Supporting Information: Label Free Screening of Enzyme Inhibitors at Femtomole Scale Using Segmented Flow Electrospray Ionization Mass Spectrometry

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1. Calibration Curve

10 solutions of the standard assay product (ZRR-OH, >99%, custom synthesized by AnaSpec Inc., Fremont, CA), ranged from 10 μM to 180 μM were prepared in 20% (v/v) methanol and 0.2% (v/v) acetic acid. Different solutions were made into plugs and directly infused into MS. The relative signal abundance (relative peak height) of each sample was used for analyzing how MS response is related to sample concentration. A linear relationship was obtained with R^2=0.991. In addition, no obvious ion suppression between analytes was observed in our experiment. The signal of the assay product was always reversely related to that of the substrate, and both of them were not affected by either samples or byproducts. All experiments were done in this calibration range.

2. Dose Response Curve of Three Cathepsin B Inhibitors

Three known inhibitors of Cathepsin B, which are E-64, leupeptin, and antipain were prepared in a series of aqueous solutions whose concentration ranged from 10^{-7} μM to 10^{3} μM to obtain the dose response relations with Cathepsin B. The final concentration of the substrate ZRR-AMC was 180 μM and Cathepsin B was at 8.1 μg/mL. The quenched reaction mixtures were analyzed using relative abundance of ZRR-OH in its XIC. The results showed sigmoidal relationship between the concentration of the inhibitor and the relative signal intensity of ZRR-OH produced. The IC_{50}s of three inhibitors under our specific experiment conditions were generally agreed with reported values (E-64: 55 nM, leupeptin: 21.3 nM, [1] antipain 0.98 μM)

3. Carry-over measurement

Carry-over of the spray emitter was measured by pumping alternating sample/blank droplets (sample droplets contained 200 μM ZRR-OH dissolved in 20% methanol and 0.2% acetic acid ESI buffer, blank droplets only contained the buffer) through the emitter tip to ESI-MS. The signal intensity of each droplet was used for analysis. < 1% carry-over occurred in the spray emitter, which was in agreement with previous results.[2] (**Figure S-3A**)

Carry-over of the PDMS tee for one addition was measured by adding quenchant (98% methanol and 2% acetic acid) into 10 nL alternating sample/blank droplets (the sample was 200 μM ZRR-OH water solution, and the blank is water), where we observed ~ 9% of carry-over. 2 additions were conducted by adding water, and then adding quenchant into the

same alternating droplets. The carry-over increased to 16% with 2 additions. In results of the real screening, 3 additions led to more than 25% of carry-over (**Figure 4B**).

We also measured the carry-over of the Teflon tee for one addition. Same quenchant was added into alternating droplets in which sample droplets contained 200 μM Ac-GFGFVGG-NH_2 and blank droplets contained water. The carry-over could be as low as 2% (**Figure S-3B**).

4. Sequence of test compounds in screenings.

(1) In-well Cathepsin B inhibitor screening using ZRR-AMC as substrate

The sequence of 23 test compounds and a non-inhibitor control is E-64, leupeptin, antipain, control, methionine, arginine, tyrosine, histamine, thyronine, proline, lysine, valine, asparagine, tryptophan, isoleucine, leucine, phenylalanine, cysteine, aspartic acid, GABA, serine, glycine, acetylcholine, adenosine.

(2) In-well screening using Ac-GFGFVGG-NH_2 as substrate

The sequence of 11 test compounds and a non-inhibitor control is E-64, leupeptin, antipain, control, methionine, arginine, tryptophan, proline, lysine, phenylalanine, aspartic acid, GABA.

(3) All-droplet screening using ZRR-AMC as substrate with PDMS tees

The sequence of 24 test compounds and the non-inhibitor control is control, antipain, cysteine, phenylalanine, leucine, lysine, proline, asparagine, E-64, aspartic acid, GABA, acetylcholine, glycine, serine, valine, adenosine, leupeptin, isoleucine, tryptophan, thyronine, histamine, tyrosine, arginine, methionine, p-nitrophenol.

(4) All-droplet screening using Ac-GFGFVGG-NH_2 as substrate with Teflon tees

One control and three test compounds are in 9 replicates, and two inhibitors are in 8 replicates. The sequence is: control, E-64, tyrosine, thyronine, leupeptin, proline.

References:

(1) Frlan, R.; Gobec, S. *Curr. Med. Chem.* **2006**, *13*, 2309-2327.
(2) Pei, J.; Li, Q.; Lee, M. S.; Valaskovic, G. A.; Kennedy, R. T. *Anal. Chem.* **2009**, *81*, 6558-6561.

Figure S-1

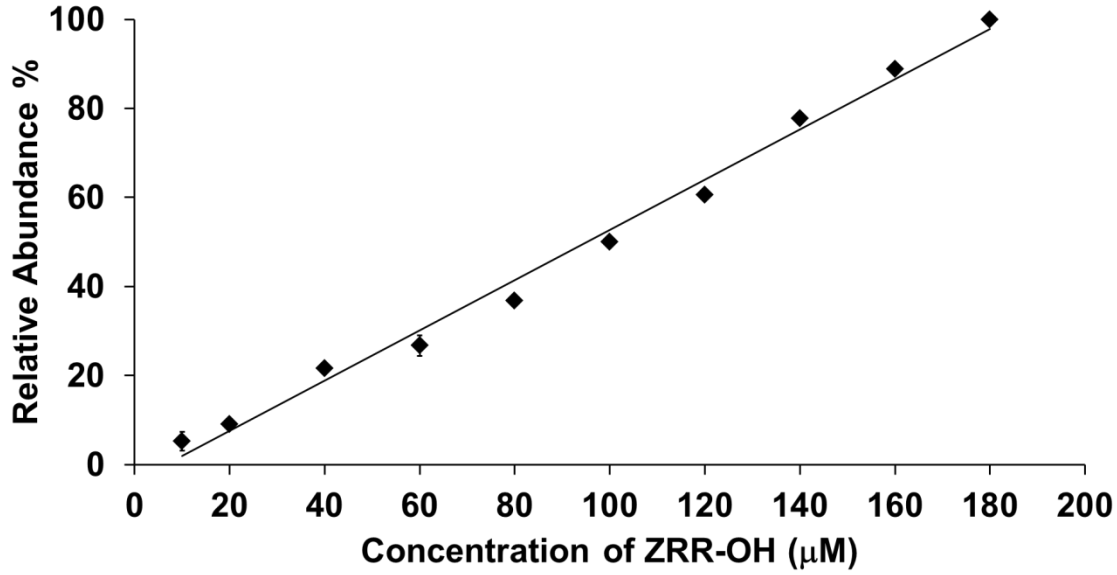


Figure S-1. Calibration curve for ZRR-OH. 10 solutions of ZRR-OH with concentration as 10, 20, 40, 60, 80, 100, 120, 140, 160, 180 μM were formatted as sample plugs and driven into ESI-MS for analysis. The ESI conditions were same with all other experiments. Using the relative abundance of each peak, the calibration curve had slope of 0.56, y-intercept of -3.6443, R^2 of 0.9912.

Figure S-2

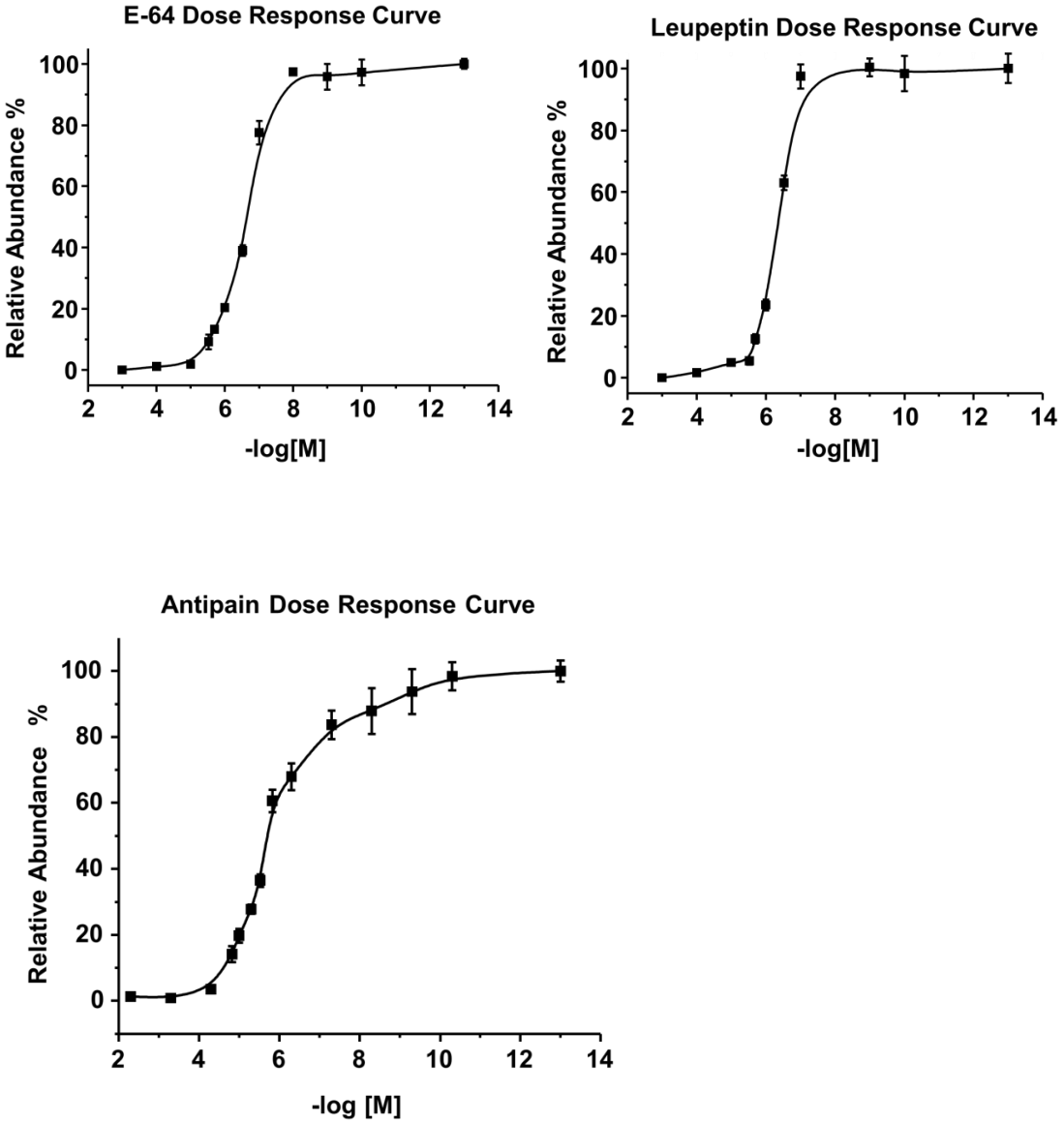
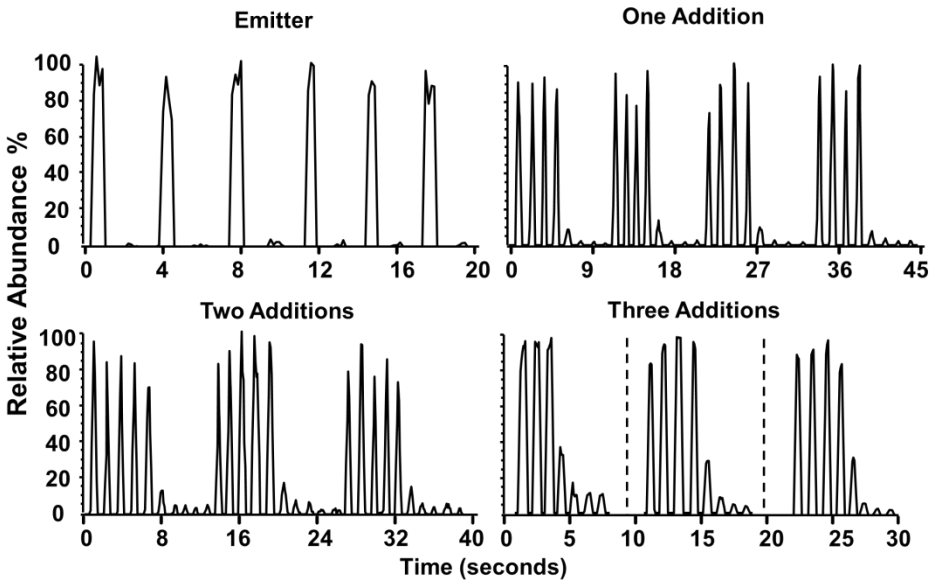


Figure S-2. Dose response curves of three known inhibitors determined by droplet-MS. Different concentrations of E-64 (10^{-7}, 10^{-4}, 10^{-3}, 10^{-2}, 0.1, 0.3, 1, 2, 3, 10, 100, 1000 μM), leupeptin (10^{-7}, 10^{-4}, 10^{-3}, 0.1, 0.3, 1, 2, 3, 10, 100, 1000 μM), antipain (5×10^{-7}, 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 0.5, 1.5, 3, 5, 10, 15, 50, 500, 5000 μM) were incubated with ZRR-AMC and the enzyme. Relative abundance of ZRR-OH peaks were used for construct the sigmoidal curves.

Figure S-3

A



B

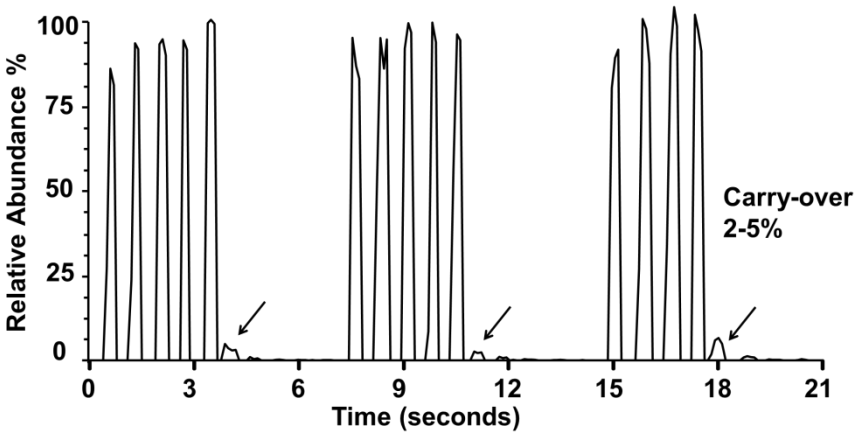


Figure S-3 (A) Carry-over of the all-droplet system. <1 % of carry-over was observed in the emitter (upper left). 9% of carry-over was generated by the tee for one addition (upper right) and 16% for two additions (lower left). The three addition result was extracted from the product trace shown in **Figure 4B**, the carry-over of which is 25-30% (lower right). (B) Carry-over of the Tefon tee for one addition.