Journal of Controlled Release 129 (2008) 11–17

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Lipidization of human interferon-alpha: A new approach toward improving the delivery of protein drugs

Liyun Yuan [a], Jeff Wang [b], Wei-Chiang Shen [a,*]

[a] Department of Pharmacology and Pharmaceutical Sciences, University of Southern, California, Los Angeles, CA 90089, USA
[b] Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA 91766, USA

ARTICLE INFO

Article history:
Received 21 November 2007
Accepted 17 March 2008
Available online 26 March 2008

Keywords:
Reversible lipidization
Interferons
Plasma half-life
Liver targeting

ABSTRACT

Human interferon-alpha (IFN-α), a 19.2 KD protein containing two disulfide bonds (cys1–cys98; cys29–cys138), was reduced and modified with a reversible lipidization agent. The product of the lipidization, PAL-IFN, was homogenous, with four palmitoyl moieties linked to the four Cys residues in the protein molecule via reversible disulfide linkages. The far-UV circular dichroism (CD) spectrum of PAL-IFN was virtually overlapped with that of IFN, indicating that the IFN structure was not altered by the modification. After iv injection in mice of 0.1 mg/kg of PAL-IFN, a low level of serum IFN activity was sustained for more than 8 h, while serum IFN activity was rapidly diminished to an undetectable level at 2 h post IFN injection at the same dose. Evidence suggested that IFN was slowly released from PAL-IFN into blood circulation upon reduction of the disulfide bonds *in vivo*. Furthermore, the liver-targeting effect of PAL-IFN was demonstrated by the observation that the level of 2′-5′ oligoadenylate synthetase (OAS) expressed in the liver of mice treated with PAL-IFN was significantly higher than that with IFN. In conclusion, reversible lipidization can potentially achieve both a prolonged plasma half-life and an enhanced liver-targeting effect of IFN in antiviral therapy.

1. Introduction

IFN-α is considered the most effective antiviral agent for chronic viral hepatitis [1]. However, the short half-life and lack of liver-specific affinity hamper the IFN-α response. Upon the administration, the blood IFN-α level reaches the peak within 1 h and quickly declines to an undetectable level after 24 h. The terminal half-life of IFN-α varies from 3 to 8 h in patients [2–4]. Even though IFN-α is given three times weekly through subcutaneous administration in clinical settings to maintain a therapeutic level, the blood IFN level waxes and wanes daily, and remains undetectable for much of the administration interval. With monotherapy of IFN-α, a sustained virologic response, which is defined as the absence of HCV RNA in serum for at least 6 months after the discontinuation of therapy, is achieved in less than one-fifth of patients with chronic hepatitis C [5,6]. Improving the therapeutic efficacy of IFN-α could be achieved through prolonging its plasma half-life or concentrating IFN-α in the liver through liver-targeting strategies.

Pegylation is most commonly used to modify IFN-α to improve the pharmacokinetics and pharmacodynamics [7]. Two pegylated forms of IFN-α have been successfully developed into the market. PEG-INTRON, developed by Schering–Plough, is a mixture of pegylated IFN-α2b with a 12 kD linear PEGs covalently-attached to the amine groups of at least 14 amino acid residues of IFN-α [8]. Although PEG-INTRON maintains only 27% of *in vitro* potency [9], the prolonged half-life of PEG-INTRON has significantly compensated for this reduced *in vitro* potency. Another pegylated form of IFN is PEGASYS, developed by Roche. PEGASYS is a pegylated IFN-α2a with a 40 kD branched-chain PEG conjugated to all possible lysine residues on the IFN surface, and contains at least four positional isomers [10]. PEGASYS maintains only 7% of *in vitro* potency, while the plasma half-life has also been significantly extended [11]. However, the increased hydrophilicity and bulkiness upon pegylation reduces the affinity of IFN-α to the liver, which may compromise the therapeutic efficacy for viral hepatitis.

Many of these deficiencies in pegylated IFNs such as molecular heterogeneity, reduced affinity to liver cells and reduced potency can be improved by reversible lipidization [12–15]. In this report, we describe the reversible lipidization of human IFN-α by conjugating palmitoyl cysteine specifically to cysteinyl residues of IFN-α via disulfide bonds. Through this synthetic strategy, we produced a homogenous single-species conjugate, PAL-IFN. More importantly, PAL-IFN maintains the conformation of native IFN which ensures the regeneration of unmodified IFN-α upon the reduction of the disulfide inside the body, possibly in the liver. Consequently, the released IFN-α will be retained locally in the liver for a prolonged period, thereby enhancing the therapeutic potential for viral hepatitis. We compared the pharmacokinetics, biodistribution, and *in vivo* biological activity of

* Corresponding author. Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, California 90089, USA. Tel.: +1 3234421902; fax: +1 3234421390.
E-mail address: weishen@usc.edu (W.-C. Shen).

0168-3659/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2008.03.014

PAL-IFN and unmodified IFN-α, proving the potential of PAL-IFN as a novel modified IFN with improved delivery and liver-targeting properties.

2. Materials and methods

2.1. Animal

Adult male CF-1 mice aged 6–8 weeks were obtained from Charles River Laboratories (Wilmington, MA, USA) and handled in accordance with the "Principles of Laboratory Animal Care" (NIH 85-23, revised 1985). The animals were allowed a standard diet and tap water *ad libitum*, and were maintained under controlled conditions (12-h light, 12-h dark schedule; 24 °C).

2.2. Regents and antigens

Human interferon alpha (hIFN-α) was obtained from BioVision (Mountain View, CA, USA); Rabbit anti-OAS1 polyclonal antibody was obtained from Abgen (San Diego, CA, USA); Mouse anti-β-actin monoclonal antibody was obtained from Abcam (Cambridge, MA, USA); BCA protein assay kits were obtained from Pierce Biotechnology (Rockford, IL, USA); CellTiter 96 Aqueous One Solution Reagent was obtained from Promega (Madison, WI, USA). All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Synthesis of palmitoylated human interferon alpha (PAL-IFN)

Fifty micrograms of IFN was dissolved into 200 μl of PBS containing 0.5% Chaps. Eight microliters of freshly prepared dithiothreitol (DTT) (1 mg/ml) was added to the solution. The tube was filled with nitrogen for 10 min. The reaction tube was then placed in a 37 °C water bath. After 40-min incubation, 24 μl of *N*-palmitoyl cysteinyl 2-pyridyl disulfide (Pal-CPD) was added to the reaction mixture and the incubation was continued for another 1 h at room temperature.

2.4. Characterization of PAL-IFN with HPLC

HPLC analysis was used to monitor the reactions. HPLC was conducted on a Hewlett-Packard 1050 HPLC system (Avondale, PA, USA) employing a 250×4.6 mm Phenomenex Jupiter® C4 column (Torrance, CA, USA). The mobile phases were: 5% acetonitrile in 0.1% trifluoroacetic acid (A) and 95% acetonitrile in 0.1% trifluoroacetic acid (B). A gradient elution was programmed, starting at 10% B, increasing to 100% B in 15 min and keeping at 100% B for an additional 5 min. The detection was made at 214 nm and 280 nm.

2.5. Characterization of PAL-IFN with LC–MS/MS

Liquid Chromatography–Ion Trap Mass Spectrometry (LC–MS) was applied to characterize the modification of PAL-IFN. 10 μg of PAL-IFN was digested with freshly prepared 1% trypsin for 2 h at 37 °C, and the digestion was repeated for another 2 h and then overnight by replenishing with fresh 1% trypsin. The tryptic digestion was stopped by adding 5% acetic acid. The sample was desalted using Protein Desalting Spin Columns (Pierce, Rockford, IL, USA).

Mass spectrometric analysis of PAL-IFN was performed using a Thermo Finnigan LCQ Deca XPPlus mass spectrometer implemented with an Ultra Plus II LC system (Micro-Tech Scientific, Brockville, Ontario, Canada) using a 150 mm×75 μm C-18 column (5 μm 300 Å particles) from Micro-Tech Scientific. Peptides were loaded onto a Michrom Bioresources Peptide Cap Trap at 95% solvent A (2% acetonitrile, 0.1% formic acid) and 5% solvent B (95% acetonitrile, 0.1% formic acid) and then eluted with a linear gradient of 5–60% solvent B for 65 min and 60–90% solvent B for 10 min. Tandem MS/MS spectra were acquired with Xcalibur® 1.2 software. A full MS scan was followed by three consecutive MS/MS scans of the top three ion peaks from the preceding full scan.

Dynamic exclusion was enabled such that after three occurrences of an ion within 1 min, the ion was placed on the exclusion list for 3 min. Other mass spectrometric data generation parameters were as follows: collision energy 35%, full scan MS mass range 400–1800 m/z, minimum MS signal 5×10^4 counts, minimum MS/MS signal 5×10^3 counts. The mass spectrometer was equipped with a nanospray ion source (Thermo Electron) using an uncoated 10 μm-ID SilicaTipTM PicoTipTM nanospray emitter (New Objective, Woburn, MA, USA). The spray voltage of the mass spectrometer was 1.9 kV and the heated capillary temperature was 180 °C.

The obtained MS Spectra was analyzed as following. A Beta test version of Bioworks (Bioworks 3.1) on a nine node (2 cpu/node) cluster computer from Thermo Electron utilizing the SEQUEST algorithm was used to determine cross correlation scores between acquired spectra and a human IFN-α protein database. To identify Cys-conjugated PAL peptides, a differential modification of +358.559 (molecular weight of PAL) was used. Other SEQUEST parameters included threshold: 1000; monoisotopic; enzyme (trypsin); and charge state (auto). For peptide identification, spectra passing a threshold of cross correlation versus charge state (1.5 for +1 ions, 2.0 for +2 ions, 2.5 for +3 ions) were then inspected to verify that all major ions were identified. MS/MS spectra were also manually validated.

2.6. Characterization of PAL-IFN with far-UV circular dichroism (CD)

PAL-IFN and IFN were eluted and collected from HPLC. To obtain Far-UV CD spectra, 1 μM of PAL-IFN in 50% acetonitrile/0.1%TFA was loaded to a cuvette with 1.0 cm pathlength. The cuvette was positioned in a Jasco J810 spectropolarimeter (Jasco Inc, Easton, MD, USA). Complete spectra were collected at room temperature with 100 scans in the wavelength range of 190–260 nm. IFN and buffer (50% acetonitrile/0.1%TFA) spectra were collected in the same condition. PAL-IFN and IFN spectra were subtracted from buffer spectra. The final spectra data was analyzed by using Origin 7 software (OriginLab Corporation, Northampton, MA, USA).

2.7. Pharmacokinetic studies

IFN and PAL-IFN were iodinated with Na ^{125}I by using the Chloramine T method [16]. Male CF-1 mice weighing 27–30 g each, with free access to food and water prior to the experiments, were used for pharmacokinetic studies. 0.023 mg/kg of ^{125}I-PAL-IFN or ^{125}I-IFN, corresponding to 1×10^6 cpm per mouse, was administered to mice via the tail vein. At 5, 15, 30 min, and 1, 2, 4, and 8 h post injection, 3 animals from each experimental group were sacrificed and blood was collected from the heart. 200 μl of plasma was collected by centrifugation of blood for 10 min at 6000 rpm. The intact proteins were precipitated with 20% ice-cold trichloroacetic acid (TCA), and the radioactivity in the precipitates was measured using a gamma counter. In ^{125}I-PAL-IFN-treated mice, the TCA precipitates of the plasma should contain both intact conjugate and the released free IFN. However, since the plasma half-life of free IFN is significantly shorter than that of PAL-IFN, the amount of radioactivity was used for estimating the pharmacokinetic parameters of PAL-IFN. The radioactivity of the whole liver, kidney, spleen, lung, stomach and colons of each mouse was measured for the determination of biodistribution of PAL-IFN and IFN.

2.8. Cell associated PAL-IFN

The Sg-PC2 cell line [17], a cell clone of the huh-7 cell line, which contains self-replicating HCV RNA, was provided by Professor J.-H. James Ou (Department of Molecular Microbiology & Immunology, University of Southern California). Sg-PC2 was seeded at a density of