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In Situ Selection of Lead Compounds by Click Chemistry: Target-Guided Optimization of Acetylcholinesterase Inhibitors

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Abstract: The target-guided, in situ click chemistry approach to lead discovery has been successfully employed for discovering acetylcholinesterase (AChE) inhibitors by incubating a selected enzyme/tacrine azide combination with a variety of acetylene reagents that were not previously known to interact with the enzyme's peripheral binding site. The triazole products, formed by the enzyme, were identified by HPLC-mass spectrometry analysis of the crude reaction mixtures. The target-guided lead discovery search was also successful when performed with reagent mixtures containing up to 10 components. From 23 acetylene reagents, the enzyme selected two phenyltetrahydroisoquinoline (**PIQ**) building blocks that combined with the tacrine azide within the active center gorge to form multivalent inhibitors that simultaneously associate with the active and peripheral binding sites. These new inhibitors are up to 3 times as potent as our previous phenylphenanthridinium-derived compounds, and with dissociation constants as low as 33 femtomolar, they are the most potent noncovalent AChE inhibitors known. In addition, the new compounds lack a permanent positive charge and aniline groups and possess fewer fused aromatic rings. Remarkably, despite the high binding affinity, the enzyme displayed a surprisingly low preference for one **PIQ** enantiomer over the other.

Introduction

By employing the biological targets themselves for assembling inhibitors within the confines of their binding sites, target-guided synthesis (TGS) promises to revolutionize lead discovery. The newly formed inhibitors usually display much higher binding affinities for their biological targets than the individual components, since they simultaneously engage in multiple binding interactions.[1,2] In principle, lead discovery by TGS is independent of the function of the target, since it relies solely on its ability to hold the reagents in close proximity until they become connected via the "arranged" chemical reaction. As long as 20 years ago, Rideout et al. reported a marked synergism between the cytotoxic effects of decanal and *N*-amino-guanidines, which they suggested to result from the self-assembly of cytotoxic hydrazones inside cells.[3,4] Since then, several approaches to target-guided synthesis have been developed: dynamic combinatorial chemistry,[5−14] stepwise target-guided synthesis,[15,16] and kinetically controlled target-guided synthesis.[17−25] The latter approach uses irreversible reactions to unite reagents inside the protein's binding pockets. Most

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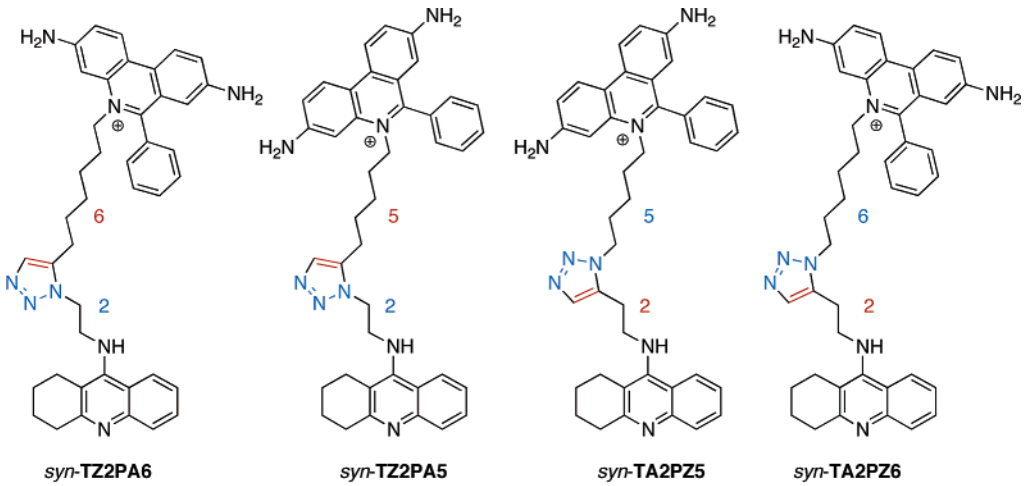

Figure 1. Four in situ click chemistry hit compounds based on tacrine and phenylphenanthridinium reagents.

approaches to TGS employ highly reactive reagents (strong electrophiles or nucleophiles, metathesis catalysts, etc.), which can cause side reactions and even destroy the biological target. To avoid such complications, we have developed an extremely reliable approach to kinetically controlled TGS, called in situ click chemistry,[17,26] which employs the completely bio-orthogonal [1,3]-dipolar cycloaddition reaction[27] between azides and acetylenes. This process is self-contained, hence no external reagents, catalysts, or byproducts that might interfere, and the "reactants" themselves are largely "invisible" in a biological milieu. Most importantly, despite its high driving force (>50 kcal/mol) the uncatalyzed reaction has a surprisingly high activation barrier of approximately 25 kcal/mol, causing it to be extremely slow at room temperature and its rate to be highly dependent on parameters that stabilize the transition state.[28] This was first exploited by Mock et al., who observed a 10^5-fold increase of the cycloaddition reaction rate when azide and acetylene groups are held together in close proximity inside the synthetic receptor, cucurbituril, leading to irreversible formation of a triazole.[29−31] In previous work we have shown that acetylcholinesterase (AChE) is able to assemble extremely potent inhibitors, which simultaneously access the enzyme's active and peripheral binding sites,[32−35] from azide and acetylene reagents, each linked to known active and peripheral site inhibitors, tacrine and phenylphenanthridinium, respectively.[17,26] Later we found that carbonic anhydrase is also capable of assembling its own inhibitors within the confines of its active center region, suggesting the in situ click chemistry technique is applicable to a broad range of targets.[36] Indeed, the scope of the method is not limited to proteins, as demonstrated by Dervan et al., who have used the azide/acetylene cycloaddition to explore the double-stranded DNA-templated interconnection of hairpin polyamides in the minor groove to produce tandem hairpin dimers in site-specific fashion, which are capable of targeting longer sequences.[25]

Recent key breakthroughs in our labs were made possible by an improved method for analyzing the in situ click chemistry reaction mixtures.[26] Instead of using MALDI/DIOS (desorption/ionization on silicon) mass spectrometry,[37,38] as done previously,[17] we now use HPLC with compound detection through electrospray mass spectrometry in the positive selected ion mode (LC/MS-SIM). For our purpose this method is more reliable, enabling identification of the product triazoles by both retention time *and* molecular weight. Additionally, chromatographic removal of the molecules that might otherwise obscure the mass spectrum of the product allows us to reduce the reaction time from 6 days to 6 hours and lower the reagent concentrations considerably. The following results were obtained under these conditions.

(1) From 52 combinations of azide- and acetylene-bearing tacrine and phenylphenanthridinium reagents, potentially giving rise to 104 products, only four were assembled inside mouse or eel acetylcholinesterase to form, with high selectivity, 1,5-disubstituted ("*syn*") triazoles (Figure 1). These enzyme-generated compounds are femtomolar inhibitors, whereas the corresponding 1,4-disubstituted ("*anti*") triazole derivatives are much less active.

(2) The triazole units of all enzyme-generated inhibitors were two methylene units away from the tacrine moiety, even though the enzyme had the opportunity to assemble products with different linker spacings. Recent X-ray crystallographic studies of complexes of *anti*- and *syn*-**TZ2PA6** and mouse AChE revealed that the formed triazole moieties are optimally positioned to contribute to protein binding through hydrogen bonding and stacking interactions.[18] This implies not only that triazoles

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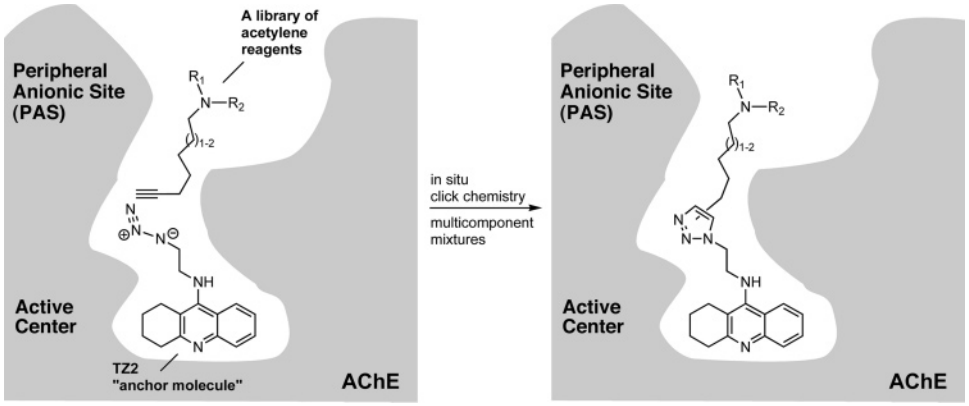


Figure 2. In situ click chemistry screening for AChE inhibitors, containing novel peripheral site ligands.

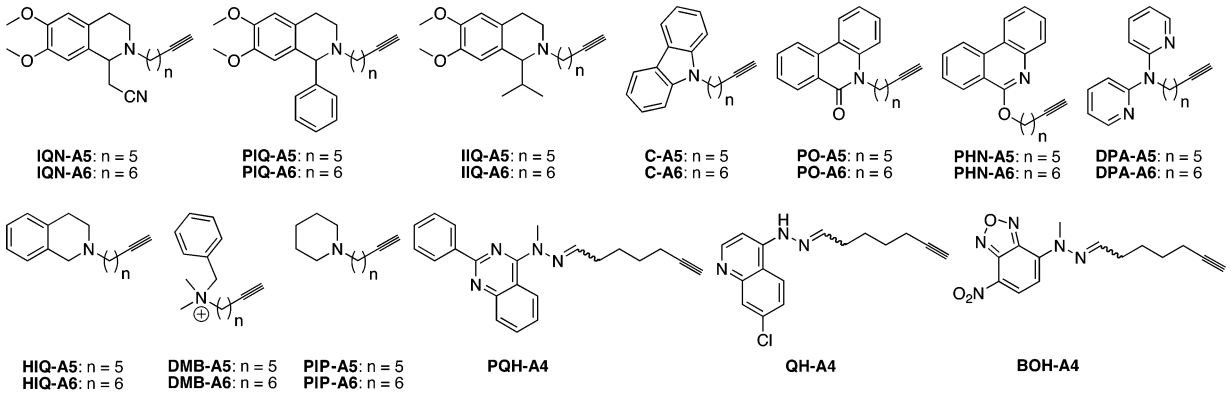


Figure 3. A library of 23 acetylene reagents for in situ click chemistry screening. All chiral compounds are racemic.

are valuable pharmacophoric units but also that the enzyme may have actively accelerated their formation by lowering the transition state energies of their formation through favorable binding interactions.

(3) Due to the chromatographic separation and greater reliability, the new LC/MS-SIM based analysis method enabled us to increase screening throughput by using multicomponent mixtures of building blocks. Thus, medium- or high-throughput in situ click chemistry screening for lead discovery is now within reach.

Results and Discussion

These results have set the stage for performing the first ever search for AChE inhibitors through in situ click chemistry based on building blocks that were not previously known to interact with the target. All previous experiments had employed known active site and peripheral binding site ligands. To minimize the number of variables, we decided to continue to use the tacrine building block **TZ2** as an "anchor molecule" that, in complex with the enzyme, would recruit and irreversibly link together novel peripheral site binders to form multivalent AChE inhibitors that simultaneously access multiple binding sites within the enzyme (Figure 2). A two-methylene spacer between tacrine and the azide was chosen, since previous experiments had proven this distance to be optimal.[26] On the basis of analogous considerations, we designed a library of complementary acetylene reagents carrying aromatic heterocyclic phenylphenanthridinium mimics with a spacing of five or six methylene units. To increase the screening throughput, we planned to test multireagent mixtures containing up to 10 acetylene reagents at a time. This multicomponent in situ click chemistry screening approach is conceptually interesting, as it addresses the question of whether an enzyme complex of one reaction partner, capable of triazole genesis (e.g., the tacrine azide **TZ2**), can find and select its "best" triazole-forming partner(s) when presented with mixtures of candidates with unknown binding affinities and so discover its own potent biligand inhibitors. In the case at hand, the goal was to replace the phenylphenanthridinium component of our previous "in situ-made" AChE inhibitors with a moiety conferring greater pharmacologic potential.

The acetylenic building blocks were readily synthesized by alkylating commercially available amines with the appropriate iodoalkynes or by forming hydrazones from 7-heptynal (cf. Supporting Information). The complete acetylene reagent library is shown in Figure 3. The heterocycles were chosen to simplify the structure and eliminate the permanent positive charge, yet retain many of the features of the phenanthridinium moiety.

For concept validation, initial in situ click chemistry screens were performed by incubating *binary* **TZ2**/acetylene mixtures with eel or mouse AChE at pH 7.4 for at least 6 h and analyzing each reaction mixture by LC/MS-SIM.[26] Most alkynes gave no detectable product, except for the phenyltetrahydroisoquinolines **PIQ-A5** and **PIQ-A6**, which formed significant amounts of triazoles. Their identity was confirmed by chromatographic comparison of the in situ click chemistry reaction mixtures with authentic samples of **TZ2PIQ-A5** and **TZ2PIQ-A6**, which were synthesized by a thermal cycloaddition reaction. Thus, two new in situ hits have been found, composed of the tacrine active site ligand and the phenyltetrahydroisoquinoline peripheral site ligands. The latter were not previously known to bind to the peripheral binding site of AChE, and they may have better

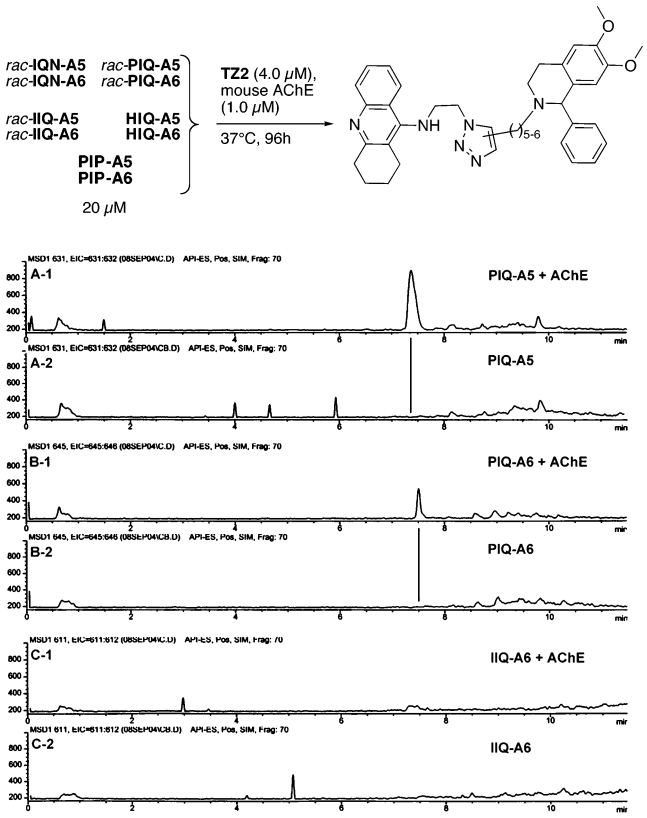


Figure 4. Multicomponent in situ click chemistry screen. Extracted ion LC/MS-SIM chromatograms for the multicomponent in situ reaction (traces A-1, B-1, C-1) and for the background reaction containing all reagents, but no enzyme (traces A-2, B-2, C-2). Traces A-1 and -2: Extracted ion chromatograms for **TZ2PIQ-A5**; note the presence of product in the enzyme reaction, while no product is formed in the absence of AChE. Traces B-1 and -2: Extracted ion chromatograms for **TZ2PIQ-A6**. Again, product is present only in the enzyme reaction. Traces C-1 and -2: Extracted ion chromatograms for **TZ2IIQ-A6**. No product is formed in either the enzyme or the control reactions. The extracted ion traces for the remaining building blocks are similar to this trace, demonstrating that the corresponding triazoles are below the detection limit in the in situ reaction mixture.

pharmacological properties than the previous phenylphenanthridinium-derived inhibitors, due to the lack of a positive charge, the absence of aniline groups, and the presence of fewer fused aromatic rings. The new hits were validated by demonstrating that no triazole was formed in the absence of AChE, or when the enzyme was replaced by bovine serum albumin (BSA) (cf. Figure 4 for results from a multicomponent screen).

After successful completion of in situ click chemistry experiments with binary **TZ2**/acetylene mixtures, we turned our attention to multicomponent screens. Incubation of a mixture of 10 structurally related alkynes (16 compounds, if all enantiomers are counted, cf. Figure 4) with **TZ2** and the enzyme gave only the expected triazole products **TZ2PIQ-A5** and **TZ2PIQ-A6**; that is, none of the other acetylenes that were present in the mixture were converted into triazoles. Thus, the **TZ2**/enzyme complex (over 99% active site saturation by **TZ2** under the reaction conditions[39]) was able to recognize subtle differences in alkyne structure (compare **IQN**, **PIQ**, and **IIQ**) and form triazole products exclusively with its preferred reagents, **PIQ-A5** and **PIQ-A6**. These results demonstrate that highly efficient multicomponent screens are practical and that

(39) AChE concentration: 1μM; **TZ2** concentration: 4−5 μM; dissociation constant of **TZ2** for mouse AChE: K_d = 23 nM.

Table 1. Inhibition Constants of Acetylene Reagents for Eel and Mouse AChE[a,b]

		K_d (μM)	
entry	compound	mouse AChE	eel AChE
1	**IQN-A5**	77	60
2	**IQN-A6**	210	74
3	**PIQ-A5**	34	18
4	**PIQ-A6**	21	7.8
5	**IIQ-A5**	>400	100
6	**IIQ-A6**	>400	67
7	**C-A5**	>400	>400
8	**C-A6**	>400	>400
9	**PO-A5**	>400	>400
10	**PO-A6**	>400	>400
11	**PHN-A5**	n.d.[c]	n.d.[c]
12	**PHN-A6**	n.d.[c]	n.d.[c]
13	**DPA-A5**	42	84
14	**DPA-A6**	83	110
15	**HIQ-A5**	99	46
16	**HIQ-A6**	190	43
17	**DMB-A5**	14	20
18	**DMB-A6**	14	20
19	**PIP-A5**	>400	320
20	**PIP-A6**	>400	170
21	**PQH-A4**	38	5.8
22	**QH-A4**	>400	>400
23	**BOH-A4**	4.1	0.50
24	**PA6**	0.36	n.d.

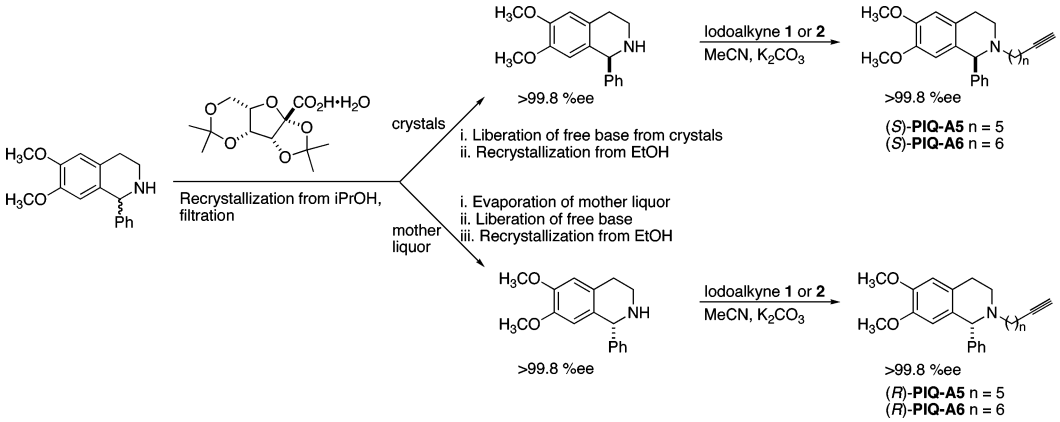
[a] The measurements were performed in 100 mM phosphate buffer pH 7.0 (+0.01% BSA to stabilize the enzyme) at 22 °C. Constants were determined in duplicate experiments using Hunter and Downs plots.[40] [b] For comparison, the dissociation constant K_d of **TZ2** for mouse AChE is 0.023 μM. [c] Not determined due to low solubility of the compound.

in situ click chemistry products are formed even when competing reagents (here **PIQ-A5** and **PIQ-A6**) are present.

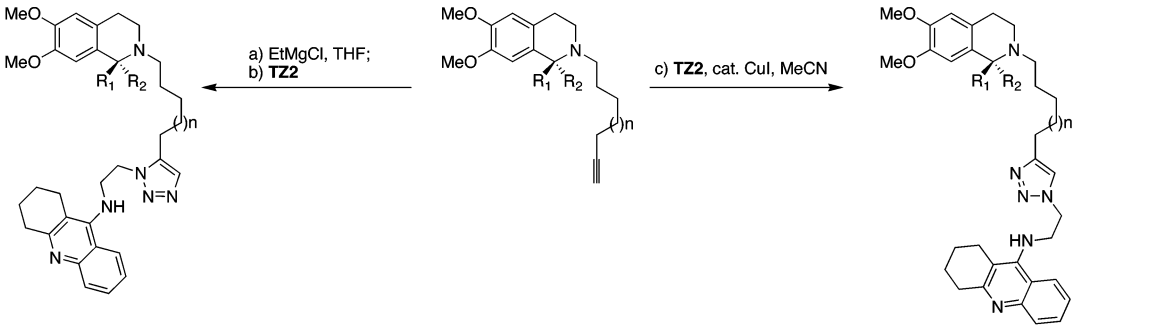
Determination of Dissociation Constants for Acetylene Reagents. The inhibition of AChE by the acetylene reagents was measured at 22 °C and pH 7.0 (Table 1). With inhibition constants in the micromolar range (8−34 μM), the phenyltetrahydroisoquinoline derivatives **PIQ-A5** and **PIQ-A6** are about 1 to 2 orders of magnitude less potent than the phenylphenanthridinium acetylenes used previously (K_d of **PA6** for mouse AChE: 0.36 μM), demonstrating that even this relatively low level of affinity is sufficient for the target-templated reaction to take place. While the **PIQ** compounds are among the higher affinity in the set, there are five other compounds (entries 13, 17, 18, 21, and 23) with an approximately equal or higher potency. Surprisingly, these compounds did not form in situ products, despite their high binding affinity to the protein and the presence of identical linker moieties, revealing a lack of correlation between a reagent's binding affinity and its ability to undergo target-templated cycloaddition. It is possible that these compounds are not peripheral site ligands, but rather active site binders, thereby preventing the in situ reaction from occurring, or that the spatial orientation of the acetylene group of the enzyme-bound reagent is suboptimal. The fact that these compounds do not show substrate-competitive inhibition expected of association with the active center appears to rule out the former possibility, and stereoelectronic factors seem to be decisive.

Enantioselectivity. Initially, all reagents were either achiral or racemic, prompting the question whether AChE would prefer one enantiomer over the other for the in situ reaction in the case of **PIQ-A5** and **PIQ-A6**. To study the relative in situ click chemistry rates, we prepared each reagent in its pure enantiomeric forms through the synthesis and optical resolution of the

Scheme 1. Synthesis of Enantiomerically Pure **PIQ** Reagents



Scheme 2. Synthesis of **TZ2**-Derived syn- and *anti*-Triazoles from **PIQ-A5** and **PIQ-A6**[a]



syn-(*S*)-**TZ2PIQ-A5** n=1, R_1=Ph, R_2=H
syn-(*R*)-**TZ2PIQ-A5** n=1, R_1=H, R_2=Ph
syn-(*S*)-**TZ2PIQ-A6** n=2, R_1=Ph, R_2=H
syn-(*R*)-**TZ2PIQ-A6** n=2, R_1=H, R_2=Ph

(*S*)-**PIQ-A5** n=1, R_1=Ph, R_2=H
(*R*)-**PIQ-A5** n=1, R_1=H, R_2=Ph
(*S*)-**PIQ-A6** n=2, R_1=Ph, R_2=H
(*R*)-**PIQ-A6** n=2, R_1=H, R_2=Ph

anti-(*S*)-**TZ2PIQ-A5** n=1, R_1=Ph, R_2=H
anti-(*R*)-**TZ2PIQ-A5** n=1, R_1=H, R_2=Ph
anti-(*S*)-**TZ2PIQ-A6** n=2, R_1=Ph, R_2=H
anti-(*R*)-**TZ2PIQ-A6** n=2, R_1=H, R_2=Ph

[a] Reagents and conditions: (a) EtMgCl (1.0 equiv), THF, rt to 60 °C, 30 min; (b) **TZ2** (0.5 equiv), rt to 60 °C, 4 h, 65−85% yield over 2 steps; (c) **TZ2** (1.0 equiv), CuI (5 mol %), MeCN, rt, 12 h, 80−90% yield.

precursor *rac*-6,7-dimethoxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline,[41,42] followed by alkylation with 7-iodohept-1-yne (**1**) or 8-iodooct-1-yne (**2**) (cf. Scheme 1). The enantiopure alkynes were also needed for kinetic and structural studies of enzyme−inhibitor complexes (vide infra).

Interestingly, the in situ click chemistry reaction rates for the enantiomers of each acetylene component are quite similar (cf. Supporting Information). In case of the mouse enzyme, the *R* isomers react slightly faster than the *S* enantiomers, whereas the opposite is true for the eel enzyme. We are in the process of investigating the molecular origin of the lack of selectivity through X-ray crystallography.

Determination of the Regioselectivity. We synthesized all regioisomers and enantiomers of the **TZ2**-derived **PIQ-A5** and **PIQ-A6** triazoles to elucidate the regioselectivity of the enzyme reaction and to develop a deeper understanding of the structure−activity relationship (Scheme 2). For the preparation of *anti*-triazoles, the recently discovered copper(I)-catalyzed process was employed,[43,44] whereas *syn*-isomers were synthesized by way of magnesium acetylides.[45−48]

The comparison of LC/MS-SIM traces of enzyme-produced triazoles and the reference compounds revealed a striking similarity to the tacrine/phenylphenanthridinium system in that all in situ reaction products are 1,5-disubstituted (*syn*) triazoles (Figure 5). The *syn* selectivity was independent of the linker length, the source of the enzyme (eel or mouse AChE), and the absolute configuration of the **PIQ** component (cf. Supporting Information for LC/MS-SIM traces).

Determination of AChE-Inhibitor Association and Dissociation Rate Constants. All kinetic parameters of inhibitor binding to and dissociation from eel and mouse AChE were measured as described previously,[26] except for one modification. For determining the first-order dissociation rate constants by measuring the return of AChE activity upon 5000-fold dilution of 50−100 nM concentrations of AChE·inhibitor complex, we employed purified inactive mouse AChE mutant Ser203Ala (20−70 nM), instead of DNA, for sequestering the inhibitors upon their release from the complex with the active wild-type AChE to prevent their reassociation at concentrations approaching or higher than their K_d. This modification was necessary, since the current set of inhibitors did not intercalate with DNA as efficiently as the phenylphenanthridinium derivatives used previously. The equilibrium dissociation constants for the *syn*- and *anti*-isomers, calculated as the ratios of their first-order

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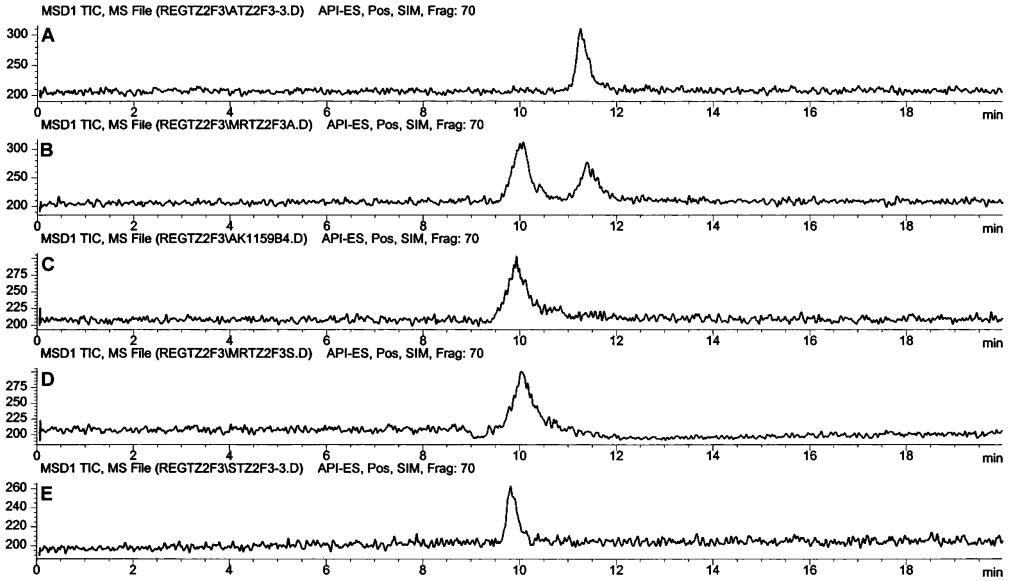

Figure 5. Regioisomer (*syn*/*anti*) determination for mouse AChE-derived in situ hits. The in situ product, (*R*)-**TZ2PIQ-A5**, was compared by LC/MS-SIM to authentic samples from the Cu(I)-catalyzed and Mg-mediated reactions. (A) *anti*-(*R*)-**TZ2PIQ-A5** prepared by the Cu(I)-catalyzed reaction; (B) co-injection of (*R*)-**TZ2PIQ-A5**, formed by AChE, and *anti*-(*R*)-**TZ2PIQ-A5**, prepared by the Cu(I)-catalyzed reaction; (C) in situ click reaction; (D) co-injection of the in situ click reaction and *syn*-(*R*)-**TZ2PIQ-A5** prepared by the Mg-mediated reaction; (E) *syn*-(*R*)-**TZ2PIQ-A5** prepared by the Mg-mediated reaction.

Table 2. Kinetic Parameters and Dissociation Constants for in Situ-Generated Inhibitors and Related Compounds

inhibitor		k_{on} (10^{10} M^{-1} min^{-1})	k_{off} (min^{-1})	K_d (fM)	AChE source
	syn-	0.70	0.00023	33	eel
(*S*)-**TZ2PIQ-A5**		0.77	0.0038	500	mouse
	anti-	0.52	1.10	210 000	eel
		0.72	6.2	870 000	mouse
	syn-	0.67	0.00024	36	eel
(*R*)-**TZ2PIQ-A5**		0.84	0.00088	100	mouse
	anti-	0.40	0.77	190 000	eel
		0.58	1.0	180 000	mouse
	syn-	0.90	0.00087	96	eel
(*S*)-**TZ2PIQ-A6**		0.97	0.0110	1100	mouse
	anti-	0.41	0.83	200 000	eel
		0.67	2.8	420 000	mouse
	syn-	0.67	0.0024	360	eel
(*R*)-**TZ2PIQ-A6**		0.73	0.012	1700	mouse
	anti-	0.50	0.82	170 000	eel
		0.64	1.4	220 000	mouse
	syn-	1.0	0.012	1200	eel
TZ2HIQ-A6		1.5	0.081	5400	mouse
	anti-	0.47	10	2 100 000	eel
		0.58	11	1 900 000	mouse
	syn-	1.5	0.0015	99	eel
TZ2PA6		1.7	0.0071	410	mouse
	anti-	1.8	0.25	14 000	eel
		2.5	0.22	8900	mouse

dissociation and second-order association rate constants, are listed in Table 2, along with the dissociation constants for the **TZ2PA6** isomers reported previously.[26]

Interestingly, despite their higher binding affinities, the **PIQ**-derived inhibitors display 2 to 3 times lower on-rates than the original **PA**-derived inhibitors. Nevertheless, the on-rates k_{on} are all very large, close to diffusion controlled, and the observed binding affinity trends are due mainly to variations in off-rates k_{off}, which are extremely slow for the tightest binding compounds.

The best inhibitors proved to be the enzyme-generated phenyltetrahydroisoquinoline derivatives (*S*)- and (*R*)-**TZ2PIQ-A5**, which do not carry a permanent positive charge and aniline groups as did the previous champion, **TZ2PA6**, while being 3 times as potent in the case of eel AChE. In fact, with a dissociation constant of less than 40 fM (eel AChE), these compounds are the most potent noncovalent AChE inhibitors known to date. As a general observation, all in situ-generated compounds tested here show a greater affinity for the eel enzyme (33−360 fM) than for mouse AChE (100−1700 fM).

Despite the tight binding of the **TZ2PIQ** triazoles, there is no clear enantiomeric preference. This observation is in line with the lack of selectivity for either one of the two enantiomeric reactants in the in situ click chemistry reaction (vide supra). Thus, in the case of **TZ2PIQ-A5**, having a 5-methylene linker between the triazole ring and the tetrahydroisoquinoline moiety, the *R* isomer is bound about 5 times more tightly than the *S* enantiomer in the case of mouse AChE, while there is no difference between the enantiomers in case of eel enzyme. The reverse trend is observed in the case of the **TZ2PIQ-A6** triazoles, having a 6-methylene linker, where the *S* isomer has a higher binding affinity for either enzyme.

As before, the in situ-generated *syn*-triazoles are several orders of magnitude more potent inhibitors than the corresponding *anti*-isomers, not formed by the enzyme. However, the extent of the difference is more than an order of magnitude larger than for phenylphenanthridinium triazoles, ranging from 600- to 5600-fold preference for the *syn*-triazoles (the average difference in the free energy of binding is about 4 kcal/mol, cf. Supporting Information for additional free energy data). In the case of the phenylphenanthridinium triazoles, the *syn*-preference was only 14- to 420-fold. In general, our data suggest that compounds that are *not* formed by the enzyme, e.g., *anti*-triazoles and **TZ2HIQ-A6**, bind more weakly than the in situ-generated triazoles.

Experimental Section

CAUTION! All of the compounds described here (and especially the most potent polyvalent inhibitors) are potentially neurotoxic. They

must be handled with extreme care by trained personnel. Azide-containing compounds, particularly those lower in saturated carbon and oxygen content, are potentially explosive and must be handled with care.

General Procedures for in Situ Click Chemistry Experiments. Determination of Acetylcholinesterase and Stock Concentrations. The enzyme concentrations were determined by quantitative measurement of AChE activity using the Ellman assay as described previously.[17,26] All in situ click chemistry reactions were performed at an active site concentration of 1 μM. The stock concentrations for all triazole compounds were determined in duplicate by titration of the inhibitor solutions with two different AChE preparations of known concentration.

In Situ Click Chemistry Screening Procedure for Binary Reagent Mixures. The tacrine azide **TZ2** was dissolved in MeOH and added to ~1 μM solutions of eel AChE (Type V-S, Sigma) or mouse AChE[49,50] in buffer (2 mM ammonium citrate, 100 mM NaCl, pH = 7.3−7.5) followed immediately by one of the acetylene components and mixed. The final concentrations were as follows: eel or mouse AChE, 1 μM; tacrine azide (**TZ2**), 4.6 μM, acetylene component, 24 μM; MeOH, 1.5%. Each reaction mixture was incubated at 37 °C for at least 6 h. Samples of the reactions were injected directly (15 μL) into the LC/MSD instrument to perform LC/MS-SIM analysis (Zorbax SB-C8 reverse-phase column, preceded by a Phenomenex C18 guard column, electrospray ionization, and mass spectroscopic detection in the positive selected ion mode, tuned to the expected molecular weight of the product). The cycloaddition products were identified by their retention times and molecular weights. Control experiments in the absence of enzyme or in the presence of bovine serum albumin (BSA, 3 mg/mL) instead of enzyme failed to produce product signals. For these control experiments, methanol (1:1 dilution) was added to the reaction mixtures prior to LC/MS-SIM analysis, to prevent possible precipitation of the expected triazole product.

In Situ Click Chemistry Screening Procedure for Multicomponent Incubations. A methanolic solution (1.0 μL) of acetylene building blocks (10 reagents at 2 mM concentration each) was added to a solution of **TZ2** and mouse AChE (99 μL of 1 μM AChE, 4.2 μM **TZ2**, 2 mM ammonium citrate, 100 mM NaCl, pH = 7.3−7.5). The final concentrations were as follows: mouse AChE, 1 μM; tacrine component (**TZ2**), 4.2 μM; acetylene component, 20 μM; MeOH, 1%. Each reaction mixture was incubated at 37 °C for at least 24 h. Samples of the reactions were injected directly (15 μL) into the LC/MSD instrument to perform LC/MS-SIM analysis, as described previously.[26] The chromatograms were analyzed for the presence of in situ reaction products by extracting single ion traces for all expected molecular weights (cf. Figure 4).

Conclusions

This study reveals that the in situ click chemistry approach has great potential for lead discovery and optimization. As established here, suitable reagents for the generation of potent inhibitors within the enzyme's binding sites can be found without prior knowledge of their affinities for the protein, provided that one of the two components has sufficient affinity to serve as an "anchor molecule". In the present study, the anchor is the azide **TZ2**, which is present at a concentration sufficient to saturate the enzyme active site. We have discovered two new potent in situ hit compounds through screening of reagent mixtures using the reliable LC/MS-SIM method for analyzing in situ click chemistry mixtures. The hit compounds **TZ2PIQ-A5** and **TZ2PIQ-A6** were made by both eel and mouse AChE with high regioselectivity for the *syn*-triazole product. With low-femtomolar dissociation constants, these compounds are the most potent noncovalent AChE inhibitors known. They lack a permanent positive charge and aniline groups and possess fewer fused aromatic rings than the original inhibitors. The corresponding *anti*-isomers, not made by the enzymes, were 2−4 orders of magnitude less potent. The affinities of the offered building block components do not always correlate well with the propensity for formation of hit compounds. The unique synergism found for formation of these hit compounds within the enzyme likely results from the proximity of the reactant moieties and their ability to adopt the appropriate orientation. In addition, the enzyme-templated cycloaddition is most likely promoted by an enthalpic stabilization of the transition state, probably through compensation of the strong dipole moment that is developed during triazole genesis. Previous crystallographic studies have revealed the unusual positioning of a tryptophan and a tyrosine at the peripheral binding site pointing toward a potential ability of the reactive building blocks to induce or stabilize a unique enzyme conformation that allows the reaction to take place. Currently, we are focusing our research on the mechanism of the in situ click chemistry involving a combination of kinetic studies and structural biology.

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Supporting Information Available: LC/MS-SIM traces for in situ click chemistry and control experiments, experimental details and LC/MS-SIM traces for regioisomer determination, comparison of reaction rates between the **PIQ** acetylene enantiomers, experimental procedures for reagent and triazole synthesis, and their characterization, and tables with free energy increments for structural modifications. This material is available free of charge via the Internet at http://pubs.acs.org.

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