Novel Temozolomide Analogs to Improve Anti-Tumor Efficacy and Overcome Resistance in Glioblastoma Multiforme

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Abstract # DDRE-25

Glioblastoma (GBM) is considered one of the most lethal forms of human cancers, and despite considerable advances in multimodality treatments, it remains an incurable disease with an overall survival of 14 to 16 months after diagnosis.[1] Even in the era of personalized medicine and immunotherapy, temozolomide (TMZ), an oral alkylating agent, remains the standard-of-care for GBM.[2] However, intrinsic or acquired resistance to TMZ due to overexpression of O6-methylguanine-DNA methyltransferase (MGMT) results in initial treatment inefficacy or tumor relapse, highlighting the significant need for improved treatment strategies.[3] Recently, much effort has been directed towards creating novel TMZ analogs to address the clinical barriers associated with TMZ. While some reported TMZ analogs showed improved brain permeability and anticancer effects in preclinical models, none of them have progressed to testing in humans. There is therefore significant room to improve the brain permeability and anticancer effect profiles of TMZ by incorporating yet unexplored functional groups to create new TMZ analogs. We have designed and synthesized a series of novel C8-substituted TMZ analogs and have evaluated their anticancer potency against a panel of GBM cell lines with variable levels of MGMT expression. Encouragingly, our analogs demonstrated promising anti-cancer effect in both MGMT low and high expressing cell lines. We then evaluated our analogs in a variety of cell-based assays to compare their activity with TMZ. In addition, we performed *in vivo* brain permeability and anti-tumor efficacy assays in mouse flank models. Our results demonstrated that several of our analogs clearly display improved anti-cancer effects and increased brain permeability over TMZ. This work points to a new direction for the development of novel TMZ analogs for improved patient survival.

Background: Temozolomide and it's Mechanism of Action

- Temozolomide is a prodrug and a DNA methylating agent. As a prodrug, it's active metabolite, MTIC, is insoluble in aqueous solutions.
- The molecule is stable at acidic pH (>5), and labile at physiological and basic pH (>7)
- In patients, it is readily absorbed with 100% oral bioavailability within 1–2 h of administration.
- Once in circulation, the slightly alkaline environment of the blood and tissues causes spontaneous hydrolysis to form the active metabolite MTIC [3-methyl-(triazen-1-yl)imidazole-4- carboxamide]; MTIC rapidly breaks down to form the reactive methyl diazonium ion (diazomethane) that alkylates DNA (**Figure 1**). This in turn triggers the DNA mismatch repair pathway, which attempts to repair the damage but results in inappropriate DNA crosslinks, G2 arrest, and ultimately leads to apoptosis (**Figure 5A**).

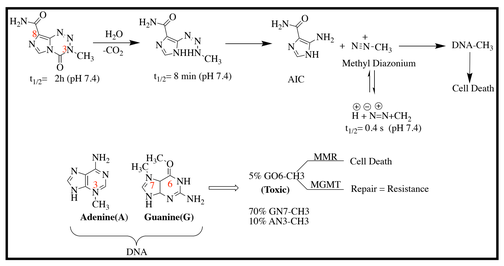

Figure 1: Schematic overview of the TMZ mechanism and formation of toxic DNA-adducts

Limitations of TMZ in the Clinic:

- Only 20% of the systemic dose of TMZ enters the brain.
- Myelosuppressive toxicity limits TMZ dose escalation.
- Intrinsic or acquired resistance to TMZ is common, due to overexpression of MGMT.
- Resistance leads to initial treatment inefficacy and tumor relapse.
- Unmethylated MGMT biomarker patients do not respond to TMZ.

Design Strategy for Novel Temozolomide Analogs (VMY-TP)

One of the main causes of tumor recurrence is a small sub-population of TMZ-resistant glioma stem cells (GSC), with capacity for self-renewal and *in vivo* tumor initiation. Once the tumor recurs, there are few treatment options available to patients. Thus, chemotherapeutic agents with greater efficacy and brain permeability than TMZ are badly needed. Considering the low efficacy of TMZ against tumors expressing MGMT, and the inevitable recurrence of GBM after multimodal combination therapy (TMZ+ Radiation), much effort has been directed towards creating novel TMZ analogs through modifications at the C-8 and C-3 positions. While some reported TMZ analogs have shown improved brain permeability with greater preclinical efficacy, none of have progressed to testing in humans. We hypothesize that there is room to improve the brain permeability and anticancer profiles of TMZ, by incorporating yet unexplored functional groups to create new analogs (**Figure 2**). These would increase the drug's brain permeability and lower systemic toxicity because the dose-limiting toxicity of TMZ (myelosuppression) is not CNS-related.

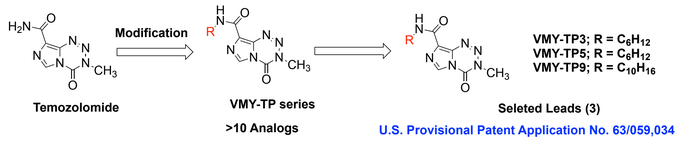

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Figure 2: Novel VMY-TP series identified through HTS campaign

Comparison of Physicochemical Properties with Guidelines for CNS Permeability

Test Agents	Mw	Log P	TPSA (A^2)	cLOGBB	MPO_{max}
TMZ	194.15	-0.84	108	-1.52	3
VMY-TP3	278.32	1.20	94	-0.86	4
VMY-TP5	276.30	0.80	94	-0.95	4
VMY-TP9	328.38	1.54	90	-0.86	5

Table 1: CNS MPO, with 0-6 with 6 being most desirable. MPO of 4-6 is consistent with passive CNS permeability; LogBB = 0.152 ClogP - 0.0148 PSA + 0.139; MPO : Multiparameter Optimization

Assessment of *in vitro* Cell Proliferation Efficacy of TMZ, VMY-TP Analogs in Glioma Cultures

Cell Lines	MGMT	IC_{50} (umol/L), 72 h			
		TMZ	VMY-TP3	VMY-TP5	VMY-TP9
U251	-	898	397	139	326
U87	-	208	399	168	99
GBM8	-	45	2	28	16
T98G	++	1145	1257	581	408

Table 2 : Cells were seeded in 96-well plates. The day after, cells were treated with various concentrations of Temozolomide or VMY-TP analogs. Cell viability was measured by Alamar Blue after 72 hours, considering the control level of cells to be 100%. Performed in triplicate, P<0.005

Assessment of Cell Proliferation Measured by BrdU

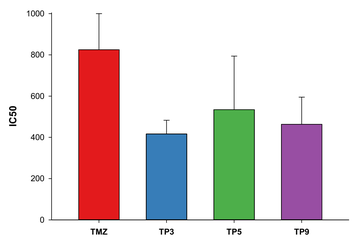
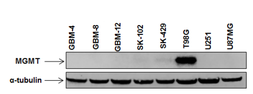


Figure 3: U87 cells were seeded at the density of 2,000 cells/well in 96-well plates and incubated overnight. The day after, cells were treated with various concentrations of Temozolomide or VMY-TP analogs for 72 h. The proliferation rate was evaluated by measuring the relative incorporation of BrdU. Values are the means ± SEM of experiments performed in triplicate. *p<0.005.

Activity of TMZ, VMY-TP analogs in MGMT positive GBM Cell lines

A.



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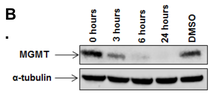


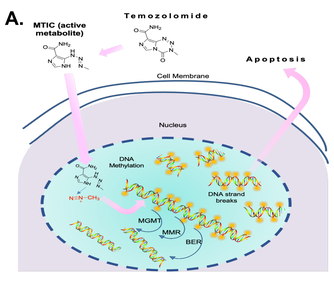
C.

Test Agents	Cell Tier Glo Assay (IC_{50}, uM), 72 h	
	Without O6-BG pretreatment	With O6-BG Pretreatment
Temozolomide	>1000	>1000
VMY-TP3	>1000	675
VMY-TP5	410	168
VMY-TP9	512	148
VAL-083	108	71

Figure 4: A. GBM-4, GBM-8, GBM-12, SK-102, SK-429, T98G, U251, and U87MG cell lysates were subjected to Western blot analyses. The blots were probed with anti-MGMT and re-probed with anti-alpha-tubulin. **B**. T98G cells were treated with O6-Benzylguanine at 100 µM for 0, 3, 6, and 24 hours or with DMSO. The T98G cell lysates were subjected to Western blot analyses. The blots were probed with anti-MGMT and re-probed with anti-alpha-tubulin. **C.** TMZ and its analogs were added to T98G cells, with or without a 5-6 h pretreatment of O^6-Benzylguanine at 100 µM, or with DMSO. IC50 values after 3 days incubation were reported above.

Measuring Expression of 53BP1 foci and gH2AX (a marker for DNA damage) in Glioma Cells treated with TMZ and its VMY-TP analogs

A.



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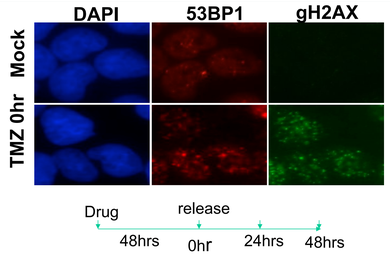

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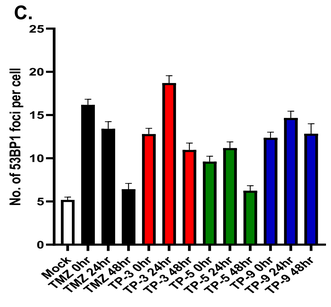

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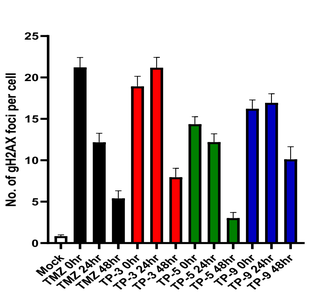
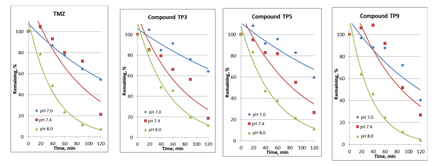


Figure 5. U251 cells were pulsed with 10 µmol/L TMZ or analog for 48 hours, fixed at the indicated time points post-treatment, immunofluorescence-stained for 53BP1 foci (red, Figure 5C), gH2AX (green, Figure 5D), and imaged at 40X magnification (**Figure 5B**). . Nuclei are stained with DAPI (blue). Average number of foci per nucleus (*y*-axis) is plotted against the corresponding time posttreatment (*x*-axis).

Hydrolytic Chemical Stability of TMZ, VMY-TP Analogs



	$T_{1/2}$ (min) (0-2 h) , 1 uM		
	pH 7.0	pH 7.4	pH 8.0
TMZ	119	59	31
TP3	119	62	39
TP5	>120	69	40
TP9	103	59	29

Table 3: Determining short-term (0-2 hours) stability of the test articles TMZ, TP3, TP5, and TP9 in aqueous media (0.1M phosphate buffer (pH 7.0), PBS (pH 7.4), 0.1M phosphate buffer (pH 8.0) using HPLC-MS. Detection of products of non-enzymatic cleavage of the tested compounds AIC-TMZ, AIC-TP3, AIC-TP5 and AIC-TP9 was performed in this study.

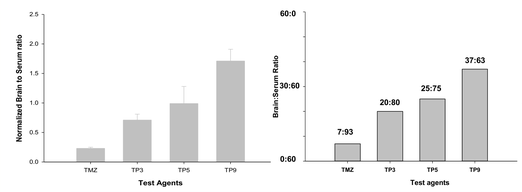
Assessment of Permeability of TMZ, VMY-TP Analogs

	P_{app} (AB) ,10^{-6} cm/s		P_{app} (BA) ,10^{-6} cm/s		BA/AB	BA/AB
	p-gp Inhibitor (-)	p-gp Inhibitor (+)	p-gp Inhibitor (-)	p-gp Inhibitor (+)	p-gp Inhibitor (-)	p-gp Inhibitor (+)
TMZ	20.2	18.1	21.0	17.2	1.0	0.9
TP3	30.7	18.6	17.0	13.2	0.6	0.7
TP5	26.6	25.7	24.3	19.4	0.9	0.8
TP9	28.3	28.0	19.5	16.7	0.7	0.6
Digoxin	1,2	5.9	15.8	6.1	13.6	1.0

Note: Verapamil, used as p-gp Inhibitor

Table 4: The purpose of this study was to evaluate the permeability of compounds **TMZ, TP3, TP5, TP9** in a bidirectional Caco-2 assay, including identification of P-glycoprotein substrate (Pgp-mediated transport) activity. The apparent permeability (Papp) was calculated for Caco-2 permeability assay using the following equation: $P_{app} = \frac{V_A}{Area \times Time} \times \frac{[drug]_{acc}}{[drug]_{initial}}$

In vivo Blood-Brain Barrier Permeability of TMZ, VMY-TP analogs



Study Design:
Method: Cassette, Intravenous, 4 animals/ group, male CD-1®IGS
Dose : 5 mg/kg/each
Formulation: 10% DMSO in PBS (v/v)
Time : 5 min
End point: Quantifying parent compound using LC-MS/MS

Test Agents	Plasma Conc (ng/ml)	Brain Conc (ng/g)	Brain/Plasma	% Brain	% Plasma	Brain: Plasma
TMZ	5537	1287	**0.23**	7	93	**7:93**
TP3	2920	2080	**0.71**	20	80	**20:80**
TP5	3116	3052	**0.99**	25	75	**25:75**
TP9	1830	3122	**1.71**	37	63	**37:63**

Figure 6: Brain samples (weight 100 mg ±1 mg) were immediately mixed with 100 µl of H_3PO_4 (4.25%) and zirconium oxide beads (115 mg ± 5 mg). Then 400 µl of IS solution was added and samples were dispersed in the Bullet Blender® homogenizer for 30 seconds at speed 8. Samples were subsequently centrifuged for 4 min at 14,000 rpm, and 2 µl of each supernatant was injected into an LC-MS/MS system. Plasma samples (50 µl) were immediately mixed with 25 µl of H_3PO_4 (8.5%). 225 µl of IS solution was then added. After mixing by pipetting and centrifuging for 4 min at 6000 rpm, 2 µl of each supernatant was injected into LC-MS/MS system. Meldonium (Md) (400 ng/ml in methanol) was used as the internal standard (**IS**) for quantification of all compounds in plasma and brain samples.

Evaluation of TMZ and TP3 in Flank SCID mice Models of GBM

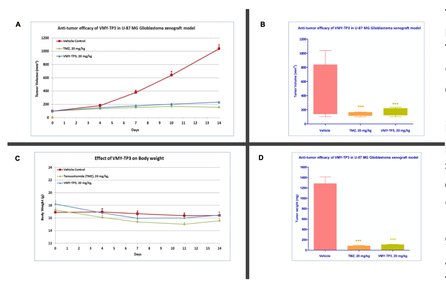


Figure 7. Treatment was started when when the average tumor size reached approximately ~100 mm^3. The test and reference compounds were formulated in 0.4 % (v/v) Tween 80, 2% (v/v) Glycerol and 97.6% (v/v) of 30% (w/v) Captisol (SβECD). The test (VMY-TP3) and reference compound (TMZ) were administered at a dose volume of 10 mL/Kg. The exact dose was administered based on individual animal body weight recorded during the study period. The test (VMY-TP3) and TMZ treatment were scheduled for once daily dosing for 14 days. However, the dose frequency for TP3 and TMZ had to be reduced from Once daily (QD) to alternative days (Q2D) due to a 10-14% loss of body weight seen in the treated mice from Day 9 until study completion. The Control group was administered with the Vehicle alone. Number of mice per treatment cohort = 8. Tumor growth was measured twice weekly using a digital Vernier caliper.

Conclusions

- Successfully synthesized novel TMZ analogs (VMY-TP) with improved CNS physicochemical properties (**Figure 2 and Table 1**)
- Superior cell killing (growth inhibition) compared to TMZ in GBM lines (**Table2 and Figure 3**)
- Alkylation-mediated cell death, similar to Temozolomide and promising anti-cancer effects in both MGMT low and high expressing cell lines (**Figure 4 and Figure 5**)
- pH-dependent hydrolytic chemical stability (**Table 3**)
- Not a p-gp protein channel substrate (**Table 4**)
- High concentrations in Brain compared to Plasma (**Figure 6**)
- Well tolerated therapeutic doses in preclinical settings, no major formulation issues, tumor growth inhibition in flank brain tumor models (**Figure 7 A and 7B & 7D**), and no measurable toxicity issues and body weight changes (**Figure 7C**).

Future Directions

Next steps include validation of our analogs in patient derived cell lines as well as orthotopic animal models, and in TMZ resistant cell models, to better recapitulate the humor tumor environment. We will also be further validating the molecular basis of anti-tumor activity resulting from our TP drugs, ideally leading to more druggable candidates for preclinical IND development.

Acknowledgement

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