Molecular Genetics and Metabolism 106 (2012) 214–220

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Rhodamine B and 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose (F-GlcNAc) inhibit chondroitin/dermatan and keratan sulphate synthesis by different mechanisms in bovine chondrocytes

Ainslie L.K. Derrick-Roberts [a,b,*], Wanda Marais [a], Sharon Byers [a,b,c]

[a] Department of Genetics and Molecular Pathology, SA Pathology, North Adelaide, SA 5006, Australia
[b] Discipline of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA 5005, Australia
[c] Department of Genetics, School of Molecular & Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia

ARTICLE INFO

Article history:
Received 2 March 2012
Received in revised form 4 April 2012
Accepted 4 April 2012
Available online 11 April 2012

Keywords:
Mucopolysaccharidosis
Glycosaminoglycan
Substrate deprivation therapy
F-GlcNAc
Rhodamine B

ABSTRACT

MPS disorders result from a deficiency or absence of glycosaminoglycan (GAG) degrading enzymes leading to an imbalance between the synthesis and degradation of GAGs and their subsequent accumulation in a range of cells. The inhibition of GAG synthesis using small chemical inhibitors has been proposed as a novel therapeutic approach to treatment. Several inhibitors have been shown to decrease heparan sulphate GAG synthesis and in this study we evaluated a novel fluorinated analog of N-acetylglucosamine (2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose (F-GlcNAc)) and rhodamine B for their ability to also inhibit the synthesis of chondroitin/dermatan and keratan sulphate GAGs present in bovine cartilage. Both inhibitors decreased GAG synthesis in chondrocyte monolayer culture and in cartilage chip explant culture in a dose dependent manner. Both inhibitors decreased the size of newly synthesised proteoglycans and in the case of F-GlcNAc this was due to a decrease in newly synthesised GAG chain size. Rhodamine B, however, did not affect GAG chain size, while both inhibitors decreased the amount of chondroitin/dermatan and keratan sulphate GAG equally. The expression of genes responsible for the initiation and elongation of chondroitin/dermatan sulphate and keratan sulphate GAGs were downregulated in the presence of rhodamine B but not in the presence of F-GlcNAc. Thus the 2 inhibitors appear to have differing effects on GAG synthesis, with F-GlcNAc inhibiting the epimerisation of UDP-GlcNAc to UDP-GalNAc thus decreasing the availability of monosaccharides for addition to the growing GAG chain, whereas rhodamine B is more likely to reduce the number of GAG chains. Together with previous data these 2 inhibitors are capable of non-specific inhibition of GAG synthesis, reducing the production of chondroitin/dermatan sulphate, keratan sulphate and heparan sulphate GAGs. As such they would be applicable to therapy in a range of MPS disorders.

1. Introduction

The mucopolysaccharidosis (MPS) disorders are a family of heritable lysosomal storage disorders that are caused by a deficiency or absence of an enzyme activity essential for the turnover of glycosaminoglycans (GAGs) [1]. The nature of the storage material is determined by the enzyme deficiency and can result in storage of undegraded or partially degraded heparan sulphate (HS), dermatan sulphate (DS), chondroitin sulphate (CS) or keratan sulphate (KS) or hyaluronan GAG chains. As a group, MPS disorders affect 1 in 22,500 Australians [2]. Symptoms vary according to the type of MPS disorder, and a range of phenotypes are observed within each MPS, but can include central nervous system (CNS) deterioration, skeletal abnormalities, corneal clouding and organomegaly [1]. Death usually occurs in the second or third decade of life in severely affected patients.

Current treatment strategies for MPS patients rely on replacement of the missing enzyme via enzyme replacement therapy (ERT) or bone marrow transplantation (BMT). ERT has been approved for clinical use in MPS VI [3] and the non-neuronal forms of MPS I and MPS II [4,5] and a phase 3 clinical trial in MPS IVA patients is underway [6]. Improved lung function, reduced urinary gag excretion and decreased liver and spleen size [4,7] are the main positive outcomes of ERT. However, sites that are not well vascularised such as the brain, cornea and bone continue to develop pathology. ERT has a small effect on alleviating skeletal disease in MPS patients with the bone not responding as well as anticipated and cartilage is non-responsive to treatment. Corneal clouding also remains problematic in a number of patients. BMT was

Abbreviations: MPS, mucopolysaccharidosis; ERT, enzyme replacement therapy; GAG, glycosaminoglycan; F-GlcNAc, 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-α-glucopyranose; SDT, substrate deprivation therapy; CNS, central nervous system; HS, heparan sulphate; CS, chondroitin sulphate; DS, dermatan sulphate; KS, keratan sulphate.

* Corresponding author at: Department of Genetics and Molecular Pathology, 4th Floor Rogerson Bldg, SA Pathology, 72 King William Road, North Adelaide, SA 5006, Australia. Fax: +61 8 8161 7100.
E-mail address: ainslie.derrickroberts@adelaide.edu.au (A.L.K. Derrick-Roberts).

1096-7192/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2012.04.002

the first multi-tissue treatment offered for MPS and has been used in a number of MPS types with variable outcomes on disease pathology. BMT is the treatment of choice for MPS I patients, especially when transplantation is undertaken at a very early age (6–12 months being the optimal age) and therefore before the onset of significant pathology [8,9]. Skeletal disease continues to progress in MPS I children who have received BMT and they require ongoing orthopaedic intervention to manage this aspect of the disorder. Major drawbacks of BMT as a treatment are the high mortality rate of 10–20% in the first year post-BMT, the rate of graft failure (15–75%) and finding a suitable donor before the onset of symptoms [10,11].

Another way to redress the balance between synthesis and degradation of GAGs is by slowing down the initial synthesis of GAGs in cells. Substrate deprivation therapy (SDT) (also known as substrate reduction therapy (SRT)) is applicable to MPS patients with residual enzyme activity, who will be able to more effectively turnover stored substrate if the synthesis of GAGs is reduced. SDT is a recently emerging therapy for MPS and lysosomal storage diseases and is currently used in the clinic for Gaucher type I patients [12] and Niemann–Pick C patients [13].

Two inhibitors, rhodamine B and genistein, are SDT therapeutics targeting GAG synthesis that have been shown to be effective *in vitro* and *in vivo* in mouse models of MPS II and III [14–16]. Both inhibitors reduced lysosomal GAG storage and improved neurological symptoms in murine MPS III [17,18]. Genistein has also been administered to MPS III patients where decreased urinary GAG and improved cognitive function were observed [19,20]. Administration of SDT has not shown any deleterious side-effects in mice and low dose rhodamine B SDT can be given long-term and during pregnancy in mice [21]. Similarly, no side-effects were observed after F-GlcNAc treatment in MPS IIIA or MPS VII mice (unpublished data). Both genistein and rhodamine B were thought to be specific inhibitors of HS synthesis and have only been tested in models where HS accumulates. Genistein acts by inhibiting tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor which activates a protein kinase cascade and activates transcription factors which are thought to regulate GAG biosynthesis [22]. The mechanism of action of rhodamine B is currently unknown, however, rhodamine B reduced lysosomal GAG storage in MPS VI patient fibroblasts [23] suggesting that it is a non-specific inhibitor of GAG. The effect of genistein on other disorders that accumulate chondroitin/dermatan sulphate (CS/DS) and keratan sulphate (KS) is currently unknown, as is the effect of rhodamine B on KS synthesis.

Other soy isoflavones [24,25] and gene silencing approaches have been used to decrease the synthesis of HS GAG *in vitro* with a positive outcome on lysosomal storage observed [26,27]. A 4-deoxy analogue of *N*-acetyl-D-glucosamine (4-deoxy-GlcNAc) has also been used to inhibit HS expression and growth factor binding *in vitro* by interfering with GAG chain elongation in melanoma cells [28]. A novel fluorinated analog of N-acetylglucosamine (2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose (F-GlcNAc) decreased HS GAG synthesis *in vitro* [29] and dampens effector T cell migration in inflammation [30,31]. Studies have also reported reduced tumour cell adhesion in colon carcinoma with F-GlcNAc treatment [32]. Recent studies in murine airway smooth muscle cells showed that F-GlcNAc inhibited CS GAG synthesis by inhibiting 4-epimerisation of UDP-GlcNAc to UDP-GalNAc, whereas HS GAG chain elongation was inhibited by incorporation of 4-F-GlcNAc at the non-reducing terminus, preventing further GAG chain elongation [33]. By reducing the length and amount of GAG chains that are synthesised in the cell, this is likely to have a positive effect on lysosomal storage.

In this study the effect on inhibiting CS/DS and KS was investigated. There is currently no report of the effect of F-GlcNAc or rhodamine B treatment on KS synthesis. Bovine chondrocytes were used to determine the effect of inhibition of KS synthesis using rhodamine B and F-GlcNAc, as they synthesise predominantly CS/DS and KS GAGs [34]. The type and amount of GAG inhibition was determined using $Na^{35}SO_4$ incorporation into bovine chondrocytes and proteoglycan size and GAG chain size and type were determined. Expression of genes involved in GAG synthesis was also analysed to determine the mechanism of action of each SDT agent. Both inhibitors decreased the size of newly synthesised proteoglycans and in the case of F-GlcNAc this was due to a decrease in newly synthesised GAG chain size. Rhodamine B, however, did not affect GAG chain size, while both inhibitors decreased the amount of CS/DS and KS GAGs equally. The expression of genes responsible for the initiation and elongation of CS/DS and KS GAGs were downregulated in the presence of rhodamine B but not in the presence of F-GlcNAc.

2. Materials and methods

2.1. Materials

Chondroitinase ABC lyase (E.C.4.2.2.4) was purchased from MP Biomedicals Australasia Pty Ltd, Australia. Keratanase (E.C. 3.2.1.103), Sepharose CL-4B, Sepharose CL-6B and Sephadex G-50 and rhodamine B were purchased from Sigma-Aldrich, Australia. $Na^{35}SO_4$ was purchased from Amersham Pharmacia, Uppsala, Sweden. F-GlcNAc was synthesised according to the method in ref. [29] at the Victorian Institute for Chemical Sciences, Monash University.

2.2. Cell and tissue culture

Articular cartilage was dissected from ankle joints of adult cows. Cartilage was minced into ~3 mm^3 chips for explant culture or digested sequentially with pronase and collagenase [35] to generate a single cell suspension. Both cartilage chip explants and monolayer chondrocyte cultures were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin G and 50 μg/ml streptomycin sulphate.

2.3. Dose response in bovine chondrocytes and cartilage chips

Bovine chondrocytes were seeded at a density of 5.2×10^4 cells per cm^2 while cartilage chips were distributed in 24-well plates at ~100–150 mg cartilage per well. Cells and tissue were pre-incubated with 0 to 1 mM F-GlcNAc or 0 to 100 μg/ml rhodamine B in DMEM + 10% FCS for 1 h at 37 °C. Cells were then incubated in the same concentration of F-GlcNAc/rhodamine B plus 5 μCi/ml $Na^{35}SO_4$ for 6 h at 37 °C. Medium was removed from the monolayer chondrocyte cultures and the cells extracted into 4 M guanidine HCl, 50 mM sodium acetate pH 5.8 buffer containing 0.1 M amino hexanoic acid, 0.05 M Na_2EDTA and 0.1% Triton X-100. The medium was removed from the cartilage explant cultures and the chips blotted dry on filter paper and weighed. Cartilage chips were extracted at 4 °C, in the same guanidine buffer as above for 48 h and then extracted in 0.5% NaOH for a further 48 h at 4 °C. Unincorporated isotope was removed by size exclusion chromatography on Sephadex G-25 equilibrated in buffer containing 2 M GuHCl, 0.1 M Na_2SO_4, 0.05 M Tris base, 0.5% Triton X-100 pH 7.5. GAG synthesis was normalised to cell protein as previously described [36].

2.4. Proteoglycan size

Bovine chondrocyte monolayer cultures were incubated in DMEM, 10% FCS containing 0.1 mM F-GlcNAc or 80 μg/ml rhodamine B and 5 μCi/ml $Na^{35}SO_4$ for 24 h at 37 °C. The medium was removed and cells were extracted into 4 M guanidine HCl, 50 mM sodium acetate pH 5.8 buffer containing 0.1 M amino hexanoic acid, 0.05 M Na_2EDTA and 0.1% Triton X 100 at 4 °C for 48 h. Both cell layer and medium fractions were dialysed against 0.1 M Tris-Acetate pH 7 followed by dialysis against distilled water for 24 h each at 4 °C. Following dialysis, each sample was lyophilised. Aliquots were resuspended in 2 M GuHCl, 0.5 M Tris, 0.1 M Na_2SO_4, pH 7.0 buffer and run on Sepharose