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Brain Content of Glycosphingolipids After Oral Administration of Monosialogangliosides GM1 and LIGA20 to Rats

A. POLO,[1] G. KIRSCHNER,[2] A. GUIDOTTI,*,[1] AND E. COSTA[1]

[1]Fidia-Georgetown Institute for the Neurosciences, Georgetown University Medical School, Washington, DC 20007; and [2]Fidia Research Laboratories, Abano Terme, Italy

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ABSTRACT

Natural (GM1) and semisynthetic [11^3-Neu-5-AcGgOse$_4$-2-D-*erythro*-1,3-dihydroxy-2-dichloroacetylamide-4-*trans*-octadecene (LIGA20)] glycosphingolipids, given parenterally, protect neurones against glutamate-induced death without producing the side effects typical of glutamate receptor antagonists. Chronic glutamate-related neurotoxicity (e.g., in recurring strokes in elderly hypertensive patients, and in Parkinson disease) could be prevented also by glycosphingolipids treatment, but this therapeutic intervention will require a protracted administration of orally active glycosphingolipids. Here we demonstrate that 3–6 h after oral administration of 68 μmol/kg of LIGA20 and GM1 to rats, the brain content of LIGA20 is 50-fold higher than that of GM1. The brain concentration for LIGA20 remains elevated for at least 12–24 h. Because the LIGA20 that reaches the brain is slowly metabolized, repeated oral administrations of this glycosphingolipid can yield to its accumulation in brain, and can yield various brain levels depending on the dose and frequency of drug administration. In contrast this is not possible with GM1, which given orally for 7 d, cannot accumulate in brain in pharmacologically significant concentrations.

Index Entries: Glycosphingolipids; GM1; LIGA20; excitotoxicity; neuronal death; stroke; Parkinson disease.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Natural (GM1, GD1a, GD1b, and GT1b) and semisynthetic [LIGA4 (11^3-Neu-5-AcGgOse$_4$-2D-*erythro*-1, 3-dihydroxy-2- acetylamide-4-*trans*-octadecene), or LIGA20 (11^3-Neu-5-AcGgOse$_4$-2D-*erythro*-1,3-dihydroxy-2-dichloroacetylamide-4-*trans*-octadecene)] glycosphingolipids protected against glutamate-induced neuronal death when added to primary neuronal cultures (Manev et al., 1990a) or when given parenterally to rats successively exposed to brain hypoxic or anoxic conditions (Karpiak et al., 1990; Seren et al., 1990; Manev et al., 1990b; Bharucha et al., 1991; Costa et al., 1992; Kharlamov et al., 1993). Recently GM1 administered parenterally to dogs has been reported to reach the brain in concentrations that reduce the glutamate-mediated neurologic injury associated with hypothermic circulatory arrest (Redmond et al., 1993).

The antiexcitotoxic action elicited by these glycosphingolipids is owing neither to an action on the glutamate channel-gating mechanisms nor to an inhibition of activation of glutamate metabotropic receptors (Favaron et al., 1988), but appears related to an inhibition of the pathological persistent activation and cell membrane translocation of protein kinase C that leads to a destabilization of $[Ca^{2+}]_i$ homeostasis and ultimately to delayed neuronal death (de Erausquin et al., 1990; Manev et al., 1990a,b). Consistent with the mechanism by which glycosphingolipids protect neurons from glutamate receptor abuse these drugs are classified as RADA, an acronym for Receptor Abuse Dependent Antagonism (Manev et al., 1990b).

Thus treatment with RADA drugs might delay the onset and attenuate the glutamate excitotoxicity operative in senile and Alzheimer dementia (Koh et al., 1991a,b), in repeated and chronically occurring convulsive episodes (Olney, 1990) and in the progressive degeneration of dopamine neurones typical of Parkinson disease (Hadjiconstantinou et al., 1986; Klockgether and Turski, 1989; Schneider et al., 1992). To optimize the therapeutic action of the natural glycosphingolipids their structure can be modified to facilitate their absorption by oral route and to increase their biological t1/2. Because the semisynthetic LIGA derivatives are more potent, and have a biological t1/2 longer lasting than that of GM1 and in addition they act more promptly than GM1 in protecting against glutamate excitotoxicity in vitro and in vivo (Manev et al., 1990b; Costa et al., 1992; Kharlamov et al., 1993), we compared the brain and plasma content of LIGA20 and GM1 after oral administration. Predictive thermodynamic-geometric correlation studies that take into account the cross-sectional molecular area of GM1 and LIGA derivatives indicate that small alterations of the substituent group of the 2-amino position of sphingosine in the LIGA compounds have a large consequence on the possibility of self aggregation and stability of the structure at the air /NaCl/ lipid monolayer interface (Sonnino et al., 1990; Perillo et al., 1993). In particular these studies predict that LIGA20 by virtue of a more condensed behavior and

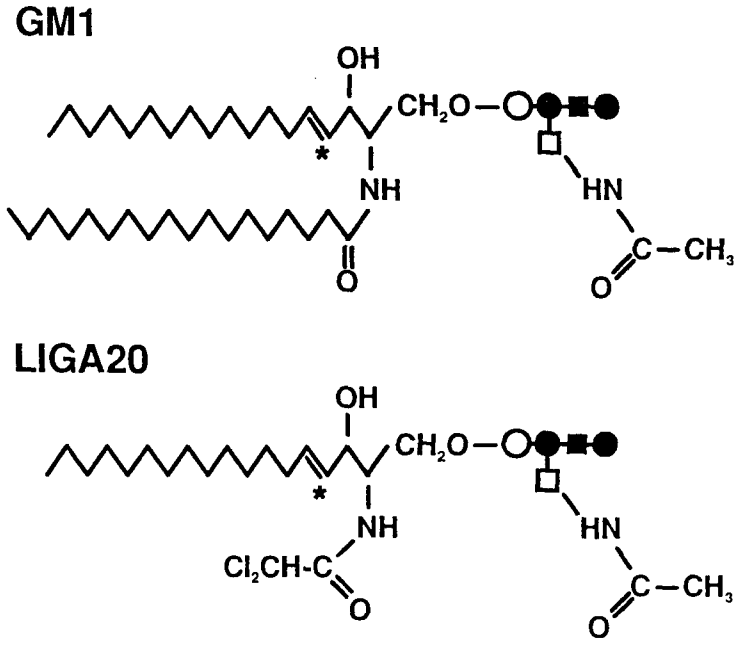

Fig. 1. Structure of GM1 and LIGA20; the asterisk represents the position of the tritium labeling. □,sialic acid; ○, glucose; ●, galactose; ■, N-acetyl galactosamine.

an increase in collapse pressure, compared to GM1, forms smaller micelles that insert promptly and certainly more rapidly than GM1 into the plasma membrane bilayers. We now report that after oral administration of LIGA20 or GM1 to rats, the plasma and brain concentration of LIGA20 is about 50-fold greater than that of GM1.

MATERIALS AND METHODS

Chemicals of analytical grade or of the highest purity available were purchased from Aldrich (Milwaukee, WI). Silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μM thick, 20 × 10 cm, Merck) were purchased from VWR Scientific (Bridgeport, NJ). Sep-Paks were from Waters (Milford, MA), and Centricons were from Centricons Amicon (Beverly, MA).

Radiolabeling Methods

Radioactive glycosphingolipids GM1 and LIGA20 were a gift from FIDIA (Abano Terme, Italy). They were tritium radiolabeled by catalytic reduction at the sphingosine double bond following the method of Schwarzmann (1978) (Fig. 1). The specific radioactivities of GM1 and LIGA20 were 2.1 and 2.05 Ci/mmol, respectively, and their radiochemical

purity was 97%. On thin-layer HPTLC [^{3}H]LIGA20 and [^{3}H]GM1 migrate as a single radioactive peak with the nonradioactive standard materials (Fig. 2).

Rats Treatment and Sample Collection

Single Oral Administration

Male Sprague Dawley rats (200–250 *g*) (Zivic-Miller, Zelianopole, PA), kept without food from the previous night, were given [^{3}H]LIGA20 or [^{3}H]GMI diluted in 2 mL of water by oral gavage using an enteral feeding tube (Corpak, Wheeling, IL, size 6 fr). At the indicated time the brain was removed, washed in ice-cold saline solution and frozen for analysis at a later time. The blood was collected in a glass heparinized tube and the plasma obtained by 2000*g* centrifugation was collected and stored at −70°C.

Repeated Daily Administrations

Rats kept without food for 12 h were given [^{3}H]LIGA20 and [^{3}H]GMI by oral gavage every day for 7 d. The animals were sacrificed 24 h after the last drug administration and brains and plasma were collected for analysis.

Extraction and Identification of Radiolabeled Lipids

Total lipids, including glycosphingolipids, were extracted and partitioned from rat brains and plasma according to Tettamanti et al. (1973). The brain, without cerebellum (approx 1.5 g) was homogenized with 1 mL of 0.01*M* potassium phosphate buffer (pH 6.8) and 8 mL of tetrahydrofuran (THF). The homogenate was mixed with vortex and centrifuged at 5,000*g* for 5 min. The supernatant was collected in a graduated glass tube. The pellet was resuspended in 1 mL of the same phosphate buffer and 8 mL THF, centrifuged at 5,000*g* for 5 min, and the resulting supernatant was collected. The procedure was repeated once more. To the pooled supernatants, a volume of ethyl ether corresponding to 30% of the total volume was added. The solution was vigorously shaken and centrifuged as before. The aqueous phase, which contains [^{3}H]GMI and [^{3}H]LIGA20, and the organic phase, which contains glycolipids and lipid metabolites of LIGA20 or GM1, were lyophilized, resuspended in a small volume of H_2O. An aliquot of the radioactivity was counted by scintillation spectrophotometry and another aliquot was subjected to HPTLC separation for the identification of various radioactive compounds. Before HPTLC, the salts were removed from the aqueous phase containing the gangliosides either by Sep-Pak or by Centricon (Centricon-10 for GM1 and Centricon-500 for LIGA20). The radioactive compounds were separated with HPTLC and identified by comparison with authentic glycosphingolipids standards under the following conditions: chloroform/methanol/0.3% aqueous $CaCl_2$, 50:42:11 by volume. The radioactivity on HPTLC was measured using a

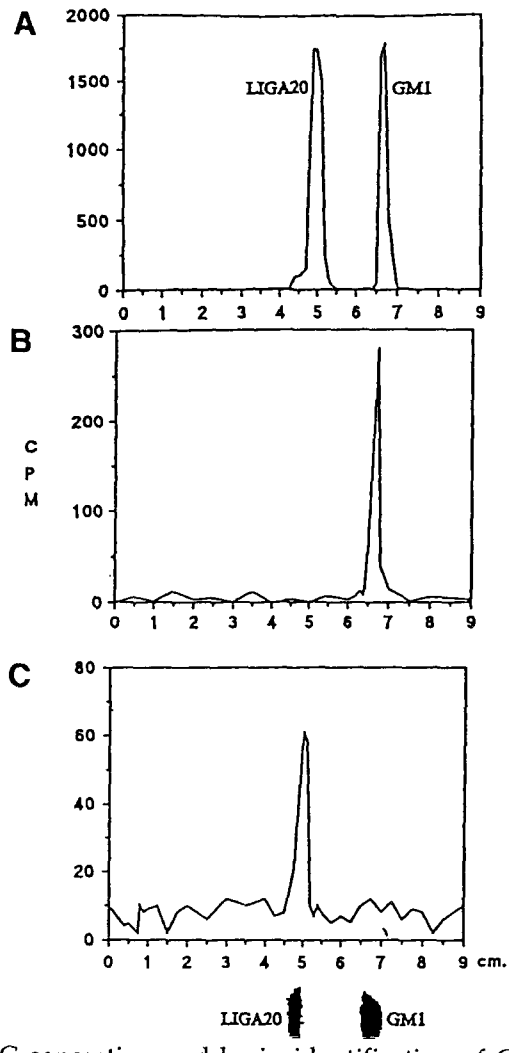

Fig. 2. HPTLC separation and brain identification of GM1 and LIGA20. (A) Standard ^{3}H-GM1 and ^{3}H-LIGA20. (B) ^{3}H-GM1 extracted from the brain of a rat treated orally per 7 d with 68 μmol/kg of ^{3}H-GM1. Extract obtained from the entire brain homogenate. (C) ^{3}H-LIGA20 extracted from the brain of a rat treated orally per 7 d with 68 μmol/kg of ^{3}H-LIGA20. Extract obtained from 100 mg of brain homogenate. The two spots below panel C depict the position of non-radioactive standard of GM1 and LIGA20 detected by colorimeteric method (Svennerholm, 1957).

quantitative radioisotope analyzer with an imaging proportional counter (Bioscan, Washington, DC). The position of the nonradioactive standard was determined by a colorimetric reaction with resorcinol as described by Svennerholm (1957). In each experiment [^{3}H]GMI or [^{3}H]LIGA20 in concentrations comparable to those found in brain and plasma of treated rats, were added to brain homogenates or plasma of untreated rats and

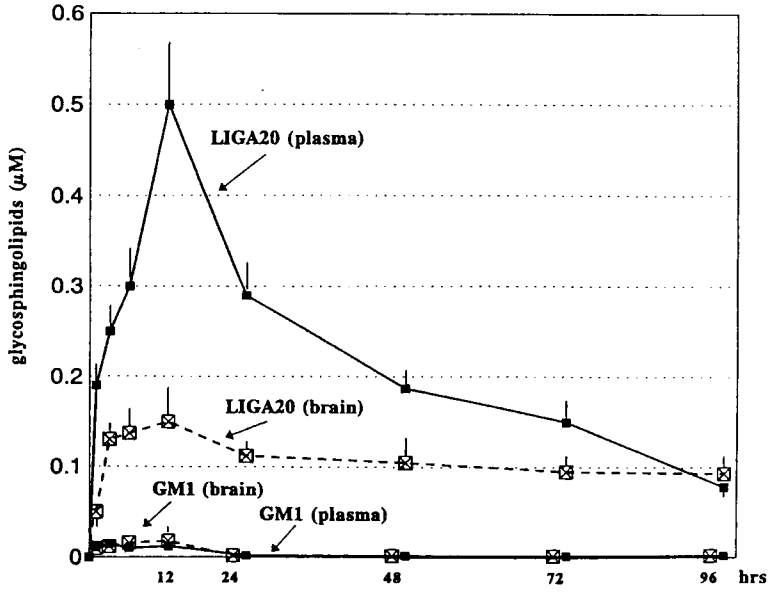

Fig. 3. Time course changes of brain and plasma glycosphingolipids content after oral administration of GM1 and LIGA20 (68 μmol/kg). Each value is the mean ±SEM of 3–5 rats.

extracted as described before. The recovery of the radioactive standard was approx 70%. For the final calculations of the concentration of LIGA20 and GM1 in brain and plasma the experimental values were corrected for the recovery of the respective standards.

RESULTS

Time Dependent Changes in the Content of Glycosphingolipids in Brain and Plasma of Rats Receiving a Single Oral Administration of [^{3}H]GM1 and [^{3}H]LIGA20

In rats receiving 68 μmol/kg of either [^{3}H]LIGA20 or [^{3}H]GMI by oral gavage measurable amounts of the two compounds were detected in plasma and brain. However, the plasma and brain content of LIGA20 is severalfold (10–50-fold) higher than that of GM1 (Fig. 3). For example, 12 h after oral administration the plasma concentration of LIGA20 is approx 500 n*M*, whereas that of GM1 is approx 10 n*M* (*see* Fig. 3). The plasma content of LIGA20 reaches its nadir between 6 and 24 h and, thereafter, declines during 96 h with an apparent half-life of approx 48 h. In the same rats the brain content of LIGA20 reaches its nadir between 3 and 12 h and the level persists virtually unabated for 96 h. Interestingly, the brain content of LIGA20 is approx one-third of the plasma level during the first 12

Table 1
Glycosphingolipids Brain Content 6 h After Oral Administration of Different Doses of GM1 and LIGA20[a]

Glycosphingolipids, GM1 or LIGA20, μmol/kg/os	Brain content, μM[b]	
	LIGA20	GM1
22	46 ± 5	—
44	78 ± 15	—
68	130 ± 10	10 ± 1.8
112	270 ± 30	—
500	600 ± 75	37 ± 4

[a] Each value is the mean ± SEM of three to five rats,—not determined.
[b] μM refers to the concentration of glycosphingolipids in 1 kg of brain tissue.

h, but approaches and surpasses the level of plasma between 24–96 h. Since we have not removed blood from the brain before LIGA20 analysis it can be assumed that plasma may contribute to the brain content of LIGA20. However, because blood represents approx 10% of the brain volume and plasma levels of LIGA20, with the exception of the initial 6–12 h are similar to those of brain, the contribution of blood to the brain content of LIGA20 must be considered marginal. The GM1 concentrations in brain and plasma are measurable only during the first 12 h, they are barely detectable after 24 h. The LIGA20 brain content appears to be dose-related between 22 and 112 μmol/kg/os (Table 1). At doses of 500 μmol/kg/os the efficacy of absorption tend to decrease at least at the time interval we studied. In addition to authentic LIGA20 and GM1, 24 h after the oral administration, the brain and plasma contain a significant amount of nonvolatile radioactivity that localizes in the organic phase and presumably represents GM1 or LIGA20 metabolites (Fig. 4). The amount of these putative metabolites found in the organic phase is virtually unmeasurable during the first few hours after the oral administration of gangliosides but increases with time.

Glycosphingolipids Content in Brain and Plasma of Rats After Repeated Daily Oral Treatments with [^{3}H]GM1 and [^{3}H]LIGA20

In rats treated orally once daily with 68 μmol/kg of LIGA20, the brain content of this glycosphingolipid, measured 24 h after the last dose, increases from approx 10 n*M* the first day to approx 600 n*M* at d 7 (Fig. 4A). Also the brain content of GM1 increases with repeated daily treatment with GM1 (68 μmol/kg/os), but never exceeds 12% of the brain content of LIGA20 (*see* Fig. 4A). In the same rats comparable differences were

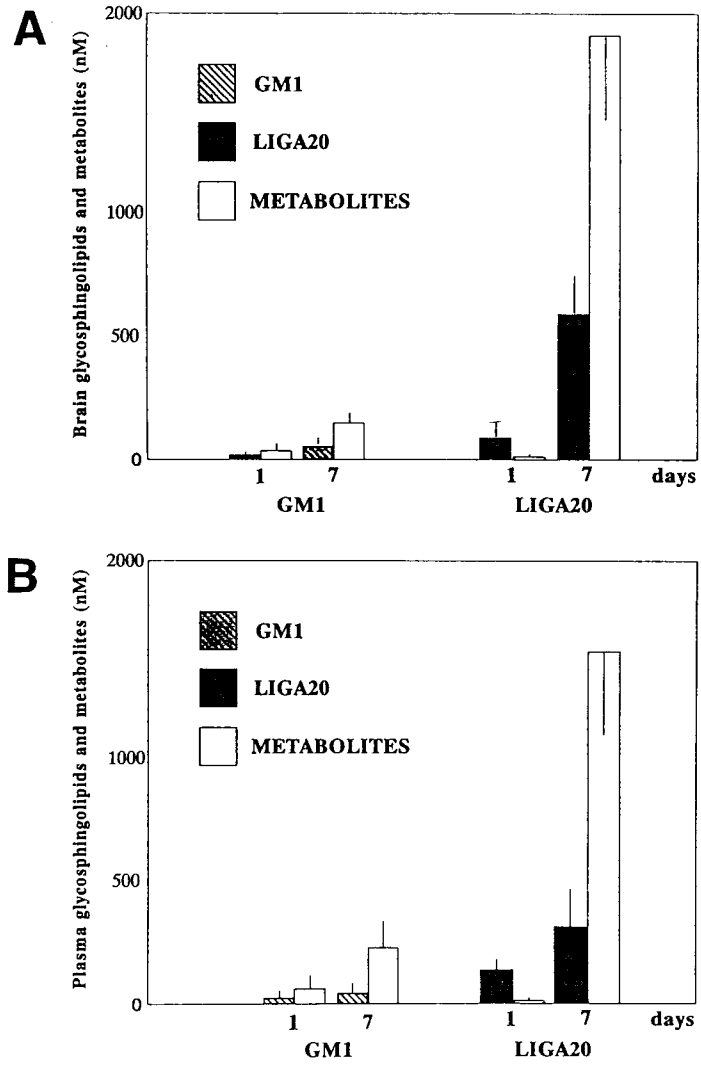

Fig. 4. Brain (Panel A) and plasma (Panel B) glycosphingolipids and metabolites after single (1 d) and repeated (7 d) oral administration of 68 μmol/kg of ^{3}H-GM1 or ^{3}H LIGA20. Each value is the mean ± SEM for 5 rats. The measurements were performed 24 h after the last administration of the glycosphingolipids.

observed in the plasma content of LIGA20 and GM1 (Fig. 4B). Interestingly, in rats receiving repeated oral doses of LIGA20 and GM1 the plasma levels of the two glycosphingolipids were below those determined in the brain. Therefore, contributing minimally to the evaluation of the concentration of the glycosphingolipids in the brain.

An analysis of the radioactivitay found in brain and plasma of rats receiving daily oral doses of [^{3}H]LIGA20 or of [^{3}H]GMI for 7 d indicated that in the aqueous phase the nonvolatile material is represented by

authentic GM1 or LIGA20 (Fig. 2), respectively, whereas the organic phase includes a significant amount of nonvolatile material (Fig. 4) which on HPTLC migrates with a kinetic different from that of LIGA20 or GM1, and presumably represents putative metabolites of the two glycosphingolipids.

DISCUSSION

The beneficial effects reported by various investigators in a number of animal models of Parkinson disease (Hadjiconstantinou et al., 1986; Schneider et al., 1992) and ischemic brain damage (Hoermann, 1988; Karpiak et al., 1990; Seren et al., 1990; Somjen et al., 1990; Bharucha et al., 1991; Costa et al., 1992; Kharlamov et al., 1993) following parenteral administration of GM1 prompted us to investigate whether an oral treatment with semisynthetic gangliosides such as LIGA20 could become a suitable therapeutic strategy to prevent neuronal damage owing to recurrent release of excitotoxins as it may occur in epilepsy (Olney, 1990; Chapman, 1992) or in minor thrombosis (McCulloch et al., 1991; Zivin and Choi, 1991) in elderly hypertensive patients that have already experienced a first episode of stroke, and in Parkinson disease (Klockgether and Turski, 1989), or in cardiosurgery during prolonged extracorporeal circulation or prolonged hypothermic circulatory arrest (Redmond et al., 1993).

It is known that orally administered natural gangliosides (i.e., GM1) are poorly absorbed and a protracted oral treatment with these compounds fails to yield appropriate therapeutic brain levels. Parenteral administration of GM1 is the route of choice when a rapid action is necessary as in the treatment of acute brain damage, but this treatment cannot be used in the protracted treatment of neurodegenerative diseases such as Parkinson disease, epilepsy, recurrent minor thrombosis, or Alzheimer disease. To develop orally absorbed glycosphingolipids we examined a large series of compounds structurally related to GM1, including the sphingosine derivative D-*erythro*-1,3-dihydroxy-2-dichloroacetylamide-4-*trans*-octadecene (PKS3) and the lyso GM1 derivative LIGA4 and LIGA20, which incorporate in the neuronal membrane rapidly and can protect neurones against glutamate toxicity in vitro (Manev et al., 1990a,b).

The physicochemical analysis of a large group of LIGA derivatives has revealed that the critical micellar concentration of LIGA derivatives is several orders of magnitude higher than that of GM1 (Perillo, 1992; Sonnino et al., 1990), therefore enhancing the availability of LIGA monomers in LIGA solutions. The micellar aggregation and the micellar size might be hindering the oral adsorption of natural gangliosides.

Moreover, the absence of the long chain fatty acyl moiety and the presence of chloracetylation at the 2-amino position in the LIGA20 structure (*see* Fig. 1) produces a more condensed behavior of this glycosphingolipid and an increase in its collapse pressure compared to GM1 (Perillo et al., 1993). These two physicochemical properties of LIGA20 reduce the

possibility of self aggregation and limit the molecular area of these aggregates, thereby facilitating and accelerating the LIGA20 insertion in lipid monolayers (Perillo et al., 1993).

Previous pharmacological studies detected that LIGA20 is more potent and longer lasting than GM1 or PKS3 in protecting against excitotoxicity in vitro; moreover, the onset of LIGA20 neuroprotective action is almost instantaneous, whereas that of the other GM1 derivatives requires 30–60 min of preincubation (Manev et al., 1990a).

Encouraged by these in vitro results we decided to study whether LIGA20, the most potent and faster acting semisynthetic derivative in vitro, is also absorbed faster and better than GM1 after oral administration to rats. LIGA20, when administered by oral gavage to fasted animals, in order to minimize adsorption to food components in the digestive tract, crosses the gastrointestinal wall, penetrates into the blood and reaches the brain rapidly. High brain concentrations of LIGA20 are observed already 3–6 h after its oral administration and maximal concentrations persisted for at least 96 h. When we compared the brain content of GM1 and LIGA20 at different time intervals after oral administration of equimolar single oral doses of these two compounds, the brain concentrations of LIGA20 were approx 10–50-fold greater than that of GM1. Moreover, the brain concentrations of LIGA20 were much higher than those of GM1 after repeated oral administrations to rats (Fig. 4). In rats receiving repeated doses of LIGA20 the plasma levels of this glycosphingolipid were below those determined in the brain (Fig. 4B). This supports the view that the plasma contribution to the brain content of this glycosphingolipid is marginal.

Because the LIGA20 is about tenfold more active than GM1 and at 1 μM concentration this glycosphingolipid is pharmacologically active (Manev et al., 1990a), with repeated oral administration of LIGA20, brain concentrations in the range required for protection against glutamate excitotoxicity can be obtained. A general problem with the measurement of glycosphingolipids in brain is whether the brain content represents uptake into neurons and glial cells after passage of the blood-brain barrier or uptake into the brain including the blood-brain barrier and the blood vessels. We have been unable to determine histochemically the location of LIGA20. However, we have demonstrated that LIGA20 administered orally in doses of 68 μmol/kg protects rats from neuronal damage induced by thrombotic stroke even if this glycosphingolipid is administered several hours after the occurrence of the stroke (Kharlamov, 1993) indicating that LIGA20 is taken up in pharmacologically active concentrations in the brain tissue itself. Because LIGA20 has a long biological half-life (more than 48 h, *see* Fig. 3) the desired drug concentration in the brain probably could be maintained with only two or three oral doses per week. Interestingly, after protracted treatment with LIGA20 a significant amount of ganglioside lipid metabolites accumulate in brain and plasma. In primary cultures of cerebellar neurones, Pitto et al. (1991) reported that a signifi-

cant percentage (30%) of LIGA20 added to the culture is transformed to PKS3. We have not been able to determine the molecular nature of the LIGA20 metabolite(s) present in the organic phase of brain extracts, but if the major metabolite of LIGA20 found in brain will turn out to be PKS3 then such a metabolite will favor the therapeutic action of LIGA20 because PKS3 in vitro has been shown to protect against glutamate neurotoxicity, with an efficacy comparable to that of GM1. Moreover, PKS3 contains a dichloroacetyl group, and this indicates that LIGA20 during its metabolism in vivo might not release the dichloroacetyl group, which may be toxic.

In summary, there are a number of chronic neurodegenerative disorders in which glutamate may be a factor in the progression of the disease. Thus, LIGA20, an orally active RADA drug (Manev et al., 1990b), which has a long lasting biological half-life, could become an important therapeutic agent in Parkinson disease, dementia, epilepsy, and other neurodegenerative disorders mediated by the production of excitotoxins.

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