SPECIAL REPORT

Hypericin: A Potential Antiglioma Therapy

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HYPERICIN, A POLYCYCLIC aromatic dione isolated from plants, is presently being clinically evaluated as an antiviral agent in the treatment of human immunodeficiency virus (HIV) infection. In addition, it is known to be a potent protein kinase C inhibitor. To evaluate its potential as an inhibitor of glioma growth, an established (U87) and low-passage glioma line (93-492) were treated with hypericin in tissue culture for a period of 48 hours after passage. Hypericin inhibited the glioma growth in a dose-related manner, with a marked inhibition of growth in the low-micromolar concentration range (e.g., in line U87 and low-passage line 93-492, a concentration of hypericin of 10 μmol/L produced 62 and 76% decreases in [^{3}H]thymidine uptake, respectively). Because the reported inhibitory effects of protein kinase C are enhanced by visible light, [^{3}H]thymidine uptake was measured in both the presence and the absence of visible light. In glioma line A172, the presence of light slightly increased the inhibitory effect of hypericin. Moreover, an apoptosis (i.e., programmed cell death) assay was performed to determine whether the treatment of glioma cells with hypericin was cytostatic or cytocidal. Cells were harvested, and purified deoxyribonucleic acid (DNA) was analyzed by agarose gel electrophoresis. DNA from cells treated with hypericin for 48 hours exhibited a classical "ladder" pattern of oligonucleosome-sized fragments characteristic of apoptosis. These data suggest that the proven safe drug hypericin may have potential as an antiglioma agent; we suggest clinical trials. (Neurosurgery 35:705–710, 1994)

Key words: Brain neoplasm, Glioma, Hypericin, Protein kinase C

Two aromatic polycyclic diones, hypericin and pseudohypericin, are derived from plants of the *Hypericum* family (Saint Johnswort, *H. perforatum*). They have recently been reported to be highly effective in preventing viral-induced manifestations that follow a variety of retrovirus infections both in vitro and in vivo (16, 19, 20, 23). These compounds probably interfere with viral infection and/or spread by direct inactivation of the virus or by preventing virus shedding, budding, or assembly at the cell membrane (23, 32). Their administration can completely prevent the rapid splenomegaly induced by aggressive murine viruses; in vitro studies with infected cell cultures demonstrate that the compounds effectively inhibit the release of reverse transcriptase activity into the growth medium but do not affect total viral messenger ribonucleic acid levels or the expression of viral antigens within the cells (23). Such work has indicated very direct cellular targets for the activity of these drugs with limited systemic toxicity. This property distinguishes their mode of action from that of the major antiretrovirus group of nucleoside analogues (23). Although the precise intracellular mechanisms for its antiviral effects are unknown, this encouraging laboratory work has propagated an evaluation of hypericin in a phase I trial for patients with HIV infection (14, 25, 34).

Malignant gliomas possess very high protein kinase C (PKC) activity when compared with nontransformed glia, which is strongly correlated to the growth rates of these tumor cells in vitro (5, 6). Surgically resected frozen human glioma specimens also display elevated PKC activity within the range of activity measured in established glioma lines (5). This elevated PKC activity may function as a target for the inhibition of glioma growth in vitro (1, 4, 27) and is currently being evaluated with some success in clinical trials for use as a chemotherapeutic target in vivo (2, 7, 8, 35). The molecular structures of hypericin (*Fig. 1*) and pseudohypericin are similar to calphostin C, a relatively specific inhibitor of PKC (30, 31). Hypericin has been previously demonstrated to be a potent PKC inhibitor (30), a mechanism that has been hypothesized

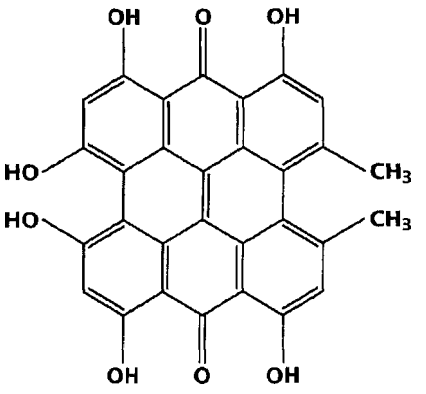

FIGURE 1. The structure of hypericin: 1,3,6,8,13-hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-opqra]perylene-7,14-dione.

to explain some of its antiviral activity. Hypericin, like calphostin C, interacts with the regulatory domain of the PKC enzyme (17, 18). These compounds, therefore, represent an alternative class of inhibitors of PKC, to be differentiated from those agents such as staurosporine, which are speculated to compete for adenosine 5'-triphosphate on the catalytic subunit of the enzyme.

Hypericin effectively crosses the blood-brain barrier, having previously been administered to humans as an antidepressant as long as 4 decades ago with apparent salutary effects (9). The ubiquitous availability of the Saint Johnswort plant worldwide and the relatively convenient and inexpensive bioextraction make this compound particularly attractive for use in HIV-related disease and for central nervous system antineoplastic applications.

We studied the effects of hypericin on the growth of both established and low-passage malignant glioma lines in vitro. We show here that hypericin inhibits the growth of malignant gliomas in vitro in a dose-related manner; furthermore, we demonstrate that the compound induces a programmed cell death (apoptosis) signal in these cells.

MATERIALS AND METHODS

Glioma cell cultures

The previously characterized human malignant glioma cell lines A172 and U87-MG were used in this study (obtained from the American Type Culture Collection). The low-passage primary glioma culture (93-492) was established at our institution; this was derived from glioblastoma tissue removed from a 27-year-old patient. Lines were maintained in 25-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units/ml; 10 μg/ml), and 10 mmol/L Hepes buffered to a pH of 7.0 (all medium constituents were purchased from Sigma Chemical Co., St. Louis, MO). Cells were grown at 37°C in a humidified 5% CO_2 incubator. The established lines U87 and A172 were routinely tested for mycoplasma contamination and were regularly passaged.

Glioma proliferation [^{3}H]thymidine uptake assay

Our method for determining the rates of glioma proliferation has been previously published (4, 6). After passage, each cell line was seeded at a density of 10^4 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 the cells to adhere to the coverslips, the wells were flooded and washed with phosphate-buffered saline. After an addition of 1 ml of feeding medium (containing 10% fetal bovine serum) to each well, the PKC inhibitor hypericin (*Fig. 1*) (LC Service Corp., Woburn, MA) was then added to the wells at predetermined concentrations in replicates of three. After a 48-hour incubation, wells were pulsed with 1 μCi of [^{3}H]thymidine/ml for a period of 6 hours before harvest. To measure thymidine incorporation, the coverslips with adherent cells were washed 4 times with phosphate-buffered saline and were placed in scintillation vials containing 5 ml of scintillation fluid (Cytoscint; ICN Chemical, Irvine, CA) for determination in a β-counter. It has been previously demonstrated that uptake of [^{3}H]thymidine into cells by this technique is a reliable index of DNA synthesis (1).

DNA fragmentation (apoptosis) assay

After treatment with hypericin for 48 hours in 25-cm^2 tissue culture flasks at the same cell density and medium as above, washed cell pellets were resuspended in lysis buffer (0.5% sodium dodecyl sulfate, 0.1 mol/L NaCl, 10 mmol/L *N,N'*-1,2-ethanedylbis[*N*-(carboxymethyl)glycine], 0.01 mol/L Tris-HCl, pH 8.0) in the presence of 0.1 mg/ml proteinase K for 18 to 24 hours at 37°C. The samples were extracted twice with phenol:chloroform/isoamyl alcohol and precipitated by the addition of 0.10 volume of 2 mol/L sodium acetate and 2.5 volumes of ethanol. After resuspension in 10 mmol/L Tris-1 mmol/L *N,N'*-1,2-ethanedylbis[*N*-(carboxymethyl)glycine], pH 7.4, samples were treated with DNase-free RNase for 2 hours at 37°C. After reextraction and reprecipitation, samples were dissolved in 10 mmol/L Tris, 0.1 mmol/L *N,N'*-1,2-ethanedylbis[*N*-(carboxymethyl)glycine], pH 7.4. DNA concentration was determined by A_{260} measurements using a Beckman DU-640 spectrophotometer. Equal amounts of DNA were electrophoresed on a 1.2% agarose gel containing 0.5 mg/ml ethidium bromide and visualized by UV fluorescence.

RESULTS

Hypericin effects upon glioma proliferation

To assess the effects of hypericin on the proliferation of gliomas, the drug was added to glioma cultures for a period of 48 hours, after which time [^{3}H]thymidine uptake was determined (*Fig. 2, A* and *B*). Hypericin inhibited the growth of both the established and low-passage tumors in a dose-related manner.

To determine the effects of visible light on the growth inhibition produced by hypericin, cultures were treated under dark and in the presence of visible light. Representative [^{3}H]thymidine uptake is shown for glioma line A172 in *Figure*

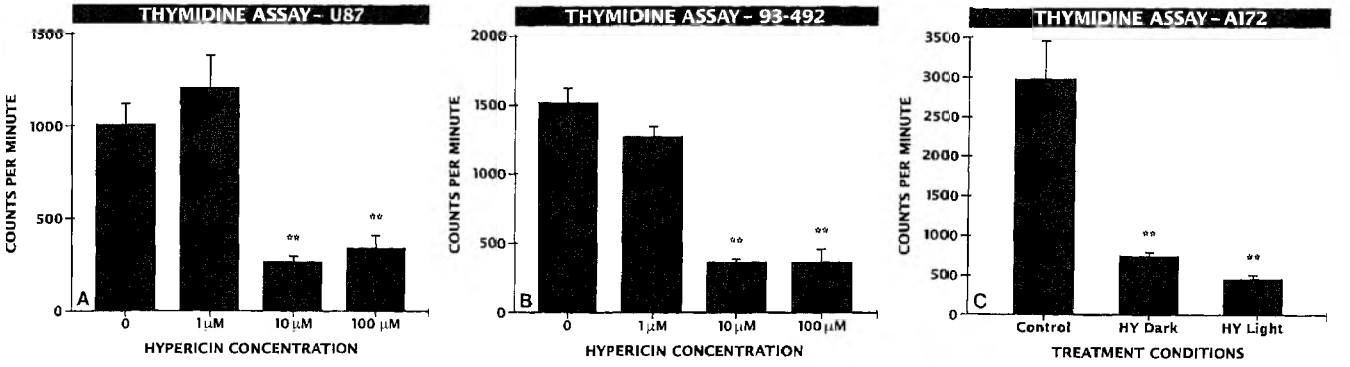

FIGURE 2. [^{3}H]Thymidine uptake after the addition of hypericin to the glioma-established cell line U87 (*A*) and low-passage malignant glioma line 93-492 (*B*) cultures for a period of 48 hours in vitro. Note the dose-related significant decrease in thymidine uptake after the treatment of each of the glioma cell lines. In a similar manner, the [^{3}H]thymidine uptake of glioma line A172 is significantly inhibited by hypericin in the dark but is facilitated under light conditions (*C*). Values represent mean + standard error of the mean of triplicate values (, significantly different from control values, using one-way analysis of variance, Duncan's multiple comparison with $P < 0.01$).**

2C. Growth inhibition was somewhat facilitated in the presence of visible light.

Hypericin induces programmed cell death (apoptosis) in malignant glioma cell lines

Treatment of A172 glioma cells with 10 μmol/L hypericin for a 48-hour period resulted in significant cell detachment. Floating cells were permeable to trypan blue dye, indicating that they had lost viability. After adherent and floating cells were collected and pooled, DNA was purified and analyzed by agarose gel electrophoresis. The results in *Figure 3* reveal a classical ladder pattern of oligonucleosomal-sized fragmented DNA, the molecular hallmark of apoptosis, for glioma cells treated with hypericin. No significant degradation of DNA was observed in untreated cells, as evidenced by the presence of an intensely stained band of high molecular weight DNA and the lack of ethidium bromide-stained material in the region of the gel corresponding to low-molecular weight DNA. As a positive control for the induction of apoptosis, glioma cells treated with 100 ng/ml tumor necrosis factor-α also displayed a prominent ladder of fragmented DNA. Comparable results were observed when glioma cells were treated with hypericin in the dark (data not shown).

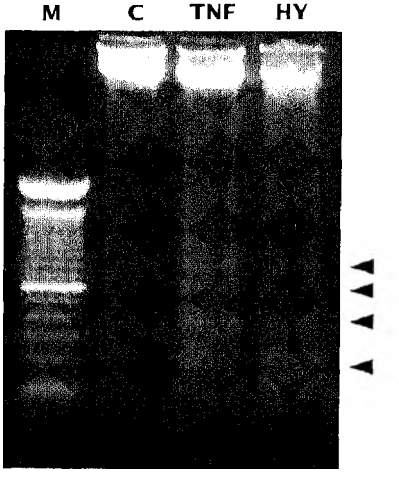

FIGURE 3. Hypericin induces apoptosis in malignant glioma cell lines. Treatment of glioma cell line A172 with hypericin and DNA isolation are described under Materials and Methods. DNA isolated from A172 cells in the absence of hypericin (*lane C*) and in the presence of 10 μmol/L hypericin (*lane HY*) were electrophoresed in 1.2% agarose and stained with ethidium bromide. Note the presence of oligonucleosome-sized fragments producing a classic ladder pattern in the lane containing DNA from treated cells (*arrows*). As a positive control for apoptosis, A172 cells were treated with 100 ng/ml tumor necrosis factor-α (*lane TNF*). The sizes of oligonucleosomal fragments were determined by comparison to DNA size markers in *lane M*.

DISCUSSION

Hypericin is currently being evaluated as an antiviral agent in the treatment of HIV infection (14, 25, 34). Hypericin has been regarded as a promising potential antiacquired immunodeficiency syndrome drug since 1988, when it was reported that a single injection of this compound prevented splenomegaly and death in mice infected with Friend leukemia virus. Administration of this compound to mice at the low doses sufficient to prevent retroviral-induced disease appears devoid of undesirable side effects (23). The exact mechanism of the antiviral activity of the aromatic polycyclic diones has not been elucidated; however, hypericin and pseudohypericin have recently been identified as relatively specific inhibitors of PKC (30), which may represent a potential candidate for such activity. Both of the compounds inhibited PKC from rat brain with IC_{50} values of 1.7 and 15 μg/ml for hypericin or pseudohypericin, respectively, with little activity against PKA or calmodulin-dependent protein kinases at much higher dosages (30). The antiretroviral activity of hypericin is greater than that of pseudohypericin, which is also consistent with their inhibitory activity against PKC (30).

It has previously been demonstrated that PKC activity and the rate of proliferation of glioma lines are highly coupled in vitro (5, 6). The very high activity characteristic of gliomas may provide a target for novel therapies using PKC inhibitors. Initial studies using two well-characterized nonspecific PKC inhibitors, tamoxifen and staurosporine, revealed a potent inhibitory effect of these drugs on the proliferation of low-passage primary and established glioma cell lines (1, 6, 27). These findings prompted the current investigation of hypericin, another anti-PKC agent, on glioma cells.

The results from the present study indicate that hypericin may inhibit the growth of glioma cell lines in vitro in a dose-related manner as measured by thymidine uptake. We also find that hypericin is a potent inducer of glioma cell death, which is accompanied by extensive degradation of genomic DNA into discrete oligonucleosomal fragments characteristic of apoptosis. Hypericin previously has been reported to exert both antiproliferative and cytotoxic effects on tumor cells (23, 30), these actions being attributed to inhibition of PKC activity by hypericin. Our studies confirm and extend this hypothesis in that PKC had been implicated as a negative regulator of apoptosis (12), which could account for our observation that hypericin induced an apoptotic response in glioma cells. Additional studies are warranted to establish that the induction of apoptosis by hypericin is not the result of other described actions of the drug, including membrane damage (11), generation of singlet oxygen and free radicals (10), and inactivation of mitochondrial enzymes and impairment of mitochondrial function (33). However, the cytotoxic activity of the compound in the present study is within the same concentration range as its PKC inhibitory activity, which suggests that the cytotoxic activity may be the result of inhibition of PKC and subsequent induction of apoptosis in these cells.

Hypericin has a long and well-documented history as a photodynamic compound. In previous studies, both the antiviral (22, 28) and antitumor (33) effects of hypericin are markedly enhanced by visible light. Data from the present study indicate that the inhibitory effect on glioma growth is increased approximately 13% by visible light, indicating that light may facilitate, but is not essential for, the antitumor effect in vitro. Although the doses necessary for antiviral and anti-PKC activity are less than those associated with clinical hypericism (ingestion of large amounts of hypericin from plants of the *Hypericum* genus by grazing animals causes skin irritation and fever), the photosensitization effect must be considered with clinical use (13, 15).

Data from the present study indicate that the drug inhibits glioma growth at least as well as tamoxifen in vitro (IC_{50} values of <10 vs 10 μmol/L) (1), an agent currently being evaluated for efficacy against malignant gliomas. An initial phase I trial of very high dose tamoxifen at our institution indicates that approximately 35 to 40% of patients with recurrent malignant gliomas (anaplastic astrocytoma or glioblastoma multiforme) stabilize or respond to high-dose tamoxifen therapy (7, 8). These encouraging results suggest potential for PKC-directed therapy and have led to the search for other well-tolerated compounds with increased potency and specificity for PKC. Tamoxifen may be considered an only moderately potent agent, as studies have illustrated that other kinase inhibitors, such as staurosporine, possess approximately 1000-fold higher activity against PKC and glioma growth (1, 4). The previously published antiviral activity of hypericin is in the same concentration range as the growth inhibitory effects upon glioma cell in vitro demonstrated in the present study, suggesting that this agent may be suitable for future chemotherapeutic trials in humans with malignant gliomas. Hypericin, like many other anticancer drugs, may kill tumor cells by initiating a programmed molecular pathway for cell suicide, designated apoptosis (3). Its photodynamic effect may potentiate its efficacy in such applications.

Hypericin is also a potent antidepressant that has been clinically used in the past, has been well tolerated, and has not caused significant side effects (9, 26, 29). In a study of patients with clinical depression who were administered hypericin, the excretion of urinary metabolites of noradrenaline and dopamine increased significantly, which paralleled a quantitative improvement in major depressive symptoms (24). The mechanism for its antidepressant action is unknown, but this work has indicated that the drug effectively crosses the blood-brain barrier. A distribution half-life of 2.0 hours and an elimination half-life of 38.5 hours have been calculated in humans; in addition, a method for rapid and specific measurement of the drug by high-performance liquid chromatography for clinical and pharmokinetic studies is available (21). Hypericin has also been synthesized, which offers an alternative supply of the compound. Given the results of the present study, the agent is proposed for phase I study for safety and possible efficacy in patients harboring recurrent malignant gliomas after standard therapy.

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COMMENTS

These results are preliminary, and we think that more experiments should be performed. In this article, Couldwell, et al. describe the effect of hypericin, a polycyclic aromatic dione and known antiviral agent, on two glioma cell lines. Specifically, they describe inhibition of [^{3}H]thymidine uptake of glioma cells after treatment with hypericin for 48 hours. This inhibition of [^{3}H]thymidine uptake was a dose-related phenomenon with a maximum effect at 10 μmol/L and minimal effect at 1 μmol/L concentration. The authors report that this effect was cytocidal, because they analyzed the DNA of cells after treatment and found a specific ladder pattern of oligonucleosome-sized fragments characteristic of cell death. They suggest that the hypericin effect is probably because of its function as a protein kinase C inhibitor.

It is unclear whether the observed effects are specific effects of hypericin on the glioma tumor cells or whether they represent nonspecific toxic effects that would be common to any cell type. In addition, this could be (as the authors briefly discussed) a membrane effect of hypericin rather than inhibition of protein kinase C activity. To better examine these possibilities, further experiments should be done with the same

concentrations of hypericin in control normal glial cells or other normal cells, such as fibroblasts, to show that there is a specific effect on the tumor cells. In addition, a time course of hypericin at lower doses, such as 1 μmol/L, which is not immediately toxic to the cells, should be performed and the numbers of cells should be counted. More thorough experiments would determine the following: 1) whether there is an inhibitory effect at lower doses for longer periods of time, and 2) the effect of longer incubations at the same low dose on morphology (phenotype) of these cells.

These data are preliminary; the effectiveness of hypericin in glioma cell cultures has not been established. Clinical studies would be premature until more cell culture studies in more glioma cell lines, as well as normal glial or other normal cell types, are performed.

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This article provides preliminary preclinical support for the phase I study of hypericin in patients with recurrent malignant gliomas. The drug is novel and has a demonstrated capacity to inhibit protein kinase C, which seems to be consistently overexpressed in malignant gliomas. A clinical trial will need further support from the laboratory, including studies in one of the many rodent brain tumor models that are readily available. Years ago, Dr. Charles Wilson said something to the effect that we will never cure a human brain tumor with chemotherapy until we cure one in rats. Perhaps this is as true now as it sounded then. It will be important to have strong data to support the use of this apparently nontoxic drug in humans.

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Couldwell and collegues continue their work on inhibitors of protein kinase C (PKC). Elevated levels of PKC are found in gliomas both in vitro and in vivo, and inhibitors of PKC appear to modulate growth in a dose-dependent manner. Promising phase I and II data regarding tamoxifen, a nonspecific inhibitor of PKC, in the setting of recurrent malignant gliomas provide compelling reasons to continue to evaluate other such agents. Hypericin, an antiviral agent just beginning phase I clinical testing in patients with acquired immunodeficiency syndrome, has been shown to be a potent inhibitor of PKC, possibly by interactions with the regulatory domain of the PKC enzyme. Data already existed to support that this agent crosses the blood-brain barrier; all that remained was to demonstrate that hypericin indeed modulated glioma growth. Couldwell et al. have demonstrated that hypericin inhibits glioma proliferative cell growth in vitro, as measured by tritiated thymidine uptake studies. Programmed cell death appeared to be induced by hypericin as well. The mechanism of apoptosis in this experiment is uncertain but is postulated to be secondary to PKC inhibition as well, with PKC acting as a negative regulator of apoptosis. The authors discuss the role of hypericin as a photodynamic compound and show a small enhancement in the antiproliferative effect with the addition of visible light.

Given the lack of significant toxicities in previous work with patients with depressive disorders, this agent does appear to be a candidate drug for phase I or II testing in recurrent gliomas. Further work is proposed to further elucidate the specific mechanisms of apoptosis and PKC inhibitory activity. Although the photodynamic effect appears to be measurable in the laboratory, one wonders how to exploit this feature in clinical trials. It is reasonable to hope that newer, nontoxic therapies will emerge as a consequence of straightforward, translationally based work, such as that presented by Couldwell et al. Combination therapies that include antiproliferative agents with agents that selectively induce apoptosis or agents that "differentiate" tumor cells toward a more normal phenotypic state would be a welcomed change from nonselective, highly toxic chemotherapies that have marginal efficacy.

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The need for effective agents for the treatment of malignant gliomas is apparent. It is remarkable how few chemotherapeutic agents have been shown to have therapeutic efficacy for this tumor type. There is major interest in the development of novel agents and new insights that will offer additional therapy options.

This work well fits these needs. A new type of agent, using a novel mechanism with a different end point, has been studied in a human cellular-based assay system. This approach is a model for a variety of research fronts.

The agent hypericin appears to have its effects via the PKC pathway, and its effect is achieving apoptosis rather than cytotoxicity. Apoptosis, or programmed cell death, is in many ways a more appealing chemotherapy. Agents such as carmustine cause immediate cell death, even on contact in culture studies. Hypericin has an apoptotic effect.

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