Potent Pleiotropic Anti-angiogenic Effects of TM601, a Synthetic Chlorotoxin Peptide

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Abstract. Chemically synthesized chlorotoxin (TM601) has been studied as a tumor targeting peptide. In this study, the anti-angiogenic properties of TM601 are reported. Materials and Methods: In vitro and in vivo models of angiogenesis and tumor growth were used to characterize the anti-angiogenic effects of TM601. Results: TM601 bound to proliferating vascular endothelial cells, decreased human umbilical vein endothelial cell (HUVEC) invasion, and reduced secretion of bioactive matrix metalloproteinase-2 (MMP-2). Using the chick chorioallantoic membrane assay (CAM), TM601 inhibited angiogenesis stimulated by any of eight pro-angiogenic factors, and when TM601 was co-administered with bevacizumab, the combination was significantly more potent than a ten-fold increase in bevacizumab dose. TM601 did not alter tumor or vascular endothelial cell growth in vitro, but TM601 treatment of tumors grown on the CAM decreased tumor growth and intra-tumoral hemoglobin levels. Intravenously injected TM601 was also shown to significantly decrease new blood vessel growth in mice. Conclusion: TM601 inhibits angiogenesis stimulated by many factors and potentiates the anti-angiogenic effect of bevacizumab.

Chlorotoxin is a 36 amino acid peptide that was first purified from the venom of the scorpion Leiurus quinquestriatus (1). Chlorotoxin shares homology and structural similarity with other venom peptides and more broadly with a family of small disulfide-rich proteins with a knotted topology (2). For clinical studies, chlorotoxin has been manufactured using solid-phase chemical synthesis, and named TM601 to distinguish it from the naturally occurring peptide (3).

The selective binding properties of TM601 to glioma cells and other tumors of neuroectodermal origin, but not to normal non-transformed cells and tissues, was initially described by Lyons et al. (4). Further, more than 15 normal human tissues were shown to be negative for chlorotoxin binding. More recently, the in vitro and in vivo tumor targeting properties of the peptide have been shown to be retained following conjugation to a fluorescent dye (5), nanoparticles (6-8), and polymers (9). TM601 by itself is not cytotoxic to tumor cells and is currently being evaluated in Phase II clinical studies as a 131-I-iodine radioconjugate (3). Following local delivery of 131I-TM601 to the resection cavity in patients with recurrent glioblastoma, gamma camera and Single Photon Emission Computed Tomography (SPECT) scans have shown that the peptide is retained at the tumor cavity site for up to 8 days after drug administration (3, 10). Other activities of the non-radiolabeled peptide include inhibitory effects on the in vitro invasion of glioma cells and the reduction of matrix metalloproteinase-2 (MMP-2) activity (11, 12).

While studying the binding properties of TM601 to tumor cells in our laboratory, the finding that TM601 does not bind to non-transformed cells such as human neurons, astrocytes and fibroblasts (4) has been confirmed. However, in the present investigation, we have found that TM601 bound to and was transported into non-transformed proliferating vascular endothelial cells. The possible anti-angiogenic properties of TM601 were therefore explored.

Materials and Methods

Chlorotoxin synthesis. The chlorotoxin peptide, TM601, was synthesized using solid-phase chemical synthesis by Peptisyntha Inc. (Torrance, CA, USA). Proper folding and formation of four disulfide bonds was achieved using proprietary conditions and the peptide was purified to greater than 95% purity.

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measured using a plate-based colorimetric kit (GE Healthcare, activity analysis. Secreted MMP-2 activity from the HUVEC was harvested, centrifuged and snap-frozen for subsequent MMP-2 conditions for 16 hours, in triplicate. Conditioned media was then either left untreated or were treated with either 50 ng/ml bFGF (R&D Systems Inc.) or 50 μM TM601 under serum-free media. Untreated, control HUVEC were also incubated with PBS (control), VEGF (2 μg/ml), bFGF (1 μg/ml), lipopolysaccharide (LPS) (5 μg/ml), TNFα (10 μg/ml), interleukin-6 (IL-6) (10 μg/ml), platelet-derived growth factor-AB (PDGF-AB) (10 μg/ml), hepatocyte growth factor (HGF) (10 μg/ml), or epidermal growth factor (EGF) (100 μg/ml). The growth factors, cytokines and LPS were from R&D Systems Inc. Twenty-four hours later, either PBS or TM601 at various doses was added topically to the CAM. Alternatively, bevacizumab (Avastin®) or ranibizumab (Lucentis®, Genentech Inc., South San Francisco, USA) was added topically to the CAM. The CAMs were harvested on the third day of stimulation and the blood vessel branch points in the area of the filter disk were counted. The mean percent inhibition relative to the number of branch points of the unstimulated PBS control was calculated as a measure of the degree of neovascular inhibition.

**Tumor growth in the CAM model.** Fertilized chick eggs were incubated at 37°C until 10 days post-fertilization at which time a window was opened in the egg shell and 1 million tumor cells were applied to the CAM. Tumor cell lines included SK-Mel-28 (melanoma), PC-3 (prostate tumor), U87-MG (glioblastoma) and Panc-1 cells (pancreatic carcinoma) (American Type Culture Collection, ATCC, Manassas, VA, USA) and D54-MG (glioma) (kindly supplied by D. Bigner, Duke University, Durham, NC, USA). Next, 10 μg per CAM of TM601 or PBS were added directly to the tumor cells on the CAM surface. Tumors were allowed to continue to grow for 7 days, at which time the tumor mass was removed and weighed.

To measure the hemoglobin content within the tumors, 1 million tumor cells were mixed with growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA). After applying the cells to the CAM surface, TM601 was applied at a total dose of 10 μg per CAM. As a control, an equivalent volume of the PBS vehicle used to dissolve TM601 was applied to the CAM region containing the tumor cells. To measure hemoglobin in the absence of tumor cells, matrigel alone was applied to the CAM, plus an equivalent volume of the PBS vehicle. Tumors or control matrigel were harvested 7 days later, homogenized and the hemoglobin content was measured using Drabkin’s reagent (Sigma, St Louis, MO, USA).

**Proliferation assay.** Tumor cells and HUVEC were cultured in the presence of TM601 (100 μM), in triplicate, for 72 hours. Then, the viable cell number was measured with CellTiter Aqueous One Solution (Promega, Madison, WI, USA). The mean values were expressed as a percent of the untreated control value.

**Mouse Matrigel plug model.** Human recombinant VEGF-165 and bFGF (R&D Systems Inc.) were added to BD Matrigel High Concentration Basement Membrane Matrix (BD Biosciences) at a final concentration of 100 ng/ml. Five-hundred microliters of the growth factor supplemented Matrigel was injected bilaterally into the flank of female C57BL/6 mice (twelve Matrigel plugs per experimental group). Dosing with TM601 at 2, 10 or 100 mg/kg began on the day of Matrigel implantation using intravenous injection three times per week. Control animals received no treatment. The Matrigel plugs were removed after two weeks and fixed in formalin for histological analyses using hematoxylin and eosin stain and anti-CD31 immunostaining according to manufacturers instructions (BD Biosciences). The number of microvessels in the cross-sectional area of each Matrigel plug was counted. Mean microvessel counts from each treatment group were compared to the no treatment group using the Student’s t-test (p<0.05). All the animal experiments were conducted in accordance with protocols reviewed and approved by the animal care committees at Southern Research Institute (Birmingham, AL, USA).
Results

Effect of TM601 on proliferating vascular endothelial cells, cell migration and MMP-2 activity. The non-transformed proliferating human vascular endothelial cells isolated from umbilical cord, brain and dermal sources bound and internalized TM601 (Figure 1). Using the Transwell migration assay to measure migration of HUVEC toward VEGF, TM601 was found to inhibit invasion in a dose-dependent manner (Figure 2A). In addition to VEGF-stimulated invasion of HUVEC, TM601 (10 μm) also inhibited invasion in response to bFGF by approximately 50% (Figure 2B). In addition, TM601 treatment of the endothelial cells blocked MMP-2 activation in response to bFGF (Figure 2C).

Effect of TM601 on angiogenesis and in combination with bevacizumab. In light of the above results, the CAM assay was used to determine whether the in vitro effects on vascular endothelial cells translated into reductions in vessel formation using a more complex model system. Using VEGF to stimulate new blood vessels on the CAM, TM601 was shown to inhibit angiogenesis in a dose-dependent manner (Figure 3A). Interestingly, TM601 also inhibited blood vessel formation stimulated by all of a wide range of pro-angiogenic factors which act via different pathways (Figure 3B). Focusing on the potency of TM601 against VEGF-stimulated angiogenesis, the dosage of TM601 was compared with two anti-VEGF antibody therapeutics, bevacizumab and ranibizumab, and similar dose-response curves in the CAM model were observed (Figure 3C). To simulate a more complex stimulatory environment, VEGF, bFGF and TNFα were co-administered. Using this combination, the anti-angiogenic effect of bevacizumab plateaued whereas TM601 reached higher inhibitory levels (Figure 3D). Co-administration of TM601 and bevacizumab also showed increased potency (Figure 3E). Low concentrations of each compound yielded approximately 30% inhibition of vessel growth. Increasing the bevacizumab dose by 10-fold increased the inhibitory effect by only an additional 10%. However, the combination of low levels of bevacizumab and TM601 resulted in greater than a 70% inhibitory effect on vessel growth.

Tumor growth inhibition caused by inhibition of intra-tumoral vasculature. Tumor cells implanted on the surface of the chick CAM grow and stimulate neovascularization. Without
TM601 treatment of the implanted tumor cells, new blood vessels formed during the 7 day study and invaded into the tumor mass as it grew (Figure 4A, upper panels). In contrast, with a single application of TM601, neovascularization was greatly reduced and the size of the tumors appeared smaller (Figure 4B, lower panels). To quantitate this effect, tumors were removed from the CAM and weighed or analyzed for hemoglobin content. Both tumor growth and hemoglobin levels were significantly decreased by the TM601 treatment in all the tumor cell lines tested (Figure 4B and 4C). To rule out possible cytotoxic effects of TM601 on the tumors or vascular endothelial cells, in vitro cell proliferation assays were performed. TM601 was shown to have no cytostatic or cytotoxic effect on either the tumors or the vascular
Figure 3. Effect of TM601 on new blood vessel growth in the CAM assay. A, Representative VEGF-stimulated blood vessel growth on the surface of the CAM. B, Quantitative evaluation of the inhibition of new blood vessel sprouting by TM601 in the presence of pro-angiogenic factors. C, Comparison between TM601 and bevacizumab and ranibizumab using the CAM assay stimulated by VEGF. D, VEGF, bFGF and TNFα stimulated CAM. E, Co-administration of TM601 and bevacizumab in the CAM assay stimulated by VEGF. Error bars indicate the standard error. *p<0.05.
endothelial cells used in the tumor CAM experiment, as none of the mean values decreased below the saline control level (Table I).

**Effect of systemically administered TM601 on neovascularization.** In the Matrigel plug assay, TM601 caused a significant reduction in vascular growth into the Matrigel plug with all the dosing regimens tested (Figure 5). The effect appeared to reach a maximal response at 10 mg/kg, as 100 mg/kg did not further inhibit neovascularization. Thus, TM601 also inhibited angiogenesis with intravenous dosing in mice.

**Discussion**

For the first time, in addition to tumor-specific binding, TM601 has been shown to bind to proliferating vascular endothelial cells in vitro and to have anti-angiogenic activity both in vitro and in vivo. It is not clear yet whether the tumor-specific binding and anti-angiogenic effects are mechanistically related. Interestingly, human vascular endothelial cells represent the only normal tissue or cell to which TM601 has been shown to bind.

MMP2, a key mediator of extracellular matrix degradation and cell migration, appears to be a target of TM601 as
TM601 blocked HUVEC invasion towards VEGF or bFGF, and decreased the levels of active MMP-2 present in the HUVEC culture medium. The exact molecular mechanism for these effects is currently under investigation.

Out of the three model systems used in this study, the CAM and Matrigel plug assays most clearly showed that TM601 was acting to block neovascularization. The effect of TM601 in the tumor CAM assay is consistent with this conclusion whereby TM601 acted primarily to limit vessel growth stimulated by the tumor cells which in turn reduced the expansion of the tumor mass. In addition, this hypothesis was supported by the observation that TM601 bound to tumor cells in vitro but did not directly affect the in vitro cell proliferation of the tumor cells used in the tumor CAM assay. However, the possibility that tumor cells grown on the CAM were somehow sensitive to TM601 in a way that was not mimicked by the in vitro growth conditions cannot be ruled out.

Using the CAM assay TM601 acted on all the pro-angiogenic factors tested. The potency for inhibiting angiogenesis using each of these factors varied by approximately 300-fold, but showed that TM601 has a pleiotropic effect on blood vessel growth. The concentration of factors used to stimulate angiogenesis in the CAM assay was not optimized for maximal inhibitory effect by TM601, and ranged from 1 μg to 100 μg per ml. Therefore, the range in effectiveness of TM601 in this model would be expected and may not necessarily correlate with the potency of TM601 for each factor.

As is well-known, tumor cells directly secrete or indirectly cause other cells to secrete a large number of pro-angiogenic growth factors and cytokines, and reducing a single factor is unlikely to completely prevent angiogenesis (14, 15). Therefore, TM601 may have an advantage over more directed therapies that target one particular receptor or pathway. TM601 inhibited VEGF-stimulated angiogenesis in a similar fashion as bevacizumab and ranibizumab, two antibody therapies directed against VEGF. However, when VEGF, bFGF and TNFα were used together to stimulate angiogenesis, TM601 showed a dose-dependent inhibitory effect that reached 80% at a dose of 2.5 nmol. In contrast, bevacizumab reached a plateau of approximately 60% inhibition between 0.13 nmol and 6.7 nmoles (50-fold range) which might have been because bevacizumab did not effectively inhibit neovascularization induced by bFGF and TNFα whereas TM601 affected multiple pathways. TM601 given in combination with bevacizumab was also more potent then either drug alone, suggesting that TM601 and bevacizumab have different mechanisms of action and could benefit therapeutically from co-administration.

The novel observation that TM601 can inhibit angiogenesis in multiple animal models suggests a wider utility of TM601 in both oncology and other diseases of aberrant neovascularization. TM601 is already being developed clinically as a radiopharmaceutical using $^{131}$I-labeled peptide (10), and pre-clinically has been shown to retain tumor targeting potential after conjugation to a fluorescent dye (5), nanoparticles (6, 7), and polymers (9). In addition, nanoparticles with multiple chlorotoxin molecules on their surface have been shown to more effectively reduce invasion compared to monomeric chlorotoxin (16). These data raise the possibility that TM601 could more broadly be used to specifically deliver conjugated cytotoxic drugs to tumors (17) and, as a non-conjugated molecule, could inhibit angiogenesis. As a result of the anti-angiogenic properties of TM601, a phase I clinical trial has been initiated using perfusion MRI to monitor anti-angiogenic effects of TM601 in patients with recurrent glioblastoma.
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References


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