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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, AtT-20 and GH_4C_1, 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 μM 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**

Activation of specific cell surface receptors results in the hydrolysis of phosphoinositides and the generation of diglycerides and inositol phosphates. One of these diglycerides, diacylglycerol, activates protein kinase C (PKC), a family of closely related isoenzymes involved in signal transduction within the cell. Protein kinase C is involved in the regulation of a number of cellular events, including growth and differentiation in a wide range of tissues. Protein kinase C activity has been examined in a variety of tumor cells, including colon and breast carcinomas, malignant gliomas, and pituitary adenomas. In pituitary adenoma cell lines, levels of PKC activity and expression were found to be greater than those found in normal human and rat pituitary cells; in invasive adenoma cells, levels were even greater than those in noninvasive adenoma cells, suggesting that PKC activity may have a functional role in the rate of growth and the invasive properties of these tumor cells.[4,12,24,36] In this regard, a point mutation has been recently reported in the α-PKC complementary DNA sequences of four invasive adenomas that was absent in the noninvasive adenomas analyzed.[3]

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.[6,10,11] 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 GH_4C_1 (American Type Culture Collection, Rockville, MD) in this study. The AtT-20 pituitary adenoma culture was maintained in 25-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml:10 μg/ml), and 10 mmol/L Hepes buffered to a pH of 7.0. The GH_4C_1 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% CO_2 incubator.

Protein Kinase C Assay

Flasks (25 cm^2) 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 phenylmethylsulfonylfluoride, and 2 mM ethyleneglycol-*bis*-(beta-aminoethylether) 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 particulate fraction. These 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.[9] For each cell culture condition, three separate cultures were assayed. Mean and standard error values from the separate experiments were then calculated and are presented below.

Cellular Viability and Proliferation Assays

Tumor cell viability and proliferation were tested using the assays specified below. Regardless of the assay performed, all control and treatment cultures were maintained under similar cell density and culture conditions in the dark following addition of hypericin.

Methylthiotetrazole Assay. A modified colorimetric assay based on the selective ability of living cells to reduce the yellow salt MTT (3-[4,5-dimethylthiozol-2-yl]-2,5 diphenyl tetrazolium bromide or methylthiotetrazole) to formazan was used to quantitate hypericin-mediated cytotoxicity. After the cells were cultured in the presence of hypericin (LC Service Corp., Woburn, MA) for various lengths of time, 5 mg/ml MTT (Sigma Chemical Co., St. Louis, MO) was added to each well for a period of 4 hours. Following formation of the formazan crystals, the culture medium supernatant was removed from the wells without disruption of the formazan precipitate. The formazan crystals were then dissolved in 150 μl/well dimethyl sulphoxide (100%). The absorbance was measured at 570 nm using a microplate spectrophotometer (MR700; Dynatech, Chantilly, VA) interfaced with a Macintosh computer. It has previously been shown that viable cell numbers correlate with optical density as determined by the MTT assay.[13,28,37,38]

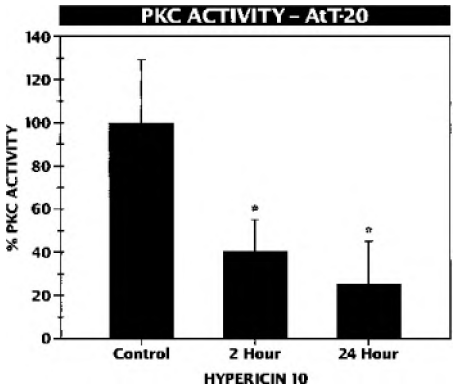

FIG. 1. Graph displaying hypericin inhibition of protein kinase C (PKC) activity in the established pituitary adenoma cell line AtT-20. A marked inhibition of total PKC activity occurs in AtT-20 cells following incubation with 10 μM hypericin. The PKC activity units are expressed as nanomoles of adenosine triphosphate transferred per minute per milligram of protein.[9] Measurements of total PKC activity obtained as early as 2 hours after treatment indicate significantly decreased PKC activity in the treated cultures. All values represent the mean of triplicate treated cultures plus the standard error of the mean. *Asterisks* indicate values significantly different from controls (one-way ANOVA, Duncan's multiple comparison; *$p < 0.05$).

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.

[^{3}H]Thymidine Uptake Assay. To offer data complementary to those yielded by the MTT viability and trypan blue exclusion assays described above, [^{3}H]thymidine uptake was determined as a measure of cellular proliferation. Our method for determining rates of cell proliferation using [^{3}H]thymidine uptake 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*.[6,8,32] It has already been shown that uptake of [^{3}H]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 2.5×10^5 cells per 5 ml of medium in 25-cm^2 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 mmol/L N,*N*′-1,2-ethanedyl-*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/isoamyl 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 mmol/L N,*N*′-1,2-ethanedyl-*bis*-(*N*-(carboxymethyl)glycine), at a pH of 7.4,

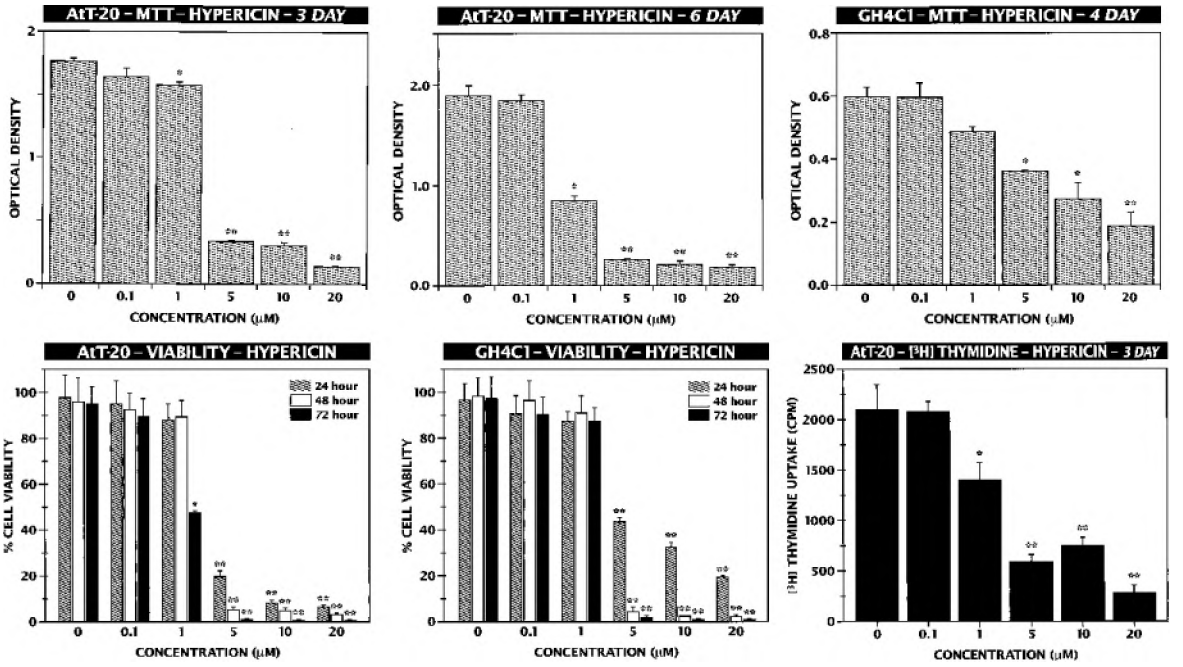

FIG. 2. Graphs showing hypericin inhibition of pituitary adenoma cell growth. Results of a methylthiotetrazole (MTT) assay indicate a dose-related decrease in the absorbance of AtT-20 cells (correlating with viable cell numbers) following administration of hypericin for a period of 3 *(upper left)* or 6 *(upper center)* days. The inhibition of growth was facilitated slightly by the longer incubation period. Similarly, the absorbance of GH_4C_1 cells decreased in a dose-related manner in the presence of hypericin for 4 days *(upper right)*. All values represent the mean of quintuplicate wells + the standard error of the mean (SEM). Similar to the results of the MTT assay, cell viability, as determined by the trypan blue dye exclusion test, was decreased in cell cultures treated with increasing concentrations of hypericin in both AtT-20 *(lower left)* and GH_4C_1 cell lines *(lower center)*. All values represent the mean of triplicate cultures + SEM. To complement the cell viability assays, [^{3}H]thymidine uptake was determined in AtT-20 cells following incubation with hypericin for 72 hours *(lower right)*. Thymidine uptake was inhibited significantly at doses of 1 μM hypericin and greater for this time period. All values represent the mean of quadruplicate wells + SEM. For all plots in Fig. 2, *asterisks* indicate values that are significantly different from controls (one-way ANOVA, Duncan's multiple comparison; *$p < 0.05$, **$p < 0.01$).

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 μM 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 GH_4C_1 (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 *lower left* and *center*) the viability of cells after treatment with hypericin at concentrations greater than 1 μM 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 μM during this treatment period.

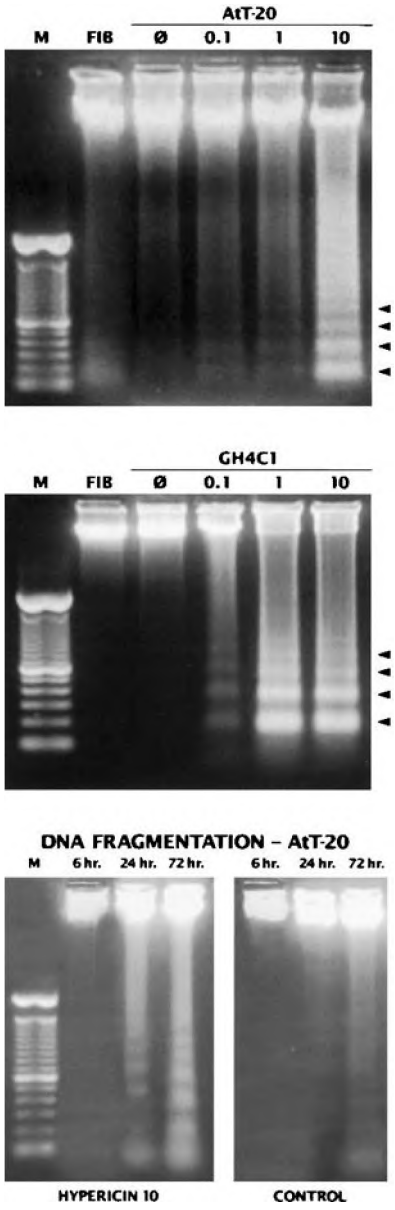

FIG. 3. Ultraviolet fluorescence photographs obtained after agarose gel electrophoresis showing that hypericin induces apoptosis in established pituitary adenoma cell lines. Treatment of pituitary adenoma cells with hypericin and DNA isolation are described in *Materials and Methods*. The DNA that was isolated from the established adenoma cell lines AtT-20 and GH_4C_1 in the absence of hypericin (Lane Ø) and in the presence of varying concentrations of hypericin (values given in micromolar concentrations) were electrophoresed in 1.2% agarose and stained with ethidium bromide (*upper* and *center* gels). Note the presence of oligonucleosome-sized fragments, which produce a classic ladder pattern in the lanes containing DNA from treated cells at doses of 0.1 μM and higher *(arrowheads)*. The human fibroblast control cell culture demonstrated no evidence of DNA fragmentation after treatment with 10 μM hypericin on either gel (Lane FIB). The sizes of oligonucleosomal fragments were determined by comparison to DNA size markers in Lane M. The DNA isolated from line AtT-20 at various time periods after addition of 10 μM hypericin indicates the presence of apoptosis after 24 and 72 hours of treatment (*lower* gel). No evident DNA fragmentation was seen at the 6-hour time 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 GH_4C_1, 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.[1,2,19,23,25–27,30,35] 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, β_1, β_2 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, ζ 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 ζ) with PKCη being expressed in rat GH_4C_1 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 GH_4C_1 cells has recently been proven to be dependent on levels of PKCϵ 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

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.[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.[16,32] Differing from more classic PKC inhibitors, such as staurosporine, Ro31-8220, and tamoxifen, which competitively 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.[6] The drug's irreversible inhibition of the PKC enzyme, disruption of cellular membranes,[18,31,34] or formation of photoactive intermediates[14,17,22] 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 antiglioma activity of hypericin is currently being evaluated;[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.

References

1. Akita Y, Ohno S, Yajima Y, et al: Overproduction of a Ca^{2+}-independent protein kinase C isozyme, nPKCε, increases the secretion of prolactin from thyrotropin-releasing hormone-stimulated rat pituitary GH_4C_1 cells. **J Biol Chem 269:**4653–4660, 1994
2. Akita Y, Ohno S, Yajima Y, et al: Possible role of Ca^{2+}-independent protein kinase C isozyme, nPKCε, in thyrotropin-releasing hormone-stimulated signal transduction: differential down-regulation of nPKCε in GH_4C_1 cells. **Biochem Biophys Res Commun 172:**184–189, 1990
3. Alvaro V, Lévy L, Dubray C, et al: Invasive human pituitary tumors express a point-mutated α-protein kinase-C. **J Clin Endocrinol Metab 77:**1125–1129, 1993
4. Alvaro V, Touraine P, Raisman Vozari R, et al: Protein kinase C activity and expression in normal and adenomatous human pituitaries. **Int J Cancer 50:**724–730, 1992
5. Andersen DO, Weber ND, Wood SG, et al: *In vitro* virucidal activity of selected anthraquinones and anthraquinone derivatives. **Antiviral Res 16:**185–196, 1991
6. Anker L, Gopalakrishna R, Jones KD, et al: Hypericin in adjuvant brain tumor therapy. **Drugs Fut 20:**511–517, 1995
7. Baltuch G, Couldwell WT, Villemure JG, et al: Protein kinase C inhibitors suppress cell growth in established and low-passage glioma cell lines. A comparison between staurosporine and tamoxifen. **Neurosurgery 33:**495–501, 1993
8. Couldwell WT, Antel JP, Apuzzo MLJ, et al: Inhibition of growth of established human glioma cell lines by modulators of the protein kinase-C system. **J Neurosurg 73:**594–600, 1990
9. Couldwell WT, Antel JP, Yong VW: Protein kinase C activity correlates with the growth rate of malignant gliomas: Part II. Effects of glioma mitogens and modulators of protein kinase C. **Neurosurgery 31:**717–724, 1992
10. Couldwell WT, Gopalakrishna R, Hinton DR, et al: Hypericin: a potential antiglioma therapy. **Neurosurgery 35:**705–710, 1994
11. Couldwell WT, Hinton DR, He S, et al: Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. **FEBS Lett 345:**43–46, 1994
12. Couldwell WT, Law RE, Hinton DR, et al: Protein kinase C and growth regulation of pituitary adenomas. **Acta Neurochir (Suppl) 65:**22–26, 1996
13. Couldwell WT, Weiss MH, Law RE, et al: Paradoxical elevation of Ki-67 labeling with protein kinase inhibition in malignant gliomas. **J Neurosurg 82:**461–468, 1995
14. Daniel C: Further communications on the photodynamic substance hypericin. **Hippokrates 19:**526–530, 1949
15. Dekker LV, Parker PJ: Protein kinase C—a question of specificity. **Trends Biochem Sci 19:**73–77, 1994
16. de Witte P, Agostinis P, Van Lint J, et al: Inhibition of epidermal growth factor receptor tyrosine kinase activity by hypericin. **Biochem Pharmacol 46:**1929–1936, 1993
17. Diwu Z, Lown JW: Photosensitization with anticancer agents 17. EPR studies of photodynamic action of hypericin: formation of semiquinone radical and activated oxygen species on illumination. **Free Radic Biol Med 14:**209–215, 1993
18. Durán N, Song PS: Hypericin and its photodynamic action. **Photochem Photobiol 43:**677–680, 1986
19. Fearon CW, Tashjian AH Jr: Thyrotropin-releasing hormone induces redistribution of protein kinase C in GH_4C_1 pituitary cells. **J Biol Chem 260:**8366–8371, 1985
20. Hartwell JL: **Plants Used Against Cancer. A Survey.** Lawrence, MA: Quarterman Publications, 1982, pp 239–241
21. Hudson JB, Lopez-Bazzocchi I, Towers GHN: Antiviral activities of hypericin. **Antiviral Res 15:**101–112, 1991
22. Hudson JB, Towers GHN: Therapeutic potential of plant photosensitizers. **Pharmacol Ther 49:**181–222, 1991
23. Ison AJ, MacEwan DJ, Johnson MS, et al: Evidence for a distinct H7-resistant form of protein kinase C in rat anterior pituitary gland. **FEBS Lett 329:**199–204, 1993
24. Jin L, Maeda T, Chandler WF, et al: Protein kinase C (PKC) activity and PKC messenger RNAs in human pituitary adenomas. **Am J Pathol 142:**569–578, 1993
25. Johnson MS, MacEwan DJ, Simpson J, et al: Characterisation of protein kinase C isoforms and enzymic activity from the αT3-1 gonadotroph-derived cell line. **FEBS Lett 333:**67–72, 1993
26. Kiley SC, Parker PJ, Fabbro D, et al: Differential regulation of protein kinase C isozymes by thyrotropin-releasing hormone in GH_4C_1 cells. **J Biol Chem 266:**23761–23768, 1991
27. Kiley SC, Parker PJ, Fabbro D, et al: Hormone- and phorbol-ester-activated protein kinase C isozymes mediate a reorganization of the actin cytoskeleton associated with prolactin secretion in GH_4C_1 cells. **Mol Endocrinol 6:**120–131, 1992
28. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods 65:**55–63, 1983
29. Nishizuka Y: The molecular heterogeneity of protein kinase C and its implications for cellular recognition. **Nature 334:** 661–665, 1988
30. Senogles SE: The D2 dopamine receptor mediates inhibition of

growth in GH_4ZR_7 cells: involvement of protein kinase-Cε. **Endocrinology 134:**783–789, 1994

31. Suzuki O, Katsumata Y, Oya M, et al: Inhibition of monoamine oxidase by hypericin. **Planta Med 50:**272–274, 1984
32. Takahashi I, Nakanishi S, Kobayashi E, et al: Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. **Biochem Biophys Res Commun 165:**1207–1212, 1989
33. Tang J, Colacino JM, Larsen SH, et al: Virucidal activity of hypericin against enveloped and non-enveloped DNA and RNA viruses. **Antiviral Res 13:**313–326, 1990
34. Thomas C, MacGill RS, Miller GC, et al: Photoactivation of hypericin generates singlet oxygen in mitochondria and inhibits succinoxidase. **Photochem Photobiol 55:**47–53, 1992
35. Thomson FJ, Johnson MS, Mitchell R, et al: The differential effects of protein kinase C activators and inhibitors on rat anterior pituitary hormone release. **Mol Cell Endocrinol 94:** 223–234, 1993
36. Todo T, Buchfelder M, Thierauf P, et al: Immunohistochemical expression of protein kinase C type III in human pituitary adenomas. **Neurosurgery 32:**635–642, 1993
37. Twentyman PR, Luscombe M: A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. **Br J Cancer 56:**279–285, 1987
38. van de Loosdrecht AA, Beelen RHJ, Ossenkoppele GJ, et al: A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. **J Immunol Methods 174:**311–320, 1994

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