Supplemental Material for:

Molecular Pharmacology: MOL #93070

Identification and characterization of novel inhibitors of mammalian aspartyl aminopeptidase

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Supplementary Figure 1. Purification of DNPEP and the HTS assay optimization. A total of 12 mg of DNPEP was purified from 3 L of T7 Express *E. coli* culture induced with 20 μM IPTG. DNPEP was stored at 1.3 mg/mL in 10 mM Tris-HCl, pH 8.0, 300 mM NaCl and 10% glycerol, as 1 mL aliquots. **A**) A Coomassie Blue-stained SDS-PAGE gel of purified DNPEP. **B**) Fluorescence intensity (E_{ex}/E_{em} = 380 nm/460 nm) versus AMC concentration curve showed a linear correlation. **C**) The steady-state kinetic parameters for DNPEP-catalyzed Asp-AMC hydrolysis showed the screening concentration of Asp-AMC at 250 μM is greater than 3x the K_m (80.9 μM) value. **D**) The presence of DMSO in the Asp-AMC hydrolysis assay showed no effect on DNPEP activity up to a 1% volume ratio. The red dot indicates the concentration used in the HTS **E**) DNPEP concentration in the Asp-AMC hydrolysis assay was optimized and 80 nM was used as final screening concentration in each well. **F**) Time course of fluorescence generated by Asp-AMC hydrolysis (in triplicate) showed linear correlation of fluorescence with time, suggesting the two-point reading (at t=0 and 30 min) was reliable for calculation of reaction rate. **G**) Minimal day-to-day and plate-to-plate variability demonstrated the reliability of the Asp-AMC hydrolysis assay. **H**) Two freeze-thaw cycles or up to 7.5 hours of incubation at 4 $^{\circ}$C did not affect DNPEP activity in the Asp-AMC activity assay demonstrating the enzyme stability.

Supplementary Figure 2. The pre-test of the activity of 33 compounds selected by virtual screening in the Asp-AMC hydrolysis assay. A) Chart of DNPEP enzymatic activity in the presence of tested compounds. 100% activity refers to the activity of the control sample with enzyme only. Three compounds inhibited the reaction by more than 50% compared to enzyme alone. **B**, **C** and **D** are the dose-response curves of compounds 1001928, 263560 and 413019 identified as DNPEP inhibitors. Their chemical structures and IC_{50} values are shown in the insets of each dose response chart.

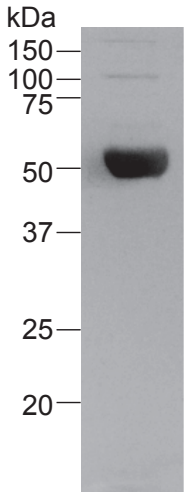
Supplementary Figure 3. Activity summary of neighboring molecules to compounds 241320 and 347749. Compounds labeled in red are DNPEP inhibitors with activity score ≤ -60 %. The activity score in the Asp-AMC hydrolysis assay and ID number of each compound are shown under its chemical structure. IC_{50} values for compounds exhibiting ≤ -60 % inhibition of DNPEP-catalyzed Asp-AMC hydrolysis are listed to the right of the respective activity scores.

Supplementary Figure 4. Activity summary of neighboring molecules to compounds 98897,769078 and 702610. Compounds labeled in red are DNPEP inhibitors with activity scores ≤ -60 %. The activity score in the Asp-AMC hydrolysis assay and ID number of each compound are shown under its chemical structure. IC_{50} values for compounds exhibiting ≤ -60 % inhibition of DNPEP-catalyzed Asp-AMC hydrolysis are listed to the right of the respective activity scores.

Supplementary Figure 5. Chemical structures of two Pfm18AAP inhibitors. A) The top lead inhibitor from the Pfm18AAP HTS (Schoenen et al., 2010). **B)** Base compound for SAR analysis in the Pfm18AAP HTS. The three variable regions in the SAR analysis are delineated by grey rectangles.

Supplementary Figure 1

A



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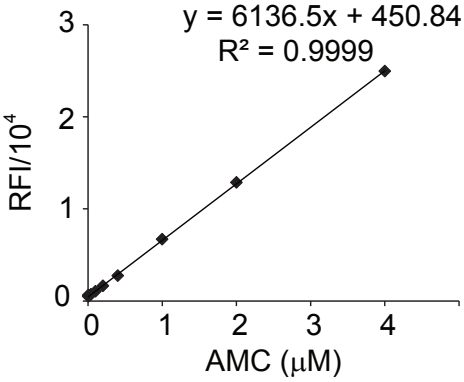

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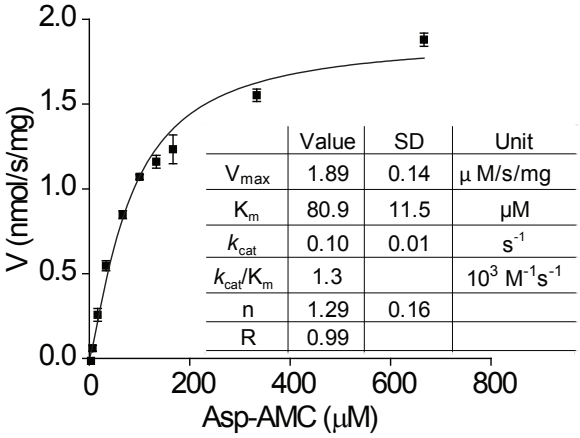
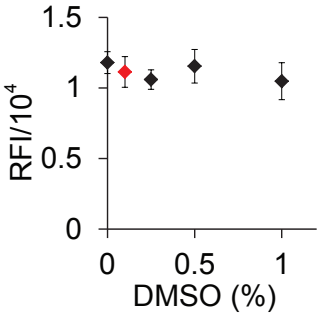


	Value	SD	Unit
V_{max}	1.89	0.14	μ M/s/mg
K_m	80.9	11.5	μM
k_{cat}	0.10	0.01	s^{-1}
k_{cat}/K_m	1.3		10^3 $M^{-1}s^{-1}$
n	1.29	0.16	
R	0.99		

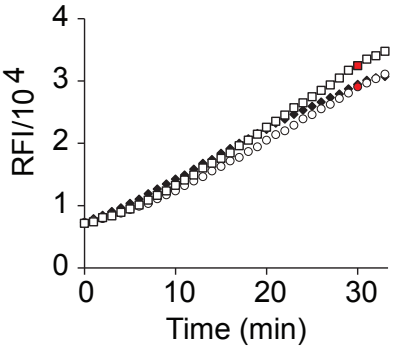
D



E

[En] (μM)	[S_0]/[En]	FI-BG	SD	%V
0.22	450	35113	189	0.54
0.17	600	27689	467	1.69
0.11	900	17980	951	5.29
0.08	1200	11210	1004	8.95
0.06	1800	6565	715	10.89
0.03	3600	3822	318	8.31
0.01	9000	588	108	18.37

F



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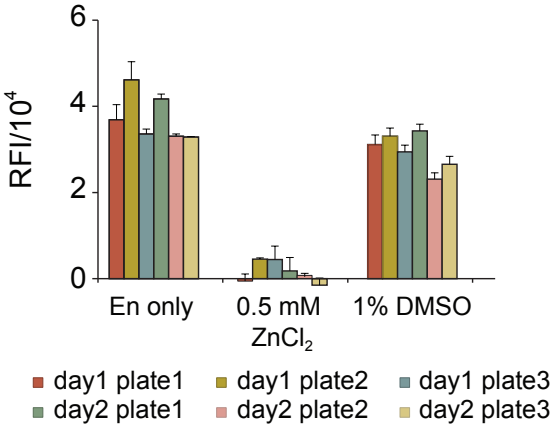

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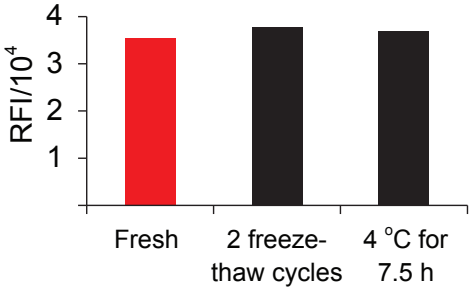
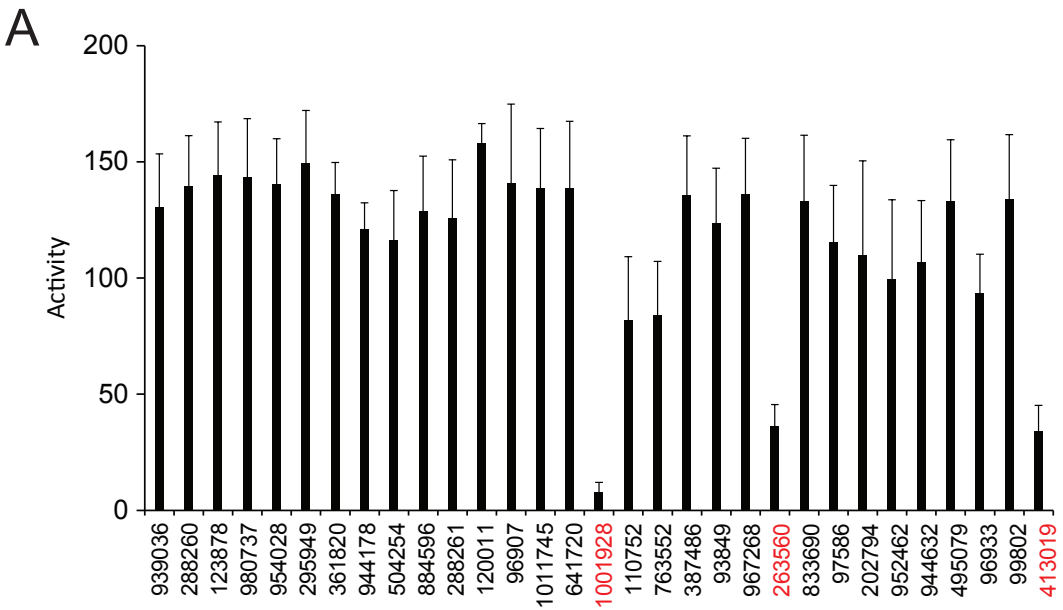
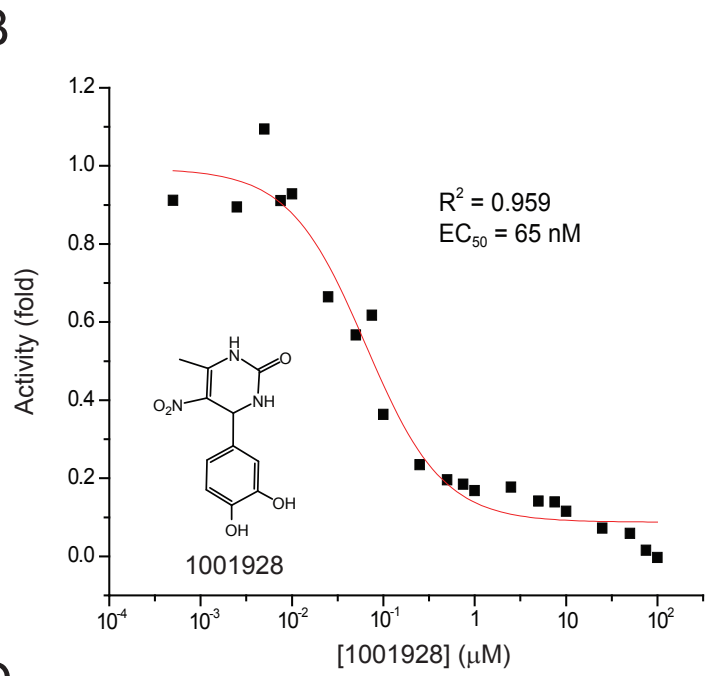
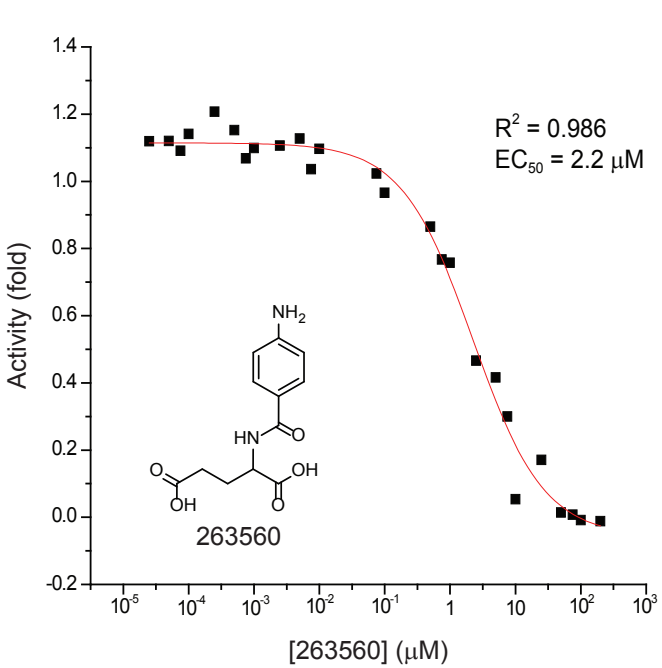
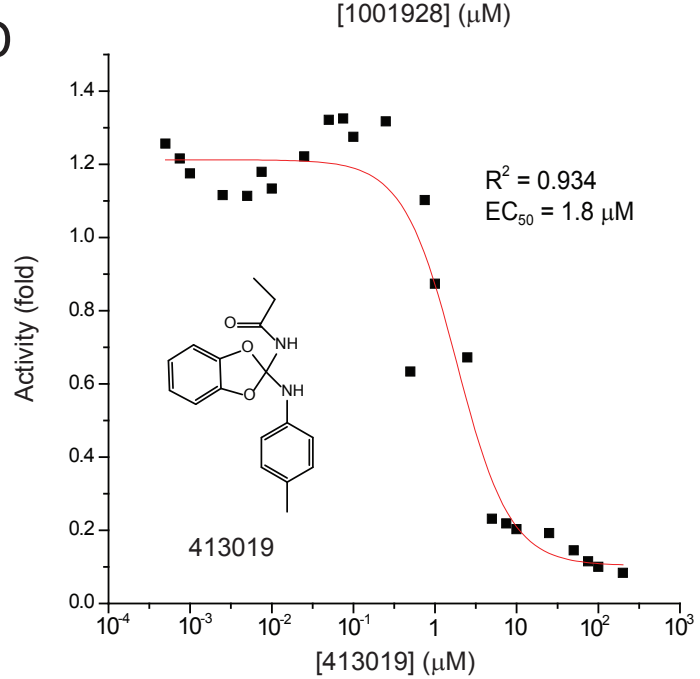


Supplementary Figure 2

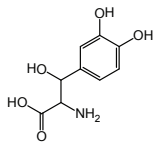
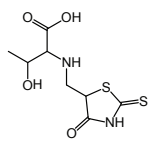
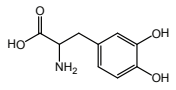
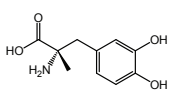
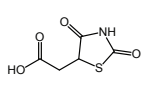
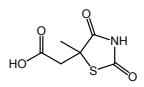
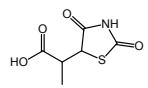
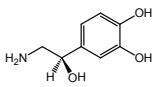
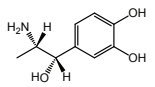
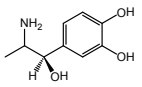
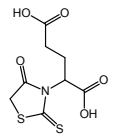
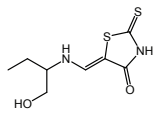
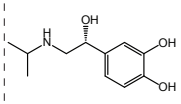
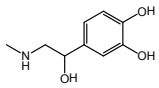
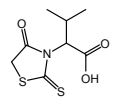
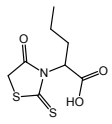
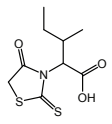
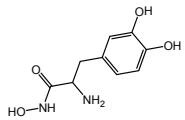
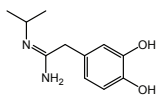
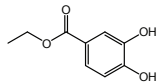
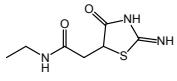
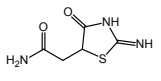
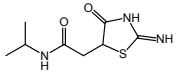
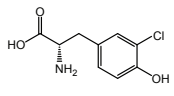
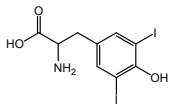
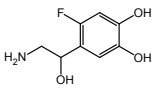
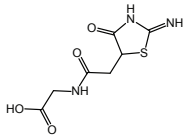
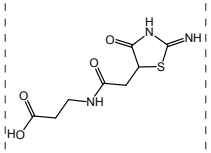
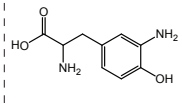
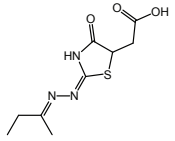
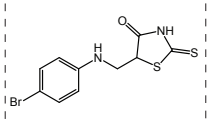
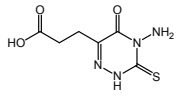




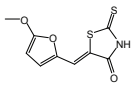
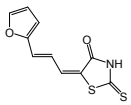
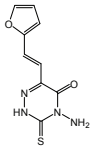
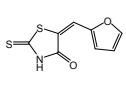
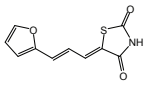
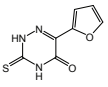
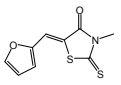
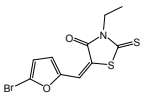
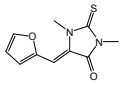
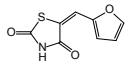
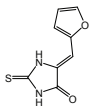
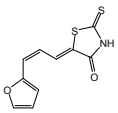
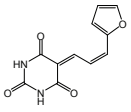
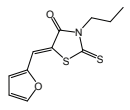
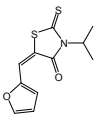
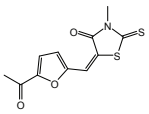
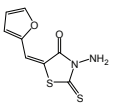
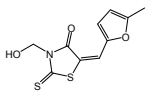
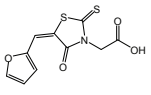
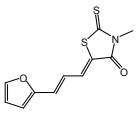
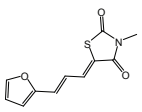
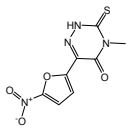
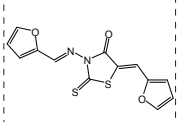
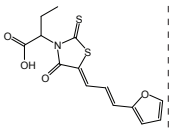
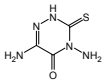
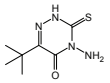
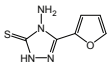
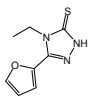
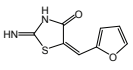
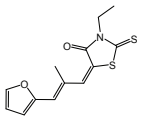
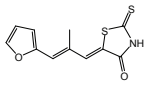
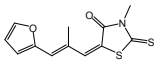
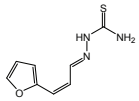
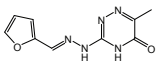
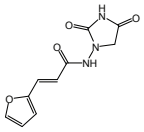
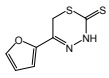
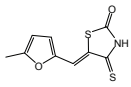
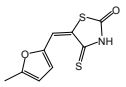
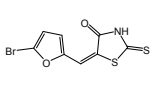




Supplementary Figure 3

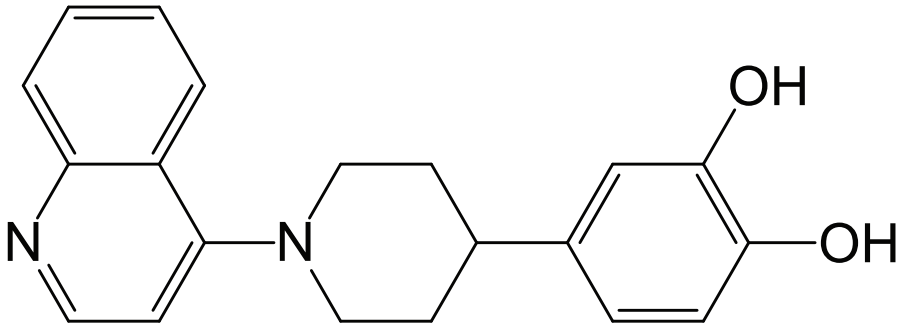
Seed	 241320, -74.5, 4.4 μM			Seed	 347749, -72.3, 2.0 μM	
Deletion of 3-hydroxyl	 503659, -86, 1.0 μM	 247266, -61, 8.5 μM		Deletion of amine and hydroxyl/ring substitution	 497467, -9	 963622, 2	 947750, -6
Deletion of carboxylate and alkylation	 247275, -75, 9.3 μM	 247255, -42	 361558, -2	Change extension position on rhodanine ring	 124943, -2	Deletion of carboxylate	 858862, -25
	 517092, -15	 104441, 0			 774463, -18	 374608, -26	 856595, -20
Side chain substitutions	 93984, -75, 3.80 μM	 9226, 47	 300234, -10	Threonine substitution with amide	 767433, -6	 951173, -1	 951174, -11
Halogen addition or replacement on catechol	 520266, -15	 130024, -4	 361522, -29	Threonine substitution with amide plus carboxylate	 948555, 1	 963980, -3	
Amine replacement of catechol hydroxyl	 281488, -1			Other modifications	 779262, -8	 279700, -54	 759849, -4

Supplementary Figure 4

Seeds	 98897, -96, 0.54 µM	 769078, -64, 5.8 µM	 702610, -84, 2.4 µM				
Isomerization and/or change in alkene linker length	 96155, -71, 8.6 µM	 994193, -76, 4.3 µM	 96379, -55	Non-polar substitution on rhodanine or triazine	 938713, -16	 782598, 0	 925567, -16
 381466, -69, 10 µM	 96292, -78, 2.2 µM	 98896, -78, 1.2 µM	 123141, -73, 2.2 µM		 920776, -32	 917820, -24	 526840, 0
Polar substitution on rhodanine nitrogen	 910179, -73, 7.1 µM	 966958, -70, 9.4 µM	 521685, -65, 9.8 µM		 374601, -52	 771512, -39	 95325, -39
 943988, -61, 11 µM	 994188, -78, 7.0 µM			Loss of furan ring and alkene linker	 966697, -57	 259097, -15	 962876, 4
Rhodanine or triazine ring substitution	 946097, –62, 7.2 µM	 778159, -67, N/A	 394747, -52	Alkylation of alkene linker or rhodanine nitrogen and isomerization	 921127, -4	 926399, -26	 921314, -1
 96270, -52	 492188, -4	 97417, -39	 96409, -72, 4.3 µM				
Furan ring derivativization	 770885, -62, 2.3 µM	 911299, -62, 1.7 µM	 98809, -73, 17 µM	Thiophene substitution for furan	980083, -17	496193, -24	240994, -18
98875, -62, 13 µM	769080, -71, 13 µM	1001360, -73, 18 µM	98871, -59		1001827, -27	917362, -17	964792, -20
98894, -5	370242, -21	100812, 6	95394, -58	Pyridine substitution for furan	240991, -35	702295, -8	
769641, -88, 3.0 µM	98866, -18	397172, -59	390117, -18	954647, -57	Other substitutions for furan	526103, -2	381558, -27, N/A

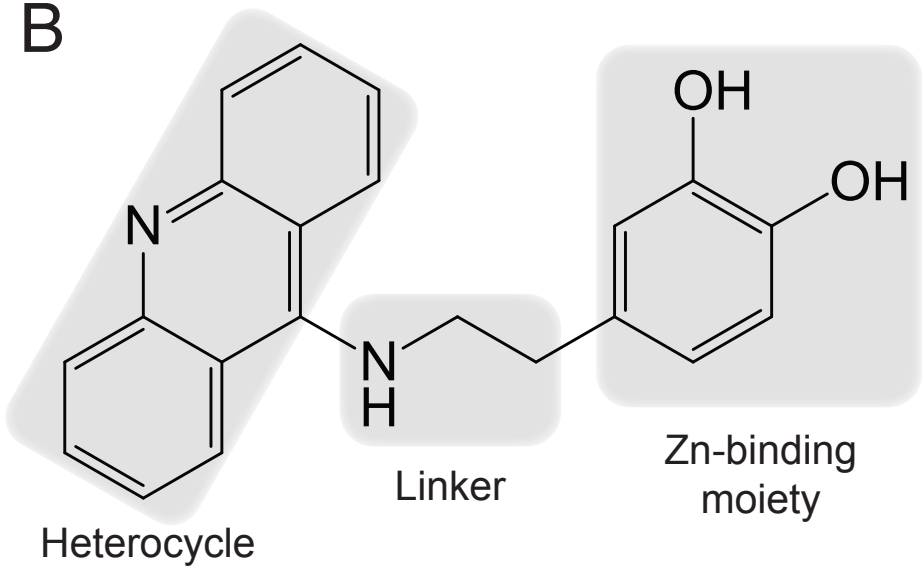
Supplementary Figure 5

A



ML369

B



CID 23724194

Supplementary Table 1. The effects of 26,386 tested compounds (12.3 μM) on DNPEP activity by the HTS (Asp-AMC hydrolysis assay)

% Activity	# Compounds	Cumulative # compounds
-90 to -100	**15**	**15**
-80 to -90	**65**	**80**
-70 to -80	**147**	**227**
- 60 to -70	**170**	**397**
-50 to -60	168	565
-40 to -50	281	846
-30 to -40	526	1,372
0 to -30	12,180	13,552
20 to 0	11,508	25,060
30 to 20	1,104	26,164
40 to 30	**173**	**26,337**
50 to 40	**33**	**26,370**
60 to 50	**7**	**26,377**
70 to 60	**2**	**26,379**
80 to 70	**5**	**26,384**
90 to 80	**2**	**26,386**

Supplementary Table 2. Summary of quality control parameters (signal/background ratio (S/B ratio) and Z'-factor) for DNPEP primary HTS.

S/B Ratio		**Z'-Factor**	
Mean ± S.D.	Range	Mean ± S.D.	Range
3134 ± 5091	346 – 3638	0.74 ± 0.03	0.66 – 0.80

Supplementary Table 3. Summary of compound effects (12.3 μM) on DNPEP activity in the Asp-AMC hydrolysis HTS performed in triplicate.

% Activity	# Compounds	Cumulative # compounds
-90 to -100	**18**	**18**
-80 to -90	**91**	**109**
-70 to -80	**120**	**229**
- 65 to -70	**34**	**263**
- 60 to - 65	**19**	**282**
-50 to -60	21	303
-40 to -50	11	314
-30 to -40	19	333
0 to -30	143	476
20 to 0	103	579
30 to 20	**4**	**583**
50 to 40	**1**	**584**
80 to 70	**1**	**585**
100 to 90	**1**	**586**

Supplementary Table 4. Summary of quality control parameters (signal/background ratio (S/B ratio) and Z'-factor) for the Asp-AMC hydrolysis HTS performed in triplicate.

S/B Ratio		**Z'-Factor**	
Mean ± S.D.	Range	Mean ± S.D.	Range
1321 ± 1337	257 – 3793	0.79 ± 0.05	0.71 – 0.87

Supplementary Table 5. Summary of 280 DAP inhibitors IC_{50} and 7 activators AC_{50} values (μM) obtained by Asp-AMC hydrolysis dose-response HTS assays

IC_{50} (μM)	**# Compounds**	**Cumulative # compounds**
0 to 1	36	36
1 to 10	219	255
10 to 20	25	280
AC_{50} (μM)	**# Compounds**	**Cumulative # compounds**
1 to 10	1	1
10 to 20	1	2
20 to 100	1	3
> 100	4	7

Supplementary Table 6. Summary of quality control parameters (signal/background ratio (S/B ratio) and Z'-factor) for the DNPEP dose-response screen.

S/B Ratio		**Z'-Factor**	
Mean ± S.D.	Range	Mean ± S.D.	Range
3029 ± 4824	418 – 23610	0.78 ± 0.03	0.72 – 0.83

Supplementary Table 7. Summary of hit effects (10 μM) on DNPEP activity in the orthogonal, Ang II hydrolysis screen.

Activity (%)	**Number of Compounds**	**Cumulative Number of Compounds**
-100 to -90	1	**1**
-90 to -80	5	**6**
-80 to -70	2	**8**
-70 to -60	5	**13**
-60 to -50	22	**35**
-50 to -40	28	63
-40 to -30	22	85
-30 to -20	48	133
-20 to 20	149	282
20 to 25	1	283

Supplementary Table 8. Summary of quality control parameters (S/B ratio and Z'-factor) for DNPEP orthogonal, Ang II hydrolysis screen.

S/B Ratio		**Z'-Factor**	
Mean ± S.D.	Range	Mean ± S.D.	Range
185 ± 75	103 – 339	0.89 ± 0.05	0.82 – 0.98

Supplementary Table 9. Summary of orthogonal triplicate confirmation screen in a single experiment. Each hit was tested in triplicate at 10 μM concentration in the Ang II hydrolysis assay. Quality control statistics are listed at the bottom of the table.

% Activity	# Compounds	Cumulative # compounds	
-100 to -90	13	**13**	
-90 to -80	4	**17**	
-80 to -70	2	**19**	
-70 to -60	1	**20**	
-60 to -50	6	**26**	
-50 to -40	1	27	
-40 to -30	1	28	
-30 to -20	0	28	
-20 to 20	4	32	
Z’	0.63	S/B	21.9

Supplementary Table 10. Quality control parameters (S/B ratios and Z’) for the dose-response Ang II hydrolysis screen.

S/B Ratio		**Z’-Factor**	
Mean ± S.D.	Range	Mean ± S.D.	Range
248 ± 104	69 – 459	0.87 ± 0.09	0.55 – 0.94

Supplementary Table 11. Quality control parameters (S/B ratios and Z’) for the DNPEP neighboring search and activity test with the Asp-AMC hydrolysis assay.

S/B Ratio		**Z’-Factor**
Exp 1.	143.8	0.96
Exp 2.	151.1	0.65