Inhibition of Melanoma Tumor Growth by a Novel Inhibitor of Glucosylceramide Synthase

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ABSTRACT

Tumor ganglioside metabolism has been implicated in modulating tumor formation and progression. We found previously that transient ganglioside depletion by inhibition of glucosylceramide synthase of MEB4 melanoma cells in vitro reduced their tumorigenic capability. Here, we have established that treatment of the host with a novel p.o. inhibitor of glucosylceramide synthase, the imino sugar OGT2378, inhibits MEB4 melanoma tumor growth in a syngeneic, orthotopic murine model. The effectiveness of p.o. OGT2378 in culture were reduced by 93 and >95%, respectively, without either cytotoxic or antiproliferative effects. Administered in the diet to C57BL/6 mice, 2500 mg/kg/day OGT2378 was well tolerated in vivo and biologically active, depleting host tissue (hepatic) gangliosides by 82% and tumor gangliosides by >98%. p.o. treatment with OGT2378 starting 3 days before intradermal tumor inoculation of 4 × 106 MEB4 cells, and continuing for 4 weeks, resulted in a 10-fold lower mean tumor volume at the end of treatment (60 versus 538 mm3, P < 0.0001). Even when OGT2378 treatment was initiated 7 days after tumor inoculation, tumor growth was similarly impeded (61 versus 620 mm3, P < 0.0001), demonstrating an effect on an established tumor. The effectiveness of p.o. OGT2378 in this murine model suggests that inhibition of glycosphinoglycolipid synthesis is a promising and now feasible novel therapeutic approach to inhibit tumor progression.

INTRODUCTION

Multiple factors influence the formation, progression, and metas- tasis of tumors in vivo. It is increasingly recognized that tumor-derived molecules may play an important role in enhancing tumor formation either by acting directly on the tumor cell or on the host. In these ways, tumor-derived factors may alter the tumor microenvironment in such a manner as to enhance tumor cell survival or modify the host responses to the tumor. One class of tumor-derived molecules that has received recent attention is the tumor-derived gangliosides (1). These sialic acid-containing glycosphinoglycolipids, which are components of the outer leaflet of the cell membrane, are actively shed by tumor cells (2–5) and taken up by the host cells in the tumor microenvironment (6). The biological properties and actions of tumor gangliosides have now been well documented. These include potent immunosuppressive activity (2, 7–9), proangiogenic properties (10–12), and, more recently, enhancement of growth factor-mediated fibroblast and vascular endothelial cell proliferation (13–15). Increased expression of gangliosides has been associated with enhanced tumor formation (16), and a correlation has been established between the expression and/or shedding of certain gangliosides and accelerated tumor progression in human tumors, such as neuroblastoma (17).

These combined findings implicating tumor gangliosides as having important roles in tumor formation and progression have led to our hypothesis that reduced ganglioside synthesis and shedding could result in decreased tumor incidence, impeded tumor progression, and/or decreased metastasis. This was documented in vivo in an experimental system in an indirect manner, by demonstrating that the addition of purified tumor gangliosides enhanced the tumorigenicity of a poorly tumorigenic, ganglioside-deficient murine lymphoma cell line (18). Adding further support to this concept, pharmacologically induced cellular ganglioside deficiency resulted in a reduction of melanoma (19) and brain tumor (20) formation in mice. Most recently, we have demonstrated that transfection of an antisense sequence to glucosylceramide synthase, the enzyme catalyzing the initial step in the synthesis of all glucosylceramide-based glycosphingo- lipids, inhibits tumor formation in syngeneic murine orthotopic MEB4 melanoma (21). These data demonstrate high ganglioside expression in tumors, a biological role of gangliosides, and the possibility of inhibition of expression by certain strategies. This suggests that a practical strategy for pharmacologic induction of cellular ganglioside deficiency could be a promising and novel therapeutic approach to human cancer.

Here, we have systematically evaluated this possibility in the above orthotopic model of murine melanoma (19). We studied a novel inhibitor of glucosylceramide synthase that can be administered systematically, the imino sugar OGT2378, a molecule related to NB-DNJ (OGT918), which has been shown to be effective in the treatment of type 1 Gaucher’s disease (22). We demonstrated both a potent inhibitory effect on glycosphinoglycolipid synthesis in the MEB4 melanoma cell line, without antiproliferative or cytotoxic effects in vitro, and marked inhibition of melanoma tumor growth in vivo after p.o. administration of OGT2378.

MATERIALS AND METHODS

Glucosylceramide Synthase Inhibitor. The imino sugar OGT2378 (Fig. 1), a novel inhibitor of glucosylceramide synthase, was obtained from Oxford Glycosciences (Abingdon, United Kingdom). OGT2378 is conformationally distinct from the imino sugar NB-DNJ, which was shown previously to inhibit glucosylceramide synthase (23, 24). OGT2378, as a salt, was stored as a dry powder. It was dissolved in basal medium for in vitro treatment of melanoma cells and mixed with powdered chow for p.o. administration, as a dietary supplement.

Cell Culture. MEB4 cells (25), a subline of B16 murine melanoma cells, were obtained from the RIKEN Cell Bank (Saitama, Japan). The cells were maintained in DMEM enriched in glucose, glutamine, and sodium pyruvate (Life Technologies, Inc.) and supplemented with 10% heat-inactivated fetal bovine serum. Cultures were maintained at 37°C in humidified air containing 5% CO2.

Cell Treatment. MEB4 melanoma cells were exposed to 20 μM OGT2378 for 4–5 days. At the end of the incubation period, the cells were harvested and analyzed for glycosphinoglycolipid metabolism, cell proliferation kinetics, and cell morphology.

Glycosphinoglycolipid Quantification. To assess the effect of OGT2378 on the synthesis of glycosphingolipids, cells were cultured to subconfluence and then labeled with 1 μCi/ml [3H]-serine for 18 h, harvested, and lyophilized (26). Unlabeled GM1 ganglioside (10 nmol) was added to the cells as a cold carrier to inhibit ganglioside synthesis and shedding could

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2 To whom requests for reprints should be addressed, at the Center for Cancer Research, Children’s Research Institute, 111 Michigan Avenue, NW, Washington, DC 20010.

3 The abbreviations used are: NB-DNJ, N-butyldexoynorjirimycin; PPPP, (D,L)-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol HCl; LBSA, lipid-bound sialic acid; HPTLC, high-performance thin layer chromatography.

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before extraction twice with 5 ml of chloroform:methanol (1:1). The combined extracts were dried under N₂, lyophilized, and partitioned twice with 2 ml of diisopropyl ether:1-butanol (60:40, by volume) and 1 ml of 0.1% aqueous NaCl (27). The upper organic phases, which contain most of the neutral glycosphingolipids, were combined and dried down under N₂, lyophilized, and redissolved in chloroform:methanol (1:1) before aliquoting for β-scintillation counting and HPTLC analysis.

Equivalent amounts of radiolabeled lipid extracts and unlabeled ceramide or glucosylceramide standards were analyzed by HPTLC. After resolution in either chloroform:acetic acid (9:1) for ceramide or chloroform:methanol:ammonium hydroxide (65:25:5) for glucosylceramide analysis, the HPTLC plate was split to separate the unlabeled standards, which were visualized by iodine vapor or charring (28). The portion of the plate containing the radioactive glucosylceramide was sprayed with En-hance (NEN Life Sciences, Boston, MA) and analyzed by HPTLC autoradiography by exposure of the plate to XRP X-ray film (Eastman Kodak, Rochester, NY). Densitometric scanning analysis of the samples was performed using a Scan Maker 5 scanner (Microtek) and Scion Image Analysis (NIH Image 160) software. In the case of ceramide, after development of the HPTLC and identification of the ceramide standard as above, the area corresponding to the ceramide band was scraped from the plate and eluted, and the radioactivity was quantified by scintillation counting (29). Total cellular protein was quantified by a modification of the Lowry method (30).

Gangliosides from cell pellets, hepatic tissue, and tumor tissue were isolated as follows: the gangliosides were recovered from the final lower aqueous phase of the diisopropyl ether:1-butanol partition, as described above but without the addition of cold carrier. This aqueous phase was lyophilized and redissolved in a small volume of distilled water. Salts and low molecular weight impurities were removed from the total ganglioside fraction by Sephadex G-50 gel filtration. Gangliosides, recovered in the void volume, were lyophilized and quantified as nanomoles of lipid-bound sialic acid (LBSA) by the colorimetric resorcinol assay (31). For HPTLC analysis, they were spotted on 10 × 20 cm precoated Silica Gel-60 HPTLC plates, which were then developed in chloroform:methanol:0.25% aq. CaCl₂×2H₂O (60:40:9, by volume). Gangliosides were visualized as purple bands with resorcinol-HCl reagent (31) and quantified by densitometry.

Assessment of Cell Proliferation. To assess the effect of OGT2378 on the proliferative capacity of MEB4 cells, the cells were cultured in 96-well plates with or without OGT2378 for 48 h. [³H]-thymidine (NEN Life Sciences) at a final concentration of 2.5 μCi/ml was added to the wells for 3 h at 37°C. The medium was removed, and the cells were washed twice with cold PBS. The cells were harvested, and the incorporated [³H]-thymidine was quantified in quadruplicate cultures by β-scintillation counting.

Flow Cytometry. To quantitate apoptosis, cells were assessed for expression of annexin V and uptake of 7-aminoactinomycin D (32, 33). Apoptotic cells were identified as annexin V positive, 7-aminoactinomycin D negative. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Tumor Formation. Female syngeneic C57BL/6 mice (Taconic, Germantown, NY), 6–8 weeks old, cared for in accordance with institutional guidelines, were used. Cultured MEB4 cells were washed in PBS and resuspended in 0.9% aq. NaCl. Cell viability was assessed by trypan blue dye exclusion and consistently >95%. Cells (4 × 10⁷) resuspended in 20 μl of PBS were injected intradermally on the back of each shaved mouse after halothane anesthesia. In one experimental group of the first experiment, the MEB4 cells were cultured with 50 μM OGT2378 for 4 days before injection and prepared as above.

OGT2378 was administered p.o., in the powdered chow, at a dose of 2500 mg/kg/day, corresponding to 35–40 mg of OGT2378 per mouse per day. Mice were examined for tumor formation and size three times weekly. Tumor size was measured in three dimensions using calipers, and tumor volume was estimated using the formula a × b × c/2.

Statistical Analysis. Differences in tumor size were evaluated for statistical significance using an ANOVA/repeated measurements model and JMP software, and differences in survival were evaluated for statistical significance using the Log-rank tests.

RESULTS

Inhibition of MEB4 Cell Sphingolipid Synthesis by OGT2378 in Vitro. In initial studies, we evaluated the effect of a 4-day exposure of MEB4 melanoma cells to 20 μM OGT2378 in vitro. As assessed by metabolic radiolabeling, the synthesis of glucosylceramide was reduced by 93% in OGT2378-treated compared with untreated MEB4 cells (Table 1). This inhibition of glucosylceramide synthesis was accompanied by a slight elevation of intracellular ceramide concentration. The striking finding was the almost complete expression inhibition of the major ganglioside of MEB4 melanoma cells, GM₃, which was reduced to <3% of normal concentration of 37 nmol LBSA/10⁶ cells. Inhibition was rapidly reversible, with ganglioside levels recovering within 48 h of removal of the drug (data not shown). These findings of striking reduction of ganglioside content led to additional investigations of the effect of the inhibitor on MEB4 cell proliferation in vitro, to determine whether alteration of autonomous cell proliferation could be a potential cause of any effect observed in vivo.

Effect of OGT2378 on MEB4 Melanoma Cell Proliferation Characteristics in Vitro. MEB4 melanoma cells were cultured for 4 days with 2 or 20 μM OGT2378, as above. After 4 days, there was no demonstrable effect on cell proliferation, as measured by thymidine incorporation (Fig. 2). Similarly, there was no inhibitory effect of OGT2378 on either direct cell counts or total cell protein determinations. These findings are consistent with our previous observations that inhibition of glucosylceramide synthase does not in itself result in an antiproliferative effect on the tumor cell (21). Lack of toxicity was reinforced by the fact that cell morphology was unaffected by exposure of MEB4 cells to ≤50 μM OGT2378 for 4 days in vitro (Fig. 2).

Because it has been postulated that increased ceramide concentrations may increase the susceptibility to apoptosis, we also evaluated the effect of OGT2378 exposure on apoptosis of MEB4 cells. There was no effect of OGT2378 exposure on the degree of apoptosis, which was ~3% in both control (untreated) and OGT2378-treated cells. As in our previous studies with another inhibitor of glucosylceramide synthase, PP19 (19), these data ensure that any subsequent effect observed in vivo was not caused by a direct inhibitory effect on cell survival or cell proliferation kinetics.

Feasibility of p.o. Administration of OGT2378. In the present studies, we observed that mice were very receptive to the addition of OGT2378 as a supplement to powdered chow. Furthermore, they appeared healthy, active, and without any gastrointestinal symptoms.

Table 1: Modulation of glycosphingolipid metabolism of MEB4 melanoma cells by OGT2378

<table>
<thead>
<tr>
<th>Glycosphingolipid</th>
<th>Control</th>
<th>OGT2378</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramide</td>
<td>130</td>
<td>10</td>
<td>-92</td>
</tr>
<tr>
<td>GM₃</td>
<td>110</td>
<td>175</td>
<td>+59</td>
</tr>
<tr>
<td>Glucosylceramide</td>
<td>37</td>
<td>&lt;1</td>
<td>-97</td>
</tr>
</tbody>
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* OGT2378 was administered p.o., in the powdered chow, for 4 days in 20 μM OGT2378, and glycosphingolipids were quantified as described in “Materials and Methods.”

** Relative concentrations, as determined by metabolic radiolabelling.

*** Relative concentrations determined by HPTLC densitometry.

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The mean tumor volume in the control group was 538 mm$^3$, whereas in the group receiving MEB4 cells that had been pretreated for 5 days with 50 $\mu$m OGT2378 was reduced to 47 mm$^3$ (mice receiving p.o. OGT2378 and OGT2378 pretreated cells, it was 0.0001). These data demonstrate that p.o. administration of OGT2378 was highly effective in impeding melanoma tumor growth in vivo.

Having found that the p.o. administration of OGT2378 markedly impeded melanoma tumor growth, a second experiment assessed whether administration of OGT2378 after tumor implantation would also affect growth of the tumor. This is an important aspect of our studies because the ability of OGT2378 to impact an established tumor would thereby strengthen the ultimate clinical utility of this therapeutic approach. As the first step in this direction, we began treatment of the mice 7 days after the intradermal administration of MEB4 melanoma cells and compared the effect with that of initiation of the drug 3 days before tumor implantation, as in the first experiment. Fig. 4 demonstrates tumour growth during the 4-week period of drug administration. Highly statistically significant differences were observed. On day 31, just after the end of the treatment (day 28), both experimental groups had much smaller tumors (4-week treatment, 101 mm$^3$; 3-week treatment, 61 mm$^3$) than did the controls (mean tumor size 620 mm$^3$). The difference between the control and treated animals was highly significant ($P < 0.0001$). It is interesting that very similar inhibition of tumor growth was observed, regardless of whether the treatment was started 3 days before or 7

### Table 2: In vivo effects of p.o. administration of OGT2378

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>OGT2378</th>
<th>% change</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight$^a$ (grams)</td>
<td>16.0 ± 0.2$^b$</td>
<td>14.3 ± 0.5</td>
<td>−11%</td>
<td>&lt;0.01$^c$</td>
</tr>
<tr>
<td>Food intake per day (grams/mouse)</td>
<td>3.7 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>−9%</td>
<td>Not significant</td>
</tr>
<tr>
<td>As % of body weight</td>
<td>23%</td>
<td>24%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic G$\text{Mg}$</td>
<td>277 ± 73</td>
<td>50 ± 22</td>
<td>−82%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Tumor G$\text{Mg}$</td>
<td>590 ± 60</td>
<td>&lt;10</td>
<td>−98%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^a$ Assessed at the end of the 3rd week of OGT2378 administration of the experiment shown in Fig. 4.

$^b$ Mean ± SE.

$^c$ Student’s t test, two tailed.

$^d$ nmol LBSA/gram protein.

$^e$ nmol LBSA/gram wet weight.

which were noted as side effects of the imino sugar studied previously, NB-DNJ (24). This tolerance to the drug was confirmed by an almost identical food intake (on a body weight basis) of control and OGT2378 fed mice (Table 2). Subsequent to an initial mild degree of weight loss (Table 2), the continuing weight gain of the mice was equivalent in the control and treated groups (data not shown). Importantly, the initial mild weight reduction did not influence the apparent health of the mice or their survival.

**Effect of Systemic, p.o. Administration of OGT2378 on MEB4 Melanoma Cell Tumor Development.** In the initial experiment to assess the effect of p.o. administration of OGT2378, we determined whether treatment of C57BL/6 mice, by p.o. administration of OGT2378, could alter the incidence or growth rate of the orthotopically (i.d) administered syngeneic MEB4 melanoma. Two experimental groups were analyzed in this experiment. One group received OGT2378 p.o. (2500 mg/kg/day) in the diet, beginning 3 days before the injection of 4 $\times$ 10$^6$ MEB4 tumor cells. The second experimental group received MEB4 cells that had been pretreated for 5 days with 50 $\mu$m OGT2378 in vitro. This group also received p.o. OGT2378, also beginning 3 days before the tumor cell injection. The control group received untreated MEB4 tumor cells. Tumors developed in all mice in each group, with a slight (but not significant) temporal delay in tumor formation when cells had been pretreated with OGT2378 to reduce their ganglioside content. The striking finding was that both groups of mice receiving p.o. OGT2378 (i.e., whether or not the cells were pretreated) had a marked reduction in the rate of tumor growth (Fig. 3). These differences reached a 10-fold level at the end of the 4-week treatment (day 27 after tumor cell injection, $P < 0.0001$). The mean tumor volume in the control group was 538 mm$^3$, whereas in mice receiving p.o. OGT2378 and OGT2378 pretreated cells, it was reduced to 47 mm$^3$ ($P = 0.008$) and to 61 mm$^3$ in mice receiving p.o. OGT2378 and nonpretreated cells ($P = 0.02$). The mean tumor volume of the two treated groups combined was 54 mm$^3$ ($P < 0.0001$). These data demonstrate that p.o. administration of OGT2378 was highly effective in impeding melanoma tumor growth in vivo.

Having found that the p.o. administration of OGT2378 markedly impeded melanoma tumor growth, a second experiment assessed whether administration of OGT2378 after tumor implantation would also affect growth of the tumor. This is an important aspect of our studies because the ability of OGT2378 to impact an established tumor would thereby strengthen the ultimate clinical utility of this therapeutic approach. As the first step in this direction, we began treatment of the mice 7 days after the intradermal administration of MEB4 melanoma cells and compared the effect with that of initiation of the drug 3 days before tumor implantation, as in the first experiment. Fig. 4 demonstrates tumour growth during the 4-week period of drug administration. Highly statistically significant differences were observed. On day 31, just after the end of the treatment (day 28), both experimental groups had much smaller tumors (4-week treatment, mean tumor volume 101 mm$^3$; 3-week treatment, 61 mm$^3$) than did the controls (mean tumor size 620 mm$^3$). The difference between the control and treated animals was highly significant ($P < 0.0001$). It is interesting that very similar inhibition of tumor growth was observed, regardless of whether the treatment was started 3 days before or 7

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**Fig. 2.** Effect of OGT2378 on MEB4 murine melanoma cell proliferation and morphology. Thymidine incorporation (mean ± SD of triplicate cultures) of MEB4 cells treated with 2 or 20 $\mu$m OGT2378 is shown in the left panel. The right panel depicts cell morphology of control (top) and 50 $\mu$m OGT2378-exposed cells (bottom).

**Fig. 3.** Effect of p.o. administration of OGT2378 on MEB4 melanoma tumor growth. The mean ± SE tumor volumes are shown.
days after the tumor cells were injected. Thus, future studies should address the potential of OGT2378 to impede the growth of tumors, which have been allowed to establish for longer periods before administration of the drug.

**Inhibition of Ganglioside Synthesis in Vivo.** To assess the metabolic effects of OGT2378 on glycosphingolipid metabolism, we analyzed the ganglioside content of both normal and tumor tissue 24–27 days after tumor cell inoculation. Analysis of hepatic gangliosides demonstrated an 82% reduction, from 277 to 50 nmol LBSA/gram protein (Table 2). The ganglioside pattern, predominantly G_{M2}, was not altered.

We also analyzed the ganglioside content of tumors obtained from control and OGT2378-treated mice. Three tumors of different sizes were harvested from each group and analyzed for ganglioside content. Ganglioside composition of all of the tumors was identical, consisting exclusively of G_{M3}. However, the p.o. OGT2378 treatment markedly reduced the ganglioside concentration of the tumors; the mean G_{M3} content of the control tumors was 590 nmol/gram wet weight. In contrast, G_{M3} was almost completely (>98%) depleted in tumors from OGT2378-treated mice, to <10 nmol/gram wet weight (Table 2; Fig. 5).

**DISCUSSION**

The rationale for using inhibitors of glycosphingolipid synthesis as a therapeutic approach to tumors derives from substantial data supporting a role for gangliosides in the process of tumor formation. From the initial description of tumor cell ganglioside shedding and immunosuppressive activity (2), to the correlation and direct evidence for a role in tumor formation in mice (18), and a link with tumor progression in humans (17), the accumulated findings of many laboratories have now established a biological role for gangliosides in a number of tumor systems (1).

The possibility that inhibition of ganglioside synthesis by tumor cells may decrease tumor cell ganglioside content and impede tumor progression has been of recent interest (34). *In vitro* studies have shown that the inhibition of ganglioside synthesis, *e.g.*, by inhibitors of glucosylceramide synthase, results in decreased ganglioside shedding (19), which in turn has been associated with a reduced inhibitory effect on the host immune response and reduced enhancement of normal growth factor-induced endothelial cell and fibroblast proliferation (14, 35). Treatment of tumor cells *in vitro* with two inhibitors of glucosylceramide synthase, N-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and PPPP, has resulted in decreased ganglioside content and decreased tumor formation (19, 36, 37). However, PPPP is highly toxic and cannot be administered *in vivo*. Recently, glucosylceramide synthase antisense clones, which have decreased ganglioside content but only marginally increased ceramide and no significant increase in apoptosis (21), have also shown inhibited tumor formation, compared with untransfected or sense-transfected control cells. Together, these studies have suggested that this inhibition of tumor cell ganglioside expression may be a viable approach to cancer treatment.

Whether systemic treatment with an inhibitor of glucosylceramide synthase would be feasible in this context has not been well studied; there is one report that NB-DNJ reduced tumor cell proliferation *in vitro* and tumor formation *in vivo* in a murine brain tumor (20). On the other hand, there has already been substantial interest in the use of inhibitors of glucosylceramide synthase to treat glycolipid storage diseases by substrate reduction (*e.g.*, Gaucher’s disease; Ref. 38), and NB-DNJ has been used with success in this manner in humans (22). Thus, inhibition of glycosphingolipid synthesis has been identified as a therapeutic target in glycolipid storage disease.

In the present work for the first time administering a new imino sugar, OGT2378, we observed highly effective depletion of gangliosides *in vitro* and particularly of tumor tissue gangliosides. To study its effect on tumor formation, we have used a relevant tumor system, namely an orthotopic model of melanoma, generated by intradermal injection of MEB4 melanoma cells (19, 21). In this syngeneic tumor model, we have shown that the continuous p.o. administration of OGT2378, in the diet, was well tolerated and resulted in highly significant inhibition of tumor growth, even when treatment was initiated only after establishment of the tumor. In addition, the fact that in the present study we found no inhibition of tumor cell proliferation *in vitro* is of importance because it reduces the likelihood of side effects *in vivo* caused by a direct cytotoxic or antiproliferative effect of the drug. These findings underscore the potential significance of ganglioside metabolism as a new therapeutic target and suggest promise for OGT2378 as an example of a potentially very effective new approach to interfering with tumor progression.

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osphingolipid depletion in the liver and lymphoid organs of mice treated with N


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