Inhibiting Human Astrocytoma Growth: Structure−Activity Relationships in Neurostatin Related Glycolipids

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Abstract: Neurostatin, a mammalian brain inhibitor of division of astroblast and astrocytoma cells, was characterized as the disialoganglioside GD1b, 9-O-acetylated on the outer sialic acid residue (Galβ1→3GalNAcβ1→4(9-O-Ac-NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1′-ceramide). Using semisynthetic approaches, we prepared and tested different gangliosides O-acetylated in the sialic acid and compared them to non-O-acetylated partners as inhibitors of U-373 glioma cells. Athough the O-acetylation of the sialic acid was the most important molecular feature for the antiproliferative activity of O-acetylated gangliosides, monosaccharide links Galβ1→3GalNAcβ1 and NeuAcα2→8NeuAcα2 enhanced the inhibitory activity.

Gliomas are the most common tumors of the mature nervous system. Although the improvements in therapeutic treatments have increased the survival time slightly, no successful therapy for gliomas is available.

The presence in brain extract of inhibitors of astroblast and astrocytoma division was first described by our group.[1] One of the inhibitors, called neurostatin, was characterized as O-acetylated disialoganglioside GD1b.[2] Neurostatin is cytostatic for rat glioma cells and various human astrocytoma cell lines at very low concentrations.[2,3] Oligosaccharide analogues of the carbohydrate moiety of neurostatin inhibited the proliferation of various rat and human glioma cell lines in vitro[4,5] and in vivo.[6,7] Here, we examine the portions of neurostatin structure required for inhibitory activity, an essential step for developing new synthetic analogues more active against brain gliomas.

Given the low abundance of O-acetylated gangliosides in nervous tissue, we synthesized and purified them by various methods, starting from commercial nonacetylated gangliosides. Compounds **1** and **2** were O-acetylated in the outer sialic acid using the enzyme subtilisin and vinyl acetate as the *O*-acetyl donor, at 37 °C, yielding 38% (**5**) and 17% (**6**), respectively. Compound **3** was O-acetylated in the sialic acid with trimethyl orthoacetate and *p*-toluenesulfonic acid at room temperature, yielding 15% of O-acetylated ganglioside (**7**). Compound **8** was purified from pig or cow brain.

The activity of the O-acetylated semisynthetic compounds on [^{3}H]thymidine incorporation into human glioma cell lines U-373 and T98G, driven by EGF or PDGF−B, was compared to the effects of **4** (GD1b) and **8** (neurostatin). The results are summarized in Table 1. Compound **4** was mitogenic or slightly inhibitory, depending on the growth factor that induced proliferation and the cell line tested. Compound **8**, however, inhibited cell proliferation in all cases with ID_{50} values between 1 and 2 μM. The nature of its activity did not depend on the growth factor or cell line tested. Similarly, O-acetylation changed drastically the activity of **4** from mitogenic or slightly inhibitory to very inhibitory in **8**.

Table 1. Influence of O-Acetylation on the Activity of Compound **8** on Human Glioma Lines U-373 and T98G[a]

compd	U-373 EGF	U-373 PDGF-B	T98G EGF	T98G PDGF−B
4	+	1.3 ± 0.2	+	**21.9 ± 0.7**
8	**1.8 ± 0.1**	**1.4 ± 0.1**	**1.0 ± 0.2**	**1.3**

[a] + represents compounds that are slightly mitogenic. Values in bold are the ID_{50} values (μM) for compounds that inhibit the proliferation induced by peptide mitogens. The other values are for compounds that increase the mitogenicity of peptide growth factors (EC_{50} in μM). All the values are the mean ± SD of at least two experiments in triplicate.

Table 2. Inhibitory Activity (ID_{50}, μM) of O-Acetylated Gangliosides (**5**−7) Compared to Non-O-Acetylated Compounds (**1**−**3**) on Human Glioma Line U-373[a]

compd	EGF	PDGF-B
1	**43.0**	>**25**
2	+	1.5 ± 0.5
3	8.0 ± 2.3	6.4
5	**21.4**	>**25**
6	**14.4 ± 2.4**	**22.6 ± 3.6**
7	>**25**	**37.0**

[a] + represents compounds that are slightly mitogenic. Values in bold are the ID_{50} values (μM) for compounds that inhibit the proliferation induced by peptide mitogens. The other values are for compounds that increase the mitogenicity of peptide growth factors (EC_{50} in μM). All the values are the mean ± SD of at least two experiments in triplicate. + represents compounds that are slightly mitogenic. Peptide mitogens are EGF and PDGF.

The biological activity of gangliosides mono-O-acetylated in sialic acid position 9, in promoting or inhibiting [^{3}H]thymidine incorporation in U-373 human glioma cells line with EGF or PDGF-B as mitogens, was compared to each other and non-O-acetylated parent compounds. The results are summarized in Table 2. Whereas **6** had 8- to 16-fold less inhibitory activity than **8**, the non-O-acetylated parent **2** and **4** showed similar mitogenic activities. The monosaccharide link Galβ1→3GalNAcβ1 in **8** was important for its inhibitory activity but not for the mitogenic activity of **4**. This result suggests that the sugar structure and O-acetylation cooperate in improving the antimitotic activity of O-acetylated compounds.

Compound **1** was the only non-O-acetylated compound with inhibitory activity for U-373 glioma cells. Although the inhibitory activity of **1** improved with O-acetylation (**5**), the improvement was the lowest of all gangliosides tested. Comparing the inhibitory activities of **5** and **6**, we notice that the loss of the outer sialic acid reduced it by half. These data suggest that without the Galβ1→3GalNAcβ1 link, the loss of a sialic acid is not so important for the inhibitory activity of the O-acetylated gangliosides. However, the loss of the same sialic acid in non-O-acetylated parent compounds transformed **2** from slightly mitogenic to inhibitory (**1**) in EGF treatments, suggesting a different role in the activity of this sialic acid in O-acetylated and non-O-acetylated compounds.

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Compound **3** was mitogenic when used together with EGF or with PDGF-B at low concentration. The comparison between the activities of **1** and **2** shows that the loss of the Galβ1→3GalNAcβ1 link is very important for the inhibitory activity of **1** with EGF as mitogen. If we compare the activities of **5** and **7**, the loss of the same link did not significantly improve the inhibitory activity of the O-acetylated monosialogangliosides. However, the loss of a sialic acid from **8** to **7** reduced almost 20-fold its inhibitory activity, showing that the NeuAcα2→8NeuAcα2 link is important for the inhibitory activity on U-373 cells when it is together with the Galβ1→3GalNAcβ1 link (compare the activity of **6** and **8**). Whereas the NeuAcα2→8NeuAcα2 and Galβ1→3GalNAcβ1 links together do not improve the inhibitory activity of non-O-acetylated compouds, the cooperation of these links is very important for the inhibitory activity of **8**. These data suggest that O-acetylation is inducing a change in **8** modifying the structure of both links and increasing the inhibitory activity on U-373 cells. A study of the conformational changes is in progress to detect intramolecular interactions that could predict the increased inhibitory activity of these O-acetylated compounds

These preliminary studies indicate that the structure of **5**, (9-O-Ac-NeuAcα2→3Galβ1→4Glcβ1→1′-ceramide), is common to all *O*-acetyl compounds that inhibit proliferation of U-373 cells. Further work using this core structure is in progress to find new synthetic compounds with improved inhibitory activity against human gliomas.

As additional information, ganglio-series gangliosides are named according to Svennerholm.[8] Although we found 7- and 8-*O*-acetyl esters of the sialic acid in O-acetylated compounds, they were named as 9-O-acetylated compounds. In nature, 9-*O*-acetyl esterification is the most common sialic acid substitution, since at physiological pH, 7- and 8-*O*-acetyl esters migrate to the 9 position.[9]

In this manuscript, the following abbreviations are used: EC_{50}, concentration that increases by 50% peptide mitogen-induced proliferation; EGF, epidermal growth factor; ID_{50}, dosage that reduces by 50% peptide mitogen induced proliferation; PDGF-B, platelet-derived growth factor B; SD, standard deviation.

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Supporting Information Available: Experimental section containing preparation, purification, and characterization of all O-acetylated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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