p53 and WAF1 Are Induced and Rb Protein Is Hypophosphorylated during Cell Growth Inhibition by the Thymidylate Synthase Inhibitor ZD1694 (Tomudex)

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SUMMARY

In a previous study, we found that treatment of HCT-8 cells with ZD1694, a specific antifolate-based thymidylate synthase inhibitor, resulted in DNA fragmentation. In this study, we have demonstrated the dose- and time-dependent induction of DNA fragmentation accompanied by elevation of p53 and WAF1 protein expression by ZD1694. WAF1 mRNA showed a time-dependent increase, whereas p53 mRNA was not found to be significantly overexpressed. The initial increase in WAF1 mRNA was detected at 4 hr, but increased WAF1 protein expression was detected 8–24 hr after a 2-hr exposure. The amount of total and hypophosphorylated pRb seems to be rising greatly after ZD1694 exposure. The effects of ZD1694 on the expression of E2F1 and formation of the E2F1-Rb complex were investigated after a 2-hr drug exposure (IC_{90}). The results showed a time-dependent decrease in E2F1 mRNA and protein expression; an increase in the abundance of the E2F-Rb complex could be demonstrated beginning 4 hr after drug exposure by a gel shift assay. Kinetic analysis showed increased availability of hypophosphorylated pRb for inhibition of E2F, which could indirectly result from WAF1-induced inhibition cyclin-dependent kinase activity. Whereas thymidylate synthase inhibition by ZD1694 was rapid in onset and maintained for at least 24 hr after drug treatment, drug-induced cellular growth inhibition was significant 24 hr after drug exposure. The increased abundance of hypophosphorylated pRb and binding to transcription factor E2F-1 is consistent with ZD1694-induced cell growth inhibition in HCT-8 cells. Therefore, the observed effect on downstream events after effective inhibition of thymidylate synthase may offer the critical determinants of response to ZD1694.

TS is a target for the treatment of patients with solid tumors. ZD1694 [*N*-{5-[*N*-(3-, 4-dihydro-2-methyl-4-oxo-quinazoline-6-yl-methyl)-*N*-methylamine]-2-theroyl}-L-glutamic acid], an analog of the folate cofactor, was identified as a potent TS inhibitor with cell growth inhibition *in vitro* and significant antitumor activity *in vivo* (1). The rapid intracellular uptake of ZD1694 and its retention in the form of higher chain length polyglutamate derivatives contribute to its antitumor potency (2). Previous studies in this laboratory have shown that ZD1694 treatment induced extensive fragmentation of genomic and newly synthesized DNA (3, 4). Preliminary results obtained in collaboration with Dr. W. Aherne and colleagues (The Institute of Cancer Research, Royal Cancer Hospital, Surrey, UK) demonstrated that this effect correlated with a significant increase in dUTP pools and a concomitant decrease in dTTP pools.

The possible roles of WT p53 in the DNA damage response have been investigated previously (5, 6). WT p53 recently has been found to activate a number of genes, including a cyclin-CDK inhibitor, WAF1 (7, 8), which inhibits the activity of cyclin-associated kinases that normally phosphorylate the Rb protein (9, 10). These observations suggest that there is a relationship between the functional status of suppressor gene products (p53 and WAF1) and Rb protein phosphorylation after DNA damage. The data reported implicate p53, WAF1, and Rb protein phosphorylation to be molecular factors associated with ZD1694-induced DNA damage.

This laboratory demonstrated that a TS inhibitor, FdUrd, was capable of decreasing c-*myc* gene expression in association with the induction of high-molecular-weight DNA fragments (11). These data suggest that events subsequent to the TS inhibition and DNA breakage may be responsible for the observed growth arrest by TS inhibitors. A study by Chu *et al.* in colon cancer cells (12) has shown that human recombinant TS protein binds to the human c-Myc mRNA, forming a

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ABBREVIATIONS: TS, thymidylate synthase; SRB, sulforhodamine B; dThyd, thymidine; CFGE, constant field gel electrophoresis; DSBs, double-strand breaks; WT, wild-type; WAF1, p21$^{WAF1/CIP1}$; Rb, retinoblastoma; DHFR, dihydrofolate reductase; G3PDH, human glyceraldehyde-3 phosphate dehydrogenase; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ribonucleoprotein complex. These data suggest that TS protein may be involved in the regulation of c-Myc mRNA.

Transcription factor E2F is now recognized as an important mediator of cell proliferation control (13). E2F is a family of transcription factors that heterodimerizes with DP1 or DP2 and binds DNA. E2F1-E2F3 form complexes with hypophosphorylated forms of pRb (14), which inhibit transcriptional transactivation of gene expression (15). Overexpression of E2F-1 has been shown to induce quiescent cells to enter S phase, providing further evidence that E2F-1 functions as a transcriptional regulator of cell cycle progression (16). Overexpression of E2F-1 also drives cells into apoptosis (17). Recent studies have shown that direct binding of pRb to E2F-1 is capable of inhibiting E2F-1-mediated transactivation (18). Because the central role of E2F is transcriptional regulation of many genes involved in cell growth control (19, 20), we reasoned that E2F-1 might be involved in cell growth inhibition by ZD1694 (21).

In this study, we investigated the possible relationship between p53/WAF1 expression and pRb phosphorylation in the initial response to treatment with ZD1694 before cell growth arrest. We demonstrated that the accumulation of hypophosphorylated pRb correlated with the abundance of the E2F-pRb complex, suggesting a possible mechanism in which pRb hypophosphorylation may contribute to the transcription control during cell growth inhibition induced by ZD1694.

Materials and Methods

Drugs and chemicals. ZD1694 (Tomudex) was kindly supplied by Zeneca Pharmaceuticals (Macclesfield, UK). The compound was dissolved in 0.1 M sodium bicarbonate, pH 8.3, at 10 mM with final dilutions made in the growth medium. Proteinase K and RNase A were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). [^{14}C]dThyd (specific activity, 56 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). SRB, dThyd, and sarkosyl were purchased from Sigma Chemical (St. Louis, MO).

Cell culture. The human ileocecal adenocarcinoma cell line HCT-8 was obtained from American Type Culture Collection (Rockville, MD) and maintained as monolayer in Roswell Park Memorial Institute 1640 medium supplemented with 10% dialyzed horse serum (GIBCO, Grand Island, NY) and 1 mM sodium pyruvate. The doubling time of the cells is approximately 20 hr. All treatments were carried out using exponentially growing cell cultures.

Cell growth inhibition assay. Cell growth inhibition was determined using the total protein SRB assay as described elsewhere (22). Briefly, 1000 cells/well were seeded in 96-well plates. After 24 hr, exponentially growing cells were treated with drug for 2 hr. At 72 hr after drug exposure, the cells were fixed with 10% trichloroacetic acid and further processed according to the published SRB procedure. The absorbance was measured at 570 nm using an automated Bio Kinetics reader (Model EL 340; Bio Tek Instruments, Winooski, VT).

Preparation of agarose plugs. The procedure for preparation of DNA plugs is a modification of those by Schwartz *et al.* (23) and Giaccia *et al.* (24). Approximately 5×10^6 cells were washed using HBSS and resuspended in 0.1 ml of HBSS. An equal volume of 2% low-melting-point agarose prepared in HBSS was added at 50°. The mixture was immediately poured into the molds. Cells embedded in agarose were digested for 24 hr in more than 20 volumes of lysis buffer, containing 0.5 M EDTA, pH 8.0, 10 mM Tris, 1% sarkosyl, and 1 mg/ml proteinase K, and then incubated for 1 hr in Tris/EDTA buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA) containing 0.2 mg RNase A/ml. Each plug contained approximately 1×10^6 cells.

CFGE analysis for DNA DSBs. For CFGE, DNA plugs were inserted into wells of a 1.8% agarose gel, sealed with a small amount

of agarose and electrophoresed in Tris/acetate/EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) at room temperature. After electrophoresis, gels were stained with ethidium bromide (5 μg/ml in H_2O) and then photographed on a UV transilluminator. For quantitative analysis of DNA fragmentation, the cells used were prelabeled with [^{14}C]dThyd (0.025 μCi/ml) for 24 hr. After CFGE, each band was cut out and the quantity of [^{14}C]-labeled DNA was determined by scintillation counting. For estimation of DNA fragment size, 1 kb DNA ladder (GIBCO BRL, Gaithersburg, MD) was used.

Western blotting. Analysis of p53, WAF1, E2F-1, and Rb protein expression was performed by Western blot. HCT-8 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris·HCl, pH 8, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 trypsin inhibitor units of aprotinin) and the protein content was determined by the BioRad DC protein assay (BioRad, Hercules, CA). Samples containing 50 μg of total protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membrane (BioRad). Western blotting was performed with anti-p53 monoclonal antibody IgG$_{2a}$ (DO-1), anti-E2F-1 monoclonal antibody IgG$_{2a}$ (KH95), and anti-Rb monoclonal antibody IgG$_1$ (IF8) (Santa Cruz Biochemicals, Santa Cruz, CA), and anti-WAF1 monoclonal antibody IgG$_1$ (Ab-1) (Oncogene Research Products, Cambridge, MA) using the Renaissance chemiluminescence reagent kit (DuPont-New England Nuclear, Boston, MA).

RNA extraction. Total cellular RNA was extracted from HCT-8 cells using TRIzol Reagent (GIBCO BRL) according to manufacturer's instructions. RNA was precipitated in 75% ethanol at −20° and resuspended in autoclaved diethylpyrocarbonate-treated water.

Northern blotting. RNA (20 μg) was separated by electrophoresis through 1.2% agarose-formaldehyde gels, transferred onto the Hybond-N membranes (Amersham Life Science, Clearbrook, IL) by capillary blotting and hybridized to the following [^{32}P]-labeled cDNA: WAF1 reverse transcription-polymerase chain reaction product (HCT-8 cells); E2F-1 cDNA, which was cloned into the pRcCMV vector, kindly provided by Dr. W. Kaelin (Dana Farber Cancer Institute and Harvard Medical Center, Boston, MA); and human G3PDH control probe (Clontech, Palo Alto, CA). The membranes were exposed to DuPont film at −70° using DuPont lighting plus intensifying screens (DuPont-New England Nuclear).

Gel mobility shift assay. Whole-cell extracts were prepared from HCT-8 cells by freeze-thaw lysis in buffer containing 500 mM KCl and inhibitors of phosphatase and protease as described previously (25). Gel mobility shift assay was performed as described elsewhere (26). The reaction mixture contained 0.2–0.5 ng of labeled DHFR promoter fragment (spanning from nucleotide position −13 to −103, relative to ATG initiation codon at +1) (18) in 20 μl of binding buffer (20 mM HEPES, pH 7.8, 1 mM MgCl$_2$, 0.1 mM EDTA, 40 mM KCl, 0.5 mM dithiothreitol, 2 μg of salmon sperm DNA, 60 μg of bovine serum albumin, 1% Ficoll, and 3–5 μg of whole cell extracts). For deoxycholate experiments, samples were incubated with 0.25% deoxycholate, after which Nonidet P-40 was added to a final concentration of 1.5%. When indicated anti-pRb antibody (Santa Cruz Biochemicals) or anticyclin A antibody (gift from Dr. J. Nevins) was added to the reaction before the DNA probe. In some reactions, WT DHFR sequence, including the E2F site or the same region with a double point mutation abolishing E2F binding (18), was added at a 20-fold molar excess for competition. Binding reactions were performed at room temperature for 20 min and gels (4% polyacrylamide) were electrophoresed for 60–80 min at 4° and then dried and exposed for autoradiography.

Results

Cell proliferation inhibition. To identify the onset of significant cell growth inhibition after a 2-hr drug exposure of HCT-8 cells to ZD1694, analyses of cell growth kinetics
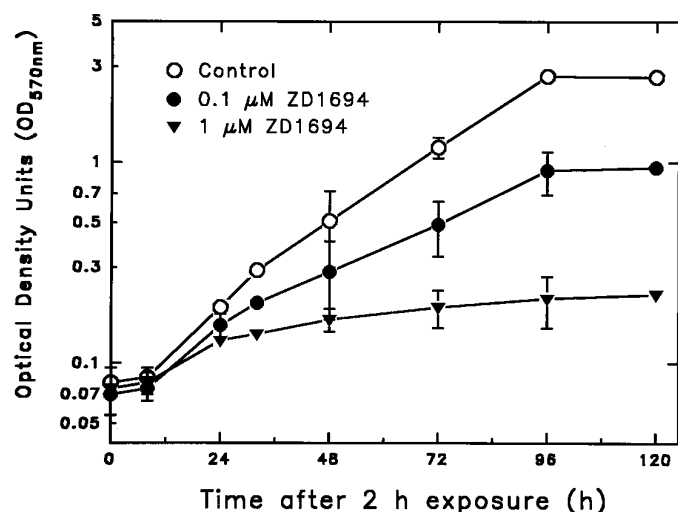
Fig. 1. Dose-time relationship of *in vitro* cytotoxicity. Exponentially growing cells were treated with indicated concentrations of ZD1694 for 2 hr and then incubated in drug-free medium for the indicated times after drug exposure. The inhibition of cell growth was based on the measurement of total protein assay (SRB assay). *Symbols*, mean ± standard deviation of at least three experiments.

were performed. Exponentially growing cells were exposed for 2 hr to 0.1 and 1 μM ZD1694, washed twice, and then either fixed with trichloroacetic acid or incubated in drug-free medium up to 120 hr. The data in Fig. 1 indicate that treatment with 1 μM ZD1694 resulted in an S-phase block (27) and significant growth suppression beginning 24 hr and persisting up to 120 hr after drug treatment.

Dose- and time-dependent relationship between DNA double-strand breaks, p53 mRNA, and protein expression. Treatment of HCT-8 cells with ZD1694 led to a dose- and time-dependent induction in DNA DSBs as shown in Fig. 2. Under the same experimental conditions, expression of p53 proteins was increased in a dose- and time-dependent manner as well (Fig. 3A). Moreover, both the extent of DNA damage and p53 protein abundance were maximal 16–24 hr after 2-hr drug exposure. In contrast, an inverse relationship between p53 mRNA and protein expression was observed; p53 protein expression increased in a dose- and time-dependent manner, whereas p53 mRNA levels decreased (Fig. 3B).

WAF1 gene and protein overexpression after ZD1694 exposure. To investigate the molecular alterations downstream of p53, expression of a putative p53 downstream effector, WAF1 (cyclin-dependent kinase inhibitor), was examined. The data in Fig. 4 indicate that the increase in the p53 protein expression after ZD1694 exposure coincided with increased WAF1 mRNA and protein expression. Similar to p53, the increase in WAF1 protein expression was concentration- and time-dependent. The increase in WAF1 mRNA levels was detected after 4 hr, whereas the increased WAF1 protein expression was detected at 8–24 hr after 2-hr exposure.

Phosphorylation status of Rb protein. As shown in Fig. 5, detection of Rb protein in whole-cell lysates by immunoblot reveals a complex pattern of Rb protein species, including the more rapidly migrating hypophosphorylated form (pRb107) and a number of more slowly migrating hyperphosphorylated forms (ppRb110–114). To investigate the expres-
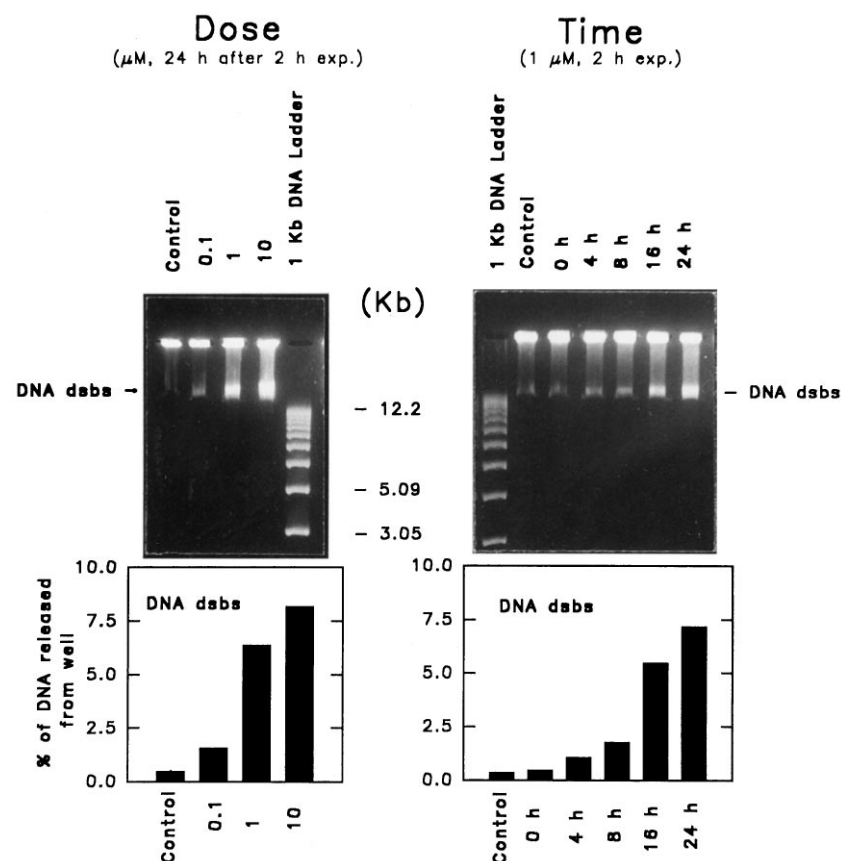
Fig. 2. Dose- and time-dependent induction of DNA DSBs. *Left*, exponentially growing cells were treated with 0.1, 1, and 10 μM ZD1694 for 2 hr and then incubated in drug-free medium for an additional 24 hr. *Right*, to identify a time-dependent drug effect, the cells were exposed to 1 μM ZD1694 for 2 hr and then incubated in drug-free medium for 0, 4, 8, 16, and 24 hr. *Bottom*, DNA damage assay was performed using CFGE with quantitation of the percentage of DNA released from the well. Agarose plugs containing DNA were processed as described in Materials and Methods.
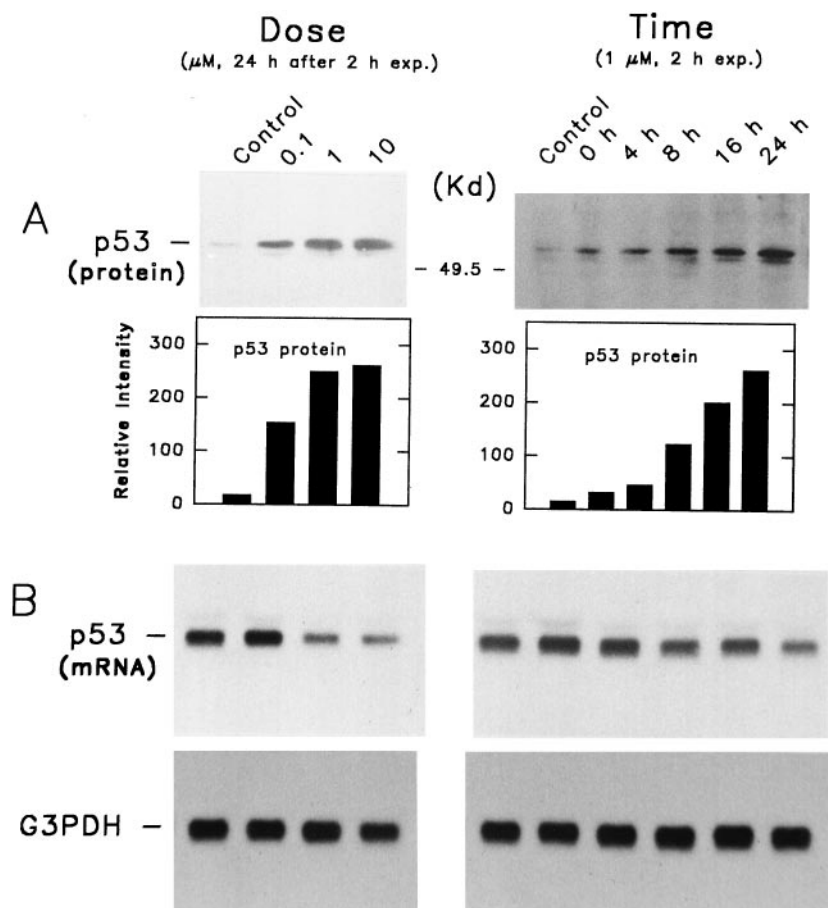
Fig. 3. p53 expression. Exponentially growing cells were exposed to ZD1694 for 2 hr and then incubated in drug-free medium as described in Fig. 2A. For Western blotting, 50 μg of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The anti-p53 monoclonal antibody IgG_{2a} (DO1) was used for detection of p53 protein. Relative levels of p53 protein were measured by using densitometry. B. For Northern blot, 20 μg RNA was separated by electrophoresis through 1.2% agarose-formaldehyde gels, transferred onto the Hybond-N membranes, and hybridized to the ^{32}P-labeled p53 and G3PDH cDNA.

sion and phosphorylation status of Rb protein in relation to functional p53 and WAF1 activity, we examined Rb protein expression and phosphorylation after treatment of cells with ZD1694. The total levels of pRb seemed to be rising greatly. Hypophosphorylation of Rb protein was increased by 4 hr and continued to increase until 24 hr after treatment.
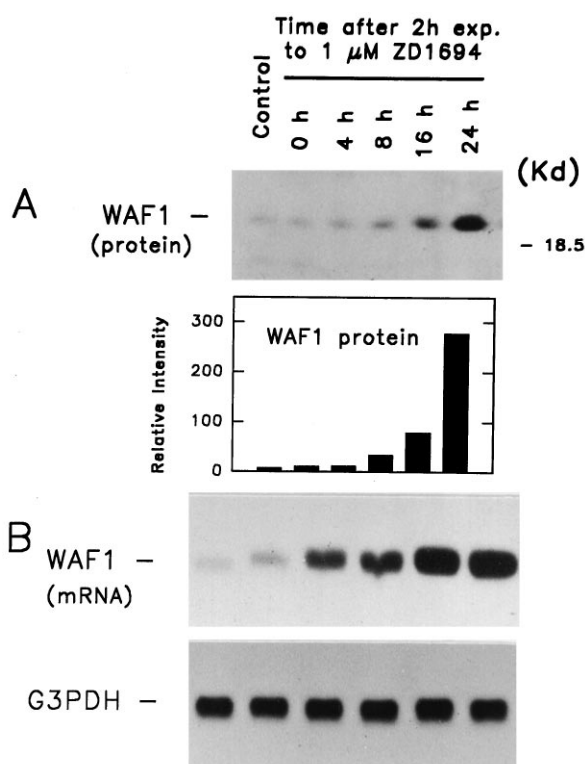
Kinetics of E2F-1 mRNA and protein expression. The previous data suggest a possible relationship between p53 protein levels, WAF1 protein and mRNA levels, and Rb phosphorylation status. Because E2F exerts an important function in Rb-mediated transcription regulation, it was of interest to examine E2F levels subsequent to ZD1694 exposure. Treatment of HCT-8 cells with ZD1694 resulted in a time-dependent decrease in E2F-1 mRNA and protein expression (Fig. 6). A marked decrease in E2F-1 mRNA and protein expression was observed 16 hr after 2-hr exposure to 1 μM ZD1694. The most pronounced suppression occurred 24 hr after drug treatment. The decrease in E2F-1 protein expression was concomitant with the increased hypophosphorylation of Rb protein (Figs. 5 and 6).

Interaction between Rb protein and E2F-1. The phosphorylation status of Rb is known to regulate the complex formation of E2F and Rb. Hypophosphorylated Rb forms an E2F-Rb complex, which suppresses the transcription of genes important for DNA replication and cell-cycle progression through S phase. This model might provide an explanation for the cell growth arrest observed at 24 hr after ZD1694 exposure. Therefore, to test whether hypophosphorylation of Rb protein subsequent to drug treatment leads to increased

binding of pRb protein to E2F-1, gel mobility shift assays were conducted. Whole-cell lysates of HCT-8 cells at various time points after drug exposure were incubated with DHFR probes containing the E2F-1 binding site. Gel mobility shift assay was then performed as described in Materials and Methods. Fig. 7 demonstrates an increase in the abundance of the pRb-E2F complex, beginning 4 hr after ZD1694 exposure.

Discussion

ZD1694 produced extensive DNA DSBs in a dose- and time-dependent fashion (3, 4) and DNA DSBs seem to be the critical molecular lesions associated with cell arrest and/or death (28). A single unrepaired or misrepaired DNA DSB in the functional copy of a required gene can result in cell death (29). Preliminary results, in collaboration with Dr. W. Aherne, demonstrated that the pools of dUTP increased significantly with a concomitant decrease in dTTP pools after ZD1694 exposure (unpublished data). This imbalance in the dUTP/dTTP pool ratio may lead to repetitive misincorporation of dUTP into repair patches (30). In addition, drug-induced DNA fragmentation in mature DNA may be associated with inhibition of the repair of spontaneous DNA damage (31). We recently reported contrasting patterns of DNA fragmentation induced by ZD1694, demonstrating extensive DSBs in nascent DNA at 24 hr after drug exposure (4). Our preliminary studies also showed that aphidicolin protection of the secondary DNA fragmentation was shown to

Fig. 4. WAF1 expression. Exponentially growing cells were treated with ZD1694 for 2 hr and then incubated in drug-free medium as described in Fig. 3. The determination of WAF1 mRNA and protein expression was performed by Western (A) and Northern blotting (B) as described in Materials and Methods. The anti-WAF1 monoclonal antibody IgG$_1$ (Ab-1) was used for detection of WAF1 protein levels. *Bottom*, relative levels of WAF1 protein were measured by using densitometry.
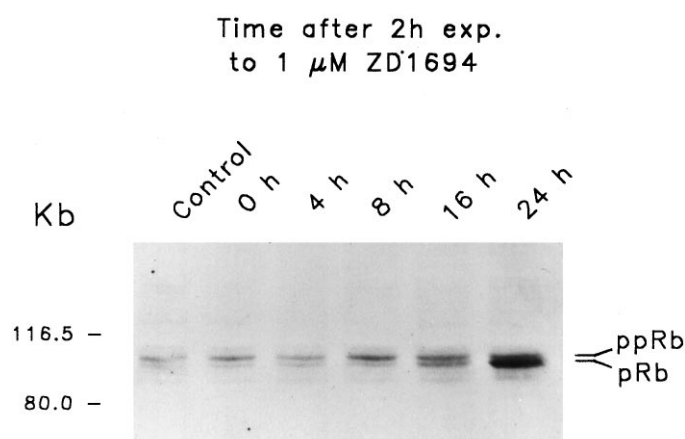
Fig. 5. Phosphorylation status of Rb protein after ZD1694 exposure. Exponentially growing cells were exposed to 1 μM ZD1694 for 2 hr followed by incubation in drug-free medium for 0, 4, 8, 16, and 24 hr after drug exposure. Rb protein was detected by Western blot as described in Materials and Methods. The molecular weights (in kDa) are indicated on the left: 107 kDa, hypophosphorylated Rb protein (*pRb*); 110–112 kDa, phosphorylated Rb protein (*ppRb*).

be minimal. This demonstrates that the secondary DNA fragmentation may be derived primarily from DNA repair synthesis.

Previous data implicate the tumor suppressor protein p53 as a major regulator of cell cycle arrest in mammalian cells
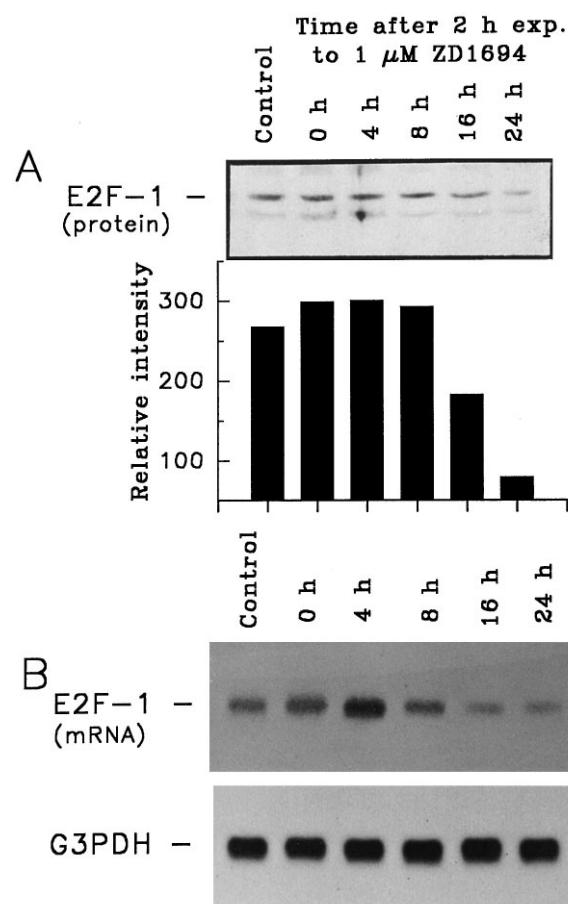
Fig. 6. Temporal alterations of E2F mRNA and protein levels after ZD1694 exposure. Exponentially growing cells were exposed to 1 μM ZD1694 for 2 hr followed by incubation in drug-free medium for 0, 4, 8, 16, and 24 hr. Levels of E2F protein (A) and mRNA (B) were determined by Western and Northern blotting as described in Materials and Methods. The anti-E2F-1 rabbit polyclonal antibody IgG (C-20) was used for detection of E2F-1 protein levels. Relative levels of E2F-1 protein were measured by using densitometry.

(5, 32). WT p53 protein levels were increased in a dose-dependent manner after ZD1694 exposure. Time-course studies of DNA damage by ZD1694 (3) showed a concomitant induction of p53 protein expression with a maximum increase in WT p53 gene product occurring at 24 hr after drug exposure. p53 mRNA levels, however, were found to be decreased after ZD1694 exposure (Fig. 3). The magnitude of increase in the p53 protein levels was not as high as that seen by other laboratories using gel mobility shift assays to examine response to the same agents (33, 34). These results may reflect some qualitative changes in p53 activity causing increased DNA binding in addition to quantitative changes in protein abundance. Moreover, these results suggest that the increase in the p53 protein levels after ZD1694 treatment probably is due to post-transcriptional regulation involving the increased translation or protein stabilization (34, 35).

WT p53 may play a central role in the induction of cell growth inhibition after exposure to ZD1694 in HCT-8 cells. WT p53 protein overexpression after DNA damage transactivates a battery of other effector genes (6), the products of which also might be involved in signal transduction. Data obtained in this laboratory showed that the levels of both WAF1 mRNA and protein were elevated after ZD1694 expo-

Time after 2 h exposure to 1 μM ZD1694
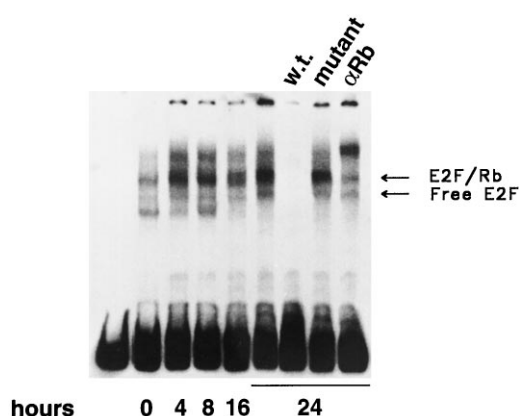
Fig. 7. DNA binding of E2F from cells treated with ZD1694. E2F complexes were assayed using gel mobility shift assay on cellular lysates from HCT-8 cells exposed to 1 μM ZD1694 followed by incubation in drug-free medium for 0, 4, 8, 16, and 24 hr. E2F-specific fragment from the DHFR promoter was used as the DNA probe. Gel mobility shift analysis was performed as described in Materials and Methods. Antibody to pRb was added to the reaction in *lane 9*.

sure and reached a peak 24 hr after 2-hr drug exposure. The magnitude of the increase in the WAF1 protein levels was similar to that seen for the WT p53 protein. In contrast, no induction of WAF1, mRNA, or protein expression occurs in response to ZD1694 treatment of HL-60 cells lacking WT p53 protein (data not shown). Therefore, these data suggest that WAF1 protein induction after ZD1694 exposure may be correlated with WT p53 accumulation in HCT-8 cells.

Recently, WAF1 has been reported to inhibit phosphorylation of Rb protein through its interaction with several cyclin-CDKs *in vitro* (10, 36). To investigate a possible role for Rb protein in the response to ZD1694, we measured Rb protein expression and phosphorylation after ZD1694 exposure. The results showed that the amount of total and hypophosphorylated Rb protein seemed to be rising greatly after ZD1694 exposure. Because E2F exerts an important function in Rb-mediated transcription regulation, it was of interest to detect possible alterations of E2F protein levels and E2F-Rb complex subsequent to exposure to ZD1694. In the present study, Northern and Western blot analyses were performed and revealed that E2F-1 mRNA and protein expression levels were decreased in drug-treated cells compared with levels in untreated cells. Kinetic analysis showed a significant decrease in E2F-1 mRNA and protein expression observed at 16 hr, and the most pronounced suppression occurred 24 hr after exposure to 1 μM ZD1694. This result probably is because E2F-1 transcription is regulated by E2F binding sites in its promoter.

It has been shown that the phosphorylation status of Rb regulates its interaction with E2F; E2F is released when pRb is phosphorylated (37–39). Furthermore, identification of a complex between pRb and cellular transcription factor E2F-1 suggested a direct linkage between pRb and transcription control (40–41). To further examine the role of the phosphorylation status of Rb protein in the transcription regulation, we analyzed the relationship between the formation of the complex of pRb-E2F-1 and pRb phosphorylation status by gel mobility shift assay and Western blot. Our results showed an increase in the total amount of Rb species with the increased

abundance of the pRb-E2F complex after ZD1694 exposure. The results are consistent with dependence of the ability to bind and inhibit E2F on the amount of the hypophosphorylated Rb proteins, not on the ratio of species. Preliminary studies showed that increased WAF1 binding to cyclin E/CDK2 was observed 24 hr after drug exposure (data not shown); the main cause of the increased availability of Rb for inhibition of E2F could be the increased amount of total Rb proteins, in addition to WAF1-induced inhibition cyclin-dependent kinase activity. It is postulated that ZD1694-induced cell growth inhibition is mediated by transcription inhibition by pRb-E2F complex.

In conclusion, as the result of sustained and prolonged inhibition of the target enzyme TS by ZD1694, several downstream cascade events, subsequent to the imbalance in the dUTP/dTTP pool ratio and DNA fragmentation, could provide the basis for the potency of ZD1694. These events in turn affect the regulation of cell proliferation and/or death in response to DNA damage. Although other proteins in addition to pRb can participate in ZD1694-induced cellular growth inhibition, the critical role of E2F-1 and its binding to Rb protein in control of cell cycle proliferation suggests a linkage between the antiproliferative response, transcription factors, and the change in Rb expression and phosphorylation. The study of the downstream molecular events induced by ZD1694 may improve our understanding of the contribution of altered signals to cell proliferation and/or death induced by TS inhibitors. Therefore, only time measurements of the degree of TS inhibition, although necessary, may not be sufficient to predict the outcome of therapy to TS inhibitors without the documented effects on downstream factors.

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