Molecular Mechanisms of Taxol Induced Cell Death in Glioblastomas

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Summary

Glioblastomas, especially glioblastoma multiforme are the most frequent and devastating brain tumors in adults. Molecular and cytogenetic studies of glioblastomas revealed a wide variety of deregulated genes associated with cell cycles, DNA repair, apoptosis, cell migration, invasion and angiogenesis with little translational success. Understanding the molecular mechanisms of these deregulated genes provides rationale for targeting specific pathways of repair, signaling, and angiogenesis. Taxol, one of the most potent anti-neoplastic drugs, strongly binds to the N-terminal region of β-tubulin to prevent tumor cell division and induce cell death. The effects of taxol may vary depending on cell type and drug concentration. At lower concentrations ranging from 10-100 nM, taxol induces phosphorylation of Bcl-2, which in turn triggers mitochondrial release of cytochrome c, cleavage of procaspases and poly (ADP-ribose) polymerase (PARP), leading to apoptotic cell death. Phosphorylation of Bcl-2 also inhibits the ability of Bcl-2 to lower intracellular Ca²⁺, which triggers calpain-mediated apoptosis. At higher concentrations, taxol induced cell death is associated with stabilization of microtubules and mitochondrial collapse leading to G2/M cell cycle arrest. Disruption of the mitotic spindle activates a number of signaling pathways, with consequences that may protect the cell. The cells arrested in mitosis exhibit no signal for apoptosis but have an increased expression of survivin, an inhibitor of apoptosis. A strong understanding of the molecular signaling events associated with taxol mediated cell cycle arrest is essential, particularly in regard to its potential for combination therapy—the use of multiple agents to enhance the efficacy of cancer treatment. Here we present a critical review of research on the molecular signaling mechanisms of taxol, its relevance to apoptosis, and its potential for combination with chemotherapy and emerging gene therapy.

Key words: Glioblastomas, taxol, paclitaxel, apoptosis, gene therapy
Malignant brain tumors

Malignant brain tumors (malignant gliomas or glioblastomas) are highly invasive and aggressive primary brain tumors and are associated with a dismal prognosis (1). As per American Cancer Society statistics (Cancer Facts & Figures, 2008), there will be an estimated 21,810 new cases of primary brain tumors, with an estimated 13,070 deaths in the United States in 2008. Glioblastomas comprise 23% of primary brain tumors and are the most commonly diagnosed brain tumor in both adults and children (2). Glioblastomas remain highly refractive to therapy, and current treatments produce no long-term survivors in patients with these tumors. The mean survival time of patients with glioblastoma treated with surgery, radiotherapy, and chemotherapy is from 9 to 12 months (3). Since malignant brain tumor cells often infiltrate deep into the normal tissue, complete surgical removal of the brain tumor is almost impossible, contributing to the high incidence of recurrence (4). The traditional means of glioma therapy are plagued with numerous side effects and subsequent poor quality of life during the course of treatment. Although understanding of the glioblastoma pathophysiology has increased significantly over the past few years, an effective treatment has not been developed for this devastating cancer. Limits to the efficacy of current treatment modalities call for the development of novel therapeutic strategies targeting the specific biological features of glioblastomas.

Causes of Brain Tumors

Generally, cancers are associated with one or more risk factors, but the only known environmental risk factor for brain tumor is ionizing radiation (5,6). People receiving radiotherapy (high-dose ionizing radiation) to the head during childhood are at increased risk for developing brain tumors. Primary brain tumors may also result from specific genetic diseases or cancer-causing chemicals such as vinyl chloride, N-nitroso compounds and polycyclic aromatic hydrocarbons (7,8). Brain tumors sometimes occur in several members of the same family, which suggests the involvement of some genetic cause. Nevertheless, the exact causes of most primary brain tumors remain a mystery. It is clear that primary brain tumors are not contagious. There is no relation between primary brain tumors and smoking (9), diet (10), use of cellular phones (11) or electromagnetic fields (12). Brain tumors occur more often among white people than among people of other races (13). A single cell gene mutation or deletion of a tumor suppressor gene may trigger an abnormal cell division, which finally forms an intracranial tumor. The risk of developing brain cancer increases with age. The rate for people under age 65 is 4.5 for every 100,000 people in the United States compared to 17.8 for persons 65 and older. Patients with a history of metastatic cancers, such as melanoma, lung, breast, colon, or kidney cancer, are at risk for secondary brain tumors.

Treatments for Glioblastomas

Glioblastomas are difficult to treat because of the location of the tumors. Moreover, malignant brain tumor cells are highly resistant to chemotherapy and other conventional therapies. None of the present three treatment regimens—surgery, chemotherapy and radiation—is effective for any types of brain tumor. Since the highly invasive malignant glioma cells penetrate deep inside normal tissue, it is practically impossible to remove the tumors completely through surgery. Almost all malignant glioma will recur within 3 months after surgery. Chemotherapy may not be effective for brain tumors because the chemotherapeutic agents do not pass through the blood brain barrier effectively (14). Additionally, chemotherapy has numerous undesirable side effects, resulting in poor quality of life for the patients. Radiation therapy for glioblastomas also affects normal cells adversely, causing severe side effects to the patient and further inducing the formation of primary brain tumors. However, advances in microsurgery techniques, radiotherapy and chemotherapy are slowly increasing the survival time of patients diagnosed with
Chemotherapy for Glioblastomas

Chemotherapy is moderately effective in controlling the growth of malignant gliomas. At present, several different types of chemotherapeutic agents are available for the treatment of glioblastomas. However, chemotherapy results in serious clinical problems that adversely affect both the quality of life and ability of patients to continue treatment. Generally, chemotherapy for primary brain tumors begins only after surgery and radiation. Almost all cancer chemotherapeutic agents are based on the principle of impairing mitosis, effectively targeting fast-dividing cells. Although chemotherapy is targeted against fast-dividing tumor cells, it also affects normal cell division and may lead to several side effects. The specific features of tumor cells that make them uniquely targetable to chemotherapeutic agents have yet to be identified. Specifically, the cells that can be affected with chemotherapy are those in the bone marrow and the cells that line the gastrointestinal tract. Chemotherapy may also affect both male and female gametogenesis and can produce defective sperm or ovum (15). Certain chemotherapeutic agents may induce permanent sterility in males. Furthermore, many chemotherapeutic agents are also neurotoxic, nephrotoxic and ototoxic. The toxicity of the anticancer agents also arises from the solvents used to dissolve them (16). Another drawback of chemotherapy is the development of drug resistant cells within the tumors and inadequate drug delivery into the brain due to the presence of the blood brain barrier. Temodar (temozolomide) and taxol (paclitaxel) are two chemotherapeutic agents currently in use for the treatment of glioblastomas. It has been demonstrated that temozolomide is more effective and powerful than taxol for the treatment of malignant brain tumors. Since temozolomide is an alkylating agent, it demethylates the promoter region of the gene for O-6-methylguanine-DNA methyltransferase (MGMT), an important DNA repair enzyme that removes methyl adducts at the O-6-position of guanine, one of the most prominent and biologically important targets of alkylating agents. MGMT function is frequently lost due to the hypermethylation of CpG islands in the promoter region of this enzyme in many types of human anaplastic astrocytomas, including malignant gliomas (17). Temozolomide treatment has further advantages because several other important genes involved in cell cycle regulation and apoptosis are also silenced in malignant gliomas due to hypermethylation of their promoter CpG islands (18-20).

Taxol

Taxol (paclitaxel) was first isolated from the bark of the Pacific yew tree, Taxus brevifolia in 1967 by Monroe E. Wall and Mansukh C. Wani (21) at the Research Triangle Institute, NC, USA. Figure 1 illustrates the molecular structure of taxol. In 1979, Susan B. Horwitz, at Albert Einstein College of Medicine, Bronx, New York, showed that the mechanism of action of taxol involves the stabilization of microtubules (22). Robert A. Holton at Florida State University first succeeded in the total synthesis of taxol in 1994 (23,24). Taxol strongly binds to the N-terminal region of the β-subunit of tubulin and promotes the formation of highly stable microtubules that resist depolymerization, thus preventing active tumor cell division and arresting the cell cycle at the G2/M phase (25,26). The arrest of microtubules inhibits the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitosis. Even though it has been well established that taxol inhibits cell division through mitotic arrest, it is unclear whether taxol-induced cell death also represents a secondary event resulting from mitotic arrest or involves a novel mechanism of action.

Molecular mechanisms of taxol induced cell death

A) Mechanism of cell cycle arrest
The exact mechanism of taxol cytotoxicity against tumor cells is not entirely clear. Unlike classical antimicrotubule agents, such as colchicines and vinblastine that induce microtubule disassembly and/or paracrystal formation (27), taxol inhibits microtubule depolymerization and promotes the formation of highly stable microtubules, thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation (28,29).

Tubulin is a member of the family of globular proteins, which mainly includes α-tubulin and β-tubulin. Microtubules are polymers assembled from dimers of α- and β-tubulin. During polymerization, the heterodimer formed from α- and β-tubulin binds to two molecules of guanosine triphosphate (GTP): a non-exchangeable GTP molecule at the α-subunit, which plays a structural stability role (30); and an exchangeable GTP molecule bound to the β-subunit.

Upon assembly of the α/β-tubulin heterodimer, GTP bound to β-tubulin is hydrolyzed to GDP, reaching a steady-state equilibrium between free tubulin dimers and microtubules (31). In the GDP-bound state, the protein is in an inactive conformation, forming double rings (32), whereas in the GTP state, it is active for microtubule assembly. GTP-GDP hydrolysis in the heterodimers controls the assembled state of tubulin (33). Taxol drives inactive GDP-tubulin into microtubules, replacing the need of the γ-phosphate of GTP to activate the protein (34). Taxol stabilizes microtubules by binding preferentially to assembled tubulin with an exact 1:1 stoichiometric ratio (34). Unpolymerized tubulin has no significant affinity for taxol (35), indicating that the binding site is formed during the polymerization process.

We treated U-251MG human glioblastoma cells (National Cancer Institute, Frederick, MD) with 100nM taxol (Bristol-Myers Squibb, Princeton, NJ) for 48 h in culture and analyzed them by flow cytometry. Figure 2 shows the FACS histogram of U-251MG cells after treatment with 100 nM taxol. In untreated U-251MG cultures, 75% of cells were in G1 phase and 6% were in G2/M phase, leaving 19% of cells in the S phase. However, after taxol treatment, exactly 75% of cells were arrested in G2/M phase with 18% in the G1 phase, leaving only 7% in the S phase. Since most of the apoptotic/dead cells are removed during processing for flow cytometry, the percentage of apoptotic cells after taxol treatment is lower compared to our previous reports (36).

B) Mechanisms of taxol induced apoptosis

Apoptosis, the terminal end of programmed cell death, is well characterized by morphological and biochemical features (37). Several lines of evidence from recent studies have suggested that taxol-induced apoptosis may occur through a signaling mechanism independent of microtubule and mitotic arrest (38,39). Dziadyk et al. showed that paclitaxel-induced apoptosis is mediated or regulated through the NF-kappaB/IkappaB signaling pathway (40). The c-Jun N-terminal kinase (JNK) signaling pathway also plays an important role in taxol mediated apoptosis (41,42). It is now well established that taxol triggers apoptosis by both caspase dependent (43-45) and caspase-independent pathways (46,47).

We treated U-251MG glioblastoma cells with 100 nM taxol for 72 h and stained for m-calpain (calpain-2) and cleaved fragment (active subunit) of caspase-9 using double immunofluorescence staining (Figure 3). There was a marked upregulation in the expression of both m-calpain and the cleaved fragment of caspase-9 with several apoptotic cells. The upregulation of calpain-2 and the cleaved fragment of caspase-9 after taxol treatment indicates that taxol triggers caspase dependant apoptotic signaling pathways. It has been demonstrated that taxol treatment upregulates tumor necrosis factor-α (TNF-α) (48) and tumor necrosis factor-related apoptosis, inducing ligand (TRAIL) (49). In one of our experiments (36), we have shown an increase of intracellular free Ca^{2+} after taxol treatment in U-251MG cells in culture. An increase of intracellular free Ca^{2+} upregulates m-calpain, which in turn triggers caspase
mediated pathway and apoptosis. The binding of taxol to microtubules exerts ER stress, which causes an influx of free Ca\textsuperscript{2+} into the cytoplasm. Taxol also phosphorylates Bcl-2, which accelerates the release of cytochrome c from mitochondria to cytosol (50,51) and initiates the formation of an apoptosome along with apoptotic protease-activating factor-1 (apaf-1) in the presence of adenosine nucleotides (52). In non-neuronal cells, taxol-induced apoptosis requires activation of N-terminal c-Jun protein kinase (JNK) that phosphorylates and inactivates Bcl-2. Figure 4 shows a schematic representation of the molecular mechanisms of taxol’s triggering both caspase dependent and caspase independent apoptotic signaling pathways and apoptosis. We have also demonstrated increased cleavage of DNA Fragmentation Factor-45 (DFF-45) and poly (ADP-ribose) polymerase (PARP), which released their active subunits after U-251 MG cells in cultures were treated with taxol (36).

**Formulation of Nanotaxol**

Several attempts to formulate nanotaxol have been undertaken to deliver the drug more efficiently for the treatment of various cancers, including glioblastomas (53,54). However, none of the available techniques efficiently delivers taxol into the brain. Recently, Eugene R. Zubarev and his group from Rice University, Texas, discovered a method to load dozens of molecules of paclitaxel onto tiny gold spheres many times smaller than living cells (55). Figure 5 demonstrates the formulation of nanotaxol, where several molecules of paclitaxel are covalently functionalized with 2 nm gold nanoparticles (AuNP). Here a flexible hexaethylene glycol linker is attached to the paclitaxel at the C-7 position, and the resulting linear analogue is coupled to phenol-terminated gold nanocrystals. There are about 70 molecules of paclitaxel covalently linked per 1 gold nanoparticle. This technique provides a rare opportunity to prepare hybrid particles with a well-defined amount of paclitaxel and offers a new alternative for the design of nanosized drug-delivery systems. This approach also allows a more accurate measurement of therapeutic activity as a result of the increased ability to quantify the amount of drug present.

**Molecular Gene Therapy for Glioblastomas**

Gene therapy involves the use of nucleic acids, which include both DNA and RNA for treatment. Gene therapy could modify the genetic make-up of the target cells, which is not possible to achieve with any other treatment modality. This novel therapeutic strategy will be used in combination with traditional treatment techniques to prolong the lifespan of patients and, ultimately, control and/or cure brain tumors. Novel, more efficient and less toxic molecular techniques are under development: for example, mammalian expression and viral vectors to deliver small interfering RNAs (siRNAs) that silence oncogenes and antiapoptotic molecules; or putative therapeutic genes delivered into the central nervous system. Such advances would constitute a new treatment paradigm and alternative modalities to control the devastating glioblastoma. Since the traditional treatment strategies for glioblastoma or any other cancer are not effective and are plagued with undesirable side effects, gene therapy is a particularly promising approach for the treatment of all cancers, including malignant gliomas. Since now we have better understanding of the pathophysiology of glioblastomas, including the mechanism of tumor invasion and angiogenesis, it is possible to target the pathogenetic oncogenes or reactivate the silenced tumor suppressor genes through the delivery of functional genes or nucleotide sequences via efficient synthetic vectors carrying powerful promoters. A large number of mammalian expression vectors carrying the gene for coral green fluorescent protein (cGFP) and luciferase are available for the efficient monitoring of gene delivery, both in vitro and in vivo. With the introduction of effective and powerful non-invasive animal imaging systems (eg: Xenogen, Bioscan), it is possible to monitor the regression of tumors in experimental animals following the successful delivery of mammalian expression or viral vectors carrying the nucleotide sequences or the gene of interest. With the advent of positron emission tomography
(PET) in conjunction with single photon emission computed tomography (SPECT), it could be possible to track the delivery of plasmid and viral vectors into human organs or tissues for various gene therapeutic applications. Tumor cells evade immunosurveillance through active participation in inducing tumor-specific immunosuppression, which facilitates easy entrance of plasmid and viral vectors into tumor cells. However, the high level of heterogeneity that exists among tumor cells may present significant challenges to the uniform delivery of such vectors into cancer cells.

Gene therapy for cancer mainly involves either expressing the silenced tumor suppressor genes (eg: p53, PTEN) or suppressing the oncogenes (eg: Ras, cMyc). Gene therapy also involves silencing several molecules that promote tumor cell invasion (eg: MMP-9) and angiogenesis (eg: VEGF) through antisense oligonucleotides or through mammalian expression plasmid vectors carrying cDNA for specific siRNAs. Furthermore, gene therapy is used to express several important genes silenced in tumor cells due to hypermethylation of promoter regions (56,57). However, expression or suppression of a single gene or protein will do little to help in the treatment malignant gliomas or any other type of cancer. Gene therapy must therefore include simultaneous use of several genes/molecules involved in the pathogenesis of cancers in conjunction with traditional therapeutic approaches. There are few reports of combining gene therapy with conventional treatment modalities. Combination treatment of synthetic Bcl-2 siRNA and a low-dose of cisplatin resulted in a massive induction of apoptotic cell death, with almost complete suppression of cell growth in malignant melanoma (58). Plasmid vectors expressing Bcl-2 and Bcl-xL shRNAs sensitize human hepatoblastoma cells to the chemotherapeutic drugs 5-fluorouracil and 10-hydroxycamptothecin. These results suggest that Bcl-2 and Bcl-xL siRNA-mediated gene silencing in combination with chemotherapy would be a potential therapeutic strategy against human hepatoblastoma (59). A patient with glioblastoma multiforme who is alive and disease-free 13 years following aggressive treatment with multiple surgeries, radiotherapy, chemotherapy, and gene therapy has been reported recently (60). Studies in our laboratory demonstrated that sequential intraperitoneal administrations of mammalian expression plasmid vector carrying Bcl-xL siRNA/cDNA (50 μg DNA/injection/mouse) in conjunction with low doses of taxol (50 μg/injection/mouse) resulted in notable regression of tumor formation in the intracerebrum of immunosuppressed mice (Figure 6). Even though the treatments with either Bcl-xL siRNA or taxol resulted in significant reduction of tumor volume compared with the scrambled Bcl-xL siRNA treated animals, the combination treatment of both agents resulted in a synergistic effect. We have also observed complete inhibition of in vivo angiogenesis (dorsal skinfold chamber model) and remarkable regression of both intracranial and subcutaneous tumorigenesis in nude mice after combination treatment with taxol and Bcl-2 siRNA (61). Previous studies demonstrated that intraperitoneal administration of taxol is more effective than intravenous administration both in patients and experimental animals (62,63). Our studies along with the previous reports indicate that the intraperitoneal route is appropriate for gene therapy in conjunction with anticancer drugs. However, the requirement of a large amount of substances is a rate limiting factor.

Even though many different anticancer gene therapy approaches are being developed, it is unlikely that any of these strategies would effectively treat or cure cancer. Gene therapy will likely be successful when several different strategies are used in combination. Furthermore, gene therapy should be used in conjunction with traditional cancer therapeutic approaches, such as surgery, chemotherapy and radiation. Along with recent advances in conventional cancer treatment modalities and effective non-invasive imaging systems such as PET, gene therapy is a promising tool for the effective treatment and cure of devastating cancers, including glioblastomas.
Figure 1. Chemical structure of paclitaxel (C_{47}H_{51}NO_{14} MW 853.91).

Figure 2. Flow cytometry (FACS) histogram of U-251MG human glioblastoma cells after treatment with 100 nM taxol for 48 h. Untreated and treated cells (Corning 6-well culture plates) were harvested using TrypLE (Invitrogen, Carlsbad, CA) after washing twice with serum free media. The cells were centrifuged and washed again with PBS. The cells were then dispersed in 1 ml of propidium iodide (50 μg/ml) (Biosure, Grass Valley, CA) with gentle vortex and incubated for 30 min in darkness at 4°C. The cells were sorted on a FACS machine (FACSCalibur, Becton and Dickinson, Franklin Lakes, NJ) based on the red fluorescence at 488 nm. (A) Untreated cells. Note 75% of cells are in G1 phase. (B) Cells after taxol treatment. Note 75% of cells are arrested at G2/M phase after taxol treatment. Most of the apoptotic/dead cells are removed during washing. Data are representative of 5 independent experiments.
Figure 3. Double immunofluorescence staining to examine the expression of m-calpain and cleaved fragment of caspase-3 after treating U-251MG human glioblastoma cells with 100 nM taxol for 72 h. The cells cultured on chamber slides were fixed with 95% ethanol and blocked with 2% goat and 2% donkey serum in PBS (50:50) for 1 h. The cells were washed and treated with rabbit polyclonal m-calpain (Cell Signaling Technology, Danvers, MA) and goat polyclonal cleaved caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies simultaneously and incubated overnight at 4°C. The cells were washed and incubated with FITC conjugated goat anti-rabbit and Texas red conjugated donkey anti-goat secondary antibodies (Biomeda, Foster City, CA) at room temperature for 1 h for the detection of m-calpain and caspase-3, respectively. Electronic merging of the stained images demonstrated the simultaneous expression and co-localization of m-calpain and caspase-3. Arrows indicate apoptotic cells.
Figure 4. Molecular mechanisms of taxol induced cell death in glioblastomas. Taxol strongly binds to the β-subunit of tubulin and promotes the formation of highly stable microtubules, which results in cell cycle arrest at the G2/M phase and induces cell death. Taxol also triggers death signals that cause endoplasmic reticulum (ER) stress and upregulation of Bax. Taxol binding to the microtubules also leads to ER stress and increases intracellular free [Ca^{2+}] that upregulates calpain. Furthermore, taxol upregulates tumor necrosis factor-α (TNF-α) and TRAIL, which triggers the extrinsic caspase pathway through TNF receptor-1 associated death domain (TRADD). Bcl-xL phosphorylation accelerates the release of cytochrome c from mitochondria to cytosol. The association of cytosolic cytochrome c with procaspase-9 and Apaf-1 processes procaspase-9 to its active form, which then triggers the intrinsic pathway of apoptosis. Procaspase-3 is cleaved to its active form by calpain, caspase-9, and caspase-8. The active caspase-3 in turn cleaves α-fodrin, DFF45, and PARP leading to DNA fragmentation and apoptosis.
Figure 5. Formulation of nanotaxol. The anticancer drug paclitaxel is covalently functionalized with 2 nm gold nanoparticles (AuNPs). The synthetic strategy involves the attachment of a flexible hexaethylene glycol linker at the C-7 position of paclitaxel followed by coupling of the resulting linear analogue to phenol-terminated gold nanocrystals. (Reproduced with permission from Prof. Eugene R. Zubarev, Department of Chemistry, Rice University, Texas).
Figure 6. Synergistic effect of Bcl-xL siRNA and taxol in the inhibition of intracranial tumor in immunosuppressed mice. U-251MG human glioblastoma cells were stably transfected with a mammalian expression vector (phCMV-FSR, Genlantis, San Diego, CA) carrying the luciferase gene and propagated in media containing G-418 (Mediatech, Manassas, VA) at a concentration of 500 μg/ml. About 1 x 10^6 cells suspended in 10 μl of serum free media were injected intracerebrally with the help of a stereotactic instrument after drilling a small hole in the cranium of the mice. Beginning from day 3 after implantation of the tumor cells, the mice were injected intraperitoneally with either a mammalian expression vector carrying Bcl-xL siRNA cDNA (pRNAT-CMV3.2/Neo, GenScript, Piscataway, NJ), (50μg DNA/injection/mouse) or taxol (50 μg/injection/mouse) or both agents together for 28 days on alternate days. On day 30, the mice were injected with 100 μl of luciferin (Genlantis, San Diego, CA) at a concentration of 50 mg/ml. After 10 min, the mice were visualized for luciferase activity using Xenogen IVIS-200 (Xenogen, Hopkinton, MA) imaging system. The combination treatment with Bcl-xL siRNA and taxol resulted in complete inhibition of intracranial tumorigenesis in nude mice. The data are representative of 4 sets of animals in each group. Note: The absence of a visible tumor image in the mouse after combination treatment with Bcl-xL siRNA and taxol doesn’t mean that the tumor is regressed completely. Tumor is still inside the intracerebral region of the brain, indicated by the large number of photons (3.498e+06) detected by the in vivo imaging system. The background signal from a normal mouse is about 1.5e+05 photons on Xenogen IVIS-200 imaging machine.
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