



PEGylation of interferon $\alpha 2$ improves lymphatic exposure after subcutaneous and intravenous administration and improves antitumour efficacy against lymphatic breast cancer metastases



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ABSTRACT

The efficacy of protein-based therapeutics with indications in the treatment of lymphatic diseases is expected to be improved by enhancing lymphatic disposition. This study was therefore aimed at examining whether PEGylation can usefully be applied to improve the lymphatic uptake of interferon $\alpha 2$ and whether this ultimately translates into improved therapeutic efficacy against lymph-resident cancer. The lymphatic pharmacokinetics of interferon $\alpha 2b$ (IFN, 19 kDa) and PEGylated interferon $\alpha 2b$ (IFN-PEG12, 31 kDa) or $\alpha 2a$ (IFN-PEG40, 60 kDa) was examined in thoracic lymph duct cannulated rats. IFN was poorly absorbed from the SC injection site (F_{abs} 36%) and showed little uptake into lymph after SC or IV administration ($\leq 1\%$). In contrast, IFN-PEG12 was efficiently absorbed from the SC injection site (F_{abs} 82%) and approximately 20% and 8% of the injected dose was recovered in thoracic lymph over 30 h after SC or IV administration respectively. IFN-PEG40, however, was incompletely absorbed from the SC injection site (F_{abs} 23%) and showed similar lymphatic access after SC administration to IFN-PEG12 (21%). The recovery of IFN-PEG40 in thoracic lymph after IV administration, however, was significantly greater (29%) when compared to IV IFN-PEG12. The anti-tumour efficacy of interferon against axillary metastases of a highly lymph-metastatic variant of human breast MDA-MB-231 carcinoma was significantly increased by SC administration of lymph-targeted IFN-PEG12 when compared to the administration of IFN on the ipsilateral side to the axillary metastasis. Optimal PEGylation may therefore represent a viable approach to improving the lymphatic disposition and efficacy of therapeutic proteins against lymphatic diseases.

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1. Introduction

The lymphatic system is the primary site of proliferation for infectious diseases that collectively affect greater than 150 million people worldwide, and is a major route for the metastatic spread of cancer [1–4]. Treatment of these diseases commonly involves the administration of small molecule drugs that display only limited lymphatic affinity, which in turn has the potential to restrict *in vivo* efficacy [5]. The increasing role of protein- and peptide-based therapeutics as adjuvants and drugs in the treatment of lymphatic diseases, however, has the potential to better target and treat these ailments, since macromolecules display improved lymphatic access after interstitial (i.e. subcutaneous [SC] or intramuscular [IM]) administration.

Previous studies have shown that the transport of therapeutic proteins into the lymph from interstitial injection sites is largely dependent on molecular weight. Thus, proteins smaller than approximately 20 kDa are primarily absorbed into the blood, whereas larger proteins, by virtue of reduced vascular permeability and greater availability for lymphatic transport, are preferentially absorbed via the lymph [6]. With the exception of monoclonal antibodies, however, many of the protein- and peptide-based therapeutics that are currently indicated for lymph-resident diseases are smaller than 20 kDa [7]. For example, interleukin-2 and interferon $\alpha 2$ are 15 to 19 kDa immunomodulatory cytokines that are approved for, or are being explored as, adjuvant treatments for leukaemias, lymphomas and lymph-metastatic cancers including melanoma. The primary anti-cancer effect of these immunomodulators results from their ability to either increase the proliferation of cytotoxic T-cells (interleukin-2) or to promote 'immunosurveillance' and cancer-directed attack by NK cells, T-cells and macrophages (interferons) [8,9]. Interferons also

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have some ability to inhibit tumour-mediated angiogenesis and inhibit cancer cell proliferation. Augmenting the lymphatic delivery of proteins with potential application in the treatment of lymph-resident diseases may therefore improve therapeutic outcomes. Furthermore, given that the lymphatic system is integrally involved in the production and storage of lymphocytes, improving the lymphatic access of immunomodulatory proteins may also promote a more rapid and/or effective therapeutic immune response against these diseases.

One approach that may be employed to improve the lymphatic delivery of therapeutic proteins is conjugation with polyethylene glycol (PEGylation). PEGylation has been used extensively to prolong the plasma retention of therapeutic proteins by improving in vivo stability and reducing urinary elimination [10]. Whilst PEGylation also reduces receptor binding affinity, therapeutic efficacy is typically improved by increasing the exposure of the protein to target receptors [10]. Furthermore, the parenteral administration of therapeutic proteins via non-intravenous routes can give low and highly variable bioavailability, in part as a result of biodegradation of the protein at the injection site [11]. This pharmacokinetic variability can ultimately have a negative impact on therapeutic success and can also lead to unwanted side effects. By reducing the biodegradation of proteins at the injection site and increasing hydrophilicity and convection through interstitial water channels, PEGylation may therefore also improve protein absorption and limit pharmacokinetic variability.

To this point, however, the impact of PEGylation on the lymphatic disposition and trafficking of proteins has been poorly described. The only published study to have explored the impact of PEGylation on the lymphatic transport of proteins was conducted by Chen and colleagues [12]. This study showed that conjugation of 3 to 4 PEG7000 chains to interleukin 2 improved subcutaneous bioavailability, but PEGylation had a more significant impact on improving uptake via the blood than via the lymph. In contrast, a more recent study from our laboratories, using polylysine dendrimers with similar dimensions and metabolic properties to small proteins, showed that increasing molecular weight through PEGylation with 200–2000 Da PEGs markedly improved absorption via the lymph in rats [13]. Interestingly, PEGylation also appeared to promote extravasation and subsequent reabsorption of the dendrimer into the lymph across peripheral capillary beds after intravenous [IV] administration (lymphatic redistribution). The impact of PEGylation on the absorption and lymphatic transport of proteins is therefore complex and warrants further examination. This is potentially significant, not only from the perspective of optimising the development of protein-based therapeutics for lymph-resident diseases, but also for better understanding the impact of PEGylation on the absorption, biodistribution and trafficking of therapeutic proteins.

The objective of this study was therefore to firstly examine how PEG loading with differing PEG molecular weights influences the lymphatic disposition of proteins after IV and SC administration in lymph-cannulated rats. This was achieved by examination of the lymphatic uptake and transport of the three clinically available forms of human recombinant interferon $\alpha 2$: native interferon $\alpha 2b$ (Intron A®, 19 kDa), mono-PEGylated interferon $\alpha 2b$ (PEG-Intron®, ~31 kDa conjugated with a single linear 12 kDa PEG), and mono-PEGylated interferon $\alpha 2a$ (PEGASYS®, ~60 kDa conjugated with a single branched 40 kDa PEG). The two isoforms of interferon $\alpha 2$ have a high degree (>95%) of sequence identity, allowing broad comparison of the effects of PEGylation on interferon pharmacokinetics (Fig. 1). An orthotopic mouse model of human lymph-metastatic breast cancer was also employed to examine the likely therapeutic benefits gained by improving the lymphatic disposition of protein therapeutics. The data suggest that PEGylation provides significant advantage, not only in improving systemic exposure, but also in promoting lymphatic targeting and therefore access to lymph resident disease sites.

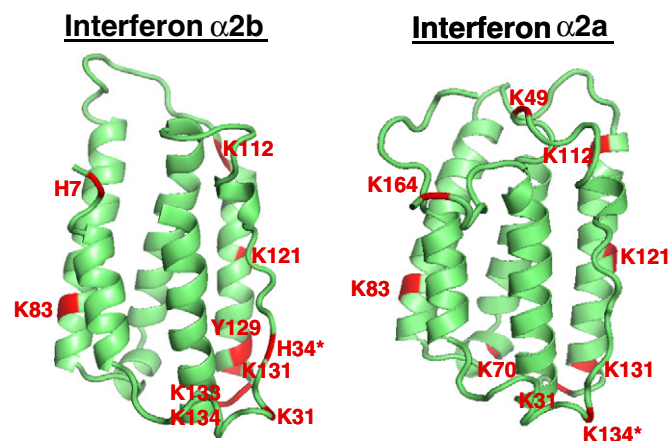


Fig. 1. Ribbon structures of mono-PEGylated interferon $\alpha 2$ examined. The location of PEGylated amino acids is indicated in the figure [14,15]. The main positional isomers of PEG-Intron® and PEGASYS® are H³⁴ and K¹³⁴ respectively (*).

2. Methods

2.1. Materials and reagents

Native recombinant human interferon $\alpha 2b$ (Intron A®, [IFN] 19.3 kDa) and PEGylated interferon $\alpha 2b$ (PEG-Intron®, [IFN-PEG12] ~31 kDa) were obtained from Schering-Plough (North Ryde, NSW, Australia). PEG-Intron® contains mono-PEGylated interferon $\alpha 2b$ that is conjugated with a single 12,000 Da linear PEG chain. The positional isomers of mono-PEGylated interferon were reported previously (Fig. 1) [14]. PEGASYS® (IFN-PEG40, ~60 kDa) was purchased from Roche (Dee Why, NSW, Australia) and contains mono-PEGylated interferon $\alpha 2a$ that is conjugated with a single 40,000 Da branched PEG chain. PEG conjugation is via one of 9 lysine residues (Fig. 1) that give a mixture of positional isomers [15]. Each interferon construct was diluted to provide 10 $\mu\text{g/ml}$ working solutions (for rat pharmacokinetic studies) in sterile saline and stored at 4 °C for up to 1 month. Reconstituted IFN-PEG12 (300 $\mu\text{g/ml}$) was diluted in sterile saline to a 286 $\mu\text{g/ml}$ working solution for mouse anti-tumour efficacy studies. Supplied stock solutions of IFN (at 10 mil IU/ml) were concentrated to 20 mil IU/ml working solutions using Amicon Ultracentrifugal filters (Millipore, VIC, Australia) with a nominal molecular weight limit of 5 kDa at 4° and 3000 $\times\text{g}$ for mouse anti-tumour efficacy studies. Sterile saline was purchased from Baxter Pty Ltd (Melbourne, VIC, Australia). Medical grade polyethylene, polyvinyl and silastic tubing (0.58 mm internal diameter, 0.96 mm external diameter) were obtained from Microtube Extrusions (NSW, Australia). RPMI media, foetal bovine serum (FBS), Glutamax, Hanks balanced salt solution and 0.25% trypsin-EDTA were purchased from Invitrogen (VIC, Australia). D-Luciferin was obtained from Choice Analytical (NSW, Australia). An ELISA kit for the quantification of human interferon- α (subtype 2) was purchased from MabTech Australia Pty Ltd (Macleod, VIC, Australia). Bovine serum albumin (BSA), Tween-20, 30% hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) tablets were purchased from Sigma Chemical Co (NSW, Australia).

2.2. Animals

Male Sprague Dawley rats (250–320 g) were supplied by Monash Animal Services (Monash University, VIC, Australia). Female athymic nude mice (15–20 g) were purchased from the Animal Resources Centre (WA, Australia). Animals were maintained at all times on a 12 h light/dark cycle and were supplied with water ad libitum.

Food was withheld after surgical implantation of cannulae in rats and for 8 h after dosing, but food was freely available at other times. Food was freely available to mice at all times. All protocols were approved by the Institutional Animal Ethics Committee.

2.3. Determination of lymphatic pharmacokinetics in rats

The experimental design involved the parallel examination of interferon pharmacokinetics in four groups of animals: (1) an IV dosed control group (without thoracic lymph duct cannulation), (2) an SC dosed control group (without thoracic lymph duct cannulation), (3) an IV dosed lymph-cannulated group (containing a thoracic lymph duct cannula) and (4) an SC dosed lymph-cannulated group (containing a thoracic lymph duct cannula). The IV dosed control group was cannulated via the right carotid artery to enable blood sampling and the right jugular vein to allow for IV dosing. The SC control group was cannulated only via the right carotid artery. Rats with thoracic lymph duct cannulas were also cannulated from the right carotid artery (to enable blood sampling) and the right jugular vein (to enable IV dosing and the replacement of fluid lost via the thoracic duct cannula via the constant infusion of sterile saline at 1.5 ml/h). The surgical implantation of cannulae was conducted as described previously [13]. All cannulae were exteriorised to the back of the neck to enable sample collection via a swivel-tether apparatus from freely moving animals housed in individual metabolism cages.

Plasma pharmacokinetic data from the IV control group provided background systemic pharmacokinetic parameters from which absolute bioavailability was calculated in the SC dosed animals. Data from IV dosed, lymph-cannulated rats allowed an evaluation of the lymphatic transfer of native and PEGylated interferon from the systemic circulation to the lymphatics. Rats in the IV dosed groups were administered native or PEGylated interferon via rapid infusion of a 1 ml bolus over 2 min at a dose of 5 µg/kg as previously described [13].

Comparison of the plasma pharmacokinetic data from the SC control group with the IV control group allowed estimation of the absolute bioavailability of each of the interferon constructs after SC administration. Data from SC lymph-cannulated rats enabled determination of the proportion of the subcutaneously administered dose of native or PEGylated interferon that was absorbed via the lymph versus via the blood. Rats in the SC groups were administered 5 µg/kg doses of native or PEGylated interferon in a volume of 5 ml/kg saline into the inner left hind leg approximately 0.5 cm above the ankle.

The IV and SC control groups were sampled for 3 days for IFN dosed rats and for 5 days for IFN-PEG dosed rats. At the end of the experiment, primary and secondary draining lymph nodes (popliteal, iliac and inguinal nodes) were collected and assayed for interferon as described in the supplementary information. Blood and lymph samples were collected from thoracic lymph duct cannulated rats for only 30 h post-dose, since animal ethics requirements precluded sample collection in this cannulated model beyond this time.

2.4. Quantification of interferon $\alpha 2$ in plasma and lymph

Interferon $\alpha 2$ was quantified using a commercially available ELISA kit for human interferon subtype 2 according to the manufacturer's instructions. The ELISA quantified both native interferon as well as PEGylated interferon constructs. The concentrations reported here therefore represent the concentrations of the dosed construct. The ELISA was validated for each of the three interferon products examined in blank heparinised rat plasma, lymph and lymph node homogenate. The validated concentration range was between 10 and 1250 pg/ml. Briefly, 96 well microplates (Medisorp) were coated with primary antibody (100 µl per well at a dilution of 1:500 in PBS) at 4 °C overnight. Wells were washed twice with PBS and blocked with 200 µl dilution buffer (0.4% bovine serum albumin, 0.05% Tween-20 in 50 mM PBS)

for at least 1 h at room temperature. Wells were then washed four times with PBS containing 0.05% Tween-20 prior to the addition of samples or standards.

Lymph and plasma samples or standards were thawed on ice on the day of analysis and briefly vortexed prior to preparation in dilution buffer (1:10–1:1000 for samples). Diluted samples and standards were then added to wells in duplicate and incubated at room temperature for 2 h. After washing the wells five times as described above the secondary antibody (100 µl at 1:1000 v/v in dilution buffer) was added and incubated at room temperature for 1 h. After washing, 100 µl streptavidin–horseradish peroxidase (1:1000 v/v in dilution buffer) was added to the wells for 1 h. After a final washing step, 100 µl TMB solution was added to each well for 10 min and the reaction stopped via the addition of 100 µl 1 M H₃PO₄. The optical densities of the wells were then read on a FluoStar plate reader at 450 nm.

2.5. Non-compartmental pharmacokinetic calculations

Pharmacokinetic parameters were calculated based on data obtained from the plasma or lymph concentration–time profiles as reported previously [13]. C_{max} and T_{max} were obtained directly from the plasma concentration vs time curves. The terminal elimination rate constants (k) were calculated from the individual post-distributive plasma concentration vs time profiles for each rat. The areas under the plasma concentration vs time profiles ($AUC^{0-\infty}$) were determined using the linear trapezoidal method and were extrapolated to infinity by dividing the last measured plasma concentration by k . In the SC lymph-cannulated IFN-PEG40 group, k could not be calculated from the post distributive plasma concentration vs time profiles since plasma concentrations did not show a clear decline by 30 h post dose. In this case $AUC^{0-\infty}$ was extrapolated using the mean value of k obtained from the IV lymph-cannulated group to enable a prediction of the bioavailability of the PEGylated protein in this group of animals as described previously [13]. The post distributive volume of distribution for the IV control groups ($V_{D\beta}$) was calculated by dividing the dose by $k \times AUC^{0-\infty}$. Clearance in the IV control groups (Cl) was calculated by dividing the dose by $AUC^{0-\infty}$. The total fraction of the dose absorbed after SC administration in control (non lymph-cannulated) animals (F) was calculated by dividing the dose normalised $AUC^{0-\infty}$ for the SC control group by the mean dose normalised $AUC^{0-\infty}$ for the IV control group. Similarly, the fraction of the dose absorbed via the blood in the SC lymph-cannulated group (F_{blood}) was calculated by dividing $AUC^{0-\infty}$ by the mean $AUC^{0-\infty}$ for the IV control group. The total fraction of the dose transported into lymph over the 30 h collection period (F_{lymph}) was calculated by dividing the mass of interferon recovered in lymph by the mass administered to the rat. The total fraction of the dose absorbed from the SC injection site in lymph cannulated animals (F_{abs}) was calculated by adding F_{blood} and F_{lymph} .

2.6. Antitumour efficacy of IFN and IFN-PEG12 against lymph node resident metastases

In order to test the hypothesis that the improved lymphatic delivery of protein therapeutics leads to enhanced therapeutic efficacy, the antitumour activities of IFN and IFN-PEG12 were compared in a mouse model of human lymphatic metastasis. Ideally, comparison of the antitumour efficacy of IFN and IFN-PEG12 would be conducted in models where therapeutic activity is derived from both the immunomodulatory and antiproliferative effects of interferon. However, the sequence identity between human and murine interferon $\alpha 2$ is low, and our preliminary studies showed that human interferon $\alpha 2$ was ineffective in promoting tumour immunosurveillance by murine peripheral white blood cells. This prohibited examination of the antitumour activity of interferon in syngeneic rodent models of lymph-metastatic cancer. The present study was therefore designed to provide proof of concept data by quantifying the antiproliferative activity of interferon

against human cancer metastases. We therefore used an orthotopic nude mouse model of human lymph-metastatic breast cancer that was derived from a highly lymph metastatic variant of human breast MDA-MB-231 carcinoma cells (MDA-MB-231HM). The use of this athymic nude mouse model enabled the establishment of lymph node metastases of the human breast cancer cell line which was sensitive to the antiproliferative effects of human interferon. It should be noted, however, that it is not known whether the lymphatic transport of human immunomodulatory proteins differs between athymic and immunocompetent animals.

MDA-MB-231HM cells were obtained from Prof. Zhou Luo Ou (Fudan University Shanghai Cancer Center, China) [16,17] and were transfected with a lentiviral vector for firefly luciferase. Cells were cultured in RPMI media containing 10% FBS and 2 mM glutamine (as Glutamax) and were maintained by twice weekly passage in trypsin/EDTA. For induction of primary mammary tumours in mice, cells were suspended in $\text{Ca}^{+}/\text{Mg}^{+}$ free HBSS at 12.5×10^6 cells/ml and maintained on ice. Mice were anaesthetised with 3% isoflurane and 20 μl of the cell suspension was injected into the fourth left mammary fat pad. Primary tumours were allowed to develop for 2 weeks before beginning weekly bioluminescent imaging (IVIS Lumina II, Caliper Life Sciences, MA, USA) to identify the formation of axillary lymph node metastases after intravenous injection of 150 mg/kg D-luciferin. Once mice developed axillary lymph node micrometastases, they were administered 3 \times weekly SC injections of saline, IFN or IFN-PEG12 in a volume of 50 μl , 0.5 cm below the 3rd mammary fat pad for 2 weeks. The antiproliferative activity of IFN was compared to IFN-PEG12 since they are derived from the same interferon subtype ($\alpha 2b$) and because the small differences in molecular weight and activity enabled the administration of relatively similar masses of protein. This enabled a more robust comparison of the effect of improved lymphatic transport on the antiproliferative activity of interferon against lymphatic metastases in the absence of factors which may confound pharmacokinetic behaviour in this model. On average, $\sim 20,000$ cells were present in the lymph node immediately before the dosing regimen began and there were no significant differences in the number of cells at this point between the groups. Injection at this site enables the lymphatic absorption of the interferon dose into lymphatic capillaries that drain towards the axillary node. The growth of axillary metastases was monitored on the Lumina on days 7 and 14 after the first dose. Dosing groups included (1) saline injected SC beneath the 3rd mammary fat pad on the ipsilateral side to the tumour, (2) 1×10^6 IU IFN beneath the 3rd mammary fat pad on the ipsilateral side, (3) 1×10^6 IU IFN-PEG12 beneath the 3rd mammary fat pad on the ipsilateral side and (4) 1×10^6 IU IFN-PEG12 beneath the 3rd mammary fat pad on the contralateral side to the tumour. Group 4 provided a control for the improved systemic exposure of SC administered IFN-PEG12 when compared to the administration of IFN. Based on the reported activity of IFN and IFN-PEG12, 1×10^6 IU of each protein construct was equivalent to a mass of 3.8 and 14.3 μg of protein respectively. This relative activity was also confirmed via assessment of inhibitory activity against the proliferation of MDA-MB-231HM cells (supplementary information).

2.7. Statistical analyses

Individual non-compartmental pharmacokinetic parameters were compared between IFN, IFN-PEG12 and IFN-PEG40 via 1 way ANOVA with Tukey's test for significant differences between groups. AUC and k in lymph-cannulated groups were compared to the respective non lymph-cannulated control values using a two-tailed unpaired t-test. Plasma concentration vs time curves in control and lymph cannulated groups were compared via 2 way ANOVA with Bonferroni test for significant differences at each time point. The growth of axillary lymph node metastases for the 4 dosing groups was similarly compared via 2 way ANOVA with a Bonferroni test. Significance was at a level of $p < 0.05$.

3. Results

3.1. Pharmacokinetics and lymphatic uptake of PEGylated interferon $\alpha 2$ in rats

IFN was rapidly cleared from non-lymph cannulated control rats after IV administration with a terminal half life of approximately 45 min (Fig. 2A, Table 1). Plasma concentrations of IFN were below the detection limit of the assay after 4 h. The IV plasma pharmacokinetics of IFN in control and lymph-cannulated rats were similar (Fig. 2A, Table 1). After SC administration, IFN was rapidly absorbed with a T_{max} of approximately 25 min, although the SC bioavailability was incomplete (36%, Fig. 2B, Table 1). This low bioavailability for native interferon $\alpha 2b$ has similarly been observed for other interferons in rodents [18–20].

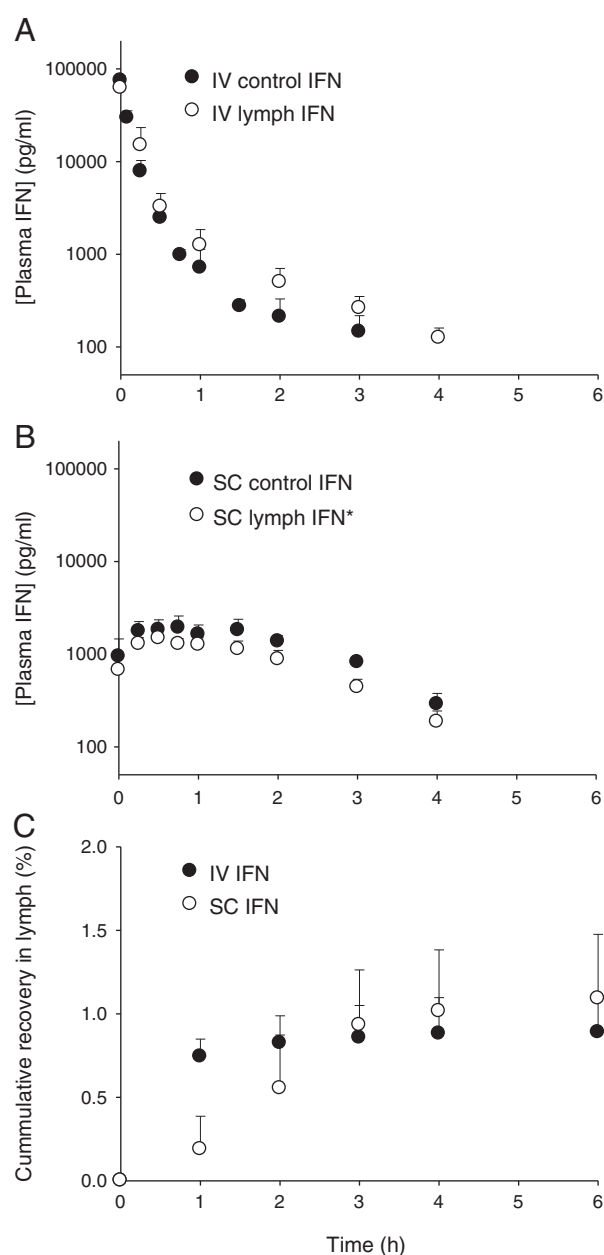


Fig. 2. Plasma concentration–time profile of IFN following 5 $\mu\text{g}/\text{ml}$ IV (panel A) or SC (panel B) dosing in non-lymph cannulated and lymph cannulated rats. The cumulative recovery of IFN in thoracic duct lymph after IV and SC dosing in rats is shown in panel C. Data represent mean \pm s.d. ($n = 4$). *Represents a significant difference between lymph cannulated and control groups via 2 way ANOVA.

Table 1

Non-compartmental pharmacokinetics of native and PEGylated interferon $\alpha 2$ following IV and SC dosing in rats. Data are represented as mean \pm s.d. (n = 4–5).

		IFN	IFN-PEG12	IFN-PEG40
<i>IV control</i>				
k	h^{-1}	1.097 \pm 0.509	0.046 \pm 0.014 ^a	0.083 \pm 0.017 ^a
AUC ^{0–∞}	pg/ml·min	13.0 \pm 3.5	155 \pm 48	2467 \pm 702 ^b
V _{Dβ}	ml	126 \pm 64	0.40 \pm 0.19 ^a	0.05 \pm 0.01 ^a
Cl	ml/h	115 \pm 28	8.4 \pm 2.1 ^a	0.6 \pm 0.2 ^a
<i>IV lymph</i>				
K	h^{-1}	0.661 \pm 0.114	0.079 \pm 0.014 ^{a,c}	0.045 \pm 0.008 ^{a,c}
AUC ^{0–∞}	pg/ml·min	14.3 \pm 2.5	235 \pm 22 ^c	1657 \pm 282 ^b
F _{lymph}	%	0.9 \pm 0.2	8 \pm 3 ^a	29 \pm 5 ^b
<i>SC control</i>				
k	h^{-1}	0.747 \pm 0.231	0.061 \pm 0.031 ^a	0.067 \pm 0.044 ^a
T _{max}	h	0.4 \pm 0.3	6 \pm 2 ^a	26 \pm 3 ^b
C _{max}	pg/ml	1611 \pm 520	5489 \pm 763	12,115 \pm 5849 ^a
AUC ^{0–∞}	pg/ml·min	4.7 \pm 1.5	127 \pm 32	567 \pm 236 ^b
F	%	36.2 \pm 5.6	82 \pm 21 ^b	23 \pm 10
<i>SC lymph</i>				
k	h^{-1}	0.787 \pm 0.222	0.081 \pm 0.025	NA
T _{max}	h	0.4 \pm 0.1	7 \pm 4	24 \pm 8 ^b
C _{max}	pg/ml	1738 \pm 514	4966 \pm 1772	4508 \pm 3877
AUC ^{0–∞}	pg/ml·min	4.2 \pm 0.5	80 \pm 16 ^c	176 \pm 152 ^c
F _{blood}	%	32 \pm 11	51 \pm 10 ^a	7 \pm 6 ^b
F _{lymph}	%	1 \pm 0.4	20 \pm 5 ^a	21 \pm 4 ^a
F _{abs}	%	33 \pm 12	72 \pm 15 ^b	28 \pm 7

^a Represents p < 0.05 cf. IFN.

^b Represents p < 0.05 cf. both interferon groups.

^c Represents p < 0.05 cf. control group.

The cumulative lymphatic recovery vs time profile for IFN showed that the native protein appeared rapidly in lymph after IV administration, albeit at only a very low level (Fig. 2c). This was followed by slower lymphatic uptake after 1 h, with approximately 1% of the dose recovered in thoracic lymph over 30 h. Uptake into the lymph after SC administration was slower, however the total amount recovered over 6 h (1% of the SC dose) was similar.

In contrast to the 19 kDa IFN, the larger (31 kDa) IFN-PEG12 displayed significantly more prolonged plasma exposure, and plasma concentrations of IFN-PEG12 were quantifiable for up to 4 days post dose (Fig. 3A). Clearance was therefore significantly slower for IFN-PEG12 compared to IFN (Table 1). The IV plasma pharmacokinetic profiles for the IV control and lymph-cannulated groups were similar, although AUC was slightly and significantly higher in the lymph-cannulated group (Fig. 3A, Table 1). After SC administration of IFN-PEG12, the dose was absorbed with a T_{max} of approximately 6 h (Table 1, Fig. 3B) and maximal plasma concentrations of IFN-PEG12 were approximately 3 fold higher after SC administration when compared to IFN (Table 1). In terms of interferon equivalents, however, SC administration of IFN-PEG12 gave an approximately 2 fold higher concentration of interferon when compared to the SC administration of native IFN. The bioavailability of IFN-PEG12 was also significantly higher when compared to IFN (82% vs 36% respectively, Table 1). Although plasma concentrations of IFN-PEG12 in SC lymph-cannulated animals were not significantly different to those in SC control animals, (Fig. 3B), the plasma AUC in SC lymph-cannulated animals was significantly lower when compared to the SC control group (Table 1).

The fraction of an IV dose of IFN-PEG12 recovered in thoracic lymph over 30 h was approximately 8%, and this was significantly higher than the lymphatic recovery of IFN after injection by the same route (Fig. 3C). In contrast to the almost exclusive absorption of IFN into the blood from the SC injection site, approximately 20% of the SC dose of IFN-PEG12 was absorbed via the lymph over 30 h (Fig. 3C). Lymphatic access of IFN-PEG12 was therefore higher after SC administration when compared to IV administration.

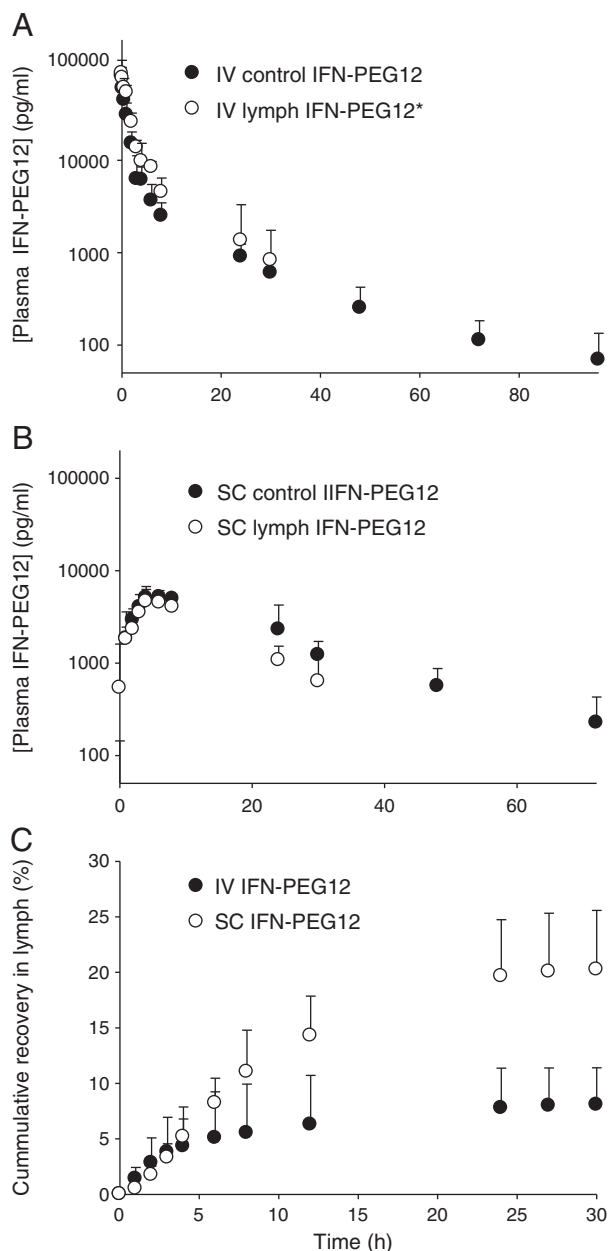


Fig. 3. Plasma concentration–time profile of IFN-PEG12 following 5 μ g/ml IV (panel A) or SC (panel B) dosing in non-lymph cannulated and lymph-cannulated rats. The cumulative recovery of IFN-PEG12 in thoracic duct lymph after IV and SC dosing in rats is shown in panel C. Data represent mean \pm s.d. (n = 4–5). *Represents a significant difference between lymph cannulated and control groups via 2 way ANOVA.

Plasma concentrations of IFN-PEG40 appeared to largely decrease in a mono-exponential manner after IV administration with a terminal half life of 9 h in IV control rats (Fig. 4A). At the 5 μ g/ml dose employed here, however, some evidence of convexity in the IV plasma concentration vs time profile was apparent, suggesting the potential for non-linear pharmacokinetics which have previously been observed for native and PEGylated interferons in rodents and monkeys [18,21]. IFN-PEG40 also displayed more prolonged plasma exposure when compared to IFN-PEG12, although clearance was not significantly slower (Table 1). A two-way ANOVA revealed that overall, plasma concentrations of IFN-PEG40 were significantly lower in the lymph-cannulated groups when compared to the control groups (Fig. 4A, B) and the AUC values in lymph-cannulated groups were significantly lower (Table 1).

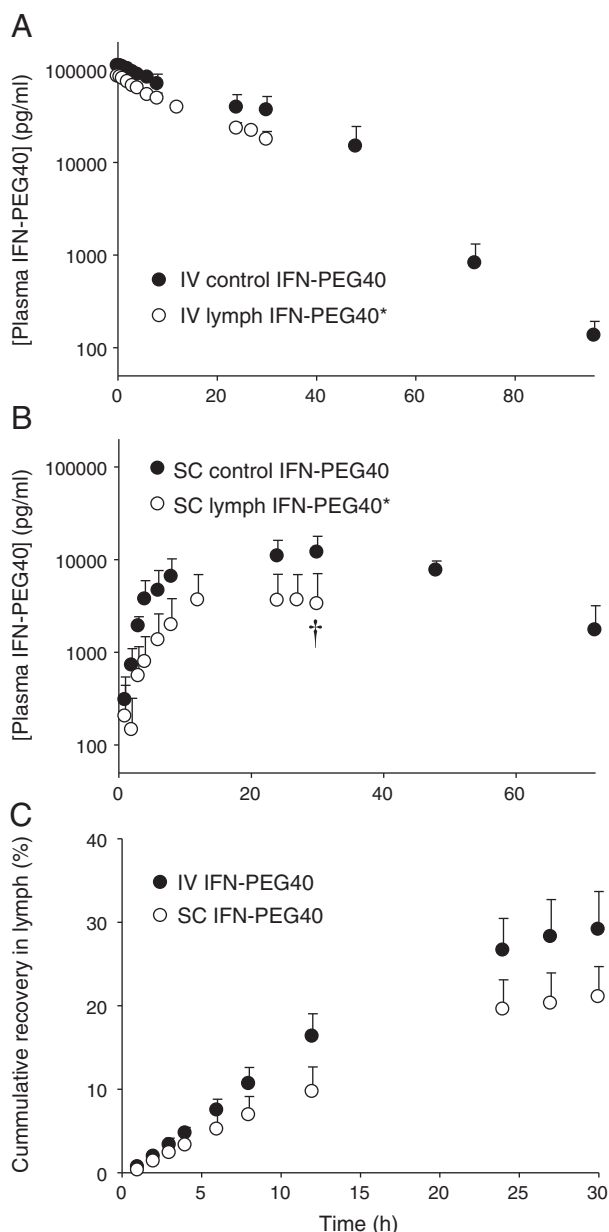


Fig. 4. Plasma concentration–time profile of IFN-PEG40 following 5 $\mu\text{g}/\text{ml}$ IV (panel A) or SC (panel B) dosing in non-lymph cannulated and lymph-cannulated rats. The cumulative recovery of IFN-PEG40 in thoracic duct lymph after IV and SC dosing in rats is shown in panel C. Data represent mean \pm s.d. ($n = 4\text{--}5$). *Represents a significant difference between lymph-cannulated and control groups via 2 way ANOVA. †Represents a significant difference between the lymph-cannulated and control group via Bonferroni post test at the 30 h time point.

IFN-PEG40 was absorbed more slowly than IFN-PEG12, with a T_{max} of 26 h (Fig. 4B, Table 1). By virtue of the marginally slower clearance of IFN-PEG40 when compared to IFN-PEG12, C_{max} for IFN-PEG40 after SC administration was significantly higher than for IFN-PEG12 (12,115 vs 5489 pg/ml respectively, Table 1), despite the reduced SC bioavailability (23%). The C_{max} of interferon equivalents, however, was similar after SC administration of IFN-PEG12 and IFN-PEG40. The reduced bioavailability likely reflects non-linear pharmacokinetics which was not examined in this study.

Since the T_{max} for SC IFN-PEG40 occurred approximately 24 h after dosing, a clear plasma elimination phase was not evident in SC lymph-cannulated animals (where data could only be collected to 30 h). As a result, k could not be calculated. An estimate of AUC was therefore generated using k from the IV lymph-cannulated

group [13]. Using this estimation, and realising that it may underestimate total availability, the fraction of the SC dose absorbed via the blood was $\sim 7\%$ (Table 1). In contrast, approximately 21% of the SC dose was recovered in thoracic duct lymph over 30 h, suggesting that IFN-PEG40 was absorbed from interstitial injection sites mainly via the lymph (Fig. 4C, Table 1). Interestingly, the lymphatic uptake of SC IFN-PEG40 and IFN-PEG12 was similar despite the higher molecular weight of IFN-PEG40. In contrast, the recovery of IFN-PEG40 in the thoracic lymph of IV dosed animals was significantly higher than the recovery of IFN-PEG12 after IV administration (29% vs 8% respectively). This suggests that the more highly PEGylated IFN-PEG40 was absorbed primarily via the lymph and that lymphatic redistribution was a feature of the prolonged plasma exposure profile.

Primary and secondary draining lymph nodes were collected at the end of the study (3–5 days after dosing) and assayed for interferon content. By 3 days after SC or IV administration of IFN or 5 days after SC or IV administration of the PEGylated constructs, the levels of these proteins in lymph nodes were generally lower than the quantifiable limit of the assay (100 pg/g wet weight of lymph nodes, supplementary information). The highest levels of interferon quantified in lymph nodes were in the IV and SC IFN-PEG12 groups, however less than 0.16% of the dose was retained per gram of wet node. It is not known, however, why the highly PEGylated IFN-PEG40 construct did not show higher levels of lymph node retention as seen previously for other large PEGylated proteins [22]. However, this may in part reflect the low reported bioavailability for this construct or reduced long term stability of the construct in rats.

3.2. Antitumour efficacy of PEGylated interferon $\alpha 2$ against lymph node resident metastases

In light of the significant improvement in lymphatic exposure for PEGylated interferon, we sought to examine whether IFN-PEG12 was able to more effectively inhibit the proliferation of lymph node-resident cancers when compared to IFN. Thrice weekly SC administration of IFN on the ipsilateral side to the primary tumour appeared to slow axillary tumour growth when compared to vehicle injected mice, although this was not significantly different due to the large variability in the growth rate of the axillary metastases (Fig. 5). SC administration of IFN-PEG12 on the contralateral side to tumour growth had a similar impact on axillary tumour growth to that obtained after administration of IFN, indicative of limited lymphatic access on the ipsilateral side to axillary tumour growth. In contrast, SC administration of IFN-PEG12 on the ipsilateral side, where local drainage into the lymph and access to the draining lymph nodes was expected, led to a significant decrease (approximately 90%) in axillary lymph node tumour burden when compared to the vehicle control (Fig. 5).

4. Discussion

The primary objective of the current study was to examine the impact of PEGylation on the lymphatic disposition and trafficking of proteins. The lymphatic uptake of interferon $\alpha 2$ from an SC injection site was significantly improved by the conjugation of a single 12 or 40 kDa PEG (by a factor of ~ 20). This is consistent with previous studies that have explored the impact of PEGylation on the lymphatic uptake and transport of colloids and polymers [13,23,24] and previous studies in sheep that suggest that the lymphatic uptake of native proteins from interstitial injection sites is generally proportional to molecular weight [11]. However, in the present study, increasing the degree of PEG loading on interferon (from 12 to 40 kDa), and therefore increasing the molecular weight of the construct from 31 kDa to 60 kDa, did not increase the fraction of the dose absorbed into lymph over 30 h. This may reflect limitations in the rate of lymphatic uptake of the larger PEGylated species, or the

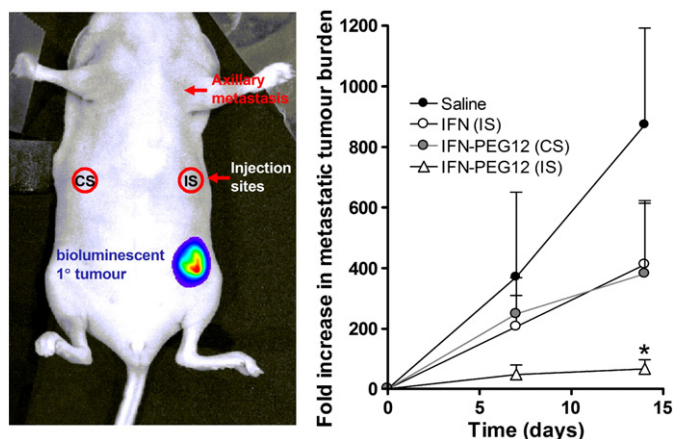


Fig. 5. Photographic and bioluminescent image of a mouse showing the injection sites (left image) and the antitumour efficacy of IFN and IFN-PEG12 against axillary lymph node MDA-MB-231HM metastases (right image). IFN, IFN-PEG12 or saline vehicle control (1×10^6 IU in 50 μ l) was injected 0.5 cm below the 3rd mammary fat pad on the ipsilateral side to the primary tumour (IS) on days 0, 2, 4, 7, 9 and 11 once axillary nodes reached on average 20,000 MDA-MB-231HM cells. In addition, a separate set of mice was dosed with IFN-PEG12 on the contralateral side (CS) to primary tumour growth to control for the more prolonged plasma exposure of IFN-PEG12 when compared to native IFN. Data are reported as fold increase in tumour growth from day 0 (first dose) \pm s.e.m. ($n = 8$ –11). *Represents $p < 0.05$ cf. saline control group.

seemingly lower SC bioavailability of IFN-PEG40 at the dose examined. Furthermore, given the ~ 20 fold increase in lymphatic uptake for IFN-PEG12 (~ 31 kDa) when compared to the only moderately smaller IFN (~ 19 kDa) it is likely that the augmentation in lymphatic uptake for the PEGylated proteins was a result not only of increases in molecular weight, but also of other (as yet unidentified) factors, at least in rats. This suggestion is supported by data showing that the lymphatic uptake of a 22 kDa polylysine dendrimer containing a fully PEGylated surface after SC administration to rats (29% of dose) was even higher than that of IFN-PEG12, despite having a comparable molecular weight to IFN [13]. Kagan and colleagues [25] also previously reported that only 2% of an SC dose of albumin (68 kDa) was absorbed via the lymph over 8 h, in contrast to approximately 8% over the same time period for the similarly sized 60 kDa IFN-PEG40 examined here. It is apparent therefore that the impact of PEGylation on promoting the lymphatic absorption of SC administered proteins is complex and is a function of the net effects of several factors. These may include improved solubility, stability at and drainage from the injection site, and convective transport through interstitial water channels. PEGylation is also expected to mask surface charges and protein binding sites, thereby reducing interaction with the interstitial matrix. In this regard, the relative simplicity of the previous relationship between molecular weight and lymphatic protein absorption observed in sheep may reflect injection into the interdigital space, where issues of instability and drainage from the injection site may have been minimised. It is unknown, however, whether PEGylated or native interferons display differing degrees of metabolic stability in lymph or interstitial fluid when compared to plasma, although the protein composition of lymph and plasma can vary significantly [26].

Another interesting finding from this work, and one that has potential to improve the clinical efficacy of protein therapeutics against more widespread lymphatic disease, was the demonstration that PEGylated proteins also displayed varying degrees of lymphatic redistribution. Lymphatic redistribution results from extravasation of the PEGylated macromolecule across capillary beds, followed by drainage into the lymphatic system and delivery of the macromolecule back into systemic circulation. This has been observed previously for highly PEGylated, and high molecular weight (≥ 22 kDa) polylysine dendrimers [13], indirectly for iron oxide nanoparticles [27] and for 150 kDa antibodies (unpublished data from this laboratory), but has not been reported for smaller proteins. In addition, Lamka and colleagues [22] previously explored the

lymphatic bioavailability of 58 to 300 kDa IV administered highly PEGylated ribonuclease, superoxide dismutase and catalase in unconscious thoracic lymph duct cannulated rats. They reported lymphatic bioavailabilities (calculated by division of the lymphatic AUC by the plasma AUC) of between 0.096 to 0.138 over 2 h, suggesting higher plasma concentrations over the 2 h post dose period when compared to lymphatic concentrations. The lymphatic recovery of drugs in the lymph of unconscious animals, however, is typically lower than in conscious freely moving animals.

In the current study, the lymphatic redistribution of IFN was extremely low (0.9% of the dose). The lymphatic redistribution of PEGylated interferon, however, was significantly higher, and more consistent with differences in molecular weight than the data obtained after SC administration. Thus 8% of IFN-PEG12 was recovered in thoracic lymph after an IV dose compared to 29% for IFN-PEG40. This may suggest that extravasation after IV administration was more consistent for the two PEGylated proteins than was drainage from the SC injection site and therefore that the molecular weight effect on lymphatic 'sieving', as seen previously in sheep, was therefore more apparent after IV when compared to SC administration. The increase in lymphatic recovery of the PEGylated proteins likely reflects ongoing systemic exposure, and therefore ongoing re-perfusion of the interstitial space and the repeated opportunity for drainage into the lymph. Whilst this provides evidence of continuing circulation of these materials between the blood and the lymph, the cumulative collection of lymph in the current studies provides an artificial mechanism of systemic clearance that is absent in non-lymph cannulated animals (where lymph is returned to the systemic circulation). The absolute values for lymphatic protein recovery after IV administration should therefore be evaluated with care. Nonetheless, the relative differences between e.g. IFN, IFN-PEG12 and IFN-PEG40 provide a good indication of the differences in expected lymphatic exposure across the three constructs, and suggest significant improvements in lymphatic access for the PEGylated species.

Although lymphatic recirculation after IV administration provides an opportunity for widespread lymphatic access and exposure, the highest local lymph concentrations are expected to be attained in the afferent lymphatic capillaries that drain a subcutaneous injection site and the sentinel lymph nodes into which these afferent lymphatics drain. From a therapeutic standpoint therefore, the treatment of focal lymph-resident disease is expected to be best achieved through the SC administration of protein therapeutics upstream of the disease site. The lymphatic transport data obtained here after IV administration,

however, suggest that there is also scope to enhance the concentrations of therapeutic proteins in lymphatic capillaries that are remote from the SC injection site.

The surgical removal of sentinel lymph nodes is one of the first treatment strategies for cancers that have the potential to metastasise via the lymph. While this intervention is aimed at removing existing lymph node metastases, and therefore preventing further spread, it has only limited success in preventing the metastatic dissemination of cancer [28,29]. The reasons for this are unclear, but may relate to the fact that this approach does not remove cancer cells present within lymphatic capillaries, or micrometastases that have already lodged in more disseminated lymph nodes. Recent evidence also suggests that metastatic cancers have an intrinsic affinity for specific tissues that may lead to deposition in remote lymph nodes despite the route of metastatic spread [30,31]. There is therefore considerable clinical utility in improving the lymphatic exposure of chemotherapeutic drugs and immunomodulators throughout the body. The data reported here suggests that optimal PEGylation provides the opportunity to enhance local lymphatic targeting downstream of an SC injection site, but that exposure to, and treatment of, lymphatic diseases present elsewhere in the body is also likely after SC or IV administration.

To our knowledge, this is the first study to demonstrate that by increasing the concentration of a therapeutic protein in lymph fluid flowing past a site of lymphatic disease, therapeutic efficacy can be significantly improved. We examined the antiproliferative activity of SC administered IFN and IFN-PEG12 against lymph node metastases of human breast MDA-MB-231HM carcinoma in a mouse xenograft model. IFN-PEG12 was utilised instead of IFN-PEG40 in part because of potential limitations to the efficiency of drainage of the larger IFN-PEG40 from the SC injection site and also since IFN-PEG12 and IFN employ the same interferon subtype (α 2b). SC administration of the non-lymphatically available IFN on the ipsilateral side to the primary tumour and SC administration of the more lymph-targeted IFN-PEG12 on the contralateral side to the primary tumour (which only marginally improved concentrations of interferon in lymph fluid flowing past the metastasis), had no significant effect on metastasis burden. The effect of these treatment groups on tumour burden were also indistinguishable. This is consistent with the results of a recent study in humans which compared metastasis-free and distant metastasis-free survival of adjuvant chemotherapy with SC IFN and IFN-PEG12 in melanoma patients [32]. In this study, IFN and IFN-PEG12 were administered SC at prescribed injection sites, regardless of the location of the primary melanoma. No significant differences were found between any of the study endpoints, suggesting that 31 kDa IFN-PEG12 may not display sufficient lymphatic redistribution to significantly improve cancer therapy at remote lymphatic metastases. In contrast, in the current study, by significantly increasing the exposure of lymph fluid flowing past the affected node (via the SC administration of IFN-PEG12 on the ipsilateral side to tumour growth) metastasis burden was significantly reduced when compared to saline treated control animals. In this study, the *in vivo* antiproliferative effects of interferon could only be measured in the absence of immunomodulatory activity, since human interferon α 2 has no effect on the mouse immune system. The *in vitro* antiproliferative activity of human interferon α 2, however, is stronger when MDA-MB-231HM cells are co-cultured with human peripheral white blood cells (supplementary information). The data therefore suggest that by improving the exposure of lymphocytes and NK cells in lymph nodes and the lymphatic system to interferon, the anti-cancer activity of the protein may be enhanced against lymph-resident cancers. This suggestion is consistent with the vaccine literature which indicates that improving the lymphatic disposition of vaccines significantly augments the immune response by better exposing the antigen to antigen presenting cells and naïve T cells [33].

In summary, the current study has demonstrated that there is scope to improve the treatment of lymph-resident diseases with protein-based therapeutics by using PEGylation to promote enhanced lymphatic disposition of the protein. The data suggest that

higher PEG loading may more optimally promote lymphatic redistribution after systemic (IV) administration, but that even relatively moderate PEGylation may have beneficial effects on SC bioavailability, uptake into the lymphatic capillaries draining the injection site and activity against lymph or lymph node resident disease. The effect of PEG loading on the receptor affinity of the protein, however, also needs to be considered in order to allow improved lymphatic exposure, without negatively impacting on activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2013.03.006>.

References

- [1] G. Pantaleo, C. Graziosi, J.F. Demarest, L. Butini, M. Montrone, C.H. Fox, J.M. Orenstein, D.P. Kotler, A.S. Fauci, HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease, *Nature* 362 (1993) 335–358.
- [2] K. Dowlatshahi, M. Fan, H.C. Snider, F.A. Habib, Lymph node micrometastases from breast carcinoma: reviewing the dilemma, *Cancer* 80 (1997) 1188–1197.
- [3] S. Babu, T.B. Nutman, Immunopathogenesis of lymphatic filarial disease, *Semin. Immunopathol.* 34 (2012) 847–861.
- [4] W. Melrose, J.M. Goldsmid, Infections of the Lymphatic System: Primer of Tropical Medicine, Australas. Coll. Trop. Med. 2005. 24.21–24.17.
- [5] J. Chen, L. Wang, Q. Yao, R. Ling, K. Li, H. Wang, Drug concentrations in axillary lymph nodes after lymphatic chemotherapy on patients with breast cancer, *Breast Cancer Res.* 6 (2004) R474–R477.
- [6] D.N. McLennan, C.J.H. Porter, S.A. Charman, Subcutaneous drug delivery and the role of the lymphatics, *Drug Discov. Today: Technol.* 2 (2005) 89–96.
- [7] B. Leader, Q.J. Baca, D.E. Golan, Protein therapeutics: a summary and pharmacological classification, *Nat. Rev. Drug Discov.* 7 (2008) 21–39.
- [8] G. Malaponte, E. Passero, S. Leonardi, V. Monte, C. Lauria, C. Mazzarino, A. Sciotto, G. Russo Mancuso, F. Di Gregorio, S. Musumeci, Effect of alpha-interferon on natural killer cell activity and lymphocyte subsets in thalassemia patients with chronic hepatitis C, *Acta Haematol.* 98 (1997) 83–88.
- [9] H. Ikeda, L.J. Old, R.D. Schreiber, The roles of IFN gamma in protection against tumor development and cancer immunoeediting, *Cytokine Growth Factor Rev.* 13 (2002) 95–109.
- [10] J.S. Kang, P.P. Deluca, K.C. Lee, Emerging PEGylated drugs, *Expert Opin. Emerg. Drugs* 14 (2009) 363–380.
- [11] C.J.H. Porter, S.A. Charman, Lymphatic transport of proteins after subcutaneous administration, *J. Pharm. Sci.* 89 (2000) 297–310.
- [12] S.A. Chen, R.J. Sawchuk, R.C. Brundage, C. Horvath, H.V. Mendenhall, R.A. Gunther, R.A. Braeckman, Plasma and lymph pharmacokinetics of recombinant human interleukin-2 and polyethylene glycol-modified interleukin-2 in pigs, *J. Pharmacol. Exp. Ther.* 293 (2000) 248–259.
- [13] L.M. Kaminskas, J. Kota, V.M. McLeod, B.D. Kelly, P. Karellas, C.J. Porter, PEGylation of polylysine dendrimers improves absorption and lymphatic targeting following SC administration in rats, *J. Control. Release* 140 (2009) 108–116.
- [14] M. Grace, S. Youngster, G. Gitlin, W. Sydor, L. Xie, L. Westreich, S. Jacobs, D. Brassard, J. Bausch, R. Borden, Structural and biologic characterization of pegylated recombinant IFN- α 2b, *J. Interferon Cytokine Res.* 21 (2001) 1103–1115.
- [15] S. Foser, A. Schacher, K.A. Weyer, D. Brugger, E. Dietel, S. Marti, T. Schreitmuller, Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon alpha-2a (PEGASYS), *Protein Expr. Purif.* 30 (2003) 78–87.
- [16] X.Z. Chang, D.Q. Li, Y.F. Hou, J. Wu, J.S. Lu, G.H. Di, W. Jin, Z.L. Ou, Z.Z. Shen, Z.M. Shao, Identification of the functional role of AF1Q in the progression of breast cancer, *Breast Cancer Res. Treat.* 111 (2008) 65–78.
- [17] D.Q. Li, L. Wang, F. Fei, Y.F. Hou, J.M. Luo, R. Zeng, J. Wu, J.S. Lu, G.H. Di, Z.L. Ou, Q.C. Xia, Z.Z. Shen, Z.M. Shao, Identification of breast cancer metastasis-associated proteins in an isogenic tumor metastasis model using two-dimensional gel electrophoresis and liquid chromatography-ion trap-mass spectrometry, *Proteomics* 6 (2006) 3352–3368.
- [18] A. Greischel, P. Tanswell, U. Busch, K. Schumacher, Pharmacokinetics and biodisposition of recombinant human interferon- α 2C in rat and marmoset, *Arzneimittelforschung* 38 (1988) 1539–1543.

- [19] A. Basu, K. Yang, M. Wang, S. Liu, R. Chintala, T. Palm, H. Zhao, P. Peng, D. Wu, Z. Zhang, J. Hua, M.C. Hsieh, J. Zhou, G. Petti, X. Li, A. Janjua, M. Mendez, J. Liu, C. Longley, M. Mehlig, V. Borowski, M. Viswanathan, D. Filpula, Structure–function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation, *Bioconjug. Chem.* 17 (2006) 618–630.
- [20] R.B. Pepinsky, D.J. LePage, A. Gill, A. Chakraborty, S. Vaidyanathan, M. Green, D.P. Baker, E. Whalley, P.S. Hochman, P. Martin, Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-beta-1a with preserved in vitro bioactivity, *J. Pharmacol. Exp. Ther.* 297 (2001) 1059–1066.
- [21] C. Yongming, Z. Zhang, K. Fan, J. Zhang, W. Shen, M. Li, D. Si, H. Luo, Y. Zeng, P. Fu, C. Liu, Pharmacokinetics, tissue distribution, excretion, and antiviral activity of pegylated recombinant human consensus interferon-alpha variant in monkeys, rats and guinea pigs, *Regul. Pept.* 173 (2012) 74–81.
- [22] J. Lamka, O. Schiavon, M. Laznickek, P. Caliceti, F.M. Veronese, Distribution of catalase, ribonuclease and superoxide dismutase modified by monomethoxy (polyethylene glycol) into rat central lymph and lymphatic nodes, *Physiol. Res.* 44 (1995) 307–313.
- [23] A.E. Hawley, L. Illum, S.S. Davis, Preparation of biodegradable, surface engineered PLGA nanospheres with enhanced lymphatic drainage and lymph node uptake, *Pharm. Res.* 14 (1997) 657–661.
- [24] C. Oussoren, G. Storm, Lymphatic uptake and biodistribution of liposomes after subcutaneous injection: III. Influence of surface modification with poly(ethyleneglycol), *Pharm. Res.* 14 (1997) 1479–1484.
- [25] L. Kagan, P. Gershkovich, A. Mendelman, S. Amsili, N. Ezov, A. Hoffman, The role of the lymphatic system in subcutaneous absorption of macromolecules in the rat, *Eur. J. Pharm. Biopharm.* 67 (2007) 759–765.
- [26] C.C. Clement, D. Aphkhaia, N. Nieves, M. Callaway, W. Olszewski, O. Rotzschke, L. Santambrogio, Protein expression profiles of human lymph and plasma mapped by 2D-DIGE and 1D SDS-PAGE coupled with nanoLC/ESI-MS/MS bottom-up proteomics, *J. Proteomics* 78 (2012) 172–187.
- [27] S.M. Moghimi, B. Bonnemain, Subcutaneous and intravenous delivery of diagnostic agents to the lymphatic system: applications in lymphoscintigraphy and indirect lymphography, *Adv. Drug Deliv. Rev.* 37 (1999) 295–312.
- [28] W.H. McCarthy, H.M. Shaw, N. Cascinelli, M. Santinami, F. Belli, Elective lymph node dissection for melanoma: two perspectives, *World J. Surg.* 16 (1992) 203–213.
- [29] K. Fife, J.F. Thompson, Lymph-node metastases in patients with melanoma: what is the optimum management? *Lancet Oncol.* 2 (2001) 614–621.
- [30] B. Psaila, R.N. Kaplan, E.R. Port, D. Lyden, Priming the 'soil' for breast cancer metastasis: the pre-metastatic niche, *Breast Dis.* 26 (2006) 65–74.
- [31] R.N. Kaplan, B. Psaila, D. Lyden, Bone marrow cells in the 'pre-metastatic niche': within bone and beyond, *Cancer Metastasis Rev.* 25 (2006) 521–529.
- [32] J.J. Grob, T. Jouary, B. Dreno, J. Asselineau, R. Gutzmer, A. Hauschild, M.T. Leccia, M. Landthaler, C. Garbe, B. Sassolas, R.A. Herbst, B. Guillot, G. Chene, H. Pehamberger, Adjuvant therapy with pegylated interferon alfa-2b (36 months) versus low-dose interferon alfa-2b (18 months) in melanoma patients without macrometastatic nodes: an open-label, randomised, phase 3 European Association for Dermato-Oncology (EADO) study, *Eur. J. Cancer* 49 (2012) 166–174.
- [33] X. Zhan, K.K. Tran, H. Shen, Effect of the Poly(ethylene glycol) (PEG) Density on the Access and Uptake of Particles by Antigen-Presenting Cells (APCs) after Subcutaneous Administration, *Mol. Pharm.* (2013) (in press).