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Research Article

Identification of acetylcholinesterase inhibitors using homogenous cell-based assays in quantitative high-throughput screening platforms

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Acetylcholinesterase (AChE) is an enzyme responsible for metabolism of acetylcholine, a neurotransmitter associated with muscle movement, cognition, and other neurobiological processes. Inhibition of AChE activity can serve as a therapeutic mechanism, but also cause adverse health effects and neurotoxicity. In order to efficiently identify AChE inhibitors from large compound libraries, homogenous cell-based assays in high-throughput screening platforms are needed. In this study, a fluorescent method using Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) and the Ellman absorbance method were both developed in a homogenous format using a human neuroblastoma cell line (SH-SY5Y). An enzyme-based assay using Amplex Red was also optimized and used to confirm the potential inhibitors. These three assays were used to screen 1368 compounds, which included a library of pharmacologically active compounds (LOPAC) and 88 additional compounds from the Tox21 program, at multiple concentrations in a quantitative high-throughput screening (qHTS) format. All three assays exhibited exceptional performance characteristics including assay signal quality, precision, and reproducibility. A group of inhibitors were identified from this study, including known (e.g. physostigmine and neostigmine bromide) and potential novel AChE inhibitors (e.g. chelerythrine chloride and cilostazol). These results demonstrate that this platform is a promising means to profile large numbers of chemicals that inhibit AChE activity.

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Abbreviations: AChE, acetylcholinesterase; **ACh**, acetylcholine; **BW284c51**, 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; **DTNB**, 5,5'-dithiobis-(2-nitrobenzoic acid); **FBS**, fetal bovine serum; **LOPAC**, library of pharmacologically active compounds; **qHTS**, quantitative high-throughput screening; **TNB**, 5-thio-2-nitrobenzoic acid; **Tox21**, Toxicology in the 21st Century

1 Introduction

Acetylcholine (ACh) is a neurotransmitter that controls essential neurobiological processes, mainly muscle movement and cognition [1, 2]. In order to sustain these vital functions, endogenous ACh levels are regulated through its metabolism, especially through its hydrolysis that is catalyzed by acetylcholinesterase (AChE), an enzyme found in soluble or membrane-bound forms [3]. Chemical compounds that inhibit AChE activity, therefore, lead to elevated endogenous levels of ACh, which has both pharmacological and toxicological implications on human health. Diseases such as Alzheimer's, myasthenia gravis,

and glaucoma are ameliorated by increasing ACh levels, which is accomplished using drugs that reversibly inhibit AChE activity [4]. Conversely, adverse health effects and toxicity may also result from inhibition of AChE activity, which is associated with pesticides and nerve agents used in chemical warfare [4]. Similarly, drugs can inadvertently inhibit AChE activity as an off-target effect, thereby posing safety liabilities [5, 6]. In addition to these recognized functions of AChE, there is emerging evidence that AChE is involved in other "non-classical" roles [7], such as, apoptosis [8] and carcinoma [9–13].

Despite AChE inhibition having significant consequences in human health, large numbers of compounds in the chemical space [14] have unknown inhibitory effects on AChE activity. These uncharacterized compounds include synthesized drug candidates, natural products (e.g. phytochemicals) [15–17], food additives [18], and industrial chemicals that pose environmental health hazards [19–21]. The ability to identify AChE inhibitors in a large chemical space, therefore, requires sensitive, in vitro, high-throughput methods. Such methods can provide an efficient means to screen and prioritize vast numbers of compounds for subsequent pharmacological and toxicological research.

There are a variety of biochemical methods available for measuring AChE activity [22, 23]. Many of these techniques, however, focus on detection of pesticides in environmental samples or clinical applications using blood or tissue matrices. There is a need to adapt AChE inhibition assays to quantitative high-throughput screening (qHTS) platforms, which employ small sample volumes (<10 μL) in 1536-well plates [24–27] while yielding comprehensive concentration-response profiles. Furthermore, use of cultured human cell lines are advantageous for qHTS assays since an unlimited supply of human AChE is available and multiplexing with other assays is possible, thereby increasing throughput and efficiency. Cell-based qHTS assays also benefit from being performed in a homogenous format, avoiding reagent removal and washing steps, thereby achieving higher assay efficiency, signal quality, and precision.

In this study, complementary qHTS assays without washing were developed for identifying AChE inhibitors. A human neuroblastoma cell line (SH-SY5Y) [28, 29] and purified AChE from electric eel were both used in conjunction with two assay detection schemes. The first detection scheme relies on measuring fluorescence of resorufin produced from a series of enzyme reactions including Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), choline oxidase, horseradish peroxidase, and ACh [30, 31]. A second detection method employed the traditional Ellman assay [32], in which the absorbance of 5-thio-2-nitrobenzoic acid (TNB) product was measured from reactions including acetylthiocholine and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB). The assays were developed and validated in a homogenous, qHTS format by evaluating their performance and ability to identify AChE inhibitors among a collection of 1368 compounds, which included a library of pharmaologically active compounds (LOPAC) and 88 compounds from the Tox21 program [20].

2 Materials and methods

2.1 Reagents

F12 and Eagle's minimum essential media were from American Type Culture Collection (Manassas, VA, USA). DMEM/F-12 media and a customized formulation of DMEM/F-12 medium without choline and phenol red were obtained from Thermo Fisher Scientific, Inc. (Grand Island, NY, USA). HyClone fetal bovine serum (FBS) was from Life Sciences/GE Healthcare (Logan, Utah, USA). Amplite fluorimetric acetylcholinesterase assay kit (red fluorescence; "Amplex Red assay"), Amplite colorimetric acetylcholinesterase assay kit (Ellman assay), and Amplite fluorimetric peroxidase assay kit (red fluorescence) were purchased from AAT Bioquest, Inc. (Sunnyvale, CA, USA). Amplex Red acetylcholine/acetylcholinesterase assay kit was from Thermo Fisher Scientific, Inc. (Grand Island, NY, USA). Chlorpyrifos-oxon was purchased from Chem Service, Inc. (West Chester, PA, USA). The following were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA): 1,5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51), dimethylsulfoxide (DMSO), library of pharmacologically active compounds (LOPAC 1280), and 88 additional compounds from Tox21 program.

2.2 Cell culture

A human neuroblastoma cell line (SH-SY5Y, CRL-2266) was obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were cultured in a mixture of Eagle's minimum essential medium (45%), F-12 medium (45%) and heat inactivated FBS (10%) at 37°C under a humidified atmosphere and 5% CO_2.

2.3 Cell-based Amplex Red and Ellman assays

An overview of the cell-based assay detection schemes is illustrated in Fig. 1. Briefly, SH-SY5Y cells were suspended in assay media, a customized formulation of DMEM/F-12 media (without choline or phenol red) supplemented with FBS (1%). SH-SY5Y cells were plated at 2000 cells per well in 4 μL of assay medium into black-clear bottom 1536-well plates (Greiner Bio-One, Monroe, NC, USA) using a Multidrop Combi 8-channel dispenser (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The assay plates were incubated overnight (18–20 h) to allow cell attachment. Test compounds or controls (23 nL) were transferred into assay plates via a Wako Pintool station (Wako Automation, San Diego, CA, USA), and the assay plates were incu-

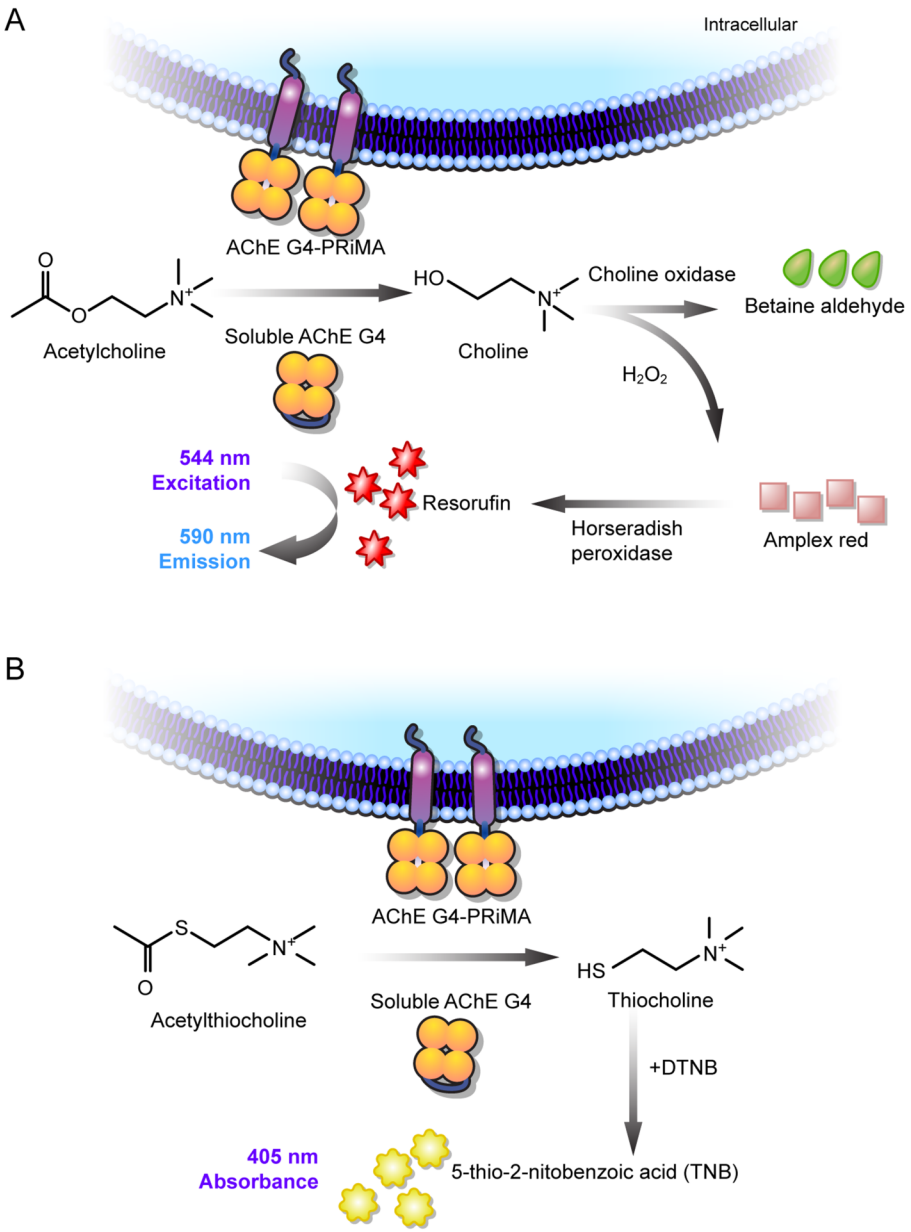


Figure 1. (**A**) Amplex Red and (**B**) Ellman cell-based assay schemes for measuring AChE activity. AChE is in soluble form or linked to cell membrane rafts by a proline-rich membrane anchor, PRiMA [3]. (**A**) Acetylcholine is hydrolyzed to choline by AChE. Choline is then oxidized by choline oxidase, producing hydrogen peroxide. Hydrogen peroxide reacts with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), catalyzed by horseradish peroxidase, to produce resorufin, which is quantified by its fluorescence. (**B**) Acetylthiocholine is hydrolyzed to thiocholine by AChE. Thiocholine reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), producing 5-thio-2-nitrobenzoic acid (TNB), which is quantified by its absorbance at 405 nm.

bated for 1 h at 37°C. Control plate layout (Supporting information, Fig. S1) shows the placement of negative controls (DMSO), positive controls (chlorpyrifos oxon and BW284c51), and test compounds.

The Amplex Red (Fig. 1A) or Ellman (Fig. 1B) assays were then performed to measure AChE activity. For the Amplex Red assay, 4 µL of detection cocktail solution containing acetycholine, Amplex Red, choline oxidase and horseradish peroxidase was added to each well using a Bioraptr FRD (Beckman Coulter, Inc., Brea, CA, USA). Assay plates were incubated at room temperature for 40 min, followed by measuring fluorescence of resorufin (λ_{ex} = 544 nm; λ_{em} = 590 nm) using ViewLux plate reader (PerkinElmer Inc., Waltham, MA, USA).

For the Ellman assay, 4 µL of detection cocktail solution containing DTNB and acetylthiocholine was added to each well, and the assay plates were incubated at room temperature for 40 min. Absorbance (λ = 410 nm) of TNB was measured using Envision plate reader (PerkinElmer Inc., Waltham, MA, USA).

2.4 Enzyme-based Amplex Red assay

The enzyme-based AChE assay was also optimized in 1536-well plates using the Amplex Red acetylcholine/acetylcholinesterase assay kit. AChE from electric eel was dispensed at 4 µL per well into black-clear bottom 1536-well plates using a Multidrop Combi 8-channel dispenser.

Test compounds or controls (23 nL) were transferred into the assay plates using a Wako Pintool station and the assay plates were incubated for 1 h at room temperature. The control compounds and plate layout are the same as those used for cell-based assays. Next, 4 μL of detection cocktail solution containing acetycholine, Amplex Red, choline oxidase and horseradish peroxidase was added to each well using a Bioraptr FRD. The final concentrations of acetycholine and AChE were 50 μM and 50 mU, respectively. Assay plates were incubated at room temperature for 40 min, followed by measuring fluorescence of resorufin (λ_{ex} = 544 nm; λ_{em} = 590 nm) using ViewLux plate reader.

2.5 Horseradish peroxidase assay

To eliminate the false positives of AChE inhibitors identified from the Amplex red assays, which include horseradish peroxidase in the reaction, Amplite fluorimetric peroxidase assay was used to identify inhibitors of horseradish peroxidase. Horseradish peroxidase (2 mU) was dispensed at 4 μL per well into black-clear bottom 1536-well plates. Test compounds or controls (23 nL) were transferred into the assay plates using a Wako Pintool station; DMSO was transferred to the first four columns of the control plate (negative controls). Assay plates were incubated at room temperature for 1 h, followed by adding 4 μL of detection cocktail solution containing Amplex Red and hydrogen peroxide using a Bioraptr FRD. Thereafter, assay plates were incubated at room temperature for 40 min before fluorescence (λ_{ex} = 544 nm; λ_{em} = 590 nm) was measured using ViewLux plate reader.

2.6 qHTS assay data analysis

Analysis of compound concentration-response data was performed as previously described [24, 33]. Briefly, raw plate measurements for each titration point were first normalized relative to the positive control compound (chlorpyrifos-oxon for cell based assays and BW284c51 for enzyme based assays; –100%) and DMSO-only wells (0%) as follows: Activity (%) = $([V_{compound} - V_{DMSO}]/[V_{DMSO} - V_{pos}]) \times 100$, where $V_{compound}$ denotes the compound well values, V_{pos} denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO-only wells. The data set was then corrected using the DMSO-only compound plates at the beginning and end of the compound plate stack by applying an in-house pattern correction algorithm [34]. The half-maximum inhibitory values (IC_{50}) for each compound and maximum response (efficacy) values were obtained by fitting the concentration-response curves of each compound to a four-parameter Hill equation [35]. Compounds were designated as class 1–4 according to the type of concentration–response curve observed [24, 33]. For the present study, antagonists were defined as compounds that inhibited AChE activity. Compounds with curve classes -1.1, -1.2, -2.1, or -2.2 (efficacy <–50%) were considered active, compounds with class 4 curves were considered inactive, and compounds with all other curve classes were defined as inconclusive. The potential AChE inhibitors were selected based on the common active compounds identified from the cell-based Amplex Red assay, cell-based Ellman assay as well as the enzyme-based Amplex Red assay. In addition, the peroxidase counter-screen was also considered to eliminate false-positives. The identified AChE inhibitors in the current study all passed chemical quality control tests for identity and purity. Data were analyzed and depicted using OriginPro 2015 (OriginLab Corp., Northampton, MA, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 Optimization of Amplex Red AChE assays

The Amplex Red assay for measuring AChE activity was developed and optimized using a human neuroblastoma cell line (SH-SY5Y). The reaction scheme for this method is presented in Fig. 1A, which depicts soluble AChE along with PRiMA-linked AChE in the cellular membrane [3]. First, the effects of assay media composition were investigated by measuring the concentration-response profiles of two known AChE inhibitors (positive controls), BW284c51 and chlorpyrifos-oxon. When regular DMEM/F12 assay media was used in a homogenous assay format, no inhibition of AChE activity was observed for BW284c51 or chlorpyrifos oxon (Fig. 2A and 2B). DMEM/F12 assay medium contains choline, which participates in the Amplex Red detection scheme (Fig. 1A), along with phenol red, which could interfere with fluorescence of resorufin. Together, these two components could have been responsible for high fluorescence intensity, resulting in a lack of observed AChE inhibition. When a customized assay medium was used (DMEM/F12 without choline or phenol red), both positive controls, BW284c51 and chlorpyrifos oxon, inhibited AChE activity in the nanomolar range as expected (Figs. 2A and 2B). Therefore, this customized assay medium formulation was chosen for subsequent screening campaigns.

The effect of FBS concentrations on AChE inhibition potency was also studied. The IC_{50} values for BW284c51 (Fig. 2C) and chlorpyrifos oxon (Fig. 2D) increased as FBS concentration increased over the range 0.5–10% since serum proteins can bind to inhibitors, thereby lowering their free concentrations in solution [36]. Based upon these data, FBS (1%) in assay medium was chosen as an optimal condition since it offered an acceptable compromise for both potency (IC_{50}) and signal-to-background ratio. An additional assay was also developed using AChE from eel in 1536-well format. The concentration of sub-

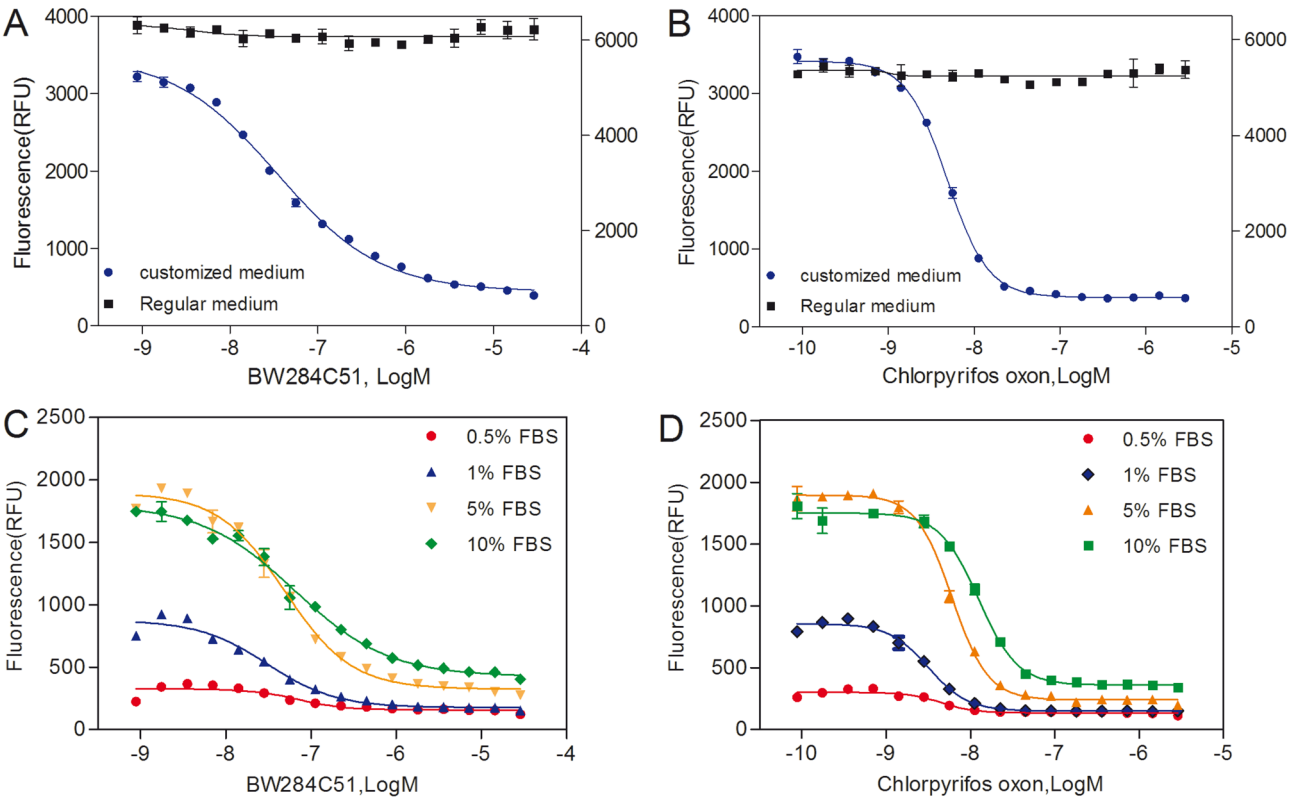


Figure 2. Optimization of assay media used for the cell-based Amplex Red AChE assay. Inhibition of AChE activity by two known AChE inhibitors, (**A**) BW284c51 and (**B**) chlorpyrifos oxon, using regular assay media and a customized formulation that excludes both choline and phenol red. Inhibition of AChE activity by (**C**) BW284c51 and (**D**) chlorpyrifos oxon using customized assay media with different concentrations of fetal bovine serum (FBS). Each data point represents mean ± standard deviation from three experiments.

strate, acetylcholine, was determined by calculating K_m of 51 µM, while optimal AChE concentration was determined by serial dilutions of the enzyme (Supporting information, Fig. S2).

3.2 qHTS assay performance and reproducibility

To evaluate the screening performance of three qHTS assay formats, a total of 1368 compounds were screened, which included a library of pharmacologically active compounds (LOPAC; 1280 compounds), along with 88 additional compounds from the Tox21 program. The screen was run three independent times with each compound evaluated at seven concentrations ranging from 1.84 nM to 28.75 µM. Additionally, BW284c51 and chlorpyrifos-oxon were included as positive controls in each assay plate, while DMSO was used as a negative control. A collection of concentration-response data generated by triplicate runs of cell-based Amplex Red and Ellman screening assays are illustrated in Fig. 3.

Positive controls BW284c51 and chlorpyrifos-oxon inhibited AChE activity in a concentration-dependent manner. There was a high concordance regarding potency (IC_{50}) of BW284c51 determined by cell-based Amplex Red (23.74 ± 4.76 nM), cell-based Ellman (39.79 ± 8.51 nM) and eel enzyme-based Amplex Red assays (18.15 ± 3.67 nM). Similarly, the potency of chlorpyrifos oxon was comparable among cell-based Amplex Red (3.98 ± 0.28 nM), cell-based Ellman (6.06 ± 0.40 nM), and eel enzyme-based Amplex Red assays (17.24 ± 1.81 nM).

Table 1. AChE assay reproducibility for identification of inhibitors.

Assay format	Active match[a)] (%)	Inactive match[a)] (%)	Inconclusive[a)] (%)	Mismatch[a)] (%)	IC_{50} (fold change)
Cell-based Amplex Red assay	12.87	84.43	2.70	0.00	1.14
Cell-based Ellman assay	4.75	94.37	0.88	0.00	1.12
Enzyme-based Amplex Red assay	16.67	79.97	3.36	0.00	1.16

a) Definitions for various match categories are provided elsewhere [33, 38].

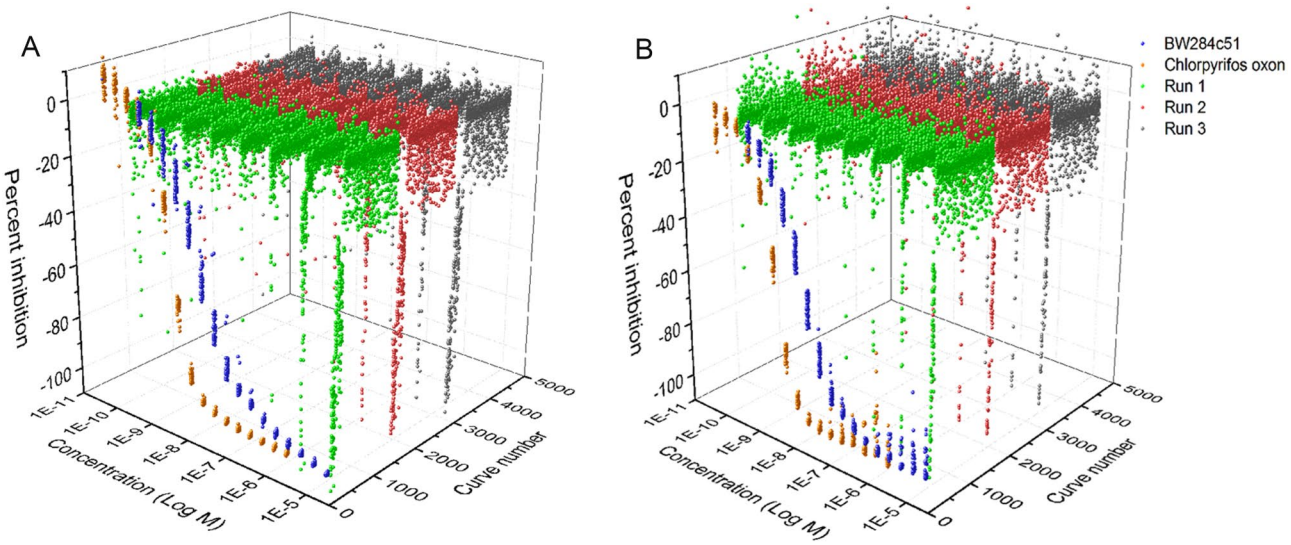


Figure 3. qHTS concentration-response data for cell-based (**A**) Amplex Red and (**B**) Ellman assays. Concentration-response data represents a total of 1368 compounds screened, each of which was tested in triplicate (run numbers 1, 2, and 3). Selected data for positive controls BW284c51 and chlorpyrifos oxon are highlighted.

Next, assay performance parameters were evaluated. As shown in Supporting Information Table S1, all three assay formats exhibited exceptional values for coefficients of variation (CV, 2.3–4.6%), signal-to-background ratio (S/B, 7.8–16.7), and assay quality (Z', 0.83–0.92) [37]. Furthermore, these performance parameters were similar among the three assay formats. Reproducibility of compound activity was also calculated [38]. The active match, inactive match, inconclusive, and mismatch rates, along with potency differences among the three runs, are listed in Table 1. For all three assays, the reproducibility was high, with no activity mismatches and IC_{50} differences among the three replicates within 1.2-fold (Table 1).

3.3 Identification of AChE inhibitors using three qHTS assay methods

Of the 1,368 compounds screened for AChE inhibition, there were 73, 57 and 55 potential inhibitors identified by cell-based Amplex Red, cell-based Ellman, and enzyme-based Amplex Red assays, respectively. A comparison of the number of inhibitors identified by each method is illustrated in a Venn diagram found in Fig. 4A. There were 45 common compounds identified as AChE inhibitors by all three assay formats. These 45 compounds exhibited IC_{50} values ranging from 46 nM to 98 μM (Fig. 4B). Among these 45 compounds, 19 potent inhibitors ($IC_{50} \leq 10$ μM) were identified; their potencies determined by the three assay formats are provided in Table 2. Many of these compounds are known AChE inhibitors, but several potential novel AChE inhibitors were also identified from this study, which are also provided in Table 2. Of the 45 inhibitors identified by all three assay methods, 40 belong to the commercially available library of pharmacologically active compounds (LOPAC). This library contains compounds with known biological activities and targets. In Fig. 4C, the 40 AChE inhibitors identified in this study were classified based upon their known biological activities and targets. These biological activities and targets include cholinergic and serotoninergic functioning, along with various other targets. Concentration response curves for two potential novel inhibitors, dequalinium dichloride and metergoline, are displayed in Fig. 4D.

4 Discussion

In this paper, qHTS assays using two detection schemes (absorbance and fluorescence) were employed to identify AChE inhibitors using a human neuroblastoma cell line (SH-SY5Y) and AChE from electric eel. These methods were conducted in a homogenous manner, where reagents were not removed and washing steps were not performed. Homogenous formats ensure higher levels of assay signal quality and precision necessary for qHTS screening. Indeed, coefficients of variation were low (CV <4.6%) for all three qHTS assays. Furthermore, it is imperative that concentration-response curves are reproducible when screening large compound libraries. All three methods in this study yielded no mismatch in compound activities, low rates of inconclusive outcomes, and IC_{50} value differences within 1.2-fold among replicated runs, all of which demonstrate high reproducibility for identifying AChE inhibitors. Finally, signal-to-background ratios (S/B) and *Z'* factor were high for all three

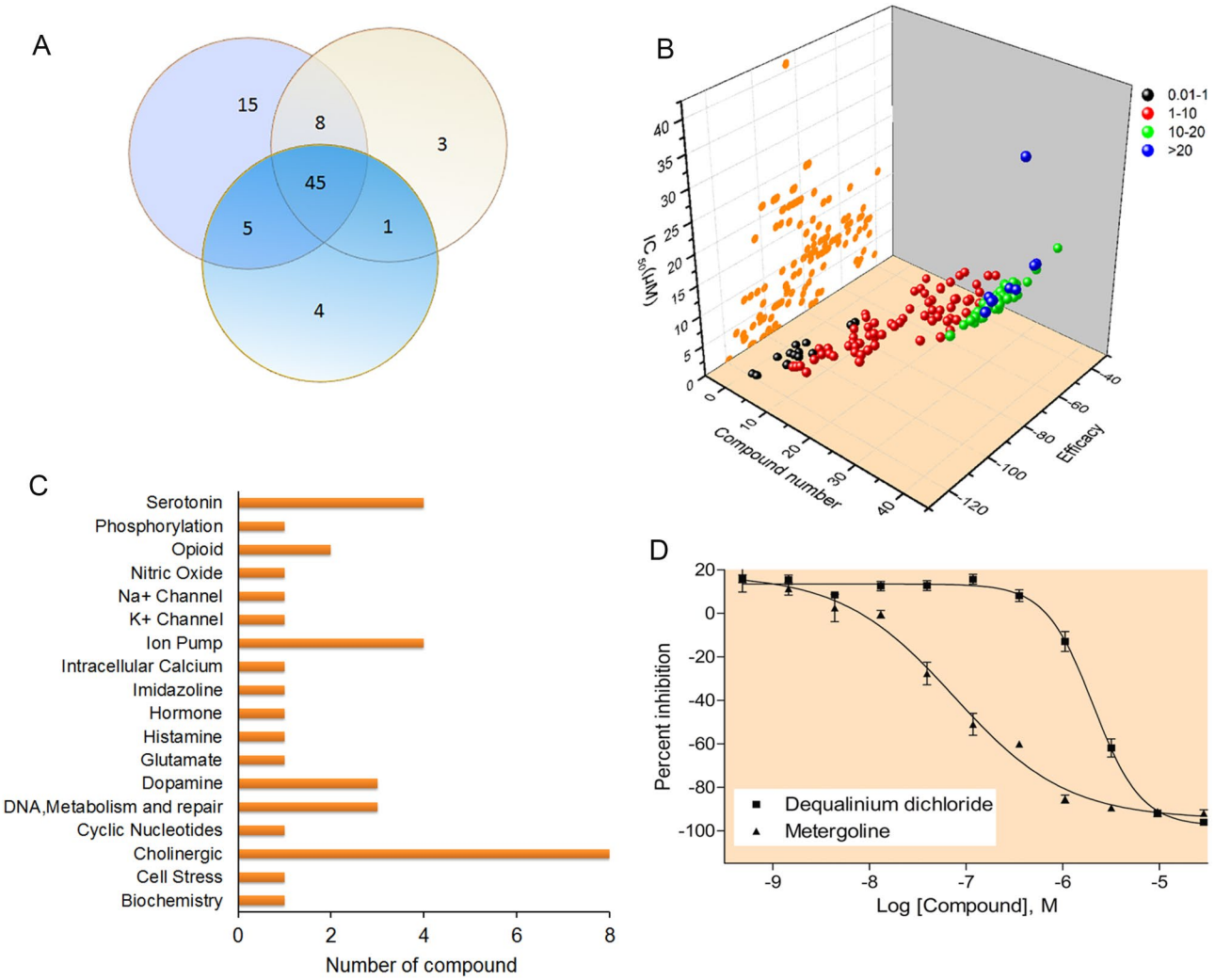


Figure 4. Potential AChE inhibitors identified from the three assays. (**A**) Venn diagram of the compounds identified from cell-based Amplex Red assay (purple), cell-based Ellman assay (yellow) and enzyme-based Amplex Red assay (blue). (**B**) The scatter cluster of the 45 compounds based on IC_{50}. (**C**) Functional classification of 40 AChE inhibitors identified in the library of pharmacologically active compounds (LOPAC). (**D**) Representative concentration-response curves of two novel AChE inhibitors, dequalinium dichloride and metergoline. Each data point represents mean ± standard deviation from three experiments.

assays, with the cell-based Amplex Red assay having slightly better values than the other two formats.

Two orthogonal assay detection schemes were used for cell-based AChE inhibition assays: fluorescence (Amplex Red assay) [30, 31] and absorbance (Ellman assay) [32]. Each assay has key distinctions. Although the Ellman assay involves a simpler reaction scheme, it can yield false positives if the screened compounds include thiols [39] or oximes [40], which participate in spurious reactions that interfere with the Ellman reagents. Likewise, botanical extracts, which are important sources of substances with unknown pharmacological and toxicological activities, may interfere with yellow absorbance wavelengths used in the Ellman assay. To circumvent some of these challenges, the Amplex Red assay uses resorufin fluorescence [31, 41] to quantify AChE activity, which is useful if AChE inhibition assays are multiplexed with parallel fluorescence assays involving other cellular targets. Furthermore, test compounds are less likely to exhibit orange autofluorescence that can interfere with that of resorufin [42]. However, the Amplex Red assay also poses challenges. For example, the Amplex Red assay involves coupled enzymatic reactions susceptible to inhibition, especially peroxidase, resulting in false positives [43]. Therefore, in this study, a counter-screening to identify peroxidase inhibitors was performed to eliminate these compounds.

Among the compounds screened (especially within LOPAC), several are known inhibitors of AChE activity, and all three qHTS assays accurately identified them.

Table 2. Compounds identified as AChE inhibitors (IC_{50}<10 μM) from a collection of 1368 compounds screened using three qHTS assays[a)]

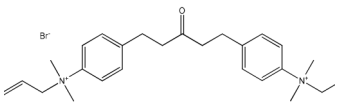
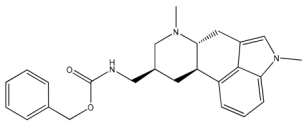
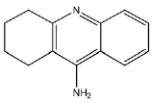
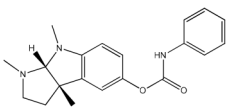
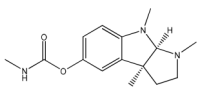
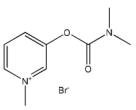
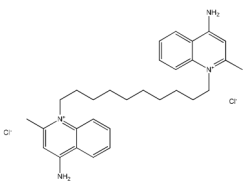
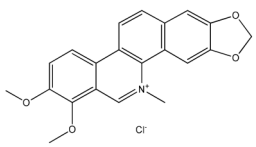
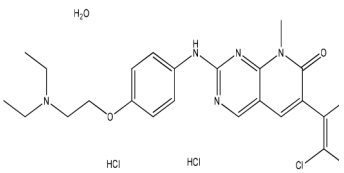
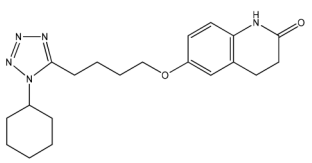
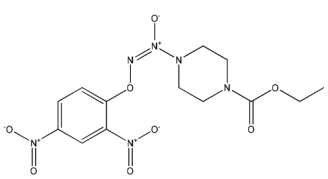
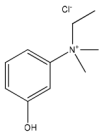
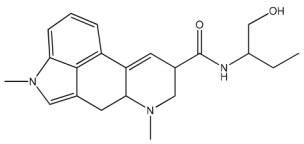
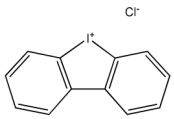
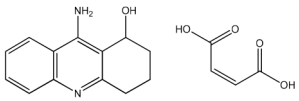
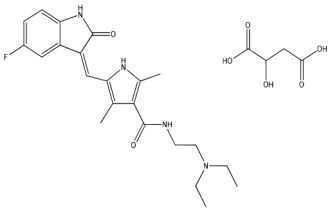
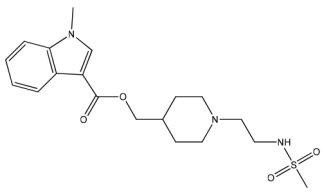
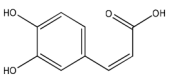
Compound	Structure	Cell-based Amplex Red assay (IC_{50}, μM)	Cell-based Ellman assay (IC_{50}, μM)	Enzyme-based Amplex Red assay (IC_{50}, μM)
BW284c51[b)]		0.10 ± 0.03	0.14 ± 0.05	0.05 ± 0.02
Metergoline		0.78 ± 0.10	1.67 ± 0.23	16.77 ± 0.00
Tacrine[b)]		0.84 ± 0.14	1.12 ± 0.13	0.17 ± 0.02
Phenserine[b)]		0.85 ± 0.16	0.47 ± 0.03	1.50 ± 0.17
Physostigmine[b)]		1.01 ± 0.07	0.31 ± 0.00	0.14 ± 0.01
Pyridostigmine bromide[b)]		1.19 ± 0.39	1.15 ± 0.09	1.85 ± 0.46
Dequalinium dichloride		2.77 ± 0.19	2.73 ± 0.00	4.38 ± 0.30
Chelerythrine chloride		3.13 ± 0.56	11.49 ± 5.30	0.72 ± 0.05
PD-166285 hydrate		4.73 ± 0.00	5.66 ± 0.38	3.35 ± 0.00
Cilostazol		5.33 ± 0.61	27.40 ± 3.15	10.25 ± 1.41
NSC-724771		7.49 ± 0.00	14.23 ± 0.96	8.10 ± 0.53

Table 2. Compounds identified as AChE inhibitors (IC_{50}<10 μM) from a collection of 1368 compounds screened using three qHTS assays[a] (continue)

Compound	Structure	Cell-based Amplex Red assay (IC_{50}, μM)	Cell-based Ellman assay (IC_{50}, μM)	Enzyme-based Amplex Red assay (IC_{50}, μM)
Edrophonium chloride[b]		7.28 ± 1.24	7.41 ± 0.48	1.13 ± 0.32
Methysergide		7.83 ± 1.0	7.47 ± 1.27	3.10 ± 0.21
Dibenziodolium chloride		9.43 ± 0.01	9.19 ± 2.60	8.10 ± 0.53
Hydroxytacrine maleate[b]		9.09 ± 0.59	7.41 ± 0.48	1.44 ± 0.09
Sunitinib malate[b]		8.21 ± 1.72	3.55 ± 0	3.98 ± 0.99
GR 113808		7.52 ± 2.47	5.89 ± 0.38	11.23 ± 0.91
Caffeic acid[b]		21.43 ± 2.46	Inactive	5.58 ± 0.38

a) Each value represents mean ± standard deviation from three experiments.
b) Known AChE inhibitors.

Many of these compounds are drugs or their derivatives used for treating Alzheimer's disease or myasthenia gravis. However, in addition to these well studied compounds, several potential novel AChE inhibitors were identified using these methods, including chelerythrine chloride, a plant alkaloid, and cilostazol, a drug used to treat peripheral vascular disease. Metergoline which acts as a ligand for various serotonin and dopamine receptors [44, 45], was also identified as an AChE inhibitor.

It is important to note that compounds inhibit enzyme activity via different mechanisms or modes [46, 47]. These mechanisms depend on molecular interactions between enzyme and inhibitor (e.g. covalent binding) along with binding site location on the enzyme (e.g. active site). Irreversible inhibitors usually bind covalently to AChE and their potencies are time dependent. Therefore, cells were pre-incubated with test compounds for 1 h and a wide range of compound concentrations were screened (1.84 nM–28.8 μM). Together, these two conditions ensure that any irreversible or time-dependent inhibitors are detected at biologically relevant concentrations. Chlorpyrifos oxon, for example, is an irreversible inhibitor and was successfully detected by qHTS assay. Reversible inhibitors, on the other hand, do not change potency over time, and a pre-incubation step has no effect on their potency. Reversible inhibitors can be sub-classified as competitive, non-competitive, or uncompetitive depending on the enzyme binding site location. A substrate concentration near K_m was used (ACh, 100 μM), which provided a comprehensive means to detect all types of reversible

inhibitors, regardless of their sub-classified modes [46]. Ultimately, a combination of employing a biologically relevant range of test compound concentrations, pre-incubation step, and substrate concentration near K_m all aid in comprehensive detection of AChE inhibitors regardless of their binding mechanism. An exact mechanism could be identified in subsequent orthogonal assays. These mechanistic assays may involve using multiple concentrations of enzyme, substrate, and inhibitor, along with a series of pre-incubation times or dilution steps.

Some IC_{50} values measured using electric eel AChE were lower than those obtained by the two cell-based methods. The enzyme-based assay media did not include fetal bovine serum (FBS), whereas both cell-based techniques required FBS (1%). High protein concentrations in FBS can bind to test compounds, lowering their free concentrations and therefore reducing their inhibitory potency towards AChE activity [36], and this may be responsible for some of the differences observed. In addition to differences between enzyme and cell-based formats, there were eight inhibitors detected by the two cell-based assays but not by the eel enzyme-based assay. These compounds may specifically inhibit the human form of AChE instead of enzyme from electric eel. Although human and eel AChE share the same acyl pocket that is characterized by two Phe residues (Phe288 and Phe290), there are still structural differences that may lead to potency differences [48]. For example, the potency of CPT-11 tested in electric eel AChE was greater than those in human recombinant and erythrocyte preparations [49]. Species differences regarding potency have also been observed for organophosphates paraoxon and malaoxon [50]. Metabolic activity present in cells may also be responsible for differences between cell and enzyme-based formats. Cytochrome P450 (CYP) 2D6, for example, is expressed in SH-SY5Y cells, which metabolizes neurotoxic compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1,2,3,4-tetrahydroisoquinoline, and β-carbolines [51]. Similarly, SH-SY5Y cells contain dehydrogenases [52] and reductases [53] which could metabolize test compounds, yielding potency differences between cell-based and enzyme-based assay formats. Characterization of these compounds as AChE inhibitors may be useful in better understanding their therapeutic or adverse effects. Overall, both Amplex Red and Ellman cell-based assays are promising tools used to identify inhibitors of AChE activity, a key metabolic enzyme with pharmacological and toxicological relevance to human health. The methods are homogenous, sensitive, reproducible, and are compatible with qHTS platforms, which can be used for future screening campaigns of large compound libraries.

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No conflicts of interest were disclosed by the authors.

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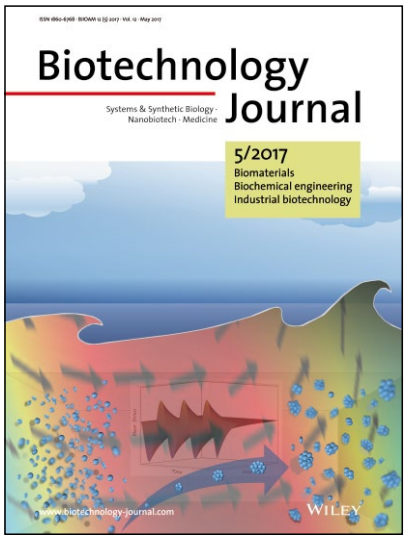

Cover illustration

The cover shows a cartoon of wave motion that provides an ideal hydrodynamic environment to induce cell-cell collision and subsequent aggregation of human mesenchymal stem cells with controlled aggregate size in suspension. The cover is prepared by Ang-Chen Tsai, Yijun Liu, Xuegang Yuan, Ravindran Chella and Teng Ma authors of the article "Aggregation kinetics of human mesenchymal stem cells under wave motion" (https://doi.org/10.1002/biot.201600448).

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