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Targeted diazeniumdiolates: Localized nitric oxide release from glioma-specific peptides and proteins

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ABSTRACT

Nitric oxide (NO) is a small yet important biological messenger, which at sufficient concentrations has been shown to induce apoptosis as well as increase radiosensitization in tumor cells. However, the short half-life of NO gas itself has limited its utility as a therapeutic agent. The objective of this study was the development of targeted NO donors and we illustrate their utility as a potential therapeutic for treatment of glioblastoma multiforme, the most common and aggressive malignant primary brain tumor in adults. We have synthesized two diazeniumdiolate NO donors by reacting NO gas with glioma-specific targeting sequences, VTWTPQAWFQWVGGGSKKKKK (VTW) and chlorotoxin (CTX), and achieved repeatable NO release from both donors. FITC-labeled biomolecules, when incubated with glioma and control cells preferentially bound to the glioma cells and showed only minimal binding to the control cells. Additionally, tumor cell viability was significantly decreased when cells were incubated with the NO donors whereas control cell viability was not affected.

1. Introduction

Nitric oxide (NO) is a small, easily diffusible gaseous molecule that plays numerous roles in human physiology. NO is soluble in water and lipids and has an *in situ* half-life of a few seconds (Shinoda and Whittle, 2001). In the body NO acts as a signaling molecule in vascular, neurological, and cytotoxic functions (Fukumura et al., 2006). A variety of NO donors have been developed for the treatment of numerous diseases, since NO gas itself has a short half-life and therefore limited utility as a therapeutic agent (Muscara and Wallace, 1999; Ignarro et al., 2002). NO donors are characterized in the following categories: (1) organic nitrates and nitrite esters, (2) metal–nitrosyl complexes, (3) *S*-nitrosothiols, (4) hybrid NO donors, and (5) diazeniumdiolates (Katsumi et al., 2007; Wang et al., 2002).

Diazeniumdiolates, also called NONOates, are a class of NO donors which have a diolate group (N(O−)N=O) bound to an amine group (Keefer et al., 1996). The NO first reacts with the amine to form a radical which then reacts with another NO molecule to form the NO donating complex (Taylor et al., 1995). These compounds decompose to release NO in acidic or neutral solutions, and under basic, freeze-dried, or frozen states diazeniumdiolates are stable (Dutton et al., 2004). Release rates may vary with temperature, pH, and the molecular structure of the donor (Taylor et al., 1995). The by-product of the reaction is the original amine compound (Davies et al., 2001); this is of great importance as many NO donors, such as sodium nitroprusside, produce toxic by-products as NO is released. Diazeniumdiolates, on the other hand, may be designed to leave an innocuous peptide or protein as a by-product (Fig. 1; Hrabie et al., 1993). Though previous studies have demonstrated that NO donors can be successfully conjugated to proteins such as bovine serum albumin (BSA) (Hrabie et al., 1999), our goal is the cell-specific delivery of NO using bioactive targeting ligands without making significant alterations to the protein or peptide structure.

Targeted drug delivery has been the focus of many cancer therapies since it results in the reduction or even elimination of drug accumulation in the non-target organ which diminishes the toxic side effects of drugs to healthy tissue (Patri et al., 2005; Sudimack and Lee, 2000). The ubiquitous role NO plays in human physiology makes it necessary that exposure to NO be limited to the therapeutic target (Saavedra et al., 1997), but thus far, no tumor selective NO donors have been developed. To illustrate the value of such a targeted NO-donating molecule, we chose to derivatize small proteins and peptides specific to glioblastoma multiforme (GBM), a Grade IV astrocytoma which is the most common and most deadly malignant primary brain tumor in adults (Robins et al., 2007; Parsons et al., 2008). Patients diagnosed with the disease have a median survival of only 12 months and five-year survival rates are less than 5% (Nakada et al., 2007) due to the aggressive and invasive nature of the tumor cells. Single cells often escape surgical removal, resulting in the reoccurrence of high grade gliomas in 90% of cases, usually

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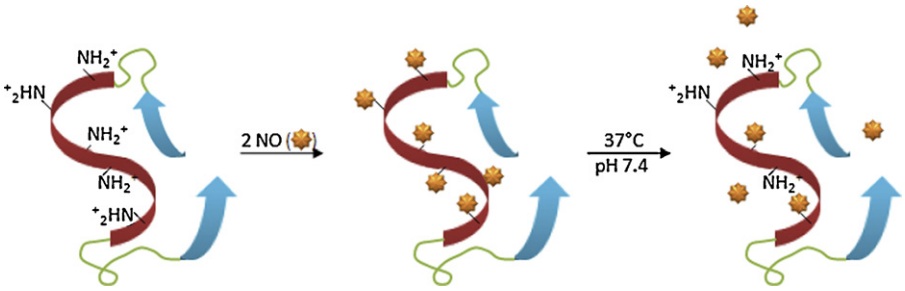

Fig. 1. The reaction of biomolecules with NO to form bioactive diazeniumdiolates. The NO first reacts with free amines to form radicals which then react with an additional NO molecule to form the diazeniumdiolate compound.

within 2 cm of the original site (Hochberg and Pruitt, 1980). Additionally 10–20% of gliomas develop lesions at further distances from the original site (Germano et al., 2010).

At sufficient concentrations, NO has been shown to induce apoptosis and increase radiosensitization in tumor cells (Kurimoto et al., 1999). Furthermore, NO has been shown to inhibit the activity of T4 DNA ligases, which repair single-stranded discontinuities in DNA, explaining the increase in single DNA strand breaks found in cells exposed to NO (Graziewicz et al., 1996). In addition to these tumoricidal effects, NO is able to increase the permeability of the blood–brain barrier, allowing larger therapeutic molecules to reach the tumor (Yin et al., 2008). Herein we report the synthesis of glioma-specific NO donors by converting the free amines of targeting biomolecules into diazeniumdiolates. By coupling the targeting ability of these biomolecules with NO-releasing capabilities we were able to develop a more efficient NO delivery method which not only reduced glioma proliferation but also had limited effects on astrocytes and brain microvascular endothelial cells.

2. Materials and methods

2.1. Chemicals

Minimum Essential Media (MEM), fetal bovine serum (FBS), L-glutamine–penicillin–streptomycin (GPS), and non-essential amino acids were obtained from Mediatech, Inc. (Manassas, VA). NO gas was obtained from Airgas (Atlanta, GA). VTWTPQAWFQWVGGGSKKKKK (VTW, 98.3% purity) was obtained from Genscript USA Inc. (Piscataway, NJ) and chlorotoxin (CTX, purity > 87%) was obtained from Bachem Chemicals (Torrance, CA). Unless otherwise mentioned all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell maintenance

T98G and U-87MG human glioblastoma cells (American Type Cell Culture, Manassas, VA), were cultured in MEM with 10% FBS, 1% GPS and 1% non-essential amino acids. Normal human astrocytes (NHAs; Lonza Inc., Walkersville, MD) were cultured in astrocyte basal media (ABM) supplemented with astrocyte growth medium SingleQuots (Lonza Inc., Walkersville, MD). Human brain microvascular endothelial cells (HBMECs; ScienCell Research Laboratories, Carlsbad, CA) were cultured in Endothelial Cell Medium (ECM) supplemented with endothelial cell growth supplement (ScienCell Research Laboratories, Carlsbad, CA) at 37 °C and 5% CO_2. For experiments NHA passages 3–5, HBMEC passages 3–6 and T98G and U-87MG passages 4–9 were used.

2.3. Synthesis and characterization of NO donors

Our first step in developing glioma specific NO-releasing therapeutics was the identification of glioma targeting peptide sequences that would make suitable diazeniumdiolates. The first biomolecule identified was chlorotoxin (CTX), a 36-amino acid protein isolated from the venom of the Deathstalker scorpion (*Leiurus quinquestriatus*) (Soroceanu et al., 1998; DeBin et al., 1993). CTX has a high affinity for surface-bound matrix metalloproteinase-2 (MMP-2) which is highly expressed in glioma cells but normally absent in brain tissue (Soroceanu et al., 1998). CTX conjugated nanoparticles have been used to image GBM, illustrating not only the specificity of CTX towards GBM cells but also its ability to cross the blood–brain barrier without damaging it (Veiseh et al., 2009b).

To form NO donors, CTX was dissolved in nanopure water (18 mΩ cm resistance) at room temperature and pH 7.4. The solution was then placed in a round bottom flask and the atmosphere was evacuated to limit the presence of oxygen in the reaction environment. The flask was then filled with NO gas and stirred for 1 h, after which the pH of the solution was adjusted to 7.4 using 1 M sodium hydroxide, thereby preventing the decomposition of the newly formed diazeniumdiolates at low pH. Subsequently the atmosphere was evacuated again, and the solution was exposed to additional NO gas for another 24 h (Bohl and West, 2000; Jun et al., 2005) to ensure conversion of remaining amines to diazeniumdiolates. After 24 h, the pH of the solution was again adjusted to 7.4 and the samples were frozen, lyophilized and stored at −20 °C until use. These samples were designated CTX-NO.

The second biomolecule identified was a derivative of the 12 amino acid peptide sequence VTWTPQAWFQWV, which was identified using a 12-mer phage display library by Wu et al. (2008). To decrease the hydrophobicity of the peptide, a GGGS spacer sequence and ten lysine residues were added to the sequence. Wu et al. demonstrated that the modified peptide binding was significantly higher to glioma cells in comparison to normal human astrocytes (Wu et al., 2008). In order to reduce the potential for nonspecific binding of unreacted positively charged molecules, the number of lysine residues was reduced to five and the sequence used in this paper was VTWTPQAWFQWVGGGSKKKKK (VTW). No changes were made to the CTX protein as there are 8 free amines inherent in the sequence. VTW-NO was synthesized using the same method as described for CTX-NO, where solutions of VTW were reacted with NO gas for 24 h.

A colorimetric Ninhydrin assay was performed in order to determine the concentration of free amines in the CTX-NO and VTW-NO samples, thus measuring the extent of the reaction with NO (Moore, 1968). Briefly Ninhydrin solution (Spectrum Chemicals, Gardena, CA) was added to solutions of CTX, CTX-NO, VTW, VTW-NO, as well as solutions of known amine concentrations. These mixtures were then placed in a boiling water bath for 15 min and then allowed to cool for 30 min. Using Beckman DTX 880 Multimode Plate Reader (Beckman Coulter, Drea, CA) sample absorbances were measured at a wavelength of 570 nm.

2.4. Characterization of NO release

Lyophilized samples of CTX-NO and VTW-NO were each dissolved in nanopure water and NO release at 37 °C from both species was quantified using an ISO-NOPF nitric oxide sensor (World Precision Instruments Sarasota, FL) connected to an APOLLO 1000 Free Radical Detector (World Precision Instruments Sarasota, FL). The ISO-NOPF sensor is a combination of a NO-sensing element and a reference electrode coated with a NO selective membrane. NO gas diffuses through the membrane and is oxidized at the surface of the working electrode generating a redox current (Zhang, 2004).

2.5. Verification of glioma targeting ability

Tumor targeting abilities of VTW and CTX subsequent to the reaction with NO were assessed using fluorescence labeling techniques. For this purpose VTW and CTX were labeled with fluorescein isothiocyanate (FITC) as per the manufacturer's protocol. Briefly, the biomolecules were dissolved in nanopure water and reacted with 0.1 mM solution of FITC in 0.1 M sodium bicarbonate buffer (pH 9.0). The FITC solution was then added dropwise to the solution of CTX or VTW in nanopure water and allowed to react for 2 h in the dark. The FITC labeled biomolecules were then dialyzed for 2 h against nanopure water and subsequently reacted with NO for 24 h to neutralize the charge on any unreacted amines on the biomolecules. The final products were frozen, lyophilized and stored at −20 °C until use. At the time of experiments these lyophilized powders were dissolved in nanopure water to form solutions such that each cell type was exposed to 5 μM of FITC-conjugated amines.

T98G, U-87MG, NHAs, and HBMECs were seeded in black-walled 96-well plates at a density of 25,000 cells/cm^2. After allowing the cells to adhere for 24 h, they were incubated for 30 min with FITC-labeled biomolecules. After 30 min, the medium was removed, and the cells were washed three times with phosphate buffered saline (PBS, pH 7.4) before fresh media was added. The ability of the biomolecules to target the different cell types was visualized using a Leica DMI 4000B fluorescent microscope equipped with a Hamamatsu ORCA-ER digital camera (Leica Microsystems, Inc., Bannockburn, IL). A minimum of three pictures were taken per well. The fluorescence within each field of view was quantified using Image J software. Additionally, cell proliferation was monitored to ensure that there would not be significant differences in the cell populations observed during this study. 24 h after cell seeding, adherent cells were removed from the culture surface using trypsin-EDTA and counted using a Beckman z1 Particle Counter (Beckman Coulter, Drea, CA).

2.6. Effect of NO on cell viability

T98G, U-87MG, NHAs, and HBMECs were seeded in 96-well plates at a density of 25,000 cells/cm^2. After allowing cells to adhere for 24 h, the cells were incubated with varying concentrations of CTX-NO or VTW-NO, such that each cell type was exposed to 0, 10, 20 and 40 μM of NO. After 48 h the cultures were rinsed thoroughly to remove dead cells; the remaining adherent cells were removed from the culture surface using trypsin-EDTA and counted using a Beckman z1 Particle Counter (Beckman Coulter, Drea, CA). Cell viability was calculated according to the following formula:

$$\text{Cell viability} = \frac{\text{Cells counted after incubation with NO donor}}{\text{Cells counted after incubation with DI}} \times 100.$$

2.7. Statistical analysis

All experiments were carried out minimally in triplicate. Statistical comparisons were conducted using an analysis of variance (ANOVA), with p-values less than 0.01 considered statistically significant.

3. Results

3.1. Characterization of NO release

The number of amines detected before and after the reaction of the biomolecules with NO was quantified using the Ninhydrin assay. Before the reaction with NO gas 6.9 ± 0.26 amines/mole CTX (85.7 ± 3.31%) were detected, whereas after the 24 h reaction with NO gas 0.00 ± 0.00 amines/mole CTX (0.00 ± 0.00%) were detected. Similarly prior to the reaction of VTW with NO gas 3.6 ± 0.07 amines/mole VTW (72.5 ± 1.42%) were detected, whereas after the reaction 0.05 ± 0.08 (0.91 ± 1.58%) amines/mole VTW were detected. The results from the Ninhydrin assay showed that at the end of 24 h, at least 97.5% of the detectable amines had reacted with the NO.

As previously discussed, diazeniumdiolates are able to dissociate in aqueous basic and neutral environments to release 2 mole of NO (Taylor et al., 1995). To measure this phenomenon, samples of CTX-NO and VTW-NO were dissolved in nanopure water and the NO release rates were measured using an NO probe, which because of its NO selective membrane, senses only NO and not byproducts of NO degradation that may be in the solution. The measurements from the NO specific microsensor showed that both biomolecules, CTX-NO and VTW-NO have similar release profiles with CTX-NO having the highest release at 3.93 h and VTW-NO having the highest release at 4.45 h (Fig. 2A and C).

Using the instantaneous release profiles measured by the NO probe as well as the results from the Ninhydrin assay, cumulative release profiles for both CTX-NO and VTW-NO were calculated (Fig. 2B and D). CTX-NO released 91.05 ± 1.30% of the expected NO in the first 72 h while VTW-NO released 87.9 ± 0.26% of the expected NO in that same time period (Fig. 2B and D). From the release profiles it was also determined that CTX-NO has a half-life of 19.2 h and VTW-NO has a half-life of 24.3 h.

3.2. Verification of targeting

To visually assess whether the utilization of amines influenced the ability of the biomolecules to target glioma cells, FITC was reacted with each peptide, fluorescently tagging the biomolecules, followed by a reaction with NO to neutralize the charge on any unreacted amines and prevent non-specific binding. Fluorescent microscopy showed that the FITC-labeled biomolecules, when incubated with T98G, U-87MG, NHA and HBMEC cells, bound preferentially to the glioma cell lines and showed only minimal binding to the NHAs and HBMECs (Fig. 3), indicating that the reaction of the amines with NO, a much smaller molecule than FITC, also does not hinder the ability of the biomolecules to target glioma cells. The relative fluorescence detected for each cell type was quantified using Image J, and both glioma cell types retained significantly more fluorescence when exposed to FITC-labeled CTX and VTW (Fig. 4). Relative to the fluorescence detected in NHA cultures (normalized to 1.00 ± 0.21), labeled CTX bound to HBMECs had an intensity of 1.01 ± 0.15 while T98G cultures had a fluorescence intensity of 5.78 ± 1.07 and U-87MGs had an intensity of 3.46 ± 0.99. To confirm targeting of labeled VTW, the relative fluorescence was quantified similarly for NHAs (again normalized to 1.00 ± 0.11), HBMECs (1.30 ± 0.16), T98Gs (4.30 ± 0.99), and U-87MGs (3.42 ± 0.73). Cell

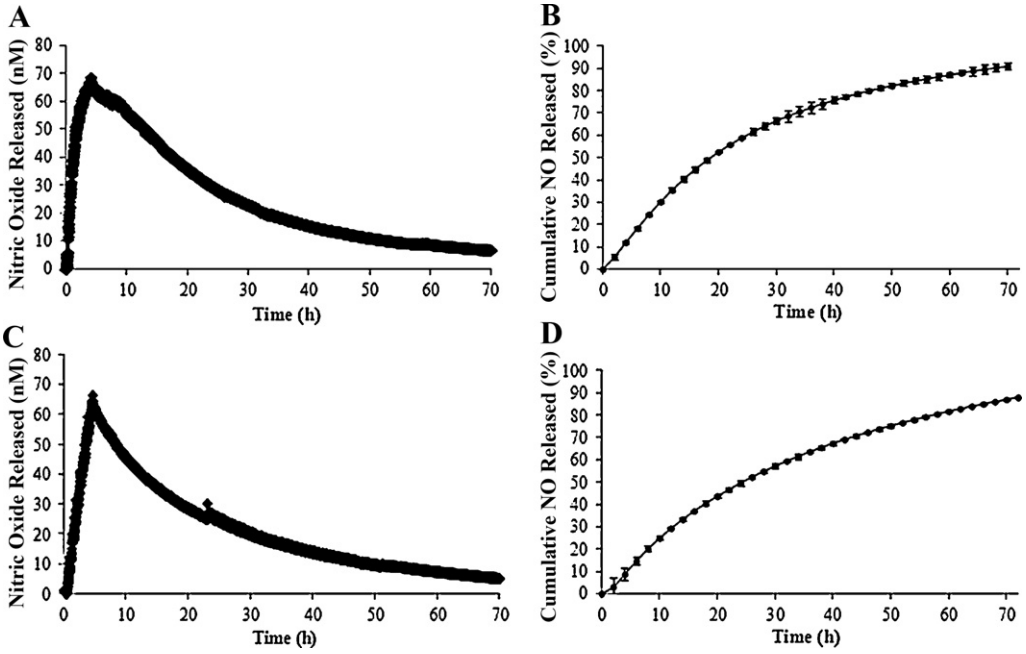

Fig. 2. NO release from targeted diazeniumdiolates: (A) instantaneous release of NO from CTX-NO at pH 7.4 in DI; (B) cumulative NO release from CTX-NO at pH 7.4; (C) instantaneous release of NO from VTW-NO at pH 7.4 in DI; and (D) cumulative NO release from VTW-NO at pH 7.4 in DI.

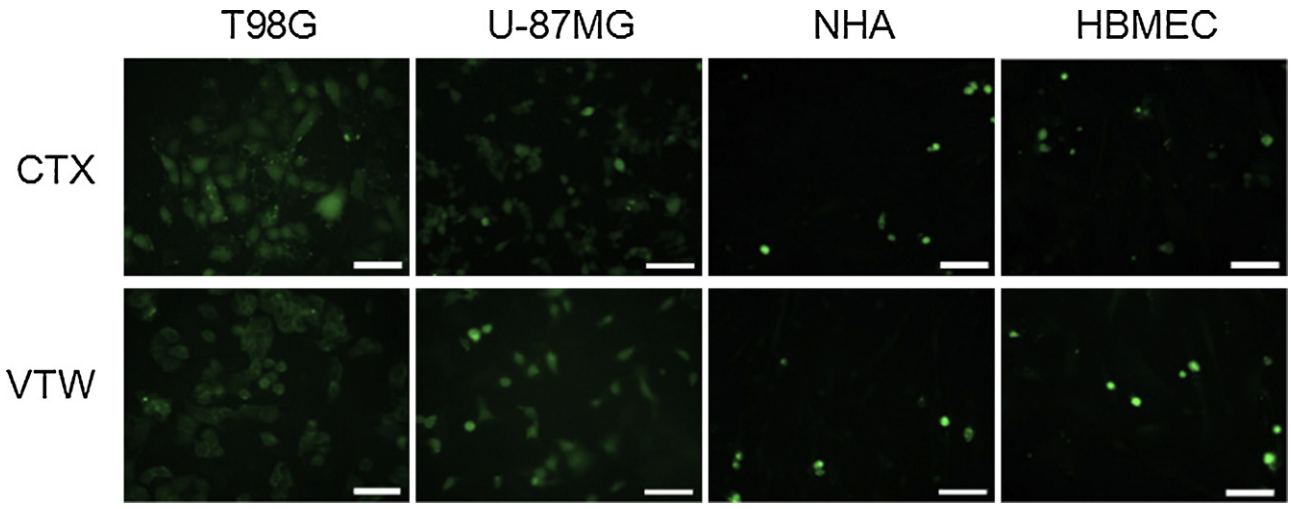

Fig. 3. Fluorescence images of cell cultures verifying the targeting abilities of CTX and VTW after the amines have been reacted. Digital pictures of glioma cells exposed to FITC labeled CTX (top row) and VTW (bottom row) show significantly greater fluorescence in comparison to the control cell lines, NHA and HBMECs. Scale bar = 10 μm.

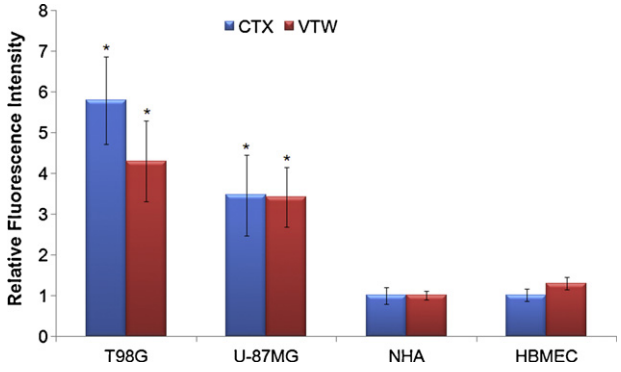

Fig. 4. Relative fluorescence intensities after targeting FITC-labeled CTX and VTW to cells in culture. Each labeled biomolecule was able to bind glioma cells more efficiently than NHAs and HBMECs. $^{*}p < 0.001$ compared to both NHA and HBMEC, $n = 5$.

proliferation was also monitored during this experiment, and no significant increase in cell number was observed; the increased fluorescence is only due to the preferential binding of CTX-NO and VTW-NO to glioma cells.

3.3. Cell viability

To assess the effect of NO on cell viability, cells were incubated with CTX-NO or VTW-NO for 48 h, trypsinized, and counted. At the lowest concentration of NO, 10 μM, CTX-NO was effective in reducing glioma cell viability to less than 72.7 ± 6.5% of the control cell number, while NHA and HBMEC viability remained above 92.5 ± 3.0%. At a higher NO concentration of 20 μM, CTX-NO reduced T98G cell viability to 27.4 ± 5.4% and U-87MG cell viability to 26.8 ± 5.1%. At the same NO concentration NHA cells have a viability of 86.8 ± 16.9% and HBMECs have a viability of 87.5 ± 2.9% (Fig. 5A). At the highest NO concentration of 40 μM, donated by

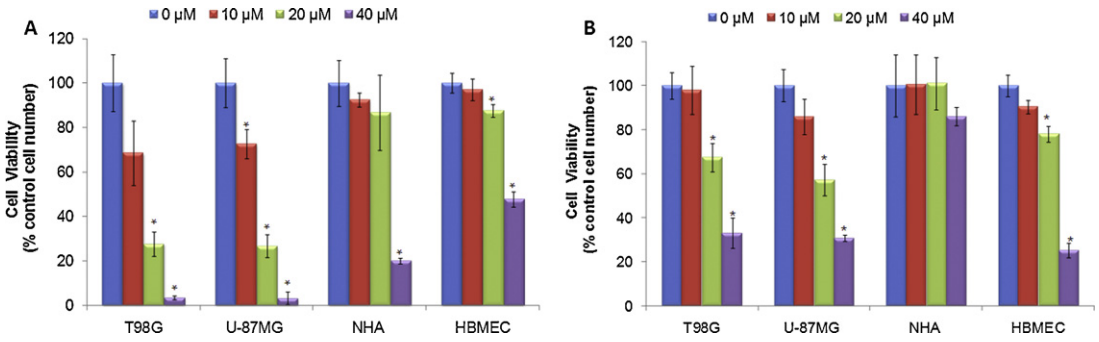

Fig. 5. Effect of targeted NO donor on the cell viability of T98G cells, U-87MG, NHA and HBMEC. Data is presented as a percentage of the number of cells that received no treatment with NO donors. Tumor cell viability is decreased dramatically in the presence of increasing concentrations of (A) CTX-NO and (B) VTW-NO. Viability of control cell types, astrocytes and endothelial cells, decreased only at the high concentrations of NO. $^{*}p < 0.01$, $n = 4$.

CTX-NO, viability of T98G cells was further reduced to $3.60 \pm 0.9\%$ and U-87MG viability was reduced to $3.1 \pm 2.9\%$. However at the highest concentration of NO, NHA and HBMEC viability was also reduced to $20.1 \pm 1.3\%$ and $47.7 \pm 3.5\%$ respectively.

VTW-NO was not as efficient as CTX-NO in decreasing tumor cell viability. At the lowest concentration of NO, 10 μM, VTW-NO was ineffective in significantly reducing glioma cell viability which remained above $85.9 \pm 7.9\%$. At a higher NO concentration of 20 μM, VTW-NO was able to reduce the cell viability of T98G cells and U-87MG cells to $67.4 \pm 6.5\%$ and $57.1 \pm 7.2\%$ respectively (Fig. 5B). However, it is to be noted that the NHA viability was not reduced and the astrocytes showed a viability of $101 \pm 11.9\%$ at a NO concentration of 20 μM. When the VTW-NO concentration was doubled, such that the cells were exposed to 40 μM of NO, the viability of the T98G cells is further reduced to $33.1 \pm 6.9\%$ and U-87MG viability was reduced to $30.8 \pm 1.5\%$. Unlike the lower concentrations, at an NO concentration of 40 μM donated by VTW-NO the viability of the NHA viability was also reduced to $86.0 \pm 4.1\%$ (Fig. 5B).

4. Discussion

In order to demonstrate the chemotherapeutic potential of targeted long-term NO donors, we developed diazeniumdiolates specific to GBM (glioma) cells and studied their effects on both tumor and control cells. In the past three decades no significant improvement in the prognoses of GBM patients has been achieved (Yamanaka, 2008). The two most promising new treatments, temozolomide and intercavity carmustine wafer implantation, have shown only modest improvements in patient median survival, averaging 2.5- and 2-month increases in patient survival time respectively (Sathornsumetee et al., 2007). Alternative treatment options currently being researched include gene therapy and cell or peptide-based immunotherapeutic approaches (Yamanaka, 2008; Lam-Himlin et al., 2006). Although various gene therapy attempts have shown antitumoral effects, they have failed to have significant impact in clinical trials (Lam-Himlin et al., 2006; Alavi and Eck, 2001), since the blood–brain barrier prevents efficient gene delivery to the tumor site (Benedetti et al., 2000; Pulkkanen and Yla-Herttuala, 2005). Furthermore, despite the fact that immunotherapeutic approaches induce antitumoral effects, the variations in genetic abnormalities reduce the efficacy of this approach (Yamanaka, 2008).

Although it is well established that NO and nitric oxide synthases (NOS) play a ubiquitous role in malignant gliomas, it is often debated whether NO can induce or inhibit tumor progression and metastasis (Fukumura et al., 2006). In this study we have shown definitively that NO can inhibit glioma cell proliferation in two different brain tumor cell lines, T98G and U87-MG (Fig. 5). This corroborates previous work that has shown that host cells, including macrophages, natural killer cells and microglia, in response to cytokines within the glioma, produce large amounts NO and either cause apoptosis or DNA damage in glioma cells (Shinoda and Whittle, 2001). It has additionally been shown that NO inhibits the activity of the T4 DNA ligases that repair DNA strands, explaining the increase in single DNA strand breaks found in cells exposed to NO (Graziewicz et al., 1996).

In order to balance this double-edged role NO plays in tumor physiology, we have designed targeted NO donors to ensure that tumor cells receive the majority of the localized dosage, limiting the exposure of non-tumor cells to NO. In order to effectively target tumor cells as well as cross the blood–brain barrier, a short peptide sequence (VTW) and a small protein (CTX) that are able to specifically bind to glioma cells were transformed into NO donors by reacting them with NO gas. The NO donors formed after this reaction, CTX-NO and VTW-NO, had peak NO release after 3.93 h and 4.45 h respectively, at pH of 7.4. Additionally when the NO donors were exposed to a lower pH of 5.5, similar to the microenvironment of tumors, a bimodal release of NO was seen with a burst release of NO within 3 min and subsequent peak NO release similar to that observed at pH 7.4 (data not shown). This is in agreement to previous studies that have shown that diazeniumdiolates dissociate to release NO by protonation and homolytic cleavage of the NO dimer (Taylor et al., 1995), thus at lower pH when more protons are available an initial burst release of NO is seen. This is of increased benefit in terms of glioma treatment since the tumor cells will receive an initial high dosage of NO only within the confines of the tumor environment followed by a more sustained dose of NO, ensuring efficacy of the treatment.

The use of targeted NO donors for glioblastoma therapy is of added benefit because complete tumor resection is almost impossible and micro-tumors form from single glioma cells which escape surgical removal (Yamanaka, 2008). By fluorescently tagging CTX-NO and VTW-NO we have demonstrated that these biomolecules retain their tumor targeting abilities and can target single glioma cells even after the reaction with NO (Fig. 3). Our chosen targeting molecules have been previously shown to efficiently target glioma cells through specific interactions. CTX has a high affinity for cell-surface MMP-2, which is highly upregulated in gliomas and related cancers, but not expressed in normal brain tissues (Deshane et al., 2003). Work by Zhang and colleagues (Conroy et al., 2008; Veiseh et al., 2009a) has demonstrated receptor mediated endocytosis of CTX-conjugated nanoparticles within an hour of incubation and the intracellular localization of their nanoparticle probes. VTW, on the other hand, has been suggested to have an affinity for gp130, a transmembrane glycoprotein that forms

one unit of cytokine receptors in the interleukin (IL) receptor family. Interactions between gp130 and the IL-11 receptor alpha chain (IL-11RA), which is also overexpressed in gliomas, are suggested as the mechanism by which VTW binds to glioma cells (Wu et al., 2008), and VTW-targeted gene delivery complexes have also been shown to bind to glioma cells within 1 h of incubation *in vitro*. We demonstrate that CTX-NO and VTW-NO are able to bind to cells within 30 min of exposure, much before the peak release of either of the NO donors, thus ensuring that the majority of the NO is released after the localization of the donors to glioma cells. Uptake of the bound molecules by endocytosis, though not the original goal of this study, is most certainly occurring early in our incubation period and may be enhancing the effects of our NO donors through intracellular delivery of NO to tumor cells.

In vitro studies showed that when glioma cells were exposed for 48 h to 20 μM of NO released from CTX-NO, glioma cell viability is reduced to less than 30% whereas NHA and HBMEC viability remained above 80% (Fig. 5). Conversely, when glioma cells were exposed to 20 μM of NO released from VTW-NO, glioma cell viability was only reduced to 57%, but NHA and HBMEC viability remained above 75%. The high viability of NHAs and HBMECs at a NO concentration of 20 μM, donated by either CTX-NO or VTW-NO, provides evidence that the biomolecules are targeting the tumor cells and the control cells only have limited exposure to NO. In contrast, previous studies that investigated the effects of non-targeted diazeniumdiolates on the viability of glioma cells found that cell viability could only be significantly reduced by spermine NONOate (SPER/NO) at NO concentrations of 200 μM or higher (Weyerbrock et al., 2009). At these concentrations the viability of astrocytes was also significantly reduced. Other non-targeted diazeniumdiolates, proline NONOate (PROLI/NO) and diethylamine NONOate (DEA/NO) were only able to significantly reduce glioma cell viability at an NO concentration of 20 mM (Weyerbrock et al., 2009). NO, as a result of its reactive nature, has a relatively small sphere of influence, extending approximately 100 μm from its origin (Miller and Megson, 2007). Thus by using targeted NO donors, we are able to deliver NO within a closer sphere of influence to the tumor cells, and the amount of NO required to produce cytotoxic effects is decreased ten-fold. Additionally, adverse effects on astrocytes and endothelial cells are limited because the donors are not able to bind the cell surface.

At a NO concentration of 40 μM of NO released from CTX-NO the glioma cell viability was reduced to less than 5%, where as when the NO donor was VTW-NO the glioma cell viability was 30–35%. Thus it appears that CTX-NO is more efficient at reducing tumor cell viability in comparison to VTW-NO. Previous studies by Veiseh and colleagues has shown that CTX alone does not induce toxic effects (Veiseh et al., 2009a). Therefore, we contend that CTX-NO is able to deliver NO more effectively to the glioma cells as compared to VTW-NO or acts synergistically with NO to decrease cell viability. In comparison to previously reported diazeniumdiolates used in studies with glioblastoma, which release NO only for a few hours (Weyerbrock et al., 2009), the NO donors developed in this project have the ability to release NO for more than 3 days. Through these studies we are able to glean the effects of different concentrations of NO as a potential therapeutic on its own; however, we envision using targeted NO in conjunction with previously validated forms of glioma therapy (surgical resection followed by localized chemotherapy and/or radiation). Our sustained release profile provides the opportunity for synergistic therapeutic action with chemotherapeutics following tumor removal. Further studies are ongoing using lower doses of NO with chemotherapeutics to further reduce any toxic effects on surrounding brain tissues.

5. Conclusions

The ability to deliver NO to very specific sites of disease can drastically expand the current knowledge of NO's interactions with specific tissues and lead to the formation of NO delivery systems with the potential for increased therapeutic benefit. The ambiguous role NO plays in glioma physiology has resulted in skepticism of NO as a treatment for GBM. Using two different glioma targeting biomolecules, we have demonstrated that peptides and proteins can be converted into NO-releasing diazeniumdiolates in a simple reaction with NO gas without additional alterations to the structure of the biomolecules, and that targeted NO donors can inhibit glioma cell viability without significant harm to human astrocytes and brain microvascular endothelium. Coupling the targeting ability of these biomolecules with their NO releasing capabilities, we have developed a more efficient NO delivery method which reduced glioma cell viability, indicating that localized NO delivery may be a feasible strategy for treatment of glioma that can be easily implemented with current treatment modalities. Additionally by ensuring that only glioma cells are targeted by the NO donor the effective dose of NO is also drastically reduced. The techniques reported in this paper can be easily modified to develop targeted NO donors for a variety of different diseases that may be used individually or coupled to enhance the effect of current treatment strategies.

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