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CHLORPROMAZINE TREATMENT INDUCED INHIBITION OF INTRACELLULAR BIOCHEMICAL ACTIVITY OF MOUSE BRAIN TISSUE

RADENOVIĆ LIDIJA [1] and KARTELIJA GORDANA [2]

[1] *Department of Biochemistry and Physiology, Faculty of Biology, University of Belgrade, Yugoslavia*
[2] *Institute for Biological Research, 29 novembra 142, 11060 Belgrade, Yugoslavia*

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Chlorpromazine (CPZ), an antipsychotic drug, was found to inhibit intracellular carboxylesterases (CarbEs). As intracellular target carboxylesterases we used alpha-naphthyl acetate esterase (alpha-NA), naphthol AS-D chloroacetate esterase (AS-D) and alpha-naphthyl butyrate esterase (alpha-NB) in mouse polymorphonuclear neutrophils (PMN), hepatocytes (HC) and neuronal brain cells (NC).

The impact of CPZ on the cells ranged from no effect to death, with intermediary effects of decreased CarbEs activities without either morphological changes or structural changes. The results of our study indicate that intracellular CarbEs activity inhibition by CPZ was dose-dependent, though the drug concentration required to bring about 50% inhibition of the initial activity (ID-50) varied between the mouse cell types, under the same experimental conditions. CarbEs activity was decreased or completely inhibited at CPZ concentrations ranging from 0.5 to 5.0 mg/ml (1.4 to 14.08 mmol/l). The impact maximum concentration of CPZ 5 mg/ml (14.08 mmol/l) on mouse brain cells resulted in 46.58% inhibition for AS-D, 54.26% for α-NA and 99.52% for α-NB.

Our studies established a clear relationship between the increasing concentrations of CPZ and the extent of inhibition of the intracellular esterases of mice. Correlation of the inhibitory effects in all the cell types was demonstrated. The polymorphonuclear neutrophils - leukocytes were the most sensitive (ID-50 = 0.42 mg CPZ/ml) and the hepatocytes most resistant to the CPZ effect (ID-50 = 2.45 mg CPZ/ml). Since leukocytes are human cells much more readily available than hepatocytes or neuronal cells, we presume that CarbEs in peripheral blood leukocytes could be used as markers for the indication of intracellular biochemical damage of hepatocytes and neuronal brain cells by CPZ.

Key words: chlorpromazine, carboxylesterase

INTRODUCTION

Chlorpromazine (CPZ) is an antipsychotic drug commonly used in chronic treatment of hard psychotic disorders, achieving its basic effects by blocking D_2 receptors. However, since data on intracellular biochemical effects of CPZ are scarce this study has been carried out.

We investigated the *in vitro* effect of CPZ on intracellular carboxylesterase (EC 3.1.1.1.) as a target enzyme. In searching for a more sensitive method for the detection of early intracellular biochemical damage, probably still in a reversible phase, the CPZ-induced change in carboxylesterase (CarbEs) localised inside live and morphologically intact cells was measured by the Method for Intracellular Measurement of Drug-Enzyme-Cell Interaction - MIMDECI (Marković, 1987). The advantage of MIMDECI is the provision of two markers (enzyme activity and cell morphology) for measuring the effect of drugs before specific damage to cells occurs. As intracellular target CarbEs we used alpha-naphthyl acetate esterase (alpha-NA), naphthol AS-D chloroacetate esterase (AS-D) and alpha-naphthyl butyrate esterase (alpha-NB).

Carboxylesterases (CarbEs) are characterised by a broad substrate specificity for aliphatic and aromatic esters as well as for aromatic amides. They are important in the hydrolytic transformation of many toxic pesticides, insecticides and drugs, and in detoxification of organophosphorus compounds (Kitahara and Eyre, 1980; Sterri 1989; Huang et al. 1993; Gupta and Dettbarn. 1993; Chambers et al. 1994) by covalent binding to the active sites of the enzymes. In this way, CarbEs considerably eliminate organophosphorus compounds from the circulation and decrease inhibition of AChE in vital tissues, such as respiratory muscles. The unusually broad substrate specificity of the CarbEs is due, in part, to multiple CarbEs isoenzymes. Many of these isoenzymes have now been partially purified, but the number of forms and the diversity of their structure are only beginning to be clarified.

CarbEs are a heterogeneous group of enzymes differing in isoelectric point and substrate-specificity. Most investigations have been made on rat liver esterases. In the last years, six CarbEs have been isolated from rat liver microsomal fractions (Mentlein et al. 1980; Hosokawa et al. 1990). However, all the esterases examined probably exist in microheterogeneous forms.

Since the target enzyme is a cytoplasmic constituent of many types of cells, we have measured the toxic effect of CPZ on mouse hepatocytes, polymorphonuclear neutrophils and neuronal cells in parallel *in vitro.*

MATERIALS AND METHODS

Animals - Female C.B.A. mice (20 gr), six months old were from ICN-Galenika. The animals were given a standard laboratory diet and water *ad libitum*. In this study 50 animals were used. From each sample 100 cells were tested so that the final number of tested cells was 5000.

Enzymes - We studied the following intracellular enzymes:

* Naphthol AS-D chloroacetate esterase (AS-D), measured with a modified technical procedure (Sigma No.90), based on the method of Moloney et al. (1960). The modification included fixation for 20s with counterstaining for 10 min and optimal incubation time for each type of cells.

* Alpha-Naphthyl acetate esterase (α-NA), measured with a modified technical procedure (Sigma No.90), based on the method of Gomory (1953). The modification included fixation for 20s, counterstaining, if required, for 10 min and optimal incubation time for each type of cells.

* Alpha-Naphthyl butyrate esterase (alpha-NB), measured with a modified technical procedure (Sigma, No.180), based on the method of Yam et al. (1971). The modification included fixation for 5s at room temperature, optimal incubation time for each type of cells and counterstaining, if required, 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 are visible under a microscope. The carboxylesterases, measured in this study, catalyze the hydrolysis of the substrate in the presence of diazonium salt, producing a coloured granular deposit. The free naphthol compound liberated by the hydrolysis of the different carboxylesterases, was coupled with a diazonium salt to form an insoluble, coloured compound at the sites of the enzyme reactions.

Methods - The effect of CPZ *in vitro* was measured by MIMDECI. This procedure integrates two methods, one for measurement of non-specific damage of cells (cytological anomalies) and one specific for the measurement of intracellular activity of the target enzyme. Namely the advantage of MIMDECI is the provision of two markers (enzyme activity and cell morphology) for measuring the effect of the drug before specific damage to cells occurs.

The experimental procedure - included the following steps:

1) microscope slide preparation
2) fixation
2a) MGG control staining
3) drug treatment
3a) control preparations
4) incubation
5) counterstaining
6) evaluation

Each animal was sacrificed by chloroform anaesthesia and the tissues (liver and brain) were kept on ice. Afterwards, tissues were sliced and spread, along with whole blood smears, on microscope slides. This way of preparation provided a monolayer of separate cells, 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 the drug (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 the drug.

After the cells were exposed to CPZ enzyme reactions were initiated directly on slides. We followed and measured 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. For the concentration effect study on *in vitro* CPZ treatment all samples were exposed to different CPZ concentrations (0.5

mg/mL-1.41 mmol/L; 2.5 mg/mL-7.04 mmol/L and 5.0 mg/mL-14.08 mmol/L) for a fixed period of time (20 min) at room temperature.

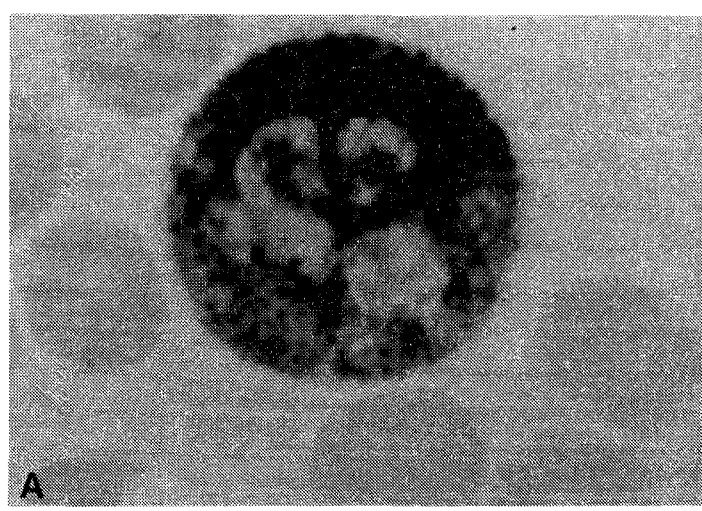
Enzyme activity before and after CPZ pre-treatment was compared only in cells that appeared to be morphologically intact by light microscopy. Basic morphological information was obtained by light microscopy of counterstained slides. Enzyme activity was measured on the slides without counterstaining,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

Measurement of enzyme activity was performed simultaneously at two different levels. A semiquantitative estimation (from 0-3) was done by light microscopy (Axioplan), and a quantitative estimation by densitometric microscopy (Axiophot),which measures the density of the reaction product inside the cells.

RESULTS

The impact of CPZ on the cells ranged from no effect to death, with intermediary effects of decreased CarbEs 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 enzyme reaction had taken place. We assessed their morphological integrity subjectively, using Wright/Giemsa staining of cells on microscope slides. The results are presented in the form of tables, including statistical evaluation and graphs.

Enzyme activity - The end products of the AS-D reaction were large, discrete, red granules, diffusely scattered throughout the cytoplasm (Fig. 1A). The product of the alpha-NA reaction were black granules, diffusely spread throughout the cytoplasm (not presented). The product of the alpha-NB reaction was a dark-brown, fine granular deposit, spread throughout the cytoplasm (Fig. 2A). Optimal incubation time for each type of cell was estimated.



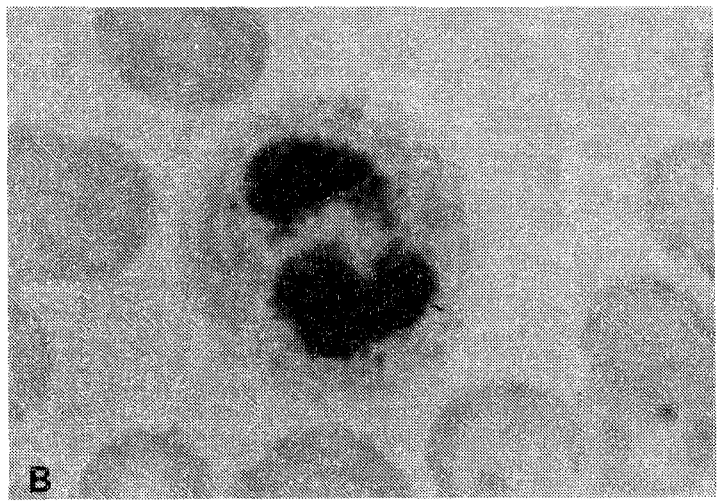
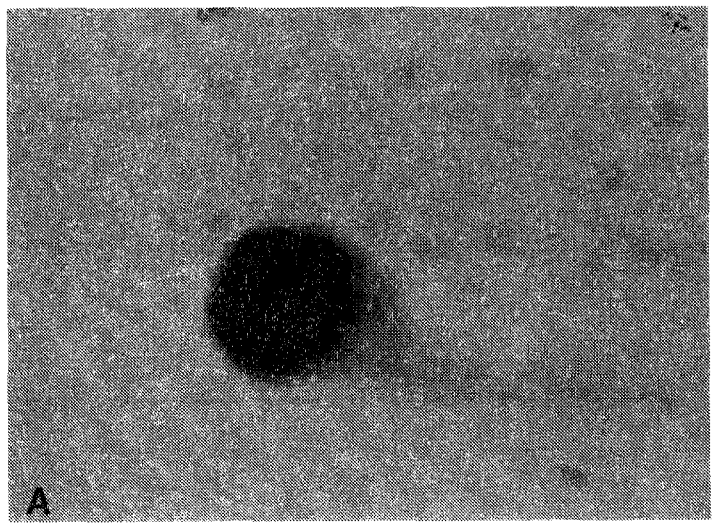


Figure 1. Light-microscopic picture of CPZ-treated, morphologically intact polymorphonuclear neutrophils. The effect of the drug on AS-D activity: A - control; B - CPZ-treated

AS-D activity in polymorphonuclear neutrophils (PMS) - The effect of different CPZ concentrations on AS-D activity in PMS, *in vitro*, is shown in Tables 1 and 2. The gradual increase in enzyme activity inhibition correlated well with the increase



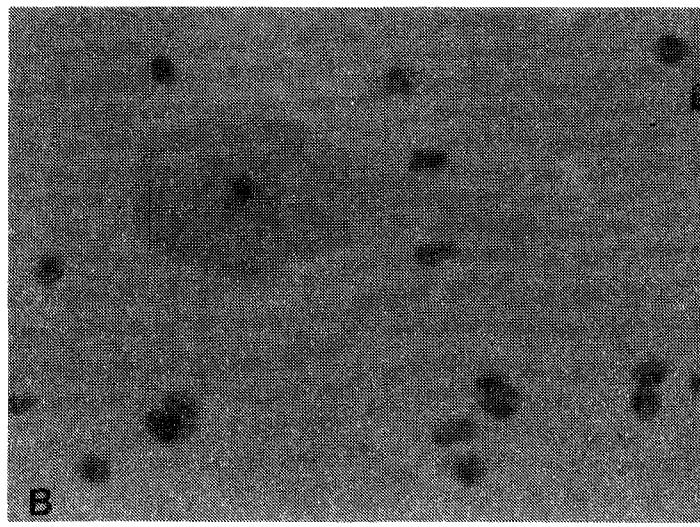


Figure 2. Alpha-NB activity in the murine neuronal cells before (A) and after (B) CPZ treatment.

in drug concentration, reaching a maximum of 91.95% (quantitative estimation) and 94.6% (semiquantitative estimation) with 2.5 mg CPZ/mL. That CPZ reduced AS-D activity in PMS was demonstrated by the decreased amount of final reaction product in CPZ pre-treated cells (Fig. 1). The amount of reaction product declined as the CPZ concentration increased. AS-D esterase activity in PMS was inversely proportional to CPZ concentration. ID-50 parameters (drug concentration that inhibits enzyme activity by 50%) were: ID-50 (semiquan.) = 0.42 mgCPZ/mL ID-50

Table 1 : Results of a semiquantitative estimation of AS-D esterase inhibition after treatment with CPZ in murine polymorphonuclear neutrophils

Conc. CPZ (mg/ml)	n	$\bar{x}$	SD	c.v.(%)	I (%)
C (0)	50	291.4	± 8.00	2.746	0
0.5	50	132.6	± 12.09	9.124	54.5
2.5	50	15.8	± 9.21	58.342	94.6

(quan.) = 0.50 mgCPZ/mL. These ID-50 parameters allowed us to compare enzyme activity in different types of cells.

Table 2 : Results of a quantitative estimation of AS-D esterase inhibition after treatment with CPZ in murine polymorphonuclear neutrophils

conc. CPZ (mg/mL	n	$\bar{x}$	SD	c.v.(%)	I (%)
C (0)	50	2.213	± 0.185	8.379	0
0.5	50	1.116	± 0.1678	14.994	49.58
2.5	50	0.842	± 0.168	19.97	91.95

AS-D activity in hepatocytes (HC) - Tables 3 and 4 illustrate the effect of different concentrations of CPZ on AS-D activity in hepatocytes, *in vitro*. The gradual increase in the enzyme activity inhibition paralleled the increase in CPZ concentration reaching a maximum of 94.54% (quantitative estimation) and 99.43% (semiquantitative estimation) with 5.0 mg CPZ/mL. There was almost a linear relationship between CPZ concentrations and enzyme activity. In other words, AS-D activity in HC was almost directly proportional to CPZ concentration.

Table 3 : Results of a semiquantitative estimation of AS-D esterase inhibition after treatment with CPZ in murine hepatocytes

conc. CPZ (mg/mL)	n	$\bar{x}$	SD	c.v.(%)	I (%)
C (0)	50	291	± 7.228	2.484	0
0.5	50	241.13	± 21.82	9.049	17.14
2.5	50	143.9	± 19.54	13.582	50.55
5.0	50	1.66	± 1.413	85.097	99.43

ID-50 parameters were: ID-50 (semiquan.) = 2.45 mgCPZ/mL ID-50 (quan.) = 2.78 mgCPZ/mL.

Table 4 : Results of a quantitative estimation of AS-D esterase inhibition after treatment with CPZ in murine hepatocytes

conc. CPZ (mg/mL)	n	$\bar{x}$	SD	c.v.(%)	I (%)
C (0)	50	2.837	± 0.177	6.262	0
0.5	50	2.339	± 0.154	6.59	17.56
2.5	50	1.846	± 0.1745	9.447	44.93
5.0	50	0.921	± 0.195	21.183	94.54

AS- D ACTIVITY IN VARIOUS TYPES OF MURINE SELLS - As seenfrom Table 5. CPZ inhibited AS-D activity in a dose-dependet manner in all examined cell types of mice. The drug concentration required to bring about 50% inhibition of the initial activity varied significantly between of cell types.

Table 5 : Results of a quantitative estimation of AS-D esterase inhibition after treatment with CPZ in different types of murine cells;PMN - polymorphonuclear neutrophils; HC - hepatocytes: NC - neuronal cells

conc. CPZ (mg/Ml)	PMN I(%)	HC I(%)	NC I(%)
C (0)	0	0	0
0.5	54.5	17.14	——
2.5	94.6	50.55	44.45
5.0	——	99.43	46.58

Table 6 : Comparison of CPZ-dependent CarbEs activity and inhibition in NC of murine cells

conc. CPZ (mg/ml)	AS - D		alpha - NA		alpha - NB	
	A (%)	I (%)	A (%)	I (%)	A (%)	I (%)
K (0)	100	0	100	0	100	0
0.5	——	——	——	——	76.2	23.8
2.5	55.55	44.45	——	——	19.05	80.95
5.0	53.42	46.58	45.74	54.26	0.48	99.52

COMPARISON OF CarbEs ACTIVIITES IN MURINE NEURONAL CELLS (NC)
- Carboxylesterases (AS-D, α-NA α-NB) were visualised in a single murine neuronal cell and the inhibitory effect of CPZ was demonstrated (Table 6). It was suspected that AS-D and α-NA were not specific enzymes for murine neuronal cells, which we demonstrated with some experimental difficulties. AS-D and α -NA

Table 7 : Comparison of CPZ-dependent inhibition of intracellular target CarbEs in different types of murine cells (PMN - polymorphonuclear neutrophils; HC - hepatocytes; NC - neuronal cells)

Conc. CPZ (mg/mL)	PMN	HC	NC
	I(%)	I(%)	I(%)
C (0)	0	0	0
0.5	54.5	17.14	23.8
2.5	94.6	50.55	80.95
5.0	——	99.43	99.52

were not suitable for intracellular biochemical evaluation in murine neuronal cells, because they were present only in minute amounts. The CPZ concentration required to bring about 50% inhibition of the initial enzyme activity for each esterase varied significantly between the esterases.

Comparison of CarbEs activities in various types of murine cells - The effect of CPZ on specific intracellular esterases for each type of murine cells is shown in Table 7. The specific intracellular esterase for PMN and HC was AS-D and for neuronal cells it was α-NB. The CPZ-induced inhibition in all the cell types was compared and the ID-50 parameters correlated. It was found that ID-50 (PMN) = 0.42 mgCPZ/mL ID-50 (HC) = 1.25 mgCPZ/mL and ID-50 (NC) = 2.45 mgCPZ/mL.

As shown in Fig.3 sensitivity for CPZ-induced inhibition of target murine enzymes was various and cell-specific.

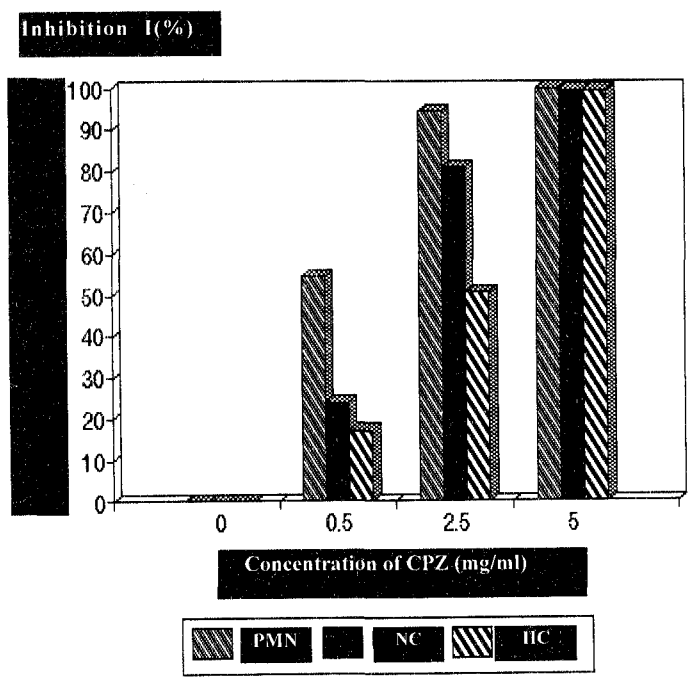


Figure 3. Comparison of CPZ-dependent intracellular CarbEs inhibition in various types of murine cells *in vitro*. PMN - polymorphonuclear neutrophils; NC - neuronal cells; HC - hepatocytes.

DISCUSSION

The results of our study indicate that inhibition of intracellular CarbEs activity by CPZ is dose-dependent, though the drug concentration required to bring about 50% inhibition of the initial activity (ID-50) varies between mouse cell types. CarbEs

activity progressively decreased with CPZ concentration increase. CarbEs (AS-D, α-NA, α-NB) activity was decreased or completely inhibited at CPZ concentrations ranging from 0.5 to 5.0 mg/mL (1.41 to 14.08 mmol/L). The extent of the decrease was dependent both on the duration of incubation with CPZ (results not shown) and drug concentration. In the present study it was shown, for the first time, that CPZ inhibits the activity of intracellular CarbEs. These enzymes catalyze hydrolysis of carboxyl esters and carboxylamide. Alpha-NA, also known as non-specific esterase, is present in many cells, including leukocytes, hepatocytes and neuronal cells. It is actually a heterogeneous group of several enzymes with different specificities for several substrates, including naphthols. AS-D is a specific esterase but also has proteolytic activity. The enzyme has been used to identify neutrophilic granulocytes and it is present in hepatocytes and neuronal cells, as well. Alpha-NB has not been previously measured in neuronal cells.

In the present study we demonstrated a comparable degree of inhibition of the same esterases but in different cells, substantiating our contention that the effects are those of an inhibitor on specific enzymes and not a non-specific drug effect on cells *in vitro*. 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 intracellular enzyme and not by the effect of the drug on the cell membrane.

Our studies established a clear relationship between the increasing concentrations of CPZ and the extent of inhibition of the intracellular esterases in the mouse. From the concentration/inhibition curve one can determine the drug concentration at which 50% of the enzyme activity is inhibited. The concentration of CPZ at which 50% of AS-D activity in polymorphonuclear neutrophils was inhibited was 0.42 and 0.50 mg/mL. In hepatocytes the CPZ concentration (ID-50) was 2.45 and 2.78 mg/mL, as judged by semiquantitative and a quantitative estimation respectively. Corresponding results for both ways of estimation of the enzyme activity refute the subjectivity of semiquantitative estimation. This ID-50 value provides a reference point for comparing the inhibitory or toxic effect of a wide variety of substances under clearly defined reproducible conditions (pH, temperature etc.).

It has been previously shown that other drugs (quinidine, quinine, chloroquine, primaquine) containing a quinoline ring structure, like CPZ, inhibit leukocyte esterases (Marković et al. 1988; 1988a). Since these drugs did not affect several other enzymes, the inhibitory effects on esterases are probably specifically linked to the chemical structure containing the quinoline ring. It has been reported also that the inhibition of Na^+,K^+-ATP-ase by CPZ occurred due to the formation of semiquinone free radicals of CPZ by light (Akera et al. 1974, Akera and Brody, 1977). In contrast, it has been found that CPZ itself inhibits transport enzyme activities both *in vitro* and *in vivo* (Mazumder et al. 1990).

Correlation of the inhibitory effect was demonstrated in all the cell types, whereby the leukocytes (PMN) were shown to be the most sensitive and the hepatocytes most resistant to CPZ effects. Since human leukocytes are much

more readily available than hepatocytes or neuronal cells, we have assumed that CarbEs in peripheral blood leukocytes (neutrophils and monocytes) could be used as markers for the indication of pending liver damage by CPZ.

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

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HRONIČNO UNOŠENJE HLORPROMAZINA IZAZIVA INHIBICIJU INTRACELULARNE BIOHEMIJSKE AKTIVNOSTI MOŽDANOG TKIVA MIŠA

RADENOVIĆ LIDIJA i KARTELIJA GORDANA

SADRŽAJ

Hlorpromazin (CPZ) je neuroleptik koji se koristi za dugotrajni tretman najteih psihijatrijskih bolesti. Osnovni mehanizam dejstva CPZ je blokada D_2 receptora ali se malo zna o efektima CPZ na intracelularnu biohemijsku aktivnost modanog tkiva. U ovom radu ispitivan je uticaj CPZ-a na intracelularne karboksilesteraze (EC 3.1.1.1.), kao marker biohemijske aktivnosti. Primenom osetljive *in vitro* metode - MIMDECI detektovane su rane intracelularne biohemijske promene u morfološki očuvanim ćelijama, indukovane primenom CPZ-a. MIMDECI je tehnologija koja koristi dva markera (za enzimsku aktivnost i morfologiju ćelija) i meri delovanje leka na enzim pre pojave nespecifičnog oštećenja ćelija. Intracelularna biohemijska aktivnost praćena je pomoću alfa-naftil acetat karboksilesteraze, naftol AS-D hloroacetat karboksilesteraze i alfa-naftil butarat karboksilesteraze. Karboksilesteraze su citoplazmatski konstituenti mnogih tipova ćelija, tako da su paralelno praćeni efekti CPZ-a u polimorfonuklearima periferne krvi, hepatocitima i nervnim ćelijama mozga sivog miša, u *in vitro* uslovima. Naši rezultati ukazuju da CPZ inhibira karboksilesteraze u sva tri tipa ćelija i da stepen inhibicije zavisi od vremena aplikacije i primenjene koncentracije. Intracelularna karboksilesterazna aktivnost je smanjena ili potpuno inhibirana primenom CPZ-a u rastućim koncentracijama od 0.5 - 5.0 mg/ml. Korelacijom inhibitornog efekta u sva tri tipa ćelija ustanovili smo da su polimorfonuklearni leukociti periferne krvi najosetljiviji na dejstvo CPZ-a (ID_{50} = 0.42 mg/ml) dok su hepatociti najrezistentniji (ID_{50} = 2.45 mg/ml).