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# Implication de la phostine 3.1a sur l'angiogenèse, le métabolisme endothélial et les pathologies associées

Simon Bousseau

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L'UNIVERSITE D'ANGERS  
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Par

**Simon BOUSSEAU**

## **Implication de la phostine 3.1a sur l'angiogenèse, le métabolisme endothélial et les pathologies associées**

Thèse présentée et soutenue à Angers, le 12 Octobre 2018

Unités de recherche : INSERM 1063 SOPAM « Stress Oxydant et Pathologies Métaboliques »  
INSERM 1083 Institut MitoVasc

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# Table des matières

Table des matières.....	1
Remerciements.....	3
Communications et publications.....	5
Abréviations.....	7
Liste des figures et tableaux .....	9
Introduction.....	11
1. Le réseau vasculaire humain.....	12
1.1. Composition des vaisseaux.....	12
1.2. Expansion du réseau vasculaire lors du développement embryonnaire.....	13
1.2.1. Généralités.....	13
1.2.2. Découverte de l'angiogenèse.....	14
1.3. Expansion du réseau vasculaire à l'état adulte.....	14
1.4. Pathologies liées au réseau vasculaire.....	15
2. Glycosylation as new pharmacological strategies for diseases associated with excessive angiogenesis.....	16
3. Glioblastome.....	48
3.1. Origine du GBM.....	48
3.2. Agressivité du GBM.....	49
3.3. Traitements anti-tumoraux actuels.....	50
4. Les phostines.....	51
4.1. Les glycomimétiques.....	51
4.2. La famille des phostines .....	52
4.3. Synthèse des phostines.....	52
4.4. Criblage.....	53
4.5. Phostine 3.1a.....	54
3.5.1. Structure.....	54
3.5.2. Activité anti-tumorale.....	55
3.5.3. Mécanisme d'action .....	56

Objectif de travail.....	58
Résultats expérimentaux.....	60
Phostine 3.1a as a pharmacological compound with anti-angiogenic properties to fight diseases with excess vascularization.....	62
Discussion générale .....	94
Bibliographie.....	101

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Vivement la suite !

# Communications et Publications

## Communications

- Orales

1. Bousseau S., Lenaers G., Andriantsitohaina R. *Phostine implication in angiogenesis, mitochondrial functions and associated pathologies*. Meeting de l'école doctorale Biologie-Santé Nantes/Angers, 15-16 décembre 2016.
2. Bousseau S., Lenaers G., Andriantsitohaina R. *Implication des phostines sur l'angiogenèse, les régulations mitochondriales et pathologies associées*. Séminaire MitoVasc, Angers, 02 Février 2017.
3. Bousseau S., Lenaers G., Andriantsitohaina R. *Implication des phostines sur l'angiogenèse, le métabolisme endothélial et les pathologies associées*. Séminaire MitoVasc, Angers, 12 Avril 2018.
4. Bousseau S., Lenaers G., Andriantsitohaina R. *Implication des phostines sur l'angiogenèse, le métabolisme endothélial et les pathologies associées*. Journée de la SFR, Angers, 29 Mai 2018.

- Posters

1. Bousseau S., Soleti R., Vergori L., Le Mao M., Benabbou N., Clarion L., Bakalara N., Lenaers G., Andriantsitohaina R. *In vitro and in vivo anti-angiogenic properties of Phostine 3.1a as a potential pharmacological compound to fight against diseases with excess vascularization*. Printemps de la Cardiologie, Nantes, 06-07 Avril 2017.
2. Bousseau S., Soleti R., Vergori L., Le Mao M., Benabbou N., Clarion L., Bakalara N., Lenaers G., Andriantsitohaina R. *In vitro and in vivo anti-angiogenic properties of Phostine 3.1a as a potential pharmacological compound to fight against diseases with excess vascularization*. Journée de la SFR, Angers, 30 Mai 2017.

## **Publications**

1. Bousseau S., Vergori L., Soleti R., Lenaers G., Martinez M. C., Andriantsitohaina R. (2018). *Glycosylation as new pharmacological strategies for diseases associated with excessive angiogenesis*. Jun. pii: S0163-7258(18)30105-0). Publié dans *Pharmacology & Therapeutics*.
2. Bousseau S., Marchand M., Soleti R., Vergori L., Hilairet G., Le Mao M., Gueguen N., Khiati S., Clarion L., Bakalara N., Germain S., Lenaers G., Andriantsitohaina R. (2018). *Phostine 3.1a as a pharmacological compound with anti-angiogenic properties against diseases with excess vascularization*. En révision positive dans *Faseb Journal*.

# Abréviations

2-deoxy-D-glucose (2DG)

3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO)

3-Hydroxy-4,5-bis-benzyloxy-6-benzyloxymethyl-2-phenyl-2-oxo-2λ5-[1,2]oxaphosphinane  
(PST 3.1a)

6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3)

Acide désoxyribonucléique (ADN)

Acide ribonucléique (ARN)

Adénosine triphosphate (ATP)

Agence américaine des produits alimentaires et médicamenteux (FDA)

Benzyl (Bn)

Cellule endothéliale (CE)

Cellule musculaire lisse (CML)

Cellule progénitrice endothéliale (CPE)

Cellule tumorale (CT)

Cellule endothéliale humaine de la veine ombilicale (HUVEC)

Concentration inhibitrice médiane (IC50)

Facteur de croissance de l'endothélium vasculaire (VEGF)

Facteur de croissance des fibroblaste (FGF)

Facteur de croissance épithélial (EGF)

Galectine (Gal)

Glioblastome multiforme (GBM)

Imagerie par résonance magnétique (IRM)

Institut national du cancer (NCI)

Isocitrate déshydrogénase (IDH)

Kinase d'adhésion focale (FAK)

Kinase dépendante des cyclines (CDK)

N-acetylglucosaminyltransférase (GnT)

Organisation mondiale de la santé (OMS)

Oxygène (O)

Phényle (Ph)

Phosphore (P)

Phostine (PST)

Récepteur du facteur de croissance de l'endothélium vasculaire (VEGFR)

Transcriptase reverse des télomères (TERT)



# Liste des figures et tableaux

## Introduction

Fig.1 : Different subtypes of angiogenesis.....	19
Fig. 2 : Contribution of lymphatic vessels to tumor progression.....	24
Fig. 3 : Classical angiogenic mechanisms and targets of anti-angiogenic agents.....	25
Fig. 4 : Compounds with anti-angiogenic properties targeting mitochondria.....	29
Fig. 5 : Anti-angiogenic treatments targeting glycolysis and fatty acid metabolism .....	32
Fig. 6 : New anti-angiogenic therapies targeting glycosylation .....	38
Fig. 7 : IRM d'un GBM au niveau de la substance blanche de l'hémisphère cérébral gauche .....	50
Fig. 8 : Structure d'une phostine .....	52
Fig. 9 : Criblage haut débit des phostines.....	54
Fig. 10 : Structure de PST 3.1a.....	55
Fig. 11 : Représentation schématique des principaux objectifs.....	59
Tableau 1 : Clinical trials using anti-angiogenic therapy combined with other treatments.....	26
Tableau 2 : Adverse effects of anti-angiogenic drugs used in clinical trials .....	27
Tableau 3 : New anti-angiogenic therapies targeting glycosylation .....	37

## Résultats

Fig.1 : PST 3.1a inhibits angiogenesis steps <i>in vitro</i> .....	81
Fig. 2 : PST 3.1a decreases angiogenesis <i>in vitro</i> .....	82
Fig. 3 : PST 3.1a decreases blood flow in zebrafish.....	83
Fig. 4 : PST 3.1a decreases tumor development.....	84
Fig. 5 : PST 3.1a treatment reduces protein expression of key angiogenesis factors in HUVECs.....	85

Fig. 6 : PST 3.1a modifies HUVEC cell cycle.....	86
Fig. 7 : PST 3.1a decreases GnT-V and Gal-1 expression.....	87
Suppl. Fig. 1 : PST 3.1a did not display any toxicity on HUVECs.....	90
Suppl. Fig. 9 : PST 3.1a did not disrupt zebrafish heartbeats.....	91
Suppl. Fig. 10 : PST 3.1a did not alter mitochondria physiology and glycolysis .....	92
Suppl. Fig. 11 : Summary of the biological effects on PST 3.1a on HUVEC mechanisms.....	93

# Introduction

## 1. Le réseau vasculaire humain

Le réseau vasculaire humain est un circuit fermé, qui permet le transport du sang depuis le cœur vers l'ensemble des tissus et organes, et son retour vers la pompe cardiaque. Il est composé des artères, des veines et des capillaires. Les artères amènent le sang oxygéné du cœur vers les organes et tissus, et se divisent en artérioles, puis en capillaires. Ces derniers permettent d'augmenter la surface d'échange avec les tissus environnants et relient directement les artérioles aux veinules, qui se rejoignent au niveau des veines, dont le rôle est le retour du sang pauvre en oxygène vers le cœur. Les vaisseaux lymphatiques complètent ce réseau vasculaire et assurent le transport de la lymphe, directement dans la circulation sanguine. L'ensemble de cet arbre vasculaire représente dans son ensemble plus de 100 000 km, soit 7 fois le diamètre de notre planète. L'oxygène ne diffusant passivement qu'à une distance maximale de quelques mm, sa mise en place au cours de l'évolution fut essentielle pour l'oxygénation des différents tissus et organes, et par conséquent pour la formation d'organismes multicellulaires complexes (Folkman, 1971).

### 1.1. Composition des vaisseaux

Les artères sont les vaisseaux les plus complexes du corps humain et sont composées de trois tuniques circulaires distinctes. La tunique la plus externe du vaisseau est l'adventice, elle correspond à un tissu conjonctif formé à la fois de cellules adipeuses, de fibres de collagène, d'élastine et de fibroblastes assurant l'ancrage du vaisseau dans le tissu.

La media est la tunique située sous l'adventice, et correspond à une limitante élastique externe en contact avec différentes couches de cellules musculaires lisses (CMLs), permettant la contraction vasculaire nécessaire à la vasomotricité (vasoconstriction et vasorelaxation du vaisseau). Le nombre de couches de CMLs est directement proportionnel au calibre des vaisseaux.

La tunique la plus interne et la plus fine du vaisseau est l'intima. Elle est constituée d'une limitante élastique interne, qui sépare la media d'une monocouche de cellules endothéliales (CEs), appelée endothélium. Directement en contact avec la lumière du vaisseau et le sang circulant, l'endothélium représente un acteur majeur dans l'homéostasie vasculaire et cette barrière régule de nombreux processus vasculaires,

- en conservant le sang à l'intérieur des vaisseaux, l'endothélium exerce une fonction de barrière mécanique.
- en favorisant la diffusion des ions, gaz et autres molécules de faible poids moléculaire, en régulant de façon sélective le passage de macromolécules et protéines de haut poids

moléculaire, de cellules immunitaires et solutés du sang vers les tissus environnants, et également en assurant l'élimination des déchets métaboliques, l'endothélium est également une barrière perméable.

- en se déformant sous l'action de différents stimuli, l'endothélium est une barrière dynamique qui régule notamment la vasomotricité en agissant sur les cellules musculaires de la média sous-jacente, et l'angiogenèse en induisant la formation de nouveaux vaisseaux à partir du vaisseau préexistant.

Les veines possèdent également les trois tuniques présentées ci-dessus, mais leurs tissus élastiques et musculaires sont moins développés, tandis que le tissu conjonctif domine.

Enfin, les capillaires sont les plus petits vaisseaux sanguins dans l'organisme et forment un réseau vasculaire complexe. Ils possèdent une structure simplifiée composée d'une monocouche endothéliale ainsi que d'une fine membrane basale.

À l'état adulte, les vaisseaux sanguins sont constitués de 1 à  $6.10^{13}$  CEs, ce qui représente un poids d'environ 1 kg (Andre et al., 1998). Chez l'adulte en condition physiologique, la grande majorité de ces cellules restent à l'état quiescent, créant une véritable homéostasie vasculaire. Il est estimé qu'à tout moment, seules 0.01 % des CEs se divisent, et leur durée de renouvellement est de 1000 jours (Andre et al., 1998). L'expansion du réseau vasculaire a principalement lieu lors de l'embryogenèse, et bien qu'elle s'étende sur l'ensemble de la vie, elle reste hétérogène temporellement.

## **1.2. Expansion du réseau vasculaire lors du développement embryonnaire**

### **1.2.1. Généralités**

Le système cardiovasculaire est le premier système qui se développe chez l'embryon. Lors du développement embryonnaire, l'émergence de l'arbre vasculaire résulte de deux processus conjoints ; la vasculogenèse, puis l'angiogenèse.

La vasculogenèse consiste en la formation de vaisseaux sanguins *de novo* à partir de cellules progénitrices endothéliales (CPEs). Elle intervient principalement lors du développement embryonnaire, durant lequel les hémangioblastes, cellules précurseurs issues du mésoderme, migrent au niveau des sites de vascularisation et se différencient en CPEs, qui sont des cellules angioblastiques (Risau & Flamme, 1995 ; Choi et al., 1998). Par la suite, les CPEs se différencient en CEs et fusionnent pour former des vaisseaux sanguins primitifs, pas encore, ou très peu fonctionnels (Risau & Flamme, 1995 ; Semenza, 2007).

L'angiogenèse, qui se définit littéralement par « genèse » (formation) et « angio » (vaisseaux), correspond à la formation de nouveaux vaisseaux sanguins à partir de vaisseaux pré-existants (Risau, 1997). Durant l'embryogenèse, l'angiogenèse permet le remodelage du réseau vasculaire primitif précédemment formé par vasculogenèse, en un réseau vasculaire mature. Sa mise en place dépend de la balance entre les facteurs pro-angiogéniques, tels que le facteur de croissance de l'endothélium vasculaire (VEGF) ou le facteur de croissance des fibroblastes (FGF), et les facteurs anti-angiogéniques, tels que la thrombospondine ou l'interleukine-12, capables d'agir directement sur les CEs. L'angiogenèse peut se dérouler via différents mécanismes : par intussusception (splitting), par septation (bridging) ou par bourgeonnement (sprouting), et conduit à la croissance et au remodelage du réseau vasculaire en un réseau plus complexe (Andre et al, 1998). Les mécanismes cellulaires et moléculaires associés à l'angiogenèse seront détaillés par la suite.

### **1.2.2. Découverte de l'angiogenèse**

Le terme d'angiogenèse a été utilisé pour la première fois par le chirurgien britannique John Hunter en 1794, pour décrire la croissance des vaisseaux sanguins dans les bois des rennes, bien que cette découverte reste débattue encore aujourd'hui (Lenzi et al., 2016).

Le professeur américain Judah Folkman a développé cette idée quelques siècles plus tard. Lors de ses recherches sur la progression tumorale, en 1970, il a émis l'hypothèse selon laquelle la néovascularisation était indispensable pour la croissance du tissu cancéreux et la dissémination métastatique (Folkman, 1971 ; Folkman, 1972 ; Brem et al., 1972). De plus, il a proposé que bloquer l'angiogenèse pourrait devenir une stratégie complémentaire afin d'inhiber la progression tumorale. Il a mis en place par la suite les premiers modèles d'angiogenèse *in vitro* sur CEs, et ses travaux permettront de découvrir il y a 40 ans les premiers facteurs pro-angiogéniques endogènes, tel que le FGF (Gospodarowicz, 1976), ou le VEGF, initialement nommé facteur de perméabilité vasculaire (Senger et al., 1983 ; Ferrara and Henzel, 1989). Ces découvertes furent rapidement suivies par l'identification de leur récepteur, tel que le récepteur au VEGF (VEGFR) (Millauer et al., 1993).

### **1.3. Expansion du réseau vasculaire à l'état adulte**

Principalement décrite comme se déroulant pendant l'embryogenèse, la vasculogenèse peut également se dérouler à l'état adulte, où les CPEs, suite à leur différenciation en CEs, peuvent promouvoir la formation de vaisseaux sanguins, notamment au niveau de sites ischémiques

(Asahara et al., 1997).

L'angiogenèse intervient également à l'état adulte, dans quelques processus physiologiques, tels que les cycles menstruels de l'appareil reproducteur féminin, la grossesse ou encore lors des phénomènes de réparation physiologique, tel que la cicatrisation suite à une blessure. Beaucoup plus restreinte que lors de l'embryogenèse, elle dépend à la fois du contexte physiologique et du lit vasculaire (Hillen & Griffioen, 2007 ; Potente & Carmeliet, 2017).

Pour la suite de cette introduction, nous nous concentrerons principalement sur l'angiogenèse.

#### **1.4. Pathologies liées au réseau vasculaire**

Le processus d'angiogenèse est impliqué dans différentes situations pathologiques, qu'il soit en excès ou en défaut. L'angiogenèse pathologique fut initialement décrite comme seulement restreinte à quelques maladies telles que le cancer, la polyarthrite rhumatoïde et le psoriasis, mais dans les dernières décennies, il fut découvert que l'angiogenèse anormale, excessive ou insuffisante participait à la genèse de bien plus nombreuses pathologies (Carmeliet, 2003 ; Lavaud et al, 2012).

En particulier, l'excès d'angiogenèse joue un rôle indispensable dans la croissance tumorale, son invasivité et le développement des métastases. L'oxygène ne diffusant qu'à une distance maximale de 2 mm (Folkman, 1971), la formation de vaisseaux sanguins intra-tumoraux est indispensable pour l'oxygénation des cellules tumorales (CTs), mais également l'apport de nutriments, et l'élimination des déchets cellulaires tumoraux. De ce fait, l'hypoxie représente un stimulus majeur de l'angiogenèse tumorale et conduit au développement rapide de capillaires intra-tumoraux.

La mise en place des premières études cliniques anti-angiogéniques se déroula au début des années 1990, avec pour principaux objectifs de développer des marqueurs pronostics chez les patients atteints de cancer, ainsi que de développer les premières thérapies anti-angiogéniques, avec pour but d'« asphyxier » la tumeur (Folkman, 1995 ; Couffinhal et al., 2001). Ces travaux ont permis, en 2004, l'approbation du bevacizumab (avastin®), anticorps monoclonal dirigé contre le VEGF, comme premier inhibiteur de l'angiogenèse par l'agence américaine des produits alimentaires et médicamenteux (FDA) (Ferrara et al., 2004). De nos jours, et malgré les progrès évidents réalisés grâce aux traitements anti-tumoraux et anti-angiogéniques actuels, ceux-ci restent peu efficaces contre certaines tumeurs, et presque 9 millions de personnes dans le monde décèdent d'un cancer chaque année, ce qui représente presque un décès sur 6 à l'échelle mondiale en 2015, selon l'organisation mondiale de la santé (OMS).

## **2. Glycosylation as new pharmacological strategies for diseases associated with excessive angiogenesis**

Afin de trouver des traitements plus adaptés dans le cadre des pathologies associées une angiogenèse excessive et notamment la progression tumorale, une meilleure compréhension des mécanismes sous-jacents est essentielle. Par conséquent, la partie suivante comprend une revue intitulée « Glycosylation as new pharmacological strategies for diseases associated with excessive angiogenesis » (Bousseau et al., 2018) et publiée dans *Pharmacology & Therapeutics*. Elle présente dans un premier temps les différents types d'angiogenèse ainsi que la lymphangiogenèse, et les mécanismes moléculaires et cellulaires impliqués. Par la suite, elle décrit plus précisément les différentes pathologies associées à une perturbation de l'angiogenèse, en se concentrant sur la croissance tumorale. Les thérapies anti-angiogéniques actuelles, ainsi que leurs limites sont rappelées, et le nouveau paradigme anti-angiogénique qui vise à inhiber les modifications métaboliques mises en place pas les CEs pour initier l'angiogenèse est spécifiquement abordé. Le métabolisme énergétique (activité mitochondriale, glycolyse et voie des acides gras) est notamment décrit. La dernière partie de ce manuscrit se concentre sur la glycosylation, qui est une modification post-traductionnelle régulant le métabolisme, dont l'implication est peu décrite dans l'angiogenèse, et pourtant fortement pressentie.

Contribution personnelle :

- 1. Introduction
- 2.1. Different subtypes of vessel formation
- 2.4.3. Tumor progression
- 3.1.1. Mitochondria
- 3.1.2. Glycolysis
- 3.2. Glycosylation
- 4. Conclusion





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## Glycosylation as new pharmacological strategies for diseases associated with excessive angiogenesis

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## ABSTRACT

Angiogenesis is a complex process describing the growth of new blood vessels from existing vasculature, and is triggered by local pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), which increase the metabolism of endothelial cells (ECs). Angiogenesis takes part in various physiological conditions such as embryogenesis, placental growth, and in pathological conditions such as tumor growth, diabetic retinopathy, rheumatoid arthritis (RA) and ischemic diseases. Current therapies against excessive angiogenesis target vascular growth signaling. However, tumors often counteract these therapies through adaptive mechanisms, thus novel alternative anti-angiogenic strategies are needed. Targeting metabolism is a new anti-angiogenic paradigm, especially through the inhibition of energy metabolism and glycosylation, with the perspective of maintaining the delicate balance between the beneficial and deleterious effects of excessive angiogenesis in patients. Recent studies described a role for EC glycolysis and its main regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) in the regulation of angiogenesis, but only few studies are related to the role of the hexosamine biosynthesis pathway during angiogenesis. Glycosylation allows the formation of glycoproteins, glycolipids and proteoglycans and impacts many pathways. The addition of glycans to N-linked proteins is catalyzed by the enzymatic activity of *N*-acetylglucosaminyltransferases (GnTs), which regulates the glycosylation status of key angiogenic factors such as VEGF receptor 2 (VEGFR2) and Notch. In addition, glycan-galectin (Gal) interactions regulate vascular signaling programs and may contribute to tumor adaptations to anti-angiogenic strategies. Herein, we review novel pharmacological strategies targeting glycosylation, which could be used to decrease excessive angiogenesis in pathological conditions.

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**Abbreviations:** 2DG, 2-deoxy-D-glucose; 3PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (PFKFB3 inhibitor); ANG, angiotensin; ATP, adenosine triphosphate; CCL, chemokine ligand; CCR, chemokine receptor; cGMP, cyclic guanosine monophosphate; CoA, coenzyme A; COUP-TFII, orphan nuclear receptor encoded by the nuclear receptor subfamily 2 group F member 2; CPT1, carnitine palmitoyltransferase 1; CRD, carbohydrate-recognition domain; DLL, ligand Delta-like; EC(s), endothelial cell(s); ECM, extracellular matrix; eNOS, endothelial NO synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinases; F6P, fructose-6-phosphate; FA, fatty acid; FABP, fatty acid-binding proteins; FAK, focal adhesion kinase; FAO, fatty acid oxidation; FASN, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport proteins; FGF, fibroblast growth factor; FLT, fms-related tyrosine kinase; FOXO2, forkhead box protein C2; G6P, glucose-6-phosphate; GAG, glycosaminoglycan; Gal, galectin; GlcNAc, acetylglucosamine; GLUT(s), glucose transporter(s); GnTs, *N*-acetylglucosaminyltransferases; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HBP, hexosamine biosynthesis pathway; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; IL, interleukin; LECs, lymphatic endothelial cells; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; MMP, matrix metalloproteinase; MSCs, mesenchymal stem cells; NO, nitric oxide; NRP, neuropilin; O<sub>2</sub>, superoxide anion; ONOO<sup>-</sup>, peroxynitrite; OXPHOS, oxidative phosphorylation; PDGF, platelet-derived growth factor; PFK15, 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFKFB3 inhibitor); PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3K, phosphatidylinositol 3-kinase; Prox1, transcription factors like prospero homeobox 1; RA, rheumatoid arthritis; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; RTKI, receptor tyrosine kinase inhibitor; RWPC, red wine polyphenolic compounds; SMC, smooth muscle cells; Sox18, SOX family of transcription factors; TAM, tumor-associated macrophages; TCA, tricarboxylic acid; TGF, transforming growth factor; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; α-TEA, α-tocopheryloxyacetic acid; α-TOS, α-tocopheryl-succinate.

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## Contents

1. Introduction . . . . .	0
2. Angiogenesis. . . . .	0
3. Targeting metabolism: The new anti-angiogenic paradigm . . . . .	0
4. Conclusion. . . . .	0
Conflict of interest statement . . . . .	0
Acknowledgments. . . . .	0
References . . . . .	0

## 1. Introduction

The vascular system is one of the largest organ in the human body and the first becoming functional during human development. It allows nutrient and oxygen supply, metabolic waste removal and surveillance by immune cells to maintain tissue homeostasis (Udan, Culver, & Dickinson, 2013). The vascular tree forms a highly structured network that perfuse all organs in vertebrates, and its expansion is controlled through a complex process called angiogenesis. This term was introduced by John Hunter to describe the process of new blood vessel growth (Hunter, 1861; Lenzi, Bocci, & Natale, 2016). The notion of angiogenesis has now been refined and represents the formation of new blood vessels from a preexisting vascular network. In adults, angiogenesis only occurs during menstruation, pregnancy and wound healing. It differs from vasculogenesis that occurs during embryogenesis, in which blood vessels are formed *de novo* from mesodermal endothelial angioblasts to create the primary vascular plexus. Blood vessels are lined by a monolayer of endothelial cells (ECs) to assist the blood transport. Small capillaries only contain ECs, while larger vessels are also surrounded by mural cells (smooth muscle cells (SMCs) and pericytes). ECs are characterized by a phenotypic plasticity allowing them to differentiate into either arterial or venous ECs (Carmeliet, 2003). Although mostly remaining quiescent or phalanx during adulthood, ECs can quickly proliferate and form new blood vessels in response to physiological or pathological stimuli (Eelen, de Zeeuw, Simons, & Carmeliet, 2015; Martin, Favot, Matz, Lugnier, & Andriantsitohaina, 2003).

Whereas angiogenesis has been extensively studied, lymphangiogenesis has been subjected to concerted investigation only during the last 20 years. The lymphatic vascular system is a network of lymphatic capillaries, collecting vessels, the thoracic duct and interspersed lymph nodes (Jussila & Alitalo, 2002). Lymphatic capillaries are composed of a single layer of overlapping lymphatic endothelial cells (LECs), which are connected by “button-like” intercellular junctions that facilitate the entry of interstitial fluid macromolecules and cells. Lymph originates in the lymphatic capillaries by draining the interstitial spaces. It flows through the collecting vessels and then enters the subclavian vein to re-join the blood circulation (Schmid-Schönbein, 1990). The lymphatic system plays a critical role for tissue fluid homeostasis as well as in metabolism and immunity.

Both angiogenesis and lymphangiogenesis mechanisms are known to be deleterious in numerous disorders such as diabetic retinopathy or cancer growth, and targeting them has emerged as a novel emerging therapeutic approach. Although traditional anti-angiogenic treatments have provided initial enthusiasm by decreasing pathological vascularization, tumors often counteracted these therapies by several adaptive mechanisms, highlighting the need for novel strategies. Consequently, emerging antiangiogenic treatments are taking into account the metabolic aspects of ECs. Some are focusing on endothelial metabolism by targeting the adenosine triphosphate (ATP) synthesis by glycolysis, oxidative phosphorylation (OXPHOS) and fatty acid (FA) oxidation (FAO). Other strategies are targeting protein modifications like glycosylation, defining new anti-angiogenic paradigms (Potente & Carmeliet, 2017).

This review provides an overview of the pathways and associated molecules implicated in angiogenesis and lymphangiogenesis. After a

preliminary outline of the current treatments and their limits, we discuss the new angiogenic perspectives, including energy production by mitochondria, glycolysis and FA metabolism. We then discuss the implication of glycosylation in the formation of vessels, and the novel pharmacological approaches targeting this process to modulate angiogenesis.

## 2. Angiogenesis

### 2.1. Different subtypes of vessel formation

Angiogenesis can occur *via* several distinct ways, depending on the vascular bed and the pathophysiological context (Potente & Carmeliet, 2017). The different subtypes of angiogenesis are summarized in Fig. 1.A.

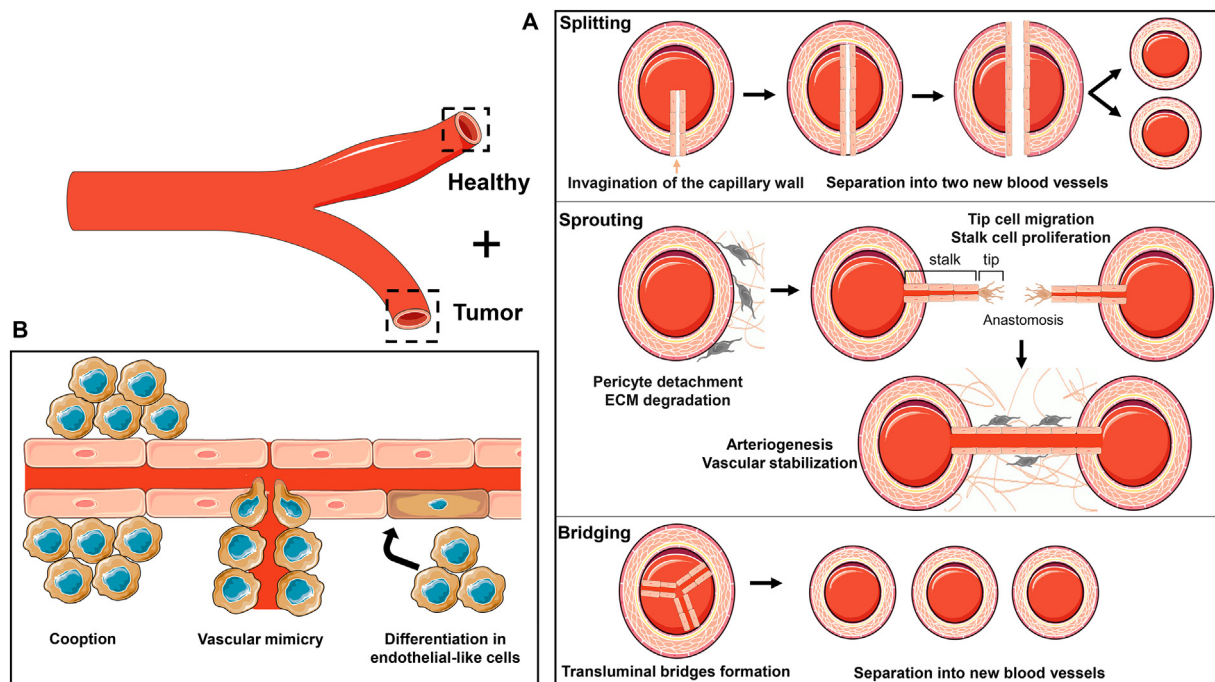
#### 2.1.1. Splitting

Angiogenesis by splitting or intussusception is the enlargement and separation of a pre-existing blood vessel into two new blood vessels. This mechanism was first observed on a post-natal remodeling of capillaries in rat lungs (Caduff, Fischer, & Burri, 1986). It is a fast process that does not require EC activation, neither proliferation nor basal membrane degradation (Djonov, Baum, & Burri, 2003). Intussusception involves the formation of tissue pillars by invagination of the capillary wall into the vessel lumen in order to create new blood vessels. Because this type of angiogenesis is a low-energy-consuming process, its implication is essential during embryonic development, and can also be observed in tumor vascularization. Intussusception is described to be complementary to the sprouting process and both mechanisms occur during the vascularization of various organs, such as the lungs, the heart or the eyes, and during tissue repair (Burri & Tarek, 1990; Djonov, Schmid, Tschanz, & Burri, 2000; Hillen & Griffioen, 2007; van Groningen, Wenink, & Testers, 1991).

#### 2.1.2. Bridging

Mother vessels are the first new angiogenic vessel type that appear, both in tumors and in response to adenoviral vector expressing vascular endothelial growth factor (VEGF-A-164). Mother vessels derive from preexisting normal venules and capillaries by a 3-step process that involves proteolytic degradation of the basal membrane, pericyte detachment, and a 4- to 5-fold vessel enlargement (Dvorak, 2015). Bridging consists in the formation of endothelial cytoplasmic exvagination into and across mother vessel lumens, creating transluminal bridges, eventually fusing with the opposite cytoplasm, designing multiple smaller-sized channels. Subsequently, these smaller channels separate from each other to form individual, capillary-sized vessels that are normal in structure and are not hyper permeable (Egginton, Zhou, Brown, & Hudlická, 2001; Nagy et al., 1995; Pettersson et al., 2000; Ren, Michael, Entman, & Frangogiannis, 2002).

Initially defined as a peculiar form of vascular remodeling, intraluminal bridging was first observed in tumor vessels (Nagy et al., 1995). Bridging has also been reported (i) in angiogenesis of normoxic tissues (such as cardiac, leg muscles and ear skin) (Pettersson et al., 2000), (ii) in the formation of transluminal endothelial projections



**Fig. 1. Different subtypes of angiogenesis.** (A) Healthy angiogenesis occurs in different ways. Splitting angiogenesis is defined by the enlargement of the mother vessel, followed by the capillary wall invagination, leading to the separation into two daughter vessels. Sprouting is the most described angiogenic mechanism. After endothelial cell (EC) activation, it requires degradation of the extracellular matrix (ECM) and the detachment of pericytes and cell-cell adhesions to initiate the sprout. ECs migrate at the tip position, followed by the proliferation of ECs. When two tip cells encounter each other, both fuse and the new vessel is remodelled, and then, vascular quiescence is re-established. Bridging is characterized by the formation of transmural bridges, allowing the formation of multiple channels. The separation of the different channels conducts to the formation of new smaller-sized vessels. (B) Tumor angiogenesis also occurs by several additional manners. Cooption represents the migration of tumor cells along the pre-existing vessels of the host organ. Tumor cells also use vascular mimicry to create a tumor channel without the presence of ECs. Finally, tumor cells can also differentiate into “endothelial-like” cells.

after chronic vascular dilatation (Egginton et al., 2001) and (iii) during wound healing following myocardial infarction (MI) (Ren et al., 2002).

An important characteristic of bridging angiogenesis is the lack of EC proliferation (Egginton et al., 2001). The origin of ECs involved in bridging, whether from locally-activated endothelium or from circulating bone marrow precursors, is still a matter of debate (Dvorak, 2003).

### 2.1.3. Sprouting

Sprouting is the most described and prevalent form of angiogenesis. It allows vascular expansion in development, regeneration and tissue repair. It is a common mechanism observed during embryonic development, and can also occur occasionally in adulthood, during wound healing, pregnancy and the menstrual cycle. Sprouting is widely described as essential in some diseases with excess vascularization such as tumor growth, diabetic retinopathy and RA (Favot, Martin, Keravis, Andriantsitohaina, & Lugnier, 2003; Lavaud et al., 2012; Xu et al., 2008). Sprouting requires the activation of ECs, followed by their coordinated migration and proliferation to form vascular sprouts. The final stage of blood vessels formation includes arterio-genesis and vascular stabilization for lumenization and vessel perfusion.

Sprouting angiogenesis is regulated by a balance between the pro-angiogenic factors such as VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and matrix metalloproteinases (MMPs), and anti-angiogenic ones, like angiostatin, thrombospondin and tissue inhibitor of MMPs. Different stimuli such as hypoxia or inflammation activate the angiogenic switch due to the release of pro-angiogenic factors, in which ECs quickly swap from a quiescent metabolism to an intense active state triggering neovascularization. ECs differentiate into migratory tip cells or proliferating stalk cells. Tip cells are located at the front of the sprout and guide it towards the angiogenic signal thanks to the extension of cellular membranes to form cellular protrusions. Then, stalk cells allow the elongation of the sprout thanks to their potent proliferative ability (Potente, Gerhardt, & Carmeliet, 2011; Stapor, Wang, Goveia, Moens, & Carmeliet, 2014).

EC specifications in tip or stalk cells are tightly regulated, and mainly driven by VEGF and Notch pathways. VEGF fixation on VEGF receptor 2 (VEGFR2) upregulates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a master regulator of glycolysis. PFKFB3 activation impedes ATP production by glycolysis, allowing the formation of cellular protrusions for migration. ECs differentiate in tip migratory cells and extend lamellipodia and filopodia to afford sprout migration. Meanwhile, VEGFR2 activation stimulates Notch ligand Delta-like 4 (DLL4) that activates the Notch intracellular domain of adjacent cells. In turn, the Notch intracellular domain inhibits VEGFR2 expression and replaces it by the decoy receptor VEGFR1, predestining these ECs to become proliferative (Eelen et al., 2015; Stapor et al., 2014). Thus, tip and stalk cells rapidly drive the sprout in a coordinated and directional manner. The tip/stalk specification is dynamically regulated by modulating the VEGFR2/VEGFR1 ratio, which controls the determinism of the cell at the top of the sprout. When two tip cells encounter each other, both vessels fuse by a process called anastomosis. VE-cadherin junctions are created between ECs, the new vessel becomes lumenized and mural cells are recruited to cover and tighten the nascent vessel. Pericytes are crucial to stabilize the new vessel and support the blood flow. Finally, extracellular matrix (ECM) is secreted by ECs and surrounding stromal cells, and the vessel becomes perfused. PDGF also contributes to vessel stabilization by recruiting PDGFR- $\beta$ -positive mesenchymal progenitors (Carmeliet, 2003). Then, ECs re-adopt a quiescent phenotype. Shear stress triggers PFKFB3 inhibition by the transcription factor kruppel-like factor 2 to decrease glycolytic flow, and VEGFR1 expression stays significant to keep a low VEGF-A response that preserves a regular quiescent metabolism (Eelen et al., 2015).

### 2.2. Molecular mechanisms of angiogenesis

Under both physiological and pathological conditions, different cell types including ECs, endothelial progenitors and stem cells from different origin produce mediators able to orchestrate angiogenesis. The



traditional and best-known promoters of vessel formation are the VEGF family members (Tunyogi-Csapo et al., 2007), the hypoxia-inducible factor (HIF-1) (Rey & Semenza, 2010) and the nitric oxide (NO) (Lau & Ma, 1996). Other angiogenic players belonging to the family of FGF (Jacobs, 2007), hepatocyte growth factor (HGF) (Tong et al., 2006), and angiopoietins (ANG) (Asahara et al., 1998) also participate to this process. Furthermore, non-traditional mediators such as microRNAs (miRNA), short noncoding RNAs, and extracellular vesicles can also regulate angiogenesis (Martinez & Andriantsitohaina, 2011).

Among the mammalian VEGF family members are the VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, the placenta growth factor, VEGF-A being the key growth factor for angiogenesis. These ligands bind to partially to three different tyrosine kinase receptors: VEGFR1 also known as FLT1, the fms-related tyrosine kinase-1 (de Vries et al., 1992; Shibuya et al., 1990), VEGFR2, also known as Flk1, fetal liver kinase 1 in mice (Matthews, Jordan, Wiegand, Pardoll, & Lemischka, 1991; Quinn, Peters, De Vries, Ferrara, & Williams, 1993) or as kinase insert domain receptor in humans (Terman et al., 1991; Terman, Jani-Sait, Carrion, & Shows, 1992), and VEGFR3 or FLT4, the fms-related tyrosine kinase-4 (Galland et al., 1993; Pajusola et al., 1992).

VEGF-A binds to VEGFR1 and VEGFR2 and the co-receptor of the neuropilin (NRP) family NRP1, while VEGF-B and PlGF specifically bind to VEGFR1 and NRP1. Finally, VEGF-C and VEGF-D bind to VEGFR3 and to the co-receptor NRP2, and in a proteolytically processed form also VEGFR2 (Koch, Tugues, Li, Gualandi, & Claesson-Welsh, 2011). NRPs do not have internal catalytic activity (Pellet-Many, Frankel, Jia, & Zachary, 2008), but instead modulate VEGFR internalization, intracellular trafficking and downstream signaling (Prahst et al., 2008).

VEGF-A exists as multiple isoforms named according to the total number of amino acids in the mature form of the protein: nine polypeptides of 111, 121, 145, 148, 162, 165, 183, 189 and/or 206 amino acids can be generated. Most VEGF-A producing cells appear to preferentially express VEGF-A121, VEGF-A165 and VEGF-A189 (Arcondéguy, Lacazette, Millevoi, Prats, & Touriol, 2013).

Although VEGF-A binds VEGFR1 with a higher affinity than VEGFR2 (Kd = 10–20 pM for VEGFR1 vs 75–125 pM for VEGFR2) (de Vries et al., 1992; Terman et al., 1992), its tyrosine kinase activity is weaker. Indeed, it mainly acts as decoy receptor for VEGF-A. In this way, it limits VEGF-A availability for VEGFR2, and thereby restricts activation and signaling of the VEGF-A/VEGFR2 axis (Hiratsuka et al., 2005; Shibuya, 2006). Another function of VEGFR1 is to regulate monocyte and macrophage migration (Barleon et al., 1996).

VEGFR1 can undergo alternative splicing to form a truncated soluble version (sVEGFR1 or sFLT-1). sVEGFR1 inactivates VEGFR2 signaling by binding and sequestering VEGF-A (Kendall, Wang, & Thomas, 1996). Moreover, sVEGFR1, when expressed by ECs adjacent to nascent vessel sprout, can shape the tissue VEGF-A gradient, facilitating a straight out-growth of the vessel sprout, and limiting the formation of new vessel sprouts in the direct vicinity (Chappell, Taylor, Ferrara, & Bautch, 2009).

VEGFR2 is critical for multiple aspects of normal and pathological EC biology including hematopoiesis, vasculogenesis, angiogenesis, and vascular permeability. As VEGF-A, VEGFR2 is essential for embryonic development of the vasculature. Indeed, a genetic deletion of these genes results in embryonic lethality (Shibuya, 2015). A soluble truncated form of VEGFR2 (sVEGFR2) has been detected in mouse and human plasma (Ebos et al., 2004). The role of this alternatively spliced VEGFR2 variant and its *in vivo* relevance has however remained elusive. It has been suggested that not all aspects of VEGFR2 activity require its kinase domain and the activation of downstream signaling (Álvarez-Aznar, Muhl, & Gaengel, 2017). In contrast, VEGFR3 is essential for lymphatic development and function (Karkkainen, Jussila, Ferrell, Finegold, & Alitalo, 2001; Mäkinen et al., 2001).

Many growth factors and cytokines, such as PDGF-B, transforming growth factor- $\beta$  (TGF- $\beta$ ), insulin-like growth factor-1, FGF, HGF, tumor necrosis factor- $\alpha$  and interleukin (IL)-1 (Ferrara, 2004), as well as mechanical forces of shear stress and cell stretch (Li, Hampton,

Morgan, & Simons, 1997; Milkiewicz, Brown, Egginton, & Hudlicka, 2001) up-regulate VEGF-A expression. Similarly, hypoxia enhances VEGF-A expression via up-regulation of HIF-1, as observed in acute human skeletal muscle ischemia (Tuomisto et al., 2004).

VEGF-A stimulates concomitant endothelial production of NO and prostacyclin (Kroll & Waltenberger, 1999; Wheeler-Jones et al., 1997), inducing angiogenesis and vascular permeability. VEGF-A via the activation of VEGFR2 upregulates expression of inducible and constitutive endothelial NO synthase (eNOS) isoforms, leading to short-term NO production (Hood, Meininger, Ziche, & Granger, 1998). Moreover, VEGF-A stimulates EC survival via (i) the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, (ii) the association with the  $\alpha_v\beta_3$  integrin and (iii) the activation of focal adhesion kinase (FAK) (Zachary, 2003). VEGF-A possesses the ability to activate different cascades involved in the proliferation and the migration of ECs, through the activation of the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), p38 and c-jun N-terminal kinase, and Rho-GTPase family members (Zachary, 2003). Because of its major role in vascular biology and because of its high angiogenic potency, VEGF-A has been the prime candidate target for *in vivo* therapeutic applications (Ylä-Herttuala & Alitalo, 2003).

Another key inductor of angiogenesis is hypoxia (Silvestre, Smadja, & Lévy, 2013), which promotes the accumulation of HIF, a ubiquitous transcription factor regulating low-oxygen environment. HIF is heterodimeric and composed of two subunits,  $\alpha$  and  $\beta$ , but can be found under three different isoforms, HIF-1, HIF-2 and HIF-3. HIF-1 $\alpha$  is the active subunit and is highly regulated by oxygen, while HIF-1 $\beta$  activity is unaffected by hypoxia (Salceda & Caro, 1997). HIF-1 $\alpha$  is involved in the re-establishment of blood vessels in hypoxic areas, as in localized ischemia and tumors (Wang, Jiang, Rue, & Semenza, 1995; Ziegler, Jovin, & Huang, 2007). Moreover, it regulates the expression of genes implicated in vascular reactivity, angiogenesis, arteriogenesis, and into the mobilization and homing of bone marrow-derived angiogenic cells (Rey & Semenza, 2010).

HIF can directly activate the expression of a number of pro-angiogenic factors, including VEGF and its receptors, plasminogen activator inhibitor-1, angiopoietin (ANG)-1 and -2, PDGF-B, the TIE-2 receptor, and MMP-2 and -9 (Hickey & Simon, 2006), which are secreted by hypoxic cells and stimulate ECs, leading to angiogenesis. In addition, HIF-1 activates compensatory and adaptive mechanisms for delivery of oxygen and nutrients to the healing site allowing restoration of tissue function. In fact, HIF-1 activates genes encoding for erythropoietin, glucose transporters and glycolytic enzymes, cell survival factors, cell surface receptors, ECM proteins and transcription of different genes implicated in angiogenesis (Semenza, 2000; Shweiki, Itin, Soffer, & Keshet, 1992). Consequently, HIF-1 plays a crucial role in the healing mechanisms through the activation of genes implicated in the modulation of oxygen and nutrients delivery to the tissue at the site of injury.

NO plays a role for angiogenesis during wound healing and tumor development (Fukumura, Kashiwagi, & Jain, 2006; Isenberg, Martin-Manso, Maxhimer, & Roberts, 2009). In ECs, VEGF signaling through VEGFR2 activates the PI3K/Akt pathway, and then phosphorylates eNOS at Ser1177 (Dimmeler et al., 1999; Fulton et al., 1999), activating eNOS and decreasing its Ca<sup>2+</sup> dependence. The kinase Src, stimulated by VEGF, also activates eNOS (Duval, Le Boeuf, Huot, & Gratton, 2007; Fulton et al., 2008). Simultaneously, VEGFR2 signaling through phospholipase-C $\gamma$  mobilizes intracellular Ca<sup>2+</sup>, which further activates eNOS in a calmodulin (CaM)-dependent manner, and increases AMP-activated protein kinase-mediated eNOS phosphorylation at Ser1177 (Reihill, Ewart, Hardie, & Salt, 2007). NO stimulates cyclic guanosine monophosphate (cGMP) synthesis via the guanylate cyclase pathway and thereby activates cGMP-dependent protein kinase and cGMP-gated channels to regulate downstream targets that increase EC proliferation, migration, survival, and permeability (Ignarro, 2002). Additional parallel signaling through Src, Akt, and the protein kinase C-MAPK pathway synergizes with NO/cGMP signaling to support each of these EC responses.

The vascular responses to NO occur at different time scales. Hemostasis and vessel tone are regulated acutely, whereas angiogenesis requires sustained elevation of NO levels (Isenberg et al., 2009). Moreover, NO exerts different properties depending on its level. At low concentrations, NO promotes vascular cell survival, proliferation and migration. At high concentrations, NO directly or following its conversion in other reactive nitrogen species, triggers additional signaling pathways, and are deleterious for ECs (Thomas et al., 2008).

The members of the FGF family are other potent inducers of angiogenesis. FGF-2 regulates EC expression of VEGF, which is an autocrine mediator of FGF-2-induced angiogenesis (Seghezzi et al., 1998). Receptor activation induces cell differentiation and promotes proliferation and matrix dissolution, thus initiating a process of mitogenic activity particularly for ECs, fibroblasts, and SMCs (Kanda, Landgren, Jungström, & Claesson-Welsh, 1996).

HGF is a strong stimulator of new vessel formation and an important angiogenic factor in vascular retinopathies (Boulton, 1999; Colombo, Menicucci, McGuire, & Das, 2007; Lashkari et al., 2000). Activation of HGF signaling leads to increased cell growth, cell motility, angiogenesis, wound healing, and tissue regeneration, in part because of the activation of the VEGF pathway by the endothelium (Reisinger, Kaufmann, & Gille, 2003; Zhang, Su, Volpert, & Vande Woude, 2003), although HGF can induce angiogenesis independently of the VEGF pathway (Sengupta et al., 2003). Another effect of HGF is the negative regulation of thrombospondin-1 (Zhang et al., 2003), which promotes anti-angiogenic signaling.

ANG-1 and ANG-2 and their tyrosine kinase receptors TIE-1 and -2, control sprouting angiogenesis, vascular remodeling and the transition from the quiescent to the activated EC phenotype (Saharinen, Bry, & Alitalo, 2010; Thurston, 2003). ANG-2 is abundantly expressed by angiogenic ECs (Stratmann, Risau, & Plate, 1998) and is found in tissues that undergo transient or periodic growth and vascularization, followed by regression, such as in ovary, uterus, and placenta (Maisonpierre et al., 1997).

### 2.3. Lymphangiogenesis

#### 2.3.1. Formation of lymphatic structure

In adults, LECs are normally quiescent, but the lymphatic developmental program can be reactivated during wound healing and in various pathological contexts, including cancer.

The primary effectors altering transcription factor profiles and the fate of LECs during embryogenesis are still unknown. Transcription factors like *prospero* homeobox 1 (Prox1), *COUP-TfII* and *Sox18* play essential roles in the cells committed to lymphatic endothelial fate (Francois, Harvey, & Hogan, 2011; Petrova et al., 2004).

Sox18, a member of the SOX transcription factor family, activates the expression of Prox1, the most important regulator of lymphatic cell differentiation. Indeed, deficiency of Prox1 blocks the differentiation and budding of lymphatic ECs and leads to absence of lymphatic vessels and edema (François et al., 2008). Furthermore, *COUP-TfII*, an orphan nuclear receptor encoded by the nuclear receptor subfamily 2 group F member 2, has also been shown to interact with Prox1 to maintain LEC phenotype (Srinivasan et al., 2010). Indeed, during LEC differentiation, Prox1 interacts with *COUP-TfII* to upregulate the expression of further lymphangiogenic signaling molecules such as VEGFR3, integrin  $\alpha 9$  and FGFR-3 (Aranguren et al., 2013).

Three potential LEC sources have been reported (Duong, Koopman, & Francois, 2012). The first one is a pre-existing lymphatic vessel in which LECs proliferate and migrate leading to neolymphatic growth (Hägerling et al., 2013). A second mechanism is the transdifferentiation of ECs from preexisting blood vessels into LECs. In this process, VEGF-C plays an essential role similar to the ones of VEGFR3 and Prox1 (Wilting, Papoutsis, Buttler, & Becker, 2007). Upregulation of various lymphatic specific genes and downregulation of blood vascular specific genes support the concept that Prox1 could reprogram ECs into LECs (Hong et al.,

2002; Johnson et al., 2008; Kim et al., 2010). Not only transcription factors decide of lymphatic fate, but also Notch1 expression tightly regulates the differentiation of ECs into LECs. Inhibition of Notch signaling, both *in vivo* and *in vitro*, promotes lymphangiogenesis (Zheng et al., 2011). The loss of Notch activity leads to excessive Prox1 and *COUP-TfII* expressions that attenuate VEGF signaling by repressing VEGFR2 and NRP1 (Kang et al., 2010). A third theory proposes that bone marrow-derived cells may also contribute to lymphangiogenesis. Indeed, two populations of stem and progenitor cells have been identified in human blood that either co-express VEGFR3, CD133, and CD34 or VEGFR3, CD31, and CD14. Both cell types are able to differentiate into LECs *in vitro*, indicating that lymphatic endothelial precursors might also contribute to lymphangiogenesis in humans (Salven, Mustjoki, Alitalo, Alitalo, & Rafii, 2003). Recently, it has been shown that lymphatic vessels could also be formed from transdifferentiation of mesenchymal stem cells (MSCs) or tumor-associated macrophages (TAMs) (Zhan et al., 2015).

LEC differentiation is followed by the formation of lymph sac, which needs to be separated from blood vessels. An essential factor involved in this process is the sialoglycoprotein podoplanin, whose transcription and expression at the LEC surface is induced by Prox1 (Pan, Wang, & Yago, 2014). The functions of the sialoglycoprotein podoplanin on LECs include the binding to CLEC-2 receptors expressed on platelets in the circulating blood. CLEC-2-podoplanin interaction leads to formation of platelet aggregates at points of contact between blood and lymphatic vasculature (Cueni et al., 2010). This mechanism is important for the separation of blood and lymphatic vessels and for the stability of high endothelial venule integrity within lymph nodes (Hess et al., 2014).

#### 2.3.2. Expansion of the lymphatic vascular network

The sprouting of LECs from the preexisting structures depends on VEGFR2 and VEGFR3 signaling driven either by VEGF-C or VEGF-D. The mechanisms involve the activation of protein kinase C, ERK 1 or 2 signaling cascade and the phosphorylation of Akt (Mäkinen et al., 2001; Shibuya, 2013). Sprouty-related EVH1-domain-containing protein SPRED-1 and SPRED-2 are negative regulators of VEGFR3 signaling, inasmuch they suppress VEGFR3-mediated ERK activation (Taniguchi et al., 2007). NRP2 acts as a co-receptor for VEGFR3 and may regulate the affinity of VEGF-C and VEGF-D for their receptors (Harris et al., 2011; Karpanen et al., 2006).

Collagen and  $\text{Ca}^{2+}$ -binding EGF domain-containing protein 1 are required for lymphatic development via VEGF-C pathway (Hogan et al., 2009). DLL4 and Notch1-mediated signaling are also essential for lymphatic vessel growth by regulating EphrinB2 expression and FGFR-3 signaling, respectively (Niessen et al., 2011).

#### 2.3.3. Maturation of lymphatic network

After the establishment of the initial lymphatic vasculature, the vessels undergo maturation to form capillaries, collecting vessels and valves. Several proteins have been identified to play a role in this process. For example, ANG-2/TIE-2 receptor tyrosine kinase signaling is implicated into lymphatic vessel remodeling (Augustin, Koh, Thurston, & Alitalo, 2009). Other factors have been described to participate in the regulation of SMC recruitment in collecting lymphatic vessels, including forkhead box protein C2 (FOXC2), EphrinB2, and Akt (Petrova et al., 2004). FOXC2 interacts with the nuclear factor of activated T cells cytoplasmic 1, and this coordination is required for basement membrane deposition, SMCs coverage and valves formation (Norrmen et al., 2009). Further studies suggest that FOXC2 maintains lymphatic integrity, through the coordination of cell-cell junction maturation and shear stress responses (Sabine et al., 2015). GATA2, a zinc finger transcription factor, is also implicated in lymphatic valve development (Kazenwadel et al., 2012).

### 2.4. Pathologic angiogenesis

Abnormal angiogenesis is the cause of a number of diseases. Typical diseases known to be associated with failed angiogenesis include MI,

peripheral ischemia, cerebral ischemia, ulcer and some neurologic disorders (Carmeliet, 2003; Chung & Ferrara, 2011). Diseases with excessive angiogenesis include cancers, psoriasis, arthritis, neuropathies, endometriosis and atherosclerosis. Lymphangiogenesis imbalance has an important role in the pathogenesis of several diseases, such as cancer, lymphoedema and various inflammatory conditions (Alitalo, Tammela, & Petrova, 2005; Baluk et al., 2007).

#### 2.4.1. Diseases associated with failed angiogenesis

Failed angiogenesis is defined by a lack or reduction of vessel formation. Although less described than excessive angiogenesis, insufficient angiogenesis is implicated in diseases like MI, neurodegeneration, hypertension, peripheral and cerebral ischemia, and wound healing (Carmeliet, 2003). There are many reviews dealing with this topic (Browne & Pandit, 2017; Inampudi, Akintoye, Ando, & Briasoulis, 2018; Thiagarajan, Thiagamoorthy, Shanmugham, Dharmalingam Nandagopal, & Kaliyaperumal, 2017), thus only one aspect will be detailed with regard on diseases associated with MI, wound healing, osteoporosis and failed lymphangiogenesis.

MI is characterized by the occlusion of a coronary artery, resulting in a reduction in oxygen supply. In the ischemic myocardium, some pro-angiogenic growth factors are released such as VEGF, FGF, ANGs or PDGF, leading to a stimulation of the perfusion in the ischemic area. Indeed, during MI, VEGF is upregulated in the myocardium and participates in collateral vessel growth probably through the (i) release of endothelial PDGF-B and (ii) the maturation of recently formed vessels by recruiting SMCs (Maulik & Thirunavukkarasu, 2008). In addition, both VEGF and FGF synergistically act to induce a local upregulation of NO production (Bougioukas et al., 2007). However, this natural compensatory process is often insufficient and/or too slow when compared to the development of the occlusion (Thiagarajan et al., 2017). In this respect, the treatment with pro-angiogenic factors could decrease the infarct size of injured area (Hao et al., 2007; Xaymardan et al., 2004).

During wound healing, neovascularization is indispensable to recover the damaged wound and to restore the oxygen and nutrients input to the site of the injury. This process is mediated by the hypoxia-regulated pro-angiogenic factors VEGF, PDGF and FGF (Guo & DiPietro, 2010). In chronic wounds, angiogenesis is insufficient and can be caused by a variety of factors, often associated with host comorbidity such as diabetes mellitus. Under these conditions, the decrease in pro-angiogenic factor production (VEGF and ANG-1, mainly) is accompanied by the increase of anti-angiogenic factor secretion (like the pigment epithelium-derived factor), leading to a decrease of capillary maturation in wounds (Okonkwo & DiPietro, 2017). Moreover, important levels of MMPs locally present in chronic wounds are described to be responsible of the degradation of ECM proteins and growth factors, resulting in insufficient angiogenesis (Bauer, Bauer, & Velazquez, 2005).

Additionally, diverse pathologies such as osteoporosis are also characterized by a reduction of angiogenesis. In the osteoporosis associated to the postmenopausal period or to diabetes, a reduced number of sinusoidal and arterial capillaries in the bone marrow is described in mice, leading to decreased bone perfusion (Zhao et al., 2012). As shown in ovariectomized mice, this is associated with downregulation of HIF-1 $\alpha$ , HIF-2 $\alpha$ , and VEGF proteins in bones (Zhao et al., 2012). Moreover, in a type 1 diabetes osteoporosis mouse model, the expression of platelet endothelial cell adhesion molecule (CD31), NGF-nerve growth factor, HIF and VEGF are decreased (Peng et al., 2016).

Regarding lymphangiogenesis, lymphedema is the condition of lymphatic insufficiency due to dysfunctional or absent lymphatic vessels leading to accumulation of interstitial fluid. Lymphedema can be classified into primary hereditary lymphedema or secondary acquired lymphedema (Norrmén, Tammela, Petrova, & Alitalo, 2011). Primary lymphedema results from heritable defects in lymphatic vascular development or function. Heterozygous mutation of *FLT4* gene that encodes for VEGFR3 causes the congenital hypoplastic lymphedema, known as Nonne-Milroy lymphedema (Karkkainen et al., 2000). Mutations of a

number of other genes that are involved in the VEGF-C/VEGFR3 axis, such as Ca<sup>2+</sup>-binding EGF domain-containing protein 1, FOXC2, and tyrosine-protein phosphatase non-receptor type 14 have also been shown to cause primary lymphedema (Aspelund, Robciuc, Karaman, Makinen, & Alitalo, 2016). Mutations involved in LEC differentiation, such as Sox-18, have also been reported to cause primary lymphedema (Irrthum et al., 2003).

Secondary lymphedema is characterized by obstruction or disruption of the lymphatic vessels. Surgery, radiation, or obesity provokes lymphatic injury particularly in patients with cancer, and induce an immune response that includes Th1-, Th2-, and Th17-mediated T cell immunity, and macrophage- and neutrophil-mediated innate immunity (Ogata et al., 2016). Insufficient regulatory T cell activity and insufficient production of lymphatic reparative factors, such as VEGF-C, lead to lymphatic vascular dysfunction (Zampell et al., 2012). Prolonged lymphatic vascular dysfunction causes interstitial fluid retention and tissue edema, accumulation of protein-rich fluid combined with inflammation, adipose tissue hypertrophy, and progressive fibrosis (Warren, Brorson, Borud, & Slavin, 2007). The approaches targeting diseases with failed angiogenesis will not be further developed here. We will now focus on diseases with excessive angiogenesis.

#### 2.4.2. Diseases associated with excessive angiogenesis

Abnormal increase of angiogenesis is a hallmark in the pathology of several diseases. Among the most described diseases associated with exacerbated angiogenesis are cancer development, retinopathies, age-related macular degeneration, psoriasis and other autoimmune disorders, atherosclerosis but also endometriosis and asthma (Carmeliet, 2003; Chung & Ferrara, 2011).

An uncontrolled vascularization is largely described in the development of retinal vasculopathies and causes severe vision impairment. In particular, diabetic retinopathy is a microvascular complication of diabetes mellitus in adults, and the principal cause of vision loss after 50 years (Chaurasia et al., 2018; Luty, 2013). The stimulation of angiogenesis during diabetic retinopathy disrupts the normal eye functions affecting the central retinal structure and conducts to opacity of the cornea, resulting in progressive loss of visual acuity (Capitão & Soares, 2016). The pathophysiological mechanisms of diabetic retinopathy genesis involve pericytes and EC apoptosis, which is preceded by an increase of ANG2 expression due to exposure to high glucose concentration (for review see Watson, Grant, & Coultas, 2017). The retinal hypoxia resulting from the progressive impairment of retinal capillary perfusion leads to the increase of hypoxia-induced pro-angiogenic factors release such as VEGF. Thus, this adaptive response induces new blood vessel formation on the retinal surface and into the vitreous (Wang et al., 2017). In this context, inhibiting the excessive angiogenesis appears to be the best therapeutic approach to target neuropathies.

Psoriasis is a chronic inflammatory skin disease in which the cytokine Th1/Th17 axis is disturbed. Several data report an excess of angiogenesis in psoriasis (Armstrong, Voyles, Armstrong, Fuller, & Rutledge, 2011): (i) histopathologic analysis of psoriasis lesions have shown abnormal vessel architecture, (ii) hypoxia, as a result of the decreased oxygen supply due to the longer diffusion distance in a thickening epidermal plaque, stimulates the production of pro-angiogenic factors such as IL-8, HIF-1 $\alpha$ , and VEGF by keratinocytes, (iii) plasmatic levels of VEGF and VEGFR1 are increased in psoriasis patients and (iv) polymorphisms within the *VEGF* gene have been detected in patients with early onset psoriasis (Young, Summers, Bhushan, Brenchley, & Griffiths, 2004). Taken together, these data underline the potential therapeutic approach of anti-angiogenic agents in this pathology.

Cardiometabolic alterations as atherosclerosis are also related with an excess of angiogenesis. In atherosclerotic lesions, as described above for psoriasis lesions, hypoxia triggers activation of HIF-1 $\alpha$  and VEGF leading to neovascularization and increasing plaque instability (Jain, Nikolopoulou, Xu, & Qu, 2018). In this process, HIF-1 $\alpha$  not only acts on SMCs and on infiltrated macrophages favoring their proliferation



and migration but also on foam cell transformation. Recently, several drugs were reported to reduce intra-plaque neovascularization in mouse models. However, adverse effects such as hypertension and an increased arterial thromboembolism risk have been described in clinical trials using VEGFR inhibitors in cancer treatment (for review see Camaré, Pucelle, Nègre-Salvayre, & Salvayre, 2017), suggesting that further investigations are needed to obtain new anti-angiogenic drugs targeting atherosclerosis.

Endometriosis is a common gynecological disorder defined by the proliferation of endometrial glands and stroma outside the uterus. Endometriosis can be compared with tumor metastasis because endometriosis implants require new vessel formation by sprouting, elongation, and intussusception to proliferate and invade into ectopic sites (Taylor et al., 2009). In normal endometrium, VEGF reaches its maximal production in the late proliferative and early-mid secretory phases of the cycle (Gambino et al., 2002; Shifren et al., 1996). However, in endometriosis, VEGF accumulates in endometriosis implants and in pelvic fluids (for review see Taylor et al., 2009). Little is known about the stimuli inducing the increase in VEGF production although hypoxia, local pro-angiogenic cytokine secretion (IL-6, IL-1 and IL-8) and hormones (estrogens and progesterone) have been proposed as key actors of the exacerbated angiogenesis in endometriosis (Becker & D'Amato, 2007). Taking into consideration these findings, the inhibition of angiogenesis may represent a new opportunity to decrease pain associated with endometriosis. For instance, quinagolide, an agonist of dopamine receptor 2 is able to reduce or eliminate peritoneal endometriosis lesions in women with endometriosis, by blocking the VEGFR-2 and downstream angiogenesis (Gómez et al., 2011).

Bronchial asthma is a chronic disease of the airways; the predominant underlying mechanisms of bronchial asthma are bronchial inflammation and hyper responsiveness, and bronchial wall remodeling consisting of sub-epithelial layer thickening, airway smooth muscle hyperplasia and angiogenesis. Indeed, bronchial *lamina propria* of severe asthma patients exhibits an increased blood vessel density compared to healthy controls (Salvato, 2001). As in the other pathologies presenting an excess of angiogenesis, hypoxia and local pro-inflammatory environment can induce angiogenesis. Accordingly, enhanced productions of HIF-1 $\alpha$ , VEGF, tumor necrosis factor- $\alpha$  and TGF- $\beta$  have been described in asthma patients. Although the inhalation of corticosteroids is the most effective approach against chronic asthma by acting directly on inflammation, corticosteroids are also able to decrease the number of vessels as well as inducing a decrease of HIF-1 $\alpha$  and VEGF concentrations in lungs on a mouse model of asthma (Sun et al., 2013). These results open new therapeutic perspectives against asthma by countering angiogenesis.

Inflammation has been shown to be associated with increased angiogenesis and lymphangiogenesis. This process is important to create adequate fluid and antigen drainage to lymph nodes in order to allow antigen processing and maintenance of tissue fluid homeostasis (Liao & von der Weid, 2015). During inflammation, macrophages can be transformed from native monocytes into VEGF-C and VEGF-D-producing cells, which can induce the growth of existing lymphatic vessels (Schoppmann et al., 2002). Macrophages have also been shown to transdifferentiate into LECs, and to be incorporated into lymphatic endothelium contributing to lymphatic vessel growth (Maruyama et al., 2005; Schoppmann et al., 2002).

Numerous inflammatory diseases are associated with lymphatic activation. For example, increased numbers of lymphatic vessels are found in ulcerative colitis in the muscularis mucosa (Kaiserling, Kröber, & Geleff, 2003). In addition, increased VEGF-C expression is observed in the joint synovium of RA patients (Paavonen et al., 2002). In these patients, the number of lymphatic vessels is positively related to the severity of synovial inflammation (Xu et al., 2003). Furthermore, emerging studies of the role of lymphatic system in inflammatory responses by modulating lymphangiogenic factors, VEGF-C and VEGFR3, support the development of therapeutic strategies targeting the lymphatic vessels to control chronic inflammation (Huggenberger et al., 2010).

#### 2.4.3. Tumor progression

Excessive angiogenesis is well described to be a prerequisite for tumor growth, invasion and metastasis. Cancer cells are metabolically very active and formation of new blood vessels is crucial to feed oxygen and nutrients for tumor growth, and for the elimination of cellular wastes (Couffignal, Dufourcq, Daret, & Duplaà, 2001). The suppression of tumor vascularization directly leads to necrosis or apoptosis, confirming the relevance of the mechanism for tumor inhibition (Brem, Brem, Folkman, Finkelstein, & Patz, 1976; Parangi et al., 1996).

There are two phases of tumor growth, an avascular growth for tumor <2 mm in diameter, and a vascular phase needed for larger tumor, in which the outmost cells are in metabolic stress and hypoxic conditions. Several factors are involved for the vascular phase of the tumor: (i) oxygen deprivation (hypoxia) or acidic conditions, (ii) induction of oncogenes such as H-ras, v-Src and HER-2, (iii) silencing of tumor suppressor genes such as p53 (Perrone et al., 2004; Clere et al., 2007) or reactive oxygen species (ROS) production (Clere, Faure, Martinez, & Andriantsitohaina, 2011); (iv) glycolysis of ECs and cancer cells producing elevated amount of lactate, described to stimulate angiogenesis by increasing HIF-1 $\alpha$ ; and (v) nutrient depletion, especially, glucose concentration which is 50-fold lower in cancer cells and is a powerful stimulator of angiogenesis (Nardo et al., 2011; Urasaki, Heath, & Xu, 2012). Overproduction of pro-angiogenic factors leads to angiogenic imbalance and a constant angiogenic switch.

For a long time, formation of the tumor vasculature was largely thought to be comparable to the physiological processes of neovascularization, with endothelial sprouting and proliferation as the principal routes of new blood vessel formation. However, it has recently been accepted that tumors have multiple, often unexpected, mechanisms of neovascularization. Six mechanisms of vessel formation in tumors are now recognized. These include post-natal vasculogenesis by endothelial precursor cells and different angiogenic mechanisms such as endothelial splitting, sprouting, bridging (although poorly described), cooption of existing vasculature, vascular mimicry, a process of tumor vessels formation to mimic the function of vascular vessels, and tumor cell differentiation into “endothelial-like” cells (for review see Dunleavy & Dudley, 2012). Vascular mimicry-forming tumors are more virulent than their non-vascular mimicry counterparts and vascular mimicry-lined channels may not respond predictably to conventional anti-angiogenic therapies (Dunleavy & Dudley, 2012). The diverse subtypes of tumor angiogenesis are recapitulated on Fig. 1.B.

Tumor vessels are distinct in several aspects compared to normal vasculature as they are disorganized and tortuous and that their spatial distribution is significantly heterogeneous, resulting in uneven drug distribution in the tumors (Carmeliet & Jain, 2011). Tumor vessels do not follow the hierarchy of arterioles, capillaries and venules. In addition, tumor vessels are more permeable than normal ones since tumor-associated ECs are widened and loosely connected. Some studies suggest that tumor ECs have cytogenetic abnormalities including aneuploidy, abnormal chromosome composition and centrosomes, raising the possibility that such instabilities favor resistance to anti-angiogenic therapies (Hanahan & Weinberg, 2011). Moreover, pericytes in tumor vessels are abnormal since they may not be in close association with ECs, and their cytoplasmic processes might extend to tumor parenchyma (Morikawa et al., 2002). Thus, tumor microenvironment composed by ECM, fibroblasts, ECs and pericytes can constitute novel targets to inhibit tumor growth (Clere et al., 2011).

Although both tumor cells and ECs exhibit many differences, both are also intertwined and have even been named “brothers in arms” (Verdegem, Moens, Stapor, & Carmeliet, 2014). Firstly, both cell types favor cell growth; blood vessels and ECs feed tumor cells in nutrients and oxygen, and in return, tumor cells release pro-angiogenic factors in the extracellular medium to enhance angiogenesis. Concerning metabolic demands, both cell types possess the particularity to be considerably glycolytic and ECs are even more glycolytic than tumors cells. As a consequence, both cells are able to produce energy without using

mitochondrial respiration. This anaerobic ATP production allows cell proliferation even under hypoxic conditions, regulating their invasiveness and proliferation rates (Verdegem et al., 2014). Glycosylation is deleterious in both cell types, described as aberrant in all cancer cells, but also in ECs by enhancing the activity of several proangiogenic factors (Hassani et al., 2017; Pinho & Reis, 2015).

Tumor cell transmigration *via* the blood or lymphatic vessels may depend on the ratio of the different inducing factors, including chemokines, growth factors and soluble receptors present within the local tumor microenvironment (Dobner, Riechardt, Jousen, Englert, & Bechrakis, 2012; Van den Eynden et al., 2007). The proliferation and migration of LECs contribute to the growth of lymphatic vessels and these mechanisms occur in various primary human cancers (Farnsworth, Achen, & Stacker, 2006). The mechanisms representing the contribution of LECs to tumor growth are summarized on Fig. 2.

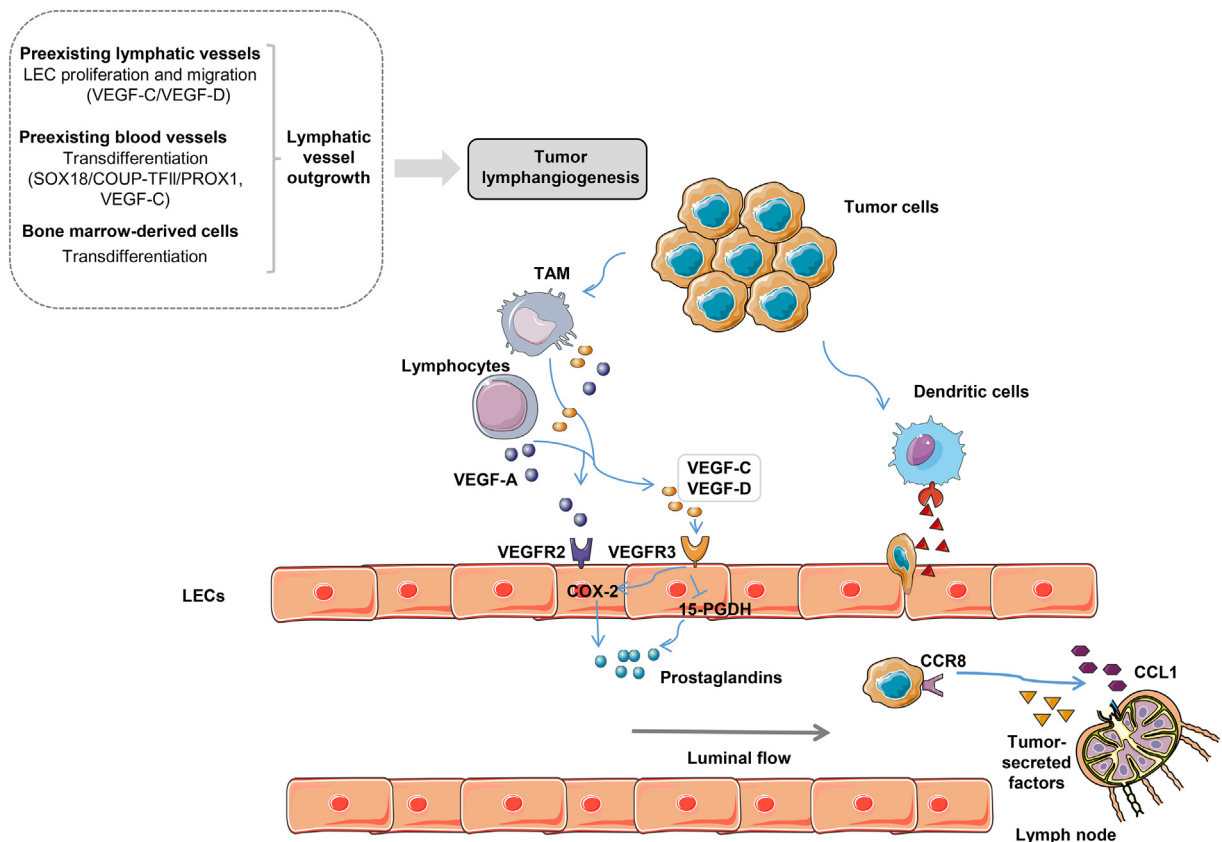
The VEGF-C and VEGF-D/VEGFR3 axis is considered as pivotal for tumor lymphangiogenesis (Tammela & Alitalo, 2010). Various signaling pathways modulate VEGF axis. As an example, cyclooxygenase 2 up-regulates the expression of VEGF-C in the tumor microenvironment *via* EP<sub>1</sub> receptor-dependent pathway (Su et al., 2004). VEGF-D modulates the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) leading to prolonged exposure of LECs to prostaglandins and enlargement of lymphatic vessels, which are associated with increased tumor cell dissemination (Karnezis et al., 2012). Under heat shock stress conditions, VEGF-D expression is controlled by nucleolin, a nucleolar protein involved in the maintenance of lymphatic vessel plasticity (Morfoisse et al., 2016).

VEGF-A (Björndahl et al., 2005), ANG (Fagiani, Lorentz, Kopfstein, & Christofori, 2011) and PDGF-B (Cao et al., 2004) are also involved in lymphangiogenesis. HIF-1 $\alpha$  expression is up-regulated in macrophages

in response to decreased oxygen tension and inflammatory microenvironment, inducing the growth of lymphatic vessels through the secretion of VEGF-A and VEGF-C, thereby facilitating cancer cell invasion (Cursiefen et al., 2004; Ji, 2014). Tumor cells can alter the surrounding microenvironment and influence the tumor progression and metastasis through crosstalk with dendritic cells, macrophages and lymphocytes (Gomes, Nedel, Alves, Nör, & Tarquinio, 2013).

Other cells, such as MSC or TAMs, may also participate in tumor dissemination. In primary tumor under hypoxia, MSCs differentiate into ECs, thereby contributing not only to angiogenesis but also to lymphangiogenesis (Zhan et al., 2015). Several factors secreted by tumors, including IL-10, IL-4, TGF- $\beta$ , and colony-stimulating factor, switch TAMs into polarized M2 macrophages (Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). Macrophages in tumor inflammation can release VEGF-A, VEGF-C and VEGF-D to initiate lymphangiogenesis *via* both LEC proliferation and recruitment of TAMs (Duong et al., 2012; Ji, 2012; Sundliisæter et al., 2007).

Dissemination of cancer cells to regional lymph nodes is the first step in metastasis and this step is a useful tool for cancer prognosis (Al-Rawi & Jiang, 2011). Factors such as microlymphatic vessel density and high expression levels of VEGF-C, VEGF-D, CXCR4, and VEGFR3 have been proven to contribute to lymphatic involvement and nodal metastasis (Fukunaga et al., 2006). Additionally, tumor-secreted factors may precede arrival of metastatic cells in the lymph node through the drainage of fluid from the periphery. These factors promote enlargement of the lymphatic networks inside the node (Hirakawa et al., 2007). Furthermore, chemokines CCL1, expressed in lymph node lymphatic sinuses, and CCR8 (CCL1 receptor), expressed by tumor cells, could represent a checkpoint for the entry of tumor cells into lymph nodes, thereby regulating metastasis (Das et al., 2013). The lymphangiogenesis into lymph



**Fig. 2. Contribution of lymphatic vessels to tumor progression.** New lymphatic vessels may originate from three alternative sources and take part in the outgrowth of tumor mass. Here, we present a schematic representation of molecular mechanisms that regulate tumor lymphangiogenesis. The VEGF-C- and VEGF-D/VEGFR-3 axis is shown. Various molecules participate in lymphatic development, such as cyclooxygenase 2 (COX2), 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The interaction of tumor cells with lymphatic vessels can be promoted through crosstalk with dendritic cells, macrophages, and lymphocytes. Furthermore, chemokines CCL1, expressed in lymph node, and CCR8 (CCL1 receptor), expressed by tumor cells can promote the entry of tumor cells into lymph nodes.



nodes may also create lymphovascular niche for cancer stem cell. This process might generate permissive conditions for the expansion of micrometastases (Hirakawa, 2009). Although drugs acting on different steps of lymphangiogenesis are therapeutically relevant, this part will not be developed here, as it has been reviewed recently by Dieterich and Detmar (2016).

## 2.5. Current anti-angiogenic therapies and limits

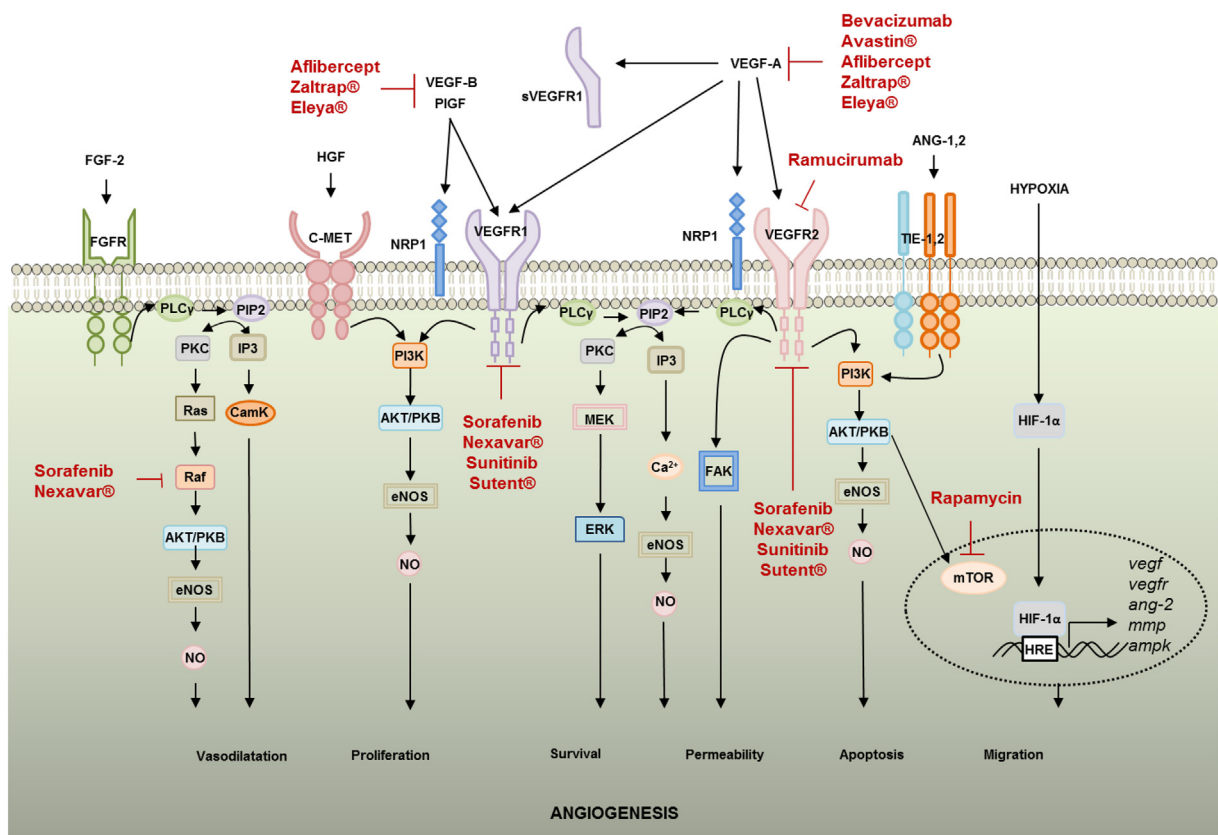
### 2.5.1. Anti-angiogenic therapies and clinical trials

The inhibition of the VEGF pathway by acting on (i) VEGF receptors, (ii) tyrosine kinase receptors of other growth factors or (iii) binding of antibodies to VEGF leads to the reduction of the tumor vessels growth limiting the expansion of cancer cells. Thus, since 2004 when the first humanized antibody (bevacizumab, Avastin®) blocking VEGF function was validated by the Food and Drug Administration, several molecules targeting the VEGF pathway have been developed to fight against solid tumor development, such as colorectal cancer, renal cell carcinoma, breast cancer, and non-small cell lung cancer (Bäuerle et al., 2008; Chebib et al., 2017; Chung & Pherwani, 2013; del Puerto-Nevado et al., 2014). These molecules preferentially target the extracellular domain of VEGFR2, such as the antibody ramucirumab, or the family of the tyrosine kinase receptors involved in the signaling cascade of VEGF and other growth factors including EGF, FGF and PDGF (for review see Berretta et al., 2016). Inhibition of tyrosine kinase receptors allows reducing the development of resistance because the non-specific action of receptor tyrosine kinase (RTK) inhibitors (RTKIs) avoids the compensatory pro-angiogenic actions of other multiple growth factors (Sennino & McDonald, 2012). Among the RTKIs, sorafenib (Nexavar®) and sunitinib (Sutent®) are the most used against hepatocellular carcinoma

and renal cell carcinoma (Crocì, Cerliani, Pinto, Morosi, & Rabinovich, 2014; Dimova, Popivanov, & Djonov, 2014). More recently, the synthesis of fusion proteins able to act as decoy receptors has allowed increasing the arsenal of anti-tumor agents (Scartozzi, Vincent, Chiron, & Cascinu, 2016). Indeed, aflibercept (Zaltrap®, Eleya®) is now used as treatment of metastatic colorectal cancer (Scartozzi et al., 2016) and neovascularization associated with retinopathy (Kiziltoprak et al., 2018; Matsumoto et al., 2018). Moreover, the development of an antibody raised against the VEGFR3 (LY3022856/IMC-3C5), considered as the mediator of tumor lymphangiogenesis, is in a phase I clinical trial. This antibody is well tolerated, but with minimal anti-tumor activity in colorectal cancer (Saif et al., 2016). Rapamycin analogues that block both mTOR complex 1 (mTORC1) and mTORC2 - OSI-027- have a more potent antiangiogenic action in preclinical models than agents that block only mTORC1 (Bhagwat et al., 2011; Falcon et al., 2011). However, no data is available for the clinical use of these rapamycin analogues. The effects of these classical anti-angiogenic drugs for tumor development are illustrated on Fig. 3. Several ongoing clinical trials are designed to assess the evolution of tumors after initiation of different therapies able to inhibit tumor angiogenesis, and concomitantly, the escape to drug resistance (see below) (www.clinicaltrials.gov and Table 1). The described adverse effects of these anti-angiogenic compounds used in clinical trials are summarized in Table 2.

### 2.5.2. Limits of traditional treatments

The major limitations of the “traditional” anti-angiogenic factors are the development of resistance and/or toxicity. Often administrated as adjuvant, it is intriguing why the anti-angiogenic therapies loss their efficacy, although two potential mechanisms are known to participate to the development of resistance (Bergers & Hanahan, 2008). Firstly, the



**Fig. 3. Classical angiogenic mechanisms and targets of anti-angiogenic agents.** Main angiogenic factors and cross talk between pathways involved in vasodilatation, proliferation, survival, permeability apoptosis and migration leading to angiogenesis are illustrated. General mechanisms of action of (i) antibodies targeting VEGF family members, extracellular domain of VEGFR2, or VEGFR tyrosine kinase receptors, (ii) non-specific tyrosine kinase receptor inhibitors and (iii) decoy receptors are shown.

**Table 1**  
Clinical trials using anti-angiogenic therapy combined with other treatments.

Title of the clinical trial	Treatment	Phase	NCT	Estimated enrollment	Administration route	Status
Angiogenic switch in patients with colorectal cancer	Xelox or Xeliri and bevacizumab	II	NCT02075086	100	Intravenous	Active, not recruiting
Sequential angiogenic blockade for the treatment of recurrent mullerian malignancies	First, bevacizumab and then, addition of cyclophosphamide	II	NCT00856180	20	Intravenous and Oral	Completed
Peptide vaccine targeting to cancer specific antigen combined with anti-angiogenic peptide antigen in treating patients with non-small cell lung cancer	HLA-A*2402 restricted epitope peptides derived from URLC10, CDCA1, VEGFR1 and VEGFR2	I	NCT00874588	6	Subcutaneous	Completed
Bevacizumab with or without cyclophosphamide and methotrexate: a pilot study in women with operable breast cancer	Bevacizumab, cyclophosphamide, methotrexate, capecitabine		NCT00121134	164	Intravenous and Oral	Completed
Hydroxychloroquine, capecitabine, oxaliplatin, and bevacizumab in treating patients with metastatic colorectal cancer	Bevacizumab, XELOX regimen, hydroxychloroquine	II	NCT01006369	39	Intravenous and Oral	Completed
AG-013736 in combination with gemcitabine versus gemcitabine alone for patients with metastatic pancreatic cancer	AG-013736 in combination with gemcitabine	II	NCT00219557	111	Intravenous and Oral	Completed
Study with AG-013736 combined with chemotherapy and bevacizumab in patients with metastatic colorectal cancer	AG-013736, AG-013726 and bevacizumab	I, II	NCT00460603	187	Intravenous and Oral	Completed
Dual rectal angiogenesis or MEK inhibition radiotherapy trial	AZD2171, a RTKI and AZD6244, a potent MEK inhibitor	I	NCT01160926	31	Oral	Terminated
Levocetirizine + Capecitabine + Bevacizumab for patients with refractory colorectal cancer	Levocetirizine + Capecitabine + Bevacizumab	II	NCT01722162	47	Intravenous and Oral	Completed
A comparison of ranibizumab and aflibercept for the development of geographic atrophy in (Wet) AMD patients	Ranibizumab versus aflibercept	IV	NCT02130024	280	Intravitreal	Completed
Uncovering the difference between ranibizumab and aflibercept, focusing on systemic anti-vascular endothelial growth factor (VEGF) effects in patients with neovascular age-related macular degeneration	Ranibizumab versus aflibercept	IV	NCT01988662	205	Intravitreal	Completed
Efficacy of ranibizumab therapy with aflibercept in patients with diabetic macular edema	Ranibizumab versus aflibercept and their combination	IV	NCT02646670	20	Intravitreal	Completed
The TIME-2 Study: A phase 2 study of AKB-9778, a novel Tie-2 activator, in patients with diabetic macular edema	AKB-9778 with or without ranibizumab	II	NCT02050828	144	Subcutaneous	Completed
Study of oral OSI-027 in patients with advanced solid tumors or lymphoma	OSI-027, a mammalian target of rapamycin (mTOR) kinase inhibitor	I	NCT00698243	128	Oral	Completed
Carboxyamidotriazole in treating patients with advanced kidney cancer	Carboxyamidotriazole, an inhibitor of non-voltage-gated Ca <sup>2+</sup> channels	II	NCT00005045	55	Oral	Completed
A phase I, first-in-man study of OTX008 given subcutaneously as a single agent to patients with advanced solid tumors	OTX008	I	NCT01724320	20	Subcutaneous	Completed
Neoadjuvant itraconazole in non-small cell lung cancer	Itraconazole	I	NCT02357836	15	Oral	Recruiting

specific characteristics of the intratumoral blood vessels - expression levels and/or single nucleotide polymorphisms of growth factor receptors including VEGFR, and activation of other VEGF-independent growth factor pathways - can lead to the intrinsic resistance. Also, adaptive resistance results from the specific effects induced by the anti-angiogenic drugs directly on the tumor and their blood vessels and are generally associated with the hypoxia generated into the tumor and the subsequent metabolic switch (for review McIntyre & Harris, 2015). For instance, changes in the dominant isoform of VEGF (VEGF121, VEGF165 or VEGF189) as well as a downregulation of VEGFR2 and subsequently a loss of endothelial cell VEGF dependence may explain, at least partly, the tumor escape of VEGF-targeted treatment (Arao et al., 2011; Sitohy, Nagy, Jaminet, & Dvorak, 2011). Furthermore, resistant tumors to bevacizumab treatment exhibit an increased expression of HIF-1 $\alpha$ , carbonic anhydrase IX, a robust HIF target, and glycolytic HIF targets, illustrating an increased glycolytic utilization of glucose, whereas a decrease of the expression of genes governing the entry of pyruvate into the tricarboxylic acid (TCA) cycle is observed (Kumar et al., 2013). This is associated with the downregulation of mitochondrial OXPHOS complexes (Kumar et al., 2013), suggesting that the regulation of the metabolic switch may be a new target for cancer therapy. Other studies also underline the metabolic adaptation of tumors driven by hypoxia and, more exactly, by HIF and HIF-related genes (Hartwich et al., 2013; Shukla et al., 2017; Wilson & Hay, 2011). Furthermore, preclinical studies have shown that severe inhibition of angiogenesis using a function-blocking antibody against VEGFR2 increases invasiveness and metastasis, likely by a mechanism involving the hypoxia/HIF-1 $\alpha$  axis. Also, the RTKI sunitinib evokes similar effects (Pàez-Ribes et al., 2009). Altogether, these data suggest that the

application of personalized cancer medicine is necessary to improve results obtained with the actual anti-angiogenic drugs.

### 3. Targeting metabolism: The new anti-angiogenic paradigm

As traditional anti-angiogenic therapies exhibited limited efficacy on angiogenesis, new alternatives have been proposed to counteract excessive angiogenesis. Although the relation between EC metabolism and angiogenic phenotype has been widely overlooked, new anti-angiogenic strategies focused on the inhibition of EC metabolism have emerged. Indeed, angiogenesis and metabolism are interconnected, and the metabolic properties of quiescent and angiogenic ECs are considerably different. Metabolic demands in angiogenic ECs are clearly higher due to the biosynthesis of proteins, nucleotides and lipids, which is essential to initiate proliferation and migration (Potente & Carmeliet, 2017). Moreover, ECs need a robust metabolism to maintain the ability to initiate sprouting in diverse pathological environmental conditions with fluctuating levels of oxygen, nutrients, or pH. Thus, EC metabolism is highly related to angiogenesis and plays a role in tumor escapement in anti-angiogenic therapies (Jiménez-Valerio & Casanovas, 2017; Vandekerke, Dewerchin, & Carmeliet, 2015).

As a consequence, numerous reports are now focused on the study of EC metabolism. In particular, the bioenergetic pathways glycolysis, OXPHOS and FAO have been newly intensively studied (De Bock, Georgiadou, & Carmeliet, 2013; Duluc et al., 2013; Schoors et al., 2015; Treps, Conradi, Harjes, & Carmeliet, 2016). However, other pathways such as protein glycosylation have been less regarded and nevertheless represent exciting approaches to target angiogenesis.

**Table 2**  
Adverse effects of anti-angiogenic drugs used in clinical trials.

Treatment	Hemorrhage	Cutaneous	Gastrointestinal	Neurological	Cardiovascular	Respiratory	Muscular
Bevacizumab	Sores	Sore, itching	Diarrhea	Fainting, dizziness, confusion, loss of vision, seizure	Heartburn	Choking, chest pain, shortness of breath, loss vision	Muscles pain
Xelox	Bleeding gums/nosebleeds	Numbness/tingling in hands and feet, mouth sores	Diarrhea, constipation	Tiredness/weakness, feeling sick		Breathlessness	
Xeliri	Bleeding gums/nosebleeds	Strong sweating, soreness, redness, peeling on hands/ft	Abdominal cramps, diarrhea	Tiredness/Weakness, feeling sick		Breathlessness	
Cyclophosphamide	Sores and bleeding	Changes in skin color, rash, hives	Nausea, vomiting, diarrhea, pain			Cough, chest pain	
Methotrexate		Rash, hives, itching	Vomiting	Dizziness, drowsiness, headache, seizure, memory loss		Difficulty breathing	Joint or muscle pain, difficulty walking
Capecitabine		Rash, peeling of skin	Stomach pain, constipation, nausea, vomiting, diarrhea	Weakness, dizziness, headache		Chest pain, fast heartbeat	
Hydroxychloroquine	Bruising and bleeding		Vomiting, diarrhea, nausea, pain	Dizziness, headache, vision and hearing difficulties, convulsions		irregular heartbeat	
Gemcitabine		Rash, hives, itching	Nausea, vomiting, diarrhea, constipation	Headache, weakness		Cough, difficulty breathing	Sore and painful
AG-013736 (Axitinib)	Bruising and bleeding	Rash, itching, definitive wounds	Nausea, vomiting, diarrhea, constipation, hemorrhoids, severe pain	Headache, weakness, dizziness, loss of vision, seizure	Heartburn	Cough, shortness of breath, chest pain	Muscles pain
AZD2171 (Cediranib)		Numbness, tingling, redness, soreness on hands and feet	Diarrhea	Tiredness, feeling sick	Raised blood pressure		
Levocetirizine	Nosebleed	Rash, hives, itching		Sleepiness, tiredness		Cough	
Aflibercept	Sore	Darkening, cracking, blistering, sore	Hemorrhoids, diarrhea, foamy urine	Tiredness, headache, dizziness, confusion, vision perturbations		Chest pain, shortness of breath	
Ranibizumab	Bleeding in/around eye		Nausea	Headache, eye and vision perturbation		Chest pain, shortness of breath	
AKB-9778		Edema	Nausea	Dizziness			Arthralgia, myalgia
Carboxyamidotriazole		Rash	Constipation, nausea, vomiting	Depression, dizziness, fatigue, headache			Muscles and joint pain
Itraconazole	Bleeding gums	Numbness, burning, creeping, rash, hives, itching	Diarrhea, constipation, nausea, pain, vomiting	Headache, dizziness, depression, tiredness	Heatburns	Difficulty breathing	

Adverse effects are collected from: <https://medlineplus.gov>; <https://find.cancerresearchuk.org>; Campochiaro et al., 2015; Hussain et al., 2003.

### 3.1. Energy metabolism

In a normal quiescent vasculature, only 0.01% of ECs are proliferative (Coutelle et al., 2014). Due to low energetic needs, quiescent ECs are mostly sustained by glycolysis. Under angiogenic conditions, ECs need to quickly increase their ATP production to adapt their metabolism to angiogenic requirements. Around 85% of total cellular ATP production in ECs is produced by the oxygen-independent glycolysis (De Bock, Georgiadou, Schoors et al., 2013). Although less implicated in ATP generation, OXPHOS and FA metabolism are also concerned, in particular under stress conditions such as glucose deprivation or oxidative stress (Caja & Enríquez, 2017). Given that ECs need to abundantly increase their bioenergetic pathways in response to metabolic modifications essential to initiate angiogenesis, targeting energy metabolism in excessive angiogenesis associated pathologies emerges as a promising approach.

#### 3.1.1. Mitochondria

Mitochondria are dynamic organelles, present in the cytosol of all eukaryotic cells, except peripheral red blood cells, and are organized in a network. They continuously fuse and split, underlying the plasticity of these organelles (Chao De La Barca et al., 2016). Their amount, size and shape depend on the cellular type and on the cellular activity. In ECs, despite their direct contact with important levels of oxygen in blood, mitochondria represent only 4% of the intracellular volume, far

less than in other cell types such as hepatocytes (about 28%) or cardiomyocytes (about 32%) (Barth, Stämmler, Speiser, & Schaper, 1992; Blouin, Bolender, & Weibel, 1977). Mitochondrial content also differs between endothelial origins: brain ECs possess more mitochondria than ECs from other tissues (Oldendorf, Cornford, & Brown, 1977). Nonetheless, despite the low mitochondria content in ECs, they are essential for endothelial function and homeostasis, and regulate a wide range of physiological processes. Beside their role in ATP generation, these organelles are notably implicated (i) in the storage of  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  ions, (ii) the steroid synthesis (testosterone, progesterone and cortisol) modulating cellular homeostasis and also several metabolic processes regulating angiogenesis.

**3.1.1.1. Implication in angiogenesis.** Although mitochondria represent <5% of the EC volume, these organelles are specifically implicated in angiogenesis. As an example, in PolgA mouse model, which presents a severe mitochondrial dysfunction due to defective mitochondrial DNA polymerase, angiogenic growth factors are potently reduced, with no adverse effects on established vascular networks, confirming a mitochondrial implication in the neovascularization (Marcu, Zheng, & Hawkins, 2017).

In most cells, mitochondria regulate ATP production by OXPHOS, an aerobic process requiring TCA cycle, FAO, amino acid oxidation and also the electron transport chain. Although ECs have direct access to oxygen from the blood (Locasale & Cantley, 2011), OXPHOS only contributes to a little part of all the ATP formation process. *In vitro*, <1% of the pyruvate



formed by glycolysis is converted into acetyl-coenzyme A (CoA) in mitochondria, while 99% is converted to lactate. ECs mainly produce ATP by glycolysis, whereas OXPHOS is at least 20-fold more efficient in generating ATP per mole of glucose (Quintero, Colombo, Godfrey, & Moncada, 2006; Verdegem et al., 2014). This reduced oxygen utilization by ECs is beneficial to provide oxygen to the perivascular tissues, and minimize endothelial oxidative damage, further sustaining angiogenic ability during hypoxia (Clementi, Brown, Foxwell, & Moncada, 1999; Marcu et al., 2017). The precise regulation of mitochondrial activity in ECs remains unclear. However, in ECs, the rate of oxygen consumption varies with its concentration, suggesting the existence of an intracellular sensor that controls cell respiration (Clementi et al., 1999). Indeed, electron transport chain and, as consequence, basal mitochondrial respiration are controlled by NO through the inhibition of complex IV. Thus, endogenous generation of NO plays a physiological role in adjusting the capacity of complex IV to use oxygen, allowing ECs to adapt to acute changes in their environment. Despite the low oxygen consumption during physiological condition, EC mitochondria are able to increase respiration under stress conditions or high metabolic demand (Dranka, Hill, & Darley-Usmar, 2010; Polet & Feron, 2013). Also, ECs display the Crabtree effect, characterized by an increased respiration in low glucose levels (~1 mM) and a reduced respiration under high glucose condition (e.g., 25 mM) (Eelen et al., 2018; Kozziel, Woyda-Ploszczyca, Kicinska, & Jarmuszkiewicz, 2012; Krützfeldt, Spahr, Mertens, Siegmund, & Piper, 1990).

Mitochondria, apart from their implication in ATP production, are implicated in the regulation of angiogenesis. Mitochondria are implicated in the maintenance of intracellular  $\text{Ca}^{2+}$  levels (Hajnoczky et al., 2006; Rossier, 2006), and contain 25% of EC  $\text{Ca}^{2+}$ . Mitochondrial  $\text{Ca}^{2+}$  homeostasis controls many cellular functions, such as the vascular permeability, motility, proliferation and angiogenesis (Marcu et al., 2017). Mitochondrial ATP production is directly related to  $\text{Ca}^{2+}$  levels, and an increase in  $\text{Ca}^{2+}$  induces ATP production *in vitro* by rising mitochondrial enzyme activity, suggesting that  $\text{Ca}^{2+}$  homeostasis is crucial for OXPHOS (Denton & McCormack, 1990; Kluge, Fetterman, & Vita, 2013). Moreover,  $\text{Ca}^{2+}$  binds to numerous signaling molecules such as FAK (Groschner, Waldeck-Weiermair, Malli, & Graier, 2012). A prolonged FAK association at the site of focal adhesions will favor the disassembly of these structures through a  $\text{Ca}^{2+}$  dependent mechanism, required for cell spreading and migration (Giannone et al., 2004).

Besides these energetic considerations, mitochondria are one of the main sources of ROS. In low-to-moderate concentrations, ROS are essential for the cell homeostasis and have physiological roles as signaling molecules in various cellular and developmental processes (Andriantsitohaina et al., 2012). Mitochondrial ROS are produced during oxidative metabolism, predominantly at complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome *c* oxidoreductase) of the electron transport chain, and approximately 0.15 to 2% of cellular oxygen consumption results in the  $\text{O}_2^-$  (superoxide anion) formation (Andriantsitohaina et al., 2012; Tahara, Navarete, & Kowaltowski, 2009). ROS are critical in physiological and pathological processes, and are also implicated in angiogenesis. The  $\text{O}_2^-$  released diffuse into the cytosol and interact with NO to form ONOO<sup>-</sup> (peroxynitrite), or will be transformed in hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) by the superoxide dismutases (Li & Shah, 2004). It has been reported that high levels of  $\text{H}_2\text{O}_2$  induce cellular damages, whereas low levels have pro-angiogenic properties by up-regulating VEGF and activation of VEGFR2 signaling. More precisely, ROS produced by NADPH oxidase 2 and 4 and by mitochondria mediate VEGFR2 activation and phosphorylation through the inhibition of the tyrosine phosphatase activity, resulting in an induction of angiogenesis (Andriantsitohaina et al., 2012; Chua, Hamdy, & Chua, 1998; Kim et al., 2017). In addition, both experimental *in vitro* and *in vivo* approaches abolishing mitochondrial ROS confirm the depletion of HIF-1 $\alpha$  and the suppression of pro-angiogenic factors like VEGF and the downstream blockade of angiogenesis. Interestingly, this anti-angiogenic model is described in ECs but also in

tumor models, without affecting mitochondrial respiration (Jung et al., 2010; Lee, Kim, Jung, & Kwon, 2012). Lastly, VEGF also favors ROS production *in vitro*, reinforcing the ROS implication in angiogenesis (Raaz et al., 2014; Verdegem et al., 2014).

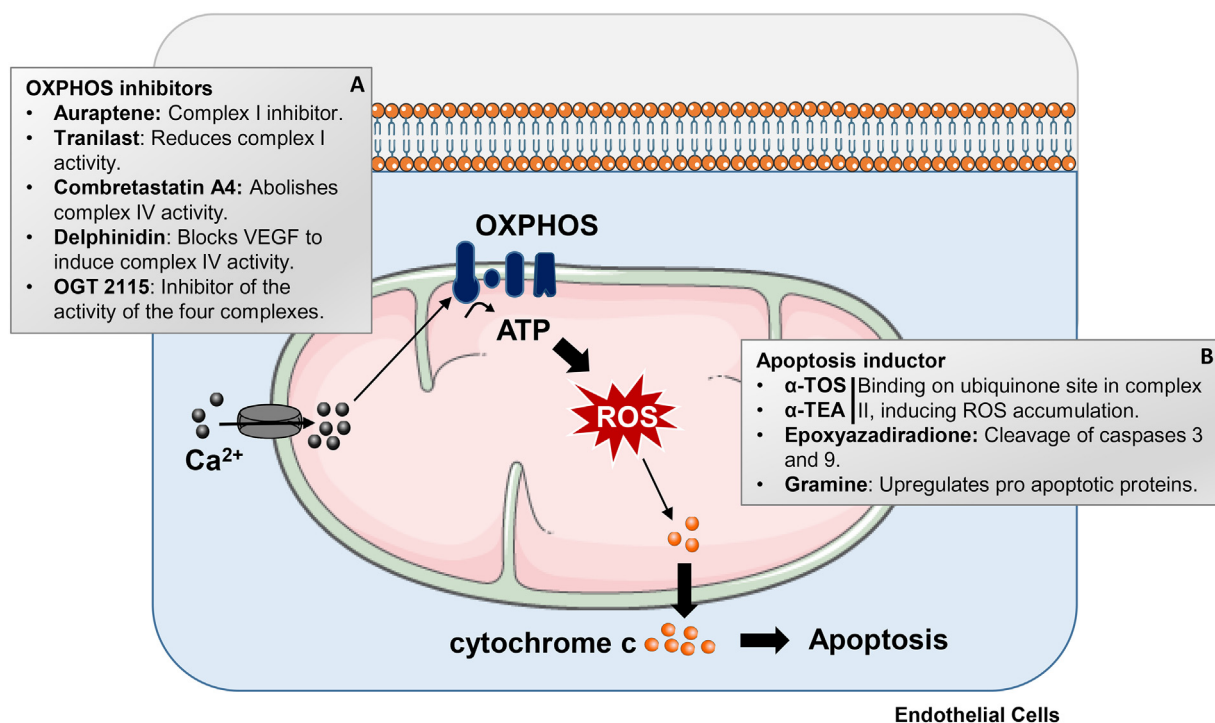
Moreover, and linked to the ROS generation, mitochondria also regulate the induction of apoptosis in response to intracellular stress or extracellular stimuli. Mitochondria release cytochrome *c* into the cytosol, triggering the activation of caspases and the induction of apoptosis (Green, 1998; Vaux, 2011). Mitochondria enable the modulation of endothelial survival during migration by regulating the release of survival or pro-apoptotic signals (Mallat & Tedgui, 2000). It is interesting to note that mitochondrial fusion and fission are major regulators of mitochondrial apoptosis. Mitochondrial fusion favors survival, whereas fission stimulates apoptosis. Besides this, they also regulate mitochondrial respiration and cell growth, and are thereby implicated in the regulation of angiogenesis (Chen & Chan, 2005).

Finally, angiogenesis is a process associated with an increase of mitochondrial capacity and biogenesis (Duluc et al., 2013; Wright et al., 2008). Specifically, the mitochondrial biogenesis is partly regulated by VEGF and it has been underlined that VEGF increases peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) expression. PGC-1 $\alpha$  is one of the key mitochondrial biogenesis regulators and activates nuclear respiratory factors 1 and 2 and increases the expression of mitochondrial biogenesis genes. By increasing PGC-1 $\alpha$  pathway in ECs, VEGF regulates mitochondrial biogenesis and metabolism (Wright et al., 2008). Interestingly, PGC-1 $\alpha$  is also a regulator of VEGF expression and angiogenesis in skeletal muscle, via an estrogen-related receptor  $\alpha$ -dependent trafficking (Arany et al., 2008). Conversely, depletion of mitochondrial biogenesis using the Sirtuin 1 silencer impedes vessel branching (Csiszar et al., 2009), confirming the close link between angiogenesis and mitochondrial biogenesis (Arany et al., 2008). Finally, PGC-1 $\alpha$  exerts protective effects against oxidative stress by upregulating the mitochondrial antioxidant defense system (Valle, Alvarez-Barrientos, Arza, Lamas, & Monsalve, 2005).

**3.1.1.2. Compounds with anti-angiogenic properties targeting mitochondria.** Considering the low relevance of ATP production in angiogenesis, few anti-angiogenic studies have considered a mitochondrial ATP inhibition as a potential therapeutic target. However, EC mitochondria are involved in diverse non-energy functions such as apoptosis regulation, ROS generation and  $\text{Ca}^{2+}$  storage, suggesting that mitochondria could impact angiogenesis. Subsequently, numerous compounds inhibiting mitochondrial functions display anti-angiogenic properties (as summarized on Fig. 4).

In particular, auraptene, a natural coumarin from *Citrus* species specifically inhibits mitochondrial respiratory complex I and displays anti-cancer properties by altering tumor neovascularization (Jang et al., 2015). Auraptene impairs HIF-1 $\alpha$  translation inducing a reduction of VEGF levels and consequently to an angiogenesis blockade (Jang et al., 2015). Nonetheless, it has been reported that specific inhibitors of mitochondrial respiration do not impede vessel branching (De Bock, Georgiadou, Schoors et al., 2013). 2-[4-[[3-(4-Bromophenyl)-1-oxo-2-propenyl]amino]-3-fluorophenyl]-5-benzoxazoleacetic acid (OGT 2115) is another anti-angiogenic drug that induces a concentration-dependent reduction in the activity of mitochondrial complex I, complex II–III and complex IV (Quayle et al., 2017). The combretastatin A4, a stilbenoid compound with established anti-angiogenic properties, is able to decrease mitochondrial oxygen consumption by preventing mitochondrial complex IV activity (Quayle et al., 2017; Tozer et al., 2001). Finally, tranilast is an *in vitro* and *in vivo* angiogenesis inhibitor acting via the decrease of mitochondrial membrane potential and the reduction of complex I activity leading to reduced oxygen consumption (Isaji, Miyata, Ajisawa, Takehana, & Yoshimura, 1997; Quayle et al., 2017).

Given that apoptosis of ECs is a physiological process that inhibits proliferation and subsequently angiogenesis, stimulating apoptosis



**Fig. 4. Compounds with anti-angiogenic properties targeting mitochondria.** (A) One current anti-mitochondrial approach aims to act on OXPHOS mechanism leading to a diminution of ATP production and ROS generation. Auraptene and tranilast inhibit complex I, whereas combretastatin A4 and delphinidin target complex IV. OGT 2115 decrease all complex activities. (B) Inducing EC mitochondrial apoptosis is another approach leading to an alteration of angiogenesis.  $\alpha$ -TOS and  $\alpha$ -TEA, by blocking the ubiquinone site in OXPHOS complex II, favor the release of ROS triggering apoptosis. Upregulation of pro-apoptotic effectors caspases 3 and 9 by epoxyazadiradione also conducts to the cell death. Gramine also favors both caspases release as well as other apoptotic inducers Bax, cytochrome c and apaf-1.

could represent an encouraging approach to target angiogenesis (Dimmeler & Zeiher, 2000). In this aim, vitamin E analogs are a class of pro-apoptotic compounds used against tumor development to selectively trigger apoptosis. In particular,  $\alpha$ -tocopheryl-succinate ( $\alpha$ -TOS) and its ether analog  $\alpha$ -tocopheryloxyacetic acid ( $\alpha$ -TEA) display anticancer features against a number of murine tumor models (Zhao, Neuzil, & Wu, 2009). This anti-tumor activity is attributed to a simultaneous inhibition of the tumor proliferation and vascularization. Indeed, these agents specifically disable angiogenesis in proliferating ECs by triggering mitochondria-related apoptosis (Dong et al., 2007).  $\alpha$ -TOS and  $\alpha$ -TEA directly bind the ubiquinone in the complex II of the inner-mitochondrial membrane, resulting in the displacement of ubiquinone and accumulation of ROS (Dong et al., 2007; Dong et al., 2008). The selective toxicity of  $\alpha$ -TOS and  $\alpha$ -TEA against proliferative ECs make them particularly efficient to target and affect only pathological neovessels but not the pre-existing vasculature (Dong et al., 2007). Furthermore, the mitochondrial targeting  $\alpha$ -TOS interacts with the ubiquitin-binding site of complex II to block the electron transfer to complex III, thus promoting ROS production and apoptosis (Dong et al., 2007; Dong et al., 2011; Park & Dilda, 2010).

Epoxyazadiradione, a limonoid that is an epoxy derivative of azadiradione, suppresses breast tumor growth and notably angiogenesis. The mechanism includes the inhibition of PI3K/Akt-dependent mitochondrial depolarization, and the induction of apoptosis via the cleavage of caspase 9 and 3 (Kumar et al., 2018). In an experimental model of oral squamous cell carcinoma, gramine displays anti-angiogenic property via its ability to induce apoptosis by upregulating the pro apoptotic proteins Bax, cytochrome c, apaf-1, caspase-9 and caspase-3 (Ramu, Kathiresan, & Ali Ahmed, 2017).

Targeting mitochondrial capacity and biogenesis to inhibit angiogenesis underscores a key role for mitochondrial function in neovascularization. Of particular interest, red wine polyphenolic compounds (RWPC) have been reported to exert vasculoprotective properties on ECs, and modulate angiogenesis in a concentration-dependent manner;

a low concentration (0.2 mg/kg/day) has shown pro-angiogenic properties, whereas a high concentration (20 mg/kg/day) RWPC inhibits angiogenesis in a model of post-ischemic neovascularization in rats (Baron-Menguy et al., 2007). When investigating the underlying mechanisms of this angiogenic modulation, a low concentration enhances mitochondrial biogenesis factors, mitochondrial respiration and capacity, and mtDNA content in order to enhance angiogenesis. On the contrary, high concentrations of RWPC do not affect these parameters. Moreover, inhibition of mitochondrial protein synthesis abolishes the pro-angiogenic capacity of low RWPC concentration, confirming a link between mitochondrial capacity and angiogenesis (Duluc et al., 2013). More specifically, delphinidin, an anthocyanin present in RWPC, displays anti-angiogenic properties both on *in vitro* and *in vivo* models, and prevents the VEGF from inducing mitochondrial respiration and complex IV activity, and increasing mtDNA content in ECs. These results highlight a likely association between inhibition of VEGF-induced mitochondrial biogenesis by delphinidin and its potent anti-angiogenic effect (Duluc, Jacques, Soleti, Andriantsitohaina, & Simard, 2014). The later effect probably participates to the ability of delphinidin to reduce tumor growth of melanoma cell *in vivo* by acting specifically on EC proliferation. Indeed, in addition to its effect on mitochondrial biogenesis, the mechanism implies an association between the inhibition of VEGF-induced proliferation via MAPK, PI3K, and at a transcription level, on CREB/ATF1 factors, and the inhibition of phosphodiesterase 2 (Keravis et al., 2015).

**3.1.1.3. Limits of the strategies targeting mitochondria.** As indicated above, therapeutic agents targeting mitochondrial functions, notably focusing on ROS and apoptosis, have shown promising results and are considered to be possible future efficient anti-angiogenic compounds. However, the exact role of mitochondria in angiogenesis is still debated and the regulation of the mitochondrial activity in ECs is not completely understood. Whereas targeting mitochondrial ROS is an encouraging strategy, manipulating them is complex by the fact that drug uptake and delivery

in mitochondria is less efficient than expected (Murphy & Smith, 2000). Furthermore, even though low concentrations of mitochondrial ROS act as pro-angiogenic effectors in ECs, excessive levels are toxic and trigger EC dysfunction, complicating the therapeutic approaches targeting mitochondrial ROS (Missiaen, Morales-Rodriguez, Eelen, & Carmeliet, 2017). Moreover, the limit level at which ROS become deleterious is still poorly understood (Potente & Carmeliet, 2017; Wellen & Thompson, 2010). Consequently, further studies are required to better understand mitochondrial ROS precise functions in ECs.

Also, targeting the regulator of mitochondrial biogenesis PGC-1 $\alpha$  has been thought as a possible strategy to improve vessel formation, due to reciprocal capacity of modulation between VEGF and PGC-1 $\alpha$  (Csiszar et al., 2009; Wright et al., 2008). Unexpectedly, in high glucose condition as in diabetes, PGC-1 $\alpha$  induction leads to the inhibition of angiogenesis (Pangare & Makino, 2012; Sawada et al., 2014). Therefore, although the implication of mitochondrial biogenesis is observed during vessel sprouting, its exact role remains to be assessed.

Finally, carboxyamidotriazole is a synthetic small molecule inhibitor of non-voltage-gated Ca<sup>2+</sup> channels that possess anti-angiogenic properties by blocking both the store-operated Ca<sup>2+</sup> entry and the intracellular Ca<sup>2+</sup> release. This drug has entered in phase I and II clinical trials both as a single cytostatic agent and in combination with cytotoxic therapies (Lodola et al., 2012). Recently, Pafumi et al. (2017) introduced the anti-angiogenic properties of naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one), a natural flavonoid as a novel inhibitor of the intracellular two-pore channel 2, implicated in the intracellular Ca<sup>2+</sup> signaling. By altering the intracellular Ca<sup>2+</sup> responses of human ECs stimulated with VEGF, naringenin inhibits *in vivo* angiogenesis. Even though inhibitors of Ca<sup>2+</sup> channels have been developed, the functional interplay between mitochondrial function and Ca<sup>2+</sup> signaling and homeostasis in ECs remains unclear (Stapor et al., 2014). Therefore, despite the fact that different mitochondrial-targeted compounds have been reported and developed, further investigations are needed to better understand the exact role of mitochondria in tumor angiogenesis, in order to improve their efficacy and specificity.

### 3.1.2. Glycolysis

**3.1.2.1. Angiogenesis regulator.** Glycolysis is a cellular mechanism allowing the formation of pyruvate from glucose, which can be metabolized and excreted into lactate, or penetrate into the mitochondria and integrate the TCA cycle to be oxidized. The glucose entry into the cell is allowed by the glucose transporters (GLUTs), in order to be converted by hexokinase into glucose-6-phosphate (G6P). G6P is then metabolized by the glycolytic pathway resulting in the formation of 2 pyruvates and 2 ATP. In comparison with mitochondrial respiration allowing the formation of 36 molecules of ATP from one glucose molecule, glycolysis contributes to a low ATP production. As a consequence, most differentiated cells generate ATP using OXPHOS, and glycolysis only becomes the primary ATP source in anaerobic conditions (Vander Heiden, Cantley, & Thompson, 2009). However, in ECs, glycolysis drives the production of >80% of ATP, even in the presence of sufficient oxygen levels for OXPHOS, and ECs are known to be the most glycolytic cells under physiological conditions (Dobrina & Rossi, 1983; Krützfeldt et al., 1990; De Bock, Georgiadou, Schoors et al., 2013). It is estimated that glycolysis flow is 200-fold more than that of mitochondrial OXPHOS and fatty acid oxidation in ECs (Marcu et al., 2017). This paradoxical characteristic is reinforced by the fact that ECs are directly in contact with oxygen present in blood.

Glucose is crucial for EC function for several reasons: (i) fast ATP generation by glycolysis allows ECs to quickly react to pro-angiogenic stimulations to rapidly form cellular protrusions, (ii) by shifting mitochondrial OXPHOS, ECs generate less ROS and therefore are preserved from a high and toxic ROS formation, (iii) glycolysis is an anaerobic mechanism allowing ECs to synthesize ATP under hypoxic conditions as long as glucose is accessible, (iv) in presence of high glucose levels,

the glycolytic pathway can synthesize higher ATP amounts and faster than when using the OXPHOS pathway (Locasale & Cantley, 2011; Vander Heiden et al., 2009), (v) glycolysis does not require oxygen, allowing the transfer of oxygen to perivascular cells, and (vi) glycolysis results in lactate production as a pro-angiogenic effector (Eelen et al., 2015; Treps et al., 2016). Accordingly, glycolysis is primordial for EC homeostasis, and a blockade of glycolytic activity triggers EC apoptosis and inhibits pathological angiogenesis (Merchan et al., 2010; Schoors et al., 2014; Xu et al., 2014). Microvascular ECs are more glycolytic than arterial ECs but less oxygen-consumer, highlighting the heterogeneity of glycolysis flow for each EC phenotype (Parra-Bonilla, Alvarez, Al-Mehdi, Alexeyev, & Stevens, 2010; De Bock, Georgiadou, & Carmeliet, 2013).

Quiescent phalanx ECs possess modest energetic requirements, and glycolysis is sufficient to support their needs (Marcu et al., 2017). Nevertheless, angiogenic ECs need a powerful glycolytic flow to increase their metabolic activity, and this flow is doubled in response to angiogenic stimuli such as VEGF stimulation, to initiate proliferation and migration (De Bock, Georgiadou, & Carmeliet, 2013; Stapor et al., 2014). Thus, ATP generation by glycolysis allows the development of cellular protrusions and the regulation of actomyosin contractility in intercellular junctions (De Bock, Georgiadou, Schoors et al., 2013; Moreno et al., 2014). The high ATP demand in lamellipodia and filopodia is illustrated by the elevated local presence of glycolytic enzymes in order to focus the ATP production at the level of these protrusions (De Bock, Georgiadou, & Carmeliet, 2013). In particular, GLUT1 and PFKFB3 are significantly overexpressed by proangiogenic effectors to accelerate glycolysis (Yeh, Lin, & Fu, 2008; De Bock, Georgiadou, & Carmeliet, 2013). Angiogenesis modulation by glycolysis is direct, without any genetic modulation, and angiogenic signals directly stimulate glycolytic proteins, confirming the close association between the two processes (Stapor et al., 2014). Finally, blood flow promotes EC quiescence by reducing the expression of the glycolytic enzymes PFKFB3 and phosphofructokinase-1 and consequently affecting glucose uptake and glycolysis (Eelen et al., 2015). Together, these findings highlight the pivotal and key role of glycolysis in the regulation of angiogenesis.

Cancer development is associated with high proliferation activity and an increased need for energy. Cancer cells are metabolically highly active and most of them use glycolysis as their main energetic supply, through the *Warburg effect*, which is a mechanism that relies on the uncoupling of glycolysis from oxygen levels (Warburg, 1956). Indeed, they increase their glycolytic flux even in presence of oxygen, while mitochondrial respiration is reduced (Keunen et al., 2011; Xu et al., 2014). Remarkably, cancer cells are able to adapt their metabolism to survive under conditions of reduced oxygen availability, low levels of nutrients and acidic extracellular pH, by increasing glycolysis to maintain the ATP production (Alfarouk et al., 2014). At a molecular level, the oncogenic genes *Ras*, *Akt* and *Myc* are implicated in the stimulation of the glucose consumption to promote glycolysis. Moreover, the tumor suppressor p53, which is inactivated in many cancers, is implicated in the modulation of glycolysis, in particular by regulating GLUT expression (Berkers, Maddocks, Cheung, Mor, & Vousden, 2013). It is well known that hypoxia and HIF-1 $\alpha$  related to tumor microenvironment dramatically increase various glycolysis regulators, including GLUT1 and glycolytic enzymes such as the hexokinase, phosphofructokinases, aldolases, phosphoglycerate kinases, enolases and lactate dehydrogenases (Shirato et al., 2011). Thus, the glycolytic energy metabolism of cancer cells is highly related to tumor initiation and progression (Jiménez-Valerio & Casanovas, 2017).

Therefore, cancer cells and ECs share the same glycolysis dependence with regard to energy metabolism. The glycolytic flux is comparable between both cell types, or even higher in ECs than in various cancer types (Verdegem et al., 2014). Finally, the link and similarities between these two cell types is attractive, considering that both can be targeted by anti-glycolytic compounds (Verdegem et al., 2014).

Among the glycolytic regulators, PFKFB3 is a bifunctional enzyme belonging to the PFKFB family possessing both kinase and phosphatase



activities. It allows the phosphorylation of fructose-6-phosphate (F6P) in fructose-2,6-biphosphate and the respective dephosphorylation. Nevertheless, the kinase activity is 700-fold stronger, and the underlying product fructose-2,6-biphosphate is an allosteric activator of PFK-1, being itself a glycolytic enzyme regulating one key glycolysis checkpoint (Sakakibara et al., 1997; Van Schaftingen, Lederer, Bartrons, & Hers, 1982). As a result, PFKFB3 is a rate-limiting glycolytic enzyme, and given that glycolysis is a major regulator of angiogenesis, PFKFB3 has been documented to be a powerful angiogenic regulator (De Bock, Georgiadou, Schoors et al., 2013). Actually, PFKFB3 influences EC differentiation into tip cells by increasing glycolytic flow, and PFKFB3 overexpression allows ECs to become endothelial tip cells. Accordingly, this enzyme regulates angiogenesis by controlling the balance between tip and stalk cells (Stapor et al., 2014). Interestingly, PFKFB3 overexpression induces a competition between stalk and tip cells to reach the tip position (De Bock, Georgiadou, Schoors et al., 2013). At a molecular level, VEGF enhances the expression of this glycolytic regulator (De Bock, Georgiadou, Schoors et al., 2013). Furthermore, PFKFB3 is a hypoxia inducible gene that is stimulated through HIF-1 $\alpha$  interaction with the promoter region of the gene (Fukasawa et al., 2004; Obach et al., 2004). Besides its glycolysis regulation, PFKFB3 also modulates the cyclin-dependent kinase 1, linking glucose metabolism with cell proliferation and survival in tumor cells (Yalcin et al., 2014). Chesney et al. (2005) have confirmed the importance of this glycolysis regulator using PFKFB3 null mice, which are embryonically lethal at embryonic day 8.

**3.1.2.2. Anti-angiogenic treatment targeting glycolysis.** Considering the pertinence of glycolysis and its key regulator PFKFB3 in controlling angiogenesis, inhibiting this pathway and this protein emerge as a promising anti-angiogenic approach against pathologies associated with an excessive angiogenesis. As only 2.5% of ATP in the body is synthesized by glycolysis, strategy inhibiting this pathway should contribute to minor adverse systemic effects than therapies targeting ATP production.

Thereby, the 2-deoxy-D-glucose (2DG) is one of the most extensively studied inhibitor of glycolysis. It is a glucose analog in which the hydroxyl group in position 2 is replaced by a hydrogen, in order to inhibit subsequent glycolysis (Aft, Zhang, & Gius, 2002). 2DG diffusion is facilitated into cells via glucose transporters and is phosphorylated by hexokinase, trapped inside the cell, but then not further metabolized by glycolysis. 2DG accumulates in the cell and inhibits phosphoglucose isomerase, until its dephosphorylation by phosphorylase (Aft et al., 2002). As a consequence, 2DG permanently and maximally inhibits not only glycolytic pathway, but also all downstream glycolytic trafficking. Through this mechanism, 2DG can suppress proliferation, induce apoptosis and reduce migration of murine ECs in an *in vitro* model. 2DG, by inhibiting the formation of cellular protrusions and by inducing the disorganization of F-actin filaments (Huang et al., 2015), reduces angiogenesis in both *in vitro* and *in vivo* models (Merchan et al., 2010; Schoors et al., 2014). Moreover, 2DG can target both ECs and cancer cells in mouse models of retinoblastoma and neuroblastoma. Indeed, ECs is sensitive to 2DG as shown by the reduced number of tumor vessels, but also through its ability to inhibit tumor proliferation by down-regulating cancer cell growth and metabolism, acting on HIF-1 $\alpha$ , PDK1, c-Myc and phospho-BAD (Huang et al., 2015; Merchan et al., 2010). Many reports have been completed evaluating the anti-tumor properties of 2DG in preclinical settings, and clinical trials have been initiated for multiple cancers (Cheong et al., 2011; Dasgupta et al., 2017; Goldberg, Israeli, & Kloog, 2012; Malm, Hanke, Gill, Carbajal, & Baker, 2015). Due to its permanent glycolysis inhibition and pleiotropic properties, 2DG also alters several intracellular mechanisms, notably decreasing ATP content and triggering EC autophagy, ROS formation, endoplasmic reticulum (ER) stress and inducing EC apoptosis via CHOP activation (Merchan et al., 2010; Missiaen et al., 2017; Wang, Liang, Shirwany, & Zou, 2011). Nevertheless, 2DG effects on the tumor

vasculature are poorly studied (Huang et al., 2015; Merchan et al., 2010). Recently, in a model of human pancreatic ductal adenocarcinoma, it has been described that the inhibition of TAM glycolysis by 2DG induces a reversion of the pro-angiogenic properties of these cells (Penny et al., 2016).

Among the emergent anti-glycolytic strategies, PFKFB3 inhibition appears to be a novel and attractive approach as it reduces the hyperglycolysis needed for EC sprouting, without affecting the basal glycolysis necessary for EC homeostasis. Notably, the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) reduces glycolysis *in vivo* by approximately 40%. This reduction impairs EC proliferation and migration, without inducing EC death, but favors cell quiescence (Schoors et al., 2014). On the contrary to other glycolytic inhibitors such as the 2DG, this reduction is temporary and incomplete, allowing the targeting of pathologic angiogenesis without affecting basal EC homeostasis and inducing extensive damages (Schoors et al., 2014). This transient glycolysis reduction can be explained by the short half-life (30 min) and rapid clearance (2312 ml/kg/min) of 3PO (Clem et al., 2013). Interestingly, the use of 3PO does not affect protein glycosylation, energy charge, or oxygen consumption (Schoors et al., 2014). 3PO displays also promising anti-angiogenic effects in ocular disease models in mice representative of human age-related macular degeneration, and also against intersegmental vessel branching in zebrafish embryos (Schoors et al., 2014). Furthermore, 3PO has been studied in inflammation models such as skin epithelial hyperplasia and inflammatory bowel disease (Schoors et al., 2014). Due to this property, studies regarding the effect of 3PO against cancer development are now developed. In particular, Cantelmo et al. (2016) have shown that 3PO allows the normalization of tumor vessels and improves vessel maturation and perfusion. Indeed, decreasing glycolysis induces a reduction of the hyper proliferation and migration of ECs, and thereby conducts to phalanx metabolism. This approach could induce therapeutic benefits and could increase the delivery and response to chemotherapy (Cantelmo et al., 2016). Therefore, 3PO could represent a potential tool to inhibit tumor growth. Indeed, it has been shown that administration of 3PO to tumor-bearing mice markedly reduces (i) the intracellular concentration of fructose-2-6-biphosphate, (ii) glucose uptake and (iii) growth of established tumors (Clem et al., 2008).

PFK15 (1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one) is another potent and selective PFKFB3 inhibitor (Zhu et al., 2016). Abnormal glycolysis contributes to joint inflammation in various pathophysiological processes such as RA. PFKFB3 is overexpressed in RA patients and PFK15 has shown promising results to attenuate the expression of key proinflammatory cytokines associated with a potential joint destruction (Zou et al., 2017). Moreover, aberrant glycolysis is implicated in several chronic inflammatory processes, and PFKFB3 inhibition has been underscored to fight against different pathologies such as inflamed skin or colon and sepsis-related acute lung injury (Gong et al., 2017; Schoors et al., 2014). With regard to tumor growth, PFK15 displays antitumor activity in various models. For example, in gastric cancer models, Lewis lung carcinomas and lung adenocarcinoma, PFK15 has been described to induce cell apoptosis, cell cycle block and cell invasion inhibition (Clem et al., 2013; Li et al., 2018; Zhu et al., 2016). Furthermore, in neck squamous cell carcinoma, PFK15 suppresses tumor growth and metastasis (Li et al., 2017). PFK15 also serves as a platform for the synthesis of a derivative, PFK158, which is used in phase I clinical trials (NCT02044861) (Clem, O'Neal, Klarer, Telang, & Chesney, 2016). However, the direct implication of PFK15 and PFK158 on tumor angiogenesis is still unknown.

Pharmacological inhibition or genetic deletion of PFKFB3 reduces both the sprouting and the capacity of ECs to reach the sprout tip (De Bock, Georgiadou, Schoors et al., 2013). PFKFB3 inhibition using the *Cre-Lox recombination* inhibits pathological angiogenesis in various murine models, such as oxygen-induced retinopathy (Xu et al., 2014). By the same way, PFKFB3 knockdown reduces protrusions formation in ECs (De Bock, Georgiadou, Schoors et al., 2013).

Finally, PFKFB3 is particularly an attractive target to treat cancer development because it has been demonstrated to be overexpressed in most of the tumors (Atsumi et al., 2002). Accordingly, EC-specific genetic silencing of PFKFB3, as well as pharmacological inhibition reduces tumor glycolysis and growth (Chesney et al., 1999; Clem et al., 2008; De Bock, Georgiadou, Schoors et al., 2013). PFKFB3 blockade in cancer development has shown exciting anti-angiogenic properties and is currently being tested as a novel anticancer treatment in phase I clinical trials (Conradi et al., 2017). Further pre-clinical data of PFKFB3 inhibitors are required to clarify its potential application for clinical use. The mechanisms depicting the anti-angiogenic treatments targeting glycolysis are recapitulated on Fig. 5.

**3.1.2.3. Limits of anti-glycolytic treatments.** Although anti-glycolytic compounds have shown promising results against angiogenesis, several limits do emerge. By inhibiting the glycolytic pathway, 2DG and other anti-glycolytic compounds also considerably alter downstream vital glucose metabolism, such as the hexosamine biosynthesis pathway and the pentose phosphate pathway. Furthermore, 2DG reduces the cellular ATP content and favors the mitochondrial ROS formation, leading to autophagy in EC models (Culic, Gruwel, & Schrader, 1997; Wang et al., 2011). To improve their effects, high levels of compounds are necessary raising toxicity issues, in particular for 2DG that has to compete with high mM level of glucose in the blood (Schoors et al., 2014). Even though 2DG has first shown promising anti-tumor results in pre-clinical models, further studies have demonstrated that it activates many survival pathways in a model of non-small cell lung cancer through the insulin-like growth factor-1 receptor/PI3K/Akt trafficking (Zhong et al., 2009). In addition, tumor cells develop a chemo-resistance against 2DG mediated by HIF-1 $\alpha$ , and 2DG is only effective under normoxic conditions (Maher, Wangpaichitr, Savaraj, Kurtoglu, & Lampidis, 2007; Niccoli, Boreham, Phenix, & Lees, 2017). Moreover, Kurtoglu et al.

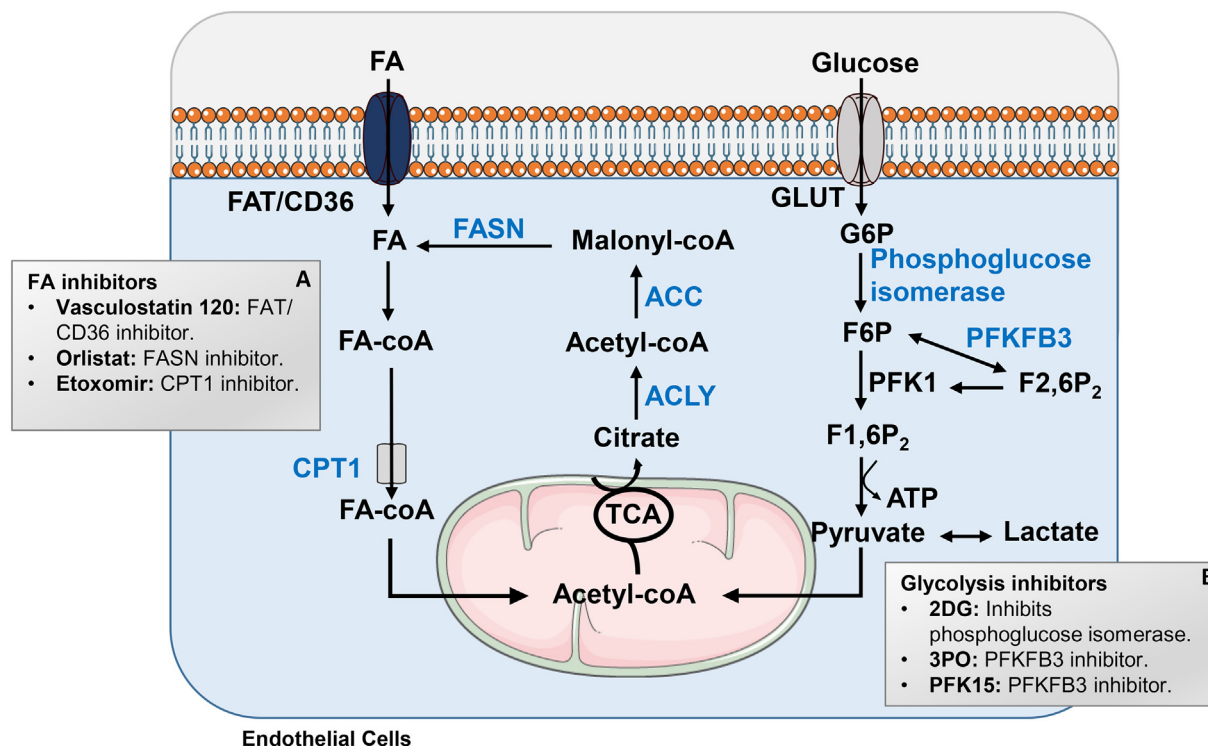
(2007) suggest that the mechanism of action of 2DG differs depending on cancer cell types, and could rather be mediated by N-linked glycosylation than glycolysis. Finally, anti-glycolytic compounds such as 2DG are publicly contested, limiting their exploitation. However, in combination with other agents, they could be more effective and represent new anti-angiogenic strategies (Granchi & Minutolo, 2012).

Targeting PFKFB3 is another anti-glycolytic strategy that allows the modulation of the hyper glycolysis in a transient manner. In this respect, 3PO affects pathological cells without altering healthy ECs, leading to a decreased angiogenesis. Nonetheless, it has recently been shown that used at high concentrations, 3PO aggravates tumor hypoxia and compromises vascular barrier integrity, thereby rendering tumor vessels leakier and facilitating cancer cell intravasation and dissemination (Conradi et al., 2017). Besides, 3PO treatment has immunosuppressive functions, and PFKFB3 inhibition can reduce the *in vitro* activation of T cells inducing *in vivo* suppression of immunity (Telang et al., 2012). Furthermore, some debates around the most effective PFKFB3 inhibitor between 3PO and PFK15 emerge, and Zhu et al. (2016) recently underline that the pharmacological activity of 3PO cannot be uniquely attributed to the inhibition of PFKFB3.

### 3.1.3. Fatty acid metabolism

FA metabolism, which is regulated by FA synthesis, oxidation, import and export, is essential for ECs mostly during angiogenesis (Schoors et al., 2015). However, the relative contribution of FA metabolism to the function of both quiescent and angiogenic ECs is not completely elucidated (Teuwen, Geldhof, & Carmeliet, 2017).

**3.1.3.1. Fatty acid synthesis.** ECs possess the machinery allowing *de novo* FA synthesis, although FAs from blood stream are continuously accessible. Indeed, ECs express (i) ATP citrate lyase that produces acetyl-CoA from citrate, (ii) acetyl-CoA carboxylase that converts acetyl-CoA into



**Fig. 5. Anti-angiogenic treatments targeting glycolysis and fatty acid metabolism.** (A) The inhibition of FA metabolism leads to an alteration of angiogenesis. Vasculostatin 120 decreases the active transport of FA into the cell through its inhibitory activity on FAT/CD36 transporter, whereas orlistat specifically inhibits FASN enzyme, resulting in a reduction of free FA levels into the cell. Targeting FAO is another alternative strategy. In this aim, etoxomir has been specifically synthesized to inhibit the enzyme responsible for the transport of FA into the mitochondria, CPT1, leading to a reduction of angiogenesis. (B) One of the most emerging strategy aims to block angiogenesis using anti-glycolytic drugs. 2DG specifically inhibits phosphoglucose isomerase, the enzyme responsible of the conversion of G6P into F6P, leading to a persistent and maximal alteration of all glycolytic pathways. Inhibition of PFKFB3 by both 3PO and PFK15 compounds also decreases glycolysis and subsequent angiogenesis, in a transient and incomplete manner.



malonyl-CoA and (iii) fatty acid synthase (FASN), a multi-enzyme complex that catalyzes the synthesis of long-chain FA such as palmitate from malonyl-CoA and acetyl-CoA. However, the relevance of FA synthesis for vascular sprouting and angiogenesis is poorly investigated (Browne, Hindmarsh, & Smith, 2006; Seguin et al., 2012; Wei et al., 2011).

It has been described that endothelial FASN is required for normal palmitoylation of eNOS and its absence results in chronic inflammation and defective angiogenesis (Wei et al., 2011). Indeed, ECs lacking FASN show deficient migratory capacity and mice with endothelial inactivation of FASN exhibit impaired reparative angiogenesis when subjected to hindlimb ischemia (Wei et al., 2011).

**3.1.3.2. Fatty acid uptake and transport.** ECs uptake FAs from the bloodstream as fatty esters in lipoproteins or albumin-bound FAs. Intracellularly, FAs are bound by tissue-specific cytoplasmic FA-binding proteins (FABPc), which act as an intracellular counterpart of plasma albumin (Glatz & Luiken, 2017). The uptake of FAs occurs either *via* passive diffusion (Stahl, Evans, Pattel, Hirsch, & Lodish, 2002) or through active transport (Ehehalt et al., 2008). Passive diffusion, which is still debated, consists in the ability of protonated and therefore uncharged FA molecules to flip from the external to the internal membrane face (Mashek & Coleman, 2006). It is dependent on a gradient of free FAs, determined by their levels inside and outside the cell (Harjes, Kalucka, & Carmeliet, 2016). In contrast, the active transport is more described and involves several transporters including FABPc, plasma membrane FABP (FABPpm), FA translocase (FAT)/CD36, long-chain fatty acyl-CoA synthase, and FA transport proteins (FATP) (Mashek & Coleman, 2006). The vascular endothelium expresses FAT/CD36, FABP4, FATP3, FATP4, FABP3, and FABP5 (Antohe et al., 1998; Elmasri et al., 2009; Greenwalt, Watt, Hasler, Howard, & Patel, 1990; Masouyé et al., 1997; Watanabe, Wakabayashi, Veerkamp, Ono, & Suzuki, 1993).

FAT/CD36 is a multifunctional transmembrane protein acting as a receptor for long chain FAs that can also bind thrombospondin, collagen and low-density lipoproteins (Endemann et al., 1993; Watanabe et al., 1998). In comparison to other FA transporters, its expression is high in cardiac ECs, indicating a relevant FA uptake in the heart (Coppello et al., 2015; Harjes et al., 2016).

FABP4 role is well described in angiogenesis. In ECs, its expression is induced by the activation of VEGF-A/VEGFR2 in a Notch-DLL4 signaling dependent manner (Harjes, Bridges, McIntyre, Fielding, & Harris, 2014). Loss of FABP4 reduces proliferation, migration and sprouting of ECs (Cataltepe, Arian, Liang, Smith, & Cataltepe, 2015; Elmasri et al., 2009; Elmasri et al., 2012; Harjes et al., 2014); and increases ROS production. Indeed, FAs deprived of their chaperone FABP4 enhance FAO caused by the need to reduce unbound FAs, leading to mitochondrial respiration enhancement and subsequent ROS production (Harjes et al., 2017).

VEGF-B induces expression of FATP3 and FATP4 in ECs, indicating that VEGF-B is involved in trans-endothelial delivery of FAs in the heart and skeletal muscles (Hagberg et al., 2010; Hagberg, Mehlem, Falkevall, Muhl, & Eriksson, 2013).

Finally, FABP5 expression is not regulated by VEGF-A or FGF-2 (Eelen et al., 2018). Its deficiency reduces EC proliferation and chemotactic migration and confers resistance to apoptotic cell death (Yu et al., 2016).

**3.1.3.3. Fatty acid oxidation.** FAO is the process by which imported FAs are metabolized. In ECs, <5% of generated ATP comes from FAO (De Bock, Georgiadou, & Carmeliet, 2013), which is not essential for maintaining energy homeostasis. Nevertheless, stalk cells are dependent on FAO for sprouting angiogenesis (Schoors et al., 2015).

The addition of an acetyl-CoA moiety to FAs allows their import into mitochondria *via* the rate limiting enzyme carnitine palmitoyltransferase 1 (CPT1). In the mitochondria, FAs undergo  $\beta$ -oxidation, producing acetyl-CoA which further integrates the TCA cycle. Entry of FA-derived acetyl-CoA, in conjunction with an anaplerotic substrate, sustains the TCA cycle to produce aspartate, used for deoxynucleotide triphosphates

synthesis and essential for DNA replication in proliferating stalk cells (Draoui, de Zeeuw, & Carmeliet, 2017; Schoors et al., 2015). EC-specific deletion of CPT1 decreases EC proliferation and triggers sprouting defects both *in vitro* and *in vivo* (Schoors et al., 2015). Furthermore, FAO and CPT1 have been shown to regulate lymphangiogenesis (Wong et al., 2017). Indeed, fully functional FAO in LECs promotes lymphatic vessel growth and stimulate LECs proliferation by fueling the TCA cycle and promoting deoxynucleotide triphosphates synthesis for DNA replication. Also, FAO stimulates differentiation from vessel to LECs through histone acetylation and epigenetic mechanisms (Wilting et al., 2002).

**3.1.3.3.1. Targeting fatty acid metabolism for angiogenic therapies.** Novel therapeutic approaches aim to exploit recently uncovered key targets involved in FA synthesis, transport and oxidation to modulate pathological vascular outgrowth (as summarized on Fig. 5).

Orlistat, a FASN inhibitor currently approved for obesity treatment, reduces proliferation and sprouting of ECs, by decreasing phosphorylation of VEGFR2 and its localization to the plasma membrane (Browne et al., 2006). Other FASN inhibitors, orlistat and cerulenin, decrease lung colonization of melanoma cells and the peri-tumor blood vessels by inducing VEGF splice variants with putative anti-angiogenic properties (Seguin et al., 2012). Different FASN inhibitors have been shown to reduce tumor size in several cancer types (Flavin, Peluso, Nguyen, & Loda, 2010). Moreover, FASN silencing in colorectal cancer cells decreases tumor vascularization, along with the reduction of MMP9 and VEGF expression levels (Zaytseva et al., 2014). However, the therapeutic potential of FASN blockade has been challenged by the induction of cachexia (Yang et al., 2015), and to date, only the FASN inhibitor TVB-2640 led to clinical trials for anti-cancer therapy (Jones & Infante, 2015; Peck & Schulze, 2016).

Inhibition of FAT/CD36 with vasculostatin 120 reduces angiogenesis both *in vitro* and *in vivo*, but it is not clear whether this anti-angiogenic effect is due to specific inhibition of long chain FA transport (Missiaen et al., 2017).

Etomoxir, a small 2-oxiranecarboxylate derivative, is an irreversible inhibitor of CPT1 (Agius, Meredith, & Sherratt, 1991) and has beneficial therapeutic effects by reducing EC proliferation and pathological angiogenesis in a mouse model of ocular retinopathy (Schoors et al., 2015). Another report showed that etomoxir increases the leakiness of vessels as a result of perturbed  $Ca^{2+}$  homeostasis (Patella et al., 2015).

Whether targeting FA synthesis, transport and oxidation might be a valuable therapeutic strategy to inhibit pathological angiogenesis require further work.

### 3.2. Glycosylation

Glucose is not only an essential source for ATP generation, but is also a critical precursor of nucleotide sugars implicated in glycosylation pathways. The glycome comprises a large diversity of molecules. Glycans are one of the basic components of cells, with many fundamentals roles in many biological processes, from cellular communication to differentiation, immunity, and vascularization. The complexity of the glycome reflects its functional importance in the homeostasis of biological systems (Bard & Chia, 2016; Moremen, Tiemeyer, & Nairn, 2012; Ohtsubo & Marth, 2006).

#### 3.2.1. Glycosylation: General overview

Glycosylation is a nutrient-sensitive protein modification allowing the formation of abundant and diverse glycans (mono- or oligosaccharides) that are attached to proteins and lipids, to form glycoconjugates (Ohtsubo & Marth, 2006). This modification is typically post-translational and irreversible, apart from the O-linked  $\beta$ -N-acetylglucosamine (O-GlcNAc) modification (Stowell, Ju, & Cummings, 2015). The regulation of glycosylation is monitored by both glycosyltransferases and glycosidases linking glycans to polypeptidic chains, by adding and hydrolyzing glycosidic linkages. At least 9 of the 20 amino acids composing proteins can be modified by a variety of carbohydrates, ranging

from a single monosaccharide to glycan chains containing hundreds of monosaccharides (Stowell et al., 2015). Glycosylation contributes to the stability, solubility, localization, trafficking and function of the glycoproteins, glycolipids, and proteoglycans. The glycosylation process begins in the ER by adding monosaccharide or polysaccharide linkage and continues in the Golgi apparatus to undergo secondary tissue-specific modifications (Boscher, Dennis, & Nabi, 2011). Such protein modifications are complex mechanisms that influence many cellular processes, including the cell survival, differentiation and angiogenesis. Several environmental stimuli, including hypoxia, nutrients and inflammation, can regulate glycosylation by masking the glycoepitopes of endogenous lectins (Cerliani, Blidner, Toscano, Croci, & Rabinovich, 2017; Johnson, Jones, Ryan, & Cobb, 2013). In eukaryotes, half of all known proteins are glycosylated and most of glycoproteins are secreted or integrated into the cell membrane, such as growth factors receptors, cytokines receptors, nutrient transporters, integrins and cadherins (Gu & Taniguchi, 2008; Li et al., 2014). It is estimated that about 80% of the cell membrane is composed of glycoconjugates including glycoproteins, glycolipids and proteoglycans (Allam et al., 2017).

**3.2.1.1. The hexosamine biosynthesis pathway.** The hexosamine biosynthesis pathway (HBP) derives from the glycolysis pathway and produces substrates for glycosylation.

After its uptake in ECs by GLUT transporters, glucose is converted into G6P by glycolysis. Whereas the majority of G6P remains in the glycolytic pathway, a portion is metabolized in the pentose phosphate pathway that produces nucleotides that are structural components of diverse nucleotide sugars, such as uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) (Shirato et al., 2011). Most of G6P is converted to F6P and metabolized by the glycolysis pathway. Between 1 and 3% of F6P integrates the HBP under physiological conditions (Cheng et al., 2006). Indeed, HBP branches out from the glycolysis pathway at the level of F6P, which is then converted into glucosamine-6-phosphate by the glutamine-fructose-6-phosphate amido-transferase. Glucosamine-6-phosphate is then rapidly acetylated in GlcNAc-6-phosphate (GlcNAc-6-P) by glucosamine-6-phosphate *N*-acetyltransferases and isomerized to *N*-GlcNAc-1-phosphate (GlcNAc-1-P). Finally, GlcNAc-1-P is uridinylated by the action of UDP-GlcNAc pyrophosphorylases to form UDP-GlcNAc. UDP-GlcNAc is then translocated into the Golgi apparatus and serves as a substrate donor for *N*- and *O*-linked glycosylation reactions for the synthesis of glycoconjugates (Schleicher & Weigert, 2000; Shirato et al., 2011).

**3.2.1.2. Glycosylation subtypes.** Glycosylation can be divided into different subtypes, depending on the glycans anchoring site on proteins, producing diverse glycosylation patterns.

*C*-glycosylation is the addition of an  $\alpha$ -mannopyranosyl residue to tryptophan residue via a C—C bond (Krieg et al., 1998). Weakly documented, it was first thought to concern isolated proteins such as IL-12 and ribonuclease 2 (Doucey, Hess, Blommers, & Hofsteenge, 1999; Doucey, Hess, Cacan, & Hofsteenge, 1998). A wide variety of natural products are *C*-glycosylated, and display numerous biological activities. However, the potential implication of *C*-glycosylation in angiogenesis remains unknown (Hultin, 2005; Yang & Yu, 2017).

*N*-glycosylation is the specific binding of glycans to the amide residue of an asparagine in Asn- Xaa-Ser/Thr motifs (Schachter, 2000). It begins in the ER where a precursor composed of 9 mannoses, 3 glucoses and 2 GlcNAc is synthesized on dolichol phosphate and then transferred to the asparagine residue of a polypeptidic chain by the oligosaccharide transferase. In the Golgi, neoglycoproteins undergo other posttranslational modifications including secondary glycosylations. The final core structure of the *N*-linked chains is composed by 2 GlcNAc and 3 mannoses residues, which can be supplemented by other glycans, depending on glycoprotein subtype. The addition of galactose to the terminal GlcNAc allows the production of a minimal disaccharide ligand (Boscher et al., 2011). The importance of *N*-glycosylation in angiogenesis is well documented (Croci, Cerliani, Dalotto-Moreno et al., 2014;

Elola, Blidner, Ferragut, Bracalente, & Rabinovich, 2015; Chandler, Leon, Meyer, Rahimi, & Costello, 2017).

*O*-glycosylation is the specific and covalent bond of glycans onto an oxygen atom of a Ser or a Thr residue (Schachter, 2000). The first monosaccharide that connects with this oxygen atom is the *N*-acetylglucosamine (GalNAc). This initiating GalNAc core can be extended into various different structures with galactose, GlcNAc, or sialic acid to generate the core 1, core 3, or sialylated GalNAc respectively (Bard & Chia, 2016; Clausen & Bennett, 1996). The addition of a galactose in a  $\beta$ 1,3-linkage, followed by the addition of a GlcNAc creates a core *O*-glycan widely expressed in mammalian cells (Ju, Brewer, D'Souza, Cummings, & Canfield, 2002; Schwientek et al., 1999; Schwientek et al., 2000). Although alterations of *O*-glycosylation are well described in tumor development, its implication in angiogenesis is still poorly investigated.

In addition, mammalian cells contain other subtypes of glycoconjugates. Among them, proteoglycans are characterized by the binding of one or more glycosaminoglycan (GAG) to the core of the neoprotein. GAGs are composed by the repetition of a disaccharide containing a hexosamine (GlcNAc, GlcNS or GalN) and another ose (glucuronic acid, iduronic acid, or galactose). Glycosphingolipids are composed of a glycan structure linked to a lipid tail that contains the sphingolipid ceramide. The core structure composed by a glucose or a galactose can be next extended with the addition of further monosaccharides, allowing the formation of amphiphilic molecules (Pinho & Reis, 2015). Gangliosides, a class of glycosphingolipids carrying one or several sialic acid residues, are known to regulate VEGFR signaling, and could interfere with angiogenesis (Julien, Bobowski, Steenackers, Le Bourhis, & Delannoy, 2013).

**3.2.1.3. Regulation of glycosylation.** *O*-linked and *N*-linked glycosylation are the most described glycosylation pathways. They are strongly regulated at different stages, including (i) the control at transcriptional/epigenetic levels of glycosyltransferases and glycosidases synthesis, (ii) the regulation of glycosyltransferases or glycosidases access to their specific substrates (iii) and the spatial compartmentalization of glycosylation-associated components (Cerliani et al., 2017).

Glycosyltransferases catalyze the addition of glycans to proteins to form neoglycoproteins. Their abundances as well as their localization are key to regulate glycosylation and each branch of the glycan tree may be modified by a particular glycosyltransferase. Conversely, a given glycosyltransferase may be responsible for impacting the glycosylation of many different glycoproteins (Stowell et al., 2015). In particular, the attachment of GlcNAc to *N*-glycans using UDP-GlcNAc donor is catalyzed by the enzymatic activity of *N*-acetylglucosaminyltransferases (GnTs or Mgat) localized in the medial-Golgi apparatus. The GlcNAc-branching of *N*-glycans successively depends on GnT-I, GnT-II, GnT-IV and GnT-V, each enzyme possessing a decline affinity for UDP-GlcNAc (Boscher et al., 2011). Each GnT has a high specificity for a hydroxyl in the core glycan, and favors the branching in a sequential and ordered manner (Schachter, 1986). In particular, mannosyl- $\alpha$ 1,6-glycoprotein  $\beta$ 1,6-*N*-acetylglucosaminyltransferase (GnT-V or Mgat5) encoded by the *Mgat5* gene, is crucial for the 4th branch to catalyze the linkage of GlcNAc to a core  $\alpha$ 1,6-mannose in *N*-glycan intermediates (Boscher et al., 2011). Localized in the medial-Golgi, and then in the trans-Golgi on newly synthesized glycoproteins, this branching selectively generates tri (2,2,6)- and tetra (2,4,2,6) antenna-like oligosaccharides, intermediates of complex type *N*-glycans (Hassani et al., 2017).

**3.2.1.4. The *N*-acetylglucosaminyltransferases galectins axis.** Currently, three main subtypes of glycan-binding proteins are described to regulate immunity and vascularization: the *C*-type lectins, sialic acid-binding Ig-like lectins, and galectins (Gals) (Cerliani et al., 2017). While the first two modulate glycoproteins and glycolipids on the cell membrane of immune cells and ECs, Gals can act in both intra- and extracellular pathways of these cells (Cerliani et al., 2017).

Gals are a family of 15 endogenous glycan-binding proteins in mammals, which can be localized at the plasmic membrane surface, in the cytoplasm or into the nucleus. These lectins share consensus amino acid sequences and possess a high affinity for  $\beta$ -galactoside residues in glycans attached to N-glycans, O-glycans and glycolipids (Thijssen, Rabinovich, & Griffioen, 2013). The interaction with glycan structures on glycoconjugates is allowed by their carbohydrate-recognition domain (CRD), a consensus sequence of 130 amino acids. The orientation, rotational flexibility, and spacing of the CRDs facilitate the specific detection of glycans (Elola et al., 2015). Gals possess one or two CRDs within a single polypeptidic chain and are classified into three families. Gal-1, Gal-2, Gal-5, Gal-7, Gal-10, Gal-11, Gal-13, Gal-14 and Gal-15 are the common Gals, possessing one CRD which can be active as a monomer or as a homodimer. Gal-4, Gal-6, Gal-8, Gal-9 and Gal-12 belong to the 'tandem-repeat' subtype and display two CRDs which are active in monomeric and oligodimeric forms (Elola et al., 2015). Gal-3 is the only Gal to possess a CRD connected to a non-lectin N-terminal region responsible for oligomerization (Rabinovich & Conejo-García, 2016). They can either be released in the extracellular medium and specifically interact with glycosylated partners in the cell membrane, or act intracellularly. Gals activity is directly dependent of GnT: silencing GnT-V selectively eliminates responsiveness to Gal-1 in ECs. This Gal-glycan interaction occurs with a glycoprotein localized in the same cell or in a neighboring one. It allows either a limiting receptor internalization and the maintenance of downstream signaling sensitivity at the cell membrane or the monitoring of intracellular signaling pathways (Boscher et al., 2011). Gals preferentially bind to the glycoprotein disaccharide *N*-acetyl-lactosamine and can also recognize different modifications of this saccharide (D'Haene et al., 2013).

Gals are commonly reported to regulate immunity. Critical immune factors are modulated by Gals (such as TGF- $\beta$  receptor, pre-B cell receptor or T cell receptor), influencing the activation, signalization and survival of immune cells (Bonzi et al., 2015; Demetriou, Granovsky, Quaggin, & Dennis, 2001; Partridge et al., 2004). Cerliani et al. (2017) have recently listed the implication of Gals in both myeloid and lymphoid immune programs. The immunosuppressive activity of Gals induces local and systemic immunosuppression and represents a major obstacle to cancer treatment by slowing down the progress of cancer immunotherapy (Labrie, De Araujo, Communal, Mes-Masson, & St-Pierre, 2017).

### 3.2.2. Glycans: Relevance in angiogenesis

Lately, much attention has been given to the glycosylations of Gals in angiogenesis. Gals arose as key regulators of neovascularization, but how this is done is not yet fully elucidated. A distinct set of glycosylation related genes has been linked to the angiogenesis process and it is now increasingly admitted that glycans are integral to different events in the angiogenesis cascade (Munkley & Elliott, 2016).

Glycosylation is known to influence vascular biology by controlling several EC processes including trafficking, endocytosis, as well as connection between blood and lymphatic vessels (Fu et al., 2008; Xu, Fuster, Lawrence, & Esko, 2011). By maintaining EC survival and controlling vascular permeability, glycans also control neovascularization (Kitazume et al., 2010; Xu et al., 2011). The relevance of glycosylation in ECs can be illustrated by the significant expression of L-Phytohemagglutinin-L-reactive GnT-V-modified *N*-glycans and poly-LacNAc ligands in primary human umbilical vein endothelial cells (HUVECs) after FGF-2 stimulation (Elola et al., 2015). Indeed, GnT-V is a strong mediator of angiogenesis and its removal completely abolishes EC proliferation, migration and tube formation (Deng et al., 2017). Interestingly, the inhibition of both N- and O-glycosylation does not affect VEGF-A-mediated neovascularization, highlighting the VEGF-A-independent regulation of angiogenesis by GnT-V (Elola et al., 2015).

Glycosylation frequently occurs on master angiogenic regulators such as platelet endothelial cell adhesion molecule, integrins and VEGFR2. The extracellular domain of VEGFR2 contains seven

immunoglobulin (Ig)-like domains, each one with at least one N-glycosylation sites. N-glycosylation of these domains plays a central role in the regulation of the stability of the extracellular domain as well as VEGF binding and trafficking (Chandler et al., 2017). Specifically, Chandler et al. (2017) demonstrated that at least 15 of the 17 N-glycosylation motifs in the VEGFR2 extracellular domain are glycosylated. Among them, domains 2 (sites N145, N160) and 3 (sites N247) are implicated in VEGF binding. Similarly, VEGFR2 Ig-like domains 4 and 7, which enable the homodimer stabilization after ligand binding, receptor dimerization and allosteric regulation, have been described to be strongly glycosylated (Chandler et al., 2017). Markowska, Liu, and Panjwani (2010) showed that these glycosylation sites regulate VEGFR2 membrane retention and pathway independently of VEGF activation.

The Notch pathway mediates EC sprouting and proliferation by regulating the tip and stalk balance *via* a mechanism potentially influenced by glycosylation. In particular, the Notch1 extracellular domain is O-glycosylated with different types of carbohydrates, including Asp-linked N-glycans and several O-glycans such as O-fucose (Moloney et al., 2000). This glycosylation status regulates Notch activity and modifies the responsiveness of DLL4 and Jagged 1 ligands (Benedito et al., 2009; Takeuchi & Haltiwanger, 2014). Also, the glycosylation mediated by GnT-III is described to increase Notch receptor expression and activity. It has been recently demonstrated that suppression of GnT-III results in a relocation of Notch receptor to the lysosome, resulting in reduced Notch receptor expression levels and an impairment of gamma secretase-mediated activation (Allam et al., 2017). Recently, the Notch signaling has been shown to regulate FAs transport across the endothelium and to act as an essential repressor of angiogenesis in the adult heart (Jabs et al., 2018).

In addition, the glycosylation pattern of cell surface glycoproteins plays relevant roles in mediating cell-cell and cell-matrix interactions and regulating migration and adhesion of ECs during angiogenesis (Abbott et al., 2008). Indeed, ECs are closely linked to the extracellular matrix which is a rich source of glycoproteins such as laminin and fibronectin that are the scaffold for angiogenesis processes like cellular adhesion. The implication of MMPs in angiogenesis and the degradation of all kinds of extracellular matrix proteins is now well described, and both MMP2 and MMP9 are regulated by GnT-V (Deng et al., 2015). MMP9 is glycosylated on two positions, Asn38 and Asn120; the glycosylated MMP9 with Asn38 interacts with Gal-8 that could facilitate the temporal activation of pro-MMP9 in MMP9 (Kumar & Cieplak, 2018). Another Gal, Gal-3 was described to accumulate at cell-cell junctions with neural cadherin and the ganglioside GM1, disrupting them and thereby enhancing neovascularization (Boscher et al., 2012). On the other hand, numerous analyses have indicated that GnT-V dramatically impacts cell behavior *via* the regulation of  $\beta$ 1,6-GlcNAc branching on integrins (Granovsky et al., 2000; Hakomori, 2002). Accordingly, oxidative stress induces the overexpression of GnT-V *via* the regulation of the FAK-ERK signaling pathway, which in turn affects the function of ECs (Deng et al., 2017).

The glycan-Gal interaction is widely designated to regulate vascular signaling programs, and Gals emerged like potent angiogenic modulators. Gal-1, Gal-3, Gal-8 and Gal-9 are the most expressed in ECs. Whereas Gal-8 is still poorly studied, Gal-1 and Gal-3 are the most described to promote angiogenesis and are implicated in pathological vascularization (Méndez-Huergo, Blidner, & Rabinovich, 2017; Stanley, 2014). Both lectins favor all steps leading to angiogenesis *in vitro*, including activation, proliferation, migration, tube formation and sprouting (Griffioen & Thijssen, 2014). The importance of Gal-1 in angiogenesis is illustrated in the zebrafish model, in which Gal-1 knock-down results in impaired vascular guidance (Thijssen et al., 2006). Furthermore, hypoxia, the main angiogenic stimulus is a direct activator of Gal-1, and controls EC signaling (Crocì et al., 2012; Hsieh et al., 2008).

Angiogenesis regulation by Gals is mediated by their direct control of several angiogenic factors such as Notch1, integrins  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 5 $\beta$ 1 and



VEGFR2 (Croci, Cerliani, Dalotto-Moreno et al., 2014; Chen et al., 2016). Gal-1 is reported to retain VEGFR2 on the plasma membrane of HUVECs and to promote segregation of the receptor into membrane micro domains. This prolongs its cell-surface residency, either in the presence or in the absence of VEGF and enhances its intracellular pathway (Croci, Cerliani, Pinto et al., 2014). VEGFR2 possesses many potential N-glycan sites. In particular, domains 3, 4, and 7 of the receptor are necessary for Gal-1 to optimally bind to the receptor (Stanley, 2014). The GnT-V enzyme is also described to enhance the bond between both proteins. Gal-1 also interacts with NRP1, activating VEGFR2 signaling and modulating EC migration and angiogenesis (Hsieh et al., 2008). VEGFR2 phosphorylation is also improved in the presence of Gal-1 and correlates with the activation of the Akt/ERK1/2 pathway, by the same mechanism than the VEGF-A. Thus, in the absence of VEGF-A, Gal-1 delivers pro-angiogenic signals through a glycosylation-dependent pathway involving complex N-glycans (Elola et al., 2015).

Gal-3 has also been demonstrated to modulate angiogenesis in several models, such as in Gal-3-deficient (Lgals3<sup>-/-</sup>) mice, in which VEGF-A failed to induce angiogenesis (Markowska et al., 2010). In an identical manner to Gal-1, Gal-3 is reported to retain VEGFR2 at the plasma membrane after its linkage to N-glycan receptors and to regulate receptor internalization in a carbohydrate-dependent manner (Markowska, Jefferies, & Panjwani, 2011). VEGF- and FGF-2-mediated angiogenesis is also modulated by Gal-3 after its binding to  $\alpha\text{v}\beta\text{3}$  integrin (D'Haene et al., 2013; Markowska et al., 2010; Markowska et al., 2011). Besides its action on VEGFR2 segregation, the addition of Gal-3 also enhances the phosphorylation of the receptor in a time- and concentration-dependent manner (Elola et al., 2015).

### 3.2.3. Aberrant glycosylation in cancer growth

Given the complexity and importance of glycosylations in cellular homeostasis, it is not surprising that alterations of these mechanisms are involved in diverse pathological processes, including tumor development. Aberrant glycosylation patterns are well described in initial oncogenic transformation and further cancer progression (Stowell et al., 2015). Indeed, glycosylation alterations can significantly impact the localization and stability of cell surface receptors and their sensitivity to a broad range of signaling molecules and ligands, leading to dysregulations of many tumor processes including adhesion, migration, cellular division, proliferation, differentiation, metastasis and angiogenesis (Häuselmann & Borsig, 2014; Li et al., 2014; Pinho & Reis, 2015; Saito et al., 2002).

#### 3.2.3.1. Abnormal glycosylation in cancer growth.

Characterization of the biological functions of each glycan, glycosidases and glycan-binding proteins is of major interest to the cancer field (Pinho & Reis, 2015). The most frequently reported glycosylation modifications in tumor development concern O- and N-glycans, which occur both in early and late stages of cancer progression and metastasis. The alteration of glycans can be attributed to a disruption of the expression or the activity of glycosyltransferases, leading either to the generation of different core glycans or to the modification of the degree of core glycan branching (Stowell et al., 2015). Increased branching evoked by GnT is the most frequent alteration in tumor progression and correlates with metastatic potential (Yamamoto et al., 2000). Among them, GnT-V overexpression is the main GnT subtype described in numerous tumor models, which is regulated by oncogene expression (Asada, Furukawa, Segawa, Endo, & Kobata, 1997; Chen, Zhang, Fregien, & Pierce, 1998; Partridge et al., 2004; Pocheć, Lityńska, Amoresano, & Casbarra, 2003). The correlation between increment of  $\beta\text{1,6-GlcNAc}$ -branched N-glycans products of GnT-V activity and tumor invasiveness has been largely described. GnT-V overexpression is associated with malignancies and correlates with cell migration, invasion, and metastatic properties (Demetriou, Nabi, Coppolino, Dedhar, & Dennis, 1995; Guo, Lee, Kamar, Akiyama, & Pierce, 2002; Yamamoto et al., 2000). Gliomas notably express highly variable levels of GnT-V mRNA, and the

corresponding enzymatic activity changes through the course of glioma genesis (Hassani et al., 2017). Furthermore, GnT-V-deficient mice display suppression of tumor growth and metastasis (Elola et al., 2015). Interestingly, GnT-V<sup>-/-</sup> tumor cells display a reduction of responsiveness to several growth factors like PDGF, FGF-2, epidermal growth factor, insulin-like growth factor and fetal bovine serum when compared with GnT-V<sup>+/+</sup> cells (Partridge et al., 2004). These authors also demonstrated that the number of N-glycosylation sites is correlated to the diminution of receptor sensitivity in GnT-V<sup>-/-</sup> cells. However, these correlations do not apply to all types of tumors. In fact, in hepatocellular carcinoma, low levels of GnT-V are associated with a high recurrence rate of the tumor (Ito et al., 2001; Li et al., 2014). GnT-V is also associated with low malignant potential and good prognosis of different tumor models (Dosaka-Akita et al., 2004; Inamori et al., 2006; Ishimura et al., 2006; Wang et al., 2013). In the same line, Shirato et al. (2011) disclosed that intracellular levels of UDP-GlcNAc are reduced after exposition to hypoxia, confirming that regulation of HBP by hypoxia is still largely unknown. GnT-III is also upregulated in epithelial ovarian cancer and this leads to an increase of growth and metastasis, favoring the expansion of the ovarian cancer stem cell population (Abbott et al., 2008; Allam et al., 2015; Allam et al., 2017). Besides GnT, other glycosylation enzymes correlate with tumor growth. In a pancreatic tumor model, inhibition of the oncogene Kras decreases glutamine-fructose-6-phosphate amidotransferase expression, HBP flux, and subsequent protein glycosylations. The latter leading to a decrease of tumor volume, suggesting a potent regulation of tumor growth by glycosylation (Verdegem et al., 2014). An aberrant expression of the O-linked glycosylation enzyme polypeptide N-acetyl-galactosaminyl-transferase 6 has also recently been observed in several cancer types, while the biological role of this enzyme *in vivo* remains unknown (Lavrsen et al., 2018; Zhang & Ten Hagen, 2018). Finally, the mannosidase MAN1A1, by affecting the adhesive properties of breast cancer cells, correlates with a diminution of survival in patients (Legler et al., 2018).

The tumor microenvironment disrupts many recognizing sites by masking or unmasking specific glycol-epitopes for endogenous Gals (Cerliani et al., 2017). Thus, altered expression of different Gals has been reported in several cancer types. Gal-1 is the most highly expressed Gals in many tumors (Demidenko & Berest, 2009), and notably in human prostate cancer cell lines where Gal-1 expression increases with the prostate cancer stage and tumor progression (Stanley, 2014). Gal-1 also influences tumor cell migration of glioma both *in vitro* and *in vivo* (Camby et al., 2001; Camby et al., 2002; Camby, Le Mercier, Lefranc, & Kiss, 2006). Comparatively, Gal-3 and Gal-9 expressions are more tumor type-dependent. A restricted expression of Gal-3 is observed in well-differentiated thyroid carcinomas compared to normal and benign thyroid conditions (Bartolazzi, Sciacchitano, & D'Alessandria, 2018), whereas Gal-9 is correlated with malignancy in chronic lymphocytic leukemia patients (Taghiloo et al., 2017). Finally, overexpression of Gals in cancer induces pro-tumor processes like proliferation, epithelial to mesenchymal transition, tumor immune evasion and angiogenesis (Martinez-Bosch, Vinaixa, & Navarro, 2018).

#### 3.2.3.2. Dysregulation in tumor angiogenesis.

Recently, much attention has been given to glycosylation and its role in tumor angiogenesis. Although its exact role in sprouting and angiogenesis is not well established, endothelial glycans are disrupted after changes in cytokines, growth factors and hypoxic conditions and correlate with the activation of pro-angiogenic signaling (Croci, Cerliani, Pinto et al., 2014).

Lately, the role of Gals in the modulation of tumor neovascularization is a growing interest in the cancer field (Funasaka, Raz, & Nangia-Makker, 2014; Thijssen & Griffioen, 2014). Among Gals, Gal-3 is disrupted in the tumor stroma, leading to a decrease of macrophage-induced angiogenesis dependent on VEGF and TGF- $\beta$  signaling (Machado et al., 2014). Gal-3 is overexpressed in several cancer cell lines, recruited to cell-cell junctions to directly interact with N-glycans on neural

cadherin, destabilizing cell-cell junctions and focal adhesions, thus promoting tumor EC migration. Moreover, Gal-3 overexpression has recently been linked to the JAG1/Notch1 signaling pathway, with potential direct implications for the development of tumor angiogenesis (Dos Santos et al., 2017). An increased Gal-9 expression levels in the vasculature of different tumors is also demonstrated, including carcinomas of the lung, liver, breast and kidney tissues (Heusschen, Schulkens, van Beijnum, Griffioen, & Thijssen, 2014). In contrast with other Gals, Gal-1 expression is specifically triggered by hypoxia, anti-VEGF therapy and EC activation with serum or tumor conditioned medium (Croci, Cerliani, Pinto et al., 2014; Thijssen & Griffioen, 2014). Gal-1 is overexpressed in ECs of different human tumors and correlates with increased tumor vascularization (Stanley, 2014; Thijssen et al., 2006). Moreover, Gal-1 silencing in prostate cancer cells reduces angiogenesis in a matrigel plug model (Laderach et al., 2013). Gal-1 is overexpressed under hypoxic conditions and modulates the expression of 7 hypoxia-related genes implicated in angiogenesis (Le et al., 2005; Le Mercier et al., 2008). Interestingly, in tumors refractory to current anti-VEGF treatments, glycosylation-dependent interaction between VEGFR2 and Gal-1 is implicated in perpetuating VEGFR2-dependent angiogenic activity, independently of VEGF (Stanley, 2014; Croci, Cerliani, Pinto et al., 2014). This observation is illustrated by the deficit of  $\beta$ 1,6-GlcNAc-branched N-glycans in ECs or the silencing of tumor-derived Gal-1, which converted refractory into anti-VEGF-sensitive tumors (Croci, Cerliani, Pinto et al., 2014).

Gals activities are connected to glycosyltransferase and even if glycosyltransferase implication on angiogenesis is not well documented, several clues argue for their implication. In fact, GnT-V and Gal-1 are closely linked, it is therefore not surprising that the two proteins are implicated in pathological angiogenesis, while the regulation of glycosyltransferase expression determines the susceptibility to the linkage of Gal-1. It was first suggested that GnT-V implication in angiogenesis could be related to a glycosylated-independent pathway (Nakahara et al., 2006; Saito et al., 2002). It is now described that N-glycosylation mediated by GnT-V activates VEGFR2 pathway in tumors in the absence of ligand binding (Very, Lefebvre, & El Yazidi-Belkoura, 2018). In absence of GnT, there is an insufficient linkage of Gal-1 to VEGFR2 that reduces its capacity to promote angiogenesis and therefore reduce tumor growth (Stanley, 2014). In GnT-V knockout mice, refractory tumors become sensitive to anti-VEGF treatment, reinforcing the implication of this enzyme in the aggressivity of tumors (Croci, Cerliani, Dalotto-Moreno et al., 2014). The hypoxic microenvironment in tumors shifts the pattern of intracellular glucose metabolism and increases the amounts of  $\beta$ 1,6-GlcNAc-branched N-glycans structures which are products of GnT-V. All of these lead to the expression of the entire repertoire of glycans by ECs, critical for the induction of pathological angiogenesis (Elola et al., 2015). Indeed, the overexpression of GnT-V in tumor ECs enhances the glycosylation status of VEGFR2 and thus tumor angiogenesis.

Finally, the heparan sulfate proteoglycans also play a pivotal role in angiogenesis by facilitating the binding of cell surface pro-angiogenic

growth factors (Munkley & Elliott, 2016). Cole, Rushton, Jayson, and Avizienyte (2014) have described that heparan sulfate is a major regulator of the angiogenic program in ovarian cancer cells, impacting EGF receptor signaling and subsequent expression of angiogenic cytokines by cancer cells.

### 3.2.4. New anti-angiogenic therapies targeting glycosylation

Dysregulated glycosylation is associated with exacerbated angiogenesis and targeting glycosylation may then represent a good candidate to develop new anti-angiogenic therapies. Considering the ubiquity of glucidic structures in biologic processes and their bioactive conformation and molecular interactions, many mimicking derivatives with potential therapeutic properties have been synthesized in the past decades (Magnani & Ernst, 2009). Several glycomimetics already display promising therapeutic results in various pathologies, independently of angiogenesis regulation, such as type 2 diabetes, viral and bacterial infectious diseases and cardiovascular pathologies (Hudak & Bertozzi, 2014). Recently, some studies reported that these drugs act as potent therapeutic agents against different pathologies with excessive angiogenesis (Table 3).

Two main modifications of glycosides have been extensively performed (i) the replacement of an intracyclic oxygen by another heteroatom, which has been extensively documented in the synthesis of phosphor-sugars and imino-sugars (Dale, Ensley, Kern, Sastry, & Byers, 1985; Hanaya & Yamamoto, 2013; Ichikawa & Ichikawa, 1995); and (ii) the replacement of the hydroxyl residue in the anomeric position by a carbon group (C-glycosid and in particular C-aryl glycosid) (Dondoni, Zuurmond, & Boscarato, 1997; Palmacci & Seeberger, 2001; Parrish & Little, 2002; Rousseau & Martin, 2003). Indeed, many enzymatic reactions involving the anomeric carbon and the generation of glycomimetic drugs with the replacement of this carbon by another heteroatom display potential beneficial effects.

Gal-1 is now a common target to alter pathological angiogenesis and several studies have highlighted its therapeutic potential. Knockdown of Gal-1 using siRNA administration has shown promising results especially in glioblastoma, in which intranasal siGal-1 nanoparticle injection reduces the vascular diameter of the tumor microenvironment. The authors hypothesize that this effect could be directly the consequence of the anti-angiogenic effects of siGal-1 (Van Woensel et al., 2017). Beside Gal-1 inhibition in glioblastoma, several others inhibitors have emerged for the targeting of melanoma, ovarian, breast and colon cancers (Ito et al., 2011; Ito et al., 2012; Le et al., 2005). Among them, Anginex is a cytokine-like peptide ( $\beta$ pep-25) designed from the structure of the  $\beta$ -sheet domains of anti-angiogenic agents (platelet factor-4, IL-8, and bactericidal-permeability increasing protein 1). Anginex inhibits tumor growth and angiogenesis in human hepatoma (Dings et al., 2007; Dong et al., 2009) by directly binding Gal-1 and then enhancing its affinity for glycoproteins. However, this drug possesses a short stability, is difficult to synthesize and only sensitizes ECs to radiotherapy in newly forming tumor vessels and not in tumor cells (Wdowiak et al., 2018). Anginex reduces also endometriosis-like lesion formation

**Table 3**  
New anti-angiogenic therapies targeting glycosylation.

Compounds	Targets	Effects	Reference
siRNA	Gal-1	Decrease vessel diameter in glioblastoma	Van Woensel et al., 2017
Anginex	Gal-1	Decrease angiogenesis in human hepatoma	Dong et al., 2009
Anginex	Gal-1	Decrease vessel densities in endometriosis-like lesions	Nap et al., 2005
OTX008	Gal-1	Tumor vessel normalization in human head and neck squamous cell carcinoma models	Koonce et al., 2017
Swainsonine	GnT-V	Decrease <i>in vitro</i> EC angiogenesis	Croci, Cerliani, Dalotto-Moreno et al., 2014
Tunicamycin	UDP-N-acetylglucosamine:dolichyl-phosphate N-acetylglucosaminophosphotransferase	Decrease angiogenesis in mouse breast tumor	Banerjee et al., 2011
Itraconazole	VEGFR2	Decrease vascular density and tumor growth	Bae et al., 2017
Itraconazole	VEGFR2	Antiangiogenic properties in a laser-induced choroidal neovascularization in rats	Bae et al., 2017

through the decrease of vessel densities in the surrounding chorioallantoic membrane in a chicken model (Nap et al., 2005). Most recently, OTX008, an allosteric inhibitor of Gal-1 binding to a site away from the lectin's carbohydrate binding site, was shown to attenuate lactose binding to the lectin. OTX008 promotes tumor vessel normalization, increases tumor oxygenation and improves pericyte coverage in human head and neck squamous cell carcinoma models (Koonce, Griffin, & Dings, 2017). Based on these data, a phase 1 clinical trial using OTX008 is initiated on advanced solid tumors (NCT01724320).

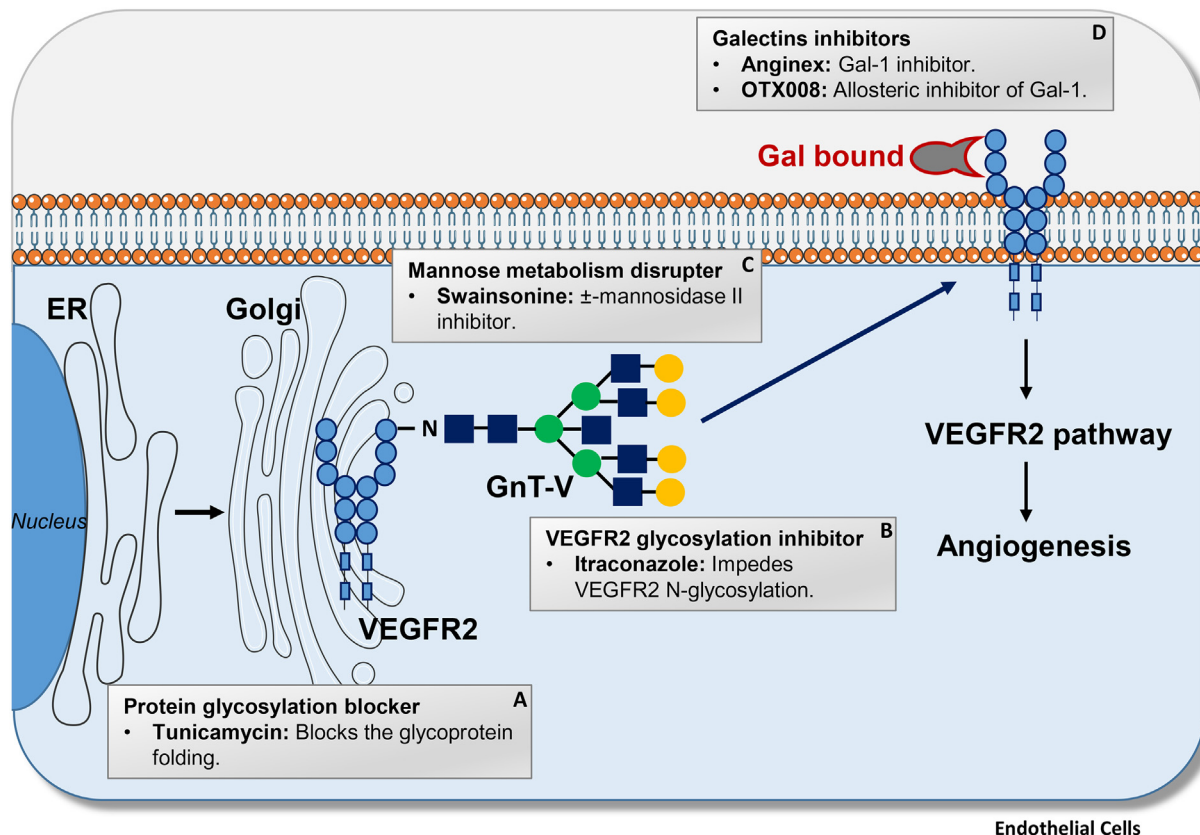
Swainsonine is an indirect GnT-V inhibitor that targets Golgi  $\alpha$ -mannosidase II, resulting in the blockade of the N-glycan GlcNAc branching and preventing the processing by GnT-V. Consequently, swainsonine abrogates almost completely the Gal-1 binding to ECs. The addition of swainsonine results in a decrease in capillary density in a model of caprine placenta, associated with a great distortion to the vasculature (Hafez, Caceci, Freeman, & Panter, 2007). Furthermore, this treatment prevents almost completely the *in vitro* EC proliferation, migration, and tube formation induced by Gal-1 (Croci, Cerliani, Dalotto-Moreno et al., 2014). Interestingly, swainsonine is able to reverse the radio resistance of the human nasopharyngeal carcinoma CNE-2R cells, suggesting that it may be used as an adjuvant to decrease radio resistance (Wu et al., 2015). However, swainsonine treatments can also cause reproductive and developmental toxicities in mice in both parents and offspring (Wu et al., 2016).

Tunicamycin is a glucosamine-containing pyrimidine nucleoside known to alter the lipid-linked oligosaccharide synthesis in the ER and thereby to block the glycoprotein folding in the ER (Brewer, Hendershot, Sherr, & Diehl, 1999). Tunicamycin inhibits the enzyme UDP-N-acetyl-glucosamine:dolichyl-phosphate N-acetyl-glucosamine-phosphotransferase, which catalyzes the first step of N-linked

glycosylation in the ER membrane (Yoo et al., 2018). This alteration leads to a stable and irreversible inhibition of *in vivo* angiogenesis due to the down-regulation of phosphorylated VEGFR1, VEGFR2 and VEGF (165)-specific phosphor-tyrosine kinase activity leading to reduced breast tumor growth in mice (Banerjee et al., 2011). More broadly, inducing ER stress triggers the activation of the unfolded protein response leading to the activation of numerous pathways and in particular the release of angiogenesis factors, as well as MMP extracellular matrix components or cytokines (Wang & Kaufman, 2014).

Itraconazole is an antifungal drug with anti-angiogenic properties that affect multiple endothelial pathways including VEGF and FGF-2-mediated angiogenic stimulation, modulation of the mammalian target of rapamycin signaling and impeding the maturation of N-linked sugars of VEGFR2 (Shi et al., 2011). A clinical trial using itraconazole in non-small cell lung cancer is in early phase 1 (NCT02357836). Recently, itraconazole has been encapsulated into albumin nanoparticles to increase its solubility and to therefore enhance the tumor inhibition activity of paclitaxel, highlighting the therapeutic potential of this drug in combination with current treatments (Zhang et al., 2017). Itraconazole displays also antiangiogenic properties in a laser-induced choroidal neovascularization in rats reflected by a downregulation of VEGFR2 mRNA and protein abundances (Bae et al., 2017).

In conclusion, glycomimetics form a novel class of therapeutics (Magnani & Ernst, 2009), their targets being summarized on Fig. 6. These drugs have higher affinities and drug-like properties with a longer half-life in serum compared to functional carbohydrates. They also are characterized by metabolic stability, low toxicity and oral bioavailability (Magnani & Ernst, 2009). Although glycomimetic drugs offer a variety of advantages, several limitations should be considered. For instance the variations of physicochemical properties of glycosylated



**Fig. 6. New anti-angiogenic therapies targeting glycosylation.** (A) Tunicamycin directly acts at the endoplasmic reticulum (ER) level by blocking the subsequent folding of neoglycoproteins, leading to a ER stress and inhibiting angiogenesis. (B) VEGFR2 N-glycosylation is altered by itraconazole which disrupts the maturation of N-linked sugars on this receptor. (C) Alterations on mannose metabolism also disrupt glycoprotein branching. Swainsonine inhibits  $\alpha$ -mannosidase II enzyme and thus alters the N-glycan GlcNAc branching. (D) Galectins (Gals) are potent angiogenic mediators and emerging drugs target these glycan-binding proteins. Anginex specifically inhibits Gal-1 although it possesses a short stability, whereas OTX008 reduces the lactose binding to the Gal-1.



drug delivery, such as the surface charge, size, distribution and biocompatibility (Kawakami & Hashida, 2014). Moreover, the lack of cell specificity hampers their application and causes unexpected side effects (Yu, Zhang, & Li, 2009). Nevertheless, glycomimetics are becoming a promising tool for achieving effective therapy for cancer and other pathologies characterized by aberrant angiogenesis.

For clinical use, further innovations are essential regarding the discovery of many pathophysiologically important carbohydrate-protein interactions and the development of more-practical preparation methods for glycosylated drugs (Cai et al., 2018).

#### 4. Conclusion

Throughout this review, we highlighted the emerging role of endothelial metabolism in angiogenesis-related pathologies with emphasis on tumor vessel growth. Among EC metabolism pathways, glycosylation could play a central role in the development of new anti-angiogenic strategies. By acting on glycosylation, these new drugs are able to reduce the vessel density in tumors and importantly, to normalize tumor vessels, favoring the improvement of tumor oxygenation and the facilitation of immuno- and chemotherapies (Batchelor et al., 2013). New molecules specifically targeting glycosylation may open novel possibilities for treatment of diseases associated with excessive angiogenesis.

Lymphangiogenesis therapy is another attractive therapeutic approach. Several preclinical anti-lymphangiogenesis therapies have been devised such as siRNA against VEGF-C (Chen et al., 2005), monoclonal antibodies against VEGF-C (e.g. VGX-100; Falchook et al., 2013), VEGFR31-Ig (Zhang et al., 2010), and monoclonal antibodies against VEGFR3 (Roberts et al., 2006) or against its co-receptor NRP2 (Caunt et al., 2008).

However, further *in vivo* studies need to be performed to evaluate potential side effects and drug resistance. In this respect, the combination of anti-angiogenic therapies with drugs directly acting on tumor cells are needed to improve the efficacy of the current treatments. The idea of exploiting EC and LEC metabolism is opening new avenues in pharmacological treatment of pathologies with abnormal angiogenesis.

#### Conflict of interest statement

The authors declare that there are no conflict of interest.

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### 3. Glioblastome

A travers cette revue, nous avons pu observer la difficulté pour trouver des traitements anti-angiogéniques efficaces, notamment dû à une toxicité importante, et au développement fréquent d'une résistance tumorale face à ces thérapies (Vandekeere et al., 2015 ; Brandes et al., 2015). En adaptant leur métabolisme, de nombreuses tumeurs deviennent insensibles à ces thérapies, et restent hautement agressives et meurtrières. Ceci est particulièrement vrai pour les tumeurs cérébrales qui sont associées à un faible pronostic chez les patients, et constituent une forte cause de mortalité. Parmi celles-ci, les gliomes, qui sont les tumeurs issues des cellules gliales, représentent la moitié des tumeurs cérébrales primaires chez l'adulte (Ricard et al., 2012). Au sein des gliomes, les astrocytomes correspondent aux tumeurs prenant naissance dans les astrocytes, cellules de support et de protection des neurones. La forme la plus commune et la plus sévère d'astrocytome est le glioblastome multiforme (GBM) (Schwartzbaum et al, 2006, Babouri et al, 2015). Il représente une forme hautement maligne de gliome, et est classé par l'OMS au plus haut grade d'agressivité parmi les gliomes, le grade IV. Particulièrement malin et invasif, il représente 12 à 15 % des tumeurs cérébrales (Schwartzbaum et al, 2006). Il touche environ 3 à 5 cas sur 100 000 personnes avec une prédominance chez les hommes. L'âge médian lors du diagnostic est de 64 ans et les patients âgés de plus de 70 ans sont touchés dans 25 % des cas de GBM (Ung et al., 2015).

#### 3.1. Origine du GBM

Le GBM est une tumeur décrite comme sporadique dans la grande majorité des cas, apparaissant de façon isolée. Dans de rares situations en revanche, ce cancer a été corrélé avec la présence de certains syndromes génétiques chez les patients, tels que le syndrome de Turcot, la neurofibromatose, ou encore le syndrome Li Fraumeni (Kyritsis et al., 2010).

Plusieurs mutations dans les astrocytes ont été proposées comme étant à l'origine du développement du GBM. En particulier, ce cancer a récemment été corrélé dans 80 % des cas avec des mutations sur le promoteur du gène *TERT*, codant la transcriptase reverse des télomères, impliquée dans le maintien de la taille des télomères (Killela et al., 2013). En absence de *TERT*, les télomères se raccourcissent à chaque division cellulaire, induisant une sénescence cellulaire. Les mutations observées dans le cadre des GBM, en créant un site de fixation aux facteurs de transcription E-twenty six, restaurent l'expression de *TERT* (Nencha et al., 2016). Il a également été proposé que le développement du GBM pourrait trouver sa source au niveau d'une délétion

du gène *NFKB1A* (codant le facteur nucléaire du polypeptide  $\kappa$ -light, gène activateur de l'inhibiteur- $\alpha$  dans les cellules B), inhibiteur de la voie du récepteur du facteur de croissance épidermal (EGF) (Bredel et al., 2011). Enfin, plusieurs études ont proposé des mutations au niveau des gènes *IDH1* et *IDH2* codant pour les isocitrate déshydrogénases 1 et 2. Ces enzymes catalysent la formation d' $\alpha$ -cétoglutarate à partir d'isocitrate au niveau du cycle de Krebs. En particulier, la mutation d'*IDH1* inhiberait son activité catalytique, ce qui réduirait les niveaux d' $\alpha$ -cétoglutarate et conduirait à une stabilisation du facteur induit par l'hypoxie HIF-1 $\alpha$ , un puissant médiateur pro-angiogénique (Zhao et al., 2009).

### 3.2. Agressivité du GBM

Le GBM est une tumeur généralement localisée au niveau des hémisphères cérébraux, dans la substance blanche (Fig. 7). Elle peut se situer aux niveaux temporal, pariétal, frontal, et occipital. De plus, elle est de nature multicentrique, à savoir qu'elle est souvent associée dans plusieurs foyers, typiquement aux niveaux temporal et frontal. Cette tumeur possède un aspect irrégulier (d'où son appellation multiforme), et est très diffuse, sans réelle démarcation avec les tissus voisins.

Le GBM est particulièrement invasif, il se diffuse très rapidement dans le cerveau, et peut envahir le corps calleux, permettant de s'étendre aux ganglions de la base, au thalamus, ou encore à l'hémisphère opposé. Une des particularités du GBM par rapport aux autres gliomes concerne son caractère nécrotique important (Raza et al., 2004). Celui-ci génère une importante hypoxie, qui régule les propriétés invasives et agressives de cette tumeur, en particulier au moyen de l'angiogenèse tumorale. Le GBM fait partie des tumeurs solides les plus vascularisées, ce qui participe fortement à son agressivité, et une augmentation de la vascularisation aggrave de manière significative le pronostic des patients (Brem et al., 1972 ; Lebelt et al., 2008). L'hypervascularisation du GBM survient majoritairement grâce à l'angiogenèse, mais peut également se manifester par plusieurs autres alternatives telles que, (i) le mimétisme vasculaire, correspondant à la différenciation des CTs en CEs (Das & Marsden, 2013), (ii) la co-option vasculaire des CTs autour du réseau vasculaire, (iii) la vasculogenèse, impliquant la différenciation des CPEs en CEs, et enfin (iv) la transdifférenciation de CTs en CEs, bien que ce dernier mode soit encore mal décrit (Hardee & Zagzag, 2012). L'ensemble de ces mécanismes démontre l'hétérogénéité de la vascularisation du GBM, et mettent en avant son importance pour la progression tumorale.

Le GBM se manifeste par différents symptômes incluant nausées, vomissements, céphalées, somnolence ou encore crises d'épilepsies. Selon l'emplacement de la tumeur, les patients peuvent également développer d'autres symptômes tels que la faiblesse d'un côté du corps, une perte de la mémoire progressive et/ou d'élocution, ainsi que des perturbations visuelles. Le diagnostic du GBM nécessite un scanner ou une imagerie par résonance magnétique (IRM) du cerveau (Fig. 7).

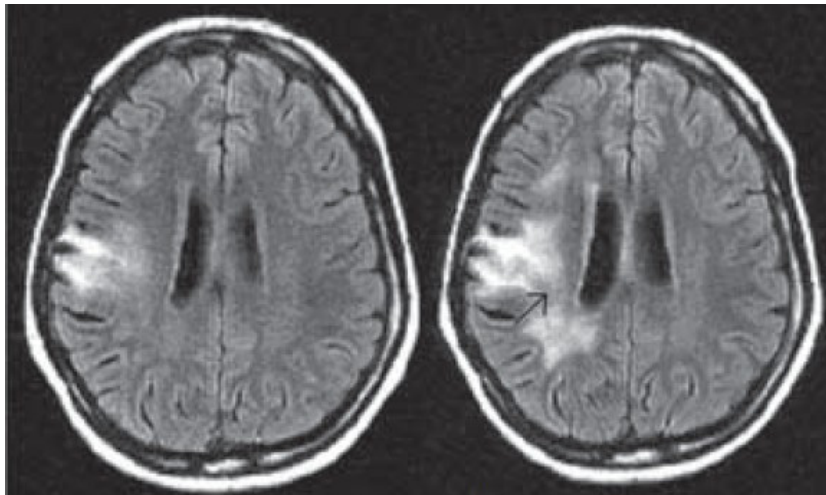


Fig. 7 : IRM d'un GBM au niveau de la substance blanche de l'hémisphère cérébral gauche (Anand et al., 2012).

### 3.3. Traitements anti-tumoraux actuels

Les traitements actuels sont palliatifs et reposent sur une résection de la tumeur, qui doit être la plus totale possible, mais souvent rendue impossible par la localisation profonde du GBM, son caractère diffus et hautement invasif. Cette ablation tumorale est complétée post-opératoire par des thérapies adjuvantes. La chimiothérapie permet l'administration locale ou systémique d'un agent cytostatique ou cytotoxique. Le principal composé utilisé actuellement contre le GBM est le témozolomide (Temodar®), qui est un agent alkylant de l'acide désoxyribonucléique (ADN), entraînant l'apoptose des CTs. Cette chimiothérapie est combinée à une radiothérapie, qui vise à détruire les CTs par irradiations (Ung et al., 2015). Les traitements approuvés par la FDA incluent également le bevacizumab, prescrit en cas de récurrence pour altérer l'angiogenèse tumorale, ainsi que le carmustine (gliadel®), et le lomustine (CCNU, Ceenu®), agents alkylants nitrosourées de l'ADN et de l'acide ribonucléique (ARN).

Pour l'ensemble de ces traitements, et malgré les avancées en médecine et en chirurgie ces dernières décennies, la médiane de survie est de 12 à 15 mois, et le taux de survie est seulement de 5 % à 5 ans (Clarion et al, 2014). Le caractère récidiviste du GBM dû à la migration et l'invasivité des CTs, le faible passage des drogues à travers la barrière hémato-encéphalique, mais également le développement d'une résistance tumorale et la forte toxicité des traitements utilisés en traitement clinique représentent différents obstacles à leur utilisation (Bergers & Hanahan, 2008). Il apparaît donc crucial de développer de nouvelles thérapies anti-tumorales et anti-angiogéniques contre le GBM.

#### **4. Les phostines**

##### **4.1. Les glycomimétiques**

Dans le cadre du GBM, les thérapies actuelles ne permettent donc pas un traitement efficace et définitif de la progression tumorale. Afin de créer des thérapies à la fois plus spécifiques et moins toxiques, nous avons pu observer à travers la revue précédente que cibler le métabolisme endothélial et notamment la glycosylation représente une stratégie prometteuse. C'est une régulation métabolique surexprimée dans les CTs et dans les CEs activées, et dont dépendent moins les cellules quiescentes.

Afin d'interférer avec la glycosylation, l'utilisation de glycomimétiques (structures chimiques mimant des glucides biologiques/endogènes) a été testée pour lutter contre différentes pathologies telles que le diabète de type II, la maladie d'Alzheimer, ou la maladie de Gaucher (Hudak & Bertozzi, 2014). Bien que peu d'approches concernant les glycomimétiques soient développées pour un traitement anti-tumoral, le processus de glycosylation est étroitement lié à la progression tumorale, la migration et l'échappement au système immunitaire (Stowell et al., 2015).

La synthèse de certains glycomimétiques comme l'ertugliflozine (steglatro®), récemment approuvé par la FDA contre le diabète de type II (Markham, 2018), repose sur la formation de composés avec une conformation de type bicyclo. Celle-ci correspond à la greffe d'un second cycle sur le sucre, afin de favoriser sa stabilité à la fois chimiquement et biologiquement. Physiologiquement, la cyclisation d'un sucre est un état transitoire, durant lequel un carbone devient asymétrique ou anomérique. Le sucre peut ainsi se présenter sous deux configurations ou anomères au niveau de ce carbone anomérique. Cependant, cet équilibre est instable, et le carbone anomérique s'ouvre et se ferme en passant par la forme linéaire. Afin de répondre à cette problématique et synthétiser des composés cycliques plus stables, le carbone anomérique du sucre

a été remplacé par un groupement phosphoré dans ces glycomimétiques, afin d'augmenter leur stabilité et ainsi perturber plus efficacement la glycosylation (Haas et al., 2014).

#### 4.2. La famille des phostines

En se basant sur le rationnel glycomimétiques présenté ci-dessus, une nouvelle famille de composés a été spécifiquement synthétisée afin d'évaluer leurs activités dans le cadre d'un traitement anti-tumoral contre différentes lignées, et en particulier contre le GBM.

Les phostines ou phosphinosucres sont une famille de glycomimétiques, basés sur une structure de type hexose, sucre à 6 atomes de carbone. Ce sont des composés cycliques, analogues des hexopyranoses (forme pyranose cyclique d'un hexose), qui possèdent un squelette hétérocyclique 1,2-oxaphosphinane (Fig. 8). En particulier, un atome de phosphore (P) est introduit en position 2 en lieu et place du carbone anomérique, afin de favoriser la stabilité des phostines. A partir de cette structure de base, plusieurs centaines de phostines ont été synthétisés, les groupements R, R' et R'' variant entre chaque composé, et le groupement X correspondant à un atome d'oxygène, d'azote ou de soufre, décrits comme les plus biocompatibles (Clarion et al, 2012 ; Clarion et al., 2014).

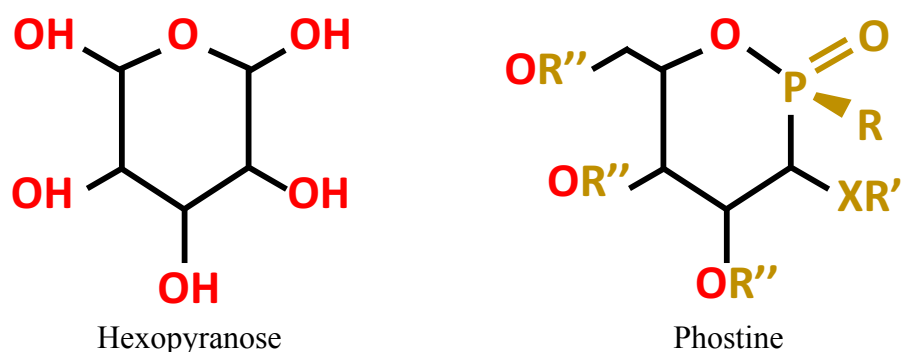


Fig. 8 : Structure d'une phostine.

Un groupement phosphinate comprenant un atome de phosphore (P) est ajouté en position 2 sur le carbone anomérique. Le groupement X correspond soit à un atome d'oxygène, d'azote ou de soufre, et les groupements R, R' et R'' varient entre chaque composé.

#### 4.3. Synthèse des phostines

La synthèse des phostines est réalisée par réaction d'un sucre protégé avec différents phosphinates, en présence de potassium t-butoxide (Clarion et al., 2012). La cyclisation est



obtenue par interaction entre le phosphinate et le groupement aldéhyde provenant de l'ouverture du sucre, conduisant à la création d'un groupe hémiacetal, qui referme la molécule en une structure cyclique. Par conséquent, le carbone anomérique du groupe hémiacetal est remplacé par un atome de phosphore issu du phosphinate. Ceci permet de stabiliser la molécule synthétisée et créer un composé administrable *in vivo* capable de perturber la glycosylation (Clarion et al., 2012). Cette voie de synthèse a permis la création d'une bibliothèque de 400 composés phostines.

#### 4.4. Criblage

L'ensemble des phostines synthétisées ont subi un criblage préliminaire, pour définir un composé présentant les meilleures activités anti-tumorales (Fig. 9). Pour cela, les propriétés antiprolifératives des phostines ont été testées dans un premier temps contre différentes lignées cellulaires tumorales. La phostine 3.1a (PST 3.1a) a montré une activité antiproliférative supérieure aux autres phostines, en particulier contre les cellules de gliome C6 avec une concentration efficace médiane de 0.5  $\mu\text{M}$  (Clarion et al., 2012). De façon intéressante, PST 3.1a réduit la prolifération de cette lignée C6 de façon plus importante que le témozolomide, médicament de référence. Dans un second temps, et afin de déterminer la spécificité d'action des composés, un screening a été établi à l'aide du panel « the human National Cancer Institute » (NCI), pour évaluer l'effet antiprolifératif contre 60 lignées tumorales humaines (Clarion et al., 2012). Ce screening a permis de confirmer la plus forte activité antiproliférative de PST 3.1a (10  $\mu\text{M}$ ) sur le cluster système nerveux central, et particulièrement contre les cellules de GBM SF-295 et SNB75, présentant un pourcentage de croissance médian inférieur à 50 % (Clarion et al., 2012). Il est à noter que ce composé inhibe également la prolifération des cellules Hs-578T, issues du cancer du sein. La toxicité de PST 3.1a a par la suite été mesurée contre les astrocytes, population cellulaire majeure du cerveau. PST 3.1a n'a montré aucune toxicité contre ces cellules à des concentrations allant jusqu'à 100  $\mu\text{M}$ , permettant de conclure à un index thérapeutique positif de ce composé (Clarion et al., 2012).

Ce screening a permis d'identifier plusieurs phostines actives, et en particulier le composé 3.1a choisi comme lead, présentant les meilleures propriétés antiprolifératives *in vitro* contre le GBM, couplé à une faible toxicité et une plus forte stabilité. Cette étude a également permis de lier l'efficacité aux phostines avec :

- i) une structure cyclique à 6 chaînons (analogue des pyranoses),
- ii) une configuration spatiale analogue du glucose (glucose-like),
- iii) la nécessité d'un groupement protecteur R'' sur la fonction alcool,

iv) ajout d'un groupement aryl (ou aromatique) sur l'atome de phosphore (Clarion et al., 2012).

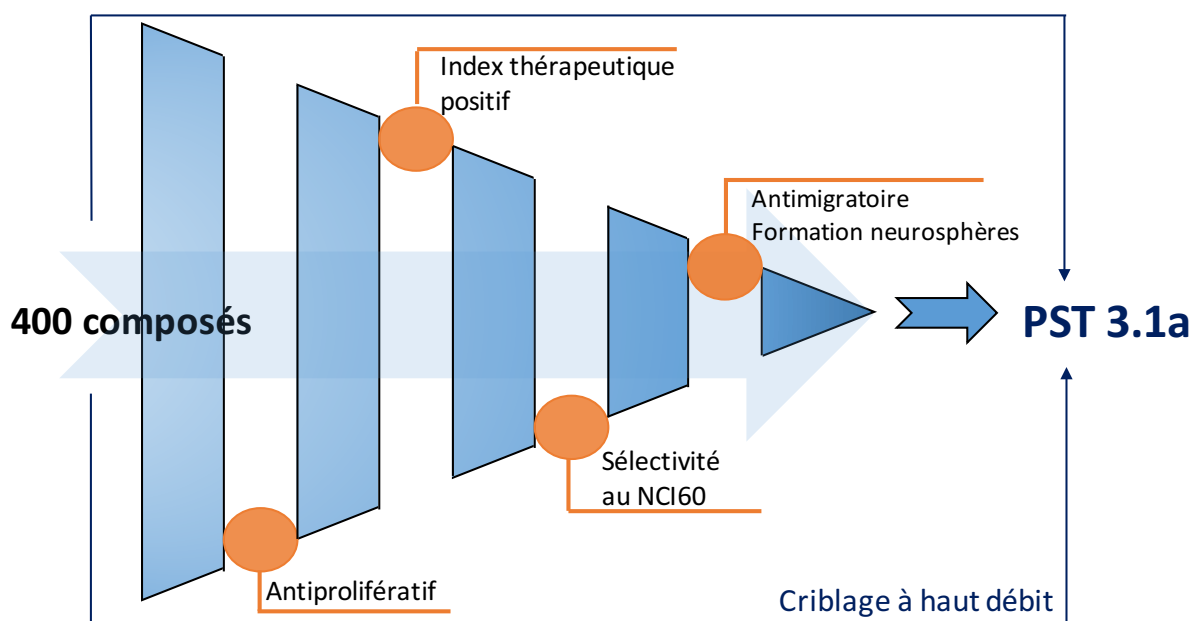


Fig. 9 : Criblage à haut débit des phostines.

Les 400 phostines ont été testées pour leurs propriétés antiprolifératives, toxiques, sélectives et antimigratoires. La phostine 3.1a (PST 3.1a) a été choisie comme composé *lead*, présentant les meilleures activités antitumorales, avec une activité prononcée contre le GBM.

## 4.5. Phostine 3.1a

### 4.5.1. Structure

Le composé PST 3.1a (3-Hydroxy-4,5-bis-benzyloxy-6-benzyloxyméthyl-2-phényl-2-oxo-2λ5-[1,2]oxaphosphinane) possède un groupement phényle (Ph) en position 2 sur l'atome de phosphore, et trois groupements benzyle (Bn), en position 4, 5 et 6 du cycle (Fig. 10). Les groupements phényle et benzyle sont des groupements stables, hydrophobes, qui favorisent la diffusion du composé à travers les tissus, et décrit comme protecteur.

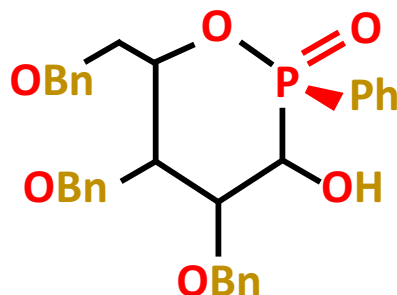


Fig. 10 : Structure de PST 3.1a.

PST 3.1a possède un groupement Phényl (Ph) en position 2 sur l'atome de phosphore (P), un atome d'oxygène (O) en position 3, et 3 groupements Benzyl (Bn) en position 4, 5 et 6.

#### 4.5.2. Activité anti-tumorale

Suite au screening précédemment décrit, l'activité anti-tumorale de PST 3.1a s'est poursuivie sur des cultures primaires issues de l'exérèse de patients atteints de GBM ; Gli4 et GliT (Guichet et al., 2013). PST 3.1a possède une activité inhibitrice sur la migration et l'invasivité des cellules Gli4 avec une concentration inhibitrice médiane (IC50) respective de 5 à 7 nM, ainsi qu'une activité antiproliférative sur Gli4 et GliT, avec une IC50 de 1,8  $\mu$ M (Hassani et al., 2017). Enfin, la formation de neurosphères (clones multipotents de cellules souches de GBM) *in vitro* est réduite de 50 % suite au traitement avec PST 3.1a (Hassani et al., 2017). Les expérimentations *in vivo* par utilisation d'une PST 3.1a sous forme de nanosuspension (15 mg/kg pendant 10 jours) ont montré une réduction significative de la densité cellulaire, ainsi que de l'invasivité de 80 % au niveau des hémisphères ipsilatéraux et contralatéraux (Hassani et al., 2017). Ces résultats sont corrélés avec une amélioration de la médiane de survie, de l'ordre de 108 jours pour les animaux traités avec PST 3.1a, contre 79 jours pour les animaux contrôle (Hassani et al., 2017).

Par ailleurs, PST 3.1a (50  $\mu$ M) ne possède pas toxicité *in vitro* contre les astrocytes non prolifératifs et les cellules mononuclées du sang périphérique. Les expériences *in vivo* ont confirmé une absence de toxicité, de perte de poids ou de modification comportementale chez les animaux, traités à une dose de 50 mg/kg pendant 20 jours (Hassani et al., 2017). Cette absence de toxicité a été confirmée chez la souris avec un traitement quotidien de 900 mg/kg pendant 28 jours (non publié).

### 4.5.3. Mécanisme d'action

Les expérimentations *in vitro* sur des cultures primaires de GBM ont démontré que PST 3.1a altère l'ajout des motifs de N-glycosylation mis en place par l'enzyme N-acetylglucosaminyltransférase V (GnT-V), celle-ci étant particulièrement décrite comme corrélée avec la progression tumorale (Chen et al., 1998 ; Hassani et al., 2017). Plus spécifiquement, PST 3.1a exerce dans ces cellules une activité inhibitrice hautement sélective contre GnT-V, avec une IC50 de 2  $\mu$ M *in vitro* (Hassani et al., 2017). Par conséquent, PST 3.1a bloque l'ajout du  $\beta$ -1,6-N-acetylglucosamine par GnT-V, qui régule entre autres l'adressage, le temps de rétention, ainsi que l'activité de différentes protéines membranaires. Ainsi à titre d'exemple, l'inhibition de GnT-V au niveau des cellules Gli4 et GliT différenciées est corrélée à une réduction de la phosphorylation de la protéine kinase d'adhésion focale (FAK), impliquée dans la migration, ainsi qu'une perturbation de la fonctionnalité de différentes molécules d'adhésion comme les cadhérines et intégrines (Hassani et al., 2017). Par ailleurs, PST 3.1a réduit la liaison membranaire des galectines (Gal) Gal-1 et Gal-3, qui ont pour site de fixation les motifs de glycosylation mis en place par GnT-V. Ces deux protéines, en reconnaissant spécifiquement leurs motifs cibles, vont participer à réguler la stabilité, l'activité, la signalisation, ou encore l'endocytose de différentes protéines membranaires. De plus, l'expression de ces deux Gal est également réduite par PST 3.1a, résultant d'un mécanisme d'autorégulation cellulaire en absence de leur motif de glycosylation cible.

Brièvement, l'altération du profil de N-glycosylation causée par PST 3.1a au niveau de GnT-V perturbe l'ajout de son motif  $\beta$ -1,6-N-acetylglucosamine sur les glycoprotéines néoformées. En particulier, cela perturbe la fonction, la morphologie ou le temps de rétention à la membrane de nombreuses protéines membranaires et sécrétées, conduisant à une inhibition de la progression tumorale.

En parallèle de ces résultats, et comme vu précédemment, le GBM est la tumeur cérébrale la plus vascularisée, ce qui participe fortement à son agressivité (Brem et al., 1972). Ses propriétés hyperprolifératives (Burger et al., 1986) et nécrotiques génèrent une importante hypoxie, qui est un moteur de l'angiogenèse (Raza et al., 2004). La croissance tumorale et l'invasivité des tissus voisins est dépendante de cette formation de vaisseaux sanguins, et cibler cette composante vasculaire présente un intérêt considérable pour réduire l'agressivité du GBM. De plus et comme décrit préalablement, une glycosylation aberrante est pressentie pour être associée avec l'angiogenèse tumorale, et le ciblage spécifique de cette voie représente une approche anti-angiogénique peu décrite et particulièrement prometteuse. Étudier l'impact de PST 3.1a sur



l'angiogenèse permettrait une meilleure connaissance du mécanisme d'action de ce composé, et faciliterait ainsi le développement de nouvelles alternatives pour le traitement du GBM, et des pathologies associées à une angiogenèse excessive.

# Objectif de travail

Nous avons pu observer dans cette introduction que les thérapies anti-angiogéniques actuelles contre la progression tumorale restent trop peu efficaces, et le nouveau paradigme anti-angiogénique vise à inhiber les modifications métaboliques mises en jeu par les CEs pour initier l'angiogénèse. Afin de trouver un composé avec des propriétés anti-angiogéniques plus spécifiques, cette étude a pour objectif de caractériser l'implication du glycomimétique PST 3.1a sur l'angiogénèse physiologique et tumorale, ainsi que sur le métabolisme endothélial, en ciblant en particulier la N-glycosylation (Fig. 11).

Pour réaliser cette étude, des objectifs spécifiques ont été développés :

Dans un premier temps, nous avons caractérisé les potentielles propriétés anti-angiogéniques de PST 3.1a. Pour cela, l'impact de ce composé a été étudié (i) *in vitro* sur les différentes étapes menant à l'angiogénèse, (ii) *in vivo* chez la souris et le zebrafish, et enfin (iii) sur un modèle de vascularisation tumorale du GBM, en sous-cutané.

Dans un second temps, nous avons étudié les mécanismes moléculaires par lesquels PST 3.1a régule l'angiogénèse.

Dans la dernière partie de ce travail, les modifications métaboliques perturbées par PST 3.1a dans les CEs ont été caractérisées. Nous avons évalué les effets de PST 3.1a sur (i) le métabolisme énergétique, en étudiant l'activité mitochondriale et la glycolyse, et sur (ii) la N-glycosylation, en se concentrant plus spécifiquement sur les perturbations précédemment observées par PST 3.1a sur les cellules de GBM.

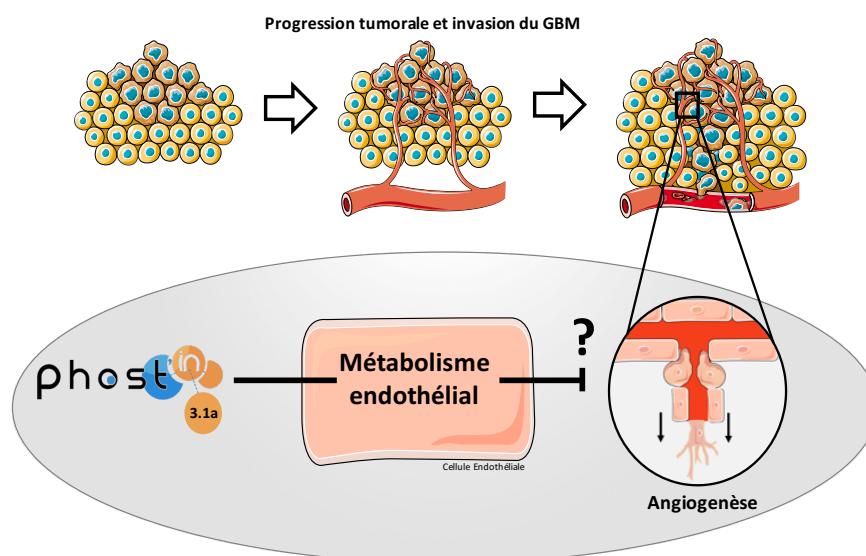


Fig. 11 : Représentation schématique des principaux objectifs.

L'implication de PST 3.1a sur l'angiogénèse et sur les modifications métaboliques endothéliales associées a été mesurée, en se concentrant sur la glycosylation et le métabolisme énergétique.

# **Résultats expérimentaux**



Les résultats de notre étude sont présentés dans le manuscrit suivant, en révision positive dans *Faseb Journal* (Bousseau et al., 2018), et intitulé « Phostine 3.1a as a pharmacological compound with anti-angiogenic properties against diseases with excess vascularization ».

Ce papier présente dans un premier temps les propriétés anti-angiogéniques de PST 3.1a. Les expérimentations *in vitro* démontrent que ce composé inhibe les différentes étapes conduisant à l'angiogenèse, incluant la migration, la prolifération, l'adhésion et la formation de tubules, sur des cellules endothéliales humaines de la veine ombilicale (HUVECs). Par la suite, les résultats témoignent également d'une réduction de l'angiogenèse *in vivo* par PST 3.1a, dans un modèle de matrigel plug chez la souris. L'étude sur le zebrafish permet de renforcer ces observations, et illustre d'une inhibition de la vascularisation développementale chez les embryons de zebrafish. Enfin, l'inhibition de l'angiogenèse est démontrée dans un modèle murin de xénogreffe de GBM, associée à une réduction de la croissance tumorale.

En parallèle de ces résultats, les données présentées attestent d'une réduction de l'expression de plusieurs facteurs pro-angiogéniques majeurs, tels que VEGFR2, FAK et 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) dans les HUVECs traitées avec PST 3.1a.

Finalement, cette étude démontre que PST 3.1a ne perturbe pas l'activité mitochondriale, ni la glycolyse dans les HUVECs. En revanche, plusieurs acteurs majeurs impliqués dans la N-glycosylation sont inhibés par PST 3.1a dans ces cellules, tels que GnT-V et Gal-1. En particulier, l'interaction entre VEGFR2 et Gal-1, régulée par GnT-V, est réduite par ce composé. Cette interaction est décrite comme clé dans la résistance tumorale aux traitements anti-angiogéniques actuels, et de ce fait, la cibler représente une approche innovante et peu décrite pour inhiber l'angiogenèse tumorale.

**Phostine 3.1a as a pharmacological compound with anti-angiogenic properties against diseases with excess vascularization**

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**Running Title:** Anti-angiogenic properties of PST 3.1a.

## Nonstandard Abbreviations

3-Hydroxy-4,5-bis-benzyloxy-6-benzyloxymethyl-2-phenyl-2-oxo-1,2-oxaphosphinane (PST 3.1a)

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3)

Basic fibroblast growth factor (bFGF)

Bovine serum albumin (BSA)

Cyclin-dependent kinase 1 (CDK1)

Dimethylsulfoxide (DMSO)

Endothelial cells (ECs)

Focal adhesion kinase (FAK)

Galectin (Gal)

Glioblastoma (GBM)

Human Umbilical Vein Endothelial Cells (HUVECs)

Intersegmental vessels (ISV)

N-acetylglucosaminyltransferases (GnTs)

Phostine (PST)

Sodium dodecyl sulfate (SDS)

Sub-intestinal venous (SIV)

Vascular endothelial growth factor (VEGF).

Vascular endothelial growth factor receptor 2 (VEGFR2)

## Abstract

Angiogenesis is a complex process leading to the growth of new blood vessels from existing vasculature, triggered by local pro-angiogenic factors such as VEGF. An excess of angiogenesis is a recurrent feature of various pathological conditions such as tumor growth. Phostines are a family of synthetic glycomimetic compounds, exhibiting anti-cancer properties, and its lead compound phostine 3.1a (PST 3.1a) shows anti-glioblastoma properties both *in vitro* and *in vivo*. In the present study, we assessed the effect of PST 3.1a on angiogenesis and endothelial metabolism. *In vitro*, PST 3.1a (10  $\mu$ M) inhibited all steps that regulate angiogenesis including migration, proliferation, adhesion and tube formation. *In vivo*, PST 3.1a reduced intersegmental vessel formation and vascularization of the sub-intestinal plexus in zebrafish embryos, and also altered pathological angiogenesis and glioblastoma progression *in vivo*. Mechanistically, PST 3.1a altered interaction of VEGF receptor 2 and glycosylation-regulating protein galectin-1, a key component regulating angiogenesis associated to tumor resistance. Thus, these data show that PST 3.1a is an innovative approach to target angiogenesis.

**Keywords:** Angiogenesis, Cancer growth, Pharmacotherapy, Endothelial metabolism, Glycosylation.



## Introduction

Angiogenesis is a complex process related to the growth of new blood vessels from existing vasculature, triggered by local pro-angiogenic factors such as the VEGF. In healthy adult, angiogenesis contributes to menstruation, pregnancy and wound healing, and can be activated in various pathological conditions such as tumor growth, diabetic retinopathy, rheumatoid arthritis, and ischemic diseases (1, 2). Endothelial cells (ECs) form the innermost layer of blood vessels and remain quiescent for years in healthy adults in physiological conditions. In contrast, ECs can rapidly increase their metabolism to initiate angiogenesis in pathological conditions. During this process, quiescent ECs quickly switch to an angiogenic phenotype and differentiate to migratory “tip” and proliferating “stalk” cells (3), which display specific metabolic phenotypes (4, 5).

Current standards of care against excessive pathological angiogenesis aim to inhibit vascular growth signals, such as VEGF. Indeed, the VEGF pathway is widely recognized as critical in angiogenesis, and most current anti-angiogenic therapies targeting VEGF have been clinically approved (6). However, in cancer therapy, anti-VEGF treatments provide less success than expected, due to restricted efficacy, intrinsic refractoriness and resistance acquisition, implying that alternative strategies are needed (7, 8). As a matter of fact, EC metabolism is a common underlying mechanism towards which proangiogenic vascular growth signals converge, and consequently represents an innovative anti-angiogenic opportunity based on an entirely distinct novel strategy (5, 9). In this respect, new targets involved in EC metabolism, energy production and protein modifications, such as glycosylation, are emerging (10). Although energy production and its main regulator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) are now well described to regulate neovascularization (11, 12), the impact of glycosylation on angiogenesis is less clear. This post-translational modification allows the addition of glycans to glycoconjugates on growth factor receptors and nutrient transporters. In particular, glycosylation of vascular endothelial growth factor receptor 2 (VEGFR2) and Notch contributes to their stability, solubility, localization, trafficking and function (13, 14). Addition of glycans on N-linked glycoproteins is modulated by the enzymatic activity of N-acetylglucosaminyltransferases (GnTs), which catalyze the addition of N-acetylglucosamine (GlcNAc) to N-linked proteins. GnT-V is particularly associated with malignancies and correlates with cell migration, invasion, and metastatic properties in numerous cancer cell lines (15-17). Furthermore, galectins, a family of endogenous glycan-binding proteins specifically interact with  $\beta$ -galactoside partners in the cell membrane to regulate stability and signaling. Galectin-1 (Gal-1) was shown to regulate angiogenesis and its interaction with VEGFR2 could thus be responsible

for tumor adaptations to anti-angiogenic strategies (6,18).

Phosphinosugars or Phostines, a class of glycomimetic compounds in which the hemiacetal group of a hexopyranose is replaced by a chemically and configurationally stable phosphinolactone group (19, 20), have been synthesized to test their capacity to alter tumor growth by interfering with N-glycosylation. In particular, the lead compound, Phostine 3.1a (3-Hydroxy-4,5-bis-benzyloxy-6-benzyloxymethyl-2-phenyl-2-oxo-2λ5-[1,2]oxaphosphinane, PST 3.1a) displays antitumoral activity against glioblastoma (GBM), the most common primary malignant brain tumor. By specifically inhibiting GnT-V enzymatic activity, PST 3.1a has shown promising properties to inhibit proliferation, migration and invasiveness of GBM initiating cells (17). It is now described that hyper-vascularization is an important feature of GBM tumors that also regulates invasion, migration, and proliferation of the tumor cells (21, 22). However, the effect of PST 3.1a on angiogenesis has never been addressed. Thus, the aim of this study was to investigate the effect of PST 3.1a on angiogenesis under physiological conditions both *in vitro* and *in vivo* as well as in a mouse model of glioblastoma. In this study, we show that PST 3.1a is an innovative approach to efficiently target angiogenesis and might constitute a new relevant therapeutic tool against glioblastoma.

## **Materials and Methods**

### **Cell culture**

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Lonza (Belgium) and grown on plastic flasks in EGM-2 medium supplemented with 0.1% human epidermal growth factor (hEGF), 0.1% ascorbic acid, 0.1% GA-1000 (Gentamicin, Amphotericin-B), 0.1% heparin, 2% fetal bovine serum (FBS), 0.1% VEGF, 0.4% human basic fibroblast growth factor basic (hFGF-B), 0.04% hydrocortisone, 0.1% R3-IGF-1, and 1% streptomycin/penicillin (all chemicals were purchased from Lonza). HUVECs were grown at 37 °C in a humidified incubator gassed with 5% CO<sub>2</sub> in air and were used for experiments at the third to seventh passage. PST 3.1a (provided from Phost'in SAS, France) solutions were prepared in 100% dimethylsulfoxide (DMSO). Cells were grown for 24 h in the absence or presence systematically, or otherwise stated, of 1, 5 and 10 μM PST 3.1a with a final DMSO (0.1 %).

### **Animal model**

The local ethics committee approved the animal protocol followed in the current study (CEEA.PdL 00509.01). This study was carried out in strict accordance with the guidelines and authorization with French Ministry of Agriculture regulations based on European Community. Six week-old male NMRI-nude mice (Janvier labs, France) were maintained at room temperature of 23 ± 2 °C under a 12h light/12h dark cycle (lights on, 8:00 AM). The animals were allowed free access to water and standard diet.

### **Wound healing assay**

HUVECs ( $6 \times 10^4$ ) were seeded in 12-well plates. When the confluence of cells reached about 90%, cells were scratched with a sterile pipet tip to make a gap. Cells were then rinsed with medium to remove any floating cells and debris, then treated with PST 3.1a for 24 h. Photographic images of the plates were taken under a microscope immediately after healing and 24 h after. The area of the scratch was measured using the T-scratch software.

### **Proliferation assay**

Evaluation of PST 3.1a on HUVEC proliferation was performed using Trypan blue exclusion. For this,  $1.2 \times 10^5$  cells per well were seeded into 6-well plates overnight, then treated in absence or presence of PST 3.1a during 24 h, and counted using Trypan blue solution (Sigma–

Aldrich, MO).

### **Adhesion assay**

Effect of PST 3.1a on HUVEC adhesion was analyzed by crystal violet staining. For this,  $10^4$  cells per well were seeded into 96-well plates overnight, then PST 3.1a was added. After 24 h of incubation, the plate was shaken for 15 sec. The supernatant with non-adherent cells was removed by three washes with the washing buffer (0.1% bovine serum albumin (BSA) in medium without serum; Sigma–Aldrich). Attached cells were fixed with 4% of paraformaldehyde (Fisher Scientific, NJ) for 15 min at room temperature. Cells were rinsed twice with washing buffer, stained with crystal violet (Sigma-Aldrich) (1 mg/mL in 2% of ethanol) for 10 min at room temperature protected from light, and extensively washed with distilled water. Then, sodium dodecyl sulfate (SDS) 2% was added and incubated for 30 min at room temperature. Absorbance was evaluated using a microplate reader at 550 nm (Synergy HT Biotek, VT).

### ***In vitro* capillary network formation on ECM gel**

Endothelial capillary network formation was assessed using matrigel (ECM gel<sup>®</sup> Sigma-Aldrich), as previously described (23). Briefly, 10  $\mu$ L of ECM gel substrate was added into a 15  $\mu$ -slide Angiogenesis plate (Ibidi, Germany), and allowed to solidify for 1 h at 37 °C. Then, HUVECs were seeded with a density of  $10 \times 10^4$  cells per well and allowed to adhere for 1 h after which the different treatments were added. Photographic images of the plates were taken under a microscope after 24 h. Tube formation was assessed using ImageJ software.

### ***In vivo* matrigel plug assay**

For *in vivo* studies of angiogenesis, HUVECs were treated or not with PST 3.1a (10  $\mu$ M) for 24 h. After treatment, cells were detached and mixed with 500  $\mu$ L of matrigel (ECM gel<sup>®</sup> Sigma-Aldrich) with recombinant bFGF-2 (300 ng/mL, Peprotech, NJ). This mixture was subcutaneously injected on the back of mice fed with normal chow. At day 7, mice were sacrificed and matrigel plugs were removed. Plugs were homogenized in RIPA lysis buffer and incubated for 24 h 4°C, then disrupted with a Polytron (PRO Scientific, CT). Hemoglobin levels were determined using Drabkin's reagent (Sigma-Aldrich) according to the manufacturer's instructions.

### **Zebrafish experiments**



General maintenance of zebrafish was carried out according to common practices at 28 °C. PST 3.1a (50, 75 or 100 μM) was added in one-cell eggs water during 72, 96 or 120 h. Embryos were anesthetized with 0.01% tricaine (Sigma-Aldrich) and mounted in 1% agarose. GFP-expressing ECs and DsRed-expressing red blood cells were visualized in Tg(fli1a:EGFP/gata1a:dsRed) zebrafish with a Spinning disk confocal microscope (Zeiss Axio Observer, Germany) as previously shown (24). Stacks of 20-50 focal planes were processed with MetaMorph.

### **Tumor Xenograft**

SNB75 glioblastoma cell line (Stanford University, CA) were dissociated and resuspended in 50% PBS and 50% matrigel. Cells ( $4.5 \times 10^6$ ) were subcutaneously injected into the back of 6 weeks-old male NMRI-nude mice (Janvier labs) under isoflurane anesthesia. Transplanted animals were allocated randomly to two treatment groups by the end of the surgery. Six-week post-surgery, animals were treated daily for 14 consecutive days, with one *per os* administration of a nano-suspension of PST 3.1a (12 mg/kg in accordance with Hassani et al (17)), or with control solution (sham-operated). Mice were then weighted every two days and tumor volume was measured using a caliper. The animals were sacrificed at the end of the treatment and the xenograft removed.

### **Echography examination**

*In vivo* echography was assessed in mice anesthetized with isoflurane using a VEVO 770 ultrasound machine from FUJIFILM Visualsonics (Canada) equipped with a 30-MHz imaging transducer. Three-dimensional M-mode data was acquired along the entire length of the xenograft at day 0, day 7 and day 14 in order to evaluate tumor growth.

### **Immunohistochemistry**

After the surgery, tumors were embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, CA), and serial frozen sections 10-μm thick were obtained using a Leica CM3050S cryostat (Leica, France). Tumor sections were incubated with rat anti-mouse CD31 antibody (BD Biosciences, NJ) followed by incubation with goat anti-rat IgG-FITC (Southern Biotech, AL). Confocal microscopy and digital image recording were performed.

### **Western blot**

After treatment, cells were homogenized and lysed. Proteins (20 µg) were separated on 4-12% precast SDS-polyacrylamide gel electrophoresis (Invitrogen, CA). Blots were probed with anti-P-VEGR2 (Y1175), anti-VEGFR2, anti-phosphorylated focal adhesion kinase (FAK) (Y925), anti-FAK, anti-phosphorylated cyclin-dependent kinase 1 (CDK1), anti-CDK1 (Cell Signaling, MA), anti-PFKFB3 (Proteintech, United Kingdom), anti-Galectin-1 and anti-Galectin-3 (Abcam, United Kingdom), anti-GnT-V (R&D systems, MN), anti-GnT-III (Thermo Fisher, MA) antibodies. Monoclonal anti-β-actin antibody (Sigma–Aldrich) was used to visualize protein gel loading. After washing, bound antibodies were detected with a secondary peroxidase-conjugated anti-rabbit antibody (US Biological, MA) or anti-mouse antibody (US Biological). The protein–antibody complexes were detected by enhanced chemiluminescence system and quantified by densitometry (Amersham Biosciences, United Kingdom).

### **Cell cycle analysis**

HUVECs ( $1.5 \times 10^5$ ) were seeded in 6-well plates and treated with or without PST 3.1a for 24 h. Cells were then trypsinized, washed in PBS and fixed with 70% ethanol at 4 °C overnight. The cells were first incubated with RNase A (50 µg/mL, Sigma-Aldrich) at 37 °C for 10 min and then labelled with propidium iodide (0.1 mg/mL, Sigma-Aldrich) and incubated at room temperature in the dark for 30 min. DNA content was then analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter, CA).

### **Immunofluorescence on cells**

After treatment, HUVECs were fixed with 4 % paraformaldehyde for 15 min at room temperature, permeabilized with 0.2 % Triton X-100 and 0.2 % BSA in PBS and then blocked with 5 % BSA in PBS for 30 min at room temperature. Cells were treated with anti-VEGFR2 (R&D systems) and anti-Galectin-1 (Abcam) antibodies in 5 % BSA in PBS overnight at 4 °C. After washing with PBS, cells were respectively treated with goat anti-mouse R-phycoerythrin antibody (Invitrogen) and goat anti-rabbit fragment-FITC antibody (Sigma–Aldrich) in 5 % BSA in PBS for 1 h at room temperature.

### **Co-immunoprecipitation**

Anti-VEGFR2 antibody (R&D systems) or anti-Gal-1 antibody (Abcam) were incubated with protein A-G Sepharose beads (Santa Cruz Biotechnology, TX) at 4 °C for 2 h to form beads-antibody complex. Then, HUVEC protein extracts treated or not with PST 3.1a (10 µM) were

added at 4 °C overnight to allow antibody interaction with VEGFR2 or Gal-1 protein. Subsequently, the tubes containing the immunoprecipitates were centrifuged, and the supernatants were eliminated. Pellets were washed two times with 100 µL lysis buffer diluted to 1:5 with PBS. Gel-loading buffer (15 µL) was then added to the pellets. The tubes were heated for 10 min at 70 °C, after which proteins were separated on 4-12% SDS-PAGE gel. The gel was trans-blotted to perform Western analysis for VEGFR2 and Gal-1 proteins as described above.

### **Data analysis**

Data are represented as mean  $\pm$  SEM; n represents the number of repeated experiments. For *in vivo* experiments, n represents the number of mice. Statistical analyses were performed either using one-way ANOVA (parametric) for all *in vitro* angiogenesis experiments, or using Mann–Whitney U-tests (non-parametric) for other experiments, using Prism software package 5.00 (GraphPAD Software, CA).  $p < 0.05$  was considered to be statistically significant.

## Results

### PST 3.1a inhibits angiogenesis steps *in vitro*

First, the effects of PST 3.1a were assayed *in vitro* on HUVECs on collective migration, proliferation, adhesion and tube formation. Using a wound healing assay, we showed that PST 3.1a decreased migration of HUVECs in a concentration-dependent manner (Fig. 1A). In addition, PST 3.1a significantly decreased EC proliferation at 10  $\mu\text{M}$ , as assayed by trypan blue exclusion test (Fig. 1B). It should be noted that PST 3.1a (10  $\mu\text{M}$ ) did modify neither apoptosis nor ATP/ADP ratio (Supplemental Fig. 1A and 1B), demonstrating that PST 3.1a did not display any toxicity under the experimental conditions used. PST 3.1a at 10  $\mu\text{M}$  also reduced HUVEC adhesion as compared to controls (Fig. 1C).

On matrigel, HUVECs reorganized and formed capillary-like structures. PST 3.1a did modify neither the areas nor the number of branching of capillary-like structures at 1 and 5  $\mu\text{M}$  (Fig. 1D). Interestingly, PST 3.1a (10  $\mu\text{M}$ ) reduced both areas and number of branching by 50% compared to controls. Overall, these results indicate that PST 3.1a reduces the angiogenic processes *in vitro* at 10  $\mu\text{M}$ .

### PST 3.1a inhibits neovascularization *in vivo*

We next characterized whether PST 3.1a might also affect angiogenesis *in vivo* using the matrigel plug assay. Basic FGF-containing plugs with HUVECs treated or not with PST 3.1a at 10  $\mu\text{M}$  for 24 h were subcutaneously injected into mice. After one week, the plug hemoglobin content was reduced in samples containing PST 3.1a-treated HUVECs as compared to control plugs (Fig. 2A and 2B). In order to study the *in vivo* effects of PST 3.1a in more detail, we next investigated the impact of PST 3.1a on the vascular development of zebrafish embryos. We first studied formation of the sub-intestinal venous (SIV) plexus that sprout from the posterior cardinal vein as it is well established that VEGF promotes initial sprouting and proliferation of the vessels of the plexus. Our results showed that 100  $\mu\text{M}$  PST 3.1a inhibited SIV formation at 72 hpf. Lower concentrations such as 75  $\mu\text{M}$  showed a similar inhibitory effect of PST 3.1a on SIV but at later time points (96 hpf and 120 hpf) (Fig. 2C). No effects were observed at a concentration of 50  $\mu\text{M}$ . The number of SIV compartments was significantly reduced at 120 hpf using 75  $\mu\text{M}$  and 100  $\mu\text{M}$  of PST 3.1a (Fig. 2D). Quantification of SIV area confirmed alteration of SIV development, as early as 72 hpf using 100  $\mu\text{M}$  of PST 3.1a, and as 96 hpf and 120 hpf using PST 3.1a at 75  $\mu\text{M}$  and 100  $\mu\text{M}$  (Fig. 2E).



In addition, whereas no effects were observed on intersegmental vessels (ISV) formation, PST 3.1a treatment reduced perfusion in ISV at 75  $\mu$ M and 100  $\mu$ M at 72 hpf, 96 hpf and 120 hpf. Fifty  $\mu$ M of PST 3.1a also reduced ISV perfusion at 120 hpf (Fig. 3A and 3B). Lack of ISV circulation is associated with a reduction of vessels diameter at 72 hpf and 120 hpf using both 75  $\mu$ M and 100  $\mu$ M PST 3.1a. (Fig. 3C). We further investigated whether ISV defects may be explained by an alteration of heart function. We thus analyzed heartbeats in embryos and revealed no difference whatever the treatment time and the PST 3.1a concentrations that were used (Supplemental Fig. 2A and 2B).

We next explored whether PST 3.1a also affects angiogenesis in a murine model of subcutaneous glioblastoma tumors generated by the injection of SNB75 cells in NMRI-Nude mice. After tumor development, animals were fed for 14 days either with PST 3.1a or with control solution. Animals treated with PST 3.1a displayed a significant decrease of tumor volume in comparison to controls (Fig. 4A, 4B and 4D). This effect was not associated with a modification of mouse weight during the treatment (Fig. 4C). Further histological analyses using CD31 immunostaining showed that PST 3.1a induced a significantly reduction of tumor vascularization (Fig. 4E and 4F).

### **PST 3.1a decreases the expression of master angiogenic regulators**

We then explored whether the vascular defects caused by PST 3.1a are associated with changes in the expression and phosphorylation levels of key angiogenic regulators such as VEGFR2 and FAK. PST 3.1a (10  $\mu$ M) reduced by more than 50% VEGFR2 expression but did not modify its phosphorylation level on Tyr. 1175 that regulates both cell proliferation and migration (Fig. 5A). Moreover, FAK expression, but not its phosphorylation on Tyr. 925, was significantly decreased upon PST 3.1a treatment (Fig. 5B). Finally, PFKFB3 expression, a potent glycolytic and angiogenic regulator, was strongly reduced in the presence of PST 3.1a (Fig. 5C).

### **PST 3.1a does not affect energy production in ECs**

We next wondered whether PST 3.1a could reduce the energy production in ECs. We first measured the effects of PST 3.1a on mitochondrial activities. PST 3.1a treatment did not disrupt mitochondrial DNA content, mitochondrial biogenesis and respiration (Supplemental Fig. 3A to 3C). Moreover, EC glycolysis, analyzed by measuring the ratio between glucose consumption and lactate production, and the abundance of the GLUT1 protein was not disturbed by PST 3.1a (Supplemental Fig. 3D and 3E). Together, these results suggest that PST 3.1a reduces angiogenesis without a significant effect on EC energetic metabolism.

### **PST 3.1a impairs HUVEC cell cycle progression via inhibition of G2/M phase transition**

Besides its direct involvement in glycolysis, PFKFB3 has also been described to regulate the cell cycle progression by a mechanism involving CDK1 (25). To investigate whether PST 3.1a affects progression through the cell cycle, HUVECs treated or not by PST 3.1a (10  $\mu$ M) were evaluated by flow cytometry. PST 3.1a treatment significantly reduced the number of cells at the G2/M phase transition, in comparison with control cells, while no significant modification was observed on G0/G1 and S phases (Fig. 6A). We next assessed the effect of PST 3.1a on CDK1 expression, a major cell cycle regulator of the G2/M transition, and found that its expression was significantly reduced with 10  $\mu$ M of PST 3.1a, without modification of its inhibitory phosphorylation site on Tyr 15 (Fig. 6B). Furthermore, the abundances of cyclins A and B, the main CDK1 partners, were not affected (Fig. 6C).

### **PST 3.1a decreases GnT-V and disrupts VEGFR2-Gal-1 interaction**

We next investigate the effect of PST 3.1a on the expression of GnT-III and GnT-V, the enzymes involved in N-glycosylation linkage. Whereas we did not find any changes on GnT-III expression, we found a significant reduction of GnT-V expression by PST 3.1a, in comparison to control (Fig. 7A). In addition, PST 3.1a did significantly reduce the glycan-binding proteins Gal-1 expression by 30%, but not that of Gal-3 (Fig. 7B). Since VEGFR2 interacts with Gal-1, and both protein abundances are decreased by PST 3.1a, we tested whether PST 3.1a affects the interaction between both proteins. Using confocal microscopy, we found that PST 3.1a treatment reduced VEGFR2/Gal-1 co-localization, compared to control cells (Fig. 7C and 7D). Moreover, co-immunoprecipitation revealed that the physical association between Gal-1 and VEGFR2 was reduced in the presence of PST 3.1a treatment (Fig. 7E and 7F).

## Discussion

Herein, we evaluated PST 3.1a activity on the angiogenesis process. PST 3.1a, which is a small glycomimetic inhibitor with an efficient activity on GBM initiating cell invasiveness and proliferation, showed a remarkable ability to alter angiogenesis *in vitro* as well as *in vivo*. Importantly, we demonstrated that PST 3.1a intervenes *in vitro* on all the different steps leading to the angiogenesis process, such as migration, adhesion, proliferation and tube formation, and alters angiogenesis in two distinct *in vivo* models. Of particular interest, we highlight a novel mechanism that modulates the interaction of VEGFR2 with its glycan-binding protein Gal-1 in ECs, leading to a reduction of the metabolic process associated with the inhibition of the master glycolytic regulator PFKFB3, and reducing the cell cycle timing at the G2/M transition.

As a first step, we found that PST 3.1a at a concentration of 10  $\mu$ M decreases HUVEC migration, proliferation, adhesion and tube formation, all of them being required in angiogenesis. Furthermore, and in accordance with these results, PST 3.1a reduced the expression of key angiogenic factors such as VEGFR2 and FAK that are implicated in proliferation and migration of ECs, respectively. In addition, PST 3.1a reduced the expression of the glycolytic regulator, PFKFB3, which is a potent angiogenic modulator. This observation fits with the fact that other inhibitors of PFKFB3, as the 3PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one), have powerful anti-angiogenic capacities (11).

Angiogenesis is a highly energetic process associated with an increase of mitochondrial respiration capacity and biogenesis (26). However, we observed that the treatment with PST 3.1a does not modify either the mitochondrial biogenesis, or the mitochondrial DNA content in HUVECs. Moreover, PST 3.1a did not modify the mitochondrial respiration, suggesting that the regulation of mitochondrial activities do not contribute to the anti-angiogenic property of PST 3.1a. These results reinforce the critical involvement of the glycolytic pathway, via the modulation of PFKFB3, as an anti-angiogenic target of the compound.

Indeed, besides its implication in controlling the energetic process, PFKFB3 also traffics to the nucleus to promote cell cycle progression by regulating CDK1 activity (25). In accordance with these evidences, treatment with PST 3.1a reduced CDK1 expression, but not its phosphorylation nor the abundances of its partner cyclins A and B, and consequently the accumulation of cells at the G2/M transition. However, unlike other reports, the inhibition of PFKFB3 expression by PST 3.1a did not induce apoptosis in HUVECs (27). Altogether, these results suggest that PST 3.1a has an anti-proliferative action on ECs, which could be mediated by a cell cycle alteration at the G2/M transition, but without promoting apoptosis as summarized on

Supplemental Fig. 4.

Furthermore, we found that, in HUVECs, PST 3.1a reduces GnT-V expression. Although the influence of glycosylation on tumor growth is nowadays well recognized, its implication in angiogenesis remains poorly documented (10, 28). Indeed, it was reported that master angiogenic regulators, such as VEGFR2 and Notch can be glycosylated, in order to modulate their functions. In this respect, galectins that are directly connected to GnT have emerged as powerful angiogenic modulators. Notably, Gal-1 and Gal-3 are among the most powerful galectins regulating angiogenesis and pathological vascularization (18, 29, 30). Consequently, Gal-1 inhibitors were synthesized to target melanoma, ovarian, breast and colon cancers, but none of them were reported until now to be efficient on GBM (31-33). Interestingly, Gal-1 mediates the resistance to a number of anticancer therapies, therefore the finding that PST 3.1a treatment reduces Gal-1 expression in HUVECs parallels a similar observation in GBM (17) and is of particular interest for increasing anti-angiogenic treatment efficiency. It was further shown that glycosylation controls the interaction between Gal-1 and VEGFR2 to sustained tumor angiogenesis (30). Indeed, the extracellular portion of VEGFR2 is composed by seven Ig-like domains which are highly N-glycosylated, a post-translational process crucial for its function, VEGF binding and stabilization (34). VEGFR2 glycosylating on 3, 4 and 7 sites may promote a Gal-1 dependent retention of VEGFR2 on plasma membrane, therefore enhancing VEGF signaling, even in the presence of VEGF inhibitors (29). Therefore, the finding that PST 3.1a reduces Gal-1-VEGFR2 interaction in HUVECs suggests that the anti-angiogenic properties of PST 3.1a may be related to this process. This may represent a promising anti-angiogenic approach in combination with the one currently used to treat tumors growth related to excess angiogenesis.

In conclusion, we provide evidences that PST 3.1a inhibits angiogenesis in cell culture and in both mice and zebrafish models, and reduces GBM progression associated with an alteration of tumor vascularization. Of particular interest is that we highlight a novel mechanism by which PST 3.1a modulates the interaction of VEGFR2 with its glycan-binding protein Gal-1. This leads to a reduction of the metabolic process associated with the inhibition of the master glycolytic regulator PFKFB3 and the cell cycle transition at G2/M check-point. These results provide information on an innovative approach to target angiogenesis-related diseases including cancer, which could eventually be extended to further diseases with excess vascularization. Most importantly, the present study underscores the potential use of PST 3.1a as new perspective in GBM anti-angiogenic treatments.

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## **Author's contribution**

S.B.: conception and design, acquisition, analysis and interpretation of data, and writing the manuscript. M.M., R.S., L.V., G.H., S.K., N.G.: technical and analysis support, interpretation of data. M.L.M., L.C., N.B.: material support. S.G., G.L. and R.A.: conception and design, interpretation of data, revision of the manuscript, study supervision.



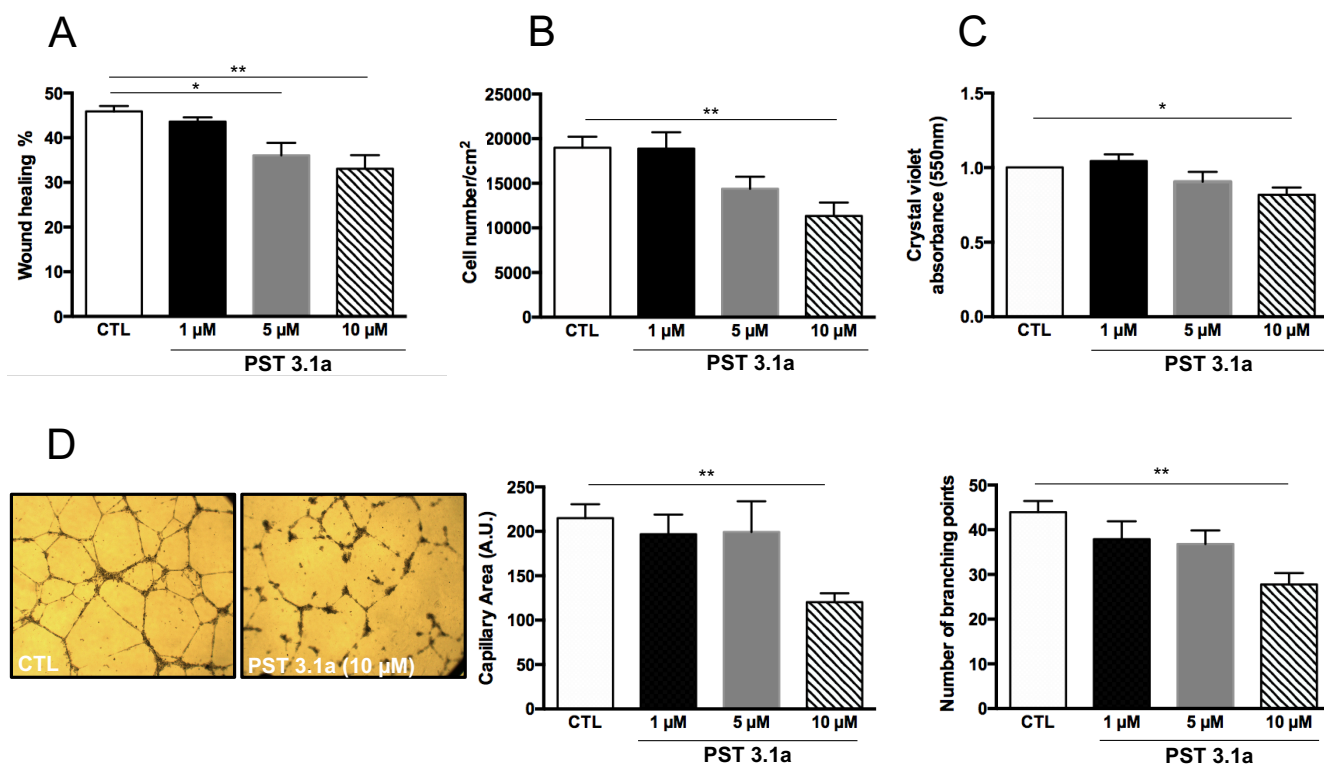
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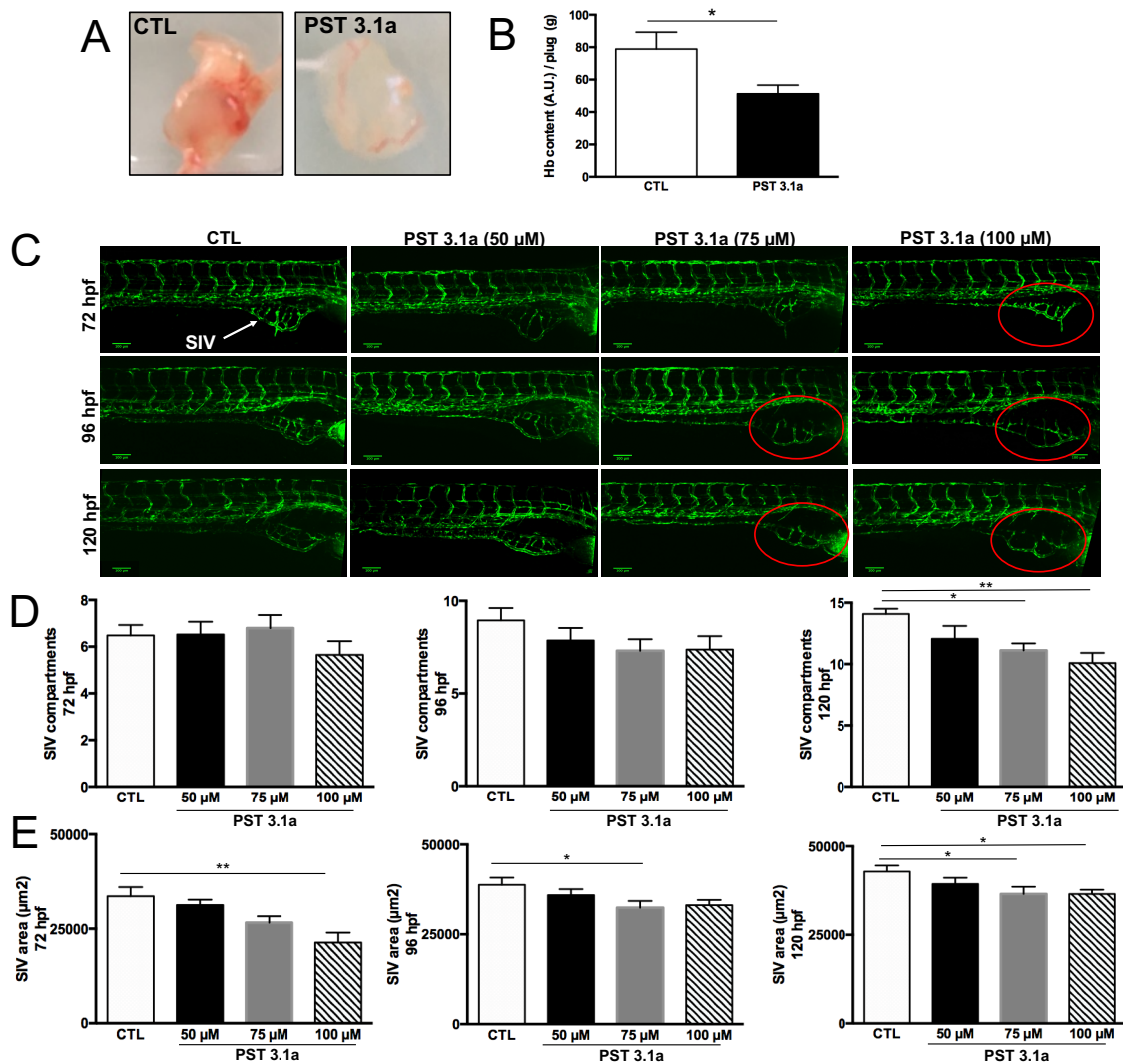
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**Figures:**



**Fig. 1: PST 3.1a inhibits angiogenesis steps *in vitro*.**

HUVECs were incubated in the absence or in the presence of different concentrations of PST 3.1a (1, 5 or 10  $\mu$ M) during 24 h. **A.** Wound healing was performed to evaluate anti-migratory properties of PST 3.1a. **B.** HUVECs were counted using Trypan blue exclusion to evaluate proliferation. **C.** Adhesion was estimated using crystal violet. **D.** *In vitro* angiogenesis using matrigel was performed, allowing HUVECs to reorganize and form capillary-like structures. Data represent the mean  $\pm$  SEM (n=3/8) (\* p<0.05; \*\* p<0.01; versus control).



**Fig. 2: PST 3.1a decreases angiogenesis *in vivo*.**

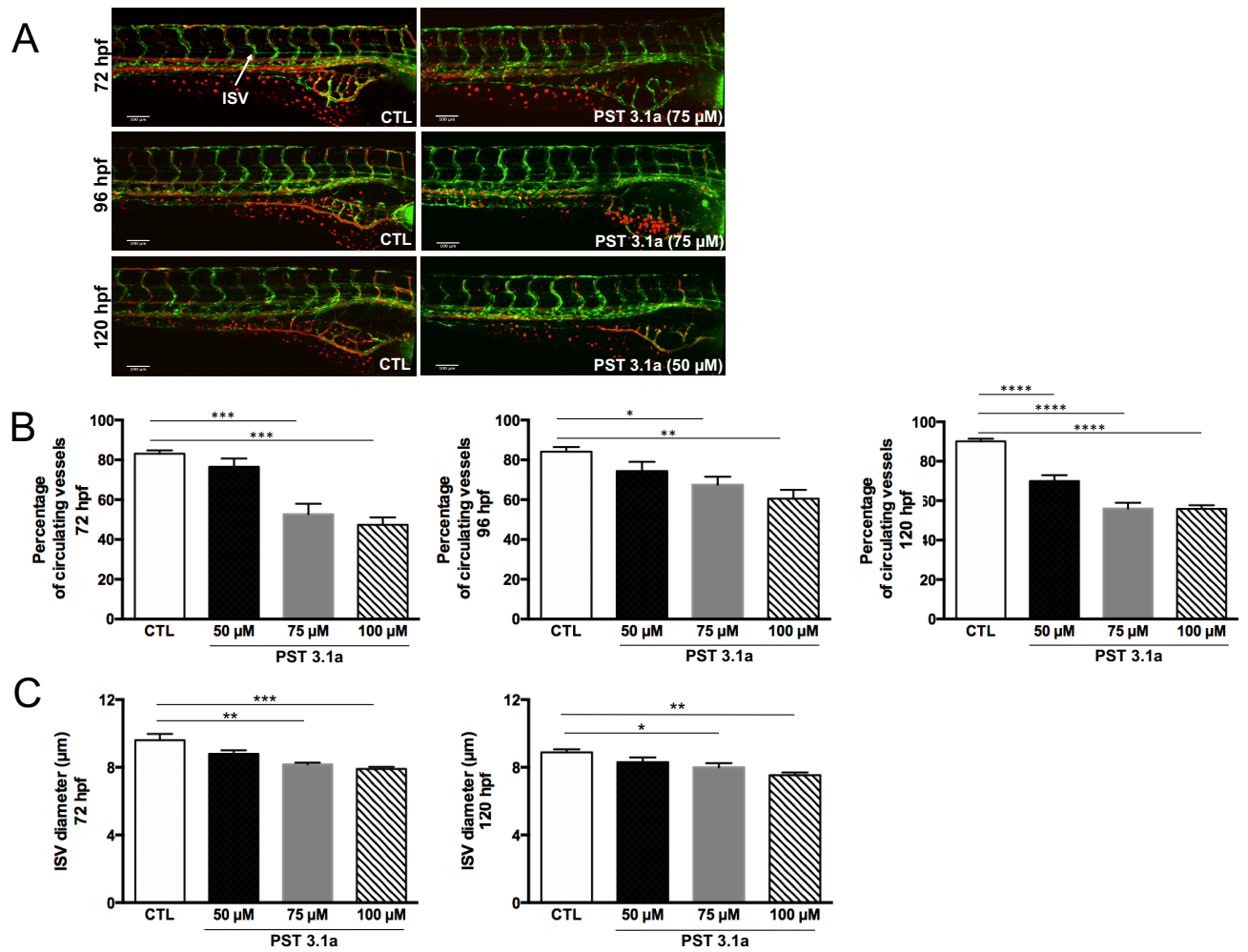
**A.** HUVECs were incubated in the absence or in the presence of PST 3.1a (10  $\mu$ M) during 24 h. Cells were mixed with Matrigel and bFGF (6  $\mu$ g/mL) and subcutaneously injected into nude mice for 1 week. Pictures show the matrigel plugs of control (left) and PST 3.1a-treated (right) cells.

**B.** Quantitative measurement of hemoglobin (Hb) was reported as absorbance/weight of plugs.

**C.** Zebrafish Tg (fli1a:EGFP/gata1a:dsRed) eggs were exposed to 50, 75 or 100  $\mu$ M of PST 3.1a and SIV formation was analyzed at 72, 96 or 120 hpf using spinning disk confocal microscopy. Green fluorescence represents ECs. Scale bars represent 100  $\mu$ m.

**D.** The mean number of compartments per sub-intestinal plexus was calculated at different times.

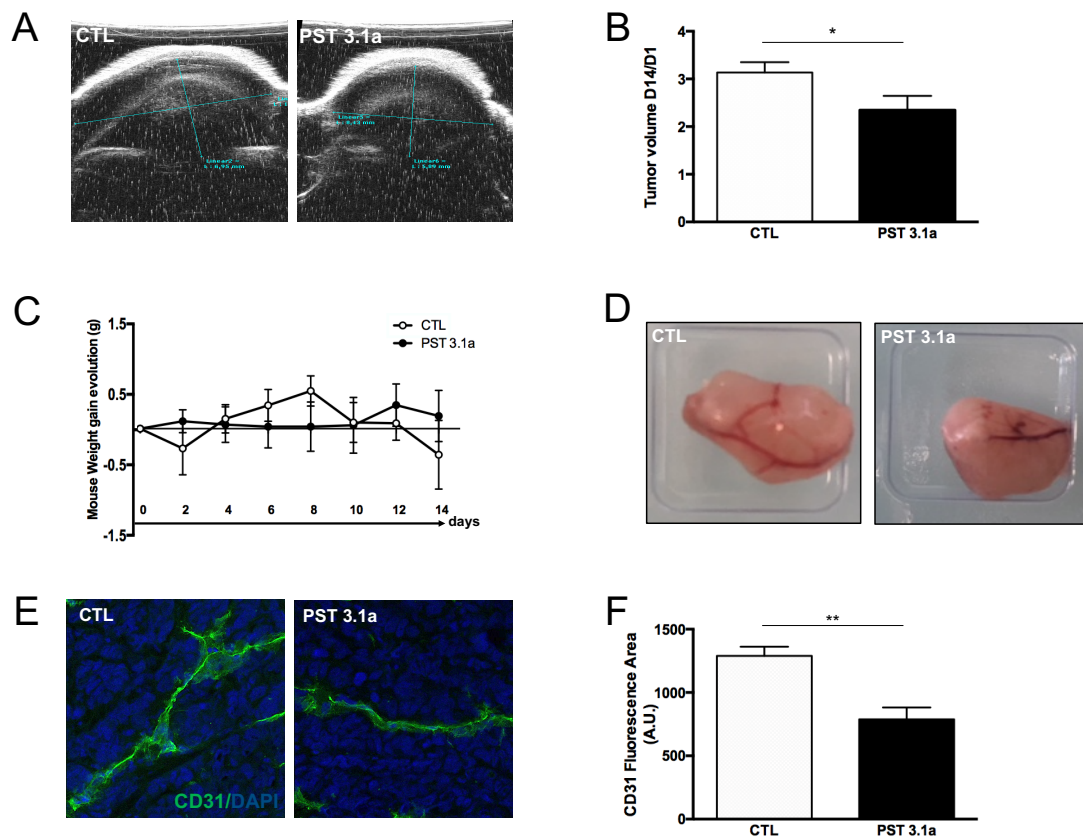
**E.** The measure of the SIV area coverage over the yolk was performed at different times. Data represent the mean  $\pm$  SEM (n=4 for matrigel plug and n=16/21 embryos per condition) (\* p<0.05; \*\* p<0.01; versus control).



**Fig. 3: PST 3.1a decreases blood flow in zebrafish.**

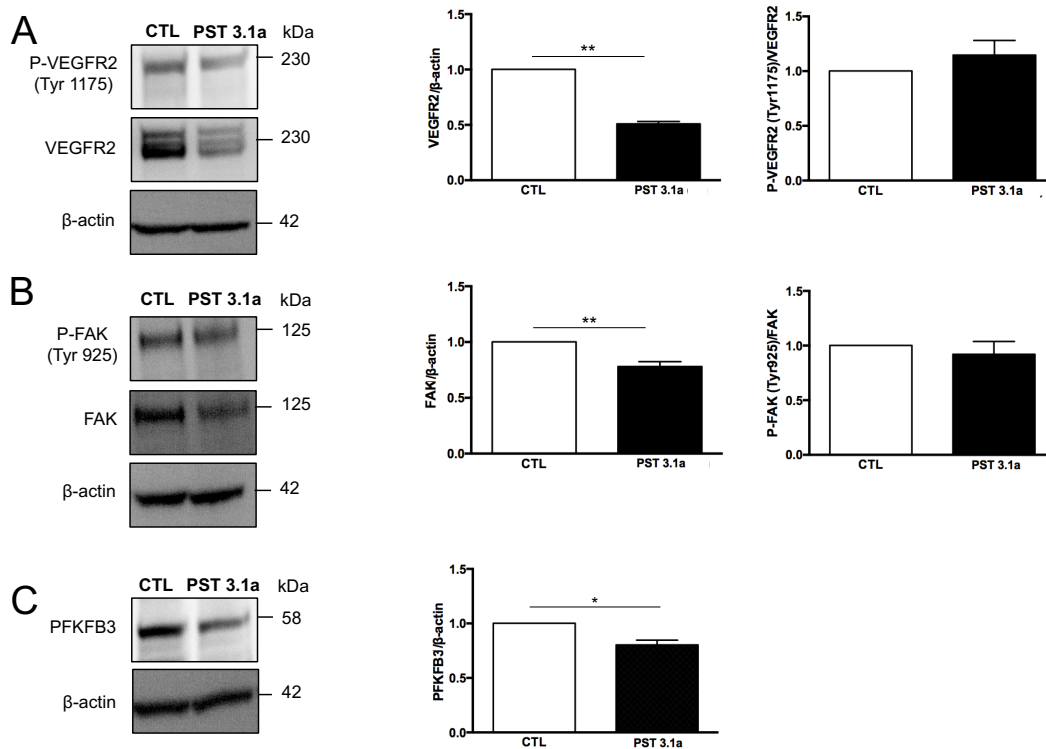
**A.** Zebrafish *Tg(fli1a:EGFP/gata1a:dsRed)* eggs were exposed or not to PST 3.1a at 50, 72 and 100  $\mu$ M for 72, 96 or 120 hpf. Representative photos showing ISV circulation (see Video Fig. 3 for corresponding videos). Green fluorescence represents ECs and red one represents red blood cells. Scale bars represent 100  $\mu$ m. **B.** The number of perfused ISV was quantified in zebrafish embryos. **C.** Quantification of ISV diameters in zebrafish embryos. Data represent the mean  $\pm$  SEM (n=16/21 embryos per condition). (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$  versus control).





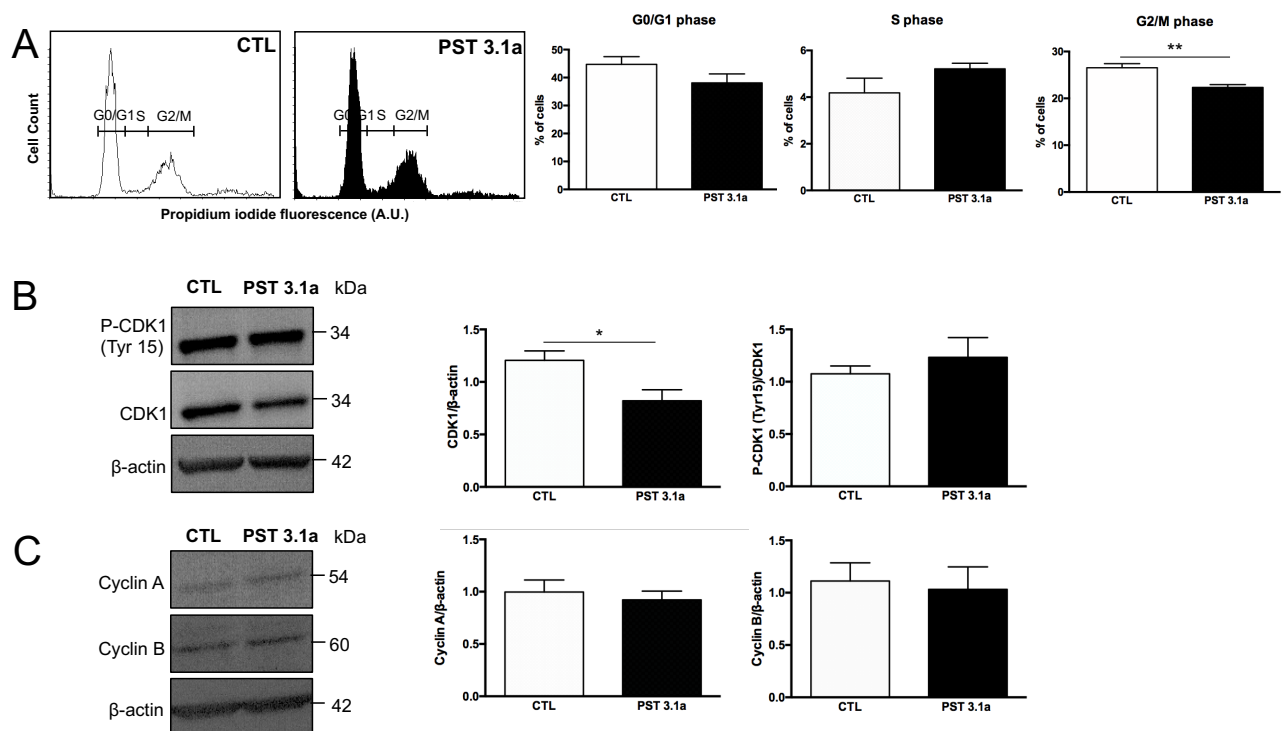
**Fig. 4: PST 3.1a decreases tumor development.**

**A.** After SNB75 injection and tumor growth, mice were fed or not with PST 3.1a (12 mg/kg) during 14 days; representative echography profiles show mouse tumors. **B.** Histogram shows the tumor volume evolution between the end and the beginning of treatment. **C.** Curves represent the weight gain evolution of mice during the treatment. **D.** After the 14 days of treatment, mice were sacrificed and tumors were extracted. Pictures show the tumor plugs of control (left) and PST 3.1a-treated (right) cells. **E.** Representative images of tumors vessel sections. Blue fluorescence represents DAPI (nuclei) and green one represents endothelial marker CD31 labelling. **F.** Histograms show the CD31 fluorescence area. The relative quantity of ECs was estimated by calculating the area occupied by green fluorescence. Data represent the mean  $\pm$  SEM (n=6/8) (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; versus control).

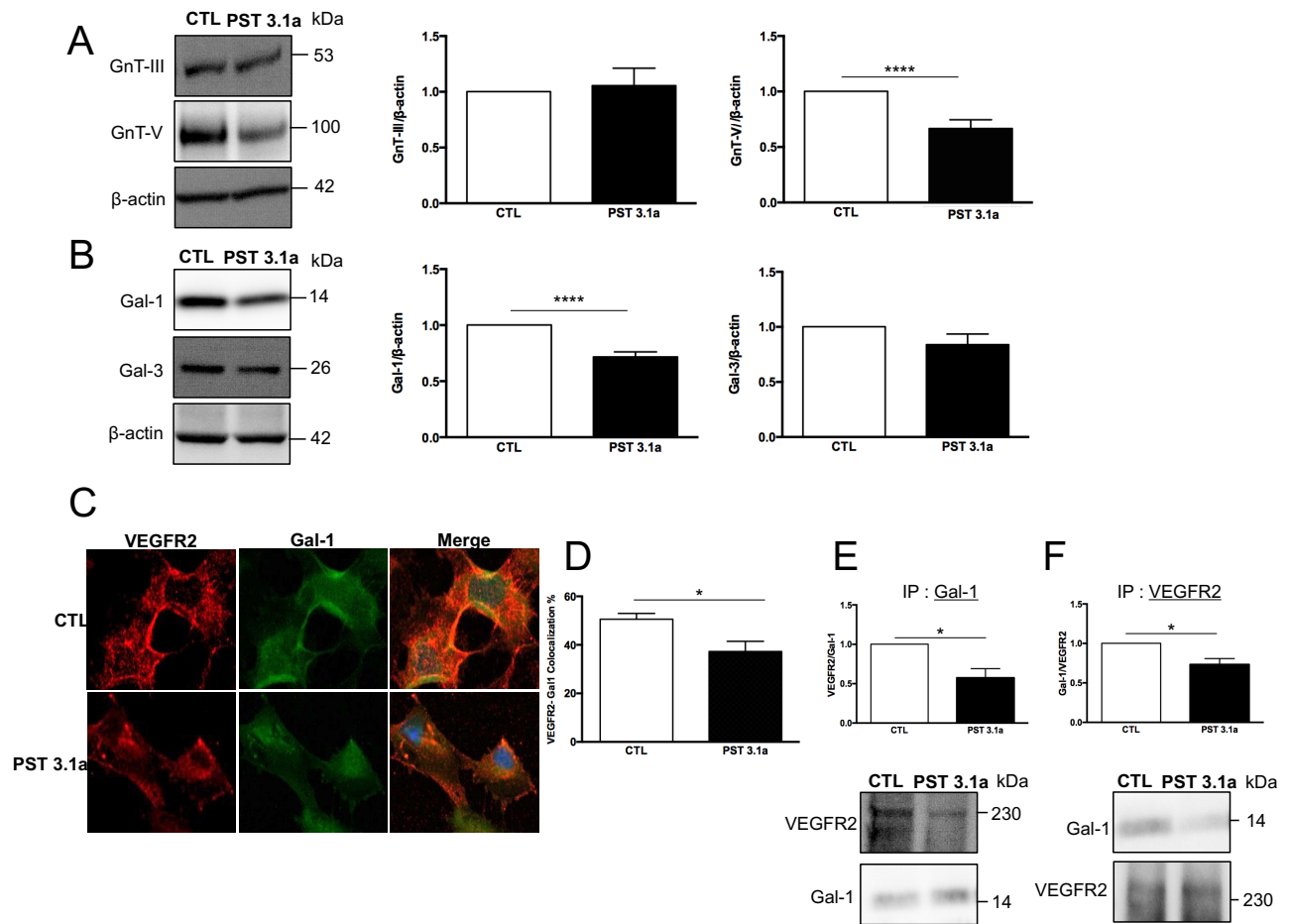


**Fig. 5: PST 3.1a treatment reduces protein expression of key angiogenesis factors in HUVECs.**

HUVECs were treated with 10  $\mu$ M of PST 3.1a for 24 h. **A.** Immunoblots show VEGFR2 phosphorylation (Tyr 1175) and expression in presence of 0.1% VEGF in the medium. Histograms show densitometric analysis of protein phosphorylation and expression.  $\beta$ -actin is used as a loading control. **B.** Western-Blots show FAK phosphorylation (Tyr 925) and expression. Histograms show densitometric analysis of protein phosphorylation and expression.  $\beta$ -actin is used as a loading control. **C.** Immunoblots show PFKFB3 expression. Histograms represent densitometric analysis of protein expression.  $\beta$ -actin is used as a loading control. Data represent the mean  $\pm$  SEM (n=5/7) (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; versus control).



**Fig. 6: PST 3.1a modifies HUVEC cell cycle.** Cells were exposed to control medium or to PST 3.1a 10  $\mu$ M during 24 h. **A.** Representative histograms showing the percentage of cells in the G0/G1, S and G2/M phases determined by flow cytometric analysis. **B.** Immunoblot shows CDK1 phosphorylation (Tyr 15) and expression. Histograms represent quantification of CDK1 phosphorylation and expression.  $\beta$ -actin is used as a loading control. **C.** Western-blot representing cyclin A and cyclin B expression in comparison with control. Histograms show densitometric analysis of protein expression.  $\beta$ -actin is used as a loading control. Data represent the mean  $\pm$  SEM (n=4/7) (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; versus control).



**Fig. 7: PST 3.1a decreases GnT-V and Gal-1 expression.**

HUVECs were treated with PST 3.1a during 24 h at 10  $\mu$ M. **A.** Western-blots represent GnT-III and GnT-V expression. Histograms show the quantitative measurement of GnT-III and GnT-V expression.  $\beta$ -actin is used as a loading control. **B.** Immunoblots show Gal-1 and Gal-3 expressions. Histograms represent the quantitative measurement of proteins expression.  $\beta$ -actin is used as a loading control. **C.** Immunofluorescence detection of VEGFR2 (red) and Gal-1 (green) was performed and colocalization of both proteins was measured using co-immunofluorescence. **D.** Representative histograms of co-immunofluorescence showing the colocalization of VEGFR2 and Gal-1. **E.** Western-blot revealing content of Gal-1 and VEGFR2 after Gal-1 immunoprecipitation in HUVECs treated with or without PST 3.1a (10  $\mu$ M). **F.** Western-blot revealing Gal-1 and VEGFR2 expression after VEGFR2 immunoprecipitation. Data represent the mean  $\pm$  SEM (n=4/10) (\*  $p < 0.05$ ; \*\*\*\*  $p < 0.0001$  versus control).

## **Supplemental data**

### **Apoptosis analysis**

HUVECs ( $1.5 \times 10^5$ ) were exposed or not to PST 3.1a (10  $\mu$ M) for 24 h and then fixed in 70 % ethanol at 4°C for at least 4 h. After a centrifugation at 15,000 g for 5 min, cells were re-suspended in PBS containing 50  $\mu$ g/mL RNase (Sigma-Aldrich) and 10  $\mu$ g/mL propidium iodide (Sigma-Aldrich). Cellular DNA content was analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter).

### **ATP/ADP measurements**

HUVECs were plated in 96-well microplates treated or not with PST 3.1a (10  $\mu$ M) for 24 h. The ATP/ADP ratio was performed using bioluminescent detection (ADP/ATP Ratio Assay Kit, Abcam) according to the manufacturer's instructions. The bioluminescent intensities were measured on a multi-mode microplate reader (Clariostar, BMG labtech, NC).

### **Quantification of the mitochondrial DNA content**

Cells ( $1.2 \times 10^5$  per well) were seeded into 6-well plates overnight, then treated in absence or presence of PST 3.1a during 24 h. Total DNA was isolated from HUVECs according to the manufacturer's protocol (Qiagen, Germany). The mitochondrial DNA copy number was determined by qPCR using a mitochondrial gene coding for the NADH dehydrogenase subunit 1 (ND1) and a nuclear gene coding for  $\beta$ 2 microglobulin (B2M) as respective markers of mitochondrial and genomic DNA. Total DNA was measured on a Light cycler 480 II (Roche, Switzerland).

### **Citrate synthase activity measurement**

HUVECs ( $2.5 \times 10^5$ ) were harvested and resuspended in cell buffer containing 250 mM sucrose, Tris 20 mM, EDTA 2 mM, BSA 1 mg/ml, pH 7.2. Analysis of citrate synthase activity was performed by measuring absorbance of citrate synthase product, the thionitrobenzoic acid (412 nm), on cell lysates, thermostatically maintained at 37° C, using Safas UVmc2<sup>®</sup> spectrophotometer (Safas, Monaco). Protein dosage was finally made to normalize the quantity of cells.

### **Measurement of HUVEC respiration**

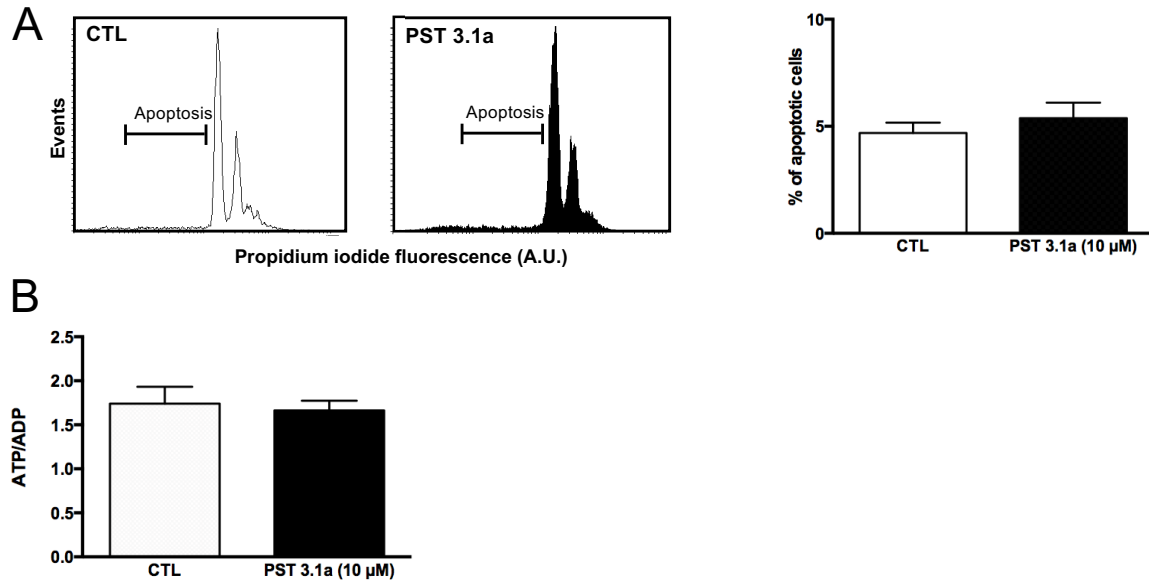
Respiration rates were measured in the HUVEC cell line, stimulated with or without PST 3.1a (1, 5 or 10  $\mu$ M). The cells were collected by trypsinization, washed once in culture medium and centrifuged (500 g  $\times$  5 min). The pellet was resuspended in respiratory medium (DMEM, Ham's F-12 1:1, 1% l-Glutamine, 1% aminopterin/thymidin/hypoxanthin, 1% non-essential amino acids). Mitochondrial oxygen consumption was recorded at 37°C using a high-resolution Oxygraph-2K respirometer (Oroboros, Austria). The cell's basal respiration rate was determined by measuring the linear rate of oxygen consumption, which reflects the cell's aerobic metabolic activity with physiological substrates in culture medium. Oligomycin (4 mg/ml) was then added to determine the respiration uncoupled to ATP synthesis. The uncoupled respiratory rate was assessed by stepwise additions of FCCP (carbonilcyanide *p*-triflouromethoxyphenylhydrazone, 200  $\mu$ M) up to an optimal concentration that represents the capacity of the respiratory chain without the regulation of ATP synthase. Finally, non-mitochondrial oxygen consumption rate was determined by adding antimycin A (1 mg/ml) and rotenone (2.5 mM). Protein concentrations were determined using the Lowry method. Respiration rate was expressed in nanomoles of O<sub>2</sub> consumed per minute and per milligram of protein. Respiratory ratios were determined as described below. The respiratory control ratio (RCR) is the ratio between the FCCP and the oligomycin respiration, and reflects the component of the maximal respiratory chain capacity that is used for the compensation of the proton leak. The phosphorylating respiratory control ratio (RCRp) was obtained by subtraction of oligomycin to basal respiration, and then by dividing with the FCCP respiration. The RCRp ratio reflects the part of the maximal respiratory chain capacity used for ATP synthesis. Finally, the uncoupling control ratio (UCR) is the ratio between the FCCP and the basal respiration and reflects the reserve of capacity of the respiratory chain.

### **Glucose and lactate measurements**

Glucose and lactate concentrations in the culture media were determined by spectrophotometry, using appropriate enzymatic kits (Boehringer, Germany) on a Hitachi-Roche apparatus (Roche).

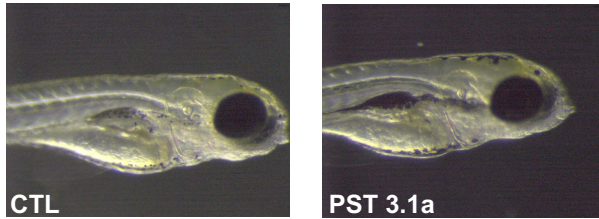
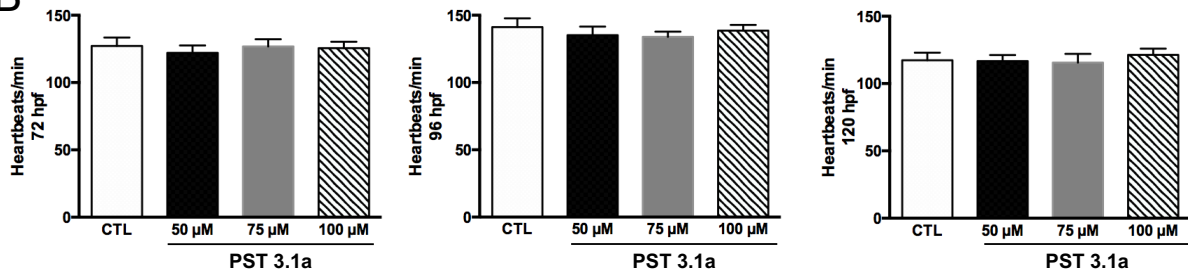


Supplemental figures:

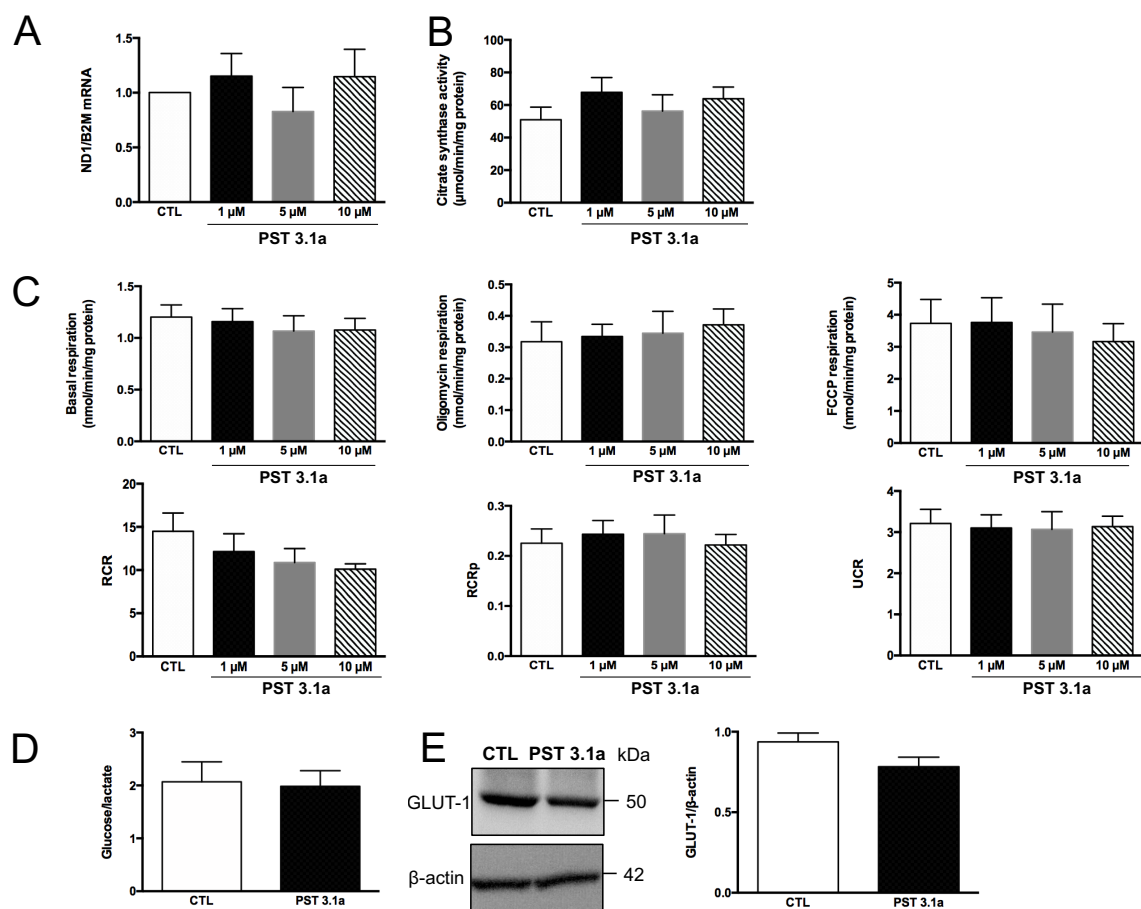


**Suppl Fig. 1: PST 3.1a does not display any toxicity on HUVECs.**

**A.** Representative cytometric profiles showing percentage of cells with hypodiploid DNA. Histograms represent the quantification of apoptotic cells. **B.** HUVECs were treated or not with PST 3.1a (10  $\mu$ M) and the ATP/ADP ratio was measured using bioluminescence. Data represent the mean  $\pm$  SEM (n=4/6).

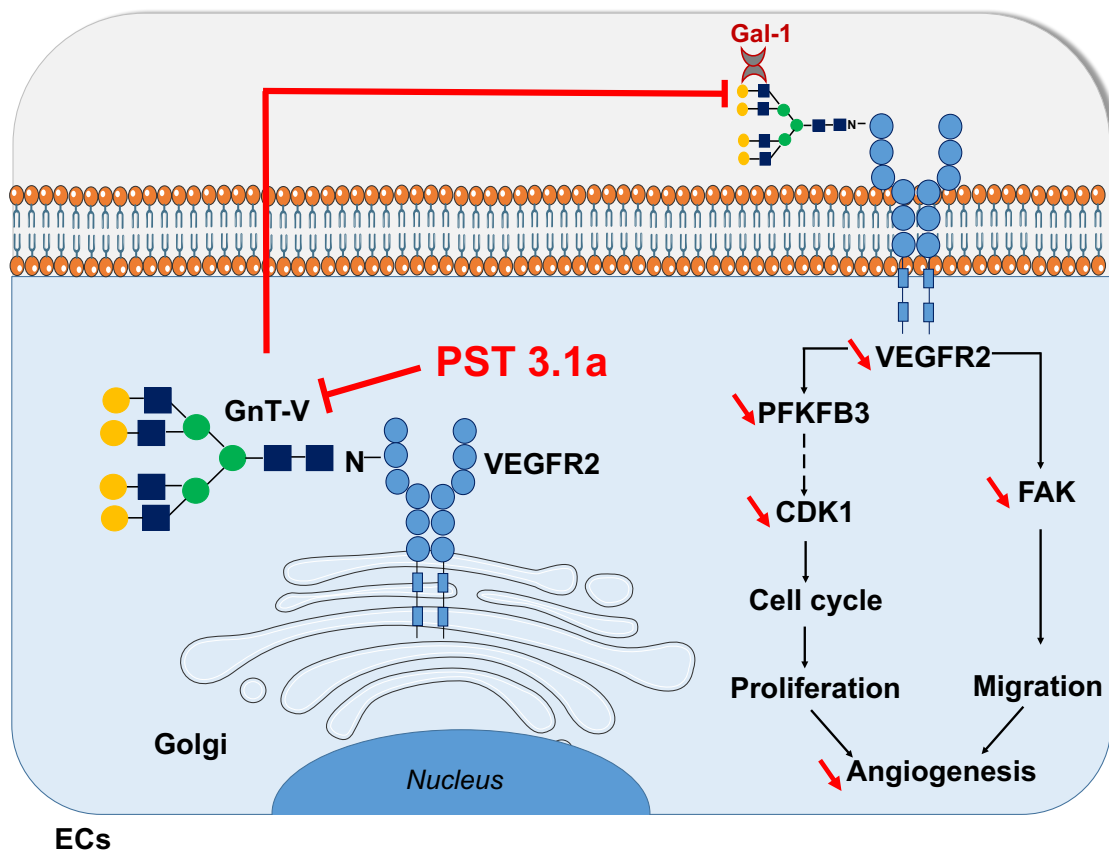
**A****B****Suppl Fig 2: PST 3.1a does not disrupt zebrafish heartbeats**

Zebrafish Tg(fli1a:EGFP/gata1a:dsRed) eggs were exposed or not to PST 3.1a at 50, 72 and 100  $\mu$ M for 72, 96 or 120 hpf. **A.** Representative pictures showing Zebrafish embryos heartbeats (see Video Suppl Fig. 2 for corresponding videos) in the absence or in the presence of PST 3.1a. **B.** Quantitative measurement of zebrafish heartbeats was reported as number of heartbeats per min. Data represent the mean  $\pm$  SEM (n=16/21 embryos per condition).



**Suppl Fig. 3: PST 3.1a did not alter mitochondria physiology and glycolysis.**

HUVECs were incubated in the absence or in the presence of PST 3.1a (1, 5 or 10 μM) during 24 h. **A.** Measurement of mitochondria DNA was performed using qPCR. NADH-ubiquinone oxidoreductase chain 1 (ND1) reflects mitochondrial DNA and β2 microglobulin (B2M) reflects nuclear cell DNA. **B.** Citrate synthase (CS) activity, which represents mitochondrial biogenesis was evaluated by measuring absorbance of CS product, the thionitrobenzoic acid (412 nm). **C.** HUVEC rate of O<sub>2</sub> consumption was assessed using an Oxygraph-2k respirometer. The respiratory control ratio (RCR) is the ratio between the FCCP and the oligomycin respiration. The phosphorylating respiratory control ratio (RCRp) was obtained by subtraction of oligomycin to basal respiration, and then by dividing with the FCCP respiration. Finally, the uncoupling control ratio (UCR) is the ratio between the FCCP and the basal respiration. **D.** Glucose and lactate concentrations were determined by spectrophotometry in the HUVEC culture media. **E.** Immunoblot showed GLUT-1 expression, and histogram represents the quantification of protein expression. β-actin is used as a loading control. Data represent the mean ± SEM (n=4/6).



**Suppl. Fig. 4: Summary of the biological effects of PST 3.1a on HUVEC mechanisms.**

PST 3.1a reduces GnT-V expression, concurring to a reduction of VEGFR2 and Gal-1 interaction at cell membrane. These effects lead to an alteration of the VEGFR2 pathway, including PFKFB3 and FAK expressions, resulting in a disruption of subsequent angiogenesis.

# Discussion générale

Ce travail de thèse a donc porté sur l'implication du glycomimétique PST 3.1a sur l'angiogenèse physiologique et tumorale, ainsi que sur le métabolisme endothélial associé. Lors de cette étude, nous avons dans un premier temps caractérisé les propriétés anti-angiogéniques de PST 3.1a, *in vitro* puis *in vivo*. Nous avons également démontré que PST 3.1a réduit la vascularisation tumorale et la progression du GBM. Dans un second temps, nous nous sommes intéressés aux perturbations métaboliques endothéliales mises en jeu. Nos résultats prouvent que PST 3.1a réduit l'expression de PFKFB3, enzyme clé pour réguler la glycolyse et la différenciation des CEs lors de l'angiogenèse. De plus, PST 3.1a inhibe l'expression de l'enzyme GnT-V qui régule la N-glycosylation, ainsi que l'interaction entre VEGFR2 et Gal-1, décrite comme primordiale dans la résistance tumorale aux traitements anti-angiogéniques. L'ensemble de ces résultats démontrent que PST 3.1a réduit l'angiogenèse par une approche innovante et prometteuse.

L'excès d'angiogenèse est dorénavant bien décrit comme étant impliqué dans la genèse de nombreuses maladies, et les traitements anti-angiogéniques représentent un enjeu thérapeutique crucial. Afin d'étudier l'implication de PST 3.1a sur l'angiogenèse, nous avons dans un premier temps caractérisé son impact *in vitro* sur les différentes étapes menant à l'angiogenèse. PST 3.1a inhibe significativement la migration, la prolifération, l'adhésion et la formation de tubules des HUVECs. Bien que l'inhibition de prolifération soit inférieure à celle du bevacizumab, anticorps anti-VEGF de référence (Carneiro et al., 2009), les étapes de migration et de formation de tubules sont réduites dans des proportions comparables dans les HUVECs, mais à une concentration de PST 3.1a largement inférieure (0.25 mg/mL pour le bevacizumab contre 10  $\mu$ M, soit 5.4  $\mu$ g/mL de PST 3.1a) (Carneiro et al., 2009). Par la suite, le modèle murin nous a permis de confirmer ces effets anti-angiogéniques *in vivo* par mesure du contenu en hémoglobine de matrigel plugs. Afin de compléter ces résultats, nous avons étudié l'impact de PST 3.1a sur le développement vasculaire chez l'embryon de zebrafish. De par ses similarités vasculaires avec les autres vertébrés, la transparence des alevins et son développement rapide, le zebrafish est un excellent modèle pour étudier la formation des néo-vaisseaux chez l'embryon (Isogai et al., 2011). Nos résultats illustrent que PST 3.1a réduit à la fois le développement vasculaire au niveau du plexus subintestinal, mais également la fonctionnalité des vaisseaux intersegmentaux, sans perturber le rythme cardiaque des alevins. Il est intéressant de noter qu'au contraire de PST 3.1a, le bevacizumab n'inhibe pas le développement des vaisseaux subintestinaux dans ce modèle, ce qui renforce le potentiel de PST 3.1a (Chimote et al., 2014). L'ensemble de ces résultats *in vitro* et *in vivo* démontrent avec robustesse les propriétés anti-angiogéniques de PST 3.1a.



L'angiogenèse est une étape essentielle pour la croissance tumorale, l'invasivité et la dissémination métastatique. Bien qu'ils soient principalement utilisés en tant qu'adjuvants à la chimio- et radiothérapie, les traitements anti-angiogéniques représentent pourtant un enjeu majeur dans le cadre des thérapies anti-tumorales. Parmi les tumeurs, le GBM est caractérisé d'un point de vue physiopathologique par son caractère très invasif et hautement angiogénique (Jain et al., 2007). Dans le cadre du GBM, les traitements anti-angiogéniques actuels reposent principalement sur l'utilisation du bevacizumab, mais il provoque une forte toxicité chez les patients (Brandes et al., 2015). Dans notre projet, bien que le composé PST 3.1a possède une activité inhibitrice contre plusieurs cancers, son étude s'est concentrée sur le GBM, pour lequel, aucun traitement, autre que palliatif, n'existe à ce jour. Les travaux précédemment publiés (Hassani et al., 2017) font état d'une activité anti-tumorale *in vivo* de PST 3.1a et nos résultats le confirment. De façon intéressante, PST 3.1a n'induit pas de mortalité ni de perte de poids chez les souris traitées avec 15 mg/kg pendant 14 jours. La même observation est effectuée à très forte dose chez les souris (900 mg/kg pendant 28 jours, non publié), ainsi que chez le zebrafish aux doses utilisées (100  $\mu$ M pendant 5 jours). Dans cette étude, nous démontrons en plus que PST 3.1a réduit fortement l'angiogenèse tumorale, de presque 50 %. Même si nos résultats ne représentent pas l'effet de PST 3.1a sur les autres modes de vascularisation du GBM, l'angiogenèse représente le principal moteur de la vascularisation du GBM et PST 3.1a la réduit de manière significative. Ces résultats sont particulièrement intéressants car le microenvironnement vasculaire tumoral est très différent du microenvironnement vasculaire physiologique ; les vaisseaux tumoraux sont décrits comme tortueux, instables, peu perméables, hétérogènes, et les CEs qui les composent possèdent de grandes et lâches jonctions inter-cellulaires (Carmeliet & Jain, 2011). L'inhibition d'angiogenèse dans ce modèle renforce d'avantage les propriétés anti-angiogéniques de PST 3.1a dans des microenvironnements hétérogènes, et sans induire de toxicité. L'utilisation potentielle de ce composé pourrait ainsi s'inscrire en tant qu'alternative au bevacizumab.

Une seconde importante limite à l'utilisation du bevacizumab concerne son efficacité insuffisante, due à la mise en place de mécanismes compensatoires par les tumeurs. Dans la perspective de mettre en place des traitements davantage spécifiques, cibler le métabolisme endothélial est considéré comme le nouveau paradigme anti-angiogénique (Vandekeere et al., 2015). En plus d'agir plus spécifiquement sur les modifications métaboliques mises en jeu par les CEs, cette stratégie vise également les CTs. Ces deux types cellulaires sont décrits comme « brothers in arms » (Verdegem et al., 2014), et possèdent plusieurs similitudes métaboliques. Le blocage de ces voies métaboliques communes est stratégique pour les futures thérapies anti-

tumorales et anti-angiogéniques, afin de développer des alternatives efficaces inhibant ces deux types cellulaires.

La glycolyse est la voie majeure de production d'adénosine triphosphate (ATP) pour les CTs et les CEs. En conséquence, l'inhiber représente une stratégie anti-angiogénique séduisante et spécifique, mais également destructrice pour les CEs quiescentes. Différentes études ont en effet montré que certaines drogues tel que le 2-deoxy-D-glucose (2DG), qui inhibe de façon permanente et maximale la glycolyse en ciblant l'enzyme glycolytique phosphoglucose isomérase, entraîne une forte toxicité cellulaire (Schoors et al, 2014). Le 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) est une seconde molécule, actuellement utilisée en phase I d'essais cliniques (Conradi et al., 2017), qui permet une inhibition plus modérée de la glycolyse, de l'ordre de 40 %. En ciblant PFKFB3, enzyme directement activée par VEGFR2 et conduisant à la différenciation des CEs en tip ou stalk cells (Stapor et al., 2014), le 3PO entraîne de plus modestes effets secondaires et une inhibition d'angiogénèse plus spécifique (Stapor et al., 2014 ; Treps et al., 2016). Cependant, différentes limites à l'utilisation du 3PO ont récemment émergé, comme ses propriétés immunosuppressives, ou son effet pro-métastatique à forte concentration (Telang et al., 2012 ; Conradi et al., 2017). Bien que nos résultats ne plaident pas en faveur d'une réduction de la glycolyse par PST 3.1a, nous observons une diminution d'expression de PFKFB3 de l'ordre de 20 %, qui semble a priori trop faible pour inhiber la glycolyse. En revanche, PFKFB3 est également un régulateur du cycle cellulaire au niveau de la kinase dépendante des cyclines (CDK)-1, régulant la transition G2/M (Yalcin et al., 2009). Dans notre étude, PST 3.1a réduit l'expression de CDK1, ainsi que le taux de cellules en phase G2/M. Ces résultats confirment l'intérêt de PFKFB3 comme cible anti-angiogénique majeure, et mettent en avant le potentiel thérapeutique de PST 3.1a.

L'impact du métabolisme endothélial sur l'angiogénèse s'exerce à travers la production d'énergie, mais également à travers les modifications protéiques régulant les voies de signalisation nécessaires à l'angiogénèse (Potente & Carmeliet, 2017). Dans cet objectif, cibler la glycosylation représente une alternative crédible pour inhiber l'angiogénèse. En effet, c'est une régulation post-traductionnelle surexprimée dans les CTs et dans les CEs impliquées dans le processus d'angiogénèse. Au contraire, les CEs quiescentes en sont moins dépendantes, et pour ces cellules, cette stratégie se révélerait potentiellement moins toxique. Nos résultats *in vitro* démontrent que PST 3.1a réduit l'expression de la glycosyltransférase GnT-V de l'ordre de 40 %, ainsi que celle de Gal-1 dans des proportions similaires. Les expérimentations démontrent également que PST 3.1a ne provoque pas d'apoptose et ne perturbe pas le ratio ATP/ADP dans

les HUVECs, et les études *in vivo* confirment cette absence de toxicité. Ces résultats nous confortent dans l'hypothèse selon laquelle cibler la glycosylation permet de réduire l'angiogenèse, tout en générant une faible toxicité.

La glycosylation est une régulation métabolique surexprimée dans les tumeurs, et celle-ci est encore accrue dans les tumeurs résistantes aux thérapies anti-angiogéniques actuelles. En augmentant leur dépendance vis-à-vis du métabolisme anaérobie, et notamment la glycosylation, de nombreuses tumeurs arrivent désormais à contrer l'utilisation du bevacizumab. En particulier, un des principaux mécanismes de résistance tumoral passe par la stimulation de l'interaction entre VEGFR2 et Gal-1, permettant d'induire la signalisation VEGFR2 indépendamment de la présence du VEGF (Crocì et al., 2014 ; Stanley, 2014). En bloquant cette interaction, PST 3.1a inhibe donc une voie majeure de résistance aux thérapies anti-angiogéniques actuelles.

En résumé, cette étude s'inscrit dans la nouvelle perspective de mise en place de traitements anti-angiogéniques, en ciblant spécifiquement les perturbations métaboliques mises en jeu lors de la vascularisation tumorale. Notre étude démontre que PST 3.1a inhibe à la fois l'angiogenèse et la progression du GBM. Le mécanisme d'action de ce composé passe par une inhibition de la N-glycosylation, ce qui génère *in fine* une moindre toxicité. De plus, PST 3.1a bloque un des principaux mécanismes de résistance tumorale et laisse présager d'une potentielle utilisation seul, ou combiné avec les traitements anti-angiogéniques en vigueur.

Certaines limites à notre étude peuvent cependant être soulignées. L'absence de contrôle positif dans les différentes expérimentations représente une limite notable. Il aurait été intéressant de comparer les propriétés anti-angiogéniques de PST 3.1a par rapport aux drogues de référence (bevacizumab) ou celles inhibant la glycosylation (tunicamycine, swainsonine). Également, bien que nos résultats démontrent une réduction de l'expression de GnT-V, nous n'avons pas prouvé que cette enzyme est la cible directe de PST 3.1a dans les HUVECs. Une étude de glycoprofile permettrait de définir avec plus de précision l'inhibition du glycome causée par PST 3.1a, et confirmer que notre composé cible spécifiquement GnT-V. Enfin, au niveau des expérimentations *in vivo*, la spécificité d'action de PST 3.1a contre l'angiogenèse tumorale n'a pas été démontrée dans notre étude. Pour réponse à cela, nous pourrions réaliser une blessure chez nos animaux traités afin d'observer si PST 3.1a inhibe également ou non cette angiogenèse physiologique.

Plusieurs axes pourraient permettre de prolonger le travail proposé lors de cette étude. Dans un premier temps, il serait intéressant de découvrir l'ensemble des voies altérées suite à l'inhibition de GnT-V par PST 3.1a, et ainsi potentiellement élargir son champ d'action. En particulier, étudier son impact sur différentes glycoprotéines impliquées dans l'angiogenèse, telles

que l'angiopoïétine like 4 (Cazes et al., 2006 ; Gomez Perdiguero et al., 2016), ou Wnt/Frizzled (Peghaire et al., 2016) pourrait être une approche prometteuse.

Une seconde perspective potentielle viserait à étudier l'impact de PST 3.1a sur la voie du FGF. Comme décrit précédemment, un des principaux mécanismes de résistance tumorale vise à augmenter l'interaction VEGFR2 et Gal-1. Afin de contrer les thérapies actuelles, certaines tumeurs mettent en place un autre mécanisme de résistance, en stimulant la sécrétion d'autres facteurs pro-angiogéniques et notamment le FGF, pour favoriser la vascularisation par d'autres voies indépendantes (Missiaen et al., 2017). Bien que nous n'ayons pas étudié la signalisation FGF, le récepteur du FGF est N-glycosylé et il a été prouvé que la voie FGF est totalement inhibée dans les cellules GnT-V<sup>-/-</sup> (Partridge et al., 2004). Etant donné que PST 3.1a réduit l'expression de GnT-V, il serait intéressant d'étudier si PST 3.1a perturbe également la signalisation du FGF.

Enfin, une dernière perspective conduirait à étudier l'implication de PST 3.1a sur d'autres pathologies caractérisées par une angiogenèse excessive (Carmeliet, 2003 ; Chung & Ferrara, 2011). En particulier, il a récemment été proposé que la surexpression de GnT-V pourrait être corrélée avec la pathogenèse du psoriasis (Aterido et al., 2016), et explorer l'implication éventuelle de PST 3.1a sur cette pathologie représenterait une perspective innovante.

Face aux nombreuses limites des traitements anti-angiogéniques actuels, plusieurs stratégies ont émergé ces dernières années, notamment celle visant à cibler le métabolisme endothélial. Ainsi, bien que de nombreux composés soient limités par leur toxicité, certaines molécules inhibant le métabolisme endothélial sont désormais utilisées en essais cliniques. Le glycomimétique PST 3.1a s'inscrit dans cette approche grâce à ses propriétés anti-angiogéniques et anti-tumorales contre le GBM, associées à une absence de toxicité dans différents modèles précliniques. Par conséquent, exploiter les propriétés anti-angiogéniques de PST 3.1a permettrait d'ouvrir une nouvelle voie dans le traitement pharmacologique des pathologies associées à une angiogenèse excessive.

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**Titre :** Implication de la phostine 3.1a sur l'angiogenèse, le métabolisme endothélial et les pathologies associées

**Mots clés :** Angiogenèse, Croissance tumorale, Pharmacothérapie, Métabolisme endothélial, Glycosylation

**Résumé :** Les thérapies anti-angiogéniques actuelles sont limitées, et cibler le métabolisme endothélial représente une nouvelle stratégie prometteuse. Parmi la famille de glycomimétiques Phostines, le composé PST 3.1a possède des propriétés anti-tumorales contre le glioblastome à la fois *in vitro* et *in vivo*. L'objectif de ce projet est d'étudier l'impact de PST 3.1a sur l'angiogenèse et le métabolisme endothélial. L'angiogenèse est un processus complexe décrivant la formation de nouveaux vaisseaux sanguins à partir d'une vasculature préexistante, dirigée par des facteurs pro-angiogéniques locaux tel que le facteur de croissance endothélial vasculaire (VEGF). L'angiogenèse intervient dans différentes conditions pathologiques, et notamment la croissance tumorale.

PST 3.1a (10  $\mu$ M) inhibe les principales étapes menant à l'angiogenèse *in vitro*, parmi lesquelles la migration, la prolifération, l'adhésion et la formation de tubes. PST 3.1a réduit également l'angiogenèse *in vivo* dans les modèles murins et du poisson zèbre, ainsi que l'angiogenèse tumorale et la progression du glioblastome. En particulier nos résultats démontrent une diminution d'interaction entre le récepteur au VEGF 2 et la galectine 1, cette interaction étant décrite comme cruciale dans le cadre des résistances tumorales face aux thérapies anti-angiogéniques actuelles. Ces résultats fournissent une ouverture thérapeutique sur une approche innovante et originale contre les pathologies associées à une angiogenèse excessive, tel que le cancer.

**Title :** Phostin 3.1a implication in angiogenesis, endothelial metabolism and associated pathologies

**Keywords :** Angiogenesis, Cancer growth, Pharmacotherapy, Endothelial metabolism, Glycosylation.

**Abstract:** Actual anti-angiogenic therapies are limited, and targeting endothelial metabolism is a new promising strategy. One of the lead compound of the Phostin family, PST 3.1a has anti-glioblastoma properties both *in vitro* and *in vivo*. The objective of the present study was to assess the effect of PST 3.1a on angiogenesis and endothelial metabolism. Angiogenesis is a complex process describing the growth of new blood vessels from existing vasculature, triggered by local pro-angiogenic factors such as the vascular endothelial growth factor (VEGF). Angiogenesis takes part in various pathological conditions and particularly in tumor growth.

PST 3.1a (10  $\mu$ M) inhibited the main steps leading to angiogenesis *in vitro* including migration, proliferation, adhesion and tube formation. PST 3.1a also reduced physiological angiogenesis in both mice and zebrafish models, and pathological angiogenesis and glioblastoma progression *in vivo*. In addition, our results highlight the alteration of the interaction between VEGF receptor 2 and galectin-1, a binding known as a key component of the regulation of angiogenesis associated to tumor resistance.

These results provide a new route towards an innovative and original approach to target angiogenesis related diseases, including cancer.