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Microtubule targeting agents in glioma

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Microtubule targeting agents: mechanisms of action and classification

Microtubules are dynamic tubular polymers of α - and β -tubulin which provide structural integrity, promote migration, transport of molecules, vesicles and organelles and play essential roles during cell division by forming the mitotic spindles, orchestrating the transport and separation of chromosomes and cytokinesis. Blocking the microtubule polymerization-dynamics leads to disruption of cellular division, mitotic arrest at the metaphase-anaphase transition and induction of apoptosis (1). Microtubules are thus a good target for anticancer therapy. Many tubulin-binding agents (TBAs) have been purified from natural products and synthesized to be used as therapy for a variety of cancers (2).

Based on their binding-site and their mechanism of action TBAs can be classified in three categories (3): (I) the Vinca alkaloid group including vinblastine and vincristine, vindesine, vinorelbine, vinflunine, cryptophycin, dolastatins and others, compounds which bind to high-affinity sites at the microtubule plus end (Vinca binding domain) and induce microtubule destabilization. These drugs have long been used in the treatment of pediatric and adult hematological malignancies and continue to be employed for the treatment of various solid tumors in several combinations; (II) taxane compounds, including paclitaxel, docetaxel, cabazitaxel and the epothilone group, bind to β -tubulin on the inside of the microtubule (Taxane site) (4), induce microtubule polymerization at high doses and suppress microtubule dynamics at lower doses; (III) the Colchicine group includes colchicine (historically used to treat gout), combretastatins and its many synthetic derivatives (AVE8062A, CA-1-P, CA-4-P, ZD6126) (5), 2-Methoxyestradiol, ABT-751, E7010, etc.); these compounds bind to the colchicine site of free β -tubulin molecules generating tubulin-colchicine complexes which polymerize into the microtubule, altering its structure and suppressing microtubule dynamics. The chemical structure of several TBAs is presented in Figure 1. Vinka alkaloids and members of the colchicine group are also categorized as microtubule polymerization inhibitors, or destabilizing agents, whereas the taxane compounds are thought of as microtubule stabilizing agents (Figure 2).

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Interestingly TBAs, particularly members of the colchicine group, but also vinka alkaloids, exhibit anti-vascular effects, inducing hemorrhagic necrosis and decrease of blood flow within tumors in preclinical cancer models (6,7). This effect is thought to be due to the action of TBAs on cytoskeletal microtubules and is primarily evident in the tumor vasculature and not in the vessels of healthy organs. The tumor specificity of the anti-vascular effects of TBA's is attributed to the differences between the cytoskeletal architecture of endothelial cells in newly formed tumor vessels and established normal blood vessels (8). It has been proposed that Combretastatin A-4 (CA-4) induces depolymerization of cytoskeletal microtubules, activation of stress signaling pathways, alterations of endothelial cell membrane permeability, membrane blebbing, detachment and necrosis, increased vascular permeability, red blood cell stacking, edema and loss of blood flow (5). Since CA-4 has limited solubility and bio-availability, several water soluble analogs (BPR0L075, BNC105, etc.) have been synthesized and tested in preclinical cancer models (5) and also in glioblastoma (GBM) (9–11).

Side effects and multi drug resistance

Drawbacks for the use of TBAs in oncology include side effects induced by these drugs, as they are inherently non-specific, targeting microtubules in cancerous and non-cancerous cells, and by the development of drug resistance. Most commonly observed are peripheral neuropathies and autonomic neuropathies, neuronal activity being highly dependent on proper functioning of microtubules (12,13). Frequent side effects are also caused by myelosuppression, bone marrow cells relying heavily on cell division to maintain blood cell homeostasis (14).

Other limitations related to the use of TBA are represented by the heterogeneity within tumors, which, unlike cancer cell lines used *in vitro*, display lower and variable rates of mitosis and are thus able to escape targeting by anti-mitotic drugs. A concern is represented by the “mitotic slippage” of cancer cells targeted with TBA, which upon mitotic arrest, do not undergo apoptosis, but remain viable, acquiring multiploidic states, increased genetic instability and malignancy (15,16).

Resistance to microtubule-targeting agents is often due to drug efflux by ATP-binding cassette (ABC) transporters such as P-glycoprotein (Pgp). These transporters are energy-dependent membrane pumps involved in the transport of a wide variety of substrates across the plasma membrane and are integral members of specialized compartments like the blood-brain, the blood-testis, and the placental barriers. In the context of drug treatment these pumps actively eliminate drugs from cells, showing preference for many of the naturally occurring anticancer agents like taxanes, the vinca alkaloids, anthracyclines, epipodophyllotoxins and others, and are responsible for the development of multidrug resistance (17,18). Other described mechanisms of resistance include: altered expression of microtubules associated proteins (MAP) (19,20), aberrant and overexpression of specific β -tubulin isotypes, mutation within the β -tubulin protein itself (21) or impaired apoptotic signaling downstream of the mitotic arrest induced by TBAs. In the brain, β -III tubulin is not normally found in glial cells, but rather is a neuronal marker with high expression in young neurons. Expression of β -III tubulin in glial tumors is however aberrant, and correlates with

resistance to taxanes and vinca alkaloids and also with increased malignancy, being found primarily in high grade glioma: astrocytoma (WHO-III) and glioblastomas (GBM: WHO-IV) (22). β -III tubulin is thus a more specific target for TBAs in glioma and studies have sought to find compounds which can target it directly. Ixabepilone, a derivate of epothilone B, has high antitumor activity in taxane resistant tumors by preferentially targeting $\alpha\beta$ III-microtubules in cells expressing high levels of β III-tubulin, however this drug cannot cross the blood brain barrier (BBB) (23), highlighting the numerous aspects to consider when designing therapies for central nervous system (CNS) malignancies with anti-tubulin agents.

Current use of microtubule targeting agents in the treatment of glioblastoma (GBM)

The current treatment for GBM, the deadliest and most common form of primary malignant brain cancer can only offer patients a 5-year survival rate of 5.1% (24). While immunotherapy is increasingly viewed as the fourth arm in the standard of care for GBM, added to maximal possible surgical rejection, radiation and chemotherapy with temozolamide (TMZ) (25–27), so far, large scale clinical trials have not shown long term survival benefit in GBM patients treated with immunotherapy (28). Therefore, the development of new antimitotic TBAs with increased specificity, decreased toxicity, capable of crossing the BBB and overcoming mechanisms of drug induced resistance is an attractive prospect (29). Employing digital modeling algorithms, several new compounds designed to specifically down-regulate β -III tubulin have been discovered: IDN5390111 and other seco-taxanes (30) as well as epothilones, such as ixabepilone and patupilone (EpothiloneB). These drugs are active against taxane resistant epithelial tumors and act by both suppressing the dynamic instability of microtubules in the mitotic spindle and by antagonizing the assembly of cytoskeletal microtubules leading to inhibition of migration and invasion of GBM cells (31,32).

Results from a phase I/II clinical trial which tested Patupilone in recurrent GBM, reported that the drug was relatively well tolerated, with no hematological side-effects, no cognitive changes, but with development of reversible sensory neuropathy in two of nine patients (33). The study demonstrated that the drug accumulates within the tumor tissue and that two patients benefited from long-term recurrence free survival (9.75 and 22 months) (33). Patupilone was also tested in preclinical studies of medulloblastoma, as a less toxic alternative to vincristine (34). Sagopilone (ZKEPO), an analogue of epothilone B, which can cross the BBB, having demonstrated promising results in rodent models of GBM (35), has been tested in 15 patients with recurrent GBM (36). After four cycles of treatment, five patients achieved the 6 months progression free survival, however six patients developed neuropathy (36). A phase II multicenter follow-up trial in 37 patients with recurrent GBM, was unable to demonstrate antitumor activity, progression free survival at 6 months being only 6.7% and 46% of patients developed moderate to severe neuropathy (37).

Verubulin (MPC-0827, Azixa) is a quinazoline-derived small molecule, which has been demonstrated to inhibit microtubule formation, to cross the BBB and to induce mitotic arrest and apoptosis in several cancer cell lines (38). Verubulin has also been tested in a phase II

clinical trial of 56 patients with recurrent GBM, either refractory or naïve for bevacizumab. While the drug was relatively well tolerated, this single agent treatment had little to no anti-tumor activity. Nonetheless, bevacizumab-naïve patients seemed to have fared better than the refractory group (median survival: 9.5 months compared to 3.4 months).

During the last three years, aided by the increasing availability and sophistication of *in silico* modeling algorithms and of high-throughput cell based screening assays, intense efforts to discover new antimitotic therapeutic agents have led to the discovery of a vast array of novel analogs of colchicine and combretastatin-A, some displaying both anti-mitotic as well as anti-vascular activity like: MDS-11P (39), indole compounds II and IAT (40), cis-constrained analogs of combretastatin A4: compounds 11 and 19 (41), cis 3-b (42), DAT-230 (43), ketone 24-b (44), phenothiazine derivative 21 (45), 1,1-diaryl-2-methoxyethylenes 4b and 4e (46), combretazet-3 (CAZ-3) (47), benzoimidazole analog 5 (48), compound 3-HCl (AG119) (49), furocoumarin analogs of combretastatins: compounds 2 and 3 (50), and the IsoCombretaQuinazolines (isoCoQ) compounds 4b, 4c and 4d (51).

In an effort to design antimitotic agents which can act on multiple targets on cancer cells and starting from the structural similarity of tubulin binding agents with receptor tyrosine kinase (RTK) inhibitors, Gangjee et al. have designed several analogs of combretastatin A to potentially act as inhibitors of both microtubule dynamics and RTKs (49). The authors showed that compound 3-HCl (later called AG119), a water soluble derivate of pyrrolo [3,2-d] pyrimidine, previously reported to have antiangiogenic, antimetastatic, and antitumor activity (52), when modified to incorporate a benzyl group acquires anti VEGFR2 activity, comparable to sunitinib. This was documented in an *in vitro* assay of tyrosine phosphorylation following stimulation with VEGF. The authors also showed that blood vessel formation in the chicken chorio-allantoic membrane is inhibited by AG119 at concentrations comparable to sunitinib (IC_{50} for 3-HCl = 2.9 μ M, and for sunitinib 1.3 μ M). *In vitro*, AG119 bound to the colchicine site on microtubules and inhibited tubulin assembly, albeit 18-fold less than combretastatin A-4P (CA-4P). Tested on the NCI 60 tumor cell panel this compound induced growth inhibition in all cell lines at a nanomolar concentration including in the astrocytoma cell line SF-268, the gliosarcoma cell line SF-539 and the GBM cell lines SF295, SNB-19, SNB-75 and U251. 3-HCl was also effective in β -III tubulin overexpressing HeLa cells, resistant to paclitaxel, and in PgP overexpression ovarian cancer cells. The authors demonstrate that the compound induced G2/M mitotic arrest similar to CA-4P and docetaxel. Furthermore, AG119 inhibited tumor growth and tumor vascular density with higher efficacy than sunitinib, docetaxel or temozolomide (TMZ) when tested in *in vivo* flank tumors of either MDA-MB-435 breast cancer cells or U251 GBM cells. This compound was also effective in the 4T1 orthotopic breast cancer model where it also inhibited the formation of lung metastases. The authors conclude that AG119 is a microtubule depolymerization anti-cancer agent, superior to TMZ, docetaxel and sunitinib in preclinical cancer models, with concurrent an anti-angiogenic anti-VEGFR2 effect.

Subsequently, AG119 was tested, in an orthotopic, syngeneic GBM model, using GL261 cells. The *in vivo* anti-tumor effect of AG119 was compared with three other treatments: anti-VEGF antibodies, antibodies against c-Met, a RTK increased in GBMs and TMZ (53). Survival analysis of these four treatment groups (5–7 animals per group) indicated that the

median survival was 20 days for untreated animals, 27 days for anti-VEGF treated, 28 days in the c-Met antibody group, 29 days in the AG119 treated animals and 34 days in the TMZ treated group. All treatment groups significantly increased survival when compared to the untreated group. MRI analysis showed a significant decrease of the tumor volume for all treatment groups. Analysis of blood perfusion into the tumors demonstrated significantly decreased blood flow in both AG119 and TMZ treated animals when compared to untreated controls. The inhibitory effect of AG119 and TMZ on TMZ sensitive and resistant human cell lines (U251 and T98G) was also analyzed, showing that the IC₅₀ of AG119 wasn't significantly different between U251 and T98G, suggesting that the anti-tumor effect of AG119 is not dependent of the MGMT methylation status. The authors conclude that AG119 significantly increases survival of GL261 glioma-bearing animals and decreases tumor vascularity warranting further explorations of this small molecule therapeutic in GBM. Comparing the results of the U251 flank tumors treated with AG119 (49 with the orthotopic GL26 tumor model in this study (53 one can notice that TMZ fared better in the orthotopic model, whereas AG119 seemed to have a more potent effect in flank tumors with respect to both tumor growth and tumor vascularity. This difference could be accounted for by the difference in BBB penetration or, possibly by differences in the immune response to treatment, since one model uses immune compromised mice, whereas animals in the syngeneic orthotopic model are immuno-competent. The myelo-suppressive effect of antimetabolic agents is well documented; however, it was not explored in this study. The authors suggest that the decrease in perfusion following AG119 treatment is due to the inhibition of VEGFR2, based on data from the previous study, however, the anti-vascular effect of AG119 could also be a consequence of altering the cytoskeletal microtubules in vascular endothelial cells, as it has been shown before for several compounds derived from CA-4, or by increased necrosis within the tumor. This study highlights the need to test novel drugs for GBM, in models which more closely resemble the human disease, i.e., orthotopic location, also the use of immuno-competent animals, given the major role played by the immune system in modulating response to treatment.

An exciting group of new Combretastatin A-4 derivatives has been recently developed, in which activity can be switched “on” and “off” by visible light. These drugs are represented by the photoisomerizable azo-Combretastatin A4 (54) and the closely related photostatins (55). Upon exposure to light, these compounds isomerize from an inactive *trans*, into an active *cis* conformation, allowing for a precise control of the activity on microtubule dynamics. These drugs open up the possibility of directed, specific, temporally and spatially controlled activation of anti-tubulin agents allowing for targeted antimetabolic and anti-vascular therapy in various cancers, an elegant avenue to pursue and overcome the many drawbacks associated with systemic side-effects of TBAs.

In summary, our knowledge of the mechanisms of action, resistance and side-effects as well as our toolbox of small molecular compounds with tubulin binding activity has increased exponentially in the last decade. We are getting closer to the design of therapeutic agents with increased specificity, decreased toxicity, capable of crossing the and overcoming mechanisms of drug induced resistance. Efforts so far have shown that single agent therapies for GBM fail, thus more successful therapies should entail a combination of agents, including surgery, radiation, multi-drug chemotherapy and immunotherapy with immune

checkpoint blockade. Inhibitors of microtubule dynamics, if made selective, effective and safe, represent an important class of molecules to consider for GBM treatment. Nonetheless, rigorous, large scale, objective studies which evaluate all aspects of treatment: anti-tumor activity, BBB permeability, resistance mechanisms, side effects are required in preclinical trials before translating these treatments into the clinic.

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Figure 1.
Chemical structures of selected microtubule-binding agents used for the treatment of CNS malignancies. CNS, central nervous system.

Figure 2.

Schematic depicting proposed mechanism of action of the different classes of microtubule targeting agents. At high dose combretastatins and vinca alkaloids induce microtubule (MT) depolymerization, whereas taxols and epothilones promote microtubule polymerization; at low dose, all microtubule-binding agents inhibit microtubule dynamics, prevent the proper alignment of chromosomes at the metaphase plate and segregation of chromosomes in anaphase, leading to mitotic arrest and apoptosis.