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Biological effect of β-(1,3)-polyglucuronic acid sodium salt on lipid storage and adipocytes differentiation

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ABSTRACT

β-(1,3)-Polyglucuronic acid sodium salt produced by the regioselective oxidation of β-(1,3)-glucan have been studied for its biological impact as putative slimming agent. This β-(1,3)-polyglucuronic acid sodium salt was synthesized using the conventional 2,2,6,6,-tetramethylpiperidine-1-oxyl radical (TEMPO)/NaBr/NaClO systems at pH 10 and 4 °C. A transcriptomical study using DNA microarray analysis, demonstrated that this heparan like sodium salt locally induced an over-expression of the gene *Angiopoietin-like 4* (ANGPTL4 or fasting induced adipose factor (FIAF)) leading to the increase in Adipokine ANGPTL4 synthesis and the inhibition of Lipoprotein Lipase (LPL). *In vitro* analysis using 3T3-L1 cells have clearly revealed that β-(1,3)-polyglucuronic acid sodium salt could act in key steps of lipid metabolism by inhibiting the differentiation of pre-adipocytes to mature adipocytes.

1. Introduction

Lipid metabolism is a complex phenomenon composed of the specific absorption of lipids, their transport and finally their abilities to be accessible for our organism (Nordøy, Davenas, Ciavatti, & Renaud, 1985). After digestion and intestinal absorption fatty acids are transferred through the body as triglycerides in lipoproteins, particular vesicles. These fatty acids are used in the liver or in muscles as energetic fuel or are reserved in adipocytes forming the adipose tissue (Nilsson-Ehle, Garfinkel, & Schotz, 1980). This physiological mechanism of lipogenesis appears thanks to the action of the Lipoprotein Lipase (LPL) which is located at the capillary endothelium of adipose tissue, skeletal muscle, heart, and other tissues (Goldberg, 1996). The Angiopoietin-like 4 (ANGPTL4, or fasting-induced adipose factor, FIAF) is a protein synthesised by adipocytes, able to inhibit LPL activity and to stimulate the Adipose Triglyceride Lipase (ATGL) (Macfarlane, Forbes, & Walker, 2008; Seckl, Morton, Chapman, & Walker, 2004). It was clearly mentioned that increasing adipokine Angiopoietin-like 4 activities could favorize adipocyte lipolysis (Hato, Tabata, & Oike, 2008; Kersten, 2005; Li, 2006). LPL were well known to play an important key role in lipoprotein metabolism by the regulation of plasma triglyceride (Bensadoun, 1991). As mentioned in literature (Eckel, Fujimoto, & Brunzell, 1977; Fried, Russell, Grauso, & Brolin, 1993; Olivecrona, Liu, Hultin, & Bengtsson-Olivecrona, 1993), the regulation of LPL in adipocytes has shown very important biological relationships for understanding the key role of this specific enzyme non-covalently bound to cell membrane heparan sulfates (Saxena, Klein, & Goldberg, 1991). It was largely described that glycosaminoglycan such as heparin could influence and stimulate the release of LPL into the cells medium (Bensadoun, 1991). Therefore, the lipase releasing activity of heparin and others heparinoïds polysaccharides has been correlated to the molecular weight and the global charge density of the polysaccharide chains (Merchant, Erbe, Eddy, Patel, & Linhardt, 1986). It has been well established that in the heparinoid, sugar composition, the degree of substitution (by carboxylates, sulfates, etc.), the specific glycosidic bonds, the high conformation and the molecular weight distribution of glycosaminoglycans (GAGs) polysaccharides have a great modulation power on the biological activities (Melo, Feitosa, Freitas, & De Paula, 2002; Petit, Delattre, Papy-Garcia, & Michaud, 2006). Then, biomimetic analogues made up of monosaccharide repeating units adopting the same conformation in solution could be proposed as heparan surrogates (Van Boeckel & Petitou, 1993; Cross, Petitou, Sizun, Perez, & Imberty, 1996). In literature, lot of works reported the anti-hyperlipidemic effect of polysaccharides, isolated from different algae or plant species. For example, the influence of *Sargassum* sp. polysaccharide on lipid metabolism was proposed (Raghavendran, Sathivel, & Devaki, 2005). Ulvan, an hetero-polyanionic polysaccharide isolated from *Ulva pertusa*, was

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found to be beneficial as an anti-hyperlipidemic agent (Pengzhan et al., 2003). More, it was shown that oral administration of polysaccharides from *Auricularia auricula* presented effect on blood metabolism (Chen et al., 2008). In fact, these polysaccharides significantly improved LPL activity and consequently, revealed the beneficial effects of polysaccharide from *A. auricula* on the preventive actions against hypercholesterolemia (Chen et al., 2008). Finally, note to mention the effect of oat glucan on the human serum lipoprotein profile (Kerckhoffs, Brouns, Hornstra, & Mensink, 2002) and the hyper-cholesterolemic properties of oxidized glucan (Park, Bae, Lee, & Lee, 2009).

Consequently, according to anti-hyperlipidemic properties described for heparinoid polysaccharides, the objective of this study was to investigate the biological effect on lipid metabolism of polyanionic heparan-like made up of β-(1,3)-glucuronic acid sodium salt and synthesized by regioselective oxidation of polysaccharides using stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Delattre, Michaud, Elboutachfaiti, Courtois, & Courtois, 2006; Delattre et al., 2009; Delattre, Wada, & Isogai, 2009; Elboutachfaiti, Petit, & Michaud, 2011). 3T3-L1 cells have been differentiated in the presence and absence of β-(1,3)-polyglucuronic acid sodium salt and therefore adipocyte differentiation was monitored using fluorescence microscopy, and aP2/FABP4 protein adipogenesis marker were assayed.

2. Material and methods

2.1. Industrial production of β-(1,3)-glucan from Agrobacterium sp. strain (ATCC 31750)

β-(1,3)-Glucan (curdlan) was produced by the *Agrobacterium* sp. strain (ATCC 31750) in Yeast Broth media (30 °C, 600 rpm) in a 750 L bioreactor (Bioflo® 110 Newbrunswick Scientific) according to industrial procedure adapted from Delattre et al. (2009). After 96 h of cultivation, curdlan was recovered from cultures by dissolution in NaOH (1 M) followed by centrifugation to remove bacteria (15,000 × *g* for 20 min at 4 °C). Finally, curdlan in supernatant was precipitated by neutralization with HCl (5 M), collected by centrifugation (15,000 × *g* for 30 min at 4 °C), abundantly washed with distilled water to remove salts, and finally dried under vacuum during 48 H.

2.2. Industrial production of β-(1,3)-polyglucuronic acid sodium salt

β-(1,3)-Polyglucuronic acid sodium salt was prepared according to the oxidation procedure described by Delattre et al. (2009). Briefly, curdlan (1000 g) was dissolved in distilled water (100 L) at pH 11.5 by adding NaOH (4 M) and was kept below 4 °C during oxidation step. TEMPO (8.66 g), NaBr (192 g) and NaOCl (15 L at 9.6%) were added to start oxidation. The reaction was quenched by adding ethanol (5 L) after 120 min of NaOCl addition, neutralized with HCl (5 M) and concentrated. β-(1,3)-Polyglucuronic acid sodium salt was precipitated with cold ethanol (3 volumes), washed with ethanol and dried under vacuum.

2.3. SEC MALLS analysis of β-(1,3)-polyglucuronic acid sodium salt

Average molecular weights and molecular weight distributions were determined by high-pressure size exclusion chromatography (HPSEC) with on line multi-angle laser light scattering (MALLS) and differential refractive index (DRI) detectors. The MALLS apparatus is the EOS from Wyatt Technology (Ca, USA) filled with a K5 cell and a Ga–As laser (λ = 690 nm). The DRI detector is the ERC7515A from Erma Cr., Inc. (Japan). Columns [OHPAK SB-G guard column, OHPAK SB804 and 806 HQ columns (Shodex)] were eluted with $LiNO_3$ 0.1 M at 0.6 mL min^{-1}. Solvent was filtered through 0.1 μm filter unit (Millipore), degassed (ERC-413) and filtered through a 0.45 μm filter upstream column. The sample, filtered on 0.45 μm unit filter (Millipore) was injected through a 100 μL full loop. The collected data were analysed using the Astra V-4-81-05 software package from Wyatt technology (Ca, USA).

2.4. Sugar assays

Glucuronic acid and glucose contents of β-(1,3)-polyglucuronic acid was assayed with *meta*-hydroxyldiphenyl (Van Den Hoogen et al., 1998) and resorcinol (Monsigny, Petit, & Roche, 1988) in order to quantify the oxidation level. D-Glucose (Sigma) and D-glucuronic acid (Sigma) were used as standards. Quantification of neutral sugars was done according to the corrective formula described by (Spick & Montreuil, 1964). The glucuronic acid ratio (GA %) of polysaccharides obtained was expressed as the amount of glucuronic acid compared with this of all sugars (glucose and glucuronic acid).

2.5. Determination of carboxylate content by conductimetry titration

In order to quantify the degrees of oxidation of β-(1,3)-polyglucuronic acid sodium salt, conductimetry titration method was used according to literature (Saito & Isogai, 2004). Dried polysaccharides (50 mg) were dissolved in 0.01 M HCl (15 mL) to set the pH value in the range of 2.5–3.0. The titration was started by addition of a 0.01 M NaOH solution at the rate of 0.1 mL min^{-1} up to pH 11 and the conductivity was measured using CDM 210 Meterlab conductimeter. The degree of oxidation was given by Eq. (1):

$$DO = 162\, M_{NaOH}(V_2 - V_1)/(m - (36\, M_{NaOH}(V_2 - V_1))) \quad (1)$$

V_1 and V_2 are respectively the volumes (mL) of NaOH solution necessary to neutralize HCl, the strong acid and to neutralize the polyglucuronic acid (weak acid).

2.6. NMR analysis

NMR analyses were performed at 80 °C with a Bruker Avance 400 spectrometer of 400 MHz equipped with $^{13}C/^{1}H$ dual probe. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 μs, a relaxation time of 1 s and a number of 1500 scans. Oxidized polysaccharides were dissolved in D_2O at a 50 g/L concentration. Curdlan was dissolved in NaOD (1 M) at a 50 g/L concentration.

2.7. Screening activities of β-(1,3)-polyglucuronic acid sodium salt by transcriptomic

DNA Array method has been used to screen the biological activities of the β-(1,3)-polyglucuronic acid sodium salt. Firstly, the cytotoxicity of the β-(1,3)-polyglucuronic acid sodium salt on human fibroblast cell cultures has been evaluated. In a second step, we incubated human fibroblast cell cultures with 5 mg/mL of the β-(1,3)-polyglucuronic acid sodium salt (with a coating method, until the cell confluence). RNA were extracted and analysed with the DNA Array method from Agilent Technologies (GeneSpring GX software (Agilent Technologies)). Briefly, DNA were marked by specific fluorochrome: Cys5 (control) and Cys3 (β-(1,3)-polyglucuronic acid sodium salt). Over-expressions of genes were estimated with the relation: Over-expression = log(Cys5/Cys3) > 1. (Note to mention that each experiment was done at least 3 times ($p < 0.05$)).

The biological responses disturbed by the individual treatments of human fibroblast by β-(1,3)-polyglucuronic sodium salt has been investigated and correlated by genes ontology and analysed by bioinformatic using GeneSpring GX software (Agilent Technologies) and FatiGO softwares [http://www.fatigo.bioinfo.cipf.es/].

2.8. In vitro analysis of β-(1,3)-polyglucuronic acid sodium salt on lipid storage

2.8.1. Biological model

Cellular type: Pre-adipocytes 3T3-L1 cultivated at 37 °C, 5% CO_2.

Growth media: DMEM (Invitrogen 21969035) supplemented with L-glutamine 2 mM (Invitrogen 25030024), Penicillin 50 UI/mL, streptomycin 50 μg/mL (Invitrogen 15070063), Amphotericine B 0.25 μg/mL (Sigma A2942), and fetal calf Serum 10% (v/v, Invitrogen 10270098).

Differenciation media: DMEM (Invitrogen 21969035) supplemented with L-glutamine 2 mM (Invitrogen 25030024) Penicillin 50 UI/mL, streptomycin 50 μg/mL (Invitrogen 15070063), fetal calf Serum 10% (v/v, Invitrogen 10270098), Methyl-isobutyl xanthine (IBMX, Sigma I7018) 500 μM, Insulin (Sigma I1882) 170 nM, and Dexamethazone (Sigma D1756) 500 nM.

Post-differentiation media: DMEM (Invitrogen 21969035) supplemented with L-glutamine 2 mM (Invitrogen 25030024) Penicilline 50 UI/mL, streptomycin 50 μg/mL (Invitrogen 15070063), fetal calf Serum 10% (v/v, Invitrogen 10270098) and insulin (Sigma I1882).

Test media: MEM without red phenol (PolyLabo 5503401) supplemented with Bicarbonate 1.87 mg/mL (Invitrogen 25080060), Penicillin 25 UI/mL, streptomycine 25 μg/mL (Invitrogen 15070063), Glutamine 2 mM (Invitrogen 25030024), Bovine Serum Albumine (BSA) and free fatty acid 0.5% (p/v) (Sigma A6003).

2.8.2. Culture and treatment

The cells were grown to confluence in growth medium with a medium change after 2 days (96-well plates). At high confluence, the cells were transferred into differentiation medium and incubated for 48 h at 37 °C, 5% CO_2. Control cultures were performed in growth medium 3T3-L1 throughout the duration of the experiment (controls undifferentiated). After cell differentiation, the medium was replaced by medium post-differentiation (growth medium + insulin) and then incubated for 48 h at 37 °C and 5% CO_2. After incubation, the post-differentiation medium was removed and the cells were placed in growth medium for 120 h at 37 °C and 5% CO_2. The growth medium was then removed and replaced with test medium containing or not (control) the β-(1,3)-polyglucuronic acid sodium salt. The cells were incubated for 48 h at 37 °C and 5% CO_2.

2.8.3. Quantification of lipid content with adipored analysis by immunofluorescence

Lipid content was measured using a commercially available kit (Adipo-Red assay Reagent; Cambrex Bio Science, Walkersville, MD). AdipoRed, a solution of the hydrophilic stain Nile Red, is a reagent that enables the quantification of intracellular lipid droplets in a high-throughput manner. In brief, after incubation time, the supernatants were removed and the cells were rinsed with phosphate buffered saline solution (PBS). Intracellular lipids were then labelled with Adipored® (Biowhittaker ref. PT-7009) following the supplier's recommendations. Image acquisition was performed using an automated microscope Incell AnalyzerTM1000 (GE Healthcare). For each well, 5 captured digitized images were performed. The labelling was quantified by measuring the fluorescence intensity overall fat density (a measure of the intensity per surface area) and the number of medium and large vesicles. The cells were then fixed and cell nuclei revealed with Hoechst dye to determine the number of cells per field of analysis.

2.9. In vitro analysis of β-(1,3)-polyglucuronic acid sodium salt in the differentiation of pre-adipocytes into mature adipocytes

2.9.1. Biological model

Cellular type: Pre-adipocytes 3T3-L1 cultivated at 37 °C, 5% CO_2.

Growth media: DMEM high glucose (Invitrogen 41965062) supplemented with L-glutamine 2 mM (Invitrogen 25030024), Penicillin 100 UI/mL, streptomycin 100 μg/mL (Invitrogen 15070063), and fetal calf Serum 10% (v/v, Invitrogen 10270098).

Differentiation media: DMEM high glucose (Invitrogen 41965062) supplemented with L-glutamine 2 mM (Invitrogen 25030024) Penicillin 100 UI/mL, streptomycin 100 μg/mL (Invitrogen 15070063), fetal calf Serum 10% (v/v, Invitrogen 10270098), Methyl-isobutyl xanthine (IBMX, Sigma I7018) 500 μM, Insulin (Sigma I1882) 170 nM, and Dexamethazone (Sigma D1756) 500 nM.

Protein extraction: Hepes 20 mM, EDTA 0.2 mM, $MgCl_2$ 1.5 mM, NaCl 0.42 M, Glycerol 25%, and pH 7.6.

2.9.2. Culture and treatment

The cells were cultured at 37 °C under 5% CO_2 atmosphere in 6-well plates (353046 Elvetec). The cells were cultured in growth medium until confluence (day-2). At this stage (day-2) of treatment with β-(1,3)-polyglucuronic acid sodium salt at 5 mg/mL is provided (Condition A) by adding the compound to the growth medium until day 0 (mid-point growth is replaced by differentiation medium containing no β-(1,3)-polyglucuronic acid sodium salt), cells were left at confluence for a further 48 h then growth medium was replaced by differentiation medium containing β-(1,3)-polyglucuronic acid sodium salt at 5 mg/mL (condition B).

The cells were then cultured until day 6 (6 days after induction of differentiation) in differentiation medium changed every day. Cells are harvested at different times of differentiation in a well by trypsinization (5 min at 37 °C) and centrifugation (5 min at 800 g). Proteins were extracted by addition of the cell pellet 60 μL of extraction buffer proteins. The various samples were taken for control cells and cells treated with β-(1,3)-polyglucuronic acid sodium salt day-2 (for condition A), day 0 (at the time of the addition of differentiation medium), then after 24 h (D1), 48 h (D2), 72 h (D3), 96 h (D4), 120 h (D5) and 144 h (D6).

2.9.3. Analysis of adipocyte differentiation by Western blot

The proteins extracted from cells were quantified by spectrophotometric assay at 595 nm by the method of Bradford. 30 μg proteins from each condition were analysed by Western blot technique. The differentiation of 3T3-L1 cell was assessed by the accumulation in these cells of a marker of adipocyte differentiation, the protein aP2/FABP4. This protein is a transporter of fatty acids that accumulate in cells during adipocyte differentiation. AP2/FABP4 detection is performed using a rat monoclonal antibody (RD Systems, MAB1443). Controlling the amount of protein in each sample is performed by detecting the protein β-actin using a rabbit polyclonal antibody (Sigma, A2066-2ML).

2.9.4. Analysis of lipid accumulation in adipocyte by coloration with red oil method

3T3-L1 cells were grown on glass slides placed in 6-well plates. At different times of differentiation (D0–D6), the cells were fixed by incubation for 15 min in a solution of 4% paraformaldehyde in PBS 1X. The glass slides are then treated with 1 min of 1,2-propanediol, 4 min with red oil at 60 °C and 1 min with 1,2-propanediol. The cells are then rinsed with water for 1 min. An anti-coloring is then performed for 30 s with Harris haematoxylin to highlight the cell areas

unstained by oil red (nucleus and cytoplasm). Excess haematoxylin was removed by several washes with water. The glass slides are then placed on microscope slides (Glycerol-PBS 1X 50/50, v/v).

3. Results and discussion

3.1. Production of β-(1,3)-polyglucuronic acid sodium salt by oxidation of curdlan mediated by TEMPO–NaBr–NaOCl system

As mentioned on Fig. 1, anionic polysaccharide such as β-(1,3)-polyglucuronic acid sodium salt could be easily industrially produced using specific catalytic TEMPO/NaBr/NaOCl oxidation. By this industrial process, we synthesized a very pure soluble β-(1,3)-polyglucuronic acid sodium salt in a good yield higher than 75% after 2 h of process. These results are in accordance to laboratory process as already described in the literature (Delattre et al., 2009; Tamura et al., 2009). As we can observe in Table 1, the average molecular weight of β-(1,3)-polyglucuronic acid sodium salt were estimated to be 500 kDa. When curdlan was subjected to regioselective and elsewhere oxidation using TEMPO–NaOCl media, the C-6 of glucose were oxidized to carboxyl groups. Thus, the presence of carboxylate can be easily confirmed in oxydized curdlan by FT-IR. These FT-IR analyses were conformed to previous work (Delattre et al., 2009; Tamura et al., 2009). In fact by using FTIR analysis of oxidized curdlan isolated at pH 7.5, we observed the specific characteristic vibrations for carboxylate group at 1420 and 1612 cm^{-1}. The intense absorption band at 1612 cm^{-1} was assigned to the asymmetrical COO^- stretching vibration, whereas the band at 1420 cm^{-1} was assigned to the symmetrical COO^- stretching vibration. Note to mention that these bands were not observed for curdlan. To confirm the complete transformation of curdlan into β-(1,3)-polyglucuronic acid sodium salt ^{13}C NMR analysis was investigated. As observed in Fig. 2, six characteristics resonance peaks (see Table 2) were obtained for the β-(1,3)-polyglucuronic acid sodium salt. In fact, ^{13}C-NMR analysis (Fig. 2B) established, according to literature (Delattre et al., 2006, 2009; Tamura et al., 2009), the absence of the C-6 resonance at around 65 ppm and the presence of a new signal due to the carboxyl group around 175 ppm after oxidation step, which indicated the total conversion of curdlan to β-(1,3)-polyglucuronic acid sodium salt. Consequently, the ^{13}C NMR spectrum of β-(1,3)-polyglucuronic acid sodium salt (Fig. 2B) is in perfect agreement with the above mentioned FT-IR analysis, then supporting a complete oxidation of curdlan into β-(1,3)-polyglucuronic acid sodium salt by using TEMPO/NaOCl/NaBr system.

3.2. Transcriptomic analysis

It is well detailed that DNA microarray technology is currently used to propose the comprehensive profiling of genes that are up- or down-regulated in response to environmental stimuli (Bogard, Ameziane, & Lamoril, 2008). Therefore, in order to elucidate the physiological functions and the molecular functions that are affected by stimulation of human fibroblasts with β-(1,3)-polyglucuronic acid sodium salt, transcriptomic analysis allowed to determine the percentage of down and up-regulated genes (Fig. 3). To determine whether genes expression could be stimulated and regulated by β-(1,3)-polyglucuronic acid sodium salt at the adipocytes level, we treated cells with the synthetic β-(1,3)-polyglucuronic acid sodium salt obtained by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr media. After harvesting the cells to prepare total RNA, cDNA was synthesized and quantitative PCR (qPCR) performed using specific primers. We first confirmed that β-(1,3)-polyglucuronic acid sodium salt did not induce the down-regulation of essential genes or the up-regulation of endogens. If we consider lipid metabolism it was important to mention over-expression of important genes known to be implicated in the lipid metabolism (Fig. 3). Then, β-(1,3)-polyglucuronic acid sodium salt stimulated the over-expression of genes such as: PTGS2 (prostaglandin Endoperoxide synthase 2 which is implicated in biosynthesis of lipid); THC2373524 which is implicated in lipid transport; EGR1 (Early Growth Response 1) which is implicated in the activation of Low Density Lipoprotein (LDL) gene receptor and APOE (Apolipoprotein E = ligand of LDL receptor) which is implicated in lipid transport.

Interestingly, β-(1,3)-polyglucuronic acid sodium salt treatment increased ANGPTL4 mRNA levels, indicating that ANGPTL4 expression could be regulated by β-(1,3)-polyglucuronic acid sodium salt *in vitro* (Fig. 3). Consequently, this transcriptomic study demonstrated that this synthetic β-(1,3)-polyglucuronic acid sodium salt could be implicated in the process of the metabolism of the lipids. In fact, the Angiopoietin-like 4 adipokine synthesized by adipocytes induce a reduction of their volumes and masses (Mandard et al., 2006). As it was related in lipid literature (Mandard et al., 2006; Sukonina, Lookene, & Olivecrona, 2006), ANGPTL4 stimulated decreasing of the entry of fatty acids in the subcutaneous adipocyte by inhibiting LPL enzyme (lipoprotein lipase) (Sukonina et al., 2006) and favor their liberation (lipolysis), stimulating the activity of the Adipose Triglyceride Lipase (Mandard et al., 2006).

3.3. Effect of β-(1,3)-polyglucuronic acid sodium salt on Lipid Storage

In vitro study using Adipored® techniques has been carried out on adipocytes cultivated with the active β-(1,3)-polyglucuronic acid sodium salt at the concentration of 0.5%. Intracellular lipids were specifically marked by fluorescence, with Adipored® technique according to recommendation described in Section 2. This labelling was quantified measuring the fluorescence intensity (5 captured images independently of each analysis) and analysed statistically. Then, considering the control (non treated cells), this Adipored® study demonstrated that β-(1,3)-polyglucuronic acid sodium salt induced an interesting reduction of 26% in the amount of intracellular lipids when it was added at the concentration of 0.5% on the adipocytes culture. As confirmed in Fig. 4, the decrease of fluorescence intensity clearly demonstrated that β-(1,3)-polyglucuronic acid sodium salt could play important role in the reduction of lipogenesis and in the stimulation of lipolysis.

3.4. Action of β-(1,3)-polyglucuronic acid sodium salt in the differentiation of pre-adipocytes into mature adipocytes

The transcriptomic analysis investigated on human cells cultivated with β-(1,3)-polyglucuronic acid sodium salt has revealed its up-regulation of implicated genes in pre-adipocyte differentiation pathway. *In vitro* study was carried out on the pre-adipocytes differentiation in order to demonstrate the influence of β-(1,3)-polyglucuronic acid sodium salt in the pre-adipocyte differentiation to mature adipocytes. For this purpose, pre-adipocytes were cultivated until confluence, in the absence ("control" cells) or in the presence of β-(1,3)-polyglucuronic acid sodium salt at the concentration of 0.5% (treated cells). During 6 days, samples of "control cells" and cells treated with β-(1,3)-polyglucuronic acid sodium salt were collected for analysis. Each sampling was analysed by Western blot technique, to study the aP2/FABP4 protein in order to check the eventual differentiation of pre-adipocytes into mature adipocytes. In fact, the aP2/FABP4 protein is involved in free fatty acid transport and is predominantly expressed in mature adipocytes and therefore represented a very interesting protein marker in pre-adipocytes differentiation.

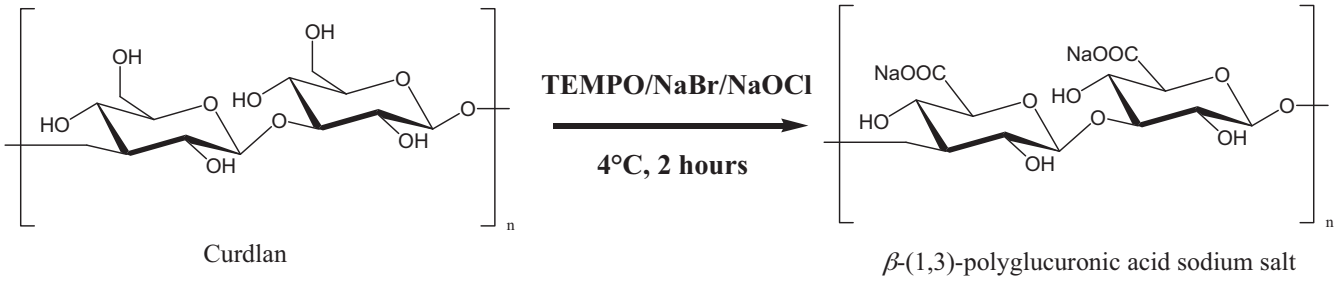


Fig. 1. Chemical process to produces β-(1,3)-polyglucuronic acid sodium salt by regioselective oxidation of β-(1,3)-glucan (curdlan) using TEMPO/NaOCl/NaBr system.

Table 1
Characterization of β-(1,3)-polyglucuronic acid sodium salt synthesized by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr catalytic system.

Derivatives	Glc (%)	UA (%)	Mw (kDa)	Mn (kDa)	Ip
β-(1,3)-Polyglucuronic acid sodium salt	0	100	520	300	1.7

Glc, glucose; UA, uronic acid estimated by colorimetric assay (Van Den Hoogen et al., 1998); Mw, weight average molecular weight; Mn, number average molecular weight; Ip, index of polydispersity.

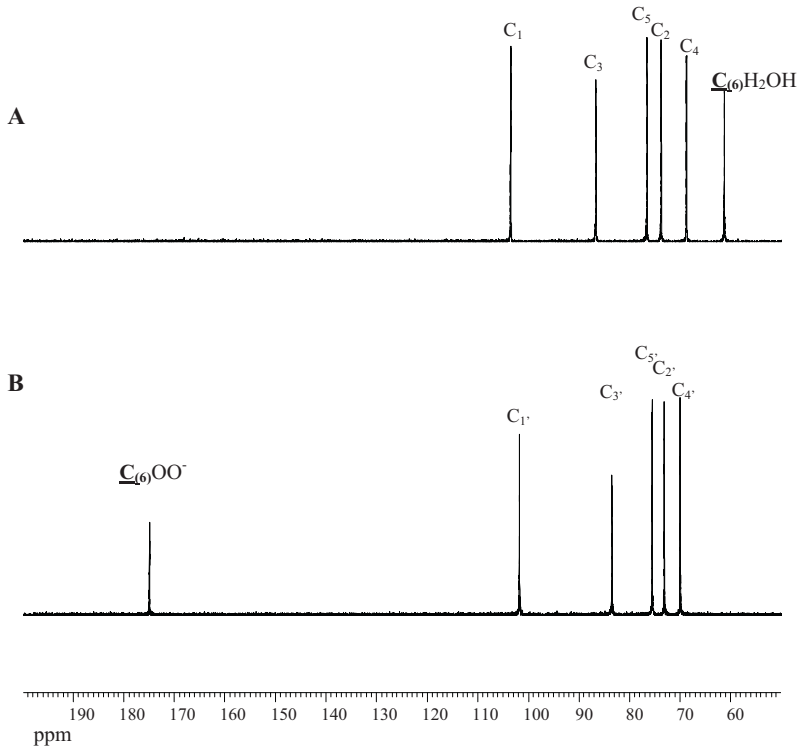
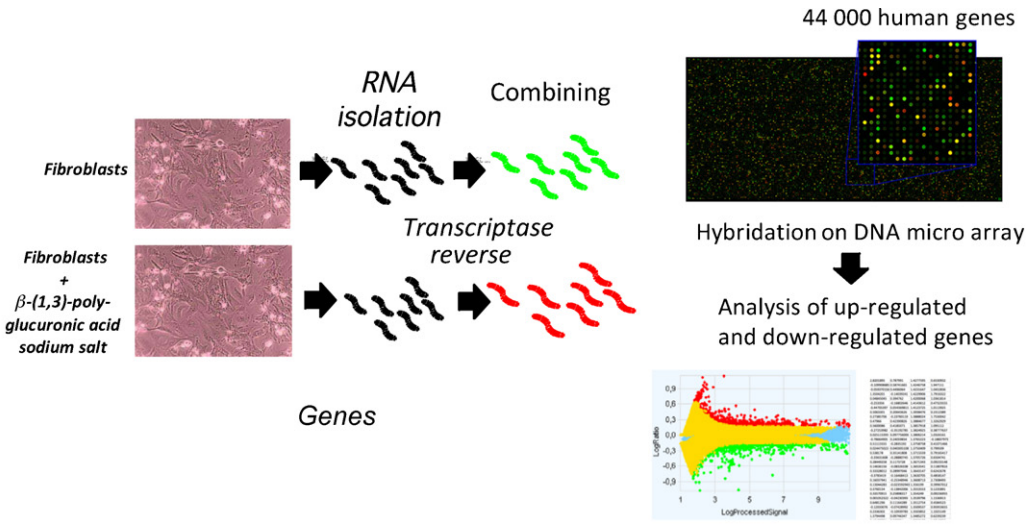


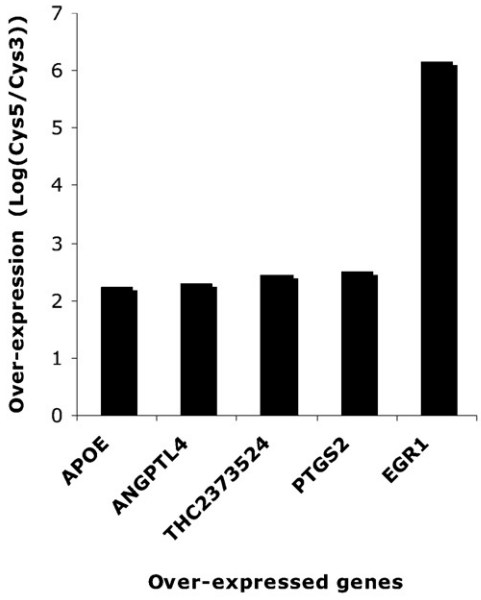
Fig. 2. ^{13}C analysis of (A) β-(1,3)-glucan (curdlan) and (B) sodium β-(1,3)-polyglucuronic acid sodium salt generated by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr system.

In condition A (Section 2) we observed (Fig. 5 condition A) decreasing in the aP2/FABP4 marker level when cells were treated with β-(1,3)-polyglucuronic acid sodium salt (0.5%) two days before induction of the differentiation. This result show that the treatment of pre-adipocytes with β-(1,3)-polyglucuronic acid sodium salt at the concentration of 0.5% induced a slow-down in differentiation of pre-adipocytes into mature adipocytes. On the other hand, it was important to mention that in condition B (Section 2), the Western blot analysis (Fig. 5 condition B) revealed a blockage in the differentiation of pre-adipocytes into mature adipocytes. In fact, when pre-adipocytes was treated with β-(1,3)-polyglucuronic acid sodium salt at the concentration of 0.5% at the same day of

Table 2
Chemical shifts (ppm) of ^{13}C (80 °C) for β-(1,3)-polyglucuronic acid sodium salt synthesized by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr catalytic system.

	Chemical shifts (δ ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Curdlan	103.64	73.96	86.77	68.88	76.67	61.48
β-(1,3)-Polyglucuronic acid sodium salt	101.96	73.35	82.86	70.14	75.65	175.56





Over-expressed Genes	Biological fonctions
APOE(+2.23*)	Lipid Transport, cell growth and cell differentiation
ANGPTL4(+2.30*)	Inhibition of LPL
THC2373524 (+2.45*)	Lipid Transport
PTGS2 (+2.51*)	Lipid Biosynthesis
EGR1 (+6.16*)	Activation of low density lipoprotein gene receptor

*=Log(Cys5/Cys3)

Fig. 3. Transcriptomic approach using DNA-array to screen the putative biological activities of β-(1,3)-polyglucuronic acid sodium salt. The effect of hydro-stimulation on genes expression in human fibroblasts was classified by Gene Ontology. The data present the percentage of responsive genes in biological process and molecular function. DNA were marked by specific fluorochrome: Cys5 (control) and Cys3 (β-(1,3)-polyglucuronic acid). Over-expressions of genes were estimated with the relation: Over-expression = log(Cys5/Cys3) > 1. Note to mention that each experiment was done at least 3 times ($p < 0.05$).

differentiation induction, we observed an inhibition of aP2/FABP4 marker secretion.

To complete this experiment, the adipocyte differentiation was analysed by microscopy (Fig. 6) after specific coloration of cells using red oil method (Section 2). In fact, pre-adipocytes do not store fatty acids (as triglycerides in vacuoles) in contrast to mature adipocytes. Thus, only matures adipocytes will stain with red oil while nucleus and cytoplasms were colored in blue by using haematoxylin colorant. As we could observed, at day 2 of treatment, control cells (non treated with β-(1,3)-polyglucuronic acid sodium salt) accumulated lipids indicating the pre-adipocyte differentiation into mature adipocytes. Concerning cells treated with β-(1,3)-polyglucuronic acid sodium salt, few amount of lipid was observed (low red coloration). At day 4, it should be noted that lipids were accumulated as lipid droplets in cells not treated with β-(1,3)-polyglucuronic acid sodium salt. In contrast, no or very few cells

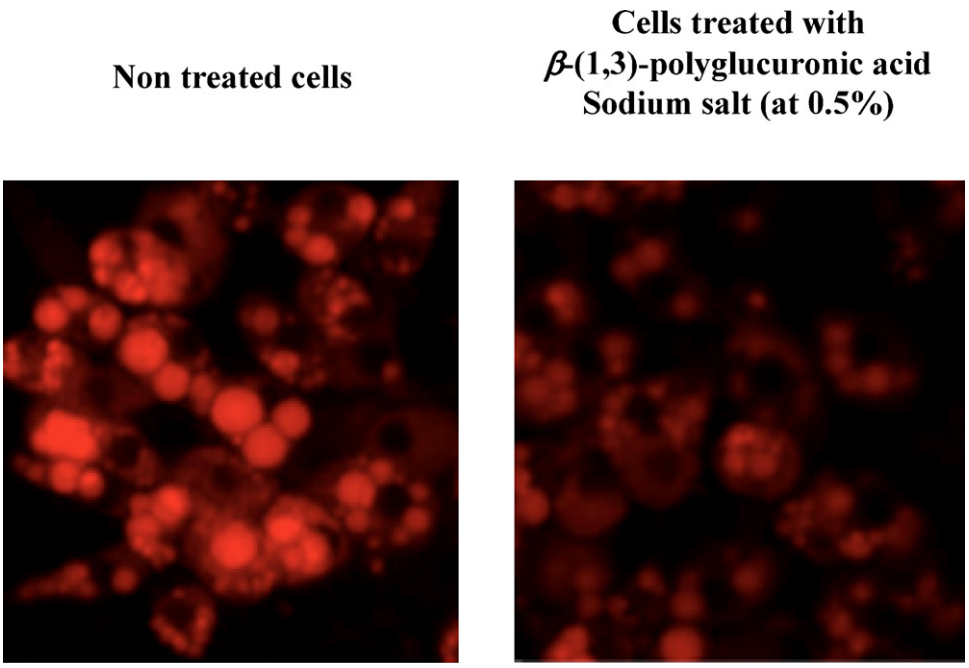


Fig. 4. Adipored® assay: morphological study of untreated and treated isolated adipocytes with the polyglucuronic acid sodium salt at 0.5% (by analysis of the reduction of red color intensity). Intracellular lipids were then labelled with Adipored® (Biowhittaker ref. PT-7009) following the supplier's recommendations. Image acquisition was performed using an automated microscope Incell AnalyzerTM1000 (GE Healthcare). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treated with β-(1,3)-polyglucuronic acid sodium salt accumulated lipids.

Finally, note to mention that at day 6, untreated cells strongly accumulate lipids indicating that many pre-adipocyte have been differentiated into mature adipocytes. In addition, we could observe a sharp increase in the volume of lipid droplets in cells already differentiated. For cells treated with β-(1,3)-polyglucuronic acid sodium salt, very few contained fatty acid and those containing lipid droplets are much smaller in size than those present in untreated cells. Consequently, we have shown that β-(1,3)-polyglucuronic acid sodium salt could induce a significant decrease in the differentiation of pre-adipocytes into mature adipocytes at the cell level. This result demonstrated that this β-(1,3)-polyglucuronic acid sodium salt could constitute an innovative slimming active blocking the differentiation of pre-adipocytes into mature adipocytes. Nevertheless, it is important to mention

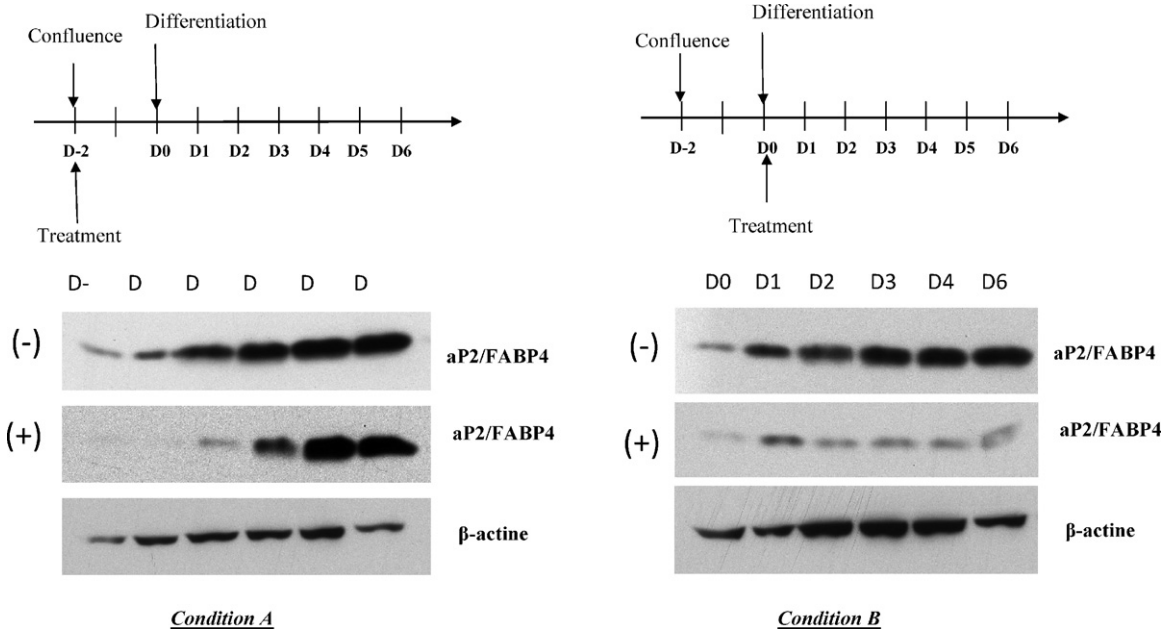


Fig. 5. Analysis of aP2/FABP4 protein by Western Blot in 'control' cells sample and treated cells sample with β-(1,3)-polyglucuronic acid sodium salt. The detection of aP2/FABP4 was carried out thanks to a monoclonal antibody. The method is validated by the detection of the protein ß-actine due to a polyclonal antibody (Sigma). (−) Control cells sample without treatment and (+) treatment with β-(1,3)-polyglucuronic acid sodium salt. All experiments were performed in triplicate.

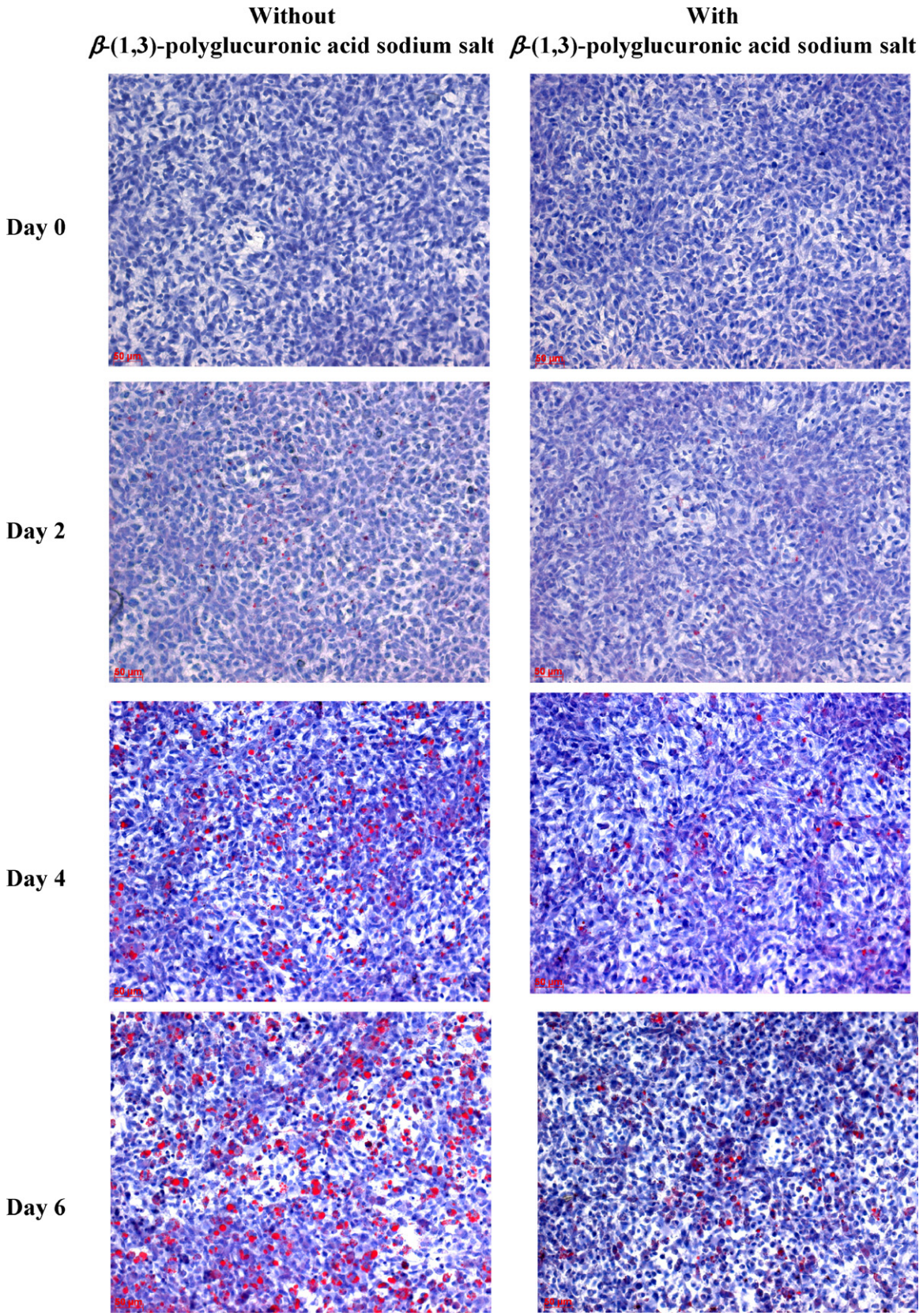


Fig. 6. Adipored® assay: microscopic analysis of pre-adipocytes 'control' and treated with β-(1,3)-polyglucuronic acid sodium salt. Red oil is the specific marker for fatty acids and the haematoxylin (a blue colorant) is a specific for the nucleus. During experimentation, cells were photographed at original magnification $\times$100. The experiment was performed three times. Representative images are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that inhibition of differentiation of pre-adipocytes into mature adipocytes may not be totally desirable. Indeed, such inhibition of adipogenesis may cause storage of lipids in non-adipose organs and tissues leading to lipotoxicity in liver, muscle and cardiovascular system (Xu et al., 2005). Consequently, *in vivo* test are in progress in order to study the impact and toxicity of this β-(1,3)-polyglucuronic acid sodium salt. Moreover, administration of this β-(1,3)-polyglucuronic acid sodium salt to C57BL/6J mice with high fat diet will be performed to test its potential effect as new drug for anti-obesity therapy.

4. Conclusion

In this present work, we have clearly demonstrated by *in vitro* studies that β-(1,3)-polyglucuronic acid sodium salt acts on 3T3-L1 cells by reducing pre-adipocyte differentiation, promoting adipocyte delipidation at low concentrations. Consequently, this β-(1,3)-polyglucuronic acid sodium salt could be used as potential therapeutic bio molecule for the treatment of lipid metabolism diseases by regulating fat cell number and size.

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