Orijinal Makale [Original Article]

The Effects of Some Anti-Tumoral Drugs on Rat Intestinal Butyrylcholinesterase

[Bazı Anti-Tümoral İlaçların Sıçan İnce Barsak Butirilkolinesterazına Etkileri]

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ABSTRACT

Cysplatin (CDDP), cyclophosphamide (CY), methotrexate (MTX) and vinblastine (VINB) are chemotherapeutic agents used respectively in uterus, breast and lung, digestive tract and kidney cancer treatment, in different combinations with other chemotherapeutic agents.

Butyrylcholinesterase (BChE) is a major detoxification enzyme found in many tissues and body fluids of different organisms. Here, we investigated the effects of these drugs on soluble BChE purified from rat small intestine, at the concentration in ranges used in cancer therapy.

The time-dependent inhibitions of BChE by the drugs studied were found to be rapid. The initial inhibition percentage for each drug at zero time was stable for 60 minutes displaying that these drugs might be the reversible inhibitors of BChE.

BChE activity was decreased by increasing the drug concentrations. Comparison of inhibitory effects of the corresponding drugs, at 1 mM concentration, revealed the inhibition percentages of 15%, 25%, 60% and 75% for MTX, VINB, CY and CDDP, respectively. MTX and VINB, at the concentrations used in therapy, 1-25 nM, will have no inhibitory effect on BChE. CY and CDDP are used at therapeutic concentrations of 100-200 μM at which BChE was inhibited approximately by 30% thus these drugs may inhibit BChE in vivo.

Among studied drugs, cysplatin was found to be the most effective inhibitor within its chemotherapeutic range. Hence, inhibition kinetics of CDDP was studied in detail. CDDP was found as a pure competitive inhibitor of BChE with a K_i value calculated as 0.080 ± 0.013 mM.

Key Words: Butyrylcholinesterase, cysplatin, cyclophosphamide, methotrexate, vinblastine, inhibition kinetics, rat intestine

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ÖZET

Cisplatin (CDDP), siklofosfamit (CY), metotreksat (MTX) ve vinblastin (VINB) tümör tipine bağlı olarak, farklı kombinasyonlar içinde kullanılan kemoterapötik ajanlardır. CDDP, uterus kanserinde; CY, meme ve akciğer kanserlerinde; MTX , sindirim sistemi kanserinde; VINB ise böbrek kanserlerinde kullanılan kemoterapotik kokteyllerin bileşenleridir.

Butirilkolinesteaz (BChE; E.C. 3.1.1.8) farklı organizmaların birçok dokusu ve vücut sıvında bulunan bir detoksifikasyon enzimidir. Bu çalışmamızda, dört anti-tumoral ilacın kanser tedavisinde kullanılan dozlarında, sıçan ince barsağından saflaştırdığımız BChE'nin çözünür formuyla moleküler seviyedeki etkileşimlerini araştırdık.

Çalışılan dört ilacın enzim üzerinde zamanla oluşturduğu inhibisyonun, sıfırıncı dakikada oluşan hızlı bir inhibisyon olup, 60 dak. boyunca değişmeden korunduğu tespit edildi. Bu sonuçlar, ilaçların BChE'nin tersinir inhibitörleri olduğunu gösterir.

İlaçların artan derişimlerine karşılık BChE aktivitesinin giderek azaldığı bulundu. Her ilacın 1mM'lık derişimlerinin enzim üzerindeki inhibisyon yüzdeleri kıyaslandığında, MTX'in % 15, VINB'nin % 25, CY'nin % 60 ve CDDP'nin % 75 oranında BChE aktivitesini azalttığı tespit edildi. Kanser terapisinde hastalara MTX ve VINB, 1-25 nM derişimde verildiğinden, bu aralıkta BChE üzerinde inhibitör etkilerinin görülmeyeceği söylenebilir. Diğer yandan, CY ve CDDP 100-200 μM derişimlerde kullanılmaktadır ve bu aralıkta BChE aktivitesini yaklaşık % 30 azaltırlar. Bu yüzden bu iki ilacın *in vivo* olarak BChE aktivitesini azaltması beklenebilir.

Çalışılan ilaçlar içinde, cisplatin diğerlerine göre en etkin inhibitör olarak tespit edildiği için inhibisyon kinetiği de çalışıldı ve CDDP'nin enzimin saf kompetitif inhibitörü olduğu saptandı. K_i değeri 0.080 ± 0.013 mM olarak hesaplandı.

Anahtar Kelimeler: Butirilkolinesteraz, cisplatin, siklofosfamit, metotreksat, vinblastin, inhibisyon kinetiği, sıçan incebarsağı

INTRODUCTION

Butyrylcholinesterase (BChE; acylcholine acylhydrolase, E.C. 3.1.1.8) is found in many tissues and body fluids of the vertebrates (1-3). Although its native substrate(s) and its biological significance are still unknown, it has been shown that BChE is involved in the extra-hepatic hydrolytic transformation of a number of ester-containing drugs and xenobiotics. As a detoxifying enzyme, BChE bioscavenges succinyldicholine (muscle relaxant) (4), organophosphate and carbamate esters (pesticides, insecticides and drugs for treatment of glaucoma, Alzheimer Disease etc.)(5), cocaine (6), aspirin (7), amitriptyline (antidepressant)(8), benactyzine and drofenine (anticonvulsants)(9) etc., however it converts some prodrugs to their active forms such as bambuterol (antiasthmatic) (10), heroin (11), irinotecan (anticancer prodrug) (12) etc.

Cancer patients are treated with many drugs and prodrugs that are metabolized or eliminated by esterases in the extra-hepatic tissues and body fluids. Cysplatin (CDDP) which is used for treatment of uterus cancer, cyclophosphamide (CY) which is used for breast and lung cancer, methotrexate (MTX) which is used for digestive tract cancer and vinblastine (VINB) which is used for kidney cancer are chemotherapeutic agents that are used in different combinations depending on the type of tumor. Puche, E. and Perea, M. (13) declared that BChE activity was clearly decreased within 24 hrs after the first session of chemotherapy.

In this study, we investigated the mechanism of the interaction of these four anticancer drugs on BChE purified from rat small intestine within their chemotherapeutic concentration ranges used in anticancer therapy.

MATERIALS AND METHODS

Materials

3-(N-Morpholino) propanesulfonic acid (MOPS), 5-5'-dithiobis (2-nitrobenzoic acid)(DTNB), procainamide hydrochloride, Sepharose 4B, N-N' methylen bis-acrylamide, Trizma base, ethopropazine were purchased from Sigma (USA). S-n-butyrylthiocholine ioide (BTCh), acrylamide, bovine serum albumin (BSA), Coommassie Brilliant Blue R-250 were from BDH (UK). Coommassie Brilliant Blue G-250 was purchased from Serva (Germany). All other chemicals used were of the best analytical grade.

Purification of BChE from rat small intestine

In this study, small intestines were obtained from female Wistar rats killed for students' laboratory coursework at Hacettepe University Medical School. Soluble BChE isoform was purified about 260 fold from rat intestine with a procedure previously reported including Sephadex G-25 chromatography and procainamide-Sepharose 4B affinity chromatography (14).

Enzyme Assay

BChE activity was measured on Shimadzu UV-1601 spectrophotometer by the method of Ellman G.L. *et.al* (15) by using butyrylthiocholine (BTCh) as substrate. Initial velocities were measured at 37°C, in one mL assay mixture, composed of 100 mM MOPS buffer, pH 7.4, 0.25 mM DTNB, 182.5 ng purified enzyme and various concentrations of BTCh; from 0.25 to 4.0 mM. CDDP, CY, MTX dissolved in 100 mM MOPS buffer, pH 7.4 and VINB dissolved in 100 mM MOPS buffer, pH 7.4 containing 10 % dimethyl sulfoxide were used in kinetic studies. In the measurements, the spontaneous hydrolysis of BTCh and the effects of CDDP, CY, MTX, VINB and dimethyl sulfoxide on substrate have been eliminated using different blank tubes containing the necessary combinations of compounds. One unit of BChE is defined as the amount of enzyme which catalyzes the formation of 1μmole product per min under the conditions mentioned above.

Protein determination

Protein contents in the chromatography steps during purification procedure were followed by measuring the absorbance at 280 nm. In the pooled samples at each purification step, protein concentrations were determined by the micro method of Bradford, M.M. (16) using BSA as standard.

Analysis of the kinetic data

The kinetic data were analyzed and the kinetic constants were calculated by means of the non-linear curve fitting module of Systat (version 9.0) statistical software.

RESULTS AND DISCUSSION

Butyrylcholinesterase is one of the major detoxification enzymes that hydrolyze the natural and the synthetic carboxylic or phosphoric acid ester-bond containing compounds (1). The enzyme plays an important role in the bioscavenging of some drugs such as succinyldicholine, organophosphate and carbamate esters, cocaine, aspirin, amitriptyline, benactyzine and drofenine; or activating of some drugs such as bambuterol heroin, irinotecan (4-12). Puche, E. and Perea, M. (13) reported that plasma BChE activity decreased within 24 hrs in the patients diagnosed as having initial stage primary carcinoma of breast, digestive tract, lung, uterus or kidney, after the first treatment with chemotherapy cocktails. This effect was also independent of sex, age, location of the tumor or type of anti-tumoral chemotherapy given. Therefore,

we wonder what the effects of individual drug of chemotherapy cocktails on BChE at molecular level are. In this study, we used BChE purified from rat small intestine and we chose the four chemotherapeutic agents used for different tumor types: Cysplatin (CDDP) which is used for treatment of uterus cancer, cyclophosphamide (CY) which is used for breast and lung cancer, methotrexate (MTX) which is used for digestive tract cancer and vinblastine (VINB) which is used for kidney cancer.

Time-Dependent Effects of Cancer Drugs on Rat Intestinal BChE

To investigate the effect of 25 μM CDDP or CY and 50 μM VINB or MTX, they are preincubated with the enzyme at 37^0C and at indicated times shown in the Figure 1, aliquots were transferred to assay mixture containing 4 mM BTCh as substrate.

As seen in the Fig.1, the time-dependent inhibitions of the enzyme by cancer drugs were rapid and, the initial inhibition percentage obtained for each drug at zero time did not change for 60 minutes. These results showed that cancer drugs might be reversible inhibitors of BChE.

The Concentration Dependent Effects of Cancer Drugs on BChE activity

The increasing concentrations of these drugs decreased the rat intestinal BChE activity. When the inhibitory effects of 1 mM of these drugs were compared, inhibitory percentages were found to be 15%, 25%, 60% and 75% for MTX, VINB, CY and CDDP, respectively (Fig. 2).

In cancer therapy, MTX and VINB are generally used at 1-25 nM concentrations. In vitro, at these drug concentrations, no inhibitory effect on BChE activity will be expected. However, when CY and CDDP were used at therapeutic concentrations (100-200 μM), approximately 20-30% inhibition in the BChE activity was observed (Fig.2).
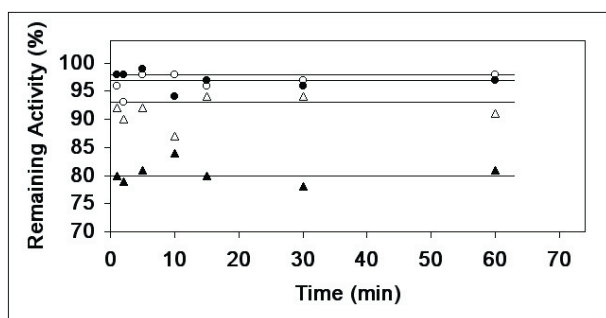
Kinetic Behavior of Rat Intestinal BChE with CDDP

Among the studied drugs, cysplatin was seen as a more effective inhibitor than cyclophosphamide, methotrexate and vinblastine for rat intestinal BChE within its chemotherapeutic concentration ranges (Fig.2). Therefore, inhibition kinetics of CDDP on the enzyme was studied.

In the Lineweaver- Burk plot, the control and inhibitory lines intersected in $1/V_m$ value (Fig. 3). Application of the data to the non-linear regression module of Systat (version 9.0) software fitted the kinetic modeling to pure competitive inhibition. The kinetic parameters, Ks and V_m of rat intestinal BChE were found to be 0.700 ± 0.123 mM and 0.035 ± 0.002 μmole/min/mg protein, respectively. K_i was calculated as 0.080 ± 0.013 mM (17).

The soluble isoform of rat intestinal BChE has tetrameric globular structure similar to that of serum BChE (14). Each monomer of the enzyme molecule contains a 20Å deep and narrow active site cavity lined with approximately 55 residues (18). It is known that two regions of this cavity involve Zn^{2+}-binding site. One of these regions is near by peripheral anionic site (PAS), which is found at the rim of active site cavity and responsible of first binding of a positively-charged substrate. The other is localized close to the esteratic site of active center, which is located at the bottom of cavity. Ni^{2+}, Co^{2+} and Mn^{2+} are competitive for the zinc binding site (19). Similarly, as a competitive inhibitor, CDDP might be bound to Zn^{2+}-binding site at PAS and block the binding of positively charge BTCh to active center.

CONCLUSION

The aim of the present study was to investigate the interactions between rat intestinal BChE and four antitumoral drugs (CDDP, CY, MTX or VINB) within their
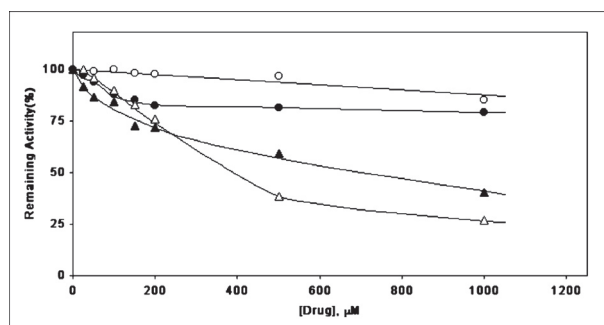

FIGURE 1. Time-dependent changes in the activities of the rat intestinal BChE, when it is treated with 50 μM VINB (○), 50 μM MTX (●), 25 μM CDDP (Δ) or 25 μM CY (▲) at 37°C. Initial velocities were measured at 4.0 mM BTCh.

FIGURE 2. The effects of increasing concentrations of VINB (●), MTX (○), CDDP (Δ) or CY (▲) on rat intestinal BChE activity at 37°C. Initial velocities were measured at 4.0 mM BTCh.
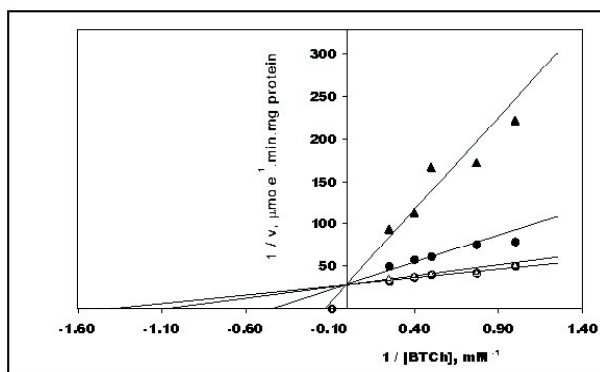
FIGURE 3. The reciprocal plots in the absence of CDDP (○) and in the presence of 0.1 mM CDDP (Δ); 0.2 mM CDDP (●); 0.4 mM CDDP (▲). Initial velocities were determined in 100 mM MOPS buffer, pH 7.4, using 0.025 mM DTNB, 0.25- 4.0 mM BTCh, and 182.5 ng purified enzyme in a final volume of 1 mL activity mixture, at 37°C.

chemotherapeutic concentration ranges. It was found that MTX and VINB do not have any important inhibitory effects on BChE activity at 1-25 nM concentrations range, in vitro. However, approximately 20-30% inhibition in the BChE activity will be observed when CY and CDDP are used at therapeutic concentrations (100-200 μM). It is clear that intestinal BChE constitutes the first line of defense for orally introduced drugs, whereas serum BChE is affected by intravenously introduced drugs. Therefore, investigating the effect of CDDP, CY, MTX or VINB on human serum BChE will be more informative than intestinal BChE, because these drugs are given intravenously to cancer patients.

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