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Research paper

The pharmacokinetic and pharmacodynamic properties of site-specific pegylated genetically modified recombinant human interleukin-11 in normal and thrombocytopenic monkeys

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ABSTRACT

In order to improve the pharmacokinetic and pharmacodynamic properties of recombinant human interleukin-11 mutein (mIL-11) and to reduce the frequency of administration, we examined the feasibility of chemical modification of mIL-11 by methoxy polyethylene glycol succinimidyl carbonate (mPEG-SC). PEG-mIL-11 was prepared by a pH controlled amine specific method. Bioactivity of the protein was determined in a IL-11-dependent in vitro bioassay, its pharmacodynamic and pharmacokinetic properties were investigated by using normal and thrombocytopenic monkey models. N-terminus sequencing and peptide mapping analysis revealed that Lys33 is the PEGylated position for PEG-mIL-11. Bioactivity of PEG-mIL-11 assessed by B9-11 cell proliferation assay was comparable to that of mIL-11. More than 79-fold increase in area-under-the curve (AUC) and 26-fold increase in maximum plasma concentration (C_{max}) was observed in pharmacokinetic analysis. Single dose administration of the PEG-mIL-11 induced blood platelets number increase and the effect duration were comparable to that of 7 to 10 consecutive daily administration of mIL-11 to the normal and thrombocytopenic monkey models. PEG-mIL-11 is a promising therapeutic for thrombocytopenia.

1. Introduction

Hematological toxicity, as manifested by neutropenia, anemia and thrombocytopenia, is often associated with cancer chemotherapy, restricting the dose of anti-tumor drugs being used to patients [1]. Although the cancer therapy-related hematological toxicity can be adequately controlled by use of some currently available hematopoietic growth factors, such as granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO) and interleukin 11 (IL-11), most of the growth factors are degraded within hours or even minutes by blood-borne enzymes, or are rapidly excreted. They must be administered daily consecutively for 2 weeks or even longer time to keep the effects [2,3], which causes poor patient compliance.

PEGylation of growth factors can increase the proteins' circulation half-life time and PEGylated versions of G-CSF and EPO (pegfilgrastim and mPEG-epoetin beta) have been commercialized for decades of years with improved bioavailabilities. However, common version of recombinant human interleukin-11 (rhIL-11, Neumega) is still the first and the only commercially available thrombocytopoietic cytokine [4]. Loss of biological activity is the biggest problem in modification process of rhIL-11. When rhIL-11 (Neumega) was non-specifically modified with water-soluble polymer polyethylene glycol (PEG), the in vitro cell proliferative activity of PEGylated rhIL-11 was dramatically decreased to 14–16% of rhIL-11 [5]. In order to site-specifically modlify rhIL-11, a cysteine analogue of rhIL-11 was modified at N-terminus, and the remaining biological activity of PEGylated form was 30% of the native

Abbreviations: mIL-11, interleukin-11 mutein; PEG-mIL-11, PEGylated mIL-11; mPEG-SC, methoxy polyethylene glycol succinimidyl carbonate; AUC, area-under-the curve; C_{max}, maximum plasma concentration; G-CSF, , granulocyte colony stimulating factor; EPO, erythropoietin; IL-11, interleukin 11.

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rhIL-11 [6]. We developed a rhIL-11 mutein (mIL-11) by the genetic engineering with improved stability [7]. The first nine amino acids (Met-Gly-Pro-Pro-Pro-Gly-Pro-Pro-Arg) of recombinant human IL-11 at the N-terminus were deleted, and substitutions of two amino acids (Ala→Val at position 10 and Asp→Asn at position 135) were introduced, generating mIL-11. The phase II clinical trial study showed that the mIL-11 is well tolerated and has thrombopoietic activity equivalent to one third of the clinical dose of rhIL-11, indicating the potential of the mIL-11 for use in the treatment of chemotherapy-induced thrombocytopenia (CIT) [8].

In this study, mIL-11 was PEGylated at Lys33 specifically using methoxy polyethylene glycol succinimidyl carbonate (mPEG-SC), generating PEG-mIL-11.Then, its pharmacodynamic and pharmacokinetic properties were investigated by using normal and thrombocytopenic monkey models. Our results showed that PEG-mIL-11 has a longer half-life in the plasma, and the same biological activity as mIL-11, therefore the efficacy for the treatment and prevention of thrombocytopenia is greatly improved.

2. Materials and methods

2.1. Materials

mIL-11. drug product was produced in genetically altered *Escherichia coli* and manufactured by Northland Biotech (Beijing, China) in compliance with regulations set by the Chinese government. Methoxy polyethylene glycol succinimidyl carbonate (mPEG-SC, 20 kDa) were purchased from Jen Kem Technology Corporation (Beijing, China). All other chemicals were of reagent grades and purchased from commercial sources.

Cynomolgus monkeys (3–5 years old) were purchased from Nanning FuZe Wild Animal Breeding (Nanning, China). Monkeys were maintained under conventional housing conditions. Top water and laboratory food were provided ad libitum. Monkeys were acclimated to these conditions for a period of 28 days prior to the experiment. All the experiments adhered to the Institutional Animal Care and Use Committee (IACUC, No. ACU12-188).

B9-11 cell line stably expressing human IL-11 receptors was donated by ViroMed (Seoul, Korea), the cell line was used for the biological activity assay of PEGylated mIL-11.

2.2. Preparation mono-PEGylated mIL-11

A pH controlled amine specific method was performed for preparation of PEGylated mIL-11. mIL-11 (0.8 mg/mL) was reacted with a 5.5 M excess of mPEG-SC in PBS (pH 8.2) for 1 h at 2–8 °C with gentle agitation. The reaction solution was then diafiltered against 50nM sodium acetate (pH 5.0) using a 10K membrane (Vivaspin2, 30 K MWCO, Vivascience, Germany) to remove non-reacted mPEG-SC polymers.

The remnant was loaded onto a cation exchange chromatography (CaptoS, GE Healthcare, USA) and equilibrated with sodium acetate buffer, pH5.0). The column was eluted with a linear salt gradient to separate di-PEGylated, mono-PEGylated (PEG-mIL-11) and non-PEGylated mIL-11. The PEG-mIL-11 fraction was then collected, desalted and changed into a formulation buffer. Concentration of PEG-mIL-11 was determined by using the Lowry assay. The final concentration was adjusted to be 1 mg/ml and stored at -80 °C until used.

2.3. Physicochemical characterization and site identification of PEG-mIL-11

The molecular weight of PEG-mIL-11 was determined by SDS-PAGE under non-reduced condition. The purity of PEG-mIL-11 was analyzed by SDS-PAGE (4–12% bis-tris gel) and size-exclusion HPLC. Isoelectric point (pI) of PEG-mIL-11 was determined by isoelectric focusing (IEF) gel electrophoresis (GE Healthcare Bio-Sciences, USA). PEGylation site of PEG-mIL-11 was identified by N-term sequencing (Procise 491 HT protein sequencer, Applied Biosystems, USA) and peptide mapping using Lys-C proteolysis / HPLC assay (Agilent 1200, Agilent, USA).

2.4. In vitro biological activity assay

The biological activity of PEG-mIL-11 was determined by an in vitro non-radioactive method as described previously [7]. Ten-fold serial dilutions of PEG-mIL-11 and mIL-11 from 1 μg/ml to 1 pg/ml, respectively, was put in 96-well plates. One hundred microliters of B9-11 cells (3×10^4 cells/ml) were added to the wells and incubated at 37 °C and 5% CO_2 for 72 h. At the end of cultivation, cells were treated with MTT agent for 5 h in the incubator. The optical densities of samples at 570 nm were measured with a microplate reader (Molecular Devices, USA). All the assays were performed in triplicate.

2.5. Measurements of pharmacodynamic and pharmacokinetic properties using normal monkey model

Male cynomolgus monkeys weighing 3–5 kg (3 monkeys/-group) were administered subcutaneously. PEG-mIL-11 (treament group) was dosed at 350 μg/kg with the single injection, while mIL-11 (control group) was dosed at 50 μg/kg/day for 7 days, with saline as vehicle group.

To evaluate the pharmacokinetic property, blood samples were collected at 0.5, 1, 2, 3, 6, 8, 10, 12, 24, 30, 48, 72, 96, 120, 144 and 168 h after dosing in PEG-mIL-11 group, and blood samples were collected at 0.083, 0.5, 1, 2, 3, 6, 8 and 24 h after the first dosing (day 0) in mIL-11 group. Plasma samples were prepared by centrifugation and stored at -80 °C until used. PEG-mIL-11 or mIL-11 concentration in the plasma was measured using commercially available human IL-11 ELISA kit (R&D system, Minneapolis, USA) and anti-PEG-Biotin (Epitomics, Eugene, USA), according to the manufacturer's instructions. Each PEG-mIL-11 and mIL-11 was used as the standard proteins. The pharmacokinetic parameters were analyzed with WinNonlin software version 5.2 (Pharsight Corp., Cary, N.C., USA) using non-compartmental approaches. All data were expressed as the mean ± standard deviation (SD), except median (range) for Tmax. For statistical analysis of pharmacokinetics, an unpaired t-test was used.

To evaluate the pharmacodynamic effect on platelet counts, blood samples were collected on 0 (predose, as the baseline value), 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 21 and 23 days after the first dose administration. Platelet counts were measured using an automatic hematology analyzer (Siemens Advia 2120 Hematology System, Bayer, Leverkusen, Germany). The relative platelet amounts were calculated with the pre-dosing platelet counts. One way ANOVA and LSD test multiple comparison test were used for evaluation of significant differences, and p values less than 0.05 were considered to be significant.

2.6. Pharamcodynamic and pharmacokinetic properties using thrombocytopenic monkey model

Thrombocytopenia was induced in cynomolgus monkeys by i.v. administration of carboplatin (Qilu Pharm, Jinan, China) at a dose of 12 mg/kg/day on day 1, 2 and 3 before PEG-mIL-11 or mIL-11 administration.

On day 4, carboplatin-induced thrombocytopenic cynomolgus monkeys weighing 3–5 kg (6 monkeys/group, 3 of each sex) were randomly assigned to 5 groups. Monkeys in three test article