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Cytotoxicity decreasing effect and antimycobacterial activity of chitosan conjugated with antituberculotic drugs

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ABSTRACT

Water-soluble chitosan conjugates were prepared by connection with isoniazid, pyrazinamide and ethionamide across the *O*-carboxymethyl and *N*-succinyl bridge followed by phosphorylation. Their structures were characterized by FTIR and ^{1}H NMR spectroscopy. Degree of drug substitution and molecular weight of prepared compounds have been investigated. Antimycobacterial activity was determined against *Mycobacterium tuberculosis* and three non-tuberculosis strains. Chitosan derivatives showed significant MIC 125 μg/mL against all tested strains which can be explained by contribution of the presence of antituberculotic drugs and original structure of chitosan. Cytotoxicity of prepared compounds was evaluated in human liver cell line Hep G2 and human peripheral blood mononuclear cells (PBMC). Toxicity of antituberculotic drugs on Hep G2 cells were compensated by connection with chitosan and tested compounds have not exhibited significant cytotoxic effect on PBMC cells. Chitosan conjugates with antituberculotic drugs could be potentially effective in the non-toxic chemotherapy of tuberculosis.

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1. Introduction

Chitosan is known as a biological active polymer having many interesting properties, low toxicity, biocompatibility, biodegradability and low cost (Uraganu & Tokata, 2006; Vinsova & Vavrikova, 2008). Chitosan is a linear polysaccharide, derived from naturally abundant chitin, composed from D-glucosamine (GluN) and *N*-acetyl-D-glucosamine (GluNAc) units bonded by β-1,4-glycosidic linkages. Degree of deacetylation of commercially prepared chitosan is usually in the range between 60 and 100% and has influence on the solubility, swelling index, wound dressing and antimicrobial properties (Qin, 2008). It is used as a carrier material in various drug delivery systems with a broad range of therapeutic application (Dodane & Vilivalam, 1998).

Chemical modifications of chitosan molecule can improve water solubility and connection with drugs. For introduction of carboxylic group there is very useful *O*-carboxymethylation or *N*-succinylation. Water solubility of *O*-carboxymethyl chitosan (OCMC) depends on reaction conditions of carboxymethylation, especially on reaction temperature and ratio of water/propan-2-ol as the reaction solvent (Chen & Park, 2003). *N*-Succinyl chitosan (NSCS) is well known as a drug carrier with a long circulating effect in the body (Kato, Onishi, & Machida, 2000). Main utilisation of *N*-succinyl chitosan is in cancer therapy. A conjugate of mitomycin C and *N*-succinyl chitosan exhibited good antitumor activities against various tumours (Kato, Onishi, & Machida, 2004; Song, Onishi, Machida, & Nagai, 1996).

Tuberculosis (TB) is leading infection disease and serious world health problem due to which 1.3 million people died in 2008 (Global tuberculosis control, 2009). Current anti-tuberculosis drugs isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA) are potential hepatotoxic drugs. They are metabolized and detoxified in the liver. Toxic metabolites develop drug-induced hepatotoxicity (DIH). Antituberculosis drug-induced hepatotoxicity can be fatal when is not recognized at an early stage, after which the therapy should be interrupted. Development of DIH depends on main risk factors as age, sex, ethnic, acetylator phenotype and HIV infection (Sharma, 2004). Incidence of drugs combination can have synergic hepatotoxic effect. It was proved that the *in vitro* hepatotoxicity of PZA is increased by pre-treatment of cells with INH (Tostmann et al., 2008). On the other hand, INH is able to increase activity of CYP2E1, the enzyme responsible for metabolism of xenobiotics to

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production of free radicals. Simultaneously, INH decreases activities of glutathione S-transferases (GSTs) function responsible for the protection of cytoplasm against free radicals, but RIF normalizes the production of ROS by CYP2E1 and GSTs. It indicates that RIF has antagonistic effect (Yue, Peng, Chen, Liu, & Dong, 2009). Co-administration of chitosan had a tendency to prevent antituberculardrug-induced hepatotoxicity in rats. The hepatoprotective effect of chitosan is probably due to a counteraction against free radicals by its antioxidant nature and/or to its ability to inhibit lipid accumulation due to its antilipidemic properties (Santhosh, Sini, Anandan, & Mathew, 2007).

Mostly, treatment of TB is based on the cellular immunity and chemotherapy. Protective immune response involves a phagocytosis of *Mycobacterium tuberculosis* by macrophages which are activated by antigen-specific T cells (Cooper & Flynn, 1995). Chemotherapy kills the majority of bacteria during few days, but subpopulation in stationary phase could persist in aerobic or anaerobic sites (Rook, Seah, & Ustianowski, 2001). Therefore, administration of antituberculotic drugs must be continued for at least 6 months. During this time immune cell system should protect body of patient. It is essential to find out if used drugs are not toxic for cells which play important role in immune response. PBMC are a preparation of blood cells which contains macrophages, monocytes and lymphocytes as an experimental model, PBMC may be used for this purpose.

In the current investigation, we have bonded first or second line antituberculotics such as INH, PZA, ethionamide (ETA) on chitosan as a carrier through the short linkage by carboxymethyl group or succinyl-bridge with assumption of its hepatoprotective activity. The water solubility of prepared conjugates was increased by phosphorylation. Synthesis, characterization of derivatives, determination of antimycobacterial activity and evaluation of cytotoxicity are reported.

2. Experimental

2.1. Synthesis

Two main approaches were used. First type of reaction was based on the formation of *O*-carboxymethylated chitosan (OCMC) **1**. The first step was the preparation of an intermediate product *O*-carboxymethyl chitosan by the reaction of chitosan and chloroacetic acid in propan-2-ol (Fan et al., 2006). The intermediate was precipitated in acetone. The following reaction was based on the linkage of OCMC with antituberculotic drug. 500 mg of OCMC (**1**) was dissolved in water and 200 mg of appropriate drug (INH, PZA or ETA) was added. The mixture was cooled to 0–5 °C. The carboxylic group was activated with 220 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Scheme 1) and the reaction mixture was stirred at 0–5 °C for 3 h, then the temperature was gradually increased to the room temperature. After 24 h without further isolation 2 mL of methanesulfonic acid and 300 mg of phosphorus pentoxide was added to the mixture (Nishi, Nishimura, Ebina, Tsutsumi, & Tokura, 1984). The reaction mixture was stirred at 0–5 °C for 3 h. The mixture was cooled overnight in a freezer and then the product **3** was precipitated with acetone.

Succinylation of amino group of chitosan was the second approach to the synthesis. Chitosan reacted with succinyl anhydride in dimethyl sulfoxide (Yan et al., 2006) to produce *N*-succinyl chitosan **4** (Scheme 2). The intermediate was precipitated in acetone. The following reaction was based on the linkage of NSCS with antituberculotic drug. 500 mg of NSCS (**4**) was dissolved in water and 200 mg of appropriate drug (INH, PZA or ETA) was added. The mixture was cooled at 0–5 °C. The carboxylic group was activated with 220 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and the reaction mixture was stirred at 0–5 °C for 3 h, then the temperature gradually increased to the room temperature. After 24 h without isolation 2 mL of methanesulfonic acid and 300 mg of phosphorus pentoxide was added to the mixture. The reaction was stirred at 0–5 °C for 3 h. The mixture was cooled overnight in a freezer and then the product **6** was precipitated with acetone.

2.2. Characterization

The chemicals were obtained from Sigma–Aldrich Co. Elemental analyses (C, H, N) were performed with a CHNS-O CE elemental analyzer (Fisons EA 1110). Infrared spectra were recorded on Nicolet Impact 400 IR spectrometer in ATR. NMR spectra were measured in D_2O on a Bruker Avance 300 (300 MHz for ^1H).

2.3. Determination of degree of deacetylation

Degree of deacetylation (DD) of chitosan was found out by calculation from ratio C/N of prepared compounds according the formula $DD = 100 - \{[(C/N) - 5.145]/(6.861 - 5.145) \times 100\}$ (Kasaai, Charlet, Paquin, & Arul, 2003). Number 5.145 corresponds to completely *N*-deacetylated chitosan and 6.861 exhibits fully acetylated chitosan. Resulting degree of deacetylation for original chitosan is 20.04% which is in accordance with the information of provider.

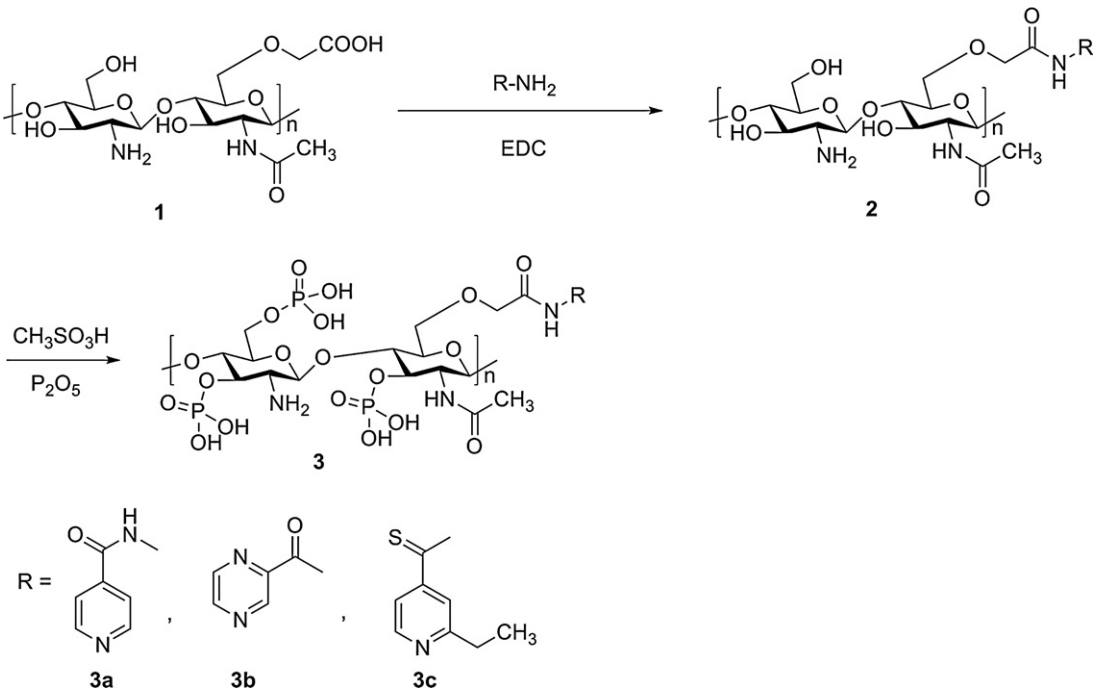
2.4. Determination of molecular weight

Molecular weight (MW) of products was calculated from viscosity measurement. As an aqueous solution system was chosen 0.3 M acetic acid/0.2 M sodium acetate at 25 °C. Stock solutions were prepared from chitosan and prepared compounds **1**, **3a**–**3c**, **4**, **6a**–**6c**. Ubbelohde viscosimeter with average of capillary 0.64 ± 0.02 mm was used for the measurement. MW was calculated from the Mark–Houwink equation formula, $[\eta] = K.MW^a$; $[\eta]$ is intrinsic viscosity, MW is molecular weight, *K* and *a* are viscometric constants depending on the degree of deacetylation of chitosan. Accordingly to Rinaudo, Milas, and Le Dung (1993), values for constants are $K = 0.074\ cm^3\ g^{-1}$ and $a = 0.76$. Original chitosan had MW 29,972 Da but the synthetic procedure resulted in the significant degradation of the polymer backbone. This effect has been previously reported (Holappa et al., 2005; Masson et al., 2008). Prepared compounds **1**, **3a**–**3c**, **4**, **6a**–**6c** exhibited half values of MW.

2.5. Determination of degree of substitution

Degree of drug substitution (DS) on prepared compounds **3a**–**3c**, **6a**–**6c** was determined by UV spectrophotometric technique on the base of calibration curve method. Calibration curve was expressed for each antituberculotic drug (INH, PZA, ETA). Degree of substitution was calculated as a percentage of drug from whole polymeric molecule of product. Calibration solutions of INH and PZA were dissolved in water, calibration solutions of ETA were dissolved in methanol. Samples of compounds **3a**–**3c**, **6a**–**6c** were dissolved in water.

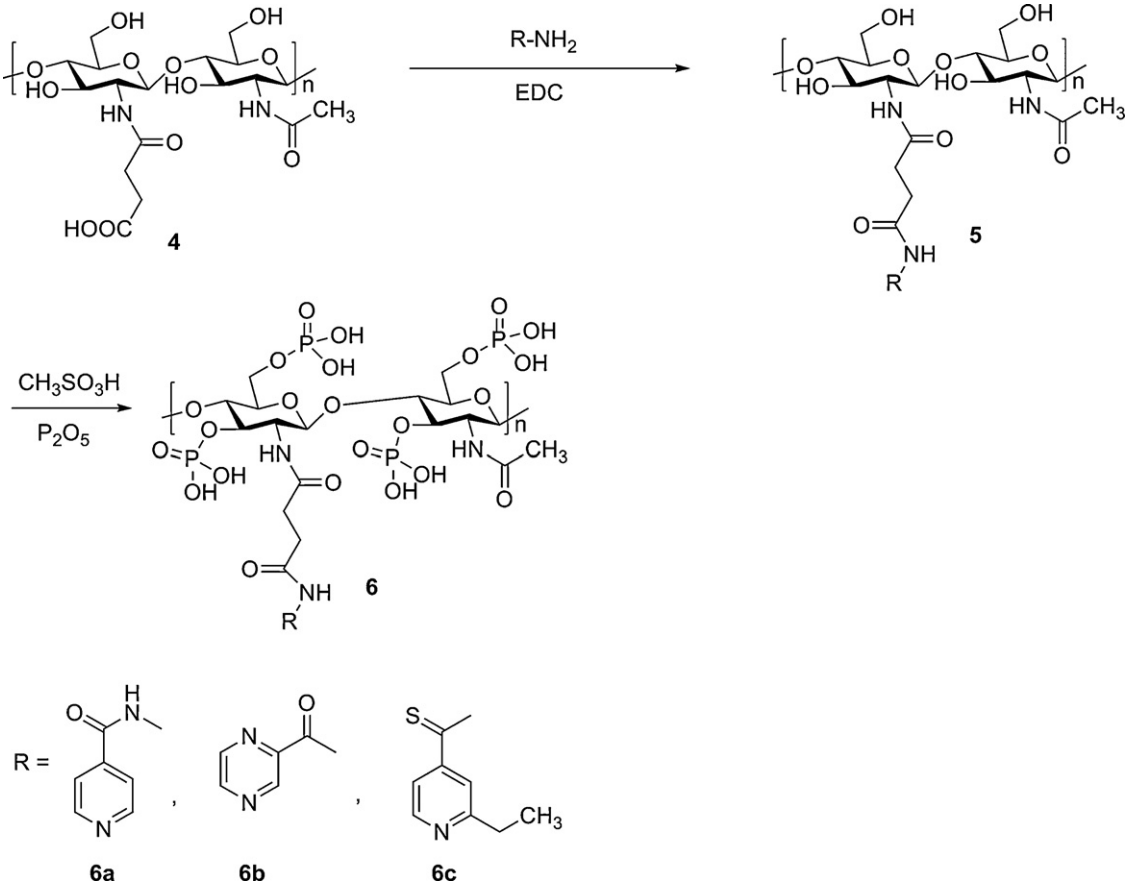
The highest values of substitution exhibited compounds **3b**, **6a** and **6b**. In general, percentage of the content of antituberculotic drugs was low (0.3–1.5%), which was confirmed as well by the reverse procedure of synthesis. Thus the suspicion of possible amide bond degradation during phosphorylation was disproved. The phosphorylation was made as the first step and the linkage of antituberculotic drugs was the following reaction step. The degree of substitution was also in the range of 0.3–1.5%.



Scheme 1. Conjugation of OCMC and antituberculotic drugs.

2.6. Characterization of prepared compounds

Chitosan and its derivatives were confirmed by FT-IR ATR and ^{1}H NMR spectroscopy. Infrared spectra of chitosan and carboxymethyl derivatives **1**, **3a**–**3c** are compared in Fig. 1 and infrared spectra of chitosan and succinylated derivatives **4**, **6a**–**6c** are compared in Fig. 2.



Scheme 2. Conjugation of NSCS and antituberculotic drugs.

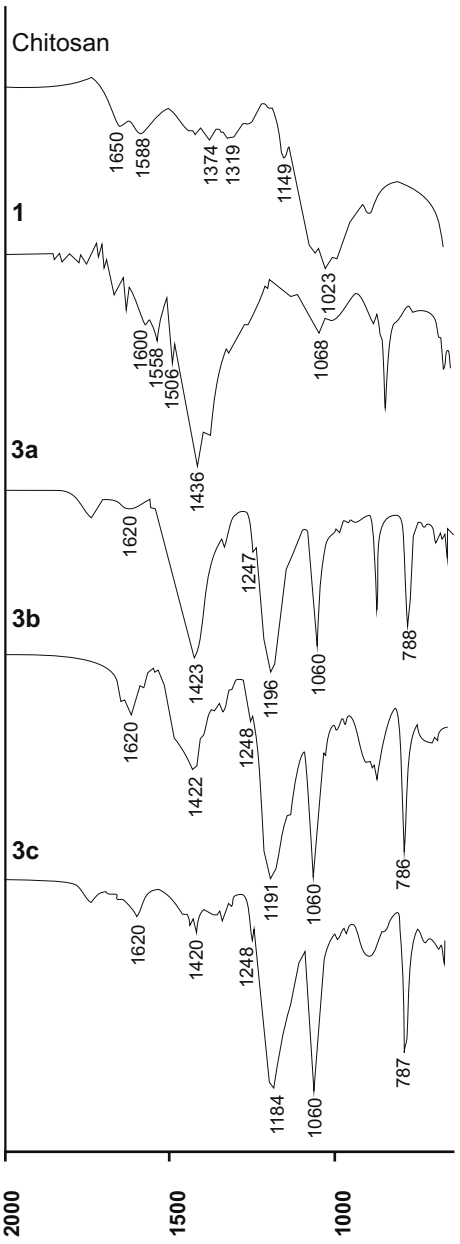


Fig. 1. FTIR spectra of chitosan, **1** and **3a**–**3c**.

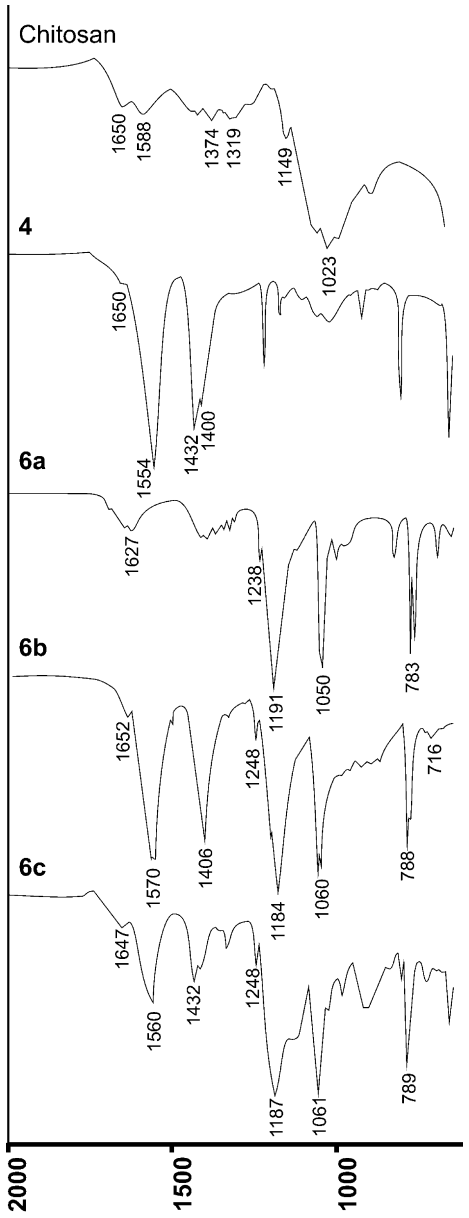


Fig. 2. FTIR spectra of chitosan, **4** and **6a**–**6c**.

OCMC (**1**): IR (ATR) 1600 ($\nu(NH_2)$), 1558 (ν_{as}(O=C–O)), 1506 ($\nu(NH_2)$), 1436 (ν_s(O=C–O)), 1068 (ν(O–H)) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 4.93 (s, 1H, H1 (GluN)), 4.67 (s, 1H, H1 (GluNAc)), 3.93 (s, 1H, CH_2), 3.73–3.31 (4H, H3, H4, H5, H6), 2.69 (s, 1H, H2 (GluNAc)), 2.22 (s, 1H, H2 (GluN)), 1.90 (m, 3H, CH_3). Anal. Calcd: C, 46.65; H, 6.58; N, 5.79. Found: C, 45.82; H, 7.86; N, 2.21. MW 15,126 Da.

OCMC connected with INH (**3a**): IR (ATR) 1683–1621 (ν(C=O) amid I), 1423 (ν_s(O=C–O)), 1247 (ν(P=O)), 1196 (ν(C–N) sec. amine), 1061 (ν(C–O–C)) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 8.60 (m, 1H, NH), 7.81 (d, J = 6.14 Hz, 2H, arom. H), 7.73 (d, J = 6.10 Hz, 2H, arom. H), 4.90 (s, 1H, H1 (GluN)), 4.67 (s, 1H, H1 (GluNAc)), 3.93 (s, 1H, CH_2), 3.72–3.47 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.22 (s, 1H, H2 (GluN)), 1.90 (m, 3H, CH_3). Anal. Calcd: C, 47.89; H, 6.37; N, 8.43. Found: C, 39.07; H, 6.69; N, 7.08. MW 14,394 Da; DS 0.89%.

OCMC connected with PZA (**3b**): IR (ATR) 1665–1620 (ν(C=O) amid I), 1422 (ν_s(O=C–O)), 1248 (ν(P=O)), 1191 (ν(C–N) sec. amine), 1060 (ν(C–O–C)), 786 (δ(C–H) arom. ring) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 9.10 (s, 1H, NH), 8.68 (m, 1H, arom. H), 8.31 (m, 1H, arom. H), 7.73 (s, 1H, arom. H), 4.89 (s, 1H, H1 (GluN)), 4.69 (s, 1H, H1 (GluNAc)), 3.90 (s, 1H, CH_2), 3.76–3.46 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.22 (s, 1H, H2 (GluN)), 1.91 (m, 3H, CH_3). Anal. Calcd: C, 47.49; H, 6.28; N, 8.52. Found: C, 39.17; H, 6.49; N, 6.80. MW 12,600 Da; DS 1.32%.

OCMC connected with ETA (**3c**): IR (ATR) 1650–1620 (ν(C=O) amid I), 1420 (ν_{as}(O=C–O)), 1375 ($\delta_s(CH_3)$), 1248 (ν(P=O)), 1184 (ν(C–N) sec. amine), 1060 (ν(C–O–C)), 787 (δ(C–H) arom. ring) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 9.33 (s, 1H, NH), 8.46 (m, 1H, arom. H), 8.34 (s, 1H, arom. H), 7.63 (m, 1H, arom. H), 4.82 (s, 1H, H1 (GluN)), 4.73 (s, 1H, H1 (GluNAc)), 3.93 (s, 1H, CH_2), 3.74–3.49 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.22 (s, 1H, H2 (GluN)), 2.10 (m, 2H, CH_2), 2.01 (m, 3H, CH_3), 1.91 (m, 3H, CH_3). Anal. Calcd: C, 48.63; H, 6.46; N, 7.22. Found: C, 37.48; H, 6.20; N, 6.51. MW 12,632 Da; DS 0.85%.

NSCS (**4**): IR (ATR) 1650 (ν(C=O) amid I), 1554 (δ(N–H) sec. amine), 1432 (δ(C–H) CH_2), 1400 (ν(C=O) amid III) cm^{-1}. ^1H NMR

(D_2O-d_6, 300 MHz): δ 4.74 (s, 1H, H1 (GluN)), 4.53 (s, 1H, H1 (GluNAc)), 3.64–3.30 (4H, H3, H4, H5, H6), 2.54 (s, 2H, CH_2), 2.22 (s, 1H, H2 (GluN)), 2.04 (m, 3H, CH_3). Anal. Calcd: C, 47.40; H, 6.32; N, 5.42. Found: C, 33.61; H, 5.01; N, 2.78. MW 17,163 Da.

NSCS connected with INH (**6a**): IR (ATR) 1627 (ν(C=O) amid I), 1238 (ν(P=O)), 1191 (ν(C–N) sec. amine), 1050 (ν(C–O–C)), 783 (δ(C–H) arom. ring) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 8.60 (m, 1H, NH), 7.80 (d, J = 6.18 Hz, 2H, arom. H), 7.73 (d, J = 6.20 Hz, 2H, arom. H), 4.91 (s, 1H, H1 (GluN)), 4.71 (s, 1H, H1 (GluNAc)), 3.80–3.48 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.59 (s, 2H, CH_2), 2.21 (s, 1H, H2 (GluN)), 1.91 (m, 3H, CH_3). Anal. Calcd: C, 48.51; H, 6.14; N, 7.94. Found: C, 42.55; H, 6.03; N, 9.79. MW 13,255 Da; DS 1.50%.

NSCS connected with PZA (**6b**): IR (ATR) 1652 (ν(C=O) amid I), 1570–1560 (δ(N–H) sec. amine), 1406 (ν_{as}(O=C–O)), 1060 (ν(O–H)), 1050 (ν(C–O–C)) cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 9.32 (s, 1H, NH), 8.68 (m, 1H, arom. H), 8.34 (m, 1H, arom. H), 7.69 (s, 1H, arom. H), 4.93 (s, 1H, H1 (GluN)), 4.71 (s, 1H, H1 (GluNAc)), 3.81–3.48 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.43 (s, 2H, CH_2), 2.22 (s, 1H, H2 (GluN)), 1.90 (m, 3H, CH_3). Anal. Calcd: C, 48.14; H, 6.06; N, 8.02. Found: C, 43.05; H, 6.19; N, 7.72. MW 11,789 Da; DS 1.48%.

NSCS connected with ETA (**6c**): IR (ATR) 1647 (ν(C=O) amid I), 1560 (δ(N–H) sec. amine), 1432 (δ(C–H) CH_2), 1248 (ν(P=O)), 1187 (ν(C–N) sec. amine), 1061 (ν(C–O–C)), 789 (δ(C–H) arom. ring), cm^{-1}. ^1H NMR (D_2O-d_6, 300 MHz): δ 8.45 (m, 1H, arom. H), 8.27 (s, 1H, arom. H), 4.93 (s, 1H, H1 (GluN)), 4.70 (s, 1H, H1 (GluNAc)), 3.90–3.54 (4H, H3, H4, H5, H6), 2.80 (s, 1H, H2 (GluNAc)), 2.39 (s, 2H, CH_2), 2.22 (s, 1H, H2 (GluN)), 1.90 (m, 3H, CH_3). Anal. Calcd: C, 49.20; H, 6.23; N, 6.81. Found: C, 39.84; H, 5.50; N, 4.78. MW 13,139 Da; DS 0.31%.

2.7. Antimycobacterial activity

The prepared chitosan derivatives **1**, **3a**–**3c**, **4**, **6a**–**6c** and their standards INH, PZA, ETA were tested *in vitro* for antimycobacterial activity in the Laboratory for TBC, Health Institute in Ostrava, against *Mycobacterium tuberculosis* 331/88 and some non-TB strains such as *Mycobacterium avium* (330/88) and *Mycobacterium kansasii* (235/80 and 6509/96). Antimycobacterial activity was measured in Sula's semisynthetic medium (SEVAC, Prague) at 37 °C. The compounds **1**, **3a**–**3c**, **4**, **6a**–**6c** were dissolved in the same medium. INH, PZA and ETA were dissolved in DMSO. The following concentrations were used: 500, 250, 125, 64, 32, 16, 8, 4, 2 and 1 μg/mL. MICs values were determined after incubation at 37 °C for 14 and 21 days, for *M. kansasii* for 7, 14, and 21 days. MIC was the lowest concentration of a substance, at which the inhibition of the growth of mycobacterium occurred.

2.8. In vitro cytotoxicity

2.8.1. In vitro cytotoxicity in PBMC

Human PBMC (Jurcevic et al., 1996) were cultured in RPMI-1640 medium without phenol red supplemented with 10% FCS, 2 mM of L-glutamine and 160 μg/mL of gentamycin. Cell cultures were maintained at 37 °C, 5% CO_2 in water-saturated atmosphere.

Cells were plated into 96-well plate with initial cell number of 5×10^3 per well (PBMC 1.5–2.0 $\times$ 10^5 cells/well). After 24 h incubation at 37 °C prior to the experiment, cells were treated with compounds **1**, **3a**, **3b**, **6a**, **6b** and INH in 100 μL serum free medium overnight. A concentration range between 1.67 and 9.35 mg/mL was used for incubation of the tested compounds. Control cells were treated with serum free medium. Four parallel measurements were performed in all cases.

After overnight incubation at 37 °C, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay (Mosmann, 1983; Slater, Sawyer, & Sträuli, 1963). 45 μL of MTT-solution (2 mg/mL) was added to each well. The respiratory chain (Slater et al., 1963) and other electron transport systems (Liu, Peterson, Kimura, & Schubert, 1997) reduce MTT and thereby form non-water-soluble violet formazan crystals within the cell (Altman, 1976). The amount of these crystals can be determined spectrophotometrically and serves as an estimate for the number of mitochondria and hence the number of living cells in the well (Denizot & Lang, 1986). After 4 h of incubation; cells were centrifuged for 5 min (2000 rpm) and supernatant was removed. The obtained formazan crystals were dissolved in 50 or 100 μL of DMSO and optical density (OD) of the samples was measured at λ = 540 and 620 nm using ELISA Reader (iEMS Reader, Labsystems, Finland). OD_{620} values were subtracted from OD_{540} values. The percent of cytotoxicity was calculated using the following equation: cytotoxicity (%) = $[1 - (OD_{treated}/OD_{control})] \times 100$; where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of the treated and the control cells, respectively. In each case two independent experiments were carried out with 4–8 parallel measurements. The 50% inhibitory concentration (IC_{50}) values were determined from the dose–response curves. The curves were defined using MicrocalTM Origin1 (version 6.0) software.

2.8.2. In vitro cytotoxicity in HepG2 cells

The synthesised compounds **1**, **3a**–**3c**, **4**, **6a**–**6c** and standards INH, PZA, ETA were tested on cytotoxicity in human liver cell line Hep G2 (passage 26–28; ECACC, UK). A standard colorimetric method measuring a tetrazolium salt reduction (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA) was used for evaluation. 10,000 cells per well were incubated at 37 °C for 3 h in 5% CO_2 atmosphere. The chitosan derivatives were dissolved in the cell medium without fetal bovine serum. INH, PZA, ETA were dissolved in DMSO. Each compound was tested using six increasing concentrations. A concentration range between 0.5 and 3.0 mg/mL was used for incubation of the tested compounds. The treated cells were incubated together with controls at 37 °C for 24 h in 5% CO_2 atmosphere. Than solution of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) was added according to recommendation of the producer of the kit. The tested plate was incubated for 2 h at 37 °C and 5% CO_2. The quantity of formazan formed in metabolically active cells was measured by the absorbance at 490 nm using a 96-well plate reader. Measured results were statistically evaluated in programs Microsoft Excel 2007 and GraphPad Prism 5.02. The standard cytotoxic parameter IC_{50} was determined in each tested compound.

3. Results and discussion

3.1. Antimycobacterial activity

To the best of our knowledge no studies aimed to antimycobacterial activity of chitosan or chitosan derivatives against complex *M. tuberculosis* have not been published yet. The anti-TB screening results of the compounds **1**, **3a**–**3c**, **4**, **6a**–**6c** and standards are summarized in Table 1. Compounds **3a**–**3c** and **6a**–**6c** have the same MIC of 125 μg/mL. Even if, **1** is without substitution of antituberculotic drug, its MIC is lower – 62.5 μg/mL (*M. tuberculosis*) and 31.25 and 62.5 μg/mL (*M. avium*). *N*-Succinyl chitosan **4** has considerably lower activity (500 μg/mL) against these strains. **4** Exhibited good activity against *M. kansassi* after 7 days (62.5 μg/mL). In general, it seems that there are two factors influencing the activity of compounds. Firstly, presence of first or second line antituberculotic drugs (INH, PZA, ETA) which is important for the inhibition of mycobacteria. Although, the degree of substitution is not high,

Table 1
Values of antimycobacterial activity and *in vitro* cytotoxicity.

	MIC [μg/mL]										[mg/mL]	
	M. tbc 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			HepG2 IC_{50}	PBMC IC_{50}
	14 days	21 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days		
1	62.5	62.5	31.25	62.5	125	125	125	125	125	125	2.83	>1.67
3a	125	125	125	125	125	125	125	125	125	125	2.13	>1.78
3b	125	125	125	125	125	125	125	125	125	125	>3	>9.35
3c	125	125	125	125	125	125	125	125	125	125	2.32	NT
4	>500	>500	500	>500	62.5	500	>500	250	>500	>500	>3	NT
6a	125	125	125	125	125	125	125	125	125	125	>3	>3.54
6b	125	125	125	125	125	125	125	125	125	125	2.72	>8.86
6c	125	125	125	125	125	125	125	125	125	125	2.96	NT
INH	0.14	0.14	>34	>34	>34	>34	>34	0.27	0.55	0.55	1.04	~0.64
PZA	>123	>123	61	>123	61	>123	>123	15.4	123	123	0.44	NT
ETA	0.66	1.33	10.4	20.8	1.33	2.66	5.32	0.66	2.66	5.32	3.25	NT

NT = not tested.

products **3a**–**3c** and **6a**–**6c** have exhibited good antimycobacterial activity. The second factor is probably an antibacterial activity of original chitosan structure which corresponds with the inhibition values of **1** against *M. tuberculosis* and *M. avium* and probably contributes to mycobacterial growth inhibition. The explication of this activity could be high degree of deacetylation of chitosan. It means that the amino group as the active functional group (chelating divalent cations) was found to be essential for the antibacterial activity of chitosan. MIC values of **3a**–**3c** and **6a**–**6c** are equal for all tested strains, this implies that amount of free amino groups in chitosan derivatives should be the same. It means that the degree of substitution of antituberculotic drugs was very similar. It is in agreement with the low degree of substitution of linked drugs.

3.2. *In vitro cytotoxicity*

Results of the experiments on cytotoxicity are presented as inhibitory concentration which is necessary to decrease viability of the cell population to 50% from the maximal viability (IC_{50}). A comparison of the found cytotoxic concentrations and MIC values demonstrates that the prepared compounds in concentrations comparable to MICs exert very low toxicity for human hepatocytes and PBMC cells (Table 1).

3.2.1. *In vitro cytotoxicity in PBMC*

Current long duration treatment of TB is among others connected with immune cell system and its protective immune response. Using of antitubercular drugs which are toxic for lymphocytes can be an encumbrance. Therefore we evaluated cytotoxicity of the prepared chitosan derivatives in *in vitro* immune cellular model, PBMC. The obtained results document that all prepared compounds in examination range of concentrations have not exhibited obvious cytotoxic effect on PBMC cells. Although, maximal possible concentrations of chitosan derivatives **1**, **3a**, **3b**, **6a**, **6b** were used, IC_{50} obtained could not be calculated due to very low altering of cell viability. Low toxicity of the studied compounds also confirmed the found value of IC_{50} for INH in PBMC, 0.64 mg/mL, which was at least several times lower than that for the tested chitosan derivatives.

3.2.2. *In vitro cytotoxicity in HepG2 cells*

5–10% prevalence of DIH led us to the idea to prepare conjugates of antibacterial drugs with chitosan as a carrier that has supposed hepatoprotective activity. We have expected that the hepatotoxicity of products should be very low. The results on HepG2 toxicity were in accordance with our hypothesis. Since MW of all tested compounds are very similar, IC_{50} concentrations can be compared on mg/mL basis. IC_{50} values ranged from 2.13 to 2.96 mg/mL. In the cases of **4**, **3b** and **6a**, IC_{50} values were higher than 3 mg/mL. Toxicity curves did not reached values enabling calculation of IC_{50}. The found INH and PZA IC_{50} values in HepG2 cells were 1.04 and 0.44 mg/mL, respectively. Such values were significantly lower than those of the corresponding chitosan derivatives. Cytotoxicity of compounds **3a**, **3b**, **6a**, **6b** was influenced by the presence of the chitosan part in the molecules. In the case of ethionamide derivative, IC_{50} 3.25 mg/mL for ETA alone was comparable to the parameters of correspond derivatives **3c** and **6c**.

4. Conclusions

Our main goal was to prepare a new carrier for antimycobacterial active drugs having lower hepatotoxic potential than original antituberculotics which could be used as a water-soluble antimycobacterial prodrug with a preventive action against antitubercular drug-induced hepatotoxicity. Thus we have synthesised chitosan derivatives with carboxymethylated and succinylated linkage used for the conjugation of some anti-tuberculosis drugs. For the first time, chitosan and its derivatives were evaluated as potential antimycobacterial agents. The performed tests have demonstrated powerful inhibitory effect against one TB strain and three non-TB strains. It is interesting that the lowest MIC has shown *O*-carboxymethylated chitosan (compound **1**). Toxicity studies showed that chitosan derivatives are toxic only in very high concentrations; both in case of hepatocytes and HepG2 toxicity was more pronounced and IC_{50} values were higher than 2.13 mg/mL. Chitosan and its derivatives may be a promising biomaterial which could be used for compensation of toxicity and for liver protection of hepatocytes during administration of antituberculotic drugs.

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