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Inhibition of Angiogenesis using Glycolysis Inhibitors: an *in vitro* study

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To my mother, father, brother and sister…

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Abbreviations

2DG	2-Deoxy-glucose
3PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
ANG1/2	Angiopoietin 1⁄2
CA	Carbonic anhydrase
CCL2	CC-chemokine ligand 2
CHC	α-cyano-4-hydroxynnamic acid
CPT1	Carnitine palmitoyl transferase 1
Dll4	Delta like ligand 4
EC	Endothelial cell
ECM	Extracellular matrix
EHEC	EA.hy926 endothelial cell
EPC	Endothelial progenitor cell
F6P	Fructose-6-phosphate
FAO	Fatty acid oxidation
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
HIF	Hypoxia inducible factor
HK	Hexokinase
HUVEC	Human umbilical vein endothelial cell
IL	Interleukin
KLF2	Krüppel-like factor 2
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
MCT	Monocarboxylate transporter
MMP	Matrix metalloprotease
NO	Nitric oxide
OXPHOS	Oxidative phosphorylation
PP	Pentose phosphate pathway
PA	Phenoxindazole analogue
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PFK1	Phosphofructokinase 1
PFKFB3	Phosphofructokinase-fructose-2,6-bisphophatase 3
PK	Pyruvate kinase
SMC	Smooth muscle cell
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor beta

TKI	Tyrosine kinase inhibitor
TNF-α	Tumor necrosis factor alpha
VCAM	Vascular adhesion molecule
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER 1

Summary

Summary

Aberrant angiogenesis contributes to many pathophysiological conditions such as cancer and diabetes mellitus. Current anti-angiogenic therapies aim at targeting key angiogenic growth factors or their endothelial cell-expressed receptors including VEGF and VEGFRs. This strategy, however, often fails to render sustained responses with minimal increased survival rate in treatment of several cancers due to toxicity and drug resistance. A novel approach in the angiogenic field is by indirect and partial inhibition of glycolysis by targeting phosphofructokinase-fructose-2,6-bisphophatase 3 (PFKFB3).

Neo-angiogenesis by endothelial cells (ECs) is regulated by metabolism: mainly glycolysis. The lead anti-glycolytic compound, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), was identified as a promising compound to reduce pathological angiogenesis and tumor growth. However, doubts have risen about its true mechanism of action. We confirm that 3PO partially reduced glycolysis, EC migration enough to impair vessel sprouting. Further, we demonstrate that these effects cannot be attributed to its' suggested binding to the target PFKFB3. 3PO does not bind to PFKFB3 and does not inhibit PFKFB3 kinase activity. Finally, 3PO caused intracellular acidification caused by lactate accumulation in ECs. In another study we have observed a strong transcriptional regulation of these transporters by 3PO. For these reasons, we speculate that 3PO regulate activity of lactate transporters, monocarboxylate transporter 1 (MCT1) and MCT4.

We next identified two selective PFKFB3 inhibitors, PA-1 and PA-2, with validated binding and potent inhibitory activity towards PFKFB3. We demonstrate that these inhibitors reduced glycolysis to similar levels as silencing of PFKFB3 or using 3PO. In turn, formation capillary-like structures were impaired by inhibiting EC proliferation and migration. PFKFB3-mediated inhibition of glycolysis resulted in inhibition of transcription and activity of matrix metalloproteases (MMP)-2 and MMP-9. Furthermore, blockade of PFKFB3 with PA compounds also acted on the VEGFA/VEGFR2 axis and reduced angiogenic activation in inflamed ECs. These insights offer promising opportunities to treat aberrant angiogenesis and vasculogenesis.

Taken together, our research has provided two selective PFKFB3 inhibitors to suppress neo-angiogenesis. Derivatives of these compounds are being studied in preclinical atherosclerotic

models characterized by intraplaque angiogenesis. Future research will provide insight into their potential to promote atherosclerotic plaque stability.

Versione Italiana

Un'angiogenesi alterata contribuisce a molte condizioni patofisiologiche come il cancro e il diabete mellito. Le attuali terapie anti-angiogeniche hanno come bersaglio i principali fattori di crescita angiogenici o i loro recettori espressi dalle cellule endoteliali, inclusi VEGF e VEGFR. Questa strategia, tuttavia, spesso non riesce a fornire delle risposte sostenute con un aumento minimo del tasso di sopravvivenza nel trattamento di diversi tumori a causa della tossicità e della resistenza ai farmaci che si sviluppa. Un nuovo approccio nel campo dell'angiogenesi è l'inibizione indiretta e parziale della glicolisi mediante andando a bloccare l'enzima chiave fosfofructochinasi-fruttosio-2,6-bisfofatasi 3 (PFKFB3).

La neo-angiogenesi da parte delle cellule endoteliali (ECs) è regolata da diverse vie metaboliche, in particolare dalla glicolisi. Il principale inibitore della glicolisi, 3- (3-piridinil) -1- (4-piridinil) -2-propen-1-one (3PO), è stato identificato come un composto promettente per ridurre l'angiogenesi patologica e la crescita del tumore. Tuttavia, sono sorti dubbi sul suo vero meccanismo d'azione. Nei nostri studi, abbiamo confermato che 3PO riduce parzialmente la glicolisi e la migrazione delle EC andando a compromettere la formazione di nuovi vasi. Inoltre, abbiamo dimostrato che questi effetti non sono attribuibili al suo legame a PFKFB3. Infatti, 3PO non si lega a PFKFB3 e non inibisce neanche l'attività chinasica di PFKFB3. Infine, 3PO ha causato l'acidificazione intracellulare in seguito all'accumulo di lattato nelle EC. In un altro studio abbiamo osservato una forte regolazione trascrizionale di alcuni trasportatori del lattato (trasportatore del monocarbossilato 1 (MCT1) e MCT4) da parte di 3PO.

Successivamente abbiamo sintetizzato e studiato due inibitori selettivi di PFKFB3, PA-1 e PA-2, che si legano a PFKFB3 con una potente attività inibitoria. Abbiamo dimostrato che questi inibitori sono in grado di ridurre la glicolisi a livelli simili a quelli ottenuti con il silenziamento di PFKFB3 o l'utilizzo di 3PO. Inoltre, inibendo la proliferazione e la migrazione delle EC, compromettono la formazione di strutture simili a capillari. L'inibizione della glicolisi mediata dal blocco di PFKFB3 ha portato all'inibizione della trascrizione e dell'attività delle metalloproteasi della matrice (MMP) -2 e MMP-9. Inoltre, il blocco di PFKFB3 con i composti PA ha inibito anche l'asse VEGFA / VEGFR2 e ha ridotto l'attivazione angiogenica nelle EC in condizione di infiammazione. Queste osservazioni offrono delle opportunità promettenti per trattare l'angiogenesi e la vasculogenesi aberranti.

Nel suo insieme, la nostra ricerca ha descritto e studiato due inibitori selettivi di PFKFB3 in grado di bloccare la neo-angiogenesi. Derivati di questi composti sono in fase di studio in

modelli preclinici di aterosclerosi caratterizzati da angiogenesi intraplacca. La ricerca futura fornirà informazioni sul loro eventuale potenziale nel promuovere la stabilità della placca aterosclerotica.

Nederlandse versie

Afwijkende angiogenese draagt bij aan veel pathofysiologische aandoeningen zoals kanker en diabetes mellitus. Huidige anti-angiogenese therapieën zijn gefocust op het blokkeren van essentiële angiogenese groeifactoren of endotheel cel-specifieke receptoren waaronder VEGF en VEGFR. Deze strategie, daarentegen, slaagt er echter vaak niet in langdurige reacties te geven met een minimaal verhoogd overlevingspercentage bij de behandeling van verschillende soorten kanker als gevolg van toxiciteit en resistentie tegen geneesmiddelen. Een nieuwe strategie in de wereld van angiogenese is via remming van phosphofructokinase-fructose-2,6-bisphophatase 3 (PFKFB3) het indirect en partieel blokkeren van glycolyse.

Neo-angiogenese door endotheel cellen wordt gereguleerd door het metabolisme: voornamelijk glycolyse. De meest toegepaste anti-glycolyse samenstelling, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), was geïdentificeerd als een veelbelovende samenstelling om pathologische angiogenese en tumor groei te reduceren. Echter, er zijn twijfels met betrekking tot zijn werkingsmechanisme. We bevestigen dat 3PO de glycolyse en endotheel cel migratie gedeeltelijk heeft verminderd voldoende om bloedvatvorming te belemmeren. Verder demonstreren we dat deze effecten niet kunnen worden toegeschreven aan de 'gesuggereerde binding aan het doelwit PFKFB3. 3PO bindt niet aan PFKFB3 en remt de PFKFB3-kinase-activiteit niet. Uiteindelijk veroorzaakte 3PO intracellulaire verzuring veroorzaakt door lactaataccumulatie in endotheel cellen. In een andere studie hebben we een sterke transcriptionele regulatie van deze transporters door 3PO waargenomen. Om deze redenen speculeren we dat 3PO de activiteit van lactaat transporters, monocarboxylaat transporter 1 (MCT1) en MCT4 reguleert.

Vervolgens, identificeerden we twee selectieve PFKFB3-remmers, PA-1 en PA-2, met gevalideerde binding en krachtige remmende activiteit tegen PFKFB3. We demonstreren dat deze remmers glycolyse verminderden tot vergelijkbare niveaus als gen deletie van PFKFB3 of door gebruik van 3PO. Als gevolg werd vorming van capillair-achtige structuren beschadigd door de remming van endotheel cel proliferatie en migratie. PFKFB3-gemedieerde remming van glycolyse resulteerde in remming van transcriptie en activiteit van matrix-metalloproteasen (MMP)-2 en MMP-9. Bovendien werkte blokkade van PFKFB3 met PA-verbindingen ook op het VEGFA/VEGFR2-as en verminderde activering van angiogenese in ontstoken endotheel cellen. Deze inzichten bieden veelbelovende mogelijkheden om afwijkende angiogenese en vasculogenese te behandelen.

In conclusie, ons onderzoek heeft twee selectieve PFKFB3-remmers opgeleverd om neo-angiogenese te onderdrukken. Derivaten van deze verbindingen worden bestudeerd in preklinische atherosclerotische modellen die worden gekenmerkt door intraplaque angiogenese. Toekomstig onderzoek zal inzicht verschaffen in hun potentieel om de stabiliteit van atherosclerotische plaques te bevorderen.

CHAPTER 2

General introduction

2.1 Angiogenesis

2.1.1 Physiological and pathological angiogenesis

The vasculature is the organ that supplies the body with oxygen and nutrients to maintain the metabolic homeostasis. Blood vessel formation is an essential process during embryonic growth, but also in wound healing in adults. Two distinct mechanisms of blood vessel formation exist: vasculogenesis and angiogenesis (Grant and Janigro, 2006).

Vasculogenesis, or embryonic neovascularization, refers to the process of *de novo* blood vessel formation from endothelial progenitor cells (EPCs). It was presumed that vasculogenesis only occurred during the embryonic development, but EPCs were also identified in adult peripheral blood which were shown to contribute to neovessel formation (Velazquez, 2007).

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vasculature. This process occurs primarily during embryonic development, wound healing, and pregnancy. However, a disrupted regulation of this process may lead to pathological angiogenesis. Excessive and pathological angiogenesis have been implicated in the pathogenesis of numerous pathological disorders including cancer, diabetic retinopathy, rheumatoid arthritis and atherosclerosis (Otrock et al., 2007; Potente et al., 2011). A big interest in angiogenesis research has proven the high therapeutic relevance of targeting angiogenesis. The role of neovascularization in atherosclerosis will be further discussed in 1.3.2.

2.1.2 Angiogenic process

In quiescent state, blood vessels regulate the supply of oxygen and nutrients and the removal of waste products. The lumen of the blood vessel consists of a layer of endothelial cells (ECs), the key regulators of angiogenesis. These are supported by the basement membrane, smooth muscle cells (SMCs) and pericytes. In hypoxic or nutrient-limiting conditions, such as ischemia, the surrounding tissue promotes the formation of neovessels by secreting several pro-angiogenic growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), and platelet-derived growth factor (PDGF) (Folkman, 2003; Nishida et al., 2006).

During the initiation of angiogenesis, quiescent ECs differentiate into two distinct phenotypes: tip and stalk cells. Angiogenic sprouts are led by tip cells that are migratory and have numerous filopodia. Stalk cells are proliferative and elongate the sprout. Cross-talk between VEGF and Notch signaling regulates the formation of tip and stalk cells. The phenotypic switch is dynamic and reversible and is regulated by a balance between pro-angiogenic factors such as VEGF and anti-proliferative delta-like ligand 4 (DLL4)-Notch activity (Stapor et al., 2014). VEGF activates VEGF receptors (VEGFR), tyrosine kinase receptors that mediate the mitogenic, angiogenic and chemotactic effects of VEGF. Out of the three VEGFRs (VEGFR1-3), VEGFR2 is the main receptor on the endothelium and is the most important for vessel sprouting that binds the ligand VEGFA. Hypoxia induces the release of VEGF, which promotes the formation of tip cells and filopodia that allow them to migrate toward the VEGFA gradient. In response to high levels of VEGF, the expression of DLL4 ligand is increased. DLL4-mediated activation of Notch receptor 1 blocks the formation of filopodia, allowing neighboring ECs to turn into stalk cells and form vascular tubes and a lumen (Abhinand et al., 2016; Treps et al., 2016). This process ends by the endothelium returning to a quiescent, non-proliferating and immobile state also called a phalanx phenotype. During this transition, the VEGF levels lower and the vessels become mature and stabilized by the recruitment and tight adhesion of pericytes along the new basement membrane (Figure 1) (Ribatti and Crivellato, 2012).

Besides the VEGFR2/Notch crosstalk, other pro-angiogenic signals contribute to the EC differentiation into tip cell phenotype. The angiopoietin (Ang)-Tie system is a second endothelial specific signaling system that regulates angiogenesis. In this system, Ang1 and Ang2 are endothelial growth factor ligands of Tie2. Although they have similar affinities to Tie2, they support different behavior of ECs. Whilst Ang1 is a regulator of EC adhesion and maturation, Ang2 disrupts cellular adhesion and survival. However, in the presence of VEGF, Ang2 supports migration and neovascularization (Saharinen and Alitalo, 2011; Fagiani and Christofori, 2013).

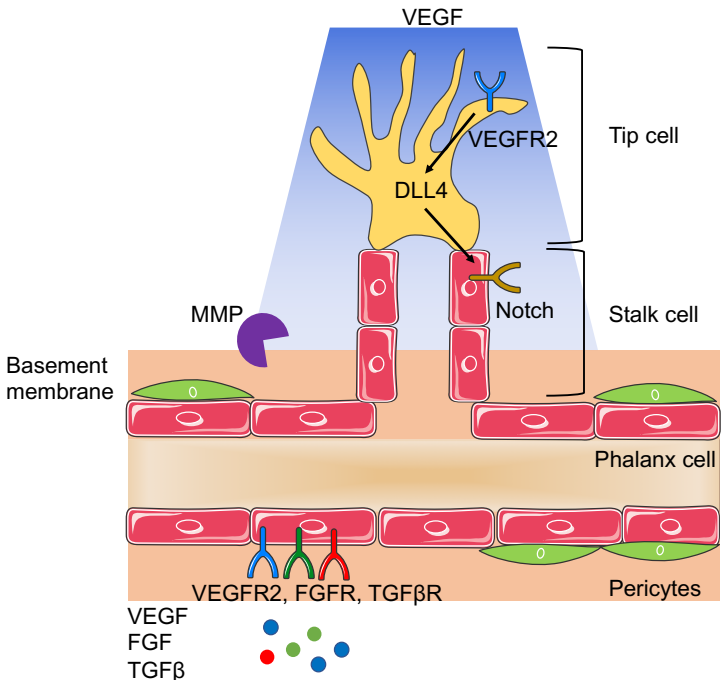


Figure 1. Endothelial tip-stalk differentiation during angiogenesis. *Hypoxia-induced VEGF stimulates formation of filopodia and the phenotypic switch to tip cells. In response to VEGFR2 activation, DLL4 expression is increased and activates Notch receptors on adjacent endothelial cells. In turn, formation of filopodia is blocked and endothelial cells switch into stalk cell phenotype.*

2.2 Role of glycolysis in angiogenesis

Under physiological and pathological conditions, angiogenesis is a highly energy demanding process. Hypoxia or tissue injury forces ECs to change phenotype and to be able to proliferate, migrate and form neovessels. Therefore, ECs need a metabolic system that can provide them with energy rapidly and in a hypoxic environment. Despite having immediate access to blood stream oxygen, ECs, like cancer cells, derive their energy primarily from glycolysis (>85%). This phenomenon is also called the Warburg effect (aerobic glycolysis) (De Bock et al., 2013b). Blocking glycolytic activity up to 80% with 2-deoxy-D-glucose (2-DG) is toxic for ECs (Wang et al., 2011). This highlights the pivotal role of glycolysis in endothelial cell function.

2.2.1 Glycolytic pathway

Glycolysis starts with the uptake of glucose into the cell, after which this is converted through multiple steps into lactate. This metabolic pathway can occur aerobically or anaerobically depending on whether oxygen is available. The rate limiting enzymes of glycolysis are hexokinase (HK) and phosphofructokinase-1 (PFK-1). As illustrated in figure 2, HK converts glucose into glucose-6-phosphate (G6P) and PFK-1 converts fructose-6-phosphate (F6P) to fructose-1,6-phosphate. Major regulators of PFK-1 are the phosphofructokinase-fructose-2,6-bisphosphatase (PFKFB) enzymes, which belongs to the PFK-2 family. By converting F6P into fructose-2,6-bisphosphate (F2,6BP) PFKFBs directly activate PFK-1 (van Schaftingen et al., 1982; Shi et al., 2017). Of the four PFKFB isoenzymes, PFKFB3 has the highest kinase (than phosphatase) activity (740-fold vs phosphatase) (Yalcin et al., 2009). Besides the production of adenosine triphosphate (ATP), glycolysis also serves other metabolic pathways with metabolites. For instance, glucose is involved in gluconeogenesis, and like G6P is an intermediate for nucleotide synthesis. By using acetyl-CoA, fatty acids and amino acids can be synthesized from pyruvate. More importantly, once acetyl-CoA is fed into the TCA cycle and subsequent oxidative phosphorylation (OXPHOS), it is further converted into CO_2 (and H_2O) and ATP within the mitochondria. Overall, the TCA cycle is a major source for biosynthesis (Berg et al., 2002; Bolaños et al., 2010).

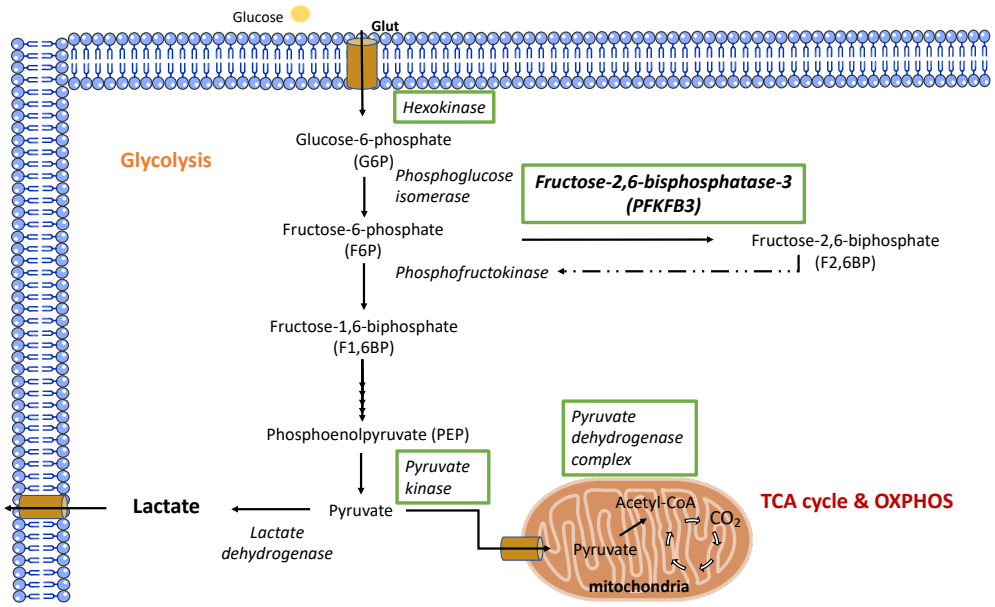


Figure 2. Schematic representation of the reactions in the glycolytic pathway. *Upon entry in the cytoplasm, glucose is converted into pyruvate. This may either be reduced into lactate (end-product of glycolysis) or oxidized into acetyl-coA which can enter the TCA cycle and oxidative phosphorylation and in turn be a source of ATP, amino acids and nucleotides.*

2.2.2 Glycolysis in endothelial cells

ECs like cancer cells display enhanced glycolytic activity (Polet and Feron, 2013). However, depending on the glucose amount in the environment ECs can reversibly switch between glycolytic and oxidative metabolism. A lowering in glucose amount, induces ECs to switch to mitochondrial metabolism. This adaptation mechanism of ECs is also called the Crabtree effect (Diaz-Ruiz et al., 2011). ECs may switch to aerobic glycolysis when glucose supply increases (Warburg effect), such as during pathological conditions pulmonary hypertension and Kaposi's Sarcoma-associated Herpes Virus (Delgado et al., 2010; Fijalkowska et al., 2010). The fact that ECs are in close contact with oxygen, makes it paradoxical for them to be highly glycolytic. It also seems counterproductive to rely on glycolysis, when OXPHOS renders more energy and provides metabolic intermediates for biosynthesis (Diaz-Ruiz et al., 2011). However, ECs migrate through avascular areas and therefore (anaerobic) glycolysis is a more efficient energy supplier in low oxygen environment. Furthermore, nitric oxide (NO) produced by endothelial NO synthase (eNOS) controls the aerobic respiration of ECs in an oxygen-dependent and reversible manner. This means that when local oxygen levels drop, NO inhibits OXPHOS of ECs (Groschner et al., 2012).

Use of glycolysis has many advantages over oxidative metabolism. Besides using minimal oxygen, it provides energy faster which is convenient during rapid dynamic changes in tip cell motility (actin cytoskeletal remodeling) (Verdone et al., 2015). Further, glycolysis offers ECs to rely on anaerobic metabolism in conditions where oxygen is depleted. By keeping the oxidative metabolism low, the production of reactive oxygen species is minimized and thereby facilitating cell survival (Zheng, 2012). Finally, glycolytic side pathways, such as the pentose phosphate pathway (PPP), provide macromolecules essential for cell proliferation (Lunt and Vander Heiden, 2011).

Endothelial phalanx cells were described to be in a quiescent and non-proliferating state. However, glycolysis is required to maintain the endothelial and vascular barrier function. Upon activation by factors such as VEGF, ECs are forced to double their glycolytic flux to meet the increased energy and biomass demand of sprouting ECs (Stapor et al., 2014). ECs increase the expression of the key glycolytic enzyme PFKFB3. This enzyme catalyses both the production and the breakdown of F2,6BP, which is the most important downstream metabolite in control of the rate-limiting enzyme PFK1 (De Bock et al., 2013b; Xu et al., 2014).

Reducing glycolysis by either silencing or pharmacologically inhibiting PFKFB3 impairs EC migration, proliferation and vessel sprouting *in vitro*. This results in a partial reduction of glycolysis by no more than 40%. Furthermore, PFKFB3 deficiency in EC also impedes vessel growth in several *in vivo* models of angiogenesis, without causing systemic effects (De Bock et al., 2013b; Schoors et al., 2014). As described above, VEGF and Notch signaling render the tip and stalk cell phenotype, respectively. Overexpression of PFKFB3 overrules Notch signaling, thereby pushing ECs into tip cell phenotype (Treps et al., 2016). Blood flow may also contribute to the EC phenotype. Interestingly, laminar shear stress reduces glucose uptake and mitochondrial content which, together with reduced expression of PFKFB3 and PFK-1, lowers the glycolysis in the endothelium. This contributes to a metabolically quiescent state of phalanx cells. This response was found to be dependent of the transcription factor Krüppel-like factor 2 (KLF2), a suppressor of PFKFB3 transcription (Doddaballapur et al., 2015). This underscores the role of glycolysis in EC plasticity and the anti-angiogenic potential of PFKFB3 inhibition.

2.2.3 Role of pH and lactate in regulating glycolysis

It is long known that pH changes influence glycolysis (Ui, 1966). Cancer cells commonly display a dysregulation in pH: increased intracellular pH and a decreased extracellular pH (White et al., 2017). A small decline in intracellular pH is sufficient to powerfully inhibit the activity of glycolytic enzymes such as lactate dehydrogenase, PFK-1 (Figure 3) and PFK-2 (Erecińska et al., 1995; White et al., 2017). As discussed earlier, PFK-2 generates F2,6BP, which is a potent allosteric stimulator of PFK-1. It has not been studied whether the activity of PFKFB3 isoenzyme is sensitive to pH changes. Recent data confirmed that fluctuations in pH dynamics enable alterations in cancer cell phenotype such as proliferation, migration, and cell survival. An increase in cytosolic pH is reported to regulate cell cycle progression and, in this manner, enables an increase in cell proliferation. The underlying mechanism however remains unknown. Interestingly, an increase in intracellular pH promotes actin remodeling and extracellular acidosis promotes cell migration, invasion and metastasis (White et al., 2017). Ion transporters such as carbonic anhydrases (CAs), monocarboxylate transporter 1 (MCT1) and MCT4, and Na^+-H^+ exchanger 1 (NHE1) regulate the cellular pH. Extra- and intracellular pH differences is determined by the gradients of both lactate anions and protons, which is mediated by MCTs. High expression of these transporters is strongly correlated with metastasis and poor

prognosis of cancer (Hong et al., 2016; Payen et al., 2017). As a consequence, the metabolic path of lactate and pH balance is influenced (Hertz and Dienel, 2005).

Lactate is not merely an end-product of glycolysis, but also acts as a fueler for oxidative metabolism and tumor/angiogenic growth factor (Végran et al., 2011; Ruan and Kazlauskas, 2013). Endothelial cells do take up lactate up in a MCT1-facilitated way, however are less efficient in serving as precursor for mitochondrial metabolism. Extracellular acidosis by lactate accumulation was suggested to correlate with tumor aggressiveness and poor prognosis. Pharmacological inhibition or RNA interference of MCTs have proven to be promising avenues of therapeutic options for inhibiting glycolysis(Doherty et al., 2014) angiogenesis (Végran et al., 2011)and growth (Floch et al., 2012; Noble et al., 2017). Interestingly, MCT1 inhibition with AZD3965 induced changes in the glycolytic pathway. It was found that MCT-1 inhibited cancer cells to display intracellular lactate accumulation, reduced glycolysis and possibly induced a shift to oxidative metabolism. As a consequence, cancer cell proliferation and tumor growth was markedly inhibited (Noble et al., 2017). Evidence suggests that glycolysis can be controlled by negative feedback mechanisms related to pH fluctuations and intracellular lactate accumulation can work (Parks et al., 2013).

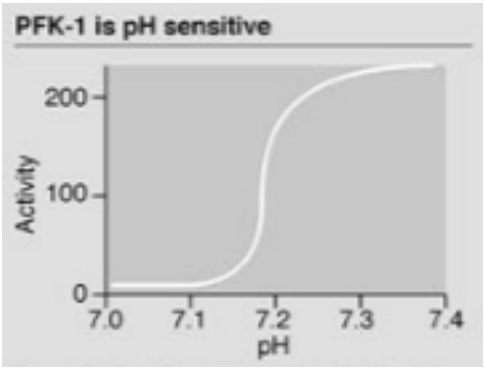


Figure 3. pH dynamics influence activity of glycolytic enzyme PFK-1. *Schematic representation of sensitivity of PFK-1 activity to changes in pH between 7.0 and 7.4. Adapted from (White et al., 2017)*

2.3 Intraplaque angiogenesis: a possible trigger of plaque rupture

2.3.1 Atherosclerosis: definition

Atherosclerosis is a chronic inflammatory disease of the large and medium-sized arteries that is characterized by the formation of plaques in the arterial wall. Plaques develop owing to the accumulation of cholesterol-rich lipoproteins, inflammatory and vascular cell infiltration and proliferation, and matrix remodeling in the subendothelial space. Over the course of years, large thrombogenic necrotic cores and weak fibrous caps may develop which ultimately can lead to plaque disruption and trigger acute thrombosis and vascular occlusion. These events can cause acute cardiovascular events such as myocardial infarction and stroke (Insull, 2009; Camaré et al., 2017).

2.3.2 Atherosclerosis: etiology and pathophysiology

Atherosclerosis is the leading cause of morbidity and mortality in the Western world (Lusis, 2000). The exact causes of atherosclerosis are not fully unraveled, however certain characteristics determine the risk of developing the disease. Established and modifiable risk factors for atherosclerosis include hyperlipidemia, diabetes mellitus, obesity, smoking and hypertension. Risk factors such as older age, genetic predisposition, and male gender may contribute to the atherosclerotic plaque development (Rafieian-Kopaei et al., 2014).

Atherogenesis evolves slowly and progressively and may start as early as in childhood stages (McGill et al., 2000). Figure 4 illustrates the development of atherosclerotic plaques. Hypercholesteremia is one of the main triggers of atherogenesis. Abnormal levels of cholesterol in the plasma may alter endothelial permeability allowing the migration and infiltration of lipoproteins, such as low-density lipoprotein (LDL), in the subendothelial space. Subsequent accumulation and oxidation of LDL in the intima activates ECs, which instigate a series of events involving cellular interactions between vascular cells and inflammatory cells. ECs enhance their expression of pro-inflammatory cytokines, adhesion molecules such as vascular adhesion molecule-1 (VCAM-1) and selectins and of chemokines (CC-chemokine ligand 2 (CCL2) to recruit circulating monocytes and, to a lesser extent, lymphocytes into the growing plaque (Singh et al., 2002). Once migrated in the plaque, monocytes differentiate into macrophages or dendritic cells. This allows macrophages to ingest native and modified

lipoproteins, resulting in the transformation into foam cells characterized by the intracellular accumulation of lipids. These foam cells release several cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α and chemokines (such as CCL2), that perpetuate the inflammatory response, and advancement of the lesion. Foam cell formation bridges the innate and adaptive responses in the lesion (Moore et al., 2013). Therefore, several subtypes of T lymphocytes as wells as B lymphocytes can be found. ECs, macrophages and T lymphocytes secrete cytokines and growth factors in the plaque that allow SMCs to proliferate and to produce a dense extracellular matrix. These events result in the development of a lesion with a thick fibrous plaque composed of a lipid core surrounded by SMCs and connective tissue fibers (e.g. collagen) (Falk, 2006; Moore and Tabas, 2011).

During the progression of the plaques, extensive apoptosis of several cell types may have harmful consequences by developing a destabilizing necrotic core and rupture-prone fibrous cap. For instance, apoptosis of SMCs leads to a loss of collagen, which is the most abundant extracellular matrix (ECM) protein providing stability to the plaque. Simultaneously, calcification may occur which is considered to be a marker of plaque burden and stability (Falk, 2006; Yla-Herttuala et al., 2013). Next to apoptosis of important cell types, proteases may contribute to the weakening of plaques by degrading components of the ECM. Matrix-metalloproteases (MMP) such as gelatinases (MMP-2 and MMP-9) and collagenases (MMP-13) are commonly found in plaques (Hansson and Libby, 2006; Moore and Tabas, 2011). In addition, MMP-mediated breakdown of ECM allows neovessel sprouting from the adventitial *vasa vasorum* into the plaque to form a microvascular network. This process, also called neo-angiogenesis or neovascularization, is promoted to meet the increasing oxygen and nutritional demands of the growing plaque. Intraplaque neovessels are often lined with a leaky endothelium, thereby creating an easy entry point for lipids, or exit point for erythrocytes (hemorrhages), leukocytes and lymphocytes (Virmani et al., 2005; Pasterkamp and Van Der Steen, 2012). Therefore, intraplaque neovascularization may contribute to the growth and destabilization of atherosclerotic plaques. The increase in blood pressure and dilatation of the arteries leads to rupture of the plaque.

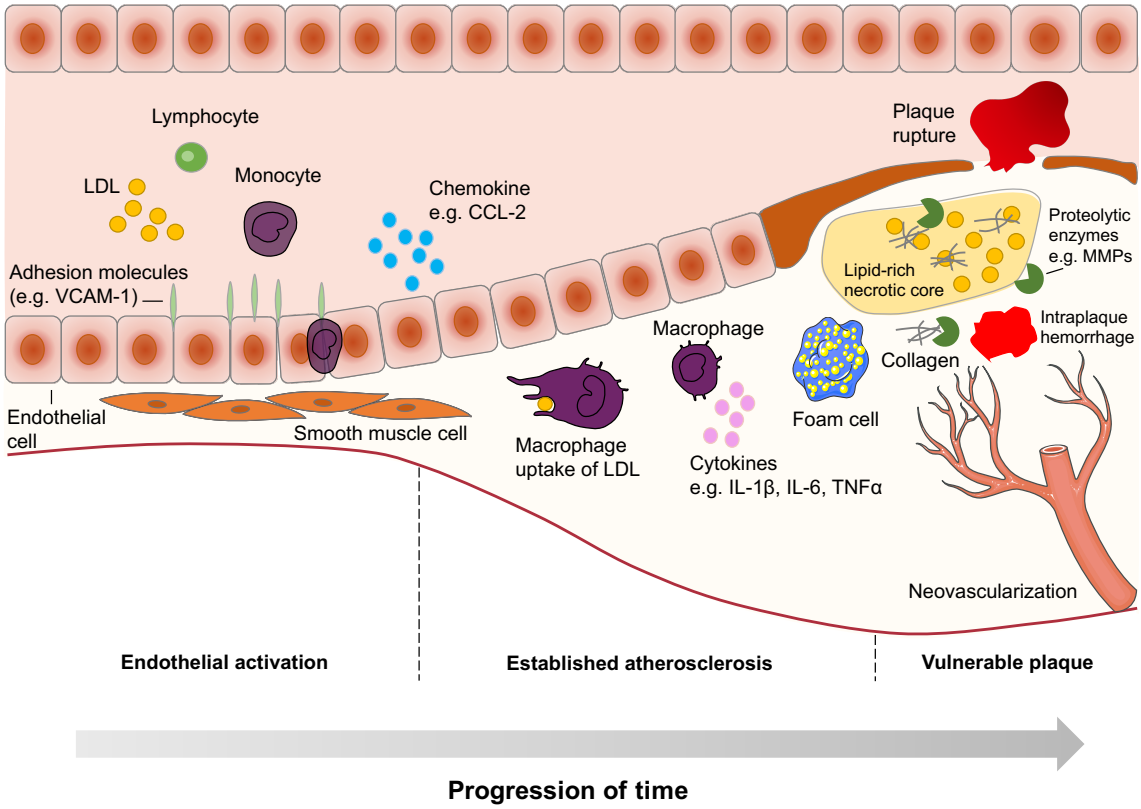


Figure 4. Unstable atherosclerotic plaque formation in the artery. *Illustrative diagram of how atherosclerotic plaques progress into an inflammatory and unstable phenotype with a lipid-rich necrotic core and thin fibrous cap. Intraplaque hemorrhage and neovascularization can further destabilize the plaque, leading to plaque rupture and thrombus formation.*

2.4 Anti-angiogenic therapy

2.4.1 Current and emerging approaches

Since angiogenesis is a hallmark of cancer and atherosclerosis (Nishida et al., 2006; Camaré et al., 2017), blocking angiogenesis is an attractive therapeutic approach. The basic principle of traditional anti-angiogenic therapy is to prune the vessels by impairing the perfusion with oxygen and nutrients. Current approaches aim at targeting VEGF and its signaling pathways using monoclonal antibodies and tyrosine kinase inhibitors (TKIs) (Loges et al., 2010; Al-Husein et al., 2012). There are several reasons for targeting VEGF. First, VEGF is the key driver of angiogenesis. Second, the VEGF family is overexpressed in a wide variety of cancers (Carmeliet, 2005; Gerhardt, 2008). Third, VEGF-targeted therapy suppresses tumor growth in mouse tumor models (Crawford and Ferrara, 2009).

TKIs, inhibitors of TK receptors such as VEGFR and FGFR, are the most common anti-angiogenics for cancer therapy. Four agents have been FDA approved for the treatment of metastatic renal cell carcinoma, namely *sorafenib*, *sunitinib*, *pazopanib* and *axitinib*.

Another approach is to target angiogenic ligands or receptors. *Bevacizumab*, a humanized monoclonal anti-VEGFA antibody, has been clinically used for several cancers (Al-Husein et al., 2012; Al-Abd et al., 2017). Although promising data have been shown, there are still cancers that do not respond to anti-VEGF therapies or develop resistance due to secretion of alternate growth factors. As a result, agents have been developed against other angiogenic ligands/receptors or multi-kinase inhibitors. Several multi-targeting TKIs are being developed in clinical trials that inhibit various VEGFR, FGFR, and PDGFR family members, such *cediranib, dovitinib, linifanib, brivanib,* and *lenvatinib* (Al-Abd et al., 2017). Further, anti-Ang2 antibodies (e.g. *trebananib*) are currently under development which block its binding to Tie-2 (Chiu et al., 2016). Also, a number of Dll4 inhibitors are being evaluated in phase I and II trials, yet no results have been reported (Clarke and Hurwitz, 2013). Finally, nonspecific MMP inhibitors like *marimastat* showed great promises in inhibiting angiogenesis in preclinical animal models but patients experienced significant side effects and is therefore not used clinically (Cathcart et al., 2015). Although these agents show promising results in preclinical studies, clinical trials reveal that the greatest challenge of anti-angiogenic drugs remains the occurrence of resistance to these approaches (Bergers and Hanahan, 2008).

A novel approach based on EC metabolism has emerged and appears efficacious in the treatment of pathological angiogenesis and suppressing tumor growth (Goveia et al., 2014). It was postulated that glycolysis in ECs regulate angiogenesis. Accumulating evidence suggests that the glycolytic enzyme PFKFB3 is associated with key processes of cancer such as cell migration, proliferation, vessel sprouting and metastasis (De Bock et al., 2013b). This makes PFKFB3 an attractive target for a pharmacological therapeutic intervention. Since targeting PFKFB3 is independent of growth factor signaling pathways, it is suggested to confer less resistance (Goveia et al., 2014).

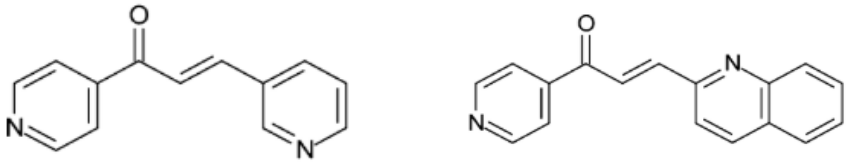
2.4.2 Modes of resistance to anti-VEGF therapy

Several mechanisms mediate two main categories of resistance from VEGF-targeted therapy: intrinsic and evasive. First, evasive (adaptive) resistance to angiogenesis inhibitors include several escape mechanisms by targeted angiogenic cells to revascularize through upregulation of alternative pro-angiogenic factors, such as FGF-2 and Ang-1. Therapy-induced hypoxia also seems to attract angiogenic cytokine-producing cells including endothelial (progenitor) cells,

pericytes and myeloid cells that may further contribute to neovascularization (Loges et al., 2010). Intrinsic resistance involves similar molecular and cellular mechanisms rendering unresponsiveness to the therapy. This could be explained by the different growth patterns of angiogenic cells (Al-Abd et al., 2017). For example, pancreatic ductal adenocarcinoma cells are able to expand in a hypoxic, hypovascularized environment and therefore lack the demand for angiogenesis (Yuen and Díaz, 2014). Further, compensatory action of other angiogenic pathways may also contribute to this type of resistance. Late stage breast tumors are less responsive to bevacizumab for the reason that they usually secrete several other growth factors like FGF-2 and TGF-β (Rugo, 2004). Other growth factors may also derive from inflammatory cells (Loges et al., 2010).

2.4.3 Glycolysis inhibitors

The widely studied glycolysis modulators, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one 1 (3PO) and PFK15 (Figure 5), have shown significant biological activity in several cancer studies (Clem et al., 2013; Li et al., 2017). Specifically, these inhibitors have been found to reduce glycolytic flux both *in vitro* and *in vivo*. As a consequence of treatment, inhibition of glycolysis with 3PO or PFK15 suppressed tumor growth and angiogenesis by inhibiting cell proliferation and migration (Clem et al., 2013; Schoors et al., 2014; Zhu et al., 2016). However, these compounds have shown anti-tumor efficacy at only maximum doses in preclinical models (Conradi et al., 2017) and is even used in phase I clinical trials as a novel anti-cancer treatment (Redman et al., 2015). Further, high doses of 3PO demonstrated to increase hypoxia and damage vascular integrity causing more influx and spreading of cancer cells (Conradi et al., 2017). This, together with the suggested off-target effects of 3PO indicates the need for effective PFKFB3 inhibitors with minimal toxicity for treatment of angiogenesis or intraplaque neovascularization. Therefore, we have selected the most potent PFKFB3 inhibitors from a previous study with known binding to kinase domain and activity (Table 1) (Boyd et al., 2015). The selected chemical structures are shown in Figure 6 and were used in this study.

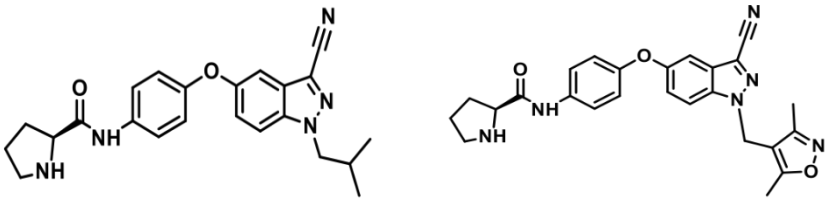


1. 3PO 2. PFK15

Figure 5. Chemical structures of commercially available PFKFB3 inhibitors

Table 1. Phenoxindazole analogues and their IC_{50} (in µM). *Adapted from (Boyd et al., 2015)*

	PFKFB1 IC_{50}	PFKFB2 IC_{50}	PFKFB3 IC_{50}	Cell F1,6BP IC_{50}	Cell Lactate IC_{50}
PA-1	0.294	0.015	0.004	0.297	15.4
PA-2	0.191	0.025	0.003	0.067	***x***



1. PA-1 2. PA-2

Figure 6. Chemical structures of synthesized PFKFB3 inhibitors

2.4.4 Potential challenges of anti-angiogenic therapy for atherosclerosis

Inhibition of angiogenesis may affect vessel formation and function. By promoting vessel maturation, atherosclerotic lesion regression or reduced risk of intraplaque haemorrhage might occur. However, angiogenesis inhibitors might also cause great potential risk to promote lesion progression. Plausible risks include: (1) further increasing the existing ischemic injury, (2) improving oxygen and nutrient transport or (3) cardiotoxicity (Doyle and Caplice, 2007). Aggravating ischemia may promote cell death, production of reactive oxygen species (ROS) and inflammatory cytokine production (Kalogeris et al., 2012). As a final note, several anti-angiogenics have been linked with clinical cardiovascular toxicities such as cardiac ischemia, hypertension, and thromboembolism (Hedhli and Russell, 2011). For instance, hypertension or haemorrhage have been described as a complication of sorafenib or bevacizumab therapy (Hedhli and Russell, 2011). Therefore, this underlines the need for accurate preclinical evaluation of the efficacy and safety profile of anti-angiogenics before moving to clinical trials.

2.5 Aims of the thesis

The glycolysis modulator 3PO is being used as a PFKFB3 inhibitor, although recently it has been demonstrated not to bind to PFKFB3 (Boyd et al., 2015). Currently, maximum doses of 3PO are needed to prune vessels and impair tumor growth. However, the anti-tumor effects of 3PO cannot be attributed to inhibition of PFKFB3 activity (Boyd et al., 2015). This thesis aims at unravelling the possible mechanism of action/working mechanism of 3PO beyond its possible interference with glycolysis/PFKFB3 and studying the anti-angiogenic potential of more potent and selective PFKFB3 inhibitors.

How can we explain the anti-angiogenic effect of 3PO?

In *chapter 3*, we describe our study on the working mechanism(s) underlying the known inhibitory effect of 3PO on endothelial cell glycolysis and vessel sprouting. So far, it is not known what the effects of 3PO treatment are on the intracellular condition of the cells. What does 3PO do with lactate intracellularly? Is the reduction of glycolysis caused by a regulation of lactate transporters, such as monocarboxylate transporters? Does 3PO affect the intracellular pH?

Can the novel PFKFB3 inhibitors impair different pro-atherogenic properties such as angiogenesis *in vitro*?

In *chapter 4,* we studied the anti-angiogenic potential of novel PFKFB3 inhibitors. This includes the effect on glycolysis, endothelial cell migration and proliferation and accordingly vessel sprouting. Additionally, expression of adhesion molecules VCAM-1 and VE-cadherin together with the expression of angiogenic molecules VEGFA, VEGFR2 and TGF-β were examined. Moreover, we studied whether these inhibitors might affect matrix metalloprotease (MMP-2 and MMP-9) expression and activity, which is essential to allow endothelial cell migration.

Chapter 5 provides a discussion of the most relevant findings of this thesis and gives an overview of future perspectives.

CHAPTER 3

3PO inhibits glycolysis but does not bind to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3)

Manuscript in preparation

3PO INHIBITS GLYCOLYSIS BUT DOES NOT BIND TO 6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE-3 (PFKFB3)

Introduction

Glycolysis is an essential bioenergetic pathway in endothelial cells (ECs) generating up to 85% of total cellular ATP. A key regulating enzyme in the glycolytic pathway is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), which is involved in both synthesis and degradation of fructose-2,6-bisphosphate. Among the four known PFKFB isoforms, only PFKFB3 reveals a kinase to phosphatase ratio of about 740:1, which favors the formation of intracellular Fru-2,6-P_2 and enhanced glycolysis (Sakakibara et al., 1997; Yalcin et al., 2009). Interestingly, expression of PFKFB3 is upregulated in response to hypoxia and inflammatory stimuli (Minchenko et al., 2003; Obach et al., 2004). Because silencing or inactivation of PFKFB3 reduces glycolysis and impairs vessel sprouting (De Bock et al., 2013b), PFKFB3 has become an attractive therapeutic target in preventing pathological angiogenesis. Recently, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) has been reported as a novel compound, which by competitively inhibiting PFKFB3 reduces glycolytic flux. It causes a rapid reduction in Fru-2,6-P_2 levels, is selectively cytotoxic in transformed cells, and inhibits tumorigenic growth *in vivo*. It also diminishes ^{18}F-2-DG uptake within xenografts (Clem et al., 2008) and inhibits EC proliferation and migration, resulting in reduced vessel sprouting in endothelial cell spheroids, zebrafish embryos and the postnatal mouse retina. Notably, all these effects are attributable to only partial and transient inhibition of glycolysis by 3PO (Schoors et al., 2014).

Although 3PO and the more potent analogue PFK15 are considered to act as PFKFB3 inhibitors, thorough experimental evidence is currently lacking. Moreover, recent data indicate that 3PO is inactive in a PFKFB3 kinase assay (IC_{50} > 100 μM) and no crystal structure is available, confirming binding of 3PO to PFKFB3 kinase (Boyd et al., 2015). It should be noted that 3PO does not inhibit the enzymatic activity of most other enzymes involved in the glycolytic pathway such as hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD), transketolase (TK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Schoors et al., 2014), but might exert other biological activities including inhibition of glucose uptake and induction of cell death (Telang et al., 2012; Klarer et al., 2014). In this chapter, we demonstrate the possible working mechanism of 3PO. It causes lactate-inducing inhibition of glycolysis by possibly regulating lactate transporters:

monocarboxylate transporters 1 (MCT1) and MCT4. We present that 3PO promotes intracellular lactate accumulation and in turn acidification.

Materials and methods

Glycolysis measurements

Glycolysis was measured in vitro using a Glycolysis Cell-Based Assay Kit (Cayman) following the manufacturer's instructions. Briefly, human umbilical vein endothelial cells (HUVECs) were seeded in a 96-well culture plate at a density of 15.000 cells/well and incubated overnight at 37°C in M199 culture medium, supplemented with 0.25% heat-inactivated fetal calf serum (FCS), 1% non-essential amino-acids (NEAA) and antibiotics. Thereafter, cells were treated with 3PO (0-100 μM) and MCT inhibitor α-cyano-4-hydroxynnamic acid (CHC: 2 mM) for 24 hours (3PO) or 18 hours (CHC) and assayed to determine the L-lactate concentration. A neutral red viability assay was performed as described to test cell viability.

Alternatively, glycolytic flux was assessed in HUVECs by measuring the extracellular acidification rate (ECAR) using a Seahorse XFp Analyzer (Agilent). ECAR values were measured in units of mpH, which is the change in pH in the media surrounding the cells due to proton flux in glycolysis. Assays were performed prior to experiments to determine optimal cell seeding density, and optimal concentrations of each compound. Briefly, 10.000 HUVECs/well were plated into XF8 polystyrene cell culture plates. After 16 hours incubation at 37°C, cells were stimulated with 3PO (0, 20 or 40 μM) for 5 hours. Prior to performing a glycolysis stress test, growth medium in the wells of XF cell plates was exchanged with the appropriate Seahorse assay medium (Agilent Technologies,103335-100). Baseline rates were measured three times prior to any injections. Firstly, glucose (Sigma Aldrich, G8270; 10 mM final concentration) was injected into the medium to provide a measure of glycolytic rate. Subsequently, oligomycin (Sigma Aldrich,75351; 2 μM final concentration) was injected, giving an estimate of glycolytic capacity. Finally, 2-DG (Sigma Aldrich, D8375; 50 mM final concentration) was injected, which is a glucose analogue that inhibits glycolysis, providing an estimate of non-glycolytic acidification. All compounds were prepared in assay medium and adjusted to pH 7.4. Glycolytic capacity was calculated as the difference between ECAR following the injection of 2 μM oligomycin and the basal ECAR reading (Divakaruni et al., 2014).

Intracellular lactate measurement

Intracellular lactate levels were measured in vitro using a L-Lactate Assay Kit (Cayman) according to the manufacturer's protocol. HUVECs were plated in T75 culture flasks until 80% confluency in M199 growth medium supplemented with 20% FCS, 1% non-essential amino-acids and antibiotics. Next, HUVECs were treated with 3PO (20 μM), CHC (2 mM) or vehicle (DMSO) for 18 hours in M199 with 0.25% FCS, 1% NEAA and antibiotics. A cell count was performed with an automated cell counter (Countess® II FL, Life Technologies). After deproteinization with 0.25M metaphosphoric acid, potassium carbonate (5M) was added to the cell pellet to neutralize the acid. Followed by centrifugation at 10.000x g for 5 minutes at 4˚C, the supernatant was used for assaying. Fluorescence was measured and normalized to the cell number.

Intracellular pH assay

Intracellular pH changes were measured with the Fluorometric Intracellular pH assay kit from Sigma-Aldrich (MAK150) following the manufacturer's protocol. In brief, HUVECs were plated overnight in M199 growth medium in a 96-well culture plate and subsequently the fluorescent pH indicator BCFL-AM was added for 30 minutes in a 5% CO2, 37˚C incubator. 3PO, CHC and U104 was diluted in Hank's Buffer (HHBS) with 20 mM HEPES and added to the cells. Five hours after addition of 3PO, fluorescence was measured at an excitation wavelengths of 490 nm and an emission wavelength of 535 nm.

Western blotting

HUVECs were plated in 12-well culture plates till confluency in M199 growth medium. Thereafter, cells were incubated with 3PO for 18 hours. Whole cells were then lysed in Laemmli buffer (Biorad). Equal amounts of protein were separated on 4-12% Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene fluoride membranes (Millipore) at 100 V for 1 hour. The membranes were blocked for 1 hour in Odyssey blocking buffer (LI-COR), and incubated with primary antibodies against MCT1 (Abcam ab85021, 54 kDa), MCT4 (Proteintech 22787-1-AP, 42 kDa) and β-actin (Sigma-Aldrich, A5441, 42 kDa). Membranes were then incubated with appropriate infrared dye labelled secondary antibodies (LI-COR, IgG926-68070 and IgG926-68071) for 1 hour. Antibodies were detected using an infrared

imaging system (Odyssey SA, LICOR) and densitometric analysis was performed using Image Studio v4.0 (LI-COR).

In vitro angiogenesis assay

Inhibition of endothelial tube formation by 3PO was monitored using an in vitro angiogenesis assay kit (Millipore) following the manufacturer's instructions. Briefly, HUVECs were cultured in M199 medium supplemented with 20% FCS, 1% non-essential amino-acids and antibiotics. After addition of 3PO (0-20 μM), changes in tube formation and formation of cellular networks were evaluated. Capillary tube branching points were counted in 5 random fields per concentration.

Endothelial cell migration assay

Murine immortalized heart endothelial cells (H5V) were cultured on 12-well plates in DMEM medium (Life Technologies) supplemented with 10% FCS and antibiotics. After reaching confluence, H5V were starved overnight in DMEM containing 0.5% FCS. Each well was marked below the plate surface by drawing a vertical line. Five different scratches intercepting the marked line were done in each well using a 200-μl sterile tip. Pictures of scratches were taken before and after 18 hours incubation with 3PO (20 μM) and/or VEGF (10mg/ml). Next, the mean closure of five different scratches was analyzed using ImageJ software. EC migration was expressed as the percentage of scratch closure after 18 hours versus the initial area by using the following formula: % closure = [(scratched area at 0 hours–scratched area at 18 hours)/scratched area at 0 hours]×100.

Aortic sprouting

An aortic ring assay was performed as described previously (Baker et al., 2012). In brief, murine thoracic aortas were dissected, cleaned under sterile conditions, transferred to 10-cm culture dishes, and cut into 0.5-mm thick rings with a sterile scalpel. After overnight starvation in serum-free Opti-MEM at 37°C, ring segments were transferred into wells of a 96-well plate coated with 50 μL of a freshly prepared collagen type I solution 1mg/mL. The aortic rings remained in Opti-MEM (supplemented with 2.5% FCS and antibiotics) in the presence or absence of 3PO and/or VEGF (50 ng/mL) (R&D Systems). Medium was replaced

every 2 days. On day 6, rings were fixed with 4% paraformaldehyde, stained with von Willebrand factor antibodies (anti-vWF, PC054, Binding Site) and DAPI prior to imaging with a fluorescence microscope. The number of sprouts was counted for each ring and sprout numbers per ring were averaged for each group and graphed.

Kinase inhibition assay

ADP-Glo Kinase assays (Promega) were performed on recombinant PFKFB3 using a slightly modified protocol as previously described (Boyd et al., 2015). A Tecan Magellan spectrophotometer with enhanced luminescence module was used to measure the luminescence signal. IC_{50} estimates were calculated using a commercially available software package from GraFit5.

3PO binding assay

Recombinant PFKFB3 was labelled with microscale thermophoresis (MST) RED-NHS dye using a Monolith NT Protein Labeling kit (Nanotemper Technologies). The labelled protein was diluted with MST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween-20) to 100 nM concentration prior to the experiment. A 100 mM DMSO stock solution of 3PO was prepared. 3PO stock was diluted to 16 different concentrations, starting from 1 mM and serially diluted to 31.25 nM. The labelled protein and 3PO were mixed in a 1:1 ratio and MST measurements with 20% LED power were performed on a Monolith NT. 115 instrument (Nanotemper Technologies).

Isothermal Titration Calorimetry (ITC)

Binding of 3PO to PFKFB3 was analyzed via calorimetry using a MicroCal ITC isothermal titration calorimeter. Before ITC analysis, a PFKFB3 sample was dialyzed against 2 L buffer (40 mM Tris, 500 mM NaCl, 5 mM MgCl2, 2 mM DTT at pH 7.4), for 2h at 4 °C under constant stirring, followed by switching to a new 2 L buffer vial and overnight dialysis. For this, Slide-A-Lyzer Dialysis G2 cassettes (Thermo Scientific, MA, USA) were used. Concentration of the sample after dialysis was determined using UV-absorbance determination at 280 nm using a Spectramax Plus 384 (Molecular Devices, UK). A small portion of the

second dialysis buffer volume was kept for matching the ligand solutions (AZ67 or 3PO). All buffers were prepared in ultrapure water (18.2 MΩ.cm), equilibrated to room temperature and degassed for 10 min in an ultrasonication bath before usage. All titrations were performed with the same PFKFB3 sample, diluted to a working concentration of 3 μM, and with the same titration conditions (except for the ligand concentration) in order to allow mutual comparison between the different runs. The ITC measurements were performed on a Peaq-ITC (non-automated version, Malvern Pananalytical, Malvern, UK). The reference cell was filled with degassed ultrapure water. The PFKFB3 solution was always put in the sample cell, after a 2-minute pre-equilibration with assay buffer (same recipe as dialysis buffer). The ligand solution was administered in the injection syringe. Titration conditions were always as follows: 1 initial injection of 0.4 μL was followed by 14 injections of 2.5 μL. The initial spacing was set to 180s, while the remaining spacing was set to 180s. The sample cell was continuously stirred at 750 rpm. Temperature was set to 37 °C before loading and kept constant during the complete run. DP was set to 5. In order to determine the dilution heats for each PFKFB3-ligand concentration combination, control titrations were performed consisting of injection of ligand into the buffer-filled cell (so in the absence of PFKFB3, without binding). Thermograms were analyzed using the Microcal Peaq-ITC Analysis software, using the 'one set of sites' binding model, by including the corresponding control titration. In order to visually compare the different titrations with completely different molar ratios, we transformed the data to generated heat (ΔH) per injection and incorporated everything in a GraphPad Prism 6.

Statistics

Results are expressed as mean ± SEM. An ordinary one-way ANOVA test was used when comparing two groups, 1-way ANOVA (Bonferroni post-hoc test) was used where indicated to compare three or more groups. *, $P < 0.05$, **, $P < 0.01$ or ***, $P < 0.001$ was considered statistically significant in the different experiments.

Results

3PO inhibits glycolysis in endothelial cells

Lactate measurements in the culture medium of human umbilical vein endothelial cells (HUVECs) showed that 3PO inhibits glycolysis in a concentration-dependent manner (Figure 1A). Viability of HUVECs was not affected if cells were treated with 3PO up to 20 µM (Figure 1B). However, cytotoxic effects were noticed after exposure to higher 3PO concentrations (100 µM: Figure 1B). By using Seahorse technology, we could show that 3PO inhibits glycolysis in a concentration-dependent manner in HUVECs. The glycolytic rate of the cells diminished by more than 50% after treatment with 20 µM 3PO (Figure 1C).

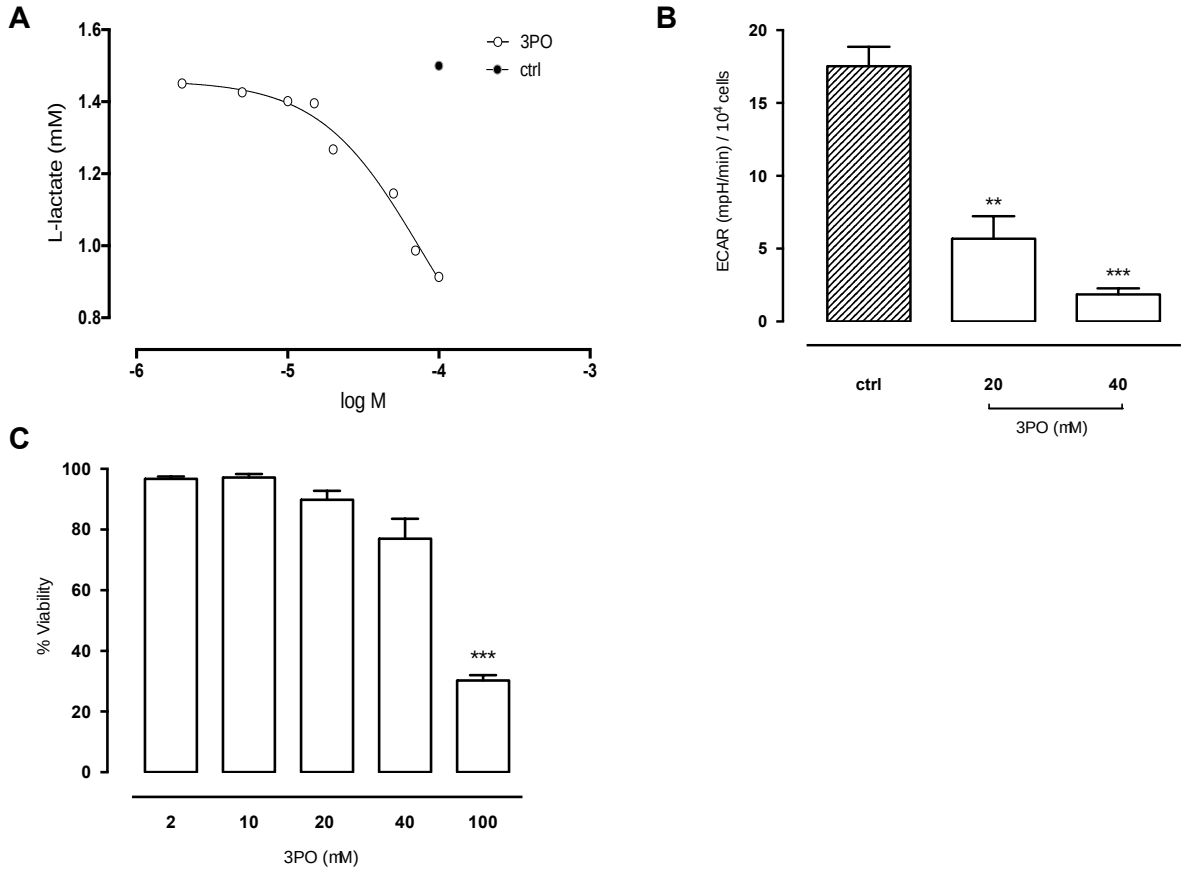


Figure 1. 3PO inhibits glycolysis in vitro. *(A-B) 3PO at 20µM concentration achieved optimal glycolysis inhibition, (C) 3PO 2, 10, 20µM did not show cytotoxicity effects compared to ctrl (cells with DMSO), however 3PO 100µM displayed toxicity. Data represent mean ± SEM. *P < 0.05. Data are representative of an experiment repeated three times. *P < 0.05.*

3PO inhibits capillary tube formation, EC migration and formation of aortic sprouts

To confirm whether glycolysis inhibition by 3PO in ECs affects neoangiogenesis, an *in vitro* matrigel assay with HUVECs was performed. This assay is based on the ability of ECs to form cord and mesh structures when seeded on a growth factor-enriched matrix. Different concentrations of 3PO were tested (Figure 2A). 3PO fully inhibited cord formation at 20 µM. At lower concentrations (5 -10 µM), mesh structures consisting of EC chords were visible but their numbers were reduced in a concentration-dependent-manner. A healthy network of chord structures was formed in the absence of 3PO (Figure 2A). Branch point counting demonstrated an inverse correlation between the amount of branch points and the concentration of 3PO (Figure 2B). Apart from inhibition of capillary tube formation, 3PO inhibited EC migration after scratching EC monolayers (Figure 3) and prevented aortic sprouting (Figure 4) both under basal conditions and when stimulated with VEGF.

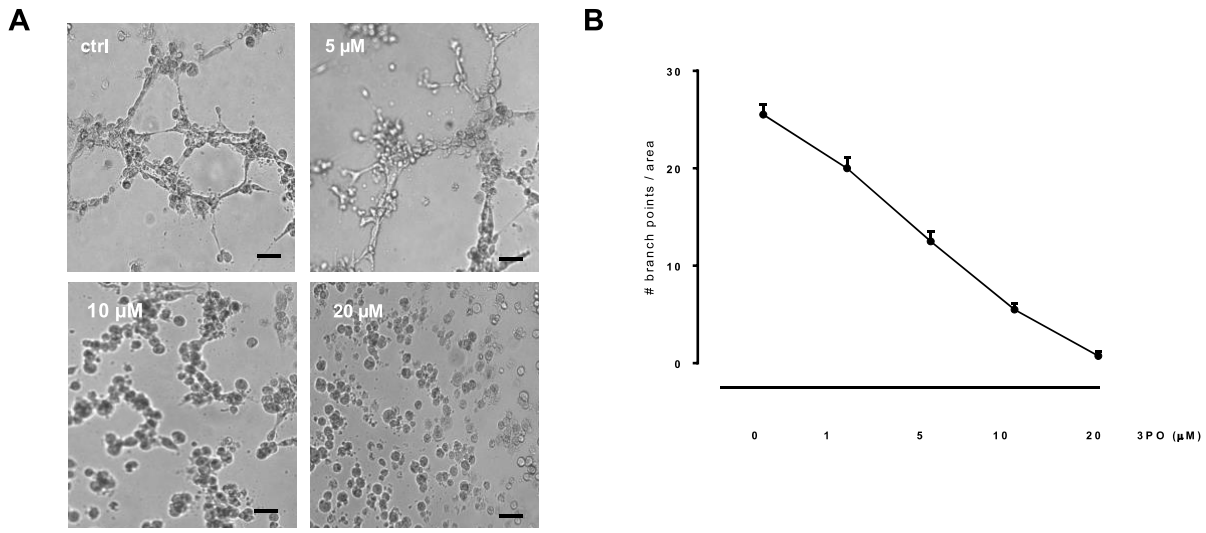


Figure 2. 3PO inhibits tube formation in an in vitro angiogenesis assay. *Quantitation of efficacy of 3PO was evaluated via two different methods. (A) Different concentrations of 3PO have a distinct effect on tube formation and formation of cellular networks. Scale bar = 100 µm (B) The progression of angiogenesis is quantified by counting the amount of capillary tube branch points at different concentrations. Data represent mean ± SEM. * P < 0.05.*

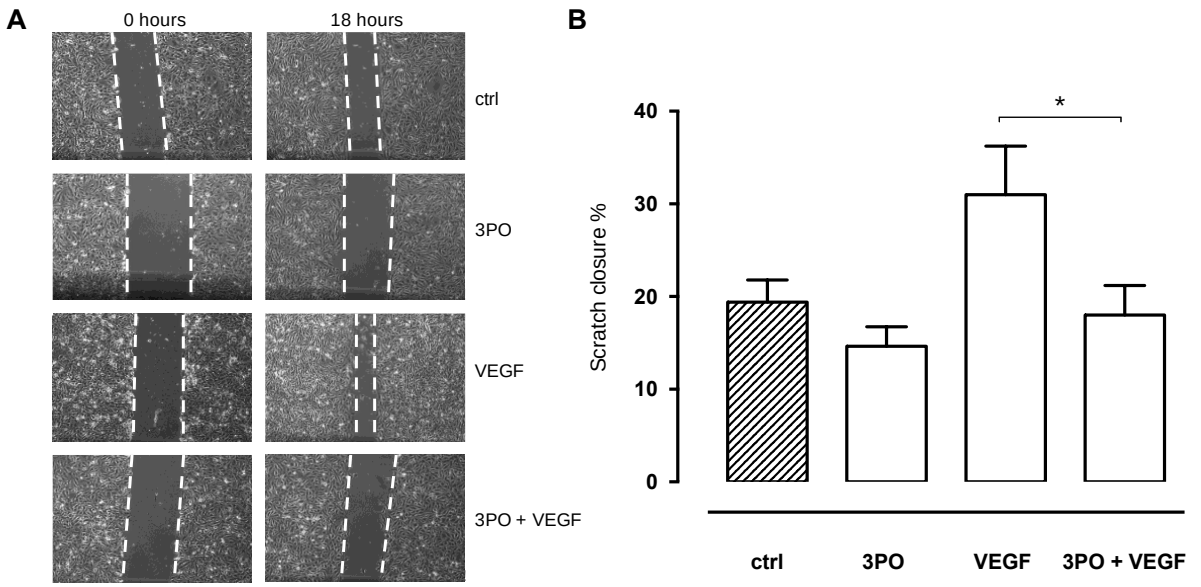


Figure 3. 3PO inhibits EC migration in scratch assay. *(A) Representative images of scratch assay. Murine immortalized heart endothelial cells (H5V) were starved, then wounded and treated with DMEM (2.5% FBS) plus 20 µM 3PO with or without 10 ng/mL VEGF for 18 h. (B) The wound area was measured in captured images at 0 and 18 h, and migration was quantified. Data are obtained from two independent experiments. Data represent mean ± SEM. *P < 0.05, ordinary one-way ANOVA.*

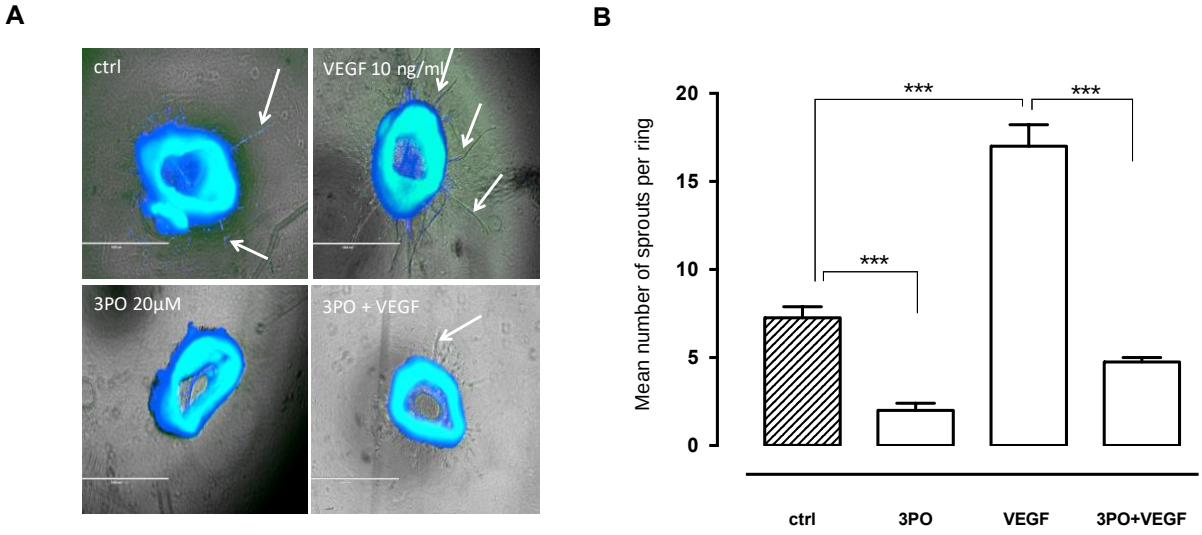


Figure 4. 3PO inhibits aortic sprouting. *(A) Aortic rings from ApoE $^{-/-}$ mice were embedded in collagen type 1 and treated with +2.5% (vol/vol) FBS or VEGF 50 ng/mL for 6 d. At day 6, rings were fixed with PFA (4%) and stained with BS1 Lectin-FITC to delineate ECs. Images of ring sprouting were obtained and quantified. (B) Data are representative of five or six mice per group, and sprouts from eight or nine rings per mouse were quantified. Data represent mean ± SEM. *P < 0.05, one-way ANOVA with Bonferroni's posttest. Data are representative of an experiment repeated three times and conducted in triplicate. *P < 0.05.*

3PO does not bind PFKFB3, nor inhibit PFKFB3 activity

To provide a complete thermodynamic profile of the molecular interaction, we used the small-molecule compound 3PO and PFKFB3 protein. Key findings were confirmed by using AZ PFKFB3 67, a potent and specific PFKFB3 inhibitor. 3PO did not show any binding affinity towards PFKFB3 while control compounds did (Figure 5A). This was further confirmed with microscale thermophoresis, where 3PO again did not show any affinity (Figure 5A). This was further confirmed using MST method (Figure 5B). AZ67, however, did show strong binding affinity to PFKFB3 (Figure 5A). Finally, we could demonstrate that 3PO was inactive in a PFKFB3 kinase activity assay: no IC_{50} could be deducted (Figure 5C). In contrast, AZ67 showed potent inhibitory activity of PFKFB3 with an IC_{50} of 1.30 ± 0.09 µM (Figure 5D).

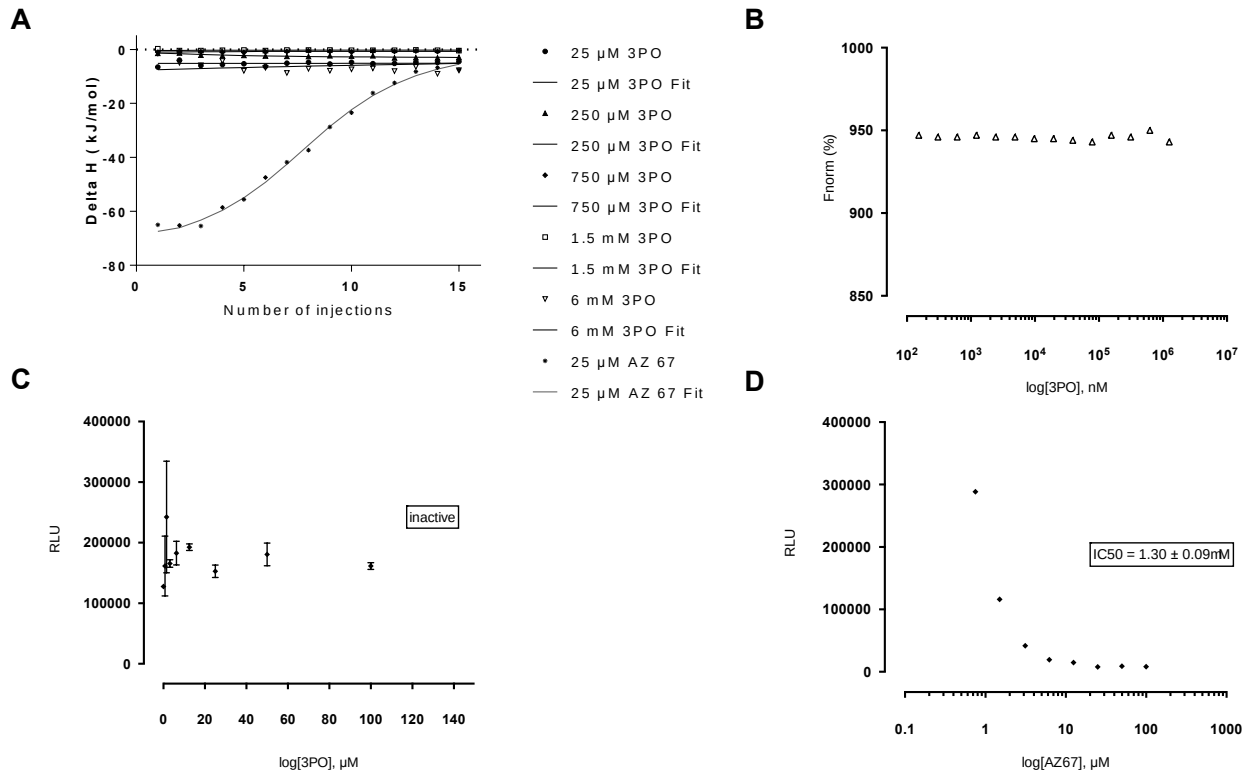


Figure 5. 3PO does not bind to PFKFB3 nor inhibit its activity. *(A) ITC data show no binding of 3PO between range of 25µM-6mM, whereas positive control AZ67 does. (B) MST data also confirm that 3PO does not have any affinity towards the target in the tested concentration range. (C) 3PO does not show kinase inhibitory activity against PFKFB3. RLU: relative luminescence units. (D) However, positive control AZ67 shows potent inhibitory kinase activity against PFKFB3. Data represent mean ± SEM.*

3PO induces intracellular acidification and decrease in pH

Disruption in intracellular pH (pH_i) has been shown to play an important role in regulating cell motility and angiogenesis (White et al., 2017) as well as regulating glycolysis (Hoa Thien Quach et al., 2016; White et al., 2017). Therefore, we have studied the effect of 3PO treatment on the pH_i in ECs. We have demonstrated that 3PO induced intracellular acidification, as shown by a significant decrease in pH_i in HUVECs (Figure 6A). Given that lactate is an important regulator of pH (Huber et al., 2017), we have measured intracellular lactate levels in HUVECs after treatment with 3PO. In accordance with our previous finding, 3PO increased the lactate concentration intracellularly (Figure 6B). These results suggest that 3PO might regulate the transport of lactate in ECs. Therefore, we investigated the effect of 3PO on protein expression of two main lactate transporters: monocarboxylate transporter 1 (MCT1) and MCT4. In Figure 6D, we demonstrate that 3PO and a known inhibitor of MCTs, CHC, did not affect protein expression of MCT1 and MCT4. These data suggest that 3PO and CHC both promote acidification by lactate accumulation without affecting protein expression of MCT1 and MCT4. The question remains whether 3PO can influence MCT activity.

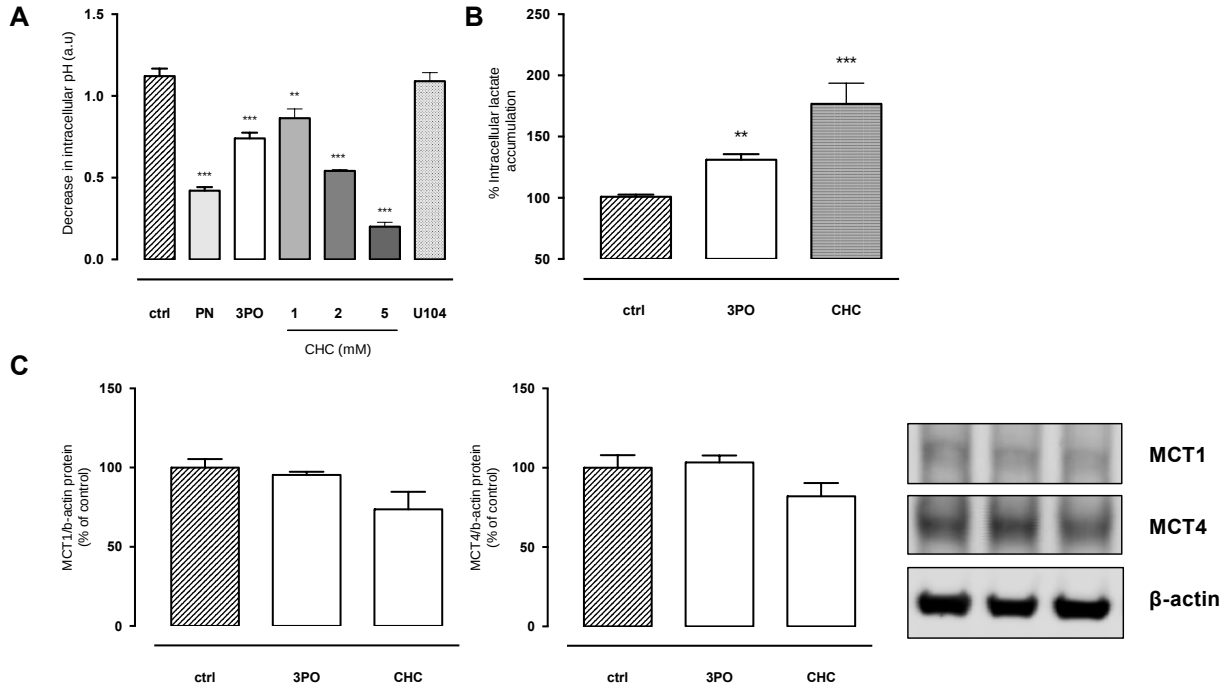


Figure 6. *Possible working mechanism of 3PO. (A) 3PO (20μM), induce intracellular acidification. PN: probenecid, nonspecific inhibitor of anion exchange transporters. CHC, a known inhibitor of MCTs, promotes acidification from a range between 1-5mM. U104 (20μM), a potent carbonic anhydrase inhibitor, does not cause acidification in endothelial cells. (B) Also, 3PO promotes lactate accumulation in endothelial cells as compared to control. Next to that, CHC (2mM) induces*

*accumulation of lactate intracellularly. (C) Both 3PO (20μM) and CHC (2mM) do not affect MCT1 and MCT4 protein expression. Data was obtained from at least 3 independent experiments. Data represent mean ± SEM. * P < 0.05, one-way ANOVA with Bonferroni's posttest.*

Discussion

Although believed to be a PFKFB3 inhibitor, current research demonstrates that 3PO interferes with glycolysis and angiogenesis in a PFKFB3-independent manner. This was uncovered by two independent enzyme binding assays, i.e. isothermal titration calorimetry and microscale thermophoresis. Even though the current study and previous scientific literature (Schoors et al., 2014; Conradi et al., 2017) could detect a biological effect of 3PO at a concentration of 20 μM, neither assays could observe PFKFB3 binding at concentrations well beyond 20 μM. This raises considerable doubt on the molecular mechanism of 3PO working through PFKFB3 inhibition. Ever since this interesting target was unveiled, attempts were made to target the F-6-P site instead of targeting the ATP, since the ATP site is shared by thousands of other kinases (Hanks et al., 1988; Davies et al., 2000) and it may diminish compounds' specificity. In our hands, there is no direct binding of 3PO to PFKFB3, but the fact that it does not affect its enzymatic activity leads to another question of the exact mechanism of action of this compound. Of importance is the fact that 3PO does not inhibit the enzymatic activity of other enzymes involved in glycolytic pathway, like GAPDH, LDH, PK, PFK, and G6PDH (Schoors et al., 2014).

Using computational identification, 3PO was first described as a small-molecule inhibitor of PFKFB3 by Clem. et al in 2008. By competitive inhibition of PFKFB3 isozyme, it was shown to reduce glycolytic flux and to cause a rapid reduction in Fru-2,6-P2 levels (Clem et al., 2008). Since then, 3PO has been implicated as a therapeutic strategy in cancer research, acute lung injury and lung fibrosis (Xie et al., 2015; Conradi et al., 2017; Gong et al., 2017). The role of 3PO in glycolysis inhibition was further confirmed in the present study. By using a Seahorse XFp Extracellular Flux Analyzer we could demonstrate that 3PO inhibits glycolysis in concentration-dependent manner in HUVECs, resulting in up to 50% reduction in the glycolytic rate. Together with the novel findings from the PFKFB3 binding assay, these data imply that although 3PO does not bind PFKFB3, its anti-glycolytic activity is not called into question.

The glycolysis level in endothelial cells is much higher than in any other healthy cell, and the majority of ATP produced in these cells is through an anaerobic pathway. These facts have led

researchers to investigate a possible role of glycolysis inhibition as an anti-angiogenic strategy. In this context, 3PO treatment to inhibit glycolysis has been shown to impair vessel sprouting in different experimental models, including zebrafish embryos, and the post-natal mouse retina (Schoors et al., 2014). In the oncological field, 3PO treatment at 25 mg/kg body weight has been shown to induce tumor vessel normalization (Conradi et al., 2017). We confirm the effect of 3PO on angiogenesis in our cell culture model by using a functional assay, where the inhibition is detected within the range of 20 μM.

Increased anaerobic glycolysis and resultant acidification are strongly correlated with angiogenesis (Kato et al., 2013; Zuazo-Gaztelu and Casanovas, 2018). Cellular pH changes are described to regulate cell functions including proliferation, migration and metabolic adaptation (White et al., 2017). For instance, it is known that some glycolytic enzymes including lactate dehydrogenase and phosphofructokinase-1 (PFK-1) are pH-sensitive. A change of less than a pH unit may reduce the activity of PFK-1 by more than 10-fold (Trivedi and Danforth, 1966; Andrés et al., 1990; Kamp et al., 2007; White et al., 2017). Additionally, PFK-2 also known as a family member of PFKFB3 has been shown to be regulated by pH changes (Putney and Barber, 2004). Whether PFKFB3 activity can be controlled by fluctuations in pH has not been studied. In the present study, we have demonstrated that 3PO induces intracellular acidification in endothelial cells, possibly by an accumulation of lactate within these cells. Besides the fact that this effect may regulate the activity of lactate transporters, one could wonder whether 3PO can indirectly inhibit glycolysis by causing a dysregulation in pH. Manipulating acidification has been considered to have therapeutic utility in tumors. One of the strategies include the inhibition of monocarboxylate transporters (MCTs), mainly MCT1 and MCT4. These transporters are pH dependent and are adapted to supply cells with lactate for energy production and releasing lactate under hypoxic conditions. Studies documented that MCT1 or MCT4 inhibition conferred antiangiogenic effects and block tumor growth (Sonveaux et al., 2012; Hong et al., 2016; Voss et al., 2017). It's worth noting that multiple studies indicated the possible proapoptotic effects of 3PO (Clem et al., 2013; Klarer et al., 2014), which are also regulated by alterations of intracellular pH (White et al., 2017). We demonstrate that 3PO and the general MCT inhibitor, CHC, do not influence the expression of MCT1 and MCT4. This does not exclude the fact that these compounds may interact with MCT activity. However, this was not studied in the present study.

Taken together, the present study has revealed that the effect of 3PO on glycolysis is not mediated through direct binding to PFKFB3 protein. To our knowledge, this is the first study

that could report these findings, since no previous study has investigated the thermodynamic profile and biomolecular interactions of 3PO and PFKFB3. We have demonstrated that 3PO could have an indirect inhibitory effect on glycolysis by inducing intracellular acidification and lactate accumulation. This leaves an obvious open question as to whether 3PO can target MCTs directly. The identification of this target may have an important impact on angiogenesis research and the development of specific anti-angiogenic treatments, which has implications in a variety of pathologies.

CHAPTER 4

Angiogenesis inhibition with selective compounds targeting the key glycolytic enzyme PFKFB3

Manuscript in preparation

ANGIOGENESIS INHIBITION WITH SELECTIVE COMPOUNDS TARGETING THE KEY GLYCOLYTIC ENZYME PFKFB3

IDENTIFICATION OF PFKFB3-MEDIATED ANGIOGENESIS INHIBITORS

Introduction

Angiogenesis is a highly dynamic and coordinated process consisting of the growth of new blood vessels from existing vasculature and is triggered by pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), which increase the metabolism of endothelial cells (ECs). This is required for the supply of oxygen and nutrients, removal of waste, and immune surveillance. A balance of angiogenic and angiostatic factors regulates the growth and regression of new sprouts (Ferrara, 2010). In response to injury/oxygen deprivation or pathological conditions, such as inflammation and tumor growth, ECs rapidly switch from a quiescent to a highly migratory and proliferative state (Adair and Montani, 2010; Vandekeere et al., 2015) as a result of release of VEGF and matrix metalloproteinases (MMPs; in particular MMP-2 and MMP-9)(Van Hinsbergh and Koolwijk, 2008; Adair and Montani, 2010). Current therapies targeting VEGF and its receptors have some limitations due to the development of resistance and/or toxicity (Bergers and Hanahan, 2008; Kumar et al., 2013). Therefore, alternative anti-angiogenic strategies are needed. New anti-angiogenic approaches focused on the inhibition of endothelial metabolism and glycolysis (Vandekeere et al., 2015; Jiménez-Valerio and Casanovas, 2017).

ECs have high glycolytic activity that is comparable to that of tumor cells. Out of this process, 85% of the total cellular ATP content is generated (De Bock et al., 2013). The conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is one of three rate-limiting steps regulated by 6-phospho-fructokinase-1 (PFK-1). The bifunctional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase-3 (PFKFB3) is a direct and powerful activator of PFK-1 (De Bock et al., 2013). Of all PFKFB isoenzymes, PFKFB3 is most abundantly expressed in ECs (Xu et al., 2014). Recent studies have shown the efficacy of partially reducing glycolysis, via PFKFB3 inhibition, as a way to impair vessel sprouting and reduce tumor growth (De Bock et al., 2013; Pisarsky et al., 2016; Schoors et al., 2014; Xu et al., 2014). Specifically, PFKFB3 was shown to be important for EC migration, proliferation and to be a powerful angiogenic regulator (De Bock et al., 2013; Schoors et al., 2014). Given its key role as a glycolytic activator, pharmacological inhibition of PFKFB3 represents a promising anti-angiogenic strategy for targeting pathological angiogenesis, overcoming resistance and insufficient

efficacy of classic anti-angiogenic therapies (Schoors et al., 2014). Although the anti-glycolytic compound 3-(pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) has shown promising results against angiogenesis, at high concentrations it compromises vascular barrier integrity (Conradi et al., 2017) and has immunosuppressive functions (Telang et al., 2012). In fact, 3PO facilitates cancer cell intravasation and dissemination thus leading to metastasis (Conradi et al., 2017). Furthermore, pharmacological activities of 3PO cannot be uniquely attributed to the inhibition of PFKFB3 and there are no evidences of binding to the PFKB3 kinase domain (Boyd et al., 2015; Zhu et al., 2016). Therefore, the development of selective inhibitors of PFKFB3, with low general toxicity, is needed.

Herein we tested the anti-angiogenic potential of two phenoxindazole analogues (PA-1 and PA-2) self-synthesized based on a previous paper by Boyd (Boyd et al., 2015). They demonstrated a greater potency of these compounds in inhibiting PFKFB3, as compared to the most widely studied agents, namely 3PO (Clem et al., 2013; Schoors et al., 2014; Boyd et al., 2015; Pisarsky et al., 2016) and PFK15 (Clem et al., 2013).

Results presented here demonstrate the ability of these more potent and selective inhibitors of PFKFB3 to block pathological angiogenesis in basal and inflamed conditions. Furthermore, we also investigated the possible mechanism(s) of action of these compounds in angiogenesis.

Materials and Methods

Cell Culture

The human endothelial EA.hy926 cell (EHEC) line (ATCC CRL-2922) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; EuroClone) supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-Glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% HAT supplement (Sigma Aldrich) (Bernini et al., 1991; Giunzioni et al., 2014). Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in endothelial growth medium (EBM™; Lonza) supplemented with (EGM™SingleQuots™, Lonza), 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and were used between passage 3-6.

Compounds

The PFKFB3 inhibitor 3PO was purchased from Merck Millipore. The other two PFKFB3 inhibitors, called phenoxindazole analogue 1 (PA-1) and analogue 2 (PA-2) (Boyd et al., 2015), were synthesized by our colleagues in Aberdeen. The formal names of the compounds are listed below. All compounds were dissolved in dimethylsulphoxide (DMSO). EHECs were treated with 20 μM 3PO, PA-1 and PA-2, while HUVECs were treated with 20 μM 3PO, 5 μM PA-1 and 20 μM PA-2 for 24h. 20 μM of PA-1 was cytotoxic in HUVECs.

3PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2E-propen-1-one
PA-1	(2S)-N-(4-{[3-cyano-1-(2-methylpropyl)-1H-indazol-5-yl]oxy}phenyl) pyrrolidine-2-carboxamide
PA-2	(2S)-N-[4-({3-cyano-1-[(dimethyl-1,2-oxazol-4-yl)methyl]-1H-indazol-5-yl}oxy)phenyl] pyrrolidine-2-carboxamide

Viability test: MTT

Cell viability was measured using the MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) reduction assay. EHECs were treated for 24h with 3PO, PA-1 and PA-2. Following the treatment, cells were incubated with 0.5 mg/mL of MTT for four hours at 37°C. After a wash with PBS, the generated formazan crystals were solubilized with acidic isopropanol (0.1N HCL in absolute isopropanol). Absorbance spectra of the MTT formazan

dye were measured at 550nm. Any sign of drug toxicity was detected after 24h treatment in EHECs.

Lactate measurements

The levels of secreted lactate in EHECs were measured using the Glycolysis Cell-Based Assay Kit (Cayman Chemical), and the intracellular lactate concentration were measured with the L-Lactate Assay Kit (Cayman Chemical), following the manufacturer's protocol. Data were normalized to the protein concentration as quantified with the Pierce BCA Protein assay kit (Thermo Fisher Scientific).

Cell proliferation

EHECs were seeded in 35mm dishes in triplicate at an equal density. Next day, the medium was replaced with DMEM containing 0.4% FBS to starve and synchronize the cells. After 48 hours of starvation, ECs were treated with or without 20 μM 3PO, PA-1 or PA-2 in culture medium for 24 hours. Cell numbers were counted using a counter analyzer (Beckman Coulter, Life Scientific), and compared to time-point zero.

In vitro directional migration (wound healing assay)

EHECs were seeded in a 12-well plate and grown to confluence. To evaluate the responses in absence of proliferation, cells were pretreated with mitomycin C (1 μg/mL: Sigma) for 24h. Monolayers were treated with or without the compounds in culture medium for 24h. Immediately after addition of the treatments, a scratch was made on the cell monolayer with a 200-μL pipet tip through the middle of the well. After the wounding, images of the wounded area were captured of three to four random fields at 0h and 24h using an inverted microscope (Axiovert 200; Carl Zeiss, 20x objective lens) equipped with a digital camera. Quantification of the wound area was performed using ImageJ. All experiments were performed in triplicate and repeated two to three times.

Chemotaxis

EHEC chemotaxis was assayed using a modified microchemotaxis Boyden chamber. Polycarbonate filters (pore size: 8 μM; Transwell; Corning) were coated with 10 μg/mL fibronectin (Sigma). Cells were starved for 24h in DMEM containing 0.4% FBS and seeded at 10^6 cells/mL in the upper compartments together with the compounds. In the lower

compartment, wells were filled with medium with the chemoattractant, serum. Endothelial cells were allowed to migrate toward 10% FCS for 18h at 37°C and 5% CO_2. Migrated cells were fixed and stained with the Diff-Quik staining set (Medion Diagnostics). Pictures were taken from at least three random fields for each experimental group with an inverted microscope (Axiovert 200; Carl Zeiss, 20x objective lens) equipped with a digital camera. Chemotaxis was quantified by counting the number of stained cells that migrated to the lower side of the filter. Stained cells without visible nucleus were excluded from the study.

Gelatin gel zymography

The effect of compounds on gelatinolytic activity was measured as previously described (Bellosta et al., 1998). Briefly, EHECs were seeded in 48-well plates and treated with or without 3PO, PA-1 and PA-2 in culture medium without serum for 24h. Then, conditioned medium was collected, and cell monolayers processed for evaluating cell protein content or mRNA extraction. Aliquots of conditioned medium (30μL per lane) were run at 4°C on 7.5% polyacrylamide gels containing 10% SDS and 1 mg/mL gelatin (Sigma-Aldrich) under non-reducing conditions and without boiling. After electrophoresis, the proteins were renatured by washing the SDS away in 2 washes with Triton X-100 (2.5%) at room temperature. Hereafter, the gels were incubated overnight at 37°C in developing buffer (50 mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$, 150 mM NaCl, and 1 μM $ZnCl_2$) to activate the gelatinases. Gels were subsequently stained with a solution of Coomassie brilliant blue (0.1% Coomassie R250 in 25% methanol, 7% acetic acid; Sigma-Aldrich) for 2 hours with gentle agitation, followed by destaining (25% methanol, 7% acetic acid) until areas of gelatinolytic activity appeared as transparent bands against a blue background. Finally, the gels were scanned using Chemidoc MP Imaging System (Biorad) and quantified by densitometric analyses using ImageJ software (National Institues of Health). Values obtained were normalized to protein content, using the Pierce BCA Protein assay kit (Thermo Fisher Scientific), and expressed as a percentage of control.

Western blotting

Endothelial cells were treated with 3PO, PA-1 and PA-2 in culture medium for 24h. After an hour incubation, human TNF-α (10 ng/mL; Sigma, T0157) was added to induce inflammation. For the preparation of total cell lysates, cells were washed in cold PBS and lysed in ice-cold buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet (NP-

40), and protease inhibitors. Molecular mass marker (Sharpmass VI Prestained Protein Marker, Euroclone S.p.A) and proteins were separated through 4-12% SDS-PAGE gel. Proteins were then transferred on to a nitrocellulose membrane and blocked with buffer containing 5% milk in 0.05% TBS-Tween. Membranes were probed with antibodies specific for PFKFB3 (1:1000, Abcam, ab96699), VE-cadherin (1:1000, Cell Signalling, 2158S), VEGFR2 (1:1000, Thermo Fisher Scientific, PA5-16487), MCT1 (Abcam ab85021, 1:1000), MCT4 (Proteintech 22787-1-AP, 1:1000) and β-actin (1:1000, Sigma, A2228) overnight at 4 °C. Next day, membranes were then incubated with goat anti-mouse 680RD and goat anti-rabbit 800CW IRDye secondary antibodies (1:10000, LI-COR Biosciences) for 1 hour at room temperature. Immunoreactive bands were detected by acquiring images with Odyssey® (LI-COR Biosciences). Densitometric readings were evaluated using the Image Studio Software.

RNA isolation and real-time RT-PCR

Endothelial cells were treated for 24h with or without 3PO, PA-1 or PA-2 in culture medium. To induce inflammation, after an hour incubation with the treatment the cells were stimulated with human TNF-α (10 ng/mL; Sigma, T0157). Total RNA was isolated using the Direct-zol™ RNA MiniPrep Pus kit (Zymo Research) and cDNA was generated by reverse transcription with the iScript gDNA Clear cDNA Synthesis Kit (Biorad), following the manufacturer's instructions. Real-time PCR was performed using iTaq™ Universal SYBR® Green supermix (Biorad) with gene-specific forward and reverse primers in an ABI PRISM 7000 Sequence detection system (Applied Biosystems). Relative gene expression was converted using the $2^{-\Delta\Delta CT}$ method against the internal control large ribosomal phosphoprotein P0. The primer sequences are listed in Table 1.

Table 1. List of real-time PCR primer sequences

Target gene	Forward primer (5' ➛ 3')	Reverse primer (5' ➛ 3')
RPLP0	TCG ACA ATG GCA GCA TCT AC	ATC CGT CTC CAC AGA CAA GG
PFKFB3	GAT GCC CTT CAG GAA AGC CT	GAA CAC TTT TGT GGG GAC GC
MCT1	CAC CGT ACA GCA ACT ATA CG	CAA TGG TCG CCT CTT GTA GA
MCT4	GAG TTT GGG ATC GGC TAC AG	CGG TTC ACG CAC ACA CTG
MMP-2	TCT GTG TTG TCC AGA GGC AA	GCC TAA CAT CAT CAC TAG GCC A
MMP-9	GTA CTC GAC CTG TAC CAG CG	AGA AGC CCC ACT TCT TGT CG
IL-1B	ATG CAC CTG TAC GAT CAC TG	ACA AGG ACA TGG AGA ACA CC
TGF-β	CAG CAA CAA TTC CTG GCG ATA	AAG GCG AAA GCC CTC AAT TT
VEGFA	ACG AAA GCG CAA GAA ATC CC	GGA GGC TCC AGG GCA TTA G
VEGFR2	ATG CAT CCT TGC AGG ACC AA	GGT TTC CTG TGA TCG TGG GT
VCAM	GGA CCA CAT CTA CGC TGA CAA	GAC TGT GAT CGG CTT CCC AG
VECADHERIN	ACC AGG ACG CTT TCA CCA TT	GGC TCA TGT ATC GGA GGT CG

In vitro tube formation

The effects of 3PO, PA-1 and PA-2 on endothelial cell ability to form capillary-like structures on basement membrane matrix was assessed *in vitro*, using the morphogenesis assay. EHECs and HUVECs were grown till 80% confluency in complete culture medium and serum starved overnight. Using pre-cooled tips and 96-well plates, 75 μL/well of liquid matrigel (GFR Membrane Matrix; Corning #356230) was distributed and allowed to solidify at 37°C for at least 30 minutes. Endothelial cells were cultured on the matrigel at a density of 1.5-2 x 10^5 in DMEM/EBM2 medium containing 100 ng/mL VEGF and 100 ng/mL FGF2 alone or with 3PO or PA-1 or PA-2 each. Pictures were captured after six hours for HUVECs, and 24h for EHECs using a Zeiss Microscope equipped with a Nikon camera (Axio Observer A1, Zeiss, Germany), and quantified using ImageJ software and the "Angiogenesis Analyzer" tool.

Flow cytometry

HUVECs were treated with the three compounds, 3PO, PA-1 and PA-2 in culture EBM-medium for 24h. Where after cells were detached, and cells were fixed, and permeabilized with CytoFix/Cytoperm kit (BD Biosciences) accordingly to manufacturer. Cells were washed and stained with PE-conjugated VEGFa (IC2931P, R&D Systems) for 30 minutes at 4°C.

Fluorescence intensity was measured using a BD FACSCanto II flow cytometer. Experiments were performed on HUVECs activated with TNF-α (10ng/mL). Flow data were analyzed with FlowJo (Treestar Inc.). VEGFa-positive cells were quantified by the mean fluorescence intensity (MFI).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Presented data were derived from at least three independent experiments and are expressed as mean ± SEM. For the quantitative analysis of immunoblots and mRNA expression, normalization was performed. A Student's t-test (two-tailed) was applied to compare two groups, whereas a one-way ANOVA test was performed to evaluate three or more groups to control. A P-value less than 0.05 was considered significant. All data shown represent results from three or more independent experiments, and are expressed as mean ± SEM. Statistical analysis and graphical presentations were performed using GraphPad Prism 2016.

Results

Novel PFKFB3 inhibitors suppress PFKFB3 and affect lactate metabolism

The impact of pharmacological inhibition of the kinase activity of PFKFB3 on glycolysis levels were assessed in both an EC line (EHEC) and in primary ECs (HUVEC) which have been both used previously as a standard for cell based assays in the field of *in vitro* angiogenesis research (Bouïs et al., 2001). In our study, key findings were confirmed by using 3PO as a reference compound. From dose-response studies we assessed the most active concentration of all compounds able to induce functional changes in EC behavior, including wound healing (date not shown).

In order to test the effectiveness of PA compounds (Figure 1) in suppressing PFKFB3, their effects on gene expression and protein level were evaluated. Treatment with the PA compounds resulted in a significant reduction in PFKFB3 mRNA levels in EHECs (Figure 2a). Immunoblot analyses confirmed reduction in PFKFB3 protein levels (Figure 2b). In contrast to these results, no inhibitory effect of 3PO on PFKFB3 transcripts was observed with a slight decrease at a protein level (Figure 2a, b). These results were confirmed in human umbilical vein endothelial cells (HUVEC: Figure 2c, d).

An elevated lactate concentration represents a good indicator of the metabolic adaptation of pathological angiogenesis and is actually correlated to clinical outcome in a variety of human cancers (Hirschhaeuser et al., 2011; Romero-Garcia et al., 2016). PFKFB3 regulates high glycolytic activity in ECs. Therefore, we examined the metabolic effects of PFKFB3 inhibition on lactate production. Following reduced PFKFB3 expression, we assessed the release of lactate. PA-1 markedly reduced the secretion of lactate (Figure 2e). PA-2 was less effective and a similar effect was observed with 3PO (Figure 2e). We observed also a marked accumulation of lactate in EHECs upon treatment with both PA compounds comparable to the effect of the well-established PFKFB3 inhibitor 3PO (Figure 2f). These results suggest a potent regulatory effect of these newly synthesized compounds on the lactate transport capacity in ECs.

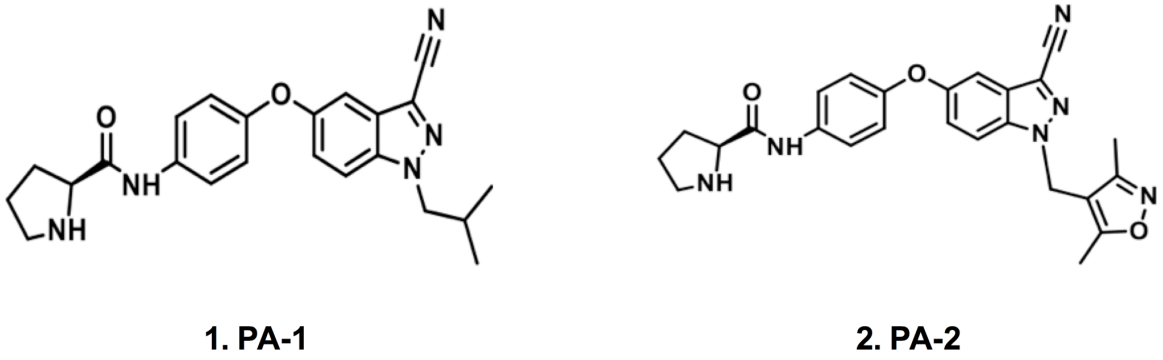


Figure 1. *Chemical structures of PFKFB3 inhibitors: PA-1 and PA-2.*

PFKFB3 inhibition regulates lactate transport through modulation of MCT1 and MCT4

The main controllers of the lactate fluxes in ECs are the monocarboxylate transporters (MCT) 1 and MCT4. MCT1 is known to be mainly a lactate importer to fuel the Krebs cycle, but can also act as an exporter, whereas MCT4 is known as a lactate exporter (Végran et al., 2011; Pérez-Escuredo et al., 2016). Therefore, we analyzed whether the observed effects on lactate levels are due to an alteration of the expression of MCT1 and/or MCT4. Expression analyses suggest that treatment with PA compounds significantly affected the expression of both MCT1 (Figure 2g, i) and MCT4 (Figure 2h, j) in both EC lines. The western blot analysis is less conclusive, since treatment with PA compounds did not affect MCT1 protein levels, while PA-1 reduced and 3PO increased MCT4 levels (Figure 2k, l). These results collectively, suggest

that PA compounds not only inhibit glycolysis by directly inhibiting PFKFB3 expression, but also by indirectly regulating lactate levels through a reduced expression of MCT1 and MCT4.

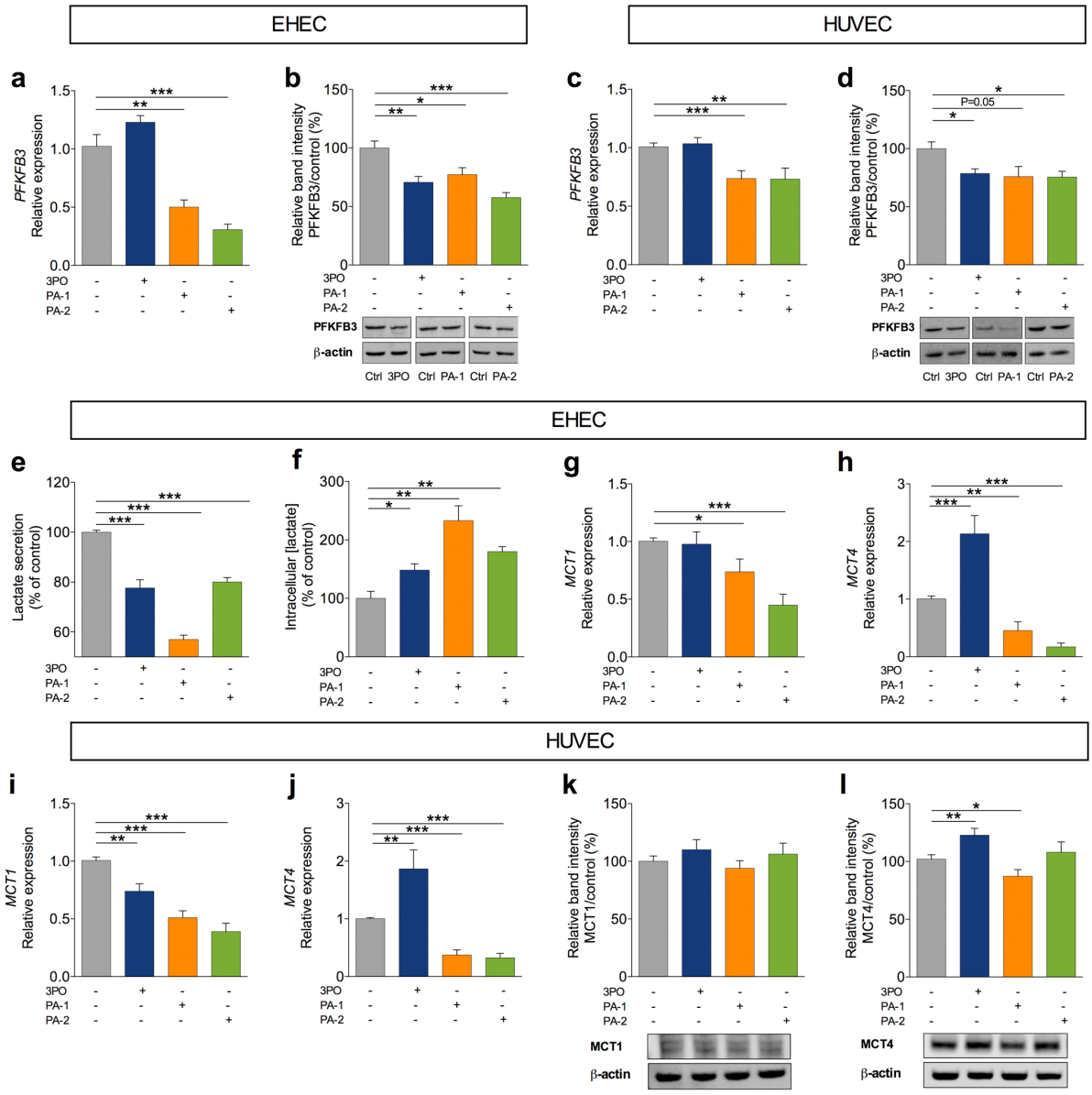


Figure 2. *PA compounds reduce PFKFB3 expression and glycolysis in endothelial cells.* ***(a)*** *Treatment with PA-1 and PA-2 reduced the mRNA expression of PFKFB3 in EHECs (n=5-7) and* ***(b)*** *HUVECs (n=3).* ***(c)*** *PA-1 and PA-2 inhibited the protein levels of PFKFB3 in EHECs (n=3) and in* ***(d)*** *HUVECs (n=3).* ***(e)*** *Partial reduction of glycolysis (measured by extracellular lactate production) by 3PO, PA-1 and PA-2 in EHECs (n=4-6).* ***(f)*** *Simultaneously, intracellular lactate concentration was elevated (n=3).* ***(g)*** *RT-PCR results show reduced expression of MCT1 (n=3) and* ***(h)*** *MCT4 in EHECs (n=3) as well as in* ***(I, j)*** *HUVECs (n=3-4) by PA-1 and PA-2.* ***(k, l)*** *Western blot analysis of MCT1 (n=8-9) and MCT4 amount (n=6-7) in HUVECs showing reduction of MCT1 upon treatment with PA-1. Data are presented as mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001*

PFKFB3 inhibitors interfere with key steps of inflammatory angiogenesis: proliferation, migration and protease activity

Proliferation and migration are crucial steps in inflammation-induced neovascularization. Previous studies already showed that PFKFB3 inhibition or knockdown suppress EC migration and proliferation (De Bock et al., 2013a; Schoors et al., 2014; Xu et al., 2014; Cruys et al., 2016).

In line with these findings, we confirmed that treatment with the PA compounds is capable of inhibiting EC proliferation (assessed by counting cell numbers) to a similar level (Figure *3*a) without affecting cell viability (Figure *4*). Further, PFKFB3 suppression with PA compounds impaired EC motility, as it reduced EC migration in a modified Boyden chamber (Figure *3*b, c), and directional migration in the absence and presence of the mitomycin C (MitoC: an inhibitor of cell proliferation) (Figure *3*d, e).

Notably, ECs treated with PA compounds expressed lower levels of endothelial activation markers such as vascular cell adhesion molecule 1 (VCAM-1: Figure *3*f, g) and vascular endothelial cadherin (VE-cadherin) (Figure *3*i, j). However, no significant changes were observed in VE-cadherin protein levels (Figure *3*h). These data indicate that endothelial PFKFB3 inhibition regulate adhesion molecules production at a transcriptional level, which may have as a consequence an impaired migration. 3PO gave contrasting results, since it reduced VCAM-1 and VE-cadherin expression in EHECs, but it induced VCAM-1 in HUVECs (Figure *3*f, i).

Due to the important role that MMPs (MMP-2 and MMP-9) have during angiogenesis (Nguyen et al., 2001), we sought to investigate whether PA compounds are capable of regulating the expression and activity of MMPs in ECs. As assessed by quantitative RT-PCR, we observed decreased MMP-2 and MMP-9 mRNA levels upon treatment with PA compounds (Figure *5*a, b). Alongside with these results, gelatin zymography revealed that treatment of ECs with PA compounds significantly reduced MMP-2 and MMP-9 activity (Figure *5*c-e). 3PO had a similar effect on the gelatinolytic activity (Figure *5*c, d).

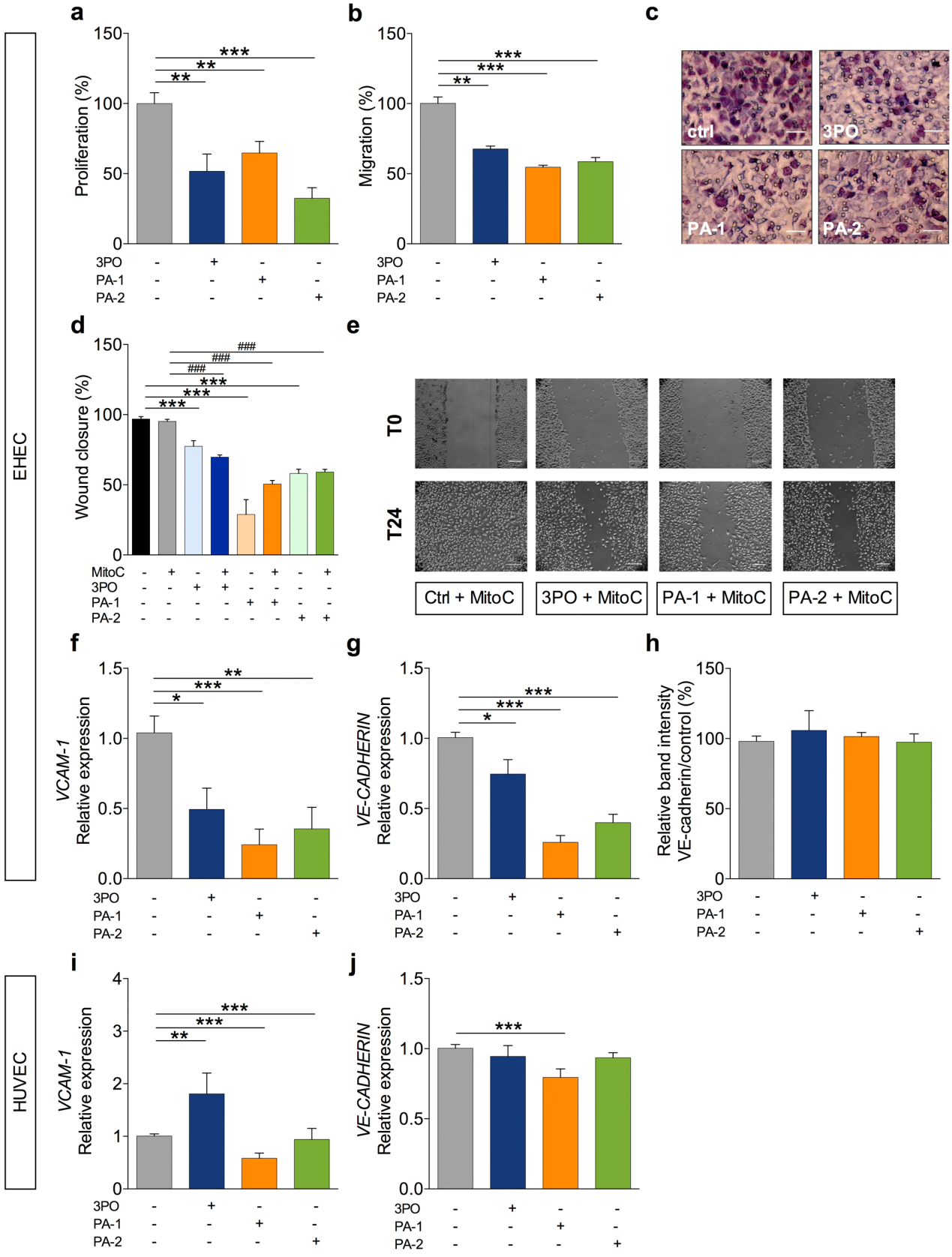


Figure 3. *PFKFB3 inhibition by PA-1 and PA-2 impairs endothelial cell proliferation and migration.* ***(a)*** *PFKFB3 inhibition with 3PO, PA-1 and PA-2 reduced EC proliferation (n=7-8).* ***(b)*** *Modified Boyden chamber migration assay showing that PFKFB3 inhibition by the three compounds reduced*

*migration of ECs (n=4). **(c)** Representative phase-contrast images of migrated ECs upon eosin & tiazine staining (scale bar: 20 µM). **(d)** Quantification of EC migration in scratch wound assay showing reduced migration upon treatment with 3PO, PA-1 and PA-2 in absence and presence of MitoC. **(e)** Representative images of MitoC-treated EC migration in scratch wound assays showing reduced migration upon PFKFB3 inhibition by 3PO, PA-1 and PA-2 (n=5-6) (scale bar: 200 µM). **(f, g)** PA-1 and PA-2 reduced mRNA expression of migration-related genes: VCAM-1 and VE-cadherin in EHECs (n=3-4). **(h)** No change was observed in VE-cadherin protein expression EHECs treated with 3PO, PA-1 and PA-2 (n=3). **(i, j)** qRT-PCR analysis showing reduced mRNA expression of VCAM-1 and VE-cadherin in HUVECs (n=3). Data are represented as mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, ### P < 0.001*

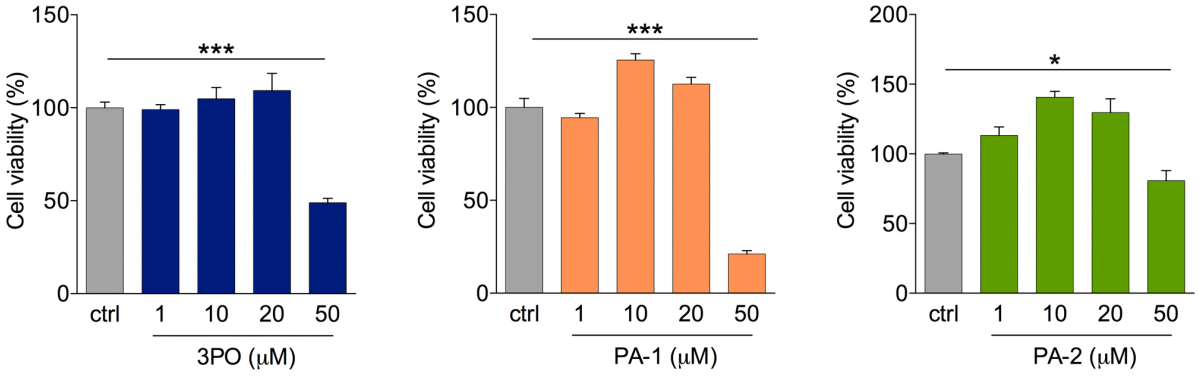


Figure 4. *Dose-range effects of 3PO, PA-1 and PA-2 on endothelial cell viability. Concentration response curve showing no cytotoxic effects at used concentration (20 µM) of 3PO, PA-1 and PA-2 in EHECs. At a concentration of 50 µM, however, significant cytotoxicity was observed (n=6-8). Data are presented as mean ± SEM. * P < 0.05, *** P < 0.001*

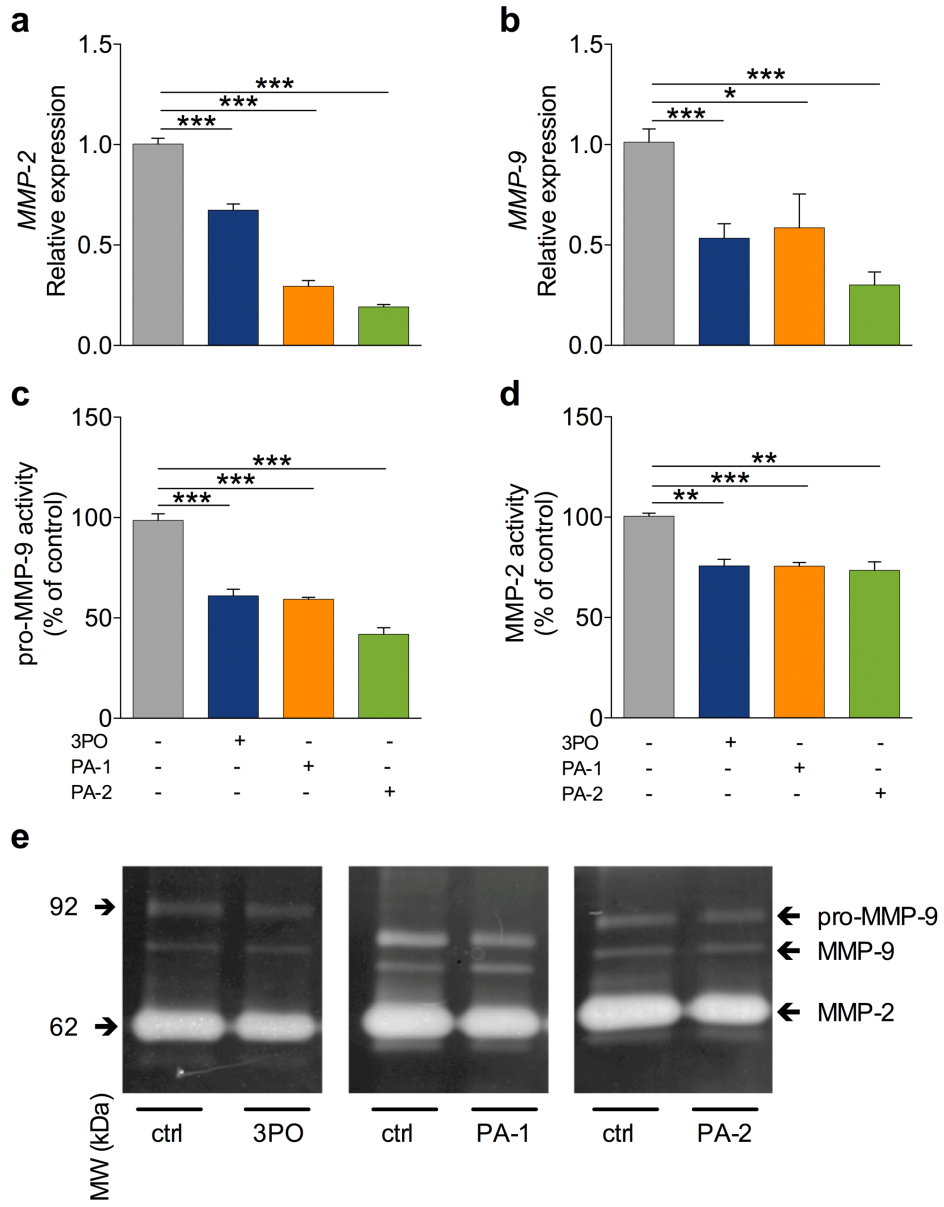


Figure 5. *PFKFB3 inhibition with PA-1 and PA-2 reduce gelatinolytic expression and activity in endothelial cells.* ***(a, b)*** *qRT-PCR results showing reduction of MMP-2 and MMP-9 mRNA levels in EHECs treated with 3PO, PA-1 and PA-2 (n=3).* ***(c, d)*** *Gelatin zymography analysis of conditioned medium from EHECs showing reduction of MMP-2 and proMMP-9 activity upon treatment with PA compounds (n=3-4).* ***(e)*** *Representative zymograms of the inhibitory effect of 3PO and PA compounds on gelatinolytic activity. Data are presented as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.*

Novel PFKFB3 inhibitors suppress capillary-like tube formation *in vitro*

Angiogenesis and inflammation are important hallmarks of several pathological situations (Costa et al., 2007; Hanahan and Weinberg, 2011). Mimicking the scenario occurring in a pro-angiogenic environment, we exposed ECs to VEGF and FGF2 and examined whether PFKFB3 inhibition with PA compounds may affect the capacity of ECs to induce the formation of

capillary-like structures on matrigel. We demonstrated that both PA-1 and PA-2 significantly impaired VEGF-induced endothelial sprouting, as shown by the decreased number and length of master segments together with a reduced number and surface area of formed meshes in HUVECs (Figure *6*a, b). Similar effects were observed in EHECs, although these cell lines appear to be more sensitive to the treatment with PA compounds (Figure *7*).

PFKFB3 inhibition interferes with VEGFA/VEGFR2 axis and suppresses proinflammatory cytokines/chemokines production

The endothelial VEGFA/VEGFR2 axis is the major pathway that regulates angiogenesis (Abhinand et al., 2016; Basagiannis et al., 2016; Shibuya, 2011). We therefore investigated whether the anti-angiogenic activity of PA compounds is regulated through this pathway. RT-PCR and western blot analysis revealed that PA-treated ECs expressed reduced mRNA and protein levels of VEGF (Figure *6*c, f) and VEGFR2 (Figure *6*d, g). The cytokine tumor necrosis factor (TNF)-α is a potent mediator of inflammation and angiogenesis (Vanderslice et al., 1998; Sainson et al., 2008), as it has been demonstrated to activate VEGFR2 and downstream signaling transduction molecules such as PI3K and AKT known to regulate EC motility and angiogenesis (Zhang et al., 2003; Karar and Maity, 2011).

Next, we identified the potential effect that PFKFB3 inhibition might have on the gene expression of important genes related to glycolysis, migration and angiogenesis in TNF-stimulated ECs. Similarly, to previous observation, expression analyses demonstrated that PA compounds inhibited the induction of PFKFB3, MCT1 and MCT4 in TNF-α stimulated ECs (Figure *6*h). VCAM-1 and VE-cadherin expression was also markedly reduced (Figure *6*h). Transforming growth factor beta (TGF-β) have been shown to promote the expression of VEGF and protect ECs from apoptosis (Ferrari et al., 2009; Guerrero & McCarty, 2017). Therefore, we analyzed the expression of TGF-β and observed a marked reduction upon treatment with PA compounds in ECs and inflammatory ECs, respectively (Figure *6*e, h). Together with this, the pro-inflammatory cytokine interleukin (IL)-1β was significantly reduced in inflammatory ECs (Figure *6*h).

Taken together, these results indicate that PFKFB3 inhibition with PA compounds impairs angiogenesis in basal and inflammatory conditions by affecting the VEGF/VEGFR2 pathway and interfering with crucial steps such as cell migration and proliferation.

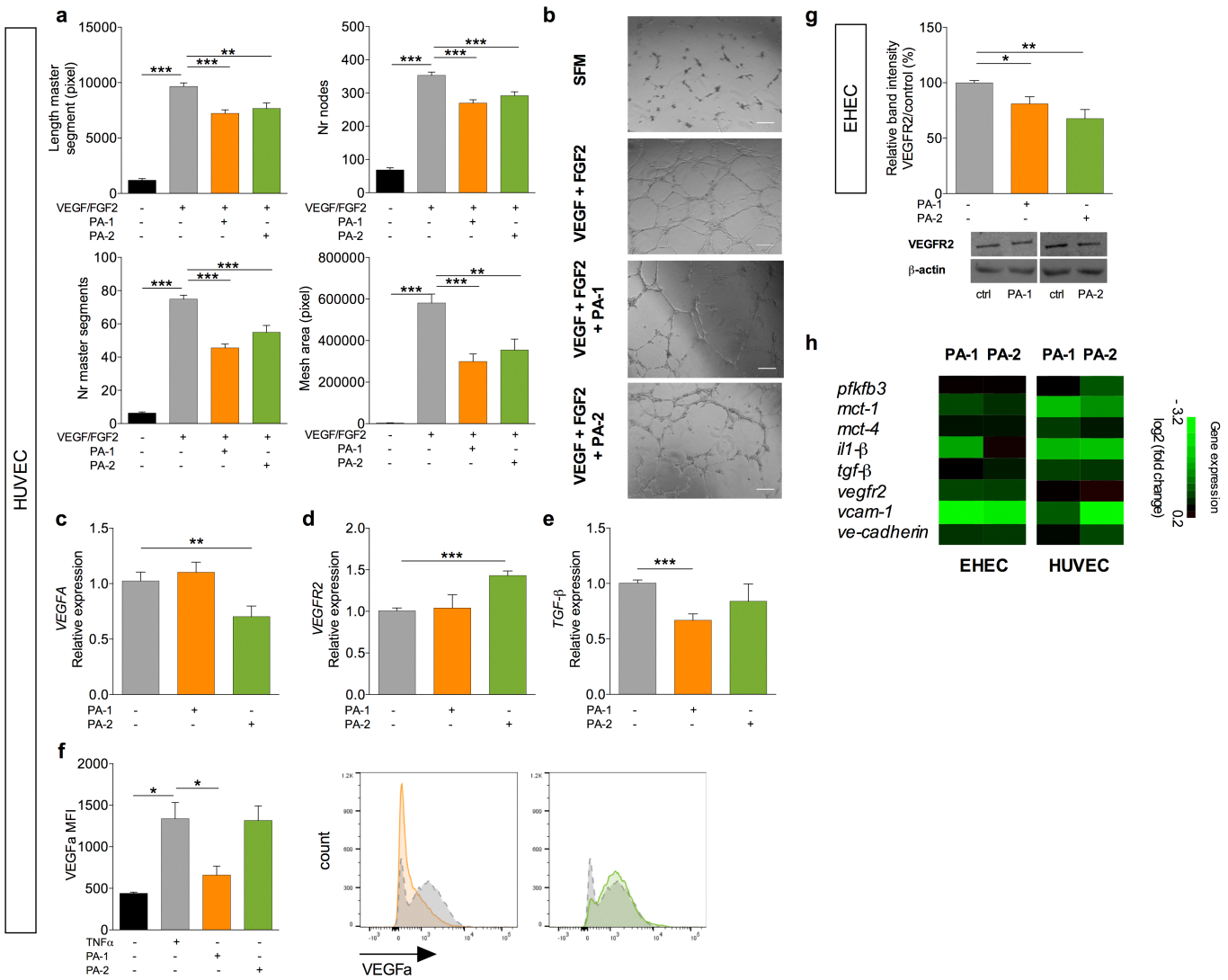


Figure 6. *PFKFB3 inhibition with PA compounds suppress capillary-like structure formation in vitro in endothelial cells.* ***(a)*** *Morphometric analysis revealing reduced effect of PA-1 and PA-2 on the total length of master segments, number of master segments and number of meshes in HUVECs.* ***(b)*** *Representative images showing suppressing effects of PA-1 and PA-2 on in vitro sprouting by HUVECs (scale bar: 200 µM), SFM = serum free medium, i.e. negative control. qRT-PCR analysis revealing downregulation of angiogenic genes* ***(c)*** *VEGFA,* ***(d)*** *VEGFR2 and* ***(e)*** *TGF-β expression in HUVECs treated with PA-1 and PA-1 (n=3-4).* ***(f)*** *By flow cytometry, a marked reduction of intracellular VEGFA production was observed in HUVECs treated with PA-1 (n=3). Representative histograms showing reduced VEGF–positive ECs by PA-1 (orange) as compared to control (grey). No change was observed in ECs treated with PA-2 (green).* ***(g)*** *Relative protein expression of VEGFR2 in EHECs showing reduction upon treatment with PA-1 and PA-2 (n=3).* ***(h)*** *Heatmap indicating fold changes in expression levels of genes related to glycolysis, migration, and angiogenesis upon treatment with PA-1 and PA-1 together with TNF-α in EHECs (left) and HUVEC (right) (n=3-4). Green color represents downregulation of mRNA levels. Data are represented as mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001*

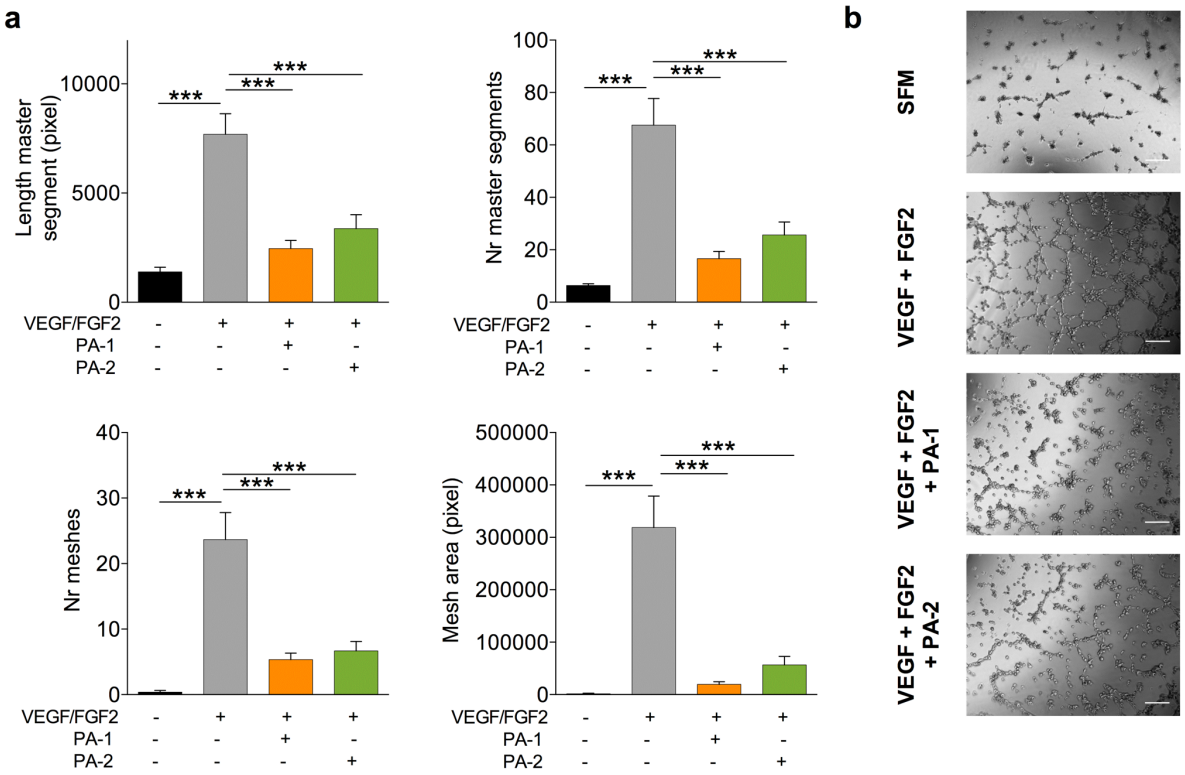


Figure 7. *PFKFB3 inhibition with PA compounds suppress formation of in vitro capillary-like structures by endothelial cells.* ***(a)*** *Quantification of morphometric analysis of in vitro angiogenesis assay showing reduction of several parameters upon treatment with PA-1 and PA-2 in EHECs.* ***(b)*** *Representative images showing suppressing effects of PA-1 and PA-2 on in vitro sprouting by EHECs (scale bar: 200 µM). Data are presented as mean ± SEM, *** P < 0.001*

Discussion

Since angiogenesis is essential in the pathophysiology of various diseases such as cancer (Nishida et al., 2006) and atherosclerosis (Camaré et al., 2017), several anti-angiogenic treatment strategies have emerged and been applied in the clinic (Potente et al., 2011). Traditional VEGF-targeted therapy, such as the monoclonal antibody bevacizumab, often fails to render sustained responses with minimal increased survival rate in treatment of several cancers due to the development of drug resistance (Kerbel, 2009; Potente et al., 2011; Clarke and Hurwitz, 2013; Gramatzki et al., 2018). This underlines the need for novel anti-angiogenic therapeutic approaches with fundamentally different mechanisms of action.

A new paradigm for treatment of pathological angiogenesis is by targeting EC metabolism with an anti-glycolytic therapy through the inhibition of PFKFB3 (Verdegem et al., 2014). Mono and combination therapy with the glycolytic inhibitor 3PO significantly reduced pathological angiogenesis in models of ocular disease and inflammatory bowel disease by partly lowering glycolysis (up to 40%) (Schoors et al., 2014). Through an indirect inhibition of glycolysis, the

metabolism of activated ECs can be normalized to a quiescent state. This approach has shown no harmful consequences at a systemic level, as opposed to direct and maximal inhibition of glycolysis (e.g. with 2-deoxyglucose) which elicited cell death (Aft et al., 2002; Ben Sahra et al., 2010). Hence, a moderate inhibition of glycolysis promises to be an appropriate therapeutic strategy for angiogenesis.

As observed by others (Boyd et al., 2015), we also confirm (data not shown) that 3PO does not bind to PFKFB3 and does not show any activity in a PFKFB3 kinase assay (IC50 > 100 μM). This, together with the immunosuppressive effects of 3PO (Telang et al., 2012), suggests unidentified off-target effects of 3PO. Therefore, we have selected two highly specific and potent PFKFB3 inhibitors, with validated PFKFB3 kinase binding activity (Boyd et al., 2015) and studied their anti-angiogenic potential in ECs. Our findings show potent inhibitory activity of PA-1 (IC_{50} = 3 nM) and PA-2 (IC_{50} = 21 nM) in a PFKFB3 kinase assay. The PA compounds are very effective in inhibiting PFKFB3 activity and their IC_{50} is much lower than other known PFKFB3 inhibitors, such as PFK158 which has an IC_{50} of 137 nM (Chand and Tapolsky, 2013). ECs rely on glycolysis, both in resting and activated state, rather than oxidative phosphorylation. As compared to macrophages and other vascular cell types including fibroblasts, and cardiomyocytes, ECs have a much higher level of glycolysis (85%) to generate energy. PFKFB3 is an essential enzyme in EC glycolysis, and the knockdown of PFKFB3 decreases glycolysis up to 40% (De Bock et al., 2013).

We first reported that pharmacological blockade of PFKFB3 with PA analogues reduced PFKFB3 levels. Consequently, glycolysis was reduced up to 40% and the formation of intracellular lactate was enhanced. ECs cells co-express the lactate transporters MCT1 (importer) and MCT4 (exporter) that can passively and bidirectionally transport several monocarboxylate metabolites, such as lactate, pyruvate, D-β-hydroxybutyrate and acetoacetate across the plasma membrane. Interestingly, it has been demonstrated that MCT4 predominates over MCT1 for lactate export in highly glycolytic cells (Baek et al., 2014; Pérez-Escuredo et al., 2016). Overexpression of MCTs have been associated with enhanced cell proliferation, migration, invasion and angiogenesis and therefore have been considered to be promising targets for cancer therapy (Pérez-Escuredo et al., 2016). We have demonstrated that glycolysis inhibition via PFKFB3 lead to accumulation of intracellular lactate. Here, we also show shat PFKFB3 inhibition markedly reduce the expression of MCTs, which modulate the intracellular accumulation of lactate and glycolysis (Doherty et al., 2014; Tan et al., 2015).

In line with previous reports showing that PFKFB3 drives EC proliferation and migration (De Bock et al., 2013b; Xu et al., 2014), we demonstrated that PFKFB3 inhibition with more selective inhibitors impaired vessel sprouting in ECs, by reducing EC proliferation, migration, and gelatinolytic activity. In conclusion, PFKFB3 inhibition impaired essential processes in angiogenesis induction.

Cytokines and growth factors can stimulate classical angiogenic signaling pathways, such as the VEGF/VEGFR2 pathway (Harjes et al., 2012; Abhinand et al., 2016). We found that PFKFB3 inhibition with PA compounds resulted in the reduction of this pathway at a transcript and protein level. Furthermore, to better mimic the vascular microenvironment in pro-inflammatory environment we assessed the effect of PFKFB3 in TNFα-activated ECs. Here, we found that PFKFB3 inhibition with PA compounds also acted on the VEGF/VEGFR2 pathway, and consequently reduced transcripts of essential drivers of cell migration.

Whether PFKFB3 inhibition could induce a switch in ECs to aerobic metabolism, i.e. oxidative phosphorylation, has not been studied yet. However, studies do indicate a shift in tumor cells from glycolysis toward the NADPH-producing pentose phosphate pathway (PPP) upon S-glutathionylation and demethylation of PFKFB3 that is induced by high levels of reactive oxygen species (Seo and Lee, 2014; Yamamoto et al., 2014). Also, 3PO was shown to induce PPP flux (Schoors et al., 2014) which promotes the angiogenic progression (Vizán et al., 2009; Hao, 2018).

Development of anti-angiogenic agents is still an ongoing task. Standard glycolytic or PFKFB3 inhibitors, like 3PO and PFK15, have been the focus of a number of studies showing their therapeutic effects. However, these compounds are poorly specific or may lead to cell death. They are usually described to be active in the micromolar range, thereby necessitating maximum doses in preclinical models (Granchi et al., 2014; Conradi et al., 2017). PA-1 and PA-2 were disclosed by AstraZeneca (Boyd et al., 2015) and described to selectively inhibit lactate production by lung cancer cells. Big advantages of these PA compounds are their higher specificity and their ability to inhibit PFKFB3 activity and expression. Moreover, advantageous is their ability to inhibit expression of MCTs, adhesion and angiogenic molecules. We present that PFKFB3 inhibition also reduce MMP-2 and MMP-9 activity (and expression), which suggest a multimodal treatment of angiogenesis by PA compounds (Figure S3). Future in vivo evaluation of these compounds will provide information about their therapeutic activity and whether they qualify for clinical evaluation.

Since the effect of (pharmacological) PFKFB3 inhibition is not limited to endothelial cells, monocytes/macrophages and T cells are probably also affected in vivo and this may be in part responsible for the reduced tumor growth when applied to cancer models. This study provides insight into the anti-angiogenic and anti-inflammatory properties of PA compounds in endothelial cells, revealing a potential therapeutic application of these highly selective PFKFB3 inhibitors to treat aberrant angiogenesis and vasculogenesis.

CHAPTER 5

General discussion and future perspectives

General discussion and future perspectives

The role of PFKFB3 has been described extensively in cancer and more recently in pathological angiogenesis (Clem et al., 2013; Schoors et al., 2014; Xu et al., 2014). The understanding that PFKFB3 is an important regulator of endothelial cell metabolism has led to the concept that PFKFB3 is potential anti-cancer and anti-angiogenic target. In the present study, we have investigated the possible working mechanism of the well-known glycolytic inhibitor 3PO and studied the anti-angiogenic potential of selective PFKFB3 inhibitors, PA-1 and PA-2, through experimental *in vitro* studies.

Genetic silencing or pharmacological inhibition (3PO) of PFKFB3 reduced glycolysis to the same level of no more than 30-40%. This was sufficient to impair vessel sprouting, suppress tumor growth and metastasis (Schoors et al., 2014; Cantelmo et al., 2016). This was described to be caused by defects in EC proliferation and migration (De Bock et al., 2013b). However, we show that the anti-glycolytic effect of 3PO cannot be attributed to its binding to PFKFB3 kinase domain nor to inhibition of the kinase activity (Chapter 2: figure 5). The major finding of our study was that 3PO induced intracellular lactate accumulation, which resulted in intracellular acidification (Chapter 2: figure 6). Changes in intracellular pH and lactate concentration are indirect regulators of glycolysis, cell motility and proliferation (Rovetto et al., 1975; White et al., 2017). These findings lead to the question whether 3PO could act on lactate transporters: MCTs. Therefore, we investigated the effect of 3PO on the main lactate transporters, MCT1 and MCT4. Treatment with 3PO affected the expression of MCTs, as assessed by RT-qPCR (Chapter 3: figure 2). Notably, the expression of MCT1 was significantly lowered whereas MCT4 expression was enhanced upon 3PO stimulation indicating that 3PO reduces lactate import, but could also induce its export. This made us wonder whether 3PO could control MCT activity. It was not feasible for our lab to perform a binding assay with MCTs. This would be of great interest for us and for a better understanding of 3PO's mechanism of action. A recent publication of Quanz *et al.* in 2018 demonstrated a technique to determine binding of MCT1 inhibitors by performing a radioligand-binding assay (Quanz et al., 2018).

Although 3PO is the lead compound to treat pathological angiogenesis, its non-specificity could serve as a disadvantage for further use in other diseases characterized by aberrant angiogenesis such as progressive atherosclerosis. A reason is that mis-targeting or non-specific interaction with the healthy vasculature could lead to toxicity (Clem et al., 2013; Tawakol et

al., 2015). Inadequate dosing of 3PO can make the difference of this glycolytic inhibitor as an anti-cancer drug (Conradi et al., 2017). On the other hand, 3PO was shown to be effective in combination therapy to overcome resistance mediated by VEGFR inhibition (Schoors et al., 2014) or multi-kinase inhibitors such as *nintedanib* (Pisarsky et al., 2016). Recently, it has been shown that 3PO could reduce intraplaque neovascularization and hemorrhages in atherosclerotic mice as well as induce apoptotic death of proinflammatory macrophages and T cells (Telang et al., 2012; Tawakol et al., 2015; Veken et al., 2017). This demonstrates the potential of 3PO to delay plaque rupture and to suppress inflammation within the plaque. However, it is worth to note that traditional anti-angiogenic drugs induce adverse cardiovascular effects (Hedhli and Russell, 2011). This is something to consider when using 3PO in a cardiovascular disease model. Altogether, 3PO demonstrated great potential as an anti-glycolytic drug to treat aberrant angiogenesis in cancer models, yet additional studies are needed to assess its true potential to be used as an anti-angiogenic drug.

AstraZeneca has described a series of selective PFKFB3 kinase inhibitors with great potency in inhibiting glycolysis as measured by: production of glycolytic product of PFK-1, fructose-1,6-biphosphate, and lactate secretion in lung carcinoma cells (Boyd et al., 2015). In accordance, we demonstrate potent inhibition of PFKFB3 with selected inhibitors: PA-1 and PA-2. In comparison to PFK15, these compounds are 50-fold more potent in inhibiting PFKFB3 kinase activity. Therefore, we investigated the effect of these compounds on endothelial cell metabolism and neovessel sprouting. Previously, strong evidence indicated that PFKFB3 regulates 40% of the glycolytic flux in endothelial cells (De Bock et al., 2013b). PFKFB3 inhibitors in the present study partially lowered glycolysis to the same level as genetic inactivation of the enzyme. Importantly, we demonstrate that PFKFB3 inhibition with PA compounds impairs vessel sprouting by reducing endothelial cell proliferation and migration. Endothelial cell activation markers such as VCAM-1 and VE-CADHERIN were markedly inhibited. The importance of PFKFB3 in regulating cell migration was shown previously. It was shown that PFKFB3 interacts with F-actin in filopodia and lamellipodia, thereby providing fuel for migration (De Bock et al., 2013b). As for PFKFB3's role in proliferation, it was demonstrated that PFKFB3 acts as a regulator of cell cycle progression and survival. Specifically, when present in the nucleus, PFKFB3 activates cyclin-dependent kinase 1 (Cdk1) and thereby prevents apoptosis (Yalcin et al., 2014).

An interesting observation was that PFKFB3 inhibition acts on the VEGF/VEGFR2 axis in basal and inflamed endothelial cells but can also affect other angiogenesis inducers such as

TGF-β. It was reported that tip cells display high levels of VEGFR2 signaling activity for their migratory functions (Ribatti and Crivellato, 2012). Our data suggest that selective PFKFB3 inhibition represents a negative feedback mechanism in ECs to maintain reduced glycolytic activity and reduced activation of VEGFR2 to obey a stalk cell phenotype.

Moreover, PFKFB3 inhibition caused marked accumulation of intracellular lactate without inducing acidification (data not shown). This could perpetuate a negative feedback loop to inhibit PFK-1 and to keep glycolysis low (Noble et al., 2017). Besides that, lactate is a promotor of angiogenesis via activation of hypoxia-inducible factor 1-alpha (HIF-1α) and upregulation of VEGFR2 (Ruan and Kazlauskas, 2013). Therefore, glycolysis reduction (with PA compounds) by reducing extracellular lactate levels inhibits VEGF-dependent angiogenesis.

Furthermore, we demonstrate that PFKFB3 inhibition reduced transcription of MMP (-2 and -9) and its secretion in ECs. This is not a surprise, since lactate itself is a regulator of MMP expression and PFKFB3 controls the lactate production. Kamenisch *et al.* demonstrated that lactate alone increases the expression of proteases such as MMP-1, MMP-2 and MMP-9 and is able to increase gelatinase release in cancer cells (Kamenisch et al., 2016). In conclusion, this study demonstrates that pharmacological blockade of PFKFB3 with PA compounds is an effective approach to prune neovessels and lower the inflammatory activity in endothelial cells.

Is glycolysis, however, the only bio-energetic pathway needed for angiogenesis? Till very recently it was believed that OXPHOS is of little importance for ECs during angiogenesis. De Bock *et al.* showed the important role of fatty acid oxidation (FAO) during angiogenic processes. While glycolysis is used for ATP production, ECs utilize FAO as a source for nucleotide synthesis. The enzyme carnitine palmitoyltransferase 1a (CPT1a) which acts as FA transporter into TCA cycle, is pivotal for DNA replication and thus a regulator of EC proliferation (Vandekeere et al., 2015). However, blocking mitochondrial metabolism does not affect EC sprouting, blocking or silencing CTP1 impaired EC proliferation and in turn vessel sprouting (Schoors et al., 2015). This indicates that different metabolic pathways direct different EC functions essential for angiogenesis.

The interaction between metabolism and angiogenesis is an interesting line of investigation providing new therapeutic strategies for treating a wide array of pathologies. Our consortium has already identified promising outcomes of glycolysis inhibition and factors contributing to atherosclerotic plaque stability such as intraplaque neovascularization and hemorrhage (Veken

et al., 2017). Currently, studies are ongoing investigating the possible effect of silencing or pharmacologically inhibiting PFKFB3 as a way to promote plaque stability.

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Appendices

Acknowledgements

CV

“Peaceful

is the one

who’s not concerned

With having more or less.

Unbound by name and fame

he is free from sorrow

from the world and

mostly from

himself”

-Rumi

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Dear Denisa; I am very thankful for the time we spent together at MultiMedica. You have taught me a lot about angiogenesis and helped me in the times that I needed you most. You taught me how to perform experiments in an efficient and productive way. You have great work ethics and I am sure you will pursue the things you want in life.

Dear Paola, "Ciao Bella!". This made me smile every day. During my time in Antwerp, we have worked really great together. You have taught me the ins and outs of in vivo work and included me in one of your projects. You are great scientist and I wish you all the best in your career and life.

T2.28 office: Arthur, Alessandro, and the Iranian squad with Hadis and Farnaz – you guys, made me enjoy going to work. You girls have become sisters to me! I loved being spoiled by you: you are such great cooks. Hadis Jan, your kindness and willingness to help others is something that I greatly admire. Farnaz Jan, you have shown how honest you are in friendships. I am confident we will keep in contact. Dastetoon dard nakone doostane khoob! Behtarin arezooharo daram baratoon (Thank you dear friends! All the best wishes for you). Arthur (Tukkie), you will be remembered as the guy that helped everyone! Alessandro, you have been my buddy in crime in Antwerp. I will remember our good times in the weekends either at T2 or chilling in Antwerp. The rest of T2 lab: you are a great team and I have enjoyed spending time with you.

Rest of the Moglynet consortium, including the main coordinators Prof. Maria Luisa Gelmi, Prof. Francesca Clerici, and Sabrina Pavan; Thank you for your pleasant collaborations and contribution during my career development.

Laura and Fabiana, my friends from Quax lab in Leiden; I would see you at our regular Moglynet meetings and have extremely funny times together. I admire your enthusiasm and hard-working mentality. You will achieve great things for sure!

Dear Lina, my buddy from DiLuca Lab and in private life! Both from Amsterdam and the only Dutchies at DISFEB, you were my other shoulder to “cry” on. What a PhD and foreigner struggles we had! After or even during-work aperitivo’s were our moments of joy. I greatly respect your hard work and I am sure it will be paid off. Thank you for being the great friend you are ☺.

Last but not least….. Sweet Goran, without your open-mindedness, compassion and understanding I would not have been able to enjoy this PhD experience. Thank you for your listening ears, and creative ideas! You have been a great contribution to my personal and professional development. You are a great scientist, friend and partner in crime. I look forward to what the future holds up for us…. ♡

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Experienced biomedical scientist in the field of cardiovascular diseases and pharmacology. Solid experience in cell culture and molecular biology techniques.

RESEARCH EXPERIENCE

PhD fellow

Dept. Pharmaceutical Sciences, Laboratory of Physiopharmacology, University of Antwerp, Belgium

06/2018 – 01/2019

Researcher in the Pharmacology group – Prof. Guido de Meyer & Prof. Wim Martinet

Metabolic (glycolytic) inhibitors for treatment of angiogenesis and atherosclerosis. Experience with mouse model for atherosclerosis, protein characterization, and cell based assays: angiogenesis, metabolic and cytotoxicity

PhD fellow

Dept. of Pharmacological and Biomolecular Sciences, Laboratory of Cellular Pharmacology of Atherosclerosis, University of Milan, Italy

06/2016 – 05/2018

Researcher in the Atherosclerosis group – Prof. Alberto Corsini & Dr. Stefano Bellosta

Identification of metabolic (glycolytic) inhibitors for treatment of angiogenesis. Experience with various in vitro assays using a variety of mammalian cell lines: gene expression, protein characterization, cell-based and biochemical assays for characterization of cellular activation/proliferation/apoptosis/cytotoxicity, functional assays for measuring migration and angiogenesis

PhD fellow, Industrial secondment

Biomax Informatics, Munich, Germany

03/2014 – 05/2016

Researcher - Dr. Dieter Maier

Design of disease map using new knowledge management system (BioXM™). Training in basics in programming and project management.

Research Assistant

Clinical Research Center, Dept. of Clinical Sciences, Lund University, Malmö, Sweden

10/2015 – 03/2016

Managed own project: "Role of Myd88 deletion on macrophage polarization and plaque stability". Assisted and performed assays for researchers. Experience in RNA isolation, RT-PCR, various immunoassays and analytical techniques: fluorescent and confocal microscopy. SOP development and optimization.

EDUCATION

Joint PhD, Pharmaceutical Sciences

University of Antwerp, Belgium

02/2016 – 02/2019

Thesis title: "Inhibition of Angiogenesis using Glycolysis Inhibitors: an in vitro study". Supervisor: Wim Martinet

Joint PhD, Pharmacological Experimental and Clinical Sciences

University of Milan, Italy

02/2016 – 02/2019

Thesis title: "Inhibition of Angiogenesis using Glycolysis Inhibitors: an in vitro study". Supervisor: Stefano Bellosta

MSc, Biomedical Sciences

Dept. of Clinical Sciences, Faculty of Medicine, Lund University, Sweden

01/2015 – 06/2015

Thesis title: "Role of Myd88 in plaque formation and stability in atherosclerotic mice". Supervisor: Harry Björkbacka

MSc, Biomedical Sciences

Dept. of Physiology, Faculty of Medicine, VU University, Amsterdam, the Netherlands

02/2014 – 08/2014

Thesis title: "Diaphragm muscle fiber contractility in mechanically ventilated ICU patients". Supervisor: Pleuni Hooijman

BSc, Biomedical Sciences

Clinical Laboratory, St. Lucas Andreas Hospital, Amsterdam the Netherlands

03/2013 – 07/2013

Thesis title: "Diagnostics of Haemoglobinopathies with the LightCycler® LC480 II". Supervisor: Martine Deckers Developed and optimized new protocol for detection of Haemoglobinopathies in the clinic.

LANGUAGES

English	Dutch	Persian
Fluent	Native	Advanced

PUBLICATIONS/CONFERENCES

- **Anahita Abdali**, Helena Macut, Sara Pellegrino, Carlo de Dominicis, Matteo Zanda, Maria Luisa Gelmi, Stefano Bellosta: "Novel MMP-inhibiting peptides for stabilizing atherosclerotic plaques" 85th European Atherosclerosis Society (EAS) Congress, Prague, Czech Republic: Science at a Glance, 23-27 April 2017 ; Oral presentation
- Stefano Bellosta, Alberto Corsini, Carlo de Dominicis, Matteo Zanda, Maria Luisa Gelmi, **Anahita Abdali**: "Novel compounds targeting PFKFB3, the key glycolytic enzyme, as a way to inhibit angiogenesis", SISA Palermo, Italy, 19-21 November 2017 ; Oral presentation
- S. Bellosta, A. Corsini, D. Baci, C. De Dominicis, M. Zanda, M. L. Gelmi, **A. Abdali**: "Angiogenesis inhibition with novel compounds targeting the key glycolytic enzyme PFKFB3" 86th Congress of the European Atherosclerosis Society EAS, Lisbon, Portugal, 5-8 May 2018 ; Oral presentation
- **Anahita Abdali**, Alberto Corsini, Denisa Baci, Carlo De Dominicis, Matteo Zanda, Maria Luisa Gelmi, Stefano Bellosta: "Inhibition of the Key Glycolytic Enzyme PFKFB3 with Novel Compounds Suppresses Vessel Sprouting", at AHA - Vascular Discovery: From Genes to Medicine, May 10-12, 2018, San Francisco, USA ; Poster presentation
- S.B. Nukala , O. Tura , L. Regazzoni , C. Marinello , **A. Abdali** , G. Aldini , V.F. Smolders, I. Blanco , J.A. Barbera , M. Cascante , M. Carini "Differentially Expressed Proteins in Pulmonary Endothelial Cells in Chronic Thromboembolic Pulmonary Hypertension" American Journal of Respiratory and Critical Care Medicine 2018; 197:A3738 ATS International conference, 18-23 May 2018, San Diego, USA ; Poster presentation
- Stefano Bellosta, Denisa Baci, Alberto Corsini, Carlo De Dominicis, Matteo Zanda, Maria Luisa Gelmi, **Anahita Abdali**: "Inhibition of the Key Glycolytic Enzyme PFKFB3 as a Way to Inhibit Angiogenesis", Knight Cardiovascular Institute, Oregon Health & Science University, Portland, USA, November 13th 2018; Oral presentation
- S. Bellosta, D. Baci, C. De Dominicis, M. Zanda, M.L. Gelmi, A. Corsini, **A. Abdali** "Glycolysis Inhibition with Novel Compounds Suppresses Angiogenesis" 32° Congresso Nazionale Società Italiana per lo studio dell'Aterosclerosi SISA, Bologna, Italy, 25-27 November 2018 ; Oral presentation
- **Anahita Abdali**, Denisa Baci, Carlo De Dominicis, Maria Luisa Gelmi, Alberto Corsini, Stefano Bellosta. Angiogenesis inhibition with selective compounds targeting the key glycolytic enzyme PFKFB3 ; Manuscript in submission

CERTIFICATES

Laboratory animal science for research workers, FELASA C