# Assessment of the Immunogenicity of Mechanically Induced Interferon Aggregates in a Transgenic Mouse Model

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*Received 2 September 2014; revised 27 October 2014; accepted 29 October 2014*

*Published online 1 December 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24292*

**ABSTRACT:** Pump delivery of human interferon alpha-2B (IFNα2b) has the potential for inducing immunogenic drug aggregates. We therefore evaluated the immunogenicity of mechanically induced IFNα2b aggregates to assess this risk. Transgenic human-IFNα2b (TG) and wild-type (WT) FVB/N mice ($n = 8$ and $n = 9$/group, respectively) were administered mechanically agitated drug [45 Hz for 6 h (LLA) or 24 h (HLA)], chemically modified drug [low pH (pH 4.0) or metal oxidized (OXD)] or unstressed drug (native). Mice received IFNα2b (50 μg; 100 μg/mL; s.c.) formulations on days 0, 7, 14, and 21. Drug-binding and neutralizing antibody titers were determined after 28 d. Aggregate concentrations were highest in OXD and HLA formulations but OXD had more dimers/trimers. Geometric mean titers were 1:131, 1:728, 1:1573, 1:871, and 1:10,240 for WT mice ($n = 9$) and 1:207, 1:587, 1:1810, 1:571, and 1:2,153 for TG mice ($n = 8$) for native, LLA, HLA, pH4, and OXD groups, respectively. Mechanical agitation of IFNα2b induced equivalent titers of immunoglobulin to that of metal oxidation, both capable of binding to or neutralizing the drug in WT and TG mice. Thus, by limiting metal contamination and by inclusion of a stabilizing agent to mitigate drug aggregation, the risk of anti-drug immunoglobulin may be reduced in a pump delivery scenario. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:722–730, 2015

**Keywords:** drug delivery systems; HPLC (high performance/pressure liquid chromatography); capillary electrophoresis; immunology; mechanical activation; protein aggregation; protein delivery; stability; transgenics

## INTRODUCTION

Protein and peptide therapeutics represent the backbone of the biotechnology industry, steadily encroaching on sales positions previously held by non-biological drugs. Almost 50% of the 30 top grossing pharmaceutical products in the fourth quarter of 2013[1] were biological.

Whether these drugs represent recombinant copies of endogenous proteins, such as human interferon (IFN) or insulin, analog modifications, or conjugated proteins, such as PEGylated IFN, many possess significant immunogenic potential via an assortment of mechanisms. These include, but are not limited to, increased drug aggregation, formation of partially denatured intermediates or fragments, and/or the loss of volatile formulation components with the effect of compromising physical stability of the drugs.[2,3] When administered via delivery systems such as external or implantable programmable pumps to control the pharmacokinetics of their payload therapeutics, external stimuli such as temperature, mechanical agitation, and liquid–surface interactions further enhance this risk.[4,5]

Protein drugs identical to endogenous molecules, or other therapeutics which have a high degree of homology with their human equivalent protein, intrinsically carry a low degree of immunogenicity.[6] During packaging and subsequent administration to the patient, either due to contaminant particulate matter and/or trace amounts of syringe lubricant, these solutions can be potentially predisposed to aggregate formation and/or other more subtle changes in the physical structure of the molecule. The use of polymeric reservoirs, such as during deployment via automated pumps, hypothetically exacerbates this risk, especially when the drug, by necessity, is loaded at high concentrations. The aggregation process may be further accelerated because of the exposure to physiological or ambient temperatures and when combined with agitation due to patient ambulation, especially when unprotected by stabilizing agents. These protein aggregates have the potential to induce an immune response that may affect a more rapid clearance of the drug from the body, neutralize the action of the drug through binding of drug-specific immunoglobulin to active sites, and/or substantially change the pharmacokinetics of the drug.[7] Even in the case of endogenous molecules, the potential for breaking immune tolerance to self therefore exists, leading to the development of an autoimmune state with potentially catastrophic consequences for the patient. In 2002, for example, widespread cases of pure red cell aplasia were reported following replacement in 1998 of human serum albumin with polysorbate 80 (Tween 80) as a stabilizing agent in preparations of recombinant human erythropoietin (EPO) analog Epoetin alpha[8–10] resulting in the generation of neutralizing antibody to EPO.

Although stringent testing of a drug formulation in such a delivery device is a necessary requirement prior to widespread

**Abbreviations used:** IFNα2b, interferon-alpha 2b; native, unmodified IFNα2b formulation; LLA, low-level aggregate IFNα2b formulation; HLA, high-level IFNα2b aggregate formulation; pH4, low pH stressed IFNα2b formulation; OXD, metal oxidized IFNα2b formulation; TG, transgenic; WT, wild-type; ND, not detectable; EPO, erythropoietin; MPA, mobile phase A; MPB, mobile phase B; TFA, trifluoroacetic acid; TTBS, Tween Tris-buffered saline; RT, room temperature; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

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Journal of Pharmaceutical Sciences, Vol. 104, 722–730 (2015)

application, we propose that preclinical models offer a modicum of screening for such risk. Human interferon-alpha 2b (IFNα2b) represents an endogenous drug with highly beneficial antiviral potential and which is successfully used in the treatment of certain leukemias, lymphomas, and malignant melanoma as well as hepatitis B and C.[11] Until development of a hepatitis C vaccine proves to be as successful as in the case of the hepatitis A and B viruses, treatment with IFNα2b continues to offer a viable alternative. Continuous subcutaneous delivery of this drug via an external pump offers a means of increasing the efficacy of the therapy[12] while potentially avoiding the side-effects otherwise associated with bolus administration of the drug, which includes mild to severe flu-like symptoms in as many as 50% of patients. Other methods of extending the half-life of the drug in the circulation, such as PEGylation, do not appear to offer a reduction in the incidence of adverse effects of the native drug.[13] More importantly, clinically relevant neuropsychiatric side effects have been observed in 20%–40% of patients, with between 3% and 6% of patients entertaining suicidal thoughts,[14] although it remains unclear whether this is attributable to the drug itself or to the underlying disease.[15] Certainly, discontinuation of treatment because of the intolerability of the drug has been reported to occur in up to 17% of patients.[11] Nevertheless, a more stabile pharmacological profile free of the supratherapeutic blood levels associated with bolus administration, as effected through pump administration, offers to minimize this risk.

Hermeling et al.[16] previously demonstrated aggregate formation and breaking of tolerance in a mouse model transgenic for human IFNα2b under accelerated storage conditions. These conditions involved chemically induced changes in the form of either metal oxidation or storage under different pH conditions and at elevated temperature. In contrast, and to attempt crude evaluation of the risk imposed by physical manipulation of the drug, as might be experienced in a pump delivery scenario, we investigated the role of mechanical agitation[17] at physiological temperature alone, in the generation of immunogenic drug aggregates. Although the nature of the pump reservoir could be assumed to be an equally important predictor of aggregate formation, this study evaluated the contribution of agitation at physiological temperature alone. Using Hermeling's *in vivo* transgenic IFNα2b mouse model, both the potential for specific drug-binding immunoglobulin and its neutralizing capability were investigated. For comparison, we evaluated the response to unmodified as well as metal oxidized and low pH IFNα2b similar to that used by Hermeling.

## MATERIALS AND METHODS

### Aggregate Preparation

Carrier-free human recombinant IFNα2b (BioVision, Mountain View, California) was used to produce aggregates according to three different methods. All samples were in sodium phosphate buffer (PB, 20 mM, pH7.2). The IFNα2b concentration used for all methods of aggregation was 300 μg/mL.

Each completed formulation preparation was divided into aliquots (3 mL each into four separate cryovials) and stored at −80°C. Immediately prior to administration, formulations were diluted with PB to produce a final concentration of 100 μg/mL IFNα2b.

#### *Chemically Modified Formulations*

A low pH stressed formulation (pH4) was produced by reconstituting lyophilized IFNα2b in acetate buffer (10 mM, pH 4.0) and incubated at room temperature (RT°) for 30 min, whereas a metal oxidized formulation (OXD) was produced by incubating IFNα2b in PB containing 0.020 mM $CuCl_2$ and 2 mM ascorbic acid for 20 min at RT°. The reaction mixture was subsequently quenched by adding 100 mM EDTA to a final concentration of 1 mM. The metal oxidized and pH stressed samples were dialyzed (5°C against PB for 24 h).

#### *Mechanically Agitated Formulations*

Mechanically agitated formulations were produced using a temperature controlled sonic vibration system produced in-house. IFNα2b was reconstituted in PB and loaded into HPLC vials each containing three sterilized clean Teflon balls. IFNα2b was added to overflowing to limit headspace within the tubes. The vials were agitated (45 Hz) at 37°C for 6 h (low level aggregate IFNα2b formulation; LLA) or for 24 h (high-level IFNα2b aggregate formulation; HLA).

#### *Non-Aggregated Control Formulation*

Unstressed IFNα2b (native) was produced by reconstituting lyophilized drug in PB without further manipulation.

### Aggregate Characterization

#### *UV–VIS Spectroscopy*

UV spectra (200–500 nm) of the samples (300 mg/mL) were recorded on a Lambda 850 UV-VIS Spectrophotometer (PerkinElmer, Waltham, Massachusetts) in 1 cm quartz cuvettes.

#### *Size-Exclusion HPLC with On-Line Multi-Angle Laser Light Scattering, Absorbance, and Refractive Index Detectors*

Size-exclusion chromatography performed using three 5 mm, 7.8 × 300 mm SEC Protein columns in series for MALS (Wyatt Technology, Santa Barbara, California): 300 Å (Catalog No. WTC-030S5), 150 Å (Catalog No. WTC-015S5), and 100 Å (Catalog No. WTC-010S5) to analyze the aggregate composition of each sample. The mobile phase, 100 mM sodium phosphate buffer, pH 7.2, with 300 mM sodium chloride, freshly prepared with nanopure water (~18 MΩ cm) and filtered through a 0.02 μm filter (Anodisc 47, Catalog No. 6809–5002; Whatman, Maidstone, Kent, UK), was delivered to the column at a flow rate of 0.25 mL/min by a binary pump (1100 Series; Agilent, Santa Clara, California). The system was equipped with an automatic sampler (1100 Series; Agilent) and an in-line 0.2 μM filter. Detection was performed using a photodiode array detector (1100 Series; Agilent), a triple-angle light scattering detector (mini DAWN™ TREOS; Wyatt Technology), a quasi-elastic light scattering detector (DynaPro™ NanoStar; Wyatt Technology), and a differential refractometer (Optilab® rEX; Wyatt Technology). Bovine serum albumin standard (Catalog No. P5619; Sigma, St Louis, Missouri) was injected into the columns at a concentration of 2 mg/mL for normalization of the light scattering detectors, band broadening and adjusting the interdetector delay. Molecular weight determination was performed using ASTRA V software (Wyatt Technology) and a d*n*/d*c* value of 0.185 mL/g.