Cancer Chemother Pharmacol (2003) 51: 271–283
DOI 10.1007/s00280-002-0562-z

ORIGINAL ARTICLE

Fumiyuki Yamasaki · Seiji Hama
Hiroyuki Yoshioka · Yoshinori Kajiwara
Kaita Yahara · Kazuhiko Sugiyama · Yuji Heike
Kazunori Arita · Kaoru Kurisu

Staurosporine-induced apoptosis is independent of *p16* and *p21* and achieved via arrest at G2/M and at G1 in U251MG human glioma cell line

Received: 12 August 2002 / Accepted: 8 November 2002 / Published online: 20 March 2003

Abstract *Objective*: The mechanisms involved in the cell cycle and cell death remain unresolved despite much investigation. Staurosporine induces cell death and G1 or G2/M arrest in a dose-dependent manner, but the mechanisms remain unknown. *Methods*: In the present study an adenovirus vector expressing *p16* or *p21* genes in human glioma cell lines was used to examine cell cycle regulation and cell death induced by staurosporine. *Results*: A low concentration (≤ 10 n*M*) of staurosporine induced G1 arrest of U251MG cells, whereas a high concentration (≥30 n*M*) induced G2/M arrest and finally induced apoptosis via a caspase-3-activated pathway from both the G2/M and G1 phases. However, pRb was dephosphorylated and cdc2 was inhibited at both the low and the high concentrations of staurosporine, indicating that the mechanisms of cell cycle regulation are not simply p53-Rb- or cdc2-dependent pathways. *Conclusions*: Forced G1 arrest by transfection with *p16* or *p21* genes did not alter the rate of staurosporine-induced cell death. This implies that an unknown pathway of apoptosis occurs from the G1 phase.

Keywords Staurosporine · p16 · p21 · Apoptosis

F. Yamasaki (✉) · S. Hama · Y. Kajiwara · K. Yahara
K. Sugiyama · K. Arita · K. Kurisu
Department of Neurosurgery, Faculty of Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, Japan
E-mail: fyama@hiroshima-u.ac.jp
Tel.: +81-82-2575227
Fax: +81-82-2575229

H. Yoshioka
Department of Neurosurgery, National Hospital, Kure Medical Center, Kure, Japan

Y. Heike
Division of Clinical Research, National Shikoku Cancer Center Hospital, Matsuyama, Japan

Introduction

Primary malignant brain tumors are the most prevalent of solid tumors among children, and represent an important cause of cancer mortality in children under the age of 15 years. Malignant brain tumors also cause a significant number of deaths among adolescents and adults [13]. The most frequent primary brain tumors are gliomas, particularly those of astrocytic origin [30]. Almost all malignant gliomas are refractory to current treatment strategies and patients eventually die from brain herniation due to unrestrained growth of the tumor [22]. Factors that regulate the cell cycle such as p53, p16, and p21 are disordered and checkpoint mechanisms of the cell cycle are always disrupted in gliomas. These factors are associated with tumorigenesis and the malignant transformation of gliomas. Clinical and experimental studies have shown that the expression of cell cycle related proteins and their kinases are important in the growth and development of gliomas.

Cells in the G0/G1 phase are more resistant to chemotherapy and radiotherapy [4]. Few cells remain in the G0/G1 phase in vitro, whereas almost all clinical glioma cells are in this phase. We demonstrated that G1 arrest induced by *p16* gene expression prevents cell death induced by chemotherapy, which appears to occur after the G1/S checkpoint [25]. To treat gliomas, strategies should be targeted to glioma cells while in the G0/G1 phase.

Protein kinase C (PKC) activity is high in gliomas and may contribute to their growth [6, 7, 11, 41]. The PKC family of serine/threonine kinases catalyzes numerous biochemical reactions critical to the function of many cellular constituents [35]. Although their exact mechanisms remain unclear, protein kinase inhibitors can cause apoptosis [11]. Protein kinases are thought to regulate proliferation and cell cycle progression by phosphorylating critical proteins. A cascade of successive proteins mediates the exit of cells from quiescence upon stimulation with growth factors [15].

Staurosporine is a microbial alkaloid (indolcarbazole produced by *Streptomyces* spp.) that potently inhibits a wide range of protein kinases, including various serine/threonine and tyrosine forms by competing with the ATP-binding region of the kinase catalytic domain [16, 49]. Staurosporine exerts various pharmacological actions involving PKC both in vivo and in vitro [16, 19, 33, 49] and inhibits both glioma cell proliferation and PKC activity [2, 3, 6, 49]. Staurosporine induces G1 [34, 40] and G2 arrest [42, 45] depending on the concentration [1, 8, 12, 20]. The mechanistic basis underlying the response of staurosporine-induced G1 or G2/M arrest remains unresolved, as are details of the relationship between the cell cycle and cell death.

We transfected the U251MG glioma cell line that lacks p16 and p21, with full-length human p16 cDNA or full-length human p21 cDNA using a replication-defective recombinant adenovirus (AxCA-hp16, AxCA-hp21). We examined the effects of *p16* and *p21* gene expression on cell cycle regulation and on proteins induced by staurosporine. We also investigated whether *p16* or *p21* gene expression could be synergistic or additive to the cytocidal effect of staurosporine. The results of our previous study had shown that *p16* or *p21* gene expression due to AxCA-hp16 or AxCA-hp21 infection in U251MG cells is cytostatic but not cytocidal in the G1 phase [25]. The aim of the present study was therefore to verify the key factor(s) of cell cycle regulation and cell death using the cytostatic effect of *p16* and *p21* gene expression, and to establish an effective treatment against glioma cells in the G0/G1 phase.

Materials and methods

Recombinant adenovirus vectors

Replication-defective adenovirus vectors were based on the human adenovirus type 5(Ad5) serotype. The full-length human p16 cDNA was a gift from Dr. T. Nobori (Department of Medicine, University of California, San Diego, Calif.) and the full-length p21 cDNA was purchased from the Meiji Institute of Health [37, 38]. Recombinant adenovirus vectors containing the full-length human *p16* gene (Ax-hp16) or *p21* gene (Ax-hp21) were generated using the COS-TPC method as described previously [25], and exogenic *p16* or *p21* gene expression after Ax-hp16 or Ax-hp21 infection was confirmed by Western blotting as described previously [25, 32, 36]. Control vectors consisted of recombinant adenoviruses containing an expression cassette without a genome (mock adenovirus vector, Ax-mock). Vector titers were determined by plaque assay as described previously [17]. The optimal conditions for virus infection of each cell line to ensure adequate gene transfer were evaluated as described previously [25].

Cell lines and cell culture

We used the human glioma cell lines U251MG (*p16* homozygous deletion, *p21* undetectable protein expression, *p53* mutated at codon 273, CGT/CAT Arg/His), T98G (*p16* undetectable protein expression, *p53* mutated) and D54MG (*p16* undetectable protein expression, *p53* wild-type) [18, 23, 24, 26, 28, 29, 52]. U251MG cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), T98G cells were obtained from the American Type Culture Collection (Manassas, Va.), and D54MG cells were provided by Dr. D.T. Curiel (University of Alabama at Birmingham, Ala.) [31]. The transfection efficiency of recombinant adenoviruses into glioma cells [25] was highest in the U251MG line so we used this in subsequent experiments. U251MG cells were cultured in minimum essential medium (MEM; Nissui, Tokyo) supplemented with non-essential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.2 mg/ml L-glutamine and 10% fetal bovine serum.

Effects of AxCA-hp16, AxCA-hp21 or AxCA-mock infection on cell growth

An asynchronous population of U251MG human glioma cells was trypsinized and reseeded at a density of 5×10^3 cells per well in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). The cells were infected on the following day with AxCA-hp16, AxCA-hp21 or AxCA-mock at a multiplicity of infection (MOI) of 5. The culture medium including recombinant vectors was not changed until the cells were counted. The average of each group of triplicate wells was calculated. The number of viable cells was periodically evaluated by Trypan blue exclusion until 5 days after infection. These experiments were performed three times.

Adenovirus infection and staurosporine administration

We examined the effect of *p16* gene expression on chemosensitivity to staurosporine as follows. In one schedule, staurosporine was added 6 h after, and in another 6 h before, *p16* gene transfer as described previously [25]. Staurosporine inhibited the growth of U251MG cells after 3–6 h, while Ax-hp16 and Ax-hp21 inhibited cell growth from 12 to 24 h after infection as reported previously [25]. Therefore, in a third schedule, staurosporine was added 24 h after *p16* or *p21* gene transfer.

Cell lysis and Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 25 m*M* Tris-HCl (pH 7.4), 50 m*M* NaCl, 0.5% Na deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 m*M* phenylmethylsulfonyl fluoride and 50 μg/ml aprotinin. Cell lysates were clarified by centrifugation for 20 min at 14,000 *g*, and the total protein concentration was determined in the resultant supernatants using the BCA protein assay reagent kit according to the instructions provided by the manufacturer (Bio-Rad, Philadelphia, Pa.). The lysates were size-fractionated by SDS-PAGE and transferred onto a polyvinylidene-difluoride membrane (Millipore, Bedford, Mass.). The membrane was blocked with TBS containing 0.05% Tween-20 and 5% skimmed milk, then incubated with primary antibody. Signals were detected using ECL Western blotting reagents (Amersham, Arlington Heights, Ill.) and the blots were stained to ensure equal protein loading. Mouse monoclonal antibodies to $p16^{INK4}$ (554070) and $p21^{WAF1/CIP1}$ (65951A) and RB (14001A), and rabbit polyclonal antibody to caspase-3 (65906E), were obtained from Pharmingen (Pharmingen, San Diego, Calif.). Mouse monoclonal antibodies to actin (I-19, sc-1616) and p53 (N-19, sc-1314) and cdc2 (17, sc-54) were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

MTT assay

Noninfected control cells were seeded in 96-well plates at a density of 800 cells per well, cultured overnight and incubated with staurosporine at final concentrations of 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 n*M* for 5 days. Cells were also seeded in 96-well plates at the same density, cultured overnight and infected with AxCA-hp16, AxCA-hp21 or AxCA-mock at a MOI of 5 (U251MG and D54MG) or at a MOI of 30 (T98G) before or after adding staurosporine at the same final

concentrations as described above. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. The results are shown as percent cell growth calculated as follows: (optical density of treated wells–optical density of cell-free control wells)/(optical density of cell-only control wells–optical density of cell-free control wells)×100.

Analysis of cell cycle by flow cytometry

U251MG cells were plated in 100-mm dishes (Falcon), cultured overnight and infected with AxCA-hp16, AxCA-hp21 or AxCA-mock at an MOI of 5 before or after adding staurosporine at a final concentration of 10 or 30 n*M*. The staurosporine concentration of 10 n*M* was the IC_{50} value and 30 n*M* was the IC_{25} value for noninfected cells, as determined by a 5-day MTT assay. The cells were collected by trypsinization after 12, 24, 36, 48, 72 and 120 h (5 days) of incubation, washed twice with PBS, fixed with 75% ethanol and stored at 4°C for 48 h. After centrifugation at 400 *g* for 10 min in a KS-5300C rotor (Kubota, Tokyo, Japan), the cells were washed with PBS and resuspended in 1 ml lysis buffer (0.1% Triton X-100, 0.1% RNase A) at 4°C overnight to release the nuclei. Immediately before analysis, 1 ml 50 μg/ml propidium iodide (PI, final concentration 25 μg/ml) was added to the cells. The PI fluorescence of individual nuclei was measured using a FACScan (Becton Dickinson), and the data were analyzed using the ModFit LT program (Becton Dickinson). These experiments were repeated three times.

The flow-cytometric and cytotoxicity data did not seem to be consistent, since cellular fragments were not demonstrated. The sub-G1 cellular peak cannot be distinguished from dead cells, cellular fragmentation and other cellular debris in the culture medium on the basis of flow cytometry. Since cellular debris interferes with analyses of the proportion of cells in each phase of the cell cycle (G1, S and G2/M phases), sub-G1 cells and cellular fragments were gated out of the flow-cytometric procedure as described previously [25].

Trypan blue exclusion

Cells were seeded in 24-well plates at a density of 5000 cells per well and cultured overnight. Trypan-blue staining was used to determine total cell counts and viable cells after 1, 3 and 5 days of infection with AxCA-hp16, AxCA-hp21 or AxCA-mock at an MOI of 5 before or after adding staurosporine at a final concentration of 10 or 30 n*M*. These concentrations of staurosporine were the IC_{50} or IC_{25} values, respectively, of noninfected cells, as determined by a 5-day MTT assay. Floating and adherent cells were collected at the indicated times, sedimented by centrifugation and resuspended in MEM. The cells were then diluted 1:9 with 0.4% Trypan blue (Sigma) and scored under a light microscope. Viable (unstained) and nonviable (blue-stained) cells were counted and the total numbers of living and dead cells were calculated. The results are presented as means ± SD of four independent experiments, with a minimum of 500 cells being scored. These experiments were repeated at least three times.

Clonogenic assay

U251MG cells were seeded in 60-mm culture dishes (Falcon) at a density of 200 cells per dish and incubated overnight. The cells were then infected with AxCA-hp16 or AxCA-mock at an MOI of 5 before or after adding staurosporine at final concentrations of 0.03, 0.1, 0.3, 1, 3, 10 and 30 n*M*. The culture medium including drug and recombinant vectors was removed, then the cells were washed twice with PBS and allowed to proliferate in fresh medium for 2 weeks. Colonies were counted when they reached 50–100 cells by staining with 0.1% crystal violet in 0.9% saline for 30 min at room temperature. The number of colony-forming units in treated cultures is expressed as the percentage in relation to untreated controls. These experiments were repeated three times.

Results

Effect of *p16* or *p21* gene transfer on chemosensitivity to staurosporine

The MTT assay at 5 days after drug treatment revealed similar resistance to staurosporine in U251MG, T98G and D54MG cells infected with AxCA-hp16 and -hp21 either before or after drug exposure (Fig. 1). At 3 days after drug treatment, the relative resistance ratios to staurosporine of *p16* gene-infected cells and cells not gene-infected were similar (data not shown).

Analysis of p16 and p21 expression

We examined p16 protein expression by Western blotting in U251MG cells infected with AxCA-hp16 (Fig. 2). We detected p16 expression in AxCA-hp16-infected cells from 1 to 5 days after infection (lanes 7–9), but not in uninfected (lanes 1–3) or AxCA-mock-infected (lanes 4–6) cells or in AxCA-hp21-infected cells (lanes 10–12), regardless of the presence or absence of staurosporine.

We likewise examined p21 protein expression in U251MG cells infected with AxCA-hp21. Figure 2 shows p21 expression in cells from 1 to 5 days after AxCA-hp21 infection (lanes 10–12), but not in uninfected (lanes 1–3) or AxCA-mock-infected (lanes 4–6) cells or in AxCA-hp16-infected cells (lanes 7–9), regardless of the presence or absence of staurosporine.

Effect of staurosporine on pRb status of U251MG cells transfected with *p16* or *p21* genes

We investigated the expression of phosphorylated retinoblastoma protein (pRb) in U251MG cells by Western blotting using an antibody that recognizes phosphorylated pRb (Fig. 2). Uninfected (lanes 1–3) and AxCA-mock-infected (lanes 4–6) cells expressed pRb and the level did not change over 5 days. However, the expression of pRb was decreased in AxCA-hp16-infected cells 1 day after infection (lane 7) and remained decreased for 5 days (lanes 8 and 9). The expression of pRb was also decreased in AxCA-hp21-infected cells for 5 days (lanes 10–12). Staurosporine inhibited pRb phosphorylation. The expression level of pRb was decreased after adding either 10 or 30 n*M* staurosporine, with or without AxCA-hp16 or AxCA-hp21 infection.

Effect of staurosporine on cdc2 in U251MG cells transfected with *p16* or *p21* genes

We investigated the expression of cdc2 in U251MG cells by Western blotting (Fig. 2). Uninfected (lanes 1–3) and AxCA-mock-infected (lanes 4–6) cells expressed cdc2 over 5 days. However, the expression of cdc2 was

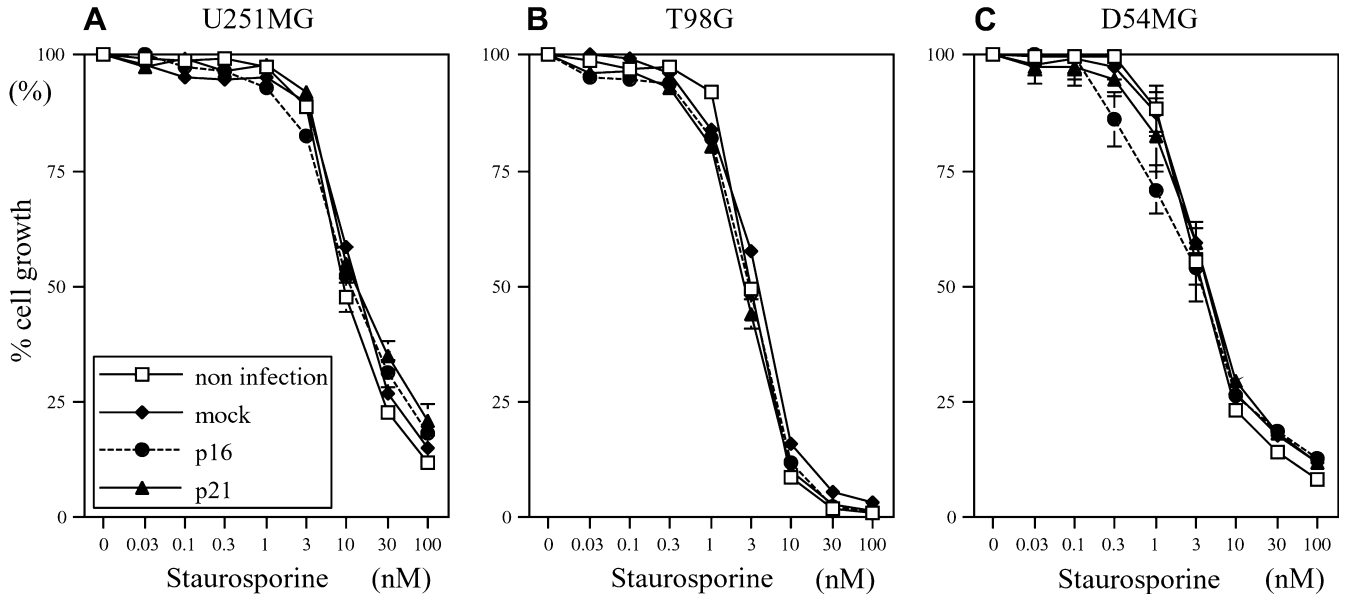


Fig. 1A–C Dose response curves of U251MG (**A**), T98G (**B**) and D54MG (**C**) cells infected with or without AxCA-mock, AxCA-hp16 or AxCA-hp21 at 5 MOI (U251MG and D54MG) or at 30 MOI (T98G). Surviving fractions were determined by the MTT assay as described in Materials and methods. Results are shown as percentage cell growth at each point, with the standard deviation of four wells (□ noninfected cells, ◆ cells infected with AxCA-mock, ● cells infected with AxCA-hp16, ▲ cells infected with AxCA-hp21)

decreased in AxCA-hp16-infected cells 1 day after infection (lane 7) and remained decreased for 5 days (lanes 8 and 9). The expression of cdc2 protein was also decreased in AxCA-hp21-infected cells over 5 days (lanes 10–12). The expression level of cdc2 was decreased after adding either 10 or 30 n*M* staurosporine, with or without AxCA-hp16 or AxCA-hp21 infection.

Effect of administration of staurosporine on the cell cycle with *p16* or *p21* gene transfer

Figure 3 shows histograms of the cell cycle status after adding 10 n*M* (IC_{50}) or 30 n*M* (IC_{25}) staurosporine and/or infection with AxCA-mock, AxCA-hp16 or AxCA-hp21. AxCA-hp16- and Ax-hp21-infected cells were arrested in the G1 phase for 5 days after infection as compared with uninfected or Ax-mock-infected cells. U251MG cells accumulated in the G1 and G2/M phases for 5 days in the presence of 10 and 30 n*M* staurosporine, respectively, compared with untreated cells.

When cells were infected with AxCA-hp16 after adding staurosporine, the proportion and progress of the cell cycle was similar to that in the presence of staurosporine alone. When infected with AxCA-hp16 before adding 10 n*M* staurosporine, most of the cells remained in G1 arrest for 5 days. However, U251MG cells infected with AxCA-hp16 before adding 30 n*M* staurosporine, particularly those infected 24 h beforehand, accumulated not in the G2/M, but in the G1 phase. The results of AxCA-hp21 and AxCA-hp16 infection were the same. The results following AxCA-mock infection were the same as those in noninfected controls.

Cell viability determined by Trypan blue exclusion

At 1, 3 and 5 days after adding 10 n*M* staurosporine, the viability of U251MG cells infected with AxCA-hp16 24 h before drug exposure was similar to that of uninfected control cells (Fig. 4). The effects of AxCA-mock and of AxCA-hp21 infection were also similar, and cells infected with AxCA-hp16 6 h before or after drug exposure were equally viable. These experiments were repeated three times with similar results.

Cell viability by clonogenic assay

Staurosporine caused clonogenic inhibition of U251MG cells (Fig. 5). Staurosporine added 6 or 24 h before or 6 h after AxCA-hp16 infection did not affect colony formation. The results of AxCA-mock and of AxCA-hp21 infection were also similar. These experiments were repeated three times.

Apoptotic assay

The apoptosis signal was detected by Western blotting as the expression of cleaved (activated) caspase-3 (Fig. 6). Cellular protein was extracted after 3 days in the presence of staurosporine. Cleaved caspase-3 was not evident in control (lane 3), AxCA-mock-infected (lane 6), AxCA-hp16-infected (lane 9) or AxCA-hp21-infected (lane 12) cells in the absence of staurosporine. Caspase-3 was cleaved in the absence of infection (lane 4), and with AxCA-mock infection (lane 7), with AxCA-hp16 infection (lane 10) and with AxCA-hp21 infection

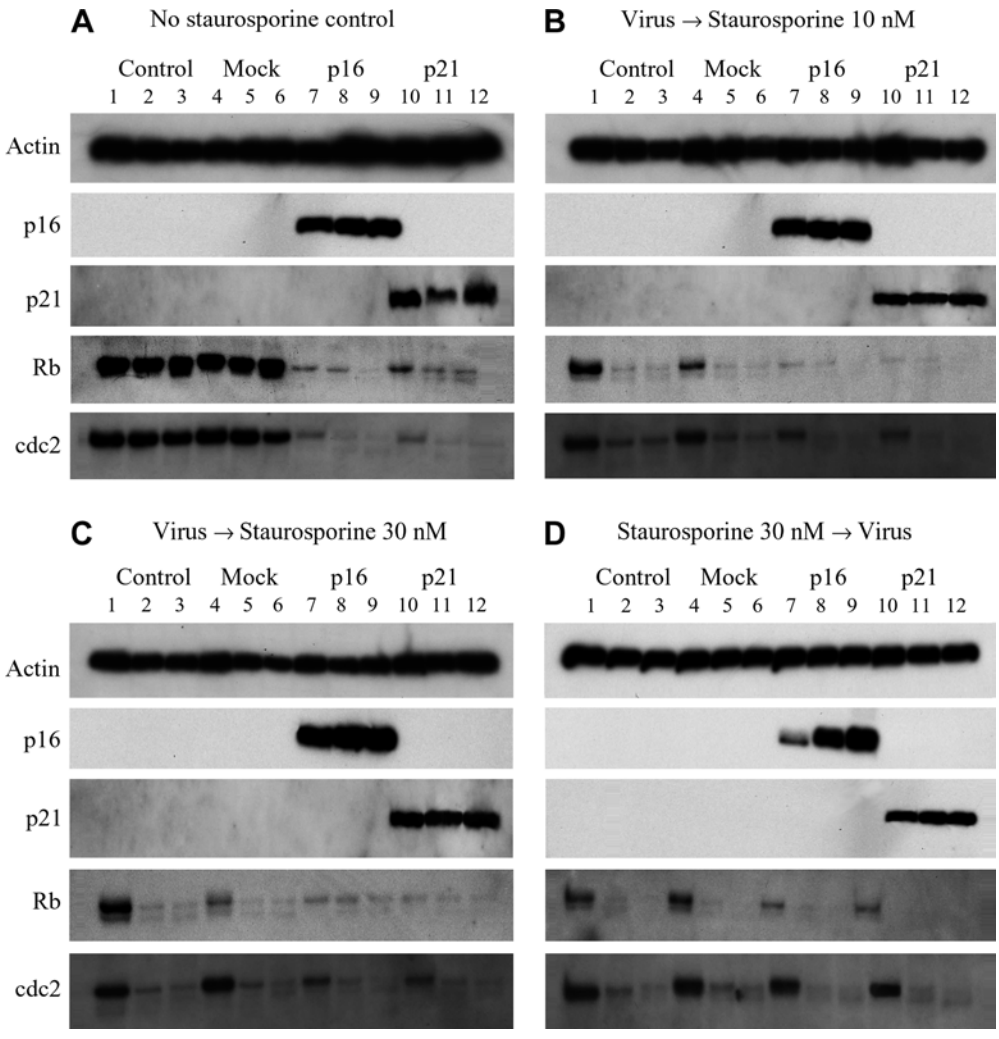


Fig. 2A–D Immunoblot analysis. Cellular protein (10 μg) extracted from cells incubated under each condition was blotted against the indicated antibodies. Actin demonstrates equal loading. Blots were visualized as described in Materials and methods. Sample loading: *lanes 1–3* noninfection control, day 1 (*lane 1*), day 3 (*lane 2*) and day 5 (*lane 3*); *lanes 4–6* AxCA-mock infection, day 1 (*lane 4*), day 3 (*lane 5*) and day 5 (*lane 6*); *lanes 7–9* AxCA-hp16 infection, day 1 (*lane 7*), day 3 (*lane 8*) and day 5 (*lane 9*); *lanes 10–12* AxCA-hp21 infection, day 1 (*lane 10*), day 3 (*lane 11*) and day 5 (*lane 12*). **A** Without staurosporine, **B** 10 n*M* staurosporine 24 h after infection, **C** 30 n*M* staurosporine 24 h after infection, **D** 30 n*M* staurosporine 6 h before infection. The effects of 10 n*M* staurosporine 6 h before infection were the same as those of 10 n*M* staurosporine 24 h after infection (data not shown)

(lane 13) 24 h before adding 10 n*M* staurosporine. Caspase-3 was cleaved without infection (lane 5), and with AxCA-mock infection (lane 8), with AxCA-hp16 infection (lane 11) and with AxCA-hp21 infection (lane 14) 24 h before adding 30 n*M* staurosporine. Infection 6 h before or 6 h after adding staurosporine yielded similar results (data not shown).

Discussion

PKC is frequently overexpressed in neoplastic, compared with non-neoplastic astrocytes and may contribute to the growth and inhibition through programmed cell death of these tumors. Staurosporine is a microbial alkaloid produced by the *Streptomyces* bacteria, and it is a potent broad-spectrum kinase inhibitor [2, 49]. In fact, staurosporine is one of the most powerful PKC inhibitors in models in vitro [2], as it inhibits cell growth at both the G1 and G2/M phases, and/or induces programmed cell death in human cancer cells [5].

The *p16* and *p21* gene products are thought to negatively control the progression of eukaryotic cells through G1 arrest of the cell cycle, and transfection with these genes causes significant growth inhibition and G1 arrest in *p16*-null human glioma cell lines [18, 24]. We have demonstrated that G1 arrest induced by *p16* gene expression decreases sensitivity to alkylating agents. These and other findings indicate that cancer cells arrested in the G1 phase acquire resistance against many anticancer agents. In the present study, the mechanism of the effect of staurosporine on the cell cycle, especially at the G1-S boundary, and the relationship between the cell cycle and cell death, were therefore investigated.

We initially constructed adenovirus vectors expressing p16 (AxCA-hp16) or p21 cDNA (AxCA-hp21) and examined their effect on the growth of U251MG, T98G and D54MG cells. U251MG, T98G and D54MG cells in the presence of staurosporine with or without AxCA-hp16 or AxCAhp21 infection were equally viable (Fig. 1). U251MG cells were used in the following experiments because they had the highest transfection efficiency among the glioma cell lines tested [25]. Most of the cells infected with AxCA-hp16 or AxCA-hp21 were viable at 5 days after infection (data not shown), even

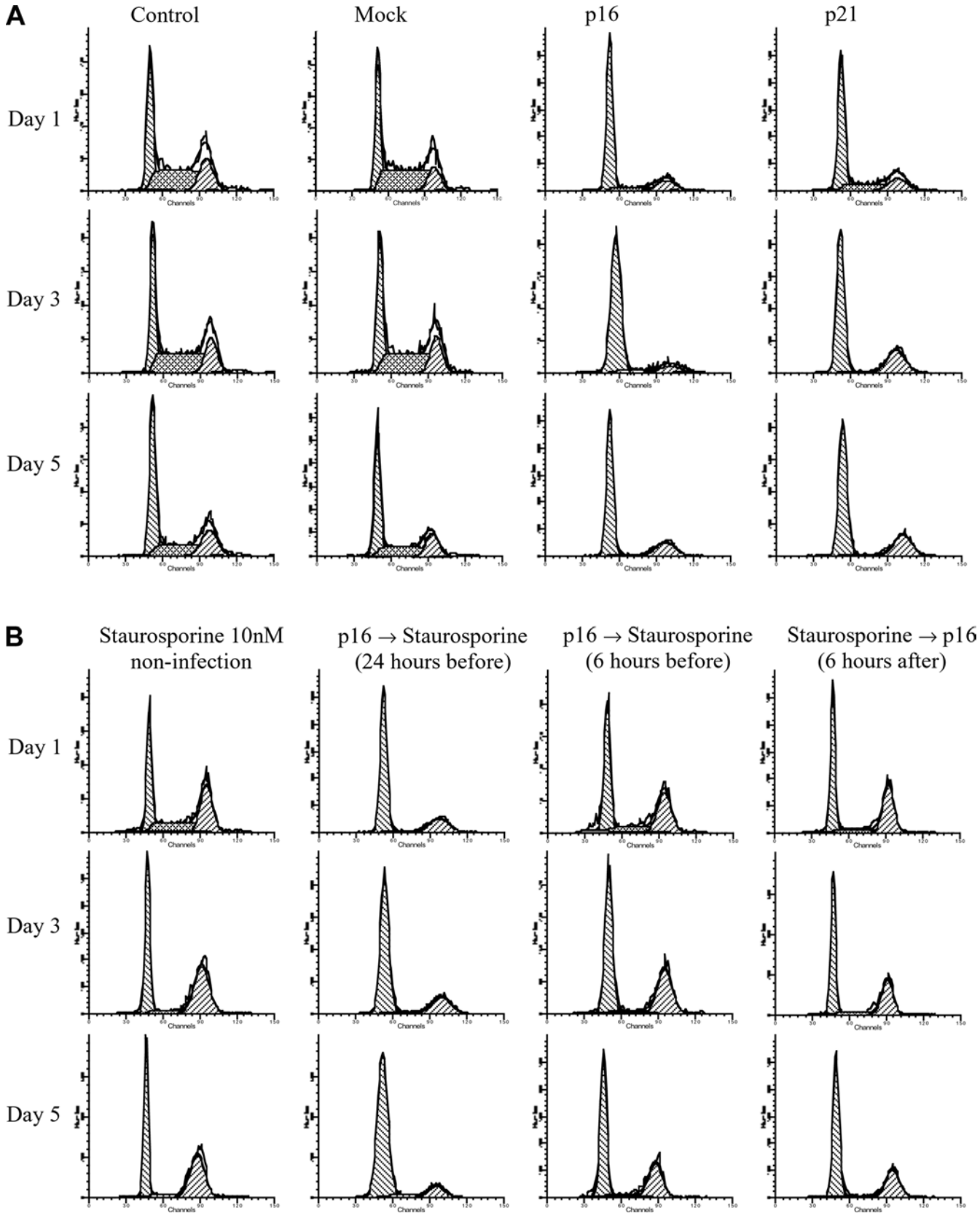
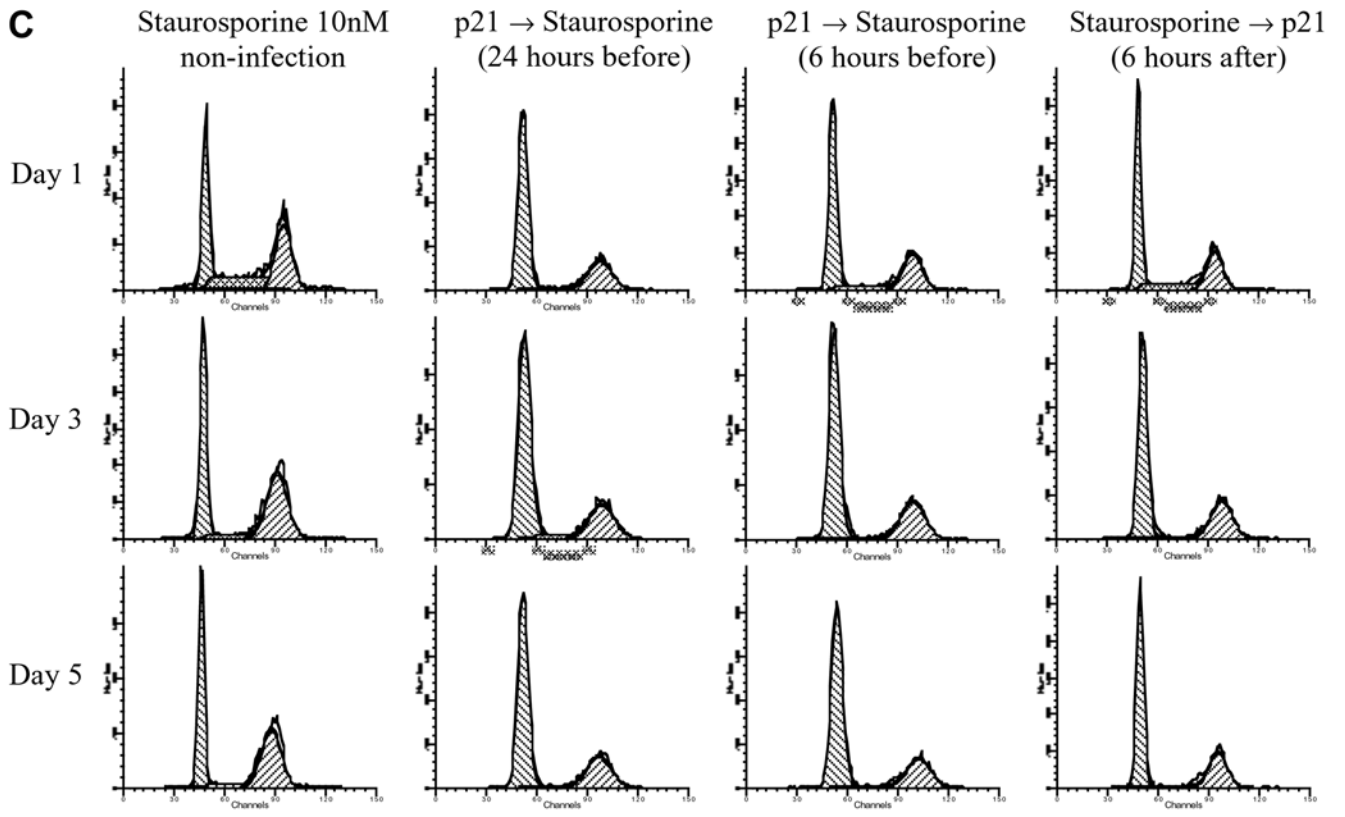


Fig. 3A–G (Contd.)



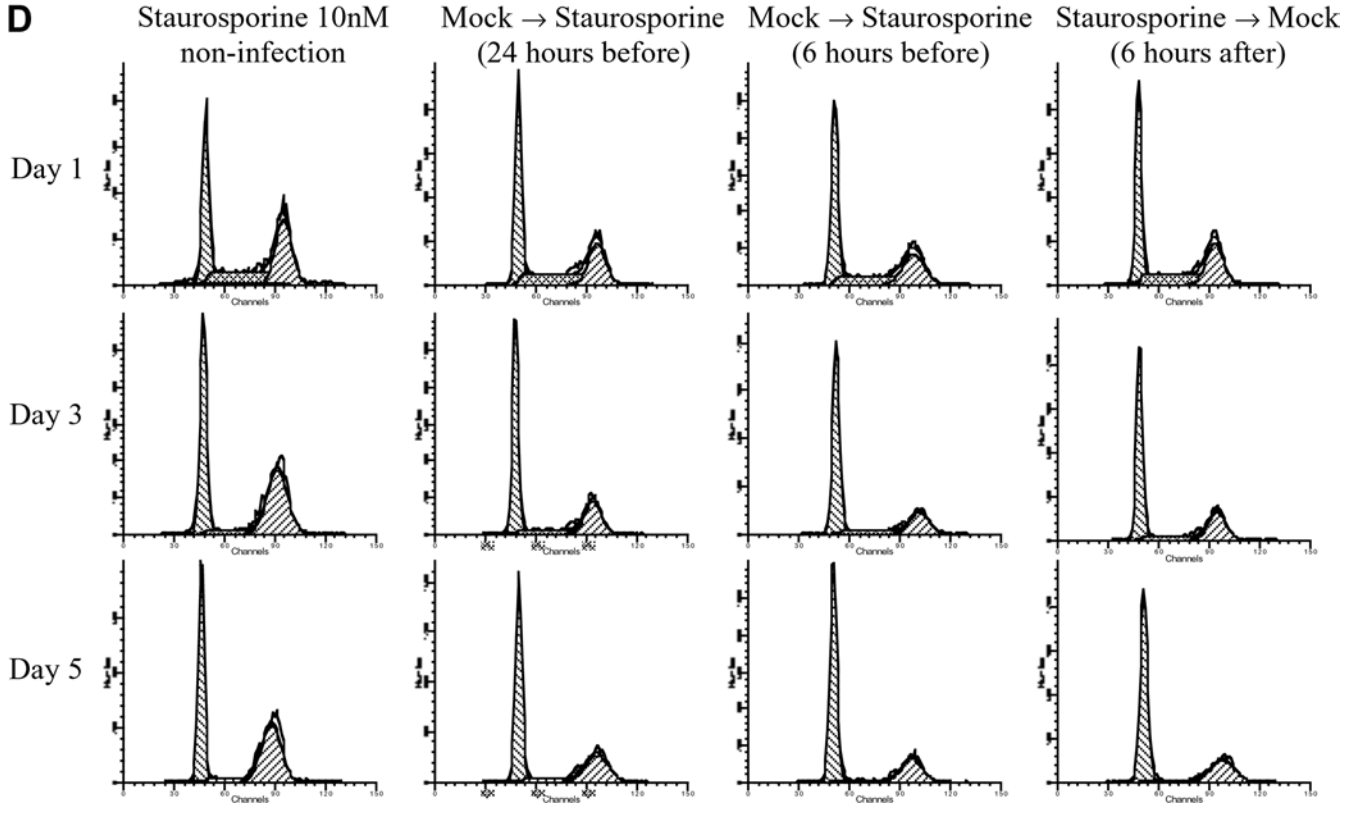
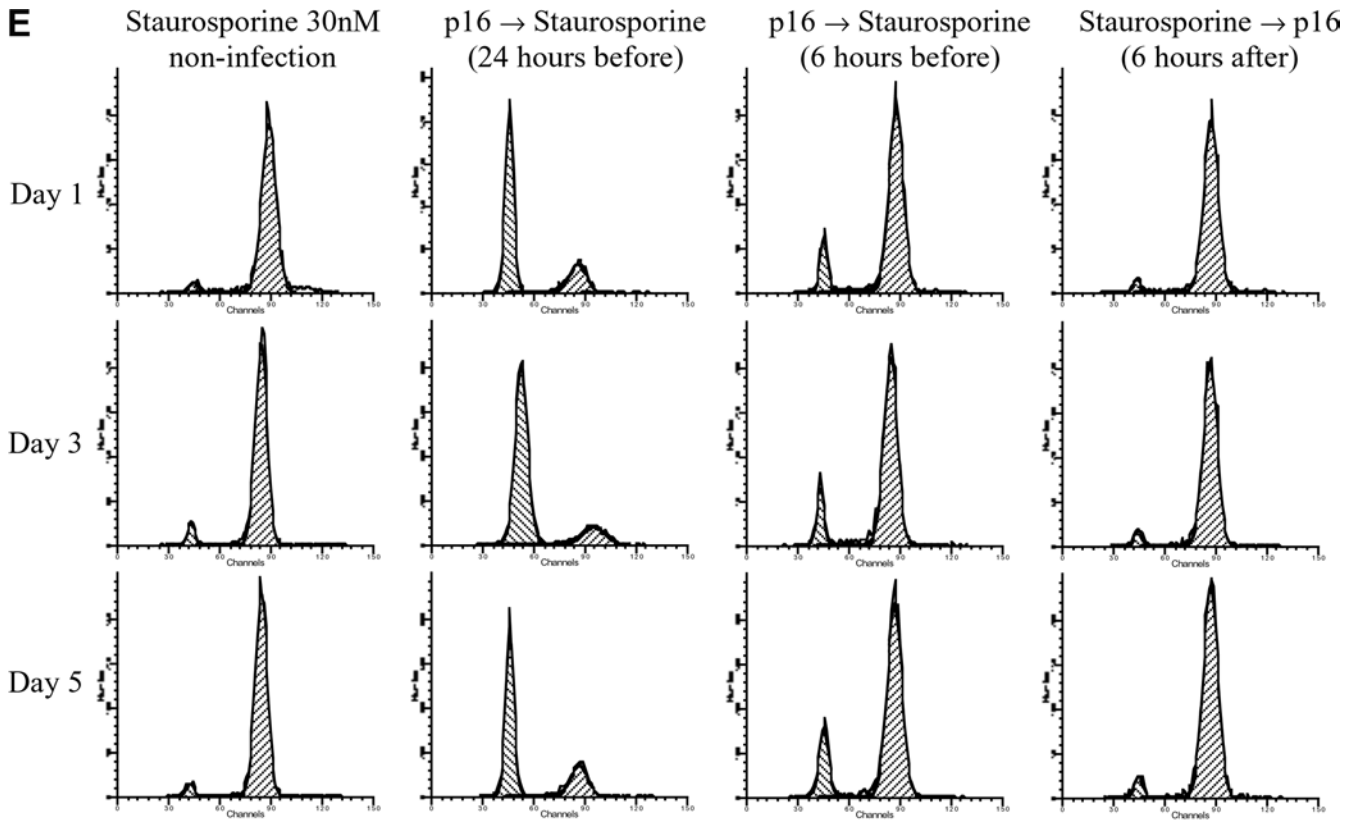


Fig. 3A–G (Contd.)



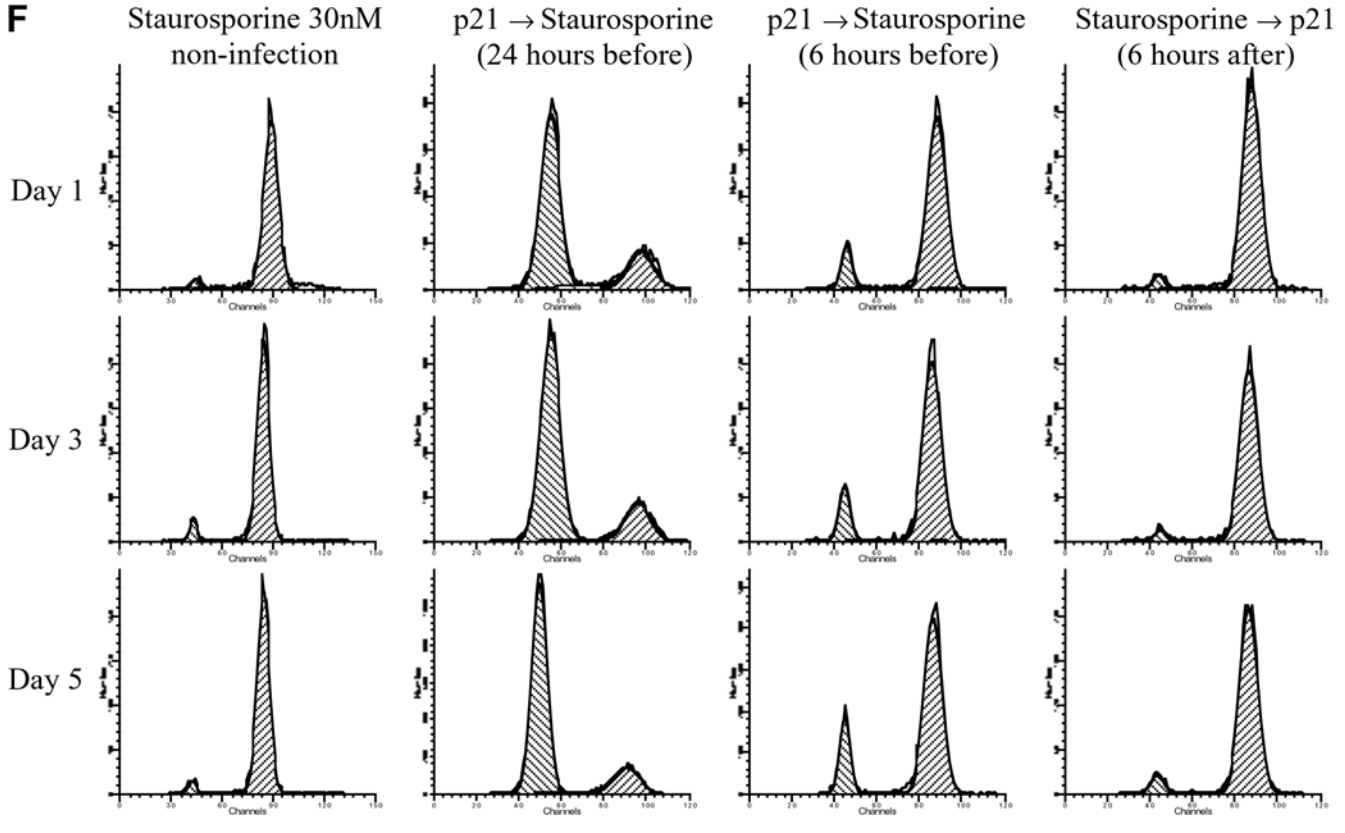


Fig. 3A–G (Contd.)

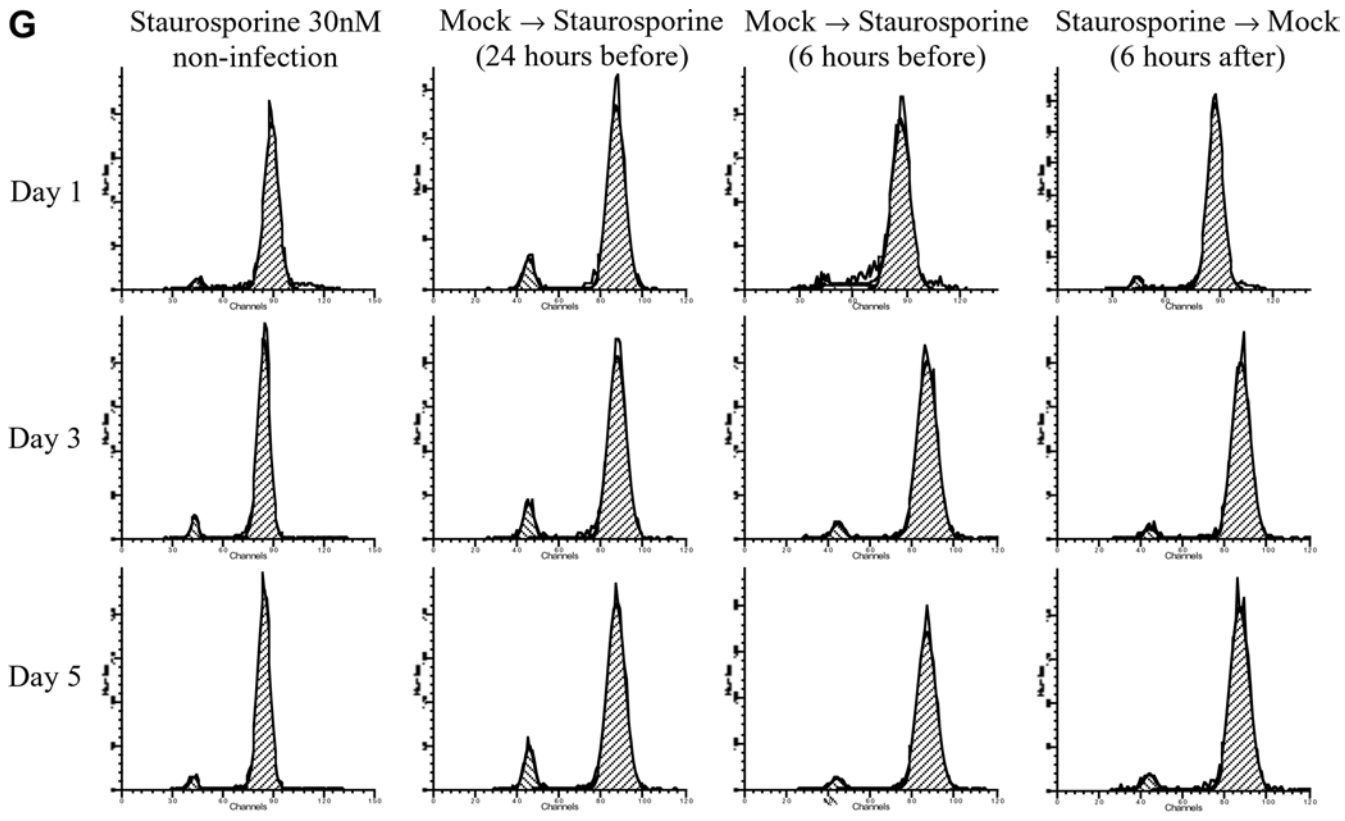


Fig. 3A–G Effects of AxCA-hp16 (**B**, **E**), AxCA-hp21 (**C**, **F**) or AxCA-mock (**D**, **G**) infection on cell cycle of U251MG treated without (**A**), or with 10 n*M* (**B**, **C**, **D**) or 30 n*M* staurosporine (**E**, **F**, **G**). U251MG cells were incubated as described in Materials and methods and cell-cycle status was analyzed by flow cytometry. Data are presented as histograms in which cell number (*y*-axis) is plotted against DNA content (*x*-axis)

though growth was arrested, suggesting that the effects of *p16* and of *p21* gene expression were cytostatic and not cytocidal under these conditions.

We then examined cell cycle regulation by staurosporine, which induces G1 and G2 arrest depending on the concentration. The low concentration of staurosporine (10 n*M*) was determined to be the IC_{50} value and the high concentration (30 n*M*) to be the IC_{25} value, according to 5-day MTT assays of uninfected cells. Staurosporine at 10 and at 30 n*M* induced G1 and G2/M arrest, respectively, in U251MG cells, which agrees with reported findings. The low concentration was under 10 n*M* and the high concentration was over 30 n*M* under our conditions.

Issues raised by these observations relate to how the staurosporine-treated cells undergo dose-dependent arrest in the G1 or G2 phases. The effects of staurosporine and its derivatives on cell cycle progression are complex and concentration-dependent, and might be attributable to diverse interference with the cyclin-dependent kinase (cdk) system [21]. The Rb gene product pRb is a substrate for G1-cdk kinases and its hypophosphorylated form inhibits cell cycle progression from G1 to S phase [50]. The pRb protein is considered to play an important role in staurosporine-induced G1 arrest [40, 44]. Staurosporine potently inhibits partially purified cdc2 and cdc2-like kinases, and cell cycle arrest in the G2 phase induced by high concentrations of staurosporine may be due largely to the inhibition of cdc2 and other similar kinases [1, 14, 20, 43, 48].

We surmise that the dephosphorylation of pRb forces G1 arrest and that the inactivation of cdc2 forces G2 arrest at low and high concentrations of staurosporine, respectively. We found that pRb was dephosphorylated and that cdc2 was inhibited during G1 and G2/M arrest induced by low and high concentrations of staurosporine, respectively. The dephosphorylation of pRb may induce G1 arrest and the inhibition of cdc2 activity may induce G2/M arrest, but staurosporine-induced G1 or G2/M arrest could not be determined by either pRb phosphorylation status or cdc2 activity.

We then examined the activity of pRb and cdc2 after infection with AxCA-hp16 or AxCA-hp21. Transfection with *p16* or *p21* genes dephosphorylated pRb and inhibited cdc2. In addition, pRb was dephosphorylated and cdc2 was inhibited in U251MG cells regardless of AxCA-hp16 or AxCA-hp21 infection after adding staurosporine. This means that staurosporine can dephosphorylate pRb or inhibit cdc2 activity without p16 or p21 expression. In other words, the regulation of staurosporine-induced G1 arrest or G2/M arrest is not associated with p16 or p21 in the U251MG glioma cell line.

Staurosporine inhibits cell cycle progression by affecting protein kinases, and also by inducing cell

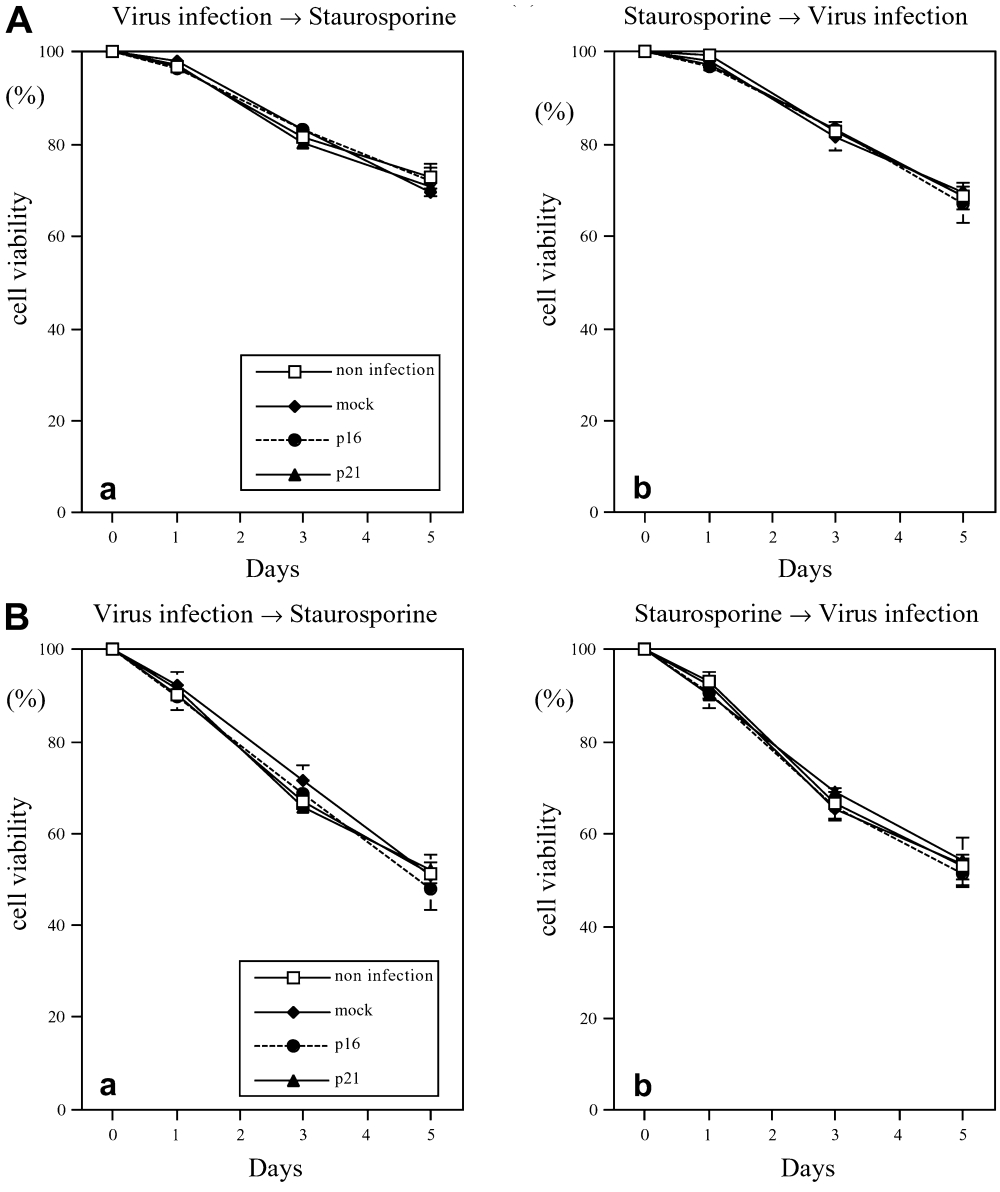


Fig. 4A, B Effect of 10 n*M* staurosporine (**A**) or 30 n*M* staurosporine (**B**) in the presence or absence of *p16* or *p21* gene transfection 24 h before (*a*) or 6 h after (*b*) the addition of staurosporine on U251MG cell viability determined by Trypan blue exclusion. The results presented are the mean numbers of cells at each point with standard deviation of three wells (□ noninfected cells, ◆ cells infected with AxCA-mock, ● cells infected with AxCA-hp16, ▲ cells infected with AxCA-hp21)

death. G1 arrest induced by staurosporine is cytostatic, but not cytocidal in normal cells [9] and staurosporine-induced G1 and G2/M arrest is reversible [1, 20]. We investigated cell viability using Trypan blue exclusion and by clonogenic assays to clarify whether staurosporine-induced G1 (low concentration) or G2/M arrest (high concentration) is cytostatic under our conditions with or without AxCA-hp16 or AxCA-hp21 infection.

Our results showed that cell death was induced by both low and high concentrations of staurosporine with or without Ax-hp16 or Ax-hp21 infection and regardless of whether cells accumulated in the G1 or G2/M phase. Moreover, cell viability in the presence of staurosporine was the same according to both the clonogenic assay and Trypan blue staining. Other glioma cell lines gave the same results (data not shown). Our previous study and those of others show that G1 arrest prevents sensitivity to cell death induced by anticancer drugs. However, forced G1 arrest does not prevent staurosporine-induced cell death. The mechanisms of cell death induced by DNA-damaging agents such as alkylating agents, and by PKC inhibitors such as staurosporine, are different. Cell death caused by DNA-damaging agents is induced at any phase after the G1-S checkpoint, while that caused by PKC inhibitors is induced specifically in the G1 and G2/M phases.

We investigated the nature of the cell death induced by staurosporine. Since staurosporine induces apoptosis, we investigated the expression of caspase-3 by Western blotting. Caspase-3 was cleaved after adding both low and high concentrations of staurosporine with or without AxCA-hp16 or AxCA-hp21 infection. These results showed that staurosporine induced apoptosis not only at G2/M arrest, but also at G1 arrest. Thus, we demonstrated that staurosporine causes apoptosis through two pathways, one in the G1 and the other in the G2/M phase.

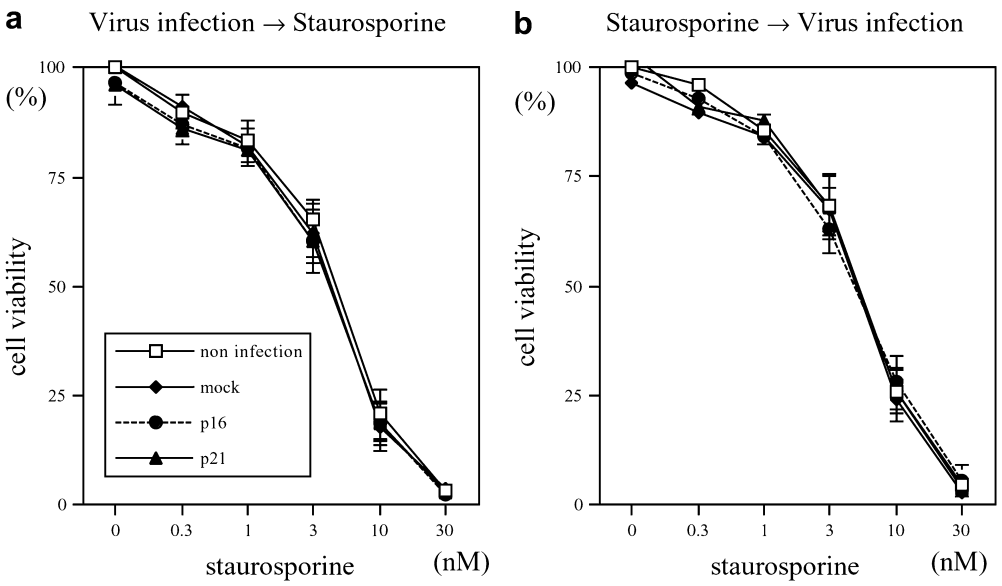


Fig. 5 Effect of administration of staurosporine on clonogenic inhibition in U251MG cells with or without *p16* or *p21* gene transfection 24 h before (*a*) or 6 h after (*b*) the addition of staurosporine. The results presented are the mean numbers of colonies with standard deviation of three dishes (□ noninfected cells, ◆ cells infected with AxCA-mock, ● cells infected with AxCA-hp16, ▲ cells infected with AxCA-hp21)

The role of cdc2 and pRb in initiating apoptosis remains controversial [10, 27, 39, 46, 47, 51]. Our results showed that apoptosis could arise when cdc2 activity is inhibited and/or pRb phosphorylation is prevented. We suggest that exhaustion of these factors is associated with cell-cycle arrest induced by staurosporine and that this finally induces apoptosis. Moreover, none of p16 or p21 transfection or p53 status was associated with apoptosis under our conditions, indicating the presence of p16-, p21- and p53-independent pathway(s). The apoptotic pathway and cell cycle arrest could not be caused by p53, p21, p16, pRb and cdc2, so a novel pathway remains to be discovered.

Our study has some limitations. First, the response to some agents such as staurosporine, that influence cell cycle regulation, may be affected by the genotypic features of the cell lines. Therefore, further studies are necessary using other glioma cell lines. Second, staurosporine inhibits many kinases including PKC. Other selective PKC

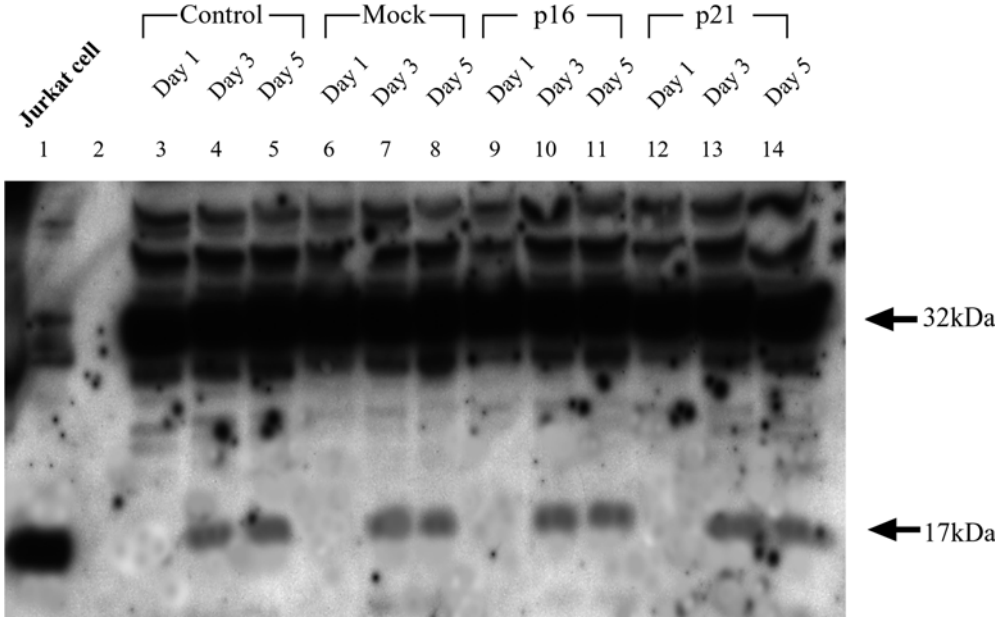


Fig. 6 Immunoblot analysis. Cellular protein (20 μg) extracted from cells under each condition was blotted against anti-caspase-3 antibody that recognizes both 32 kDa unprocessed pro-caspase-3 and the 17 kDa subunit of active caspase-3. Sample loading: *lane 1* positive control, Jurkat cell lysate; *lane 2* blank; *lanes 3–5* noninfected control U251MG cells, without staurosporine (*lane 3*), with 10 nM staurosporine (*lane 4*), with 30 n*M* staurosporine (*lane 5*); *lanes 6–8* AxCA-mock infection, without staurosporine (*lane 6*), with 10 n*M* staurosporine (*lane 7*), with 30 n*M* staurosporine (*lane 8*); *lanes 9–11* AxCA-hp16 infection, without staurosporine (*lane 9*), with 10 n*M* staurosporine (*lane 10*), with 30 n*M* staurosporine (*lane 11*); *lanes 12–14* AxCA-hp21 infection, without staurosporine (*lane 12*), with 10 n*M* staurosporine (*lane 13*) with 30 n*M* staurosporine (*lane 14*)

inhibitors should be used to examine the association between PKC inhibition and G0/1 cytotoxicity.

Low and high concentrations of staurosporine therefore regulated U251MG cells under G1 and G2/M arrest, respectively, and finally induced apoptosis via a caspase-3-activated pathway from both phases. However, pRb was dephosphorylated and cdc2 was inhibited at both low and high concentrations of staurosporine, indicating mechanisms of cell cycle regulation more complex than simple p53-Rb- or cdc2-dependent pathways. In conclusion, forced G1 arrest by transfection with *p16* or *p21* genes did not alter the rate of staurosporine-induced cell death, implying the existence of an unknown apoptotic pathway from the G1 phase.

Acknowledgements We acknowledge and thank Dr. T. Nobori for providing human full-length p16 cDNA and Dr. A. Noda for providing human full-length p21 cDNA.

References

1. Abe K, Yoshida M, Usui T, Horinouchi S, Beppu T (1991) Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp Cell Res 192:122
2. Baltuch GH, Couldwell WT, Villemure JG, Yong VW (1993) Protein kinase C inhibitors suppress cell growth in established and low-passage glioma cell lines. A comparison between staurosporine and tamoxifen. Neurosurgery 33:495
3. Baltuch GH, Dooley NP, Couldwell WT, Yong VW (1993) Staurosporine differentially inhibits glioma versus non-glioma cell lines. J Neurooncol 16:141
4. Belizario JE, Dinarello CA (1991) Interleukin 1, interleukin 6, tumor necrosis factor, and transforming growth factor beta increase cell resistance to tumor necrosis factor cytotoxicity by growth arrest in the G1 phase of the cell cycle. Cancer Res 51:2379
5. Bossy-Wetzel E, Newmeyer DD, Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 17:37
6. Bredel M, Pollack IF (1997) The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. Acta Neurochir (Wien) 139:1000
7. Bredel M, Pollack IF, Freund JM, Rusnak J, Lazo JS (1999) Protein kinase C inhibition by UCN-01 induces apoptosis in human glioma cells in a time-dependent fashion. J Neurooncol 41:9
8. Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z (1992) Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res 52:470
9. Chen X, Lowe M, Herliczek T, Hall MJ, Danes C, Lawrence DA, Keyomarsi K (2000) Protection of normal proliferating cells against chemotherapy by staurosporine-mediated, selective, and reversible G1 arrest. J Natl Cancer Inst 92:1999
10. Chin LS, Murray SF, Doherty PF, Singh SK (1999) K252a induces cell cycle arrest and apoptosis by inhibiting Cdc2 and Cdc25c. Cancer Invest 17:391
11. Couldwell WT, Hinton DR, Law RE (1994) Protein kinase C and growth regulation in malignant gliomas. Neurosurgery 35:1184
12. Crissman HA, Gadbois DM, Tobey RA, Bradbury EM (1991) Transformed mammalian cells are deficient in kinase-mediated control of progression through the G1 phase of the cell cycle. Proc Natl Acad Sci U S A 88:7580
13. DeAngelis LM (2001) Brain tumors. N Engl J Med 344:114
14. Donna A, Betta PG, Ribotta M, Maran E, Mazzucco G, Mollo F, Bellingeri D, Libener R (1992) Mitogenic effects of a mesothelial cell growth factor: evidence for a potential autocrine regulation of normal and malignant mesothelial cell proliferation. Int J Exp Pathol 73:193
15. Egan SE, Weinberg RA (1993) The pathway to signal achievement. Nature 365:781
16. Fallon RJ (1990) Staurosporine inhibits a tyrosine protein kinase in human hepatoma cell membranes. Biochem Biophys Res Commun 170:1191
17. Feng M, Cabrera G, Deshane J, Scanlon KJ, Curiel DT (1995) Neoplastic reversion accomplished by high-efficiency adenoviral-mediated delivery of an anti-ras ribozyme. Cancer Res 55:2024
18. Fueyo J, Gomez-Manzano C, Yung WK, Clayman GL, Liu TJ, Bruner J, Levin VA, Kyritsis AP (1996) Adenovirus-mediated p16/CDKN2 gene transfer induces growth arrest and modifies the transformed phenotype of glioma cells. Oncogene 12:103
19. Fujita-Yamaguchi Y, Kathuria S (1988) Characterization of receptor tyrosine-specific protein kinases by the use of inhibitors. Staurosporine is a 100-times more potent inhibitor of insulin receptor than IGF-I receptor. Biochem Biophys Res Commun 157:955
20. Gadbois DM, Hamaguchi JR, Swank RA, Bradbury EM (1992) Staurosporine is a potent inhibitor of p34cdc2 and p34cdc2-like kinases. Biochem Biophys Res Commun 184:80
21. Gescher A (1998) Analogs of staurosporine: potential anticancer drugs? Gen Pharmacol 31:721
22. Giangaspero F, Burger PC (1983) Correlations between cytologic composition and biologic behavior in the glioblastoma multiforme. A postmortem study of 50 cases. Cancer 52:2320
23. Gomez-Manzano C, Fueyo J, Kyritsis AP, Steck PA, Roth JA, McDonnell TJ, Steck KD, Levin VA, Yung WK (1996) Adenovirus-mediated transfer of the *p53* gene produces rapid and generalized death of human glioma cells via apoptosis. Cancer Res 56:694
24. Hama S, Sadatomo T, Yoshioka H, Kurisu K, Tahara E, Naruse I, Heike Y, Saijo N (1997) Transformation of human glioma cell lines with the p16 gene inhibits cell proliferation. Anticancer Res 17:1933
25. Hama S, Heike Y, Naruse I, Takahashi M, Yoshioka H, Arita K, Kurisu K, Goldman CK, Curiel DT, Saijo N (1998) Adenovirus-mediated p16 gene transfer prevents drug-induced cell death through G1 arrest in human glioma cells. Int J Cancer 77:47
26. He J, Allen JR, Collins VP, Allalunis-Turner MJ, Godbout R, Day RS 3rd, James CD (1994) CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma cell lines. Cancer Res 54:5804
27. Heald R, McLoughlin M, McKeon F (1993) Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. Cell 74:463
28. Kawabe S, Roth JA, Wilson DR, Meyn RE (2000) Adenovirus-mediated p16INK4a gene expression radiosensitizes non-small cell lung cancer cells in a p53-dependent manner. Oncogene 19:5359
29. Kawada M, Uehara Y, Mizuno S, Yamori T, Tsuruo T (1998) Up-regulation of p27Kip1 correlates inversely with anchorage-independent growth of human cancer cell lines. Jpn J Cancer Res 89:110
30. Kleihues P, Ohgaki H (1997) Genetics of glioma progression and the definition of primary and secondary glioblastoma. Brain Pathol 7:1131
31. Miller CR, Buchsbaum DJ, Reynolds PN, Douglas JT, Gillespie GY, Mayo MS, Raben D, Curiel DT (1998) Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. Cancer Res 58:5738

32. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C, Saito I (1996) Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc Natl Acad Sci U S A 93:1320
33. Niggli V, Keller H (1991) On the role of protein kinases in regulating neutrophil actin association with the cytoskeleton. J Biol Chem 266:7927
34. Nishi K, Schnier JB, Bradbury EM (1998) The accumulation of cyclin-dependent kinase inhibitor p27kip1 is a primary response to staurosporine and independent of G1 cell cycle arrest. Exp Cell Res 243:222
35. Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607
36. Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193
37. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA (1994) Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368:753
38. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res 211:90
39. Norbury C, MacFarlane M, Fearnhead H, Cohen GM (1994) Cdc2 activation is not required for thymocyte apoptosis. Biochem Biophys Res Commun 202:1400
40. Orr MS, Reinhold W, Yu L, Schreiber-Agus N, O'Connor PM (1998) An important role for the retinoblastoma protein in staurosporine-induced G1 arrest in murine embryonic fibroblasts. J Biol Chem 273:3803
41. Pollack IF, Randall MS, Kristofik MP, Kelly RH, Selker RG, Vertosick FT Jr (1990) Response of malignant glioma cell lines to activation and inhibition of protein kinase C-mediated pathways. J Neurosurg 73:98
42. Qiao L, Koutsos M, Tsai LL, Kozoni V, Guzman J, Shiff SJ, Rigas B (1996) Staurosporine inhibits the proliferation, alters the cell cycle distribution and induces apoptosis in HT-29 human colon adenocarcinoma cells. Cancer Lett 107:83
43. Rialet V, Meijer L (1991) A new screening test for antimitotic compounds using the universal M phase-specific protein kinase p34 cdc2/cyclin B cdc13, affinity-immobilized on $p13^{SUC1}$-coated microtitration plates. Anticancer Res 11:1581
44. Schnier JB, Nishi K, Goodrich DW, Bradbury EM (1996) G1 arrest and down-regulation of cyclin E/cyclin-dependent kinase 2 by the protein kinase inhibitor staurosporine are dependent on the retinoblastoma protein in the bladder carcinoma cell line 5637. Proc Natl Acad Sci U S A 93:5941
45. Sedlak J, Hunakova L, Sulikova M, Chorvath B (1997) Protein kinase inhibitor-induced alterations of drug uptake, cell cycle and surface antigen expression in human multi-drug-resistant (Pgp and MRP) promyelocytic leukemia HL-60 cells. Leuk Res 21:449
46. Shan B, Lee WH (1994) Deregulated expression of E2F1 induces S-phase entry and leads to apoptosis. Mol Cell Biol 14:8166
47. Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW, Greenberg AH (1994) Premature p34cdc2 activation required for apoptosis. Science 263:1143
48. Tam SW, Schlegel R (1992) Staurosporine overrides checkpoints for mitotic onset in BHK cells. Cell Growth Differ 3:811
49. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phospholipid/Ca++ dependent protein kinase. Biochem Biophys Res Commun 135:397
50. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81:323
51. Wu X, Levine AJ (1994) p53 and E2F1 cooperate to mediate apoptosis. Proc Natl Acad Sci U S A 91:3602
52. Yokozaki H (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 50:767