

## Expression of recombinant G-CSF receptor domains and their inhibitory role on G-CSF function

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### Abstract

**Background and purpose:** Granulocyte colony-stimulating factor (G-CSF) is routinely used in combination with chemotherapy to battle neutropenia. However, studies suggest that this chemokine may increase the risk of metastasis and malignancy in many cancers. To counteract the adverse effects of G-CSF in cancer, antibodies have been used to block its action. However, antibodies are large and complex molecules which makes their production expensive. Thus in this study, we aim to construct different structure variants of the G-CSF receptor containing different domains and select the best variant that prevents the adverse actions of this chemokine. These novel structures are smaller than antibodies and easier to produce.

**Experimental approach:** Different domains of the G-CSF receptor were designed and cloned into the pET28a expression vector. These recombinant receptor subunits were then expressed in *Escherichia coli* and purified using standard affinity chromatography techniques. Interaction of recombinant receptor subunits with G-CSF was assessed using enzyme-linked immunosorbent assay and NFS60 cells.

**Findings / Results:** Two recombinant receptor subunits containing D1 + D2 + D3 domains and D2 domain showed the strongest inhibitory activity to G-CSF.

**Conclusion and implications:** These novel recombinant receptor variants could be candidates for further studies in the development of novel therapeutics.

**Keywords:** G-CSF antagonist; G-CSF-R domains; NFS60.

### INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) promotes the survival, proliferation, and differentiation of progenitor blood cells such as eosinophils, basophils, and neutrophils into mature and functional cells (1,2). Thus, it is routinely used for the treatment of patients with congenital

neutropenia (G-CSF deficiency), acquired immune deficiency syndrome (AIDS), and those who underwent intensive chemotherapy or radiotherapy for bone marrow transplants or cancer. G-CSF can also boost the host immune system against pathogenic microorganisms (2,3).

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G-CSF receptor (G-CSF-R) is predominantly expressed on neutrophils, however, expression in various tumor cells has been reported (4,5). Moreover, cancer cells also express and secrete G-CSF. This was first reported in lung cancers, and soon after, other cancer cells including non-hematopoietic tumors such as hepatomas, ovarian, bladder, astrocytoma, glioblastomas, mesothelioma oropharyngeal squamous cell, melanomas, and sarcomas have been detected to secrete G-CSF in large amounts causing increase hematopoietic activity and worsening of the disease (4,5). Various reports have attributed aggressive phenotypes of tumors such as increased malignancy and resistance to treatments to G-CSF expression, leading to complications and adverse side-effects (5). G-CSF promotes these adverse effects by stimulating angiogenesis and neovascularization or prevention of cancer cell apoptosis. Thus, there is a direct relationship between high G-CSF levels and resistance to anti-vascular endothelial growth factor (VEGF) treatments (5,6).

Expression of G-CSF and its receptor is one of the main pathways employed by tumor cells to escape treatments and stimulate growth and survival (7). This was supported by the reports indicating that the administration of antibodies against this cytokine can prevent cancer cell growth and metastasis (8,9). Recent studies show that certain inflammatory diseases such as rheumatoid arthritis are also associated with G-CSF and blocking the cytokines pathway leads to improvement of the disease symptoms (10,11). Consistent with this, anti-G-CSF therapy has also been shown to play a crucial role in the suppression of growth and angiogenesis in refractory tumors (5,9).

Conventional antibodies have been vastly used for this purpose, however, antibodies are large and complex molecules that require eukaryotic expression systems which makes their production difficult and expensive (12). On the other hand, G-CSF-R subunits can naturally bind to G-CSF with high specificity and affinity (13) and can be easily engineered and expressed in the bacterial host, thus removing limitations associated with the use of antibodies in anti-G-CSF therapy.

The ligand-receptor interaction happens through extracellular domains of the receptor often known as D1, D2, and D3. The D1 domain has a similar structure to the immunoglobulin (immunoglobulin-like domain) and the D2 and D3 domains create a conformation loop similar to the cytokine receptor homologous (14).

Activation of the receptor requires binding of two G-CSF molecules to two receptors forming a tetramer leading to phosphorylation of the cytoplasmic domains of the receptors and activation of the downstream pathways through Janus kinase (JAK) family such as JAK1 and JAK2 (15).

In this study we aimed to develop different recombinant receptor subunits comprise of D1, D2, and D3 domains of human G-CSF-R and investigate their efficiency in blocking G-CSF function. Thus, providing a more convenient solution for anti-G-CSF therapy, bypassing the complexity and the cost associated with the production of large conventional monoclonal antibodies.

## MATERIALS AND METHODS

### *Cloning the extracellular domains of the G-CSF-R*

cDNA sequence of human G-CSF-R was obtained from Gene Bank NCBI. The coding sequence was optimized for expression in *Escherichia coli* (*E. coli*). *EcoRI* and *XhoI* restriction sites were included at 5' and 3', respectively to facilitate the cloning process.

The final sequence had 930 bp and was synthesized by Biomatik (Canada). Specific primers were designed to amplify the nucleic acid sequence of various domains of the receptors including D1, D2, D3, D1 + D2, D2 + D3, and the extracellular region of the receptor consisting of D1 + D2 + D3 domains (Table 1). Each sequence was then cloned into the pET28a(+) expression vector and the final construct was used for transformation of *E. coli* BL21 expression host. Cloning and transformation were confirmed using enzymatic digestion, polymerase chain reaction (PCR) with specific primers and sequencing.

**Table 1.** Sequences of specific primers to amplify the nucleic acid sequence of various granulocyte colony-stimulating factor receptor domains.

Domains	primers	
	Forward sequences	Reverse sequences
D1 + D2 + D3	GATGAATTCTCTGCGGAATGCGGTC	AATCTCGAGCGCACGTTCCGGTAGT
D1 + D2	GATGAATTCTCTGCGGAATGCGGTC	GGTCTCGAGCGGCTCCAGTTTAA
D2 + D3	CATGAATTCGCAATCCCGCACAA	AATCTCGAGCGCACGTTCCGGTAGT
D1	GATGAATTCTCTGCGGAATGCGGTC	TTTCTCGAGGATTGCCGGCGGATAA
D2	CATGAATTCGCAATCCCGCACAA	GGTCTCGAGCGGCTCCAGTTTAA
D3	GCTGAATTCATGCTGCGTACTATGGA	AATCTCGAGCGCACGTTCCGGTAGT

### **Expression and purification of different recombinant subunits derived from G-CSF-R**

The transformed *E. coli* BL21 with each of the six constructs were cultured in Luria-Bertani (LB) with 70 µg/mL kanamycin and incubated with shaking (200 rpm) at 37 °C until the optical density (OD) reached 0.7. Expression was then induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the incubation continued at 25 °C overnight. Cells were collected by centrifugation and disrupted by sonication in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0).

Cell debris were removed by centrifugation and the expression of the proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant protein was purified using Ni-nitrilotriacetic acid (NTA) affinity chromatography resin column (Qiagen, USA). Five mg of total protein were loaded on the column, followed by the addition of 20 mL of washing buffer (NaH<sub>2</sub>PO<sub>4</sub> 50 mM, NaCl 300 mM, imidazole 20 mM, pH 8.0). Recombinant proteins were removed from the column using 3 mL of elution buffer (NaH<sub>2</sub>PO<sub>4</sub> 50 mM, NaCl 300 mM, imidazole 250 mM, pH 8.0). The purity of the recombinant proteins was confirmed by 14% SDS-PAGE. The Bradford method was used for measuring protein concentration.

### **Enzyme-linked immunosorbent assay of recombinant G-CSF-R**

Each well of the enzyme-linked immunosorbent assay (ELISA) plate was coated with 0.5 µg of G-CSF (filgrastim, Neupogen<sup>®</sup>) or bovine serum albumin (BSA, Sigma, Germany) as a control, in 100 µL

coating buffer (carbonate-bicarbonate buffer pH 9.6) and then incubated at 4 °C overnight. After the incubation period, coated wells were washed with phosphate-buffered saline + 0.05% Tween<sup>®</sup> 20 and blocked with 5% skimmed milk at 37 °C for 1.5 h. After washing the plate, different concentrations of each purified recombinant subunits (0.5, 2.5, 5, 10 µg/mL) were added to each well (in triplicates) and incubation continued at 37 °C with shaking (250 rpm) for another 1.5 h. The wells were then washed three times as before, followed by the addition of horseradish peroxidase (HRP) conjugated mouse anti-his tag monoclonal antibody (Roche, Germany) at a final dilution of 1:5000 to each well. The plate was then incubated at 37 °C with shaking (250 rpm) for 1 h. Washing was done as before and 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) substrate (supersensitive, Sigma, Germany) was added to each well. After 15 min, the reaction was stopped by addition of 100 µL of 2M H<sub>2</sub>SO<sub>4</sub>, and the OD was measured at 450 nm.

### **Proliferation and differentiation inhibition assay**

Blocking the growth stimulation of G-CSF using the recombinant receptor subunit was done using MTT assay. NFS60 cells were cultured in RPMI supplemented with 10% phosphate-buffered saline (PBS) and interleukin (IL)-3 as a growth factor. After 72 h, cells were collected and rinsed three times with RPMI to remove excessive IL-3. A total of 15,000 cells were cultured in each well of a 96-well plate. G-CSF (200 IU, Neupogen<sup>®</sup>) along with different concentrations of each recombinant subunit (0.5, 2.5, 5, 10 µg/mL)

was added to the corresponding wells. In addition, specific anti-G-CSF-R nanobody as a positive control and BSA as negative control were used. Then the plate was incubated for 48 h, after which, 20  $\mu$ L of MTT solution (Sigma, Germany) was added to each well and the plate was incubated for 4 more hours. The supernatant was removed and DMSO (Sigma, Germany) was added to each well to solve the formazan crystals. The OD was measured at 450 nm. The test was repeated three times.

### Statistical analysis

The data are presented as mean  $\pm$  SD. After coming up the normal distribution of data through Shapiro-Wilk test, the mean differences between diverse compositions of recombinant G-CSF receptor domains were analyzed using analysis of variance (ANOVA) test. Bonferoni correction post hoc was recruited to compare study groups at the baseline and relationship between variables.  $P \leq 0.05$  was considered as significant statistical difference.

## RESULTS

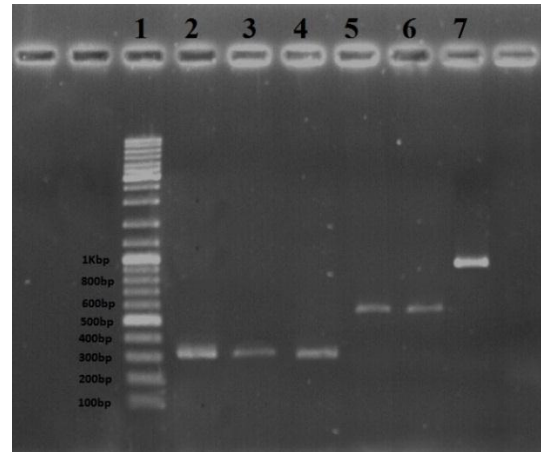
### Cloning the extracellular domains of G-CSF-R

The extracellular domains of G-CSF-R which bind to the ligand comprise three domains, each with the coding sequence of 300 bp in length. The sequences of these domains were PCR amplified, cloned, and expressed in *E. coli*. To identify the domain with the most contribution in ligand binding, a total of six structures were constructed; each domain of D1, D2, and D3 separately, D1 + D2, D2 + D3, and the entire binding structure consisted of D1 + D2 + D3. PCR amplicon of each of these structures showed the correct size on agarose gel electrophoresis (Fig. 1). Cloning and transformation of the *E. coli* expression host were further confirmed using restriction digestion using *Eco*RI and *Xho*I as well as PCR using specific primer for each construct. These constructs were further confirmed using sequencing. The sequence of each recombinant G-CSF receptor subunit is shown in Table 2.

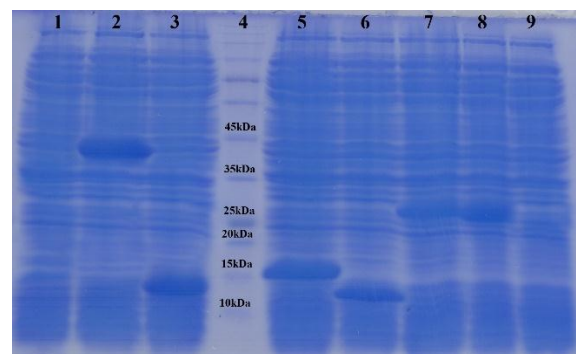
### Expression and purification of the extracellular domains of G-CSF-R

The expression of the recombinant G-CSF-R subunits was done at 25 °C overnight. The

molecular weight of each recombinant receptor subunit was estimated by ExPASy to be 11, 15, 13, 26, 28, and 36 KDa for D1, D2, D3, D1 + D2, D2 + D3, and D1 + D2 + D3 domains, respectively. The estimated molecular weights were further confirmed by SDS-PAGE (Fig. 2). Ni-NTA column affinity purification showed a single band with an estimated purity of > 95% for each recombinant structure representing a correct molecular weight (Fig. 3).



**Fig. 1.** PCR products of different constructs of G-CSF-R. Line 1, Ladder; line 2, D1 domain (300 bp); line 3, D2 domain (300 bp); line 4, D3 domain (300 bp); line 5, D1 + D2 domains (600 bp); line 6, D2 + D3 domains (600 bp); and line 7, D1 + D2 + D3 domains (900 bp). PCR, Polymerase chain reaction; G-CSF-R, granulocyte colony-stimulating factor receptor.



**Fig. 2.** SDS-PAGE analysis of different recombinant G-CSF-R domains expression. Lane 1, non-induced cells transformed with D1 + D2 + D3 construct; lane 2, induced cells transformed with D1 + D2 + D3 construct; lane 3, induced cells transformed with D3 construct, lane 4, molecular weight marker; lane 5, induced cells transformed with D2 construct; lane 6, induced cells transformed with D1 construct; lane 7, induced cells transformed with D2 + D3 construct; lane 8, induced cells transformed with D1 + D2 construct; lane 9, induced cells transformed with the empty vector. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; G-CSF-R, granulocyte colony-stimulating factor receptor.

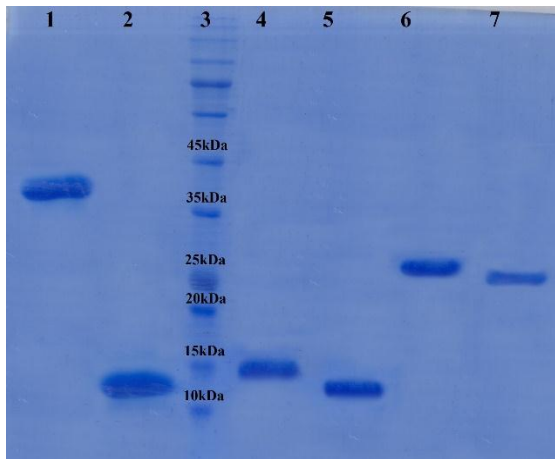
**Table 2.** Nucleic acid sequences of recombinant granulocyte colony-stimulating factor receptor.

Domains	Sequences
D1 + D2 + D3	TCTGCGGAATGCGGTCACATCTCTGTTTCTGCTCCGATCGTTACCTGGGCGATCCGATCACCGCGTCTTGCATCATCAAACAGAAGCTGTTCTCATCTGGA <sub>c</sub> CCGGAACCGCAGATCCTGTGGCGCCTGGGTGCGGAACTGCAGCCGGGTGGTTCGTCAGCAGCGTCTGTCCGACGGTACCCAGGAATCTATCATTACCCTGCCGCATCTGAACCACACTCAGGCGTTTCTGTCTTGTCTCTGAACTGGGGTAACAGCCTGCAGATTCTGGACCAGGTTGAAGTGCCTGCGGGTTATCCGCCGGCAATCGCAATCCCGCACAACTGTCTGCCTGATGAACCTGACCACTCTCTCTGATCTGCCAGTGGGAACCGGGTCCGGAAACCCACCTGCCGACCTCTTTCACCCTGAAATCTTTCAAATCTCGTGGTAACTGCCAGACCCAGGGCGACTCCATCCTGGATTGTGTGCCGAAAGACGGCCA GTCTCACTGCTCTATTCCGCGTAAACACCTGCTGCTGTACCAGAACATGGGTATCTGGGTTTCAAGGCTGAA AACGCGCTGGGCACCTCTATGTCTCCGACGCTGTGCCTGGACCCGATGGACGTTGTTAAACTGGAGCCG CCGATGCTGCGTACTATGGA <sub>c</sub> CCGTCTCCGGAAGCGGCACCGCCGACGGCGGGTGTCTGCAGCTGAGCT GGGAGCCGTGGCAGCCGGCCCTGCACATTAACCAGAAATGCGAGCTGCGTCATAAGCCGCAGCGTGGT GAGGCATCTTGGGCGCTGGTTGGTCCGCTGCCGCTGGAGGCTCTGCAGTACGAACTGTGCGGTCTGCTGC CGGCTACCGCGTACACCCTGCAGATTCGCTGTATCCGTTGGCCGCTGCCGGGTCATTGGTCTGATTGGAG CCCGTCCCTGGAGCTGCGCACTACCGAACCTGCGCCG
D1 + D2	TCTGCGGAATGCGGTCACATCTCTGTTTCTGCTCCGATCGTTACCTGGGCGATCCGATCACCGCGTCTTGCATCATCAAACAGAAGCTGTTCTCATCTGGA <sub>c</sub> CCGGAACCGCAGATCCTGTGGCGCCTGGGTGCGGAACTGCAGCCGGGTGGTTCGTCAGCAGCGTCTGTCCGACGGTACCCAGGAATCTATCATTACCCTGCCGCATCTGAACCACACTCAGGCGTTTCTGTCTTGTCTCTGAACTGGGGTAACAGCCTGCAGATTCTGGACCAGGTTGAAGTGCCTGCGGGTTATCCGCCGGCAATCGCAATCCCGCACAACTGTCTGCCTGATGAACCTGACCACTCTCTCTGATCTGCCAGTGGGAACCGGGTCCGGAAACCCACCTGCCGACCTCTTTCACCCTGAAATCTTTCAAATCTCGTGGTAACTGCCAGACCCA GGGCGACTCCATCCTGGATTGTGTGCCGAAAGACGGCCAGTCTCACTGCTCTATTCCGCGTAAACACCTG CTGCTGTACCAGAACATGGGTATCTGGGTTTCAAGGCTGAAAACGCGCTGGGCACCTCTATGTCTCCGCA GCTGTGCCTGGACCCGATGGACGTTGTTAAACTGGAGCCGCCGATGCTGCGTACTATGGA <sub>c</sub> CCGTCTCCGG AAGCGGCACCGCCGACGGCGGGTGTCTGCAGCTGAGCTGGGAGCCGTGGCAGCCGGCCCTGCACATTA ACCAGAAATGCGAGCTGCGTCATAAGCCGCAGCGTGGTGAGGCATCTTGGGCGCTGGTTGGTCCGCTGC CGCTGGAGGCTCTGCAGTACGAACTGTGCGGTCTGCTGCCGGCTACCGCGTACACCCTGCAGATTCGCTG TATCCGTTGGCCGCTGCCGGGTCATTGGTCTGATTGGAGCCCGTCCCTGGAGCTGCGCACTACCGAACGT GCGCCG
D2 + D3	GCAATCCCGCACAACTGTCTGCCTGATGAACCTGACCACCTCTCTCTGATCTGCCAGTGGGAACCGG GTCCGGAAACCCACCTGCCGACCTCTTTCACCCTGAAATCTTTCAAATCTCGTGGTAACTGCCAGACCCA GGGCGACTCCATCCTGGATTGTGTGCCGAAAGACGGCCAGTCTCACTGCTCTATTCCGCGTAAACACCTG CTGCTGTACCAGAACATGGGTATCTGGGTTTCAAGGCTGAAAACGCGCTGGGCACCTCTATGTCTCCGCA GCTGTGCCTGGACCCGATGGACGTTGTTAAACTGGAGCCGCCGATGCTGCGTACTATGGA <sub>c</sub> CCGTCTCCGG AAGCGGCACCGCCGACGGCGGGTGTCTGCAGCTGAGCTGGGAGCCGTGGCAGCCGGCCCTGCACATTA ACCAGAAATGCGAGCTGCGTCATAAGCCGCAGCGTGGTGAGGCATCTTGGGCGCTGGTTGGTCCGCTGC CGCTGGAGGCTCTGCAGTACGAACTGTGCGGTCTGCTGCCGGCTACCGCGTACACCCTGCAGATTCGCTG TATCCGTTGGCCGCTGCCGGGTCATTGGTCTGATTGGAGCCCGTCCCTGGAGCTGCGCACTACCGAACGT GCGCCG
D1	TCTGCGGAATGCGGTCACATCTCTGTTTCTGCTCCGATCGTTACCTGGGCGATCCGATCACCGCGTCTTGCATCATCAAACAGAAGCTGTTCTCATCTGGA <sub>c</sub> CCGGAACCGCAGATCCTGTGGCGCCTGGGTGCGGAACTGCAGCCGGGTGGTTCGTCAGCAGCGTCTGTCCGACGGTACCCAGGAATCTATCATTACCCTGCCGCATCTGAACCACACTCAGGCGTTTCTGTCTTGTCTCTGAACTGGGGTAACAGCCTGCAGATTCTGGACCAGGTTGAAGTGCCTGCGGGTTATCCGCCGGCAATC
D2	GCAATCCCGCACAACTGTCTGCCTGATGAACCTGACCACCTCTCTCTGATCTGCCAGTGGGAACCGG GTCCGGAAACCCACCTGCCGACCTCTTTCACCCTGAAATCTTTCAAATCTCGTGGTAACTGCCAGACCCA GGGCGACTCCATCCTGGATTGTGTGCCGAAAGACGGCCAGTCTCACTGCTCTATTCCGCGTAAACACCTG CTGCTGTACCAGAACATGGGTATCTGGGTTTCAAGGCTGAAAACGCGCTGGGCACCTCTATGTCTCCGCA GCTGTGCCTGGACCCGATGGACGTTGTTAAACTGGAGCCG
D3	CCGATGCTGCGTACTATGGA <sub>c</sub> CCGTCTCCGGAAGCGGCACCGCCGACGGCGGGTGTCTGCAGCTGAGCT GGGAGCCGTGGCAGCCGGCCCTGCACATTAACCAGAAATGCGAGCTGCGTCATAAGCCGCAGCGTGGT GAGGCATCTTGGGCGCTGGTTGGTCCGCTGCCGCTGGAGGCTCTGCAGTACGAACTGTGCGGTCTGCTGC CGGCTACCGCGTACACCCTGCAGATTCGCTGTATCCGTTGGCCGCTGCCGGGTCATTGGTCTGATTGGAG CCCGTCCCTGGAGCTGCGCACTACCGAACCTGCGCCG

**ELISA of the extracellular domains of G-CSF-R**

ELISA was used to determine the binding potency of the recombinant receptor subunits to G-CSF. G-CSF was coated in the wells of an ELISA plate and the purified recombinant receptor subunits were used as the antigen. ELISA was performed as described above. Results indicated that the extracellular receptor region consisted of all

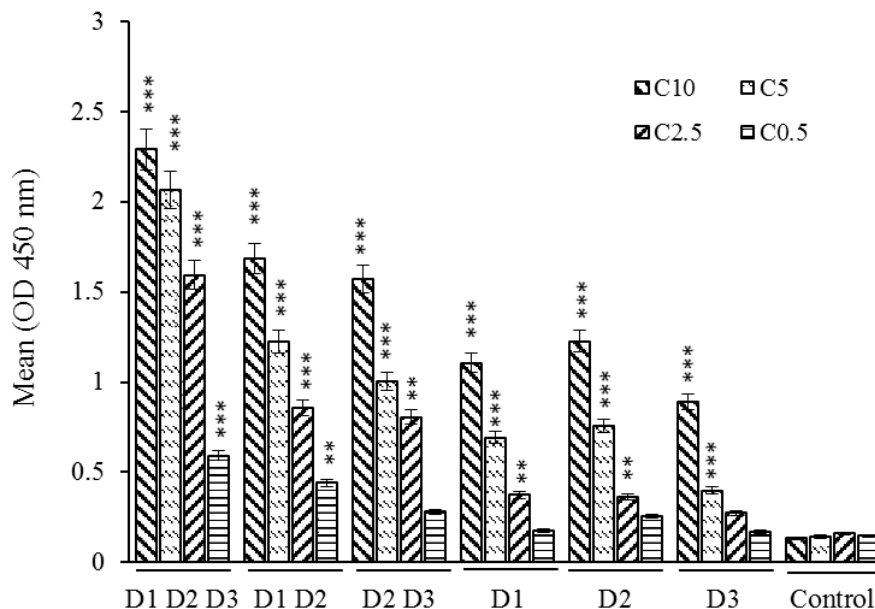
three domains (D1 + D2 + D3) has the most binding efficiency when used at 10 µg/mL (OD: 2.4) and the recombinant receptor consisting of the D3 domain only shows the weakest efficiency when used at the same concentration (OD: 0.85; Fig. 4). According to the results shown in Fig. 4, the efficiency of the receptor binding was as follows: D1 + D2 + D3 > D1 + D2 > D2 + D3 > D2 > D1 > D3.



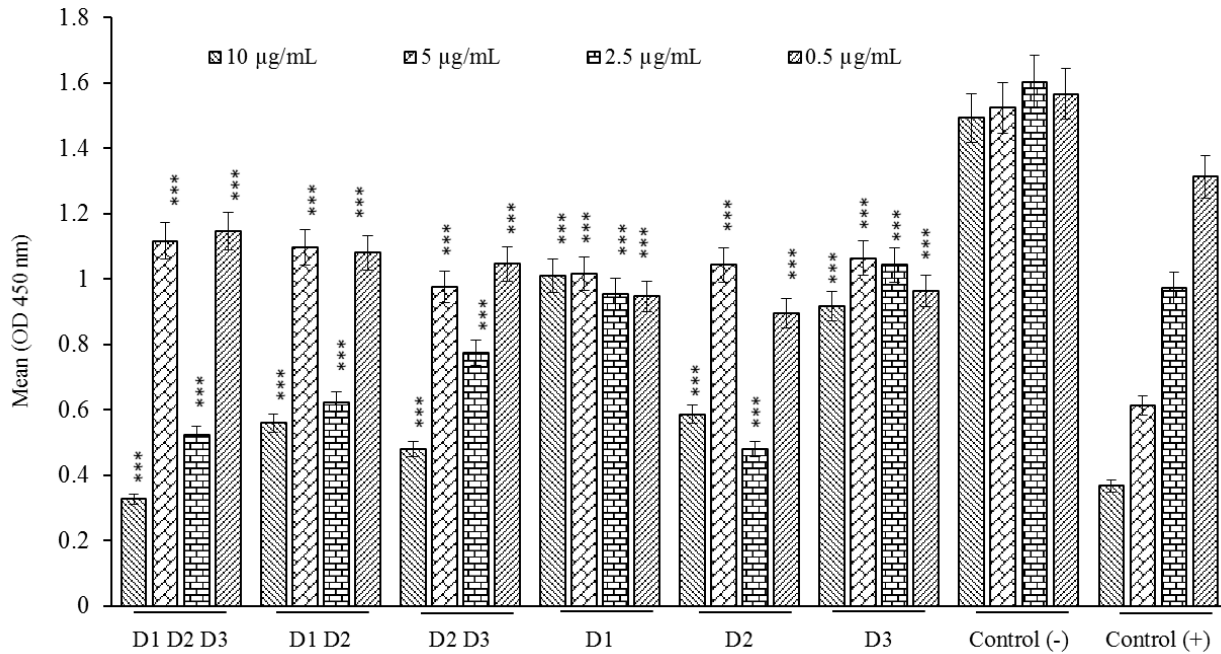
**Fig. 3.** SDS-PAGE analysis of different constructs derived from the G-CSF-R purified by the Ni-NTA column. Lane 1, D1 + D2 + D3 recombinant receptor; lane 2, D3 recombinant receptor; lane 3, molecular weight marker; lane 4, D2 recombinant receptor; lane 5, D1 recombinant receptor, lane 6, D2 + D3 recombinant receptor, lane 7, D1 + D2 recombinant receptor. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; G-CSF-R, granulocyte colony-stimulating factor receptor; NTA, nitrilotriacetic acid.

**Proliferation and differentiation inhibition assay on NFS60 cell line**

NFS60 cells were used to assess the efficacy of each six recombinant receptor subunit in inhibiting the effects of G-CSF on growth and differentiation. The antagonist activity of these recombinant receptors was assessed at different concentrations with the maximum concentration of G-CSF (200 IU) as described in the previous study (4). MTT assay results indicated that the recombinant receptor consisted of the D2 domain had a 90% inhibitory effect on G-CSF growth stimulation with a concentration of 2.5  $\mu\text{g}/\text{mL}$ , while the extracellular receptor region with all three domains showed 85% inhibitory effect at the same concentration. Complete blocking of the G-CSF stimulation was seen at 10  $\mu\text{g}/\text{mL}$  concentration of the recombinant receptor subunit consisted of D1 + D2 + D3 domains. Similarly, the anti-G-CSF-R specific nanobody neutralized this growth stimulation at 10  $\mu\text{g}/\text{mL}$  while BSA showed no effect (Fig. 5).



**Fig. 4.** Determination of the reactivity of recombinant purified G-CSF-R domains to the G-CSF by ELISA. The plate was coated with G-CSF and reactivity of different constructs of G-CSF-R against G-CSF was determined using various concentrations of the recombinant receptor domains. The results are demonstrated as mean  $\pm$  SD from triplicate tests. \*  $P < 0.05$  indicates a significant differences compared to the control group (BSA). G-CSF-R, granulocyte colony-stimulating factor receptor; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.



**Fig. 5.** The proliferation inhibitory effect of recombinant purified G-CSF-R on G-CSF stimulated NFS60 cells. Cultured NFS60 cells were incubated in the RPMI medium supplemented with G-CSF and various concentrations of each six recombinant receptors. BSA was used as the negative control and anti-GCSF-R nanobody was used as the positive control. Following 48 h of incubation, cell growth was examined by MTT assay. The data are demonstrated as mean  $\pm$  SD from triplicate tests. \*  $P < 0.05$  indicates a significance differences compared to the negative control group; G-CSF-R, granulocyte colony-stimulating factor receptor; BSA, bovine serum albumin

## DISCUSSION

G-CSF is a cytokine that stimulates the bone marrow to increase the proliferation and differentiation of white blood cells. G-CSF deficiency increases the risk of various infections. This cytokine also functions as the key regulator of growth and migration of non-blood cells such as endothelial cells. Moreover, it can stimulate regeneration in various tissues (5). There is an increasing body of evidence indicating that G-CSF can boost the growth, migration, and angiogenesis in cancer cells and tissues causing resistance in anti-VEGF therapy (4,5). Thus, patients with high levels of G-CSF are at higher risk of progression, metastasis, and decreased likelihood of improvement (4,16-18). Many tumors, such as mesothelioma, sarcoma, bladder, hepatoma, squamous cell carcinoma, melanoma, and glioblastoma show increased expression of G-CSF, therefore, the negative side-effects can be expected regardless of the exogenous use as a part of therapeutic regimen (4,5). In addition, increased expression of this cytokine has been reported in

autoimmune diseases such as acute and chronic rheumatoid arthritis and plays a role in the worsening of the disease symptoms (10,11).

It has been previously shown that the administration of anti-G-CSF antibody can reduce these adverse effects (5). Furthermore, blocking G-CSF in bladder and lung cancers leads to a dramatic decrease in the rate of metastasis (8). In gastric adenocarcinoma, antibodies against G-CSF inhibit the growth of cancer cells and decrease the metastasis potential (19). Similar trends have also been reported in other cancers. Likewise, G-CSF antagonists significantly decrease the severity of symptoms in inflammatory arthritis (20).

Antibodies are large molecules consisting of many disulfide bonds and glycosylation sites that require expression in eukaryotic cells. Therefore, large production of functional antibodies requires a complex process of expression and purification resulting in a significant increase in the cost (12). Moreover, due to the large size of antibodies, absorption, and diffusion into various tissues including tumors is limited (12).

G-CSF-R has a dissociation constant (Kd) of 100-500 pM and binds to G-CSF with higher affinity and specificity compared to conventional antibodies (14). Thus, in this study, six different constructs were produced from human G-CSF-R domains and were tested for their binding activity to G-CSF. These recombinant receptor subunits are small and simple in structure, thus, they could be easily produced in bacterial cells. The advantages of these recombinant receptors are their small size, specificity, and high affinity to G-CSF which leads to faster and better tissue penetration into solid tumors compared to conventional antibodies (21). Furthermore, these recombinant receptors are from human origin hence are not immunogenic which facilitates their clinical application.

We tested the binding activity of these recombinant receptors to G-CSF and their inhibitory function on G-CSF induced growth and differentiation *via* ELISA and functional cell assays. The addition of G-CSF to NFS60 cells caused proliferation and differentiation (4), while further addition of these recombinant receptors blocked G-CSF and prevented cell growth, proliferation, and differentiation. These structures showed the most inhibitory effects at 10 and 2.5 µg/mL.

The recombinant receptor subunit consisted of D1 + D2 + D3 showed the most inhibition at 10 µg/mL, surprisingly the recombinant receptor subunit consisted of only D2 domain could inhibit the effect of the ligand on cells at 2.5 µg/mL. All recombinant receptor subunits showed concentration-dependent inhibition of G-CSF effects which indicate their specificity toward the ligand. Our primary results suggest that the two structures consisted of D1 + D2 + D3 and D2 are good candidates for future studies to construct recombinant fusion proteins of anti-G-CSF therapeutics and have the potential for clinical application.

## CONCLUSION

In this study, two recombinant G-CSF-R consisted of D1 + D2 + D3 and D2 domain showed potency to inhibit G-CSF function in a concentration-dependent manner. Expression of G-CSF and its receptor is one of the main

pathways employed by tumors to escape treatments and stimulate growth and survival. Many reports indicating that the administration of inhibitory factors against this hormone can prevent cancer cell growth and metastasis. These novel recombinant constructs can potentially be new candidates for the development of anti-G-CSF therapy.

## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest in this study.

## AUTHORS' CONTRIBUTION

H. Bakherad carried out most of the experiments leading to the made of constructs, contributed to the design and the supervision of the study, analyzed the data, prepared the figures, and wrote the manuscript; N. Setayesh supervised the study and corrected the manuscript; S. L. Mousavi Gargari and Z. Sepehrizadeh supervised the study and Scientific supported the project; W. Ebrahimizadeh modified text and corrected the manuscript; F. Mavandadnejad carried out some cloning steps and wrote the manuscript; E. Faghfuri carried out the cell culture and preparation of cell ELISA; S. Ebrahimi carried out some cloning steps; M. Heiat analyzed and interpreted of data and helped prepare the figures, and M. Shahpari executed some ELISA tests.

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