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In vivo evaluation of an oral drug delivery system for peptides based on S-protected thiolated chitosan

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ABSTRACT

The aim of the present study was the development and evaluation in vitro as well as in vivo of an oral delivery system based on a novel type of thiolated chitosan, so-called S-protected thiolated chitosan, for the peptide drug antide. The sulfhydryl ligand thioglycolic acid (TGA) was covalently attached to chitosan (CS) in the first step of modification. In the second step, these thiol groups of thiolated chitosan were protected by disulfide bond formation with the thiolated aromatic residue 6-mercaptonicotinamide (6-MNA). Absorptive transport studies of antide were evaluated ex vivo using rat intestinal mucosa. Matrix tablets of each polymer sample were prepared and their effect on the absorption of antide evaluated in vivo in male Sprague–Dawley rats. In addition, tablets were examined in terms of their disintegration, swelling and drug release behavior. The resulting S-protected thiomer (TGA–MNA) exhibited 840 μmol of covalently linked 6-MNA per gram thiomer. Based on the implementation of this hydrophobic ligand on the thiolated backbone, the disintegration behavior was reduced greatly and a controlled release of the peptide could be achieved. Furthermore, permeation studies with TGA–MNA on rat intestine revealed a 4.5-fold enhanced absorptive transport of the peptide in comparison to antide in solution. Additional in vivo studies confirmed the potential of this novel conjugate. Oral administration of antide in solution led to only very small detectable quantities in plasma with an absolute and relative bioavailability (BA) of 0.003 and 0.03%, only. In contrast, with antide incorporated in TGA–MNA matrix tablets an absolute and relative BA of 1.4 and 10.9% could be reached, resulting in a 421-fold increased area under the plasma concentration time curve (AUC) compared to the antide solution. According to these results, S-protected thiolated chitosan as oral drug delivery system might be a valuable tool for improving the bioavailability of peptides.

1. Introduction

For the efficient delivery of peptides, proteins and other biopharmaceuticals via the gastrointestinal (GI) tract, novel concepts are needed to overcome significant enzymatic and diffusion barriers. Strategies to overcome these barriers include the use of enzyme inhibitors [1] and permeation enhancers [2]. Moreover, the use of multifunctional polymers seems to represent another promising strategy providing a protective effect for incorporated peptides towards enzymatic digestion [3]. Multifunctional polymers show excellent properties regarding mucoadhesion and permeation enhancement [4,5]. Owing to their close contact with the absorption membrane, a steep concentration gradient serving as the driving force for drug uptake via passive diffusion can be guaranteed [4]. In addition, multifunctional polymers are promising candidates within the group of enzyme inhibitors as they provide a protective effect towards secreted as well as membrane-bound enzymes [6,7]. Based on all these encouraging features, a presystemic degradation of peptides and proteins in the GI-tract between the dosage form and the absorption membrane can be strongly reduced as it is regarded as one of the main reasons for their limited bioavailability after oral administration. There is increasing evidence that the interaction between various types of mucoadhesive polymers and epithelial cells has direct influence on the permeability of mucosal epithelia. Within these multifunctional polymers, thiolated polymers — designed thiomers are of particular interest, as they exhibit high mucoadhesive and permeation enhancing features [8,9]. In contrary to this, thiomers display as well certain shortcomings such as they are subject of thiol oxidation at physiological pH unless sealed under inert conditions [10]. The design and development of thiomers being stable at this pH value would therefore be highly advantageous, opening the door for numerous additional applications. Within this study a thiolated chitosan, whose thiol groups are protected by a pyridyl substructure (6-mercaptonicotinamide) should be designed, offering the advantage

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of not being subject of oxidation at all. The theory behind this conjugate is based on covalent chromatography, where, for instance, peptides and proteins are linked to thiol bearing resins, when they are protected via pyridyl substructures [11,12]. The protection of thiol groups of the thiomer prevents an early oxidation before coming into contact with the mucosa, whereby more active thiol groups are available for a long time intimate contact with mucosal membranes, resulting in a prolonged residence time. Consequently, a higher drug concentration gradient can be offered at the absorption site, facilitating the transport and moreover enhancing the bioavailability of drugs, especially for peptides and proteins. First in vitro studies with an S-protected thiomer revealed promising results regarding mucoadhesive properties [13]. So far, however, their potential has not been shown in an animal model. Therefore, the aim of the present study was the development and evaluation of a drug carrier system based on S-protected thiolated chitosan as promising tool for non-invasive peptide drug delivery. The therapeutic peptide antide was chosen as model peptide drug. Antide is a decapeptide with sequence of Ac-(D-Nal)-(D-p-Cl-Phe)-(D-Pal)-Ser-Lys(nicotinoyl)-[D-Lys(nicotinoyl)]-Leu-Lys(isopropyl)-Pro-[D-Ala]-NH_2 and an antagonist of gonadotropin hormone-releasing hormone (GnRH), which has been studied for the treatment of various disorders including hormone dependent prostate and breast cancer, endometriosis and uterine fibrosis [14]. The development of an oral delivery system for this peptide would be highly beneficial as pain, infections and further side effects of injections can be avoided. On the contrary, when being orally administered with state-of-the-art delivery systems, the peptide does not at all reach the systemic circulation. Utilizing the thiomer-technology in previous studies with pigs, an absolute and relative BA of 1 and 3% was obtained, respectively [15]. Within this study comparatively higher values should be achieved by this novel type of thiolated chitosan, tested in matrix tablets as oral peptide delivery system. To our knowledge, so far, no other promising oral delivery system including this peptide was developed and examined within the last years. In order to evaluate the BA of antide in matrix tablets, all formulations were orally administered to rats and the drug concentration in plasma determined as a function of time. In addition, permeation studies of antide were evaluated ex vivo using rat intestinal mucosa and tablets were characterized regarding their swelling, disintegration and drug release behavior.

2. Materials and methods

2.1. Materials

Antide acetate (Mw: 1591.32 g/mol) was purchased from Anawa AG, Switzerland. Chitosan with an average Mw of 100 kDa and a deacetylation degree of 85% was obtained from Heppe Medical Chitosan GmbH. Dimethyl sulfoxide (DMSO), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent), sodium borohydride ($NaBH_4$), hydrogen peroxide (H_2O_2), thioglycolic acid, thiourea, 6-chloronicotinamide, glutathione in reduced form (GSH), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were obtained from Sigma Aldrich, Austria. All other chemicals were of analytical grade.

2.2. Synthesis and characterization of thiolated chitosan

Thiolated chitosan can be synthesized by derivatization of its primary amino groups with coupling reagents bearing thiol functions. The first modification in this study was achieved via the covalent attachment of thioglycolic acid (TGA) to chitosan as described previously [10].

The degree of modification was determined by quantifying the total amount of thiol groups, which is a composition of free and oxidized thiol groups in the form of disulfide bonds. The amount of free thiol groups was determined photometrically with Ellman's reagent. A calibration curve of TGA was used to calculate the amount of free thiol groups. Absorbance was measured at a wavelength of 450 nm with a microplate reader (FluoStar Galaxy, BMG Offenburg, Germany). To determine the degree of disulfide bonds, the reaction with Ellman's reagent was performed after reducing disulfides with $NaBH_4$. Measurement of absorbance and quantification of the total amount of thiol groups was performed as described above.

2.3. Synthesis and characterization of the S-protecting ligands

As 6-mercaptonicotinamide (6-MNA) is commercially not available, it was synthesized according a method developed by Forrest et al. [16]. In brief, 6-chloronicotinamide and thiourea were suspended in ethanol and refluxed for 6 h. Over time, the suspension turned light yellow. The reaction mixture was allowed to cool down to room temperature and stirred over night. The resulting salt *S*-(5-carbamyl-2-pyridyl)thiouronium chloride was then separated by filtration and brought to dryness. The dry thiouronium salt was decomposed through addition of alkaline solution and stirred for 45 min at room temperature. Acetic acid was added to adjust the pH to 4, whereby the light yellow suspension turned to a dark yellow color. The resulting 6-MNA was isolated by filtration, washed with water and brought to dryness.

In order to avoid any oxidation of 6-MNA itself, the dimeric reagent 6,6′-dithionicotinamide (6,6-DTNA) was synthesized. In brief, 6-MNA was suspended in water and pH was adjusted to 7 before adding a hydrogen peroxide solution. The mixture was incubated for 1 h under continuous stirring, whereby the yellow color of 6-MNA suspension turned to white induced by formation of the dimeric reagent. The resulting 6,6-DTNA was isolated by filtration, washed with water and brought to dryness. Both reagents exhibit solubility in DMSO only.

To control all further reactions, both aromatic reagents were analyzed by an UV-spectrometer (UV-mini1240, Shimadzu Co., Japan).

2.4. Synthesis and characterization of S-protected thiolated chitosan

The covalent attachment of the aromatic ligand to thiolated chitosan was achieved by disulfide bond formation between thiol groups of 6-MNA and free thiol groups of CS–TGA. Coupling of the thiomer with the S-protected ligand was implemented under various conditions with the dimeric reagent 6,6′-DTNA according a method used to reactivate covalent chromatography resins [11,12]. In brief, the lyophilized thiomer was dissolved in a mixture of DMSO and water. The dissolution of hydrophilic thiomer on the one hand and hydrophobic ligand on the other hand in the same medium was a major obstacle. Due to the insolubility and precipitation of 6-MNA in aqueous solutions, the thiomer had to be dissolved in a mixture of DMSO and water with a ratio of 7:3. The dimer was dissolved in DMSO and added to the thiomer solution in a molar ratio of 2:1. After addition of the ligand, pH was adjusted to 6. The reaction was stirred over 6 h and analyzed by an UV-spectrometer to control the coupling of 6-MNA. For purification, the conjugate solution was first dialyzed against mixtures of DMSO and water to eliminate all unbound aromatic ligand, followed by exhaustive dialysis against water until all DMSO was removed. Dialysis was controlled step by step using an UV-spectrometer concerning the elimination of 6-MNA as well as of DMSO. Subsequently, the conjugate was lyophilized and stored in a desiccator for further use. In order to determine the amount of conjugated aromatic ligand, samples of 0.1% (m/v) S-protected thiomer solution were analyzed by addition of reduced GSH (0.1%) for release of 6-MNA, which in turn can be quantified at 307 nm. Calculations were done by interpolation from a standard curve obtained with increasing amounts of 6-MNA. The amount of remaining free thiol groups and disulfide bonds fixed on the S-protected thiomer was determined photometrically with Ellman's reagent as described above.