Cancer Letters 377 (2016) 25–31

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) augments the therapeutic effect of pegfilgrastim on gemcitabine-induced neutropenia

Nina Yoo [a,b,c,1], Ha-Reum Lee [a,c,1], Su-Hyun Shin [a], Ki-Young Sohn [c], Heung-Jae Kim [c], Yong-Hae Han [c], Saeho Chong [c], Myung-Hwan Kim [d], Sun Young Yoon [c,2,*], Jae Wha Kim [a,b,2,**]

[a] Cell Factory Research Center, Division of Systems Biology and Bioengineering, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea
[b] Department of Functional Genomics, University of Science and Technology, Daejeon, Republic of Korea
[c] ENZYCHEM Lifesciences, 103-6, KAIST-ICC F741, Munjidong, Daejeon 305-732, Republic of Korea
[d] Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 12 January 2016
Received in revised form 14 April 2016
Accepted 14 April 2016

Keywords:
G-CSF
Pegfilgrastim
Gemcitabine
Neutropenia
Diacylglycerol

ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is widely used for preventing neutropenia during chemotherapy. Polyethylene glycol-conjugated granulocyte colony-stimulating factor (PEG-G-CSF, pegfilgrastim) serves the same purpose but has a longer half-life and greater stability than G-CSF. In this study, we investigated whether 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol, acetylated diglyceride (PLAG), augments the therapeutic effect of pegfilgrastim on chemotherapy-induced neutropenia. We compared neutrophil counts in four groups of mice: control mice, gemcitabine-treated mice, gemcitabine/pegfilgrastim-treated mice, and gemcitabine/pegfilgrastim/PLAG-treated mice. PLAG (50 mg/kg) was orally administered every day during the treatment course. CBC analysis showed that the group treated with PLAG experienced a dramatically increased neutrophil counts on the third day following pegfilgrastim treatment. PLAG had no effect on blood cell apoptosis and neutrophil release from bone marrow. Additionally, pegfilgrastim-induced CXCR2 expression in neutrophils was markedly decreased in PLAG-treated animals. These results suggest that PLAG plays a role in inhibiting neutrophil extravasation, giving rise to an increased number of circulating neutrophils when used with pegfilgrastim during gemcitabine treatment. These data support the potential for PLAG to be used with pegfilgrastim to treat or prevent chemotherapy-induced neutropenia by modulating neutrophil transmigration.

Introduction

Neutrophils are major players in the innate immune responses against inflammation and infection by pathogens [1]. Neutrophils are recruited to the infection site, cross the endothelium, and proceed to destroy the pathogens [2]. The number of neutrophils in the blood is abnormally increased in response to infections or inflammatory disorders. A low number of neutrophils in the blood, or neutropenia, often indicates severe damage to the body's defense systems. Because neutrophils play a critical role in the innate immune response, a patient's neutrophil count indicates their condition and should thus be monitored closely in patients with various types of infection or those undergoing chemotherapy or radiation therapy.

Neutropenia is a common side effect of chemotherapy and is associated with a higher risk of serious infections [3]. Thus, patients are typically given treatment for neutropenia following chemotherapy [4]. Anti-cancer drugs, including DNA analogs, work by non-selectively inhibiting highly uncontrolled cell division typical of malignant cells, thus influencing the cell regenerative process. This lack of specificity causes toxicity to healthy blood cells [5]. For this reason, cytotoxic chemotherapy leads to side effects such as severe and febrile neutropenia [6–8]. In addition, most chemotherapeutic agents cause DNA damage, which eventually triggers the danger-associated molecular pattern molecules (DAMPs) and chemokine release [9]. Circulating neutrophils will migrate toward the chemokine- and DAMPs-releasing lesions and subsequently travel to surrounding tissues. This active neutrophil extravasation will abruptly decrease the number of circulating neutrophils. Limited

* Corresponding author. Tel.: +82 42 864 2845; fax: +82 42 864 2844.
E-mail address: syyoon@enzychem.com (S.Y. Yoon).
** Corresponding author. Tel.: +82 42 860 4238; fax: +82 42 860 4593.
E-mail address: wjkim@kribb.re.kr (J.W. Kim).
[1] The first authors Nina Yoo and Ha-Reum Lee contributed equally to this work.
[2] Jae Wha Kim and Sun Young Yoon contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2016.04.025
0304-3835/

neutrophil production caused by chemotherapy and exaggerated transendothelial migration caused by treatment-induced inflammation may also be the major causes of transient neutropenia.

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) is a nucleoside analog that is widely used in chemotherapy for pancreatic cancer, non-small cell lung cancer, and metastatic breast cancer, among others. Granulocyte colony-stimulating factor (G-CSF) is the standard treatment for gemcitabine-induced neutropenia. G-CSF is a major regulator of granulopoiesis, survival, proliferation, and maturation of myeloid progenitor cells of the neutrophil lineage and induces neutrophil release from the bone marrow [10,11]. Two forms of G-CSF are available: a short-half-life G-CSF and a second-generation polyethylene glycol (PEG)-conjugated G-CSF, pegfilgrastim. Short-half-life G-CSF requires repeated administration during chemotherapy, and pegfilgrastim was developed to address this problem. Pegfilgrastim has a 20 kDa monomethoxy-PEG molecule conjugated to the N-terminal methionyl residue of G-CSF. Because of its long half-life, pegfilgrastim is administered only once per chemotherapy cycle [12]. Pegfilgrastim is used most often in patients at risk of failure with short-half-life G-CSF. Pegfilgrastim is both a cost-effective and a therapeutically effective treatment for neutropenia [13].

PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) was originally isolated from the antlers of Sika deer and has since been chemically synthesized, with the synthesized version confirmed to be identical to the naturally occurring protein [14,15]. The diverse biological roles of PLAG have been studied in mouse models of sepsis, metastasis, and atopic dermatitis and have been shown to mediate anti-inflammatory effects [16,17]. Moreover, PLAG has been shown to have a better therapeutic effect for the treatment of gemcitabine-induced neutropenia for 39 h longer than lenograstim (recombinant G-CSF) early in the treatment course (Supplementary Fig. S1). In this study, we investigate the extent to which PLAG augments the therapeutic effect of pegfilgrastim on chemotherapy-induced neutropenia. Our data suggest that the combination of PLAG with pegfilgrastim-induced production of neutrophils in the setting of exaggerated neutrophil extravasation has a greater therapeutic effect on chemotherapy-induced neutropenia than pegfilgrastim alone.

Materials and methods

Animals and treatment reagents

BALB/c mice were obtained from Koatech Co. (Pyeongtaek, Republic of Korea) and preserved under specific pathogen-free conditions. The mice were 6–8 weeks of age and 20–22 g at the time of the experiments. The experiments were conducted with the approval of the Korea Research Institute of Bioscience and Biotechnology, Institutional Review Committee for Animal Care and Use (Daejeon, Republic of Korea). The mice were injected intraperitoneally with 10 mg/kg gemcitabine (Selleck Chemicals, Boston, USA) to induce neutropenia. Pegfilgrastim (Amgen, CA, USA) was diluted in phosphate-buffered solution (PBS) and administered subcutaneously at a dose of 150 μg/kg. Mice treated with PLAG (*Enzychem*, Daejeon, Republic of Korea) were given 50 mg/kg/day orally.

Peripheral blood and bone marrow cell analysis

Mouse whole blood was collected from the orbital sinuses using heparinized capillary tubes (Kimble Chase Life Science and Research Products LLC, FL, USA) and collection tubes containing K3E-K3EDTA (Greiner Bio-One International, Frickenhausen, Germany). To obtain bone marrow cells, the femurs and tibias were removed from the mice. After both ends of the femoral bones were cut, the bone marrow was flushed with 2 ml of Roswell Park Memorial Institute 1640 media (Life Technologies, Karlsruhe, Germany). The flushed cells were centrifuged at $300 \times g$ for 10 min and suspended in 500 μl of PBS (Welgene, Daegu, Republic of Korea). The collected cells were counted by CBC analysis using a BC 5300 Mindray analyzer (Shenzhen Mindray Bio-medical Electronics, China).

Flow cytometric analysis

For flow cytometric analysis, additional steps were required to remove erythrocytes from the collected cells. After incubation with ACK Lysing Buffer (Life Technologies, Karlsruhe, Germany) for 10 min, the collected cells were washed with PBS containing 1% fetal bovine serum (FBS) (Life Technologies) and were subsequently subjected to specific antibody staining.

Apoptosis in whole blood cells was assessed using fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Pharmingen, NJ, USA) and 7-amino-actinomycin D (7AAD) (BD Pharmingen) according to the manufacturer's instructions. To assess the neutrophil population, cells were washed with PBS and stained with Alexa Fluor® 488-conjugated rat anti-mouse Gr-1 (BD Pharmingen) and PE-Cy™7-conjugated rat anti-mouse CD11b (eBioscience) for 30 min on ice. CXCR2 expression was analyzed by staining with PE-conjugated rat anti-mouse CXCR2 (R&D Systems, MN, USA). The mean fluorescence intensity was analyzed with a FACSCalibur flow cytometer (BD Biosciences, NJ, USA), and the data were processed with FlowJo software (Tree Star, OR, USA).

Statistical analyses

Statistical analyses were performed using a paired *t*-test. $P < 0.00$, $P < 0.01$ and $P < 0.05$ were considered statistically significant.

Results

Schematic overview of gemcitabine, pegfilgrastim, and PLAG administration

Gemcitabine was administered intraperitoneally to induce neutropenia (Fig. 1A). On Day 0, pegfilgrastim was administered subcutaneously. PLAG was orally administered every day. The proper gemcitabine dose for inducing neutropenia, which then responded to pegfilgrastim, was determined using 1, 10, and 100 mg/kg doses of gemcitabine. Neutrophil counts on Days 0, 3, and 5 were measured using a CBC analyzer. As shown in Fig. 1B, 10 mg/kg of gemcitabine successfully induced neutropenia, which subsequently responded to treatment with pegfilgrastim, increasing the circulating neutrophil count to approximately 6000/μl. We show that high-dose gemcitabine with G-CSF can effectively increase circulating neutrophils in a dose-dependent manner.

Combined PLAG and pegfilgrastim is more effective at increasing circulating neutrophils in gemcitabine-induced neutropenia than pegfilgrastim alone

To investigate whether combined PLAG and pegfilgrastim increases the number of circulating neutrophils more than pegfilgrastim alone in gemcitabine-induced neutropenia, mice were treated with PLAG, gemcitabine, and pegfilgrastim as described in the Materials and Methods section. On the third day following the pegfilgrastim injection, circulating neutrophils were counted. Fig. 2A shows that neutrophil counts were significantly decreased in mice treated with 10 mg/kg gemcitabine (0.64 k/μl) compared to the control group (1.66 k/μl). Mice that received gemcitabine and subsequent pegfilgrastim had an increased neutrophil count (4.84 k/μl) when compared to mice that received gemcitabine without pegfilgrastim. Moreover, mice also treated with PLAG showed a significantly increased neutrophil count (approximately 6.11 k/μl). The hematological indices and the absolute cell counts are presented in Table 1. The increased therapeutic effect of combined PLAG and pegfilgrastim over that of pegfilgrastim alone was confirmed by flow cytometry analysis (Fig. 2B). The presence of neutrophils was determined by double-positive staining of Gr1 and CD11b. Mice also treated with PLAG had more neutrophils than mice treated with pegfilgrastim alone on Day 3. Neutrophil counts in mice treated with pegfilgrastim reached the level of control mice on the fifth day (Fig. 2C). The half-life of pegfilgrastim is different for humans and mice. The half-life may depend on renal clearance and degradation by G-CSF receptors or neutrophil elastase [18–20]. These findings indicate that PLAG plays a role in increasing the number of circulating neutrophils in chemotherapy-induced neutropenia.

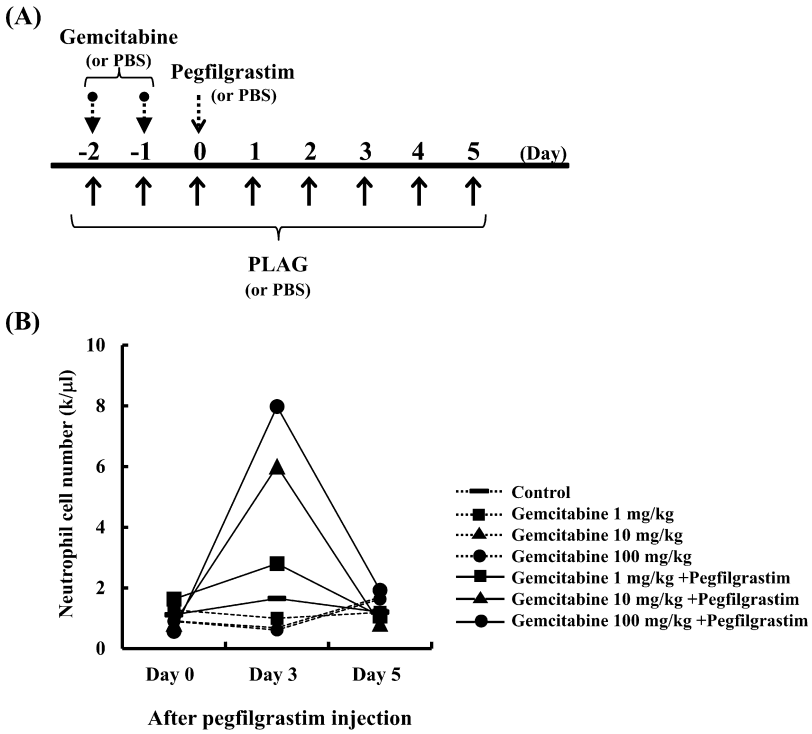

Fig. 1. Experimental design. (A) Mice were divided into eight groups: (1) control, (2) PLAG-treated, (3) gemcitabine (Gem)-treated, (4) Pegfilgrastim-treated, (5) Gem/PLAG-treated, (6) Pegfilgrastim/PLAG-treated, (7) Pegfilgrastim/Gem-treated, and (8) Pegfilgrastim/Gem/PLAG-treated. Gemcitabine was administered intraperitoneally in a dose of 10 mg/kg on Days –2 and –1. After gemcitabine injection, pegfilgrastim was administered at 150 μg/kg once subcutaneously on Day 0. PLAG was administered orally at 50 mg/kg once daily. (B) Mice were injected intraperitoneally with gemcitabine (1, 10, or 100 mg/kg) once daily for 2 days. After 24 h, one group of mice was given a single 150 μg/kg dose of pegfilgrastim subcutaneously, and the other group received no pegfilgrastim. On Days 0, 3, and 5, peripheral blood neutrophils were counted. Control or gemcitabine-treated mice are represented by the dotted line. The solid line indicates neutrophil numbers from the pegfilgrastim/gemcitabine combined treatment group. ▬, control; ■, 1 mg/kg gemcitabine-treated group; ▲, 10 mg/kg gemcitabine-treated group; ●, 100 mg/kg gemcitabine-treated group. Each group contained three mice.

PLAG has no effect on apoptosis and release of neutrophils from bone marrow

The increase in circulating neutrophils could be achieved by increasing neutrophil release from bone marrow or inhibiting neutrophil apoptosis. The anti-apoptotic activity of PLAG was investigated in an apoptosis assay. Leukocytes were obtained from the orbital sinuses of mice on Day 3 and were stained with Annexin V and 7AAD as described in the Materials and Methods section. No apoptotic leukocyte death was observed in any of the treatment groups, including those treated with pegfilgrastim alone or with combined pegfilgrastim and PLAG (Fig. 3A). Next, PLAG influence on the release of neutrophils from bone marrow was investigated by CBC analysis on Days 1 and 3. The results show that the number of neutrophils was significantly decreased in gemcitabine-treated mice compared with control mice. However, we found no statistically significant differences in bone marrow–derived neutrophil counts in gemcitabine-treated, gemcitabine/pegfilgrastim-treated, and gemcitabine/pegfilgrastim/PLAG-treated mice on Day 1 (Fig. 3B). Neutrophil counts in bone marrow were analyzed on Day 3 in the groups treated with pegfilgrastim (Fig. 3C). These data indicate that PLAG had no effect on neutrophil counts in the bone marrow or on neutrophil apoptosis, suggesting that the increased number of circulating neutrophils in PLAG-treated mice is not caused by an increase in neutrophil release from the bone marrow or by the inhibition of neutrophil apoptosis.

PLAG decreases the exaggerated extravasation of circulating neutrophils by attenuation of pegfilgrastim-induced CXCR2 expression

Increased circulating neutrophils associated with PLAG treatment may be achieved by modulating the neutrophil extravasation. Neutrophil mobility is regulated by various chemokines, some of which are induced by chemotherapeutic agents. The chemokine receptor CXCR2 plays a critical role in the recruitment of neutrophils from blood into tissues [21]. To determine whether PLAG regulates CXCR2 expression, peripheral blood leukocytes were obtained and analyzed on Day 3. Cells were stained with neutrophil-specific markers, Gr1 and CD11b, and CXCR2-specific markers. The results show that pegfilgrastim increases CXCR2 expression and the addition of PLAG significantly decreases the pegfilgrastim-induced CXCR2 expression (Fig. 4A). The control population was set as approximately 5% of CXCR2 expression in the M1 region, and the populations of the other groups were calculated accordingly. The pegfilgrastim/gemcitabine-treated mice exhibited 53.15% positive CXCR2 expression, and the PLAG/pegfilgrastim/gemcitabine-treated mice exhibited 18.5% positive CXCR2 expression (Fig. 4B). These results suggest that PLAG increases the number of circulating neutrophils and enhances the therapeutic effect of pegfilgrastim on chemotherapy-induced neutropenia by controlling neutrophil transmigration through downregulation of CXCR2.

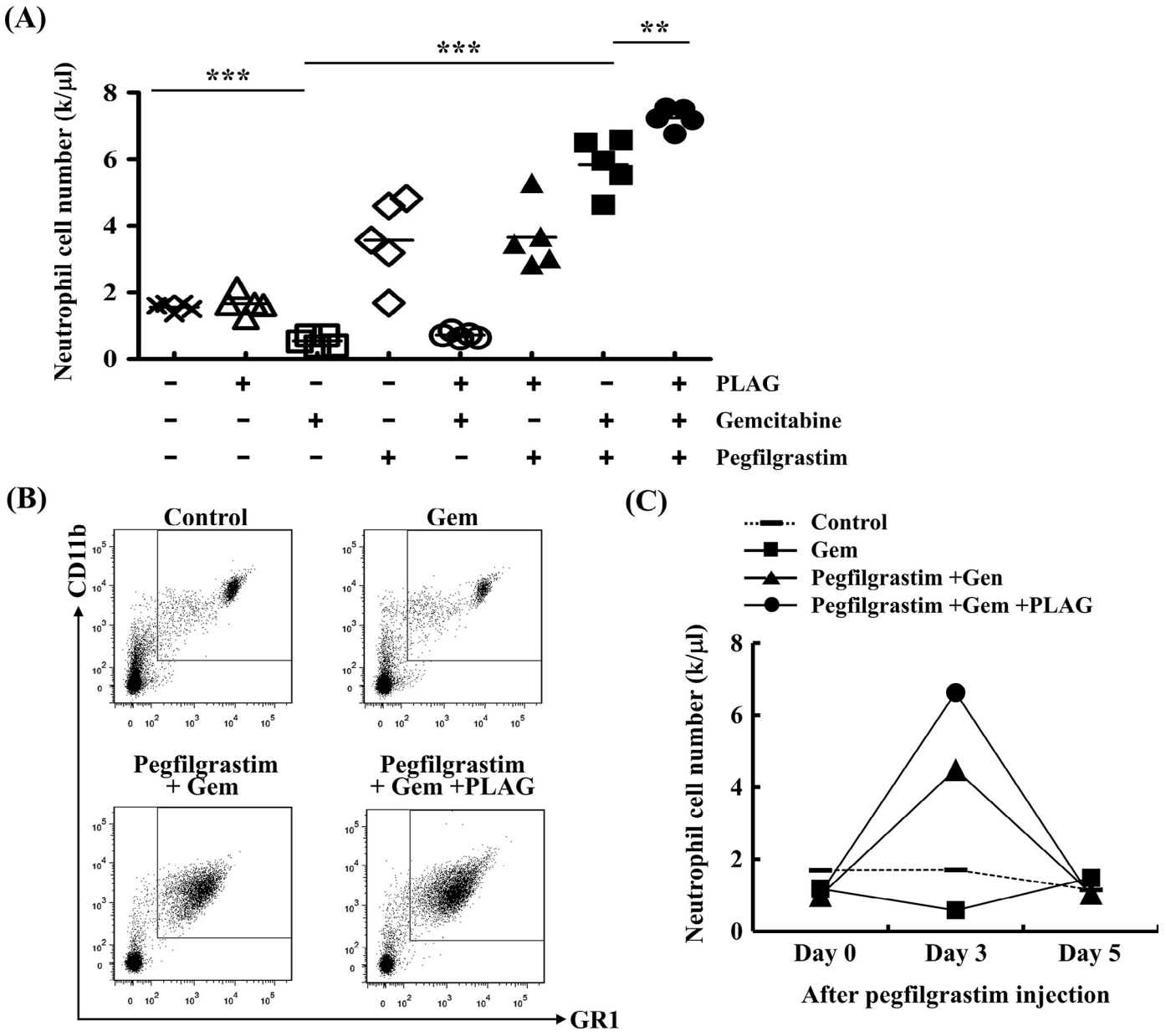

Fig. 2. PLAG with pegfilgrastim increased the number of circulating neutrophils in blood. (A) Mice were divided into eight groups as described in Fig. 1A. On Day 3, whole blood was collected from the orbital sinuses and neutrophils were counted. ×, control; △, PLAG-treated group; □, gemcitabine-treated group; ◇, pegfilgrastim-treated group; ○, gemcitabine/PLAG-treated group; ▲, pegfilgrastim/PLAG-treated group; ■, pegfilgrastim/gemcitabine-treated group; ●, pegfilgrastim/gemcitabine/PLAG-treated group. Each group contained five mice, and the bars represent the mean. Student's *t*-test was performed to determine statistical significance. $^{***}p < 0.00$ and $^{**}p < 0.01$. (B) Whole blood was analyzed by flow cytometry on Day 3. Blood was stained with Alexa Fluor 488-conjugated anti-GR1 and PE-Cy™7-conjugated anti-CD11b to determine neutrophil expression. The experiments were performed in triplicate, and representative images are displayed. (C) Blood was taken from the orbital sinuses on Days 0, 3, and 5, and neutrophils were counted. ▬, control; ■, gemcitabine-treated group; ▲, pegfilgrastim/gemcitabine-treated group; ●, pegfilgrastim/gemcitabine/PLAG-treated group. Each group contained three mice, and the average values were calculated.

Discussion

Recent studies suggest that pegfilgrastim treatment for neutropenia during chemotherapy has unfavorable side effects. G-CSF may promote tumor growth and increase neutrophil myeloperoxidase and the expression of vascular endothelial growth factor in tumor cells [22]. Large amounts of G-CSF have been detected in various tumors and tumor microenvironments. In colorectal cancer, G-CSF has been found to affect proliferation and migration of carcinoma cells, lead to accumulation of regulatory T cells, and create a favorable environment for tumor cell growth [23]. Fibroblasts neighboring colorectal tumors also secrete G-CSF. Pegfilgrastim induces mechanical hyperalgesia via spinal activation and activates peripheral cytokines, such as tumor necrosis factor alpha and interleukin (IL)-1 beta [24–29]. Moreover, G-CSF recruits neutrophils to tumor sites and induces unexplained blast proliferation. Novel strategies to prevent or treat neutropenia with fewer side effects are necessary.

In this study, PLAG augmented pegfilgrastim's therapeutic effect on gemcitabine-induced neutropenia, as shown in Fig. 2. These data suggest that PLAG plays a role in preventing the loss of circulating neutrophils by exaggerated transmigration. Given these results, PLAG, when used with pegfilgrastim, could be a therapeutic option for the treatment of gemcitabine-induced neutropenia. The interaction between CXCR2 and IL-8 is an important mechanism in the func-

Table 1
Hematological indices and absolute blood cell counts (n = 5).

	Groups							
	Control	PLAG	Gem	Pegfilgrastim	Gem + PLAG	Pegfilgrastim + PLAG	Pegfilgrastim + Gem	Pegfilgrastim + Gem + PLAG
(A)								
Erythrocyte (1×10^6/μl)	9.05 ± 0.31	9.09 ± 0.09	8.62 ± 0.32	9.13 ± 0.24	8.77 ± 0.47	9.05 ± 0.17	8.87 ± 0.26	8.80 ± 0.26
Hemoglobin (g/dl)	14.28 ± 0.41	14.88 ± 0.26	13.52 ± 0.65	14.85 ± 0.44	14.08 ± 0.69	14.80 ± 0.25	13.92 ± 0.28	13.86 ± 0.43
Platelet (k/μl)	1046.00 ± 53.85	1116.50 ± 26.01	554.80 ± 68.85	892.00 ± 58.72	743.00 ± 22.85	833.00 ± 45.23	429.20 ± 119.24	485.60 ± 39.15
(B) (Unit: k/μl)								
WBC	9.58 ± 0.86	9.28 ± 1.32	5.70 ± 0.74	12.94 ± 2.03	5.87 ± 0.86	13.71 ± 2.01	12.50 ± 0.82	15.76 ± 1.58
Neutrophil	1.62 ± 0.04	1.66 ± 0.32	0.62 ± 0.07	3.57 ± 1.26	0.63 ± 0.07	3.66 ± 0.97	4.38 ± 0.77	6.96 ± 0.55
Lymphocyte	7.52 ± 0.75	7.21 ± 1.01	4.88 ± 0.64	8.09 ± 0.84	5.09 ± 0.82	8.95 ± 1.21	7.39 ± 0.73	7.94 ± 1.35
Monocyte	0.31 ± 0.13	0.27 ± 0.03	0.10 ± 0.05	0.95 ± 0.71	0.10 ± 0.03	0.69 ± 0.49	0.36 ± 0.24	0.47 ± 0.13
Eosinophil	0.12 ± 0.04	0.14 ± 0.03	0.09 ± 0.08	0.32 ± 0.10	0.05 ± 0.01	0.40 ± 0.05	0.37 ± 0.19	0.39 ± 0.15
Basophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.01

Separate groups of mice were treated as described in Fig. 1A. Blood cells were counted by CBC analysis on Day 3 and are represented as mean ± standard error.

tional activation and regulation of neutrophils. The inhibition of CXCR2 expression leads to decreased neutrophil transmigration and chemotaxis by IL-8 [30]. Inhibition of CXCR2 expression by PLAG suggests that extravasation of circulating neutrophils could be efficiently blocked, preserving the number of circulating neutrophils in the blood. Furthermore, PLAG could be utilized as a therapeutic agent in inflammatory diseases, dramatically improving the progression of inflammation via regulation of neutrophil migration. It is well known that the inflammatory microenvironment may promote cancer malignancy, and chemotherapy often induces chronic inflammation [31,32]. We investigated whether PLAG-suppressed neutrophil transmigration influences pathogenesis using a bacteria-induced inflammation model. When PLAG exhibits a therapeutic effect, which is defined as increased circulation of neutrophils in the CIN model, *Pseudomonas aeruginosa* was administrated intranasally. After 3 h, bacterial CFUs were counted by agar plating of complete bronchoalveolar lavage fluid (BALF) obtained from infected animals. PLAG actively improved the clearance of PA and

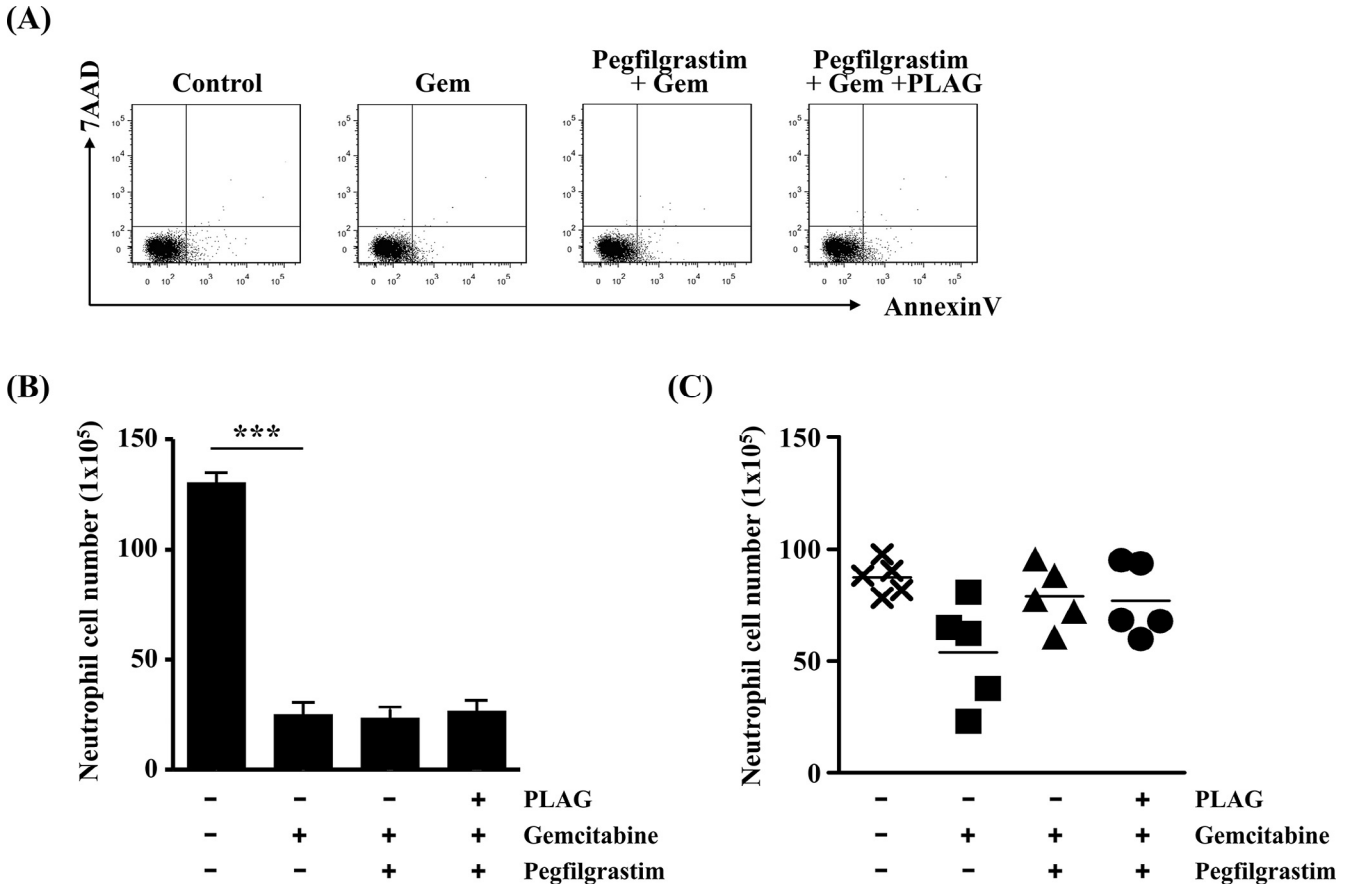

Fig. 3. PLAG had no effect on blood cell apoptosis and neutrophil release from bone marrow. (A) Experimental mouse cohorts were treated as described in Fig. 1A and analyzed on Day 3. The collected blood was stained with FITC-conjugated Annexin V and 7-amino-actinomycin D to assess apoptosis. The experiments were performed in triplicate, and representative images are displayed. (B) After the mice were treated as described in Fig. 1A, bone marrow cells were isolated from femurs and tibias on Day 1. Neutrophils were counted with CBC analysis. The data represent one experiment performed in triplicate. Each group contained three mice, and the averaged values were calculated. ***$p < 0.00$. (C) Neutrophils in bone marrow were counted on Day 3 following treatment as described in Fig. 1A. ×, control; ■, gemcitabine-treated group; ▲, Pegfilgrastim/gemcitabine-treated group; ●, Pegfilgrastim/gemcitabine/PLAG-treated group. Each group contained five mice, and the bars represent the means.

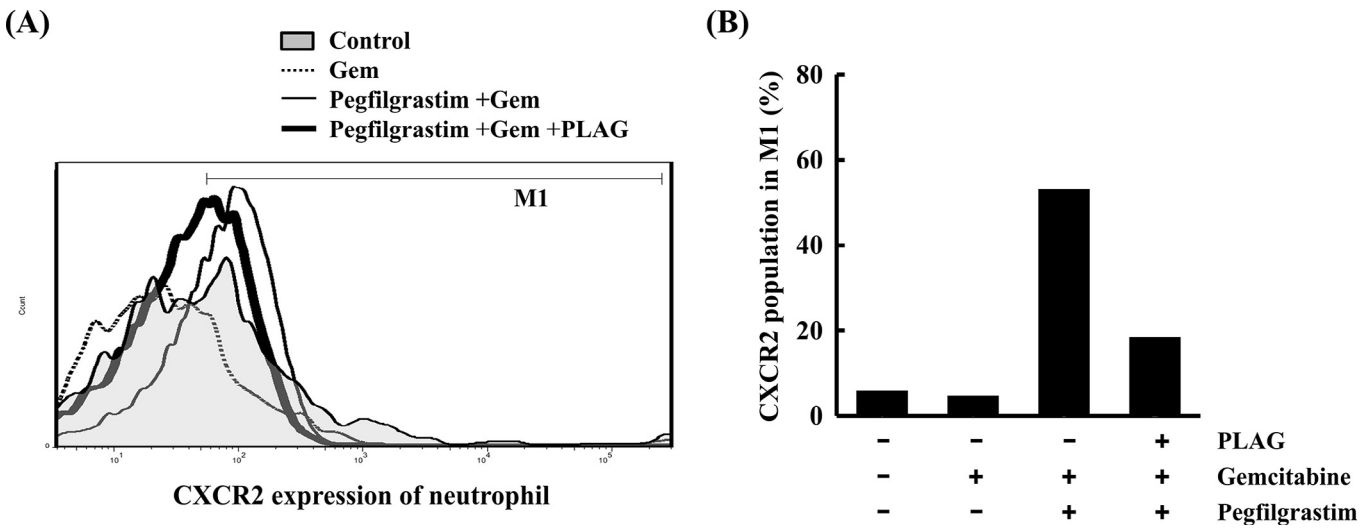

Fig. 4. PLAG decreased CXCR2 expression on neutrophils in blood. (A) After the mice were treated as described in Fig. 1A, flow cytometry analysis was performed using Alexa Fluor 488-conjugated anti-Gr-1, PE-Cy7-conjugated anti-CD11b, and PE-conjugated anti-CXCR2 antibodies. GR-1^{+}CD11b^{+} cells were analyzed for CXCR2 expression. The gray-shaded area represents the control group, and the dotted line represents the gemcitabine-treated group. The pegfilgrastim/gemcitabine-treated group is represented by the thin line. The bold line represents the pegfilgrastim/gemcitabine/PLAG-treated group. (B) CXCR2 population in the M1 region is shown.

without attenuating neutrophil transmigration into the infection site (data not shown). This suggests that the neutrophils maintained in the blood by PLAG treatment were ready to transmigrate into the infection site. From these data, we show that PLAG administration enhances the efficacy of chemotherapy in mice.

As shown in Fig. 5, gemcitabine-induced tissue damage led to increased chemokine levels, resulting in chemotaxis and neutrophil recruitment. Neutrophil extravasation began when gemcitabine was administered, which caused the loss of circulating neutrophils and, therefore, neutropenia. Treatment with pegfilgrastim promoted neutrophil synthesis and motility, including neutrophil release, which resulted in an increased number of circulating neutrophils. PLAG augmented pegfilgrastim's therapeutic effect by inhibiting neutrophil transmigration through CXCR2 modulation. When combined with pegfilgrastim, PLAG may be a therapeutic option for chemotherapy-induced neutropenia that works through modulating neutrophil transmigration.

Acknowledgments

This work was supported by the KRIBB Research Initiative Program (KGM4701511 and KGM5251611), Contract Based Department Program (KFM0191511) by University of Science and Technology, and a grant (IGM0161411 and IGM0081511) from ENZYCHEM Lifesciences.

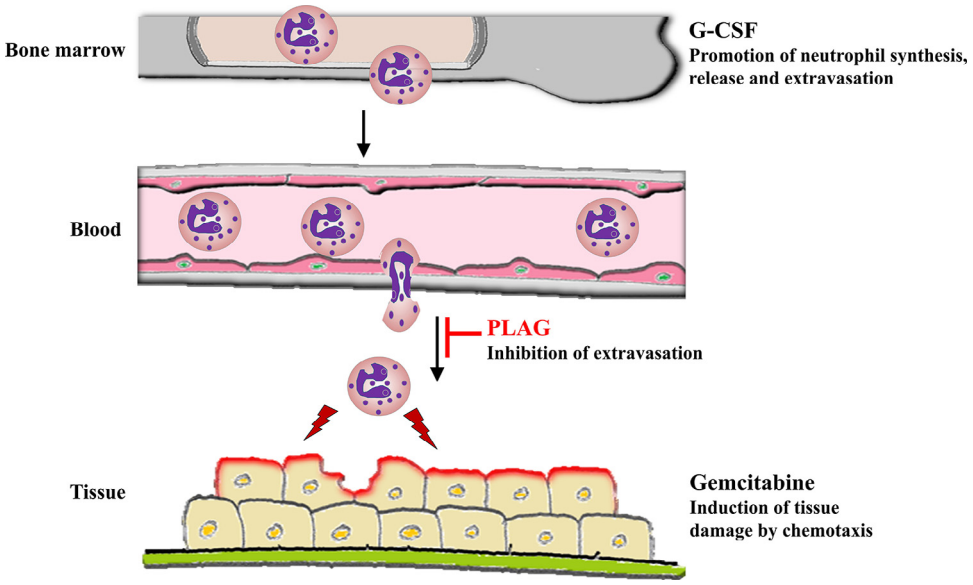

Fig. 5. PLAG protected circulating neutrophils from exaggerated extravasation. Gemcitabine-induced tissue damage triggered neutrophil extravasation and neutropenia. Pegfilgrastim promoted neutrophil synthesis and motility, resulting in an increase in the number of circulating neutrophils. PLAG augmented pegfilgrastim's therapeutic effect by inhibiting neutrophil transmigration through CXCR2 modulation.

Conflict of interest

The authors have declared no conflicting interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.04.025.

References

[1] P.H. Leliefeld, L. Koenderman, J. Pillay, How neutrophils shape adaptive immune responses, Front. Immunol. 6 (2015) 471.
[2] Z. Bian, Y. Guo, B. Ha, K. Zen, Y. Liu, Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions, J. Immunol. 188 (2012) 844–853.
[3] M.L. Pastor, C.M. Laffont, L. Gladieff, E. Chatelut, D. Concordet, Model-based approach to early predict prolonged high grade neutropenia in carboplatin-treated patients and guide G-CSF prophylactic treatment, Pharm. Res. 32 (2015) 654–664.
[4] M. Craig, A.R. Humphries, F. Nekka, J. Belair, J. Li, M.C. Mackey, Neutrophil dynamics during concurrent chemotherapy and G-CSF administration: mathematical modelling guides dose optimisation to minimise neutropenia, J. Theor. Biol. 385 (2015) 77–89.
[5] O.O. Shevchuk, C.A.C. Posokhova capital Ka, I.N. Todor, N.Y. Lukianova, V.G. Nikolaev, V.F. Chekhun, Prevention of myelosuppression by combined treatment with enterosorbent and granulocyte colony-stimulating factor, Exp. Oncol. 37 (2015) 135–138.
[6] M.S. Aapro, J. Bohlius, D.A. Cameron, L. Dal Lago, J.P. Donnelly, N. Kearney, et al., 2010 update of EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphoproliferative disorders and solid tumours, Eur. J. Cancer 47 (2011) 8–32.
[7] J. Crawford, C. Caserta, F. Roila, ESMO Guidelines Working Group, Hematopoietic growth factors: ESMO Clinical Practice Guidelines for the applications, Ann. Oncol. 21 (Suppl. 5) (2010) v248–v251.
[8] E. Petru, C.F. Singer, S. Polterauer, A. Galid, C. Schauer, J. Klocker, et al., Prophylactic long-acting granulocyte-colony stimulating factors (G-CSF) in gynecologic malignancies: an oncologic expert statement, Wien. Med. Wochenschr. 165 (19–20) (2015) 387–394.
[9] G. Srikrishna, H.H. Freeze, Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer, Neoplasia 11 (2009) 615–628.
[10] B.R. Avalos, A. Lazaryan, E.A. Copelan, Can G-CSF cause leukemia in hematopoietic stem cell donors?, Biol. Blood Marrow Transplant. 17 (2011) 1739–1746.
[11] C.L. Semerad, F. Liu, A.D. Gregory, K. Stumpf, D.C. Link, G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood, Immunity 17 (2002) 413–423.
[12] M. Wadhwa, C. Bird, T. Dougall, P. Rigsby, A. Bristow, R. Thorpe, et al., Establishment of the first international standard for PEGylated granulocyte colony stimulating factor (PEG-G-CSF): report of an international collaborative study, J. Immunol. Methods 416 (2015) 17–28.
[13] M. Lambertini, A.R. Ferreira, L. Del Mastro, R. Danesi, P. Pronzato, Pegfilgrastim for the prevention of chemotherapy-induced febrile neutropenia in patients with solid tumors, Expert Opin. Biol. Ther. (2015) 1–19.
[14] H. O. Yang, S.H. Kim, S.H. Cho, M.G. Kim, J.Y. Seo, J.S. Park, et al., Purification and structural determination of hematopoietic stem cell-stimulating monoacetyldiglycerides from Cervus nippon (deer antler), Chem. Pharm. Bull. 52 (2004) 874–878.
[15] H. O. Yang, J.S. Park, S.H. Cho, J.Y. Yoon, M.G. Kim, G.J. Jhon, et al., Stimulatory effects of monoacetyldiglycerides on hematopoiesis, Biol. Pharm. Bull. 27 (2004) 1121–1125.
[16] J.J. Hong, Y. Koh, J.S. Park, H.D. Jung, S.H. Kim, T.S. Lee, et al., Enteral administration of a synthetic monoacetyldiglyceride improves survival in a murine model of abdominal sepsis, J. Trauma 68 (2010) 62–68.
[17] S.Y. Yoon, H.B. Kang, Y.E. Ko, S.H. Shin, Y.J. Kim, K.Y. Sohn, et al., 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (EC-18) modulates Th2 immunity through attenuation of IL-4 expression, Immune Netw. 15 (2015) 100–109.
[18] M. Scholz, M. Ackermann, C. Engel, F. Emmrich, M. Loeffler, M. Kamprad, A pharmacokinetic model of filgrastim and pegfilgrastim application in normal mice and those with cyclophosphamide-induced granulocytopaenia, Cell Prolif. 42 (2009) 813–822.
[19] B.B. Yang, P.K. Lum, M.M. Hayashi, L.K. Roskos, Polyethylene glycol modification of filgrastim results in decreased renal clearance of the protein in rats, J. Pharm. Sci. 93 (2004) 1367–1373.
[20] F. El Ouriaghli, H. Fujiwara, J.J. Melenhorst, G. Sconocchia, N. Hensel, A.J. Barrett, Neutrophil elastase enzymatically antagonizes the in vitro action of G-CSF: implications for the regulation of granulopoiesis, Blood 101 (2003) 1752–1758.
[21] Y. Wu, S. Wang, S.M. Farooq, M.P. Castelvetere, Y. Hou, J.L. Gao, et al., A chemokine receptor CXCR2 macromolecular complex regulates neutrophil functions in inflammatory diseases, J. Biol. Chem. 287 (2012) 5744–5755.
[22] J.S. Kim, Y. Son, M.J. Bae, M. Lee, C.G. Lee, W.S. Jo, et al., Administration of granulocyte colony-stimulating factor with radiotherapy promotes tumor growth by stimulating vascularization in tumor-bearing mice, Oncol. Rep. 34 (2015) 147–154.
[23] K.T. Morris, E.F. Castillo, A.L. Ray, L.L. Weston, R.A. Nofchissey, J.A. Hanson, et al., Anti-G-CSF treatment induces protective tumor immunity in mouse colon cancer by promoting protective NK cell, macrophage and T cell responses, Oncotarget 6 (2015) 22338–22347.
[24] M. Schweizerhof, S. Stosser, M. Kurejova, C. Njoo, V. Gangadharan, N. Agarwal, et al., Hematopoietic colony-stimulating factors mediate tumor-nerve interactions and bone cancer pain, Nat. Med. 15 (2009) 802–807.
[25] K. Obata, H. Yamanaka, Y. Dai, T. Mizushima, T. Fukuoka, A. Tokunaga, et al., Differential activation of MAPK in injured and uninjured DRG neurons following chronic constriction injury of the sciatic nerve in rats, Eur. J. Neurosci. 20 (2004) 2881–2895.
[26] S. Pezet, F. Marchand, R.D. Mello, J. Grist, A.K. Clark, M. Malcangio, et al., Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions, J. Neurosci. 28 (2008) 4261–4270.
[27] C.I. Svensson, M. Marsala, A. Westerlund, N.A. Calcutt, W.M. Campana, J.D. Freshwater, et al., Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing, J. Neurochem. 86 (2003) 1534–1544.
[28] J.T. Xu, H.Y. Tu, W.J. Xin, X.G. Liu, G.H. Zhang, C.H. Zhai, Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats, Exp. Neurol. 206 (2007) 269–279.
[29] T.T. Carvalho, S.M. Borghi, F.A. Pinho-Ribeiro, S.S. Mizokami, T.M. Cunha, S.H. Ferreira, et al., Granulocyte-colony stimulating factor (G-CSF)-induced mechanical hyperalgesia in mice: role for peripheral TNFalpha, IL-1beta and IL-10, Eur. J. Pharmacol. 749 (2015) 62–72.
[30] J.R. White, J.M. Lee, P.R. Young, R.P. Hertzberg, A.J. Jurewicz, M.A. Chaikin, et al., Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration, J. Biol. Chem. 273 (1998) 10095–10098.
[31] F. Balkwill, A. Mantovani, Inflammation and cancer: back to Virchow?, Lancet 357 (2001) 539–545.
[32] A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation, Nature 454 (2008) 436–444.