The course Drug Discovery (CHEM-4330) was initiated at Rensselaer Polytechnic Institute in the 2001 spring semester as part of a Howard Hughes Medical Institute-funded curriculum development of an undergraduate bioinformatics–molecular biology major. Drug Discovery (DD) is a required course for that major and serves as an elective for chemistry, biochemistry–biophysics, biology, chemical engineering, and related majors. Undergraduates take DD in their junior or senior years; two semesters of sophomore organic chemistry are prerequisites. Graduate students in chemistry, biology, and other disciplines also participate.

The goal of the course is to study applications of bioinformatics and genomics to the discovery of new drugs. DD is divided into a lecture component (1 hour 20 minutes, twice weekly) and a weekly three-hour laboratory. The objective of the lectures is to examine how modern biotechnologies are used in research to speed the discovery of new drugs, especially those small molecules to treat diseases with large unmet therapeutic need (e.g., cancer). Topics include high-throughput screening (HTS), combinatorial chemistry and molecular target (MT) validation using proteomics, and DNA and protein microarrays. Case studies such as the discovery of captopril (1–5) and imatinib (6) are interspersed through these lectures to illustrate successful approaches.

The objective of the lab component is to reduce to practice some of the HTS procedures used by researchers to discover drugs. Criteria used to select a MT for this lab were the following:

1. High relevance to human disease or to the discovery of useful drugs.
2. MT is amenable to 96-well format bioassay (e.g., no wash or filter steps) using absorbance or fluorescence microplate readers and can be accomplished in the three hours allotted for the lab.
3. MT is commercially available and relatively stable.
4. Inhibitors of the MT are commercially available or easily made.
5. X-ray crystal structures of MT-inhibitor complexes are available to students to rationalize the elements of molecular recognition and to develop a pharmacophore hypothesis.

The enzyme, carboxypeptidase A (CPA; EC 3.4.17.1; ref 7), is ideally suited for our purpose. CPA fulfilled the criteria noted above including the fact that Squibb scientists used CPA-related biochemistry and inhibition in the 1970s to discover captopril (Figure 1), the first clinically useful inhibitor of angiotensin converting enzyme (ACE; refs 1–5). Captopril and successor agents are important components of the physician's war chest to treat hypertension (1).

The case study of captopril is considered a "classic" because it is one of the first successful applications of rational drug design. In this approach, the structure of the natural substrate and the molecular mechanism of catalysis were used to aid the design of inhibitors. The Squibb team also recognized the close structural and biochemical relationship between ACE and the well-studied enzyme CPA; both are Zn-metalloproteinases that cleave an amide bond releasing a dipeptide (ACE) or single amino acid (CPA) from the C-terminus of substrate peptide. Using 2-benzylsuccinic acid (Figure 1), a byproduct inhibitor of CPA (8), the structure of angiotensin I (decapeptide substrate for ACE) and a snake venom nonapeptide ACE inhibitor screening hit, Squibb scientists discovered captopril in rapid fashion.

With a strong rationale in hand for using 2-benzylsuccinic acid inhibition of CPA as a DD lecture tool, we developed an appropriate assay to determine its inhibition...
potency in 96-well format to illustrate how HTS is used in modern drug discovery to identify bioactive molecules. Numerous absorbance (8, 9) and fluorescence (10) assays for CPA inhibition have been reported; however, we could not find reference to any performed in 96-well format. We report our efforts in developing a colorimetric 96-well plate assay for determination of the \( K_i \) for inhibition of CPA by (±)-2-benzylsuccinic acid using Ac-Phe-Thiaphe-OH (Figure 1) as substrate.

### Reagents and Equipment

The reagents used in this experiment are commercially available and described in the Supplemental Material. Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) and were used without further purification. Precautions were taken during handling the reagents used in digestion and (±)-2-benzylsuccinic acid. Students used a hood and wear gloves and goggles when handling these and other reagents. MSDS sheets of inhibitors of CPA, students use a hood and wear gloves and goggles when handling these and other reagents. MSDS sheets of all reagents are made available to students.

### Experimental Procedure

A known procedure (11) performed in cuvettes (final volume 1 mL) for determination of the Michaelis constant \( (K_m) \) of Ac-Phe-Thiaphe-OH was first modified for 96-well plate format. We then developed a new 96-well assay to determine the \( K_i \) for inhibition of CPA by performing kinetic experiments in the presence of various concentrations of (±)-2-benzylsuccinic acid.

Following grading of the prelab, students worked individually or in groups of two or three per plate, depending on the size of that lab section. A multichannel pipettor was used for addition of enzyme; standard single channel or repeater pipettor was used at the student’s discretion. Because adding enzyme in the last step initiated the reaction, students had to be very organized (i.e., plate reader reserved and software ready to go) before this last addition step. Assays were run in duplicate with the total volume of each well equal to 200 µL. The layout of wells, instrument settings, and order of addition are found in the Supplemental Material. Formulas used by the software to generate \( K_m \) and \( K_i \) values were preprogrammed into each group table; however, students had to understand how each formula was derived.

After 25 min of plate reader time, students removed their raw data *pda file to allow the next group to use the plate reader. Students then analyzed and refined the data (deleting an outlier well from the analysis), saving the *pda file under a different name if it was modified in any way. Printouts of raw and refined (if needed) data *pda file(s) were included in their final report along with discussion.

### Hazards

Since CPA is a pancreatic-derived mammalian enzyme used in digestion and (±)-2-benzylsuccinic acid is a potent inhibitor of CPA, students use a hood and wear gloves and goggles when handling these and other reagents. MSDS sheets of all reagents are made available to students.

### Results and Discussion

The Lineweaver-Burk plots generated by the software from raw data of an actual student-run experiment are shown in Figure 2. As would be expected from an enzyme–substrate–inhibitor system that displays Michaelis–Menten kinetics (11) and competitive inhibition (8), all plots in Figure 2 converge on the y axis at the nearly the same point (x = 0). The intersection of the plots on the x axis \((1/[S])\) is related to the \( K_m \) (see Supplemental Material for all formulas). For plot 1, \( K_m \) is the same as the \( K_m \) of substrate since \([I] = 0 \mu M \) (Table 1). For plots 2–4, each \( K_i \) was calculated from the corresponding \( K_m \) and \([I] \). Results are shown in Table 1. Despite the fact that this experiment was performed only once by this student, low variability \((R^2 = .946–.995)\) was observed and these data are in excellent agreement with literature values of \( K_m = 0.22 \mu M \) (11) and \( K_i = 1.1 \mu M \) (8).

Besides being commercially available and stable, a major advantage of using Ac-Phe-Thiaphe-OH as substrate is the fascinating organic chemistry involved in the indirect detection of hydrolysis products at 405 nm (11). Detection in the visible region is preferred because inexpensive polystyrene 96-well plates (versus UV transparent plates) can be used. As part of the prelab (see Supplemental Material), students are asked to provide a scheme for all reactions involved in substrate hydrolysis, including release and breakdown of the colorless “unstable amine” product and subsequent detection with Ellman’s reagent (Figure 1) to give yellow Ellman’s anion.

### Table 1. Linear Fit of the Lineweaver-Burk Plots Shown in Figure 2

<table>
<thead>
<tr>
<th>Plot</th>
<th>([I]) µM</th>
<th>A</th>
<th>B</th>
<th>(R^2)</th>
<th>(K_i) µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.134</td>
<td>0.028</td>
<td>.946</td>
<td>210</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.144</td>
<td>0.055</td>
<td>.975</td>
<td>0.61</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.150</td>
<td>0.085</td>
<td>.995</td>
<td>0.59</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>0.134</td>
<td>0.139</td>
<td>.951</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*Plots fitted to \( y = A + Bx \)

*K\(_m\)

*K\(_i\)
Conclusions

In this experiment, students learn principles and techniques used by drug discovery HTS researchers including enzyme inhibition, Michaelis-Menten kinetics, Lineweaver-Burk plots, use of microplate readers and associated software, layout of bioassays, and multichannel and repeater pipetting. Because of the close ties to the lecture and the use of modern equipment, we find that experiments in DD genuinely ignite students' interest in biomedical science and enable them to make more informed decisions about their career paths. When working in groups and sharing common goals, they learn firsthand the benefits of teamwork, something that is a necessity in drug discovery research. We also observe a healthy sense of competition among groups to generate the best plots, values, et cetera. We plan to implement experiments where students resolve (±)-2-benzylsuccinic acid into its enantiomers and conduct inhibition assays to assess enantioselectivity of binding and inhibition.

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Supplemental Material

Prelab instructions, guidelines for lab reports, questions for students, notes for instructors, and printouts of sample *.pda files are available in this issue of JCE Online.

Literature Cited