Chapter 6

Acetylcholinesterase Inhibition Assays for High-Throughput Screening

Shuaizhang Li, Andrew J. Li, Jinghua Zhao, Michael F. Santillo, and Menghang Xia

Abstract

Acetylcholinesterase (AChE) hydrolyzes acetylcholine (ACh), a vital neurotransmitter that regulates muscle movement and brain function, including memory, attention, and learning. Inhibition of AChE activity can cause a variety of adverse health effects and toxicity. Identifying AChE inhibitors quickly and efficiently warrants developing AChE inhibition assays in a quantitative, high-throughput screening (qHTS) platform. In this chapter, protocols for multiple homogenous AChE inhibition assays used in a qHTS system are provided. These AChE inhibition assays include a (1) human neuroblastoma (SH-SY5Y) cell-based assay with fluorescence or colorimetric detection; (2) human recombinant AChE with fluorescence or colorimetric detection; and (3) combination of human recombinant AChE and liver microsomes with colorimetric detection, which enables detection of test compounds requiring metabolic activation to become AChE inhibitors. Together, these AChE assays can help identify, prioritize, and predict chemical hazards in large compound libraries using qHTS systems.

Key words Acetylcholinesterase (AChE), AChE inhibitors, Cell-based AChE inhibition assay, Enzyme-based AChE inhibition assay, High-throughput screening, Liver microsomes, Pesticides

1 Introduction

Acetylcholine (ACh) is a neurotransmitter associated with cognitive, autonomic, and neuromuscular functions. Acetylcholinesterase (AChE), an enzyme found at neuromuscular junctions and cholinergic synapses, is essential for maintaining normal function of the central and peripheral nervous systems by hydrolyzing ACh [1]. However, chemicals that inhibit AChE activity interfere with this process, leading to an accumulation of ACh and numerous adverse effects, such as smooth muscle contractions, skeletal muscle twitching, glandular secretions, and flaccid paralysis. Some examples of AChE inhibitors that cause adverse effects include pesticides

Hao Zhu and Menghang Xia (eds.), *High-Throughput Screening Assays in Toxicology*,
Methods in Molecular Biology, vol. 2474, https://doi.org/10.1007/978-1-0716-2213-1_6,

(organophosphates and carbamates), chemical warfare agents (nerve agents), drugs, and phytochemicals [2–4].

Given the toxicological effects due to AChE inhibition, in vitro AChE inhibition assays are important for identifying potential chemical hazards and prioritizing these chemicals for further toxicological testing. Additionally, AChE inhibition assays may facilitate development of predictive toxicology models such as quantitative structure–activity relationships (QSAR). Although AChE inhibition assays have been used extensively in manual, lower-throughput formats (e.g., 96-well plates), AChE inhibition assays used in quantitative high-throughput screening (qHTS) platforms [5] (e.g., 1536-well plates) will improve the ability to identify chemical hazards and develop predictive models when dealing with large chemical libraries. Tox21 (Toxicology in the twenty-first Century), a research consortium among multiple federal agencies uses a battery of high-throughput screening in vitro assays for determining toxicity [6, 7]. The ultimate goals of Tox21 are identifying, prioritizing, and predicting chemical safety, which require high-throughput in vitro toxicology assays to screen a library containing 10,000 chemicals with human health relevance. The in vitro assays cover a variety of toxicological mechanisms and pathways, including AChE inhibition. [8–10]

In this chapter, protocols are provided for multiple in vitro AChE inhibition assays used in automated qHTS systems. Since a single AChE inhibition assay may be prone to interference, false positives or negatives, multiple complementary assay formats and detection schemes are provided to address these challenges. First, a protocol for a cell-based AChE inhibition assay is described. This method uses a human neuroblastoma cell line (SH-SY5Y) with a choice of Amplite Red fluorimetric (Fig. 1) or colorimetric (Fig. 2) assay. The cell-based qHTS assay is conducted in a homogenous format without reagent removal and washing steps, which improves assay performance [5, 11–13]. The second protocol describes a cell-free, human recombinant AChE inhibition assay that uses a choice of colorimetric or Amplite Green fluorimetric (Fig. 3) assay. Finally, a third protocol is outlined, which uses a combination of human recombinant AChE and liver microsomes in a colorimetric assay. Many organophosphate pesticides themselves do not inhibit AChE activity but once transformed into metabolites, they become potent AChE inhibitors. The addition of liver microsomes in the AChE inhibition assay can detect many of these compounds that undergo bioactivation. An assay that incorporates metabolic activity may enhance the ability to detect AChE inhibitors such as organophosphates. Together these three AChE inhibition assays serve as high-throughput screening methods for identifying chemicals that may affect an important toxicological target.

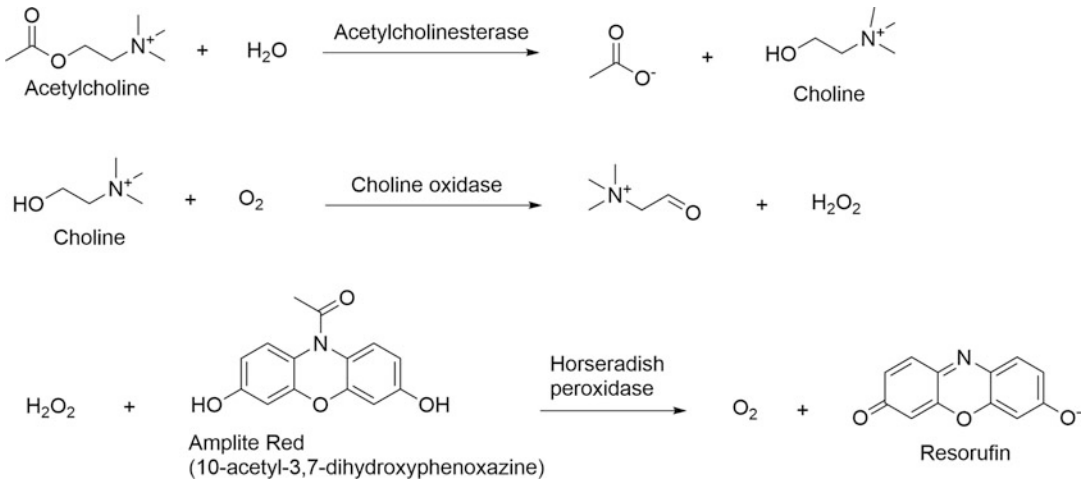

Fig. 1 Amplite Red fluorimetric AChE assay principle. AChE hydrolyzes acetylcholine to choline. Next, choline is oxidized to betaine aldehyde and hydrogen peroxide (H_2O_2) by choline oxidase. Finally, horseradish peroxidase converts H_2O_2 and Amplite Red to resorufin. The concentration of resorufin is quantified by its fluorescence intensity (excitation, 540 nm; emission, 590 nm), which reflects AChE activity

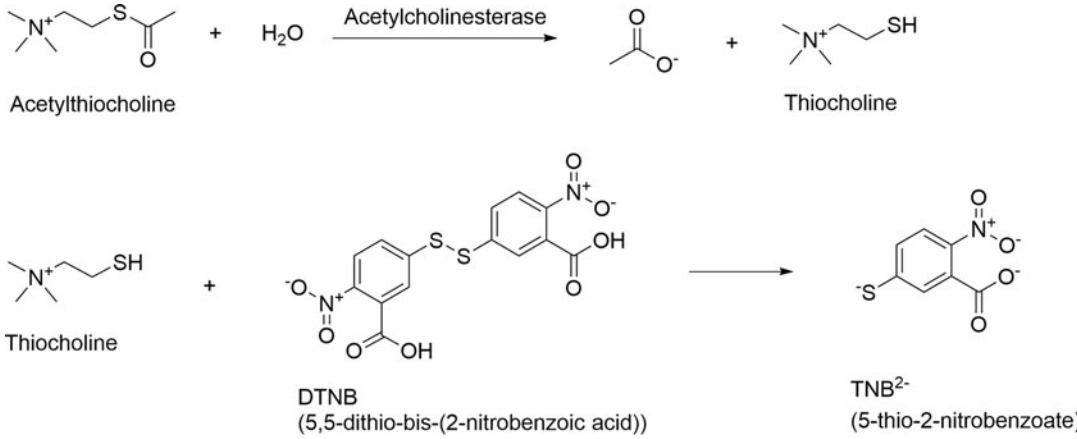

Fig. 2 Colorimetric AChE assay principle. AChE hydrolyses acetylthiocholine to thiocholine, which reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce 5-thio-2-nitrobenzoate (TNB). The concentration of TNB is quantified by its absorbance (405 nm), which reflects AChE activity

2 Materials

2.1 Equipment

1. Purifier Logic + Class II, Type A2 biosafety cabinet.
2. Steri-Cult CO_2 incubator.
3. BioRAPTR Flying Reagent Dispenser (FRD) workstation (Beckman Coulter Inc., Brea, CA).
4. Multidrop Combi 8-Channel Reagent Dispenser (Thermo Scientific, Waltham, MA).

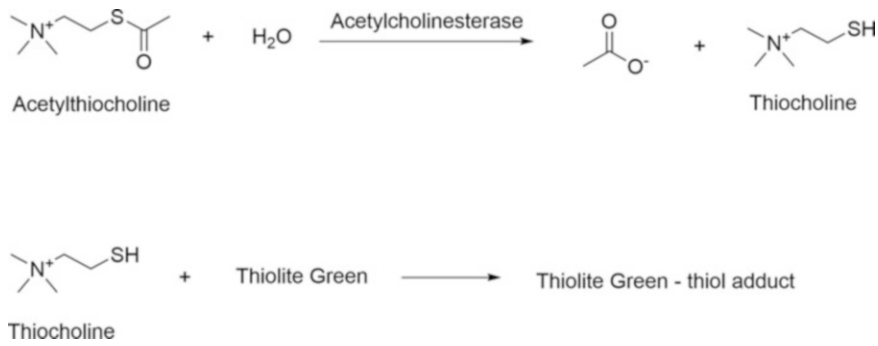

Fig. 3 Amplite Green fluorometric AChE assay principle. AChE hydrolyses acetylthiocholine to thiocholine, which reacts with a proprietary probe, Thiolite Green, to produce a fluorescent adduct. The concentration of the adduct is quantified by its fluorescence intensity (excitation, 490 nm; emission, 520 nm), which reflects AChE activity

5. Pintool workstation (Wako Automation, San Diego, CA).
6. EnVision Multilabel Plate Reader (PerkinElmer, Shelton, CT).
7. ViewLux uHTS Microplate Imager (PerkinElmer).
8. PHERAstar plate reader (BMG LABTECH, Cary, NC).

2.2 Reagents and Supplies

1. Human neuroblastoma cell line (SH-SY5Y) from American Type Culture Collection (ATCC, Manassas, VA).
2. Cell culture medium: A mixture of 45% F-12 and 45% Eagle's minimum essential media from ATCC, supplemented with heat-inactivated 10% FBS (Hyclone Laboratories, Logan, UT) and 50 U/mL penicillin and 50 μg/mL streptomycin (Thermo Fisher Scientific, Grand Island, NY).
3. Cell assay medium: A customized formulation of DMEM/F-12 medium without choline and phenol red from Thermo Fisher Scientific, Inc. supplemented with 1% premium FBS (Thermo Fisher Scientific).
4. Positive control compounds: Chlorpyrifos-oxon from Chem Service (West Chester, PA), chlorpyrifos and 1,5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51) from Sigma-Aldrich (St. Louis, MO) (*see* **Note 1**).
5. Assay kits from AAT Bioquest (Sunnyvale, CA): Amplite Red fluorimetric acetylcholinesterase assay kit, Amplite Green fluorimetric acetylcholinesterase assay kit, Amplite colorimetric acetylcholinesterase assay kit, and Amplite Red fluorimetric peroxidase assay kit.
6. 1536-well black wall/clear-bottom plates, cell culture treated, and sterile (Greiner Bio-One, Monroe, NC)
7. Recombinant human AChE from Sigma-Aldrich Co.

8. InVitroCYP 150-D human liver microsomes (HLM), prepared from 150 donor human liver tissue fraction pools with mixed gender, from BioIVT (Baltimore, MD).
9. Acroclor 1254-induced male Sprague-Dawley rat liver microsomes (RLM) from Molecular Toxicology (Boone, NC).
10. NADPH from Sigma-Aldrich.
11. Cell culture flasks (225 cm^2).
12. Test compound stock solutions in DMSO.

3 Methods

3.1 Cell-Based Assays

1. Detach SH-SY5Y cells from flasks using 0.25% trypsin, followed by centrifugation for 4 min at 900 rpm.
2. Suspend SH-SY5Y cells (500,000 per mL) in customized cell assay media (without choline and phenol red).
3. Apply the cell suspension through a cell strainer prior to dispensing the cells at 2000 cells/4 μL/well in 1536-well plates using a Multidrop Combi 8-channel reagent dispenser.
4. Incubate SH-S5Y cells overnight (18 h) at 37 °C and 5% CO_2.
5. Prepare detection solutions for colorimetric (*see* **Note 2**) or Amplite Red fluorimetric assays (*see* **Note 3**).
6. Transfer 23 nL of test compounds, negative controls (DMSO), and positive control compounds (chlorpyrifos oxon and BW284c51) to the assay plate via a Wako Pintool station. In addition to chlorpyrifos oxon and BW284c51, other known AChE inhibitors may also be used as positive controls (*see* **Note 1**). For an example of a 1536-well plate layout, *see* Fig. 4.
7. Incubate the assay plates for 1 h at 37 °C, 5% CO_2.
8. Add 4 μL of Amplite Red detection solution or colorimetric detection solution to each well using BioRAPTR FRD and incubate assay plates for 40–90 min at room temperature.
9. For Amplite Red assay, measure fluorescence intensity (excitation, 544 nm; emission, 590 nm) using a ViewLux plate reader. For colorimetric assay, measure absorbance (405 nm) using a by an EnVision plate reader. To identify potential false positives for the Amplite Red fluorimetric assay, perform a counter-screen for peroxidase inhibitors (*see* **Note 4**).
10. Analyze fluorescence or absorbance measurements (*see* Subheading 3.4).

3.2 Human Recombinant AChE-Based Assays

1. Prepare detection solutions for colorimetric (*see* **Note 2**) or Amplite Green fluorimetric assays (*see* **Note 5**).

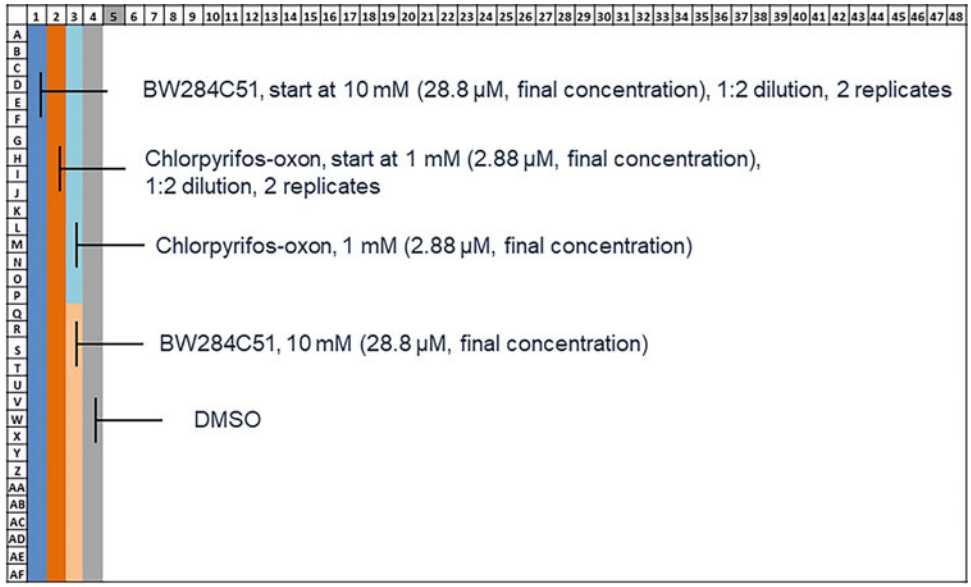

Fig. 4 Plate layout with two positive control compounds (chlorpyrifos oxon and BW284c51) and negative controls (DMSO). For each of the two selected compounds, 1:2 dilution is performed with two replicates

2. Dispense 4 μL human recombinant AChE (50 mU/mL for colorimetric assay or 20 mU/mL for Amplite Green assay) into 1536-well plates using a Multidrop Combi 8-channel dispenser.
3. Transfer 23 nL of test compounds, negative controls (DMSO), or positive controls immediately into the assay plates using a Wako Pintool station. The plate layout is similar to that of cell-based assays.
4. Incubate the assay plates for 30 min at room temperature.
5. Add 4 μL of colorimetric detection solution or Amplite Green detection solution using a BioRAPTR FRD.
6. Incubate the assay plates at room temperature for 10–30 min.
7. Measure absorbance (405 nm) for colorimetric assay or fluorescence intensity (excitation, 490 nm; emission, 520 nm) for Amplite Green assay using an EnVision plate reader.
8. Analyze absorbance or fluorescence measurements (*see* Subheading 3.4).

3.3 Human Recombinant AChE-Based Assays with Liver Microsomes

1. Prepare detection solutions for colorimetric (*see* **Note 2**) assay and NADPH solution.
2. Dispense 3 μL mixture containing human recombinant AChE (50 mU/mL) and liver microsomes (0.25 mg/mL) into 1536-well plates using a BioRAPTR FRD. Additionally, dispense a

mixture containing AChE and heat-inactivated liver microsomes as a control for protein binding.

3. Transfer 23 nL of test compounds, negative controls (DMSO), or positive control compound (chlorpyrifos) into the assay plates using a Wako Pintool station. The plate layout is the same as those used for cell-based assays except the positive control compound.
4. Dispense 1 mg/mL NADPH (1 μL) into the assay plate using a BioRAPTR FRD for a final concentration of 0.25 mg/mL.
5. Incubate the assay plates for 30 min at room temperature.
6. Add 4 μL colorimetric detection solution using a BioRAPTR FRD.
7. Incubate assay plates at room temperature for 10–30 min.
8. Measure absorbance (405 nm) using an EnVision plate reader.
9. Analyze absorbance measurements (*see* Subheading 3.4).
10. Compare IC_{50} values from assay incubations containing AChE and (1) liver microsomes, (2) heat-inactivated liver microsomes, and (3) no microsomes. The human recombinant AChE-based assay (*see* Subheading 3.2) serves as a control for no microsomes.

3.4 Data Analysis

1. Construct concentration–response curves by plotting signal (fluorescence or absorbance) on y-axis and log of test compound concentration on the *x*-axis. Fit the concentration–response data to a nonlinear four parameter logistic regression using software such as GraphPad Prism. Examples of concentration–response curves are presented in Figs. 5 and 6.
2. Evaluate assay performance by calculating coefficient of variation (CV), signal-to-background ratio (S/B), assay quality (*Z*′-factor), and fold differences of IC_{50} values for positive control compounds (*see* **Note 6**). For representative AChE assay performance data, *see* Table 1.

3.5 Confirmation of K_m and V_{max} for Colorimetric Recombinant AChE-Based Assay (Optional)

1. To ensure that AChE and acetylthiocholine substrate concentrations are appropriate for measuring AChE inhibition, this optional experiment can be carried out to confirm the substrate half-saturation concentration (K_m), maximum velocity (V_{max}), and linearity of absorbance over time.
2. Incubate a series of acetylthiocholine substrate concentrations (0.0–2.0 mM) with AChE and DTNB to measure the absorbance at multiple time points for each incubation.
3. Plot absorbance on the *y*-axis and time on the *x*-axis for each acetylthiocholine concentration tested. Fit each dataset by linear regression. The slope of each line is the enzyme velocity.

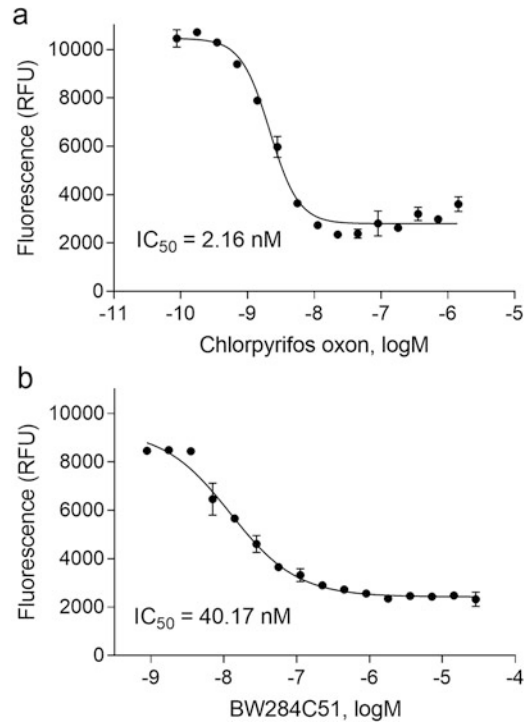

Fig. 5 Concentration–response curves demonstrating AChE inhibition by (**a**) chlorpyrifos oxon and (**b**) BW284c51 in cell-based Amplite red fluorimetric assay. Each data point represents mean ± standard deviation from three experiments

4. Plot the enzyme velocity on the *y*-axis and concentration of acetylthiocholine substrate on the *x*-axis. Fit the data to Michaelis-Menten equation by nonlinear regression using software such as GraphPad Prism (Fig. 7a). The K_m concentration for acetylthiocholine derived from the Michaelis-Menten equation should be used when performing AChE inhibition assays.
5. Confirm there is a linear relationship between acetylthiocholine substrate concentration and velocity up to the K_m concentration, which indicates initial velocity conditions (<10% of substrate depletion).
6. Incubate a series of AChE concentrations with acetylthiocholine substrate (K_m concentration) and DTNB and measure the absorbance.
7. Plot absorbance on the *y*-axis and AChE concentration on the *x*-axis. The AChE concentration used for inhibition assays should be within the linear range (Fig. 7b).

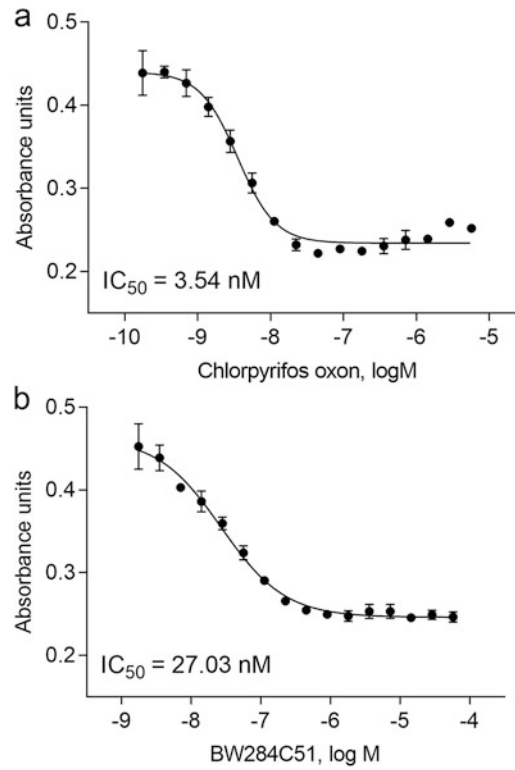

Fig. 6 Concentration–response curves demonstrating AChE inhibition by (**a**) chlorpyrifos oxon and (**b**) BW284c51 using cell-based colorimetric assay. Each data point represents mean ± standard deviation from three experiments

Table 1
Representative assay performance parameters for cell-based AChE inhibition assays

Readout	Amplite Red	Colorimetric
CV (%)	6.98 ± 0.03	1.32 ± 0.24
S/B	4.29 ± 0.14	1.96 ± 0.04
Z' factor	0.69 ± 0.01	0.75 ± 0.02

Data were calculated from 32 wells per 1536-well plate across three independent experiments

4 Notes

1. Positive controls: In addition to chlorpyrifos oxon and BW284c51, there are many other well-known AChE inhibitors that can also serve as positive controls reported in the literature [8–10, 14].
2. Amplite colorimetric AChE assay kit. DTNB stock solution (20×): add 600 μL of assay buffer to the vial of DTNB.

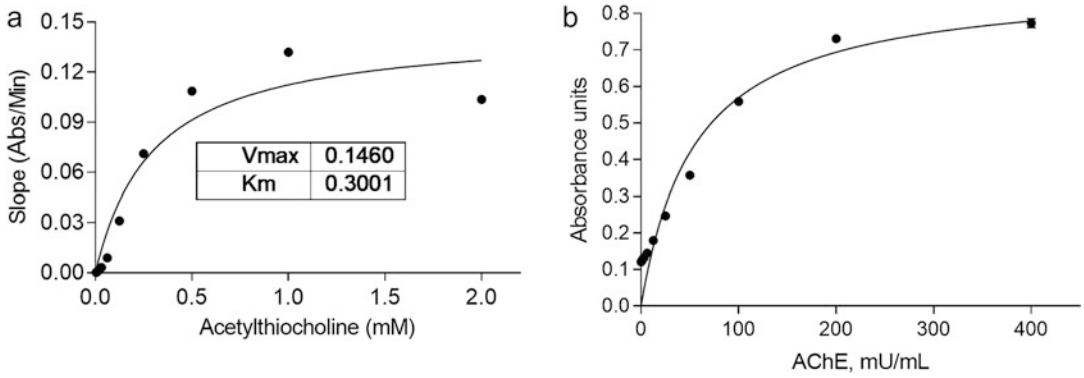

Fig. 7 Kinetics of the colorimetric AChE inhibition assay. K_m and V_{max} were determined at various (**a**) acetylthiocholine substrate concentrations and (**b**) AChE enzyme concentrations. Each data point represents mean ± standard deviation from three experiments. (Figure reproduced from reference 9)

Acetylthiocholine stock solution (20×): add 600 μL of water to the vial of acetylthiocholine. Colorimetric detection solution: add 250 μL of DTNB stock solution and 250 μL of acetylthiocholine stock solution into 4.5 mL of assay buffer. Protect all solutions from light.

3. Amplite Red fluorimetric AChE assay kit. Amplite Red stock solution (250×): add 40 μL of DMSO to the vial of Amplite Red. Acetylcholine stock solution (1000×): add 100 μL of assay buffer into the vial of acetylcholine. Amplite Red detection solution: add 5 mL of assay buffer to acetylcholinesterase probe, followed by adding 5 μL of acetylcholine stock solution and 20 μL of Amplite Red stock solution. Protect all solutions from light.
4. False positives: Perform a counter-screening to identify peroxidase inhibitors (Amplite Red fluorimetric peroxidase assay kit) that may have yielded false positives in the Amplite Red AChE assay.
5. Amplite Green fluorimetric AChE assay kit. Thiolite Green stock solution (200×): add 50 μL of DMSO into the vial of Thiolite Green. Acetylthiocholine stock solution (500×): add 600 μL of water to the vial of acetylthiocholine. Amplite Green detection solution: add 10 μL of acetylthiocholine stock solution and 25 μL of Thiolite Green stock solution into 5 mL of assay buffer. Protect from light and use the working solution within 30 min of preparation.
6. Assay quality parameters: The following are recommended criteria for adequate assay performance and quality (C_{DMSO} is the signal from negative controls and $C_{positive}$ is signal from positive controls):

(a) Coefficient of variation (CV) less than 10%, where $CV = SD\ (C_{DMSO})/Mean\ (C_{DMSO}) \times 100$.

(b) Signal-to-background ratio (S/B) greater than 3, where $S/B = Mean\ (C_{positive})/Mean\ (C_{DMSO})$.

(c) Assay quality (Z' factor) greater than 0.5, where $Z' = 1 - ([3 \times SD(C_{positive}) + 3 \times SD\ (C_{DMSO})]/|Mean\ (C_{positive}) - Mean\ (C_{DMSO})|)$.

(d) IC_{50} for positive controls within a threefold range.

Acknowledgments

This work was supported in part by the Intramural research program of the NCATS, NIH.

References

1. Soreq H, Seidman S (2001) Acetylcholinesterase - new roles for an old actor. Nat Rev Neurosci 2(4):294–302. https://doi.org/10.1038/35067589
2. Imbimbo BP (2001) Pharmacodynamic-tolerability relationships of cholinesterase inhibitors for Alzheimer's disease. CNS Drugs 15(5):375–390. https://doi.org/10.2165/00023210-200115050-00004
3. Mirjana BC, Danijela ZK, Tamara DL-P, Aleksandra MB, Vesna MV (2013) Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol 11(3):315–335. https://doi.org/10.2174/1570159X11311030006
4. Ali TB, Schleret TR, Reilly BM, Chen WY, Abagyan R (2015) Adverse effects of cholinesterase inhibitors in dementia, according to the pharmacovigilance databases of the United-States and Canada. PLoS One 10(12):e0144337. https://doi.org/10.1371/journal.pone.0144337
5. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. Proc Natl Acad Sci U S A 103(31):11473–11478. https://doi.org/10.1073/pnas.0604348103
6. Attene-Ramos MS, Miller N, Huang R, Michael S, Itkin M, Kavlock RJ, Austin CP, Shinn P, Simeonov A, Tice RR, Xia M (2013) The Tox21 robotic platform for the assessment of environmental chemicals--from vision to reality. Drug Discov Today 18(15–16):716–723. https://doi.org/10.1016/j.drudis.2013.05.015
7. Thomas RS, Paules RS, Simeonov A, Fitzpatrick SC, Crofton KM, Casey WM, Mendrick DL (2018) The US federal Tox21 program: a strategic and operational plan for continued leadership. ALTEX 35(2):163–168. https://doi.org/10.14573/altex.1803011
8. Li S, Huang R, Solomon S, Liu Y, Zhao B, Santillo MF, Xia M (2017) Identification of acetylcholinesterase inhibitors using homogenous cell-based assays in quantitative high-throughput screening platforms. Biotechnol J 12(5). https://doi.org/10.1002/biot.201600715
9. Li S, Zhao J, Huang R, Santillo MF, Houck KA, Xia M (2019) Use of high-throughput enzyme-based assay with xenobiotic metabolic capability to evaluate the inhibition of acetylcholinesterase activity by organophosphorous pesticides. Toxicol In Vitro 56:93–100. https://doi.org/10.1016/j.tiv.2019.01.002
10. Li S, Zhao J, Huang R, Travers J, Klumpp-Thomas C, Yu W, MacKerell AD, Sakamuru S, Ooka M, Xue F, Sipes NS, Hsieh J-H, Ryan K, Simeonov A, Santillo MF, Xia M (2021) Profiling the Tox21 chemical collection for acetylcholinesterase inhibition. Environ Health Perspect 129(4):047008. https://doi.org/10.1289/EHP6993
11. Inglese J, Johnson RL, Simeonov A, Xia M, Zheng W, Austin CP, Auld DS (2007) High-throughput screening assays for the identification of chemical probes. Nat Chem Biol 3(8):466–479. https://doi.org/10.1038/nchembio.2007.17

12. Pereira DA, Williams JA (2007) Origin and evolution of high throughput screening. Brit J Pharmacol 152(1):53–61. https://doi.org/10.1038/sj.bjp.0707373
13. Sipes NS, Martin MT, Kothiya P, Reif DM, Judson RS, Richard AM, Houck KA, Dix DJ, Kavlock RJ, Knudsen TB (2013) Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays. Chem Res Toxicol 26(6):878–895. https://doi.org/10.1021/tx400021
14. Santillo MF, Liu Y (2015) A fluorescence assay for measuring acetylcholinesterase activity in rat blood and a human neuroblastoma cell line (SH-SY5Y). J Pharmacol Toxicol Methods 76:15–22. https://doi.org/10.1016/j.vascn.2015.07.002