A comparative study of the hypolipidaemic effects of a new polysaccharide, mannan Candida albicans serotype A, and atorvastatin in mice with poloxamer 407-induced hyperlipidaemia

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Abstract

Objectives We evaluated the hypolipidaemic effect of mannan Candida albicans serotype A, relative to atorvastatin, in a mouse model of hyperlipidaemia.

Methods Mannan serotype A was investigated in vitro and in vivo to determine its effects on macrophage proliferation, nitric oxide (NO) production by cultured macrophages, serum and liver lipids, changes in liver morphology and serum chitotriosidase activity and its expression in the liver.

Key findings Mannan serotype A stimulates the macrophage proliferation and NO production in murine peritoneal macrophages in vitro. The activity of serum chitotriosidase (an enzyme released from the activated macrophages) was found to be significantly increased in P-407-induced hyperlipidaemic mice pretreated with low-dose mannan compared with mice administered P-407 only. Mannan treatment in mice was shown to significantly increase the chitotriosidase expression in the liver of both non-hyperlipidaemic and P-407-induced hyperlipidaemic mice. Lastly, mice pretreated with mannan before the induction of hyperlipidaemia with P-407 showed a significant reduction in the serum concentration of atherogenic LDL cholesterol, total cholesterol, triglycerides and liver triglycerides.

Conclusions It is suggested that mannan serotype A, like β-glucan, may represent another hypolipidaemic agent, which could potentially be used as an adjunct therapy with conventional antihyperlipidaemic drugs (statins and fibrates) in humans.

Introduction

Hyperlipidaemia is an important problem of modern medicine leading to the development of atherosclerosis and, as a result, increasing the risk of myocardial infarction and stroke.[1,2] Statins are well-known hypolipidaemic agents that are widely used to prevent or treat existing coronary heart disease due to atherosclerosis.[3] However, the use of statins can be associated with severe adverse side effects to the skeletal muscle (rhabdomyolysis) and the development of statin resistance/intolerance. New lipid-lowering drugs include PCSK9 inhibitors[4] and compounds of plant origin (phytonutrients).[5] The water-insoluble, wall-yeast polysaccharide known as zymosan has been shown to decrease the atherogenic serum lipids in experimental lipidaemia induced in mice, although the hypolipidaemic effects of zymosan, which is composed primarily of β-glucan and mannan, are still poorly understood.[6] The
former component, namely β-glucan, has previously been shown to exhibit a hypolipidemic effect in poloxamer 407 (P-407)-induced hyperlipidaemic mice. Thus, besides the use of the statin class of drugs for lowering LDL cholesterol, biological response modifiers, such as the partially water-soluble β-1,3-glucans, may be used as adjunctive therapy with the statins. These natural polysaccharides are much less expensive than statin therapy and have no adverse side effects on the skeletal muscle.

Mannan, which belongs to a class of immunomodulators of polysaccharide origin, has been shown to stimulate macrophages in vivo through its interaction with the mannose receptor. Polysaccharides, unlike statins, are natural stimulators of macrophages, which cause the macrophages to increase their endocytic activity. Following endocytosis, these branched polysaccharides have the capacity to activate LDL and scavenger receptors and increase the uptake of atherogenic LDL cholesterol, as well as other lipoproteins with modified chemical structures. It has also been suggested that excess cholesterol may potentially be removed from the atherosclerotic plaque by the activated macrophages.

Due to the fact that polysaccharides cause an increase in the activity of macrophages, they consequently promote an increase in the secretion of an enzyme called chitotriosidase by macrophages, which may then serve as a biomarker of macrophage stimulation/activation. In fact, chitotriosidase has been suggested to represent a new, non-lipid biomarker for the development of early atherosclerosis and has been demonstrated in individuals with established atherosclerosis.

By utilizing new biomarkers (e.g. chitotriosidase), clinicians will be able to identify those at risk of developing atherosclerosis when the changes in serum lipid profiles are unremarkable and would otherwise not suggest a patient at risk for the development of coronary heart disease secondary to atherosclerosis. More importantly, these same biomarkers may provide insight into the effectiveness of various therapeutic (hypolipidaemic) interventions – the administration of standard hypolipidaemic drugs used either alone, or in combination with various phytonutrients such as β-glucan, mannan and other natural compounds. Thus, one of our aims in this study was to evaluate the activity of serum chitotriosidase, as well as its expression, by liver cells following the administration of mannan to mice, although it was our primary aim to determine the hypolipidaemic effects of mannan, relative to atorvastatin, in a well-established mouse model of hyperlipidaemia and atherosclerosis known as the P-407-induced mouse model in a well-established mouse model of hyperlipidaemia and hypolipidaemic effects of mannan, relative to atorvastatin, mice, although it was our primary aim to determine the activity of serum chitotriosidase, as well as its expression, by liver cells following the administration of mannan to mice, although it was our primary aim to determine the hypolipidaemic effects of mannan, relative to atorvastatin, in a well-established mouse model of hyperlipidaemia and atherosclerosis known as the P-407-induced mouse model of atherogenesis.

The P-407-induced mouse model of dyslipidaemia allows for a dose-dependent elevation in plasma cholesterol and triglycerides (TG) and offers an advantage over classic diet-induced mouse models of hyperlipidaemia, because the latter models are unable to increase the plasma TG. Continuous administration of P-407 results in the formation of aortic atherosclerotic lesions (fatty streaks) after 1 month, with atheroma formation reaching a maximum in terms of the number and size of formed lesions after 4 months of P-407 administration.

Materials and Methods

Microorganisms and cultivation conditions

Candida albicans serotype A (CCY 29-3-100) and C. albi-
cans serotype B (CCY 29-3-103) yeast strains from the Culture Collection of Yeasts (CCY; Institute of Chemistry of Slovak Academy of Sciences, Bratislava, Slovakia) were cultivated in YPD medium containing 0.5% yeast autolysate, 1% peptone and 2% D-glucose for 48 h at 28 °C using a rotatory shaker (100 rpm).

Mannan preparation

Mannoprotein was extracted from a wet biomass by autoclaving three-times for 1 h at 120 °C with 0.2 m NaCl. Autoclaved mixtures were centrifuged and the supernatants were pooled. Mannoprotein was subsequently precipitated from the supernatants with 0.1% CH₃COONa in ethanol (1 : 4). The freeze dried mannoprotein was suspended in 2% KOH and heated for 1 h at 100 °C. Mannan was precipitated from the supernatant with Fehling reagent and the sediment was dissolved in 3 m HCl and added dropwise to methanol-acetic acid.

Physicochemical characterization of mannans

Physicochemical characterization of mannans was conducted using mannans from C. albicans serotypes A and B. Both mannans were analysed for carbon, hydrogen and nitrogen contents using a EA 1108 device (FISONS Instruments, Uckfield, England, UK). The monosaccharide composition of mannans was determined using gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), a temperature programme of 110–125 °C (20 °C/min) to 165 °C (20 °C/min) and a hydrogen flow rate of 20 cm³/min. Additionally, both mannans were identified on 1H NMR spectra and 1H–13C TOCSY NMR spectra using a VNMR 400 MHz Varian spectrometer equipped with a 1H–13C–31P PFG AutoX DB NB probe head. The spectra were recorded using 2% (w/v) solution of mannans at 45 °C with acetone (2.217 ppm) as...
an internal standard. The previously published chemical shift assignments of individual mannannyl residues found in intact *C. albicans* mannan and side-chain fragments of both serotypes were used.[25,26]

**Peritoneal macrophage proliferation and nitric oxide production**

Peritoneal macrophages were isolated from 10- to 12-week-old male CBA/Lac mice using standard techniques. The well-known MTT assay was used to quantify the macrophage proliferation in the absence and presence (0, 10, 50, 250 and 500 μg/ml) of mannann A and B. Absorbance of each well was detected spectrophotometrically at 570 nm on a StatFax 2100 microplate reader (Palm City, Florida, USA). Briefly, nitric oxide (NO) production was quantified using the Griess reaction after collecting the culture supernatant of peritoneal macrophages following incubation with either mannan A or mannan B (0, 10, 50, 250 and 500 μg/ml) or LPS (1 and 10 μg/ml) (positive control) for 24 h. The absorbance of each well was detected spectrophotometrically at 540 nm on the same microplate reader described above and the NO content calculated using a least-squares linear regression analysis of a sodium nitrite standard curve.

**Animal protocols**

Male CBA/Lac mice having a body mass of 25–30 g were obtained from the breeding station of the Institute of Physiology and Basic Medicine, Novosibirsk, Russia. Poloxamer 407 (Pluronic F-127; Sigma, St. Louis, Missouri, USA) was administered to mice as an intraperitoneal injection at a dose of 300 mg/kg.[22,27] The mice were decapitated at 24 h after P-407. Control mice received the appropriate volume of saline. The rationale for animal sacrifice 24 h after P-407 administration is based on the fact that we have previously reported that serum lipids reach a maximum at this time point.[20–22]

Mannans of *C. albicans* serotype A was administered to mice by intraperitoneal injection in doses of 50 mg/kg, five times, or 100 mg/kg, twice, 24 h before P-407 treatment. Due to our extensive experience with mannan A, we used only mannan A in the subsequent in-vivo experiments. To determine the effects of mannans in the non-hyperlipidaemic state, a second group of mice received mannan A in the corresponding doses and manner of administration as described above, but no P-407 to induce hyperlipidaemia. For comparative purposes, a third group of mice was administered atorvastatin (75 mg/kg) (Atorisoris; KRKA, Novo mesto, Slovenia) by oral gavage at 24 and 48 h before an injection of P-407.[21] Control mice received an equivalent volume (0.5 ml) of saline only.

Mice were deprived of food, but had free access to water 15 h before euthanasia. All procedures for drug administration, blood and tissue collection were in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals published in 2011 by the United States National Academy of Sciences, and the treatment protocol (animal protocol #9) was also approved by the Animal Care and Use Committee at the Institute of Physiology and Fundamental Medicine on 27 May 2014. The in-vivo experiments were also conducted in compliance with Institute of Physiology and Basic Medicine Ethical Committee Recommendations pertaining to research involving laboratory animals.

**Serum and liver lipid determinations**

Serum was obtained after the centrifugation of blood samples at 3000g for 20 min at 4 °C (Eppendorf Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) and stored at −70 °C until the analysis of total cholesterol, triglycerides and LDL cholesterol (commercial kit for LDL cholesterol; Biosystems, Costa Brava, Spain) and expressed in mmol/l.

The method of Le Guezenece et al.[28] was employed to quantify both liver cholesterol and triglycerides using a 96-well microplate procedure. After the addition of working reagent (Olvex Diagnosticum, Total Cholesterol kit, St. Petersburg, Russia), the optical density was measured at 530 nm using a multimodal reader (TriStar; Berthold Inc., Bad Wildbad, Germany) and the results were expressed as mg of cholesterol per 1 g of liver tissue.

**Serum chitotriosidase activity**

Serum chitotriosidase (CHITI, EC 3.2.1.14) activity assay was determined using a fluorometric method against the fluorescent substrate, 4-methylumbelliferyl-β-D-N-N″-triacetylchitotrioside (i.e. 4-MUF-β-D-N-N″-triacetyl-chitotrioside) (Sigma), at pH 5.2 as previously described.[16,18]

**Expression of chitinases using RT-qPCR**

Total RNA was purified from the mouse liver (for comparison purposes, RNA was also purified from stomach, tongue, lung, intestine and brain cortex) using the RNasey Plus mini kit (Qiagen, Hilden, Germany), including on-column DNase treatment to remove genomic DNA. The concentration and the purity (A260/A280) of RNA were measured using the NanoPhotometer P-Class (Implen, Munich, Germany). cDNA was synthesized from 1 μg of total RNA using ABI High Capacity RNA to cDNA kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, California, USA) with an oligo(dT), primer.
Real-time PCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) using HS-qPCR Mix SYBR Green (Biosan, Novosibirsk, Russia), 200 nM real-time PCR primers (Table 1) and 5 μl of template (1 : 50 diluted cDNA). All samples were analysed in triplicate. Non-template controls were run together with samples. The fold-change values were determined using the $2^{-\Delta\Delta Ct}$ relative quantification method according to Livak and Schmittgen.\[^{29}\]

**Morphological study of liver**

For electron microscopy, liver specimens with a size up to 1 mm$^3$ were fixed in a 4% paraformaldehyde solution prepared using Hanks’ buffer, fixed within 1 h in a 1% OsO$_4$ solution in phosphate buffer (pH 7.4), dehydrated in ethyl alcohol of increasing concentrations and then finally embedded in Epon media (Serva, Heidelberg, Germany). Ultrathin slices of 35–45 nm in thickness were contrasted by saturated water solution of uranyl acetate and lead citrate and evaluated using a model JEM 1400 electron microscope (Japan). Morphometric analysis was conducted by means of a computer ImageJ program (WayneRasband, Bethesda, Maryland, USA) for the determination of the percentage volume density of lipids in the cytoplasm of hepatocytes.

**Statistical analysis**

The results were analysed by one-way analysis of variance (ANOVA) with post-hoc analysis [least significant difference (LSD) method] using the statistical program STATISTICA 10.0 (Dell Statistica, Tulsa, OK, USA).

**Results**

**Effect of mannans on isolated peritoneal murine macrophages in vitro**

In the in-vitro study with cultured mouse peritoneal macrophages, mannan serotype A induced a significant increase in both macrophage proliferation ($P < 0.01$) (Figure 1a) and NO production ($P < 0.05$) (Figure 1b) relative to control (basal) levels. There was also a significant increase in both macrophage proliferation ($P < 0.05$) (Figure 1a) and NO production ($P < 0.05$) (Figure 1b) when mannan serotype B was evaluated in the macrophage cell culture and compared with the respective mean values for control (Figure 1a and 1b).

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**Table 1** Real-time PCR primers

<table>
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<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size bp</th>
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<tbody>
<tr>
<td>Chia1</td>
<td>TCCTGGTAGAGGAAATGCTG</td>
<td>AAATCCCACGCTACAGCA</td>
<td>96</td>
</tr>
<tr>
<td>Chit1</td>
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<td>TTCCAGGAGCCACCTGTTAT</td>
<td>101</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GCTTCTCCCTGTCCAGAGAC</td>
<td>CCAATACGGCCAATCCCAGTCA</td>
<td>103</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TCTACAATGAGCTGCTGTTG</td>
<td>GGGGTGTTGAAGGTCTCAAA</td>
<td>121</td>
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</table>

Figure 1 Effect of mannan serotype A and serotype B on murine macrophage proliferation (a) and NO production (b) in vitro. Basal (control) = basic macrophage proliferation; LPS-1 = lipopolysaccharide (1 mcg/ml); LPS-10 = lipopolysaccharide (10 mcg/ml); Man A 50 = mannan serotype A (50 mcg/ml); Man B 50 = mannan serotype B (50 mcg/ml); Gly 50 = β-glucan (50 mcg/ml). Macrophages were cultured with the various treatments for 48 h. Filled circles represent the median values, whereas the upper and lower points of the vertical lines (‘whiskers’) represent the maximum and minimum values. Horizontal hash marks (lines) through the vertical lines represent the values of the 1st quartile, or 25th percentile (lower) and 3rd quartile, or 75th percentile (upper). $^aP < 0.05$ and $^bP < 0.01$ vs basal (control), $^cP < 0.05$ vs LPS-1 and LPS-10, $^dP < 0.05$ vs Man A and Man B.

Effect of mannans on liver weight

Both doses of mannan (50 and 100 mg), whether dosed alone (without P-407) or when mice were made hyperlipidaemic with P-407, resulted in a significant ($P < 0.05$) increase in the relative weight of excised livers when compared with controls (Figure 2). Additionally, in P-407-induced hyperlipidaemic mice predosed with 100 mg of mannan (Figure 2; Bar 6), there was a significant ($P < 0.05$) increase in the relative weight of excised livers when compared with P-407-induced hyperlipidaemic mice dosed with P-407 only, but no mannan (Figure 2; Bar 4).

Effect of mannan on the serum lipid profile during acute P-407-induced hyperlipidaemia

When administered to non-hyperlipidaemic (i.e. non-P-407-treated) mice, neither treatment with atorvastatin nor both doses of mannan resulted in a significant change in either total or LDL cholesterol relative to controls (Figure 3a and 3b), although mannan at both doses did cause a significant ($P < 0.05$) decrease in serum triglycerides when compared with controls (Figure 3c). Not unexpectedly, P-407 induced a significant ($P < 0.001$) elevation in serum total cholesterol, LDL cholesterol and TG when compared with controls (Figure 3a–3c). As a reference for the hypolipidaemic effects of mannan, pretreatment of P-407-induced hyperlipidaemic mice with atorvastatin resulted in a significant reduction in serum total cholesterol ($P < 0.05$), LDL cholesterol ($P < 0.001$) and TG ($P < 0.001$) (Figure 3a–3c) when compared with P-407 only (Bar 5). A very noteworthy finding was that pretreatment with mannan at a dose of 50 mg/kg × 5 was just as effective as atorvastatin (75 mg/kg × 2) at reducing the serum total cholesterol ($P < 0.05$), LDL cholesterol ($P < 0.001$) and TG ($P < 0.001$) in P-407-treated hyperlipidaemic mice (Figure 3a–3c) when compared with P-407 only (Bar 5).
Interestingly, pretreatment of mice with mannan at a dose of 100 mg/kg × 2 was not as effective at reducing the serum total cholesterol in P-407-induced hyperlipidaemic mice as either atorvastatin (75 mg/kg × 2) or low-dose mannan (50 mg/kg × 5) (Figure 3a), although both serum LDL cholesterol and TG were found to be significantly (P < 0.001) decreased relative to P-407-only-treated (hyperlipidaemic) mice (Bar 5) for all three treatments (Figure 3b and 3c).

Effect of mannan on liver cholesterol and triglycerides

The administration of atorvastatin alone, P-407 alone, mannan at either the low or high dose of mannan or pretreatment with either the low or the high dose of mannan + P-407 to induce the hyperlipidaemic state produced no change in the liver concentrations of cholesterol when compared with controls (Figure 4a). Administration of either atorvastatin, low-dose mannan or high-dose mannan to P-407-induced hyperlipidaemic mice resulted in a significant (P < 0.001) reduction in liver TG when compared with controls (Figure 4b; Bars 6, 7 and 8, vs Bar 1), as well as when compared with P-407-only-treated mice (Figure 4b; Bars 6, 7 and 8, vs Bar 5).

Effect of mannan on serum chitotriosidase activity

With the exception of low-dose mannan, both atorvastatin and high-dose mannan administration to non-P-407-induced hyperlipidaemic mice had no effect on serum chitotriosidase activity when compared with controls (Figure 5). However, all three treatments [atorvastatin (P < 0.01) and both low-dose (P < 0.001) and high-dose (P < 0.05) mannan] caused a significant increase in serum chitotriosidase activity in P-407-induced hyperlipidaemic mice (Figure 5; Bars 6, 7 and 8), as did the administration of P-407 only (P < 0.05) (Figure 5; Bar 5), when each was compared with the mean value of serum chitotriosidase activity when P-407 only was treated (Figure 5; Bar 5).

![Figure 4](image_url) Influence of atorvastatin or mannan A pretreatment on liver cholesterol and triglycerides in hyperlipidaemic (poloxamer 407-induced) and non-hyperlipidaemic mice. (a) Total cholesterol; (b) triglycerides. Cont. = controls; Ator. = treatment with 75 mg/kg × 2 of atorvastatin; Man 50 = treatment with 50 mg/kg × 5 of mannan; Man 100 = treatment with 100 mg/kg × 2 of mannan; P-407 = 300 mg/kg P-407; Ator. + P-407 = pretreatment with 75 mg/kg × 2 of atorvastatin + 300 mg/kg P-407; Man 50 + P-407 = pretreatment with 50 mg/kg × 5 of mannan + 300 mg/kg P-407; Man 100 + P-407 = pretreatment with 100 mg/kg × 2 of mannan + 300 mg/kg P-407. Bars represent the mean value ± SD (n = 8–10 mice/group). aP < 0.05 vs control; bP < 0.01 vs control, cP < 0.001 vs control (Bar 1) and P < 0.01 vs P-407 only (Bar 5).

![Figure 5](image_url) Influence of atorvastatin or mannan A pretreatment on serum chitotriosidase activity in hyperlipidaemic (poloxamer 407-induced) and non-hyperlipidaemic mice. Cont. = controls; Ator. = treatment with 75 mg/kg × 2 of atorvastatin; Man 50 = treatment with 50 mg/kg × 5 of mannan; Man 100 = treatment with 100 mg/kg × 2 of mannan; P-407 = 300 mg/kg P-407; Ator. + P-407 = pretreatment with 75 mg/kg × 2 of atorvastatin + 300 mg/kg P-407; Man 50 + P-407 = pretreatment with 50 mg/kg × 5 of mannan + 300 mg/kg P-407; Man 100 + P-407 = pretreatment with 100 mg/kg × 2 of mannan + 300 mg/kg P-407. Bars represent the mean value ± SD (n = 8–10 mice/group). aP < 0.05 vs control; bP < 0.01 vs control, cP < 0.001 vs control (Bar 1) and P < 0.01 vs P-407 only (Bar 5).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Relative quantification of Chit1 and Chia1 mRNA levels normalized by GAPDH and beta-actin in select mouse tissues</th>
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<tbody>
<tr>
<td>Organ</td>
<td>Chit1 (%)</td>
</tr>
<tr>
<td>Stomach</td>
<td>70 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tongue*</td>
<td>100 ± 5.5</td>
</tr>
<tr>
<td>Lung</td>
<td>2.0 ± 0.03</td>
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<tr>
<td>Intestine</td>
<td>0.6 ± 0.11</td>
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<tr>
<td>Liver</td>
<td>2.4 ± 0.12</td>
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<tr>
<td>Brain</td>
<td>2.2 ± 0.12</td>
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<sup>*The expression level in the tongue was considered 100% for the Chit1 gene and 100% in the lung for the Chia1 gene. **Values represent the mean value ± SD.</sup>
activity determined in controls (Figure 5; Bar 1). Only low-dose mannan resulted in a significant (P < 0.01) increase in the activity of serum chitotriosidase when compared with hyperlipidaemic mice treated with P-407 only (Figure 5; Bar 7 vs Bar 5).

**Chitotriosidase expression study**

Table 2 lists the relative expression of Chit1 and Chia1 in various mouse organs, while Table 3 demonstrates a statistically significant increase in the mean values of the fold-change in the hepatic expression of Chit1 and Chia1 relative to control following the administration of mannan to both non-hyperlipidaemic and P-407-induced hyperlipidaemic mice.

**Electron microscopic analysis of liver**

Accumulation of lipids in the liver was observed as soon as one hour after P-407 administration to mice (Figure 6a–c, 6f). The structure of the sinusoid, as well as sinusoidal cells, did not undergo significant changes following the

<table>
<thead>
<tr>
<th>Group</th>
<th>Chit1 Fold-change</th>
<th>Chia1 Fold-change</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.575</td>
<td>1.00 ± 0.258</td>
</tr>
<tr>
<td>P-407 (1 h)</td>
<td>1.68 ± 0.145</td>
<td>1.63 ± 0.736</td>
</tr>
<tr>
<td>P-407 (24 h)</td>
<td>0.87 ± 0.665</td>
<td>2.01 ± 1.255</td>
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<tr>
<td>Mannan (24 h)</td>
<td>3.87 ± 1.910</td>
<td>3.97 ± 0.424</td>
</tr>
<tr>
<td>Mannan + P-407 (24 h)</td>
<td>6.08 ± 2.561</td>
<td>2.66 ± 1.234</td>
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</table>

The number of mice in each group was 4–5. Chia1, chitinase, acidic 1; acidic mammalian chitinase (AMCase); Chia. Chit1, chitinase 1 (chitotriosidase). aTime in parentheses indicates time following treatment at which gene expression was determined. bIndicates the fold-change compared with control. cIndicates the mean value ± the standard error of the mean (SEM). dIndicates P < 0.01 vs control. eIndicates P < 0.05 vs control. fIndicates P < 0.01 vs P-407 (24 h).

Figure 6 Ultrastructure of hepatocytes in mice with P-407-induced hyperlipidaemia and pretreated with mannan A. (a) A fragment of a hepatocyte from control mice. (b) Accumulation of lipids (arrows) in the cytoplasm of a hepatocyte 1 h after a single injection of P-407 (300 mg/kg). (c) Accumulation of lipids (arrows) in the cytoplasm of a hepatocyte 24 h after a single injection of P-407 (300 mg/kg). (d) A fragment of a hepatocyte 24 h after the treatment of mice with mannan. (e) Mild accumulation of lipids (arrows) in the cytoplasm of a hepatocyte 24 h after the combined administration of mannan and P-407. (f) Ito’s cells containing large lipid droplets in the liver of mice (arrows) 24 h after a single injection of P-407 (300 mg/kg). Magnification = 6000 x.
administration of P-407 only (Figure 6a and 6b). Lipid deposits were also noted in the cytoplasm of macrophages following the treatment with P-407 only (Figure 7a–7c).

Overall, electron microscopy confirmed that the liver tissue of P-407-treated mice displayed the accumulation of lipids within the hepatocytes, macrophages and Ito’s cells, as well as considerable expansion and vacuolization of sinusoids and macrophages. In summary, mannan administration resulted in a decrease in the accumulation of lipids within the hepatocytes, but expanded (enlarged) sinusoids still remained with an increased number of lymphocytes and macrophages present (Figures 6d and 6e and 7e and 7f).

**Morphometric analysis of liver lipids observed using electron microscopy**

The percentage volume density of lipids in the liver tissue from P-407-treated hyperlipidaemic mice was found to be significantly ($P < 0.001$) greater, and approximately 50%, when compared with this same parameter in controls (~1%) (Figure 8). Most significant to this study is that while mannan-pretreated, P-407-induced hyperlipidaemic mice (Bar 5 of Figure 8) did have an estimated percentage volume density of liver lipids (~23%) that was found to be significantly ($P < 0.01$) greater than mice treated with mannan only (Bar 4 of Figure 8), nevertheless, and importantly, liver lipids are found to be significantly ($P < 0.05$) reduced by about one-half with mannan treatment when compared with P-407-induced hyperlipidaemic mice (Bars 2 and 3 in Figure 8).

**Discussion**

The main finding in this study is that mannan *C. albicans* serotype A functioned as an effective stimulator of murine macrophages and exerted a hypolipidaemic effect in P-407-induced hyperlipidaemic mice following parenteral...
administration. Interestingly, we have previously shown that chemically modified (partially water-soluble) yeast polysaccharides, such as carboxymethylated β-glucans, also exerted a hypolipidaemic effect in the P-407-induced hyperlipidaemic mouse model.\[17,19\] Thus, a brief summary of our current findings using mannan C. albicans serotype A will be presented. Mannan C. albicans serotype A significantly decreased the atherogenic LDL cholesterol fraction of the serum lipids, as well as total cholesterol and triglycerides, in mice with P-407-induced hyperlipidaemia following a single dose of P-407. Additionally, a lipid-lowering effect was shown in the liver, where mannan (and atorvastatin) decreased the TG concentration in P-407-induced hyperlipidaemic mice, as well as decreased (as shown by electron microscopy and accompanying morphometric analysis) the number of lipid droplets; the latter effect may possibly have resulted from an increased rate of autophagy (lipophagy). In general, mannan’s hypolipidaemic effect was very similar to atorvastatin at the doses used in our experiments. Therefore, it is concluded that polysaccharides (like mannan) may potentially represent a useful class of hypolipidaemic agents from the category known as yeast immunomodulators.

We have also shown that serum chitotriosidase activity is significantly increased in mannan-pretreated, P-407-induced hyperlipidaemic mice, possibly as a result of both macrophage stimulation (which causes the release of chitotriosidase from macrophages) and increased hepatic expression of chitotriosidase, which we have determined in this investigation. It is interesting that we also detected a simultaneous increase in the expression of another chitinase (AMCase), which is responsible for an increased expression of chitotriosidase. Humans and mice have two genes that encode active chitinases, chitotriosidase (Chit1) and acidic mammalian chitinase (Chia, AMCase).\[30,31\] Chit1 was the first mammalian chitinase to be purified and cloned. AMCase, which is the second most-active chitinase in mammals, was identified as a compensatory enzyme for Chit1 and named for its optimal activity in acidic conditions.\[32\] Interestingly, Chit1 expression in the liver was highest in the case where mice were administered mannan + P-407, which is in concordance with the data on serum chitotriosidase activity in the mannan + P-407 groups. Perhaps, as suggested by Kitamoto et al.,\[33\] Chit1 has a protective role against atherosclerosis by regulating the macrophage lipid uptake and efflux and an enhanced expression and activity of Chit1 locally in the liver is the body’s attempt to maintain homeostasis rather than to create an imbalance in macrophage lipid turnover and, consequently, initiate an atherogenic biochemical cascade.

Along the lines of macrophage activation/stimulation, yeast polysaccharides categorized as immunomodulators have been shown to stimulate macrophages in vivo (e.g. β-glucans – mainly through dectin and scavenger receptors; mannan – primarily through the mannose receptor) and often exhibit antihypercholesterolaemic and anticoagulant properties; both are important in the treatment for atherosclerosis. However, in some experimental studies, water-insoluble polysaccharides of a bacterial origin have demonstrated not only moderate hypolipidaemic effects in vivo, but also slightly toxic effects, which have subsequently led to mild inflammation.\[34,35\] This is important in view of the fact that atherosclerosis is now widely accepted to be a chronic inflammatory disease with an impaired balance between macrophage uptake and efflux of cholesterol, which leads to the accumulation of free cholesterol in the cell and the formation of foam cells.\[36\] In consideration of these findings, it is imperative that further testing be undertaken to determine any long-term toxicity of mannan, as it relates to low-grade inflammation, when being considered for use as an adjunct hypolipidaemic agent.

The antihypercholesterolaemic effects of mannans of different origins have been shown previously; however, the mechanisms associated with their protective effects are still poorly understood. It has been reported that Kluyveromyces marxianus YIT 8292 elicited a more potent antihypercholesterolaemic effect than other yeasts containing Saccharomyces cerevisiae.\[37\] The greater antihypercholesterolaemic activity of K. marxianus YIT 8292 was suggested to arise from the side-chain structure of the cell surface.
polysaccharide (i.e. mannan). The connection between chemical structure and antihypercholesterolaemic activity was investigated by comparing mutants of S. cerevisiae mannan for their antihypercholesterolaemic activity in rats fed a high-cholesterol diet.\textsuperscript{[37]} It was concluded that the length and phosphate content of mannan side chains had a significant effect on the antihypercholesterolaemic activity.\textsuperscript{[37]}

According to the recent literature, glucomannan has been introduced for the treatment of hyperlipidaemia associated with atherosclerosis.\textsuperscript{[17,38,39]} In humans, glucomannan (Konjac mannan from the tuber Amorphophallus konjac; a highly branched viscous glucomannan) incorporated into the diet to increase fibre content has demonstrated cholesterol-lowering effects.\textsuperscript{[38,39]} The antihypercholesterolaemic effects of glucomannan were evident with even small doses and have been documented in both healthy persons and in populations of hyperlipidaemic patients.\textsuperscript{[17]} The mechanisms responsible for glucomannan’s protective hypolipidaemic effects are still not fully understood.

**Conclusion**

Based on this study, which has demonstrated the hypolipidaemic effects of mannan A in an experimentally induced hyperlipidaemic mouse model, it would therefore seem that future work aimed at evaluating the potential hypolipidaemic effects of polysaccharides should include several of the following goals. To begin, perhaps the polysaccharides (e.g. β-glucans and mannans) should be more fully investigated as adjunctive antihyperlipidaemic therapy in combination with specific classes of both well-established and newer antihyperlipidaemic agents (e.g. statins, fibrates, PCSK9 inhibitors) in humans. However, due to inflammation concerns, long-term toxicity studies of mannan in experimental animal models should precede any studies of mannans used as an adjuvant hypolipidaemic agent in humans. Additionally, it is suggested that the process of macrophage activation and the subsequent release of chitotriosidase should be more fully investigated in the context of hyperlipidaemia so as to clearly define the benefits of using chitotriosidase as a biomarker to stratify those patients most likely to develop future atherosclerosis. It would also appear worthwhile to investigate how different sizes and branching patterns associated with β-glucans and mannans (serotype A and serotype B) affect their antihypercholesterolaemic properties.

**References**


