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Interferon, in particular interferon$\alpha$2a, is a pharmaceutically active protein which has antiviral and antiproliferative activity. For example interferon is used to treat hairy cell leukemia and Kaposi's sarcoma, and is active against hepatitis. In order to improve stability and solubility, and reduce immunogenicity, pharmaceutically active proteins such as interferon may be conjugated to the polymer polyethylene glycol (PEG).

The bioavailability of protein therapeutics are often limited due to their short plasma half-life, thus preventing them from attaining their maximum clinical potency. In recent years, PEG conjugated biomolecules have been shown to possess clinically useful properties (Inada et al., J. Bioact. and Compatible Polymers 5, 343 (1990); Delgado et al., Critical Reviews in Therapeutic Drug Carrier Systems 9, 249 (1992); Katre, Advanced Drug Delivery Systems 10, 91 (1993)). Among these are better physical and thermal stability, protection against susceptibility to enzymatic degradation, increased solubility, longer *in vivo* circulating half-life, decreased clearance and enhancing potency. It has been reported that branched PEG conjugates exhibit increased pH and thermal stability and greater stability towards proteolytic digestion than linear PEG conjugates. (Monfardini et al., Bioconjugate Chem. 6, 62 (1995)). Other properties of PEG proteins are reduced immunogenicity and antigenicity, as well as reduced toxicity. Another effect of PEGylation of certain proteins may be reduced *in vitro* activity accompanied by enhanced *in vivo* activity. This has been observed in G-CSF (Satake-Ishikawa et al., Cell Structure and Function 17, 157-160 (1992)), IL-2 (Katre et al., Proc. Natl. Acad. Sci. USA 84, 1487 (1987)), TNF-$\alpha$ (Tsutsumi et al., Jpn. J. Cancer Res. 85, 9 (1994)), IL-6 (Inoue et al., J. Lab. Clin. Med. 124, 529 (1994)) and CD4-IgG (Chamow et al., Bioconj. Chem. 5, 133 (1994)), among others.

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It has been now observed that in the case of interferon, PEGylation reduces *in vitro* antiviral activity but increases antiproliferative activity in human tumor cells. However the new PEG interferon conjugate of this invention has surprising properties in that the antiproliferative activity of the PEG interferon is much higher than that not only of interferon but of other PEG interferon conjugates. Although the antiproliferative activity of the conjugate is much increased over other PEG interferon-α conjugates, yet the reduction in antiviral activity is similar. In addition, the PEG interferon-α conjugate of this invention is non-immunogenic, it elicits virtually no antibody formation. In contrast, other PEG interferon-α conjugates do elicit limited antibody formation.

Accordingly, the invention is a new class of PEG derivatives of interferonα (IFNα). The conjugate of this invention has a branched PEG structure, as can be seen below. The branched PEG has the advantage of allowing the attachment of 2 linear PEG molecules at a single site, thus doubling the attached PEG mass without multiple sites of PEGylation.

Compared to unmodified IFNα (i.e. IFNα without a PEG attached), the conjugate has an increased circulating half-life and plasma residence time, reduced immunogenicity, decreased clearance, and increased antiproliferative activity, concomitant with decreased *in vitro* antiviral activity. Compared with other PEG-IFNα conjugates, the conjugate of this invention has a much greater antiproliferative activity, disproportionate to the enhancement or reduction that occurs in its other characteristics, and virtually no immunogenicity.

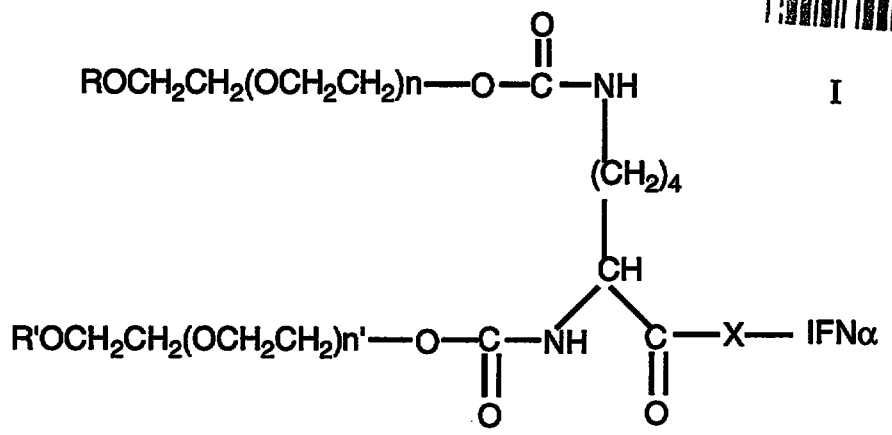
The physiologically active PEG-IFNα conjugate species of this invention has the formula:

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The conjugate of this invention has the same uses as IFNα, for example, antiproliferative uses. In particular, the PEG interferon-α conjugates of this invention are useful to treat immunomodulatory disorders such as neoplastic diseases, for example, hairy cell leukemia, CML, and Kaposi's sarcoma, and infectious diseases, in the same way IFNαs (especially IFNα2a) are used to treat these diseases. However, the conjugate of this invention has improved properties including superior stability, greater solubility, enhanced circulating half-life and plasma residence times. In addition, these conjugates have anti-proliferative activity which is superior to IFNα. Also as noted the conjugate shows a surprising dissociation of antiviral and anti-proliferative effects. This property is additionally useful to enhance a desired activity of a conjugate, while decreasing or eliminating an undesired activity. For example, if an undesired side effect is associated with the antiviral activity, eliminating this activity would eliminate the side effect, while retaining the antiproliferative activity. Therefore, the present invention also comprises the pharmaceutical compositions on the basis of the compounds of formula I or their salts and to methods for producing them.

The pharmaceutical compositions of the present invention used in the control or prevention of illnesses comprises an interferon conjugate of the general formula I and a therapeutically inert, non toxic and therapeutically acceptable carrier material. The pharmaceutical compositions to be used can be formulated and dosed in a

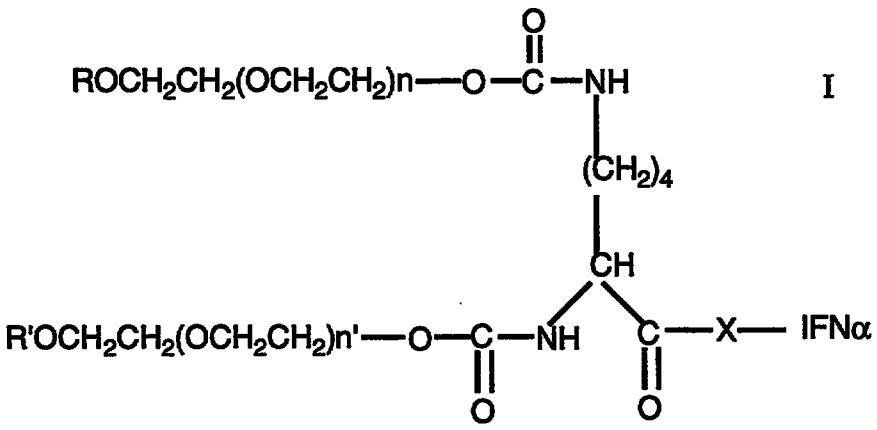
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fashion consistent with good medical practice taking into consideration the disorder to be treated, the condition of the individual patient, the site of delivery of the protein conjugate, the method of administration and other factors known to practitioners.

The claimed conjugate is a physiologically active PEG-IFNα conjugate having the formula




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where R and R' are independently lower alkyl; X is NH or O (X is at least one of the functional groups in the IFNα molecule selected from $NH_2$ or OH); n and n' are integers having a sum of from 600 to 1500; and the average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons. The conjugate of formula I has a branched structure, in that two PEG moieties are attached to the protein via a single linkage.

The numbers n and n' are selected such that the resulting conjugate of Formula I has a physiological activity of IFNα, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified IFNα. n and n' (n and n' may be the same or different) represent the number of ethylene glycol units in the PEG. A single PEG unit of $OCH_2CH_2$ has a molecular weight of about 44 daltons. The molecular weight of the conjugate (excluding the molecular weight of the IFNα) depends on the numbers n and n'. The sum of n and n' for the conjugate of Formula I is from 600 to

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1500, producing a conjugate having a total average molecular weight of PEG units of from about 26,000 to 66,000 and preferably from about 35,000 to 45,000 daltons, and especially about 39,000 to 45,000 daltons, with 40,000 daltons especially preferred. A preferred sum of n and n' is from about 800 to 1200, with the average sum being from about 850 to 1000, and a preferred sum being about 910. Either of n or n' may individually be 420 or 520, or both may be 420 or 520, or both may be 455. The preferred ratio of n to n' is from about 0.5 to 1.5, with an especially preferred ratio of from about 0.8 to about 1.2. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques.

Also preferred is a conjugate of Formula I where IFNα is IFNα2a, a conjugate where R and R' are methyl, a conjugate where X is NH, and a conjugate where n and n' are individually or both either 420 or 520. Such a conjugate having all the above characteristics is especially preferred.

R and R' may be any lower alkyl, by which is meant an alkyl group having from one to six carbon atoms such as methyl, ethyl, isopropyl, etc. Branched alkyls are included. A preferred alkyl is methyl. With regard to the two PEG groups of Formula I, R and R' may be the same or different.

By IFNα (interferon α) and its species IFNα2a is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of IFNα, such as muteins or otherwise modified proteins, is encompassed. Obtaining and isolating IFNα from natural or recombinant sources is well known (Pestka, Arch. Biochem. Biophys. 221, 1 (1983)). A preferred IFNα is IFNα2a, which as stated above, is obtained by known methods (Pestka, Sci. Am. 249, 36 (1983); European Patent No. 43 980)).

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The physiologically active conjugate of Formula I has IFNα activity, by which is meant any fraction or multiple of any known IFNα activity, as determined by various assays known in the art. In particular, the conjugates of this invention have IFNα activity as shown by antiproliferative activity against tumor cells and antiviral activity against cells infected with a virus. These are known activities of IFNα. Such activity in a conjugate can be determined by assays well known in the art, for example the assays described below (see also Rubinstein et al., J. Virol. 37, 755 (1981); Borden et al., Canc. Res. 42, 4948 (1982)). Part of this invention is a conjugate of Formula I which has greater antiproliferative activity and less antiviral activity than unmodified IFNα.

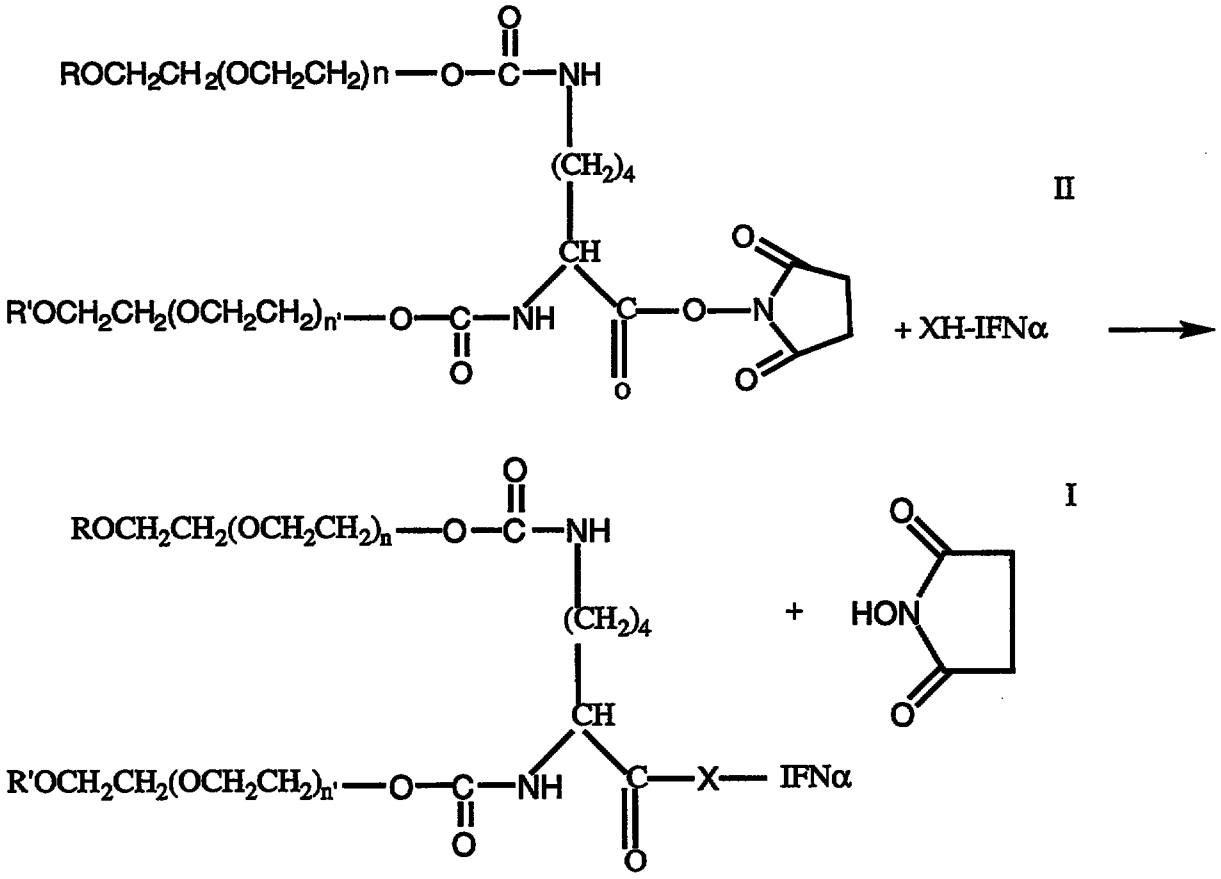
The conjugate of Formula I is produced by covalent linkage of IFNα to PEG which has been activated by replacement of the PEG hydroxyl with a linking group, forming a reagent which is an N-hydroxy succinimide ester derivative of PEG (in particular monomethoxy PEG) of Formula II. The reagent may be obtained by conventional methods (Monfardini et al., supra). Linkage is via an amide or ester bond. In a preferred conjugate, linkage is via an amide bond (X is NH). Part of this invention is a method for increasing the antiproliferative activity of IFNα while reducing the antiviral activity of the IFNα, by linking the IFNα as described above to a reagent of Formula II to produce a PEG-IFN conjugate.

X represents the attachment site on IFNα by which the PEG reagent of Formula II is covalently attached to the IFNα. The reagents attach to primary amino groups (XH = $NH_2$) on for example lysine or to the N-terminus of the IFNα. The reagents can also attach to a hydroxyl (XH = OH) on for example serine.

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The reagent of formula II (PEG2-NHS), in which a total of 2 mono-methoxy PEG (m-PEG) chains are linked to lysine, one each at the α and ε amino groups via carbamate (urethane) bonds and having the lysine carboxyl group activated to a succinimidyl ester, may be obtained by conventional methods, according to known procedures (Monfardini et al., supra) applicable to a reagent with R as lower alkyl, and a desired n. The reagent may be obtained from Shearwater Polymers, Inc. (Huntsville, Alabama). The preferred average MW of the PEG obtained is about 20,000 daltons, providing a total PEG mass of about 40,000 daltons in PEG2-NHS (other MWs may be obtained by varying n for the PEG-alcohol starting materials for the reagent of Formula II, by conventional methods).

The reagent of formula II may be conjugated to IFNα by conventional methods. Specifically, the reagent of Formula II

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primarily reacts with one or more of the primary amino groups (for example N-terminus and lysine side chains) of IFNα (for example IFNα2a) to form an amide linkage between the IFNα and the polymer backbone of PEG. The PEGylation reaction can also take place between PEG2-NHS and the free (if any) hydroxyl groups (for example serine) of IFNα to form an ester linkage. The reaction mechanism is shown above. The reaction conditions are conventional to a skilled person, and are provided in detail below. The PEG reagent is combined with IFNα under mildly basic conditions at low temperature under conditions suitable for a nucleophilic substitution which will produce the conjugate of Formula I. This is also shown in the above reaction mechanism.

Attaching the reagents to IFNα may be accomplished by conventional methods. PEGs of any selected MW of this invention may be used. Reaction conditions may be selected to provide the claimed conjugate with one reagent attached. The conjugate of Formula I, which has a single reagent of Formula II attached, is separated from unmodified IFNα and conjugates having attached more than one reagent molecule by conventional methods. Purification methods such as cation exchange chromatography may be used to separate conjugates by charge difference, which effectively separates conjugates into their various molecular weights. The content of the fractions obtained by cation exchange chromatography may be identified by molecular weight using conventional methods, for example, mass spectroscopy, SDS-PAGE, or other known methods for separating molecular entities by molecular weight. A fraction then is accordingly identified which contains the conjugate of Formula I purified free from unmodified IFNα and from conjugates having more than one reagent attached. In addition, the reagents of Formula II release one lysine per reagent upon acid hydrolysis, so that the number of lysines in the hydrolysis indicates the number of PEGs attached to the protein, thus the number of reagent molecules attached to a conjugate may be verified.

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The following Examples are provided to illustrate the invention and do not limit it in any way. IFNα2a is used in these examples. Other species of IFNα may also be conjugated to PEG by the methods exemplified.

DESCRIPTION OF THE DRAWINGS

Figure 1: Antitumor activity of the PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal A498 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal A498 cells on Study Day -33. On Study Day 0 PEG-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 μg) of PEG2-IFN alpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a four week period.

Figure 2: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal A498 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal A498 cells on Study Day -33. On Study Day 0 IFNalpha2a treatment was initiated. The indicated amount (10, 20, 40 or 100 μg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a four week period.

Figure 3: Antitumor activity of PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal ACHN cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal ACHN cells on Study Day -25. On Study Day 0 PEG2-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 μg) of PEG2-IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a five week period.

Figure 4: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal ACHN cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal ACHN cells on Study Day -25. On Study Day 0 IFNalpha2a treatment was initiated. The

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indicated amount (10, 20, 40 or 100 µg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a five week perid.

Figure 5: Antitumor activity of PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal G402 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal G402 cells on Study Day -45. On Study Day 0 PEG2-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 µg) of PEG2-IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a five week period.

Figure 6: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal G402 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal G402 cells on Study Day -45. On Study Day 0 IFNalpha2a treatment was initiated. The indicated amount (10, 20, 40 or 100 µg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a five week period.

## Example 1

## Preparation of conjugate of Formula I

Materials

Interferonα2a was prepared by known methods (Pestka, supra). Polyethylene glycol (PEG) reagent of formula II was purchased from Shearwater Polymers, Inc. (Huntsville, Ala). Fractogel® EMD CM 650(S) resin, with particle sizes 25-40µm, were supplied by EM Separations (Gibbstown, MA). Concentrated (10X) phosphate buffered saline (PBS), pH 7.3, was purchased from BioWhittaker (Walkersville, MD). Sodium dodecyl (laurel) sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) pre-cast gels and electrophoresis units were obtained from NOVEX (San Diego, CA). Concentrated Fast Stain for protein staining of PEG conjugates on SDS-PAGE was purchased

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from Zoion Research, Inc. (Newton, MA). The LAL endotoxin assay kit was purchased from Associates of Cape Cod, Inc. (Woods Hole, MA). All other reagents used were of the highest quality available. The jugular cannulated rats and BDF-1 mice were supplied by Charles River Laboratories (Wilmington, MA).

Experimental Procedures

A. Small scale preparation of conjugate of Formula I

Two hundred-eight milligrams (5.2μmol) of the reagent of Formula II (average MW of 40,000 daltons) were added to 50 mg (2.6μmol) of IFNα in 10ml of 100mM borate, pH 8.0. Final protein reagent molar ratio was 1:2. The reaction mixture was stirred at 4°C for 2 hours. The reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid.

The reaction mixture was diluted 50-fold with water, filtered through a 0.2μ filter and applied onto an Amicon column packed with 100ml (3.2x13cm) Fractogel EMD CM 650(S), at a flow rate of 20ml/min. The column was previously equilibrated with 10mM ammonium acetate, pH 4.5. The column effluent was monitored by UV absorbance at 280nm. The column was then washed with the equilibration buffer until UV absorbance returned to baseline. PEG-IFN conjugates having more than one reagent of Formula II attached (PEG-IFN oligomers) were eluted with 40 mM ammonium acetate, pH 4.5 and the conjugate of Formula I was eluted with 0.12M NaCl in the 40 mM ammonium acetate buffer. The unmodified IFN remaining in the column was eluted with 0.5M NaCl in the same buffer. The column was regenerated by a 1.0M NaCl wash followed by the equilibration buffer wash. The pooled fractions of the conjugate of Formula I were concentrated in an Amicon stirred cell concentrator fitted with a YM10 membrane to approximately 1mg/ml concentration.

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The Fractogel CM 650(S) cation exchange resin used for purification, adsorbed the PEG and unmodified IFN effectively. The strength of adsorption was dependent upon the degree of PEGylation. The conjugates bound less tightly than the unmodified IFN. The PEG-IFN oligomers were eluted with 40mM ammonium acetate, while the conjugate of Formula I eluted with 0.12M NaCl. The unmodified IFN eluted with 0.5M NaCl. All preparations contained <5EU/mg endotoxins. The resulting preparation contained >99% of conjugate of Formula I and was free of unmodified IFN.

B. Large-Scale Preparation of conjugate of Formula I

Six thousand two hundred and forty milligrams (156 μmol) of the reagent of Formula II (average molecular weight of 40,000 daltons) was dissolved in 63 ml of 1mM HCl at 4°C and quickly added to 125 ml of a solution containing 1000 mg (52 μmol) of interferon in 50 mM borate buffer, pH 9.0. The final protein/reagent ratio was 1:3 and the final reaction mixture protein concentration was 5.3 mg/ml. The reaction mixture was stirred for 2 hours at 4°C. The reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid.

The reaction mixture was diluted 10-fold with water and applied onto a column packed with 600 ml Fractogel EMD CM 650(M) previously equilibrated with 20mM sodium acetate, pH, 4.5 at a linear velocity of 1.3cm/min. The column was washed with the equilibration buffer followed by 10 mM NaCl to remove excess reagent, reaction byproducts and PEG-IFN oligomers. The conjugate of Formula I was eluted with the equilibration buffer containing 200mM NaCl. The unmodified interferon still adsorbed to the column was removed by washing with 0.75 M NaCl in the equilibration buffer. The conjugate of Formula I, which was eluted at 0.3-0.5mg/ml was further concentrated and diafiltered into the final formulation buffer, 20 mM sodium acetate, pH, 5.0, containing 150mM NaCl. The overall yield of the conjugate of Formula I was 40-45%.

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The purified PEG-IFN from the large-scale preparation consists of >99% conjugate of Formula I. The average molecular weight of the conjugate of Formula I of this example is 62,000 daltons, including the molecular weight of IFNα2a which is 19,241 daltons, and the average molecular weight of the reagent which is between 40,000 and 45,000 daltons, about 43,000 daltons.

## Example 2

### Characterization of conjugate of Formula I

Protein Determination

Protein concentrations were determined using an $A_{280}$ value of 1.0 for a 1mg/ml solution of IFNa α2a.

SDS-PAGE Analysis

The conjugate was analyzed by sodium dodecyl (lauryl) sulfate/polyacrylamide (8-16%) gel electrophoresis, under reducing conditions, according to the methods of Laemmli (Nature 227, 680 (1970)). SDS-PAGE containing PEG-conjugates were stained for protein using Fast Stain (Zoion Research, Inc.) according to the manufacturer's instructions.

Determination of Endotoxin Levels

Endotoxin levels were determined using the LAL method, according to the manufacturer's instructions. All preparations contained <5 EU/mg endotoxins.

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Example 3

*In Vitro* Bioactivities of conjugate of Formula I

Antiviral Activity in Bovine Kidney Cells

The *in vitro* antiviral activity of IFNα2a and the conjugate of Formula I as prepared in Example 1.A. were determined in a cell culture bioassay employing Madin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus (Rubinstein et al., supra). The antiviral activities are listed in Table 1, along with their corresponding residual activities as a percentage of the starting IFN.

Table 1

Anti-Viral Activities

| Samples | PEG Type | Total PEG Mass (kDa) | # Lys Modified | Specific Activity (U/mg) | Residual Activity (%) |
|---|---|---|---|---|---|
| IFNα2a | - | - | - | $2.00 \times 10^8$ | 100 |
| Conjugate of Formula I | Branched | 40 | 1 | $1.40 \times 10^7$ | 7 |

*In Vitro* Antiproliferative Activity in Human Tumor Cells

The *in vitro* antiproliferative activities were assayed in human Daudi (Burkitt's Lymphoma) cells, as described by Borden et al. Human Daudi cells were maintained as stationary suspension cultures in RPMI 1540 supplemented with 10% fetal bovine serum and 2 mM glutamine (Grand Island Biologicals, Grand Island, NY). The cells were screened and found to be free of mycoplasma. Cells ($2 \times 10^4$) were added to wells of microtiter plates (Costar, MA) in 100 µl of medium. Various concentrations of IFN and the conjugate of Formula I as prepared in Example 1.A. were added to the wells in a volume of 100 µl. The plates were incubated at 37°C in 5% $CO_2$ for 72 hours.

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Cells were pulsed with 0.25μCi/well of $^3$H-thymidine (New England Nuclear, Boston, MA), sixteen hours before cell harvesting. The cells were harvested onto glass filters and counted in a liquid scintillation counter. The results were expressed as % inhibition calculated using the formula:

% Inhibition = [ (A - B / A] x 100, where;

A = cpm in control culture (cells incubated in medium alone)
B = cpm in experimental culture

Samples were run in quadruplicate and standard deviation was less than 20% of the mean of all cases. Experiments were run at least twice with comparable results.

The antiproliferative activities ($IC_{50}$) of IFN and the conjugate are listed in Table 2. The data indicate that there is a 28-fold increase in antiproliferative activity for the conjugate of Formula I, as compared to that of IFN.

Table 2

In Vitro antiproliferative activities in human Daudi (Burkitt's lymphoma) cell lines.

| Sample | Antiproliferative IC50 (ng/ml) | Activity Increase |
|---|---|---|
| IFNα2a | 0.56 | 1x |
| Conjugate of Formula I | 0.02 | 28x |

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Example 4

Pharmacokinetics

Female Sprague Dawley rats, surgically implanted with jugular cannulas, with an average body weight of 240 - 260g were housed individually, allowed free access to food and water and maintained in a 12 hour light -dark cycle. Within 4 - 6 hours after arrival, jugular cannulas were flushed with PBS. The following day, after flushing with 0.15 - 0.2ml PBS, $2x10^6$ units of IFNα in 0.2 - 0.4ml PBS were injected, followed by injection of 0.15 - 0.2ml PBS to assure that all drug was washed into the animal. Thus each animal received a dosage of $8x10^6$ IFNα units/kg body weight.

Blood samples were drawn at 5, 15 and 30 minutes, as well as, 1, 3, 5, 12 and 24 hours after injection of IFN and, the conjugate of Formula I. At all time points, after discarding the first 0.15 - 0.2ml of blood, an aliquot of 0.5ml blood was withdrawn using a fresh syringe via the jugular cannula. The samples were discharged into serum separating tubes at room temperature. Once all the samples were collected for the time points, the tubes were centrifuged at 14,000 x g in a refrigerated Eppendorf centrifuge for 10 minutes. The separated serum was transferred into 1.5ml microfuge tubes and frozen at -80°C, until ready for bioassay. Serum samples were diluted appropriately and the antiviral activity at each time-point was determined as described. From the plot of time vs. activity, the terminal half-life of the conjugate of Formula I and IFNα were determined and listed in Table 3, which also include plasma residence times.

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Table 3

Terminal Half-Lives ($t_{1/2}$) and Mean Plasma Residence Time

| Sample | $^t$1/2 (hours) | Plasma Residence Time (hours) |
|---|---|---|
| IFNα2a | 2.1 | 1.0 |
| Conjugate of Formula I | 15.0 | 20.0 |

Terminal $^t$1/2 estimated by log linear regression.

Example 5

Immunogenicity

Normal BDF-1 mice (ten per group) were injected intraperitonially once per day five times per week with various interferon preparations having 300,000 units of antiviral activity. Some mice were also injected with aggregated form of IFNα2a which is more immunogenic than the monomer form. Blood samples were taken 19 days following the last injection and the serum was evaluated for neutralizing antibodies.

As seen in Table 4, mice injected with IFNα2a produced neutralizing antibodies and this response was greatly increased in mice injected with interferon aggregates. No antibodies were detectable in the majority of animals injected with the conjugate of this invention.

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Table 4

Immunogenicity

| Treatment | Antibody (INU/ml) * Median | Range |
|---|---|---|
| IFNα2a | 2,400 | 217-8,533 |
| IFNα2a Aggregates | 42,667 | 8,000-768,000 |
| Conjugate of Formula I | 0 | 0-1,133 |

* Interferon neutralizing units/ml

Example 6

Antitumor activity *In Vivo*

The *in vivo* antitumor activity of a conjugate of Formula I (PEG2-IFNalpha2a) and unmodified IFNalpha2a were evaluated by determining their ability to reduce the size of various human tumor cells implanted subcutaneously into mice. Results are shown in Figures 1-6.

Procedure: Athymic nude mice (Harlan) received a subcutaneous implant under the left rear flank of 2 x $10^6$ human renal A498 cells (Figures 1 and 2), human renal ACHN cells (Figures 3 and 4), or human renal G402 cells (Figures 5 and 6). 3 to 6 weeks were allowed for the tumors to become established, as indicated. The size criteria for acceptance into the study was 0.05 to 0.50 cubic centimers. The mice were given total weekly doses of PEG2-IFNalpha2a or unmodified IFNalpha2a of 30, 60, 120 or 300 μg. In the case of PEG2-IFNalpha2a the mice were treated one time per week (Monday) with 30, 60, 120 or 300 μg of PEG2-IFNalpha2a per treatment. In the case of unmodified IFNalpha2a the mice were

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treated three times per week (Monday, Wednesday, Friday) with 10, 20, 40 or 100 μg of IFNalpha2a per treatment. The duration of treatment was 4 to 5 weeks depending on tumor aggressiveness. Tumor volumes were measured every Monday prior to treatments.

Results: PEG2-IFNalpha2a showed a marked reduction in A498 tumor size as compared to unmodified IFNalpha2a for all weekly dosage levels tested, at 7 days, 14 days, 21 days and 28 days after the beginning of treatment (Figures 1 and 2). Treatment continued for four weeks. Seven days after treatment was discontinued three mice in each group were sacrified. In the three mice treated with PEG2-IFNalpha2a no residual tumor was observed. In mice treated with unmodified IFNalpha2a the A498 tumor weight was 1.28 grams, 0.62 grams, and 1.60 grams respectively in each of three mice. The A498 tumor weight was 2.32 grams, 2.37 grams, and 1.94 grams in each of three control mice. At 80 days after the end of the four week treatment period the existence of tumors was determined by palpation in seven mice. All seven mice were free of tumor tissue by palpation.

PEG2-IFNalpha2a showed a significant reduction in ACHN tumor size as compared to unmodified IFNalpha2a for weekly dosage levels of 60, 120, and 300 μg, at 14 days, 21 days, 28 days and 35 days (Figures 3 and 4).

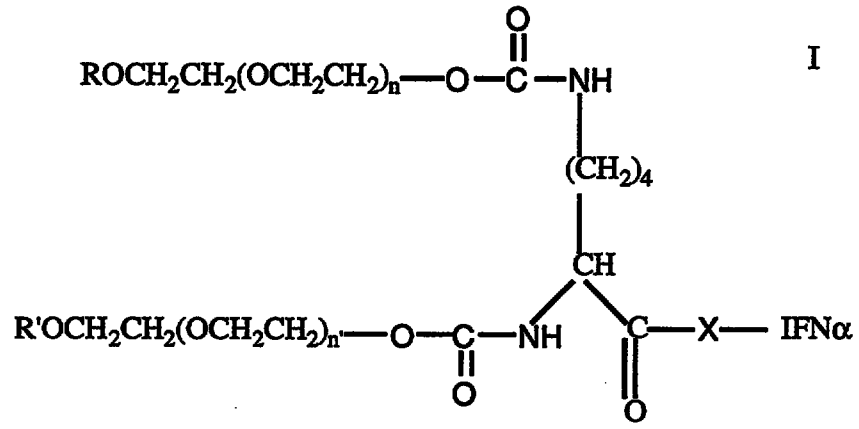
PEG2-IFNalpha2a showed a significant reduction in G402 tumor size as compared to unmodified IFNalpha2a for weekly dosage levels of 60 and 120 μg, at 14 days, 21 days, 28 days and 35 days (Figures 5 and 6).

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## Claims

1. A physiologically active PEG-IFNα conjugate having the formula



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wherein R and R' are independently lower alkyl; X is NH or O; n and n' are integers having a sum of from 600 to 1500; and the average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons.

2. A conjugate of claim 1 wherein the molecular weight of the polyethylene glycol units is from about 35,000 to about 45,000 daltons.

3. A conjugate of claim 2 wherein the molecular weight of the polyethylene glycol units is about 40,000 daltons.

4. A conjugate of claim 1 wherein R and R' are methyl.

5. A conjugate of claim 1 wherein X is NH.

6. A conjugate of claim 1 wherein the IFNα is IFNα2a.

7. A conjugate of claim 1 wherein the average sum of n and n' is 850 to 1000.

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8. A conjugate of claim 1 wherein R and R' are methyl; X is NH; IFNα is IFNα2a; and one or both of n and n' is 420.

9. A conjugate of claim 1 wherein R and R' are methyl; X is NH; IFNα is IFNα2a; and one or both of n and n' is 520.

10. A conjugate of claim 1 which has greater antiproliferative activity than IFNα and less antiviral activity than IFNα.

11. A method for producing a PEG-IFNα conjugate having an increased antiproliferative activity and decreased antiviral activity as compared to IFNα, which method consists of covalently linking a reagent of Formula II to IFNα to produce said PEG-IFNα conjugate.

12. Pharmaceutical compositions comprising a PEG-IFNα conjugate as claimed in any one of claims 1-10 and a therapeutically inert carrier.

13. Pharmaceutical compositions for the treatment or prophylaxis of immunomodulatory disorders such as neoplastic diseases or infections diseases comprising a PEG-IFNα conjugate as claimed in any one of claims 1-10 and a therapeutically inert carrier.

14. The use of a PEG-IFNα conjugate according to any one of claims 1-10 for the manufacture of medicaments for use in the treatment or prophylaxis of illnesses.

15. A PEG-IFNα conjugate as claimed in claims 1-10 whenever prepared according to the method as claimed in claim 11.

16. A PEG-IFNα conjugate as claimed in claims 1-10 for the manufacturing of medicaments for use in the treatment or prophylaxis of illnesses.

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17. The products, pharmaceutical compositions, processes and methods substantially as described hereinbefore.

18. A method for the treatment or prophylaxis of immuno-modulatory diseases comprising administering a PEG-IFNα conjugate according to any one of claims 1-10.

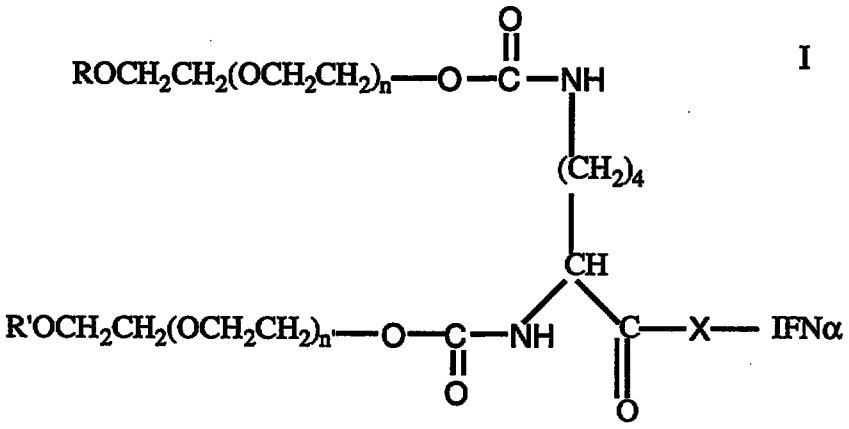
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## Abstract

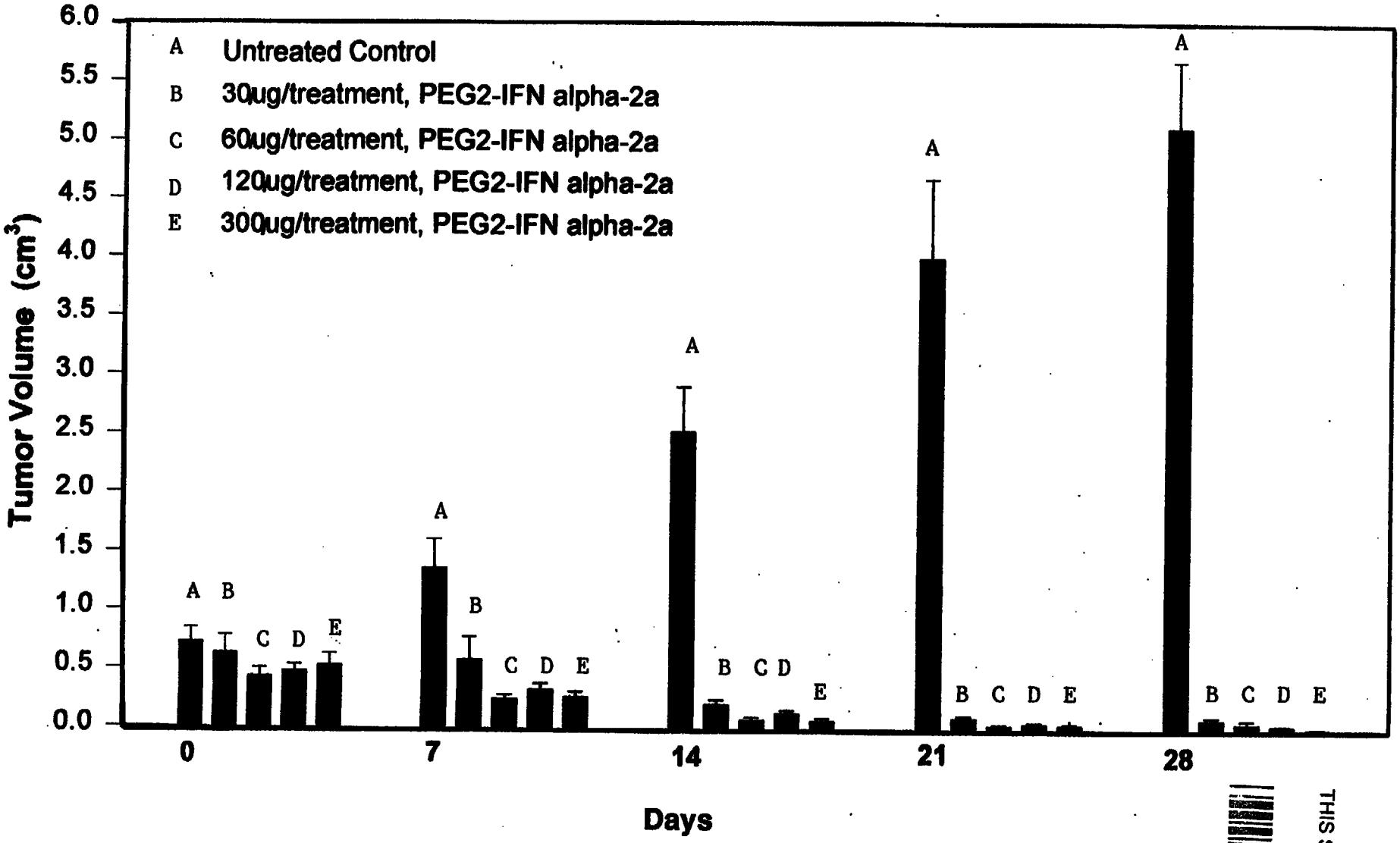
Physiologically active PEG-IFNα conjugates having a formula as follows:



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FIGURE 1

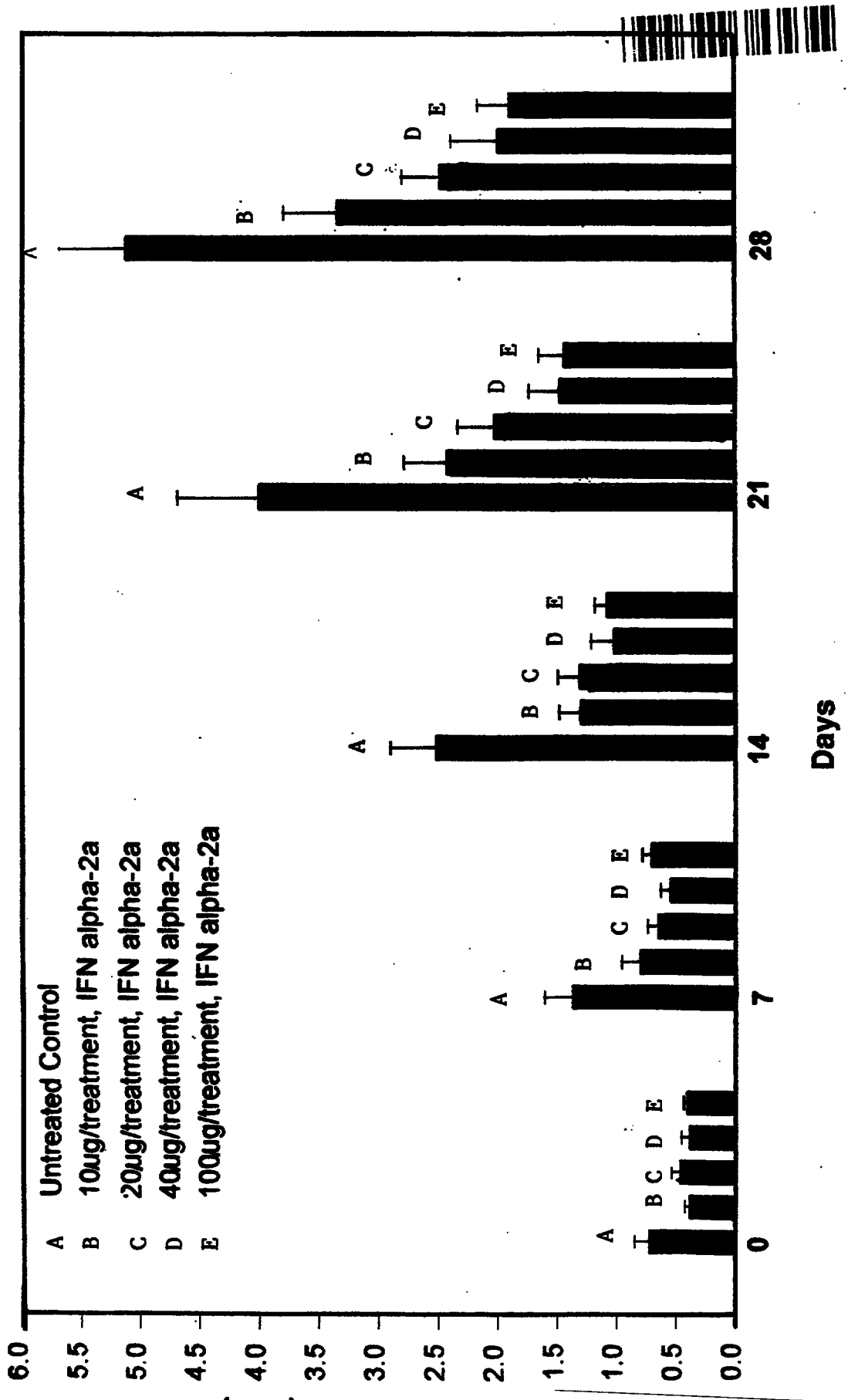



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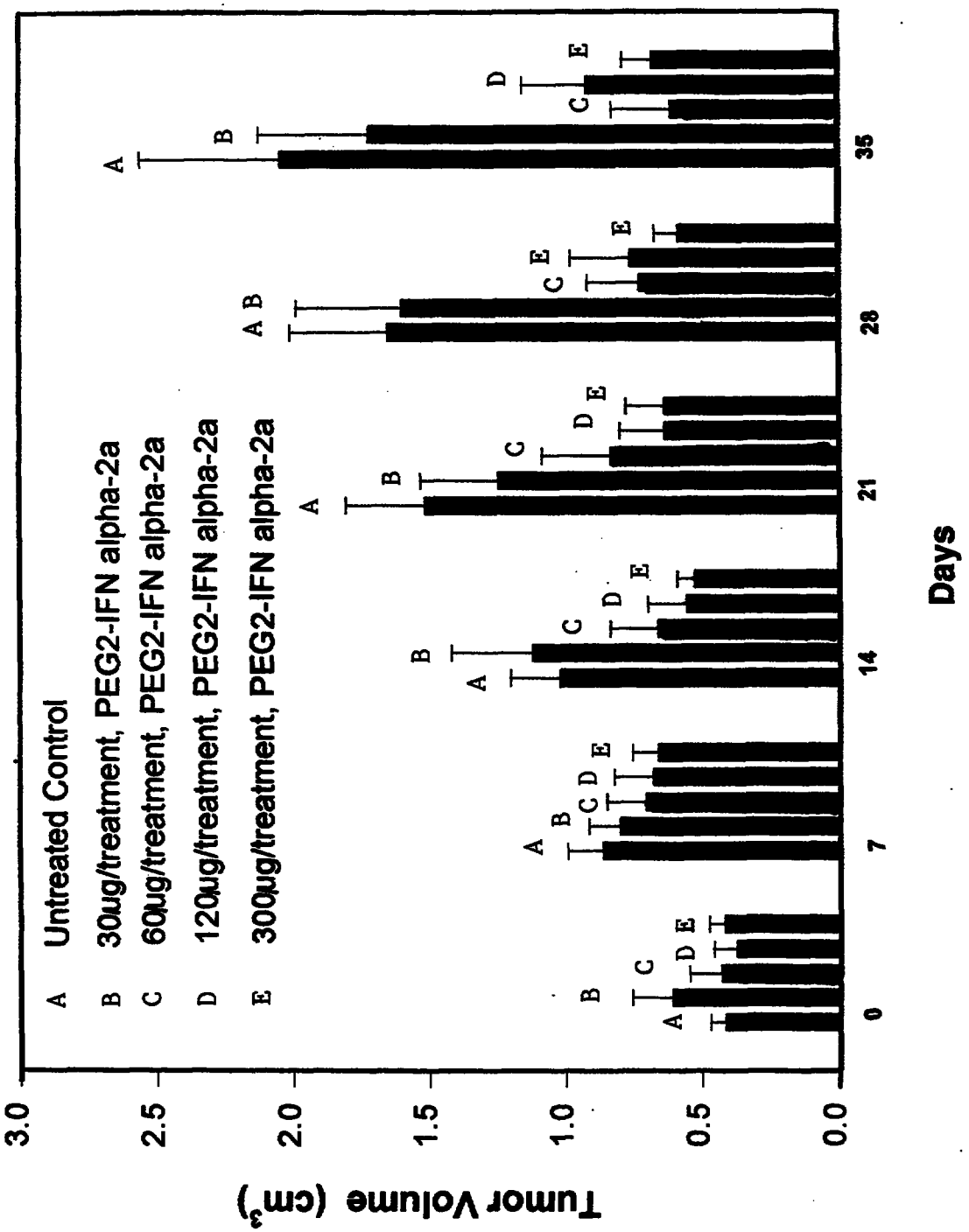
FIGURE 2




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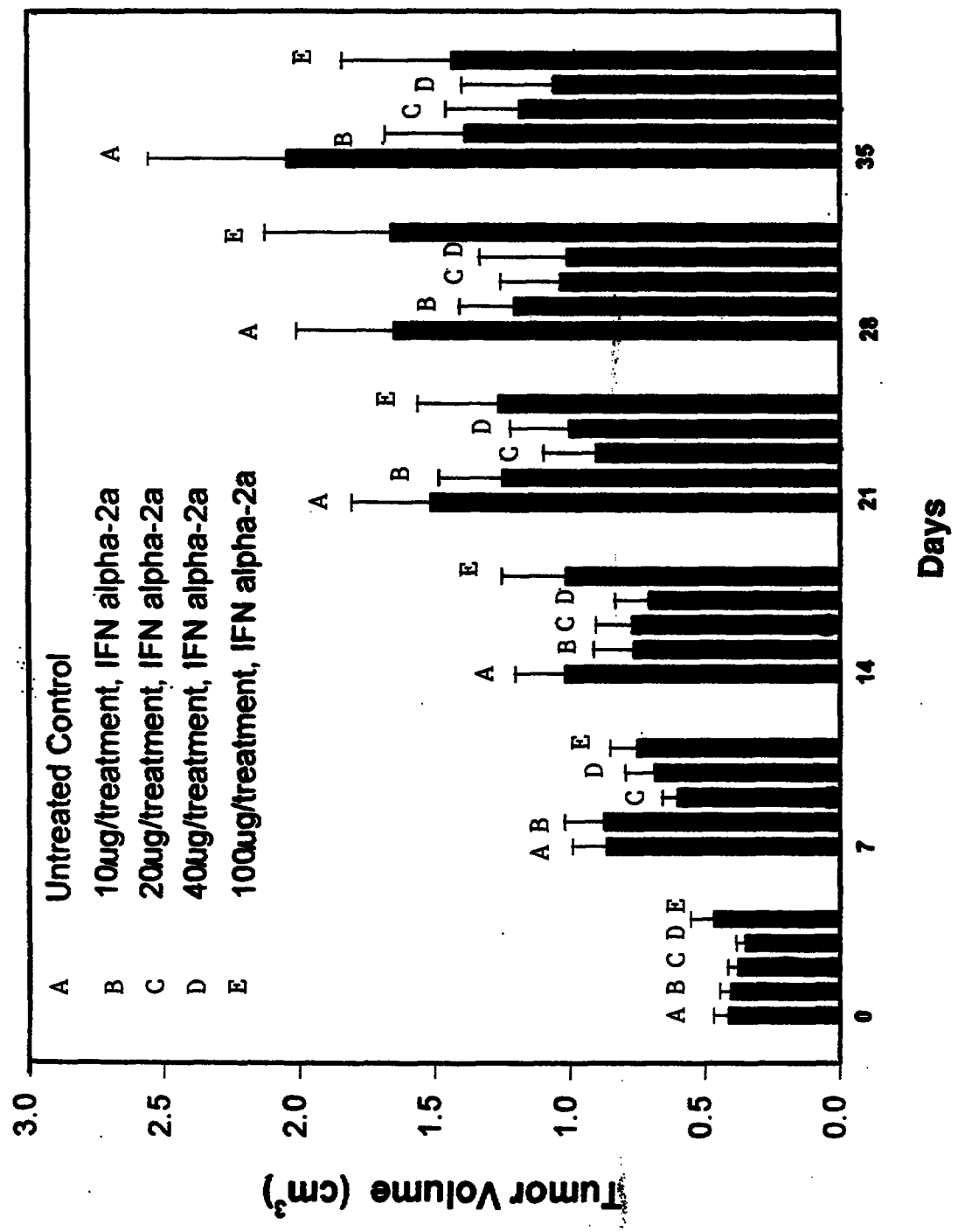
FIGURE 3




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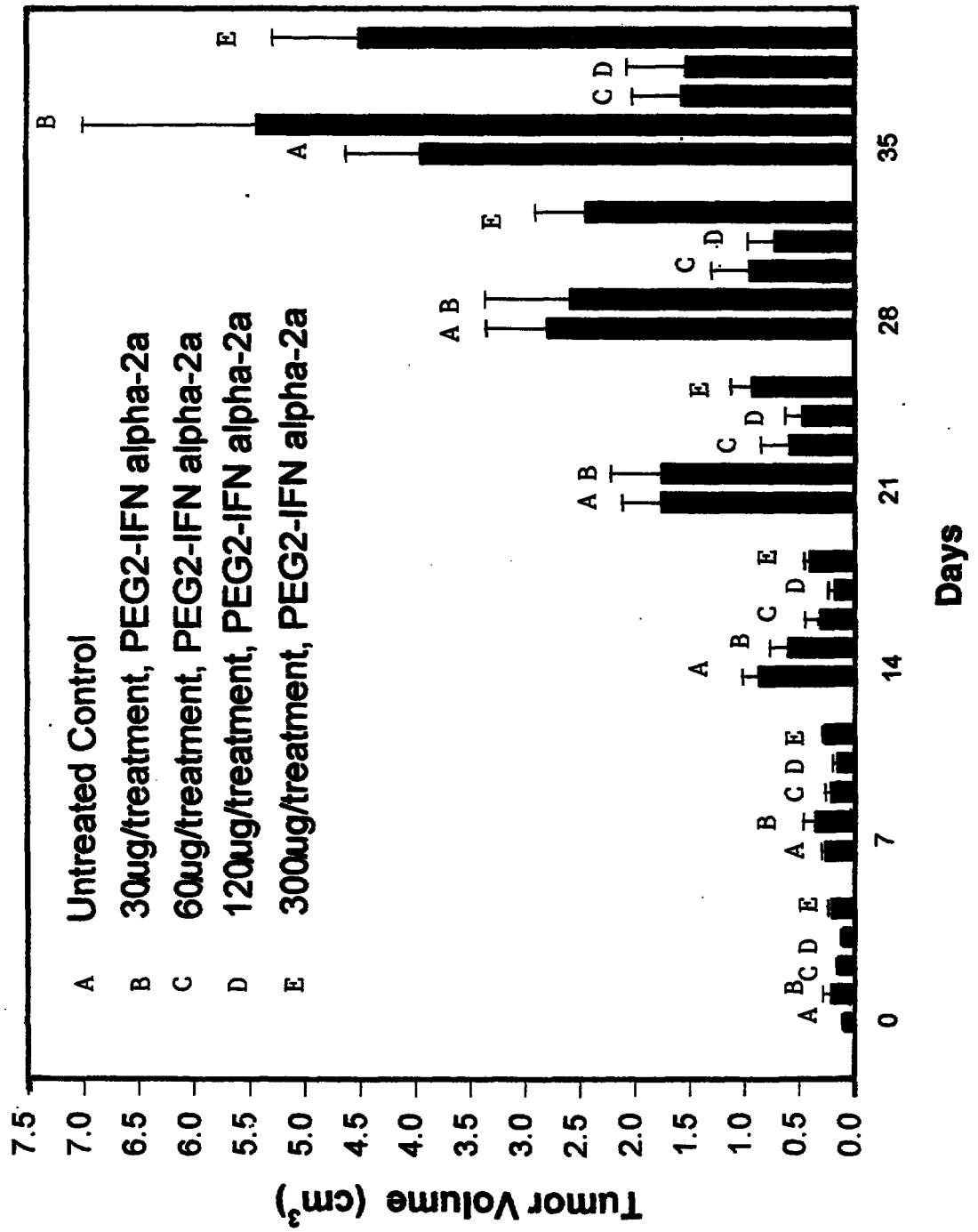
FIGURE 4




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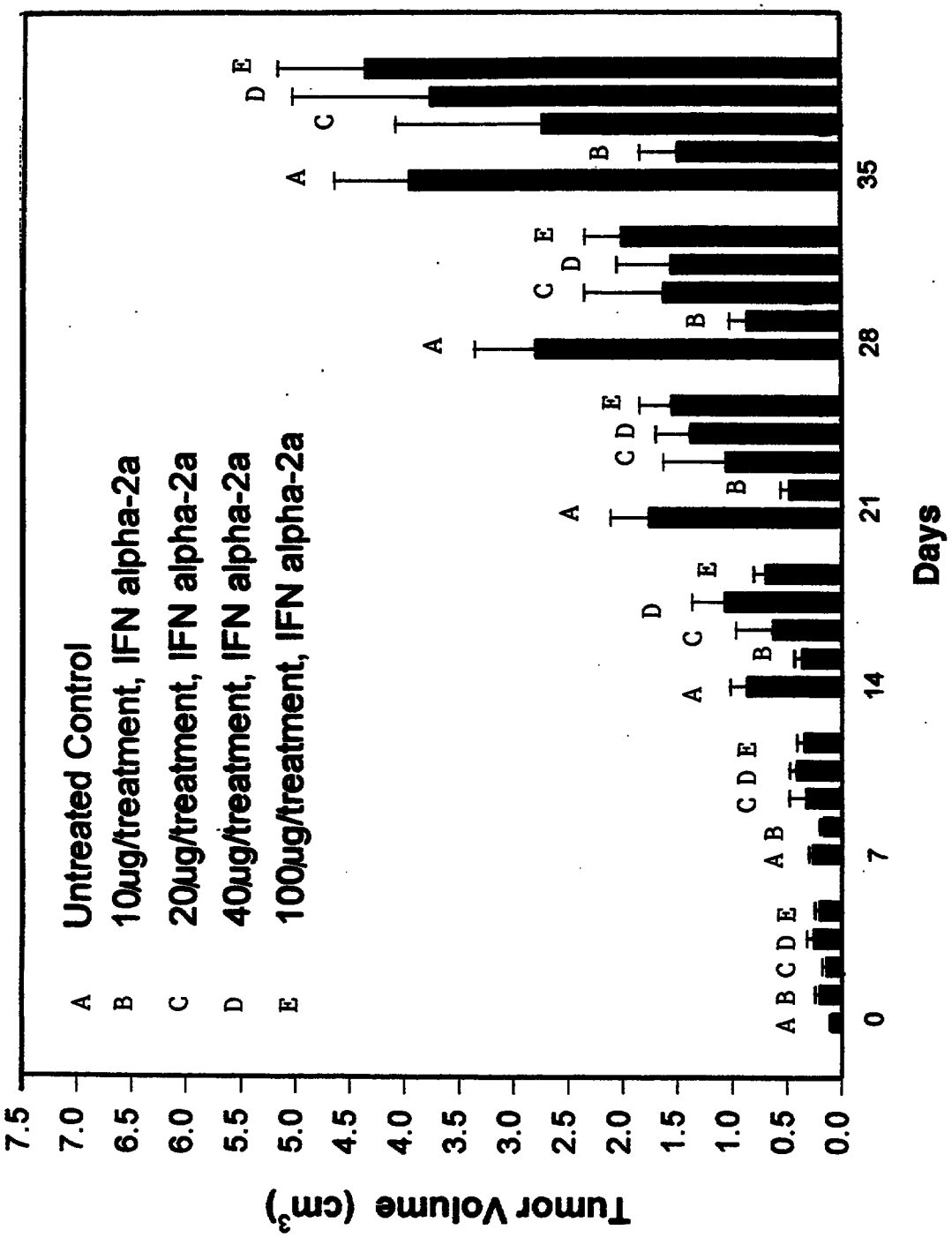
FIGURE 5




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FIGURE 6




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☑ Description : paged 1 to paged 19
☑ Claim(s) : paged 20 to paged 22
☑ Drawing(s) : paged 1 to paged 2
☑ Abstract : paged 23 to paged
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# INTERFERON CONJUGATES

Interferon, in particular interferonα2a, is a pharmaceutically active protein which has antiviral and antiproliferative activity. For example interferon is used to treat hairy cell leukemia and Kaposi's sarcoma, and is active against hepatitis. In order to improve stability and solubility, and reduce immunogenicity, pharmaceutically active proteins such as interferon may be conjugated to the polymer polyethylene glycol (PEG).

The bioavailability of protein therapeutics are often limited due to their short plasma half-life, thus preventing them from attaining their maximum clinical potency. In recent years, PEG conjugated biomolecules have been shown to possess clinically useful properties (Inada et al., J. Bioact. and Compatible Polymers 5, 343 (1990); Delgado et al., Critical Reviews in Therapeutic Drug Carrier Systems 9, 249 (1992); Katre, Advanced Drug Delivery Systems 10, 91 (1993)). Among these are better physical and thermal stability, protection against susceptibility to enzymatic degradation, increased solubility, longer *in vivo* circulating half-life, decreased clearance and enhancing potency. It has been reported that branched PEG conjugates exhibit increased pH and thermal stability and greater stability towards proteolytic digestion than linear PEG conjugates. (Monfardini et al., Bioconjugate Chem. 6, 62 (1995)). Other properties of PEG proteins are reduced immunogenicity and antigenicity, as well as reduced toxicity. Another effect of PEGylation of certain proteins may be reduced *in vitro* activity accompanied by enhanced *in vivo* activity. This has been observed in G-CSF (Satake-Ishikawa et al., Cell Structure and Function 17, 157-160 (1992)), IL-2 (Katre et al., Proc. Natl. Acad. Sci. USA 84, 1487 (1987)), TNF-α (Tsutsumi et al., Jpn. J. Cancer Res. 85, 9 (1994)), IL-6 (Inoue et al., J. Lab. Clin. Med. 124, 529 (1994)) and CD4-IgG (Chamow et al., Bioconj. Chem. 5, 133 (1994)), among others.

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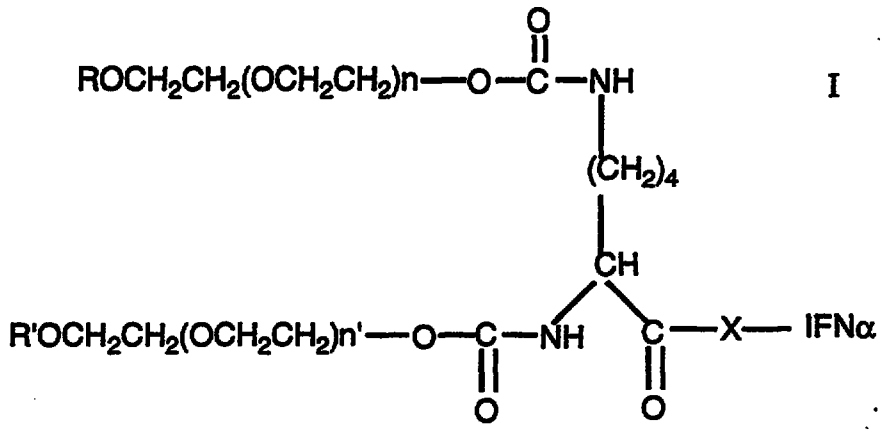
It has been now observed that in the case of interferon, PEGylation reduces *in vitro* antiviral activity but increases antiproliferative activity in human tumor cells. However the new PEG interferon conjugate of this invention has surprising properties in that the antiproliferative activity of the PEG interferon is much higher than that not only of interferon but of other PEG interferon conjugates. Although the antiproliferative activity of the conjugate is much increased over other PEG interferon-α conjugates, yet the reduction in antiviral activity is similar. In addition, the PEG interferon-α conjugate of this invention is non-immunogenic, it elicits virtually no antibody formation. In contrast, other PEG interferon-α conjugates do elicit limited antibody formation.

Accordingly, the invention is a new class of PEG derivatives of interferonα (IFNα). The conjugate of this invention has a branched PEG structure, as can be seen below. The branched PEG has the advantage of allowing the attachment of 2 linear PEG molecules at a single site, thus doubling the attached PEG mass without multiple sites of PEGylation.

Compared to unmodified IFNα (i.e. IFNα without a PEG attached), the conjugate has an increased circulating half-life and plasma residence time, reduced immunogenicity, decreased clearance, and increased antiproliferative activity, concomitant with decreased *in vitro* antiviral activity. Compared with other PEG-IFNα conjugates, the conjugate of this invention has a much greater antiproliferative activity, disproportionate to the enhancement or reduction that occurs in its other characteristics, and virtually no immunogenicity.

The physiologically active PEG-IFNα conjugate species of this invention has the formula:

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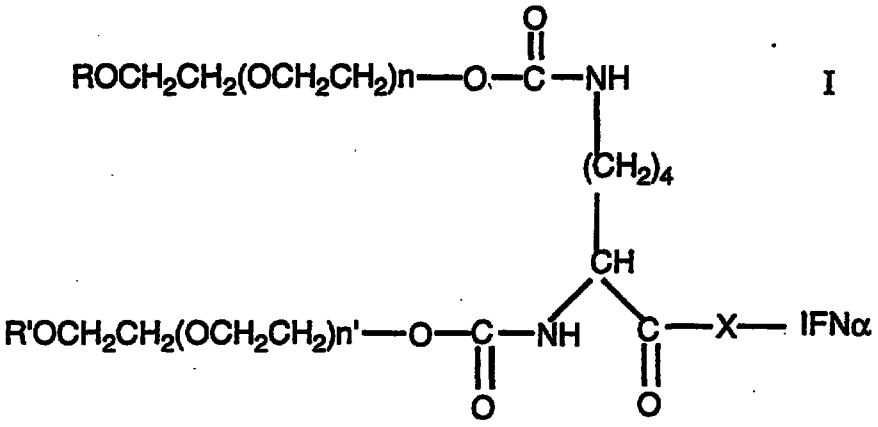
The conjugate of this invention has the same uses as IFNα, for example, antiproliferative uses. In particular, the PEG interferon-α conjugates of this invention are useful to treat immunomodulatory disorders such as neoplastic diseases, for example, hairy cell leukemia, CML, and Kaposi's sarcoma, and infectious diseases, in the same way IFNαs (especially IFNα2a) are used to treat these diseases. However, the conjugate of this invention has improved properties including superior stability, greater solubility, enhanced circulating half-life and plasma residence times. In addition, these conjugates have anti-proliferative activity which is superior to IFNα. Also as noted the conjugate shows a surprising dissociation of antiviral and anti-proliferative effects. This property is additionally useful to enhance a desired activity of a conjugate, while decreasing or eliminating an undesired activity. For example, if an undesired side effect is associated with the antiviral activity, eliminating this activity would eliminate the side effect, while retaining the antiproliferative activity. Therefore, the present invention also comprises the pharmaceutical compositions on the basis of the compounds of formula I or their salts and to methods for producing them.

The pharmaceutical compositions of the present invention used in the control or prevention of illnesses comprises an interferon conjugate of the general formula I and a therapeutically inert, non toxic and therapeutically acceptable carrier material. The pharmaceutical compositions to be used can be formulated and dosed in a

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fashion consistent with good medical practice taking into consideration the disorder to be treated, the condition of the individual patient, the site of delivery of the protein conjugate, the method of administration and other factors known to practitioners.

The claimed conjugate is a physiologically active PEG-IFNα conjugate having the formula




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where R and R' are independently lower alkyl; X is NH or O (X is at least one of the functional groups in the IFNα molecule selected from $NH_2$ or OH); n and n' are integers having a sum of from 600 to 1500; and the average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons. The conjugate of formula I has a branched structure, in that two PEG moieties are attached to the protein via a single linkage.

The numbers n and n' are selected such that the resulting conjugate of Formula I has a physiological activity of IFNα, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified IFNα. n and n' (n and n' may be the same or different) represent the number of ethylene glycol units in the PEG. A single PEG unit of $OCH_2CH_2$ has a molecular weight of about 44 daltons. The molecular weight of the conjugate (excluding the molecular weight of the IFNα) depends on the numbers n and n'. The sum of n and n' for the conjugate of Formula I is from 600 to

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1500, producing a conjugate having a total average molecular weight of PEG units of from about 26,000 to 66,000 and preferably from about 35,000 to 45,000 daltons, and especially about 39,000 to 45,000 daltons, with 40,000 daltons especially preferred. A preferred sum of n and n' is from about 800 to 1200, with the average sum being from about 850 to 1000, and a preferred sum being about 910. Either of n or n' may individually be 420 or 520, or both may be 420 or 520, or both may be 455. The preferred ratio of n to n' is from about 0.5 to 1.5, with an especially preferred ratio of from about 0.8 to about 1.2. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques.

Also preferred is a conjugate of Formula I where IFNα is IFNα2a, a conjugate where R and R' are methyl, a conjugate where X is NH, and a conjugate where n and n' are individually or both either 420 or 520. Such a conjugate having all the above characteristics is especially preferred.

R and R' may be any lower alkyl, by which is meant an alkyl group having from one to six carbon atoms such as methyl, ethyl, isopropyl, etc. Branched alkyls are included. A preferred alkyl is methyl. With regard to the two PEG groups of Formula I, R and R' may be the same or different.

By IFNα (interferon α) and its species IFNα2a is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of IFNα , such as muteins or otherwise modified proteins, is encompassed. Obtaining and isolating IFNα from natural or recombinant sources is well known (Pestka, Arch. Biochem. Biophys. 221, 1 (1983)). A preferred IFNα is IFNα2a, which as stated above, is obtained by known methods (Pestka, Sci. Am. 249, 36 (1983); European Patent No. 43 980)).

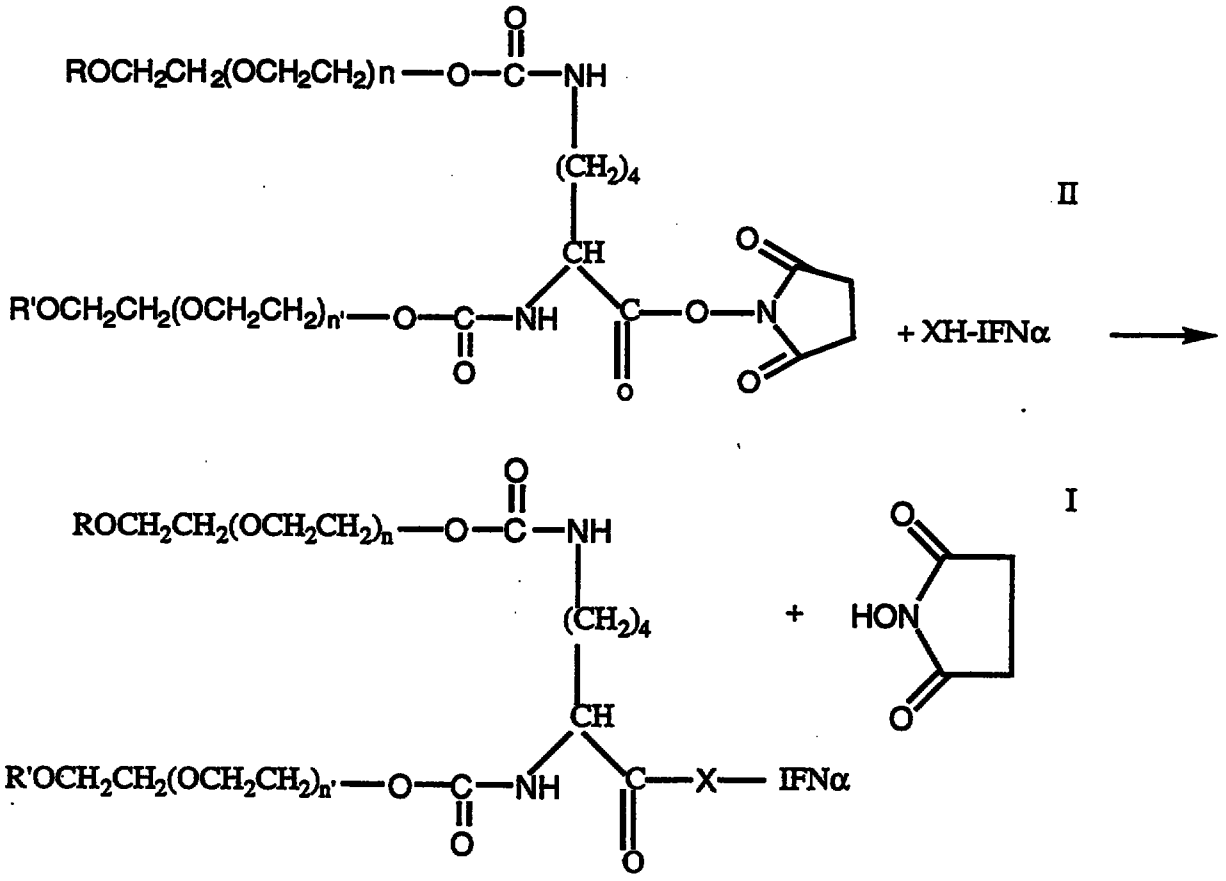
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The physiologically active conjugate of Formula I has IFNα activity, by which is meant any fraction or multiple of any known IFNα activity, as determined by various assays known in the art. In particular, the conjugates of this invention have IFNα activity as shown by antiproliferative activity against tumor cells and antiviral activity against cells infected with a virus. These are known activities of IFNα. Such activity in a conjugate can be determined by assays well known in the art, for example the assays described below (see also Rubinstein et al., J. Virol. 37, 755 (1981); Borden et al., Canc. Res. 42, 4948 (1982)). Part of this invention is a conjugate of Formula I which has greater antiproliferative activity and less antiviral activity than unmodified IFNα.

The conjugate of Formula I is produced by covalent linkage of IFNα to PEG which has been activated by replacement of the PEG hydroxyl with a linking group, forming a reagent which is an N-hydroxy succinimide ester derivative of PEG (in particular monomethoxy PEG) of Formula II. The reagent may be obtained by conventional methods (Monfardini et al., supra). Linkage is via an amide or ester bond. In a preferred conjugate, linkage is via an amide bond (X is NH). Part of this invention is a method for increasing the antiproliferative activity of IFNα while reducing the antiviral activity of the IFNα, by linking the IFNα as described above to a reagent of Formula II to produce a PEG-IFN conjugate.

X represents the attachment site on IFNα by which the PEG reagent of Formula II is covalently attached to the IFNα. The reagents attach to primary amino groups (XH = $NH_2$) on for example lysine or to the N-terminus of the IFNα. The reagents can also attach to a hydroxyl (XH = OH) on for example serine.

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The reagent of formula II (PEG2-NHS), in which a total of 2 mono-methoxy PEG (m-PEG) chains are linked to lysine, one each at the α and ε amino groups via carbamate (urethane) bonds and having the lysine carboxyl group activated to a succinimidyl ester, may be obtained by conventional methods, according to known procedures (Monfardini et al., supra) applicable to a reagent with R as lower alkyl, and a desired n. The reagent may be obtained from Shearwater Polymers, Inc. (Huntsville, Alabama). The preferred average MW of the PEG obtained is about 20,000 daltons, providing a total PEG mass of about 40,000 daltons in PEG2-NHS (other MWs may be obtained by varying n for the PEG-alcohol starting materials for the reagent of Formula II, by conventional methods).

The reagent of formula II may be conjugated to IFNα by conventional methods. Specifically, the reagent of Formula II

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primarily reacts with one or more of the primary amino groups (for example N-terminus and lysine side chains) of IFNα (for example IFNα2a) to form an amide linkage between the IFNα and the polymer backbone of PEG. The PEGylation reaction can also take place between PEG2-NHS and the free (if any) hydroxyl groups (for example serine) of IFNα to form an ester linkage. The reaction mechanism is shown above. The reaction conditions are conventional to a skilled person, and are provided in detail below. The PEG reagent is combined with IFNα under mildly basic conditions at low temperature under conditions suitable for a nucleophilic substitution which will produce the conjugate of Formula I. This is also shown in the above reaction mechanism.

Attaching the reagents to IFNα may be accomplished by conventional methods. PEGs of any selected MW of this invention may be used. Reaction conditions may be selected to provide the claimed conjugate with one reagent attached. The conjugate of Formula I, which has a single reagent of Formula II attached, is separated from unmodified IFNα and conjugates having attached more than one reagent molecule by conventional methods. Purification methods such as cation exchange chromatography may be used to separate conjugates by charge difference, which effectively separates conjugates into their various molecular weights. The content of the fractions obtained by cation exchange chromatography may be identified by molecular weight using conventional methods, for example, mass spectroscopy, SDS-PAGE, or other known methods for separating molecular entities by molecular weight. A fraction then is accordingly identified which contains the conjugate of Formula I purified free from unmodified IFNα and from conjugates having more than one reagent attached. In addition, the reagents of Formula II release one lysine per reagent upon acid hydrolysis, so that the number of lysines in the hydrolysis indicates the number of PEGs attached to the protein, thus the number of reagent molecules attached to a conjugate may be verified.

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The following Examples are provided to illustrate the invention and do not limit it in any way. IFNα2a is used in these examples. Other species of IFNα may also be conjugated to PEG by the methods exemplified.

DESCRIPTION OF THE DRAWINGS

Figure 1: Antitumor activity of the PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal A498 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal A498 cells on Study Day -33. On Study Day 0 PEG-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 μg) of PEG2-IFN alpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a four week period.

Figure 2: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal A498 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal A498 cells on Study Day -33. On Study Day 0 IFNalpha2a treatment was initiated. The indicated amount (10, 20, 40 or 100 μg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a four week period.

Figure 3: Antitumor activity of PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal ACHN cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal ACHN cells on Study Day -25. On Study Day 0 PEG2-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 μg) of PEG2-IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a five week period.

Figure 4: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal ACHN cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal ACHN cells on Study Day -25. On Study Day 0 IFNalpha2a treatment was initiated. The

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indicated amount (10, 20, 40 or 100 µg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a five week perid.

Figure 5: Antitumor activity of PEG2-IFNalpha2a in nude mice implanted subcutaneously with human renal G402 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal G402 cells on Study Day -45. On Study Day 0 PEG2-IFNalpha2a treatment was initiated. The indicated amount (30, 60, 120 or 300 µg) of PEG2-IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 1 time per week for a five week period.

Figure 6: Antitumor activity of IFNalpha2a in nude mice implanted subcutaneously with human renal G402 cells. All animals received a subcutaneous implant of 2 x $10^6$ human renal G402 cells on Study Day -45. On Study Day 0 IFNalpha2a treatment was initiated. The indicated amount (10, 20, 40 or 100 µg) of IFNalpha2a was administered subcutaneously under the opposite flank of the tumor, 3 times per week for a five week period.

## Example 1

### Preparation of conjugate of Formula I

Materials

Interferonα2a was prepared by known methods (Pestka, supra). Polyethylene glycol (PEG) reagent of formula II was purchased from Shearwater Polymers, Inc. (Huntsville, Ala). Fractogel® EMD CM 650(S) resin, with particle sizes 25-40µm, were supplied by EM Separations (Gibbstown, MA). Concentrated (10X) phosphate buffered saline (PBS), pH 7.3, was purchased from BioWhittaker (Walkersville, MD). Sodium dodecyl (laurel) sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) pre-cast gels and electrophoresis units were obtained from NOVEX (San Diego, CA). Concentrated Fast Stain for protein staining of PEG conjugates on SDS-PAGE was purchased

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from Zoion Research, Inc. (Newton, MA). The LAL endotoxin assay kit was purchased from Associates of Cape Cod, Inc. (Woods Hole, MA). All other reagents used were of the highest quality available. The jugular cannulated rats and BDF-1 mice were supplied by Charles River Laboratories (Wilmington, MA).

Experimental Procedures

A. Small scale preparation of conjugate of Formula I

Two hundred-eight milligrams (5.2μmol) of the reagent of Formula II (average MW of 40,000 daltons) were added to 50 mg (2.6μmol) of IFNα in 10ml of 100mM borate, pH 8.0. Final protein reagent molar ratio was 1:2. The reaction mixture was stirred at 4°C for 2 hours. The reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid.

The reaction mixture was diluted 50-fold with water, filtered through a 0.2μ filter and applied onto an Amicon column packed with 100ml (3.2x13cm) Fractogel EMD CM 650(S), at a flow rate of 20ml/min. The column was previously equilibrated with 10mM ammonium acetate, pH 4.5. The column effluent was monitored by UV absorbance at 280nm. The column was then washed with the equilibration buffer until UV absorbance returned to baseline. PEG-IFN conjugates having more than one reagent of Formula II attached (PEG-IFN oligomers) were eluted with 40 mM ammonium acetate, pH 4.5 and the conjugate of Formula I was eluted with 0.12M NaCl in the 40 mM ammonium acetate buffer. The unmodified IFN remaining in the column was eluted with 0.5M NaCl in the same buffer. The column was regenerated by a 1.0M NaCl wash followed by the equilibration buffer wash. The pooled fractions of the conjugate of Formula I were concentrated in an Amicon stirred cell concentrator fitted with a YM10 membrane to approximately 1mg/ml concentration.

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The Fractogel CM 650(S) cation exchange resin used for purification, adsorbed the PEG and unmodified IFN effectively. The strength of adsorption was dependent upon the degree of PEGylation. The conjugates bound less tightly than the unmodified IFN. The PEG-IFN oligomers were eluted with 40mM ammonium acetate, while the conjugate of Formula I eluted with 0.12M NaCl. The unmodified IFN eluted with 0.5M NaCl. All preparations contained <5EU/mg endotoxins. The resulting preparation contained >99% of conjugate of Formula I and was free of unmodified IFN.

B. Large-Scale Preparation of conjugate of Formula I

Six thousand two hundred and forty milligrams (156 μmol) of the reagent of Formula II (average molecular weight of 40,000 daltons) was dissolved in 63 ml of 1mM HCl at 4°C and quickly added to 125 ml of a solution containing 1000 mg (52 μmol) of interferon in 50 mM borate buffer, pH 9.0. The final protein/reagent ratio was 1:3 and the final reaction mixture protein concentration was 5.3 mg/ml. The reaction mixture was stirred for 2 hours at 4°C. The reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid.

The reaction mixture was diluted 10-fold with water and applied onto a column packed with 600 ml Fractogel EMD CM 650(M) previously equilibrated with 20mM sodium acetate, pH, 4.5 at a linear velocity of 1.3cm/min. The column was washed with the equilibration buffer followed by 10 mM NaCl to remove excess reagent, reaction byproducts and PEG-IFN oligomers. The conjugate of Formula I was eluted with the equilibration buffer containing 200mM NaCl. The unmodified interferon still adsorbed to the column was removed by washing with 0.75 M NaCl in the equilibration buffer. The conjugate of Formula I, which was eluted at 0.3-0.5mg/ml was further concentrated and diafiltered into the final formulation buffer, 20 mM sodium acetate, pH, 5.0, containing 150mM NaCl. The overall yield of the conjugate of Formula I was 40-45%.

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The purified PEG-IFN from the large-scale preparation consists of >99% conjugate of Formula I. The average molecular weight of the conjugate of Formula I of this example is 62,000 daltons, including the molecular weight of IFNα2a which is 19,241 daltons, and the average molecular weight of the reagent which is between 40,000 and 45,000 daltons, about 43,000 daltons.

## Example 2

### Characterization of conjugate of Formula I

Protein Determination

Protein concentrations were determined using an $A_{280}$ value of 1.0 for a 1mg/ml solution of IFNa α2a.

SDS-PAGE Analysis

The conjugate was analyzed by sodium dodecyl (lauryl) sulfate/polyacrylamide (8-16%) gel electrophoresis, under reducing conditions, according to the methods of Laemmli (Nature 227, 680 (1970)). SDS-PAGE containing PEG-conjugates were stained for protein using Fast Stain (Zoion Research, Inc.) according to the manufacturer's instructions.

Determination of Endotoxin Levels

Endotoxin levels were determined using the LAL method, according to the manufacturer's instructions. All preparations contained <5 EU/mg endotoxins.

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## Example 3

### *In Vitro* Bioactivities of conjugate of Formula I

Antiviral Activity in Bovine Kidney Cells

The *in vitro* antiviral activity of IFNα2a and the conjugate of Formula I as prepared in Example 1.A. were determined in a cell culture bioassay employing Madin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus (Rubinstein et al., supra). The antiviral activities are listed in Table 1, along with their corresponding residual activities as a percentage of the starting IFN.

Table 1

Anti-Viral Activities

| Samples | PEG Type | Total PEG Mass (kDa) | # Lys Modified | Specific Activity (U/mg) | Residual Activity (%) |
|---|---|---|---|---|---|
| IFNα2a | - | - | - | $2.00 \times 10^8$ | 100 |
| Conjugate of Formula I | Branched | 40 | 1 | $1.40 \times 10^7$ | 7 |

*In Vitro* Antiproliferative Activity in Human Tumor Cells

The *in vitro* antiproliferative activities were assayed in human Daudi (Burkitt's Lymphoma) cells, as described by Borden et al. Human Daudi cells were maintained as stationary suspension cultures in RPMI 1540 supplemented with 10% fetal bovine serum and 2 mM glutamine (Grand Island Biologicals, Grand Island, NY). The cells were screened and found to be free of mycoplasma. Cells ($2 \times 10^4$) were added to wells of microtiter plates (Costar, MA) in 100 µl of medium. Various concentrations of IFN and the conjugate of Formula I as prepared in Example 1.A. were added to the wells in a volume of 100 µl. The plates were incubated at 37°C in 5% $CO_2$ for 72 hours.

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Cells were pulsed with 0.25μCi/well of $^3$H-thymidine (New England Nuclear, Boston, MA), sixteen hours before cell harvesting. The cells were harvested onto glass filters and counted in a liquid scintillation counter. The results were expressed as % inhibition calculated using the formula:

% Inhibition = [ (A - B / A] x 100, where;

A = cpm in control culture (cells incubated in medium alone)
B = cpm in experimental culture

Samples were run in quadruplicate and standard deviation was less than 20% of the mean of all cases. Experiments were run at least twice with comparable results.

The antiproliferative activities ($IC_{50}$) of IFN and the conjugate are listed in Table 2. The data indicate that there is a 28-fold increase in antiproliferative activity for the conjugate of Formula I, as compared to that of IFN.

Table 2

In Vitro antiproliferative activities in human Daudi (Burkitt's lymphoma) cell lines.

| Sample | Antiproliferative IC50 (ng/ml) | Activity Increase |
|---|---|---|
| IFNα2a | 0.56 | 1x |
| Conjugate of Formula I | 0.02 | 28x |

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## Example 4

## Pharmacokinetics

Female Sprague Dawley rats, surgically implanted with jugular cannulas, with an average body weight of 240 - 260g were housed individually, allowed free access to food and water and maintained in a 12 hour light -dark cycle. Within 4 - 6 hours after arrival, jugular cannulas were flushed with PBS. The following day, after flushing with 0.15 - 0.2ml PBS, $2x10^6$ units of IFNα in 0.2 - 0.4ml PBS were injected, followed by injection of 0.15 - 0.2ml PBS to assure that all drug was washed into the animal. Thus each animal received a dosage of $8x10^6$ IFNα units/kg body weight.

Blood samples were drawn at 5, 15 and 30 minutes, as well as, 1, 3, 5, 12 and 24 hours after injection of IFN and, the conjugate of Formula I. At all time points, after discarding the first 0.15 - 0.2ml of blood, an aliquot of 0.5ml blood was withdrawn using a fresh syringe via the jugular cannula. The samples were discharged into serum separating tubes at room temperature. Once all the samples were collected for the time points, the tubes were centrifuged at 14,000 x g in a refrigerated Eppendorf centrifuge for 10 minutes. The separated serum was transferred into 1.5ml microfuge tubes and frozen at -80°C, until ready for bioassay. Serum samples were diluted appropriately and the antiviral activity at each time-point was determined as described. From the plot of time vs. activity, the terminal half-life of the conjugate of Formula I and IFNα were determined and listed in Table 3, which also include plasma residence times.

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Table 3

Terminal Half-Lives ($t_{1/2}$) and Mean Plasma Residence Time

| Sample | $t_{1/2}$ (hours) | Plasma Residence Time (hours) |
|---|---|---|
| IFNα2a | 2.1 | 1.0 |
| Conjugate of Formula I | 15.0 | 20.0 |

Terminal $t_{1/2}$ estimated by log linear regression.

Example 5

Immunogenicity

Normal BDF-1 mice (ten per group) were injected intraperitonially once per day five times per week with various interferon preparations having 300,000 units of antiviral activity. Some mice were also injected with aggregated form of IFNα2a which is more immunogenic than the monomer form. Blood samples were taken 19 days following the last injection and the serum was evaluated for neutralizing antibodies.

As seen in Table 4, mice injected with IFNα2a produced neutralizing antibodies and this response was greatly increased in mice injected with interferon aggregates. No antibodies were detectable in the majority of animals injected with the conjugate of this invention.

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Table 4

Immunogenicity

| Treatment | Antibody (INU/ml) * Median | Range |
|---|---|---|
| IFNα2a | 2,400 | 217-8,533 |
| IFNα2a Aggregates | 42,667 | 8,000-768,000 |
| Conjugate of Formula I | 0 | 0-1,133 |

* Interferon neutralizing units/ml

Example 6

Antitumor activity *In Vivo*

The *in vivo* antitumor activity of a conjugate of Formula I (PEG2-IFNalpha2a) and unmodified IFNalpha2a were evaluated by determining their ability to reduce the size of various human tumor cells implanted subcutaneously into mice. Results are shown in Figures 1-6.

Procedure: Athymic nude mice (Harlan) received a subcutaneous implant under the left rear flank of 2 x $10^6$ human renal A498 cells (Figures 1 and 2), human renal ACHN cells (Figures 3 and 4), or human renal G402 cells (Figures 5 and 6). 3 to 6 weeks were allowed for the tumors to become established, as indicated. The size criteria for acceptance into the study was 0.05 to 0.50 cubic centimers. The mice were given total weekly doses of PEG2-IFNalpha2a or unmodified IFNalpha2a of 30, 60, 120 or 300 µg. In the case of PEG2-IFNalpha2a the mice were treated one time per week (Monday) with 30, 60, 120 or 300 µg of PEG2-IFNalpha2a per treatment. In the case of unmodified IFNalpha2a the mice were

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treated three times per week (Monday, Wednesday, Friday) with 10, 20, 40 or 100 µg of IFNalpha2a per treatment. The duration of treatment was 4 to 5 weeks depending on tumor aggressiveness. Tumor volumes were measured every Monday prior to treatments.

Results: PEG2-IFNalpha2a showed a marked reduction in A498 tumor size as compared to unmodified IFNalpha2a for all weekly dosage levels tested, at 7 days, 14 days, 21 days and 28 days after the beginning of treatment (Figures 1 and 2). Treatment continued for four weeks. Seven days after treatment was discontinued three mice in each group were sacrified. In the three mice treated with PEG2-IFNalpha2a no residual tumor was observed. In mice treated with unmodified IFNalpha2a the A498 tumor weight was 1.28 grams, 0.62 grams, and 1.60 grams respectively in each of three mice. The A498 tumor weight was 2.32 grams, 2.37 grams, and 1.94 grams in each of three control mice. At 80 days after the end of the four week treatment period the existence of tumors was determined by palpation in seven mice. All seven mice were free of tumor tissue by palpation.

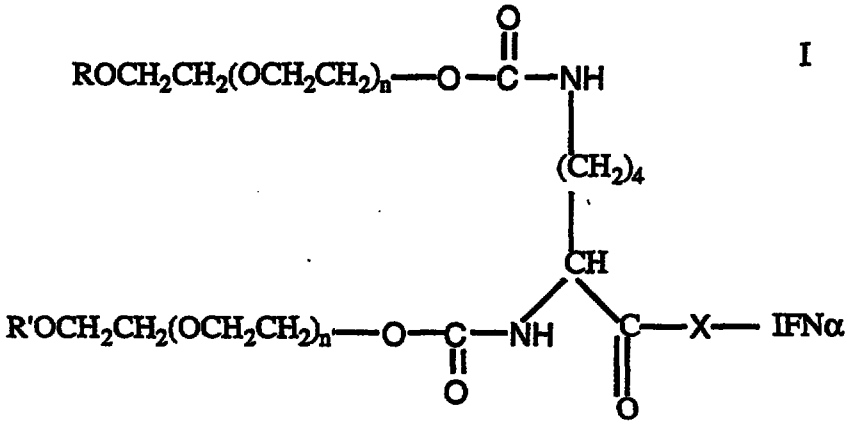
PEG2-IFNalpha2a showed a significant reduction in ACHN tumor size as compared to unmodified IFNalpha2a for weekly dosage levels of 60, 120, and 300 µg, at 14 days, 21 days, 28 days and 35 days (Figures 3 and 4).

PEG2-IFNalpha2a showed a significant reduction in G402 tumor size as compared to unmodified IFNalpha2a for weekly dosage levels of 60 and 120 µg, at 14 days, 21 days, 28 days and 35 days (Figures 5 and 6).

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## Claims

1. A physiologically active PEG-IFNα conjugate having the formula



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wherein R and R' are independently lower alkyl; X is NH or O; n and n' are integers having a sum of from 600 to 1500; and the average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons.

2. A conjugate of claim 1 wherein the molecular weight of the polyethylene glycol units is from about 35,000 to about 45,000 daltons.

3. A conjugate of claim 2 wherein the molecular weight of the polyethylene glycol units is about 40,000 daltons.

4. A conjugate of claim 1 wherein R and R' are methyl.

5. A conjugate of claim 1 wherein X is NH.

6. A conjugate of claim 1 wherein the IFNα is IFNα2a.

7. A conjugate of claim 1 wherein the average sum of n and n' is 850 to 1000.

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8. A conjugate of claim 1 wherein R and R' are methyl; X is NH; IFNα is IFNα2a; and one or both of n and n' is 420.

9. A conjugate of claim 1 wherein R and R' are methyl; X is NH; IFNα is IFNα2a; and one or both of n and n' is 520.

10. A conjugate of claim 1 which has greater antiproliferative activity than IFNα and less antiviral activity than IFNα.

11. A method for producing a PEG-IFNα conjugate having an increased antiproliferative activity and decreased antiviral activity as compared to IFNα, which method consists of covalently linking a reagent of Formula II to IFNα to produce said PEG-IFNα conjugate.

12. Pharmaceutical compositions comprising a PEG-IFNα conjugate as claimed in any one of claims 1-10 and a therapeutically inert carrier.

13. Pharmaceutical compositions for the treatment or prophylaxis of immunomodulatory disorders such as neoplastic diseases or infections diseases comprising a PEG-IFNα conjugate as claimed in any one of claims 1-10 and a therapeutically inert carrier.

14. The use of a PEG-IFNα conjugate according to any one of claims 1-10 for the manufacture of medicaments for use in the treatment or prophylaxis of illnesses.

15. A PEG-IFNα conjugate as claimed in claims 1-10 whenever prepared according to the method as claimed in claim 11.

16. A PEG-IFNα conjugate as claimed in claims 1-10 for the manufacturing of medicaments for use in the treatment or prophylaxis of illnesses.

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17. The products, pharmaceutical compositions, processes and methods substantially as described hereinbefore.

18. A method for the treatment or prophylaxis of immuno-modulatory diseases comprising administering a PEG-IFNα conjugate according to any one of claims 1-10.

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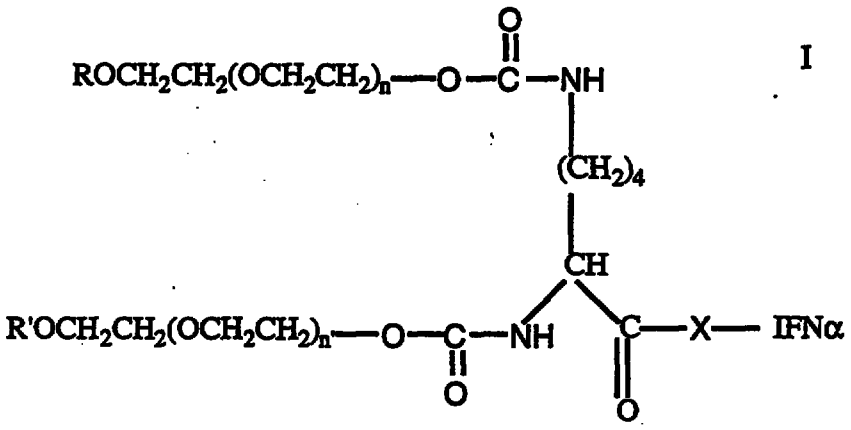
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# INTERFERON CONJUGATES

## Abstract

Physiologically active PEG-IFNα conjugates having a formula as follows:




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Figure 1

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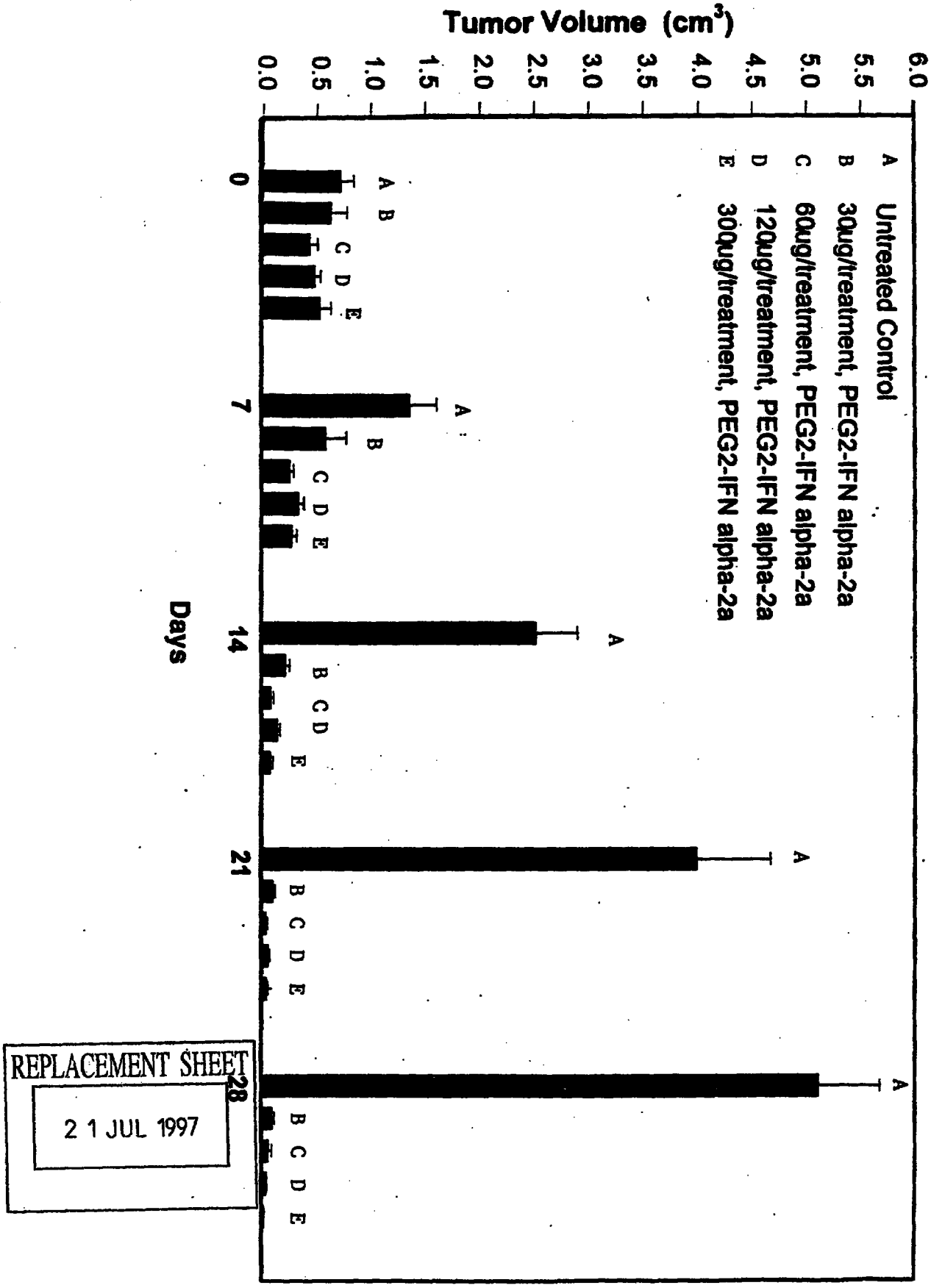



FIGURE 1

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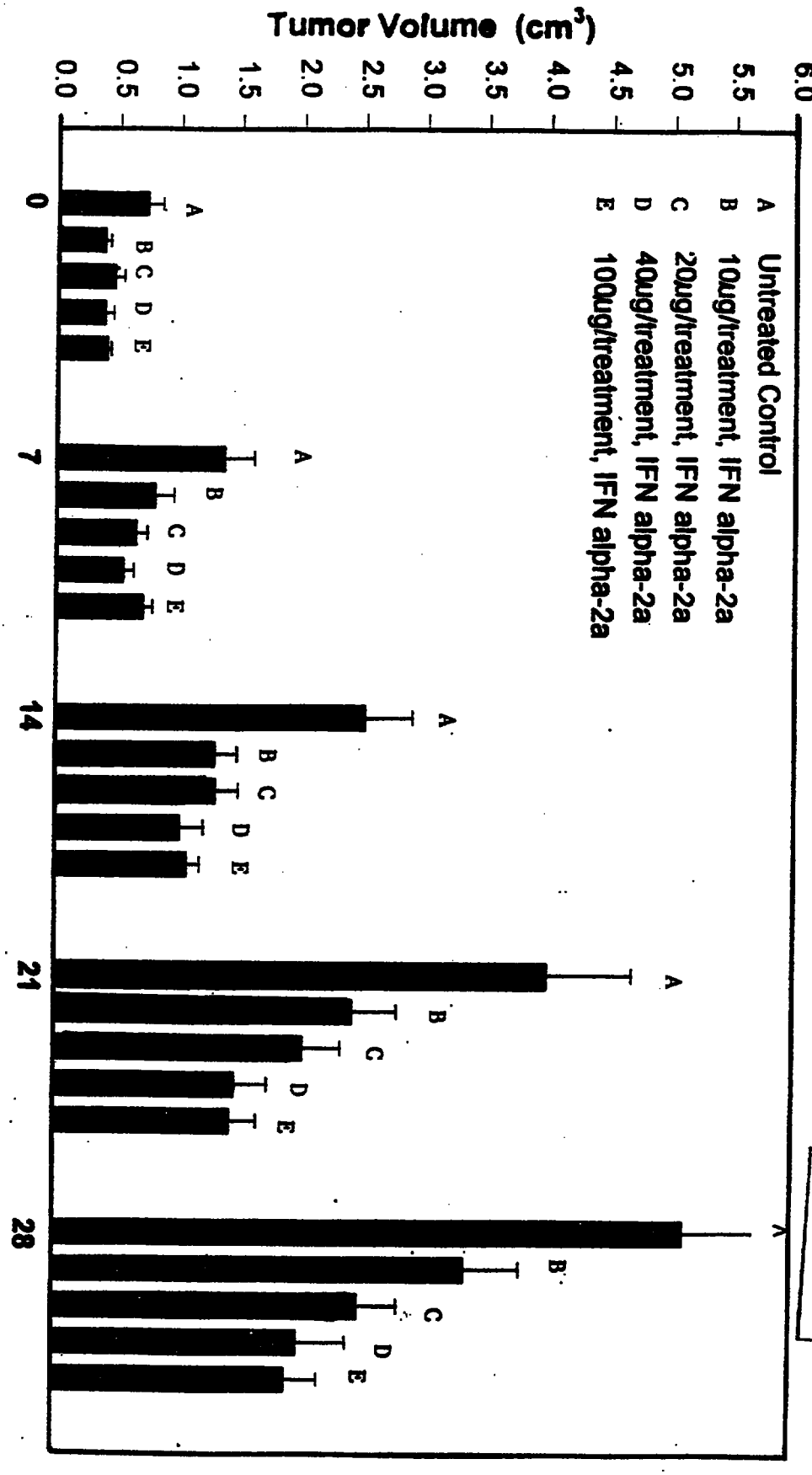



FIGURE 2

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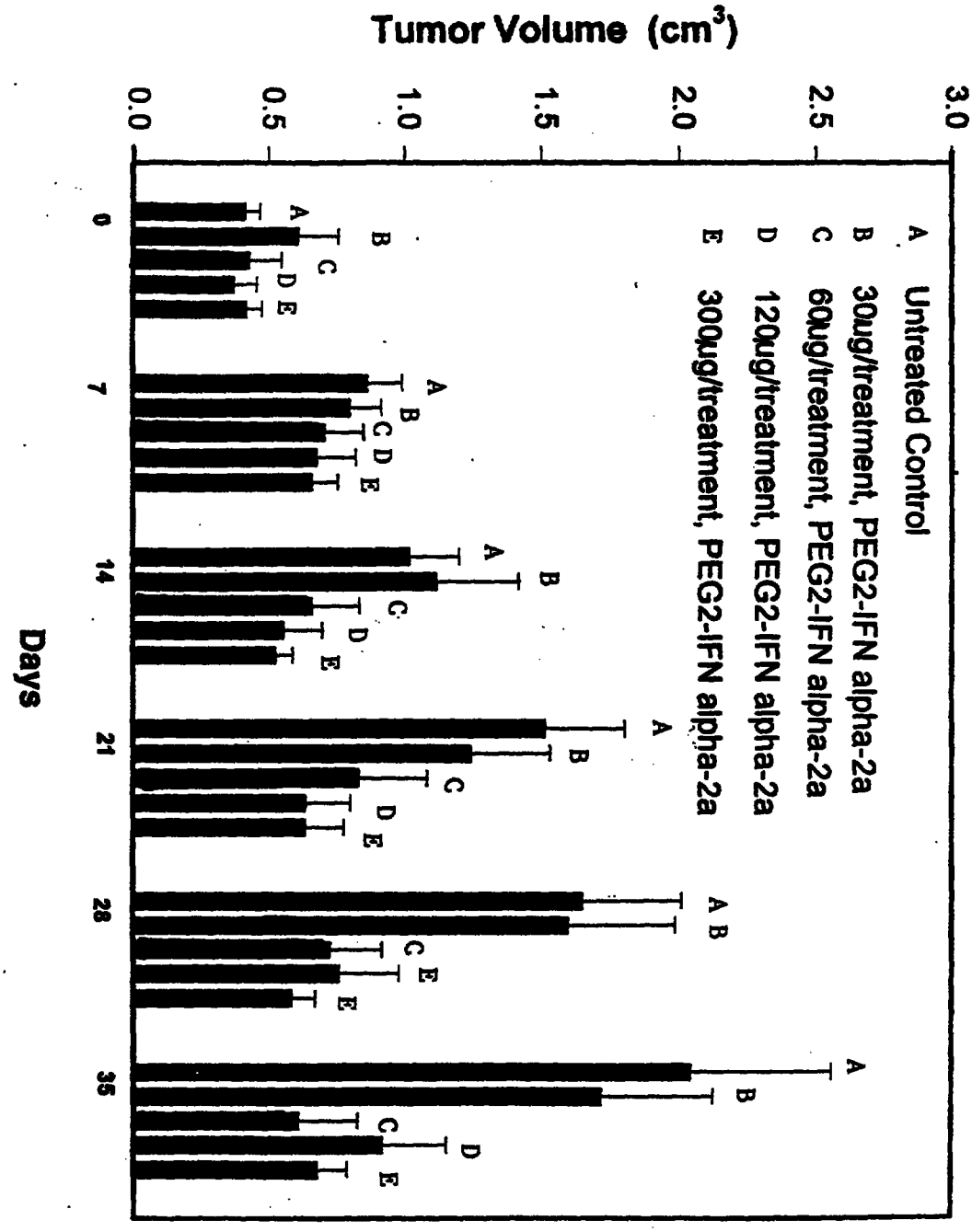



FIGURE 3

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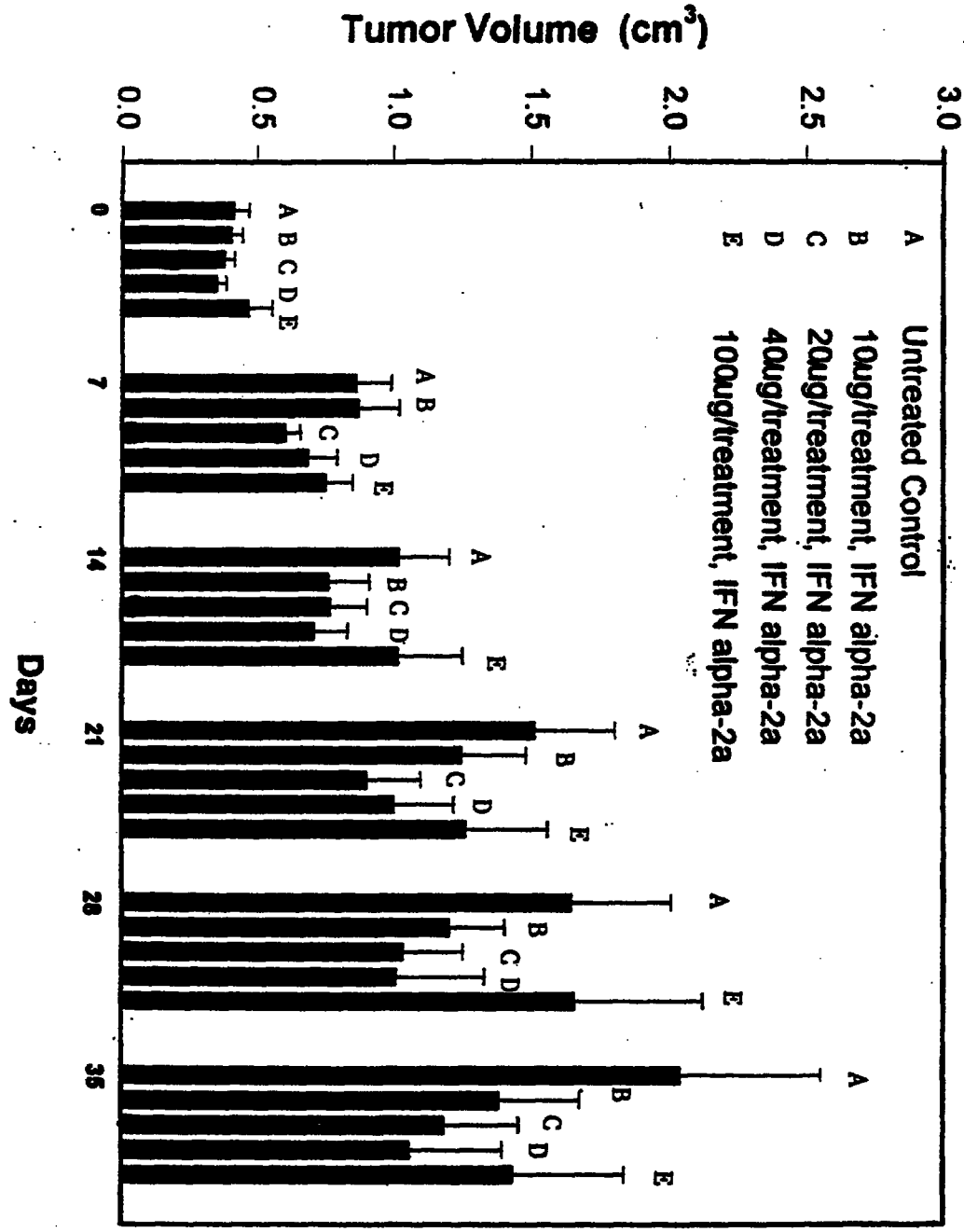



FIGURE 4

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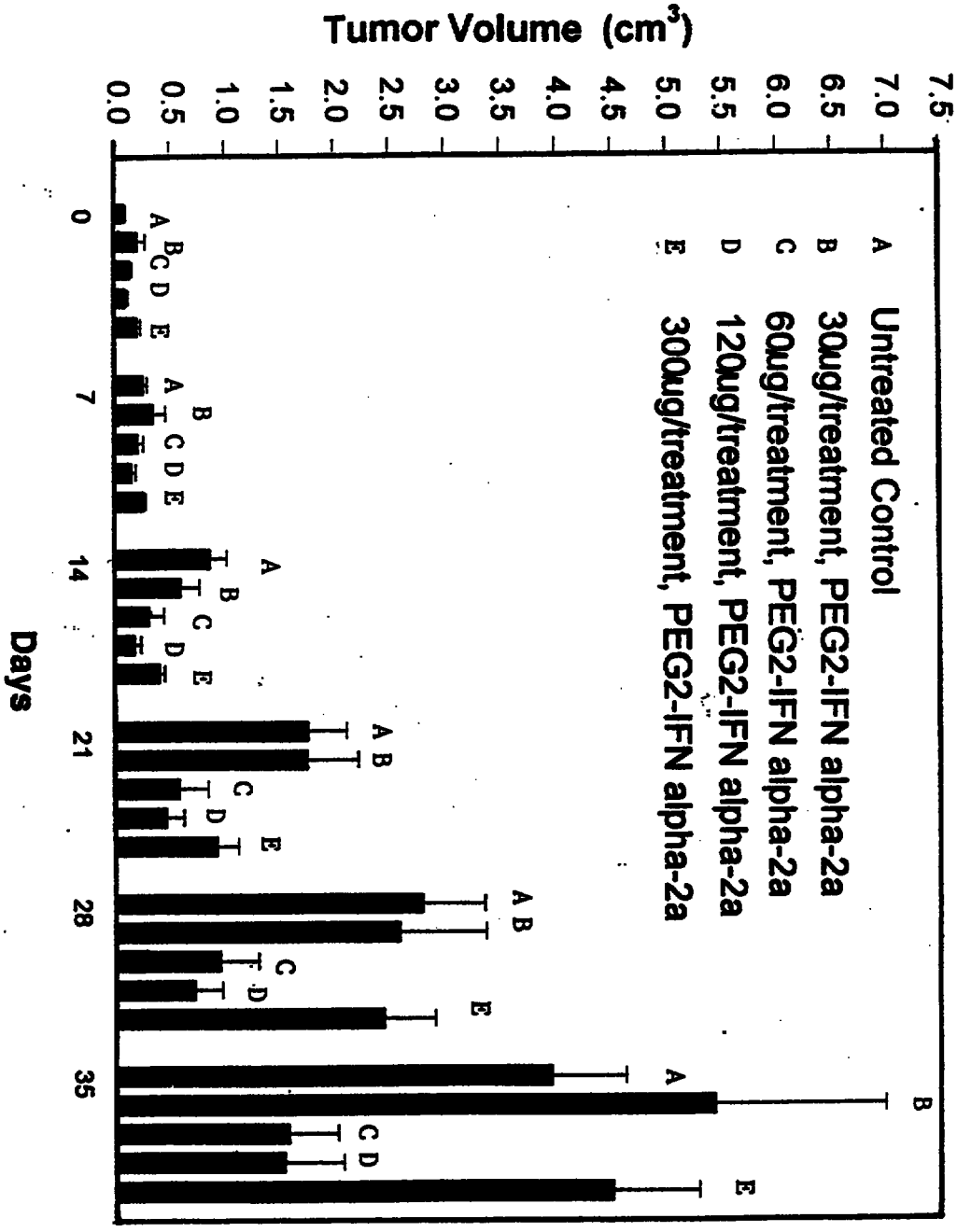



FIGURE 5

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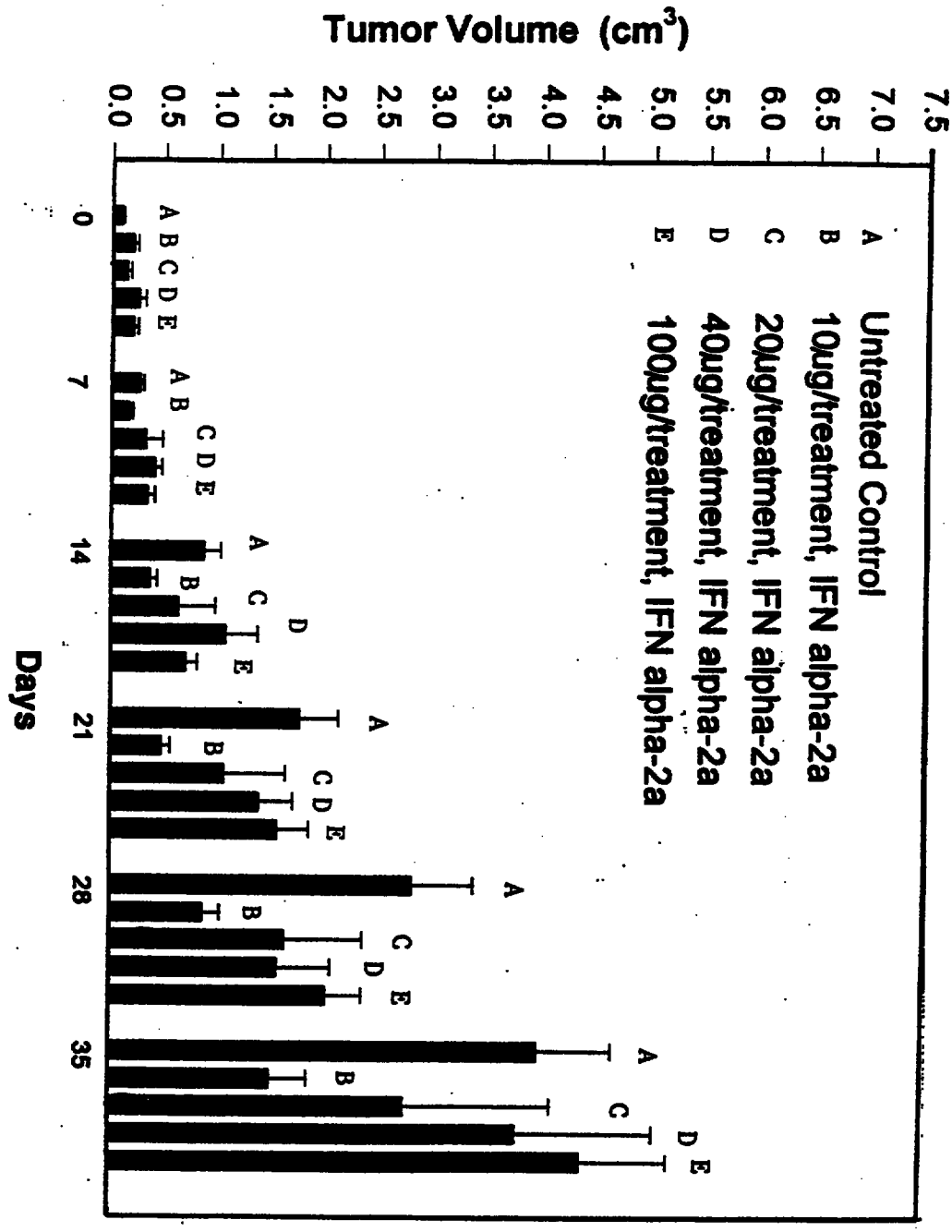



FIGURE 6

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17. The products, pharmaceutical compositions, processes and methods substantially as described hereinbefore.

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