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Investigation Into Efficiency of a Novel Glycol Chitosan–Bestatin Conjugate to Protect Thymopoietin Oligopeptides From Enzymatic Degradation

Yong Zhang [1, 2, 3, *], Jiao Feng [1], Lili Cui [4], Yuebin Zhang [5], Wenzhao Li [2], Chunlei Li [3], Nianqiu Shi [2, 6], Yan Chen [1, 2, *], Wei Kong [1, 2]

[1] National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun 130012, China
[2] Key Laboratory for Molecular Enzymology and Engineering the Ministry of Education, School of Life Sciences, Jilin University, Changchun 130012, China
[3] State Key Laboratory of New Pharmaceutical Preparations and Excipients, China Shijiazhuang Pharmaceutical Group Co., Ltd., Shijiazhuang 050051, China
[4] School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China
[5] State Key Laboratory of Molecular Reaction Dynamics, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[6] Department of Pharmaceutics, School of Pharmacy, Jilin Medical College, Jilin 132013, China

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ABSTRACT

In this study, a novel glycol chitosan (GCS)–bestatin conjugate was synthesized and evaluated to demonstrate its efficacy in protecting thymopoietin oligopeptides from aminopeptidase-mediated degradation. Moreover, the mechanism and relative susceptibility of three thymopoietin oligopeptides, thymocartin (TP4), thymopentin (TP5), and thymotrinan (TP3), to enzymatic degradation were investigated and compared at the molecular level. Initial investigations indicated that formation of the GCS–bestatin conjugate, with a substitution degree of 7.0% (moles of bestatin per mole of glycol glucosamine unit), could significantly protect all 3 peptides from aminopeptidase-mediated degradation in a concentration-dependent manner. The space hindrance and loss of one pair of hydrogen bonds, resulting from the covalent conjugation of chitosan with bestatin, did not affect the specific interaction between bestatin and aminopeptidase. Moreover, TP4 displayed a higher degradation clearance compared with those of TP5 and TP3 under the same experimental conditions. The varying levels of susceptibility of these 3 peptides to aminopeptidase (TP4 > TP5 > TP3) were closely related to differences in their binding energies to enzyme, which mainly involved Van der Waals forces and electrostatic interactions, as supported by the results of molecular dynamics simulations. These results suggest that GCS–bestatin conjugate might be useful in the delivery of thymopoietin oligopeptides by mucosal routes, and that TP3 and TP5 are better alternatives to TP4 for delivery because of their robust resistance against enzymatic degradation.

Introduction

Thymopentin (TP5, Arg–Lys–Asp–Val–Tyr), corresponding to amino acid residues 32–36 in thymopoietin, was first synthesized by Gideon Goldstein et al.[1] in 1979 and has been shown to retain the biological activity of thymopoietin, such as induction of early T cell differentiation, inhibition of B cell differentiation, and modulation of mature lymphocytes. Subsequently, analogs of thymopoietin, thymocartin (TP4, Arg–Lys–Asp–Val) and thymotrinan (TP3, Arg–Lys–Asp), were created as therapeutic substitutes and found to exhibit significant immunostimulating potencies exceeding those of TP5.[2] In preclinical animal studies, TP5, TP4, and TP3 all have been demonstrated to be safe at dosage levels up to 500 times those administered to humans, which may be explained by their rapid degradation in human plasma.[2-4] TP5 has been used as an immunomodulating agent for treatment of rheumatoid arthritis, cutaneous T-cell lymphoma, severe atopic dermatitis, acquired immunodeficiency syndrome, cancer-associated immunodeficiency, and other primary immunodeficiencies.[5-9] Although

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Lili Cui current address: Silence Therapeutics GmbH, Robert-Rössle-Strasse 10, 13125 Berlin, Germany.

* *Correspondence* to: Yong Zhang (Telephone: +86-431-85167674; Fax: +86-431-85167751) and Yan Chen (Telephone: +86-431-85167674; Fax: +86-431-85167751).

E-mail addresses: zhangyongking1@gmail.com (Y. Zhang), chen_yan@jlu.edu.cn (Y. Chen).

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TP5 is currently available in a parenteral dosage form for clinical therapy, reasonable bioavailabilities after other patient-friendly routes of administration of TP5, TP4, and TP3 (i.e., oral, buccal, nasal, rectal) are not expected because of their susceptibility to rapid degradation by luminal and membrane-bound enzymes.[10-12]

Because of the abundance of enzymes present in noninvasive routes, it is important to determine the type of enzymes to which candidate peptides are sensitive, their enzymatic degradation kinetics as well as the related mechanisms. Such knowledge would help to develop strategies to minimize the degradation of peptides used in therapies. Metabolic studies at the nasal mucosa have revealed that both TP5 and TP4 primarily undergo stepwise degradation beginning at the N-terminus by aminopeptidases.[12,13] For all three peptides (TP5, TP4, and TP3), similar degradation kinetics also were found in the presence of brush-border membrane vesicles prepared from pig mid-jejunum. Further investigation has shown that both aminopeptidase (EC 3.4.11.2) and carboxypeptidase (EC 3.4.17.1) are responsible for the rapid inactivation of TP5, whereas TP4 and TP3 mainly undergo aminopeptidase (EC 3.4.11.2)-mediated degradation in the gastrointestinal tract.[10] Generally, the loss of the arginine residue from thymopoietin oligopeptides results in a biologically inactive compound.[1,2] Therefore, it is essential to protect thymopoietin oligopeptides from enzymatic degradation during their delivery by the mucosal route. Aside from the extensive metabolism, low membrane permeability is also a key barrier to developing alternatives to needle injections.[13-15]

To overcome these barriers, many different strategies have been developed, including chemical modification,[16-18] co-administration of enzyme inhibitors[10,12] and absorption enhancers,[14] as well as encapsulating these peptides in appropriate carriers.[19-22] For example, aminoisobutyric acid substitution at the second position or N-terminal acetylation coupled with substitution at the second position by proline (Ac-Pro2-TP5 and Aib2-TP5) can enhance enzymatic stability of TP5 without loss of its activity.[16] Ester derivatives or conjugation of TP5 with lipoamino acid residues also exerts a positive effect on both stability and lipophilicity.[17,18] In addition, the co-perfusion of the inhibitor boroleucine in the rat nasal cavity leads to a significant increase in the half-life of TP5 from 12 to 37 min at an inhibitor to substrate molar ratio of 0.015:1.[12] Furthermore, the aminopeptidase inhibitor puromycin was found to effectively protect TP4 and TP3 from degradation in the presence of pancreatic extract.[10]

In the present study, based on the above-mentioned knowledge of enzymatic degradation, a novel polymer-inhibitor conjugate was developed to improve the stability of thymopoietin oligopeptides during delivery by mucosal routes (i.e., oral, nasal). As all three peptides (TP5, TP4, and TP3) mainly undergo aminopeptidase-mediated degradation by both oral and nasal deliveries, the inhibitor bestatin (Fig. 1a) was selected in this study because of its competitive inhibition of most of the aminopeptidases, including leucine aminopeptidase (EC 3.4.11.1), alanine aminopeptidase (EC 3.4.11.2), arginine aminopeptidase (EC 3.4.11.6), and proline aminopeptidase (EC 3.4.11.9) at relatively low concentrations.[23] Bestatin is currently in clinical use as an anti-tumor drug with low toxicity by oral administration. However, bestatin itself is sparingly soluble in water and cannot be formulated into an aqueous solution with a neutral pH. In addition, in order to avoid rapid systemic absorption and because of the requirement for high concentrations resulting from dilution and clearance during passage, the peptide should be retained in the luminal mucosa, such as by conjugation with a biodegradable and bio-adhesive polymer. In general, reducing the concentration of an enzyme inhibitor introduced into the body will result in fewer side effects, especially with long-term therapy. The polymer selected in this study was glycol chitosan (GCS; Fig. 1b), a linear unbranched

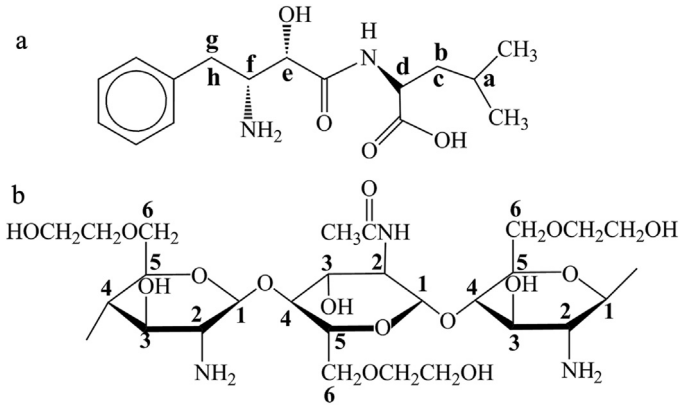

Figure 1. Structures of bestatin (a) and GCS (b). The protons along the backbones of bestatin and GCS are numbered for subsequent peak assignments in NMR spectra.

cationic polysaccharide consisting of D-glucosamine-6-glycol and N-acetyl-D-glucosamine-6-glycol through β (1–4) linkages. As one of the chitosan derivatives, GCS has low toxicity, high biodegradability and biocompatibility and mucoadhesive properties, as well as a permeation enhancing effect. Thus, it has been considered a useful material for biomedical and pharmaceutical applications, such as the delivery of drugs, proteins, and genes.[24,25] However, unlike chitosan, GCS is water-soluble at all pH values because of the pendant glycol branches on the polymer. Although various chitosan-enzyme inhibitor conjugates have been developed,[26] there is no report to our knowledge of one with the capacity to specifically inhibit aminopeptidases. Thus, the aim of this study was to synthesize and characterize a novel GCS–bestatin conjugate and to demonstrate its efficacy in protecting thymopoietin oligopeptides from aminopeptidase-mediated degradation. Moreover, the relative susceptibility of all three thymopoietin oligopeptides to enzymatic degradation and associated mechanisms were investigated and compared at the molecular level.

Materials and Methods

Materials

Peptides TP3 (Arg–Lys–Asp), TP4 (Arg–Lys–Asp–Val), and TP5 (Arg–Lys–Asp–Val–Tyr) and their metabolites were synthesized and purchased from GL Biochem Ltd. (Shanghai, China). The purity of each peptide as determined by reversed-phase HPLC (RP-HPLC) was higher than 98%. [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (bestatin), GCS (MW 250 kDa, degree of deacetylation ca. 80.0%), N-acetyl-L-leucine (purity, ≥99%), leucine aminopeptidase (EC 3.4.11.2, microsomal from porcine kidney), N-hydroxy succinimide (NHS) and N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDAC) were purchased from Sigma (St. Louis, MO). Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). These peptides and compounds were used as received without further purification. Deionized double-distilled water was used throughout the study unless otherwise prescribed. All other chemicals were of analytical reagent grade or purer.

Synthesis of GCS–Bestatin Conjugates

The covalent attachment of bestatin to GCS was achieved by the formation of amide bonds between the carboxylic acid group of bestatin and amino groups of the polymer. The crosslinking procedure involving EDAC and NHS-mediated activation reaction