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Synergic effects of inhibition of glycolysis and multikinase receptor signalling on proliferation and migration of endothelial cells

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Abstract. Activated endothelial cells play a crucial role in the formation of new blood vessels, a process known as angiogenesis, which can underlie the development of several diseases. Different antiangiogenic therapies aimed against vascular endothelial growth factor (VEGF), the dominant pro-angiogenic cytokine, have been developed. Because the treatment is limited in its efficiency and has side effects, new approaches are currently being evaluated. One of them is aimed at blocking glycolysis, the dominant energetic pathway of activated endothelial cells during vessel sprouting. In the present study we investigated the efficiency of a combined strategy to inhibit glycolysis and block VEGF action on proliferation and migration in human endothelial cells. Human endothelial cells (HUVECs) were treated with different doses of the glycolysis inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) in combination with the multikinase inhibitor sunitinib l-malate. Our results show that HUVECs with reduced glycolytic activity are more sensitive to co-administered sunitinib. Analysis of post-receptor pathways controlling proliferation and migration of HUVECs showed suppression of phosphorylated PI3K/Akt and ERK1/2 after exposure to sunitinib but not to 3PO in 10 μM concentration. Our results suggest that simultaneous inhibition of energy metabolism and blocking of pro-angiogenic growth factor signalling pathways can be a promising strategy to inhibit the pathological form of angiogenesis.

Key words: Metabolism — Angiogenesis — Endothelial cells — 3PO — Sunitinib

Introduction

Angiogenesis, the process of creating new blood vessels from pre-existing structures, is involved in many physiological and pathological processes (Carmeliet and Jain 2011). Angiogenesis is a highly coordinated process: upon induction of sprouting by pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), quiescent endothelial cells become active and increase their level of glycolysis. Subsequently, the cells begin to proliferate, migrate and form new vessels (De Bock et al. 2013).

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The majority of ATP generated by endothelial cells for proliferation and migration is obtained from glycolysis (Xu et al. 2014). Despite the fact that endothelial cells are exposed to a high concentration of oxygen in circulating blood, they prefer glycolysis over oxidative phosphorylation (Gatenby and Gillies 2004). There are several reasons why endothelial cells favour glycolysis: 1) the consumption of oxygen by endothelial cells is not so high, enabling oxygen to diffuse to the surrounding tissues (Verdegem et al. 2014); 2) endothelial cells are exposed to lower levels of reactive oxygen species, so they are partially protected from oxidative stress; 3) endothelial cells can also migrate under conditions of hypoxia and are able to use glycolysis to form new vessels under hypoxic conditions (Mertens et al. 1990); 4) although ATP production *per* mole of glucose in oxidative phosphorylation is higher, ATP production in glycolysis is faster. The production of ATP during glycolysis

is comparable with oxidative phosphorylation as far as there is a sufficient amount of glucose, which is always present in circulating blood (Vander Heiden et al. 2009); 5) side branches of glycolysis are important for the biosynthesis of macromolecules (Leopold et al. 2003).

Antiangiogenic therapy is one of the supplementary strategies for cancer treatment and is aimed at inhibition of angiogenic signals, such as VEGF, leading to destruction of the vasculature and starving of the tumour (Gatenby and Gillies 2004). Inhibition of VEGF signalling using antibodies against VEGF (Ferrara et al. 2004) or VEGF receptor antagonists (Shaheen et al. 2001) proved its potent antiangiogenic effects. The first approved angiogenesis inhibitor was the humanized monoclonal VEGF-antibody Bevacizumab (Avastin, Pfizer). Bevacizumab binds VEGF and inhibits its interaction with the VEGF receptor, thus preventing cell proliferation and migration (Herbst et al. 2005). In addition to VEGF receptors, other tyrosine kinase receptors (platelet-derived growth factor receptor and fibroblast growth factor receptor) have important roles in tumour progression and blood vessel formation (Kerbel and Folkman 2002). Simultaneous blocking of several growth factors by multitargeted tyrosine kinase inhibitor sunitinib l-malate was shown as a potent antitumor and anti-angiogenic strategy (Mendel et al. 2003).

Blocking of VEGF has become a clinically attractive strategy, since it interferes with different cell control pathways. Activation of tyrosine kinase receptors leads to up-regulation of several post-receptor pathways, including the Ras/Raf/MEK/ERK1/2, which regulates proliferation and migration of endothelial cells (Gotink and Verheul 2010). Moreover, phosphatidylinositol 3′-kinase (PI3K) and its downstream activated serine/threonine kinase Akt/protein kinase B (PKB) are associated with several processes involved in angiogenesis control. This pathway includes endothelial cell migration, proliferation and survival (Engelman et al. 2006). However, both insufficient efficacy and the development of resistance limit the clinical use of VEGF-blocking therapy.

Increased levels of glycolysis during proliferation and migration of endothelial cells point to glycolysis as another attractive therapeutic target for the inhibition of angiogenesis (De Bock et al. 2013). Silencing *in vitro* or inactivation *in vivo* of the key glycolytic enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase-3 (PFKFB3) reduced the formation of new vessels (De Bock et al. 2013, Schoors et al. 2014).

The aim of our study was to explore the effects of simultaneous inhibition of glycolysis as the dominant metabolic pathway in activated endothelial cells together with inhibition of post-receptor signal cascades by the multiple-kinase inhibitor sunitinib. We hypothesized that such combined treatment can have a synergic inhibitory effect on the proliferation and migration of endothelial cells. Moreover, we analysed post-receptor signal pathways that mediated proliferation and survival of endothelial cells to identify which intracellular pathways are predominantly affected and might be targets for development of new inhibitors with higher efficiency and lower side effects when co-administered with glycolysis inhibitors. In our recent study (Murár et al. 2018) we proposed that 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) could be a multi-targeted inhibitor; therefore, the additional aim of the present study was to explore if it directly interacts with pathways controlling proliferation and migration of HUVECs.

Material and Methods

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium (ECGM; PromoCell, Germany), containing endothelial cell growth supplements (ECGS; PromoCell) and were maintained at 37°C in humidified incubator (Heal Force Bio-meditech, China) containing 5% CO_2. Cells were used between passages 1–6. HUVECs were isolated from fresh umbilical cords digested by collagenase. The umbilical vein was cannulated and rinsed with Earle's Balanced Salt Solution (EBSS). The rinsed vein was filled with 5 ml of collagenase NB4 (7 mg/ml) (Serva, Germany) dissolved in EBSS. After incubation for 20 min at 37°C cells were washed from the vein with Hank's Balanced Salt Solution (HBSS) and the suspension was spin at 300 × *g* for 15 minutes. Subsequently HUVECs were cultured in ECGM supplemented with ECGS and antibiotics (Biosera, France).

Drug preparation

Stocks solutions of tyrosine kinase receptors inhibitor sunitinib l-malate (Pfizer, USA) and glucose metabolism inhibitor 3PO, synthetized as described in Murár et al. (2018), were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) in a concentration of 10 mM. Subsequently, stock solutions were diluted in ECGM to required concentration. All inhibitors were used at concentration, which induced no cytotoxicity.

Cell proliferation assay

HUVECs were seeded in a 96-well plate at a density 5×10^3 cells/well. After the cells reached 80% confluence, the medium was removed, and the cells were treated with different doses of inhibitors. Cells were incubated in the absence (control) or in the presence of inhibitors for 24 hours. Cell proliferation was performed by the MTT assay (Sigma-Aldrich) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 590 nm (Elisa reader Elx800 ^{TM}; Bio-Tek Instruments, USA). Proliferation was evaluated as the percentage of absorbance of the samples treated with different doses of inhibitors compared with

untreated controls ($A_{590\ treated}/A_{590\ untreated} \times 100$). Data are presented as the mean ± standard error of the mean.

Migration assay

Cells were seeded in 24-well plates coated with 1.5% gelatine (Sigma-Aldrich) at a density 5×10^5 cells/well. After reaching a confluent monolayer, the medium was replaced with starvation medium, and the cells were incubated for further 17 hours. Each well was wounded using the tip of a pipette. Subsequently, the cells were incubated in starvation medium containing 20 ng/ml of $VEGF_{165}$ (Peprotech, USA) in the presence or absence of inhibitors for 8 hours. Migration of HUVECs was observed with an Olympus IMT2 inverted optical microscope (Olympus, Japan) and recorded by a Moticam 1000 camera system (Motic Incorporation, Hong Kong) at time zero and 8 hours after treatment. Changes in cell migration were evaluated by using the software Motic Images 2.0 ML (Motic incorporation).

Immunoblotting

For Western blot analysis, HUVECs were used at passage 1–2. Cells were cultured in 6-well plates at a density of 1.2×10^5 cells/well until they reached 80% confluence. Subsequently, cells were pre-incubated with medium containing the inhibitor 3PO at two different concentrations (10 and 20 μM) for 24 hours. Then, the medium was removed, and cells were incubated with different doses of 3PO and sunitinib for 1 hour. Afterwards, 2 μl of $VEGF_{165}$ were added to each well (stock solution of $VEGF_{165}$ 10 ng/μl; Peprotech). Following treatment, cells were lysed in a lysis buffer. The total protein concentration was determined using the bicinchoninic acid assay (BCA assay kit; Sigma-Aldrich). Equal amounts of protein were separated by SDS-PAGE (Owl P8DS; Owl Separation systems, USA) and transferred to a nitrocellulose membrane (Thermo Scientific, Germany). The membrane was blocked for 1 hour at room temperature using bovine serum albumin (BSA; Serva) to prevent nonspecific binding of antibodies. Afterwards, blots were incubated with anti-Akt, anti-phospho-Akt, anti-p 44/42 MAPK (ERK1/2) and anti-phospo-p 44/42 MAPK (ERK1/2) antibodies followed by incubation with goat anti-rabbit HRP and horse anti-mouse HRP secondary antibodies (all antibodies were obtained from Cell Signaling Technology, USA) at concentrations which were previously tested. For protein visualization, the ECL Substrate Clarity™ (BioRad, USA) was used. The ratio of phosphorylated to total forms of protein was determined using the software Image Studio Lite Ver. 5.2 (Li Cor, USA).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using STATISTICA 7.0 (StatSoft Inc.). Data were analysed by one-way ANOVA followed by a Tukey *post hoc* test. The value $p < 0.05$ was considered as significant.

Results

Effect of sunitinib and 3PO on cell migration

Cell migration ability was quantified after treatment with 3PO and sunitinib alone or in combination (Figure 1). Suni-

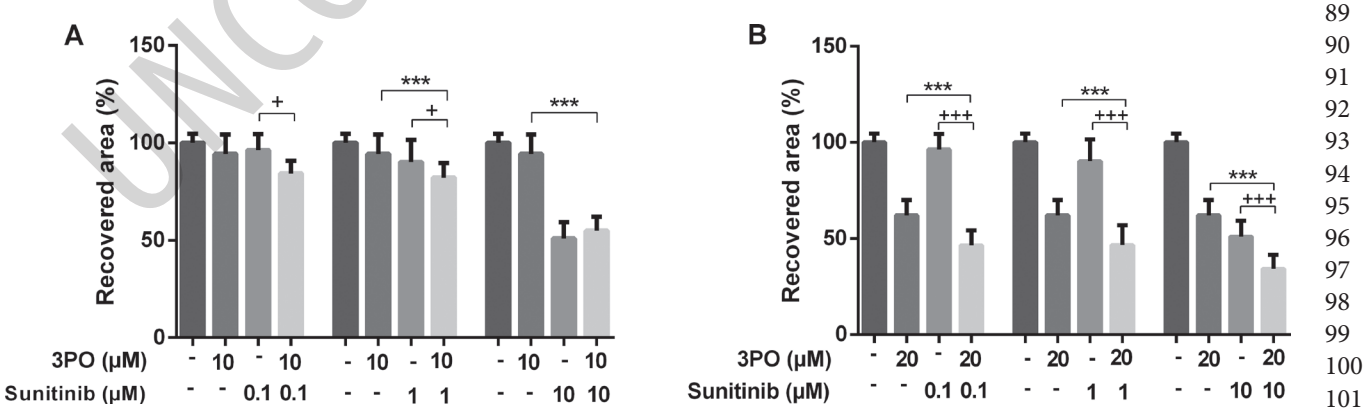

Figure 1. Inhibitory effect of sunitinib and 3PO on cell migration. Confluent cell monolayers were wounded, and endothelial cells were treated with vehicle (control), different concentrations of sunitinib and 3PO at 10 μM (**A**) and 20 μM (**B**). The wounded areas were photographed at the beginning and after 8 hours of incubation with inhibitors. Graphs represent the mean percentage of recovered areas ± SEM from three different experiments. $^{+}p < 0.05$ combined effect of sunitinib + 3PO compared to 0.1 and 1 μM sunitinib; $^{+++}p < 0.001$ combined effect of sunitinib + 3PO compared to sunitinib at 0.1–10 μM; *** $p < 0.001$ combined effect of sunitinib + 3PO compared to 3PO at 10 μM or 20 μM.

tinib at 0.1 μM had no effect, but higher doses decreased cell migration compared to control cells incubated in starvation medium supplemented with VEGF. At 20 μM, 3PO reduced cell migration (Figure 1B), but a lower concentration of 3PO had no effect on recovery of wounded areas (Figure 1A).

The combined action of 3PO with sunitinib in lower concentrations (1 μM) more efficiently inhibited cell migration in comparison with 3PO or sunitinib alone (Figure 1A). In contrast, the combined effect of 3PO and sunitinib at a higher concentration (10 μM) did not exhibit a synergistic inhibitory effect on cell migration compared to sunitinib alone. Interestingly, lower concentrations of sunitinib (0.1–1 μM) in combination with 3PO at 10 μM reduced cell migration into wounded areas. Simultaneous administration of sunitinib at 0.1–10 μM with 3PO at 20 μM significantly decreased HUVEC cell migration compared with inhibitors applied alone (Figure 1B).

Effect of sunitinib and 3PO on cell proliferation

Effects of both inhibitors on cell proliferation were assayed with 3PO at 10 or 20 μM and different concentrations of sunitinib. Treatment with 3PO at 10 μM did not affect cell proliferation (Figure 2A), whereas the higher dose of 3PO reduced cell proliferation (Figure 2B).

Treatment of cells with 3PO at 10 μM and sunitinib at 0.1–1 μM did not induce changes in cell proliferation, but the combined action of sunitinib and 3PO at 10 μM substantially decreased cell proliferation in comparison with cells treated with inhibitors applied individually (Figure 2A).

Administration of 3PO at 20 μM and sunitinib in the range of 0.1–10 μM negatively affected cell proliferation compared to the inhibitory effect of sunitinib or 3PO applied alone (Figure 2B).

Effect of 3PO and sunitinib on Akt and ERK1/2 phosphorylation

Changes in VEGF-induced phosphorylation of the PI3K/Akt and ERK1/2 signalling pathways were evaluated by Western blot analysis after treatment with sunitinib in the concentration range from 0.1 to 10 μM and 3PO at 10 and 20 μM administered alone or in combination. Inhibitors applied individually decreased VEGF-induced phosphorylation of protein kinase PI3K/Akt and ERK1/2 in a dose-dependent manner except 3PO at 10 μM. Simultaneous administration of 3PO at 10 μM and sunitinib in the range of 0.1–10 μM reduced phosphorylation of ERK1/2 (Figure 3A) and PI3K/Akt (Figure 3C). Interestingly, the combined effect of the two compounds did not decrease phosphorylation of PI3K/Akt and ERK1/2 compared with inhibitors applied individually (Figure 3). The inhibitor 3PO at 20 μM showed stronger negative effects on phosphorylation of PI3K/Akt and ERK1/2 after VEGF-induced phosphorylation (Figure 3B, 3D). Simultaneous treatment with sunitinib in the concentration range of 0.1–10 μM dose-dependently decreased phosphorylation of PI3K/Akt and ERK1/2 compared with the total protein form.

Discussion

In the present study, we explored the possibility of inhibiting pathological angiogenesis *via* suppression of glucose metabolism and blocking of growth factor receptors, either individually or simultaneously, as a novel antiangiogenic and cancer treatment strategy.

The inhibitory effect of 3PO on proliferation and migration in HUVEC cells was dose-dependent. Cells incubated in the presence of lower 3PO concentrations (≤10 μM) did

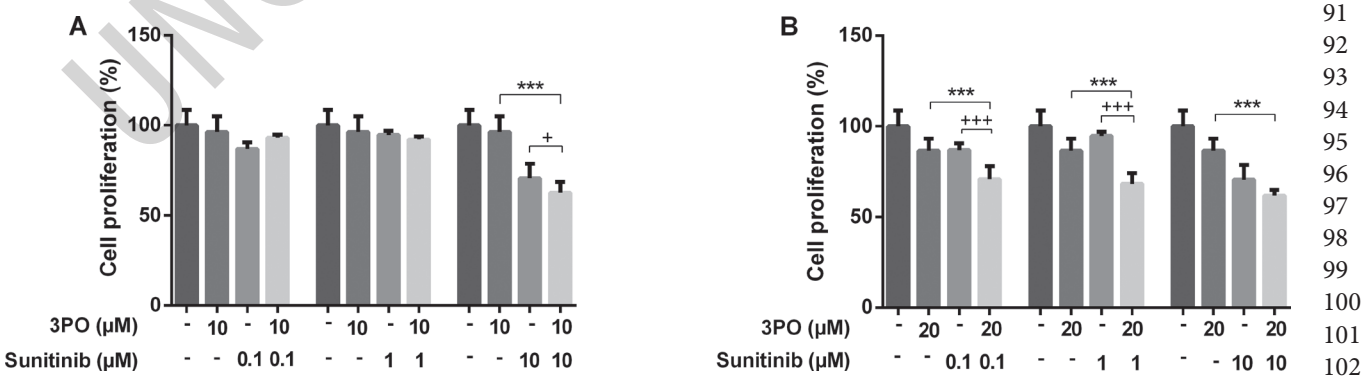

Figure 2. Inhibitory effect of sunitinib and 3PO on cell proliferation. After reaching confluent monolayer endothelial cells were treated with vehicle (control), 3PO at 10 μM (**A**) and 20 μM (**B**) and different doses of sunitinib for 24 hours. Changes in cell proliferation were evaluated after 4 hours of incubation with MTT. Data represent the mean ± SEM of three independent experiments. $^{+}p < 0.05$ combined effect of sunitinib + 3PO compared to 10 μM sunitinib; $^{***}p < 0.001$ combined effect of sunitinib + 3PO compared to 3PO at 10 μM or 20 μM; $^{+++}p < 0.001$ combined effect of sunitinib + 3PO compared to sunitinib at 0.1–10 μM.

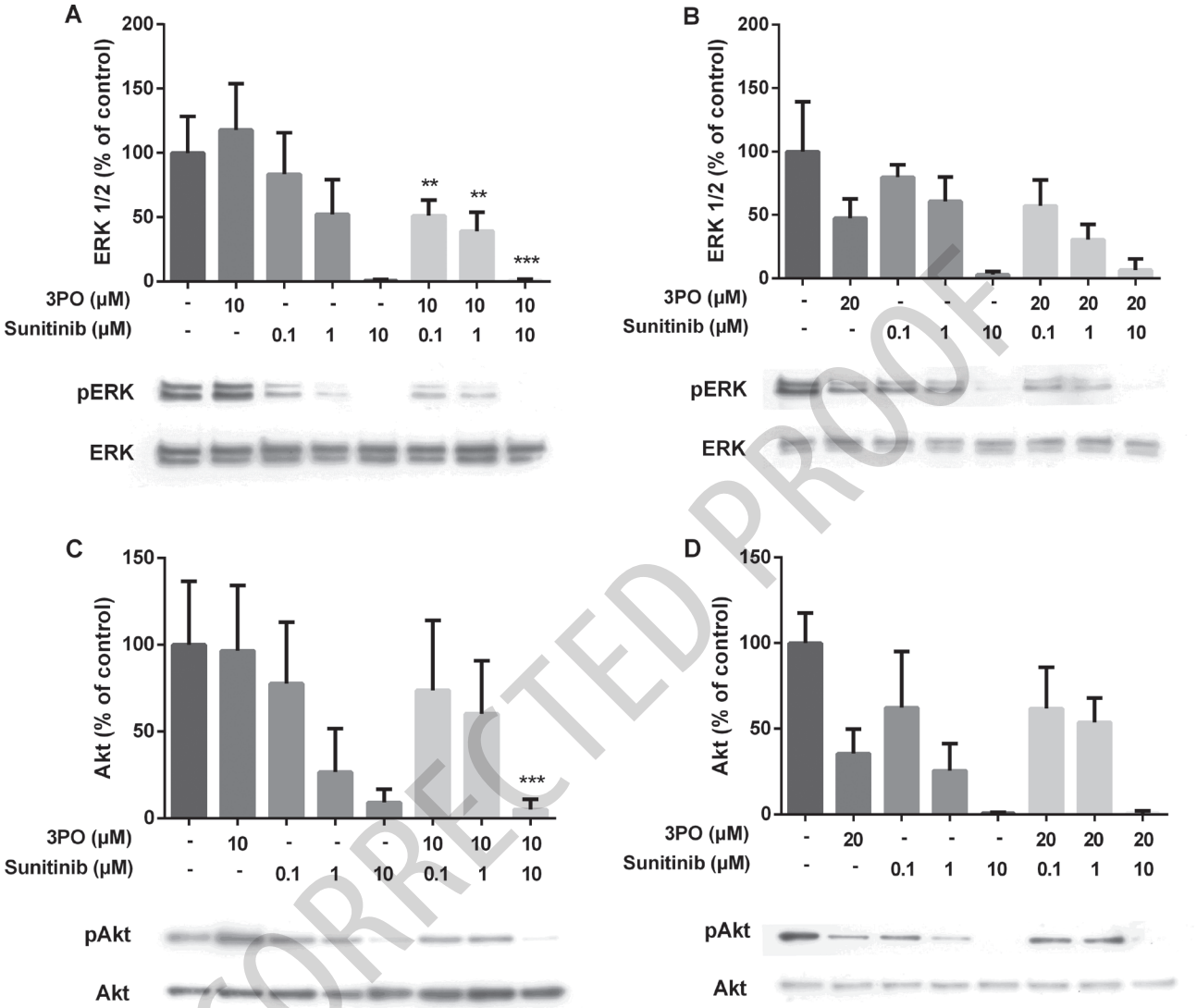

Figure 3. Quantification of PI3K/Akt and ERK1/2 phosphorylation after treatment with different doses of 3PO and sunitinib administered alone or in combination. The ratio between phosphorylated and total forms of PI3K/Akt (**A, B**) was evaluated as the mean percentage of groups ± SEM ($n = 3$). Western blots illustrating density of phosphorylated form compared with total form of Akt are shown below the graph. The ratio between phosphorylated and total forms of ERK1/2 (**C, D**) was evaluated as the mean percentage of groups ± SEM (n = 3). Western blots illustrating density of phosphorylated form compared to total form of ERK1/2 are shown below the graph. ** $p < 0.01$, *** $p < 0.001$ combined effect of sunitinib + 3PO compared to 3PO.

not show any morphological changes, and cell proliferation and migration were not suppressed. However, 3PO at a concentration of 20 μM efficiently inhibited cell migration, and the inhibitory effect was much more pronounced in comparison with 10 μM. Our data are in accordance with previous studies. The inhibitor 3PO in the concentration range of 15–20 μM led to a reduction of glycolysis in endothelial cells, resulting in decreased proliferation and migration (Schoors et al. 2014). Moreover, blockade of glycolysis by 3PO reduced vessel sprouting in zebra fish embryos by inhibiting endothelial cell proliferation and migration (De Bock et al. 2013). In our recent study (Murár et al. 2018), in which cell proliferation was estimated on the basis of bromodeoxyuridine incorporation into newly synthetized DNA in living cells, the inhibitory effects were observed even with lower doses of 3PO, probably reflecting the higher sensitivity of the assay in comparison with the MTT test.

The importance of glycolysis for energy metabolism of endothelial cells was documented in several previous studies (Clem et al. 2008; De Bock et al. 2013). The complete

inhibition of glycolysis using glycolysis inhibitors, such as 2-deoxy-D-glucose, results in cell death. However, blockade of the key glycolytic enzyme PFKFB3 leads to a reduction of vessel sprouting, while the cells remain alive (De Bock et al. 2013). The inhibitor 3PO lowers the activity of PFKFB3 and subsequently reduces glycolysis, resulting in suppression of endothelial cell growth (Clem et al. 2008; Murár et al. 2018).

Our results proved the expected inhibitory effects of sunitinib on endothelial cell migration and proliferation. Sunitinib administered in concentrations from 0.1 to 10 μM dose-dependently decreased the proliferation and migration of endothelial cells. These effects are in line with previously published data on endothelial and cancer cells (Mendel et al. 2003; Pla et al. 2014), as well as the clinical use of sunitinib in the treatment of solid cancers (Socinski et al. 2008).

In the present study, we explored if the simultaneous inhibition of glucose metabolism and growth factor receptor signalling has synergistic effects, is more efficient and enables the administered doses of both drugs to be decreased for clinical use. Indeed, we found that treatment with 3PO and sunitinib in combination resulted in more pronounced inhibition of HUVEC migration and proliferation, and the effect was enhanced when a higher dose of 3PO was administered. Our experiments demonstrated that the inhibitory effect of multikinase inhibitor sunitinib may be amplified by simultaneous inhibition of glycolysis. Endothelial cells treated with different doses of sunitinib in combination with 3PO (20 μM) significantly decreased their migration and proliferation. Several animal studies support the possibility that the simultaneous inhibition of growth factor receptors and glucose metabolism has the potential to be a new antiangiogenic strategy. Treatment of zebrafish embryos with 3PO and sunitinib at different doses resulted in vessel defects, suggesting that PFKFB3 blockade can enhance the antiangiogenic effect of VEGFR inhibition (Schoors et al. 2014). Moreover, potent anti-angiogenic efficacy was demonstrated for another multikinase inhibitor, nintedanib, in combination with the glycolysis inhibitor 3PO in a mouse model of breast cancer (Pisarsky et al. 2016).

From a translational point of view, it is important that cancer cells exhibit similar metabolic characteristics as activated endothelial cells and use predominantly glycolysis for their metabolism. Inhibition of the glycolytic enzyme PFKFB3 in cancer cells is responsible for decreased proliferation and migration (Conradi et al. 2017). A low dose of 3PO (25 mg/kg) reduced the level of glycolysis by 15–20%, caused tumour necrosis, negatively influenced cancer cell invasion, and induced normalization of tumour vessels. A high dose of 3PO (70 mg/kg) substantially impaired proliferation of cancer cells and subsequently increased cell death. Therefore, the combined inhibition of glycolysis and growth factor receptors can inhibit both cancer and activated endothelial cells and more efficiently inhibit tumour progression. Such a strategy enables decreasing the doses of administered drugs and affects cells which are resistant to a single treatment.

Signalling pathway

Activation of the protein kinases PI3K/Akt and ERK1/2 is necessary for regulation of cell proliferation and migration. In line with previously published data, our results showed that sunitinib significantly inhibited the phosphorylation of p-Akt and p-ERK1/2 in human endothelial cells (Moravčík et al. 2016). Moreover, our recent study (Murár et al. 2018) suggested that 3PO may inhibit other important biological targets in addition to PFKFB3. Therefore, we explored whether the simultaneous action of sunitinib and 3PO is mediated through the inhibition of phosphorylation of protein kinases PI3K/Akt and ERK1/2. Although sunitinib dose-dependently inhibited phosphorylation of protein kinase PI3K/Akt and ERK1/2, the simultaneous actions of both inhibitors did not always amplify the inhibitory effects on phosphorylation in comparison with the inhibitor applied individually. A similar stimulatory effect was observed in cancer cells. The selective tyrosine kinase inhibitor sorafenib applied at low concentrations (<1 μM) increased human bladder cancer cell proliferation and migration, which could be mediated through activation of the ERK1/2 signalling pathway (Rose et al. 2010).

Since simultaneous treatment with sunitinib and 3PO did not result in additional inhibition of PI3K/Akt and ERK1/2 phosphorylation, the effects of 3PO are not mediated *via* RISK pathways. Therefore, additional possibilities and other signalling pathways must be considered in future studies. In summary, our study confirmed the dose-dependent inhibitory effects of the glycolytic inhibitor 3PO and the multikinase inhibitor sunitinib on the migration and proliferation of endothelial cells. Simultaneous treatment with both inhibitors resulted in almost all cases a more pronounced decrease in cell migration and proliferation in comparison with individually administered drugs. Molecular data suggest that the higher efficiency of combined administration of these two inhibitors is not mediated by additional up-regulation of the PI3K/Akt and ERK1/2 signalling pathways, which are involved in the control of migration and proliferation of HUVECs. Our results indicate a novel strategy for inhibition of cell migration and proliferation with future prospects for cancer treatment.

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