ORIGINAL ARTICLE

AHI EVRAN MEDICAL JOURNAL
E-ISSN: 2619 - 9203

Cyclophosphamide Exerts an Anti-Metastatic Effect by Reducing the Expression of MMP-2 and -9 in Saos-2 Osteosarcoma Cells

Siklofosfamid, Saos-2 Osteosarkom Hücrelerinde MMP-2 ve -9'un Ekspresyonunu Azaltarak Anti-Metastatik Etki Gösterir

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ÖZ

Amaç: Osteosarkoma, ergenler ve genç yetişkinler arasında en sık rastlanan primer malign kemik tümörü olmakla birlikte, kanser gelişiminin altında yatan moleküler mekanizmalar tam anlamıyla aydınlatılamamıştır. Siklofosfamid (CYC), çeşitli kanserlerin ve kronik inflamatuvar hastalıkların tedavi rejimlerinde oldukça yaygın kullanılan alkilleyici antineoplastik bir ajandır. Bu çalışmanın amacı, CYC'nin Saos-2 hücreleri üzerine olan etkisini kanser progresyonu ilişkili genlere ait bazı matriks metalloproteaz (MMP-2 ve MMP-9) proteinlerinin ekspresyon seviyelerini belirleyerek moleküler düzeyde ortaya koymaktır.
Araçlar ve Yöntem: Geliştirilen insan osteosarkoma (Saos-2) hücre kültürlerinde CYC uygulanarak sitotoksisite analizleri yapıldı. Belirlenen dozda CYC, iki boyutlu hücre hatlarına 12, 24 ve 48 saatlik sürelerde uygulandı. Ajan uygulaması sonucunda bu kültürlerdeki metastatik belirteçler olan MMP'lerin ekspresyon seviyeleri immünositokimyasal yöntem ile belirlendi.
Bulgular: MMP2 ve MMP-9 protein ekspresyon seviyeleri oluşturulan hücre kültürlerinde artan süre ile korele olarak kontrol (PBS) gruplarında artarken; CYC uygulaması ile birlikte azalmıştır.
Sonuç: Saos-2 hücrelerine CYC uygulaması ile MMP-2 ve MMP-9 gibi metastazda rol oynayan genlerin ekspresyon düzeylerini azaltıldığı belirlendi. Bu sonuçlar osteosarkoma kanseri tedavisi için moleküler çalışmalara ışık tutacak niteliktedir.

Anahtar Kelimeler: kanser; Saos-2; siklofosfamid; MMP; osteosarkoma

ABSTRACT

Purpose: Although osteosarcoma is the most common primary malignant bone tumor among adolescents and young adults, the molecular mechanisms underlying the development of cancer are not fully elucidated. Cyclophosphamide (CYC) is an alkylating antineoplastic agent widely used in the treatment regimens of various cancers and chronic inflammatory diseases. The aim of this study is to reveal the effect of CYC on Saos-2 cells at the molecular level by determining the expression levels of some matrix metalloprotease proteins (MMP-2 and MMP-9) of cancer progression-related genes.
Materials and Methods: Cytotoxicity analyzes were performed by applying CYC to the developed human osteosarcoma (Saos-2) cell cultures. The determined dose of CYC was applied to the 2D cell lines for 12, 24 and 48 hours. As a result of agent application, the expression levels of MMPs, which are metastatic markers in these cultures, were determined by the immunocytochemical method.
Results: While MMP-2 and MMP-9 protein expression levels increased in control (PBS) groups in correlation with the extent of duration in the cell cultures created, decreased with CYC administration.
Conclusion: It was determined that the expression levels of genes that play a role in metastasis, such as MMP-2 and MMP-9, were decreased by CYC application to Saos-2 cells. These results shed light on molecular studies for the treatment of osteosarcoma cancer.

Keywords: cancer; cyclophosphamide; Saos-2; MMP; osteosarcoma

Received: 03.07.2022; Accepted: 28.08.2022

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How to cite: Sarıbaş GS. Cyclophosphamide exerts an anti-metastatic effect by reducing the expression of MMP-2 and -9 in Saos-2 osteosarcoma cells. Ahi Evran Med J. 2023;7(1):69-74. DOI: 10.46332/aemj.1140022

INTRODUCTION

Cancer is a pathological condition that occurs as a result of the disruption of the balance between cell death and proliferation due to excessive cell proliferation or reduced apoptosis. It has been reported that suppressed or decreased apoptosis plays an important role in the cancer formation process. The data from the WHO show that as of 2018, 18.1 million new cancer cases have been diagnosed worldwide, and an average of 600.000 deaths due to cancer have occurred. In line with these data, cancer is defined as the second most common cause of death among non-communicable diseases, with an average of 30% death rate.[1] Because different types of cancers grow at different rates and show different ways of spreading, each type of cancer responds differently to treatments. Therefore, different treatments can be applied in the treatment of cancer patients, depending on the type of cancer today. Three main treatment approaches are used in cancer treatment: surgery, radiotherapy and chemotherapy. Sarcoma is a type of cancer originating from the connective tissue (bone, cartilage, fat, nerve) and each develops from cells of mesenchymal cell origin outside the bone marrow.[2,3]

Osteosarcoma (OS) is the most common primary bone cancer in children and adolescents, and its diagnosis is difficult due to the known heterogeneous nature of its pathogenesis. OS has been associated with dysfunctional mutations in tumor suppressors and genetic and cytogenetic abnormalities such as the activation of oncogenes.[4] Osteosarcomas are very common primary malignant bone tumors, containing cells with unusual cellular functions. Typically occurs in the middle of the long bones.[5]

Cyclophosphamide (CYC) is a nitrogenous mustard derivative antineoplastic chemotherapeutic agent, which is basically in the alkylating group. It has a place in clinical use as an immunosuppressive agent at different application doses.[6] CYC is used especially in the treatment of hematological malignancies such as lymphoma, multiple myeloma, and lymphocytic leukemia. It is also used in the treatment of some solid tumors such as neuroblastoma, retinoblastoma and breast cancer.[7]

The aim of this study is to contribute to the elucidation of tumor progression by investigating the effect of CYC on the osteosarcoma cell line Saos-2. In accordance with this purpose, after determining the appropriate dose for CYC, this dose was applied in Saos-2 cultures for 12, 24 and 48 hours. Then, the effects of these applications on the protein expressions of the determined genes were revealed by the immunocytochemical method.

MATERIALS and METHODS

Cell Culture

The osteosarcoma cell lines (Saos-2) were purchased from ATCC (USA). Human osteosarcoma cell lines were grown in 88% RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% Gentamicin and 1% Penicillin antibiotic in flasks at 37°C and 5% CO_2 incubator. When 80% of the flask surface was covered by cells, cells were passaged using trypsin.

Cytotoxicity Assay

XTT assay (Biological Industries, USA) was performed to determine the effects of CYC on viability in Saos-2 cells. $5x10^4$ cells were seeded in a flat-bottom 96 well plate and incubated at 37 °C for 72 h. Then, different doses of the drug were applied to each well, and an activated XTT solution was added after incubation. The formazan dye formed in the wells was measured with the BioTek ELx880 Absorbance Microplate Reader (absorbance measured at wavelength 450 nm). Finally, the dose-response curve was plotted with the calculated viability rates relative to the negative and positive controls. The IC50 value, a measure of drug resistance, was calculated and immunocytochemical experiments were performed at this concentration.

Immunocytochemical Assay

Immunocytochemical staining of cultured Saos-2 cells was performed by Streptavidin-Biotin-Peroxidase method using polyclonal primary antibodies against antigens. Briefly, cells treated with optimal concentrations of agents for different durations (12, 24, 48 hours) were fixed in 4% paraformaldehyde for 20 minutes. After washing the cells with PBS with 0.1% Triton X-100, they were incubated with rabbit polyclonal MMP-2 (bs-0412R, Bioss, USA) and rabbit polyclonal MMP-9 (bs-41146R, Bioss, USA) primary antibodies overnight at 4°C. At the end of the incubation, cells washed with PBS were incubated with the

HRP secondary kit (TP-125-BN, Lab Vision, Thermo Scientific) specific for primary antibodies. Next, cells were stained with aminoethyl carbazole (AEC) chromogen (TA-125-HA, Lab Vision, Thermo Scientific). Nuclear labeling of cells was then performed with hematoxylin. Preparations were photographed under a computer-aided microscope. The regions where the primary antibody reacts with the AEC chromogen are marked in red-brown tones; nuclei of the cells were observed as blue-violet by ground staining with hematoxylin. Immunocytochemical analyzes were performed in 24-well plates. Six wells were allocated for each group. Images were taken randomly from 4 fields from each well. Immune positivity values in the images were determined as a percentage using the Image J program.

Statistical Analysis

Data distribution was evaluated by the Shapiro-Wilk test. Mean, standard deviation (SD), median, minimum, and maximum values were used to define the variables. Column charts were created with the mean and SD values of the immunopositivity of all groups. Independent data that did not show normal distribution were evaluated with the Kruskal-Wallis test. When there was a statistically significant difference among the groups, post-hoc pairwise comparisons were used to identify the different groups. Statistical analyzes were performed using IBM SPSS Statistics 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY). $p<0.05$ was considered statistically significant.

RESULTS

Cytotoxicity Findings

MTT analysis was performed to determine the toxic doses of the CYC. As a result of this analysis, the IC_{50} values of the CYC in Saos-2 cell cultures were determined to be 56.51 µmol/L (Figure 1).

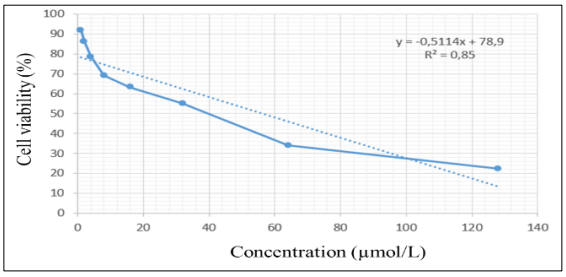

Figure 1. Effect of CYC on cell viability after 24 h incubation in Saos-2 cell line (IC_{50}: 56.51 µmol/L)

Immunocytochemical Findings

When MMP-2 immunopositivity was evaluated in 12-, 24-, and 48-hour Saos-2 cell cultures immunocytochemically (Table 1 and 2, Figures 2 and 3), MMP-2 positivity increased as the incubation period extended in the control (PBS) group. In the CYC-treated groups, MMP-2 immunopositivity levels decreased with the extend in duration. While no significant difference was observed between CYC groups in different time applications; it was statistically significant only between the 12- and 48-hour groups in the control group ($p=0.021$). The 24-hour CYC group showed a statistically significant reduction compared to the control group ($p<0.001$). Likewise, the 48-hour CYC group showed a statistically significant decrease compared to the control group ($p<0.001$).

Table 1. Immunopositivity descriptive values of MMP-2 and MMP-9 in Saos-2 cells of diffe-rent durations (%)

	Groups		Mean±SD	Median	Minimum	Maximum
MMP-2	PBS	12h	60.67±10.89	61	39	82
		24h	76.88±12.13	78.5	53	90
		48h	86.88±7.41	88	63	97
	CYC	12h	36.33±12.57	35	20	59
		24h	20.58±9.16	19.5	9	41
		48h	16.13±8.15	14.5	8	35
MMP-9	PBS	12h	62.96±11.67	62	46	86
		24h	70.83±13.96	73	46	91
		48h	78.54±12.46	81	56	94
	CYC	12h	52.29±14.41	54.5	28	75
		24h	25.33±12.32	24	9	48
		48h	21.25±11.54	16.5	9	42

Table 2. Kruskal-Wallis post-hoc pairwise comparisons

Pairwise groups	Adjusted *p*-value	
	MMP-2	MMP-9
PBS-12h vs. PBS-24h	0.865	1.000
PBS-12h vs. PBS-48h	**0.021**	0.266
PBS-24h vs. PBS-48h	1.000	1.000
CYC-12h vs. CYC-24h	0.690	**0.006**
CYC-12h vs. CYC-48h	0.068	**0.001**
CYC-24h vs. CYC-48h	1.000	1.000
PBS-12h vs. CYC-12h	0.157	1.000
PBS-24h vs. CYC-24h	**<0.001**	**<0.001**
PBS-48h vs. CYC-48h	**<0.001**	**<0.001**

Bolded values remain significant after correction for multiple comparisons

When MMP-9 immunopositivity was evaluated in 12-, 24- and 48-hour Saos-2 cell cultures immunocytochemically (Table 1 and 2, Figures 2 and 4), MMP-9 positivity increased as the incubation period extended in the PBS group. However, in the CYC-treated groups, MMP-9 immunopositivity levels decreased with the extension in duration. The difference between the control groups was not statistically significant in different time applications. This difference was statistically significant between the 12- and 24-hour CYC groups and between the 12- and 48-hour CYC groups (p=0.006; 0.001, respectively). The 24-hour CYC group showed a statistically significant decrease compared to the control group (p<0.001). Likewise, the 48-hour CYC group showed a statistically significant decrease compared to the control group (p<0.001).

In summary, MMP-2 and MMP-9 protein expression levels, which we determined as metastatic markers in cancer development, decreased with CYC application in correlation with the prolonged time in cell cultures.

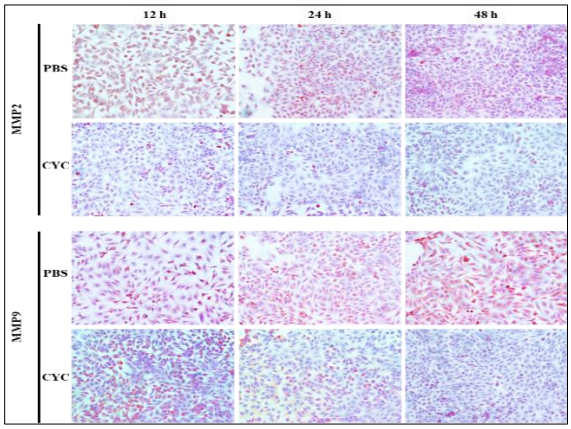

Figure 2. Immunoreactivity of MMP-2 and MMP-9 in Saos-2 cells of different durations (AEC&Hematoksilen) (X100)

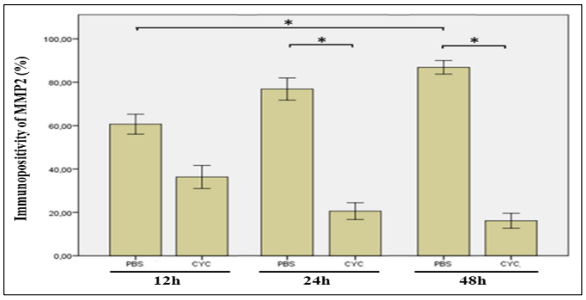

Figure 3. Immunopositivity values of MMP-2 in Saos-2 cells of different durations (n=24, for each group) (*: p<0.05; Bonferroni correction post-hoc tests)

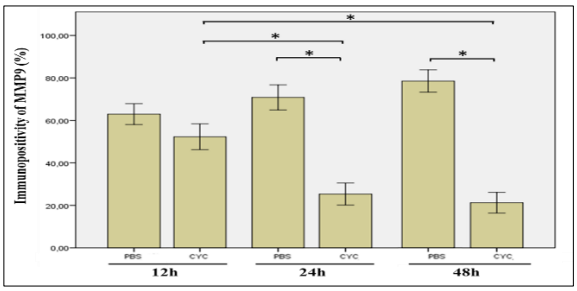

Figure 4. Immunopositivity values of MMP-9 in Saos-2 cells of different durations (n=24, for each group) (*: p<0.05; Bonferroni correction post-hoc tests)

DISCUSSION

Cancer occurs as a result of cells in different parts of our body gaining growth advantage and uncontrolled division as a result of errors at the genetic and epigenetic levels. Genetic changes in oncogenes or tumor suppressors cause abnormalities in various mechanisms such as proliferation, signal transduction, regulation of cell cycle, and apoptosis. Cancer is the second cause of death after cardiovascular diseases both in the world and in our country, and it is becoming a serious public health problem day by day.[2,3]

Osteosarcoma is the most common primary malignant bone tumor among adolescents and young adults. However, the molecular mechanisms underlying disease development have not been fully elucidated. New approaches and alternative applications in disease treatment are promising for treatment success. And many approaches are being studied for this purpose.[8,9]

Cyclophosphamide is a biologically inactive substance and requires metabolic activation. It has two main active metabolites, phosphoramide mustard (FAM) and acrolein. Both of these metabolites are strongly cytotoxic by binding to the macromolecules of the cell. It is thought that FAM inhibits cell division by binding to DNA, and in this way, Cyclophosphamide also mediates its anti-tumor effects. Also, acrolein is thought to play a role in suppressing the immune system. Different effects of formed intermediates

or end metabolites, such as mutagenicity, teratogenicity, genotoxicity, and carcinogenicity were also emphasized.[10,11]

Singh et al.[12] showed that administration of 5 mM CYC in breast cancer cell line MCF-7 cells increased their susceptibility to exposure to apoptosis using flow cytometry time-dependent analysis. In the study, it was also shown that control cells only exhibited basal apoptosis. In addition, a significant increase ($p<0.01$) in the apoptotic fraction was shown after 12 hours in the MCF-7 cell line treated with 5 mM CYC. Also, this apoptotic index increased proportionally with the increase in the application time.

Matrix metalloproteases (MMPs) play a role in tumor metastasis. MMPs can degrade most of the proteins in the extracellular matrix to destroy the tissue barrier against tumor metastasis. MMP-9 and MMP-2 are important subgroups of MMP. MMP-9 and -2 are isozymes with similar mechanisms of action in most cancer tumorigenesis, invasion, and metastasis. In many studies, it has been shown that MMP-2 and MMP-9 levels are significantly higher in patients with various cancers than in healthy individuals or those with benign breast disease.[13-15] In many studies in the literature,[16-18] a significant reduction in tumor weight and size was observed in mice treated with CYC. In the study published by Sun et al.,[16] a significant decrease in protein and mRNA levels of MMP-9 was also revealed in CYC groups. Another report showed that MMP-2 was upregulated and TIMP-2, an inhibitor of MMP-2, was downregulated in the breast cancer cell line with CMF (100 mg/kg cyclophosphamide, 50 mg/kg methotrexate, 100 mg/kg 5-fluorouracil) combined therapy.[19] In our study, the effects of 56.51 μmol/L CYC concentration on the expression levels of MMPs in osteosarcoma SaOs2 cells were examined.

Despite all these study reports, some studies emphasize that CYC contributes to the metastasis process with the synergistic effect of MMPs and some proteins create confusion.

For instance, in a study by Man et al., [20] lung and liver tissues from CYC-treated mice expressed high levels of MMP-2, while tissues from untreated mice expressed high levels of the MMP inhibitor TIMP2. As a result of these findings, the researchers emphasized that CYC may be associated with the secretion of some metastasis proteases.

Hung et al.[21] stated that CYC could increase the metastasis pathway in breast cancer. Hung and team evaluated the metastasis outcomes of CYC in cancer cells and its association with the cancer metastasis marker chemokine receptor 4 (CXCR4). The result of these evaluations showed that increasing CYC concentrations in breast cancer cells induce CXCR4 expression and thus facilitate cell migration. Also, the expression of MMP-9 was elevated in cells treated with CYC.

Zhang et al.[22] also stated in their study that CYC increased MMP-9 levels. They studied the effect of CYC on kidney tissue in rats with diabetes. As a result, they showed that the expression of TGF-β1 was decreased, but the level of MMP-9 increased.

Izdebska et al.[23] observed that CYC could increase MMP-9 expression. In addition, the researchers observed that the administration of CYC alone did not result in a statistically significant anti-tumor effect. In the study, It was emphasized that if the treatment is used together with an agent targeting MMP-9, it may be more effective for CYC treatments.

Although there are studies examining the anticarcinogenic effects of CYC in the literature, studies on MMP-2 and MMP-9, which are the precursors of metastatic markers, are contradictory. The increased metastasis results of CYC, which is widely used for cancer treatment, in some cancer cells are troubling. In our study, in which we investigated the effects of CYC on Saos-2 cells, an immunocytochemical method was used to determine the expression levels of MMP-2 and MMP-9 proteins in cell cultures. Despite conflicting study findings in the literature, we confirmed the antimetastatic effects of 56.51 μmol/L CYC concentration in osteosarcoma Saos-2 cells with reduced expression of MMP-2 and MMP-9. A statistically significant reduction in the expression of MMPs was observed in CYC-treated cells, especially at the 24- and 48-hour periods.

In conclusion, in our study, it was determined that CYC application to Saos-2 cells significantly reduced the expression levels of proteins that play a role in tumorigenesis, such as MMP-2 and MMP-9. The results of this study will shed light on the molecular studies necessary to cope with cancer and will enable to development of current treatment approaches.

Conflict of Interest

The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

Acknowledgments

This work was supported by the Scientific Researches Project Unit at Kirsehir Ahi Evran University [grant number TIP.A4.20.002].

Ethics Committee Permission

Ethics committee approval is not required for the study.

Authors' Contributions

Concept/Design: GSS. Data Collection and/or Processing: GSS. Data analysis and interpretation: GSS. Literature Search: GSS. Drafting manuscript: GSS. Critical revision of manuscript: GSS.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 2018;68(6):394-424.
2. Koutsogiannouli E, Papavassiliou AG, Papanikolaou NA. Complexity in cancer biology: is systems biology the answer? Cancer Med. 2013;2(2):164-177.
3. Yokota J. Tumor progression and metastasis. Carcinogenesis. 2000;21(3):497-503.
4. Ram Kumar RM, Boro A, Fuchs B. Involvement and clinical aspects of microRNA in osteosarcoma. Int. J. Mol. Sci. 2016;17(6):877.
5. Pautke C, Schieker M, Tischer T, et al. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. 2004;24(6):3743-3748.
6. Coggins PR, Ravdin RG, Eisman SH. Clinical evaluation of a new alkylating agent: Cytoxan (cyclophosphamide). Cancer. 1960;13(6):1254-1260.
7. Koyama H, Wada T, Nishizawa Y, et al. Cyclophosphamide-induced ovarian failure and its therapeutic significance in patients with breast cancer. Cancer. 1977;39(4):1403-1409.
8. Messerschmitt PJ, Garcia RM, Abdul-Karim FW, Greenfield EM, Getty PJ. Osteosarcoma. JAAOS- J Am Acad Orthop Surg. 2009;17(8):515-527.
9. Lindsey BA, Markel JE, Kleinerman ES. Osteosarcoma overview. Rheumatol Ther. 2017;4(1): 25-43.
10. Lopez SG, Luderer U. Effects of cyclophosphamide and buthionine sulfoximine on ovarian glutathione and apoptosis. Free Radic. Biol. Med. 2004;36(11):1366-1377.
11. Kawabata TT, Chapman MY, Dong-Hyun K, Stevens WD, Holsapple MP. Mechanisms of in vitro immunosuppression by hepatocyte-generated cyclophosphamide metabolites and 4-hydroperoxy cyclophosphamide. Biochem. Pharmacol. 1990;40(5): 927-935.
12. Singh N, Nigam M, Ranjan V, et al. Resveratrol as an adjunct therapy in cyclophosphamide-treated MCF-7 cells and breast tumor explants. Cancer Sci. 2011;102(5):1059-1067.
13. Sliwowska I, Kopczyński Z. Zymography--method for quantitation of activity on gelatinase A (pro-MMP-2, 72 kDa) and gelatinase B (pro-MMP-9, 92 kDa) in serum of patients with breast cancer. Wiad. Lek. 2007;60(5-6):241-247.
14. Wu ZS, Wu Q, Yang JH, et al. Prognostic significance of MMP-9 and TIMP-1 serum and tissue expression in breast cancer. Int. J. Cancer Res. 2008;122(9):2050-2056.
15. Provatopoulou X, Gounaris A, Kalogera E, et al. Circulating levels of matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL) and their complex MMP-9/NGAL in breast cancer disease. BMC cancer. 2009;9(1):1-7.
16. Sun Z, Yzu T, Chen J, et al. Effects of ulinastatin and cyclophosphamide on the growth of xenograft breast cancer and expression of CXC chemokine receptor 4 and matrix metalloproteinase-9 in cancers. Int. J. Med. Res. 2010;38(3):967-976.
17. Guo H, Ge Y, Li X, et al. Targeting the CXCR4/CXCL12 axis with the peptide antagonist E5 to inhibit breast tumor progression. Signal Transduct. Target. Ther. 2017;2(1):1-8.
18. Qin Y, Zhang Q, Lee S, et al. Doxycycline reverses epithelial-to-mesenchymal transition and suppresses the proliferation and metastasis of lung cancer cells. Oncotarget. 2015;6(38):40667.
19. Paulus P, Stanley ER, Schäfer R, Abraham D, Aharinejad S. Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. Cancer Res. 2006;66(8):4349-4356.
20. Man S, Zhang Y, Gao W, Yan L, Ma C. Cyclophosphamide promotes pulmonary metastasis on mouse lung adenocarcinoma. Clin. Exp. Metastasis. 2008;25(8):855-864.
21. Hung CM, Hsu YC, Chen TY, Chang CC, Lee MJ. Cyclophosphamide promotes breast cancer cell migration through CXCR4 and matrix metalloproteinases. Cell Biol. Int. 2017;41(3):345-352.
22. Zhang Y, Wu G, Hu X, Zhang J. Effect of Cyclophosphamide on Expression of MMP-9 and TGF-β1 in Renal Tissue of Rats with Diabetes Mellitus. Cell Biochem. Biophys. 2015;72(2):399-403.
23. Izdebska M, Zielińska W, Krajewski A, et al. Downregulation of MMP-9 enhances the anti-migratory effect of cyclophosphamide in MDA-MB-231 and MCF-7 breast cancer cell lines. Int. J. Mol. Sci. 2021;22(23):12783.