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Cancer Letters 210 (2004) 27–33
CANCER Letters
www.elsevier.com/locate/canlet

Hypericum perforatum methanolic extract inhibits growth of human prostatic carcinoma cell line orthotopically implanted in nude mice

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Received 5 November 2003; received in revised form 15 January 2004; accepted 16 January 2004

Abstract

The antiproliferative effect of serotonin-reuptake inhibitors (SSRI) and serotonin antagonists has been demonstrated in prostate tumors. Since *Hypericum perforatum* components act as serotonin-reuptake inhibitors and exert cytotoxic effects on several human cancer cell lines, in this work we analyzed the effect of a treatment with *Hypericum perforatum* extract (HPE) on the growth of human prostate cancer cells in vitro and in vivo. This study highlighted a significant reduction of tumor growth and number of metastasis suggesting that this natural compound may be useful in the treatment of prostate cancer.
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Keywords: Prostate cancer; *Hypericum perforatum*; Serotonin; Neuroendocrine differentiation

1. Introduction

Prostate carcinoma is the most common cancer among men in the US, representing the second cause of cancer death [1]. Current treatments mainly involve radical prostatectomy or radiotherapy for localized disease, whereas androgen ablation is used for advanced metastatic tumors.

Androgen withdrawal causes apoptosis of prostate cancer cells [2,3] and a high number of patients positively respond to hormonal therapy. With time, however, cancer cells undergo a neuroendocrine differentiation related to an androgen-independent tumor growth that is characterized by neovascularisation and metastasis [4,5]. So far, no effective treatments exist at this stage of the tumor.

Serotonin acts as a growth factor for several cell types and is a marker of neuroendocrine differentiation in prostatic carcinomas [4]. The inhibition of serotonin autocrine and paracrine communications leads to a decrease of prostate cancer growth as the antiproliferative effect of serotonin-reuptake inhibitors and serotonin antagonists on human prostate cancer cell lines has been demonstrated in vitro and in vivo [6,7].

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doi:10.1016/j.canlet.2004.01.031

Androgen-independent human prostate cancer cells are more sensitive to serotonin signal inhibition compared to androgen-responsive cells. In fact, the IC_{50} values for the antiproliferative effects of the potent 5-HT_{1A} antagonist Pindobind on PC-3 and DU-145 cells were 0.5 and 0.6 μM respectively, whereas the IC_{50} for the LNCaP were 1.6 μM. Similar results were obtained using serotonin-reuptake inhibitors and fluoxetine, which has been widely used for the treatment of depression, was the most potent inhibitor of the serotonin-reuptake in the prostate cancer cell line studied [6].

Hypericum perforatum is a serotonin-reuptake inhibitor endowed with antidepressant-like action in human and laboratory animals, and several works showed that the *Hypericum* preparations, at doses therapeutically equivalent to fluoxetine, exert fewer side effects [8,9,10]. At the same antidepressant activity, the incidence of side effects was 15% for the synthetic SSRI and only 1–3% for the HPE tested [11].

A variety of neurochemical and biochemical effects have been reported for HPE. It reduces serotonin reuptake [12–14] and causes MAO inhibition [15,16]. HPE has been shown to reduce noradrenaline, dopamine and L-glutamate reuptake [17–19].

Hyperforin and Hypericin are two important component of HPE and their involvement in 5-HT regulation has been widely investigated. In particular, Hyperforin seems to inhibit the serotonin-reuptake [20,21].

Treatments with *Hypericum perforatum* extract and its constituents were also tested to inhibit cancer growth. Several studies have shown the cytotoxic activities of Hyperforin and Hypericin on different human cancer cell lines by inducing apoptosis [22–24].

As far as prostate cancer, it has been demonstrated in vitro that Hypericin exerts cytotoxic effects on the three human prostate cancer cell lines PC-3, DU-145 and LNCaP [25]. Based upon this finding, in this work we analyzed the effect of a treatment with *Hypericum perforatum* extract on the growth of human prostate cancer cells in vitro and in vivo after orthotopic implantation in nude mice.

2. Materials and methods

2.1. Prostate cancer cell line

PC-3 human caucasian prostate adenocarcinoma cell line from Sigma was used. PC-3 cells were maintained in Minimal Essential Medium (MEM) and 10% fetal bovine serum, L-glutamine, MEM vitamin solution, MEM sodium pyruvate solution, penicillin, and MEM non essential amino acid solution (GIBCO).

Cell cultures were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO_2 in air.

2.2. Animals

Athymic male nude mice Nu/Nu (Harlan, Italy), 6 weeks old were used. Mice were kept in laminar-flow cage in a standardized environmental condition. Sterilized food (Harlan, Italy) and water were supplied ad libitum.

2.3. Hypericum perforatum extract

The *Hypericum perforatum* methanolic extract (HPE) employed in the present study was a generous gift of Indena S.p.A., Milan, Italy. It was a dry extract containing 0,3% hypericin and 3,8% hyperforin.

2.4. In vitro proliferation

The test was performed following the NCI guidelines. Cells were plated in serum supplemented growth medium at 5000 cells/well in 96 well plates. The following day, cells were treated with HPE dissolved in DMSO at various concentrations as indicated. After 3 days, cell numbers were determined using the MTT assay. Briefly, after incubation with HPE, the solution were discarded and 20 μl of a solution of 5 mg/ ml of MTT (Sigma, Italy) was added and the cultures were incubated for an additional 3 h at 37 °C. The supernatant was removed and the precipitate was dissolved in DMSO (100 μl/well). After a few minutes the plate was read on a microtitre plate reader at a wavelenght of 570 nm.

2.5. Orthotopic inoculation

At near confluence, cells were harvested with trypsin/EDTA solution and finally suspended in Ca^{++} and Mg^{++} free Hank's balanced salt solution (HBSS). Only cell suspensions of >90% viability (trypan blue exclusion) were used.

Mice were anaesthetized with Tiletamina chlorohydrate and Zolazepam chlorohydrate and placed in supine position.

A midline incision was made in the lower abdomen. A tumor cells suspension (6×10^5 in 20 μl of HBSS) was injected into the left dorsal prostatic lobe using a 27 gauge needle and a 1000 μl syringe. A slight elevation of the right dorsal prostate capsule was considered indicative of correct deposition of tumor cells.

The abdomen was then closed with clips.

2.6. Therapeutic procedures

Three groups of animals (10 animals each group) were used. Treatments (100 μl) started 10 days after tumor cells injection. The first group was treated intraperitoneally at 10.00 am with a dose of 15 mg/kg of HPE dissolved in DMSO 1% prepared just before treatment.

Two groups of animals received an intraperitoneal administration of vehicle and saline solution, respectively. The treatment lasted 25 consecutive days.

2.7. Autopsy and histology

The mice were sacrificed by CO_2 after the 36th day from cells injection. Primary tumors (including the entire prostate) were weighed. Presence of metastasis was macroscopically checked.

The samples were removed and immediately frozen on powdered dry ice and stored at −70 °C.

2.8. Immunohistochemistry

For neuroendocrine differenziated cells detection a policlonal Goat anti-human Cga (Santa Cruz Biotechnology) was used.

After autopsy tumors were placed in OTC compound and snap frozen in liquid nitrogen. Frozen section (10–20 μm) and cells were fixed with cold acetone for 5 min, acetone/chlorophorm 1/1 (5 min) and cold acetone (5 min). The samples were then rinsed with PBS and treated with 3% hydrogen peroxide in methanol (vol/vol). The treated slides were incubated in a blocking solution (5% normal horse serum + 1% normal goat serum in PBS) and then over night at 4 °C in a humidified chamber with the primary antibody.

The slides were then rinsed and incubated, first with the blocking solution and then with a Bovine anti-goat horse radish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology). Positive reactions were visualized by incubating the slides for 5 min with stable DAB (Sigma, Italy) followed by counterstaining with Mayer's hematoxylin (Sigma, Italy). The slides were dried and mounted with Universal Mount.

2.9. Statistical analysis

The significance of the in vitro and in vivo data was analyzed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test.

3. Results

3.1. Effect of HPE on PC-3 cells growth in vitro

We tested the effect of HPE on PC-3 cells in vitro. Results are shown in Fig. 1. The HPE inhibited PC-3 growth by ~80% at a concentration of 1.41 mg/ml, which corresponds to 100 μM hyperforin and 8.38 μM hypericin. The IC_{50} value was 0.42 mg/ml

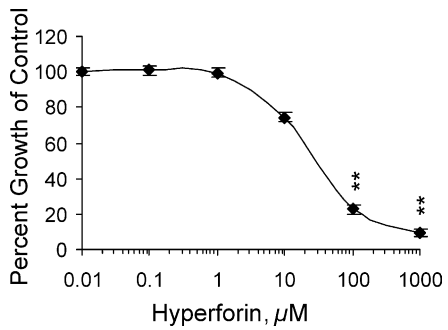

Fig. 1. Inhibitory effects of HPE on the in vitro proliferation of the androgen-independent human prostate cancer cell line PC-3. Values shown are mean ± SEM ($n = 3$). $^{**}P < 0,01$.

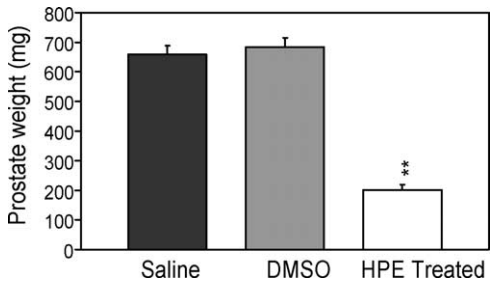

Fig. 2. Inhibitory effects of HPE on the growth of PC-3 cells orthotopically implanted in athymic nude mice. Values shown are mean ± SEM ($n = 10$). $**P < 0,01$. The prostate tumors were removed and weighed. Normal mouse prostate weights are 45 ± 5 mg.

which corresponds to 30 μM hyperforin and 2.5 μM hypericin.

3.2. Effect of HPE on PC-3 cells growth in vivo

We also tested the effects of HPE on the growth of PC-3 cells after orthotopic implantation in athymic nude mice. PC-3 cells (6×10^5) were implanted into the dorsal prostatic lobes. After ten days, mice daily received an intraperitoneal dose of HPE or vehicle or saline solution. Results are shown in Fig. 2. Tumors incidence was 100% in all groups and treatment with HPE inhibited tumor growth by 70%. Regional lymph node metastasis was observed in 100% of the controls mice compared to 30% of mice treated with HPE ($P < 0,01$). There were no side effects observed in any of the treated mice.

3.3. Immunohistochemical analysis

PC-3 cells were implanted into the prostate of nude mice. Ten days later mice received a daily intraperitoneal administration of 15 mg/kg of HPE. Tumor samples were collected after 25 days of treatment for immunohistochemical analysis. Anti-CgA antibody staining revealed the presence of NE cells in both controls and treated tumors confirming that orthotopic implantation of PC-3 cells is a suitable model for the study of NE prostate tumors.

4. Discussion

The incidence of prostate cancer increases with aging and 75% of the cases occur in men 75 years old and older [26,27]. The complex and multiple steps involved in prostate cancer growth are not completely known and the differences in clinical behavior of this tumor emphasize the need for a search of more effective therapies. Prognosis depends on the stage of the cancer: low stage cancers have good prognosis, with about 90% of 5 years disease-free survival after radical prostatectomy, whereas high stage cancers have poor prognosis regardless of therapy [28].

Currently, the serum detection of prostate-specific antigen (PSA) as a prostate cancer marker allows diagnosis of the disease at a low stage, but a high percentage of advanced cancers at time of clinical check-ups is found and metastases can also occur after treatment.

Based upon these findings, the present studies are aimed at developing therapies for high stage and high grade prostate tumors, characterized by tumor cells colonization of tissues close to prostate and metastases. At present, hormonal therapy is the conventional treatment for metastatic prostate cancers resulting only in palliative effects and leading to neuroendocrine differentiation and to androgen-independent growth [5].

In this work we studied the effect of a treatment with HPE on the androgen-independent human prostate cancer cell line PC-3 orthotopically implanted in nude mice. This model is suitable for the study of advanced human prostate cancer and the presence of stronger 5-HT staining in the PC-3 cells compared to DU-145 and LNCaP was demonstrated [29]. Moreover, NE differentiation of PC-3 cells in our model was confirmed by immunohistochemical determination of Chromogranin A.

Neuroendocrine differentiation is a common step in prostate adenocarcinomas, occurring in 30–100% of the tumors [4] and has been associated with androgen-independent growth [5]. Androgen withdrawal leads to a rapid tumor regression as well as to an induction of neuroendocrine cells formation which generally seems to be not mitotic and do not express androgen receptors [30,31]. In absence of normal hormones, neuroendocrine

differentiation can develop as an alternate regulatory pathway for prostate cancer cells proliferation as NE and proliferating cancer cells seems to be topographically related [32]. Moreover, immunohistochemical studies showed a correlation between NE differentiation and angiogenesis in prostatic carcinoma [33].

Serotonin and Chromogranin A are the best markers for NE differentiation as most if not all the NE cells contain 5-HT [5]. Serotonin is a monoamine derived from tryptophan and it is well know as a neurotransmitter implicated in numerous physiological functions such as the regulation of circadian rhythms, sexual behaviour, mood changes, pain perception and appetite [34]. Growth stimulatory effects of serotonin have been described on several cell types [35–38] and the use of serotonin reuptake inhibitors to decrease the proliferation of human prostate cancer cell lines was demonstrated in vitro and in vivo [6,7].

In our work, we used the *Hypericum perforatum* extract to block the serotonin paracrine communication between the prostate cancer cells. However, a number of different substances are part of the *Hypericum perforatum* extract and several of them possesses cytotoxic activity. Beyond Hyperforin and Hypericin, also flavonoids such as quercetin inhibit cancer cells growth [39]. Our data can be thus explained considering the serotonin reuptake inhibition and the cytotoxic properties of the *Hypericum perforatum* extract components that synergistically contributed to the reduction of the PC-3 cells proliferation.

The differences highlighted in the in vitro studies between the doses needed for the PC-3 growth inhibition and those responsible for the neurochemical effects demonstrated by other researchers, can also be explained considering the different characteristics of the HPE compounds.

The decrease of PC-3 proliferation we found in vitro and in vivo by HPE is consistent with the results obtained using synthetic SSRI. [6]. As for fluoxetine, daily administration of HPE markedly inhibited the growth of PC-3 cells in vivo and a lower incidence of regional lymph node metastases were found in all treated mice compared to control group (Table 1). Moreover, the concentration of HPE used to inhibit the PC-3 cells proliferation in vitro contained an amount of hypericin and hyperforin comparable with those used to contrast the growth of other human cancer cell lines [22–24].

The use of HPE for the treatment of prostate cancer can be interesting also on the basis of its antidepressant effects. A number of studies have shown a clear relationship between mood disorders and the response to therapies as well as the incidence of cancer has been shown to be related to depression [40–42]. Furthermore, the increase of circulating serotonin caused by the antidepressant drugs can result in an higher production of melatonin [43,44] which inhibits the prostate cancer growth as reported by several studies [45–47]. In our model, significant growth inhibition of PC-3 cells was observed at a dose of 15 mg/Kg/day that has been administered for 25 consecutive days. This is equivalent to a human dose of about 1050 mg per day which is similar to the HPE oral dose used for the treatment of depression in humans, generally ranging between 600 and 1200 mg per day [48].

Since we used an intraperitoneal administration of the drug and due to the differences between murine and human physiology, more studies are necessary to develop a protocol for the treatment of prostate cancer with the HPE in humans.

Our results support previous findings on the inhibitory activity of HPE and its components on prostate cancer cells growth, thus suggesting a possible use of the this plant in the prostate tumor therapy.

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