Potentiation of paclitaxel-induced apoptosis by the novel cyclin-dependent kinase inhibitor NU6140: a possible role for survivin down-regulation

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Abstract

Cyclin-dependent kinases (CDK) play a crucial role in the control of the cell cycle. Aberrations in the control of cell cycle progression occur in the majority of human malignancies; hence, CDKs are promising targets for anticancer therapy. Here, we define the cellular effects of the novel CDK inhibitor NU6140, alone or in association with paclitaxel, with respect to inhibition of cell proliferation and cell cycle progression and induction of apoptosis in HeLa cervical carcinoma cells and in comparison with purvalanol A. Both CDK inhibitors induced a concentration-dependent cell cycle arrest at the G_2-M phase and an increase in the apoptotic rate, with a concomitant down-regulation of the antiapoptotic protein survivin, a member of the inhibitors of apoptosis protein family. Notably, the addition of NU6140 to paclitaxel-treated cells resulted in markedly increased cytotoxic effect and apoptotic response in comparison with the paclitaxel-purvalanol A combination (86 ± 11% and 37 ± 8%, respectively). Similarly, the extent of caspase-9 and caspase-3 activation in paclitaxel-NU6140–treated cells was ~4-fold higher than after the paclitaxel-purvalanol A combination. Moreover, an almost complete abrogation of the expression of the active, Thr^{34}-phosphorylated form of survivin was observed in cells exposed to the paclitaxel-NU6140 combination. A synergistic effect of the paclitaxel-NU6140 combination, as a consequence of survivin inhibition and increased activation of caspase-9 and caspase-3, was also observed in OAW42/e ovarian cancer line but not in the derived OAW42/Surv subline ectopically expressing survivin. Results from this study indicate that NU6140 significantly potentiates the apoptotic effect of paclitaxel, with inhibition of survivin expression/phosphorylation as the potential mechanism. [Mol Cancer Ther 2005;4(9):1328–37]

Introduction

Cell progression through the cell cycle is controlled by serine/threonine cyclin-dependent kinases (CDK) that form complexes with cyclins and operate in distinct phases of the cell cycle (1, 2). Aberrant control of CDKs is a central feature of the molecular pathology of cancer (3–5); hence, CDKs and their related pathways represent attractive targets in the development of anticancer therapeutics. Small-molecule CDK inhibitors, including flavopiridol, roscovitine, UCN-01, and purvalanol A (Fig. 1), which act by competing with ATP for binding at the catalytic site of the kinases, have been generated (6). In addition to arresting cell cycle progression through the inhibition of multiple CDKs (6), some of these inhibitors exhibit a variety of activities, including induction of apoptosis (7), promotion of cell differentiation (8), interference with transcription (9, 10), and antiangiogenic effects (11). Furthermore, the possibility of enhancing the activity of conventional chemotherapeutic agents and radiation by combination with CDK inhibitors has also been shown (12–17). The purine-based CDK inhibitor NU6140 [4-(6-cyclohexylmethoxy-9H-purin-2-ylamino)-*N,N*-diethylbenzamide; Fig. 1] was derived from NU6102 [6-cyclohexylmethoxy-2-(4′-sulfamoylanilino)purine], a potent and selective CDK2 inhibitor developed using structure-based drug design (18), as a CDK inhibitor with greater cellular potency.

Survivin is a member of the inhibitor of apoptosis gene family (19), which is involved in control of cell division and inhibition of apoptosis (20). This protein, which is expressed in the most common human cancers (21), exerts its antiapoptotic activity by interfering with the processing and activity of caspases (21). Survivin is expressed in a cell cycle–dependent manner, and during mitosis, it localizes to various components of the mitotic apparatus and associates with microtubules of the mitotic spindle (22). Survivin expression is controlled at the transcriptional (23) and post-translational (24) levels. In this context, it has been

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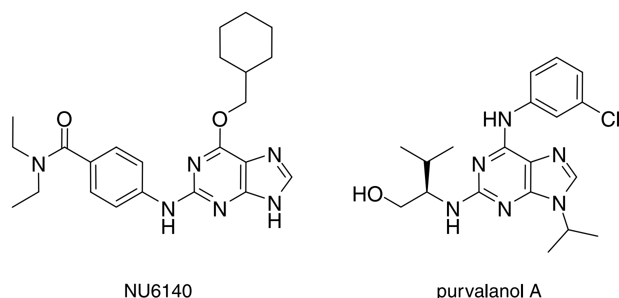

Figure 1. Chemical structures of NU6140 and purvalanol A.

shown that CDK1-mediated phosphorylation of survivin on Thr^{34} is essential for the cytoprotective function of the protein (24). Moreover, previous evidence indicated that targeting survivin by means of purvalanol A resulted in an increased sensitivity to paclitaxel in HeLa cells (25).

In this study, we investigated the cellular effects of the novel CDK inhibitor NU6140 in relation to inhibition of cell proliferation and cell cycle progression and ability to potentiate the apoptotic response to paclitaxel in HeLa cells in comparison with purvalanol A. Results indicated that, when given in combination with paclitaxel, NU6140 significantly increased the apoptotic rate (to a greater extent than that observed in the paclitaxel-purvalanol A combination), with inhibition of survivin expression/phosphorylation as the potential mechanism. A possible direct link between NU6140 effect on paclitaxel cytotoxicity and survivin expression was also suggested by results we collected in a pair of isogenic ovarian cancer cell lines. Specifically, a synergistic effect of the paclitaxel-NU6140 combination, which was paralleled by an increased caspase activation, was observed in the parental line OAW42/e but not in the derived subline ectopically expressing survivin.

Materials and Methods

Cell Line and Tissue Culture

The HeLa cervical carcinoma cell line and a pair of isogenic human ovarian carcinoma cell lines (OAW42/e, which was transfected with an empty pCI Neo vector, and OAW42/Surv, which was transfected with the pCI Neo vector carrying the full-length human survivin cDNA; ref. 26) were used in the study. Cells were maintained in the logarithmic growth phase at 37°C in a 5% CO_2 humidified atmosphere using RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% (v/v) FCS and 0.25% (w/v) gentamicin.

Drugs

Paclitaxel and purvalanol A were purchased from Sigma (St. Louis, MO) and Tocris (Bristol, United Kingdom), respectively. NU6140 was prepared as described below. Drugs were dissolved in DMSO, stored at −20°C, and diluted in complete culture medium immediately before use.

Synthesis of NU6140

To a stirred suspension of 6-cyclohexylmethoxy-2-fluoro-9H-purine (1.66 g; 6.5 mmol), prepared as described previously (27), and *N*,*N*-diethyl-4-aminobenzamide (2.50 g; 13.0 mmol) in trifluoroethanol (54 mL) under nitrogen was added trifluoroacetic acid (2.50 mL; 32.5 mmol). The resulting mixture was allowed to stir at reflux for 48 hours. The reaction mixture was concentrated under reduced pressure and the resulting yellow oil was dissolved in dichloromethane (100 mL) and washed with 5% (w/v) sodium hydrogen carbonate solution (4 × 25 mL). The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to give a yellow oil. Column chromatography with ethyl acetate/petrol (7:3) as eluent gave an off-white crystalline solid (1.34 g; 48.1%). ^1H nuclear magnetic resonance (300 MHz, DMSO-d_6) δ 1.10 [6 H, t, J = 7.0 Hz, $N(CH_2CH_3)_2$], 1.23 (5 H, m, cyclohexyl), 1.80 (6 H, m, cyclohexyl), 3.15 [4 H, br s, $N(CH_2CH_3)_2$], 4.33 (2 H, d, J = 6.3 Hz, CH_2O), 7.28 (2 H, d, J = 8.6 Hz), 7.86 (2 H, d, J = 8.6 Hz), 8.00 [1 H, s, C(8)H], 9.54 (1 H, s, NH). ^{13}C nuclear magnetic resonance (75 MHz, DMSO-d_6) δ 26.1, 26.8, 30.2, 37.7, 72.6, 116.2, 119.0, 127.8, 130.3, 138.7, 141.8, 154.1, 155.9, 161.5, 172.2. Liquid chromatography-mass spectrometry ES^+ (methanol): m/z 423.2 (M + 1), 424.3 (^{13}C M + 1).

Inhibition of CDKs by NU6140

Inhibition of CDK1-cyclin B was determined according to the manufacturer's instructions (New England BioLabs, Beverly, MA) and CDK2-cyclin A was assayed as described previously (28). Inhibition of CDK4-cyclin D1 was determined in a similar assay using an assay buffer composed of 50 mmol/L HEPES, 10 mmol/L $MnCl_2$, 1 mmol/L DTT, 100 μmol/L NaF, 100 μmol/L sodium vanadate, 10 mmol/L sodium glycerophosphate, 5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 100 μmol/L phenylmethylsulfonyl fluoride, and a recombinant retinoblastoma peptide (encoding residues 792–928) as substrate. Cyclin D1-CDK4 was prepared as a glutathione *S*-transferase–tagged complex from baculoviral-infected insect cell lysate and was a gift from AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). Inhibition of CDK5-p25 was determined according to the manufacturer's protocols (Upstate Cell Signalling Solutions, Dundee, United Kingdom). CDK7-cyclin H MAT1 kinase was purchased from the Upstate Cell Signalling Solutions and assayed using the synthetic peptide YSPTSPSYSPTS-PSYSPTSPS-KKKK corresponding to amino acids 1,745 to 1,765 of mouse RNA polymerase II (29) as a substrate and synthesized by the Molecular Biology Unit (University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom). The final ATP concentration in each assay was as follows: CDK1, CDK2, and CDK4, 12.5 μmol/L; CDK5 and CDK7, 100 μmol/L. The IC_{50} concentration for each inhibitor is the concentration required to inhibit enzyme activity by 50% under the assay conditions used.

Colony Formation Assay

HeLa, OAW42/e, and OAW42/Surv cells were plated at appropriate density (250–1,000 cells per well) in six-well plates. Twenty-four hours later, cells were treated with increasing concentration of paclitaxel, NU6140, and purvalanol A singly administrated or with paclitaxel and CDK inhibitors in combination. Specifically, two schedules were tested: (*a*) a 24-hour paclitaxel treatment followed by a 24-hour incubation with NU6140 or purvalanol A and

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(*b*) a 24-hour NU6140 or purvalanol A treatment followed by a 24-hour incubation with paclitaxel. Control cells were exposed to 1% (v/v) DMSO. At the end of the treatments, cells were incubated in drug-free medium for 10 days to form colonies. Colonies consisting of at least 50 cells were stained with 0.5% (w/v) crystal violet and counted. The plating efficiency was calculated from the number of colonies counted and the number of cells seeded. The surviving fraction was calculated as follows: surviving fraction = (plating efficiency of treated sample) / (plating efficiency of control).

Median Effect Analysis

The method described by Chou and Talalay (30) was used to determine the nature of the interaction between NU6140 or purvalanol A and paclitaxel. Drugs were always combined at a constant ratio of paclitaxel and NU6140 or purvalanol A concentrations (i.e., 1:1,000). The interaction of drugs was quantified by determining a combination index (CI). CI values of <1 or >1 indicated synergy or antagonism, respectively, whereas a CI value of 1 indicates additivity.

Cell Cycle Analysis

The effect of CDK inhibitors on cell cycle progression was analyzed by flow cytometry. Cells were incubated with NU6140 (1–100 μmol/L) or purvalanol A (1–100 μmol/L) alone for 24 hours or with paclitaxel (50 nmol/L) for 24 hours followed by a 24-hour exposure to each CDK inhibitor (10 μmol/L). Cells were then trypsinized, washed with PBS, permeabilized with 70% (v/v) ethanol at −20°C for 30 minutes, stained with a solution containing 50 μg/mL propidium iodide, 50 mg/mL RNase, and 0.05% (w/v) NP40 for 30 minutes at 4°C, and analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). The cell cycle distribution was evaluated on DNA plots using the CellFit software according to the SOBR model (Becton Dickinson).

Apoptosis Analysis

Cells were exposed to NU6140 (1–100 μmol/L) or purvalanol A (1–100 μmol/L) alone for 24 hours or with paclitaxel (50 nmol/L) for 24 hours followed by a 24-hour exposure to each CDK inhibitor (10 μmol/L) and then scored for nuclear morphology of apoptosis (chromatin condensation and DNA fragmentation) by labeling with a solution containing 50 μg/mL propidium iodide, 50 mg/mL RNase, and 0.05% (w/v) NP40. After staining, the slides were examined using fluorescence microscopy. The percentage of cells with an apoptotic nuclear morphology was determined by scoring at least 500 cells in each sample.

In the same cellular samples, caspase-9 and caspase-3 activation was analyzed. Caspase-9 and caspase-3 proteolytic processing was analyzed by Western blot analysis as described below. In addition, caspase-9/Mch6 Fluorometric Protease Assay kit (MBL Ltd., Nagoya, Japan) and the Caspase-3 Assay kit (BD Biosciences, PharMingen, San Diego, CA) were used for quantitative determination of caspase activity. Briefly, cells were washed, pelleted, and lysed according to the manufacturer's instructions. Total protein and the specific fluorogenic substrate (Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin for caspase-9 and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin for caspase-3) were mixed for 1 hour at 37°C and transferred to 96-well microtiter plates. Hydrolysis of the specific substrates for caspase-9 and caspase-3 was monitored by spectrofluorometry at 505 and 440 nm, respectively.

Western Blot Analysis

For protein analysis, total cellular lysates were separated on a 12% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose using standard protocols. The filters were blocked in PBS with 5% skim milk and incubated overnight with primary antibodies specific for survivin (Novus Biologicals, Littleton, United Kingdom), caspase-9 and caspase-3, Bcl-2, and Bcl-x_L (Abcam Ltd., Cambridge, United Kingdom). The filters were then incubated with the secondary peroxidase-linked whole antibodies (Amersham Biosciences Europe GmbH, Freiburg, Germany). Bound antibodies were detected using the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences Europe). An anti–proliferating cell nuclear antigen monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used on each blot to ensure equal loading of protein on the gel. The results were quantified by densitometric analysis using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

For the assessment of the Thr^{34}-phosphorylated form of survivin, precleared, detergent-solubilized cell extracts (100 μL) were immunoprecipitated with the anti-human survivin antibody (20 μg/mL) for 16 hours at 4°C, with precipitation of the immune complex by addition of 50 μL of a 50:50 protein A slurry. After separation by SDS gel electrophoresis, samples were transferred to nitrocellulose and incubated with the antibody (0.1 μg/mL) to Thr^{34}-phosphorylated survivin (kindly provided by Prof. D. Altieri, Department of Cancer Biology and the Cancer Center, University of Massachusetts Medical School, Worcester, MA; of ref. 24) followed by secondary anti-rabbit antibody and chemiluminescence.

Reverse Transcription-PCR

Total RNA isolated from exponentially growing cells was reverse transcribed using a GeneAmp RNA PCR Core kit (Perkin-Elmer, Wellesley, MA) according to the manufacturer's instructions. To analyze survivin expression, the resultant cDNA was amplified using specific primers designed based on the nucleotide sequence of *survivin* gene: the sense primer was 5′-AGCCCTTTCTCAAGGAC-CAC-3′ and the antisense primer was 5′-TGACAGATAAG-GAACCTGCA-3′ (M-Medical-Genenco, Firenze, Italy). Cycling conditions were as follows: initial denaturation at 95°C for 2 minutes followed by 30 cycles at 95°C for 60 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. A fragment corresponding to β-actin was coamplified and used as the standard of the amplification reaction. The PCR products were verified by gel electrophoresis.

Statistical Analysis

Statistical evaluation of data was done with two-tailed Student's *t* test. *P*s < 0.05 were considered statistically significant.

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Results

Inhibition of CDKs by NU6140 and Purvalanol A

The activity of NU6140 against purified CDKs was as follows [IC_{50} concentration ± SD (μmol/L; $n \geq 3$), the concentration required to inhibit the CDK by 50%]: CDK1-cyclin B, 6.6 ± 1.1 μmol/L; CDK2-cyclin A, 0.41 ± 0.05 μmol/L; CDK4-cyclin D, 5.5 ± 1.1 μmol/L; CDK5-p25, 15 ± 4 μmol/L; and CDK7-cyclin H, 3.9 ± 0.5 μmol/L. Thus, NU6140 is 10- to 36-fold selective for CDK2-cyclin A over the other CDKs tested. For comparison, the corresponding values for purvalanol A under the same assay conditions are as follows: CDK1-cyclin B, 0.4 ± 0.09 μmol/L; CDK2-cyclin A, 0.021 ± 0.002 μmol/L; CDK4-cyclin D, 8.2 ± 1.2 μmol/L; CDK5-p25, 0.24 ± 0.01 μmol/L; and CDK7-cyclin H, 0.1 ± 0.01 μmol/L.

Effect of CDK Inhibitors, Alone or in Combination with Paclitaxel, on Tumor Cell Growth

Clonogenic survival assays were done to assess the antiproliferative activity of NU6140 and purvalanol A alone or in combination with paclitaxel. In a preliminary set of experiments, HeLa cervical carcinoma cells were treated with increasing concentrations of each CDK inhibitor alone. A concentration-dependent decline in cell survival was observed after treatment with both compounds (Table 1), although NU6140 was somewhat more potent than purvalanol A as indicated by the 3-fold lower concentration required to kill 50% of cells (IC_{50}s, 2.3 ± 0.2 versus 8.7 ± 0.4 μmol/L, respectively).

We further assessed the cytotoxic effects of the CDK inhibitors in combination with paclitaxel. HeLa cells were treated with paclitaxel for 24 hours followed by a

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Table 1. Cytotoxic effect of NU6140 and purvalanol A against HeLa cells as single agents and in combination with paclitaxel

Agent/combination	Concentration	Surviving fraction		Expected/observed ratio*	CI†
		Observed	Expected‡		
Paclitaxel (nmol/L)	1	0.76			
	2.5	0.34			
	5	0.12			
NU6140 (μmol/L)	1	0.70			
	2.5	0.47			
	5	0.14			
Purvalanol A (μmol/L)	1	0.97			
	2.5	0.89			
	5	0.75			
Paclitaxel (nmol/L) + NU6140 (μmol/L)	1				
	1	0.50	0.53	1.06	1.14
	2.5				
	2.5	0.05	0.16	3.2	0.47
	5				
	5	0.002	0.02	10	0.29
Paclitaxel (nmol/L) + purvalanol A (μmol/L)	1				
	1	0.62	0.74	1.19	0.90
	2.5				
	2.5	0.18	0.30	1.7	0.60
	5				
	5	0.02	0.09	4.5	0.44
NU6140 (μmol/L) + paclitaxel (nmol/L)	1				
	1	0.64	0.53	0.87	1.51
	2.5				
	2.5	0.09	0.16	1.78	0.76
	5				
	5	0.012	0.02	1.67	0.45
Purvalanol A (μmol/L) + paclitaxel (nmol/L)	1				
	1	0.66	0.74	1.12	0.95
	2.5				
	2.5	0.18	0.30	1.67	0.70
	5				
	5	0.03	0.09	3.00	0.53

*(Surviving fraction observed) / (surviving fraction expected).
†CI according to Chou and Talalay (ref. 30).
‡Calculated as the product of the surviving fractions observed for individual drugs.

24-hour exposure to NU6140 or purvalanol A, and the opposite sequence was also tested. As shown in Table 1, both CDK inhibitors cooperated with paclitaxel to inhibit clonogenic cell survival. Specifically, sequential treatment with paclitaxel followed by each CDK inhibitor was more effective than the reverse treatment sequence, and when drugs were combined at the two highest concentrations, a synergistic effect was consistently observed. However, the most marked synergistic interaction was obtained with the paclitaxel-NU6140 combination as indicated by the lowest CI values (Table 1). A marked synergistic effect of the paclitaxel-NU6140 combination was also confirmed in OAW42/e ovarian cancer cells. Conversely, a subadditive effect of the paclitaxel-NU6140 combined treatment was found in OAW42/Surv cells ectopically expressing survivin (Table 2).

Effects of CDK Inhibitors on Cell Cycle Progression and Apoptosis

Flow cytometric analysis of propidium iodide–stained HeLa cells showed that a 24-hour treatment with NU6140 and purvalanol A resulted in a concentration-dependent decrease in the number of cells in the G_1 phase and a concomitant increase in the sub-G_1 apoptotic cell population (Fig. 2A). Moreover, chromatin condensation and DNA fragmentation, which are common features of

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Table 2. Cytotoxic effect of NU6140 against OAW42 and OAW42/Surv cells as single agent and in combination with paclitaxel

Agent/combination	Concentration	Surviving fraction		Expected/observed ratio*	CI †
		Observed	Expected ‡		
A. OAW42/e					
Paclitaxel (nmol/L)	1	0.81			
	2.5	0.56			
	5	0.14			
NU6140 (μmol/L)	1	0.83			
	2.5	0.29			
	5	0.17			
Paclitaxel (nmol/L) + NU6140 (μmol/L)	1				
	1	0.82	0.67	2.09	1.33
	2.5				
	2.5	0.08	0.16	2.00	0.57
	5				
	5	0.0023	0.023	10	0.36
NU6140 (μmol/L) + paclitaxel (nmol/L)	1				
	1	0.43	0.67	1.55	0.95
	2.5				
	2.5	0.13	0.16	1.23	0.74
	5				
	5	0.014	0.023	1.64	0.60
B. OAW42/Surv					
Paclitaxel (nmol/L)	1	0.99			
	5	0.75			
NU6140 (μmol/L)	1	0.89			
	2.5	0.30			
	5	0.18			
Paclitaxel (nmol/L) + NU6140 (μmol/L)	1				
	1	0.85	0.88	1.03	1.19
	2.5				
	2.5	0.30	0.27	0.90	1.28
	5				
	5	0.16	0.13	0.81	1.34
NU6140 (μmol/L) + paclitaxel (nmol/L)	1				
	1	0.87	0.88	1.01	1.25
	2.5				
	2.5	0.32	0.27	0.84	1.33
	5				
	5	0.18	0.13	0.72	1.40

*(Surviving fraction observed) / (surviving fraction expected).
† CI according to Chou and Talalay (ref. 30).
‡ Calculated as the product of the surviving fractions observed for individual drugs.

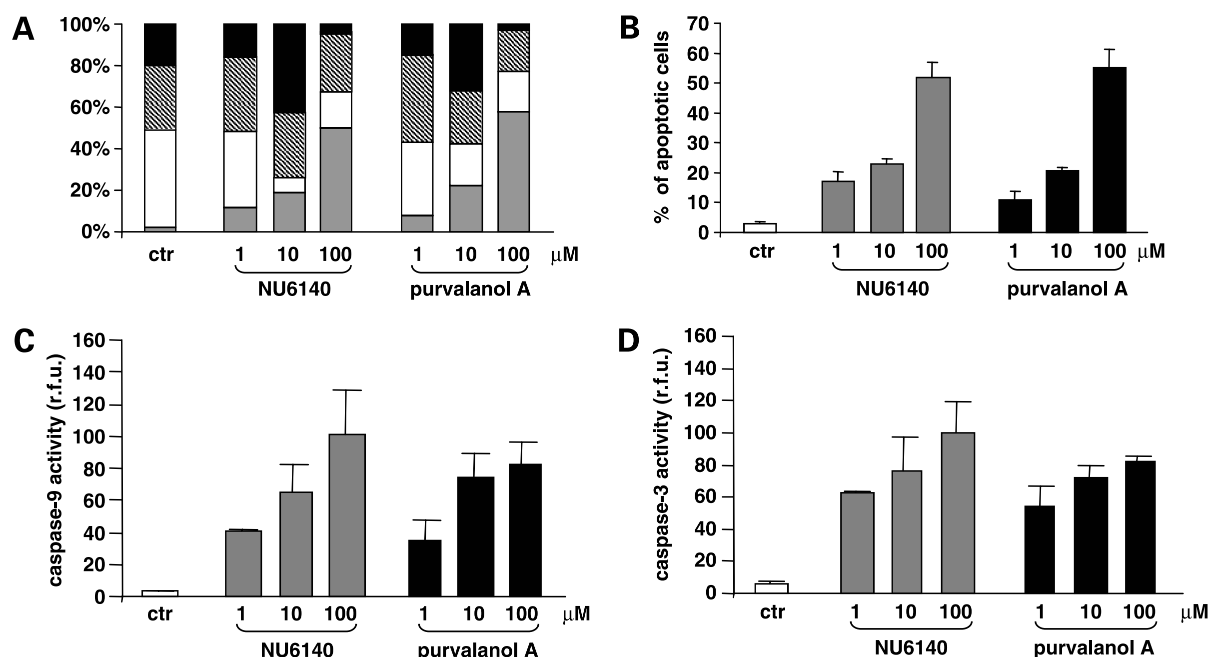

Figure 2. Effect of NU6140 and purvalanol A on cell cycle and apoptosis. **A,** HeLa cells were exposed to 1% (v/v) DMSO (control cells; *ctr*) or 1, 10, and 100 μmol/L NU6140 and purvalanol A. Cell cycle distribution was assessed as described in Materials and Methods. The percentage of cells in sub-G_1 (*gray*), G_1 (*white*), S (*hatched*), and G_2-M (*black*) phases are shown. *Columns,* mean of three independent experiments; SDs were always within 5%. **B,** percentage of cells with an apoptotic morphology was assessed by fluorescence microscopy after exposure of cells to 1% (v/v) DMSO (*white column*) or 1, 10, and 100 μmol/L NU6140 (*gray column*) and purvalanol A (*black column*) for 24 h. *Columns,* mean of three independent experiments; *bars,* SD. Caspase-9 (**C**) and caspase-3 (**D**) catalytic activity was determined by hydrolysis of the fluorogenic substrates Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin, respectively, in cells exposed to 1% (v/v) DMSO (*white column*) or treated with 1, 10, 100 μmol/L NU6140 (*gray column*) and purvalanol A (*black column*) for 24 h. *Columns,* mean relative fluorescence units (*r.f.u.*) of three independent experiments; *bars,* SD.

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apoptosis, were detected by fluorescence microscopy after staining with propidium iodide of HeLa cells treated with NU6140 and purvalanol A. The percentage of cells with an apoptotic nuclear morphology, as determined on the overall cell population, increased in a concentration-dependent fashion after treatment with both inhibitors (Fig. 2B).

At the molecular level, treatment with NU6140 and purvalanol A resulted in a concentration-dependent increase in the catalytic activity of capase-9 and caspase-3 as assessed by the *in vitro* hydrolysis of specific fluorogenic substrates (Fig. 2C and D).

Effects of CDK Inhibitors on Survivin Expression

To investigate the mechanisms by which NU6140 and purvalanol A induced apoptosis, we evaluated the expression of the antiapoptotic gene *survivin* in cancer cells after drug exposure. As shown in Fig. 3A, treatment of HeLa cells with NU6140 or purvalanol A induced a concentration-dependent decrease in the levels of survivin protein. A NU6140-induced inhibition of survivin expression was also seen in OAW42/e and OAW42/Surv cells. However, in OAW42/Surv cells, which displayed a 2.5-fold higher basal level of survivin protein compared with OAW42/e cells, the protein abundance was consistently higher than in parental cells also after exposure to different NU6140 concentrations (Fig. 3A). Furthermore, reverse transcription-PCR analysis in HeLa cells showed that survivin mRNA levels were reduced by both CDK inhibitors (Fig. 3C), suggesting that NU6140 and purvalanol A modulate survivin expression at the transcriptional level.

The possibility that NU6140 and purvalanol A inhibit survivin activation by interfering with CDK1, a kinase that is known to activate survivin through phosphorylation of its Thr^{34} residue (25), was also studied. Results from immunoblotting experiments using a phosphospecific antibody indicated that the levels of the active, Thr^{34}-phosphorylated form of survivin were reduced in a concentration-dependent fashion following treatment of HeLa cells with NU6140 and purvalanol A (Fig. 3D). Conversely, exposure to NU6140 failed to modulate the expression of other antiapoptotic proteins, such as Bcl-2 and Bcl-x_L, in all three cell lines (Fig. 3B).

Effects of Combined Treatment with Paclitaxel and CDK Inhibitors on Cell Cycle Phase Distribution and Apoptosis

Next, we examined cell cycle perturbations induced by paclitaxel alone or paclitaxel followed by the CDK inhibitors (Fig. 4). HeLa cells treated with paclitaxel alone for 24 hours accumulated in the G_2-M phase, and a fraction of sub-G_1 apoptotic cells, corresponding to 19 ± 1.2% of the overall cell population, was observed. When cells were treated sequentially with paclitaxel and

NU6140, a reduction in G_2-M arrested was observed and 86 ± 11% of cells underwent apoptosis. Such an apoptosis rate was significantly ($P < 0.01$) higher than that observed after sequential treatment of cells with paclitaxel and purvalanol A (37 ± 8%). However, it must be stressed that results of cell cycle analysis of cells undergoing apoptosis have to be considered with some caution, because partial loss of DNA during the apoptotic process can make it difficult to distinguish between S-phase and G_2-M–phase cells.

We then assessed whether the increased apoptotic response induced by combined treatments was sustained by caspase activation in HeLa cells. Immunoblotting results indicated that treatment with paclitaxel and purvalanol A, singly given or in combination, did not induce any proteolytic processing of the enzymes. Caspase-9 cleavage was detected only after the sequential treatment of cells with paclitaxel and NU6140. Moreover, in response to NU6140, the 32-kDa pro-caspase-3 was partially cleaved to a 20-kDa intermediate form, whereas a complete activation of caspase-3 was observed after sequential treatment with paclitaxel followed by NU6140 as indicated by the presence of both 20- and 17-kDa active cleaved products (Fig. 5A).

The proteolytic processing of caspase-9 and caspase-3 coincided with an increased catalytic activity of both enzymes. Specifically, caspase-9 and caspase-3 activity was ~3-fold ($P < 0.001$) and 4-fold ($P < 0.01$) higher, respectively, in HeLa cells treated with paclitaxel followed by NU6140 than in cells exposed to single agents or paclitaxel-purvalanol A combination (Fig. 5B). Similarly, a 3-fold increase ($P < 0.001$) in caspase-9 and caspase-3 catalytic activity was observed in OAW42/e cells exposed to paclitaxel followed by NU6140 compared with cells treated with individual agents (Fig. 5C). Conversely, a modest and nonstatistically significant increase in caspase catalytic activities was found in OAW42/Surv cells treated with paclitaxel-NU6140 combination (Fig. 5C).

Effects of Combined Treatment with Paclitaxel and CDK Inhibitors on Survivin Activation

Finally, the effects of paclitaxel, alone or in combination with NU6140 and purvalanol A, on survivin activation, as indicated by the level of the Thr^{34}-phosphorylated form of the protein, was assayed by immunoblotting. As expected, treatment with paclitaxel alone increased the level of phospho-survivin, whereas combined treatment with paclitaxel and CDK inhibitors appreciably inhibited survivin phosphorylation, which was almost completely abrogated in cells exposed to the paclitaxel-NU6140 combination (Fig. 6).

Discussion

Because most human malignancies show aberrations in cell cycle control, the pharmacologic modulation of CDKs may be an important approach for the therapy of cancer. In the present study, we evaluated the cellular effects of the purine derivative NU6140, a novel CDK inhibitor, in comparison with purvalanol A, in HeLa cervical carcinoma and in OAW42/e and OAW42/Surv ovarian cancer cell lines. Both CDK inhibitors induced an impairment of cell cycle progression with a concentration-dependent accumulation of cells in the G_1 compartment, which was accompanied by a decline of cell growth and the

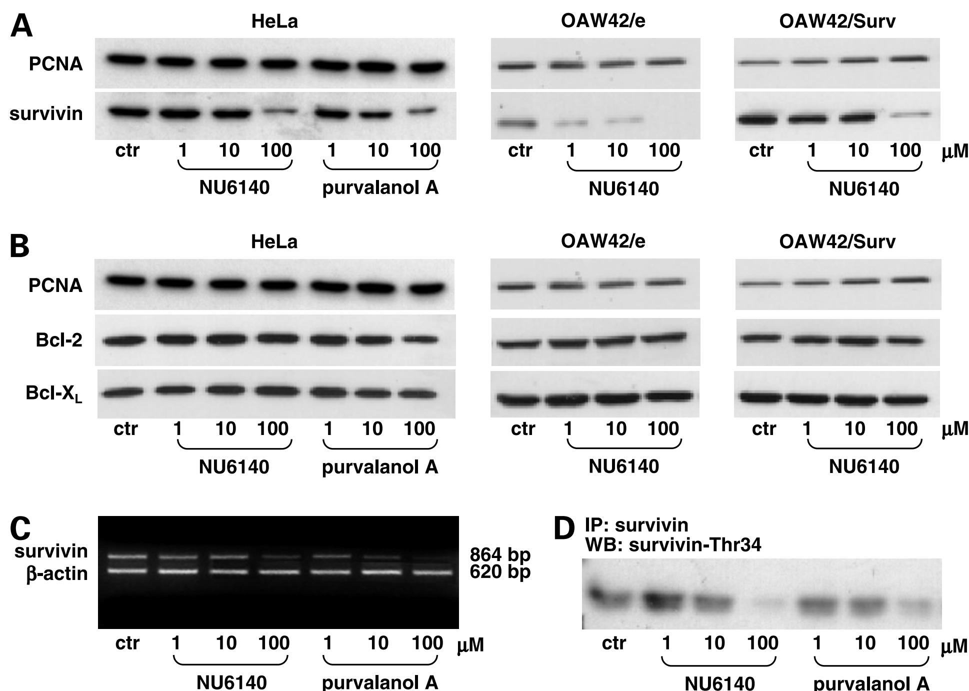

Figure 3. Down-regulation of survivin by CDK inhibitor treatment. Tumor cells were exposed to 1% (v/v) DMSO (control cells) or treated with 1, 10, 100 μmol/L NU6140 and purvalanol A for 24 h. Survivin (**A**) and Bcl-2 and Bcl-x_L (**B**) protein expression was assessed by Western blot. Proliferating cell nuclear antigen (*PCNA*) was used as a control for loading. **C,** survivin mRNA expression was assessed by reverse transcription-PCR. β-actin was used as a control for amplification. **D,** survivin phosphorylation on Thr^{34} as evaluated by Western blot. Survivin was immunoprecipitated using the antihuman survivin antibody and analyzed with the antibody to phosphorylated Thr^{34}.

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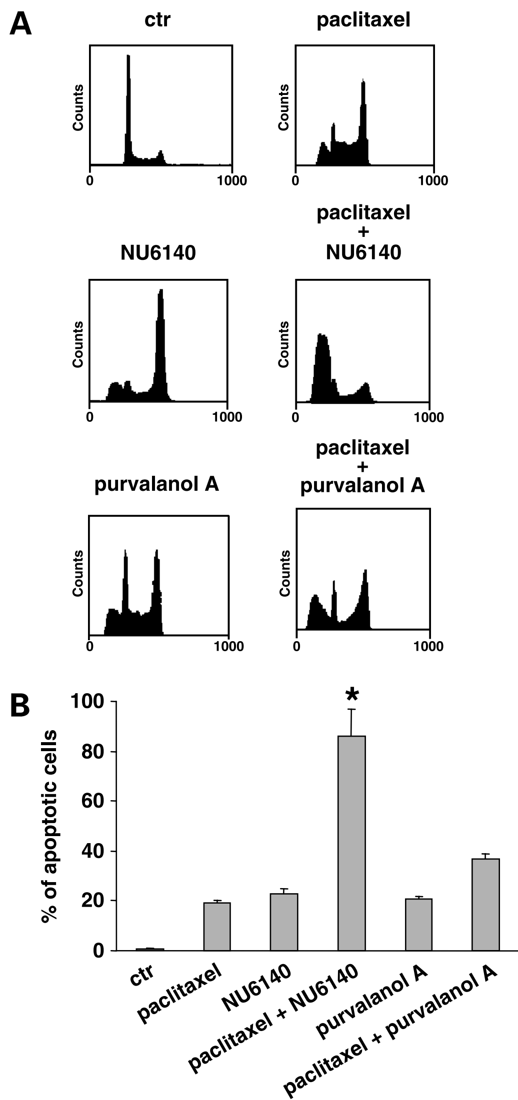

Figure 4. Effect of combined treatment with paclitaxel followed by CDK inhibitors on cell cycle progression and apoptosis. **A,** HeLa cells were incubated with paclitaxel (50 nmol/L), NU6140 (10 μmol/L), or purvalanol A (10 μmol/L) alone for 24 h or with paclitaxel (50 nmol/L) for 24 h followed by a 24-h exposure to CDK inhibitors (10 μmol/L). Following treatment, cells were analyzed by flow cytometry. **B,** quantification of sub-G_1 apoptotic cells. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ (Student's t test).

induction of an apoptotic response. Treatment with NU6140 and purvalanol A also inhibited the expression of survivin by negatively interfering with the transcription of the gene. Moreover, NU6140- and purvalanol A–treated cells exhibited a lower abundance of the Thr^{34}-phosphorylated form of survivin possibly as a consequence of drug-induced inhibition of CDK1 catalytic activity. It has been shown that during mitosis survivin physically associates with CDK1 and that phosphorylation on its Thr^{34} residue by CDK1 is a requirement for survivin-mediated cytoprotection (24). Moreover, one of the mechanisms by which CDK1-mediated phosphorylation regulates survivin seems to be by increasing its stability at mitosis (25). No appreciable interference with the expression of other antiapoptotic proteins, such as Bcl-2 and Bcl-x_L, was observed after exposure of tumor cells to NU6140.

CDK-mediated down-regulation of survivin observed in the present study is consistent with previous evidence indicating that interference with survivin function by the use of specific inhibitors (including antisense oligonucleotides, ribozymes, or survivin dominant-negative mutants) leads to increased apoptotic cell death in different human tumor models (31–33). Furthermore,

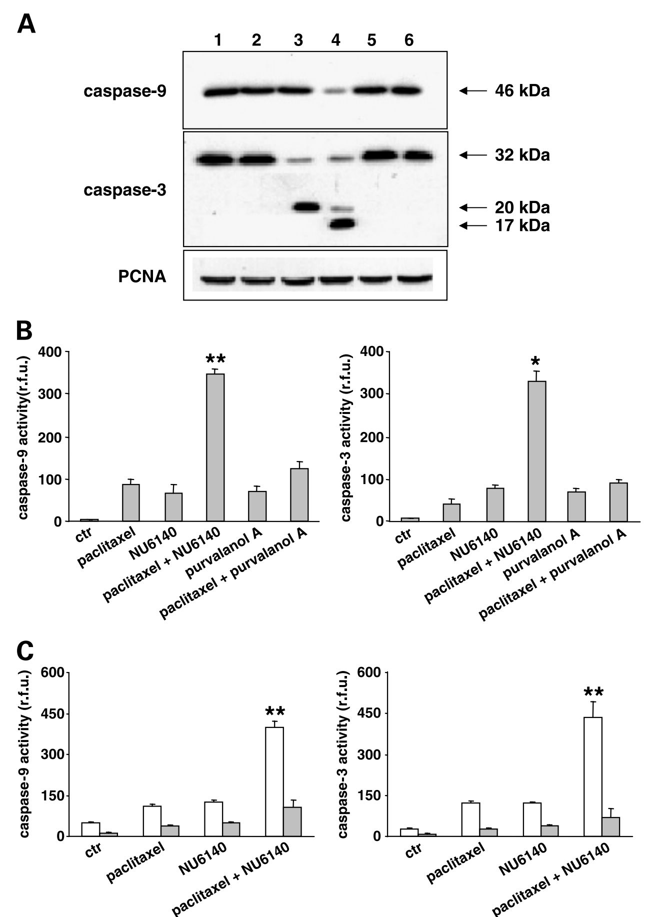

Figure 5. Activation of caspases by combined treatment of tumor cells with paclitaxel followed by CDK inhibitors. Cells were incubated with paclitaxel (50 nmol/L), NU6140 (10 μmol/L), or purvalanol A (10 μmol/L) alone for 24 h or with paclitaxel (50 nmol/L) for 24 h followed by a 24-h exposure to CDK inhibitors (10 μmol/L). **A,** proteolytic processing of caspase-9 and caspase-3 was assessed by Western blot assay in HeLa cells. *Lane 1,* control; *lane 2,* paclitaxel; *lane 3,* NU6140; *lane 4,* paclitaxel + NU6140; *lane 5,* purvalanol A; *lane 6,* paclitaxel + purvalanol A. **B,** caspase-9 and caspase-3 catalytic activity as determined by hydrolysis of the fluorogenic substrates Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin, respectively, in HeLa cells. **C,** caspase-9 and caspase-3 catalytic activity in OAW42/e (*white column*) and OAW42/Surv (*gray column*). *Columns,* mean relative fluorescence units of three independent experiments; *bars,* SD. **, $P < 0.001$; *, $P < 0.01$ (Student's t test).

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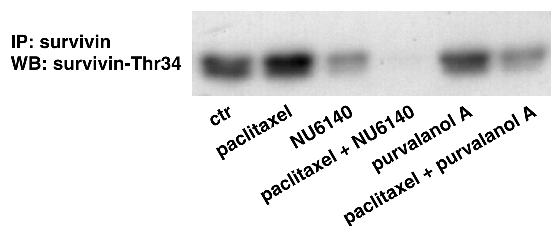

Figure 6. Effect of combined treatment of HeLa cells with paclitaxel followed by CDK inhibitors on survivin phosphorylation. The phosphorylation of survivin on Thr^{34} residue was evaluated on HeLa cells treated with paclitaxel (50 nmol/L), NU6140 (10 μmol/L), or purvalanol A (10 μmol/L) alone for 24 h or with paclitaxel (50 nmol/L) for 24 h followed by a 24-h exposure to CDK inhibitors (10 μmol/L) as described in Fig. 3D legend.

CDK inhibitor–induced activation of caspase-9 and caspase-3 points to a selective role of survivin in antagonizing mitochondrial-dependent apoptosis. In this context, a possible direct interaction of survivin with caspase-9 has been reported by O'Connor et al. (24). More recently, Song et al. (34) suggested an alternative model for indirect inhibition of caspases by survivin, which requires Smac/Diablo as an intermediate protein. However, the possibility that survivin down-regulation also results in caspase-independent apoptosis was recently suggested by Liu et al. (35). Specifically, they found that the earliest proapoptotic event in melanoma cells transfected with a survivin dominant-negative mutant was nuclear translocation of mitochondrial apoptosis-inducing factor known to trigger both apoptotic mitochondrial events and caspase-9-independent DNA fragmentation.

Taking into account the ability of NU6140 and purvalanol A to down-regulate survivin, we tested the ability of the inhibitors to increase paclitaxel cytotoxicity. Results from preclinical studies as well as clinical data suggest a direct link between survivin expression and tumor cell susceptibility to paclitaxel. Initial reports showed that the forced expression of survivin was able to counteract apoptosis induced by paclitaxel in NIH3T3 fibroblasts (36). More recently, we showed that stable transfection of human ovarian carcinoma cells with survivin cDNA caused a 4- to 6-fold increase in resistance to paclitaxel with a concomitant reduction in the apoptotic response of the cells to the drug (26). The increased resistance of ovarian cancer cells ectopically expressing survivin to paclitaxel was also confirmed in the present study. These findings were indirectly supported by similar observations obtained analyzing clinical tumor material where high levels of survivin protein expression, as detected by immunohistochemistry in advanced ovarian carcinomas, were significantly associated with clinical resistance to a paclitaxel/platinum–based regimen. Specifically, in these patients, survivin overexpression correlated with a lower clinical or pathologic complete remission rate than absent/low protein expression (26).

The current article shows that combination treatment of HeLa cells with paclitaxel and CDK inhibitors produced a sequence-dependent synergistic effect. Specifically, exposure of paclitaxel-treated cells to NU6140 or purvalanol A strongly decreased clonogenic cell survival and increased caspase-9-mediated apoptotic response. However, such an enhancement was significantly greater for NU6140 than for purvalanol A. A synergistic effect of the paclitaxel-NU6140 combination, which was accompanied by a significantly enhanced caspase-9- and caspase-3-mediated apoptotic response, was also observed in OAW42/e ovarian cancer cells. Conversely, a subadditive effect of the paclitaxel-NU6140 combined treatment and a lack of significant caspase activation was found in OAW42/Surv cells, which ectopically expressed survivin and showed a considerably higher level of survivin protein expression compared with OAW42 parental cells. Sequential treatment of HeLa cells with CDK inhibitors greatly reduced (or almost completely abrogated, in the case of NU6140) the expression of the Thr^{34}-phosphorylated form of survivin, which conversely was significantly increased after treatment with paclitaxel alone. These data are consistent with a recently proposed model according to which microtubule stabilization by poisons, such as paclitaxel, engages a survival pathway that depends on elevated activity of CDK1 kinase activity and increased phosphorylation and expression of survivin (25). These authors showed that sequence-specific inhibition of CDK1 kinase with purvalanol A after paclitaxel treatment in HeLa cells removed this survival checkpoint and promoted strong antitumor activity *in vitro* an *in vivo*. As shown in the studies described here, NU6140 induces greater potentiation of paclitaxel cytotoxicity than purvalanol A despite the generally lower potency of NU6140 as an inhibitor of CDKs in purified enzyme inhibition assays. Studies with NU6140 in the SKUT1B uterine tumor cell line have shown that, following exposure to an extracellular concentration of 10 μmol/L NU6140 for 4 hours, cellular levels can exceed 200 pmol per 10^6 cells (i.e., ~200 μmol/L).[3] Hence, the greater potentiation of paclitaxel cytotoxicity induced by NU6140 could in part be due to the preferential cellular accumulation of the compound by yet undefined mechanisms. Moreover, although it has not been formally investigated in this study, the possibility that NU6140 inhibits the phosphorylation of the carboxyl-terminal domain of RNA polymerase II and interferes with DNA transcription and RNA processing, as already shown for the CDK inhibitor flavopiridol (37), cannot be excluded.

Overall, results from our study indicate that NU6140 can potentiate the apoptotic effect of paclitaxel in cancer cells and support the hypothesis that inhibition of survivin phosphorylation, with the consequent disruption of the CDK1-mediated survival checkpoint, is a major mechanism.

[3] H. Thomas and D.R. Newell, unpublished results.

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This may provide a rational approach to enhance the therapeutic efficacy of paclitaxel in the clinical setting. In fact, based on the evidence derived from preclinical studies, which show the concept of cell cycle–mediated drug resistance, the combination of CDK inhibitors with standard cytotoxic agents is now emerging as a promising approach to cancer therapy.

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