Hyperforin, a bio-active compound of St. John’s Wort, is a new inhibitor of angiogenesis targeting several key steps of the process

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Hyperforin, a phloroglucinol derivative found in St. John’s wort related mainly to its antidepressant effects, has been reported recently to induce apoptosis in tumour cells and to inhibit cancer invasion and metastasis. We show that hyperforin inhibits angiogenesis in vitro in bovine aortic endothelial cells and in vivo in the chorioallantoic membrane assay. In a variety of experimental systems representing the sequential events of the angiogenic process, hyperforin treatment of endothelial cells resulted in strong inhibitory effects. Hyperforin inhibited the growth of endothelial cells in culture. Capillary tube formation on Matrigel was abrogated completely by addition of hyperforin at the low micromolar range. Hyperforin also exhibited a clear inhibitory effect on the invasive capabilities of endothelial cells. Zymographic assays showed that hyperforin treatment produced a complete inhibition of urokinase and a remarkable inhibition of matrix metalloproteinase 2. Our data indicates that hyperforin is a compound that interferes with key events in angiogenesis, confirming the recent and growing evidence about a potential role of this compound in cancer and metastasis inhibition and making it a promising drug for further evaluation in the treatment of angiogenesis-related pathologies.

Key words: hyperforin; St. John’s wort; angiogenesis inhibitor; endothelial cells

Angiogenesis, the formation of new blood vessels from the existing vascular bed, has been described as one of the hallmarks of cancer, playing an essential role in tumour growth, invasion and metastasis. Because tumour blood vessels are different from normal vessels and are not genetically unstable, they are potential targets in therapy for all types of cancer. In many other pathological conditions, including diabetic retinopathy, hemangiomas, arthritis, psoriasis and atherosclerosis, cancer seems to be driven by persistent unregulated angiogenesis. This is a complex process in which several key steps are involved. When dormant endothelial cells are activated by an angiogenic signal, they are stimulated to release degrading enzymes allowing endothelial cells to migrate, proliferate and finally differentiate to form new vessels. Any of these steps may be a potential target for pharmacological intervention in angiogenesis-dependent diseases.

St. John’s wort (Hypericum perforatum) has been used as a medicinal plant for centuries. St. John’s wort preparations are increasingly popular in the treatment of mild to moderate depression. The traditional use of this wort also includes the treatment of a range of ailments, including bacterial and viral infections, respiratory conditions, skin wounds, peptic ulcers and inflammation. Interestingly, the latter 3 uses are related to angiogenesis.

Among the different active compounds of St. John’s wort, the phloroglucinol derivative hyperforin (Fig. 1) is the main active component with antidepressant effects. Hyperforin has been proposed recently as being an innovative anticancer drug that induces apoptosis in tumour cells and inhibits metastasis in vivo. An inhibitory effect of hyperforin on the neovascularization of an experimental murine tumor model has also been suggested. To our knowledge, however, the direct effect of hyperforin on typical in vitro or in vivo angiogenesis assays has not been reported.

Our study shows that hyperforin inhibits key steps of angiogenesis in vitro, as well as in vivo angiogenesis. We open the way for the possibility of a new pharmacological use of hyperforin for the treatment of angiogenesis-related malignancies.

Material and methods

Materials

Cell culture media were purchased from Gibco (Grand Island, NY) and BioWhittaker (Walkersville, MD). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Matrigel was purchased from Becton Dickinson (Bedford, MA) and Calcein-AM was from Molecular Probes (Eugene, OR). Highly purified hyperforin was supplied by ChromaDex (St. Ana, CA). Stock solution (10 mM) was prepared in DMSO. In all the assays, the vehicle (DMSO) was at <1% (v/v) and controls with the vehicle alone were carried out in parallel. Supplements and other chemicals not listed in this section were obtained from Sigma Chemical Co. (St. Louis, MO). Plasticware for cell culture was supplied by NUNC (Roskilde, Denmark).

Cell culture

Bovine aorta endothelial (BAE) cells were isolated as described previously and were maintained in DMEM containing glucose (1 g/L), glucose (2 mM), penicillin (50 IU/mL), streptomycin (50 µg/mL) and amphotericin (1.25 µg/mL) supplemented with 10% FBS (DMEM/10% FBS). MDA-MB231 human breast cancer and NIH-3T3 immortalized mouse fibroblast cell lines were supplied by the American Type Culture Collection and maintained in RPMI-1640/10% FBS and DMEM/10% calf serum (containing 4.5 g/L of glucose), respectively.

In vivo chorioallantoic membrane assay

The assay was carried out as described elsewhere. Fertilized chick eggs, provided by Granja Santa Isabel (Córdoba, Spain), were incubated horizontally at 38°C in a humidified incubator, windowed at Day 3 of incubation and processed at Day 8. Hyperforin stock solution was added to a 0.7% solution of methylcellulose in water and 10 µL drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood. The methylcellulose discs were implanted on the chorioallantoic membrane assay (CAM), the eggs were sealed with adhesive tape and returned to the incubator for 48 hr. Negative controls were always carried out with DMSO and mixed with methylcellulose. Six eggs were used for each tested dosage of hyperforin. After reincubation, CAM were examined under a stereomicroscope. The assay was considered as positive when 2 independent observers reported a significant reduction of vessels in the treated area.

Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue

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formazan product, which can be measured spectrophotometrically. BAE and tumor cells (3 × 10^5 cells in a total volume of 100 μL of complete medium) were incubated in each well with 1:1 serial dilutions of hyperforin, beginning with 0.1 mM of the compound and down to concentrations in the submicromolar range. After 3 days of incubation (37°C, 5% CO2 in a humid atmosphere), 10 μL of MTT (5 mg/ml in PBS) were added to each well and the plate was incubated for a further 4 hr (37°C). The resulting formazan was dissolved in 150 μL of 0.04 N HCl/2-propanol and read at 550 nm. All determinations were carried out in triplicate. IC50-values were calculated as those concentrations of hyperforin yielding a 50% cell survival.

Cell viability assay

To check the viability of endothelial cells after the treatment with hyperforin in the tubulogenesis, migration or invasion assays, BAE cells were incubated in 96-well plate with hyperforin in the same conditions used for the aforementioned assays (that means, higher cell densities and shorter incubation times than those employed in the cell growth assay). After the maximum incubation time for these assays (4–24 hr), cell viability in comparison to untreated control cells was determined by the addition of MTT as described for cell growth assay.

Tube formation by endothelial cells on Matrigel

Matrigel (50 μL of about 10.5 mg/mL) at 4°C was used to coat each well of a 96-well plate and allowed to polymerize at 37°C for a minimum of 30 min. BAE cells (5 × 10^4) were added with 200 μL of DMEM. Finally, different amounts of hyperforin were added and incubated at 37°C in a humidified chamber with 5% CO2. After 7 hr incubation, cultures were observed (40× and 100× magnifications) and photographed with a NIKON inverted microscope DIAPHOT-TMD (NIKON Corp., Tokyo, Japan). Each concentration was tested in duplicate and 2 different observers evaluated the inhibition of tube formation. Only those assays where no tubular structure could be observed were evaluated as positive in the inhibition of morphogenesis of endothelial cells on Matrigel.

Conditioned media and cell lysates

To prepare conditioned media and cell lysates, BAE cells were grown in 6-well plates. When the cells were at 75% confluency, medium was aspirated, cells were washed twice with phosphate-buffered saline (PBS) and each well received 1.5 mL of DMEM/0.1% BSA containing 200 KIU of Trasylol/mL. Media and cell lysates were centrifuged at 1,000g and 4°C for 20 min. Supernatants were collected and used for zymography. Duplicates were used to determine cell number with a Coulter counter.

Zymographies

Assays of urokinase-type plasminogen activator (uPA) activity in gel were carried out as follows. Aliquots of cell lysates normalized for equal cell numbers were subjected to sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 4°C under non-reducing conditions, with 5% stacking gel and 10% resolving gel. Gels were washed for 10 min twice with 50 mM Tris/HCl, pH 7.4, supplemented with 1% Triton X-100 and twice with 50 mM Tris/HCl, pH 7.4 and laid over a substrate gel prepared with agar (0.8%), plasminogen (40 μg/ml) and skimmed milk (1.5% in PBS). Gels were incubated under a moist atmosphere overnight at 4°C and then incubated at 37°C. After 4–8 hr, bands of proteolysis due to uPA activity were photographed under dark field.

The gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) delivered to the conditioned media or present in cell lysates was detected in gelatinograms. Aliquots of conditioned media and cell lysates normalized for equal cell numbers were subjected to non-reducing SDS-PAGE as above but with gelatin (1 mg/mL) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mM Tris/HCl, pH 7.4, supplemented with 0.1% Triton X-100 and twice with 50 mM Tris/HCl, pH 7.4. Each wash with continuous shaking lasted 10 min. After the washes, the gels were incubated overnight at 37°C and immersed in a substrate buffer (50 mM Tris/HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mM CaCl2 and 0.02% NaN3). In some experiments, 10 μM hyperforin was added to the substrate buffer. Finally, the gels were stained with Coomassie blue R-250 and the bands of gelatinase activity could be detected as non-stained bands in a dark, stained background.

MMP-9 activity assay

The gelatinolytic activity of purified MMP-9 from HT-1080 human fibrosarcoma cells was fluorometrically measured by using a fluorogenic substrate specific for MMP-9 (described elsewhere).15

Endothelial cell migration assay

The migratory activity of BAEC was assessed using a wounded migration assay. Confluent monolayers in 6-well plates were wounded with pipet tips after 2 perpendicular diameters, giving rise to 2 acellular 1 mm-wide lanes per well. After washing, cells were supplied with 1.5 mL complete medium in the absence (controls) or presence of 10 μM hyperforin. Wounded areas were photographed. After additional 4, 9 and 24 hr of incubation, plates were observed under microscope and photos were taken from the same areas as those recorded at zero time. Acellular surface was determined by image analysis in both controls and treated wells and normalized respect to their respective values at zero time.

Endothelial cell invasion assay

Invasion of fluorescence-labeled endothelial cells was assessed by using a 24-well fluorescence-opaque membrane insert. This assay allows for a real-time monitoring of the process because it eliminates the need to remove non-invading cells before quantifying invading cells.

BAE cells were grown to 80–90% confluence in DMEM/10% FBS and then labeled in situ with 5 μg/mL Calcein-AM in complete culture medium for 2 hr at 37°C. After washing, the cell monolayer was briefly trypsinized to lift the cells, which were
washed and suspended in DMEM supplemented with 0.1% BSA (DMEM/0.1% BSA). BAE cells were added to 8 μm FALCON HTS FluoroBlok inserts (Becton Dickinson, Bedford, MA) and filters were coated with Matrigel (25 μg/filter) at a density of 2 × 10⁶ cells/insert in the absence or presence of 10 μM hyperforin. DMEM/10% FBS was used as chemottractant in the lower wells. The inserts were incubated at 37°C and the real time kinetics of cell invasion were determined by taking readings at different times. Fluorescence of cells that had migrated through the inserts was measured on the Fluorescence Microplate Reader (FL600FA, BIO-TEK Instruments, Winooski, VT, USA) in the bottom read mode using excitation/emission wavelengths of 485/530 nm and a gain setting of 75. Relative velocities of invasion for control and treated cells were compared.

Statistical analysis and image analysis

All data are expressed as means ± SD. Two-tailed Student’s *t*-test was used for the evaluation of pairs of means to establish groups that differed from the control group. Quantitative analysis of images was carried out with the NIH Image 1.6 Program.

Results

Hyperforin inhibits in vivo angiogenesis

The CAM assay is used frequently to determine the ability of test compounds to inhibit *in vivo* angiogenesis. In controls, blood vessels form a dense and spatially oriented, leaf-like branching network of vascular structures of a progressively smaller diameter as they branch (Fig. 2a). Treatments with hyperforin yielded inhibitory effects with a dose-response pattern: 17, 33 and 50% of the CAM were scored as positive at hyperforin doses of 10, 20 and 30 nmol/egg, respectively. No effect was induced by the vehicle alone. In all the cases scored as positive, the treated area showed decreased vascular density and the peripheral vessels grew centrifugally (Fig. 2b–d, arrows). At higher concentrations, disorganization of the pre-existing vessels (Fig. 2c,d, asterisks) and tissue inflammation (Fig. 2d, diamonds) could be observed.

Hyperforin inhibits culture cell growth

Angiogenesis involves local proliferation of endothelial cells. We investigated the ability of hyperforin to inhibit the growth of endothelial cells (Fig. 3a). The estimated IC₅₀-value was 7 ± 3 μM. For treated non-endothelial cells, similar survival curves were obtained (Fig. 3b,c) but with higher IC₅₀-values: 12 ± 4 μM for MDA-MB231 human breast cancer cells and 49 ± 12 μM for NIH-3T3 immortalized mouse fibroblasts.

Hyperforin inhibits capillary tube formation by endothelial cells

The final event during angiogenesis is the organization of endothelial cells in a 3-D network of tubes. *In vitro*, endothelial cells plated on Matrigel align themselves forming cords, already evident a few hours after plating. The minimal concentration of hyperforin yielding inhibition of endothelial morphogenesis on Matrigel was 2.5 μM (Fig. 4). The concentrations required to inhibit the differentiation of BAE cells did not affect their viability after 7 hr (results not shown).

Hyperforin inhibits extracellular matrix degrading potential of endothelial cells

Angiogenesis involves the acquisition by endothelial cells of the capability to degrade the basement membrane and, in general, to remodel the extracellular matrix. Gelatin zymography of conditioned media (CM) and cell extracts (CE) of BAE cells untreated and treated for 24 hr with 10 μM hyperforin shows a remarkable inhibition of MMP-2 production by BAE cells (Fig. 5a). This inhibitory effect is evident even at an order of magnitude lower concentration of hyperforin (results not shown). This does not seem to be a direct effect on the gelatinolytic activity of MMP-2 because the addition of 10 μM hyperforin to the substrate buffer produced no effect on the gelatinolytic band yielded by control samples (Fig. 5b). Furthermore, 10 μM hyperforin did not inhibit...
the other gelatinolytic activity (MMP-9) in an in vitro fluorogenic assay (results not shown).

Urokinase (uPA) is also involved in extracellular matrix remodeling. Figure 6 shows that 1 μM hyperforin induced an important decrease in BAE cell conditioned media uPA levels. Image analysis showed that this inhibition was >60%. Furthermore, media conditioned by BAE cells treated with 10 μM hyperforin expressed no band of uPA activity detectable by zymography.

Hyperforin inhibits slightly the migrating potential of endothelial cells

Migration of endothelial cells is required for angiogenesis to proceed. In the wound assay for BAE cells, 10 μM hyperforin seemed to produce a slight inhibitory effect, as observed 4 hr after wounding (Fig. 7). According to quantitative image analysis, the wounding surface reoccupied by treated BAE cells after 4 hr of incubation was 20% smaller than control values. This slight inhibition could also be detected at higher incubation times (7 and 9 hr, results not shown). Twenty-four hours was a long incubation time,
However, allowing for the full reoccupation of the wounded area in control and treated cells (results not shown). This points to the weakness of the inhibitory effect caused by hyperforin on migration.

Hyperforin inhibits Matrigel invasion by endothelial cells

A key feature of angiogenic endothelial cells is its ability to invade the surrounding space. Figure 8 shows that 10 μM hyperforin produced a significant inhibition of the invasive capability of BAE cells through a Matrigel layer.

Discussion

Hyperforin has been proposed recently as an innovative anti-cancer and antimetastatic drug.11–13 Because cancer progression is angiogenesis-dependent and several of the traditional uses of this wort include the treatment of angiogenesis-dependent processes, we wanted to test the hypothesis that hyperforin could modulate angiogenesis. The results with the in vivo CAM assay show that hyperforin can indeed inhibit angiogenesis.

Once the antiangiogenic potential of hyperforin in the in vivo CAM assay was demonstrated, we carried out a systematic in vitro study to identify which key steps of angiogenesis were targeted by hyperforin. Angiogenesis inhibitors can block any of the following steps of the angiogenic process: degradation of the basement membrane, migration and proliferation of endothelial cells and the formation of capillary-like tubes. Our in vitro results show clearly that hyperforin is able to inhibit several of these key steps.

IC50-values derived from growth assays allow the comparison of relative toxicity effects of different compounds on a cell line growth or relative inhibition of several cell lines growth by a given compound. IC50-value obtained for hyperforin is similar or lower than those found for other natural compounds with anti-angiogenic properties.14,15 This antiproliferative effect does not seem to be endothelial-cell specific because the IC50-value is similar to those shown for MDA-MB231 human breast cancer cells and those reported previously for this compound in cytotoxicity assay against several other tumour cell lines.11–13 It is well documented that, this cytotoxic effect of hyperforin is related to induction of apoptosis in tumour cells.11–13 It is noteworthy that the IC50-value obtained for hyperforin on non-tumoral, immortalized NIH-3T3 fibroblasts (cells with proliferation rates similar to those of BAE cells under the culture conditions of our lab) is almost an order of magnitude higher than that obtained on BAE cells.

The formation of tubular-like structures on Matrigel is an assay used commonly to test the effects of treatments on one of the key steps of angiogenesis.16–18 Our results for this assay show that hyperforin inhibits endothelial cell differentiation at concentrations that are lower than or in the same range as those required for other known inhibitors.14,18–20 This inhibitory effect is not due to cytotoxicity. Criteria to reasonably consider whether a chemotherapeutic agent might have meaningful anti-angiogenic activity have been suggested recently.21 According to these criteria, hyperforin could be considered an anti-angiogenic compound because it interferes with endothelial cell function at concentrations that do not cause cell death.

The assays to test effects on extracellular matrix proteases, migration and invasion were carried out at a fixed 10 μM hyperforin concentration (some zymographies were also carried out with samples obtained from cells treated with 1 μM hyperforin), which caused no detectable effect on the viability of BAE cells under the assay conditions.

A net-positive proteolytic balance is required for capillary sprout elongation and lumen formation during angiogenesis. MMP-2 is a gelatinase that has been shown to modulate the morphology of endothelial structures, as well as their invasive behavior.22–25 Our results show that hyperforin does not inhibit the activity of gelatinase. It does, however, inhibit the production of MMP-2 by BAE cells. This is also the case for curcumin.19 This is not a key step of angiogenesis and is not a target for all the antiangiogenic compounds. For instance, anti-angiogenic puupehenone-related compounds do not inhibit the production and secretion of MMP-2 by endothelial cells.25

Urokinase is another protease that plays a central role in extracellular matrix remodeling associated with angiogenesis. MMP-2 is a gelatinase that has been shown to modulate the morphology of endothelial structures, as well as their invasive behavior.22–25 Our results show that hyperforin does not inhibit the activity of gelatinase. It does, however, inhibit the production of MMP-2 by BAE cells. This is also the case for curcumin.19 This is not a key step of angiogenesis and is not a target for all the antiangiogenic compounds. For instance, anti-angiogenic puupehenone-related compounds do not inhibit the production and secretion of MMP-2 by endothelial cells.25

How hyperforin interferes with MMP-2 and urokinase expression are interesting questions that warrant future experimental efforts.

Migration and invasion of endothelial cells are 2 other key steps of angiogenesis.26 Several anti-angiogenic compounds have been shown to inhibit endothelial cell migration invasion.14,15,23,30–33 Our results show that 10 μM hyperforin produces a remarkable and significant inhibition of the invasive capability of BAE cells and only a slight inhibition of their migratory capability. These data are consistent with those reported recently on the effects of hyperforin on metastatic tumour cells.11–13 It seems reasonable to assume that the inhibition of endothelial cell invasion, triggered by hyperforin, is the result of a remarkable downregulation of the release or synthesis of MMP-2 and of a complete inhibition of urokinase.

In conclusion, we have shown that the phloroglucinol hyperforin inhibits in vivo angiogenesis and is able to inhibit several key steps of angiogenesis in vitro, including endothelial cell proliferation, differentiation and invasion, as well as extracellular matrix degradation by MMP-2 and urokinase. Therefore, hyper-
forin seems a promising antiangiogenic compound with multiple targets. Further evaluations in preclinical and clinical trials, as well as the determination of the molecular mechanisms related with its antiangiogenic effects seem highly recommendable.

References


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