

Development of Pegylated Interferons for the Treatment of Chronic Hepatitis C

Antoni Kozlowski, Stephen A. Charles and J. Milton Harris

Shearwater Corporation, Huntsville, Alabama, USA

Abstract

The chemical attachment of poly(ethylene glycol) [PEG] to therapeutic proteins produces several benefits, including enhanced plasma half-life, lower toxicity, and increased drug stability and solubility. In certain instances, pegylation of a protein can increase its therapeutic efficacy by reducing the ability of the immune system to detect and mount an attack on the compound.

A PEG-protein conjugate is formed by first activating the PEG moiety so that it will react with, and couple to, the protein. PEG moieties vary considerably in molecular weight and conformation, with the early moieties (monofunctional PEGs; mPEGs) being linear with molecular weights of 12kD or less, and later moieties being of increased molecular weights. PEG2, a recent innovation in PEG technology, involves the coupling of a 30kD (or less) mPEG to lysine that is further reacted to form a branched structure that behaves like a linear mPEG of much larger molecular weight. These compounds are pH and temperature stable, and this factor along with the large molecular weight may account for the restricted volume of distribution seen with drugs utilising these reagents.

Three PEG-protein conjugates are currently approved for clinical use in the US, with more under clinical development. Pegademase is used in the treatment of severe combined immunodeficiency disease, pegaspargase for the treatment of various leukaemias, and pegylated interferon- α for chronic hepatitis C virus infections. As illustrated in the case of the 2 pegylated interferon- α s, all pegylated proteins are not equal. The choice of PEG reagent and coupling chemistry is critical to the properties of the PEG-protein conjugate, with the molecular weight of the moiety affecting its rate and route of clearance from the body, and coupling chemistry affecting the strength of the covalent attachment of PEG to therapeutic protein.

1. Introduction to Protein Pegylation: Properties of Poly(ethylene glycol) (PEG) Moieties

Pegylation was developed in the 1970s by Abuchowski and colleagues^[1] to enhance the delivery of therapeutic proteins. The changes in pharmacokinetics and pharmacodynamics produced by pegylation have prompted research on the use of

PEG-protein conjugates in several therapeutic areas, including oncology and infection.

Both protein and nonprotein molecules can be pegylated, although research efforts have focused primarily on PEG-protein conjugates. Pegylation, by increasing the molecular weight (MW) of the protein, typically reduces its excretion, permitting a decrease in dosage frequency. Thermal and pH stability is enhanced, helping to increase the termi-

nal half-life of the protein. By forming a 'shell' around the protein, pegylation can also decrease the immunogenicity of the protein and protect it from proteolytic degradation. Drug toxicity is reduced, presumably as a result of the reduction in plasma concentration fluctuations induced by multiple daily administrations.

The specific characteristics of poly(ethylene glycol) [PEG] moieties relevant to pharmaceutical applications are:

- water solubility
- high mobility in solution
- lack of toxicity
- lack of immunogenicity
- ready clearance from the body
- altered distribution in the body.^[2,3]

Studies of PEG moieties in solution have shown that each ethylene oxide unit is tightly associated with 2 to 3 water molecules. This hydration, coupled with the rapid motion of the PEG molecule in aqueous medium, gives the PEG molecule the solubility properties of a molecule 5 to 10 times as large as a soluble protein of comparable MW. This size effect is readily revealed by several techniques such as size exclusion chromatography and gel electrophoresis.

The lack of toxicity of PEG has been established through many years of experience in the food, cosmetic and pharmaceutical industries.^[4] The observed low immunogenicity of PEG is supported by several studies (reviewed by Harris^[2,3]). A point of interest is the recent demonstration of the generation of anti-PEG antibodies under extreme conditions in rabbits.^[5] This immune response, however, is not considered to be clinically significant, as there are no known examples of antibodies against PEG being generated from injection of therapeutic proteins into humans. The clearance of PEG moieties of differing MWs has been studied. Results show that the PEG moiety is cleared, without structural change, with clearance being proportional to the MW. Below a MW of about 20kD, the molecule is cleared via the kidney and excreted in the urine. Higher MW PEGs are cleared more slowly through the kidney, with liver clearance increasing as the PEG MW increases.^[6]

Many properties of the native polymer are transferred to PEG-protein conjugates, which therefore exhibit a variety of desirable characteristics, some of which are mentioned above. All of these effects are dependent on the MW of the attached PEG moiety. The effect of PEG moiety attachment on solubility can be dramatic and is frequently observed with small molecule pharmaceuticals. The shielding ability of a covalently attached PEG moiety can lead to reduction or elimination of protein immunogenicity.^[1,7] Unfortunately, insufficient knowledge of protein structure or the inability to selectively place the PEG moiety may hinder attempts to eliminate protein immunogenicity. The general approach is to attach the PEG moiety to some of the available lysines on the protein surface and hope that the desired result is achieved. Similarly, the shielding effect of the PEG moiety leads to reduced sensitivity to proteolysis for PEG proteins, and this effect is especially strong when high MW, branched PEG moieties are used.^[8] Attachment of PEG moieties to a small protein will lead to a larger PEG-protein conjugate with a reduced rate of renal clearance. In sum, these properties enhance the circulation half-lives of proteins. PEG proteins also exhibit altered distribution in the body,^[9] and when injected subcutaneously exhibit absorption into the bloodstream that is dependent on the MW of the PEG moieties. PEG proteins have enhanced stability that leads to a longer shelf life.

2. Chemistry for Pegylation

To couple a PEG moiety to a protein it is first necessary to activate the polymer by converting the hydroxyl terminus to some functional group capable of reacting with the functional groups found on the surface of proteins. The most common method has been to activate the PEG moiety with functional groups suitable for reaction with lysine and N-terminal amino groups. The PEG used is typically monofunctional PEG (mPEG) because this polymer resists cross-linking and aggregation. One early example of this approach is the reaction of mPEG tresylate with alkaline phosphatase [fig. 1].^[10]

Several lysine residues are typically substituted

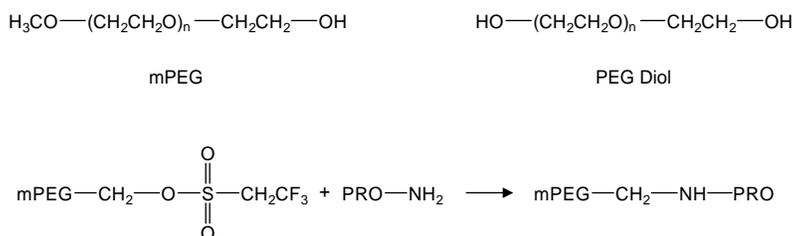


Fig. 1. Reaction of mPEG tresylate with alkaline phosphatase (represented as PRO-NH₂).

during these reactions and each molecule of the protein can have a different pattern of lysine substitution. PEG moieties are polydisperse, which means that there is a distribution of MWs about the mean. The usual measure of polydispersity is the ratio of weight average MW to number average MW (M_w/M_n), with a larger ratio indicating a broader distribution. Compared with many polymers, PEG moieties have an unusually narrow polydispersity that is typically less than 1.05, and frequently close to 1.01; higher MWs typically have broader distributions.

The heterogeneity in lysine substitution and in PEG MWs is of some importance for PEG protein pharmaceuticals, and it is generally necessary to demonstrate that the pattern for a particular pharmaceutical can be measured and reproduced. It is possible in some instances, however, to reduce or eliminate heterogeneity in the position of substitution.

A major problem faced in PEG chemistry is that mPEG-OH is often contaminated with PEG diol (HO-PEG-OH). Some manufacturers produce low-diol mPEG, but some of this impurity is always present. Diol content can range as high as 10 to 15%.^[11] Activation of the diol leads to a difunctional contaminant that is associated with cross-linking and aggregation. Diol arises from the presence of water contamination during the base-catalysed polymerisation of ethylene oxide to form mPEG, and given that there is a lower concentration of initiator (methoxide) in preparation of high MW mPEGs, water contamination and diol formation is a more

serious problem in this case. Also, because the diol chain can grow at each end, the contaminating diol typically has a higher average MW than the desired mPEG.

One characteristic of pegylation chemistry is that the diol and the resulting difunctional PEG are not removed. Consequently, one must be prepared to accept some protein cross-linking in the final product or make the effort to remove cross-linked product. An alternative is now available to purify the activated PEG reagent and remove the difunctional material. This route has the advantage of minimising the loss of expensive protein.

2.1 Monofunctional PEG (mPEG)

A wide range of PEG derivatives has been used for protein pegylation. Until recently these reagents were linear PEGs with MWs of 12kD or less. Examples of 2 PEG reagents are mPEG succinimidyl succinate (SS-PEG) and mPEG succinimidyl carbonate (SC-PEG) [fig. 2]. Although these reagents are widely used, including use in US Food and Drug Administration (FDA)-approved PEG-protein products, they are associated with several disadvantages including weak linkages between the PEG moiety and protein, potential unwanted side reactions, contamination, and restriction to low MW PEGs.

2.1.1 mPEG Succinimidyl Succinate

Activation of acids as succinimidyl esters has long been a popular chemistry, and SS-PEG has

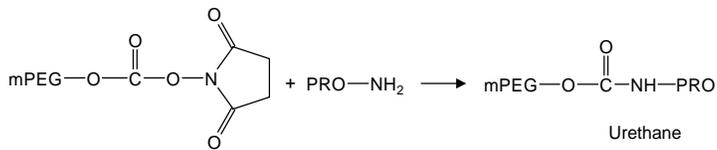


Fig. 4. The linkage formed between succinimidyl carbonate (SC) and a protein lysine is a urethane linkage.

because the acetaldehyde is very susceptible to dimerisation via aldol condensation. A key property of mPEG-propionaldehyde, as disclosed by Kinstler et al.^[17] in work on pegylation of granulocyte colony-stimulating factor (G-CSF), is that the aldehyde is highly selective for the N-terminus of G-CSF. Although complete selectivity is not observed, the extensive heterogeneity normally seen with lysine chemistry is greatly reduced. Coupling of aldehyde proceeds through a Schiff base that is reduced *in situ* to give a stable secondary amine linkage (fig. 5).

Kinstler et al.^[17] utilised mPEG 20 000 for coupling to G-CSF. Because of the size of G-CSF (18kD), this coupling resulted in an approximate doubling of the absolute MW of the molecule, and the heavy hydration and rapid motion of PEG resulted in an effective MW that is at least twice that of the native protein. It is expected that G-CSF with a single mPEG of 20kD would exhibit improved pharmacokinetic properties and enhanced efficacy relative to the native molecule. Clinical trials will ultimately determine the effects of pegylation on G-CSF. High MW PEG-propional-

dehyde has also been used for pegylation of the tumour necrosis factor receptor for the treatment of rheumatoid arthritis.^[18] In all these instances it was necessary to have a robust purification procedure to remove small amounts of contamination produced from PEG-diol-associated chemistry.

2.1.4 mPEG Vinylsulfone

Total selectivity in protein pegylation can be achieved by the introduction of a cysteine into the amino acid sequence of a protein by site-directed mutagenesis. In this way it is possible to position the mPEG in a location on the protein remote from the active or binding site. For example, mPEG vinylsulfone can be used to give selective conjugation with a cysteine group, with no lysine modification.^[19] Another derivative that is useful for selective thiol pegylation is mPEG maleimide. Generally, this derivative is more reactive and easier to use than the mPEG vinylsulfone. Similarly, thiol pegylation can also be accomplished with orthopyridyldisulfide (OPSS) mPEG (OPSS-PEG). El Tayar et al.^[20] recently prepared a highly active pegylated IFN β using this derivative. In this case the available thiol was located in a sterically

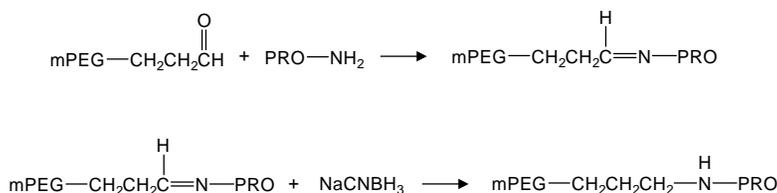


Fig. 5. Coupling of propionaldehyde proceeds through a Schiff base that is reduced *in situ* to give a stable secondary amine linkage in the resulting mPEG-protein conjugate.

crowded region and little modification could be achieved with mPEG maleimide or mPEG vinyl-sulfone of high MW. The approach ultimately adopted was to couple a low MW di-OPSS-PEG (MW 2000) to the IFN and then couple a high MW PEG thiol to the remaining, terminal OPSS group.

2.1.5 mPEG Carboxylic Acid

The final hurdle to overcome with pegylation chemistry is removal of the difunctional derivative formed from diol contamination. This can be accomplished by preparing a mPEG carboxylic acid and purifying this acid by ion exchange chromatography.^[21] By using this route it is possible to remove the di-acid formed from the diol. Also, we have found that there is some improvement in the polydispersity of the mPEG carboxylic acids because low MW PEG acids bind more tightly to the ion-exchange column than do the high MW PEG acids.

The first mPEG carboxylic acid that was produced was the succinate discussed earlier (see section 2.1.1). The ester linkage present in this molecule, however, leads to hydrolysis and degradation in an ion exchange column. Carboxymethylated PEG has been known for some time. Unfortunately, the succinimidyl ester of this compound is so reactive that it is difficult to use. To correct these problems, Harris and Kozlowski^[21] prepared mPEG propionic acid, and then converted this compound into the succinimidyl active ester (SPA). This SPA-PEG compound has ideal reactivity and can be prepared with a diacid content of less than 1% for MW 20 000 PEG. Note that the starting mPEG for this product had about 5% of diol, but ion exchange chromatography of the intermediate acid reduced the difunctional material to less than 1%. SPA-PEG has been used for pegylation of human growth hormone receptor antagonist for the treatment of acromegaly. Clinical trials are complete and the PEG-protein conjugate is under regulatory review for marketing approval.^[22-24]

2.2 PEG2

Another approach to preparing a pure, high MW, monofunctional PEG moiety is to couple crude mPEG-benzotriazole carbonate (BTC), of a MW up to 30kD, to lysine. This yields a high MW PEG acid with no hydrolytically degradable linkages. The crude PEG2 acid is contaminated with unreacted mPEG-BTC, 'PEG1', in which only a single mPEG is coupled to lysine, and the 'PEG3' that is formed from the reaction of activated PEG diol (or BTC-PEG-BTC) with 2 lysines followed by the coupling of 2 mPEG-BTC molecules to the remaining 2 amino groups (thus PEG3 is a diacid). During aqueous work-up, the unreacted mPEG-BTC is converted back to mPEG-OH, greatly simplifying subsequent ion exchange chromatography. Thus the reaction mixture contains a diacid, a zwitterion, a neutral mPEG and the desired monoacid. Careful ion exchange chromatography permits large-scale production of the desired pure PEG2-CO₂H. Activation of the succinimidyl ester (PEG2-NHS) is straightforward (fig. 6).

The PEG2 compound turns out to be a very exciting protein pegylation reagent that can be prepared in monofunctional form up to MW 60kD. Experiments have shown that PEG2, attached to a protein, 'acts' much larger than a corresponding linear mPEG of the same MW. This is due to the branching that occurs with the PEG2 moiety. Branched chain PEG-protein conjugates have increased pH and thermal stability compared with linear PEG conjugates.^[8] Furthermore, the branched

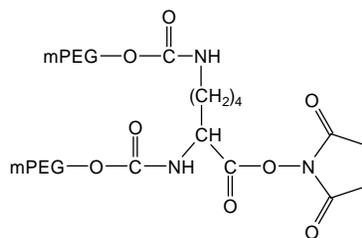


Fig. 6. PEG2-NHS is a pure, high molecular weight (MW), monofunctional PEG moiety created by coupling mPEG-benzotriazole carbonate of MW up to 30 kD to lysine.

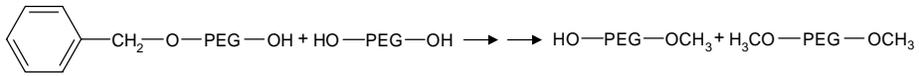


Fig. 7. Benzyloxy-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus, the diol is converted to the inert dimethyl ether.

structure of the PEG moiety may contribute to the restricted volume of distribution observed with PEG molecules. The availability of the very large PEG2-NHS means that only a single point of attachment is required to achieve the desired pegylation effect.^[25] In addition, the pure PEG2-NHS can be converted into a range of other pure, mono-functional PEG derivatives including aldehyde and maleimide compounds that previously were always contaminated with difunctional material.

Another approach to the solution of the diol problem has recently been demonstrated by Bentley et al.^[26] In this work, a crude PEG benzyloxy, containing diol impurity, was methylated and then hydrogenated to remove the benzyl group. Thus, the diol was converted to the inert dimethyl ether, which could be removed after activation and protein attachment (fig. 7).

3. Pharmaceutical PEGs

3.1 Pegademase

In light of the properties described above, several PEG-protein conjugates are been approved for clinical use by the US FDA or are currently under clinical development. Pegademase was approved in 1990 for use in severe combined immunodeficiency disease (SCID), a disease associated with an inherited deficiency of adenosine deaminase (ADA).^[27] Before the availability of pegademase, patients with SCID were treated with partial exchange transfusions of red blood cells that contain ADA. These transfusions placed patients at risk for iron overload and transfusion-related viral infections.^[28,29] Pegademase has approximately

1800-fold more ADA activity per millilitre than red blood cells; therefore, the drug produces higher concentrations of ADA activity than partial exchange transfusion.^[30]

3.2 Pegaspargase

Pegaspargase is available for the treatment of acute lymphocytic leukaemia, acute lymphoblastic leukaemia and chronic myelogenous leukaemia. The native asparaginase compound is associated with a high incidence of allergic reactions and the development of neutralising antibodies that shorten its half-life, making it difficult to maintain effective plasma concentrations.^[31] Pegaspargase reduces the tendency of the enzyme to induce an immune response, thus allowing patients with hypersensitivity to the native enzyme to tolerate pegaspargase without further incident.^[32]

3.3 Other Therapeutic PEG Conjugates in Development

There are several important PEG proteins in advanced stage clinical trials, and it is expected that some of these proteins will receive approval within the next year. Included in this list of probable approvals are PEG-growth hormone receptor antagonist for treatment of acromegaly,^[33] free radical scavengers, blood derivatives, antineoplastic agents, cardiovascular agents and antigens.^[28]

Further evidence of the suitability of PEG-protein conjugates for human therapeutics is given by the FDA approval of 2 other PEG-containing products: stealth PEG-liposomes for delivery of doxorubicin for the treatment of Kaposi's sarcoma

(pegylated liposomal doxorubicin; Doxil^{®1}) and PEG-lactide-glycolide-acrylate (FocalSeal[®]) used as a sealant in lung surgery.

3.4 Pegylated Interferon- α

Chronic hepatitis C virus (HCV) infection is a rapidly emerging disease that is the leading cause of liver cirrhosis and hepatocellular carcinoma.^[34] The protein IFN α has been used extensively to treat HCV; however, monotherapy with IFN is associated with a sustained response in only a small percentage of patients.^[34,35] This poor response is probably the result of multiple host (e.g. viral load) and viral factors (e.g. HCV genotype) as well as the inadequate pharmacokinetics of IFN. In particular, serum concentrations of IFN fall below the limit of detection within 24 hours of a single subcutaneous dose. Thus, even though the current treatment regimen calls for subcutaneous injection of IFN 3 times weekly, the virus has extended periods during which it is not exposed to a sustained therapeutic concentration of the drug. The addition of ribavirin to IFN in the treatment of HCV has increased the number of patients achieving a sustained virological response (38 to 43%).^[35,36] This increase in response, however, is accompanied by a higher incidence of adverse events, some of which lead to discontinuation of therapy.

Pegylation of IFN improves the pharmacokinetics and, as a result, the pharmacodynamics of the drug, most likely leading to an increase in response. In 1999, clinical trials were conducted to evaluate the efficacy of IFN α -2a modified by attachment of mPEG of MW 5kD in patients with chronic HCV. Once weekly administration of PEG(5kD) IFN α -2a at doses of 15, 45, 90 and 135 μ g did not show improved efficacy over unmodified IFN α -2a 3 million international units (MIU) 3 times weekly.^[37] More recently, clinical trials have evaluated a pegylated IFN α -2a prepared by coupling a branched PEG of MW 40kD to IFN α -2a.^[38] The results of these trials are highly

encouraging.^[39,40] Additionally, a linear pegylated IFN α -2b of 12kD is currently approved in the European Union and the US for the treatment of HCV.

Further development of pegylated IFN α -2a has involved examination of various pegylation chemistries. A detailed report of these studies will be published elsewhere.^[41] After investigation, the moiety chosen for coupling with IFN α -2a was PEG2-NHS 40 000. Since the protein has a MW of about 19kD, the conjugate is a much larger molecule. Preclinical studies and modelling indicated that this conjugate, PEG(40kD) IFN α -2a would have desirable pharmacokinetics and activity. In particular, it was predicted that the steady-state blood concentration would be essentially constant (fig. 8). In practice, the desired result was achieved, and nearly constant blood concentrations are attained with once weekly subcutaneous injections.^[42] Pegylation with PEG2-NHS 40 000 reduces renal clearance 100-fold relative to native IFN α -2a, and gives an elimination half-life of 77 vs 9 hours. PEG(40kD) IFN α -2a exhibits sustained absorption and a restricted volume of distribution when administered subcutaneously. This allows for a more convenient administration schedule (once weekly vs 3 times weekly for unmodified IFN α -2a).

Clinical results are now available for PEG (40kD) IFN α -2a. An open-label, parallel dose, global phase III trial randomised 531 patients to receive either PEG(40kD) IFN α -2a 180 μ g once weekly for 48 weeks (n = 267) or unmodified IFN α -2a 6 MIU 3 times weekly for 12 weeks followed by 3 MIU 3 times weekly for 36 weeks (n = 264). Results showed that PEG(40kD) IFN α -2a is associated with a greater virological response at week 48 (69 vs 28%, p = 0.001) and sustained virological response at week 72 (39 vs 19%, p = 0.001) compared with unmodified IFN α -2a.^[40] Additionally, an open-label randomised, parallel dose study was performed in 271 patients with chronic HCV and biopsy-proven cirrhosis or bridging fibrosis. Patients were randomised to receive 48 weeks of treatment with IFN α -2a 3 MIU 3 times weekly

1 Use of tradenames is for product identification only and does not imply endorsement.

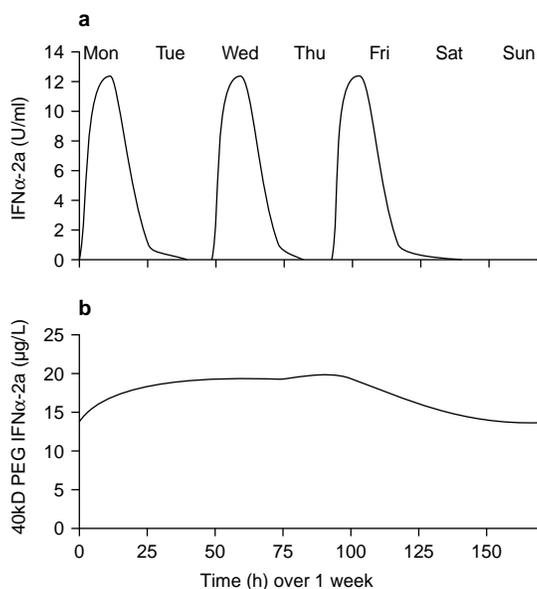


Fig. 8. Pharmacokinetic profiles for interferon (IFN)- α -2a and PEG(40kD)-IFN α -2a.

($n = 88$) or PEG(40kD) IFN α -2a 90 μ g ($n = 96$) or 180 μ g ($n = 87$) once weekly followed by a 24-week treatment-free period. 44% of patients who were treated with 180 μ g PEG(40kD) IFN α -2a had a virological response at the end of 48 weeks' treatment ($p = 0.001$ vs IFN α -2a), and 30% achieved a sustained virological response at 72 weeks ($p = 0.001$ vs IFN α -2a). Among the 88 patients who received IFN α -2a 3 MIU 3 times weekly, only 14% achieved a virological response at 48 weeks and 8% had a sustained virological response at 72 weeks.^[39]

It is well known that combination therapy with IFN and the antiviral agent ribavirin leads to enhanced efficacy compared with IFN alone.^[34-36] Thus, it is of interest to combine PEG(40kD) IFN α -2a with ribavirin in the hope that improved results will be seen. Clinical trials to address this point are currently under way and the results of a small pilot study on PEG(40kD) IFN α -2a plus ribavirin in 20 patients have been announced.^[43] Sustained virological and biochemical responses

were observed in 9 of 20 patients. Five of 16 patients infected with HCV genotype 1 achieved a sustained virological response, as well as all 4 patients infected with other HCV genotypes.

A pegylated form of IFN α -2b has also been approved by the FDA.^[44,45] Pegylation technology for preparation of a linear 12kD form of pegylated IFN α -2b with mPEG-SC 12 000 has been patented (PEG interferon- α -2b).^[15] A special feature of this work, as noted above, is that pegylation at low pH (around pH 5) leads to extensive coupling to histidine via a hydrolytically unstable linkage.

Results from a phase III clinical trial comparing pegylated IFN α -2b once weekly with unmodified IFN α -2b 3 times weekly^[45] showed that at the optimal dose (1.0 μ g/kg), pegylated IFN α -2b gave sustained HCV clearance in 25% of 297 patients, whereas 12% of 303 patients who received native IFN α -2b showed sustained viral clearance ($p < 0.001$).

Results were recently reported for a phase III trial involving 1530 patients who were randomised to receive either pegylated IFN α -2b 1.5 μ g/kg once weekly plus ribavirin 800 mg/day for 48 weeks (PEG α -2b 1.5/R); pegylated IFN α -2b 1.5 μ g/kg once weekly plus ribavirin 1000 to 1200 mg/day for 4 weeks followed by pegylated IFN α -2b 0.5 μ g/kg once weekly plus ribavirin 1000 to 1200 mg/day for 44 weeks (PEG α -2b 0.5/R); or IFN α -2b 3 MIU 3 times weekly plus ribavirin 1000 to 1200 mg/day for 48 weeks. A 54% sustained virological response was found overall in the pegylated IFN α -2b 1.5/R group compared with 47% in the IFN α -2b/ribavirin group ($p = 0.01$ vs interferon- α -2b + ribavirin), whereas 42% ($p = 0.02$ vs IFN α -2b/ribavirin) and 82% of patients in the PEG α -2b 1.5/R group with HCV genotype 1 and non-1, respectively, achieved a sustained virological response.^[46] It is important to note, however, the difference between the highest response rate with pegylated IFN α -2b and IFN α -2b/ribavirin was only 7%.

Based on available data, it is obvious that both pegylated IFN α -2b and PEG(40kD) IFN α -2a have

superior efficacy when compared with unmodified IFN α -2b and IFN α -2a, respectively. The higher MW, branched pegylated IFN α -2a may have a better pharmacokinetic and pharmacodynamic profile than the lower MW pegylated IFN α -2b, offering a true once weekly administration regimen with the ability to maintain adequate serum IFN concentrations and possibly achieving improved sustained responses even in more difficult-to-treat patients. Confirmation of this supposition will require a head-to-head clinical trial between PEG(40kD) IFN α -2a and pegylated IFN α -2b. Clearly, pegylation of therapeutic proteins in general is valuable, and pegylation of IFN promises to increase treatment success in a growing population of patients diagnosed with HCV infection.

Acknowledgements

The development of this manuscript was supported by Shearwater Corporation. Shearwater Corporation manufactures the PEG reagents used in much of the work described in this review.

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Correspondence and offprints: Dr *J. Milton Harris*, Shearwater Corporation, 1112 Church St., Huntsville, AL 35801, USA.

E-mail: jmharris@swpolymers.com