Letters

Identification of Promiscuous Small Molecule Activators in High-Throughput Enzyme Activation Screens

David R. Goode,[†] Ryan K. Totten,[†] James T. Heeres,[‡] and Paul J. Hergenrother*,[†,‡]

Departments of Chemistry and Biochemistry, Roger Adams Laboratory, University of Illinois, 600 S. Mathews Avenue, Urbana, Illinois 61801
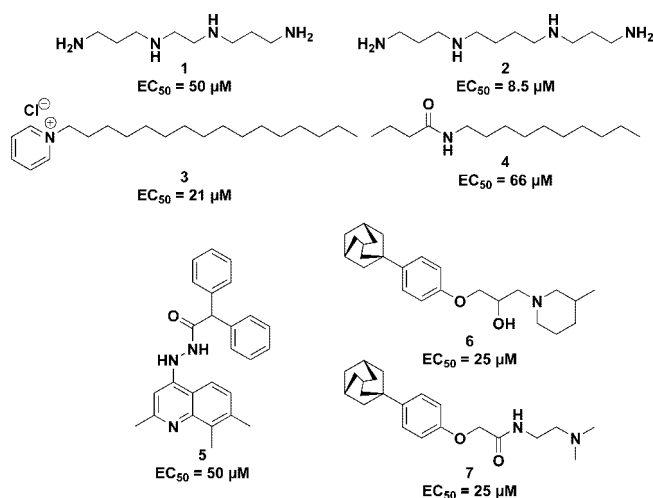
Abstract: It is recognized that high-throughput enzyme inhibition screens often return nonspecific inhibitors as "hits". Recently, high-throughput screens for enzyme activators have led to the identification of several compounds with novel and potent biological activity. Here, we show that enzyme activation screens can also uncover compounds that activate multiple enzymes in a nonspecific fashion. Described herein are the general structural features of such compounds and methods to differentiate between specific and general enzyme activation.

High-throughput screening (HTS) is now a major method for the discovery of new small molecule leads.[1–3] It is increasingly recognized that in high-throughput enzyme inhibition assays some compounds will inhibit the enzyme of interest in a nonspecific fashion.[4] This promiscuous inhibition appears to be the result of compound aggregation into colloidal particles.[5,6] The nonspecific inhibition effect is well documented and has allowed for the early identification of potential aggregators via the development of structural filters.[7] Importantly, there now exists a battery of experiments that can be used to distinguish specific from nonspecific enzyme inhibition.[8,9]

Although high-throughput screens for the identification of enzyme inhibitors comprise the majority of HTS efforts, there is a growing interest in enzyme activation assays, where small molecules that "turn on" enzymatic activity are sought. These can be compounds that replace biological ligands that are known to enhance enzymatic activity, or they can be compounds that enhance enzyme/proenzyme activity for which there is no known endogenous biological activator. For instance, the activation of glucokinase to alter glucose homeostasis has been proposed to treat type 2 diabetes,[10] and multiple allosteric activators are currently being studied.[11] Similarly, small molecule activators of soluble guanylate cyclase are also being pursued to control cGMP signaling.[12] HTS campaigns were used to discover novel activators of RNase L[13] and SIRT1.[14] Finally, a small molecule activator of procaspase-3 (called PAC-1) was recently identified through HTS; PAC-1 enhances the activity of procaspase-3 in vitro and induces apoptosis in cancer cells in cell culture and in vivo.[15]

On the basis of these and other success stories, the use of HTS for identification of compounds that enhance the activity of specific enzymes will likely increase. As such enzyme activation screens become more prevalent, it will be important

* To whom correspondence should be addressed. Phone: (217) 333-0363. Fax: (217) 244-8024. E-mail: hergenro@uiuc.edu.
  † Department of Chemistry.
  ‡ Department of Biochemistry.

Figure 1. Structures of hits and their potency in the procaspase-2 activation assay.

to determine if certain compounds induce a nonspecific activation effect, analogous to the effect of nonspecific inhibitors. A brief mention of such an activation effect was noted during a high-throughput screen for promiscuous enzyme inhibitors, although the effect was attributed to low-volume conditions of HTS formats.[16] Here, we document compounds that show promiscuous enzyme activation; as described below, these compounds were initially discovered during a high-throughput screen for procaspase-2 activation.

Compounds from an in-house library[17] (~22 000 compounds) were screened for their ability to increase the catalytic activity of procaspase-2. Procaspase-2 was incubated with compound (~20 μM) for 16 h at 37 °C in 384-well plates. At this point, the known caspase-2 substrate *N*-acyl-Val-Asp-Val-Ala-Asp-*p*-nitroanalide (Ac-VDVAD-pNA)[18] was added and the absorbance at 405 nm was monitored for 2 h. Compounds were considered hits if they induced an activity greater than 2 standard deviations above the average of vehicle treated controls. This screen produced 147 hits, a hit rate of 0.7%. Through secondary evaluations in the same assay, it was determined that seven compounds enhanced procaspase-2 enzyme activity in a dose-dependent manner and with reasonable potency. Potency was evaluated by the concentration required to induce half-maximal activation; the seven compounds shown in Figure 1 have EC_{50} values for procaspase-2 activation in the low micromolar or mid-micromolar range.

Several hits from this screen showed very unusual structural features, with some having polyamine structures (**1** and **2**) and others appearing surfactant-like (**3** and **4**). The remaining library hits (**5**–**7**) showed more druglike characteristics, two of which (**6** and **7**) had considerable structural similarity. Thus, further chemical derivatization was focused on compounds such as **6** and **7**, with the goal of identifying even more potent activators of procaspase-2.

The synthesis of **6** commenced with the functionalization of epichlorohydrin to generate epoxide **8**, followed by amine opening of the epoxide to provide **6** (Scheme 1). The efficient synthesis of **6** inspired a parallel synthesis strategy whereby

Scheme 1. Synthesis of **6**
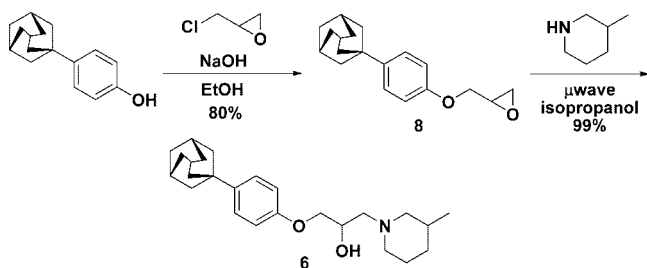
adamantyl-linked epoxides **8** and **9** were crossed with 42 different amines to provide an 84-member library (Scheme 2). The library members were synthesized in an average yield of 63% to give an average mass of 19.5 mg after purification by SiO$_2$ column chromatography. After this purification, all final products appeared as a single spot upon analysis by thin-layer chromatography (see Supporting Information Figure S1).

Interestingly, upon evaluation in the procaspase-2 activation assay, none of the derivatives were found to be more potent activators than the parent **6**, although the vast majority of the compounds retained the ability to enhance the activity of procaspase-2.

Compounds found to enhance the activity of procaspase-2 were generally surfactant-like, containing a charged headgroup and a hydrophobic tail. This observation and the inability to identify more potent compounds from the parallel synthesis library led us to suspect that the activation may be occurring through a nonspecific mechanism. To investigate the general effect of these compounds on enzyme activity, we investigated the ability of the original seven hits in Figure 1 to activate another protease, chymotrypsin. The activity of chymotrypsin was monitored by cleavage of the *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanalide (Suc-AAPF-pNA) substrate.[19] Known surfactants (CHAPS, Triton-X, Tween-20, and SDS) were also investigated for their ability to enhance the activity of this enzyme. The

surfactants and surfactant-like compounds showed activation of chymotrypsin. The effect of different concentrations of CHAPS, **6**, and **7** on chymotrypsin activity is shown in Figure 2A, and the activity vs time progress curves for **7** are shown in Figure 2B. The activating effect of surfactants on enzymatic activity has been previously documented and is thought to be due to prevention of oligomerization or loosening of protein secondary structure.[20] This effect could also be due to the surfactant-like molecules masking hydrophobic patches on the protein surface, as has been seen previously for CHAPS.[21]

In order to investigate the mechanism of activation by the HTS hits, the chymotrypsin assay was repeated with 0.1% (w/v) CHAPS added to the assay buffer. In all cases, the addition of CHAPS abrogated the activation conferred by the HTS hits. Maximal enzymatic activity was obtained when CHAPS was added to the buffer, regardless of the presence of the HTS hits; this is seen when comparing the highest activation of a HTS hit to the activity of enzyme alone in the CHAPS buffer (shown for **7** in Figure 2B,C). This nonadditive effect of CHAPS with the compounds points to their behaving in a similar manner, likely as surfactants in the buffer.

The generality of the nonspecific activation phenomenon was further examined by assaying multiple enzymes with different catalytic activities: β-lactamase, β-galactosidase, horse radish peroxidase, alkaline phosphatase, trypsin, and caspase-3. Standardized assay conditions were used for most enzymes, namely, 50 mM phosphate buffer, pH 7.0, and known substrates (see Supporting Information for assay details). CHAPS was also included in the compound set as the known surfactant control. As shown in Table 1, surfactant activation is not seen for every enzyme, and some of the compounds identified herein actually inhibit the enzymes as the compound concentrations approach the solubility limit in the specified assay buffer (see Supporting Information Figure S4). However, every enzyme that was activated by CHAPS was also activated by some HTS hits, supporting the notion of a nonspecific surfactant effect for these

Scheme 2. Parallel Synthesis of Derivatives of Compound **6**
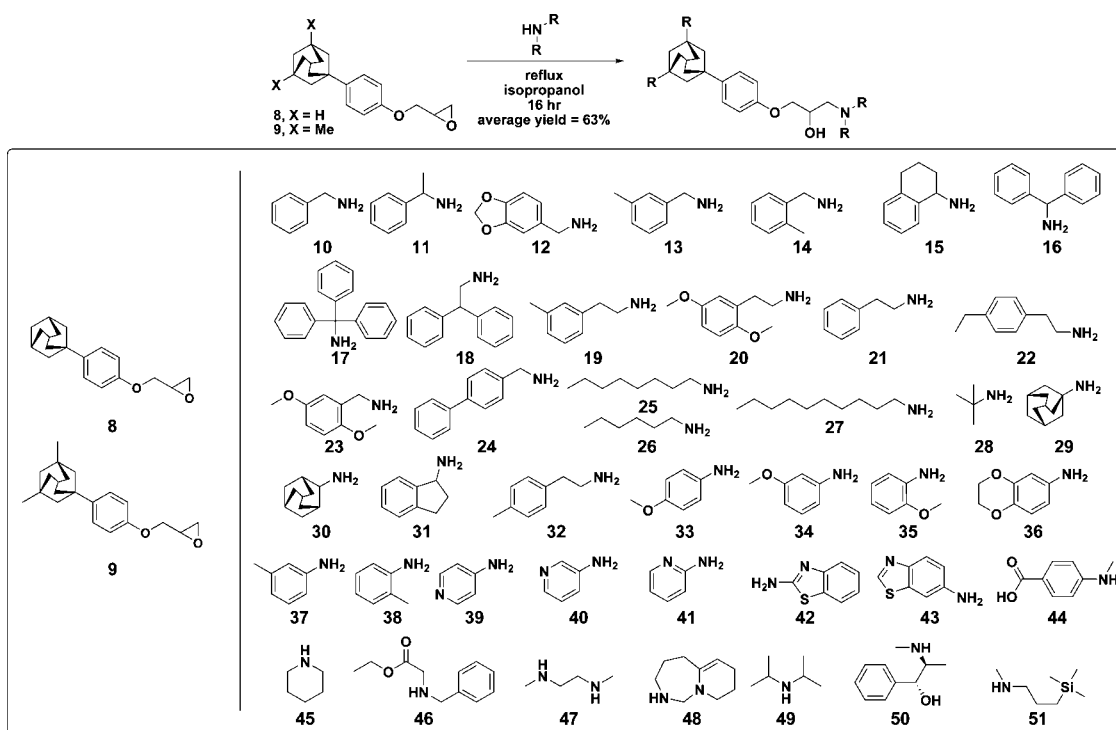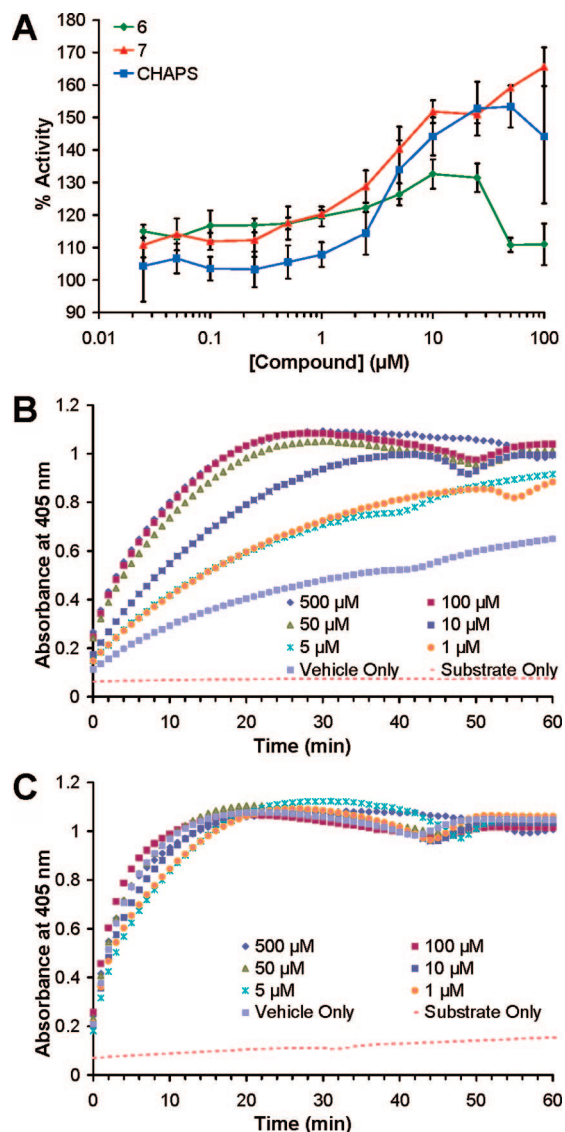
Figure 2. Activation of chymotrypsin by small molecules: (A) representative dose response activation of chymotrypsin by **6**, **7**, and CHAPS, where error bars represent standard deviations from the mean; (B) progress curves showing activation of chymotrypsin by **7** in phosphate assay buffer; (C) progress curves showing no activation of chymotrypsin by **7** in phosphate assay buffer + 0.1% CHAPS.

Table 1. Activation of Enzymes by **1**–**7** and CHAPS[a]

enzymes	1	2	3	4	5	6	7	CHAPS
procaspase-2	+	+	+	+	+	+	+	+
chymotrypsin	−	+	+	+	−	+	+	+
β-lactamase	−	−	+	+	+	+	+	+
β-galactosidase	−	−	−	−	−	−	−	−
HR peroxidase	−	−	−	−	−	−	−	−
alkaline phosphatase	+	−	−	−	−	−	−	−
trypsin	−	−	+	−	+	+	−	+
caspase-3	+	+	−	−	−	+	+	+

[a] Minus sign denotes no effect or inhibition, and plus sign denotes activation. See Supporting Information for full activation curves for each compound and enzyme.

compounds. This data are consistent with a report by Shoichet and co-workers, whereby multiple compounds in a large high-throughput screen (70 000) showed enzyme activation that was detergent-sensitive.[16] Importantly, addition of BSA to the assay buffer also prevents chymotrypsin activation by CHAPS and **7** (see Supporting Information Figure S5). Of the enzymes surveyed thus far, trypsin, caspase-3, chymotrypsin, and β-lac-

tamase appear to be the most sensitive to this surfactant activation (Table 1; see Supporting Information Figure S4 for full activation curves for each compound and enzyme).

These results show the presence of promiscuous activators in HTS libraries, which pose a danger as nuisance compounds in future high-throughput enzyme activation screens. A search of PubChem reveals that **1**–**3** and **5**–**7** have been tested in multiple high-throughput screens, and several of these compounds are members of commercial screening libraries. The structural features of the promiscuous activators identified herein are the same as those of surfactants: a polar or charged headgroup and a large hydrophobic tail. However, these structural features may be embedded within more druglike compounds, as is the case for **6** and **7**. In addition, these compounds induce activation at concentrations relevant in high-throughput screens (low micromolar), which is not always the case for classical surfactants. While this activation effect is not completely general across all enzyme classes, similar compounds will likely appear as hits in other enzyme activator screens, and thus, any small molecule enzyme activator should be examined for this nonspecific effect.

The promiscuous activation may be due to some combination of enzyme stabilization in solution, loosening of the protein secondary structure, or alteration of adsorption of the enzymes to surfaces that they make contact with during the course of the experiments. To investigate the latter possibility, an experiment examining the effect of preincubation time on chymotrypsin activity was performed. The results from this experiment show that simple incubation of chymotrypsin in a 384-well plate for 1 h causes the enzyme to lose significant activity (Supporting Information Figure S6). This effect is abrogated, however, by the addition of CHAPS or **7**. Thus, it appears that CHAPS and **7** allow enzymes to retain their full activity, at least in part by preventing their adsorption onto surfaces commonly used in enzymatic assays.

Shoichet has recommended the inclusion of detergents in primary HTS for enzyme inhibition to eliminate the identification of compounds that inhibit enzymes through formation of colloidal aggregates.[8] As shown herein, when HTS for enzyme activators is conducted, the inclusion of detergent (or BSA) in the enzyme assay buffer should also reduce the number of "hits" that are actually nonspecific activators. However, time is a variable in these experiments as well. For example, we have found that in the presence of CHAPS, **7** will not enhance the activity of procaspase-2 when incubated for short (30 min) times. When **7** is incubated with procaspase-2 for long times (16 h), however, enhanced activity is observed, even in the presence of CHAPS.

Described herein is the unexpected discovery of general enzyme activators in HTS assays. Also described are facile methods for identification of such promiscuous activators, namely, counter-screening hits against a readily available protease (such as chymotrypsin) and screening for elimination of compound activation by addition of a detergent (such as CHAPS) to the assay buffer. These experiments have allowed us to authenticate that the compounds in Figure 1 are promiscuous enzyme activators. Importantly, similar experiments have confirmed that one reported enzyme activator, PAC-1, does not act through this nonspecific mechanism (see Supporting Information Figure S7). Analogous to promiscuous inhibitors,[7] a computational filter set could be developed to flag possible promiscuous activators among HTS collections. Avoiding promiscuous activators by these simple screens or filters will save time and effort in future HTS endeavors.

Supporting Information Available: Experimental details, including biochemical assay conditions, compound synthesis, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pereira, D. A.; Williams, J. A. Origin and evolution of high throughput screening. *Br. J. Pharmacol.* **2007**, *152*, 53–61.

(2) Carnero, A. High throughput screening in drug discovery. *Clin. Transl. Oncol.* **2006**, *8*, 482–490.

(3) Posner, B. A. High-throughput screening-driven lead discovery: meeting the challenges of finding new therapeutics. *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 487–494.

(4) Shoichet, B. K. Screening in a spirit haunted world. *Drug Discovery Today* **2006**, *11*, 607–615.

(5) McGovern, S. L.; Helfand, B. T.; Feng, B.; Shoichet, B. K. A specific mechanism of nonspecific inhibition. *J. Med. Chem.* **2003**, *46*, 4265–4272.

(6) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* **2002**, *45*, 1712–1722.

(7) Seidler, J.; McGovern, S. L.; Doman, T. N.; Shoichet, B. K. Identification and prediction of promiscuous aggregating inhibitors among known drugs. *J. Med. Chem.* **2003**, *46*, 4477–4486.

(8) Feng, B. Y.; Shelat, A.; Doman, T. N.; Guy, R. K.; Shoichet, B. K. High-throughput assays for promiscuous inhibitors. *Nat. Chem. Biol.* **2005**, *1*, 146–148.

(9) Feng, B. Y.; Shoichet, B. K. A detergent-based assay for the detection of promiscuous inhibitors. *Nat. Protoc.* **2006**, *1*, 550–553.

(10) Grimsby, J.; Sarabu, R.; Corbett, W. L.; Haynes, N. E.; Bizzarro, F. T.; et al. Allosteric activators of glucokinase: potential role in diabetes therapy. *Science* **2003**, *301*, 370–373.

(11) Guertin, K. R.; Grimsby, J. Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. *Curr. Med. Chem.* **2006**, *13*, 1839–1843.

(12) Evgenov, O. V.; Pacher, P.; Schmidt, P. M.; Hasko, G.; Schmidt, H. H.; et al. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat. Rev. Drug Discovery* **2006**, *5*, 755–768.

(13) Thakur, C. S.; Jha, B. K.; Dong, B.; Das Gupta, J.; Silverman, K. M. Small-molecule activators of RNase L with broad-spectrum antiviral activity. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9585–9590.

(14) Milne, J. C.; Lambert, P. D.; Schenk, S.; Carney, D. P.; Smith, J. J.; et al. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* **2007**, *450*, 712–716.

(15) Putt, K. S.; Chen, G. W.; Pearson, J. M.; Sandhorst, J. S.; Hoagland, M. S., et al. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. *Nat. Chem. Biol.* **2006**, *2*, 543–550.

(16) Feng, B. Y.; Simeonov, A.; Jadhav, A.; Babaoglu, K.; Inglese, J., et al. A high-throughput screen for aggregation-based inhibition in a large compound library. *J. Med. Chem.* **2007**, *50*, 2385–2390.

(17) Hergenrother, P. J. Obtaining and screening compound collections: a user's guide and a call to chemists. *Curr. Opin. Chem. Biol.* **2006**, *10*, 213–218.

(18) Talanian, R. V.; Quinlan, C.; Trautz, S.; Hackett, M. C.; Mankovich, J. A., et al. Substrate specificities of caspase family proteases. *J. Biol. Chem.* **1997**, *272*, 9677–9682.

(19) DelMar, E. G.; Largman, C.; Brodrick, J. W.; Geokas, M. C. A sensitive new substrate for chymotrypsin. *Anal. Biochem.* **1979**, *99*, 316–320.

(20) D'Auria, S.; Di Cesare, N.; Gryczynski, I.; Rossi, M.; Lakowicz, J. R. On the effect of sodium dodecyl sulfate on the structure of beta-galactosidase from *Escherichia coli*. A fluorescence study. *J. Biochem. (Tokyo)* **2001**, *130*, 13–18.

(21) Gall, A. L.; Ruff, M.; Moras, D. The dual role of CHAPS in the crystallization of stromelysin-3 catalytic domain. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2003**, *59*, 603–606.

JM701583B