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# Tetrandrine Induces Early $G_1$ Arrest in Human Colon Carcinoma Cells by Down-Regulating the Activity and Inducing the Degradation of $G_1$-S–Specific Cyclin-Dependent Kinases and by Inducing p53 and $p21^{Cip1}$

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## ABSTRACT

**Tetrandrine is an antitumor alkaloid isolated from the root of *Stephania tetrandra*. We find that micromolar concentrations of tetrandrine irreversibly inhibit the proliferation of human colon carcinoma cells in MTT and clonogenic assays by arresting cells in $G_1$. Tetrandrine induces $G_1$ arrest before the restriction point in nocodazole- and serum-starved synchronized HT29 cells, without affecting the $G_1$-S transition in aphidicolin-synchronized cells. Tetrandrine-induced $G_1$ arrest is followed by apoptosis as shown by fluorescence-activated cell sorting, terminal deoxynucleotidyl transferase–mediated nick end labeling, and annexin V staining assays. Tetrandrine-induced early $G_1$ arrest is mediated by at least three different mechanisms. First, tetrandrine inhibits purified cyclin-dependent kinase 2 (CDK2)/cyclin E and CDK4 without affecting significantly CDK2/cyclin A, CDK1/cyclin B, and CDK6. Second, tetrandrine induces the proteasome-dependent degradation of CDK4, CDK6, cyclin D1, and E2F1. Third, tetrandrine increases the expression of p53 and $p21^{Cip1}$ in wild-type p53 HCT116 cells. Collectively, these results show that tetrandrine arrests cells in $G_1$ by convergent mechanisms, including down-regulation of E2F1 and up-regulation of p53/$p21^{Cip1}$.**

## INTRODUCTION

$G_1$ is the cell cycle phase in which cells respond to extracellular cues that ultimately determine whether cells make the decision to replicate DNA and divide or alternatively to exit the cell cycle into a quiescent state ($G_0$). The time late in $G_1$ phase when the decision is made has been designated the "restriction point" by Pardee (1). At the molecular level, the restriction point is mediated by the retinoblastoma protein (Rb)-E2F pathway under the control of two families of regulatory enzymes, the cyclin D– and cyclin E–dependent kinases (CDKs; refs. 2–5).

The D-type cyclins act as growth factor effectors. Their expression depends more on extracellular signals than on the cell's position in the cell cycle (6). The D cyclins interact combinatorially with two distinct catalytic CDK partners, CDK4 and CDK6 (7, 8). The cyclin D1/CDK4 and cyclin D1/CDK6 complexes phosphorylate Rb (p-Rb) on serine and threonine residues, thereby canceling Rb's growth-repressive functions (9), which are caused by Rb's negative control of a family of heterodimer transcriptional regulators, collectively termed the E2F (10). Unphosphorylated Rb prevents cell proliferation by binding to and inactivating E2Fs (11). Phosphorylation of Rb by CDK/cyclin complexes results in the release of active E2F species that stimulate the transcription of genes whose products are necessary for the $G_1$-S transition and S-phase progression. Although this process is initially triggered by the cyclin D–dependent kinases, it is amplified by Rb phosphorylation by the cyclin E-CDK2 complex (12). In proliferating cells, the expression of cyclin E is normally periodic and maximal at the $G_1$-S transition. Throughout this interval, cyclin E is bound to and activates its catalytic partner CDK2. Because the *cyclin E* gene is itself E2F responsive, a positive feedback cyclin E-CDK2 loop acts to facilitate progressive rounds of Rb phosphorylation and E2F release (4, 5, 13). An additional positive feedback is exerted at the level of E2F1, which stimulates its own transcription. Thus, positive feedback on E2F1 and cyclin E produces a rapid increase of both activities as cells approach the $G_1$-S boundary (5). In concert with the irreversible commitment to enter S phase, inactivation of Rb shifts from being mitogen dependent (cyclin D driven) to mitogen independent (cyclin E driven). Decreased requirements for mitogens and decreased sensitivity to antiproliferative signals are among the hallmarks of human cancer. These attributes are, at least partly, caused by overexpression of cyclin D1 and CDK4, and mutations that directly or indirectly compromise the function of p-Rb (2, 14). Proteins that participate in the cell cycle control have become targets for cancer therapy (3, 15–17).

Tetrandrine, a bis-benzylisoquinoline alkaloid with an interesting structure (Fig. 1), was isolated from the root of *Stephania tetrandra* and is used in traditional Chinese medicine as an antirheumatic, anti-inflammation, and antihypertensive agent (18). Several studies recently have shown that tetrandrine possesses antitumor activity in cultured tumor cells and animal models (19–27). Tetrandrine induces $G_1$-phase arrest in cells containing wild-type p53 (20, 25) and apoptosis in p53 wild-type and null cells (20, 22, 25). However, the molecular mechanisms by which tetrandrine induces $G_1$-phase arrest remain poorly understood. In this study, we investigated the molecular and cellular pharmacology of tetrandrine in the human colon carcinoma p53-mutant HT29 cells and in the p53 wild-type HCT116 cells (28). We find that tetrandrine induces early $G_1$-phase arrest by down-regulating several key $G_1$ regulatory proteins, such as CDK4, CDK6, cyclin D1, p-Rb, and E2F1. We also find that tetrandrine induces p53 and $p21^{Cip1}$ in p53/wild-type HCT116 cells and ultimately induces apoptotic cell death.

## MATERIALS AND METHODS

**Cell Culture.** Human colon carcinoma HT29 cells were obtained from American Type Culture Collection (Manassas, VA). Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) provided HCT116 cells. Cells were grown at 37°C in the presence of 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD), 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin.

**Drugs, Chemicals, and Tetrandrine Structure Minimization.** Tetrandrine, aphidicolin, nocodazole, and MG132 were purchased from Sigma Chemical Co. (St. Louis, MO). Tetrandrine was dissolved in 0.1 mol/L HCl and neutralized with $Na_2CO_3$ to pH 6 to achieve a concentration of 20 mmol/L. Aphidicolin and nocodazole were dissolved in dimethylsulfoxide at a concentration of 10 mmol/L and 2 mg/mL, respectively. All of the drugs were diluted to desired concentrations in medium immediately before each experiment.

Tetrandrine structure minimization (Fig. 1*B*) was performed using Chem3D Pro version 5.0 for Macintosh (CambridgeSoft Corporation, Cambridge, MA). The minimization parameters used were minimum root mean square gradient, 0.100; step interval, 2.0 fentoseconds; and frame interval, 10 fs.

**MTT Assays.** HT29 cells were seeded at a density of 3000 cells/well in 96-well microplates. The next day, cells were treated with tetrandrine for 18,

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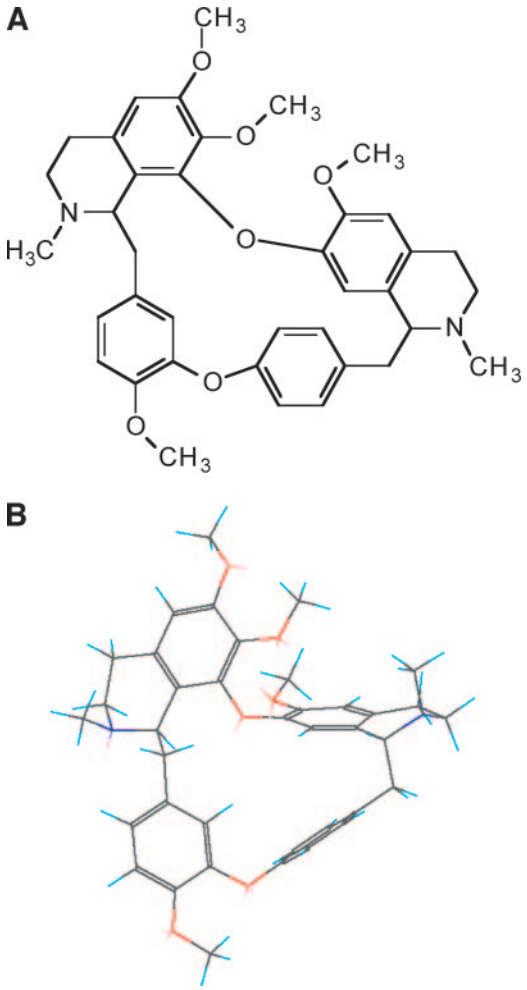


Fig. 1. Structure of tetrandrine. *B,* the minimized drug structure.

24, 48, or 72 hours. At the end of culture, 20 μL of MTT (5 mg/mL) were added to each well, and plates were placed at 37°C for 4 hours. One hundred microliters dimethylsulfoxide were added to each well to lyse the cells. Absorbance was measured at 570 nm using a multiwell spectrophotometer (Emax; Molecular Devices, Sunnyvale, CA).

**Clonogenic Assays.** Cells were treated with various concentrations of tetrandrine for 18 hours or with 10 μmol/L of tetrandrine for 1, 4, 8, and 18 hours. Following treatment, cells were washed in PBS and trypsinized. Cells were seeded in six-well plate in 5 mL medium at two densities (2000 and 200 cells/well). Colonies were grown for 2 weeks. Plates were washed in PBS, and colonies were fixed with methanol. Staining was with methylene blue (0.04%).

**Flow Cytometry Analysis of DNA Content.** Cells were seeded and treated in six-well plates. They were harvested after treatment and were fixed in 70% EtOH at 4°C for at least 30 minutes. Before analysis, cells were washed once in PBS and stained with PBS containing 50 μg/mL propidium iodide (PI) and 50 μg/mL RNase for 30 minutes at 37°C. Analyses were performed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Cell cycle distributions were calculated on DNAplots by ModFit *LT* software (Verity Software House, Inc., Topsham, ME).

**Determination of Apoptosis.** Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay and translocation of phosphatidyl serine to the cell surface with the annexin V–Fluos kit (both from Roche, Indianapolis, IN). Fraction of annexin V–positive cells was measured with CellQuest software (BD Biosciences, San Jose, CA).

**Western Blot Analyses.** Treated and untreated cells were washed three times with ice-cold PBS before being lysed in 300 μL freshly prepared extraction buffer [1% SDS, 1 mmol/L $Na_3VO_4$, 0.1 mol/L Tris (pH 7.4), protease inhibitor mixture (Roche, Indianapolis, IN), and phosphatase inhibitor mixture (Upstate Inc, Lake Placid, NY)] at 4°C. Protein detection was performed using a protein assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Proteins were resolved at 40 μg/lane on 8 or 12% SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 2 hours at 15 V. Membranes were blocked for 1 hour in PBS-Tween (0.1%) containing 5% nonfat dried milk and probed for 1 hour with primary antibodies, including Rb, E2F1, cyclin D1, CDK1, CDK2, CDK4, CDK6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p-Rb, and cyclin E (Upstate Inc). Membranes then were incubated with antimouse IgG or antirabbit IgG (Amersham Biosciences, Piscataway, NJ) and were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's instructions. Shown are representative data from individual experiments that were repeated at least twice.

**CDK1/Cyclin B, CDK2/Cyclin A, CDK2/Cyclin E Kinase, and CDK6/Cyclin D3 Assays.** Recombinant active CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, and CDK6/cyclin D3 were purchased from Upstate Inc. Phosphorylation of histone H1 was measured by incubating the CDK complexes with 20 μL of "hot" kinase buffer [20 mmol/L Tris/HCl (pH 7.5), 10 mmol/L $MgCl_2$, 10 μmol/L ATP, 1 μCi [$\gamma$-$^{32}$P]ATP, and 3 μg histone H1] containing different concentrations of tetrandrine as indicated for 30 minutes at 30°C. Reactions were stopped by boiling the samples in SDS sample buffer [63 mmol/L Tris/HCl (pH 6.8), 10% glycerol, 2% SDS, 0.1 mol/L DTT, and 0.02% bromphenol blue] for 5 minutes. The samples were separated on 12% SDS-PAGE. After drying the gels, histone H1 phosphorylation was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Shown are representative data from an individual experiment that was repeated at least twice.

**Immunoprecipitation and CDK4 Kinase Assay.** CDK4-associated kinase activities were determined as described previously (7) with minor modifications. Briefly, HT29 cells were lysed in 500 μL lysis buffer (PBS containing 1% NP40, 1 mmol/L $Na_3VO_4$, 5 mmol/L NaF, and protease inhibitor mixture) on ice for 30 minutes and sonicated at 4°C for 5 seconds. Lysates were clarified by centrifugation at 10,000 × *g* for 15 minutes. One milligram of the cell protein per sample was used for immunoprecipitation. Immunocomplexes were resuspended in kinase buffer [50 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, and 1 mmol/L DTT] containing 10 μmol/L unlabeled ATP and 10 μCi [$\gamma$-$^{32}$P]ATP with 1 μg Rb peptide in the presence of different concentrations of tetrandrine as indicated and incubated at 30°C for 30 minutes. Reactions were stopped, and samples were separated and visualized as described previously. Shown are representative data from individual experiments that were repeated at least twice.

**Immunoprecipitation Assays.** Treated or untreated HT29 cells were incubated with 500 μL ice-cold lysis buffer (PBS containing 1% NP40, 1 mmol/L $Na_3VO_4$, 5 mmol/L NaF, and protease inhibitor mixture) on ice for 30 minutes and sonicated at 4°C for 5 seconds. Lysates were clarified by centrifugation at 10,000 × *g* for 15 minutes, and 300 μg of total protein were used for immunoprecipitation. Lysates were precleared with 20 μL 25% protein A/G agarose slurry for 1 hour at 4°C with rotation. Lysates were spun at 5,000 rpm for 5 minutes, and the supernatants were transferred to another tube and incubated at 4°C with 1 μg monoclonal anti-CDK6 or anti-Rb and 40 μL 25% protein A/G agarose slurry for 2 hours. The protein A/G agarose was recovered by centrifugation at 5,000 rpm and washed four times with ice-cold lysis buffer. Proteins were eluted with 20 μL of SDS loading buffer by boiling for 5 minutes and subjected to immunoblot analysis with antiubiquitin antibody.

## RESULTS

**Irreversible Growth Inhibition Induced by Tetrandrine.** The antiproliferative effect of various concentrations of tetrandrine on HT29 cells is shown in Fig. 2*A*. Tetrandrine inhibited cell growth in a concentration- and time-dependent manner in MTT assays with an $IC_{50}$ ~4 and 14 μmol/L for 72- and 18-hour exposures, respectively. Comparable activity was observed in clonogenic assays (Fig. 2*B* and *C*; $IC_{50}$ = 8 μmol/L for 18-hour exposure) and in HCT116 (data not shown).

**Irreversible $G_1$ Block Induced by Tetrandrine.** To determine the phase of the cell cycle at which tetrandrine exerts its growth-inhibitory effect, exponentially growing HT29 cells were treated with different concentrations of tetrandrine for 18 hours and analyzed by

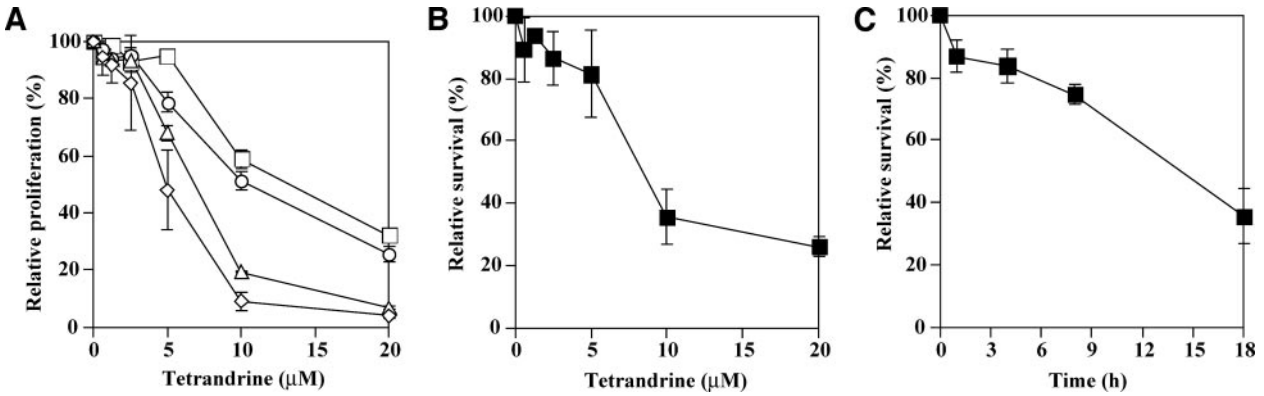


Fig. 2. Effect of tetrandrine on HT29 cell proliferation. *A,* MTT assay: Cell viability was determined by MTT assays after continuous exposure to tetrandrine for 18, 24, 48, or 72 hours. —□— , 18 h; —○— , 24 h; —△— , 48 h; —◇—, 72 h. *B.* Cell survival was determined by clonogenic assays after 18-hour treatments with the indicated tetrandrine concentrations. *C.* Cell survival was determined by clonogenic assays after 1, 4, 8, and 18 hours of exposure to 10 μmol/L tetrandrine. Data are means (*bars,* SD) of three independent experiments.

flow cytometry (Fig. 3*A*). $G_1$ arrest was apparent after treatment with 10 μmol/L tetrandrine (51%) and increased to 64% in the presence of 30 μmol/L tetrandrine (*A* in Fig. 3*D*).

To differentiate between tetrandrine-induced $G_1$ arrest and early S-phase arrest, HT29 cells were first synchronized in early S phase with aphidicolin for 24 hours. After chasing in drug-free medium for 1 hour, cells were further incubated in the presence of tetrandrine alone or tetrandrine plus nocodazole for 18 hours (*B* in Fig. 3*D*). As shown in Fig. 3*B*, all of the cells (in the presence of tetrandrine or not) went through S phase and stopped at the $G_2$-M peak in the presence of nocodazole. We conclude that tetrandrine has no effect on the S-phase progression once the cells have passed the $G_1$-S transition (where aphidicolin acts). An additional effect visible in Fig. 3*B* in the aphidicolin-synchronized cells is that the cells went through the first cycle and accumulated in the second $G_1$ phase in the presence of tetrandrine alone, which shows that tetrandrine also induced $G_1$ arrest in the second cell cycle after tetrandrine addition.

To further explore tetrandrine-induced $G_1$ arrest in the first cell cycle, HT29 cells were first synchronized in the $G_0$ phase by transient culture in low serum (0.04% FBS starvation for 24 hours), followed by incubation and release in complete medium (containing 10% FBS) in the presence of tetrandrine alone or tetrandrine plus nocodazole. As shown in Fig. 3*C*, normal cell cycle progression was observed after 15 hours in the absence of tetrandrine treatment (see cells treated with nocodazole alone). Tetrandrine caused an apparent early $G_1$-phase arrest as its concentration was increased (*C* in Fig. 3*D*). This early $G_1$ arrest was more clearly shown in the tetrandrine- and nocodazole-cotreated cells. Cells went through $G_1$-S phase and stopped in $G_2$-M phase in the presence of nocodazole alone, whereas most cells remained in early $G_1$ phase in the presence of nocodazole plus tetrandrine. Collectively, these results show that tetrandrine induces early $G_1$-phase arrest without arresting the S-phase progression.

To determine whether tetrandrine-induced $G_1$-phase arrest is reversible, HT29 cells were treated for 18 hours (T18) to induce $G_1$ arrest (Fig. 4). Tetrandrine then was washed out, and cells were further incubated in fresh medium with or without nocodazole (0.2 μg/mL) for an additional 24 hours (R24) or 48 hours (R48). Tetrandrine-treated cells remained in $G_1$, showing that tetrandrine-induced $G_1$-phase arrest is irreversible. Furthermore, a sub-$G_1$ peak emerged 24 and 48 hours after tetrandrine removal, suggesting that tetrandrine induces apoptosis following $G_1$-phase arrest. The irreversibility of $G_1$ arrest induced by tetrandrine is consistent with the clonogenic assays (see Fig. 2*B*).

**Induction of Apoptosis by Tetrandrine Follows $G_1$ Arrest.** To show that tetrandrine induces apoptosis, TUNEL assays were performed, and cell morphology was examined under fluorescent microscope after 24 hours of treatment of HT29 cells with tetrandrine. As

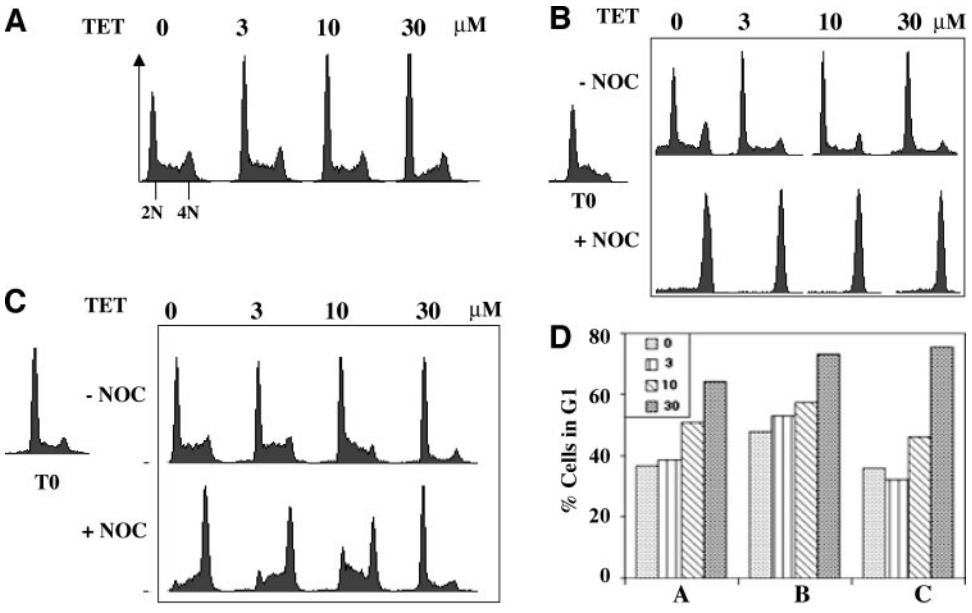


Fig. 3. Tetrandrine induces early $G_1$-phase arrest. *A.* Exponentially growing HT29 cells were treated with the indicated concentrations of tetrandrine for 18 hours. *B.* HT29 cells were first synchronized in early S phase with 1 μmol/L aphidicolin for 24 hours (T0). After aphidicolin removal (by rinsing the cultures in drug-free medium) and further incubation in fresh medium for 1 hour, cultures were further incubated in the presence of the indicated tetrandrine concentrations alone or tetrandrine plus nocodazole (0.2 μg/mL) for 18 hours. *C.* HT29 cells were first synchronized in $G_0$ in medium containing 0.04% FBS for 24 hours. Cells then were incubated in normal medium containing 10% FBS in the presence of tetrandrine alone or tetrandrine plus nocodazole for 15 hours. *D,* quantification of the $G_1$-phase population corresponding to protocols *A, B,* and *C.* The DNA content distribution histograms of fluorescence-activated cell-sorting analysis were measured by flow cytometry. The different phases of the cell cycle were determined with ModFit DNA analysis software. Data shown are representative of at least three independent experiments.

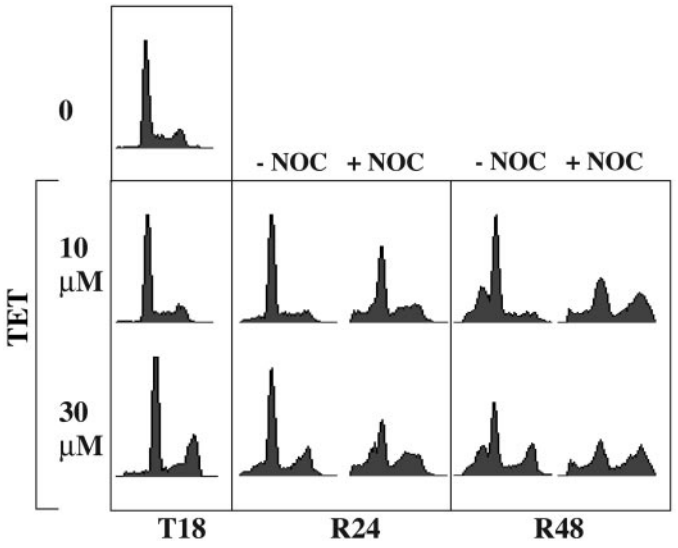


Fig. 4. Tetrandrine-induced $G_1$-phase arrest in HT29 cells is irreversible. Exponentially growing HT29 cells were treated with tetrandrine for 18 hours (T18). Tetrandrine was washed out, and the cells were further incubated in fresh medium with or without 0.2 μg/mL of nocodazole for 24 hours (R24) or 48 hours (R48). Cells were collected at each time point, and DNA content was analyzed by fluorescence-activated cell sorting.

shown in Fig. 5*A*, 10 μmol/L tetrandrine for 24 hours induced the appearance of a few TUNEL-positive cells, and treatment with 30 μmol/L tetrandrine resulted in a significant increase in TUNEL-positive cells. Induction of apoptosis was further confirmed and quantified by annexin V and PI double staining. Annexin V binding reveals the phosphatidylserine molecules have been flipped out from the inner to the outer cell surface during apoptosis. As shown in Fig. 5*B* and *C*, each plot can be divided into four regions. The bottom left quadrant contains viable cells, which exclude PI and are negative for annexin V binding. The bottom right quadrant contains early apoptotic cells, which are positive for annexin V binding and exclude PI. The top right quadrant contains late apoptotic cells or necrotic cells, which are positive for PI and annexin V. Annexin V–positive cells increased in dose- and time-dependent manners (Fig. 5*B* and *C*). Apoptosis was clearly detectable with 30 μmol/L tetrandrine for 24 hours (Fig. 5*B*). At this concentration, apoptosis occurred between 8 and 18 hours following the initiation of the tetrandrine treatment (Fig. 5*C*).

**Selective Inhibition of $G_1$-Specific CDK Activities by Tetrandrine.** To elucidate the mechanisms of tetrandrine-induced $G_1$ arrest, we tested the effect of tetrandrine on cyclin/CDKs in cell-free systems. Kinase activities of CDK1/cyclin B1, CDK2/cyclin A, CDK2/cyclin E, and CDK6/cyclin D3 were assayed using purified active complexes. CDK4 activity was assayed using immunoprecipitated complexes prepared from whole cell lysates from exponentially growing HT29 cells. As shown in Fig. 6, tetrandrine inhibited CDK2/cyclin E and CDK4 in a concentration-dependent manner with $IC_{50}$s of ~3 μmol/L and ~15 μmol/L, respectively. By contrast, tetrandrine had no effect on CDK6 and little inhibition on CDK1/cyclin B1 and CDK2/cyclin A in the pharmacologic concentration range. These results indicate that tetrandrine can selectively inhibit some $G_1$-S transition-related CDKs, which should contribute to $G_1$ arrest.

To further investigate the molecular mechanism of CDK4 inhibition by tetrandrine, CDK4 kinase assays were performed with different concentrations of ATP. As shown in Fig. 6*C* and *D*, increasing the ATP concentration had no significant effect on the inhibitory activity of tetrandrine, which indicates that tetrandrine probably does not compete with ATP and does not interfere with the ATP binding pocket of CDK4.

**Selective Degradation of $G_1$-Specific Cell Cycle Proteins by Tetrandrine.** To correlate the effect of tetrandrine on cell cycle progression with some molecular effectors of the restriction point,

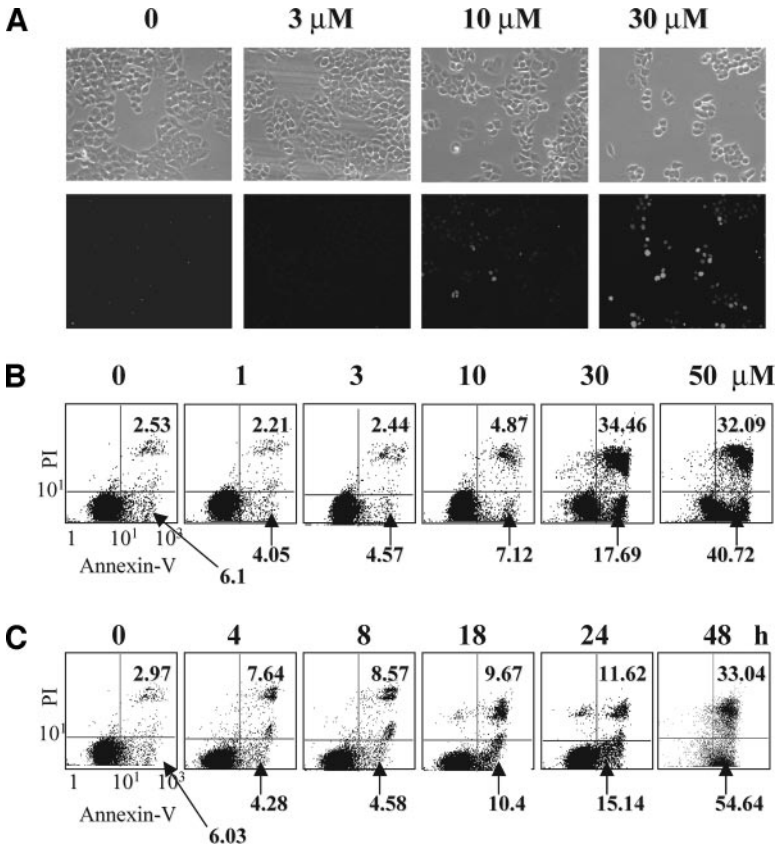


Fig. 5. Tetrandrine-induced apoptosis in HT29 cells. *A,* detection of apoptosis by TUNEL assay. Cells were treated with the indicated concentrations of tetrandrine for 24 hours (0, control untreated cells). *Top,* phase contrast microscopy; *bottom,* fluorescence detection. *B* and *C,* detection of apoptosis by annexin V and PI staining. *B,* Cells were treated with the indicated concentrations of tetrandrine for 24 hours. *C,* time course in cells treated with 30 μmol/L tetrandrine for 4, 8, 18, 24, and 48 hours, respectively. The percentage of annexin V–positive cells in the top (PI negative) and bottom (PI positive) right quadrants is indicated.

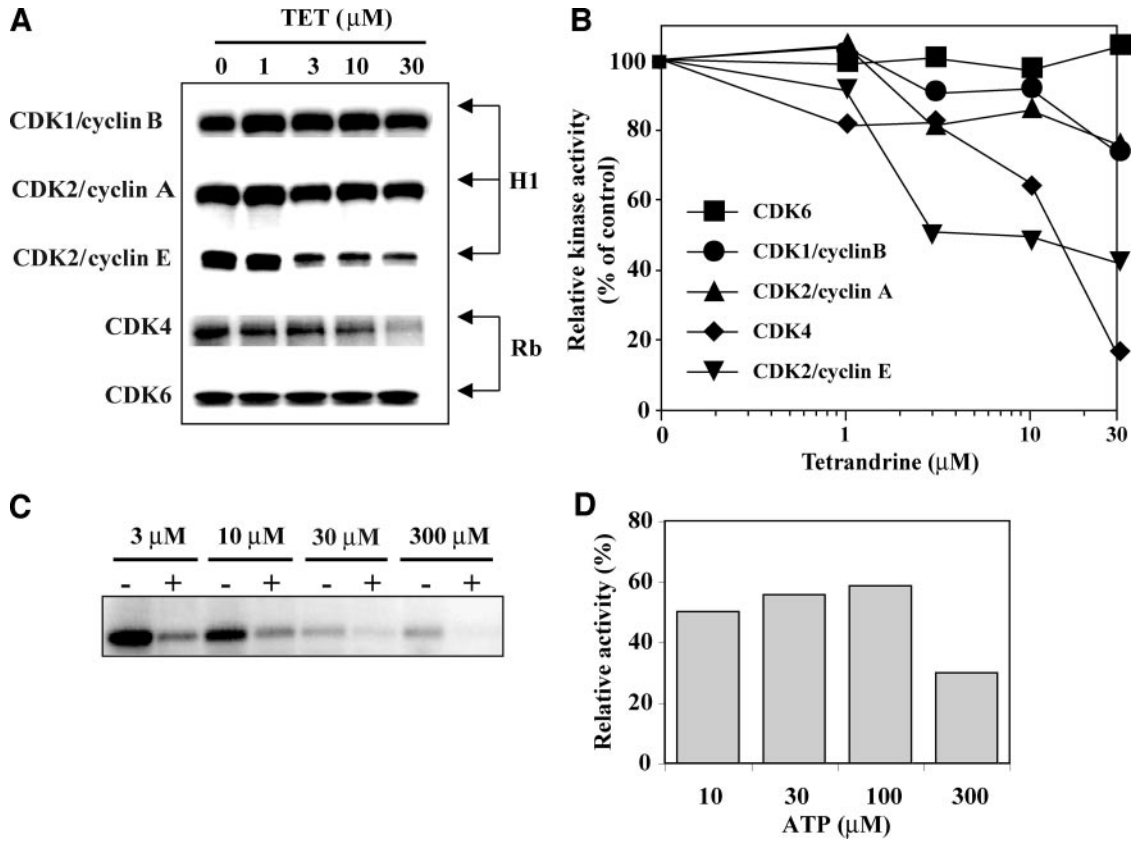


Fig. 6. Effect of tetrandrine on the activities of CDKs *in vitro*. *A.* Purified CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, or CDK6/cyclin D3 active complexes were incubated with [$\gamma$-$^{32}$P]ATP, histone H1, and the indicated concentrations of tetrandrine for 30 minutes at 30°C. CDK4 immune complexes were prepared from exponentially growing HT29 cells and reacted with [$\gamma$-$^{32}$P]ATP, a Rb polypeptide, and tetrandrine for 30 minutes at 30°C. *B,* quantification of the inhibitory activity of tetrandrine from the data shown in *A* using ImageQuant software (Molecular Dynamics). Data shown are representative of at least two independent experiments. *C,* effect of ATP on inhibition of CDK4 by tetrandrine. CDK4 immunoprecipitation kinase assays were performed in the presence of the indicated ATP concentrations. *D,* quantification of relative CDK4 activity in the presence of tetrandrine and increasing ATP concentration.

HT29 cells were first synchronized in $G_2$-M phase with nocodazole for 15 hours and then released from $G_2$-M phase by washing out nocodazole and further incubated in fresh medium in the presence or absence of tetrandrine. As shown in Fig. 7, tetrandrine-treated or -untreated cells went through mitosis after nocodazole release. Tetrandrine-treated cells were arrested in $G_1$ phase for up to 12 hours after nocodazole release, whereas untreated cells were already in S and $G_2$ phase 10 hours after nocodazole release. Protein levels were detected at the same times by Western blot analysis. One of the most important substrates of the $G_1$-specific CDKs is Rb, which sequesters the E2F transcription factor when it is underphosphorylated. p-Rb by cyclin D–dependent CDKs is critical for the $G_1$- to S-phase transition (see Introduction). We tested the effect of tetrandrine on p-Rb using an anti-phospho–p-RB antibody specifically recognizing the cyclin D–specific phosphoserine 780 residue of p-RB (29). Fig. 7 shows that tetrandrine induced a marked decrease in p-Rb levels. Correspondingly, the top band of total Rb, which represents p-Rb, also decreased, whereas the bottom Rb band remained relatively unchanged. CDK4, CDK6, and cyclin D1, which are critical for Rb phosphorylation, also decreased within 4 hours of tetrandrine exposure. E2F1 began to decrease after 8 hours of treatment. Contrarily, CDK1, which is not involved in the $G_1$-S transition, remained relatively unchanged. These results show that tetrandrine selectively reduces the expression of key proteins regulating the $G_1$-S transition.

**Tetrandrine Selectively Suppresses CDK4 and CDK6 by Proteasome-Mediated Degradation.** To explore whether the 26S proteasome is involved in the down-regulation of CDK4 and CDK6, the effects of tetrandrine were studied in the presence of the 26S proteasome

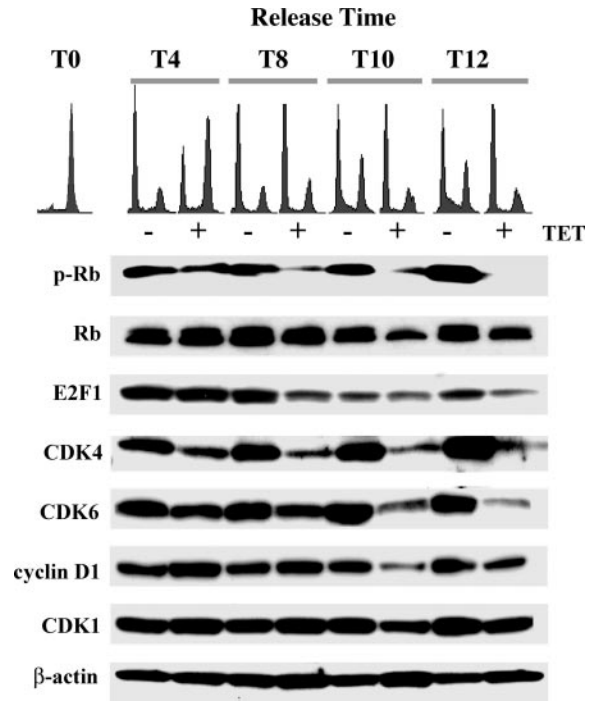


Fig. 7. Effect of tetrandrine on Rb phosphorylation and levels of cyclin D1 and CDKs in HT29 cells following synchronization in $G_2$-M phase with 0.2 $\mu$g/mL nocodazole for 15 hours (T0). Times after nocodazole release (by washing out nocodazole) and further incubation in fresh medium with or without tetrandrine are indicated at the top of the figure.

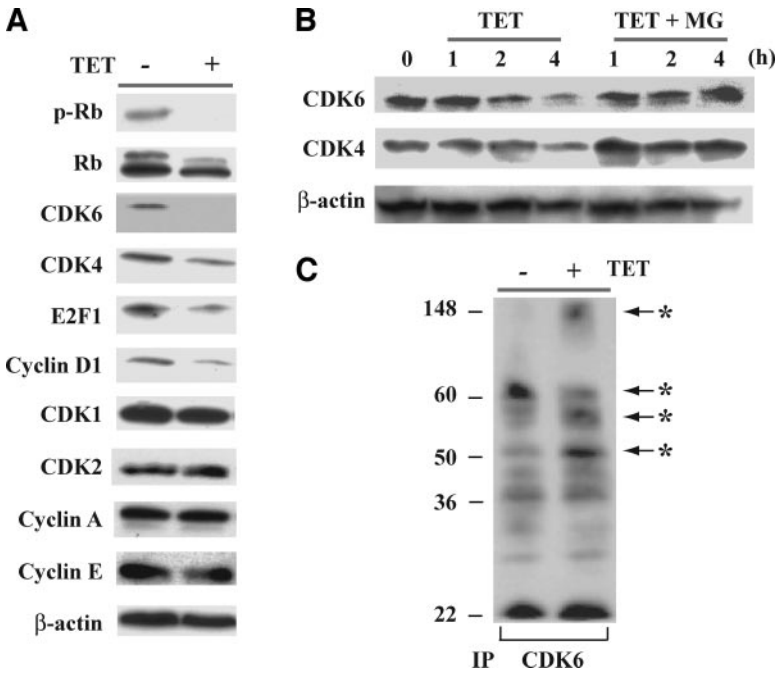


Fig. 8. Tetrandrine enhances the proteasome-mediated degradation of CDK4 and CDK6 in exponentially growing HT29 cells. *A,* Western blot analysis of the indicated cell cycle–related proteins in cells treated with 30 $\mu$mol/L tetrandrine (+) for 8 hours. *B,* Western blot analysis of CDK6, CDK4, and $\beta$-actin in cells were treated with 30 $\mu$mol/L tetrandrine for 1, 2, and 4 hours in the absence and presence of MG132 (5 $\mu$mol/L, 15 minutes pretreatment and cotreatment with tetrandrine). *C,* changes in CDK6 ubiquitination induced by tetrandrine. Cells were treated with 5 $\mu$mol/L MG132 alone or plus 30 $\mu$mol/L tetrandrine for 8 hours. Cell lysates were immunoprecipitated with anti-CDK6 and then probed with antiubiquitin.

inhibitor MG132 (30). Because MG132 itself is cytotoxic, exponentially growing HT29 cells were used in the following experiments. We first detected the protein levels of the indicated cell cycle–related proteins in cells treated with 30 $\mu$mol/L tetrandrine alone for 8 hours. As shown in Fig. 8*A*, tetrandrine treatment decreased hyperphosphorylated Rb (p-Rb), CDK6, CDK4, E2F1, and cyclin D1 but not CDK1, CDK2, cyclin A, and cyclin E, which is consistent with the results described in Fig. 7. More detailed kinetics of CDK6 and CDK4 down-regulation were studied at earlier time points in cells treated with tetrandrine alone or tetrandrine plus MG132. As shown in Fig. 8*B*, CDK6 and CDK4 started to decrease after 2 hours of exposure to tetrandrine alone. Pretreatment with MG132 blocked the tetrandrine-induced reduction of CDK6 and CDK4, suggesting the involvement of the 26S proteasome in the degradation of CDK6 and CDK4. MG132 also prevented the E2F1 and cyclin D1 reduction in tetrandrine-treated cells (data not shown). We next tested whether tetrandrine-induced proteasomal degradation of CDK6 was associated with changes in ubiquitination. MG132 was added to block degradation of ubiquitinated proteins. The cell lysates were immunoprecipitated with anti-CDK6, and ubiquitination of the immunoprecipitated complexes was analyzed by antiubiquitin immunoblotting (Fig. 8*C*). Analysis of CDK6 immunoprecipitates showed a series of bands corresponding to different ubiquitin conjugates. Thus, ubiquitinated CDK6 increased after exposure of HT29 cells to tetrandrine, which is consistent with the consecutive proteasomal degradation of CDK6 (see Figs. 7 and 8*A* and *B*). These results show that CDK6 is degraded by an ubiquitin-mediated pathway after exposure to tetrandrine.

**Induction of p53 and $p21^{Cip1}$ by Tetrandrine in HCT116 Cells.** The $p53/p21^{Cip1}$ pathway plays a critical role in regulating the $G_1$-S transition in response to a variety of cellular stresses. Because p53 is mutated in HT29 cells, we examined the effects of tetrandrine on p53 and $p21^{Cip1}$ levels in colon carcinoma HCT116 cells containing wild-type p53 (28). Tetrandrine induced comparable $G_1$ arrest in HCT116 and HT29 cells (data not shown). As shown in Fig. 9, p53 protein increased after 8 hours of treatment and kept increasing up to 18 hours in HCT116 cells. By contrast, p53 levels were much higher in HT29 cells than in HCT116 cells, as expected for p53-mutant cells (28), and remained unchanged in tetrandrine-treated cells. $p21^{Cip1}$ was induced in HCT116 cells and remained undetectable in HT29 cells under the same condition. These results show that p21 activation also can contribute to tetrandrine-induced $G_1$ arrest in p53-competent cells.

## DISCUSSION

Cell cycle is regulated by a series of checkpoints monitoring genomic integrity and ensuring that DNA replication proceeds in a coordinated manner (5, 14). Different combinations of CDK and cyclin subunits operate at checkpoint controls during the cell cycle to integrate mitogenic and antiproliferative signals. Aberrations in cell cycle progression occur in the majority of human malignancies (see refs. 2, 3, 5, 17). Whereas cell proliferation and differentiation are specifically controlled in the $G_1$ phase and the $G_1$-S transition in the cell cycle, oncogenic progresses exert their greatest effect by targeting particular regulators of $G_1$ phase progression (31). In this study, we show that tetrandrine induces early $G_1$-phase arrest, followed by apoptosis in human colon carcinoma HT29 cells. Similar results were found in p53/wild-type HCT116 cells (data not shown).

Abnormalities of $G_1$-S transition regulators and more specifically of the Rb pathway have been recognized as significant factors in the development of human cancers (2). Rb is an important tumor suppressor gene, commonly inactivated in tumors by the hyperactivation of CDKs (2). Consequently, modulation of CDKs is a major target for tumor prevention and therapy. There are two main strategies to

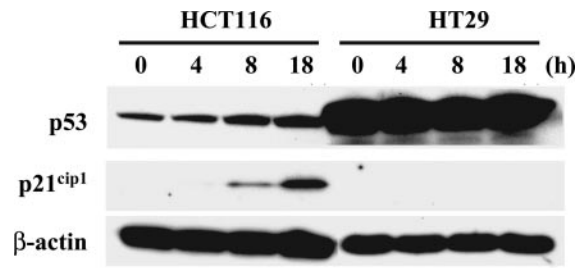


Fig. 9. Activation of p53 and $p21^{Cip1}$ by tetrandrine. Exponentially growing HCT116 and HT29 cells were treated with 30 $\mu$mol/L tetrandrine. Western blot analysis for p53, $p21^{Cip1}$, and $\beta$-actin were performed at the indicated times after the addition of tetrandrine.

modulate CDK activities: directly and indirectly (32, 33). Direct modulators target the catalytic subunit of CDKs. Most of CDK inhibitors interact specifically with the ATP-binding site of CDKs (33, 34). Examples of this class include flavopiridol, roscovitine, aminothiazole, UCN-01, and alsterpaullone. Indirect modulators target the upstream pathways that regulate CDK activity. Examples for this class of compounds include perifosine, lovastatin, and UCN-01 (34). Tetrandrine differs from other CDK inhibitors because it reduces the catalytic activity of specific CDKs and the levels of key $G_1$ regulatory proteins. First, tetrandrine selectively inhibits CDK4 and CDK2/cyclin E, which play essential roles in $G_1$-S transition. However, no significant inhibitory effect was observed on CDK1/cyclin B, CDK2/cyclin A, and CDK6 in the pharmacologic concentration range. CDK4 inhibition was not competitive with ATP, indicating that tetrandrine might induce $G_1$ arrest through direct CDK4 and CDK2/cyclin E inhibition but without targeting the ATP binding site of CDKs.

Tetrandrine also down-regulates the hyperphosphorylated form of Rb and E2F1 and suppresses the expression CDK4, CDK6, and cyclin D1 proteins. Therefore, tetrandrine-induced $G_1$ arrest can be caused by an additional mechanism: suppression of CDK4, CDK6, cyclin D1, and E2F1 in association with dephosphorylation of Rb. We found that the 26S proteasome inhibitor MG132 prevents the down-regulation of CDK4, CDK6, cyclin D1, E2F1, and dephosphorylation of Rb induced by tetrandrine and that tetrandrine increases CDK6 ubiquitination. These results indicate that tetrandrine selectively activates the ubiquitin-proteasome pathway, which specifically degrades some $G_1$-phase proteins, resulting in dephosphorylation of Rb. Unphosphorylated Rb sequesters E2F transcription factors and prevents them from activating critical genes for the $G_1$-S transition. In this way, tetrandrine arrests cell cycle progression by activating the degradation of critical proteins involved in the $G_1$-S transition.

Cell cycle control is tightly regulated physiologically by CDK inhibitors (CKIs). CKIs consist of two families, the INK4 and Cip/Kip families (3, 35). Members of the INK4 family specifically target the cyclin D–dependent kinases. They include $p16^{INK4a}$, $p15^{INK4b}$, $P18^{INK4c}$, and $p19^{INK4d}$. Members of the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind to cyclin-CDK complexes and inhibit their activities. Because a defect in CKI activity is one of the factors causing uncontrolled proliferation of tumor cells (2), one possible strategy to control cancer cell proliferation is to induce CKI expression, which would lead to $G_1$ arrest and inhibit tumor growth. Although the tumor suppressor p53 itself is not a CKI, it has been implicated in a variety of cellular processes, including induction of $G_1$ arrest and apoptosis by transactivating a number of downstream genes (4, 36). Among the transcription targets of p53, $p21^{Cip1}$ plays a key role in mediating $G_1$ arrest (37). We observed that tetrandrine induces $G_1$-phase arrest in HCT116 cells, which possess functional p53, and increases p53 and $p21^{Cip1}$ protein level in a time-dependent manner. Thus, $p21^{Cip1}$ could contribute to $G_1$-phase arrest produced by tetrandrine in cells with normal p53.

In summary, we show that tetrandrine possesses antiproliferative activity at micromolar concentrations and induces early $G_1$-phase arrest in p53-dependent and -independent ways. At least three mechanisms contribute to tetrandrine-induced $G_1$-phase arrest: first, tetrandrine acts as a direct inhibitor of CDK4 and CDK2/cyclin E; second, tetrandrine acts as an indirect CDK modulator by inducing the degradation of the $G_1$-phase proteins CDK4, CDK6, cyclin D1, and E2F1 by the ubiquitin-proteasome pathways; and third, in p53 wild type cells tetrandrine can inactivate CDK/cyclin complexes by inducing p53, which in turn activates $p21^{Cip1}$. The tetrandrine-induced $G_1$-phase arrest is irreversible and followed by apoptotic death.

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