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 Lys^{31}, Lys^{121}, Lys^{131}, and Lys^{134} 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, antitumor, 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

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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 (PBMNs), 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 (PEG_2-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 (PEG_2-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). Super Signal ULTRA Western blotting kit was provided by Pierce (Rockford, IL). The LAL endotoxin kit was obtained from Associates of Cape Cod (Woods Hole, MA). 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 PEG_2-IFN. Pegylation reaction mixture was comprised of interferon α-2a and 40K PEG_2-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 gel. Column was washed with the equilibration buffer to remove excess PEG reagent, reaction byproducts and PEG_2-IFN oligomers. The desired mono-pegylated PEG_2-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 PEG_2-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 PEG_2-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 PEG_2-IFN as 1.05.

SDS−PAGE Analysis. Reaction mixture and the purified PEG_2-IFN was subjected to sodium dodecyl (lauryl) 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 PEG_2-IFN conjugates were specifically stained using Titrisol iodine solution (EM Science, Gibbstown, New Jersey) according to a modified procedure of Kürfurst (*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 0.1 N Titrisol iodine solution for another 10 min. Titrisol was washed off with distilled water. The PEG stained (orange brown bands) SDS−PAGE gel containing PEG_2-IFN samples was stored in distilled water in a heat-sealed Kapak/Scotchpak bag.

Western Blotting with Anti-PEG AGP3 Monoclonal Antibody. PEG_2-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 chemilluminescent 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 PEG_2-IFN (*13*).

Determination of Pegylation Sites. Positional isomers of PEG_2-IFN were separated as described (*13*) using cation exchange HPLC. Separated positional isomers of PEG_2-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 PEG_2-IFN preparation was determined using the lumulus amebocyte lysate (LAL) method according to the manufacturer's (Associates of Cape Cod) instructions.

In vitro Anti-Viral Activity. The in vitro anti-viral activities of interferon α-2a and PEG_2-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

[1] Abbreviations: IFN, interferon; PEG, poly(ethylene glycol); HCV, hepatitis C virus; PEG_2-NHS, branched succinimidyl ester derivative of PEG; PEG_2-IFN, 40K PEG conjugated interferon α-2a; PEGASYS, registered name of 40K branched PEG-conjugated IFN-α-2a.

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. $= L \times W^2$). Mice were treated once weekly (Monday) with each of the dosages of 30, 60, 120, or 300 μg of PEG_2-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, 2×10^6 units of interferon α-2a and PEG_2-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 8×10^6 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 14000*g* 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 intraperitonially once per day five times a week with interferon α-2a, aggregates of interferon α-2a, 5K PEG-IFN and PEG_2-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. PEG_2-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 urethane bonds, one at the α-amino group and the other at the ϵ-amino group of lysine. The carboxyl group of lysine in turn is derivatized to an *N*-hydroxysuccinimide ester (*16*).

Synthesis of Pegylated Interferon α-2a. PEG_2-IFN was formed as a result of the reaction between an *N*-hydroxysuccinimide ester derivative of a 40K branched PEG molecule and the free amino group of interferon α-2a forming an amide bond. The reaction mechanism is illustrated in Figure 1.

Under the experimental conditions used, pegylation reaction products were a mixture containing approximately 45−50% monomer, 5−10% oligomers (predominantly dimer), and 40−50% unmodified interferon α-2a, as determined by densitometric measurements and by mass spectrometry (data not shown). Purified PEG_2-IFN was ∼95−99% mono-pegylated, as determined by SDS−PAGE analysis (both protein and PEG staining, Figure 2, respectively) and mass spectrometry, as well as by Western blotting with a newly discovered high-affinity monoclonal antibody which recognizes PEG (*21*) (Figure 3). PEG_2-IFN preparation contained <2.5 endotoxin units/mg.

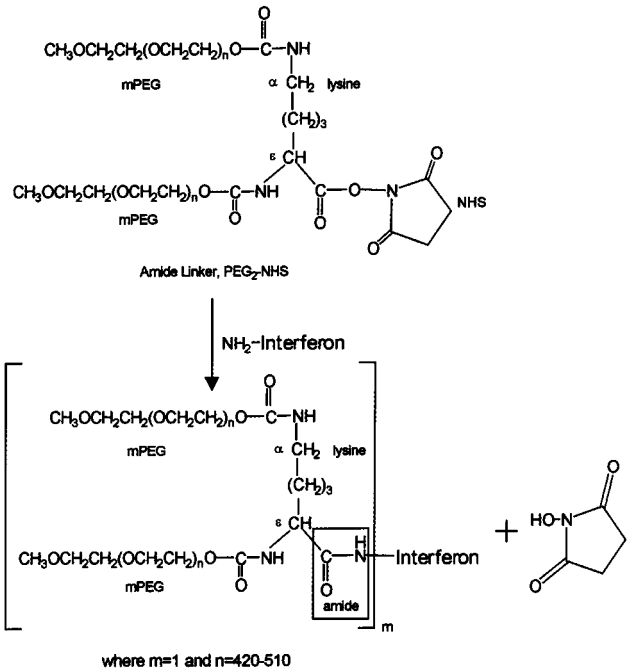

Figure 1. Reaction mechanism of the synthesis of PEG_2-IFN. The free amino groups of interferon α-2a react with the *N*-hydroxysuccinimide ester derivative of a 40K branched poly(ethylene glycol) forming an amide bond between the two.

Characterization of PEG_2-IFN. Upon the basis of the 40K average molecular weight of the branched PEG moiety and the 19.2K molecular weight of interferon α-2a, it is estimated that the molecular weight of PEG_2-IFN is ∼59.2K. However, the actual molecular weight of PEG_2-IFN is dependent upon the average molecular weight (39−43K) of PEG_2-NHS reagent used in the pegylation reaction.

Figure 2 illustrates the SDS−PAGE profiles of the pegylation reaction mixture, purified PEG_2-IFN and interferon α-2a as determined by protein specific and PEG-specific staining. In addition to the various PEG_2-IFN conjugates (mono-, di-, tri-, etc.), reaction mixture contains unmodified interferon α-2a and excess PEG reagent (Figure 2). PEG_2-IFN's electrophoretic mobility is considerably slowed because of PEG's large hydrodynamic volume (3 mol of water form an adduct with each ethylene oxide subunit of PEG) (*25*). In addition, branched PEG could get trapped into the polyacrylamide gel matrix, further slowing down the electrophoretic mobility. Thus, PEG_2-IFN appears to have a higher molecular weight (∼96K, Figure 2A) than the calculated molecular weight when compared to molecular weight marker proteins. However, in Figure 2B, PEG_2-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 PEG_2-IFN is authentic, predominantly mono-pegylated and did not contain fragments of PEG or significant amounts of oligomeric forms of PEG_2-IFN (Figure 3).

Both interferon α-2a and PEG_2-IFN have identical amino acid sequence and amino acid composition (data not shown). The only difference in the composition of

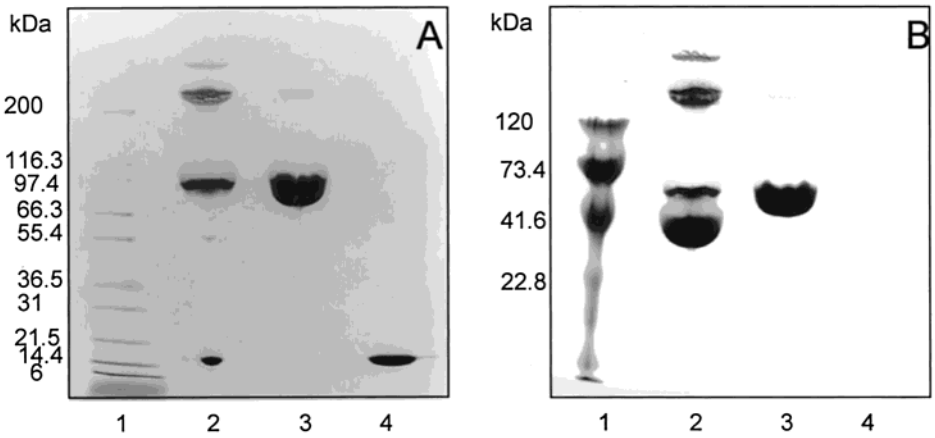

Figure 2. SDS−PAGE analysis. (A) Specifically stained for protein with Coomassie blue. Lanes: 1, molecular weight marker proteins; 2, pegylation reaction mixture; 3, purified PEG_2-IFN; and 4, interferon α-2a. (B) Specifically stained for PEG with iodine. Lanes: 1, molecular weight marker PEGs; 2−4, same as in Figure 2A. Note that lane 4 containing interferon α-2a in gel B is not stained by iodine.

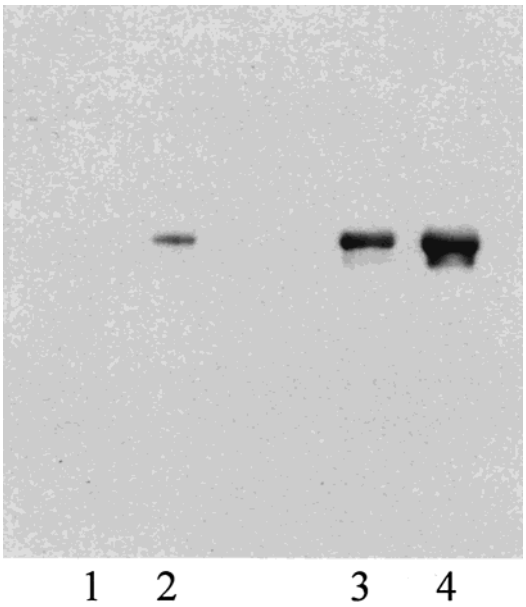

Figure 3. Western blotting of PEG_2-IFN with AGP3 monoclonal antibody directed against PEG. Five second exposure of the film. Lanes: 1, interferon α-2a (1 μg), as expected, no recognition by AGP3 antibody; 2, PEG_2-IFN (0.1μg); 3, PEG_2-IFN (0.5 μg); and 4, PEG_2-IFN (1μg).

native and modified proteins is due to the formation of an amide bond between the amino group of interferon α-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 Lys^{31}, Lys^{121}, Lys^{131}, or Lys^{134}. Collectively, ~94% of the PEG attachment takes place in these four sites. The remaining ~6% of the pegylation takes place at Lys^{70} and Lys^{83}. The N-terminal cysteine, which is involved in a disulfide bond (Cys^1−Cys^{98}), does not appear to be a pegylation site.

In Vitro Anti-Viral Activity. The anti-viral activities of interferon α-2a and PEG_2-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. PEG_2-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 PEG_2-IFN[a]

protein	anti-viral activity (IU/mg)	residual activity (%)
interferon α-2a	2×10^8	100
PEG_2-IFN	1.4×10^7	7

[a] Antiviral activity determined in MDBK cells infected with vesicular stomatitis virus.

In Vivo Antitumor Activity. The in vivo antitumor activity of PEG_2-IFN and interferon α-2a were evaluated by studying their ability to reduce the size of various human tumor cells implanted subcutaneously into mice. PEG_2-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 PEG_2-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 PEG_2-IFN treatment, all seven remaining mice were free of tumor as determined by palpation in the 4-week treatment group. Similarly, PEG_2-IFN effected a significant reduction in the size of ACHN and G402 human renal tumors (data not shown).

Pharmacokinetic Parameters. Pharmacokinetic parameters of PEG_2-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 PEG_2-IFN occurred 24 h after the injection. PEG_2-IFN's peak activity was 3-fold greater

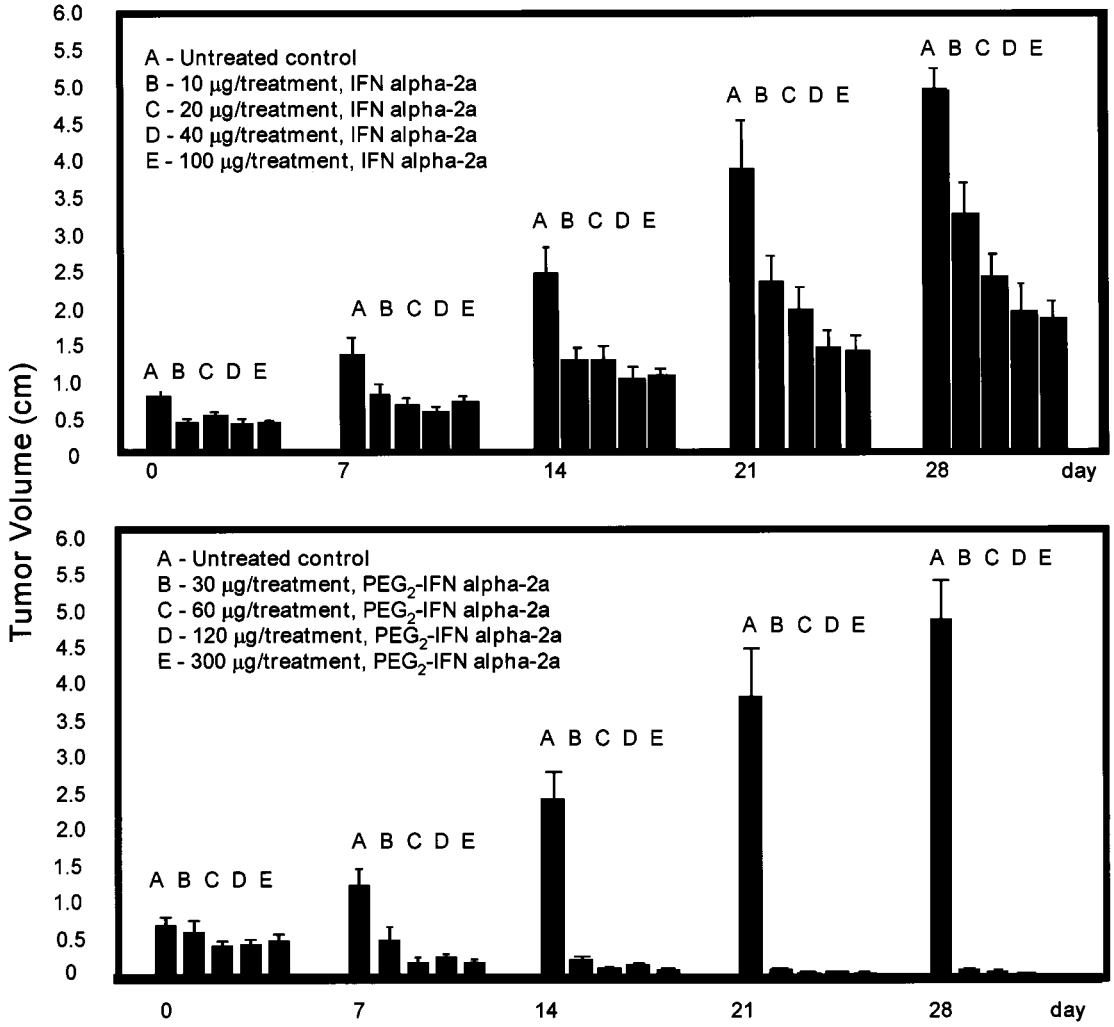

Figure 4. In vivo antitumor activity of interferon α-2a (top panel) and PEG_2-IFN (bottom panel) in athymic nude mice subcutaneously implanted with human renal A498 cells. Insert shows the amount of interferon α-2a and PEG_2-IFN used in the treatment of the mice implanted with the tumor. *x*- and *y*-axes indicate the days and the corresponding tumor volumes, respectively.

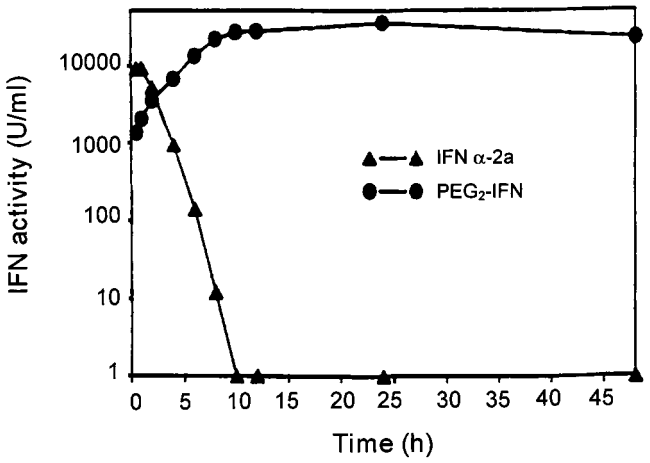

Figure 5. Mean serum activity versus time of interferon α-2a and PEG_2-IFN after subcutaneous injection in rats. PEG_2-IFN has a 51-h half-life compared to 0.7-h for interferon α-2a concomitant with mean plasma residence time of 80 and 1.6 h, respectively. Logarithmic regression analyses were used to calculate the pharmacokinetic parameters.

than that of interferon α-2a. Area under the curve, up to the last measurable time point of PEG_2-IFN, was 6-fold greater than that of interferon α-2a. After subcutaneous injection of PEG_2-IFN, a 70-fold increase in half-life and a 50-fold increase in mean residence time (MRT) were observed. C_{max} and AUC of the PEG_2-IFN were 3.5- and 59-fold increased, respectively compared to those of interferon α-2a. Consequently, PEG_2-IFN has a significantly increased plasma exposure.

Neutralizing Antibodies. As seen in Table 4, mice injected with PEG_2-IFN produced no detectable amount of neutralizing antibodies in 9 of the 10 animals tested. In contrast, mice injected with interferon α-2a produced neutralizing antibodies and this response was greatly pronounced in mice injected with interferon α-2a aggregates. A much-reduced response was observed with the first generation PEG-interferon α-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. PEG_2-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 PEG_2-IFN and other pegylated protein therapeutics.

Table 2. Summary of Interferon Pharmacokinetic Parameters

	interferon pharmacokinetic parameters								
rat no.	T_{max} (h)	C_{max} (units/mL)	T_{last} (h)	C_{last} (units/mL)	AUC_{last} (units h/mL)	half-life (h)	AUCinf (units h/mL)	AUC_% extrap	MRT (h)
1	1	10 726	6	113	22 202	0.7	22 318	0.5	1.5
2	0.5	12 299	4	401	20 840	0.7	21 250	1.9	1.4
3	1	11 786	6	28	22 833	0.5	22 854	0.1	1.4
4	1	5888	8	44	18 402	0.7	18 448	0.2	2.2
mean	0.9	10 175	6	147	21 069	0.7	21 217	0.7	1.6
SD	0.3	2932	1.6	174	1963	0.1	1963	0.8	0.4

Table 3. Summary of PEG_2-IFN Alfa-2a Pharmacokinetic Parameters

	PEG_2-IFN alfa-2a pharmacokinetic parameters								
rat no.	T_{max} (h)	C_{max} (units/mL)	T_{last} (h)	C_{last} (units/mL)	AUC_{last} (units h/mL)	half-life (h)	AUCinf (units h/mL)	AUC_% extrap	MRT (h)
1	24	42 220	48	21 110	1 530 853	45	2 911 253	47	66
2	24	36 200	48	27 215	1 277 506	58	3 566 959	64	94
3	24	27 190	48	19 225	912 794.5	48	2 243 867	59	81
mean	24	35 203	48	22 517	1 240 384	51	2 907 360	57	80
SD	0	7564	0	4177	310 697	7	661 555	9	14

Table 4. Summary of Immunogenicity Data

	antibody (INU/mL)[a]	
protein	median	range
interferon α-2a	2400	217−8533
interferon α-2a aggregates	42 667	8000−768533
first generation 5 kDa PEG2-IFN α-2a	75	50−750
PEG_2-interferon α-2a	0	0−1133[b]

[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 nuetralizing antibody.

PEG_2-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 PEG_2-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 PEG_2-IFN is less heterogeneous than the linear low molecular weight PEG-IFN.

It is postulated that the chemical attachment of PEG moieties 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 PEG_2-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 PEG_2-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 Cys^{29}−Asp^{35} and Phe^{123}−Trp^{140} in interferon α-2a as responsible for receptor binding interactions, as well as constituting the receptor recognition site. These two domains are located on the surface of interferon α-2a and are in close spatial proximity. The four major pegylation sites in PEG_2-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 PEG_2-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.

Contrary to the reduced in vitro anti-viral activity of PEG_2-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 PEG_2-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 several forms of cancer, such as renal carcinoma, malignant melanoma, chronic myelogenous leukemia, among others.

PEG_2-IFN provides extraordinary protection against production of antibodies against interferon α-2a in mice compared to the linear low molecular weight first generation PEG-interferon α-2a. Since interferons are species-restricted, human interferons are generally immunogenic in animals. 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 PEG_2-IFN in noncirrhotic patients. The Phase II clinical trial results (*33*) demonstrated that hepatitis C patients treated with PEG_2-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

treatment and only 3% maintained undetectable levels of virus for 24 weeks following the completion of treatment.

A PhaseII/III clinical trial of PEG_2-IFN in cirrhotic hepatitis C patients had shown that 29% of the patients experienced a sustained response of nondetectable levels of hepatitis C virus, compared to only 6% of patients treated with interferon α-2a. Sustained response is measured for 24 weeks following 48 weeks of treatment (*34*).

Finally, in a large Phase III clinical trial (*35*), PEG_2-IFN exhibited superior efficacy than standard interferon. Highlights of the trial include (1) at the end of treatment virological response was 69% for PEG_2-IFN compared to only 29% for interferon treated group, (2) sustained virologic response was observed in 39% of patient population, (3) in the toughest to treat patient population; those with cirrhosis, 39% experienced sustained virological response, (4) histology was significantly improved, (5) tolerability was similar to standard interferon, and (6) dosing regimen included convenient once weekly dosing compared to thrice weekly dosing of standard interferon.

It is remarkable that although PEG_2-IFN used in the clinical trials had only 5−10% of the original in vitro antiviral activity of interferon α-2a, yet it was significantly more effective in treating HCV infection in humans than the standard interferon. Thus PEG_2-IFN, with the registered name of PEGASYS is a well-tolerated and effective treatment for hepatitis C patients, including those with difficult to treat liver cirrhosis.

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LITERATURE CITED

(1) Pestka, S. (1983) The human interferons- From protein purification and sequence to cloning and expression in bacteria: Before, between and beyond. *Arch. Biochem. Biophys. 221*, 1−37.

(2) Baron, S., Tyring, S. K., Fleischmann, W. R., Jr., Coppenhaver, D. H., Niesel, D. W., Klimpel, G. R., Stanton, G. J. and Hughes, T. K. (1991) The interferons. Mechanisms of action and clinical applications. *J. Am. Med. Assoc. 266*, 1375−1383.

(3) Spiegel, R. J. (1989) Alpha interferons: A clinical overview. *Urology 34*, 75−79.

(4) Wadler, S., and Schwartz, E. L. (1990) Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: A review. *Cancer Res. 50*, 3473−3486.

(5) Lippman, S. M., Parkinson, D. R., Itri, L. M., Weber, R. S., Schantz, S. P., Ota, D. M., Schusterman, M. A., Krakoff, I. H., Gutterman, J. U., and Hong, W. K. (1992) 13-cis-retinoic acid and interferon α-a: Effective combination therapy for advanced squamous cell carcinoma of the skin. *J. Natl. Cancer Inst. 84*, 235−241.

(6) McHutchison, J. G., Gordon, S. C., Schiff, E. R., Shiffman, M. L., Lee, W. M., Rustgi, V. K., Goodman, Z. D., Ling, M. H., Cort, S., and Albrecht, J. K. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N. Engl. J. Med. 339*, 1485−1492.

(7) Wills, R. J. (1990) Clinical pharmacokinetics of interferons. *Clin. Pharmacokinet. 19*, 390−399.

(8) Barouki, F. M., Witter, F. R., Griffin, D. E., Nadler, P. I., Woods, A., Wood, D. L., and Lietman, P. S. (1987) Time course of interferon levels, anti-viral state, 2′,5′-oligoadenylate synthetase and side effects in healthy men. *J. Interferon Res.* 7, 29−39.

(9) *Physician's Desk Reference* (1999) Vol. 53, pp 2697−2701 and 2850−2858, Medical Economics Co. Inc., Montvale, NJ.

(10) Fuerteges, F., and Abuchowski, A. (1990) The clinical efficacy of poly(ethylene glycol)-modified proteins *J. Controlled Release 11*, 139−148.

(11) Katre, N. V. (1993) The conjugation of proteins with poly(ethylene glycol) and other polymers; altering properties to enhance their therapeutic potential. *Adv. Drug Delivery Sys. 10*, 91−114.

(12) Bailon, P., and Berthold, W. (1998) Poly(ethylene glycol)-conjugated pharmaceutical proteins, *Pharm. Sci. Technol. Today 1*, 352−356.

(13) Monkarsh, S. P., Ma, Y., Aglione, A., Bailon, P., Ciolek, D., DeBarbieri, B., Graves, M. C., Hollfelder, K., Michel, H., Palleroni, A., Porter, J. E., Russoman, E., Roy, S., and Pan, Y. C. (1997) Positional isomers of mono-pegylated interferon α-2a: Isolation, characterization, and biological activity. *Anal. Biochem. 247*, 434−440.

(14) Fung, W.-J., Porter, J. E., and Bailon, P. (1997) Strategies for the preparation and characterization of poly(ethylene glycol) (PEG) conjugated pharmaceutical proteins. *Polym. Prep. 38*, 565−566.

(15) Caliceti, P., Schiavon, O., and Veronese, F. M. (1999) Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers. *Bioconjugate Chem. 10*, 638−646.

(16) Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M., Veronese, F. M. (1995) A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjugate Chem. 6*, 62−69.

(17) Bendele, A., Seely, J., Richey, C., Senello, G., and Shopp, G. (1998) Renal tubular vacuolation in animals treated with polyethylene-glycol-conjugated proteins. *Toxicol. Sci. 42*, 152−157.

(18) Xu, C. Z−X., Rakhit, A., and von Brummelen, P. (1998) PK/PD modeling approach to support clinical development of a long acting interferon (RO 25−3036) for the treatment of hepatitis C. *Hepatology 28* (Suppl.), 702A.

(19) Laemmli, U. K. (1970) Cleavage of structural protein during assembly of the head bacteriophage T4. *Nature 227*, 680−685.

(20) Kurfurst, M. K. (1992) Detection and molecular weight determination of poly(ethylene glycol)-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem. 200*, 244−248.

(21) Cheng, T. L., Chen, B. M., Chern, J. W., Wu, M. F., and Roffler, S. R. (1999) Accelerated clearance of poly(ethylene glycol)-modified proteins by anti-poly(ethylene glycol) IgM. *Bioconjugate Chem. 10*, 520−528

(22) Pan, Y.-C. E., and Stein, S. (1986) Methods of Protein Characterization (J. E. Shively, Ed.) pp 155−166, The Humana Press Inc., Clifton, NJ.

(23) Rubinstein, S., Familletti, P., and Pestka, S. (1981) Convenient assay for interferons. *J. Virol. 37*, 755−758.

(24) Hennes, U., Jucker, W., Fischer, E. A., Krummenacher, T., Palleroni, A. V., Trown, P. W., Linder-Ciccolunghi, S., and Rainisio, M. (1987) The detection of antibodies to recombinant interferon alfa-2a in human serum. *J. Biol. Stand. 15*, 231−244.

(25) Maxfield, J., and Shepherd, W. (1975) Conformation of poly(ethylene oxide) in the solid state, melt and solution measured by Raman scattering. *Polymer 10*, 585−589.

(26) Fish, E. N. (1992) Definition of receptor binding domains in interferon α. *J. Interferon Res. 12*, 257−266.

(27) Klaus, W., Gsell, B., Labhardt, A. M., Wipf, B., and Senn, H. (1997) The three-dimensional high resolutions structure of human interferon alpha-2a determined by heteronuclear NMR spectroscopy in solution. *J. Mol. Biol. 274*, 661−675.

(28) Satake-Ishikawa, R., Ishikawa, M., Okada, Y., Kakitani, M., Kawagishi, M., Matsuki, S., and Asano, K. (1992) Chemical modification of granulocyte colony stimulating factor by poly(ethylene glycol) increases its biological activity in vivo. *Cell Struct. Funct. 17*, 157−160.

(29) Katre, N. V., Knauf, M. J., and Laird, W. J. (1987) Chemical modification of interleukin-2 by poly(ethylene glycol) increases its potency in the murine Meth A Sarcoma model. *Proc. Natl. Acad. Sci. U.S.A. 84*, 1487−1491.

(30) Tsutsumi, Y., Kihira, T., Yamamoto, S., Kubo, K., Nakagawa, S., Miyake, M., Horisawa, Y., Kanamori, T., Ikegami, H., and Mayumi, T. (1994) Chemical modification of natural human tumor necrosis factor-α with poly(ethylene glycol) increases its potency. *JPN. J. Cancer Res. 85*, 9−12.

(31) Inoue, H., Kadoya, T., Kabaya, K., Tachibana, K., Nishi, N., Sato, M., Ohsawa, M., Mikayama, T., and Mori, K. J. (1994) A highly enhanced thrombopoietic activity by poly-(ethylene glycol)-modified human interleukin-6. *J. Lab. Clin. Med. 124*, 529−536.

(32) Chamow, S. M., Kogan, T. P., Venuti, M., Gadek, T., Harris, R. J., Peers, D. H., Mordenti, J., Shak, S., and Ashkenazi, A. (1994) Modification of CD4 immunoadhesion IgG with monomethoxypoly(ethylene glycol) aldehyde via reductive alkylation. *Bioconjugate Chem. 5*, 133−140.

(33) Heathcote, J. (1998) *New therapy may offer advance in the treatment of chronic hepatitis C*, American Association for the Study of Liver Diseases Annual Meeting, Chicago, IL.

(34) Heathcote, J. (1999) *A controlled, randomized, multicenter, Phase II/III standard interferon α-2a versus standard interferon α-2a for treatment of patients with chronic hepatitis C and cirrhosis*, American Association for the Study of Liver Diseases Annual Meeting, Dallas, TX.

(35) Zeuzem, S., Feinman, S. V., Rasenack, J., Heathcote, E. J., Lai, M.-Y., Gane, E., O'Grady, J., Reichen, J., and Brunda, M. J. (2000) *Evaluation of the safety and efficacy of once-weekly PEG Interferon α-2a (PEGASYS) for chronic hepatitis C. A multinational, randomized study*, European Association for the Study of Liver Annual Meeting, Rotterdam.

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