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Original Research Article

EFFECTS OF CERTAIN DRUGS ON DIHYDROPYRIMIDINE DEHYDROGENASE ENZYME PURIFIED FROM BOVINE LIVER

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Abstract: The inhibitory effects of certain drugs on dihydropyrimidine dehydrogenase from the bovine liver have been investigated. Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: NADP+ oxidoreductase, EC 1.3.1.2; DPD] enzyme was purified from the bovine liver. The purification was performed by preparation of homogenate, ammonium sulphate precipitation, and affinity chromatography. Moreover, some important modifications were made in the purification procedure. Purification of bovine liver DPD enzyme was obtained with a yield of 12.5%. SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern is discussed in this article. In addition, the effects of certain drugs on bovine liver dihydropyrimidine dehydrogenase enzyme activity were investigated. Oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin, were used as drugs. All the drugs indicated the inhibitory effects on the enzyme. IC_{50} values of the drugs were determined by plotting activity % vs [I]. IC_{50} values of oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin 0.030, 0.046, 0.140, 0.610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920, and 6.300, mM; the Ki constants were 0.050±0.01, 0.090± 0.06, 0.130±0.045, 0.185±0.057, 2.010±0.55, 2.096±1.06, 2.115±1.00, 2.700±0.56, 3.730±1.48, 5.240±1.04, and 9.570±2.84, mM for bovine liver DPD, respectively. Ki constants for dihydropyrimidine dehydrogenase were determined by Lineweaver-Burk graphs. All drugs showed non-competitive inhibition patterns.

Key words: dihydroprimydin dehydrogenase; bovine liver; drug; inhibition

Introduction

Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: $NADP^+$ oxidoreductase, EC 1.3.1.2; DPD] the initial rate-limiting enzyme in pyrimidine catabolism, catalyses the conversion of pyrimidine and NADPH, to dihydropyrimidine and $NADP^+$. The enzyme DPD has an important function in the synthesis of the neurotransmitter β-alanine. It is the first enzyme in a series of enzymatic reactions converting Uracil to β-alanine, 5-florouracil to α-fluoro-β-alanine and thymine to β-amino isobutyrate (1, 2). DPD is a cytosolic enzyme that is present in various mammalian tissues, especially liver, kidney, pancreas, and lung (3). It has been purified from different species, including from human, rat, porcine and bovine livers. The molecular size of homodimeric DPD is roughly 220 kDa. It is known that each monomer of DPD contains a flavin adenine dinucleotide (FAD), a flavin mononucleotide (FMN), and four FeS groups (4, 5, 6). Recently the importance of the DPD enzyme has been more and more appre-

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ciated. Especially in cancer therapy, it has been found that the function of DPD is related to the efficacy of chemotherapy. Drugs used in chemotherapy such as 5-fluorouracil (5FU), a widely used anticancer drug (7), inhibit thymidylate synthetase which catalyses the synthesis of thymine. Inhibition of thymidylate synthetase prevents DNA synthesis in cancer cells. However, accumulation of 5FU causes toxic effects in the organism. In liver 5FU is normally catabolised and thus removed from the system. In this process DPD is essential as it first converts 5FU to 5-fluoro 5, 6-dihidrouracil and then into α-fluoro-β-alanine in a two-step mechanism. Genetic deficiency or inhibition of DPD might cause a lethal effect in cancer patients. Therefore, it is crucially important to know the inhibitors and activators of this enzyme (8).

The aim of this study was to purify bovine liver dihydropyrimidine dehydrogenase and the determination of inhibition or activation effects of some drugs (oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin) on the purified DPD.

No reports could be found in the literature on the *in vitro* and *in vivo* effects of the above drugs on bovine liver DPD.

Materials and methods

Materials

Uracil, dithiothreitol (DTT), NADPH, Tris and the other chemicals were purchased from Sigma, and the drugs were purchased from Hoechst Marian Roussel (Turkey).

Activity determination

DPD activity was measured spectrophotometrically at 25 °C as described by Podschun et al. (9). Briefly, the enzyme sample was added to a 1mL (final volume) incubation mixture containing 280 mM K_3PO_4 (pH 7.4), 8 mM DTT, 20 mM $MgCI_2$, 600 μM NADPH and 1.5 mM uracil and the decrease in absorption at 340 nm measured due to the oxidation of NADPH at 25 °C. One enzyme unit represents the oxidation of 1μmol of NADPH min^{-1} at 25 °C, pH 7.4.

Preparation of the homogenate

Bovine liver (60 g) was obtained from a slaughterhouse in Erzurum, Turkey. A liver sample was cut with a knife. Excess blood, foreign tissues and membranes were removed from the samples. The livers were washed three times with buffer A (35 mM K_3PO_4, 2.5 mM $MgCl_2$, 0.25 M sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DTT, 1 mM aminoetilizotiyoürebrobür, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) and were homogenized by liquid nitrogen, transferred to the same buffer, and centrifuged at 4 °C, 13,500 g for 120 min. Supernatant was used in further studies (9).

Ammonium sulphate precipitation

The homogenate was subjected to orderly precipitation with ammonium sulphate (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70-80% and 80–90%). Ammonium sulphate was slowly added to the haemolysate for complete dissolution. This mixture was centrifuged at 4 °C, 10,000 g for 15 min and the precipitate was dissolved in buffer A containing 1 mM DTT. For each respective precipitation, the enzyme activity was determined both in the supernatant and in the precipitate. The enzyme was observed to precipitate at 40–60% saturation. It was then dialysed at 4 °C in buffer A containing 0.5 mM DTT for 2 h with two changes of buffer (9).

Purification of bovine liver DPD

The column was equilibrated with buffer A. The ammonium sulphate fraction (40–60%) of the homogenate obtained above was loaded onto a 2', 5'-ADP Sepharose 4B affinity column, and the flow rate was adjusted to 20 mL/h. The column was then sequentially washed with buffer A. Washing continued until an absorbance of 0.05 at 280 nm was obtained. Some important modifications had been made during the purification process. The protein fractions within the solution were precipitated with ammonium sulphate and loaded onto an affinity column. However, high DPD enzyme activity was observed in the washing fractions. We suspected that Glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and

glutathione reductase (GR) enzymes have higher affinities than DPD to the used column, DPD enzyme was unable to bind to the column; thus, it was present at very high levels within washing fractions. To solve this problem, firstly G6PD, 6PGD and GR enzymes were removed from the column by using their own eluting solutions. Then, these elutes, which were free of G6PD, 6PGD and GR enzymes, were reloaded onto the same column, which had been washed and regenerated. The washing solution was collected and subsequently analysed. No DPD activity was detected indicating that DPD enzyme was bound to the column. Then, elution of the DPD enzyme was carried out with buffer A containing 0.5 mM DTT, 0.2 mM NADPH and 1 M KCl. Enzyme activity was measured in all fractions, and the activity-containing fractions were pooled, then dialysed in buffer A containing 1 mM DTT for 2 h with two changes of buffer. All procedures were performed at 4 °C (9).

Protein determination

The protein content in all samples was quantified spectrophotometrically at 595nm according to Bradford's (10) method using bovine serum albumin as standard.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme purity was examined using Laemmli's (11) procedure with 3% and 8% acrylamide concentrations for running and stacking gel, respectively. *E. coli* β-galactosidase (116,000 Da), bovine albumin (66,000 Da), chicken ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), and stokrom c (12,400 Da), were used as standards (Sigma) (See Fig. 1).

In vitro effect of drugs

In order to determine the effects of some drugs on bovine liver DPD, concentrations of oxytetracycline (0.022–0.154 mM), ciprofloxacin (0.018–0.12 mM), ceftazidime (0.078–0.47 mM), cefoperazone (1.49–7.45 mM), amikacin, (1.28–3.40 mM), ornidazole, (0.038–0.303 mM), metronidazole, (0.87–2.04 mM), cefuroxime, (2.94-–14.70 mM), cefepime, (1.74–8.70 mM), ampicillin, (9.12–27.36 mM), and amoxicillin, (2.05–20.50 mM), were added to the reaction mixture and the enzyme activity was measured. An experiment in the absence of drug was used as a control (100% activity). The IC_{50} values were obtained from activity (%) vs. drug concentration plots (Fig. 2). In order to determine the K_i values, the substrate (Uracil) concentrations were 0.0075, 0.015, 0.075, 0.15, 0.225mM for drugs. Inhibitor (drug) solutions were added to the reaction mixture at 3 different fixed concentrations. Lineweaver-Burk graphs were drawn using 1/V vs. 1/[S] values and the K_i values were calculated from these graphs (see Fig. 3).

Results

Purification of the enzyme led to a specific activity of 1.66 EU/mg proteins, a yield of 12.5% and 1660-fold purification (Table 1). SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern is shown in Fig. 1. IC_{50} values of oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin 0.030, 0.046, 0.140, 0.610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920, and 6.300, mM, respectively, and the K_i constants were 0.050±0.01, 0.090±0.06, 0.130±0.045, 0.185±0.057, 2.010±0.55, 2.096±1.06, 2.115±1.00, 2.700±0.56, 3.730±1.48, 5.240±1.04, and 9.570±2.84, mM for bovine liver DPD, respectively. Whole drugs showed non-competitive inhibition. Representative graphs are shown for oxytetracycline (Fig. 2 and 3).

Discussion

Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: $NADP^+$ oxidoreductase, EC 1.3.1.2; DPD] the initial rate-limiting enzyme in pyrimidine catabolism, catalyses the conversion of pyrimidine and NADPH, to dihydropyrimidine and $NADP^+$. The enzyme DPD has an important function in the synthesis of the neurotransmitter β-alanine. It is the first enzyme in a series of enzymatic reactions converting Uracil to β-alanine, 5-florouracil to α-fluoro-β-alanine and thymine to β-amino isobutyrate (1, 2). In the liver, 5FU is normally catabolised and thus removed from the system. In this process, DPD is essential as it first converts 5FU to 5-fluoro 5, 6-dihidrouracil. Genetic deficiency or inhibition of DPD might cause

Table 1: Purification scheme of DPD from bovine liver

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	0.020	35	19.26	672	0.70	0.001	100	1
Ammonium sulphate precipitation (40-60%)	0.055	11	4.90	53.90	0.605	0.011	86.4	11
2',5'-ADP Sepharose 4B affinity hromatography	0.011	8	0.0066	0.048	0.088	1.66	12.5	1,660

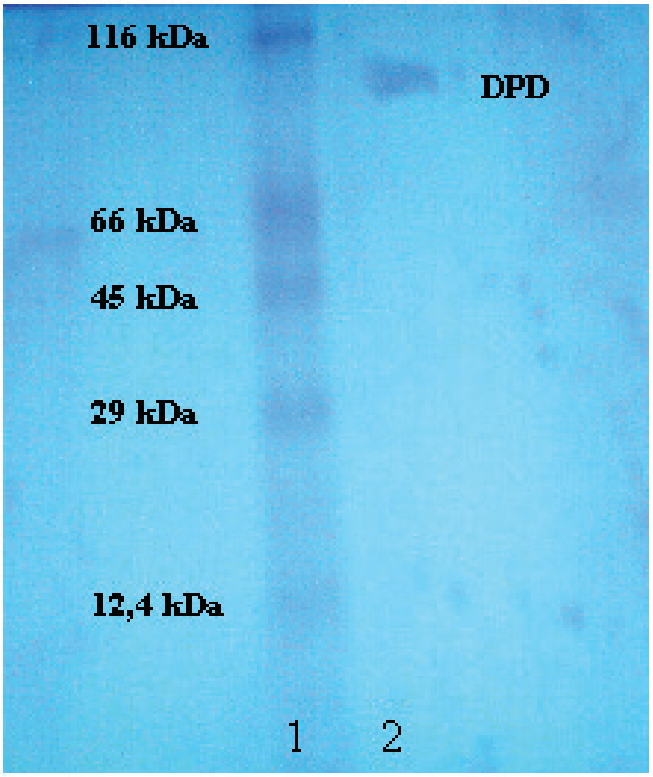

Figure 1: SDS-PAGE bands (Lane 1: Standards: E. coli β-galactosidase (116,000 Da), bovine albumin (66,000 Da), chicken ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000), and stokrom C (12,400 Da) Lane 2: Bovine liver DPD

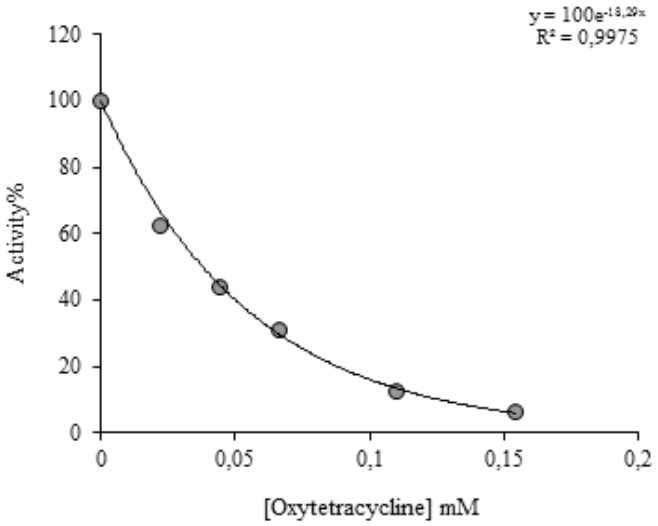

Figure 2: Activity % *vs* [Oxytetracycline] regression analysis graphs for bovine liver DPD in the presence of 5 different oxytetracycline concentrations

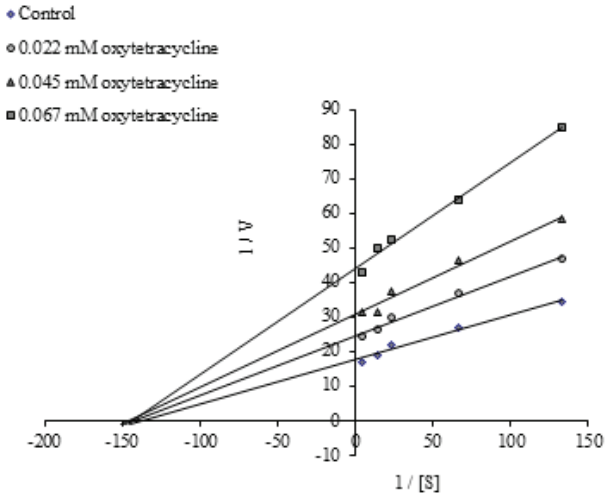

Figure 3: Lineweaver-Burk graph for 5 different substrate concentrations and 3 different oxytetracycline concentrations for determination of Ki

lethal effects in cancer patients. Therefore, it is crucially important to know the inhibitors and activators of this enzyme (8).

In order to determine the activators and inhibitors of this enzyme, it is necessary to investigate the effects of those drugs on *the enzyme's* activity. For this purpose, the enzyme was purified using affinity chromatography on a 2', 5'-ADP-Sepharose column. Some important modifications had been made during the purification process. The protein fractions within the solution were precipitated with ammonium sulphate and loaded onto an affinity column. However, high DPD enzyme activity was observed in the washing fractions. We suspected that G6PD, 6PGD, and GR enzymes have higher affinities than DPD to the used column, DPD enzyme was unable to bind to the column; thus, it was present at very high levels within washing fractions. To solve this problem, firstly G6PD, 6PGD and GR enzymes were removed from the column by using their own eluting solutions. Then, these elutes, which are free of G6PD, 6PGD, and GR enzymes, were reloaded onto the same column, which had been washed and regenerated. The washing solution was collected and subsequently analysed. No DPD activity was detected, indicating that that DPD enzyme was bound to the column. Then, elution of the DPD enzyme was carried out with buffer A containing 0.5 mM DTT, 0.2 mM NADPH, and 1 M KCl. Thus, the DPD enzyme was then purified 1660-fold and with a 12.5% yield.

The effects of many drugs such as antibiotics, analgesics, and anaesthetics on human G6PD, 6PGD, GR and CA have been investigated (12, 13, 14, 15). Another study shows that anaesthetics inhibited Na^+, K^+-ATPase, Mg^{+2}-ATPase and acetylcholinesterase activity from rat cerebral cortexes (16).

However, to the best of our knowledge, the inhibitory effects of the drugs examined here on DPD in the bovine liver have not been studied. In order to show inhibitory effects, while the most suitable parameter is the Ki constant, some researchers use the IC_{50} value (17, 18). Therefore, in this study, both the Ki and IC_{50} parameters of these drugs for DPD were determined. Whole drugs showed non-competitive inhibition. In this study, the drugs inhibited DPD activity in comparison with the control group. The drugs can cause non-competitive inhibition by binding to other sites affecting the three-dimensional structure of the enzyme (19).

Ki values show that oxytetracycline had the highest inhibitory effect, followed by ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin. IC_{50} values showed the same trend. According to Ki constants and IC_{50} values, *the enzyme is mostly inhibited by* oxytetracycline, ciprofloxacin, ceftazidime, and cefoperazone drugs. The chemical structures of all these drugs contain an active group of carbonyl, hydroxyl, and nitrogen.

Ki and IC_{50} parameters show that the inhibitory effect of oxytetracycline, ciprofloxacin, ceftazidime, and cefoperazone are highly potent. Therefore, it is thought that if these drugs inhibit DPD activity, they would cause significant problems in metabolism. Pyrimidine catabolism is obstructed, and β-alanine cannot be synthesized. Patients using antibiotics that inhibit DPD activity together with chemotherapy drugs, such as 5-fluorouracil, might experience poisoning and a lethal effect because of the accumulation of 5FU. In conclusion, DPD is the initial and the rate-limiting enzyme of pyrimidine catabolism. Moreover, it functions in the synthesis of β-alanine and also removes the toxic effects of 5FU from the system. Therefore, the dosage of antibiotics which inhibit DPD activity might be of great significance in cancer therapy.

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VPLIVI NEKATERIH ZDRAVIL NA ENCIM DIHIDROPIRIMIDIN DEHIDROGENAZO, PRIDOBLJEN IZ GOVEJIH JETER

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Povzetek: Proučevani so bili zaviralni učinki nekaterih zdravil na encim dihidropirimidin dehidrogenazo, pridobljen iz govejih jeter. Encim dihidropirimidin dehidrogenaza [5, 6-dihydrouracil: NADP + oksidoreduktaza, ES 1.3.1.2; DPD] so pridobili iz govejih jeter s pripravo homogenata, obarjanjem z amonijevim sulfatom in afinitetno kromatografijo. Poleg tega so bile v postopek prečiščevanja vnešene nekatere dodatne manjše prilagoditve. S postopkom prečiščevanja je bilo iz govejih jeter pridobljeno 12,5 % encima DPD. Po čiščenju encima je bila opravljena poliakrilamidna gelska elektroforeza z SDS. Raziskani so bili tudi učinki nekaterih zdravil na encimsko aktivnost dihidropirimidin dehidrogenaze v govejih jetrih. Kot zdravila so bili uporabljeni oksitetraciklin, ciprofloksacin, ceftazidim, cefoperazon, amikacin, ornidazol, metronidazol, cefuroksim, cefepim, ampicilin in amoksicilin. Vsa zdravila so na encim delovala zaviralno. Vrednosti IC_{50} proučevanih zdravil so bile določene z grafičnim modelom, pri katerem je prikazan odstotek aktivnosti v odvisnosti od [i]. IC_{50} vrednosti oksitetraciklina, ciprofloksacina, ceftazidima, cefoperazona, amikacina, ornidazola, metronidazola, cefuroksima, cefepima, ampicilina in amoksicilina so bile 0,030, 0,046, 0,140, 0,610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920 in 6.300 mM; konstante Ki pa 0,050 ± 0.01, 0.090 ± 0,06 0,130 ± 0,045, 0,185 ± 0,057, 2.010 ± 0,55, 2.096 ± 1.06, 2.115 ± 1,00, 2.700 ± 0,56, 3.730 ± 1,48, 5.240 ± 1.04 in 9.570 ± 2,84 mM za DPD govejih jeter. Konstante Ki so bile za dihidropirimidin dehidrogenazo določene z grafom Lineweaver-Burk. Vsa zdravila so pokazala nekompetitivne zaviralne vzorce delovanja.

Ključne besede: dihidroprimidin dehidrogenaza; goveja jetra; zdravila; zaviranje