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Research Article

The Mutant p53-Conformation Modifying Drug, CP-31398, Can Induce Apoptosis of Human Cancer Cells and Can Stabilize Wild-Type p53 Protein

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KEY WORDS

CP-31398, p53, Apoptosis, Cancer, Affymetrix Gene Chip

ABSTRACT

CP-31398, a styrylquinazoline, emerged from a screen for therapeutic agents that restore a wild-type DNA-binding conformation of mutant p53 to suppress tumors in-vivo (Science 286, 2507, 1999). We investigated the growth inhibitory mechanism of CP-31398 using nine human cancer cell lines containing wild-type, mutant or no p53 expression. Six of nine cell lines underwent apoptosis after exposure to CP-31398, while two cell lines, DLD1 colon cancer and H460 lung cancer, underwent exclusively cell cycle arrest. Cell cycle arrest preceded the apoptosis in some cases. CP-31398 did not inhibit growth of the p53 non-expressing ovarian cancer cell line SKOV3. Interestingly, we found that wild-type p53 protein is stabilized upon CP-31398 exposure. p53 target genes such as $p21^{WAF1/Cip1}$, and KILLER/DR5 were upregulated by CP-31398, but their expression did not correlate with arrest or apoptosis induction. Combination of CP-31398 and TRAIL or chemotherapeutic agents enhanced cancer cell killing effect possibly through upregulation of p53-regulated genes such as KILLER/DR5. Bax-/-, wild-type p53-expressing cells displayed reduced susceptibility to killing by CP-31398. An Affymetrix GeneChip Array screen revealed that CP-31398 alters expression of non-p53 target genes in addition to p53-responsive genes. Although our preliminary data suggest that CP-31398 does not alter wild-type p53:MDM2 interaction, further efforts are required to elucidate the mechanism of wild-type p53 stabilization by CP-31398. The results increase our understanding of CP-31398 action, and suggest strategies for improving its specificity, possibly through use of microarrays to screen related compounds with higher mutant p53-specificity.

INTRODUCTION

p53 is the most commonly mutated gene in human cancer. It has become clear that cancer cells escape from wild-type p53 function thus avoiding its pivotal role in regulating cell growth and death.[1,2] A number of stressful conditions (such as DNA damage, hypoxia, changes in the redox potential, oncogene expression, etc.) can stabilize wild-type p53 protein, which in turn transcriptionally activates specific target genes leading to cell cycle arrest or apoptosis.[1,2] The underlying mechanism of cell cycle arrest by p53 is well characterized[3] but the mechanism of inducing apoptosis is less well understood. Several potential target genes have been proposed as mediators of p53-dependent apoptosis through sequence-specific transcriptional control.[3,4] These include Bax[5-7] and Fas/APO-1,[8] the best characterized targets of p53 in apoptosis, but neither is required for p53-dependent apoptosis in vivo.[9,10] Other genes such as PERP,[11] Noxa,[12] KILLER/DR5,[13,14] p53AIP1,[15] PIDD,[16] and p53DINP1[17] have been recently reported to mediate p53-dependent apoptosis. Despite the growing number of p53-regulated apoptotic genes, the extent to which sequence-specific transactivation contributes to apoptosis remains controversial, although it is clear that p53-dependent apoptotic signals converge upon mitochondrial cytochrome c release and apoptosome activation. A recent study has demonstrated the importance of p53-dependent transcriptional activation towards tumor growth suppression in vivo.[18]

It has been suggested that pharmacological agents reducing specific free energy of a protein's active conformation may have utility in cancer, cystic fibrosis and neurodegeneration.[19-22] Because of its frequency of mutation in cancer cells, p53 is a good candidate for therapeutic targeting. CP-31398 emerged from an in-vitro screen for drugs that can modify a mutant p53 to a wild-type p53.[23] CP-31398 was shown to promote the stabilization of the DNA binding domain of p53 and restore the transactivation function of certain mutant-p53 proteins both in vitro and in vivo. It was also demonstrated that CP-31398 inhibits tumor

xenograft growth in mice, suggesting its further development as a therapeutic agent. However, little is known about the mechanism(s) by which CP-31398 inhibits cell growth. We used several cancer cell lines carrying wild-type or various mutants of p53 to examine the effect of CP-31398 on cancer cell growth inhibition. Upon exposure of cancer cells to CP-31398, we observed apoptotic cell death in six out of nine cell lines. Two cell lines displayed cell cycle arrest and one p53-null cell line showed no phenotype upon treatment with CP-31398. We further analyzed the activity of CP-31398 by examining gene expression patterns among apoptotic and arrested cells following drug exposure. The results suggest that CP-31398 can alter expression of non-p53 regulated genes in addition to p53-responsive genes. The results further illuminate our understanding of the mechanism of action of the novel CP-31398 mutant p53 conformation modifying drug, and suggest possible strategies for improving its specificity.

Table 1 **Summary of Effect of CP-31398 on the Various Cancer Cell Lines**

Cell Line	Origin	p53 Status	Effect of CP-31398
DLD-1	colon cancer	mutant	G1 arrest
SW480	colon cancer	mutant	apoptosis
SKBr3	breast cancer	mutant	apoptosis
H460	lung cancer	wild type	G1 arrest
PA1	ovarian cancer	wild type	apoptosis
U20S	osteosarcoma	wild type	apoptosis
HCT116	colon cancer	wild type	apoptosis
Saos-2	osteosarcoma	null	apoptosis
SKOV3	ovarian cancer	null	no effect

MATERIALS AND METHODS

Cell Lines and Cell Killing Assay. All the cell lines used in the experiments are summarized in Table 1. The human osteosarcoma cell line Saos2 and U2OS, human colon cancer cell line SW480, human lung cancer cell line DLD-1, human breast cancer cell line SKBr3, and human ovarian cancer cell lines SKOV3, PA1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The human lung cancer cell line H460 and the human colon cancer cell lines HCT116 and SW480 were maintained as described previously.[24] U2OS-Neo and E6 were obtained by transduction of U2OS cells with either pCMV-Neo or E6 after the selection with 400 μg/ml of G418. Both H460 and PA1 transduced with pCMV-Neo or -E6 were described previously.[25] HCT116- *Bax*-/- cells[26] were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University). The MTT method was used to analyze growth inhibition in some experiments as described previously.[27]

Antibodies and Western Blot Analysis. Western blot analysis was carried out as previously described.[28] Blotted membranes were immunostained with anti-p53 (Ab-2, 1:500, Oncogene Science, Cambridge, MA), anti-p21 (Ab-1, 1:200, Calbiochem, San Diego, CA), anti-Rb (Ab-5, 1:1000, Oncogene Science), anti-PARP (1:2000, Boehringer Mannheim, Mannheim, Germany), anti-Bax (C-19, 1:1000; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), anti-DR5 (1:500; IMGENEX, San Diego, CA), anti-Mdm2 (C-18 and N-20; 1:500 Santa Cruz,) or anti-Actin (I-19, 1:200; Santa Cruz) antibody.

Electrophoretic Mobility Shift and Supershift Assays. The mouse anti-human p53 monoclonal antibody pAb421 (Ab-1, Oncogene Science) was used to activate sequence-specific DNA-binding by p53 in electrophoretic mobility shift assays as previously described[14] with the following modification. The double-stranded DNA probes which were used in the experiment contained the following sequences from the p21 promoter: 5′-CAGGAACATGTCC-CAACATGTTGAGC-3′; site 1.

Flow Cytometry, Mitochondrial Membrane Potential Assay and Active-Caspase 3 Assay. After plating 5 x 10^5 cells in a 6-well culture plate, various cell lines were treated with various doses of CP-31398 for a variable number of hours as indicated in the figure legends. Preparation of cells for flow cytometric analysis was performed as described.[29] Flow cytometry was performed on a Coulter Epics Elite counter. DNA content analysis was performed using MacCycle software (Phoenix Flow Systems, San Diego, CA). In order to measure mitochondrial membrane potential, we used $DiOC_6$ (Molecular Probes). In brief, after plating 5 x 10^5 cells per well in a 6-well plate, cells were exposed to CP-31398 for 16 hours, and harvested. Cells were subsequently incubated with 100 nM $DiOC_6$ for 30 min at 37°C and analyzed by flow cytometry. In the case of the active-Caspase 3 assay, harvested-cells were fixed with fixation buffer according to manufacturer's instruction (Cytofix/Cytoperm™ kit, PharMingen, San Diego, CA) and incubated with anti active-Caspase 3 antibody as the primary antibody (PharMingen), and then probed by using anti-rabbit IgG conjugated with phycoerythrin (PE). The intensity of PE was quantitated by Flow cytometry and positive cells were counted as described.[30]

Gene Array Screening. Affymetrix GeneChip Array screening was performed as recommended by the Manufacturer (AFFYMETRIX, Inc., Santa Clara, CA). Eight μg of total RNA isolated from DLD1 and SW480 colon cancer cell lines treated with 15 μg/ml of CP-31398 for 4 hours was used for generating biotin labeled-cRNA probes. The hybridization intensity of each dot on the array was measured and the data was analyzed using the Affymetrix Microarray Suite software (Version four, AFFYMETRIX, Inc., CA).

Northern Blotting. Total RNA isolation and Northern blotting was carried out as previously described.[31] A Not I fragment of 1.2 kilobases in size from the plasmid pCEP4 carrying the $p21^{WAF1}$ coding sequence was used as the probe for $p21^{WAF1}$ mRNA expression.[31] A 1.3-kilobase Hind III fragment from the plasmid pMV60 carrying the Bax coding sequence was used as a probe for Bax mRNA expression.[30] A 2.1-kilobase Hind III fragment from pCEP4-KILLER was used as the probe for human KILLER/DR5 mRNA expression.[13] Probes for other p53-target genes, such as p53AIP1, p53R2, Pidd, PERP, and NOXA were amplified by polymerase chain reaction. Quantitation of the hybridization intensity of each band was performed using Imagequant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

CP-31398 Restores Sequence-Specific DNA-Binding Ability to p53 and Induces $p21^{WAF1}$ Gene Expression in Mutant p53-Expressing SW480 Human Colon Cancer Cells. We examined the effect of CP-31398 (Fig. 1A) on p53-DNA binding activity using mutant-p53 (A273H, P309S) expressing SW480 cells. After 16 hours of treatment with 15 μg/ml of CP-31398, mutant p53 in SW480 regained the ability to bind to the p53 consensus sequence as revealed by an electrophoretic mobility shift assay (Fig. 1B). Western blot analysis revealed that $p21^{WAF1}$ protein expression was increased following treatment with CP-31398 (Fig. 1C, second panel). In addition, hypophosphorylation of pRB occurred and cleavage of PARP was noted within 20 hours of CP-31398 exposure, suggesting that CP-31398 could induce apoptosis and cell cycle arrest in SW480 cells (Fig. 1C, third panel). To confirm these results, we investigated the effects of CP-31398 using additional tumor cell lines expressing either mutant or wild-type p53.

CP-31398 Increases Wild-Type p53 Protein Levels and Induces $p21^{WAF1}$ in Both Wild-Type and Mutant-p53 Expressing Human Cancer Cell Lines. In our initial studies to investigate the effects of CP-31398, we varied drug dose and time of drug exposure using several cancer cell lines, expressing wild-type or mutant p53 (Fig. 2). As shown in Figure 2A, CP-31398 induced $p21^{WAF1}$ at a concentration of 15 μg/ml in all cell lines (containing wild-type or mutant-p53 expression) studied. However, in some cell lines, e.g., SW480, U2OS, and HCT116, lower doses of CP-31398 appeared to elevate p21 expression (Fig. 2A). In the case of wild-type p53 expressing cell lines (i.e., PA1, U2OS, and HCT116), we noted that p53

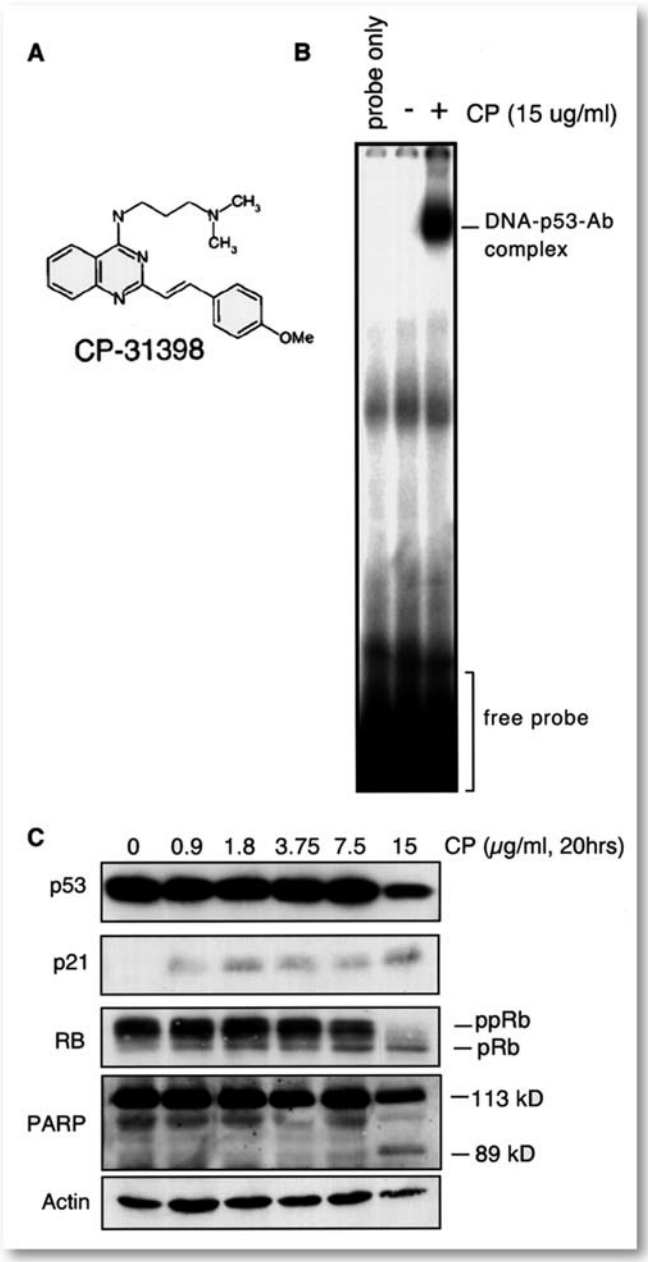

Figure 1. CP-31398 restores DNA-p53 binding activity and induces $p21^{WAF1}$ in endogenous mutant p53-expressing SW480 colon cancer cells. A) Structure of CP-31398. B) DNA binding activity and supershift analysis of SW480 cells treated with 15 μg/ml of CP-31398 for 16 hours. 10 μg of nuclear extract was harvested and incubated with ^{32}P-labeled double stranded p53 DNA consensus sequence, and an electromobility shift assay was performed in the presence of anti-p53 antibody (pAb421) as described in "Materials and Methods". C) Western analysis of cells exposed to CP-31398. Total cellular extract was prepared from SW480 cells after treatment with 15 μg/ml of CP-31398 for 20 hours, and the protein expression was analyzed by Western blotting. ppRb and pRb represent phosphorylated pRb and hypophosphorylated pRB, respectively. Cleaved- and uncleaved-PARP are indicated by 89 kD and 113 kD, respectively. $p21^{WAF1}$ and actin protein expression are shown.

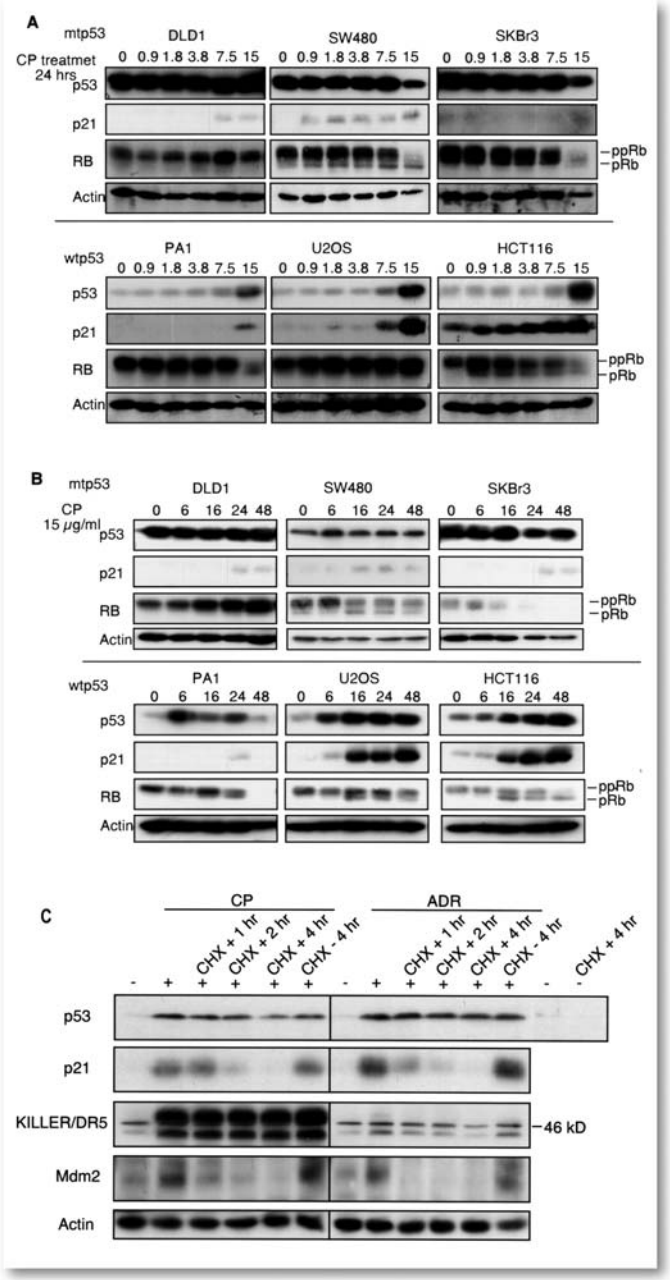

Figure 2. CP-31398 induces $p21^{WAF1}$ expression in wild-type and mutant p53-expressing cancer cell lines. A) Cell lines were treated with the indicated concentrations of CP-31398 (0-15 μg/ml) for 20 hours. After exposure to CP-31398, 50 μg of total cell lysate were subjected to 10% (for pRb western blot) or 15 % SDS-PAGE (p53, p21 and actin), and analyzed as described in "Materials and Methods". B) Cells were treated with 15 μg/ml of CP-31398 and harvested at the indicated time points (hr). Western blotting was performed as described above (A). ppRb and pRb represent phosphorylated pRb and hypophosphorylated pRb, respectively. C) Wild-type p53 can be stabilized by CP-31398 exposure. Wild-type p53 expressing cell, U2OS was exposed to 15 μg/ml of CP-31398 or 0.2 μg/ml of Adriamycin for 16 hours. After intensive washing, cells were incubated with (+) or without (-) 10 μg/ml of Cycloheximide (CHX) for indicated hours and harvested for Western blot analysis.

protein levels accumulated following exposure to CP-31398 (Fig. 2A and 2B). In some cell lines examined such as DLD1, SW480, PA1, U2OS and HCT116, hypophosphorylation of pRb following CP-31398 exposure was noted to occur whether cells expressed endogenous wild-type or mutant p53 (Fig. 2A and 2B). In some cell lines, most notably SKBr3 breast cancer cells, exposure to CP-31398 appeared to inhibit pRb expression (Fig. 2A and 2B). Following CP-31398 exposure, upregulation of $p21^{WAF1}$ protein expression was observed within 16 hours (Fig. 2B), and this was accompanied by hypophosphorylation of pRb in most cases. The observation that following CP-31398 exposure both PARP cleavage and hypophosphorylation of pRb

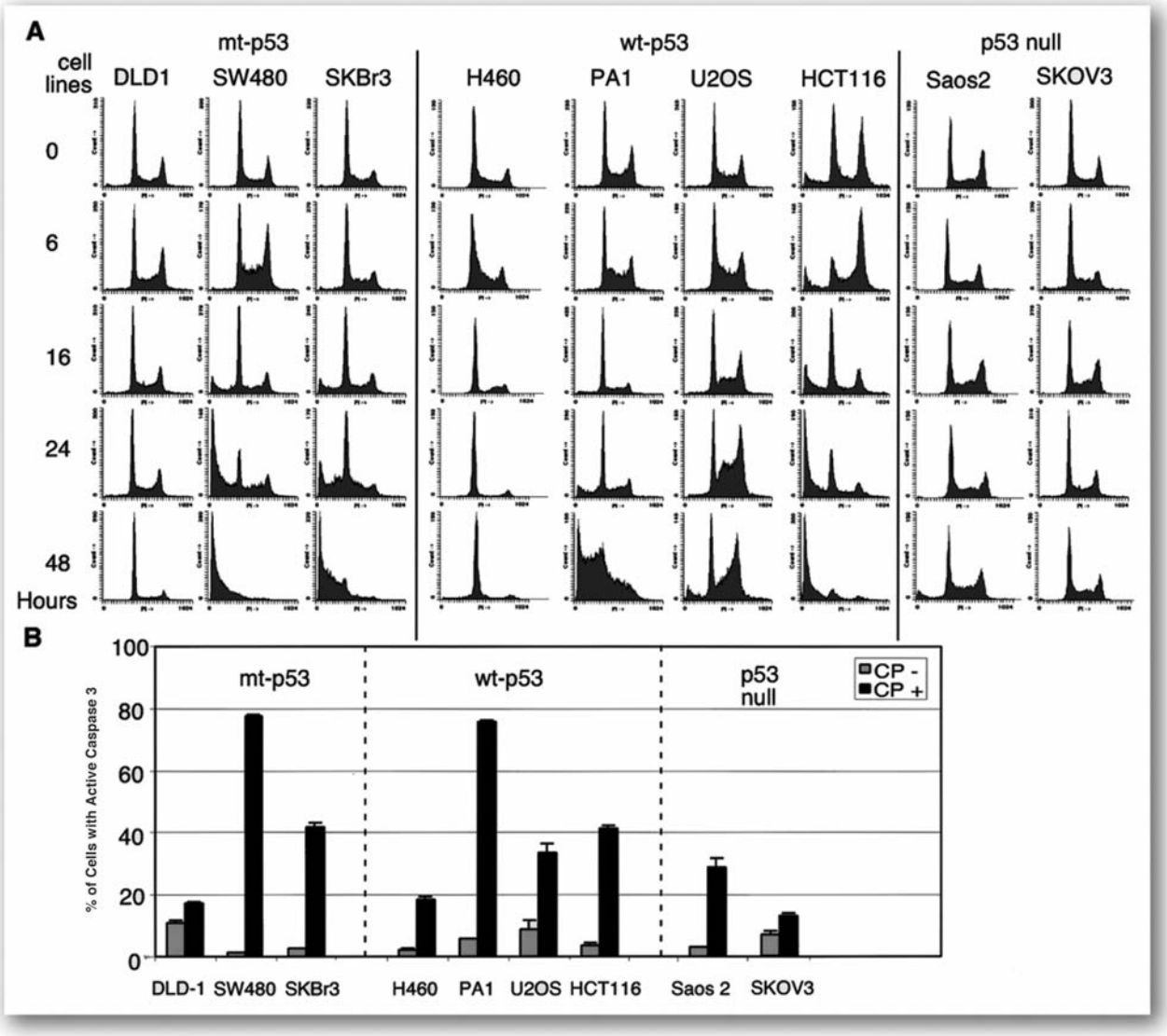

Figure 3. CP-31398 induces apoptosis in cancer cell lines. A) Various cancer cell lines (as indicated) were treated with 15 μg/ml of CP-31398 for 48 hours. At each time point, cells were harvested and fixed for cell death analysis. Flow cytometric analysis was performed as described in "Materials and Methods". B) The Active-caspase 3 assay was performed using cancer cell lines treated with or without 15 μg/ml of CP-31398 for 20 hours. At least two experiments with triplicate samples were performed.

could occur simultaneously suggested that cancer cell growth inhibition may be due to a combination of cell cycle arrest and apoptosis. We further investigated this possibility.

We also examined whether the accumulation of p53 induced by CP-31398 in wild-type p53 expressing cells was due to protein stabilization. We exposed U2OS cells to CP-31398 or adriamycin for 16 hours and then added cycloheximide (CHX) to inhibit new protein synthesis to evaluate the half-life of p53 protein. We observed that the p53 protein level was not significantly altered up to 2 hours after CHX treatment (Fig. 2C, left). The sustained expression of p53 following CP-31398 exposure in CHX-treated cells was nearly the same as compared to adriamycin treatment (Fig. 2C right). We concluded that, in wild-type expressing cells, CP-31398 can stabilize p53, to a similar extent as the chemotherapeutic agent adriamycin. We are currently investigating the mechanism of stabilization of p53 induced by CP-31398, and our preliminary results suggest that the drug does not alter p53:MDM2 interaction in some cell lines (W. Wang, R. Takimoto and W.S. El-Deiry, unpublished observations).

CP-31398 Induces Apoptosis Regardless of p53 Status in Cancer Cells. In order to examine the effects of CP-31398 on cell cycle progression and cell death, we performed flow cytometric analysis of CP-31398 treated tumor cell lines (Fig. 3). As shown in Figure 3A, cell cycle analysis revealed that six out of nine cell lines appear to undergo apoptosis after 48 hours of exposure to CP-31398, regardless of p53 status, as evidenced by the accumulation of sub-G1 cells (Fig. 3A). Both DLD1 and H460 cells seemed to undergo G1 cell cycle arrest. Although the compound had no appreciable effect on the cell cycle progression of SKOV3 cells, which expresses no detectable p53, p53-null Saos2 cells appeared sensitive to killing by CP-31398 by 48 hours post drug exposure. These results suggest that CP-31398 may exhibit its effects on the cell cycle and the induction of apoptosis in cancer cells through p53-dependent and -independent pathways. To confirm that cells with accumulation of SubG1 peaks in fact underwent apoptosis, we performed an assay to detect active-caspase 3 in CP-31398 treated cell lines. The cell lines which showed subG1 fraction after exposure to CP-31398 demonstrated the activation of caspase 3, indicating that CP-31398 can induce apoptosis of cancer cells (Fig. 3B).

P53 Target-Genes Were Induced by CP-31398. To begin to explore the mechanism underlying differences between apoptosis and cell cycle arrest induced by CP-31398, we examined the expression of various p53-target genes involved in mediating p53 effects. As shown in Figure 4, all the cell lines examined, except for SKOV3 cells, showed an induction of $p21^{WAF1}$

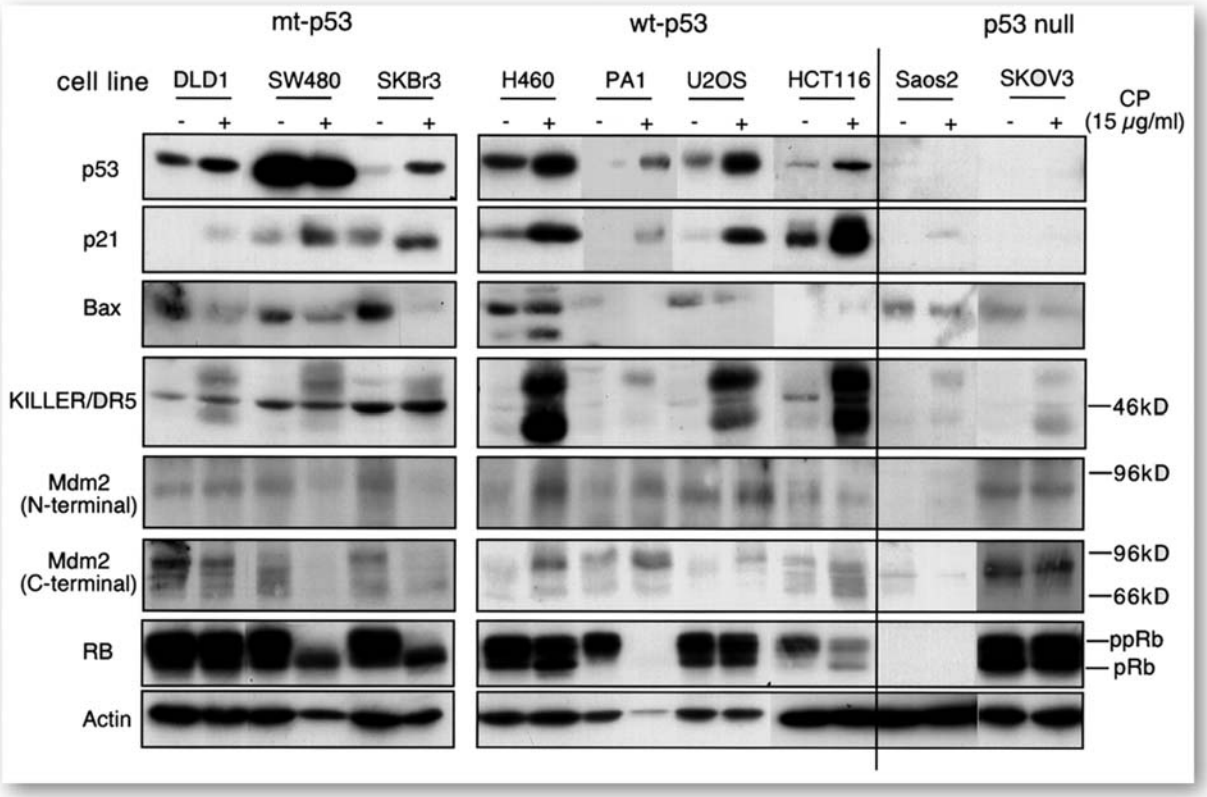

Figure 4. CP-31398 induces the expression of p53-regulated proteins. Western blot analysis was performed to determine whether CP-31398 could induce p53-dependent target molecules. Each sample was prepared from total cell lysates harvested following treatment of cell lines (as indicated) with 15 μg/ml of CP-31398 for 16 hours. KILLER/DR5 expression was observed as two molecular weight forms indicated by arrows. "-" and "+" represent untreated or CP-31398-treated cells, respectively.

following CP-31398 treatment. Interestingly, KILLER/DR5 was also slightly upregulated by CP-31398 even in p53-deficient SKOV3 and Saos2 cells, probably due to a p53-independent pathway.[32] Furthermore, CP-31398 suppressed the expression of Bax, a proapoptotic molecule, in almost all the cell lines examined, suggesting that Bax may not be involved in the apoptosis induction by CP-31398 through transcriptional activation. However, when we exposed HCT116 *Bax-/-* cells to CP-31398, the cells underwent apoptosis with delayed kinetics compared to parental HCT116 cells (Fig. 5). This result suggests other models for the involvement of Bax, e.g. possible effects on its mitochondrial translocation contributing to apoptosis following CP-31398 exposure. In the case of Saos2 cells, we observed weak induction of both $p21^{WAF1}$ and KILLER/DR5 (Fig. 4), indicating that CP-31398 may have p53- and p73-independent mechanisms to upregulate these genes. Interestingly, the induction of Mdm2 was not observed in mutant-p53 expressing cells after exposure to CP-31398 revealed by use of two different antibodies, indicating that CP3198 may not generically modify a mutant-p53 conformation to one capable of upregulating all p53 targets (Fig. 4). These results reveal that not all known p53 targets are upregulated by CP-31398, a p53-independent increase of some p53 targets occurs, and there appears to be a lack of correlation between regulation of known death inducers and observed apoptosis following CP-31398 exposure.

CP-31398 Fails to Kill the E6-Expressing U2OS Cells. In order to investigate whether CP-31398-induced apoptosis requires p53 in wild-type expressing cells, we established an E6-expressing U2OS cell line. When the E6-expressing U2OS cells, designated as E6-F6 and -F8, were exposed to CP-31398, the cells did not undergo apoptosis as determined by sub-G1 content within the cell cycle profile (data not shown) or by the active-caspase 3 assay (Fig. 6A). We also tested the other E6-expressing cell lines, PA1-E6 and H460-E6, and observed that CP-31398-induced apoptosis was decreased as compared to Neo-expressing control cell lines (Fig. 6C). Western blot analysis revealed that in E6-transfected cell lines p53 stabilization was abrogated in PA1-E6 cells and partially decreased in H460-E6 cells (Fig. 6D). These results suggest that while the mechanism(s) of CP-31398-induced apoptosis isn't fully understood, p53 may be required for inducing apoptosis in some cell lines. Moreover, CP-31398 appears incapable of blocking the effects of E6 protein on the ubiquitin-mediated proteolysis of wild-type p53 protein.

Combination of CP-31398 with Chemotherapeutic Agents or TRAIL Enhances Cancer Cell Killing. In order to test the possibility for amplification of cancer cell killing by the combination of CP-31398 with chemotherapeutic drugs, we exposed cancer cell lines to several DNA damaging agents with or without CP-31398. We chose two cell lines, H460 and DLD1, that underwent cell cycle arrest after CP-31398 exposure, in order to determine if the presence of a chemotherapeutic agent might lead to cell death as the dominant phenotype. We first assessed cell viability by using an MTT assay. As shown in Figure 7A, the combination of CP-31398 enhanced the observed cell growth inhibitory effect of chemotherapeutic agents in an additive manner. After exposure to DNA damaging agents alone, such as VP-16, Adriamycin, and Cisplatin, flow cytometric analysis revealed that both cell lines were undergoing G2/M arrest. However, the combination of CP-31398 with these drugs appeared to induce apoptosis of both cell lines (Fig. 7B). The results suggest that CP-3198 may enhance the cell killing effects of chemotherapeutic agents possibly through augmentation of p53 function. The induction of apoptosis by the addition of the chemotherapeutic agents to CP-31398 under conditions where CP-31398 alone caused cell cycle arrest suggests that a second signal may permit cell death.

We also examined the effect of combination of CP-31398 with TRAIL because we observed strong induction of KILLER/DR5 after CP-31398 exposure. As expected, the enhancement of TRAIL-mediated cell killing effect was observed in H460 cells (Fig. 7C). It remains to be determined if with the combination of CP-31398 plus chemotherapeutic agents or TRAIL the resulting cell death is still p53-dependent or whether the upregulation of KILLER/DR5 is the underlying cause for sensitization.

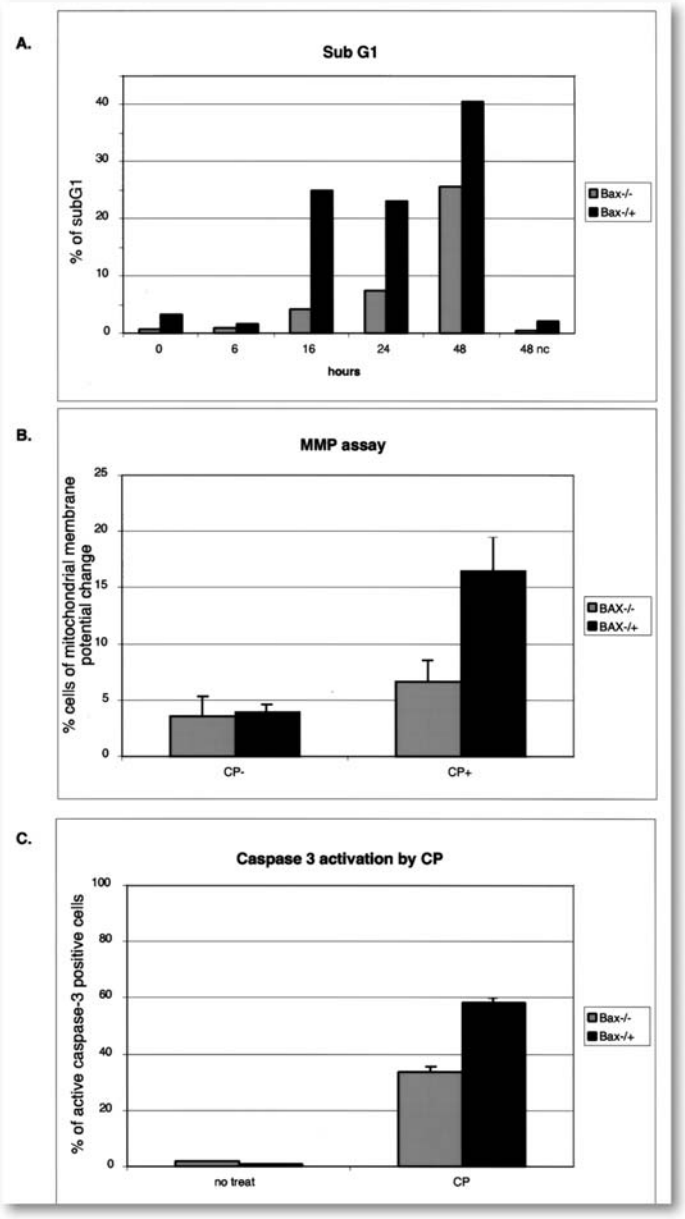

Figure 5. Induction of apoptosis by CP-31398 in Bax null cells is delayed. A) Effect of CP-31398 on the subG1 phase. After treatment with CP-31398, HCT116-Bax +/- or HCT116-Bax -/- cells were harvested and analyzed by flow cytometry as described in Material and Methods. Percent of subG1 phase is shown. B) Effect of Mitochondrial membrane potential change in Bax -/- cells. C) Active-caspase 3 assay for Bax-/- and Bax-/+ cells exposed to CP-31398.

Early Induction of p21WAF1 mRNA May Contribute to CP-31398-Induced Cell Cycle Arrest. To more globally investigate differences between arresting and apoptotic cells following CP-31398 treatment, we examined the expression of 1100 genes using Affymetrix GeneChip Array screening. Among several upregulated genes out of 1100 genes, $p21^{WAF1}$ was found to be induced in both arresting and dying cells (Table 2). This induction of p21 during cell cycle arrest or apoptosis has been previously observed.[33] As shown in Figure 8A, the induction of $p21^{WAF1}$ following CP-31398 exposure was observed early consistent with a role in promoting cell cycle arrest. However, upon CP-31398 exposure, expression of Bax was suppressed in DLD1 and SW480 cells and KILLER/DR5 was upregulated in all cell lines tested in this experiment, suggesting that neither of them appeared to be a determinant for cell cycle arrest and apoptosis induced by CP-31398. We

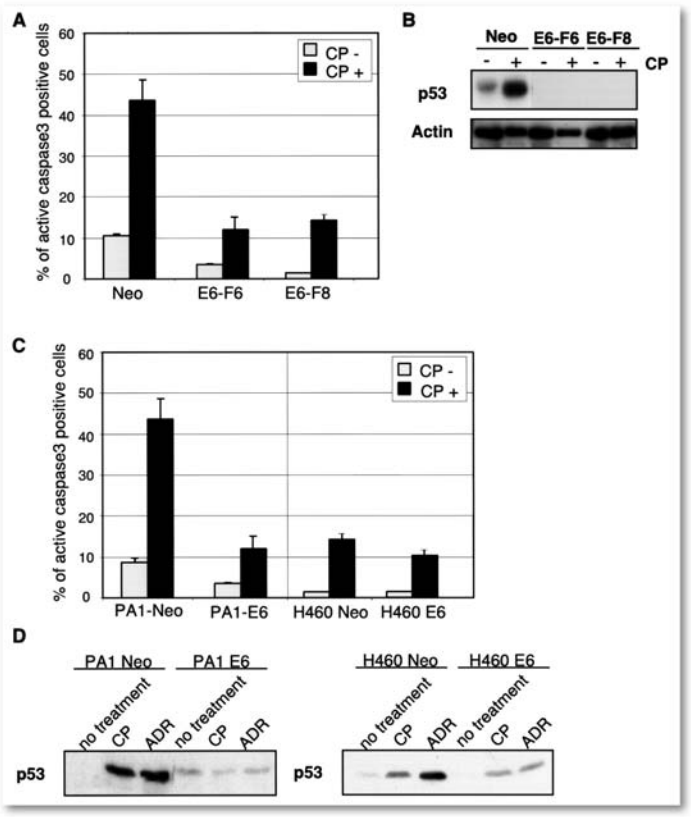

Figure 6. Human Papillomavirus E6 prevents cells from undergoing apoptosis following CP-31398 exposure. A, C) Active-Caspase 3 assay for determination of apoptosis induced by CP-31398. Cells were treated with 15 μg/ml of CP-31398 for 16 hours and harvested as described in Materials and Methods. B) Total cell lysates from U2OS-Neo (Neo), E6-F6 (F6) and E6-F8 (F8) were prepared at 16 hours after CP-31398 (15 μg/ml) exposure. "-" and "+" represent untreated or CP-31398-treated cells, respectively. Actin was used as a loading control. D). Either Neo- or E6- transfectant of PA1 and H460 cells were exposed to 15 μg/ml of CP-31398 or 0.2 μg/ml of Adriamycin for 16 hours, and then harvested for p53 Western blot analysis.

also tested other p53 target genes, such as p53R2, PERP, PIDD, Noxa, and p53AIP1 which have been reported as p53-dependent apoptosis inducing genes, but we were unable to observe significant changes of gene expression using the two cell lines (Fig. 8B). It should be noted that some of the transcriptional regulators, such as TBP-associated factor, and TATA binding protein-associated phosphoprotein (DR1), were upregulated in SW480 and DLD1 cells at an early time point following CP-31398 exposure (Table 2). Interestingly, in the case of SW480 cells that undergo apoptosis, the inhibitor of apoptosis protein 1 (IAP1) was suppressed by CP-31398 exposure, suggesting that inhibition of IAP1 may be involved in apoptosis induced by CP-31398. Furthermore, although the role of Cyclin G2 in the apoptotic pathway has not yet been clarified, the strong induction of Cyclin G2 by CP-31398 may indicate a new function other than cell cycle regulation.

DISCUSSION

Administration of CP-31398, a modifier of mutant-p53 DNA-binding domain conformation, to tumor-bearing mice appeared safe and suppressed tumor growth in-vivo.[23] However, it remained to be determined how CP-31398 inhibits cancer cell growth and the range of p53 mutants that can be modified by CP-31398. In this respect, we tested the effect of CP-31398 on a panel of nine human cancer cell lines. Among the cell lines carrying either mutant or wild-type p53, six were found to undergo apoptosis following CP-31398 exposure. This

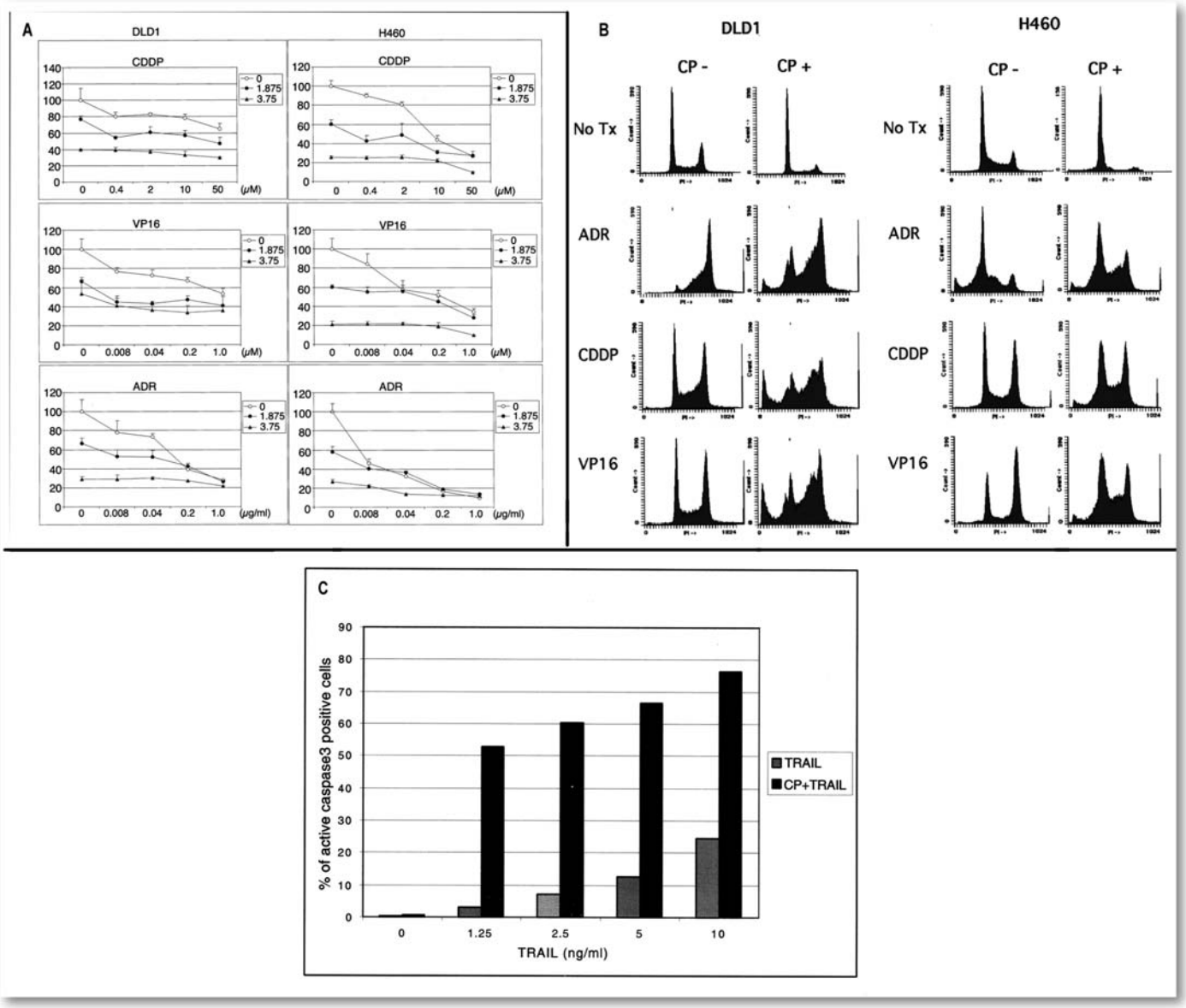

Figure 7. CP-3198 enhances cancer cell killing induced by chemotherapeutic drugs or TRAIL. A) Effect of combination treatment of chemotherapeutic drugs and CP-31398 on the viability of H460 and DLD1. Cells were treated with increasing doses of chemotherapeutic agents (as indicated) in the absence (open circle) or presence (solid circle, 3.75 μg/ml; solid triangle, 1.875 μg/ml) of CP-31398 for 48 hours. B) Cell cycle profile of H460 and DLD1 treated with various DNA damaging agents in the presence or absence of 15 μg/ml CP-31398. C) H460 cells were exposed to CP-31398 at a final concentration of 15 μg/ml for 4 hours, and then TRAIL was added at final concentrations of 1.25, 2.5, 5 and 10 ng/ml respectively together with anti-HIS antibody (1 μg/ml). After 4 hours of incubation at 37°C, the cells were collected and subjected to Active caspase-3 assay.

observation led us to further investigate the induction of p53-downstream molecules associated with the apoptotic signaling pathway. As shown Figure 4, the proapoptotic molecule KILLER/DR5, but not Bax, was upregulated by CP-31398 and DR5 was especially highly induced in wild-type p53 expressing cells. This implies that the combination of TRAIL and CP-31398 may provide a new therapeutic strategy for enhancing cell killing effects.

We speculate that the downregulation of Mdm2 in the mutant-p53 expressing cells after CP-31398 exposure might be due to insufficient modification of p53 to induce Mdm2 or possibly altered activities of coactivators such as p300 or other factors.[28,34] The downregulation of Mdm2 may enhance the transactivation potential of p53 in some cell lines. As the case of MDM2, Bax downregulation may be due to insufficient modification of p53 to transactivate the Bax gene, or possibly a modification that leads to repression. The observation that CP-31398 induced apoptosis is reduced in *Bax*-null cells suggests that *Bax* may play a role in the observed cell death through mechanisms other than transcriptional induction by p53, i.e., translocation of preexisting *Bax* to the mitochondrial membrane. It has been reported that p73-dependent apoptosis is required for E2F-1.[35,36] In addition, the p53 homologue p63 has been shown to play an important role in apoptosis induction upon some forms of DNA damage.[37] However, we found no evidence that E2F-1, p73 or p63 proteins are stabilized after CP-31398 exposure (data not shown). We are currently investigating whether the activity of these proteins might be altered by CP-31398, independent of protein stabilization.

Table 2 **GENE EXPRESSION PROFILE OF CP-31398-EXPOSED CELLS**

	repressed genes		induced genes	
	Description of genes	fold induction*	Description of genes	fold induction*
Arrest (DLD1)	Tumor necrosis factor receptor 2 related protein	-3.7	Wild-type p53 activated fragment-1 (WAF1)	17.1
	c-myb	-2.4	jun-B	6.3
	Cytochrome P(1)-450	-1.8	Histone H1	5.7
	Type 3 inositol 1,4,5-trisphosphate receptor (ITPR3)	-1.8	Insulin-like growth factor binding protein 4 (IGFBP4)	5.3
	Lipocortin II	-1.5	Type 2 inositol 1,4,5-trisphosphate receptor	4.3
			TBP-associated factor (hTAFII100)	4.0
			c-fos	3.8
			Keratinocyte growth factor	3.6
			FGF-9	3.6
			Dual-specificity protein phosphatase	3.1
			Thrombospondin-4	3.0
			KDR/flk-1 protein	2.6
			Inositol polyphosphate 1-phosphatase	2.4
			Protein tyrosine kinase	2.0
			Ubiquitin-Conjugating Enzyme Ubch5	1.8
			TATA binding protein-associated phosphoprotein (DR1)	1.7
			Myeloid cell differentiation protein (MCL1) mRNA	1.7
			MAP kinase kinase 3 (MKK3)	1.7
			TGF-beta superfamily protein	1.4
			Human protein synthesis factor (eIF-4C)	1.3
Apoptosis (SW480)	Tumor necrosis factor receptor	-4.7	Interleukin 8 (IL8)	11.5
	TGF-betaIIR alpha	-3.1	Cyclin G2	10.2
	Int-2 proto-oncogene	-3.1	Multidrug resistance-associated protein homolog (MRP5)	7.1
	Inhibitor of apoptosis protein 1 (IAP1)	-2.8	Heat shock protein HSPA2	5.9
	Homeobox protein (HOX7)	-2.7	Melanoma growth stimulatory activity (MGSA)	5.5
	Kinesin-related protein, partial cds	-2.6	Transcription factor (TFIIB) mRNA, complete cds	4.4
	c-myc	-2.4	Cdk-inhibitor p57KIP2 (KIP2)	4.0
	Urokinase-type plasminogen activator receptor	-2.2	Cytochrome P(1)-450	4.0
	Serine/threonine protein kinase	-2.2	Homeobox protein (HOX-1.3)	3.6
	MAD-related gene SMAD7 (SMAD7)	-2.1	Human TR3 orphan receptor	3.0
	CDK8	-2.0	c-fos	2.8
	Protein tyrosine phosphatase (PTPase)	-2.0	Prostate carcinoma tumor antigen (pcta-1)	2.3
	NF-kappa-B	-2.0	TGF-beta superfamily protein	2.1
	Translation initiation factor 5 (eIF5)	-2.0	TBP-associated factor (hTAFII100)	2.1
	BRCA1-associated RING domain protein (BARD1)	-2.0	Insulin receptor substrate-1	2.0
	Histone H1(0)	-2.0	Histone H2A	1.9
	Human steroid receptor (TR2-11)	-1.9	14-3-3 epsilon	1.7
	phosphatidylinositol-glycan-class C (PIG-C)	-1.9	Tumor susceptiblity protein (TSG101)	1.5
	NF-kappa-B transcription factor p65 subunit	-1.8		
	Ret Transforming Gene	-1.8		
	Casein kinase I delta	-1.8		
	GAP binding protein p62dok (DOK)	-1.8		
	Heat shock protein 40	-1.8		
	Farnesyltransferase alpha-subunit	-1.7		

*Fold induction was obtained by subtraction of CP31398 treated-gene profile from untreated-one using AffyMetrix software.

The results showing an enhanced cell killing effect of chemotherapeutic drugs by CP-31398 imply that this agent may be useful in reducing the effective dose of certain drugs, possibly reducing their toxicity. The effect of addition of chemotherapeutic drugs to induce apoptosis in cancer cells that undergo cell cycle arrest following CP-31398 exposure is of interest. This result suggests that mutant or wild-type p53 present in those cells has the potential to induce apoptosis given appropriate signals. We are currently investigating whether cells exposed to CP-31398 plus chemotherapeutic agents undergo apoptosis that is p53-dependent.

Gene array profiles indicate there may be p53-independent effects of CP-31398 as well as p53-dependent gene induction. Further examination will be necessary to clarify the mechanism of p53 modification and/or a possible p53-independent cell killing effect of CP-31398. Taken together, although CP-31398 seems to be a potent therapeutic agent for cancer therapy, it appears not to be totally specific for p53 modification. We propose that array screening may be a powerful tool to examine derivatives of CP-31398 and screen for p53 conformation modifying agents with higher specificity for p53.

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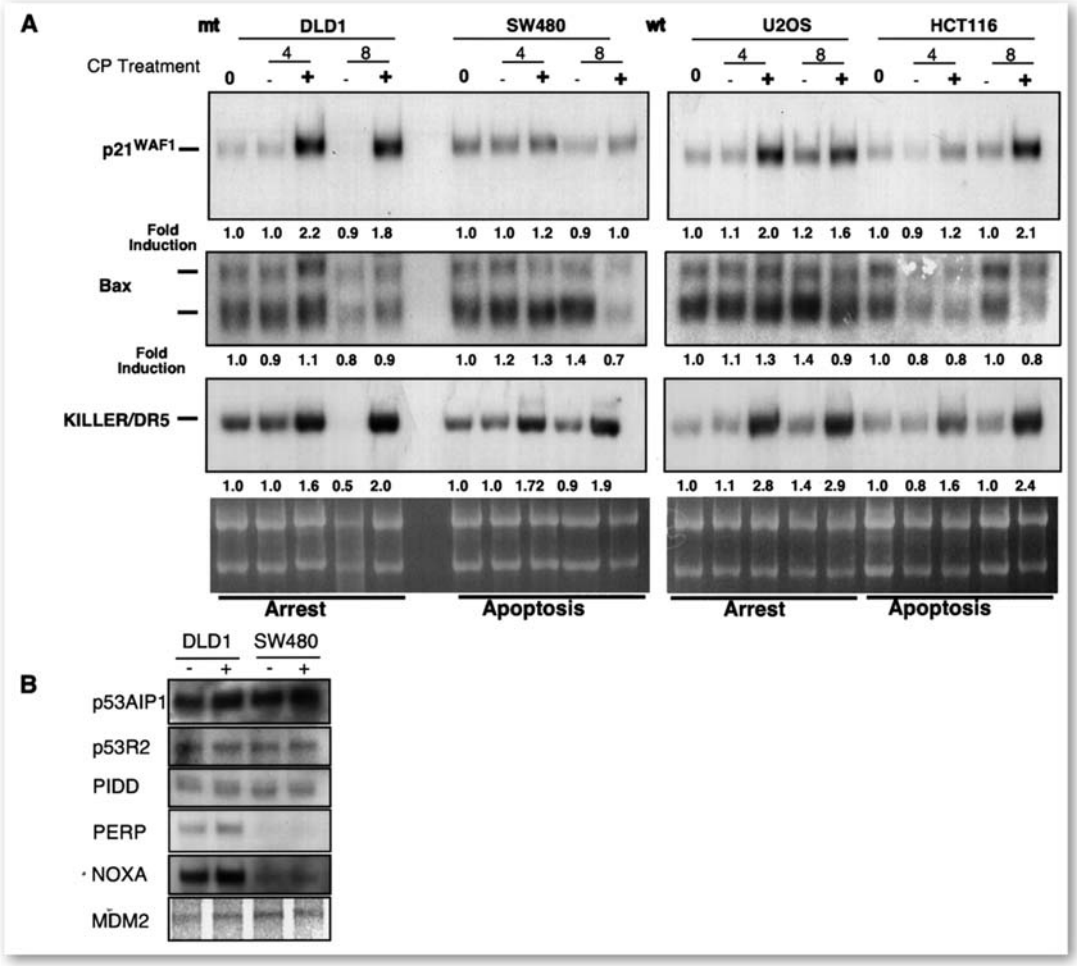

Figure 8. p21^{WAF1} is an early response gene induced by CP-31398. Several p53-response genes were examined by Array screening to understand the underlying mechanism of p53–dependent cell cycle arrest and apoptosis induced by CP-31398 (Table 2). A) Cells were harvested after treatment with CP-31398 and total RNA was isolated as described in "Materials and Methods". Left two cell lines, DLD1 and SW480, are expressing mutant p53 (indicated as "mt"), whereas right two cells, U2OS and HCT116, are expressing wild-type p53 (indicated as "wt"). After transferring total RNA to membrane, mRNA expression was detected by ^{32}P-labeled cDNA probes as indicated. B) p53-dependent apoptosis inducing genes were not affected by CP-31398 exposure. mRNA expression was quantified by Imagequant software and shown as a fold induction.

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