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Mechanisms of Hyperforin as an anti-angiogenic angioprevention agent

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ABSTRACT

Hyperforin, the major lipophilic compound contained in extracts of *Hypericum perforatum*, is responsible for the antidepressant activity associated with the extract. Recently, several other biological properties of Hyperforin have been unveiled including inhibition of tumour invasion and angiogenesis. The mechanism of the anti-angiogenic activity of Hyperforin remains to be fully elucidated. We show that treatment with non-cytotoxic concentrations of Hyperforin restrains, in a dose-dependent manner, the capacity of endothelial cells to migrate towards relevant chemotactic stimuli. Hyperforin inhibits the organisation of HUVE endothelial cells in capillary-like structures *in vitro*, and potently represses angiogenesis *in vivo* in the Matrigel sponge assay in response to diverse angiogenic agents. Immunofluorescent staining shows that in cytokine-activated endothelial HUVE cells Hyperforin prevents translocation to the nucleus of NF-κB, a transcription factor regulating numerous genes involved in cell growth, survival, angiogenesis and invasion. Under Hyperforin treatment *in vivo*, the growth of Kaposi's sarcoma – a highly angiogenic tumour – is strongly inhibited, with the resultant tumours remarkably reduced in size and in vascularisation as compared with controls. Hyperforin has also been reported to have anti-inflammatory properties. Here we show that Hyperforin inhibits neutrophil and monocyte chemotaxis *in vitro* and angiogenesis *in vivo* induced by angiogenic chemokines (CXCL8 or CCL2). These results highlight the potential for Hyperforin as an anti-inflammatory angioprevention agent, acting as a strong inhibitor of inflammation- or tumour-triggered angiogenesis, and provide new therapeutic approaches to halting pathology-associated angiogenesis.

1. Introduction

Extracts of St. John's wort (*Hypericum perforatum*, Guttiferae) have been in use since the time of Hippocrates for treating wounds, and was known to Paracelsus for the treatment of psychiatric disorders long before depression was recognised as a well-described pathology.[1,2] Today, controlled trials confirm the efficacy of this plant extract over placebo in the treatment of mild to moderately severe depression, through inhibition of neurotransmitter reuptake, such as dopamine,

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noradrenaline and gamma-aminobutyric acid.[3] Of the different classes of *H. perforatum* secondary metabolites, the prenylated acylphloroglucinol Hyperforin (Hyp) – isolated first by Bystrov in 1975[4] – has emerged as a key player in anti-depressant activity.[5]

In addition to its anti-depressive properties, Hyp has other beneficial effects, including anti-inflammatory activity through inhibition of the proliferation and induction of apoptosis of peripheral blood mononuclear cells (PBMC)[6]; blocking of 5-LOX and COX-1, i.e. two crucial enzymes in the biosynthesis of pro-inflammatory eicosanoids[7]; and significant relief of mild to moderate atopic dermatitis in topical treatment.[8] Hyp thus appears to act as an *in vivo* anti-inflammatory agent[9], though underlying mechanisms are still under debate. It has been demonstrated that Hyp inhibits the generation of reactive oxygen species (ROS) as well as the release of leukocyte elastase (degranulation) and Ca^{2+} mobilisation in human isolated polymorphonuclear (PMN) leucocytes.[10] Quiney et al. have shown that Hyp promotes apoptosis and capacity to secrete MMP-9 on neoplastic B lymphocytes,[11] while for T lymphocytes, Schempp et al. have demonstrated that Hyp suppresses the proliferation of PBMC in a dose-dependent manner.[12]

Further, Hyp is able to lower the proliferation rate of different mammalian cancer cell lines, such as squamous cell carcinoma, malignant melanoma, chronic myeloid leukaemia and lymphoma, by altering the balance between tumour cell proliferation and cell death rates.[13] Similar activities were found for a soluble derivative of Hyp, aristoforin.[14] Moreover, Hyp has been shown to effectively inhibit cancer growth and metastatic spreading without inducing adverse toxic effects.[15]

Recent studies show that Hyperforin and several synthetic derivatives also have anti-angiogenic properties[16–20]; however, the mechanisms of action of this anti-angiogenic action remain to be fully elucidated. Based on the anti-angiogenic and anti-inflammatory properties of Hyp, we hypothesised that Hyp could have direct actions on endothelial cells as well as the potential to inhibit inflammation-associated angiogenesis and innate immune cell activities.

In order to create new blood vessels, endothelial cells must migrate, differentiate into tubular structures and colonise the target tissue. Previous studies have found that Hyp is able to inhibit matrix metalloproteinase 9 (MMP-9),[11,19,20] a principally leucocyte-derived metalloprotinase involved in cellular invasion. We investigated the ability of Hyp as the stable and crystalline dicyclohexylammonium salt (Hyp-DCHA; Fig. 1),[21] a convenient storage form, to inhibit endothelial cell growth, migration and morphogenesis *in vitro* in the absence of apoptosis and to restrain angiogenic tumour growth *in vivo* in the Kaposi's sarcoma xenograft model.

It is now well recognised that inflammation-triggered angiogenesis plays a crucial role in cancer proliferation, growth and spread. Innate immune cells – tightly interplaying with endothelial cells – act as promoters of the angiogenic process, and their recruitment correlates with increased malignant phenotype of the tumours. Contrasting inflammation-triggered angiogenesis could thus be a crucial additional front of attack against the carcinogenesis process of solid tumours.[22,23] Here we show that Hyp inhibits migration of monocytes and PMNs *in vitro* and inflammation-triggered angiogenesis *in vivo*. Finally, we show that Hyp inhibits nuclear translocation of NF-κB, a central hub in both angiogenesis and inflammation. Our findings reveal that Hyperforin acts as a broad-spectrum anti-angiogenic compound and, given its anti-angiogenic anti-inflammatory properties and low toxicity, it may be an effective chemopreventive agent.

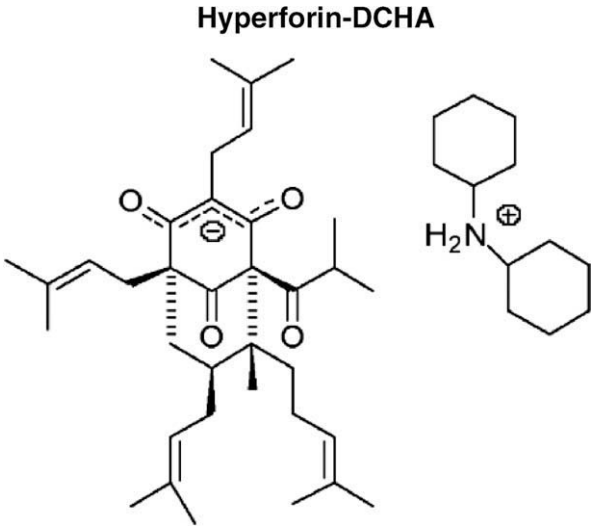

Fig. 1 – The chemical structure of Hyperforin-DCHA (dicyclohexylammonium) salt.

2. Materials and methods

2.1. Chemicals and cell cultures

The stable dicyclohexylammonium salt of Hyperforin (Hyp-DCHA, Fig. 1) was prepared according to the method described in patent PCT WO 99/41220 1999 Aug 19,[4] and solubilised 10 mM in DMSO, the latter also included in appropriate controls without Hyp-DCHA. Human recombinant VEGF-A, EGF, acid and basic FGFs, and TNF-alpha were obtained from Peprotech, heparin and hydrocortisone from Sigma, and Matrigel from the Engelbreth-Holm-Swarm sarcoma as previously described.[24] Monocytes and PMNs (essentially neutrophils) were freshly isolated (>96% pure) from human peripheral blood of healthy donors using standard Ficoll and Percoll gradients, and grown in 10% heat-inactivated foetal bovine serum (FBS), supplemented with 1% L-glutamine in RPMI medium (Sigma). Human umbilical vascular endothelial cells (HUVEC) were obtained from Interlab Cell Line Collection, IST Genoa, freshly isolated from umbilical veins and grown on 0.1% gelatine-coated tissue culture plates in M199 endothelial growth medium (Sigma), supplemented with 10% heat-inactivated FBS supplemented with 1% L-glutamine, FGF (1 μg acid-FGF plus 1 μg basic-FGF/100 ml), EGF (1 μg/100 ml), heparin (10 mg/100 ml) and hydrocortisone (0.1 mg/100 ml). In all *in vitro* experiments, cells were used between the 8th and 10th *in vitro* passages. Human Kaposi's sarcoma cells (KS-Imm) were derived from a spontaneously immortalised cell line from the iatrogenic form of KS,[25] and were routinely grown in 10% heat-inactivated FBS, supplemented with 1% L-glutamine in DMEM medium (Sigma).

2.2. *In vitro cell proliferation and apoptosis assay*

Cytostatic and toxic effects of Hyp were tested using the crystal violet assay. At day 0, 1500 cells/well were seeded in 96-multiwell plates in complete medium and treated with Hyp as specified. After the indicated times of incubation, the plates were processed and the absorbance read at 595 nm wavelength; indirect OD quantification was obtained by fixing and staining the cells for 20 min with a solution of 75% crystal violet, 0.35% sodium chloride, 32% ethanol and 3.2% formaldehyde, and then dissolved in 50% ethanol, 0.1% acetic acid for the spectrophotometric analysis.

The effects on apoptosis were determined using the Cell Death Detection ELISA assay (Roche) according to the manufacturer's instructions. To measure any enrichment of cytoplasmic histone-associated-DNA-fragmentation after Hyp treatment, HUVE (24,000 cells/ml/well) were plated in complete medium and allowed to grow for 12 h in 24-multiwell plates. After this stabilisation period, the culture medium was changed and supplemented with either Hyp and/or Vincristine (10 μM). After the indicated incubation periods, the plates were processed and the absorbance read at 504 nm wavelength.

2.3. *Neutrophils, monocytes, and HUVE cell chemotaxis*

Neutrophil and monocyte Chemotaxis was performed in 48-microwell chemotaxis chambers (Costar-Nucleopore) using 5 μm pore-size polyvinylpyrrolidone-free polycarbonate filters (Costar-Nucleopore). The lower compartment of each chamber was filled with chemoattractant stimulus (IL-8 50 ng/ml or MCP-1 250 ng/ml) diluted in RPMI-0.1% BSA. Simple RPMI-0.1% BSA (serum-free medium (SFM)) was used as the control for unstimulated random migration. Each upper compartment well was filled with neutrophils or monocytes in suspension in SFM (3×10^6 and 5×10^6 cells/ml respectively), with or without Hyp, and each experimental procedure was performed in sextuplicate. The filled chambers were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 45 min. The filters were then removed and the cells fixed with 100% ethanol and stained with toluidine blue. Non-migrated cells were removed from the upper surface of the filter, using filter paper, and the migrated cells were counted by scanning the filter surface, followed by densitometric analysis using the National Institutes of Health Image Analysis program.

Chemotaxis assays of HUVE cells were performed using the Boyden chambers, as previously described.[24,26] Cells (5×10^4) were extensively washed with PBS, resuspended in SFM and placed in the upper compartment with or without Hyp. Upper and lower compartments of the Boyden chambers were separated by 12-μm pore-size polycarbonate filters coated with 5 μg/ml of collagen IV for the chemotaxis assay. Supernatants from NIH3T3 cells (NIH3T3-CM) were used as chemoattractants. After 6 h incubation at 37 °C in a 5% CO_2 humidified atmosphere, the filters were recovered and the cells on the upper surface removed (as above). Migrated cells were fixed with 100% ethanol and stained with toluidine blue and then counted in five to eight unit fields for each filter under a microscope. Each test was performed in triplicate and repeated three times.

2.4. *In vitro Matrigel morphogenesis assay*

The effects of Hyp on the ability of endothelial HUVE cells to reorganise and differentiate into capillary-like networks were assessed in the *in vitro* Matrigel morphogenesis assay as described previously.[27] Matrigel (300 μl/well) thawed at 4 °C was added with a cold pipette to a pre-chilled 24-multiwell plate. After polymerisation of Matrigel at 37 °C, 5×10^4 cells/well were suspended in 1 ml of complete endothelial cell growth medium with or without Hyp at the indicated concentrations, and carefully layered on top of the polymerised Matrigel. The effects on morphogenesis of the cells were photographed after 6 h incubation with a Leitz DR-IMB inverted microscope equipped with CCD optics.

2.5. *In vivo angiogenesis: Matrigel sponge assay*

The ability of Hyp to inhibit the formation of new blood vessels was tested using the Matrigel sponge model of *in vivo* angiogenesis as described previously.[28] Inflammatory/angiogenic chemokines (50 ng/ml IL-8 or 50 ng/ml MCP-1) and 26 U/ml Heparin were added to unpolymerised Matrigel at 4 °C to a final volume of 600 μl/pellet. The suspension obtained was slowly injected subcutaneously (s.c.) into the flanks of C57/Bl6 N male mice (Charles River Laboratories) with a cold syringe, then the Matrigel polymerises *in vivo* forming solid gel.

In one series of experiments the anti-angiogenic effect of Hyp was tested by direct addition of the compound at different concentrations to the Matrigel mixture before s.c. injection into mice. In other experiments, animals received Hyp systemically by intraperitoneal (i.p.) injection at 5.4 mg/kg body weight (150 μl final volume of 1 mM solution, 10% DMSO), whereas control animals received the same volume of vehicle alone (10% DMSO in PBS solution) each day following Matrigel injection. After 4 days the animals were sacrificed and the gels were recovered, weighed, and either fixed in formalin and embedded in paraffin for histological examination, or minced and diluted in water for haemoglobin content measurement with a Drabkin reagent kit (Sigma). Final haemoglobin concentration was calculated from a standard calibration curve after spectrophotometric analysis at 540 nm.

2.6. *In vivo xenograft tumour growth and histochemistry*

KS xenograft tumours were obtained by s.c. injection of 5×10^6 human KS-Imm cells/mouse, mixed with liquid Matrigel (final volume 250 μl), into the flanks of 7-week-old male Nude *nu/nu* (CD-1)BR mice (Charles River Laboratories) as described previously.[25] Treated animals received Hyp systemically by i.p. injection at 5.4 mg/kg (150 μl final volume of 1 mM solution, 10% DMSO), whereas control animals received the same volume of vehicle alone (10% DMSO in PBS solution), or by a local peritumour (p.t.) injection of Hyp at the indicated doses, three times a week starting at day 7 when tumours became palpable. Animals were weighed and tumour growth

was monitored at regular intervals by measuring two tumour diameters with callipers and calculating the tumour volumes using the following formula: length × width^2/2. On day 28 animals were sacrificed and tumours were removed, weighed, fixed in formalin, and paraffin embedded. Three-μm sections were stained with haematoxylin and eosin (H&E) for histological examination.

2.7. Immunofluorescence microscopy

HUVE cells were seeded (2×10^4/well) on 0.1% gelatine-coated multiwell chamber slides (LabTek, Nunc). After 24 h the medium was changed and the cells were treated for 3 h with Hyp; human TNF-α (50 ng/ml) was added for the last 30 min where indicated. At the end of incubation, the cells were fixed and permeabilised in cold methanol for 10 min at –20 °C, blocked in PBS with 10% horse serum (Sigma) for 10 min, and stained for 1 h with anti-NF-κB (p65 subunit) monoclonal antibody (Zymed) at a 1:100 dilution in PBS with 1% horse serum. The cells were then washed three times with PBS, incubated 30 min with TRITC-conjugated secondary anti-mouse antibody (DakoCytomation) at 1:200 dilution in PBS with 1% horse serum, counterstained 5 min with DAPI (Sigma) at 1 μg/ml, and washed in PBS. Slides were mounted and viewed in a CCD-optics equipped Leica DML epifluorescence microscope at ×10 magnification.

2.8. Statistical analyses

The statistical analyses performed were two-tailed *t* tests for comparison of two datasets, one-way ANOVA for multiple datasets, and two-way ANOVA for growth curves, using the Graph Pad Prism statistics and graphing program.

3. Results

3.1. Effects of Hyp on endothelial cell growth, survival and apoptosis

Hyp was tested for its ability to modulate HUVE cells *in vitro* growth, measured by the crystal violet assay. On HUVE cells, induced to proliferate by angiogenic stimuli, a cytostatic activity of Hyp was observed at doses of 3 and 9 μM; and significant reduction of growth was registered after 72 h, with doses from 9 to 27 μM Hyp ($P < 0.001$, two-way ANOVA) (Fig. 2). Lower concentrations of Hyp caused no evident or significant effects even at long exposure times.

The potential apoptotic or cytotoxic activity of Hyp was then investigated. Cells were exposed to different μM concentrations of Hyp for 24, 48 and 72 h. After incubation, cells were processed for detection of oligonucleosomes in the cytoplasmatic fraction of cell lysates, with a commercial cell death detection ELISA kit. The basal levels of DNA fragmentation – as an indicator of apoptosis – were not modified by treatment for 24 h with Hyp in the 1–9 μM range, in HUVE cells (data not shown). Furthermore, Hyp had no additive effect on the strong apoptotic rate induced by 10 μM vincristin. We found cytostasis with low levels of cytotoxicity at 27 μM Hyp in HUVE cells; these were almost unaffected after 24 h treatment, with a minor increase of the basal apoptotic rate (data not shown).

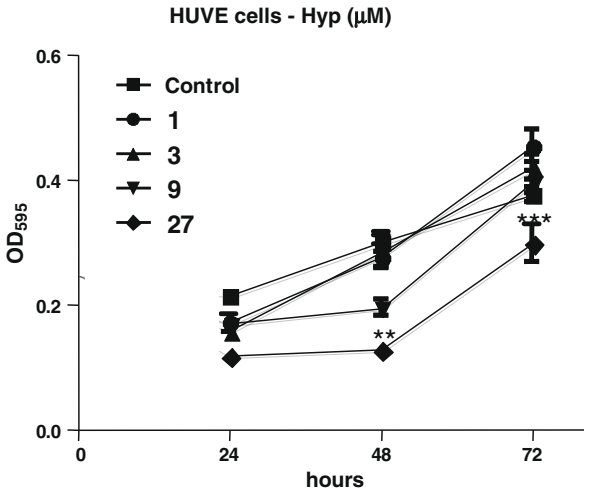

Fig. 2 – The effect of Hyp on growth of HUVE cells *in vitro* over time. At 48 and 72 h with 27 μM the growth of HUVE cells is significantly different from controls ($P \leqslant 0.01$; ***$P < 0.001$, two-way ANOVA). Means ± SE are shown.**

3.2. Effects of Hyp HUVE cell migration and morphogenesis

Tumour angiogenesis requires endothelial cell migration to enable endothelial cells to cross basement membranes in their move toward angiogenic stimuli. A chemotaxis assay was used to measure the effects of Hyp on HUVE cells *in vitro* in response to potent angiogenic factors such as those present in the conditioned medium of NIH/3T3 cells, used as a chemoattractant. Addition of 1 to 27 μM Hyp resulted in significant and dose-dependent inhibition of HUVE cell migration (Fig. 3A).

An important step in endothelial cell modulation during angiogenesis is differentiation and reorganisation to make new vessels. HUVE cells, when plated on a three-dimensional Matrigel layer, are able in 6 h to organise themselves into capillary-like networks, mimicking *in vitro* the events that occur *in vivo* during the angiogenic process.[27] Consistent with the data on cell viability and the effects on *in vitro* migration, the addition of μM Hyp dose-dependently interfered with growth and morphogenesis of HUVE cells, thus restraining their ability to organise a capillary-like network (Fig. 3B).

3.3. Effects of Hyp on xenograft tumour growth in vivo

We then examined whether Hyp was able to affect *in vivo* vascular tumour growth in the KS-Imm cell xenograft model. These tumour cells form highly angiogenic tumours, when injected s.c. in *nu/nu* nude mice, characterised by evident vascularisation similar to human KS lesions.[25] In these experiments, mice were divided into four groups: one untreated control group (with the DMSO vehicle alone), two groups receiving 1 or 3 μg of Hyp locally injected peri-tumourally, respectively, and the fourth group systemically treated with i.p. injection at 5.4 mg/kg of Hyp three times weekly.

Tumour growth in animals receiving Hyp was strongly and significantly reduced ($P < 0.001$, two-way ANOVA) compared

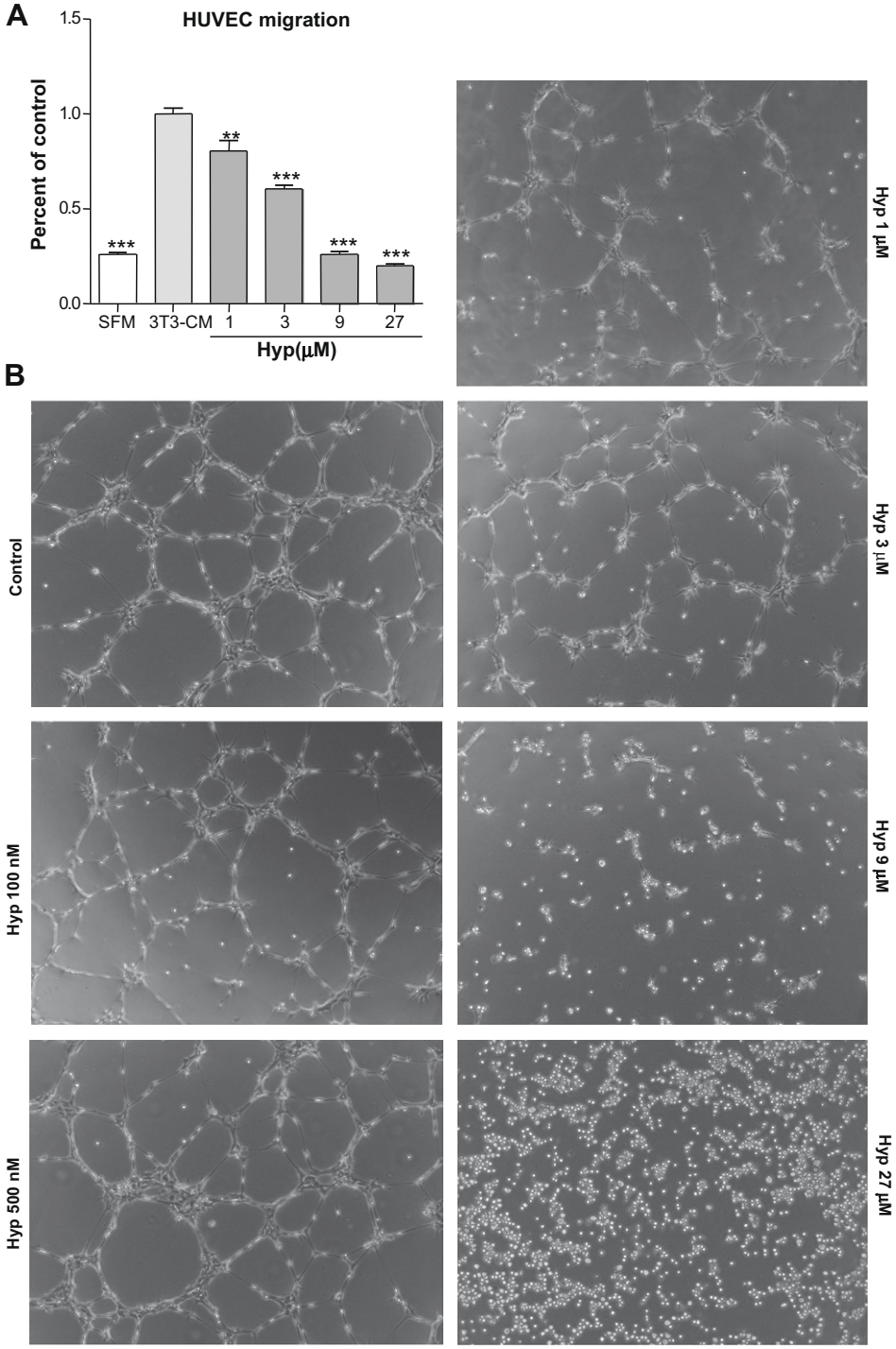

Fig. 3 – (A) The inhibition of HUVE cell migration by increasing doses of Hyp. Serum-free medium (SFM) was used as a negative control; NIH-3T3 fibroblast conditioned medium (CM) was used as a positive control. Experiments were performed in triplicate and repeated three times with similar results. Means ± SE are shown. $^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$ (two-tailed t test). (B) Effects of Hyp on organisation of HUVE cells on Matrigel. HUVE cells spontaneously organise into capillary-like networks after 6 h incubation at 37 °C in the presence of angiogenic factors. Hyp dose-dependently inhibited this differentiation of HUVE cells.

with the untreated controls in all three Hyp-treated groups; a strong effect was also found with the systemic (i.p.) administration (Fig. 4A). Substantial differences were noted as the control-tumour growth exceeded 100 mm^3, the differences statistically significant from day 24 onwards. No differences were noted in the body weight or general health parameters in the treated animals compared with untreated controls, again indicating limited or no toxicity of Hyp treatment, even after long-term administration. Upon removal of the tumours at the end of the experiment (day 28), mice receiving Hyp had

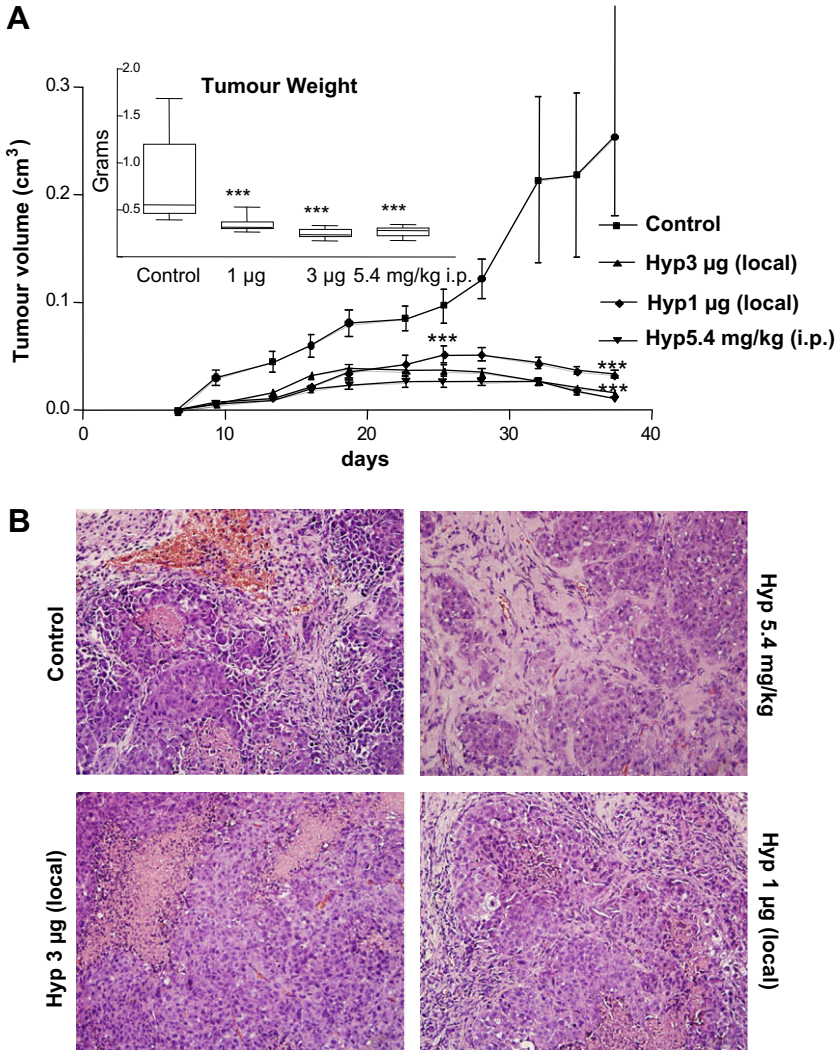

Fig. 4 – Effects of Hyp on angiogenic tumour growth *in vivo*. (A) The growth of highly vascularised KS-Imm tumour xenografts was significantly reduced by Hyp treatment (*$P < 0.001$, two-way ANOVA) by both local peritumoural injection (1 or 3 µg, as indicated) and systemic (i.p.) administration (5.4 mg/kg body weight). At the end of the experiment, a significantly lower weight (***$P < 0.001$, two-tailed *t* test) of the tumours from Hyp-treated animals was observed (A, inset). (B) Histological analysis of tumour sections (haematoxylin and eosin, H&E) shows vascularised areas in vehicle-treated animals, while more extensive necrotic areas with matrix deposition and very few vessels were found in the tumours from Hyp-treated animals.**

significantly smaller tumours than the untreated ones, as determined by tumour weight (Fig. 4A, inset). Histological analysis of the KS tumours showed extensive areas of vascularisation interspersed with occasional zones of necrosis in control tumours, while in tumours from Hyp-treated animals, extensive areas of necrosis and residual fibrosis were found with fewer and smaller vessels and degenerating tumour cells with pycnotic nuclei (Fig. 4B).

3.4. *Effects of Hyp on angiogenesis in vivo*

In addition to angiogenic factors, KS cells also produce significant quantities of cytokines and chemokines associated with inflammatory infiltrate within the tumours.[29] We therefore used the Matrigel sponge model as a rapid and quantitative system for measuring the *in vivo* anti-angiogenic activity of Hyp. Subcutaneous injection of Matrigel produces a three-dimensional pellet which, when angiogenic factors are present, becomes rapidly vascularised.[27] The extent of vascularisation can be indirectly estimated by measuring the haemoglobin content of the recovered gels, and by histological examination of paraffin-embedded samples. CXCL8 (IL-8) (Fig. 5) and CCL2 (MCP-1) (Fig. 6) were used as inflammatory/angiogenic stimuli mixed with the Matrigel, inducing an intense angiogenic reaction.[30,31] Addition of increasing concentrations of Hyp to this mixture significantly inhibited the *in vivo* angiogenic response to both of these chemokines (Figs. 5A and 6A).

We therefore tested whether – in the same model-system – systemic administration of Hyp could repress *in vivo* angiogenesis. Animals received Hyp by i.p. injection at 5.4 mg/kg body weight daily following Matrigel injection (Figs. 5B and 6B), a dose shown to be effective in restraining tumour growth *in vivo* but without any appreciable toxicity. Stereomicro-

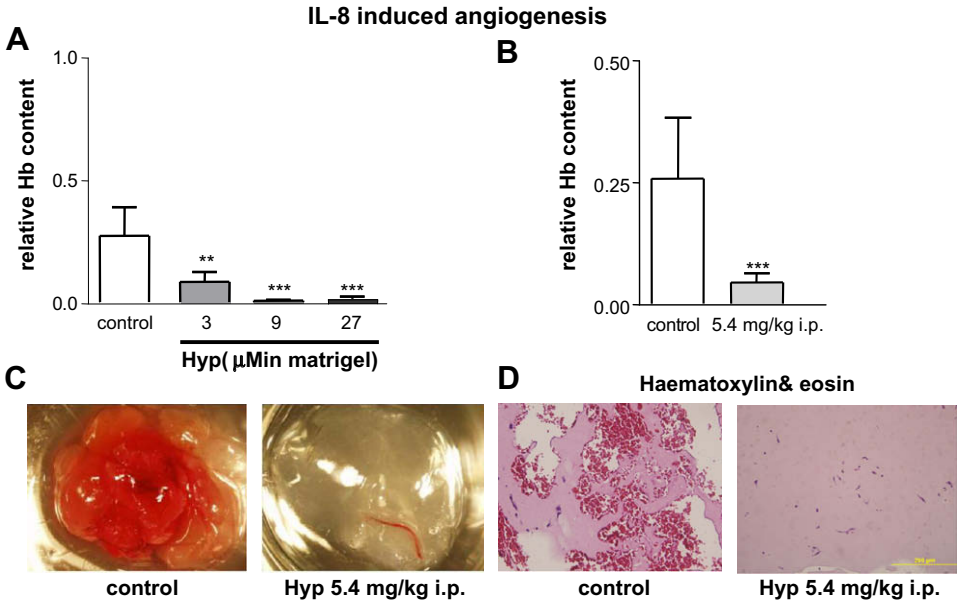

Fig. 5 – Inhibition of angiogenesis induced by IL-8 (CXCL8). (A) As compared to controls, the IL-8 induced vascularisation of the s.c.-injected Matrigel implants was significantly inhibited by addition of Hyp to the gels. (B) A remarkable reduction of the angiogenic response was also seen using a systemic (i.p.) treatment (5.4 mg/kg body weight). (C) Stereomicroscopic images and (D) histological analysis show examples of the inhibition of the vascular and cellular-infiltrates of the recovered implants from Hyp-treated animals. The angiogenic response was measured as the haemoglobin (Hb) content of the gels; gel sections were stained by haematoxylin and eosin (H&E); $^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$ (two-tailed *t* test).

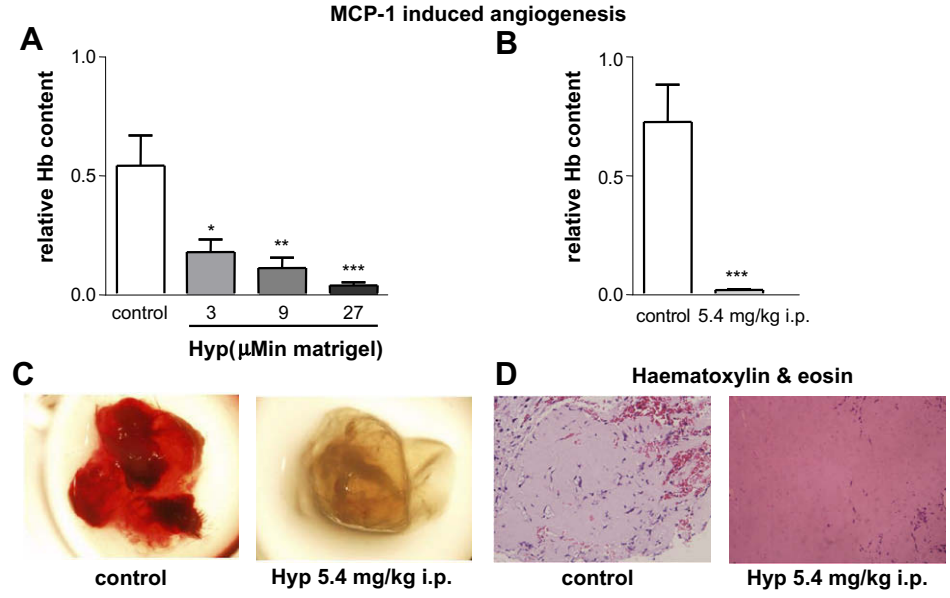

Fig. 6 – Inhibition of angiogenesis induced by (CCL2) MCP-1. (A) As compared to controls, the MCP-1 induced vascularisation of the s.c.-injected Matrigel implants was significantly inhibited by addition of Hyp to the gels. (B) A remarkable reduction of the angiogenic response was also seen using a systemic (i.p.) treatment (5.4 mg/kg body weight). (C) Stereomicroscopic images and (D) histological analysis show examples of the inhibition of the vascular and cellular-infiltrates of the recovered implants from Hyp-treated animals. The angiogenic response was measured as the haemoglobin (Hb) content of the gels; gel sections were stained by haematoxylin and eosin (H&E); $^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P<0.05$ (two-tailed *t* test).

scopic images and histological examination of the recovered implants, stained with haematoxylin and eosin, showed a strong reduction of vessel colonisation and a variable reduction of the inflammatory cellular infiltrate (Figs. 5C and 6C).

3.5. *Effects of Hyp on PMN and monocyte migration*

Given the ability of Hyp to block angiogenesis in response to the myeloid cell associated chemokines IL-8 (CXCL8) and

MCP-1 (CCL2) *in vivo*, we examined whether Hyp directly affects the primary target cells of these chemokines, neutrophils and monocytes, respectively. The ability of neutrophils and monocytes to migrate *in vitro* towards these specific chemotactic stimuli was markedly restrained by non-cytotoxic concentrations of Hyp in a dose-dependent manner (Fig. 7A and B). On collagen IV-coated filters, Hyp was able to inhibit the *in vitro* migration of these inflammatory cells within the range of concentrations tested (3–27 μM) with a two-fold decrease at the highest concentration and no significant differences at the lowest concentration (1 μM), as compared to the positive control (Fig. 7A and B).

3.6. *Effects of Hyp on NF-κB nuclear translocation in HUVE cells*

Hyp was able to inhibit angiogenesis induced by inflammatory/angiogenic factors such as IL-8 (CXCL8) and MCP-1 (CCL2), inflammatory stimuli known to be angiogenic,[32,33] and has been previously suggested to be an effective anti-inflammatory agent.[9] We had previously noted that the NF-κB pathway is frequently targeted by angioprevention agents in endothelial cells,[34] thus we hypothesised that Hyp could affect the NF-κB pathway and, as a consequence, induction of angiogenesis by endothelial and innate immune cells. Immunofluorescent staining with Texas Red indicated that NF-κB was largely localised in the cytoplasm of unstimulated HUVE cells and that Hyp treatment had little effect on this localisation. Stimulation of endothelial cells for 30 min with 50 ng/ml TNF-α resulted in translocation of NF-κB to the nucleus (Fig. 8, arrows); pre-treatment with 3 μM Hyp inhibited this translocation (Fig. 8, arrowheads), with the p65 subunit remaining in the cytoplasm of HUVE cells.

4. Discussion

The tumour microenvironment is a complex society of many cell types which have been recognised as playing a crucial role in the progression of cancer. Among these, endothelial cells and inflammatory components have recently been shown to provide crucial angiogenic stimuli and to modulate the microenvironment that favours constant tissue remodelling within the tumour.[35–37] Angiogenesis is in fact a rate-limiting step in progression to solid tumour malignancy, and without host support cancer cells lose much of their capacity to form tumour masses and disseminate destructive metastases.[38,39]

Cancer chemoprevention holds the promise of being able to halt or hinder the carcinogenesis process, a potentially highly effective approach to the morbidity of cancer.[40] Plant derived products appear to be particularly adapt for chemoprevention approaches.[41,42] Given the recognised role of angiogenesis and inflammation in tumour development and promotion, new therapies or prevention strategies targeting the tumour microenvironment have recently been developed, and show promising properties and lower toxicity compared with conventional chemotherapies.[22,40] Recent advances in the comprehension of mechanisms involved in tumour angiogenesis are providing the necessary background in order to develop even more effective anti-angiogenic strategies for cancer chemoprevention and therapy.[39] The identification of pharmacologically active compounds of natural origin is opening new perspectives in chemoprevention of tumours,[22] as well as in cardiovascular disease, and here we show a possible use of *Hypericum* extracts for its anti-inflammatory and anti-angiogenic potential.[19]

The present work investigates the activity of Hyperforin (Hyp) – the major lipophilic constituent of St. John's wort (*Hypericum perforatum*) – as an inhibitor of inflammation-triggered angiogenesis in both *in vitro* and *in vivo* experimental models. The results show that this revisited active plant-derived compound[19] is effective in hampering or cutting off the blood supply essential for tumour growth, opening new perspectives in the chemoprevention of solid cancers.

We previously reported that Hyp restrains inflammation and eventual lung fibrosis[20] and inhibits cancer invasion

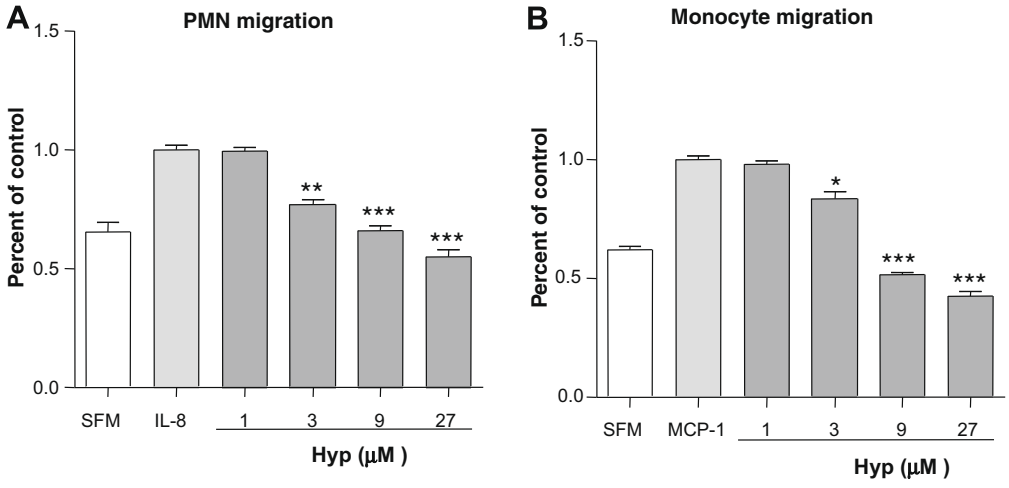

Fig. 7 – Inhibition of (A) PMN and (B) monocyte migration by increasing doses of Hyp. Serum-free medium (SFM) was used as a negative control; as chemotactic stimuli, Interleukin-8 (IL-8, CXCL8) was used for neutrophils, and monocyte chemotactic protein-1 (MCP-1, CCL2) was used for monocytes. Experiments were performed in triplicate and repeated three times with similar results. Means ± SE are shown. $^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$ (two-tailed *t* test).

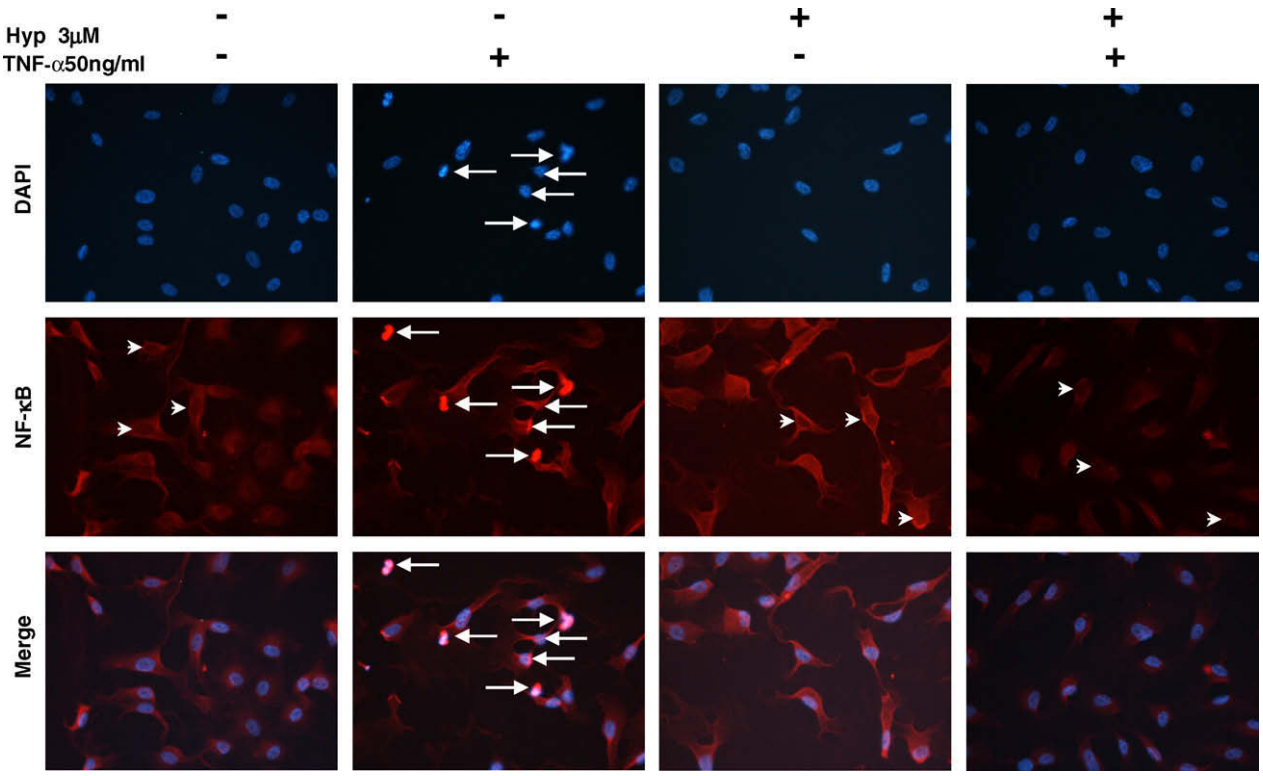

Fig. 8 – The effect of Hyp on NF-κB nuclear translocation in HUVE cells. Nuclei are stained blue with DAPI. Immunofluorescent staining with a Texas Red labelled anti-NF-κB p65 subunit shows that 3 μM Hyp inhibits translocation to the nucleus upon stimulation with TNF-α. Similar inhibition was observed with Hyp at 1 μM (not shown). Arrows indicate nuclei positive for NF-κB, arrowheads indicate examples of nuclei negative for NF-κB.

and metastasis[15] in murine preclinical models. Previous studies have indicated that Hyp harbours a significant anti-angiogenic activity,[16,18,20] potentially through inhibition of MMP-9.[11,19,20] Here we further investigate the mechanisms of this natural product on the inflammation-angiogenesis-tumour scenario. Hyp, at concentrations around one order of magnitude greater than the haematic concentrations registered in individuals using *Hypericum perforatum* extracts,[43] restrains the proliferation of endothelial (HUVE) cells at concentrations similar to that reported previously.[18] Clinical use of *Hypericum perforatum* extracts has been found to be remarkably well tolerated,[44] suggesting that this compound could be used in clinical chemoprevention settings, although interactions with numerous other pharmaceuticals is a concern.[44] Importantly, Hyp exerts its anti-angiogenic activity without substantially inducing apoptosis or cytotoxicity in endothelial cells, indicating a largely cytostatic effect. Moreover, at the same concentrations, it blocks the chemotactic motility of endothelial cells (on basement-membrane-collagen-coated filters) and the capacity to reorganise on Matrigel. We also demonstrate that Hyp inhibits the growth of a highly angiogenic tumour, Kaposi's sarcoma, with both local and systemic treatment without apparent toxicity. These *in vivo* and *in vitro* studies suggest that the anti-angiogenic activity Hyp is not detrimental to the existing vascular system, further indicating lack of toxicity.

In addition to the direct effect on the endothelial cells, the anti-angiogenic effect of Hyp was also indirectly mediated through its efficacious anti-inflammatory properties, as our *in vitro* and *in vivo* data show that hyp effectively repressed recruitment and activation of innate immune cells. The presence of an extensive leucocytic inflammatory infiltrate in the tumour stroma is common, and plays an important role in the release of inflammatory and angiogenic cytokines, chemokines and growth factors.[23,35–37] This chronic inflammatory status is often favourable for the initial phases of angiogenic process and neoplastic progression.[23,35–37] The pro-angiogenic activity of Interleukin-8 and MCP-1 is exploited through a double signalling system, which acts directly on neutrophils and monocytes, respectively, to stimulate release of pro-angiogenic factors like VEGF, and indirectly on the endothelial cells.[30,45,46] The inhibition of PMN and monocyte migration by Hyp could lead to inhibition of the release of chemotactic factors and to direct inhibition of pro-angiogenic factors targeting the vascular tumour endothelium. Furthermore, potent inflammatory molecules – such as TNFα and CC-family chemokines, MCP-1 (CCL2) – can act as strong promoters of VEGF and VEGF-Receptor expression: mice lacking the TNF-Receptor1 show impaired angiogenesis,[47] confirming the role of this molecule in promoting angiogenesis. Furthermore, these inflammatory stimuli can induce production of proteases that mediate the invasion by both monocytes/macrophages and tumour cells, which often use these enzymes produced by stroma cells for their invasion.[11,20,48] The combined action of Hyp in inhibiting the migration of the main phagocytic elements can therefore exert an efficient multi-factor

counter-offensive against the angiogenic promotion triggered by these inflammatory cells.

To seek support for this hypothesis, we also investigated the mechanism by which Hyp inhibits inflammatory angiogenesis. Inflammation activates the NF-κB pathway in endothelial cells, which is also one of the main molecular transactivators of angiogenesis.[34] Numerous downstream mediators under the transcriptional control of NF-κB are known to influence tumour invasion.[49,50] Targeting NF-κB may represent a potential mechanism for controlling endothelial cell activity and inhibiting angiogenesis, as demonstrated by similar effects observed with numerous molecules associated with suppression of angiogenesis.[34] The NF-κB transcription factor is required in the regulation of many genes required for cell growth, survival and invasion, and is a pro-inflammatory factor promoting tumourigenesis.[51,52] Previous studies have shown that Hyp and its derivatives can inhibit NF-κB[53,54] or related activities.[55] Here we show that Hyp blocks NF-κB activation by TNFα in endothelial cells, providing additional support to the concept that this is a key regulatory mechanism of angiogenesis and a common target of anti-angiogenic drugs.[34]

In conclusion, inflammation-dependent angiogenesis appears to be a pivotal force in tumour growth and progression, since inhibition of inflammation prevents angiogenesis, and this in turn blocks tumour expansion. Inflammation, angiogenesis and tumour growth must thus be viewed as three corners of cancer pathology.[35,56] In this context, Hyp is able to hinder specific crucial mechanisms at each of these corners, however, without inducing apoptosis of the normal endothelial cells, providing a rationale for safe application in the chemoprevention of tumour angiogenesis and other inflammatory angiogenesis-associated pathologies such as arthritis and cardiovascular pathologies in whose pathogenesis the inflammatory component plays a crucial role.

Conflict of interest statement

None declared.

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