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Cell cycle arrest and cytochrome *c*-mediated apoptotic induction by MCS-5A is associated with up-regulation of $p16^{INK4a}$ in HL-60 cells

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ABSTRACT

MCS-5A, an analog of sangivamycin, selectively inhibits the cyclin-dependent kinases CDK1 and 4 in HL-60 cells in vitro (IC_{50}: 9.6 and 8.8 μM, respectively), while weakly inhibiting other housekeeping protein kinases. MCS-5A effectively induces HL-60 cell cycle arrest at the G_1 and G_2/M phases through direct inhibition of CDK1 and 4 activity. In addition, elevated expression of $p16^{INK4a}$ and a reduction in the level of hyperphosphorylated pRb showed that 3 μM MCS-5A also induces $p16^{INK4a}$-mediated cell cycle arrest at the G_1 phase. Furthermore, apoptotic induction in MCS-5A-treated HL-60 cells is associated with the release of cytochrome *c* from mitochondria, which, in turn, results in the activation of procaspase-8, -9 and -3, and the cleavage of poly(ADP-ribose) polymerase (PARP). In addition, the involvement of $p16^{INK4a}$ in this apoptotic induction was demonstrated using A549 cells with a homozygous deletion of $p16^{INK4a}$. Based on these results, we conclude that MCS-5A is a candidate therapeutic agent for the treatment of human promyelocytic leukemia via the up-regulation of $p16^{INK4a}$.

Serine/threonine protein kinases play pivotal roles in the cellular signaling pathways that control the proliferation and differentiation of eukaryotic cells.[1,2] The cyclin-dependent kinases (CDKs), which belong to this protein kinase family, are regulators of eukaryotic cell cycle progression in cooperation with various endogenous cyclins and cyclin-dependent kinase inhibitors (CKIs), including $p21^{CIP1}$, $p27^{KIP1}$, and $p16^{INK4a}$.[3,4] Achieving cell cycle control is an important goal in the treatment of diseases characterized by uncontrolled cell proliferation. Consequently, the CDKs could be important molecular targets for such therapeutic intervention, and inhibition of CDK activity may be particularly useful in the treatment of cancer.

Apoptosis has been characterized as a fundamental cellular event to maintain the physiological balance and homeostasis of the organism.[5] The relationship between dysregulation of apoptosis and cancer formation has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways.[5,6]

Accordingly, many small molecular inhibitors of the CDKs have been described during the last decade.[7–18] Previously, we showed that sangivamycin and toyocamycin isolated from *Streptomyces* sp. LDL931 inhibit CDK1 and CDK2.[19] Sangivamycin was originally found to have antiviral activity.[20,21] To search for a novel antiproliferative and antitumor compound that specifically inhibits the CDKs and has minimal inhibitory effects on other protein kinases, we synthesized L-derivatives of sangivamycin.[22] One of these is MCS-5A (also known as xylocydine[23]), which was elucidated as 4-amino-6-bromo-7-(β-L-xylofuranosyl)pyrrolo[2,3-*d*]pyrimidine-5-carboxamide (Fig. 1).

In the present study, we demonstrate that treatment of human promyelocytic leukemia (HL-60) cells with MCS-5A is associated with an accumulation of cells in the G_1 and G_2/M phases of the cell cycle and with the induction of apoptosis via a cytochrome *c*-mediated intrinsic pathway, both effects of which are caused by a key molecular event, the up-regulation of $p16^{INK4a}$.

To examine whether MCS-5A can selectively inhibit CDKs activity, we used in vitro kinase assays employing the specific peptide substrates histone H1 for CDK1 and GST-Rb for CDK4 to determine IC_{50} values. CDK1 and 4 were immunoprecipitated from total cell

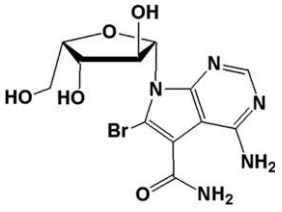

Figure 1. The chemical structure of MCS-5A, 4-amino-6-bromo-7-(β-L-xylofuranosyl)pyrrolo[2,3-*d*]pyrimidine-5-carboxamide.

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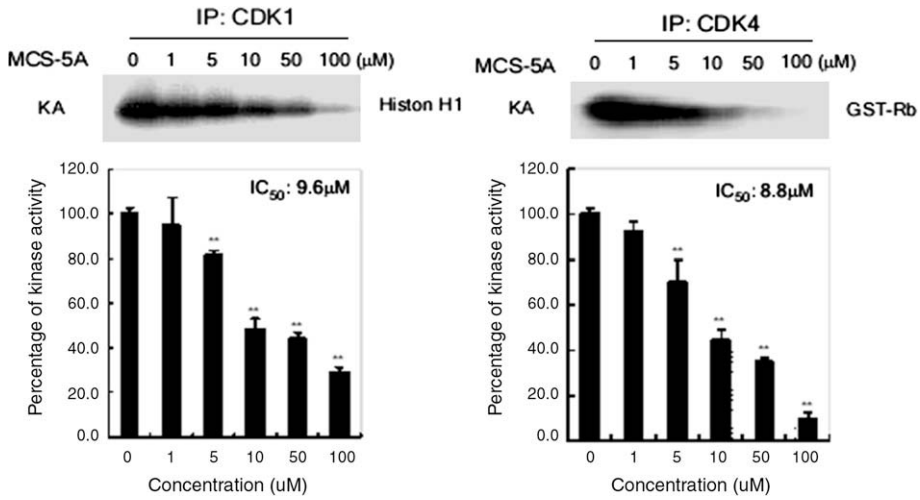

Figure 2. Inhibitory effect of MCS-5A on CDK1 and 4 activity. CDKs were immunoprecipitated (IP) with antibodies, and kinase activity was measured. As shown in the upper panels, kinase assays (KA) were performed at 30 °C for 15 min in a reaction buffer containing a synthetic peptide as a specific substrate for each kinase together with 1 μCi of [γ-^{32}P] ATP and each kinase in a final volume of 25 μl. The reaction was stopped by adding 6 μl of 100% trichloroacetic acid and then trapped on P-81 paper. Trapped radioactivity was measured by a liquid scintillation counter. Data are expressed as percent kinase activity compared to untreated control and are representative of three independent experiments. ($^{**}p < 0.01$ vs control).

Table 1
Inhibitory effect of 10 μM MCS-5A on housekeeping kinases

Protein kinase	Inhibition* (%)
Jun N-terminal kinase 1 (JNK1)	15 ± 6
Jun N-terminal kinase 2 (JNK2)	19 ± 1
Jun N-terminal kinase 3 (JNK3)	8 ± 0
Protein kinase A (PKA)	15 ± 2
Protein kinase C (PKC)	13 ± 5
Spleen tyrosine kinase (SYK)	0
Zeta-chain-associated protein kinase 70 (ZAP70)	0
Leukocyte-specific protein tyrosine kinase (LCK)	6 ± 1
c-Src tyrosine kinase (CSK)	5 ± 3
MLK-like MAP triple kinase (MLTK-α)	10 ± 2
Phosphatidylinositol-3-kinase (PI3K)	4 ± 1
CDK1/Cyclin B	70 ± 4
CDK4/Cyclin D1	85 ± 2

* Data are the mean ± SD of three independent experiments.

Table 2
Flow cytometric analysis of the cell cycle population (%) in HL-60 cells treated with 0.5 μM MCS-5A.

	MCS-5A (0.5 μM)				
	0 h	1 h	4 h	8 h	24 h
G_1	38.6 ± 1.3	23.8 ± 2.1	20.4 ± 2.1	42.9 ± 1.3	35.0 ± 2.9
S	4.8 ± 0.9	2.1 ± 0.7	4.3 ± 0.2	2.9 ± 0.4	4.3 ± 0.5
G_2	30.6 ± 2.1	39.1 ± 1.9	43.8 ± 0.6	28.2 ± 1.5	1.5 ± 0.2
AP	2.1 ± 0.5	1.0 ± 0.7	2.46 ± 0.2	8.9 ± 1.3	35.8 ± 3.3

AP: Apoptotic population in the sub-G_1 phase. Values are the mean ± SD calculated from three independent experiments.

lysates using the appropriate polyclonal anti-CDK1 and -CDK4 antibodies, respectively. As shown in Figure 2, MCS-5A inhibits CDK1 and 4 in a dose-dependent manner, with IC_{50} values of 9.6 and 8.8 μM, respectively.

Furthermore, we examined whether MCS-5A can inhibit other housekeeping protein kinases,[24] including PKA, PKC, PI3K, and JNKs (Table 1). We found that 10 μM MCS-5A inhibits the activity of CDK1 and 4 to a much greater degree (70% and 85%, respectively) than that of these other protein kinases (<20%). Thus, MCS-5A can inhibit CDK1 and 4 activity in vitro with significant selectivity over other types of protein kinases.

To examine the pattern of inhibition of the cell cycle progression by MCS-5A, we determined the cell population ratio in 0.5 μM MCS-5A-treated HL-60 cells using flow cytometry (Table 2). We found that the G_1 and G_2/M phase arrest was induced 8 and 4 h, respectively, after treatment with 0.5 μM MCS-5A. The percentage of cells in the G_1 phase increased from 23.8 ± 2.1% (1 h) to 42.9 ± 1.3% after 8 h, and that of cells in the G_2/M phase also increased slightly from 30.6 ± 2.1% (0 h) to 43.8 ± 0.6% after 4 h. Furthermore, the sub-G_1 phase population, that is, the apoptotic population (AP), increased significantly from 2.1% (0 h) to 35.8% 24 h after drug treatment.

CDKs are activated at various checkpoints after specific intervals during the cell cycle progression, but can also be induced and regulated by exogenous factors. CDKs are thus subjected to negative regulation by the binding of a class of proteins known

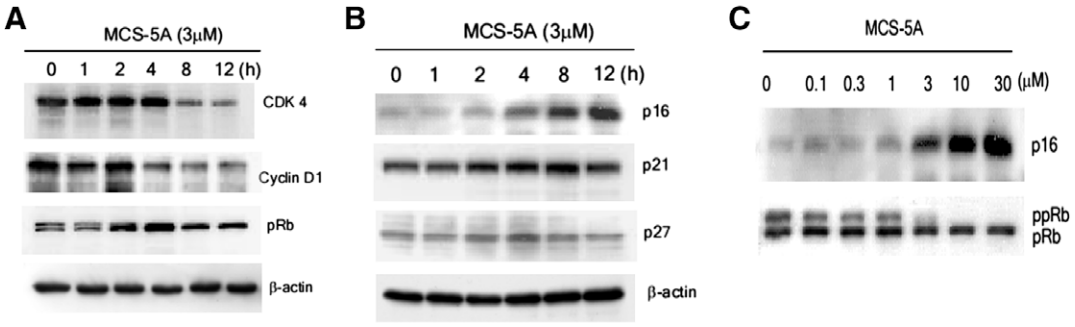

Figure 3. Effect of MCS-5A on expression of cell cycle-associated proteins in HL-60 cells. (A) Effect of 3 μM MCS-5A on expression of CDKs and cyclins and (B) the CKIs $p16^{INK4a}$, $p21^{CIP1}$, and $p27^{KIP1}$. (C) Effect of MCS-5A at various concentrations on the G_1 phase-associated proteins $p16^{INK4a}$ and pRb 12 h after drug treatment. β-actin was used as a loading standard.

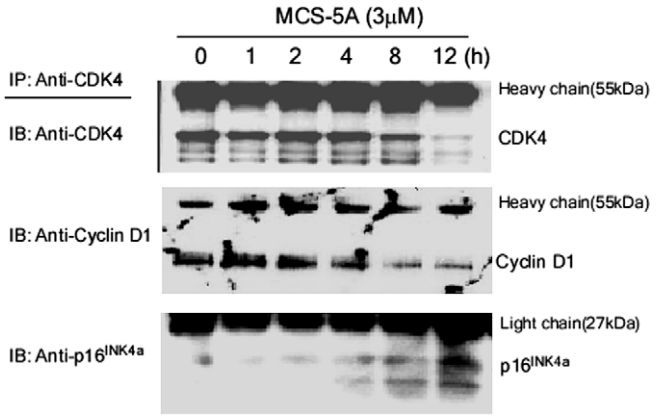

Figure 4. Effect of MCS-5A on protein–protein interaction between CDK4, cyclin D1 and $p16^{INK4a}$ in HL-60 cells. IP: Immunoprecipitation. IB: Immunoblotting.

as cyclin-dependent kinase inhibitors (CKIs). Research data suggest that cancer cells harbor defects that exert their effects at various checkpoints regulated by CDK/cyclin/CKI machinery.[25]

To determine the pattern of regulation of expression of the CKI genes *INK4a*, *CIP1*, and *KIP1* and of retinoblastoma protein (pRb) phosphorylation in MCS-5A-treated HL-60 cells, we performed a Western blot analysis,[24] as shown in Figure 3.

We found that induction of cell cycle arrest in 3 μM MCS-5A-treated HL-60 cells was associated with time-dependent down-regulation of CDK4 and cyclin D1 (Fig. 3A) as well as up-regulation of $p16^{INK4a}$ (Fig. 3B), which play key roles in regulating the entry of cells at the G_1/S transition checkpoint, and was followed by hypo-phosphorylation of pRb in a dose-dependent manner (Fig. 3C). The other notable CKIs p21 and p27 were not affected by treatment with MCS-5A (Fig. 3B).

To determine whether the up-regulation of $p16^{INK4a}$ can explain the down-regulation of CDK4 and cyclin D1 due to its competing

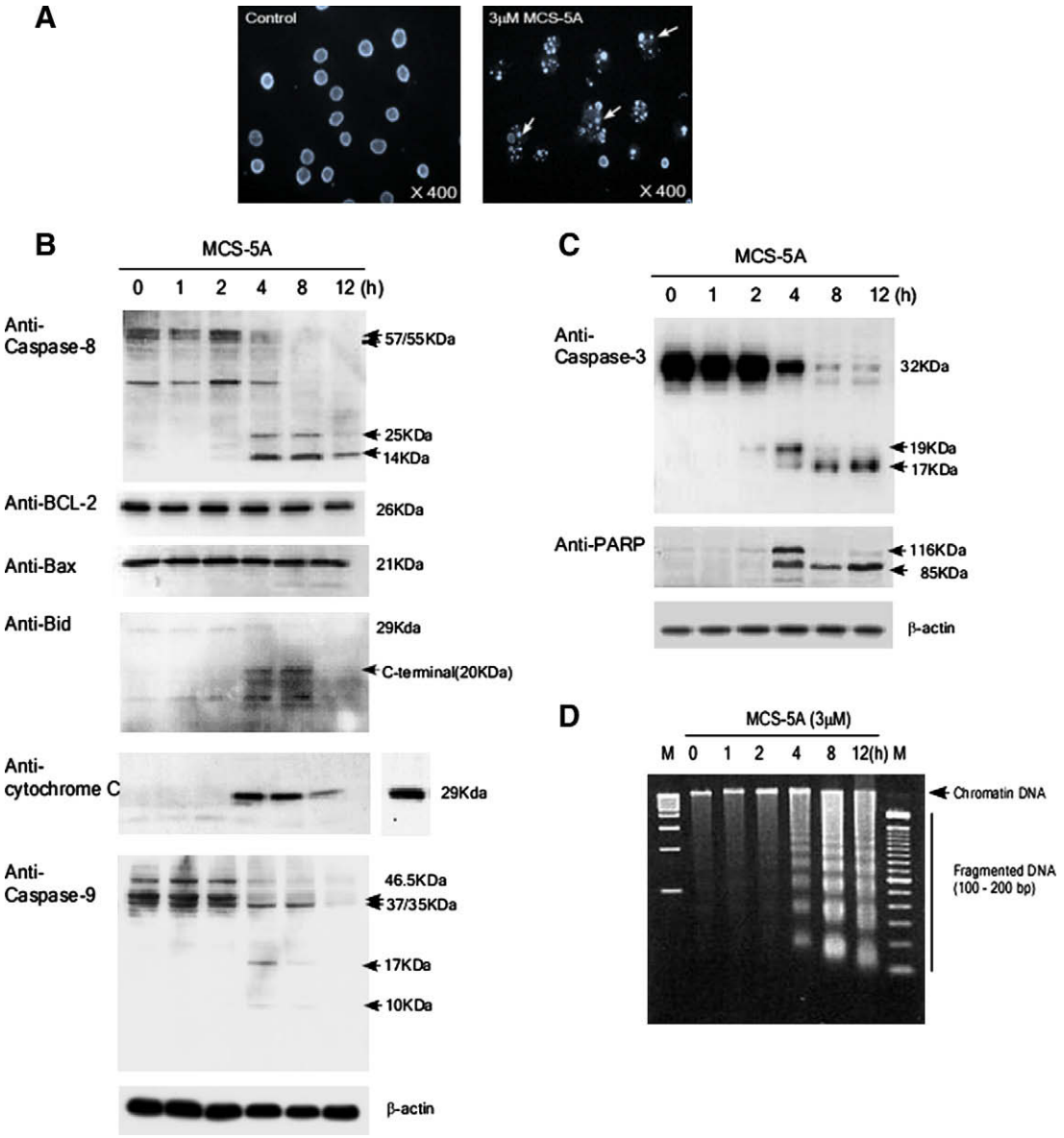

Figure 5. Effect of MCS-5A on expression of apoptosis-associated proteins in HL-60 cells. (A) Fluorescence microscopic examination of DAPI-stained untreated cells (left) or cells treated with 3 μM MCS-5A for 8 h (right). (B) Effect of MCS-5A on cleavage of procaspase-8, Bid (formation of *t*Bid), and procaspase-9. The release of mitochondrial cytochrome *c* to cytosol was demonstrated. (C) MCS-5A induced cleavage of procaspase-3 and PARP. (D) DNA fragmentation analysis that revealed a typical DNA laddering induced by treatment with MCS-5A.

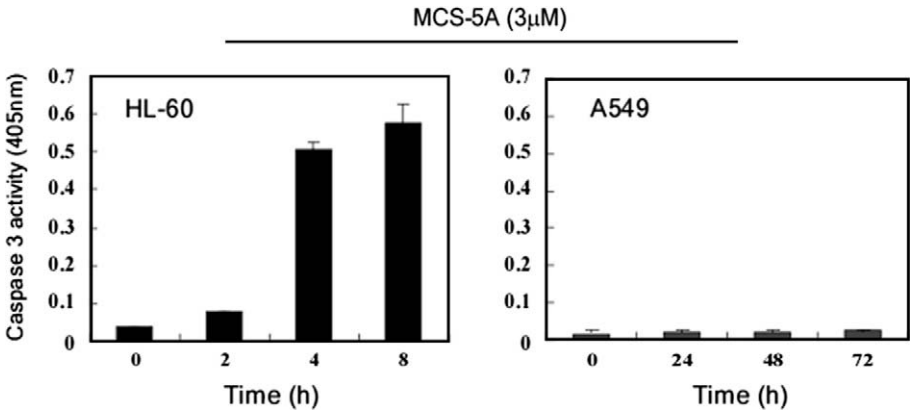

Figure 6. Effect of MCS-5A on activation of procaspase-3 in HL-60 and A549 cells. The fluorometric immunosorbent enzyme assay (FIENA) is highly specific for detection of caspase 3 activity by the use of an anti-caspase 3-specific monoclonal capture antibody in combination with a specific caspase substrate. The samples were analyzed using a fluorometer (Molecular Devices, CA, USA) at 405 nm for excitation and 510 nm for emission. Each data point represents the mean ± SD from three independent experiments (**p <0.01).

with cyclin D1 for CDK4 via a protein–protein interaction (PPI), we performed Western blotting. Five hundred-microgram lysates of HL-60 cells treated with 3 μM MCS-5A were immunoprecipitated with polyclonal rabbit anti-CDK4 antibody. As shown in Figure 4, we found that PPI between $p16^{INK4a}$ and CDK4 gradually increased in a time-dependent manner. However, in contrast, PPI between CDK4 and cyclin D1 decreased significantly 4–8 h after treatment with MCS-5A and was followed by cell cycle arrest at the G_1 phase in HL-60 cells.

Thus, we concluded that MCS-5A effectively induces cell cycle arrest at the G_1 and G_2/M phases through direct inhibition of CDK1 and 4 activity in HL-60 cells. In addition, the elevated level of $p16^{INK4a}$ and reduced level of hyperphosphorylated pRb showed that 3 μM MCS-5A induces $p16^{INK4a}$-mediated cell cycle arrest at the G_1 phase through a PPI in which $p16^{INK4a}$ competes with cyclin D1 for CDK4.

To assess whether MCS-5A induces apoptosis in HL-60 cells, we performed DAPI staining to examine the effect of the drug on nuclear morphology. As shown in Figure 5A, 3 μM MCS-5A induced formation of fragmented apoptotic bodies, which are typically observed in apoptosis. Morphological observation of apoptosis was confirmed by caspase activation, release of cytochrome *c* from mitochondria, and cleavage of PARP using Western blot analysis. As shown in Figure 5B, 3 μM MCS-5A induced activation of procaspase-8 and -9, 4 h after drug treatment.

The cleavage of Bid, a pro-apoptotic Bcl-2 protein, to truncated Bid (*t*Bid, 20 kDa) by caspase-8, and subsequent release of cytochrome *c* from mitochondria induced activation of procaspase-9, which, in turn, resulted in activation of procaspase-3 and subsequent cleavage of PARP in HL-60 cells after treatment with 3 μM MCS-5A for 4 h (Fig. 5C). The morphologic observation of apoptosis was confirmed by DNA fragmentation analysis that revealed a typical DNA laddering induced by treatment with MCS-5A (Fig. 5D).

To investigate whether apoptotic induction of MCS-5A is due to the up-regulation of $p16^{INK4a}$, we conducted a fluorometric immunosorbent enzyme assay (FIENA) in 3 μM MCS-5A-treated HL-60 ($p53^{-/-}$, $p16^{+/+}$) and non-small cell lung cancer A549 ($p53^{+/+}$, $p16^{-/-}$) cells, a dominant-negative $p16^{INK4a}$ mutant cell line. Interestingly, as shown in Figure 6A, FIENA revealed that MCS-5A-mediated apoptosis in HL-60 cells was apparently associated with the up-regulation of $p16^{INK4a}$, through activation of caspase-3, 4 h after drug treatment. In contrast, activation of caspase-3 and apoptotic induction were not detected in A549 cells treated with 3 μM MCS-5A for 72 h (Fig. 6B). Moreover, we previously reported that over-expression of $p16^{INK4a}$ using transfection of exogenous $p16^{INK4a}$ into A549 cells apparently induces caspase-3 activation and apoptotic induction.[26] Thus, it has been suggested that up-regulation of $p16^{INK4a}$ plays a pivotal role in MCS-5A-mediated and p53-independent apoptotic induction in HL-60 cells.

In conclusion, in the present study, we showed that MCS-5A can inhibit CDK1 and 4 activity with significant selectivity over other types of housekeeping protein kinases in vitro. In addition, we have demonstrated that treatment of HL-60 cells with MCS-5A is associated with an accumulation of cells in the G_1 and G_2/M phases of the cell cycle and with the induction of apoptosis via a cytochrome *c*-mediated intrinsic pathway, both effects of which are induced by a key molecular event, the up-regulation of $p16^{INK4a}$. MCS-5A seems to have multiple mechanisms of antiproliferative activity against promyelocytic leukemia. More detailed studies are needed to establish both the cancer preventive and therapeutic efficacy of MCS-5A in this and other types of leukemia.

Acknowledgments

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24. Protein kinases used in Table 2: JNK1 (Cat. No.14-327), JNK2 (14-329), JNK3 (14-501), PKA (12-257), PKC (14-497), Syk (14-314), ZAP-70 (14-404), LCK (14-442), CSK (14-458), PI3K (14-602), CDK1 (14-450) were purchased from Millipore (Upstate), MA, USA and CDK4 from Sigma–Aldrich Corp., MO, USA. In contrast, MLTK-α was obtained by immunoprecipitation.
Antibodies used in Western blots: primary antibodies were used to detect β-actin, CDK4, CDK2, CDK6, CDK1, cyclin D1, cyclin A, cyclin B1, cyclin E, caspase-3, caspase-9, Rb, E2F1, E2F2, PARP, p15, and p16 (Santa Cruz Biotechnology, Inc., CA, USA). Appropriate mouse and rabbit antibodies were used for secondary detection (Santa Cruz Biotechnology). Other primary antibodies included Bid, Bax, Bad, Bcl-2, Cytochrome C, Fas, FasL (Neomarker, CA, USA), $p21^{waf1}$, and caspase-8 (Zymed, CA, USA).
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