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14. ABSTRACT

We have synthesized a library of compounds based on a previously identified procaspase-2 activator. These compounds were then tested for their ability to activate procaspase-2 in vitro. The results from these tests indicated that several of the compounds did indeed activate procaspase-2. Testing of these active compounds against a panel of enzymes then revealed, surprisingly, that the compounds are general enzyme activators. We have now explored this phenomena of general enzyme activation more thoroughly and have submitted a manuscript on the topic. The data and knowledge acquired through this work now allows us to move confidently to a high-throughput screen to identify truly specific procaspase-2 activators.

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High-throughput screening, proenzyme activation

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Introduction

The goal of this research was to identify compounds that selectively activate a proapopotic protein (called procaspase-2) in breast cancer cells, which was hypothesized to lead to cell death. Due to the crucial position of caspases in the apoptotic pathway, their activation would likely ignite the apoptotic signal, leading to cellular death. As an initiator caspase, procaspase-2 activation would likely lead to apoptosis induction. Procaspase-2 has been shown to be activated to caspase-2 when recruited to a protein complex of RAIDD and PIDD through its CARD domain.(Tinel and Tschopp, 2004) Once activated, caspase-2 has been shown to cleave caspase-8, which ignites other caspases leading to apoptotic death.(Lin et al., 2004) Caspase-2 has also been shown to cleave Bid, which leads to mitochondrial depolarization by freeing pro-apoptotic Bcl-2 protein Bax.(Bonzon et al., 2006, Wagner et al., 2004) This activity has been shown in apoptosis induction in media starvation, β-amyloid induced cell death, and DNA damage.(Zhivotovsky and Orrenius, 2005, Troy and Shelanski, 2003) However, caspase-2 null mice show no phenotypic or developmental alterations, pointing to redundant caspases or parallel death induction pathways.(Troy and Shelanski, 2003)

Nonetheless, procaspase-2 activation can lead to apoptosis, and procaspase-2 has been shown to be upregulated in certain cancers. In a screen of the National Cancer Institute cell lines, multiple cell lines from various cancer originations overexpress caspase-2, including breast cancer.(Svingen et al., 2004) Also, caspase-2 was found to be overexpressed in certain leukemia cell lines.(Droin et al., 1998, Fink et al., 2001) With these promising results, procaspase-2 may be a promising target as a personalized cancer treatment.

Promising initial results showed that compounds **1** and **2** (in Figure 1) activated procaspase-2 *in vitro*. In the work, we sought to optimize those compounds through chemical synthesis and to determine the precise mechanism by which these compounds activate procaspase-2. Interestingly and importantly, we found these compounds to be *general* enzyme activators, a result that will have a significant impact on future high-throughput enzyme activation screens. As a result of this discovery, the compounds were not tested for their ability to induce death in breast cancer cell lines; we await the discovery of specific procaspase-2 activators before performing these cell culture experiments. A manuscript describing these results was recently submitted to *J. Med. Chem.* and this data is described below.

Body

As outlined in the Statement of Work, combinatorial libraries of our procaspase-2 activating compounds (compounds **1** and **2** in the initial grant application) were synthesized. The retrosynthetic analysis scheme for these syntheses is shown in Figure 1, below, and the forward scheme that was used to create the compounds is shown in Figure 2.

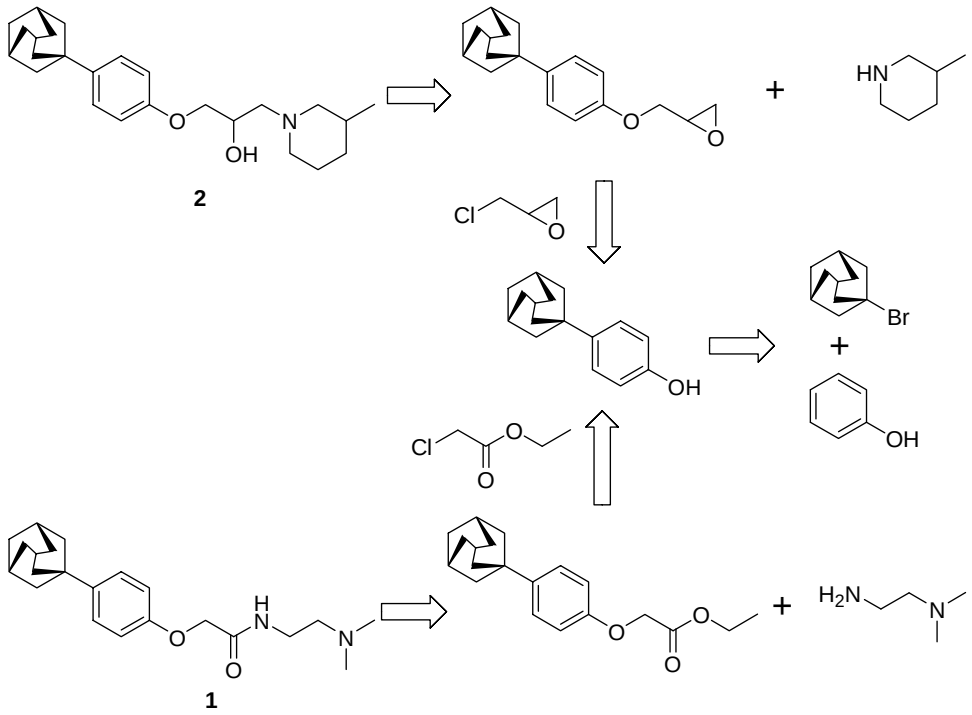

Figure 1. Retrosynthetic analysis of compounds **1** and **2**.

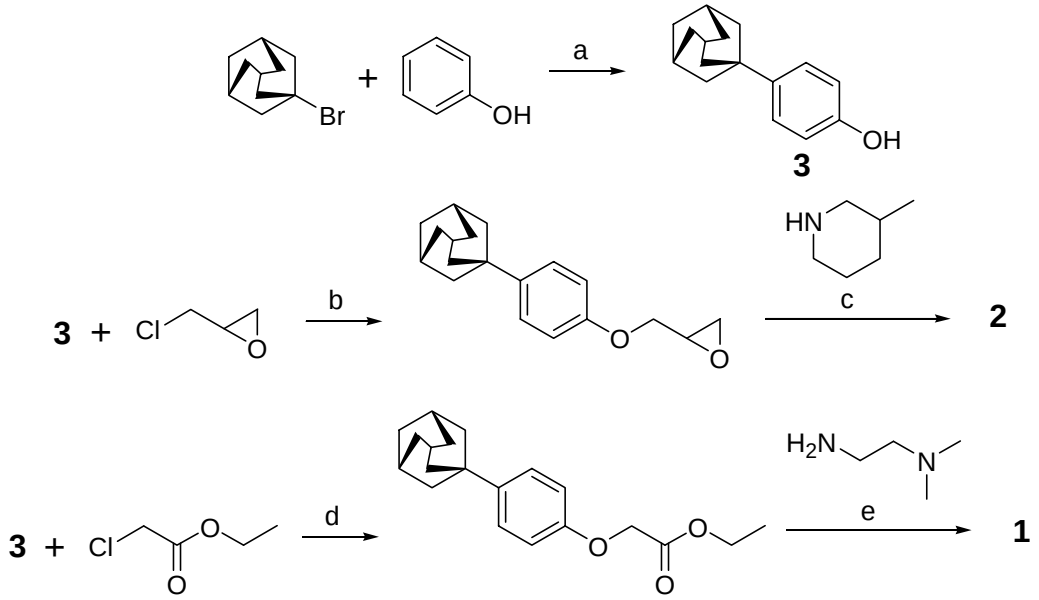

Figure 2. Synthesis of compounds **1** and **2**. a) microwave 300W, 185 °C, 30 min, 95% yield. b) NaOH, ethanol, 80% yield. c) isopropanol, microwave 300W, 110 °C, 30 min, 99% yield. d) K_2CO_3, KI, acetone, reflux, 20 hrs, 65% yield. e) Ti(OiPr)4, HOBt, toluene, 90 °C, 2 hours, 90 % yield.

Based on the highly efficient synthetic scheme shown in Figure 2, a combinatorial library of compounds was constructed in the hopes of identifying even more potent procaspase-2 activators. As the adamantyl motif seemed to be important to procaspase-2

activation, a significant amount of effort was spent attempting to incorporate the adamantyl moiety into a wide variety of compounds. The key reaction for the library was the epoxide opening on the 4-(1-adamntyl)phenyl glycidyl ether by various amines. A second glycidyl ether, in which the adamantyl group was methylated at the 3 and 5 positions, was also included. These two epoxides were synthesized as previously described above on a ~3 g scale. Fourty-four amines were then selected to react with the 2 epoxides, giving a total library with 88 members (Figure 3).

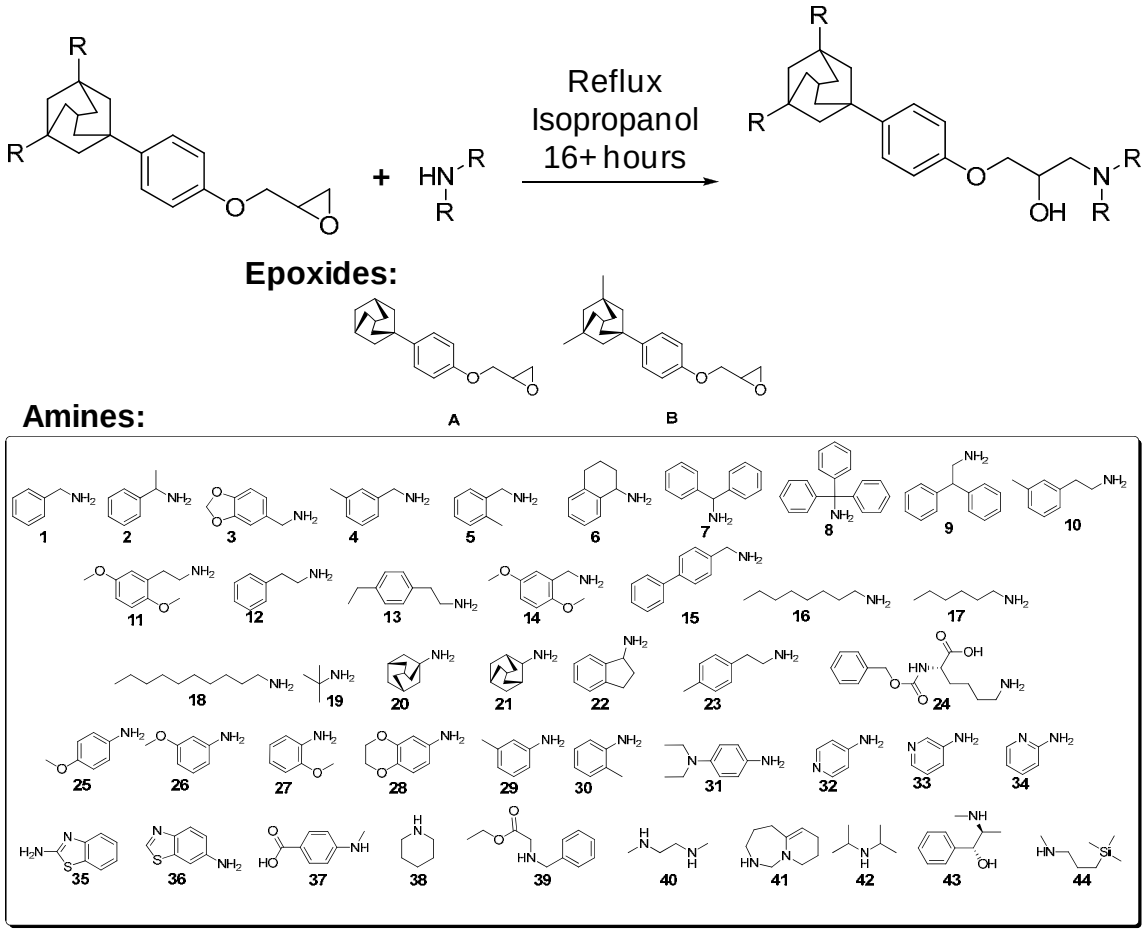

Figure 3. Combinatorial library synthesized based on compounds **1** and **2**.

The library was synthesized in a Buchi parallel synthesizer using the 96 tube platform and 16 x 125 mm tubes. Each amine was added to two tubes followed by a solution of the appropriate epoxide in isopropanol. The platform was then fitted with the condenser block and the airtight caps, and all 88 reactions were heated to 90 °C while shaking at 300 rpm. After 16 hours, the reactions were cooled to 25 °C and monitored by TLC (Figure). The reactions showed complete consumption of the epoxide starting material. All 88 reactions were then concentrated to dryness using the evaporation block of the parallel synthesizer. Each product was then isolated via flash column chromatography, and the purity assessed by TLC and LC-MS. The overall results of the library synthesis are 63% average yield, 95% average purity. A picture of the TLC evaluation of all 88 compounds is shown in Figure 4.

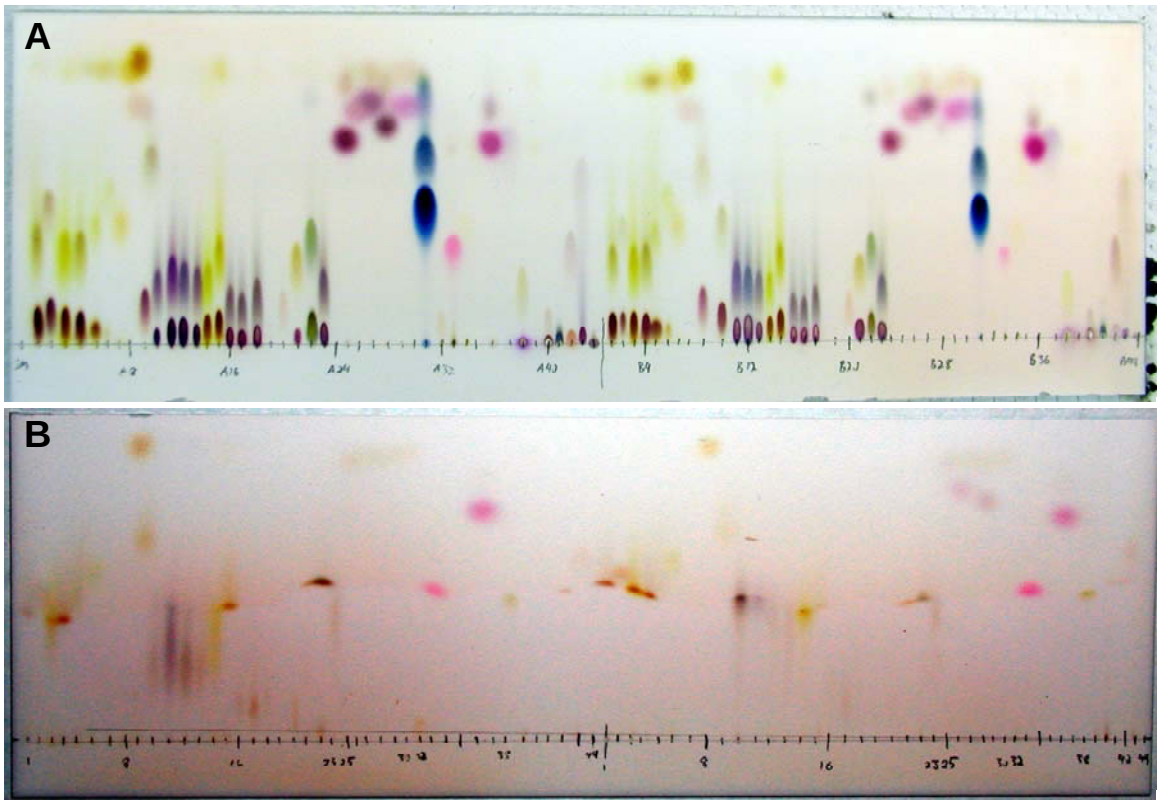

Figure 4. Evaluation of all 88 compounds from Figure 3 by Thin-Layer Chromotography (TLC) (a) as reaction mixtures or (b) after purification, visualized by Ninhydrin stain

The ability of these synthesized derivatives to activate procaspase-2 was then assessed. Procaspase-2 was expressed and purified recombinantly in *E. coli.* The activation assay indicated that several of the derivatives synthesized did indeed activate procaspase-2. The library compounds were assayed with procaspase-2 at 4 different concentrations. Most of the library compounds showed activation of procaspase-2, with only a few being inactive. The inactive compounds were generally composed of aniline-like amines or amines with steric bulk one or two carbons away. Between the two adamantyl derivatives, more of the dimethylated compounds were inactive; however, the differences were slight. Focusing on the dose response of the hits from the four point assay, all the active compounds showed a distinct jump in activation from 10 μM to 50 μM. This would place the EC_{50} of these compounds around 25 μM, in the same range as the parent compounds. Full EC_{50} curves on a ten compound subset of the active compounds verified this result. Thus, it appears that the amine functionality has little effect on the compounds' ability to activate procaspase-2. The inactivity of the aniline like amines might possibly due to a change in pK_A of the amino functionality in the final structure, and thus the charge of the compound under the assay conditions.

Summary of Derivative Results. Over 88 derivates of **1** and **2** were synthesized and assayed for procaspase-2 activation. Many other compounds were found that showed similar activation as the parent compounds, although none were any better. The final SAR shows the requirement for a bulky hydrophobic group opposite a positively charged

functionality. This SAR is very similar to the definition of a surfactant. The similarities of this SAR and the odd features of the other hits led to the question if there is a group of compounds in activation similar to the promiscuous aggregating inhibitors. It was hypothesized that compounds with a detergent-like structure, such as the HTS hits and derivatives, cause general activation of enzymes.

Identification of promiscuous activators in HTS compound collections. Due to these observations, the HTS hits were assayed against an unrelated enzyme to see if the activation effect of the compounds was general. Chymotrypsin, a serine protease that is commonly used in aggregation compound screening, was chosen for the initial off target test. The chymotrypsin enzymatic activity is monitored in a similar fashion as the caspases—via cleavage of a peptide substrate producing free *p*-nitroanaline, which is observable by absorbance at 405 nm. Chymotrypsin recognizes and cleaves after aromatic amino acids, particularly phenylalanine. Thus, the peptide substrate chosen for this assay was *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA).

The assay was carried out in 50 mM potassium phosphate buffer at pH 7.0. Appropriate dilutions, controlled for DMSO content, were placed into wells of a 384 well plate followed by addition of a solution of chymotrypsin in the assay buffer. The compounds and enzyme were incubated at room temperature for 30 minutes prior to addition of Suc-AAPF-pNA substrate solution to give a final concentration of 200 µM. The absorbance at 405 nm was then monitored every minute for 30 minutes. The slopes of the linear portions of the resulting plots were converted to percent activity based upon a vehicle treated control. In order to test the detergent hypothesis, the known detergents CHAPS, succinimide monolaurate, Trition-X, Tween-20, and sodium dodecylsulfate (SDS) were included in the compound set.

The results show that most of the HTS hits along with some of the selected detergents activate chymotrypsin under the assay conditions (Figure 5). Chembridge hit **1** (32-M8) shows inhibition of chymotrypsin, possibly due to its aggregation as mentioned earlier. No effect was seen from the polyamines M3981 or M4836, who lack the typical surfactant structure but maintain some similarities to other detergents. All of the compounds with surfactant-like structures cause activation of chymotrypsin except for SDS, which is known to be a more abrasive detergent. It appears that the effect of the HTS hits is similar to the detergent effects and is not specific to procaspase-2.

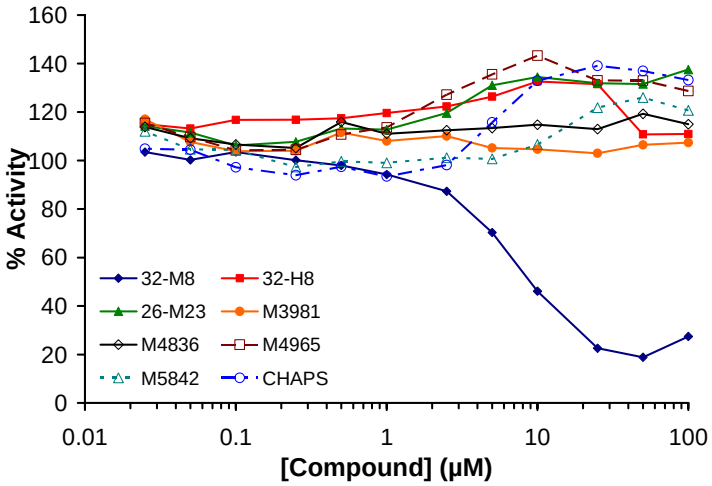

Figure 5: The effect of the HTS compounds and CHAPS on chymptrypsin activity.

Abrogation of Activation by CHAPS. To see if the effects were manifested through a similar mechanism, the chymotrypsin assay was repeated with 0.1% (w/v) CHAPS added to the buffer. If the compounds are acting as detergents, then the addition of the large excess of CHAPS should remove the activation due to saturation. However, if the mechanisms are different, an additive effect would be expected and activation would still be observed. Therefore, the same assay was run above with the HTS hits in buffer containing 0.1% (w/v) CHAPS.

In each case, CHAPS was completely able to remove the activation effect of the compounds (Figure 6). This result confirmed that the compounds were acting similar to detergents. Detergents are generally added to assay buffers in order to add stability to the enzymes in use. The detergents can help solvate the hydrophobic patches of proteins and prevent oligimerization, which can contribute to a loss of overall activity. This was noted in a ST3 crystal structure where CHAPS was found in the crystal interacting with the hydrophobic patch on the protein surface.(Gall et al., 2003) It has also been postulated that detergent stabilization of hydrophobic patches can allow for the loosening of protein secondary structure, which may allow for more efficient catalysis. In either case, the results indicate that the HTS hits act via a similar mechanism as detergents.

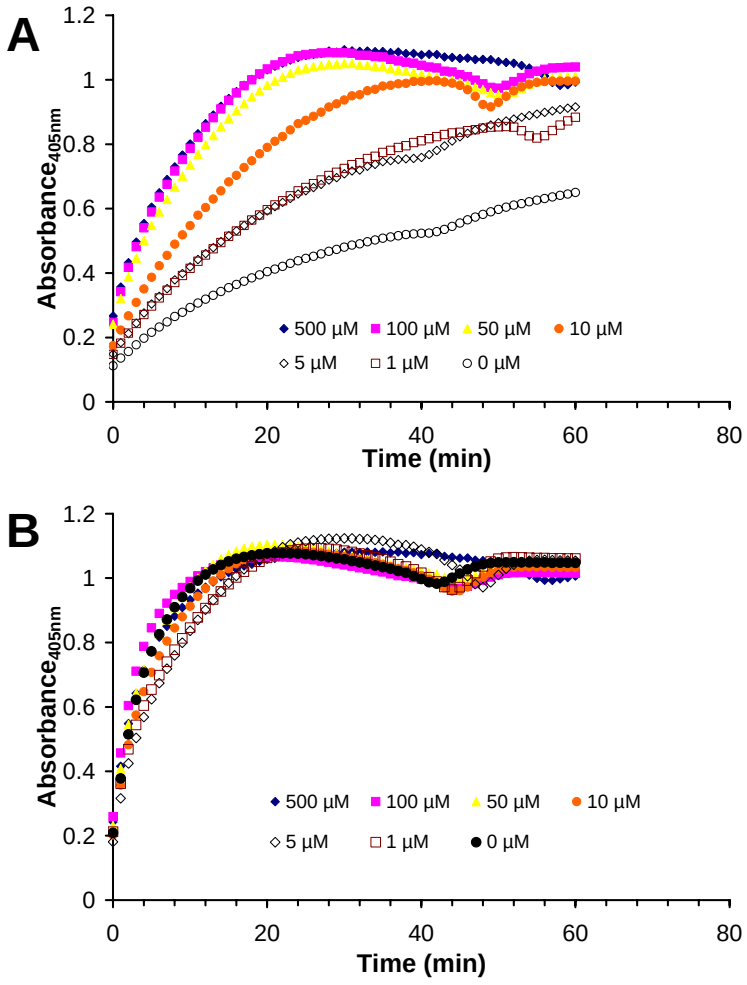

Figure 6. Chymotrypsin progress curves with various **2** concentrations; (a) assay buffer alone, (b) assay buffer with 0.1% CHAPS added.

Screening HTS Hits Against Other Enzymes. In order to see the generality of this activation effect, the HTS hits and CHAPS were tested against different enzymes. The enzymes chosen for examination were caspase-3 (C3), trypsin, β-lactamase (betaLac), β-galactosidase (betaGal), alkaline phosphatase, and horse radish peroxidase (HRP). The enzymes were chosen for their mechanism of catalysis as compared to PC2 and chymotrypsin, as well as their ease of access. All enzymes were tested in 50 µM potassium phosphate buffer at pH 7.0 except for alkaline phosphatase, which required

basic assay conditions. All assays were conducted in a similar fashion to the chymotrypsin assay. The colorimetric substrates used for each enzyme were standard: Ac-DEVD-pNA for C3, N_{α}-benzoyl-L-arginine 4-nitroanilide for trypsin, CENTA for betaLac, 2-Nitrophenyl β-D-galactopyranoside (ONPG) for betaGal, 4-nitrophenyl phosphate for alkaline phosphatase, and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (AzBTS) for HRP. The appropriate dilution of compound in assay buffer (controlled for DMSO content) was placed into each well of a 384 well plate followed by addition of enzyme solution in assay buffer. The enzymes and compounds were incubated at room temperature for 30 minutes prior to addition of the appropriate substrate to give a final concentration of 200 μM. The activity was monitored by measuring the required absorbance for each substrate every minute for 30 minutes. The slope from the linear portion of the resulting graphs was used to compare activities based upon a vehicle treated control.

Table 1: The effect of compounds on various enzymes by derivatives of compounds **1** and **2**. Minus (-) denotes no effect, plus (+) denotes activation, and brackets ([ ]) denote inhibition seen at high concentrations.

	32-M8	**32-H8**	**26-M23**	**M3981**	**M4836**	**M4965**	**M5842**	**CHAPS**
Alkaline Phosphatase	-	-	-	+	-	-	-	-
β-galactosidase	[-]	[-]	-	-	-	-	-	-
β-lactamase	+	+	+	-	-	+	+	+
Horse Radish Peroxidase	[-]	[-]	-	-	-	-	-	-
Chymotrypsin	-	+	+	-	+	+	+	+
Trypsin	+	+	-	-	-	-	-	+
Caspase-3	[-]	[+]	+	+	+	[-]	[-]	+

As seen in Table 1, activation by the HTS hits and CHAPS was not seen for all of the tested enzymes (β-galactosidase, HRP, and alkaline phosphatase not activated). However, every enzyme that was activated by CHAPS was also activated by some HTS hits (C3, trypsin, betaLac, and chymotrypsin), supporting the non-specific detergent effect of the compounds. While this effect is not completely general, the compounds are promiscuous activators and would likely appear as hits in other activator HTSs.

The enzymes tested herein only cover two of the four known protease mechanisms—serine protease and cysteine protease. In both of these mechanisms, an activated nucleophile in the protein active site (serine or cysteine) reacts with the carbonyl of the amide bond, forming a tetrahedral covalent intermediate, which collapses releasing the amine and leaving behind a (thio)ester. This (thio)ester intermediate is then cleaved by water, which is activated by the enzyme as well. The tetrahedral intermediate is also present in the betaLac mechanism, but absent from the other two protease mechanisms. Thus, it remains to be seen if metalloproteases or aspartic proteases, both of which use an activated water as the initial nucleophile, are activated by detergents or

not. Nonetheless, the presence of promiscuous activators among HTS libraries is important information to those screening for specific protease activators.

Promiscuous activator conclusions. During the course of HTS for an activator of procaspase-2, hits were identified with interesting structural features similar to those of detergents. Investigation of the SAR of two of these compounds lead to structural requirements that describe a surfactant and no compounds showing improved activation. This "flat" SAR was similar to compounds found in inhibitor HTS termed promiscuous inhibitors. Thus, the HTS hits were assayed against multiple enzymes for activation, which was only found among serine and cysteine proteases. This promiscuous activator effect was also seen with known detergents, and addition of a known detergent to assay buffer abrogated the compounds' activation effect. Thus, the mechanism of promiscuous activation is most likely through enzyme stabilization or secondary structure relaxation, both of which are known for detergents.

Promiscuous activators are present among HTS libraries, and pose a danger as nuisance compounds in future activator HTS. The structural features of the promiscuous activators are the same as the description of surfactants, a polar or charged head group and a large hydrophobic tail. As with promiscuous inhibitors, a computational filter set could be developed for promiscuous activators. However, in the absence of such filters, promiscuous activators can be identified through counter screening. The best screen would be for activation of an unrelated serine protease, such as chymotrypsin or trypsin. While the effect of promiscuous activators can be abrogated by detergents such as CHAPS or Tween-20, this abrogation is only for short term incubations with the abrogation diminishing over time as seen in the present work for the PC2 screen. Thus, counter screening in the presence of detergents with short incubation times is also a viable test for promiscuous activation. Avoiding promiscuous activators by these simple screens or filters will save time and money in future HTS endeavors.

Detailed procaspase-2 activation experiments were then performed. The evidence now indicates that our procaspase-2 activators are general enzyme activators; that is, they activate many different enzymes in a non-specific manner. This is a very interesting finding, and something that we are now writing a manuscript on. This finding has implications not only for enzyme activation, but also for screens that search for enzyme activators. No compounds were identified that were specific procaspase-2 activators, thus no activity data in cell culture against breast cancer cells was gathered.

Key research accomplishments

We are excited to have discovered some fundamental aspects of enzyme activation, especially as it relates to high-throughput screening. This data will assist us and others that are interested in high-throughput screens for enzyme activators. Specifically, the key research accomplishments are:

- The development of efficient synthetic routes to compounds **1** and **2**.
- The development of an efficient method for the parallel synthesis of derivatives of compounds **1** and **2**
- The implementation of the method above to generate a library of 88 derivatives of compounds **1** and **2**.

- The evaluation of this library in procaspase-2 activation assays.
- The development of the hypothesis that compounds **1** and **2** and their derivatives were *general* enzyme activators.
- The development of methods to test this hypothesis, including the use of orthogonal enzymes such as chymotrypsin, alkaline phosphatase, β-lactamase, etc.
- The discovery that CHAPS will abrogate the activation from a promiscuous activator, but not a specific activator.
- The submission of a manuscript describing, for the first time, the prevalence and identity of small molecule enzyme activators in HTS collections. Also, a methodology for eliminating these false positives was developed and optimized.

Reportable outcomes

We have submitted a manuscript describing this promiscuous activation phenomenon:

Goode, D. R.; Totten, R. K.; Hergenrother, P. J. "The Identification of Promiscuous Small Molecule Activators in High-Throughput Enzyme Activation Screens" submitted to *J. Med. Chem.*

Conclusion

With the information and knowledge that we have gathered through this process, we are now in a good position to identify truly specific procaspase-2 activators. Our original compounds, as it turns out, are promiscuous activators, a phenomenon that has never been described in the literature. We are now set to screen a much larger (~150,000) compound library in the search for specific procaspase-2 activators.

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Appendices

None.