Effectiveness of Bispecific Cytotoxin (DTEGFATF) in Ameliorating Human Glioblastoma Tumors

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Abstract

Glioblastoma brain tumors are the third leading cause of cancer-related deaths, with a survival rate of less than 20% even with aggressive chemotherapy. Current glioblastoma chemotherapy treatments target brain-tumor cells; however, safe dosages of the cytotoxins used in chemotherapy do not completely eradicate the tumors.

I ran a proliferation assays to compare effectiveness of a newly engineered bispecific cytotoxin, DTEGFATF, to its monospecific counterparts, DTEGF and DTAT, in eradicating glioblastoma brain-tumor cells. I chose to study DTEGFATF because previous studies have shown that bispecific cytotoxins are more effective at eradicating prostate cancer cells than monospecific cytotoxins. I also ran proliferation assays to determine if DTEGFATF targets vascular cells that supply blood to brain-tumor cells and to ensure that DTEGFATF does not kill non-targeted cells. Finally, I ran blocking assays to determine if DTEGFATF specifically targets desired receptor sites on glioblastoma tumors because, if chemotherapy drugs disperse throughout tumors, dangerously high dosages must be used.

Results showed that DTEGFATF was10-fold more effective than DTEGF and 95-fold more effective than DTAT in eradicating glioblastoma brain-tumors cells and in targeting vascular cells than its monospecific counterparts. However, results showed that DTEGFATF did not specifically target glioblastoma brain-tumor receptors. This suggests that DTEGFATF may disperse throughout tumors rather than targeting specific receptor sites. The next step is to verify if DTEGFATF disperses throughout tumors to ensure that DTEGFATF can be used safely in chemotherapy against glioblastoma brain tumors.

Introduction

Glioblastoma brain tumors are the third leading cause of cancer-related deaths for patients between the ages of 15 and 34 years old, and the two-year survival rate for these patients is less than 20% (*1*). Current glioblastoma chemotherapy treatments target brain-tumor cells; however, safe dosages of these drugs often do not completely eradicate glioblastoma tumors (*2*). The first goal of my study was to investigate the potential of a newly engineered bispecific cytotoxin, DTEGFATF, and compare it to its monospecific counterparts, DTEGF and DTAT, in targeting and eradicating glioblastoma brain-tumor cells. (See Glossary of Terms in Appendix for names of abbreviated terms.)

DTEGFATF is a bispecific ligand-directed cytotoxin that is synthesized with two cytokine receptor-binding ligands on a single-chain molecule. These cytokine ligands are designed to selectively bind to overexpressed receptors on human cancer cells (*2*). The monospecific counterparts of DTEGFATF, which are DTEGF and DTAT, are ligand-directed cytotoxins that are constructed with only one cytokine ligand each (*2, 3*). In addition to the ligands, diphtheria toxin is incorporated into both cytotoxin structures to inhibit protein synthesis, which leads to cell death (*4*).

I chose to study DTEGFATF because its monospecific counterparts have been shown to target glioblastoma brain-tumor cells. A study by Stish et. al (2008) showed that DTEGF contains a ligand that binds to epidermal growth factor receptors found on glioblastoma brain tumors, and, in doing so, eradicates glioblastoma brain-tumor cells (*3*). A study by Vallera et. al (2002) stated that DTAT contains a ligand, that inhibits the formation of endothelial vasculature, which supplies nutrient-rich blood to brain-tumor cells. DTAT binds to amino-terminal fragments of urokinase plasminogen activator receptors on endothelial vasculature cells, effectively cutting off blood supplies to cells

(*2*). Based on the Stish study, I hypothesized that because epidermal growth factor receptors are found on glioblastoma brain-tumor cells, DTEGF would be effective in targeting glioblastoma cells. In addition, based on the Vallera study, I hypothesized that DTAT would be effective therapy in eradicating glioblastoma brain tumors because urokinase fragments are found exclusively on glioblastoma brain-tumor cells and related endothetial vasculature cells.

I chose to compare effectiveness of DTEGFATF to DTEGF and DTAT because studies have shown that bispecific ligand-directed cytotoxins are more effective in targeting receptors than monospecific cytotoxins (*4*). A prior study by Stish et al. (2007) showed that a bispecific ligand-directed cytotoxin called DTEGF13 was more effective in killing human prostate cancer cell lines than its monospecific counterparts DTEGF and DTIL13. This Stish study showed that the bispecific cytotoxin was more effective because it simultaneously targeted two established receptors in human prostate cancer cells—epidermal growth factor receptor and interleukin-13 receptor (*4*). A similar study by Vallera et al. (2005) showed that a bispecific cytotoxin called DT2219 had greater anticancer activity against leukemia/lymphoma cells than its monospecifics CD19 and CD22 (*5*). Based on these studies, I hypothesized that the bispecific cytotoxin that I investigated, DTEGFATF, would be more effective in killing cancer cells, than its monospecific counterparts DTEGF and DTAT.

My second goal was to run a proliferation assay on human umbilical vein endothelial cells and compare effectiveness of DTEGFATF, DTEGF, DTAT, and a combination of DTEGF + DTAT in targeting human umbilical vein endothelial cells that supply blood to brain-tumor cells. Based on the study by Vallera et. al (2002), which showed that DTAT is effective in killing human endothelial cells, I hypothesized that

DTEGFATF would be effective in eradicating human umbilical vein endothelial cells because DTAT is part of the composition of DTEGFATF (*2*).

My third goal was to run a control proliferation assay on HPB-MLT cells to be sure that DTEGFATF does not kill non-targeted cells at the same rate that it kills glioblastoma brain-tumor cells. HPB-MLT cells contain epidermal growth factor receptors and amino-terminal fragments of urokinase plasminogen activator receptors but have fewer of these receptors than glioblastoma brain-tumor cells (*2*). Because HPB-MLT cells have fewer of these receptors, I hypothesized that DTEGFATF would have less effect on HPB-MLT cells than on glioblastoma brain-tumor cells.

My fourth goal was to run a blocking assay to ensure that DTEGFATF specifically binds only to epidermal growth factor and amino-terminal fragments of urokinase plasminogen activator receptors on human glioblastoma brain-tumor cells. It is important for cancer chemotherapy drugs to target specific receptors on tumors because, if chemotherapy drugs disperse throughout a tumor, much higher dosages must be used, which will kill healthy cells (*4*). So, I conducted a blocking assay to block the receptors that DTEGFATF was expected to target: epidermal growth factor receptors and amino-terminal fragments of urokinase plasminogen activator receptors. I used antibody epidermal growth factor receptor (anti-EGFr) and antibody urokinase plasminogen activator receptor (anti-μPAR) because these antibodies have been shown to block the receptors that DTEGFATF targets (*2*, *4*).

Materials and Methods

Cell Culture: Human glioblastoma brain-tumor cell lines U-87 were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L

L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. U-87 and U-87/Luc cells were held in suspension using culture flasks. Cell cultures were incubated in a humidified 37 °C with 5% CO_2 atmosphere. Adherent cells that were 80% to 90% confluent were removed using trypsin-EDTA for detachment. Only cells with viability > 95%, as determined by trypan-blue exclusion, were used for experiments.

Proliferation Assays to measure* in vitro *cytotoxin activity: To determine effects of DTEGFATF on U-87, proliferation assays that measured thymidine incorporation were used. Cells (1 x 10^4 cells/well) were transferred to a 96-well flat-bottomed plate. Cells were incubated overnight at 37 °C with 5% CO_2 to allow cells to adhere to the plate. Each cytotoxin (100μL) in varying concentrations (10^{-4} to 10 nM) were added to each well in triplicate. Cells were then incubated at 37 °C with 5% CO_2 for 24, 48, 72, or 96 hours, depending on the assay. [Methyl-^{3}H]thymidine was added to each well (1 μCi/well) by laboratory personnel[1] for the final eight hours of incubation. Plates were frozen at –80 °C to detach cells from the wells. Cells were harvested onto a glass fiber filter, washed with dH_2O, dried under a heat lamp, and counted using standard scintillation methods. Assays were repeated using leucine-free media and were labeled with [^{3}H]leucine, rather than [^{3}H]thymidine, for last 24 hours of incubation. Results from assays were reported as percentage of control counts. This procedure was repeated for human umbilical vein endothelial cells and HPB-MLT cells. Results were reported as inhibitory concentrations, IC_{50}, a concentration of a cytotoxin that is lethal to 50% of

[1] Because I was not 18 when conducting my research, I was not eligible to use [Methyl-^{3}H]thymidine because it is a radioactive isotope. So laboratory personnel added the tritium isotope and completed the scintillation count. I conducted all other steps of the protocol.

cells in cell culture. Cytotoxin Bic3 was used as a control because it does not target glioblastoma cells but does target HPB-MLT cells.

Blocking Assay to measure* in vitro *cytotoxin activity: Blocking assays were conducted to test specificity of DTEGFATF. Anti-EGFr and anti-μPAR were added to Dulbecco's modified eagle's medium + 7.5 blastocidin media containing 1-nM DTEGFATF. Resulting mixtures were added to wells containing U-87 cells, and proliferation was measured by [^{3}H]thymidine uptake. Antibody Ly 5.2 was used as a negative control.

Results

Figure 1 compares effectiveness of bispecific cytotoxin to monospecific counterparts in eradicating glioblastoma brain-tumor cell line U-87. IC_{50} was figured at 50% of thymidine response, which is the point where half of the assayed cells were still living. The IC_{50} of DTEGF was 0.34417 nM compared to IC_{50} of DTAT that was 3.39280 nM. IC_{50} of the combination drug, DTEGF + DTAT, was 1.7848 nM, while the IC_{50} of the bispecific cytotoxin DTEGFATF was 0.03562 nM.

Figure 1. Effects of DTEGFATF, DTEGF, DTAT, Bic3, DTEGF + DTAT on proliferation of U-87 cells.

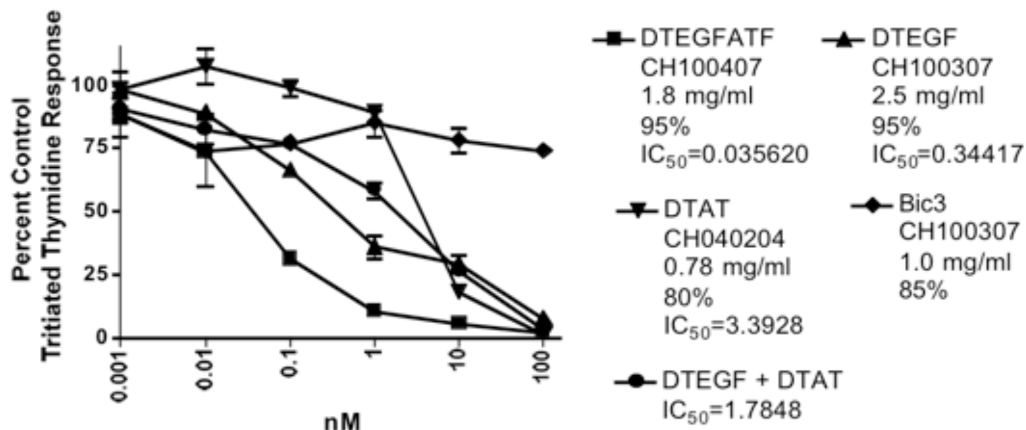


Figure 2 shows effects of DTEGFATF on human umbilical vein endothelial cells compared to its monospecific counterparts. The IC_{50} of DTAT was 0.15113 nM, while the IC_{50} of DTEGF + DTAT was 0.92028 nM, and the IC_{50} of DTEGFATF was 0.03353 nM.

Figure 2. Effects of DTEGFATF, DTEGF, DTAT, Bic3, DTEGF + DTAT on proliferation of human umbilical vein endothelial cells.

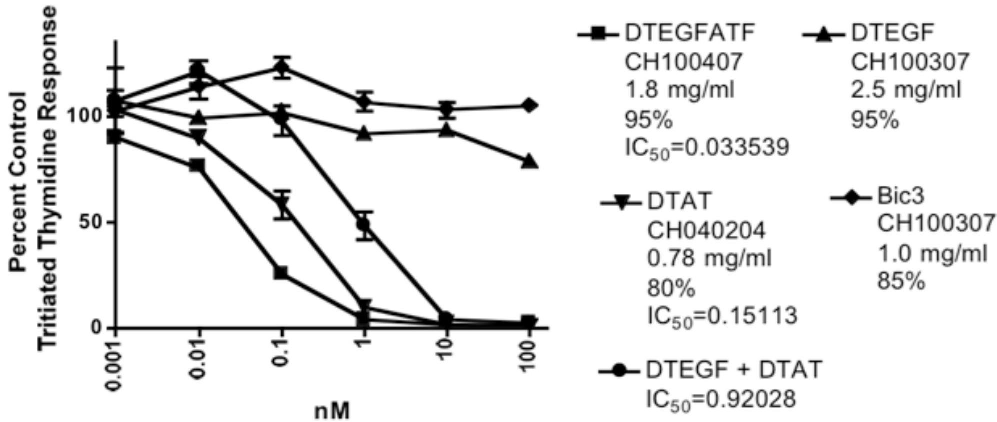


Figure 3 shows the effects of DTEGFATF on HPB-MLT cells compared to monospecific counterparts. The IC_{50} of DTAT was 2.645 nM, while the IC_{50} of DTEGF + DTAT was 2.352 nM, and the IC_{50} of DTEGFATF was 3.589 nM. The IC_{50} for the control Bic3 was below 0.0001 nM.

Figure 3. Effects of DTEGFATF, DTEGF, DTAT, Bic3, DTEGF + DTAT on proliferation of HPB-MLT cells.

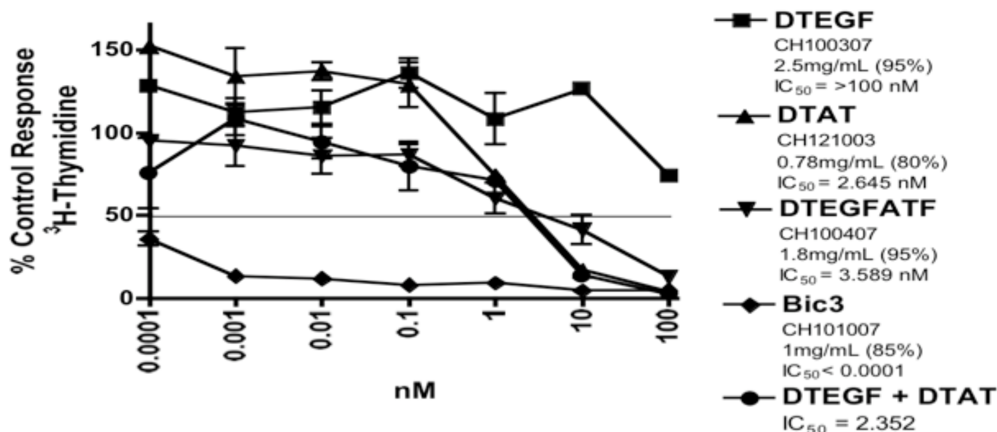
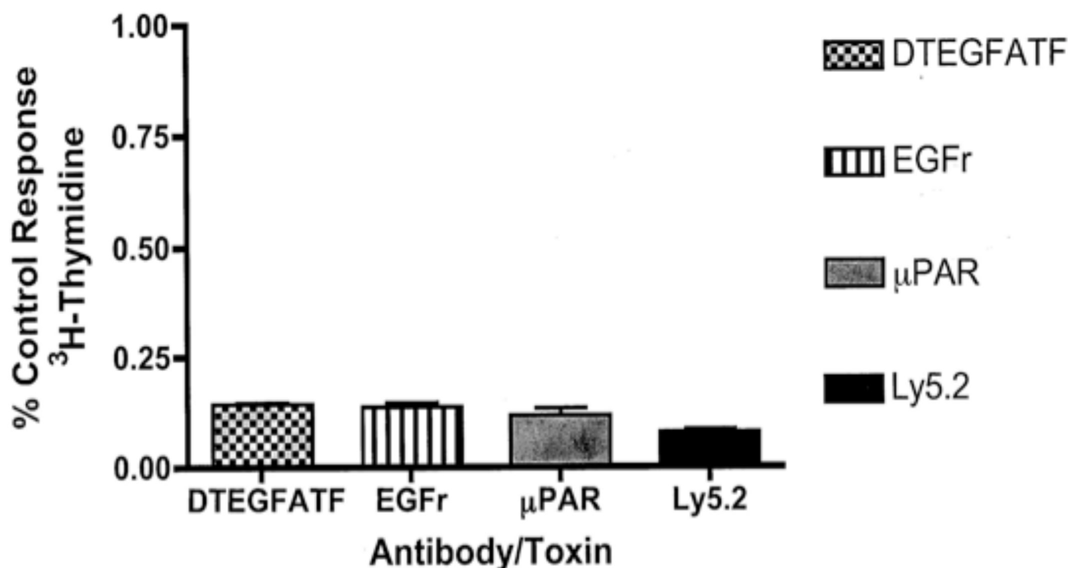


Figure 4 shows results from the blocking assay, comparing abilities of anti-EGFr, anti-µPAR, and control anti-Ly 5.2 antibodies to block effects of DTEGFATF on U-87 cells. Anti-EGFr and anti-µPAR blocked 15% of 1-nM DTEGFATF, while the control, anti-Ly 5.2, blocked 13% of 1-nM DTEGFATF.

Figure 4. Abilities of EGFr, µPAR, Ly5.2 to block effects of DTEGFATF on proliferation of U-87 cells.



Discussion

Results supported my hypothesis that DTEGFATF is more effective in eradicating human glioblastoma brain-tumor cells than its monospecific counterparts. Proliferation results showed that DTEGFATF inhibited glioblastoma brain-tumor cells 10-fold more than DTEGF and 95-fold more than DTAT. Additionally, DTEGFATF inhibited glioblastoma tumor-cells 50-fold more than monospecific combination of DTEGF + DTAT (Figure 1).

Results supported my second hypothesis that DTEGFATF is more effective than its monospecific counterparts at selectively binding to vascular cells that supply blood to brain-tumor cells. Proliferation results showed that DTEGFATF inhibited human

endothelial vasculature cells 5-fold more than DTAT. Results also showed that DTEGFATF eradicated more endothelial cells than DTEGF at every concentration. While 1-nM DTEGFATF eradicated close to 100% of endothelial cells, 1-nM DTEGF did not kill more than 20% of endothelial cells. Additionally, DTEGFATF inhibited glioblastoma tumor-cells 27-fold more than monospecific combination of DTEGF + DTAT (Figure 2).

Results also supported my third hypothesis that DTEGFATF would have less effect on HPB-MLT cells than glioblastoma cells. The IC_{50} of DTEGFATF when targeting glioblastoma cell line U-87 was 0.035620 nM (Figure 1) compared to an IC_{50} value of 3.589 nM when targeting HPB-MLT cells (Figure 3). DTEGFATF targeted less than 90% of HPB-MLT cells below 10 nM. However, above 10-nM DTEGFATF did target more than 50% of HPB-MLT cells (Figure 3). These results indicated that DTEGFATF does have an effect on HPB-MLT cells at high concentrations; however, in clinical trials, much lower dosages than 10 nM are used.

Results did not support my final hypothesis that DTEGFATF would specifically bind only to epidermal growth factor and amino-terminal fragments of urokinase plasminogen activator receptors on human glioblastoma brain-tumor cells. Results from the blocking assay showed that DTEGFATF was blocked less than 20% (Figure 4). This suggests that DTEGFATF may disperse throughout the brain tumor rather than targeting specific receptor sites.

Conclusion

My study showed that DTEGFATF is a more effective cytotoxin to use against glioblastoma brain tumors than its monospecific counterparts (Figures 1 and 2). My study also showed that DTEGFATF targeted a higher percentage of glioblastoma brain-tumor

cells than HPB-MLT cells, suggesting that DTEGFATF will not target cell lines that do not contain epidermal growth factor receptors and amino-terminal fragment of urokinase plasminogen receptors. However, specificity testing to ensure that DTEGFATF solely attacks epidermal growth factor receptors and amino-terminal fragment of urokinase plasminogen receptors was not conclusive (Figure 4).

Limitations to my study centered around the blocking assay. While results suggest that DTEGFATF disperses throughout the tumor rather than targeting specific receptor sites, there are other factors that must be considered. The blocking assay may not have worked because the antibodies used to block epidermal growth factor and amino-terminal fragment of urokinase plasminogen activator receptors were not effective. These antibodies blocked only 20% of the epidermal growth factor and amino-terminal fragment of urokinase plasminogen activator receptors. In addition, the concentration of DTEGFATF that I used against the antibodies may have been high enough to overpower blocking antibodies. Better results may be obtained by running additional blocking assays at concentrations below 10 nM and adding higher concentrations of antibodies.

Because the blocking assay did not give conclusive results, the next step of this project will involve an *in vivo* study to verify results that suggest DTEGFATF is an effective cytotoxin for eradicating glioblastoma brain tumors. If the *in vivo* study is conclusive, then another blocking assay of DTEGFATF will be conducted. Based on the results of the blocking assay, DTEGFATF will be further investigated as a possible clinical trial drug.

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Appendix. Glossary of Terms

Abbreviations	**Chemical Name**
Anti-EGFr	Antibody epidermal growth factor receptor
Anti-μPAR	Antibody urokinase plasminogen activator receptor
Anti-Ly 5.2	Control antibody
CD19	Clusters of differentiation 19
CD22	Clusters of differentiation 22
DT2219	Diphtheria toxin 2219
DTEGFATF	Diphtheria toxin epidermal growth factor amino terminal fragment
DTEGF	Diphtheria toxin epidermal growth factor
DTAT	Diphtheria toxin amino acid transfer
DTEGF13	Diphtheria toxin epidermal growth factor 13
DTIL13	Diphtheria toxin interleukin-13
HPB-MLT	Human T-cell leukemic cell line
U-87	Human glioblastoma brain-tumor cell line