Site of Pegylation and Polyethylene Glycol Molecule Size Attenuate Interferon-α Antiviral and Antiproliferative Activities through the JAK/STAT Signaling Pathway*

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Therapeutic pegylated interferon-αs (IFN-α) are mixtures of positional isomers that have been monopegylated at specific sites on the core IFN-α molecule. The pegylation results in lower in vitro specific activity associated with the core IFN-α molecule that is related to the site of pegylation and size of polyethylene glycol (PEG) attached. We prepared purified, homogeneous, positional pegylation isomers of IFN-α2b that were monopegylated using 5–30-kDa linear PEG molecules attached at 7 primary reactive amino acid residues: Cys1, His34, Lys31, Lys121, Lys131, and Lys134. The isomers were evaluated for STAT translocation and antiviral activity. The site of pegylation strongly influenced activity relative to an IFN-α2b control. The highest residual activity was observed with the His34 positional isomers, and the lowest was observed with the Cys1 positional isomers. The Lys positional isomers demonstrated intermediate activity, with a general order of Lys134 > Lys31 > Lys121 > Lys83. The progressive relationship between decreased activity and increased PEG size suggests that pegylation may interfere with interaction and binding of IFN-α to the IFNAR1-IFNAR2 heterodimeric receptor. The higher specific activity associated with the His34 positional isomer suggests that this site may be favorable for pegylating IFN-α2b molecules.

Chronic hepatitis C is considered one of the major causes of chronic liver disease, cirrhosis, and hepatocellular carcinoma and is the most common reason for liver transplantation in the United States (1). It is estimated that there are 3 million chronically infected individuals in the United States and over 170 million worldwide (1). Treatment of hepatitis C has evolved from the use of interferon-α (IFN-α), either alone or in combination with ribavirin, to the newer pegylated interferons (PEG-IFNs), which have provided a dramatic increase in virological response, especially in combination with ribavirin. Standard IFN-α therapy has a short (≤12-h) half-life that requires subcutaneous injection three times weekly to maintain effective levels in the blood (2). The short half-life of IFN-α has led to the development of longer lasting preparations achieved by the attachment of a large polyethylene glycol (PEG) molecule directly to IFN-α. Two different commercial preparations of PEG-IFN-α have been developed for clinical use, PEG-IFN-α2a (PEG-INTRON®) and PEG-IFN-α2a (Pegasys®); both have long half-lives (40 and 80 h, respectively) that permit once weekly administration (3). Both of these preparations have been demonstrated to be effective for the treatment of patients with hepatitis C (4), and clinical trial results have shown further that both of the pegylated molecules produce sustained viral response rates superior to those achieved with their respective standard IFN-αs (5–7).

Whereas pegylation has proven to be highly effective for slowing the clearance of biological molecules, including IFN-α, and thus increasing serum half-life, it has been shown to also modify in vitro biological activity (8). For instance, we have reported that pegylation of IFN-α2b with a 12-kDa linear PEG molecule results in a preparation that has a specific activity of 28% relative to IFN-α2b; the loss in activity was not due to structural perturbation of the core IFN-α2b core protein (9). Other groups have reported that pegylation of IFN-α2a with a 40-kDa branched PEG molecule results in a preparation that contains from 1 to 7% relative specific activity compared with IFN-α2a (10, 11). These two pegylated interferon-αs (PEG-IFN-αs) differ substantially in their postpegylation constituent properties. PEG-IFN-α2b has a 12-kDa linear PEG molecule attached using succinimidyl carbonate polyethylene glycol (SC-PEG) chemistry via a covalent urethane-like bond to the IFN-α2b protein (12). The pegylation linkage process results in a heterogeneous mixture of pegylation positional isomers that occur predominantly (~50%) at the His34 amino acid residue, with the remaining positional isomers pegylated at various lysines, the N-terminal cysteine, a serine, tyrosine, and an alternate histidine residue (12). PEG-IFN-α2a has a 40-kDa branched PEG molecule attached using N-hydroxysuccinimide-PEG chemistry via a covalent amide bond to the IFN-α2a protein (11). PEG-IFN-α2a is also a heterogeneous mixture of pegylated positional isomers consisting of four major positional isomers at Lys31, Lys83, Lys121, and Lys134 (10).

The different relative specific activities reported for the IFN-α preparations suggested that the significant differences in the size of the PEG molecule or the distribution of pegylation positional isomers or both might be accountable. Fractionation

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1 The abbreviations used are: IFN, interferon; PEG, polyethylene glycol; PEG-IFN, pegylated interferon; SC-PEG, succinimidyl carbonate polyethylene glycol; HPSEC, high performance size exclusion chromatography; HPIEX, high pressure ion exchange chromatography; SP, sulfopropyl; HPLC, high pressure liquid chromatography; STAT, signal transducers and activators of transcription; ANOVA, analysis of variance; JAK, Janus-activated kinase; M, apparent M.

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of specific pegylation positional isomers for 12-kDa PEG-INF-α2b have demonstrated that positional isomers had differential relative specific activities, with the His34 positional isomer retaining higher relative activity (37%) than the mixture of positional isomers (9). Thus, changes in the activity of pegylated proteins appear to be influenced by the site of pegylation and, in some cases, the molecular weight of the PEG moiety (13).

Our study was undertaken to directly determine the effects of size and site of pegylation on in vitro activity for IFN-α2b. This information is important for understanding how specific characteristics of PEG-INF-αs influence their in vitro activity that may be translated to their in vivo efficacy.

**MATERIALS AND METHODS**

IFN-α2b (Intron® A) and 12-kDa PEG-INF-α2b (PEG-INTRON) were manufactured by Schering-Plough Corp. (Kenilworth, NJ). The IFN-α2b international reference standard (95/566) was kindly provided by the National Institute for Biological Standards and Control (NIBSC, South Mimms, Hertfordshire, UK) (14). The specific activity for IFN-α2b, used as starting material in these studies, was 2.6 × 10^9 IU/mg, as calculated in Brigande IFN-α2a and 42-kDa IFN-α2a (Pegasys) were manufactured by Hoffmann-La Roche Inc. (Nutley, NJ). IFN-α2a was purchased and used directly from the commercial stock solution of 36 MIU/ml, which has a protein concentration of 133.3 µg/ml. For this study, the specific activity of IFN-α2a was determined empirically to be 2.4 × 10^10 IU/mg when calibrated against the IFN-α2b reference standard in the antiviral assay. The 40-kDa PEG-INF-α2a was purchased and used directly from the commercial stock solutions of 135 and 180 µg/ml. Protein concentrations for IFN-α2a and 40-kDa PEG-INF-α2a were confirmed prior to use. SC-PEG linkers with average PEG molecular masses of 5, 12, 20, and 30 kDa were purchased from Shearwater Polymers (Nektar Therapeutics, San Carlos, CA).

**Production and Isolation of Pegylated Interferon Isomers for Activity Studies**—The primary amino acid residues of IFN-α reactive to pegylation are Cys4, Lys4, His134, Lys135, Lys137, His138, and Cys34 most reactive at a neutral pH, whereas the ε-amino groups of lysine residues are most reactive at basic pH (15). Therefore, pegylation reactions utilizing SC-PEG 5 kDa, SC-PEG 12 kDa, SC-PEG 20 kDa, and SC-PEG 30 kDa were performed at room temperature at pH 6.5 to produce His4, and Cys34-modified positional isomers and pH 10 to produce the ε-amino-modified lysine positional isomers.

Reactions at pH 6.5 were performed in 10 mM sodium phosphate monobasic monohydrate, pH 6.0. To optimize resolution of the effect of pegylation on Stokes radius. Branched 20-kDa PEG-INF-α2b and di-20-kDa PEG-INF-α2b for Estimation of Stokes Radius—Two additional preparations of pegylated IFN-α2b were made solely for the study of the effect of pegylation on Stokes radius. Branched 20-kDa PEG-INF-α2b was produced by using PEG2-N-hydroxysuccinimide 20 kDa at a 2:1 molar ratio. Pegylation was performed at pH 6.8 at 22 °C in 40 mM sodium tetraborate with quenching at 60 min using 1 M glycine. The reaction mixture was isolated and purified as described for the positional isomers used for activity measurements. Di-20-kDa PEG-INF-α2b was produced as a secondary product from the SC-PEG reactions at pH 10. The mixture was purified from the pegylation reaction by collecting the unbound fraction obtained from anion exchange chromatography on Q HyperD (BioSepra). The reaction mixture was isolated and purified as described for the positional isomers used for activity measurements. Di-20-kDa PEG-INF-α2b was purified from diPEGylated IFN-α2b by 2D-PAGE under conditions identical to those used for the purification of monopegylated interferon α. The purity of diPEGylated IFN-α purified by this column was >99% by size exclusion HPLC.

**STAT1 Translocation Assay**—Human hepatoma (HuH-7) cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM GlutaMax-1, 100 units/ml penicillin/streptomycin, and nonessential amino acids. Cells (10,000 cells/well) were seeded overnight in 96-well Packard black view plates (Packard Instrument Co.). 3-Fold serial dilutions of the test IFN-αs were prepared in Dulbecco’s modified Eagle’s medium and incubated with cells at 37 °C in 5–6% CO2 for 30 min. The cells were washed with phosphate-buffered saline, fixed with 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100. The cells were then incubated with either a polyclonal anti-STAT1 p84/p91 (clone E-23; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a polyclonal anti-STAT2 (clone C-20; Santa Cruz Biotechnology) primary antibody. After 1 h at room temperature, the cells were initially washed with phosphate-buffered saline and 0.01% Tween 20. They were then incubated for 1 h with a mixture of Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) and Hoechst 33342 (Molecular Probes). The cells were again washed with 0.01% Tween 20 and phosphate-buffered saline. The plates were imaged on the ArrayScan® II High Content Screening System (Cellomics, Pittsburgh, PA) with a ×10 objective using the ArrayScan version 2.1 soft-
The PEG-IFN-ISONs in the antiviral assay were also performed. In selective cases, additional comparisons were also conducted looking at the effect of translocation measurement using this instrumentation and software (16). The test preparation, which induced a 50% decrease in formazan-deter-

ard. The IFN-

across a suitable response range. Specific activities for 12-kDa PEG-IFN-

were (Cellomics). The resulting images were processed using the Cyto-

plasm to Nucleus Translocation Application software (Cellomics). The cytoknucleus difference, defined as the difference in fluorescence intensity of the target STAT in the nuclear region minus the cytoplasmic region, was used as a measure of STAT translocation. Cytoplasmic and nuclear STAT1 levels were also examined for each cytoknucleus difference determination. We confirmed that the cytoknucleus difference was primarily caused by an increase in nuclear STAT1, whereas cytoplasmic STAT1 levels remained constant or only decreased slightly. This observation was consistent with data reported for TNF-α-mediated NF-κB cytoknucleus translocation measurement using this instrumentation and software (16).

**Antiviral and Antiproliferation Assays**—The antiviral assay was performed by titrating serial 2-fold dilutions in 96-well microtiter assay plates seeded with either human foreskin fibroblast cells (FS-71) or non-small cell lung carcinoma cells (A549) infected with encephalomyo-

carditis virus as described previously (17). The relative potency of IFN-α across a suitable response range was determined by titrating serial 2-fold dilutions in 96-well microtiter assay plates with a calibrated IFN-α2b reference standard. The mean titer (IU/ml) was determined at the 50% point of the titration curve and was an average of curves from three different experimental days (n = 9 curves). Specific activity was calculated using the concentration of the IFN-α2 protein, independent of weight contribution from PEG. The relative percentage of IFN-α2b activity was calculated using the specific activity of IFN-α2b at 2.6 × 10^8 IU/mg.

**TABLE I**

|                      | Mean titer IU/ml | Specific activity IU/mg/mg | Percentage of IFN-α2b activity % |
|----------------------|------------------|---------------------------|----------------------------------|
| FS-71 antiviral protection assay | 1.65 × 10^7       | 6.61 × 10^7               | 25.4                             |
| 12-kDa PEG-IFN-α2b   | 5.90 × 10^6       | 3.15 × 10^6               | 1.2                              |
| 40-kDa PEG-IFN-α2a   | 1.37 × 10^7       | 9.00 × 10^7               | 34.6                             |
| A549 antiviral protection assay | 5.85 × 10^6       | 3.13 × 10^6               | 1.2                              |
| 12-kDa PEG-IFN-α2b   | 1.35 × 10^6       | 7.52 × 10^6               | 28.9                             |
| 40-kDa PEG-IFN-α2a   | 1.78 × 10^6       | 0.7                       | 0.7                              |

Influence of Pegylation on Interferon Efficacy

**RESULTS**

**Confirmation of Differential Activity between 12-kDa Monopegylated IFN-α2b and 40-kDa Monopegylated IFN-α2a**—Because the reported activity for 12-kDa monopegylated IFN-α2b and 40-kDa monopegylated IFN-α2a was generated from different laboratories using different antiviral assays, we first measured the differential activity directly. 12-kDa PEG-IFN-α2b was consistently more than 25-fold more active than 40-kDa PEG-IFN-α2a in an antiviral protection assay using either FS-71 or A549 cells (Table I). The activity of 12-kDa PEG-IFN-α2b ranged between 25 and 35% of that for IFN-α2b control, values comparable with those previously reported (9). The activity of 40-kDa PEG-IFN-α2a was ~1% of that for IFN-α2b control. This value is lower than that from one previous report (10), but consistent with those from another recent evaluation of the activity of PEG-IFN-α2a in the Madin-Darby bovine kidney antiviral assay (11). IFN-α2a, the core interferon protein for 40-kDa PEG-IFN-α2a, was titrated in the FS-71 antiviral assay with IFN-α2b and was found to be equivalent in activity within the variation of the assay. Thus, the lower activity seen with the 40-kDa PEG-IFN-α2a versus 12-kDa PEG-IFN-α2b cannot be accounted for by differences in the core proteins in the assay. In addition, circular dichroism spectroscopy revealed no perturbation in the near or far UV for the core protein of 40-kDa PEG-IFN-α2a (data not shown).

Results obtained in a Daudi cell antiproliferation assay were similar to those for the antiviral assay (Table I). The specific activity of 12-kDa PEG-IFN-α2b was 28.9% of that for IFN-α2b as compared with a value of 0.7% obtained for 40-kDa PEG-IFN-α2a.
Effect of PEG Molecule Size on PEG-IFN-α2b Stokes Radius and Apparent Molecular Weight—Because of the hydrophilic nature of PEG polymers, increased polymer length can significantly increase the relative Stokes radius of a pegylated protein (18). In addition, due to the linear nature of the PEG moiety, the increase in the apparent molecular weight of a globular protein such as IFN-α2b is much greater than the sum of the molecular weights of the protein and the attached PEG group. Using SC-PEG chemistry, we purified mixed positional isomer preparations of 5-kDa, 12-kDa, linear 20-kDa, branched 20-kDa, and di-20-kDa PEG-IFN-α2b and measured the apparent molecular weight (aM) and Rg using HPSEC analysis. The addition of a 5-kDa PEG group to IFN-α2b increased the aM, to 73,300, a value of more than 3 times the additive molecular mass of the 23.1-kDa protein and the 5-kDa PEG group and the Rg was increased by 58% (Table II). With the addition of the 12-kDa PEG, the aM increased by 119% relative to IFN-α2b, and the aM increased to 165,600. The branched and linear 20-kDa PEGs increased Rg by 166 and 184% relative to IFN-α2b, and aM increased to 272,300 and 320,600, respectively. For the di-20-kDa PEG (with a total molecular mass of 63.1 kDa), the Rg was 275% higher than IFN-α2b, and the aM was 639,700. This increase in Rg as pegylated moieties increase in size or position might have implications for receptor binding, activation, and clinical efficacy.

Production and Isolation of Pegylated Positional Isomers of IFN-α2b—Pegylated positional isomers at Cys1, His34, Lys83, Lys121, Lys131, and Lys134 were chosen for study because they have been reported as being major positional isomers for 12-kDa PEG-IFN-α2b or 40-kDa PEG-IFN-α2a (10, 12). All reaction products were resolved by size exclusion chromatography to generate mono-PEG-IFN-α that was >99% pure by HPSEC. These monopegylated reaction products were then resolved into individual positional isomers by HPIEX (Fig. 1, A and B) to 98% purity by HPSEC and 91–99% purity by analytical HPIEX (15). Peptide mapping analysis can usually detect >5% impurities. However, one exception is the inability to resolve by HPIEX the neighboring peaks of 5-kDa Lys131 and 5-kDa Lys134, hence the lower purity estimation by analytical HPIEX relative to HPSEC. The HPIEX chromatography profiles and positional isomer elution order in this study matched those reported previously (12, 15, 19). In addition, the identity and purity of the purified positional isomers were confirmed by peptide mapping analysis.

STAT1 Translocation Studies—To study the effect of pegylation size and site on Jak/STAT IFN-α signal transduction, an assay was developed to assess STAT1 translocation from the cytoplasm to the nucleus in HuH-7 cells. The STAT1 translocation activity for the titration curves of each of the seven positional isomers at each respective PEG molecule size is shown in Fig. 2. The ED50 (infection points) for the positive control curves of 12-kDa PEG-IFN-α2b and 40-kDa PEG-IFN-α2a ranged from 2.0 to 4.8 and from 33.0 to 76.0 ng/ml, respectively, across the experiments in Fig. 2; a statistical analysis (USP 27 (111)) determined that none of the control infection points were outliers. This variation is consistent with newly developed cell-based bioassays. Assay results showed that the 12-kDa PEG-IFN-α2b was more active than the 40-kDa PEG-IFN-α2a. The STAT1 translocation activity for the His34 positional isomer was consistently similar to that for 12-kDa PEG-IFN-α2b. There was also little significant reduction in STAT1 translocation activity for the His34 positional isomers from 5 through 30 kDa (Fig. 2A). The Lys31 positional isomers exhibited less STAT1 translocation activity, demonstrated by a rightward shift in the titration curve, as the size of the PEG molecule increased from 5 to 30 kDa (Fig. 2B). The 5-kDa Lys31 PEG-IFN-α2b isomer had less translocation activity than the 12-kDa PEG-IFN-α2b control curve (Fig. 2B). Similar trends of decreased translocation activity associated with increasing PEG molecule size were observed with the other Lys and Cys positional isomers (Fig. 2, C-G).

The ED50 was calculated for each of the site-pegylated IFN-α2b isomers at PEG molecule sizes from 5 through 30 kDa PEG (Fig. 3). The His34 positional isomers at 5–30 kDa were the most active for STAT1 translocation, and increasing PEG molecule size at His34 had only a small effect on STAT translocation activity. Cys1 and Lys31 had the lowest translocation activity across all PEG molecule sizes, and both were very sensitive to increasing PEG molecule size above 5 kDa. The STAT1 translocation activity for each differently sized PEG positional isomer for both Cys3 and Lys31 was significantly different (p < 0.05, Student’s t test) compared with the respective His34 positional isomer. The Lys34 positional isomer trended to be the most active for STAT translocation of the lysine isomers studied, whereas the Lys83, Lys121, and Lys131 isomers were roughly equivalent in their respective STAT translocation activities. These four lysine positional isomers also appeared to trend to lower STAT1 translocation activity compared with the His34 positional isomer, although statistical significance was observed only with the 5-kDa Lys83 and 20-kDa Lys121. For all of these positional isomers, there was a consistent decrease in translocation activity associated with increased PEG molecule size to 20 or 30 kDa.

Antiviral Protection Activity of Pegylated Positional Isomers—The same site and size pegylation isomers were also studied using an antiviral protection assay in FS-71 cells. Overall, the results from the antiviral assay (Fig. 4) were consistent with the STAT1 translocation results. The most active positional isomer was His34, and the least active were Cys1 and Lys31. The most active lysine positional isomer was Lys34, whereas Lys83, Lys121, and Lys131 were roughly comparable in activity. Of note, all differently sized PEG positional isomers, compared against the respective His34 positional isomer, were significantly lower (p < 0.05) in antiviral activity. However, the Lys34 positional isomer, although lower in activity than His34, was statistically the most active of the lysine positional isomers studied. In addition, significant decreases occurred (p < 0.05) in the antiviral protection activity associated with increasing PEG molecule size for each of the positional isomers studied. These results confirm the significant differences and trends that were observed in the STAT1 translocation assay.
DISCUSSION

Our initial studies were performed using unfractionated PEG-IFN-α2a and PEG-IFN-α2b, which are known to be mixtures of positional isomers (10, 12). These results confirmed that there was a consistent 25–35-fold difference between the in vitro antiviral and antiproliferative activities of the two different PEG-IFN-α mixtures. The differences in relative activities between 12-kDa PEG-IFN-α2b and 40-kDa PEG-IFN-

FIG. 1. A, separation of His<sup>34</sup> and Cys<sup>1</sup> positional isomers from mono-PEG-IFN-α2b produced at a reaction pH of 6.5 by preparative scale cation exchange chromatography. B, separation of lysine positional isomers from mono-PEG-IFN-α2b produced at a reaction pH of 10 by preparative scale cation exchange chromatography. IFN-α2b was pegylated with SC-PEG 5-kDa, SC-PEG 12-kDa, SC-PEG 20-kDa, and SC-PEG 30-kDa linkers at a reaction pH of 6.5 to produce His<sup>34</sup> and Cys<sup>1</sup> positional isomers and reaction pH of 10 to produce lysine-modified positional isomers. The monopegylated reaction products were isolated by size exclusion chromatography and then resolved into individual positional isomers by HPIEX. The elution profiles of the ion exchange chromatographies are shown in A and B.
alpha2a may result from the different sizes of the PEG moieties, the different distribution of site-pegylated isomers, differences in the core IFN-alpha proteins, and/or differences in the bonds linking the PEG molecules to their respective core protein (4, 20). It is unlikely that differences between the activities of the core proteins, IFN-alpha2a and IFN-alpha2b, contribute to the observed differences between the two pegylated preparations, since their activities have been reported to be similar. In addition, prior studies have shown that pegylation does not detectably alter the secondary or tertiary conformation of the IFN-alpha2 core protein as well as for both the 12-kDa PEG-IFN-alpha2b and 40-kDa PEG-IFN-alpha2a (9). Thus, the site of pegylation and size of PEG moiety would appear to be the critical elements influencing the relative activities of 12-kDa PEG-IFN-alpha2b and 40-kDa PEG-IFN-alpha2a.

The size of the PEG moiety increased the Mr of the pegylated molecule in excess of the additive empirical molecular weight of the PEG to IFN-alpha2 core protein. This is due, in part, to the effect of the hydrophilic nature of PEG polymers on the Stokes radius of the pegylated molecule. Increases in linear PEG molecules from 5 to 20 kDa resulted in increased Mr from 23,100 for unpegylated IFN-alpha2b to 320,600 for 20-kDa PEG-IFN-alpha2b. Modification in PEG structures also impacted Mr. Branching two 10-kDa PEG molecules to make 20-kDa PEG decreased the Mr from 320,600 to 272,300, whereas conjugating two 20-kDa PEG molecules as separate dipegylated moieties onto a single IFN-alpha2b core protein increased the Mr to 639,700. Thus, increasing PEG moiety size could potentially impact the IFN-alpha2 core protein when the PEG IFN-alpha2 is presented to the interferon receptor.

The relative difference in STAT1 translocation activity that we observed between the 12-kDa PEG-IFN-alpha2b and the 40-kDa PEG-IFN-alpha2a was 30:1 and consistent with their relative antiviral and antiproliferative activities. Several laboratories have reported that IFN-alpha antiviral and antiproliferative activity in vitro is dependent upon JAK/STAT signaling through a potentially rate-limited interaction with the IFNAR1-IFNAR2 heterodimeric receptor complex (21–23). IFN-alpha binds to the IFNAR1-IFNAR2 heterodimeric complex, activating the JAK1 and Tyk2 kinases. This leads to phosphorylation and dimerization of STAT1 and STAT2 and subsequent translocation of the dimer to the nucleus with IRF-9 (24–27). Translocation is required for the dimer to form the ISGF-3 complex and bind to the ISRE element that in turn initiates the transcription of IFN-alpha-inducible genes (28). The advantage of using a STAT1 translocation assay for signaling assessment is that the
translocation event is linked to both the receptor-ligand interaction and the ultimate expression of IFN-α gene transcription, which is necessary for antiviral and antiproliferative activity.

It seems likely that the very different distributions of positional isomers for 12-kDa PEG-IFN-α2b and 40-kDa PEG-IFN-α2a may contribute substantially to their differences in \textit{in vitro} activity. The His\textsuperscript{34} positional isomer is the major isomer in 12-kDa PEG-IFN-α2b due to the chemical conditions used for the SC-PEG conjugation (29). The remaining positional isomers for 12-kDa PEG-IFN-α2b are predominantly lysine conjugates (9). In contrast, 40-kDa PEG-IFN-α2a comprises almost completely lysine positional isomers and includes no histidine positional isomers (11). The 12-kDa PEG-IFN-α2b and 40-kDa PEG-IFN-α2a have positional isomers at Lys\textsuperscript{31}, Lys\textsuperscript{83}, Lys\textsuperscript{121}, Lys\textsuperscript{131}, and Lys\textsuperscript{134}, although in different distributions. Significantly, the His\textsuperscript{34} positional isomer demonstrated a consistently lower ED\textsubscript{50} for induction of STAT1 translocation than any of the other positional isomers studied. Conversely, the Lys\textsuperscript{31} positional isomer had one of the highest ED\textsubscript{50} values for induction of STAT1 translocation than any of the other positional isomers studied. Conversely, the Lys\textsuperscript{31} positional isomer had one of the highest ED\textsubscript{50} values for induction of STAT1 translocation than any of the other positional isomers studied. Conversely, the Lys\textsuperscript{31} positional isomer had one of the highest ED\textsubscript{50} values for induction of STAT1 translocation than any of the other positional isomers studied.

There are several potential explanations for the difference in activity of the His\textsuperscript{34} and Lys\textsuperscript{31} positional isomers. One potential explanation for the higher activity of His\textsuperscript{34} is depegylation either prior to or associated with binding to IFNAR2. This would result in free IFN-α2 that could have more favorable reaction kinetics with the receptor. Two results argue against this possibility. Measurements of pegylated and free His\textsuperscript{34} IFN-α2, in culture medium under conditions used for the STAT1 translocation assay, revealed that free IFN-α2 levels were too low to account for the significantly higher activity of the His\textsuperscript{34} isomer and that no significant free IFN-α2 formed during the experiment. Second, the attenuation of His\textsuperscript{34} positional isomer antiviral activity with 20- and 30-kDa PEGs would not have been observed if the signal had been transmitted by depegylated, free IFN-α2b. The more likely reason for higher activity with the His\textsuperscript{34} isomer is that the site of pegylation may reduce the impact of steric hindrance from the PEG molecule. Roisman \textit{et al.} (31) described the free energy of association interaction between IFN-α2 and IFNAR2 through two domains: the deep insertion of the 45–52 loop of IFNAR2 into a groove in IFN-α2 formed around Ala\textsuperscript{145} on the E-helix (amino acids 137–156) and an interaction with the central part of the IFN-α2 E-helix at Arg\textsuperscript{149} and Ser\textsuperscript{152} with the 76–82 loop of IFNAR2. Arg\textsuperscript{33} may make two potential hydrogen bonds on the AB1 loop of IFN-α2 through its side chain with the backbone oxygens of Ser\textsuperscript{49} and Lys\textsuperscript{50} on the 45–52 IFNAR2 loop. Additional interactions at Phe\textsuperscript{27} and Asp\textsuperscript{55} in the AB1 loop of IFN-α2 may also exist with the 45–52 IFNAR2 loop (31, 34). However, the

FIG. 2—continued

![Graph E](image1)

![Graph F](image2)

![Graph G](image3)
striking discrepancy in activity between two closely related positional isomers on the AB1 loop, His34 and Lys31, suggests that tertiary positioning of the pegylation site may be involved in disrupting interaction with the receptor. A recent model of IFN-α2b dimer binding to IFNAR1-IFNAR2 based on x-ray crystallography suggests that His34 is not located at the receptor interface but rather at the IFN-α2b dimer interface (34). Whereas pegylation at the His34 site may appear favorable for retaining the activity of IFN-α2b, the addition of larger PEG molecules (i.e., >12 kDa) can quickly limit antiviral activity for this isomer. This suggests that there is an upper limit of PEG size at His34 around 3545 Å², much of the PEG molecule would have to be directed away from interacting into the binding domains. However, as the surface area for 12- and 20-kDa His34 PEG-IFN-α2b increases to 6760 and 10,023 Å², respectively, the PEG molecule may begin to significantly encroach into the binding domain. Alternatively, increased steric hindrance at the dimer binding interface may also contribute to the decreased activity observed with increased PEG molecule size, either cooperatively with or independently of the receptor binding interaction.

The Lys121, Lys131, and Lys134 positional isomers reside on the D-helix of IFN-α2b. The low STAT1 translocation and antiviral activity observed with the Lys121 and Lys131 positional isomers suggest that pegylation at these sites is also unfavorable. These two sites are close to both the AB1 loop area and E-helix groove of IFN-α2b. Interestingly, the Lys134 positional...
isomer had higher STAT1 translocation and antiviral activity. This site is located in the DE loop of IFN-α2b, suggesting that steric hindrance from a PEG molecule at the Lys314 site is less than at either Lys125 or Lys131. Lys131 is reported to be located at an interaction point between IFN-α2b and IFNAR1, so it is reasonable to observe diminished activity with this positional isomer (34). Finally, the Cys1 site was found to be particularly sensitive to pegylation. The structural relevance of this site is important, since it is absolutely conserved; in the NH2 terminus portion of IFN-α, this has been shown to be important in mediating antiviral activity (33, 35).

Another possible explanation for diminished activity based on the site of pegylation is differential influence on IFN-α2b and IFNAR1/IFNAR2 interaction kinetics (21, 36). For example, it has been postulated that the charge loss associated with His34 pegylation may perturb the electrostatic potential at or near the receptor binding site less than with ε-amine pegylation (15). However, it should be noted that Pielker and Schreiber have observed that the IFN-α binding site on IFNAR2 is not a highly negatively charged area on the protein surface, and this limits the potential role for electrostatic forces in determining the rate of association (37). Nevertheless, a cooperative interaction, first through electrostatic steering, followed by docking of the IFN-α2b and IFNAR2 and IFNAR1, might be impacted by the pegylation.

It is also possible that the site of pegylation in conjunction with size-mediated steric hindrance might cause the core IFN-α protein to be presented in a different contextual interaction with the heterodimeric receptor such that unique recognition domains are not engaged. There have been many reports of differential activity conferred by different IFN-α species with the intact receptor (35). Hybrid IFN-α recombinants have been shown to have specific domains for antiviral activity (35, 38), antiproliferative activity (39), and cytotoxic activity (40–42). Studies using human IFN-α2a/2c hybrids have shown that the N terminus region and the hydrophobic residues on the C-helix region are particularly important for antiproliferative activity (43). Splice variants of the human IFNAR1 have been shown to differentially recognize different IFN-α through unique receptor subdomains for major histocompatibility complex class I antigen expression (44). PEG-IFN-α2b has been shown to have roughly equivalent reductions in the antiviral, antiproliferative, cytotoxic, and major histocompatibility complex class I expression activities, suggesting that the sites of pegylation and the 12-kDa PEG molecule impact these potential recognition domains on the heterodimeric receptor equivalently (9). In these studies, we confirmed that PEG-IFN-α2a lost approximately equivalent activity in the antiviral and antiproliferative assays, although significantly more activity was lost than that observed for PEG-IFN-α2b. This suggests that similar domain recognition may exist even with the differences in site of pegylation and size of the PEG molecule for PEG-IFN-α2a and PEG-IFN-α2b.

In light of our results, it is interesting to note that both antiviral and antiproliferative activity in vitro is transduced by a relatively small receptor occupancy that appears to be independent of mass action but dependent on continuity of signal induction (22, 37). Site of pegylation and PEG molecule size both significantly affect in vitro signaling of IFN-α2b, suggesting that a discontinuity in signaling may be occurring as a result of a decreased $K_m$ rate. This may have important clinical implications, since the $K_m$ rate is critical for receptor-mediated signaling. Another clinically important component is the serum residency time of the molecule. Pegylation of IFN-α significantly increases the serum residency and thus the number of potential interactions with the receptor. However, at the same time, pegylation decreases the ability of IFN-α to interact with its receptor once it reaches the cell surface. These consequences of pegylation are likely to have opposing effects on the clinical utility of the resulting preparations, and they must be carefully balanced in the design of PEG-IFN-α.

**Conclusion**—The higher in vitro specific activity of 12-kDa PEG-IFN-α2b relative to 40-kDa PEG-IFN-α2a can be attributed to differences in the respective size of the PEG moiety and the distribution of positional isomers. This study provides evidence that the overall in vitro activity of PEG-IFN-α is governed by both PEG moiety size and by specific positional isomers. In particular, the 12-kDa PEG-His34 positional isomer, a major constituent of 12-kDa PEG-IFN-α2b, retained the highest postpegylation specific activity. This activity was significantly higher for in vitro antiviral activity compared with all other isomers studied. The higher activity for 12-kDa PEG-IFN-α2b was observed to occur as early as the STAT1 translocation step within the IFN-α-mediated JAK/STAT signaling pathway. However, increasing the PEG moiety size significantly attenuated the in vitro antiviral activity of all pegylation sites studied. The correlative effects of site and size of pegylation observed with the antiviral, antiproliferation, and STAT1 translocation activity point to a receptor-mediated mechanism. Unfortunately, extant reported clinical studies have not been appropriately balanced by protein weight to help clearly elucidate the impact of any receptor-mediated activity on in vivo efficacy. Further study of the in vitro effects of pegylation and the subsequent impact on in vivo efficacy are needed to improve our understanding of the optimal balance of receptor-mediated activity against extended serum half-life.

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