NATURAL AND SYNTHETIC GANGLIOSIDES AMELIORATE CALCIUM-INDUCED CYTOTOXICITY

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ABSTRACT

Apoptosis, or cell death, occurs as a result of calcium influx in the neuronal cell. To prevent cell apoptosis, one must control the entrance and exit of calcium ions. This study was conducted to compare the ability of two different compounds, LIGA-20 and GM1, to control calcium ion levels within two neuroblastoma cells, NG108 and NG-CR72. While the NG108 cell line contains the naturally occurring GM1 ganglioside, this compound is absent from the mutant NG-CR72 cells. However, NG108 cells have properties more similar to the neurons found in the brain. Different types of cells were used in order to determine whether an unnatural amount of GM1 ganglioside affects control of calcium ion levels. Intracellular calcium ion levels were observed by staining the cells with fluo-4 dye and observing the intensity of the resulting florescence. LIGA-20 was found to have better control over the calcium-ion level within both NG108 and NG-CR72 cells. This can be attributed to the smaller size of LIGA-20, a property that enables this ganglioside to pass through the plasma membrane and insert itself into the nuclear membrane. In effect, LIGA-20 provides a second line of defense from the influx of calcium ions. Also, the smaller size of LIGA-20 enables it to quickly travel through the plasma membrane. This results in a shorter time for LIGA-20 to attach itself to the nuclear membrane and could be the difference between cell survival and apoptosis. Finally, unnaturally increased levels of GM1 had a negative effect on NG108 cells; the calcium ion level increased when only GM1 was added to the cell culture. The results of the study therefore suggest that LIGA-20 is a more effective controller of intracellular calcium ion levels.

Keywords: Apoptosis, gangliosides, GM1, LIGA-20, neuroblastoma cells, NG108, NG-CR72, intracellular calcium ion levels

INTRODUCTION

Acute spinal cord injury (SCI) is a life-altering event in which the extent of resultant disability depends on the initial site and severity of injury as well as whether medical therapy may intervene in a timely fashion to prevent the subsequent secondary degeneration. Globally, acute SCI has an annual occurrence rate of twenty to forty persons per million; the estimated economic impact in the United States alone is over $4 billion per year [1]. Considering the financial and emotional burdens that afflict acute SCI patients and their families, development of effective treatment would be highly beneficial.

SCI is known to have both a primary and secondary process. A blunt contusion, compression, or transection initiates trauma to the spinal cord neurons. This is often followed by

secondary tissue damage in neighboring regions in the days and months following the primary injury. The initial trauma gives rise to necrotic cell death in the immediate area surrounding the lesion. Energy loss, passive cell swelling, and intense mitochondrial damage disrupts internal homeostasis and leads to membrane rupture as well as cell lysis. Considerable evidence suggests that the extracellular environment of the spinal cord neurons is altered by infiltration of inflammatory mediators and cytoplasmic leakage of excitatory neurotransmitters such as glutamate [2].

These cytoplasmic contents have the ability to move to different levels of the spinal cord, where they proceed to harm the other nerve cells. The region of surrounding nerve cells, harmed by the neurotransmitters released by the primary injury site, is known as the area penumbra, the secondary site of injury. Damage to the area penumbra usually occurs within a few hours of the injury [2]. This is considered a serious problem, since it increases consequences of neuronal damage and thus greater functional deficit. Subsequent damage to neurons up to two spinal segments above and below the initial lesion often renders a patient more disabled than the primary injury alone would have [2].

The contents of the lysed nerve cells can offset the natural homeostasis of the surrounding nerve cells. The abnormal levels of glutamate cause excitotoxicity in the area penumbra. This excitatory amino acid neurotransmitter binds to its complementary target receptors, causing an influx of calcium ions into the cell. Compared to the immediate shock involved in primary cell death, the cells in the area penumbra are slow to react to the glutamate. Nevertheless, the amount of Ca^{2+} within the cell eventually accumulates to a toxic level and initiates apoptosis, or programmed cell death.

Apoptosis is a normal and necessary part of the cell life cycle. A prevalent example of this would be during the development of the central nervous system in embryos. In this process of differentiation, cells are sloughed off in the retina, the regio olfactoria, and the regio respiratoria [3]. The mechanism for cleaving these cells is apoptosis. When apoptosis does not function properly, major problems may arise for an organism. Cancer is a primary example of this. Normally, cells acting in the same manner as cancer cells would initiate apoptosis. However, for some reason, cancer cells, avoid the signal and process of apoptosis, and continue to grow and harm the body. Cells that have sustained genetic damage are not destroyed but divide to give rise to cancerous tumors. Too little apoptosis has also been associated with immune system disorders such as rheumatoid arthritis and systemic lupus erythematodes [3]. On the other hand, an excess of apoptosis leads to out-of-control cell death that has been linked with neurodegenerative diseases associated with spinal cord injury, Alzheimer's disease and stroke damage to neurons [4].

Several mechanisms exist for apoptosis: one generated by signals sent from within cells, and one created by death activators such as TNF-α, lymphotoxin, and Fas ligand (FasL) [5]. Signals from within the cell are often caused by internal damage to the cell, such as an excess of intracellular Ca^{2+} concentration. Calcium is a critical component in normal neuron function, because it regulates the activation of numerous cytoplasmic enzymes [5]; Cells can only be supported when intracellular Ca^{2+} concentration is within the window of 320-390 nM [6]. Concentration of intracellular Ca^{2+} is controlled by membrane potassium-calcium pumps, Ca^{2+}

ATPases, and organelle sequestration especially by mitochondria and the endoplasmic reticulum (ER) [5]. Upon the incidence of Ca^{2+} cytotoxicity, this ion may enter the nucleus in excessive amounts and interfere in normal transcription. As a result, transcription of mRNA for apoptosis-specific proteins begins and eventually causes a complete halt of normal cell metabolism, ending in complete cell death.

Few pharmacological agents, both natural and synthetic, have demonstrated a clear and more effective role in mediating influx and efflux of neuronal Ca^{2+} than ganglioside GM1 (monosialoglycosylceramide) [7,8]. The presence of this ganglioside results in the rescue of cells that would otherwise die via glutamate excitotoxicity [9,10]. GM1, regularly present in both the plasma and nuclear membrane, has been shown to control Ca^{2+} movement in and out of the cell. Wu & Ledeen (1991) proved that neuritogenesis in many different neuroblastoma cells depends on calcium influx into the cell [7]. Later, they showed the relationship between GM1 and the nuclear membrane Na+/ Ca^{2+} exchanger in NG-108 neuroblastoma cells [8]. Calcium cytotoxicity studies have also shown significant neuroprotection by GM1 and LIGA-20 as measured by MTT assay [11].

However, it has been questioned as to whether exogenously administered GM1 may function to its fullest potential. Physicochemical properties of exogenously applied GM1 provide a challenge to its insertion into the plasma membrane, and it does not insert at all into the nuclear membrane [11].

In contrast, LIGA-20 is a semisynthetic derivative of GM1 in which the fatty acid tail at the 2-amino position is replaced by a dichloroacetyl group [12], which has a greater capacity for membrane passage and insertion; it is permeable to the blood brain barrier which makes it much more useful than GM1 in clinical scenarios (Fig 1). For this reason, LIGA-20 is expected to be an important molecule used to treat Ca^{2+} -induced secondary cell death in nerve cells.

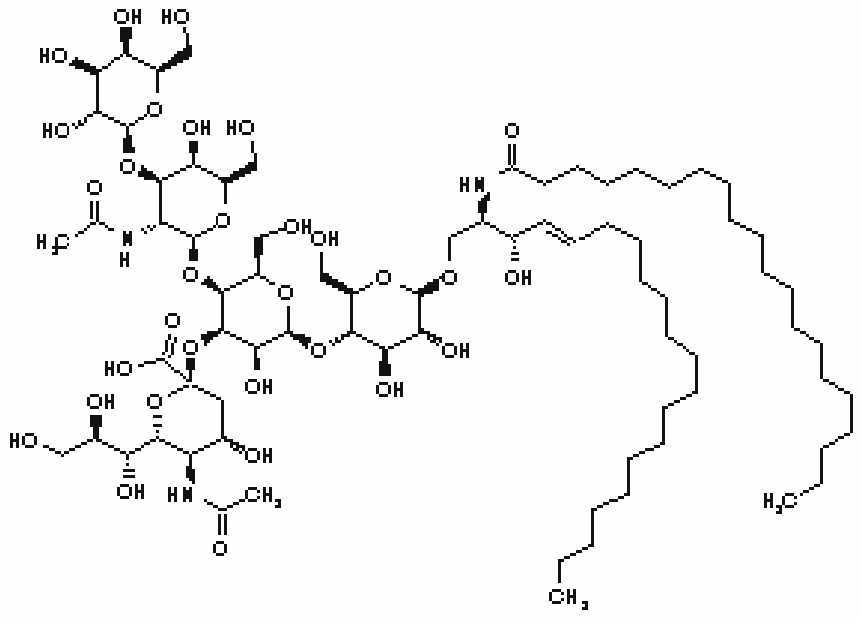

Fig. 1: Structure of GM1 ganglioside (Courtesy of NIAID)
<http://apps1.niaid.nih.gov/struct_search/class/class_many.asp?class=GLYCOLIPIDS>

Various neuroblastoma cells have been used to study ganglioside function. Two types of neuroblastoma cells are known for their relatively high content of a series of gangliosides. Neuroblastoma Glioma 108 (NG-108) cells are commonly used for neurotogenic studies and experiments. This variety of neuroblastoma usually contains GM1 along with GD1a, both in the gangliotetraose family of gangliosides, in both cell membranes and nuclear envelopes. NG-108 cells are able to respond to an assortment of stimuli; one common response is neurite germination. Neurites are the long ends and projections extending from the neuron's cell body, which include dendrites and usually a single axon, used for connecting with other neurons and to pass information through changes in membrane potential. In the presence of axonogenic substances, which would normally cause the outgrowth of axons, NG-108 cells acquire an excess of gangliosides in the nuclear membrane [11].

When there is an overabundance of calcium in the extracellular space, calcium diffuses into each cell through ion channels. The neuron cannot handle high levels of calcium influx, so it initiates a signal-transduction pathway that leads to apoptosis. In neurons of living organisms, this abundance of calcium ions usually occurs when glutamate binds to its receptor and opens ion channels [5]. Ionomycin, a compound similar to glutamate, also causes increased calcium ion influx. In order to recover from such an overload of calcium, protection is needed, which may be provided by gangliosides, particularly GM1 and its derivative LIGA-20.

In previous research, NG-108 cells were cultured in the presence of cholera toxin B, which is specific for GM1 [11]. After exposure to the pathogen, the survivor cells were analyzed, and a specific sub-clone of the neuroblastoma NG-108 was identified, sequestered, and named. The sub-clone, Neuroblastoma Glioma-CR 72 (NG-CR 72), is a mutated variety whose identifying characteristic is its lack of a series of gangliosides, specifically GM1 and GD1a in the plasma and nuclear membranes. In addition, NG-CR 72 cells lack UDP-Gal: GMZ galactosyltransferase, and GM1 synthase [11]. As a result, these neuroblastoma cells have a deleterious response to axonogenic stimuli (i.e. potassium chloride, ionomycin), substances which would normally cause an increased influx of Ca^{2+} with subsequent outgrowth of axons. NG-CR 72 cells respond by producing and extending aberrant dendrites from the central body. Furthermore, when treated with substances allowing for elevated influx of Ca^{2+}, the levels of the ions, which were dangerously high, were maintained. In turn, calcium-induced apoptosis occurred among the cells.

These NG-CR 72 cells are a good model to study secondary cell processes because they lack GM1 ganglioside and in essence, serve as a knock-out model. Since there is no endogenous GM1, the amount of the exogenously applied ganglioside used in testing serves as a more precise quantification.

The experiments described below explore the abilities of GM1 ganglioside and its derivative, LIGA-20, to protect cells from calcium-ion influx. Originally, it was hypothesized that both GM1 and LIGA-20 would be capable of maintaining calcium homeostasis. However, it was also theorized that LIGA-20 would be more effective than GM1 and perform more efficiently in maintaining calcium homeostasis.

METHODS AND MATERIALS

NG-108 and NG-CR 72 neuroblastoma /glioma hybrid cells were grown in T-25 flasks in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). This medium was supplemented with gentamycin (50 µg/mL), penicillin (50 units/mL), and streptomycin (50 µg/mL) The cell cultures were then placed into a 37^{o} C incubator containing 5% CO_2/95% humidified air. For all conducted studies, the cells were seeded at a density of 4 X 10^4 cells/mL in 24-well culture plates. After 24 hours, cells were treated with either 100 µM GM1 or 50 nM LIGA-20 in 1.5% FBS / DMEM with N2 supplements (experimental medium). After twenty-four hours, these cells received 1 µM ionomycin over a period of two hours in the following manner: ionomycin alone, GM1 + ionomycin, and LIGA-20 + ionomycin. The controls setups were as follows: untreated cells, GM1 alone, and LIGA-20 alone (see Fig 2).

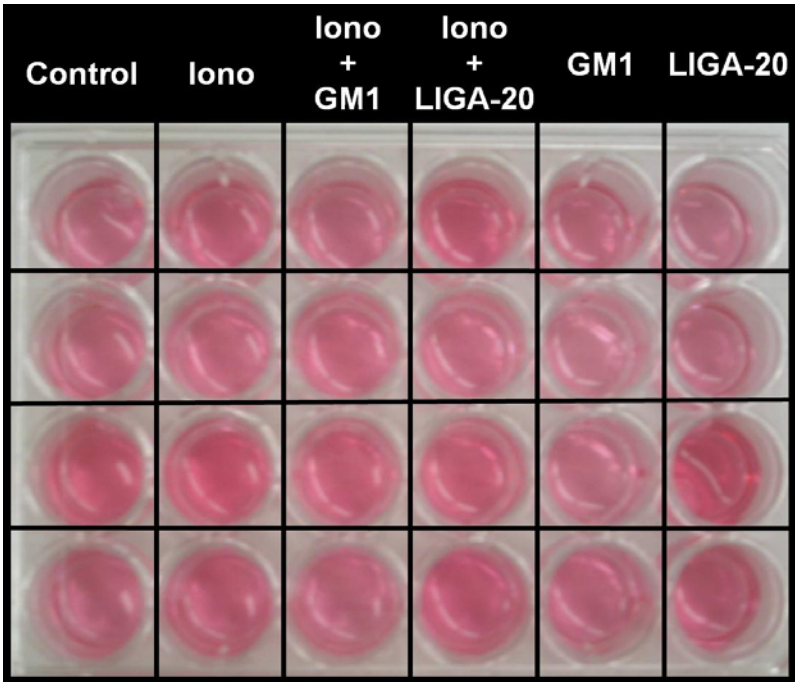

Fig 2: 24 well culture plates were seeded with either NG-108 cells or NG-CR 72 cells. They were then exposed to the chemicals and organic compounds as demonstrated above.

In the experiments involving the differentiated NG-108 line, cells were seeded as above, then incubated for 24 hours. After completion of the incubation period, the cells were treated with the two differentiating agents: 30 mM KCl and 1 mM cAMP in experimental medium as per Kozireski-Chuback *et al* (1999) [14]. After five days, differentiated cells were treated for two hours with several combinations of agents. One group of neurons was exposed to LIGA-20 and 1

μM of ionomycin, while the other received only ionomycin. The control treatment was untreated cells. Preliminary work was completed on GM1 + 1 μM ionomycin and a favorable result was obtained; however, time did not allow for a complete evaluation of this application.

CALCIUM IMAGING

After incubation with ionomycin, the cells were incubated with experimental medium + 1 μM fluo-4 (molecular probes) for ten minutes. This treatment was followed by a wash-out with experimental medium for thirty minutes. Cells were first observed under phase contrast microscopy through use of a Nikon fluorescence microscope (Eclipse TE300). Afterwards, fluorescence imaging was conducted with an FITC filter.

DATA ACQUISITION

Computer software Image Pro Plus, Version 4.1 was employed to measure the fluorescence intensity of fluo-4 in the cells as well as the background. Relative fluorescence was calculated as the difference between the mean values of cell fluorescence intensity and background intensity.

Microsoft Excel was then utilized to perform statistical analysis on the data. The t-test: two sample assuming unequal variances was used to determine the t-statistic and p-value for each of the following pairs:

Table 1: Pairs of Cell Types Statistically Compared

	Undifferentiated NG-108 Cells	**Differentiated NG-108 Cells**	**NG-CR 72 Cells**
Control vs. Ionomycin	**X**	**X**	**X**
Control vs. Ionomycin + GM1	**X**		**X**
Control vs. Ionomycin + LIGA-20	**X**	**X**	**X**
Ionomycin vs. Ionomycin + GM1	**X**		**X**
Ionomycin vs. Ionomycin + LIGA-20	**X**	**X**	**X**

Table illustrates which pairs of cell types were statistically compared with Microsoft Excel. With the differentiated NG-108 cells, there were only three comparisons completed since there was no data for NG-108 differentiated cells treated with GM1 alone.

RESULTS

In terms of gross morphology, mutant NG-CR 72 cells were somewhat smaller than wild type NG-108 neuroblastoma cells and extended small, retractable neurites in the undifferentiated state (Fig. 3a and 3b Showing phase contrast micrographs).

Their doubling time was twenty-five 5 hours in comparison to twenty-two hours in NG-108. Upon treatment with Ca^{2+} ionophore, ionomycin, NG-CR 72 morphology changed within ninety minutes such that cell bodies began to shrink, round up and retract their small neurites (Fig 3d). NG-108 cells were somewhat more hardy in that similar changes in cell morphology began occur after two hours post-ionophore treatment (Fig 3c).

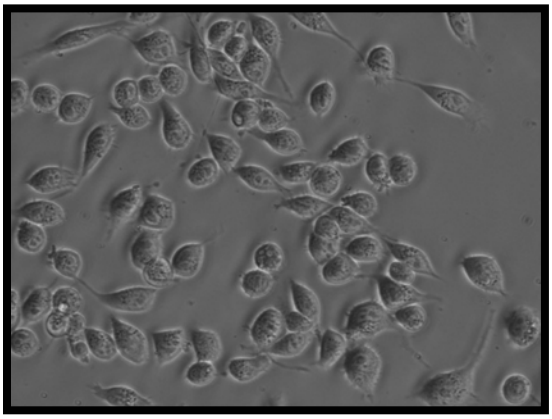

Fig 3a: NG-CR 72 Control (200 X)

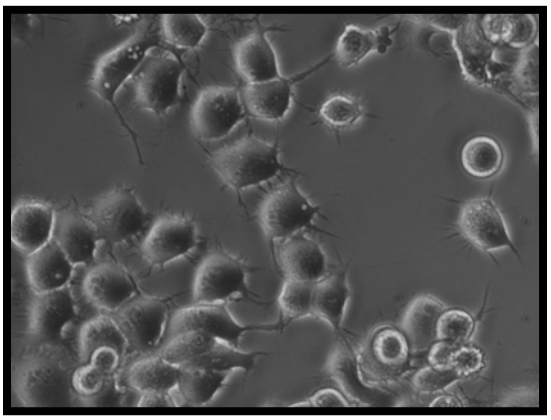

Fig 3b: NG-108 Undifferentiated Control (200 X)

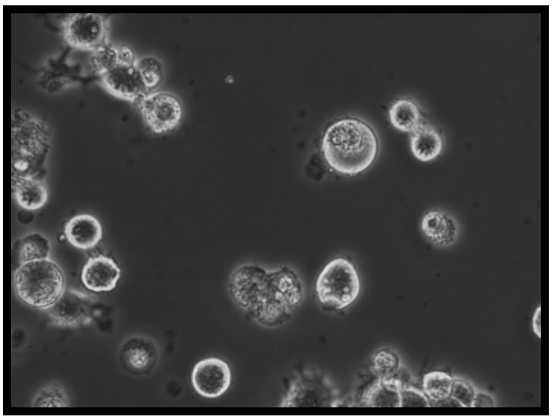

Fig 3c: NG-108 Undifferentiated Ionomycin (200 X)

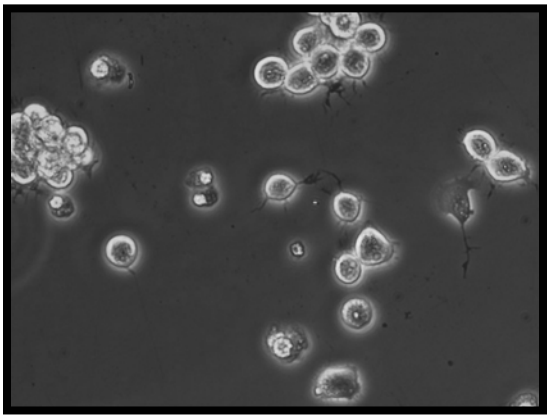

Fig 3d: NG-CR 72 Undifferentiated Ionomycin (200 X)

Ca^{2+} imaging in NG-108 revealed qualitatively higher fluorescence intensity in ionophore-treated cells than in controls and in either of the experimental groups (Fig 4a and 4b). Mean intensity levels were significantly higher in the ionophore alone group compared to the group pretreated with GM1 before ionophore exposure ($p<0.001$) (Table 2, Fig. 5). Similarly,

those cells pretreated with LIGA-20 before ionophore exposure had an even lower mean fluorescence than cells treated only with ionophore ($p<0.001$).

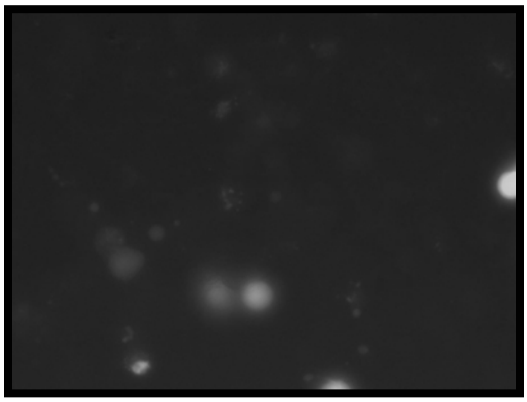

Fig 4a: NG-108 Control cells (Fluorescence Imaging)

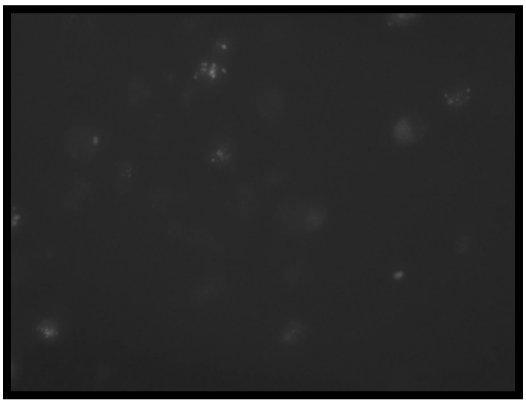

Fig 4b: NG-108 Ionophore-treated cells (Fluorescence Imaging)

Table 2: NG-108 Cell Treatment Data

Cell Type	Control	Ionomycin	Ionomycin + GM1	Ionomycin + LIGA-20	GM1	LIGA-20
Undifferentiated NG-108 Cells	41.5732	99.5720	45.8084	19.8881	57.2733	23.4642

Intensity of Fluo-4 staining of undifferentiated NG-108 cells. Their just means were then recorded after running histogram interpretations of their fluorescence imaging. The results are justified by the research and hypothesis with only few deviations.

The cells treated only with ionomycin had an increase of almost two hundred percent that of the control in calcium concentration. Cells treated with GM1 and LIGA-20 gangliosides along with the ionomycin were shown to bring the concentration down to normal and below normal calcium levels, respectively. An anomaly in this particular experiment was that of the GM1 alone. The cells exposed only to GM1 were shown to have higher calcium concentrations than the control and the GM1 + ionophore wells. However, this anomaly can be attributed to the fact that this group erroneously received a higher GM1 in the first experiment. As a result, the GM1 levels may have been slightly toxic to the cell, thus leading to higher calcium fluorescence.

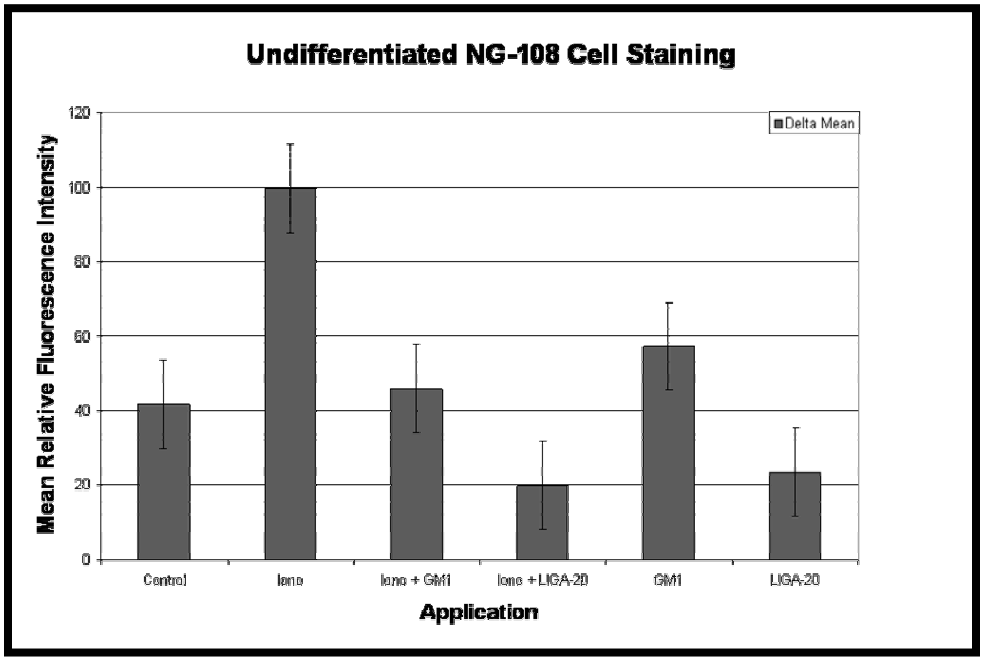

Fig 5: Delta means taken from fluorescence imaging and histogram results. The numbers represent the intensity of the calcium in the cells. This graph of the data from Table 2 clearly shows that cells treated purely with ionomycin were more susceptible to Ca^{2+} (n=2).

The levels of calcium within cells treated with both ionomycin and GM1 were brought down to about 45 relative intensity, which is not statistically different from the control cells, showing that the extra exogenous GM1 had a positive effect on the cells. The GM1 alone, however, was only able to bring calcium concentration down to the upper edge of the control range, showing that GM1 had essentially no effect on unstressed neuroblastoma cells. The effects of LIGA-20 were much more than the effects of GM1. Calcium levels in LIGA-20 treated cells were brought down to twenty-three relative intensity, which is well below the calcium concentration level of the control cells. This may have occurred because the wells containing cells treated with only LIGA-20 may have not had a chance to be incubated for a sufficient period of time. Consequently, these cells did not contain enough fluo-4 calcium ion indicator to show up underneath the fluorescent light in the inverted microscope.

Ca^{2+} imaging in mutant NG-CR 72 cells also revealed higher fluorescence intensity in ionophore-treated cells than in control or experimental cells. (Fig 6a and 6b). Mean intensity levels were once again significantly higher in ionophore treated cells. A similar trend of concentrations was observed once again; however, the effect of GM1 compared to controls is more apparent in the NG-CR 72 cells ($p<0.001$) (Table 3, Fig. 7). Cells treated with both LIGA-20 and GM1 displayed much lower levels of calcium than the control ($p<0.001$).

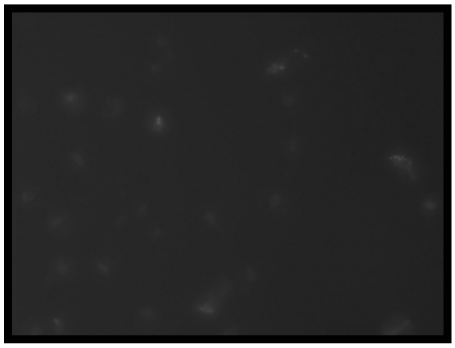

Figure 6a: NG-CR 72 Control cells (Fluorescence Imaging)

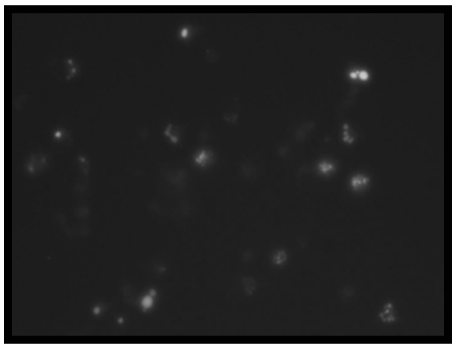

Figure 6b: NG-CR 72 Ionophore-treated cells (Fluorescence Imaging)

Table 3: NG-CR 72 Cell Treatment Data

Cell Type	Control	Ionomycin	Ionomycin + GM1	Ionomycin + LIGA-20	GM1	LIGA-20
NG-CR 72 Cells	20.9116	149.2610	46.7138	31.7322	10.9438	9.2250

Delta mean intensity values from calcium imaging of fluo-4 treated NG-CR 72 cells. The ionomycin is much more drastically deleterious to the mutant cells here than the wild type NG-108 cells in the previous well example. A similar number trend is observed as well.

Here as well, the cells treated with ionomycin had a greater increase in calcium concentrations than the NG-108 cells treated with ionomycin. Cells treated with both GM1 and LIGA-20 along with the ionomycin had calcium concentrations brought down near the normal control value, showing an effective job of both GM1 and LIGA-20 in protecting the cells from calcium. Almost on the verge of hypo-calcium concentrations, the cells treated with GM1 and LIGA-20 alone had such miniscule traces of calcium that the stained calcium was hardly visible during fluorescent data collection.

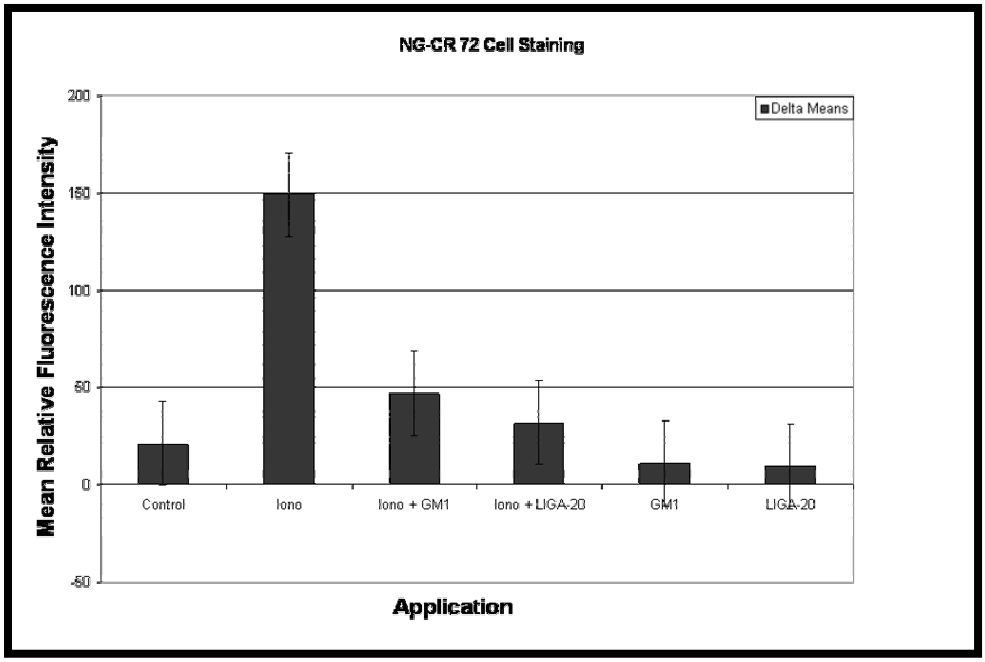

Figure 7: Fluorescence imaging and histogram results recorded in the data table show the predicted trend of the calcium in cells. There is a deviation in the activity of GM1 in that it seemed to act as predicted opposed to the effect it had demonstrated in the NG-108 cells.

Once again, the levels of calcium in the ionomycin + GM1 cells were as low as the range of the control cells, however, they were relatively higher than the NG-108 cells, probably due to the absence of naturally occurring GM1. Another major difference between the NG-108 cells and the NG-CR 72 cells lies in the GM1 alone wells. GM1 managed to significantly lower the level of calcium within the NG-CR 72 cells, whereas in NG-108, the levels of calcium for the purely GM1 wells were not lowered below that of the control.

The NG-CR 72 cells that were treated purely with ionomycin also proved to be different from the NG-108 cells. Calcium concentration within the mutant cells treated with ionophore was brought up to nearly 150 fluorescence per pixel, as opposed to the NG-108 cells, where the concentration was only brought up to about 100. This is yet another indication that the lack of inherent endogenous GM1 has a negative effect on NG-CR 72's ability to extrude Ca^{2+} from the cell.

Since the NG-CR 72 cells had no GM1 to begin with, they serve as good basis for the comparison of GM1 and LIGA-20. LIGA-20 was more effective in the NG-CR 72 cultures. LIGA-20 was about 15 relative fluorescence per pixel more effective in extracting the calcium ionophores from the cell in NG-CR 72 cells than were the GM1. This is evidence that LIGA-20 is more effective in the prevention of calcium induced apoptosis.

DIFFERENTIATED NG-108 DATA AND ANALYSIS

The differentiated NG-108 cells, which were used to simulate a more realistic SCI situation, revealed similar results in that the ionophore-treated cells showed the highest concentration of calcium relative to the control and LIGA-20/ionomycin–treated cells (Fig 8a and 8b). Mean intensity levels in the ionophore treated cells were greater than those of the LIGA-20 and control cells for obvious reasons: the control cells were not stimulated to accept Ca^{2+} influx, and the LIGA + ionomycin cells were able to block calcium influx with the effects of LIGA-20 (Table 4, Fig 9).

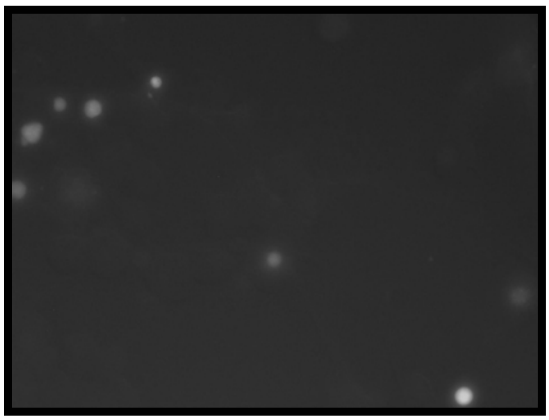

Fig 8a: Differentiated NG-108 cells with Ionomycin
(Fluorescence Imaging)

Fig 8b: Differentiated NG-108 cells with Ionomycin and LIGA-20
(Fluorescence Imaging)

Table 4: Differentiated NG-108 Cell Treatment Data

Cell Type	Control	Ionomycin	Ionomycin + LIGA-20
Differentiated NG-108 Cells	41.5732	76.2260	35.0461

The differentiated NG-108 cells were tested with Ionomycin alone, and Ionomycin + LIGA-20. The Ionomycin still managed to make the calcium concentration rise above normal levels. LIGA-20 was once again successful in helping to ameliorate the situation.

The differentiated NG-108 cells also showed that LIGA-20 is effective in extruding calcium. When the cells were treated with ionomycin alone, the mean was 76.226 fluorescence per pixel, and when treated with ionomycin and LIGA-20, the mean value lowered to 35.046 fluorescence per pixel. Thus, even differentiated cells, which have a natural defense against calcium compare to undifferentiated cells, are still less efficient in extruding Ca^{2+} as cells treated with LIGA-20.

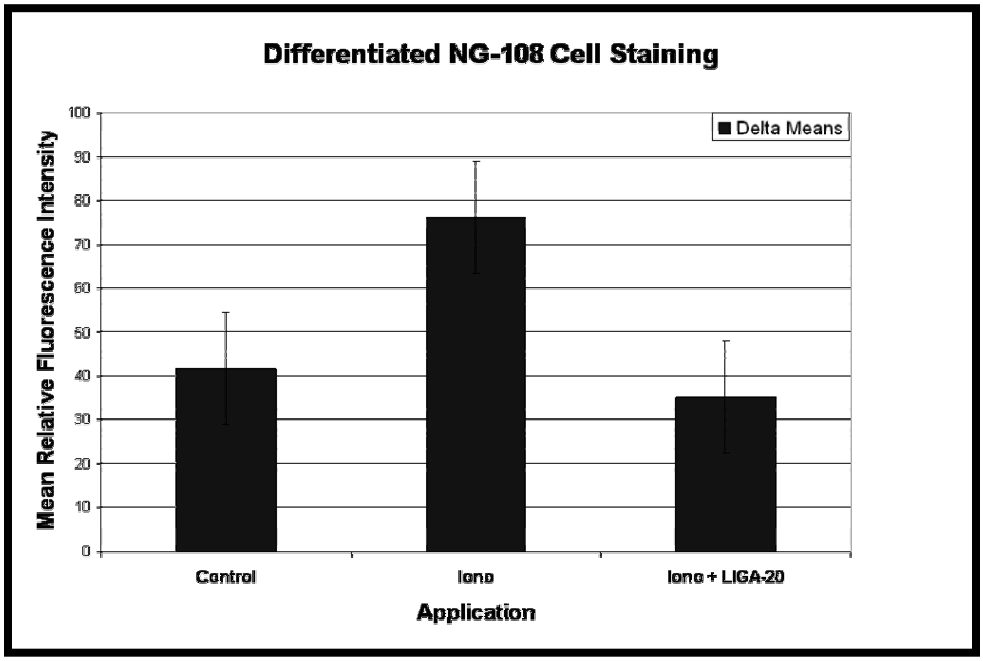

Fig 9: Calcium intensity in differentiated NG-108 cells. Here, ionomycin-treated cells expressed a higher concentration as usual; however, it wasn't as drastically high as the other NG-108 cells. This was probably due to the fact that the differentiated NG-108 cells are more mature and have more inherent gangliosides to defend against cytotoxic levels of Ca^{2+}.

The hypothesis is further supported by the data from the NG-CR 72 cells, which do not contain the ganglioside GM1. When the cells were treated with LIGA-20 alone, the results showed that more calcium was ejected than when it was treated with any other combination of compounds. As expected, GM1 worked the second best in expelling calcium, followed by ionomycin and LIGA-20 which had a mean value of 31.732 fluorescence per pixel. As mentioned above, GM1 did not work as efficiently as LIGA-20 but was still able to halve the control mean value to 10.944 fluorescence per pixel. Finally, when only ionomycin was added, calcium entered the cells in great quantities and the mean value shot up to 149.261 fluorescence per pixel.

STATISTICAL ANALYSIS

As aforementioned, statistical analysis of the data was conducted using Microsoft Excel with the t-test analysis, using two samples with unequal variances. The following data was calculated using the analysis:

Table 5: Statistical Examination of Data

Statistical Comparison Pair	t-statistic	p-value
Undifferentiated NG-108: Control vs. Iono.	-3.68	**0.001**
Undifferentiated NG-108: Iono. vs. Iono. + GM1	3.62	**0.001**
Undifferentiated NG-108: Iono. vs. Iono. + LIGA-20	5.38	**1.21777E-05**
Undifferentiated NG-108: Control vs. Iono. + GM1	-0.41	0.344
Undifferentiated NG-108: Control vs. Iono.+ LIGA-20	2.11	**0.024**
NG-CR 72: Control vs. Iono.	-5.27	**0.017**
NG-CR 72: Iono. vs. Iono. + GM1	4.00	**0.014**
NG-CR 72: Iono. vs. Iono. + LIGA	4.89	**0.020**
NG-CR 72: Control vs. Iono. + GM1	-2.39	**0.020**
NG-CR 72: Control vs. Iono. + LIGA	-1.73	0.055
Differentiated NG-108: Iono. vs. Iono. + LIGA-20	2.21	**0.046**
Differentiated NG-108: Control vs. Iono.	-1.96	0.072
Differentiated NG-108: Control vs. Iono. + LIGA-20	0.50	0.315

Table 5: Statistical analysis of all cell types. Using an α level of 0.05, all significant p-values are emboldened.

As shown above, almost all t-tests resulted in significant p-values with an α level of 0.05. However, 4 p-values were insignificant. These results indicate that the LIGA-20 and GM1 were statistically successful in regulating calcium influx into neurons.

DISCUSSION

As predicted, results from our experiments show that, in general, the semi-synthetic organic compound LIGA-20 is more efficient at preventing ionomycin induced calcium influx than its natural counterpart, GM1 ganglioside. This corroborates previous studies which show that LIGA-20 inserts itself into the nuclear membrane as well as the plasma membrane, thus providing a second line of defense against cytotoxic calcium ion influx [13,11]. GM1 and other gangliosides are naturally incorporated into the nuclear envelope during neurite development [14], but the concentrations are perhaps too low to deal with sudden calcium influx induced by overstimulation of glutamate receptors.

Surprisingly, when GM1 was used alone in NG-108 undifferentiated cells, intracellular calcium levels increased by about 15.700 relative intensity (Table 2). Such an increase could potentially force the cell into apoptosis. An explanation for the aforementioned phenomenon is that there are limitations to the amount of GM1 ganglioside that may be used to control calcium levels inside the cell. As the levels of GM1 reach this toxic level, the effect of GM1 backfires, and more calcium could flow in. This hypothesis is supported by the finding that GM1 alone is more effective in the mutant NG-CR 72 cells, which normally do not have GM1 in the membrane.

When analyzing the statistics of NG-CR 72 cells, it was discovered that when comparing the two treatments of the control cells versus the ionomycin + LIGA-20, no statistical significance was found. This indicates that the LIGA-20 brought sick neurons

back to the same calcium level as control cells, which illustrates that LIGA-20 is an extremely well-equipped ganglioside. LIGA-20 brings unhealthy cells back to the same homeostatic calcium level as the healthy cells in the control group.

Another unexpected result was the effect of LIGA-20 alone in NG-108 undifferentiated cells. At a 100 nM concentration, there was no visible fluorescence although there were healthy cells visible in the phase-contrast view of the field (Fig 10a and 10b).

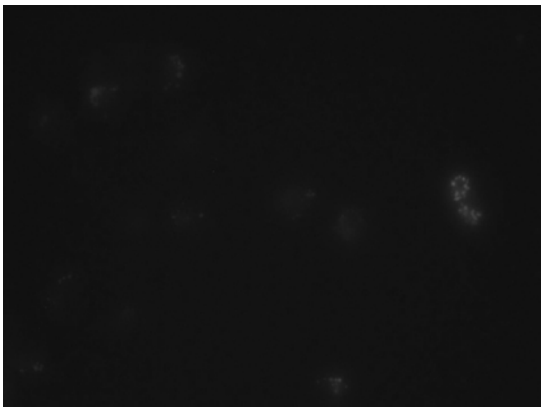

Fig 10a: Undifferentiated NG-108 treated with LIGA-20 (Fluorescence Imaging) (200 X)

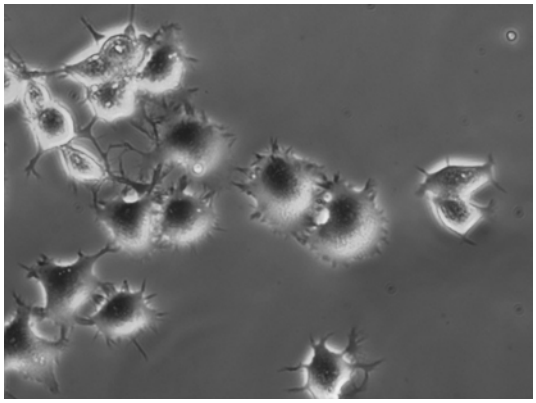

Fig 10b: Undifferentiated NG-108 treated with LIGA-20 (Phase-Contrast View) (200 X)

There were many possible sources of error. One problem involved low cell density in solution, thereby leading to varying cell counts in culture. Nevertheless, the project was not adversely affected by this factor. In the worst case scenario, the group could have had another case of excess concentration of GM1 ganglioside per cell, and, as mentioned before, high levels of GM1 could have led to severely toxic levels of the compound, thus creating the potential for cell toxicity before the application of any ionomycin.

Incomplete fluorescent staining also occurred due to uncontrollable factors such as variable cell uptake of the dye, florescent calcium indicator-4 (fluo-4). Also, time constraints limited the optimal incubation time for the NG-108 and NG-CR 72 cells. Therefore, the cells may not have been ready for examination, and perhaps the group was not seeing the entire effect of the compounds on each cell culture.

Indeed, the results are supported by previous research done in this area. Research done in 2001 has already confirmed that LIGA-20 has aided in controlling calcium-induced apoptosis in NG-CR 72 cells [11]. Additional studies in the effectiveness of LIGA-20 and GM1 gangliosides have suggested that both will control the calcium levels within the cell; however, LIGA-20 tends to be more efficient in this process and will be able to limit the site of injury [13]. Gangliosides have also been found to play an

important role in regulating intercellular calcium levels, and therefore are critical to neuronal development [6].

Possible future experiments could consider factors such as relative concentrations of GM1 and LIGA-20. For example, GM1 may have demonstrated greater efficiency than LIGA-20 if there had been higher concentrations of GM1 in the extracellular environments, although concentrations in the aforementioned experiments were derived from previously published data [6]. Such experiments could have similar setups, but with increased numbers of cell cultures to allow for more variables such as time and concentration. In addition, further research into the possibly detrimental nature of LIGA-20 could be conducted. Because real neurons must use glutamate in controlling calcium influx, research is still needed in other cell models containing glutamate receptors to investigate the effects of LIGA-20. Because the results of clinical trials of the use of GM1 in spinal cord injury proved to be marginal [15], the clinical application of Liga-20 could be more significant and effective. LIGA-20 can be administered orally, and it can cross the blood-brain-barrier (BBB). Thus, further basic science research must be done so that clinical trials can be justified, and perhaps LIGA-20 can be utilized in humans.

However, other research could be conducted by reversing the process. Instead of adding LIGA-20 to the cell culture before treating it with ionomycin, cells could be pretreated with ionomycin before the addition of LIGA-20. This way, the optimal method for implanting LIGA-20 would be found.

Additional studies into the nature of NG-CR 72 differentiated cells are needed as well. Because of time restraints on this project, investigation into the effects of LIGA-20 and GM1 in NG-CR 72 differentiated cells could not be conducted. However, the db and KCL treatment used to differentiate NG108 cells does so through a calcium ion deposit process [16]. Preliminary experiments have shown this method of differentiation to be toxic to NG-CR 72. Many suppose that this is a result of the lack of GM1. Future research could address the ability of NG-CR 72 to differentiate in this way after having been pre-treated with LIGA-20 first. On the other hand, continued research on the topic could yield other compounds that would have a more beneficial result than LIGA-20. Such compounds could provide even greater control of calcium ion influx by inserting themselves not only into the nuclear membrane. Additionally, studies examining the distribution of calcium levels in healthy versus stressed cells should be done through mitochondrial staining.

In fact, use of the information obtained could have numerous applications in the medical field. For example, instead of using GM1 ganglioside to control cell apoptosis after spinal cord injury, the medical profession could employ LIGA-20 to perform the same task. Perhaps LIGA-20 could be used to treat patients suffering from epileptic seizures and other similar neurological disorders by inhibiting the misfiring of ion potential.

In conclusion, semi-synthetic LIGA-20 is clearly more efficient than GM1 ganglioside in preventing ionomycin-induced calcium influx in both NG-108 and NG-CR

72 neuroblastoma cells. More research in the area of LIGA-20 may result in possible future use of the compound in medical fields.

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