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Effect of Chlorpromazine on Human and Murine Intracellular Carboxylesterases

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Abstract—Clinical use of chlorpromazine (CPZ), an antipsychotic drug, is limited due to its hepatotoxicity. CPZ is found to inhibit *in vitro* intracellular carboxylesterases (CE), such as α-naphthyl acetate esterase, naphthol AS-D chloroacetate esterase, and α-naphthyl butyrate esterase in polymorphonuclear neutrophils, hepatocytes, and neuronal brain cells from mice. CPZ inhibits CE of all these cell types, whereby the degree of the inhibition depends on the incubation time and CPZ concentration. The polymorphonuclear neutrophils are most sensitive to CPZ. Comparable results were obtained with polymorphonuclear neutrophils from mice and humans. Since leukocytes are much more available than hepatocytes or neuronal cells in humans, we assume that CE in peripheral blood leukocytes (neutrophils and monocytes) can be used as markers for indication of pending liver damage by CPZ.

Key words: carboxylesterase, chlorpromazine, hepatocytes, intracellular enzymatic activity, liver damage, neuron, polymorphonuclear neutrophils

Chlorpromazine (CPZ) is an antipsychotic agent commonly used in chronic treatment of severe psychotic disorders, realizing its basic effects by blocking dopamine D_2 receptors. It is a phenothiazine that has been found to express many side effects during its therapeutic use [1]. Clinical use of CPZ can be limited due to its hepatotoxicity [2]. This side effect is usually reflected by an increased level of hepatic enzymes in blood plasma [3]. The release of enzymes results from damage to living hepatocytes and a considerable change in membrane permeability indicating an initiation of lethal cell necrosis. Since data on CPZ intracellular biochemical effects are scarce, this study has been carried out.

In searching for a more sensitive method for the detection of early hepatocyte damage, the CPZ-induced change in activity of intracellular carboxylesterases (CE; EC 3.1.1.1), localized inside living and morphologically intact hepatocytes, was evaluated.

Abbreviations: α-NA) α-naphthyl acetate esterase; α-NB) α-naphthyl butyrate esterase; AS-D) naphthol AS-D chloroacetate esterase; CE) carboxylesterases; CPZ) chlorpromazine; HC) hepatocytes; NC) neuronal brain cells; PMN) polymorphonuclear neutrophils.

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Hepatic microsomal CE plays an important role in drug and lipid metabolism in mammals [4]; therefore, changes in CE activity have both physiological and pharmacological significance [5]. Recently, several authors have reported multiple isoenzymes of hepatic microsomal CE in various animal species [6]. The unusually broad substrate specificity of the CE is due, in part, to multiple CE isoenzymes. Many of these isoenzymes have now been partially purified, but the number of forms and the diversity of their structure are still unknown. However, all CE examined may exist in microheterogeneous forms. Mammalian CE represents a multi-gene family, the products of which are localized in the endoplasmic reticulum of many tissues [7]. So far, distinct CE isoforms have been described in human liver, brain, and placenta [8].

We have evaluated CPZ effect *in vitro* on the activity of CE in hepatocytes (HC), polymorphonuclear neutrophils (PMN), and neuronal brain cells (NC) in mice, as well as in human PMN.

MATERIALS AND METHODS

Chemicals. Substrates naphthol AS-D chloroacetate, α-naphthyl acetate, and α-naphthyl butyrate; di-

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azonium salts of Fast Blue RR, Fast Corinth V, and Pararosaline; buffers Trizma 6.3 and Trizma 7.6; drug chlorpromazine hydrochloride were purchased from Sigma (USA). Fixatives citrate concentrate and methanol were from Alkaloid (Macedonia). Solvents dimethyl formamide and ethylene glycol monomethyl ether were from ICN-Galenika (USA). May-Grunwald, Giemsa, Hariss's hematoxylin, and Mayers's hematoxylin were from Kemika (Croatia). Chemicals were of analytical grade. All CPZ solutions were prepared on the day of experiment.

Animals. Female CBA mice (20 g), six-month-old, were from ICN-Galenika. The animals were given a standard laboratory diet and water *ad libitum*. In this study, 50 animals were used, and the number of samples (experiments) was repeated twice. All measurements were performed in triplicate. From each sample, 100 cells were tested. The final number of tested cells was 5000. Animals used for procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

The experimental procedure includes the following steps: 1) preparation of microscope slides; 2) fixation; 2a) MGG control staining; 3) drug treatment; 3a) control preparations; 4) incubation; 5) counterstaining; 6) evaluation.

Each animal was sacrificed by giving an intraperitoneal injection of pentobarbital sodium (0.045 g/kg body weight) anesthesia, and the tissues (liver and brain) were kept on ice. Afterwards, tissues were cut to thin slices and spread, along with whole blood smears, on microscope slides. The tissue slides are prepared with the "touch" technique. This way of preparation provided a monolayer separate, clusters, or groups of cells adhering to the slides. Single cells and groups of cells were used for the study. Microscope slides were subsequently air-dried, fixed, washed, and subjected to the appropriate cytochemical reaction. Standard cytochemical procedures were used to study the inhibitory effect of CPZ. The slides were also used in the pre-treatment of the cells with CPZ, in the cytochemical reactions, and in the counterstaining. Finally, the slides were viewed under a microscope to evaluate cell reactions. For each component of the study, control specimens were included, following the same procedures in the absence of CPZ. The enzymatic reactions were initiated directly on slides. We followed and evaluated them by viewing with the microscope. The activity of each enzyme after CPZ incubation was compared with the activity of the same but not pre-treated enzyme. All samples were exposed to different CPZ concentrations (50, 100, and 150 μM dissolved in phosphate buffer, pH 6.6) for a fixed period of time (20 min) at room temperature (20-22°C).

Enzymatic activity before and after CPZ pre-treatment was compared only in cells that appeared to be morphologically intact by light microscopy. Basic morphological information was obtained by light microscopy on counterstaining slides. Enzyme activity was measured on the slides without counterstaining, but these slides were also examined morphologically by light microscopy.

Determination of enzymatic activity *in situ*. *Naphthol AS-D chloroacetate esterase* (AS-D) was measured according to a modification of Sigma technical procedure No. 90. The modification includes fixation for 20 sec at room temperature with counterstaining for 10 min and optimal incubation time for each type of cells (PMN human, 10 min; PMN mice, 15 min; HC, 20 min; NC, 60 min).

α-Naphthyl acetate esterase (α-NA) was measured according to a modification of Sigma technical procedure No. 90. The modification includes fixation for 20 sec at room temperature with counterstaining for 10 min and optimal incubation time for each type of cells (PMN human, 60 min; PMN mice, 60 min; HC, 30 min; NC, 60 min).

α-Naphthyl butyrate esterase (α-NB) was measured according to a modification of Sigma technical procedure No. 180. The modification includes fixation for 5 sec at room temperature and optimal incubation time for each type of cells (PMN human, 60 min; NC, 120 min) and counterstaining for 5 min.

Cytochemical reaction. In cytochemical enzymatic reactions, the final reaction product is precipitated within those parts of the cell containing the enzyme. These deposits contrast with the rest of the cell and in the presence of diazonium salt produce a colored granular deposit visible under a microscope. The CEs examined in this study, catalyze the hydrolysis of specific artificial substrates. The free naphthol compound liberated by the hydrolysis was coupled with a diazonium salt to form an insoluble, colored compound at the sites of the enzymatic reactions.

MIMDECI. The *in vitro* effect of CPZ was measured by the Method for Intracellular Measurement of Drug–Enzyme–Cell Interaction (MIMDECI) [9]. This method integrates two methods: the first one, for measurement of non-specific damage to cells (cytological anomalies), and the second one, specific for the measurement of intracellular activity of target enzyme. Namely, the advantage of MIMDECI is that it provides two markers (cell morphology and enzymatic activity) for measuring the effect of drug before specific damage to cells occurs.

Data presentation and statistical analysis. Measurement of enzymatic activity was performed simultaneously in two different ways: a semi-quantitative estimation (arbitrary scale from 0-3) was made by light microscopy (Axioplan) and a quantitative estimation by densitometric microscopy (Axiophot), measuring density of reaction product inside the cells. The results are presented in tables including statistical evaluation (mean value, standard deviation, coefficient of variation, standard error of the estimate of the mean value using Microsoft Excel 97) and graphs.

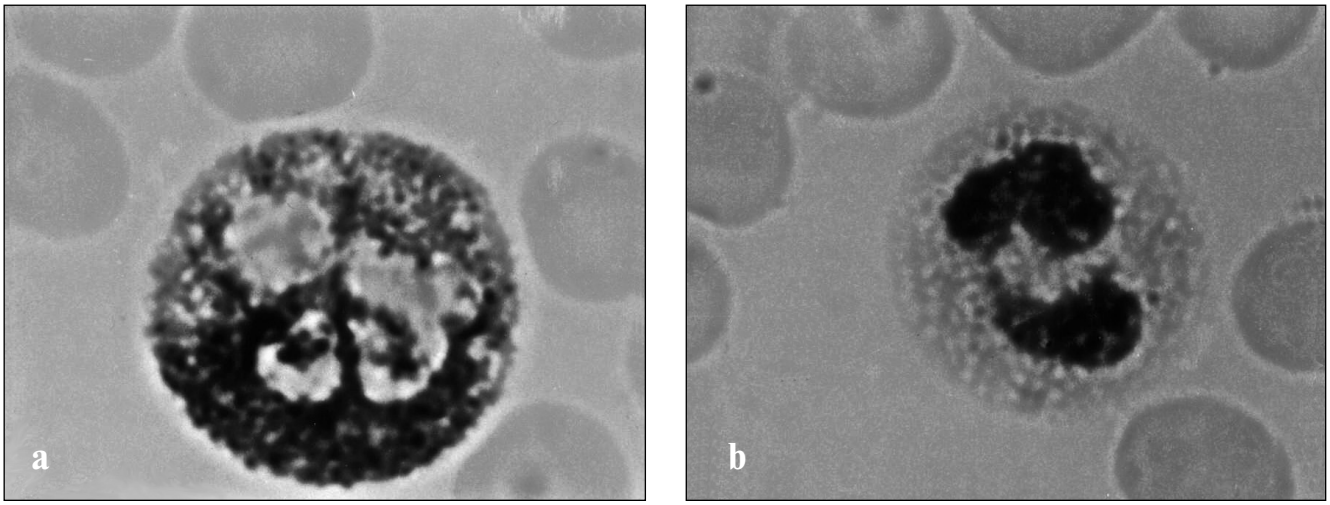

Fig. 1. High-power light micrographs of intact and CPZ-treated morphologically intact murine polymorphonuclear neutrophils. The effect of CPZ on AS-D activity: a) control; b) CPZ-treated (100 μM, 20-min incubation, room temperature).

RESULTS

The effect of different CPZ concentrations (50, 100, and 150 μM) on cells ranged from no effect to death, with intermediary effects of decreased CE activities without either morphological or structural changes. The CPZ effect was compared only in morphologically intact cells, i.e., in those with normal morphology both after the drug treatment and after the enzymatic reaction had taken place.

Enzymatic activity. The end product of the AS-D esterase reaction was represented by large discrete red granules, diffusely scattered throughout the cytoplasm (Fig. 1a). The basic AS-D activity in human PMNs was found to be higher than that in mice PMNs (table). Mouse blood contains a lesser number of PMNs than human blood, but it contains a large number of lymphocytes and platelets. Lymphocytes and platelets are particularly interesting because they possess higher activity of the α-NA esterase than of the AS-D esterase. HC from mice display high activity of the AS-D esterase, and NC from mice display very weak activity of the AS-D esterase (data not shown).

The product of the α-NA esterase reaction was represented by black granules diffusely spread throughout the cytoplasm (not shown). The activity of the α-NA

Quantitative estimation of AS-D esterase inhibition (I, %) *in vitro* after CPZ treatment in murine polymorphonuclear neutrophils (PMN_m) and hepatocytes (HC) as well as in human polymorphonuclear neutrophils (PMN_h) (C, control; δ, standard deviation; CV, coefficient of variation; SE, standard error)

	CPZ, μM	ξ ± δ (*n*)	CV, %	SE, %	I, %
PMN_h	C (0)	2.9 ± 0.15 (50)	6.9	2.0	0
	50	1.6 ± 0.17 (50)	10.1	1.9	52.7
	100	0.9 ± 0.16 (50)	18.4	1.8	98.2
PMN_m	C (0)	2.2 ± 0.18 (50)	8.4	2.0	0
	50	1.1 ± 0.17 (50)	14.3	1.8	54.5
	100	0.8 ± 0.17 (50)	19.7	1.8	94.6
HC	C (0)	2.8 ± 0.18 (50)	6.2	1.9	0
	50	2.3 ± 0.15 (50)	6.6	1.6	17.1
	100	1.8 ± 0.17 (50)	9.4	1.2	50.5
	150	0.9 ± 0.19 (50)	19.2	2.1	99.4

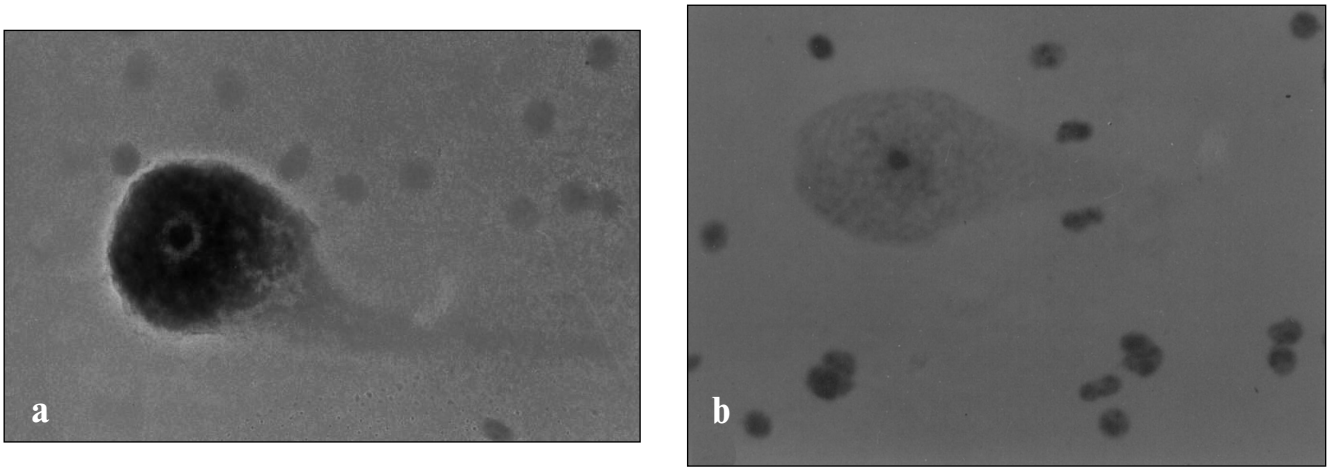

Fig. 2. High-power light micrographs of the product of α-NB esterase reaction in mice neuronal brain cells before (a) and after (b) CPZ treatment (150 μM, 20-min incubation, room temperature).

esterase was detected in monocytes, HC, and NC of mice.

The product of the α-NB esterase reaction was a dark-brown fine granular deposit spread throughout the cytoplasm (Fig. 2a). The activity of the α-NB esterase was detected in monocytes, HC, and particularly in NC of mice.

Optimum incubation time for each type of cells was estimated after a series of different incubation times (ranging from 5 to 150 min) under the same experimental conditions. We repeated same procedure for each CE, and estimated optimal incubation time: for the AS-D esterase in PMN (human) it was 10 min; in PMN (mice), 15 min; in HC, 20 min; and in NC, 60 min; for the α-NA esterase in PMN (human) it was 60 min; in PMN (mice), 60 min; in HC, 30 min; and in NC, 60 min; for the α-NB esterase in PMN (human) it was 60 min; in NC (mice), 120 min.

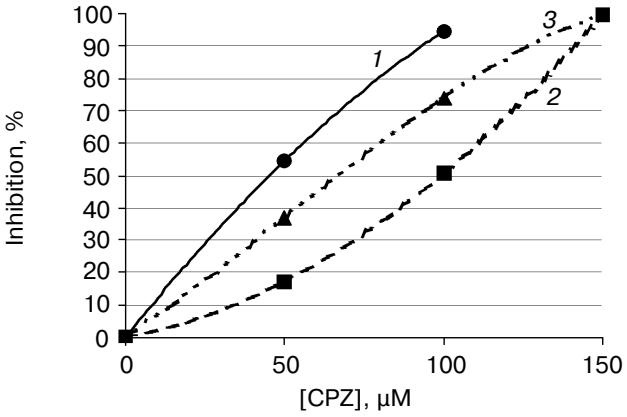

Fig. 3. Comparison of inhibition of intracellular target carboxylesterases by CPZ in polymorphonuclear neutrophils (AS-D esterase (*1*)), in hepatocytes (AS-D esterase (*2*)), and in neuronal brain cells (α-NB esterase (*3*)). PMN, polymorphonuclear neutrophils; NC, neuronal cells; HC, hepatocytes.

AS-D inhibition in various mouse and human cell types. The effect of different concentrations of CPZ on AS-D activity in PMN originating from mice is demonstrated by the decreased amount of final reaction product in CPZ pre-treated cells (Fig. 1b). The amount of reaction product declines as the CPZ concentration increases for all cell types tested here (such as human PMN (PMN_h), murine PMN (PMN_m), and HC from mice) (table). A gradual increase in inhibition of the enzymatic activity in PMN_m correlates well with increase in CPZ concentration reaching its maximum of 94.6% (quantitative estimation for PMN_m) or 92.0% (semi-quantitative estimation for PMN_m) with 100 μM CPZ. I_{50} values (CPZ concentration that inhibits enzymatic activity by 50%) were: I_{50} (quant.) = 42 μM CPZ and I_{50} (semi-quant.) = 45 μM CPZ. These values of I_{50} parameter allowed us to compare enzyme sensitivity in different cell types.

We also evaluated the effect of different CPZ concentrations on the AS-D esterase activity in human PMN (PMN_h). As can be seen from the table, the results are comparable with those for PMN_m—a gradual increase in inhibition of the enzyme activity correlates well with increase in CPZ concentration reaching its maximum of 98.2% (quantitative estimation) and 96.7% (semi-quantitative estimation) with 100 μM CPZ. Basic levels of the AS-D activity were higher in human PMN. I_{50} values were estimated: I_{50} (quant.) = 46 μM CPZ and I_{50} (semi-quant.) = 49 μM CPZ.

The CPZ effect on AS-D esterase activity in HC from mice is also presented in the table. A gradual increase in inhibition of the enzymatic activity parallels the increase in CPZ concentration reaching its maximum of 99.4% (quant.) and 94.5% (semi-quant.) with 150 μM CPZ. There is almost a reverse relationship between CPZ concentrations and enzyme activity (Fig. 3). I_{50} values

were estimated: I_{50} (quant.) = 99 μM CPZ and I_{50} (semi-quant.) = 93 μM CPZ.

The activities of α-NA and α-NB esterases in the human and mouse PMN and in HC were detectable, but the basic level of their activities was substantially lower than that of AS-D esterase in the same cells (not shown), so a statistical evaluation was impossible.

Comparison of CE inhibition in neuronal brain cells (NC) of mice. The mouse neuronal brain cells (NC) demonstrated high activity of the α-NB esterase (Fig. 2a) which was completely (99.5%) inhibited by 150 μM CPZ (Fig. 2b). AS-D and α-NA esterases are not suitable for intracellular biochemical evaluation in mouse neuronal cells because their activities are extremely low.

Comparison of target CE inhibition in various mouse cell types. The effect of CPZ on target intracellular carboxylesterases for each tested cell type was demonstrated. Target intracellular carboxylesterases for PMN and HC were AS-D esterases. Within NC, the target intracellular CE was the α-NB esterase. The CPZ-induced inhibition in all cell types was compared and the I_{50} parameters correlated. It was found that I_{50} (PMN) = 42 μM CPZ, I_{50} (HC) = 99 μM CPZ, and I_{50} (NC) = 63 μM CPZ.

As shown in Fig. 3, the sensitivity for CPZ inhibition of the target enzymes is variable and cell-specific.

DISCUSSION

Several carboxylesterases (CE) play an important role in the hydrolytic biotransformation of a number of structurally diverse endogenous compounds and medications. These enzymes are major determinants of the pharmacokinetic behavior of most therapeutic agents containing ester or amide bonds. CE activity can be directly influenced by interactions of a variety of compounds. Since a significant number of substances effect CE, changes in the activity of these enzymes have important clinical implications and require caution. Drug elimination decreases and the incidence of drug–drug interaction increases when two or more drugs compete for hydrolysis by the same CE isoenzymes. It is therefore important to study the mechanism responsible for CE-induced hydrolysis, which can be accomplished through the use of potent and selective inhibitors.

In the present study, it was shown that CPZ inhibits the activity of intracellular CE. The extents of decrease depended on both the incubation time (data not shown) and drug concentration. CPZ concentrations resulting in ~50% inhibition of the initial activity (I_{50}) vary between murine cell types. This I_{50} value provides a reference point for comparing the inhibitory effects of various substances under clearly defined reproducible conditions (pH, temperature, etc.). CE activity was progressively decreased or completely inhibited at CPZ concentrations ranging from 50 to 150 μM. Corresponding results for both ways of estimation of the enzyme activity refute the subjectivity of semi-quantitative estimation. α-NB esterase has not been previously measured in mouse neuronal cells, although rat and human brain CE were recently investigated [10, 11]. These CE isozymes may have a role to protect the central nervous system from toxic ester or amide compounds and perhaps being a component of a blood–brain barrier system.

In the present study, we have demonstrated a comparable degree of inhibition of carboxylesterases (AS-D, α-NA, α-NB) in different cells. Given the direct time- and concentration-dependent inhibitory effect, it seems likely that the observed reduction in enzyme activity was caused by the inhibition of the intracellular enzyme (CE) but not by the effect of the CPZ on the cell membrane. In contrast, other CE inhibitors, such as eserine [12] or diazinon [13], caused mitochondrial dysfunction and cytotoxicity accompanied by losses of cellular ATP, total adenine nucleotide pools, and reduced glutathione, independently of lipid peroxidation and protein thiol oxidation.

The correlation (by I_{50} parameter) of inhibitory effect in all studied here mouse cell types, has been demonstrated whereby the leukocytes (PMN) were most sensitive and the hepatocytes most resistant to CPZ effect. We also demonstrated comparable results obtained with human and mouse PMN. Since leukocytes are much more readily available than hepatocytes or neuronal cells in humans, we have assumed that CE in peripheral blood leukocytes (neutrophils and monocytes) could be used as markers for the indication of pending liver damage by CPZ. Yang et al. [14] concluded that the α-NA esterase from human plasma provided significant protection against paraoxin intoxication and that the inhibition of this enzyme prevented the development of tolerance seen with repeated paraoxin treatment.

This study demonstrates that measurable biochemical changes in the cell may be observed earlier than morphological changes and, certainly, before cell death occurs. This suggests that monitoring of biochemical effects would provide a more sensitive measure of assessing drug toxicity than determining cell death.

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