

Paradoxical elevation of Ki-67 labeling with protein kinase inhibition in malignant gliomas

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✓ The monoclonal antibody Ki-67 recognizes a nuclear antigen expressed in the G₁, S, G₂, and M phase of the cell cycle and has been used extensively as an indicator of cellular proliferation in malignant gliomas, both in the laboratory and clinically. Recently, protein kinase C (PKC) inhibitors have been demonstrated to inhibit malignant glioma growth both in *in vitro* and *in vivo*. This study was undertaken to determine whether Ki-67 could function as an indicator of cellular proliferation rate after PKC inhibition in gliomas and to explore cell cycle specificity of such inhibition. Both established and low-passage malignant glioma cell lines have previously been shown to be sensitive to growth inhibition by the PKC inhibitors staurosporine and tamoxifen *in vitro* (IC₅₀ in the nanomolar and micromolar ranges, respectively), as measured by cell numbers, [³H]thymidine uptake, and flow-cytometric DNA analysis. However, in the same cells that are inhibited by staurosporine and tamoxifen on these assays, and on the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay in the present study, the Ki-67 labeling index paradoxically increased in a dose-related manner with the same treatments, as measured by immunohistochemistry and confirmed by flow cytometry. For example, in established line U-87, a 20.5% decrease in thymidine uptake and a 28.5% decrease in absorbance on the MTT assay produced by tamoxifen at 1 μM was associated with an increase in Ki-67 labeling from 42% to 62%; staurosporine, which produces a 78.8% decrease in thymidine uptake in cell line A-172 at 10 nM, produced an increase in Ki-67 labeling from 19% to 32%. In this regard, Ki-67 labeling of glioblastoma tissue from a patient treated with high-dose tamoxifen yielded results within the range of 10% to 15% (consistent with values seen in untreated glioblastoma), despite tumor regression with treatment. The authors' interpretation of these results is that these PKC inhibitors are halting the cell cycle in the G₁ phase or the G₁-S transition (beyond G₀ but before S-phase), resulting in a paradoxical increase in labeling while arresting growth. Two important implications from these observations are that Ki-67 is not a reliable indicator of cellular proliferation after treatment with PKC inhibitors and that these inhibitors used at the doses given above halt cell growth in a phase-specific manner.

KEY WORDS • brain neoplasm • chemotherapy • glioma • monoclonal antibody • Ki-67 • protein kinase C • tamoxifen

MALIGNANT gliomas possess very high protein kinase C (PKC) activity when compared to non-transformed glia, and PKC activity is strongly correlated to the growth rates of these tumor cells *in vitro*.³⁻⁵ Surgically resected frozen human glioma specimens also display elevated PKC activity within the range measured in established glioma lines.⁴ This elevated PKC activity may function as a target for inhibition of glioma growth *in vitro*,^{1,3-5,14} and is currently being evaluated with some success in clinical trials for use as a chemotherapeutic target *in vivo*.^{2,6,18}

The monoclonal antibody Ki-67 recognizes a nuclear antigen expressed in the G₁, S, G₂, and M phases of the cell cycle.^{9,11,13,15-17,19} It has been used extensively in recent years both as a laboratory and a clinical index of tumor proliferation. The present study was undertaken to evalu-

ate the accuracy of Ki-67 as an indicator of tumor proliferation following treatment with PKC inhibitors and to explore the cell-cycle specificity of the growth inhibition produced by these agents. The authors demonstrate that the use of Ki-67 following PKC inhibition is an unreliable indicator of glioma proliferation *in vitro* and *in vivo*. Clinical evaluation of tumor kinetics following treatment with PKC inhibitors may require the use of alternative measures of proliferation.

Materials and Methods

Glioma Cell Cultures

The previously characterized human malignant glioma cell lines A-172 and U-87 were used for the purposes of

this study.* Cell lines were maintained in 25-cm² tissue culture flasks, in medium consisting of Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS), gentamicin (20 µg/ml), glutamine, pyruvate (1 mM), dextrose (0.1%) and essential amino acids, buffered to a pH of 7.0.† Cells were grown at 37°C in a humidified 5% CO₂ incubator. All cell lines were routinely tested for mycoplasma contamination and passaged regularly.

In Vitro Glioma Proliferation Assays

[³H]Thymidine Uptake. Our method for determining rates of glioma proliferation has been previously reported.^{3,4} Following passage, each cell line was seeded at a density of 10⁴ cells in 40 µl of medium on 12-mm glass coverslips placed in 24-well culture plates. Coverslips were previously coated with 10 µg/ml poly-L-lysine to facilitate cell adherence. Twelve hours later, after adequate time for cells to adhere to the coverslips, the wells were flooded and washed with phosphate-buffered saline (PBS). After addition of 1 ml feeding medium (containing 10% FCS) to each well, each PKC inhibitor was then added to the wells at predetermined concentration in triplicate. Following a 48-hour treatment period, wells were pulsed with 1 µCi/ml [³H]thymidine for a period of 6 hours prior to harvest. To measure thymidine uptake, the coverslips with adherent cells were washed four times with PBS and placed in scintillation vials containing 5 ml scintillation fluid‡ for determination in a β-counter. It has been previously demonstrated that uptake of [³H]thymidine into cells by this technique is a reliable index of deoxyribonucleic acid (DNA) synthesis.¹

MTT Assay. As an additional method of measuring cellular proliferation, a colorimetric assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was performed following the addition of the PKC inhibitors. Glioma cells were seeded at a density of 2 × 10³ cells/well in 100 µl feeding medium in flat-bottomed 96-well plates. Following adherence in 100 µl medium, the test agents were added in predetermined concentrations, in quadruplicate, to achieve a total volume of 200 µl/well. After incubation for 48 hours as above, 20 µl MTT prepared at a concentration of 5 mg/ml in PBS was added to each well for 6 hours. The supernatant was decanted, and the MTT formazan precipitate was solubilized by addition of 150 µl 100% dimethyl sulfoxide and placed on a plate shaker for 10 minutes.§ Absorbance at 570 nm was determined on a spectrophotometric microplate reader.||

Staining With Ki-67. The Ki-67 staining was quantitated immunohistochemically and by flow cytometry. For microscopic immunostaining of glioma cell lines, cells

were seeded and grown on coverslips, treated for 48 hours with the PKC-inhibiting agents, and fixed with acetone for 5 minutes at room temperature. Following rehydration with PBS, the Ki-67 antibody* (diluted 1:30) was added to cover the cells for 2 hours at room temperature. Following three washes with PBS, anti-mouse biotinylated antibody† (1:10 dilution in PBS) was added at room temperature and allowed to incubate for 20 minutes. Following three washes in PBS, streptavidin-peroxidase reagent (1:20 dilution in PBS) was added to the coverslips and incubated for 20 minutes. After an additional wash, aminoethyl carbazole chromogen solution was added.‡ (All reagents except for the primary antibody were obtained in kit form and were diluted from the stock solution as recommended by the manufacturer.) The coverslips were then washed in PBS, counterstained with hematoxylin solution (10 minutes), and mounted for viewing.

For flow cytometric staining, cell lines were treated for 48 hours with the agents as detailed above. Then the cells were removed using gentle trypsinization (0.05% for 10 minutes) and washed three times in PBS after inactivation of the trypsin with FCS. Cells were fixed for a period of 1 hour in a solution of 4% paraformaldehyde at 4°C. Following three washes in PBS, immunocytochemical Ki-67 staining was performed in 1.8-ml Eppendorf tubes using 30 µl primary antibody for 1 hour (mouse anti-Ki-67; volume to achieve saturation of antigen)§ followed with equivalent volumes of anti-mouse secondary antibody conjugated with fluorescein isothiocyanate for 1 hour. Each stain was followed by three washes with PBS. The samples were analyzed with the flow cytometer to quantitate the amount of immunofluorescence specifically related to Ki-67 staining. Gating for acquisition of single cells was performed, and 10⁴ events were acquired from each culture for analysis.

The Ki-67 antigen staining of glioma tissue obtained from a patient treated with high-dose tamoxifen was performed using MIB-1 antibody.|| Five 1-µm paraffin sections were deparaffinized and stained as previously described¹⁰ using the avidin-biotin complex method and an automated immunostaining device* after microwave treatment of tissue sections in antigen retrieval solution.†

DNA Cytofluorometry of Glioma Cultures. To confirm the results of the [³H]thymidine incorporation, DNA cell-cycle analysis was performed by propidium iodide staining with analysis by flow cytometry. Cells were initially plated following passage at a density of 5 × 10⁵ cells per 25-cm² tissue culture flask, in 5 ml glioma feeding medium described above (containing 10% FCS). After ade-

* Malignant glioma cell lines A-172 and U-87 obtained from American Type Culture Collection, Rockville, Maryland.

† Medium constituents obtained from GIBCO, Grand Island, New York.

‡ Cytoscent scintillation fluid obtained from ICN Chemical, Irvine, California.

§ MTT and dimethyl sulfoxide obtained from Sigma Chemical Co., St. Louis, Missouri.

|| Spectrophotometric microplate reader, Model MR700, manufactured by Dynatech, Chantilly, Virginia.

* Monoclonal antibody Ki-67 obtained from Dakopatts, Glostrup, Denmark.

† Anti-mouse biotinylated antibody obtained from Biogenex Laboratory, San Ramon, California.

‡ Streptavidin-peroxidase reagent and aminoethyl carbazole chromogen solution obtained from Zymed, South San Francisco, California.

§ Mouse anti-Ki-67 antibody obtained from Dakopatts, Glostrup, Denmark.

|| MIB-1 antibody obtained from AMAC, Westbrook, Maine.

* Automated immunostaining device, Model Techmate 1000, obtained from Techmate Inc., Goleta, California.

† Antigen retrieval solution obtained from Biogenex, San Ramon, California.

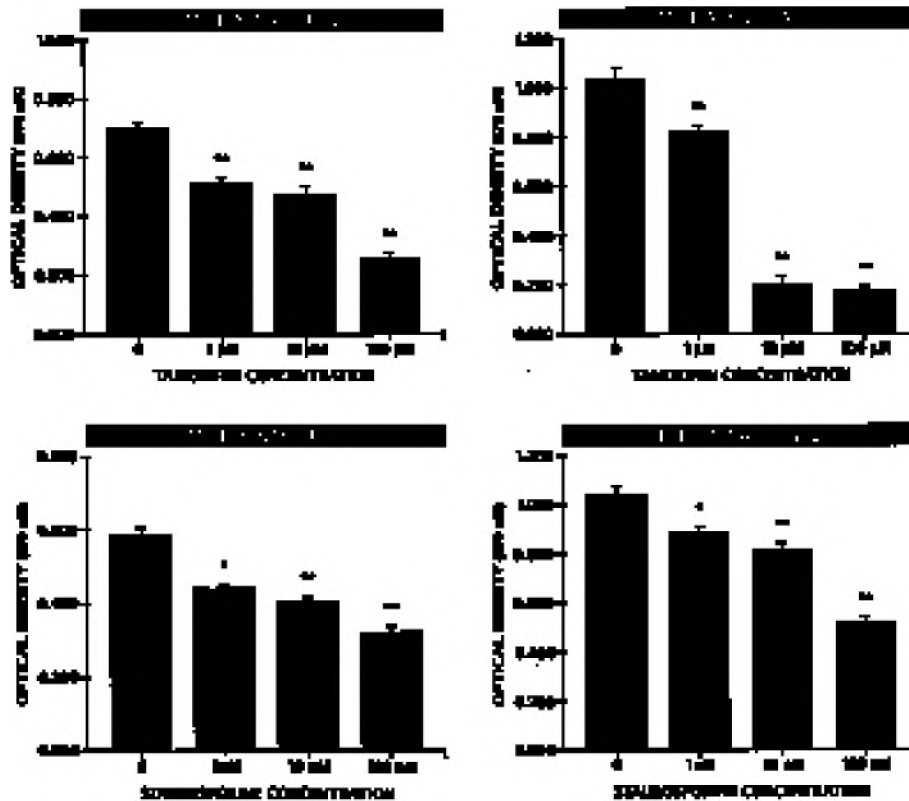


FIG. 1. Graphs showing growth inhibition of glioma cell lines U-87 and A-172 by the protein kinase C (PKC) inhibitors staurosporine and tamoxifen, using [³H]thymidine assay. The PKC inhibitors reduced [³H]thymidine uptake in a dose-dependent manner in both cell lines. Values for glioma uptake of [³H]thymidine represent counts per minute and are the mean ± standard error of the mean for triplicate wells. * = p < 0.05, ** = p < 0.01 (one-way analysis of variance, Duncan's multiple comparison).

quate time for cell adherence (12 hours), the medium was changed and fresh medium containing the PKC inhibitors in the concentrations outlined below was added. The cultures were maintained for a period of 48 hours, after which the cells were trypsinized, washed three times in PBS, and fixed by resuspension (10⁶ cells/ml) in a solution of PBS and ice-cold methanol (1:2 final concentration, vortexing the cells to avoid clumping) for a period of 1 hour at 4°C. The staining protocol is adapted from that of Hurley.‡ After fixation, cells were centrifuged, supernatant was decanted, and 0.5 ml propidium iodide stain solution was added. This stain solution contained 10 mg propidium iodide, 0.1 ml Triton X-100, and 3.7 mg ethylenediamine tetraacetic acid in 100 ml PBS. Immediately following addition of the stain solution, 0.5 ml ribonuclease solution (10 mg ribonuclease mixed with 5 ml PBS and heated to 75°C for 30 minutes before use) was added to eliminate double-stranded ribonucleic acid that would interfere with DNA quantification.§ Samples were analyzed on a fluorescent-activated cell sorter|| after a 1-hour staining period

‡ Preparation of alcohol-fixed whole cells from suspensions for DNA analysis. Becton Dickinson Source Book, Section 1.11, 1989.

§ Propidium iodide obtained from Calbiochem, San Diego, California; other reagents obtained from Sigma Chemical Co., St. Louis, Missouri.

|| FACSscan fluorescent activated cell sorter manufactured by Becton Dickinson, San Jose, California.

at 37°C in the dark. Ten thousand events were acquired for analysis.

Results

Glioma Cell Line Proliferation Assays

As has been demonstrated in previous reports,^{1,4,5} [³H]thymidine incorporation into the established glioma cell lines U-87 and A-172 decreased in a dose-related manner after the addition of staurosporine or tamoxifen for a 48-hour period (Fig. 1). Similarly, by MTT assay these cell lines exhibited decreased absorbance (correlating with cell numbers) following the addition of these agents (Fig. 2). The MTT assay demonstrated a linear relationship between cell counts and optical density (Fig. 3).

Staining With Ki-67

Under treatment conditions identical to those described in the growth assays above, staurosporine and tamoxifen were added to cultures of the U-87 and A-172 glioma lines in logarithmic phase growth in medium containing 10% FCS. In contrast to the assay results given above for [³H]thymidine and MTT, Ki-67 staining increased with the treatments (Fig. 4). Demonstrative photomicrographs of cell line U-87 stained with Ki-67 are presented in Fig. 5. The relative amount of Ki-67 staining among a popula-

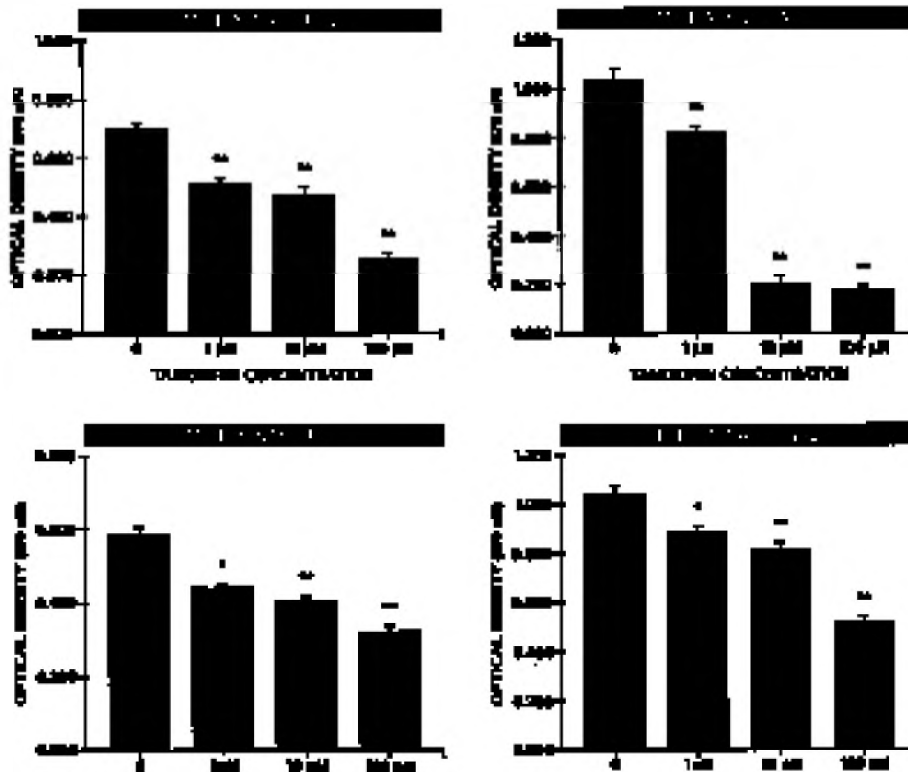


FIG. 2. Graphs showing growth inhibition of glioma cell lines U-87 and A-172 by the protein kinase C (PKC) inhibitors staurosporine and tamoxifen, using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The PKC inhibitors decreased optical absorbance in a dose-dependent manner in both cell lines. Optical density values represent the mean \pm standard error of the mean for quadruplicate wells. * = $p < 0.05$, ** = $p < 0.01$ (one-way analysis of variance, Duncan's multiple comparison).

tion of A-172 cells was compared by flow cytometry (Fig. 6); similar results were obtained, demonstrating an increase in relative Ki-67 staining among the cultures treated with the PKC inhibitors.

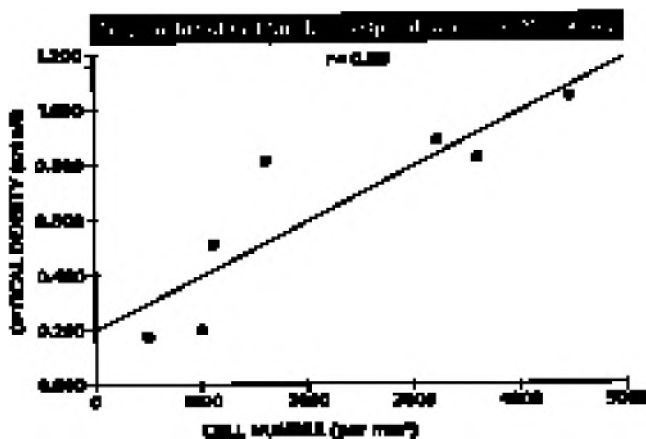


FIG. 3. Graph illustrating standardization of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay and demonstrating the linear relationship between cell counts and optical density as measured by this technique.

DNA Flow Cytometry of Glioma Cultures

Figure 7 shows DNA profiles of glioma line U-87 grown in medium containing 10% FCS, either alone or in the presence of PKC inhibitors for 48 hours. In accordance with the [³H]thymidine assay results, tamoxifen and staurosporine produced a dose-dependent decrease in the DNA synthesis phase (S-phase) of the tumor compared to the untreated control, indicating that the arrest of the cycle was preferentially prior to entrance into the S-phase, as previously described.⁵ At very high doses of staurosporine (100 nM), an increase was also noted in the G₂/M peak (Fig. 7 upper right).

Increased Ki-67 Staining in Glioma Tissue Treated With High-Dose Tamoxifen

Figure 8 demonstrates Ki-67 antigen staining of glioblastoma tissue resected from a patient treated with high-dose tamoxifen. Note the high index of Ki-67 staining in this tumor (consistent with values seen in untreated glioblastoma^{16,17,19}). The patient has demonstrated a dramatic clinical response to treatment, having survived 36 months thus far on tamoxifen therapy (100 mg twice daily) with continuing tumor regression.⁶

Discussion

The PKC signal transduction system activity is overex-

Elevation of Ki-67 labeling with PKC inhibition in gliomas

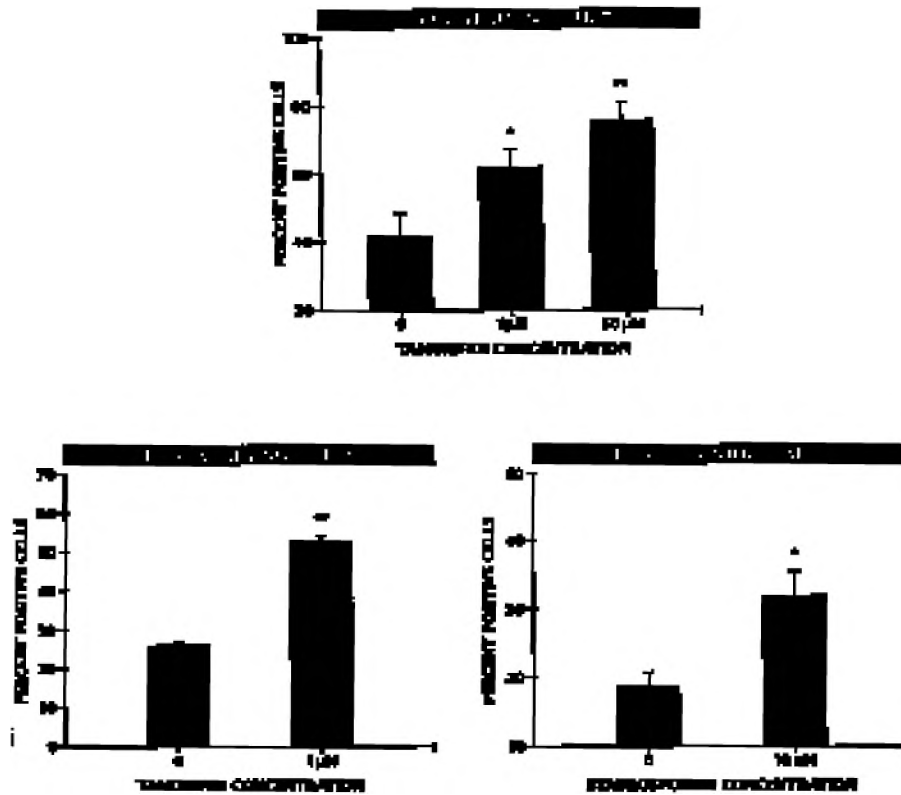


FIG. 4. Graphs showing amount of immunohistochemical staining for Ki-67 antigen in glioma lines U-87 and A-172. In contrast with the results of the [³H]thymidine and MTT assays (Figs. 1 and 2), the number of cells that stained positive for the Ki-67 antigen increased in a dose-related manner with tamoxifen treatment of glioma line U-87 (*upper*) and similarly increased with both tamoxifen and staurosporine treatment of glioma line A-172 (*lower*). Values represent the mean of three separate microscopic fields, with a total of 1000 cells counted per treatment group. All counts were performed blind on coded specimens. * = $p < 0.05$, ** = $p < 0.01$ (one-way analysis of variance, Duncan's multiple comparison).

pressed in gliomas in comparison to nontransformed glia and correlates strongly with proliferation rates of gliomas *in vitro*.³⁻⁵ Tamoxifen, in a property distinct from estrogen receptor blockade, inhibits PKC in established and some

low-passage glioma cell lines in the low-micromolar concentration range.¹ Although not a particularly potent inhibitor of PKC, tamoxifen is currently being investigated for therapeutic efficacy in patients harboring recurrent

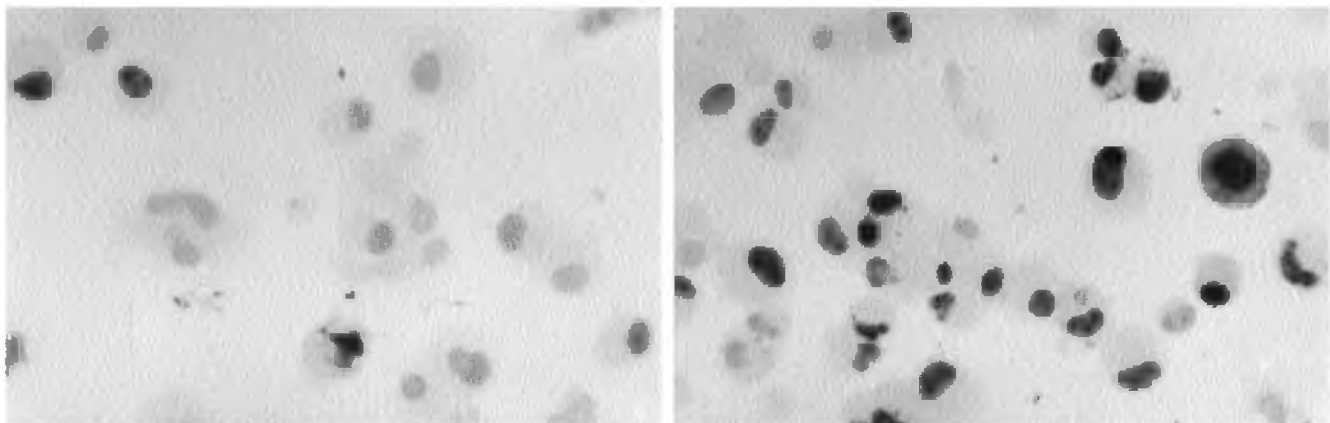


FIG. 5. Photomicrographs of cell line U-87 showing immunohistochemical staining for Ki-67 antigen. The control culture (*left*) demonstrates few stained nuclei, whereas administration of staurosporine (10 nM) increased the staining markedly (*right*). Hematoxylin, original magnification $\times 200$.

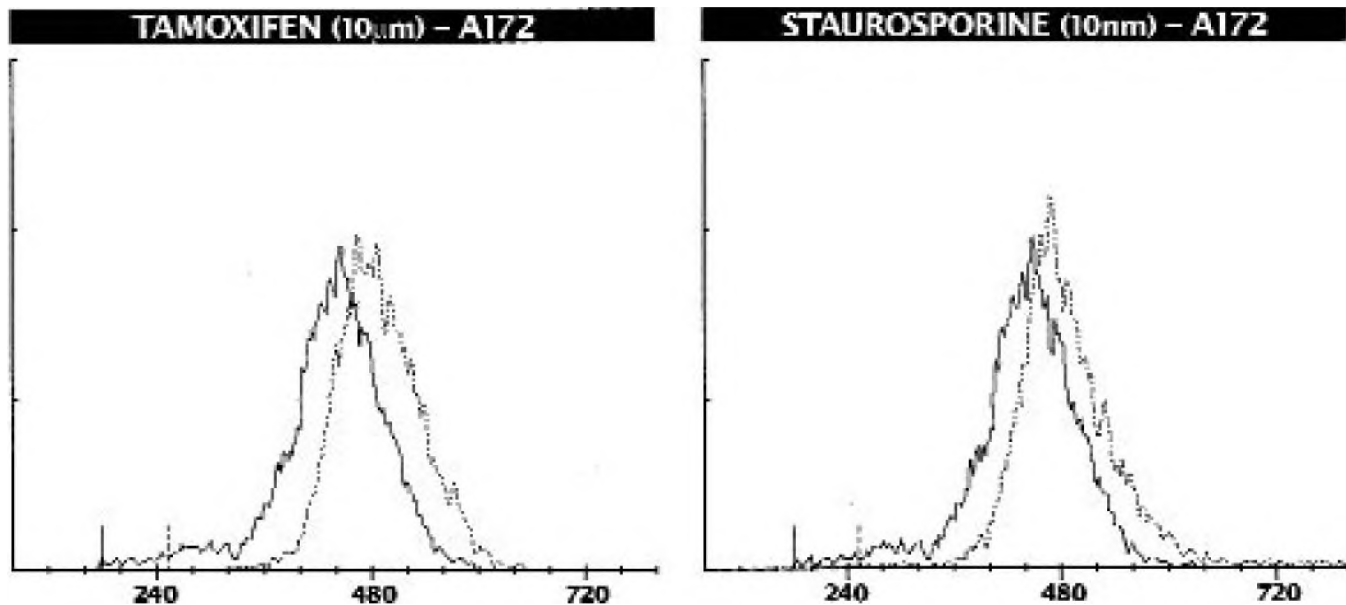


FIG. 6. Overlay histogram plots demonstrating cytofluorometric analysis of Ki-67 staining. X-axis = logarithmic fluorescence units. *Solid lines* represent A-172 control culture and *dotted lines* 48 hours of incubation with either 10 μ M tamoxifen (*left*) or 10 nM staurosporine (*right*). Both plots demonstrate a shift in fluorescent intensity related to increased Ki-67 staining.

malignant gliomas.^{2,6,18} The potential for this type of therapy has engendered clinical trials utilizing more potent and specific inhibitors of PKC for patients harboring malignant gliomas.

In the present study, the relatively specific PKC inhibitor staurosporine and the less selective inhibitor tamoxifen both inhibited the growth of malignant glioma cell lines in a dose-related manner, as has been noted in previous reports.^{1,5} In contrast, both agents paradoxically increased Ki-67 labeling in the glioma cell lines within the same concentration range that produced growth inhibition as measured by cell counting, [³H]thymidine uptake, and MTT assay. Although PKC inhibition is a likely candidate for the mechanism of this growth inhibition and is consistent with the concentrations necessary to inhibit the enzyme in these cells,^{1,3,4} we cannot exclude the possibility of other kinase inhibition or other pleiotropic effects of these agents that might contribute to the phase-specific growth arrest produced by these agents in this study.

The monoclonal antibody Ki-67 recognizes a nuclear antigen expressed in the G₁, S, G₂, and M phases of the cell cycle.^{9,11,15,18} This antigen has recently been cloned and sequenced, and is a high-molecular-weight short-lived nonhistone nuclear protein, which may be an absolute requirement for maintaining cell proliferation.¹⁵ In the present study, measures of glioma cell proliferation using [³H]thymidine uptake, MTT assay, and cytofluorometric DNA analysis yielded consistent results, in that the PKC inhibitors produced an inhibition of cell growth. Paradoxically, under identical conditions, the Ki-67 index increased after these treatments; these apparently diametric results may be rationalized by examination of the DNA cytofluorometric results, which indicate phase-specific cell growth arrest following these treatments (Fig. 7). The

DNA histograms indicate that cell growth is arrested prior to initiation of the S-phase,¹² in the G₀/G₁ peak, producing a reduction in the proliferation index (defined as %S + %G₂/M) as noted previously.⁵ As the antigen recognized by Ki-67 is expressed in the G₁ phase, in addition to the S, G₂, and M phases,^{15,16} the staining would actually increase as a result of growth arrest in this phase. This interpretation is supported by an increased population of lightly stained cells in the staurosporine- and tamoxifen-treated cells in the present study (Fig. 5) indicative of growth arrest in the early cell cycle. In this regard, Crissman, *et al.*⁷ have also recently noted G₁-phase growth arrest of nontransformed cells by staurosporine. In a subsequent study,⁸ the same group reported that staurosporine may induce a G₂ block in a mouse mammary carcinoma line when used in higher concentrations, presumably mediated by blocking of p34^{cdc2} and p34^{cdc2}-like kinase activity.⁸ This nonspecific kinase inhibition at higher concentrations may also explain the increase in the number of cells arrested in the G₁/M peak noted in the present study when the glioma cells were treated with 100 nM staurosporine (Fig. 7). It is noteworthy that blockage of the cells at either of these points in the cell cycle could paradoxically increase Ki-67 staining, although this assay was not performed by Crissman and colleagues. Shiraishi,¹⁶ using an immunoelectron microscopic analysis of Ki-67 staining, has noted staining patterns that were phase-specific (starting with early G₁ phase staining in the nucleoplasm); this work indicated that the antigen recognized by the antibody may be related to the processing and assembly of preribosomal particles.

In conclusion, the data from the present study underline a potential pitfall with the use of a single labeling study for determining the proliferation rate of malignant gliomas *in*

Elevation of Ki-67 labeling with PKC inhibition in gliomas

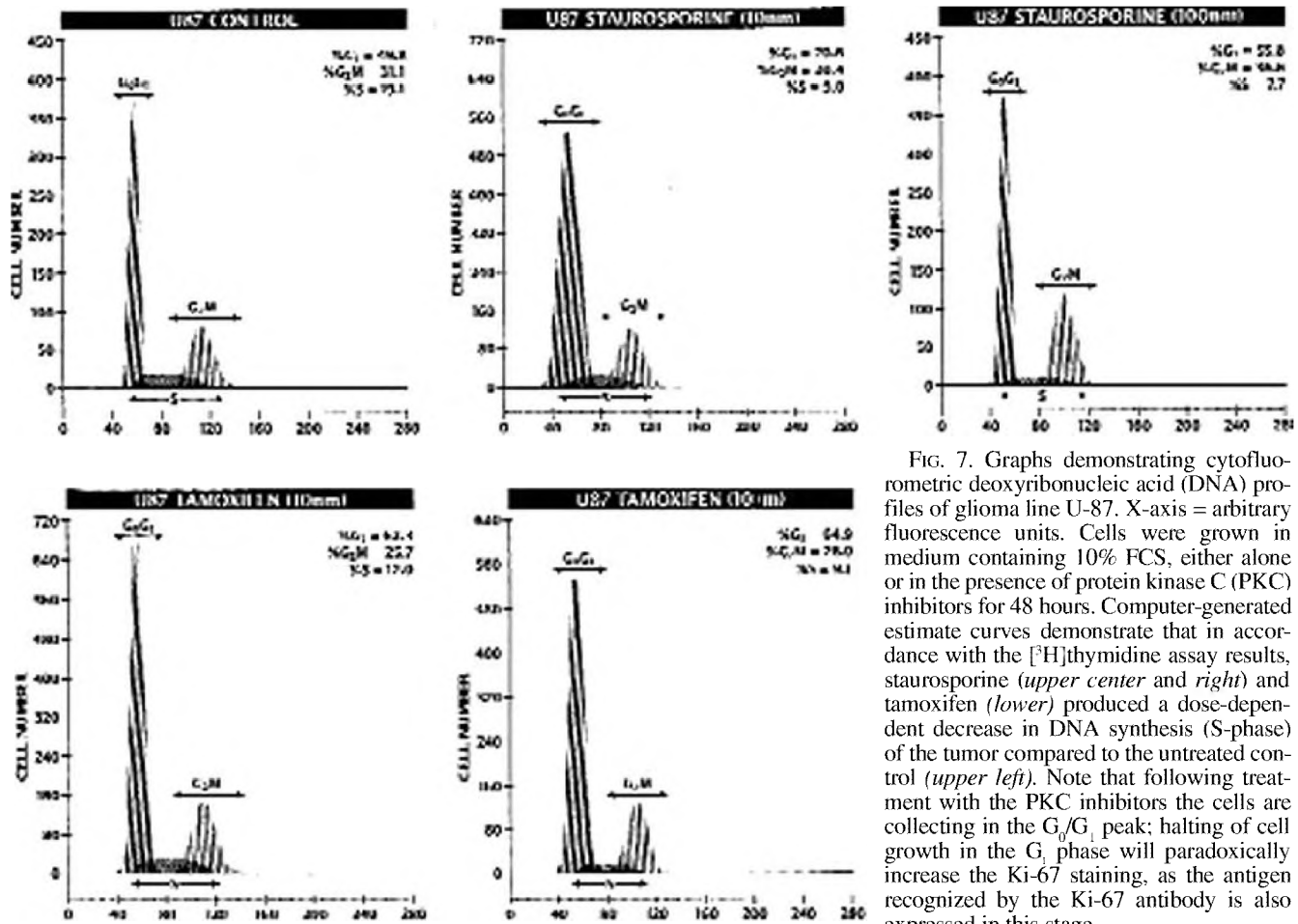


FIG. 7. Graphs demonstrating cytofluorometric deoxyribonucleic acid (DNA) profiles of glioma line U-87. X-axis = arbitrary fluorescence units. Cells were grown in medium containing 10% FCS, either alone or in the presence of protein kinase C (PKC) inhibitors for 48 hours. Computer-generated estimate curves demonstrate that in accordance with the [³H]thymidine assay results, staurosporine (*upper center and right*) and tamoxifen (*lower*) produced a dose-dependent decrease in DNA synthesis (S-phase) of the tumor compared to the untreated control (*upper left*). Note that following treatment with the PKC inhibitors the cells are collecting in the G₀/G₁ peak; halting of cell growth in the G₁ phase will paradoxically increase the Ki-67 staining, as the antigen recognized by the Ki-67 antibody is also expressed in this stage.

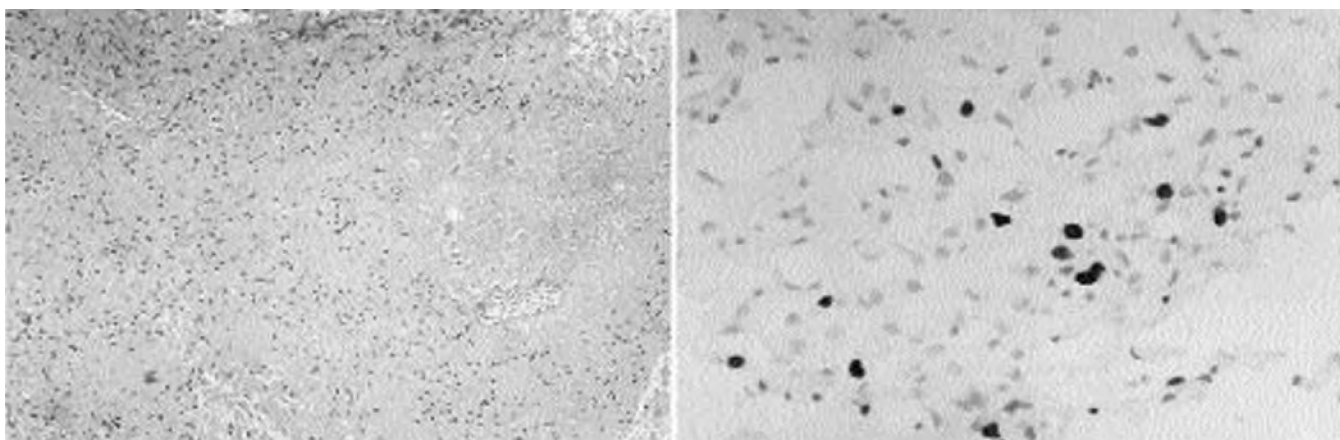


FIG. 8. Photomicrograph showing Ki-67 antigen staining of a recurrent astrocytoma in a patient treated with high-dose tamoxifen. The original biopsy from this patient revealed glioblastoma multiforme (Grade IV astrocytoma). H & E. *Left:* The current biopsy reveals a predominantly gemistocytic malignant astrocytoma with necrosis. Original magnification $\times 16$. *Right:* The five most proliferative fields were chosen to avoid areas of necrosis. All tumor cell nuclei were counted (a minimum of 1000 cells) and the percentage of positively stained nuclei determined. Immunoperoxidase stain for Ki-67 using MIB-1 shows that 10% to 15% of cells are immunoreactive despite dramatic tumor regression on tamoxifen therapy. Original magnification $\times 160$.

vitro. Moreover, clinical use of Ki-67 staining from *in vivo* surgical specimens must be interpreted with caution following treatment with kinase inhibitors, given the high labeling index demonstrated in the patient in this report in whom a dramatic clinical response has been obtained. In consideration of the Ki-67 staining results, together with the DNA cytometric analysis following treatment with PKC inhibitors, it appears that the inhibitors slowed tumor growth in a phase-specific manner.

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