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The metabolic and biochemical impact of glucose 6-sulfonate (sulfoquinovose), a dietary sugar, on carbohydrate metabolism

Juliana L. Sacoman *, Lauren N. Badish, Thomas D. Sharkey, Rawle I. Hollingsworth

Department of Biochemistry and Molecular Biology, 603 Wilson Rd., Room 116, Michigan State University, East Lansing, MI 48824, United States

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ABSTRACT

Increased activity of the main carbohydrate pathways (glycolysis, pentose phosphate, and hexosamine biosynthetic pathways) is one of the hallmarks of metabolic diseases such as cancer. Sulfoquinovosyl diacylglycerol is a sulfoglycolipid found in the human diet that possesses anticancer activity that is absent when its carbohydrate moiety (glucose 6-sulfonate or sulfoquinovose) is removed. This work used bacterial systems to further understand the metabolism of this sugar through three main carbohydrate processing pathways and how this could influence its biological activity. Using ^{13}C NMR spectroscopy and enzyme assays, we showed that glucose 6-sulfonate cannot enter the pentose phosphate pathway, hence decreasing pentose and nucleotide biosyntheses. In glycolysis, glucose 6-sulfonate only provides one pyruvate per monosaccharide molecule, decreasing the flux of this pathway by half when compared to glucose 6-phosphate. Glucose 6-sulfonate can enter the hexosamine biosynthetic pathway by producing glucosamine 6-sulfonate, which is a reported antibacterial agent that competitively inhibits hexosamine production. All these interactions with carbohydrate routes might help explain the observed anticancer activity that glucose 6-sulfonate has in vitro. This adds to our knowledge of how vegetables rich in glucose 6-sulfonate can also act as metabolic inhibitors of pathways that are increased in metabolic diseases.

1. Introduction

There is a rising tide of evidence linking the long-term activity in certain carbohydrate processing pathways to the susceptibility to various diseases. In recent times, the focus has been placed on the association between the consumption of large amounts of simple sugars and the prevalence of metabolic disorders[1–4] in which there are alterations in the metabolic activity through the main carbohydrate-processing routes. The three main pathways usually in focus are glycolysis, the pentose phosphate pathway, and the hexosamine biosynthetic pathway. For instance, increased flux through the hexosamine biosynthesis pathway is linked to type 2 diabetes, Alzheimer's, and cancer.[5–7] Hyperactivity in the pentose phosphate pathway and glycolysis is present in cancer.[8–11] Understanding the dynamics of the processes between these various pathways and determining ways to apportion the activities between the various branches are critical goals for developing a metabolism-based approach to disease management. An overview of the interconnectivities between these three pathways is depicted in Figure 1 and reinforces how central carbohydrate chemistry is in cellular biochemistry.

An important way of regulating the level of activity in certain segments of these interconnected chemical processes is through the use of specific chemical inhibitors to reduce flux into particular pathways. A broader strategy is to utilize a dietary carbohydrate that is capable of generating intermediates that inhibit several steps or skewing the activity through one or more pathways.

Glucose 6-sulfonate (sulfoquinovose) is an important sugar present in the human diet. It is found in all plants, mostly in the form of sulfoquinovosyl diacylglycerol (SQDG) and it is present in high amounts in spinach, green tea, and other green leaves.[12–14] The availability of glucose 6-sulfonate from some plants can be 10 times larger than some amino acids.[15] In these amounts it can exert a profound influence on carbohydrate metabolism in humans.

Not much is known about the metabolism of glucose 6-sulfonate in mammals besides the participation of the microbial flora in the digestive tract being responsible for most of the degradation of SQDG.[16] In bacteria, glucose 6-sulfonate follows a catabolic path in which it is converted to fructose 6-sulfonate and then follows a pathway that is similar to the glycolytic breakdown of fructose 6-phosphate. An important difference is that sulfolactate is formed from one half of the molecule instead of glyceraldehyde 3-phosphate.[17] There is not much known about the metabolism of glucose

Abbreviations: DNB-Cl, dinitrobenzoyl chloride; GFAT, glutamine: fructose-6-phosphate amido transferase; G6PDH, glucose-6-phosphate dehydrogenase; NMR, nuclear magnetic resonance; PPP, pentose phosphate pathway; SQDG, sulfoquinovosyl diacylglycerol.

* Corresponding author. Tel.: +1 517 4321113; fax: +1 517 3539334.
E-mail address: sacomanj@msu.edu (J.L. Sacoman).

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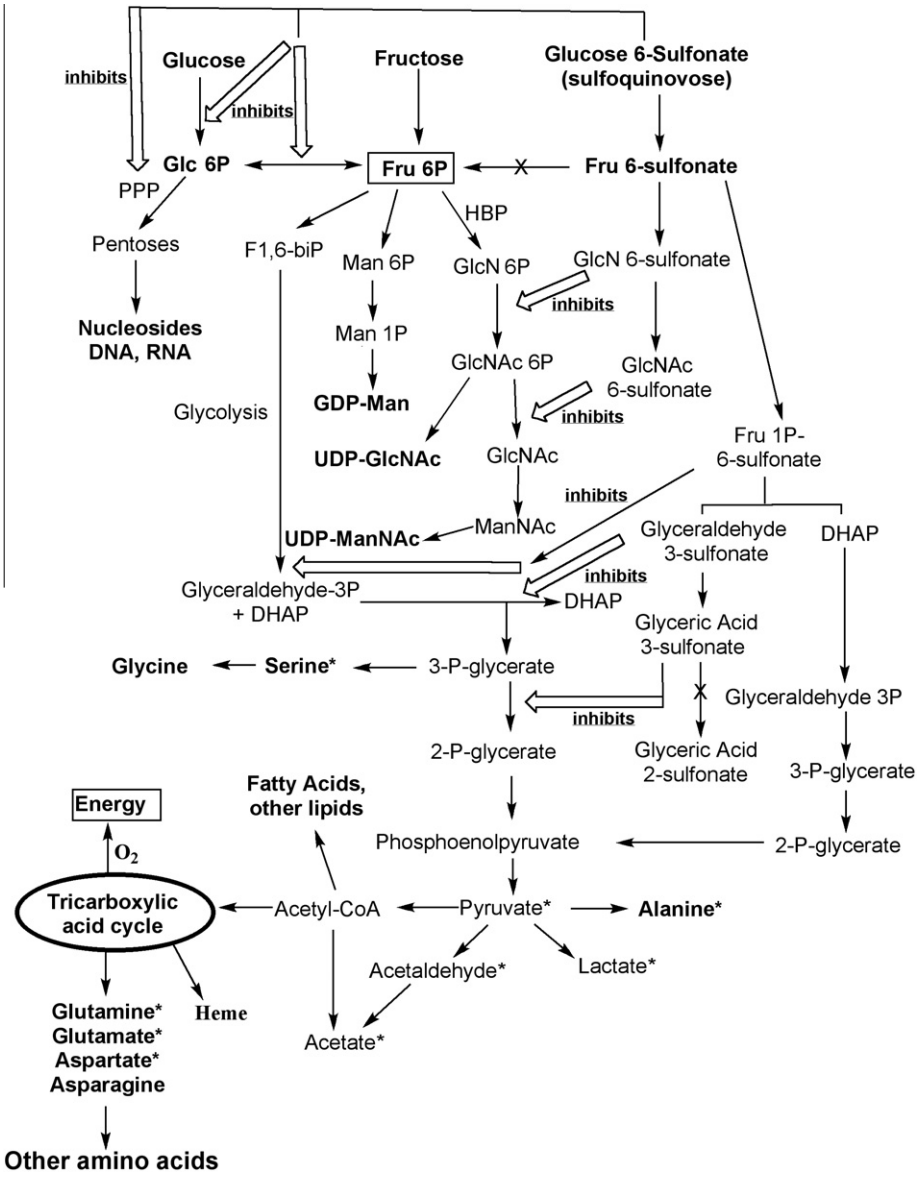

Figure 1. Metabolic web of carbohydrate pathways affected by glucose 6-sulfonate. Glucose 6-sulfonate can be isomerized to fructose 6-sulfonate, which can enter the HBP and competitively inhibit its enzymes. Many derivatives of glucose 6-sulfonate can also enter glycolysis and also inhibit a variety of enzymes in this pathway. PPP: pentose phosphate pathway; HBP: hexosamine biosynthetic pathway; DHAP: dihydroxyacetone phosphate. *Represents the molecules detected in the ^{13}C NMR studies.

6-sulfonate in other important carbohydrate pathways (pentose phosphate and hexosamine biosynthetic pathways) and which intermediates are actually formed in these processes. Our idea was to evaluate the status of this complex biosynthetic web by a non-destructive analytical method that can give information on the identity of analytes. Nuclear Magnetic Resonance (NMR) spectroscopy[18–21] could be useful provided the isotopic abundance of the nucleus being probed is high enough. We employed this technique to follow the catabolism of fructose 6-phosphate and glucose 6-sulfonate alone and in combination through glycolysis and pentose phosphate pathway. For intermediates from pathways (hexosamine biosynthetic pathway) that were below the detection limit of ^{13}C NMR techniques, other methods to probe them directly in vivo were used. This was the case for glucosamine 6-sulfonate (2-amino-2,6-dideoxy-6-sulfo-D-glucose), previously described[22] as an inhibitor of hexosamine biosynthesis, which confers its antibacterial activity by inhibiting cell wall synthesis. The idea is that glucosamine 6-sulfonate would originate from glucose 6-sulfonate isomerization to fructose 6-sulfonate, followed by amination of fructose 6-sulfonate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) on the hexosamine biosynthetic pathway. This is a reasonable assumption given the demonstrated tolerance of enzymes in the hexosamine pathway for substitution of phosphate groups by sulfonates in their substrates.[22]

As illustrated in Figure 1, there are several points at which glucose 6-sulfonate or its metabolic products can modulate activity through several other elements of carbohydrate metabolism. This potential modulation opens up a path for potentially tailoring metabolic activity to confine it within certain regimes as a strategy for disease management.

2. Results and discussion

2.1. NMR studies

Both ^{13}C-1-glucose 6-sulfonate and ^{13}C-1-fructose 6-phosphate synthesized by chemical or enzymatic reactions were used for profiling the metabolites after 0, 1, 2, 4, 5, and 144 h of incubation

with *Escherichia coli* cell lysate. Small peaks started to appear in the NMR profiles after 5 h of incubation, but the most significant changes in the profile were established after 144 h; therefore, the signals listed in Table 1 refer to this last time point (144 h).

The incubation of the cell lysate with ^{13}C-1-fructose 6-phosphate generated large quantities of alanine, lactic acid and significant amounts of acetic acid, glutamine, glutamate, aspartate, serine, and 6-phosphogluconate (Table 1). The production of alanine, lactic acid, acetic acid, and serine is directly related to the entry of fructose 6-phosphate into glycolysis. Glutamine, glutamate, and aspartate are derived from entry of pyruvate into the Krebs cycle, in which intermediates can be formed for amino acid biosynthesis as well.

Entry into the pentose phosphate pathway is also detected by the signal at 181 ppm, which corresponds to the formation of 6-phosphogluconolactone from glucose 6-phosphate. High instantaneous concentrations of fructose 6-phosphate can cause a shift toward the production of high quantities of glucose 6-phosphate due to the equilibrium constant of the phosphoglucose isomerase ($K_{eq[Glu6P/Fru6P]} \sim 3$).[23] By increasing glucose 6-phosphate, elevated concentrations of fructose 6-phosphate induce a higher activity down the pentose phosphate pathway.[24] Fructose can induce an increase of 250% in transketolase activity (an enzyme from the non-oxidative stage of the PPP) in comparison to glucose in pancreatic cancer cells.[24] This important link shows that fructose is a stronger inducer of nucleotide biosynthesis and therefore, cell division and proliferation, in comparison to glucose.

When ^{13}C-1-glucose 6-sulfonate is used as carbon source, it produces many of the same metabolites as ^{13}C-1-fructose 6-phosphate, with direct evidence of the biosynthesis of alanine, pyruvate, glutamine, acetic acid, aspartate, asparagine, and cysteine (Table 1). Many of these metabolites are derived from the activity of glycolytic enzymes. Our ^{13}C NMR data corroborate the report[17] that show that sulfoquinovose does go through glycolysis by the detection of methyl derivatives such as acetic acid, pyruvate, and alanine in the region 15–30 ppm.

The formation of 6-phosphogluconolactone (which was detected at 181 ppm) was not observed, indicating that glucose 6-sulfonate may not be metabolized by glucose 6-phosphate dehydrogenase, the first step of pentose phosphate pathway. When cell lysates were mixed with both ^{13}C-1-fructose 6-phosphate and ^{13}C-1-glucose 6-sulfonate, the majority of the products formed were the same as if fructose 6-phosphate was incubated alone. Glucose 6-sulfonate does not participate nor impede in fructose 6-phosphate metabolism at the concentration used as observed by the formation of intermediates of the pentose phosphate pathway (6-phosphogluconolactone) and glycolysis (alanine, lactic acid) (Table 1). A comparison of the metabolic routes and intermediates observed when fructose 6-phosphate or glucose 6-sulfonate is the sole carbon sources incubated with cell lysates is depicted in Figures 2 and 3.

2.2. Effect on glycolysis

The data described in Table 1 show evidence for sulfoquinovose metabolism through glycolysis by the detection of compounds such as ethanol, acetic acid, and lactic acid. The involvement of sulfoquinovose in the glycolytic pathway has been previously described and our data corroborate these previous findings.[17] An important point about sulfoquinovose metabolism through glycolysis is that sulfoquinovose could possibly act as an inhibitor of glycolysis as well. While each molecule of fructose 6-phosphate can form two molecules of pyruvate, one molecule of sulfoquinovose only provides one molecule of pyruvate. Due to the inability of the cell to break the carbon–sulfur bond between the sulfonate and C6 of glucopyranose, only half of the molecule can be converted to glyceraldehyde 3-phosphate. A proposed route of metabolic conversion for sulfoquinovose and its ability to act as an inhibitor of carbohydrate-related pathways is illustrated in Figure 1.

2.3. Effect on the pentose phosphate pathway

The pentose phosphate pathway is responsible for the biosynthesis of riboses, purines, and pyrimidines, which makes this pathway one of the most important control points of nucleic acid biosynthesis through the regulation of the availability of substrates involved in their formation.[25] An important reaction that regulates the flux through this pathway is the one catalyzed by glucose-6-phosphate dehydrogenase. Suppression of the glucose-6-phosphate dehydrogenase activity can cause inhibition of proliferation and cell death.[26] On the other hand, a hyper activation of pentose biosynthesis is very characteristic of cancer cells due to the intense demand of nucleic acid biosynthesis for cell proliferation.[27] Therefore, drugs that can inhibit this pathway by competitively blocking enzyme activity, and therefore, blocking the elevated level of nucleic acid biosynthesis, are promising molecules for cancer therapy.

To better understand the possible role of glucose 6-sulfonate in the pentose phosphate pathway (PPP), an enzymatic assay using sulfoquinovose as substrate was performed. The Hanes–Woolf plot for the K_m determination of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49, the enzyme from the first step of PPP) is depicted in Figure 4 and it is estimated at 108 μM in presence of increasing amounts of glucose 6-phosphate. When glucose 6-sulfonate is incubated alone with G6PDH, less than 1% of enzyme activity is detected. This result confirms that the absence of a signal for 6-phosphogluconolactone in the NMR study is because glucose 6-sulfonate is not a substrate for the dehydrogenase and, therefore, cannot form 6-sulfogluconolactone and enter pentose biosynthesis.

Given the similarity in structure we tested whether glucose 6-sulfonate could be a competitive inhibitor of G6PDH. Glucose 6-sulfonate was added to a G6PDH assay with 50 μM G6P (approximately half the K_m concentration to increase the chance of seeing an inhibition if glucose 6-sulfonate is indeed a competitive inhibitor). At 100 μM, glucose 6-sulfonate inhibition of G6PDH was not

Table 1
Resonances obtained from different carbon sources after 144 h of incubation with cell lysate.

Fructose 6-phosphate		Glucose 6-sulfonate		Fructose 6-phosphate + glucose 6-sulfonate	
Chemical shift ^{13}C	Compound identified	Chemical shift ^{13}C	Compound identified	Chemical shift ^{13}C	Compound identified
19.7	Alanine	19.6	Alanine	19.6	Alanine
22.5	Lactic acid	23.6	Acetic acid	22.8	Lactic acid
23.2	Acetic acid	26.7	Cysteine	25.9	Cysteine
32.4	Glutamine	27.1	Pyruvate	35	Glutamic acid
33.6	Glutamic acid	30.4	Glutamine	36.2	Asparagine
38.5	Aspartate	36.7	Asparagine	39	Aspartate
63.8	Serine	37.6	Aspartate	63.9	Serine
180.6	6-Phospho gluconolactone	62.8	Serine	182.0	6-Phospho gluconolactone

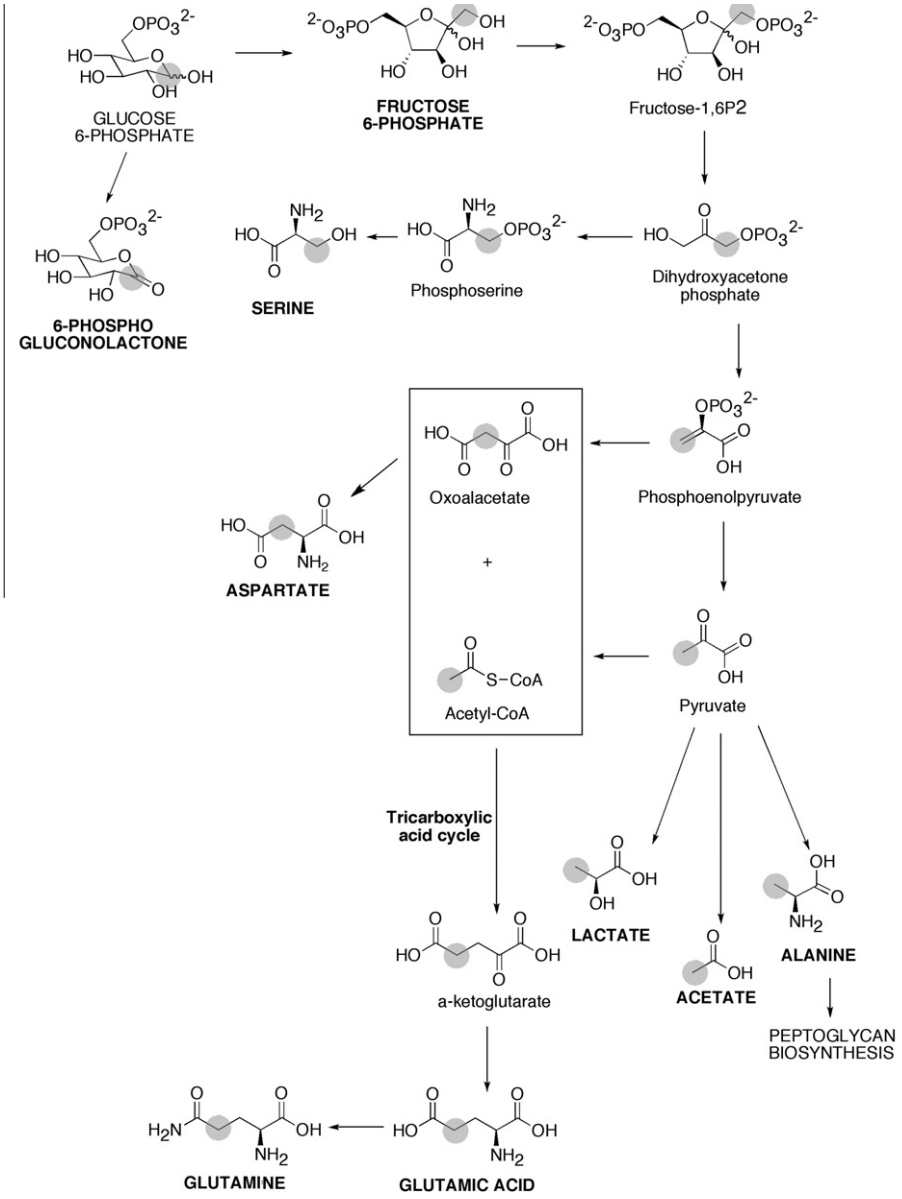

Figure 2. Fructose 6-phosphate metabolic pathways observed by ^{13}C NMR after 144 h of incubation with cell lysate. Metabolites observed in this study are highlighted in bold.

statistically significant ($p = 0.145$). However, at 400 μM a 5% decline was found and this was significant ($p = 0.02$, two tailed t-test of the difference of the means).

The percentage of SQDG in spinach is 1.7% of dry weight,[28,29] meaning that the ingestion of 100 g of spinach by a person weighing 75 kg would result in blood levels of 19 μg/mL (assuming that 60% of body weight is water). For achieving concentrations that could act as an effective inhibitor of G6PDH, the concentration of sulfoquinovose in the blood would need to be 690 μg/mL. Although these numbers show that the use of sulfoquinovose as an inhibitor of pentose biosynthesis cannot be achieved by diet, it is possible that high levels of glucose 6-sulfonate could be used as a pharmacological drug to slow the pentose phosphate pathway. More studies are needed to determine its toxicity and pharmacokinetics.

When analyzing the quaternary structure of glucose-6-phosphate dehydrogenase,[30] it is noticeable that three residues are especially important to stabilize the charge and accommodate the phosphate group of glucose 6-phosphate in the active site. These residues are His178, Tyr179, and Lys182. This is supported by site directed-mutagenesis studies showing that the substitution of these residues, in particular H178N, decreases the ability of the enzyme to discriminate between glucose and glucose 6-phosphate.[30] It is proposed that His178 interacts to the phosphate moiety through hydrogen bonds and charge–charge interactions. Apparently, the substitution of this amino acid increases the distance between the three residues and to the phosphate moiety, which affects the binding of glucose 6-phosphate to the enzyme. If the distance between the H-bond donor ligands and the phosphate group is longer than 9 Å, the interaction is disrupted.[30]

In glucose 6-sulfonate the sulfonate group is directly attached to the C6 of the glucopyranose, without an oxygen molecule bridging the bond between SO_3^- and the C6 in glucose 6-phosphate. The lack of this oxygen increases the distance between the three residues mentioned above (His178, Tyr179, and Lys182) and also between these residues and the substrate's charged group. Therefore, the increased distance between the SO_3^- group and the enzyme residues should also be an important factor in decreasing the interaction between the glucose-6-phosphate dehydrogenase and glucose

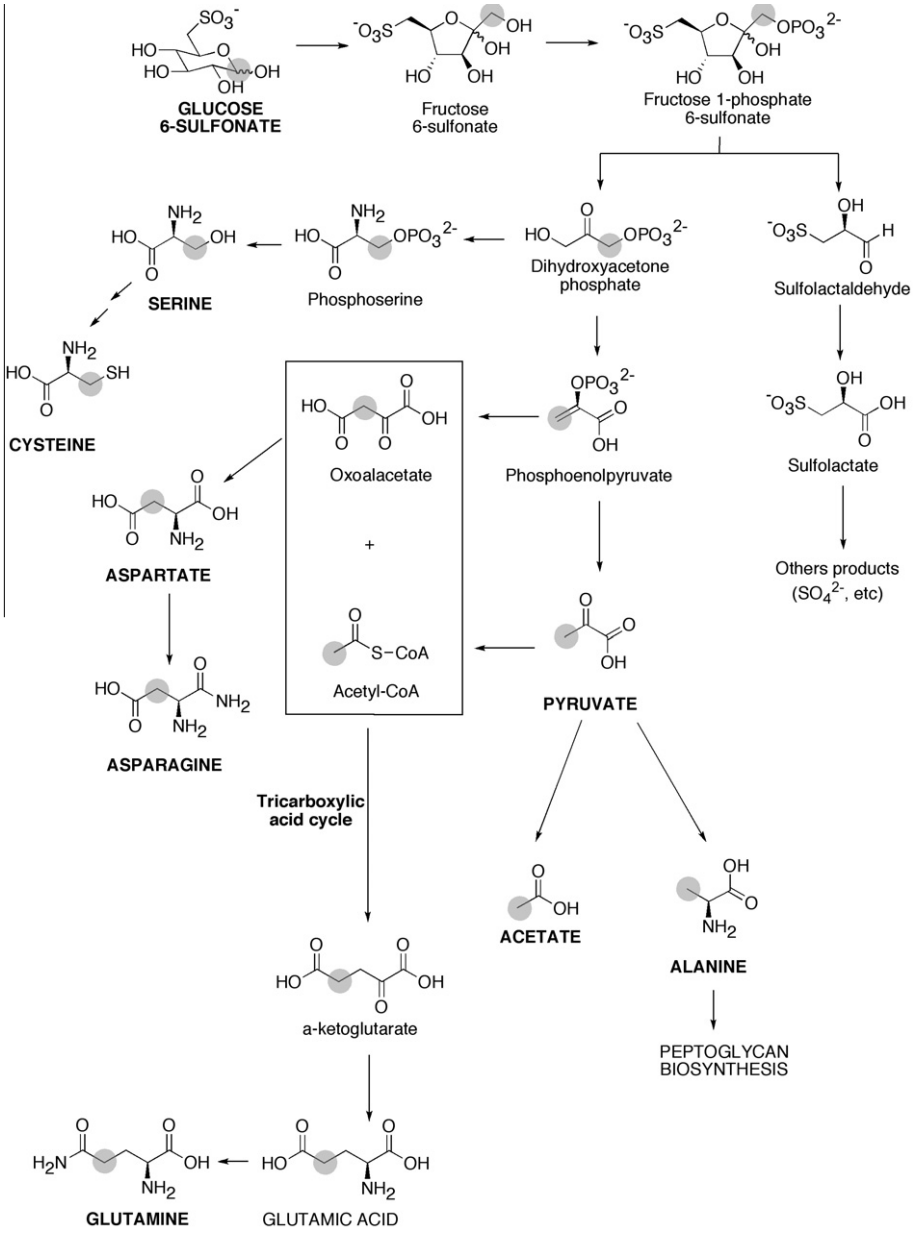

Figure 3. Glucose 6-sulfonate (sulfoquinovose) metabolic pathways observed by ^{13}C NMR after 144 h of incubation with cell lysate. Metabolites observed in this study are highlighted in bold.

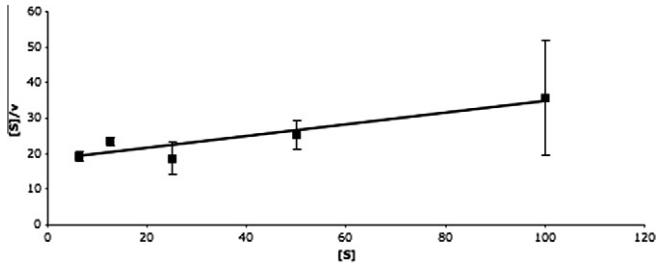

Figure 4. Hanes–Woolf plot for K_m determination of glucose 6-phosphate dehydrogenase. Glucose 6-phosphate is the substrate of this reaction [S] and was used in 6.25, 12.5, 25, 50, and 100 μM. From the linear regression, the K_m of G6PDH is 108 μM.

6-sulfonate. This may explain why glucose 6-sulfonate was unable to serve as substrate to glucose 6-phosphate dehydrogenase to form 6-phosphogluconolactone.

Another characteristic apparently important for glucose 6-phosphate interaction with glucose-6-phosphate dehydrogenase is the divalent charge of its phosphate group (PO_4^{-2}). It is reported that double charged anions such as phosphate (PO_4^{-2}), carbonate (CO_3^{-2}), or sulfate (SO_4^{-2}) are able to inhibit the enzyme activity by occupying the site pertinent to the phosphate moiety in the active site. Single charged ions (such as Cl^-), however, inhibit the enzyme very poorly.[31,32] Glucose 6-sulfonate has a valence of 1 because of the replacement of the phosphate (PO_4^{2-}) by the sulfonate (SO_3^-) group. This is probably another reason contributing for the inability of this enzyme to use glucose 6-sulfonate as substrate and why it is a poor inhibitor.

2.4. Effect of glucose 6-sulfonate on cancer cell lines

The ability of glucose 6-sulfonate to be metabolized through glycolysis but not the PPP makes it an attractive molecule to selectively

inhibit cells that require high rates nucleotide biosynthesis, for example cancer cells. Because of that, the potential activity of glucose 6-sulfonate as cytostatic or cytotoxic agent was evaluated in different breast cancer cell lines. Glucose 6-sulfonate at 1, 2, 4, and 8 times the molar concentration of glucose in the media was added to two breast cancer cell lines and one non-tumorigenic breast line. Figure 5 shows that glucose 6-sulfonate has a cytostatic effect on all lines from 11 to 44 mM and starts to show an intense cytotoxic effect in one cancer cell line (MCF-7) at 88 mM.

SQDG is reported to have many biological properties, acting as an anti-viral,[33] anti-tumoral,[34,14,35] anti-angiogenic,[36] and antibacterial[37] molecule. In most of these studies, the carbohydrate portion (glucose 6-sulfonate) from SQDG is shown to be essential for the attributed activity and our findings help to explain why the glucose 6-sulfonate moiety is important for the described activities of SQDG. The inability of a cell to have a proper rate of nucleic acid biosyntheses (as shown by the absence of 6-phosphogluconolactone formation when glucose 6-sulfonate is the carbon source) and energy (through glycolysis) helps explain why SQDG possesses cytotoxicity and the other biological activities. Due to the remarkable activity that molecules containing sulfoquinovose have, further knowledge of the metabolic pathways sulfoquinovose per se is involved is crucial in order to explore this molecule and its derivatives as potential drugs.

2.5. Effect on hexosamine metabolism

The ^{13}C NMR studies were able to identify intermediates or final products from glycolysis and the pentose phosphate pathway due to the generally high flux of carbohydrates through these pathways. Intermediates of the third pathway (the hexosamine biosynthetic pathway), however, were not identified by NMR spectroscopy. This is probably due to the lower flux of sugars through this pathway when compared to glycolysis and the pentose phosphate pathway. It is known that only approximately 2–3% of the total carbohydrates go through the hexosamine pathway,[37] which makes the concentration of its intermediates very low for detection by NMR. Because of that, the potential entry of glucose 6-sulfonate through the hexosamine pathway was studied by verifying the potential conversion of glucose 6-sulfonate to glucosamine 6-sulfonate (through fructose 6-sulfonate) in vivo in cow and human blood samples derivatized with 3,5-DNB-Cl and monitoring it by HPLC with UV spectra. The peak corresponding to the desired derivative (3,5-DNB-glucosamine 6-sulfonate) was identified based on its retention time compared to the derivatized synthetic standard by mass spectrometry (m/z 436) and UV spectrometry (Fig. 6). Based on UV absorbance measurements compared to a standard it is estimated that the concentrations of glucosamine 6-sulfonate in the cow and human blood samples were approximately 2–4 μg/mL (Fig. 7). The presence of glucosamine 6-sulfonate produced from metabolism of glucose 6-sulfonate can probably also contribute to the cytotoxic activity of glucose 6-sulfonate in breast cancer cell lines (Fig. 5). This is reasonable speculation since it is well established that cancer cells have an elevated hexosamine biosynthesis[7] and glucosamine 6-sulfonate is shown to be an effective inhibitor of the formation of glucosamine and other hexosamines.[22]

As shown in Figure 7, the concentration of glucosamine 6-sulfonate in human blood is approximately 2 μg/mL. The human blood sample was taken from the subject more than 16 h after ingestion of any plant material and the levels could possibly be much higher if assayed earlier. The kinetics of elimination from the blood stream as a function of time after ingestion of known amounts of sulfolipid in several subjects is a study that should be performed in the future.

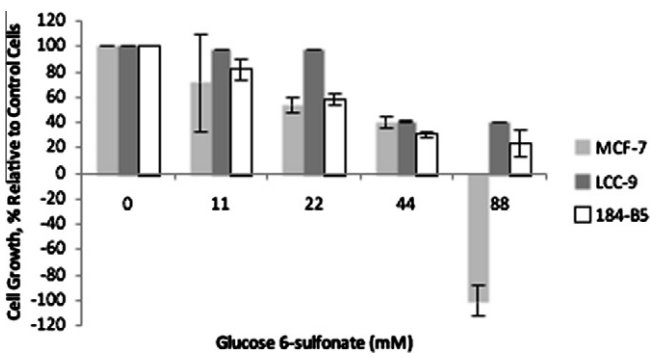

Figure 5. Cell growth of MCF-7, LCC9, and 184B5 lines in presence of 0, 50, 100, 200, and 400 μM of glucose 6-sulfonate for 48 h. Cell viability was determined by the sulforhodamine B colorimetric assay. In the *Y*-axis, negative cell growth represents cell death.

2.6. Conclusion

In summary, this study reports a natural component of the diet, sulfoquinovose, which is obtained by consumption of plants, algae, and certain cyanobacteria as food or in supplements, inhibits the hexosamine and pentose phosphate biosynthetic pathways and glycolysis. This study adds to the body of knowledge in the field of biochemical nutrition of why the consumption of vegetables containing high amounts of sulfonate sugars can have chemo protective activities. This is even more evident in studies showing that consumption of these vegetables are also associated with lower rates of disorders of carbohydrate metabolism, such as cancer and type 2 diabetes.[38,39]

3. Experimental procedures

3.1. NMR studies

3.1.1. Cell culture

Escherichia coli strain DH5α (50 mL of culture) was grown in minimal media M9 containing 4% glucose at 30 °C until logarithmic phase was reached. Cells were centrifuged at 3000 rpm for 20 min and the pellet disrupted at 4 °C by sonication in 50 mM Tris–HCl buffer pH 7.6. This procedure for obtaining the bacterial lysate was employed for all NMR experiments. The volume of the lysate was reduced 10 times and used directly in subsequent studies.

3.1.2. Synthesis of ^{13}C-1-fructose 6-phosphate and ^{13}C-1-glucose 6-sulfonate

^{13}C-1-fructose 99% (250 mg) (Sigma) was converted to ^{13}C-1-fructose 6-phosphate by incubation with 3 units of hexokinase (Sigma–Aldrich) in 50 mM Tris–HCl 13.3 mM $MgCl_2$ (600 μL) buffer pH 7.4 for 48 h at 37 °C. The conversion was monitored by ^{1}H and ^{13}C NMR. The overall scheme of synthesis of ^{13}C-1-glucose 6-sulfonate is illustrated in Figure 8. ^{13}C-glucose was first converted to its methyl glucoside by Fisher glycosidation.[40] ^{13}C-1-glucose (Sigma) (250 mg), methanol (100 mL), and sulfuric acid (0.2 mL) were refluxed overnight to form compound **2** (^{13}C-1-methyl-glucose). Sodium bicarbonate (1 g) was added to the methanol solution of **2** and the mixture stirred, filtered, and concentrated under reduced pressure to give crude **2**, which was used without further purification. Compound **2** (250 mg) was mixed with pyridine (5 mL), triphenylphosphine (0.6 g), and carbotetrabromide (0.6 g). The mixture was stirred overnight at room temperature to form the bromo compound **3**. Compound **3** was dried, ressuspended in water (10 mL) at 4 °C to precipitate triphenylphosphine and triphenylphosphine oxide. The supernatant was decanted and

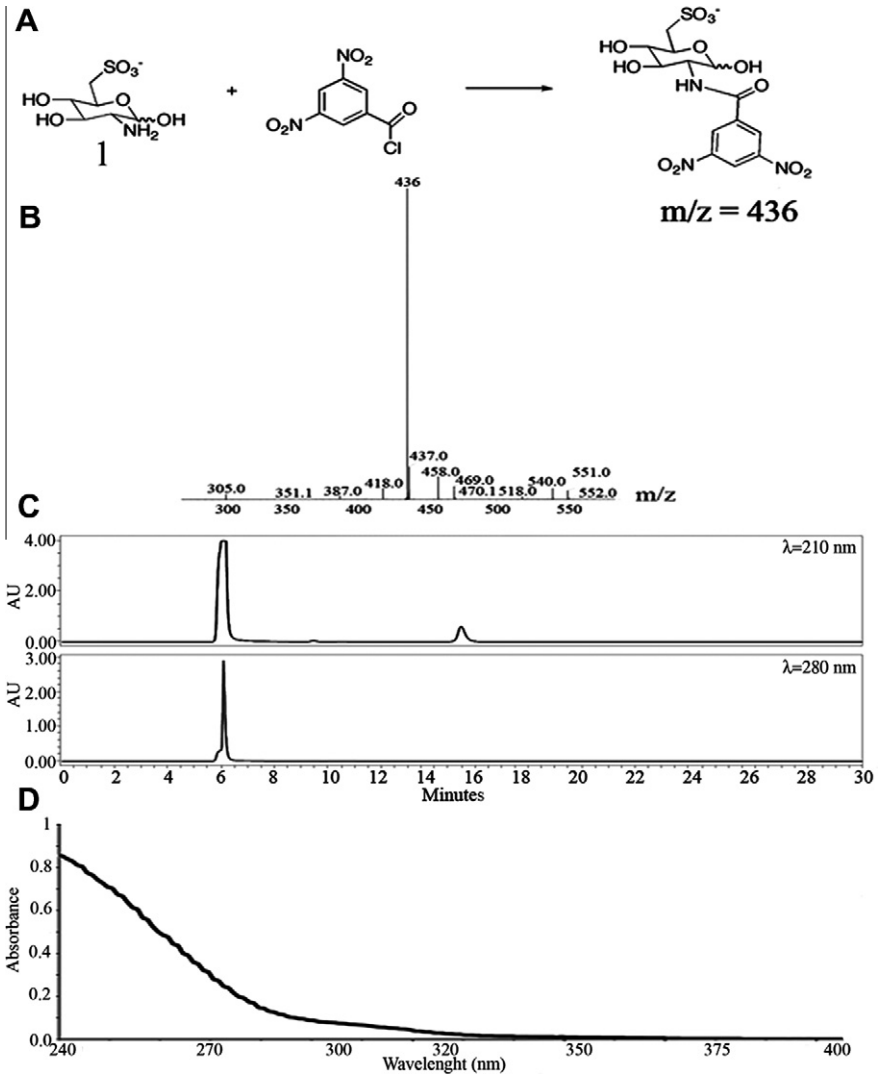

Figure 6. (A) Route of derivatization of the synthetic glucosamine 6-sulfonate (standard). (B) Electrospray mass spectrum of the dinitrobenzoyl derivative of 3,5-DNB-glucosamine 6-sulfonate. (C) HPLC trace at 210 and 280 nm showing peak corresponding to derivative at 6.2 min. (D) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate.

passed through a mixed bed ion exchange resin column (H^+/OH^-) and concentrated under reduced pressure. The residue compound **3** was mixed with sodium sulfite (0.5 g) in water (10 mL) and stirred for 3 hr at 80 °C to form the sulfonate compound **4**, which was passed through a strong acid ion exchange resin (H^+ form) to remove pyridine and other cation ionic impurities. The solution was dried and mixed with 2 M HCl in water (10 mL) and heated for 2 h at 120 °C to give compound **1** (^{13}C-1-6-deoxy-6-sulfo-D-glucopyranose).

The acid was removed before **1** was used for biological assays by stirring in methanol containing excess sodium bicarbonate, checking for neutrality, filtering, and concentrating. The synthesis of ^{13}C-glucose 6-sulfonate was confirmed by ^{1}H NMR (Fig. 3 of Supplementary data).

3.1.3. NMR analyses

NMR spectra were measured at ambient temperature on a Varian Anova 600 MHz equipped with a 5 mm Pulse-Field-Gradient switchable broadband probe operating at 599.804 MHz (^{1}H) and 150.83 MHz (^{13}C). One-dimensional ^{1}H spectra were referenced to the solvent residual peak and ^{13}C were referenced to standards of known frequency. Semi quantitative ^{13}C was obtained with a relaxation delay of 5 s and inverse-coupled gated. ^{1}H NMR spectra were obtained using a spectral width of 8000 Hz over 64,000 data points and ^{13}C in a spectral width of 36,200 Hz over 94,000 points and multiplied by an exponential function corresponding to a 0.50 Hz broadening prior to Fourier transformation. Spectra were obtained in buffered water as indicated containing 13% D_2O in order to obtain a stable lock signal.

3.1.3.1. ^{13}C-1-Fructose 6-phosphate studies.

^{13}C-1-fructose 6-phosphate (20 mg) was mixed with glutamine (40 mg), *E. coli* lysate (300 μL), and 50 mM Tris–HCl pH 7.6 (400 μL). The reaction incubated at 37 °C and monitored by ^{13}C NMR at 0, 1, 2, 3, 4, 5, 24, 48, and 144 h.

3.1.3.2. ^{13}C-1-Glucose 6-sulfonate (sulfoquinovose) studies.

^{13}C-1-glucose 6-sulfonate (20 mg) was mixed with glutamine (40 mg), *E. coli* lysate (300 μL), and 50 mM Tris–HCl pH 7.6 buffer (400 μL) and the reaction incubated at 37 °C and monitored by ^{13}C NMR at 0, 1, 2, 4, 24, 48, and 144 h.

To analyze if ^{13}C-1-glucose 6-sulfonate is an inhibitor of enzymes involved in the metabolism of fructose 6-phosphate, 20 mg of ^{13}C-1-fructose 6-phosphate and 20 mg of ^{13}C-1-glucose 6-sulfonate were mixed with glutamine (40 mg), *E. coli* lysate (300 μL), and 50 mM Tris–HCl pH 7.6 buffer (400 μL) and the

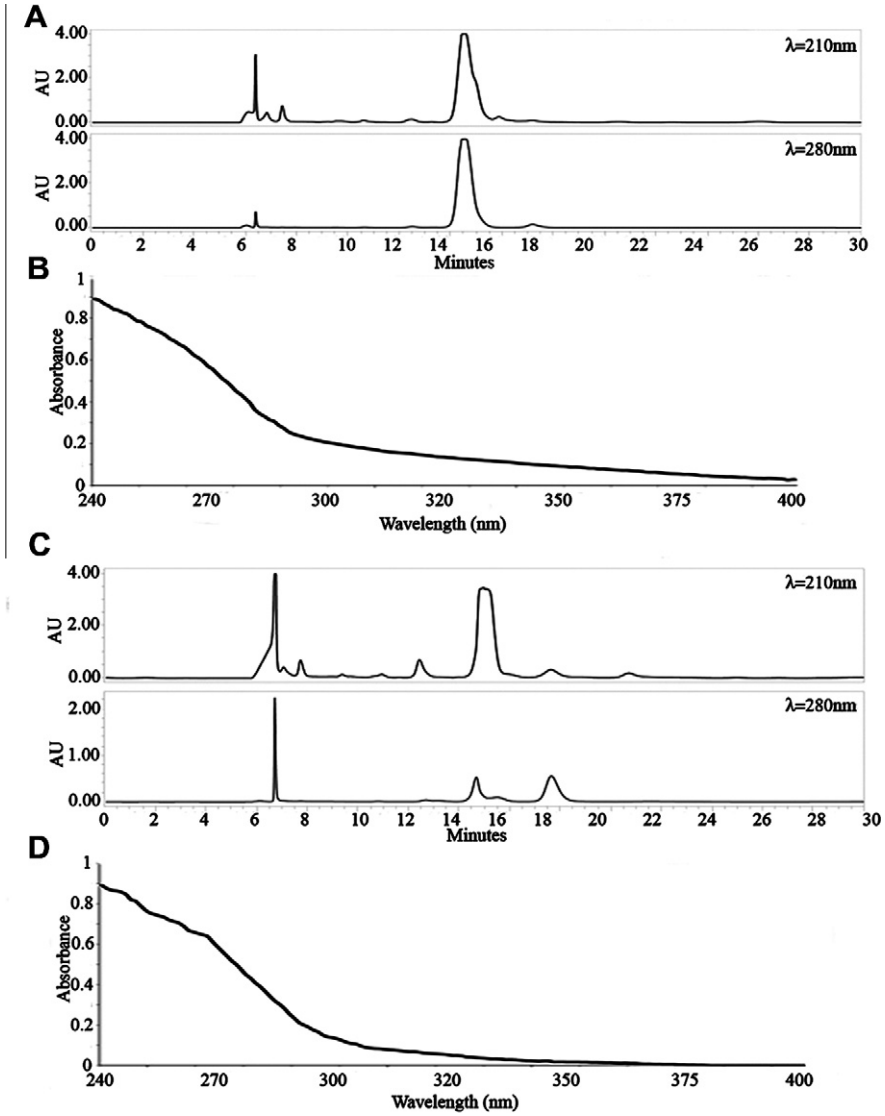

Figure 7. (A) HPLC profile and (B) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate from cow serum. (C) HPLC profile and (D) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate found in human serum. The peak eluting just after 6 min was collected in order to obtain the UV spectra.

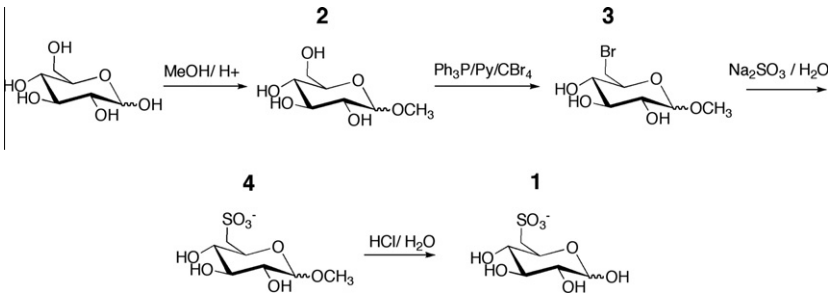

Figure 8. Synthetic route to ^{13}C-1-D-glucose 6-sulfonate (**1**).

reaction incubated at 37 °C and monitored by ^{13}C NMR at 0, 2, 4, 24, 48, and 144 h.

3.2. Glucose 6-phosphate dehydrogenase enzyme activity assay

Glucose 6-phosphate at various concentrations (6.25, 12.5, 25, 50, and 100 μM) was mixed with 488 μM NADP and 150 mM HEPES buffer pH 7.2 up to a volume of 250 μL. The absorbance was monitored at 340 nm. Once the background was established, 0.25 units of glucose 6-phosphate dehydrogenase (Sigma G8529-1KU, *Leuconostoc mesenteroides* recombinant enzyme grown in *E. coli*, 700 U/mg) were added and the conversion to 6-phosphogluconolactone was monitored at 340 nm by the coupled formation of NADPH. All the experiments were done in triplicate. The reaction rate at each time point was calculated as follows: OD (observed) × volume (250 μL)/6270 × minutes (0.4). K_m was

calculated from the linear regression obtained from a Hanes–Woolf plot (Fig. 4).

To evaluate if glucose 6-sulfonate is a substrate for glucose-6-phosphate dehydrogenase, 100, 200, 300, and 400 μM of glucose 6-sulfonate, 488 μM of NADP, and 150 mM HEPES buffer pH 7.2 were mixed up to a total volume of 250 μL and the reaction monitored by the same method described above.

To evaluate the potential inhibition of glucose 6-phosphate dehydrogenase by glucose 6-sulfonate, 50 μM of glucose 6-phosphate, 488 μM of NADP, and 150 mM HEPES buffer pH 7.2 were mixed to glucose 6-sulfonate at 100 and 400 μM up to a total volume of 250 μL.

3.3. Glucose 6-sulfonate cytotoxicity assay

MCF-7, LCC9, and 184B5 cell lines were cultured in Iscove's Modified Eagle Medium (IMEM) in 10% fetal bovine serum, 1% Penicillin/Streptomycin. The first two are breast cancer lines while the last one is a non-tumorigenic epithelial breast cell line. All cell lines were plated in 96-well plates at densities experimentally determined (MCF-7: 5×10^3 cells/mL; LCC9 and 184B5: 1×10^5 cells/mL). Glucose 6-sulfonate at 11, 22, 44, and 88 mM were added to the cell lines and incubated for 48 h at 37 °C at 5% CO_2. These concentrations were chosen to result in ratios between glucose (present in the media at 11 mM) and glucose 6-sulfonate of 1:1, 1:2, 1:4, and 1:8. Cells were fixed with 50% trichloroacetic acid for 1 h and stained with sulforhodamine B as previously described.[41] All samples were done in triplicate.

3.4. Design and detection of glucosamine 6-sulfonate in vivo

3.4.1. Development of method

For detection of glucosamine 6-sulfonate, we used glucosamine 6-sulfonate that was previously synthesized by our group as a standard.[22] Glucosamine 6-sulfonate (10 mg) (standard) was derivatized by mixing it with methanol (100 μL), water (100 μL), 3,5-dinitrobenzoyl chloride (3,5-DNB-Cl) (30 mg), and sodium bicarbonate (30 mg) with vigorous stirring for 48 h at 25 °C. Acetic acid (50 μL) was added, the mixture dried and eluted through a reverse phase column (250 mg C18 resin) in 2:1 water/methanol (total volume 500 μL). This solution (20 μL) was injected on a HPLC column (Aminex HPX-97H) using 0.018 M H_2SO_4 as eluent. The UV spectroscopy measurements were obtained in aqueous solution in the range of 240–400 nm. The mass spectrometry profile was obtained by electrospray ionization in negative ion mode.

3.4.2. Detection of glucosamine 6-sulfonate in vivo

For investigation of the possible presence of glucosamine 6-sulfonate in vivo, cow (10 mL) and human (10 mL) blood were used. Both samples were clotted for 48 h at 4 °C and centrifuged at 3500 rpm for 20 min. Each was mixed with EtOH 100% (40 mL), sonicated for 20 min, and centrifuged at 3500 rpm for 20 min. The supernatants were collected, dried, and derivatized and analyzed under identical conditions as described for the standard.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.09.014.

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