

Electronic Supporting Information (ESI†)

Evaluation of binding Competition and group epitopes of Acetylcholinesterase inhibitors by STD NMR, Tr-NOESY, DOSY and Molecular Docking: An old approach but new findings.

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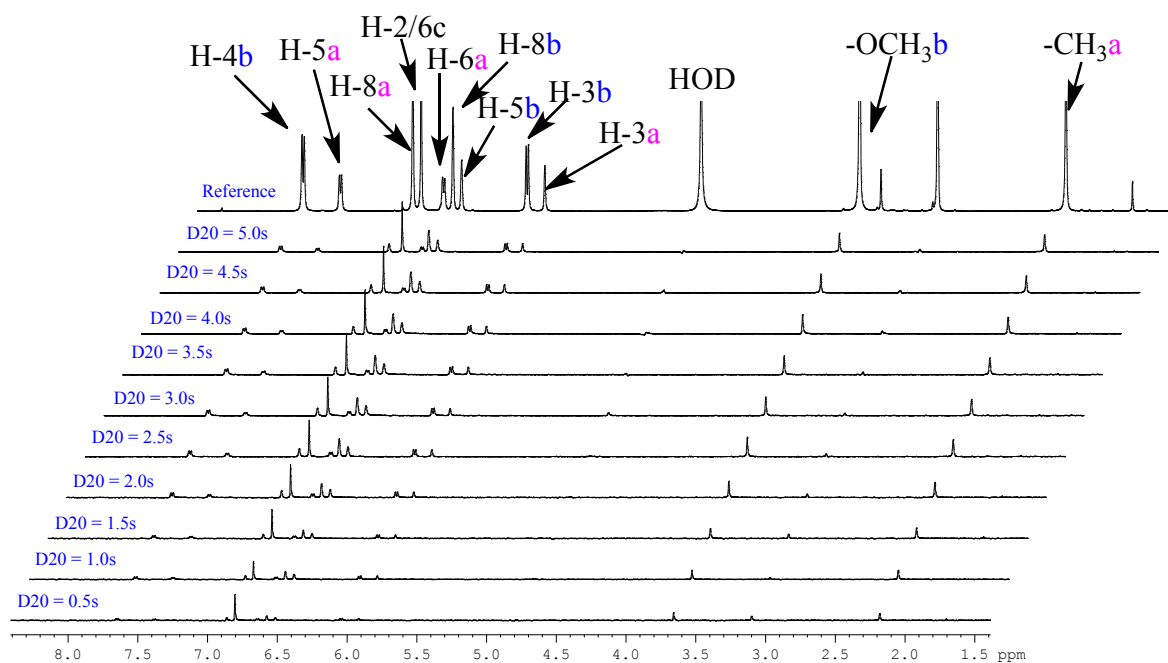


Figure: S-1. Stacked plot of time-dependent saturation STD NMR spectra, where, All STD experiments were recorded on a Bruker 600 MHz (for Hydrogen nucleus) AVANCE III NMR spectrometer at a temperature of 298 K. For more information, see the experimental details.

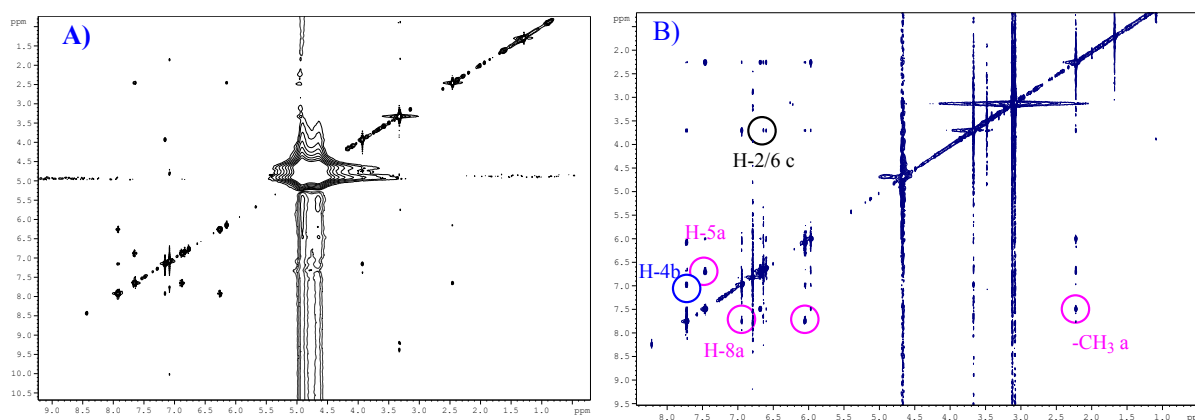


Figure S-2: 2D-NOESY (A) and 2D Tr-NOESY (B) spectra of targeted compounds (scopoletin and 4-methylumbelliferone and gallic acid) without (A) and with (B) adding AChE. Where, pink, blue and black circles are shown for 4-methylumbelliferone, scopoletin and gallic acid signals. Standard NOESY pulse sequence with gradient phase-sensitive spectrum was acquired in deuterated PBS and CD₃OD (95:5% v/v respectively) solution for each inhibitor at 295-298K temperature, whereas, Tr-NOESY spectrum in AChE (50μM, in the deuterated PBS buffer, pH 7.2).

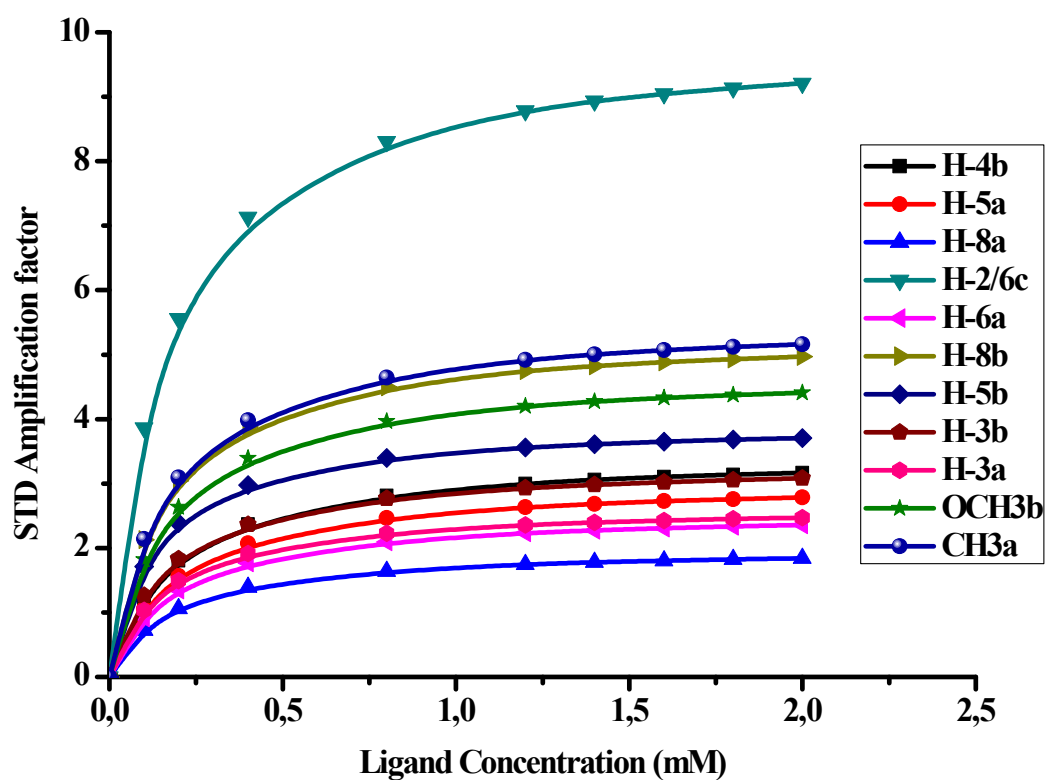


Figure S-3. STD amplification factor curve plot of **A**, **B** and **C** as a function of varying concentrations ranging from 0.10, 0.20, 0.40, 0.80, 1.20, 1.40, 1.80, and 2.0 mM for each single parabolic line that determined from the STD titration spectra as shown in Figure 4 in the main text.

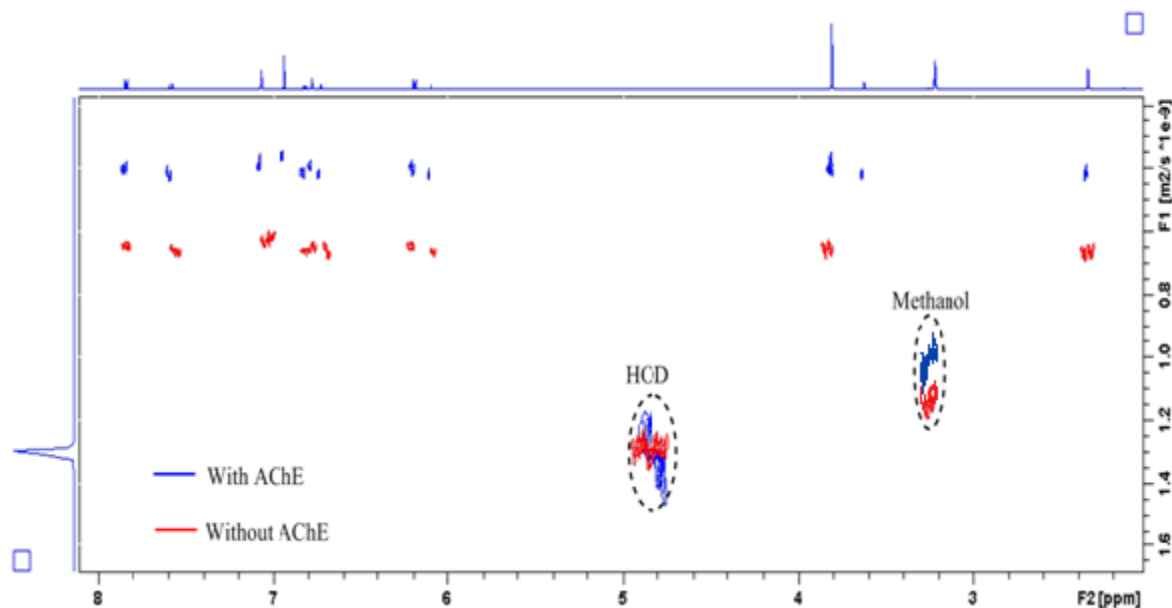


Figure S-4: A double display of 2D-DOSY NMR spectrum of **A**, **B**, and **C** with Acetylcholinesterase, where the X-axis contains the standard ^1H NMR chemical shifts values, while the y-axis contains the diffusion coefficient values in $1 \times 10^{-9} \text{ m}^2/\text{s}$. Spectra produced because of total diffusion time of 60ms, 5 ms of compensation of eddy current and $1400 \mu\text{s}$ gradient length. Where, the red signals presenting the diffusion without AChE and blue with the addition of AChE, similarly, the area in the circle, corresponding to the diffusion coefficient of the solvent (D_2O) and CD_3OD .

S-5: Dissociation constant calculations by diffusion NMR experiment

If we consider the rapid equilibrium between the ligand and the AChE system then the dissociation constant values can be achievable through the following equation.

$$K_D = \frac{[P][L]}{[PL]} \quad (1)$$

In the above equation 1, the [P], [L] and [PL] are the initial total concentrations of the protein, ligands, and the ligand-protein complex respectively. If we make a speculation about this system such that; the system is in equilibrium, all the ligands and protein interact reversibly, having independent binding sites with similar binding constants than to solve the equation 1 for the dissociation constant it is necessary to know all concentrations. ¹

However, diffusion experiment is the best option to observe the dissociation constants of a small molecule in the presence of macromolecule, in a solution with ligand fast-exchange. The low-molecular-mass compounds diffuse faster due to rapid moving as opposed to the macromolecules. Therefore, the low-molecular-mass compounds while binding to the larger macromolecule show a similar behavior of slow moving. ² So the observe diffusion is probably the weight averages of the free and bound states of the ligands. In a consequence of the interactions, the smaller molecule would show changes in their diffusion coefficient values in the presence of macromolecule but, on the other hand, macromolecules do not show observable changes in their diffusion values. ^{3,4}

By using the strategies employed by the Laravie *et al.*, one would be able to calculate the K_D accurately while using the diffusion experiment ^{2,5} as below, where the diffusion coefficient value ($8.1 \times 10^{-12} \text{ m}^2/\text{s}$) for AChE is adopted from an earlier report. ⁶

$$K_D = [P]_o \frac{(D_{bn} - D_{obs})}{(D_{obs} - D_{fr})} + [L]_o \frac{(D_{obs} - D_{bn})}{(D_{bn} - D_{fr})} \quad (2)$$

Where, D_{bn} , D_{obs} and D_{fr} represent the diffusion of the protein, diffusion of the ligand in the presence of protein and diffusion of free ligands, respectively. So by taking help of the values (presented in the table below) resulting from DOSY experiments, the K_D calculation can be done.

Compounds	Diffusion ($10^{-10} \text{ m}^2/\text{s}$) in absence of AChE	Diffusion ($10^{-10} \text{ m}^2/\text{s}$) in presence of AChE	K_D (10^{-6} M)
A	4.29	6.72	92.7
B	4.10	6.47	93.5

C	3.59	6.22	75.8
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The discrepancies seen between the dissociation constant values calculated by STD NMR (as presented in the main text) and DOSY NMR (in the above table) might be due to the rebinding effect, which is a common factor, sometimes, underestimate, the affinity. In STD NMR the signal intensities depend upon the saturation time and concentration of the ligands, hence, a larger saturation time might implicate in rebinding.⁷ Conversely, the DOSY experiments rely on the molecular size, shape and geometry, etc., where the saturation time has as such no malicious effect. Secondly, a ligand with larger concentration can occupy additional binding sites of the protein that might affect the actual protein-ligand affinity. Signal to noise ratio is another factor that might have some implications in underestimating the dissociation constant values in the STD experiments, for instance.^{7,8}

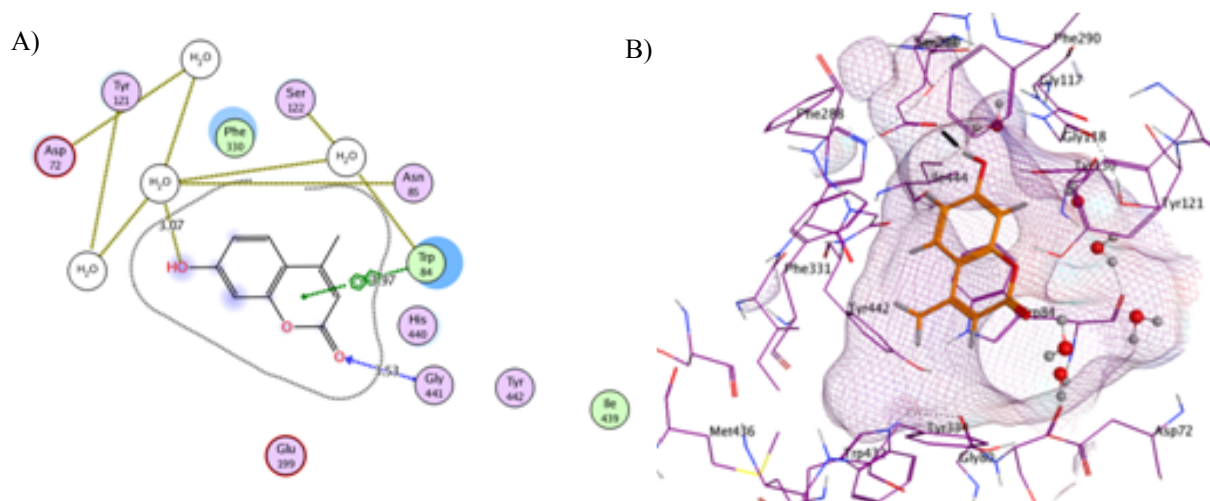


Figure S-6: A 2D (A) and 3D (B) molecular docking model of inhibitor **B**, showing interactions between the Acetylcholinesterase (AChE) and Scopoletin, generated by using Molecular Operating Environment (MOE) 2011.10. The AChE PDB file was taken from protein data bank with the code (PDB code: 1ACJ). The classical binding sites were marked in corresponding subdomain cations. See more details in the experimental section.

