Rational Design of a Potent, Long-Lasting Form of Interferon: A 40 kDa Branched Polyethylene Glycol-Conjugated Interferon α-2a for the Treatment of Hepatitis C

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A potent, long-lasting form of interferon α-2a mono-pegylated with a 40 kilodalton branched poly(ethylene glycol) was designed, synthesized, and characterized. Mono-pegylated interferon α-2a was comprised of four major positional isomers involving Lys31, Lys121, Lys131, and Lys134 of interferon. The in vitro anti-viral activity of pegylated interferon α-2a was found to be only 7% of the original activity. In contrast, the in vivo antitumor activity was severalfold enhanced compared to interferon α-2a. Pegylated interferon α-2a showed no immunogenicity in mice. After subcutaneous injection of pegylated interferon α-2a, a 70-fold increase in serum half-life and a 50-fold increase in mean plasma residence time concomitant with sustained serum concentrations were observed relative to interferon α-2a. These preclinical results suggest a significantly enhanced human pharmacological profile for pegylated interferon α-2a. Results of Phase II/III Hepatitis C clinical trials in humans confirmed the superior efficacy of pegylated interferon α-2a compared to unmodified interferon α-2a.

INTRODUCTION

Interferon α is comprised of a family of closely related, species-restricted proteins that exhibit anti-viral, anti-tumor, and immunomodulatory activities (1). The recombinantly produced human interferon α-2a and interferon α-2b (1) have undergone extensive clinical investigations and have become useful drugs for both viral and oncological indications (2–6).

The reported terminal half-life of interferon α ranges from 4 to 8 h, with peak serum concentrations occurring at 3–8 h following intravenous (i.v.) or subcutaneous (s.c.) administration (7). Little or no interferon α is detected 24 h following i.v. or s.c. administration (7, 8), necessitating frequent dosing of interferon α to achieve sustained efficacy. The frequency of interferon α treatment is dependent upon the disease being treated. For example, certain oncological indications require daily dosing, while viral indications may require only three times weekly dosing. Interferon α treatment for these diseases at the above-mentioned intervals may range from periods of several months to a year or longer. Furthermore, frequent treatment with interferon α produces several dose-dependent side effects (9).

In recent years, poly(ethylene glycol) (PEG) conjugated biomolecules have been shown to exhibit superior clinically useful properties than their corresponding unmodified parent molecules (10–12). These include better physical and thermal stability, greater protection against proteolytic degradation, higher solubility, longer in vivo circulating half-lives, and lower clearance, thus enhancing efficacy. Additional qualities of pegylated proteins are reduced immunogenicity and antigenicity, as well as reduced toxicity.

Hepatitis C virus (HCV) infection is a blood-borne disease of the liver. In the United States alone, an estimated four million Americans are infected with HCV and up to 180 000 new infections occur each year. Currently, the only available treatment for HCV is with interferon, injected three times per week. However, the patient population experiencing sustained response of undetectable levels of HCV is only 10–15%.

In the early 1990s, an attempt was made to develop a pegylated form of interferon α-2a for hepatitis C. The desired target profile was once a week dosing with comparable efficacy to interferon α-2a given three times weekly. Interferon α-2a was pegylated with a linear 5K PEG via urea linkage (13). In 1994, development of first generation PEG-interferon α-2a with once a week dosing was discontinued during Phase II clinical trial because efficacy equivalent to interferon α-2a given three times weekly was not achieved.

Since then, many new insights have been gained on the pegylation of protein therapeutics. Among them are (1) in vitro activity (cell-based) of a number of cytokines, whose pharmacological effects involve receptor interactions, is shown to be inversely proportional to the attached PEG mass and is not predictive of in vivo biological activity (12, 14, unpublished data); (2) one large PEG at a single site is preferable to several small PEGs attached at multiple sites (12); (3) linear PEG is distributed throughout the body with larger distribution volume (15); (4) branched PEG is distributed with a smaller distribution volume and early on delivered to the liver.
and spleen (15); (5) branched PEG protein conjugates exhibit increased pH and thermal stability, as well as greater stability toward proteolytic digestion (16); (6) smaller linear PEGs may deposit in the kidney vacuoles (17).

Another major development was the elucidation of a model, which predicts efficacy based upon combined pharmacokinetic and pharmacodynamic analyses (8, 18). Of great importance, the model predicts a correlation between efficacy and the trough levels of interferon-induced surrogate marker protein, 2′,5′-oligoadenylate synthetase (2′,5′-OAS) in peripheral blood mononuclear cells (PBMs), but not with peak levels or area under the curve of 2′,5′ OAS. Pharmacokinetic data obtained from animal studies can also be used to develop simulations that will indicate what might occur in patients who receive various doses and regimens of PEG-interferon α-2a.

The ability to predict the efficacy of PEG-interferon α-2a, coupled with the latest advancements in pegylation technology convinced us to pursue the development of a second generation PEG-interferon α-2a with hepatitis C virus (HCV) as the first target indication.

A 40K branched PEG was selected because of the possibility that it would facilitate the delivery of PEG-interferon α-2a to the liver and its ability to circulate for a long period of time in the blood. Thus, interferon α-2a conjugated with a branched 40K PEG (PEG2-IFN) would have a better chance of eradicating hepatic and extrahepatic HCV infection.

EXPERIMENTAL PROCEDURES

Materials. Interferon α-2a (IFN) and 5K PEG-IFN were obtained from the Biopharmaceuticals Department of Hoffmann-La Roche Inc. A branched 40K succinimidyl derivative of PEG (PEG2-NHS) was purchased from Shearwater Polymers Inc. (Huntsville, AL). Fractogel EMD CM 650(M) was supplied by EM Separations (Gibbstown, NJ). Precast SDS–PAGE gels and electrophoresis units were purchased from Novex (San Diego, CA). Titrisol, concentrated iodine solution, was from EM Science (Gibbstown, NJ). Anti-PEG AGP3 monoclonal antibody was acquired from Academia Sinica (Taipei, Taiwan). All other reagents used were of highest quality available. The cannulated rats and BDF-1 mice were supplied by Charles River Laboratories (Wilmington, MA).

Preparation of PEG2-IFN. Pegylation reaction mixture was comprised of interferon α-2a and 40K PEG2-NHS reagent at 1:3 molar ratio in 50 mM sodium borate buffer, pH 9.0. The final protein concentration was ~5 mg/mL. Reaction mixture was stirred for 2 h at 4 °C. Reaction was stopped by adjusting the pH of the mixture to 4.5 with glacial acetic acid.

Reaction mixture was diluted 10-fold with water and applied onto a column packed with Fractogel EMD CM 650(M) resin, previously equilibrated with 20 mM sodium acetate, pH 4.5, at a linear velocity of 1.3 cm/min. Protein loading onto the column was 2 mg/mL. Column was washed with the equilibration buffer to remove excess PEG reagent, reaction byproducts and PEG2-IFN oligomers. The desired mono-pegylated PEG2-IFN was then eluted with 200 mM sodium chloride in the equilibration buffer. Unmodified interferon α-2a still adsorbed onto the column was removed by washing with 750 mM sodium chloride in the equilibration buffer. Mono-pegylated PEG2-IFN eluate was further concentrated to approximately 1 mg/mL and diafiltered into the final storage buffer, 20 mM sodium acetate, pH 5.0, containing 150 mM sodium chloride. Concentrated PEG2-IFN was sterile filtered with a 0.2 μm filter and stored at 4 °C.

Protein Determination. Protein concentrations were determined using an A 280 value of a 1 mg/mL solution of PEG2-IFN as 1.05.

SDS–PAGE Analysis. Reaction mixture and the purified PEG2-IFN was subjected to sodium dodecyl sulfate/polyacrylamide (8–16%) gel electrophoresis according to the methods of Laemmli (19) and stained for protein using Coomassie blue dye.

PEG moieties in the PEG2-IFN conjugates were specifically stained using Titrisol iodine solution (EM Science, Gibbstown, New Jersey) according to a modified procedure of Küffürst (20). The SDS–PAGE gel was rinsed with distilled water and placed in 5% barium chloride solution. After 10 min, the above gel was washed with distilled water and placed in 0.1N Titrisol iodine solution for another 10 min. Titrisol was washed off with distilled water. The PEG stained (orange brown bands) SDS–PAGE gel containing PEG2-IFN samples was stored in distilled water in a heat-sealed Kapak/Scotchpak bag.

Western Blotting with Anti-PEG AGP3 Monoclonal Antibody. PEG2-IFN was transferred from SDS–PAGE gel to nitrocellulose membrane using standard techniques. An anti-PEG monoclonal antibody, denoted AGP3 (21), was used as the primary antibody. Development was performed according to the manufacturer’s (Pierce) instructions using SuperSignal ULTRA Western blotting kit for the detection of mouse IgG (Pierce). Secondary antibody used was goat anti-mouse IgG conjugated with horseradish peroxidase. An ultra sensitive chemiluminescent compound provided as part of the Western blotting kit (Pierce) was used as the enzyme substrate.

Amino Acid Analysis. N-Terminal sequence and amino acid composition analyses were performed with a postcolumn ninhydrin amino acid analyzer (22).

Mass Spectrometry. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI TOF MS) was used to determine the true molecular weight of PEG2-IFN (13).

Determination of Pegylation Sites. Positional isomers of PEG2-IFN were separated as described (13) using cation exchange HPLC. Separated positional isomers of PEG2-IFN were then analyzed by a combination of analytical methods including, peptide mapping, sequencing and mass spectrometry, as described (13).

Determination of Endotoxin Levels. Endotoxin level in PEG2-IFN was determined using the LAL endotoxin kit. Separated positional isomers of PEG2-IFN were analyzed by a combination of analytical methods including, peptide mapping, sequencing and mass spectrometry, as described (13).

In vitro Anti-Viral Activity. The in vitro anti-viral activities of interferon α-2a and PEG2-IFN were determined in a cell culture bioassay employing Mardin-Darby bovine kidney cells challenged with vesicular stomatitis virus as described (23).

In Vivo Antitumor Activity. Athymic nude mice (Harlan) received a subcutaneous implant of 2 × 10^6 human renal A498 cells or human renal ACHN cells or human renal G402 cells under the rear flank. Three to
six weeks were allowed for the tumors to get established. The size criteria for acceptance into the study was 0.05–0.50 cubic centimeters (Vol. = L × W²). Mice were treated once weekly (Monday) with each of the dosages of 30, 60, 120, or 300 μg of PEG2-IFN. In the case of interferon α-2a the mice were treated thrice weekly (Monday, Wednesday, and Friday) with 10, 20, 40, or 100 μg of interferon α-2a. The duration of treatment was 4–5 weeks depending on tumor growth. Tumor volumes were measured every Monday prior to treatments.

Pharmacokinetics. Female Sprague Dawly rats, surgically implanted with jugular cannulas with an average body mass of 240–260 g were housed individually, allowed free access to food and water and maintained in a 12 h light/dark cycle. Within 4–6 h after arrival, jugular cannulas were flushed with PBS (phosphate buffered saline). The following day, after flushing with 0.15–0.2 mL PBS, 2 × 10⁶ units of interferon α-2a and PEG2-IFN were injected, followed by injection of 0.15–0.2 mL of PBS to ensure that all drug was washed into the animal. Thus, each animal received a dosage of 8 × 10⁶ interferon units/kg body weight.

Blood samples were drawn at 0.5, 1, 3, 5, 12, and 24 h after injection of the drugs. At all time points, after discarding the first 0.15–0.2 mL of blood, an aliquot of 0.5 mL of blood was withdrawn using a fresh syringe via the jugular cannula. Samples were discharged into serum separator tubes at room temperature and centrifuged at 14000g in a refrigerated Eppendorf centrifuge for 10 min. The separated sera were then transferred into 1.5 mL microfuge tubes and kept frozen at −80 °C until ready for the determination of anti-viral activity. Serum samples were diluted appropriately and the anti-viral activity was determined as described previously (23).

Neutralizing Antibodies. Normal BDF-1 mice (10/group) were injected intraperitoneally once per day five times a week with interferon α-2a, aggregates of interferon α-2a, 5K PEG-IFN and PEG2-IFN, each having 300 000 units of anti-viral activity. Blood samples were taken 19 days following the last injection and the serum was evaluated for neutralizing antibodies as described (24).

RESULTS

Description of Branched Pegylation Reagent. PEG2-NHS (Figure 1) consists of two monomethoxy poly(ethylene glycol) chains each having an average molecular weight of 20K, linked to a lysine molecule via (ethylene glycol) chains each having an average molecular weight of 20K, linked to a lysine molecule via (ethylene glycol) forming an amide bond between the two.

Synthesis of Pegylated Interferon α-2a. PEG2-IFN was formed as a result of the reaction between an N-hydroxysuccinimide ester derivative of a 40K branched poly(ethylene glycol) forming an amide bond between the two. Thus, PEG2-IFN appears to have a higher molecular weight than the calculated molecular weight when compared to standard PEG molecular weight marker proteins. However, in Figure 2B, PEG2-IFN has a molecular weight corresponding to its expected molecular weight of 59–60K when compared to standard PEG molecular weight markers. This result is consistent with that obtained by MALDI-TOF mass spectrometry (data not shown).

Western blotting with AGP3 antibody directed against PEG (21) demonstrated that the purified PEG2-IFN is authentic, predominantly mono-pegylated and did not contain fragments of PEG or significant amounts of oligomeric forms of PEG2-IFN (Figure 3).

Both interferon α-2a and PEG2-IFN have identical amino acid sequence and amino acid composition (data not shown). The only difference in the composition of
native and modified proteins is due to the formation of an amide bond between the amino group of interferon R-2a and the PEG molecule at the point of attachment (Figure 1).

A combination of high performance cation exchange chromatography, peptide mapping, amino acid sequencing and mass spectrometric analyses were performed to identify the pegylation sites (data not shown). A single unit of branched poly(ethylene glycol) was found to be attached to either one of Lys31, Lys121, Lys131, or Lys134. Collectively, ~94% of the PEG attachment takes place in these four sites. The remaining ~6% of the pegylation takes place at Lys70 and Lys83. The N-terminal cysteine, which is involved in a disulfide bond (Cys1-Cys98), does not appear to be a pegylation site.

In Vitro Anti-Viral Activity. The anti-viral activities of interferon α-2a and PEG2-IFN are compared in a cell culture bioassay utilizing Mardin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus. This assay measures interferon's inhibitory effect on virus induced cell lysis (cytopathic effect). Procedure involves the addition of serially diluted interferon samples MDBK cells followed by a challenge with virus. The endpoint of the assay is approximately a 50% protection of MDBK cells. The quantity of interferon present is determined relative to a reference preparation of interferon. The unit of anti-viral activity is an arbitrarily established value by the World Health Organization. PEG2-IFN had an anti-viral specific activity of approximately 7% of that of interferon α-2a. Results are listed in Table 1.

Table 1. In Vitro Antiviral Activities of Interferon α-2a and PEG2-IFN

<table>
<thead>
<tr>
<th>protein</th>
<th>anti-viral activity (IU/mg)</th>
<th>residual activity (%)</th>
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<tr>
<td>interferon α-2a</td>
<td>2 \times 10^8</td>
<td>100</td>
</tr>
<tr>
<td>PEG2-IFN</td>
<td>1.4 \times 10^7</td>
<td>7</td>
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</table>

*Antiviral activity determined in MDBK cells infected with vesicular stomatitis virus.

In Vivo Antitumor Activity. The in vivo antitumor activity of PEG2-IFN and interferon α-2a were evaluated by studying their ability to reduce the size of various human tumor cells implanted subcutaneously into mice. PEG2-IFN showed a marked reduction in human renal A498 tumor size (Figure 4, bottom panel) compared to interferon α-2a (Figure 4, top panel) for all dosage levels tested, at 7, 14, 21, and 28 days after the beginning of treatment. After 4 weeks of treatment and 7 days after treatment was discontinued, three mice in each group were sacrificed. In the three mice treated with PEG2-IFN, no residual tumor was observed. In the mice treated with interferon α-2a, tumor weight was 1.28, 0.62, and 1.6 g, respectively. In the three control mice, the A498 tumor weights were 2.32, 2.37 and 1.94 g, respectively. Eighty days following termination of PEG2-IFN treatment, all seven remaining mice were free of tumor as determined by palpation in the 4-week treatment group. Similarly, PEG2-IFN effected a significant reduction in the size of ACHN and G402 human renal tumors (data not shown).

Pharmacokinetic Parameters. Pharmacokinetic parameters of PEG2-IFN were determined after subcutaneous injection into female Sprague–Dawley rats. Figure 5 illustrates the mean serum activity versus time. Summary and individual pharmacokinetic parameters are found in Tables 2 and 3. Serum activity of interferon α-2a peaked 1 h after the subcutaneous injection, whereas the peak activity of PEG2-IFN occurred 24 h after the injection. PEG2-IFN's peak activity was 3-fold greater.
than that of interferon R-2a. Area under the curve, up to the last measurable time point of PEG 2-IFN, was 6-fold greater than that of interferon R-2a. After subcutaneous injection of PEG2-IFN, a 70-fold increase in half-life and a 50-fold increase in mean residence time (MRT) were observed. $C_{\text{max}}$ and AUC of the PEG2-IFN were 3.5- and 59-fold increased, respectively compared to those of interferon α-2a. Consequently, PEG2-IFN has a significantly increased plasma exposure.

Neutralizing Antibodies. As seen in Table 4, mice injected with PEG2-IFN produced no detectable amount of neutralizing antibodies in 9 of the 10 animals tested. In contrast, mice injected with interferon R-2a produced neutralizing antibodies and this response was greatly pronounced in mice injected with interferon R-2a aggregates. A much-reduced response was observed with the first generation PEG-interferon R-2a in which the PEG moiety was linear and had a molecular weight of 5K.

DISCUSSION

To take full advantage of the clinically useful properties of branched poly(ethylene glycol) and to meet the minimum threshold PEG mass requirement, we chose a 40K branched poly(ethylene glycol) moiety for the pegylation of interferon α-2a. PEG2-IFN was well-characterized using various analytical methods including immunoblotting with a newly discovered high-affinity AGP3 monoclonal antibody directed against PEG. The discovery and availability of this antibody opens whole new avenues to follow the metabolic fates of PEG2-IFN and other pegylated protein therapeutics.
in mice for example, are much longer (days) and are reach peak activity. On the other hand, in vivo bioassays as well as constituting the receptor recognition site. These

R

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Contrary to the reduced in vitro anti-viral activity of PEG2-IFN, a significantly enhanced in vivo antitumor activity was observed in various transplanted human tumors. Reduced in vitro activity accompanied with increased in vivo biological activity has been observed with other pegylated protein therapeutics. Examples are G-CSF (28), IL-2 (29), TNF-α (30), IL-6 (31), and CD4-IgG fusion protein (32), among others. The enhanced antitumor activity suggests that PEG2-IFN is potentially a far superior antitumor agent than interferon α-2a. Consequently, there are a number of clinical trials ongoing or planned for the treatment of severe human interferons are generally immunogenic restriction PEG-interferon

production of antibodies against interferon α-2a in mice. Therefore, the lack of immunogenicity in mice is a good indication that it will be less immunogenic in humans.

A Phase II clinical trial was initiated in 1998 with PEG2-IFN in noncirrhotic patients. The Phase II clinical trial results (33) demonstrated that hepatitis C patients treated with PEG2-IFN once weekly showed undetectable levels of the virus in 76% of the patients, 12 weeks after treatment. Another 36% of patients had similar response, 24 weeks following completion of treatment. On the other hand, only 17% of the patients who had undergone the standard interferon α-2a treatment experienced undetectable levels of hepatitis C virus after 12 weeks of

two domains are located on the surface of interferon α-2a and are in close spatial proximity. The four major pegylation sites in PEG2-IFN also fall within these domains. In the cell-based in vitro assay with the relatively short incubation time combined with the sites of pegylation plays a major role in how many chances PEG2-IFN gets to interact with its receptor. However, in vivo biological effect is not expected to be affected in a similar fashion. Though there may be some interference in receptor binding due to pegylation, the very large circulating half-life combined with the flexibility of poly-(ethylene glycol) provides enough opportunities for interferon α-2a to come into contact with the receptor to exert its biological effect.

PEG2-IFN is comprised of mono-pegylated interferon α-2a (1 PEG/mol of interferon α-2a) but distributed among four major pegylation sites. In addition to the N-terminus, there are 11 lysines available for pegylation. It should be noted that when a low molecular weight poly-(ethylene glycol) (e.g., 5K) was used for pegylation of interferon α-2a all 11 lysines were pegylated (13), thus more heterogeneous than the PEG2-IFN in terms of positional isomers. Not all pegylation sites are readily accessible to the 40K branched PEG due to its larger size and shape. The end-result is fewer sites are pegylated. Thus PEG2-IFN is less heterogeneous than the linear low molecular weight PEG2-IFN.

It is postulated that the chemical attachment of PEG to the polypeptide backbone of interferon α-2a produces some form of conformational perturbances, which may adversely affect receptor/ligand interactions and thus lower binding affinity (12). This is one reason in vitro anti-viral activity is considerably decreased compared to interferon α-2a. In addition, the relatively short incubation time (hours) of the in vitro assay is probably insufficient for the low affinity PEG2-IFN to reach peak activity. On the other hand, in vivo bioassays in mice for example, are much longer (days) and are terminated after several days after the injection of the drug. The extended exposure time afforded by the longer half-life of the PEG2-IFN (Tables 2 and 3) compensates for any slack in the binding interactions due to pegylation. The end-result is maximum biological effect. A sustained release prodrug mechanism is ruled out because the amide bond between PEG and interferon α-2a is stable.

Fish (26) and Klaus et al. (27) define amino acid domains Cys29–Asp35 and Phe123–Trp140 in interferon α-2a as responsible for receptor binding interactions, as well as constituting the receptor recognition site. These

Table 2. Summary of Interferon Pharmacokinetic Parameters

<table>
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<td>SD</td>
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Table 3. Summary of PEG2-IFN Alfa-2a Pharmacokinetic Parameters

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Table 4. Summary of Immunogenicity Data

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<th>antibody (INU/mL)</th>
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<tr>
<td>interferon α-2a</td>
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<td>first generation 5 KDa PEG2-IFN α-2a</td>
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<tr>
<td>PEG2-IFN interleaver α-2a</td>
<td>0–1136</td>
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a Interferon neutralizing units/mL. INU/mL = (units of interferon neutralized × serum dilution)/(final test volume). b One of 10 animals in the group produced neutralizing antibody.


