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Cisplatin and 5-Fluorouracil Inhibits 6-Phosphogluconate Dehydrogenase Activity in Human Erythrocytes *in vitro* and *in vivo*

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The antineoplastic agents cisplatin and 5-fluorouracil, have been used frequently in the medical treatments of various cancers. Cisplatin is an important drug that is administrated in the treatment of metastatic tumours of the testis, ovaries and solid tumors. 5-Fluorouracil is used in combination therapy for the treatment of some cancers including head and neck. In this study, *in vitro* effects of these drugs on human erythro-cytes 6-phospogluconate dehydrogenase (6PGD) and *in vivo* effects on rabbit erythrocytes 6PGD activity were investigated. For this purpose, human erythrocyte 6PGD was purified with 0.46 U/mg protein specific activity, 50 % yield and approximately 742-fold using 35-65 % ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity chromatography. The enzyme activity was determined by Beutler's method. To check the purity of enzyme, SDS-polyacrylamide gel electrophoresis was performed. For the drugs, *in vitro* inhibition studies were performed and activity % - [Drug] graphs were drawn. IC_{50} values were calculated as 1.49 mM for cisplatin and 62.5 mM for 5-fluorouracil from these graphs. Then, K_i values for cisplatin and 5-fluorouracil were also calculated from Lineweaver-Burk graphs as 1.35 ± 0.206, 53.8 ± 10.53 mM, respectively and inhibition types of the drugs were found out to be uncompetitive manner. In addition, time-dependent *in vivo* studies were executed for the drugs in New Zealand-albino rabbits. Cisplatin at 1 mg/kg inhibited the enzyme activity significantly ($p < 0.01$) 3 and 5 h after dosing. 5-fluorouracil at 25 mg/kg inhibited the enzyme activity significantly ($p < 0.01$) 1, 3 and 5 h after dosing. As seen from obtained IC_{50} and K_i values that the purified human 6PGD has been quite inhibited by these drugs.

Key Words: 6-Phospogluconate dehydrogenase, Erythrocyte, Cisplatin, 5-Fluorouracil.

INTRODUCTION

The new possibilities in cure of cancer and improvement of the life quality of patients have come by using of chemotherapy in cancer treatment. However, some anti-cancer drugs, applied in widely treatment, provide a number of symptoms of direct toxicity.

Cisplatin (*cis*-diammine-dichloro-platinum, CDDP) is an effective anti-cancer drug. This drug has been used in the treatment solid human cancers such as, ovary, lung, head, neck, bladder and testis cancer[1]. Cisplatin reacts with nucleophilic sites in cellular macromolecules in cell[2]. The use of cisplatin in the treatment of cancer causes a side-effect known as nephrotoxicity.

One of the most effective chemotherapeutic agents that used in combination therapy for the treatment of head and neck carcinomas is 5-fluorouracil[3-5]. It is reported that 5-fluorouracil has some cytotoxic effects in metabolism. For instance, the best known mechanism for the cytotoxic effect of 5-fluorouracil is the inhibition of thymidylate synthase[6].

Enzyme activities display significant variations under various conditions such as environmental conditions, genetic disorders, oxidative stress and many chemical substances including drugs[7]. There are many literatures related to changes of enzyme activities. A few reports have indicated some increases and decreases in human and animal tissue enzyme activity levels such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and carbonic anhydrase[7]. In addition, *in vitro* and *in vivo* inhibitory effects of ampicillin, amikacin sulfate, netilmicin sulfate and metamizole on rat erythrocyte 6PGD have been previously determined in our laboratory. Besides, *in vitro* and *in vivo* inhibitory effects of dantrolene sodium on glucose 6-phosphate dehydrogenase from human erythrocytes have also been investigated[8].

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44; 6PGD) is the third enzyme of pentose phosphate metabolic pathway (PPP), catalyzing the conversion of 6-phosphogluconate to D-riboluse-5-phosphate in the presence of $NADP^+$. The reaction, catalyzed by 6PGD, yields NADPH which protects the cell against the oxidant agents by producing reduced glutathione (GSH)[9]. For this reason, 6PGD can be defined as an antioxidant enzyme[10,11]. Reduced glutathione form contains tripeptide which has a free thiol group. This form acts as antioxidant, which keeps cystein residue of hemoglobin and proteins of erythrocyte as reduced form. Normally, the ratio of reduced glutathione to oxidized glutathione is *ca.* 500. Reduced glutathione also plays a role in some detoxification reactions reducing anorganic and organic peroxides[12,13]. Because of this, 6PGD has vital importance in living organisms.

In this study, the *in vitro* and *in vivo* effects of cisplatin and 5-fluorouracil on human erythrocytes 6PGD and the *in vivo* effects of this drug on the rabbit erythrocytes enzyme activity were investigated. Also, for cisplatin and 5-fluorouracil, K_i values and the type of inhibition were determined by means of Lineweaver-Burk graphs.

EXPERIMENTAL

Cisplatin and 5-fluorouracil, were obtained from Hospital of Medical Faculty (Erzurum). New Zeland-albino rabbits were used in the *in vivo* experiments. 2',5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, 6-phosphogluconate, protein assay reagent were purchased from Sigma Chem. Co. All other chemicals used were analytical grade and purchased from either Sigma or Merck.

The haemolysate preparation and hemoglobin estimation: Fresh blood samples from the healthy subjects were collected to EDTA-containing tubes. The haemolysate was prepared according to our previous studies[14]. Hemoglobin (Hb) concentration in hemolysate was determined by cyanmethemoglobin method. All steps were carried out at + 4 ºC.

Ammonium sulfate fractionation and dialysis: The hemolysate was subjected to precipitation with ammonium sulfate. Ammonium sulfate fractionation was done according to the previous study[15]. The enzyme was observed to precipitate at 35-65 % precipitation step. The resultant solution was clear and contained partially purified enzyme. The enzyme solution was dialyzed at + 4 ºC against 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0), for 2 h with two changes of buffer.

Enzyme assays: 6-PGD activity was routinely assayed by measurement of the increase in absorbance at 340 nm of the reaction product, NADPH[16]. A volume of 1 mL of the reaction mixture contains: 0.1 mM *Tris*-HCl (pH = 8.0) with 0.5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$ and 0.6 mM (6-PGA) and the enzyme. One unit of enzyme (U) activity was defined as the enzyme amount reducing 1 mmol $NADP^+$ per min at 25 ºC, pH 8.0.

2',5'-ADP Sepharose 4B affinity chromatography: Preparation of the affinity column was performed according to Beydemir *et al.*[17]. The dialyzed sample was loaded on 2',5'-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 mL/h. Then, the column was sequentially washed with 25 mL of 0.1 M potassium acetate + 0.1 M potassium phosphate, (pH: 6.0) and 25 mL 0.1 M potassium acetate + 0.1 M potassium phosphate (pH: 7.85). The washing with 0.1 M potassium chloride + 0.1 M potassium phosphate, (pH: 7.85) was continued until the final absorbance difference became 0.05. Elution was carried out with 80 mM potassium phosphate + 0.5 mM KCl + 5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in final fractions and the activity-containing tubes were collected together. All of the procedures were performed at + 4 ºC[18,19].

Protein assay: During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to Bradford method, using bovine serum albumin as the standard[20].

SDS polyacrylamide gel electrophoresis (SDS-PAGE): The control of enzyme purity was carried out using Laemmli's procedure[21] with 3 and 8 % acrylamide concentrations for running and stacking gel, respectively. The gel solution was supplemented with 10 % SDS.

***in vitro* drug studies:** In order to determine the effects of cisplatin and 5-fluorouracil on 6-PGD, five different concentrations of cisplatin (0.033, 0.16, 0.66, 1.0, 1.33 and 1.66 mM) and 5-fluorouracil, (7.6, 19, 38, 76, 115 and 154 mM) were added to separate tubes containing purified enzyme, respectively. The enzyme activity was measured in these tubes taking the tubes containing no drug as control (100 % activity). The IC_{50} values were obtained after activity in % was plotted *vs.* drug concentration. Drug concentration producing 50 % inhibition (IC_{50}) was calculated from the graphs.

***in vivo* drug studies:** 10 Adult New Zealand-albino rabbits (1200-1500 g) were selected for each drug and intraperitoneally administration of cisplatin (1 mg kg^{-1}) and 5-fluorouracil 25 mg kg^{-1}. Blood samples (0.5 mL) were taken from each rat prior to drug administration as well as at 1, 3 and 5 h intervals thereafter. They were placed into test tubes containing EDTA (2 mM) and haemolyzed[8] 6PGD activity was determined spectrometrically as described above[16]. Statistical analyses of the data obtained were made by test of t and were given as X SD.

RESULTS AND DISCUSSION

The occurrence of 6PGD activity in the human erythrocyte hemolysate was determined according to preliminary enzyme assays. The purification of the enzyme was performed with 35-65 % ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity gel chromatography. The enzyme was purifed *ca.* 742-fold with a specific activity of 0.46 U × mg^{-1} and overall yield of 50 % (Table-1).

TABLE-1
PURIFICATION SCHEME OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM HUMAN ERYTHROCYTES

Purification steps	Activity (U/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Hemolysate	0.0035	25	5.630	140.750	0.0875	0.00062	100	1.0
AA	0.0048	15	0.220	3.300	0.0720	0.02200	82	35.5
BB	0.0088	5	0.019	0.095	0.0440	0.46000	50	742.0

AA = Ammonium sulfate precipitation (35-65) %.
BB = 2´,5´- ADP Sepharose 4B chromatography .

Inhibitory effects of cisplatin and 5-fluorouracil on the enzyme activity were tested under *in vitro* conditions. For each drug, IC_{50} value was determined by activity % - [Drug] graphs. Drug concentrations that produce 50 % inhibition (IC_{50}) were calculated from graphs as 1.49 mM for cisplatin and 62.5 mM for 5-fluorouracil (Table-2, Fig. 1). In addition, K_i values were calculated as 1.35 mM for cisplatin and 53.8 mM for 5-fluorouracil from Lineweaver-Burk graphs (Table-2, Fig. 2). The result of *in vivo* effects of cisplatin and 5-fluorouracil are presented in Table-3. In cisplatin-treated group of rabbits, the control enzyme activity was 1.70 ± 0.23 U/gHb, while the respective values determined 1, 3 and 5 h after drug administration were: 1.54 ± 0.20, 0.40 ± 0.08 and 0.88 ± 0.13 U/gHb. On the other hand, in 5-fluorouracil-treated group of rabbits, the control enzyme activity was 2.80 ± 0.44 U/gHb, while the respective values determined 1, 3 and 5 h after drug administration were: 0.20 ± 0.03 U/gHb ($p < 0.01$), 0.67 ± 0.04 U/gHb ($p < 0.01$) and of 1.21 ± 0.17 U/gHb ($p < 0.01$).

TABLE-2
IC_{50}, K_i VALUES AND INHIBITION TYPES FOR
2 INHIBITORS OF HUMAN ERYTHROCYTE 6-PGD

Inhibitors	IC_{50} (mM)	K_i	Average values of K_i (mM)	Inhibition type
Cisplatin	1.49	1.57 1.28 1.20	1.35 ± 0.206	Uncompetitive
5-Fluorourasil	62.5	67.8 59.4 34.2	53.8 ± 10.53	Uncompetitive

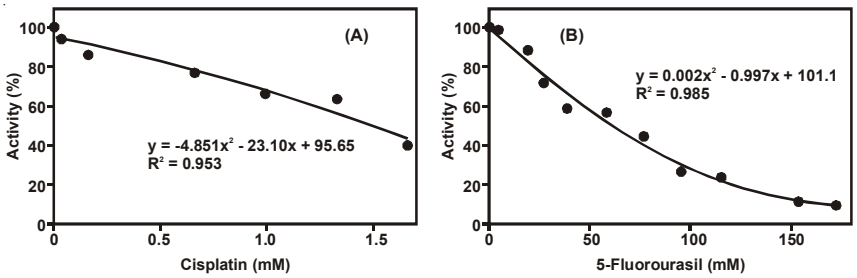

Fig. 1. Activity % *vs.* [Cisplatin] (1a) and Activity % *vs.* [5-Fluorouracil] (1b) regression analysis graphs for 6-PGD

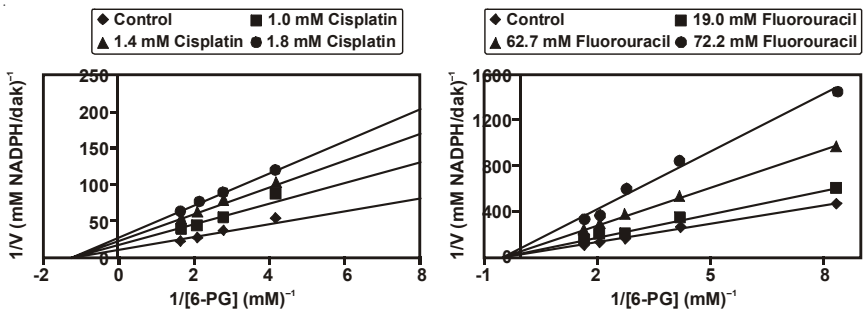

Fig. 3. Lineweaver-Burk graphs in different sustrate (6-PG) concentrations for determination of K_i of cisplatin (2a) and 5-fluorouracil (2b)

Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme[7]. The effects can be dramatic and systemic[7]. *in vitro* and *in vivo* effects of some chemicals and drugs on different enzymes from different sources including human erythroctes have been mentioned in the previous studies[7,22-26]. For example, Akyuz *et al.*[26] stated that netilmycin sulfate, cefepime, amikacin, isepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, ofloxacin, levofloxacin, cefotaxime, penicillin G, gentamycin sulfate, ciprofloxacin inhibited *in vitro* human erythrocyte 6-PGD activity. They found that each drug inhibited erythrocyte 6-PGD activity in *in vivo* studies for netilmicin sulphate and cefepime, significantly. However, cefozin, decefin, streptomycin, combisid and meronem did not have any effect on the enzyme. In addition, it has been reported that vitamin C stimulates 6-PGD[27]. Additionally, we investigated the inhibitiory effects of some sulfonamide derivatives on the activity of carbonic anhydrase from rainbow trout erythrocytes, *in vitro* and *in vivo*. Sulfonamides were the effective chemotherapeutic agents used systematically in the cure and prevention of bacterial infections. They were most important and popular medicines against bacterial infections before the advent of antibiotics and the development of bacterial resistance to sulfonamides in the course of time[28].

Although the inhibitory effects of many chemicals and therapeutic drugs on the enzyme have been studied in most tissues and red blood cells, no study has been done on the effects of cisplatin and 5-fluorouracil, anticancer drugs, on human 6PGD, yet. This enzyme, an important and third enzyme of the penta phosphate metabolic pathway, forms a supramolecular complex in human neutrophils[29]. The enzyme catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate and CO_2 with a concomitant reduction of $NADP^+$ to NADPH which protects the cell against oxidative agents by producing reduced glutathione[30]. NADPH is also a coenzyme participating in the synthesis of a number of biomolecules

such as fatty acids, steroids and some amino acids[26]. In the case of NADPH deficiency, the concentration of reduced glutathione in living systems declines, resulting in cell death. As explained above, the enzyme has an important role in glucose metabolism. For this reason, 6-PGD was purified with 50 % yield and *ca.* 742-fold by 2',5'-ADP Sepharose 4B affinity chromatography from human erythrocyte. Then *in vitro* effects of cisplatin and 5-fluorouracil on the 6-PGD activity were investigated. Both the IC_{50} and K_i parameters of these drugs for 6-PGD were determined. It was important that inhibition type of the drugs were determined as uncompetitive. From the result it was understood that these drug molecules have been binding the active site of the enzyme. *in vivo* studies on rabbits showed that cisplatin and 5-FU inhibited significantly the enzyme activity. Cisplatin inhibited the enzyme by 76.4 % at 3rd h and 48.23 % at 5rd h. 5-Fluorouracil inhibited the enzyme by 92.8 % at 1st h, 76.07 % at 3rd h and 56.7 % at 5th h (Table-3). So, the *in vitro* and *in vivo* results confirm each other.

TABLE-3
in vivo EFFECTS OF CISPLATIN AND 5-FLUOROURACIL ON HUMAN RED BLOOD CELL 6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY (n = 6)

Drug	Time (h)	X ± SD (U/gHb)	p
Cisplatin	Control	1.70 ± 0.23	–
	1	1.540 ± 0.20	> 0.05
	3	0.400 ± 0.08	< 0.01
	5	0.881 ± 0.13	< 0.01
5-Fluorourasil	Control	2.80 ± 0.44	–
	1	0.20 ± 0.03	< 0.01
	3	0.67 ± 0.04	< 0.01
	5	1.21 ± 0.17	< 0.01

In conclusion, due to of these findings, 6PGD deficieny should be investigated before administration these drugs at patients. Especially, for many patients with G6PD and 6PGD deficiency the uncontrolled usage of these drugs may be hazardous. Thus dosages of the drugs should be arrangement according to this situation.

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