Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 300 (2005) 125–130
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Improving the therapeutic efficacy of peptides and proteins: A role for polysialic acids

Gregory Gregoriadis [a,b,*], Sanjay Jain [a,b], Ioannis Papaioannou [b], Peter Laing [b]

[a] *School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK*
[b] *Lipoxen Technologies Ltd, 29-39 Brunswick Square, London WC1N 1AX, UK*

Accepted 1 June 2005

Abstract

Peptide and protein drugs are a growing class of therapeutics. However, their effective application in the clinic is compromised by problems, for instance proteolysis in the circulating blood, premature clearance through the kidneys, and immunogenicity. A number of approaches have been used to circumvent such shortcomings including changes in the primary peptide structure, entrapment into nanoparticles (e.g. liposomes) and conjugation to polymers. Polysialylation, namely, conjugation of peptides and proteins to the naturally occurring, biodegradable α-(2→8) linked polysialic acid is a recent development, which promises to be at least as effective as PEGylation but without its potential toxicity. Polysialylation of a range of peptide and protein therapeutics has led to markedly reduced proteolysis, retention of their activity in vivo, prolongation of their half-life in the circulation and reduction in immunogenicity and antigenicity. It is anticipated that polysialylation will lead to a new generation of peptide and protein constructs with significantly improved pharmacological profiles.

Keywords: Polysialic acid; Peptide drugs; Protein drugs; Insulin interferon; Erythropoietin; Granulocyte colony stimulating factor

1. Introduction

The therapeutic use of peptide and protein drugs, for instance insulin, growth hormone and the interferons has a history of several decades. However, the potentially huge impact of this class of drugs in therapy has become apparent only recently, as a result of advances in genomics and proteomics. These have led to the discovery of numerous protein and peptide drugs of therapeutic potential, a number of which are already applied clinically (Walsh, 2003).

Effective use of peptide and protein drugs in the patient can, however, be compromised by their instability in the body, rapid rates of clearance, premature uptake by tissues (for instance the reticuloendothelial system), loss through the kidneys, and immunogenicity or antigenicity (Harris and Chess, 2003). Concerted efforts made over the years to circumvent such prob-

* Corresponding author. Tel.: +44 20 7753 5822; fax: +44 20 7753 5820.
E-mail address: gregoriadis@ulsop.ac.uk (G. Gregoriadis).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.06.007

lems include changes in the primary peptide structure to render it less prone to degradation, introduction of glycons into the structure or conjugation to polymers in order to improve residence in the circulating blood and, also, reduce immunogenicity, and entrapment into nanoparticles such as liposomes.

By far the most successful approach to date has been conjugation to monomethoxy poly(ethyleneglycol) (mPEG) (Mehwar, 2000). PEGylation (as conjugation to mPEG is commonly referred to) endows protein and peptide drugs with certain advantages which include longer circulatory half-lives and reduction of immunogenicity. An increasing number of PEGylated drugs are now used clinically (e.g. asparaginase, interferon α, tumour necrosis factor and granulocyte-colony stimulating factor) (Harris and Chess, 2003). However, PEG is not biodegradable, and although there is some evidence (Caliceti and Veronese, 2003) of enzyme driven low rate oxidation generating aldehydes and ketones, this is not a normal detoxification mechanism. When conjugated to therapeutic proteins that are large enough to escape kidney clearance, PEG will end up in the tissues participating in the uptake of the PEGylated constructs where it will accumulate intralysomally. Moreover, PEGylated proteins have been found to generate anti-PEG antibodies that could influence the residence time of the conjugate in the circulating blood. So far, however, no adverse effects of PEG immunogenicity have been observed, possibly because of the very small amounts of injected PEGylated drugs currently in use (Caliceti and Veronese, 2003).

2. Polysialic acids

As already mentioned, peptide and protein drugs interact with the biological milieu in ways that can curtail their therapeutic efficiency. An approach to circumvent this difficulty would be to modify drugs in a way that renders them “unnoticeable” in the body and yet allows them to retain their activity. One such approach is to be found in certain bacteria that have evolved to foil the body’s defences by coating their walls with polysialic acid. Arguably nature’s ultimate stealth technology, polysialic acids (PSA) (Fig. 1) are linear polymers of *N*-acetylneuraminic acid (sialic acid) abundantly present on the surface of cells and many proteins. Interestingly, the role of PSA in protecting invading bacteria by interfering with host complement activation and phagocytic activity is extended to such functions in the body as modulating cell to cell inhibition thus facilitating neural tissue development, or helping cancer cells to metastasise by reducing their adherence and thus promoting migration.

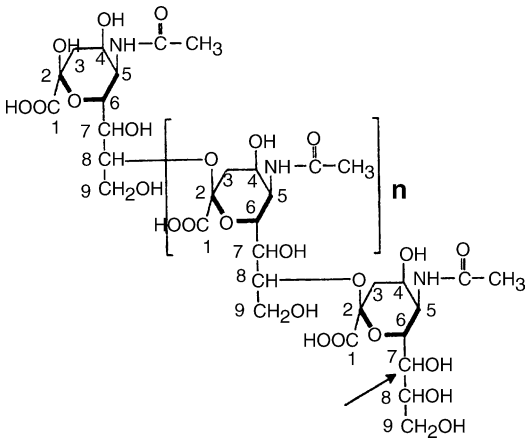

Fig. 1. Structure of polysialic acid (colominic acid). *N*-acetylneuraminic acid units are linked via α-(2→8) glycosidic linkages. The arrow indicates the carbon atom (C_7) at the non-reducing end of the sugar where periodate oxidation introduces an aldehyde group.

It was proposed in 1993 (Gregoriadis et al., 1993) that the unique ability of polysialic acid to insulate microbes and cells alike from external insults could be used to protect therapeutic molecules from the biological milieu and improve their pharmacokinetics. The rationale was that by forming a “watery” cloud around the therapeutic molecule by virtue of the extreme hydrophilicity of PSA, interaction with other molecules (e.g. proteolytic enzymes, opsonins, neutralizing antibodies or receptors on phagocytic cells), would be interfered with, thus allowing the therapeutic to preserve both its structure integrity and activity and prolong its presence in the body. In the case of small size therapeutics (e.g. short peptides and conventional drugs), a considerable increase in size as a result of conjugation with PSA, together with the highly ionic state of PSA could contribute to reduced loss of therapeutic through the kidneys. A schematic representation of polysialylated constructs in Fig. 2 shows two different types. In the case of relatively large peptides and proteins, a number of polymer chains of appropriate length attached randomly or strategically, would

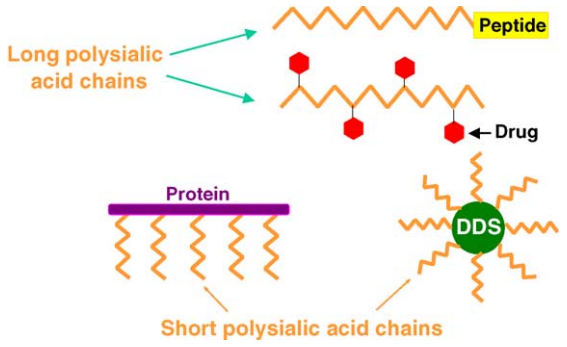

Fig. 2. Schematic representation of polysialylated constructs.

ensure protection. With small molecules on the other hand (e.g. short peptides), a long PSA chain would not only protect, it could also determine the molecule's circulatory half-life. Indeed, it has been shown that the longer the PSA chain the longer the PSA circulates in the blood. For instance, PSA half-lives of up to 40 h (mouse) have been observed after intravenous injection (Gregoriadis et al., 1993). Subsequent work (Fernandes and Gregoriadis, 1996, 1997, 2001; Gregoriadis et al., 2000; Jain et al., 2003a) with a number of therapeutic peptides and proteins and also a small drug molecule has confirmed some of the anticipated advantages of polysialylation.

There is considerable amount of knowledge on the structure and function of polysialic acid of which several types exist (Mühlenhoff et al., 1998). However, in terms of employing the polymer in the polysialylation of drugs, the α-(2→8)-linked senogroup B capsular polysaccharide from *Escherichia coli* K1 (Fig. 1) and its shorter derivatives (also known as colominic acids) are the most appropriate. Bacterial PSA, being chemically and immunologically identical to PSA in the host organism, is by virtue of this structural mimicry, completely non-immunogenic even when conjugated to proteins. Moreover, it does not react with the low affinity antibodies to PSA known to exist at trace levels in the blood. Compared to other polymers such as dextran and PEG, PSA is biodegradable, this being an important advantage when polymers are used to improve the pharmacokinetics profiles of therapeutics administered chronically in relatively large doses (e.g. insulin, antibody fragments).

3. Polysialylation of therapeutics

Work on polysialylation has been carried out mostly by the use of *E. coli*-derived α-(2→8)-linked polysialic acid. Conjugation of polysialic acid to protein therapeutics can be achieved through a variety of simple and gentle procedures, which preserve much of the activity of the therapeutic (Gregoriadis et al., 1993; Fernandes and Gregoriadis, 1996, 1997, 2001; Jain et al., 2003a,b). Conjugation techniques involve modification (activation) of either of the terminals units of the (linear) polymer to structures that can interact with pendant groups of the therapeutic to generate constructs with an average of one or more PSA chains per molecule. Activation procedures include periodate oxidation of the non-reducing end of the PSA followed by interaction with the ε-aminogroups or the N terminal of the protein drug and reductive amination (Fernandes and Gregoriadis, 1996, 1997), as well as other, as yet unpublished, procedures using a variety of chemistries. From the practical point of view, it is convenient to produce large batches of activated PSA, which is then freeze-dried and stored until required. Polysialylation techniques have been recently optimized by the attachment of PSA chains to regions in the molecule that are away from its active site (unpublished results).

Polysialylation has been tested with a wide range of therapeutics so as to demonstrate that criteria that are deemed important in their application in the treatment of disease are satisfied. These include preservation of stability and function, optimal pharmacokinetics and pharmacodynamics, and reduced immunogenicity or antigenicity.

3.1. Preservation of stability

Therapeutic peptides and proteins are often vulnerable to proteolysis in the circulating blood, thus necessitating their administration in increased amounts. We have shown that polysialylation of asparaginase (an enzyme used in the treatment of certain forms of leukaemia) preserves its activity in the presence of serum at 37 °C. Fig. 3 shows that the almost complete loss of native asparaginase activity observed in the presence of serum is avoided when the enzyme is polysialylated (Fernandes and Gregoriadis, 1997). Preservation of enzyme activity is probably effected by PSA chains restricting access of

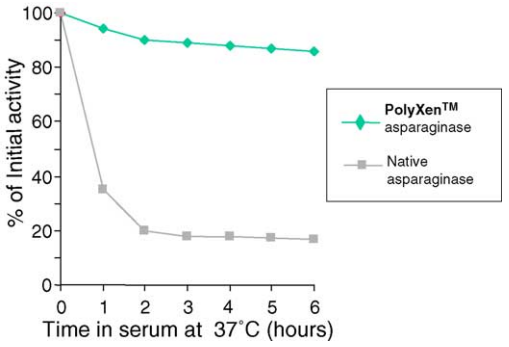

Fig. 3. Stability of intact and polysialylated asparaginase in blood serum (mouse). PolyXen™ is the trademark for polysialylated molecules.

proteolytic enzymes to protein sites that are liable to proteolysis.

3.2. Preservation of function

Interference of polymer chain with the activity of peptides and proteins causing as a result severe loss of activity with PEGylated growth hormone (Clark et al., 1996) and interferon α (Bailon et al., 2001), is a potential drawback that could exclude important therapeutics from polymer-mediated optimisation. Thus, a prime consideration in employing polysialylation of peptide and protein therapeutics is maintenance of their function, for instance in terms of enzyme activity or ability to bind to relevant receptors. Examples of therapeutics chosen to test functionality on polysialylation were asparaginase and interferon α-2b. In the former case, enzyme activity was shown to be fully maintained as evidenced by the identical K_m values of the native and polysialylated protein (Fernandes and Gregoriadis, 1997). It was of interest to note that quantitative preservation of function was also observed (Fig. 4) with polysialylated interferon where the "substrate" (receptor) is a much larger molecule on the surface of cells, confirming its unhindered access to the receptor-binding site (Hirst et al., 2002). More importantly, retention of activity also appears to occur in vivo. This was clearly demonstrated with intravenously injected polysialylated asparaginase (Fernandes and Gregoriadis, 1997) and polysialylated insulin (Jain et al., 2003a) in subcutaneously treated animals. In the latter case (Fig. 5), glucose levels were reduced similarly to those seen with intact insulin although,

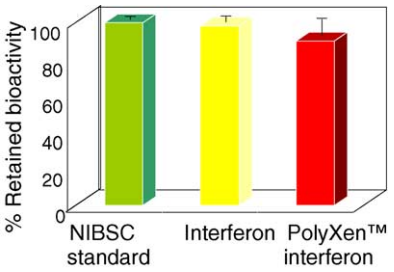

Fig. 4. Receptor (Daudi cells) binding ability of polysialylated interferon α-2b.

as discussed later, the hypoglycaemic effect of the polysialylated hormone lasted for much longer.

3.3. Prolongation of pharmacological action

A crucial feature of polysialylation is that it extends the presence of the active drug in the blood circulation, an event that should help maintain therapeutic concentrations of the active for prolonged periods, in turn reducing dosages and frequency of injections. Molecules on which the concept was tested in vivo were fluorescein (a small model drug molecule) (Gregoriadis et al., 1993), a tumour specific antibody Fab fragment (Epenetos et al., 2002), asparaginase (Fernandes and Gregoriadis, 1997) and insulin (Jain et al., 2003a). With all drugs where plasma concentrations were measured, half-lives in the blood circulation and areas under

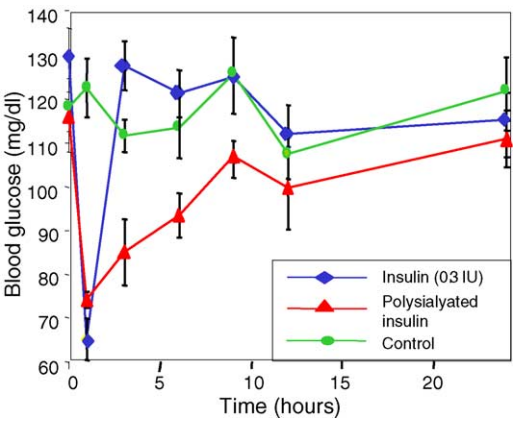

Fig. 5. Hypoglycaemic action of intact and polysialylated insulin in subcutaneously injected mice. Animals were injected with 0.3 international units of intact or polysialylated insulin. Control animals received saline.

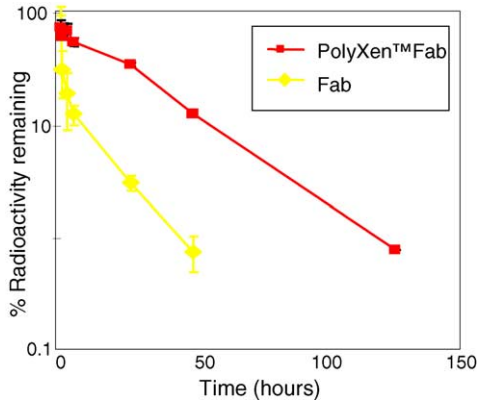

Fig. 6. The effect of polysialylation on the circulatory half-life of an antibody Fab fragment injected intravenously into mice.

the curve increased considerably when drugs were polysialylated. Interestingly, when a tumour-specific polysialylated Fab fragment was used, its prolonged circulation (Fig. 6) was associated with improved localization in the relevant tumour (Epenetos et al., 2002) suggesting that, inspite of PSA chains on the molecule, the antigen-recognising region of Fab was able to bind to the relevant antigen on the cell surface.

3.4. Reduced immunogenicity and antigenicity

Therapeutic use of peptide and protein drugs, especially when repeated injections over prolonged periods are required, is often compromised by the formation of antibodies against the drugs. These can neutralize the activity of the therapeutic and also lead to anaphylactic reactions. Such antibody-mediated loss of activity affects some of the peptides and proteins currently in use (e.g. insulin, erythropoietin and interleukin-2) even those made recombinantly using the human genes (Caliceti and Veronese, 2003).

It has been shown that polysialylation can abrogate both the immunogenicity and antigenicity of peptides and proteins. With asparaginase, for instance, the polysialylated enzyme exhibited similar (greatly improved) activity half-lives in naïve mice and previously immunized (with asparaginase) mice. As expected, activity half-lifes of the native enzyme were much lower in the naïve mice. Importantly, however, they were lower still in immunized mice, presumably because of unhindered antigen–antibody com-

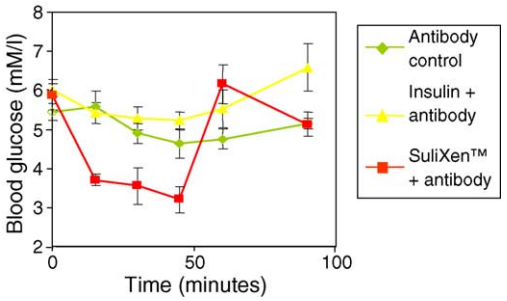

Fig. 7. Hypoglycaemic action of intact and polysialylated insulin in subcutaneously injected mice following incubation with anti-insulin antibody. Note retention of activity for the polysialylated hormone. Antibody control denotes animals injected with antibody only.

plex formation (Fernandes and Gregoriadis, 2001). Although antibody formation was only slightly reduced by polysialylation (Fernandes and Gregoriadis, 2001), the presence of PSA chains on the surface of the enzyme prevented complexing with its antibodies in the circulating blood. Work with insulin on the other hand, shows significant reduction in both immunogenicity and antigenicity. Thus, whereas one or two subcutaneous injections of insulin generated an immune response in two different strains of mice (C57; high responders and Balb/c; low responders), four injections were needed for the elicitation of a (modest) response to polysialylated insulin (Jain et al., 2003a). Moreover, injection of mice with polysialylated insulin that had been previously incubated with anti-insulin antibody led to hypoglycaemia, presumably because of the inability of the antibody to interact with (and inactivate) the relevant antigenic site on the hormone (Fig. 7). In contrast, native insulin incubated with the antibody failed to induce hypoglycaemia (Fig. 7).

4. Conclusions

Polysialylation of peptides and proteins can circumvent many of the problems encountered in their direct use as therapeutics. However, the future of polysialylation as a means to produce new drug entities that will improve the quality of life in patients treated with a wide range of peptide and protein drugs will depend on whether the present approach is as efficient as existing technologies. To that end, evidence amassed so far is promising. There is a huge variety of therapeutics

either in clinical use or in the preclinical stage with many of these in need of improvement. It is anticipated that polysialylation will contribute significantly to the optimization of peptide and protein drugs.

References

Bailon, P., Palleroni, A., Schaffer, C.A., Spence, C.L., Fung, W.J., Porter, J.E., Enrich, G.K., Pan, W., Xu, Z.X., Modi, M.W., Farod, A., Berthold, W., 2001. Bioconjug. Chem. 12, 195–202.

Caliceti, P., Veronese, F.M., 2003. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. Adv. Drug Delivery Rev. 55, 1261–1277.

Clark, R., Olson, K., Fuh, G., Marian, M., Mortense, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M., Wells, J.A., 1996. Long-acting growth hormones produced by conjugation with polyethylene glycol. J. Biol. Chem. 271, 21969–21977.

Epenetos, A.A., Hreczuk-Hirst, D.H., McCormack, B., Gregoriadis, G., 2002. Polysialylated proteins: a potential role in cancer therapy. Clin. Pharm. 21, 2186.

Fernandes, A., Gregoriadis, G., 1996. Synthesis, characterization and properties of sialylated catalase. Biochim. Biophys. Acta 1293, 92–96.

Fernandes, A., Gregoriadis, G., 1997. Polysialylated asparaginase: preparation, activity and pharmacokinetics. Biochim. Biophys. Acta 1341, 26–34.

Fernandes, A., Gregoriadis, G., 2001. The effect of polysialylation on the immunogenicity and antigenicity of asparaginase: implications in its pharmacokinetics. Int. J. Pharm. 217, 215–224.

Gregoriadis, G., McCormack, B., Wang, Z., Lifely, R., 1993. Polysialic acids: potential in drug delivery. FEBS Lett. 315, 271–276.

Gregoriadis, G., Fernandes, A., Mital, M., McCormack, B., 2000. Polysialic acids: potential in improving the stability and pharmacokinetics of proteins and other therapeutics. Cell. Mol. Life Sci. 57, 1964–1969.

Harris, J.M., Chess, R.B., 2003. Effect of pegylation on pharmaceuticals. Nat. Rev. 2, 214–221.

Hirst, H.D., Jain, S., Genkin, D., Laing, P., Gregoriadis, G., 2002. Preparation and properties of polysialylated interferon α-2b. In: AAPS Annual Meeting, Toronto, Canada, p. M1056.

Jain, S., Hirst, D., McCormack, B., Mital, M., Epenetos, A.A., Laing, P., Gregoriadis, G., 2003a. Biochim. Biophys. Acta 1622, 42–49.

Jain, S., Bacon, A., Hreczuk-Hirst, D.H., McCormack, B., Caparros-Wanderley, W., Epenetos, A.A., Laing, P., Gregoriadis, G. 2003b. The effect of polysialylation on the immunogenicity and antigenicity of insulin: implications for its in vitro and in vivo activity. Controlled Release Society 30th Annual Meeting Proceedings, Abstract 441.

Mehwar, R., 2000. J. Pharm. Pharm. Sci. 3, 125–136.

Mühlenhoff, M., Eckhardt, M., Gerardy-Schahn, R., 1998. Polysialic acid: three-dimensional structure, biosynthesis and function. Curr. Opin. Struct. Biol. 8, 558–564.

Walsh, G., 2003. Pharmaceutical biotechnology products approved within the European Union. Eur. J. Pharm. Biopharm. 55, 3–10.