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REVIEW ARTICLE

TARGETED ANTITUMOR THERAPY WITH THE SCORPION VENOM CHLOROTOXIN

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SUMMARY

Chlorotoxin (CTX) is a 36-amino-acid neurotoxin isolated from the venom of the giant yellow Israeli scorpion Leiurus quinquestriatus. The peptide preferentially binds to human malignancies, but not to normal human tissues. Annexin A2 is a receptor for CTX. CTX binding results in internalization and subsequent downregulation of matrix metalloproteinase-2 (MMP-2) and chloride channel protein 3 (CIC-3) on malignant cells. In turn, this inhibits migration of glioma cells, induces antiangiogenic effects and potentially increases the efficacy of other therapies. A synthetic version of this peptide, TM-601 (Morphotek, Inc.), has been covalently linked to iodine 131 ([^{131}I]-TM-601) as a means of targeting radiation to tumor cells. Phase I-II clinical trials in patients with recurrent glioma indicate that [^{131}I]-TM-601 binds malignant glioma with high affinity and a long duration. The therapy appears safe, minimally toxic and may improve survival. The fluorescent bioconjugate CTX:Cy5.5 has been developed as a "tumor paint" to detect tumors and maximize the extent of removal during surgery. CTX has also been conjugated to magnetic nanoparticles for MRI detection and intracellular delivery of DNA for gene therapy. Due to its small size, selective tumor binding properties, minimal toxicity and relative ease of manipulation, CTX represents a potentially important targeting agent for many cancers.

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OVERVIEW OF THE PROBLEM

Approximately 1.5 million malignant cancers were diagnosed in the U.S. in 2009 and an estimated 562,340 patients died from cancer in 2009, the equivalent of more than 1,500 deaths per day (1). Cancer is now the leading cause of death in the U.S., surpassing heart disease for all people below the age of 85. Despite major advances in cancer treatments, meaningful long-term therapy remains elusive for many cancers, including tumors of the brain, pancreas and lungs.

In the U.S., an estimated 22,000 new cases of malignant brain tumors were diagnosed in 2010 (2). The incidence of primary brain tumors appears to be on the rise, although it is unclear if this is attributable to better reporting or the influence of environmental or genetic factors. Approximately 13,000 brain cancer deaths attributable to glioma occur annually in the U.S. The median survival for WHO grade IV glioma (the most common form) is approximately 18 months. For WHO grade III the survival is approximately 3 years, and for low-grade (WHO grade II) astrocytoma 5-7 years (3). These grim statistics make gliomas among the most deadly form of cancer.

The lack of meaningful therapies with long-term impact on disease control and survival has led to continued research for better treatments. This paper will focus primarily on the role of chlorotoxin (CTX) in the treatment of malignant glioma, because this tumor is the primary disease platform in which the technology has been evaluated to date. Nonetheless, increasing laboratory and clinical studies suggest that this agent is also applicable for many other cancers.

CURRENT STANDARD OF CARE

Surgery for primary brain tumors

Open surgical removal of brain tumors has been a mainstay of glioma management for decades (4-7). Unfortunately, total excision of glioma is rarely possible. Glioma cells are highly invasive and are commonly observed 4 cm or more away from the primary tumor mass (8). Most of these cells are interdigitated with normal functioning brain parenchyma, and resection of these regions can result in unacceptable neurological deficits. Furthermore, while gliomas rarely metastasize outside the central nervous system (CNS), they frequently disseminate widely in both hemispheres of the brain.

Tools to aid surgeons in differentiating normal tissue from glioma cells at the periphery of tumors can improve the extent of micro-

scopic removal. These methods include the use of tumor fluorescence (9, 10) and infrared imaging (11). Gross total excision of low-grade gliomas, especially pilocytic tumors, is curative in upwards of 80% of cases (6). In contrast, gross total removal of all other gliomas and most primitive neuroectodermal tumors (PNETs) is not clearly correlated with a higher cure rate. Cytoreductive surgery, which removes at least 85% of the enhancing volume of high-grade glioma, has been correlated with improved length of survival and improved quality of life (7, 12), with this effect especially evident for > 98% resection (12, 13). These results indicate that methods to improve the extent to which all macroscopic and microscopic tumors can be removed should result in better outcomes. Surgical resection remains the most effective single therapy for gliomas, especially when tumors recur or progress (12).

Radiation therapy

Gliomas are not particularly radiosensitive tumors. Radiation doses in excess of 50 Gy are effective in retarding glioma progression, but do not generally produce cure or long-term control of disease (14). Increasing doses of radiation have improved efficacy in higher-grade tumors, although most dose-escalation studies have failed to demonstrate improved survival. Unfortunately, normal brain parenchyma is also sensitive to radiation effects. The tolerance for brain injury escalates quite rapidly above 70 Gy, effectively limiting total radiation doses to the 60 Gy range. The use of radiosensitizers has not been proven to improve the effects of radiation or long-term outcome (15). Furthermore, radiation injury and radiation-induced cell death (radiation necrosis) are often as damaging as the primary tumor. Despite these limitations, radiation therapy is used almost uniformly in the treatment of gliomas, and is likely to remain a mainstay of therapy in the foreseeable future.

Chemotherapy

Systemic chemotherapy has proven to be disappointing in the treatment of gliomas. This is partially attributable to the poor distribution of drugs in the brain due to the blood–brain barrier (BBB). Many agents, including BCNU (carmustine), CCNU (lomustine), procarbazine and temozolomide, have demonstrated responses against high-grade gliomas (16, 17). However, these agents tend to have fairly limited or partial responses in both an upfront and recurrent setting. Most recently, the combination of temozolomide with radiation therapy in patients with newly diagnosed glioblastoma multiforme (GBM), followed by a course of temozolomide, has been found to increase the median survival by 2 months when compared to radiation alone, with approximately 35% of patients surviving beyond 18 months (18). Bevacizumab, a targeted vascular endothelial growth factor receptor (VEGFR) antibody, has demonstrated efficacy, especially in recurrent or progressive disease, extending progression-free survival by about 4-12 months in 35% of patients, with up to 63% demonstrating some response (19, 20). However, overall survival has not been changed. A significant number of chemotherapy trials are under way for glioma, although a thorough review is well beyond the scope of this paper. Similarly, a number of antiangiogenic agents are being tested.

In light of the limits of the treatments described above, tremendous attention has focused on the development of tumor-specific targeted therapies. The broad goal of this technology platform is to directly bind a cytotoxic agent to the tumor cells while avoiding or preventing any therapy delivery to normal brain cells. While this concept is broadly applicable to many malignancies, it is particularly important and challenging for gliomas, due to the fact that they so heavily infiltrate into and interdigitate with normal brain tissue. Unlike other solid organs, the destruction of the normal brain parenchyma results in specific loss of neurological functions. Therefore, secondary damage to noncancerous cells is poorly tolerated. Furthermore, the BBB functionally excludes the majority of large molecules from entering the brain, making the ability to actually deliver the therapy key to its success.

PRINCIPLES OF TARGETED THERAPY

Targeted cancer therapies principally depend upon receptor-mediated selective binding to tumor cells. The success of this approach requires high specificity or selectivity of binding to tumor cells or other tumor-related cells, such as tumor-induced neovascular blood vessels. The targeting agent should take advantage of either the overexpression of receptors by tumor cells or the preferential expression of receptors not found on normal brain tissues. The targeting agents for these receptors are either: 1) antibody or antibody-like ligands; 2) proteins that bind the receptor; 3) proteins that bind the cell and are internalized; or 4) large molecules with receptor-specific binding properties.

Targeted cytotoxin therapy

Targeted therapy of glioma with tumor-specific cytotoxins has generated great interest of late. The basic strategy employed is to identify a cellular toxin, most commonly one derived from bacteria, modify that toxin to maximize antitumor activity, and deliver the toxin directly to the tumor with a tumor-specific ligand acting as the carrier molecule (see 21 for a review). The most notable of these is a modified variant of *Pseudomonas* exotoxin (PE) joined to interleukin-13 (IL-13). The IL-13 receptor is preferentially expressed in many gliomas. When this fusion protein binds to glioma cells, it is internalized and results in cell death. Phase I and phase II studies with this agent were encouraging (22). A phase III clinical trial of this agent was recently completed. Unfortunately, the results indicated minimal survival benefit, with significant toxicity. An alternate strategy employs diphtheria toxin (DT), which has been fused to a fragment of the transferrin receptor antibody and delivered via direct brain infusion (convection-enhanced delivery) (23). This agent is currently undergoing phase III clinical trials, but the ubiquitous nature of transferrin in the brain is a potential limiting factor. Fusion of DT to human plasminogen activator, as well as IL-13, has also been accomplished (24), but these agents have not yet undergone clinical testing.

Targeted radioimmunotherapy and radiopeptide therapy

Antibodies to cell surface antigens have been most commonly employed in clinical settings, and several of these compounds are FDA-approved for clinical use. Examples include trastuzumab (Herceptin®), an antibody to the receptor tyrosine-protein kinase erbB-2 (HER2/Neu) found in many breast cancer cells, and ibritu-

momab tiuxetan (Zevalin®), a radiolabeled antibody to the CD20 antigen on lymphoma cells.

This approach has been tested for brain tumors. Examples of this approach in glioma therapy include targeting the epidermal growth factor receptor (EGFR) (25), fibronectin (26) and the extracellular matrix molecule tenascin with [^{131}I]-radiolabeled ligands (27). However, imaging studies demonstrate that the antibodies were too large to penetrate beyond a few millimeters into the surrounding brain parenchyma, essentially neutralizing any potential targeting advantage (28). Furthermore, tenascin is widely expressed throughout the nervous system, and EGFR, while upregulated in some gliomas, is also expressed by normal brain cells, limiting the specificity of these approaches. Both of these compounds are unlikely to have widespread impact on the disease or treatment strategies in the foreseeable future.

CTX – A TUMOR-SPECIFIC PEPTIDE FOR TARGETED THERAPY

CTX is a small neurotoxin isolated from the venom of the giant yellow Israeli scorpion *Leiurus quinquestriatus.* CTX is a 36-amino-acid (Mol wt.: 3,950 D) peptide containing a single tyrosine residue which is available for radioiodination, eight cysteine residues and four disulfide bonds, yielding a tightly folded tertiary structure (Fig. 1). In nature, CTX functions as a paralytic agent for small insects and other invertebrates. The scorpion bites an insect, which induces paralysis so the scorpion can either eat the insect or potentially escape a dangerous situation. When crayfish are injected with CTX, they become completely immobilized for a period of approximately 2 minutes and then resume normal locomotion. This response is dose-dependent and highly reproducible, thus providing an important biological assay for CTX and any engineered modifications of its structure. The human response to this venom in nature is generally bland and does not result in paralysis or death. The lack of a paralytic response in humans underlies the species-selective nature of CTX binding, and the absence of a large number of binding targets in normal human tissues.

CTX was first identified as having tumor-specific targeting during patch clamp recordings of cells derived from cultures of normal and malignant human astrocytes (astrocyte = glial cell; malignant astrocytes = glioma) (29-32). These studies demonstrated that CTX blocked a voltage-activated chloride channel in malignant glioma, but not in normal glia, and the degree of response correlated with the degree of malignancy of the primary cell type. This finding was subsequently confirmed in an extensive assortment of tissue explants obtained from human gliomas (29, 33, 34) and other tumors. Subsequent studies demonstrated that CTX facilitates the downregulation of cell surface levels of the chloride transporter CIC-3 via internalization, and therefore CTX was initially described as a glioma-specific chloride channel ligand (29).

Tissues that bind CTX

A host of immunostaining studies have been performed to better elucidate which cell types bind to CTX and to what degree (29, 33, 34; and internal data, TransMolecular, Inc). A summary of findings is listed in Table I. Basically all tumors of neuroepithelial origin, such as glioma, medulloblastoma, PNET and oligodendroglioma, avidly bind CTX, with increasing receptor density as tumor grade increases

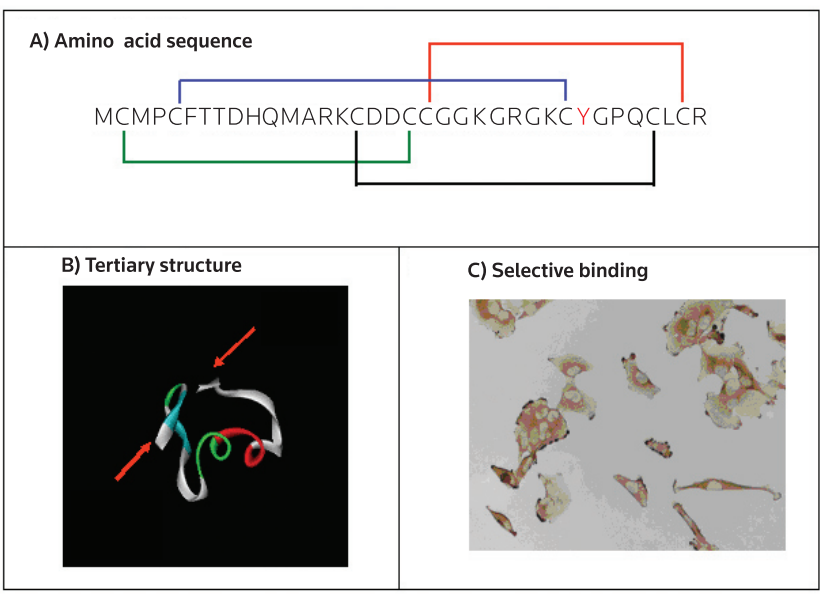

Figure 1. Chlorotoxin structure and binding. **A**) Amino acid sequence demonstrating four disulfide bridges. **B**) Tertiary structure of CTX. **C**) Cell surface binding of biotinylated TM-601 to fixed cervical HeLa cancer cells. Mamelak, A., Jacoby, D. Expert Opin Drug Deliv 2007, 4(2): 175-86, copyright © 2007, Informa Healthcare. Reproduced with permission of Informa Healthcare.

Table I. *Summary of various human tissues stained with TM-601.*

Tissue type	Cases	Results
Primary brain tumors (glioma)		
Glioblastoma multiforme (GBM)	31	31 positive
WHO grade IV		
Anaplastic astrocytoma	7	7 positive
WHO grade III		
Low-grade astrocytoma	4	4 positive
WHO grade II		
Pilocytic astrocytoma	14	13 positive
WHO grade I		1 negative
Other ungraded gliomas	5	4 positive
		1 negative
Oligodendroglioma	8	8 positive
Gliosarcoma	2	2 positive
Ganglioglioma	5	5 positive
Meningioma	25	20 positive
		5 negative
Ependymoma	3	3 positive
Other normal or diseased brain tissue		
Alzheimer's brain	8	8 negative
Parkinson's/schizophrenic brain	4	4 negative (2 each)
Normal brain or uninvolved tissue	29	21 negative
of brain cancer patients		8 positive[1]
Epilepsy/gliosis/stroke brain	6	6 negative[2]
Peripheral neuroectodermal tumors		
Medulloblastoma	4	4 positive
Neuroblastoma	9	8 positive
		1 negative
Ganglioneuroma	4	4 positive
Melanoma (metastatic)	11	11 positive
Melanoma (primary)	3	3 positive
Pheochromocytoma	6	5 positive
		1 negative
Ewing's sarcoma	2	2 positive
Primitive PNET	1	1 negative
Small cell lung carcinoma	5	4 positive
		1 negative
Schwannoma	3	3 positive
Other brain tumors		
Epidermoid cysts	5	1 positive
		4 negative
Brain tumors of unknown pathology	9	9 positive
Pituitary gland of GBM patients	2	2 positive
Metastatic tumors to brain	17	15 positive
		2 negative[3]
Other tumors		
Breast cancer	14	14 positive
Breast cancer metastases	11	11 positive
Kidney cancer	3	3 positive
Liver cancer	3	3 positive
Lung cancer	3	3 positive
Lymphoma	2	2 positive
Ovarian cancer	3	3 positive
Pancreatic cancer	3	3 positive
Prostate cancer	9	8 positive
		1 negative
Normal human tissues		
Breast	2	1 positive
		1 negative
Colon	2	2 negative
Endometrium/myometrium	3	3 negative
Eyeball (cross-section)	1	1 negative
Heart	2	2 negative
Kidney	3	3 negative[4]
Adrenal gland	3	3 negative
Liver	2	2 negative
Lung	3	3 negative
Lymph node	3	1 positive
		2 negative
Meninges	3	3 negative
Muscle (skeletal)	2	2 negative
Thyroid	1	1 negative
Pancreas	3	1 positive
		2 negative
Prostate	3	1 positive
		2 negative
Spleen	2	2 negative
Stomach	2	2 negative

[1]Samples from normal brains or from areas of GBM patient's brain diagnosed not to be involved in GBM; [2]areas of glial cell proliferation show a few cells binding TM-601; [3]metastatic tumors of unknown tissue origin; some may not be related to neuroectodermal tissue; [4]a few positive cells were observed.

(Fig. 2). Similarly, robust binding was observed in the overwhelming majority of malignancies tested, including melanoma and lung cancer (Fig. 3). Minimal or absent binding was observed in normal brain and other solid organs. The only normal cells in which appreciable binding was detected were proliferating human vascular endothelial cells, such as those in human umbilical vein (HUVEC) (33).

Identification of the cell surface binding partner for CTX

While in vitro cell culture and immunostaining data supported the view that CTX binds a tumor cell surface receptor, the exact receptor has proven much more difficult to identify. Initial studies demonstrated that a human glioma cell line contained 1,300 high-affinity binding sites per cell (29). Given that electrophysiological experiments showed that CTX inhibits chloride ion fluxes across the cell membrane of glioma cells, it was initially suggested that the receptor was associated with the chloride channel (29-31, 35). Subsequent analysis using a recombinant polyhistidine-tagged version of CTX indicated that the binding site may be a matrix metalloproteinase, i.e., MMP-2 (36). MMP-2 is considered to be a chloride channel activator and is believed to indirectly regulate the chloride channels expressed by gliomas, but not normal human cells (29-31, 35). MMP-2 stimulates the breakdown of the basement membrane and facilitates cellular migration of glioma cells and invasion of normal brain parenchyma. Application of CTX inhibits cellular migration in vitro, consistent with the idea that it interacts with MMP-2. Subsequent studies have shown that CTX more likely binds a macromolecular complex associated with a lipid raft on cell surfaces containing MMP-2, matrix metalloproteinase-14, metalloproteinase inhibitor 2 (TIMP-2) and the ClC-3 channel. The net result of this binding is internalization and subsequent downregulation of both MMP-2 and ClC-3 (36, 37). In this way, CTX decreases MMP-2 enzyme activity, which is important for glial cell migration and a variety of other functions.

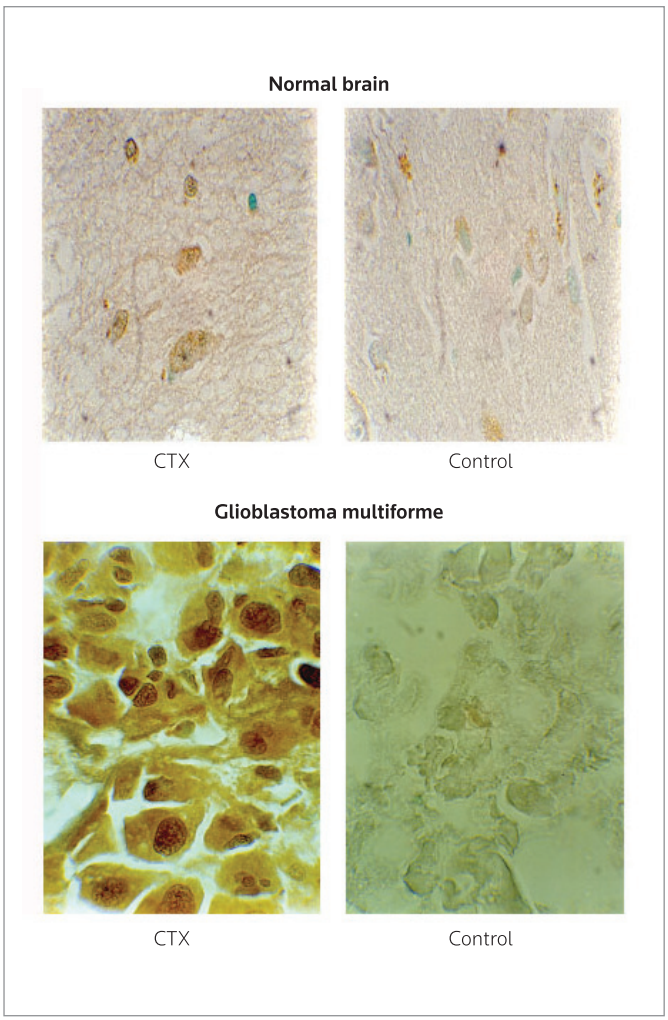

Figure 2. Representative examples of normal human brain (upper) and high-grade glioma (lower) stained with biotinylated TM-601 (left column) or buffered saline (right column). Normal brain tissue demonstrates no chlorotoxin (CTX) binding, whereas gliomas demonstrate avid binding. Mamelak, A., Jacoby, D. Expert Opin Drug Deliv 2007, 4(2): 175-86, copyright © 2007, Informa Healthcare. Reproduced with permission of Informa Healthcare.

Demonstrating that CTX specifically interacts with MMP-2 as initially postulated has proven more difficult. Using a fluorescent conjugate of CTX (CTX:Cy5.5), Veiseh et al. demonstrated that human breast adenocarcinoma MCF7 cells that normally express small amounts of MMP-2 on their cell surface exhibited a marked increase in CTX binding when transfected with a plasmid encoding for MMP-2, reinforcing the concept that MMP-2 clearly mediated the binding of CTX, either directly or indirectly. However, pulldown assays of recombinant MMP-2 incubated with either CTX:Cy5.5 or albumin and then anti-MMP-2 antibodies did not confirm MMP-2-specific binding for CTX (34). Taken together, these data support the view that, while CTX cell binding modulates MMP-2 activity, MMP-2 is unlikely to be the direct binding partner.

More recently, annexin A2 has been identified as a likely molecular target and binding partner for CTX (38). Utilizing the pancreatic carcinoma cell line PANC-1 and HUVEC, it was demonstrated that the presence of annexin A2 on the cell surface was required for CTX-mediated inhibition of angiogenesis mediated by vascular endothelial growth factor (VEGF) and several other growth factors. In the presence of normal or elevated levels of annexin A2, CTX markedly reduced tumor-induced neovascularity in the chick chorioallantoic membrane (CAM) assay. Reduction of annexin A2 on cell surfaces using a single-strand RNA (siRNA) knockdown technique resulted in a marked reduction of this effect. Furthermore, CTX inhibition of platelet-derived growth factor (PDGF)-CC-induced human glioblastoma U-373 MG and HUVEC cell migration was blocked with reduction of annexin A2 levels. Irreversible cross-linking of cellular proteins followed by affinity pulldowns and mass spectrometry identified annexin A2 as a binding partner for CTX. It is unknown whether other binding partners exist.

EFFECTS OF CTX ON HUMAN CELLS

Inhibition of migration

Using in vitro invasion assays, CTX has been shown to inhibit invasion in a concentration-dependent fashion (36). Several studies suggest that, through its inhibitory affect on ion efflux, CTX prevents cell shrinkage, thereby diminishing the ability of glioma cells to migrate through tight extracellular spaces in brain tissue. Furthermore, by

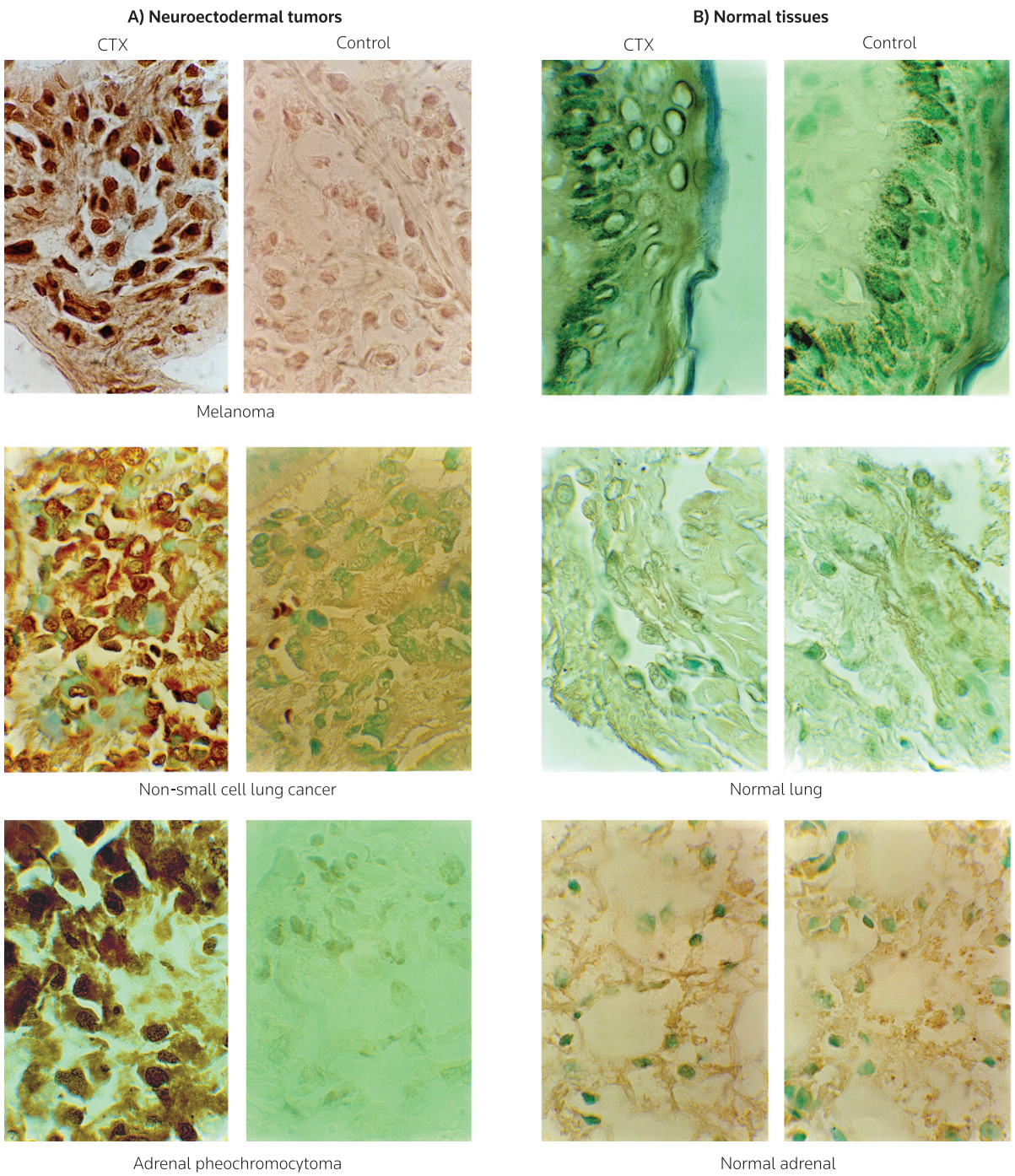

Figure 3. Representative examples of: **A**) human tumor tissues matched to **B**) normal tissues stained with biotinylated TM-601 (left column) or buffered saline (right column). Intense brown color indicative of positive staining is only seen in tumor tissues exposed to biotinylated TM-601 and not normal tissues. Mamelak, A., Jacoby, D. Expert Opin Drug Deliv 2007, 4(2): 175-96, copyright ©, Informa Healthcare. Reproduced with permission of Informa Healthcare.

decreasing MMP-2 activity, CTX prevents proteolytic degradation of extracellular matrix (ECM), thus preventing the release of glioma cells from the constraints of cellular interactions with ECM (37).

Anti-angiogenesis

Using the transwell migration assay, CTX was demonstrated to exert profound inhibition against agents that normally promote angiogenesis. CTX blocked the invasion of proliferating HUVEC exposed to VEGF and basic fibroblast growth factor (bFGF) by approximately 50%. Furthermore, CTX blocked MMP-2 activation in response to bFGF. CTX was shown to inhibit the formation of neovasculature by VEGF in a concentration-dependent fashion. This effect was observed for a wide range of angiogenesis-promoting factors, including bFGF, epidermal growth factor (EGF), IL-6, PDGF, TNF-α and hepatocyte growth factor (HGF). When CTX was coadministered with bevacizumab –an anti-VEGF antibody used in clinical cancer treatment– neovascularity was reduced by more than the effect observed with a 10-fold increase in bevacizumab dose alone. This observation suggests that CTX may inhibit more angiogenesis pathways than the VEGF-mediated pathway, and therefore provide an important synergistic effect in clinical treatment. As such, CTX may be an important antiangiogenic therapy, especially when used in conjunction with other treatments.

CLINICAL STUDIES

CTX was synthetically produced using solid-phase synthesis methods by TransMolecular, Inc., a biotechnology company that was subsequently acquired by Morphotek, Inc. The product, TM-601, can be produced in large quantities at reasonable cost, and the amino acid structure can be modified as needed. Mass spectrometry and HPLC assays indicate that the compound is identical to the naturally occurring compound in shape and structure. GMP-grade TM-601 has received FDA approval for clinical testing. The crayfish biological assay shows an identical response with the synthetic compound as with the natural compound. Therefore, all clinical testing in the past and anticipated future will likely be performed with TM-601 rather than naturally purified CTX. For the purpose of this review, the terms CTX and TM-601 may be used interchangeably.

Despite its selective binding properties and numerous cellular interactions noted above, CTX does not kill glioma cells in vitro when given as a single agent. Therefore, TM-601 was initially developed clinically as a targeting agent for delivering a therapeutic payload. For initial studies, the therapeutic payload utilized was [^{131}I] because it had been demonstrated to have efficacy against gliomas and had been utilized in previous radiolabeled antibody trials. Furthermore, the use of a radioactive isotope as a tracer facilitated dosimetry and biodistribution studies.

A phase I study to evaluate the safety, tolerability, biodistribution and dosimetry of intracavitary [^{131}I]-TM-601 in adult patients with recurrent high-grade glioma has been completed (39). In this trial, the dose of [^{131}I] was kept fixed at 10 mCi, while the dose of CTX was escalated from 0.25 mg to 0.50 mg and 1.0 mg in three dosing panels containing six patients each.

[^{131}I]-TM-601 was injected into the resection cavity of 18 patients with recurrent high-grade gliomas via an Ommaya reservoir 2 weeks after surgery. Radiation doses to normal organs were clinically insignificant. In contrast, mean radiation dose to within 2 cm of the cavity wall was 81 cGy/mCi (median 49) and ranged from 12 to 275 cGy/mCi. These values are substantially higher than doses to the whole body (mean 0.4 cGy/mCi) and to any other organs. Furthermore, the biological half-life of [^{131}I]-TM-601 in the tumor cavity margin was longer than in any other organ, including the normal brain, indicating long-term retention of the drug in and around the injection site. The median biological half-life in the cavity margin was 70 (range 32-193) hours, 80 (range 25-86) hours and 55 (range 41-62) hours for patients receiving 0.25, 0.50 and 1.0 mg peptide, respectively.

Total body and brain SPECT imaging showed that [^{131}I]-TM-601 localized to and remained primarily concentrated in and around the patient's surgical cavity for all 5 days imaged (Fig. 4).

[^{131}I]-TM-601 was well tolerated by all patients. There were no grade III or IV toxicities related to the study drug or method of administration in the immediate- and/or long-term follow-up period. There were no patient complaints related to the study drug or method of administration. Three patients had serious adverse events possibly or probably related to study medication and reported within 22 days of administration. These included the following: fever, chills, upper extremity paresthesias accompanied by mild cerebral edema on CT; progression in left-sided weakness; and infection of the tumor resection cavity and osteomyelitis. Additional serious adverse events reported by investigators to be possibly attributed to the study agent beyond the initial 22-day observation period included 1 case of generalized seizure and 1 case of headache, face droopiness, dysarthria and instability. Over the course of the 180-day observation period, there were a total of 7 deaths. Two patients survived over 30 months. Median survival was 27.0 weeks from surgery for all three dosing groups. Histochemistry of the tumor tissue from all patients tested thus far stained intensely positive for TM-601.

An analysis of the imaging data obtained for a subset of the patients enrolled in this trial was also performed (40). The purpose of this analysis was to determine if radiolabeled CTX might be a useful tool for imaging the extent of glioma invasion since it is tumor-specific and small enough to diffuse away from the primary tumor site. This study demonstrated that [^{131}I]-TM-601 SPECT scan estimates of tumor volume were midway between estimates on T2- and T1-weighted contrast-enhanced MRI scans, a result consistent with known glioma invasion patterns. This study provides promise that CTX may be useful for tumor imaging, as well as tumor therapy.

A phase II trial of this agent was also completed in patients with recurrent glioma. The goals of this trial were to determine the safety of multiple-dose administration at 40 mCi/0.8 mg/dose and to test the potential efficacy of the agent. The first portion of the study consisted of a dose-escalation scheme in which individual patients each received three doses of [^{131}I]-TM-601, spaced 1 week apart. The specific activity of the radiolabeled peptide was fixed, with the dose varying between 20 mCi/0.4 mg and 40 mCi/0.8 mg. There were no major toxicities observed. In the later phase, patients were randomized to receive either three or six weekly intracavitary injections of 40 mCi/0.8 mg [^{131}I]-TM-601. This trial was conducted at 17 centers in

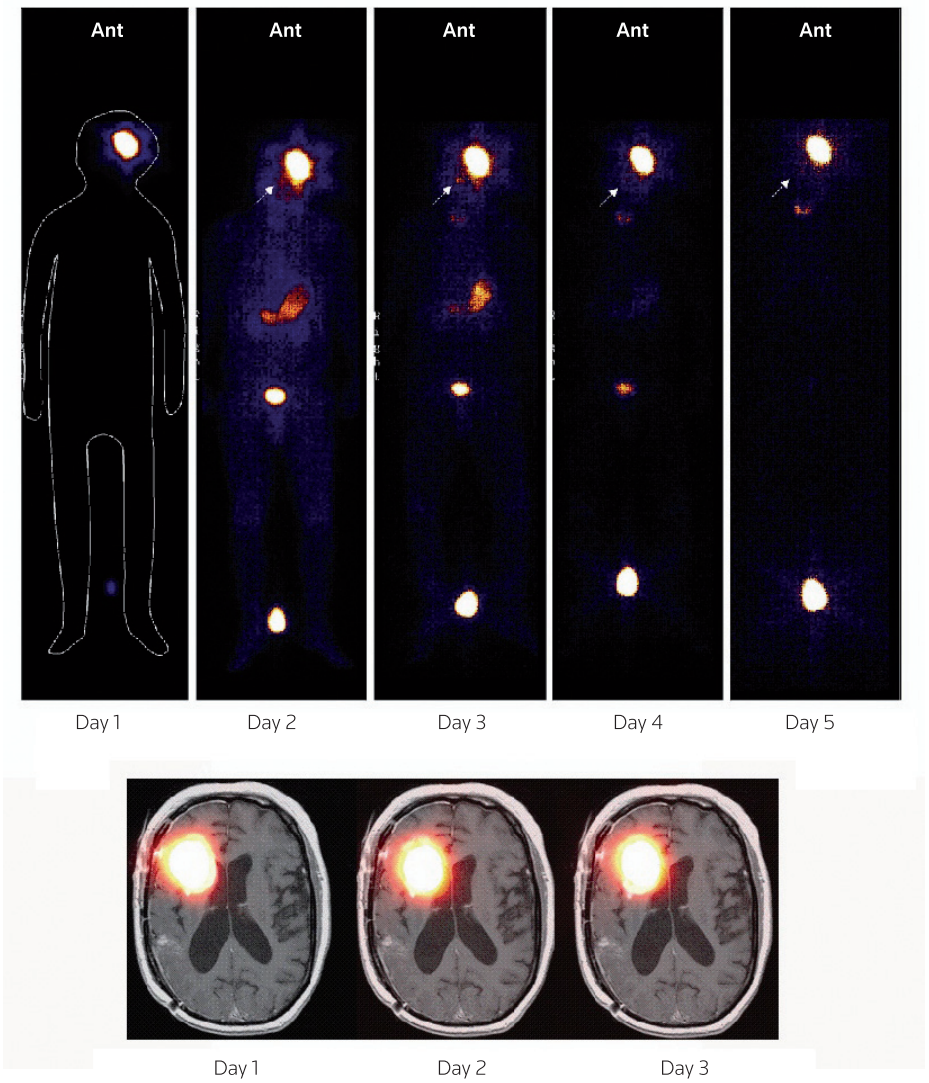

Figure 4. Total body planar and brain SPECT imaging of [^{131}I]-TM-601 following a single intracavitary dose of 10 mCi. The bright region noted between the legs in the total body planar images is a 1 mCi standard. Retention of the peptide at the tumor cavity site (left side of head) is evident on days 1-5, with minimal uptake anywhere else in the body. The loss of signal in the images roughly approximated the physical decay constant of [^{131}I], indicating long-term retention and minimal biological elimination at the tumor site, with rapid elimination elsewhere. Axial SPECT scans co-registered to MRI indicate long-term retention at the tumor cavity site with a volume of distribution that well approximates the tumor volume. Mamelak, A., Jacoby, D. Expert Opin Drug Deliv 2007, 4(2): 175-96, copyright ©, Informa Healthcare. Reproduced with permission of Informa Healthcare.

the U.S. and completed accrual in 2007. Although the final results have not been published, preliminary data (A. O'Neil, personal communication) suggest improved survival for patients receiving six doses compared to those receiving three doses. As with the initial phase of the trial, no deaths or major toxicities directly attributable to the therapy have been reported.

Simultaneously, an intravenous imaging trial was performed in patients with progressive cancer of any origin (CNS or systemic). The primary endpoint was the ability to visualize specific uptake of the agent on total body imaging. If so, this would suggest that [^{131}I]-TM-601 or variants could be used as both cancer imaging agents and as systemic targeted therapy. To date, uptake in regions of metastatic

disease has been documented, but formal dosimetry analyses have not been completed; therefore, the data are encouraging but still preliminary (J. Fiveash, personal communication).

OTHER USES OF CTX

In vivo tumor imaging

CTX demonstrates many of the properties ideally suited for use as a cancer imaging agent. Due to its small size, it can cross the BBB and penetrate deep into solid organs. It appears to demonstrate almost no binding to nonmalignant human tissues and is therefore highly tumor-selective. Furthermore, it appears to be nonimmunogenic and is rapidly cleared by the kidneys (40).

CTX has been bioconjugated to the fluorescent probe Cy5.5, a fluorophore that emits in the near-infrared range (34, 41). This compound, CTX:Cy5.5, was demonstrated to bind multiple tumor cell lines in vitro, with the extent of binding significantly inhibited by blocking MMP-2. Subsequent in vivo biophotonic imaging in an s.c. rat 9L gliosarcoma model, as well as spontaneous medulloblastoma, prostate cancer, intestinal cancer and sarcoma animal models, documented accurate detection of the tumors. Ex vivo imaging of the intestines from a mouse model of intestinal adenomatous familial polyposis also documented discrete and focal detection of tumor nodules and the ability to discriminate normal tissue from tumor (34). These studies indicate that fluorescently tagged CTX may be able to be used as a "tumor paint" given prior to surgery and identified during surgery by infrared fluorescence imaging. This method could aid in achieving more complete surgical resections and tumor-negative margins, both of which have been correlated with improved survival for many cancers. One of the technical limitations of this work is the need to demonstrate the safety of this specific fluorophore for clinical use. Nonetheless, based on encouraging data reported for the use of aminolevulinic acid (5′-ALA)-induced cellular fluorescence to detect tumor cells during glioma resection (10, 42), this technology appears promising.

5′-ALA is an orally administered agent that induces mitochondrial production and accumulation of protoporphyrin IX (pPIX), a compound that is fluorescent at 635 nm (near infrared) when excited in the ultraviolet range (405 nM). Because tumor cells have a higher rate of metabolic activity than normal glia and neurons, glioma cells accumulate significantly greater amounts of pPIX and can be detected by illuminating the tumor in vivo with a UV light source. This technique has been employed to maximize the extent of surgical resection in glioma, with some encouraging results (10, 42). However, cellular lysis and leakage of pPIX during surgery result in suboptimal resolution and a limited time window for use. Therefore, direct tumor targeting with a covalently bound fluorophore that is not dependent on tumor metabolism is very appealing. A comparison of the advantages and disadvantages of 5′-ALA- and CTX-based fluorescence for tumor resection is presented in Table II. However, to date, no human testing of CTX:Cy5.5 or other CTX-based fluorescent probes has been performed.

CTX nanoparticles for MRI-based tumor detection

CTX has also been bound to iron oxide magnetic nanoparticles for in vivo tumor detection via MRI (41, 43). In animal studies, nanoparticles coated with CTX targeted brain tumor cells with high affinity and were internalized despite an intact BBB (41, 44). Subsequent studies in which CTX nanoparticles were also linked to green fluorescent protein (GFP) DNA and given to animals harboring tumors identified the presence of fluorescence in tumor cells removed several weeks later (45). These studies demonstrated the feasibility of the concept that CTX-coated nanoparticles would be internalized by tumor cells and lead to activation of a gene transcript, thereby suggesting that CTX nanoparticles may provide a novel delivery system for gene therapy. These studies are in preliminary phases and require significant clarification of the purity and stability of the compounds, as well as clinical safety testing. However, they suggest that the tumor-specific targeting observed with CTX is robust and provides a novel platform for the development of a wide range of target-based imaging and therapy for tumors.

SOURCE OF CTX

GMP-grade CTX was produced and clinically tested by TransMolecular, Inc., a Cambridge, Massachusetts-based biotechnology company. TransMolecular developed the GMP synthesis process, as

Table II. *Comparison of 5′-ALA and fluorescently tagged chlorotoxin (CTX) for in vivo imaging of tumors during surgical resection.*

	CTX	5′-ALA
Method of fluorescence	– Extrinsic fluorophore (Cyc5.5) covalently linked to CTX – CTX-dependent cellular binding	– Cellular production of fluorophore driven by enzymatic synthesis
Excitation range	Ultraviolet	Ultraviolet
Emission range	Near infrared	Near infrared
Method of delivery	Direct intracavitary or i.v.	Oral
Bioavailablity	> 24-48 hours	6-12 hours
Technical obstacles	– FDA approval of extrinsic fluorophore for clinical use – Toxicity of UV light	– FDA approval of 5′-ALA – Variability of pPIX production – Toxicity of UV light
Other uses	Unknown	Photodynamic therapy

Table III. *Advantages and disadvantages of chlorotoxin as a targeted therapy for tumors.*

Advantages	Disadvantages	Other issues
Small size	Does not kill tumor alone	Currently only peptide in clinical trial for glioma
– Diffusible	– Needs "payload" therapy or coadministration of other therapy	May be effective against many other malignancies
– Crosses BBB	– Mechanism of action unclear	Basic science needs to better define peptide function
– Lack of immunogenicity		
Synthetic manufacturing	Clears very rapidly from systemic circulation	Multiple platforms or options for clinical use, difficult to define the "best strategy"
– Easily modified	– May impede use as i.v. agent	
– Can add linkers and payloads more easily	– May require repeated administration	
No binding to normal human tissues		
– No obvious toxicities		
– Tumor-specific action		
Useful for imaging and therapy applications		

BBB, blood–brain barrier.

well as the radiolabeling techniques that were used in all human studies, and obtained FDA approval for all clinical trials. Recently, the company was purchased by Morphotek, Inc., a subsidiary of Eisai Pharmaceuticals. As such, Morphotek currently owns the patent rights to this compound and subsequent modifications of its structure. Non-GMP-grade CTX can be obtained from a variety of chemical suppliers.

CONCLUSIONS

CTX represents a novel and exciting platform for cancer therapy and imaging. CTX meets many of the criteria of an ideal targeting agent (Table III). It is small and highly compact in structure. This means that it could cross the BBB and is far more likely to diffuse deep into solid tumors than antibodies, minibodies or other such larger molecular targeting agents. It is synthetically manufactured under GMP conditions and can be easily modified by amino acid substitution and pre- or post-folding modification. It contains a single tyrosine residue for iodination and can also be modified by covalent linkage for the conjugation of other imaging or therapy payloads. Because it is a naturally occurring biological peptide that was evolutionarily derived from an invertebrate with no human biology "design", it fortuitously demonstrates binding to human malignancies, but is otherwise alien to human tissues. Therefore, it appears to have no innate toxicity in humans despite repeated administration. The underlying interaction of CTX with tumor cells remains to be fully elucidated, and is currently the subject of much research. It is likely that CTX has antitumor and antiangiogenic activity even in the absence of a therapeutic payload, and as such, may be used as a stand-alone therapy or in conjunction with other cancer therapies as a treatment modulator. The ability of CTX to selectively bind to tumor cells may be exploited by linkage to a fluorescent or radioactive ligand for intraoperative tumor identification. Similarly, the agent may find use as a means to deliver target gene therapy or cellular toxin to tumor cells. Overall, the small size, lack of antigenicity and lack of toxicity make CTX an extremely attractive peptide for ongoing development in the field of cancer therapy and imaging.

The identification and testing of CTX further bolster the search for similar naturally occurring toxins and venoms that may play equally intriguing roles in the treatment of human diseases. This is likely to provide a promising pathway for new drug discovery over the next several decades.

DISCLOSURES

The author states no conflicts of interest.

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