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The Influence of Some Polyphenols on Human Erythrocytes Glutathione *S*-Transferase Activity

Mustafa Erat

***Abstract*—**Glutathione *S*-transferase was purified from human erythrocytes and effects of some polyphenols were investigated on the enzyme activity. The purification procedure was performed on Glutathione-Agarose affinity chromatography after preparation of erythrocytes hemolysate with a yield of 81%. The purified enzyme showed a single band on the SDS-PAGE. The effects of some poliphenolic compounds such as catechin, dopa, dopamine, progallol and catechol were examined on the *in vitro* GST activity. Catechin was determined to be inhibitor for the enzyme, but others were not effective on the enzyme as inhibitors or activators. IC_{50} value -the concentration of inhibitor which reduces enzyme activity by 50%- was estimated to be 10 mM. K_i constants were also calculated as 6.38 ± 0,70 mM with GSH substrate, and 3.86 ± 0,78 mM with CDNB substrate using the equations of graphs for the inhibitor, and its inhibition type was determined as non-competitive.

***Keywords*—**Drug resistance, Glutathione *S*-transferase, Inhibition.

I. Introduction

CHEMOTERAPY is a method applied to the treatment of cancer targeting to kill the tumor cells by the agents called as antineoplastic. Many of those agents inhibit the growth and proliferations of malignant cells by cytotoxic effects and lead to their death. However, the drug reduced the sensitivity of tumor cells; in other words, the development of resistance to the drug is an important factor limiting the therapeutic efficacy of an antineoplastic agent. However, the development of tumor resistance, such as some types of cancer can be spontaneous may also develop after chemotherapy [1]-[3].

The development of resistance to antineoplastic agents were associated with increasing reduced glutathione (GSH) and glutathione S-transferase (GST) levels in cells and changes in permeability to the drug. Although the GSH concentration is 0.03-3 mM in plasma, it has reached up to 10 mM in the tumor cells. This increase in concentration has attracted the attention of researchers to the enzymes of glutathione reductase (GR) and GST [1], [4].

Glutathione *S*-transferases (EC 2.5.1.18) are a family of multifunctional enzymes that involve in the detoxification processes through several different mechanisms. These enzymes can catalyze the conjugation of endogenous and exogenous electrophilic xenobiotics to reduced glutathione (GSH), and remove toxic compounds from circulation through covalent and non-covalent binding [5], [6].

It is indicated that GSTP1-1, the most ubiquitous and prevalent of the GST isozymes is secreted heavily in many different types of human tumors such as lung, breast, colon, kidney, ovary, esophagus, and stomach [7], [8]-[11]. By accelerating metabolism of many drugs (adriamycin, chlorambucil, melphalan and other nitrogen mustards) metabolized in this system and used in chemotherapy treatment of increased GSH/GST levels, it is shown that the point targeted with drug has not been reached, in other words, it has caused the development of resistance to the drug [12], [13]. For this reason, in regulating the efficiency of conventional electrophilic cancer drugs used in chemotherapy. It came to mind that the use of GST inhibitors may be useful. For this purpose, various compounds were developed and were tested both experimentally and in clinic [14]-[16].

The purpose of this study is to determine GST inhibitors of polyphenolic compounds, especially determining those which are GSTP inhibitors, and to find out the inhibition types of the agents determined to be inhibitors. If the results obtained from this in vitro study are supported by in vivo studies, the new inhibitors may be given to cancer patients together antineoplastic drugs.

II. Materials and Methods

A. Material

Human blood obtained from volunteer donors, Glutathione-Agarose, Sephadex G-100, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), polyphenolic compounds, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich.

B. Purification of Human GST

Human blood was collected into centrifuge tube with 0.1 M Na-sitrate, 0.16 M glucose, 0.016 M Na-phosphate, 2.59 mM adenine for anticoagulation. It was centrifuged at 2,500g for 10 min and the plasma and leukocyte coat were removed. The erythrocytes were washed three times with isotonic KCl solution including 1 mM EDTA, the sample was centrifuged at 2,500g each time and supernatant was removed. The washed erythrocytes were hemolysed with 5 volumes of ice-cold distilled water containing 2.7 mM EDTA and 0.7 mM β-mercaptoethanol and centrifuged at 4°C, 15,000g for 60 min to remove residual intact cells and membranes [17]. Purification of the enzyme was performed on Glutathione-Agarose affinity gel. For this aim, the lyophilized powder was incubated a

M. E. is with the Atatürk University, Erzurum Vocational College, Chemistry and Chemical Processing Technologies, 25240 Erzurum-Turkey (phone: +904422311966; fax: +90 442 2360948; e-mail: eratm@atauni.edu.tr).

night in deionized water and swelled. After swelling, the gel was washed thoroughly with 10 ml volumes of deionized water to remove the lactose present in the lyophilized product and packed in a column. After precipitation of the gel, it was equilibrated 10 mM K-phosphate buffer including 150 mM NaCl, pH 7.4, (equilibration buffer) by means of a peristaltic pump. The flow rates for washing and equilibration were adjusted 20 mL/h. The previously prepared hemolysate was loaded onto the Glutathine-agarose column and washed with equilibration buffer until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted successively with a gradient of 0 to 10 mM GSH in 50 mM Tris-HCl, pH 9.5, buffer. Active fractions were collected and dialyzed with equilibration buffer. All of the procedures were performed at 4^oC [15], [18].

C. Activity and Protein Assay

GST activity was determined as described by Habig et al. [19]. The reaction medium contained 0.1 M potassium phosphate buffer pH 6.5, 1.0 mM GSH, 1.0 mM CDNB, and 1% absolute ethanol in a total volume of 1.0 mL. The reaction was monitored by increase in A_{340} with a Beckman Spectrophotometer (DU 730). All reactions were initiated by the addition of the enzyme solution. One unit of activity is defined as the formation of 1.0 μmol product min^{-1} (extinction coefficient at 340 is 9.6 mM^{-1} cm^{-1} for GSDNB). Quantitative protein determination was measured spectrophotometrically at 595 nm according to the method of Bradford, with bovine serum albumin as a standard [20].

D. SDS-PAGE

To check the purity of the enzyme, SDS-PAGE (*Sodium dodecil sulfate-polyacrylamide gel electrophoresis)* was performed by Laemmli's procedure [21] as was done in our previous report [22]

E. In vitro Inhibition Studies

In order to determine the effects of the polyphenols on human erythrocytes GST, enzyme activities were assayed for catechin, dopa, dopamin, progallol, and catechol at the cuvette concentrations of1 to 20 mm. An experiment in the absence of inhibitor was used as a control for each polyphenol. Control cuvette activity was taken as 100%. For each poliphenolic agent having inhibitory effect, an [Inhibitor]-Relative Activity graph was drawn and inhibitor concentration causing 50% inhibition (IC_{50}) was calculated from this graph.

For determining K_i constants (dissociation constant of enzyme-inhibitor complex), 3 fixed inhibitor concentrations (1.25 mM, 1.88 mM, and 2.50 mM for the inhibitor) were tested. In these experiments, both GSH and CDNB were used as substrates with their 5 different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mM of GSH and 0.8, 0.4, 0.2, 0.1, and 0.05 mM of CDNB). Three assays were performed for each data point. Analysis of data obtained was made by t-test and they were given as X ± SD. The Lineweaver-Burk graphs were obtained for the inhibitor by using 1/V and 1/[S] values [23]. K_i constants and its inhibition type were estimated from these graphs.

III. Results

Glutathione S-transferase enzyme, after preparing the haemolysate from human erithrocytes, was purified 1,286 fold with a 81% recovery of the total activity having a specific activity of 18 EU/mg proteins by using glutathione-agarose affinity column as shown in Table I. The purity of the enzyme was confirmed by SDS-PAGE. As highlighted in our previous report, the enzyme was thought to be isozyme GSTP owing to the substrate/inhibitor interaction properties. Inhibitory effect of five selected polyphenols (catechin, dopa, dopamine, progallol and catechol) was detected on the purified enzyme. Four polyphenols (dopa, dopamine, progallol and catechol) were not changed enzyme activity as an activator or an inhibitor, but catechin was estimated to be inhibitor for the enzyme. [I]-% Activity graph was drawn by measuring the enzyme activities in different inhibitor concentrations. IC_{50} value was calculated as 10 mm for the inhibitor from equations of graphic (Fig. 1). To explain the effect of inhibition mechanisms of catechin, its inhibition type and Ki constant was determined. For this purpose, the three reasonable fixed inhibitor concentrations were detected and activities were measured in each fixed inhibitor concentration by decreasing substrate concentrations. Lineveawer-Burk graphs were drawn using the data obtained. Ki constants were calculated using the equations of these graphs. When GSH was used as the variable substrate, the mean K_i constants of 6.38 ± 0,70 mM for catechin was found. When the variable substrate was the CDNB, the mean Ki constant was 3,86 ± 0,78 mM for the inhibitor. The data obtained from the studies performed with two substrates showed that catechin was non-competitive inhibitor for the enzyme.

IV. Discussion

GSTs are important cellular drug metabolizing enzymes, catalyzing conjugation of some electrophiles to the tripeptide glutathione with the formation of compounds that are generally less reactive, but effective in resistance to anticancer agents [24].

The over-expression of GST in tumors should in principle be linked to enhanced detoxification of akylating agents, and therefore be responsible for the development of resistance [25], [26]. In fact, many acquired akylating agents and cisplatin resistant tumor cells have a high GSH concentration and/or an increase in the activity of enzymes such as GST [27]. In particular the isoform GSTP has been associated with early stages of human carcinogenesis and there is an expanding body of evidence that overexpression of this iso enzyme plays an important role in the malignant cellular transformation of various tissues [28].

TABLE I
PURIFICATION OF GLUTATHIONE S-TRANSFERASE FROM HUMAN ERYTHROCYTES

Purification step	Total volume (mL)	Activity (EU/mL)	Total Activity (EU)	Protein (µg/mL)	Total Protein (µg)	Specific activity (EU/ µg)	Yield (%)	Purification Fold
Hemolysate	50	0.08	4.00	5.92	296.00	0.014	100	1
Affinity Chromatography	6	0.54	3.24	0.03	0.18	18.000	81	1286

GST inhibitors are emerging as promising therapeautic agents for managing the development of resistance amongst anticancer agents. For this aim, various compound groups have been determined targeting this system as GST inhibitors and investigated its effects experimentally and clinically [29]. These groups are combined with etakrinik acid and its derivatives, glutathione analogues, plant polyphenolic compounds, bifunctional inhibitors, antimalarial drugs, tocopherols, haloenol lactones, 7-nitro-2,1,3-benzoksadiazole derivatives, and prodrugs activated by GST such as, TLK 286, purine analogues, and nitric oxide donors [3].

The importance of identified GST inhibitor stems from the possible use of it with antineoplastic agents to eliminate the resistance caused by GST activity. Because of the fact that GSTs play a pivotal role in the detoxification system, inhibition of their all isozymes is not desirable. Isoenzyme selective GST inhibitors may therefore be used to improve drug response and decrease resistance [30].

Ethacrynic acid is the first compound studied as an inhibitor of the GST. This compound and its derivatives were not used as antineoplastics because of having a diuretic effect and not being isozim specificity [31]. Glutathione analogues are another most studied group. The most important of these compounds is γ-glutamyl-*S*-benzylcysteinyl-phenylglycyl diethylester (TLK 199).

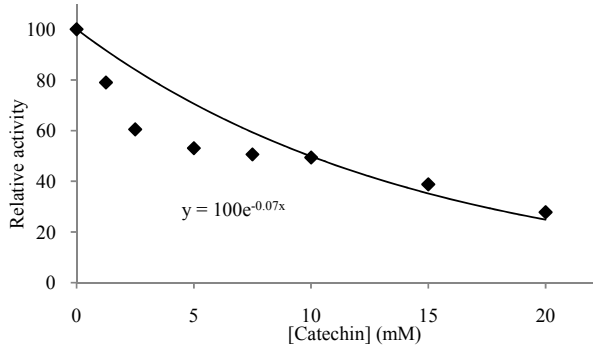

Fig. 1 [Catechin]-Activity (%) graphs for GST in the seven different catechin concentrations

It was reported that the compound transforms to its active form after entering into the cell hydrolyzed by intracellular esterases and its new form (TLK 117) inhibits GSTP1-1 [32] and that it increased the efficiency of alkylating agent in many cell lines secreting this enzyme more. Glutathione conjugates, despite being good GST inhibitors, cannot be used in vivo, because they are eliminated rapidly. Some flavonoids, such as galangin, kaempferol, eriodictyol and quercetin have been reported to inhibit the activity of GSTP1-1 [18]. Of all flavonoids, 25 µM concentration of galanginin was observed to inhibit the activity of in almost all cellular GSTP1-1 [33].

A group of compounds having haloenol lactone structure were indicated as mechanism-based inhibitors of the GSTs. It was reported that this group of compounds are compounds like substrates transforming into reactive electrophills inactivating the enzyme by binding covalently to essential aminoacids through the normal catalytic mechanism of the enzyme [34]. New inhibitor compounds, able to pass through the cell membrane due to their lipophilic properties and effectively connected with GSTs, were designed. These compounds are not similar to GSH. For this purpose, a group of compounds, having the sutructure of 7-nitro-2,1,3-benzoxadiazole derivatives have been synthesized [35]. Of the synthesized compounds, 6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol has shown to be the most effective inhibitor for GSTP1-1 with an IC_{50} value of 0.8 µM.

Catechins, antioxidant compounds from flavonoids, present in fruits and vegetables, especially green tea. There are a lot of forms of catechin in green tea. (-)-epigallocatechin-3-gallate (EGCG) is one of the forms having the most antioxidant properties.

The compounds prevent the death of brain cells and repair damaged neurons. In particular, its main catechin polyphenol constituent EGCG has been shown to exert neuroprotective/neurorescue activities in a wide array of cellular and animal models of neurological disorders [36].

Catechins have also thermogenic effect on adipose tissue. catechin-polyphenols and caffeine with sympathetically released noradrenaline (NA). Since catechin-polyphenols are known to be capable of inhibiting catechol-O-methyl-transferase (the enzyme that degrades NA), and caffeine to inhibit trancellular phosphodiesterases (enzymes that break down NA-induced cAMP), it is proposed that the green tea extract, via its catechin-polyphenols and caffeine, is effective in stimulating thermogenesis by relieving inhibition at different control points along the NA-cAMP axis [37].

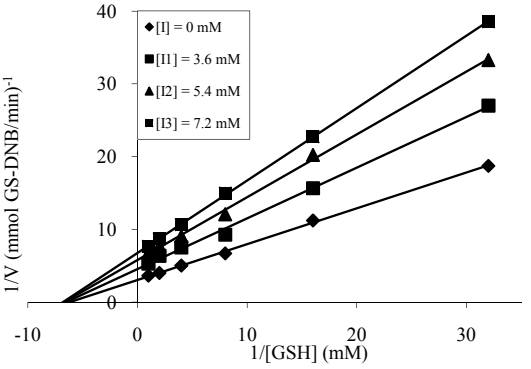

Fig. 2 Lineweaver-Burk graph in different substrate (GSH) concentrations and in 3 fixed inhibitor concentrations for the determination of K_i value

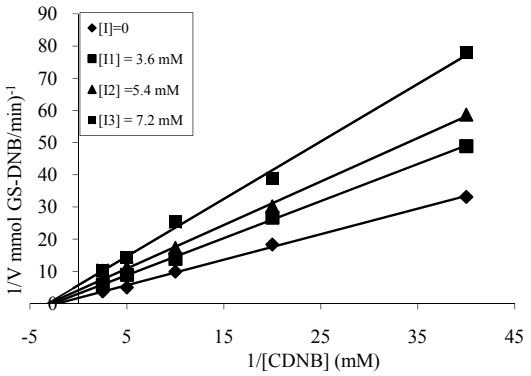

Fig. 3 Lineweaver-Burk graph in different substrate (CDNB) concentrations and in 3 fixed inhibitor concentrations for the determination of K_i value

TABLE II
IC50, KI VALUES AND INHIBITION TYPE OF CATECHIN ON HUMAN ERYTHROCYTE GST

Inhibitor	Fixed Substrate	IC_{50} values (mM)	K_i constants (mM)	Avarage K_i constants (mM)	Inhibition Type
Catechin	CDNB	10	7.29 5.98 5.89	6.38 ± 0.70	Non-competitive
Catechin	GSH	10	4.66 3.83 3.09	3.86 ± 0.78	Non-competitive

In this work, the effects of some polyphenolic compounds on GSTP were investigated. The enzyme was purified from human erythrocytes by the procedure mentioned in our previous report [22]. Of the tested polyphenolic compounds, catechin was determined to be noncompetitive inhibitor of the enzyme. If the influence of catechin studied *in vitro* in this report is supported by in vivo studies, this compound can be used in cancer chemoterapy together other antineoplastic drugs.

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