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In Vitro Effects Of Some Chemotherapeutic Drugs On Rat Erythrocytes Glutathione S-Transferase (GST) Enzyme

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Abstract: Cancer is the leading cause of death worldwide after heart disease. Currently, breast, lung, bowel, and prostate cancer are the most common cancers in the worldwide. By stopping cancer cells from dividing, spreading, growing, making more cells, and then destroying them, chemotherapy drugs are used to treat diseases caused by cancer. The glutathione S-transferase enzyme is responsible for the detoxification of xenobiotic molecules produced by the body during cancer treatment. In this study, glutathione S-transferase enzyme (GST) was purified from the erythrocytes of rats by affinity column chromatography in one step. The SDS-PAGE (gel electrophoresis) was used to verify the GST enzyme's purity, A single protein band was obtained. The GST enzyme was purified with 22.5 EU/mg specific activity, 237.14 purification-fold, and 48.98% purification yield. Then, the *in vitro* effects of chemotherapy drugs 5-fluorouracil (5-FU) and cyclophosphamide (CP) on purified GST enzyme activity were investigated. The research results showed that both 5-fluorouracil and cyclophosphamide increased GST activity in the concentration ranges of (0.385 to 15.4 mM) and (19.15 to 191.5 mM), respectively.

Bazı Kemoterapötik İlaçların Sıçan Eritrositleri Glutatyon S-transferaz (GST) Enzimi Üzerine *İn Vitro* Etkileri

Anahtar Kelimeler
Sıçan eritrosit, Glutatyon S-transferaz, Saflaştırma, Kemoterapötik ilaçlar, Enzim aktivitesi

Öz: Kanser dünya çapındaki ölümlerin kalp hastalıklarından sonra önde gelen nedenidir. Günümüzde meme, akciğer, bağırsak ve prostat kanseri dünya çapında en sık görülen kanserlerdir. Kemoterapi ilaçları, kanser hastalığının tedavisinde kullanılan ve kanser hücrelerinin bölünmesini, yayılmasını, büyümesini ve daha fazla hücre oluşturmasını durduran ilaçlardır. Glutatyon S-transferaz enzimi, kanser tedavisi sırasında vücudun ürettiği ksenobiyotik moleküllerin detoksifikasyonundan sorumludur. Bu çalışmada sıçan eritrositlerinden glutatyon S-transferaz enzimi (GST, EC: 2.5.1.18) afinite kolon kromatografisi ile tek adımda saflaştırıldı. GST enziminin saflığını control etmek için SDS-PAGE (jel elektroforezi) kullanıldı. Jelde tek bant elde edildi. Saflaştırma işlemi sonucunda GST enzimi 22,5 EU/mg spesifik aktiviteyle ve %48,98 verimle 237,14 kat ile saflıkta elde edildi. Daha sonra 5-florourasil (5-FU) ve siklofosfamid (CP) kemoterapi ilaçlarının saflaştırılan GST enzim aktivitesi üzerindeki *in vitro* etkileri araştırıldı. Araştırma sonuçları, hem 5-florourasil hem de siklofosfamidin sırasıyla (0,385 ila 15,4 mM) ve (19,15 ila 191,5 mM) konsantrasyon aralığında GST aktivitesini arttırdığı belirlendi.

1. INTRODUCTION

Cancer is a large category of illnesses that includes the unnatural growth and division of cells, Cancer is one of the most prevalent diseases, and it causes a lot of deadliness. Advances in cancer prevention and treatment have resulted in longer lifespans or even healing for some patients who have cancer diseases. However, chemotherapy drugs are still required for most patients, and they often cause severe side effects [1]. There are many different chemotherapy drugs used to treat various kinds of cancer diseases, 5-fluorouracil (5-FU) is an anticancer drug used to treat several types of cancer such as breast, lung, skin, and head [2]. 5-FU can enter cells through the uracil transport system, inhibiting thymidylate synthase enzymes and RNA synthesis function, and acts on the S-phase of the cell cycle to cause DNA damage [3]. Cyclophosphamide (CYP) is a chemotherapy drug widely used to treatment of various neoplastic diseases and chronic autoimmune diseases. Cyclophosphamide chemotherapy can damage normal cells in the body, such as the heart, bladder, and testicle. This can lead to multiple organ toxicity [4,5].

Glutathione molecule is a natural antioxidant that plays a major role in neutralizing xenobiotic compounds, this impact is attributable to the capacity of the sulfhydryl (-SH) group on cystine amino acid by donating more electrons and preventing tissue cells from being damaged as associated with the defense of cellular against toxicity. There are two main sources formation of xenobiotic molecules. First, endogenous factors of normal cellular metabolism such as endoplasmic reticulum oxidation, electron transport chain, and most enzymatic activity. Second, exogenous factors such as chemotherapy, radiation, cigarette, and oxygen themselves. The body detoxification process works to out most of the xenobiotic molecules produced by both above sources through the use of group enzymes [6-9]. Glutathione S-transferases (GSTs) are a multigene family of enzymes with about 223 amino acids in total. They are categorized, as alpha, zeta, theta, kappa, mu, pi, sigma, and omega GST isoforms based on their amino acid sequence and specificity of substrates [10]. Glutathione S-transferase can be detected in both eukaryotic and prokaryotes, and work to detoxify xenobiotic compounds from exogenous and endogenous living cells by catalyzing glutathione natural anti-oxidant molecule reactions with xenobiotics, changing toxic molecules to non-toxic metabolizable molecules and excretion from the body. Glutathione S-transferase is an important enzyme that helps to detoxify harmful compounds by catalyzing the conversion of glutathione-toxic compounds into non-harmful substances [8,11–13].

The purpose of this study is to purify glutathione S-transferase (GST) enzyme from rat erythrocytes and investigate the effects of 5-fluorouracil and cyclophosphamide chemotherapy drugs on enzyme activity from in vitro, possibly the results of this study could help improve the treatment of cancer disease and have benefits in toxicology systems, the clinical cancer research community must collaborate and focus on new research that uses comprehensive results to determine the best way to treat cancer diseases.

2. MATERIAL AND METHOD

2.1 Materials

Reduced glutathione (GSH), 5-FU (5-Fluorouracil), ethylene diamine tetra acetic acid (EDTA), β-mercaptoethanol, 1-chloro-2,4-dinitrobenzene (CDNB), TEMED (N, N, N, N-tetramethyl-ethylenediamine), acrylamide, Tris (Trihydroxy methyl amino methane), and glutathione-agarose affinity gel got from Sigma-Aldrich (Sigma-Aldrich and MERCK, Darmstadt, Germany). Ammonium persulfate (Chem Solute Bio). CYP (Endoxan) was purchased from a pharmacy (Istanbul, Turkiye). Glycerol, isopropanol, Ammonium sulfate, stacking gel, separation gel, separation buffer, Coomassie Brilliant Blue R-250, paint solution, and fixing solution (Fishcer Scientific).

2.2 Methods

2.2.1 Preparation of homogenate

The blood sample of the rat was obtained from the Bingöl University Experimental Research Center and brought the sample to the biochemistry laboratory in anticoagulant tubes, it was centrifuged at (4 oC, 2,500 Xg, for 15 min), discarded plasma and saved rat erythrocytes were in the refrigerator at -20 °C according to the cold chain rule. The rat erythrocyte sample was washed with KCl solution (0.16 M) and centrifuged at (4 °C in 2,500 Xg for 15 min) three times repeating this step, and the erythrocyte cells were hemolyzed with ice water in the ratio of (1: 5 = erythrocyte: ice water), then centrifuged at (4oC in 10,000 Xg for 60 min), finally, 3.5 mL supernatant was saved for purification of GST and the precipitate was discarded [14–16]. The study was designed and conducted according to ethical norms approved by the Animal Experimentation Ethics Committee of the Bingol University (Bingol, Turkiye) (Protocol No. 2019-85680299/020).

2.2.2 GST enzyme activity determination

The activity of glutathione S-transferase enzyme was measured by monitoring the absorbance of 2,4-dinitrophenyl glutathione product of a reaction between 1,2-dichloro-4-nitrobenzene (CDNB) and reduced glutathione (GSH) at 340 nm in a spectrophotometer (Shimadzu UV-1601, Australia) [17–19].

2.2.3 Applied glutathione-agarose affinity chromatography for Purification of GST enzyme

The affinity column chromatography of glutathione-agarose was prepared and the flow rate of the column was adjusted by using a peristaltic pump to 20 mL/h. Next using (10 mM KH_2PO_4 and 150 mM NaCl) equilibration solution to adjust the pH = 7.4 of the column. Then the hemolytic erythrocyte sample was placed on the column and washed column with (10 mM KH_2PO_4 and 0.1M KCl,

pH = 8.0) buffer solution. Then, continued washing process until the absorbance of the column eluate at 280 nm was 0.05. Finally, using gradient elution purifies the GST enzyme. The gradient elution solution consists of (1.25-10 mM GSH and 50 mM Tris-HCl, pH = 9.5) and using Eppendorf tubes (1.5 mL) to collect eluates and, absorbance was measured spectrophotometrically at 340 nm [13, 18, 20].

2.2.4 Protein assay

The qualitative protein was tested by measuring the absorbance of tyrosine and tryptophan amino acids in the protein structure at 280 nm. standard bovine serum albumin was used to determine quantitative protein by measuring absorbance spectrophotometrically at 595 nm based on the Bradford method [21, 22].

2.2.5 Applied SDS-PAGE to control enzyme purity

The pure glutathione S-transferase enzyme was examined by gel electrophoresis (SDS-PAGE) method, and the purified GST enzyme was seen as a single band of protein on the SDS-PAGE, according to the Laemmle procedure [23].

2.2.6 *In vitro* investigation of chemotherapeutic drugs

The *in vitro* effect of chemotherapy drugs on the Glutathione S-transferase enzyme activity in rat erythrocytes was determined by adding different concentrations of 5-fluorouracil (0.385 to 15.4 mM) respectively and different concentrations of Cyclophosphamide (19.15 to 191.5 mM) respectively into the reaction medium in the cuvette and measured absorbance spectrophotometrically at 340 nm. The absorbance of absence drugs used as control (%100 activity). Graphs of % activity against drug concentrations were drawn using MS Excel program.

3. RESULTS

Glutathione S-transferase (GSTs) enzyme of phase II detoxification process that works to protect cellular organs from attack xenobiotic reactive molecule, It acts as a catalyst for the conjugation of the glutathione (GSH) molecule to numerous electrophilic molecules, both endogenous and exogenous, the conjugation reaction of glutathione involve in the first step of the mercapturic acid pathway causes the elimination of the toxic compound [24].

The G6PD enzyme functions in the pentose phosphate pathway and produces NADPH [25,26]. NADPH ensures that GSH in the cell remains in its reduced form [27]. Glutathione is linked to defense against some cancer etiology as it is the principal intracellular antioxidant, detoxifies several carcinogens through phase II conjugation, and maintains immunological function by controlling the mitogenic response and lymphocyte proliferation [28]. Among the enzymes that shield organ cellular structures from damage caused by carcinogens and toxic chemicals, glutathione S-transferase is the most significant enzyme, It is important for detoxifying endogenous and exogenous toxic substances. GST enzyme catalyzes the reaction between glutathione and electrophile toxic molecules to form glutathione S-conjugates, which are crucial for the deactivation and subsequent excretion of xenobiotic molecules [29].

The purification process of the Glutathione S-transferase enzyme from rat erythrocytes was carried out by applying the GSH-agarose affinity column chromatography method. It is a one-step process that is powerful, simple to perform, inexpensive, and takes less time. Portable and very effective for large quantities of enzymes that have been purified. The same method was applied to purify GST enzyme from human hepatoma [29], catfish intestinal mucosa [30], rainbow trout liver [31], and the freshwater fish Monopterus albus's liver [32], erythrocytes of children with Down syndrome (DS) and healthy children [33], from rat liver [34], Van Lake fish muscle tissue [23], and the human erythrocyte [12]. GST Enzyme activity measured during the purification process is a significant key and helps to continue the study.

In this study, purification of rat erythrocyte glutathione S-transferase (GST) enzyme was carried out with 22.5 EU/mg specific activity, 237.14 purification-fold, and 48.98% purification yield, by a one-step of GSH-agarose affinity column chromatography (Table 1).

Table 1. Purification table of GST enzyme in the rat erythrocyte

Purification Steps	Total Volum (mL)	Activity (EU/mL)	Protein (mg/mL)	Total Activity (EU)	Total Protein (mg)	Specific Activity (EU/mg)	Yield %	Purification Fold
Hemolaysate	3.5	0.315	3.32	1.105	11.62	0.095	100	1
Glutathione agarose affinity chromatography	2	0.27	0.012	0.541	0.024	22.569	48.98	237.142

The results were compared with GST purification from the human placenta tissues with 23.7 EU/mg specific activity, 11% yield, and 1107 folds [35], from the turkey liver 164.31 U/mg specific activity, 45% yield and 252.7-folds [36] and human erythrocytes 16.2 EU/mg specific activity, 35% yield and 265.97-folds [12]. The result of this study is near to the result of the human erythrocytes study it is a significant point.

The SDS-PAGE method was used to verify the GST enzyme's purity. A single protein band of the purified GST enzyme appeared on the SDS-PAGE, which revealed the enzyme was successfully purified and allowed the study to continue (Figure 1).

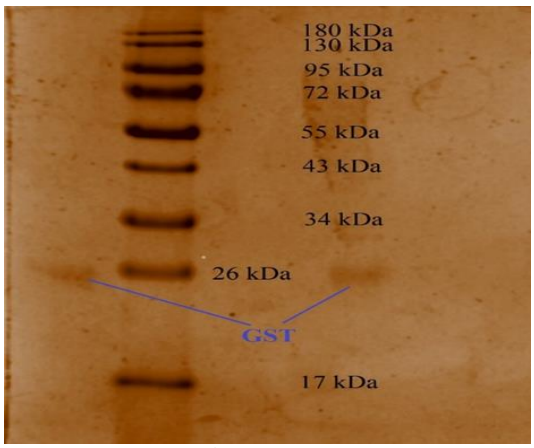

Figure 1. SDS-PAGE of rat erythrocytes GST enzyme.

The same way is used in the human erythrocytes [12], human hepatoma [29], rat liver [34] and brain cytosol of rats [37]. The study's findings demonstrate that GST enzymes in rat erythrocytes have been successfully separated from other enzymes, which is a positive result that should encourage researchers to continue with additional research steps.

In vitro, study to investigate the chemotherapy drug effects on the purified GST enzyme activity from the rat erythrocyte was performed, and the results show both 5-Fluloruracil and Cyclophosphamide chemotherapy drugs increased the GST enzyme activity from the concentration range 0.385 to 15.4 mM (Figure 2) and 19.15 to 191.5 mM (Figure 3) respectively.

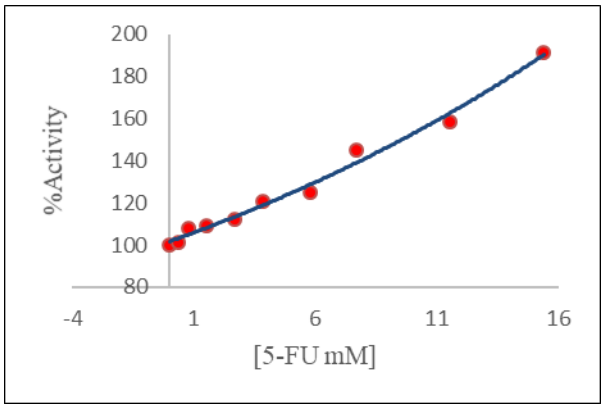

Figure 2. In vitro effect of 5-Fluorouracil on GST enzyme activity

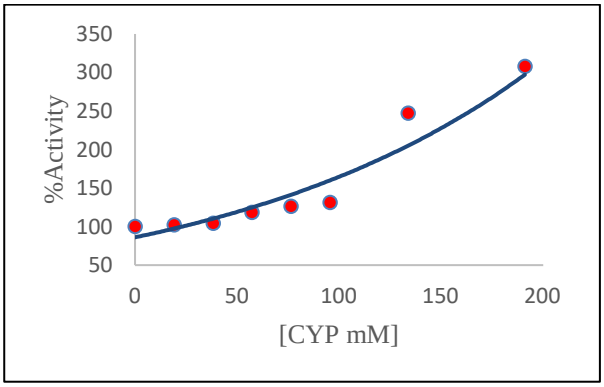

Figure 3. *In vitro* effect of cyclophosphamide on GST enzyme activity

4. DISCUSSION AND CONCLUSION

The results indicated that both drugs have a positive effect by increasing GST enzyme activity when the concentration of the drugs is increased. This is crucial to the field of pharmacology because it helps scientists to understand how drugs affect the biological system in living cells and how the body's living cells react particularly chemotherapy drugs, which have a long list of side effects when used to treat cancer. These side effects make patients more likely to experience complications and eventually destroy their bodies. Researchers must work with utmost sincerity, patience, and honesty to get information about drugs and treatment ways to improve pharmacologist science. Comparing this study's findings to those of other studies, it was found both 5-FU and tamoxifen chemotherapy drugs increase the GST enzyme activity that is purified from erythrocytes of humans [12]. 5-FU and tamoxifen chemotherapy drugs increase the activities of 6PGD and G6PD enzymes [38]; the GST enzyme from human erythrocytes was found to be inhibited by the effects of paclitaxel, cyclophosphamide, and gemcitabine [39].

Glutathione S-transferase enzyme purification was carried out in the rat erythrocytes in one step by using GSH-agarose affinity column chromatography. The purified enzyme was examined using SDS-PAGE. In the second phase, the effects of 5-fluorouracil and cyclophosphamide chemotherapeutic drugs on enzyme activity were investigated. The findings indicate that both drugs increase GST enzyme activity. This study results may be a guide for purification studies on the GST enzyme and chemotherapy approaches whose target is the GST enzyme.

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