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Differential Roles of $p21^{Waf1}$ and $p27^{Kip1}$ in Modulating Chemosensitivity and Their Possible Application in Drug Discovery Studies

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ABSTRACT

In this study, the differential role of the cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ in cell cycle regulation was proposed for use in screening natural or synthetic compounds for cell cycle-dependent (particularly M phase-dependent) antineoplastic activity. $p21^{Waf1}$ or $p27^{Kip1}$ was ectopically expressed with an ecdysone-inducible mammalian expression system in a human colon adenocarcinoma cell line. Induction of $p21^{Waf1}$ or $p27^{Kip1}$ expression inhibited the activities of CDK2 and completely arrested cells at G_1 phase of the cell cycle by $p27^{Kip1}$ and at G_1 and G_2 phases by $p21^{Waf1}$. We examined the sensitivity of these cells to several antineoplastic agents known to be cell cycle-dependent or -independent. Substantially increased resistance to cell cycle-dependent antineoplastic agents was found in the cells when the expression of $p21^{Waf1}$ or $p27^{Kip1}$ was induced. In contrast, only a desensitization to cell cycle-independent antineoplastic agents was found in the cells arrested by $p21^{Waf1}$ or $p27^{Kip1}$. Because $p21^{Waf1}$ induces an additional block at G_2 phase that inhibits cell entry into M phase, we further examined the difference between $p21^{Waf1}$- and $p27^{Kip1}$-induced cells in their sensitivity to D-24851, a novel M phase-dependent compound. We found that induction of $p21^{Waf1}$ after exposure of the cells to D-24851 conferred stronger resistance than did induction of $p27^{Kip1}$. Taken together, our results suggest that the differential effect of $p21^{Waf1}$ and $p27^{Kip1}$ on cell cycle regulation may be advantageous for screening chemical libraries for novel antineoplastic candidates that are cell cycle-dependent, and M phase-dependent in particular.

The identification of a group of nuclear enzymes called cyclin-dependent kinases (CDK) has profoundly advanced our understanding of cell cycle progression (Garrett and Fattaey, 1999; Johnson and Walker, 1999). CDK activity is primarily stimulated by the binding of G_1 cyclins in response to the effects of both mitogenic growth factors and the extracellular matrix; CDK activity is opposed by the so-called CDK inhibitors, such as $p21^{Waf1}$ and $p27^{Kip1}$ (Sherr and Roberts, 1999). $p21^{Waf1}$ was initially identified as a p53-dependent gene product in cells with wild-type p53 after exposure of the cells to DNA-damaging agents (Harper et al., 1993; Dulic et al., 1994), and it has since been shown that $p21^{Waf1}$ can also be induced in a p53-independent manner (Michieli et al., 1994; Macleod et al., 1995). In contrast, $p27^{Kip1}$ was first discovered in Mv1Lu mink lung epithelial cells arrested at G_1 phase of the cell cycle by contact inhibition or by treatment with transforming growth factor-β (Toyoshima and Hunter, 1994; Polyak et al., 1994).

We and others have recently reported that, in contrast to $p27^{Kip1}$, which arrests cell cycle traversal solely at G_1 phase (Coats et al., 1996; Rivard et al., 1996), $p21^{Waf1}$ arrests the cell cycle at both G_1 phase and G_2 phase (Cayrol et al., 1998; Medema et al., 1998; Schmidt et al., 2000). The latter arrest at G_2 phase is caused by $p21^{Waf1}$-mediated inhibition of CDC2 activity, which is required for cells to enter M phase of the cell cycle. The differential modulation of cell cycle traversal by $p21^{Waf1}$ and $p27^{Kip1}$ was correlated with their effects on the cytotoxicity of paclitaxel, an M phase-dependent antineoplastic drug used to treat a variety of human cancers (Rowinsky, 1997; Aisner and Cortes-Funes, 1997). When ectopically expressed, both $p21^{Waf1}$ and $p27^{Kip1}$ conferred resistance to paclitaxel-mediated apoptosis on the cells. However, induction of $p21^{Waf1}$ after exposure to paclitaxel

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ABBREVIATIONS: CDK, cyclin-dependent kinase; Rb, retinoblastoma; GST, glutathione *S*-transferase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; D-24851, (*N*-[pyridin-4-yl]-[1-[4-chlorbenzyl]-indol-3-yl]-glyoxyl-amid).

produced much greater resistance to the drug than did expression of $p27^{Kip1}$ (Schmidt et al., 2000). This result was attributed to the additional G_2 phase block by $p21^{Waf1}$, which prevented cells from entering M phase of the cell cycle and becoming committed to apoptosis after paclitaxel treatment (Schmidt et al., 2000).

Given the remarkable chemoresistance toward paclitaxel in $p21^{Waf1}$- or $p27^{Kip1}$-arrested cells, we examined a panel of chemotherapeutic agents that were categorized for their cell cycle-dependent or -independent antineoplastic activities. Cell cycle dependence in this context refers to the fact that active cell cycling is required for an antineoplastic agent to exert a cytotoxic effect (Myers and Chabner, 1990; Perry, 1992). DNA-intercalating, cross-linking, or alkylating agents (such as cisplatin and melphalan) do not necessarily require their targeted cells to be actively cycling; in contrast, antimetabolites and topoisomerase inhibitors (such as 5-fluorouracil and camptothecin) may be toxic to the cells only during the S phase of a cell cycle, and agents that interfere with the mitotic spindle (such as paclitaxel) may kill the cells primarily during M phase. We observed a substantially increased resistance to several known cell cycle-dependent antineoplastic agents in $p21^{Waf1}$- or $p27^{Kip1}$-induced cells, compared with uninduced cells, whereas only a desensitation of the arrested cells was observed upon treatment with several known cell cycle-independent agents. Additionally, we found that $p21^{Waf1}$ and $p27^{Kip1}$, when induced after drug exposure, conferred differential chemoresistance to the cytotoxicity of D-24851, a novel M phase-dependent compound (Bacher et al., 2001) in a manner very similar to what we reported of their effects on the cytotoxicity of paclitaxel, a well-known antineoplastic agent with M phase-dependent action (Schmidt et al., 2000). Our results suggest that this $p21^{Waf1}$- and $p27^{Kip1}$-inducible expression system might be a useful tool for determining not only the cell cycle dependence but also the M phase dependence of candidate compounds after the antineoplastic activity of the candidate compounds has been established during a primary high-throughput screening assay.

Experimental Procedures

Stable RKO $p21^{Waf1}$- and $p27^{Kip1}$-Inducible Expression Clones. The RKO human colon carcinoma cells containing an ecdysone-inducible expression vector of $p21^{Waf1}$ or $p27^{Kip1}$ (RKO-p21 or RKO-p27) were described previously (Schmidt et al., 2000). Briefly, human waf1 and kip1 cDNAs were amplified by polymerase chain reaction techniques using MCF10A human nonmalignant mammary epithelial cell cDNA as a template and subcloned into pIND (Invitrogen, Carlsbad, CA) via restriction sites included in the waf1- and kip1-specific oligonucleotide primers. The $pINDp21^{Waf1}$ or $pINDp27^{Kip1}$ vector was transfected with the Fugene-6 kit (Roche Diagnostic Corp., Indianapolis, IN) into an RKO cell clone that was previously transfected with the pIND-regulatory vector pVgRXR (Invitrogen); the latter contains a transgene carrying an ecdysone-responsive regulatory sequence. Stable $pINDp21^{Waf1}$-transfected or $pINDp27^{Kip1}$-transfected clones (RKO-p21 or RKO-p27) were kept in double-selection culture medium containing 200 μg/ml Zeocin (Invitrogen, Carlsbad, CA) and 500 μg/ml neomycin (G418). Expression of $p21^{Waf1}$ or $p27^{Kip1}$ was induced by exposure to 3 μM muristerone A for 24 h and examined by Western blot analysis with specific antibodies.

Antineoplastic Agents. The antineoplastic agents used in this study were as follows: cisplatin (Platinol-AQ) and etoposide (VePesid) were from Bristol Laboratories (Princeton, NJ); doxorubicin (Adriamycin) was from Gensia Sicor Pharmaceuticals (Irvine, CA); melphalan (Alkeran) was from GlaxoSmithKline (Research Triangle Park, NC); 5-fluorouracil (Adrucil) was from Pharmacia (Kalamazoo, MI); camptothecin was from Sigma Chemical Co. (St. Louis, MO); and D-24851 (*N*-[pyridin-4-yl]-[1-[4-chlorbenzyl]-indol-3-yl]-glyoxylamid) was from ASTA Medica AG (Frankfurt, Germany) (Bacher et al., 2001).

Western Blot Analysis. Western blot analysis was performed as described previously (Fan et al., 1995). Briefly, RKO-p21 or RKO-p27 cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin, and the lysates were sonicated at 4°C. Equal amounts of lysate were separated on SDS-polyacrylamide gels and subsequently blotted onto nitrocellulose membranes for incubation with antibodies against $p21^{Waf1}$ (Neo Markers Biotechnology Inc., Union City, CA), $p27^{Kip1}$, Rb, or the Rb-related p130 (Santa Cruz Biotechnology Inc. Santa Cruz, CA). Specific signals were visualized using the enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech Inc., Piscataway, NJ).

CDK Assays. The CDK assay with GST-Rb (Santa Cruz Biotechnology) as a substrate was performed as reported previously (Fan et al., 1995; Wu et al., 1996). Briefly, cells were lysed as described above. CDK2 was immunoprecipitated from sonicated lysates with corresponding antibodies (Santa Cruz Biotechnology) and subjected to an in vitro kinase reaction in the presence of the CDK substrate GST-Rb and [γ-^{32}P]ATP, followed by separation with SDS-polyacrylamide gel electrophoresis and autoradiography.

Flow Cytometric Analysis. After completion of the desired treatment, RKO-p21 or RKO-p27 cells were harvested by trypsinization, and an aliquot of 1×10^6 cells was washed once with cold phosphate-buffered saline and then fixed with cold 70% ethanol. The DNA was stained with a solution containing 25 μg/ml propidium iodide and 10 μg/ml RNase A (Sigma Chemical Co) in phosphate-buffered saline for 6 h. Cell cycle distribution was analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA) at an excitation wavelength of 488 nm (Fan et al., 1995).

Cytotoxicity Analysis (MTT Assay). Cell viability was measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Schmidt et al., 2000). The cytotoxic effects of selected drugs on RKO-p21 or RKO-p27 with and without induction of $p21^{Waf1}$ or $p27^{Kip1}$ were determined after drug treatment, and the results were expressed as a percentage of relative cell numbers of control cells that were not exposed to the drugs. Briefly, after desired treatment, cells were incubated with 1 mg/ml MTT (Sigma) in 0.5 ml of culture medium for 3 h in a 37°C CO_2 incubator, followed by cell lysis with 0.5 ml of lysis buffer containing 20% SDS in dimethyl formamide/H_2O, pH 4.7, at 37°C for more than 6 h. Optical absorbance of the cell lysate was determined at a wavelength of 595 nm.

Determination of Mitotic Index. Upon completion of the desired treatment, cells were harvested by trypsinization. An aliquot (5×10^3 cells) was spun onto glass slides using a Cytospin and subsequently fixed and stained with the Hema-3 kit (Biochemical Sciences, Inc., Swedesboro, NJ). Two hundred cells were counted each time under a microscope from different areas for a total of five times. Cells in mitotic phase were scored individually, and the numbers were expressed as the percentage of the total cell number counted. Representative areas for each treatment condition were photographed (Schmidt et al., 2000).

Results

The $p21^{Waf1}$- or $p27^{Kip1}$-Inducible Expression RKO Cell Clones. RKO cells are human colon cancer cells that contain wild-type p53 and Rb and express very low levels of endogenous $p21^{Waf1}$ or $p27^{Kip1}$ (Boyd et al., 1988; Kessis et

al., 1993; van Bree et al., 1999; Schmidt et al., 2000). Figure 1A presents Western blot analysis data showing the expression of $p21^{Waf1}$ or $p27^{Kip1}$ in representative RKO-p21 and RKO-p27 transfectant clones upon gene induction. Expression of $p21^{Waf1}$ or $p27^{Kip1}$ was detectable approximately 2 h after the gene expression inducer muristerone A was added to the cell culture medium. The expression level reached a plateau approximately 24 h after induction, and expression persisted for at least 72 h without further supplementation with muristerone A (Schmidt et al., 2000). Induction of $p21^{Waf1}$ or $p27^{Kip1}$ strongly inhibited CDK2 activity (Fig. 1B). This inhibition of CDK2 activity was accompanied by dephosphorylation of Rb and the Rb-related p130 protein (Fig. 1C). Flow cytometric analysis further indicated that induction of $p27^{Kip1}$ completely arrested the cells at G_1 phase of the cell cycle within 24 h, whereas induction of $p21^{Waf1}$ arrested the cells at both G1 and G_2, although most cells were arrested in G_1 phase. Cell proliferation was completely inhibited upon the expression of $p21^{Waf1}$ or $p27^{Kip1}$. No signs of cytotoxic effects were observed up to 5 days after the induced expression of $p21^{Waf1}$ or $p27^{Kip1}$ (data not shown).

Effect of $p21^{Waf1}$- and $p27^{Kip1}$-Inducible Expression on the Cytotoxicity of Antineoplastic Agents. Fig. 2A shows the treatment schedule of RKO-p21 and RKO-p27 cells with three representative antineoplastic agents known to be cell cycle-dependent (5-fluorouracil, camptothecin, and etoposide). RKO-p21 and RKO-p27 cells showed a concentration-dependent response to the cytotoxic effects mediated by these three drugs (Fig. 2B). Continuous expression of either $p21^{Waf1}$ or $p27^{Kip1}$ conferred on these cells nearly complete resistance to cell death induced by 5-fluorouracil, camptothecin, or etoposide. The IC_{50} values in $p21^{Waf1}$- or $p27^{Kip1}$-uninduced cells versus $p21^{Waf1}$- or $p27^{Kip1}$-induced cells shifted from approximately 40 μM to >1,000 μM for 5-fluorouracil, from 160 nM to >10,000 nM for camptothecin, and

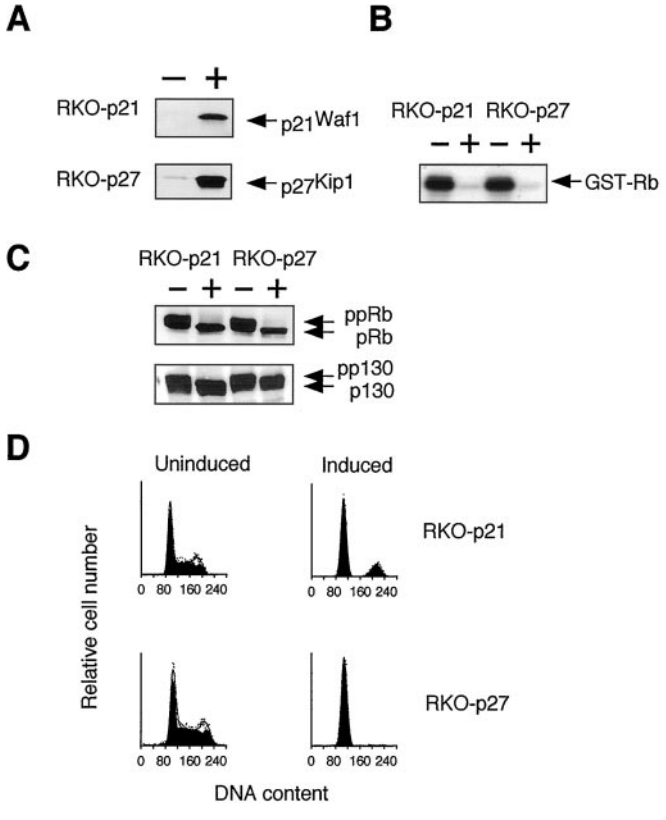

Fig. 1. Inducible expression of $p21^{Waf1}$ and $p27^{Kip1}$ in RKO cells. A, induction of $p21^{Waf1}$ and $p27^{Kip1}$ in representative RKO-p21 and RKO-p27 clones, respectively. The cells were incubated for 24 h in culture medium in the absence (−) or presence (+) of 3 μM muristerone A. The samples were analyzed by Western blotting with specific antibodies against $p21^{Waf1}$ or $p27^{Kip1}$. B, inhibition of cyclin-dependent kinase activity by ectopically expressed $p21^{Waf1}$ and $p27^{Kip1}$. RKO-p21 and RKO-p27 cells were incubated as described in A. The samples were subjected to CDK2 kinase assay using GST-Rb as a substrate. C, dephosphorylation of Rb and the Rb-related p130 after inducible expression of $p21^{Waf1}$ or $p27^{Kip1}$ in RKO-p21 and RKO-p27 cells. The cells were incubated for 24 h in culture medium in the absence (−) or presence (+) of 3 μM muristerone A. The samples were analyzed by Western blotting with specific antibodies against Rb or p130. D, cell cycle distribution analysis of RKO cells upon induction of $p21^{Waf1}$ or $p27^{Kip1}$. RKO-p21 and RKO-p27 cells were kept for 24 h in culture medium in the absence or presence of 3 μM muristerone A. The cell cycle distribution of the cells was analyzed by flow cytometric analysis after propidium iodide staining of the DNA.

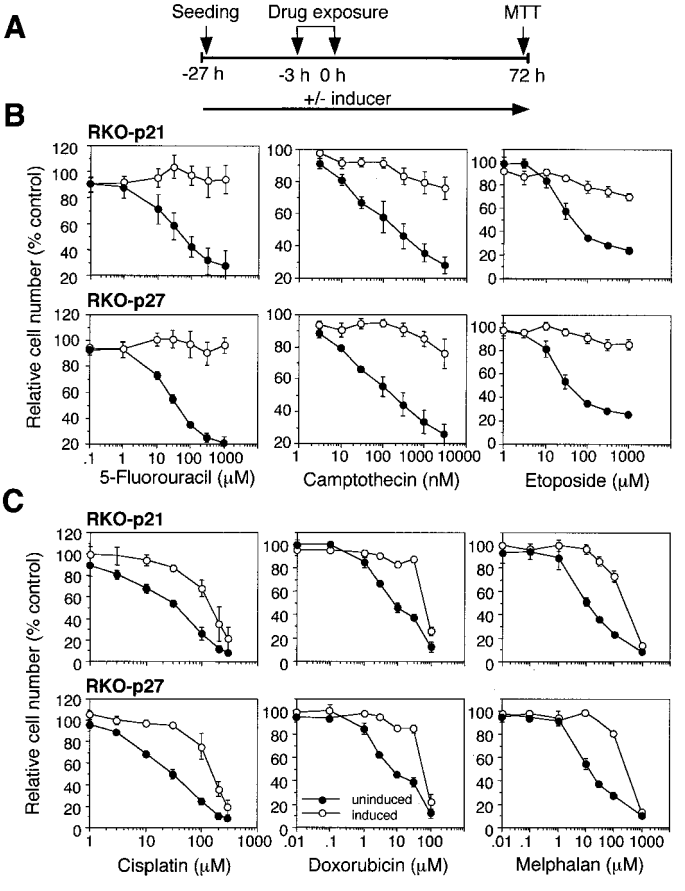

Fig. 2. Differential responses of $p21^{Waf1}$- and $p27^{Kip1}$-arrested RKO cells to known cell cycle-dependent and -independent antineoplastic agents. A, schematic illustration of the treatment schedule. RKO-p21 or RKO-p27 cells were seeded in medium in the presence or absence of 3 μM muristerone A for 24 h. After being exposed to various concentrations of chemotherapeutics for 3 h, the cells were cultured for an additional 72 h with or without 3 μM muristerone A in the culture medium. An MTT assay was then performed as described under *Experimental Procedures*. B, treatment of RKO-p21 or RKO-p27 cells with the cell cycle-dependent antineoplastic agents 5-fluorouracil, camptothecin, and etoposide. Cells were treated as described in A, and the relative cell number after treatment was determined in comparison with untreated cells. ●, uninduced cells; ○, induced cells. Values are means ± S.D. for three independent experiments. Each experiment had triplicate wells for each treatment group. C, treatment of RKO-p21 or RKO-p27 with the cell cycle-independent antineoplastic agents cisplatin, doxorubicin, and melphalan. See B for details.

from 35 μM to >1,000 μM for etoposide. Induction of $p21^{Waf1}$ and $p27^{Kip1}$ did not produce any noticeable difference in conferring resistance on the RKO-p21 and RKO-p27 cells to cell cycle-dependent antineoplastic agents.

Parallel experiments were performed with three representative antineoplastic agents known to be cell cycle-independent (cisplatin, doxorubicin, and melphalan). Although we found a shift in the IC_{50} value for all three drugs in both $p21^{Waf1}$- and $p27^{Kip1}$-induced RKO cells (the IC_{50} values increased from ~30 μM to 180 μM for cisplatin, from 8 μM to 60 μM for doxorubicin, and from 15 μM to 250 μM for melphalan), the cells were all killed by a higher concentration of each drug regardless of $p21^{Waf1}$ or $p27^{Kip1}$ expression; again, there was no noticeable difference in drug concentration response between RKO-p21 and RKO-p27 cells. Thus, in contrast to the results with the cell cycle-dependent drugs, which rendered the cells nearly completely resistant to the drugs upon induction of either CDK inhibitor, induction of $p21^{Waf1}$ or $p27^{Kip1}$ caused only desensitization to the toxic effects of these cell cycle-independent drugs. These remarkable differential results confirmed our hypothesis that this CDK inhibitor-based cellular system can distinguish between cell cycle-dependent and -independent antineoplastic agents and thereby might have potential application in screening natural or synthetic candidate compounds for cell cycle-dependent antineoplastic activity.

Cell Cycle-Dependent Antineoplastic Activity of D-24851. On the basis of our results with known antineoplastic agents, we next evaluated the cytotoxicity of *N*-[pyridin-4-yl]-[1-[4-chlorbenzyl]-indol-3-yl]-glyoxyl-amid (D-24851), a novel investigational synthetic compound. D-24851 is a successful candidate for antineoplastic activity, having been selected by a cell-based, high-throughput screening assay by ASTA Medica AG, Germany. The compound is a novel synthetic microtubule inhibitor, and we have shown that D-24851 has potent cytotoxic properties in vitro and in vivo with much less neurotoxicity than occurs with paclitaxel (Bacher et al., 2001).

RKO-p21 and RKO-p27 cells were exposed to increasing concentrations of D-24851, using the schedule shown in Fig. 3A. We found that D-24851 had concentration-dependent cytotoxic effects on RKO-p21 and RKO-p27 cells (Fig. 3B). Compared with D-24851-induced cytotoxicity in $p21^{Waf1}$- or $p27^{Kip1}$-uninduced RKO-p21 and RKO-p27 cells, expression of $p21^{Waf1}$ or $p27^{Kip1}$ conferred strong resistance on both RKO-p21 and RKO-p27 cells to D-24851-induced cytotoxicity (the IC_{50} value increased from 0.2 μM to >100 μM in RKO-p21 cells and from 0.35 μM to >100 μM in RKO-p27 cells). This result suggests that the mechanism by which D-24815 exerts its cytotoxic effects is cell cycle-dependent.

Flow cytometric analyses of the DNA content further indicated that, in $p21^{Waf1}$- or $p27^{Kip1}$-uninduced RKO-p21 or RKO-p27 cells, there was a predominant G_2/M cell population after D-24815 exposure (Fig. 3C). The accumulation of cells in G_2/M phase was accompanied by a marked increase in the percentage of DNA in the pre-G_1 peak (an indication of apoptosis). Expression of $p27^{Kip1}$ in RKO-p27 cells prevented the cells from accumulating in G_2/M phase and also prevented the appearance of the pre-G_1 peak, with cells arrested in G_1 phase of the cell cycle (Fig. 3C, bottom). Similarly, induction of $p21^{Waf1}$ in RKO-p21 cells prevented both cell accumulation in G_2/M phase and the pre-G_1 peak, with cells arrested in both G_1 phase and G_2 phase (Fig. 3C, top). These patterns are similar to the cell cycle distribution shown in Fig. 1D, which shows a complete G_1 arrest following induction of $p27^{Kip1}$ in RKO-p27 cells and a G_1 and G_2 dual block after induction of $p21^{Waf1}$ in RKO-p21 cells.

M Phase-Dependent Antineoplastic Activity of D-24851. We took the advantage of the additional G_2 block produced by induction of $p21^{Waf1}$ in the RKO-p21 cells (but not by induction of $p27^{Kip1}$ in the RKO-p27 cells) to further confirm that the antineoplastic activity of D-24851 depends on arrest of the cells in M phase of the cell cycle. In contrast to the experiments shown in Fig. 3, in which cell cycle arrest was achieved by induction of either $p21^{Waf1}$ (a dual G_1 and G_2 arrest) or $p27^{Kip1}$ (a complete G_1 arrest) before the exposure of RKO-p21 or RKO-p27 to D-24851 and continued during the 72-h postdrug period, $p21^{Waf1}$ and $p27^{Kip1}$ were induced only during the 72-h postdrug period (Fig. 4A). In this experimental setting, the cell cycle distribution in both the RKO-

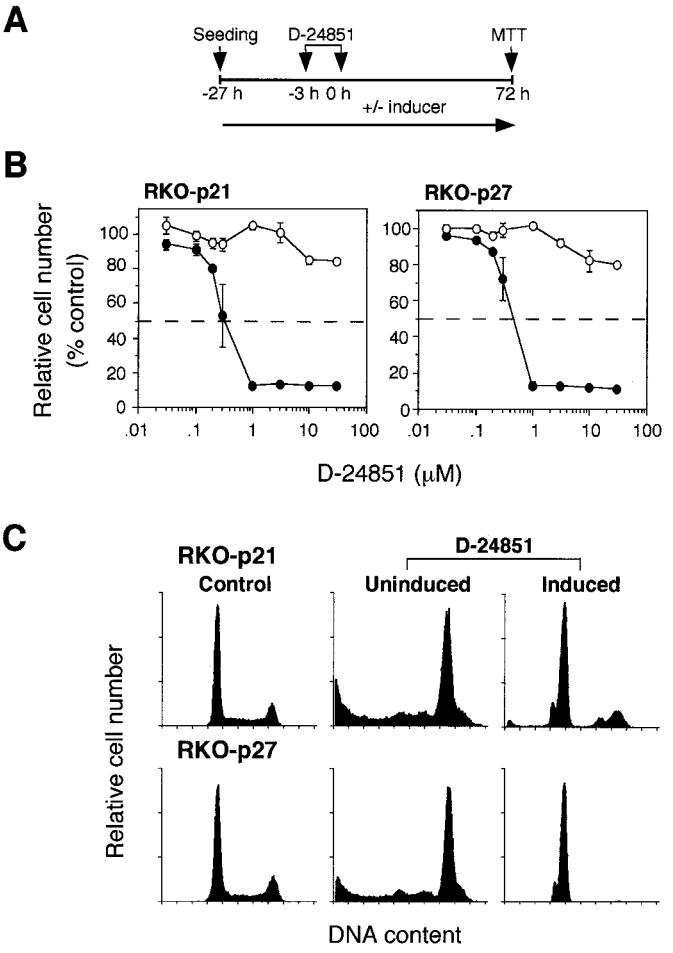

Fig. 3. Cell cycle-dependent cytotoxicity of the investigational compound D-24851. A, schematic illustration of the treatment schedule, similar to that described in the legend to Fig. 2. B, RKO-p21 or RKO-p27 cells were seeded in medium in the presence or absence of 3 μM muristerone A for 24 h. After being exposed to various concentrations of D-24851 for 3 h, the cells were cultured for an additional 72 h with or without 3 μM muristerone A in culture medium. An MTT assay was performed to determine cytotoxicity. ●, uninduced cells; ○, induced cells. Values are means ± S.D. for three independent experiments. Each experiment had triplicate wells for each treatment group. C, Flow cytometric analysis of RKO cells treated with D-24851 with or without induction of $p21^{Waf1}$ or $p27^{Kip1}$. RKO-p21 cells or RKO-p27 cells were treated with 1 μM D-24851 for 3 h. Twenty-four hours later, the cells were harvested, stained with propidium iodide, and subjected to cell cycle distribution analysis.

p21 and RKO-27 cells was supposed to be identical to that in their parental RKO cells before drug exposure; thus, equal percentages of cells in different phases of the cell cycle would be exposed to D-24851 during the 3-h pulse treatment period. Compared with the results shown in Fig. 3B, induction of $p21^{Waf1}$ or $p27^{Kip1}$ after exposure to D-24851 produced a less strong resistance of the RKO-p21 and RKO-*p*-27 cells to D-24851-induced cytotoxicity (Fig. 4B), which is to be expected because in this setting, both the RKO-p21 and RKO-p27 cells were still actively progressing through the cell cycle during the drug exposure. However, this change in the sequence of $p21^{Waf1}$ or $p27^{Kip1}$ induction with reference to drug exposure produced differential effects between $p21^{Waf1}$ and $p27^{Kip1}$ on mediating cell resistance to D-24851-mediated cytotoxicity measured 72 h later. Notably, induction of $p27^{Kip1}$ resulted in much less chemoresistance to D-24851 than did induction of $p21^{Waf1}$ (a >30-fold difference). It took approximately 24 h after D-24851 treatment for the cell cycle distribution pattern to transit from a normal pattern to an M phase-dominant pattern (Fig. 3C). The result shown in Fig. 4B suggested that the additional effect of $p21^{Waf1}$ at G_2 phase that impeded the entrance of cells into M phase during the period after D24851 treatment may have contributed to this difference in conferring D-24851 resistance to cells. The data therefore indicate that entry into M phase might be critical for D-24851–induced cytotoxicity (M phase-dependent toxicity).

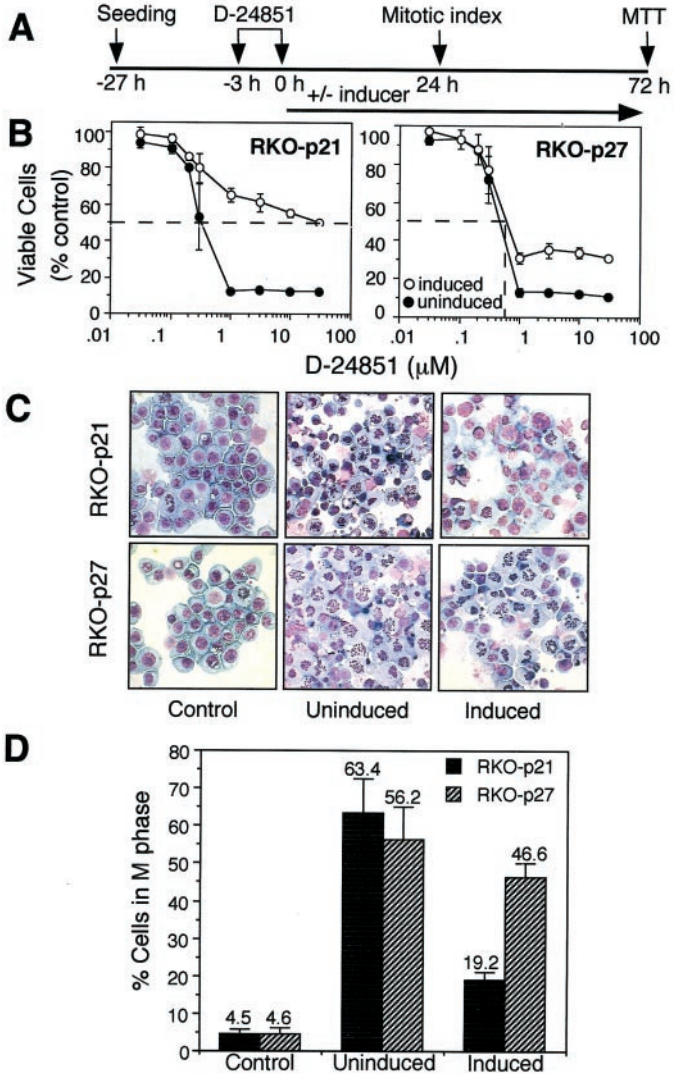

Fig. 4. M phase-dependent cytotoxicity of D-24815 on RKO cells. A, schematic illustration of the new treatment schedule. B, in contrast to the procedure described in Fig. 3B, RKO-p21 or RKO-p27 cells were seeded in muristerone A-free medium for 24 h. After a 3-h exposure to various concentrations of D-24851, the cells were divided into two groups and cultured for an additional 72 h with or without 3 μM muristerone A in the medium. An MTT assay was performed to determine cytotoxicity. ●, uninduced cells; ○, induced cells. Values are means ± S.D. for three independent experiments. Each experiment had triplicate wells for each treatment group. C, cytochemical staining of the RKO-p21 and RKO-p27 cells. Cells were exposed to 1 μM D-24851 for 3 h without (middle) or with (right) subsequent induction of $p21^{Waf1}$ or $p27^{Kip1}$ for 24 h. The cells were then fixed and stained with methylene blue and eosin. Control cells (left) were left untreated. Individual representative areas were photographed. D, determination of the mitotic index. Two hundred cells each time were blindly counted from the experiments shown in C, with cells counted for a total of five times in different areas under a microscope. Cells in mitotic phase with condensed chromosomes were counted and are expressed as the percentage of total cells. The experiment was repeated for three times; similar results were obtained.

To prove that induction of $p21^{Waf1}$ in RKO-p21 cells caused fewer cells to enter into M phase after D-24851 treatment, whereas induction of $p27^{Kip1}$ in RKO-p27 cells did not affect the number of cells entering into M phase, we compared the percentages of mitotic cells in RKO-p21 and RKO-p27 cells after D-24851 treatment with or without subsequent induction of $p21^{Waf1}$ or $p27^{Kip1}$. Figure 4C shows the results of cytochemical staining of the RKO-p21 and RKO-p27 cells after D-24851 treatment. Exposure of RKO-p21 or RKO-p27 cells to D-24851 without subsequent induction of $p21^{Waf1}$ or $p27^{Kip1}$ produced a substantial number of mitotic cells 24 h after drug exposure (Fig. 4C, 'uninduced'). In contrast, exposure of RKO-p21 cells to D-24851 with subsequent induction of $p21^{Waf1}$ markedly reduced the number of mitotic cells (Fig. 4C, top right), whereas exposure of RKO-p27 cells to D-24851 with subsequent induction of $p27^{Kip1}$ failed to substantially reduce the number of mitotic cells (Fig. 4C, bottom right). Quantitative determination of mitotic cell indices showed that exposure of the RKO-p21 cells to D-24851 resulted in 63.4% of the cells being mitotic without induction of $p21^{Waf1}$ and 19.2% of the cells being mitotic with induction of $p21^{Waf1}$. In contrast, exposure of the RKO-p27 cells to D-24851 resulted in 56.2% of the cells being mitotic without induction of $p27^{Kip1}$ and 46.6% of cells still being mitotic with $p27^{Kip1}$ induction (Fig. 4D). This observation is reminiscent of the results for our recently published study with paclitaxel, a well-known M phase-dependent antineoplastic drug (Schmidt et al., 2000). In that study, $p21^{Waf1}$ and $p27^{Kip1}$ differentially modulated paclitaxel-mediated cytotoxicity, a result very similar to that found in the current studies with D-24851. Using the same assay, we showed in our previous study that exposure of RKO-p21 cells to paclitaxel resulted in 87.5% of the cells being in mitotic phase without induction of $p21^{Waf1}$, with the percentage dramatically lower at 23% when $p21^{Waf1}$ was induced. In contrast, exposure of RKO-p27 cells to paclitaxel resulted in a similar 87.4% of the cells being in mitotic phase without induction of $p27^{Kip1}$ and the percentage only slightly lower at 74.5% when $p27^{Kip1}$ was induced (Schmidt et al., 2000). Our current results are in agreement with those from our another recent report showing that D-24851 interferes with tubulin assembly and prevents chromosomal segregation in tumor cells (Bacher et al., 2001).

Taken together, these results suggest that cytotoxicity of D-24851 is M phase-dependent and that our $p21^{Waf1}$- and $p27^{Kip1}$-inducible expression system may therefore have potential application in identifying novel M phase-dependent antineoplastic agents in drug discovery studies.

Discussion

Screening for potential antineoplastic compounds from chemical libraries is often hampered by the lack of a system that can distinguish a potential antineoplastic compound from nonspecific toxins, such as poisons of the respiratory chain. In this study, the CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, which were ectopically expressed in a human colon adenocarcinoma cell line and showed differential effects on cell cycle arrest (a complete G_1 phase arrest of the cell cycle by $p27^{Kip1}$ and a dual G_1 and G_2 phase arrest by $p21^{Waf1}$), were evaluated for their application in drug discovery studies by investigating their modulation of cell sensitivity to several antineoplastic agents known to be cell cycle-dependent and -independent and to D-24851, a novel investigational compound.

We first showed that the $p21^{Waf1}$ and $p27^{Kip1}$-inducible expression system could distinguish between cell cycle-dependent and -independent antineoplastic agents. Induction of $p21^{Waf1}$ in the RKO-p21 or $p27^{Kip1}$ in the RKO-p27 cells conferred marked resistance to cell cycle-dependent drugs but only moderately desensitized the cells to cell cycle-independent drugs. We performed similar studies with another cell line, A431 human epidermoid carcinoma cells. We found that inducible expression of either $p21^{Waf1}$ or $p27^{Kip1}$ caused markedly increased resistance of the cells to chemotherapeutic agents, but we did not find substantial differences in drug resistance between paclitaxel (cell cycle-dependent) and cisplatin (cell cycle-independent) in A431 cells, presumably because A431 cells contain a mutated p53, whereas RKO cells have wild-type p53 (Schmidt and Fan, 2001).

In Fig. 2C, the relative cell number pattern reflecting the cytotoxicity after exposure of the cells to lower concentrations of doxorubicin and melphalan seems similar to the pattern after exposure of the cells to the cell cycle-dependent compounds. This is probably because doxorubicin inhibits DNA topoisomerase II at lower concentrations and intercalcates with DNA at higher concentrations (Bodley et al., 1989). We speculate that this may also be the case with melphalan.

Our current results lead us to propose the use of this RKO cell-based system for screening natural or synthetic compounds that have cell cycle-dependent antineoplastic activities, after the antineoplastic activities of these candidate compounds have been established during a primary high-throughput screening assay.

We further demonstrated that the differential effects of $p21^{Waf1}$ and $p27^{Kip1}$ on the cell cycle presented an additional advantage for the identification of candidate compounds that are M phase-dependent. We showed recently that the G_2 block produced by $p21^{Waf1}$, but not by $p27^{Kip1}$, contributed to their unequal modulation of sensitivity to paclitaxel-mediated apoptosis (Schmidt et al., 2000). In the current study, we used this system to demonstrate that the cytotoxicity of the investigational compound D-24851 is M phase-dependent. Our results suggest that, by comparing the cytotoxicity of candidate compounds from chemical libraries in the RKO-p21 and RKO-p27 cells with and without postdrug induction of $p21^{Waf1}$ or $p27^{Kip1}$, we may be able to determine whether the antineoplastic activities of those candidate compounds are M phase-dependent. Thus, this cell-based system presented here may be used not only for screening for cell cycle-dependent antineoplastic compounds, but also for determining which candidate compounds are M phase-dependent.

The therapeutic index of a potential antineoplastic compound is in principal based on its preferential activity to rapidly dividing cells. Screening of chemical libraries with the described $p21^{Waf1}$- and $p27^{Kip1}$-inducible system will not only provide information on the mode of antineoplastic activity but also predict possible adverse effects of candidate compounds on normal cells. Our system compares the antineoplastic activity of candidate compounds on proliferating cells versus quiescent cells of the same type and genetic background, thus providing unbiased information on both the therapeutic index and the mode of action.

Cell cycle synchronization can also be achieved by several known chemical compounds such as aphidicolin or nocodazole; however, these compounds are all very toxic to target cells and are therefore unfit for drug-screening studies. Additionally, serum deprivation is frequently used to synchronize cells; however, as has been observed in many cancerous cells, serum deprivation was not successful in inducing cell cycle synchronization in the RKO cells. In fact, it has been suggested that the mechanism of serum deprivation-induced cell cycle synchronization is mediated primarily by induction of $p27^{Kip1}$ (Coats et al., 1996). Induction of $p21^{Waf1}$ in RKO-p21 cells or $p27^{Kip1}$ in RKO-p27 cells does not cause any notable cytotoxic effects in RKO cells and thus avoids any overlay of cytotoxic effects on a candidate compound; therefore, any observed cytotoxicity could be attributed primarily to the effect of the candidate compound. Other approaches, such as analysis of fluorescence-activated cell sorting, can provide information on the cell cycle distribution caused by candidate compounds but cannot indicate in which phase such compounds may be effective in killing cancer cells or inhibiting cancer cell proliferation.

Although inducible or constitutive expression of $p21^{Waf1}$ or $p27^{Kip1}$ in cancer cells has been described in the literature during the last several years (St Croix et al., 1996, 1998; Rivard et al., 1996; Niculescu et al., 1998; Ruan et al., 1998; Yamamoto et al., 1999), these previous studies have not demonstrated a complete cell cycle arrest by $p21^{waf1}$ or $p27^{kip1}$ and a differential cell cycle arrest by $p21^{waf1}$ or $p27^{kip1}$ as shown in our system (Fig. 1D). Our system can be used in a highly standardized setting, allowing for very reproducible results. This $p21^{Waf1}$- and $p27^{Kip1}$-inducible expression system may also be used to examine the requirement or correlation of an active cell cycle with reference to the cytotoxic effects of a variety of other known cytotoxic agents, such as cytokines, and other anticancer treatments, such as radiation therapy.

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