Title: Localized Targeted Antiangiogenic Drug Delivery for Glioblastoma

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Abstract:
Systemic delivery of antiangiogenic agents has been ineffective in improving the overall survival of patients with both primary and recurrent glioblastoma, in part due to dose-limiting toxicities. With the development of new and efficient localized delivery methods and vehicles, an otherwise lethal dose of antiangiogenic chemotherapy can be used to treat tumors while minimizing systemic side effects. Current in-vitro and in-vivo animal studies have shown promising results that encourage the pursuit towards human clinical trials for localized antiangiogenic treatment in the near future.

Key Words: glioblastoma, glioma, VEGF, angiogenesis, local drug delivery
**Introduction**

Glioblastoma multiforme (GBM), recognized as one of the most aggressive primary malignant brain tumors, has an incidence rate of three cases per 100,000 persons in the middle-aged population [1-3]. After maximal surgery, chemotherapy, and radiation, the median survival is between 14-16 months [4, 5]. The outlook is even worse for recurrent GBM, with a progression free survival of only 6.9 months after tumor recurrence [3, 6]. Treatment options for recurrent GBM are varied, and include assorted chemotherapeutic regimens, re-resection, and recently, tumor treatment fields with radio waves [7]. Despite these standard treatment options, recurrence is almost inevitable [2]. Being a highly vascular tumor, GBM is characterized by extensive proliferation of blood vessels through various cellular pathways such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), platelet derived growth factor (PDGF), stromal cell-derived factor-1 (SDF-1) and interleukin 8 (IL-8) [8, 9]. Studies have considered a number of novel treatment approaches over the years such as the application of antiangiogenic agents like Bevacizumab, a humanized monoclonal antibody targeting VEGF, for systemic GBM therapy. Results yielding prolonged progression free survival was promising, but unfortunately minimal improvements in overall survival were achieved in patients receiving Bevacizumab as a single agent or in combination therapy [10-13]. In addition, dose limiting systemic toxicities including hypertension, arterial thromboembolic events, proteinuria, bowel perforation, reversible posterior leukoencephalopathy syndrome, wound complications and hemorrhage were seen among Bevacizumab treatment groups [14]. A factor that commonly contributes to poor treatment response in the central nervous system is the limited permeability of the blood brain barrier (BBB) [3]. In GBM, the BBB is partially disrupted in the tumor core but the majority remains intact, forming a strong protective barrier between the tumor and the outside environment. This makes it difficult to deliver these treatment agents at a sufficient dose for effectiveness without also creating serious toxicity [15, 16].

With the advent of various localized drug delivery strategies being investigated in other non-GBM cancers, this concept of localized therapy is being highly considered as a possible solution for bypassing BBB. Current strategies that circumvent the BBB include: local delivery - depot of drug directly placed/injected into the extracellular space or by convection enhanced technique using a pressure head to drive material through the extracellular space; targeted delivery – receptor-mediated delivery; blood brain barrier disruption -opening the tight junctions in the BBB endothelium, thereby allowing larger or lipid insoluble agents to pass into the CNS parenchyma for a defined period of time and in a specific area. These strategies may enhance the clinical value of antiangiogenic agents, by allowing sustained local delivery in the tumor region while minimizing their systemic toxicity. This review discusses the current state of locally delivered angiogenesis inhibitors for GBM.

**Mechanisms of delivery**
Local Delivery

Similar to systemic GBM treatments, current therapies using antiangiogenic inhibitors for non-GBM cancers have their own set of shortcomings that include systemic toxicity and low drug dose arrival at the tumor site [17]. Local drug delivery, via intratumoral administration or adjacent to the cancerous tissue (loco-regional administration), using novel methods like drug delivery depot systems, have shown great potential at addressing the problems seen in not only non-GBM cancers but also as possible mechanisms of drug delivery in GBM [17, 18]. Gliadel wafers, a biodegradable polymer embedded with the chemotherapeutic carmustine (BCNU), are FDA approved for both primary and recurrent GBM, and have shown modest improvement in survival [19]. Since the advent of Gliadel in the 1990’s, options for local delivery of chemotherapeutics to the site of GBM have expanded, and various nanocarrier vehicles are currently under investigation. These methods have allowed the use of drugs that were previously limited due to poor availability in the brain and/or dose limiting systemic toxicity.

Polymer vehicles

One type of carrier used for local drug delivery is biodegradable polyanhydride polymers. Angiogenesis inhibitors are incorporated into polymers based on a ratio by weight basis and in accordance to the target of treatment. Drug carrying polymers are dissolved in methylene chlorides, and placed onto discs for surgical implantation. In glioma studies, drug carrying polymers are intracranially implanted by first surgically excising majority of the tumor and placement of polymer around the surface area of the resection cavity [20].

Microspheres (MS) are a form of polymer used for targeted cancer treatment. Specifically, a subtype that has shown promising results is polylactic co-glycolic acid microspheres (PLGA). PLGA is considered favorable due to its biodegradability and biocompatibility, FDA and EMA approval for parenteral administration, well characterized formulations and production methods suitable for various drugs, protection of the loaded drugs from degradation and serum inactivation, and most importantly, its facilitation of controllable sustained release [21]. An in-vitro study demonstrated the versatile loading capacity of PLGA with two endogenous inhibitors PEX and a fragment of PF-4 (PF-4/CTF). It was shown that the formulation procedure of PLGA microspheres did not affect or alter the biological activity of the incorporated drugs [22]. There was also evidence that PLGA microspheres injected adjacent to tumors were able to diffuse and arrive at the target site just as well as an injection of treatment directly into the tumor. PLGA’s have also shown promising results with new anti-angiogenic inhibitors like AZD2171. With a short half-life and high toxicity, local administration of PLGA’s enables the inhibition of tumor growth through VEGF signaling and proliferation [23]. The results from these studies justify the further testing of PLGA’s in future investigations.
**Convection Enhanced Delivery**

The utilization of a pressure head to drive infusate through extracellular matrix of peritumoral regions, termed convection enhanced delivery (CED), is one of the most widely-investigated techniques in local drug delivery currently. Though a detailed description of the technique, shortcomings, and current status are beyond this review, CED trials have been investigated in preclinical and clinical trials utilizing conjugated toxins, chemotherapeutics, viral vectors, and liposomes for treatment of GBM, and continue to be a promising technique for future investigation [24].

**Gene Therapy**

Genetic therapy has also been considered among investigators as an approach for continuous local delivery of antiangiogenic inhibitors. In a recent study, intrapleural administration of an Adeno-associated virus (AAV) vector encoding the murine monoclonal antibody equivalent of bevacizumab demonstrated sustained expression and effectiveness of this antibody in lung cancer treatment [25]. Transgene expression lasted for 40wk after vector administration, the highest levels being found in the lungs and little to no expression being seen in major organs like liver and spleen. All AAVrh.10aVEGF encoded the anti-human VEGF light chain and heavy chain sequence derived from the protein sequence for the antibody A.4.6.1. The benefit of this protocol in this study came from the fact that the coding sequences for human VEGF-A binding site were identical to that of bevacizumab. These vectors showed low toxicity compared to previously tested model vectors, such as adenoviruses, rendering the usage of AAV vectors as a novel alternative platform for drug delivery. Another approach for genetic therapy was proposed through the usage of plasmids. In order for the angiogenesis inhibitor endostatin to be effective, prolonged administration and high concentrations of this antiangiogenic factor are necessary. A study investigating whether intratumoral injection of endostatin plasmid inhibits mammary tumor growth was conducted by administering treatment once a week for two weeks in mice murine mammary tumor models [26]. Data suggested that local delivery of endostatin plasmid was able to efficaciously suppress murine mammary carcinoma growth, and reduce angiogenesis and perfused tumor vessel density. Therefore, plasmid delivery of endostatin gene serves as a plausible drug delivery system for treatment for this cancer and further investigations among other carcinomas.

**Targeted Delivery**

**Ligands**

GBM cells, like many other cancers, exhibit overexpression of various proteins that makes the use of specific ligand-binding therapeutics an attractive area of study. Though systemically administered, chemotherapeutics conjugated to specific ligands will tend to accumulate at the sites of high concentration of tumor cells, effectively increasing the effective
dose and specificity of the drug. The overexpression of VEGF-A ligand on glioma cells, among others, has allowed for investigation of therapeutic targeting for GBM, as will be discussed in later sections.

Blood Brain Barrier Disruption

Focused Ultrasound

Osmotic agents, inflammatory cytokines, and blood vessel modulators have all been used as ways to induce changes in BBB permeability [27]. Though these methods are successful for BBB disruption, they are nonspecific and do not address issues of drug availability to tumor sites. In order to address these issues, focused ultrasound with microbubbles (MBs) has evolved as a technique for selective BBB disruption for localized drug delivery. The technique usually consists of magnetic resonance image (MRI)-guided ultrasound beams that disrupt tight junctions in the BBB at select sites, either reversibly or irreversibly, depending on desired effect. In conjunction with the focused ultrasound beam, microbubbles are used to encourage the opening of the tight junctions by several mechanisms after local excitation by the ultrasound waves [28]. Preparations of these micro sized carriers involve physical methods such as the thin-film hydration method. The advantage of using this technique is the ability to yield small and uniform particles in a simplistic and replicable way [29]. The phospholipid shell composition of microbubbles allows for both electrostatic and/or hydrophobic interactions to occur with the drug of interest, allowing for its encapsulation within the vehicle. MBs have been incorporated into focused ultrasound approaches as a way of mitigating any damage to surrounding brain tissue, all while ensuring the desired effects of FUS: selective disruption of tight junctions and increased permeability of the BBB. A variety of therapeutic agents such as doxorubicin [30] and temozolomide [31] have been successfully incorporated in MB-FUS induced disruption of BBB without causing negative effects like hemorrhage [32]. Using carriers like MBs along with FUS appear to be work well with most antitumor drugs [33] and provides favorable preclinical evidence of increased dose concentrations at the site of the tumor and decreased tumor growth. While few studies have considered incorporating antiangiogenic inhibitors to MBs, these results warrant future studies to attempt incorporating these agents to MBs-FUS approaches in order to solve the systemic toxicities that arise from their use and enhancing their effects in GBM treatment.

Current use of angiogenesis inhibitors in local drug delivery for glioma
Local delivery of angiogenesis inhibitors has been investigated for use in other cancers and diseases (Table 1). Similarly, local administration of various angiogenesis inhibiting agents, alone or in combination with other therapy, are under investigation for GBM; however, studies have been limited to in vitro and animal models thus far. Table 2 summarizes the study designs and outcomes for local angiogenesis delivery to glioma.

**Encapsulation**

In rats that received transplants of BT4C glioma cells, an in-vivo experiment studied the effect of genetically engineered cells that delivered endostatin locally [36]. In order to protect the cell from host immune system, the cells were encapsulated in an immunoisolating substance called sodium alginate. Alginate is composed of L-guluronic and D-mannuronic acid, both of which are able to form a gel network that can immunoisolate the cells that are residing within. According to the data, encapsulated endostatin producing cells stayed viable for at least four months following intracerebral implantation. It was noted that the group that received the alginate-encapsulated endostatin survived 84% longer than the control group.

**Polymer Delivery**

Local delivery of synthetic endostatin fragments can also be used in conjunction with other systemic chemotherapeutic drugs to increase survival in animal models. Synthetic endostatin peptides are shorter peptides derived from sequence of endostatin. Specifically, synthetic endostatin peptides corresponding to sequences 6-49 and 134-178 of the full-length human endostatin have been shown to preserve the antiangiogenic activity. Both in vitro and in vivo studies show that the synthetic 6-49 and 134-178 peptides have similar or sometimes greater potency and efficacy when compared to full-length human endostatin [37]. An in vitro rat 9L gliosarcoma model was used for local delivery of synthetic endostatin using a biodegradable (bis-[carboxyphenoxy-propane]-sebacic-acid) polymer (pCPP:SA) [38]. The study removed a small section of parietal cortex and placed the polymer into the brain parenchyma approximately 1 mm below the dura. In addition to using local synthetic endostatin, the study also used systemic administration of BCNU (Carmustine), an alkylating antineoplastic drug that has good penetration into the blood brain barrier. The study (n=64) found no significant survival difference between the group with endostatin polymer alone and control group, suggesting that local delivery was not sufficient. However, the group with local endostatin polymer on day 0 and systemic BCNU delivery on day 5 had significantly improved long term survival (p<0.001) when compared to control group and groups with either treatment alone. The results suggest that local antiangiogenic properties of synthetic endostatin fragments have synergistic effect when given in conjunction with systemic BCNU.

Delivery mechanisms such as PGLA microspheres combined with AZD2171 (Cediranib), a VEGF tyrosine kinase inhibitor, have shown promising results for glioblastoma treatment. An in-vivo study used a mice model with subcutaneous U87 glioma tumor order to identify the
effect of AZD2171-loaded PLGA microspheres [23]. The tumor size was measured every 2-3 days for 14 days before the sacrifice. Post-sacrifice histological (H&E) and immunohistochemical analysis for vascularization (CD31) showed that the tumors in the treatment group with AZD2171 microsphere administration were less vascularized and had more defining edges compared to the tumors of control group with empty microsphere. Additionally, the tumor growth was completely halted in the group with AZD2171 microsphere administration, compared to an average of 15-fold increase in tumor-size in control groups (PBS injection or empty microsphere).

Even though many antiangiogenic treatments for glioblastoma involve local inhibition of VEGF, additional models have been proposed that target PDGF receptors. A 2009 study was the first to show therapeutic effect of local imatinib mesylate delivery using PLGA microspheres [39]. Imatinib is a small molecule kinase inhibitor that targets the receptors for PDGF, hence blocking angiogenesis. The study consisted of in vivo subcutaneous rat model and in vivo intracranial rat model with human U87-MG glioblastoma cells. One of the groups in each model was treated with PLGA microspheres containing imatinib mesylate. The results for the subcutaneous model showed significant inhibition of U87-MG cell growth, suppression of tumor volume by 88%, and reduction in tumor weight by 77% in the group treated with imatinib mesylate delivered through PLGA microspheres. In the intracranial model, imatinib-loaded microspheres resulted in significant (79%) reduction in tumor volume when compared to empty microspheres. The immunohistochemical analysis showed a significant increase in tumor-cell apoptosis, but no significant decrease in microvessel count.

Heparin combined with cortisone is an anti-angiogenesis treatment that does not target VEGF or PDGF pathways directly. Local therapy for heparin combined with cortisone has shown improved survival in glioma by inhibiting angiogenesis [40]. Heparin inhibits angiogenesis by affecting HGF/SF while cortisone changes the turnover of basement membrane in developing capillary blood vessels in the presence of heparin [41]. A model of 50 Fischer 344 rats with subcutaneous 9L glioma was used to test the effect of localized heparin and cortisone delivery using biodegradable polyanhydride polymer matrix. The results showed a 4.5-fold reduction in growth of 9L glioma in rats treated with local heparin and cortisone compared to a 2.3-fold decrease in local delivery of cortisone alone. Local heparin administration by itself neither inhibited nor enhanced the growth of the tumor [40].

Angiogenesis can be inhibited by basement membrane disruption of pre-existing blood vessels. Minocycline is a tetracycline derivative that has inhibitory effects on matrix proteases and therefore has been shown to inhibit tumor angiogenesis [42]. The activity of a locally delivered polymer containing minocycline and pCPP:SA with a 50:50 ratio by weight and systemic BNCU was measured in a study using a 9L glioblastoma rat model [43]. In the protocol measuring the simultaneous implantation of minocycline polymer and the tumor, the treatment group received minocycline implantation along with the tumor while the control received tumor only. All the control animals died within 21 days of tumor implantation, with histological analysis concluding the cause of death as a large tumor mass in their brain. On the other hand,
100% of treatment rats had long-term survival and a representative sample was euthanized after 120 days and there was no tumor found. In clinical setting, however, malignant gliomas have extensive vascular proliferation before they are identified. In order to mimic this clinical setting, another protocol was established with a delayed treatment. In this protocol measuring the effect on established tumor, the treatments were given 5 days after the tumor implantation and results still pointed towards a synergistic activity of minocycline when combined with BCNU. In the protocol, local minocycline + systemic BCNU resulted in a statistically significant increase in median survival (42 days) compared to systemic BCNU alone (23 days), intracranial minocycline alone (19 days) and control group (14 days).

Local minocycline delivery for glioma has also been tested in combination with radiotherapy and oral temozolomide (TMZ) chemotherapy in a 9L glioma rat model. The study tested the effectiveness of combining minocycline with radiotherapy and temozolomide chemotherapy [44]. It is important to note that the three different treatments were not given in a synergistic manner. Minocycline was loaded in a biodegradable polymer (minocycline CPP: SA polymer) and surgically implanted in female rats, whereas radiotherapy and oral temozolomide were given according to their mode of administration. Results showed that minocycline delivered locally heightened the effects of both radiotherapy and oral temozolomide (TMZ) in increasing the length of survival in rat glioma models.

**CED**

Convection-enhanced local delivery of angiogenesis inhibitors such as bevacizumab has shown promising results in mouse model with U87 and U251 human glioma xenografts [45]. For local delivery, Alzet micro-osmotic pump filled with the desired dose of reagent was implanted into the animals seven days after tumor cell implantations. A plastic tube was used to connect the infusion kit to the brain and the treatment was locally delivered using pump mediated delivery in order to achieve desirable drug concentration. In terms of monotherapy, the results show 30% longer survival rate of mice treated with local CED-bevacizumab compared to the ones treated with IV bevacizumab. For human U87 glioma cells, the combination therapy of local CED-bevacizumab with chemotherapeutic drug CPT-11 (irinotecan) nearly doubled the survival times of mice that were treated with IV bevacizumab and CPT-11.

**Gene Therapy**

Next, genetic modification of neurons to facilitate local anti-angiogenesis treatments is a possible avenue towards glioblastoma treatment due to its ability to persistently deliver anti-angiogenesis monoclonal antibodies locally. Similar to the intrapleural viral delivery of AAVrh.10 gene therapy for lung tumors [25], a study measured the effectiveness of AAVrh.10BevMab gene transfer vector coding for bevacizumab to suppress glioblastoma multiforme growth in immunodeficient mice. Along with increased survival, the mice treated with AAVrh 10 gene presented with reduced tumor volume under MRI and reduced tumor blood
vessel density under histology [46]. Clinically, the administration of the viral vector can be done
during surgery after the surgeon has removed as much tumor as possible.

**Focused Ultrasound with BBB Disruption and Targeted Delivery**

In addition to the local endostatin combined with systemic BCNU approach, an
alternative approach for the treatment of glioma has proposed local delivery of
chemotherapeutics to the regions of VEGF-A overexpression in order to suppress the vascular
proliferation in GBM. Using a C-6 glioma rat model, the study used VEGF-A ligand conjugated
to microbubbles (MBs) encapsulating BCNU [47]. The hypothesis was that the VEGF-BCNU-
MBs will localize to sites with overexpression of VEGFR-2 receptor, resulting in localization at
the site of the glioma. Following tumor and treatment administration, the study used focused
ultrasound (FUS) to open the blood brain barrier at the site of glioma for improved BCNU
penetration. Not only was the blood brain barrier disrupted by FUS-BBB opening, but results
also showed an increase in tumor-specific targeting, enhanced delivery, and improved median
survival time in the group with VEGF-BCNU-MB followed by FUS.

**Drug dose and distribution**

Studies have shown a dose dependent effect of bevacizumab on glioma cells such that
low doses affect the vascularity of the tumor cells but higher doses may have additional specific
antitumoral effects, independent of vascular regression [48]. Overall, optimal dosing of
bevacizumab has not been established and, moreover, the specific doses required to trigger
decreased tumor growth have not been studied in clinical trials. Given the prevalence of systemic
adverse effects of current intravenous Bevacizumab dosing regimens (10 mg/kg every two weeks
for glioblastoma [49]), it is reasonable to believe that local administration would be a more
viable option to explore high concentration efficacy of the drug. In fact, pharmacokinetic data
from Gliadel wafer studies, perhaps the most extensively clinically studied local delivery vehicle
for GBM, have shown peak systemic levels of BCNU 600 times lower compared to
chemotherapeutic administered intravenously [50]. Tumoral and per-tumoral drug penetration in
local delivery has been examined through mathematical modeling and some animal studies,
showing advantages in achieving significantly higher concentrations of drug in brain tissue when
compared with systemic administration [51]; despite these preliminary studies, drug distribution
and real-time monitoring with local delivery is an area in desperate need of continued
investigation.

**Conclusion**
The failure of systemic antiangiogenic therapy to improve overall survival in GBM demands for a more targeted approach for disease treatment. Over the past two decades, targeted drug delivery modalities, including various carrier vehicles and drug combinations have been developed to prevent angiogenesis and, therefore, tumor proliferation. In addition to increased efficacy, localized drug targeting could prevent adverse effects from systemic drug administration. Although the current landscape of research for localized antiangiogenic therapy for GBM is in its infancy, in-vitro and animal studies have shown promising results. Still, further investigative approaches will require large animal studies to be done before the initiation of phase 1 clinical trials.

References


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<td>Minimal plasmatic sunitnib concentration (0.002 mg/mL on day 1), high intratumoral sunitnib concentration (40.4 mg/g on day 1 and 27.4 mg/g on day 14) and no VEGFR2 phosphorylation was detected.</td>
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<td>In-vivo</td>
<td>Decreased tumor growth (p&lt;0.05) and number of blood vessels and mitotic nuclei in the tumor (p&lt;0.05) was observed AAVrh.10αVEGF treated group. In addition, AAVrh.10αVEGF demonstrated effective long-term expression of anti-VEGF-A antibody in lung and increased survival among treatment group (p&lt;0.005).</td>
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<tr>
<td>Mammary carcinoma [26]</td>
<td>Intratumoral injection of endostatin plasmid</td>
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<td>Treatment group tumor weights were 51% of controls (P&lt;0.01). Increased distance between tumor cells and vessels demonstrated reduction of total vascular density. Treatment group tumors also experienced an increased apoptotic index (P&lt;0.05), along with decrease in tumor perfused vessels.</td>
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<td>Type of study</td>
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<td>Drug/treatment</td>
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<td>In-vivo [36]</td>
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<td>In-vivo [23]</td>
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<td>Minocycline; temozolomide (TMZ); radiotherapy</td>
<td>pCPP:SA</td>
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<td>Minocycline locally heightened the effects of radiotherapy and oral temozolomide in increasing the length of survival.</td>
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<td>In-vivo[45]</td>
<td>Mice</td>
<td>Bevacizumab; Irinotecan (CPT-11)</td>
<td>None</td>
<td>Convection-enhanced delivery</td>
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<td>Monotherapy: 30% longer survival rate of mice treated with local bevacizumab compared to IV bevacizumab. Combination therapy: Nearly double survival time of mice treated with local bevacizumab + CPT-11 when compared to IV bevacizumab + CPT-11.</td>
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<td>In-vivo[46]</td>
<td>Mice</td>
<td>Bevacizumab-coding gene AAVrh.10BevMab</td>
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<td>Increased survival, reduced tumor volume, and reduced tumor blood vessel density.</td>
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<td>In-vivo[47]</td>
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<td>BCNU loaded microbubbles followed by FUS resulted in weakened blood brain barrier, tumor specific activity, enhanced delivery, and improved median survival time.</td>
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