Emerging Microtubule Targets in Glioma Therapy

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Major advances in the genomics and epigenomics of diffuse gliomas and glioblastoma to date have not been translated into effective therapy, necessitating pursuit of alternative treatment approaches for these therapeutically challenging tumors. Current knowledge of microtubules in cancer and the development of new microtubule-based treatment strategies for high-grade gliomas are the topic in this review article. Discussed are cellular, molecular, and pharmacologic aspects of the microtubule cytoskeleton underlying mitosis and interactions with other cellular partners involved in cell cycle progression, directional cell migration, and tumor invasion. Special focus is placed on (1) the aberrant over-expression of βIII-tubulin, a survival factor associated with hypoxic tumor microenvironment and dynamic instability of microtubules; (2) the ectopic overexpression of γ-tubulin, which in addition to its conventional role as a microtubule-nucleating protein has recently emerged as a transcription factor interacting with oncogenes and kinases; (3) the microtubule-severing ATPase spastin and its emerging role in cell motility of glioblastoma cells; and (4) the modulating role of posttranslational modifications of tubulin in the context of interaction of microtubules with motor proteins. Specific antineoplastic strategies discussed include downregulation of targeted molecules aimed at achieving a sensitization effect on currently used mainstay therapies. The potential role of new classes of tubulin-binding agents and ATPase inhibitors is also examined. Understanding the cellular and molecular mechanisms underpinning the distinct behaviors of microtubules in glioma tumorigenesis and drug resistance is key to the discovery of novel molecular targets that will fundamentally change the prognostic outlook of patients with diffuse high-grade gliomas.

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anatomical location, circumscribed gliomas (WHO grade I) can be amenable to gross total or subtotal surgical resection and hence potential cure. In contrast, diffuse gliomas, which span the histologic spectrum of grades II-IV tumors, are therapeutically challenging given their highly infiltrative nature and potential to undergo anaplastic transformation to become high-grade gliomas. Glioblastoma (WHO grade IV) is the most frequent and most malignant form of primary brain cancer in adults, whereas pilocytic astrocytoma, a low-grade tumor (WHO grade I), is the most common type of glioma in children. That said, high-grade gliomas, including glioblastoma, can also be encountered in children. In the pediatric setting, deep-seated diffuse thalamic gliomas and diffuse intrinsic pontine gliomas pose a formidable therapeutic challenge given their infiltrative growth pattern and propensity for anaplastic change. Overall, the prognosis of glioblastoma remains dismal for all age groups, with most patients dying within 1 year after diagnosis. In adults, primary and secondary glioblastomas constitute clinically and genetically distinct disease subtypes. Primary glioblastomas develop rapidly de novo, affect mainly the elderly, and are genetically characterized by loss of heterozygosity, epidermal growth factor receptor (EGFR) amplification, p16INK4a deletion, and PTEN mutations. Secondary glioblastomas manifest usually in younger patients, frequently exhibit P53 mutations, and develop through tumor progression from low-grade diffuse astrocytoma or anaplastic astrocytoma. Genetically, pediatric gliomas differ from adult gliomas. In recent years, emphasis has been placed on stratifying brain tumors by molecular subtype based on the presence of specific mutations. This approach is gaining increasingly clinical acceptance in the classification and typing of gliomas and medulloblastomas. Although significant strides have been made in the genomics and epigenomics of brain tumors during the past decade, these discoveries have not been translated at the therapeutic level.

Currently, the standard approach to the treatment of glioblastoma combines surgical resection, chemotherapy, and radiotherapy. Beyond initial surgery aimed at reducing the tumor burden, the mainstay of therapy is based on the use of concurrent and adjuvant temozolomide, a DNA-binding agent, in conjunction with radiotherapy (radiochemotherapy). Combined radiotherapy and chemotherapy with temozolomide have increased median survival time of 9-15 months, compared with radiosurgery alone. However, increases in survival have been negligible (ie, 6-9 months for median progression-free survival and 14.6 months for overall survival times). Hypermethylation (and thus functional inactivation) of the O\(^\text{6}\)-methylguanine-DNA-methyltransferase (MGMT) gene enhances chemosensitivity to temozolomide in this clinical setting. Currently, temozolomide treatment is administered regardless of MGMT methylation status. Treatment challenges are multifold and may be linked to, and compounded by, drug resistance and suboptimal drug delivery owing to hindrance from the blood-brain barrier. Moreover, significant variations regarding responsiveness to treatment, or lack thereof, are observed among patients with tumors of the same histologic type and tumor grade. This necessitates the elucidation of new molecular targets that are biologically linked to cancer behaviors in gliomas and an urgent mandate for alternative innovative approaches to glioma therapy.

This review offers a critical appraisal of the current knowledge on the subject of the microtubule cytoskeleton in cancer stemming from an interdisciplinary effort undertaken by basic and clinical researchers to develop new treatment strategies in high-grade gliomas based on a rigorous interrogation of altered microtubule behavior in brain cancer cells.

### Microtubules: Time-Honored Targets in Cancer Chemotherapy

One of the core strategies used in cancer pharmacology is to disrupt the integrity of microtubules in the mitotic spindle thus blocking and restraining mitotic division. Microtubules in cancer cells have also emerged as a target aimed at countering tumor cell motility and invasion given the involvement of interphase microtubules in directional tumor cell migration and metastasis acting in concert with the actin cytoskeleton. To that end, recent studies have raised awareness about control of Rho family GTPases by microtubules, which bears critical importance in the regulation of the actin cytoskeleton during cell migration, and microtubule dynamics. In view of the fact that microtubules constitute the main target of numerous agents used in cancer chemotherapy, new insights into the mechanisms of dysregulation of microtubules in cancer cells would be crucial in the development of new molecularly targeted approaches for the rational treatment of diffuse gliomas and, in particular, the highly aggressive and devastating glioblastoma.

Microtubules, which are composed of \(\alpha\beta\)-tubulin heterodimers, are the targets of some of the most widely used and time-honored anticancer natural-product small molecule inhibitors, collectively referred to as tubulin-binding agents (TBAs). TBAs are broadly classified as microtubule-stabilizing drugs and microtubule-stabilizing drugs. The former are further subdivided into vinca domain–binding agents (vinca alkaloids and dolastatins) and colchicine domain–binding agents (colchicine and analogues) but also encompass other microtubule-polymerizing compounds such as estramustine, noscapine, and certain psychoactive drugs (phenytoin). The microtubule-polymerizing drugs are principally represented by the taxanes and epothilones (Box 1).

TBAs are also commonly referred to as antimitotic drugs because they cause mitotic arrest and produce cell death. TBA-induced tumoricidal action occurs either at the G1 phase of the cell cycle or after mitotic arrest; however, such action may escape by virtue of “mitotic slippage.” In addition to anticancer effects, TBAs exert antitumor angiogenic effects. A major hurdle accounting for treatment
failures at the clinical level is the development of drug resistance.10,13,15 Mechanisms of TBA resistance are multifold and include potentially overexpression of the MDR-1 gene; point mutations of β-tubulin at the paclitaxel-binding site; polymorphisms in β-tubulin isotypes; selective overexpression of class I, III, and IVa β-tubulin isotypes; and impaired apoptotic signaling downstream of the microtubule-associated insults to which tumor cells are exposed.10,13,14 A case in point is the widely used anticancer drug taxol (paclitaxel), the prototype in the family of potent microtubule-polymerizing compounds known as taxanes, chemoresistance to which has been linked to selective overexpression of βIII-tubulin in certain human epithelial cancers (see below).10,13,15,22

Significant strides in the sphere of cancer pathobiology of microtubules during the past decade coupled with rapid advances in computer modeling and docking (in silico) approaches to rational drug design have rekindled a keen interest in new microtubule-driven molecular targets in cancer therapeutics.

**Microtubules At a Glance**

Microtubules are dynamic cytoskeletal structures involved in diverse and essential cell functions such as mitotic spindle formation, ensuring proper chromosome segregation and cell division, cellular architecture, intracellular transport, and signal propagation.14,15,23-26 Microtubules interact with diverse cellular organelles, including the endoplasmic reticulum, Golgi apparatus, lysosomes, and mitochondria.13,26

During mitosis in diploid cells, there is assembly of the mitotic spindle whereby microtubules align chromosomes at the spindle equator in metaphase, and ensure the fidelity of an equal distribution of chromatids to the 2 daughter cells in anaphase.13,27 This highly specific process rests on the physical interaction between growing microtubules and the kinetochores.13 The mitotic spindle itself is a macromolecular complex comprising dynamic microtubules and associated proteins, which include a host of molecular motor proteins that use the energy of adenosine triphosphate (ATP) hydrolysis to organize and transport microtubules.27 To ensure the fidelity of mitosis, these motors must function in a highly coordinated and specific temporospatial manner.27 The differential organization, sorting, and use of microtubules in different cellular processes rest in part on their dynamics.

Microtubules in the mammalian brain make up approximately 20% of total protein compared with 3%-4% of total protein in somatic tissues. Polymerized microtubules have an outer diameter of 25 nm and consist of αβ-tubulin dimers arranged in a head-to-tail fashion forming 13 protofilaments. Microtubules are polar structures in which α-tubulin is exposed at the slow-growing minus (−) end whereas β-tubulin is exposed at the fast-growing plus (+) end.15,24 The current structural and functional model of microtubules is based on docking of the high-resolution structure of brain tubulin by electron crystallography28,29 and also on lower-resolution microtubule maps imaged by electron cryomicroscopy.30,31 The outer and inner surfaces of microtubules are endowed with a large number of binding sites for a host of proteins and small ligands.32

Assembly (polymerization) and disassembly (depolymerization) of microtubules is driven by the binding, hydrolysis, and exchange of guanosine-5′-triphosphate (GTP) on the β-tubulin monomer. GTP hydrolysis is necessary for switching between alternating phases of growth and shrinkage separated by “catastrophe” (transition from growth to shrinkage) and “rescue” (transition from shortening to growth) events. Polymerization is typically initiated from a pool of GTP-loaded tubulin subunits (Fig. 1C-1).33 Growing microtubule (+) ends fluctuate between slightly bent and straight protofilament sheets. GTP hydrolysis occurs shortly after incorporation, and it has been postulated that GTP hydrolysis changes the conformation of protofilament from a slightly curved tubulin-GTP to a more profoundly curved tubulin-guanosine diphosphate (GDP) structure.34 The curved tubulin-GDP is forced to remain straight when it is part of the microtubule wall. Growing microtubule sheets maintain a “cap” of tubulin-GTP subunits. A loss of this cap results in rapid depolymerization. A closure of the terminal sheet structure generates a metastable, blunt-ended microtubule intermediate (Fig. 1C-2). A shrinking microtubule is characterized by fountainlike arrays of ring and spiral protofilament structures (Fig. 1C-3). This conformational
change, presumably directed by tubulin-GDP, may destabilize lateral contacts between adjacent protofilaments. The polymerization-depolymerization cycle is completed by exchanging GDP of the disassembly products with GTP (Fig. 1C-4). These characteristics result in dynamic instability, an essential feature of microtubules that allows them to search through the cell for targets, such as the chromosomal kinetochores, the cell cortex, and the actin cytoskeleton. Within cells, microtubule minus (−) ends are anchored in microtubule organizing centers (MTOCs), which often correspond to centrosomes. Microtubules may be also released from their MTOCs and then redistributed and organized by molecular motor proteins.

Microtubules are modulated by a host of regulatory proteins, which include microtubule-stabilizing proteins, such as the (−) end-binding γ-tubulin and γ-tubulin complex proteins (GCPs), the side-binding microtubule-associated protein 2 (MAP2) and τ (tau), and (†) end-stabilizing proteins such as +TIPs (EB1 and CLIP170), and microtubule-destabilizing or depolymerizing proteins, such as the severing enzymes (spastin and katanin), the (†) end depolymerizing motor protein kinesin-13, and the αβ-tubulin dimer–binding protein stathmin (Fig. 2).

Microtubules also interact with proteins that are involved in the intracellular transport (kinesins and dyneins); regulation of the cell cycle and apoptosis, including the tumor suppressor protein p53, which is physically associated with dynem; and also with prosurvival proteins, such as Bcl-2 and survivin. However, the nature of these interactions in diploid and cancer cells merits further mechanistic elucidation.

**Tubulin Isotypes**

Both tubulin subunits are encoded by multiple genes, which exhibit a significant degree of phylogenetic conservation. Mammalian β-tubulin comprises 7 isotypes, designated as βI (TUBB), βII (TUBB2A, TUBB2B, βIII (TUBB3), βIVa (TUBB4), βIVb (TUBB2C), βV (TUBB6), and βVI
(TUBBI1), each of which is a product encoded by distinct genes (designated in italics) without alternative splicing. 38–40 For the most part, the β-tubulin isotypes differ within the C-terminus that is located on the exterior of the microtubule, 13 which represents the binding domain for MAPs. 41 Different β-tubulin isotypes display differential patterns of cellular expression and distribution, which may be cell type– or tissue-specific or be developmentally defined. In normal diploid cells, βΙ-tubulin (encoded by the TUBB1 gene) is constitutively expressed in most cells and tissues. In contrast, βΙΙΙ-tubulin (encoded by the TUBB3 gene) is predominantly expressed in neurons of the CNS and the peripheral nervous system (PNS) as well as in testicular Sertoli cells and spermatozoa; βΙΙΙ-tubulin (encoded by the TUBB1 gene) is associated with cells of the hematopoietic system. 38,40 Given the highly conserved sequences of β-tubulin isotypes across phylogeny, individual isotypes are endowed with certain unique characteristics that may account for the functional diversity of microtubules. 38,40 That said, tubulin isotypes are thought to be freely interchangeable and capable of coassembling into all classes of microtubules. 42 Still, the dominance of certain tubulin isotypes may contribute to differences in microtubule dynamics and binding of antimicrotubule drugs. 43

The differential expression, function, and cell type distribution of β-tubulin isotypes have been extensively investigated and mapped in the developing mouse brain. 44–47 It is thought that βΙ is critical for cell survival, βΙΙ for neurite outgrowth, and βΙΙΙ for survival under stress conditions. 46 In the developing and mature human cells and tissues, βΙΙΙ-tubulin is highly expressed in both CNS and PNS, where it is, for the most part, neuronal associated, albeit not neuronal specific. 4,14,23,51,52 In the human fetal CNS, βΙΙΙ-tubulin is widely expressed in the germinal matrix neuroepithelium of the telencephalic subventricular zones, which consist of putative restricted glial precursor cells or bipotential neural stem cells or both. 53 Expression of βΙΙΙ-tubulin isotype is also detected in fetal astrocytes in vitro. 53

The distinctive function(s) βΙΙΙ-tubulin may derive from certain distinct biochemical properties, which distinguish it from other β-tubulin isotypes. 59,54 Compared with other isotypes, βΙΙΙ-tubulin lacks the widely conserved and oxidation-sensitive residue cys239, which is substituted by ser239. 59,54,55 Thus, at variance with other β-tubulin isotypes, βΙΙΙ-tubulin is phosphorylated at a serine residue in the C-terminus 54,56 and has a threonine residue Thr (429), which is associated with microtubule assembly. 57

**Figure 2** Families of microtubule-binding proteins. (A) Proteins that stabilize microtubules. Microtubule-binding proteins can bind to the ends or the side of polymer or to tubulin dimer. (Adapted with permission from Pollard Ernshaw. 17) (Color version of figure is available online.)

**Differential Expression of βΙΙΙ-tubulin in Cancer**

**Cellular Distribution**

The βΙΙΙ-tubulin is by far the most extensively studied isotype in human cancer. Although βΙΙΙ-tubulin expression in normal cells and tissues is predominantly neuronal associated, 48,50 it is widely expressed in a broad range of human malignancies. Early studies conducted in our laboratory defined a differential pattern of βΙΙΙ-tubulin expression in neuronal or neuroblastic versus nonneuronal tumors. 14,15,23,51,52 In neuronal or neuroblastic tumors such as medulloblastomas, retinoblastomas, sympathoadrenal neuroblastomas, and pheochromocytomas, βΙΙΙ-tubulin expression conforms to morphologic changes of neuronal differentiation characterized by development of neuritelike processes elaborated by neuroblastlike tumor phenotypes and decreased tumor cell proliferation. 51,52,58 On the contrary, overexpression of βΙΙΙ-tubulin in nonneuronal solid tumors, such as in epithelial tumors (carcinomas) of the lung, including small cell lung cancer (SCLC), 59 non–SCLC (NSCLC), 59,65 and ovary, 66–72 breast, 13,60,73 alimentary tract, 74–76 pancreas, 77,78 prostate, 79,82 and kidney cancers, 83 as well as in gliomas, 84–87 is associated with a tendency toward a higher histologic tumor grade and unfavorable clinical outcomes. Accordingly, βΙΙΙ-tubulin expression in neuronal tumors is constitutive and differentiation dependent, whereas in nonneuronal tumors, it is fundamentally aberrant, associated with cancer phenotypes 14,23,51,52,58 and triggered by toxic factors related to tumor microenvironment (stated later). 72,88,89

It is noteworthy that βΙΙΙ-tubulin may exhibit complementary patterns of expression with βΙ-tubulin at the protein level. However, despite certain similarities shared...
between the βIII and βV isotypes at the biochemical level, their distribution differs in both normal and neoplastic cells and tissues, pointing to the existence of functional differences. Expression of βV isotype has been described in NSCLC and breast cancer cell lines. The elucidation of the role of differential tubulin isotype expression in tumor cell responses to microtubule-targeting compounds merits future studies.

**Functional Considerations**

There is general agreement that abnormal overexpression of βIII-tubulin in nonneurogenic cancers is associated with an overall proclivity for aggressive tumor behavior and adverse clinical outcomes. Kavallaris et al. were the first to report that aberrant expression of βIII-tubulin was associated with paclitaxel resistance in NSCLC cell lines and clinical resistance in ovarian cancer. Mutations in β-tubulin that affect microtubule polymer levels or drug binding are also associated with resistance to tubulin-binding agents such as paclitaxel, albeit to a lesser degree. Currently, it is widely held that expression of βIII-tubulin is associated with resistance to taxanes or vinorelbin in a host of epithelial cancer types including adenocarcinomas of pulmonary, ovarian, mammary, and gastrointestinal origins as well as in metastatic tumors of unknown origin.

Silencing the expression of βIII-tubulin or TUBB3 sensitizes human NSCLC cells to several TBAs, including paclitaxel. Conversely, such a sensitization effect is not present following silencing of βI- or βIVb-tubulin isotypes in paclitaxel-treated NSCLC cells. Nevertheless, the question of whether βIII-tubulin constitutes a far-reaching predictive biomarker of taxane resistance in cancer is a subject of debate (described later). Some investigators uphold the view that although βIII-tubulin is a prognostic marker in certain cancers, it is not a predictive biomarker of taxane resistance per se. With this in mind, it should be reiterated that high βIII-tubulin expression is not—a priori—an indicator of aggressive disease, for it could be a sign of cellular differentiation signifying a less aggressive tumor behavior as exemplified in the case of neuronal tumors described previously.

Moreover, in melanomas, which are neoplasms of putative neuroectodermal origin that basally express the βIII isotype, loss of expression of βIII-tubulin is associated with tumor progression with chemosensitivity to taxanes. Collectively, βIII-tubulin overexpression is a prognostic factor of unfavorable outcomes in certain tumor types and oncologic settings. However, its significance should be interpreted with caution and in a clinicopathologic context.

**βIII-Tubulin as a Survival Factor Triggered by Hypoxic Microenvironment**

In recent years, βIII-tubulin has emerged as an important survival factor. It has been suggested that βIII-tubulin (TUBB3) is part of a complex molecular pathway that is activated on cellular exposure to a toxic microenvironment characterized by hypoxia and nutritional deprivation. At the clinical level, activation of this prosurvival pathway is associated, for the most part, with an aggressive cancer phenotype, albeit with some notable exceptions. It is noteworthy that in the 3' flanking region of TUBB3, there is an E-box (5'-RCGTG-3') that binds HIF-1 and HIF-2. Recent studies have provided further validation for the regulation of TUBB3 gene expression by HIF-2alpha and Sox9 in aggressive ovarian cancer. A direct linkage between HIF-2alpha and overexpression of βIII-tubulin has also been recently demonstrated in glioblastoma. Thus, the hypoxic microenvironment in tumors sets in motion prosurvival pathways, rendering cells resistant to taxanes and other chemotherapeutic agents. GBP-1, which is a GTPase, directly binds to βIII-tubulin and facilitates the incorporation of additional prosurvival factors into microtubules including PIM-1 (proviral integration site 1) along with a host of kinases. GBP-1 together with HIF-1 and HIF-2, as well as miR-200c, which are upstream βIII-tubulin regulators, along with other downstream effectors, can be used potentially as biomarkers to define a subset of patients with cancer exhibiting activation of this prosurvival pathway. Interestingly, βIII-tubulin suppression in NSCLC cells results in increased sensitivity to anakin, a form of programmed cell death that is induced by anchorage-dependent cellular detachment from the surrounding extracellular matrix.
βIII-Tubulin and Signaling Pathways Associated With Malignant Tumor Behavior

Recently, proteomic studies have unraveled that βIII-tubulin regulates the expression of proteins associated with malignant growth and metastatic behavior including the adhesion-associated tumor suppressor, maspin. In the context of NSCLC, the PTEN/AKT signaling axis has been defined as a critical pathway regulated by βIII-tubulin. Earlier studies have shown that overexpression of βIII-tubulin (TUBB3) in NSCLC is also regulated by the K-RAS signaling pathway. In prostate cancer, βIII-tubulin overexpression is associated with both TMPRSS2-ERG rearrangement and ERG expression. The link of βIII-tubulin overexpression with key genomic alterations, such as TMPRSS2-ERG fusions and PTEN deletions, indicate that the βIII isotype interacts with divergent signaling pathways involved in prostate tumorigenesis.

A comprehensive elucidation of βIII-tubulin interactions, both upstream and downstream, can provide further mechanistic insights into the role of βIII-tubulin in tumorigenesis, tumor progression, and metastasis and facilitate the development of novel anticancer drugs beyond the realm of traditional TBAs.

βIII-Tubulin in Gliomas

In the human developing and mature CNS, βIII-tubulin is expressed in postmitotic neurons but not in glial, meningothelial, vascular endothelial, or other mesenchymal cells. This neuronal lineage-associated phenotypic expression is recapitulated in neuroblast embryonal tumors of the CNS such as cerebellar medulloblastoma and retinoblastoma as well as other CNS neuronal tumors according to a differentiation-dependent manner. To the degree that βIII-tubulin is not normally expressed in normal resting (mature) glia, the expression of this isotype in glial tumors (gliomas) is considered to be aberrant or a “dedifferentiation” phenomenon associated with the acquisition of progenitor cell– or stem cell–like phenotypes. Although βIII-tubulin is variously expressed in all histologic types and grades of human gliomas, including diffuse astrocytomas and oligodendrogliomas, the expression of this protein is significantly increased in high-grade gliomas, particularly in glioblastoma. βIII-Tubulin was 1 of 6 marker proteins to show significant differences between high-grade and low-grade gliomas in an immunohistochemical study on 378 brain tumors using 37 antibodies in tissue microarrays.

The lack of cell type specificity of βIII-tubulin in the context of cancer underscores its limitation as a diagnostic marker for histologic typing of brain tumors. As exemplified in the rare group of mixed glioneuronal tumors, the detection of βIII-tubulin by immunohistochemistry cannot extricate a neuronal from a glial tumor phenotype nor can it reliably predict aggressive tumor behavior.

Although βIII-tubulin expression in glioblastoma is detected across different morphologic phenotypes, special attention is placed on βIII-tubulin localization in poorly differentiated, small (anaplastic) cells akin to glial precursor cells, oligodendrocyte progenitor cells, or bipotential neural stem cells and also on the increased βIII-tubulin staining in hypoxic tumor areas bordering palisading necrosis. Interestingly, a direct linkage between HIF-2α and increased expression of βIII-tubulin has been recently reported in glioblastoma. However, other studies have failed to validate a relation between hypoxia and βIII-tubulin expression in gliomas.

Targeting βIII-Tubulin in Glioblastoma Therapy

Tackling Dynamic Instability of Microtubules With Microtubule-Stabilizing Drugs

Given the role of βIII-tubulin in drug resistance in solid tumors outside the CNS, a number of approaches were explored, including high-throughput screening and in silico modeling, aimed at downregulating this isotype. This led to the discovery of antitubulin agents such as IDN5390 and other seco-taxanes, as well as epothilones, such as ixabepilone and patupilone (Epothilone B). Epothilones represent a class of microtubule-polymerizing (stabilizing) TBAs developed for the treatment of taxane-resistant epithelial tumors. Similar to taxanes, epothilones alter microtubule dynamics by binding to β-tubulin and stabilizing microtubule polymers, as well as by causing mitotic arrest and apoptosis. Although epothilones target selectively β-tubulin, the factors underpinning microtubule susceptibility to epothilones remain unclear. Both epothilones and taxanes share overlapping, albeit not identical, binding sites on β-tubulin. Initial clinical trials using epothilone B (Patupilone; Novartis) or a novel analogue of epothilone B (Ixabepilone; Bristol-Myers Squibb) had shown promising tumoricidal activity in patients with metastatic breast cancer and NSCLC. It is thought that the significant antineoplastic activity of epothilone B in taxane-resistant tumors may be because of its selective suppression of the dynamic instability of microtubules that are enriched in α/βIII-tubulin dimers enriched in cancer cells, as evidenced by overexpression of βIII-tubulin. Beyond the expected suppression of dynamic instability of microtubules in the mitotic spindles of glioblastoma cells, patupilone antagonizes glioblastoma cell migration and invasion by divergent mechanisms, which include stimulation of “microtubule catastrophes” through EB1 accumulation at microtubule (+)-ends and disruption as well as reorganization of the actin-binding protein α-actinin 4.

In ovarian cancer, patupilone is known to produce a remarkable therapeutic response in subset of patients with ovarian cancer. However, the demonstration of significant downregulation of TUBB3 in patupilone-resistant ovarian
and endometrial cancers coupled by failure of this agent to achieve its primary end point in a large multicenter ovarian cancer study has dampened enthusiasm about patupilone use in this clinical setting.

In brain tumors, patupilone has shown promise in a phase I or II clinical trials for glioblastoma, given its ability to cross the blood-brain barrier. In recurrent glioblastoma, patupilone can be given safely preoperatively and postoperatively, and the treatment has apparently resulted in long-term progression-free survival in some patients. Thus, patupilone is a compound whose potential usefulness deserves further evaluation in combination with currently standard radiochemotherapy in patients with glioblastoma. Interestingly, patupilone has also been proposed as a promising candidate aimed at replacing vincristine as part of a combined treatment strategy with radiotherapy in medulloblastoma.

Another synthetic analogue of epothilone B that crosses the blood-brain barrier is sagopilone (ZK-EPO, Bayer Schering Pharma AG), which has demonstrated preclinical activity in orthotopic rodent models of human glioblastoma lines or metastatic brain tumors. Although sagopilone (ZK-EPO, ZK 219477) was well tolerated, albeit with evidence of peripheral neuropathy, there was no demonstrable antitumor activity in cases of recurrent glioblastoma in a phase II multicenter trial by the European Organisation for Research and Treatment of Cancer Brain Tumor Group. Similarly, ixabepilone was found also not to have a clinically measurable antitumor activity in patients with recurrent high-grade gliomas.

**Targeting Partners and Downstream Effectors of βIII-Tubulin**

It is well established that βIII-tubulin functions like a survival factor rescuing tumor cells from cell death signals triggered by diverse classes of DNA-targeting chemotherapeutic agents, such as cisplatin, doxorubicin, and etoposide. Current treatment strategies exploit the knowledge that βIII-tubulin is part of a prosurvival macromolecular complex that activates a cytoskeletal gateway mediated by GBP-1, a GTPase, with part of a prosurvival macromolecular complex that activates treatment strategies exploit the knowledge that stemness maintenance, miR-210 might be a potential therapeutic target to eliminate GSCs located in hypoxic niches.

**γ-Tubulin and γ-Tubulin Complex Proteins as Targets in Gliomas**

One of the key components required for microtubule nucleation and stabilization is γ-tubulin, a quantitatively minor but functionally important member of the tubulin superfamily, concentrated in interphase cells at the pericentriolar material of centrosomes, the conventional MTOCs. There is approximately 30% identity between γ-tubulin, with molecular weight approximately 48 kDa, and tubulin dimers. In mitotic cells, γ-tubulin is localized predominantly on spindle poles (Fig. 3), but it is also distributed along spindle fibers. During cytokinesis, it is found in midbodies.

In the context of microtubule nucleation, γ-tubulin forms complexes with other proteins that are embedded in the MTOC matrix (Fig. 3). The human γ-tubulin small complex (γTuSC) comprises 2 molecules of γ-tubulin and 1 molecule each of GCPs 2 and 3. Large γ-tubulin ring complexes (γTuRCs) are formed by γTuScs together with other cytoplasmic complex proteins, including GCPs 4, 5, and 6. Besides MTOC-driven microtubule nucleation, γTuRCs are also involved in the regulation of microtubule (+)-end dynamics and in spindle assembly checkpoint signaling. γ-Tubulin is associated also with cellular membranes where it can be involved in microtubule nucleation outside the centrosomes.

The γTuRC is essential for microtubule nucleation in eukaryotic cells, but the elucidation of mechanisms by which γTuRC is activated either at centrosomes or other potential sites of microtubule nucleation in healthy diploid cells or in the context of centrosome abnormalities in cancer cells is evolving. Although a large number of proteins bind to cytoplasmic γTuRCs in both interphase and mitotic cells, only a selected number of proteins endowed with a γTuRC-mediated nucleation activator motif are capable of initiating microtubule-nucleating activity.

Beyond centrosomes, cell membranes, and cytosol, γ-tubulin is also compartmentalized in the nucleus and nucleolus where it modulates the activity of the tumor suppressor protein C53 (cyclin-dependent kinase 5 regulatory subunit-associated protein 3). Nuclear γ-tubulin modulates also the activity of the transcription factor E2F.

Nuclear localization of GCP2 and GCP3 has been also recently detected in the human glioblastoma cell line T98G (E. Drabová, M. Sobol, P. Hozák, and P. Dráber, unpublished data).

In contrast to the α- and β-tubulins, only 2 functional genes (TUBG1 and TUBG2) exist in mammalian cells that code very similar γ-tubulins. Although γ-tubulin 1 exhibits a ubiquitous expression, γ-tubulin 2 is principally distributed in the CNS. Previous studies have shown significant alterations in the expression profile of human γ-tubulins in various human cancers. High levels of...
γ-tubulin have been reported in breast cancer, primary gliomas and glioblastoma cell lines, medulloblastomas and medulloblastoma cell lines, laryngeal carcinomas, and NSCLC. Overexpression of γ-tubulin in tumors may be linked to increased microtubule-nucleating capacity through ectopic or supernumerary MTOCs as well as abnormal centrosome function. The latter may be accompanied by a gamut of structural and functional abnormalities of centrosomes potentially leading to mitotic spindle abnormalities, accounting for chromosomal breaks and missegregation, as well as ultimately culminating in aneuploidy in populations of transformed daughter cells. This process known as “centrosome amplification” could activate or otherwise alter the expression of an array of centrosome-associated molecules, including a number of kinases and tumor suppressor gene products. The docking of numerous key molecules involved in cell cycle progression on the centrosome makes this organelle a critical site for cell cycle regulation. Moreover, γ-tubulin overexpression might affect regulation of microtubule (+)-end dynamics or intracellular signaling or both as it interacts with various protein kinases. γ-Tubulin also modulates DNA damage G2/M checkpoint activation in human glioblastoma cell lines.

In the context of gliomas, we and others have demonstrated that γ-tubulin is overexpressed in glioblastoma and that silencing of γ-tubulin through siRNA inhibition leads to severe mitotic spindle abnormalities and mitotic arrest in T98G human glioblastoma cells. A statistically significant relationship has been reported between high levels of γ-tubulin expression and high tumor grade, poor patient performance status after surgical resection, and a less favorable overall survival status. We have also recently demonstrated overexpression of γTuSC proteins, GCP2 and GCP3, in both glioblastoma tissue specimens and human glioblastoma cell lines. Consequences of amplified expression of proteins involved in microtubule nucleation can include changes in cellular architecture and control of focal adhesion dynamics in terms of force generation for directional cell migration.

![Figure 3](image-url) Localization of βIII-tubulin and γ-tubulin in mitotic DAOY human medulloblastoma cells. Cells in metaphase (A and B), anaphase (C), and telophase or cytokinesis (D) were stained for βIII-tubulin (green, A-D) and γ-tubulin (red, B-D). DNA staining with DAPI highlights cell nuclei (blue). Note βIII-tubulin immunolabeled mitotic spindle microtubules of cells in metaphase and anaphase flanked by 2 γ-tubulin immunoreactive centrosomes (B and C) as well as in the midbody of cytokinesis (D). Formaldehyde fixation followed by Triton X-100 extraction. Scale bar: 10 μm. (Adapted with permission from Caracciolo et al.) (Color version of figure is available online.)
and tumor invasion.19,162,163 An interaction between γ-tubulin complex proteins and kinesin-like proteins is exemplified in the context of regulation of bipolar spindle assembly whereby, in fission yeast, the kinesin-14 Pkl1 binds the γ-TuRC MTOC at spindle poles altering its structure and function.164 The kinesin-14–triggered inhibition of microtubule nucleation is offset by the kinesin-5 protein, Cut7.168 Intriguingly, a yeast kinesin-14 peptide blocks microtubule nucleation in human breast cancer cell lines, denoting that this mechanism is evolutionarily conserved and may represent a potential antimitic target in cancer therapeutics.164

**Interaction of γ-Tubulin and Complex Proteins With Tumor-Activated Signaling Pathways**

The overexpression and abnormal cytoplasmic accumulation and distribution of γ-tubulin and GCP2/GCP3 in glioblastoma cells transcend microtubule nucleation, as it may involve possible interactions of these proteins with tumor-activated signaling pathways or other cellular organelles such as mitochondria, endoplasmic reticulum, and Golgi complexes, which are also activated in cancer.22,26 γ-Tubulin is posttranslationally modified165–167 by phosphorylation166,170 and monoubiquitination.171 Potential partners include tyrosine kinases that may serve as regulators of γ-tubulin function and hence have a role in signal transduction and the regulation of microtubule protein interactions.168,172,173 γ-Tubulin forms complexes with a host of kinases such as Plk1, Wee1, MARK4 (microtubule affinity–regulating kinase 4), Syk tyrosine kinase, Src family tyrosine kinases (Lyn, Src, and Fyn), BubR1 kinase, and phosphoinositide 3-kinase (PI3K).174 Also, γ-tubulin directly interacts with the C-terminal Src homology 2 domain of p85α (regulatory p85α subunit of PI3K) and signaling-cascade proteins such as tyrosine kinases and PI3K could modulate noncentrosomal microtubule nucleation by membrane-associated γ-tubulin.141 A recent study has shown that γ-tubulin forms complexes with GIT1 and βPIX signaling proteins that regulate nucleation of microtubules in mast cells.175

The PI3K/AKT signaling pathway is frequently activated in glioblastoma because of tumorigenic mutations involving key components of the pathway or through regulatory derangements leading to uncontrolled cell growth, increased cell survival and proliferation, tumor angiogenesis, and tumor cell migration and invasion.176 Molecular alterations in this regard include overexpression and mutations of receptor tyrosine kinases, mutations and deletions of the PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene, encoding a lipid kinase that directly antagonizes PI3K activity, and derangements in RAS signaling.176 In glioblastoma, the PI3K/AKT signaling pathway is one of the most extensively studied pathways in terms of therapeutic targeting176,178 and EGFR drug inhibitor resistance.179 Similarly, Src family kinases are implicated in glioblastoma tumorigenesis and are currently therapeutically exploited as molecular targets.180,181

It is noteworthy that γ-tubulin is coexpressed with βIII-tubulin in human glioblastoma cells.86 As stated previously, the βIII isotype of tubulin, which is principally distributed in neurons in the normal human CNS, is aberrantly expressed in neoplastic glial phenotypes (Fig. 4A). Although γ-tubulin and βIII-tubulin are differentially sorted in the mitotic spindles and cytoplasm of interphase tumor cells (Fig. 4B), both proteins form complexes pointing to a functional interaction.86 The possible involvement of γ-tubulin and complex proteins in prosurvival pathways, in a manner analogous to βIII-tubulin (stated previously), remains a tempting conjecture.

**Nuclear and Nucleolar γ-Tubulin: Functional Considerations**

Previous studies have shown that γ-tubulin may be involved in nuclear- and nucleolar-specific functions beyond its *bona fide* role in microtubule nucleation and spindle formation. These functions encompass modulation of transcriptional activity of E2 promoter binding factor (E2F),146,182 complementary interaction with retinoblastoma protein (Rb1) in the regulation of E2F activity in the context of a γ-tubulin/Rb1 signaling network,146,182 a regulatory role in the anaphase-promoting complex or cyclosome, which is a key mitotic and cell cycle regulatory complex,183 and modulation of the activity of tumor suppressor protein C53.145 Suppression of γ-tubulin protein levels in tumors with nonfunctional Rb1 has a proapoptotic effect.182 The C53 protein, which is a caspase substrate, is involved in the regulation of apoptosis triggered by genotoxic stress by way of modulating cyclin-dependent kinase 1-cyclin B1 function.164 Ectopic expression of C53 makes cells susceptible to genotoxins that trigger G2/M arrest, whereas C53 deficiency gives partial resistance to genotoxic agents such as etoposide and ionizing irradiation.184

In summary, the ectopic overexpression of γ-tubulin and γTuSC proteins in high-grade gliomas may be linked to both abnormal microtubule nucleation and pro-oncogenic events involving interactions with tumor suppressor genes and key signaling pathways. Collectively, γ-tubulin and complex proteins emerge as new targets in cancer therapy especially in light of a renewed interest in the mechanisms and signaling networks involved in centrosome amplification in cancer.

**Therapeutic Targeting of γ-Tubulin and Complex Proteins in Glioblastoma**

Because microtubules are nucleated in large amounts during spindle assembly, the development of anticancer drugs targeting microtubule-nucleating complexes emerges as a rational approach in cancer therapy.174 Depletion of γ-tubulin or γTuRC proteins induces changes in microtubule dynamics and spindle defects that resemble the effects of microtubule...
Importantly, depletion of components of the γTuRC by small interfering RNA (siRNA) inhibition sensitizes NSCLC cells at 1000-fold reduced doses of paclitaxel.187

Computer Modeling in Drug Design: Colchicine and Combretastatin A-4

As previous studies have shown an overexpression of γ-tubulin86,150,153 (Fig. 4B and D) and γTuSC proteins GCP2161 (Fig. 4C and E) and GCP3161 (Fig. 4D and F) in human glioblastomas and glioblastoma cell lines, a computational effort was undertaken to identify through virtual screening if inhibitors of γ-tubulin could be found and validated. To this end, molecular dynamics simulations and docking studies were used to analyze the hypothesized γ-tubulin–binding domain (Fig. 5).188 Computational prediction was validated by experimental evidence in protein-based assays that colchicine and combretastatin A-4 bind to γ-tubulin (Fig. 6A-C).188 The suitability of the potential binding modes was evaluated and suggests the subsequent rational design of novel targeted inhibitors of γ-tubulin.188 Subsequently, in a virtual screening study, the interface between GCP4 and γ-tubulin in a multiprotein complex γ-TuRC was targeted using molecular dynamics simulations.189

A stable complex was predicted in silico, and the existence of a binding pocket at the interface between the 2 proteins demonstrated upon complex formation. By combining virtual screening using a fragment-based approach and biophysical screening, several small molecules were found that bind to this pocket. Validation of the inhibitory properties of the obtained compounds was provided by differential scanning fluorimetry. More work is needed to optimize drug design for specific binding locations on γ-tubulin regarding inhibiting the formation of protein complexes involved in microtubule nucleation.

Noscapine Revisited

A recent study has demonstrated that compounds based on the plant alkaloid, such as noscapine, bind with high affinity in a pocket close to γ-tubulin-GCP4 interface; the most robust and stable interaction was observed with the synthetic derivative bromo-noscapine followed by noscapine and amino-noscapine.190 The elucidation of a novel chemical scaffold for γ-tubulin–binding drugs near γ-tubulin-GCP4 interface190 has rekindled interest in the potential role of noscapinoids in glioma therapy, particularly in light of the relatively mild toxicity associated with these compounds. Noscapine is a microtubule poison that alters the dynamic
instability of microtubules causing mitotic arrest and cell death and hence inhibiting cell proliferation.\textsuperscript{191} Accordingly, noscapine and its synthetic derivatives, known as noscapinoids, have potential antineoplastic properties.\textsuperscript{191} Previous studies have shown that noscapine crosses the blood-brain barrier and inhibits glioblastoma growth.\textsuperscript{192} It also exerts an antiangiogenic effect and enhances radiosensitivity of glioma xenograft, resulting in a significant tumor growth delay.\textsuperscript{193}

Posttranslational Modifications of Tubulin: Altered Expression Profiles in Cancer

The functional heterogeneity of the microtubules is attributed to both genetically encoded and posttranslationally generated variations mainly of tubulin C-terminals.\textsuperscript{194} Extensive posttranslational modifications involve both tubulin subunits.\textsuperscript{44,195} Most of them are especially enriched on stable microtubules, which are most notably present in centrioles, cilia, and axons. To date, the most well-characterized posttranslational modifications of tubulin include acetylation, detyrosination, polyglutamylation, and polyglycylation.\textsuperscript{34,196} Detyrosination of $\alpha$-tubulin, polyglutamylation of both tubulin subunits, and polyglycylation of both tubulin subunits occur on the C-terminal domains of tubulins exposed on the outer surface of microtubules, whereas acetylation occurs on the N-terminal domain located in the interior of microtubules.\textsuperscript{30,197,198} The diverse posttranslational modifications are governed by a “tubulin code” that can be read by other proteins or ligands that interact with microtubules.\textsuperscript{199-201} Such modifications could guide the targeting of molecular motors and MAPs to defined subsets of microtubules in various subcellular compartments (stated later). For example, subsets of microtubules that orient toward the leading edge of the polymer are more stable and thus are enriched in acetylation and detyrosination.\textsuperscript{202} Polyglutamylation has the highest potential for generation of complex signals in microtubules.\textsuperscript{203} Glutamylation can also have a microtubule-destabilizing effect by way of regulation of microtubule-severing proteins, such as spastin and katanin (described later). Accordingly, hyperglutamylated microtubules are more sensitive to spastin-mediated severing in vitro.\textsuperscript{204}

In support of the “tubulin code” concept, it has been shown recently that different molecular motors recognize distinctive tubulin “signatures.”\textsuperscript{205} Some kinesin motor proteins associate preferentially with microtubules that are enriched with acetylation and detyrosination enabling kinesin motors to distinguish membrane trafficking events between stable and dynamic microtubules.\textsuperscript{206} Tubulin isotypes and posttranslational modifications govern, in a highly selective manner, motor velocity, processivity, and microtubule depolymerization rates.\textsuperscript{207} The importance and specificity of posttranslational modifications is exemplified by the observations that kinesin-1 motility on $\beta$II-tubulin is increased by polyglutamylation and that kinesin-2 motility requires detyrosination of $\alpha$-tubulin.\textsuperscript{208}
Polyglutamylation of tubulin is a reversible posttranslational modification generated by tubulin-tyrosine ligase–like enzymes.\textsuperscript{207} Altered polyglutamylation is linked to tumorigenesis and resistance to TBAs and represents a potential target in cancer chemotherapy.\textsuperscript{207} To date, polyglutamylase inhibition in the treatment of brain cancer represents uncharted territory. A small number of well-characterized inhibitors of polyglutamylases have been identified, including the phosphinic acid–based inhibitors of Ttll7\textsuperscript{207} (also, described later under the heading of microtubule-severing proteins).

The effect of tubulin acetylation on mitotic progression and cell proliferation has been recently reappraised.\textsuperscript{208} A histone deacetylase class II enzyme (HDAC6), which is principally cytoplasmic,\textsuperscript{209} interacts with microtubules and deacetylates α-tubulin both on preassembled microtubules in vitro and in cells.\textsuperscript{210,211} In the context of breast cancer, this HDAC6-dependent tubulin deacetylation also increases cell motility and contributing to the malignant properties of breast cancer cells.\textsuperscript{210,211} Cellular exposure to HDAC6 inhibition results in hyperacetylated microtubules, which exhibit low dynamics and are accompanied by increased adhesion and reduced cell motility.\textsuperscript{212} Compared with normal mammary ductal epithelial cells, ductal carcinoma cells of the breast exhibit increased HDAC activity accounting for the hypoacetylation of histones and of nonhistone proteins, including tubulin.\textsuperscript{213} The level of protein acetylation may serve as a tumor biomarker predicting the sensitivity of different cancer cell types to HDAC inhibitors used as antineoplastic agents, such as trichostatin A.\textsuperscript{214} Multiple HDAC inhibitors are currently undergoing extensive clinical evaluation as single agents or in combination with other chemotherapeutic agents. A number of HDAC inhibitors including vorinostat, romidepsin, and belinostat have received approval in the United States by the Food and Drug Administration for the treatment of lymphoma. Valproic acid, a commonly used antiepileptic drug, which is also an HDAC inhibitor, was shown to increase overall survival of patients with glioblastoma in combination with radiation therapy.\textsuperscript{215} In vitro, treatment of glioma cells with HDAC inhibitors causes cell death by a G2 checkpoint defect culminating in mitotic catastrophe-induced apoptosis.\textsuperscript{216} A number of clinical trials are underway using HDAC inhibitors in combination with radiation therapy for the treatment of high-grade gliomas (ClinicalTrials.Gov). In addition, tubulin tyrosination and deacetylation inhibitors

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**Figure 6** (A) Colchicine binding to γ-tubulin. (B) Comparison of position of colchicine binding in γ-tubulin (white) and β-tubulin (purple). In both, a ring penetrates furthest into protein. (C) Comparison of γ-tubulin and β-tubulin crystal structures. Root-mean-square deviation of 1.18 Å in the intermediate β-sheets. There is 33% sequence identity and 75% structure similarity of the 2 structures. (Adapted with permission from Friesen et al.\textsuperscript{188}) (Color version of figure is available online.)
have started to emerge as potential therapeutic compounds in cancer.\textsuperscript{207,217,218}

**Microtubule-Based Motor Proteins: Kinesin Targets in Cancer**

Microtubule-based motor proteins play key roles in mitosis including bipolar spindle assembly and delineation of the cell division axis as well as chromosomal alignment and segregation.\textsuperscript{27,219} Bipolar spindle assembly requires force to organize the microtubule network, and to that end, various motor proteins, including Eg5, Kif15, and dynein, act together to build the bipolar spindle.\textsuperscript{220} Excessive inward force results in monopolar spindle formation, whereas excessive outward force generation results in unstable spindles with splayed spindle poles.\textsuperscript{220} Other mitotic motors include the so-called chromokinesins that interact with chromosomes, and the depolymerizing kinesins that promote the shortening of microtubules from their ends.

Other than cytoplasmic dynein, the mitotic microtubule-based motors are members of the kinesin superfamily.\textsuperscript{210} Kinesins are microtubule-based ATP-powered motors, which besides their role in cell division are involved in diverse cellular functions, including intracellular trafficking and transport.\textsuperscript{221,222} Although all kinesins share a conserved catalytic core, individual kinesin members are functionally distinct and have different subcellular localizations.\textsuperscript{219} These divergent behaviors are related to variations in the enzymatic domain as well as the nonconserved regions that lie outside the kinesin motor domains.\textsuperscript{219,223} The latter domains are subject to multiple adaptations that enable them to adjust their mechanochemical properties in accordance with different functional needs.\textsuperscript{223}

The manner by which microtubule binding stimulates the corresponding ATPase and controls force generation is not fully understood. Microtubule binding of kinesin-1 and kinesin-3 motor domains promotes ordered conformations of conserved loops that stimulate ADP release, enhance microtubule affinity, and prime the catalytic site for ATP binding.\textsuperscript{222} In turn, ATP binding induces large conformational changes that permit force generation and neck linker docking toward the microtubule (+) end.\textsuperscript{222} Ultimately, the unique properties of each kinesin motor rest on family-specific differences accounting for different kinesin-microtubule interactions governed by conserved ATP-driven mechanisms.\textsuperscript{223}

Different molecular motors recognize distinctive tubulin “signatures,” which lends credence to the importance and specificity of posttranslational modifications in the context of the aforementioned tubulin code hypothesis.\textsuperscript{194,205} Hence, posttranslational modifications can modulate the behavior of selected molecular motors.\textsuperscript{194,205} For example, kinesin-1 motility on βIII-tubulin is increased by polyglutamylation, and robust kinesin-2 motility requires deetyrosination of α-tubulin.\textsuperscript{205}

Mitotic motor proteins became a favored target for drug development over a decade ago, with the idea being that inhibition of mitotic kinesins would interfere with mitosis without having the side effects on neurons and other cells of the body compared with conventional microtubule-active drugs. Since then, however, it has become clear that mitotic kinesins are repurposed in neurons and other cell types, hence necessitating caution in terms of potentially adverse effects on noncancerous host cells.\textsuperscript{224}

**Anti-Kinesin Drugs**

Dysregulation of mitotic motors in cancer provides the rationale for exploiting these molecules as targets for antitumor drug development and underscores the importance of elucidating the molecular regulatory mechanisms underpinning their function.\textsuperscript{27} In recent years, mitotic kinesins have emerged as potential therapeutic targets in cancer.\textsuperscript{221} Several small molecules that inhibit kinesin family member 11 (Kif11, also known as Eg5 or kinesin-5) and the centromere-associated protein E (CENPE) have entered phase I and II clinical trials, either as monotherapies or in combination with other drugs in the treatment of solid tumors and hematologic malignancies.\textsuperscript{223,225} Additional mitotic kinesins are currently being validated as drug targets, expanding the repertoire of kinesin-based compounds.\textsuperscript{221}

In the context of gliomas, the Kif11 (Eg5 or kinesin-5), which is critical for proper mitotic spindle assembly, has been investigated therapeutically in preclinical studies as a target for cell cycle inhibition in glioblastoma by small molecules such as ispinesib (Monastrol, Merck) and monastrol analogues enastron, dimethylenastron, and vasastrol VS-83.\textsuperscript{226,227} Ispinesib analogue compounds, predicted to cross the blood-brain barrier based on in silico selection, showed an antiproliferative and proapoptotic effects in human glioblastoma cell lines by virtue of causing blockade in the G2/M phase at a nonneurotoxic concentration range and by showing increased caspase 3/7–induced apoptosis in U87MG cells.\textsuperscript{221} In a preclinical study conducted as part of the Pediatric Preclinical Testing Program at Children’s Cancer Institute Australia for Medical Research, ispinesib demonstrated broad but variable in vivo antitumor activity in evaluable solid tumor xenografts in mice, including glioblastoma xenografts, and produced complete responses in rhabdoid tumor, Wilms tumor, and Ewing tumor xenografts, albeit with high toxicity rates at the doses studied.\textsuperscript{228}

Recently, LaSOM 65, a monastrol-derived compound, has emerged as a promising new small molecule inhibitor targeting Kif11 (Eg5 or kinesin-5) in the treatment of glioblastoma as it has antiproliferative and proapoptotic effects on glioblastoma cells in vitro and displays less neurotoxic effects compared with other antimitotic drugs.\textsuperscript{229,230} A robust synergistic antineoplastic effect of Kif11 (Eg5 or kinesin-5) siRNA silencing and hemagglutinating virus of Japan envelope (HVJ-E) vector, a drug delivery system with direct tumoricidal properties, has been
Microtubule targets in glioma therapy

Microtubule-Severing Protein Spastin: A Target of Invasion in Gliomas

Dynamic remodeling of microtubules is essential in cell division, migration, and differentiation. Microtubules in living cells undergo alterations in dynamic assembly and disassembly, bundling and splaying, severing and rapid transport, as well as interactions with other cytoskeletal components. These behaviors may be altered in cancer cells, and in doing so, they may affect the expression and distribution of microtubule-regulatory proteins, including microtubule-severing enzymes.

The microtubule-severing enzymes such as spastin and katanin are part of the family of AAA proteins (ATPases associated with various cellular activities). These ATPases control microtubule organization by severing existing microtubules or by removing tubulins directly from the microtubule ends. The generation of short microtubule fragments through the action of these proteins is critical in the reorganization of microtubules without the need of going through complete microtubule disassembly. One of the functions of the microtubule-severing ATPase katanin is through its involvement in mitosis as evidenced by the higher activity of this enzyme in extracts from mitotically active cells compared with cells in interphase. In the mitotic (M) phase, katanin severs microtubules from the centrosome to permit tubulin subunits to flux through microtubules during chromosome segregation. Spastin is another member of the AAA family, which also severs microtubules. Mutations of the spastin gene are the major cause of hereditary spastic paraplegia. Additional microtubule-severing proteins include fidgetin and VPS4.

In a previous study, we have demonstrated an increased expression of the microtubule-severing ATPase spastin in glioblastoma, making this molecule a potential therapeutic target at the bedside. Supporting this idea is our discovery that spastin depletion substantially inhibits cell motility of glioblastoma cells in vitro accompanied by an enrichment of spastin in the leading edges of cultured glioblastoma cells (Fig. 7A) and populations of neoplastic cells from surgically resected tumor specimens (Fig. 7B). Another microtubule-severing protein called katanin-like-1 has also been reported to concentrate at the leading edge of migratory cells and play a role in cell movements.

The functional profiles of microtubule-severing ATPases may differ among diverse cell types across phylogeny and between nonneoplastic (healthy) and neoplastic (cancerous) cells. To this example, downregulation of katanin in migratory D17 cells, resembling Drosophila hemocytes, isolated from cultures of dissected imaginal discs from Drosophila melanogaster, leads to increased motility. Conversely, silencing of spastin in human glioblastoma cells inhibits cell motility. Herein, we propose that targeting microtubule-severing proteins, such as spastin in vivo, may represent a novel mechanism and strategy to treat diffuse high-grade gliomas, including glioblastoma. Given that microtubule-severing proteins are ATPases, the synthesis of drugs that will specifically inhibit them would seem to be a surmountable challenge, as drugs that specifically inhibit kinesins have been successfully developed.

Aside from abrogating tumor cell proliferation, kinesin inhibitors also adversely affect vascular endothelial function. KiF11 (Eg5 or kinesin-5) and 4 other mitotic kinesins, including KiF20A or Mklp2, are overexpressed in the context of angiogenesis, and KiF11 suppression robustly inhibits tumor angiogenesis in experimental tumor models. Several other members of the kinesin family have been identified as prognostic markers and therapeutic targets in gliomas. Increased expression of KiF2C and KiF14 is associated with high histologic grade and with clinically aggressive behavior in patients with glioma. KiF14 overexpression has been reported in surgically resected high-grade astrocytic gliomas (Fig. 7B). In human gliomas, TPX2 overexpression has been found in various human cancer cell lines by KIF14 and the KIF5 protein, Cut7, which may be exploited potentially as targets in cancer therapy (described later).

The targeting protein for Xenopus kinesin-like protein 2 (TPX2) is a cell cycle–associated protein, and increased TPX2 expression has been found in various human cancers. In human gliomas, TPX2 overexpression has been reported in surgically resected high-grade astrocytic gliomas where it is significantly associated with decreased patient survival. Silencing of the TPX2 gene had antiproliferative and proapoptotic effects on U87MG human glioblastoma cells.

Another emerging target relates to dysregulation (overexpression) of the human Ndc80/Hec1 outer kinetochore protein, which has been linked to aneuploidy and tumorigenesis. Recent studies have identified a ubiquitous Ndc80 internal loop as a protein-protein interaction platform with binding partners including, but not limited to, the TACC-TOG MAPs and kinesin motors, underscoring the role kinetochore-microtubule attachment in cancer cells.

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to be inhibitors of ATP-dependent ligases and carboxypeptidases. An important caveat is that phosphinic acid inhibitors may exhibit target selectivity. For example, the active inhibitor synthesized by Liu et al was selective for glutamylase Ttll7, a β-tubulin-specific polyglutamylase but was devoid of activity against the ATP-dependent enzyme tubulin-tyrosine ligase (Ttl), which targets α-tubulin. The extent to which different phosphinic acid inhibitors influence the activity of other ATPases including microtubule-severing enzymes and molecular motors remains to be determined. However, it is potentially attainable to modify the chemical structures of the phosphinic acid inhibitors to enhance their efficacy by virtue of embedding the inhibitors into tubulin C-terminal peptides to potentiate their action.

Targeting Tubulin-Binding Site on the Neurofilament Light Protein

A strategy for the peripheral delivery and internalization of drug compounds into brain tumor cells was developed through the use of lipid nanocapsules coated with a cell-internalizing peptide (NFL-TBS.40-63 peptide), an active ligand that interacts with tubulin-binding sites (TBSs). Previous studies have shown that glioma cells maintained in cell culture internalize the NFL-TBS.40-63 peptide corresponding to the sequence of a TBS on the neurofilament light (NFL) protein, which disrupts the microtubule network, inhibits migration and proliferation, and leads to apoptosis. The antineoplastic effects of this small molecule have been validated in an orthotopic xenograft rodent model, whereby a single intratumor injection significantly attenuates tumor growth, while neither peptide uptake nor adverse effects are observed elsewhere in the host nervous system. Mechanistically, the uptake of NFL-TBS.40-63 peptide by glioblastoma cells occurs mainly through endocytosis and does not entail transmembrane translocation or involvement of glycosaminoglycans and αVβ3 integrins in the peptide recognition and internalization by tumor cells. In contrast, there is involvement by tyrosine kinase receptor signaling, especially via EGFR overexpression in tumor cells, denoting that the uptake of NFL-TBS.40-63 peptide by glioblastoma cells relates to high proliferative activity.

Because of its tumor-selective uptake, the NFL-TBS.40-63 peptide is a potentially promising treatment modality in the context of glioblastoma based on its dual microtubule-disrupting and tumorcidal actions as well as its capacity to selectively target glioma cells as a vector containing small molecule inhibitors.

Stathmin: A Target of Glioma Invasion and Neoangiogenesis

Stathmin is a microtubule-destabilizing phosphoprotein, which is involved in the crosstalk and signaling between microtubules and the actin cytoskeleton (reviewed in). Stathmin has a key role in cell motility and migration of normal and neoplastic cells and is involved in tumor metastasis. Stathmin-induced cell motility rests on the action of this protein on the leading edge of growing microtubules. Moreover, this protein is involved in the RhoA/ROCK signaling pathway by way of interacting with p27kip1, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. In the context of peripheral (sympathoadrenal) neuroblastoma, stathmin overexpression has been linked to advanced and aggressive disease. Similarly, a statistically significant relationship between stathmin overexpression and metastatic tumor
dissemination has been reported in medulloblastoma. Suppression of stathmin expression modulates the phosphorylation of the actin-regulatory proteins, coflin and MLC via ROCK signaling. Suppression of stathmin expression inhibits tumor cell migration and invasion in vitro and blocks metastatic disease to the lungs in an orthotopic xenograft model of neuroblastoma.

In the context of gliomas, stathmin (gene: STMN1) is a target of microRNA-9, a CNS-associated microRNA; in glioblastoma cell lines, microRNA-9 modulates cell proliferation, vasculogenic mimicry, and tumor volume growth by virtue of controlling STMN1 expression. Silencing of stathmin expression through siRNA inhibition mitigates invasion and increases chemosensitivity of cancer stem cells derived from glioma cell lines. In addition to its role in glioma tumorigenesis, stathmin is involved in mechanisms underpinning neoangiogenesis in glioblastoma. Previous studies have shown that the p27-mediated blockade of neoangiogenesis is linked with the ability of p27Kip1 to bind to and inhibit stathmin. Downregulation of stathmin suppresses tumor angiogenesis in glioblastoma.

Mechanistically, STMN1 negatively modulates LRRC4, a tumor suppressor gene that is commonly inactivated epigenetically in gliomas. Induction of LRRC4 expression has an antiproliferative effect in glioma cells. Previous studies have shown that LRRC4 inhibits the proliferation of human glioma cells by modulating the expression of STMN1 and microtubule polymerization. In this regard, LRRC4 acts as a major glioma suppressor causing cell cycle arrest and modulating microtubule dynamics.

Given that anaplastic oligodendrogliomas with loss of heterozygosity on chromosome 1p (1p+/−) frequently respond to procarbazine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and vincristine, the possible role of the 1p-encoded protein stathmin in this regard was previously investigated. Decreased stathmin expression in tumors was significantly associated with loss of heterozygosity in 1p and increased recurrence-free survival in human patients with anaplastic oligodendroglioma, suggesting that loss of heterozygosity for the stathmin gene may be associated with improved outcomes of patients with 1p+/− anaplastic oligodendroglioma tumors. Stathmin expression was found to be inversely associated with overall survival of nitrosourea-treated mice carrying xenograft tumors; moreover, the nitrosourea-induced mitotic arrest in glioblastoma cells was potentiated in cells with decreased stathmin expression. It is also worthy of note that 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea inhibits the stathmin-mediated cell migration and invasion of glioblastoma cells in vitro. Nitrosoureas, which are traditionally viewed as DNA alkylating agents, can also covalently modify proteins such as stathmin.

Collectively, microRNA-9 and stathmin have emerged as molecular targets in glioma therapy. Although the mechanism(s) by which stathmin affects tumor cell migration, invasion, and metastasis require further elucidation, one envisaged strategy is to target the actin cytoskeleton via its interacting partners of the microtubule cytoskeleton; this may conceivably circumvent the significant toxicity associated with actin-targeting therapies.

### End-Binding Proteins as Therapeutic Targets in Glioblastoma

The roles of the microtubule-stabilizing proteins end-binding protein 1 (EB1) and ATIP3 in therapeutic targeting in cancer have been critically reviewed elsewhere. EB1 is a microtubule (+)-end-binding protein that colocalizes and interacts with both cytoplasmic and spindle microtubules in interphase and mitotic cells respectively, effectively acting as a negative inhibitor of microtubule stability. EB1 promotes tumor cell migration by contributing to the generation of stable detyrosinated microtubules. ATIP3 is another potent microtubule-stabilizing protein whose depletion increases microtubule dynamics. ATIP3 is a prognostic marker for overall survival in breast cancer, where low ATIP3 levels in metastatic tumors are associated with decreased patient survival. Functionally, ATIP3 was found to mediate cell motility and directionality as well as influence the number and size of metastases.

In the context of gliomas, EB1 expression and subcellular distribution is affected by the action of TBAs. Previous studies have shown that patupilone (epothilone B) at non-cytotoxic concentrations inhibits migration of glioblastoma cells by inducing microtubule catastrophes and causing reduced accumulation of EB1 and other microtubule (+)-end-tracking proteins at microtubule (+) ends without significantly affecting microtubule growth rate or other microtubule dynamic instability parameters. Vinflunine, a microtubule-targeting drug of the vinca alkaloid family exerted its antiangiogenic or antimigratory activities through an increase in microtubule dynamics and an inhibition of microtubule targeting to adhesion sites. Such effect was associated with a reduction of EB1 comet length at microtubule (+) ends and inhibition of cell migration. Thus, vinflunine inhibits endothelial cell migration through alterations of EB1 comet length and detyrosination-retyrosination cycle.

Collectively, these data indicate that EB proteins may represent another potential target in the therapy of the notoriously invasive diffuse gliomas and glioblastoma.

### Hypersensitization to Tubulin-Binding Agents and Multitarget Hits

As exemplified in NSCLC, tumor cell hypersensitization to TBAs by way of RNA silencing of βIII-tubulin expression has emerged as an attractive model of targeted cell therapy. Although βIII-tubulin knockdown by itself may not result in significant alterations in microtubule dynamic instability, it may significantly enhance the effectiveness of TBA by virtue...
of 2 distinct mechanisms, namely, suppression of microtubule dynamics at low drug concentrations and a mitosis-independent mechanism of cell death at higher drug concentrations. This was confirmed in a subsequent study using NSCLC cell lines, which demonstrated that βIII-tubulin knockdown induced increased sensitivity to epothilone B in contrast to knockdown of βII-tubulin that did not produce a similar sensitization effect. Moreover, βIII-tubulin knockdowns exhibited a marked proapoptotic effect in a dose-dependent manner after treatment with epothilone B as compared with βIV-tubulin knockdowns, which required higher concentrations of epothilone B to induce apoptosis. The fact that βIII-tubulin knockdown renders tumor cells more sensitive to epothilone B and such knockdown does not significantly alter microtubule dynamics lends credence to the notion that βIII-tubulin is a prosurvival protein, which exerts its effects independent of the microtubule structure.

Recent evidence suggests that βIII-tubulin expression in the human glioblastoma cell line U87VI contributes to chemoresistance to the DNA-damaging agent temozolomide, which is currently the first-line therapy for glioblastoma, and tubulin-binding agents epothilone B and paclitaxel. Suppression of βIII-tubulin in glioblastoma cells confers increased sensitivity to these drugs.

Similar sensitization effects can be produced potentially following silencing of genes encoding other microtubule proteins of interest described in this review. For example, depletion of the microtubule-severing protein spastin, which inhibits tumor cell motility in glioblastoma cells in vitro, may synergize with chemotherapeutic agents currently used in the treatment of glioblastoma, known to suppress proliferation, and may also synergize with other Food and Drug Administration-approved cancer drugs. One approach is to decipher the antineoplastic effects of downregulation of specifically targeted molecules of interest in combination with currently used mainstay therapies including radiotherapy and chemotherapy (temozolomide and cisplatin) and other microtubule-acting compounds. Another experimental therapeutic approach is to elucidate the sensitization effect on specific classes of TBAs or other compounds, such as ATPase inhibitors, by way of double or multiple silencing hits aimed at potentiating a combined inhibitory effect on the growth and migration of glioma cells in vitro and also in preclinical in vivo models (orthotopic xenografts).

**Microtubule Toolbox in Cancer and Health**

The notion that cells use a common toolbox to perform an array of functions has gained appeal among cell biologists for some time. The microtubule toolbox contains molecular motors, stabilizers, severing proteins, and various other associated proteins that shape and sway the properties and behaviors of microtubules. Different cell types might reach for a different tool to accomplish a cellular task. For example, some cell types may use the microtubule-severing enzymes predominantly to cut microtubules in their more stable regions whereas other cells may use it to trim the (+) ends of highly dynamic microtubules. Attention is drawn to the fact that the use and function of diverse microtubule proteins may significantly differ in healthy (nonneoplastic) glia and their neoplastic (gliomatous) counterparts. Thus, the differential use of a broad gamut of proteins within the versatile microtubule toolbox becomes further complicated in cancer cells that are genetically and epigenetically dysregulated and in need to deploy alternate mechanisms to meet their altered requirements for cell cycle progression, survival, invasion, and metastasis. An illustrative example in this regard relates to the microtubule-nucleating protein γ-tubulin, which is overexpressed and differentially sorted in various subcellular compartments of cancer cells where it can function also as a transcription factor and interact with oncogenes and kinases. Molecular targeting of microtubule proteins and its potential translation to the bedside require critical insights into the complex pathobiology and pharmacology of microtubules in cancer cells. Emerging interdisciplinary work opens new exciting opportunities to the microtubule field, which may eventuate in the discovery of novel and tangible modalities in the treatment of diffuse gliomas and glioblastoma.

**References**

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