# Novel Approach for Treating Neural Crest Derived Tumors: Selective Inhibition of Ganglioside Biosynthesis With Small Molecules

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# Abstract

**The primary goal of this novel research is to selectively inhibit ganglioside biosynthesis with small molecules to treat neural crest derived tumors.**

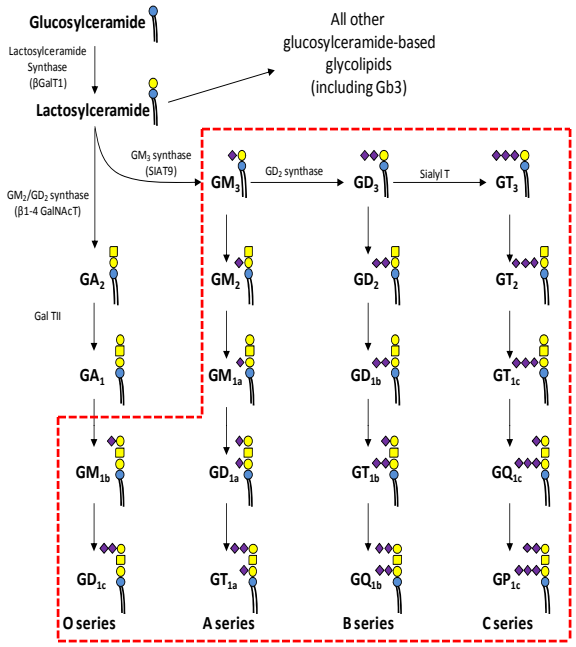
Neural crest derived tumors express high levels of a unique class of lipid linked glycan known as gangliosides. Gangliosides are involved in growth factor signaling by regulating complexes in lipid rafts. Genetic studies show that through aberrant expression of gangliosides, these tumors acquire aggressive growth properties. Prior to this research the only ganglioside inhibitors identified were non specific and broadly blocked virtually all glycolipid classes. These nonspecific glycolipid inhibitors demonstrated anti-cancer activity in animal models of neural crest tumors. However, due to substantial off target dose limiting toxicity from lack of specificity for the ganglioside sub class, they effectively cannot be used in humans for cancer treatment.

Selective inhibitions of gangliosides without affecting other glycan classes could potentially avoid these problems and provide an effective treatment for neural crest and other ganglioside dependent tumors. To identify the first known selective inhibitors of gangliosides, we developed a novel molecular screening strategy for identifying selective small molecule ganglioside inhibitors. This platform identified the first drug like selective inhibitors of gangliosides.

ZP10395, a lead compound, selectively and dose-dependently reduces gangliosides in multiple tumor cell lines and is 10-15 fold more potent than the existing non-specific inhibitors. Importantly, it does not inhibit other glycolipid classes associated with dose-limiting toxicity. Administering ZP10395 to a mouse xenograph melanoma model significantly reduced ganglioside production and slowed tumor growth in the presence of a reduced T-cell response. These results demonstrate the potential of specific ganglioside inhibitors for treating ganglioside dependent tumors.

# Ganglioside Biosynthesis

## Ganglioside Biosynthesis is Catalyzed by a Set of Enzymes in the ER and Golgi



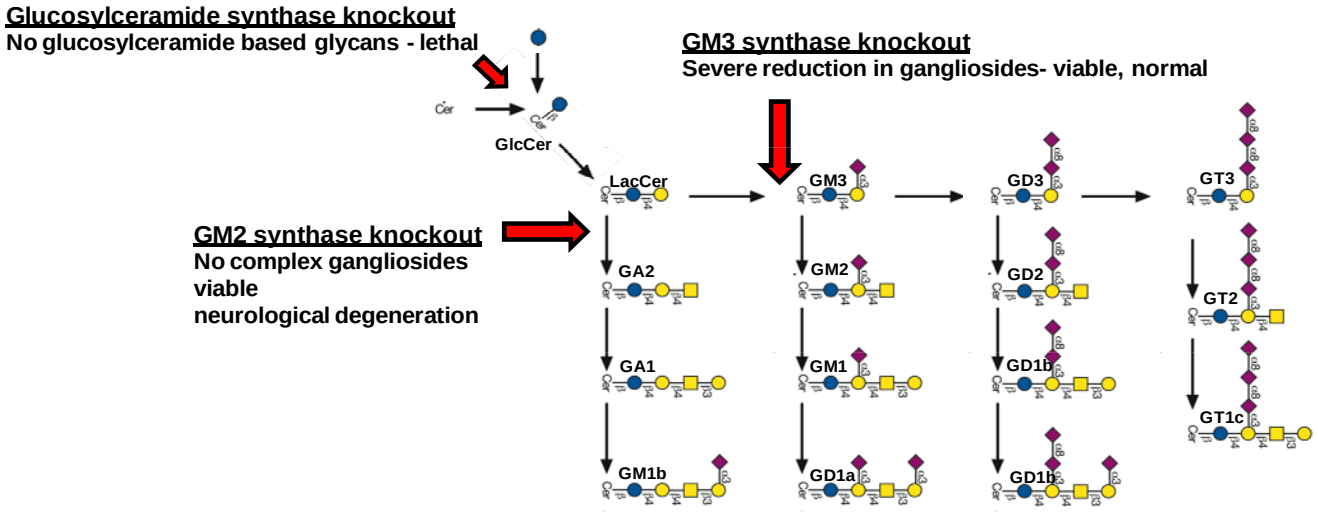

**Gangliosides.** Gangliosides are a subset of lipid linked glycans modified by sialic acid that are present on the surface of all mammalian cells (shown within red dotted line). They are most abundant in the nervous system; however, they are present in most tissues at lower levels.

**Ganglioside Biosynthesis.** Gangliosides are synthesized by a series of glycosyltransferases from a glucosylceramide core. The biosynthesis proceeds down four distinct "series" of gangliosides. The composition of gangliosides produced by a cell is a reflection of the biosynthetic machinery and regulators expressed by the cell.

**Ganglioside Normal Function: Gangliosides Play an Important Role in Cell Signaling.**
Studies have shown that gangliosides modulate the activity of receptor tyrosine kinases. For example, activation of the EGF receptor can be regulated by gangliosides. Similarly, gangliosides affect the NGF receptor in neuritogenesis. The entire ganglioside family also functions in the structural organization of the lipid bilayer. Through co-localization, gangliosides also regulate EGF and FGF signaling by regulating receptor localization. Overall, gangliosides play an important role in cell signaling.

# Are Gangliosides Safe to Inhibit?

**Genetic Data Indicate that Gangliosides are Safe to Inhibit.** A series of mice deficient in key ganglioside biosynthetic enzymes have been produce
These mice revealed that lipid linked glycans based on glucosylceramide are essential for embryonic development; however, gangliosides are not.
shown below, knocking out key ganglioside biosynthetic enzymes is tolerated.




**Conclusions: High levels of tolerance for inhibitions of gangliosides. Functional redundancy between specific gangliosides.**

The goal of this program is to develop inhibitors that reduce the synthesis of gangliosides. Based on the knockout mice, we expect these to be well tolerated in vivo.

# Ganglioside Over Expression Correlates with Tumor Progression

**Human Clinical Studies.**

The progression of some human tumor types can be correlated with tumor ganglioside expression. **Below is a Kaplan-Meier analysis of tumor progression in Neuroblastoma patients based on ganglioside levels at diagnosis.**

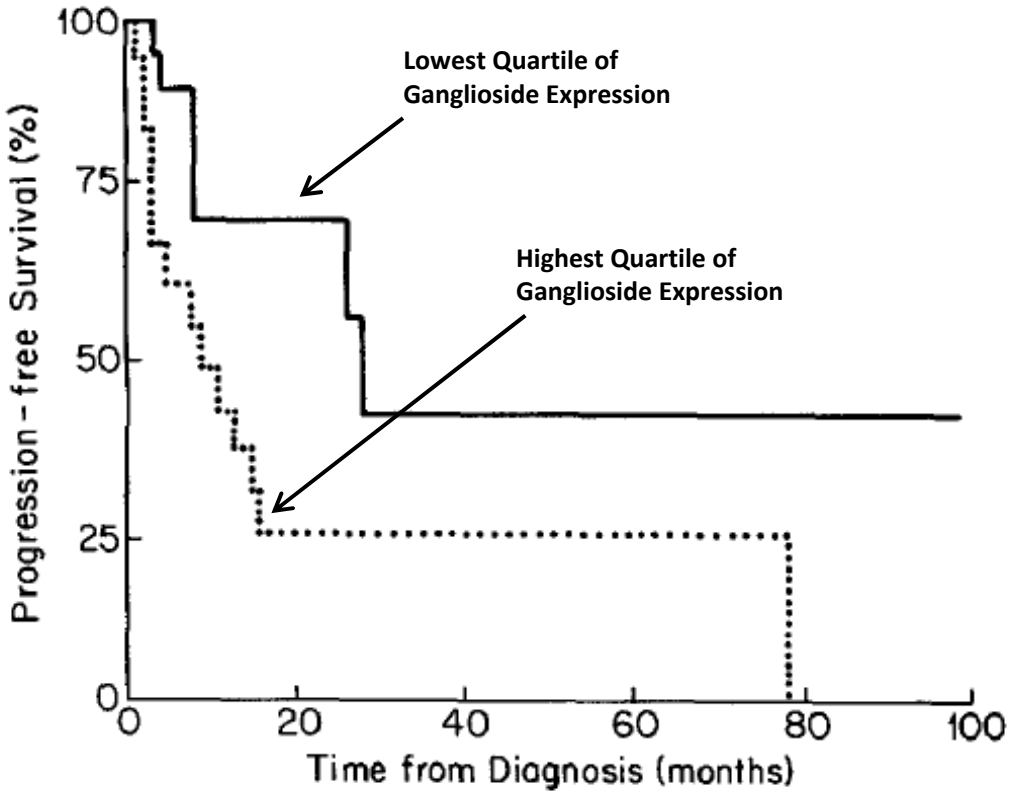



**Fig 3. Kaplan-Meier analysis of PFS of neuroblastoma patients. The survival of the quartiles of patients with the lowest (—) and highest (· · ·) $G_{D2}$ levels at diagnosis are compared.**

**Figure from:**
Valentino et al.
**Shed tumor gangliosides and progression of human neuroblastoma,**
Blood, 1990 75: 1564-1567

# Ganglioside Inhibition Blocks Tumor Growth

**Published In Vivo Studies Support Gangliosides as a Modulator of Tumor Growth.** The table below summarizes published genetic and pharmacological studies (with known glucosylceramide synthase inhibitors) that demonstrate that gangliosides play a key role in the growth of neural crest as well as other tumor types.

| Model | Approach | Experimental System, Treatment | Ref. |
|---|---|---|---|
| **Neuroblastoma** | Genetic | F11 rat neuroblastoma, GD3 antisense | [1, 2] |
| **Astrocytoma** | Pharmacological | CT-2A mouse astrocytoma, 2,400 mg/kg ND-DNJ | [3] |
| | Genetic | Mouse CT-2A astrocytome, GM2 synthase antisense | [4] |
| **Melanoma** | Genetic | Human melanoma (SK-Mel-28), GM3 deficient | [5] |
| | Pharmacological | B16F10 subclone, glucosyceramide synthase inhibitor | [6] |
| | Genetic | B16F10 subclones, high and low ganglioside expression | [6] |
| | Genetic | B16F10 subclone, glucosylceramide synthase antisense | [7] |
| | Pharmacological | B16F10 subclone, glucosylceramide synthase inhibitor | [8] |
| | Genetic | Human melanoma (SK-Mel-28), transfection | [9] |
| **Ependymoblast** | Pharmacological | EPEN mouse ependymoblastoma, 2,400 mg/kg ND-DNJ | [3] |
| **Ovarian Cancer** | Genetic | Human A2780, GM3 synthase transfection | [10] |
| **Fibroblast** | Genetic | Transformed fibroblasts, ganglioside KO mice | [11] |
| **Breast Cancer** | Genetic | MDA-MB-231, GM3 synthase transfection | [12, 13] |
| | Genetic | 4T1 and 67NR, siRNA and transfection | [14] |
| **Lung Cancer** | Genetic | SK-LS-17, GD3 synthase transfection | [15] |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# Ganglioside Inhibition Blocks Tumor Growth, cont.

**Known Glycolipid Inhibitors Block Tumor Formation in the B16F10 (MEB4) Mouse Melanoma Model.** Compound 2378 is an amino-sugar based inhibitor of the glucosylceramide synthase which is an undesirable target to inhibit (due to the global inhibition of most glycolipids); however, it illustrates the potential for a selective ganglioside inhibitor. In these published studies, compound 2378 was dosed at 2.5 grams/kg/day.

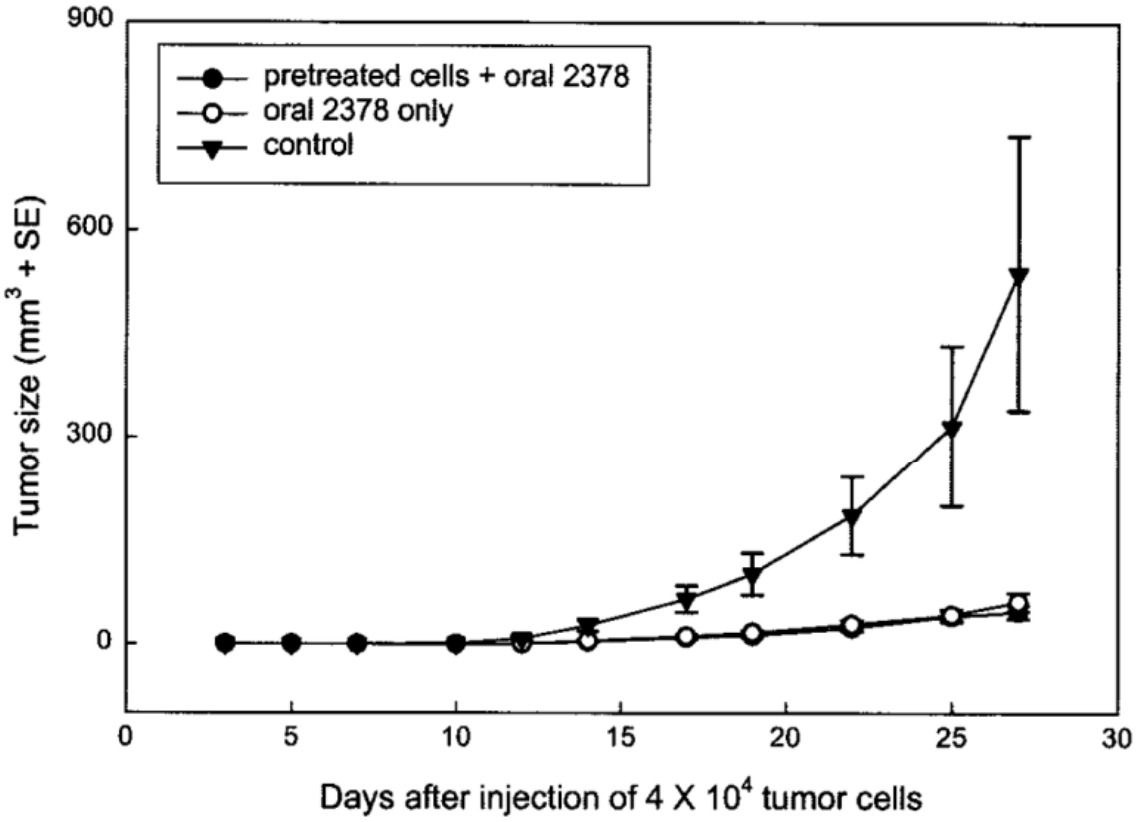
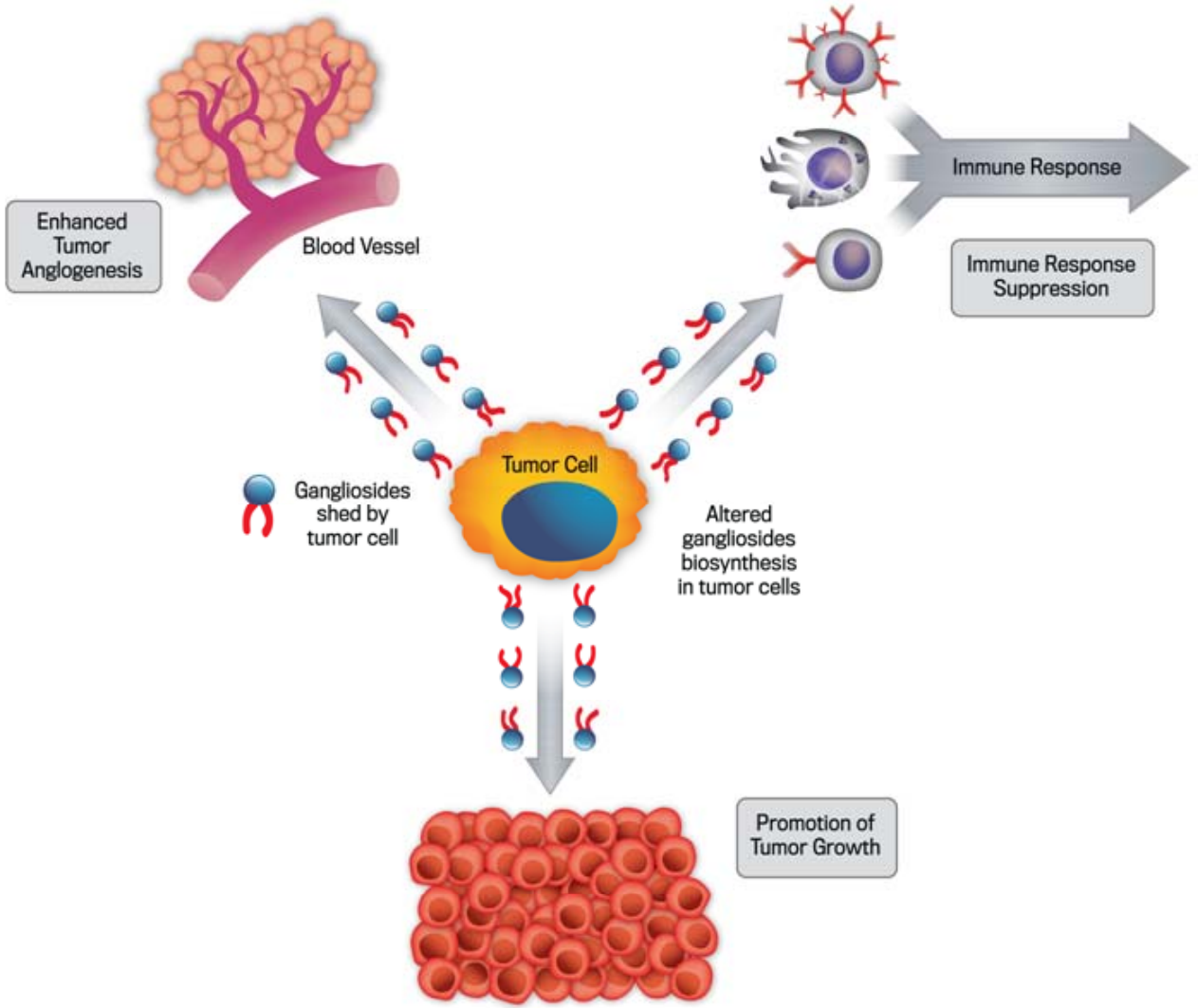



Fig. 3. Effect of p.o. administration of OGT2378 on MEB4 melanoma tumor growth. The mean ± SE tumor volumes are shown.

**Figure from:** Weiss et al.. **Inhibition of Melanoma Tumor Growth by a Novel Inhibitor of Glucosylceramide Synthase,** CANCER RESEARCH 63, 3654–3658, July 1, 2003.

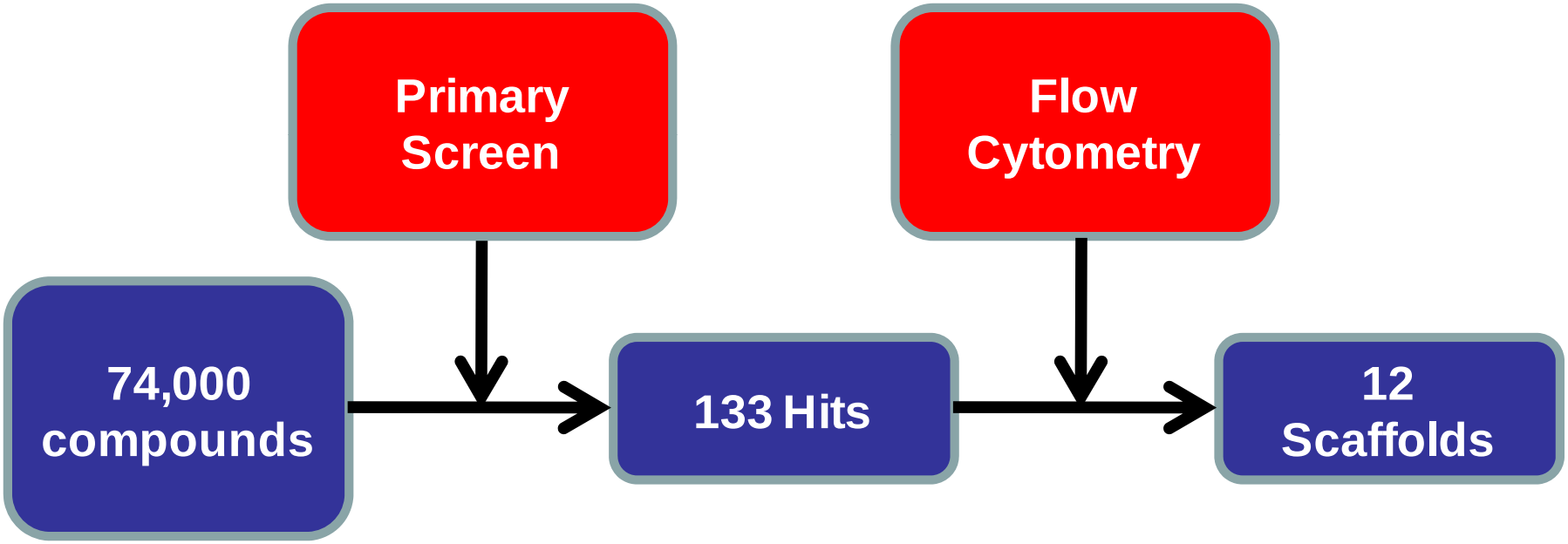
# Mechanisms for Efficacy of Ganglioside Inhibitors

**Critical Roles Played by Gangliosides in the Pathogenesis of Cancer.**
Human clinical data and in vivo mouse models indicate that Gangliosides play critical roles in the growth of many tumor types. The potential mechanisms that gangliosides use to modulate tumor growth are described in the figure below.



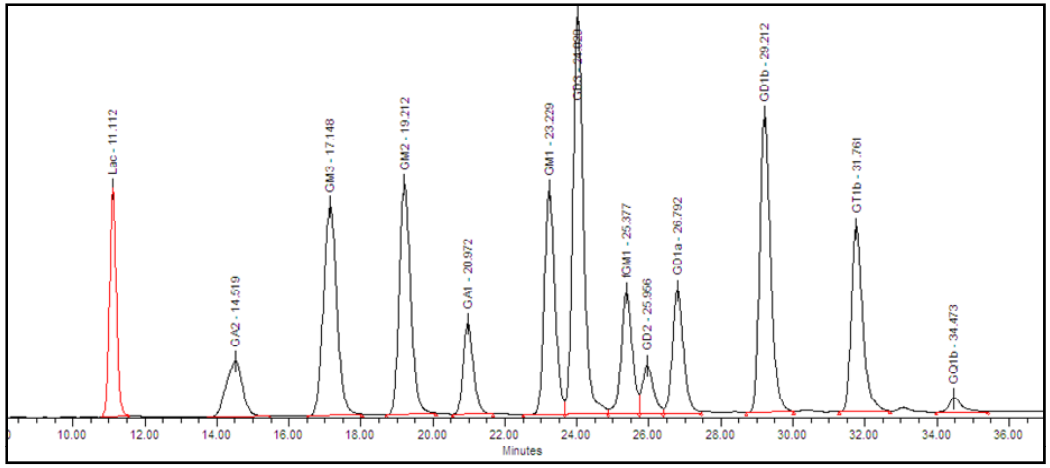
# Ganglioside Inhibitor Discovery

**High Throughput Screening.** We have developed a proprietary cell-based assay capable of screening for compounds that interfere with ganglioside biosynthesis using ganglioside binding lectins. Using this assay, we screened over 74,000 drug-like compounds and identified 133 hit compounds. These hit compounds reduce binding of ganglioside dependent lectins without affecting lectins that bind to unrelated glycans (such as heparan sulfate). 12 compounds were identified that dose-dependently reduced CTB binding by flow cytometry.



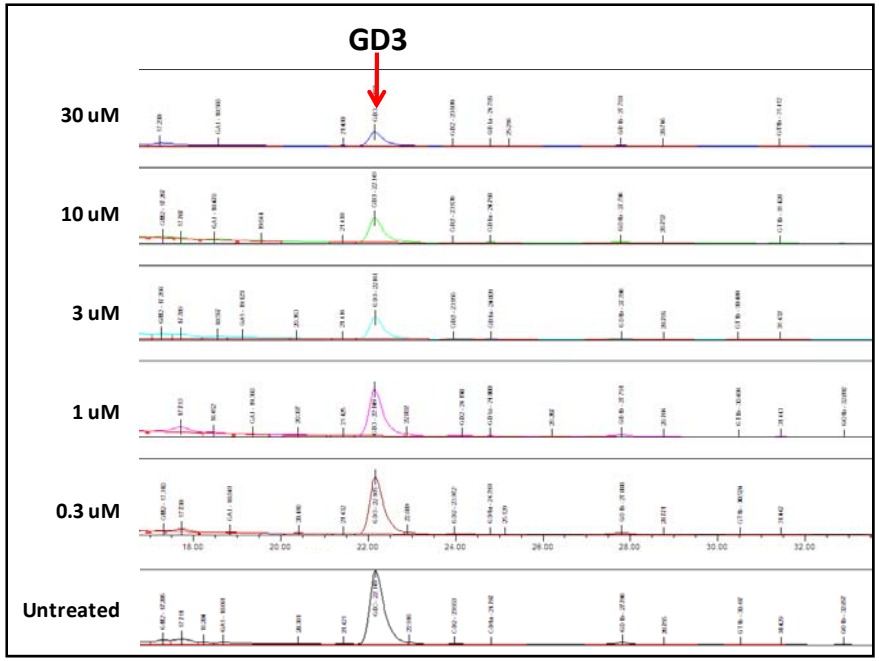
# In Vitro Ganglioside Inhibition

**Bovine Brain Gangliosides – HPLC analysis**




**Quantitation of Ganglioside Synthesis in Vitro.**
In order to quantify the effect of the test compounds on ganglioside biosynthesis, we developed a 96-well plate based UPLC assay to characterize ganglioside content from cellular samples (based in the method of Wing, et al. Analytical Biochemistry 298, 207-217, 2001). As shown below, this method is able to quantify the gangliosides typically found in cultured tumor cell lines.

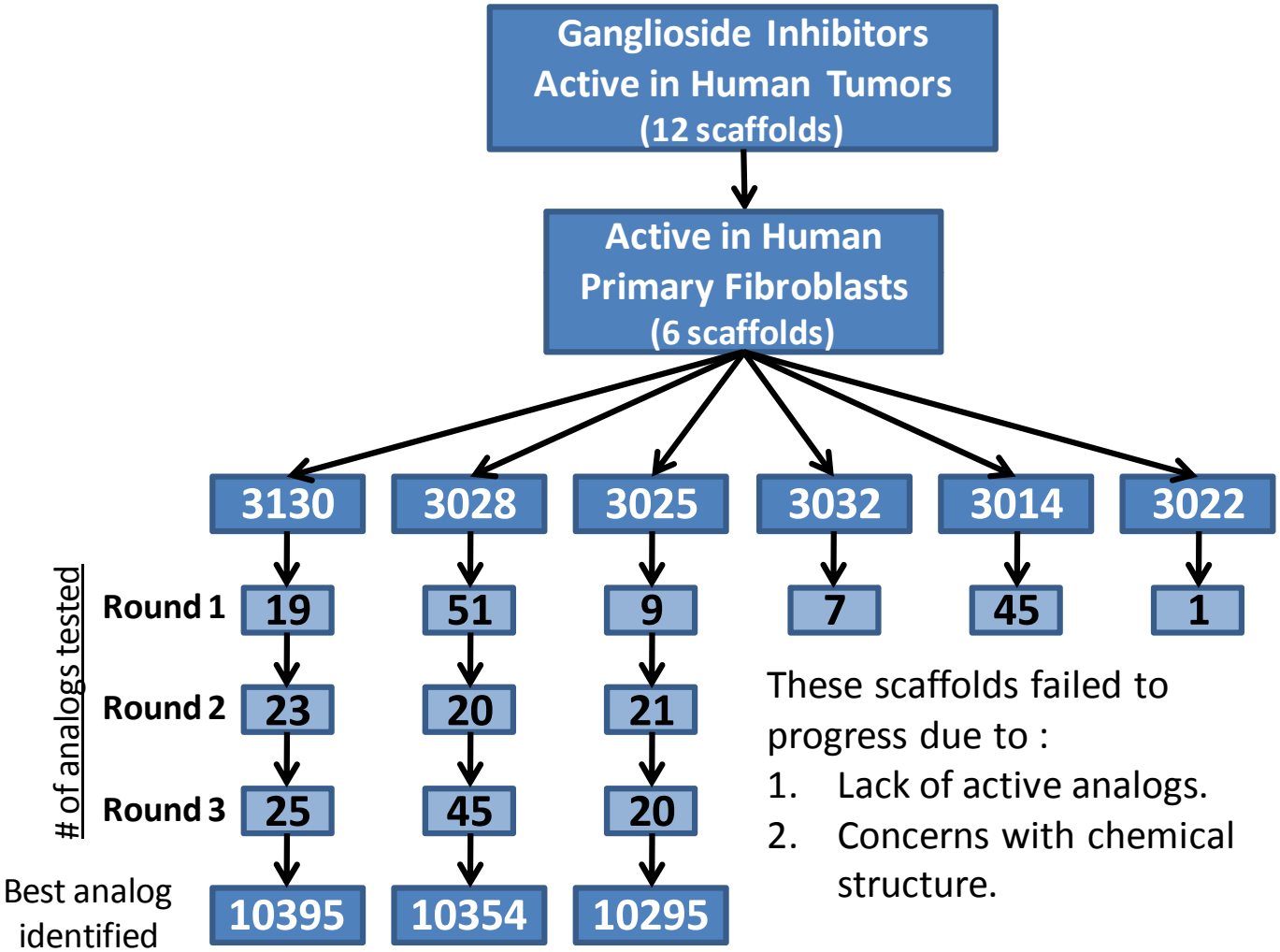
**SKMEL28 - PDMP Dose Response**




**Assay Validation Using Known Glucosylceramide Synthase Inhibitors.**
In order to demonstrate the ability of the cellular assay to detect ganglioside inhibition, we used a known glucosylceramide synthase inhibitor (PDMP). As shown below, this assay is able to detect the dose-dependent reduction of gangliosides in SKMEL-28 cells.

# Hit Expansion

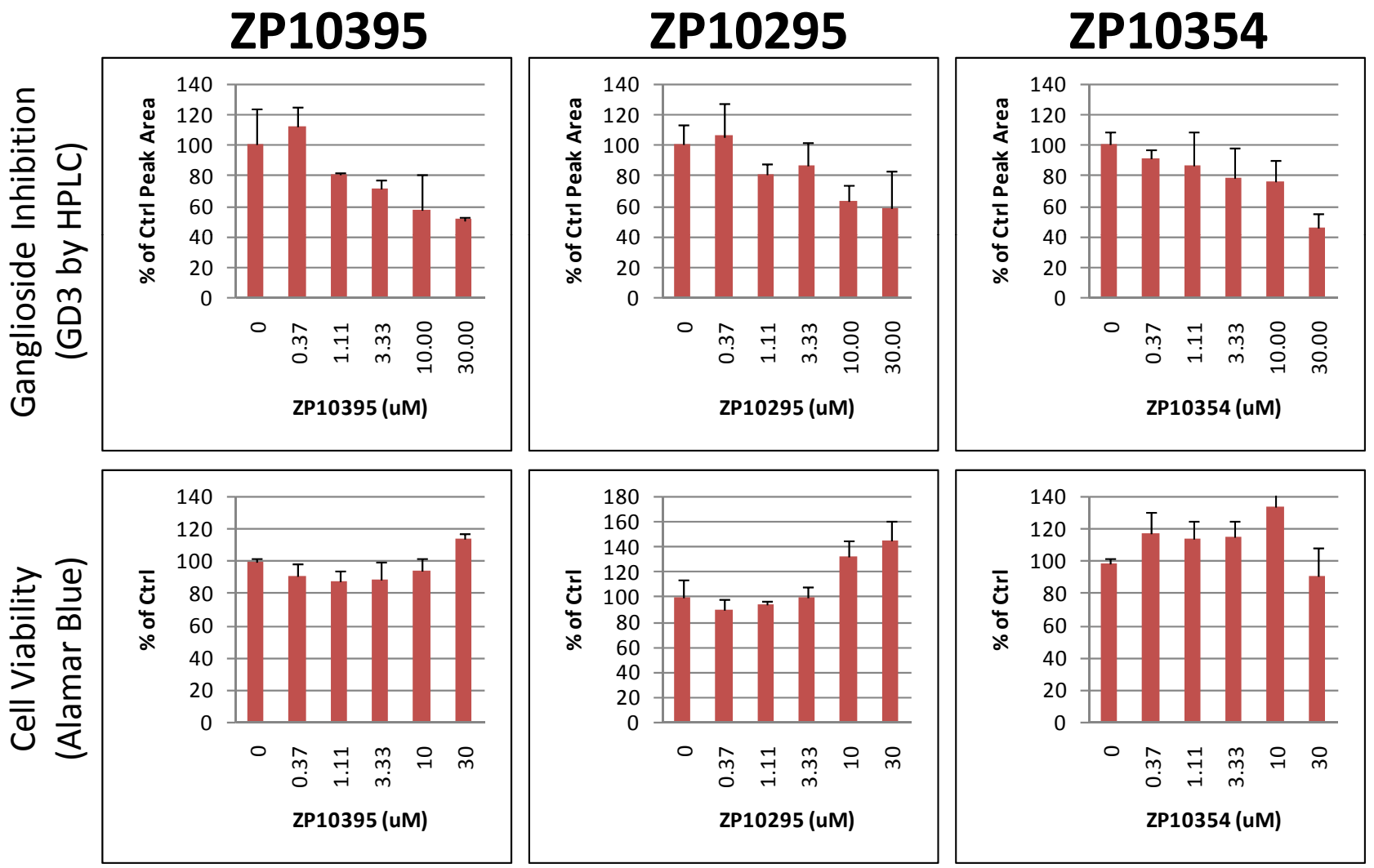
**Scaffold Selection.** Based on potency, activity in primary human fibroblasts, and structural features, we selected six scaffolds for further development. We tested a series of analogs of each scaffold and identified three scaffolds (ZP3130, ZP3028, and ZP3025) which demonstrated structure-activity relationship and features amenable for further lead optimization. This effort led to the identification of ZP10395, ZP10354, an ZP10295 as the best analogs of these three scaffolds.



# Hit Expansion, cont.

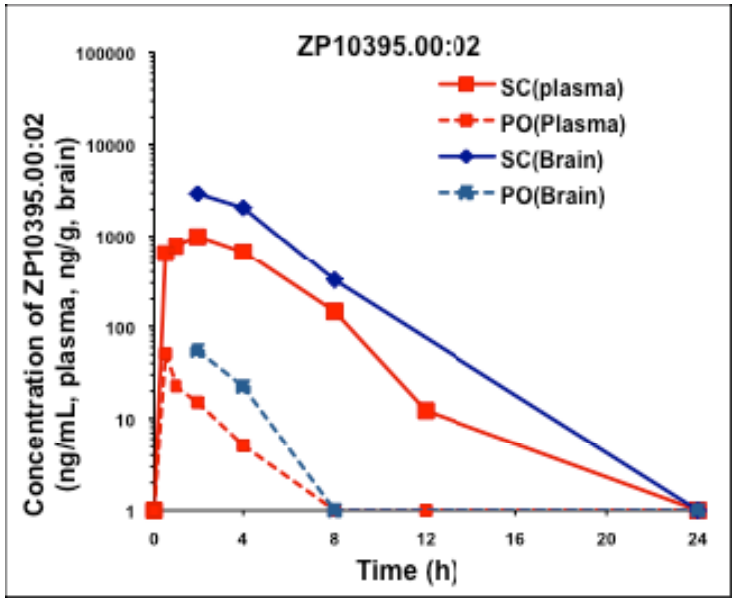
The data for three of these scaffolds in SKMEL28 (a human tumor line that over-expresses GD3) is shown below (top panels). The inhibition of ganglioside synthesis was accomplished without general toxicity as determined by Alamar blue oxidation (bottom panels).

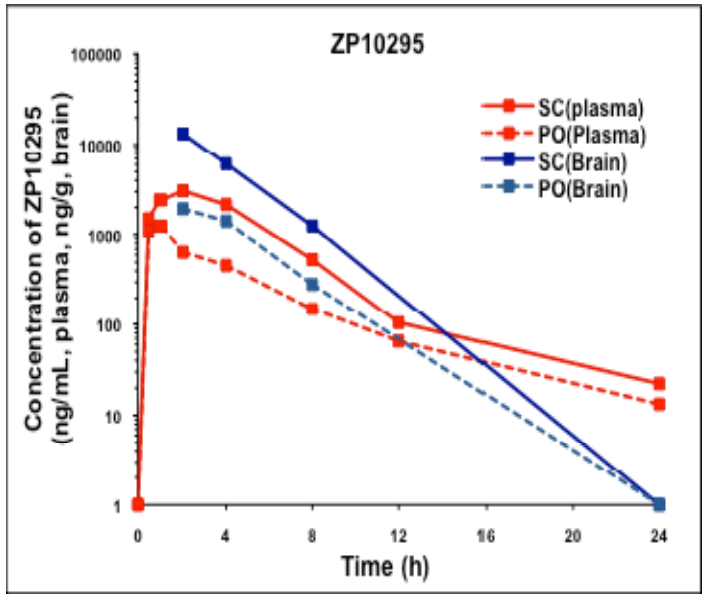
**Inhibition of Ganglioside Biosynthesis in Cultured Cells**

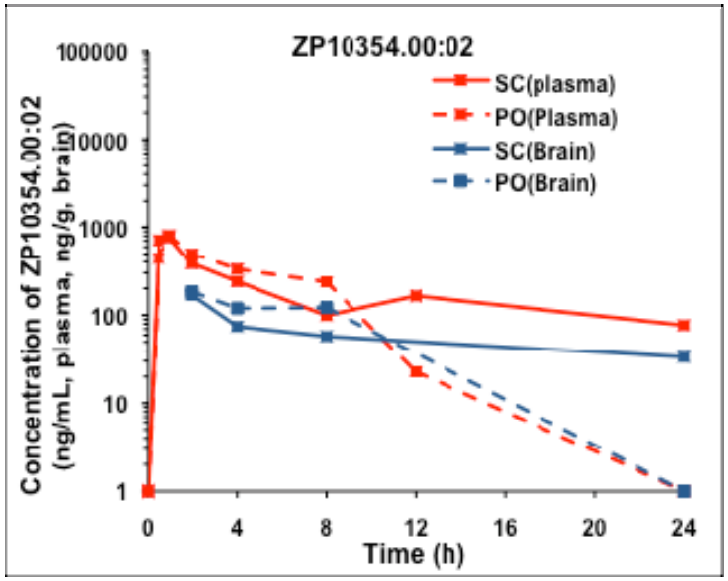


# Mouse PK Analysis

**The Ganglioside Inhibitors are CNS Penetrant and Have Acceptable PK for In Vivo Testing**



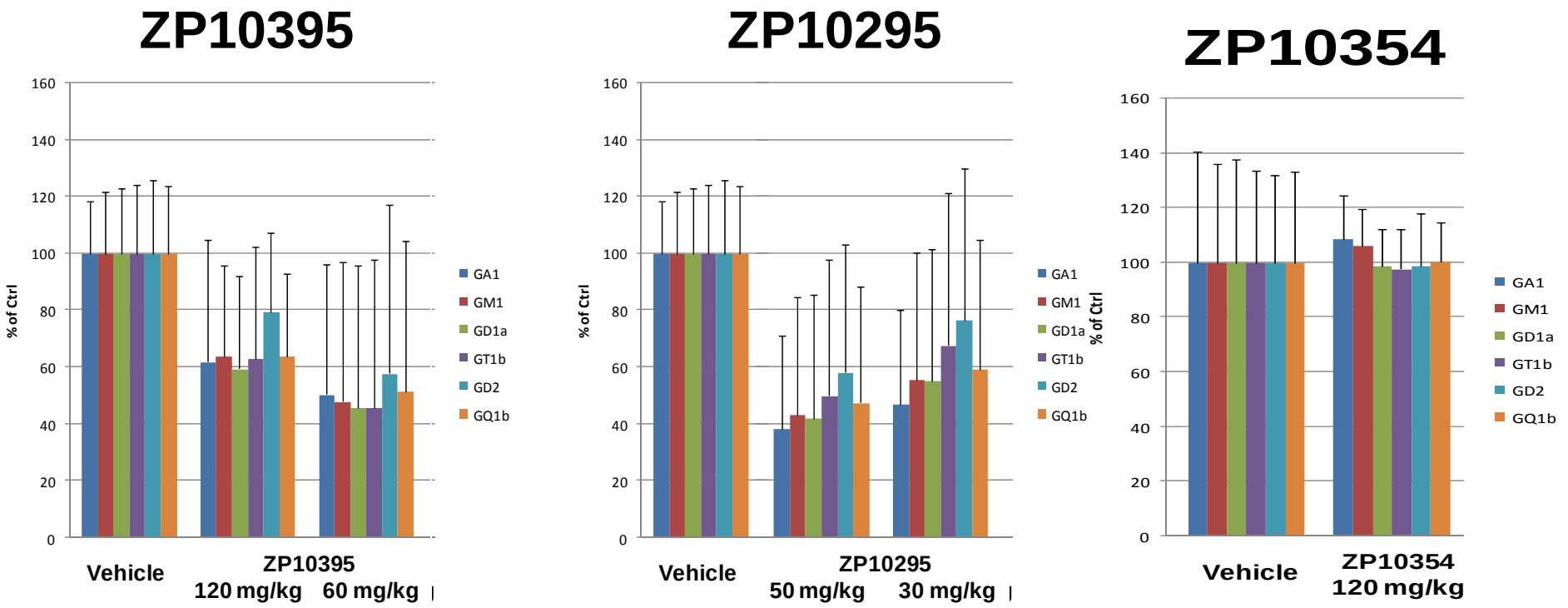




**All Three Ganglioside Inhibitor Scaffolds Penetrate the CNS and Have Acceptable PK Properties for In Vivo Testing.**

The active ganglioside inhibitors were evaluated in mouse PK studies using subcutaneous and oral administration at 30 mg/kg. ZP10395 and ZP10295 scaffolds have excellent BBB penetration and reached blood and CNS levels at or close to the level required to inhibit ganglioside biosynthesis (based on in vitro assays).

# In Vivo Inhibition of Ganglioside Biosynthesis

**Two of the Top Three Scaffolds are Effective In Vivo**

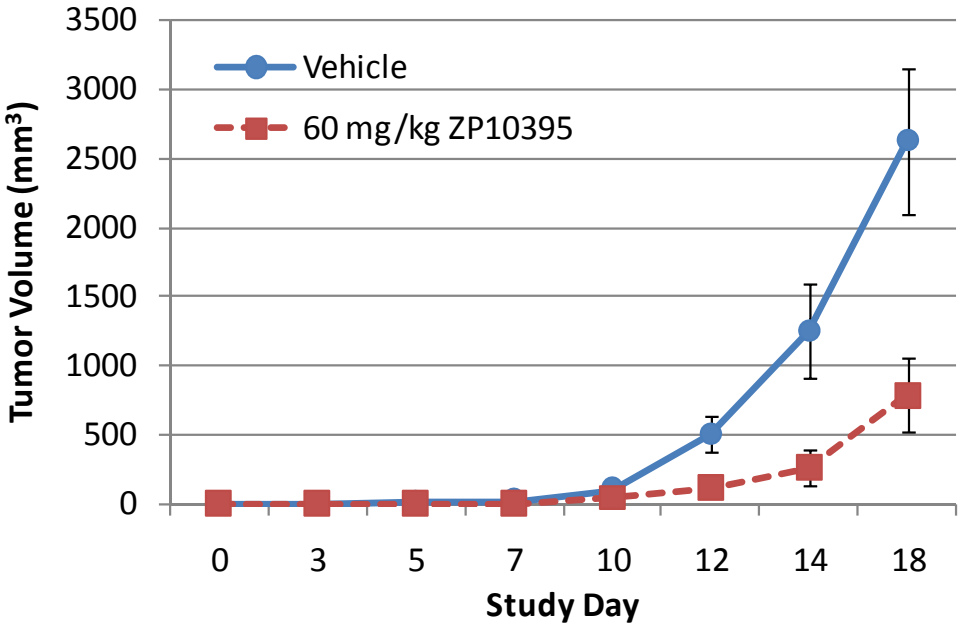


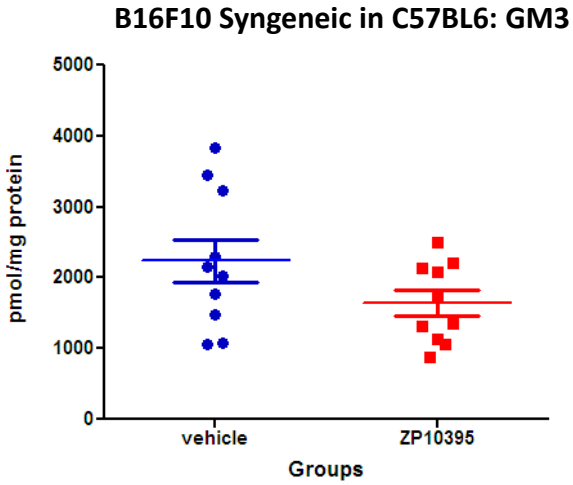

**In Vivo Inhibition of Ganglioside Biosynthesis in the Brain**

Based on the PK and BBB penetration of the lead scaffolds, an in vivo efficacy study was run in wild type C57Bl6 mice. The mice were dosed at the indicated doses for 40 days and brain gangliosides were quantified by HPLC. ZP10395 and ZP10295 had a significant effect on the ganglioside content in the brain. At this dose, ZP10354 was not effective.

# In Vivo Melanoma Efficacy Model

**ZP10395 Inhibits Ganglioside Biosynthesis and Slows Tumor Growth In Vivo**




ZP10395 was used as the candidate scaffold for in vivo testing in the B16F10 mouse melanoma model. In this study, groups of 15 C57Bl6 mice were treated with 60 mg ZP10395/kg or vehicle SC BID . B16F10 cells were injected into the flank of the mice and tumor growth was monitored. Tumor associated gangliosides were extracted at the end of the study which indicated ~30% reduction of ganglioside biosynthesis.

# Conclusion

## The Ganglioside Inhibition is a Novel Therapeutic Strategy for the Treatment of Cancer

Gangliosides are required by many tumors for rapid growth. Despite the strong evidence validating gangliosides as an anti-cancer target, no clinically viable ganglioside inhibitors exist.

Zacharon's program is the first to demonstrate reduced tumor growth in mouse models with their selective ganglioside biosynthesis inhibitors. **The small molecules inhibit the biosynthesis of the ganglioside subset of glycolipids. The novel ganglioside inhibitors characterized by Zacharon are currently being optimized to identify a clinical candidate for human trials.** The Ganglioside Inhibitor program also will enable a new clinical biomarker, the quantification of ganglioside levels. This biomarker may prove useful in the clinical management of patients with neural-crest tumors.