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Direct Effects of Anti-Angiogenic Therapies on Tumor Cells:

A Focus on the VEGF Signaling

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Abstract

Over the past decades, anti-angiogenic therapies (AAT) have shown promising results in the treatment of different malignancies. Unfortunately, resistance often develops and patients ultimately relapse. In an attempt to elucidate the causes of recurrence, most studies have focused on the tumor responses to the hypoxic conditions induced by AAT. However, strategies against those mechanisms of resistance are still failing to improve treatments. Furthermore, a potential direct impact of the AAT on tumor cells cannot be overlooked. For the first time, this review provides an overview of the different aspects of tumor cells' response to AAT. Conflicting data about the nature of this effect are discussed and reconciled, providing new insights on tumor recurrence to AAT.

Anti-Angiogenic Therapies Act on Tumor Cells, Independently of their Anti-Vascular Effects

Using Anti-Angiogenic Therapies for Cancer Treatment is of Limited Benefit

The growth of a tumor is strongly related to **neo-angiogenesis** (see **Glossary**) (1, 2), which has led to the development of anti-angiogenic therapies (AAT) that can kill cancer cells by ‘starving them to death’ (3, 4). Amongst the most well-known AAT is **bevacizumab** (see **Glossary**), a monoclonal antibody that neutralizes one of the main pro-angiogenic factors, the vascular endothelial growth factor-A (VEGF-A) (4, 5). Bevacizumab is a US food and drug administration (FDA) approved drug used for the treatment of metastatic renal cell carcinoma (mRCC), non-small cell lung cancer (NSCLC), metastatic colorectal cancer (mCRC) and recurrent glioblastoma (6-10). Receptor tyrosine kinase inhibitors (RTKIs), including sorafenib, sunitinib, pazopanib and axitinib, which inhibit the vascular endothelial growth factor-receptors (VEGF-Rs), platelet-derived growth factor-receptors (PDGF-Rs) and c-Kit activities respectively, have also been approved by the FDA for the treatment of mRCC (11, 12) (**Table 1**).

However, many patients become resistant to AAT and eventually relapse (3, 13, 14). Studies using mouse xenograft models have shown that hypoxia induced by AAT can promote the emergence of resistance mechanisms such as **vascular mimicry** (see **Glossary**), stromal cells infiltration or increasing tumor cell invasion (4, 15-18), which can lead to tumor recurrence (19-21) (**Figure 1**). However, considering the fact that new multi-targeted AAT are still failing to improve patients’ survival, it appears that a complete understanding of tumor response to such drugs is still missing.

Anti-Angiogenic Therapies Can Act Directly on Tumor Cells through Inhibition of Autocrine Signaling

Over the last decade, the role of angiogenic factors and associated receptors in tumor cells has also been investigated. In 2008, Knizetova et al. described a VEGF-mediated autocrine regulation of glioblastoma aggressiveness (22). The involvement of the VEGF/VEGF-R signaling in tumor growth/survival has also been implicated in other cancers including ovarian, CRC and small cell lung cancer (SCLC) (22-26). Based on these studies, it has been proposed that AAT could also have a direct effect on tumor cells, which can trigger resistance (4, 27-29). Nevertheless, the direct effect of AAT on tumor cells is still hardly understood. In addition, the

limited amount of studies around this research field can explain the lack of a review manuscript on this topic so far.

Hence, this article aims to provide a comprehensive overview of the literature about the unknown direct effects of AAT on tumor cells, their underlying mechanisms and their relevance in resistance. As using AAT remains a relevant and promising strategy to treat highly aggressive cancers and improve patients' quality of life (1), we believe that a precise description and a complete understanding of the consequences of the direct effect of AAT on tumor cells can bring new insights to counteract tumor refractoriness to AAT.

Conflicting Data about the Nature of the Direct Effect of Anti-Angiogenic Therapies on Tumor Cells

Expected Decrease of Tumor Cells' Survival in Response to Anti-Angiogenic Therapies

The 'predictable' direct anti-tumoral effect of AAT relies on the hypothesis that if pro-angiogenic factors and their receptors are expressed by tumor cells, neutralizing such factors or inhibiting these receptors is expected to suppress tumor growth and survival (4, 30-34).

Wedam's laboratory conducted one of the first studies demonstrating that bevacizumab has a direct effect on tumor cells (29). In tumor biopsies from breast cancer patients, they observed an increase of tumor cells' apoptosis following treatment with bevacizumab, even when the proliferation status remained unchanged. The authors attributed this effect to the disruption of VEGF-A paracrine (on endothelial cells) and autocrine activity (on tumor cells) (29). Similarly, another report demonstrated an Akt-dependent direct anti-tumor effect of bevacizumab on human multiple myeloma (MM) cell lines and primary cells through a decrease of tumor cells' viability and proliferation (35).

More recently, sunitinib has been shown to decrease viability and proliferation while enhancing apoptosis in human adrenocortical carcinoma cell lines (36, 37). In the same way, vandetanib (VEGF-R, epidermal growth factor-receptor (EGF-R) and RET inhibitor) was able to directly inhibit neuroblastoma cells' growth *in vitro* and tumor growth in a human neuroblastoma mouse model (38). Depletion of the VEGF-dependent autocrine signaling has also been reported to have an anti-tumor effect in a K5-son of sevenless (SOS)-dependent mouse skin tumor model (38, 39). According to those data, it appears that AAT are able to directly affect tumor cells, independently of their deleterious effects on endothelial cells.

Refractoriness of Tumor Cells upon Anti-Angiogenic Treatment

Despite promising data showing a direct anti-tumor effect of AAT on cancer cells, other reports have failed to demonstrate similar results. More precisely, *in vitro* studies showed no direct inhibitory effect of sunitinib, when used at clinically relevant concentrations, on the viability of metastatic cells from a renal cell carcinoma patient (40).

Interestingly, some cancer patients were totally unresponsive to anti-angiogenic strategies, without even a transitory benefit (3). Tumor cells, such as glioblastoma, can express a plethora of different growth factors (i.e. VEGF-A, epidermal growth factor (EGF), (tumor growth factor- β (TGF- β)) that can directly promote aggressiveness through autocrine mechanisms (22). Hence, for the same reasons that certain tumors can be insensitive to the anti-vascular effects of AAT, they can also be intrinsically resistant to a direct anti-tumor effect of AAT (4, 41). By comparing bevacizumab-resistant (HT-29) versus -sensitive (DLD-1) CRC cells, Mesange et al. suggested that the intrinsic resistance to bevacizumab could be associated with a pre-existing strong VEGF-VEGF-R signaling both *in vitro* and *in vivo* (41).

As proposed by Rahman et al., it is also possible that such intrinsic resistance to AAT could be linked to the presence of stem cells features, namely the expression of the membrane **ATP-binding cassette (ABC) drug transporters** (see **Glossary**) in cancer cells. By expulping drugs out of tumor cells, the ABC transporters can avoid the direct AAT anti-tumor effects (4, 42). Similarly, an increased lysosomal sequestration of sunitinib has been observed *in vitro* in human renal and colon cancer cells that are resistant to the drug, compared to the sensitive ones. In addition, prolonged exposure to sunitinib did not change the levels of p-Akt / p-ERK and the viability of sunitinib-resistant cells, further demonstrating the decreased effectiveness of the AAT (**Figure 2**) (43). Another group observed a similar lysosomal accumulation of sunitinib in mRCC cells, while sunitinib treatment was able to increase the expression of the ATP-binding cassette, subform B [MDR/TAP] member 1 (ABCB1), leading to the drug efflux from the tumor cells (44). Such a ‘defense mechanism’ against AAT could also be involved in patients’ refractoriness to drugs (4). Furthermore, increased expression of **autophagy** (see **Glossary**) markers has been reported in MM cells that did not respond to bevacizumab (35). Autophagy is a cell-protective mechanism that can render tumor cells resistant to AAT through the promotion of cell growth arrest. Consequently, when inhibiting the formation of autophagosomes involved in the autophagy process, a decrease in tumor cell viability could be observed in response to bevacizumab (35).

Hence, it appears that the AAT-mediated inhibition of a predominant autocrine signaling pathway in tumor cells, such as the VEGF/VEGF-R by bevacizumab or sunitinib, does not necessarily impede tumor cells' viability and aggressiveness. In addition, the failure of AAT in cancer patients followed by tumor recurrence has led to the consideration of a potential pro-tumoral effect of AAT on tumor cells.

Un-expected effect of Anti-Angiogenic Therapies on Tumor Cells

- Bevacizumab Decreases Tumor Cells' Survival only at Clinically Non-relevant Concentrations

In the case of bevacizumab, it is worth mentioning that studies showing a direct anti-tumor effect used very high drug dosages, which can be considered unsuitable for patients' treatment. For example, the anti-tumor effect of bevacizumab on MM cells *in vitro*, was obtained only with clinically un-relevant doses (2 mg/ml) (35). Similarly, *in vivo* experiments in animals bearing xenografts of the human glioblastoma cell line U87 revealed that only high doses of bevacizumab have anti-tumor activity independently of vascular regression (45). FDA-approved bevacizumab doses (5-15 mg/kg) correspond to a concentration between 100 ug/mL and 500 ug/mL in cell culture experiments (46). Therefore, surprisingly, other reports described a direct pro-tumoral effect of bevacizumab at 250 ug/mL and 100 ug/mL respectively (27, 47). Furthermore, these studies evaluated the chronic / long-term exposure of cancer cells to bevacizumab (3 months and 14 days respectively), which is more comparable / analogous to the long-term treatments of cancer patients.

This 'time-dependent' effect of bevacizumab has already been described in cancer patients that initially respond to the treatment but ultimately relapsed (3). Therefore, it seems possible that an initial anti-tumor effect of bevacizumab could be quickly replaced by a direct pro-tumoral effect as a result of newly developed resistance mechanisms.

- Increase of Tumor Cells' Aggressiveness in Response to Anti-Angiogenic Therapies

The hypothesis of a pro-tumoral direct effect of AAT has emerged mainly during the last five years. Studies have argued that the unexpected resistance to AAT cannot be solely due to a response to the inhibition of the tumor vascularization, especially when considering the failure of strategies targeting mechanisms of hypoxia-mediated escape (30, 32). Consequently,

some reports proposed that AAT could eventually promote tumor cells' aggressiveness (1, 48, 49), contradicting the logical view to date of a direct anti-tumor effect of such therapies.

Different groups have previously evaluated the migration and invasion properties, gene/protein expression and phosphorylation status of tumor cells treated with AAT (21, 50). Studies have shown that AAT can enhance the expression and activation of alternative pathways stimulating tumor cells' invasion, survival and aggressiveness in the 'absence' of their targeted factor(s) (47, 48). Bevacizumab has been shown to stimulate the expression of tumor-growth associated factors such as matrix metalloproteases (MMPs), basic-fibroblast growth factor (b-FGF) or interleukins in human glioblastoma cells *in vitro*, while promoting their invasion capabilities both *in vitro* and *in vivo* in a glioblastoma xenograft mouse model (48). Moreover, an upregulation of VEGF-C and VEGF-D followed by increased cell proliferation has been noticed in human glioblastoma cell lines following a 14 days-treatment with bevacizumab (47). Similarly, expression levels of VEGF-A and VEGF-Rs could be stimulated *in vitro* following treatment with bevacizumab, as shown in human bladder cancer cell lines (46). In line with these findings, another report documented that chronic inhibition of tumor cell-derived VEGF-A, using an anti-human VEGF-A antibody, decreases hypoxia-induced apoptosis, promotes the expression of VEGF-A, placental growth factor (PlGF), VEGF-R1 and VEGF-R2, while it enhances the ability of human colorectal cancer cell lines to form **spheroids** (see **Glossary**) *in vitro* (51). The authors suggested that the over-expression of the VEGF/VEGF-R signaling in response to anti-VEGF-A treatment might contribute to drug resistance (51). Furthermore, Tomida et al. proposed that a direct effect of bevacizumab and foretinib, a RTKI targeting Met and VEGF-R2, on human colorectal cancer cells can confer resistance to hypoxia-induced apoptosis (52). Interestingly, they also observed that both therapies could enhance the expression of VEGF family members in tumor cells (52).

At the same time, it has been reported that the expression of stromal derived factor-1 α (SDF-1 α) and its receptor (CXC chemokine receptor type-4 (CXCR-4)) were increased in human rectal cancer cells from patients' biopsies following a 12 days-treatment with bevacizumab (33). As SDF-1 α can promote cell invasion and survival by binding to CXCR-4 (4), the authors concluded that a SDF-1 α / CXCR-4-dependent mechanism could underlie the increased tumor aggressiveness observed in cancer patients following bevacizumab treatment (33).

Collectively, based on these results, it appears that tumor cells, such as glioblastoma or colorectal, are able to develop escape mechanisms to the inhibitory activity of AAT on pro-

tumoral signaling. Interestingly, in those cases, AAT can actually directly promote tumor cells' invasiveness and aggressiveness.

- Mechanisms Underlying the Pro-Tumoral Effect of Anti-Angiogenic Therapies on Tumor Cells

Deciphering the mechanisms through which AAT can promote tumor cells' aggressiveness is still in its infancy, making this unexpected effect difficult to understand. Recently, our work on human glioblastoma cells brought new insights for describing the intracellular downstream signaling pathways involved in glioblastoma cell response to the direct pro-tumoral effects of bevacizumab (50). Using a tridimensional hyaluronic acid (HA) hydrogel, we showed an increase of glioblastoma cells' invasion following treatment with bevacizumab. We observed that this effect could be due to an activation of the tumor growth mediators, Akt and extracellular signal-regulated kinase (Erk). Additionally, we noticed an over-expression of PlGF and VEGF-R1 in response to therapy. According to the *in vitro* studies cited above and our own data, we proposed that bevacizumab could directly promote glioblastoma cells' invasion through the establishment of a new PlGF-dependent autocrine loop activating the Akt and Erk pathways (**Figure 3**) (27, 50). Similarly, a recent phosphoproteomic profiling reported that sunitinib can enhance the activation of the Axl and p21 activated kinase (PAK) signaling in human renal cell cancer cells (53). Furthermore, others have reported an increase of ERK1/2 phosphorylation in human adrenocortical carcinoma cells following sunitinib treatment (54).

Unfortunately, the origins of such escape mechanisms are still unclear. Feedback mechanisms could be activated in tumor cells as a result of neutralization of an important pro-tumoral autocrine signaling (i.e. VEGF/VEGF-Rs) by AAT (50). According to this hypothesis, it has been suggested that inhibiting the VEGF signaling could have an “antiangiogenesis-independent effect” on human colorectal cells leading to increased expression of the hypoxia-inducible factor-1 α (Hif-1 α). Hence, it was also observed that such a Hif-1 α up-regulation could promote VEGF family members expression in cancer cells in response to the anti-VEGF antibody (51).

Other mechanisms have been proposed to explain the direct pro-tumoral effect of AAT *in vitro*. A recent report described an *in vitro* proliferative effect of bevacizumab on human malignant melanoma cell lines (55). The authors suggested that bevacizumab could form a

complex with VEGF-A and VEGF-R2 at the cell surface, leading to VEGF-R2 clustering and subsequent auto-activation (55). They also proposed that the auto-activated VEGF-A-bevacizumab-VEGF-R2 complex can be internalized into tumor cells, promoting melanoma cell proliferation even in the presence of bevacizumab (55). Conversely, other reports oppose this theory suggesting that bevacizumab is unable to enter cells (41).

Moreover, recent studies proposed that the interactions between VEGF-R2 and MET on the cell surface could underlie the direct pro-tumoral effect of AAT (56). Indeed, it has been demonstrated that activation of MET, which is involved in invasion, is inhibited in cells that also express VEGF-R2 (23). Interestingly, in human glioblastoma cells, VEGF-R2 and MET have been observed to physically and mechanistically interact with each other: upon VEGF-R2 activation by VEGF-A, a non-receptor protein tyrosine phosphatase 1B (PTPB1) can be recruited to the VEGF-R2/MET complex, de-phosphorylating MET. Consequently, VEGF blockade or VEGF-R2 inhibition may promote MET activation and invasion of tumor cells in their micro-environment (23), which could explain the direct pro-tumoral effect of AAT targeting VEGF-A and VEGF-R2 (51, 57). Nevertheless, such a theory is conflicting to the well-described pro-angiogenic and pro-tumoral role of VEGF-A during tumor growth. Accordingly, Lu et al. observed that VEGF-A has an inhibitory effect on human glioblastoma cells' invasiveness through the VEGF-R2/MET association, despite being a well-described pro-angiogenic factor *via* its action on endothelial cells that express VEGF-R2 (23). The authors then suggested that VEGF-A can promote or inhibit different cell signals depending on the molecular partner involved in a complex with VEGF-R2 (23).

Based on these data, it appears that various mechanisms can trigger the unexpected direct pro-tumoral effect of AAT on cancer cells. Consequently, a full description of the underlying phenomenon that could link the neutralization of a pro-angiogenic factor, or the inhibition of a RTK, with an increase of tumor cell aggressiveness is still needed.

Specific Treatment and Cancer Growth Conditions Affect the Tumor Cells Response to Anti-Angiogenic Therapies

Receptor Tyrosine Kinase Inhibitors vs Neutralizing Agents

It is worth mentioning that most of the studies reporting a direct anti-tumor effect of the AAT are based on data obtained with treatments targeting receptors of pro-angiogenic factors, such as RTKIs. On the contrary, the direct pro-tumoral effects of an anti-angiogenic strategy

are mostly revealed in studies using **neutralizing agents** (see **Glossary**), such as bevacizumab or aflibercept, when focusing on the VEGF-A. In human glioma and melanoma cells, it has been observed that sunitinib decreases cell proliferation while bevacizumab enhances it (27, 55, 58). Such drugs have a larger field of activity, neutralizing different signaling pathways at once. For example, sunitinib can inhibit VEGF-R, PDGF-R and c-Kit activation simultaneously in human cancer cells (59). Consequently, the observed higher efficacy of RTKIs on the inhibition of cancer cells' aggressiveness could be due to their capability to counteract alternative autocrine and paracrine signaling in tumor cells, compared to neutralizing agents which can target only one signaling pathway at a time (1, 3).

Various responses of tumor cells to Anti-Angiogenic Therapies according to the cell origin

As the mechanisms of tumor development and neo-angiogenesis vary depending on the cell type of origin, so are the mechanisms of resistance to different therapies. As it happened with cancer patients, some tumor cell types are sensitive to AAT, others are intrinsically indifferent while certain ones can become more aggressive during prolonged AAT (42). For instance, human breast cancer cells are sensitive to a direct anti-tumor effect of AAT while gliomas or colorectal cancer cells are more prone to an increasing aggressiveness in response to AAT (27, 29, 47, 51, 60, 61). Furthermore, even for the same tumor cell type, the nature of response to AAT could also be patient-dependent. For example, bevacizumab has been shown to increase PTEN-negative/VEGF-R2-positive human glioblastoma cell lines' invasiveness compared to PTEN-positive/VEGF-R2-negative glioblastoma cell lines (57). Consequently, it seems that tumor cells can have many different responses to the direct effect of AAT according to the tumor cell type, location and grade (1).

The effect of Anti-Angiogenic Therapies on tumor cells communication with their microenvironment

The direct effect of AAT, whatever its nature is, cannot be studied separately from the tumor microenvironment it arises from. As explained in this review, tumor-associated stromal cells (i.e. endothelial) and tumor cells share many common signal transduction pathways. Therefore, through paracrine and autocrine mechanisms, these signaling pathways are responsible for the intra-communication between vascular and tumor cell compartments (1, 62), which can be altered in response to AAT. In addition, it is worth noticing the similarity between

the mechanisms described in response to the effect of AAT on tumor vasculature and those observed in response to a direct AAT tumoral effect (27, 63). Consequently, it appears essential to consider the consequences to both the direct and anti-vascular effects of AAT as part of a whole integrating mechanism. The recently described **extracellular vesicles (EVs)** (see **Glossary**) might explain one of the plausible ways through which tumors communicate with their stromal counterparts by carrying lipids, proteins and RNAs, from one cell to the other (62). During the last years, the involvement of EVs in tumor development and metastasis has been thoroughly considered and it appears that they can be key elements in processes such as neo-angiogenesis (64). Recently, we suggested that changes in EVs' quantity and content following treatment with AAT could alter the tumor cells / endothelial cells interactions and thus partly drive resistance to AAT (62, 65).

Concluding Remarks

Overall, when targeting essential growth factors such as the VEGF-A, we need to consider the consequences of AAT not only on the vascular compartment itself (main target) but on the whole tumor system as well. The research field related to AAT resistance is quite new and still remains unexplored. To the best of our knowledge, this is the first review to summarize and organize published data about the direct effects of AAT on tumor cells and therefore it can be supportive and helpful for future *in vitro* and *in vivo* studies related to this topic (**Figure 4, Outstanding Questions, Box 1**). In aggregate, deciphering the direct effect of AAT on tumor cells will bring new insights in the aim to establish future combinatorial and personalized therapeutic strategies.

Conflict of Interest

The authors declare no conflict of interest.

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Figure captions

Fig.1: Tumor Resistance Mechanisms to Anti-Angiogenic Therapies

As a result to anti-angiogenic therapies (AAT) treatment, blood vessel network is reduced and normalized. A new hypoxic condition can therefore arise in the tumor, leading to an increase of neo-angiogenesis pathways, alternative mechanisms of neo-vascularization, recruitment of bone marrow derived endothelial cell precursors and myeloid cells, cell survival mechanisms such as autophagy and tumor cell invasiveness.

Dll4-Notch: Delta-like ligand 4-Notch; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; PDGF: Platelet derived growth factor; VEGF: Vascular endothelial growth factor.

Fig.2: Tumor Cell Resistance to Sunitinib by Lysosomal Sequestration

Sunitinib is able to inhibit the vascular endothelial growth factor (VEGF) receptors' tyrosine kinase activity. Consequently, sunitinib can reduce the activation of Akt and extracellular signal regulated kinase (Erk) signaling pathways that result in decreased cell proliferation *in vitro*. Nevertheless, in sunitinib-resistant cells, it appears that the drug can be sequestered in lysosomes, leading to a reduced effectiveness of sunitinib.

Erk: Extracellular signal regulated kinase; VEGF-R: VEGF-Receptor.

(Gotink et al., 2011)

Fig.3: Direct Effect of Bevacizumab on Glioblastoma Cells *In Vitro*

Changes in the expression profiles of components of the vascular endothelial growth factor/ vascular endothelial growth factor-receptor (VEGF/VEGF-R) pathway has been observed in glioblastoma cells *in vitro*. Bevacizumab directly acts on glioblastoma cells by activating the Akt and Erk survival signaling pathways. Bevacizumab also enhances proliferation and invasiveness of glioblastoma cells. The direct pro-tumor effect of bevacizumab on glioblastoma cells could be due to changes in the VEGF-A-dependent autocrine loop as well as in the intracellular survival pathways, leading to the enhancement of tumor aggressiveness.

Erk: Extracellular signal regulated kinase; PlGF: Placental Growth Factor; VEGF: Vascular endothelial growth factor; VEGF-R: VEGF-Receptor.

(Simon et al., 2014)

Fig.4: Direct Expected Anti-Tumoral Effect of Anti-Angiogenic Therapies vs Direct Unexpected Pro-Tumoral Effect of Anti-Angiogenic Therapies

Recent data are conflicting about the real nature of the direct effect of anti-angiogenic therapies (AAT) on tumor cells. In certain types of tumor cells (e.g. breast cancer cells), such drugs can have an anti-tumoral effect decreasing cell survival. In other tumor cell types (e.g. glioblastoma), AAT can increase cell aggressiveness. The potential consequences of targeting

the vascular endothelial growth factor/ vascular endothelial growth factor-receptor (VEGF-VEGF-R) axis using bevacizumab and sunitinib are shown here.

Erk: Extracellular signal regulated kinase; b-FGF: basic-Fibroblast growth factor; MMP: Matrix metalloproteases; SDF-1 α : Stromal derived factor-1 α ; VEGF: Vascular endothelial growth factor; VEGF-R: VEGF-Receptor

Vascularised tumour

Anti-angiogenic therapy

Reduction and normalization of
blood vessel network =
transient hypoxia

Resistance mechanisms to anti-angiogenic therapies

1- Alternative pro-angiogenic pathways:
angiopoietins, FGFs, PDGF, EGF, HGF,
VEGFs, Dll4-Notch...

**5- Tumour cell adaptation to
energetic stress**
e.g. autophagy

Endothelial cell

Endothelial-like tumour cell

**2- Neo-angiogenesis independent
mechanisms of blood vessels
formation**
e.g. vascular mimicry

3- Escape from hypoxia
vascular co-optation, invasion of the healthy tissue

**4 – Involvement of tumour-associated
stromal cells**
e.g. recruitment from the bone marrow
of CD11b+Gr1+ myeloid cells promoting
angiogenesis

Sunitinib sensitive tumor cell

Sunitinib resistant tumor cell

Name	Active Substance	Targets	Tumour	Authorization (year)		Resistance
				FDA	EMA	
Avastin	Bevacizumab	VEGF-A	Non-Small-Cell Lung Cancer, Colorectal Cancer, Renal Cell Carcinoma Ovarian Cancer, Breast Cancer* Glioblastoma #	2004	2005	Colorectal Cancer, Glioblastoma
Sutent	Sunitinib	VEGF-R, PDGF-R, c-KIT, RET	Gastrointestinal stromal tumour, Metastatic renal-cell carcinoma, Pancreatic Neuroendocrine tumour	2006	2006	Gastrointestinal stromal tumour, Renal Cell Carcinoma, Neuroendocrine tumor
Nexavar	Sorafenib	VEGF-R, PDGF-R, c-RAF, b-RAF	Renal Cell Carcinoma, Hepatocellular Carcinoma, Differentiated thyroid carcinoma	2005	2006	Hepatocellular Carcinoma Thyroid carcinoma
Caprelsa	Vandetanib	VEGF-R, EGF-R, RET	Medullary Thyroid Cancer	2010	2012	Medullary Thyroid Cancer
Votrient	Pazopanib	c-KIT, FGF-R, PDGF-R, VEGF-R	Renal Cell Carcinoma*, Soft Tissue Sarcoma	2012	2010	<i>Data not yet available</i>
Vargatef , Ofev	Nintedanib	VEGF-R, FGF-R, PDGFR	Non-Small-Cell Lung Cancer*, Idiopathic pulmonary fibrosis	2014	2014	<i>Data not yet available</i>
Lenvima	Lenvantinib	VEGF-R2, VEGF-R3	Differentiated thyroid carcinoma	2015	2015	<i>Data not yet available</i>

*EMA only, # FDA only

Table 1: Some current AAT: applications and tumor resistance EMA: European Medicine Agency; FDA: (US) Food and Drug Administration; EGF-R: Epidermal growth factor receptor; FGF-R: Fibroblast growth factor receptor; PDGF-R: Platelet derived growth factor receptor; RAF: Rapidly Accelerated Fibrosarcoma; VEGF-A: Vascular endothelial growth factor-A; VEGF-R: VEGF-Receptor.

BOX 1: OUSTANDING QUESTIONS

- What are the molecular markers that can describe the tumor cell types whose invasion is directly inhibited by anti-angiogenic therapies (AAT)?
- Is the nature (inhibition or activation) of the direct effect of AAT on tumor cells dose-dependent?
- As in the vascular compartment, does a short time-window exist during which AAT can have a direct inhibitory effect on tumor cells before promoting the emergence of escape mechanisms enhancing tumor cells' invasion?
- What are the consequences of such direct effects of AAT on tumor cells interactions with their microenvironment including stromal cells and the tumor vasculature?
- What are the mechanisms underlying the direct unexpected pro-tumoral effect of AAT on tumor cells such as colorectal cancer cells and glioblastoma cells?
- How the unexpected direct pro-tumoral effect of AAT on tumor cells can be inhibited?

TRENDS BOX

- Anti-angiogenic therapies (AAT) improved cancer patients' progression free survival and quality of life. Nevertheless, resistance mechanisms are still limiting the efficacy of AAT.
- Autophagy, ATP-binding cassette (ABC) transporter-dependent drug efflux and lysosomal sequestration allow tumor cells to escape the deleterious direct effects of AAT *in vitro*.
- Bevacizumab (anti-vascular endothelial growth factor-A (VEGF-A) humanized monoclonal antibody) activates pro-invasive mechanisms in human glioblastoma and colorectal cancer cells *in vitro*.
- Tumor cells could develop resistance mechanisms in response to a long exposure to AAT. These mechanisms can be added to those developed in response to the hypoxic condition caused by the anti-vascular effects of AAT.
- In the aim to fully describe the resistance mechanisms to AAT, effects of those drugs have to be elucidated not only in their main target, the tumor vasculature, but the whole tumor growth system, including tumor cells themselves.

BOX 2: CLINICIAN'S CORNER BOX

- The activity of anti-angiogenic therapies (AAT) is not limited to the tumor vasculature compartment and can have direct effects on tumor cells as well. These direct effects on tumor cells have to be considered during patients' treatments since their real nature (tumor inhibition or activation) is not elucidated yet.
- In addition to their inhibitory effect on tumor vasculature, receptor-tyrosine kinase inhibitors such as nintedanib seem to be more effective in inhibiting tumor cells' invasion than other types of inhibitors such as anti-circulating agents' antibodies (i.e. bevacizumab). This could be due to the wider impact of receptor tyrosine kinase inhibitors (RTKIs) that can inhibit several targets at the same time.
- The nature of the direct effects of AAT on tumor cells could be cancer type dependent. As an example, AAT seem to directly inhibit breast cancer cells' invasion while increasing glioblastoma cells' aggressiveness.
- In the case of drugs for which an unexpected direct pro-tumoral effect has been observed, it could be possible that a short therapeutic window exists during which the therapy does have an inhibitory effect on tumor cells, before promoting the emergence of compensatory mechanisms that enhance tumor cells' aggressiveness.
- For all the aforementioned reasons, in the future, it would be interesting if we could control these direct effects of AAT on tumor cells by setting up multi-targeted / personalized therapeutic strategies, using biomarkers that reflect the response of tumor cells to those drugs.

GLOSSARY

Anti-angiogenic therapies (AAT): Therapies targeting molecular mediators involved in neo-angiogenic process. AAT can trap/neutralize pro-angiogenic factors secreted by tumor and endothelial cells (i.e. bevacizumab, aflibercept, cilengitide...), or inhibit the activity of tyrosine kinase receptors for the pro-angiogenic factors (i.e. sunitinib, cabozantinib...).

Angiokinease: Receptor tyrosine kinase involved in angiogenesis process (i.e. receptors for vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)).

ATP-binding cassette (ABC) drug transporters: Proteins that can transport a large variety of molecules (proteins, sugars, drugs...) through the cell membrane in an ATP-dependent way. ABC drug transporters are also involved in cancer resistance by promoting the efflux of molecules out of tumor cells.

Autocrine loop: an autocrine loop is set up when a molecular mediator can act and trigger intracellular changes within the cell in which it was produced / released. For example, VEGF that is produced / released by a cancer cell can bind VEGF receptors at the surface of the same cancer cell and activate tumor survival and invasion pathways.

Autophagy: an intracellular self-destructive process that results in the degradation of components (i.e. proteins or organelles) in order to maintain the cell energetic status. Autophagy is very useful during cancer growth, especially in case of stress conditions such as hypoxia.

Bevacizumab: (trade name: Avastin, Roche®) humanized monoclonal antibody targeting and neutralizing VEGF-A.

Cytostasis: Arrest of cell growth.

Direct effect of AAT on tumor cells: capability of the AAT to directly affect tumor cells through the inhibition of autocrine loops depending on pro-angiogenic factors in tumor cells. This direct effect is defined as strictly independent from the AAT effect on the tumor vasculature.

Extracellular vesicles: small (10-1000 nm) membrane-enclosed vesicles containing lipids, proteins and RNAs, produced by cells in secretory micro-vesicular bodies or through cell membrane blebbing. Vesicles content can be carried from one cell to another for short or long distances mostly through blood circulation. Cancer cells produce big amounts of extracellular vesicles.

Hypoxia: a high decrease of the oxygen supply in a tissue, leading to cell death. During tumor growth, hypoxia can arise in the center of the tumor mass due to poor oxygen diffusion.

Neo-angiogenesis: emergence of new blood vessels from pre-existing ones. During tumor growth, the neo-angiogenesis process is triggered by hypoxia arising at the center of the tumor bulk. Thus, neo-angiogenesis allows the tumor to get its own vasculature, grow more than 2mm in diameter and increase its invasion capabilities.

Neutralizing agent: Agent “trapping” a circulating factor to avoid any binding of this factor to the tumor cells, thus inhibiting its activity. For instance, bevacizumab and aflibercept can neutralize/trap VEGF-A, keeping it away from binding to VEGF-Rs.

Pro/Anti-angiogenic factors: molecular mediators involved in regulating the neo-angiogenesis process. Pro-angiogenic factors promote neo-angiogenesis while anti-angiogenic factors inhibit this process. Different cell types express angiogenic factors, including normal stromal cells, endothelial cells and tumor cells. During tumor growth, the expression of pro-angiogenic factors is increased by hypoxia, stimulating the neo-angiogenesis process.

Spheroid: tridimensional structure formed *in vitro* by cells growing in a low attachment cell culture model or in a tridimensional matrix cell culture model. A spheroid-based system allows the observation of direct cell-cell interaction and extra-cellular matrix formation in conditions close to what can be observed *in vivo*.

Vascular mimicry: capability of tumor cells to form blood vessel-like structures without the presence of endothelial cells.