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# Hypericin in the dark inhibits key steps of angiogenesis in vitro

Beatriz Martínez-Poveda, Ana R. Quesada, Miguel Ángel Medina*

*Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, 29071-Málaga, Spain*

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**Abstract**

Photoactivated hypericin has a potent cytotoxic effect over a wide range of cells. However, very recently hypericin has been shown to have antitumoral and antimetastatic effects in the dark. The aim of this study was to test whether hypericin in the dark affects angiogenesis. Different in vitro assays were used to study the potential effects of this compound on key steps of angiogenesis, namely, a colorimetric assay of cell proliferation/viability, a tubular formation on Matrigel assay, zymographic assays for gelatinases and urokinase, a wound assay for migration and a fluorometric assay for invasion through Matrigel. In this report, we show for the first time that hypericin kept in the dark inhibits several key steps of the angiogenic process, namely, bovine endothelial cell proliferation, formation of tubular-like structures on Matrigel, migration and invasion, as well as extracellular matrix degrading urokinase.


*Keywords:* Hypericin; St. John's wort; Angiogenesis inhibitor; Bovine endothelial cells

## 1. Introduction

Hypericin (Fig. 1) is a naphthodianthrone found in many *Hypericum* species (Kitanov, 2001), including St. John's wort (*Hypericum perforatum*), a perennial herb that 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 (Greeson et al., 2001). However, the traditional use of this wort also includes the treatment for bacterial and viral infections, respiratory conditions, skin wounds, peptic ulcers and inflammation (Di Carlo et al., 2001).

Hypericin is the most powerful photosensitizer found in nature and, as such, there is a renewed interest in the potentials of this compound for antitumoral photodynamic therapy. In fact, upon light activation hypericin generates reactive oxygen species, behaves as a potent protein kinase C inhibitor, induces membrane lipid peroxidation and impairs mitochondrial function and antioxidant systems. In photodynamic therapy, the selective retention of the photosensitizer in neoplastic tissues and the in situ activation of hypericin by local irradiation lead to a selective photodynamic destruction of tumor cells and severe damage of tumor vasculature (Agostinis et al., 2002). However, several biological effects of hypericin also seem to occur in the dark, in the absence of photoactivation. These include catalytic inhibition of human DNA topoisomerase II, as well as antiviral and antitumor activities (Meruelo et al., 1988; Tang et al., 1990; Blank et al., 2001; Peebles et al., 2001).

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 tumor growth, invasion, and metastasis (Hanahan and Weinberg, 2000). Furthermore, many other diseases are dependent on upregulated angiogenesis (Carmeliet, 2003). 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

* Corresponding author. Tel.: +34 9 52137132; fax: +34 9 52132000.
*E-mail address:* medina@uma.es (M.Á. Medina).

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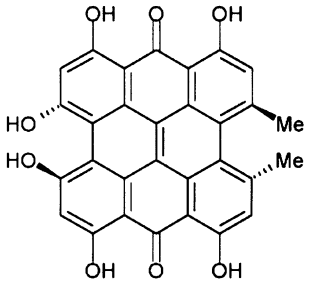


Fig. 1. Chemical structure of hypericin.

potential target for pharmacological intervention of angiogenesis-dependent diseases (Harris, 1998; Paleolog and Miotla, 1998). This is the major reason why angiogenesis has attracted recent attention in the field of pharmacological research.

Several of the traditional uses of St. John's wort in the popular pharmacopea are related to angiogenesis-dependent processes. Furthermore, migration and invasion, two features required for angiogenesis, are inhibited by hypericin in human malignant glioma cells (Zhang et al., 1997). Therefore, the hypothesis that hypericin could interfere with the angiogenic process seems reasonable. To test this hypothesis, in the present study, we determine the effects of non-photoactivated hypericin on the bovine vascular endothelial cell functions associated with key steps of angiogenesis, including proliferation, tubular formation, extracellular matrix protease production, migration and invasion.

## 2. Materials and methods

### 2.1. Materials

Cell culture media were purchased from Biowhittaker (Walkersville, MD, USA). Fetal bovine serum was a product of Harlan-Seralab (Belton, U.K.). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA), and Calcein-AM was from Molecular Probes (Eugene, OR, USA). Hypericin was obtained from ChromaDex (St. Ana, Ca, USA). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis, Mo., USA). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark).

### 2.2. Cell culture

Bovine aorta endothelial (BAE) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/l), glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 mg/l), and amphoterycin (1.25 mg/l) supplemented with 10% fetal bovine serum.

### 2.3. Photoactivation and manipulation of hypericin in the dark

For experiments with photoactivated hypericin, cell cultures in the presence of hypericin in the dark for 24 h were washed twice with phosphate-buffered saline (PBS), and after addition of new medium they were irradiated for 30 min with fluorescent lamps at a power density of light 4 J/cm$^2$. Afterwards, cells were further incubated in the dark.

For all the treatments with non-photoactivated hypericin, manipulation of hypericin and treatments were carried out in the dark, keeping hood and laboratory lights switched off. Under these conditions, ambient light fluence rate was $< 0.06$ mW/cm$^2$.

### 2.4. Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) 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 formazan product, which can be measured spectrophotometrically. BAE and tumor cells ($3 \times 10^3$ cells in a total volume of 100 μl of complete medium) were incubated in each well with serial dilutions of hypericin. After 3 days of incubation in the dark (37 °C, 5% $CO_2$ in a humid atmosphere), 10 μl of MTT (5 mg/ml in PBS) was added to each well and the plate was incubated for further 4 h (37 °C). The resulting formazan was dissolved in 150 μl of 0.04 N HCl-2 propanol and read at 550 nm. For photoactivation experiments, cells were seeded and incubated for 24 h prior to the addition of non-photoactivated hypericin. After another 24-h incubation (in the dark), cells were washed twice, new culture medium was added and photoactivation was carried out as described above. After an additional 24-h incubation in the dark, the MTT assay was carried out as described above. All determinations were carried out in triplicate. $IC_{50}$ values were calculated as those concentrations of hypericin yielding 50% cell survival, taking the values obtained for control as 100%.

### 2.5. Tube formation on Matrigel by endothelial cells

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. $5 \times 10^4$ BAE cells were added with 200 μl of DMEM. Finally, different amounts of hypericin were added and incubated at 37 °C in a humidified chamber with 5% $CO_2$. After incubation of 5 h in the dark, cultures were observed and photographed with a NIKON inverted microscope DIAPHOT-TMD (NIKON Corp., Tokyo, Japan). Each concentration was tested in duplicate, and two different observers evaluated the inhibition of tube formation. Only those assays where no tubular structure could be observed were evaluated as positive in the morphogenesis inhibition of endothelial cells on Matrigel.

### 2.6. Conditioned media and zymography

To prepare conditioned media, BAE cells were grown in 6-well plates. When the cells were at 75% confluency, cells were washed twice with PBS and each well received 1.5 ml of DMEM/0.1% bovine serum albumin containing 200 kallikrein units of aprotinin/ml. Additionally, some wells received 20 μM hypericin. After 24 h of incubation in the dark, conditioned media were collected. Media were centrifuged at $1000 \times g$ and 4 °C for 20 min. Afterwards, the supernatants were collected and used for zymography.

Zymographic assays of urokinase-type plasminogen activator (uPA) activity and the gelatinolytic activity of matrix metal-

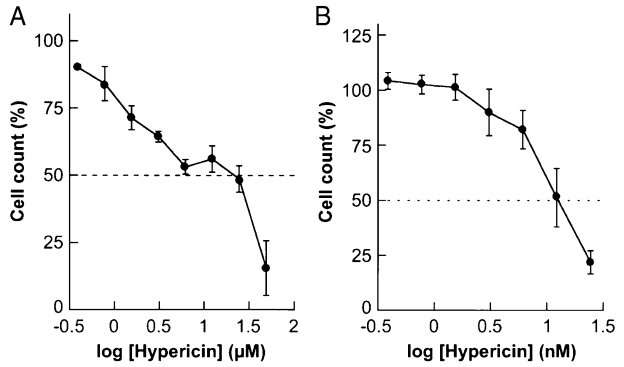


Fig. 2. Effect of hypericin on bovine aorta endothelial cell growth. Experiments with non-photoactivated hypericin in the dark (A) and photoactivated hypericin (B) were carried out as described in Materials and methods. Data are means ± S.D. of three independent experiments, each with quadriplicate samples for each tested hypericin concentration.

loproteinase-2 (MMP-2) delivered to the conditioned media were carried out as previously described (García de Veas et al., 1998; Fajardo et al., 1999).

### 2.7. 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 following two perpendicular diameters, giving rise to two 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 hypericin. Wounded areas were photographed. At different times of incubation in the dark, 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 with respect to their respective values at zero time.

### 2.8. Endothelial cell invasion assay

Invasion of fluorescence-labelled endothelial cells was assayed by using a 24-well fluorescence-opaque membrane insert. This assay allows for a real-time monitoring of the process, since 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 labelled in situ with 5 mg/ml Calcein-AM in complete culture medium for 2 h at 37 °C. After washing, the cell monolayer was briefly tripsinised to lift the cells, which were washed and suspended in DMEM supplemented with 0.1% bovine serum albumin. BAE cells were added to 8-mm FALCON HTS FluoroBlok inserts (Becton Dickinson, Bedford, MA, USA), whose filters were coated with Matrigel (25 μg/filter) at a density of $2 \times 10^5$ cells/insert in the absence or presence of 10 μM hypericin. DMEM supplemented with 0.1% bovine serum albumin was used as chemoattractant in the lower wells. The inserts were incubated in the dark at 37 °C and cell invasion was 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.

### 2.9. Statistical analysis and image analysis

All quantitative data are expressed as means ± standard deviation (S.D.). Two-tailed Student's *t*-test was used for evaluation of pairs of means, to establish which groups differed from the control group. Quantitative analysis of images was performed with the NIH Image 1.6 Program.

## 3. Results

### 3.1. Hypericin inhibits endothelial cell proliferation

We investigated the ability of hypericin to inhibit the growth of endothelial cells. Fig. 2A shows the mean survival curve obtained with the MTT assay. The estimated $IC_{50}$ value was 10 μM, three orders of magnitude higher than that obtained with photoactivated hypericin, namely, 13 nM (Fig. 2B).

### 3.2. Hypericin in the dark inhibits the capillary tube formation by endothelial cells

Using the Matrigel in vitro assay, our results show that the minimal concentration of non-photoactivated hypericin able to inhibit endothelial tube formation on Matrigel was 5 μM (Fig. 3).

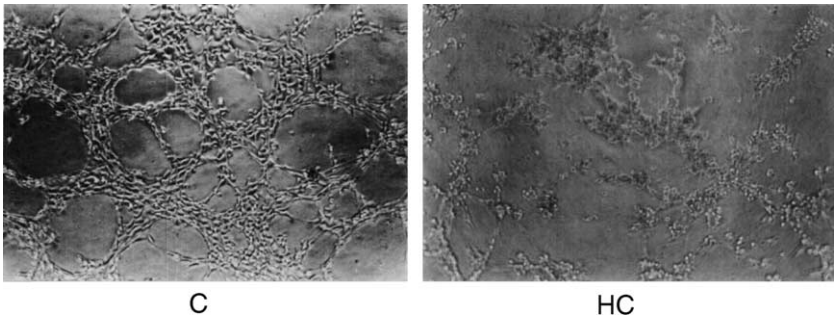


Fig. 3. Effect of hypericin in the dark on endothelial cell tubulogenesis in vitro. Experiments were carried out as described in Materials and methods. BAE cells seeded on Matrigel formed tubes (C), whereas tubulogenesis was inhibited in the presence of 5 μM hypericin (HC). Cells were photographed 5 h after seeding under an inverted microscope (× 100).

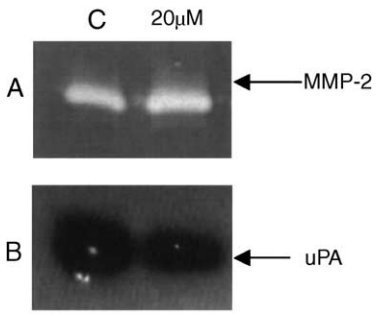


Fig. 4. Effects of hypericin in the dark on endothelial cell matrix metalloproteinase-2 and urokinase. (A) Conditioned media from BAE cells treated for 24 h with 20 μM hypericin in the dark and non-treated cells (control, C) were normalized for equal cell density and used for gelatin zymography as indicated in Materials and methods. (B) Conditioned media from BAE cells treated for 24 h with 20 μM hypericin in the dark and non-treated cells (control, C) were normalized for equal cell density and used for zymographic detection of urokinase as indicated in Materials and methods.

### *3.3. Hypericin in the dark decreases the extracellular levels of endothelial cell urokinase but not those of MMP-2*

Fig. 4A shows that 20 μM hypericin in the dark does not seem to inhibit BAE cell MMP-2, therefore excluding this extracellular matrix degrading enzyme as one of its targets. In contrast, Fig. 4B shows that 20 μM hypericin treatment in the dark produces a remarkable decrease of urokinase levels. In fact, image analysis allows us to estimate that this decrease amounts to almost 40% of the levels in conditioned media from control, untreated cells.

### *3.4. Hypericin in the dark inhibits the migration of endothelial cells, as well as their capability to invade through a Matrigel layer*

In the wound assay for BAE cells, 10 μM hypericin in the dark produced a potent inhibitory effect, observed 4–7 h after wounding (Fig. 5). In fact, comparative image analysis allows the estimation of 40% and 64% decreases in the wounding surface reoccupied by treated BAE cells after 4 and 7 h of incubation, respectively, as compared to values for control cells.

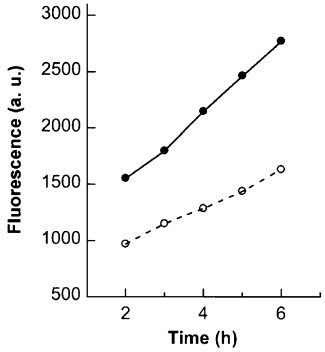


Fig. 6. Effect of hypericin on BAE cell invasion. The invasion assay was carried out as described in Materials and methods. Relative fluorescence of invading cells was followed with incubation time for both control (closed circles) and treated (10 μM hypericin, open circles) cells in the dark. Data are given in arbitrary units and they are means of triplicate samples in two independent experiments.

On the other hand, Fig. 6 shows that 10 μM hypericin in the dark produced a significant inhibition of the invasive capability of BAE cells through a Matrigel layer, decreasing the invasion velocity by almost 60%.

## 4. Discussion

Angiogenesis inhibitors can block any of the following components of the angiogenic process: degradation of the basement membrane, migration and proliferation of endothelial cells, and the formation of capillary-like tubes. When

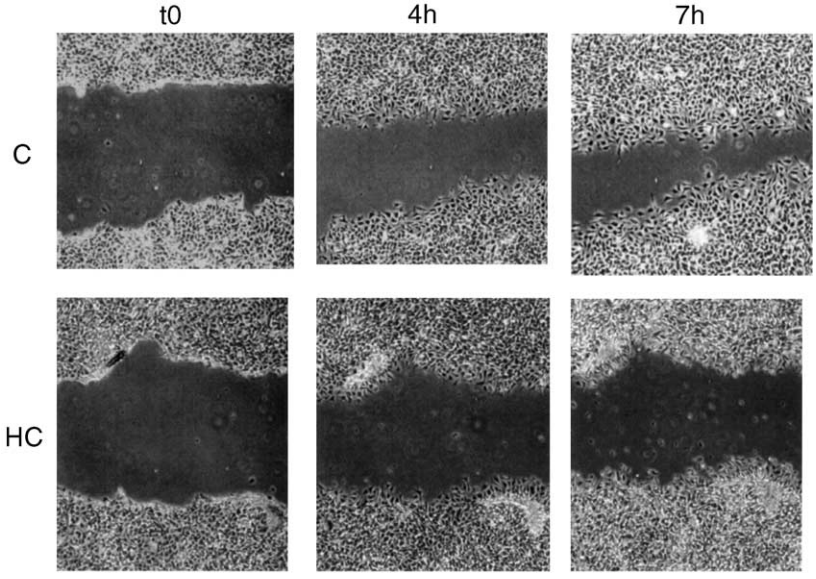


Fig. 5. Effect of 10 μM hypericin on the migration of BAE cells as revealed by the wound assay. Confluent monolayers were wounded as described in Materials and methods. After washing, fresh culture medium was added, either in the absence (control, C) or presence of 10 μM hypericin (HC). Photographs were taken at the beginning of the assay and after 4 and 7 h of incubation in the dark.

a factor can inhibit one or several of these key events in vitro, it is considered a candidate for the inhibition of angiogenesis in vivo (Moses and Langer, 1991).

Based on the traditional use of St. John's wort for the treatment of inflammatory processes and for the healing of wounds and ulcers (Di Carlo et al., 2001), and on the recent observation of the antitumoral activities of hypericin in the dark (Blank et al., 2001), we hypothesized that non-photoactivated hypericin could interfere with angiogenesis. Our results show for the first time that non-photoactivated hypericin is able to inhibit key steps of angiogenesis in vitro.

In response to an angiogenic stimulus during neovascularization, endothelial cells proliferate. Viability assays are easily adapted to carry out fast, sensitive and efficient determinations of the effects of drugs on cell survival and/or proliferation. For these reasons, these assays are usually selected for primary screening of new drugs with potential pharmacological use. In fact, several of the best characterized compounds described unambiguously as antiangiogenic were initially detected and selected for their capability to interfere with endothelial cell growth. This is the case of the extremely selective inhibitor of endothelial cell proliferation TNP-470, a synthetic analog of fumagillin with enhanced anti-angiogenic properties (Ingber et al., 1990; Rodríguez-Nieto et al., 2001b). Many other natural compounds with anti-angiogenic effects do, indeed, inhibit endothelial cell proliferation (Igura et al., 2001; Rodríguez-Nieto et al., 2001a; Shimamura et al., 2001). Our results show that hypericin in the dark inhibits BAE cell proliferation (Fig. 2A). In our hands BAE cells were extremely sensitive to photoactivated hypericin (Fig. 2B), much more than other endothelial and tumor cells (Kimura et al., 1997; Miccoli et al., 1998; Delaey et al., 2000; Blank et al., 2001; Xie et al., 2001). Nonetheless, the $IC_{50}$ value obtained for non-photoactivated hypericin in our experimental work with BAE cells is similar or lower than those found for this compound with tumor cells and for other anti-angiogenic compounds (Blank et al., 2001; Igura et al., 2001; Rodríguez-Nieto et al., 2001a; Shimamura et al., 2001).

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. In vitro, endothelial cells plated on Matrigel align themselves forming cords, already evident a few h after plating. Our results with non-photoactivated hypericin in this assay (Fig. 3) are remarkable, since previously it had been shown that tubular formation by bovine choroidal endothelial cells was inhibited by photoactivated hypericin in the same range of concentration (Kimura et al., 1997). This inhibitory effect on the morphogenesis of endothelial tubes is not due to cytotoxicity, since the concentrations required to inhibit the differentiation of BAE cells, did not affect their viability after 5 h (results not shown). Furthermore, this inhibitory effect was exerted at concentrations lower than or in the same range as those required for other known inhibitors (Hisa et al., 1998; Thaloor et al., 1998; Kim et al., 2000; Rodríguez-Nieto et al., 2001a; Castro et al., 2004).

Tumor angiogenesis and metastasis are two very complex processes that are closely allied at the mechanistic level, since both use similar operational strategies, involving changes in their adhesion to their microenvironment and activation of their capabilities to remodel extracellular matrix through a shift of the proteolytic balance towards proteolysis (Liotta et al., 1991). An essential step is the breakdown and removal of extracellular matrix, mediated by several proteases, the most important of which appear to be matrix metalloproteinases (MMP) and serine proteases. In fact the MMPs, 72 kDa and 92 kDa type IV collagenases or gelatinases (MMP-2 and MMP-9, respectively) and the serine protease urokinase are mainly involved in tumor invasion and metastasis (Pepper et al., 1990; Stetler-Stevenson, 1999; Egeblad and Werb, 2002; Rakic et al., 2003). BAE cells do express both MMP-2 and urokinase. Our results show that non-photoactivated hypericin produces a remarkable decrease in the levels of urokinase but not on those of MMP-2 (Fig. 4).

Migration and invasion of endothelial cells are two other key steps of the angiogenic process (Gourly and Williamson, 2000). Several anti-angiogenic compounds have been shown to inhibit endothelial cell migration and/or invasion (Dormond et al., 2001; Griggs et al., 2001; Igura et al., 2001; Rodríguez-Nieto et al., 2001a; Kang et al., 2003; Castro et al., 2004). Wound assays are frequently used to study the effects of drugs on the migratory capability of tested cells. Our results with this assay in short-time incubations (4–7 h) show that hypericin in the dark inhibits BAE cell migration (Fig. 5). Although we extended our observations, an additional effect on the adherence of BAE cells was evident for longer incubation times, since an increasing number of BAE cells detached from the culture plate (results not shown). For invasion, both extracellular matrix degradation and migration of endothelial cells have to happen. Since hypericin does not seem to inhibit MMP-2 but is a potent inhibitor of BAE cell migration and urokinase expression, the inhibitory effect observed in the invasion assay (Fig. 6) seems to be a consequence of these inhibitions. However, effects on adhesion and inhibitors of extracellular matrix proteases (not tested in this study) cannot be ruled out as additional partial causes of the observed global anti-invasive effect.

In conclusion, our results show that hypericin in the dark is able to inhibit key steps of angiogenesis, including endothelial cell proliferation, tubular formation on Matrigel, extracellular matrix degradation by urokinase, migration and invasion. Further evaluation in in vivo angiogenesis assays is warranted. Very recently, the

antimetastatic activity of hypericin in the dark has been demonstrated (Blank et al., 2004).

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