Molecular Interaction of the Antineoplastic Drug, Methotrexate with Human Brain Acetylcholinesterase: A Docking Study

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Abstract: This study describes molecular interactions between human brain acetylcholinesterase (AChE) and the well known anti-neoplastic drug, methotrexate (MTX) and its comparison to 'AChE-cyclophosphamide (CP) interactions' that we reported previously. Docking between MTX and AChE was performed using 'Autodock4.2'. Hydrophobic interactions and hydrogen bonds both play an equally important role in the correct positioning of MTX within the 'acyl pocket' as well as 'catalytic site' of AChE to permit docking. However, docking of CP to AChE is largely dominated by hydrophobic interactions. Such information may aid in the design of versatile AChE-inhibitors, and is expected to aid in safe clinical use of MTX. Scope still remains in the determination of the three-dimensional structure of AChE-MTX complex by X-ray crystallography to validate the described data. The current computational study supports our previous experimental study which concluded a mixed inhibition model for AChE-inhibition by MTX. Furthermore, the present report confirms that MTX is a more efficient inhibitor of human brain AChE compared to CP with reference to K_i and ΔG values.

Keywords: Methotrexate, docking, enzyme-inhibition, human brain acetylcholinesterase.

INTRODUCTION

Methotrexate (MTX) (4-amino-10-methylpteroyl-glutamate) is used in the treatment of neoplastic diseases [1], psoriasis [2] and rheumatoid arthritis [3]. However, the therapeutic benefits of MTX are achieved at the cost of post-treatment neurotoxic effects. Leukoencephalopathy is the most frequent neurologic manifestation of MTX neurotoxicity [4]. In a 2011 study, MTX-induced posterior reversible encephalopathy syndrome in an adult patient was reported [5]. In yet another 2011 study MTX-treated mice showed significant depression-like behaviors and memory defects [6]. The authors concluded that the neurotoxic effect of MTX involved inhibition of the proliferation of hippocampal progenitor cells leading to hippocampal dysfunction, such as depression and cognitive impairment [6]. In short, neuronal cell death in the brain stem, acute transient cerebral dysfunction and degeneration of autonomic nerve fibres in small intestine are some of the frequently reported examples of MTX neurotoxicity.

The present study concerns the enzyme acetylcholine-sterase (AChE, EC 3.1.1.7), which is a serine hydrolase. Silman and Sussman [7] have described 'structure-function relationship' for AChE and reported that the enzyme derives from a large family of proteins that, jointly, share a common α/β fold [7]. Such proteins comprise enzymes that are esterases, lipases and proteases, along with non-enzymatic proteins that operate as adhesion molecules and pro-hormones [7-9]. The primary physiological role of AChE involves the termination of chemical transmission at cholinergic synapses and secretory organs by catalyzing the hydrolysis of the neurotransmitter acetylcholine, ACh, at a high turnover rate ($2.5x10^4$ molecules per second). A significant number of roles have been attributed to AChE in diseases of high scientific concern e.g. cancer [10] and Alzheimer's disease [11]. Moreover, mutations at specific AChE genetic loci have been found to be associated with leukaemia and myelodysplastic syndromes [12]. Depending on their time-dependent concentration, mechanism of binding and use, inhibitors of AChE have been demonstrated to have efficacy (e.g., donepezil, rivastigmine and galantamine in Alzheimer's disease; and pyridostigmine in myasthenia gravis) [13, 14] as well as toxicity (e.g., organophosphate and carbamate pesticides in health) [15].

MTX has been reported to inhibit human erythrocyte AChE [1, 16] and camel retina AChE [17] *in vitro*. In an *in vivo* study which used a rat model, the authors concluded that different brain tumor-types might have highly variable impact on MTX-penetration in brain and brain tumor extracellular fluids [18]. It has long been established that many, if not all, anticancer drugs produce inhibitory effects on different enzymes *in vitro* and *in vivo* [19, 20]. However, there is a paucity of information about the inhibitory effect of MTX on human brain AChE and possible molecular interactions between the two in case of high dose treatment. Hence, the identification of the amino acid residues crucial to the interaction between human AChE and MTX is of scientific interest. Such information is expected to aid in optimizing the safe and efficacious use of MTX in patients. Furthermore, this study would be useful for scientists involved in drug design in their ongoing search for more potent and versatile AChE-inhibitors. Currently, no X-ray crystallographic structural data are available within the Protein Data Bank to aid in the characterization of the interaction between AChE and MTX. It is noteworthy to mention that we had proposed the molecular interaction between CTX-M-15 (a bacterial enzyme) and cefotaxime (a third generation cephalosporin antibiotic), when no crystallographic data were available [21, 22]. CTX-M-15

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was crystallized in 2011 [23]. Recently, we have described interaction between human brain AChE and cyclophosphamide (CP) [24]. The present study describes the mode of potential interactions that may underpin MTX-induced inhibition of human brain AChE.

METHODS

The 3-dimensional structure of human brain AChE which we submitted to the Protein Model Database (Accession Number: PM0077393) was used for the docking study. The Protein Data Bank co-ordinates for the structure of MTX were retrieved from 'DrugBank' database (Accession No. DB00563). Thereafter, the ligand (MTX) was docked to the enzyme (human brain AChE) using 'Autodock4.2'. The MMFF94 force field was used for energy minimization of the ligand molecule. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on the protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Conserved water molecules were duly added to the binding pocket in order to mimic the *in vivo* environment prior to docking. Affinity (grid) maps of 30×30×30 Å grid points and 0.375 Å spacing were generated using the Autogrid program aimed to target grid co-ordinates in proximity with the acyl pocket and the anionic sub site of the catalytic site (CAS) of AChE. The values of x, y and z co-ordinates used for targeting the 'acyl pocket' were 94.00, 89.00 and 3.75, respectively. The x, y and z values used in docking calculations to target the CAS were 90.81, 83.98 and -8.04, respectively. To target the peripheral anionic site (PAS), several docking experiments were performed by placing the center of the grid at different well-recognized amino acid residues known to constitute the PAS. The grid dimensions for targeting the PAS used in this study varied from 20x20x20 Å to 60x60x60 Å. AutoDock parameter set, and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the 'Lamarckian genetic algorithm' and the 'Solis & Wets local search method'. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied. Protein Data Bank co-ordinate files for the complexes showing two ligands (MTX and CP) within the same active site were generated by PyMol (http://pymol.org/educational) using its 'Align' function. The final figures were generated using Discovery Studio2.5 (Accelrys).

RESULTS AND DISCUSSION

The 3-dimensional structure of AChE can be compared to two hemispheres that sandwich the catalytic center between the acyl- and omega-loops. These loops act as the sidewalls of the active site gorge that is approximately 300 $Å^3$ in dimension [7, 9]. Three sub-sites within this active site gorge are noteworthy, (i) a PAS at the gorge mouth that is the initial binding domain encountered by a substrate or inhibitor, (ii) a deeper cationic-π site (CAS), where the quaternary ammonium of choline of ACh interacts, and, (iii) the acyl-binding pocket located at the base of the gorge [7, 9]. It is worth mentioning that the 'ligand' as well as 'protein side chains' were held flexible by the docking software throughout the study. In the present study, the acyl pocket of human brain AChE was found to interact with MTX through the amino acid residues G151, G152, G153, Y155, E233, S234, W317, L320, Q322, E323, S324, V325, F326, F328, Y368, F369, Y372 and H478 (Fig. **1**; Table **1**). The free energy of binding and estimated inhibition constant (K_i) for the 'MTX-AChE acyl pocket-interaction' were determined to be -9.08 kcal/mol, and 0.22 µM, respectively. Five carbon atoms of MTX, namely CA, C, CG, CD and CB were predicted to be involved in hydrophobic interactions with amino acid residues W317, L320, F328, Y368, F369 and Y372 of the enzyme. Total intermolecular energy of docking for MTX-AChE acyl pocket-interaction was found to be -10.72 kcal/mol. One of the nitrogen atoms of MTX was observed to make polar bonds with two amino acid residues (Y155 and H478) of AChE, and hydrogen bonds with another two (S234 and Y368). Moreover, residues E233, W317, F326, F328, and F369 were also found to make hydrogen bonds with MTX. Hence, a total of 9 hydrogen bonds were found crucial to the appropriate positioning of MTX in the acyl pocket of human brain AChE. This is in contrast to 'CP-AChE acyl pocket-interaction' where neither hydrogen bonds nor polar interactions seemed to play any significant role in docking [24]. 'Van der Waals', 'Hydrogen Bond' and 'Desolvation' energy components together contributed -10.68 kcal/mol while the 'Electrostatic' energy component was found to be -0.03 kcal/mol. Total interacting surface area for MTX-AChE acyl pocket-interaction was found to be 1060.06$Å^2$ while pi-pi and cation-pi interactions were absent. This is in contrast to CP-AChE acyl pocket-complex where a hydrogen atom (H9) of CP was found to be involved in cation-pi interaction with F331 of AChE [24].

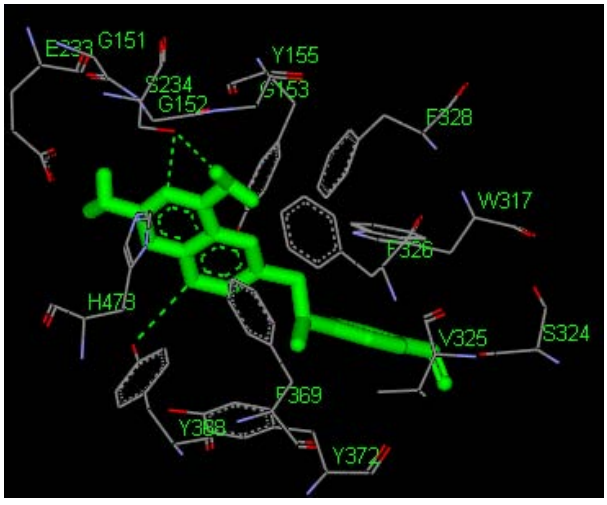


Fig. (1). Interaction of methotrexate docked to the "acyl pocket" of human brain acetylcholinesterase. The ligand (methotrexate) is shown in 'stick' representation.

The CAS site of human brain AChE was determined to interact with MTX through 14 amino acid residues, namely Y103, V104, D105, T106, L107, Y155, W317, S324, V325,

Table 1. Amino Acid Residues Involved in 'AChE-MTX' and 'AChE-CP' Interactions

Interacting Amino Acid Residues of AChE	Ligands	
	Methotrexate (MTX)	Cyclophosphamide (CP)
Acyl pocket	G151, G152, G153, Y155, E233, S234, W317, L320, Q322, E323, S324, V325, F326, F328, Y368, F369, Y372, H478	Y70, Y121, W233, V287, F288, R289, F290, Y334, F408, Y442
Catalytic site or 'CAS'	Y103, V104, D105, T106, L107, Y155, W317, S324, V325, F326 F328, Y368, F369, Y372	W84, N85, G116, G117, Y121, S122, G123, L127, Y130, E198, Y334, H443, G444

F326, F328, Y368, F369 and Y372 (Fig. **2**; Table **1**). The free energy of binding and estimated K_i for the 'MTX-AChE CAS-interaction' were determined to be -9.75 kcal/mol and 0.07 μM, respectively. Elaboration of these interactions might aid in the design of AChE nhibitors focused on the backbone of MTX. Four carbon atoms of MTX, namely CA, C, CD and CB were predicted to be involved in hydrophobic interactions with eight amino acid residues of the enzyme, namely Y103, W117, Y155, W317, V325, F328, F369 and Y372. Total intermolecular energy of docking for MTX-AChE CAS-interaction was found to be -11.74 kcal/mol. Two oxygen atoms of MTX, namely O and OE2 were observed to make four polar bonds involving three amino acid residues (S234, Y368 and H478) of AChE. Residues Y103, D105 and Y155 formed one hydrogen bond each with one of the nitrogen atoms of MTX. The oxygen atom OE1 of MTX was found to make a hydrogen bond with the residue S156 of the enzyme. Another six amino acid residues namely, Y103, D105, T106, Y155, W317 and F328 were involved in hydrogen bonding with the ligand, MTX. Hence, a total of 10 hydrogen bonds were crucial to the appropriate positioning of MTX to allow its docking to CAS of human brain AChE. Again it is noteworthy that more hydrogen bonds are observed in 'MTX-AChE CAS-interaction' compared to 'CP-AChE CAS-interaction' [24], the ratio being 10:3. 'Van der Waals', 'Hydrogen Bond' and 'Desolvation' energy components together contributed -11.85 kcal/mol, the 'Electrostatic' energy component being 0.15 kcal/mol. Total interacting surface area for MTX-AChE CAS-interaction was 999.69$Å^2$. No pi-pi or cation-pi interactions were observed, a feature similar to CP- AChE CAS-interaction [24].

The predicted K_i value for MTX-AChE acyl pocket-interaction (0.22 μM) lies between the reported K_i values for compounds known to interact and inhibit AChE, such as donepezil (K_i 0.11 μM), tacrine (K_i 0.43 μM), and galantamine (K_i 0.44 μM) [25, 26]. Furthermore, the predicted K_i value for MTX-AChE CAS-interaction (0.07 μM) is comparable to that of physostigmine (K_i 0.06 μM) and phenserine (K_i 0.05 μM) [25, 26]. It is noteworthy that all these compounds have been reported to inhibit AChE in humans and elevate ACh levels in the brain of animal models [27]. Figs. (**1**, **2**) show MTX docked to the 'acyl pocket' and 'CAS' sites of human brain AChE, respectively. The docking pose and interacting amino acid residues for the described sites are in strong agreement with X-ray crystallographic structures of known AChE complexes. These residues are highly conserved, and most have been assigned functional roles [28]. A higher (negative) free energy of binding is an indicator of efficient interaction between an enzyme and inhibitor [22]. Accordingly, the free energy of binding for the complexes shown in Figs. (**1**, **2**) were be -9.08 kcal/mol and -9.75 kcal/mol, respectively. These values fall within the same approximate range as a series of donepezil analogues [29], which suggests that MTX is an efficient inhibitor of human brain AChE.

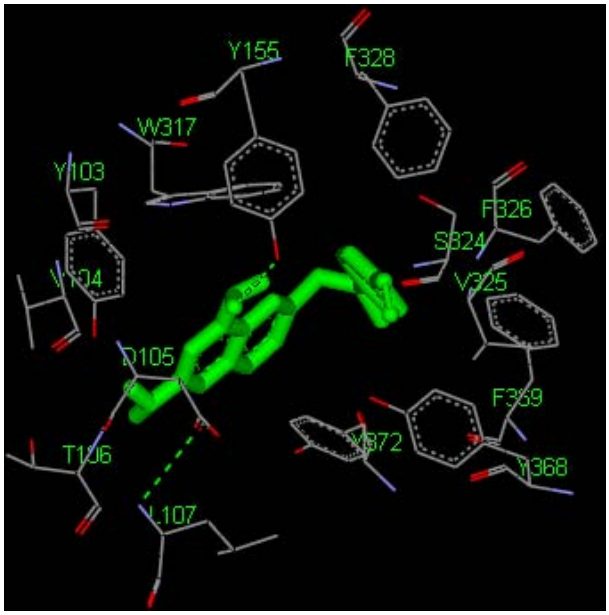
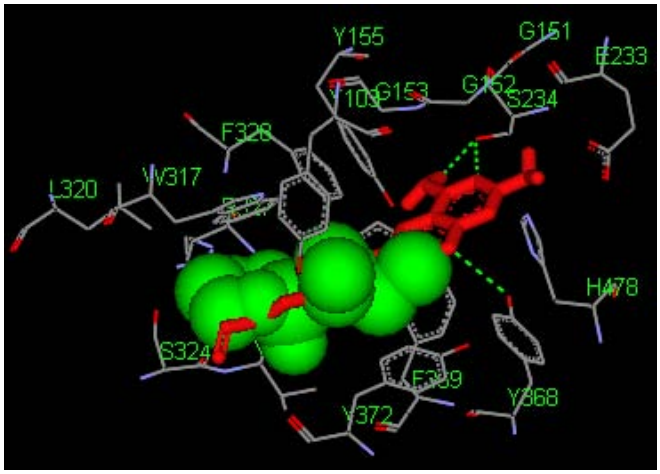


Fig. (2). Interaction of methotrexate docked to the "catalytic site" or "CAS" of the human brain acetylcholinesterase. The ligand (methotrexate) is shown in 'stick' representation.

Fig. (**3a**) represents a superimposition view whereby the ligands MTX and CP are shown occupying the same binding site (AChE acyl pocket) while Fig. (**3b**) is a close-up view of the same. Similarly, to aid in comparison of docking poses of MTX and CP, we constructed Fig. (**4a**) which represents a superimposition view of these ligands within AChE-CAS. Fig. (**4b**) gives a close-up view of the same. 'CP-AChE acyl pocket-interaction' displayed a predicted K_i of 8.49 μM and ΔG of -6.92 kcal/mol; while 'CP-AChE CAS-interaction'

displayed these values as 4.42 µM and -7.31 kcal/mol, respectively [24]. 'MTX-AChE interaction' involving either CAS (K_i = 0.07 µM; ΔG = -9.75 kcal/mol) or acyl pocket (K_i = 0.22 µM; ΔG = -9.08 kcal/mol) displayed a lower Ki and a higher negative ΔG value compared to 'CP-AChE interaction' involving the same binding sites. Hence, the present study reveals that MTX is a stronger inhibitor of human brain AChE than CP in terms of predicted K_i values and free energy of binding as well. In the case of a third docking experiment (MTX to PAS of human brain AChE), it was observed that minor changes in the grid co-ordinates resulted in a different docking pose and even changes in interacting residues, indicative of a mixed inhibition system. In this regard, this is in accord with our previous studies [16, 30].

(**a**)



(**b**)

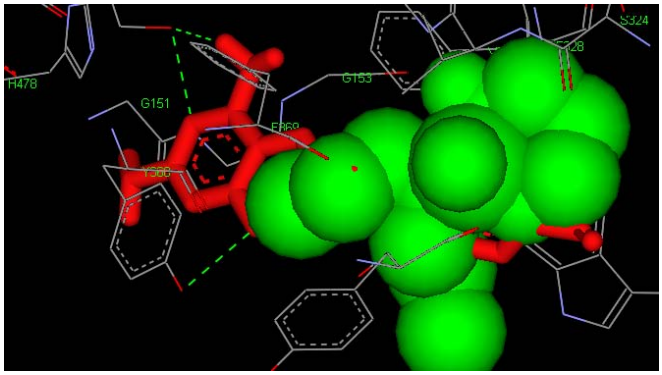
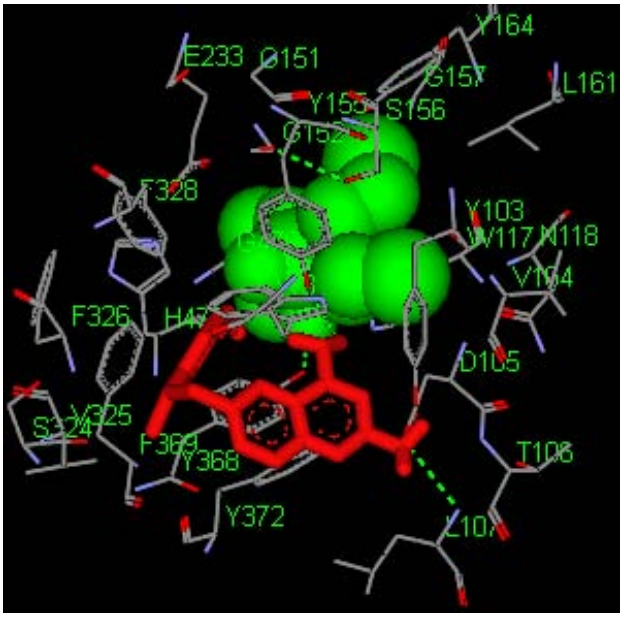


Fig. (3). (**a**) A 'superimposition view' developed by PyMol whereby the ligands (methotrexate and cyclophosphamide) are shown occupying the same binding site (acetylcholinesterase acyl pocket). Cyclophosphamide is shown in 'ball and stick' representation while methotrexate appears as a red colored 'stick'. (**b**) A 'close up view' of Fig. (**3a**).

Further investigations are needed to address the issue regarding the ready entry of MTX-concentrations into human brain tissues, high enough to inhibit AChE. However, it can be safely stated that a high-dose treatment or blood-brain barrier breakdown could elevate brain drug levels or result in the achievement of high systemic levels in the realm of MTX-inducible AChE inhibition. Moreover, the described 'MTX-AChE interactions' highlight the numerous ways in

(**a**)



(**b**)

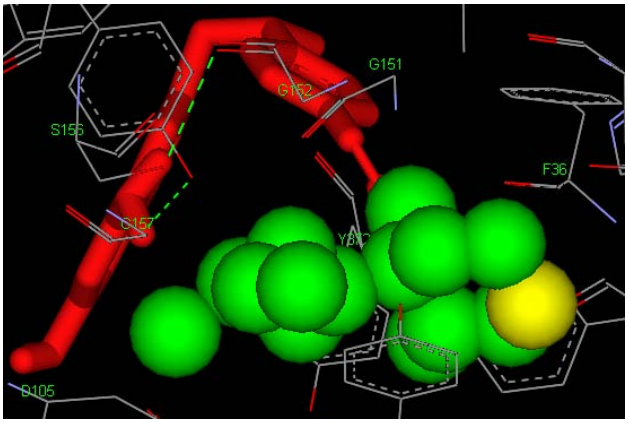


Fig. (4). (**a**) A 'superimposition view' developed by PyMol whereby the ligands (methotrexate and cyclophosphamide) are shown occupying the same binding site (acetylcholinesterase 'catalytic site' or 'CAS'). Cyclophosphamide is shown in 'ball and stick' representation while methotrexate appears as a red colored 'stick'. (**b**) A 'close up view' of Fig. (**4a**).

which structurally diverse inhibitors utilized in the focused treatment of one disease can occasionally dock at an unrelated target (e.g. at AChE) [31], thereby producing such signs and symptoms in patients undergoing treatment which clinicians find difficult to interpret. The study is expected to aid future design of more specific pharmacological compounds. It is noteworthy that the drugs (MTX and CP) discussed herein are of clear clinical significance. The future prospects of MTX and CP in the treatment of diseases like myasthenia gravis (MG) are being actively pursued. MG is a neuromuscular disorder which is characterized by a clinical course of fluctuating and painless muscle weakness [32]. In a 2011 study, the authors reported that the use of cyclophosphamide and rituximab for patients with refractory MG showed promising results [33]. In a recent single-blinded trial of methotrexate versus azathioprine as steroid-

sparing agents in generalized MG, the authors suggested that methotrexate had similar efficacy and tolerability to azathioprine and might be the drug of choice in financially constrained health systems [34].

Before closing the discussion we find it appropriate to mention that K_i and ΔG values obtained through computational studies can only *suggest* efficiency of binding for an enzyme-ligand pair. However, it has been observed that the results of computational analyses often correlate well with the outcomes of experimental studies. The reader is encouraged to see Fig. (**1**) of our previous study which strongly supports this claim [21]. Nevertheless, the *trio* consisting of 'computational', *'in vitro'* and *'in vivo'* studies with reference to the study enzyme (AChE) and ligands (MTX and CP) is expected to form the basis of future therapy against several neurological disorders.

CONCLUSION

This study explores molecular interactions between human brain AChE and the well-known anti-neoplastic drug MTX. Moreover, we have provided a comparative account of the studied interactions with 'AChE-CP interactions' that we reported previously. Hydrophobic interactions and hydrogen bonds both play an equally important role in the correct positioning of MTX within the 'acyl pocket' as well as 'catalytic site' of AChE to permit docking. However, docking of CP to AChE is largely dominated by hydrophobic interactions. Such information may aid in the design of versatile AChE-inhibitors, as well as aid in the most efficacious and safe clinical use of MTX. Scope remains in the determination of the three-dimensional structure of an AChE-MTX complex by X-ray crystallography to validate these data. The current computational study supports our previous experimental study which concluded a mixed inhibition model for AChE inhibition by MTX. This study confirms that MTX is a more efficient inhibitor of human brain AChE compared to CP with reference to K_i and ΔG values.

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CONFLICT OF INTEREST

Declared none.

ABBREVIATIONS

ACh = Acetylcholine

AChE = Acetylcholinesterase

CAS = The anionic sub-site of catalytic site of human AChE

CP = Cyclophosphamide

MG = Myasthenia gravis

MTX = Methotrexate

PAS = The peripheral anionic site of human AChE

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