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Comparative evaluation of cytotoxicity of a glucosamine-TBA conjugate and a chitosan-TBA conjugate

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Abstract

D-Glucosamine and chitosan were modified by the immobilization of thiol groups utilizing 2-iminothiolane. The toxicity profile of the resulting D-glucosamine-TBA (4-thiobutylamidine) conjugate, of chitosan-TBA conjugate and of the corresponding unmodified controls was evaluated in vitro. On the one hand, the cell membrane damaging effect of 0.025% solutions of the test compounds was investigated via red blood cell lysis test. On the other hand, the cytotoxity of 0.025, 0.25 and 0.5% solutions of the test compounds was evaluated on L-929 mouse fibroblast cells utilizing two different bioassays: the MTT assay (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide), which asses the mitochondrial metabolic activity of the cells, and the BrdU-based enzyme-linked immunosorbent assay, which measures the incorporation in the DNA of 5-bromo-2′-deoxyuridine and consequently the cell proliferation.

Results of the red blood cell lysis test showed that both thiolated compounds displayed a lower membrane damaging effect causing a significantly lower haemoglobine release than the unmodified compounds. Data obtained by the MTT assay and the BrdU assay revealed a concentration dependent relative cytotoxicity for all tested compounds. The covalent linkage of the TBA-substructure to D-glucosamine did not cause a significant increase in cytotoxicity, whereas at higher concentrations a slightly enhanced cytotoxic effect was caused by the derivatisation of chitosan.

In conclusion, the –TBA derivatives show a comparable toxicity profile to the corresponding unmodified compounds, which should not compromise their future use as save pharmaceutical excipients.

Keywords: Glucosamine; Chitosan; Thiomers; Red blood cells lysis; Cytotoxicity; L-929 cells

1. Introduction

Interest in mucoadhesive polymers is based on their potential utilization as vehicles in site-specific non-invasive drug delivery. Since mucoadhesion can prolong the residence time of formulations at the

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absorption sites, an improved absorption of the active agent can be expected (Takeuchi et al., 2001). However, as progress in pharmaceutical sciences has led to more and more sophisticated drug delivery systems, features of natural produced polymers became in many cases insufficient. A promising strategy to obtain novel polymers capable of fulfilling the new demands is represented by the chemical modification of natural produced polymers (Bernkop-Schnürch, 2000). In particular the deacetylation of chitin, leading to the poly(ß-1α-4-D-glucosamine) or so-called chitosan, has gained considerable attention. Chitosan which has displayed a very safe toxicity profile (Rao and Sharma, 1997) is widely used as pharmaceutical excipient (Illum, 1998) and further chemical modification are easy feasible due to the primary amino groups of each polymer-subunit (Kotze et al., 1997). Recently a novel chitosan derivative has been obtained by the introduction of a 4-thiobutylamidine (TBA) substructure leading to a chitosan-TBA conjugate. This conjugate is a more significant exponent of the thiolated polymers or so-called thiomer family. Alike many other thiomers, also in the case of the chitosan-TBA conjugate the covalent immobilization of sulfhydryl groups on the polymer backbone leads to strongly enhanced mucoadhesive (Roldo et al., 2004) as well as permeation enhancing features (Bernkop-Schnürch et al., 2003). The conjunction of these properties renders this chitosan derivative a promising excipient in particular for the non-invasive administration of peptide drugs. Recently, a proof of efficacy has been given for the chitosan-TBA conjugate. In vivo studies showed that the use of a chitosan-TBA carrier matrix provides a significantly enhanced oral bioavailability of an incorporated model peptide drug (Guggi et al., 2003a,b). However, the practical relevance of this thiolated polymer depends not only on its capability to guarantee a strong in vivo efficacy, but also on its biocompatibility. The in vitro evaluation of the cytotoxicity of novel compounds on immortalized cell lines is generally recognized as an essential step for the assessment of their biocompatibility (Ignatius and Claes, 1996).

Table 1
Amount of thiol/disulfide groups per gram thiolated compound

	Average MW (Da)	μmol –SH/g	μmol –S–S–/g
Glucosamine-TBA conjugate	280	666	1452
Chitosan-TBA conjugate	150000	292	37

Indicated values for thiol groups are means of at least four determinations.

It was therefore the aim of this study to investigate the influence of the covalent linkage of TBA-substructures to different D-glucosamine-derivatives on their cytotoxicity. Accordingly, a D-glucosamine-TBA conjugate (MW = 280 Da) (see Fig. 1) and a chitosan-TBA conjugate (MW = 150 kDa) were synthesized. The red blood cells lysis caused by the obtained conjugates (see Table 1) and by corresponding controls was evaluated. Furthermore, the concentration dependent cytotoxicity of these substances was investigated on L-929 mouse fibroblast cells, since this cell line is largely recommended for the testing of polymeric components (USP XXVI, 2003). Two assays basing on different principles were performed on cells in order to measure different types of cell damage: the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay (Sgouras and Duncan, 1990) and the BrdU-based enzyme-linked immunosorbent assay (Ignatius and Claes, 1996).

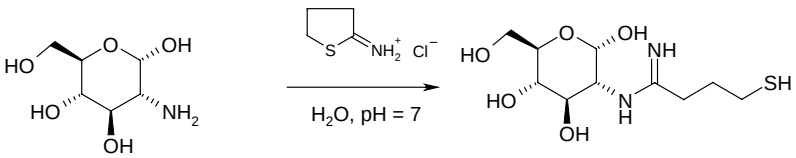

Fig. 1. Synthesis scheme for generation of glucosamine-TBA conjugate. Covalent attachment of 2-iminothiolane was achieved by the constitution of an amidine bond between the carboxylic C-atom of this reagent and the free amino group of the glucosamine.

2. Materials and methods

2.1. Synthesis and purification of glucosamine-TBA conjugate

Glucosamine-TBA conjugate was obtained by following procedure: 530 mg (2.5 mmol) (+)-D-glucosamine·HCl (Sigma, St. Louis, MO) were dissolved in 3 ml of demineralized water. The pH was adjusted to 7 by addition of 1 M NaOH. Three hundred and forty milligrams (2.5 mmol) of 2-iminothiolane·HCl (Traut's reagent) were added in one portion and the pH was readjusted to 7 with 1 M NaOH. During the time of reaction 1 M NaOH was added to keep the pH constant at 7. The reaction was performed at room temperature under thin-layer chromatography (TLC) control (ethylacetate/methanol/light petroleum ether/triethylamine, 4 + 2 + 2 + 1) and was regarded complete after 6 h based on consumption of the starting material. The obtained mixture was frozen at −20 °C and lyophilized without further purification. TLC was performed on Merck Kieselgel KGF_{254} aluminum sheets and the substances were detected by short wave (254 nm) UV-light and by spraying with ninhydrin/*n*-butanol/glacial acetic acid reagent followed by heating. Mass spectra were recorded on a API QSTAR Pulsar Hybrid-System™ spectrometer with electrospray ionization. Unmodified glucosamine·HCl (Sigma, St. Louis, MO) without immobilized thiol functions was used as corresponding control for toxicity studies.

2.2. Synthesis and purification of the chitosan-TBA conjugate

First, 0.5 g of chitosan (150 kDa; deacetylation degree 80.2%; Fluka GmbH, Buchs, Switzerland) was dissolved in 50 ml of 1% acetic acid to obtain a 1% (m/v) solution. The pH was adjusted to 6.5 with 5 M NaOH and 200 mg of 2-iminothiolane·HCl were added. The reaction mixture was stirred for 24 h at room temperature. The resulting polymer conjugate was dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. Thereafter, the polymer was adjusted to pH 7 with 5 M NaOH, freeze-dried at −20 °C and 0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4 °C until further use (Roldo et al., 2004). Control chitosan without linked thiol functions was dissolved in 1% acetic acid and then dialyzed, adjusted to neutral pH, lyophilized and stored in the same way as the chitosan-TBA conjugate.

2.3. Determination of the degree of modification of the chitosan-TBA conjugate

The total amount of sulfhydryl groups fixed on the polymer is represented by the summation of reduced thiol groups and of oxidized thiol moieties available in form of disulfide bonds.

5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Sigma, St. Louis, MO) was used to quantify photometrically the amount of free sulfhydryl groups on modified chitosan as described previously (Hornof et al., 2003).

To determine the total amount of bound thiol functions 0.5 mg of the conjugate were hydrated in 1 ml of 0.05 M tris(hydroxymethyl)-aminomethane buffer pH 6.8. After a swelling process of 30 min 1 ml of a freshly prepared 4% solution of sodium-borohydride was added to the polymer suspension. The mixture was then incubated for 1 h in an oscillating water bath at 37 ± 0.5 °C. Thereafter 200 μl of 5 M HCl were added and the reaction mixture was agitated for 10 min in order to destroy the remaining sodium-borohydride. The solution was neutralized by the addition of 1 ml 1 M phosphate buffer pH 8.5 and then immediately 100 μl of 0.4% (m/v) Ellman's reagent dissolved in 0.5 M phosphate buffer pH 8.0 were added. After incubation for 15 min at room temperature aliquots of 200 μl were transferred to a 96-well microtitration plate and the absorbance was measured at 450 nm with a microtitration-plate reader (Anthos Reader 2001, Salzburg, Austria). The quantity of bound iminothiolane was calculated using a standard curve obtained by the sulfhydryl group determination of a series of solutions containing increasing concentrations of cysteine hydrochloride (Sigma–Aldrich, Steinheim, Germany). The amount of disulfide bonds was calculated by subtracting the quantity of free thiol groups from the totality of thiol moieties present on the polymer.

2.4. Evaluation of red blood cells lysis

Blood was obtained from male wistar rats after sacrificing by cervical dislocation. Erythrocytes were

collected by centrifugation (2000 × *g*, 5 min, 4 °C, Hermle Z 323K) of the blood four times in a washing solution (175 g sorbitol + 8 g NaCl in 1 l water). The final pellet was resuspended in the washing solution to give a 2% (w/v) solution. 100 μl of the obtained erythrocyte solution were then transferred to each well of a 96-well microtitration plate. To the wells 100 μl of a 0.25 mg/ml solution of the test substances as listed in Table 1 or of corresponding controls dissolved in the washing solution were added and incubated for 1 h. The microtitration plate was then centrifuged at 1000 × *g* for 10 min and the supernatants (100 μl) transferred into another microtitration plate. Haemoglobine release was determined photometrically with a microtitration-plate reader (Anthos Reader 2001, Salzburg, Austria) at 570 nm. A 0.25 mg/ml solution of dextran 500 was used as negative control, whereas the detergent Triton X-100 (1%; v/v) was used as positive control causing 100% haemoglobine release. Results were expressed as the amount of haemoglobine release induced by the test substances in percentage of the total amount.

2.5. Cytotoxicity tests with cultured L-929 cells

L-929 mouse fibroblast cells were used to evaluate cytotoxicity. The cell line was cultured in RPMI 1640 (Sigma, St Louis, MO) supplemented by 10% fetal calf serum, L-glutamine (0.08 mM/l) and gentamycine (75 mM/l). Cells were subcultured by trypsin treatment and maintained in a humidified atmosphere (5% CO_2/95% O_2). For studies the cells were seeded in flat-bottomed Cellstar® 96-well culture plates (Greiner Bio-One, Solingen, Germany). The cytotoxicity of the tested substances, listed in Table 1, was evaluated using 0.025, 0.25 and 0.5% solutions of the test substances and of the corresponding controls in RPMI medium adjusted at pH 6.5. This pH was chosen in order to guarantee a better solubility of the polymeric components.

2.5.1. MTT test

Cell viability studies in the presence of test substances were performed via MTT-test. Cells were resuspended in culture medium at a density of 3.3×10^4 cells/ml and plated (200 μl per well) into culture plates at 6.6×10^3 cells per well. The plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2. Thereafter, the medium was replaced by 200 μl of the test or control solutions using culture medium by itself as control. After 72 h incubation solutions were removed and each well was treated with 50 μl of methylthiazololetetrazolium (1 mg MTT/ml in medium 199 without phenol red, Sigma, St. Louis, MO) and incubated for further 4 h at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2. Then, MTT solution was replaced by 100 μl per well of isopropanol in order to dissolve the formazane crystals. The plate was placed in a humidified atmosphere of 5% CO_2/95% O_2 at 37 °C for 10 min and thereafter for 15 min at 6 °C before absorbance measurement. The optical density was read on a microplate reader (Anthos Reader 2001, Salzburg, Austria) at 570 nm.

2.5.2. BrdU test

A commercially available BrdU-based enzyme-linked immunosorbent assay was used (Boehringer, Mannheim, Germany). L-929 cells (2.5×10^3 cells/ml) were plated into 96-well plates and incubated for 24 h. Growth medium was then replaced by 200 μl of the test or control solutions. Cells were exposed to the solutions for 72 h. Thereafter, the assay was performed following the manufacturer's protocol. Colorimetric staining of the microtitre plates was evaluated on a multiwell plate reader (Anthos Reader 2001, Salzburg, Austria) at 405 nm with a reference wavelength of 492 nm.

2.6. Statistical data analysis

Statistical data analysis was performed using the student *t*-test with $P < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat Version 5.0 (b8.3).

3. Results and discussion

3.1. Characterization of the glucosamine-TBA conjugate

In this study the D-glucosamine-TBA conjugate was firstly synthesized in order to obtain a single unit –TBA derivative of low molecular weight, providing information about the influence of the covalent linkage of –TBA-substructures to D-glucosamine derivatives

on cytotoxicity. By using this monomer likely disturbing interactions and phenomena due to the polymeric properties of chitosan should be excluded.

The synthesis scheme for the generation of glucosamine-TBA conjugate is depicted in Fig. 1. The lyophilized substance appeared as a white intensively smelling powder. It was easy soluble in water and methanol, insoluble in $CDCl_3$ and only poorly soluble in DMSO. ^1H and ^{13}C NMR analysis in DMSO displayed a high tendency of hydrolysis of the product (data not shown). The substance gave an intensive colorimetric reaction with Ellman's reagent, confirming the presence of high quantities of reduced thiol groups. The chemical structure of the glucosamine-TBA conjugate was confirmed by mass spectroscopy ($C_{10}H_{20}N_2O_5S + H^+$: $m/e = 281.11$, 100% relative intensity).

3.2. Characterization of the chitosan-TBA conjugate

The covalent attachment of 2-iminothiolane to the chitosan backbone occurs via an amidine bond formation between the carboxylic C-atom of this reagent and the primary amino group of the glucosamine subunit of the polymer. Therefore, the chemical structure of the glucosamine-TBA conjugate shown in Fig. 1. represents the presumptive substructure of the thiolated chitosans as well. The obtained polymer was slightly yellowish, odorless and showed a fibrous structure. A full characterization of the properties of the chitosan-TBA conjugate, including an evaluation of the disintegration behavior, the swelling behavior, the mucoadhesiveness and the permeation enhancing effect has already been performed (Bernkop-Schnürch et al., 2003; Roldo et al., 2004). The features of the polymer derivative described here were in good accordance with them. The amount of reduced thiol groups as well as the amount of thiol groups available in form of disulfide bonds per gram polymer for the glucosamine derivatives was quantified via Ellman's reagent. Results of this study are shown in Table 1.

3.3. Evaluation of red blood cells lysis

In this study haemolysis experiments were performed to investigate interactions of the cationic test compounds with the negatively charged red blood cell membrane. The membrane-damaging properties

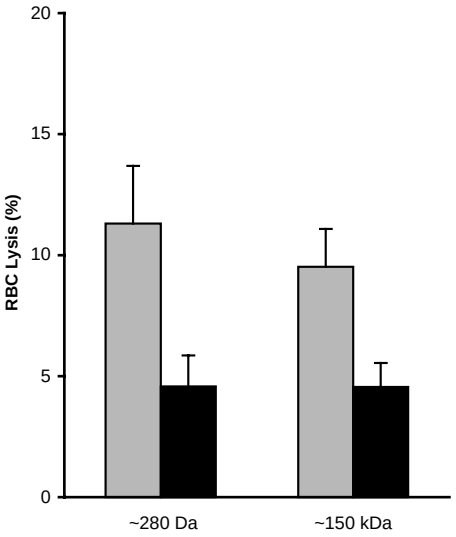

Fig. 2. Red blood cells (RBC) lysis in dependence on the molecular weight after incubation with 0.025% solutions of controls (glucosamine and chitosan) (gray bars) and of corresponding thiolated test substances (glucosamine-TBA and chitosan-TBA) (black bars). Indicated values are means (±S.D.) of at least five experiments.

of the test compounds were determined by the quantification of released haemoglobine. Results of this study are shown in Fig. 2. After 1 h of incubation, both test compounds bearing TBA-substructures displayed a lower membrane-damaging effect causing a significantly lower haemoglobine release than the unmodified compounds. Red blood cell lysis seemed not to be dependent on the molecular weight of the tested compound, as no significant difference could be detected between D-glucosamine-TBA conjugate and chitosan-TBA conjugate or between the two control compounds. The haemolytic effect of higher test compound concentrations (5 mg/ml) as well as the influence of a prolonged incubation time (24 h) were also investigated. Increasing the concentration of the test compounds as well as extending the incubation time to 24 h induced complete liberation of haemoglobine. However, interactions of the test compounds with released haemoglobine resulting in precipitation made an exact quantification of haemolysis in these high concentrations impossible (data not shown). These findings are in good agreement with previous studies asserting that compounds bearing primary amines have a significant toxic effect on red blood cells causing them to agglutinate (Dekie

et al., 2000). The erythrocyte membrane contains anionic glycoproteins able to bind the protonated amino group of glucosamine and of its derivatives. This process induces membrane curvature, leading to rupture and haemoglobine release (Carreno-Gomez and Duncan, 1997). Moreover, Ferruti et al. (1997) noted that compounds with secondary amino groups exhibit lower toxicity than those with primary amino residues, which might explain the lower haemolytic effect of the D-glucosamine-TBA conjugate in comparison to unmodified D-glucosamine. In fact, in the thiolated compound the primary amines are derivatized to secondary functionalities (see Fig. 1). However, because of its comparatively low degree of modification this explanation seems not to be applicable for the chitosan-TBA conjugate. In the case of the thiolated polymer the lower membrane-damaging effect in comparison to the corresponding unmodified polymer might be explained by the formation of intra- as well as inter- molecular disulfide bonds, thus leading to a higher rigidness of the molecule. Rigid molecules have more difficulties to attach to the cellular membrane than flexible molecules (Fischer et al., 2003; Singh et al., 1992).

3.4. Cytotoxicity tests

Two different techniques were used to measure changes in the viability of L-929 cells after incubation with solutions of the test compounds.

On the one hand, the MTT assay was performed, which is a quick effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. In recent years it has been frequently used as a preliminary screen for the evaluation of in vitro cytotoxicity of polymeric components (Sgouras and Duncan, 1990).

On the other hand, the BrdU-based enzyme-linked immunosorbent assay is used to investigate the ability of cells to proliferate (Gratzner, 1982).

Results of MTT-test (shown in Fig. 3A and B) revealed a concentration dependent decrease of mitochondrial activity for all the tested compounds. Results of BrdU assay correlated with the observations of the MTT assay: after treatment of the cells with the test compounds DNA synthesis was reduced significantly (see Fig. 4A and B). In test compounds concentrations as low as 0.025% a negligible cytotoxicity

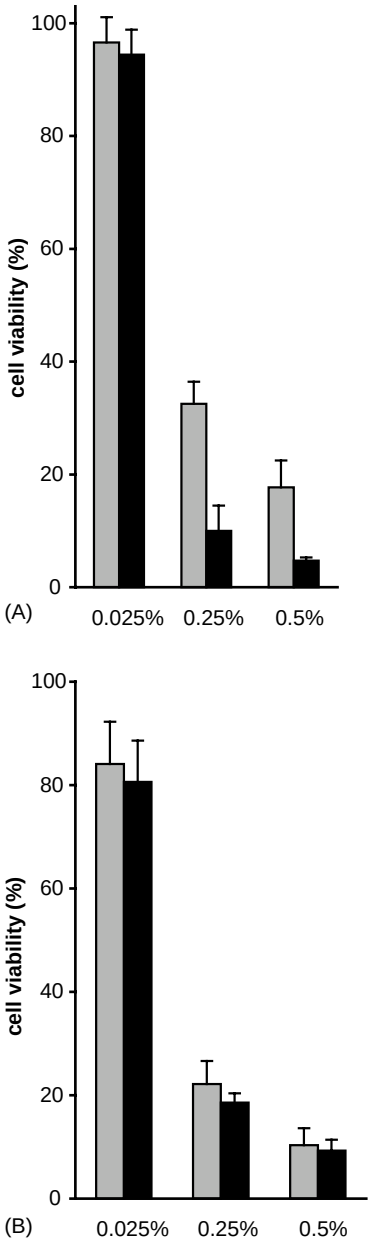

Fig. 3. Dose-dependent effects of test substances and controls on L-929 cells. Cytotoxicity of three different concentrations was quantified via MTT-assay. Each value represents the mean (±S.D.) of 4–6 determinations. (A) Cell viability after incubation with chitosan (gray bars) and with chitosan-TBA conjugate (black bars). (B) Cell viability after incubation with glucosamine (gray bars) and with glucosamine-TBA conjugate (black bars).

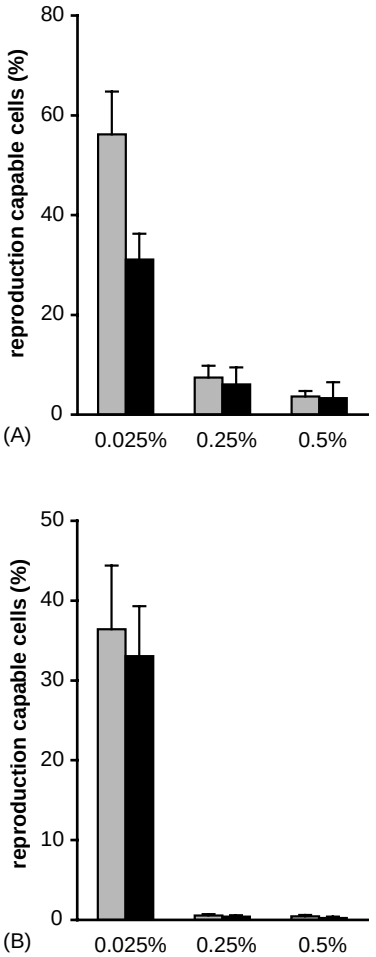

Fig. 4. Dose-dependent effects of test substances and controls on L-929 cells. The influence of three different concentrations on the reproduction capability of cells was determined via BrdU-assay. Each value represents the mean (±S.D.) of 3–5 determinations. (A) Cell proliferation after incubation with chitosan (gray bars) and with chitosan-TBA conjugate (black bars). (B) Cell proliferation after incubation with glucosamine (gray bars) and with glucosamine-TBA conjugate (black bars).

was observed, whereas already at a concentration of 0.25% a significant cytotoxicity occurred. According to the results a strongly concentration dependent direct proportional cytotoxic effect could be identified. An explanation for this effect might be given by various concentration dependent mechanism of cell resistance and/or sensitivity.

The covalent linkage of the TBA-substructure to D-glucosamine did not cause a significant increase in cytotoxicity (Figs. 3B and 4B), whereas at higher concentrations a significant enhanced cytotoxic effect was caused by the derivatisation of chitosan (Fig. 3A and B). These findings lead to the presumption that the chemical modification with 2-iminothiolane per se provokes only a slight dose-dependent effect on the viability of the cells. Similarly, Weber et al. (2000) found that the treatment of human serum albumine nanoparticles with 2-iminothiolane also resulting in a thiolated polymer shows no severe toxicity. The cross-linking of chitosan-TBA conjugate due to the formation of disulfide bonds during the 72 h of incubation and the consequent increase in molecular weight and viscosity, might explain its slightly more toxic effect in comparison to the control polymer.

Moreover, results of this study showed that unmodified chitosan displays per se some degree of cytotoxicity, thus being in accordance with the observations of Carreno-Gomez and Duncan (1997). The BrdU assay displayed a certain toxicity already at lower concentrations of the tested compounds than the MTT assay. Accordingly, the D-glucosamine derivatives tested seem to cause already in very low concentrations (0.025%) an inhibition of the cell proliferation, whereas only higher concentrations led to a decrease in metabolic activity.

In general, while red blood cell lysis test showed a lower toxicity of the –TBA conjugates, the cytotoxicity assays on cultured cells displayed a slightly lower toxicity profile of the unmodified compounds. Differences between the destructive effect of the test compounds on red blood cells and L-929 mouse fibroblasts might be due to the different composition of the membranes and glycocalyx of erythrocytes and fibroblasts (Fischer et al., 2003). Therefore, considering the data obtained on the one hand from the haemolysis test and on the other hand from cytotoxicity assays on L-929 cells, it can be assumed that the TBA derivatives have a comparable toxicity profile to the corresponding unmodified compounds.

Moreover, the in vivo deployment of the cationic chitosan as pharmaceutical excipient is, in spite of a certain in vitro toxicity, considered to be save and valuable (Illum, 1998; Takeuchi et al., 2001). Therefore, also the novel –TBA conjugates should display acceptable in vivo compatibility.

4. Conclusion

In the present study, the biocompatibility of a D-glucosamine-TBA conjugate, of a chitosan-TBA conjugate and of corresponding unmodified controls was evaluated qualitatively and quantitatively. On the one hand, red blood cells lysis studies showed a significantly lower membrane damaging effect of the –TBA derivatives in comparison to the corresponding controls. On the other hand, cytotoxicity assays on immortalized L-929 mouse fibroblasts displayed a slightly lower toxic effect of the unmodified compounds.

In conclusion, the novel conjugates display a low cytotoxicity profile comparable to that of the unmodified controls. Therefore, their future utilization as save carrier matrices for drug delivery systems in pharmaceutical technology does not seem to be compromised.

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References

Bernkop-Schnürch, A., 2000. Chitosan and its derivatives: potential excipients for peroral peptide delivery systems. Int. J. Pharm. 194, 1–13.

Bernkop-Schnürch, A., Guggi, D., Pinter, Y., 2003. Thiolated chitosans: development and in vivo evaluation of a mucoadhesive permeation enhancing oral drug delivery system. J. Contr. Rel. 94, 177–186.

Carreno-Gomez, B., Duncan, R., 1997. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. Int. J. Pharm. 148, 231–240.

Dekie, L., Toncheva, V., Dubruel, P., Schacht, E.H., Barrett, L., Seymour, L.W., 2000. Poly L-glutamic acid derivatives as vectors for gene therapy. J. Cont. Rel. 65, 187–202.

Ferruti, P., Knobloch, S., Ranucci, E., Gianasi, E., Duncan, R. 1997. A novel chemical modification of poly-L-lysine reducing toxicity while preserving cationic properties. In: Proc. Int. Symp. Control. Rel. Bioact. Mater.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Gratzner, H.G., 1982. Monoclonal antibody to 5-bromo and 5-iodo-deoxyuridine: a new reagent for detection of DNA replication. Science 218, 474–475.

Guggi, D., Kast, C.E., Bernkop-Schnürch, A., 2003a. In vivo evaluation of an oral salmon calcitonin-delivery system based on a thiolated chitosan carrier matrix. Pharm. Res. 20, 1989–1994.

Guggi, D., Krauland, A.H., Bernkop-Schnürch, A., 2003b. Systemic peptide delivery via the stomach: in vivo evaluation of an oral dosage form for salmon calcitonin. J. Contr. Rel. 92, 125–135.

Hornof, M.D., Kast, C.E., Bernkop-Schnürch, A., 2003. In vitro evaluation of the viscoelastic behavior of chitosan—thioglycolic acid conjugates. Eur. J. Pharm. Biopharm. 55, 185–190.

Ignatius, A.A., Claes, L.E., 1996. In vitro biocompatibility of bioresorbable polymers: poly(L, DL-lactide) and poly(L-lactide-co-glycolide). Biomaterials 17, 831–839.

Illum, L., 1998. Chitosan and its use as a pharmaceutical excipient. Pharm. Res. 15, 1326–1331.

Kotze, A.F., Luessen, H.L., De Leeuw, B.J., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997. *N*-Trimethyl chitosan chloride as a potential absorption enhancer across mucosal surfaces: in vitro evaluation in intestinal epithelial cells (Caco-2). Pharm. Res. 14, 1197–1202.

Rao, S.B., Sharma, C.P., 1997. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J. Biomed. Mater. Res. 34, 21–28.

Roldo, M., Hornof, M., Caliceti, P., Bernkop-Schnürch, A., 2004. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. Eur. J. Pharm. Biopharm. 57, 115–121.

Sgouras, D., Duncan, R., 1990. Methods for evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use. 1. Use of the tetrazolium-based colorimetric assay (MTT) as a preliminary screen for evaluation of in vitro cytotoxicity. J. Mater. Med. 1, 61–68.

Singh, A.K., Kasinath, B.S., Lewis, E.J., 1992. Interactions of polycations with cell-surface negative charges of epithelial cells. Biochim. Biophys. Acta 1120, 337–342.

Takeuchi, H., Yamamoto, H., Kawashima, Y., 2001. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Deliv. Rev. 47, 39–54.

Weber, C., Reiss, S., Langer, K., 2000. Preparation of surface modified protein nanoparticles by introduction of sulfhydryl groups. Int. J. Pharm. 211, 67–78.