Published in final edited form as:

Chem Biol Interact. 2008 September 25; 175(1-3): 161–165. doi:10.1016/j.cbi.2008.04.048.

Probing Gorge Dimensions of Cholinesterases by Freeze-Frame Click Chemistry

Zoran Radić¹, Roman Manetsch², Didier Fournier³, K. Barry Sharpless⁴, and Palmer Taylor¹

¹Department of Pharmacology, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093-0576, USA.

²Department of Chemistry, University of South Florida, Tampa, FL 33620-5250, USA.

³Groupe de Biotechnologie des Protéines, Institut de Pharmacologie et de Biologie Structurale (CNRS/UPS), Toulouse, France.

⁴Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037, USA.

Abstract

Freeze-frame click chemistry is a proven approach for design *in situ* of high affinity ligands from bioorthogonal, reactive building blocks and macromolecular template targets. We recently described *in situ* design of femtomolar reversible inhibitors of fish and mammalian acetylcholinesterases (EC 3.1.1.7; AChEs) using several different libraries of acetylene and azide building blocks. Active center gorge geometries of those AChEs are rather similar and identical triazole inhibitors were detected *in situ* when incubating the same building block libraries in different AChEs. *Drosophila melanogaster* AChE crystal structure and other insect AChE homology models differ more in their overall 3D structure than other members of the cholinesterase family. The portion of the gorge proximal to the catalytic triad and choline binding site has a ~50% reduction in volume, and the gorge entrance at the peripheral anionic site (PAS) is more constricted than in the fish and mammalian AChE's. In this communication we describe rationale for using purified recombinant *Drosophila* AChE as a template for *in situ* reaction of tacrine and propidium based libraries of acetylene and azide building blocks. The structures of resulting triazole inhibitors synthesized *in situ* are expected to differ appreciably from the fish and mammalian AChEs. While the latter AChEs exclusively promote synthesis of *syn*-substituted triazoles, the best *Drosophila* AChE triazole inhibitors were always *anti*-substituted. The *anti*- regioisomer triazoles were by about one order of magnitude better inhibitors of *Drosophila* than mammalian and fish AChEs. Moreover, the preferred site of acetylene + azide reaction in insect AChE and the resulting triazole ring formation shifts from near the base of the gorge to closer to its rim due to substantial differences of the gorge geometry in *Drosophila* AChE. Thus, in addition to synthesizing high affinity, lead inhibitors *in situ*, freeze-frame, click chemistry has capacity to generate species specific AChE ligands that conform to the determinants in the gorge.

Keywords

acetylcholinesterase; click chemistry; species specific inhibitors; *Drosophila* acetylcholinesterase; active center gorge; femtomolar inhibitors

Introduction

Click chemistry has recently being defined as a "chemical philosophy introduced by K. Barry Sharpless in 2001 and describes chemistry tailored to generate substances quickly and reliably by joining small units together. This is inspired by the fact that nature also generates substances by joining small modular units" (1). In recent years freeze-frame click chemistry has been tested on number of macromolecular biological templates evolving into an established approach for design *in situ* of high affinity ligands from bioorthogonal, reactive building blocks.

Representative examples of successful applications of click chemistry include *in situ* design of a 200 pM carbonic anhydrase (EC 4.2.1.1) inhibitor (2), 1.7 nM HIV-1 protease (EC 3.4.23.16) inhibitor (3), 62 nM α -1,3-fucosyltransferase (EC 2.4.1.214) inhibitor (4), and as a chronologically first and most noteworthy design of femtomolar acetylcholinesterase (AChE; EC 3.1.1.7) tight binding reversible inhibitors (5,6,7).

Joining acetylene and azide derivative building blocks in the course of a cycloaddition "click" reaction can inherently produce two different isomeric substitutions of a resulting 1,2,3-triazole heterocyclic ring, *syn*- and *anti*- (Scheme I).

In the absence of a macromolecular template or a small molecular metal catalyst both substitutions appear as equally likely reaction product. The influence of the binding site geometry of a macromolecular template or interaction with metal catalyst (8) results in preferential formation of one of the products.

In this communication we analyze products of cycloaddition "click" reaction formed in AChEs and their potency for inhibition of AChE from different species, with aim of designing species specific AChE inhibitors.

Results and Discussion

Comparison of active center gorge geometries of various AChEs reveals high level of similarity between mammalian (mouse) and fish (*Torpedo californica*) AChEs, while larger differences in both shape and volume (Figure 1) are observed for insect (*Drosophila melanogaster*) AChE.

It is therefore not surprising that *in situ* "click" chemistry screening using the same libraries of acetylene and azide building blocks yields identical products when mammalian and fish AChEs are used as reaction templates (5–7). All "*in situ*" generated triazole inhibitors of mammalian or fish AChE were "*syn*-substituted" reflecting the curvature of their long and narrow active center gorges. In contrast "*anti*-substituted" triazoles, inherently more elongated in shape, appeared as two to three orders of magnitude weaker inhibitors (Table 1). This very large difference in inhibitory potency was entirely due to a markedly compromised fit of compounds within the active center gorge shape resulting in reduction of their mean residence times in the gorge and acceleration of their dissociation rates from the enzyme (Table 1).

This similarity in substitution preference is not, however, preserved throughout the entire cholinesterase family of enzymes. Table 2 indicates that insect (*Drosophila*) AChE as well as mammalian (mouse) butyrylcholinesterase (BuChE) are preferentially inhibited with "*anti*-substituted" triazoles, and thus show an inverted preference in comparison with mammalian and fish AChEs. This is not surprising and could be predicted to result from the significant

difference in geometry of the active center gorge of insect AChE and the gorge of BuChE relative to gorges of other AChEs, as illustrated in Figure 1.

The crystal structure of mouse AChE in complex with one of the tightest binding inhibitors *syn*-TZ2PA6 (9,10) reveals a snug fit of a triazole inhibitor within the active center gorge of AChE captured in an unique, previously unseen conformation (Figure 2B). Weaker binding of this triazole to *Drosophila* AChE and to mouse BuChE (Table 2) is consistent with a less complementary fit into their active center gorges inferred from molecular modeling (Figure 2A and 2C). Binding of *syn*-TZ2PA6 to BuChE appears compromised largely because of slower association rate, likely due to the extensively solvated BuChE gorge, where water molecules need to be displaced from the gorge in the course of binding. The interaction with *Drosophila* AChE is obviously imperfect due to severe steric clashes in the peripheral site area and the lack of triazole ring support near the base of the active center gorge.

An additional and very good indicator of specific differences in the active center gorge conformations of various AChEs is achieved by overlaying a series of tacrine based inhibitors crystallized in complex with fish (*Torpedo californica*), mammalian (mouse) or insect (*Drosophila melanogaster*) AChE (Figure 3).

These structures were overlaid by finding smallest deviations of corresponding AChE protein backbone atom positions in different AChE structures. Inhibitors were not involved in the overlay process. Positions of tricyclic tacrine portions of all nine overlaid inhibitors, however, superimpose remarkably closely indicating strong intermolecular stabilization at the base of all AChE gorges and a high degree of structural similarity between AChEs in this region. Subsequent inhibitor atoms extending towards AChE peripheral site progressively diverge in the overlay, with two points of maximal deviation. One is around the intervening chain and formed triazole of the inhibitory ligands in the narrow passage of the AChE gorge defined by the sidechain of the peripheral site Tyr (121 in fish and 124 in mammalian AChEs), known as a “choke point”. The other divergence is at the end of inhibitory molecules interacting with the AChE peripheral site. Divergence in this region at the peripheral site could be compensated, but only in part, by conformational flexibility of Trp 286 (mammalian numbering) observed for tight binding complexes at the peripheral site (8,9 and Figure 2B and Figure 3B). The two sites of divergence in positions of the three groups of inhibitors (crystallized in fish, mammalian and insect AChEs) clearly indicate differences in shape of the corresponding active center gorges. The congested “choke point” located deep into the active center gorge in fish and mammalian AChEs but is shifted closer to the gorge opening and the peripheral site in the insect AChE. Thus, more space exists around the intervening chain of inhibitors. Effectively the gorge becomes less “channel like” and tortuous. The small gain in gorge volume is however compensated by severe restriction of available binding space in the area around peripheral binding site.

With details of these differences in mind, we are now designing a library of compounds aimed at species specific interactions with different AChEs to produce species selective AChE inhibitors. It is clear that a high affinity *Drosophila* AChE specific triazole inhibitor will have “anti-” substitution at the central triazole ring, and that the moiety interacting with the peripheral site will be both smaller in volume and extend over a shorter distance compared to the corresponding specific inhibitors of mammalian or fish AChEs. Additionally, a greater accessible volume creating a wider gorge at the very base of the *Drosophila* active center gorge should allow for introduction of specific tricyclic ring substitutions of tetrahydroamino acridine that would sterically preclude a tight fit of the inhibitor within the mammalian and fish AChEs, while allowing it to be accommodated in the gorge of *Drosophila* AChE.

We demonstrate here that “*anti*”-substituted triazoles have a preference for inhibition of *Drosophila* AChE. Also, inhibitors containing a peripheral site binding moiety smaller than the phenylphenanthridinium exhibit an order of magnitude greater “*syn*”/“*anti*” preference for mouse AChE inhibition (Table 1). Introducing additional steric bulk at the tacrine moiety and combining appropriate building blocks by the “*in situ*” click chemistry approach, we expect to identify potent ligands that will specifically probe AChE active center gorges in a species specific manner.

References

1. http://en.wikipedia.org/wiki/Click_chemistry
2. Mocharla VP, Colasson B, Lee LV, Röper S, Sharpless KB, Wong CH, Kolb HC. *In situ* click chemistry: enzyme-generated inhibitors of carbonic anhydrase II. *Angew Chem Int Ed Engl* 2004;44:116–120. [PubMed: 15599912]
3. Whiting M, Muldoon J, Lin YC, Silverman SM, Lindstrom W, Olson AJ, Kolb HC, Finn MG, Sharpless KB, Elder JH, Fokin VV. Inhibitors of HIV-1 protease by using *in situ* click chemistry. *Angew Chem Int Ed Engl* 2006;45:1435–1439. [PubMed: 16425339]
4. Lee LV, Mitchell ML, Huang SJ, Fokin VV, Sharpless KB, Wong CH. A potent and highly selective inhibitor of human alpha-1,3-fucosyltransferase via click chemistry. *J Am Chem Soc* 2003;125:9588–9589. [PubMed: 12904015]
5. Lewis WG, Green LG, Grynszpan F, Radić Z, Carlier PR, Taylor P, Finn MG, Sharpless KB. Click chemistry *in situ*: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angew Chem Int Ed Engl* 2002;41:1053–1057. [PubMed: 12491310]
6. Manetsch R, Krasinski A, Radić Z, Raushel J, Taylor P, Sharpless KB, Kolb HC. *In situ* click chemistry: enzyme inhibitors made to their own specifications. *J Am Chem Soc* 2004;126:12809–12818. [PubMed: 15469276]
7. Krasinski A, Radić Z, Manetsch R, Raushel J, Taylor P, Sharpless KB, Kolb HC. *In situ* selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. *J Am Chem Soc* 2005;127:6686–6692. [PubMed: 15869290]
8. Rasmussen LK, Boren BC, Fokin VV. Ruthenium-catalyzed cycloaddition of aryl azides and alkynes. *Org. Lett* 2007;9:5337–5339. [PubMed: 18052070]
9. Bourne Y, Kolb HC, Radić Z, Sharpless KB, Taylor P, Marchot P. Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation. *Proc Natl Acad Sci U S A* 2004;101:1449–1454. [PubMed: 14757816]
10. Bourne Y, Radić Z, Kolb HC, Sharpless KB, Taylor P, Marchot P. Structural insights into conformational flexibility at the peripheral site and within the active center gorge of AChE. *Chem Biol Interact* 2005;157–158:159–165.

Figure 1. Solvent accessible Connolly surfaces generated (using 1.4 Å probe radius) for *Drosophila melanogaster*, *Torpedo californica* and mouse AChEs. Cross-section through the center of each molecule reveals different geometries of the centrally located active center gorge.

Figure 2.

Syn-TZ2PA6 bound in the active center gorges of A) *Drosophila* AChE, B) mouse AChE and C) human BuChE. Panel B represents actual crystal structure of the complex (9; PDB id 1q83) while in panels A and C syn-TZ2PA6 is placed based on a protein overlays of *Drosophila* AChE (PDB id 1dx4) and mouse AChE (1q83)(A) and human BuChE (PDB id 1p0i) and mouse AChE (1q83) (C). The active serine side chain is indicated in red in each panel. Panels illustrate obvious differences in active center gorge geometries of three enzymes and a consequent lack of adequate fit of syn-TZ2PA6 in *Drosophila* AChE (A) and human BuChE (C). Red arrows indicate direction of the active center gorge entry for the each of three enzymes.

Figure 3. Overlay of nine tacrine based AChE inhibitors crystalized in complex with *Torpedo* AChE (five inhibitors in red sticks, PDB ids 1acj, 1adc, 1ut6, 1zgb, 1zgc) mouse AChE (two inhibitors in blue sticks PDB ids, 1q83, 1q84) and *Drosophila* AChE (two inhibitors in yellow sticks, PDB ids, 1dx4, 1qon), superimposed within active center gorges of A) *Drosophila* AChE (1dx4) and B) mouse AChE (1q83).

Scheme 1.

Kinetic and equilibrium interaction constants of tightest triazole inhibitors generated “*in situ*” using fish (eel *Electrophorus electricus*) and mammalian (mouse) AChE as cycloaddition reaction templates. Given are structures of *syn*- substituted triazoles generated *in situ*, and interaction constants determined for both *syn*- triazoles and their “*anti*- substituted” analogues.

Table 1

Inhibitor	k_{on} ($10^{10} M^{-1} min^{-1}$)	k_{off} (min^{-1})	K_d (nM)	AChE
(R)-TZ2PIQA5	0.57	0.00024	42	<i>eel</i> mouse
	0.72	0.00088	120
TZ2PA6	0.39	0.77	200,000	<i>eel</i> mouse
	0.56	1.0	180,000
TZ2PA6	1.5	0.0015	99	<i>eel</i> mouse
	1.3	0.0079	410
TZ2PA6	1.8	0.250	14,000	<i>eel</i> mouse
	2.4	0.300	8,900	

Table 2
Interaction constants of *syn*- and *anti*- substituted TZ2/PA6 triazole inhibitors with AChEs from several different species and with BuChE.

<i>syn</i> -TZ2/PA6				<i>anti</i> -TZ2/PA6			
k_{on} ($10^{10} \text{ M}^{-1} \text{ min}^{-1}$)	k_{off} (min^{-1})	K_d (fM)	Enzyme	k_{on} ($10^{10} \text{ M}^{-1} \text{ min}^{-1}$)	k_{off} (min^{-1})	K_d (fM)	
1.5	0.0015	99	<i>Eel AChE</i> <i>Torpedo AChE</i> <i>mouse AChE</i> <i>Drosophila AChE</i> <i>Mouse BuChE</i>	1.8	0.25	14,000	
1.3	0.0011	77		3.2	0.026	720	
1.7	0.0071	410		2.4	0.30	8,900	
2.0	0.072	3,600		3.4	0.058	1,700	
0.36	0.0026	720		0.69	0.0032	460	