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## Improving drug penetration in tumors by targeting tumor vascularization

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**Abstract** Clinical oncologists have not been paying enough attention to the fact that poor tumor penetration represents a major impediment to the efficiency of cancer chemotherapeutics. The cytokine tumor necrosis factor (TNF) was the first treatment shown to affect tumor vessel destruction and improve vascular permeability of drugs in a clinical setting. TNF produces an early increase of vessel permeability followed by a dual targeting: TNF induces apoptosis of intratumoral angiogenic endothelial cells and melphalan during the apoptosis of tumor cells. Given the systemic toxicity, TNF has to be administered by regional therapy. However, experimental data indicate that a systemic approach will be possible, thanks to TNF targeting. Three fusion proteins with TNF were shown to target tumors: anti-EDB fibronectin/TNF, Asn-Gly-Arg-TNF, and anti-gp240/TNF. Current approaches to blocking angiogenesis include strategies that target vascular endothelium growth factor (VEGF)-A. However, little attention has been paid to possible drawbacks, which may include vessel destruction and reduced penetration by chemotherapeutic agents administered simultaneously or subsequently. An antiangiogenic treatment is optimal when it is given at a critical dose and at a critical time. Current protocols seem not to take these prerequisites into consideration. Other new approaches to increased tumor vessel permeability include histamine and combretastatin analog. The current paradigm of antitumor strategy based

on the synergism of empirical drug combinations is obsolete. Instead, the design of protocols based on new pharmacodynamic concepts should provide better efficiency of cancer treatment as exemplified in the use of TNF and anti-VEGF antibody.

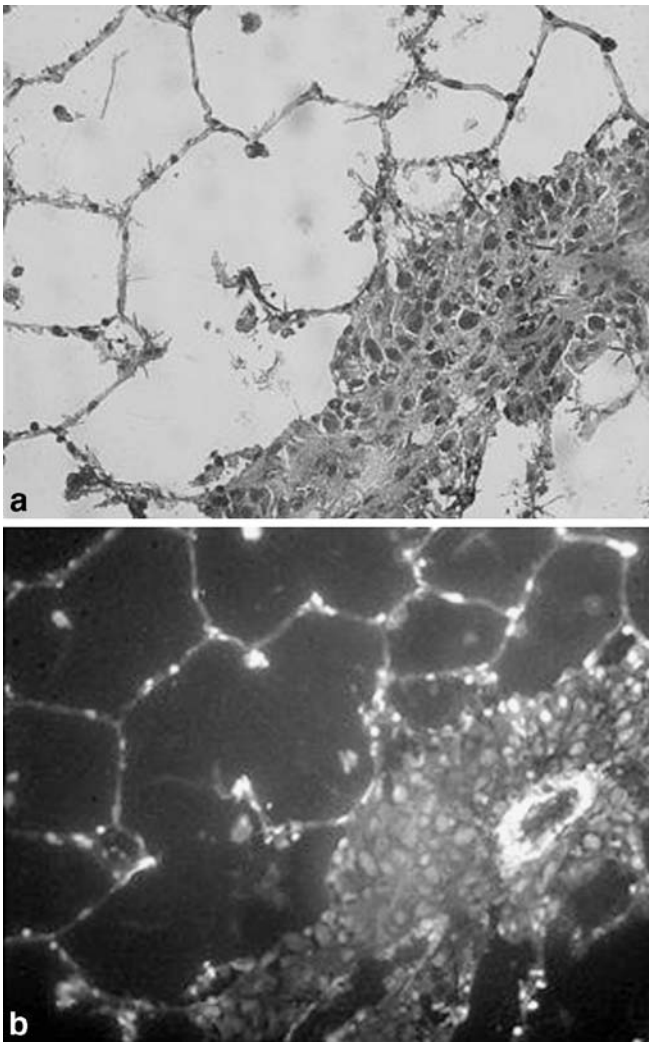
**Keywords** Tumor vessels · Permeability · TNF · Anti-VEGF · Chemotherapy

### Introduction

A quick survey of the literature shows that little fundamental progress in cancer chemotherapy has been achieved over the last 20 years. This unfortunate state of affairs may reflect the complexity of the tumor environment and our failure to target both cancer cells and their associated stroma that includes angiogenic vessels. Although the idea of the cancer cell as a “seed” that can only develop if it is supported by a good “soil” (the stroma with connective tissue and angiogenic vessels) is an old one (see recent retrospective by Fidler [1]), traditional therapies fail to account for the fact that anticancer agents must pass through the soil before they can destroy the seed. Thus, for a long time, clinical oncologists were not paying enough attention to the fact that poor tumor penetration represents a major impediment to the efficiency of cancer chemotherapeutics. A typical example is the lack of penetration of doxorubicin [2] as illustrated by its detection by fluorescence in the tissues (Fig. 1). The pioneering work of R. Jain [3] shed a new light on tumor vascularization and fluid biology. It shows that the major impediments to drug penetration in tumors are the lack of permeability and the increased interstitial pressure. It is possible to reverse the situation by acting on endothelial cells in the angiogenic vessels. The two most explored ways to do so are the use of the cytokine tumor necrosis factor (TNF) and antivascular endothelium growth factor (VEGF) strategies.

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**Fig. 1** Lack of doxorubicin penetration in sarcoma. Fischer rats bearing syngeneic soft tissue sarcoma lung metastasis were submitted to isolated lung perfusion with doxorubicin. After killing the rats, their lungs were removed and analyzed by photonic and fluorescence microscopy. Sample: sarcoma metastases surrounded by normal lung tissue assessed by **a** (top) hematoxylin & eosin staining, and **b** (bottom) fluorescence microscopy, demonstrating that doxorubicin is localized in normal tissue, including the wall of the tumor-feeding vessel, but not in the tumor itself [40]

## TNF- $\alpha$

The cytokine TNF was the first treatment shown to affect tumor vessel destruction and improve vascular permeability of drugs in a clinical setting [4, 5]. The cytokine was named after its property to produce hemorrhagic necrosis in experimental tumors but the clinical application of TNF as systemic treatment was rapidly abandoned because it exerts only a relatively weak antitumor effect and because as a mediator of septic shock, it is poorly tolerated.

To circumvent this problem, in 1988, we had the idea of using high-dose TNF in isolated limb perfusion (ILP) in combination with chemotherapy for locally advanced melanomas and sarcomas of the limbs. ILP consists of the surgical isolation of the limb vasculature connected to a

heart–lung machine, allowing the continuous circulation of high doses of cytotoxic agents over a short period of time (90 min). After perfusion, the vascular space is extensively rinsed and the vasculature reestablished (reviewed in [6, 7]). Our protocol (Fig. 2) includes the administration of high-dose TNF (3–4 mg/limb), interferon (IFN) gamma (0.2 mg/limb), followed after 30 min by melphalan (10–13 mg/1 limb volume) (TIM-ILP) under mild hyperthermia (38–40°C). In advanced melanoma and soft tissue sarcoma, TIM-ILP produces extensive tumor necrosis in the absence of significant local and systemic toxicity. This treatment, we found, yields a very high complete response (CR) rate in melanoma: 70 to 90% CRs in-transit metastases [8, 9] (Fig. 3). In soft tissue sarcomas that are not extirpable, TM-ILP produces 18 to 45% CRs and saves 80% of patients from amputation [10, 11]. Angiographic and histological studies revealed that its effect was due to selective destruction of the tumor-associated vessels and that vessels in normal tissues were spared [4] (Fig. 4). Human recombinant (tasonermin) is now registered in Europe for the treatment of advanced sarcoma of the limbs by ILP and is also registered for in-transit melanoma metastases in Switzerland.

These clinical results inspired experimental studies, in a bedside-to-bench transfer, and it emerged that either systemic or intratumoral application of TNF can increase tumor microvasculature flow and permeability, and hence, penetration of antibodies in a mouse colon cancer xenograph model [5] (Fig. 5). In a rat limb soft tissue sarcoma and osteosarcoma models, it was found that perfusion with TNF selectively enhances melphalan and doxorubicin penetration into tumors but not in muscle tissue [12, 13] (Fig. 6). TNF also reduces IFP, as evidenced by an increase in capillary filtration with no effect on capillary tone [14]. Taken together, these results indicate a selective effect of TNF on angiogenic endothelial cells.

The antitumor vasculature activity of TNF involves two events. First, a rapid increase (within minutes) in the permeability of the tumor vasculature, which favors the selective accumulation of the chemotherapeutic agent in the tumor tissue. The molecular mechanisms leading to

## rTNF +melphalan (TM)+IFN $\gamma$ (TIM) via Isolated Limb Perfusion

Day 0	Day +1	Day +2
<p><b>ILP</b></p> <p>*Continuous leakage monitoring; max 10%</p> <p>*TNF 3–4 mg, 90'</p> <p>*IFN<math>\gamma</math> 0.2 mg, 90'</p> <p>*Melphalan 10–13 mg/L, 60'</p> <p>*Hyperthermia 38–40°C</p> <p>*Washout 4–6 Litre</p>	<p><b>Post op period</b></p> <p>*Fluid loading</p> <p>*Vasopressive amines if necessary</p>	

**NB: IFN omitted in EMEA registration.**

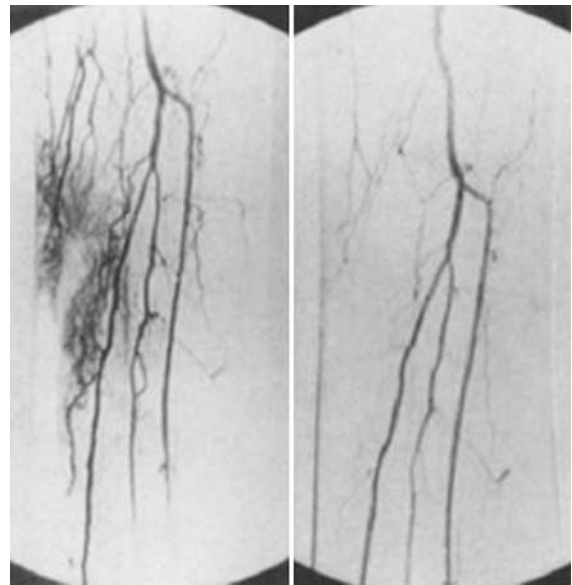
**Fig. 2** Schematic protocol description of TNF/IFN $\gamma$ /melphalan-isolated limb perfusion (TIM-ILP)



**Fig. 3** Efficacy of TIM-ILP in melanoma. Male, 50 years old, proximal melanotic melanoma in-transit metastases on left thigh. **a** Day of TIM-ILP and **b** day 26 after perfusion. Nearly complete response. Pigmentation indicates the area of high melphalan concentration during ILP, fortunately corresponding to tumor distribution

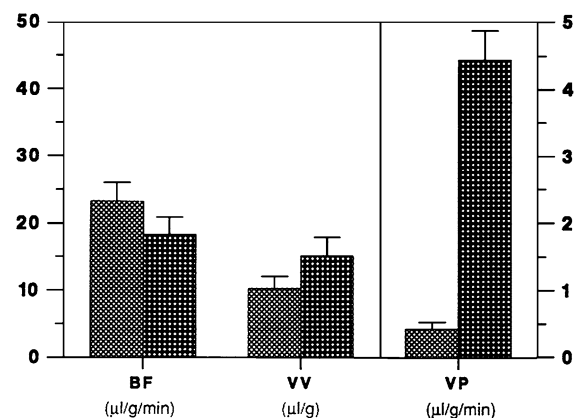
enhanced permeability involve remodeling of the cytoskeleton and redistribution of the cell–cell adhesion molecules PECAM-1 and VE-cadherin (reviewed in [7]). Second, TNF elicits late (within hours) cytotoxic damage to the tumor endothelial cells. Thus, the effectiveness of this therapy is due to the sequenced administration of TNF, followed 30 min later by melphalan for an additional time of 60 min. TNF produces an early increase of vessel permeability followed by a dual targeting: TNF induces apoptosis of intratumoral angiogenic endothelial cells and melphalan the apoptosis of tumor cells.

Our earlier ILP results in melanoma and sarcoma showed that TNF can increase the therapeutic efficiency of chemotherapeutic agents, even those of marginal activity. For example, melphalan, a drug with no activity in soft tissue sarcoma, can produce 20% CRs in large sarcomas of the limbs when administered with TNF. These events are presented in a tentative model (Fig. 7).



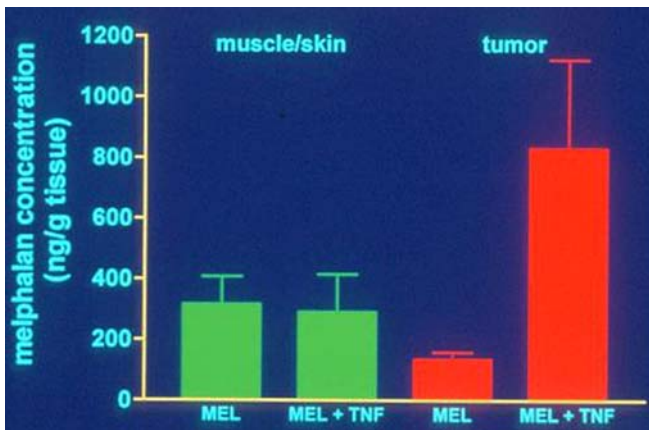
**Fig. 4** Selective tumor vessel destruction by TIM-ILP. Female, 76 years old, extensive second recurrence of malignant fibrohistiocytoma in the anterior aspect of the leg. Angiogram before (*left*) and 7 days after (*right*) TIM-ILP. Histological assessment on large biopsy taken 30 days after treatment confirmed complete response. Twenty-three months after ILP, patient was alive with no sign of recurrence nor metastasis. (Reprinted with permission from D. Lienard et al., *J Clin Oncol* 10(1):52–60. Copyright 1992 [4])

An explanation for this remarkable selectivity for growing but not quiescent vascular tissues came from *in vitro* and *in vivo* studies on endothelial biology. TNF, in combination with IFN, deactivates the integrin  $\alpha V\beta 3$ , which is only expressed by angiogenic endothelial cells. Because this integrin is essential for proliferation and survival, treated angiogenic endothelial cells fail to adhere to extracellular matrix proteins in the microvessels, and they undergo massive apoptosis [15–17].



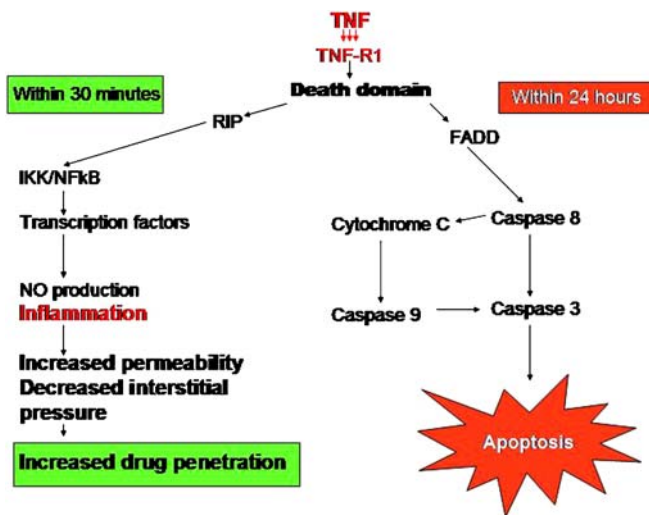
**Fig. 5** TNF increases tumor blood vessels permeability. Subcutaneous LoVo human colon carcinoma xenografts were submitted to intratumoral injection of 2  $\mu\text{g}$  of TNF. Vascular permeability (VP) in the tumors increased eight to tenfold 1 h after injection of TNF compared to control tumors injected with saline. There was no significant change in blood flow (BF) nor in vascular volume (VV). (Reprinted with permission from S. Folli et al., *Int J Cancer* 53 (5):829–836. Copyright 1993 [5])





**Fig. 6** TNF increases chemotherapy penetration in tumors. Rats bearing syngeneic soft tissue sarcoma on the leg were submitted to ILP with melphalan with or without TNF. After the procedure, measurements of melphalan were made in normal tissues and in the tumors. While there was no change in normal tissues, there was a sixfold increase of melphalan uptake in tumors. (Reprinted with permission from Macmillan Publishers Ltd: J. H. de Wilt et al., *Br J Cancer* 82(5):1000–1003. Copyright 2000 [12])

Is there a prospect for the systemic application of TNF? Several attempts were made to improve the local delivery of TNF in tumors and to decrease the systemic exposure to the cytokine. Repeated intravenous injection of stealth



**Fig. 7** Hypothetic model of the two distinct effects of TNF on angiogenic endothelial cells after high dose of TNF administered by ILP. Upon binding of TNF to TNF receptor p55/TNFR-1 and activation of death domain, two different signaling pathways are activated. *Left*: within 30 min of cleavage and activation, RIP phosphorylation leads to I transcription factors, including inducible NO synthase and NO production. The inflammatory response (see text) leads to increased permeability and decreased interstitial pressure. This results in increased drug penetration. *Right*: after 24 h, FADD activation and loss of AKT activation—due to inhibition of  $\alpha V\beta 3$  integrin—results in caspases activation and apoptosis of angiogenic endothelial cells. In parallel, melphalan induces apoptosis of tumor cells (not shown)

liposomes with TNF and doxorubicin in rats bearing soft tissue sarcoma resulted in objective tumor response without significant systemic side effects [18]. However, half-life of stealth liposomes can exceed several days, a condition of long-lasting low TNF concentration. Therefore, the risk of tumor metastasis enhancement is to be considered, although no such effect has been recorded in this setting. To the best of our knowledge, no clinical trial with this combination in liposomes has been performed.

Targeting TNF in tumor angiogenic vessels is an appealing approach. The recent approach of using phage display libraries led to the discovery of new targets for antiangiogenesis strategies. It was recently possible to isolate human antibody fragments that recognize altered extracellular matrix proteins in oncofetal tissues and to find peptides that can bind receptors expressed by angiogenic endothelial cells in tumors. This allowed the development of recombinant antibodies that specifically bind an isoform of fibronectin (FN) and of a peptide that binds an isoform of aminopeptidase N (CD13).

#### Anti-EDB FN/TNF fusion protein

The isoform containing the ED-B domain (B-FN) is highly expressed in tumors, fetal tissues, and tissues undergoing physiologic remodeling such as endometrium, ovary, and wound healing tissues, but cannot be detected in normal adult tissues. It was demonstrated that B-FN is especially expressed in intratumoral angiogenic vessels (reviewed in [19]). A single chain Fv antibody L19 (single-chain variable fragment) specific for the B-FN isoform was fused with monomeric TNF. The sequential administration of L19/TNF followed by melphalan resulted in an impressive tumor growth retardation while the same dose of construct or melphalan alone showed poor efficiency [20]. L19/TNF will be studied in phase I at Schering.

#### NGR-TNF

An isoform of aminopeptidase N (CD13) extensively expressed by angiogenic tumor vessels binds peptides containing the Cys-Asn-Gly-Arg-Cys motif [21]. A fusion protein was designed where the N terminus of TNF is coupled with the C terminus of the peptide: Asn-Gly-Arg (NGR)-TNF. Moreover, a concentration of 106 times less of NGR-TNF than TNF is still efficient. Evidence was obtained that NGR-TNF improved the penetration of melphalan and other drugs in tumors, which resulted in a high therapeutic index [22]. It is interesting to note that a critical therapeutic window was evidenced: The best antitumor effect was obtained when drugs were injected 2 h after NGR-TNF, whereas it was lower after 1 or 3 h [23]. This observation again illustrates the importance of the therapeutic window. NGR-TNF is currently studied in phase I at EORTC [24].

## Anti-gp240/TNF fusion protein

The recombinant fusion construct of single-chain anti-melanoma antibody (scFvMEL)/TNF is composed of the human TNF and a single-chain Fv recognizing gp240 present on 80% of melanoma [25] cases and 20% of breast cancer cases. The fusion protein scFvMEL/TNF was more cytotoxic to antigen-positive melanoma cells than TNF alone and, in addition, was active against melanoma cells completely resistant to TNF itself. Coadministration of scFvMEL/TNF and chemotherapeutic agents [vincristine, etoposide, cisplatin, 5-fluorouracil (5-FU), and Adriamycin] in vitro for 72 h demonstrated synergistic antitumor activity with Adriamycin or 5-FU and demonstrated additive effects in combination with vincristine, etoposide, or cisplatin [26].

Taken together, these three TNF-targeting preclinical studies strongly suggest that it is possible to efficiently, safely, and systemically administer TNF at a low dose if it is targeted to angiogenic vessels or to a tumor. The efficiency clearly resides in the improved efficacy of chemotherapy because of improved intratumoral penetration.

## Anti-VEGF

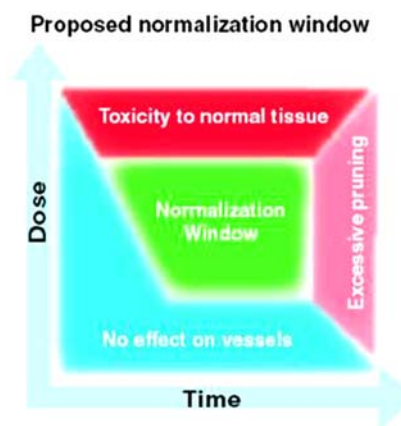
The VEGF family, especially VEGF-1 or VEGF-A, that is now widely recognized as a major mediator of tumor angiogenesis was first identified as vascular permeability factor. Current approaches to blocking angiogenesis include strategies that target VEGF-1, but little attention has been paid to possible drawbacks, which may include reduced penetration by chemotherapeutic agents administered simultaneously or subsequently. In several animal models, anti-VEGF antibodies or antisense VEGF therapy reduce tumor growth but also consistently reduce intratumoral capillary permeability by as much as sixfold as demonstrated by window chamber or contrast-enhanced magnetic resonance imaging [27–29]. Vascular permeability changes in response to VEGF result from the accumulation of NO, which is produced mainly by endothelial NO synthase [30]. Under pathological conditions, including those that prevail in tumors, NO is converted to peroxynitrite (ONOO<sup>-</sup>), which is responsible for the enhanced vascular permeability [31]. This effect is mediated partly through the activation of matrix metalloproteinases [32]. Therefore, anti-VEGF strategy is expected to decrease vessel permeability at a certain point in time. This prediction was fortunately not fulfilled in the combination of anti-VEGF (bevacizumab) and chemotherapy in metastatic colorectal carcinoma treatment. It resulted in better response and longer survival compared to chemotherapy alone. The benefit in terms of overall survival was 4.7 months, progression-free survival was 4.4 months, and objective response rate was increased from 38.8% with chemotherapy alone to 44.8% with the addition of bevacizumab [33].

This success could well be to the lucky coincidence with the “normalization window” described by R. Jain (Fig. 8)

[3]. However, a better-designed protocol that would take this concept into consideration could well give better results. Tumor vasculature is structurally and functionally abnormal. Initially, the antivascular treatment improves the structure and the function of tumor vessels. However, aggressive or sustained antiangiogenic treatment may eventually damage the vasculature, resulting in reduction of blood supply and drug penetration. Therefore, the normalization window would indicate that an antiangiogenic treatment is optimal when it is given at a critical dose and at a critical time. This concept was demonstrated using anti-VEGFR-2 antibody and chemotherapy in an animal model [34]. It seems that the current protocols combining anti-VEGF to chemotherapy are not based on the rationale of critical drug penetration and normalization window. Rather, they are based on dual targeting of vessels and tumor cells. It was demonstrated that a single infusion of 5 mg/kg of bevacizumab decreases tumor perfusion, decreases microvascular density, decreases interstitial fluid pressure, and increases pericyte coverage.

The same team that published the bevacizumab study addressed the question of tumor microvascularization changes in rectal cancer patients treated with a first dose of bevacizumab alone [35]. They found tumor blood flow and interstitial pressure reduction on day 12 after injection. It is unfortunate that subsequent bevacizumab injections were given simultaneously to chemotherapy. Indeed, the authors did not take into account the critical effect of bevacizumab on tumor vasculature and interstitial pressure.

It seems that a better rationale would be to first administer anti-VEGF antibody and follow, at an adequate time, with the administration of chemotherapy. A subsequent step will indeed be to allow the destruction of the angiogenic vessels by repeated high dosage of anti-VEGF antibody.



**Fig. 8** Proposed model of tumor vascular normalization by R. Jain when using a combination of antiangiogenic followed by cytotoxic drugs. In case of too low dose or too early administration of antiangiogenic drug, there is no effect on vessels. In the reverse situation, normal tissues and vessels are damaged and cytotoxic drug does not penetrate. The “normalization window” is the critical situation where tumor vessels tend to reverse to a normal structure and synergy between the antiangiogenic and cytotoxic drugs will be possible. (Reprinted with permission from R. Jain, *Science* 307:58–62. Copyright 2005 AAAS [3])

This strategy could be also applied when using other antiangiogenic agents. For example, sorafenib was found to decrease the transfer of gadolinium from vessels to tissues ( $K^{\text{trans}}$ ) [36]. If chemotherapy were given at this point in time, an impediment to its tumor penetration must be predicted.

## Other compounds

Histamine is able to increase blood tumor permeability [37]. ILP treatment of syngeneic rat sarcoma with histamine—instead of TNF—increased tumor melphalan uptake and improved the tumor response [38]. There was also a reduction of vascular integrity in the surrounding tissue after ILP treatment with histamine and melphalan compared with melphalan alone. Because there was in vitro evidence of tumor cell sensitization to melphalan, it seems that this approach involves histamine effects on both endothelial cells and tumor cells. A clinical trial is in preparation in Rotterdam at Erasmus MC, Daniel den Hoed Cancer Center.

A water-soluble analog of the antivascular agent combretastatin A4, tubulin polymerization inhibitor, was used in combination with docetaxel in a mammary adenocarcinoma model in the mouse. Unlike combretastatin that was found too toxic, the use of dosages as low as 13% of the highest nontoxic dose when administered before, but not after, or simultaneously to docetaxel, resulted in synergy with large therapeutic index [39]. A randomized phase II was initiated at Sanofi/Aventis.

## Conclusion

The current antitumor strategy paradigm based on the synergism of empirical drug combinations is obsolete. Instead, the design of protocols based on new pharmacodynamic concepts, such as the “therapeutic window” and the “normalization window”, should provide better efficiency of cancer treatment as exemplified in ILP with TNF and chemotherapy.

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