Inhibition of the Growth of Glioblastomas by CGP 41251, an Inhibitor of Protein Kinase C, and by a Phorbol Ester Tumor Promoter

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ABSTRACT

Protein kinase C (PKC) plays a central role in signal transduction pathways that mediate the action of certain growth factors, tumor promoters, and cellular oncogenes. To explore whether PKC might be an appropriate target for the chemotherapy of human brain tumors, cell lines were established from five glioblastomas, one mixed gliosarcoma and glioblastoma, two astrocytomas, and one choroid plexus carcinoma. The staurosponge derivative CGP 41251, an inhibitor of PKC, inhibited cell proliferation in all nine cell lines with an IC₅₀ in the range of 0.4 μM. Drug withdrawal and clonogenicity assays showed that CGP 41251 induced an irreversible growth arrest. Three cell lines were examined in detail: two human glioblastoma cell lines, GB-1 and GB-2, and one gliosarcoma cell line, GS-i. All of these three cell lines were highly aneuploid and displayed morphologies and immunohistochemical markers characteristic of the glial lineage. The compound 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter and activator of PKC, also inhibited the growth of these cell lines. CGP 41251 in combination with TPA caused further growth inhibition. Cultures treated with CGP 41251 displayed an increase in the fraction of cells in G₂-M, a decrease of cells in S phase, and no consistent effect on G₀-G₁. Immunohistochemical analyses demonstrated that growth inhibition by CGP 41251 was associated with the formation of giant nuclei with extensive fragmentation and apoptotic bodies. These effects of CGP 41251 were abrogated by withdrawal of serum from the medium or by exposure of these cells to aphidicolin, actinomycin D, cycloheximide, or TPA. In contrast to the effects seen with the glioblastoma cell lines, nontransformed astrocyte lines remained viable in the presence of 0.4 and 0.8 μM CGP 41251 and displayed only a slight increase in the fraction of giant nuclei with fragmentation. The antitumor activity of CGP 41251 was demonstrated in vivo against xenografts of the glioblastoma cell lines U87 MG and U373 MG. These findings suggest that CGP 41251 might be a useful agent for the treatment of glioblastomas.

INTRODUCTION

PKC is a family of phospholipid-dependent serine and threonine kinases that has been shown to have the highest activity in the central nervous system (1-3). About 13 different isoforms of PKC have been described today, which differ in their cofactor requirement and are differentially expressed during development and in various tissues. Most of these isoforms are activated by diacylglycerol, which is generated after activation of phospholipase C. Diacylglycerol binds to the regulatory domain of various PKC isoforms. Phorbol esters, such as TPA, also bind to the regulatory domain of these same isoforms and are potent activators of PKC. Overexpression of PKC in R6 rat fibroblasts using retroviral-mediated gene transfer demonstrates that individual isoforms of PKC have different biological effects. Thus, high level expression of PKCβ₂ leads to growth abnormalities, including increased frequency of focus formation and growth in agar (4); overproduction of PKCα does not cause any growth defects (5), whereas overexpression of PKCζ leads to malignant transformation and tumorigenicity in athymic mice (6).

Due to the central role of PKC in growth control, inhibition of this enzyme might be a particularly promising approach for cancer therapy (3). Recent studies indicate that the staurosponine derivative CGP 41251 inhibits PKC in subcellular assays and also inhibits tumor cell growth in cell culture and in athymic mice (7-9). Because several isoforms of PKC are normally expressed at high levels in the central nervous system and...
because glioblastomas are particularly refractory to treatment with conventional chemotherapeutic agents, the present study was undertaken to examine the effects of CGP 41251 on a series of human brain tumor-derived cell lines. This study demonstrates that CGP 41251 is a potent growth inhibitor of glioblastoma cells and that this inhibition is, paradoxically, enhanced by TPA. Growth curves with drug reversal as well as clonogenicity studies suggest that CGP 41251 acts both through a cytotoxic and cytostatic mode. Flow cytometry studies indicate an accumulation of cells in G2-M, and morphological studies indicate that CGP 41251 leads to the formation of giant nuclei and the formation of apoptotic bodies. Furthermore, the antiproliferative property of CGP 41251 was confirmed in xenografts of human glioblastoma cell lines U87 MG and U373 MG.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. All cell lines were established in the Brain Tumor Laboratory of the Department of Neurosurgery, Columbia-Presbyterian Medical Center. In brief, fresh tumor specimens were placed in 15% FCS in the operating room. In the tissue culture laboratory, the tumor cells were mechanically dispersed with 18-gauge needles or enzymatically with trypsin, depending on the tumor consistency. Dispersed cell explants were then placed in tissue culture dishes containing DMEM with 20% FCS. The cell lines were also maintained in DMEM supplemented with 20% FCS (Hybrimax; Sigma Chemical Co., St. Louis, MO). TPA (LC laboratories, Woburn, MA) and CGP 41251 and CGP 42700 (Ciba-Geigy, Basel, Switzerland) were dissolved in DMSO. To obtain growth curves, cells were plated at a density of 10^4/well in 6-well plates (3-cm diameter) and grown in complete medium with or without the indicated drugs (Fig. 1). Cell counts were determined on alternate days in triplicate wells during the subsequent 8 days with a Coulter Counter. Actinomycin D (Sigma) was dissolved in DMSO and used at a final concentration of 2.5 μg/ml. Cycloheximide (Sigma) was dissolved in ethanol and used at a final concentration of 10 μg/ml. Aphidicolin (Sigma) was dissolved in DMSO and used at a final concentration of 1 μg/ml. In all cases, the final concentration of DMSO in the media was less than 0.1%, which had a negligible effect on cell growth.

Protein Extraction and Immunoblotting. The various cell lines were grown to subconfluency, washed three times with PBS, and immediately lysed in a 1% SDS buffer [20 mm...
Tris-HCl (pH 7.5) and 2 mM EDTA] and boiled for 5 min. The protein content in these total extracts was determined according to the method of Bradford (Bio-Rad). The equivalent of 50 μg protein cell extracts was fractionated by electrophoresis in 7% polyacrylamide gels, transferred to nitrocellulose, and exposed to antibodies specific to various isoforms of protein kinase C as indicated [PKCs, rabbit affinity purified IgG (GIBCO-BRL); PKCs δ, ε, and ζ, rabbit polyclonal antisera; Refs. 9 and 10]. The specificity of the antisera has been demonstrated previously (9).

Clonogenicity. Cells were plated in 100-mm dishes with 10 ml DMEM supplemented with 10% FCS (GIBCO) and allowed to attach for about 24 h. The numbers of cells to be plated were chosen such that 50 to 100 colonies would survive after a specified treatment. CGP 41251 or DMSO as control were then added, and the cells were incubated for an additional 24, 48, 96, 120, and 144 h. Following these incubation periods, the medium was removed by aspiration and replenished with DMEM supplemented with 10% FCS without additives. The cultures were then incubated for 14 days. Cells were fixed in 75% methanol/25% acetic acid and stained with crystal violet.

BrdUrd Incorporation Studies. BrdUrd labeling (Amersham) was carried for 60 min at 37°C in DMEM supplemented with 20% FCS. Subsequently, the cells were washed in PBS and fixed in glacial acetic acid/ethanol for 30 min. The endogenous peroxidase activity was quenched by incubation with methanol/3% peroxide for 30 min at room temperature. Subsequently, the dishes were treated with blocking solution (horse serum, 2% BSA, 0.1% Triton X-100, and PBS) for 1 h. Then anti-BrdUrd mouse monoclonal antibodies (Amersham) were applied for 1 h, and the plates were washed in PBS. The mouse monoclonal antibody was detected with the ABC system (mouse monoclonal antibody; Accurate Scientific, NN16); neuron-specific enolase (rabbit polyclonal antibodies; ICN); NFI60 (mouse monoclonal antibody; Accurate Scientific, NN16); neurofilament 200 (rabbit polyclonal antiserum; Sigma); and MAP2 (mouse monoclonal antibody AP420; Sigma). The secondary antibodies used were goat-antimouse-conjugated FITC (Sigma) at a 1:50 dilution and goat-antirabbit FITC-conjugated antibodies (TAGO) at a 1:100 dilution. The nuclei were counterstained with DAPI (Boehringer-Mannheim) at a final concentration of 1.5 μg/ml in PBS for 30 min at room temperature and visualized with fluorescence microscopy. For quantitation of the nuclear morphology under various culture conditions, the morphologies of the nuclei were separated into six categories (ovoid, fragmented, multinucleated, mitotic, pyknotic, and multilobular). For each data point, four fields, each containing at least 150 nuclei, were evaluated.

Transmission Electron Microscopy. Cells were fixed as a pellet in 2.5% glutaraldehyde in 1,4-piperazinediethanesulfonic acid buffer overnight, rinsed in 1,4-piperazinediethanesulfonic acid buffer, followed by postfixation in 2% OsO4 for 1 h. The samples were then rinsed in distilled H2O, and this was followed by dehydration in a graded series of alcohol of 50, 75, and 95% through absolute alcohol, followed by propylene oxide, and then overnight in propylene oxide:PolyBed 812 (1:1). The samples were embedded in Poly/Bed 812 in a 60°C oven. Ultrathin sections were obtained with a Reichert Ultracut S microtome. The thin sections were photographed using a JEOL 1200-EX electron microscope.

MTT Viability Assay. The MTT assay was performed as described (11). Cells (2-5 × 105) were seeded in each well (0.38 cm2 area) of 96-well plates in DMEM plus 20% FCS. On the following day, the medium was replenished with the drugs at the indicated dosages. After various times (see “Results”), the medium was removed, and 22 μl of MTT solution (5 mg/ml in PBS; Sigma) were added to 200 μl of 20% FCS in DMEM in each well and incubated at 37°C for 3 to 4 h. This medium was then discarded, 100 μl of DMSO were added to each well, and the plates were placed for 15 to 30 min on a shaker. The color change was recorded by measuring the absorbance at 570 nm on an automated multwell reader. Each data point represents the mean of quintuplicate assays and was calculated as a fraction of the control.

In Vivo Experiments. The U87 MG (HTB-14) and U373 MG (HTB-17) human glioblastoma cell lines were obtained from the American Tissue Culture Collection (12, 13). Both tumor lines were maintained by serial passage by implanting tumor fragments (approximately 25 mg) s.c. into the left flank of female BALB/c nude mice (Bohmnholtgard, Copenhagen, Denmark) with a 13-gauge Trokar needle under Forene (Abbott, Cham, Switzerland) narcosis. Each group of mice contained six animals. Drug treatment was started on day 5 after transplantation and continued until day 17. Compounds were prepared as follows. For i.v. applications, CGP 41251 was dissolved in DMSO, Tween 80, and NaCl. In brief, a stock solution containing 150 mg/ml CGP 41251 was prepared by adding the appropriate amount of compound to DMSO containing 1% Tween 80 dissolved at room temperature. Prior to use, the clear stock
solution was diluted 1:20 (v/v) with sterile 0.9% NaCl, resulting in a milky suspension (final concentration: 7.5 mg/ml CGP 41251, 5% DMSO, 0.5% Tween 80, and 0.9% NaCl). For p.o. applications, CGP 41251 was prepared using Gelucire 44/14. In brief, a waxy solid formulation of CGP 41251 (18% w/w in Gelucire 44/14; Gattefossé, France) was obtained from CIBA Pharmaceutical. Prior to use, 16.7 mg of the active ingredient corresponding to 83.3 mg of the waxy formulation were added to 1 ml of sterile water, and the mixture was sonicated in an ultrasonic bath for 10 min, resulting in a milky suspension that was given to the mice for 13 consecutive days once daily p.o. (7). All placebo-treated animals received the matrix without CGP 41251 (gelucire/water) p.o. Tumor growth was followed by measuring perpendicular tumor diameters. Tumor volumes were calculated as described using the formula \(V = \frac{4}{3} \pi \times L \times D^2/6\), where \(L\) is the length and \(D\) is the diameter (14, 15). Mean tumor volume (± SD of six animals) was expressed in cubic centimeters.

**Statistical Methods.** For experiments on cell proliferation, the cell counts were analyzed on the log scale. A linear model was fit with a common intercept (number of cells at day 0) with a separate slope for each treatment group. The equality of slopes was tested with the regression F test. For BrdUrd incorporation studies, the data of the percentage of labeled cells were analyzed on the logistic scale. The data were analyzed with an ANOVA model incorporating a main effect of cell line, a slope for the drug dose, and an interaction of cell line and dose. In the clonogenicity studies, the surviving fractions were transformed to the log scale. The data were analyzed by a linear model with a main effect of time, a slope for drug dose, and the interaction of time and dose. We conducted separate analyses for each cell line. In all analyses, we deleted the data from dose group 0, because they were scaled to have mean SF = 1. To analyze the in vivo tumor growth data, the tumor volumes were transformed to the log scale. We fit a mixed model with a random animal effect and a separate slope for each treatment group (placebo, p.o. administration, and i.v. administration). To analyze the data on nuclear morphology, a separate two-way table of treatment by morphology was formed for each of three replicate assays. To eliminate cells with the smallest counts, our analyses combined the multinucleated, multilobular, and pyknotic categories. To compare the treatment groups, we executed a Mantel-Haenszel test for general association, stratified by replication. All analyses were carried out in SAS version 6.09 (SAS Institute, Cary, NC). We fit linear models in Proc MIXED, mixed models in Proc Mixed, and executed Mantel-Haenszel tests in Proc Freq software programs.

**RESULTS**

**Characteristics of Cell Lines.** Various properties of the three major cell lines used in this study are summarized in Tables 1 and 2. All three cell lines were positive for the glial-specific protein GFAP and negative for the neural markers MAP2 and NF160. Cultures of GB-1 and GB-2 cells were heterogeneous by morphological criteria, displaying cells with squamous, astrocytic, and polygonal morphologies. GB-1 and GB-2 cells were arranged in fascicular patterns and piled up. In contrast, cultures of GS-1 cells formed flat mosaic patterns of polygonal cells. Occasionally, cells with astrocytic morphologies were found in GS-1 cultures. All three cell lines were highly aneuploid and showed continuous chromosomal rearrangements during serial passages (data not shown). The cell lines GB-1 and GB-2 were tumorigenic when injected into athymic mice (nu/nu). The histopathology of the grafted tumors exhibited highly infiltrative tumor growth, nuclear polymorphism, pseudopalisading, and expression of GFAP. Two astrocyte lines were established from nontumorous tissue: A-1 from normal appearing brain tissue obtained from a patient with a glioblastoma multiforme; and A-2 from brain tissue removed from a patient with epilepsy. A-1 was shown to be nontumorigenic in athymic mice. A-1 and A-2 both exhibited astrocytic morphologies (data not shown).

**Table 1** Description of cell linesa

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>Protein expression</th>
<th>Tumorigenicity in nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>F</td>
<td>GB-1</td>
<td>Glioblastoma multiforme</td>
<td>+</td>
</tr>
<tr>
<td>71</td>
<td>M</td>
<td>GB-2</td>
<td>Glioblastoma multiforme</td>
<td>+</td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>GS-1</td>
<td>Glioblastoma multiforme and gliosarcoma</td>
<td>+</td>
</tr>
</tbody>
</table>

a For additional detail, see “Materials and Methods.”

**Table 2** Growth inhibition by CGP 41251 in glioblastomas, astrocytomas, and a choroid plexus carcinoma cell line

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>(IC_{50}) ((\mu)m) for CGP 41251a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB-1</td>
<td>Glioblastoma multiforme</td>
<td>0.2</td>
</tr>
<tr>
<td>GB-2</td>
<td>Glioblastoma multiforme</td>
<td>0.4</td>
</tr>
<tr>
<td>GB-3</td>
<td>Glioblastoma multiforme</td>
<td>0.6</td>
</tr>
<tr>
<td>GB-4</td>
<td>Glioblastoma multiforme</td>
<td>0.8</td>
</tr>
<tr>
<td>GB-5</td>
<td>Glioblastoma multiforme</td>
<td>0.2</td>
</tr>
<tr>
<td>GS-1</td>
<td>Glioblastoma multiforme and gliosarcoma</td>
<td>0.2</td>
</tr>
<tr>
<td>AT-1</td>
<td>Astrocytoma</td>
<td>0.1</td>
</tr>
<tr>
<td>AT-2</td>
<td>Astrocytoma</td>
<td>0.4</td>
</tr>
<tr>
<td>CP-1</td>
<td>Choroid plexus carcinoma</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a Determined after a 6- to 8-day exposure of cultures to the drug, as described in Fig. 1.
Effects of TPA and CGP 41251 on Cell Proliferation. The cell line GB-1 was first grown in the presence of increasing concentrations of TPA (Fig. 1A). TPA markedly inhibited growth with an IC_{50} of about 0.1 ng/ml. Then the effect of the PKC selective inhibitor CGP 41251 on the growth of GB-1 cells was tested either alone or in combination with TPA. CGP 42700, a close structural analogue of CGP 41251 that does not inhibit PKC (7, 9), and the solvent DMSO were used as controls. As shown in Fig. 1B, proliferation ceased at 0.4 μM of the active compound CGP 41251, and cell killing was observed with 0.8 μM of CGP 41251 (P < 0.001). CGP 42700 was not significantly different from growth medium at 0.4 mM; there was a modest but significant inhibition at 0.8 mM. Thus, both TPA (Fig. 1A) and CGP 41251, as well as CGP 42700 at a modest level (Fig. 1B), inhibit the proliferation of GB-1 in a dose-responsive manner. The inhibitory effect of 0.4 μM CGP 41251 plus 1 ng/ml TPA was greater than the effect of either agent alone (P < 0.001). The inhibitory effect of TPA plus CGP 42700 was modest but significantly greater than that of TPA alone (Fig. 1B; P < 0.001). This suggested that CGP 41251 might be cytotoxic when tested in combination with TPA. Trypan blue exclusion studies, however, did not show increased cell staining of CGP 41251 or CGP 41251 plus TPA-treated cultures when compared to untreated cultures or to CGP 42700 or DMSO-treated cultures (data not shown).

The growth-inhibitory effects of CGP 41251 were analyzed across a range of concentrations of CGP 41251 in the GB-1 and eight additional brain tumor-derived cell lines, and the results are summarized as IC_{50} (i.e., the concentration required to produce about a 50% inhibition of growth) in Table 2. The five glioblastoma-derived cell lines (GB-1 to GB-5) displayed IC_{50} values between 0.2 to 1 μM. This value was 200 nM for the GS-1 gliosarcoma cell line; 0.1 and 0.2 μM, for the two astrocytoma cell line, AT-1 and AT-2, respectively; and 0.3 μM for the choroid plexus carcinoma cell line CP-1 (Table 2).

As with the GB-1 cell line (Fig. 1B), when TPA was combined with CGP 41251, there was further inhibition of growth of the GB-2 and GS-1 cell lines, and the compound CGP 42700 caused a modest growth inhibition in GS-1 and no inhibition in GB-2. When the GB-1 or GS-1 cells were exposed to CGP 41251 at 0.8 μM for 4 days and the drug then removed, the proliferation of the cells failed to resume. However, under similar conditions, the growth inhibition obtained with the GB-2 cells was partially reversed when the drug was removed (data not shown).

Effects of CGP 41251 and TPA on the Expression of Specific Isoforms of PKC. Fig. 2 displays an immunoblot analysis of the expression profile of several isoforms of PKC in GB-1 cells and the effects of exposing these cells to various drugs. PKCs α, δ, ε, and ζ were detected in total cell extracts but not the PKC isoforms β and γ (data not shown). The molecular weights of PKCs were as follows: Mᵣ 86,000 (PKCα); Mᵣ 96,000 (PKCδ); Mᵣ 78,000 (PKCe). We also detected an approximately Mᵣ 58,000 species cross-reacting with anti-PKCζ antisera (Fig. 2, open arrow). This moiety most likely represents the COOH-terminal catalytic fragment of PKCζ termed PKM (16, 17). The expression of PKM has been demonstrated in rat brain (16, 17) and might play a role in long-term potentiation (17). After exposing GB-1 cells for 48 h to TPA at 0.01, 0.1, and 1 ng/ml, there was no significant effect on the expression levels of the four isoforms (Fig. 2), although the 1 ng/ml dose caused marked growth inhibition (Fig. 1A). Exposure to 10 and 100 ng/ml TPA caused marked down-regulation of PKCs α and ε. Exposure to CGP 41251 at 0.4 or 0.8 μM, or the control compound CGP 42700, caused no appreciable loss of these four isoforms of PKC (Fig. 2, Lanes 8–10). The combination of CGP 41251 (0.8 μM) with TPA at 1 or 10 ng/ml (Fig. 2, Lanes 11 and 12) showed complete down-regulation of PKCs α and ε. Similar profiles of these PKC isoforms were found with the GB-2 and GS-1 cells (data not shown).

BrdUrd Incorporation Studies. To further analyze the antiproliferative properties of CGP 41251, the GB-1, GB-2, and GS-1 cell lines were selected for further detailed studies. BrdUrd incorporation studies were performed to determine possible effects of CGP 41251 on DNA synthesis. Fig. 3 summa-

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**Fig. 2** Immunoblot for PKCs α, δ, ε, and ζ. GB-1 cells were cultured for 2 days in the presence of DMEM supplemented with 20% FCS alone (Lane 1) or with 0.1% DMSO (Lane 2); TPA at the following concentrations of 0.01, 0.1, 1, 10, and 100 ng/ml (Lanes 3–7); CGP 41251 at 0.4 and 0.8 mM (Lanes 8 and 9); CGP 42700 at 0.8 mM (Lane 10); and CGP 41251 (0.8 mM) plus TPA at 1 or 10 ng/ml (Lanes 11 and 12). In the PKCζ immunoblot, the solid arrow denotes the complete PKCζ, and the open arrow, the PKM form of PKCζ. For additional details, see "Materials and Methods."
Inhibition of the Growth of Glioblastomas by CGP 41251

**Fig. 3 Effects of CGP 41251 on BrdUrd incorporation.** Cultures of GS-1, GB-1, and GB-2 cells were treated for 4 days with 200, 400, or 800 nm CGP 41251. The cultures were then incubated with BrdUrd for 1 h, fixed, and prepared for staining as described in “Materials and Methods.” The figure indicates the BrdUrd labeling indices, i.e., the percentage of nuclei positive for BrdUrd. In the statistical analyses, the slope of the dose of CGP 41251 was significant ($P < 0.001$), as was the main effect of the cell line. The statistical interaction was not significant. Thus, the analyses suggest a strong inhibitory effect of CGP 41251, which is present in all three of the cell lines studied. Bars, SD.

rizes BrdUrd labeling indices obtained after a 4-day treatment with various concentrations of CGP 41251. In untreated cultures, the GB-2 cells exhibited the highest labeling index (about 33%), as compared to GB-1 and GS-1 cells (about 12%). The addition of CGP 41251 (200 to 800 nm) resulted in a dose-dependent inhibition of BrdUrd incorporation in all three cell lines. At 800 nm CGP 41251, the labeling index had decreased by about 80% in all three cell lines when compared to the untreated cultures. Time course studies with the GB-2 cell line indicated that inhibition of BrdUrd incorporation began within 24 h after exposure to the drug and continued to decline thereafter (data not shown). Inspection of nuclei revealed that those nuclei that were labeled were predominantly giant nuclei with a fragmented morphology (see below), suggesting that these cells were undergoing re-replication of DNA without entering mitosis. Exposure to TPA (100 ng/ml) for 2 days also led to a decrease in BrdUrd incorporation with both the GB-2 and GS-1 cells, and this inhibitory effect was further augmented by the addition of CGP 41251 (data not shown).

**Clonogenicity Assays.** To further analyze the antiproliferative effects of CGP 41251, clonogenicity studies were performed. GB-1, GB-2, GB-3, and GS-1 cells were plated at low density and incubated for various times with different doses of CGP 41251. Subsequently, the medium was replaced with regular growth medium, and the cells were cultured in the absence of the drugs for an additional 2 weeks, after which the surviving fractions were determined (see “Materials and Methods”). As shown in Fig. 4, all of the four cell lines showed only partial inhibition when exposed to CGP 41251 for only 24 h. When exposed to the drug for 48 h or longer, marked inhibition and a clear dose-response relationship was seen in all of the cell lines, although GB-2 cells were less responsive than the other three cell lines. In addition, at a given dose of drug, an increased duration of treatment led to decreased survival. For example, GB-1 cells treated with 1 μM CGP 41251 for 24, 48, 96, and 144 h showed survival fractions of 24, 9, 0.7, and 0.6%, respectively. The survival of the cells also declined in a dose-response fashion. Thus, the survival fractions of GB-1 cells after a 96-h exposure to 100, 200, 400, 600, 800, and 1000 nm CGP 41251 were 53, 43, 13, 1.7, 1.3, and 0.7%, respectively. Finally, the survival fractions of the particular cell lines were compared at a given dose for a given duration of exposure. At the longest exposure (120 to 144 h), the LD$_{50}$ (i.e., the concentration required to produce a 10% survival) for CGP 41251 was 360, 740, 320, and 350 nm in GB-1, GB-2, GB-3, and GS-1 cells, respectively. At the maximum dose (1000 nm) and after the longest exposure (120 to 144 h), the surviving fractions were 0.1, 0.6, 3, and 6.2% for the GB-3, GB-1, GS-1, and GB-2 cells, respectively. These results demonstrate that GB-1 and GB-3 cells are the most sensitive, GS-1 cells are intermediate, and GB-2 cells are least sensitive to inhibition by CGP 41251. In summary, the effects of CGP 41251 on cell

![Graph showing BrdUrd incorporation](image-url)

- **Ctrl**
- **CGP41251 200 nM**
- **CGP41251 400 nM**
- **CGP41251 800 nM**

% labeled nuclei

- **GS-1**
- **GB-1**
- **GB-2**
survival depends on both the dosage and the duration of exposure to the drug and the particular cell line.

**Cell Cycle Analysis.** To investigate possible effects on cell cycle progression, exponentially dividing cultures of GB-1 and GB-2 cells were treated with the DMSO solvent (control) or with CGP 41251 (800 nM) for 4 days, and then the cells were processed for flow cytometry (Table 3). With both cell lines, the drug caused a 2- to 3-fold increase in the fraction of the total cell population that was in G1-M and a decrease in the fraction of cells in S phase. With the GB-1 cells, there was no significant change in the fraction of cells in G1-M, and in GB-2 cells, there was a slight decrease in this fraction. The inactive compound, CGP 42700, had no effect on cell cycle distribution in cultures of GB-1 (Table 3) and GB-2 cells (data not shown). Additional studies, not shown here, indicated that exposure of GB-1 and GB-2 cells to TPA (100 ng/ml) also led to an increase of cells in G1-M and a decrease in S phase; the combination of TPA plus CGP 41251 further enhanced the accumulation of cells in G1-M.

**Table 3  Cell cycle analysis**

<table>
<thead>
<tr>
<th></th>
<th>GB-1</th>
<th>GB-2</th>
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<tbody>
<tr>
<td>G1-G1</td>
<td>59.2</td>
<td>52.0</td>
</tr>
<tr>
<td>S</td>
<td>30.0</td>
<td>35.2</td>
</tr>
<tr>
<td>G1-M</td>
<td>10.8</td>
<td>12.8</td>
</tr>
<tr>
<td>G1-G1</td>
<td>5.4</td>
<td>10.4</td>
</tr>
<tr>
<td>S</td>
<td>5.6</td>
<td>14.8</td>
</tr>
<tr>
<td>G1-M</td>
<td>87.5</td>
<td>85.2</td>
</tr>
<tr>
<td>G1-G1</td>
<td>7.5</td>
<td>10.4</td>
</tr>
<tr>
<td>S</td>
<td>12.5</td>
<td>18.2</td>
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<tr>
<td>G1-M</td>
<td>85.0</td>
<td>78.0</td>
</tr>
<tr>
<td>G1-G1</td>
<td>5.0</td>
<td>10.0</td>
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<tr>
<td>G1-M</td>
<td>90.0</td>
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</tbody>
</table>

*ND, not determined.*

**Formation of Giant Nuclei with Extensive Fragmentation.** To investigate possible changes at the level of cellular morphology and differentiation, cultures of GB-1, GB-2, and GS-1 cells were incubated with CGP 41251, TPA, or a combi-
of fragmented cells in the COP 41251-treated group was significantly smaller than the fraction in the group treated with TPA alone. Then the cultures were fixed and processed for staining as described in “Materials and Methods.” Denoted are the means of triplicate assays with standard variation in percent. In all three replications, there was significant association by the equal of OB-2 cells. Fig. 5 shows a typical example of a culture of GB-2 cells. Fig. 5A and B, show that all of the cells expressed the glial-specific marker GFAP. However, none of the cells expressed the neural-specific markers NEF160, or MAP2 before or after treatment with COP 41251, TPA, or the two drugs combined (data not shown). Under control conditions (Fig. 5A and B), the nuclei were polymorphic and predominantly oval, with occasional mitoses. After exposure to COP 41251 at 0.8 μM for 4 days (Fig. 5C and D) the cells were flatter and larger in size and acquired giant nuclei with extensive fragmentation and apoptotic bodies (open arrow). The giant nuclei in the COP 41251-treated cultures had the longest diameter, which was 2.7-fold that of the untreated control cultures. The cells that formed apoptotic bodies in this culture no longer expressed GFAP (Fig. 5C), nor did they express NEF160 or MAP2 when stained with the respective antibody (data not shown). However, not all of the cells underwent these changes. As shown in Fig. 5C and D, some of the cells continued to express GFAP and retained an oval nuclear morphology. In contrast to the effects of COP 41251, exposure to TPA at 100 ng/ml did not cause an increase in fragmented nuclei (Fig. 5F). Combined exposure to COP 41251 and TPA tended to resemble the effects of COP 41251 alone (Fig. 5H and data not shown). To quantify these observations, the morphologies of the nuclei were evaluated on a large number of cells and assigned to the following categories: oval, fragmented, multinucleated, mitotic, pyknotic, and multilobular. As summarized in Table 4, when compared to control untreated GB-2 cells, treatment with 0.8 μM COP 41251 for 4 days caused the following changes. The number of cells containing fragmented nuclei increased from 1.7 to 65%, and the number of mitotic nuclei decreased to 3.9 to 1.4%. On the other hand, following treatment with 100 ng/ml TPA for 4 days, there was no increase in the number of fragmented nuclei (1.7% versus 2.1%), and there was a slight increase in the number of mitotic cells, from 3.9 to 4.6%, and multinucleated cells, from 0.8% to 3.4%. Treatment of GB-2 cells with 0.8 μM COP 41251 plus 100 ng/ml TPA for 4 days resulted in an increase in the number of fragmented nuclei from 1.7 to 54% and an increase in the number of mitotic nuclei from 3.9 to 6.4%. Thus, TPA slightly inhibited the formation of fragmented giant nuclei and protected the cells against the inhibitory effects of COP 41251 with respect to mitosis.

The marked induction of nuclear fragmentation by COP 41251 and the inhibition of this process by TPA were also seen with the GB-1 and GS-1 cells. Time course studies with the GB-1 cells showed that the induction of giant nuclei with fragmentation and apoptotic bodies by 0.8 μM COP 41251 occurred in about 50% of the cells within 24 to 48 h. Dose-response studies with GB-2 cells showed that the concentration of COP 41251 required to produce 50% of the maximum induction of giant nuclei with fragmentation and apoptotic bodies was about 0.4 μM. Additional studies indicated that the induction of nuclear fragmentation by COP 41251 was markedly inhibited when the cells were grown in 0.2% rather than 20% FCS, or when the cells were treated with actinomycin D (2.5 μg/ml), cycloheximide (10 μg/ml), or aphidicolin (1 μg/ml), during the time of treatment with COP 41251 (data not shown). The treated GB-2 cells were also examined by transmission electron microscopy (Fig. 6). Various stages of apoptosis with loss of cell-cell contact, nuclear condensation, and formation of apoptotic bodies (Fig. 6B, cell b), as well as membrane blebbing, vacuolization, and cellular fragmentation (Fig. 6B, cell a), were observed. As commonly seen in cells undergoing apoptosis.

**Table 4  Nuclear morphology of GB-2 cells after exposure to COP 41251 and TPA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oval</th>
<th>Multi-nucleated</th>
<th>Fragmented</th>
<th>Mitotic</th>
<th>Multi-lobular</th>
<th>Pyknotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.4 ± 8.4</td>
<td>0.8 ± 0.5</td>
<td>1.7 ± 0.3</td>
<td>3.9 ± 0.5</td>
<td>2.4 ± 0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>COPG41251</td>
<td>28.7 ± 2.5</td>
<td>1.5 ± 0.2</td>
<td>65.2 ± 5.4</td>
<td>1.4 ± 1.1</td>
<td>2.1 ± 1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>TPA</td>
<td>84.9 ± 3.3</td>
<td>3.4 ± 2.0</td>
<td>2.1 ± 1.5</td>
<td>4.6 ± 2.6</td>
<td>4.1 ± 1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>COPG41251 + TPA</td>
<td>27.4 ± 9.6</td>
<td>3.6 ± 0.3</td>
<td>54.4 ± 8.3</td>
<td>6.4 ± 4.4</td>
<td>2.2 ± 0.2</td>
<td>6 ± 5.7</td>
</tr>
</tbody>
</table>

**Fig. 5** Morphology of GB-2 cells after exposure to COP 41251 or TPA. The cells were cultured on LabTek chamber slides with COP 41251 or TPA for 4 days and then fixed and stained with antibodies specific for GFAP (A, C, E, and G). The nuclei were stained with DAPI (B, D, F, and H). The control cultures (A and B) show GFAP-positive cells (A). Nuclei in the control culture (B) are predominantly oval and polymorphic with occasional mitoses (arrow). The majority of cells treated with 0.8 mM COP 41251 for 4 days (C and D) form giant nuclei (arrow) with apoptotic bodies (open arrow). Some of the COP 41251-treated cells are GFAP negative (C, +). Some GFAP-positive cells with oval nuclei are still present in the culture (C and D, solid arrow). After treatment with TPA (100 ng/ml) for 4 days (E and F), most of the cells still display an oval nuclear morphology with occasional mitoses (F, solid arrow). Cells treated with the combination of COP 41251 plus TPA (G and H) display oval and polymorphic nuclei, frequent mitoses (H, solid arrow), and multiple nuclei (H, open arrow). The combination led to a modest decrease of cells with giant nuclei and apoptotic bodies (H) when compared to cells treated only with COP 41251 (D). H, bar, 50 μm.
Fig. 6 Transmission electron microscopy of GB-2 cells after exposure to 0.8 mM COP 41251 for 4 days. The control culture (A) shows glioblastoma cells with typical multilobed nuclei. Exposure to COP 41251 for 4 days (B) leads to apoptosis. Cell b is in the process of apoptosis during the formation of apoptotic bodies (arrow); cell a is disintegrating and shows extensive vacuolization. A and B: bar, 2 μm.

Viability and Nuclear Morphology of Nontransformed Astrocyte Cultures. To investigate the effects of COP 41251 on nontransformed astrocytes, primary human astrocyte lines were established and examined for viability and nuclear morphology after exposure to COP 41251. One nontransformed astrocyte line (A-1) was obtained from a noninfiltrated brain sample of a patient undergoing resection of a glioblastoma multiforme, and the other (A-2) was derived from a brain sample of a patient with epilepsy. The nuclear morphology of these cell lines was evaluated after a 5-day exposure to various concentrations of COP 41251 (Fig. 7). Only about 1% of the A-1 cells displayed apoptotic bodies and giant nuclei in the absence or presence of 0.8 μM COP 41251. The A-2 cells also showed about 1% apoptotic cells in the control culture and 3, 4, and 6% giant nuclei with fragmentation and apoptotic bodies after a 5-day exposure to COP 41251 at 0.2, 0.4, and 0.8 μM, respectively. In contrast, GB-2 cells treated in parallel with similar concentrations of COP 41251 showed an increase from 5.2% under control conditions to 21, 34, and 85% after exposure to 0.2, 0.4, or 0.8 μM COP 41251, respectively. The selective increase in nuclear fragmentation in GB-2 cells was confirmed after treatment for only 24 h with COP 41251 (data not shown). Furthermore, the viability of glioblastoma cells was compared to that of the nontransformed cells after treatment with COP 41251, using the MTT assay. Cultures of GB-1, GB-2, GS-1, A-1, and A-2 cells were treated with COP 41251 at 0.8 μM and compared to DMSO-treated control cultures. There was a significant decrease in viability among the tumors cell lines, and 30, 50, and 60% in the GB-1, GB-2, and GS-1 cells, respectively. In contrast, the two astrocyte lines A-1 and A-2 showed a viability of greater than 90%. Thus, normal astrocyte cell lines are only minimally affected by exposure to COP 41251 in contrast to glioblastoma cell lines.

Antitumor Activity. The antiproliferative effect of COP 41251 was further investigated in xenografts. Two previously described human glioblastoma cell lines, U87 MG and U373 MG, were used for the tumorigenicity studies since they have been shown to grow rapidly in nude mice in contrast to the above-described GB-1 and GB-2 lines. In this study, serially passaged tumor fragments of U87 MG as well as U373 MG cells were grafted s.c. into athymic mice, and the mice were observed for tumor formation for the following 18 or 25 days, respectively. The mice were treated once daily with COP 41251, with either 75 mg/kg body weight p.o. or 10 mg/kg body weight i.v., beginning on day 5 after transplantation and continuing to day 17. As shown in Fig. 8A, the growth of the transplanted U87 MG tumor was inhibited by about 50% when the athymic mice were treated with COP 41251, either when administered p.o. or i.v. Growth of the U373 MG grafts was completely inhibited when the animals were treated with COP 41251 (Fig. 8B). Furthermore, in the group of mice carrying the U373 MG tumor, the drug was removed at day 17, and the animals were observed for
Fig. 7  Nuclear morphology of nontransformed astrocyte lines, A-1 and A2, and the GB-2 glioblastoma-derived cell line after exposure to CGP 41251. The cultures were seeded on LabTek chamber slides and maintained in the presence of the indicated concentrations of CGP 41251 for 5 days. They were then fixed, and the DNA was stained with DAPI and analyzed as described in “Materials and Methods” and Table 4. Bars, SD.

DISCUSSION
Glioblastoma multiforme, also designated grade IV astrocytoma, is a common form of primary human brain tumors (18). It is associated with a poor prognosis, even with the most advanced current types of multimodal therapy (19). Previous studies have demonstrated the expression of several isoforms of PKC in astrocytomas of various grades (20–24) and provided evidence for a role of PKC in regulating the proliferation of both astrocytes and astrocytoma cells in vitro (25, 26). This study demonstrates that both a potent activator of PKC, TPA, as well as a potent inhibitor of PKC, CGP 41251, inhibit the in vitro proliferation of human glioblastoma cells and certain other types of human brain tumor-derived cell lines (summarized in Table 2). Furthermore, the inhibition obtained with TPA plus CGP 41251 is greater than that obtained when each agent is used separately, suggesting that they inhibit growth by different mechanisms. Consistent with this conclusion is the finding that cultures treated with CGP 41251 form giant nuclei, whereas cultures treated with TPA contain multinucleated cells.

At the present time, it is not apparent why both TPA, an activator of PKCs, and the PKC inhibitor CGP 41251 cause growth inhibition of the glioma cell lines (Fig. 1). This is apparently not simply because TPA causes down-regulation of PKCs, since TPA-mediated growth inhibition was seen at doses of TPA (0.1 to 1 ng/ml; Fig. 1A) that did not produce detectable down-regulation of PKCs α, δ, ε, or ζ (Fig. 2). Alternatively, stimulation of the activity of one or more specific isoforms of PKC in these cells by TPA might lead to growth inhibition. Thus, it has been observed that overexpression of specific isoforms of PKC either stimulate or inhibit growth in the presence of TPA, depending on the cell type (4, 27). Furthermore, overexpression of PKCδ in CHO cells results in growth inhibition (28), and when these derivatives were treated with TPA, the cells arrested in G2-M and there was an accumulation of cells in telophase (28). It is of interest that although TPA is a tumor promoter in mouse skin, TPA inhibits the in vitro growth of not only human glioblastoma cells, as indicated in the present study, but also the growth of a variety of human melanoma (29), breast (30), and colon (27) cancer cell lines. In the latter cases, the

the subsequent 9 days. There was negligible regrowth of the tumor during the latter period of time. Thus, CGP 41251 is also a potent inhibitor of the tumorigenicity of human glioblastoma cells in athymic mice.
Fig. 8 Antitumor activity of CGP 41251. Antitumor activity was tested using xenografts of the human glioblastoma multiforme cells U87 MG (A) and U373 MG (B), as described in “Materials and Methods.” The compound was administered from day 5 to day 17 following s.c. transplantation of the tumor cells, as indicated. In both in vivo tumor growth experiments, administration of CGP 41251 significantly inhibited tumor growth ($P < 0.001$). In both cases, the placebo and treated curves were significantly different, and the two modes of administration of CGP 41251 were not significantly different ($A, P = 0.9; B, P = 0.6$). Bars, SD.

precise mechanism of inhibition is also not known. Other activators of PKC that lack tumor-promoting activity, for example the bryostatins (30–33), might therefore, be useful agents in cancer therapy.

The present studies establish the fact that the staurosporine derivative CGP 41251 is a potent inhibitor of the in vitro growth of nine early-passage cell lines derived from the following human tumors: five glioblastoma multiforme, one mixed glioblastoma and gliosarcoma, two astrocytomas, and one choroid plexus carcinoma. Using growth-curve studies, the $IC_{50}$ was in the range of 0.4 $\mu M$, although the GB-3 glioblastoma cell line was relatively resistant, since inhibition of growth required 0.8 to 1 $\mu M$ CGP 41251 (Table 2). However, in clonogenicity studies, this line was highly sensitive (Fig. 4). More detailed studies suggest that CGP 41251 inhibited the proliferation of these cell lines through both cytostatic and cytocidal mechanisms. However, although CGP 41251 has a high selectivity for inhibition of PKC (7), our studies do not exclude the possibility that its growth-inhibitory effects in the above-described cell types is due, at least in part, to inhibition of other protein kinases. Of particular interest might be inhibitory effects on the functions of the platelet-derived growth factor and epidermal growth factor receptors (8), since these receptors have been shown to be frequently activated in glioblastomas through mutations and amplifications of the corresponding genes (34–37).

We hypothesize that CGP 41251 inhibits the growth of glioblastoma cells, at least in part, by arresting cells at the $G_2$ to $M$ transition point, and to a variable extent during $G_2$ or $G_1$. This hypothesis is consistent with the flow cytometry data (Table 3), the inhibition of BrdUrd labeling (Fig. 3), the markedly reduced number of mitotic figures, and the increased number of giant nuclei with fragmentation and apoptotic bodies seen in the treated cultures (Fig. 5). Studies in yeast have shown that PKC is required for the $G_2$ to $M$ transition (38). Furthermore, yeast cells deficient in the cdc2/cyclin B complex are unable to transit from the $G_2$ to the $M$ phase and form giant nuclei (39). In recent studies, we have found that glioblastoma cells treated with CGP 41251 exhibit a decrease in cdc2/cyclin B histone H1 kinase activity. Other inhibitors of PKC have been found to arrest cells in $G_2$ or result in inhibition of cdc2 kinase activity (40–45). High concentrations of staurosporine lead to the arrest of transformed cells in $G_2$ but arrest nontransformed cells in both $G_1$ and $G_0$ (44, 45). However, further studies are required to determine the precise effects of CGP 41251 on cell cycle progression and cyclin-CDK activity.

A striking finding in the present study is that CGP 41251 induces nuclear fragmentation and other morphological changes characteristic of apoptosis (Fig. 5). A common feature of apoptosis is internucleosomal DNA fragmentation, which was investigated by preparation of high molecular weight DNA from treated cultures as well as through various in situ procedures. However, DNA fragmentation was not observed after treatment with CGP 41251 in these glioblastoma cells (data not shown). Nevertheless, apoptotic cell death has been shown to occur without DNA fragmentation (46–48). The absence of DNA

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*Fig. 8 Antitumor activity of CGP 41251. Antitumor activity was tested using xenografts of the human glioblastoma multiforme cells U87 MG (A) and U373 MG (B), as described in “Materials and Methods.” The compound was administered from day 5 to day 17 following s.c. transplantation of the tumor cells, as indicated. In both in vivo tumor growth experiments, administration of CGP 41251 significantly inhibited tumor growth ($P < 0.001$). In both cases, the placebo and treated curves were significantly different, and the two modes of administration of CGP 41251 were not significantly different ($A, P = 0.9; B, P = 0.6$). Bars, SD.

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4 M. Begemann, unpublished results.
fragmentation might reflect the absence of endonucleases in these particular cell types or, alternatively, different pathways leading to cell death. The CGP 41251-induced nuclear fragmentation with apoptotic bodies was partially inhibited by TPA (Fig. 5), which is consistent with the ability of certain phorbol esters to inhibit apoptosis in thymocytes (49). Serum deprivation, which is consistent with the ability of certain phorbol esters (50), has been shown to uncouple cell cycle control in rat diploid fibroblasts, leading to tetraploidization, which was inhibited also by serum deprivation, cycloheximide, and actinomycin D (43). Other investigators (50) have recently reported that tamoxifen, which also inhibits PKC, also induces apoptosis in human malignant gliomas, but they did not examine cell cycle-related effects. Nuclear fragmentation and micronucleation, as well as G2 arrest, were described in leukemic lymphocytes after exposure to staurosporine, the parental compound, from which CGP 41251 was derived (44). Immunohistochecmical studies (data not shown) provide evidence that GB-1, GB-2, and GS-1 cell lines used in the present study express a mutant p53 protein. This suggests that CGP 41251 induces apoptosis by a p53-independent pathway and, therefore, may be useful against a variety of human tumors. Furthermore, cultures of normal astrocytes were relatively resistant to the toxic effects of CGP 41251, including the induction of nuclear fragmentation (Fig. 7).

Finally, CGP 41251 when given either by the oral route or i.v. markedly inhibited the tumorigenicity of two previously characterized human glioblastoma cell lines, U87 MG and U373 MG, following s.c. transplantation into athymic mice. This finding extends previous studies indicating that CGP 41251 inhibits the growth of other types of human cancer cells in athymic mice (7). Thus, CGP 41251 might be a useful agent when administered either alone or in combination with other agents for the therapy of human brain tumors.

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