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Differential Efficiency and Pathway of Ribitol, Ribulose and Ribose for Enhancing Matriglycan Expression

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ABSTRACT

Glycosylation of alpha dystroglycan is critical for normal neural development and muscle function. Reduced or absence of glycosylation, specifically a terminal repeating biglycan units of 3GlcA-1-3Xyl-1 (matriglycan) on core M3, is the primary indication of numerous muscular dystrophies, and has a demonstrated effect in tumor development or metastasis. Fukutin-related protein (FKRP) is a glycosyltransferase using CDP-ribitol as the substrate for addition of ribitol-5-phosphate to the core M3 and is essential for completing matriglycan synthesis. Cytidine 5'-diphosphate-ribitol is synthesized with isoprenoid synthase domain containing (ISPD) using ribitol-5P and CTP as substrates. In this study, we examined a panel of pentoses and their phosphates for potential to enhance matriglycan synthesis in a cell culture system previously shown relevant to experimental therapy to dystroglycanopathy in vivo. Ribitol, ribulose, ribose and their phosphates all enhance matriglycan synthesis with highest efficiency by ribitol followed by ribulose and lowest by ribose. This is supported by metabolomics analysis showing that levels of ribitol-5P are significantly higher with ribitol and ribulose treatments than ribose. Enhanced matriglycan level by cytosine and its phosphates when combined with ribitol is demonstrated by immunocytochemistry. Ribose to ribulose to ribitol to ribitol-5P pathway is likely the predominant flow for synthesis of ribitol-5P when the pentoses are supplemented for enhancement of matriglycan expression as therapeutics. Ribitol is preferred for life-long treatment of FKRP-related dystroglycanopathy. Therapeutic potential of cytosine and its phosphates may be limited and remain to be explored in vivo.

Keywords: Matriglycan, Dystroglycanopathy, ribitol, Limb Girdle Muscular Dystrophy.

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Abbreviations

FKRP	Fukutin related protein
FKTN	Fukutin
ISPD	Isoprenoid synthase domain containing
POMT1	Protein O-mannosyltransferase 1
POMT2	Protein O-mannosyltransferase 2
POMGnT2 (GTDC2)	Protein O-Linked Mannose N-Acetylglucosaminyltransferase 2 (Glycosyltransferase-Like Domain-Containing Protein 2)
B3GALNT2	Beta-1,3-N-Acetylgalactosaminyltransferase 2
TMEM5	Transmembrane protein 5
B4GAT1	Beta-1,4-glucuronyltransferase
LARGE	Like-Acetylglucosaminyltransferase
CMP:	Cytidine 5′-monophosphate
CDP	Cytidine 5'-diphosphate
CTP	Cytidine triphosphate

Supplementary Materials

	Name	Catalog	Company
1	D-fructose	T4823	Sigma
2	D-maltose	00206	Sigma
3	D-galactose	F10950	Sigma
4	D-arabinose	W008725	Sigma
5	Ribitol/ Adonitol	A5502	Sigma
6	D-ribose	R9626	Sigma
7	D-ribulose	18893 I	Cayman Chemicals
8	D-xylitol	X3375	Sigma
9	D-xylose	X3877	Sigma
10	D-xylulose	S134209	Sigma
11	D-glucose	G5767	Sigma
12	N-acetyl-D-glucosamine	A8625	Sigma
13	D-glucuronic acid	G5269	Sigma
14	D-mannose	4440-M	Sigma
15	CDP-Ribitol		Z-biotech
16	Control		

Supplementary Table 1. List of Sugars.

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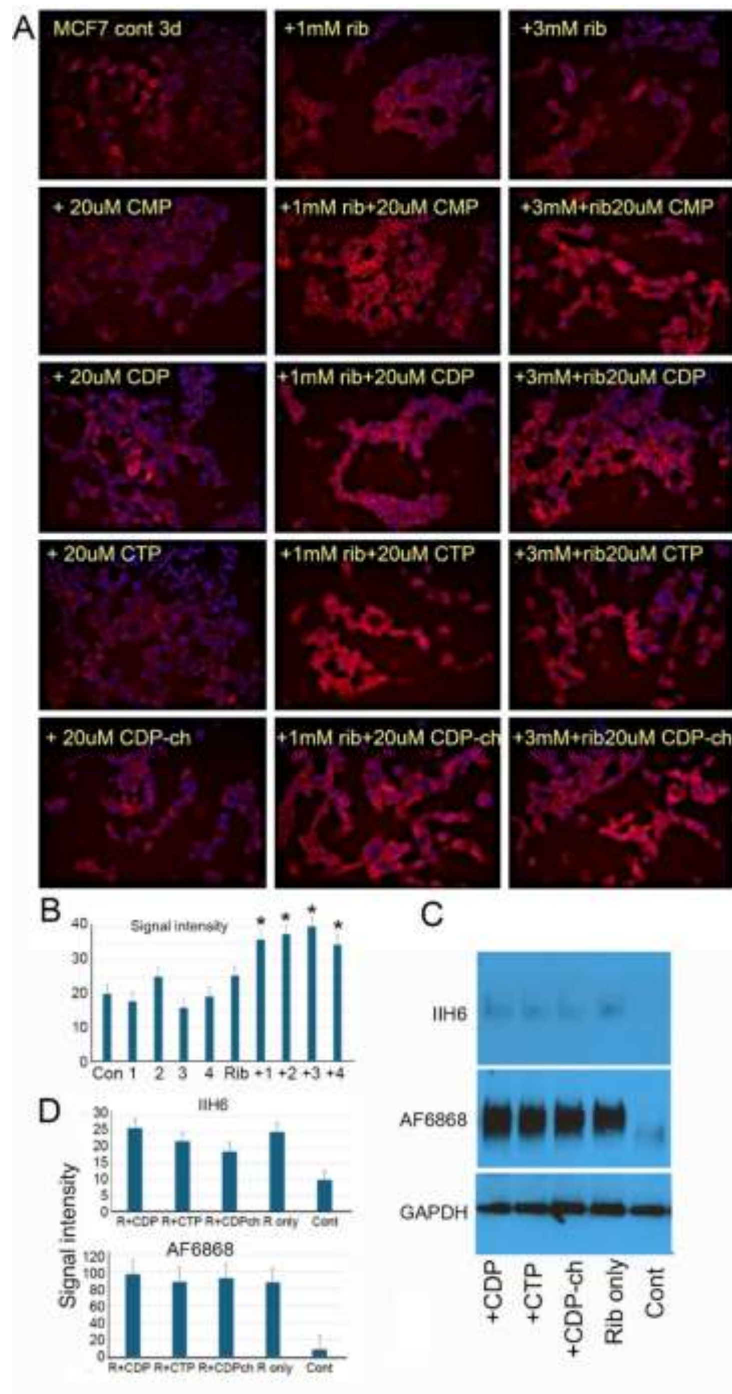

Supplementary Figure 1. Matriglycan expression with the treatment of CMP, CDP, CTP and CDP-choline in combination with ribitol for enhancing matriglycan expression.

Introduction

Muscular dystrophy is a group of muscle degenerative diseases caused mainly by loss of function mutations in genes predominantly forming the muscle membrane structure called dystrophin-glycoprotein complex (DGC). One critical member of the complex is dystroglycan (DG) which spans the sarcolemma with its two units, alpha-DG (α-**DG**) and beta-DG (**β-DG**) [1,2]. Alpha-DG acts as a cellular receptor for laminin and other extracellular matrix (ECM) proteins, including agrin and serves to connect cytoskeletal proteins and ECM proteins critical for the stability of the DGC and fiber membrane[2,3]. This laminin-binding capacity is mediated directly by the interaction of the specific *O*-mannosylated glycan (Core M3) on α-**DG**[2]. The sugar units of this glycan chain have recently been defined as **as (3GlcA-β1-3Xyl-α1)** ***n*-3GlcA- β1-4Xyl-Rbo5P-1Rbo5P-3GalNAc- β1-3GlcNAc- β1-4(P-6)** Man-1-Thr/ser [4]. Furthermore, transferases directly responsible for successive addition of individual sugars to the chain have

been identified as POMT1, POMT2 [5], POMGnT2 (GTDC2) [6,7], B3GALNT2 [8], FKTN, FKRP [4], TMEM5 [9], B4GAT1 [10], and LARGE, respectively (Figure 1 and Table 1). The actual laminin-binding domain of the glycan called matriglycan is the repeating biglycan units of 3GlcA-1-3Xyl-1 extended by the LARGE-mediated xylosyltransferase and glucuronyltransferase activities [11]. Lack of α-DG protein or matriglycan resulting from mutations in any of the above-mentioned genes cause muscular dystrophies of dystroglycanopathy. One common cause is mutations in the FKRP gene with variable disease severity, largely determined by mutation types affecting function of the protein differentially [12-14]. Complete loss of function due to non-sense or frame-shift mutations in both alleles of the gene is embryonic lethal. Therefore, almost all patients carry mutated FKRP with reduced function rather than total loss of function. This has recently been supported by several lines of evidence. Although diseased muscles largely lack matriglycan, individual muscle fibers with strong signal of detectable matriglycan can be found in nearly all biopsy samples from clinics and in mouse models of FKRP mutations [15-18]. Near normal levels of matriglycan can be achieved in regenerating fibers during disease progression or induced by injurious notexin treatment in P448L mutant mice associated with CMD in clinics [19]. Furthermore, matriglycan is easily detectable in skeletal and cardiac muscles of newborn P448L mutant mouse with homozygous P448L mutation[20]. However, matriglycan level drops to undetectable within a week. Finally, functionality of the P448L mutant FKRP has been confirmed directly by AAV meditated gene delivery using P448L mutant FKRP as transgene [21]. The ability of mutant FKRPs to support matriglycan expression up to normal levels underpins the approach of enhancing remaining function of mutant FKRPs for treating the majority of FKRP-related diseases.

Recent studies have also advanced our understanding of pathways leading to the synthesis of CDP-ribitol, the substrates for both FKRP and FKTN glycosyltransferases, leading to the sequential addition of ribitol-5-phosphate (ribitol-5P) to the growing Core M3 glycan chain [22]. Cytidine 5'-diphosphate-ribitol is synthesized by isoprenoid synthase domain containing (ISPD) as a cytidyltransferase (pyrophosphorylase) using ribitol-5P and CTP as substrates [4,23,]. Interestingly, a study from Gerin *et al.* demonstrated that ribitol treatment of HEK293 cells overexpressing ISPD (also known as CRPPA, CDP-L-ribitol pyrophosphorylase A) and patient-derived fibroblasts with ISPD-deficiency leads to increased levels of CDP-ribitol and partially corrects the matriglycan deficiency [24]. Our studies in mouse models of FKRP mutation demonstrate that ribitol treatment increases the levels of CDP-ribitol and enhances matriglycan expression [25,26]. This results in improvement of pathology and muscle function in both diseased skeletal and cardiac muscles. In vitro studies have also demonstrated the ability of ribose as well as ribitol to increase CDP-ribitol with enhancement of matriglycan expression in several cancer cell lines [27]. Currently, there are ongoing phase II-III clinical trials with ribitol and a case report of using ribose for LGMD2I patients [28].

We previously reported that expression of matriglycan in the MCF7 breast cancer cells, similar to the muscles with mutant FKRPs in vivo, can be significantly increased with treatment of ribitol and CDP-ribitol without affecting the expression of relevant glycosyltransferases including FKRP and LARGE [27]. This result suggests that pathways of ribitol-induced matriglycan expression are likely shared in both cells, and MCF7 cells can serve as an in vitro model for efficacy comparison of potential sugar metabolites. In this study, we examined a panel of pentoses and pentitols and their phosphates for effect of enhancing matriglycan synthesis in the cell line. Metabolomics analyses were also performed to probe the conversion capacity of ribitol, ribose and ribulose between each other and to ribitol-5-phosphate (ribitol-5P). The results show that ribitol, ribulose and ribose can all significantly enhance the

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matriglycan expression dose-dependently with decreasing efficiency from ribitol to ribulose and then ribose. Similarly, efficiency is highest with ribitol-5P followed by ribulose-5P and then ribose-5P. Minimal effect is observed with all other sugars including arabinose and xylose. CTP, another substrate of FKRP, as well as cytidine, CDP or CMP also have no clear effect on matriglycan expression when used alone. Immunostaining shows capacity of the cytidine-based compounds to further enhance ribitol effect when used in combination. However, this enhancement cannot be clearly demonstrated by western blot, possibly due to limited sensitivity of the method. Metabolomics analysis of the cells treated with the three pentoses at the same concentration reveals that levels of ribitol is higher with ribulose treatment when compared with ribose treatment. Both ribitol and ribulose only slightly increase the level of ribose. Ribose supplement leads to the highest level of ribose-5P, but lowest of ribulose-5P. Importantly, ribitol-5P levels are highest with ribitol treatment and lowest with ribose treatment. The overall results support the notion that ribitol to ribitol-5P pathway is predominant for contributing to the enhancement of matriglycan synthesis.

Materials and Methods

CELL LINES AND CULTURE

MCF7 (ATCC- HTB-22) human breast cancer cell line was used. Growth medium: DMEM-GlutaMAX (10569, Gibco by life technologies), 10% fetal bovine serum (FBS A5256801 ThermoFisher) and 10 µg/ml insulin (I5500 Sigma). Growth condition: 37^{0}C with 10% $CO_{2.}$ Cells seeded in 24 well culture plate and T25 flask in triplicate in the growth medium for one day. The following day, the medium was supplemented with sugars at a given concentration, and the cells were grown in the supplemented media for 3 days. The cultured vessel was washed with PBS and cells were processed for analysis by immunostaining, western blot and metabolomics.

Reagents

The following reagents were from Sigma; Ribitol/Adonitol (A5502), Ribose (R9626), D-Ribose-5-phosphate disodium salt hydrate (R7750), D-Ribulose 5-phosphate disodium salt (83899), and Cytidine (C4654), CMP-Cytidine 5′-monophosphate (C1131), CDP-Cytidine 5′-diphosphate sodium salt hydrate (C9755), CTP-Cytidine 5′-triphosphate disodium salt (C1506) and CDP Choline -Cytidine 5′-diphosphocholine sodium salt dihydrate (C0256). D-Ribulose (18893) was from Cayman Chemical. Ribitol-5-phosphate disodium salt and CDP-Ribitol disodium salt were from Z-biotech.

Immunostaining

Cell cultures were washed with PBS before fixation with ice-cold ethanol for 10 minutes. Residual ethanol was removed by washing with PBS and air dry. Cells were rehydrated prior to staining procedures with PBS and blocked with 6% bovine serum albumin (BSA), 2% normal goat serum (NGS) in PBS for 30 minutes. Primary antibody anti-a-dystroglycan IIH6C4 (IIH6), mouse monoclonal IgM (05-593, EMD Millipore) at 1:600 dilution in 1% BSA/PBS. Cells were incubated overnight at 4°C. Samples were washed three times for 10 minutes each with PBS and finally incubated with Alexa Fluor 549-conjugated or 488-conjugated goat anti-mouse IgM secondary antibodies (Life Technologies, Carlsbad, CA, USA) at 1:500 dilution. Samples stained without primary antibody were used as control. Nuclear staining was achieved with fluorescence mounting medium from Abcam (Cambridge, UK) containing 1X DAPI (4′,6′-diamidino-2-phenylindole).

Immunofluorescence was visualized using an Olympus BX51/BX52 fluorescence microscope (Opelco, Dulles, VA) and images were captured using the Olympus DP70 digital camera system (Opelco).

Western Blot

Bio-Rad Protein Assay, Electrophoresis, and Blocking: Cells were lysed in Triton lysis buffer containing 1% Triton X-100, 50 mM Tris pH 8, 150

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mM NaCl, 1 mM EDTA, and 1x Protease Inhibitor Cocktail (Sigma). After clarification of the lysates by centrifugation at 13,000 rpm for 10 minutes at 4^{0}C, protein concentration of the lysates was measured using the Bradford method (Bio-Rad Laboratories). Samples were electrophoretically separated on a 4-15% Mini-Protean TGX gels (4561084 Bio-Rad Laboratories) and transferred onto supported nitrocellulose membrane. The blots were blocked with nonfat milk (1706404 Bio-Rad) blocking buffer for 2 hours and then incubated with primary antibody overnight at 4^{0}C

Primary antibodies: IIH6C4 was used at 1:1500 dilution in 3% nonfat milk/PBS blocking buffer. Anti-human dystroglycan polyclonal sheep AF6868 (R&D Systems) was used at 1:1000 dilution and rabbit polyclonal antibody to GAPDH (PA1-988 ThermoFisher) was used at 1:3000 in 5% nonfat milk/1xTBS-0.05% Tween 20 blocking buffer.

Secondary antibodies: Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (62-6820 Invitrogen), rabbit-anti-Sheep IgG (618620 Invitrogen) and goat-anti-rabbit IgG (1706515 Bio-Rad) were used. After overnight incubation with primary antibody, membranes were washed and probed with secondary antibodies 1:3000 dilution in blocking buffer for 1 hour 30 minutes. Bands were detected using ECL Kit NEL 104001EA (PerkinElmer) on blue basic autoradiography film (USA Scientific). ImageJ1.45 software was used for IIH6 band quantification from western blot.

Measurement of signal intensity with immunostaining

Semi-quantitative measurement of membrane matriglycan signal was carried out by ImageJ1.45 Multi Point tool for comparison as described previously [29]. Briefly, after immunostaining with IIH6 antibody, all images were taken with same exposure time and magnification and stored as TIFF files of same size. The files were opened through ImageJ1.45 and Multi Point tool was chosen. The diameter of the point size was slightly wider than the width of fiber membrane. Each image was sampled for 100 fibers (n=3) (unless otherwise stated) and the signal intensity of non-membrane background was also taken within the image and deducted from fiber membrane signal intensity measurement.

Metabolomics

Cell pellets from 72-hour cultures with treatment of the pentoses, or untreated control were collected via scraping and centrifugation after PBS wash and submitted to NIH West Coast Metabolomics Center for metabolomic analysis. Primary Metabolomic analysis was performed with ALEX-CIS GCTOF MS in resuspension volume of 100 µl, with an injection volume of 0.5 µl [30]. The data of identified metabolites were processed and normalized, and the reported data are given as peak heights for the quantification ion (mz value) at the specific retention index. Statistical significance was calculated between experimental groups by t-test with $P< 0.05$ as significant.

Statistical analysis

All data are expressed as mean ±SEM unless stated otherwise. Statistical analyses were performed with individual means were compared using two-tailed t-tests. Differences were statistically significant at $p \leq 0.05$ (* or +).

Results

Differential effect of ribitol, ribose, ribulose and CDP-ribitol on matriglycan expression

We first examined 15 pentoses, pentitols and other related metabolites for their capacity to enhance the expression of matriglycan in the MCF7 cells (Supplementary Table 1). Ribitol and CDP-ribitol were included as positive controls [27]. The doses of 0.1 mM for CDP-ribitol and 1 mM for all other metabolites were chosen based on our earlier results from the same cells. After 3 days of treatment, VIA4 antibody was used to specifically detect matriglycan from the treated cells in culture plates in triplicates and the stained samples were imaged with signal intensity measured by the Tecan plate reader. Only the treatment of ribitol,

ribulose and CDP-ribitol significantly increased matriglycan signal when compared to the untreated samples. Ribose treatment also clearly increased the level of matriglycan, but the increase did not reach *p<0.05* significance over untreated control (Fig 1).

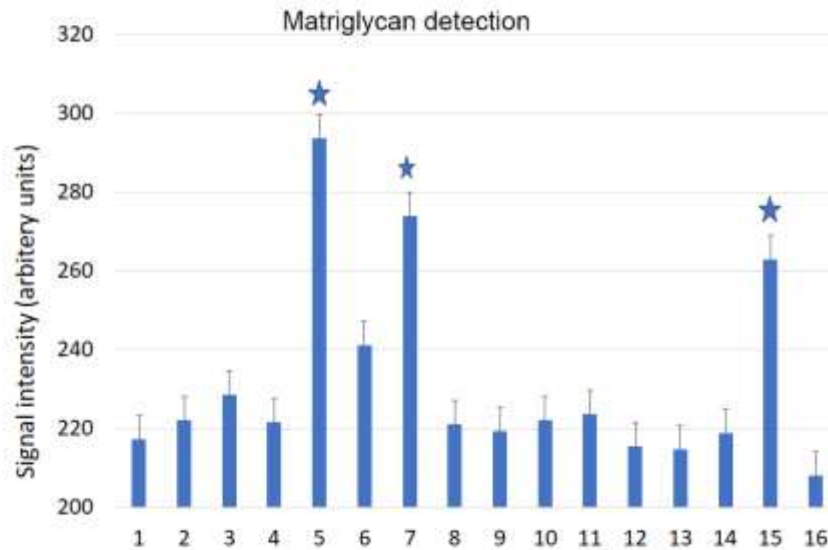

Figure 1. Matriglycan detection in MCF7 cells by immunoassay with VIA4 and Tecan plate reader. 1. D-fructose, 2. D-maltose, 3. D-galactose, 4. D-arabinose, 5. ribitol, 6. D-ribose, 7. D-ribulose, 8. D-xylitol, 9. D-xylose, 10. D-xylulose, 11. D-glucose, 12. N-acetyl-D-glucosamine, 13. D-glucuronic acid, 14. D-mannose, 15. CDP-ribitol, 16. Control. The doses are 1 mM for all sugars except for CDP-ribitol with 0.1 mM. * *p<0.05*, two-tail T.TEST between metabolite treated and control sample. n=3.

Differential effect of ribitol, ribose and ribulose on matriglycan expression

To compare the efficiency of ribitol, ribose and ribulose in enhancing matriglycan expression, we conducted a two-dose test with 1 mM and 5 mM of each compound, and the samples were examined by both immunostaining and western blots. As shown in Figure 2A, strong matriglycan signals were detected in most cells with all 3 pentoses at the highest dose with significance when compared to the control. Matriglycan levels with ribitol treatment were also higher than ribose or ribulose. At the dose of 1 mM, the difference in proportion of matriglycan positive cells became very clear between the pentoses. While more than 50% cells remained strong positive with ribitol treatment, only about 15% cells treated with ribulose were positive, and even less cells were positive with ribose treatment. Signal intensity measurement showed highly significant difference between ribitol and ribulose treatment and between ribulose and ribose treatment (Fig 2B). This difference was also clearly demonstrated by western blot (Fig 2C) showing the strongest matriglycan signal with ribitol treatment. Matriglycan signal was weaker in the cells with ribulose than ribitol treatment. Ribose treatment produced the weakest enhancement in levels of matriglycan, but still clearly higher than background signal of the control cell. Increasing matriglycan synthesis can also be indicated by the increase in molecular weight of α-DG demonstrable in western blots by polyclonal antibody AF6868 which recognizes both functionally glycosylated as well as part of the core α-DG. As shown in the Figure 2C, the molecular weight of α-DG was clearly increased with both ribitol and ribulose treatments but limited with ribose treatment when compared to the control (Fig 2D). This is consistent with the efficiency ranking demonstrated with IIH6 antibody which only recognizes matriglycan.

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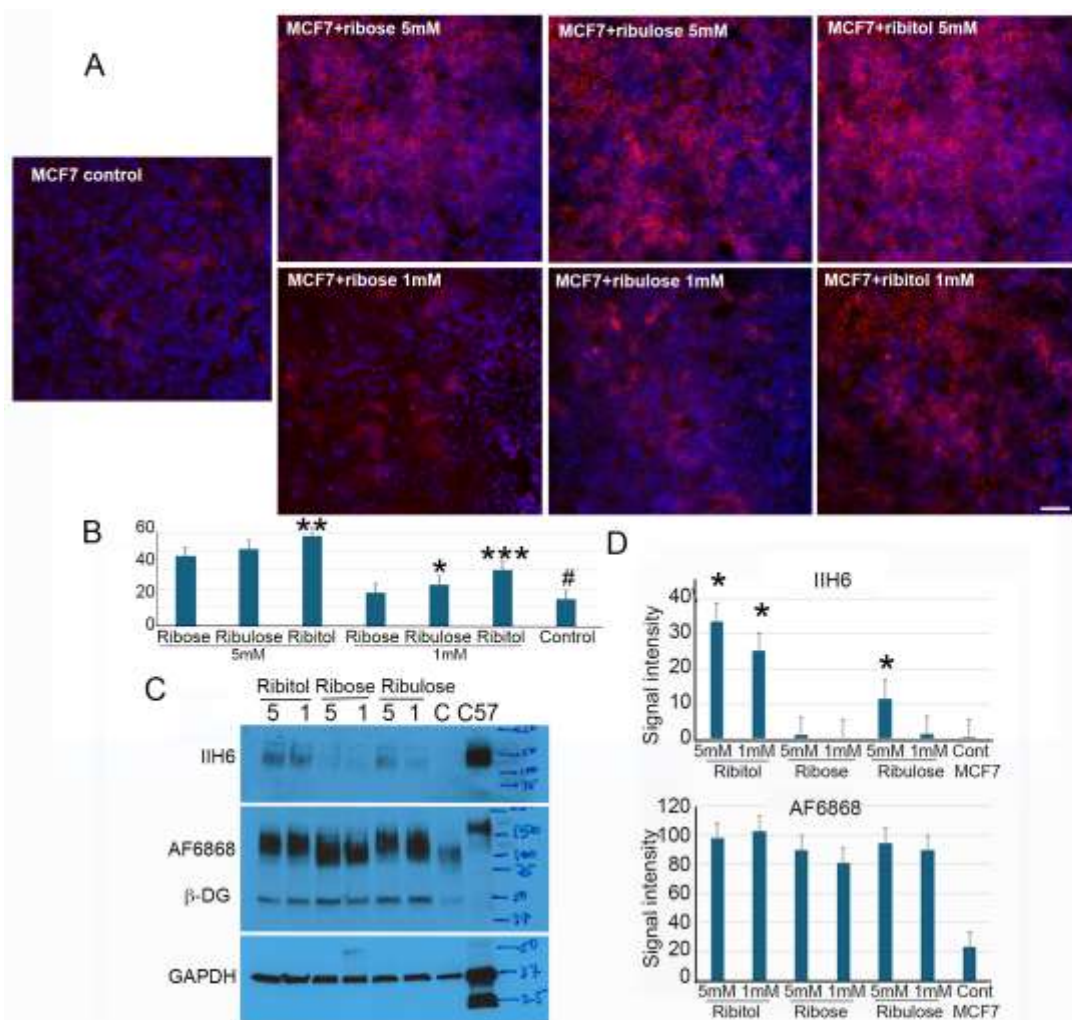

Figure 2. Matriglycan detection by immunostaining (A & B) and western blot (C &D) in cells treated with ribitol, ribose and ribulose. Cells were treated with 2 concentrations of 1 mM (1) and 5 mM (5) of the pentoses and detected by immunostaining with IIH6 antibody in (A) (scale bar = 100μM), and with antibodies IIH6, AF6868, β-DG and GAPDH (as protein loading control) in (C). (B) Membrane signal intensity of immunostaining measured by ImageJ Multipoint tool. # *$p<0.001$* for all treated samples when compared to the control. * *$p<0.05$* between ribulose and ribose treatment. ** *$p<0.001$* and *** *$p<0.0001$*, between ribitol and either ribose or ribulose treatment. two-tail T.TEST. n=100x3. (D) Band intensity of western blots measured by NIH Image J. C and Cont, untreated control sample. C57, TA muscle from C57 mouse used as positive control for matriglycan. Signal intensity for AF6868 and IIH6 is adjusted with β-DG and GAPDH respectively.

Effect of pentose phosphates on matriglycan expression

Ribitol-5 Phosphate (ribitol-5P) is the substrates of ISPD for the synthesis of CDP-ribitol, and the observed effect of ribitol on matriglycan is likely through the increase of the ribitol-5P. Similar to ribose and ribulose, ribose-5P and ribulose-5P are considered reversibly convertible to ribitol-5P. Therefore, the 2 compounds could also have similar capacity to enhance matriglycan expression. We therefore examined ribitol-5P, ribose-5P and ribulose-5P for their efficiency in enhancing matriglycan synthesis in comparison with ribitol only. Doses of 0.1 mM, 0.5 mM and 1 mM were used as toxicity was detected especially with ribulose-5P and ribose-5P when 5 mM was used. Treatment for 3 days resulted in clear enhancement with all 3 compounds dose-dependently (Fig 3A). Ribitol-5 phosphate treatment resulted in the strongest signals when compared to the other two pentose phosphates at the same dose. Ribose-5P treatment produced the weakest matriglycan expression (Fig 3A and B). Matriglycan signal remained detected in the majority of the cells at the lowest dose of ribitol-5P, ribulose-5P and ribose-5P when compared to the control (Fig 3).

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The signal intensity of matriglycan with 1 mM ribitol-5P was close to that with ribitol treatment at 5 mM and CDP-ribitol treatment at 0.5 mM which is consistent with our previous report [27]. The differential effect on matriglycan with the 3 phosphorylated pentoses was confirmed by Western blots with both IIH6 and AF6868 antibodies as shown in Figure 3C and D. Matriglycan levels detected with IIH6 were enhanced most strongly with ribitol-5P treatment at 1 mM, but weakest with ribose-5P. All 3 phosphate pentoses significantly increased signal intensity and molecular weight of α-DG when detected by AF6868 antibody. These results overall support the conclusion that ribitol-5P is the most efficient intermediate in the pathway towards matriglycan synthesis.

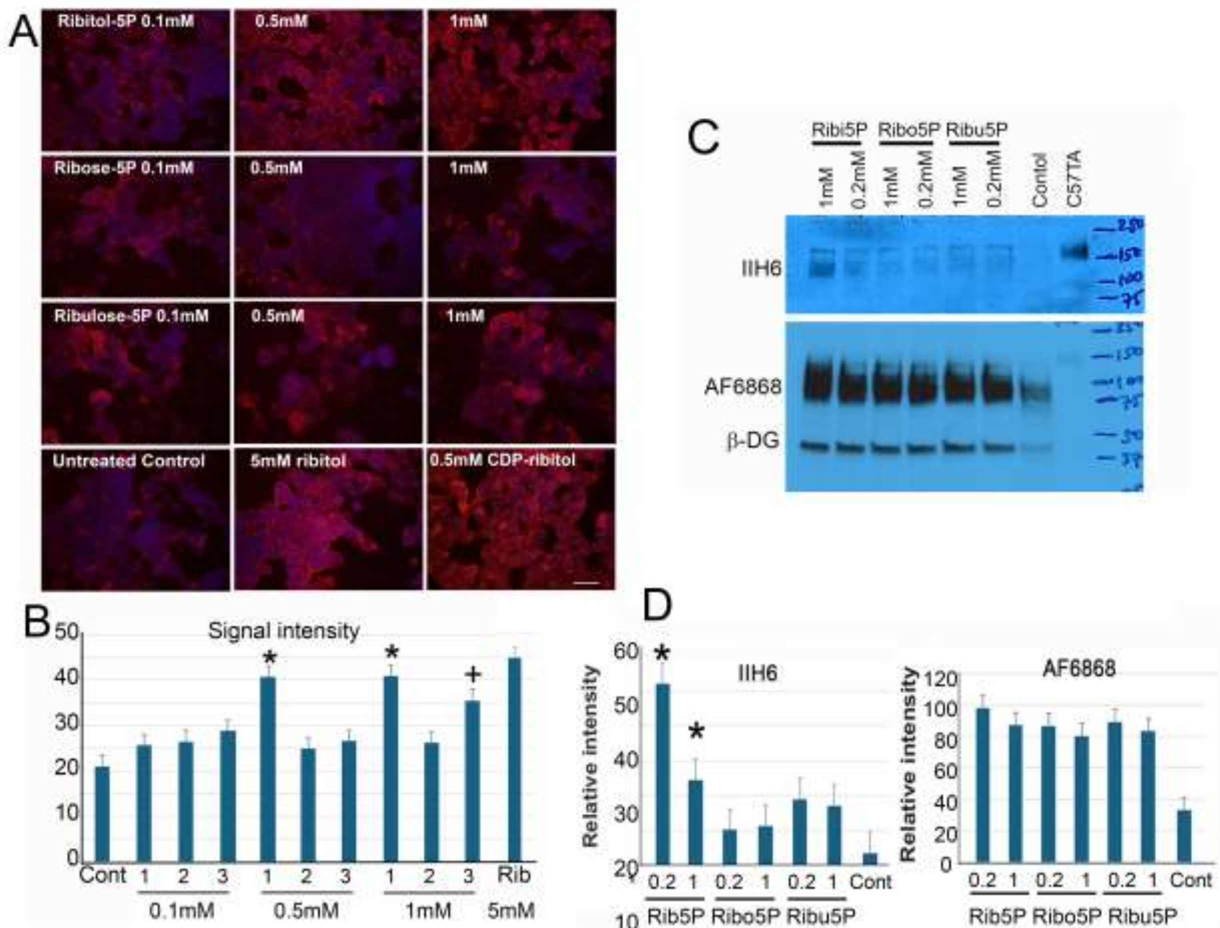

Figure 3. Dose response of MCF7 cells to the treatment of ribitol-5P, ribose-5P and ribulose-5P for enhancing matriglycan expression. A. Immunostaining with IIH6 antibody. Scale bar = 100 µM. B. Membrane signal intensity in (A) measured by ImageJ. 1, ribitol-5P; 2, ribose-5P; 3, ribulose-5P. * $P < 0.05$ when ribitol-5P is compared to the same dose of ribose-5P and ribulose-5P. + $P < 0.05$ when ribulose-5P is compared to the same dose of ribose-5P. rib, ribitol. n=100x3. C. Western blot detection of matriglycan with IIH6 and AF6868. D. Signal intensity measurement of matriglycan bands from (C). $P < 0.05$ when ribitol-5P is compared to the same dose of ribose-5P and ribulose-5P. Note that signal intensity detected by AF6868 was measured for the portion of the bands above the levels of the main band in the control sample, representing α-DG with enhanced glycosylation. Signal intensity is normalized with β-DG.

Effect of cytidine and related nucleotides on matriglycan expression

Cytidine triphosphate (CTP) is the other substrate of ISPD for the synthesis of CDP-ribitol. We therefore examined effect of CTP supplementation on synthesis of matriglycan. Considering that CTP will likely be at least partly dephosphorylated into CDP, CMP or cytidine during the path of delivery into cells or through oral route *in vivo*, their effect was also examined [31]. Cytidine triphosphate-choline, an over-the-counter supplement, was also included. It is likely that potential effect of CTP on

matriglycan expression might only be achieved when used together with ribitol, these compounds were therefore examined at 20 μM alone and in combination with ribitol at 1 mM and 3 mM concentration. As illustrated in Supplementary Figure 1, all cytidine phosphates when used alone did not result in detectable difference in IIH6 signals by immunostaining when compared to the untreated control cells. As expected ribitol treatment alone enhanced the levels of matriglycan. Signals for matriglycan were significantly stronger in the cells with combined treatment of ribitol and each of the cytidine-related compounds including CDP-choline when compared to the cells treated with ribitol alone. There was no clear difference between different combinations (Supplementary Fig 1). Consistent with immunostaining, western blot also showed no clear difference between the single treatment groups compared to the control. Surprisingly, enhancement by the combination treatments was not detected when compared to ribitol treatment alone with both IIH6 and AF6868 antibodies (Supplementary Fig 1).

To further evaluate the potential difference in matriglycan levels detected between immunostaining and western blot, we tested the combination of ribitol with cytidine and CDP at 3 doses (up to 500 μM and 100 μM respectively) by the two methods. Immunostaining showed no clear difference in matriglycan signal at any dose of both compounds alone when compared to the control. However, significantly stronger signals for matriglycan were again observed in combination with ribitol when compared to the ribitol treatment alone (Fig 4A and B). Consistent with immunostaining, Western blot showed no clear difference between single treatment groups and the control. Again, significant enhancement in matriglycan expression by combination treatments was not detected at the highest dose of 500 μM and 100 μM for cytidine and CDP respectively when compared to ribitol alone group (Fig 4C). These results overall suggest that supplement of cytidine and cytidine phosphates have limited effect on matriglycan synthesis.

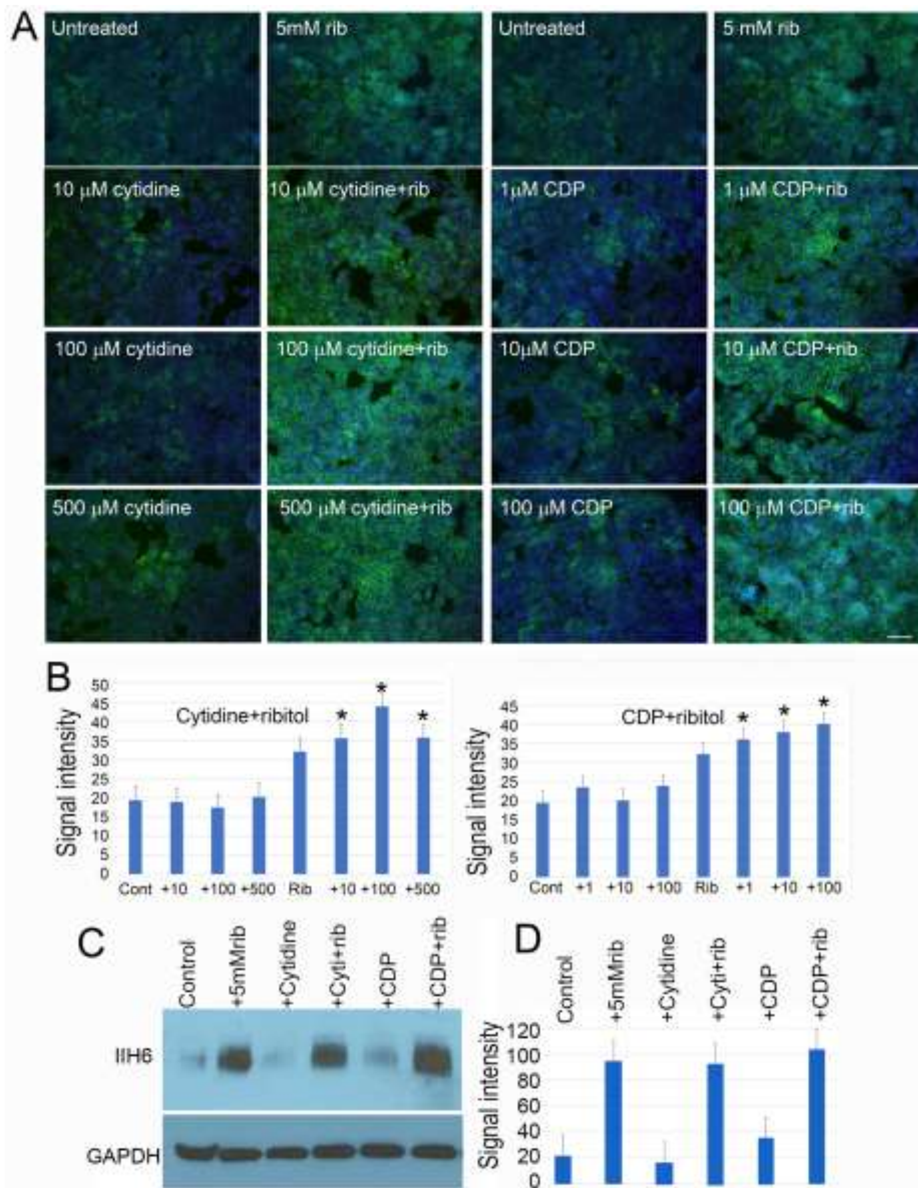

Figure 4. Dose response of cytidine and related compounds on matriglycan expression. MCF7 cells were treated with ribitol (rib) at 5 mM concentration. Cyti, cytidine. (A and B) Immunostaining with IIH6. Scale bar = 100 μM. The numbers of 1, 20, 100 and 500 represent the concentration in μM. * *P<0.05*, comparison was made between ribitol alone and combination treatment groups. N=100x3. (C and D) Western blots detection with IIH6. Rib, ribitol at 5 mM concentration; cyti, cytidine at 500 μM concentration; CDP at 100 μM concentration. Signal intensity is adjusted with GAPDH.

Effect of ribulose and ribose on levels of ribitol and ribitol-5P

To understand the potential conversion pathway of ribose, ribulose and ribitol to ribitol-5P, targeted metabolomics analysis with focus on pentose phosphate pathway related metabolites was performed for the cells treated with each of the 3 pentoses at 2 mM concentration. As expected, the levels of each pentose were correlated well with the treatment (Fig. 5). Interestingly, ribulose treatment led to 80 times higher levels of ribitol than the control. In contrast, ribose treatment only slightly increased ribitol level over untreated cells. Ribitol and ribulose treatment did not significantly increase the level of ribose, whereas ribose and ribitol treatment did not significantly increase the levels of ribulose. Consistently, ribitol-5P level was the highest with ribitol treatment followed by ribulose treatment, and lowest with ribose treatment. Interestingly all 3 pentose treatments decreased the levels of both ribose-5P and ribulose-5P when compared with the control, but there was no significant difference between the treatments.

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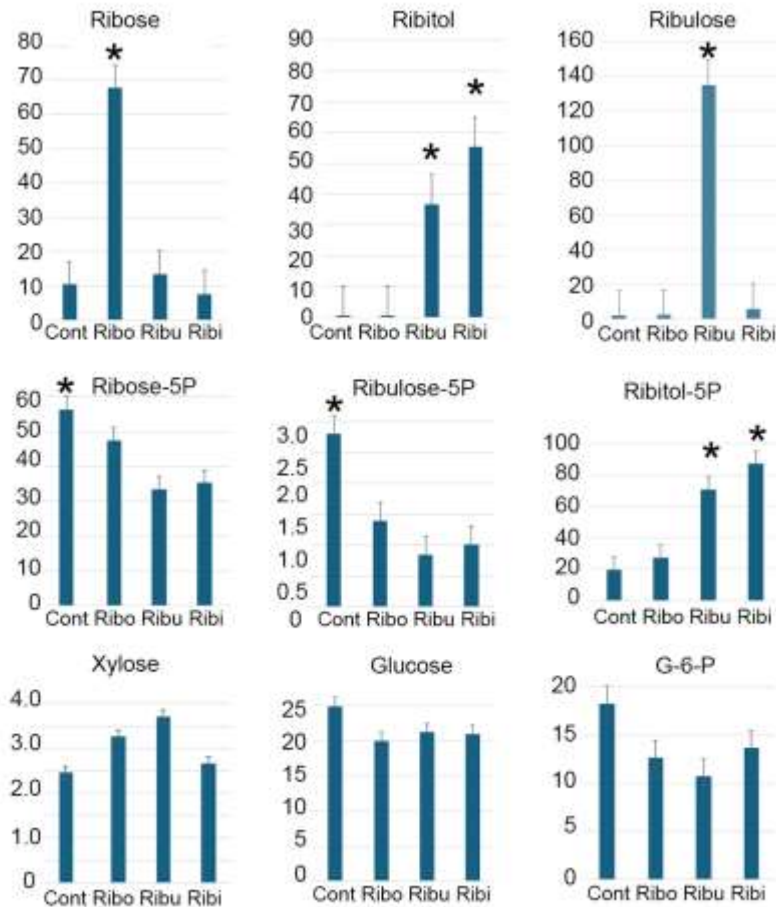

Figure 5. Comparison of metabolomics analysis of MCF7 cells treated with ribitol, ribose and ribulose. Cont, untreated cells as control. Ribo, Ribu and Ribi represent ribose, ribulose and ribitol respectively. Y axis is the number x 1000, representing relative peak intensities (peak heights) and normalized by the average mtic of the samples. * $P<0.05$. T.TEST from duplicates. Comparison was made between each pentose to the control.

Discussion

Earlier studies have established that both ribitol and ribose are capable of enhancing synthesis of matriglycan *in vitro* and *in vivo* of mouse models with FKRP mutations [25-27]. However, other pentoses or related metabolites might also be effective for the same purpose. By examining a group of metabolites on matriglycan, the results from the current study demonstrate that ribitol, ribose and ribulose and their 5-phosphates are effective metabolites capable of enhancing matriglycan synthesis significantly. The results also confirmed that ribitol and ribitol-5P are most effective in enhancing matriglycan levels when compared to ribose and ribulose. Interestingly, ribulose and ribulose-5P showed clearly higher efficiency than ribose and ribose-5P. While all the three metabolites could be used for experimental therapy, both ribose and ribulose participate in the production of advanced glycation end products (AGEs) that can lead to cell dysfunction and death [32]. In contrast, ribitol as a reduced form of ribose poses little capacity for glycation. The current results together therefore support the notion that ribitol is preferred for life-long treatment of FKRP-related dystroglycanopathy.

Ribitol, ribose and ribulose as well as their 5-phosphates are considered reversible in conversion to each other with specific reductase, dehydrogenase, kinase or isomerase largely within the non-oxidative pentose phosphate pathway [33-35]. Under normal physiological conditions, the flow direction of these pentoses and their 5-phosphates are likely controlled by demand for specific metabolites required for cellular functions or proliferation. However, the mechanisms controlling the flow direction of conversion from one pentose to another, or one 5-phosphate to another is not

clearly understood. This study focused on identification of the most effective metabolites on matriglycan synthesis for therapeutic potential, therefore, understanding the pathway is highly valuable. It has been convincingly demonstrated that CDP-ribitol is synthesized by ISPD with ribitol-5P and CTP as substrates, but little is known about ribitol-5P synthesis. Earlier studies suggested that this could come from reduction of D-ribose, or D-ribose-5-P, or D-ribulose-5-P [36]. Shunsuke Hoshino et al. recently proposed four possible pathways [37]. Both ribose and ribulose (pathway 1 and 2) can be reduced to ribitol and then phosphorylated to ribitol-5P. Both ribose-5P and ribulose-5P (pathway 3 and 4) can be reduced to ribitol-5P. By studying endogenous reductase activities for generation of ribitol-phosphate pathway in HEK293T cells, the authors reported that ribitol-5P is synthesized through the reduction of ribose or ribulose to ribitol, which is then phosphorylated to ribitol-5P by FGGY. In addition, since the ribose reduction activity constitutes 64% of the endogenous activity, the ribose reduction pathway is likely to be the dominant contributor to the production of ribitol-5P. The results from the current comparative analysis of efficiency in matriglycan levels and metabolomics of the cells treated with ribitol, ribose and ribulose allow us to propose an alternative metabolic flow under non-physiological concentration in relation to ribitol-5P and matriglycan synthesis. Ribulose is clearly more effective than ribose for enhancing matriglycan. Consistently, ribulose treatment leads to much higher levels of ribitol and ribitol-5P than ribose treatment. Furthermore, ribitol and ribulose treatment did not significantly increase the level of ribose, whereas ribose treatment slightly increases the levels of ribulose. These data together support the view that both ribose and ribulose can be converted to ribitol which is then phosphorylated into ribitol-5P as the substrate of ISPD. The higher-level increase in ribitol and matriglycan with ribulose treatment over ribose treatment supports the notion that ribulose and ribulose-5P are more effectively converted to ribitol and ribitol-5P when compared to ribose and ribose-5P. We therefore propose that ribulose to ribitol to ribitol-5P pathway is the more efficient flow of the two pentoses to the synthesis of ribitol-5P when they are supplemented (Fig. 6). Alternatively, CDP-ribitol could be directly converted from CDP-ribulose by ISPD as reported [24]. Intriguingly, cells treated with all the pentoses had reduced levels of both ribose-5P and ribulose-5P when compared to the control cells, and mechanism(s) involved remains to be explored.

Another interesting observation from this study is the difference in signal levels of matriglycan detected by immunostaining and by western blots with the same monoclonal antibody IIH6 after the treatment of cytidine and its phosphates. While significant enhancement can be detected consistently by immunostaining when cytidine and its phosphates were used in combination with ribitol, this effect cannot be convincingly demonstrated by western blots. The reason(s) behind such discrepancy is not clearly understood, but several possible explanations can be envisaged. Immunostaining especially with the IIH6 antibody to matriglycan is generally more sensitive for the detection of lower levels of protein than western blot. Thus, the limited enhancement in matriglycan consistently detected by immunostaining is not sufficient to be distinguished by western blot. Other less likely possibility is that IIH6 antibody might non-specifically bind to some cellular components in muscle sections by immunostaining. However, such binding capacity may be eliminated by sample treatment procedure of western blot which disrupts higher-level protein structures causing alteration in antibody recognition. It is also possible that weak signals binding to other cellular components may be filtered out by different sizes in western blot. Nevertheless, the nature of these metabolites, especially the safety and benefit of the over-the-counter supplement CDP-choline warrants further investigation *in vivo* to explore its potential benefit to the relevant disease.

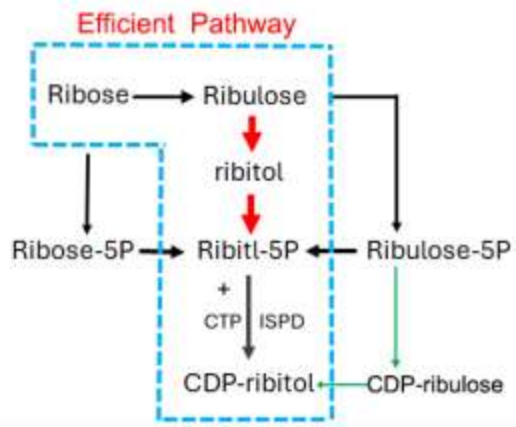

Figure 6. Predicted flow efficiency of ribose, ribulose and ribitol in the pathway towards synthesis of ribitol-5P and CDP-ribitol. Red arrows indicate the pathway with higher efficiency in the cells with ribose and ribulose supplement. The green line indicates a possible alternative route directly from ribulose-5P to CDP-ribulose and then CDP-ribitol. 5P, 5-phosphate.

Conclusions

The pathway from ribose to ribulose to ribitol and then to ribitol-5P is likely the predominant flow for synthesis of ribitol-5P when the pentoses are supplemented for enhancement of matriglycan expression as therapeutics. Therapeutic potential of cytosine and its phosphates may be limited and remain to be explored in vivo. Ribitol is preferred for life-long treatment of FKRP-related dystroglycanopathy.

Author Contributions

Conceptualization, Q.L.L., and P.J.L.; Methodology, P.J.L., Q.L.L., E.B and J.T.; Validation, P.J.L.; Formal analysis, P.J.L.,Q.L L.; Data acquisition, P.J.L., E. B., Resources, Q.L.L.; Data Curation, Q.L L, P.J.L.; Writing - Review & Editing, Q.L.L., P.J.L, and J.T.; Project Administration, Q.L.L.; Funding Acquisition, Q.L.L.

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Availability of Data and Materials

The authors declare that all data supporting the findings of this study are available within the article and its Supplemental Information Files.

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Not applicable.

Conflicts of Interests

Q.L.L. is consultant for the company ML BioSolution which is conducting clinical trials of ribitol for Limb Girdle Muscular Dystrophy 2I.

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