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Supplementary Material

Discovery of small molecule inhibitors for the snake venom metalloprotease BaP1 using *in silico* and *in vitro* tests

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Materials

BaP1 was isolated from *B. asper* venom as previously described[1, 2] . Solvents and reagents were purchased from commercial suppliers and were used without further purification. The compounds ZINC06812429 (A1) and ZINC08767570 (A2) were purchased from MolPort (https://www.molport.com).

In Silico Ligand preparation

Our approach centered on identifying a series of compounds with functional groups that can coordinate with the Zn(II) (ZBG) and groups that interact with the subsite S1´ of the BaP1 protein. Three virtual libraries were used to search for such compounds ZINC using the Drug-like physical property subsets in the .sdf file format at pH 6-8, the CHEMBL data base (CHEMBL_17, CHEMB_MMP) and PubChem. The first step was the database curation. Using Pipeline Pilot (BIOVIA/Accelrys Inc., San Diego, CA, USA), a workflow was designed as illustrated in Figure S1.

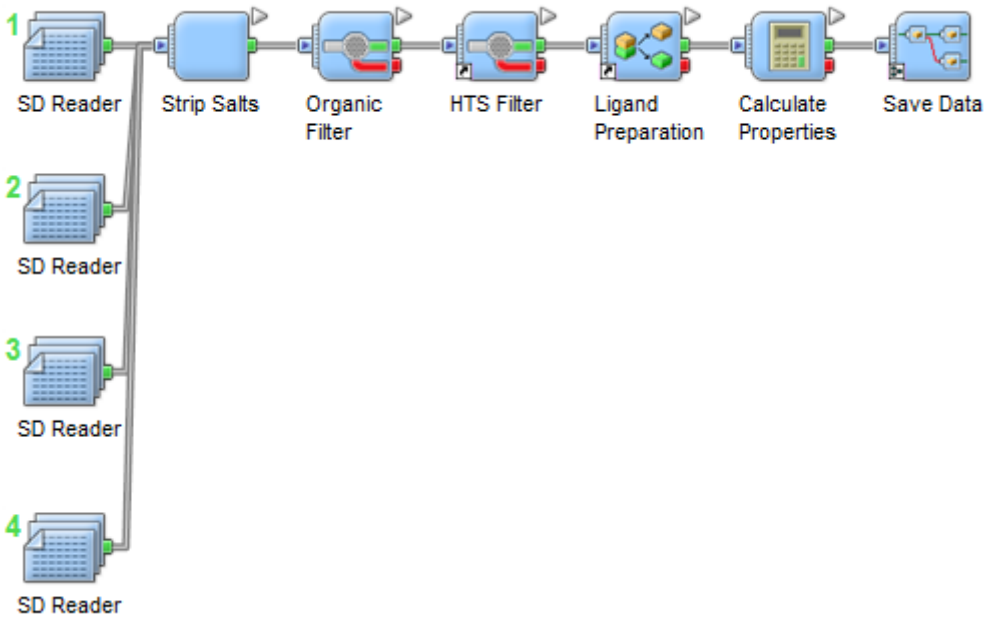

Figure S1. Pipeline Pilot workflow design for the curation of the combination virtual library (Zinc, PubChem, ChemBl and ChemBl with refine ligand for MMP).

To prepare the library, "Generate Conformation" protocol from Pipeline Pilot was applied. This protocol offers a selection of conformation and torsion search methods for creating diverse ligand conformations. Conformation search method was set to FAST.

In Silico Protein preparation

BaP1 enzyme was downloaded from Protein Data Bank (PDB code: 2W14)[3]. BaP1's structure was prepared by using "Prepare Protein" protocol available in Discovey Studio (DS) version 4.0 (BIOVIA/Accelrys Inc., San Diego, CA, USA). Default parameters were used.

Pharmacophore

Considering ligand-interacting BaP1 residues, a pharmacophore model was built applying "Interaction Generation" protocol implemented in DS 4.0 with default parameters. This protocol extracts pharmacophore queries from the *Ludi* interaction map created inside the active site sphere and assigns three main features namely HBA (Hydrogen Bond Acceptor), HBD (Hydrogen Bond Donor) and HY (Hydrophobic). The HBD feature that orients to the zinc(II) ion has been replaced by zinc feature using the option "Add Feature From Dictionary" under the pharmacophore tab in DS 4.0. The zinc feature in the present study was modified to detect the following functional groups: carboxyl, phosphate, imidazole, 1,2,3-triazole, 1,2,4-triazole, tetrazole, thiadiazole, 1-hydroxy-2-oxo derivatives[4].

Virtual High-Throughput Screening

Once prepared the ligand library and the target, the Virtual High Throughput Screening was carried on. The docking workflow was designed using Pipeline. This Workflow had a series of components to filtrate the large library as shown in the Figure S2.

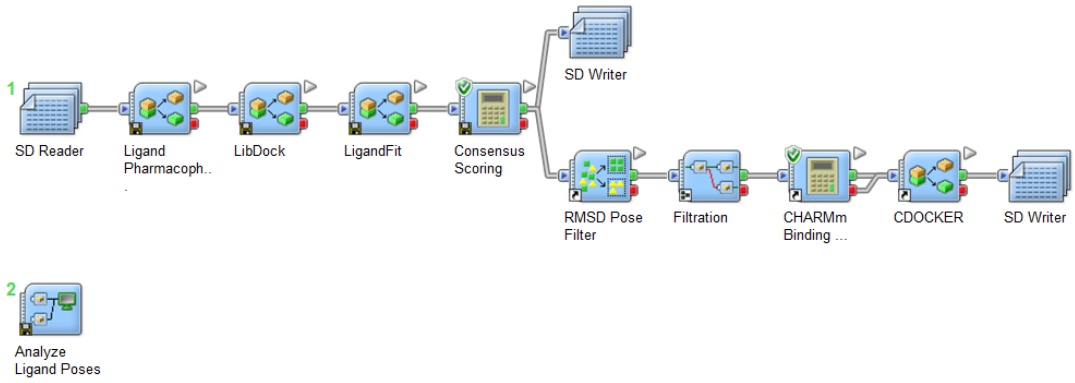

Figure S2. Pipeline Pilot workflow of Virtual High Throughput Screening used in analysis of the ligand poses of the BaP1 inhibitors.

Electrophoresis

Electrophoresis was carried out in polyacrylamide gels (SDS-PAGE, 12%) under reducing conditions[5].

Inhibition of proteolytic activity

Inhibition of the proteolytic activity of the BaP1 was assessed using the azocasein as a substrate and as described elsewhere[6, 7]. The BaP1 was diluted in the reaction buffer, mixed with the inhibitor dissolved in dimethyl sulfoxide (DMSO) and this mixture was incubated for 30 min at 37 °C. Then, 100 µL of the azocasein solution was added and incubated for 90 min at 37 °C. Finally, 200 µL of trichloroacetic acid (5% solution) were added to quench the reaction and the samples were centrifuged for 10 min at 4,000 × g. 100 µL of the supernatant was mixed with an equal amount of 0.5 $mol{\cdot}L^{-1}$ of NaOH in H_2O. The absorbance was recorded at 450 nm. The final concentration of BaP1 was 2 $\mu mol{\cdot}L^{-1}$. The **half maximal inhibitory concentrations (IC_{50})** as measures of the effectiveness of the two compounds (A1 and A2) in inhibiting BaP1[7, 8] function were determined after incubating the BaP1 at 37 °C. during 30 min with different concentrations of A1 or A2 inhibitors, which varied from 0 to 100 $\mu mol{\cdot}L^{-1}$, and then, the solution of the azocaisein was added and experiments continued as just described above. All experiments were performed in duplicate.

Circular Dichroism

The BaP1 CD spectra were obtained from 4 $\mu mol{\cdot}L^{-1}$ protein concentration in 25 $mM{\cdot}L^{-1}$ Tris buffer at pH = 8.0. The spectra were recorded from 200 to 260 nm with a Jasco J-720 spectropolarimeter (JASCO International Co. Ltd., Tokyo, Japan). For 2D structure features assessment, temperature of 25 ºC was used. Also, 1 mm path length cell, average of 8 scan at a scan speed of 20 nm/min were used. For assessing the thermal stability of the BaP1, two protein samples were heated in range from 25 to 80 ºC with a ramp of 10 ºC/h, and the water bath (ThermoFisher) was used.

Protein Fluorescence

The relative intrinsic fluorescence of BaP1 free and with the inhibitors was monitored with a Varian (Varian, Palo Alto, USA) spectrofluorimeter. The protein sample and reaction mixtures (all volumes were 500 µL) were put into 1 cm path length quartz cuvette. We used as buffer 25 $mM{\cdot}L^{-1}$ Tris-HCl (pH 8.0). These were titrated with DMSO solution of inhibitors. These experiments were performed three-times for each inhibitor (A1 and A2) concentration. Fluorescence was measured between 300 and 500 nm after excitation at 280 nm. The Stock-Volmer graphs were plot and K_d was determined applying a non-linear regression performed with Origin (OriginLab Corporation).

Virtual Screening Results

Table S1 Structure and CDDOCKER ENERGY of 50 best-ranked compounds

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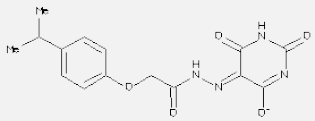
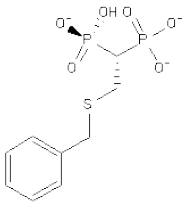
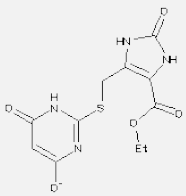
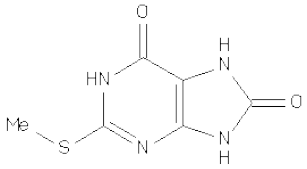
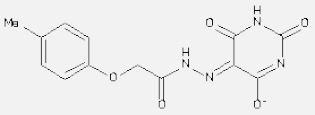
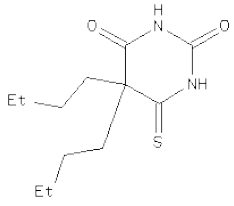
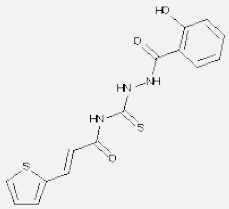
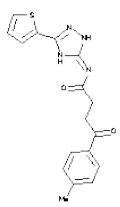
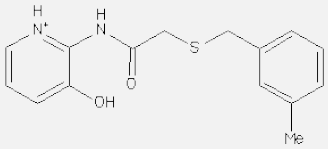
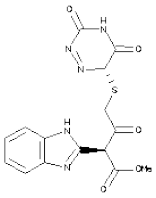
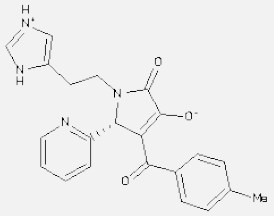
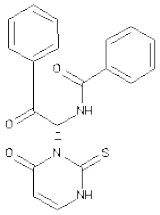
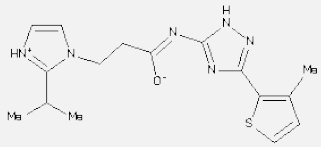
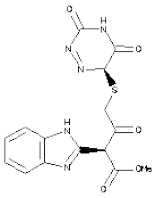
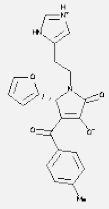
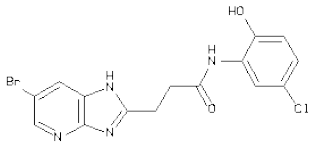
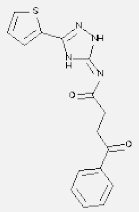
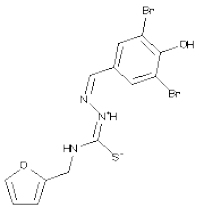
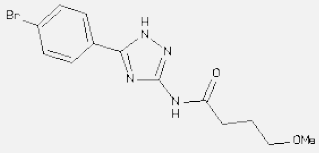
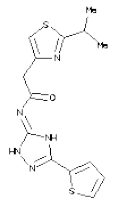
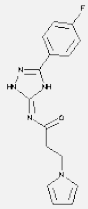
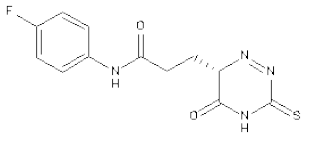
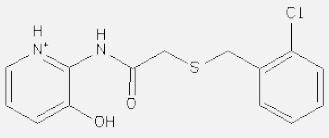
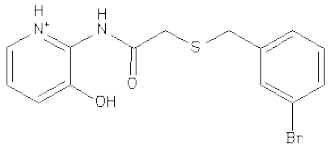
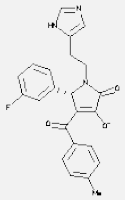
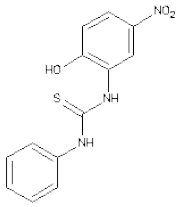
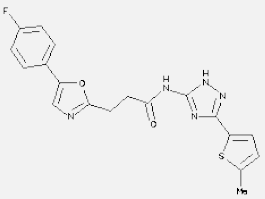
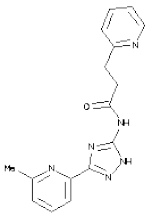
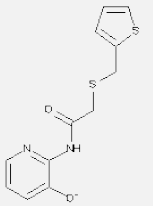
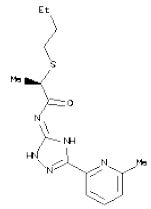
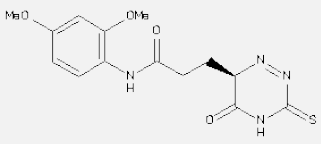
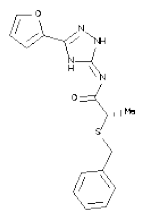
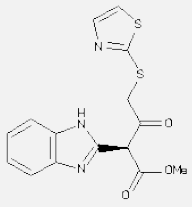
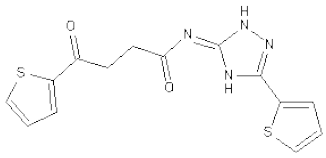
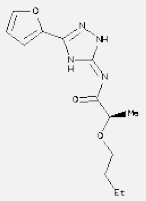
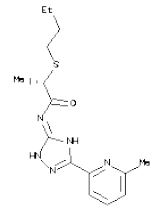
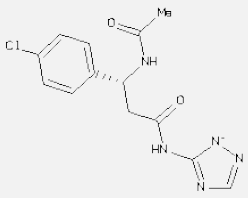
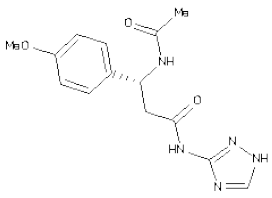
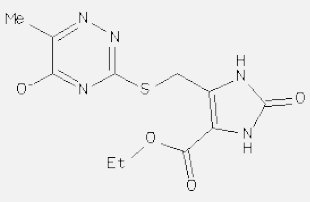
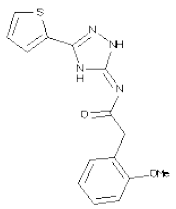
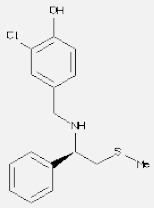
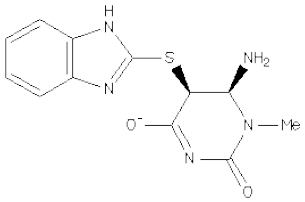
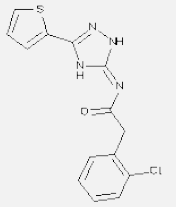
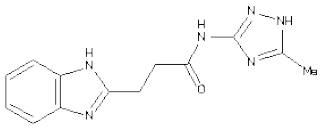
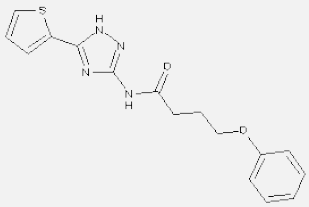
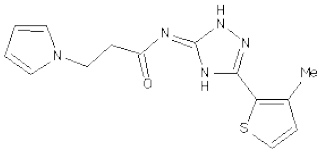
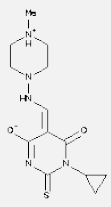
ZINC08767570		83.4334
ZINC19201999		80.684
ZINC36615464		78.1994
ZINC04429579		69.4876
ZINC06812429		69.4267
ZINC33882761		69.3729
ZINC04950591		68.6864

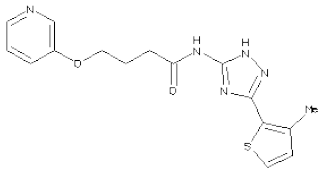
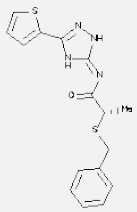
ZINC77043675		66.6792
ZINC21901592		65.8107
ZINC63864597		65.788
ZINC13640678		65.4436
ZINC00820548		65.0052
ZINC73765572		64.899
ZINC63864595		64.8686
ZINC36612940		64.086

ZINC79476872		63.1224
ZINC77043364		62.8608
ZINC59751389		62.8121
ZINC76205095		62.7797
ZINC75939883		62.6843
ZINC73567406		62.6517
ZINC39764084		62.2745
ZINC21901841		62.0672

ZINC21901619		61.9462
ZINC36612995		61.9171
ZINC13764028		61.6504
ZINC50006746		60.9812
ZINC63340125		60.8676
ZINC21901804		60.8322
ZINC80347996		60.6998
ZINC39764053		60.6541

ZINC79814733		60.443
ZINC64287586		60.3924
ZINC80013062		60.0611
ZINC65618174		60.0231
ZINC80347993		59.8911
ZINC48356335		59.5209
ZINC26259343		59.3628
ZINC03117445		58.686

ZINC77043442		58.5097
ZINC76769880		58.4699
ZINC38767349		58.1611
ZINC77043379		58.1478
ZINC72403879		58.0855
ZINC77043736		58.0223
ZINC73765612		58.0059
ZINC20328834		57.8514

ZINC80333146		57.7327
ZINC80013525		57.6794

Quantum Mechanical and Molecular Mechanical (QM/MM) Docking

Although compounds A1 and A2 are structurally similar, their best-scored poses from the virtual screening campaign were dissimilar. That's possibly due to CDOCKER's lack of force field parameterization for metalloproteinases. In order to overcome this problem and shed some light on the possible binding modes of compounds A1 and A2, re-docking studies were performed using the protocol "CDOCKER with QM Charges" as implemented in Pipeline Pilot with default parameters.

This protocol contains three main steps. First step consists of the initial placement of the ligand in the binding site using CDOCKER (MM) docking program, followed by a RMSD filter procedure to ensure that initial ligand poses (predicted binding modes) are diverse. In step two, ligand atomic charges are calculated for each pose by quantum mechanical methods (QM) in the presence of the enzyme. More specifically, QM charges calculation is performed using dmol3 with the PBE functional. Lastly, in the third step the ligand is re-docked with CDOCKER considering atomic ligand charges calculated previously.

The best scored QM/MM docked pose for each compound were depicted in Figure 1. Reported CDOCKER energies are -80.93 and -85.83 for compounds A1 and A2 respectively.

Enzyme-ligand interactions

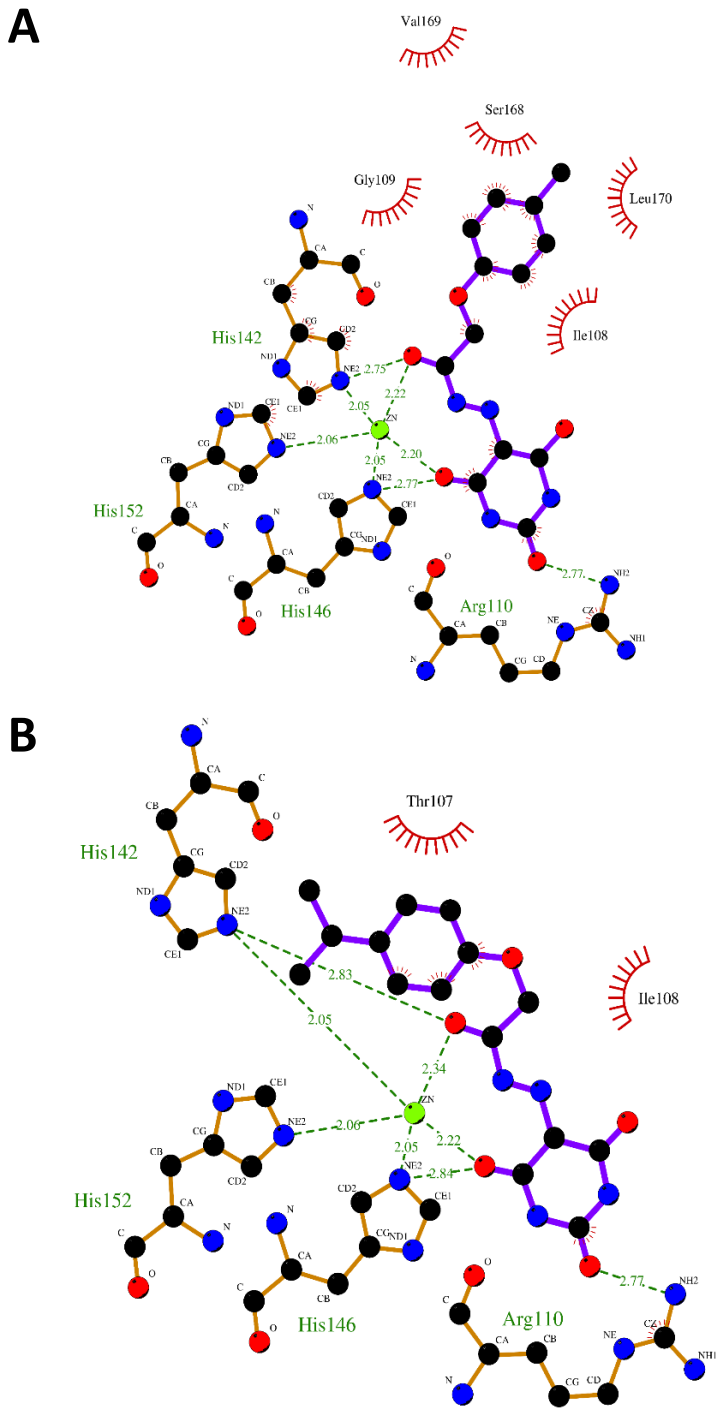


Figure S3. 2D-interaction diagram for compounds A1 and A2 plotted using LIGPLOT+[9]. Red arcs represent hydrophobic interactions. Hydrogen bonds, metal and charged interactions are indicated by green dashed lines. Ligands and enzyme amino acid residues are depicted in purple and orange, respectively. C, N, O and ZN atoms are colored in black, blue, red and green respectively. (A) A1 - ZINC06812429; (B) A2 - ZINC08767570. Π-stacking interactions are not depicted by LIGPLOT+, even though they do constitute an important interactive element among A1 ligand and enzyme.

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