Activity of VNP40101M (Cloretazine) in the treatment of CNS tumor xenografts in athymic mice

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VNP40101M, or 1,2-bis(methylsulfonyl)-1-(2-choloroethyl)-2-(methylamino)carbonylhydrazine (Cloretazine), is a bifunctional prodrug that belongs to a class of DNA-modifying agents—the sulfonylhydrazines—that has been synthesized and been shown to have activity against a wide spectrum of xenografts. The current study was designed to assess the activity of VNP40101M administered at a dose of 18 mg/kg daily for five days against a panel of human adult and pediatric CNS tumors growing subcutaneously or intracranially in athymic nude mice. The results demonstrated statistically significant ($p < 0.05$) growth delays of 15.0, 8.3, 51.0, 60+, 60+, and 60+ days in subcutaneous xenografts derived from childhood glioblastoma multiforme (D-456 MG), childhood ependymoma (D-528 EP and D-612 EP), childhood medulloblastoma (D-425 MED), and adult malignant glioma (D-245 MG and D-54 MG), respectively, with corresponding tumor regressions in 10 of 10, 4 of 10, 8 of 10, 9 of 10, 9 of 10, and 10 of 10 treated mice, respectively. Delayed toxicity was seen more than 60 days after treatment, with 23 deaths in 100 treated animals, despite a median weight loss of only 0.06%. In mice bearing intracranial D-245 MG xenografts, treatment with VNP40101M at a dose of 18 mg/kg daily for five days produced a 50% increase in median survival compared with controls. Additional experiments conducted against subcutaneous D-245 MG xenografts by using reduced doses of 13.5 or 9.0 mg/kg daily for five days demonstrated tumor growth delays of 82.2 and 53.5 days, with corresponding tumor regressions in 8 of 9 and 9 of 10 treated mice, respectively (all values, $p < 0.001$), with one toxic death. These findings suggest that VNP40101M is active in the treatment of a wide range of human central nervous system tumors and warrants translation to the clinic. *Neuro-Oncology 9, 240–244, 2007 (Posted to Neuro-Oncology [serial online], Doc. D06-00058, May 23, 2007. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2007-011)*

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Nearly 17,000 new cases of primary malignant glioma are diagnosed each year in the United States.[1] Despite several decades of intense research, the prognosis of patients with primary malignant glioma has not changed significantly and remains exceptionally dismal. The mainstay of therapy for malignant glioma has traditionally been surgical resection, followed by radiation therapy and subsequent nitrosourea-based chemotherapy[1] and, more recently, temozolomide.[2] Unfortunately, de novo or acquired resistance to these

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agents frequently occurs, with consequent tumor growth and patient death. Studies identifying newer, potentially more active agents are thus clearly warranted.

A novel class of alkylating agents, the 1,2-bis(sulfonyl)hydrazines (BCHs), has been synthesized.[3,4] The BCH compounds produce a chloroethylating species but, unlike chloroethylating agents such as chloroethyl nitrosoureas, do not lead to vinylation or hydroxyethylation, which can produce toxicity in normal organs. The therapeutic index of BCH compounds may therefore be more favorable.

VNP40101M (Cloretazine) is a derivative of the BCH compounds that generates two short-lived active species: 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine ($t_{1/2}$ = 30 s at physiological state) and a thiophilic carbamoylating methyl isocyanate species.

In the current study, we evaluated the use of VNP-40101M against a panel of tumor xenografts derived from adult high-grade glioma (D-54 MG and D-245 MG), childhood high-grade glioma (D-212 MG and D-456 MG), medulloblastoma (D-341 MED and D-425 MED), and ependymoma (D-612 EP and D-528 EP), as well as the relationship between depletion of tumor O^6-alkylguanine-DNA alkyltransferase (AGT) activity and enhanced activity of VNP40101M.

Materials and Methods

Animals and Chemicals

Male and female athymic BALB/c mice (*nu/nu* genotype, six weeks or older) were used for all studies and were maintained as previously described.[5] The study was approved by the Duke University Institutional Animal Care and Use Committee prior to commencement of study.

VNP40101M was provided by Vion Pharmaceuticals (New Haven, CT, USA). O^6-benzylguanine (BG) was provided by the National Cancer Institute.

Xenografts and Implantation

A panel of eight human (adult and childhood) CNS-derived xenografts was used for all in vivo studies: D-425 MED, D-341 MED, D-456 MG, D-245 MG, D-54 MG, D-212 MG, D-528 EP, and D-612 EP. The xenografts were maintained as previously described.[6] For the subcutaneous implantations, mice were inoculated with a volume of 50 μl into the right flank as previously described.[7] Intracranial tumor implantations into the right cerebrum were performed as previously described, with an inoculation volume of 10 μl.[7]

Tumor Measurements

Tumors were measured twice weekly with handheld calipers (Scientific Products, McGraw, IL, USA). Tumor volume was calculated in cubic millimeters, according to this formula: $([\text{width}]^2 \times [\text{length}])/2$.

Xenograft Therapy

VNP40101M was administered to animals via intraperitoneal injection at a dose of 18.0, 13.5, or 9.0 mg/kg per day for five days in a solution of 20% dimethyl sulfoxide (DMSO) in 5% dextrose solution. The initial dose of 18 mg/kg per day for five days was derived from previous animal studies in which CD_2F_1 mice were inoculated with the leukemia cell line L1210 and then treated daily with 18 mg/kg for six days.[4] The lethal single dose of VNP40101M for 10% of test subjects, with and without single-dose BG, was determined as previously described.[6]

For all subcutaneous xenograft experiments, 8–10 mice per arm were randomly selected for inoculation when the implanted tumors reached a median tumor volume of 200–300 mm^3. Mice with tumors in the acceptable starting size range were ranked from smallest to largest tumor. The mouse with the smallest tumor was assigned to group A, the second smallest to group B, and so on, until all mice had been assigned to either group A or B. Then group A was randomly assigned to treatment or control by a coin flip; group B was the other study group. In addition to ensuring that there was no selection bias in assigning animals to the groups, this method provided a reasonable balance in starting tumor sizes. In all experiments, control mice were handled in the same manner and treated with drug vehicle.

For the intracranial xenograft experiment, groups of 10 randomly selected mice bearing D-245 MG xenografts were treated using VNP40101M in a solution of 20% DMSO in 5% dextrose solution via intraperitoneal injections at a dose of 18 mg/kg per day for five days on the day representing 50% of the time elapsing between the initial tumor inoculation and the median day of death, as previously determined in the intracranial tumor-bearing mice receiving vehicle alone.

AGT Activity Assay

Cellular extracts from eight xenograft cell lines were prepared in 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol buffer. Samples were sonicated for 1 min and centrifuged at 14,000 *g* for 30 min. The assay for AGT activity was performed as described previously.[8] Briefly, AGT activity was measured as the removal of O^6-[^{3}H]methylguanine from a ^{3}H-methylated DNA substrate (18 Ci/mmol) after incubation with tissue extract at 37°C for 30 min. The DNA was precipitated by adding ice-cold 0.25 N perchloric acid and hydrolyzed by the addition of 0.1 N hydrogen chloride at 70°C for 30 min. After filtration using a microfilter apparatus, the modified bases were separated by Beckman Ultrasphere C_{18} (Beckman Coulter, Fullerton, CA, USA) reverse-phase high-performance liquid chromatography with 0.05 M ammonium formate (pH 4.5) containing 10% methanol. Protein was determined by the Bradford method,[9] and the amount of O^6-methylguanine released from the DNA substrate per milligram of protein was calculated.

Assessment of Response

The response of the subcutaneous xenografts was assessed by delay in the growth of tumor in mice treated with drug as compared with growth in control mice (T-C). The growth delay was the difference between the median times required for tumors in treated (T) and control (C) mice to reach five times the size at the initiation of therapy and at least greater than 1,000 mm^3. Tumor regression was defined as a decrease in tumor volume over two successive measurements.

Statistical analysis was performed using the Wilcoxon rank-sum test for growth delay and Fisher's exact test for tumor regressions as previously described.[6] Although the killing of the mice at 60 days did right censor the data for those animals, other steps were taken in the experimental design to ensure a valid test using the Wilcoxon rank-sum test. All mice in the control group were allowed to reach their endpoint without censoring. No mice in the treatment group were killed before the last one in the control group had reached the study endpoint. These two steps ensured that the assumptions of the Wilcoxon test were met and that the test was valid. If, in addition, fewer than half of the treated mice are killed, then the time to the endpoint and T-C can be obtained.

The response of the intracranial xenografts was assessed by the percentage increase in median survival. Statistical analysis was performed using the Wilcoxon rank-sum test as described previously.[6]

Results

AGT Activity

Tumor AGT concentrations ranged from a high of 393 fmol/mg protein in D-212 MG xenografts to undetectable levels (<10 fmol/mg protein) in D-245 MG and D-425 MED xenografts (Table 1).

Experiment 1

In experiment 1 (18 mg/kg per day for five days), the longest growth delays were seen in D-425 MED, D-245 MG, D-54 MG, D-612 EP, and D-456 EP (Table 1), with growth delays for these xenografts ranging from 8.3 to more than 60 days. Xenografts of D-341 MED and D-212 MG displayed modest growth delays of 1.2 and 3.1 days, respectively. Tumor regressions were seen in each of the xenograft lines except D-212 MG (Table 1). VNP40101M produced a 50% increase ($p < 0.001$) in the median survival of mice bearing intracranial D-245 MG xenografts (median day of death, day 72) compared with mice receiving vehicle (median day of death, day 48), with all animals dying.

Of the 80 mice treated with VNP40101M at 18 mg/kg, three died of toxic effects. The median weight loss in all animals tested was 0.06%. However, 19 deaths were seen at longer than 70 days. These animals had no significant wasting at any time after treatment. All mice used to calculate toxic deaths and median weight loss were up to 60 days after treatment or had tested out of study.

Experiment 2

In experiment 2 (9.0 and 13.5 mg/kg per day for five days), the delayed toxicity associated with a dose of 18 mg/kg for five days led us to repeat these studies in mice with D-245 MG xenografts at reduced VNP40101M doses of 13.5 or 9.0 mg/kg for five days (Table 2). Relative to controls, treated mice showed statistically significant growth delays at VNP40101M doses of 13.5 mg/kg for five days (T-C of more than 60 days, $p < 0.001$) and

Table 1. Effect of VNP40101M treatment (18 mg/kg per day for five days) on the growth of human CNS xenografts in mice

Xenograft	Derivation	T-C[a]	Tumor Regressions[b]	% Range (Median) of Tumor Regression	AGT (fmol/mg Protein)	Temozolomide T-C[a]	BCNU T-C[a]
D-425 MED	Medulloblastoma	60+*	10 of 10*	100 (100)	Below detection	ND	ND
D-245 MG	Adult high-grade glioma	60+*	9 of 10*	35–86 (56)	Below detection	108.3	56.7
D-54 MG	Adult high-grade glioma	60+*	10 of 10*	30–83 (50)	13.7 ± 16.6	40.8	ND
D-456 MG	Childhood high-grade glioma	15*	10 of 10*	30–92 (65)	94.0 ± 16.6	120+	6.1
D-612 EP	Ependymoma	51*	8 of 10*	62–86 (74.5)	236.4 ± 93.1	72.8	18.3
D-341 MED	Medulloblastoma	1.2	1 of 10	50	385.0 ± 206.5	3.5	ND
D-212 MG	Childhood high-grade glioma	3.1	0 of 10	No regressions	393.0 ± 196.6	56.2	4.1
D-528 EP	Ependymoma	8.3*	4 of 10	61–82 (71)	516	68.3	10.9

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ND, not done.

[a]The response of subcutaneous xenografts was assessed by delay in growth of tumor in animals treated with drug compared with growth in control animals (T-C). The growth delay is the difference (measured in days) between the median time required for tumors in treated (T) and control (C) animals to reach five times the size at the initiation of therapy and greater than 1,000 mm^3 or whichever was higher.[8,15]

[b]Tumor regression was defined as a decrease in tumor volume over two successive measurements.

*$p < 0.001$

Table 2. Activity of VNP40101M at three dose levels for five days in the treatment of D-245 MG xenografts

VNP40101M Dose (mg/kg)	T-C[a]	Tumor Regressions[b]*	Toxic Deaths
13.5	>60	8 of 9	1
9.0	53.5*	9 of 10	0
18.0	>60	9 of 10	0

[a]The response of subcutaneous xenografts was assessed by delay in growth of tumor in mice treated with drug as compared with growth in control mice (T-C). The growth delay is the difference (measured in days) between the median time required for tumors in treated (T) and control (C) mice to reach five times the size at the initiation of therapy and greater than 1,000 mm^3 or whichever was higher.

[b]Tumor regression was defined as a decrease in tumor volume over two successive measurements.

*$p < 0.001$ compared with control.

9.0 mg/kg for five days (T-C of 53.5 days, $p < 0.001$), as well as at a dose of 18.5 mg/kg for five days (T-C of more than 60 days, $p < 0.001$). Tumors regressed in 8 of 9 mice and 9 of 10 mice that received 13.5 and 9.0 mg/kg per day for five days, respectively, compared with regressions in 9 of 10 mice that received the 18.5-mg/kg dose ($p < 0.001$). There was one toxic death among the 20 mice evaluated with 13.5- and 9.0-mg/kg doses (a mouse that had received 13.5 mg/kg), with the condition of the mice followed for 90 days.

Experiment 3

In experiment 3 (VNP40101M and O^6-benzylguanine), mice were treated with VNP40101M alone at 10 mg/kg per day or with VNP40101M following BG administered at a dose of 30 mg/kg (Table 3). In mice bearing D-456 MG xenografts and treated with VNP40101M plus BG, there was an increase in growth delay of 2.6 days compared with mice that received only VNP40101M (2.0–4.6 days). In mice bearing D-341 MED xenografts and treated with VNP40101M plus BG, there was an increase in growth delay of 10.9 days ($p < 0.001$) compared with mice treated with VNP40101M alone (3.6–14.5 days). There were no toxic deaths, with the condition of the mice followed for 90 days.

Discussion

VNP40101M has been shown to be active in vivo against a variety of tumors, including L1210 leukemia, P388 leukemia, B16 melanoma, M109 lung carcinoma, M5076 sarcoma, C26 colon carcinoma, U251 glioma, and LX-1 human lung carcinoma.[4] Cell lines that have been resistant to such commonly used chemotherapeutic agents as melphalan, cytoxan, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) were sensitive to the cytotoxic effects of VNP40101M.[10] VNP40101M is most accurately described as a bifunctional prodrug because it generates two active species: methyl isocyanate and a chloroethylating species. In a study comparing the use of a metabolite of VNP40101M that generates only the chloroethylating species with the use of VNP40101M in the treatment of CHO cells transfected with the gene for AGT, VNP40101M was considerably more cytotoxic than this metabolite, possibly indicating that the methyl isocyanate species is important in the inhibition of AGT.[10] The results presented here suggest that VNP40101M has activity against a broad panel of xenografts derived from childhood or adult CNS tumors. Analysis of the relationship of drug activity to tumor AGT concentrations indicated that the longest growth delays were in tumors with low or undetectable AGT levels.

In experiment 1, there were several late deaths (more than 60 days after treatment), similar to reports from other investigators. In one study, late deaths were seen in CD_2F_1 mice that received a single dose of VNP40101M greater than 80 mg/kg; these mice exhibited signs of wasting prior to death.[4] In subsequent toxicity studies, VNP40101M was administered daily for five consecutive days (1, 3, 10, and 20 mg/kg) to rats, and necropsies were performed on days 15, 29, and 31 after treatment. At necropsy, these rats exhibited congestion and hemorrhage of various solid organs and lymph nodes, and the severity increased with increasing dose.[11] The only microscopic abnormality in bone marrow was mild erythroid hyperplasia that was observed in some of the rats treated with 10 and 20 mg/kg. It is noteworthy that the major hematologic toxic effects in human patients were granulocytopenia and thrombocytopenia,[12] whereas in rats the major effect was lymphocytopenia. Drug-related

Table 3. Activity of VNP40101M (10 mg/kg) and of VNP40101M plus O^6-benzylguanine (BG) in D-456 MG and D-341 MED xenografts

Xenograft	AGT (fmol/mg Protein)	Treatment	T-C[a]	Regressions[b]	Toxic Deaths
D-456 MG	94.0 ± 16.6	VNP40101M	2.0	0 of 10	0
		VNP40101M + BG	4.6	0 of 10	0
D-341 MED	385.5 6 206.5	VNP40101M	3.6	0 of 10	0
		VNP40101M + BG	14.5*	4 of 10	0

Abbreviation: AGT, O^6-alkylguanine-DNA alkyltransferase.

[a]The growth delay (T-C) is the difference (measured in days) between the median time for tumors in treated (T) and control (C) mice to reach five times the volume recorded at the beginning of treatment.

[b]Tumor regression was defined as a decrease in tumor volume over two successive measurements.

*$p < 0.001$.

late deaths in human patients receiving a single dose of VNP40101M have not been observed.[12,13] It is unclear whether we would have had similar findings if necropsies were performed on the mice that experienced late deaths in experiment 1. The etiology for these late deaths in experiment 1 is unknown but is now being explored.

Owing to the significant toxicity seen in experiment 1, we reevaluated VNP40101M at two lower dose levels (9.0 and 13.5 mg/kg) administered daily for five days. D-245 does not express detectable AGT, and the growth delays were substantial and statistically significant. In this experiment, we produced statistically significant growth delays without encountering substantial late deaths in both groups. We observed significant growth delays and responses at both dose levels (9.0 and 13.5 mg/kg per day for five days) of VNP40101M without significant late deaths. In this model, lower doses of VNP40101M seem to decrease toxicity without substantially affecting growth delays and responses.

We also evaluated the ability of BG-mediated AGT depletion to augment the activity of VNP40101M. Mice bearing D-456 MG xenografts and treated with VNP40101M plus BG exhibited only a three-day increase in growth delay compared with animals treated with VNP40101M. In contrast, animals bearing D-341 MED xenografts and treated with VNP40101M plus BG had an increase in growth delay of 10.9 days and tumor regressions in 4 of 10 mice compared with mice treated with VNP40101M alone. These results suggest that BG-mediated AGT depletion may enhance VNP40101M activity, similar to the findings with BCNU and temozolomide.[14,15]

In conclusion, VNP401010M is a bifunctional prodrug that has activity in a panel of human CNS tumor-derived xenografts. On the basis of the activity of VNP40101M in preclinical models and the limited toxicities seen in phase I studies in patients with various solid tumors, we have initiated phase II evaluation of VNP40101M in the treatment of patients with malignant glioma.

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