Anti-angiogenic effects of mangiferin and mechanism of action in metastatic melanoma

René Delgado-Hernández[a], Ivones Hernández-Balmaseda[b], Idania Rodeiro-Guerra[b], Julio Cesar Rodriguez Gonzalez[c], Olivier De Wever[d], Emilie Logie[e], Ken Declerck[e], Claudina Pérez-Novo[e] and Wim Vanden Berghe[e]

Advanced metastatic melanoma, one of the most aggressive skin malignancies, is currently without reliable therapy. The process of angiogenesis is crucial for progression and metastasis of the majority of solid tumors including melanomas. Therefore, new therapies are urgently needed. Mangiferin is a naturally occurring glucosylxanthone which exerts many pharmacological activities against cancer-inflammation. However, the effect of mangiferin on metastasis and tumor growth of metastatic melanoma remains unclear. In this study, we demonstrate that mangiferin interferes with inflammation, lipid and calcium signaling which selectively inhibits multiple NFkB target genes including interleukin-6, tumor necrosis factor, interferon gamma, vascular endothelial growth factor receptor 2, plasminogen activator urokinase, matrix metalloprotease 19, C-C Motif Chemokine Ligand 2 and placental growth factor. This abrogates angiogenic and invasive processes and capillary tube formation of metastatic melanoma cells as well as human placental blood vessel explants in-vitro and blocks angiogenesis characteristic of the chicken egg chorioallantoic membrane assay and in melanoma syngeneic studies *in vivo*. The results obtained in this research illustrate promising anti-angiogenic effects of the natural glucosylxanthone mangiferin for further (pre)clinical studies in melanoma cancer patients. *Melanoma Res* XXX: 000–000

Melanoma Research 2019, **XXX**:000–000

Keywords: angiogenesis, endothelial cell, mangiferin, melanoma, metastasis

[a]Centro de Estudios para las Investigaciones y Evaluaciones Biológicas (CEIEB), Instituto de Farmacia y Alimentos (IFAL), Universidad de La Habana (UH), [b]Laboratorio de Farmacología, Instituto de Ciencias del Mar (ICIMAR), CITMA, [c]Centro de Investigación y Desarrollo de Medicamentos (CIDEM), La Habana, Cuba, [d]Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, UZ-Gent, Gent and [e]Laboratory of Proteinchemistry, Proteomics & Epigenetic Signaling (PPES), Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium

Correspondence to Wim Vanden Berghe, PhD, Laboratory of Proteinchemistry, Proteomics & Epigenetic Signaling (PPES), Department of Biomedical Sciences, University of Antwerp, Campus Drie Eiken, S4.25, Universiteitsplein 1, 2610 Antwerp, Belgium
Tel: +32 3 265 26 57; fax +32 3 265 23 39;
e-mail: wim.vandenberghe@uantwerpen.be

Received 14 September 2018 **Accepted** 5 September 2019

Introduction

Skin cancer incidence is rapidly growing over the last decades, and melanoma represents one of the most aggressive and the deadliest forms. Current treatments include surgical resection, chemotherapy, photodynamic therapy, immunotherapy, biochemotherapy and targeted therapy. Despite the development of novel therapies, the management of malignant melanoma remains challenging [1]. The therapeutic strategy can include single agents or combined therapies, depending on the patient's health, stage and location of the tumor [2]. The efficiency of these treatments can be decreased due to the development of diverse resistance mechanisms. Various studies have shown the vascular angiogenic system to be pivotal for metastasis in melanoma. Angiogenesis, the development of new blood vessels, from pre-existing vessels is a complex multifactorial process involved in tissue development and wound healing but becomes pathologic when associated with solid tumor growth and metastasis [3]. Consequently, the effects of various anti-angiogenic experimental therapies are currently investigated in pre-clinical and clinical trials. Although most studies focus on inhibition of vascular endothelial growth factor (VEGF) signaling, others are aimed at determining the effect of multikinase inhibitors or the inhibition of angiogenic integrin activity. However, overall survival rates have not significantly improved in different clinical trials with anti-angiogenic agents. Resistance to anti-VEGF monotherapy has been observed in several studies, especially in malignant melanoma [4–6]. Thus inhibition of a single kinase or a pathway is unlikely to stop malignant angiogenesis and metastasis. Therefore, new multi-targeted approaches are urgently needed [7].

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) is a natural bioactive xanthonoid which can be purified from pulp, peel, seed, bark and leaf of many plant species, amongst which the mango tree (*Mangifera indica L.*), growing in most tropical areas of India, Africa, Asia and Central America. It has been cultivated in the

0960-8931 DOI: 10.1097/CMR.0000000000000647

Indian subcontinent for over 4000 years in Ayurvedic and indigenous traditional medical systems and is chemical constituents are receiving a growing interest in the field of cancer research worldwide [8–12]. Today, mangiferin is known for its immunomodulatory [13,14], anti-tumoral [15,16], anti-inflammatory actions [17–20] and anti-oxidant actions [21,22]. Although a wide range of pharmacological actions has already been documented, its anti-tumor mechanism of action related to neovascularization and migration of tumor cells has not yet been fully elucidated [9–12,23–25].

In the current study, we further characterize the potential anti-angiogenic action of mangiferin in melanoma and endothelial cell assays *in vitro*, the human placental blood vessel explant assay, the chicken egg chorioallantoic membrane (CAM) assay and in melanoma syngeneic studies *in vivo*. Furthermore, mangiferin specific gene expression changes in metastatic melanoma cells are analyzed by pathway analysis, related to cell migration, proliferation, survival and vasculogenesis. Results are finally validated in an experimental *in vivo* model of angiogenesis using tumor necrosis factor (TNF) or a TNF-secreting melanoma syngeneic model as proangiogenic inducers, to evaluate inhibition of capillary tube formation and neovascularization by mangiferin. Altogether, we provide novel insights in the anti-angiogenic properties of the naturally occurring glucosylxanthone mangiferin for future therapeutic applications in metastatic melanoma.

Material and methods

Chemical and biological reagents

Mangiferin was purified and characterized from the aqueous extract of the bark of *Mangifera indica L.* to 95.5% purity at the Departments of Pharmacognosy, Institute of Food and Pharmacy, Havana University and Department of Analytical Chemistry of Center of Drugs Research and Development, CIDEM [15,26,27]. Recombinant FGF, TNF, VEGF were purchased from Peprotech (Neuilly/Seine, France).

Cells for in-vitro assays

The EA.hy 926 hybridoma cell line of the human umbilical vein endothelial cell with the human epithelial cell line A549, shown to retain several native endothelial cell characteristics, was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and HAT selection supplement (Invitrogen, Carlsbad, California, USA) in mycoplasma- and endotoxin-free conditions at 37°C with humidified 95% air/5% CO_2 as previously described [28,29]. Microvascular endothelial cells were isolated by trypsinization from the dermal vessels of the vascular-rich region of the newborn human foreskin dermis obtained following routine circumcision as described previously [30,31]. The cells were cultured in MEM with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin in mycoplasma- and endotoxin-free conditions at 37°C with humidified 95% air/5% CO_2.

Metastatic B16 melanoma cells (B16F10 cell line) were routinely cultured in DMEM, supplemented with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin mixture (Pen/Strep, 10 000 IU/ml) and 2% HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] as previously described [29,32–34]. The cells were cultured by incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere. Endothelial cells from human dermal microvasculature were routinely cultured and characterized as described [30]. The cells were grown in MCDB-131 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Culture medium and serum were obtained from BRL (Life Technologies, Carlsbad, California, USA). Cells were grown to confluence on plastic tissue culture flasks (COSTAR, Cambridge, Massachusetts, USA). All studies on endothelial cells were performed on early (up to the fifth passage) cultures that were 48–72 hours post confluent at the time of the study (long-confluent cells).

Cell viability assay

Cell viability was assessed by colorimetric assay using 3-(4,5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, Missouri, USA) as previously described [35]. Briefly, B16F10 melanoma and EA.hy926 endothelial cells were plated into 96-well plates and exposed to medium with/without mangiferin (60, 120 and 240 µM) for 24 hours. Alternatively, primary endothelial cells from human dermal microvasculature were grown for 1 week in medium supplemented with proangiogenic 10 ng/ml of basic fibroblast growth factor (bFGF) or 10 ng/ml of VEGF (Peprotech) in presence or absence of mangiferin, as indicated in figure legends. Following treatment, medium was refreshed and 10 µl of MTT reagent was added into each well and the plates were incubated at 37°C for 4 hours. Upon weak vortexing, the spectrophotometric absorbance was read on a microplate reader (BioRad Model 3550-UV, Hercules, California, USA) at 595 nm. Relative absorbances in the linear range were converted into relative percent viable proliferating cells. Each individual experiment was performed at least three times.

Western blot analysis

Following treatment, cells were lysed for 15 minutes in 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM ß-glycerolphosphate, 1% Triton X-100 (w/v), 20 mM Tris HCl, pH = 7.5 and proteinase inhibitor (Complete, EDTA-free Protease Inhibitor Cocktail; Sigma-Aldrich) plus PhosphataseArrest Phosphatase Inhibitor Cocktail (phosphataseArrest; G-Biosciences, St. Louis, Missouri, USA). Then, cells were centrifuged for 15 minutes at 13 200*g* at 4°C, and supernatant containing the soluble proteins were stored at −20°C until Western analysis. Subsequently, protein lysates (20 µg) were mixed with

5× sample buffer (5% SDS, 20% glycerol, 0.2% bromophenol-blue, 250 mM DTT, 65 mM Tris HCl) all purchased from Sigma Aldrich, heated for 5 minutes at 95°C and loaded in a 12% SDS-PAGE gel. Proteins contained in the homogenates were separated during 30 minutes at 60–70 V and 1 hour at a constant voltage of 130 V. Further 10 µl of BenchMark Pre-Stained Protein Standard (Life Technologies, California, USA) were also loaded next to the samples. After separation proteins transferred onto a Nitrocellulose Membrane (BioRad, California, USA) during 2 hours at 45 V. Non-specific binding sites were blocked by incubating the membranes with blocking buffer (0.05 % Tween 20, 1 × TBS, 5% BSA) for 1 hour at room temperature. The membrane was then incubated with the primary antibodies: rabbit mAb anti-Phospho-NF-κB p65 (Ser536) (#3031; Cell Signaling Technology, Danvers, Massachusetts, USA) or rabbit polyclonal Anti-GAPDH antibody (ab9485; Abcam, Cambridge, UK) overnight at 4°C. After membranes were washed they were incubated with (1:10 000) Donkey anti-Rabbit IgG (H + L) Secondary Antibody-HRP (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 hour at room temperature. Chemiluminiscence detection was performed using the ECL detection kit (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific) in a ChemiDoc MP system (BioRad, Hercules, California, USA).

Cell scratch migration assay

Wound healing assay was carried out to determine the cell migration ability of human endothelial cells. The wound healing migration assay was performed as previously described [36,37]. Briefly, B16F10 and EAhy926 cells were plated on 6-well plates (Corning, New York) and grown to 80%–90% confluence. A sterilized 200 µl pipette tip was used to generate a scratch 'wound'across the cell monolayer, and the plates were washed with PBS. The cells were cultured in DMEM medium containing concentrations of Mangiferin (60, 120 and 240 µM) for 24 hours at 37°C. Control cells were cultured in medium alone. The untreated and treated cells were stimulated with bFGF (10 ng/ml) to evaluate the effects on migration induction. Next, the cells were washed briefly, fixed with absolute methanol, and then stained with 2% crystal violet solution [38]. Images from the region of the monolayer adjacent to the wound into which cells had migrated were taken with a camera attached to a bright-field microscope (Olympus, Hamburg, Germany). Three randomly selected views along the scraped line were photographed on each well at a magnification of 100×. A reduction in the wounded area is indicative for increase in cell motility. The experiments were performed in triplicate at different mangiferin doses (n = 3).

Human placental blood vessel explant assay

Superficial vessels, approximately 1–2 mm in diameter and 2–5 cm in length, were excised from the apical surface of human placentas within 24 hours of an elective cesarean birth [39]. The vessels were placed in Hank's balanced salt solution containing 2.5 µg/ml of fungizone and cut into 1–2 mm fragments using fine dissecting forceps and iridectomy scissors. Vessel fragments were free of residual clots and soaked in Hank's balanced salt solution before use. Dissecting and sectioning of vessels was performed with the aid of a magnifying lens. Assays were performed in 48-well culture plates (COSTAR). Fifteen microliters of thrombin (50 NIH U/ml in 0.15 M NaCl) was added to each well, followed by 0.5 ml/well of 3 mg/ml fibrinogen (bovine plasma; Calbiochem-Novabiochem Corporation, La Jolla, California, USA) in Medium 199 (Gibco, Paisley, Scotland). The thrombin and fibrinogen were mixed rapidly, and one vessel fragment was quickly placed in the center of the well before clot formation. Fibrin gel formation usually occurred within 30 seconds, and ideally, the vessel fragment remained suspended in the gel [40]. After gel formation, 0.5 ml/well of medium 199 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin were added. Vessels were cultured at 37°C in a humidified environment for 14–21 days, and the medium was changed twice weekly. Angiogenesis was quantified by computer-based image analysis using the Image 1.41 software (National Institutes of Health, Bethesda, Maryland, USA); digital images of the cultures were obtained with a Dycam 3.04 digital image camera (Dycam Inc., Chatsworth, California, USA). To test the angiogenesis modulating activity, mangiferin (20–200 µM) was diluted in Medium 199. Immediately after the embedding of vessel fragments in the fibrin gels, 0.5 ml of the medium containing the test substance was added to each well, and each treatment was performed in quadruplicate. Fresh medium containing test substance was added twice weekly. Control cultures received medium without mangiferin. Basic FGF (0.5 ng/ml) was used as promoter. In addition, each well-received 5 µg/ml of aprotinin which was added to prevent fibrinolysis by the vessel fragments.

Capillary tube formation by endothelial cells sandwiched between fibrin gels

Capillary tube formation was assessed essentially as previously described [41]. Fibrinogen (bovine plasma; Calbiochem-Novabiochem Corporation) was used at a final concentration of 1–3 mg/ml. To make the underlying fibrin gel, 225 µl of fibrinogen solution was placed into each well of a 24-well culture plate and human thrombin (10–30 U/ml in 10 × minimum essential medium) was added to a final concentration of one unit of thrombin/mg of fibrinogen. After fibrin gel polymerization (at least 5 minutes at 37°C), 1 ml aliquots of microvascular cells (250 000 cells/ml) suspended in serum-free MCDB-131, supplemented as indicated above, were seeded onto each gel. After formation of a confluent monolayer (24 hours after seeding), the culture medium was aspirated and the same procedure was used to generate a second fibrin gel overlying the apical surface

of the cells. After 5 minutes of polymerization at 37°C, 1 ml aliquots of supplemented, serum-free MCDB-131 were added to each well and tube formation was assessed. The dimensions (length in mM) of each capillary tube as a signal of the angiogenic process were quantified by computer-based image analysis using the Image 1.41 software (National Institutes of Health, Bethesda, Maryland, USA). Digital images of the cultures were obtained with a Dycam 3.04 digital image camera (Dycam Inc.).

Chorioallantoic membrane angiogenesis assay

This assay was performed as described previously [42]. Briefly, fertilized eggs were incubated for 3 days at 37 °C with a humidity of 48%. On day 4, albumen was removed to detach the shell from the developing CAM, and a window was made in the eggshell, exposing the CAM, and covered with a breathing film (Suprasorb F). The eggs were returned to the incubator until day 10, before application of the test compounds. Test compound (Mangiferin 50 µg/disc; bFGF 2 µg/ml) and control (DMEM without 10% FBS) were poured onto separate sterile discs (12 mm diameter), which were allowed to dry under sterile conditions. A solution of cortisone acetate (125 µg/disc) was poured onto all discs to prevent an inflammatory response. Test discs probed with recombinant human bFGF (Peprotech) served as a control for angiogenesis stimulation. On each CAM, the disc containing control compound and the disc containing test compound were placed at a distance of 1 cm. The windows were covered and the eggs were incubated until day 14, before assessment of angiogenesis. Therefore, the eggs were flooded with 10% buffered formalin and the eggs were kept at room temperature for at least 20 minutes. The CAM, the area of the discs included, was placed in a petri dish with 10% buffered formalin. The plastic discs were removed and phase-contrast pictures of the area of the plastic discs were taken. The vascular index was measured as described [42]. Vascular intersections on a grid containing three concentric circles (6, 8 and 10 mm diameter with as center the center of the disc) were counted. The angiogenic index = t/c, with t the number of intersections in the area covered by the test disc and c the number of intersections in the area covered by the control disc in the same egg. Photographs of each disc were taken in each experimental condition in order to determine the level of vascularization that has occurred in each case.

RNA extraction and Angiogenesis RT2 Profiler PCR Array (PAMM-024Z)

Total RNA from non-treated controls and mangiferin-treated murine melanoma B16F10 cells from three independent experiments was isolated using 1 ml of TRI Reagent (Sigma-Aldrich) per 5×10^6 cells and further proceeded according to the manufacturer's protocol till the step of phase separation. After transferring the aqueous phase to a new 1.5 ml microtube, an equal amount of 70% ethanol was added and samples were further purified on RNeasy spin columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Following extraction concentration was measured by nanodrop (NanoDrop 1000; Thermo Scientific, Waltham, Massachusetts, USA). The Mouse Angiogenesis RT2 Profiler PCR Array (PAMM-024Z) (SABiosciences; Qiagen) was used to determine the expression levels of 84 key genes involved in angiogenesis, essentially as previously described [43]. One microgram of total RNA, treated with RNase-Free DNase Set (Qiagen), was used for further processing according to the manufacturer's protocol. cDNA synthesis was performed by RT2 First Strand Kit (C-03). The RT2 SYBR Green ROX qPCR Mastermix was used for preparing the experimental cocktail according to the protocol and 25 µl was dispensed to each well of 96-well PCR Array plate. Relative mRNA levels of genes of interest were quantified by real-time quantitative PCR reaction on an ABI Prism 7300 (Applied Biosystems, Carlsbad, California, USA) and normalized against selected housekeeping genes (*HPRT*, *ACTB*). An Excel-based RT2 Profiler PCR Array Template (V3.3) (http://www.sabiosciences.com/pcrarraydataanalysis.php) was used for statistical analysis (paired *T*-test) and fold change quantification of the samples treated with mangiferin as compared to DMSO controls. A gene was considered not detectable when Ct > 35. Moreover, Ct was defined as 35 for the ΔCt calculation when the signal was under detectable limits. Fold-change and fold-regulation values >2 were indicative of upregulated genes; fold-change values <0.5 and fold-regulation values <–2 were indicative of downregulated genes.

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) (Ingenuity Systems) was used for functional enrichment and detection of significant pathways. The principal algorithm/method embedded in IPA for biological functions/pathway enrichment. Briefly, the program searches through the list of selected genes and determines genes which are involved in the respective Knowledge Base (including GO term, canonical signal transduction or metabolic pathways). Then, Fisher's Exact algorithm is used to calculate the probability of which each functional gene set is enriched. Only the biological functions/pathways (Bonferroni's corrected *P*-value <0.05) were considered significantly enriched. The activation or inhibition status of the functions/pathways was predicted using IPA Upstream Regulator Analysis Tool by calculating a regulation Z-score and an overlap *P*-value, which is based on the number of known target genes of interest pathway/function, expression changes of these target genes and their agreement with literature findings. Pathways were considered significantly inhibited (–3 <Z-score <–0,5) or activated (0.5> Z-score >3) with an overlap *P*-value ≤0.05. The detailed descriptions of IPA analysis are available under 'Upstream Regulator Analysis', 'Biological Functions Analysis' and 'Ingenuity Canonical Pathways Analysis' on the IPA website (http://www.ingenuity.com) [44].

In-vivo studies

Male 6–8-week-old C57BL/6 mice were purchased from the National Center for Animal Breeding (CENPALAB), Havana, Cuba. Animal was acclimated (25 ± 1°C) and caged in groups of five. They received a chow diet and water *ad libitum*. Animals were anesthetized in a methoxyflurane chamber before the experimental procedure and were observed until fully recovered. They were sacrificed by a lethal dose of methoxyflurane. All the experiments were carried out in compliance with the Ethical Standards for Animal Experimentation of the Institute of Pharmacy and Food of the University of Havana. The inhibitory activity of mangiferin against tumor necrosis factor-α (TNF-α) -induced angiogenesis was determined using matrigel (Becton Dickinson, Franklin Lakes, New Jersey, USA), an extract of murine basement proteins from the Engelbreth-Holm-Swarm mouse sarcoma consisting predominantly of laminin, collagen IV, heparan sulfate, proteoglycan and nidogen/entactin. The sterile solution of Matrigel was prepared as previously described [45]. Briefly, 500 µl of Matrigel with 64 U/ml of heparin (Sigma-Aldrich) and 10 ng/ml of TNF-α (from Sigma-Aldrich) were injected subcutaneously (s.c.) into the abdomens of mice. A week later, angiogenesis was examined macroscopically and the content of hemoglobin in the gels was determined using the Drabkin method and Drabkin reagent kit 525 (Sigma-Aldrich) [46]. In the same conditions, other in vivo assay was performed using as inductor of neovascularization B16F10 (10^5/ml) melanoma cells line as described [47]

Statistical analysis

The results are presented as mean ± SD or SEM as indicated and statistical significance between the groups was determined by one-way analysis of variance and Dunnett's multiple comparison post-test. All statistical analyses and figures were carried out using GraphPad Prism Version 7.00. P values less than 0.05 ($P < 0.05$) was considered significant.

Results

Mangiferin inhibits motility of metastatic B16F10 melanoma and human EA.hy926 endothelial cells

We examined the ability of mangiferin to interfere with cellular migration of human metastatic melanoma B16F10 and EA.hy926 endothelial cells using a classic *in vitro* wound healing in-vitro model. Confluent monolayers of B16F10 and EA.hy926 cells were scratched with a 200 µl yellow tip and the cell migration into the wound from adjacent areas of the monolayer was evaluated following different treatments. The cells were left untreated or treated for 24 hours with mangiferin in the presence or absence of bFGF to promote motility. As shown in Fig. 1, mangiferin treatment reveals significant inhibitory effects on bFGF-induced cellular migration of B16F10 (Fig. 1a) and EA.hy926 (Fig. 1b) cells.

Mangiferin does not affect cell viability of B16F10 melanoma and human EA.hy926 endothelial cells

Next, we evaluated potential cytotoxic effects of increasing concentrations of mangiferin treatment (24 hours) in B16F10 and EA.hy926 endothelial cells in an MTT cell viability assay. No significant cell cytotoxic effects could be observed in a concentration range of 30–240 µM mangiferin, whereas cell viability was significantly reduced to 5%–10% in presence of the reference cytotoxic anti-cancer compound withaferin A (10 µM) (Fig. 2a and b) [43],

Mangiferin inhibits proangiogenic cell proliferation of primary endothelial cells

Next, we evaluated effects of increasing concentrations of mangiferin treatment on primary human dermal microvasculature endothelial cells in presence of a proangiogenic stimulus, that is, bFGF (10 ng/ml) or VEGF(10 ng/ml) in a cell viability MTT assay. Our results in Fig. 2 show that mangiferin dose dependently inhibits bFGF as well as VEGF stimulated endothelial cell growth in a concentration range of 30–120 µM (Fig. 2c and d).

Mangiferin inhibits capillary growth and tube formation in the human placental blood vessel explant assay and endothelial fibrin gel sandwich assay

Fragments of human placental blood vessel were embedded in fibrin gel with mangiferin to evaluate mangiferin effects on capillary growth. As can be seen in Fig. 2e, mangiferin dose dependently (30–240 µM) inhibits bFGF (0.5 ng/ml) induced capillary growth with a maximal inhibition of 50% at 240 µM mangiferin. Similarly, capillary tube formation by primary endothelial cells sandwiched between fibrin gels was dose dependently inhibited by mangiferin in a similar human anti-angiogenic in-vitro assay (Fig. 2f) with a maximal inhibition of 70 % at 240 µM.

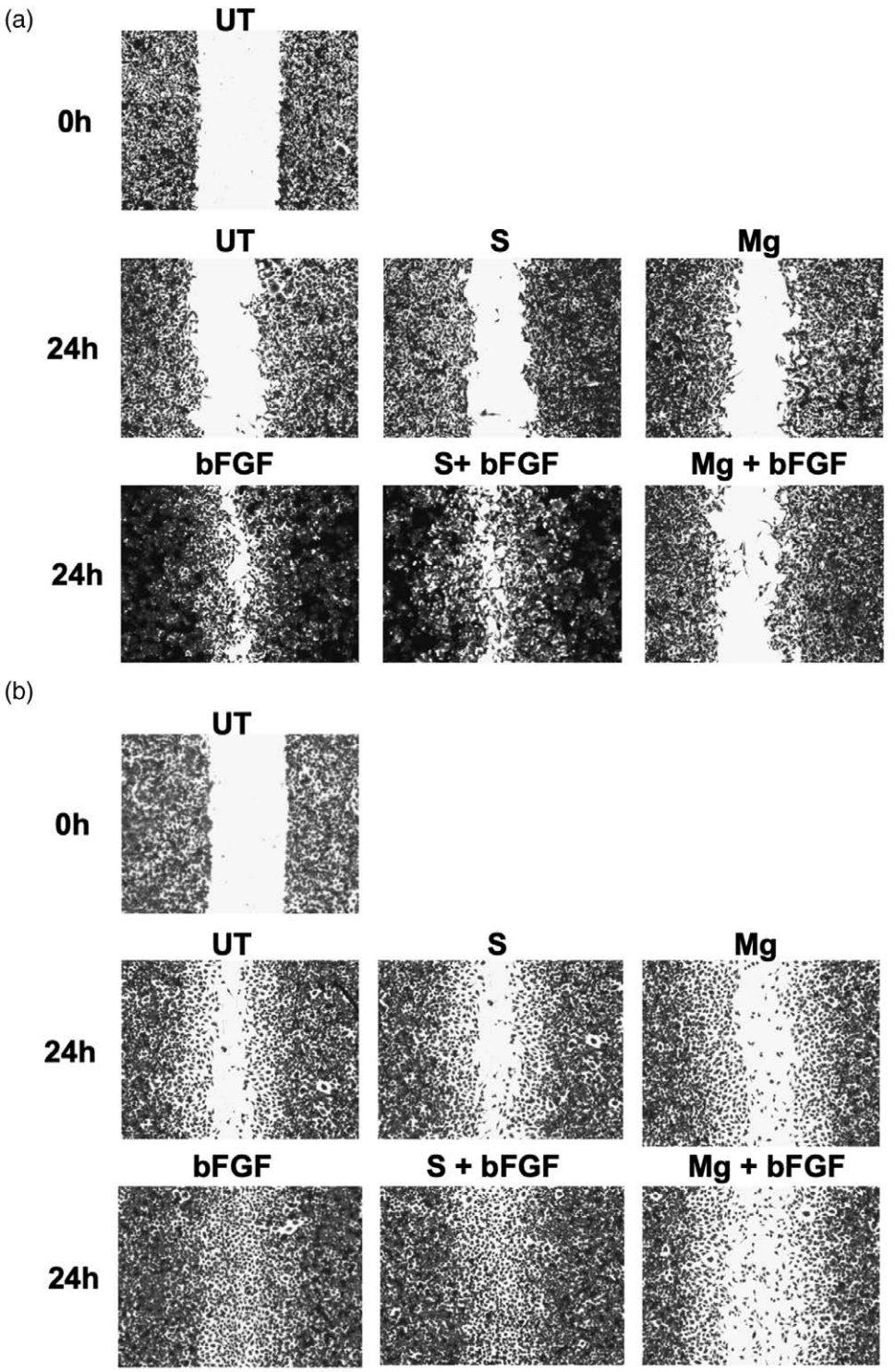
Mangiferin selectively inhibits IL6, TNF, IFNG, KDR(VEGFR2), PLAU, MMP19, CCL2 and PGF gene expression

We performed QPCR to evaluate the ability of 6 hours mangiferin treatment to modulate gene expression of 84 selected genes on the angiogenesis PCR array (Qiagen) in B16F10 cells. Of special note, only nine genes revealed significant inhibition (>2-fold change, $P < 0.05$), whereas most other genes remained unaffected (Fig. 3a and data not shown), irrespective of the observed variation in basal expression of the different genes (Fig. 3b).

Mangiferin decreased gene expression strongly inhibits angiogenesis, cell proliferation, cell motility (invasion, migration) and cell viability through upstream inhibition of calcium signaling, lipid metabolism and inflammatory pathways (NFkB, HMGB1, NO)

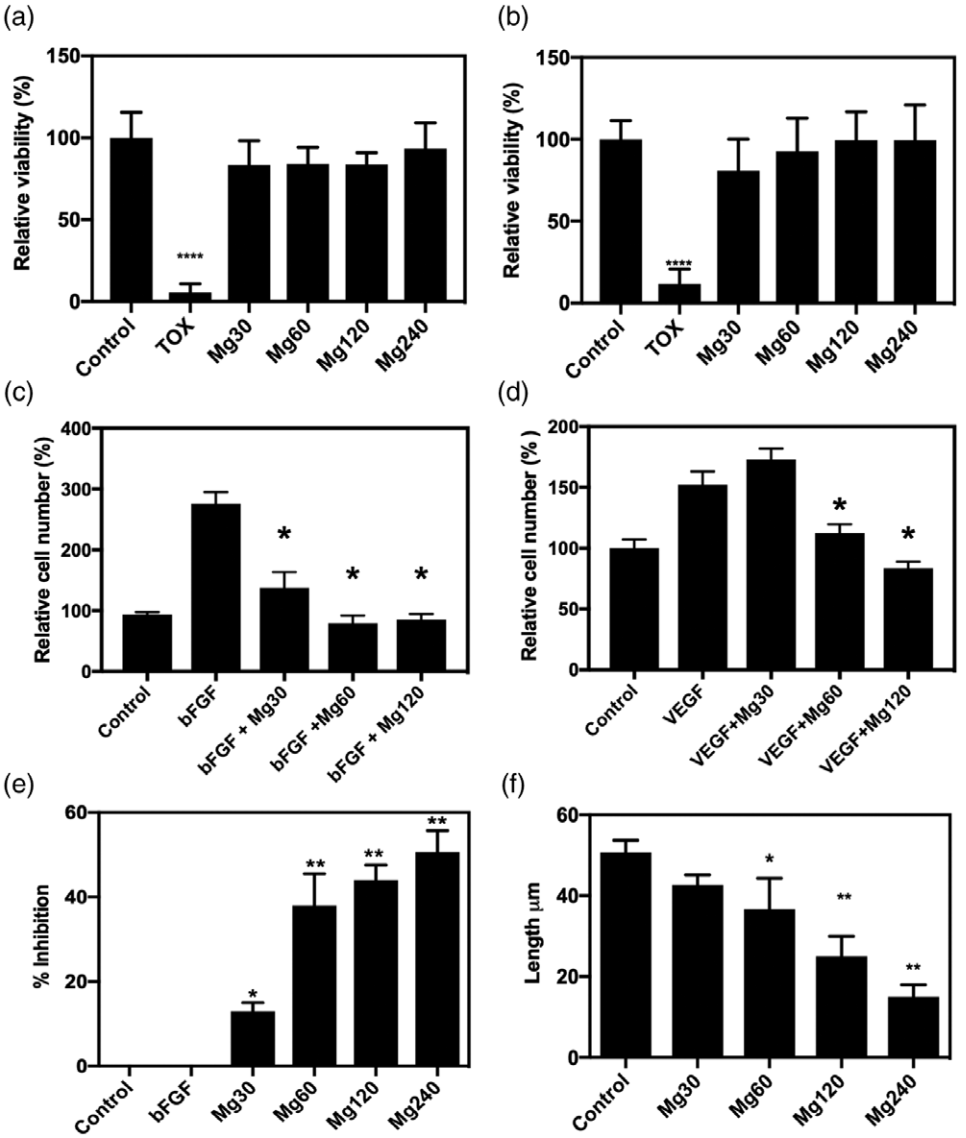
IPA core analysis of the list of genes which are significantly inhibited upon mangiferin treatment in B16F10

Fig. 1



Mangiferin inhibits bFGF-dependent migration of metastatic melanoma and endothelial cells. B16F10 melanoma (a) and EA.hy926 endothelial (b) cells were left untreated (UT) or treated with solvent (S, 0.1% DMSO control) or Mangiferin (Mg, 240 μM) in presence or absence of bFGF (10 ng/ml). The migratory cells were stained with crystal violet and photographed at 0 and 24 hours, as indicated.

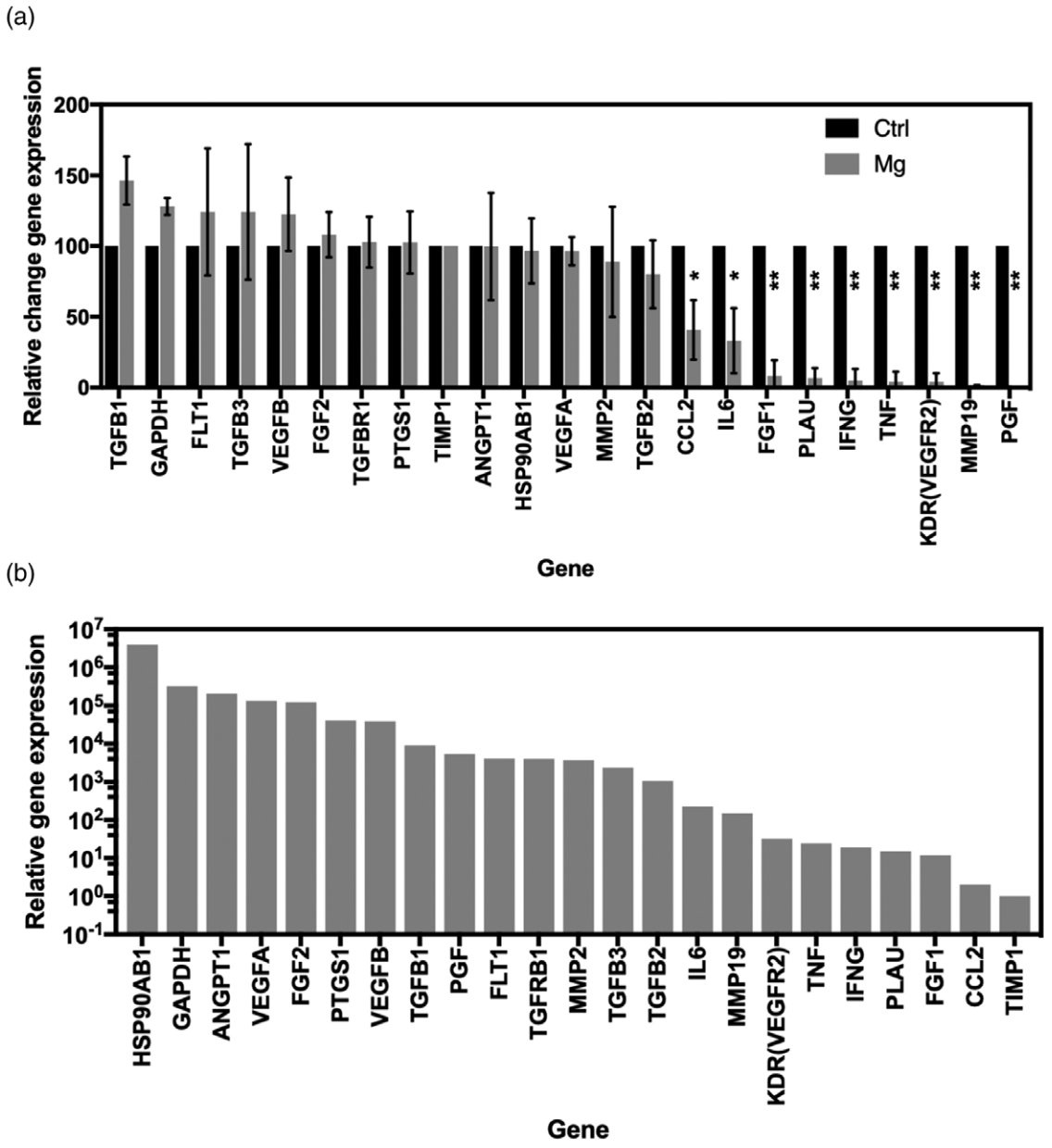
Fig. 2



Mangiferin lacks cellular cytotoxicity and inhibits proangiogenic endothelial cell proliferation and capillary tube growth. (a) Viability of B16F10 melanoma cells in presence of increasing concentrations [of mangiferin (24 hours treatment, Mg: 30, 60, 120 and 240 µM as indicated)] was evaluated in a MTT cell viability assay. As a positive control for cytotoxicity, the reference anti-cancer compound withaferin A was included. As a positive control for cytotoxicity, the reference anti-cancer compound withaferin A was included. Each group represents the mean ± SEM of three assays ****$P < 0.0001$, **$P < 0.01$ and *$P < 0.05$ compared with control value. (b) Viability of EA.hy926 endothelial cells in presence of increasing concentrations of mangiferin (24 hours treatment, Mg: 30, 60, 120 and 240 µM as indicated) was evaluated in a MTT cell viability assay. As a positive control for cytotoxicity, the reference ant-cancer compound withaferin A was included. Each group represents the mean ± SEM of three assays ****$P < 0.0001$, **$P < 0.01$ and *$P < 0.05$ compared with control value. (c and d) Human dermal microvascular endothelial cells were left untreated (Control) or treated with proangiogenic stimuli bFGF (10 ng/ml) (c) and VEGF (10 ng/ml) (d), either or not upon cotreatment with different concentrations of mangiferin (Mg: 30, 60 and 120 µM as indicated). Results are presented as relative cell number (% living cells). Each group represents the mean ± SEM of three assays. *$P < 0.05$ compared with bFGF or VEGF cell stimulation. (e) Inhibitory effects of mangiferin (Mg, 30–240 µM) on capillary growth and tube formation on ex-vivo explants of human placental blood vessel stimulated with bFGF (0.5 ng/ml) in an endothelial fibrin gel sandwich assay. Values are expressed as percentage of inhibition of new blood vessel formation respect to control group. Each group represents the mean ± SEM of three assays. *$P < 0.05$ and **$P < 0.01$ compared with bFGF control setup. (f) Effect of mangiferin (Mg, 30–240 µM) against capillary growth and tube formation by endothelial cells sandwiched between fibrin gels. Each group represents the mean ± SEM. of 10 cultures. **$P < 0.01$ and *$P < 0.05$ compared with control value. bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor.

cells (>2-fold change, $P < 0.05$) confirms highly significant enrichment (10^{-4} < P-value <10^{-12}) of pathways related to angiogenesis, cell proliferation, cell motility (invasion, migration) and cell viability (Fig. 4a). Moreover, Z-score analysis shows moderate to strong inhibition of these biological processes (Fig. 4b), in line with our in-vitro cell

Fig. 3



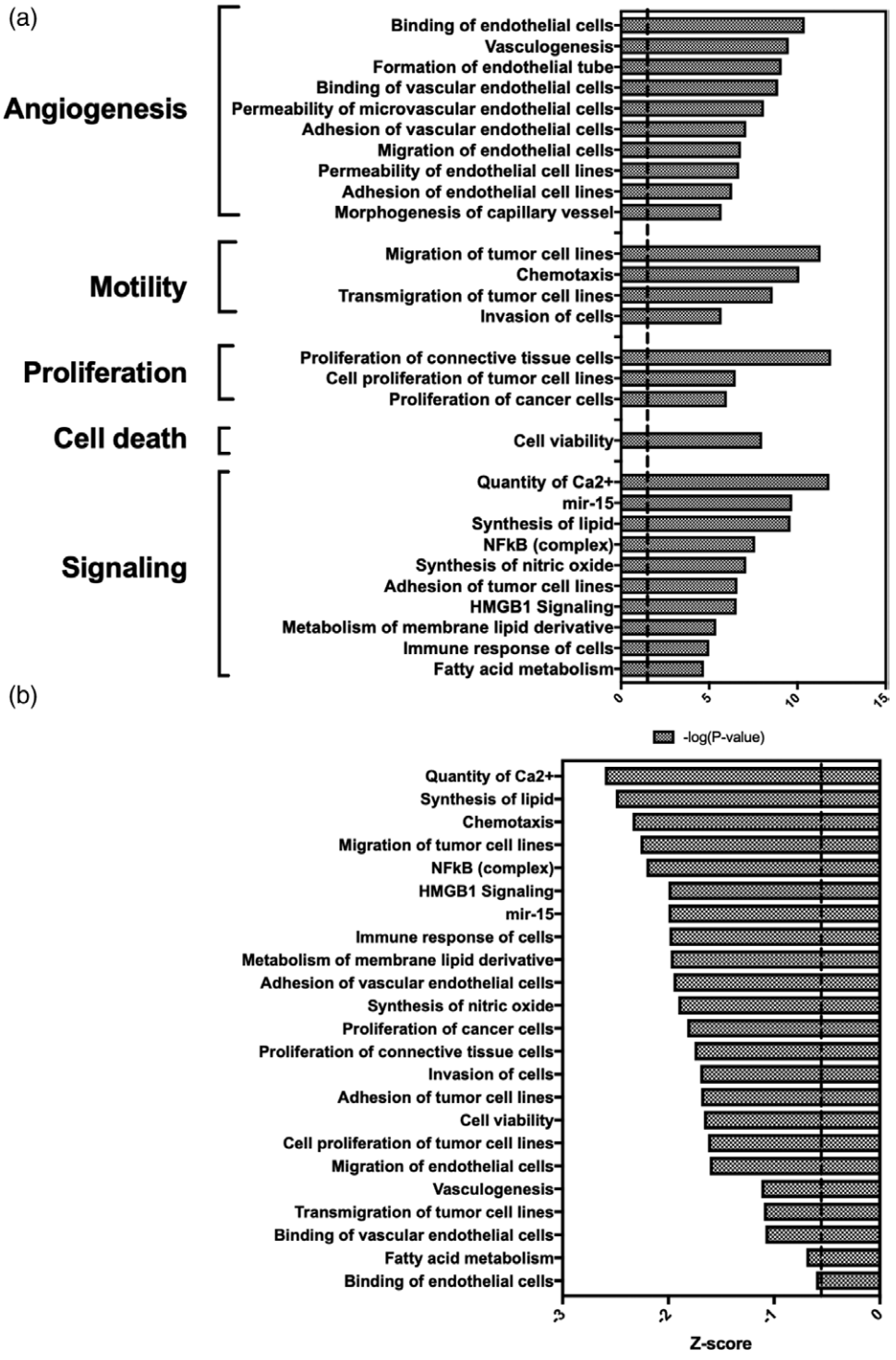
Modulation of angiogenesis pathway-related gene expression by Mangiferin in B16F10 melanoma cells. B16F10 melanoma cells were left untreated (0.1% DMSO control) or treated for 6 hours with 240 μM Mangiferin (Mg). Upon RNA isolation, gene expression of 84 known or predicted genes involved in angiogenesis was determined by QPCR array. Pairwise mRNA changes of the 84 genes were calculated according to the ΔCt method and normalized against selected housekeeping genes (*HPRT*, *ACTB*) with the Excel-based RT2 Profiler PCR Array Template (see Methods section). (a) Bargraph summarizes most significant gene expression changes upon mangiferin treatment (relative fold change to control setup), in addition to various genes which remain unaffected. Significantly inhibited genes are marked with asterisks, $^{**}P < 0.01$ and $^{*}P < 0.05$. (b) Bar graph represents relative basal gene expression levels of the different genes presented in panel A.

assay results. Of special note, upstream analysis predicts significant inhibition of NFkB signaling (Fig. 4b), which could be experimentally validated by Western analysis, revealing dose-dependent inhibition of NFkB p65 Ser536 phosphorylation in response to Mangiferin, to a similar extent as the reference NFkB inhibitor Withaferin A (Fig. 5) [48,49].

Mangiferin inhibits basal and fibroblast growth factor induced angiogenesis in the chorioallantoic membrane assay

Angiogenesis is an attractive target in cancer therapy not only because it supplies oxygen and nutrients for the survival of tumor cells but also provides the route for metastatic spread of these cancer cells. To investigate possible

Fig. 4



Ingenuity pathway analysis of mangiferin specific transcriptome changes in B16F10 cells. (a) Disease function related pathway enrichment analysis of mangiferin responsive genes (-log *P*-value). (b) Z-scores of IPA enriched pathways, disease functions and/or upstream regulators. IPA, ingenuity pathway analysis.

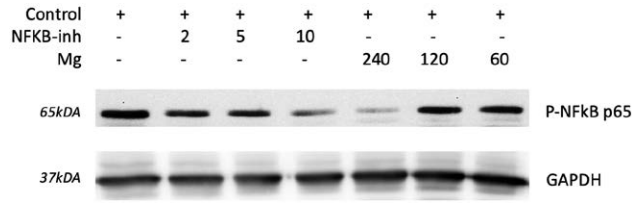
mechanisms of mangiferin action against neovascularization *in vivo*, we evaluated potential anti-angiogenic effect of mangiferin in the CAM assay involving the coordination and integration of multicellular responses during development of the chick embryo. As shown in Fig. 6a, no vascular sprouting was observed when CAM was treated with

serum-free medium (control). However, vascular sprouting was observed in the CAM assay upon treatment with bFGF (2 μg/ml), which could be strongly reversed upon cotreatment with mangiferin (50 μg/disc), in line with the in-vitro cell assays. Corresponding quantitative data from the CAM assay images are summarized in Fig. 6b, which show that bFGF (2 μg/ml) increases the angiogenic index with 40% as compared to the control setup. Moreover, Mangiferin (50 μg/disc) completely inhibits the formation of new blood vessels induced by bFGF (Fig. 7b).

Mangiferin inhibits tumor necrosis factor induced neovascularization *in vivo*

To further validate anti-angiogenic effects of mangiferin in an in-vivo mouse model, the proangiogenic cytokine TNF [50] was mixed with matrigel and heparin, and injected into the dorsal region of C57BL/6 mice to promote angiogenesis. The hemoglobin content of the gels, calculated by Drabkin's method, was well correlated with the number of new vessels formed. In line with our in-vitro results, we found that mangiferin (120 and 240 μM), dose dependently inhibited TNF-induced angiogenesis (Fig. 7a). Similarly, when TNF treatment was replaced by xenografts of TNF producing metastatic B16F10 melanoma cells (10^5 cells/ml), we demonstrate that 120 and 240 μM treatment similarly inhibit B16F10 tumor cell-induced angiogenesis (Fig. 7b).

Fig. 5



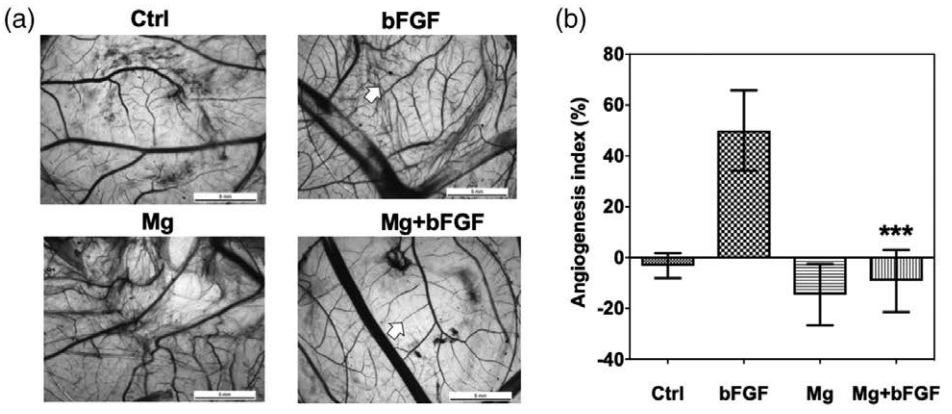
Mangiferin inhibits basal NFkB p65 Ser536 phosphorylation in B16F10 cells. B16F10 cells treated for 6 hours with Mg (60, 120, 240 μM as indicated) or the reference NFkB inhibitor Withaferin A (2, 5, 10 μM as indicated) Total cell lysates were prepared in SDS/Laemmli sample buffer and extracts were analyzed for changes in NFkB activation via expression levels of phospho-NFkB p65 Ser536. Equal protein loading is verified by Western analysis of corresponding GAPDH expression levels.

Discussion

The process of angiogenesis is crucial for progression and metastasis in melanoma. Angiogenesis is characterized by increased microvessel endothelial cell proliferation, production and/or activation of matrix degradative enzymes, migration in the subendothelial matrix and differentiation into functional new capillaries [51]. As such, angiogenesis relies on interplay of multiple genes and anti-angiogenic therapies may require a multi-targeted approach [7]. Various plant-derived polyphenols, that is, constituents of green tea, catechins and gallic acid derivatives already showed 'promiscuous'multitargeted inhibition of several key events of the angiogenic process such as proliferation and migration of endothelial cells [7,52].

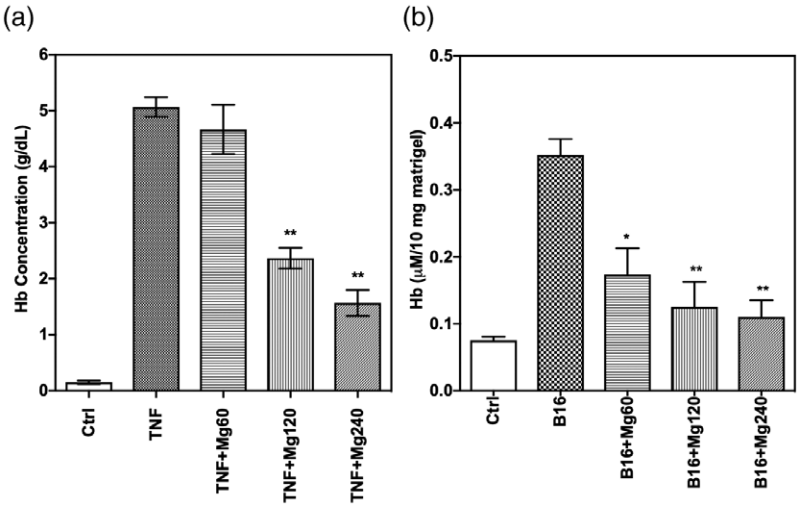
Here we further demonstrate by different in-vitro and in-vivo approaches that mangiferin, a natural glycosylated xanthone isolated from Mangifera Indica, potently inhibits bFGF- and TNF-dependent cell migration and angiogenesis as well as microvessel outgrowth and

Fig. 6



Mangiferin inhibits bFGF induced neovascularisation in the chorioallantoic membrane (CAM) assay. Mangiferin (Mg) was evaluated in a chicken CAM assay as an in-vivo model of angiogenesis. Representative photographs of each disk are shown in (a), where it is possible to appreciate the degree of vascularization that occurs with each experimental condition used. The arrow indicates areas of very high vascularization induced by FGF and areas of very low vascularization due to the anti-angiogenic effect of mangiferin. The Angiogenic index = (t − c)/c in CAM angiogenesis assay, was determined as described in materials and methods. Bars indicate angiogenic indices (%) of CAMs probed with serum-free medium. Conditions evaluated: bFGF (2 μg/ml), Mangiferin (Mg) 50 μg/disc/egg. Data represents mean ± SD. In each experiment, seven eggs were tested per each condition. The Mann–Whitney *U*-test was used for statistical analysis (***$P < 0.001$, statistically different from the mean angiogenic index of CAMs bFGF setup, which promotes vascularization). bFGF, basic fibroblast growth factor.

Fig. 7



Mangiferin inhibits neovascularisation *in vivo*. Effects of mangiferin (Mg, 60–240 μM) on (a) TNF- and (b) B16F10 (10^5 cells)-induced angiogenesis in mouse abdominal subcutaneous connective tissue. TNF-α (10 ng/ml) or B16F10 cells (10^5 cells/ml) were mixed with 64 U/ml of heparin, 0.5 ml of matrigel and mangiferin. These mixtures were injected subcutaneously into the abdominal midline of mice. Data represent the mean hemoglobin values (determined by Drabkin's method) from at least five mice per point with SEM as indicated. Statistical differences among groups were analyzed by one-way ANOVA and Dunnett's multiple comparison test (*$P < 0.05$ and **$P < 0.01$). ANOVA, analysis of variance; TNF, tumor necrosis factor.

neovascularization. Along the same line, PCR array gene expression profiling revealed that mangiferin selectively inhibits expression of IL6, TNF, IFNG, KDR(VEGFR2), FGF1, PLAU, MMP19, CCL2 and placental growth factor (PGF), to block angiogenesis, metastasis-invasion-motility, cell proliferation and viability in cancer signaling processes, according to IPA pathway enrichment analysis. Along the same line, other phytochemicals such as ferulic acid and kaempferol were also found to inhibit angiogenesis in part through inhibition of FGF1 [53] and PGF expression [54]. Furthermore, the multi-tyrosine kinase inhibitor anlotinib was found to suppress blood vessels sprouting, reduce microvessel density and endothelial migration and tube formation upon inhibition of KDR(VEGR2)-induced angiogenesis, in analogy to mangiferin [55,56]. Moreover, cell invasiveness in angiogenesis involves various extracellularly acting proteolytic enzymes, including essentially MMPs (e.g. interstitial collagenases, gelatinases and stromelysins) and serine proteases, in particular the PA/plasmin system [52]. In line with this model, our results demonstrate a clear inhibition of *MMP19* and *PLAU* protease gene expression by mangiferin, which may further decrease protein levels and protease functions [57–60]. With respect to the pro-metastatic role of IL6, various reports demonstrate that therapeutic inhibition of IL6/Stat3 signaling by natural compounds strongly reduces cell motility and invasive cancer properties of melanoma cells [43,61–64]. However, metastasis is frequently caused by a mix of tumor-derived cytokines and chemokines in the microenvironment, such as IL6, TNF or CCL2 [50,65,66]. Finally, in line with previous results obtained in our lab and of others [15,67], bioinformatic IPA analysis suggests that mangiferin inhibits NFkB signaling in metastatic melanoma cells, which could be experimentally confirmed by Western analysis showing similar inhibition of phosphoactivated NFkB p65 Ser536 as the reference NFkB inhibitor Withaferin A [48,49]. In addition to NFkB inhibition, epigenetic modulation of DNA methylation, histone marks or noncoding RNAs in response to mangiferin may further fine-tune gene selective transcription [68,69]. Of particular interest, prediction of upstream regulators also indicates mangiferin dependent regulation of calcium signaling, which is critically involved in angiogenic VEGF signaling [70,71]. Important roles for HMGB1 and mir15 have also been described in angiogenesis, which deserves further molecular investigation [72,73].

In conclusion, although modern targeted therapies have ameliorated the management of metastatic melanoma, safer, more affordable and more effective strategies for treatment of skin cancer patients are needed. We demonstrate that mangiferin hold promise as a novel drug pharmacophore candidate for cancer treatment in the battle against melanoma, as this glucosylxanthone exerts potent anti-angiogenic and metastatic effects and is widely available, highly tolerated and cost-effective. Further intervention studies and (pre)clinical trials are needed for further development into cancer therapeutic applications for melanoma patients.

Acknowledgements

We wanted to dedicate this work especially to the memories of Janet Rodríguez Morales (Cuba) and Prof. Dr. Sandra Apers (Belgium), two young researchers who died at too young age during the course of our research project. Janet was one of the initiators of research in the field of experimental angiogenesis in Cuba. Prof. Dr. Sandra Apers (Department of Pharmaceutical Sciences, University of Antwerp), exceptional promoter of the VLIR research project on mangiferin, supporting active collaborative with various research groups in Cuba. We want to dedicate these results to their memories. Their young talented love of science and life will continue to inspire us in future research. They will live forever in the memory of their friends, research colleagues and family.

This work was supported by the International Collaboration Project ZEIN2011PR383 and ZEIN2016PR418 from the Flemish VLIR collaboration program between Department of Pharmaceutical Science, University of Antwerp (Belgium) and Institute of Food and Pharmacy, Havana University and for the Project of Institute of Food and Pharmacy, University of Havana (Cuba).

Conflicts of interest

There are no conflicts of interest.

References

1 Felcht M, Thomas M. Angiogenesis in malignant melanoma. *J Dtsch Dermatol Ges* 2015; **13**:125–136.
2 Domingues B, Lopes JM, Soares P, Pópulo H. Melanoma treatment in review. *Immunotargets Ther* 2018; **7**:35–49.
3 De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer* 2017; **17**:457–474.
4 Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 2016; **388**:518–529.
5 Carbone C, Moccia T, Zhu C, Paradiso G, Budillon A, Chiao PJ, *et al*. Anti-VEGF treatment-resistant pancreatic cancers secrete proinflammatory factors that contribute to malignant progression by inducing an EMT cell phenotype. *Clin Cancer Res* 2011; **17**:5822–5832.
6 Carbone C, Tamburrino A, Piro G, Boschi F, Cataldo I, Zanotto M, *et al*. Combined inhibition of IL1, CXCR1/2, and tgfβ signaling pathways modulates in-vivo resistance to anti-VEGF treatment. *Anticancer Drugs* 2016; **27**:29–40.
7 Aggarwal BB, Prasad S, Reuter S, Kannappan R, Yadev VR, Park B, *et al*. Identification of novel anti-inflammatory agents from ayurvedic medicine for prevention of chronic diseases: "reverse pharmacology" and "bedside to bench" approach. *Curr Drug Targets* 2011; **12**:1595–1653.
8 Lauricella M, Emanuele S, Calvaruso G, Giuliano M, D'Anneo A. Multifaceted health benefits of *Mangifera indica L*. (Mango): the inestimable value of orchards recently planted in Sicilian rural areas. *Nutrients* 2017; **9**:E525.
9 Benard O, Chi Y. Medicinal properties of mangiferin, structural features, derivative synthesis, pharmacokinetics and biological activities. *Mini Rev Med Chem* 2015; **15**:582–594.
10 Rajendran P, Rengarajan T, Nandakumar N, Divya H, Nishigaki I. Mangiferin in cancer chemoprevention and treatment: pharmacokinetics and molecular targets. *J Recept Signal Transduct Res* 2015; **35**:76–84.
11 Gold-Smith F, Fernandez A, Bishop K. Mangiferin and cancer: mechanisms of action. *Nutrients* 2016; **8**:396.
12 Núñez Selles AJ, Daglia M, Rastrelli L. The potential role of mangiferin in cancer treatment through its immunomodulatory, anti-angiogenic, apoptopic, and gene regulatory effects. *Biofactors* 2016; **42**:475–491.
13 García D, Delgado R, Ubeira FM, Leiro J. Modulation of rat macrophage function by the *Mangifera indica L*. extracts vimang and mangiferin. *Int Immunopharmacol* 2002; **2**:797–806.
14 García D, Leiro J, Delgado R, Sanmartín ML, Ubeira FM. *Mangifera indica L*. extract (vimang) and mangiferin modulate mouse humoral immune responses. *Phytother Res* 2003; **17**:1182–1187.
15 García-Rivera D, Delgado R, Bougarne N, Haegeman G, Berghe WV. Gallic acid indanone and mangiferin xanthone are strong determinants of immunosuppressive anti-tumour effects of *Mangifera indica L*. bark in MDA-MB231 breast cancer cells. *Cancer Lett* 2011; **305**:21–31.
16 Guha S, Ghosal S, Chattopadhyay U. Antitumor, immunomodulatory and anti-HIV effect of mangiferin, a naturally occurring glucosylxanthone. *Chemotherapy* 1996; **42**:443–451.
17 Garrido G, González D, Lemus Y, García D, Lodeiro L, Quintero G, *et al*. *In vivo* and *in vitro* anti-inflammatory activity of *Mangifera indica L*. extract (VIMANG). *Pharmacol Res* 2004; **50**:143–149.
18 Garrido-Suárez BB, Garrido G, Castro-Labrada M, Merino N, Valdés O, Rodeiro I, *et al*. Anti-hypernociceptive effect of mangiferin in persistent and neuropathic pain models in rats. *Pharmacol Biochem Behav* 2014; **124**:311–319.
19 Garrido-Suárez BB, Garrido G, Delgado R, Bosch F, del C Rabí M. A *Mangifera indica L*. extract could be used to treat neuropathic pain and implication of mangiferin. *Molecules* 2010; **15**:9035–9045.
20 Garrido G, González D, Lemus Y, Delporte C, Delgado R. Protective effects of a standard extract of *Mangifera indica L*. (VIMANG) against mouse ear edemas and its inhibition of eicosanoid production in J774 murine macrophages. *Phytomedicine* 2006; **13**:412–418.
21 Pardo-Andreu GL, Cavalheiro RA, Dorta DJ, Naal Z, Delgado R, Vercesi AE, Curti C. Fe(III) shifts the mitochondria permeability transition-eliciting capacity of mangiferin to protection of organelle. *J Pharmacol Exp Ther* 2007; **320**:646–653.
22 Pardo-Andreu GL, Sánchez-Baldoquín C, Avila-González R, Delgado R, Naal Z, Curti C. Fe(III) improves antioxidant and cytoprotecting activities of mangiferin. *Eur J Pharmacol* 2006; **547**:31–36.
23 Khurana RK, Kaur R, Lohan S, Singh KK, Singh B. Mangiferin: a promising anticancer bioactive. *Pharm Pat Anal* 2016; **5**:169–181.
24 Imran M, Arshad MS, Butt MS, Kwon JH, Arshad MU, Sultan MT. Mangiferin: a natural miracle bioactive compound against lifestyle related disorders. *Lipids Health Dis* 2017; **16**:84.
25 Rauf A, Imranb M, Patel S. Mangiferin: a phytochemical with panacea potential. *Biomed Pharmacother* 2017; **96**:1562–1564.
26 Núñez Sellés AJ, Vélez Castro HT, Agüero-Agüero J, González-González J, Naddeo F, De Simone F, Rastrelli L. Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indica L*.) stem bark aqueous decoction used in cuba as a nutritional supplement. *J Agric Food Chem* 2002; **50**:762–766.
27 Gil E, Hernández R, Paz L, Hoogmartens J. Determination of mangiferin in *Mangifera indica L*. stem bark extract (Vimang®) and pharmaceuticals by liquid chromatography. *Emir J Food Agr* 2014;616–622.
28 Heyninck K, Sabbe L, Chirumamilla CS, Szarc Vel Szic K, Vander Veken P, Lemmens KJA, *et al*. Withaferin A induces heme oxygenase (HO-1) expression in endothelial cells via activation of the keap1/nrf2 pathway. *Biochem Pharmacol* 2016; **109**:48–61.
29 Van Hauwermeiren F, Armaka M, Karagianni N, Kranidioti K, Vandenbroucke RE, Loges S, *et al*. Safe TNF-based antitumor therapy following p55tnfr reduction in intestinal epithelium. *J Clin Invest* 2013; **123**:2590–2603.
30 Marks RM, Czerniecki M, Penny R. Human dermal microvascular endothelial cells: an improved method for tissue culture and a description of some singular properties in culture. *In Vitro Cell Dev Biol* 1985; **21**:627–635.
31 Davison PM, Karasek MA. Human dermal microvascular endothelial cells *in vitro*: effect of cyclic AMP on cellular morphology and proliferation rate. *J Cell Physiol* 1981; **106**:253–258.
32 Fang XY, Song R, Chen W, Yang YY, Gu YH, Shu YQ, *et al*. PRL-3 promotes the malignant progression of melanoma via triggering dephosphorylation and cytoplasmic localization of NHERF1. *J Invest Dermatol* 2015; **135**:2273–2282.
33 Poste G, Doll J, Hart IR, Fidler IJ. *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res* 1980; **40**:1636–1644.
34 Nakamura K, Yoshikawa N, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M. Characterization of mouse melanoma cell lines by their mortal malignancy using an experimental metastatic model. *Life Sci* 2002; **70**:791–798.
35 Suttana W, Mankhetkorn S, Poompimon W, Palagani A, Zhokhov S, Gerlo S, *et al*. Differential chemosensitization of P-glycoprotein overexpressing

K562/adr cells by withaferin A and siamois polyphenols. *Mol Cancer* 2010; **9**:99.
36 Liang CC, Park AY, Guan JL. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc* 2007; **2**:329–333.
37 Ding M, Feng R, Wang SY, Bowman L, Lu Y, Qian Y, *et al*. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J Biol Chem* 2006; **281**:17359–17368.
38 Dias PF, Siqueira JM Jr, Vendruscolo LF, de Jesus Neiva T, Gagliardi AR, Maraschin M, Ribeiro-do-Valle RM. Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed sargassum stenophyllum. *Cancer Chemother Pharmacol* 2005; **56**:436–446.
39 Palmieri D, Mura M, Mambrini S, Palombo D. Effects of pleiotrophin on endothelial and inflammatory cells: pro-angiogenic and anti-inflammatory properties and potential role for vascular bio-prosthesis endothelialization. *Adv Med Sci* 2015; **60**:287–293.
40 Brown JK, Maynes FS, Bezos A, Maguire JD, Ford DM, Parish RC. A novel "in vitro" assay for human angiogenesis. *Lab Inv* 1996; **75**:539–555.
41 Chalupowicz DG, Chowdhury ZA, Bach TL, Barsigian C, Martinez J. Fibrin II induces endothelial cell capillary tube formation. *J Cell Biol* 1995; **130**:207–215.
42 Attoub S, Arafat K, Gélaude A, Al Sultan MA, Bracke M, Collin P, *et al*. Frondoside a suppressive effects on lung cancer survival, tumor growth, angiogenesis, invasion, and metastasis. *Plos One* 2013; **8**:e53087.
43 Szarc vel Szic K, Op de Beeck K, Ratman D, Wouters A, Beck IM, Declerck K, *et al*. Pharmacological levels of withaferin A (withania somnifera) trigger clinically relevant anticancer effects specific to triple negative breast cancer cells. *Plos One* 2014; **9**:e87850.
44 Krämer A, Green J, Pollard J Jr, Tugendreich S. Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics* 2014; **30**:523–530.
45 Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, *et al*. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992; **67**:519–528.
46 Drabkin DL, Austin JH. Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J Biol Chem* 1935; **112**:51–65.
47 Yeh CH, Peng HC, Yang RS, Huang TF. Rhodostomin, a snake venom disintegrin, inhibits angiogenesis elicited by basic fibroblast growth factor and suppresses tumor growth by a selective alpha(v)beta(3) blockade of endothelial cells. *Mol Pharmacol* 2001; **59**:1333–1342.
48 Heyninck K, Lahtela-Kakkonen M, Van der Veken P, Haegeman G, Vanden Berghe W. Withaferin A inhibits NF-kappab activation by targeting cysteine 179 in ikkβ. *Biochem Pharmacol* 2014; **91**:501–509.
49 Kaileh M, Vanden Berghe W, Heyerick A, Horion J, Piette J, Libert C, *et al*. Withaferin a strongly elicits ikappab kinase beta hyperphosphorylation concomitant with potent inhibition of its kinase activity. *J Biol Chem* 2007; **282**:4253–4264.
50 Fràter-Schröder M, Risau W, Hallmann R, Gautschi P, Böhlen P. Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic in vivo. *Proc Natl Acad Sci U S A* 1987; **84**:5277–5281.
51 Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell* 2011; **146**:873–887.
52 Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. *J Burn Care Res* 2010; **31**:158–175.
53 Chen J, Zhou Z, Yao Y, Dai J, Zhou D, Wang L, *et al*. Dipalmitoylphosphatidic acid inhibits breast cancer growth by suppressing angiogenesis via inhibition of the CUX1/FGF1/HGF signalling pathway. *J Cell Mol Med* 2018; **22**:4760–4770.
54 Xu XH, Zhao C, Peng Q, Xie P, Liu QH. Kaempferol inhibited VEGF and PGF expression and *in vitro* angiogenesis of hrecs under diabetic-like environment. *Braz J Med Biol Res* 2017; **50**:e5396.
55 Lin B, Song X, Yang D, Bai D, Yao Y, Lu N. Anlotinib inhibits angiogenesis via suppressing the activation of VEGFR2, pdgfrβ and FGFR1. *Gene* 2018; **654**:77–86.
56 Yoshiji H, Kuriyama S, Hicklin DJ, Huber J, Yoshii J, Miyamoto Y, *et al*. KDR/flk-1 is a major regulator of vascular endothelial growth factor-induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Hepatology* 1999; **30**:1179–1186.
57 Montuori N, Pesapane A, Rossi FW, Giudice V, De Paulis A, Selleri C, Ragno P. Urokinase type plasminogen activator receptor (upar) as a new therapeutic target in cancer. *Transl Med Unisa* 2016; **15**:15–21.
58 Laurenzana A, Chillà A, Luciani C, Peppicelli S, Biagioni A, Bianchini F, *et al*. Upa/upar system activation drives a glycolytic phenotype in melanoma cells. *Int J Cancer* 2017; **141**:1190–1200.
59 Yu G, Herazo-Maya JD, Nukui T, Romkes M, Parwani A, Juan-Guardela BM, *et al*. Matrix metalloproteinase-19 promotes metastatic behavior *in vitro* and is associated with increased mortality in non-small cell lung cancer. *Am J Respir Crit Care Med* 2014; **190**:780–790.
60 Müller M, Beck IM, Gadesmann J, Karschuk N, Paschen A, Proksch E, *et al*. MMP19 is upregulated during melanoma progression and increases invasion of melanoma cells. *Mod Pathol* 2010; **23**:511–521.
61 Li M, Yue GG, Song LH, Huang MB, Lee JK, Tsui SK, *et al*. Natural small molecule bigelovin suppresses orthotopic colorectal tumor growth and inhibits colorectal cancer metastasis via IL6/STAT3 pathway. *Biochem Pharmacol* 2018; **150**:191–201.
62 Linnskog R, Mohapatra P, Moradi F, Prasad CP, Andersson T. Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: dual interference of this loop more effectively impairs melanoma cell invasion. *Oncotarget* 2016; **7**:37790–37802.
63 Lederle W, Depner S, Schnur S, Obermueller E, Catone N, Just A, *et al*. IL-6 promotes malignant growth of skin sccs by regulating a network of autocrine and paracrine cytokines. *Int J Cancer* 2011; **128**:2803–2814.
64 Momtaz S, Niaz K, Maqbool F, Abdollahi M, Rastrelli L, Nabavi SM. STAT3 targeting by polyphenols: novel therapeutic strategy for melanoma. *Biofactors* 2017; **43**:347–370.
65 Coniglio SJ. Role of tumor-derived chemokines in osteolytic bone metastasis. *Front Endocrinol (Lausanne)* 2018; **9**:313.
66 Zhuang H, Cao G, Kou C, Liu T. CCL2/CCR2 axis induces hepatocellular carcinoma invasion and epithelial-mesenchymal transition *in vitro* through activation of the hedgehog pathway. *Oncol Rep* 2018; **39**:21–30.
67 Takeda T, Tsubaki M, Sakamoto K, Ichimura E, Enomoto A, Suzuki Y, *et al*. Mangiferin, a novel nuclear factor kappa B-inducing kinase inhibitor, suppresses metastasis and tumor growth in a mouse metastatic melanoma model. *Toxicol Appl Pharmacol* 2016; **306**:105–112.
68 Vanden Berghe W, Ndlovu MN, Hoya-Arias R, Dijsselbloem N, Gerlo S, Haegeman G. Keeping up NF-kappab appearances: epigenetic control of immunity or inflammation-triggered epigenetics. *Biochem Pharmacol* 2006; **72**:1114–1131.
69 Vanden Berghe W. Epigenetic impact of dietary polyphenols in cancer chemoprevention: lifelong remodeling of our epigenomes. *Pharmacol Res* 2012; **65**:565–576.
70 Noren DP, Chou WH, Lee SH, Qutub AA, Warmflash A, Wagner DS, *et al*. Endothelial cells decode VEGF-mediated ca2+ signaling patterns to produce distinct functional responses. *Sci Signal* 2016; **9**:ra20.
71 Kohn EC, Alessandro R, Spoonster J, Wersto RP, Liotta LA. Angiogenesis: role of calcium-mediated signal transduction. *Proc Natl Acad Sci U S A* 1995; **92**:1307–1311.
72 Sun CY, She XM, Qin Y, Chu ZB, Chen L, Ai LS, *et al*. Mir-15a and mir-16 affect the angiogenesis of multiple myeloma by targeting VEGF. *Carcinogenesis* 2013; **34**:426–435.
73 Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol* 2014; **95**:563–574.