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In vitro effects of some drugs on human erythrocyte glutathione reductase

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Aim: The effects of dacarbazine, thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil on the enzyme activity of glutathione reductase (GR) were studied using human erythrocyte GR enzymes in in vitro studies, respectively.

Materials and methods: The enzyme was purified 3333-fold from human erythrocytes in a yield of 44.44% with 4.0 U/mg. The purification procedure involved the preparation of hemolysate, ammonium sulfate precipitation, 2', 5'-ADP Sepharose 4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. SDS-PAGE showed a single band for enzyme. Purified enzyme was used in the in vitro studies.

Results: In the in vitro studies, IC50 values and Ki constants were 0.014 mM and 0.0121 ± 0.0026 mM for dacarbazine, and 0.054 mM and 0.0135 ± 0.0024 mM for thiocolchicoside, 0.31 mM and 0.1213 ± 0.040 mM for methotrexate, 0.35 mM and 0.3173 ± 0.0981 mM for olanzapine, 0.94 mM and 0.5413 ± 0.0728 mM for pantoprazole sodium, and 98.57 mM and 69.611 ± 9.690 mM for 5-fluorouracil, showing the inhibition effects on the purified enzyme. Inhibition types were non-competitive for dacarbazine, methotrexate, olanzapine, and 5-fluorouracil and competitive for thiocolchicoside and pantoprazole sodium.

Conclusion: All the drugs indicated the inhibitory effects on the enzyme.

Key words: Glutathione reductase, dacarbazine, thiocolchicoside, methotrexate

Bazı ilaçların insan eritrosit glutatyon redüktaz enzimi üzerine etkileri

Amaç: Bu çalışmada dekarbazin, tiyokolsikosid, metotreksat, olanzapin, sodium pantoprozol ve 5-florourasil ilaçlarının insan eritrositlerinden saflaştırılan glutatyon redüktaz enzimi (GR) üzerine in vitro etkileri araştırıldı.

Yöntem ve gereç: Bu amaçla 4 EÜ/mg protein spesifik aktivitesine sahip olan enzim sırasıyla hemolizatın hazırlanması, amonyum sülfat çöktürmesi, Sephadex G-200 jel filtrasyon kromatografisi ve 2',5'-ADP Sepharose 4B afinite kromatografisi ile % 44,44 verimle 3333 kat saflaştırıldı. Bu dört basamak sonucu elde edilen saf enzimin saflık kontrolü sodyum dodesilsülfat poliakrilamid jel elektroforezzi (SDS-PAGE) ile yapıldı. Tüm kinetik çalışmalarda saflaştırılmış enzim kullanıldı.

Bulgular: Enzim üzerinde inhibisyon etkisi gösteren dekarbazin, tiyokolsikosid, metotreksat, olanzapin, sodium pantoprozol ve 5-florourasil ilaçları için sırasıyla IC50 değerleri ve Ki sabitleri 0,014 ve 0,0121 ± 0,0026; 0,054 ve 0,0135 ± 0,0024; 0,31 ve 0,1213 ± 0,040; 0,35 mM ve 0,3173 ± 0,0981; 0,94 ve 0,5413 ± 0,0728; 98,57 ve 69,611 ± 9,690; olarak hesaplandı. Ayrıca inhibisyon çeşidi çalışmaları sonucu tüm ilaçların enzimi yarışmasız olarak inhibe ettikleri tespit edildi.

Sonuç: Kullanılan tüm ilaçların enzim üzerinde inhibisyon etkisi tespit edildi.

Anahtar sözcükler: Glutatyon redüktaz, dekarbazin, tiyokolsikosid, metotreksat

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Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) has many important functions. It is an antioxidant, is involved in the detoxification of xenobiotics, and serves as a cofactor in isomerization reactions (1). Glutathione has an important role in the synthesis and degradation of proteins, regulation of enzymes, formation of the deoxyribonucleotid precursors of deoxyribonucleic acid (DNA) and protection of cells against free radicals and reactive oxygen species (2). Responsible for the reduction of GSSG to GSH is the enzyme glutathione reductase (GR; NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2), a flavoprotein which uses NADPH as electron donor for the reduction reaction. GR enables several vital functions of the cell, such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis, by maintaining a high ratio of GSH/GSSG (3). Decreased glutathione levels have also been reported in several diseases, such as acquired immune deficiency syndrome (AIDS) (4), Parkinson's disease (5), and diabetes (6,7). A major role of GSH in erythrocytes is the prevention of hemoglobin denaturation, preserving the integrity of erythrocyte membrane sulfhydryl groups and detoxification of the xenobiotics and reactive oxygen species in red blood cells (8).

Many drugs have activation or inhibition effects on the body's enzyme systems (9-11). Many drugs may show the same effects under both in vivo and in vitro conditions; however, some of them may not show the same effects on enzymes (12-14). Both fluorouracil (5-FU) and dacarbazine are used for treatments of some different cancer diseases. Methotrexate is used in cancer chemotherapy. Contrary to these 3 drugs, thiocolchicoside is used for symptomatic treatment of painful muscle spasms; and while pantoprazole sodium is a proton pump inhibitor drug used for short-term treatment of erosion and ulceration of the esophagus caused by gastroesophageal reflux disease olanzapine is a novel antipsychotic agent (15).

We have not encountered any studies on the effects of dacarbazine, thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil on human erythrocyte GR activity. The objective of the present study was to investigate any possible in vitro effects of these drugs on purified human erythrocyte GR.

Materials and methods

Materials

2', 5'-ADP Sepharose 4B was purchased from Pharmacia. Sephadex G-200, NADPH, GSSG, the protein assay reagent and the chemicals for electrophoresis were purchased from Sigma Chem. Co. (Germany). All other chemicals used were analytical grade and purchased from either Merck or Sigma (Germany).

Preparation of the hemolysate

Fresh human blood from one single subject was collected into a tube containing 0.1 M Na-citrate, 0.16 M glucose, 0.016 M Na-phosphate, and 2.59 mM adenine for anticoagulation. The sample was centrifuged at 3000 × *g* for 15 min. Plasma and leukocyte coat were removed. The erythrocytes were washed three times with isotonic NaCl solution including 1 mM EDTA, the samples being centrifuged at 3000 × *g* each time and the supernatants removed. The washed erythrocytes were hemolyzed with 5 volumes of ice-cold distilled water containing 2.7 mM EDTA and 0.7 mM β-mercaptoethanol, and then centrifuged at 4 °C, 20,000 × *g* for 30 min to remove residual intact cells and membranes (16).

Ammonium sulfate fractionation and dialysis

The hemolysate was subjected to precipitation with ammonium sulfate (between 30% and 70%). Enzyme activity was determined both in the supernatant and in the precipitate for each respective precipitation. The precipitate was dissolved in phosphate buffer (50 mM, pH 7.0). The resultant solution was clear, and contained partially purified enzyme. This solution was dialyzed at 4 °C in 1 mM EDTA + 10 mM K-phosphate buffer (pH 7.5) for 2 h with 2 changes of buffer (12). Partially purified enzyme solution was kept at 4 °C.

2',5'-ADP Sepharose 4B affinity chromatography

Dry 2', 5'-ADP Sepharose 4B (2 g) was washed and swelled in distilled and deionized water. After removal of the air in the gel, it was resuspended in 0.1 M Kacetate/0.1 M K-phosphate buffer, pH 6.0, and packed into a small column (1 × 10 cm). After precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump with the

flow rate adjusted to 20 mL/h. The dialyzed sample obtained from ammonium sulfate precipitation was loaded onto the column, and washed with 25 mL of 0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0 and 25 mL of 0.1 M Kacetate + 0.1 M K-phosphate, pH 7.85. Washing continued with 50 mM of K-phosphate including 1 mM EDTA (pH 7.0) until the final absorbance difference was 0.05 at 280 nm. Proteins bound on the gel were eluted with a gradient of 0-0.5 mMGSH and 0-1m M NADPH in 50 mM K-phosphate, containing 1 mM EDTA (pH 7.0). Active fractions were collected and dialyzed with 50 mM of K-phosphate including 1 mM EDTA (pH 7.0) at 4 °C (17-20).

Sephadex G-200 gel filtration chromatography

Dry Sephadex G-200 (5 g) was incubated in distilled water at 90 °C for 5 h. After removal of the air in the gel, it was loaded onto a column (2 × 50 cm). The flow rate was adjusted to 15 mL/h by means of a peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, and 50 mM KCl buffer (pH 7.0) until the final absorbance difference became 0 at 280 nm and the pH was the same as that of the equilibration buffer. The dialyzate from the affinity chromatography column was mixed with glycerol in a proportion of 5%. The final sample was loaded onto the column and 2 mL of each elution was collected. The absorbance values were determined at 280 nm and 340 nm in each fraction (11). Active fractions were lyophilized and stored at -85 °C for use in the in vitro studies.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the enzyme's purity, SDS-PAGE was performed according to Laemmli's method (21). The acrylamide concentration of the stacking and separating gels was 3% and 8%, respectively, and 1% SDS was also added to the gel solution. The gel was stained for 2 h in 0.1% Coomassie Brilliant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water. Then the gel was washed with many changes of the same solvent without dye. Cleared protein bands were photographed (Figure 1).

Activity assay

Glutathione reductase activity was determined by the method of Carlberg and Mannervik (22) with a Shimadzu Spectrophotometer UV-(1208) at 25 °C. The assay system contained 40 mM Tris-HCl buffer, pH 8.0, including 0.8 mM EDTA, 1 mM GSSG, and 0.1 mM NADPH in 1 mL total reaction volume. The activity was measured by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH at 25 °C. One enzyme unit is defined as the oxidation of 1 μmol NADPH per min under the assay conditions.

Protein determination

Quantitative protein determination was performed spectrophotometrically at 595 nm according to Bradford's method (23), using bovine serum albumin as a standard. Qualitative protein determination was also performed spectrophotometrically at 280 nm according to Segel's method (24).

In vitro drug studies

Dacarbazine, thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil tested to determine their effects on human erythrocyte GR activity. Assays were performed in cuvette concentrations of 0.004-0.053 mM dacarbazine, 0.035-0.14 mM thiocolchicoside, 0.022-1.1 mM methotrexate, 0.16-0.96 mM olanzapine, 0.24-1.96 mM pantoprazole sodium, and 19.22-

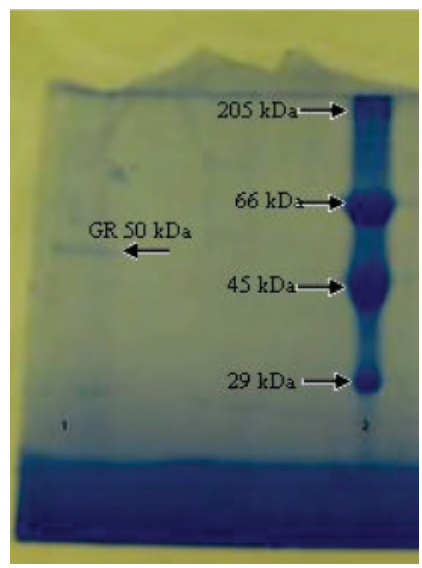

Figure 1. SDS-PAGE photograph: Lane 1, purified enzyme from Sephadex G-200 gel filtration. Lane 2, standard proteins: rabbit myosin (205 kDa), bovine carbonic anhydrase-I (29 kDa), egg albumin B (45 kDa), bovine albumin (66 kDa).

192.28 mM 5-fluorouracil. The enzyme activity in the absence of drug was used as a control (100% activity). The IC_{50} values were obtained from activity (%) vs. drug concentration plots and regression analysis graphs were drawn by a statistical package (SPSS for Windows; version 10.0) (Student's t-test; n = 3). In order to determine Ki constants for the inhibitors, the substrate (GSSG) concentrations were 0.03, 0.08, 0.14, 0.2, and 0.3 mM. Inhibitors (drugs) solutions were added to the reaction medium, at 3 different fixed concentrations of inhibitors in 1 mL of total reaction volume. Lineweaver-Burk graphs (25) were drawn by using 1/V vs. 1/[S] values and Ki constant were calculated from these graphs.

Results

The purification of the enzyme led to a specific activity of 4 EU/mg protein, a yield of 44.44% and a purification coefficient of 3333. SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern was photographed (Figure 1). IC_{50} values of dacarbazine, thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil were 0.014, 0.054, 0.31, 0.35, 0.94, and 98.57 mM, respectively, and the Ki constants were 0.0121 ± 0.0026, 0.0135 ± 0.0024, 0.1213 ± 0.040, 0.3173 ± 0.0981, 0.5413 ± 0.0728, and 69.611 ± 9.690 mM respectively (Table). Representative graphs are shown for dacarbazine (Figures 2 and 3). Inhibition types were non-competitive for dacarbazine, methotrexate, olanzapine, and 5-fluorouracil and competitive for thiocolchicoside and pantoprazole sodium.

Discussion

Enzymatic defense is provided by many enzyme systems such as glutathione reductase, glutathione peroxidase, glutathione S transferase (GST), superoxide dismutase, catalase, aldoketoreductase, and DNA repair enzymes (25). Glutathione reductase catalyzes the reduction of GSSG in the presence of NADPH, with the latter being produced in the pentose phosphate metabolic pathway. GSH is a product of the reaction catalyzed by glutathione reductase, and the GSH-related enzyme system is one of the most important protective systems in cells. Reduced glutathione is used in the detoxification of xenobiotics, protection of the thiol groups of intracellular proteins, scavenging of H_2O_2 and other organic peroxides, and counteraction of oxidative events. The first step, in the detoxification of xenobiotics, consists of GSH conjugates owing to its high nucleophilic potency (27).

Many chemicals and drugs are known to have adverse or beneficial effects on human enzyme and metabolic events, and the effects can be dramatic and systemic (28). The inhibition of some important enzymes plays a key role in a metabolic pathway, e.g. some metabolic diseases are affected by enzyme activity (29). Similarly, acetazolamide has an inhibitory effect on the carbonic anhydrase (CA) enzyme leading to diuresis (30). Additionally, epiandrosterone was found to inhibit red blood cell glucose 6-phosphate dehydrogenase (G6PD) uncompetitively and suppress hexose monophosphate shunt activity by more than 95% (31). Some chemicals and drugs, such as nitrofurazone,

Table. Ki constants and IC_{50} values obtained from regression analysis graphs for GR in the presence of different drugs.

Drugs	IC_{50} (mM)	Ki (mM)	Inhibition type
Dacarbazine	0.014	0.0121 ± 0.0026	Non-competitive
Thiocolchicoside	0.054	0.0135 ± 0.0024	Competitive
Methotrexate	0.310	0.1213 ± 0.0400	Non-competitive
Olanzapine	0.350	0.3173 ± 0.0981	Non-competitive
Pantoprazole Sodium	0.940	0.5413 ± 0.0728	Competitive
5-Fluorouracil	98.57	69.611 ± 9.6900	Non-competitive

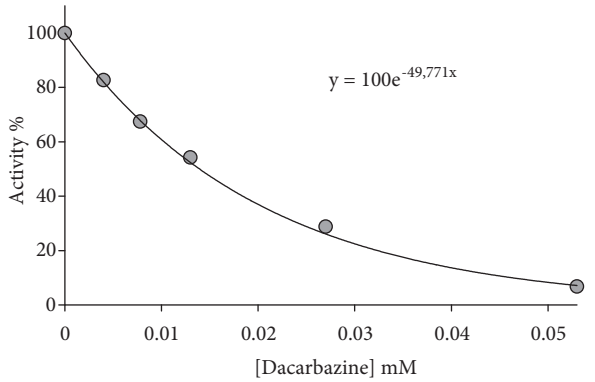

Figure 2. Activity % vs [Dacarbazine] regression analysis graphs for human erythrocytes GR in the presence of 5 different dacarbazine concentrations.

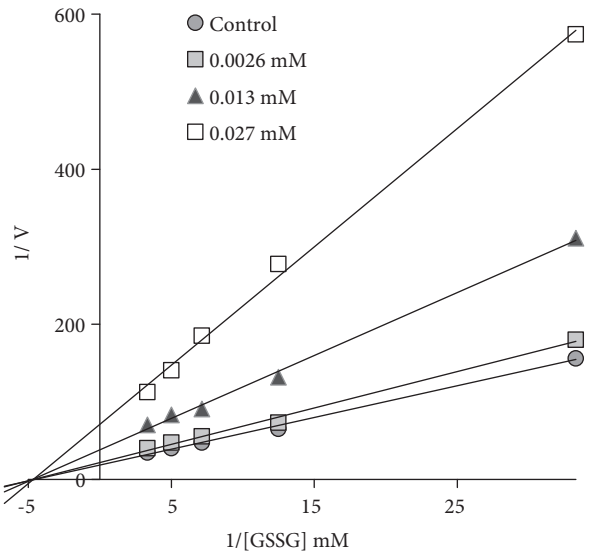

Figure 3. Lineweaver-Burk graph with 5 different substrate (GSSG) concentrations and 3 different dacarbazine concentrations for determination of Ki.

nitrofurantoin, 5-nitroindol, 5-nitro-2-furoic acid, 2, 4, 6-trinitrobenzene sulfonate (TNBS) (32), and polyphenolic compounds, also inhibit GR enzyme activity (32). Furthermore, it is reported that human erythrocyte GR, human erythrocyte CA-I and CA-II, human erythrocyte G6PD and human erythrocyte 6PGD are inhibited by some drugs and chemicals (11-14,34-39).

Dacarbazine can induce a marked reduction of blast cells as well as severe myelotoxicity in leukemic patients (40). Methotrexat inhibits the metabolism of a-oxoaldehydes in vivo in leukemic children, as a likely consequence of glyoxalase I inhibition (41). When methotrexat was used as a chemotherapy drug, it was seen that ALT rises 14% and AST rises 8%. After the drug is discontinued enzymes turn to normal (42).

In the present paper, the effects of some commonly used drugs on human erythrocyte GR enzyme were investigated. In order to show inhibitory effects, while the most suitable parameter is the Ki constant, some researchers use the IC_{50} value. Therefore, in this study, both the Ki and IC_{50} parameters of these drugs for GR were determined.

IC_{50} values of dacarbazine, thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil were 0.014, 0.054, 0.31, 0.35, 0.94, and 98.57 mM, and the Ki constants were 0.0121 ± 0.0026, 0.0135 ± 0.0024, 0.1213 ± 0.040, 0.3173 ± 0.0981, 0.5413 ± 0.0728, and 69.611 ± 9.690 mM, respectively. Ki constants show that dacarbazine had the highest inhibitory effect, followed by thiocolchicoside, methotrexate, olanzapine, pantoprazole sodium, and 5-fluorouracil, in that order. IC_{50} values showed the same trend. In this investigation, by using the obtained Ki constants and IC_{50} values, undesirable side effects of these drugs on GR activity and body metabolism and antioxidative capacity can be reduced.

The Table shows that the enzyme is mostly inhibited by dacarbazine, thiocolchicoside, methotrexate, olanzapine, and pantoprazole sodium drugs. The chemical structures of all these drugs contain an active group of carbonyl and nitrogen. Only olanzapine does not contain a carbonyl group. Moreover, thiocolchicoside and olanzapine contain sulfur groups and methotrexate contains hydroxyl groups. 5-Fluorouracil, which has a less inhibitory effect on the enzyme's activity, contains carbonyl, fluoride, and nitrogen groups, but does not contain sulfur and hydroxyl groups.

The dosages of the examined drugs (*iv*) used clinically give blood drug concentrations as follows; dacarbazine ~4, thiocolchicoside ~58, methotrexate ~315, olanzapine ~455, pantoprazole sodium ~345, and 5-fluorouracil ~532 mM (19). By taking into account these concentrations, the inhibition data calculated from plots were found to be 2%, 13%, 23%, 1%, 0.4%, 0%, and 0.1%, respectively. According to these data, if patients are required to be given

thiocolchicoside and methotrexate, their dosage should be very well controlled to decrease hemolytic and other side effects due to possible inhibition of GR.

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