Kinetics for Camel (*Camelus dromedarius*) Retina Acetylcholinesterase Inhibition by Methotrexate In Vitro

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ABSTRACT—This work addresses the kinetic analysis of the interaction of methotrexate (MTX) with camel retina acetylcholinesterase (AChE, EC 3.1.1.7). It was found that the MTX effect was reversible in nature. The IC_{50} was determined, by two methods, to be 1.362 mM. The Michaelis-Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide (ASCh) by AChE was 0.123 mM in the control system, and the MTX-treated systems showed a 10–35% decrease in this value. The V_{max} was 0.789 $\mu\text{mol}/\text{min}/\text{mg}$ protein for the control system, while it was decreased by 23–76% in the MTX-treated systems. The Lineweaver-Burk plot, Dixon plot and their secondary replots indicated that the inhibition was a linear mixed type; i.e., uncompetitive and noncompetitive. The values of K_i and K_i' were estimated as 0.782 and 0.404 mM, respectively. The use of camel retina as a model for the study of human retina may open new avenues for studying various aspects of AChE.

Keywords: Acetylcholinesterase, Kinetics, Inhibition, Methotrexate, Retina

In the central nervous system (CNS), acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7, AChE) is a very efficient enzyme because it hydrolyzes neurotransmitter acetylcholine (ACh) at a rate nearly as fast as allowed by diffusion (1). Cholinesterases are also present in non-cholinergic contexts. For example, AChE and butyrylcholinesterase (EC 3.1.1.8, BChE) are found in the early stages embryonic development, well before the apparition of cholinergic synapses (2). Therefore, AChE and BChE could be involved in the formation or regulation of ACh gradients that could guide the growth of nerve cells (3). AChE is also present at the surface of the red cells of some vertebrates or in soluble form in the plasma, together with BChE (4, 5). In blood, AChE and BChE are thought to play a detoxification role (6). The presence of AChE and ACh in vertebrate retina have been extensively documented (7, 8). The functional role of ACh in visual transmission in all mammalian retinas has been fairly well-documented (9–12). Also there is a wealth of data demonstrating the effect of cholinergic drugs and anticholinesterases on the function of the retinas (13). The effects reported by various groups of investigators appear to vary widely. Inhibition of AChE has been reported to either hasten or reduce the process of dark adaptation (14–16). Thus, although decreases in visual activity have often been noted following exposure

to anticholinesterases and a muscarinic cholinergic agonist, which may cause an increase in the flicker fusion frequency (17, 18), these effects have largely been ascribed to changes in accommodative ability (19).

Although the retina is embryologically derived from the CNS and rich in AChE, the interactions of AChE with various inhibitors have not been investigated in this tissue, especially in camel retina (7, 20). We believe that camel retina may be an ideal model for studying various aspects of human AChE such as the pharmacokinetic effects of various drugs and chemicals, because it has the specific characteristics of a centrally elongated macular area in which mainly cone photoreceptors are found. Moreover, there is no tapetum lucidum layer between the choroid and retina as found in the eyes of large nocturnal mammals (21).

A series of studies on the effect of anticancer drugs on AChE activities has begun in our laboratory (22–26). One of the studied drugs is methotrexate (4-amino-10-methyl-pteroyl-glutamate, MTX), which has been widely used in the treatment of neoplastic disease (27, 28) rheumatoid arthritis (29) and psoriasis (30), as well as for the treatment of intraocular lymphoma (31). However, its therapeutic use is affected by various types of neurotoxicity following treatment with MTX. Some of the reported examples of MTX neurotoxicity are: acute transient

cerebral dysfunction (32), death of nerve cells in the brain stem (33) and degeneration of the autonomic nerve fibers in the small intestine (34). Severe toxicity on retina resulted as evaluated by electroretinography (35). A survey of the literature reveals that many, if not all, anti-cancer drugs can inhibit different enzymes in vitro and in vivo (22–24, 36, 37). However, little is known about the inhibitory effect of MTX on retinal AChE.

It was of interest, therefore, to gain more insight into the mechanism of retina AChE inhibition by MTX in vitro. To this end, a study was carried out with a camel retina detergent soluble enzyme. In this study, we report the sensitivity of the enzyme to this anti-neoplastic drug, which manifests some of its side effects on the CNS.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide (ASCh, used as substrate), 5,5'-dithiobis-(2-nitro) benzoic acid (DTNB), MTX, adenine and citrate-phosphate-dextrose solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and bovine serum albumin (BSA) was obtained from Fluka Chemika-BioChemika, (Buchs, Switzerland). All other chemicals were of the highest grade available from commercial sources.

Methods

Enzyme preparation: Retinas of young camels (*Camelus dromedarius*) were obtained from the local abattoir. The whole retina from each eye was rapidly removed, rinsed in pre-cold 0.9% saline solution, blotted and weighed. In each batch of enzyme preparation, twenty whole retinas were prepared by homogenizing them in pre-chilled 50 mM sodium phosphate buffer. The homogenization and centrifugation processes for the extraction of retina membrane-bound AChE by 0.5% Triton X-100 were performed as described in the previous report (38).

Assay of AChE activity: AChE activity was determined by the spectrophotometric method of Ellman et al. (39). The 4-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 the reaction time and enzyme concentration employed (8).

To study the effect of MTX, the enzyme was preincubated with MTX for 4 min prior to the addition of substrate. The γK_s represents the dissociation constant of the AChE-ASCh-MTX complex into the AChE-MTX complex and ASCh. The inhibition constant K_i represents the dissociation constant of the AChE-ASCh-MTX complex into the AChE-ASCh complex and free MTX.

Dialysis: A solution of AChE (150 μ l) and MTX (1.5 mM, 2100 μ l) was prepared in 4060 μ l 50 mM sodium phosphate buffer (pH 7.4). A 1.0-ml aliquot was assayed by addition of ASCh and DTNB ($n=3$), and the remaining 3310 μ l was dialyzed against 700 ml buffer at 8 °C for 20 hr. A control solution (AChE and buffer only) was treated similarly.

Protein estimation: The protein content of the enzyme preparation was estimated according to the method of Lowry et al. (40) using BSA as a standard. The detergent Triton X-100 interfered with this assay, so the precipitate was removed by centrifugation as described previously.

Fig. 1. Camel retina AChE inhibition as a function of MTX concentration. The data are presented in two ways: i) $\log (v/v_0 - v)$ (●), where v and v_0 are the reaction rates for the experimental and control systems, respectively; ii) $\log \%$ residual activity of AChE (○) was plotted versus $\log$ MTX concentration. The correlation coefficient was 0.9502 and 0.8723 for $\log (v/v_0 - v)$ and $\log \%$ residual activity of AChE plots, respectively. Each point represents the mean $\pm$ S.D. of four determinations of triple batches of the enzyme preparation.

Table 1. Effect of MTX on kinetic parameters of camel retina AChE

MTX (mM)	K_{sapp} (mM)	% Decrease	V_{maxapp} (U/mg)	% Decrease
0.00	0.115	0.00	0.671	0.00
0.25	0.103	10.43	0.518	22.80
0.50	0.095	17.22	0.397	40.84
1.00	0.084	27.04	0.253	62.30
1.50	0.075	34.96	0.160	76.11

The K_{sapp} and V_{maxapp} were determined by their respective regression equation as well as by the Lineweaver-Burk plot (Fig. 2A). The K_{sapp} is equal to the reciprocal of the x-intercept and V_{maxapp} is equal to the reciprocal of y-intercept of each line for each MTX concentration in the Lineweaver-Burk plot (each point in this figure represents the mean of four determinations of triple batches of the enzyme preparation).

(41).

Statistical analyses: The graphs were plotted by the Grafit program (42). The values of the correlation coefficient, slope, intercept and their standard errors were obtained by the linear regression analysis of this program.

Fig. 2. Inhibition of camel retina AChE by MTX. A: Lineweaver-Burk plot of $1/v$ against $1/ASCh$ in the absence ($\circ$) and presence of 0.25 mM ($\bullet$), 0.50 mM ($\square$), 1.0 mM ($\blacksquare$) and 1.50 mM ($\triangle$) MTX. Each point represents the mean $\pm$ S.D. of four determinations of triple batches of the enzyme preparation. B: Secondary replot of $1/V_{maxapp}$ ($\circ$) and slope ($\bullet$) from the Lineweaver-Burk plot against MTX concentration for the determination of K_i and K_{iapp} , respectively. The correlation coefficient was 0.9862 and 0.9899 for replot of $1/V_{maxapp}$ and slope, respectively.

RESULTS

Dialysis data show that $57.88 \pm 1.48\%$ of the AChE activity was restored (before dialysis, the activity of the AChE-MTX solution was $16.71 \pm 0.25\%$ of the control,

Fig. 3. Inhibition of camel retina AChE by MTX. A: Dixon plot for representation at six different fixed concentrations of 0.50 mM ($\blacktriangle$), 0.25 mM ($\triangle$), 0.125 mM ($\blacksquare$), 0.0625 mM ($\square$), 0.042 mM ($\bullet$) and 0.031 mM ($\circ$) ASCh in the presence of 0–1.50 mM MTX. Each point represents the mean $\pm$ S.D. of four determinations of triple batches of the enzyme preparation. B: Secondary replot of slope ($\circ$), $1/V_{maxapp}$ ($\bullet$) and K_{1app} ($\square$) from the Dixon plot against $1/ASCh$. The correlation coefficient was 1, 1 and 0.9728 for the replot of slope, $1/V_{maxapp}$ and K_{1app} , respectively.

while after dialysis, the corresponding value was $74.59 \pm 1.13\%$, $n=3$).

The transformed data, $\log (v/v_0 - v)$ for the AChE inhibition as a function of $\log$ MTX concentration, is presented in Fig. 1 for the estimation of IC_{50} . The results show that MTX (0.25–1.50 mM) inhibits AChE activity (11.7–61.4%) in a concentration dependent manner, with IC_{50} being about 1.349 mM. The IC_{50} was also determined by plotting $\log$ % residual activity versus $\log$ [MTX] concentration, which gave a value of 1.374 mM. The mean IC_{50} obtained by the above two methods was 1.362 ± 0.0125 mM.

The data in Table 1 shows that MTX (0–1.50 mM)

Table 2. Kinetic constants estimated by various methods

Method	K_i (mM)	K_I (mM)	K_s (mM)	γK_s (mM)	V_{max} (U/mg)
Primary plot	0.784	—	0.115	0.061	0.671
Secondary replot ^{1LBP}	—	0.375	—	—	0.848
Secondary replot ^{2LBP}	0.779	—	—	—	—
Secondary replot ^{1DP}	—	—	—	0.0626	—
Secondary replot ^{2DP}	—	—	0.130	—	0.848
Secondary replot ^{3DP}	—	0.433	—	—	—
Mean value	0.782	0.404	0.123	0.062	0.789
S.E.M.	0.0025	0.0291	0.0076	0.001	0.0591

The details of the primary plot and secondary replots are given in the text; ^{LBP} means Lineweaver-Burk plot and ^{DP} means Dixon plot. ^{1LBP} represents the replot of $1/V_{maxapp}$ versus MTX concentration and ^{2LBP} represents the replot of the slope from the Lineweaver-Burk plot versus MTX concentration (Fig. 2B). ^{1DP} represents the replot of the slope from the Dixon plot versus ASCh concentration, ^{2DP} represents the replot of $1/V_{maxapp}$ versus ASCh concentration and ^{3DP} represents the replot of K_{Iapp} from the Dixon plot versus ASCh concentration (Fig. 3B).

Table 3. Kinetic data from Dixon plot for camel retina AChE inhibition by MTX

ASCh (mM)	K_{Iapp} (mM)	% Decrease	$V_{maxiapp}$ (U/mg)	% Increase
0.031	0.646	—	0.164	—
0.042	0.617	4.49	0.206	25.61
0.063	0.576	10.84	0.276	68.29
0.125	0.504	21.98	0.418	154.88
0.250	0.463	28.33	0.550	235.37
0.500	0.420	34.99	0.673	310.37

The K_{Iapp} and $V_{maxiapp}$ were determined by their respective regression equation and Dixon plot (Fig. 3A). The K_{Iapp} is equal to the x-intercept and $V_{maxiapp}$ is equal to reciprocal of the y-intercept of each line for each ASCh concentration in the Dixon plot (each point in this figure represents the mean of four determinations of triple batches of the enzyme preparation).

decreases K_m (10.43–34.96%) and V_{max} (22.8–76.11%). The type of inhibition produced by MTX was assessed by a Lineweaver-Burk plot as well as a Dixon plot. As shown in Figs. 2A and 3A, MTX inhibited AChE activity as a mixed-type inhibitor. This linear mixed-type inhibition system is considered to be composed of uncompetitive and pure noncompetitive-type inhibitions.

The K_s and γK_s values of the camel retina AChE were found to be 0.123 ± 0.008 and 0.062 ± 0.001 mM, respectively. These values were calculated from the mean of the primary double reciprocal plot (Fig. 2A), regression equations and secondary replot of the $1/\text{slope}$ (for γK_s), $1/V_{maxiapp}$ (for K_s) from a Dixon plot, against the reciprocal form of ASCh concentration (Fig. 3B). The values for these kinetic parameters such as K_i , K_I , K_s , γK_s and V_{max} calculated by these three methods are summarized in Table 2. The K_{Iapp} for each ASCh concentration was estimated by a regression equation and is presented in Table 3. The K_{Iapp} decreased from 4.49–34.99% and $V_{maxiapp}$ increased between 25.61% and 310.37% over the range of ASCh concentration used. The values of K_i and K_I were calculated from primary Dixon plots and secondary replots (43a) of slope and $1/V_{maxapp}$ values from the Lineweaver-Burk plot (Fig. 2B) and regression equation for each ASCh concentration at fixed MTX concentration in the Dixon plot.

DISCUSSION

The restoration of AChE activity ($57.88 \pm 1.48\%$) by dialysis suggests that the MTX effect is reversible (44). The IC_{50} of camel retina AChE for MTX was 1.9 times more than that of the human erythrocyte enzyme, showing that the inhibitory potency of MTX is lower in camel retina than human AChE (Table 4).

The patterns of the lines in the Lineweaver-Burk plot (Fig. 3A) did not follow the patterns expected for simple intersecting linear inhibition systems; i.e., competitive,

Table 4. Comparative data for the effect of MTX on human erythrocyte* and camel retina AChE

Parameter	Human*	Camel
K_s (mM)	0.13	0.123
γK_s (mM)	0.026	0.062
V_{max} (U/mg)	1.27	0.789
IC_{50} (mM)	0.73	1.362
K_i (mM)	1.67	0.782
K_I (mM)	0.34	0.404
Mode of inhibition	mixed (uncompetitive + noncompetitive)	mixed (uncompetitive + noncompetitive)

*Reference 48.

noncompetitive and uncompetitive. Therefore, this means that the inhibition of camel retina AChE by MTX is a mixed-type inhibition. In the case of mixed-type inhibition, there are two types: partial and linear. These two types can be distinguished on the basis of the shape of the Dixon plots; A curved plot indicates a partial mixed-type inhibition system. In our case, the Dixon plot was linear (Fig. 3A), indicating that our system exhibited linear mixed-type inhibition. The linear mixed-type inhibition system can be further categorized into two main types: in the first type, $\alpha > 1$, where the family of Dixon plots as well as Lineweaver-Burk plots intersect above the horizontal axis, while in the second case, $\alpha < 1$, where the family of straight lines (control and "plus inhibitor") in the Lineweaver-Burk as well as Dixon plots intersect below the X-axis. In the present study we found the latter case; i.e., intersection below the X-axis, indicating a mixed-type system showing both uncompetitive and noncompetitive types of inhibition.

The uncompetitive type has also two subtypes: partial and pure. Our system is the pure one, because the plot of $1/v$ -axis intercept (i.e., $1/V_{\max\text{app}}$) versus [MTX] was linear (Fig. 2B), instead of hyperbolic as in the case of partial uncompetitive inhibition (43b).

As in uncompetitive type of inhibition, the noncompetitive type of inhibition has also two subtypes: partial and pure, which can be distinguished on the basis of replots of slope $_{1/\text{ASCh}}$ and $1/V_{\max\text{app}}$ versus [MTX]. In this study we found linear (Fig. 2B) plots that indicate pure noncompetitive inhibition, instead of hyperbolic, as in the case of the partial noncompetitive subtype. There is still one point that requires clarification: in the case of pure competitive inhibition, the slope $_{1/\text{ASCh}}$ plot should also be a linear function of inhibitor concentration (43c) which was observed here; however, this is, overcome by the secondary replot of the Dixon plot, i.e., slope versus $1/[\text{ASCh}]$, in which the straight line does not pass through the origin as it does with a replot in the case of pure competitive inhibition (Fig. 3B).

The results indicate that MTX might produce a conformational change of the enzyme by binding with the AChE-ASCh complex reversibly, yielding a non-productive AChE-ASCh-MTX complex. In this way, MTX can decrease the activity of the enzyme because it can prevent the proper positioning of the catalytic center for acylation with substrate to form acylated AChE due to steric hindrance; i.e., peripheral binding of MTX might decrease acylation to a greater extent than deacylation ($\gamma = 0.504$). MTX decreases the $V_{\max}$, and K_{sapp} , although not to the same extent as in pure uncompetitive inhibition, as is very clearly observable from the pattern of the secondary plots in Fig. 2B. Moreover, MTX and ASCh are not mutually exclusive and both can bind independently of each other

to free AChE, as well as the AChE-ASCh or AChE-MTX complex.

The decrease in K_{sapp} values indicates that MTX increases the substrate affinity towards the anionic site of the active center (45). However, the decrease in K_{sapp} value with MTX occurred because the reaction ($\text{AChE-ASCh} + \text{MTX} \rightarrow \text{AChE-ASCh-MTX}$) removes some of the AChE-ASCh complex, thereby causing the reaction ($\text{AChE} + \text{ASCh} \rightarrow \text{AChE-ASCh}$) to proceed to the right.

The decrease in $V_{\max\text{app}}$ of the enzyme on increasing MTX concentration can be explained in two ways. One possibility is the indirect interaction of MTX on the esteratic site through van der Waals volumes of aromatic residues of the active-site gorge of AChE at the free stage of AChE, due to the noncompetitive component. This effect causes deformation of the esteratic site or disturbance in the quaternary structure of this site in such a way that proper catalysis will be hindered (or reduced). The second possibility for decreasing $V_{\max\text{app}}$ is that MTX binds with the peripheral anionic site (at AChE-ASCh stage) in such a way that it induces a wave of disturbance in all types of binding forces, resulting in conformational changes in the esteratic site at the active center of the AChE and thus block the deacylation process. The uncompetitive component is responsible for this latter type of inhibition pattern.

According to the accepted hydrolysis scheme for acetate substrate by AChE (46), MTX can also interact with AChE at either the AChE-ASCh complex stage or the A-AChE stage. The former possibility is more probable, because if MTX was bound to A-AChE, P_1 would not be affected, while the results show that P_1 is affected by increasing the concentration of MTX, because absorption of the color product was decreased at 412 nm. This means that MTX did not bind with A-AChE and bound instead with the AChE-ASCh complex. Now, considering the case of the AChE-ASCh complex, the anionic site is occupied by the cholinic part of the ASCh and thus is not available to a second ligand. Therefore MTX must bind with a peripheral anionic site. The low value of the K_i/K_j ratio ($\alpha = 0.517$) suggests that MTX has greater affinity towards the peripheral site than the van der Waals volumes of aromatic residues of the esteratic site (47). The comparative data for the effect of MTX on human erythrocyte (48) and camel retina AChE are presented in Table 4. From these results, it can be concluded that the MTX has a mixed-type inhibitory effect on camel retina AChE. If AChE is inhibited by MTX in vivo, neurotransmitter ACh will accumulate in the synapses and neuromuscular junctions, which will interfere with the regular sequence of the nerve impulses and, ultimately, muscle contraction and loss of normal function will appear. The mechanism of action of MTX on camel retina AChE

resembles its interaction with human erythrocyte AChE (48). On the basis of the present kinetic parameters such as K_i , K_t , γK_s , K_s , $V_{\max}$ and the nature of the inhibition (Table 4), our hypothesis that camel retina can be used as an ideal model has been proven.

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