Increased bioavailability of IFN α 2b modificed by conjugation to a two-branched polyethyleneglycol molecule

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ABSTRACT

Interferons (IFNs) are a family of cytokines of well-defined biological properties and exert immunoregulatory antiviral effects and antiproliferative activity. Nevertheless, the clinical application of biopharmaceuticals based on IFNs has been limited by the relatively restrained bioavailability of these molecules in blood. In the last years, the pegylation technology (i.e., the conjugation of polyethyleneglycol molecules to peptides and proteins) has provided great advances for the development of formulations showing prolonged bioavailability and surpassing much of the limitations for the use of therapeutic polypeptides, such as IFNs. In this work, the IFN α 2b molecule was chemically modified by conjugating it to a two-branched PEG polymer of 40 kDa molecular weight. This was the first report on this molecular combination. Moreover, a technological process was designed for the conjugation, purification and formulation of the IFN α 2b-PEG molecule. The physico-chemical and biological characterization demonstrated that this molecule complied with the international parameters enforced by regulatory authorities for those biopharmaceuticals to be used to treat human diseases such as hepatitis C. This molecule received the Sanitary Registration in Cuba in 2009. It was demonstrated that pegylation increased the mean half-time of IFN α 2b-PEG in blood either in preclinical or in clinical studies, in respect with the non-modified IFN molecule. Therefore, a technology platform was established to generate products pegylated with 40 kDa PEG. This research granted the 2014 Award of the Cuban National Academy of Sciences.

Keywords: hepatitis C, interferón, pegylation, chemical modification of proteins

RESUMEN

Incremento de la biodisponibilidad del IFN α 2b modificado por conjugación química con una molécula ramificada de Polietilenglicol. Los interferones (IFN) comprenden a una familia de citoquinas con propiedades biológicas definidas, que ejercen un efecto antiviral immunoregulador y tienen actividad anti-proliferativa. La utilidad clínica de los fármacos basados en los IFN se ha visto limitada por la relativamente restringida biodisponibilidad de la molécula en sangre. Durante los últimos años, la tecnología de Peguilación, conjugación de péptidos y proteínas a moléculas de polientilenglicol (PEG), ha tenido grandes avances en el desarrollo de formulaciones de acción prolongada, lo que elimina muchas de las limitaciones en el uso de proteínas terapéuticas, como por ejemplo los IFN. En este trabajo se logró la modificación química de la molécula de IFN α 2b mediante su conjugación a un polímero de PEG ramificado de 40 kDa de peso molecular, lo cual constituyó el primer reporte para este tipo de molécula de PEG. Además, se diseñó un proceso tecnológico para la conjugación, purificación y formulación de la molécula de IFN α 2b-PEG. La caracterización fisicoquímica y biológica demostró que cumple con los parámetros exigidos internacionalmente para poder ser usado como producto farmacéutico en el tratamiento de enfermedades como la hepatitis C, habiéndole sido otorgado el Registro Sanitario en Cuba en el año 2009. Se demostró que la peguilación incrementó el tiempo de vida media del IFN α 2b-PEG en sangre, al compararlo con la molécula de IFN no modificada, tanto en estudios preclínicos como en un ensayo clínico. De esta forma, se estableció una plataforma tecnológica para la obtención de productos con moléculas conjugadas a PEG 40 kDa. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2014.

Palabras clave: hepatitis C, interferón, Peguilación, modificación química de proteínas

*I*ntroduction

The therapeutic use of alpha interferons (IFNs) has been extensively limited by the low bioavailability of the protein molecule. Therefore, other alternatives have been explored, such as the chemical modification of the IFN molecule, its conjugation to a polyethyleneglycol (PEG, pegylation) among the most promising ones [1].

The pegylation technology successfully masks or occludes protein epitopes susceptible to immune recognition and clearance through the reticuloendothelial system, or subjected to degradation by proteolytic enzymes. Moreover, pegylation increases molecular size what reduces the molecule's renal filtration and modifies its biodistribution. The main aspects influenced by conjugation are: 1) the number of PEG chains coupled to the protein; 2) the molecular size and the structure of PEG; 3) the molecule site for PEG coupling, and 4) the conjugation technology used [2].

The first PEG-protein conjugates were obtained by using the so-called first-generation PEG [3]. They were linear molecules of less 12 kDa molecular weight and forming weak bonds when conjugated to proteins. A second generation was obtained, using branched PEG molecules of larger size, further decreasing the main undesired effects. For IFNs, they have been reported as conjugated to 5- and 12-kDa PEG molecules specifically for IFN α 2b and to 40 kDa for IFN α 2a [4]. In this last case, superior results have been reported as compared with the previous molecules conjugates to smaller molecular size PEG molecules.

In this work, we developed and implemented the technology to obtain the IFN α 2b molecule conjugated to two-branched 40 kDa PEG molecules, showing increased bioavailability and pharmacokinetics in rabbits and further tested in a bioequivalence clinical trial in healthy human subjects.

*R*esults

IFN alfa 2b (Center for Genetic Engineering, Cuba) was modified by conjugation to PEG [5]. The first phase of the working strategy comprised the theoretical aspects mentioned above; the technology for obtaining the two-branched 40 kDa PEG was designed and developed, considering the advances reported for second generation PEG molecules [6].

As shown in figure 1, the PEG molecule was functionalized for conjugation in form of N-hydroxysuccinimidyl ester. Once the PEG molecule was obtained with the molecular properties, purity ($\geq$ 95 %) and activation degree ($\geq$ 60 %) as required for the conjugation step, the technology for its conjugation to IFN alfa 2b was developed. This included the reaction under basic pH conditions to direct the binding of PEG to amine groups in lysine residues.

The developed reaction supported obtaining the product by forming highly stable amide-type bonds, generating a very stable IFN-PEG conjugate. Noteworthy, conjugation levels above 40 % were achieved through a strategy designed to guarantee the presence of solvents facilitating the structure conditions as required during the conjugation reaction, a key aspect previously unreported and responsible for an increased efficiency in the process recovery. Addition-
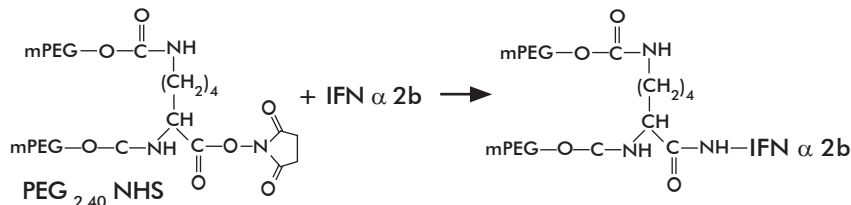

Figure 1.Reaction for the conjugation of recombinant interferon α 2b (IFN α 2b) to the 40-kDa polyethyleneglycol (PEG) functionalized as N-hydroxysuccinimidyl ester (PEG $_{2,40}$ NHS).

ally, a significant element of the development process was structuring it as complying with pharmaceutical-grade parenteral product requirements, since the final purpose of the work was to generate an IFN-PEG conjugate for therapeutic applications. Hence, the technology developed supported the obtainment of a conjugated IFN-PEG molecule complying with regulatory safety and efficacy requirements of international pharmaceutical standards.

Subsequently, a toxicological study was conducted to demonstrate the safety of the IFN-PEG molecule. The results were satisfactory. Additionally, the purity, levels of contaminants and biological activity of the molecule were monitored through the respective analytical techniques, which were jointly developed for the testing. All contaminants were below 5 % [5].

Stability studies demonstrated that the IFN-PEG molecule was stable for more than 12 months and its quality parameters remained unchanged as designed for the product. The molecule was highly resistant to proteases and of high thermal stability.

Pharmacokinetic studies demonstrated a significant increase in the bioavailability of the product, as compared with the non-conjugated molecule. In fact, the pegylated IFN formulation showed a slower clearance rate than the native molecule, as detected in the longest evaluation time tested of 168 vs 12 hours. This further corroborated that the pegylated IFN molecule formulation was obtained with the designed properties and according to the process developed. These properties are shown in figure 2. Clearance decreased considerably, indicating this mechanism as one determining the increased bioavailability of the product.

1. Reddy KR. Controlled-release, pegylation, liposomal formulations: new mechanisms in the delivery of injectable drugs. Ann Pharmacother. 2000;34:915-23.

2. Veronese FM, Harris JM. Peptide and protein PEGylation: advances in chemistry and clinical applications. Adv Drug Deliv Rev. 2008;60(1):1-2.

3. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2002;54:459-76.

4. Grace MJ, Cutler DL, Bordens RW. Pegylated IFNs for chronic hepatitis C: an update. Expert Opin Drug Deliv. 2005;2:219-26.

5. Ramon J, Saez V, Baez R, Aldana R, Hardy E. PEGylated interferon-alpha2b: a branched 40K polyethylene glycol derivative. Pharm Res. 2005;22:1374-86.

6. Fee CJ. Size comparison between proteins PEGylated with branched and linear poly(ethylene glycol) molecules. Biotechnol Bioeng. 2007;98:725-31.
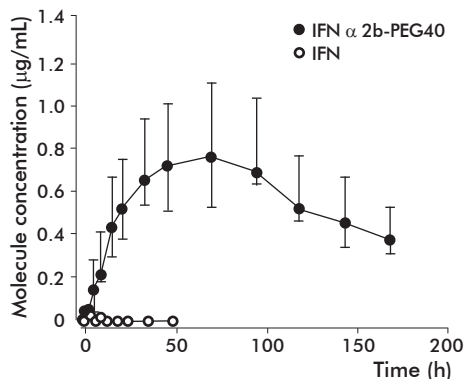
Figure 2. Blood levels of recombinant interferon α 2b (IFN α 2b), alone or conjugated to a two-branched polyethyleneglycol molecule of 40 kDa (IFN α 2b-PEG40), when administered in rabbits. Values are represented as means ± standard deviation (n = 6).

The physico-chemical stability of the 40 kDa IFN-PEG molecule was demonstrated in a liquid formulation containing Tween 80, ethylenediaminetetraacetic acid salt and phosphate-buffered at pH 7.2. This formulation was stable for 24 months upon storage at 4 ºC and for 6 months at 28 ºC. The studies under stressing conditions revealed it was quite stable even when stored at high temperature. Aggregation was identified as the relevant instability at 60 ºC. No degradation products of the pegylated molecule were detected, as opposed to the behavior of the non-pegylated IFN molecule which was very unstable in solution.

Following these results, a clinical trial was conducted in human healthy volunteers [7], following the Helsinki ethics guidelines [8], after the review and approval of the clinical protocol by the institutional Ethics Committee of the participating National Center of Toxicology (CENATOX) and the approval by the Cuban National Regulatory Authority (Center for State Control of Medicines, Medical Equipment and Devices, CECMED). The IFN α2b-PEG molecule was demonstrated as bioequivalent when compared against the biosimilar Pegasys (alpha 2a IFN-PEG 40 kDa), this last the standard available in the market. The IFN α2b-PEG molecule showed an increase half-life time in blood contrary to the behavior of the non-conjugated IFN molecule.

The product received the sanitary registration in Cuba, supporting its introduction into medical practice, with satisfactory results and improvement in the therapeutic outcome as compared to the previous treatment with the non-pegylated IFN.

Branded PEG-Heberon®, the IFN α 2b-PEG 40 kDa molecule provides a therapeutic alternative for the chronic hepatitis C virus population to increase, at least, in 9 % the therapeutic sustained virological response in respect with the response obtained with the conventional alpha IFN variant plus ribavirin. It also increases in 27 % the benefits in respect to the response obtained with the monotherapy with the conventional alpha IFN variant for the control of the chronic hepatitis C.

The satisfactory clinical results obtained with PEG-Heberon® in Cuban patients suffering from chronic hepatitis C, mainly subtype 1b which is the highly refractory to treatment with conventional IFN α, were considered as promising by the gastroenterologists involved. The percentage of SVR was within the reported range in all the evaluated timepoints, equivalent to the response demonstrated by other products available in the market.

*R*elevance of the study

A technological process was obtained for the conjugation and purification of the conjugate, which complies with the international standards for pharmaceutical products of parenteral use for the treatment of human diseases such as hepatitis C.

The IFN α 2b molecule conjugated to 40 kDa PEG was obtained stable, and proved to be safe when administered by parenteral route. This was the first time that a biosimilar pegylated IFN formulation was developed in Cuba, showing an increased half time in blood as compared to the native IFN molecule, and extending its bioavailability to further reduce the number of administrations required to achieve the therapeutic effect needed. Moreover, a higher therapeutic effect of the conjugated molecule was demonstrated, in respect to the non-conjugated molecule. Moreover, this was the first report on the conjugation of a ramified PEG molecule to IFN α 2b.

A significant therapeutic efficacy was attained, with an adequate balance between the adverse events recorded in relation to the number of PEG-Heberon® vials administered, and no severe adverse event was reported. The conjugated molecule was shown as equivalent to Pegasys®. Thus, it could be concluded that the designed and developed IFN α 2b- 40 kDa PEG formulation branded PEG-Heberon® is a therapeutic formulation with an adequate therapeutic safety and efficacy profile supporting its use for further clinical testing and further use against chronic infections requiring it or other diseases in humans.

*A*ckowledgements

7. World Medical Association. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. 2013 [cited 2015 Aug 18]. Available from: http://www.wma.net/en/30publications/10policies/b3/.

8. García I, González CA, Valenzuela C, Díaz A, Cruz M, Nodarse H, *et al*. Pharmacokinetic and pharmacodynamic comparison of two "pegylated" interferon alpha-2 formulations in healthy male volunteers: a randomized, crossover, double-blind study. BMC Pharmacology. 2010;10(1):15.