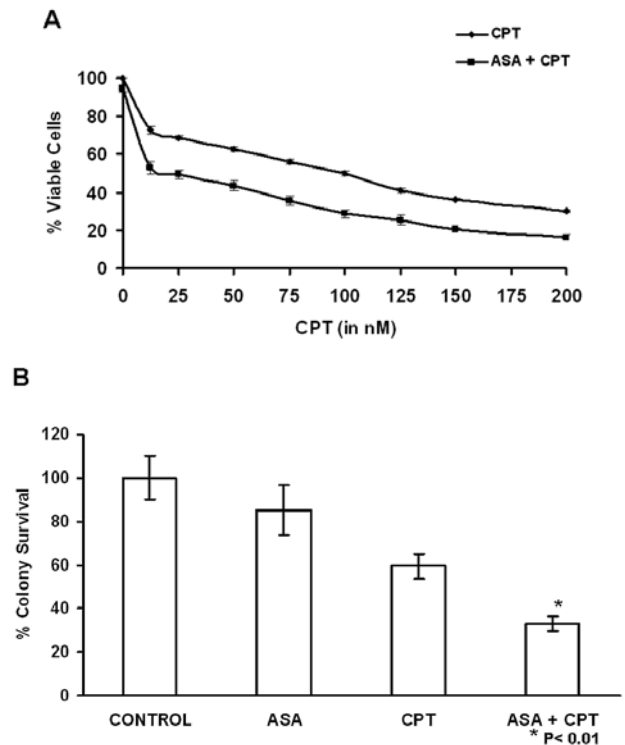


Figure 10. Aspirin potentiates CPT-induced cell death. A, MTT assay shows effect of aspirin and CPT on cell viability. Cells were treated with various concentrations of CPT from 12.5 to 200 nM alone or in combination with 100 μ M aspirin. Following 72 h of treatment, cells were treated with MTT, the formazan crystals formed were dissolved in DMSO and the absorbance was read at 570 nm as described in Materials and methods. The effect of CPT alone and in combination with aspirin on cell viability is graphically represented. All treatments were performed in triplicate. B, clonogenic cell survival assay shows the effect of aspirin and CPT on cell survival. Cells left untreated or treated with aspirin or CPT or with both drugs for 72 h were reseeded at a density of 500 cells per plate, grown for 2 weeks in normal media, and colonies stained with methylene blue as described in Materials and methods. The percentage of surviving colonies were calculated with respect to the control and represented graphically. All treatments were performed in triplicate. The data were analyzed using one-way ANOVA followed by Newman-Keuls multiple comparison test, giving a P-value of <0.01 between the CPT- and aspirin + CPT-treated groups.

Cell viability with 100 μ M aspirin alone was similar to the untreated control. Cells (73%) were viable at the lowest concentration of CPT tested (12.5 nM), however, inclusion of aspirin along with CPT reduced the cell viability to 53%. Concentration of CPT (100 nM) showed 50% cell viability, and inclusion of aspirin with CPT decreased the cell viability to 28%. These results suggest that aspirin co-treatment enhances CPT-induced cytotoxicity.

To further establish the potentiating effect of aspirin on CPT-induced cell death, we performed clonogenic cell survival assays, which determine the capability of adherent cells to survive and to replicate following exposure to cytotoxic drugs. For this, cells exposed to aspirin or CPT or with both drugs for 72 h were reseeded at low density, grown for 2 weeks and colonies stained with methylene blue. Fig. 10B demonstrates that the percentage of colonies that formed were similar between control and aspirin treatment groups. CPT treatment reduced the number of colonies formed to ~60% of the control whereas a co-treatment with aspirin and CPT further decreased the number of colonies formed to 33%. These results confirm

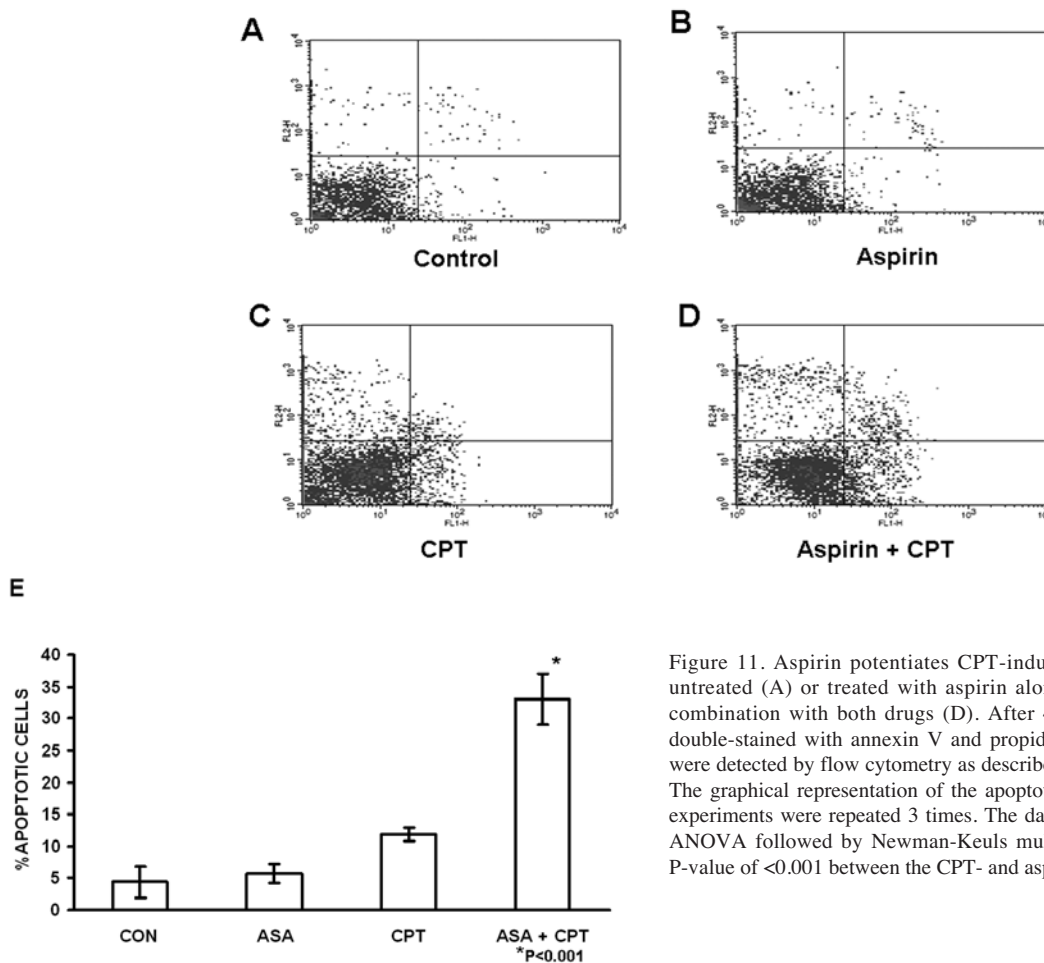


Figure 11. Aspirin potentiates CPT-induced apoptosis. Cells were left untreated (A) or treated with aspirin alone (B) or CPT alone (C) or in combination with both drugs (D). After 48 h, cells were harvested and double-stained with annexin V and propidium iodide. The apoptotic cells were detected by flow cytometry as described in Materials and methods. (E) The graphical representation of the apoptotic cells in all treatments. These experiments were repeated 3 times. The data were analyzed using one-way ANOVA followed by Newman-Keuls multiple comparison test, giving a P-value of <0.001 between the CPT- and aspirin + CPT-treated groups.

that aspirin significantly reduces cell survival when treated in combination with CPT.

The potentiating effect of aspirin on CPT-induced cell death was also confirmed by measuring apoptosis at 48 h using flow cytometry after double staining cells with annexin V and propidium iodide (PI). In this experiment we used 48 h of drug treatment to capture the early apoptotic cells in addition to late apoptotic cells. The distribution of stained cells is shown in Fig. 11A-D. In each of the histograms, the bottom left quadrant represents live cells, the bottom right quadrant represents cells in early apoptosis, the top right quadrant represents cells in late apoptosis, and the top left quadrant represents cells stained with PI alone. It is clear that the percentage of cells in the apoptotic stage in the untreated control (Fig. 11A) and in cells treated with aspirin (Fig. 11B) were similar (~7%), suggesting that aspirin at 100 μ M did not exert much apoptotic effect. In contrast, CPT (Fig. 11C) induced cell death (13%). Interestingly, in cells treated with both aspirin and CPT (Fig. 11D), we observed a significant increase in cells undergoing apoptosis (35%). These values are also represented in a bar graph shown in Fig. 11E. These results support the hypothesis that aspirin-mediated enhancement of CPT-induced cell death involves initiation of apoptosis.

Discussion

Breast cancer remains the most common malignancy affecting women worldwide, with a lethality rate of >410,000 cases

per year (53). In the last few years, several *in vivo* and *in vitro* studies showed that NSAIDs can inhibit breast cancer cells and suppress tumor growth (54,55). This was also supported by epidemiological studies which showed that regular aspirin intake may in fact reduce breast cancer risk (54,56,57). Despite these observations and reports, the pathways leading to the anticancer effects of aspirin are not well understood. Aspirin consists of two parts, an acetyl group and a salicylate group, both of which have their own distinct targets. The acetyl group of aspirin is known to acetylate COX thereby causing its inhibition; the salicylate group has been shown to bind I (κ)- β and prevent NF- κ B activation (58); thus both contribute to the anti-inflammatory properties of aspirin. Although inhibition of COX and NF- κ B may contribute to its anticancer effects (59), to date no studies have been conducted to determine whether aspirin acetylates cellular proteins involved in tumor suppression thereby modulating their activity.

In the present study, we report several novel observations including a mechanism by which aspirin may exert its anti-cancer effects. Since plasma concentrations of intact aspirin (acetylsalicylic acid) after administration of a single therapeutic dose reach a maximum of ~100 μ M (46), we used this concentration in our studies to determine its cellular effects. Using immunoblotting and immunofluorescence studies, we discovered that aspirin acetylates p53 and this was associated with the increased expression of two of its target genes namely, p21^{CIP1} and Bax. The ability of aspirin to induce p53 DNA

binding activity and subsequently increase the expression of p21^{CIP1} mRNA levels suggests that it regulates the expression of p21^{CIP1} gene at the level of transcription. Although, aspirin induces p21^{CIP1} and Bax protein levels, the kinetics of their induction appear very different. Induction of p21^{CIP1} protein by aspirin was transient, whereas the Bax expression was sustained, suggesting that unique mechanisms govern their expression in the cell.

It is to be noted that although >50% of all human cancers contain mutant forms of p53, not all mutations lead to inactivation of its function (60). MDA-MB-231 cells contain a mutant p53 (codon 280, Arg to Lys), however, limited studies exist on how this mutation affects gene expression. Previous studies show that many mutant forms of p53, including the one present in MDA-MB-231 cells are capable of binding to the high-affinity p21 promoter and induce expression of p21^{CIP1} mRNA albeit to lower levels compared to wild-type p53 (61-64). The conformational changes of the p53 mutant present in MDA-MB-231 cells or its molecular interactions with the DNA recognition sequence have not been investigated; further, a comprehensive functional characterization of this mutation has not been reported to date. Despite the presence of mutant p53 in these cells, we observed some basal levels of p53 DNA binding activity in the untreated control (Figs. 3A and 6A), and increased p53 DNA binding activity in aspirin-treated cells, suggesting that mutant p53 and its aspirin-acetylated form is to some extent functional. In our study, we have used an antibody specific for the detection of p53 acetylated at K382 and it is not clear at this stage how many of the 19 lysine residues present in p53 are acetylated by aspirin. Although MDA-MB-231 cells contain a mutant form of p53, it is possible that acetylation of other residues, if occurring, may also be responsible for restoring some functional activity. On the other hand, despite aspirin-induced acetylation of p53 and increased DNA binding activity, we cannot rule out the possibility that p21^{CIP1} and Bax expression could occur through p53-independent pathways.

Aspirin-induced p21^{CIP1} protein levels were first detected at 3 h; maximal at 6-8 h, however, the levels gradually decreased with longer time of exposure (30 h). Northern blot analysis demonstrated that aspirin-induced upregulation of p21^{CIP1} mRNA levels were higher at 8 h compared to the control; however, mRNA decreased substantially to the untreated control levels at 24 h. This suggests that the decrease in the p21^{CIP1} protein expression observed at later time points such as 24 h is at least in part due to decreased mRNA synthesis. Our data also point to additional regulatory mechanisms such as the involvement of a protease in the degradation of p21^{CIP1} protein levels. The two major pieces of evidence in support of this are: 1) aspirin potentially inhibited CPT-induced p21^{CIP1} protein levels although under the same conditions, the ability of p53 to bind its target DNA sequence and p21^{CIP1} mRNA synthesis were unaffected; 2) cytoplasmic extract prepared from aspirin-treated cells when incubated with recombinant p21 caused its degradation, and this was reversed by protease inhibitors. We also observed that the peptide aldehyde inhibitor, MG132, capable of inhibiting calpains, lysosomal cysteine proteases and proteasomes (65), also reversed the p21^{CIP1} degradation in cells exposed to aspirin (data not shown). These data suggest that a protease capable of

degrading the p21^{CIP1} protein is activated in aspirin-treated cells. The inhibitory action of aspirin on CPT-induced p21^{CIP1} protein levels was specific to intact aspirin (acetylsalicylic acid) as co-treatment with salicylic acid alone did not cause degradation of p21^{CIP1}.

Our finding that aspirin inhibits CPT-induced expression of p21^{CIP1} protein levels in cancer cells is an important observation with implications for designing improved chemotherapeutic strategies. Several recent studies show that p21^{CIP1} expression rescues cells from apoptosis and that elimination of p21^{CIP1} sensitizes cells to apoptosis (49-52). Although the molecular events underlying the p21^{CIP1} mediated anti-apoptotic effects have not been fully elucidated, it appears to have the ability to inhibit several steps such as apoptosis signal regulating kinase 1, c-Jun N-terminal kinase and caspase-3, all of which are implicated in programmed cell death (49). It was shown that in the absence of p21^{CIP1} gene, DNA damaged cells acquire grossly deformed, polyploidy nuclei, abnormal DNA synthesis and die through apoptosis (50). Other studies showed that loss of p21 gene increases sensitivity of tumor cells to apoptosis following DNA damage via an increase of both p53 levels and alteration of Bax/Bcl-2 ratio (49,51). p21^{CIP1} is known to be a negative regulator of both, p53-dependent and p53-independent apoptosis (31). In a recent study, Lazzarini *et al* demonstrated that elimination of the p21^{CIP1} expression increases chemosensitivity to anticancer agents, doxorubicin, etoposide and irinotecan (52). These studies indicated that suppression of p21^{CIP1} using specific short-hairpin RNAs induced high levels of chemosensitivity in malignant pleural mesothelioma (52). Therefore, it was suggested that small molecules that eliminate the expression of p21^{CIP1} may improve the cytotoxic activity of anticancer drugs (31,52). In this context, our findings show that aspirin has the ability to inhibit the CPT-induced p21^{CIP1} protein levels and it enhances CPT-induced apoptosis in MDA-MB-231 breast cancer cells.

Unlike the unique stimulatory and inhibitory effect on p21^{CIP1}, exposure of cells to aspirin caused a sustained increase in Bax protein levels. Bax is a pro-apoptotic protein localized to the outer mitochondrial membrane and its activity can be modulated by p53 via transcription-dependent and -independent routes (19). In the transcription-dependent route, activated p53 induces Bax expression leading to its cellular accumulation resulting in the loss of mitochondrial membrane potential, and cytochrome c release. In the transcription-independent route, p53 stabilization and accumulation in the cytoplasm causes its direct translocation to the mitochondria where it interacts with the antiapoptotic proteins Bcl2 and Bcl-XL (66), leading to oligomerization of Bax and increased permeabilization and release of cytochrome c. Cytochrome c subsequently activates caspases causing cleavage of cellular substrates producing apoptotic phenotypes. Although aspirin alone (acetylsalicylic acid) at 100 μ M increased Bax levels, it had no significant effect on cell viability suggesting that decreased p21^{CIP1} and increased Bax expression alone are insufficient to exert a cytotoxic effect. However, other investigators have used much higher concentrations of aspirin (ranging from 1 to 20 mM) and observed DNA damage and apoptosis, suggesting the activation of other pathways at higher doses (23,67,68). These investigators used high doses

of aspirin to reflect the salicylate concentrations detected in the plasma after treatment for various chronic inflammatory diseases (23). Since our primary measured effect in this study was acetylation, it was important to use only 100 μ M concentration to reflect physiologically achievable concentrations of intact aspirin in the plasma (46). Aspirin at 100 μ M potentiated the effect of CPT-induced apoptosis which suggests that its cytotoxic effect at this concentration occurs mainly in DNA-damaged cells. The pathway that is targeted or sensitized by aspirin which contributes to the potentiation of apoptosis in CPT-treated cells requires further study.

In addition to p21^{CIP1} and Bax, aspirin may also modulate the expression of other genes involved in apoptosis. For example, in a recent study it was reported that aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels (68) through a mechanism involving protein degradation. Survivin is an antiapoptotic protein and a reduction in its levels would favor the apoptotic process. Therefore, additional studies are required to establish if aspirin modulates survivin levels.

Although the chemopreventive properties of aspirin are increasingly being recognized, recent studies have argued for or against the advisability of the use of aspirin in chemoprevention (13,69). Aspirin is a systemic agent that has both beneficial and adverse effects throughout the body. Despite its major beneficial effects, it can also cause potentially fatal gastrointestinal bleeding and hemorrhagic strokes. Due to its adverse effects, some researchers have cautioned against the use of aspirin for chemoprevention in healthy individuals since its beneficial effects are observed only after prolonged use (70). In contrast, cancer patients are given anticancer drugs for short intervals which are often associated with major toxicities and adverse effects. In life-threatening diseases such as cancer, combined regimens of anticancer drugs and aspirin may prove beneficial in reducing the dose and duration of chemotherapy.

Acknowledgements

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