Brief Definitive Report

Inhibition of Proliferation of Normal and Transformed Neural Cells by Blood Group–related Oligosaccharides

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Summary

A synthetic tetrasaccharide structurally related to blood groups and selectin ligands inhibited division of astrocytes, gliomas, and neuroblastomas at micromolar concentrations. The compound was cytostatic for primary astrocytes in culture, but cytotoxic for fast proliferating cell lines.

In the mammalian central nervous system (CNS), astroglial cell division during adulthood and old age remains more a potentiality than a frequent event (1). It was long suspected that mitogen inhibitors may play a crucial role in the control of astrocyte populations and, previously, we presented evidence that an antimitotic, immunologically related to epidermal growth factor receptor (EGFR), inhibited proliferation of rat astrocytes in primary culture (2). Astrocyte proliferation after open CNS injury correlated with a decrease in the activity of this antimitotic, suggesting that the inhibitor was involved in the physiological control of astrocyte number. The following evidence suggested that the active moiety of the antimitotic is glycosidic in nature. Immunoglobulins from human blood group O, as well as a mAb to a carbohydrate epitope of EGFR crossreacting with blood group A (antibody 29.1; reference 3), blocked antimitotic activity (4). Digestion of brain antimitotic with β-glucosidase or β-glucuronidase destroyed the inhibitory activity (J. Abad and M. Nieto-Sampedro, unpublished observations). Finally, a common sugar component of blood groups, L-Fucose (Fuc), had weak antimitotic activity (ID_{50} = 35 mM; reference 4). We hypothesized that oligosaccharides with structure related to that of blood groups, may be cell division inhibitors. To test this idea we synthesized (*a*) fucosyl-lactoses; (*b*) the trisaccharide best recognized by antibody 29.1 (11); and (*c*) a tetrasaccharide that combined the structural features of the best trisaccharide inhibitors. The results of testing their ability to inhibit the division of both astrocytes in primary culture and transformed neural cells, are the subject of this report.

Materials and Methods

Synthetic Compounds, Cell Culture, [³H]thymidine Incorporation, and Cell Viability. Synthesis of oligosaccharide and primary cultures of purified rat astrocytes, were performed as described previously (5–7). All cells were maintained in a 1:1 mixture of DMEM and Ham's F12 (Sigma Chemical Co., St. Louis, MO), supplemented with 10% (vol/vol) FCS (2, 4).

Incorporation of [³H]thymidine into primary cultures of rat astrocytes (and its inhibition), was measured using the 96-well microassay previously described (2). The data are the mean and SEM of three independent experiments each performed in triplicate. Control incorporation in the absence of antimitotic sugar ranged from 6,000 to 12,000 dpm/well for astrocytes and RN22 cells (7) to 19,000 dpm/well for A7 cells. Addition of serum was not necessary for the cell lines A7 astrocytoma (8) and Neuro-2a neuroblastoma (9) to incorporate [³H]thymidine. The concentrations of antimitotic that inhibited [³H]thymidine incorporation by 50% (ID_{50}), were obtained from dose–response curves (see Fig. 1). Cell viability was determined at inhibitor concentrations above the ID_{50} value, as described by Jones and Senft (10).

Results and Discussion

Immunoglobulins from human blood group O and a monoclonal anti-EGFR that crossreacted with blood group A (11), blocked rat brain antimitotic activity (3). A common blood group constituent, L-Fucose (Fuc), was weakly antimitotic (ID_{50} = 35 mM). Taken together, these data suggested that the natural brain antimitotic could be structurally related to blood group sugars (4). Accordingly, the trisaccharide best recognized by monoclonal 29.1, *N*-acetylgalactosaminyl-lactose (11) and three fucosyl-lactoses, were synthetized (5, 6), and their antimitotic activity examined. All fucosyl-lactoses, as well as *N*-acetylgalactosaminy-lactose, were inhibitory (Table 1).

Relating the structure of the trisaccharide:

α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-D-Glc

to that of the most inhibitory fucosyllactose:

β-D-Gal-(1→4)-D-Glc
↑1–3
α-L-Fuc

we hypothesized that the tetrasaccharide:

Table 1. *Carbohydrate Inhibition of [³H]thymidine Incorporation in Astrocytes*

	Carbohydrate structure	ID_{50}
		mM
L-Fucose	L-Fuc	35.0
2′-Fucosyllactose	β-D-Gal-(1→4)-D-Glc ↑1–2 α-L-Fuc	7.8
3,2′-Difucosyllactose	β-D-Gal-(1→4-D-Glc ↑1–2 ↑1–3 α-L-Fuc α-L-Fuc	6.4
3-Fucosyllactose	β-D-Gal-(1→4)-D-Glc ↑1–3 α-L-Fuc	4.3
Trisaccharide	α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-β-D-Glc-Me	0.235
Tetrasaccharide	α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-β-D-Glc-Me ↑1–3 α-L-Fuc	0.076

*ID_{50} estimated as indicated in Materials and Methods. [³H]thymidine incorporation in the absence of antimitotic sugar ranged from 8,000 to 12,000 dpm/well.

α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-D-Glc
↑1–3
α-L-Fuc

structurally related to blood groups A and Lewis X, and referred to as TS4, would be a better antimitotic than any of the trisaccharides.

α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-GlcMe, was synthesized (6) and its antimitotic activity tested on cultures of neonatal rat astrocytes, A7 astrocytoma (8), Neuro-2a neuroblastoma (9), and RN22 Schwannoma (7). A dose-dependent inhibition of thymidine incorporation was observed, with 50% inhibition (ID_{50}) in the μM range (Fig. 1 and Table 1). All cells were viable at the ID_{50}, indicating that growth inhibition did not involve cytotoxicity. In fact, cell death was never observed with astrocytes or RN22 cells, even at doses of TS4 that totally blocked thymidine incorporation. However, a large proportion of fast dividing cells of lines A7 and Neuro-2a were not viable after 24-h contact with TS4 concentrations between 0.5 and 1.0 mM (Fig. 1, percent viability). Therefore, selective destruction of fast proliferating cells appears possible.

Some structure-activity conclusions may be drawn from the ID_{50} values in Table 1 and Fig. 1. Thus, although fucose itself inhibited astrocyte division, its presence in oligosaccharides was not essential for antimitotic activity (see *N*-acetylgalactosaminy-lactose, Table 1). *N*-acetylgalactosamine seemed more important when the target cells were primary astrocytes, whereas fucose substitution conferred oligosaccharides higher antimitotic effectiveness on fast proliferating cells, relative to primary cultures. It contributed to make TS4 three times more potent than *N*-acetylgalactosaminy-lactose on primary astrocytes, but 13 times more potent on transformed Neuro-2a cells (Fig. 1).

Known ligands of selectins, a class of Ca^{2+}-dependent lectins that contain EGF domains, contain a carbohydrate moiety similar to TS4 (12). Previously, we reported that an inhibitor of astrocyte division present in rat brain shared a carbohydrate epitope with EGFR (2). Our present results suggest that the active site of the natural EGFR-related antimitotic may be related to synthetic TS4. Compared with TS4, glycoconjugates have *N*-acetylglucosamine instead of glucose. Hence, a structure α-D-Gal*N*Ac(1→3)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-Gl*N*Ac, containing the 3-fucosyl-*N*-acetyllactosamine (FAL) epitope (13), may be more similar to the natural regulators of cell division. A similar group, sialyl-Lewis X ($SiLe^x$) seems involved in both normal and tumoral cell proliferation (14–16). Furthermore, it has been proposed that interaction of $SiLe^x$ with the selectin endothelial leukocyte adhesion molecule-1 (ELAM-1) may lead to extravasation of tumoral cells, thus mediating metastasis (17). Although fucose and sialic acid are required for ELAM-1 binding (18), the effect of a Gal*N*Ac instead of sialic acid was not tested. Synthetic TS4 analogues would be antimetastatic if they could compete with the natural ligand for the ELAM-1 binding site.

Although blood group carbohydrate groups have been known for a long time, their biological role was never established. Inhibition of cell division by blood group-related oligosaccharides, suggests that these substances may be involved in controlling cell proliferation. Blood group-related carbohydrates, also involved in cell adhesion, could underlie

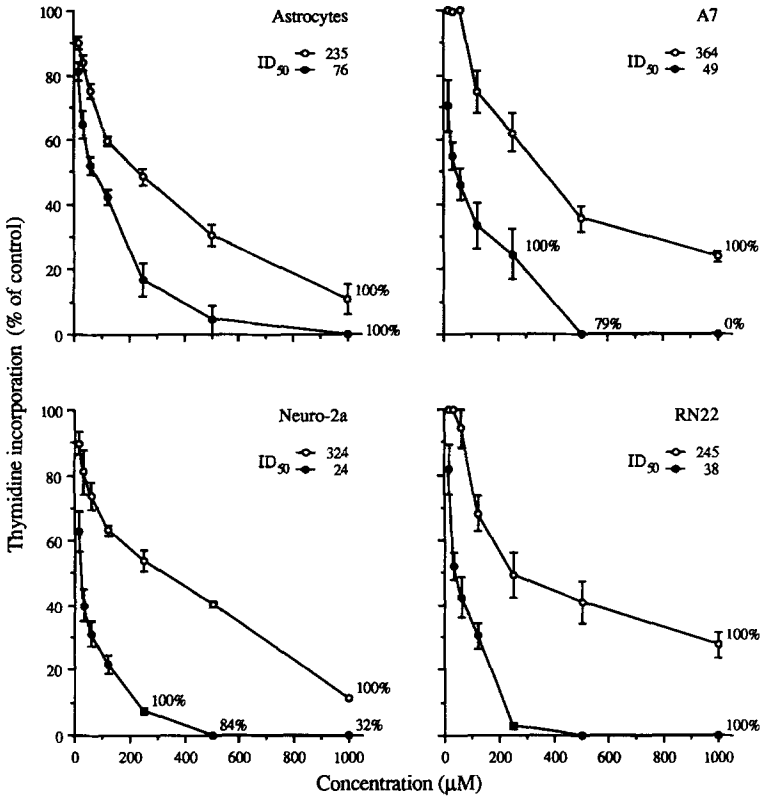

Figure 1. Dose–response curves for the antimitotic activity of (—○—), the trisaccharide α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-β-D-Glc-Me, and (—●—), the tetrasaccharide α-D-Gal*N*Ac-(1→3)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-Glc-Me. Percent values indicate percent viability. All data are the mean ± SEM of three independent experiments, each performed in triplicate.

contact inhibition of cell growth. The identification of a synthetic structure capable of inhibiting fast proliferating tumoral cells, raises the possibility of controlling pathological cell division and has practical importance. Most brain tumors are gliomas, and preliminary results in a rat model indicate that TS4 can arrest their growth.

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