

TARGETING THE TUMOR VASCULATURE STRATEGIES FOR COMBINATION THERAPY

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Abstract The tumor vasculature is a well-recognized and increasingly popular target for the therapy of solid tumors, since it is reasonable to presume that damaging tumor vessels would affect the many tumor cells that depend on them for survival. Phenotypic and functional characteristics distinguish tumor vessels from the mature vasculature in normal tissues, thus providing selective targets for therapy of antineoplastic agents designed to prevent the formation of new vessels (anti-angiogenic therapy) or to damage the already formed vessels (vascular targeting/disrupting therapy). A number of approaches have been developed and have shown their efficacy in preclinical models, and more recently, in clinical studies. Combination therapies, including one or more angiogenesis inhibitors together with conventional means, constitute the new avenue to the treatment of progressive cancer disease. The choice of the combination/s, doses and schedules, as well as the sensitivity of the tumor, are some of the issues that need to be considered in the design of trials implementing this approach. No less important are the metabolic and pharmacokinetic interactions and unexpected toxicities that play a relevant role in treatment outcome. Here we highlight the critical factors that determine the success or failure of these treatments. Also analyzed is the relevance of the mechanism of action and the intrinsic activity of the drugs, as well as the possibility that the two combined agents synergistically affect the vasculature or independently target the host and the tumor compartments. Special attention is given to the need to optimize scheduling and sequencing, through the use of reliable end points, in order to avoid adverse pharmacological interactions and to improve the antineoplastic efficacy of combination treatments.

Key words: combination therapies, chemotherapy, angiogenesis inhibitors, vascular disrupting agents, tumor stroma

Resumen *Vascularización tumoral como blanco de terapias combinadas.* Dado que se presume que dañando los vasos del tumor se afecta la sobrevida de las células tumorales, la vasculatura tumoral es un blanco terapéutico cada vez más reconocido. Características fenotípicas y funcionales distinguen a los vasos de los tumores de los vasos maduros en tejidos normales, proveyendo así un blanco selectivo para agentes antineoplásicos diseñados para prevenir la formación de nuevos vasos (terapia anti-angiogénica) o para dañar vasos ya existentes (terapias disruptivas). Varias estrategias han sido desarrolladas y han demostrado eficacia en modelos preclínicos, y más recientemente en ensayos clínicos. Terapias combinadas que incluyen uno o más inhibidores de la angiogénesis junto con otros tratamientos convencionales constituyen hoy una nueva estrategia para combatir el cáncer. La elección de las combinaciones, dosis y esquemas de administración, así como la determinación de la sensibilidad del tumor son algunos de los temas que necesitan ser considerados para diseñar ensayos clínicos que pongan a prueba esta estrategia. No menos importantes son las consideraciones respecto del metabolismo e interacciones fármaco-cinéticas y toxicidades inesperadas que juegan un papel importante en el resultado del tratamiento. En este artículo analizamos los factores críticos que determinarían el resultado positivo o negativo de estos tratamientos. También se analiza el mecanismo de acción y la actividad intrínseca de las drogas, como también la posibilidad de utilizar dos agentes combinados que sinérgicamente afecten la vasculatura o que por el contrario tengan como blancos independientes tanto al tumor como al huésped. Especial énfasis se da a la necesidad de optimizar el cronograma y secuencia de administración utilizando parámetros creíbles, en forma de evitar interacciones farmacológicas adversas y mejorar la eficacia antineoplásica con tratamientos combinados.

Palabras clave: terapias combinadas, quimioterapia, inhibidores de la angiogénesis, agentes disruptivos de la vasculatura, estroma tumoral

The conventions of anticancer therapy have been challenged by the recognition of the multi-compartment na-

ture of the tumor microenvironment. This understanding has spawned a radically different approach towards the discovery and validation of new treatments. Historically aimed at the development of cytotoxic agents to kill tumor cells, the search now endeavors to identify novel, "bio-

logical", compounds that selectively target not only the cancer cell, but also the tumor stroma.

This approach also seeks to develop target-selective agents with limited side effects and it has the theoretical advantage not to develop the classical genetically-based drug resistance induced in tumor cells by cytotoxic drugs. Various strategies are under investigation aimed to target the tumor microenvironment. Among others, the prospect of targeting the tumor vasculature has led to the development of compounds that prevent tumor vessel formation (inhibitors of angiogenesis) or that destroy the already existing vasculature (vascular disrupting agents or VDA) [Folkman, 2007; Jain, 2006; Carmeliet, 2005; Chaplin, 2006; Giavazzi, 2007; Neri, 2005].

The original concept, based on the knowledge that the progression of solid tumors depends on a functional supporting vasculature, and therefore that targeting the vessels would have deleterious effects on the whole tumor, was indeed confirmed by early preclinical findings. However, subsequent studies and, more importantly, the clinical experience, soon made it clear that monotherapies with these agents had little chance of achieving a relevant therapeutic efficacy [Jain, 2006]. For instance, clinical trials with the anti-VEGF antibody bevacizumab (Avastin®) demonstrated a significant survival benefit in combination with first-line chemotherapy in metastatic colorectal carcinoma [Ferrara, 2004]. Other promising molecules are those affecting multiple growth factors and receptor kinases, such as the recently approved Sunitinib: developed mainly as inhibitors of angiogenesis, these drugs act on multiple molecular targets, on both the stroma and the tumor cells, thereby implementing a "one molecule - multiple targets" approach. The combination of small molecule inhibitors with chemotherapy has been described to improve efficacy.

Combination therapies offer a multitude of advantages. For one, the targeting of multiple molecules, cells, and compartments promotes additive anticancer activity. Furthermore evidence now shows that pharmacokinetics and pharmacodynamics of cytotoxic drugs are influenced by tumor stroma properties, and that this impact ultimately determines tumor response to chemotherapy. Also, tumor microenvironment might induce epigenetic protective mechanisms, which lead to an impaired response to chemo- and radiotherapy, as occurs with hypoxia induction. The effective delivery of anti-cancer drugs is reduced by environmental conditions within the tumor caused by changes in the stroma, such as increased interstitial fluid pressure and changes in vascular flow. Therefore, another advantage of combination therapies is a synergistic antineoplastic effect stemming from the influence of stroma-targeting agents on the distribution or activity of chemotherapeutic agents. By the same token, the contrary is also true, and it must be borne in mind that combinations of different treatments might also result in detrimental interactions.

Current attempts at combination treatments are often empirical. What are needed, however, are rational protocols that take into account metabolic, pharmacokinetic and mechanistic drug interactions, as well as the intrinsic biological characteristics of the tumor microenvironment (cellular biochemistry, blood vessel architecture, hemodynamics and extracellular matrix). Critical factors, therefore, in optimizing the efficacy of combination approaches (and averting possible negative inhibition and side effects) are the careful design of the study (dosing, scheduling and sequencing of treatment administration) [Chaplin, 2006; Gasparini, 2005; Giavazzi 2007]. This optimization process requires reliable and robust end points to monitor the activity of the combination. Monitoring the activity of both agents becomes particularly crucial in the clinical setting: for this purpose, non-invasive procedures, such as imaging analysis modalities and the detection of soluble markers, can be used to determine efficacy and to optimize the administration of combination regimens in patients.

The relative effect on the tumor and the stroma compartments is another key factor to be considered when designing combination therapies with vascular targeting compounds (be they antiangiogenic or VDA). Indeed, three possible scenarios can be envisaged with such combinations: i) the vascular targeting agent affects the tumor vasculature by influencing the distribution of the cytotoxic drug throughout the tumor; ii) the cytotoxic and the vascular targeting agents target different compartments of the tumor (the tumor and the endothelial cells, respectively); iii) the two therapies simultaneously act on the same compartment, i.e., the endothelial cells. As mentioned above, correct scheduling, dosing and timing are - in each case - critical issues that determine the final outcome of the combination.

Paclitaxel is one of the most widely-used cytotoxic drugs employed in the treatment of several neoplasms. Here we review our experience of the tumor vasculature as a target for paclitaxel-based combination treatments in combination with inhibitors of angiogenesis or vascular disrupting agents.

Inhibitors of angiogenesis and combination treatments

Given the complexity and the multifactorial nature of the angiogenesis process, several approaches have been proposed to inhibit directly or indirectly vessel formation. Compounds developed as inhibitors of angiogenesis are: monoclonal antibodies targeting growth factors; antibodies targeting growth factor receptors (i.e. VEGFR-2); receptor tyrosine kinase inhibitors targeting multiple receptors; molecules affecting endothelial cell proliferation and other functions; inhibitors of matrix metalloproteases or integrin activity. The review of the activity of these classes of

compounds and their status of development are not the objective of this paper [Folkman, 2007; Jain, 2006].

Numerous growth factors trigger angiogenesis: VEGF is generally considered the most important in various tumor types. Others, such as FGF-2 and PDGF, have been shown, directly or indirectly, to be involved in determining the angiogenic phenotype of solid receptors that are selectively expressed on tumor endothelial cells or other vascular supporting tumors. The biological activity of these growth factors relies on the expression of tyrosine kinase cells, as well as on the complex downstream signaling cascade that leads to tumor angiogenesis [Carmeliet, 2005]. Potent antiangiogenic and antineoplastic properties have been shown by low molecular weight molecules that inhibit signaling in tumor and vascular cells, some of which, e.g. sunitinib and sorafenib, have been approved for cancer treatment (Table 1). The clinical trials with anti-VEGF molecules has been recently reviewed [Jain, 2006].

SU6668 is an inhibitor of the tyrosine kinase activity of VEGFR-2 (Flk-1/KDR), PDGFR and FGFR that affects tumor vascularization and the growth of different types of human tumor xenografts [Laird, 2000]. The molecule has been tested as a single agent in phase I clinical trials. We investigated the antitumor proprieties of its combination with paclitaxel on preclinical models of human ovarian carcinoma xenografts transplanted in nude mice. These studies, described in detail in [Garofalo, 2003; Naumova, 2006], showed that the combination, compared to single therapies, reduced ascites formation, tumor burden and invasion of the organs of the peritoneal cavity in nude mice and significantly prolonged overall survival. The same investigations demonstrated that a) the magnitude of the effect depended on the tumor type, treatment du-

ration, and the tumor burden at treatment outset; b) the addition of paclitaxel to the combination –even at a low dose– was sufficient to keep tumors from invading peritoneal cavity organs and c) the dose-schedule of the treatments influenced the final outcome. Particularly noteworthy was the combination of SU6668 with a split-low doses of paclitaxel (subtoxic dose) that gave rise to outcomes similar to those of high dose paclitaxel in monotherapy (maximum tolerated dose), suggesting that prolonged treatments with limited side effects could be achieved with this kind of schedule [Garofalo, 2003].

In our view, the effect of combination treatments is dose-schedule dependent and related to the cells' sensitivity to paclitaxel, as demonstrated in tumors that are indeed responsive to it. However, a delay in growth, albeit limited, was observed also in tumor resistant to paclitaxel. In those tumors reduced tumor vascular density was also demonstrated [Naumova, 2006], thus suggesting that this combination treatment also affects host compartments, most likely vascular cells. These findings endorse two of the original hypotheses: that angiogenesis inhibitors and cytotoxic agents can act on tumor and host cells independently (studies with PTX sensitive tumor), and that the effects of the two treatments act on the same compartment (the host), as in the studies with PTX resistant tumors. How at which extents either of these effects impacts on the final outcome remains to be seen.

The antiangiogenic effects mediated by conventional anticancer drugs have been known for some time [Belotti 1996; Miller, 2001; Kerbel, 2004]. In the case of Paclitaxel, its anticancer activity extends beyond its direct cytotoxicity against tumor cells, since it also targets the tumor stroma. Like other cytotoxic drugs [Miller, 2001; Kerbel, 2004], paclitaxel (and taxanes in general,) inhibit angio-

TABLE 1.– *Anti-VEGF agents in clinical development*

Molecule	Drug type	Main target/s
Bevacizumab (Avastin®)	Antibody	VEGF
VEGF-trap	Soluble decoy receptor	VEGF isoforms
Sunitinib (SU11248)	TKR inhibitor	VEGFR-2, PDGFR- β , FLT3, c-Kit
Sorafenib (Bay 43-9006)	TKR inhibitor	Raf, VEGFRs, PDGFR- β , c-Kit
Zactima (ZD6474)	TKR inhibitor	VEGFR-2, EGFR
Vatalanib (PTK787/ZK)	TKR inhibitor	VEGFRs, PDGFR- β
AZD2171	TKR inhibitor	VEGFRs, PDGFR- β , c-Kit
CEP-705	TKR inhibitor	VEGFRs
IMC-C1121b	TKR inhibitor	VEGFR-2
XL999	TKR inhibitor	FGFR, VEGFRs, PDGFR- β

Main VEGF blockades and low molecular weight molecules with high affinity for VEGF receptor and other kinases that have shown potent antiangiogenic and antitumor proprieties.

genesis-related endothelial cell functions at lower concentrations/doses than those required for the cytotoxic activity [Belotti, 1996].

Robert Kerbel and coworkers advanced the use of these chemotherapeutic agents at low doses on a frequent and continuous schedule (metronomic regimen) in order to optimally exploit their antiangiogenic activity [Kerbel, 2004]. The combination with VEGF inhibitors prevents endothelial cells from repairing damage induced by cytotoxic drugs, thereby maximizing the antivascular effects. We found that the combination of paclitaxel with SU6668 synergistically inhibits, *in vitro*, the proliferation of endothelial, including microvascular endothelial, cells activated by VEGF or FGF-2, and causes greater apoptosis than the single agents. SU6668 also inhibited PDGF-B induced proliferation of aortic smooth muscle cells, and the effect was significantly amplified in combination with paclitaxel. Smooth muscle cells were selected to represent a cell population, such as pericyte, that play important functions in blood vessel maturation and integrity [Bergers, 2003; Carmeliet, 2005]. Thus, simultaneous inhibition of both VEGFR-2 and PDGFR-B, as in the case of SU6668, could affect the interaction between endothelial cells and pericytes, and enforcing tumor vessel regression [Laird, 2000]. We found synergistic effects of paclitaxel with SU6668 at sub-lethal concentrations of the single agents, thus supporting the potential of the combination as a vascular targeting/angiogenesis inhibitor [Naumova, 2006]. Our results support the notion that therapies aimed at inhibiting multiple receptors, particularly those targeting perivascular and endothelial cells, and in combination with sub-toxic concentrations of chemotherapy result in more potent antiangiogenic effects.

The combined antiangiogenic activity of the drugs was confirmed by the *in vivo* studies showing inhibition of FGF-2 induced angiogenesis in the Matrigel plug transplanted sc in mice and a significant reduced number of CD31

positive vessels in tumors [Naumova, 2006]. These findings, together with the effect on endothelial cells *in vitro*, support the hypothesis that the enhanced effect exerted by the combination of paclitaxel and SU6668 on tumor growth is also mediated by an effect on the vasculature.

Vascular disrupting agents and combination treatments

Vascular disrupting agents (VDA) exploit the antigenic and functional differences between blood vessels in tumors and in normal tissues [Neri, 2005; Chaplin, 2006]. The tumor vasculature contains immature, highly-permeable, chaotic vessels with heterogeneous blood flow rates: characteristics that result in selective sensitivity to the action of VDAs, whereas vessels in normal tissues are spared [Chaplin, 2006]. Strategies to affect the tumor vasculature consist of ligand-directed vascular targeting compounds (i.e. antibodies or peptides that recognize proteins expressed selectively on the tumor vasculature, used to deliver an effector to the endothelium), or molecules directly damaging the vasculature (i.e. antagonists of junctional proteins, cytokine-inducer flavonoids and tubulin targeting agents). [Tozer, 2005; Neri, 2005; Chaplin, 2006]. Several small molecule tubulin-binding VDAs (e.g CA4P, ZD6126, AVE8062, Oxi-4503, MN-029, ABT-751, and TZT-1027) have been developed and are currently undergoing clinical testing (Table 2).

Sub-toxic concentrations of VDAs induce morphologic alterations of endothelial cells in the tumor vessels, triggering a cascade of events that ultimately leads to vessel shutdown and tumor necrosis. [Micheletti, 2003; Tozer, 2005]. Typically, the final effect of a single administration of VDAs is the induction of massive central tumor necrosis (24 h after treatment), leaving a rim of viable, proliferating cells at the tumor periphery, the hallmark of the action of

TABLE 2.- Vascular- disrupting agents (VDA) in clinical development

Molecule	Drug origin	Main mechanism
CA4-P	Cambretastatin A-4 prodrug	Tubulin binding
ZD6126	N-acetylcolchicol prodrug	Tubulin binding
AVE8062 (AC7700)	CA-4 prodrug	Tubulin binding
ABT751 (E7010)	Sulphonamide	Tubulin binding
OXI4503	CA-1 prodrug	Tubulin binding
TZT-1027	Dolastatin-10 derivative	Tubulin binding
DMXAA (AS1404) inducer	Flavonoid	Cytochine

Main small molecules antagonists of junctional proteins, cytokine-inducer flavonoids and tubulin targeting agents that have shown potent vascular disrupting activity and antitumor response.

tubulin-binding VDAs. These viable tumor cells can rapidly repopulate the tumor, which is then able to resume its growth, unless treatment with the VDA is repeated or the VDA is combined with other types of treatments.

The induction of vascular shutdown demonstrated in experimental tumor models as the mechanism of action of these compounds has been confirmed in clinical studies, where PET and MRI used to measure the effects of VDAs have shown suppressive effects on tumor perfusion and blood flow. Phase I studies have found that these compounds are in general tolerated and manageable with no significant hematological/chemical toxicity, although reversible blood pressure changes and cardiac adverse effects have been reported.

Although apparently contrary to what common sense would suggest, there are reasons to combine drugs with opposite effects on the same molecular target. Indeed, the association of the microtubule-stabilizing paclitaxel and microtubule-destabilizing VDAs provides an interesting example of how two agents sharing the same molecular target can be successfully combined. [Taraboletti, 2005]. The conditions for administration must be optimized in advance, however, so that each drug preferentially acts on a specific cell population and treatment with one agent amplifies the response to the second one.

For our studies we used the colchicine analogue ZD6126, a synthetic water-soluble phosphate prodrug that is rapidly converted *in vivo* into the microtubule destabilizing ZD6126 phenol. The effects of the compound on endothelial cells *in vitro* and on neo-vessels *in vivo* have been thoroughly documented [Blakey, 2002; Micheletti, 2003], as has its ability to induce tumor necrosis in experimental models [Blakey, 2002; Taraboletti, 2005]. ZD6126 has been reported to synergize with radiotherapy, chemotherapy as well as other inhibitors of angiogenesis, in preclinical studies [Blakey, 2002].

Given the effect of the VDAs on the vasculature (tumor necrosis), we propose the sequence of administration in combination with chemotherapy can be chosen according to two rationales. Vessel shutdown induced by the VDA given after the cytotoxic compound would cause trapping of the already present cytotoxic drug within the tumor, and, also it would prevent the possible VDA-induced impairment of drug distribution in the tumor; conversely, an inverse combination schedule, i.e., pre-administration of the VDA before chemotherapy, might generate the favorable conditions for the activity of chemotherapy by exploiting the fact that the highly proliferating cells at the periphery of VDA-treated tumors constitute ideal targets for cytotoxic drugs [Chaplin, 2006].

In the case of the combination of microtubule-destabilizing VDA (in our studies ZD6126) with the microtubule-stabilizing (in our studies paclitaxel) it is complicated by the fact that both agents interact with microtubules, but with an opposite effect. We found that

paclitaxel given shortly before ZD6126, prevented the morphologic changes induced by the VDA in endothelial cells *in vitro*. We believe this was due to an counteraction of the two drugs at the level of microtubule organization, as shown by the immunofluorescence analysis of the endothelial cell cytoskeleton [Taraboletti, 2005]. The "protective" action of paclitaxel against VDA activity was observed also *in vivo* where pretreatment of mice with paclitaxel completely prevented vascular shutdown induced by ZD6126, inhibited ZD6126-induced necrosis in transplanted tumors [Taraboletti, 2005] and failed to improve the antineoplastic efficacy of either agent alone (manuscript submitted).

Noteworthy is the observation that the counteracting effect of paclitaxel *in vitro* and *in vivo* was reversible. In order to identify the timing of treatments, we used tumor necrosis induced by ZD6126 as the readout of the VDA activity *in vivo*. This enabled us to determine that an interval longer than 24 hours after paclitaxel administrations was required to restore the vascular targeting activity of ZD6126. A combination of the two drugs based on this time interval was indeed more effective in inhibiting tumor growth than either agent alone (manuscript submitted). We also investigated the efficacy of the opposite sequence of administration, based on the assumption that the VDA given before paclitaxel would enhance tumor responsiveness to the cytotoxic drug. The combination of ZD6126 followed by paclitaxel 24h later exerted an increased antineoplastic activity compared to each single agent, leading to complete tumor remissions (manuscript submitted). It is conceivable that pretreatment with ZD6126 amplifies the response to paclitaxel by increasing the number of proliferating, paclitaxel-responsive cells in the tumor periphery. The actual target cell that homes the viable rim surrounding the necrotic area after VDA treatment, either tumor cell or endothelial progenitor cell, is still unidentified. However it represents a candidate marker helping to design combination treatments with VDA.

Conclusions

A review of the body of preclinical investigations on combinations of paclitaxel with an inhibitor of angiogenesis or a VDA affords the general conclusion that, in both cases, the combination, when optimized, is more effective than either monotherapy alone. Early clinical trials with different agents and combination protocols parallel this finding.

The design of successful combination therapies is contingent on a number of critical factors. Firstly, the intrinsic biological properties of tumors, together with their sensitivity to the single drugs, must be borne in mind, since they can determine the ultimate efficacy of the combination. Secondly, because the pharmacological properties

and mechanism of action of the two drugs can impact on the response, administration protocols must be carefully designed in order to improve the final outcome. The possible negative interactions between the two drugs must also be considered, but these, too, can be avoided by careful scheduling, sequencing and timing of drug administrations. Finally, combination protocols must be optimized through the use of robust endpoints, such as soluble factors, circulating progenitor cells, and analysis of molecular targets. For this purpose, non-invasive imaging technologies, already used in clinical protocols, provide unprecedented tools to monitor drug activity and tumor response.

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Le bonheur ce n'est pas faire ce que l'on veut sinon aimer ce que l'on fait

La felicidad no es hacer lo que uno quiere sino querer lo que uno hace

Jean-Paul Sartre (1905-1980)