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RESEARCH ARTICLE

In vitro effects of some drugs on human erythrocyte glutathione reductase

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Abstract
The effects of ketotifen, meloxicam, phenyramidol–HCl and *gadopentetic acid* on the enzyme activity of GR were studied using human erythrocyte glutathione reductase (GR) enzymes *in vitro*. The enzyme was purified 209-fold from human erythrocytes in a yield of 19% with 0.31 U/mg. The purification procedure involved the preparation of haemolysate, ammonium sulphate precipitation, 2′′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography. Purified enzyme was used in the *in vitro* studies. In the *in vitro* studies, IC_{50} values and K_i constants were 0.012 mM and 0.0008 ± 0.00021 mM for ketotifen; 0.029 mM and 0.0061 ± 0.00127 mM for meloxicam; 0.99 mM and 0.4340 ± 0.0890 mM for phenyramidol–HCl; 138 mM and 28.84 ± 4.69 mM for *gadopentetic acid,* respectively, showing the inhibition effects on the purified enzyme. Phenyramidol–HCl showed competitive inhibition, whereas the others showed non-competitive inhibition.

Keywords: Glutathione reductase, drugs, inhibition

Introduction

Oxidative stress, which refers to the unusually high presence of molecules with a high potency to abstract electrons from biomolecules, plays an important role in the pathogenesis of various diseases[1,2]. The undesirable biological effects of these highly reactive molecules are eliminated by enzymatic and nonenzymatic antioxidant defence systems. Enzymatic defence is provided by many enzyme systems such as superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase, glutathione reductase (GR), aldoketoreductases and DNA repair enzymes. Nonenzymatic antioxidant defence systems include many different agents, such as transferrin, ceruloplasmin, lactoferrin, vitamins (e.g. A, E and C), uric acid, taurine, glutathione (GSH), cysteamine and cysteine. The GSH and GSH-related enzymes responsible for glutathione metabolism make up one of the most important protective systems in cells[3].

Glutathione reductase (glutathione:$NADP^+$ oxidoreductase, EC 1.8.1.7; GR) plays a central role in glutathione metabolism. This flavin enzyme is essential for reducing glutathione disulphide (GSSG) to the tripeptide form. GSH not only counteracts oxidative agents but also protects the sulphydryl groups of intracellular proteins in the erythrocytes. The GSH concentration needs to be about 2 mM to keep red blood cells alive and not being haemolyzed[4]. On the other hand, a high GSSG concentration inhibits a number of important enzyme systems including protein synthesis[5]. Decreased GSH levels have also been reported in several diseases, such as acquired immune deficiency syndrome (AIDS)[6], adult respiratory distress syndrome,[7] Parkinson's disease[8] and diabetes[9]. Many drugs have activation or inhibition effects on the body's enzyme systems[10–12]. 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[13].

In the present study, ketotifen, meloxicam, phenyramidol–HCl and gadopentetic acid were selected. Since the effects of these drugs on human erythrocyte GR enzyme activity has not been investigated, these drugs

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are used very often for medical treatments. Considering the study from this direction, it is thought that different class and chemical structures of drugs are important to add a better sense on GR enzyme activity.

Materials and methods

Materials

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

Preparation of the haemolysate

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 and the plasma and leucocyte 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 were removed. The washed erythrocytes were haemolyzed with five 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[14].

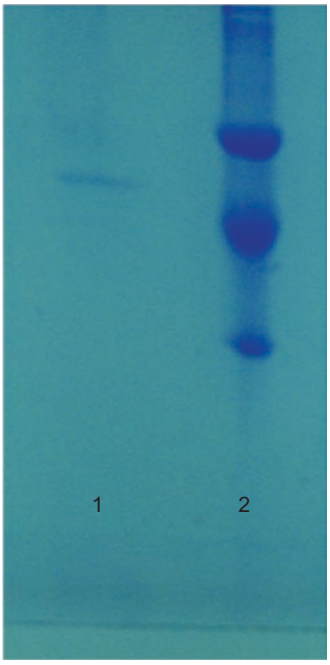

Figure 1. SDS-PAGE photograph: Lane 1, purified enzyme from Sephadex G-200 gel filtration; Lane 2, standard proteins: Bovine carbonic anhydrase (29 kDa), chicken ovalbumin (45 kDa), bovine albumin (66 kDa).

Ammonium sulphate fractionation and dialysis

Ammonium sulphate precipitation was done in the haemolysate at 30–70% saturation. Ammonium sulphate was added slowly to the haemolysate, followed by stirring until it dissolved completely. The mixture was centrifuged at 5000 *g* for 15 min. The precipitate was dissolved in 50 mM of phosphate buffer including 1 mM EDTA (pH 7.0). Precipitates in the range (30–70%) were collected and dialyzed at 4°C in the same buffer for 2 h with two changes of buffer[15,16].

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 potassium acetate/0.1 M potassium 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 sulphate precipitation was loaded onto the column, and washed with 25 mL of 0.1 M potassium acetate + 0.1 M potassium phosphate, pH 6 and 25 mL of 0.1 M potassium acetate + 0.1 M potassium phosphate, pH 7.85. Washing continued with 50 mM of potassium 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 mM GSH and 0–1 mM NADPH in 50 mM potassium phosphate, containing 1 mM EDTA (pH 7.0). Active fractions were collected and dialyzed with 50 mM of potassium phosphate including 1 mM EDTA (pH 7.0) at 4°C[15–19].

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 zero at 280 nm and the pH was the same as that of the equilibration buffer. The dialysate 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 and 340 nm in each fraction. Active fractions were lyophilized and stored at –85°C for use in the *in vitro* studies.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

To determine the purity of the enzyme, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's method[20]. The acryl amide 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 destained with many changes of the same solvent without dye. Cleared protein bands were photographed (Figure 1).

Activity assay

GR activity was determined by the method of Carlberg and Mannervik[21] 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 mmol NADPH per min under the assay conditions.

Protein determination

Quantitative protein determination was performed spectrophotometrically at 595 nm according to Bradford's method[22], using bovine serum albumin as a standard. Qualitative protein determination was also performed spectrophotometrically at 280 nm according to Segel's method[23].

In vitro drug studies

Ketotifen, meloxicam, phenyramidol–HCl and *gadopentetic acid* were tested to determine their effects on human erythrocyte GR activity. Assays were performed in cuvette concentrations of 0.0047–0.0282 mM ketotifen; 0.01425–0.142 mM meloxicam; 0.107–3.20 mM phenyramidol–HCl; 12.5–200 mM *gadopentetic acid*. The cuvette GR activity in the absence of drug was taken as 100%. For each drug, a activity% – [drug] graph was drawn (Figure 2), and drug concentrations producing 50% inhibition (IC_{50}) were graphically calculated for ketotifen, meloxicam, phenyramidol–HCl and *gadopentetic acid*. In the media with or without inhibitor, five substrate (GSSG) concentrations (0.03, 0.08, 0.14, 0.20 and 0.30 mM for drugs) were used. Three different fixed concentrations of inhibitor solution selected from activity% – [drug] graphs were added to the reaction medium in a total reaction volume of 1 mL for each fixed inhibitor concentration. Regression analysis provided the equations to be used for drawing the Lineweaver–Burk graphs (Figure 3) using $1/V$ and $1/[S]$ values. The K_i values were graphically calculated for these drugs.

Results

Human erythrocyte GR was purified 209-fold with a yield of 19% using 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography after ammonium sulphate precipitation of the haemolysate (Table 1). The purity of the enzyme was determined by SDS-PAGE and a single band was observed on the gel after the final chromatographic step (Figure 1). *In vitro* studies showed that ketotifen, meloxicam, phenyramidol–HCl and *gadopentetic acid* inhibited enzyme activity. IC_{50} values and K_i constants were 0.012 mM and 0.0008 ± 0.00021 mM for ketotifen;

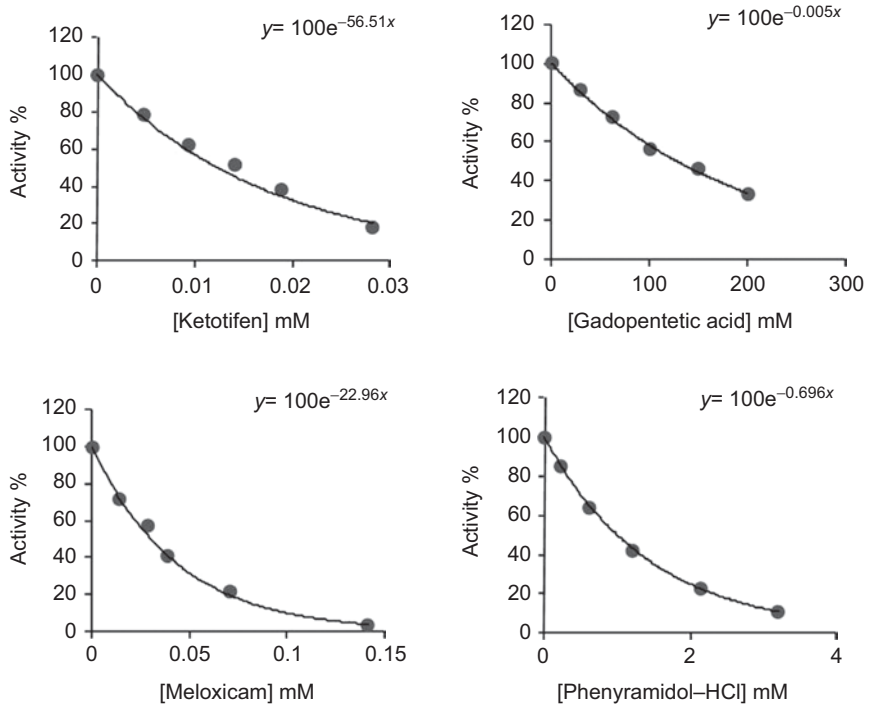

Figure 2. Activity% versus drug concentration regression analysis graphs for glutathione reductase (GR) in the presence of different drug concentrations.

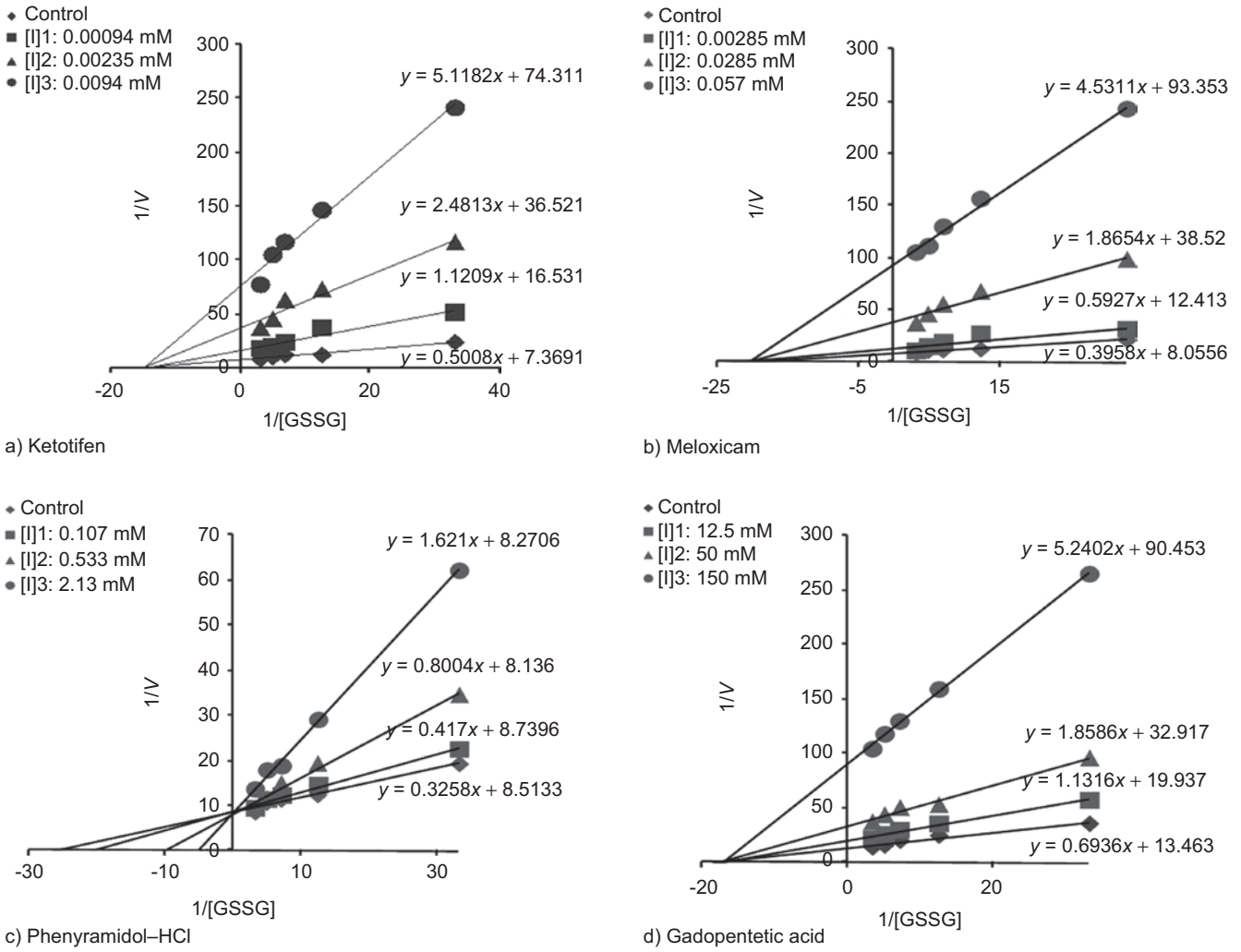

Figure 3. Lineweaver–Burk graphs using three different drugs (A: ketotifen; B: meloxicam; C: phenyramidol–HCl; D: gadopentetic acid) concentrations for determination of K_i.

Table 1. Purification scheme of glutathione reductase (GR) from human erythrocyte.

Purification step	Total volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification factor
Haemolysate	86	0.053	4.56	35.6	0.0015	100	1
Ammonium sulphate precipitation (30–70%)	37	0.064	2.368	28.9	0.002	52	1.46
2′,5′-ADP Sepharose 4B affinity chromatography	6	0.255	1.53	0.535	0.047	33.5	31.5
Sephadex G-200 gel filtration chromatography	4	0.211	0.844	0.0521	0.314	18.5	209

0.029 mM and 0.0061 ± 0.00127 mM for meloxicam; 0.99 mM and 0.4340 ± 0.0890 mM for phenyramidol–HCl; 138 mM and 28.84 ± 4.69 mM for *gadopentetic acid,* respectively (Table 2).

Inhibition types were determined to be non-competitive for ketotifen, *gadopentetic acid* and meloxicam, but competitive for phenyramidol–HCl (Figure 3).

Discussion

GR is essential for the maintenance of cellular glutathione in its reduced form, which is highly nucleophilic for many reactive electrophils[24]. GSH is either involved as a substrate in the cytosolic GSH redox cycle, or able to directly inactivate free radicals and reactive oxygen species (ROS), which are known to be effective stress agents[25]. 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[26]. The inhibition of some important enzymes plays a key role in a metabolic pathway, for example, some metabolic diseases such as diabetes mellitus are affected by enzyme activity[27]. Similarly, acetazolamide has an inhibitory effect on the carbonic anhydrase (CA)

Table 2. K_i values obtained from Lineweaver–Burk graphs for glutathione reductase (GR) in the presence of three fixed inhibitors and five substrate concentrations for different drugs.

Drugs	IC_{50} (mM)	K_i (mM)	Inhibition type
Ketotifen	0.012	0.0008 ± 0.00021	Non-competitive
Meloxicam	0.029	0.0061 ± 0.00127	Non-competitive
Phenyramidol–HCl	0.99	0.4340 ± 0.0890	Competitive
Gadopentetic acid	138	28.84 ± 4.6900	Non-competitive

enzyme leading to diuresis[28]. Additionally, epiandrosterone was found to inhibit red blood cell glucose 6-phosphate dehydrogenase (G6PD) uncompetitively and suppress hexose monophosphate shunt activity by >95%[29]. Some chemicals and drugs, such as nitrofurazone, nitrofurantoin, 5-nitroindol, 5-nitro-2-furoic acid, 2,4,6-trinitrobenzene sulphonate (TNBS)[30] and polyphenolic compounds, also inhibit GR enzyme activity[31]. Furthermore, it is reported that human erythrocyte CA-I and CA-II are inhibited by ampicillin[13], human erythrocyte G6PD by netilmicin, gentamicin, streptomycin, ampicillin,[32] metamizol and magnesium sulphate[33], and bovine and human erythrocyte GR by some drugs[15,16]. Many drugs are commonly used in the therapy of human diseases, but we have not encountered any investigations of their inhibition or activation effects on GR enzyme in the literature. In the present article, the effects of some used drugs on human erythrocyte GR enzyme were investigated, and IC_{50} values and K_i constants were estimated for the drugs showing inhibition effects.

K_i constants show that ketotifen had the highest inhibitory effect, followed by meloxicam, phenyramidol–HCl and *gadopentetic acid*, respectively. IC_{50} values showed the same trend (Table 2). In this investigation, by using the obtained K_i constants and IC_{50} values, undesirable side effects of these drugs on GR activity and body metabolism and antioxidative capacity can be reduced. Table 2 shows that *the enzyme is mostly inhibited by ketotifen and* meloxicam drugs. The chemical structures of *ketotifen and* meloxicam contain an active group of sulphur that appears to be different from the other two drugs.

It was determined that drugs such as tenoxicam, lornoxicam, thiocolchicoside and olanzapine, containing sulphur groups, efficiently inhibit GR enzyme activity.[34,35] Similar results were obtained in the present study for *ketotifen and* meloxicam. Probably, the sulphur group binds to outside of the active region of GR enzyme (non-competitive) that inhibits GR activity by changing the enzyme's three-dimensional structure. Phenyramidol–HCl and *gadopentetic acid* have a less inhibitory effect on the enzyme's activity, we anticipated both drugs contain carbonyl, nitrogen and carboxyl groups, but do not contain sulphur groups.

In this investigation, by using the obtained K_i and IC_{50} values, undesirable side effects of these drugs on GR activity and body metabolism and fatty acid synthesis can be reduced. According to this data, if it is required to give ketotifen and meloxicam to patients, its dosage should be very well-controlled to decrease haemolytic and other side effects due to possible inhibition of GR.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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