Scientific paper

The Characterization and Potential use of G-CSF Dimers and their Pegylated Conjugates

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Abstract

G-CSF successfully prevents chemotherapy-induced neutropenia. Two second-generation drugs with improved therapeutic properties are already available and the development of new forms is still ongoing. For an efficient receptor dimerization two G-CSF molecules have to bind. Development of G-CSF dimers acting as receptor dimerizers was explored and their potential use evaluated. The *in vitro* biological activities of the prepared dimers were lower than G-CSF monomer activity, presumably due to non-optimal spatial orientation of the molecules. Most likely two dimers had to bind to trigger receptor dimerization instead of one dimer acting as a dimerizer. Although significantly lower in the residual *in vitro* biological activity, the diPEG-F_{dim} conjugate exhibited pharmacokinetical (PK) and pharmacodynamical (PD) properties comparable to pegfilgrastim or even better. An interesting PD profile with the second maximum in absolute neutrophil count (ANC) and a balanced elevated ANC profile over the longer time interval was namely observed.

Keywords: G-CSF, pegylation, protein dimerizers, HPLC, biological activity, PK PD profile

1. Introduction

Chemotherapeutics, with the exception of target-specific monoclonal antibodies, act non-selectively upon cells with a high replication rate causing myelosuppression as an adverse side effect.[1] The resulting drop in neutrophil count is accompanied by the risk of severe infections and it used to be the major dose-limiting factor of systemic chemotherapy.[2] For the past two decades the chemotherapy-induced neutropenia is successfully prevented by the use of granulocyte colony-stimulating factor (G-CSF).[3] As a haematopoietic regulator of the neutrophil lineage, G-CSF controls differentiation of precursor cells to neutrophils, their proliferation, and stimulates the release of mature neutrophils from the bone marrow.[4,5] Native human G-CSF is a glycoprotein with a short glycane chain attached to Thr133 (O-glycosylation).[6] Its typical cytokine structure, a four helix-bundle, is stabilized by two disulphide bridges. It contains an additional, non-paired, partially solvent exposed cysteine.[7, 8] The main clearance pathways are renal clearance and a saturable self-regulating mechanism with neutrophils.[9, 10]

For medical treatment four variants have been developed so far.[3, 11] Filgrastim and lenograstim, both recombinant human G-CSFs, are first generation biopharmaceuticals with equivalent efficacy.[12, 13] The improved second generation therapeutic forms, nartograstim (KW-2228) and pegfilgrastim, followed. In nartograstim the amino acid sequence manipulation resulted in increased granulopoetic activity.[14–17] Protein modification of filgrastim by pegylation increased the hydrodynamic radius to the degree where renal clearance is substantially reduced.[18] Although resulting in reduced *in vitro* biological activity, the significantly prolonged elimination half-life *in vivo* allows a more sustained blood level of G-CSF and less frequent dosing: once per chemotherapy cycle for pegfilgrastim vs. daily dosage for filgrastim.[19–21]

Development of new forms of G-CSF with enhanced therapeutic properties is still ongoing. It is mainly directed into the prolongation of the elimination half-life *in vivo* by increasing the molecule's hydrodynamic radius (pegylation, glycopegylation, HESylation, conjugation with the polysialic acid and protein fusion with IgG-Fc) or developing depot formulations enabling gradual release.[3, 22–25] Recombinant G-CSF MAXY-G34 was developed by site-specific attachment of multiple PEG chains. Preclinical data suggested superior properties to pegfilgrastim regarding the reduction in the duration of neutropenia.[3]

Generally, cytokine signalization pathways are induced by a single cytokine molecule binding to its respective transmembrane receptor molecule, triggering the receptor dimerization and initiating response. It is presumed that G-CSF, when bound to the G-CSF receptor (G-CSFR) on the cell surface, has the 2:2 stoichiometry (Figure 1), meaning that binding of two G-CSF molecules is needed for receptor homodimerization and subsequent signal transduction. Several authors experimentally confirmed this 2:2 G-CSF:G-CSFR complex. Early studies suggested that G-CSF is a monovalent ligand and that receptor dimerization occurs through receptor-receptor interactions. Two receptor binding sites were later discovered and reported as both interacting with a single receptor, inducing a conformation allowing stronger receptor-receptor interactions. More recent evidence shows that G-CSF first binds to the receptor through its primary binding site, resulting in conformational changes in both G-CSF and G-CSFR and enabling binding of a second receptor molecule to the secondary binding site on G-CSF.[26–30]

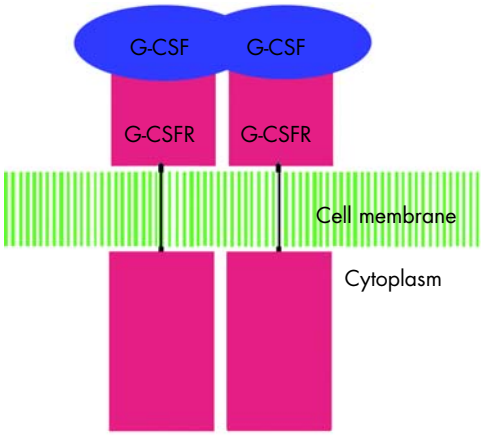

Figure 1: The 2:2 stoichiomerty of the G-CSF:G-CSFR complex.

An alternative approach to develop new forms of therapeutic molecules could lie in using protein dimerizers, molecules that, similarly to the original molecule, trigger receptor dimerization. The most well known are antibodies. Cell proliferation of a prolactin-dependent cell line was demonstrated using antibodies to the prolactin receptor.[31] Also small molecules can act as protein dimerizers. Tian et. al. reported the discovery of a small molecule SB247464 and its ability of activating the G-CSF receptor by mimicking the action of G-CSF.[32] Reviews on protein dimerization are given in Austin, 1994 and Clemons, 1999.[33, 34]

G-CSF dimers acting as protein dimerizers could represent an interesting option for the design of further improved therapeutic forms of G-CSF. In order to test this hypothesis we investigated several G-CSF dimers connected via disulphide bridges between non-paired cysteines of two G-CSF molecules. A G-CSF dimer spontaneously occurring during the G-CSF purification process and two variants of PEGylated G-CSF dimers were prepared: a dipegylated spontaneously occurring dimer obtained by dimer PEGylation and a dipegylated G-CSF dimer obtained by monomer pegylation and subsequent dimerization.

This paper presents the preparation and characterization of different G-CSF dimers and their pegylated conjugates and the possibility of obtaining a dimer with enhanced therapeutic properties. Taking into account the 2:2 stoichiometry of G-CSF receptor binding, the dimer might activate the receptor with higher efficiency (dimer:G-CSFR = 1:2). Therapeutic potential of the molecules was evaluated with the following *in vitro* and *in vivo* assays: *in vitro* biological activity and PK/PD studies in rats.

2. Experimental

Filgrastim (G-CSF) was prepared in *Escherichia coli (E. coli)* as described earlier.[35–37] **Pegfilgrastim** (G-CSF with a 20 kDa polyethylene glycol chain attached to the N-terminus) was purchased from Amgen (Neulasta). $\mathbf{F_{dim}}$ (*E. coli* produced G-CSF dimer) forms spontaneously during the filgrastim preparation process and can be isolated as a by-product in chromatographic purification.[38, 39]

2. 1. Pegylation

G-CSF and F_{dim} were N-terminally pegylated with the 10 kDa PEG-CHO reagent (NOF, Japan) as described by Kinstler et. al.[19] A 5-fold molar excess of PEG reagent and an appropriate volume of 1 M $NaCNBH_3$ solution to the final concentration of 20 mM were added to the protein solution of hG-CSF at 3–4 mg/ml, pH 5. The pegylation reaction took place at +2–8 °C for about 24 hours, with gentle stirring and was terminated by the application of the reaction mixture to the cation exchange column (isolation step).

2. 2. Isolation of Pegylated Conjugates

FN10L (monopegylated G-CSF) and **diPEG-$\mathbf{F_{dim}}$** (F_{dim} pegylated at both N-termini) conjugates were isola-

ted using cation exchange chromatography procedure as described in Kusterle et. al.: TSKgel SP-5PW with 25 mM acetic acid pH 3.8 as a loading buffer and 75 mM acetic acid pH 8.0 as an elution buffer.[40]

2. 3. Dimerization

FN10L was dimerized using copper ions for the oxidation of free cysteines. All chemicals were purchased at Sigma (USA). The oxidation process was performed under slightly denaturing conditions to expose the unpaired cysteines. Reaction conditions were 40 μM $CuSO_4$, 0.02% SDS, pH 8.0, protein concentration 0.42 mg/mL. FN10L was left to dimerize for 24 h at 4 °C with gentle mixing. Afterwards SDS was removed with Dowex ion exchange resin. 2.5 g of Dowex per 0.2 g SDS was added directly into the dimerization mixture and the mixture was left at room temperature and stirring at 220 rpm for 90 min. Dowex was removed by centrifugation at 3220 rcf for 10 min. The obtained **FN10L$_{dim}$** was isolated as described below.

2. 4. Isolation of *in vitro* Prepared Dimer

The *in vitro* prepared dimer FN10L$_{dim}$ was isolated on the chromatographic system AKTApurifier (Amersham Biosciences) using cation exchange chromatography on TSKgel SP-5PW (Tosoh Bioscience, Japan) with 50 mM CH_3COOH pH 4.5 as a loading buffer and 50 mM CH_3COOH, 1 M NaCl pH 4.5 as an elution buffer in 15 column volumes. All chemicals were purchased at Sigma (USA), buffer exchange of the purified conjugate was performed on Amicon Ultracel 10k (Millipore, USA).

An overview of the conjugates and their properties is summarized in Table 1.

2. 5. SDS-PAGE

Pegylation efficiency and isolations were monitored with non-reducing SDS – polyacrylamide gel electrophoresis (SDS-PAGE). For the characterization of the final samples non-reducing SDS-PAGE and reducing SDS-PAGE analyses were performed using 4–12% gradient gels on NuPage Novex Bis-Tris SDS-PAGE (Invitrogen, USA) system. All chemicals were purchased at Invitrogen. Samples were mixed with SDS-PAGE loading buffer and, in case of reducing SDS-PAGE, Sample Reducing Agent. All samples were denatured at 70 °C for 10 minutes. 2 g of each sample was loaded onto the gel. Novex Sharp was used as molecular weight protein marker. The gels were run for 40 min at 200 V in NuPAGE MES SDS running buffer and dyed with protein-specific Simply Blue Safe Stain.

2. 6. HPLC Chromatography

HPLC analyses were performed on Agilent 1200 system. Pegylation and isolation/purification efficiency was monitored with reversed phase chromatography (RP-HPLC). Analytical characterization of final samples was performed with RP-HPLC and size exclusion chromatography (SE-HPLC). RP-HPLC was carried out on YMC-ODS-AQ, 4.6 mm × 15 cm column (YMC, Japan) with mobile phase A (MPA) 10% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA), mobile phase B (MPB) 90% ACN, 0.1% TFA and a linear gradient from 46% – 70% MPB in 30 min, at 1 mL/min, 65 °C. Size exclusion chromatography was performed on TSK-gel G3000$_{SWXL}$ 7.8 mm × 30 cm column (Tosoh Bioscience, Japan), using isocratic elution with 20 mM Na-phosphate, 150 mM NaCl pH 6.8 + 2% ACN, 1% diethyleneglycol at 0.7 mL/min, 30 °C. Acetonitrile and diethyleneglycol were purchased at Merck (Germany). All other chemicals were purchased at Sigma (USA).

2. 7. *In vitro* Biological Activity

In vitro biological activity measurements are based on the stimulation of cell proliferation of mouse myeloide leucemic cell line M–NFS–60.[41] Briefly, cells were seeded on microtitre plates and serial dilutions of WHO reference standard (NIBSC, 88/502) and test sample were added into the culture medium. Cells were incubated for 48 hours. After incubation the MTT reagent (3–[4,5–methylthiazol–2yl]–2,5–diphenyl tetrazolium bromide) was added. During the incubation the mytochondrial dehydrogenase of live cells reduces the MTT reagent into insoluble

Table 1: An overview of dimeric G-CSF in comparison to standards Filgrastim and Pegfilgrastim

	Protein / Conjugate	Composition	MW protein	MW PEG	Total MW
1	Filgrastim (G-CSF)	G	18.8 kDa	/	18.8 kDa
2	Pegfilgrastim	20PEG–G	18.8 kDa	1 × 20 kDa	38.8 kDa
3	FN10L	10PEG–G	18.8 kDa	1 × 10 kDa	28.8 kDa
4	FN10L$_{dim}$	10PEG –G-G–10PEG	37.6 kDa	2 × 10 kDa	57.6 kDa
5	F$_{dim}$	G-G	37.6 kDa	/	37.6 kDa
6	diPEG-F$_{dim}$	10PEG –G-G–10PEG	37.6 kDa	2 × 10 kDa	57.6 kDa

Legend: G: G-CSF molecule, G-G: G-CSF dimer, 10 PEG: 10 kDa PEG chain, 20 PEG: 20 kDa PEG chain.

formazane crystals. The crystals are then solubilized and the absorbance at 570 nm is measured. The absorbance values represent a natural logarithm of the cell concentration. The test sample's activity is determined by comparing the dilutions of reference standard and test sample at which 50% of maximal proliferation stimulation is achieved.

2. 8. Pharmacokinetic and Pharmacodynamic (PK/PD) Studies in Rats

All rats received care in compliance with the European Convention for the Protection of Vertebrate Animals and European Directive 86/609/EEC. The study was approved by the Veterinary Administration of the Republic of Slovenia. Four animals were included in each study. Fifty (50) μg of the samples were injected subcutaneously (250 μg/kg) and serum samples were taken at appropriate time intervals. The content of G-CSF dimers or their pegylated conjugates in serum at different time points was measured using the ELISA test. The number of leukocytes was determined with the haematological analyzer ABC Vet (ABX Diagnostics, France) and the percentage of neutrophil count was determined manually under the light microscope. The absolute neutrophil count (ANC) was calculated by multiplying the total leukocyte count and the percentage of neutrophils.[42] The obtained results represent PK/PD profiles of the tested samples.

Serum concentrations of samples were determined using the Human G-CSF ELISA (IBL International, cat. # JP27131). The assay was performed following the manufacturer's instructions. In brief, 100 μL of standards, serum samples and blanks in the appropriate dilutions were incubated on plates for 1 hour at 37 °C. Plates were washed and 100 μL of detection antibody-HRP conjugate was added. Plates were incubated for 30 min at 37 °C. After washing, substrate was added on plates and plates were incubated for 30 min at room temperature. Reaction was stopped and absorbance read at 450 nm. The serum concentration of each conjugate was calculated from the standard curve, which was generated using the same conjugate as it was investigated in the rats. Thus we avoided an incorrect concentration determination of pegylated G-CSF, which would result from the lower ELISA sensitivity for pegylated proteins.

3. Results and Discussion

3. 1. Analytical Characterization of G-CSF Dimers and their Pegylated Forms

The dimers and their pegylated forms were prepared as described under Experimental and characterised by electrophoretic and chromatographic methods, always in comparison to G-CSF and pegfilgrastim. They originated either from spontaneously formed natural dimer F_{dim} or from artificial dimerization of pegylated G-CSF monomers, all disulphide-linked via Cys18 residues.

SDS-PAGE analyses (Figures 2a-2b) were performed under non-reducing and reducing conditions with protein-specific staining. On the non-reducing SDS-PAGE the electrophoretic mobility of the samples corresponds to their molecular weight and mobility characteristics (Figure 2, above), whereas the reducing SDS-PAGE (Figure 2, below) reveals the reducible nature of dimers and their conjugates. As expected, the electrophoretic mobility and reducibility of diPEG-F_{dim} (Lane 8) corresponds to the electrophoretic behaviour of $FN10L_{dim}$ (Lane 5).

The SE-HPLC elution order (Figure 3) corresponds to the molecules' hydrodynamic radius, which is, in case of non-pegylated and pegylated molecules, not directly correlated to the molecules' molecular weight: $FN10L_{dim}$

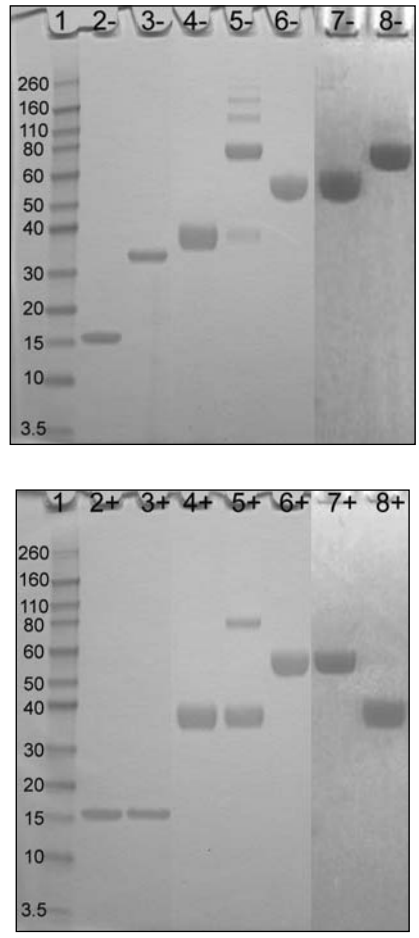

Figure 2: SDS-PAGE analysis of G-CSF dimers and their pegylated forms

Lane 1: MW protein marker [kDa], Lane 2: G-CSF, Lane 3: F_{dim}, Lane 4: FN10L, Lane 5: $FN10L_{dim}$, Lanes 6 and 7: pegfilgrastim, Lane 8: diPEG-F_{dim}. The non-reduced and reduced samples are annotated as "–" and "+", respectively.

exhibits the biggest hydrodynamic radius and thus elutes first. Pegfilgrastim, FN10L, F_{dim} and G-CSF follow. The SE-HPLC elution profile of diPEG-F_{dim} corresponds to the elution of $FN10L_{dim}$ (data not shown).

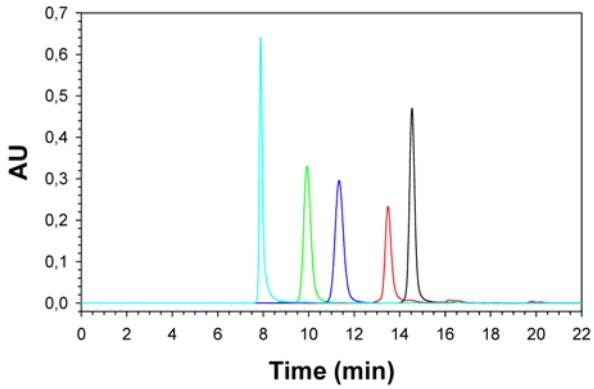

Figure 3: SE-HPLC analysis of G-CSF dimers and their pegylated forms: $FN10L_{dim}$ (cyan), pegfilgrastim (green), FN10L (blue), Fdim (red) and G-CSF (black).

RP-HPLC (Figure 4) reveals increasing PEG-related hydrophobicity. G-CSF as the most hydrophilic compound has the shortest retention time. FN10L (blue) is shifted towards longer retention times due to the attachment of the 10 kDa PEG chain. Pegfilgrastim (green) with the 20 kDa PEG chain attached follows. $FN10L_{dim}$ (cyan) elutes after pegfilgrastim. Hydrophobicity of F_{dim} (red) is significantly increased compared to G-CSF. After pegylation the diPEG-F_{dim} retention on the column is stronger compared to the unpegylated original molecule. The RP-HPLC purity of the samples is given in Table 2.

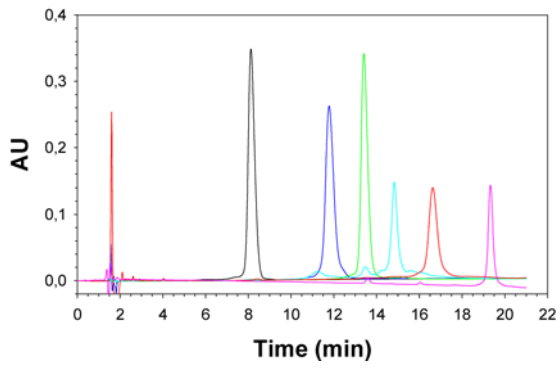

Figure 4: RP-HPLC analysis of G-CSF dimers and their pegylated forms: G-CSF (black), FN10L (blue), pegfilgrastim (green), $FN10L_{dim}$ (cyan), Fdim (red) and diPEG-F_{dim} (pink).

Although disulphide bonding between two G-CSF molecules can happen with different orientation of the molecules (Figure 5), theoretically both pathways, spontaneous and forced dimerization, could produce conjugates with the same properties. *E.g.*, diPEG-F_{dim} and $FN10L_{dim}$ are both G-CSF dimers with a 10 kDa PEG attached N-terminally to each G-CSF subunit, the first one derived from pegylation of the natural dimer and the second one obtained by artificial dimerization of the pegylated G-CSF monomer.

Table 2: RP-HPLC purity of the samples

	Protein / conjugate	Purity (area %)	Residual G-CSF (area %)
1	G-CSF	97,1	
2	FN10L	98,9	0,1
3	Pegfilgrastim	96,6	0,1
4	$FN10L_{dim}$	58,5*	0,6
5	F_{dim}	90,7	0,7
6	diPEG-F_{dim}	97,6	0,0

* $FN10L_{dim}$ main peak, the conjugate is not homogenous on RP-HPLC.

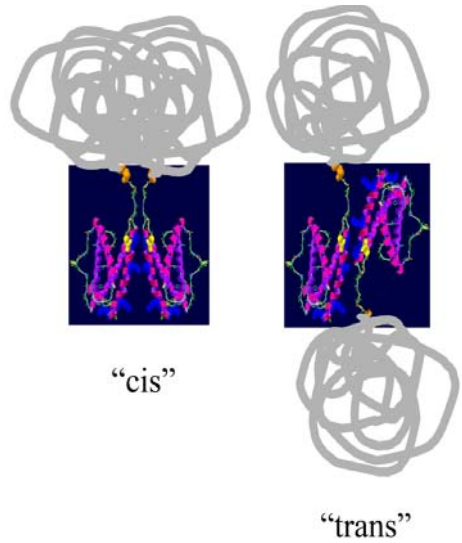

Figure 5: Possible orientations of G-CSF dimers and their pegylated conjugates. (G-CSF structure from Protein Data Bank using PDB Viewer).

On SDS-PAGE (Figure 2) these two conjugates do exhibit the same properties, which are independent of the preparation pathway: molecular weight and reducibility. Also on size-exclusion chromatography, where the elution is based on the hydrodynamic radius of the molecules, there are no differences regarding the nature of natural or artificial dimers. However, RP-HPLC analysis revealed that diPEG-F_{dim} and $FN10L_{dim}$ conjugates, with comparable molecular weight, hydrodynamic radius and reducibility, exhibit significant differences in hydrophobicity, resulting into the following RP-HPLC elution order of the

molecules: G-CSF < FN10L < pegfilgrastim < $FN10L_{dim}$ < F_{dim} < diPEG-F_{dim}. Obviously, the pegylated dimers di-PEG-F_{dim} and $FN10L_{dim}$ are not identical and possible differences will be discussed below.

3. 2. Biological Activity and PK/PD Studies

The eventual therapeutic potential of G-CSF dimers and their pegylated forms was characterized by *in vitro* and *in vivo* tests, always in comparison to G-CSF and pegfilgrastim.

Generally, the *in vitro* biological activity is reduced for pegylated molecules.[43, 44] The reason is steric hindrance for receptor binding due to the PEG attachment. FN10L with 10 kDa PEG chain retained 70% and pegfilgrastim with 20 kDa PEG chain retained 45% of G-CSF monomer initial activity, which is attributed as 100% (Figure 6, Table 3). The results confirmed the negative correlation of PEG chain length with residual biological activity.[45] Negative correlation was also observed for other protein therapeutics, such as interferon-α.[44]

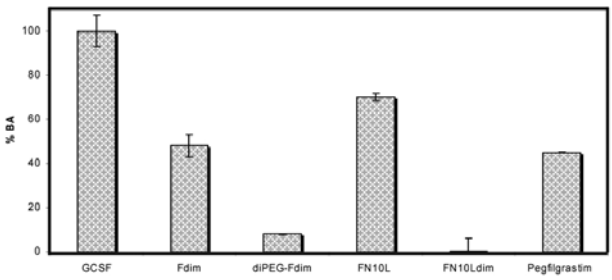

Figure 6: Residual in vitro biological activity

The capability of stimulating cell proliferation in the *in vitro* assay was reduced for all dimer molecules. The activity of F_{dim}, the non-pegylated natural dimer, was reduced to 48% and after pegylation to 8% (diPEG-F_{dim}) of initial activity. The activity of $FN10L_{dim}$ was diminished to almost 0% (Figure 6, Table 3).

As discussed above, the diPEG-F_{dim} and $FN10L_{dim}$ conjugates theoretically possess the same structural properties but differ in hydrophobicity. It is possible that the preparation of the artificial dimer resulted in the impairment of the molecule: after oxidation under mildly denaturing conditions the molecules may not have regained their complete native structure. Analytical characterization somehow supports this hypothesis. On RP-HPLC the $FN10L_{dim}$ conjugate is heterogeneous regarding hydrophobicity (Figure 4) in spite of its homogeneity on SE-HPLC (Figure 3).

Another reason may lie in different dimer conformation and / or orientation leading to unproductive receptor binding. As evident from the crystal structure of G-CSF/G-CSFR complex, the orientation of both monomers in G-CSF dimer plays a significant role in its ability to bind to the receptor molecules.[28] In our experiments, filgrastim is produced with a recombinant *E. coli* strain and F_{dim} is most probably formed during the cell disruption and further purification steps. During this process dimers in several orientations may form and it was observed that only a small amount of the dimers (F_{dim}) was retained on the IMAC column and subsequently isolated.[38] Approximately 50% *in vitro* biological activity might indicate that either only one G-CSF molecule in the dimer or half of the dimeric G-CSF molecules are capable of receptor binding. If the spontaneous dimerization led to cis and trans orientation with equal probability, after pegylation the diPEG-F_{dim} would also retain the same proportion of cis and trans oriented dimers. On the other hand, forced dimerization of the FN10L bearing a bulky PEG chain might favour the trans orientation due to steric hindrance reasons and it might not be favourable for binding to the G-CSFR.

It is reported that the *in vitro* activity determined by cell-based bioassays is not predictive of the *in vivo* therapeutic effect, because of the phenomenon that the major effect of PEGylation is steric hindrance and not conformational changes.[46] Although steric hindrance lowers the binding affinity to the receptor, there are plenty of opportunities for the receptor–ligand interactions to occur because of the prolonged circulating half-life. It is also very likely that the flexibility of PEG may play a role here. Enhanced pharmacodynamic properties are evident from the case of pegfilgrastim and other marketed pegylated products, *e.g.* pegylated interferons.[47, 48]

The selected conjugates were tested for pharmacokinetic and pharmacodynamic parameters. PK and PD profiles (Figure 7) were generated in rats using a single dose s.c. application.

G-CSF monomer is rapidly eliminated from the body ($t_{1/2}$ = 2.1 h) and F_{dim} has a comparable elimination

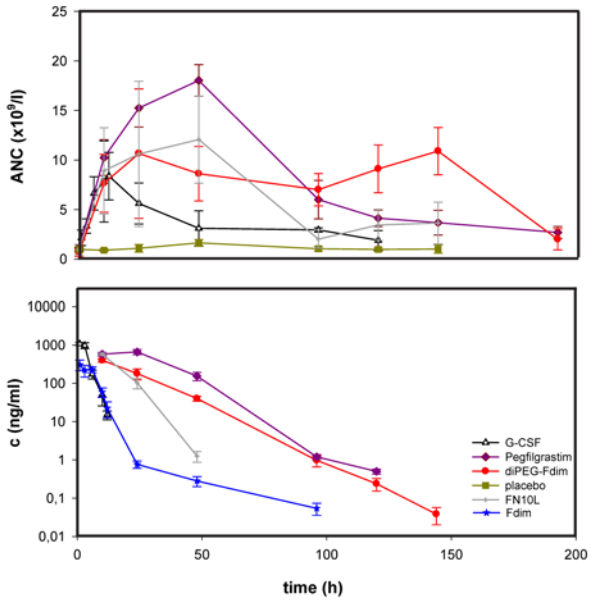

Figure 7: PD (upper panel) and PK profiles (lower panel). ANC – absolute neutrophil count.

half-life ($t_{1/2}$ = 2.2 h). The attachment of PEG chain increased the hydrodynamic radius above renal filtration: pegylated G-CSF conjugates exhibit prolonged elimination half-lives of approximately 6–10 hours (Table 3).

Table 3: Biological activity and PK (elimination half-life) results

	Protein / conjugate	Biological activity (%)	Elimination half-life (h)
1	G-CSF	100	2.1
2	FN10L	70	5.6
3	Pegfilgrastim	45	7.7
4	$FN10L_{dim}$	~ 0	n.d.
5	F_{dim}	48	2.2
6	diPEG-F_{dim}	8	9.9

The pharmacodynamic aspect of the prolonged elimination half-life is well reflected in the absolute neutrophil count (ANC) in blood serum. After filgrastim administration, the initial augmentation of neutrophil count is followed by a rapid drop due to the molecule's rapid clearance. A sustained elevation in ANC is observed in case of pegfilgrastim and diPEG-F_{dim} (Figure 7). The diPEG-F_{dim} PD profile is possibly superior to pegfilgrastim due to the second ANC maximum and a more balanced concentration profile over a longer time interval.

Having in mind that a dose dependent ANC increase in patients receiving chemotherapy was reported, this is not very likely in our case since the same doses were used for all conjugates.[49] The occurrence of the second ANC maximum in the PD profile of diPEG-F_{dim} could be explained by the G-CSF mode of action and its elimination pathways. In addition to being filtrated through the kidneys, the G-CSF removal is accelerated by a rapid ANC increase due to the self-regulation mechanism by neutrophils. A rapid ANC decrease back to baseline values is demonstrated in Figure 7. The increased hydrodynamic radius of diPEG-F_{dim} substantially reduced the renal filtration and the second clearance mechanism should prevail. Thus, it would be beneficial to avoid a rapid ANC increase to prolong the G-CSF residence time, as was speculated for pegylated conjugates: their lowered biological activity itself could act to increase the half-life by reducing self-elimination.[45] The second ANC maximum observed in the diPEG-F_{dim} PD profile could thus result from the biological activity being reduced enough to moderately stimulate the self-elimination.

3. 3. Conclusions

Since the stoichiometry of the G-CSF binding to its receptor is G-CSF:G-CSFR = 2:2 our hypothesis was that a dimeric form of the protein could activate the G-CSFR more efficiently than the monomer (dimer:G-CSFR = 1:2). The biological activities of the dimers being well below 100%, also in case of unpegylated dimer F_{dim}, are opposing this initial hypothesis. It is presumed that the spatial orientation of the molecules connected via Cys18 was not suitable for forming an effective dimer:G-CSFR = 1:2 complex and we speculate that this is the reason for not obtaining dimers with enhanced biological activities. Possibly a dimer:G-CSFR = 2:2 complex was formed and the resulting low biological activity is a consequence of one dimer obstructing the binding of the second dimer molecule. Another possibility is that due to different possible G-CSF orientations in the dimer only a portion of dimers bearing favourable orientation was capable of receptor binding. However, we were able to obtain a conjugate with attractive properties even with this suboptimal orientation: the *in vivo* PK/PD study revealed that diPEG-F_{dim} has pharmacokinetical and pharmacodynamical properties comparable to pegfilgrastim. Its *in vitro* biological activity is significantly lower (8% vs. 45%, respectively), but it possibly possesses superior properties due to the second maximum in ANC. It remains to be established whether more suitable conjugates can be obtained.

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Povzetek

G-CSF uspešno preprečuje nevtropenijo, ki je posledica kemoterapije. Na voljo sta že dve zdravili druge generacije z izboljšanimi terapevtskimi lastnostmi in razvoj novih oblik še vedno poteka. Za učinkovito dimerizacijo receptorja je potrebna vezava dveh G-CSF molekul. V tej raziskavi smo preučevali možnost razvoja dimerov G-CSF, ki bi delovali kot dimerizacijski dejavnik, in preizkušali možnost njihove uporabe. *In vitro* biološke aktivnosti dimerov so bile nižje od aktivnosti G-CSF monomera, zaradi česar predvidevamo, da prostorska orientacija molekul ni bila optimalna. Za dimerizacijo receptorja sta se najverjetneje morala vezati dva dimera namesto enega, ki bi deloval kot dimerizacijski dejavnik. Konjugat diPEG-F_{dim} ima kljub znatno nižji *in vitro* biološki aktivnosti farmakokinetične in farmakodinamične lastnosti primerljive pegfilgrastimu, ali celo boljše. Zanimiv je namreč njegov PD profil z drugim vrhom v absolutnem številu nevtrofilcev, oziroma dokaj enakomerno povišano število nevtrofilcev v daljšem časovnem intervalu.