REVIEW

The Pharmacology of PEGylation: Balancing PD with PK to Generate Novel Therapeutics

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ABSTRACT: Conjugation of macromolecules to polyethylene glycol (PEG) has emerged recently as an effective strategy to alter the pharmacokinetic (PK) profiles of a variety of drugs, and thereby to improve their therapeutic potential. PEG conjugation increases retention of drugs in the circulation by protecting against enzymatic digestion, slowing filtration by the kidneys and reducing the generation of neutralizing antibodies. Often, PEGylation leads to a loss in binding affinity due to steric interference with the drug–target binding interaction. This loss in potency is offset by the longer circulating half-life of the drugs, and the resulting change in PK–PD profile has led in some cases to enabling of drugs that otherwise could not be developed, and in others to improvements in existing drugs. Thus, whereas most approaches to drug development seek to increase the activity of drugs directly, the creation of PEGylated drugs seeks to balance the pharmacodynamic (PD) and pharmacokinetic properties to produce novel therapies that will meet with both increased efficacy and greater compliance in the clinical setting. This review examines some of the PEGylated drugs developed in recent years, and highlights some of the different strategies taken to employ PEG to maximize the overall PK–PD profiles of these compounds. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: biotechnology; conjugation; drug delivery; macromolecular drug delivery; PEGylation; pharmacokinetics/pharmacodynamics; renal clearance

INTRODUCTION

Polyethylene glycol (PEG)-conjugated drugs first appeared on the pharmaceutical scene in 1990 with the FDA approval of Adagen® (pegademase: PEGylated adenosine deaminase), as enzyme replacement therapy for patients with severe combined immunodeficiency disease (SCID), an inherited disorder in which deficiency of adenosine deaminase causes accumulation of metabolites and prevents lymphocyte maturation. The approval of Adagen followed more than a decade of research, precipitated by the first description of protein PEGylation by Abuchowski et al.[1] which documented the ability of PEG to prolong the half-life and reduce the immunogenicity of a conjugated protein. When applied to adenosine deaminase to produce pegademase, the technology yielded a drug that enabled twice-weekly intramuscular injections to replace multiple blood transfusions, and, by avoiding the transfusion-associated risks of viral infection and iron overload, provided both a better pharmacological profile and a considerable improvement in convenience for patients.

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The following years saw the approval of additional PEGylated therapeutics for a number of prevalent conditions including Hepatitis C, chemotherapy-associated neutropenia and leukemia (Tab. 1). Several more PEG-conjugated compounds are currently in clinical and preclinical development, reflecting the emergence of this platform as a dominant strategy for enabling or improving macromolecule drugs.

The currently approved PEGylated products are all macromolecules that, between them, cross a number of therapeutic classes, including oncology, metabolic diseases and infectious diseases. The first five approved products were proteins or peptides; the most recently approved, Macugen® (pegaptanib), is an RNA aptamer, while studies on PEG-conjugates of small molecules such as the α4β1 integrin inhibitor demonstrate that the technology can extend beyond biologics and macromolecules.[2] Furthermore, clinical and preclinical studies have been reported which use PEG to make prodrugs for small molecules such as irinotecan,[3,4] doxorubicin[5–7] and camptothecin.[8,9]

Pharmacodynamic (PD) properties of a drug can be measured at the molecular level by parameters such as receptor binding affinity or enzyme activity. While PEG characteristically prolongs the plasma circulating time of a drug, a seminal PK parameter, this often comes at the expense of reduced binding affinity for the target receptor or enzyme. Thus PEG operates to alter the balance between pharmacodynamic and pharmacokinetic properties, compensating for reduction in binding affinity by extension of systemic exposure (Fig. 1). The earlier PEGylated conjugates aimed simply to use PEG to increase systemic exposure of the drug or reduce adverse reactions, without optimizing the effect on potency. More recent approaches, however, integrate the pharmacological properties of the drug and of PEG to minimize the loss of potency while maximizing exposure.

This review will: (i) describe the pharmacological properties of PEGylated drugs, (ii) highlight two case-studies, Somavert® (pegvisomant) and PEGASYS® (peginterferon-α2a), and (iii) analyze some of the different classes of drug that can benefit from PEGylation, to demonstrate how incorporating PEG conjugation into the design of a drug is emerging as an effective strategy for improving or enabling novel therapeutics.

PHARMACOLOGICAL PROPERTIES OF PEGYLATED DRUGS

PEG polymers are composed of repeating units of ethylene glycol, which can be produced as linear or branched chains, with functional groups at one or more termini to enable a variety of conjugation possibilities (Fig. 2). Chemical strategies for conjugating PEG to macromolecules are beyond the scope of this review and have been described in detail recently.[8,10] Further diversity for PEG conjugation arises from the use of either stable or hydrolyzable linkages, the latter resulting in the generation of pro-drugs. In both cases, the conjugated molecule benefits from the pharmaceutical properties of PEG, which include increased solubility, stability over a wide range

Table 1. FDA Approved PEGylated Drugs

Commercial Name	Drug Name	Parent Drug	PEG Size (Da)	Indication	Year of Approval
Adagen®	Pegadamase	Adenosine deaminase	5000	SCID[d]	1990
Oncaspar®	Pegaspargase	Asparaginase	5000	Leukaemia (ALL[e], CML[f])	1994
PEG-INTRON®	Peginterferon-α2b	IFN-α2B	12000	Hepatitis C	2000
PEGASYS®	Peginterferon-α2a	IFN-α2A	40000	Hepatitis C	2001
Neulasta®	Pegfilgrastim	GCSF[a]	20000	Neutropenia	2002
Somavert®	Pegvisomant	GH[b] antagonist	4–5 × 5000	Acromegaly	2003
Macugen®	Pegaptanib	Anti-VEGF[c] aptamer	40000	Age-related macular degeneration	2004

[a]GCSF, granulocyte-colony stimulating factor.
[b]GH, growth hormone.
[c]VEGF, vascular endothelial growth factor.
[d]SCID, severe combined immunodeficiency disease.
[e]ALL, acute lymphoblastic leukemia, acute lymphocytic leukemia.
[f]CML, chronic myeloid leukemia.18pt

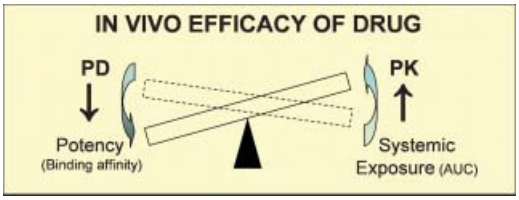

Figure 1. PEG alters the in vivo efficacy of drugs by altering the balance between their pharmacodynamic (PD) and pharmacokinetic (PK) properties. A decrease in potency caused by reduced binding affinity is compensated for by an increase in the overall systemic exposure caused by the prolonged plasma circulating time. The resulting change in the PK–PD profile provides an overall improved therapeutic efficacy that enables the generation of new drugs.

of temperature and pH, and high mobility in solution.[11] However, whereas for prodrugs the activity lies in the released parent molecule, stable conjugates constitute a new active species. This new molecule possesses different pharmacokinetic and pharmacodynamic properties from the parent drug although it may act at the same target receptor or enzyme. Steric hindrance created by the large PEG polymer often underlies the reduced binding affinity that results from PEG conjugation. Compensation for this is achieved through the prolonged circulation time, which, together with the reduced immunogenicity often afforded by PEG, creates an overall improved pharmacological profile that can translate not only to improved efficacy but also to reduced dosing frequency and increased patient compliance. Table 2 outlines comparative PK and PD parameters for PEGylated molecules and their unmodified parent drugs for a number of compounds. These data demonstrate how the combination of decreased activity at the target receptor or enzyme with increased plasma half-life can translate to increase efficacy in an *in vivo* animal model.

Linear PEG-OH $H-(OCH_2CH_2)_n-OH$

Linear mPEG-OH $CH_3-(OCH_2CH_2)_n-OH$

Branched mPEG$_2$

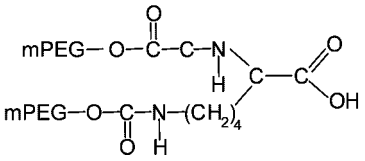

Figure 2. Structural formulae of PEG molecules. mPEG, monomethoxy PEG.

In addition, it is worth noting that PEG conjugation, by limiting diffusion across membranes, often retains drugs in the plasma compartment and results in a reduced volume of distribution.[11] For PEGylated prodrugs, therefore, controlled-release from the plasma compartment can be achieved by using the appropriate PEG linkers, as discussed in recent reviews.[8,10]

Prolonged Circulation Time

The characteristic prolonged circulation time that PEG endows on proteins and peptides arises through two principal effects; a decrease in the rate of kidney clearance and an increase in protection from proteolytic degradation, both of which decrease the overall clearance of the drug. Since PEG polymers are highly hydrated, with two water molecules per ethylene glycol unit, their hydrodynamic radii are approximately 5- to 10-fold greater than would be predicted by their nominal molecular weight,[11] underlying a dramatic increase in the effective molecular size of the PEG conjugate. At lower molecular weights of PEG, clearance occurs primarily by the kidneys; above a molecular weight of approximately 20 kDa renal filtration decreases in favor of excretion by the bile, and above approximately 50 kDa, hepatobiliary clearance dominates.[12,13] It is worth noting that PEGylation of proteins that are normally cleared by receptor-mediated endocytosis does not generally alter the route of elimination, although some slowing of this process has been observed.[14,15]

For many proteins and peptides, rapid proteolytic degradation by circulating enzymes represents one of the principal challenges in producing viable therapeutics. PEG provides protection from proteases and peptidases by impairing access for proteolytic enzymes. Despite this, the continual mobility of the PEG domain provides sufficient flexibility to enable high affinity interactions between the target receptor and the drug moiety and thus yield a biological effect. Consequently, the PEGylated drug retains efficacy while acquiring greater stability in plasma.

The most likely model to explain the protection from proteolysis involves a dynamic process in which the highly hydrated but mobile PEG moiety creates steric obstruction over the domain of the protein that serves as an enzyme substrate, thus reducing the frequency of favorable collisions. Figure 3 shows two scenarios, one involving the

Table 2. Comparative Pharmacokinetic and Pharmacodynamic Parameters for PEGylated Molecules and Their Parent Unmodified Drugs

Measured Parameter	PK				PD	*In Vivo* Efficacy	
	Half-Life ($t_{1/2}$)				*In Vitro* Activity	*In Vivo* Activity	
PEGylated Drug	PK Species[a]	Parent Drug $t_{1/2}$ (h)	PEG-Drug $t_{1/2}$ (h)	Fold Difference	% Activity Retained[b]	PEG Drug Compared with Parent Drug in Animal Models	References
Enzymes							
PEG-arginine deiminase	m	2.8	50	↑ × 18	48	Sustained reduction of plasma arginine to undetectable levels in mice	63
PEG-catalase	m	1	10	↑ × 10	95	10^3–10^4-fold reduction in immunogenic response in mice	1,64
PEG-methioninase	m	2	38	↑ × 19	70	12-fold longer depletion of plasma methionine levels; 10^4-fold decrease in IgG titer in mice	65,66
PEG-superoxide dismutase	r	0.01	38	↑ × 380	51	Protection from oxygen toxicity in rats and 10^3–10^4-fold reduction in immunogenic response in mice	20,64,67
PEG uricase	m	3	72	↑ × 24	100	Reduction in urate levels and protection of renal function in uricase-deficient mice. Reduced immunogenic response	44,68
Cytokines							
PEG-GCSF[c]	r	1.8	7.0	↑ × 3.9	41	14-fold longer effect in sustaining neutrophil count in rats	69
PEG-IFN-α2a	m	0.7	51	↑ × 73	7	Increased anti-tumor activity, elimination of neutralizing antibody response in mice	34
PEG-IFN-β1a	r	0.98	13	↑ × 13	50	2-fold increase in anti-angiogenic properties in murine tumor model	70
PEG-interleukin-6	m	0.05	48	↑ × 960	51	10-fold increase in thrombopoietic	71
PEG-TNFα[d]	m	0.07	0.7	↑ × 10	80	Increased tumor regression using lower doses of drug in mice	72
Polypeptide hormones							
PEG-calcitonin	r	3.31	15.4	↑ × 4.6	50	3–6-fold increase in hypocalcaemic efficacy in rats	51,73
PEG-GLP-1[e]	r	0.04	0.56	↑ × 14	83	Stabilization of glycemia in diabetic mouse model and prolonged glucose-lowering effect in mice	50,52
PEG-hGH[f]	r	0.34	10	↑ × 29	24	2–3-fold prolongation of growth promoting activity in hypophysectomized rats	49
Antibodies							
Fab' fragments	r	0.33	9.05	↑ × 27	100	Reduced tissue distribution and greater concentration in plasma in mice. Reduced immunogenic response	58,59
Nucleic acids							
Anti-VEGF RNA aptamer	mo	24[g]	94	↑ × 3.91	25	3-fold increased inhibition of VEGF-induced vascular permeability in guinea pigs	62,74

[a] m, mouse; r, rat; mo, monkey.
[b] Percent parent drug activity retained in *in vitro* assay (enzyme–substrate, receptor-binding, etc.).
[c] GCSF, granulocyte colony-stimulating factor.
[d] TNF, tumor necrosis factor.
[e] GLP-1, glucagon-like peptide-1.
[f] hGH, human growth hormone.
[g] Half-life values for parent and PEG refer to clearance from vitreous humor in the eye in monkeys.

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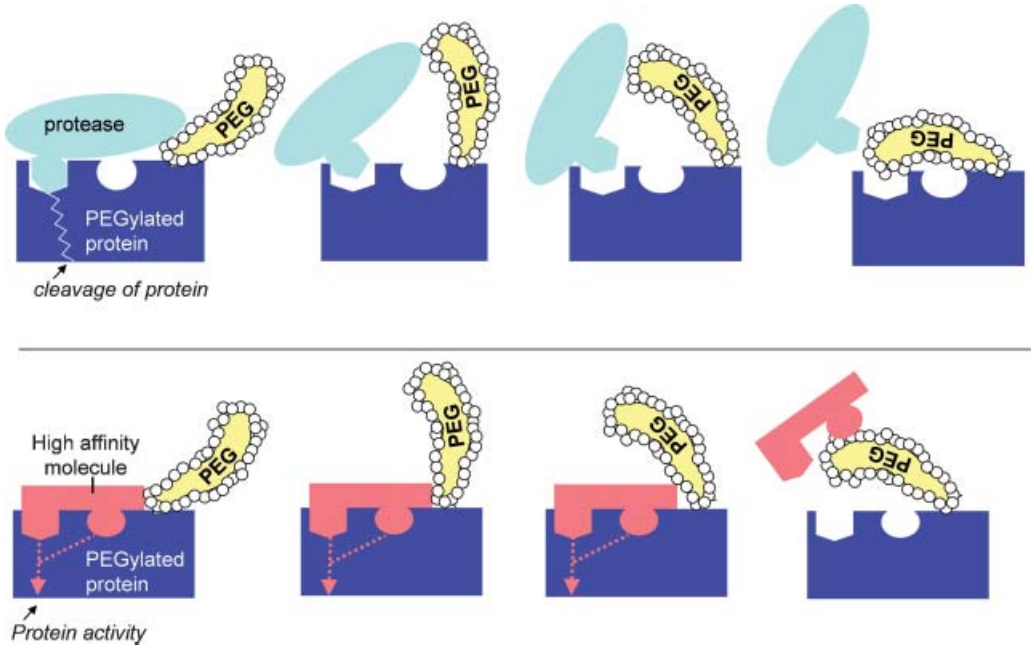


Figure 3. Model for mechanism by which PEG provides proteolytic protection. Top panel: Binding of a plasma protease (light blue) to a PEGylated protein (dark blue) is impaired by the presence of the highly hydrated PEG domain (yellow, with white circles). The mobile PEG domain generates different configurations which reduce the probability of a favorable collision leading to enzyme–substrate interaction and protein cleavage. Lower panel: For the intented target binding molecule (pink) the higher affinity of the interaction drives the equilibrium to increase the probability of a productive interaction, and thus more configurations are permissive for yielding biological efficacy.

action of a proteolytic enzyme (top panel), and the other the action of a high affinity binding molecule (bottom panel). The two are distinguished by the affinity of the interaction. Thus for the lower affinity protease, accessing the necessary configuration for binding and cleavage is made considerably harder by the presence of the PEG domain. For the target binding molecule (which could be a receptor, enzyme substrate or other macromolecule), the higher affinity of the interaction increases the probability of a productive interaction, and thus biological efficacy is still achieved. Note that the presence of PEG does create steric hindrance to some degree also for this interaction, and this is reflected in the lower intrinsic activity of PEGylated molecules compared with their parent native molecule seen in Table 2.

Reduced Immunogenicity and Toxicity

The same steric effect of the hydrated PEG chain that impairs access of proteolytic enzymes also underlies the reduced immunogenicity of PEGylated proteins. The PEG moiety minimizes the exposure of antigenic determinants, thereby reducing or preventing the generation of neutralizing antibodies. The outcome is thus not only increased circulating half-life but also reduced toxicity of the conjugated drug. PEG itself has been approved by the FDA for use in food and cosmetics, and itself is considered essentially nontoxic.[16,17] Uptake of PEGylated drugs into Kuppfer cells in the liver has also been reported,[12] and while some reports indicate that such intracellular uptake of PEGylated molecules can lead to vacuolization, no toxic consequences of this phenomenon have been observed.[18–20] Researchers have attempted to capitalize on this postulated cellular uptake property of PEG by using it to enhance uptake of enzymes such as catalase and superoxide dismutase into cells and deliver to their site of action.[21] To date, however, this has not yet proved sufficiently effective to transition to full drug development.

TWO CASE STUDIES

The traditional paradigm for creating drugs involves synthesis of new molecular entities in

the Discovery phase which are optimized according to their activity *in vitro* and in animal models *in vivo*. Formulation approaches are commonly included later in Preclinical Development, and are employed to obtain the most favorable biopharmaceutical properties and PK profile. PEGylation provides an alternative strategy in which both the efficacy and desired PK profile are built into the design of the molecule during the Discovery phase. The first of the two case-studies describes the creation of the drug pegvisomant (Somavert®) in which PEGylation was incorporated into the molecular design of the drug at the outset, and in which PEG enabled a drug whose rapid elimination would otherwise have made it not viable as a therapeutic. The second case-study describes the development of peginterferon-α2a (PEGASYS®), in which PEG conjugation was utilized on an existing drug not merely to provide a more convenient dosing regimen, but to alter the overall pharmacological efficacy of the drug as a result of its altered PK profile. Both cases highlight the potential for using PEG to alter the balance of PD and PK in a coordinated manner to provide valuable and effective new drug entities.

SOMAVERT®: Pegvisomant—A Drug "Enabled" by PEGylation

Pegvisomant was developed as a growth hormone (GH) antagonist to block the actions of hypersecreted GH in acromegaly, and represents a first-in-class therapy in the treatment of this disorder. Acromegaly is a growth disorder characterized by abnormal enlargement of the hands, feet, skull and jaw, and is caused by excessive secretion of GH, in most cases arising from a pituitary adenoma.[22] The hypersecreted GH activates signaling pathways that lead to overproduction of the insulin-like growth factor IGF-1 and related growth factors, whose mitogenic properties underlie the growth and metabolic clinical manifestations of acromegaly.

First-line therapy for acromegaly involves surgical removal of the adenoma, but this is successful in less than 60% of cases.[23] Alternative treatments, including radiation therapy and drug treatment with dopaminergic or somatostatin agonists show limited success and cause significant side-effects.[22,24] The development of pegvisomant as a GH antagonist therefore represented a breakthrough therapy and provided physicians with a potent tool for treating this endocrine disorder.

Growth Hormone is a 22 kDa protein, secreted by somatotrophs of the anterior pituitary gland, that promotes growth and affects metabolism.[25] The hormone binds to a preformed dimer of the GH receptor, leading to activation of the JAK/STAT-MAP kinase signaling pathway (Fig. 4). GH contains two distinct binding sites for the receptor, termed BS1 and BS2, which display high and low affinity binding respectively. *In vitro* binding studies suggest that high affinity binding to a single receptor via the BS1 site enables interaction of the lower affinity BS2 site with a second receptor in the preformed dimer, leading to activation of the intracellular signaling cascade.[26]

Site-directed mutagenesis experiments demonstrated that a single point glycine to lysine mutation in the BS2 site (G120K) generates an antagonist able to bind the GH receptor with high affinity but unable to activate signal transduction.[24] This G120K-GH antagonist displayed a very short plasma half-life of approximately 30 min, similar to the 15 min half-life reported for the parent GH.[24] To prolong the plasma circulating time, 5 kDa PEG was conjugated to the antagonist using a random conjugation approach that attached PEG at free amine groups on lysine residues. This yielded a protein with a dramatically longer half-life at more than 100 h, but which lost 186-fold potency in receptor binding studies compared with GH (Tab. 3). To restore some binding potency, eight additional mutations were introduced into BS1 to increase its affinity further and compensate for the loss in affinity at BS2. Two of these, K168R and K172R, mutated lysine residues to arginine to remove PEGylation sites and thus reduce the steric hindrance generated by PEG around the binding site. The other six mutations optimized binding based on structure-function analyses of the ligand–receptor interaction.[25] The resulting mutated form of GH, termed B2036, behaved as a GH antagonist with similar binding affinity to that of the parent GH. PEGylation of B2036 yielded a markedly improved profile over that of the previously PEGylated antagonist. The same prolonged plasma half-life of ~100 h was produced, but the binding affinity was reduced by only 28-fold, suggesting greater retention of activity. Interestingly, binding studies using the soluble extracellular portion of the receptor demonstrated only a 4.5-fold lower affinity for PEG-B2036 than the non-PEGylated antagonist, and comparable

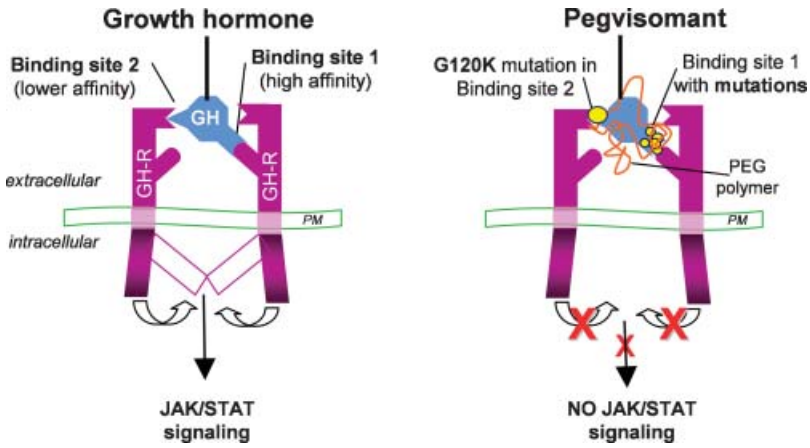


Figure 4. Model for binding of growth hormone receptor to GH (left) and pegvisomant (right). Binding of high affinity and low affinity sites on the hormone (blue shape) to sites on the extracellular domain of the GH-R in its dimer configuration causes a conformational change that activates signaling through the JAK/STAT pathway. Pegvisomant, by contrast, contains the G120K mutation (large yellow circle) and additional mutations (small yellow circles) which enable high affinity binding but cause no conformational change and hence no signal transduction, thus acting as an antagonist. GH, growth hormone; GH-R, growth hormone receptor; JAK, janus kinase; PM, plasma membrane; STAT, signal transducers and activators of transcription.

binding affinity to the parent GH,[25] strongly supporting the notion that the majority of the effect of PEG on binding occurs due to steric interference with the membrane-bound configuration of the receptor.

Clinical studies on pegvisomant demonstrated prolonged efficacy for up to 12 weeks,[27] evident as sustained suppression of total and free IGF-1, IGF Binding Protein 3 (IGFB-3) and the acid-labile subunit of IGFB-3 in patients with acromegaly (Fig. 5). Thus by combining the pharmacodynamic and pharmacokinetic properties of PEG and GH, a potent antagonist was produced that proved effective in clinical trials and which received FDA approval in 2003 as second-line therapy in treatment of acromegaly.

PEGASYS®: Peginterferon-α2a—A Drug "Improved" by PEGylation

FDA approval for peginterferon-α2a (PEG-IFN-α2a) was granted in 2001. Since that time, this drug has obtained status as first-line therapy in chronic hepatitis C infection, and is administered either alone or in combination with the broad spectrum antiviral agent ribavirin.[28] Chronic hepatitis C (HCV) infection affects approximately 4 million people in the US, and represents the leading cause of hepatocellular carcinoma and liver cirrhosis.[29] Previous treatment of HCV infection relied on interferon-α (IFN-α) monotherapy, and was succeeded by treatment with IFN-α in combination with ribavirin. The potent

Table 3. Relative Binding Affinities and Serum Half-Lives of GH Receptor Ligands Generated During the Discovery Process for Pegvisomant

Ligand	Binding Affinity (K_i) at GH Receptor nM	Fold Decrease in Binding Affinity[a]	Serum Half-Life $t_{1/2}$ (h)	Fold Increase in Serum Half-Life ($t_{1/2}$)[b]
GH	3.53 ± 0.54	1.0	0.25	1
G120K-GH	4.66 ± 0.04	1.3	0.25	1
PEG-G120K-GH	657.1 ± 0.71	186	>100	>400
B2036	3.84 ± 0.48	1.1	0.50	2
PEG-B2036	99.60 ± 6.39	28.2	>100	>400

[a]Decrease in binding affinity compared with GH.
[b]Increase in $t_{1/2}$ compared with GH.

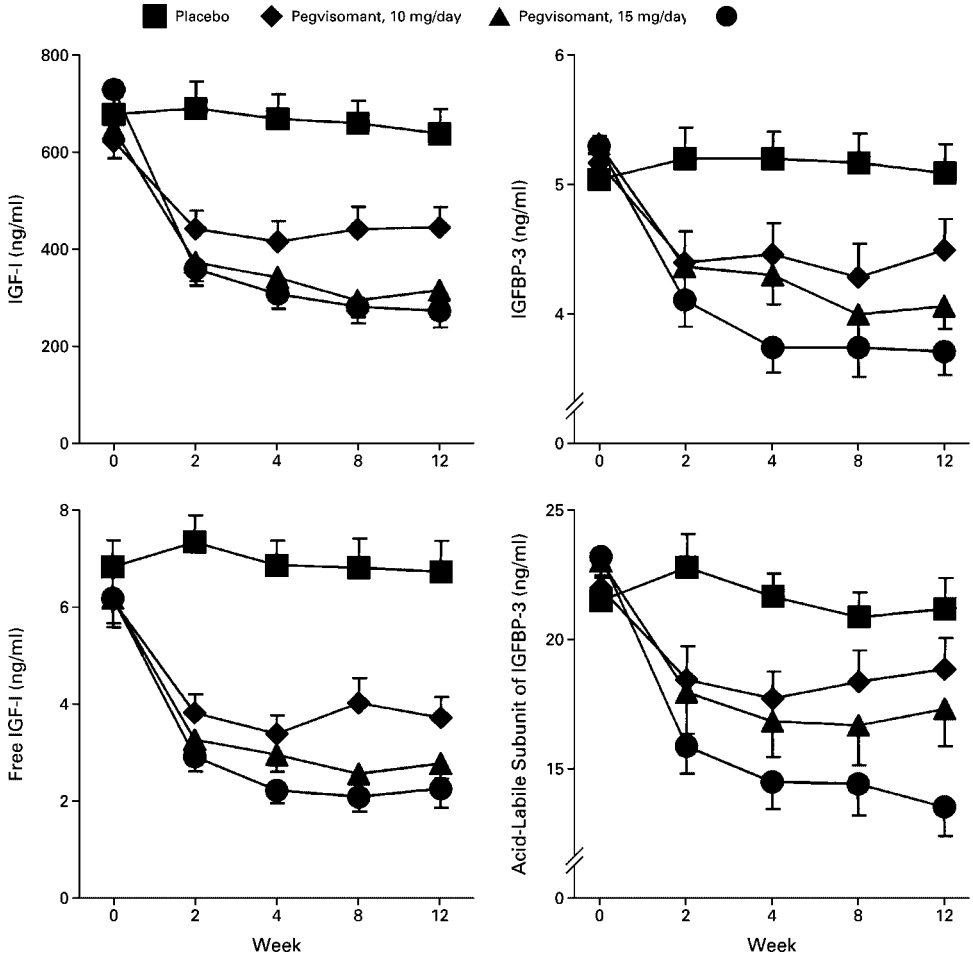
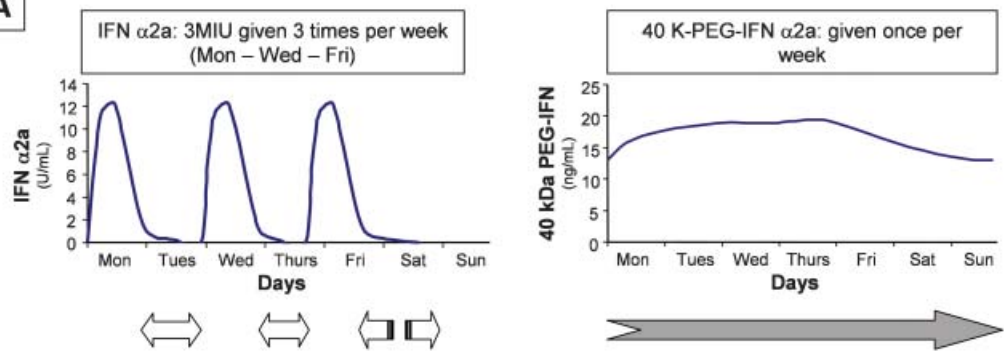


Figure 5. Clinical data show prolonged efficacy of pegvisomant. Following 12 weeks of treatment with pegvisomant, patients displayed significant, dose-dependent reductions in serum concentrations of total and free IGF-1, and the full and acid-labile subunits of IGF1-binding protein (IGF1BP-3) compared with placebo.

antiviral activity of IFN-α results from induction of interferon-stimulated genes (ISGs) via an IFN receptor-JAK/STAT mediated pathway.[30] Induced ISGs inhibit the translation of viral proteins and decrease the stability of the viral RNA. In addition, IFNs stimulate the innate cell-based immune response by enhancing memory T cell proliferation and natural-killer cell activation while inhibiting T cell apoptosis. The mechanism by which ribavirin augments the response to IFN is not well understood, but may involve a shift in the balance of T_H1 and T_H2 cells, inhibition of HCV RNA polymerase, mutagenesis of HCV RNA or GTP depletion.[30]

Treatment of HCV infection with unmodified interferons produces a sustained response only in a minority of patients however, as a result of host factors such as the viral load, viral factors such as the viral genotype, and most significantly pharmacokinetic properties of the interferons themselves.[28] The plasma half-life of 4–6 h results in undetectable levels of IFN-α2a within 24 h of a subcutaneous dose, and thus requires 3 times per week dosing by subcutaneous injection.[31] This produces continual fluctuations in plasma concentrations of IFN-α2a, in which intervals of negligible IFN-α2a creates periods of exposure to the virus, and opportunities for the virus to replicate and develop resistance mutations (Fig. 6A, left panel). The aim of PEGylation therefore, was to provide a means of obtaining a longer serum half-life, and a more consistent, "flatter" serum concentration–

A



B

	40K-PEG-IFNα2a	IFNα2a
No of patients	267	264
Dose, ROA, regimen	180 ug, s.c., 1/week, 48 weeks	6 MIU, s.c., 3 / week x 12 weeks, *followed by* 3 MIU, s.c. 3 / week x 36 weeks
Undetectable HCV RNA (< 100 copies/mL)	68%	28 %
Compliance: patients who completed course	84%	60%

Figure 6. The altered PK profile of PEG-IFN-α2a results in a dramatic improvement in efficacy compared with unmodified IFNα2a. (A) Serum concentration–time profiles for IFNα2a (left panel) and PEG-IFNα2a (right panel) demonstrating the ability of PEG to provide prolonged systemic exposure to the drug. Low serum concentrations of IFNα2a in between doses cause periods of exposure to the virus (left panel, open arrows) which create opportunities for viral replication and development of resistance. PEG conjugation creates a prolonged continual exposure to IFN-α2a (right panel, hatched arrow), during which serum concentrations are sufficiently high to minimize viral replication and mutation. (B) Clinical studies demonstrate that PEG-IFNα2a, with reduced frequency of dosing, produces a significant reduction in detection of virus particles (indicated by HCV RNA copies per mL) in addition to an improvement in compliance, evident from number of patients who completed the course. HCV, hepatitis C virus; IFN, interferon; MIU, million units; ROA, route of administration; s.c., subcutaneous.

time profile with sustained levels of interferon that would generate continual anti-viral protection (Fig. 6A, right panel).

Initial attempts at PEGylation employed a 5 kDa PEG conjugated to the 19 kDa IFN-α2a molecule, but generated an insufficient improvement over the unmodified IFN-α2a when tested in clinical trials.[11] A branched 40 kDa PEG was then used to conjugate to IFN-α2a, and produced a substantially different serum concentration–time profile (Fig. 6), displaying the desired PK behavior. PEGylation prolonged the serum half-life from 3.8 to 65 h, slowed the clearance by more than 100-fold and reduced the volume of distribution fivefold.[32] In phase III clinical trials comparing the efficacy of treatment for 48 weeks with IFN-α2a, given subcutaneously 3 times per week, with PEG-IFN-α2a, subcutaneously, once per week, the response rate to the treatment was more than doubled. 68% of patients who received PEG-IFN-α2a displayed undetectable levels (<100 copies HCV RNA/mL) of virus compared with only 28% of patients who received the unmodified IFN-α2a. In addition, compliance was improved with the PEGylated product since the proportion of patients who

finished the course rose from 60% in the IFN-α2a cohort to 84% in the PEG-IFN-α2a cohort (Fig. 6B).[33]

Thus, by slowing renal elimination of IFN-α2a and protecting from plasma proteases, PEG prolongs the circulation half-life of the molecule. Moreover, by reducing the tissue distribution and maintaining the drug primarily in the plasma where it acts on the virus, PEG increases the exposure of the virus to the drug and creates an improved therapeutic.

Interestingly, *in vitro* activity assays on the 40 kDa PEG-conjugated IFN-α2a indicate that only approximately 7% of the activity of the parent molecule is retained.[34] By contrast, a competing product, composed of a linear 12 kDa PEG-conjugated IFN-α2b (PEG-INTRON®), retains 28% of the parent compound anti-viral activity *in vitro*.[35] However, the improvement in systemic exposure offered by the 12 kDa PEG is relatively modest compared with that of the branched 40 kDa PEG, as it displays a half-life of 27–37 h, a 10-fold lower clearance and only a minor change in the volume of distribution.[32] While both forms of peginterferon are effective, these data exemplify how altering the balance between PK and PD affects the overall pharmacological profile. Thus the degree to which intrinsic activity is retained is not the sole parameter dictating the efficacy of these drugs. Equally important is the overall exposure and its relationship to the pharmacodynamics of the drug.

The current commercial formulation of PEG-IFN-α2a contains a heterogeneous mixture of monoPEGylated isomers of the IFN-α2a molecule. The PEGylation conjugation chemistry attaches the 40 kDa PEG molecule via amide linkages to one of nine reactive lysine residues in the IFN-α2a molecule. Recent studies have separated the mixture chromatographically and examined the activity of the individual positional isomers.[36,37] Using VSV-infected MDBK cells and the human melanoma cell line ME15 for antiviral and antiproliferative assays respectively, Foser et al.[36] found that two of the isomers (K31 and K134) exhibited greater activity than the original PEG-IFN-α2a, while the other seven isomers displayed less activity than the mixture. Variations were also observed between the isomers in the binding affinity for the extracellular portion of the interferon receptor IFNR2. The behavior of the different PEGylated positional isomers has been used to aid understanding of the structure-activity relationship of the IFN–receptor interaction, and may enable the selection of a more potent isomer for development of a further improved PEG-interferon.

DIFFERENT CLASSES OF DRUGS BENEFIT FROM PEGYLATION

The benefits of PEG conjugation can be leveraged differently to improve the PK–PD balance depending on the class of drug, and on its mechanism of action. This technology has been applied to multiple classes of macromolecules, including enzymes, cytokines, polypeptide hormones, antibodies and nucleic acids, as detailed in Table 4. The molecular mechanism by which PEG improves the drug is often similar within a class, resulting in similar trends for the effects of PEG on the PK and PD parameters for drugs within those classes. Therapeutic enzymes, for example, suffer relatively little loss of activity upon PEGylation whereas hormones and cytokines, which require high affinity interactions with cell surface receptors, show significant loss of binding affinity upon PEGylation (Tab. 2). For the latter, the presence of the lipid bilayer and position of the receptor allow less room for maneuver for the ligand, and the steric hindrance created by the PEG molecule thus has a greater impact on the binding affinity. For soluble enzymes which are not tethered to cellular structures, the PEG domain is sufficiently mobile and flexible to enable access of the substrate to the catalytic site, and it can more easily be conjugated at a location which has relatively little impact on the catalytic activity.

Enzymes

Therapeutic enzymes represent a growing class of biopharmaceuticals and PEGylation has played a central role in improving or enabling several of these products.[38] For most enzymes examined, reduction in immunogenicity represents the principal mechanism by which PEG prolongs the circulating half life (Tab. 4). Early work on adenosine deaminase (ADA) demonstrated the ability of PEG to reduce the generation of neutralizing antibodies, extending the plasma half-life from several minutes to approximately 24 h, and led to the development of the first PEGylated enzyme therapeutic, pegademase (Adagen®), as described earlier.[39] Perhaps most

Table 4. Different Classes of Drugs Benefit from PEGylation by Differing Mechanisms of Action

Class	Molecule	PEG size (kDa)	PEGs per Molecule[a]	PEG Mechanism of Action	Development Status	References
Enzymes	Adenosine deaminase	5	Multiple	Proteolytic protection, reduced immunogenicity	FDA approved (*Adagen*®)	39,40,75
	Asparaginase	5	Multiple	Reduce immunogenicity	FDA approved	42,76
	Arginine deiminase	20	22	Reduce immunogenicity	Phase I	63,77,[78]
	Catalase	1.9, 5	Multiple	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	1,64,79
	Methioninase	5	3–7	Reduce immunogenicity	Preclinical	65,80
	Superoxide dismutase	5	10–15	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	20,64,81
	Uricase	20	Multiple	Reduce immunogenicity	Preclinical	44,45
Cytokines	GCSF	20	1	Increase size to slow renal clearance	FDA approved (*Neulasta*®)	46
	Interferon-α2a	40	1	Increase size to slow renal clearance	FDA approved (*PEGASYS*®)	31,32
	Interferon-α2b	12	1	Increase size to slow renal clearance	FDA approved (*PEGINTRON*®)	35
	GM-CSF[b]	5, 10, 20	1	Increase size to slow renal clearance	Preclinical	82
	Interferon-β1a	20	1	Increase size to slow renal clearance	Preclinical	70,83
	Interleukin-6	5	Multiple	Increase size to slow renal clearance	Preclinical	71
	TNFα[c]	5	7–8	Increase size to slow renal clearance	Preclinical	72
Hormones	GH antagonist	5	4–5	Protection from proteases	FDA approved (*Somavert*®)	25
	Calcitonin	2	1	Protection from proteases	Preclinical	73,84
	Growth hormone	20	1	Protection from proteases	Preclinical	48,49
	GLP-1[d]	2	1	Protection from DPPIV[h] and other peptidases	Preclinical	50
Antibodies	Fab' fragments	5, 25, 40	Multiple	Increase size to slow renal clearance	Preclinical	59
	Anti-TNFα Fab'	nd[g]	1	Increase size to slow renal clearance	Filed for approval (*Cimzia*®)	57
	Anti-VEGF-R2[e] Fab'	nd	1	Increase size to slow renal clearance	Phase II (*CDP791*)	56
Nucleic acids	Anti VEGF aptamer (pegaptanib)	40	1	Slow diffusion away from site of action in vitreous humor	FDA approved (*Macugen*®)	60
	Anti PDGF[f] aptamer	40	1	Increase size to slow renal clearance	Preclinical	85

[a]Number of PEG moieties attached per molecule of parent drug.
[b]GM-CSF, granulocyte-macrophage colony-stimulating factor.
[c]TNFα, tumor necrosis factor-α
[d]GLP-1, glucagon-like peptide 1.
[e]VEGF-R2, vascular endothelial growth factor receptor-2.
[f]PDGF, Platelet-derived growth factor.
[h]DPPIV, dipeptidylpeptidase IV.
[g]nd, not disclosed.

interesting for this product is that it operates effectively despite not being able to reach the same cellular location as the endogenous enzyme which it replaces. While endogenous adenosine deaminase is primarily a cytosolic enzyme, the PEG component of pegademase limits its tissue distribution and retains it in the circulation, outside cells. ADA deficiency causes toxic accumulation of metabolites, in particular adenosine and 2′deoxyadenosine.[40] Rapid diffusion of these nucleosides across cell membranes enables them to reach the circulation, however, where pegademase can metabolize them to inosine and 2′deoxyinosine respectively, restoring the balance of metabolites necessary for recovery of immune function.[41]

Reduction in immunogenicity represents the primary benefit of PEGylation for a number of other enzymes under investigation for different chemotherapeutic applications, including pegasparaginase and PEG-uricase (Tab. 4). Pegasparaginase (Oncaspar®) was developed to avoid the hypersensitivity reactions which occurred in 5–20% of acute lymphoblastic leukemia (ALL) patients treated with unmodified L-asparaginase.[42] Treatment with L-asparaginase capitalizes on the absence of asparagine synthetase in ALL tumor cells and acts to deprive them of their external supply of the nutrient.[43] The ability of pegasparaginase to provide this benefit without significant immunogenicity led to the FDA approval of Oncaspar® in 1994 for ALL patients who displayed allergic reaction to the unmodified form of the drug.[43] Coupled with its reduced immunogenicity, the improved PK profile which enables less frequent dosing led the FDA in 2006 to expand the use of the drug for first-line treatment of patients with acute lymphoblastic leukemia (ALL) as a component of a multi-agent chemotherapy regimen.

For the enzyme uricase, immunogenicity presents a common complication and has prompted investigation of PEG-uricase for use in conditions of hyperuricemia, such as occurs during cancer chemotherapy,[44] where toxic levels of uric acid accumulate in the blood, or in gout.[45] Since humans do not express the enzyme, recombinant uricase from other species must be employed, resulting in high antigenicity due to the species difference and the nonmammalian hyperglycosylation that occurs during manufacture in yeast. By a similar mechanism to that described for proteolytic protection, illustrated in Figure 3, PEG provides a means of shielding carbohydrate and other antigenic groups, thereby reducing the immunogenicity of the enzyme and providing the possibility of a safer therapeutic.[44]

Cytokines

Cytokines are small secreted proteins involved in the regulation of immunity, inflammation and hematopoiesis, that characteristically display short plasma circulating half-lives due to their rapid clearance by the kidneys. This has complicated attempts to develop therapeutic cytokines for boosting the immune system in conditions of immunodeficiency. Since PEG conjugation to cytokines can increase their overall hydrodynamic volume, it provides a means to reduce the rate of renal filtration and extend the plasma circulating time, and has been investigated for a number of cytokines (Tab. 4).

GCSF, granulocyte-colony stimulating factor (filgrastim: Neupogen®), is a cytokine involved in stimulating production of neutrophils, whose short half-life complicates its therapeutic use for treatment of chemotherapy-induced neutropenia.[46] The short half-life of GCSF results from its rapid clearance, which is mediated by two pathways: renal filtration and receptor-mediated internalization. Thus, following GCSF-mediated stimulation of neutrophil generation, GCSF binds to its cognate receptors on the upregulated neutrophils and mediates its own clearance. The development of a PEGylated version of GCSF, called pegfilgrastim (Neulasta®), capitalized on the ability of PEG to affect the renal route of clearance while having a nominal effect on the neutrophil-mediated pathway. This was particularly important for this cytokine, since the negative-feedback mechanism operates to limit the number of neutrophils generated. Thus PEGylated GCSF displays slowed renal clearance, providing a significant improvement in plasma half-life, while retaining sufficient biological activity to stimulate production of neutrophils.[47] The newly generated neutrophils bind the circulating PEG-GCSF and remove it from the circulation by an endocytic-lysosomal pathway, thus providing a limit to the upregulation and preventing excessive formation of neutrophils which could be harmful.

Several other cytokines display benefits from the sustained plasma levels achieved by PEG conjugation. As with interferon-α2a, described

previously, the altered PK profile produced by PEGylation presents opportunities not merely for convenience improvement but provides a direct benefit to the pharmacodynamics and hence efficacy of such drugs.

Polypeptide Hormones

A number of polypeptide hormones have been investigated or developed as biotherapeutics for treatment of endocrine disorders caused by either lack of, or excessive, hormone levels. In several cases, PEGylation presents an effective strategy for providing protection from proteolytic enzymes to enable the development of viable therapeutics (Tab. 4).[24,48–50] As polypeptide hormones generally bind cell-surface receptors which are integral membrane proteins, the relatively bulky PEG moiety often interferes with high affinity binding to the target receptor and reduces the activity of the hormone. For this class in particular, therefore, considerable efforts have been targeted at selecting the most appropriate site on the molecule, or engineering appropriate sites, for conjugation of the PEG polymer. Early work on PEGylation of growth hormone, for example, employed a nonselective strategy that conjugated PEG to free amines on lysine residues, and demonstrated a direct relationship between number of PEG moieties and loss of binding affinity.[50] In this case, the increased exposure from prolonged circulation time was not sufficient to compensate for the considerable loss in activity. A more recent study, however, demonstrated that by engineering a threonine to cysteine mutation (T3C) in the GH polypeptide, a cysteine-targeted PEGylation strategy using a 20 kDa PEG could yield a monoPEGylated GH derivative that retains good binding activity and benefits from the prolonged circulation time.[49] Several other polypeptide hormones involved in metabolic disorders have been PEGylated to prolong their plasma circulating time, including calcitonin,[51] GLP-1,[50,52] insulin,[52–54] and neuropeptide Y (NPY).[55] In the case of calcitonin and insulin, nasal and pulmonary delivery respectively were attempted to provide a prolonged delivery system that would extend the PK–PD profile and reduce the frequency of administration.[51,54] In this approach, the large PEG moiety serves to slow absorption through the nasal or pulmonary epithelium, in addition to prolonging circulating time once in the systemic circulation. Here, PEG retains the drug in the nasal cavity or lung, which effectively serve as reservoirs, and choice of the appropriate PEG size controls the rate at which the drug crosses the epithelium to reach the plasma compartment.

Incorporating the PEGylation strategy into the design of a polypeptide hormone during the Discovery phase of drug development, and considering the PK and PD characteristics early on in the process, presents promising new avenues for advancing the development of improved hormone biotherapeutics.

Antibodies

Two products in development, Cimzia® (CDP870: PEG-anti-TNFα Ab) and CDP791® (PEG-anti-VEGFR-2), for treatment of rheumatoid arthritis and solid tumors respectively, are Fab' antibody fragments that employ PEG conjugation to improve the drugs' PK profile while having a minimal loss on the antibody–antigen interaction.[56,57] Studies aimed at determining the optimal site on IgG Fab' antibody fragments for conjugating PEG demonstrated that the hinge region cysteine residues can tolerate attachment of one or two PEG moieties, of up to total 40 kDa molecular weight, with little effect on antigen binding affinity. This is complemented by significant increases in plasma circulating times resulting from the reduced glomerular filtration by the kidneys and lower immunogenicity than the parent IgG,[58,59] and suggests a mechanism for successfully PEGylating antibody Fab' fragments.

These data should aid in the design of PEGylation strategies as the field of therapeutic antibody fragments continues to develop, and further antibody targets continue to be identified.

Nucleic Acids

Pegaptanib sodium (Macugen®) represents the first approved RNA aptamer and was developed as an anti-VEGF directed therapeutic for treatment of age-related macular degeneration.[60] Aptamers are oligonucleotides that bind with high affinity to target molecules;[61] for pegaptanib the target is VEGF, a principal mediator of the angiogenesis and increased vascular permeability that lead to this form of macular degeneration. For full efficacy this drug requires intravitreal injection to enable the aptamer to access the local VEGF that

causes neovascularization. Conjugation of the 40 kDa PEG serves to slow diffusion out of the vitreous humor thus maximizing efficacy and minimizing systemic exposure. PEG conjugation causes a fourfold drop in binding affinity,[62] but its prolonged retention at the site of action more than compensates for this and enables sufficient VEGF inactivation to yield good clinical efficacy.

As nucleic acids provide more tools for creating drugs in the form of antisense oligonucleotides, aptamers and siRNAs, PEGylation is likely to emerge as an effective tool for improving the pharmacological profile by producing an optimal balance between the PK and PD characteristics.

SUMMARY

PEGylation of macromolecules has advanced considerably over the last two decades, and the appearance of PEGylated drugs promises to continue to accelerate as the field of biotherapeutics expands. Understanding how PEGylation affects the pharmacology of drugs at a molecular level enables incorporation of a PEGylation strategy into drug design from the early stages in the development pathway. This optimization of the PK–PD balance early in the process may provide a more efficient approach to generating viable drug candidates, and may facilitate the creation of novel drugs from multiple classes of macromolecule.

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