**In Vitro Effects of Some Chemotherapy Drugs on Glutathione Reductase Enzyme Activity Purified from Sheep Spleen Tissue**

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| **Abstract**In this study, the in vitro effects of some drugs used in chemotherapy on glutathione reductase (GR; EC 1.8.1.7) enzyme activity purified from sheep spleen tissue were investigated. Purification was carried out using homogenate preparation, 20-70% ammonium sulfate precipitation and 2', 5' ADP-Sepharose 4B gel affinity chromatography steps. In the second stage of the study, the effects of some chemotherapy drugs such as ibandronic acid, oxaliplatin, carboplatin and cisplatin on the activity of the pure enzyme were investigated. As a result of the in vitro studies, the Activity%-[I] graph was drawn for the drug oxaliplatin, which has an inhibitory effect on the enzyme, and the IC50 value was calculated as 4.53 mM. In addition, it was detected that the drugs ibandronic acid, carboplatin and cisplatin did not have a significant effect on the enzyme. |
| ***Key Words:*** *Glutathione reductase, Ibandronic acid, Oxaliplatin, Carboplatin* |

1. **Introduction**

Glutathione reductase (E.C. 1.8.1.7; GR) enzyme, which belongs to the oxidoreductase enzyme group, is the most important enzyme that carries out the metabolic reactions of glutathione (GSH) metabolism, which forms the basis of antioxidant metabolism [1]. GSH molecule, which contains most of the free –SH groups in the cell; It consists of γ-glutamine-cysteine-glycine. By transferring the H in the –SH group in this molecular structure to the radical precursors, the radicals are broken down and transformed into water and oxygen, and it itself turns into a reducing form. The glutathione molecule is found in the cell in more than 99% reduced form. The GSH/GSSG ratio is kept at a certain level in the cell. According to research, this ratio for erythrocyte cells is approximately 500/1, and a decrease in this ratio causes hemolysis [2]. The GR enzyme is vital in keeping this ratio constant. For this, the GR enzyme catalyzes the conversion of oxidized glutathione (GSSG) into reduced glutathione (GSH) in the presence of the nicotinamide adenine dinucleotide phosphate (NADPH) molecule produced in the pentose phosphate pathway and malic enzyme step [3]. Thus, the cell is cleared of radicals that attack vital structures such as DNA, especially to prevent aging [4, 5, 6]. The fact that the GR enzyme requires the NADPH coenzyme molecule in its operation reveals the relationship of this enzyme with the glucose 6-phosphate dehydrogenase (G6PD) enzyme. Two molecules of NADPH are formed from each glucose 6-phosphate molecule oxidized in the oxidative part of the pentose phosphate pathway. Therefore, a malfunction in the functioning of the G6PD enzyme will cause the GR to be negatively affected. In this case, it directly affects GSH formation [7, 8]. Many purification and inhibition studies have been carried out on vital enzymes such as GR, which serve at key points of cell metabolism These inhibitors are antibiotics and drugs generally used in human and animal treatment [4, 5, 9]. Our research aimed to investigate the effects of some chemotherapy drugs such as ibandronic acid, oxaliplatin, carboplatin and cisplatin on GR enzyme activity. The main purpose of chemotherapy is to suppress protein production by preventing the transcription of DNA and thus stopping the spread of cancer cells. Increasingly in recent years, chemotherapy drugs have different cytotoxic effects on cells.

Among these drugs, Cisplatin is from the antineoplastic drug group and is the chemotherapy drug with the most known nephrotoxic side effects. In patients taking cisplatin, the rate of nephrotoxicity decreases significantly when hydrated with physiological saline (150-200 mL/hour). Ibandronic acid is used in the prevention and treatment of skeletal fractures associated with cancer-related hypercalcemia and osteolytic bone metastases. Carboplatin belongs to the antineoplastic drug group and is much less nephrotoxic than cisplatin. It is used instead of cisplatin in patients with renal failure. Oxaliplatin, a third-generation platinum analogue, is a cancer drug used to treat colorectal cancer [10].

1. **Materials and Methods**
	1. **Preparation of Hemogenate**

The sheep spleen tissue used in this study was obtained from Bingöl Provincial Combined Meat and Milk Institution according to cold chain rules. For the GR enzyme, 15 grams of tissue was taken and cut into small pieces and suspended in the homogenator with 45 mL of 50 mM KH2PO4 (pH = 8.0) homogenate buffer. The resulting suspension was centrifuged at 10,000xg for 1 hour, then the pellet was discarded to form homogenate [2, 9, 11].

* 1. **2', 5'-ADP Sepharose 4B Affinity Chromatography**

The enzyme sample, which was partially purified after ammonium sulfate precipitation, was purified by 2', 5' ADP Sepharose-4B affinity chromatography. For this method, first an affinity column was prepared. For a bed volume of 10 mL; 2', 5'-ADP sepharose 4B gel was weighed 2 g dry. Then, the gel was washed several times with 400 mL of distilled water to remove solids and the gel was swollen during this time. The air resulting from inflation was removed by vacuuming and the suspension was made by adding equilibration buffer (50 mM KH2PO4/1 mM EDTA, 1mM DTT, pH: 6.0) to the gel. The prepared gel was packed into a refrigerated column and the gel was waited to precipitate. It was understood that the column was balanced by approximately equalizing the absorbance and pH of the eluate and buffer at 280 nm. The enzyme sample was applied to the prepared column. Then, the column was washed with three separate washing solutions of 25 mL each. The column was first washed with 0.1 M K-acetate/0.1 M K-phosphate (pH=6) buffer. Then, 0.1 M K-phosphate/0.1 M KCl (pH=7.85) buffer was used for washing and finally it was washed with 50 mM KH2PO4/1 mM EDTA (pH=7) buffer. The washing process was monitored on a spectrophotometer and the absorbance values were determined to be approximately equal to the blank. After the washing process was completed, the enzyme was eluted with elution buffer (50 mM KH2PO4/1 mM EDTA, 1mM GSH and 0.5 mM NADPH, pH 7.3)[9, 11, 12, 13, 14]. The elutions were placed in 1.5 mL Eppendorf tubes and their activity values were checked.

* 1. **Activity Determination**

To determine the activity, 2 mM NADPH solution, 0.1 M KH2PO4 (pH 8.0) buffer, 20 mM GSSG solution were prepared and 200 μL KH2PO4 buffer, 100 μL GSSG solution, 100 μL NADPH solution, 20 μL homogenate were added to a 1000 μL cuvette. and 580 μL of pure water was added and measurements were made spectrophotometrically at 340 nm. This situation was determined by the decreasing amount of NADPH due to the oxidation of NADPH in the presence of GSSG [6, 15, 16].

* 1. **Kinetic Studies**

In kinetic studies, the effects of some chemotherapy drugs such as ibandronic acid, oxaliplatin, carboplatin and cisplatin on GR enzyme activity purified from sheep spleen tissue were investigated. These drugs were taken in different concentrations and added to the bathtub environment. As a result of activity measurements, trials were made up to the highest possible inhibitor concentration, and the % Inhibition – [I] graph was drawn and the IC50 value was calculated for the drug oxaliplatin, which showed an inhibitory effect.

1. **Results**

In kinetic studies, the effects of some chemotherapy drugs such as ibandronic acid, oxaliplatin, carboplatin and cisplatin on GR enzyme activity at different concentrations were investigated and % Activity-[I] graphs were drawn (Figures 1, 2, 3 and 4). Using the graphic equation, the IC50 value for oxaliplatin, which has an inhibitory effect, was calculated as 4.53 mM. In addition, it was determined that the drugs ibandronic acid, carboplatin and cisplatin did not have a significant effect on the enzyme (Table 1).

 **Figure 1**. Activity%-[I] graph obtained for carboplatin

 **Figure 2.** Activity%-[I] graph obtained for cisplatin

 **Figure 3.** Activity%-[I] graph obtained for oxaloplatin

 **Figure 4.** Activity%-[I] graph obtained for ibandronic acid

 **Table 1**. Obtained IC50 values

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| Drug | IC50 (mM) |
| Oxzaliplatin | 4,53  |

1. **Conclusion**

GR enzyme is a very important enzyme for glutathione metabolism, which plays a major role in the balanced and orderly conduct of biochemical events within the cell by converting oxidized glutathione (GSSG) into the reduced glutathione (GSH) molecule, which is more than 99% in reduced form in the cell [2]. In this metabolism, it is vital for the cell that the GSH/GSSG ratio remains at a certain level. Thus, the continuity of several vital functions of the cell, such as protein and DNA biosynthesis as well as detoxification of reactive oxygen species, is ensured [17]. This is especially important for erythrocytes. In a study, when erythrocytes were exposed to oxidative stress for various reasons, there was a decrease in the enzymes of antioxidant systems such as G6PD and GR. This situation was determined by the increase in malondialdehyde (MDA), which is an indicator of lipid peroxidation [18]. GR enzyme has been purified from many different tissues and its biochemical properties have been examined. In addition, inhibition studies have been conducted on enzyme activity with different drugs and chemicals [6, 13, 19]. In our study, the effects of some chemotherapy drugs such as ibandronic acid, oxaliplatin, carboplatin and cisplatin on GR enzyme activity purified from sheep spleen tissue were investigated. It was determined that the GR enzyme precipitated by 20-70% with the homogenate prepared first in the purification of the enzyme. The precipitation value we found between 20-70% is compatible with the literature review we conducted [6].Additionally, in literature reviews, the ammonium sulphate range of the GR enzyme purified from sheep liver is 0-60% [20], the ammonium sulphate precipitation range of the GR enzyme purified from human and bovine erythrocytes is 30-70% [7]. and the ammonium sulphate range of the GR enzyme purified from sheep brain is 30-70% [7]. The range was found to be 35-55% [21]. In the next stage of purification, 2', 5'-ADP Sepharose 4B affinity chromatography was applied, which provides very fast and high-yield purification [6].After the purification process, kinetic studies were carried out. Here, the IC50 value for the oxaliplatin drug based on GR enzyme activity purified from sheep spleen tissue was calculated as 4.53 mM. In addition, it was determined that the drugs ibandronic acid, carboplatin and cisplatin did not have a significant effect on the enzyme. In a literature review conducted on sheep spleen tissue, the effects of some antibiotics and anti-inflammatory drugs were investigated on GR enzyme activity. These; ampicillin, lincomycin, novamizole, gentamicin, streptomycin sulfate, cefazolin sodium, Cefoperazone sodium, prekort-lyo, amoxicillin, tylosin, cefuroxime sodium, ketogesik and clindamycin. Among these drugs, those that have an inhibitory effect on enzyme activity are prekort-lyo (1.27 mM), ampicillin (3.22 mM), streptomycin sulfate (7.95 mM), cefoperazone sodium (16.97 mM) and gentamicin (17.20 mM). ) Of these; Ampicillin and gentamicin inhibited non-competitively, while streptomycin sulfate, cefoperazone sodium and precort-lyo inhibited competitively [6].

In conclusion; Inhibitors are of great importance for enzyme therapeutic approaches. Since the GR enzyme keeps the vital GSH/GSSG ratio under control, the use of inhibitors that can disrupt this balance by inhibiting the GR enzyme must be controlled.

**References**

1. Taser P, &., Ciftci , M. (2012). Purification and characterization of glutathione reductase from turkey liver. *Turkish Journal of Veterinary ve Animal Science,* 36(5): 546-553.
2. Keha, EE., Küfrevioğlu, Öİ. Biyokimya, Aktif Yayınevi, 6. Baskı, İstanbul, 2010.
3. Demir, H., Erat, M., & Şakiroğlu, H. (2006). In vitro effects of some antibiotics on glutathione reductase obteined from chicken liver. *Turk. J. Vet. Anim. Sci*, (30):513-519.
4. Ciftci, M., Kufrevioglu, OI., Gundogdu, M., & Ozmen, I. ( 2000). Effects of some antibiotics on enzyme activity of glucose-6-phosphate dehydrogenase from human erythrocytes. *Pharmacological Research.* 41(1):109-113.
5. Sevki, A., & Ciftci, M. (2012). Purification of rat kidney glucose 6 phosphate dehydrogenase, 6- phosphogluconate dehydrogenase, and glutathione reductase enzymes using 2ˈ, 5ˈ-ADP sepharose 4B affinity in a single chromatography step. *Protein Expression and Purification.* 81(1):1-4.
6. Çoban Ç., *Glutatyon Redüktaz Enziminin Koyun Dalak Dokusundan Saflaştırılması, Karakterizasyonu ve Bazı Antibiyotiklerin Enzim Aktivitesi Üzerine Etkilerinin Araştırılması* Doktora Tezi, Bingöl Üniversitesi, Fen bilimleri Enstitüsü, Kimya Anabilim Dalı, 2022, s. 88, Bingöl.
7. Erat M., *İnsan ve sığır eritrosit glutatyon redüktaz enziminin saflaştırılması, bazı ilaç ve kimyasal maddelerin inhibisyon veya aktivasyon etkilerinin araştırılması,* Doktora tezi, Atatürk Üniversitesi, Fen Bilimleri Enstitüsü, Kimya Anabilim Dalı, 2002, 112-11, Erzurum.
8. Temel, Y., Bozkuş, T., Karagözoğlu, Y., & Çiftçi M. (2017). Glutatyon redüktaz (GR) enziminin japon bıldırcın (Coturnix coturnix japanica) eritrositlerinden saﬂaştırılması ve karakterizasyonu*. Journal of the Institute of Science and Technology,* 7.3: 143-150.
9. Çoban, Ç., & Çiftci, M. (2022). Bazı İlaçların Koyun Dalak Dokusundan Saflaştırılan Glukoz-6-Fosfat Dehidrogenaz Enzimi Üzerine *İn Vitro* Etkileri. *Türk doğa ve fen dergisi,* 11: 1, 29 – 35
10. Kayaalp, SO. (2002) *Rasyonel tedavi yönünden tıbbi farmakoloji.*10. Baskı. Kitabın muhtelif bölümleri
11. Smith, LL. (1987) Cholesterol autoxidation. *Chem. Phys.* Lipids 44: 87-125
12. Bradford, MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72(1 2): 248–254
13. Temel, Y., & M.Ş. Taysi. 2019. The Effect of Mercury Chloride and Boric Acid on Rat Erythrocyte Enzymes. *Biological Trace Element Research* 191(1).
14. Demir, H., Erat, M., & Şakiroğlu, H. (2006). In vitro effects of some antibiotics on glutathione reductase obteined from chicken liver. *Turk. J. Vet. Anim. Sci*, 30):513-519.
15. Carlberg, I., & Mannervik, B. (1981). Purification and characterization of glutathione reductase from calf liver. An improved procedure for affinity chromatography on 2’, 5’-ADP-Sepharose 4B. *Anal. Biochem*, 116:531–536
16. Çoban, Ç., Teme,l Y., & Çiftci, M. (2022). Koyun Dalak Dokusundan Saflaştırılan Glukoz-6-Fosfat Dehidrogenaz ve Glutatyon Redüktaz Enzimi Üzerine Diklofenak Sodyumun *İn Vitro* Etkileri. *Journal of the Institute of Science and Technology.*  12:3, 1624 – 1634.
17. Çakmak, R., Durdagi, S., Ekinci, D., Şentürk, M., & Topal, G. (2011). Design, synthesis and biological evaluation of novel nitroaromatic compounds as potent glutathione reductase inhibitors. *Bioorganic & medicinal chemistry letters*, 21(18): 5398- 5402.
18. Stocks, J., Offerman, EL., Modell, CB., & Dormandy, TL. (1972) The Susceptibility to Autoxidation of Human Red Cell Lipids in Health and Disease. *Brit J Haematol* 23: 713-24.
19. Ekinci, D., & Şentürk, M. (2013). Assesment of metal inhibition of antioxidant enzyme glutathione reductase from rainbow trout liver. *Journal of Enzyme Inhibition and Medicinal Chemistry* , 28(1): 11-15.
20. Ulusu, NN., & Tandogan, B. (2007). Purification and kinetic properties of glutathione reductase from bovine liver. *Mol Cell Biochem,* 303: 45-51
21. Acan L., & Tezcan, EF. (1989). Sheep brain glutathione reductase: purification and general properties. *FEBS Lett* 250(1): 72-74