Efficacy of Asparaginase *Erwinia chrysanthemi* With and Without Temozolomide Against Glioma Cells and Intracranial Mouse Medulloblastoma

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**Abstract.** Background: Anti-metabolites are less myelosuppressive than DNA-damaging anticancer drugs and may be useful against brain tumors. Materials and Methods: We evaluated the asparagine/glutamine-deaminating agent Erwinaze with/without temozolomide against brain tumor cells and mouse medulloblastomas. Results. Erwinaze treatment of cell lines and neurospheres led to dose-dependent reductions of cells (reversible by L-glutamine), with half maximal inhibitory concentrations ($IC_{50}$) of 0.12–10 IU/ml. Erwinaze at <1 IU/ml reduced temozolomide $IC_{50}$ by 3.6- to 13-fold (300-1,200 μM to 40-330 μM). Seven-week-old SMO/SMO mice treated with Erwinaze (regardless of temozolomide treatment) had better survival 11 weeks post-therapy, compared to those not treated with Erwinaze (81.25% vs. 46.15, $p=0.08$). Temozolomide-treated mice developed 10% weight loss, impairing survival. All 16 mice treated with temozolomide (regardless of Erwinaze treatment) succumbed by 40-weeks of age, whereas 5/8 animals treated with Erwinaze alone and 2/6 controls survived ($p=0.035$). Conclusion: Erwinaze enhances cytotoxicity of temozolomide in vitro, and improves survival in SMO/SMO mice, likely by reducing cerebrospinal fluid glutamine. Temozolomide-associated toxicity prevented demonstration of any potential combinatorial advantage with Erwinaze in vivo.

Brain tumors with a poor prognosis are mainly malignant gliomas in adults and medulloblastomas in children (1). Along with neurosurgical resection, DNA-damaging strategies (e.g. radiotherapy and alkylating chemotherapy) are the backbone of standard care (2). Immunotherapy and cell-signaling inhibition are promising, but mostly in clinical research – with improved outcomes in limited patients (2, 3). Two-thirds of the nearly 24,000 patients diagnosed each year with brain tumors in the U.S. succumb to these types of cancers (4). Thus, additional research is imperative in order to exploit strategies that are biologically and clinically justified. One approach is to combine partially effective therapies with potentially active agents that are not currently used for brain tumors.

Temozolomide is part of the standard of care for adjuvant therapy of glioblastoma (GBM). Temozolomide prolongs patient survival and delays disease progression (5). It showed anti-medulloblastoma activity in mice (6) and in patients with recurrent medulloblastomas or primitive neuroectodermal tumors (7). Temozolomide has dose-limiting hematological toxicities, like other DNA-alkylating agents (5, 8). We hypothesized that coupling temozolomide with the use of less myelosuppressive but potentially active agents against brain tumors may produce superior therapeutic effects, without overlapping toxicity. Anti-metabolic agents, such as bacterial asparaginase (ASNase), are good candidates for combining with temozolomide, because they are less myelosuppressive (9).

Asparaginases are very effective essential parts of childhood acute lymphoblastic leukemia (ALL) therapy and have been in use since the 1960s (10). ASNase acts through depletion of asparagine (Asn) and glutamine (Gln) (11). ALL blasts die when deprived of these compounds. This mechanism may be active against brain tumors, which have higher metabolic needs (12, 13), including demands for Gln (14-16). Although large molecules such as ASNase may not cross the blood–brain barrier (BBB), ASNase does reduce Asn and Gln levels in the cerebrospinal fluid (CSF) (17, 18). Moreover, the BBB may not be completely intact in tumors (19), theoretically allowing enzyme extravasation and penetration into the brain parenchyma.

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We showed that co-administration of ASNase from *Escherichia coli* enhances the effects of chemotherapy against malignant glioma cells *in vitro*, and against subcutaneous (SQ) medulloblastomas in mice (20). These findings favor pre-clinical testing of other ASNase formulations, such as the ASNase from *Erwinia chrysanthemi* (Erwinaze). The agent is FDA approved as second-line therapy for ALL. Moreover, Erwinaze has favorable Michaelis-Menten constant for Gln and was shown to effectively deplete plasma glutamine in leukemia patients (21). Gln is well known to be important for brain tumor growth (22).

Whether ASNase-induced enhancement of antitumor effects [as seen in the SQ model (20)] is possible for intracranial brain tumors is unknown. Therefore, testing ASNase combinations in a preclinical intracranial brain tumor model is needed, preferably with the BBB intact. Based on experience with pediatric ALL, the best synergy between ASNase and another agent occurs when ASNase is administered sequentially (23), e.g. 3-24 hours after cytarabine (24) or methotrexate (25). Thus, Erwinaze followed treatment with an anti-brain tumor agent in our experiments.

We used a diverse panel of brain tumor cell lines and neurosphere cultures (also known as gliomaspheres) to mimic brain tumor heterogeneity. For a mouse model, we used SMO/SMO genetically modified mice, which have a 90% rate of occurrence of spontaneous leptomeningeal medulloblastomas (26), without artificial disruption of the BBB.

**Materials and Methods**

**Cell lines and neurosphere cultures.** We used four brain tumor cell lines (cultured in serum supplemented media) and two primary neurosphere (NS) lines (grown in serum-free media): DAOY pediatric medulloblastoma cells with inducible alkylator resistance (27, 28); U87 and primary GBM-ES cells, both human GBM lines resistant to temozolomide and radiation (29); GL-261 cells, mouse glioma cells sensitive to a ketogenic diet (30); and NS 157 and NS 217 from primary human GBM lines.

Primary GBM cells were obtained from tumors collected at the University of California, Los Angeles [approved by Institutional Review Board protocol IRB#11-000432-AM-00010 (31)] and authenticated by morphology and growth curves. The other cell lines were from the American Type Culture Collection (Manassas, VA, USA) and verified according to standard procedures. All cell lines tested negative for mycoplasma.

**Media and drugs.** Serum supplemented Dulbecco’s modified Eagle’s medium (DMEM) for cell lines. DMEM [containing 5% fetal bovine serum (FBS)] was used with different concentrations of glutamine (Gln 1 and 10 mM). Gln-containing media was used for colorimetric assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Media with no Gln, as well as with Gln at 1 and 10 mM, were used to assess Gln effects in cell viability rescue experiments.

**Media for NS cultures.** We used serum-free media supplemented with epidermal growth factor and fibroblast growth factor, as reported previously (32).

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**Drugs and dimethyl sulfoxide (DMSO).** Erwinaze was kindly provided by Jazz Pharmaceuticals (Palo Alto, CA USA). Temozolomide was purchased commercially (CGene-Tech Inc., Indianapolis, IN, USA), as was DMSO.

**Cell viability MTT assay and neurosphere assay.** MTT assay kits (MTT 98%, ACROS Organic, Thermo Fisher Scientific, NJ, USA) were used according to the manufacturer’s instructions. DMEM (with 5% FBS) was supplemented with 1 or 10 mM of L-glutamine (15-013-CM Corning Inc. from Fisher Scientific, Corning, NY, USA) or unsupplemented. An incubation time of 72 h was used to assess IC₅₀ values for temozolomide and Erwinaze. On day 1, 2,000 to 4,000 cells/well (75 μl/well) were seeded, depending on the growth rate of the cell line. Erwinaze (at 0.01, 0.02, 0.04, 0.08, 0.16, 0.3125, 0.625, 1.25, 2.5, 5 and 10 IU/ml), and temozolomide (0.01, 15.625, 31.25, 62.5, 125, 250 and 500 μM) against four cell lines grown in serum-supplemented media.
combination assays, concentrations used for Erwinaze were 0.01, 0.02, 0.04, 0.08, 0.16, 0.3125 and 0.625 IU/ml, and temozolomide concentrations were 0.01, 0.04, 0.08, 0.16, 0.3125 and 0.625 IU/ml. The MTT reagent (5 mg/ml, 25 μl/well) was added after 72 h (day 4), and plates were incubated for 4 h more. The medium was then removed, 100 μl/well DMSO was added, and plates were read at 570 nm. Neurosphere assays were carried out as previously reported (20).

Mice. All in vivo experiments were carried out according to institutionally approved protocols. Homozygous C57BL/6-Tg(Neurod2-Smo*A1)199Jol/J mice (SMO/SMO) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). These mice express a constitutively active point mutation SmoA1 (mouse smoothened homolog gene), under the control of the specific promoter Neurod2 (mouse neurogenic differentiation 2), with transgene expression specific to cerebellar granule cells. The mice have a 90% occurrence rate of spontaneous leptomeningeal medulloblastomas by 1.5-2 months of age (26). This intracranial model has additional advantages over orthotopic GBM xenotransplants: no injection into the brain parenchyma and no artificial puncturing of the BBB is required. Four groups of SMO/SMO mice were treated with intra-peritoneal injections for three cycles of therapy: (i) Control (n=6): DMSO 10%, 50 μl injection daily; (ii) temozolomide (n=8): 0.05 mg/g/daily; (iii) Erwinaze (n=8): 2.5 IU/g/daily; (iv) combination (n=8): Erwinaze followed by temozolomide 4 h later at the same doses; all administered 5 days a week for 2 weeks every 4 weeks.

Injections started at 7 weeks of age. Animals were monitored daily by independent observers for signs of tumor progression or neurological impairment. All mice were weighed three times a week during treatment, and at least once a week afterwards until euthanized. Indications for euthanasia included any sign of neurological impairment (indicating possible tumor progression) such as: impaired ambulation, extreme lethargy, inability to remain upright, seizures, head tilting, and/or emaciation (>10% weight loss). Moribund animals were promptly euthanized in accordance with Institutional Animal Care and Use Committee guidelines (CO2 inhalation followed by cervical dislocation). All remaining mice were sacrificed at 42 weeks of age (study cut-off).

Statistical analysis. The half-maximal inhibitory concentration (IC50) calculations, in vitro synergy determinations, weight curves, and survival analyses were made using GraphPad Prism 6.0.
The in vivo study followed a 2×2 factorial design with two drug types (Erwinaze, temozolomide). Thus, the four groups were compared with respect to post-treatment survival. With 6-8 animals per group, we anticipated having the power to detect the measured survival differences between the groups with a significance level of \( p<0.05 \).

**Results**

**Erwinaze efficacy and synergy with temozolomide in vitro.** Dose-dependent brain tumor cell death was observed in vitro, induced by single agents Erwinaze and temozolomide (Figure 1A and B, respectively). IC\(_{50}\)s for Erwinaze ranged widely, from 0.12 IU/ml to >10 IU/ml for different cell lines (Figure 1A). Combining temozolomide with Erwinaze at concentrations <1 IU/ml enhanced cytotoxicity against all four cell lines, with 3.6- to 13-fold decreases in temozolomide IC\(_{50}\) (from ranges of 300-1,200 μM to 40-330 μM; Figure 2).

**Glutamine rescue of cells from Erwinaze effects.** Next, we assessed effects of Erwinaze on cell lines in the absence and presence of Gln at 1 and 10 mM. Gln reversed Erwinaze-induced cytotoxicity in a dose-dependent manner in all four lines (Figure 3).

**Erwinaze against NS.** We used two glioblastoma cell lines grown as NS, treated with Erwinaze at different concentrations. These experiments also showed dose-dependent reduction of NS numbers (IC\(_{50}\) of 0.68 and 1.5 IU/ml; Figure 4).

**Erwinaze against mouse medulloblastoma with and without temozolomide.** Seven-week-old SMO/SMO mice were treated with three cycles of 10 intraperitoneal injections of DMSO (controls; \( n=6 \)), temozolomide, Erwinaze, and a combination (\( n=8 \) each). Analyses at 18 weeks after starting therapy showed trends toward a survival advantage for mice receiving Erwinaze (regardless of temozolomide treatment; Figure 5A) compared to mice that did not (81.25% vs. 46.15%, \( p=0.08 \); Figure 5B). However, mice on temozolomide, regardless of Erwinaze treatment, gained less weight than mice not treated with temozolomide (by at least 10%; Figure 6). Moreover, the combination of Erwinaze and temozolomide did not improve survival. Thus, by 33 weeks after starting therapy (at age 40
weeks), all 16 mice receiving temozolomide, alone and with Erwinaze, had succumbed, whereas five out of eight mice receiving Erwinaze alone and two out of six control mice were still alive \((p=0.035; \text{Figure 5})\). One more mouse in the group treated with Erwinaze alone died by 42 weeks of age, and all remaining mice were euthanized.

### Discussion

Treatment of malignant brain tumors often requires adjuvant therapy, in addition to neurosurgical resection. This adjuvant therapy includes traditional systemic DNA-damaging agents, which have dose-limiting toxicities \((\text{e.g. myelosuppression})\).
Anti-metabolic strategies that are less myelosuppressive provide pre-clinical opportunities to explore potential alternatives. We reported in 2013 that the anti-leukemia agent *E. coli*-ASNase enhances chemo-cytotoxicity against brain tumors (20). Additional studies validating the anti-glioblastoma activity of ASNase in vitro and in vivo were recently reported (33, 34). Our current study shows that activity of Erwinaze against brain tumor cells is comparable to the reported in vitro effects of *E. coli*-ASNase (Figure 1A and 4). Gln supplementation reversed the antitumor cell activity of Erwinaze, confirming that amino acid depletion is the main mechanism of action (as with *E. coli*-ASNase; Figure 3).

Moreover, Erwinaze alone prolonged survival of SMO/SMO mice. Adding temozolomide to Erwinaze did not have detrimental effects up to 8 weeks post-therapy. However, temozolomide most likely contributed to the eventual death of SMO/SMO mice with the regimen used, for reasons yet to be explored. Possibly the unique biology of SMO/SMO mice caused delayed temozolomide-induced toxicity, which overrode the effect of Erwinaze in the combination group. To our knowledge, this study is the first attempt to treat SMO/SMO mice with an alkylator combined with an anti-metabolite agent.

SMO/SMO mice have sonic hedgehog (SHH) pathway activation (26). R2 Tumor Genomics Data showed that medulloblastomas with SHH pathway activation have the highest expression of asparagine synthetase (ASNS), compared to other subtypes (35, 36). ASNS catalyzes de novo biosynthesis of Asn using Gln as an amino group donor (aspartic acid + glutamine $\text{ASNS} \rightarrow$ asparagine + glutamic acid).

High ASNS expression in SHH tumors can augment Asn synthesis, and may indicate an increased Asn requirement, which we hypothesize led to sensitivity of SMO/SMO mice to Erwinaze. Moreover, one of the downstream target genes for the SHH pathway is *MYC*. The *MYC* oncogene is associated with Gln addiction (37), supporting an interpretation that Gln deamination might have contributed to the antitumor activity of Erwinaze in this medulloblastoma model.

The concept of amino acid depletion contributing to anti-brain tumor activity may not be unique to Asn and Gln, because others have demonstrated similar anti-GBM effects with manipulation of arginine metabolism (38, 39). Our study adds evidence justifying further research on amino acid manipulation for development of brain tumor therapeutics. Amino acid effects may be studied not only by depletion of these nutrients in brain tumors, but also by inhibition of up-regulated enzymes, such as branched chain amino acid transaminase 1 (35, 40, 41).

In conclusion, our study demonstrated that a spontaneous mouse medulloblastoma model can be used to test pre-clinical therapeutics with Erwinaze. Erwinaze appeared to maintain its antitumor activity in vivo, whereas the traditionally effective alkylating agent temozolomide failed to prolong survival of SMO/SMO mice. The synergistic potential of Erwinaze with conventional therapies against brain tumors is supported by our in vitro data. Erwinaze should be considered for future pre-clinical research on brain tumors.

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