Cite this: *Med. Chem. Commun.*,
2017, 8, 2248

Deciphering the role of hydrophobic and hydrophilic bile acids in angiogenesis using *in vitro* and *in vivo* model systems

Somanath Kundu,^{ab} Sandhya Bansal,^a Kalai Mangai Muthukumarasamy,^c
Chetana Sachidanandan, ^c Rajender K. Motiani ^{*c} and Avinash Bajaj ^{*a}

Bile acids have emerged as strong signaling molecules capable of influencing various biological processes like inflammation, apoptosis, cancer progression and atherosclerosis depending on their chemistry. In the present study, we investigated the effect of major hydrophobic bile acids lithocholic acid (LCA) and deoxycholic acid (DCA) and hydrophilic bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) on angiogenesis. We employed human umbilical vein endothelial cells (HUVECs) and zebrafish embryos as model systems for studying the role of bile acids in angiogenesis. Our studies revealed that the hydrophilic CDCA enhanced ectopic vessel formation as observed by the increase in the number of sub-intestinal vessels (SIVs) in the zebrafish embryos. The pro-angiogenic role of CDCA was further corroborated by *in vitro* vessel formation studies performed with human umbilical vein endothelial cells (HUVECs), whereas the hydrophobic LCA reduced tubulogenesis and was toxic to the zebrafish embryos. We validated that CDCA enhances angiogenesis by increasing the expression of vascular growth factor receptors (VEGFR1 and VEGFR2) and matrix metalloproteinases (MMP9) and by decreasing the expression of adhesion protein vascular endothelial cadherin (VE-cadherin). Our work implicates that the nature of bile acids plays a critical role in dictating their biological functions and in regulating angiogenesis.

Received 18th September 2017,
Accepted 30th October 2017

DOI: 10.1039/c7md00475c

rsc.li/medchemcomm

Introduction

Bile acids are amphipathic molecules derived from cholesterol in the liver and account for the majority of cholesterol turnover.¹ In order to maintain a homeostatic pool of bile acids, they are reabsorbed from the intestine for recycling back to the liver, a process known as enterohepatic recirculation. The key function of bile acids is to facilitate the intestinal digestion and absorption of dietary fat, steroids, drugs, and lipophilic vitamins.² Bile acids, due to their detergent-like nature, can damage tissues, cause cell death, and induce inflammation.³ However, recent findings suggest that bile acids act as important signaling molecules and regulate lipid homeostasis, glucose metabolism and xenobiotic detoxification.⁴

Bile acids have been demonstrated to act as ligands for FXR^{5,6} and G protein-coupled receptors (GPCRs), such as TGR5.⁷ Bile acids activate downstream signaling pathways

upon binding with these receptors.⁸ These receptors are largely expressed on the intestine, liver, kidney,⁶ vascular cells⁹ and liver sinusoidal endothelial cells.¹⁰ These receptors have different affinities for bile acids, for example, CDCA strongly activates FXR,¹¹ whereas LCA stimulates TGR5.⁷ Therefore, although all bile acids are involved in the emulsification of fats, the signaling roles of bile acids in biological processes like cancer cell proliferation,¹² inflammation,^{3,13} and apoptosis¹⁴ are contingent on the nature of the bile acids.^{15–17}

Angiogenesis is the process of formation of new blood vessels from existing blood vessels and it is a critical component of cancer progression, tissue repair, and wound healing.¹⁸ Although the role of bile acids in regulation of tumorigenesis¹⁹ and hepatic injury²⁰ is known, their influence on angiogenesis remains poorly characterized. Earlier studies have demonstrated that bile acids can induce endothelial dysfunction by enhancing expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), and E-selectin via stimulation of NF-κB (nuclear factor kappa of activated B cells) and p38 MAPK signaling pathways.²¹ The specific role of the different types of naturally occurring human bile acids in the process of angiogenesis and the potential impact of these bile acids on the endothelium are not clearly understood.

^a Laboratory of Nanotechnology and Chemical Biology, Regional Centre for Biotechnology, 3rd Milestone Faridabad-Gurgaon Expressway, NCR Biotech Cluster, Faridabad, Haryana-121001, India. E-mail: bajaj@rcb.res.in; Tel: +91 129 2848831

^b Manipal University, Manipal-576104, Karnataka, India

^c CSIR-Institute of Genomics and Integrative Biology, Mathura Road, New Delhi 110025, India. E-mail: rajmotiani@gmail.com

HUVECs (human umbilical vascular endothelial cells) and zebrafish are commonly used *in vitro* and *in vivo* angiogenesis model systems for understanding the effect of molecules/drugs. HUVECs are primary endothelial cells that can form capillary-like structures with a lumen when plated on a gelled basement matrix.²² Zebrafish are vertebrate organisms that appear to be an excellent model for drug toxicity screening due to their small size, transparency, and ability to absorb compounds through water. Further, zebrafish embryos develop most of the organs present in mammals at their very early development stage.²³ Transgenic zebrafish are also shown to be a promising model for understanding angiogenesis as they possess a complex mammalian-like circulatory system and its transparent nature helps in easy visualization.²⁴

Therefore, in this study, we evaluated the contribution of hydrophobic bile acids like lithocholic acid (LCA) and deoxycholic acid (DCA) and hydrophilic bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) to angiogenesis using HUVECs for *in vitro* experiments and zebrafish for *in vivo* studies (Fig. 1). The toxicity of different bile acids to HUVECs was investigated using the MTT assay. We then tested the toxicity of bile acids to zebrafish embryos. We next performed a tubulogenesis assay to study the impact of bile acids on endothelial cell tube formation. Further, we employed wound healing assays for determining the effect of different bile acids on migration of HUVECs. The zebrafish transgenic line Tg(fli1:eGFP) was used for investigating the role of these bile acids in angiogenesis. Molecularly, the effect of LCA and CDCA on expression of VEGFR1, VEGFR2, VE-cadherin and MMP9 secretion from HUVECs was investigated. Our results suggest that the hydrophilic bile acid CDCA induces angiogenesis in HUVECs and zebrafish embryos through upregulation of VEGFR1,

VEGFR2 and MMP9, whereas the hydrophobic bile acid LCA is highly toxic.

Results and discussion

The biological functions of bile acids are contingent on their chemical nature and polarity.²⁵ During pathogenic conditions like obstructive cholestasis, there is accumulation of bile acids along with the rise in their serum levels that could be harmful to the endothelium.²⁶ However, the current understanding of bile acids' role in regulating endothelium functions such as barrier function and angiogenesis remains very limited. In this study, we evaluated four bile acids, the hydrophilic primary bile acids CA and CDCA and the hydrophobic secondary bile acids LCA and DCA, for their possible role in controlling angiogenesis. We evaluated the role of these bile acids in regulating *in vitro* tubulogenesis, endothelial cell migration, and cell survival. We further screened the bile acids in the developing embryos of transgenic zebrafish line Tg(fli1:eGFP) wherein endothelial cells express GFP to examine their role in angiogenesis.

For determining the working concentration of the bile acids, we first performed toxicity screening in both HUVECs and zebrafish embryos. The bile acids were serially diluted in concentrations ranging from 100 to 6.25 μM and were tested on HUVECs for their cytotoxic effects by performing the MTT assay (Fig. 2A). All the bile acids except for LCA were found to be non-toxic to HUVECs. With LCA treatment, the cell viability was compromised with concentrations above 25 μM , as LCA at 100 μM induced $\sim 75\%$ cell death. However, at lower concentrations of 12.5 μM and below, none of the bile acids induced significant cell death (Fig. 2A). LCA and DCA are hydrophobic in nature and displayed differential toxicity wherein LCA was more toxic than DCA. In our earlier

Fig. 1 Chemical structures of bile acids, lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and CA (cholic acid), tested in the present study.

Fig. 2 Cytotoxicity assay: A) cytotoxicity of bile acids to HUVECs (human umbilical vein endothelial cells) using the MTT assay showing that LCA is the most toxic; B) toxicity of bile acids against zebrafish embryos confirming the highly toxic nature of LCA. Data in graph A represents the mean $\pm$ S.D. of three independent experiments.

studies,²⁷ we investigated the interactions of bile acids and their derivatives with cancer cells and model membranes. We showed that DCA possessing two hydroxyl groups shows poor interactions with cellular membranes as compared to the highly hydrophobic LCA. The hydration barrier between the cellular membranes and DCA does not favour the interactions resulting in poor uptake by mammalian cells. In contrast, LCA being more hydrophobic is able to penetrate the cell membranes easily and is highly toxic. The differential activity of DCA and LCA against HUVECs might also be due to their differential binding with cellular receptors.^{5–7}

We next performed similar toxicity studies in the zebrafish embryos for determining the non-toxic bile acid concentrations that could be used for performing angiogenesis studies in the embryos. We observed that LCA was the most toxic bile acid to the embryos as it killed embryos even at the concentration of 3.25 μ M, whereas all other bile acids were non-toxic to zebrafish embryos (Fig. 2B). Taken together, these data suggest that LCA is highly toxic to endothelial cells and zebrafish embryos. Further, these studies implicate that bile acids below the concentration of 25 μ M are non-toxic to HUVECs and therefore could be used for testing their potential role in angiogenesis.

In order to evaluate the contribution of these bile acids to angiogenesis, we next performed an *in vitro* tubulogenesis assay on HUVECs. In this assay, primary endothelial cells like HUVECs are seeded under 3D culture conditions on efficient extracellular matrices. HUVECs on induction in the 3D matrix differentiate to form tube-like structures with a lumen surrounded by endothelial cells.²⁸ We tested two different non-toxic concentrations (12.5 and 25 μ M) of all the four bile acids for investigating their role in angiogenesis (Fig. 3). All the tubulogenesis assays were performed in triplicate and five different fields from each well were quantified by calculating the number of nodes and tube lengths. The hydrophobic LCA inhibited endothelial tube formation at these low concentrations of 12.5 and 25 μ M (Fig. 3A). We observed a significant decrease in tube length (Fig. 3B) and number of nodes

(Fig. 3C) on treatment with LCA at 12.5 μ M that might be due to the toxic nature of LCA. In contrast, hydrophilic CDCA at 12.5 μ M significantly enhanced *in vitro* tube formation (Fig. 3A). We observed a significant increase in tube length (Fig. 3B) and a 150% increase in number of nodes on CDCA treatment at 12.5 μ M (Fig. 3C); whereas, there was no significant effect on tubulogenesis at 25 μ M. However, the other bile acids DCA and CA did not induce any change in tubulogenesis at these concentrations (Fig. 3). Just like angiogenesis, *in vitro* tubulogenesis depends upon endothelial cell proliferation, migration, and proper spatial organization so that the tubes are formed efficiently.²⁹ We therefore tested the effect of bile acids on cell migration upon treatment of HUVECs with bile acids.

We performed scratch wound assays on HUVEC monolayers for evaluating the role of CDCA and LCA in endothelial cell migration (Fig. 4). HUVECs were cultured in a 24-well plate till they became 80% confluent. After creating a wound, the cells were treated with 12.5 μ M bile acids. Pictures were taken at regular intervals and the cells were analyzed for their migration in comparison to control DMSO-treated cells. As shown in Fig. 4A, we observed a significant increase of wound healing in the case of CDCA-treated HUVECs in comparison to control treatment, whereas HUVEC migration remarkably decreased upon LCA treatment (Fig. 4A). We then quantified the percentage of migrated cells which clearly revealed a significant increase in migration of HUVECs on CDCA treatment as compared to other bile acids (Fig. 4B).

We next validated the role of CDCA in angiogenesis *in vivo* using the transgenic zebrafish line Tg(fli1:eGFP),³⁰ where all the endothelial cells are labeled with green fluorescent protein. This transgenic line is ideal for monitoring the formation of the inter-segmental vessels (ISVs) and sub-intestinal veins (SIVs) in live embryos and has been used extensively for screening of pro- and anti-angiogenic compounds for therapeutic applications.^{31,32} Angiogenesis in zebrafish usually starts after 20 hpf (hours post fertilization) and changes in sub-intestinal veins (SIVs) can be seen after 72 hpf.

Fig. 3 *In vitro* tubulogenesis assay: A) effect of different bile acids on *in vitro* tube formation of endothelial cells showing an increase in tube formation on CDCA treatment; B) quantification of tube formation in terms of tube length showing an increase in tube length on CDCA treatment at 12.5 μM as compared to control and a decrease with LCA; C) quantification of the number of nodes during the tube formation assay revealing a significant increase in number of nodes with CDCA at 12.5 μM and a decrease with LCA. Data in graphs (B and C) represents the mean $\pm$ S.D. of three independent experiments and statistical significance $*P < 0.05$ was calculated by one-way ANOVA (Dunnett's test). Tubulogenesis assays were performed in triplicate and all the quantifications were performed on five independent images of each replicate.

Therefore, for the angiogenesis assay, 48 hpf zebrafish embryos were treated with CDCA, DCA, and CA at 6.25 and 12.5 μM concentrations. LCA was not used for this assay due to its highly toxic nature. We then evaluated the effect of bile acids on SIV formation at 72 hpf. The anti-angiogenic compound LY294002, which inhibits the PI3K pathway, was used as a negative control. In the case of DMSO-treated controls, the SIV baskets developed normally and there were no lethal effects on the embryos. Intriguingly, we observed a significant increase in sprouting and branching of SIVs upon treatment with CDCA (Fig. 5A). There was a dose-dependent enhancement of the pro-angiogenic properties of CDCA in the developing zebrafish embryos. At 12.5 μM , CDCA treatment resulted in a marked increase in branching and sprouting of SIVs in most of the embryos with additional

arcades and SIV baskets extending towards the ventral side. This pro-angiogenic effect of CDCA was observed at an even lower concentration of 6.25 μM in comparison to the 0.1% DMSO control suggesting that CDCA could enhance angiogenesis *in vivo* (Fig. 5A). In contrast, treatments with LCA even at a dose of 3 μM were lethal for zebrafish embryos and caused significant embryo death.

We observed abnormality in the vasculature of developing zebrafish embryos on DCA treatment (Fig. 5B) and the highest concentration of DCA at 25 μM was lethal. We observed pronounced vascular modifications where most of the vessels did not have a regular pattern of branches at 12.5 μM . In few cases, we observed that the branching was lost which could be attributed to the toxic nature of hydrophobic DCA. At the lower concentration of 6.25 μM , the pro-

Fig. 4 Wound healing assay: A) effect of bile acids on migration of HUVECs upon treatment with different bile acids at 12.5 μM ; representative images out of 5 from triplicate experiments were presented here; B) quantitative graph from independent images showing the percentage of migrated cells as compared to control. Data is represented as the mean $\pm$ S.D. of three replicates and statistical significance between the control and treated groups was calculated using one-way ANOVA (Dunnett's test); * $P < 0.05$, *** $P < 0.001$.

angiogenic effect of DCA was evident in the thickening and sprouting out of the vessels (Fig. 5B); however, these effects were not as prominent as seen with the CDCA-treated embryos. Treatment with another hydrophilic bile acid CA resulted in no observable phenotypic changes in SIV of zebrafish after treatment suggesting that it may have no role in angiogenesis (Fig. 5C). As expected, treatment with the PI3K (phosphoinositide 3-kinase) inhibitor totally abolished the angiogenesis (Fig. 5D). Fig. 5E presents the percentage of embryos with pro-angiogenic effects upon treatment with different bile acids confirming the significant increase in angiogenesis with CDCA treatment.

In order to identify the molecular mechanism driving the changes in the extent of angiogenesis induced by CDCA and LCA, we analyzed the levels of VEGFR1 (vascular endothelial growth factor receptor-1), VEGFR2 (vascular endothelial growth factor receptor-2), and matrix metalloproteinase 9 (MMP 9) upon treatment of HUVECs with CDCA and LCA. Vascular endothelial growth factor (VEGF) is the chief regulator of angiogenesis and it is able to do so by binding and activating receptor tyrosine kinases VEGFR1 and VEGFR2.^{33,34}

We performed quantitative PCR with the mRNA isolated from HUVECs treated with 12.5 μM CDCA or LCA and compared the levels of VEGFR1 and VEGFR2 to those in the case of control DMSO-treated HUVECs. Excitingly, we observed a robust increase in the mRNA expression of VEGFR1 and VEGFR2 upon treatment with CDCA (Fig. 6A). In contrast, we noticed an ~ 2 -fold decrease in VEGFR1 and VEGFR2 expression levels with LCA treatment (Fig. 6B).

MMP 9 is a well-established regulator of angiogenesis that promotes angiogenesis by increasing the expression of VEGFR and by enhancing the association of VEGF with VEGFR.^{35,36} We therefore performed a time course experiment wherein we treated HUVECs with either 12.5 μM CDCA, LCA or DMSO (vehicle control) and measured the levels of MMP 9 secreted in the media supernatant 12 and 24 h post treatments. Our ELISA assays clearly demonstrated that the levels of secreted MMP 9 robustly increase 24 h post CDCA treatment and at the same time point secreted MMP 9 levels decrease with LCA treatment (Fig. 6C). Collectively, our data suggests that CDCA promotes angiogenesis by inducing MMP 9 secretion and increasing VEGFR expression.

Fig. 5 *In vivo* studies: A–C) representative images of Tg(fli1:eGFP) zebrafish embryos 72 h post-fertilization showing the effect of different bile acids at 6.25 and 12.5 μM concentrations; D) effect of the PI3K inhibitor (LY294002) on sub-intestinal vessel formation; E) graph showing the percentage of embryos possessing the pro-angiogenic modifications of SIVs with CDCA in a dose-dependent manner. The data in graph (E) is calculated as the mean ± S.D. of duplicate experiments ($n = 50$). The statistical significance among the treatments $**P < 0.005$ was calculated by one-way ANOVA (Dunnett's test).

Several studies suggest that receptor tyrosine kinases (RTKs) Eph and their ligands, ephrins, play an important role in angiogenesis.³⁷ A recent report demonstrated a cross-talk between ephrin/VEGF signaling pathways in vascular remodeling.³⁸ Eph-ephrin signaling helps in VEGF receptor internalization, thereby promoting endothelial cell sprouting and angiogenesis.³⁹ Numerous strategies are underway to develop anti-angiogenic therapeutics targeting the Eph-ephrin system⁴⁰ and in the process it was discovered that LCA acts as a competitive inhibitor that prevents the Eph-ephrin interaction.⁴¹ Moreover, LCA and its amino acid analogues have recently been established as EphA2 antagonists preventing Eph-kinase activation.⁴² Therefore, LCA could be inhibiting angiogenesis by both decreasing VEGF receptor expression and preventing Eph pathway activation.

Angiogenesis is also associated with enhanced endothelial cell migration and as per our data bile acids can regulate endothelial cell migration. The cytoskeletal elements undergo reorganization during cell migration and division, associated with angiogenesis.⁴³ The literature suggests that the loss of

vascular endothelial cadherin (VE-cadherin)-mediated cell adhesion plays a critical role in angiogenesis.⁴⁴ Park *et al.* have shown that tauroursodeoxycholic acid causes dissociation of CD34⁺ hematopoietic stem cells by reducing adhesion molecule expression and enhances angiogenesis in the mouse hind limb ischemia model.⁴⁵ Exposure to taurocholic acid increases the expression of angiogenic factors and taurocholic acid-treated tumors exhibit high vascularization as they enhance HUVEC migration.⁴⁶

During cell migration, endothelial cells lose contact with adjacent cells and it is associated with the decrease in the expression of VE-cadherin. We therefore examined the effect of CDCA on cell morphology and VE-cadherin expression. HUVECs were stained with FITC-conjugated phalloidin to observe the cytoskeleton behavior. As shown in Fig. 6D, CDCA-treated HUVECs showed extensive stress fiber formation in comparison to DMSO-treated cells. We observed the highest extent of stress fibers in well-aligned parallel arrangements in HUVECs upon treatment with 12.5 μM CDCA, whereas LCA-treated cells showed irregular arrangement of stress

Fig. 6 Effect of CDCA on cell adhesion: A and B) change in expression of VEGFR1 (A) and VEGFR2 (B) in HUVECs on LCA and CDCA treatment; C) relative expression of MMP9 from HUVECs on LCA and CDCA treatment; D) representative confocal microscopy images of bile acid-treated HUVECs showing an increase in actin stress fiber formation with CDCA; E) representative confocal microscopy images of HUVECs co-stained with VE-cadherin (green) and actin (red) antibodies showing loss of cell adhesion on CDCA treatment. Data in graphs (A–C) is shown as the mean $\pm$ S.D. of duplicate experiments. Statistical significance among the treated groups was calculated by one-way ANOVA (Dunnett's test); * P < 0.05, ** P < 0.005, *** P < 0.001.

fibers accompanied by the loss of cell viability (Fig. 6D). Further, VE-cadherin staining showed a marked decrease in VE-cadherin expression and loss of cell-to-cell contact in the case of CDCA-treated HUVECs (Fig. 6E). In contrast, LCA induced significant cell death and therefore the changes in cellular architecture could be associated with the cell death induced by LCA. Taken together, these studies underline that CDCA may play a potential role in angiogenesis by enhancing endothelial cell migration *via* regulation of actin dynamics and adhesion protein expression.

Conclusions

Our study demonstrated that the most hydrophobic secondary bile acid LCA inhibits *in vitro* tubulogenesis and cell migration and induces endothelial cell death at concentrations higher than 25 μ M. Similarly, the other hydrophobic bile acid DCA alters zebrafish angiogenesis, inducing coiling and shrinkage of vessels. Therefore, the hydrophobic bile acids could have detrimental effects on the endothelium. In contrast, the hydrophilic primary bile acid CDCA positively regu-

lates angiogenesis without affecting endothelial cell survival. CDCA at a moderate concentration of 12.5 μ M enhances *in vitro* tube formation by promoting cell migration. CDCA further augments zebrafish angiogenesis as revealed by ectopic vessel formation and an increase in the number of branches in SIVs. This suggests that at moderate concentrations CDCA induces angiogenesis. Angiogenesis is increasingly acknowledged as a facilitator for repair mechanisms in response to liver injury.⁴⁷ The liver repair during cholestatic liver disease has been linked with changes in bile acid levels in the liver and blood.⁴⁸ Our data suggests that CDCA could be helpful in hepatic tissue regeneration by enhancing angiogenesis, whereas at higher concentrations, hydrophobic bile acids could lead to vascular damage.

Experimental section

Reagents

All the chemicals were purchased from Sigma Aldrich. The tested chemicals cholic acid (CA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA) and deoxycholic acid (DCA)

were soluble in DMSO (Sigma-Aldrich). Stocks were maintained at 25 mM concentrations.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (USA) and maintained with EBM-2 media (Lonza, CC-3156) supplemented with a bullet kit containing all the growth supplements (Lonza, CC-3162). The passages 2–5 HUVECs were used to carry out the experiments.

Cell proliferation assay

Briefly, 5×10^3 cells per well were cultured on 96-well plates with complete EBM-2 media for 24 hours and allowed to adhere. The cells were then serum-starved for 4 hours and then treated with various bile acids in complete growth medium for 24 hours. The cells were then incubated with 20 μ L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) solution (5 mg mL⁻¹ stock) for 4 hours at 37 °C. After the incubation, formazan crystals were dissolved using DMSO and absorbance was read at 550 nm using a plate reader spectrophotometer. The experiments were performed in triplicate.

In vitro tube formation assay⁴⁹

12-well plates were coated with 500 μ L basement membrane matrix extract (1:1, Matrigel solution (BD Biosciences): plain EBM-2 media). Later, 2×10^5 cells per well along with required dilutions of bile acids and vehicle control were added to the wells and treated overnight. Images were captured in bright field at 10 $\times$ magnification using an inverted microscope (Nikon) from five different fields in each well. *In vitro* angiogenesis or tube formation is quantified by calculating the number of nodes and tube lengths formed. The experiments were performed in triplicate.

Wound healing assay⁵⁰

From a fully confluent flask, cells were trypsinized, resuspended and then reseeded as 2×10^5 cells per well were plated in a 12-well plate and returned to the incubator until the cells are completely confluent. Afterwards, the cells were serum-deprived overnight; on the next day, a wound was created on the monolayer of cells using a sterile p200 pipette tip and washed with DPBS to get rid of debris, then a reference point was created using a fine marker pen. Complete growth media (EBM-2) along with required dilutions of bile acids and vehicle control were added. The wound was imaged and after 18–24 hours of incubation the cells were imaged from the reference point created using a phase contrast microscope at 10 $\times$ magnification. Images were analysed using ImageJ software. The experiments were performed in triplicate.

Zebrafish maintenance and treatment

Zebrafish (*Danio rerio*) were raised, bred and maintained at 28.5 °C under standard conditions as described (M. Westerfield, The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*), University of Oregon Press, 2000).

All zebrafish handling and experiments were performed in accordance to protocols approved for BSC0124 by the Institutional Animal Ethics Committee (IAEC) of the CSIR-Institute of Genomics and Integrative Biology, India under the rules and regulations set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India.

The Tg(fli:eGFP) adult animals were mated as described (M. Westerfield, The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*), University of Oregon Press, 2000). The embryos were staged according to the zebrafish convention.⁵¹ Healthy embryos were selected at the 48 hpf (hours post fertilization) stage and placed in a 6-well plate at 20–25 embryos per well. All the bile acids were dissolved in DMSO and further dilutions were made in embryo water (M. Westerfield, The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*), University of Oregon Press, 2000). For the treatments, 0.1% DMSO was used as the vehicle control. The experiments were performed twice with 50 embryos per group. Three concentrations of 6.25 μ M, 12.5 μ M and 25 μ M were used for treatments. At the 72 hpf stage, the bile acid solutions were replaced by fresh embryo water. The embryos were then anesthetized with tricaine and imaged. Observation and imaging of phenotypes were conducted using a Zeiss Stemi 2000-C stereomicroscope with AxiocamICc1 and a Zeiss Axio Scope A1 fluorescence microscope with AxiocamHRc. SIV baskets were examined to determine the effect based on the following criteria: a) the presence of vessels sprouting out of the basket structure, b) extension of the SIV basket into the yolk region with more than eight vertical branches within the basket and c) thickening of the vessels and interconnecting branches.

RNA isolation

HUVECs were grown in 6-well plates and treated with 12.5 μ M CDCA and LCA along with the vehicle control (DMSO) for 12 and 24 hours. Total RNA was isolated from the cell pellets using an RNAiso Plus (TaKaRa) total RNA isolation reagent following the manufacturer's protocol. The isolated RNA was dissolved in nuclease-free water. The purity and concentration were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and the quality was assessed by running in 1% agarose gel. 1 μ g of RNA was used to prepare the cDNA using an iScript cDNA synthesis kit (Bio-Rad).

Quantitative real-time PCR

Quantitative real-time PCR was performed in a Quant Studio 6 Flex Real-Time PCR system (Applied Biosystems) using 96-well plates with the SYBER-green premix Ex Taq II (Tli RNase

H Plus, TaKaRa) for the following genes: 1) VEGFR1, forward sequence: 5'TTTGCCTGAAATGGTGAGTAAGG3', reverse sequence: 5'TGGTTTGCTTGAGCTGTGTTC3' and 2) VEGFR2, forward sequence: 5'GGCCCAATAATCAGAGTGGCA3', reverse sequence: 5'CCAGTGTCAATTCGATCACTTT3' (Sigma). The quantitative $\Delta\Delta\text{CT}$ values were calculated and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward sequence: 5'CCTGGCCAAGTCATCCATG3', reverse sequence: 5'GGAAGGCCATGCCAGTGAGC3') and the relative fold change in expression upon control was calculated. PCR thermal profiling was conducted by initial denaturation at 95 °C for 10 min, then at 95 °C for 5 s and at 60 °C for 30 s for 40 cycles. The experiments were repeated twice.

ELISA assay

The changes in the levels of MMP 9 upon treatment with CDCA and LCA were measured using an ELISA kit (Sigma; Catalog no. RAB0372). The MMP 9 levels in the media were estimated as per manufacturer's instructions. Briefly, HUVECs were plated in 6-well plates and were treated with either 12.5 μM CDCA/LCA or DMSO (vehicle control). Time course experiments were performed and the media supernatant 12 and 24 h post treatment was collected for performing ELISA. The final ELISA HRP signal was quantified by measuring the absorbance at 405 nm and the concentration of MMP 9 was estimated by interpolating the OD values of the samples on the curve obtained with the MMP 9 standards. The experiments were repeated in duplicate.

Immuno-cytochemistry⁵²

HUVECs were seeded and grown up to 70% confluency on sterile glass cover slips in 24-well plates. The cells were treated with bile acids at different concentrations overnight after which they were fixed with 4% paraformaldehyde for 5 minutes at room temperature and then washed with PBS thrice for 3 minutes each. The cells were blocked with 1% BSA (Thermo Scientific) and 0.1% Triton X-100 (Sigma) in 1 $\times$ PBS for 2 hours at RT, followed by incubation with primary antibodies, VE-cadherin anti-rabbit (1:200; Cell-Signaling) and α -tubulin anti-mouse (1:400, Sigma-Aldrich), overnight at 4 °C followed by secondary anti-rabbit Alexa-488 (1:800; Cell-Signaling) and anti-mouse Alexa-594 (1:1000; Cell-Signaling) for 1 hour at RT. Antibody dilutions were made in 0.1% BSA and 0.1% Triton X-100 in PBS. F-actin was stained with FITC-conjugated phalloidin (1:100, gifted by Dr. Sam Jacob Mathew, RCB). Nuclear staining was performed with DAPI. The glass cover slips were mounted on slides using Pro Long gold anti-fade mounting media (Thermo Scientific), imaged under a confocal microscope (Leica TCS SP5) and analysed using the Leica image analysis suite. The experiment was repeated twice.

Author contributions

AB conceived the idea and designed the experiments. SK and RM performed the zebrafish experiments. SK, SB and RM

performed the cell culture experiments. KMM helped in the zebrafish studies and CS supervised the zebrafish studies.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

We thank RCB for intramural funding. SK thanks RCB for research fellowship. RKM was supported by DST-INSPIRE Faculty fellowship LSBM-038 by the Department of Science and Technology, India. CS was supported by the Council of Scientific and Industrial Research (CSIR), Government of India (grant BSC0124).

References

- 1 A. F. Hofmann, *Hepatology*, 2008, 49, 1403.
- 2 J. Maldonado-Valderrama, P. Wilde, A. Macierzanka and A. Mackie, *Adv. Colloid Interface Sci.*, 2011, 165, 36.
- 3 M. J. Park, K. H. Kim, H. Y. Kim, K. Kim and K. J. Cheong, *Carcinogenesis*, 2008, 29, 2385.
- 4 S. M. Houten, M. Watanabe and J. Auwerx, *EMBO J.*, 2006, 25, 1419.
- 5 M. Makishima, A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf and B. Shan, *Science*, 1999, 284, 1362.
- 6 H. Wang, J. Chen, K. Hollister, L. C. Sowers and B. M. Forman, *Mol. Cell*, 1999, 3, 543.
- 7 T. Maruyama, Y. Miyamoto, T. Nakamura, Y. Tamai, H. Okada, E. Sugiyama, T. Nakamura, H. Itadani and K. Tanaka, *Biochem. Biophys. Res. Commun.*, 2002, 298, 714.
- 8 Y. Kawamata, R. Fujii, M. Hosoya, M. Harada, H. Yoshidam, M. Miwa, S. Fujusumi, Y. Habatam, T. Itoh, Y. Shintani, S. Hinuma, Y. Fujisawa and M. Fujino, *J. Biol. Chem.*, 2003, 278, 9435.
- 9 F. He, J. Li, Y. Mu, R. Kuruba, Z. Ma, A. Wilson, S. Alber, Y. Jiang, T. Stevens, S. Watkins, B. Pitt, W. Xie and S. Li, *Circ. Res.*, 2006, 98, 192.
- 10 V. Keitel, R. Reinehr, P. Gatsios, C. Rupprecht, B. Gorg, O. Selbach, D. Haussinger and R. Kubitz, *Hepatology*, 2007, 45, 695.
- 11 D. J. Parks, S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore and J. M. Lehman, *Science*, 1999, 284, 1365.
- 12 J. M. Kyriakis and J. Avruch, *Physiol. Rev.*, 2012, 92, 689.
- 13 D. Qiao, W. Chen, E. D. Stratagoules and J. D. Martinez, *J. Biol. Chem.*, 2000, 275, 15090.
- 14 S. Sato, H. Yamamoto, K. Mukaisho, S. Saito, T. Hattori, G. Yamamoto and H. Sugihara, *PLoS One*, 2014, 9, e88831.
- 15 K. Yadav, P. Bhargava, S. Bansal, M. Singh, S. Gupta, G. Sandhu, S. Kumar, V. Sreekanth and A. Bajaj, *Med. Chem. Commun.*, 2015, 6, 778.
- 16 M. Singh, S. Bansal, S. Kundu, P. Bhargava, A. Singh, R. K. Motiani, R. Shyam, V. Sreekanth, S. Sengupta and A. Bajaj, *Med. Chem. Commun.*, 2015, 6, 192.

- 17 S. Bansal, M. Singh, S. Kidwai, P. Bhargava, A. Singh, V. Sreekanth, R. Singh and A. Bajaj, *Med. Chem. Commun.*, 2014, 5, 1761.
- 18 T. A. Drixler, M. J. Vogten, E. D. Ritchie, T. J. Vroonhoven, M. F. Gebbink, E. E. Voest and I. H. Borel Rinkes, *Ann. Surg.*, 2002, 236, 703.
- 19 T. Soma, J. Kaganoi, A. Kawabe, K. Kondo, S. Tsunoda, M. Imamura and Y. Shimada, *Int. J. Cancer*, 2006, 119, 771.
- 20 S. Fischer, U. Beuers, U. Spengler, F. M. Zwiebel and H. G. Koebe, *Clin. Chem. Acta*, 1996, 251, 173.
- 21 P. Qin, X. Tang, M. M. Elloso and D. C. Harnish, *Am. J. Physiol. Heart Circ. Physiol.*, 2006, 291, 741.
- 22 K. L. DeCicco-Skinner, G. H. Henry, C. Cataisson, T. Tabib, J. C. Gwilliam, N. J. Watson, E. M. Bullwinkle, L. Falkenburg, R. C. O'Neill, A. Morin and J. S. Wiest, *J. Visualized Exp.*, 2014, 51312.
- 23 A. L. Rubinstein, *Expert Opin. Drug Metab. Toxicol.*, 2006, 2, 231.
- 24 S. Nicoli and M. Presta, *Nat. Protoc.*, 2007, 2, 2918.
- 25 M. J. Monte, J. J. Marin, A. Antelo and J. Vazquez-Tato, *World J. Gastroenterol.*, 2009, 15, 804.
- 26 G. T. Everson, *J. Lipid Res.*, 1987, 28, 238.
- 27 M. Singh, A. Singh, S. Kundu, S. Bansal and A. Bajaj, *Biochim. Biophys. Acta, Biomembr.*, 2013, 1828, 1926.
- 28 M. W. Irvin, A. Zijlstra, J. P. Wikswo and A. Pozzi, *Exp. Biol. Med.*, 2014, 239, 1476.
- 29 R. Auerbach, R. Lewis, B. Shinnars, L. Kubai and N. Akhtar, *Clin. Chem.*, 2003, 49, 32.
- 30 N. D. Lawson and B. M. Weinstein, *Dev. Biol.*, 2002, 248, 307.
- 31 M. Raghunath, Y. Sy Wong, M. Farooq and R. Ge, *Biochem. Biophys. Res. Commun.*, 2009, 378, 766.
- 32 S. Isogai, M. Horiguchi and B. M. Weinstein, *Dev. Biol.*, 2001, 230, 278.
- 33 S. Abraham, M. Yeo, M. Montero-Balaguer, H. Paterson, E. Dejana, C. J. Marshall and G. Mavria, *Curr. Biol.*, 2009, 19, 668.
- 34 S. Koch and L. Claesson-Welsh, *Cold Spring Harbor Perspect. Med.*, 2012, 2, 1006502.
- 35 S. Kanno, N. Oda, M. Abe, Y. Terai, M. Ito, K. Shitara, K. Tabayashi, M. Shibuya and Y. Sato, *Oncogene*, 2000, 19, 2138.
- 36 G. Bergers, R. Bekken, G. McMahon, T. H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb and D. Hanahan, *Nat. Cell Biol.*, 2000, 2, 737.
- 37 J. Chen, D. Hicks, D. Brantley-Sieders, N. Cheng, G. W. McCollum, X. Qi-Werdich and J. Penn, *Exp. Eye Res.*, 2006, 82, 664–673.
- 38 Y. Wang, M. Nakayama, M. E. Pitulescu, T. S. Schmidt, M. L. Bochenek, A. Sakakibara, S. Adams, A. Davy, U. Deutsch, U. Lüthi, A. Barberis, L. E. Benjamin, T. Mäkinen, C. D. Nobes and R. H. Adams, *Nature*, 2010, 465, 483.
- 39 S. Sawamiphak, S. Seidel, C. L. Essmann, G. A. Wilkinson, M. E. Pitulescu, T. Acker and A. Acker-Palmer, *Nature*, 2010, 465, 487.
- 40 W. Boyd, P. F. Bartlett and M. Lackmann, *Nat. Rev. Drug Discovery*, 2013, 13, 39.
- 41 M. Incerti, M. Tognolini, S. Russo, D. Pala, C. Giorgio, I. Hassan-Mohamed, R. Noberini, E. B. Pasquale, P. Vicini, S. Piersanti, S. Rivara, E. Barocelli, M. Mor and A. Lodola, *J. Med. Chem.*, 2013, 56, 2936.
- 42 C. Giorgio, I. Hassan Mohamed, L. Flammini, E. Barocelli, M. Incerti, A. Lodola and M. Tognolini, *PLoS One*, 2011, 6, e18128.
- 43 T. J. Mitchison and L. P. Cramer, *Cell*, 1996, 84, 371.
- 44 S. Abraham, M. Yeo, M. Montero-Balaguer, H. Paterson, E. Dejana, C. J. Marshall and G. Mavria, *Curr. Biol.*, 2009, 19, 668.
- 45 J. G. Cho, J. H. Lee, S. H. Hong, H. N. Lee, C. M. Kim, S. Y. Kim, K. J. Yoon, B. J. Oh, J. H. Kim, S. Y. Jung, T. Asahara, S. Kwon and S. G. Park, *Stem Cells*, 2015, 33, 792.
- 46 S. Sato, H. Yamamoto, K. Mukaisho, S. Saito, T. Hattori, G. Yamamoto and H. Sugihara, *PLoS One*, 2014, 9, e8831.
- 47 W. E. Naugler, *PLoS One*, 2014, 9, e97426.
- 48 G. Li and G. Guo, *Acta Pharm. Sin. B*, 2015, 5, 93.
- 49 I. Arnaoutova and H. K. Kleinman, *Nat. Protoc.*, 2010, 5, 628.
- 50 C. R. Justus, N. Leffler, M. Ruiz-Echevarria and L. V. Yang, *J. Visualized Exp.*, 2014, 88, e51046.
- 51 C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling, *Dev. Dyn.*, 1995, 203, 253.
- 52 M. G. Lampugnani, M. Resnati, M. Raiteri, R. Pigott, A. Pisacane, G. Houen, L. P. Ruco and E. Dejana, *J. Cell Biol.*, 1992, 118, 1511.