Palmitoyl Derivatives of Interferon α: Potential for Cutaneous Delivery

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Abstract □ Palmitoyl derivatives of interferon α2b (p-IFNα) were prepared by covalent attachment of the fatty acid to lysine residues in the protein through a reaction with *N*-hydroxysuccinimide palmitate ester. The p-IFNα was characterized by capillary electrophoresis (CE), mass spectrometry (MS), SDS–PAGE, and antiviral assay. Flow-through diffusion cells and human breast skins were used to measure cutaneous and percutaneous absorption. Formation of p-IFNα derivatives was demonstrated by CE to be dependent on reaction time and reagent: protein ratio. Electrospray MS of the crude p-IFNα mixture indicated three populations of IFNα derivatives with 10, 11, and 12 palmitoyl substitutions. The addition of palmitoyl residues to IFNα under the conditions described reduced the antiviral specific activity by 50%. However, the cutaneous absorption of p-IFNα was about 5–6 times greater than the parent protein. The amount of p-IFNα and IFN α in whole skin after 24 h of treatment was 2.106 ± 1.216 μg/cm^2 and 0.407 ± 0.108 μg/cm^2, respectively. Approximately two times higher flux was detected for p-IFNα compared to the nonfatty acylated IFNα. The total amount of drug diffused in 24 h was also approximately two times higher for the p-IFNα. The results indicate a potential for using fatty acylated derivatives of IFN α for dermal and transdermal delivery.

Introduction

The routine administration of protein and peptide drugs is hindered by the lack of a reliable and convenient mode of delivery. The oral route is often impractical due to the digestion of proteins in the gastrointestinal tract. Thus parenteral administration is generally the only alternative, although the frequent injections required, due to the short half-life of peptides (usually minutes), can decrease patient compliance.

Newer initiatives are being explored to deliver protein drugs by other "nonconventional" routes such as nasal, pulmonary, rectal, vaginal, ocular, and transdermal.[1,2] Encouraged by the relative success with smaller drug molecules using penetration enhancers, a number of research groups have investigated the transdermal route in more detail. The advantage of the transdermal route over the mucosal routes is that the skin has lower proteolytic activity compared to the mucosa; therefore, metabolism during transit can be minimized and the bioavailability improved if enough drug can penetrate. Bodde et al.[3] for example demonstrated that des-enkephalin-γ-endorphin (DEγE) had a long, 4.4 h, half-life in fresh whole skin compared to a plasma half-life of only a few minutes.

Proteins and peptides may exhibit very low permeability through the skin due to properties such as hydrophilicity and high molecular weight. Transdermal delivery of peptides is challenging, but nevertheless a very worthwhile effort. The various approaches to delivering peptides through the skin have utilized methods which temporarily compromise the integrity or physicochemical characteristics of the skin, including the use of penetration enhancers such as *N*-alkylazacycloheptanones (Azone) for desglycinamide arginine vasopressin (DGAVP)[4] or for DEγE by Bodde et al.[3] The nonionic surfactant, *n*-decyl methyl sulfoxide was shown to enhance the penetration of Leu-enkephalin through hairless mouse skin in vitro.[5] Iontophoresis was employed by some researchers in the past few years for the enhancement of delivery of short peptides.[6,7]

Most of the above studies demonstrated enhanced penetration of the different peptides through excised animal skin in vitro. While these studies demonstrate the feasibility of the transdermal route for peptide delivery, there is an almost total lack of knowledge concerning the delivery of sufficient quantities of a protein drug required for the desired pharmacological effect.

Interferon α2b (recombinant) is a 19.3 kDa molecular weight polypeptide consisting of 165 amino acids. Interferon α shows multiple biological effects including antiviral, antiproliferative, and immunomodulatory. The mechanism of action is through binding to specific cell surface receptors. The binding induces protein kinase and 2′5′-oligoadenylate synthetase.[8] These enzymes can inhibit protein synthesis in the cell and therefore can prevent a virus from replicating.[9]

Most topical applications of IFNα2 are intended for local (treatment of cutaneous or genital herpes or condylomata acuminata) and not for systemic effects. Very little absorption was found through either the nasal mucosa or the skin unless high concentration of surfactants or other additives were used.[10,11] The delivery of IFNα or β from various topical dosage forms such as gels and creams was tested in clinical experiments with negative or minor positive results.[12−16] The reason for the lack of efficacy was proposed to be the inadequate delivery of drug to the target sites.

The main goal of this investigation was to evaluate a novel concept for the delivery of IFN α into or through the skin using a hydrophobic molecular carrier. In this paper we report the preparation and evaluation of palmitoyl derivatives of IFNα (p-IFNα) for improved dermal delivery.

Materials and Methods

Preparation of Palmitoyl Derivatives of IFNα—Palmitoyl derivatives of IFNα2b (generously provided by Schering-Plough Research Institute, Kenilworth, NJ) were synthesized using the active ester of *N*-hydroxysuccinimide and palmitic acids (C16). In step one the synthesis of *N*-hydroxysuccinimide ester of palmitic acid (NHS-P) was carried out. Briefly, equal molar amounts of palmitic acid (Sigma Chemical Co., St. Louis, MO) and *N*-hydroxysuccinimide (Sigma) were mixed together in ethyl acetate,

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and then dicyclohexylcarbodiimide (DCC) was added. The mixture was stirred overnight at 4 °C. Dicyclohexylurea (DCU) was filtered out, and NHS-P was recrystallized from the filtrate by the addition of ethanol at 4 °C. ^{1}H NMR studies on NHS-P confirmed the expected structure. The high field ^{1}H NMR was recorded on a Bruker AMX 300 spectrometer. There was no impurity detectable in the NMR spectrum. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.86 (t, J = 7 Hz, 3H), 1.75 9m, 2H), 2.6 (t, J = 7 Hz, 2H), 1.38 (m, 2H), 1.28 br s, 22H), 2.85 (s, 4H). Step two involved the preparation of palmitoyl IFN α as follows. NHS-P was dissolved in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) and added at 1:1 to 25:1 molar ratio to the PBS buffer (7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 141.2 mM NaCl) containing IFNα at pH 7.2. The mixture was kept at room temperature for 3 h with occasional gentle agitation. After the reaction, DMF or DMSO was removed under vacuum in a lyophilizer (Labconco) at <−50 °C, and the residue was redissolved in sterile distilled water. The p-IFNα was separated from free fatty acids by chromatography on Sephadex G-25 column (Pharmacia, Uppsala, Sweden) using PBS. The fractions were tested by polyacrylamide gel electrophoresis (PAGE) and silver staining. The protein was quantitated by densitometry. The fractions containing protein were pooled, freeze-dried, and reconstituted with sterile distilled water before use.

Polyacrylamide Gel Electrophoresis of IFNα—Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was carried out in a Mini-Protean II (BioRad, Mississauga, ON) apparatus. The gel consisted of a running gel containing 14% (w/v) acrylamide and stacking gel containing 5% acrylamide. The gel thickness was 1.0 mm. The electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.01% (w/v) SDS, pH 8.6. Electrophoresis was carried out at 200 V constant voltage. The electrophoresis was conducted for 45 min. After electrophoresis, the gels were silver stained to detect the protein.

Capillary Electrophoresis—CE studies were performed using P/ACE System 5500 (Beckman, Fullerton, CA) with diode array detector and System Gold Software. Free-zone electrophoresis was carried out using an uncoated capillary (57 cmM × 75 μm) at 23 °C, 20 kV with a 5 s pressure injection. The running buffer was 0.6% w/v sodium borate ($Na_2B_4O_7{\cdot}10H_2O$) and 0.5% boric acid, pH 8.75. The detector was used at 200−300 nm. Prior to use the capillary was washed with NaOH (0.1 M) for 10 min and for 1 min between each run.

Mass Spectrometry—Four microliters of IFNα-2b (25 μg/mL in water) or 6 μL of p-IFNα prepared at 25:1 reagent:protein ratio (400 μg/mL in water) was injected.

The mass spectrometer used in this study was a Micromass VG Quattro II tandem quadruple mass spectrometer (VG Organic, Tudor Road, Altrincham, UK) operated in conjunction with VG MassLynx Software. The ionization interface used was the atmospheric pressure electrospray (ES) source operated in positive ion mode. The mobile phase (0.2% HCOOH, 49.9% H_2O, and 49.9% ACN, v/v) was delivered at a flow rate of 20 μL/min by a Model 140A dual syringe solvent delivery system (Applied Biosystems Ltd., Foster City, California). The sampling cone voltage was optimized at 45 V. Mass spectra were acquired at mass unit resolution. The molecular weights of the proteins were automatically produced from multiply charged electrospray spectra by the MaxEnt algorithm using the maximum entropy method.

Iodination of p-IFNα—Iodination of IFNα and p-IFNα was carried out using the lactoperoxidase method.[17] Briefly, 2 mCi Na^{125}I obtained from Amersham (Oakville, ON), was neutralized by adding 3 volumes of 0.03 N HCl, and the total volume was made up to 25 μL with 0.2 M sodium phosphate buffer pH 7.2. The following were added to this mixture: 50 μL of Enzymobeads (Bio-Rad), 15 μL of freshly made 2% β-D-glucose in 0.1 M sodium phosphate buffer, pH 7.2, 10 μL of IFN (approximately 10 μg of protein). The reaction mixture was incubated for 20 min at room temperature. The reaction was stopped by adding 25 μL of 1 M sodium azide and incubating for 15 min. Finally 125 μL of saturated l-tyrosine in PBS was added, and the mixture was transferred onto a Sephadex G25 column. Fractions containing the protein were pooled. As another method, the iodination mixture was transferred onto Bio-Spin columns (exclusion limit 6000) (Bio-Rad), and the iodinated protein was recovered by a brief low speed centrifugation. To remove any possible residue of unbound iodine, the protein preparation was dialyzed overnight against 1 mM sodium iodide in PBS. This procedure removed practically all acid soluble iodine as determined by trichloroacetic acid precipitation. The final preparations of ^{125}I-IFNα and ^{125}I-p-IFNα had specific activities of 2.05×10^7 cpm/μg and 1.94×10^7 cpm/μg protein, respectively. The iodinated IFN and p-IFNα were examined by PAGE for intactness, and the protein concentration was determined by densitometry.

Determination of Antiviral Activity—Antiviral activity of palmitoyl derivatives of IFNα was determined by the cytopathic effect inhibition assay using Georgia Bovine Kidney (GBK) cells and vesicular stomatitis virus as the challenge virus.[18] We are currently using GBK cells in our antiviral assays with success, since the GBK cells are sensitive to human IFNα. The reference standard was IFNα-2b, specific activity 2.24×10^8 IU/mg (Schering-Plough).

In Vitro Cutaneous and Percutaneous Absorption of p-IFNα—The rate of diffusion of p-IFNα across full thickness human breast skin (freshly obtained from plastic surgery and kept at −20 °C until used within 1 week) was investigated using Teflon, Flow-Thru Diffusion Cells (Crown Glass Co. Inc. Somerville, NJ), which have a surface area for diffusion of 0.32 cm^2. The diffusion cells are designed such that fluid may be continuously pumped through them in order to maintain sink conditions. PBS, pH of 7.2, maintained at 37 °C, was used as the perfusion fluid. The diffusion cells were mounted in a PosiBloc Diffusion Cell Heater (Crown Glass Co. Inc., Somerville, NJ), maintained at 32 °C by water circulated through a circulating water bath. Each cell was connected to a fraction collector. The flow rate was 3 mL per hour. Each experiment was conducted for a continuous period of 24 h. The test preparations [0.1 mL of solution (PBS buffer) or 0.1 g of methylcellulose 1500 cP (2.5%) gel hydrated with PBS] labeled with ^{125}I-p-IFNα, were instilled into the cells at the beginning of each experiment. The drug concentration in the preparations was 20×10^6 IU (89.3 μg) of p-IFNα/g or mL product. The average amount of drug applied was 20.7 μg/cm^2 skin surface area. The quantity of p-IFNα in the collected fractions was determined by γ counting.

After 24 h, the skin was removed from the diffusion cell and rinsed thoroughly with cold (4 °C) PBS (3 × 15 mL), and the skin was blotted with tissue paper. The skin surface was swabbed with a cotton tip applicator dipped into PBS containing 0.5% Tween 80 two times to remove surface bound drug. Care was taken not to disturb the stratum corneum. The skin was carefully folded (epidermal sides together) to avoid contamination of dermal side and placed into glass tubes. The radioactivity associated with the skin was determined by γ counting and was considered to be the "whole skin" counts. The skin was then stripped 10 times with a Scotch tape, and the radioactivity associated with each strip was determined separately. The skin after the stripping was counted again in a clean tube to obtain the counts associated with the viable layers of the skin (epidermis, dermis, and subcutaneous tissue). The skin stripping technique was validated by sectioning the paraffin embedded stripped skin to observe the complete removal of the stratum corneum in the light microscope (results not shown). Trichloroacetic acid (TCA) precipitation was used to determine free and bound iodine label in percutaneous fractions and skin homogenate prepared from treated skin samples. TCA was added to each sample to 5% w/v concentration and was incubated at 4 °C overnight. The supernatants and pellets were analyzed by γ counting after centrifugation in a Beckman Microfuge at 14000 rpm for 15 min. The experiments with TCA precipitation from skin homogenates (after tape stripping) and fractions showed that 40−50% of radioactivity was precipitated from both IFNα- and p-IFNα-treated samples.

Results and Discussion

Fatty Acylation of IFNα—Fatty acylation of IFN α utilizes the formation of an amide bond which would be fairly stable in dosage form development and in the biological environment. The synthetic steps for the preparation of the palmitoyl derivatives of IFNα are shown in Figure 1. The first step involved the preparation of NHS-P, which in turn was reacted with IFNα in a solvent. We found that DMSO was a better medium for the reaction than DMF for the preservation of the antiviral activity of IFNα. The synthetic procedure described above provides nonspecific acylation, therefore it is expected that some of

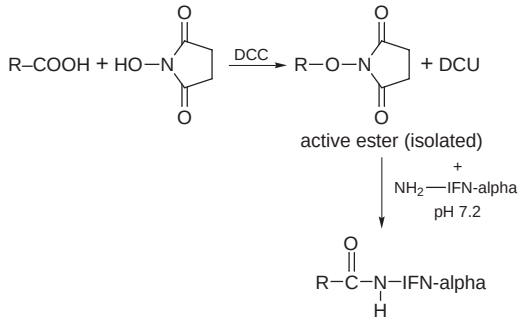
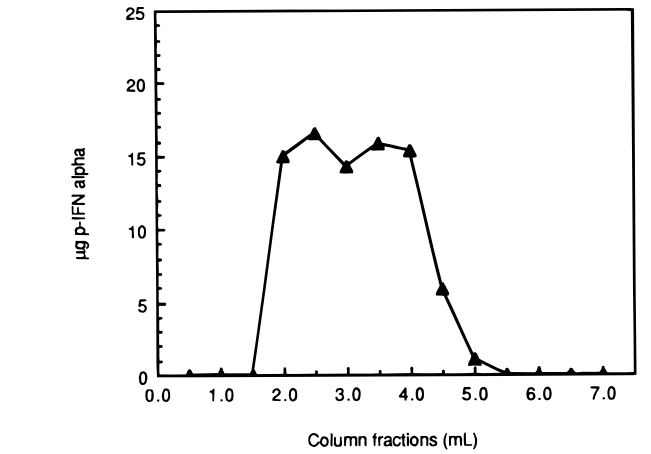


Figure 1—Synthetic process for the fatty acylation of IFN α2. R–COOH = palmitic acid



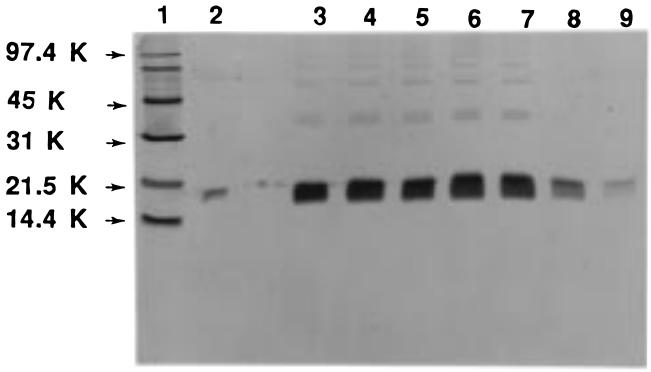
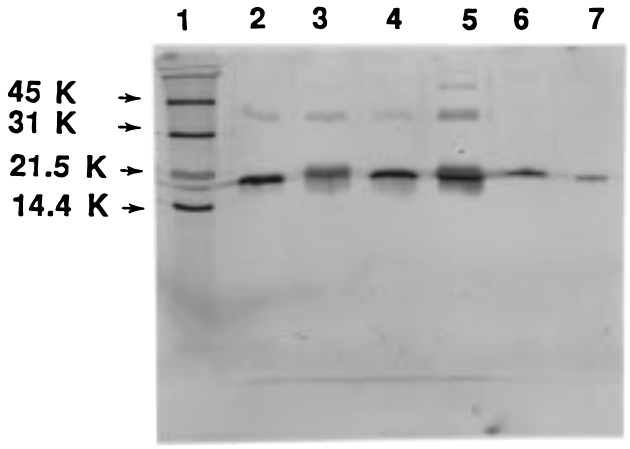


Figure 2—Chromatographic profile of p-IFN α on Sephadex G-25 column and SDS–PAGE pattern of the corresponding chromatographed fractions after silver staining. Lane 1, Bio-Rad molecular weight standard; lane 2, IFN α standard 100 ng, lanes 3–9, fractions from Sephadex column 2–5 mL.

the lysine ϵ-amino groups and the terminal amino group on the protein will be acylated. The p-IFNα preparation after gel filtration may be a mixture which contains IFN α species acylated to various degrees. For the purpose of the current study, however, the different fractions (i.e. monopalmitate, dipalmitate, etc.) were not separated or purified ("crude p-IFNα"). The SDS−PAGE profile of p-IFNα separated on Sephadex G-25 column is shown in Figure 2. The final yield of recovery of p-IFNα was dependent on the starting concentration. At 25 μg "batch" size it was 50.2% and at 100 μg it was 84.0% as determined by the densitometry of p-IFNα bands of the column fractions.

Figure 3 shows the results of the PAGE analysis in which the IFNα was palmitoylated in media containing DMF or DMSO (lanes 3 and 5, respectively). It was found that the total yield of p-IFNα was 15−20% higher in media containing DMSO compared to DMF. Identical treatment except for the addition of palmitic acid was used to evaluate the



Respective Antiviral Activity (% of IFN α 2b std)	100	0	100	50	

Figure 3—SDS–PAGE analysis of p-IFN α prepared under various conditions. Lane 1, Bio-Rad molecular weight standard; lane 2, IFN α incubated in DMF medium; lane 3, p-IFN α prepared in DMF medium; lane 4, IFN α incubated in DMSO medium; lane 5, p-IFN α – prepared in DMSO medium; lane 6, IFN α standard 100 ng; lane 7, IFN α standard 50 ng.

CYS ASP LEU PRO GLN THR HIS SER LEU *GLY SER*	11
ARG ARG THR LEU MET LEU LEU ALA GLN MET ARG	22
ARG ILE SER LEU PHE SER CYS LEU LYS ASP ARG	33
HIS ASP PHE GLY PHE PRO GLN GLU GLU PHE GLY	44
ASN GLN PHE GLN LYS ALA GLU THR ILE PRO VAL	55
LEU HIS GLU MET ILE GLN GLN ILE PHE ASN LEU	66
PHE SER THR LYS ASP SER SER ALA ALA TRP ASP	77
GLU THR LEU LEU ASP LYS PHE TYR THR GLU LEU	88
TYR GLN GLN LEU ASN ASP LEU GLU ALA CYS VAL	99
ILE GLN GLY VAL GLY VAL THR GLU THR PRO LEU	110
MET LYS GLU ASP SER ILE LEU ALA VAL ARG LYS	121
TYR *PHE GLN ARG ILE THR LEU TYR LEU LYS GLU*	132
ASP LYS TYR SER PRO CYS SLS TRP GLU VAL VAL	143
ARG ALA GLU ILE MET ARG SER PHE SER LEU SER	154
THR ASN LEU GLN GLU SER LEU ARG SER LYS GLU	165

Figure 4—Structure of IFN α-2b (as deduced from the nucleotide sequence of cDNA) (taken from Intron A Product Monograph, Schering Canada Inc., 1991).

effect of solvents on the protein (lanes 2 and 4, Figure 3). No apparent negative effect was found. Close investigations of the PAGE bands indicated an about 6−10% increase in molecular weight of IFNα due to acylation.

IFNα is a hydrophilic protein with 9 lysine amino acids (at amino acid positions 31, 49, 70, 83, 112, 121, 131, 134, and 164) available for potential covalent attachment of fatty acids in addition to the amino terminal. The amino acid sequence of IFNα-2b is shown in Figure 4. Although IFNα has two disulfide bonds between residues 1 and 19 and residues 29 and 138,[19] only the latter disulfide bond is critical for maximal antiviral activity.[20] Circular dichroism studies indicated the presence of 45−70% α helix content and the lack of β sheet configuration.[21]

Previous data on consensus IFNα indicate that there are three structurally distinct domains (10−35, 78−107, and

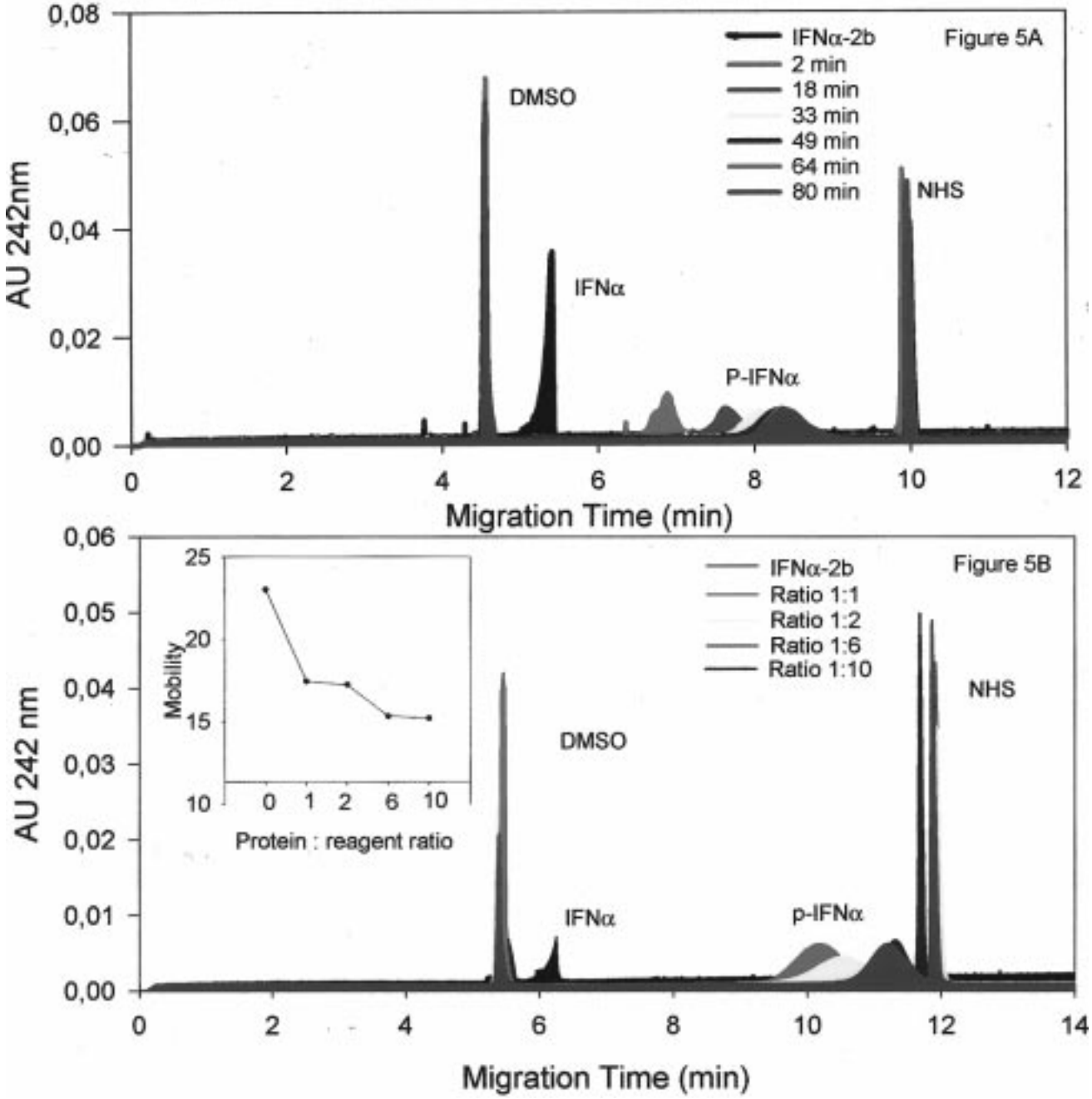


Figure 5—(A) Electropherogram of lipid-modified derivatives of IFNα: effect of incubation time. (B) Electropherogram of lipid-modified derivatives of IFNα: effect of protein:reagent ratio.

123−166) that are important for activity (see regions in italics, Figure 4).[22] It was expected that more than one acyl group would be incorporated per protein molecule since IFNα has nine lysine amino acids plus the terminal cysteine. The three-dimensional structure for IFNα was recently constructed by computer modeling for the primary amino acid sequence of consensus IFNα.[23] With the aid of this model it may be possible to predict which lysine residues are conformationally available for binding. According to the model accessible regions within the molecule are domains 29−35, 78−95, and 123−140. The two most important receptor binding domains are 29−35 and 123−140 (see underlined regions, Figure 4), while domain 78−95 may be an alternative active site.[23] This suggests that at least four lysine residues (31, 83, 131, 134) within these regions plus the terminal amino acid may bind with fatty acids.

Capillary Electrophoresis—CE analysis indicated that after reaction with NHS-P for 3 h, the migration time of IFNα changed from 5.1 to 8.5 min. More detailed investigations indicated that the degree of palmitoylation appeared to be time dependent (Figure 5A). After 2 and 18 min of incubation, the migration time changed to 7 and 7.8 min, respectively, with smaller changes in migration time up to 1 h. After 1 h reaction there was no further change in migration time.

The effect of protein: NHS-P reagent ratio on palmitoylation was also investigated (Figure 5B). At low ratios, more heterogeneous population of lipid-modified derivatives may form as indicated by the broader peaks with lower mobility. At a ratio of 1:10 or higher (e.g., 1:25 routinely used in our studies) a reproducible population of p-IFNα was obtained with an electrophoretic mobility of 9.5 min (Figure 5B insert).

Table 1—Mass Spectrometric Analysis of Palmitoyl-IFN α

peak no.	MS peak mass	calcd peak mass	no. of palmitoyl group linked	relative error %
1	21644	21640	10	0.018
2	21888	21879	11	0.041
3	22120	22117	12	0.014

Mass Spectrometry—Mass spectrometric results revealed three major peaks in the p-IFNα sample with masses of 21644, 21884, and 22120 which corresponds to 10, 11, and 12 palmitoyl groups linked to IFNα (Table 1). The parent IFNα had a molecular mass of 19256, consistent with the calculated individual amino acid composition. The palmitoyl groups are expected to be linked to nine lysine residues and the terminal cysteine under these conditions (pH 7.2). Two further linking sites may be arginine residues.

Antiviral Activity—Since our interest is to develop novel delivery systems for the efficient delivery of IFNα in the treatment of human papillomavirus infections (condylomata acuminata), we focused particularly on the changes in antiviral properties of this drug. The "crude p-IFNα" preparation was evaluated for antiviral activity as a preliminary measure of whether the acylated derivatives retained the original activity (Figure 3). The results showed a complete loss of activity when the reaction was carried out in a medium containing DMF and a 50% preservation of activity in DMSO. At this point it is not certain whether the partial loss of antiviral activity is due to "overacylation". The separation of the crude fraction into single acyl-protein fractions will contribute to the clarification of this question. The optimum degree of fatty acylation will have to be addressed. Although direct comparison is not possible, attempts have been made to prepare palmitoyl derivatives of insulin in order to improve its lipophilicity.[24] It was found that the biological activity (as measured by the hypoglycemic effect in male rats) of the mono and dipalmitoyl derivatives decreased by 12 and 52%, respectively, compared to the parent compound, and the tripalmitoyl derivative had no hypoglycemic effect. At the same time, immunoreactivity decreased by 50 and 70%, respectively, for the mono and dipalmitoyl derivative, which may be therapeutically advantageous.

Interferons have numerous functional effects including antiviral and antitumor action, immunoregulatory action, macrophage activation, enhancement of cytotoxicity of lymphocytes, hormonal interactions, influence on subsequent production of other cytokines, oncogene regulation, and cellular differentiation. It is known that some cytokine functions do not involve receptor binding and can act directly on intercellular signaling pathways.[25] It was shown that substances which increase cellular permeability may actually enhance certain activities which are not associated with receptor binding. Therefore, partial loss of antiviral activity does not exclude the possibility that some other functions of IFNα are unchanged or perhaps increased. Also, partial loss of antiviral activity may not be prohibitive if the degree of cutaneous penetration enhancement is substantially higher.

Cutaneous Absorption of p-IFNα—The main purpose of these experiments was to determine whether the increased lipophilicity of the protein or the covalently attached fatty acyl residues would enhance the penetration of IFNα into or through the skin. Similarly to IFNα, p-IFNα was successfully iodinated by the lactoperoxidase method and used for the skin absorption experiments. The results showed that both the cutaneous and percutaneous absorption of p-IFNα was greater than the original protein.

Table 2—Cutaneous and Percutaneous Absorption of IFNα and p-IFN α through Human Breast Skin in Vitro

(A) Cutaneous Delivery

preparation	whole skin μg/cm^2 $n = 6$	stratum corneum μg/cm^2 $n = 6$	viable layers μg/cm^2 $n = 6$
IFN α gel	0.407 ± 0.108 (1.76 ± 0.47%)	0.202 ± 0.075	0.228 ± 0.091 (0.98 ± 0.39%)
p-IFN α gel	2.106 ± 1.216 (11.53 ± 6.66)	0.227 ± 0.145	1.881 ± 1.160 (10.29 ± 6.35%)

(B) Percutaneous Delivery

parameters	^{125}I-IFN α	^{125}I-p-IFN α
steady-state flux (ng/cm^2/h)[a]	1.47	2.71
permeability coefficient (cm/h)[b]	1.65×10^{-5}	3.03×10^{-5}
diffusion coefficient (cm^2/s)[c]	6.85×10^{-12}	5.45×10^{-12}
total amount diffused in 24 h: (ng/cm^2) $n = 6$	23.8 ± 17.4	42.7 ± 25.70

[a] Determined by regression analysis of the linear portion of the average cumulative amount of drug diffused (Q) vs time (t) curve. [b] Permeability coefficient (P) was calculated from Fick's first law: $(dQ/dt)_{SS} = J_{SS} = P\Delta C$; where $P = K_pD/h$ [J_{SS} – steady-state flux; ΔC – concentration difference between donor and receiver compartments; K_p – partition coefficient between skin and the preparation]. [c] Diffusion coefficient was calculated from $D = h^2/6L$; where h – thickness of the stratum corneum (0.001 cm); L – lagtime (s).

The skin absorption of p-IFNα and IFNα was tested in a gel type pharmaceutical formulation. The cutaneous absorption of p-IFNα was about 5−6 times greater than the IFNα (Table 2A). The amount of p-IFNα and IFNα in whole skin after 24 h of treatment was 2.106 ± 1.216 μg/cm^2 and 0.407 ± 0.108 μg/cm^2, respectively. This represents 11.53 ± 6.66 and 1.76 ± 0.47% of total drug applied, respectively. In the viable skin layers the difference between p-IFNα and the parent protein was 8−10-fold, 1.881 ± 1.160 μg/cm^2 (10.29 ± 6.35%) and 0.228 ± 0.091 μg/cm^2 (0.98 ± 0.39%).

It is worth mentioning that both the synthesis and the cutaneous absorption of p-IFNα were found to be very reproducible.

The calculated percutaneous absorption parameters for the solutions of p-IFNα and IFNα are shown in Table 2B. Approximately two times higher flux was detected for p-IFNα compared to the nonfatty acylated IFNα. The total amount of drug diffused in 24 h was also about two times higher for the p-IFNα.

The evaluation of the effect of IFNα from various topical vehicles (gel, petrolatum, solution)[12] and from liposomes[26,27] was reported previously. These results indicated only a marginal reduction of the number of lesions, the total lesion area, and the lesion virus titer by the gel and petrolatum formulations at 300×10^6 U/g IFNα concentration when used four times a day for 3−5 days starting 24 h prior to virus inoculation in a cutaneous herpes model in guinea pigs. When treatment was initiated 24 h after virus inoculation, no reduction in the number of lesions could be observed. The reduction in any of the parameters tested was not statistically significant compared to the vehicle only control regardless whether treatment started before or after virus inoculation.[12]

The liposomal delivery of IFNα was reported by Weiner et al.[26] also in the cutaneous herpes simplex guinea pig model. When IFNα was entrapped into certain type of liposomes ("skin lipid" liposomes made by the dehydration−rehydration method) significant reduction of lesion scores could be observed.

Studies in patients diagnosed for having condylomata acuminata (genital warts) also confirmed that liposome encapsulation of IFNα enhances delivery into the wart

lesions.[27] Due to the enhanced delivery, improved clinical effect could be observed.

On the basis of the literature data and our previous results,[27] it is fairly certain that the lack of efficacy is related to a delivery problem. The elimination of the virus from the lesions is prerequisite for preventing recurrences after apparent successful treatment. The maximization of the efficiency of delivery of IFNα will be necessary in order to achieve this.

Conclusions

Our results indicate that fatty acylation may increase the cutaneous and percutaneous delivery of IFN α, and therefore there are good possibilities for therapeutic improvement. There are a number of questions which we hope to answer in the future to fully characterize the acylation sites, including the effect of acylation at different sites with different fatty acids on antiviral activity, the metabolic fate of the new derivatives in the skin, receptor binding ability, and subsequent cellular activation.

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