Protein Kinase C Inhibitors Suppress Cell Growth in Established and Low-Passage Glioma Cell Lines. A Comparison between Staurosporine and Tamoxifen

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WE HAVE PREVIOUSLY demonstrated that the proliferation of established human glioma cell lines correlated with protein kinase C (PKC) activity and that a relatively selective PKC inhibitor, staurosporine, inhibits glioma cell proliferation. The purpose of this study was to determine whether low-passage glioma cell lines were also sensitive to staurosporine and to compare the antimitotic effects of staurosporine with tamoxifen, an antiestrogen with a known PKC inhibitory effect presently being investigated in the treatment of recurrent glioma. We measured the effects of treatment with staurosporine or tamoxifen on the proliferation rate of five established glioma cell lines (A172, U251, U87, U373, U563) and four low-passage glioma cell lines. The proliferation of all cell lines was inhibited by staurosporine, at an IC_{50} value (concentration at which activity is 50% inhibited) of approximately 2 nmol/L. All established lines, but only one low-passage line, were susceptible to tamoxifen, with an IC_{50} value of 10 μmol/L. Three of the four low-passage lines were poorly inhibited by tamoxifen. The IC_{50} values for the inhibition of cellular proliferation by staurosporine and tamoxifen closely corresponds to the IC_{50} data for the inhibition of particulate PKC activity in gliomas. We conclude that staurosporine is more effective in the inhibition of glioma proliferation than tamoxifen and that staurosporine is potentially useful in the adjuvant treatment of gliomas. The correspondence in IC_{50} results for proliferation and PKC activity further strengthens the hypothesis that an aberrant PKC system in gliomas drives their hyperproliferative state. (Neurosurgery 33:495–501, 1993)

Key words: Brain neoplasm, Glioma cell lines, Protein kinase C, Staurosporine, Tamoxifen

Previous studies in this laboratory have demonstrated that 1) established glioma cell lines have very high protein kinase C (PKC) activity when compared with non-malignant adult human glia; 2) the proliferation rate of glioma cell lines is highly correlated with their particulate and total PKC activity ($r = 0.94$ and 0.98, respectively); and 3) the proliferation of the A172 glioma cell line is susceptible to inhibition by the relatively selective PKC inhibitor staurosporine (8, 9, 11). These observations have led us to postulate that an aberrant PKC system in gliomas drives their intrinsic hyperproliferative state and that PKC inhibitors may be useful as adjuvant therapy for malignant gliomas. Indeed, the intratumoral administration of staurosporine in rats harboring the C6 glioma have significantly extended survival rates and increased general motor activity, when compared with sham controls (10). A currently used chemotherapeutic agent with known PKC inhibitory action (14, 20, 25) is tamoxifen, an antiestrogenic agent widely used in the adjuvant treatment of breast carcinoma (7, 13). Pollack et al. (22) have demonstrated that tamoxifen can decrease the proliferation of established as well as low-passage glioma cell lines. Those authors proposed that the decrease in cellular proliferation was secondary to PKC inhibition, although PKC enzyme activity was not assessed. Vertosick et al. (29) have observed increased survival after treatment with tamoxifen in patients with recurrent glioma. We have recently reported that high-dose tamoxifen therapy in a patient with recurrent glioblastoma produced tumor regression and clinical stabilization (2).

In view of the reports that the IC_{50} (concentration at which activity is 50% inhibited) of tamoxifen for PKC inhibition was

in the micromolar range (20), whereas the equivalent IC_{50} for staurosporine was in the nanomolar range (15, 26), we have directly compared the efficacy of staurosporine with that of tamoxifen on the cellular proliferation of five established glioma cell lines. In order to account for the highly clonal status of established cell lines, which might not be truly representative of the cellularly heterogeneous glioblastoma multiforme, four low-passage glioma cell lines were used as a closer approximation of the in vivo state. The results demonstrate that on a molar basis, staurosporine is 3 orders of magnitude more effective than tamoxifen in inhibiting cellular proliferation and PKC activity. In addition, although the proliferation of all of the low-passage cell lines was susceptible to staurosporine, three of four low-passage lines were not significantly altered by tamoxifen. These results suggest that staurosporine (21) is potentially more useful than tamoxifen in the adjuvant treatment of patients with gliomas.

MATERIALS AND METHODS

Cell lines

The established glioma cell lines were A172, U87-MG, U251-MG, U563-MG, and U373-MG. These are well-characterized, long-term lines courtesy of V. P. Collins (Ludwig Institute for Cancer Research, Stockholm, Sweden) (6). Cells were of a relatively homogenous population, as determined by morphological criterion, presumably as the result of clonal selection with repeated passages.

Fresh glioblastoma specimens were obtained from the operating room and were histologically confirmed by a neuropathologist. Tumor specimens were minced into small pieces of 1 mm^3 or less and were allowed to adhere and grow on a culture dish coated with 10 μg of poly-l-lysine per milliliter. Cell lines were passaged at confluency via gentle trypsinization and were used for study on the second or third passage. Glial fibrillary acidic protein immunohistochemistry was done on all of the low-passage lines. All contained cells positive for glial fibrillary acidic protein.

The cell lines were maintained in feeding medium consisting of 10% fetal bovine serum; gentamicin, 20 μg/ml; glutamine, 1 mmol/L; pyruvate, 1 mmol/L; 0.1% dextrose; and essential amino acids buffered to pH 7.4 (all medium constituents were purchased from GIBCO, Grand Island, New York). The cell lines were fed twice a week and passaged when confluent by gentle trypsinization (0.05%).

Assessment of cell proliferation

The assessment of cell proliferation was performed by two techniques: [^{3}H]thymidine incorporation and counting of cell numbers. To assess [^{3}H]thymidine incorporation, cells were seeded onto 12-mm glass coverslips at a density of 10,000 per coverslip; plating was considered as Day 1 of the experiment. Six hours later, after cells had adequately adhered, the coverslips were flooded with the medium described above. Each coverslip was pulsed with 1 μCi of [^{3}H]thymidine for 6 hours before being harvested at various times postseeding (see Results). The method of harvesting coverslips for the measurement of [^{3}H]thymidine incorporation has been previo scribed; results were read as counts per minute (9). To dose-response curves, sister cultures were treated wit ent concentrations of either staurosporine or tamoxife hours before harvest at Day 5. In another set of expe the duration of staurosporine and tamoxifen treatmer from 6 to 36 hours, and the resultant rate of proliferat tabulated.

To assess cell numbers, cell line A172 was plate million cells per 100-mm culture plate. To generate response curve, sister cultures were treated with differ centrations of staurosporine for 48 hours before harves 5. At predetermined times, cells were gently trypsiniz lected and counted on a hemocytometer.

Protein kinase C assay

Measurement of the A172 cell line PKC enzyme acti by a histone phosphorylation assay where the rate of [^{32} phate transferred into lysine-rich histone was quantifi has been previously described in detail (9).

RESULTS

[^{3}H]thymidine incorporation is a good indicator of changes in cell numbers

We have previously reported that nonmalignant r mouse astrocytes treated with inhibitors or promoters tosis showed corresponding changes in their rates of p ation that could be comparably detected by three d methods: changes in cell numbers, [^{3}H]thymidine upt bromodeoxyuridine incorporation (35). Because malign oma cells can quadruple their deoxyribonucleic acid without mitosis, creating an aneuploid glioblast, it was tant to demonstrate that [^{3}H]thymidine incorporation w a reliable indicator of the proliferation of glioma cells. Th A172 cell line was plated and cellular proliferation was a at different days postseeding. *Figure 1* showed that [^{3} midine incorporation corresponds well ($r = 0.96$) with c in cell number. This correspondence was also true fo cells treated with staurosporine, counted, and correlate the dose-response curve for A172 shown in *Figure 2* ($r =$ On the basis of these observations, we have therefor [^{3}H]thymidine incorporation as an index of cell prolifera the rest of the studies.

Comparison of the inhibition of glioma cell proliferation with staurosporine and tamoxifen

The five established glioma cell lines A172, U87, U251, and U373 with different basal rates of proliferatio all sensitive to inhibition by staurosporine with IC_{50} between 1 and 4 nmol/L (*Fig.* 2). The individual IC_{50} obtained from computer-generated best-fit curves we

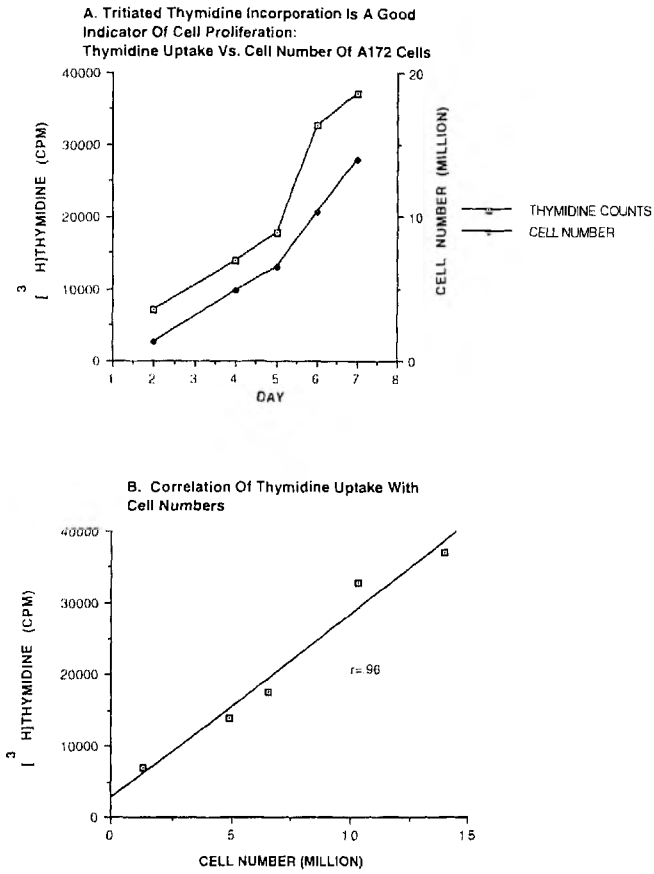


FIGURE 1. Correspondence of [^{3}H]thymidine uptake with changes in cell numbers. In *Panel A*, the amount of [^{3}H]thymidine incorporation after a 6-hour pulse at different time periods postseeding on Day 1 (n = 3 coverslips per point) was compared with changes in cell numbers (n = 4) at corresponding intervals. The standard error was less than 10% of the mean. These data were submitted to regression analysis in *Panel B* to give a correlation coefficient of 0.96 ($P < 0.001$).

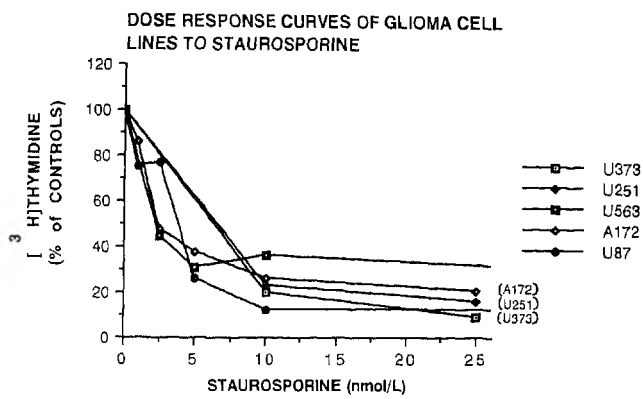


FIGURE 2. Proliferation of glioma cell lines is sensitive to staurosporine inhibition in a dose-related manner. Staurosporine was administered for 48 hours at the doses indicated. Values are the mean of four coverslips, with the standard error less than 10% of the mean. In the absence of staurosporine, control counts-per-minute (cpm) values (± standard error) were A172, 18,703 ± 1,073; U563, 14,977 ± 1,140; U251, 51,801 ± 3,037; U87, 14,460 ± 1,420; U373, 27,838 ± 1,345. IC_{50} values were taken from dose-response curves that represented the best-fit curves. Data for 100 nmol of staurosporine per liter, which were 10% or less of controls, are not shown in the graph in order that the effects at lower concentrations can be clearly discerned.

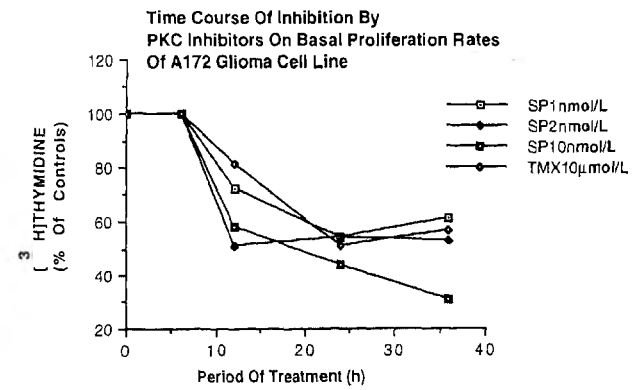


FIGURE 3. Time course of action of PKC inhibitors on the proliferation of the A172 glioma cell line. Each point is the mean of four coverslips, with the standard error less than 10% of the mean in all cases. SP, staurosporine; TMX, tamoxifen.

spectively, A172 (2.75 nmol/L), U87 (2 nmol/L), U373 (3.5 nmol/L), U251 (1 nmol/L), and U563 (1 nmol/L). The inhibition of the proliferation of cell line A172 was evident by 12 hours of treatment with staurosporine and plateaued by 24 hours (*Fig. 3*). In addition to the established cell lines, all four low-passage cell lines were sensitive to staurosporine in approximately the same concentrations as were the established cell lines (*Fig. 4*). Individual IC_{50} values were, respectively, W342, 7.5 nmol/L; W362, 1 nmol/L; W363, 6 nmol/L; and W368, 1 nmol/L.

In comparison with staurosporine, tamoxifen treatment also reduced the proliferation of all five established glioma cell lines (*Fig. 5*). However, the IC_{50} values were approximately 10 μmol/L on average. In further contrast to staurosporine, tamoxifen treatment of up to 10 μmol/L did not substantially reduce the proliferation rates of three of four low-passage cell lines (*Fig. 6*).

Correspondence of cell proliferation with protein kinase C activity

Measurements of the PKC activity of the A172 glioma cell line after staurosporine treatment revealed that, although cytosolic activity was unaffected, particulate PKC activity was substantially reduced (*Fig. 7a*). Correlation analysis of particulate PKC activity with [^{3}H]thymidine incorporation after staurosporine treatment resulted in an r value of 0.85 (*Fig. 7b*). The IC_{50} for the inhibition of particulate PKC activity was 1.5

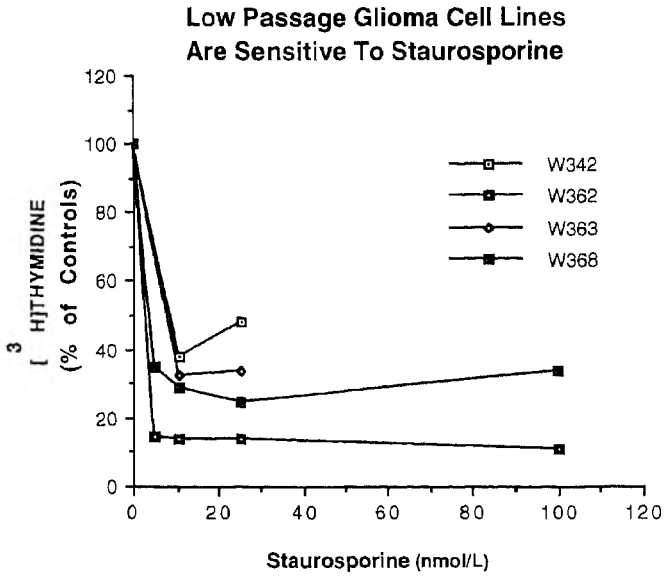


FIGURE 4. Low-passage glioma cell lines are sensitive to inhibition by staurosporine. Each point is the mean of four coverslips, with the standard error less than 20% of the mean in all cases. Control rates of proliferation (± standard error) were: W342, 1364 ± 212; W362, 4430 ± 199; W363, 1746 ± 310; and W368, 1703 ± 214.

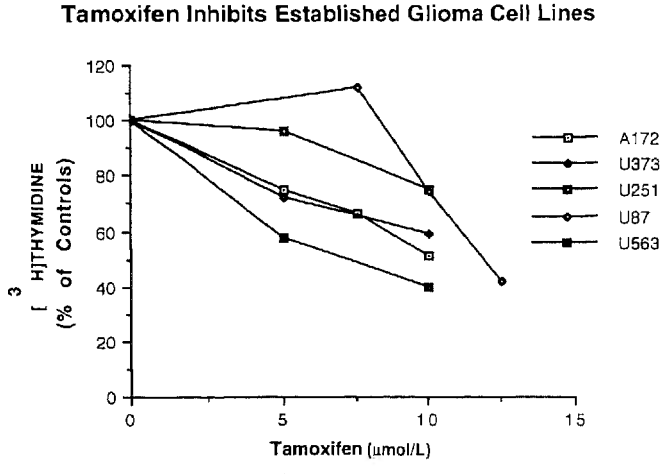


FIGURE 5. Established glioma cell lines are also sensitive to inhibition by tamoxifen. Each point is the mean of four coverslips, with the standard error less than 10% of the mean in all cases.

nmol/L, which closely corresponds to the IC_{50} for proliferation (2 nmol/L). Similarly, tamoxifen treatment of the A172 cells resulted in a diminution of particulate PKC activity, with an IC_{50} value of 5.6 μmol/L (*Fig. 8*).

Comparison of the effects of staurosporine on the proliferation of glioma cells and neonatal rat astrocytes

Proliferation rates were measured in neonatal rat astrocytes in response to staurosporine and were compared with those of

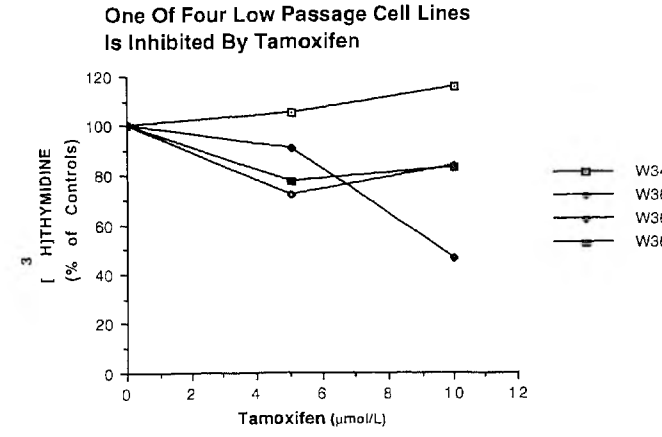
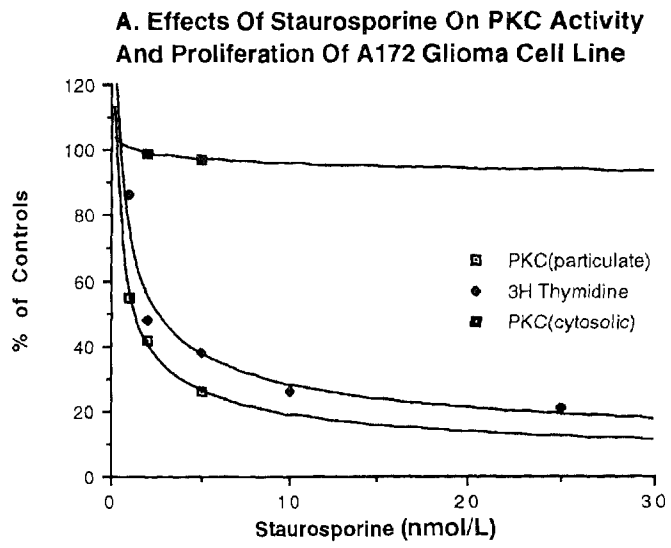


FIGURE 6. Proliferation of only one of four low-passage glioma cell lines is substantially inhibited by tamoxifen. Each point is the mean of four coverslips, with the standard error less than 10% of the mean in all cases.

the A172 glioma cell line. Glioma cell lines were marginally more sensitive to staurosporine, with an IC_{50} of 2 nmol/L, than were rat astrocytes, which had an IC_{50} of 5 nmol/L (*Fig. 9*).

DISCUSSION

The multitude of reports on the abnormal expression of growth factors such as epidermal growth factor in glioma cells (1, 31) raises the question of how extracellular signals provided by ligand-receptor interactions are transduced to the nucleus of the cell effecting proliferation. The interaction of epidermal growth factor with its receptor-tyrosine kinase complex results in numerous events, including autophosphorylation at the intracellular SH2 domain and activation of a series of enzymes including phospholipase C-γ (19). Phospholipase C-γ activation results in diacylglycerol and inositol triphosphate formation. Whereas the latter mobilizes calcium from intracellular stores, diacylglycerol goes on to activate PKC, a phospholipid-dependent serine-threonine kinase composed of at least 10 known isoforms (19). The isoforms demonstrate differences in their enzymological properties, differential tissue expression, and specific intracellular localization (19). An integrated nomenclature has been developed to categorize the PKC isoforms on the basis of their mode of activation. The group A isoforms consist of the four classical PKCs: α, βI, βII, and γ. The group B isoforms consist of four new PKCs (PKC): δ, ε, η(L), and θ. The group C isoforms consist of two atypical PKCs (PKC): ζ and λ. PKC has been implicated in various functions in the central nervous system including proliferation of astrocytes (34), process formation in oligodendrocytes (32), and long-term potentiation in neurons (3). The finding that the tumor-promoting phorbol esters acted via PKC led us to investigate its role in transformed glia (17, 18, 30). We have reported that the enzymatic activities of PKC in glioma cells are at least 3 orders of magnitude higher than those of nonmalignant adult human glia and that the proliferation rates of five established glioma cell lines are highly correlated with their PKC activity ($r = 0.98$) (9). In addition, the relatively selective PKC inhibitor stauro-



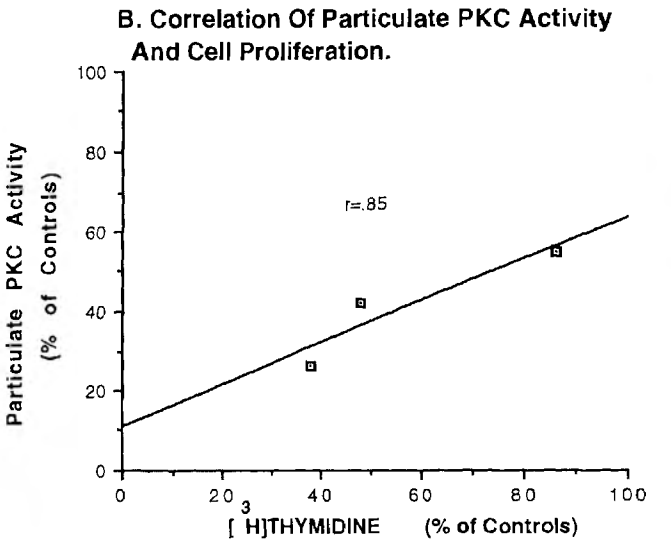


FIGURE 7. Effect of staurosporine on the proliferation rate and PKC activity of the A172 glioma cell line. PKC activity (in picomoles per minute per milligram of protein) was assayed in triplicate. Control cytosolic PKC value was 1314 ± 127, whereas control particulate PKC activity was 303 ± 86. Control [^{3}H]thymidine incorporation was 18,703 ± 1,073. In *Panel B*, the particulate PKC activity was correlated with [^{3}H]thymidine incorporation to give a correlation coefficient of 0.85.

sporine (12, 16, 21, 23) could inhibit basal proliferation as well as block the effect of mitogens of glioma cells (9, 11).

Previous studies from this laboratory had investigated the effect of staurosporine on one human (A172) and one rat (C6) glioma cell line (9). In this study, we have extended the experiments to five established glioma lines of different basal

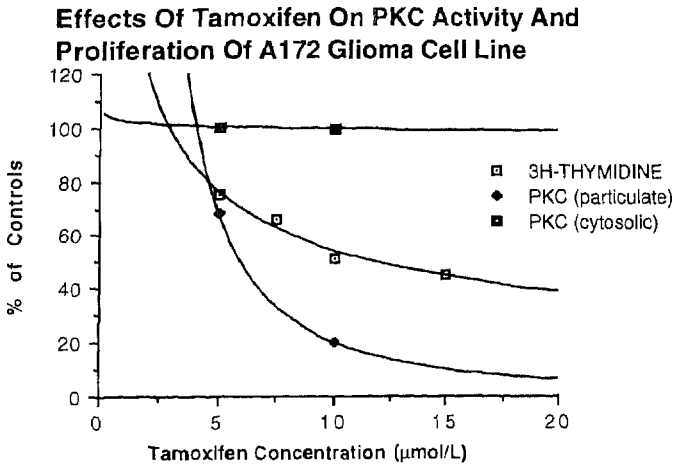


FIGURE 8. Effect of tamoxifen on the proliferation rate and PKC activity of the A172 glioma cell line. See legend to *Figure 7*.

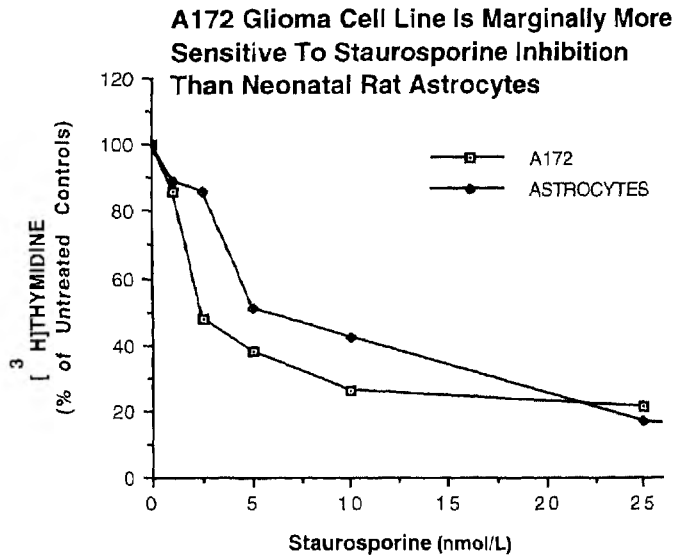


FIGURE 9. The A172 glioma cell line is marginally more sensitive to staurosporine inhibition than are neonatal rat astrocytes. Preparation of neonatal rat astrocytes has been previously described (30, 31). Each point is the mean of four coverslips, with the standard error less than 10% of the mean in all cases. Control rate of proliferation (± standard error) for neonatal rat astrocytes was 12,408 ± 508.

proliferation rates (9) and have found them all to be equally sensitive to staurosporine (*Fig. 2*). A common criticism of using established cell lines is that they are highly clonally selected and are not representative of the in vivo state. This is especially true in high-grade glioma, in which there is cellular heterogeneity, hence, the name *glioblastoma multiforme*. Therefore, we have further examined low-passage glioma cell lines as a closer approximation of the in vivo state. The results show that all of the low-passage lines were inhibited by staurosporine in approximately the same concentrations as the established cell lines (*Fig. 3*).

The inhibition of glioma cell proliferation by staurosporine occurred at concentrations that inhibited PKC (*Fig. 7*); for A172, the IC_{50} of staurosporine for proliferation (2 nmol/L) closely corresponds to the IC_{50} for the inhibition of PKC (1.5 nmol/L). Such data lend further support to the contention that PKC is intimately involved in regulating the proliferation of glioma cells. It must be noted that staurosporine is "relatively" selective, also inhibiting protein kinase A and tyrosine kinases, however, at higher concentrations than those required to inhibit PKC (15). Interestingly, we found that it was the particulate fraction of PKC that was inhibited by staurosporine (*Fig. 7a*). This correlation is in keeping with the concept that the particulate form of PKC represents the active form of the enzyme (17, 18). Further studies will determine if a particular isoform is intimately involved in regulating proliferation in these neoplasms (4, 27).

Comparisons of the effects of staurosporine on glioma cells and neonatal rat astrocytes indicate that staurosporine is marginally more effective in inhibiting proliferation of the malignant cells. Whether this may provide a therapeutic window for effective chemotherapy without much associated cerebral toxicity remains unknown; adult human astrocytes have a very low rate of proliferation in vitro when compared with neonatal rat astrocytes (33–35) and conceivably will be less susceptible to the inhibitory effects of staurosporine; however, this assumes that the toxicity of this relatively selective agent is related to its inhibition of PKC. This possibility will be examined in detail with analogs with increased selectivity for PKC.

There has been much recent interest in tamoxifen because it has been extensively used in the treatment of breast cancer and recently in the treatment of malignant melanoma (5). Tamoxifen is tolerated in high doses, giving concentrations in plasma in the micromolar range (5, 28). Its secondary effects are well known, and it crosses the blood-brain barrier. Vertosick et al. (29) have demonstrated increased survival of patients with recurrent glioma who are using tamoxifen. We have treated a patient with recurrent glioblastoma with high-dose tamoxifen and have observed tumor regression and clinical stabilization (2).

We studied the effects of tamoxifen on five established and four low-passage cell lines. Although tamoxifen inhibited cell proliferation in all of the established lines, its effectiveness was in the micromolar concentration, as opposed to the nanomolar concentration required by staurosporine. The PKC enzyme assay demonstrated an IC_{50} of 5.6 μmol/L for tamoxifen (*Fig. 7*), in close correspondence with its IC_{50} for the inhibition of glioma cell proliferation (10 μmol/L). Three of four low-passage cell lines were, however, not inhibited by tamoxifen, in contrast to staurosporine, which inhibited all of the lines. The reasons for this tamoxifen resistance is not immediately apparent; a prediction would be that some gliomas are not susceptible to tamoxifen. This was borne out by the study of Vertosick et al. (29), in which some but not all of the patients with malignant gliomas showed a response to tamoxifen. Although we have reported a case of tumor regression (2), we have subsequently treated two patients with recurrent glioma who had no response to high-dose tamoxifen. We are presently conducting a Phase 2 cooperative study on the effect of high-dose on recurrent glioma.

In conclusion, the close correspondence of the IC_{50} the inhibition of proliferation and PKC activity for t rosporine and tamoxifen supports the hypothesis th PKC activity in glioma cells drives their high rate o ation and that these drugs decrease proliferation by i the PKC system. Comparative in vitro studies of stau with tamoxifen as inhibitors of glioma proliferatio that staurosporine is a potentially more useful dr adjuvant therapy of patients with malignant gliomas sporine has been previously shown to inhibit the g human cervical carcinoma cells, as well as produc inhibition of human bladder carcinoma cells, a huma elocytic leukemia line (HL-60), and bovine endotheli cells at concentrations that correlate well with in v inhibition (15). Staurosporine has also been shown t the invasion of human bladder carcinoma cells, sugges it may play a role in other aspects of the malignant ph (24). Furthermore, staurosporine has demonstrated a activity against human carcinoma xenografts in nude m Indeed, we have demonstrated increased survival in a model of brain tumor in rats with implanted C6 glio staurosporine treatment, with little evidence of neur (10). However, this study was preliminary, and furthe trials are in progress with different animal glioma m well as various modes of staurosporine administratior human trials can be considered.

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COMMENT

In the attempt to find more effective approaches to the biochemical control of glioma growth, it is critical to establish new targets for potential therapeutic agents. The protein kinase C pathway has become one of these potentially useful targets. It is clear from many different studies that the protein kinase C pathway is somehow a factor in affecting glioma growth.

In this study, the authors extend their earlier observations by studying five low-passage human glioma lines, rather than a single high-passage line, and compare a highly selective inhibitor of protein kinase C activity, staurosporine, with a less-selective agent, tamoxifen. The findings are suggestive of a modest but apparent difference in effectiveness, with staurosporine being shown to be the more useful agent. These findings are of interest because they serve to add more data to our understanding of a new and potentially useful target for developing strategies to control glioma cell growth.

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