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Structure Based Virtual Screening for Discovery of Novel Human Neutrophil Elastase Inhibitors

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Virtual Screening Methods

The crystal structure of HNE in complex with a peptide chloromethyl ketone inhibitor at 1.84 Å resolution (PDB code: 1HNE)[22] was used for docking calculations. To prepare the enzyme for the docking studies, the inhibitor, methoxysuccinyl-Ala-Ala-Pro-Ala chloromethyl ketone, MSACK, and crystallographic waters included in the 1HNE structure were removed. Hydrogen atoms were then added and the protonation states were assigned using the Protonate-3D tool within the MOE 2009.10 software package; energy was minimized using MMFF94x forcefield.[19]

AutodockVina 1.1:

For docking calculations the Autodock Tools package was used to prepare PDBQT input files for receptor and ligands. Grid box center was defined with the coordinates from HNE Ser195 oxygen atom, and grid box size was defined x= 30.00 Å, y = 21.75 Å and z = 30.00 Å. Exhaustiveness was defined 8 and maximum number of binding modes to generate was 9. Calculations were performed at iMed.UL scientific cluster facilities. The molecules were ranked by predicted binding affinity and the best 200 000 were kept for further refinement.

GOLD 5.0:

Binding site was defined by the HNE Ser195 oxygen atom and a 15 Å radius was defined for active site. GoldScore scoring function was used with the number of GA runs set to 50 and search efficiency was defined to be 30% (screening mode) or 100% (for final refinement). Standard default settings mode was used - number of islands was 5, population size of 100, number of operations was 100 000, a niche size of 2, and a selection pressure of 1.1. Structures were selected by GoldScore ranking. Six GOLD 5.0 licenses were used for calculations.

Chemical Suppliers

The compounds were acquired from established suppliers such as Asinex (**1**-**9**), Chembridge (**10**-**13**), Chemical Block (**14**, **15**), EMC Microcollections (**16**, **17**), Enamine (**18**-**21**), InterBioScreen (**22**-**26**), Life Chemicals (**27**), and Maybridge (**28**).

Pharmacological Methods

Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Assay buffer was 0.1 M HEPES pH 7.5 at 25 °C. Assays were performed in chromogenic or fluorimetric 96 well microplates and a Microplate Reader Tecan infinite M200 (Tecan, Switzerland). Controls were performed using enzyme alone, substrate alone and enzyme with DMSO and also with positive control (MeOSuc-Ala-Ala-Pro-Ala-CMK, Calbiochem, Germany for HNE and PPE; Cathepsine G inhibitor I, Calbiochem, Germany, for Cathepsine G; and 3,4-Dichloroisocoumarin, Calbiochem, Germany, for Proteinase 3).

Computing the log of inhibitors concentrations versus the percentage of activity was performed using the GrafPad programme and the IC_{50} values were determined by non-linear regression analysis. Assays were performed in triplicate and data presented as the mean and the standard deviation.

Human neutrophil elastase activity by chromogenic 96 well microplate assay

HNE inhibition activity were carried out in 200 µL assay buffer containing 50 µL of 80 nM HNE (Merck, Germany, from stock solution 2 µM in 0.05 M acetate buffer, pH 5.5) in assay buffer, 95 µL assay buffer and 5 µL of each concentration of tested inhibitors. After a period of 30 minutes of incubation at 25ºC the reaction was initiated by the addition of 50 µL of 4mM chromogenic substrate (N-MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide, Sigma, UK) in assay buffer, and activity was monitored at 410nm for 60

min, at 25ºC. Due to solubility issues for more concentrated solutions, 90 µL assay buffer were used instead of 95 µL while 5 µL of DMSO were to all solutions used for progress curve evaluation, activity was monitored at 410nm at 25 ºC for 120 min.

Porcine pancreatic elastase (PPE) Inactivation by the Incubation Method

The inhibition of PPE was assayed by incubation method, 5 µl of inhibitor solution in DMSO was incubated at 25 ºC with 155 µL of assay buffer, and 20 µL of 50 µM PPE solution in HEPES buffer. After 30min of incubation at 25ºC the reaction was initiated by the addition of 20 µL of fluorogenic substrate to final concentration of 200 µM (MeO-Suc-Ala-Ala-Pro-Val-AMC, Merck, Germany) and activity was monitored (excitation 380 nm; emission 460 nm) for 30 min, at 25ºC .

Inhibition Assay for Cathepsin G

Inactivation of Cathepsin G (Calbiochem cat # 219373) was studied at 25 ºC using the progress curve method. A Chromogenic assay for the Cathepsin G, Human Neutrophil (Calbiochem, Germany) inhibition activity was carried out in 200 µL assay containing 5 µL of each concentration of tested inhibitors, 125 µL of assay buffer and 20 µL of 680 nM Cathepsin G (680 nM in 0.05 M acetate buffer, pH 5.5). After a period of 30 minutes of incubation at 25ºC the reaction was initiated by the addition of 50 µL of 3.4 mM chromogenic substrate (Suc-Ala-Ala-Pro-Phe-p-nitroanilide, Calbiochem, Germany) in assay buffer (stock solution 42.5 mM in DMSO), and activity was monitored at 410nm for 30 min.

Inhibition Assay for Proteinase 3

Inactivation of proteinase 3 (Calbiochem cat #539483) was studied at 25 ºC in 200 µL assay buffer containing 70 µL of 65 nM proteinase 3 in assay buffer (stock solution 650 nM in 0.05 M acetate buffer, 150 mM NaCl, pH 5.5), 50 µL assay buffer and 5 µL of

each concentration of tested inhibitors. The reaction was initiated by the addition of 75 μL of 10 mM chromogenic substrate (N-MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide, stock solution 50 mM in DMSO, Sigma, UK) in assay buffer, and activity was monitored at 410 nm for 60 min, at 25 ºC.

Chemical stability at pH 7.4

Chemical stability was determined for solutions of compound **19** (100 μM) and **27** (50 μM due to solubility issues) in phosphate buffer (pH 7.4). Aliquots were taken in regular times and analysed by HPLC.

Stability in human plasma

Human plasma was obtained from the pooled, heparinised blood of healthy donors, and was frozen and stored at -20ºC prior to use. For the stability assay, the compounds (10 μL of a 10^{-2} M stock solution for compound **19** and 5 μL of a 10^{-2} M stock solution of compound **27** due to solubility issues), were incubated at 37 ºC in human plasma that had been diluted to 80% (v/v) with phosphate buffer pH 7.4. Aliquots were taken in regular times, the reaction was stopped by addition of MeCN and the samples were vortexed and centrifugated for 10 min and analysed by HPLC.

Stability towards microsomal activity

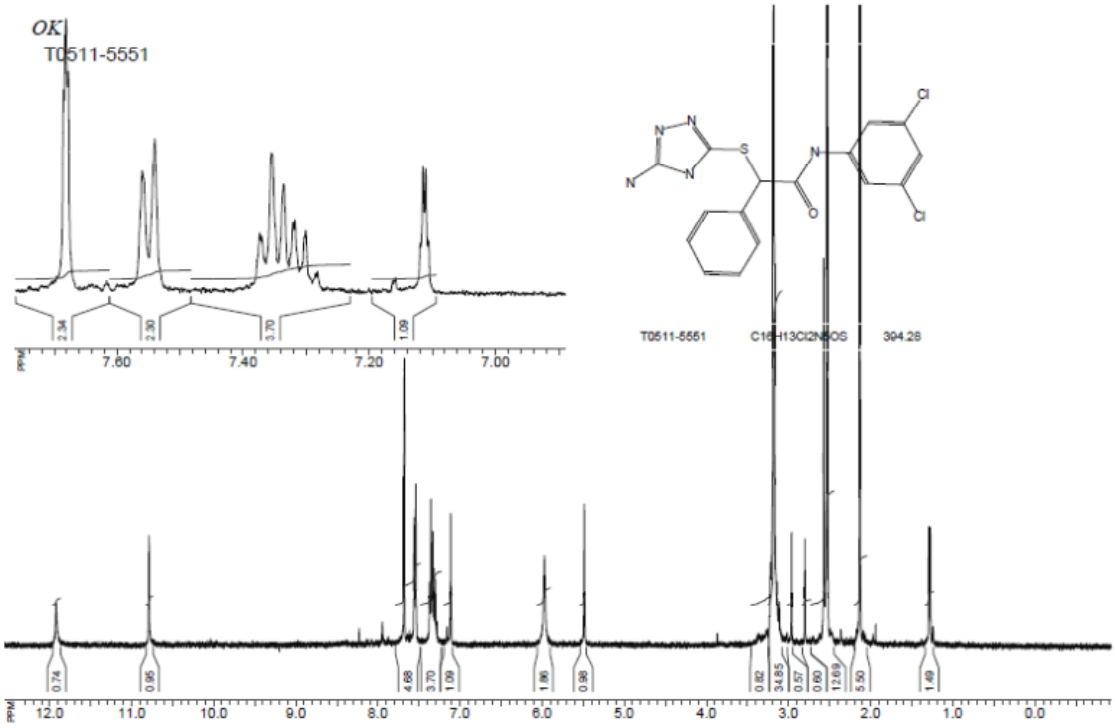
Stability was assayed against Rat Pooled Liver Microsomes Male (Sprague-Dawley) 20 mg/mL from BD Gentest™. A typical incubation medium was prepared, containing 10 μL of microsomal protein, 920 μL of phosphate buffer (pH 7.4) and NADPH generating system [50 μL of solution A ($NADP^+$ and G6P) and 10 μL of solution B (G6PDH), both from BD Gentest™], in a 37 ºC thermostatic bath. Reaction was started by addition of the substrate (10 μL of a 10^{-2} M stock solution for compound **19** and 5 μL of a 10^{-2} M stock solution of compound **27** due to solubility issues). Aliquots were taken in regular

times, the reaction was stopped by addition of MeCN and the samples were vortexed and centrifugated for 10 min and analysed by HPLC. For the assays in the absence of NADPH generating system, 980 µL of phosphate buffer (pH 7.4) were used.

HPLC system: Merck Hitachi LaChrom – L-7100 pump; Merck Hitachi D-2500A Chromato-Integrator; LiChroCart Purospher RP-18 column (5 μm, 250-4 mm); Shimadzu SPD-6AV UV-VIS spectrophotometric detector (at 220 nm); MeCN/H_2O (50/50, v/v) was used as mobile phase (1mL/min); 20 µL injection volumes.

NMR Spectra for active compounds:

Compound 19



Compound 27