Biopolymer encapsulation of a glycolipid synthesis inhibitor prolongs its antiproliferative effects

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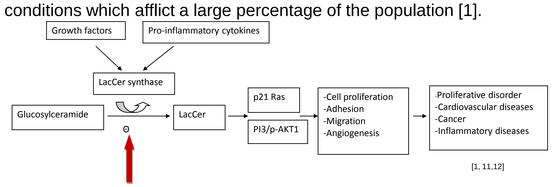
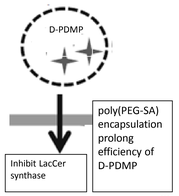
Introduction

Lactosylceramide (LacCer) plays a pivotal role in the biosynthesis of glycosphingolipids. Growth factors and pro-inflammatory cytokines converge to activate LacCer synthase to generate LacCer, which then activates downstream signaling pathway that leads to cell proliferation, adhesion, migration and angiogenesis. Dys-regulation of such phenotypic changes are a hallmark of diseases of the cardiovascular system, proliferative disorders such as cancer and inflammatory diseases. Therefore, LacCer and LacCer synthase provide a novel target to effectively address a wide variety of current health conditions which afflict a large percentage of the population [1].



Biopolymer Encapsulated D-PDMP

Previously, D-threo-1-pheny-2-decanoylamino-3-morholino propanol (D-PDMP), an inhibitor of LacCer synthase markedly inhibited platelet-derived growth factor (PDGF)-induced cell proliferation [2].



Unfortunately, D-PDMP's therapeutic benefits over a prolonged period are decreased due to rapid serum clearance and diffusion [2, 3]. We solved this problem by using a biopolymer.

FDA approved polyethylene glycol (PEG) is widely used in medical applications due to its biodegradability and biocompatibility [4, 5]. However, hydrolysis of PEG follows bulk erosion profile, which can lead to unwanted side effects [6]. Combination with sebacic acid (SA), which degrades via surface erosion [7, 8], solved this problem.

We encapsulated D-PDMP in a poly(PEG-SA) biopolymer (BP) that proved successful in delivering drugs [3, 9].

Objectives:

1) To investigate the efficacy of BP in prolonging delivery of D-PDMP in mitigating growth factor-induced proliferation in human arterial smooth muscle cells (ASMC) as compared to D-PDMP alone.
2) To determine the downstream signaling pathway in LacCer-induced cell proliferation.

Approach:

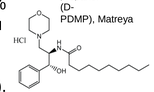
- Incubate ASMC with growth factors (GF) PDGF and tissue growth factor (TGF) for 48 hrs with various treatments:
  - No growth factor as negative control
  - GF w/ D-PDMP (10 and 20 µM)
  - biopolymer alone (33.3 µg/mL) as positive control
  - GF w/ BP encapsulated D-PDMP (BPD 10 µM/ 33.3µg/mL and BP D20 µM/66.6 µg/mL)
- Measure [3H] Thymidine incorporation into DNA
- Measure [3H] Galactose incorporation into ceramide, GlcCer, and LacCer
- Perform western immunoblot assay using ASMC extract and p-AKT1 and GAPDH (housekeeping protein) antibodies.

Materials and Methods

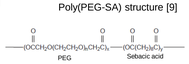
BP encapsulation

- D-PDMP (Matreya Biochemicals)
- Synthesis of poly(PEG-SA) (BP) (10% PEG and 90% SA) followed the procedure previously reported by Fu and coworkers [9] using polycondensation method
- Melt polycondensation method used to encapsulate D-PDMP in BP (starting ratio 70:30 BP/D-PDMP w/w).
- Microparticles prepared using double emulsion solvent evaporation method [3].

D-threo-1-pheny-2-decanoylamino-3-morholino propanol (D-PDMP), Matreya



Poly(PEG-SA) structure [9]



Proliferation assay [2]

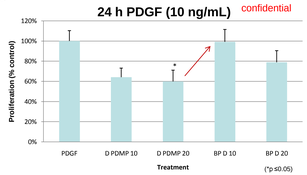
- ASMC and medium (Invitrogen)
- PDGF-BB and TGF-β (Cell Signaling Technology)
- Beckman liquid scintillation counter used to count [3H] Thymidine (5 µCi/mL, American Radio label Chemicals) incorporation into DNA and [3H] Galactose (5µCi/mL) incorporation into LacCer
- HPTLC separated the glycosphingolipids (LacCer GlcCer, and ceramide).

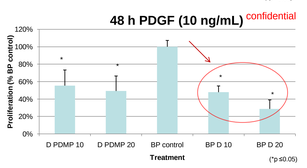
Western Blot

- p-AKT1 and GAPDH antibody (Invitrogen)
- BCA protein assay kit (Pierce)

Results

BP-D prolongs the efficacy of D-PDMP in mitigating growth factor-induced ASMC proliferation

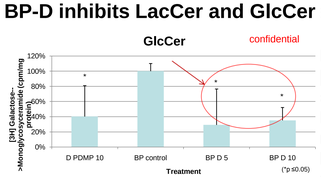


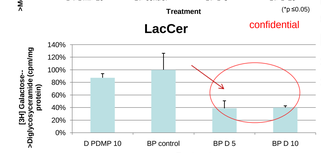


For 24 h, D-PDMP was more effective than BP-D. But for 48 h, BP D inhibits proliferation more strongly.

Effects of various treatments on human arterial smooth muscle cells proliferation. Confluent human arterial smooth muscle cells (x10⁴) were seeded in 96-well trays. After 24 h, the medium was replaced by serum free medium and incubated for 24 hours. The serum free medium is then replaced with serum free medium with 5 µCi/mL of [3H] thymidine and various treatments at a concentration calculated to deliver the same level of D-PDMP (PDGF alone (5 and 10 ng/mL), PDGF and D-PDMP (10 and 20 µM), PDGF and biopolymer alone (33.3 µg/mL), PDGF and biopolymer-D-PDMP (70:30 by weight; BP D10 10 µM/33.3µg/mL and BP D20 20 µM/66.6 µg/mL)).
Incubation was continued for another 48 h at 37ºC. The medium was then removed, and the cells washed extensively with phosphate buffered saline. Subsequently, the cells were solubilized in 100 µl of 1 N NaOH and the incorporation of [3H]-thymidine was measured by scintillation spectrophotometry. Values are from a representative independent experiment with n= 1
*Significant difference as compared to control (*p ≤0.05).
Cells growth in PDGF alone incorporated 253 cpm of [3H] thymidine in 48 h on average.
These values were used as 100% in our calculations.

BP-D inhibits LacCer and GlcCer synthesis

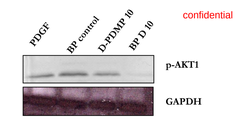


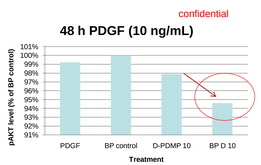


Metabolic incorporation of [3H] Galactose into ceramide, GlcCer, and LacCer revealed that BP-D markedly decreased the biosynthesis of LacCer and GlcCer as compared to D-PDMP alone, while ceramide did not show any significant decrease.

Effect of D-PDMP and biopolymer encapsulated D-PDMP on the glucosylceramide/LacCer levels in human ASMC. Confluent cultures of human ASMC grown in six-well trays were incubated for 48 h with [14C] plamitate (5 µCi/mL) and the following: PDGF alone (5 and 10 ng/mL), PDGF and D-PDMP (10 and 20 µM), PDGF and biopolymer alone (33.3 µg/mL), PDGF and biopolymer-D-PDMP (70:30 by weight; BP D10 10 µM/33.3µg/mL and BP D20 20 µM/66.6 µg/mL). Each treatment is done in triplicates. Next, the cells were washed with PBS and extracted with hexane-isopropanol (3:2 by volume). The lipid extracts were dried in N_2 and subjected to silicic acid column chromatography. The glycosphingolipid fraction was eluted using acetone:methanol (9:1 v/v). The glycosphingolipid fractions were separated further by HPTLC. Following autoradiopgraphy, gel area corresponding to glucosylceramide were excised and radioactivity was measured in a Beckman scintillation spectrometer. The data is presented as a percent control value of 1678 cpm/mg protein for average activity level for various lipids in PDGF only treatment. *p ≤ 0.05

BP-D inhibits pAKT expression when compared to D-PDMP





Western immunoblot assays demonstrated a marked decrease in the levels of phosphorylated protein kinase B (p-AKT1) in treatment with BP-D compared to D-PDMP. p-AKT protein kinase is up-regulated in human cancers [10, 11].

BP-D inhibits p-AKT1 expression when compared to D-PDMP
The levels of p-AKT1 protein were measured in human ASMC treated for 48 h with PDGF alone (10 ng/mL), PDGF and D-PDMP (10 µM), PDGF and biopolymer alone (33.3 µg/mL), PDGF and biopolymer-D-PDMP (70:30 by weight; BP D10 10 µM/33.3µg/mL). p-AKT1 protein levels were quantified by determining the ratio of the integrated density of p-AKT1 to GAPDH loading control using ImageJ v.1.45s (NIH, USA) software.

Discussion/Conclusions

We demonstrate that encapsulation of D-PDMP within a biopolymer is an effective approach to increase the efficacy of this glycolipid inhibitor/anti-proliferative compound and reduce cost of experimentation and exposure of animals to drugs.

Further Studies:
-Determining the efficacy of BP-encapsulated D-PDMP in transgenic mouse model of atherosclerosis

Supporting Data Available:
-Result showing controlled release of D-PDMP from BP by HPLC

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