Dianhydrogalactitol inhibits the growth of glioma stem and non-stem cultures, including temozolomide-resistant cell lines, *in vitro* and *in vivo*

Shaun D Fouse[1], Anne Steino[2], Nicholas Butowski[1], Jeffrey A Bacha[2], Sarath Kanekal[2], Nancy Dos Santos[3], Dennis Brown[2], Joseph F. Costello[1]

[1]Department of Neurological Surgery, University of California, San Francisco, CA, USA, [2]DelMar Pharmaceuticals, San Carlos, CA, USA, [3]BC Cancer Agency, Vancouver, BC, Canada

ABSTRACT #2562

The standard of care for glioblastoma multiforme (GBM) patients is surgical resection followed by temozolomide (TMZ) and radiation (XRT). However, TMZ-resistance has emerged as a significant unmet medical need, as DNA repair enzyme 0-6-methylguanine DNA methyltransferase (MGMT) removes the methyl-group adducts caused by TMZ. Dianhydrogalactitol (VAL-083) is a structurally unique alkylating agent causing DNA crosslinks at N7 position of guanine. Because VAL-083's N7 adducts are not subject to MGMT mediated repair, it may be an effective chemotherapeutic in the treatment of TMZ-resistant GBM. VAL-083 crosses the blood brain barrier and accumulates in brain tumor tissue. We have recently shown that TMZ activity is similar in cancer stem cells (CSC) and their paired non-CSC from primary GBM tissues, and that the activity is MGMT-dependent[1]. We thus sought to investigate how our CSC and non-CSC panel would respond to VAL-083 alone or in combination with XRT. We further investigated the activity of VAL-083 in in vivo models of drug-resistant GBM in comparison to TMZ. Rag2 mice bearing intracranial human GBM xenograft tumors of either MGMT-positive and TMZ-resistant origin (BT74), or MGMT-negative and TMZ-sensitive origin (U251) were treated. VAL-083 was given i.p. 3 times/week x 3 weeks, and the efficacy of VAL-083 in controlling tumor growth compared to TMZ (30 mg/kg). Disease progression was evaluated by overall survival, clinical observations and body weight measurements. Our in vitro results show that VAL-083 is a potent inhibitor of all tested primary GBM cultures, irrespective of MGMT status. VAL-083 causes cell cycle arrest and loss of cell viability in TMZ-resistant cells, and at lower concentrations than TMZ in TMZ-sensitive cells. Furthermore, VAL-083 is not affected by cell culture condition (Stem vs. Non-Stem). Low dose VAL-083 combined with XRT exhibited an additive effect in all cultures tested, suggesting that VAL-083 might act as a radiosensitizer. In the in vivo U251 model, the median survival time for mice treated with 4 mg/kg VAL-083 was significantly increased to 72 days compared to 48 days for controls (p<0.0001). Median survival time for 3 mg/kg VAL-083 was 54 days. Body weight loss was observed in mice treated with 5 mg/kg and treatment was stopped after 4 doses after which the animals recovered and their median survival was 57 days. Animals treated with TMZ were terminated at day 102 at the end of the study. BT74: study is ongoing, data will be presented at the meeting.In conclusion, VAL-083 is highly efficacious against both stem and non-stem GBM cell cultures in vitro, the activity is independent of MGMT and VAL-083 appears to act as a radiosensitizer in GBM. In vivo xenograft GBM models further validate the benefits of VAL-083 in the treatment of GBM and support ongoing clinical research with VAL-083, which is currently in a clinical trial for GBM patients with recurrent disease.

BACKGROUND: VAL-083 *(dianhydrogalactitol)* is a bifunctional alkylating agent causing interstrand DNA crosslinks at the N^7position of guanine.[7,8,9] In prior clinical studies sponsored by the US National Cancer Institutes, VAL-083 exhibited clinical activity against a number of tumor-types in a number of cancers including lung, brain, cervical, ovarian and hematologic malignancies. VAL-083 is approved in China for the treatment of chronic myelogenous leukemia and lung cancer and has received orphan drug designation in Europe and the U.S. for the treatment of gliomas.

We have previously demonstrated that VAL-083 is active against temozolomide-resistant cancer stem cells (CSCs) and retains cytotoxic activity independent of the repair enzyme MGMT (O^6-methylguanine methyltransferase) *in vitro*. MGMT is correlated with temozolomide resistance and poor GBM patient outcomes.[1, 4]

Figure 1: VAL-083 inhibits the growth of patient-derived CSCs that were TMZ resistant.

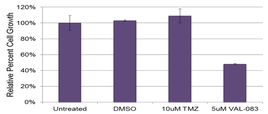
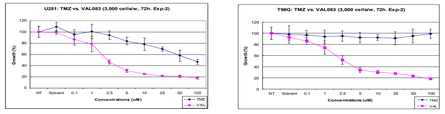


Fig 2. In vitro activity of VAL-083 vs. TMZ activity in GBM cell lines SF188 (pediatric), U251 (adult), and T98G (adult) as impacted by expression of MGMT repair enzyme[1]



Historical NCI-sponsored studies achieved promising results in patients with newly diagnosed and recurrent GBM using a dosing regimen of VAL-083 given 25 mg/m^2/day for five days every five weeks[2]. These data suggest comparable or enhanced survival and improved safety compared to other alkylating agents such as TMZ or nitrosourea-based therapy.

Table 1. Historical clinical data of TMZ or VAL-083 in GBM support the potential for comparable or enhanced survival similar to standard-of-care chemotherapy

Chemotherapeutic Agent	MGMT Promoter Status	XRT Alone	XRT + Chemotherapy	p-value
		median survival (mo.)		
LOMUSTINE[(5)]	n/a	n/a	**11.9**	n/a
CARMUSTINE[(5)]	n/a	n/a	**9.2 - 11.5**	n/a
SEMUSTINE[(5)]	n/a	n/a	**8.0**	n/a
TMZ[(6)]	Methylated *(low MGMT expression)*	15.3	**21.7**	0.007*
	Unmethylated *(high MGMT expression)*	11.8	**12.7**	0.06
VAL-083[(2)]	n/a	8.8	**16.8**	0.02*

**statistically significant improvement p-value by log-rank test*

Historically, toxic effects of VAL-083 were noted on white blood cell (WBC) and platelet counts. For example, Eagan et al (1979)[2] observed nadir of 2,100/μL (lymphopenia) and 88,000/μL (thrombocytopenia) in patients with newly diagnosed and recurrent GBM using a dosing regimen of VAL-083 given 25 mg/m^2/day for five days every five weeks. In a separate study, a dose of 40mg/m^2/day VAL-083 for five days resulted in a median platelet nadir of 31,000/μL and WBC nadir of 2,300/μL[3]. In general, nadir occurred within three weeks and returned to normal within 7 days. Anemia, nausea and vomiting were usually mild to moderate. No renal, hepatic, central nervous system, cardiac, or pulmonary toxicity was identified.

Based on promising historical clinical data, differentiated cytotoxic mechanism and data supporting activity against temozolomide-resistant GBM cell lines and cancer stem cells, DelMar Pharmaceuticals has initiated a Phase I/II clinical trial with VAL-083 as a potential new chemotherapy for the treatment of refractory GBM (clinicaltrials.gov identifier: NCT01478178).

We believe that VAL-083 may provide a viable treatment option and the potential to surpass standard-of care for patients suffering from GBM, particularly those GBM patients who fail or are unlikely to respond to the current standard-of-care.

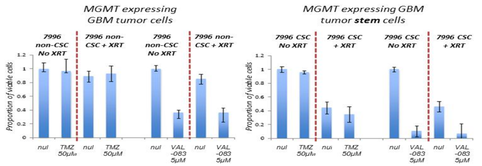
We sought to develop new non-clinical data to further explore the activity of VAL-083 in comparison to temozolomide.

VAL-083 functions as a radiosensitizer with potential superiority to temozolomide

MGMT expressing GBM tumor cells and MGMT expressing GBM tumor stem cells were treated with either TMZ at high doses (50μM) or VAL-083 at a clinically relevant dose of (5μM) either with or without 2Gy of radiation and compared to untreated control (nul). Temozolomide exhibited no activity against MGMT expressing GBM tumor cells in comparison to control with or without radiation. However, VAL-083 was active against both MGMT expressing GBM tumor cells and MGMT expressing GBM tumor stem cells.

Fig 3.

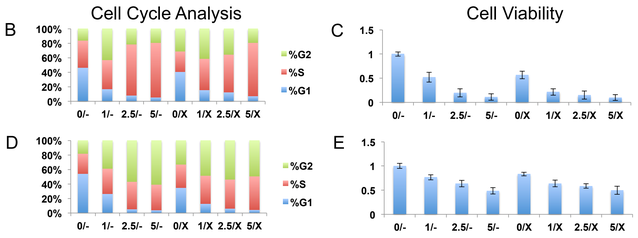
Cell viability analysis at day 6 post treatment for the paired CSC and non-CSC MGMT-expressing 7996 cultures.



VAL-083 at 5uM doses resulted in near-100% tumor cell kill; therefore, radiation had no added benefit. Notably however, when VAL-083 was added to CSC cultures at low micromolar doses (1, 2.5 μM) with or without 2Gy radiation, VAL-083 acted as a radio-sensitizer against CSC's in all cultures tested.

Fig 4.

Cell cycle profile analysis at day 4 post treatment and cell viability analysis at day 6 post treatment for two different patient-derived CSC cultures treated with VAL-083 with or without irradiation (2Gy). 7996 (graph A&B) and 8565 (graph C&D).



VAL-083 increased survival time in models of GBM *in vivo* in a dose dependent manner

U251: Female Rag2 mice bearing intracranial human GBM xenograft tumors of U251 (TMZ-sensitive) or BT74 (TMZ-resistant) origin were treated with TMZ or VAL-083.

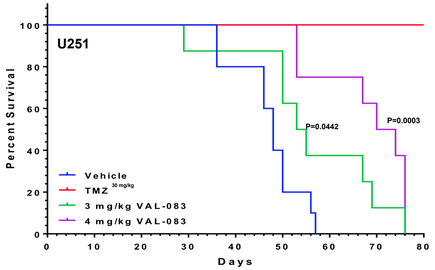
Animals were treated with a dose of 30 mg/kg of TMZ or three different doses of VAL-083 ranging from 3 mg/kg to 5 mg/kg. Disease progression is evaluated by overall survival, clinical observations and body weight measurements.

As expected, animals bearing intracranial U251 (TMZ-sensitive) tumors were sensitive to both TMZ and VAL-083. Median survival time for mice treated with 4 mg/kg VAL-083 was significantly increased to 72 days compared to 48 days for controls (p=0.0003).

Median survival time for 3 mg/kg VAL-083 was 54 days. Body weight loss was observed in mice treated with 5 mg/kg and treatment was stopped after 4 doses after which the animals recovered and their median survival was 57 days. As expected, U251 is sensitive to TMZ and animals treated with TMZ were terminated at day 102 at the end of the study.

BT74: Preliminary data suggest that VAL-083 treatment also increases survival time in mice bearing intracranial BT74 tumors compared to untreated control.

Fig. 5 Kaplan-Meier survival plot for group 1-4 animals bearing U251 intracranial tumors. The table shows the median survival and corresponding p-values.



Treatment Group	Control	VAL-083 3mg/kg	VAL-083 4mg/kg	VAL-083* 5mg/kg
Median Survival (days)	48	54 *p=0.0442*	72 *p=0.0003*	57 *p=0.0057*

*Animals in the 5 mg/kg VAL-083 group only received 4 doses due to weight loss after which the animals recovered spontaneously.

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VAL-083 causes cell cycle arrest in TMZ-resistant cultures, including CSCs

A panel of primary cancer stem cells (CSC) and their paired primary non-stem (non-CSC) cultures were characterized for MGMT expression.

Treatment with VAL-083 led to a greater degree of cell cycle arrest as indicated by fewer cells in the G1 phase

- Over 40% of the CSCs treated with DMSO control of TMZ are in G1 phase at doses of 5μm, representing a normal cell cycle distribution for viable tumor cells at doses similar to those achieved clinically in the treatment of glioblastoma.
- In contrast, than 10% are in G1 phase after a dose of 5μM with VAL-083, indicating that progression back into G1 phase has been blocked causing cell cycle arrest and apoptosis.
- Higher doses resulted in cell-cycle arrest for both compounds; however, doses exceeding CNS tissue exposure higher than 10μM of TMZ or VAL-083 are generally not achievable in a clinical setting due to toxicity.

Fig 6. MGMT western blot analysis of protein extracts from 4 pairs of CSC and non-CSC cultures derived from primary GBM tissue

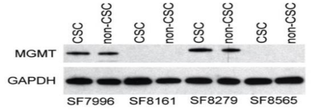
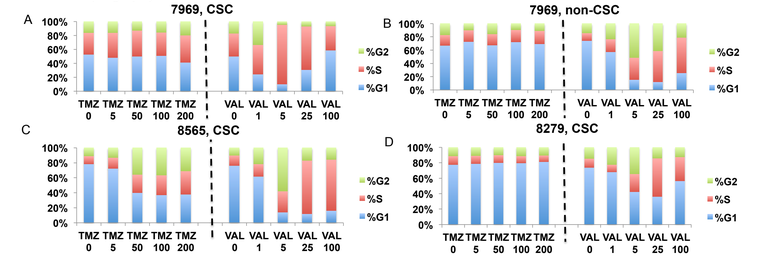


Fig 7. Cells were treated with either increasing doses of TMZ (5, 50 100 and 200 μM) or VAL-083 (1, 5, 25 and 100 μM) and cell cycle analysis was performed 4 days post treatment (cell cycle arrest is indicated by the blue portion of each column). MGMT-expressing and all CSC cultures (A, B, D) were all resistant to TMZ at clinically relevant doses; however, VAL-083 was active against all cell cultures tested even at low (single μM) doses.



In summary:

- Response to VAL-083 was not dependent on culture type. Both CSC and non-CSC cell cultures exhibit sensitivity to VAL-083.
- VAL-083 killed tumor cells at clinically relevant doses in all CSC and non-CSC cultures tested independent of MGMT expression.
- All CSCs were resistant to TMZ and in non-CSC cultures where MGMT is highly expressed.

MGMT Status	GBM Cancer Cell	GBM Cancer Stem Cell
HIGH	TMZ: ⊘ VAL-083: ✓	TMZ: ⊘ VAL-083: ✓
LOW	TMZ: ✓ VAL-083: ✓	TMZ: ⊘ VAL-083: ✓

CONCLUSIONS

- VAL-083 maintains anti-tumor activity independent of the MGMT resistance mechanism
- Low dose VAL-083 showed an additive effect when combined with radiation in all cultures tested *in vitro*, suggesting that VAL-083 might act as a radiosensitizer
- Because VAL-083's radio-sensitizing effect is seen in MGMT-expressing GBM that are resistant to TMZ + radiation, VAL-083 has the potential to replace TMZ as the chemotherapy of choice in chemo-radiation treatment of newly diagnosed GBM.
- VAL-083 was effective against GBM *in vivo* in extending survival time in U251 intracranial model in a dose dependent manner
- VAL-083 is a potent inhibitor of primary GBM cultures *in vitro*, irrespective of MGMT status or culture condition
- VAL-083 causes cell cycle arrest and loss of cell viability in TMZ-resistant cells *in vitro*
- All tested primary cultures (Stem and Non-Stem) were sensitive to VAL-083 *in vitro*
- These results suggest that VAL-083 has the potential to surpass standard of care in the treatment of GBM, particularly where fail or are unlikely to respond or intolerant to TMZ