Abstract  Filgrastim, a recombinant methionyl human granulocyte colony-stimulating factor (G-CSF) (r-metHuG-CSF), is efficacious in stimulating neutrophil production and maturation to prevent febrile neutropenia (FN) in response to chemotherapy. Because of its relatively short circulating half-life, daily filgrastim injections are required to stimulate neutrophil recovery. In an effort to develop a long-acting form of filgrastim that was as safe and efficacious as filgrastim but had a longer in vivo residence time, a number of strategies were considered. Ultimately, fusion of filgrastim to polyethylene glycol (PEG) was selected. Following extensive analysis of conjugation chemistries as well as in vitro and in vivo characterization of a panel of PEGylated proteins, a construct containing a 20 kDa PEG moiety covalently conjugated to the N-terminus of filgrastim was chosen for advancement as pegfilgrastim. Pegfilgrastim is primarily cleared by neutrophils and neutrophil precursors (rather than the kidneys), meaning that clearance from the circulation is self-regulating and pegfilgrastim is eliminated only after neutrophils start to recover. Importantly, addition of PEG did not alter the mechanism of action and safety profile compared to filgrastim. Clinical evaluation revealed that a single 6 mg dose effectively reduces the duration of neutropenia and risk of FN in patients receiving chemotherapy. This work demonstrates the benefit of using PEGylation to generate pegfilgrastim, which allows for once-per-chemotherapy cycle administration while maintaining similar safety and efficacy profiles as those for multiple daily administration of filgrastim. Approaches that may provide advances for therapeutic agonists of G-CSF receptor are also discussed.

Key Points

- Evaluation of multiple PEGylated forms of filgrastim identified pegfilgrastim, a 20 kDa polyethylene glycol (PEG) fusion protein, as the construct with the optimal balance of in vitro activity and in vivo residence time.
- Pegfilgrastim, having self-regulating clearance, remains in circulation during chemotherapy-induced neutropenia and is not eliminated until neutrophils start to recover.
- Clinical efficacy of pegfilgrastim is achieved with a single 6 mg dose once-per-chemotherapy cycle.

1 Introduction

Granulocyte colony-stimulating factor (G-CSF) is an endogenous hematopoietic growth factor that stimulates proliferation and differentiation of neutrophil precursors and increases survival and activity of mature neutrophils [1]. Neutrophils, the most abundant white blood cells in circulation, play a critical role in innate immunity and also influence the adaptive immune response. The protective role of neutrophils is demonstrated by the severe and sometimes fatal
infections that are developed by patients with congenital or induced neutrophil deficiencies; in these patients the risk of infection is proportional to the extent of neutropenia [2].

Neutrophils are short-lived cells and remain in circulation for only hours to a few days [3, 4]. To maintain supply, approximately $10^7$ neutrophils are produced each second, requiring an estimated two-thirds of the bone marrow’s hematopoietic space. This output requires the rapid proliferation of granulocyte precursors [5], making them highly susceptible to cell death in patients being treated with cytotoxic cancer chemotherapy. The reduction in neutrophil number during chemotherapy increases the risk of infection and hospitalization, as well as the risk of reducing the chemotherapy dose and/or delaying the chemotherapy treatment [6]. As a consequence, severe neutropenia can increase the risk of mortality both in the short-term (due to infections) and long-term (due to receiving insufficient intensity of chemotherapy) [7–9].

To address the risk of complications associated with neutropenia in patients receiving myelosuppressive chemotherapy, recombinant methionyl human G-CSF (r-metHuG-CSF; filgrastim) was approved by the US FDA in 1991. Filgrastim decreases the incidence of infection as manifested by febrile neutropenia (FN) in patients with non-myeloid malignancies receiving myelosuppressive chemotherapy. Filgrastim has a relatively short circulating half-life (3.5 h) [10], necessitating daily administration until neutrophil recovery. Furthermore, filgrastim requires bodyweight-based dosing (starting at 5 μg/kg/day) and frequent monitoring of neutrophil counts [11], which increase the challenge of compliance. To enhance the product characteristics, a variety of approaches to develop a long-acting version of filgrastim were considered and tested. By adopting a systematic approach and following a series of rigorous testing procedures, pegfilgrastim emerged as a G-CSF that can significantly reduce the risk of neutropenia and related complications with one administration per chemotherapy cycle [12–14].

Pegfilgrastim has been used in clinical practice since 2002, and its efficacy and safety have been demonstrated in a variety of tumor types and settings [15–18]. With the ongoing development of other long-acting G-CSF molecules and the expected arrival of pegfilgrastim biosimilars in the next few years, there is increased interest in understanding the basis of the structure–function relationship important for developing a long-acting G-CSF. Although the development program of pegfilgrastim has been described elsewhere [19], a detailed overview of the early non-clinical work and the scientific approach taken to select PEGylation as the G-CSF conjugate is lacking. The objective of this review is to describe the rationale, clinical implications, and scientific data of the PEGylation approach used in the development of pegfilgrastim.

2 Filgrastim

Clinical experience with filgrastim extends for more than 20 years, and has been reviewed extensively [1, 20]. The initial approval was based on two randomized clinical studies in patients with lung cancer that demonstrated that filgrastim treatment significantly reduced the duration of severe neutropenia, the number of days of hospitalization, the incidence of antibacterial treatment, and the number of chemotherapy dose reductions [15, 21, 22]. Filgrastim has additionally been approved for use in patients with acute myeloid leukemia, severe congenital neutropenia, AIDS-associated neutropenia, and to speed neutrophil recovery in cancer patients receiving bone marrow transplant. It is also approved for patients undergoing peripheral blood progenitor cell collection.

In patients undergoing chemotherapy, it is recommended that filgrastim dosing start 24 h after the completion of chemotherapy and be continued daily until the absolute neutrophil count (ANC) is $\geq 10 \times 10^9$ cells/L, which, in some cases, may require one injection each day for 10–14 days. To eliminate the need for patients to either return daily to their treatment center or to self-inject, a long-acting form of filgrastim was desired. Strategies to extend the duration of action include prolonged delivery (e.g., slow absorption from a drug reservoir) or half-life extension (i.e., to maintain concentrations in the blood). Filgrastim itself is relatively unstable for extended periods of time at physiologic temperature and pH [23], making a prolonged delivery option less desirable. Therefore, it was preferable to pursue a form of filgrastim with a longer elimination half-life; achievable by modulating the rate of filgrastim clearance. The desired properties of a long-acting filgrastim are summarized in Table 1.

Filgrastim, being a protein, can be cleared by proteolysis; however, its primary clearance pathways are renal- and neutrophil-mediated; for the latter, after binding of filgrastim to the G-CSF receptor (G-CSF-R) on neutrophils and neutrophil precursors, the filgrastim–G-CSF-R complex is internalized and degraded. In neutropenic individuals, neutrophil-mediated clearance is significantly reduced, and renal clearance becomes dominant. Therefore, if renal clearance could be eliminated or significantly reduced while neutrophil-mediated clearance was retained, the drug would remain in circulation during neutropenia and be cleared only when the neutrophils start to recover, effectively resulting in a self-regulating therapeutic. Strategies to reduce renal clearance include making the protein larger, more elongated, or more negatively charged as these attributes make the protein less likely to be filtered by the renal glomeruli [24, 25].

△ Adis
PEGylation of Filgrastim

3.1 Selection of PEGylation to Improve In Vivo Residence Time

Options for increasing protein size include introducing novel glycosylation sites (glycoengineering) or fusing the protein to another protein, such as an antibody or serum albumin, or fusing the protein to a polymer such as polyethylene glycol (PEG). Many of these options were considered and the factors influencing the selection of PEGylation are summarized in Table 2. In particular, there was a desire to maintain the filgrastim core protein and to preserve the means of filgrastim production such that the extensive safety profile could be preserved in a long-acting molecule.
3.2 Physicochemical Properties of Polyethylene Glycol

The PEGylation process involves the covalent attachment of PEG polymers to mature, purified protein. By starting with purified material there would be no need to change the core protein or the protein expression system to accommodate PEGylation. PEG molecules comprise repeating ethylene oxide units. The carbon–carbon and carbon–oxygen bonds are mobile, and there are no additional large or bulky groups on the polymer chain; as a result, the PEG molecules are highly flexible and tend to occupy a large space. Furthermore, because the oxygen interacts strongly with water, each ethylene oxide unit can coordinate 3–7 water molecules [26], creating a dynamic, hydrated polymer that is predicted to form a thick, flexible layer across the surface of the protein. With the addition of this layer, the PEGylated protein occupies a much larger volume than would be predicted based on the combined molecular weight of the protein and polymer alone. This effect becomes more magnified as the size of the PEG molecule increases, such that a 17 kDa PEG molecule has a similar size as a 66 kDa protein and a 50 kDa PEG molecule has a similar size as a 476 kDa protein [27]. This increase in the size of the PEG–protein conjugate increases the in vivo residence time as has been demonstrated for interferon IFN-α-2b conjugated to a panel of PEG molecules with increasing molecular weight [28]. With the addition of PEG moieties of 10, 20, 30 and 45 kDa, the hydrodynamic radius (cumulative radius of the protein and PEG polymer) increased from ~2 nm for unconjugated IFN-α-2b to 5.7, 7.4, 9.1, and 9.6 nm for the 10, 20, 30, and 45 kDa–PEG conjugates. The associated serum half-lives increased from 1.7 h for unconjugated IFN-α-2b to 7.3, 10.5, 19.9, and 23.9 h for the respective PEG conjugates. Similarly, the half-life of PEGylated human growth hormone increased from 1.4 to 6, 15, and 24 h upon conjugation to 2, 5, or 7.5 kDa PEG constructs, respectively [29].

PEG molecules are available in different molecular weight ranges (e.g., 1–100 kDa) and in both linear and branched conformations, thereby enabling generation of a protein conjugate with specific properties. PEG polymers are relatively chemically inert, and have been shown to be safe when used as fusion partners in injectable protein therapeutics [30], in food and healthcare products (toothpaste can be up to 10% PEG [31]), and as excipients in various drug formulations [32]. Additional benefits of PEGylation include reports that it reduces immunogenicity [33], proteolysis [34], and protein aggregation [35]. Because of these advantageous properties, PEGylation was chosen as the means of increasing the in vivo residence time of filgrastim.

3.3 Strategies for PEGylation of Filgrastim

The first FDA-approved PEGylated proteins, Adagen® and Oncaspar®, were heterogeneous multi-PEG mixtures that differed in the number of attached chains and the site of attachment [36]. From our work on a long-acting filgrastim therapeutic, we came to the realization that having a conjugate with a single site of attachment would be preferred as it would be easier to demonstrate consistency and provide proof of quality; however, the techniques and reagents required for such a construct were still in development. One strategy to achieve site-selective addition of a PEG molecule available at the time was to target the free thiol from an unpaired cysteine. G-CSF contains five cysteines; however, four of these are involved in disulfide bonds and would not be available for PEG conjugation. The remaining cysteine (Cys 17) is not disulfide-linked but efforts to attach a PEG molecule to this site resulted in decreased activity. Structural analysis reveals that this cysteine is located near the interior of the protein [37] and attachment of the PEG molecule was likely disrupting the protein fold.

PEGylation of free amino groups is another strategy that was evaluated. Free amino groups in proteins are present in two instances: (1) the ε-amino group from the side chain of a lysine residue; or (2) the α-amino group from the N-terminus of the protein. While there is typically only one N-terminus per protein chain (provided the protein chain is not cleaved to form multiple chains), lysines are frequently found on the surface of proteins. This abundance of accessible amino groups can lead to multiple PEG additions. For example, both Oncaspar® and Adagen® are PEGylated at multiple sites: Oncaspar® is reported to be PEGylated on 22 of its 33 free amino groups [38], and Adagen® is reported to be PEGylated on at least half of its 27 free amino groups [39], although exact figures have not been released by the Adagen® manufacturer.

G-CSF contains five free amino groups including the N-terminus and four lysines at positions 16, 23, 34, and 40. Non-specific PEGylation of free amino groups in G-CSF was an approach pursued by other research groups around the same time as Amgen’s initial work to develop a long-acting filgrastim (Ro 25-8315 and PEG-rHuG-CSF; Table 3); however, none of these other early molecules has gained regulatory approval. In one example, PEG was added to filgrastim both at the four surface-exposed lysine residues and the N-terminal methionine residue [40]. Analysis of this material revealed multiple species of modified proteins with different molecular weights. Another group used a mutated version of G-CSF (nartograstim), in which several amino acids from the recombinant G-CSF have been replaced [41]. PEGylation of this construct also resulted in multiple species of modified G-CSF with different molecular weights.
Table 3  Summary of the characteristics and studies of PEGylated granulocyte colony-stimulating factors

| Name                  | G-CSF backbone | PEGylation site and composition | Non-clinical activity                                                                 | Clinical activity                                                                                           | Stage of development | References |
|-----------------------|----------------|---------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|----------------------|------------|
| Pegfilgrastim         | Filgrastim     | Single linear 20 kDa PEG attached to N-terminus methionine of filgrastim | After IV injection of 5 or 100 μg/kg doses, the plasma AUC values for pegfilgrastim were 300 and 550 % higher, respectively, than filgrastim. PK profiles of pegfilgrastim were similar between sham-operated and nephrectomized rats. Neutrophil counts in mice increased for 5 days by a single dose of pegfilgrastim (1000 μg/kg SC) vs. 1–2 days by single-dose filgrastim (2500 μg/kg SC). Duration of chemotherapy-induced neutropenia was 2 days with a single dose of pegfilgrastim (1000 μg/kg SC) and daily doses of filgrastim (300 μg/kg SC). | Breast cancer: Pegfilgrastim (6 mg), n = 77  
Filgrastim (5 μg/kg/day), n = 75  
FN incidence 13 vs. 20%; p = NS<sup>a</sup>  
Breast cancer: Pegfilgrastim (6 mg), n = 463  
Placebo, n = 465  
FN incidence 1 vs. 17%; p < 0.001 | Approved globally since 2002 | [78] [12] [18] [13] [16] |
| Lipegfilgrastim       | Filgrastim     | Single 20 kDa PEG at Thr 134 (natural glycosylation site of G-CSF) | Plasma elimination half-life in monkeys after single 100 μg/kg SC injection was 10.5 h for lipegfilgrastim vs. 10.6 h for pegfilgrastim. Comparable PDs with pegfilgrastim in a rat model of cyclophosphamide-induced neutropenia and in monkeys | Breast cancer: Lipegfilgrastim (6 mg), n = 101  
Pegfilgrastim (6 mg), n = 101  
FN incidence: 1 vs. 3%; p = NS<sup>a</sup>  
Lung cancer: Lipegfilgrastim 6 mg, n = 250  
Placebo, n = 125  
FN incidence cycle 1: 2.4 vs. 5.6%; p = 0.1151 | Approved in Europe 2013 | [68] [69] |
| Ro 25-8315            | Nartograstim (KW-2228/ND-28) | PEG added to N-terminus and lysine residues: 1–3 units of 20 kDa PEG per G-CSF | Plasma concentration in mice 24 h after injection of 10 μg SC was 110 ng/mL for PEG-ND-28 vs. 6 ng/mL for ND-28. Increased and prolonged neutrophil response in mice with PEG–nartograstim vs. nartograstim; molecules with more PEG units elicited greater neutrophil responses | Breast cancer: Ro 25-8315 (20; 60; 100 μg/kg), n = 9; 9; 10  
Filgrastim (5 μg/kg/day), n = 8  
FN incidence: 0 vs. 11 vs. 10 vs. 0%<sup>c</sup> | Phase I study completed | [41] [44] [79] |
| Empegfilgrastim       | Filgrastim     | Single 30 kDa PEG | Not reported | Breast cancer: BCD-017 (3 mg; 6 mg), n = 21; n = 20  
Filgrastim (5 μg/kg/day), n = 19  
FN incidence: 5 vs. 5 vs. 0%<sup>a</sup> | Phase III study ongoing | [80] |
| Name          | G-CSF backbone            | PEGylation site and composition                                                                 | Non-clinical activity                                                                 | Clinical activity                                                                 | Stage of development   | References |
|---------------|--------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------------------------|------------|
| Maxy-G34      | G-CSF modified with 5 amino acid substitutions: removal of potential PEGylation sites (K16R, K34R, K40R) and addition of new PEGylation sites (T105 K, S159 K) | 3 units of 5 kDa PEG per G-CSF linked to unique amino acid substitutions resulting from amino acid substitutions | In cyclophosphamide-treated rats, pegfilgrastim had higher specific degradation and bioavailability, and lower distribution volume than Maxy-G34. There were no differences in ANC recovery | Breast cancer: Maxy-G34 (10; 30; 45; 60; 100 μg/kg), n = 6; 6; 6; 6; 3 Pegfilgrastim (6 mg), n = 8 FN incidence 2.6 vs. 4.2 %<sup>a</sup> | Phase II completed      | [81, 82] |
| PEG-rHuG-CSF  | Filgrastim               | PEG added to N-terminus and 4 lysine residues Filgrastim is mixed with 4.5 or 10 kDa PEG units to produce species with different molecular weights | Increased neutrophil counts in mice and longer serum half-life in rats for PEGylated vs. filgrastim; greater increase with larger PEG units | Not reported                                                                  | No clinical studies published | [83]       |
| BK0026        | Filgrastim               | Single 20 kDa PEG conjugated to glutamine 135                                                   | Plasma half-life in rats was 7.4 h for BK0026 vs. 8.9 h for pegfilgrastim            | Not reported                                                                  | Phase I study ongoing   | [84]       |

ANC absolute neutrophil count, \( AUC \) area under the concentration–time curve, \( FN \) febrile neutropenia, \( G-CSF \) granulocyte colony-stimulating factor, \( IV \) intravenous, \( NS \) not significant, \( PEG \) polyethylene glycol, \( PEG-rHuG-CSF \) polyethylene glycol recombinant methionyl human granulocyte colony-stimulating factor, \( PD \) pharmacodynamic, \( PK \) pharmacokinetic, \( SC \) subcutaneous

<sup>a</sup> Non-inferiority study with duration of severe neutropenia as the primary endpoint and FN as secondary endpoint

<sup>b</sup> Grade 3/4 neutropenia was the primary endpoint and grade 3/4 FN was a secondary endpoint

<sup>c</sup> Duration of severe neutropenia was the primary efficacy endpoint and FN was a secondary endpoint
While our initial efforts also involved non-specific PEGylation of free amino groups, our results suggested that PEGylation of the lysine residues interfered with receptor binding. Structural studies of the G-CSF/G-CSF-R complex subsequently showed that two of these lysine residues are located within the receptor-binding interface (Fig. 1) [41]. In comparison, PEGylation of the N-terminus did not significantly interfere with receptor binding; thus, for the long-acting filgrastim, we pursued a means of selectively PEGylating the \( \alpha \)-amino group from the N-terminus.

Although the G-CSF/G-CSF-R (domains 1–3) structure was not available when this choice was made, the co-crystal structure has confirmed that the N-terminus is not involved in the G-CSF/G-CSF-R interaction and is pointing away from the ligand/receptor interface [42]. To achieve selective addition of the PEG moiety to the N-terminal \( \alpha \)-amino group and limit reaction with the lysine \( \varepsilon \)-amino groups, it was necessary to identify conditions where the \( \alpha \)-amino group (acid dissociation constant [pKa] = 7.6–8.0 [43]) of the N-terminus was more reactive than the lysine \( \varepsilon \)-amino groups (pKa = 10.0–10.2 [43]). This was achieved by lowering the pH of the reaction and incorporating an optimized reactive PEG construct. In the end, the process that was selected involved reductive alkylation of G-CSF with a PEG aldehyde derivative at pH 5.

### 3.4 Screening and Selection of an Appropriate PEGylated Filgrastim

To select a long-acting filgrastim candidate for therapeutic use, a large panel of PEG–G-CSF conjugates was generated and characterized. The PEG moieties that were evaluated spanned a range of available molecular weight polymers (12–30 kDa) and comprised both linear and branched conformations. The extent of reaction was monitored by high-performance liquid chromatography (HPLC) to ensure a high percentage of conjugation, followed by purification, and verification of the site of addition using endoproteinase peptide mapping and additional physicochemical characterization [ultracentrifugation, MALDI (matrix-assisted laser desorption/ionization) TOF (time-of-flight) mass spectrometry, and size exclusion HPLC with associated in-line light scattering]. This characterization ensured appropriate composition, stoichiometry, and structure of the conjugates. More than 25 constructs were evaluated for in vitro and in vivo activity; the in vitro activity score was derived from results in a cell-based proliferation assay using cells stably expressing the G-CSF-R and the in vivo activity score was based on the area under the concentration–time curve (AUC) of the ANC in healthy animals.

As shown in Fig. 2, there was a positive relationship between the molecular weight of the added PEG and the in vivo activity of the modified filgrastim. Similar results were obtained by other researchers with PEGylated filgrastim or nartograstim [41, 44], whereby white blood cell counts in mice were found to increase as the size or number of PEG units was increased [55]. In those studies, like our own, it was also observed that molecules with increased in vivo activity demonstrated decreased in vitro activity. In general, these characteristics became more magnified as the molecular weight of the PEG molecule increased. The decreased in vitro activity is likely a consequence of the PEG molecule interfering directly and/or indirectly with G-CSF binding to its receptor. This is likely a consequence of the PEG molecule’s bulk and mobility which could cause periodic occlusion of the binding interface, slowing the on-rate of binding. A similar relationship between enhanced in vivo activity and decreased in vitro activity has been observed for PEG conjugates of human growth hormone [29] and asiolofetuin [45]. The result of our studies

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![Fig. 1](image-url) The N-terminus of granulocyte colony-stimulating factor (G-CSF) is distal from the receptor binding site while lysine residues 16 and 23 are located in the receptor binding site. Left panel the G-CSF-G-CSF receptor (G-CSF-R) signaling complex [42] comprises two G-CSF (green) and two G-CSF-R molecules (light and dark blue). The atoms of the G-CSF N-terminus are marked with yellow balls. The atoms of lysines 16 and 23 are marked with pink balls. Right panel a close-up of the interface providing more detail regarding the location of lysines 16 and 23.
was the selection of an r-metHuG-CSF–PEG conjugate containing a single linear 20 kDa PEG attached to the N-terminal methionine. This construct would become pegfilgrastim.

4 Pegfilgrastim

4.1 Non-Clinical Evaluation of Pegfilgrastim Activity

The in vivo activity of pegfilgrastim compared to filgrastim was evaluated in both normal and neutropenic mice by subcutaneous (SC) injection. ANC is commonly used as a pharmacodynamic marker because it is straightforward to measure and represents the target cell for eliciting clinical benefit in neutropenic patients. In normal mice a single dose of pegfilgrastim (1000 μg/kg SC) increased the ANC for 5 days, whereas a single injection of filgrastim at a higher dose (2500 μg/kg SC) increased the ANC for only 1–2 days (Fig. 3a). In a model of chemotherapy-induced neutropenia, control mice that received neither filgrastim nor pegfilgrastim experienced 7 days of neutropenia, whereas mice treated with either daily filgrastim (300 μg/kg SC, days 2–11) or a single dose of pegfilgrastim (1000 μg/kg SC) experienced only 2 days of neutropenia (Fig. 3b). These data demonstrated that a single injection of pegfilgrastim was as effective in stimulating neutrophil recovery as multiple daily injections of filgrastim.

Pegfilgrastim exhibited an acceptable non-clinical safety profile in short- and long-term studies across a range of species—including rats, rabbits, and Cynomolgus monkeys—using delivery by SC and intravenous routes. By retaining filgrastim as the core of the molecule, a predictable safety profile was possible because pegfilgrastim, like endogenous G-CSF, is specific for only one receptor. Furthermore, this safety profile shows that addition of the PEG molecule to filgrastim did not cause significant qualitative changes in the filgrastim pharmacology/safety profile and no off-target toxicities were identified.
4.2 Pharmacokinetics of Pegfilgrastim

The pharmacokinetics of pegfilgrastim have been described in recent review articles [46, 47] and are summarized briefly here. The prolonged in vivo activity of pegfilgrastim compared with filgrastim can be explained by the nearly negligible role played by the kidneys in pegfilgrastim clearance. The reduction in renal elimination was demonstrated in bilaterally nephrectomized rats. In this model, clearance of filgrastim 100 μg/kg was reduced by 75% in nephrectomized compared with sham-operated rats. By comparison, clearance of pegfilgrastim 100 μg/kg was similar in both the nephrectomized and sham-operated rats (Fig. 4a) [48]. This clearly demonstrated that renal clearance had been greatly reduced. To evaluate the role of the kidney in pegfilgrastim clearance in humans, the pharmacokinetic profile for pegfilgrastim was evaluated in subjects with varying degrees of renal function ranging from normal to mildly, moderately, and severely impaired as well as subjects with end-stage renal disease [49]. Across all groups, the pharmacokinetic profile of pegfilgrastim was similar (Fig. 4b), supporting the non-clinical observations that the kidney contribution to clearance is negligible.

Addition of the 20 kDa PEG molecule increases both the molecular weight and the size of filgrastim. The molecular weight increases from 19 kDa (filgrastim) to 39 kDa (pegfilgrastim). This is still below the 60–70 kDa molecular weight that is considered to be required to avoid glomerular filtration and subsequent renal elimination. The hydrodynamic radius, however, increases approximately 2.5- to 3-fold (calculated using equations in Fee and Van Alstine [50]). This increases the size of filgrastim from ~4 nm to ~6 nm in diameter. In general, proteins ≥6 nm in diameter (e.g., hemoglobin 6.4 nm and albumin 7 nm) avoid glomerular filtration [25, 51]. With the reduction in renal clearance, the primary means of pegfilgrastim removal from the circulation is by neutrophil-mediated clearance. Neutrophils and neutrophil precursors express G-CSF-R. This receptor binds pegfilgrastim and the drug–receptor complex is internalized and degraded inside the cell. Support for this hypothesis came from a comparison of pegfilgrastim clearance in wild-type versus G-CSF-R-deficient mice. In wild-type mice, serum concentrations of pegfilgrastim remained elevated for less than 48 h following injection of pegfilgrastim 10 μg/kg, whereas serum concentrations of pegfilgrastim remained elevated for 144 h in G-CSF-R knockout mice [52].

Another consequence of the G-CSF-R/pegfilgrastim interaction is the stimulation of neutrophil precursor proliferation and differentiation. Figure 5 demonstrates the relationship between pegfilgrastim serum concentrations and circulating neutrophils in breast cancer patients treated with doxorubicin (60 mg/m^2) and docetaxel (75 mg/m^2), followed by a single injection of pegfilgrastim (100 μg/kg).
1 day later [14]. After SC administration of pegfilgrastim, serum concentrations of pegfilgrastim were sustained during neutropenia. ANC levels started to increase after the nadir on day 7, resulting in a rapid decline of serum concentrations of pegfilgrastim due to neutrophil-mediated clearance. Thus, during chemotherapy-induced neutropenia, circulating pegfilgrastim concentrations remain elevated until there is an increase in the ANC, which leads to increased pegfilgrastim consumption and self-regulated clearance. In comparison, serum concentrations of filgrastim were rapidly cleared after each injection, and multiple daily injections of filgrastim were required to maintain its clinical efficacy [14]. A semi-mechanistic model that includes neutrophil-mediated clearance adequately describes the relationship between pegfilgrastim serum concentrations and the ANC profile in healthy subjects [53] and in patients with non-small-cell lung cancer [54].

4.3 Clinical Efficacy of Pegfilgrastim

Pegfilgrastim is indicated to reduce the risk of FN in patients with non-myeloid malignancies receiving myelosuppressive chemotherapy. Approval of pegfilgrastim for use in patients with chemotherapy-induced neutropenia was based on results from two randomized, double-blind phase III studies in patients with breast cancer, designed to test the non-inferiority of pegfilgrastim versus filgrastim [46, 47]. Similar results were observed in both trials, demonstrating that a single fixed dose of pegfilgrastim per chemotherapy cycle was at least equivalent to daily doses of filgrastim in all efficacy endpoints including the duration of severe neutropenia (ANC <0.5 × 10^9/L), the depth of the ANC nadir in all cycles, the incidence of FN (neutropenia plus fever/infection), and rates of hospitalization and infection.

Subsequent clinical studies have shown pegfilgrastim to be similarly effective across a range of regimens and tumor types [15, 16, 55, 56]. Meta-analyses of clinical studies have shown that pegfilgrastim reduces the risk of FN to a significantly greater extent than filgrastim [57, 58] and that pegfilgrastim also reduces the incidence of early mortality during chemotherapy [57, 59]. Observational studies, which include a broader range of patient populations than those eligible for clinical trials, indicate that pegfilgrastim is similarly effective in reducing FN risk in clinical practice [60, 61].

Unlike filgrastim, pegfilgrastim is not approved for stem cell mobilization, but clinical studies have shown that it is at least as effective as daily G-CSFs in mobilization of peripheral blood stem/progenitor cells and engraftment in the autologous and allogeneic setting [62–65].

4.4 Clinical Safety of Pegfilgrastim

Clinical safety data derived from the various clinical trials show that the safety profile of pegfilgrastim is comparable to that of filgrastim in terms of the total incidence of adverse events, incidence of adverse events attributed to study drug, and withdrawals due to adverse events. As with all G-CSFs, the most common adverse effect is bone pain, which is generally mild to moderate and manageable with non-narcotic analgesics. A patient-level analysis across multiple studies found that bone pain was experienced by more patients treated with pegfilgrastim than placebo, but that incidence of bone pain was similar between the pegfilgrastim- and filgrastim-treated groups [66]. Serious adverse reactions occur more rarely, and include splenic rupture, acute respiratory distress syndrome, allergic reactions, and potential for tumor growth stimulation effects on malignant cells [67]. As with all protein biologics, the potential for immunogenicity also exists, but neutralizing antibodies to pegfilgrastim have not been reported in clinical studies.

5 Future Perspectives

Pegfilgrastim was the only commercially available long-acting G-CSF for more than 10 years, until the European approval of lipegfilgrastim in 2013 through the demonstration of clinical non-inferiority to pegfilgrastim in a randomized phase III study in patients with breast cancer [68]. Lipegfilgrastim is PEGylated on the O-linked glycosylation site at Thr-134; however, because this site is heterogeneously glycosylated when G-CSF is expressed in mammalian cells, the polysaccharide is added to the G-CSF protein core enzymatically using two recombinant glycosyltransferase enzymes and activated sugar nucleotide donor substrates in vitro. Subsequently, the PEG molecule is covalently attached to the polysaccharide [69].

Other PEGylated G-CSFs have been tested (Table 3), but few examples of approaches to extend the half-life of G-CSF exist outside of PEGylation. The most clinically advanced non-PEGylated molecules are benefilgrastim, which is a recombinant fusion protein of human Fc and G-CSF produced in mammalian cells [70] and balugrastim, which is a recombinant fusion protein of human serum albumin and G-CSF produced in yeast [71]. Benefilgrastim, which comprises two chains of human G-CSF-Fc, has recently been evaluated in a phase II study in patients with breast cancer [72]. In this study, benefilgrastim administered once per cycle at 240 or 320 μg/kg SC provided neutrophil support with a safety profile similar to that of pegfilgrastim during multiple chemotherapy cycles.
Balugrastim has demonstrated non-inferiority to pegfilgrastim in a phase III study in patients with breast cancer, but it has not been approved for commercial use in either the USA or Europe. Since these molecules are administered in an identical manner to pegfilgrastim (injected once per chemotherapy cycle) and have not demonstrated superior clinical efficacy or safety, they may be better characterized as alternatives to pegfilgrastim rather than therapeutic advances.

Innovations in drug delivery provide additional possibilities for therapeutic improvements. Pegfilgrastim should be administered the day after chemotherapy [11] to avoid the risk of stimulating proliferation of myeloid progenitor cells in the presence of cytotoxic chemotherapy agents and worsening neutropenia outcomes [17]. Nonetheless, same-day administration of pegfilgrastim is observed in clinical practice [73], most likely due reasons of logistics or convenience. To overcome this problem, an on-body injector has been developed—a programmable delivery device attached to the patient’s skin to deliver pegfilgrastim the day after chemotherapy—that eliminates the requirement for a patient to return to the clinic to receive pegfilgrastim [74]. This device, which was recently approved by the FDA (tradename OnPRO), may therefore increase patient compliance and increase the efficacy of pegfilgrastim in clinical practice.

Although not necessarily long-acting, small molecules capable of stimulating the G-CSF-R signaling pathway can provide the option of orally administered G-CSF, which may appeal to some patients. Several groups are pursuing this approach and have demonstrated in vitro and in vivo activity in preclinical studies [75–77], although currently they are in the early stages of clinical development. Clearly more work is needed in this area, including studies in human subjects and clinical testing against the available protein-based G-CSF products.

6 Conclusion

Filgrastim is a widely used short-acting G-CSF with a long-standing record of clinical efficacy and safety. To eliminate the need for daily injections and to maintain a similar safety profile, a long-acting form of filgrastim was designed that retained the core protein with additions to increase the molecular size and extend the circulating half-life. PEGylation was considered useful for therapeutic proteins: it is non-toxic; it can improve properties related to immunogenicity, stability, and aggregation; and it can enhance the pharmacokinetic/pharmacodynamic profiles as a conjugation partner. In addition, technology has been developed so that it can be added to specific sites on the protein to limit the potential for interference with receptor binding, and it is possible generate PEG moieties with a range of sizes to enable selection of a modified protein with optimal pharmacokinetic/pharmacodynamic profiles. Pegfilgrastim contains a single linear 20 kDa PEG attached to the N-terminal amino acid of filgrastim, and the pharmacokinetic profile has been sufficiently improved by significantly reducing the renal clearance. As such, a single dose of pegfilgrastim effectively reduces the risk of neutropenia and neutropenia-associated complications for an entire chemotherapy cycle.

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