Levels and distribution of BCNU in GBM tumors following intratumoral injection of DTI-015 (BCNU-ethanol)

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The alkylation products formed by in vitro treatment of DNA with tritium-labeled 1,3-bis(2-chloroethyl)-1-nitrosourea (³H-BCNU) were identified and quantified. Twelve adducts were resolved by high-performance liquid chromatography (HPLC). The principal DNA adducts formed by BCNU treatment corresponded to N-7-(2-hydroxyethyl)guanine (N7-HOEtG) (26%), N-7-(2-chloroethyl)guanine (15%), and phosphotriesters (19%). In addition, several minor products were identified as 1,2-(diguano-7-yl)ethane, N-1-(2-hydroxyethyl)-2-deoxyguanosine, 1-(N-1-2-deoxyguanosinyl), 2-(N-3-2-deoxycytidyl)ethane cross-link, and O-6-(2-hydroxyethyl)-2-deoxyguanosine, and individually they represented 1% to 5% of the total alkylation. An HPLC-electrochemical method was applied to quantify the levels of N7-HOEtG in samples treated with BCNU. Treatment of either purified DNA or U87MG cells with various amounts of BCNU produced a linear increase in the amount of N7-HOEtG. These results demonstrated

that the levels of N7-HOEtG formed by BCNU treatment could be used as a molecular dosimeter of BCNU treatment dose. We measured the levels of N7-HOEtG in DNA isolated from tumor samples taken from four patients with GBM tumors following stereotactic intratumoral injection with DTI-015 (BCNU-ethanol). The level of N7-HOEtG in these samples ranged from 14.7 to 121.9 μmol N7-HOEtG/mol DNA within 1 cm of the site of injection. As the distance from the site of injection increased, the levels of N7-HOEtG in tumor DNA decreased. In two of the samples, the levels of N7-HOEtG were 0.2 to 0.3 μmol N7-HOEtG/mol DNA at 3.5 to 3.9 cm from the site of injection, demonstrating significant distribution of BCNU in the tumor. The levels of N7-HOEtG in these tumor samples corresponded to BCNU treatment concentrations of 0.02 to 43.0 mM. These studies demonstrate that stereotactic intratumoral injection of DTI-015 into human GBM tumors produces high concentrations of BCNU up to 2.5 cm from the site of injection in some of the tumors. These observations suggest that intratumoral injection of DTI-015 may be of benefit in the treatment of primary and recurrent GBM tumors. *Neuro-Oncology* 9, 12–19, 2007 (Posted to *Neuro-Oncology* [serial online], Doc. D05-00010, October 3, 2006. URL www.dukeupress.edu/neuro-oncology; DOI: 10.1215/15228517-2006-014)

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²Abbreviations used are as follows: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CNU, 1-(2-chloroethyl)-1-nitrosourea; dG-dC, 1-(N-1-2-deoxyguanosinyl), 2-(N-3-2-deoxycytidyl)ethane; EC, electrochemical; GBM, glioblastoma multiforme; ³H-BCNU, tritium-labeled BCNU; HPLC, high-performance liquid chromatography; IT, intratumoral; N1-HOEtG, N-1-(2-hydroxyethyl)-2-deoxyguanosine; N7-bis-G, 1,2-(diguano-7-yl)ethane; N7-ClEtG, N-7-(2-chloroethyl)guanine; N7-HOEtG, N-7-(2-hydroxyethyl)guanine; O⁶-HOEtG, O-6-(2-hydroxyethyl)-2-deoxyguanosine.

Keywords: BCNU, carmustine, DTI-015, ethanol, glioblastoma multiforme, intratumoral

Despite treatment programs which combine surgical resection, radiotherapy, and chemotherapy, patients with glioblastoma multiforme (GBM)² tumors have a life expectancy of approximately 6 to

24 months after initial diagnosis (Curran et al., 1993; Mahaley, 1991). The observed resistance of GBM brain tumors to long-term therapeutic response is most likely brought about by a combination of their unique physical location and biological properties. The location of GBM tumors limits the extent of any surgical resection that may be performed. In addition, astrocytic tumors infiltrate surrounding brain and become encompassed by a blood-tumor barrier, which reduces or prevents drug delivery (Groothuis, 2000; Kemper et al., 2004; Lesser and Grossman, 1994; Levin et al., 1980; Warren and Fine, 2002). GBM tumors also have numerous alterations in cell signaling pathways that can influence both their growth rate and response to treatment (Guha and Mukherjee, 2004; Kitange et al., 2003; Rutka et al., 2000; Steinbach and Weller, 2004).

A variety of approaches for overcoming the resistance of GBM tumors to treatment have been investigated (Groothuis, 2000; Kroll and Neuwelt, 1998; Wang et al., 2002; Warren and Fine, 2002). These approaches have included intra-arterial and interstitial injection of chemotherapeutic agents (Shapiro et al., 1992), use of agents to increase the permeability of the blood-tumor barrier to therapeutic agents (Doolittle et al., 2000; Gumerlock et al., 1992; Neuwelt et al., 1986; Prados et al., 2003), implantation of biodegradable matrices containing chemotherapeutic agents into the resected tumor cavity (Brem et al., 1995; Wang et al., 2002; Westphal et al., 2003), and convection-enhanced delivery of molecules (Lidar et al., 2004; Parney et al., 2005). Despite promising results from preclinical models, the results from clinical trials using these different approaches have been less encouraging, varying from increased normal tissue toxicity to only a modest increase in patient stabilization and survival.

DTI-015 is a formulation of ethanol plus carmustine (1,3-bis[2-chloroethyl]-1-nitrosourea, or BCNU). The use of water-miscible organic solvents such as ethanol allows the delivery of high concentrations of BCNU to the tumor at flow rates of 1 ml/min. This process has been described by Pietronigro et al. (2003) as solvent-facilitated drug perfusion. In preclinical models, administration of DTI-015 significantly inhibits tumor growth rates (Bodell et al., 2003; Hall et al., 2004) and increases time of survival of rats with intracerebral tumors (Hall et al., 2004; Pietronigro et al., 2003). Several lines of evidence indicate that these responses are due to increased deposition and retention of BCNU within the tumor (Bodell et al., 2003; Hamstra et al., 2005).

A phase 1/2 clinical trial of DTI-015 in recurrent inoperable GBM tumors has demonstrated a significant increase in survival time for the DTI-015 treatment group compared to the survival time for conventionally treated patients (Hassenbusch et al., 2003). A preliminary study has described a significant decrease in tumor and cerebral blood flow in primary GBM tumors following administration of DTI-015 (Jenkinson et al., 2005). This observation suggests that the increased retention of BCNU in GBM tumors following DTI-015 administration may be due in part to reduced blood flow in the tumor.

There have been few studies on the level(s) of chemotherapeutic agents achieved and their distribution in human brain tumors following administration of these agents (Bergenheim et al., 2005; Bodell et al., 2001). The paucity of information on the level and distribution of chemotherapeutic agents in human brain tumors following treatment is due to a number of factors, including instability of drugs and lack of appropriate analytical methods to make the determinations. We have developed and applied high-performance liquid chromatography (HPLC) analysis with electrochemical (EC) detection to quantify the levels of N-7-(2-hydroxyethyl)guanine (N7-HOEtG) in cellular DNA following treatment with 1-(2-chloroethyl)-1-nitrosourea (CNU) (Ye and Bodell, 1997). We have used this approach to quantify the levels of N7-HOEtG in samples from a brain tumor following intratumoral injection of DTI-015 (Bodell et al., 2001). The results of that study demonstrated that intratumoral administration of DTI-015 produced very high levels of N7-HOEtG.

In these studies, we have quantified the individual DNA alkylation products formed in DNA treated with tritium-labeled BCNU (^3H -BCNU). We have observed that the principal alkylation product formed by this treatment was N7-HOEtG. The levels of N7-HOEtG in purified DNA or U87MG cells treated with nonradioactively labeled BCNU were measured by HPLC-EC. A significant association between level of N7-HOEtG and BCNU treatment concentration was found for both treatment groups. Samples of recurrent GBM tumors were collected 40 to 60 min after stereotactic intratumoral injection with DTI-015. In these samples, very high levels of N7-HOEtG were detected in all of the tumor samples collected ≤ 1 cm from the site of injection. In two of the samples, high levels of N7-HOEtG were detected in samples collected 2 to 3 cm from the site of injection. These studies demonstrate that the levels of N7-HOEtG in brain tumor samples can be used to estimate the levels and distribution of BCNU following intratumoral injection of DTI-015.

Materials and Methods

Reaction of BCNU with DNA

Purified calf thymus DNA in 50 mM triethanolamine buffer (pH 7.0) was incubated with either 1 mCi of ^3H -BCNU (labeled in the N-1-chloroethyl position, 500 mCi/mmol; Moravek Biochemicals, Brea, Calif.) or different amounts of unlabeled BCNU. The volume of the reaction mixture was 2 ml. The reaction mixture was incubated at 37°C for 6 h, and the DNA was precipitated with ethanol and sodium acetate. The DNA was dissolved in 0.15 M sodium chloride and 0.015 M sodium citrate buffer of pH 7.0. Samples incubated with ^3H -BCNU were repeatedly precipitated until a constant specific activity (^3H cpm/mg DNA) was obtained.

Treatment of U87MG Cells with BCNU

U87MG cells were grown in 175-cm² flasks containing 25 ml of Eagle's minimum essential medium supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37°C in a humidified, 5% CO₂, 95% air atmosphere. When the cells were confluent, they were washed with Hank's balanced salt solution, trypsinized, and collected by centrifugation. The cells were suspended in 50 ml of medium, and BCNU dissolved in ethanol was added directly to achieve the final concentrations indicated. The cells were treated for 1 h at 37°C with intermittent shaking. Following treatment, the cells were collected by centrifugation and quick frozen. Samples were stored at -80°C until DNA was isolated.

Analysis of DNA Adducts Formed by ³H-BCNU

DNA from in vitro reaction with ³H-BCNU was enzymatically digested overnight with pancreatic DNase, snake venom phosphodiesterase, and alkaline phosphatase (Bodell, 1999). The mixtures were heated at 100°C for 90 s to inactivate the enzymes and to ensure that the N7 products were converted completely to the free-base form. The heated digests were passed through a centrifugal filter (0.2-µm pore size). Adduct standards corresponding to N-7-(2-chloroethyl)guanine (N7-ClEtG), N-1-(2-hydroxyethyl)-2-deoxyguanosine (N1-HOEtG), O-6-(2-hydroxyethyl)-2-deoxyguanosine (O⁶-HOEtG), and 1-(N-1-2-deoxyguanosinyl), 2-(N-3-2-deoxycytidyl)ethane (dG-dC) were added to the digests and used to identify the radiolabeled products (Bodell, 1999).

HPLC was performed with a Perkin-Elmer 250 pump (Perkin-Elmer, Norwalk, Conn.) and a 5-µm C-18 reversed-phase column (Adsorbosphere HS, 4.6 × 250 mm; Alltech Associates, Inc., Deerfield, Ill.). UV absorbance at 260 nm was monitored with a Spectra-Physics detector (Spectra Physics, Mountain View, Calif.). Radioactivity was continuously detected by using an IN/US β-RAM flow detector (IN/US Systems, Inc., Tampa, Fla.) using IN-Flow 3 (IN/US Systems, Inc.) as the scintillant. The mobile phase consisted of 10 mM NH₄H₂PO₄ of pH 5.1 (solvent A) and various amounts of methanol (solvent B). The flow rate was 1 ml/min. The gradient used to resolve the alkylation products was as follows: 95% A and 5% B for 15 min, 85% A and 15% B for 30 min, 85% A and 15% B for 10 min, 30% A and 70% B for 20 min; and 30% A and 70% B for 10 min. Under these conditions, the retention times of the standards were as follows: N7-HOEtG, 11.0 min; 1,2-(diguano-7-yl)ethane (N7-bis-G) and N1-HOEtG, 30.0 min; N7-ClEtG, 36.5 min; dG-dC, 47.5 min; and O⁶-HOEtG, 49.5 min.

Patient Samples

Four patients with recurrent GBM tumors signed a consent form from the University of California, San Francisco, or from the University of Texas M.D. Anderson

Cancer Center for intratumoral treatment with DTI-015 and for tumor sampling. The patients were treated by stereotactic intratumoral injection with up to 4 ml of DTI-015 at a DTI-015 concentration of 45 to 60 mg of BCNU/ml of ethanol. The rate of injection was 1 ml/min. At 40 to 60 min after injection, stereotactic biopsy samples were taken from the tumor at various distances from the injection site. The locations for the biopsies were determined with a neuronavigator and were recorded. The accuracy of the biopsy locations is estimated to be ±2 mm. Subtotal or total surgical resection of tumor followed collection of the biopsy samples. The samples were quick frozen and stored at -80°C until DNA isolation.

Quantitation of N7-HOEtG Levels in Patient Samples

Isolation of DNA. DNA was isolated from the individual tumor specimens by using a modified chloroform-isoamyl alcohol extraction. Following initial isolation, the DNA was digested for 1 h at 37°C with a combination of pancreatic ribonuclease A and T1 and T2 ribonuclease, followed by digestion for 1 h at 37°C with proteinase k. The DNA-containing solution was extracted with chloroform-isoamyl alcohol (24:1), and the DNA was precipitated with sodium acetate and ethanol and collected by centrifugation. The DNA was dissolved in 0.15 M of sodium chloride and 0.015 M of sodium citrate buffer of pH 7.0. DNA concentration was determined from its absorbance at 260 nm. The concentration was determined from the relationship that one absorbance unit is equivalent to a concentration of 50 µg of DNA/ml.

Thermal hydrolysis. For quantification of N7-HOEtG, up to 100 µg of DNA in 100 µl of phosphate-buffered saline, pH 8, was heated at 100°C for 30 min (Ye and Bodell, 1997). The samples were chilled in an ice bath, and 100 µl of cold 1 M HCl was added. The samples were centrifuged at 1300 × g, 0°C, for 10 min to collect the DNA. The pellet was washed once with 100 µl of cold 1 M HCl and centrifuged as described above. Both supernatants were combined and adjusted to pH 6 with the addition of 4 M sodium acetate (pH 6.4) and concentrated NH₄OH. The hydrolysates were filtered prior to prepurification.

Prepurification of N7-HOEtG. An HPLC-UV system consisting of a model 250 Perkin-Elmer solvent delivery system with a Perkin-Elmer LC 235 diode array detector was used. A 5-µm C-18 reversed-phase analytical column with column dimensions of 250 × 4.6 mm (Econosphere column, Alltech Associates) was employed for the prepurification separations. Column temperature was set at 30°C with a Perkin-Elmer column oven. For prepurification of N7-HOEtG, an isocratic mobile phase of 10 mM ammonium acetate, pH 4.5, with 2.5% methanol was used. Fractions corresponding to the elution of N7-HOEtG were collected and reduced in volume. Following injection of either standard solutions of N7-HOEtG or DNA samples, blank samples were injected,

and fractions corresponding to the elution of N7-HOEtG were collected and subsequently analyzed by HPLC-EC. Under these conditions of analysis, no carryover of N7-HOEtG between samples has been detected.

HPLC-EC detection. The HPLC-EC system consisted of a model 580 ESA solvent delivery system with a SSI pulse dampener (Scientific Systems, Inc., State College, Penn.) coupled to an ESA Coulochem II electrochemical detector with an ESA 5010 analytical cell (ESA Biosciences, Inc., Chelmsford, Mass.). A 5- μ m Econosphere column was employed for the analytical separations (Econosphere, Alltech Associates). Column temperature was set at 30°C. For quantitation of N7-HOEtG, an isocratic mobile phase of 50 mM sodium acetate, pH 5.1, with 5% methanol was used.

Electrode 1 was set at an oxidation potential of +400 mV. Electrode 2 was set at an oxidation potential of +825 mV for N7-HOEtG. Electrochemical response was digitized with a Nelson interface and analyzed by using TurboChrom 4 software (Perkin-Elmer, Cupertino, Calif.). Standard curves were generated by measuring EC response following injection of standard solutions of N7-HOEtG (Chemsyn Science Laboratories, Lenexa, Kans.) over the concentration range of 0.05 to 5.0 pmol. Levels of adducts in the individual samples were determined by comparison with the standard curve. Using DNA reacted with radiolabeled CNU, we have determined that the detection and recovery of N7-HOEtG by HPLC-EC is $\geq 80\%$ (Ye and Bodell, 1997). The levels of N7-HOEtG were expressed as micromoles of N7-HOEtG per mole of DNA.

Statistical Analysis

Statistical analysis was performed by using Sigma Stat 2.03 (SPSS Inc., Chicago, Ill.). The results are expressed as mean $\pm$ standard deviation.

Fig. 1. Radioactive profile of the individual alkylation products that were formed by incubation of ^3H -BCNU with calf thymus DNA and resolved by HPLC. The identities of the individual peaks labeled A to G are provided in Table 1.

Results

Aliquots of DNA reacted with ^3H -BCNU were digested, and the individual alkylation products were resolved by HPLC. The elution of the individual products and the amount of radioactivity were detected by online measurement of radioactivity. A representative radiochromatographic profile observed with analysis of these samples is presented in Fig. 1. At least 12 DNA alkylation products were formed by this treatment (Fig. 1). Seven of these alkylation products have been identified and are indicated as peaks A to G (Fig. 1). The levels of these individual adducts are listed in Table 1. The principal alkylation product formed in DNA incubated with ^3H -BCNU was N7-HOEtG, representing approximately 26% of the total DNA alkylation. N7-CIEtG and phosphotriesters were also significant products formed by the reaction of BCNU with DNA and represented 15% and 19% of the total alkylation, respectively. In addition to these principal products, several minor products were detected and quantified. These products were identified as N7-bis-G, N1-HOEtG, dG-dC cross-link, and O⁶-HOEtG, and individually they represented 1% to 5% of the total alkylation.

The levels of N7-HOEtG in purified DNA treated with various concentrations of BCNU were quantified by HPLC-EC. Control samples had significant levels of N7-HOEtG (60.8 ± 9.0 $\mu\text{mol/mol}$ DNA). Treatment of DNA with increasing concentrations of BCNU produced a dose-dependent increase in the level of N7-HOEtG (Fig. 2). A significant correlation between level of N7-HOEtG and BCNU concentration was observed ($r^2 = 0.80$, $P < 0.001$). The slope of the regression analysis was 0.81 μmol N7-HOEtG/mol DNA/ μM BCNU.

In similar studies, the levels of N7-HOEtG in U87MG cells treated with various concentrations of BCNU were measured. A linear increase in the level of N7-HOEtG was observed with increased BCNU treatment dose (Fig. 3). The slope of the dose-response curve was 0.0037 μmol of N7-HOEtG/mol of DNA/ μM of BCNU ($r^2 = 0.98$, $P < 0.001$). Comparison of the slopes of the dose-response curves demonstrates that the level of alkylation in purified DNA is approximately 219-fold higher than that in cellular DNA.

Patients undergoing resection for GBM tumors were injected with various amounts of DTI-015 40 to 60 min prior to the resection. Individual tumor samples were collected at or near the injection site and at various distances from the site of injection. DNA was isolated and purified from each of the tumor samples, and the levels of N7-HOEtG were quantified by HPLC-EC. The results of these analyses are presented in Table 2. The level(s) of N7-HOEtG in the samples ranged from 0.08 to 159.1 $\mu\text{mol/mol}$ of DNA. In each set of samples, the highest level of N7-HOEtG was observed at or close to the site of injection. The level of N7-HOEtG then decreased with distance from the injection site. Although this was the general pattern observed, there was considerable variation in how the level of N7-HOEtG decreased with distance from site of injection. In patients 1 and 2, significant levels of N7-HOEtG were measured up to

Table 1. Levels of individual DNA alkylation products formed by incubation of ^3H -BCNU with calf thymus DNA

HPLC Fraction ^a	Product ^b	Percent of Total (Mean $\pm$ SD)	Amount of Product (μmol Alkylation/mol of DNA) ^b
A	N7-HOEtG	26.2 $\pm$ 2.7	208.5
B and C	N1-HOEtG and N7-bis-G	4.7 $\pm$ 1.5	37.4
D	N7-ClEtG	15.5 $\pm$ 2.2	123.4
E	O ⁶ -HOEtG	1.7 $\pm$ 0.3	13.5
F	dG-dC	1.9 $\pm$ 0.3	15.1
G	PTEs	19.0 $\pm$ 0.8	151.2

Abbreviations: dG-dC, 1-(*N*-1-2-deoxyguanosinyl), 2-(*N*-3-2-deoxycytidyl)ethane; ^3H -BCNU, tritium-labeled 1,3-bis (2-chloroethyl)-1-nitrosourea; HPLC, high-performance liquid chromatography; N1-HOEtG, *N*-1-(2-hydroxyethyl)-2-deoxyguanosine; N7-bis-G, 1,2-(diguano-7-yl)ethane; N7-HOEtG, *N*-7-(2-hydroxyethyl)guanine; N7-ClEtG, *N*-7-(2-chloroethyl)guanine; O⁶-HOEtG, O-6-(2-hydroxyethyl)-2-deoxyguanosine; PTEs, phosphotriesters.

^aHPLC peaks A–G are shown in Fig. 1.

^bThe final specific activity of the DNA incubated with ^3H -BCNU was determined to be 539,837 cpm/mg of DNA. Based on the specific activity of the ^3H -BCNU, this value is equivalent to a total alkylation level of 796 μmol alkylation/mol of DNA.

3.9 and 3.5 cm from the site of injection. In contrast, in patient 3, the level of N7-HOEtG was at the limit of quantitation 1.2 cm from position of injection. In patient 4, significant levels of N7-HOEtG were detected in each of the tumor samples.

Discussion

Treatment of DNA with ^3H -BCNU produced a complex mixture of 12 DNA adducts. The levels of the individual products ranged from less than 1% to 26% of the total alkylation. Seven of the 12 adducts were identified by cochromatography with previously identified adducts (Bodell, 1999; Ludlum, 1990). Three principal adducts formed after BCNU treatment were N7-HOEtG, N7-ClEtG, and phosphotriesters. In addition, the levels of N7-bis-G, N1-HOEtG, O⁶-HOEtG, and the dG-dC cross-link were also quantified. The levels of the

individual DNA alkylation products formed by BCNU are the same as those in DNA treated with CNU (Bodell, 1999). The identification of N7-HOEtG as a principal adduct formed by BCNU treatment suggests its use as a molecular dosimeter for the level and distribution of BCNU in tumors.

Recent studies have used coinfusion of gadolinium derivatives to estimate volume of distribution of the infused agents by MRI (Mamot et al., 2004; Mardor et al., 2005; Nguyen et al., 2003). This approach assumes that the distribution of the infused agent is the same as that of gadolinium derivatives, which for molecules such as BCNU may not be the case. In addition, these approaches are unable to provide a quantitative measurement of drug concentration at different distances from the site of infusion. In contrast, since N7-HOEtG is a product of the reaction of DNA with BCNU, the levels of N7-HOEtG in tumor samples will be representative

Fig. 2. Levels of N7-HOEtG in DNA after treatment with various concentrations of BCNU.

Fig. 3. Levels of N7-HOEtG in DNA of U87MG cells treated for 1 h with various concentrations of BCNU. The levels of N7-HOEtG are expressed as the mean $\pm$ SD. For those data points where error bars are not observed, the error is smaller than the size of the symbol.

Table 2. Levels of N7-HOEtG in brain tumor samples at various distances from site of intratumoral injection with DTI-015

Patient	DTI-015	Distance (cm) ^a	N7-HOEtG Level ($\mu\text{mol/mol}$ of DNA)	BCNU (mM) ^b
1	(60 mg/ml) $\times$ 4 ml = 240 mg	0.9	121.9	32.9
		2.4	0.9	0.24
		3.9	0.3	0.08
2	(60 mg/ml) $\times$ 2.7 ml = 162 mg	2.0	159.1	43.0
		2.5	119.2	32.2
		3.5	0.2	0.05
3	(45 mg/ml) $\times$ 3 ml = 135 mg	0.5	46.9	12.7
		1.2	.08	0.02
		2.0	.08	0.02
4	(45 mg/ml) $\times$ 3 ml = 135 mg	0	82.8	22.4
		0.7	14.7	4.0
		1.2	8.5	2.3

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; N7-HOEtG, N7-(2-hydroxyethyl)guanine.

^aEstimated distance from site of injection.^bEstimated concentrations of BCNU achieved. The results for patient 1 were taken from Bodell et al. (2001).

of the BCNU concentrations that are present following administration.

The levels of N7-HOEtG in human brain tumor samples collected at various distances from the site of intratumoral injection of DTI-015 were measured. In each of the tumors, a similar pattern for level of N7-HOEtG was observed. High levels of N7-HOEtG were produced at or near the site of injection with DTI-015 and then decreased with distance from the site of injection. Although this was true for each of the tumors, there was considerable variation among the tumors with regard to levels of N7-HOEtG near and distal to the site of injection. In samples from patients 1 and 2, high levels of N7-HOEtG were observed at the points closest to the site of injection and at 2.4–2.5 cm from the site of injection. In these two patients, detectable levels of N7-HOEtG were measured 3.5 and 3.9 cm from the site of injection. The results for patient 3 show a high level of N7-HOEtG near the site of injection but levels of N7-HOEtG similar to the level in untreated brain tumor samples beyond 1 cm from injection site. The tumor samples from patient 4 show high levels of N7-HOEtG at the site of injection and at 1.2 cm.

These levels of N7-HOEtG in brain tumor samples following treatment with DTI-015 can be compared with the levels of N7-HOEtG in untreated brain tumor samples. The level of N7-HOEtG in untreated brain tumor samples is $0.14 \pm 0.09 \mu\text{mol/mol}$ of DNA (Bodell et al., 2001). Comparison of this value with the values reported in Table 2 demonstrates that most of the values of N7-HOEtG in the DTI-015 samples are significantly higher and thus due to DTI-015 treatment.

The variability in the levels of N7-HOEtG in GBM tumors following intratumoral (IT) injection of DTI-015 can be caused by a number of factors. BCNU-ethanol will be distributed through the tumor by bulk flow. This bulk flow will be determined by a combination of the

pressure developed by injection of BCNU-ethanol solution into the tumor and the interstitial pressure of the tumor. Therefore, differences in pressures developed during the injection of DTI-015 and in the interstitial pressures of the tumors likely contribute to the observed variations in the distribution of BCNU in the tumors (Groothuis, 2000; Jain, 1987; Jang et al., 2003). The BCNU levels achieved in the tumor samples are likely to be determined by different factors. Since BCNU rapidly crosses brain capillaries, differences in vascular density of the GBM tumors will contribute to differences in the BCNU levels obtained. Furthermore, a recent study has reported a significant decrease in tumor and cerebral blood flow in primary GBM tumors following administration of DTI-015 (Jenkinson et al., 2005). This reduction in blood flow should result in increased levels of BCNU being retained within the tumor.

Previous studies have investigated the distance of penetration by BCNU and other chemotherapeutic agents following either injection in an aqueous solution or release from a biodegradable matrix (Blasberg et al., 1975; Fung et al., 1996, 1998). In these cases, the levels of drug achieved and distance of penetration by the drug are determined primarily by diffusion of the agent (Fung et al., 1996, 1998; Groothuis, 2000). In these studies, high concentrations of the drug were achieved at the site of injection or implantation; however, the penetration of the drug was generally limited to 3 to 5 mm. The results in Table 2 demonstrate that IT injection of DTI-015 results in up to a fivefold increase in distance of penetration by BCNU into some of the tumors when compared to diffusion.

The dose-response relationship observed between levels of N7-HOEtG formed in U87MG cells and BCNU treatment concentrations enables us to estimate the concentrations of BCNU achieved in the human tumor samples from the measured levels of N7-HOEtG. The

result of this calculation shows that at distances ≤ 1 cm from the site of injection, the BCNU concentration ranges from 4 to 32 mM (Table 2). At distances of 1 to 2.5 cm, BCNU concentrations ranging from 0.24 to 43 mM were achieved in four of six tumor samples. At distances of 3.5 to 3.9 cm from the site of injection, the BCNU concentrations achieved were 0.05 to 0.08 μ M. A recent report (Hamstra et al., 2005) has estimated the BCNU concentration achieved in a 9L intracerebral tumor following IT injection of DTI-015 to be 40.9 ± 5.2 mM. These studies in patients, together with those in the 9L model, demonstrate that very high concentrations of BCNU are achieved in tumors following administration of DTI-015. Although the mechanism(s) by which high concentrations of BCNU are retained in human brain tumors following administration of DTI-015 have not been established, studies by Jenkinson et al. (2005) have demonstrated significant reductions both in cerebral tumor blood flow and in cerebral blood flow following injection of DTI-015 in patients. This reduced blood flow may contribute to the retention of BCNU in the tumor.

The biological consequences of these high BCNU concentrations for the treatment of brain tumors are important. In vitro studies have demonstrated that BCNU-resistant human glioma cells are effectively killed at BCNU concentrations of 250 μ M (Bodell et al., 1988). Although this concentration of BCNU cannot

be achieved by bolus i.v. administration, following IT administration of DTI-015, this and higher levels of the drug were achieved in the tumors.

A recent study by Hassenbusch et al. (2003) has demonstrated that IT administration of DTI-015 to inoperable recurrent GBM tumors produced a significant increase in time of survival for these patients. Preclinical studies have demonstrated that treatment with DTI-015 produces a significant tumor growth delay and increased time of survival. The results of this study suggest that the increased time of survival for patients following administration of DTI-015 may be due to the high concentrations and significant distributions of BCNU achieved in the tumors.

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