Inhibition of cellular growth and induction of apoptosis in pituitary adenoma cell lines by the protein kinase C inhibitor hypericin: potential therapeutic application

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Protein kinase C (PKC) is an enzyme involved in the regulation of cellular growth, proliferation, and differentiation in a number of tissues, including the anterior pituitary, in which it is also believed to play a role in hormone secretion. Protein kinase C activity and expression have been found to be greater in adenomatous pituitary cells than in normal human and rat pituitary cells and higher in invasive pituitary tumor cells than in noninvasive ones. Inhibition of PKC activity has been shown in a variety of tumor cells to inhibit growth in a dose-related fashion. The purpose of the current study was to determine whether hypericin, a potent inhibitor of PKC activity that may be administered clinically, alters the growth and proliferation in established pituitary adenoma lines and to determine if inhibition of PKC activity induces apoptosis, as reported in some other tumor cell types. Two established pituitary adenoma cell lines, A1T-20 and GH4C1, were treated with hypericin in tissue culture for defined periods following passage. Inhibition of growth was found to be dose dependent in all three cell lines in low micromolar concentrations of hypericin, as determined by viable cell counts, methylthiotetrazole assay, and \[^{3}H\]\thymidine uptake studies. Concentrations of hypericin as low as 100 nM also induced apoptosis in these established lines, whereas treatment of normal human fibroblasts with a concentration of 10 \(\mu\)M failed to induce apoptosis. The potential use of hypericin in the therapy of pituitary adenomas warrants additional in vitro investigations with the aim of later moving toward therapeutic trials in selected patients in whom surgical or medical therapy has failed.

KEY WORDS • apoptosis • hypericin • pituitary adenoma • protein kinase C

Hypericin, a conjugated quinone biosynthesized by members of the plant genus Hypericum, is a photodynamic pigment that has been found in vitro to oxidize lipids, amino acids, and proteins and to disrupt the normal function of cellular membranes.\(^{14,17,18,31,34}\) Hypericin exhibits antidepressant, antiviral, and (it has been suggested) antineoplastic properties in some cell types.\(^{5,6,10,20-22,31,33}\) Hypericin has recently been shown to be a potent inhibitor of PKC as well as of other cellular enzymes.\(^{16,31,32}\) This chemical has also been demonstrated to be a potent inhibitor of glioma growth and an inducer of apoptosis in these cells.\(^{5,10,11}\) These effects have been positively correlated with PKC activity, suggesting that inhibition of PKC is involved in these processes.

Cell growth and apoptosis are regulated by a limited set of signal transduction systems that regulate second messengers involved in the phosphorylation of proteins, ultimately controlling cellular metabolism and gene expression. Apoptosis occurs in a multitude of both normal and pathological processes. Apoptosis differs from necrosis morphologically by the presence of blebbing of plasma and nuclear membranes, by chromatin condensation and
fragmentation, and biochemically by the activation of endonucleases and proteases that result in characteristic fragmentation of nuclear DNA. Selective manipulation of this process of apoptosis may provide a mechanism by which a number of pathological conditions, including dysregulated cell growth, may be altered.

The present study was undertaken to determine if hypericin, a drug used as a clinical antidepressant and currently undergoing evaluation for use as an antiretroviral agent in treating patients with the human immunodeficiency virus, could inhibit growth and induce apoptosis in established pituitary adenoma cell lines in vitro. Here we show that the agent is a potent inhibitor of both PKC activity and adenoma growth and induces apoptosis in these cells.

Materials and Methods

Pituitary Adenoma Cell Cultures

We used the previously characterized rat pituitary adenoma cell lines AtT-20 and GH3C1 (American Type Culture Collection, Rockville, MD) in this study. The AtT-20 pituitary adenoma culture was maintained in 25-cm² tissue culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml:10 mg/ml), and 10 mM/L L-Hepes buffered to a pH of 7.0. The GH3C1 cultures were grown under identical conditions, except that the medium was supplemented with 5% fetal bovine serum. Cells were grown at 37°C in a modified 5% CO₂ incubator.

Protein Kinase C Assay

Flasks (25 cm²) containing the two established cell lines (in the presence or absence of hypericin) were rinsed with ice-cold phosphate-buffered saline, then rinsed with a homogenizing buffer containing 50 mM Tris-HCl, 2 mM dithiothreitol, 1 mM phenylmethylsulfonfluoride, and 2 mM ethyleneglycol-bis-(beta-aminoethyl ether) N,N,N’,N’-tetraacetic acid. The cells were subsequently scraped from the culture flasks, suspended in 2 ml of the solution, and homogenized with the homogenate centrifuged at 100,000 G for 60 minutes, and designated as the cytosolic fraction. The pellet was resuspended in 2 ml of the buffer containing 1% Triton X-100, homogenized, gently mixed for 30 minutes, centrifuged at 100,000 G for 60 minutes, and designated as the cytosolic fraction. The procedures were performed at 4°C, with the designated enzyme fractions then stored at −70°C prior to the PKC assay.

The method used to assay for PKC activity (adenosine triphosphate (ATP) transfer into lysine-rich histone) has been previously published.8,9 In these studies, hypericin was added to wells containing cells in midlogarithmic growth at predetermined concentrations in replicates of four. Radioactive thymidine uptake was then determined following a 72-hour incubation in concentrations of hypericin ranging from 0 to 20 μM. These concentrations previously have been demonstrated to lie within the range known to modulate PKC in vitro.8,9

Trypan Blue Dye Exclusion Assay. Following treatment of the cell cultures in varying concentrations of hypericin (0–20 μM), the culture plates were centrifuged, their medium was decanted, the cells were washed with PBS, and trypan blue dye was added to the cell suspension. Three hundred cells from each of three cultures per treatment group were counted on coded specimens by observers blinded to the groups. The percent viability (dye exclusion) was then calculated and recorded.

[3H]Thymidine Uptake Assay. To offer data complementary to those yielded by the MTT viability and trypan blue exclusion assays described above, [3H]thymidine uptake was determined as a measure of cellular proliferation. Our method for determining rates of cell proliferation using [3H]thymidine uptake has been previously published.5,8 In these studies, hypericin was added to wells containing cells in midlogarithmic growth at predetermined concentrations in replicates of four. Radioactive thymidine uptake was then determined following a 72-hour incubation in concentrations of hypericin ranging from 0 to 20 μM. These concentrations previously have been demonstrated to lie within the range known to modulate PKC in vitro.5,8

It has already been shown that uptake of [3H]thymidine into cells using this technique is a reliable index of DNA synthesis.7

Apoptosis Assay

After passage, each cell line was seeded at a density of 2.5 × 10⁴ cells per 5 ml of medium in 25-cm² tissue culture flasks and subsequently treated with hypericin for 72 hours. Washed cell pellets were resuspended in lysis buffer (0.5% sodium dodecyl sulfate, 0.1 mol/L NaCl, 10 mM/L N,N’,N’-1,2-ethanediyl-bis-(N-(carboxymethyl)glycine), 0.01 mol/L Tris-HCl, pH 8.0) in the presence of 0.1 mg/ml proteinase K for 12 to 18 hours at 37°C. The samples were twice extracted with phenol/chloroform/isooamyl alcohol and precipitated by addition of a 0.10 vol of 2 mol/L sodium acetate and 2.5 vol of ethanol. These were then resuspended in 10 mM/L N,N’,N’-1,2-ethanediyl-bis-(N-(carboxymethyl)glycine), at a pH of 7.4,
then treated with DNase-free RNase for 2 to 4 hours at 37˚C. Equal amounts of DNA were electrophoresed on a 1.2% agarose gel containing 0.5 mg/ml ethidium bromide, visualized by means of ultraviolet fluorescence, and photographed. A low-passage human fibroblast line was treated concurrently under identical conditions as a nontransformed cell control.

### Results

**Hypericin Inhibition of Protein Kinase C Activity in the Pituitary Cell Line AtT-20**

Protein kinase C activity was measured following treatment with hypericin (Fig. 1). Total AtT-20 PKC activity levels after isolation of cytosolic and particulate (membrane) fractions were found to be consistent with published levels in other pituitary tumor cells.\(^4,12\) After treatment of AtT-20 cultures with 10 \(\mu\)M hypericin, total PKC activity was inhibited significantly as early as 2 hours following treatment. Protein kinase C activity continued to decrease to approximately 25% of control activity after 24 hours of treatment.

**Hypericin Inhibition of Pituitary Adenoma Growth**

An MTT assay was performed after treatment of the pituitary established cell lines with hypericin for various periods of time over a range of concentrations to determine the effect of this drug on cellular growth. Hypericin inhibited the growth of the two established pituitary cell lines in a dose-related manner in upper nanomolar and low micromolar concentrations (Fig. 2 upper left and right). In the established line AtT-20, the inhibitory effect increased with the duration of incubation from 3 to 6 days (Fig. 2 upper left and center). Similar results were noted in line GH4C1 (Fig. 2 upper right).

To confirm the results of the MTT assay, cell viability was determined following treatment with hypericin by trypan blue dye exclusion. As shown (Fig. 2 center), the viability of cells after treatment with hypericin at concentrations greater than 1 \(\mu\)M decreased significantly as early as 24 hours in culture. Cell death increased with treatment time and reached a maximum level at 72 hours after treatment in both cell lines tested.

To explore the growth inhibition produced by hypericin further, \([^{3}H]\)thymidine uptake was determined in AtT-20 cells following treatment with various concentrations of the drug (Fig. 2 lower right). Thymidine uptake decreased in a dose-related manner after a 72-hour treatment with hypericin; significant inhibition was noted with concentrations as low as 1 \(\mu\)M during this treatment period.
Hypericin Induction of Apoptosis in Established Pituitary Adenoma Cell Lines

Following treatment of the cultures with hypericin for 72 hours, adherent and floating cells were collected and subjected to subsequent purification and analysis of DNA by agarose gel electrophoresis. In both established pituitary adenoma cell lines, AtT-20 and GH4C1, the classic ladder pattern of oligonucleosome-sized fragmented DNA, indicative of apoptosis, was noted following incubation with hypericin in nanomolar concentrations (Fig. 3 upper and center gels). In contrast, no detectable DNA degradation was found in the untreated cells, as evidenced by the absence of an ethidium bromide–stained substance in the region of the agarose gel corresponding to low-molecular-weight DNA and the presence of a band of high-molecular-weight DNA. In the low-passage human fibroblast culture, no evidence of DNA fragmentation was detected when treated with hypericin at a concentration of 10 µM.

To determine the time course of DNA fragmentation, AtT-20 cells were treated for various time periods with 10 µM hypericin. As shown in the lower gel in Fig. 3, oligonucleosomal DNA fragmentation was noted as early as 24 hours after treatment. No significant fragmentation was noted following the 6-hour treatment period.

Discussion

The PKC signal transduction system has been shown to be involved in the regulation of cellular growth, differentiation, and gene transcription.29 In pituitary cells, this enzyme is also believed to modulate hormone synthesis and secretion.1,2,19,25-27,30,35 The PKC family currently consists of a minimum of 12 structurally distinct isoforms that have been separated into three categories based on cofactor requirements.15 The classic PKCs, α, β1, β2, and γ, require calcium for activity and they are activated by the phorbol ester phorbol-12-myristate-13-acetate (PMA), whereas the novel PKCs, δ, ε, η, and θ, possess activity in the absence of calcium and remain activated by PMA, and the atypical PKCs, ξ and λ, possess activity in the absence of calcium and are not activated by PMA. Both calcium-dependent and independent PKC isoenzymes have been found in normal and adenomatous pituitary cells, suggesting their prominent role in signal transduction in anterior pituitary cells. At least five isozymes are expressed in pituitary cells (α, β, δ, ε, and ξ) with PKCη being expressed in rat GH4C1 cells.1,2,25-27 The activation by PMA indicates that the classic and novel PKC isozymes present (α, β, δ, ε, and η) may be involved in the regulation of growth and differentiation in pituitary adenomas. Moreover, prolactin secretion in GH4C1 cells has recently been proven to be dependent on levels of PKCe activity.1,2

Protein Kinase C in Growth Regulation and Induction of Apoptosis

Inhibition of the high level of PKC activity in the pituitary cell line AtT-20 by hypericin is identified in the current study, with the drug exhibiting potent inhibition of enzyme activity in these cells. Furthermore, significant PKC inhibition was noted as early as 2 hours following
Pituitary adenoma growth inhibition by hypericin.

treatment; this preceded the induction of apoptosis and the decrease in thymidine uptake and cell viability noted in these cell lines. Similarly, hypericin has been shown to inhibit malignant glioma growth and to be a potent inducer of apoptosis in these cells.\textsuperscript{10,11} The correlation of PKC activity with cell growth and apoptosis suggests that the PKC enzyme system may be involved in the control of these processes and that inhibition of PKC may provide a potential avenue for clinical tumor growth inhibition in these cell types.

**Hypericin as a Potential Antitumor Agent**

As supported by data provided in the present study, hypericin is a potent inhibitor of PKC, as well as the epidermal growth factor receptor tyrosine kinase.\textsuperscript{10,32} Differing from more classic PKC inhibitors, such as staurosporine, Ro31-8220, and tamoxifen, which competitive-ly block the ATP catalytic site, hypericin inhibits PKC by interacting with the regulatory domain of this enzyme and displays more selectivity in its inhibitory actions than either staurosporine or tamoxifen. Hypericin has also been found in vitro to oxidize lipids, amino acids, and proteins and to disrupt the normal function of cellular membranes.

The mechanism of action of hypericin in its antineoplastic effect may be multifactorial and has recently been reviewed elsewhere.\textsuperscript{9} The drug’s irreversible inhibition of the PKC enzyme, disruption of cellular membranes,\textsuperscript{10,31,34} or formation of photoactive intermediates\textsuperscript{4,17,22} may ultimately be involved in inhibition of cellular growth and induction of apoptosis in tumors with high PKC activity—in this case pituitary adenoma cells. The nontransformed human fibroblasts in the present study displayed no evidence of cytotoxicity or DNA degradation after treatment with hypericin at doses that induced apoptosis in pituitary adenoma cells. The antiangioma activity of hypericin is currently being evaluated;\textsuperscript{10} the observed effects of hypericin in the present study indicate that hypericin or related compounds may also offer therapeutic potential in selected patients with pituitary adenomas in whom surgical and medical therapies have failed.

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