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THE MODE OF INHIBITION OF HUMAN ERYTHROCYTE MEMBRANE-BOUND ACETYLCHOLINESTERASE BY CISPLATIN *IN VITRO*

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The effect of the well known anticancer drug "cisplatin" on the human erythrocyte membrane-bound acetylcholinesterase (AChE) has been investigated. It was found that cisplatin has inhibitory activity against AChE. Cisplatin (0.5–7.0 mM) inhibited AChE activity (27–82%) in a concentration dependent manner. The nature of the inhibition of AChE by cisplatin was complex and involved a partial reversible stage followed by a slow acting irreversible step. The inhibition was independent of a preincubation period up to 90 min while becoming dependent after this period. By diluting the preincubated AChE-cisplatin mixture for different times (0–24 h), the inhibition was partially reversed but again increased progressively with incubation time, ultimately reaching complete inhibition. The effect of increasing substrate concentration had the same behaviour as the dilution effect.

KEY WORDS: Anticancer drug, cisplatin, acetylcholinesterase, inhibition

INTRODUCTION

Cisplatin (cis-platinum (II) diammine dichloride or cis-dichlorodiamine platinum (II); Cdp) is a square planar complex composed of a central platinum atom with a +2 valency coordinated with two amino and two chloro ligands. Cdp can act synergistically with cyclophosphamide and other antineoplastic drugs, and is widely used in the treatment of various types of tumors,[1-8] but particularly against bladder,[9-12] ovarian[13-14] and testicular tumors.[15] To date, there have been no studies of the interaction of Cdp with acetylcholinesterase (EC. 3.1.1.7; AChE). In this study we present data on the interaction of Cdp with human erythrocyte membrane-bound AChE. Generally, AChE catalyses the hydrolysis of an acetate substrate through the formation of the enzyme-substrate complex, followed by acetylation of a serine hydroxyl of the AChE, yielding the acetylenzyme (EA) along with release of the alcohol (first product). The hydrolysis of EA leads to free enzyme and acetic acid (second product).[16]

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MATERIALS AND METHODS

Materials

Acetylthiocholine iodide (AtCh used as a substrate) was supplied by BDH Chemicals Ltd., Pool, (England); 5,5'-dithiobis-(2-nitro) benzoic acid (DTNB) and Cisplatin (Cdp) were purchased from Sigma Chemical Co. and bovine serum albumin (BSA) was obtained from Fluka Chemika-BioChemika, (Switzerland). All other chemicals were of the highest grade available from commercial sources.

Methods

Enzyme Preparation Human blood was collected from healthy males in the presence of citrate phosphate–dextrose–adenine solution as an anticoagulant. Erythrocytes were separated from plasma by centrifugation at 1000 × g (30 min, 4°C) and the plasma and the buffy coat removed by aspiration. The cells were then washed twice with 10 vol. of 310 imOsM, Tris/HCl buffer, pH 7.6, by recentrifugation as described above. The preparation of the enzyme was the same as that reported[17] except lysis buffer was without imidazole and $MgCl_2$, while 100 ug/ml erythrocyte membrane were solubilized with 1% Triton X-100 and 80% of the AChE activity was recovered in the supernatant after centrifugation.

Enzyme assays The enzyme assays were performed according to the spectrophotometric method of Ellman *et al.*[18] at 25°C. The assay mixture (3 ml) contained 0.20 mM AtCh, 0.15 mM DTNB and 31.22 ug protein (in 200 ul 1% Triton X-100 supernatant) as a AChE source. The 5 min incubation time was selected for the enzyme assay, after preliminary experiments were performed to ensure that the enzyme activity was linear with respect to reaction time and the enzyme concentration employed. Two types of blanks were also run at each time, i.e. one without Cdp and enzyme (control blank) and other without enzyme, but with Cdp (Experimental Blank).

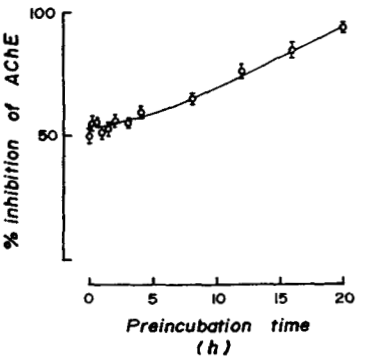

FIGURE 1 Time dependence for the inhibition of AChE by Cdp. Cdp and AChE were incubated for the times shown before addition of AtCh for enzyme assay. Each point represents the mean ± s.d. from 5 experiments.

Time dependence Cdp (7.0 mM) was preincubated with AChE for different times (0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 8, 12, 16 and 20 h) before addition of substrate. Another sample of AChE received Cdp just after addition of substrate (zero-time sample) while the control was assayed in the absence of Cdp. All 13 assays with their blanks were run in three batches. The same experiment was repeated four times under the same conditions.

Combined effect of dilution, preincubation and incubation time The rate of hydrolysis of AtCh was measured in the presence and absence of 7.0 mM Cdp respectively. The assays were run simultaneously for 5 min and the rate of change of absorbance ($\Delta A_{412} = A_{Sample} - A_{Blank}$) were recorded. A 0.3 ml aliquot of each solution was then added to 2.7 ml mixture of the same buffer + DTNB + AtCh for the dilution assay and the A_{412} of each set was recorded along with the original assay (without dilution) for up to 14 h.[19] Similarly the same dilution assay was conducted for each type of preincubated AChE sample (0–24 h) and A_{412} were recorded up to 14 h with different regular intervals of time as shown in Figure 2 for the dilution experiment.

Substrate effects The activity of AChE (zero time incubated with Cdp) in the presence and absence of 7.0 mM Cdp respectively was recorded with a 0.05 mM AtCh. Five successive 20 ul aliquots of AtCh were then added to each reaction media at 10 min intervals such that the final concentration of AtCh was 0.1, 0.2, 0.4, 0.8 and 1.6 mM respectively. The rate of change of absorbance was measured at each substrate concentration. Similarly the same experiment was conducted for the 24 h incubated AChE sample with Cdp (7.0 mM).

Protein estimation The protein content of the enzyme preparation was estimated according to the method of Lowry *et al.*[20] using BSA as standard. The detergent Triton X-100 interfered with this assay was overcomed by centrifuging the precipitate at 1000 × g (4 min, 25°C).[21]

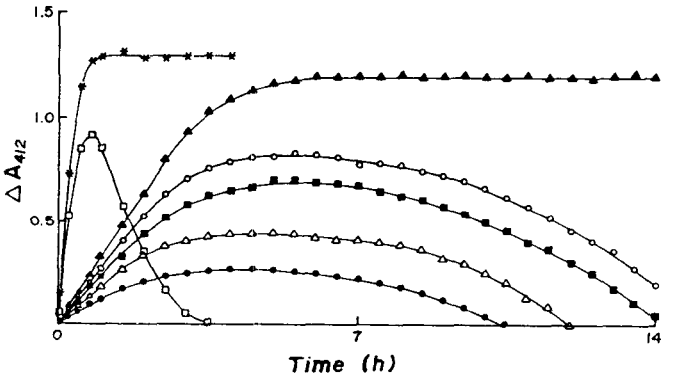

FIGURE 2 Course of product formation in the absence (∗) and presence of Cdp (□) without dilution and without preincubation. In the case of the dilution; ▲, ○, ■, Δ and ●, represents control (without Cdp), 0 h (zero time), 3 h, 9 h and 24 h preincubation of AChE with 7.0 mM Cdp before addition of AtCh respectively. Each point represents the mean of four values.

Statistical analysis The significance of differences between values of control and experimental determinants was determined by the anova test (one way analysis of variance). The straight lines were plotted from statistical calculations by employing the regression analysis programm, where R-sq represents the Hill coefficient in the form of a percentage for expressing the relationship of Y-axis data with their respective X-axis data.[22]

RESULTS

The results presented in Figure 1 show that AChE inhibition by Cdp (7.0 mM) was 99.9% independent of pre-incubation time up to 90 min (R-sq–0.1%), partially independent up to 4.0 h (R-sq-54.5% i.e, 45.5% dependency with time) and fully dependent after a 4.0 h pre-incubation time period (R-sq-97.2%; 2.8% dependency with time). The changes in percentage inhibition of AChE were non-significant between the 0–90 min and 2–4 h periods and significant for the 4–20 h time period ($P > 0.025$). The anova test showed that there were significant differences at 99.9% confidence level (d.f–6/8) for 0–20 h points but the differences were non-significant for 0–90 min or 0–4 h points.

The results shown in Figure 2 are the delta absorbance data for the without dilution and after dilution of different time preincubated AChE samples with their respective controls to show the effect of dilution on inhibition of AChE by 7.0 mM Cdp (Table 1). From the same data, the log percentage activities were plotted against the incubation time for each type of preincubated sample (Figure 3) and the results are summarized in Table 2 for the determination of t_{100} (the time required for 100% inhibition)

TABLE 1
The effect of dilution on the percentage of AChE inhibition by cisplatin at various preincubation and incubation periods

Preincubation time h	% inhibition at various incubation time periods (h) 0.25	4.0	8.0	12.0
*0.0	28.57	100.0	100.0 >	100.0 >
†0.0	15.0	28.0	35.0	63.00
†3.0	30.0	37.5	45.3	75.4
†9.0	45.5	59.4	67.0	100 >
†24.0	58.0	76.0	84.1	100 >

*Without dilution. †With dilution. Each value represents the mean of four independent experiments. The % inhibition of various AChE samples preincubated at different times intervals with cisplatin (7.0 mM) were calculated at various incubation periods by comparing them with their respective controls (without cisplatin). The controls were considered a 100% activity or zero % inhibition in both cases i.e. without dilution as well as after 10 times dilution.

TABLE 2
The t_{100} and t_{50} for AChE inhibited by Cdp with different pretreatment times

Preincubation time (h)	*t_{100} (h)	†t_{50} (h)	System
0.0	3.43	1.35	without dilution
0.0	18.20	10.15	with dilution
3.0	14.55	8.30	with dilution
9.0	11.90	0.45	with dilution
24.0	10.22	−2.38	with dilution

*Time required for 100% inhibition of AChE. †Time required for 50% inhibition of AChE. The values of t_{100} and t_{50} for various AChE samples which were preincubated at different time intervals (0–24 h) with cisplatin (7.0 mM) in the case of before dilution and after dilution were determined from Figure 3.

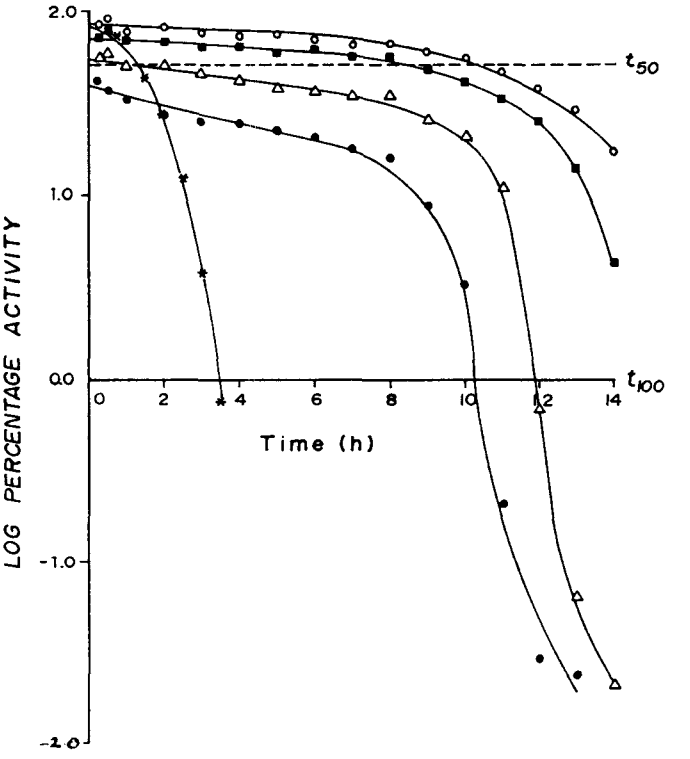

FIGURE 3 Rate of inhibition of AChE by cisplatin without preincubation and dilution (∗) and after dilution of various samples preincubated with Cdp at zero h (○), 3 h (■), 9.0 h (△) and 24.0 h (●).

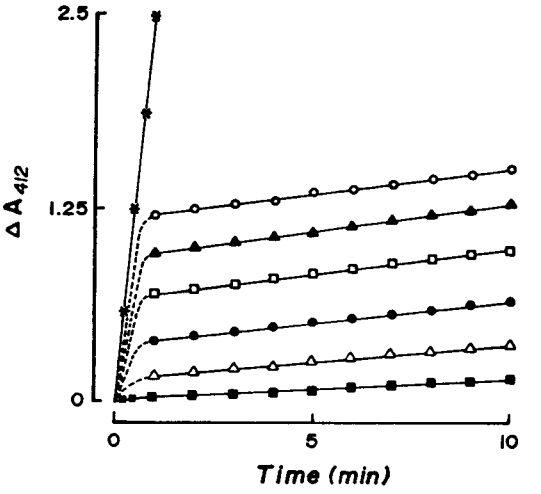

FIGURE 4 Course of hydrolysis of AtCh in the presence and absence of Cdp (∗). In the presence of Cdp the final concentrations (mM) of AtCh were 0.05 (■); 0.10 (△); 0.20 (•); 0.40 (□); 0.80 (▲) and 1.60 (○). Each point represents the mean of four independent experiments.

and t_{50} (the time required for 50% inhibition). It was found that dilution increases t_{100} (397%) as well as t_{50} (624%) and that both t_{100} and t_{50} were inversely related to the preincubation period (Table 2).

The data presented in Figure 4 shows that A_{412} increases proportionally with AtCh concentration and incubation time up to 10 min. The straight lines are parallel with each other from 1 to 10 min, while broken lines shows the extrapolated lines for 0–1 min reaction time for the experimental data.

It can be seen in Figure 5 that AChE inhibition by Cdp was reduced progressively (78.9–39.6%) with increasing concentration of AtCh (0.05–1.6 mM). The anova test was applied for $AtCh_1$ (0.05 mM) to $AtCh_6$ (1.6 mM), $AtCh_1$ to $AtCh_5$, $AtCh_1$ to $AtCh_4$, $AtCh_1$ to $AtCh_3$ and $AtCh_1$ to $AtCh_2$ and gave 99.99%, 99.95%, 99.90%, 99.00% and 95.00% level of confidence respectively. Inhibition was not reduced at all in the case of completely inhibited AChE by Cdp (due to over-night incubation) by increasing the AtCh concentration.

DISCUSSION

Cdp inhibited AChE in a reversible manner when preincubated with the enzyme for up to 90 min, but the nature of the inhibition changed when the preincubation time was increased so that after 4 h of preincubation the inhibition was irreversible. It is probable that during this 4 h period the enzyme and inhibitor slowly form a covalent bond. The effect should be compared with other irreversible inhibitors such

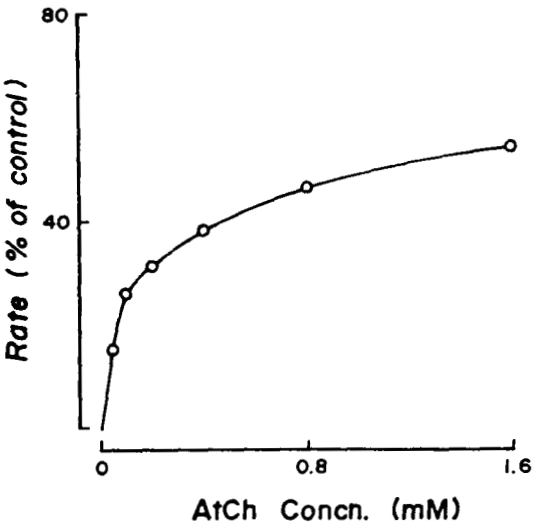

FIGURE 5 Effect of AtCh concentration on the inhibition of human erythrocyte AChE by Cdp (7.0 mM). Data are shown as the mean of four independent determinations.

as methane-sulfonates which also form reversible complex before irreversible stage.[23] Additional support for this conclusion was provided by the results of the dilution experiments (Figure 2). Dilution data shows that the inhibition of AChE by Cdp is partially reversible, the extent of reversion depending upon the preincubation period in an inversely related manner. In the case of dilution when the concentration of Cdp was reduced 10 times i.e., 7 mM to 0.7 mM that this 0.7 mM also has the ability to inhibit AChE 100% but it requires a longer incubation period. The extent of this time for t_{100} again depends upon the preincubation treatment in an inversely related manner as indicated in Table 2.

Moreover, the effect of AtCh concentration on the inhibition of AChE by Cdp also suggests that the nature of the inhibition is first reversible and then it converts to an irreversible form (Figures 4 and 5). This is because the AChE inhibition was progressively decreased by increasing the AtCh concentration up to the first hour of incubation time while an increase in AtCh concentration has no effect on the percentage of inhibition for completely inhibited AChE formed by overnight incubation with Cdp.

The possible mode of action of Cdp on AChE can be postulated in that it forms a reversible complex followed by an irreversible-like form due to covalent bonding between oxygen (hydroxyl group of the serine residue at the catalytic site of the enzyme) and the drug $Pt(NH_3)_2Cl^+$ (the residual part of the Cdp after elimination of Cl following bonding with hydrogen of the serine hydroxyl). By dilution some of the molecules of this covalent complex can be dissociated into free AChE and $Pt(NH_3)_2ClOH$ and later on the Cdp derivative ($Pt(NH_3)_2ClOH$) can again combine

with free AChE and form first a reversible complex, then an irreversible one. The displacement of the chloro ligands by hydroxyl ligands is also supported by the aquation process of cisplatin.[24] One problem arising from these studies and requiring further consideration is the reason for the biphasic nature of the Cdp inhibition of AChE. One possible explanation is initially for Cdp to form a reversible complex with the enzyme by binding to the non-catalytic site followed by formation of covalent bonds by binding to serine or histidine residues. At later incubation times, the serine which have an oxygen nucleophile and histidine which has a nitrogen nucleophile are displaced by another side chain amino acid (cysteine) which has a higher affinity for platinum due to a sulfur nucleophile.[25] This process could inhibit the esterase activity through disruption of the tertiary structure of the enzyme.

Our results concerning the anti-acetylcholinesterase activity of Cdp is also supported by the latest report on its anti-enzymatic activities for Cu-Zn-superoxide dismutase (SOD), Mn-SOD, glutathione peroxidase, glutathione S-transferase and catalase.[26] From these results it can be concluded that Cdp has an inhibitory effect on the human erythrocyte membrane-bound AChE.

References

1. Ohtsu, A., Yoshida, S., Saito, D., Shimada, Y., Miyamoto, K., Fujii, T., Yohino, M. and Yoshimori, M. (1991) *Jpn. J. Clin. Oncol.*, **21**, 120–124.
2. Teatini, G., Meloni, F., Bisail, M., Milia, V., Sanna, G., Sarobba, M.G., Massidda, M., Scotto, T. and Farris, A. (1990) *J. Chemother.*, **2**, 394–396.
3. Gebbia., V., Palmeri S., Russo, A, Gebbia, N. and Rausa, L. (1989) *J. Chemother.*, **1**, 203–206.
4. Shiu, W., Teo, P. and Wong, K. (1990) *Br. J. Clin. Pract.*, **44**, 519–520.
5. Kigawa, J., Kanamori, Y., Morishita, K., Ishihara, H., Minagawa, Y. and Maeda, K. (1990) *Nippon. Gan. Chiryo. Gakkai. Shi.*, **25**, 2665–2669.
6. Palmeri, S., Russo, A., Gebbia, V., Borsellino, N., Gebbia, N., Rustum, Y. and Rausa, L. (1990) *J. Chemother.*, **2**, 28–32.
7. Bacci, G., Picci, P., Avella, M., Dallari, D., Ferrari, S., Prasad, R., Di Scioscio, M., Malaguti C. and Caldora, P. (1990) *J. Chemother.*, **2**, 127–135.
8. Asai, A., Matsutani, M., Kohno, T., Fujimaki, T. and Takakura, T. (1989) *Neurosurgery*, **24**, 439–443.
9. Roncero, C., Flores, N., Portillo, J.A., Escudero, A., Jimenez C.F. and Isorna, S. (1990) *Prog. Clin. Biol. Res.*, **353**, 95–103.
10. Deitmer, T. and Urbanitz, D. (1988) *J. Cancer Res. Clin. Oncol.*, **114**, 644–646.
11. Sager, E.M., Fossa, S.D., Ous, S. and Johannessen, N. (1988) *Prog. Clin. Biol. Res.*, **260**, 499–510.
12. Maru, A., Akaza, H., Isaka, S., Koiso, K., Kotake, T., Machida, T., Matsumura, Y., Nakagami Y., Niijima, T., Obata, K. *et al.*, (1987) *Cancer Chemother. Pharmacol.*, **20**, 44–48.
13. Deppe, G., Malviya, V.K., Boike, G. and Young, J. (1991) *Eur. J. Gynaecol. Oncol.*, **12**, 93–97.
14. Piura, B., Glezerman, M. and Cohen, Y. (1990) *Eur. J. Gynaecol. Oncol.*, **11**, 389–393.
15. Mackintosh, J.F., Hawson, G.A., Johnston N.G. and Matar, K.S. (1989) *Aust. N. Z. J. Surg.*, **59**, 399–404.
16. Al-Jafari, A.A. (1993) *Toxicol. Lett.*, **66**, 125–131.
17. Al-Jafari, A.A. (1993) *Comp. Biochem. Physiol.*, **105C** (2), 323–327.
18. Ellman, G.L., Courtney, D., Andres, V. and Featherston, R.M (1961) *Biochem. Pharmacol.*, **7**, 88–95.
19. Dawon, R.M. (1990) *Neurosc. Lett.*, **118**, 85–87.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.

21. Chandarajan, J. and Klein, L. (1975) *Anal. Biochem.*, **69**, 632–636.
22. Daniel, W.W. (1974) (Ed.) *Health Sciences*, pp. 179–268. New York: John Wiley.
23. Kitz, R. and Wilson, I.B. (1962) *J. Biol. Chem.*, **237**, 3245–3249.
24. LeBlanc, G.A. and Waxman, J.D. (1989) *Drug Metab. Rev.*, **20**, 395–439.
25. Sundquist, W.I. and Lippard, S.J. (1990) *Coordination Chem. Rev.*, **100**, 293–322.
26. Sadzuka, Y., Shoji, T. and Takino, Y. (1992) *Biochem. Pharma. Col.*, **43**, 1872–1875.