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Improvement of in vitro stability and pharmacokinetics of hIFN-α by fusing the carboxyl-terminal peptide of hCG β-subunit

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ABSTRACT

Improving *in vivo* half-life and *in vitro* stability of protein-based therapeutics is a current challenge for the biopharmaceutical industry. In particular, recombinant human interferon alpha-2b (rhIFN-α2b), which belongs to a group of cytokines extensively used for the treatment of viral diseases and cancers, shows a poor stability in solution and an extremely short plasma half-life which determines a strict therapeutic regimen comprising high and repeated doses. In this work, we have used a strategy based on the fusion of the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin (hCG) β-subunit, bearing four *O*-linked oligosaccharide recognition sites, to each or both N- and C-terminal ends of rhIFN-α2b. Molecules containing from 5 (CTP-IFN and IFN-CTP) to 9 (CTP-IFN-CTP) *O*-glycosylation sites were efficiently expressed and secreted to CHO cells supernatants, and exhibited antiviral and antiproliferative bioactivities *in vitro*. Significant improvements in pharmacokinetics in rats were achieved through this approach, since the doubly CTP-modified IFN variant showed a 10-fold longer elimination half-life and a 19-fold decreased plasma apparent clearance compared to the wild-type cytokine. Moreover, CTP-IFN-CTP demonstrated a significant increase in *in vitro* thermal resistance and a higher stability against plasma protease inactivation, both features attributed to the stabilizing effects of the *O*-glycans provided by the CTP moiety. These results constitute the first report that postulates CTP as a tag for improving both the *in vitro* and *in vivo* stability of rhIFN-α2b which, in turn, would positively influence its *in vivo* bioactivity.

1. Introduction

Since their discovery in the 1950s (Isaacs and Lindenmann, 1957), interferons have gained ground in therapeutic applications. Type I interferons belong to a group of cytokines which regulate resistance to viral infections, enhance innate and acquired immune responses and modulate normal and tumour cell survival and death; such properties being the causes of their extensive use for treatment of viral diseases and cancers (Borden et al., 2007; Lin and Young, 2014).

Particularly, human interferon-α2 (hIFN-α2) was the first highly active IFN subtype to be cloned in the early 1980s, and the recombinant protein was also the first cytokine to be produced and commercialized by the pharmaceutical industry. Currently, rhIFN-α2a and rhIFN-α2b produced in bacteria are licensed drugs for treatment of chronic viral hepatitis B and C, and as antitumor agents for hairy cell leukemia, melanoma, Kaposi sarcoma, follicular lymphoma, renal cell carcinoma and chronic myelogenous leukemia, either as a monotherapy or in combination with other drugs (El-Baky and Redwan, 2015; Leader et al., 2008).

Widely successful therapeutic outcomes of rhIFN-α2 are hampered by the development of adverse side effects which alter the patientsí quality of life and often demand dose reduction or premature treatment interruption (Paul et al., 2015; Sleijfer et al., 2005). These range from normally occurring mild side effects (such as fever, myalgia and headache) to neurological toxicity, revealed as depression and suicide tendency, representing a major concern that should be carefully managed. Chronic side effects also include asthenia, weight loss and autoimmune manifestations that

Abbreviations: hIFN-α, human interferon alpha; hCG, human chorionic gonadotropin; CTP, carboxyl-terminal peptide; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyrotropin; EPO, erythropoietin; LV, lentiviral; LVP, lentiviral particle; C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life; AUC, area under curve; CLapp, apparent clearance; 2-AB, 2-aminobenzamide.

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can greatly limit the safety and efficacy of rhIFN-α2 treatment (Uze and Tavernier, 2015). The short half-life of the cytokine is the main driver of such complications, since high and frequent doses are required to achieve the therapeutic effect. Except in rare situations, rhIFN-α2 is administered parenterally in order to avoid proteolysis, reaching a peak level within 1 h, and quickly declining to undetectable values after 24 h post-injection (Pedder, 2003; Yuan et al., 2008). Thus, the optimization of pharmacokinetics represents a key requisite for maximizing rhIFN-α2 efficacy while reducing its harmful side effects.

The covalent conjugation of polyethylene glycol (PEG) to therapeutic proteins is the most widespread technology for extending serum half-life to reduce administration frequency (Baker, 2001; Podobnik et al., 2015; Roberts et al., 2002). Although two pegylated forms of rhIFN-α2 have been successfully introduced into the market (PEGINTRON, by Schering Plough, and PEGASYS, by Roche), there are still concerns about the immunogenicity of PEG and the possibility of generation of anti-PEG antibodies which would threaten the safety and effectiveness of treatment. Other approaches for half-life extension which have not reached clinical setting yet include Fc and albumin fusion, nanoparticle encapsulation, introduction of proteolysis-resistant mutations and incorporation of new glycosyl moieties (Ceaglio et al., 2008; Choi and Park, 2006; Elliott et al., 2003; Markert et al., 2001; Sinclair and Elliott, 2005; Subramanian et al., 2007). All of them aim to reduce the elimination mechanisms of rapidly-cleared biopharmaceuticals, such as renal clearance, receptor-mediated removal and protease degradation.

The introduction of new glycans exhibits the advantage of combining properties that influence many protein clearance pathways simultaneously. Carbohydrates increase the molecular size of glycoproteins and thus reduce the rate of glomerular filtration. Renal clearance is also delayed by repulsion between the negative charges of glycosaminoglycans situated in the glomeruli and the negative charges of protein glycans conferred by terminal sialic acids (Bocci et al., 1990; Mahmood and Green, 2005). These residues also contribute to decrease the uptake of glycoproteins from circulation by the hepatocytes by restraining the binding to hepatic asialoglycoprotein receptors that have specificity for terminal galactose or *N*-acetylgalactosamine residues (Morell et al., 1971). Additionally, the decreased receptor affinity of glycosylated proteins can reduce clearance by receptor-mediated endocytocis (Koury, 2003). Resistance to proteolysis can also be enhanced by glycans through masking of cleavage sites, thus reducing inactivation by endogenous serum or tissue proteases (Markert et al., 2001; Subramanian et al., 2007). Our group has previously applied a strategy to incorporate new *N*-linked glycans in order to improve the pharmacokinetic properties of rhIFN-α2b, rendering a molecule with four *N*-glycosylation sites (4N-IFN) with a 20-fold increased plasma half-life which was reflected in an enhanced therapeutic activity in mice in comparison with the non-glycosylated molecule (Ceaglio et al., 2010a; Ceaglio et al., 2008). The main challenge of this strategy is the rational selection of suitable positions for introducing new *N*-glycosylation sites preserving, at the same time, the functional and structural properties of the protein (Marshall et al., 2003; Samoudi et al., 2015).

An alternative approach consists in the introduction of *O*-glycosylation sites to a therapeutic protein. Unlike *N*-glycosylation, a consensus sequence for *O*-glycosylation has not been defined yet, although there is evidence that it occurs in regions rich in Ser, Thr and Pro, and so it is more difficult to predict (Bai et al., 2015; Rudd and Dwek, 1997; Van den Steen et al., 1998). Fusion of peptides containing *O*-glycosylation potential glycosylation sites to the N- or C-terminal end of proteins represents a possibility to address this issue, avoiding the extensive modification of the peptide backbone.

Regarding this last approach, a strategy based on the fusion of carboxyl-terminal peptide (CTP) of human chorionic gonadotropin (hCG) β-subunit has been successfully applied. hGC, together with pituitary gonadotropins (luteinizing hormone, LH, and follicle-stimulating hormone, FSH) and thyrotropin (TSH) constitute a family of glycoprotein hormones that are heterodimers containing a common identical α-subunit covalently bound to a β-subunit which determines the biologic specificity of each protein (Pierce and Parsons, 1981). The β-subunit of hCG is distinguished from the others because of the presence of a C-terminal extension rich in Ser and Thr, and four of these residues are responsible of the existence of *O*-linked glycans (Birken and Canfield, 1977). This extension is believed to play a role in maintaining the prolonged half-life of hCG compared to the other hormones (Matzuk et al., 1990). Previous studies have demonstrated that fusing the CTP to β-subunits of FSH (Fares et al., 1992) and TSH (Joshi et al., 1995), α-subunit of hCG (Furuhashi et al., 1995), human growth hormone (hGH) (Fares et al., 2010) and erythropoietin (hEPO) (Fares et al., 2007; Fares et al., 2011) did not affect assembly, secretion, receptor binding affinity, and *in vitro* bioactivity. Moreover, the addition of *O*-linked oligosaccharides by CTP fusion significantly increased the half-life and *in vivo* potency of such proteins.

In the present study, we evaluated the effect of fusing CTP to the N-terminal end (CTP-IFN), the C-terminal end (IFN-CTP) and both ends (CTP-IFN-CTP) of rhIFN-α2b. Therefore, IFN analogs containing 5–9 potential *O*-glycosylation sites were constructed and a physicochemical, biological and pharmacokinetic characterization was performed. Also, the influence of *O*-glycans on the stability against thermal stress and plasma protease inactivation was analyzed, comprising the first report where these properties were evaluated in order to connect the effects of CTP-derived *O*-glycans with the *in vitro* and *in vivo* stability of CTP-fused recombinant proteins.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO-K1) cells were grown in a mixture of Dulbeccoís Modified Eagleís Medium (DMEM)/Hamís F12 1:1 (Gibco, USA) supplemented with 5% (v/v) fetal calf serum (FCS) (PAA, Austria) and 2 mM glutamine (Gibco).

Human embryonic kidney (HEK293T) cells were cultured in DMEM supplemented with 10% (v/v) FCS and 2 mM glutamine.

Madin-Darby bovine kidney (MDBK) cells were grown in Minimum Essential Medium, MEM (Gibco, USA) supplemented with 10% (v/v) FCS (growth medium). For bioassays, MEM supplemented with 2% (v/v) FCS (assay medium) was employed.

The human Daudi cell line was maintained in RPMI 1640 medium (Gibco) plus 10% (v/v) FCS.

All cells were incubated at 37 °C in humidified 5% CO_2.

2.2. Design and construction of IFN variants

Three IFN variants were designed by fusing the carboxyl-terminal peptide (CTP) of hCG β-subunit to the coding sequence of hIFN-α2b: CTP-IFN (CTP was ligated to the N-terminal end), IFN-CTP (CTP was ligated to the C-terminal end) and CTP-IFN-CTP (two CTPs were ligated to IFN, one to the N-terminal and one to the C-terminal end). Recognition sites for the restriction enzymes *Sal*I and *Xba*I were added flanking both ends of the constructs. SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP, (Petersen et al., 2011)) was used to evaluate the effect of fusing CTP to the N-terminal end of hIFN-α2b on the native signal peptide cleavage site of the cytokine. As the native cleavage site was incor-