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Hands-On Approach to Structure Activity Relationships: The Synthesis, Testing, and Hansch Analysis of a Series of Acetylcholineesterase Inhibitors

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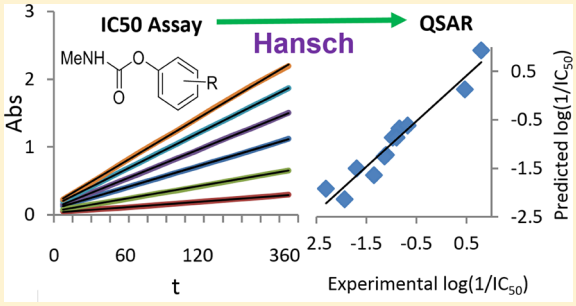
Supporting Information

ABSTRACT: This series of three practical sessions centers on drugs that inhibit the enzyme acetylcholineesterase. This enzyme is responsible for the inactivation of acetylcholine and has been the target of drugs to treat glaucoma and Alzheimer's disease and for a number of insecticides and warfare agents. These sessions relate to a series of carbamate inhibitors that are classed as slowly reversible anticholinesterases and show how structure−activity relationships can guide the development of drug molecules. In Practical 1, each student group synthesizes one carbamate inhibitor of acetylcholinesterase. In Practical 2, students measure the activity of their inhibitor to derive an IC_{50} value suitable for comparison with the other derivatives made by the class. In Practical 3, students gain firsthand experience with structure−activity methods, such as a Hansch analysis, to investigate whether these can be used to make predictions of the activity of related compounds based on structural properties. This series gives students a rare insight into a number of the important aspects of drug discovery, that of drug synthesis, activity testing, and the construction of subsequent quantitative structure−activity relationships (QSARs).



KEYWORDS: *Second-Year Undergraduate, Upper-Division Undergraduate, Hands-On Learning/Manipulatives, Laboratory Instruction, Medicinal Chemistry, Drugs/Pharmaceuticals, Organic Chemistry*

BACKGROUND

Acetylcholine (ACh) was the first neurotransmitter to be identified in 1915 and since has been shown to have an important role at all autonomic ganglia, at the neuromuscular junction and at many autonomically innervated organs. This series of experiments concerns one of the enzymes responsible for the inactivation of acetylcholine, acetylcholinesterase (AChE) (Scheme 1).[1]

Some AChE inhibitors have been used in human medicine for many years such as ecothiopate (Figure 1), used to treat glaucoma, whereas others are so toxic that they serve as chemical warfare agents (e.g., VX). More recently, tacrine, donepezil, and rivastigmine have been introduced to alleviate the symptoms of Alzheimer's disease.

Acetylcholinesterase inhibitors are toxic to insects and have found wide use as insecticides. Insecticidal AChE inhibitors are organophosphorus derivatives (e.g., malathion, Figure 2) or carbamates (e.g., carbaryl). Carbamate inhibitors are usually classed as being medium-duration anticholinesterases (slowly reversible), while organophosphorus inhibitors are often irreversible inhibitors.

The mechanism by which ACh is hydrolyzed by acetylcholinesterase involves two steps: (1) transfer of the acetate group to a serine OH located at the active site of the enzyme, resulting in release of choline, and (2) hydrolysis of this acetyl group to form acetate. When the carbamate insecticides inhibit AChE, the carbamoyl group is transferred to the serine OH group, which is much more difficult to remove by hydrolysis. As a consequence, the active form of AChE is not regenerated, and endogenous ACh cannot be hydrolyzed after activating cholinergic receptors in the insect, with resultant toxic effects.

EXPERIMENTAL OVERVIEW

There are three practical sessions in this series. In Practical 1, students gain experience in the synthetic procedures used in medicinal chemistry with each group synthesizing one acetylcholinesterase inhibitor. In Practical 2, students measure the activity of their synthesized inhibitors. This derives the IC_{50} value (the concentration of carbamate inhibitor required for 50% inhibition) that can be compared with other carbamate inhibitors. In Practical 3, students gain firsthand experience with structure−activity techniques to investigate whether predictions of the activity of inhibitors can be made based on structural properties. Assessment for these sessions is in the form of a laboratory report, the details of which have been included in the tutor guide and student handouts (Supporting Information).

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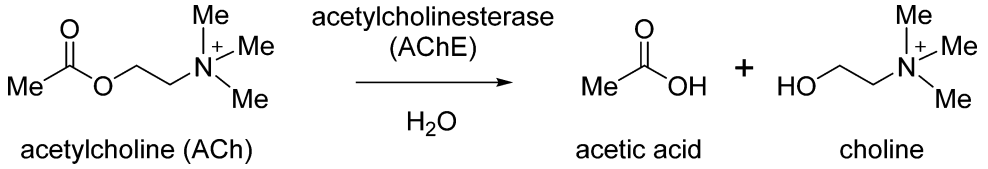
J. Chem. Educ. 2015, 92, 1745−1750

Scheme 1. Hydrolysis of ACh by AChE To Give Acetic Acid and Choline



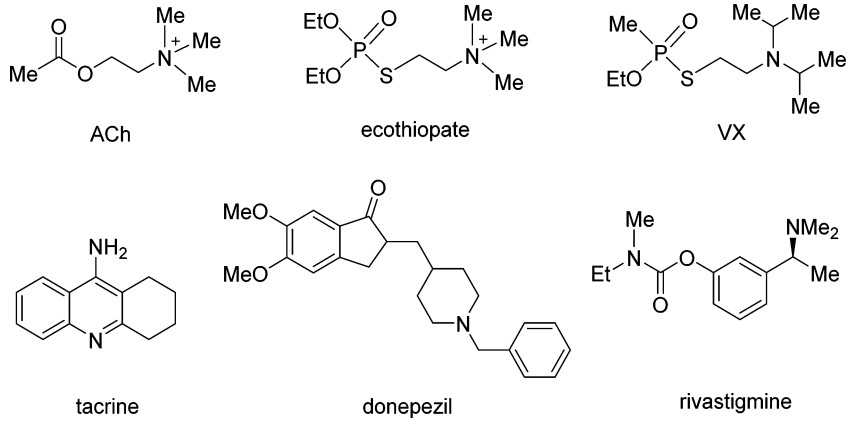


Figure 1. Structures of various AChE inhibitors used in human medicine.

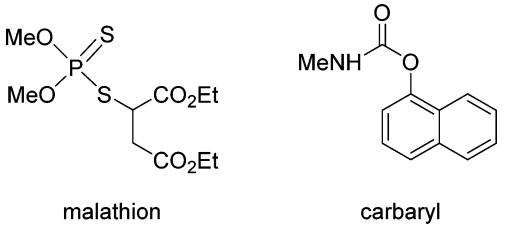


Figure 2. Structures of various AChE inhibitors.

EXPERIMENTAL SECTION

Session 1. Synthesis of Carbamate Derivatives

Students work in groups of 3 or 4 to synthesize one substituted carbamic ester. The method used is the reaction of methyl isocyanate with a substituted phenol (Scheme 2). TAKE CARE: methyl isocyanate is a poisonous compound, and demonstrators should show students how to transfer the material safely.

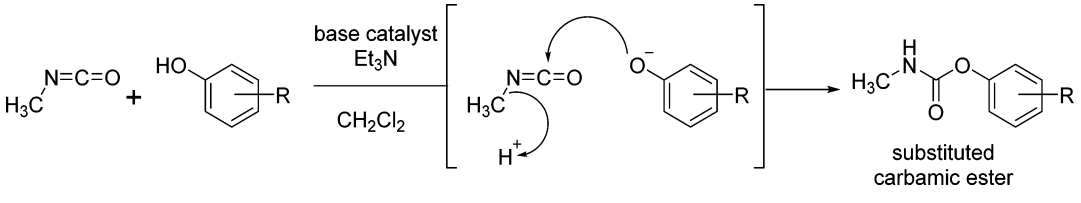
Each student group is allocated a substituted phenol (see Supporting Information Table S2 of tutor guide) with which to work, preparing a 3 mmol scale reaction. The reaction is left to proceed at room temperature for up to 30 min, during which time students are able to monitor progression using TLC (Merck silica gel 60 F_{254}, visualizing with UV). Complete instructions for this task are in the Supporting Information. Upon completion, students isolate the product via a simple extraction procedure and evaporation in vacuo. Students characterize the carbamates using ^{1}H NMR spectroscopy to identify the appropriate structure of the formed product and calculate the corresponding molecular weight and yield obtained.

Session 2. Activity as Acetylcholineesterase Inhibitors

The relative activity and potency of AChE inhibitors is measured using a standard 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (ACTI) colormetric assay that is based on the formation of a colored thiol (Scheme 3).[2] Students perform two assays, the first being a screening assay, designed to detect whether the compound has sufficient activity to be investigated further and, if the compound is active, to give an approximation of the IC_{50}. The second assay, the IC_{50} assay, more accurately determines the concentration of inhibitor needed to reduce the reaction rate by half (IC_{50}). At the end of this session, students should be able to understand and perform a colormetric assay with a microplate reader, gain experience with measuring the activity of an enzyme, and derive an IC_{50} value for use in the final session.

Part A: Screening Assay. All assays are performed using standard 96-well plates and a corresponding plate reader capable of measuring within the UV−vis range (A_{max} = 414 nm) and across the relevant time scale. If, however, such a plate reader is not available, students may be able to record manually reaction velocities in real time. Full descriptions of the methods for this section have been provided in the tutor guide.

Scheme 2. Synthesis of Carbamic Ester Derivatives



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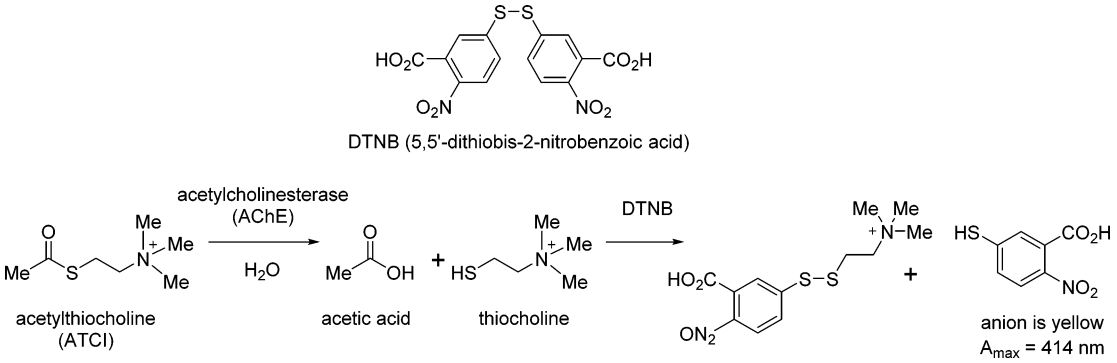
Scheme 3. Hydrolysis of ACTI in the Presence of DTNB



For Part A, the screening assay, students prepare two blank, one control and five inhibitor (0.4 mM through to 0.04 μM in 10-fold dilutions) samples. Complete details for these are provided in the Supporting Information. Students examine the results from the assay, identifying the concentration of inhibitor that provides slightly greater than 50% inhibition of the control reaction. This is the concentration that they prepare 1:2 serial dilutions for Part B, the IC_{50} assay, providing a narrow range of concentrations flanking the IC_{50} value.

Part B: IC_{50} Assay. Students prepare a series of reaction mixtures in a similar fashion to the screening assay. In total there are eight samples: two blanks, one control, and five with 2-fold decreasing inhibitor concentrations.

Part C: Calculating the IC_{50}. For each of the concentrations tested in the IC_{50} assay, students need to calculate the reaction rate as a percentage of the control value. The rate of enzymatic reaction is given by the slope of each line. The rate (slope) of each of the inhibitor reactions can be divided by the control reaction rate (slope) to give a percentage of control activity. Next, using molar inhibitor concentrations, students produce a semilog plot of the percentage control activity against the log of the inhibitor concentration. The equation governing the trendline of this can be solved for 50% to give the derived log IC_{50} value. These should be gathered by demonstrators for collation and use in the following session to determine the structure–activity of the inhibitors. Full details of these calculations are given in the Supporting Information.

Session 3. Determining Structure–Activity Relationships of Acetylcholineesterase Inhibitors

Part A. Qualitative Structure–Activity Relationships. Students begin this session with a facilitated discussion that qualitatively examines the relationship between the structure of carbamate inhibitors and measured IC_{50} values. Questions to lead the discussion are provided in the tutor guide. Students should be able to draw basic conclusions around how substitution patterns, hydrophobicity, and steric and electronic factors effect activity. These data suggest that substitution at either the 2- or 3-position with bulky, hydrophobic, and electron donating groups favors potent activity. This conclusion will help guide students through the next stage of the session, the use of a Hansch analysis to generate a quantitative structure–activity relationship (QSAR).

Part B. Quantitative Structure–Activity Relationships. In this part of the practical session, students utilize a series of parameters that describe hydrophobic, steric, and electronic properties of the given substituents to construct a QSAR in the form of a Hansch equation.[3]

Students are provided with parameters for substiuent hydrophobicity (π) (or alternatively, for an extension exercise, students calculate log P values[4]), molar refractivity (MR, a measure of substituent size) and Hammett substituent constants (σ, a measure of the electron donating or withdrawing effects for aromatic ring substituents)[5] for the three series of carbamates in the spreadsheet (Supporting Information). Students are also given a form of the Hansch equation, eq 1

$$\log\left(\frac{1}{C}\right) = -k_1(\pi^2) + k_2\pi + k_3\sigma + k_4\mathrm{MR} + k_5 \quad (1)$$

where:

C is the concentration to produce a certain effect, for example, $C = IC_{50}$.

π is a measure of the hydrophobicity.

σ is a measure of the hydrophobicity electronic effects.

MR is a measure of steric factors.

k_{1-5} are constants. These are the values that the linear regression will calculate to give a suitable fit to data.

The QSAR is formulated in the provided spreadsheet. Here, the relevant substituent parameters and measured IC_{50} values are entered and students perform a linear regression analysis to determine optimized values for required constants to give the final Hansch relationship. This is achieved by using the inbuilt regression function of Microsoft Excel called LINEST. By entering substituent parameters into this equation for each carbamate, students generate a series of predicted IC_{50} values. These are compared to values derived experimentally to ascertain the success of the Hansch equation in being able to predict AChE inhibitor activity based on substituent parameters alone. Full details of these calculations are included in the tutor guide.

■ HAZARDS

Potentially hazardous and flammable reagents are used during this practical. Methyl isocyanate is a poisonous compound which must only be handled in the fumehood. The MSDS (Material Safety Data Sheet) sheet for methyl isocyanate stipulates the use of safety glasses and gloves and the provision of good ventilation. All sources of ignition should be removed from the working area. If there is evidence of an allergic response, discontinue exposure immediately. Demonstrators should clearly show students how to transfer the material safely, and all parties should be made aware of the precautions to handle small quantities of the liquid safely. Dichloromethane is volatile and toxic, ethyl acetate is flammable and an irritant, methanol and triethylamine are flammable and toxic, hexane is

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J. Chem. Educ. 2015, 92, 1745–1750

flammable, toxic (neurotoxin), an irritant, and is toxic to aquatic life, and the phenols may be flammable, toxic, irritating, and carcinogenic. Deuterated chloroform is an irritant, toxic and suspected carcinogen/teratogen. The derived carbamate inhibitors are biologically active species and may be flammable, toxic, irritating, and carcinogenic. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) is an irritant and acetylthiocholine iodide (ATCI) is toxic and an irritant. It is recommended that staff consult relevant MSDS sources before commencing any of these exercises. Care must be taken when handling potentially hazardous materials. Contact with skin should be avoided and suitable personal protective equipment worn.

RESULTS AND DISCUSSION

These practical sessions have been run each year for 10 years in an upper-division undergraduate pharmacology course entitled "Drug Design and Development" across a total cohort of over 1200 students. Drug design efforts often iterate between experiment and computer modeling to improve the activity of a drug.[6] In this bench-active experiment, students are exposed to a hands-on drug design experiment as an extension to the molecular modeling undertaken earlier in their pharmacology courses. The sessions aim to give students a unique insight into all stages of drug development, including drug synthesis, activity assessment and the construction of quantitative structure−activity relationships. This method is robust and reproducible, with students typically obtaining results that are within an order of magnitude of literature values.

Session 1. Synthesis of Carbamate Derivatives

The chosen reaction of methyl isocyanate with a substituted phenol is easily performed using inexpensive laboratory apparatus. It progresses readily, can be performed under ambient conditions, and does not require vigorous stirring. The reaction can be undertaken in standard 20 mL glass sample vials rather than using more expensive magnetic stirring apparatus. The majority of additions can also be made using automatic pipettes, which is highly efficient and reduces the chance of exposure. Isolated yields were typically quite high (>90%) and products were of suitable purity as determined by ^{1}H NMR for use in session 2 (for representative spectra see Supporting Information). Students were generally able to assign peaks of the derived ^{1}H NMR spectra to functional groups of their carbamate products. In particular, students are able to examine the integration ratios of the *N*-methyl peak (∼2.9 ppm) with that of the aromatic peaks (∼7.0−8.0 ppm) to ascertain complete conversion of the substituted phenol into the corresponding phenyl methylcarbamate product. Students with a strong grounding in characterization may also wish to investigate further the complexities of peak splitting patterns as an extension exercise.

Session 2. Activity as Acetylcholineesterase Inhibitors

The DTNB assay used in session 2 not only provides students with a visible means of ascertaining acetylcholinesterase activity but is also robust and can be efficiently performed using a standard microplate reader. The advantages of using microplate readers for class experiments have been detailed in a number of publications,[7,8] in particular for the assay of enzyme activity[9,10] and inhibitors.[11]

All required calculations to derive final IC_{50} values can be easily performed using a basic spreadsheet program such as Microsoft Excel. An example of a student-derived plot of the data obtained in the IC_{50} assay of 3-methylphenyl *N*-methylcarbamate is given in Figure 3. This exercise allows students to explore visually how the enzyme reaction rate

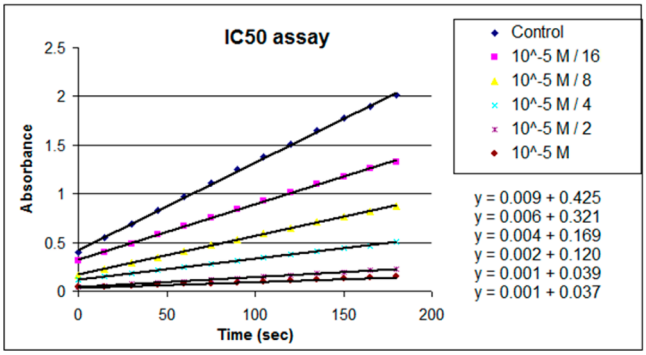

Figure 3. Example of a student-derived Excel plot of the raw data from the IC_{50} assay of 3-methylphenyl *N*-methylcarbamate. In this case, trendlines (and their equations) have been added to ascertain the slope of lines.

(slope of line) changes in the presence of increasing inhibitor concentrations. Students found a linear relationship between the normalized enzyme activity and low- to midrange molar inhibitor concentrations (Figure 4). Solving the corresponding

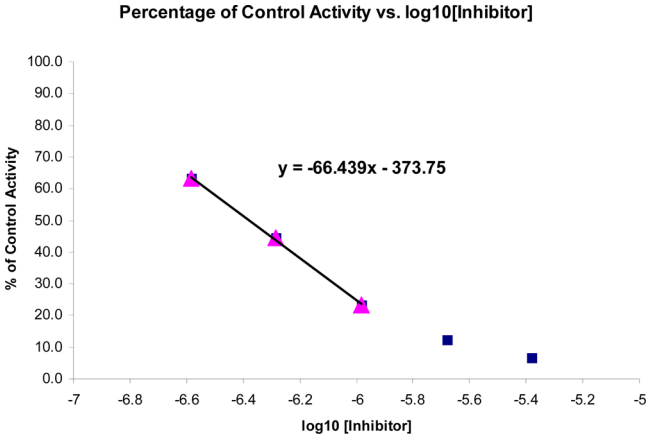

Figure 4. Example of a student-obtained semilog plot of percentage control inhibition vs log([Inhibitor]) for 3-methylphenyl *N*-methylcarbamate. Note this graph is based on a single set of student measurements and may differ to the averaged results reported in Table 1.

equation for 50% activity gave students the final IC_{50} values. IC_{50} values (Table 1) were calculated from student data obtained across a five-year period. In most cases, student-derived data were of the same magnitude as the reported literature values, thereby validating the described methods to obtain useful measures of inhibitor activity.

Session 3. Determining Structure−Activity Relationships of Acetylcholineesterase Inhibitors

This session gives students the chance to investigate how the structure of drugs can affect their activity, a process that is greatly aided with the use of computer-based methods.[13,14] There is the option to utilize measured student values or those available in the literature for inhibitors[12] (Table 1). Although student values often approximate the literature values, literature values are typically used for investigation of the structure−activity relationships. This gives a more comprehensive and

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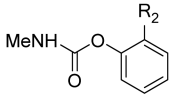
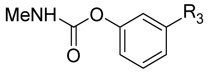
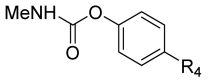
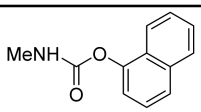
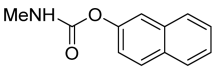
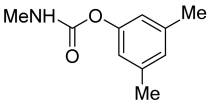
Table 1. Phenyl *N*-Methylcarbamate IC_{50} Data against Acetylcholineestierase Obtained by Undergraduate Students

Structure	Substituent	Student IC_{50} Value (μM)[a]	Literature IC_{50} Value (μM)[c]
	Br	7.1 ± 0.6	2.2
	i-Pr	0.4 ± 0.1	6.0
	H	231.0 ± 37.8	200
	F	23.5 ± 6.5	85
	Cl	54.2 ± 6.8	50
	Br	21.1 ± 14.2	13
	I	11.9 ± 2.8	7.0
	Me	7.1 ± 1.9	14
	i-Pr	0.27 ± 0.07	0.34
	OMe	60.9 ± 12.1	22
	NMe_2	1.7 ± 0.1[b]	8.0
	Br	103.5 ± 1.5	88
	Me	71± 4[b]	NR
	i-Pr	66.9 ± 17.2	70
	OMe	287.7 ± 80.2	80
	α-naphthyl	1.8 ± 0.8	0.9
	β-naphthyl	29.8 ± 10.3	14
	3,5-dimethyl	12.53 ± 3.8	6

[a]Data are mean ± SEM (n = 3–7 unless otherwise stated). [b]Value reported is from a single measurement. [c]Values from Metcalfe 1965 reference.[12]

reliable data set to work with. Using literature values and the provided substituent parameters, students derived the Hansch equation, eq 2

$$\log\left(\frac{1}{C}\right) = -0.48\pi^2 - 0.10\pi - 0.03\sigma + 0.08\mathrm{MR} - 2.21 \quad (2)$$

Students entered substituent parameters into this equation for each carbamate to generate predicted IC_{50} values. These were compared with literature values using a scatter plot (Figure 5). On the basis of the obtained goodness of fit (R^2 = 0.94), students concluded that the derived Hansch equation was able to predict the activity of the AChE inhibitors adequately based on their substituent parameters alone.

SUMMARY

The principle pedagogic goal of the experiment was to expose students to the full spectrum of activities relevant to drug development, with an emphasis on increasing understanding of structure–activity relationships. This goal was met through the 3-week experiment (3 sessions of 3 h) traversing the synthesis of the drug (week 1), testing the inhibitory activity of the drug (week 2), and undertaking a structure–activity relationship analysis using class and literature data (week 3). The achievement of this goal was evidenced by strong student performance in the associated practical report (for further details see tutor guide) and the final examination in the sections on structure–activity relationships.

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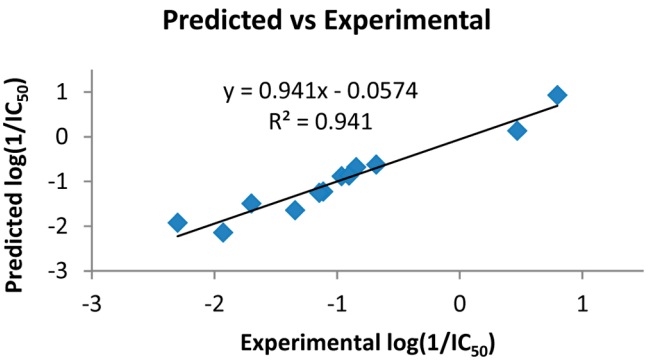

Figure 5. Example of a student-derived plot of predicted and experimental IC_{50} values for the 3-substituted carbamate AChE inhibitors.

This series was a unique and comprehensive introduction into all stages of drug design. Although a number of previously reported undergraduate laboratory experiments may deal with a subset of aspects in this process, such as the synthesis of drug-like molecules,[15,16] the use of in vitro assays to determine activity[9,10,17] or computational methods to guide drug design,[4,13,14,18,19] to the best of the authors knowledge, there have been no previous reports of a single series that effectively covers all of these aspects in such a comprehensive and integrated manner. This method is robust and reproducible, with students typically obtaining results that were within an order of magnitude of literature values.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00270.

Student handouts and tutor materials. (XLSX)
Student handouts and tutor materials. (PDF)
Student handouts and tutor materials. (PDF)
Student handouts and tutor materials. (DOCX)
Student handouts and tutor materials. (PDF)
Student handouts and tutor materials. (DOCX)

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Notes

The authors declare no competing financial interest.

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