Supporting Information: A Comparative Assessment Study of Known Small-Molecule GPVI Modulators

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Experimental Details: Chemical Synthesis

General Procedures

All chemicals used for synthesis were obtained from commercial suppliers and were used without prior purification. Analytical TLC was performed using TLC silica gel 60 F_{254} aluminium plates (Merck, Germany), and were visualised with UV radiation (254 nm). Compounds purified by normal phase chromatography were loaded onto pre-packed Biotage SNAP KP-Sil (10 – 250 g) silica columns, and were run on an Isolera Four flash purification system (Biotage, Uppsala, Sweden).^{1}H NMR spectra were recorded at 400 MHz, and ^{13}C NMR at 100 MHz using a Bruker Avance III HD spectrometer. High resolution mass spectroscopy (HRMS) was performed on an Agilent 1260 HPLC with diode array detector coupled with an Agilent 6120 single quadrupole mass spectrometer (Santa Clara, USA). Optical rotation measurements were determined using a Schmidt+Haensch polartronic H532 polarimeter (Berlin, Germany). Samples were measured ($n \geq 3$) in a 100 mm cell, at room temperature using the sodium D-line (*c* in g/100 mL).

Chiral HPLC analysis was performed on an Agilent 1290 Infinity UHPLC with diode array detection (Santa Clara, USA). Chromatographic separations were achieved using a Daicel CHIRALPAK AS-H chiral column (Chiral Technologies, Daicel Corporation, Pennsylvania, USA) with an isocratic mobile phase of 95% Hexane 5% IPA at a 1.0 $mLmin^{-1}$ flow rate.

All the final compounds were purified to >95% purity as determined by HPLC analysis, which was performed using an Agilent 1290 Infinity HPLC system (Agilent, Santa Clara, United

States), with a diode array detector. Chromatographic separations were performed using a Supeclo C18 (2.1 × 50 mm i.d., 2.7 μm particle size; Supelco, Bellefonte, United States) at 25 °C. The mobile phases were water/acetonitrile with 0.1% trifluoroacetic acid (A and B respectively). A gradient was used starting at 95 % of A and 5 % of B over 5 min, ending with 5 % A and 95 % B at a flow rate of 0.5 mL/min. The DAD recorded the chromatogram at a wavelength of 254 nm.

2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (7)

The compound was synthesised according to a previously described procedure with some deviations.[1] Methyl (3*S*)-2,3,4,9-tetrahydro-1H-β-carboline-3-carboxylate (100 mg, 0.43 mmol) and NH_3 (7M in MeOH, 12.4 mL, 86.9 mmol) were stirred and heated under N_2 for 24-36 h at 60 ˚C until TLC (R_f = 0.65 (10% MeOH in DCM) showed complete conversion. The reaction mixture was then concentrated under reduced pressure to yield the crude product as a yellow/brown gum, which was then purified by column chromatography with a gradient of 0 to 20% MeOH in DCM. The appropriate fractions were combined and concentrated under reduced pressure to furnish **6** as a gummy yellow solid (54 mg, 0.25 mmol, 58%). ^{1}H NMR (Methanol-d_4, 400 MHz) δ 7.41 (d, *J*=7.8 Hz, 1H), 7.29 (dd, *J*=10.1, 3.1 Hz, 1H), 7.07 (t, *J*=8.2, 7.1 Hz, 1H), 6.99 (t, *J*=7.3 Hz, 1H), 4.21 – 4.07 (m, 2H), 3.76 (dd, *J*=10.8, 4.8 Hz, 1H), 3.15 (m, 1H), 2.85 (m, 1H); ^{13}C NMR (Methanol-d_4, 100 MHz) δ 176.9, 138.0, 131.8, 128.2, 122.4, 120.0, 118.4, 112.0, 107.3, 57.8,

42.8, 26.1; $[\alpha]_D^{20}$ -64.30; HRMS *m/z* found: $[C_{12}H_{14}N_3O]^+$ = 216.1131; $[C_{12}H_{14}N_3O]^+$ requires = 216.1137 m/z.

*(3S)-2-(4-methoxybenzene-1-sulfonyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (**5**)*

The compound was synthesised according to a previously described procedure with minor deviations.[1] A solution of 4-methoxybenzenesulfonyl chloride (198 mg, 0.96 mmol) and anhydrous DCM (5 mL) was added dropwise to a stirred solution of 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (172 mg, 0.80 mmol), anhydrous DCM (5 mL) and TEA (0.14 mL, 1.20 mmol) at 0 ˚C under N_2. The reaction mixture was stirred at room temperature for 6-12 h until TLC (R_f = 0.42 (10% MeOH in DCM) showed complete conversion. The reaction mixture was then concentrated under reduced pressure to yield the crude product, which was then was purified using column chromatography with a gradient of 0-10% MeOH in DCM. The appropriate fractions were combined and concentrated under reduced pressure to yield a sticky yellow product, which was triturated with MeOH to furnish **5** as a white solid (61 mg, 0.16 mmol, 20%). ^{1}H NMR (Chloroform-*d*, 400 MHz) δ 7.76 – 7.67 (m, 3H), 7.38 (d, *J*=7.8 Hz, 1H), 7.27 (m, 1H), 7.14 (t, *J*=7.5 Hz, 1H), 7.06 (t, *J*=7.4 Hz, 1H), 6.84 (d, *J*=8.8 Hz, 2H), 6.58 (s, 1H), 5.43 (s, 1H), 4.98 – 4.87 (m, 2H), 4.58 (d, *J*=16.1 Hz, 1H), 3.77 (s, 3H), 3.49 (m, 1H), 2.49 (dd, *J*=15.8, 6.7 Hz, 1H);

^{13}C NMR (Chloroform-*d*, 100 MHz) δ 171.4, 163.5, 136.3, 130.4, 129.3, 127.0, 122.5, 119.9, 118.6, 114.6, 110.9, 107.2, 55.7, 55.1, 40.9, 20.1; $[\alpha]_{20}^{D}$ +30.74; chiral HPLC = 9.64 mins (96% *S* enantiomer); HRMS *m/z* found: $[C_{19}H_{19}N_3O_4SNa]^+$ = 408.0986; $[C_{19}H_{19}N_3O_4SNa]^+$ requires = 408.0994.

Purchased Compounds

All purchased compounds were reported to be >99% pure by their respective sellers. HPLC chromatograms for these compounds are presented in Supporting Information Figure S1.

4-(2-hydroxy-5-(prop-2-en-1-yl)phenyl)-2-(prop-2-en-1-yl)phenol (**1**). Purchased as Honokiol from MedChemExpress, product ID HY-N0003/CS-1696, batch ID 10058.

(7R,14R)-ent-7,14,dihydroxy-(-)-kaur-16-en-3,15-dione (**2**). Purchased as Glaucocalyxin A from MedChemExpress, product ID HY-N2112/CS-0018634, batch ID 79859.

(2-butyl-4-chloro-1-(4-(2-(2H-tetrazol-5-yl)phenyl)benzyl)-1H-imidazol-5-yl)methanol (**3**). Purchased as Losartan Potassium from Sigma Aldrich, product ID PHR1602-1G, batch ID LRAB1233.

(2E)-N-(2-((3-(dimethylamino)propyl)sulfanyl)phenyl)-3-phenylprop-2-enamide (**4**). Purchased as Cinanserin Hydrochloride from TOCRIS, product ID 0460, batch ID 2A/196614.

Experimental Details: Functional Assays

Human Blood Donation

Human whole blood was drawn after prior consent from drug-free, healthy volunteers using a 21 gauge butterfly needle from the medial cubital vein, and was collected into ACD vacutainers (trisodium citrate (22.0 mg/mL), citric acid (8.0 mg/mL) and dextrose (24.5 mg/mL)) from BD Biosciences (Becton, Dickinson and Company, New Jersey, USA) or S-Monovettes 1.6 mL coated in hirudin (SARSTEDT, Leicester, UK) with a 2 mL discard. Phlebotomist donned full PPE and donor wore a face covering in accordance with University of Leeds COVID-19 specific phlebotomy protocol. These studies were approved by the University research ethics committee, reference HSLTLM/12/045 and MREC19-006 and were conducted in accordance with the Declaration of Helsinki. Once prepared, platelets were stored at 37 ˚C, and whole blood at room temperature without rotation for a maximum of four hours before disposal. To assess donor-to-donor variability three independent assay replicates were performed using blood from different donors for each experiment.

Light Transmission Aggregometry

Isolation of Human Platelets

ACD whole human blood was centrifuged at 100 g for 20 min, acceleration 7, brake 0, at room temperature. PRP was taken using a Pasteur pipette leaving the buffy coat layer undisturbed. PRP

was split between two 15 mL conical polypropylene centrifuge tubes, 200 nM PGI_2 was added and mixed by gentle inversion. PRP with PGI_2 was centrifuged for 10 min at 1000 g, acceleration 7, brake 2 at room temperature for 10 min. Platelet pellet was resuspended in 1 mL Tyrode's Buffer supplemented with glucose (TBG) (150 mM NaCl, 5 mM HEPES (4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid), 0.72 mM Na_2HPO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$ hexahydrate, 5.6 mM D-glucose, pH 7.4) and made up to 5 mL using 0.5 mL ACD (20.5 g/L D-glucose, 8.79 g/L trisodium citrate, 4.24 g/L sodium chloride, 0.59 g/L citric acid) and TBG, and supplemented with 200 nM PGI_2. Platelets were pelleted by centrifugation at 1000 g for 10 min at room temperature, acceleration 7, brake 2. The platelet pellet was resuspended in pre-warmed (37 ˚C) TBG (1 mL), pooled and counted using a Beckman Coulter Z Series Coulter counter, and adjusted to working concentration of 2.5 x 10^8 p/mL using TBG. All centrifugation steps were carried out in a Hettich ROTANA 460 centrifuge with aerosol capped swing-out buckets. The sample was left for a further 10 min following completion of centrifugal cycle to allow any aerosols to dissipate.

Assay

A turbidimetric method was employed to assess platelet aggregation using a four channel

aggregometer (AggRAM, Helena Biosciences, Gateshead, UK). The washed platelets (250 µL, 2.5 × 10^8 platelets/mL) and compound (10, 20 or 30 µM, n =1) or an isovolumetric solvent control (0.001, 0.002 and 0.0033% DMSO respectively) were pre-mixed (37 ˚C, 1000 rpm) for 3 min. The aggregometer was then baselined with TBG, and the platelet/compound mixture was recorded for 1 min before 2 µg/mL CRP-XL (Dr. R. W. Farndale, Department of Biochemistry, University of Cambridge, UK) was added. Data was recorded for 9 min and was expressed as %aggregation relative to the TBG buffer baseline. The results were normalised against a DMSO control and were presented in GraphPad Prism (version 8.4.2.).

Flow Cytometry

Sample Preparation

Human whole blood was pre-incubated (37 ˚C, 5 min) with compounds (n = 3) diluted in TBG to a final concentration of 100 µM, before adding to agonist-stain mix (1 µg/mL CRP-XL, 150 ng/mL CD62p-PE, and 150 ng/mL CD42b-APC). Blood, compound and stain mix were incubated at room temperature for 20 min in the dark, and then samples were fixed with 10x volume of 1 % PFA-TBG. Stain-mix antibodies were sourced from BD Biosciences (Becton, Dickinson and Company, New Jersey, USA).

Data Collection and Analysis

Changes in P-selectin expression were determined by measuring the differences in the mean fluorescence intensity (MFI) of the CD62p-PE antibody using a Beckman Coulter CytoFLEX flow cytometer. Data were collected by gating for physical platelet forward and side scatter properties (10^4-10^5), and 10,000 events were recorded. Following data collection, the samples were then further gated for the GPIbα platelet positive (CD42b+) population only using the 640 660-20A channel. The median fluorescence intensity from CD62p-PE was collected from the CD42b+ gate using the 488 585-42A channel. Results were exported to excel and percentage activation for the compounds was then calculated using custom KNIME (version 4.1.3) workflows relative to the median of the DMSO controls. Data are presented in GraphPad Prism.

Multiplate Aggregometry

Human whole blood aggregation was determined by MEA using Diapharma's Multiplate analyser (Cincinnati, USA). Hirudin-whole blood samples were gently mixed to resuspend before testing. Normal saline with 100 μM compound were incubated with hirudin-whole blood for 3 min at 37 ˚C prior to agonist addition. Platelet aggregation in the presence of compound or DMSO ($n \geq 3$) was assessed using either: collagen (100 μg/mL, Helena Biosciences, Gateshead, UK); TRAP-6 (32 μM); ADP (6.5 μM) or arachidonic acid (0.5 mM) (Roche Diagnostics, Mannheim, Germany). The results were expressed as arbitrary aggregation units and plotted against time

(recorded for 6 min), defining platelet function as AUC. Percentage aggregation for the compounds was calculated using custom KNIME workflows relative to the average of the DMSO controls, and results were presented using GraphPad Prism.

Experimental Details: Binding Assays

GPVI-Fc Expression and Purification

GPVI-Fc construct was expressed and purified, as following methods previously described.[2] In short, GPVI residues S2-T183 were sub-cloned into Sigplg+ mammalian expression vector (R&D systems, Minneapolis, USA) between a CD33 signal sequence (N-terminus) and an FXa cleavage site which was followed by a human IgG1-Fc sequence (C-terminus). Transient expression of GPVI-Fc was achieved by transfection of HEK293T cells with polyethylenimine hydrochloride (Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany). GPVI-Fc was produced by transfected cells and secreted into the medium from which the protein was purified by protein-A affinity chromatography. Eluted proteins were further purified by size exclusion chromatography using Superdex S200.

MST

FITC labelled GPVI-Fc was prepared by incubating 16 μM GPVI-Fc in buffer plus 0.1 M sodium carbonate, with a 2-fold molar excess of FITC (freshly-dissolved in DMSO at 1 mg/mL) on ice for a maximum of 2 hours. Excess FITC was removed using illustra MicroSpin G-25 columns (GE

Healthcare) that were equilibrated with 10 mM HEPES, 140 mM NaCl, pH = 7.4. To determine the concentration of FITC labelled GPVI-Fc to use in the assay, serially-diluted samples of FITC labelled GPVI-Fc were prepared with the afore mentioned buffer. Fluorescent signal intensity of each sample was determined using a Monolith NT.115 Series (NanoTemper, Munich, Germany) microscale thermophoresis instrument with the excitation/emission wavelength set to 493/521 (blue channel) and 20% LED power. The sample with a signal intensity of approximately 600 and gaussian peak was chosen, typically 0.4 or 0.2 μM FITC labelled GPVI-Fc.

FITC labelled GPVI-Fc stock was diluted to the previously determined concentration, and the GPVI modulators (10 mM stock in DMSO) were serially-diluted (n = 1 for each compound concentration) using a buffer containing 10 mM HEPES, 140 mM NaCl, 2 mg/mL BSA and 5% DMSO. Binding assays were prepared by mixing 10 μL of the GPVI modulators and 10 μL of FITC labelled GPVI-Fc. Reactions were incubated at room temperature for 15 min and were then loaded into standard treated capillaries. Data were collected at room temperature with the excitation/emission wavelength set to the blue channel, 20% MST and 40% LED power. Three independent assay replicates were performed, each with a new batch of FITC labelled GPVI-Fc. To calculate FNorm, a temperature-related intensity change trace was recorded for each data point,

and was normalised to start at 1. The fluorescence value from the heated state was then divided by the cold state to give FNorm as parts per thousand (0/00). The compound FNorm results were averaged and compared against DMSO negative controls (prepared in the same manner) by plotting in GraphPad Prism.

STD NMR

GPVI-Fc was dialysed into Phosphate Buffered Saline ((PBS), pH = 7.4) using a Pur-A-Lyser mini dialysis kit (Sigma Aldrich), and then was diluted to 2.22 mg/mL using PBS. All NMR samples were 250 µL within Wilmad-328 3 mm diameter NMR tubes, and contained 100 µM ligand in deuterated DMSO, 1 µM GPVI-Fc, 10% deuterium oxide (v/v) and PBS. NMR experiments were performed at 278 K using a Bruker AV4 NEO 11.75 T (500 MHz ^{1}H) NMR spectrometer equipped with a 5 mm TXI probe. Excitation sculptured perfect-echo water suppressed ^{1}H NMR spectra were recorded with 64 scans for each sample. Saturation Transfer Difference (STD) NMR experiments used 20 ms Eburp2 pulses applied as a saturation comb to acquired data from samples in the presence and absence of protein. STD experiments were recorded in a pseudo-2D format, with a total of 5120 scans, where data from 'on' (-2 ppm) and 'off' (-30 ppm) saturation was interleaved. Data processing generates two 1D STD NMR spectra from this data; one control and one difference spectrum, each utilising 2560 scans. STD

amplification factors were calculated by dividing the integrals by the reference spectra: (STD-AF) = I/I_o. NMR data processing was completed with TopSpin (4.0.7., Bruker), and figure images were generated in MestReNova (12.0.0) with HDO referenced at 5.006 ppm.[3]

Molecular Modelling

Molecular modelling studies were performed with Schrodinger's Maestro (version 2019.12.4). Images were generated with Schrodinger's PyMOL (version 2.088) or Chemical Computing Group's MOE (version 2019.01).

GPVI crystal structures (PDB ID: 2GI7, 5OU8 and 5OU9) were downloaded from the RCSB Protein Data Bank and were prepared using Schrodinger's 'Protein Preparation Wizard'. Briefly, bond orders were assigned, hydrogen atoms added and protonation states for basic and acidic residues based on residue pKa's at pH 7.0 ± 2.0 were generated using the 'pre-process' function. The structure was then optimised using the 'optimise' function, and then an 'Impref' minimisation was performed using the OPLS3e force field to remove steric clashes and bad contacts.

Binding site predictions for GPVI were made on previously prepared monomers using Schrodinger's 'SiteMap' tool with the following parameters: at least 15 site points per reported site were required; 10 possible sites were reported; used a more restrictive definition of hydrophobicity; standard grid was used; site maps were cropped at 4 Å from nearest site point;

and shallow binding sites were detected. For 2GI7(A) only, a single binding region was evaluated by centring on the co-crystallised sulphate in the speculative GPVI ligand binding site.

The shape and properties of the active site for PDB ID: 2GI7(A) were mapped onto a grid using Schrodinger's 'Receptor Grid Generation' tool. The automatically generated grid size was used and was centred on the co-crystallised sulfate anion. The ligands were built in the 2D workspace by pasting SMILES strings, and were prepared for calculations using the OPLS3e force field at pH 7 ± 2 (for protonated ligands) or pH 7 ± 0 (for non-protonated ligands) with epik to determine ionisation states using Schrodinger's 'LigPrep'. For standard docking the 'ligand docking' wizard was used with SP/XP, and 5 poses were included in the post-docking minimization, but only 1 pose per ligand was written as an output file.

For induced fit docking the 'Induced Fit Docking' tool was used with the OPLS3e forcefield and standard protocol. The centre of the receptor was defined by picking the co-crystallised sulfate anion in PDB ID: 2GI7. Ligands with a length of ≤ 20 Å were docked, and their ring conformations were sampled. Receptor and ligand van der Waals scaling were both set to 0.5 and a maximum of 20 poses were generated in the initial round of docking. Residues within 5 Å of the predicted pose were refined and optimised. Ligands were then re-docked into the induced fit prepared protein

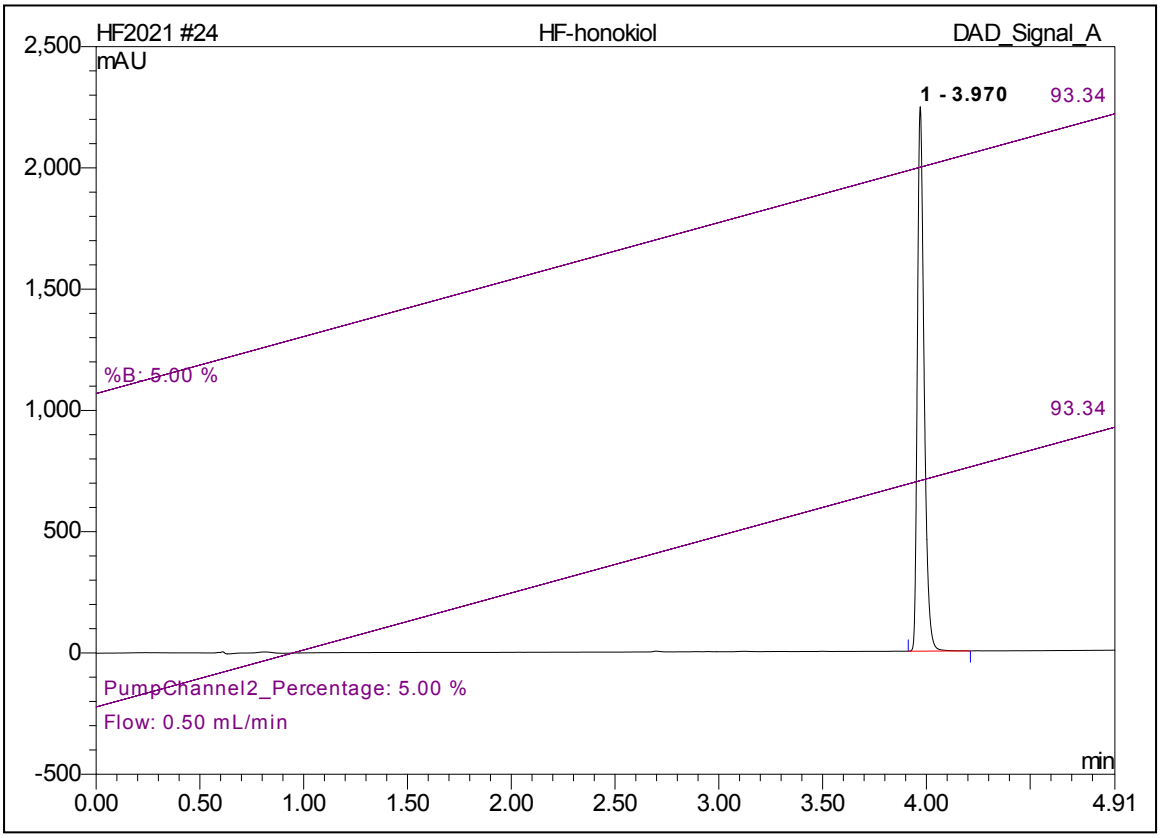
model either with SP or XP. Docking conditions were selected that yielded a pose with a potent docking score, and a reasonable distance between the GPVI residues Gln48 and Lys41, and the corresponding interacting phenyl protons of losartan (Supporting Information Figure S5).24 The distance between the docked ligands and protein residues were calculated using PyMOL. These poses were then filtered by considering the results from the STD NMR experiments reported herein.

Figure S1: HPLC Chromatograms for Purchased and Final Compounds

Honokiol

24 HF-honokiol

Sample Name:	**HF-honokiol**	*Injection Volume:*	**1.0**
Vial Number:	**P1:A1**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
Recording Time:	**12/01/2021 10:00**	*Sample Weight:*	**1.0000**
Run Time (min):	**4.91**	*Sample Amount:*	**1.0000**

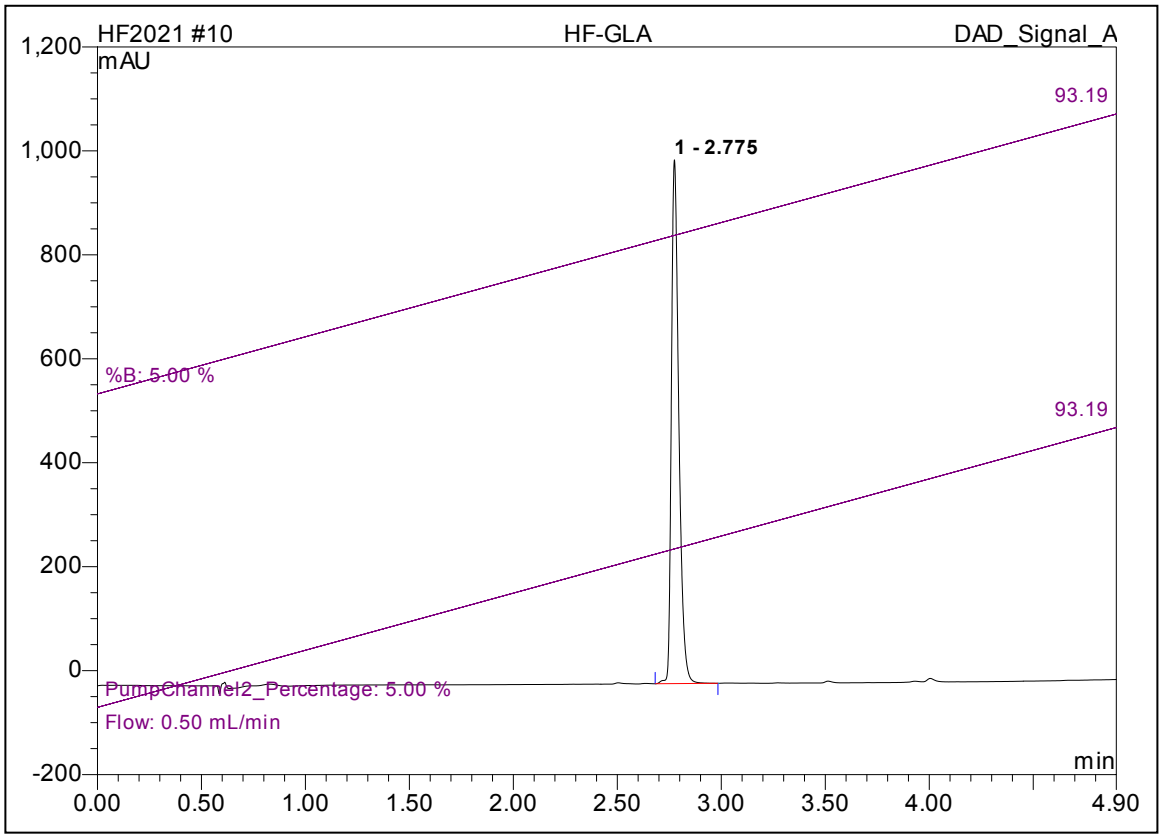


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3.97	Honokiol	2245.134	84.703	100.00	n.a.	BMB
Total:			2245.134	84.703	100.00	0.000	

GLA

10 HF-GLA

Sample Name:	**HF-GLA**	*Injection Volume:*	**5.0**
Vial Number:	**P1:A2**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
Recording Time:	**11/01/2021 11:12**	*Sample Weight:*	**1.0000**
Run Time (min):	**4.90**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.77	GLA	1007.682	41.821	100.00	n.a.	BMB
Total:			1007.682	41.821	100.00	0.000	

17 HF-Losartan

Sample Name:	**HF-Losartan**	*Injection Volume:*	**1.0**
Vial Number:	**P1:A3**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
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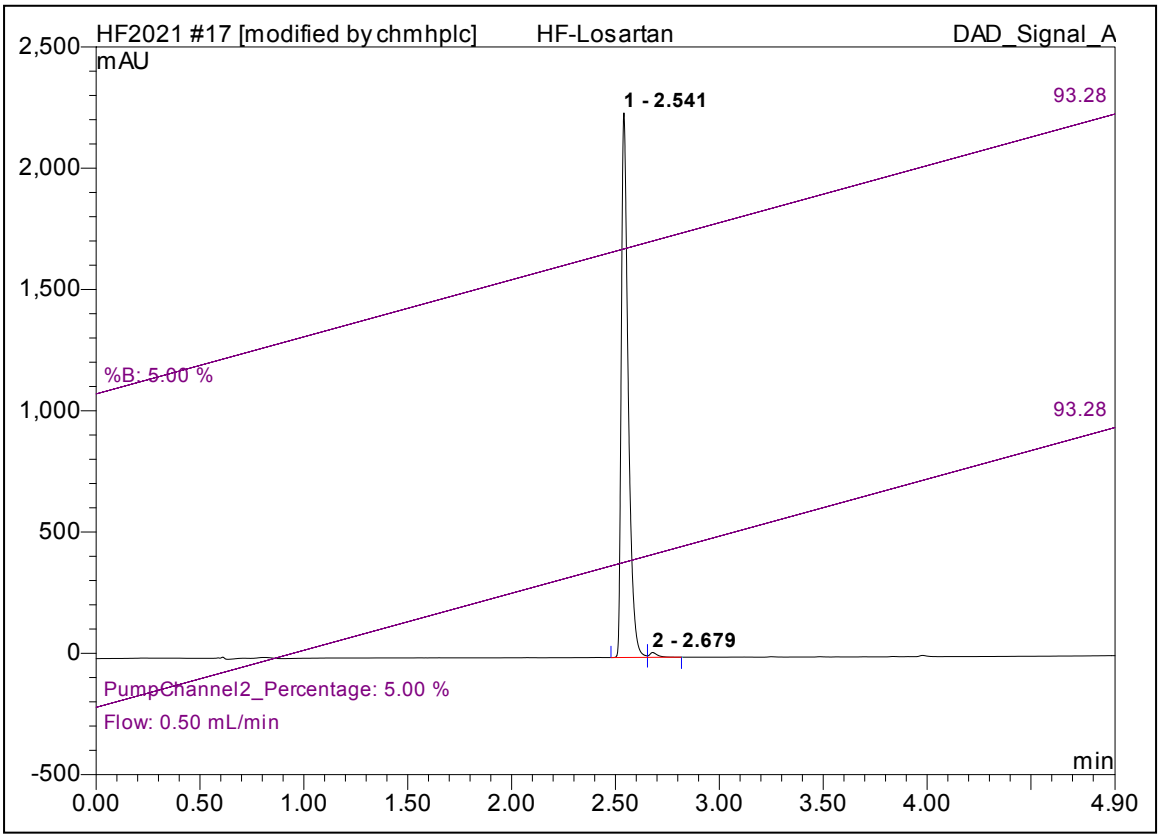
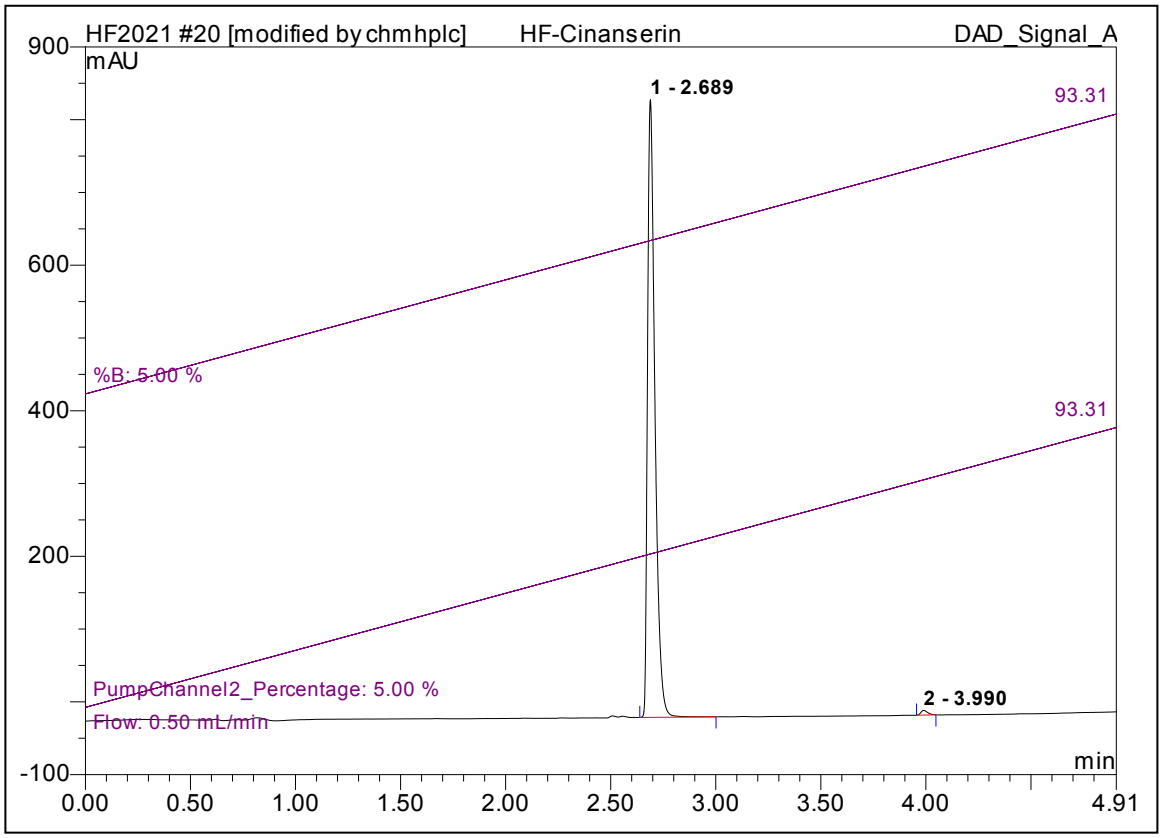
Run Time (min):	**4.90**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.54	Losartan	2245.218	87.280	99.02	n.a.	BM *
2	2.68	Impurity	19.903	0.868	0.98	n.a.	MB*
Total:			2265.121	88.147	100.00	0.000	

20 HF-Cinanserin

Sample Name:	**HF-Cinanserin**	*Injection Volume:*	**0.1**
Vial Number:	**P1:A4**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
Recording Time:	**11/01/2021 12:54**	*Sample Weight:*	**1.0000**
Run Time (min):	**4.91**	*Sample Amount:*	**1.0000**

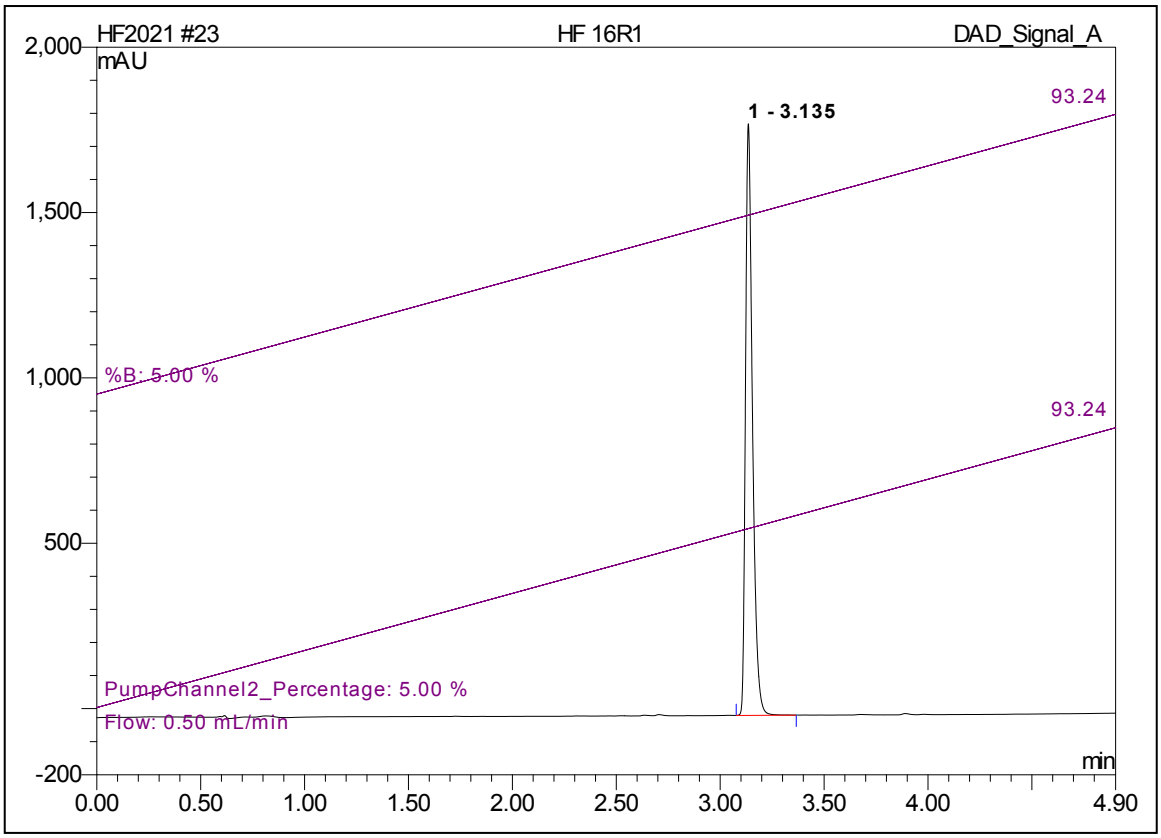


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.69	Cinanserin	849.001	34.746	99.32	n.a.	BMB
2	3.99	Impurity	6.393	0.239	0.68	n.a.	BMB*
Total:			855.394	34.985	100.00	0.000	

Compound 5

23 HF 16R1

Sample Name:	**HF 16R1**	*Injection Volume:*	**1.0**
Vial Number:	**P1:A2**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
Recording Time:	**12/01/2021 09:51**	*Sample Weight:*	**1.0000**
Run Time (min):	**4.90**	*Sample Amount:*	**1.0000**

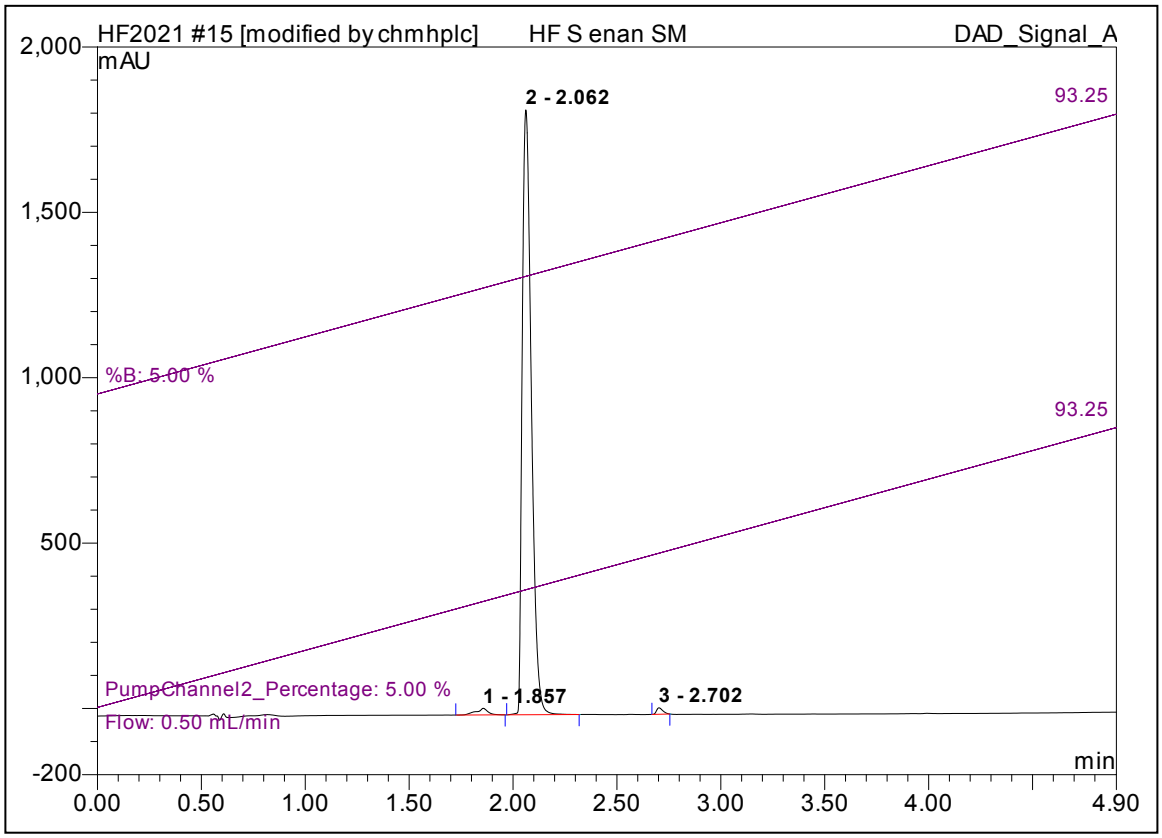


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3.14	Compound 5	1788.855	69.760	100.00	n.a.	BMB
Total:			1788.855	69.760	100.00	0.000	

Compound 6

15 HF S enan SM

Sample Name:	**HF S enan SM**	*Injection Volume:*	**5.0**
Vial Number:	**P1:A7**	*Channel:*	**DAD_Signal_A**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Rapid5-95+TFA Ascentis Express C18**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MartinH**	*Dilution Factor:*	**1.0000**
Recording Time:	**11/01/2021 11:43**	*Sample Weight:*	**1.0000**
Run Time (min):	**4.90**	*Sample Amount:*	**1.0000**



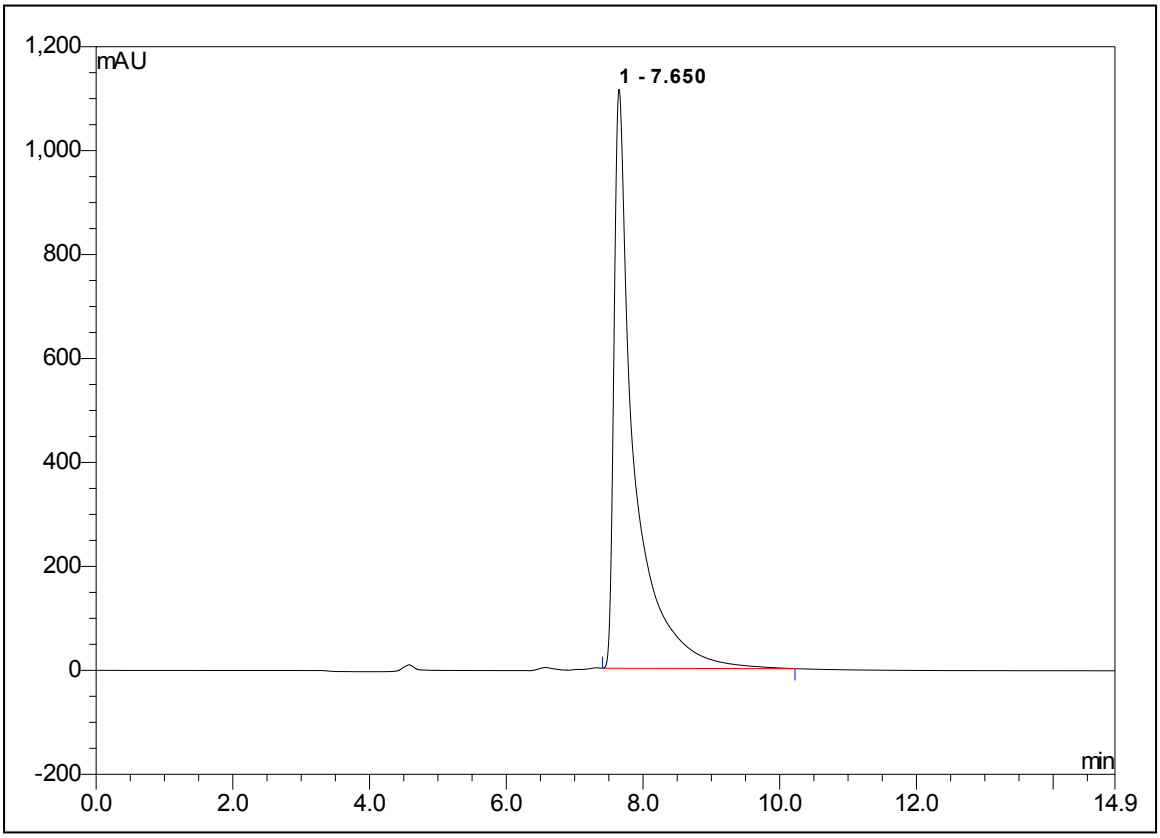
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	1.86	Impurity	20.023	1.331	1.40	n.a.	BMB
2	2.06	Compound 6	1828.705	93.201	97.89	n.a.	BMB
3	2.70	Impurity	19.463	0.682	0.72	n.a.	BMB*
Total:			1868.191	95.214	100.00	0.000	

Figure S2: Chiral HPLC Chromatograms

*Methyl (3S)-2,3,4,9-tetrahydro-1H-β-carboline-3-carboxylate (**6**)*

6 Holly Foster HF-1692-016-R1 Purchased SM 100%EtOH

Sample Name:	**Holly Foster HF-1692-016-R1 Purchased SM 100%EtOH**	*Injection Volume:*	**1.0**
Vial Number:	**P1:F5**	*Channel:*	**DAD_Signal_C**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**100%A2 15min 0,5ml min pos1 AS-H**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MH1**	*Dilution Factor:*	**1.0000**
Recording Time:	**06/07/2018 11:49**	*Sample Weight:*	**1.0000**
Run Time (min):	**14.90**	*Sample Amount:*	**1.0000**

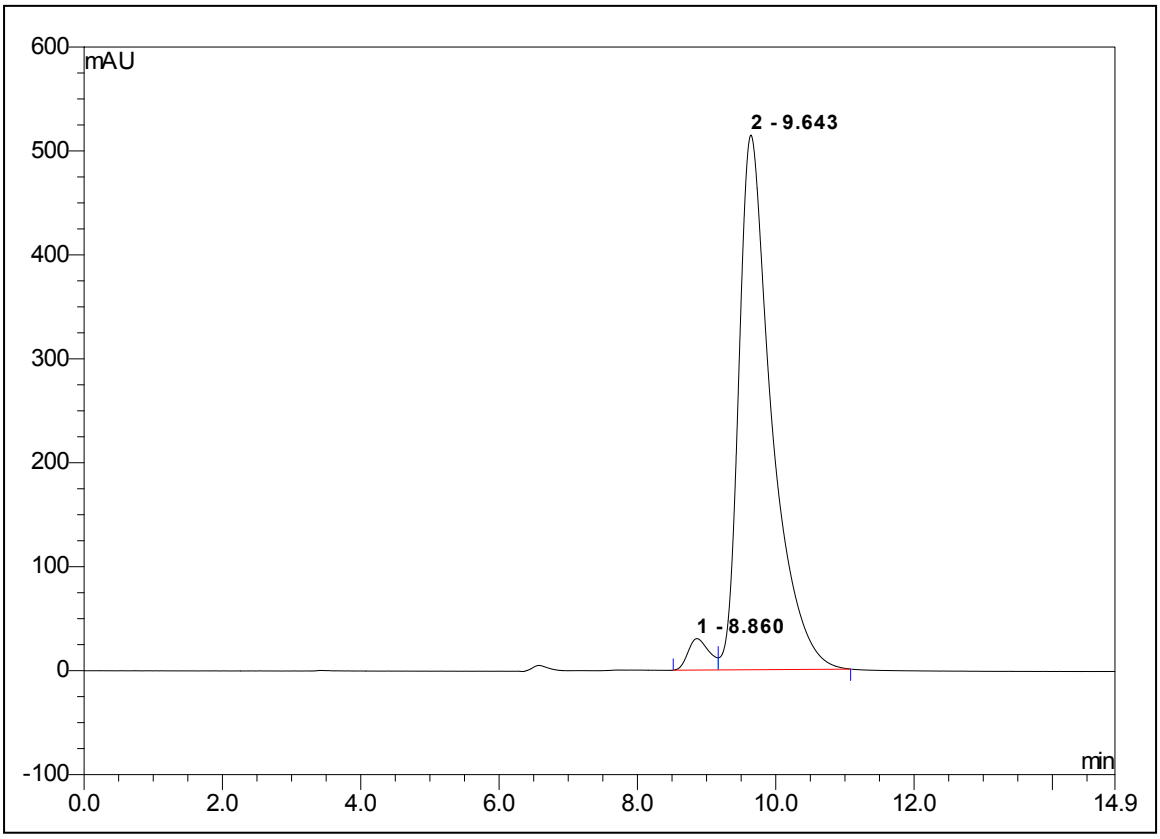


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	7.65	*S* Enantiomer	1114.018	377.089	100.00	n.a.	BMB
Total:			1114.018	377.089	100.00	0.000	

*(3S)-2-(4-methoxybenzene-1-sulfonyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (**5**)*

5 Holly Foster HF-1692-016-R1 00%EtOH

	Holly Foster HF-1692-016-R1		
Sample Name:	**100%EtOH**	*Injection Volume:*	**1.0**
Vial Number:	**P1:F4**	*Channel:*	**DAD_Signal_C**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**100%A2 15min 0,5ml min pos1 AS-H**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MH1**	*Dilution Factor:*	**1.0000**
Recording Time:	**06/07/2018 11:33**	*Sample Weight:*	**1.0000**
Run Time (min):	**14.90**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	8.86	*R* Enantiomer	30.278	10.712	3.66	n.a.	BM
2	9.64	*S* Enantiomer	514.388	281.682	96.34	n.a.	MB
Total:			544.667	292.394	100.00	0.000	

Figure S3: STD NMR No Protein Controls

STD-NMR results for the known modulators (100 μM) in the absence of the GPVI-Fc dimer. The top spectrum is a water supressed ^{1}H NMR. The protons that were found to interact with GPVI in the presence of the protein have been indicated to aid comparisons. The below spectrum is the corresponding STD NMR spectrum without the GPVI-Fc dimer. Solvent and other minor impurities have been indicated with an asterisk.

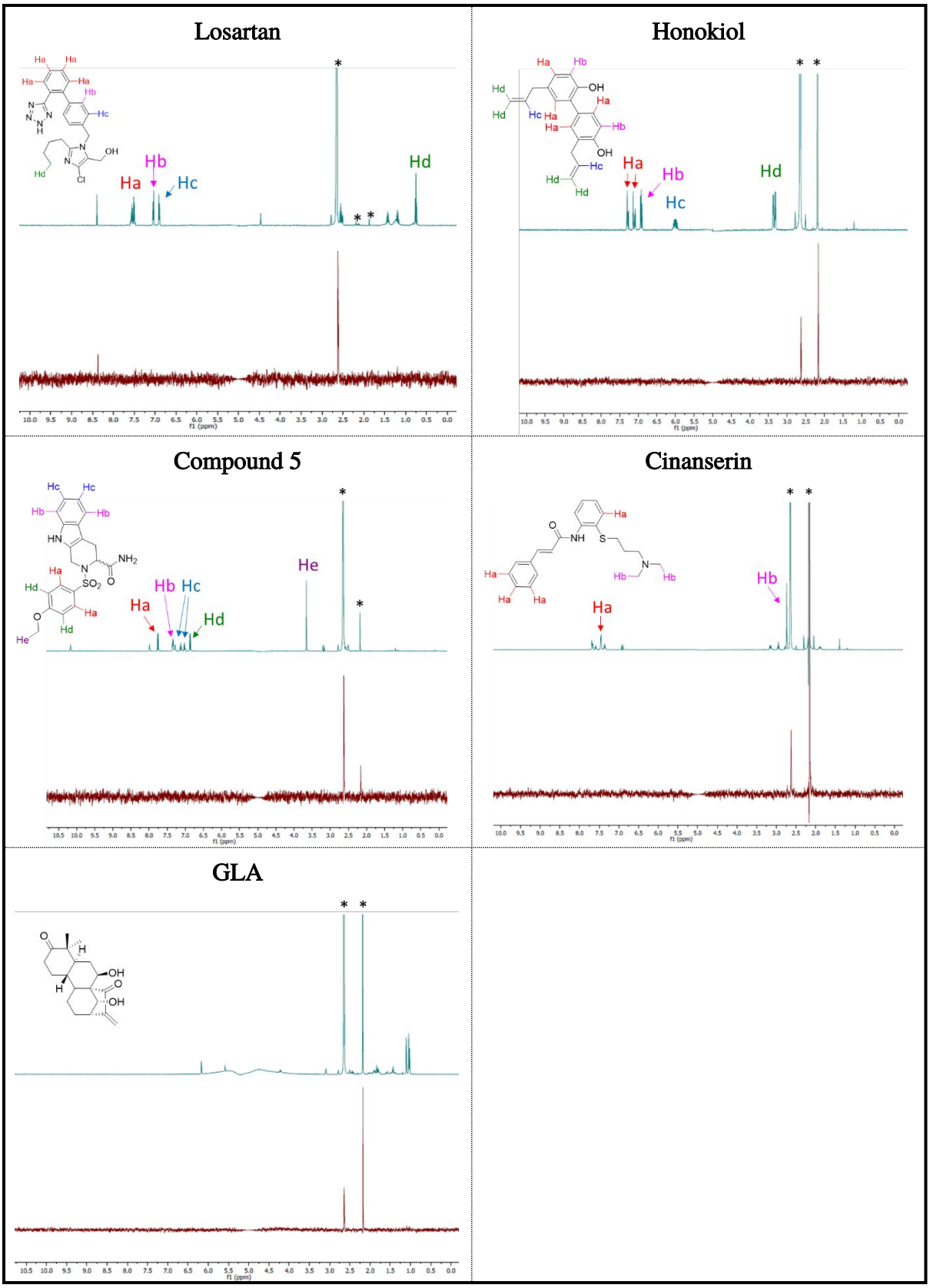
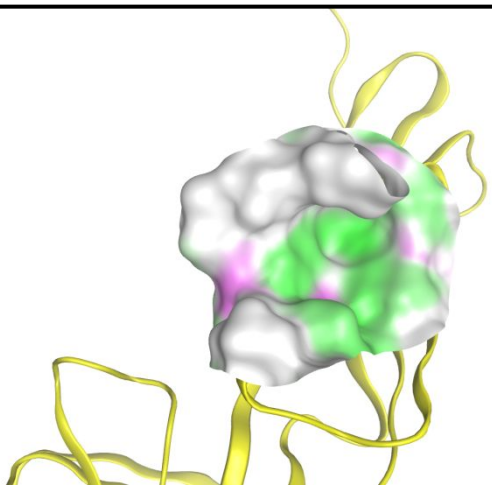

Losartan
Honokiol
Compound 5
Cinanserin
GLA

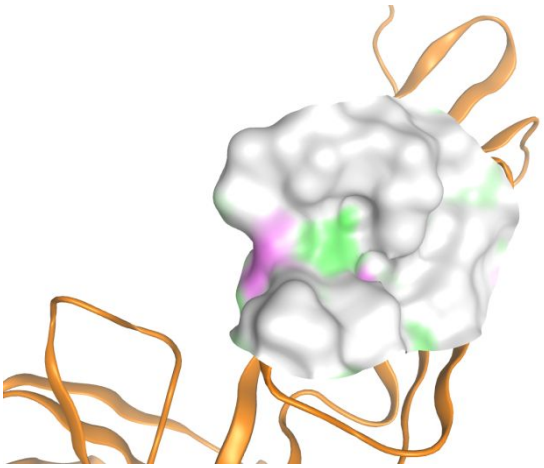
Figure S4: GPVI SiteMap Analysis

The GPVI-ligand binding region was variable amongst the known crystal structures and was quantified using SiteMap. Monomers within the same crystal structure have been labelled as chain A and B. Hydrophobic regions have been highlighted in green and polar regions are pink on the protein surface. 2GI7(A) had the most accessible structure (best SiteMap score), so this was used for modelling studies. PDB ID: 7NMU was omitted from this study as the bound inhibitory nanobody NB2 resulted in several conformational changes including: the swapping of the D2 domain and a shift of the βC' sheet in the D1 domain that may or may not be induced upon small molecule binding.[4]



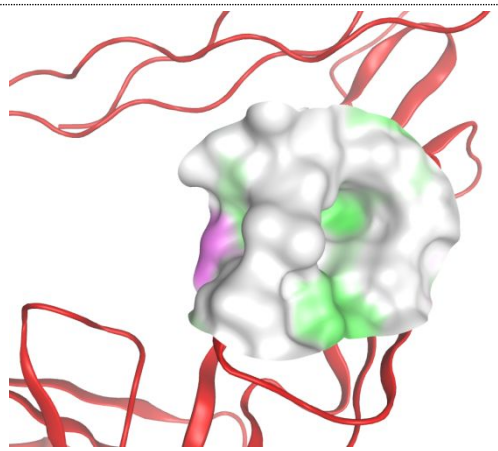
PDB ID: 2GI7(A)

SiteMap Score = 0.96



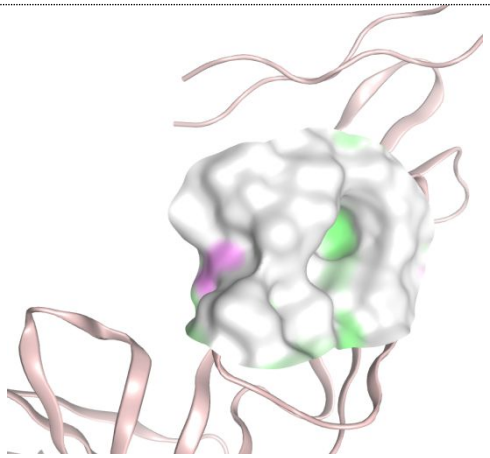
PDB ID: 2GI7(B)

SiteMap Score = 0.76



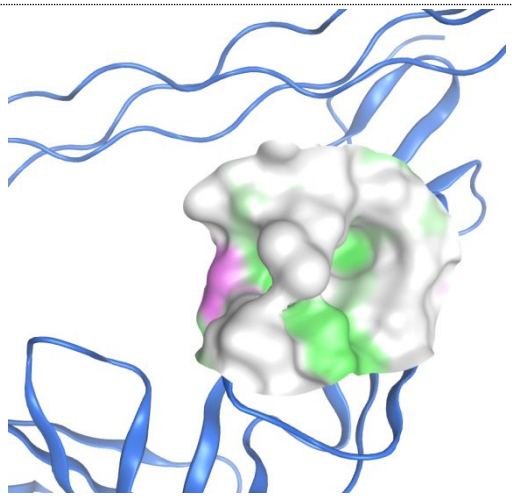
PDB ID: 5OU8(A)

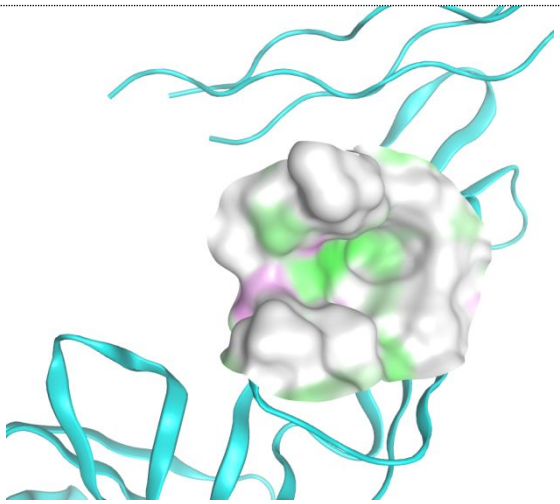
SiteMap Score = 0.92



PDB ID: 5OU8(B)

SiteMap Score = 0.60





PDB ID: 5OU9(A)	PDB ID: 5OU9(B)
SiteMap Score = 0.86	SiteMap Score = 0.79

Figure S5: Standard GLIDE Docking with Losartan and 2GI7(A)

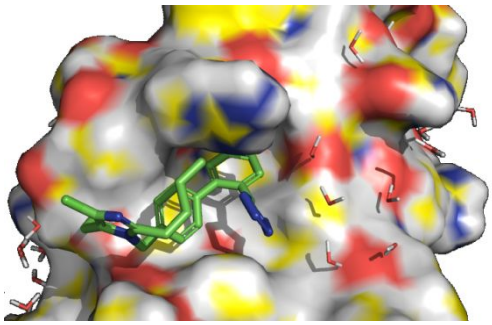
Standard GLIDE docking procedures using losartan as a tool compound were ineffective as no poses with predicted when standard precision was used and the scores for extra precision were very poor.

a) Summary of docking parameters investigated where - = no poses generated. b) Pose predicted using conditions described by entry 4 of section a. c) Pose predicted using conditions described by entry 8 of section a.

a

Entry	Ligand State	GRID	Precision	GScore (Kcal/mol)
1	P	Water	SP	-
2	NP	Water	SP	-
3	NP	Water	XP	-3.226
4	P	Water	XP	-3.226
5	P	No Water	SP	-
6	NP	No Water	SP	-
7	NP	No Water	XP	0.707
8	P	No Water	XP	0.707

b



c

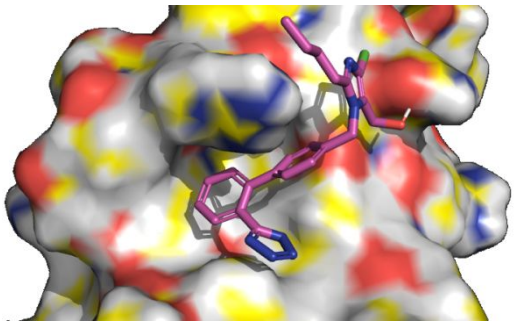


Figure S6: Induced Fit Docking with Losartan and 2GI7(A)

Induced fit docking protocols were able to predict a pose for losartan that was in agreement with the published protein-observed NMR experiments.[5]

a) Summary of the induced fit docking conditions investigated. Only poses with the highest GScore were considered. The distance between losartan's phenyl protons H_{14}, H_{15}, H_{16} and the key GPVI binding residues (Lys41 and Gln48 amide N-H's), suggested from the published protein-observed losartan binding experiments, were calculated. Results were formatted on a red-green scale where green represents a good result. The conditions described in Entry 1 were used to model other known GPVI modulators. b) Pose generated by entry 1, section a. c) Pose generated by entry 4, section a. d) Pose generated by entry 5, section a. e) Pose generated by entry 8, section a.

a

Entry	Ligand State	GRID	Re-dock Precision	Glide Gscore/ Kcal/mol	IFD Score Kcal/mol	H_{15}-Lys41 N-H (Å)	H_{16}-Lys41 N-H (Å)	H_{14}-Gln48 N-H (Å)	H_{15}-Gln48 N-H (Å)
1	P	Water	SP	-6.784	-380.24	3.9	2.6	4.8	3.5
2	NP	Water	SP	-6.784	-380.24	3.9	2.6	4.8	3.5
3	NP	Water	XP	-5.827	-379.77	3.8	2.4	4.5	3.5
4	P	Water	XP	-5.827	-379.77	3.8	2.4	4.5	3.5
5	P	No Water	SP	-6.124	-336.31	15.8	15	13.6	15.4
6	NP	No Water	SP	-6.124	-336.31	15.8	15	13.6	15.4
7	NP	No Water	XP	-4.844	-335.03	3.3	3	4.2	3.2
8	P	No Water	XP	-4.844	-335.03	3.3	3	4.2	3.2

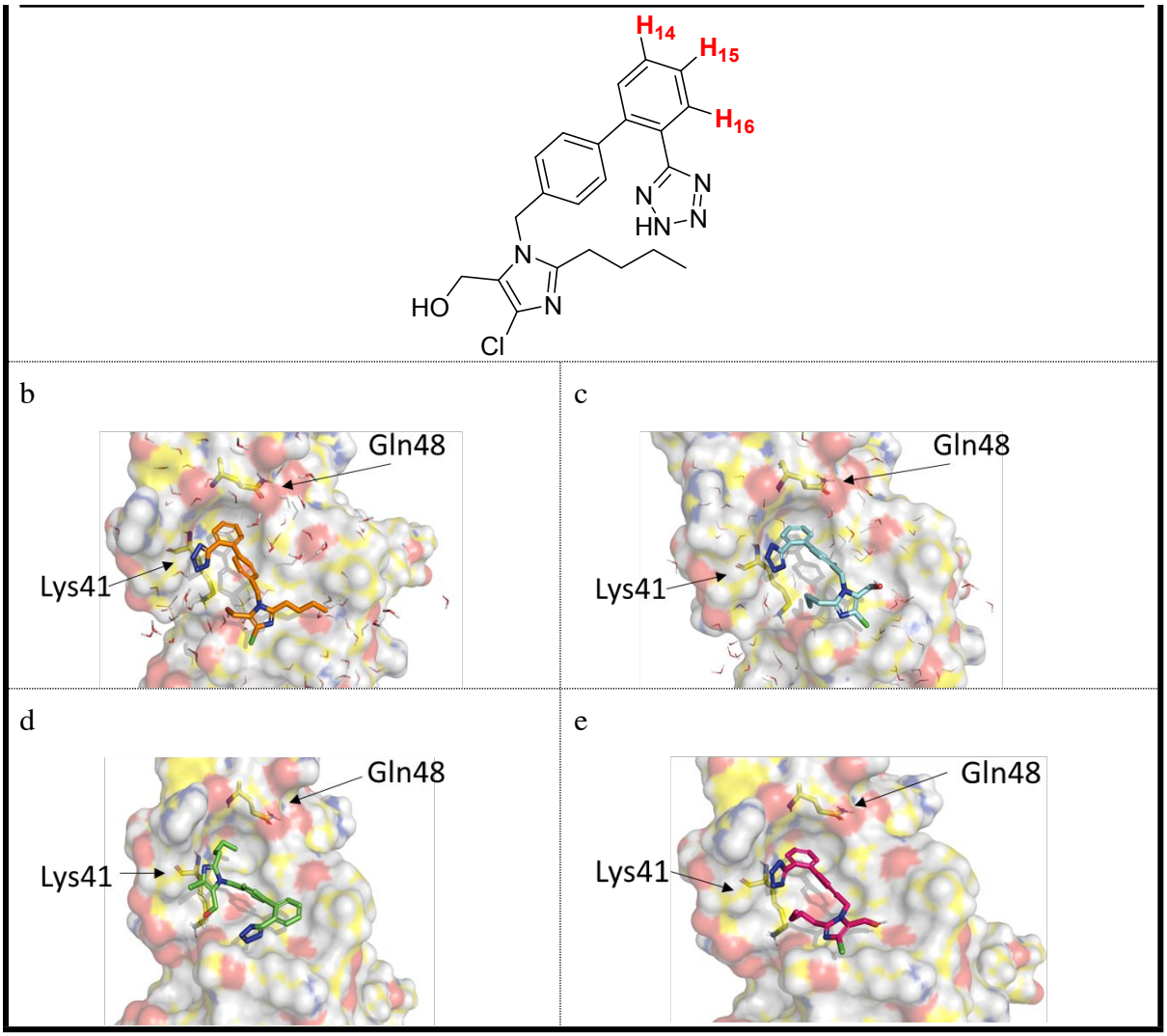


Figure S7: Losartan MST Optimisation Experiments

Thermophoresis is sensitive to several molecular properties including changes in charge, conformation and size. Therefore, alterations were made to the salt levels in the buffer and a higher concentration of losartan was utilised (1250 μM compared to the standard 250 μM), however no change in thermophoresis was observed, illustrating the need for orthogonal binding assays to verify relatively weak potential binders.

a) Performing a serial dilution with a higher top concentration of losartan 1250 µM (n = 1) compared to the usual 250 µM (n = 3) to did not make the change in thermophoresis upon losartan binding more obvious (MST power = 20%, LED power = 40%).

b) Changing the final salt concentration in the buffer (low salt = 10 mM, n = 1; high salt = 500 mM, n = 1; and normal = 140 mM, n = 3) did not make the change in thermophoresis upon losartan binding more obvious (MST power = 20%, LED power = 40%).

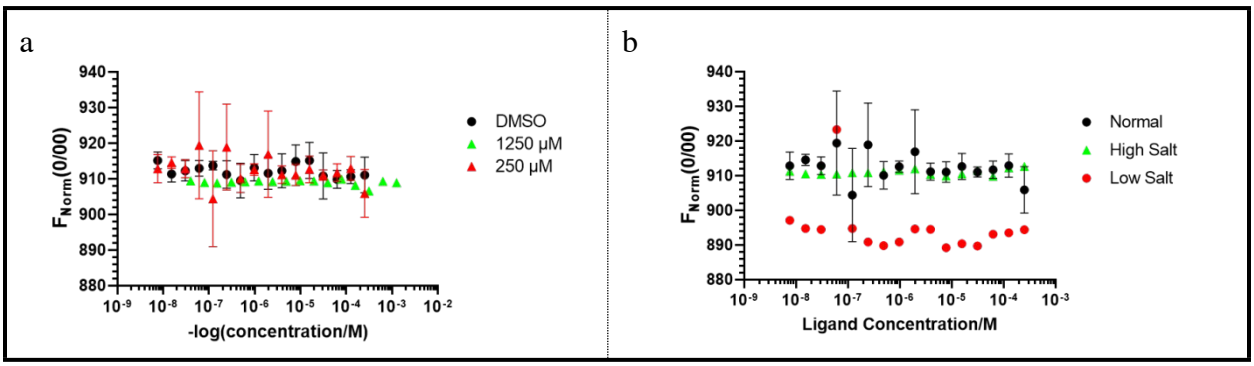
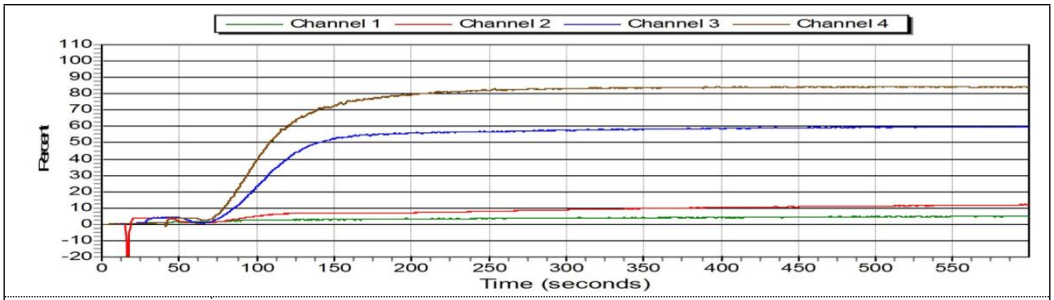
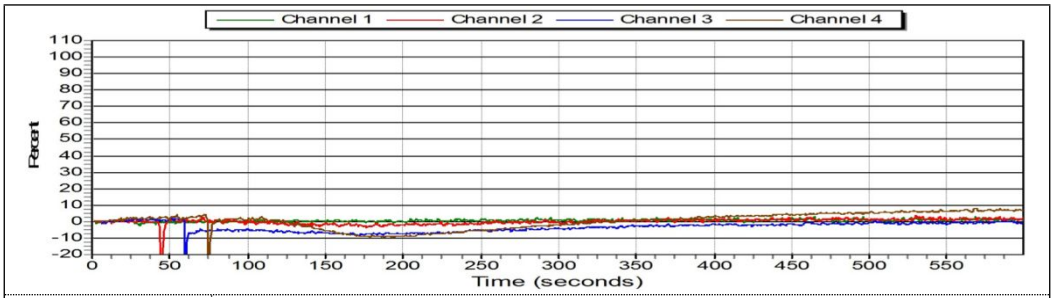


Figure S8: Additional LT Aggregometry Assay with Losartan and Compound 5

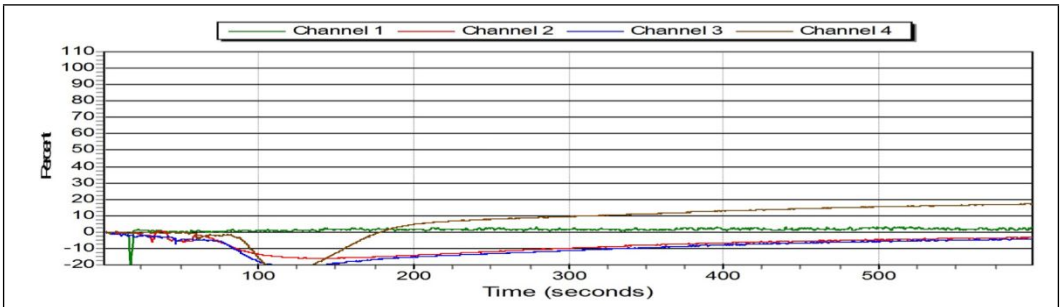
Losartan and compound **5** inhibited collagen-induced platelet aggregation when tested at 100 µM in the LT aggregometry assay. Results were collected on the same day with platelets from the same donor with n = 1 for each datapoint.



Channel 1	0 µg/mL collagen
Channel 2	1 µg/mL collagen
Channel 3	2.5 µg/mL collagen
Channel 4	5 µg/mL collagen



Channel 1	0 µg/mL collagen and losartan (100 µM)
Channel 2	1 µg/mL collagen and losartan (100 µM)
Channel 3	2.5 µg/mL collagen and losartan (100 µM)
Channel 4	5 µg/mL collagen and losartan (100 µM)



Channel 1	0 µg/mL collagen and compound **5** (100 µM)
Channel 2	1 µg/mL collagen and compound **5** (100 µM)

Channel 3	2.5 μg/mL collagen and compound **5** (100 μM)
Channel 4	5 μg/mL collagen and compound **5** (100 μM)

References

(1) Novel Glycoprotein VI Antagonists as Antithrombotics: Synthesis, Biological Evaluation, and Molecular Modeling Studies on 2,3- Disubstituted Tetrahydropyrido(3,4-b)Indoles. *J. Med. Chem.* **2017**, *60*, 322–337.

(2) Fibrin and D-Dimer Bind to Monomeric GPVI. *Blood Adv.* **2017**, *1*, 1495–1504.

(3) 1H, 13C and 15N Chemical Shift Referencing in Biomolecular NMR. *J. Biomol. NMR* **1995**, *6*, 135–140.

(4) Structural Characterisation of a Novel GPVI Nanobody-Complex Reveals a Biologically Active Domain-Swapped GPVI Dimer. *Blood* **2021**.

(5) Structural Basis for Platelet Antiaggregation by Angiotensin II Type 1 Receptor Antagonist Losartan (DuP-753) via Glycoprotein VI. *J. Med. Chem.* **2010**, *53*, 2087–2093.