Supporting Information

Complementarity Between a Docking and High-Throughput Screen in Discovering New Cruzain Inhibitors

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General Methods:

Unless otherwise stated, all reactions were carried out under an atmosphere of dry argon or nitrogen in dried glassware. Indicated reaction temperatures refer to those of the reaction bath, while room temperature (rt) is noted as 25 °C. All solvents were of anhydrous quality purchased from Aldrich Chemical Co. and used as received. Commercially available starting materials and reagents were purchased from Aldrich and were used as received.

Analytical thin layer chromatography (TLC) was performed with Sigma Aldrich TLC plates (5 x 20 cm, 60 Å, 250 µm). Visualization was accomplished by irradiation under a 254 nm UV lamp. Chromatography on silica gel was performed using forced flow (liquid) of the indicated solvent system on Biotage KP-Sil pre-packed cartridges and using the Biotage SP-1 automated chromatography system. ¹H- and ¹³C NMR spectra were recorded on a Varian Inova 400 MHz spectrometer. Chemical shifts are reported in ppm with the solvent resonance as the internal standard (CDCl₃ 7.26 ppm, 77.00 ppm, DMSO-δ₆ 2.49 ppm, 39.51 ppm for ¹H, ¹³C respectively). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants, and number of protons. Low resolution mass spectra (electrospray ionization) were acquired on an Agilent Technologies 6130 quadrupole spectrometer coupled to the HPLC system. High resolution mass spectral data was collected in-house using and Agilent 6210 time-of-flight mass spectrometer, also coupled to an Agilent Technologies 1200 series HPLC system. If needed, products were purified via a Waters semi-preparative HPLC equipped with a Phenomenex Luna® C18 reverse phase (5 micron, 30 x 75 mm) column having a flow rate of 45 mL/min. The mobile phase was a mixture of acetonitrile and H₂O each containing 0.1% trifluoroacetic acid. Samples were analyzed for purity on an Agilent 1200 series LC/MS equipped with a Luna® C18 reverse phase (3 micron, 3 x 75 mm) column having a flow rate of 0.8-1.0 mL/min over a 3-minute gradient and
a 4.5 minute run time. The mobile phase was a mixture of acetonitrile (0.025% TFA) and H$_2$O (0.05% TFA), and a temperature was maintained at 50 °C. Purity of final compounds was determined to be >95%, using a 3 µL injection with quantitation by AUC at 220 and 254 nM (Agilent Diode Array Detector).

**Preparation of compound 31:**

![Chemical structure of compound 31](image)

**tert-butyl 2-(cyclohexanecarboxamido)acetate:**

To a solution of tert-butyl 2-aminoacetate (0.6 g, 4.57 mmol) in CH$_2$Cl$_2$ (25 mL) was added triethylamine (0.64 mL, 4.57 mmol) and cyclohexanecarbonyl chloride (0.67 g, 4.57 mmol). The reaction mixture was stirred for 1 h at rt, at which time the solution was washed with water. The organic layer was extracted, dried on MgSO$_4$, filtered, and concentrated in vacuo. The resulting residue was purified directly on silica column. Gradient elution with ethyl acetate (10 → 60%) in hexanes provided the title compound as a colorless solid: yield (1.0 g, 4.14 mmol, 91%).

![Chemical structure of tert-butyl 2-(cyclohexanecarboxamido)acetate](image)

**2-(cyclohexanecarboxamido)acetic acid:**

To a solution of tert-butyl 2-(cyclohexanecarboxamido)acetate (1.0 g, 4.14 mmol) in toluene (100 mL) was added SiO$_2$ (20 grams). The reaction was heated to reflux overnight, then cooled and filtered. The SiO$_2$ was washed with 10% MeOH/CH$_2$Cl$_2$ several times. The filtrate was concentrated to yield the title compound as a colorless solid. No further purification was needed: yield (0.69 g, 3.73 mmol, 90%).
2-oxo-1,2-diphenylethyl 2-(cyclohexanecarboxamido)acetate:

To a solution of commercially available 2-hydroxy-1,2-diphenylethanone (0.15 g, 0.71 mmol) and 2-(cyclohexanecarboxamido)acetic acid (0.13 g, 0.71 mmol) in THF (7 mL) was added triphenylphosphine (0.19 g, 0.71 mmol), followed by dropwise addition of diisopropyl azodicarboxylate (0.14 mL, 0.71 mmol). The reaction mixture was stirred at rt for 2 h. Upon completion, the solvent was removed under reduced pressure and the residue was purified directly on silica column. Gradient elution with ethyl acetate (1 → 35%) in hexanes provided the title compound as a colorless solid: yield (0.21 g, 0.55 mmol, 78%). LC-MS: rt (min) = 3.68; $^1$H NMR (DMSO-d$_6$) δ 1.06-1.36 (m, 6H), 1.52-1.73 (m, 4), 2.07-2.21 (m, 1H), 3.84-4.06 (m, 2H), 7.15 (s, 1H), 7.32-7.43 (m, 3H) 7.46-7.57 (m, 4H) 7.62 (t, $J$ = 7.4 Hz, 1H) 8.05 (d, $J$ = 7.2 Hz, 2H) 8.20 (t, $J$ = 6.0 Hz, 1H); $^{13}$C NMR (DMSO-d$_6$) δ 25.39, 25.55, 29.57, 43.91, 43.99, 44.06, 77.00, 78.19, 128.53, 128.72, 129.12, 129.77, 130.06, 133.73, 133.95, 134.26, 134.30, 134.82, 135.00, 170.02, 176.12, 176.29, and 193.92; HRMS (m/z): [M$^+$] calcd. for C$_{23}$H$_{25}$NO$_4$, 379.1784; found, 379.1788.

General Scheme for compounds 32-35:
General Procedures:

To a stirring solution of t-butyl glycine (1.0 eq) in CH₂Cl₂ were added the acid chloride (1.1 eq) and triethylamine (1.1 eq). The reaction mixture was stirred at rt for 1 hr, at which time the mixture was diluted further w/ CH₂Cl₂ and washed with sat. ammonium chloride solution and brine. The organic layer was extracted, dried on MgSO₄, filtered, and the solvent was removed under reduced pressure. The residue was purified directly on silica gel. Gradient elution (20-40% EtOAc in hexanes) afforded the desired product (t-butyl amidoacetates): yield (95-99%).

A solution of the t-butyl amidoacetate (1 mmol) and SiO₂ (6 g) in toluene was refluxed for 16 h. The reaction mixture was filtered and the silica gel was washed several times with 10% MeOH-CH₂Cl₂ and the solvent was removed under reduced pressure to afford the amidoacetic acids, S1 as a colorless or pale solids: yield (90-95%). See below for specific example.
General procedure C – To a solution of the requisite aniline (1.0 eq) in CH₂Cl₂ were added triethylamine (1.1 eq) and 2-chloro-2-oxoethyl acetate (1.1 eq). The reaction mixture was stirred at rt for 1 h, at which time the mixture was diluted further w/ CH₂Cl₂ and washed with sat. ammonium chloride solution and brine. The organic layer was extracted, dried on MgSO₄, filtered, and the solvent was removed under reduced pressure. The residue was purified directly on silica gel. Gradient elution (20-40% EtOAc in hexanes) afforded the desired products (2-oxo-2-(arylamino)ethyl acetates) as a colorless or pale solid: yield (95-99%).

General procedure A – To a stirring solution of 2-oxo-2-(arylamino)ethyl acetate (1.0 eq) in a methanol with potassium carbonate (1.0 eq). The reaction mixture was stirred at rt for 1 h, at which time it was diluted w/ EtOAc and filtered through Celite. The organic layer was washed with sat. ammonium chloride and brine, extracted, dried on MgSO₄, filtered, and the solvent was removed under reduced pressure. The residue was purified directly on silica gel. Gradient elution (40-60% EtOAc in hexanes) afforded the desired product (2-hydroxy-N-arylacetamides, S2) as a colorless or pale solid or oil: yield (90-95%).

General procedure e – To a stirring solution of 2-hydroxy-N-arylacetamides S2 (1.0 eq), amidoacetic acids S1 (1.0 eq), and triphenylphosphine (1.1 eq) in THF was added diisopropyl
azodicarboxylate (DIAD, 1.1 eq) dropwise at rt. The reaction mixture was stirred for 16 h, at which time the solvent was removed under reduced pressure. The residue was purified directly on silica gel. Gradient elution (25-60% EtOAc in hexanes) afforded the desired products (benzamidoacetates or carboxamidoacetates, S3) as colorless or pale solids: yield (80-95%).

2-(2-chloro-5-(trifluoromethyl)phenylamino)-2-oxoethyl 2-(2-chlorobenzamido)acetate

LC-MS: rt (min) = 3.55; \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 4.43 (d, 2H, \( J = 5.6 \) Hz), 4.87 (s, 2H), 6.98 (m, 1H), 7.33-7.44 (m, 4H), 7.52 (d, 1H, \( J = 8.4 \) Hz), 7.71 (d, 1H, \( J = 7.6 \) Hz), 8.56 (brs, 1H) and 8.65 (s, 1H); \( ^{13}C \) NMR (CDCl\(_3\)) \( \delta \) 41.89, 63.67, 119.02 (q, F-splitting), 122.07 (q, F-splitting), 127.02, 127.67, 129.67, 130.38, 130.45, 130.55, 130.88, 131.99, 133.41, 134.06, 164.69, 166.73 and 168.17; HRMS (m/z): [M]+ calcd. for C\(_{18}\)H\(_{13}\)Cl\(_2\)F\(_3\)N\(_2\)O\(_4\), 448.0204; found, 448.0200.

2-(2-chloro-5-(trifluoromethyl)phenylamino)-2-oxoethyl 2-(cyclohexanecarboxamido)acetate

LC-MS: rt (min) = 3.61; \( ^1H \) NMR (DMSO-d\(_6\)) \( \delta \) 1.08-1.32 (m, 5H), 1.55-1.67 (m, 5H), 2.10-2.17 (m, 1H), 3.28 (s, 3H), 3.64 (s, 1H), 3.90 (d, 2H, \( J = 6.0 \) Hz), 7.53 (dd, 1H, \( J = 8.4 \) and 1.6 Hz), 7.75 (d, 1H, \( J = 8.4 \) Hz), 8.12 (d, 1H, \( J = 2.0 \) Hz), 8.15 (m, 1H) and 9.90 (s, 1H); \( ^{13}C \) NMR
(DMSO-d$_6$) $\delta$ 25.14, 25.41, 29.03, 43.51, 51.91, 60.74, 62.59, 121.73 (q, F-splitting), 122.17, 122.85 (q, F-splitting), 124.89, 127.89, 128.22, 130.15, 130.87, 135.10, 166.34, 169.69, 175.64 and 175.80; HRMS (m/z): [M]$^+$ calcd. for C$_{18}$H$_{20}$ClF$_3$N$_2$O$_4$, 420.1064; found, 420.1068.

![Chemical Structure](image)

2-(2-chlorophenylamino)-2-oxoethyl 2-(cyclohexanecarboxamido)acetate

LC-MS: rt (min) = 3.38; $^1$H NMR (DMSO-d$_6$) $\delta$ 1.1-1.35 (m, 5H), 1.58-1.70 (m, 5H), 2.15 (tt, 1H, $J = 11.2$ and 3.2 Hz), 3.93 (d, 2H, $J = 6.0$ Hz), 4.76 (s, 2H), 7.21 (td, 1H, $J = 7.6$ and 1.6 Hz), 7.33 (td, 1H, $J = 8.0$ and 1.6 Hz), 7.50 (dd, 1H, $J = 8.0$ and 1.6 Hz), 7.68 (dd, 1H, $J = 8.0$ and 1.6 Hz) and 8.20 (t, 1H, $J = 6.0$ Hz); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 25.14, 25.42, 29.04, 43.53, 126.21, 126.74, 127.49, 129.53, 134.06, 165.74, 169.64 and 175.81; HRMS (m/z): [M]$^+$ calcd. for C$_{17}$H$_{21}$ClN$_2$O$_4$, 352.1190; found, 352.1187.

![Chemical Structure](image)

2-(2-chlorophenylamino)-2-oxoethyl 2-(2-chlorobenzamido)acetate

LC-MS: rt (min) = 3.37; $^1$H NMR (DMSO-d$_6$) $\delta$ 4.15 (d, 2H, $J = 6.0$ Hz), 4.83 (s, 2H), 7.22 (td, 1H, $J = 7.6$ and 1.6 Hz), 7.33 (td, 1H, $J = 8.0$ and 1.2 Hz), 7.38-7.52 (m, 5H), 7.69 (dd, 1H, $J = 8.0$ and 1.6 Hz), 8.95 (t, 1H, $J = 6.0$ Hz) and 9.70 (s, 1H); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 40.79, 62.72, 126.24, 126.76, 127.07, 127.49, 129.02, 129.54, 129.73, 129.99, 131.09, 134.06, 135.97,
165.69, 166.85 and 169.05; HRMS (m/z): [M]+ calcd. for C_{17}H_{14}Cl_{2}N_{2}O_{4}, 380.0331; found, 380.0331.
**Supplementary Figure 1** Lineweaver-Burk plots for representative compounds for five classes of cruzain competitive inhibitors. (a) 8, cluster 1, apparent Ki = 65 nM (b) 26, Ki = 0.8 µM, (c) 5, Ki = 6 µM, (d) 27, cluster 2, Ki = 2 µM, (e) 29, cluster 31, Ki = 2 µM.
**Supplementary Figure 2** Enrichment curves. (a) Improvement of enrichment at each stage of mechanistic follow up. Curves for all hits (purple), detergent insensitive compounds (putative non aggregators) (cyan), non fluorescent compounds (red), compounds selective for cruzain (pink) and competitive inhibitors (orange) are shown. (b) Enrichments for each cluster of competitive inhibitors. Clusters 1, 2, 31 and 44 are shown in magenta, blue, orange and green respectively. Enrichment expected by random ranking of compounds shown in black.
**Supplementary Figure 3** Cruzain catalyzed degradation of cluster 1 compounds. UV trace for absorbance at 254 nm for solutions of 11 (a) solution after 1 day in the absence of cruzain, (b) fresh solution in presence of 100 nM cruzain, (c) after 40 minutes in the presence of 100 nM cruzain. The peak eluted at 4.4 minutes refers to compound 11, whereas the one eluted at 3.2 min corresponds to a product of 11 cleavage. Time-dependence of cruzain inhibition by compounds (d) 8 and (e) 11.
Supplementary Figure 4 Replacement of ester functionality in 11 yields inactive compounds.

NO ACTIVITY WAS OBSERVED

replaced ester with amide, oxadizaole, thiadiazole triazole.
**Supplementary Figure 5** Chemical stability and time-dependence cruzain inhibition data for compounds 4 and 5. UV trace for absorbance at 254 nm for fresh compound solutions of (a) 4 and (b) 5 in absence of cruzain; and for solutions incubated with 100 nM cruzain: (c) 4 after 4 h incubation and , (d) 5 after 1 day incubation. Time-course of cruzain inhibition by compounds (e) 4 and (f) 5, indicating no time-dependence over 240 min incubation.
compound 5 - 100 uM

incubation with cruzain
incubation without cruzain
**Supplementary Figure 6** Comparison between cruzain structure used for docking and crystal structure of cruzain/27 complex. Residues within 5 Å of compound 27 are shown in sticks. Conformations of active site residues are similar in both structures, except for Gln159 and double conformation of Cys25. Carbon atoms colored green in crystal structure and gray in structure used for docking. Oxygen, nitrogen and sulfur colored red, blue and yellow respectively. Figures prepared with Pymol.²⁴
Supplementary Figure 7 Superposition of conformation of 27 in complex with cruzain and closest conformation found by docking (rmsd = 1.4 Å). Carbon atoms colored cyan in crystallographic complex and green in docked conformation. Oxygen, nitrogen, sulfur and bromine colored red, blue, yellow and orange respectively. Figures prepared with Pymol.24
**Supplementary Table S1** - Follow up of qHTS Hits ranked among top 1% of the database by DOCK

| Structure | DOCK rank | IC50 (µM) | AmpC inhibition? | Detergent sensitivity? | Time-dependence? |
|-----------|-----------|-----------|------------------|------------------------|------------------|
| ![Structure 1](image1.png) | 6         | 11        | No               | No                     | No               |
| ![Structure 2](image2.png) | 20        | 0.4       | No               | No                     | No               |
| ![Structure 3](image3.png) | 97        | 38        | No               | No                     | No               |
| ![Structure 4](image4.png) | 153       | 1         | No               | No                     | No               |
| ![Structure 5](image5.png) | 173       | 7         | No               | No                     | No               |
| ![Structure 6](image6.png) | 311       | 25        | Yes              | No                     | No               |
| Compound | MW | pIC50 | HDAC | 4011 | 4013 | 4014 |
|----------|-----|-------|------|------|------|------|
| 6        | 550 | 0.7   | No   | No   | No   | No   |
| 7        | 555 | 0.5   | No   | No   | No   | No   |
| 37       | 647 | 166   | Yes  | No   | No   | No   |
| 38       | 734 | -     | No   | Yes  | No   | No   |
| 8        | 789 | 0.3   | No   | No   | No   | No   |
| 39       | 951 | -     | yes  | Yes  | yes  | yes  |
| 9        | 1151| 65    | no   | No   | No, but low inhibition | No, but low inhibition |
| 1182     | 18  | No    | No   | No   | No   | No   |
| Compound | MW | pIC50 | Selective | Recognition | Conformation |
|----------|----|-------|-----------|-------------|--------------|
| ![Molecule 10](image1.png) | 1378 | 3 | No | No | No |
| ![Molecule 11](image2.png) | 1485 | 0.7 | No | No | No |
| ![Molecule 41](image3.png) | 1623 | - | No | Yes | No |
| ![Molecule 42](image4.png) | 1825 | - | No | Yes | Yes |
| ![Molecule 43](image5.png) | 1833 | - | No | Yes | No |
**Supplementary Table S2** – DOCK ranking and experimental follow up of compound 4 analogues (Cluster 44)

| Structure | DOCK rank | IC$_{50}$ (µM) | AmpC inhibition? | Detergent-sensitivity? | Time-dependence? | Ki (µM) |
|-----------|-----------|----------------|------------------|------------------------|------------------|---------|
| ![Structure 4](image1.png) | 153 | 1 | No | No | No | 1.6 |
| ![Structure 44](image2.png) | 8593 | 25 | ND | No | No | ND |
| ![Structure 26](image3.png) | 11337 | 0.9 | No | No | No | 0.8 |
| ![Structure 45](image4.png) | 21010 | 5 | ND | No | No | ND |

ND = not determined
**Supplementary Table S3** Follow up of clusters selective for cruzain

| Cluster/compounds per cluster | Compound tested experimentally | qHTS IC$_{50}$ (µM) | Detergent sensitive? | Time-dependent? | % β-lactamase inhibition |
|-------------------------------|--------------------------------|----------------------|----------------------|-----------------|-------------------------|
| 1/88                          | ![Structure 1](attachment:structure1.png) | 7                    | No                   | no              | 0                       |
|                              | ![Structure 2](attachment:structure2.png) |                      |                      |                 | (10 µM)$^a$             |
| 2/43                          | ![Structure 3](attachment:structure3.png) | 0.4                  | No                   | no              | 0                       |
|                              | ![Structure 4](attachment:structure4.png) |                      |                      |                 | (100 µM)$^a$            |
| 12/8                          | ![Structure 5](attachment:structure5.png) | 6                    | yes                  | inconclusive    | NA                      |
| 21/2                          | ![Structure 6](attachment:structure6.png) |                      | No                   | yes             | NA                      |
| 27/5                          | ![Structure 7](attachment:structure7.png) | 2                    | No                   | yes             | NA                      |
| 28/7                          | ![Structure 8](attachment:structure8.png) | 25                   | Yes                  | no              | NA                      |
| No. | Compound | EC50 (µM) |
|-----|----------|-----------|
| 30/5| ![Compound 21](image) | 1 No yes NA |
| 31/10| ![Compound 29](image) | 13 No no 0 (100 µM)<sup>a</sup> |
| 37/3| ![Compound 19](image) | 14 No yes NA |
| 44/4| ![Compound 4](image) | 13 No no 5 (10 µM)<sup>a</sup> |
| Singleton | ![Compound 5](image) | 14 No no 6 (10 µM)<sup>a</sup> |
Singleton  

| 18 |

Singleton  

| 24 |

a compound concentration in assay.
### Supplementary Table S4 – Potential qHTS false negatives prioritized for testing by docking

| Structure | % cruzain inhibition at 200 µM |
|-----------|-------------------------------|
| ![Structure 46](image) | 0 |
| ![Structure 47](image) | 0 |
| ![Structure 48](image) | 0 |
| ![Structure 49](image) | 23 |
| ![Structure 50](image) | 0 |
| ![Structure 51](image) | 0 |
| ![Structure 52](image) | 0 |
| ![Structure 53](image) | 6 |
| ![Structure 54](image) | 1 |
| ![Structure 55](image) | 49 |
| ![Structure 56](image) | 8 |
| ![Structure 57](image) | 25 |
| ![Structure 58](image) | 0 |
| ![Structure 59](image) | 0 |
| ![Structure 60](image) | 7 |
| ![Structure 61](image) | 19 |
| Structure | % cruzain inhibition at 200 µM |
|-----------|-------------------------------|
| ![Structure 62](image) | 74 |
| ![Structure 63](image) | 32 |
| ![Structure 64](image) | 62 |
| ![Structure 65](image) | 3 |
| ![Structure 66](image) | 25 |
| ![Structure 67](image) | 2 |
| ![Structure 68](image) | 42 |
| ![Structure 69](image) |  |
| ![Structure 70](image) | High fluorescence |
| ![Structure 71](image) | 8 |
| ![Structure 72](image) | 8 |
| ![Structure 73](image) | 68 |
| ![Structure 74](image) | 40 |
| ![Structure 75](image) | 50 |
| ![Structure 76](image) | 8 |
| ![Structure 77](image) | 60 |
Supplementary Table 5 *In vitro* activity of compound 11 and representative analogues

| Compound | IC<sub>50</sub> (nM) | Against Cruzain |
|----------|---------------------|-----------------|
| ![Structure of 11](image) | 260 | |
| ![Structure of 31](image) | 30 | |
| ![Structure of 32](image) | 220 | |
| ![Structure of 33](image) | 670 | |
| ![Structure of 34](image) | 520 | |
| ![Structure of 35](image) | 250 | |
**Supplementary Table 6** Comparison of DOCK scores for crystallographic and DOCK poses of compound 27

| Cruzain structure | Compound 27 pose | Scores |  |  |  | Total score |
|-------------------|------------------|--------|--------|--------|----------------|
|                   |                  | electrostatics | van der Waals | ligand desolvation |                       |
| Same used for virtual screening | Best ranked by docking | -33.0 | -22.2 | 19.7 | -35.5 |
| Crystallographic |                  | -17.0 | -8.4 | 16.9 | -8.5 |
| From structure complex with 27 | Best ranked by docking | -12.06 | -25.9 | 11.2 | -26.7 |
| Crystallographic |                  | -11.7 | -22.5 | 16.6 | -17.8 |