

Current therapies of renal cell carcinoma (RCC), a highly vascularised tumour, mostly rely on anti-angiogenic treatment options. These include tyrosine kinase inhibitors (TKIs) and anti-VEGF monoclonal antibodies. Although these strategies aim at restraining vascularisation to control tumour growth, the effects of such therapies are much wider, as affecting the vessel structure deeply modifies the microenvironment of the tumour mass. The aim of this review is to provide an overview of current knowledge on the global effects of anti-angiogenic treatment, mostly TKIs, on the shaping of the immune component of the RCC microenvironment. The data supporting the modification of immunity by anti-angiogenic therapies are collected to reveal the potential of angiogenesis modulation as a strategy for the adjuvant anti-cancer approach in immunotherapy.

Key words: renal cell carcinoma, angiogenesis, tyrosine kinase inhibitors, vessel normalisation.

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Immune consequences of anti-angiogenic therapy in renal cell carcinoma

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Despite considerable advances in treatments, in most developed countries cancer is the second cause of death, and the occurrence of the disease is rising all over the world [1]. Renal cell carcinoma (RCC) is among the 10 most prevalent cancers, with increasing incidence worldwide. Additionally, it is a disease with a poor prognosis, as up to 30% of patients present metastases at the time of diagnosis and a further 20% will develop them despite treatment [2]. This cancer seeds mostly to the lungs, bones, lymph nodes and liver [3], and metastasis is the main cause of high mortality among RCC patients (50% of patients during 5 years after diagnosis) [4].

Renal cell carcinoma and angiogenesis

Renal cell carcinoma is considered a highly vascularised cancer, and recent advances in RCC biology have shown that angiogenesis is the key player in pathophysiology of this cancer [5, 6]. 60–80% of RCC tumours are characterised by a mutation of the *vhl* gene [7–9]. VHL, von Hippel-Lindau protein [10], is a tumour suppressor crucial for the hypoxia response pathways; it targets for degradation the α -subunit of hypoxia-inducible factor 1 α (HIF-1 α) upon normoxic/physioxic conditions. Partial pressure of oxygen (pO₂) varies in different tissues; the physiological level (physioxia) ranges from 1% in the skin to 10% in the kidney, and reaches 13% in arterial blood, yet is still below oxygen levels in atmospheric air (21%) [11]. Upon correct, physiological tissue oxygenation and constitutive expression of HIF-1 α , the factor is regulated by oxygen-dependent proteolysis. However, when oxygen is lacking (in a hypoxic microenvironment), HIF-1 α is not hydroxylated by prolyl hydroxylases (PHD), and cannot be recognised by active phosphorylated VHL (pVHL) and targeted for proteolysis, so it is stabilised and accumulates in the cell [12]. Acting as a transcription factor, HIF1 α activates the expression of many genes responsible for cell survival in low pO₂ and counteracting pathologic hypoxia. These include VEGF (vascular endothelial growth factor; regulator of angiogenesis), EPO (erythropoietin), glucose transporters (responsible for anaerobic glycolysis), TWIST and Matrix metalloproteinases (significant for epithelial-to-mesenchymal transition and metastasis), cadherins and stem-cell related markers (Oct4 and Notch) [13]. Increased secretion of VEGF and platelet-derived growth factor (PDGF) by cancer cells in hypoxia induces the formation and rearrangement of the host vasculature, which helps to sustain tumour growth and dissemination. They act as attractants for endothelial cells and induce their migration towards the tumour and subsequent proliferation [14]. Down-stream effects of HIF1 α activation and stabilisation are summarised in Fig. 1.

De-regulation of VHL in RCC tumours have a similar effect; even in the absence of hypoxia, HIF-1 α is not degraded, leading to activation of down-stream signalling as described above. Consequently, VHL truncated kidney

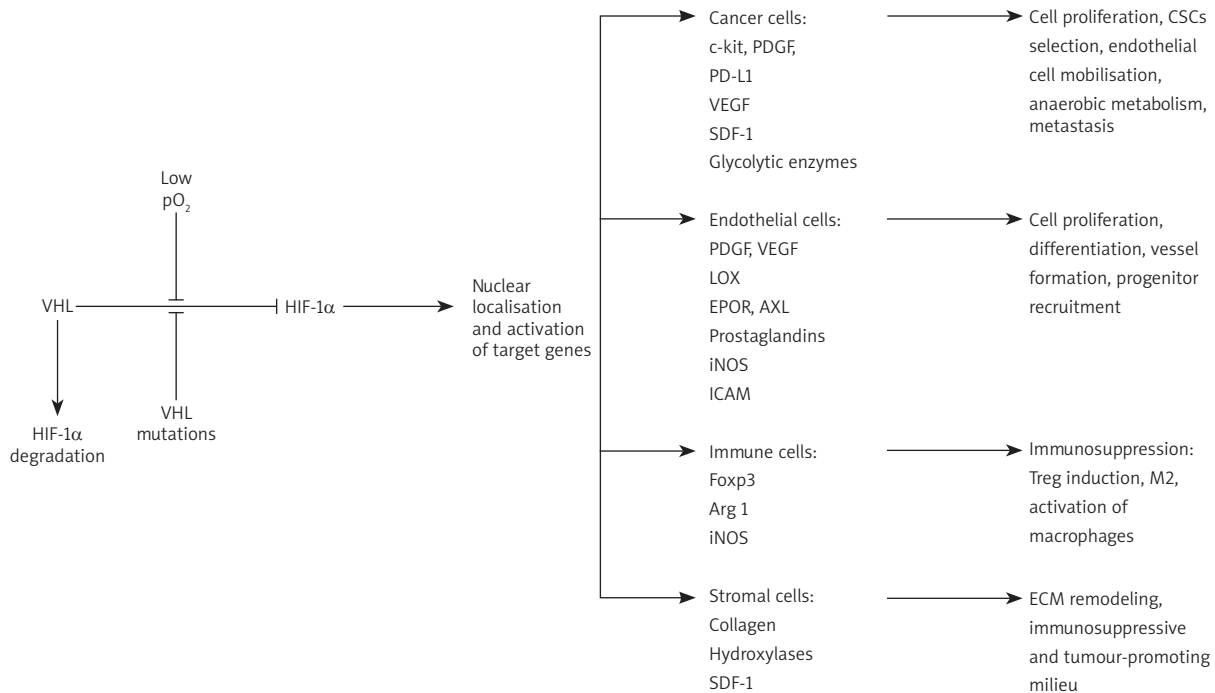


Fig. 1. Downstream effects of HIF1 α /VHL pathway

tumours are characterised by a high level of VEGF [15] and intense angiogenesis [16]. However, blood vessels in the tumour are highly abnormal; they are irregularly shaped and organised, prone to leakage and have disturbed blood flow [17]. In such a setting, the tumour is characterised by temporal and spatial heterogeneity in terms of blood flow, oxygenation and nutrient levels, creating a unique, cancer-promoting microenvironment [18]. Hypoxia, or pseudo-hypoxia related to VHL mutation, was shown to be a selection factor for cancer stem-like cells (CSCs), which are likely to represent the main driving force of cancer progression, relapse and resistance to therapies [19]. Additionally, leaky vessels facilitate cancer spreading; insufficient lining of the endothelium promotes tumour cell extravasation [20]. Additionally, malfunctioning endothelium compromises drug delivery due to incorrect blood perfusion of the tumour mass [21]. On the other hand, pathological vasculature restrains the proper migration of immune cells and the cancer microenvironment activates their suppressive phenotypes [22]. As the tumour microenvironment is now considered as the main driving force for cancer development as well as a potential means to “re-educate” tumour cells [23], pathological angiogenesis seems to be an interesting target for treatment. An indirect strike on the tumour can be sufficient to modify the processes of its growth and progression. Indeed, anti-angiogenic treatments, including tyrosine kinase inhibitors (TKIs), show significant efficacy in the clinic [24]. Many RCC patients benefit from anti-angiogenic therapy [25]; TKI treatment reduces tumour growth and vascular density [26, 27] and leads to prolonged survival [28].

Immunosuppression in renal cell carcinoma

Although RCC is to some extent an immunogenic tumour [29–31], immunosuppression in kidney cancer patients is frequent. The host is unable to develop an adequate immune response and immunity is abrogated by tumour-induced immunosuppression. This is mediated by multiple mechanisms with Treg lymphocytes and myeloid-derived suppressor cells (MDSCs) being the most widely studied in the RCC field.

Although data on Treg frequencies in RCC patients are mixed [32, 33], it was reported that CD4+CD25+Foxp3+ lymphocytes are induced in kidney cancer patients, both in blood and the tumour, which correlates with poor prognosis [33–36], and these cells possess a suppressive phenotype [37]. Kim *et al.* [38] reported that systemic Treg induction is characteristic for RCC patients with large tumours (> 7 cm), although intra-tumour presence of CD4+CD25+Foxp3+ cells was similar as in healthy kidney tissues. Tregs are important mediators of immunosuppression and mediate toleration to self-antigens in physiological conditions. However, as many cancer antigens are host proteins, activity of Treg cells strongly contributes to cancer immune evasion [39]. Treg cells produce regulatory cytokines, e.g. IL-10 and TGF- β , and directly influence other leukocytes. Treg cells were shown to hamper maturation of dendritic cells (DCs) (expression of co-stimulatory molecules) and therefore block their activator functions. What is more, Tregs suppress effector T lymphocytes (both CD4+ and CD8+), NK cells and other major players in anticancer immunity [40]. Interestingly, additional expansion of Tregs was observed in RCC patients in response to IL-2 treatment, which explains the weak efficacy of this thera-

peutic approach [36]. Treg induction in RCC can be a consequence of impaired DC activation [41] and it was shown that other CD4+ cell populations of kidney cancer patients tend to express a naïve/resting phenotype although they do respond to non-specific activation *in vitro* [42]. In RCC cases it was shown that circulatory Tregs can differ from their intra-tumoural counterparts, which had increased levels of HLA-DR, Fas, and GITR and simultaneously possessed higher suppressive properties [37]. Yet, other studies proved that blood Tregs from RCC cases are functionally suppressive and seem to be more resistant to apoptosis than the cells from healthy individuals [43]. Therefore, restriction of Treg suppressive action seems to be a promising strategy for RCC management, particularly in the case of metastatic disease. Ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) are monoclonal antibodies that target Treg-mediated immunosuppression [44]. In mRCC, immunotherapy with these drugs showed significant efficacy in clinical trials and may soon change the treatment paradigm in RCC. According to NCCN guidelines [45], anti-PD-1 antibodies are suggested as a second line treatment for RCC patients treated previously with other drugs; immunotherapy showed superior efficacy to mTOR inhibitor leading to prolonged survival [46]. What is more, anti-PD-1 therapy combined with anti-CTLA-4 antibodies was shown to be more effective first line treatment in comparison to TKIs [47]. This shows emerging role of immunotherapy and immune-modulating therapies for RCC control but also proves crucial role of Treg immunosuppression in the progression of the disease.

Target proteins of anti-CTLA4 and anti-PD-1 antibodies are immune checkpoint receptors and were shown to be negative Treg regulators. CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) is constitutively expressed in these cells and is dependent on Foxp3, a transcription factor that serves as a Treg marker both in mice and humans. CTLA-4 expression is crucial for Treg-mediated protection from autoimmunity; it is postulated that it may be a competitor to CD28 and reduce binding to co-stimulatory molecules on antigen-presenting cells (APCs), suppressing activation of naïve T cells [48]. Anti-CTLA-4 antibodies lead to activation of CD4+ and CD8+ cells regardless of specificity, promoting anti-cancer responses [49]. Also increased diversity of T cell receptors was reported in melanoma patients treated with anti-CTLA-4 antibodies, what may both mediate anti-tumour protection and toxicities. At the same time, anti-CTLA-4 therapy enhanced humoral response to vaccine antigens with no exacerbation of autoimmunity [50]. In cancer, CTLA-4 blockade cause reduction of suppressive T cell activity [51] and activate Th1 cell responses [52]. Programmed cell death-1 (PD-1; CD279) is present on effector T cells, while its ligand (PD-L1) can be found on various cells, including tumour cells. Interactions of these two molecules lead to inhibition of immune responses, both during natural tolerance and cancer immune evasion [53]. Blocking of PD-1/PD-L1 interaction by monoclonal antibodies stops negative signalling and restores T-cell responses; lymphocytes proliferate and produce IFN- γ and IL-2 [54]. Cancer-specific CD8+ lymphocytes expand upon PD-1 blockade in melanoma what coincides with in-

creased recognition and cytotoxicity against tumour cells [55]. Anti-PD-1 antibodies can modulate the immunological microenvironment in the tumour restoring protective immune responses; T cell infiltration of the tumour can be enhanced [56] with diminished Treg activity [57]. On the other hand, anti-PD-L1 therapy showed only moderate anti-cancer efficacy [58]. Importantly, PD-L1 is regulated by the HIF1 α pathway; it is overexpressed in hypoxia and in VHL-mutated RCC cells [59]. This directly links hypoxia and immunosuppression but also suggests that counteracting low pO₂ in tumours is a strategy to shape the anti-cancer immune response. Overall, blockade of CTLA-4 and PD-1 mediated Treg suppression is of great importance in restoring protective responses with proved clinical potential.

MDSCs are another population of cells with suppressive functions observed during RCC. These is a heterogeneous population of progenitors of mono- and granulocytes that are normally present in the bone marrow and blood to serve as a backup pool. However, during pathological states, such as cancer or chronic inflammation, these progenitors fail to mature but become activated and express a suppressive phenotype [60]. MDSCs suppress antigen-specific and non-specific activation of CD4+ and CD8+ lymphocytes in both a direct and indirect manner. T cells are arrested in G0/G1 phase of the cell cycle by depletion of L-arginine and cysteine from the microenvironment by extensive MDSC metabolism of these amino acids [61]. Also, leukocyte migration is affected, leading to reduced infiltration of the tumour with effector cells. Due to antigen-presenting abilities of MDSC, Treg cells can be additionally induced [62]. Production of IL-10 by MDSC further leads to an ineffective response, including M2 (or alternative) activation of macrophages [63]. This cell population is induced in blood and tumours of kidney cancer patients [64–67], and it was also reproduced in a murine model of RCC [68]. MDSCs were shown to accumulate in the tumour predominantly as compared to the healthy kidney tissues in a murine syngeneic model of kidney cancer. Moreover, both intra-tumoural and splenic cells express a suppressive phenotype [68]. The predominant subpopulation of myeloid suppressor cells in RCC patients comprises granulocytic MDSCs with enhanced arginase 1 activity [67]. In mRCC patients, the blood elevation of arginase 1 activity (low L-arginine, high L-ornithine levels) coincided with decreased ζ chain expression in T and NK cells. As *in vitro* depletion of MDSCs restores T cell responsiveness to anti-T cell receptor (TCR) stimulation, the suppressive role of these cells in RCC patients is significant [69]. Interestingly, granulocytic MDSCs from RCC were shown to be more resistant to apoptosis after activation in comparison to cells from healthy individuals [67], but they are susceptible to cell death mediated by IL-2/anti-CD40 treatment [70]. The ways in which RCC activates MDSCs are not yet elucidated; however, most likely they are induced by secretion of GM-CSF, M-CSF, IL-6, IL-10, TGF- β , COX-2 or PGE2, as shown for other cancers [61, 71]. Also VEGF blocks myeloid cell maturation, which may account for RCC induction of MDSCs [72]. High mobility group box-1 protein (HMGB1) expression in the tumour was linked with suppression by myeloid cells in RCC; blocking of the protein reduced the

progression of the disease by inhibition of MDSC induction [73].

Currently, studies performed on targeting MDSCs focus mainly on limiting their suppressive functions. PDE-5 inhibitors were shown to limit MDSC suppression in cancer by inhibition of arginase 1 and iNOS – enzymes whose activity mediates T cell suppression [74]. Also COX-2 inhibitors can be used to block PGE-2 production, which is responsible for MDSC expansion [75]. In RCC, another approach was also used: administration of retinoic acid in cancer patients, which led to differentiation of MDSCs and abrogated their suppressive phenotype [76, 77]. However, no immunotherapy based on direct counteracting MDSCs is currently used in the clinic on daily basis [78].

Additionally, RCC cells counteract protective immune responses directly by secretion of suppressive cytokines or expression of down-regulating receptors. Lymphocyte apoptosis induced by RCC cells through CD70 [79] or FasL mediated mechanisms [80] and the lack of co-activation molecules (B7-1 and B7-2) [81] contributes to ineffective immunity in kidney cancer patients. Mutation of *vhl* in RCC cells was also shown to reduce interactions of cancer and immune cells in a VCAM-mediated mechanism [82]. Immune escape is also related to disturbed antigen processing and presentation by cancer and APCs [83–86]. HLA-G, a non-classical MHC class I molecule, is frequently up-regulated in RCC [87], and this receptor protects cancer cells from recognition by immune cells and subsequently lysis [88]. Also IL-6, IL-10, TNF, TGF and VEGF secreted by RCC cells create a cytokine milieu that down-regulates immunity [89–91]. A non-protective response in RCC is also related to unfavourable immune bias – non-protective Th2 and Th17 tend to be elevated during kidney cancer [92], which is mediated, among other factors, by impaired DC activation [93].

Although limitation of tumour-induced immunosuppression seems to be a promising approach to treat cancer [94], immunotherapy is rarely treated as a single option [95]. Cancer is a complicated disease and it has to be addressed on multiple levels, as breaching subtle tumour-host interactions can lead to far-reaching consequences. Immunotherapy, like any other drug treatment, is limited by the accessibility of the tumour site where the cells to counteract are located. Changing the conditions of the vasculature by restraining the anarchic growth of inefficient vessels would help to obtain a properly functioning vascular network. This is a purpose of TKI treatment, which may change the tumour microenvironment and influence immune responses of the host and immunomodulatory action of the cancer cells. Therefore, this aspect of anti-angiogenic therapy can serve as a strategy to boost natural defence mechanisms of the host.

Anti-angiogenic therapy of renal cell carcinoma and changes in the immune response

When diagnosed early, over 50% of RCC patients are cured, but late stage disease has a poor prognosis. In the case of primary RCC, partial or radical nephrectomy without adjuvant therapy is a preferred treatment. With disease progression, apart from resection of metastatic

tumours, systemic therapy is implemented. Since 2005 targeted therapy is possible for advanced RCC, which up to now consists of 7 FDA-approved drugs, including: tyrosine kinase inhibitors (TKIs; sunitinib, sorafenib, pazopanib, axitinib), mTOR inhibitors (temsirolimus, everolimus), anti-VEGF antibodies and immunotherapy, as described earlier in this review. Additionally, combinational therapy and other targeted drugs, like cabozantinib, reported efficient in different cancers, are tested as second line treatment in mRCC patients relapsing after prior treatment [45, 96]. VEGF and many other growth factors bind to their receptors that contain tyrosine kinases in the intracellular domain that is responsible for transduction of the signal inside the cell [97]. Tyrosine kinase inhibitors are small molecules that target these intracellular domains of receptor and in-cell signalling molecules [98]. Drugs that are currently used in anti-RCC treatment and slightly differ in specificity, include sorafenib (targeting VEGFR-2, VEGFR-3), sunitinib (targeting VEGFR 1–3, PDGFR, KIT, FLT3), pazopanib (targeting VEGFR, PDGFR, KIT) and axitinib (targeting VEGFR 1–3, PDGFR, KIT) [99]. All these TKIs showed benefit in mRCC patients [100]. Despite the relatively large amount of data on TKI effectiveness in different patient groups [101–114] there are surprisingly few studies on the molecular effects of this treatment on the RCC tumour and its microenvironment.

Anti-angiogenic therapy shows a wide spectrum of effects. TKIs directly reduce tumour growth by inhibition of c-kit signalling in cancer cells of c-kit and PDGF signalling, thus regulating their proliferation [115]. At the same time, TKIs target endothelial cells by inhibition of VEGF signalling, which is crucial for recruitment of new vessels and modulates tumour vascularisation [116]. This restrains tumour expansion; its growth is tempered and angiogenesis is normalised. Blocked receptors fail to activate endothelial cells to form new vasculature in the tumour but also restore proper functions of existing cancer-induced blood vessels. Also, as a consequence of TKI treatment, tumour compactness is diminished, leading to better perfusion [116]. TKIs should then normalise the tumour microenvironment, leading to homogeneous conditions in the tumour, and reduce expression of growth factors, which may cause partial control of the disease. Additionally, TKI-mediated modification of cancer cells and angiogenesis affects the immune microenvironment; cancer-induced immunosuppression can be reversed, restoring host immunity [116]. When normal functions of blood vessels are regained, the tumour mass is again available for the action of immune cells [117] and other drugs when applied as adjuvant treatment [17]. As an example, it was observed that in cancer patients treated with TKIs, induction of immunosuppressive cell populations is reduced in comparison to untreated controls, which proves the additional benefit of anti-angiogenic treatment and makes it an adjuvant strategy for immunotherapy. Although the mechanism of this phenomenon is also weakly described, changes in the tumour microenvironment affect its immunogenic/immunomodulatory abilities (Table 1).

TKIs were shown to directly affect T cell functions, decreasing proliferation and inducing apoptosis [118], yet this study was performed on effector T cells and it is not certain that a simi-

Table 1. Immunosuppression mechanisms induced by RCC and affected by TKI treatment

Immunosuppressive mechanism	Observation in RCC patients	Effect of TKI
Treg induction	Increased percentage in blood [33, 36, 38, 121] Local accumulation in tumour [33] Unchanged accumulation [38]	Reduced percentage [66, 119, 132] Increased percentage [131] Reduced infiltration [120, 130]
MDSC induction	Increased percentage in blood [64–67] Local accumulation in tumour [64–67]	Reduced percentage [66] Reduced infiltration [122]
Th1/Th2 polarisation	Th2 biased responses [92, 93]	Restoration of Th1 immunity [66, 122, 126, 127]

lar effect is valid for Treg cells – especially as *in vivo*, TKI were shown to increase T cell mediated immunity in other cancers [116]. Sunitinib decreased the amount of circulatory [66, 119] and intra-tumoural Treg lymphocytes in RCC [120]. It was also observed to modulate Th2 immune bias and promote Th1-related responses [119] while decreasing regulatory cells [121]. Also the percentage of MDSCs in the blood of RCC patients dropped after 28 days of sunitinib therapy with a simultaneous increase in IFN- γ production in T cells [66]. In a murine model of kidney cancer it was confirmed that also intratumoural and splenic accumulation of MDSCs is reduced by sunitinib, and initial observations suggest that a similar phenomenon occurs in humans [122]. It can be mediated by Stat3 dependent inhibition of pro-angiogenic activity of MDSCs [123]. As VEGF receptors are expressed by MDSCs [124], a direct effect of TKIs on these cells is possible. Sunitinib was shown to impair proliferation and survival of MDSCs in murine cancer. However, similarly to tumour cells, this cell population may become resistant to TKIs [125]. Normal type DC distribution was recovered in RCC patients after sunitinib administration [126]. TKI therapy improved tumour infiltration with CD8+ cells [127] and increased T cell functions measured by the level of IFN [122], which supports the use of sunitinib in combination with immunotherapy.

Sorafenib's effect on the immune response in RCC is much less clear. The drug was reported to alter DC maturation, impairing T cell responses [128, 129]. Nonetheless, sorafenib treatment reduced the infiltration of the tumour with Treg cells [130] while others showed that this TKI augments Foxp3+ lymphocytes in peripheral blood [131]. Busse *et al.* [132] on the other hand observed diminished percentages of circulatory Tregs in sorafenib-treated RCC patients but no effect of the therapy could be seen on the level IL-10 or TGF β .

The systemic up-regulation of immunity during anti-angiogenic treatment of RCC is additionally striking, as TKIs were shown to have direct immunosuppressive properties ([133] for review). This suggests the predominance of the vessel normalisation effect on the overall microenvironment and disease progression. Moreover, another approach for anti-angiogenic therapy using anti-VEGF antibodies showed no effect on the development of immunosuppression as MDSCs were not affected by the treatment [67], proving the importance of the definition of the term anti-angiogenic strategies.

Conclusions

Although the nature of immunosuppression reversal observed in TKI-treated RCC patients cannot be fully ex-

plained, it seems that the drugs affect immune cells both directly, inhibiting tyrosine kinase pathways, and indirectly. This has deep consequences by modification of the malignant microenvironment. Overall, treatment with TKIs is one of the anti-angiogenic strategies because, by modifying endothelial cell growth, they reduce the anarchic angiogenesis and cause restoration of functional vasculature in the tumour. This leads to normalised pO₂ and improves cell metabolism, while phenotypically normalised endothelium regulates the functions of vessels. This enhances the efficacy of chemotherapeutics, radiotherapy and migration of immune cells. Nevertheless, the normalisation effect of TKIs is transient, and temporal TKI resistance in RCC is often observed [26, 134]; ultimately anti-angiogenic therapy leads to vascular regression and consequently hypoxia with all its cancer-promoting mechanisms [135]. Therefore, an innovative approach for the control of cancer growth could be persistent and stable vessel normalisation [136]. This could be achieved by several means, including enhanced O₂ transport by erythrocytes and/or targeted reduction of pro-angiogenic signals. Kieda *et al.* showed that administration of the allosteric haemoglobin effector myo-inositol-trispyrophosphate (ITPP) increases the oxygen level and blood flow in experimental settings of melanoma and breast cancer models [137, 138]. ITPP caused activation of PTEN in endothelial cells, which led to vessel repair, and the treatment caused suppression of HIF1 α -activated pathways and reduced the pro-angiogenic activity of the tumours. These results suggest that ITPP can be an interesting adjuvant molecule improving the vascular architecture and enhancing the efficacy of co-administered drugs. Additionally, the same group described a novel anti-VEGF therapy using epithelial precursor cells (EPCs) – tissue-specific cells which express soluble VEGF-R, consuming the excess VEGF [139]. sVEGF-R is under the control of the hypoxia response element (HRE) and thus is expressed only in hypoxic conditions. When the vessels are normalised, hypoxia is alleviated and the sVEGF-R is no longer produced. Such an approach also causes stable vessel normalisation and is tightly controlled by the treatment outcome. Re-organisation of the tumour epithelium results in proper vasculature that can mediate immune infiltration, hypoxia alleviation and drug transport. These and other strategies can potentially be an adjuvant strategy to RCC treatment. Stable normalisation of the tumour vasculature can be effective in restoration of the protective immune response, as seen in TKI-treated RCC patients, yet long-term effectiveness of the novel approach can be

more beneficial for patients, leading to prolonged control of the disease.

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