Supplemental Material to Yeung et al. "12-lipoxygenase activity plays an important role in PAR4 and GPVI-mediated platelet reactivity" (Thromb Haemost 2013; 110.3)

Supplemental Methods:

Materials:

12-LOX compounds (NCTT-956 and -694) were synthesized at the NIH Chemical Genomics Center (Rockville, MD). 12(*S*)-hydroxyeicosatetraenoic acid (12-HETE) was synthesized. U-46619 was purchased from Cayman chemicals (Ann Arbor, MI). Baicalein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). Human α-thrombin was purchased from Enzyme Research Labs (South Bend, IN). ADP was purchased from Sigma-Aldrich (Saint Louis, MO). Phycoerythrin-conjugated anti-P-selectin and FITC-conjugated PAC-1 were purchased from BD Biosciences (San Jose, CA) and processed with a C6 flow cytometer from Accuri (Ann Arbor, MI). Aggregometers, chronolume reagent, collagen, and other aggregation supplies were purchased from Chrono-Log Corp. (Havertown, PA).

Human Platelets:

For washed platelets, blood was centrifuged at 200 *g* for 15 min at room temperature. Platelet-rich plasma was transferred into a conical tube containing a 10% acid citrate dextrose solution (39 mM citric acid, 75 mM sodium citrate, and 135 mM glucose, pH 7.4) and centrifuged at 2000 *g* for 15 min at room temperature. Platelets were resuspended in Tyrode's buffer (12 mM $NaHCO_3$, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES), and the final platelet concentration was adjusted to 3 X 10^8 platelets/ml after counting with a Coulter counter (Beckman Coulter, Fullerton, CA). Reported results are the data obtained using platelets from at least three different subjects. Agonists and inhibitors were used at concentrations indicated in the figures and figure legends unless otherwise specified.

Whole Blood Aggregation. Whole blood was collected in vacutainer tubes containing sodium citrate. 500µl of blood was diluted in a 1:1 ratio with irrigation grade saline and pre-warmed for 5 minutes at 37 °C. The blood was pre-treated with or without NCTT-956 or NCTT-694 for 10 minutes. An electrode probe assembly was then placed in the cuvette. Aggregation was induced by collagen (final concentration of 5µg/ml) at 37 °C, stirring at 800 rpm. Platelet aggregation was monitored and responses were recorded as electrical impedance across paired electrodes (ohms).

Flow Cytometry. For these experiments, 50 µl aliquots of washed platelets adjusted to a final concentration of 5 x 10^5 platelets/ml were pre-treated with inhibitors for 10 min. After addition of 10 µl of the indicated antibody, platelets were stimulated with agonist for 10 min. and then diluted to a final volume of 500 µl using Tyrode's buffer. The fluorescence intensity of 10,000 platelets was immediately measured using an Accuri flow cytometer.

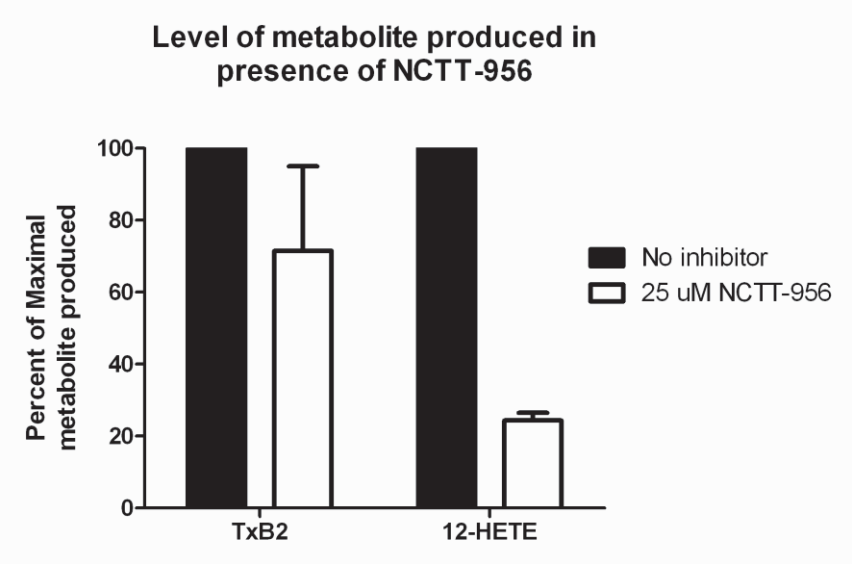
GC/MS analysis of TxB_2: 2 ng of [$^2H_4$] TxB_2 was added to the samples as internal standards. The pH of the medium was adjusted to 3 and the sample prepurified with a C18 SepPak column. TxB_2 was eluted with 10 ml heptane/ethyl acetate (1:1). The sample was dried under N_2, dissolved in acetonitrile and TxB_2 was converted to pentafluorobenzyl esters by a 30 minute, 37°C incubation with 20 µl 10% diisopropyl ethylamine and 40 µl 10% pentaflurobenzyl bromide in acetonitrile. TxB_2 was then purified on Silica TLC in chloroform : ethanol 93:7. After

extraction, the sample was again dried under N_2 before conversion to O-trimethylsilyl ether derivatives by incubation with 10 µl dimethyl formamide and 20 µl bis(trimethylsilyl)trifluoroacetamide. The reagents were dried under a stream of N_2 and the sample resuspended in undecane. The derivatives were analyzed by gas chromatography/electron capture negative chemical ionization mass spectrometry, using an SPB-1 column (15 meters), with a temperature gradient from 190°C to 300°C at 20°C/min. The ion corresponding to the derivatized TxB_2 was monitored by selected ion monitoring (SIM). The signal for TxB_2 is *m/z* = 614. The signal for the internal standard [$^2H_4$] TxB_2 is *m/z* = 618.

Analysis of [$^2H_8$] Thromboxane synthesis in platelets: [$^2H_8$] TxB_2 was extracted and derivatized for analysis by GC/NICI/MS as decribed for TxB_2 above with the difference that the ion monitored for [$^2H_8$] TxB_2 is *m/z* = 622.

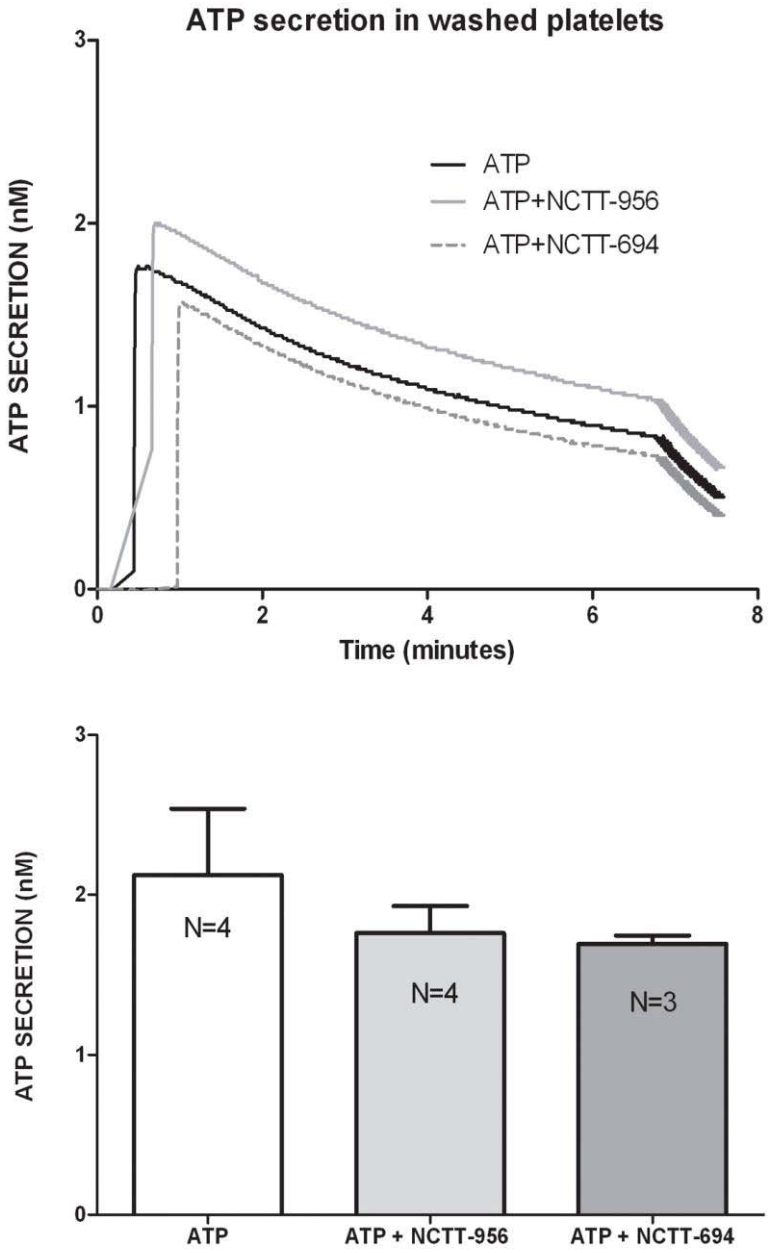
Analysis of 12-HETE: 50 µl of supernatant were diluted with 50 µl of pH 3 water containing 1 ng of [$^2H_8$] 12-HETE (internal standard) and the pH will be adjusted to 3 with 1N HCl. 12-HETE was then extracted with 1 ml of ethyl acetate and converted to pentafluorobenzyl esters by a 20 minute, 37°C incubation with 20 µl 10% diisopropyl ethylamine and 40 µl 10% pentaflurobenzyl bromide in acetonitrile. After evaporating the solvents under nitrogen stream, the derivatized 12-HETE were resuspended in 80 µl of hexane:isopropyl alcohol 99.3:0.7 (mobile phase) and analyzed by liquid chromatography (LC) /capture atmospheric pressure chemical ionization tandem mass spectrometry (-APCI/MS/MS) using a silica HPLC column in isocratic condition at a flow rate of 1 ml/min. Presence of 12-HETE was determined by measuring the transition from m/z = 319 to *m/z* = 179 (12-HETE) and from *m/z* = 327 to *m/z* = 184 ([$^2H_8$] 12-HETE) at CID energy of 20eV under collision gas pressure of 1.5 mTorr.

Supplemental Figure 1:



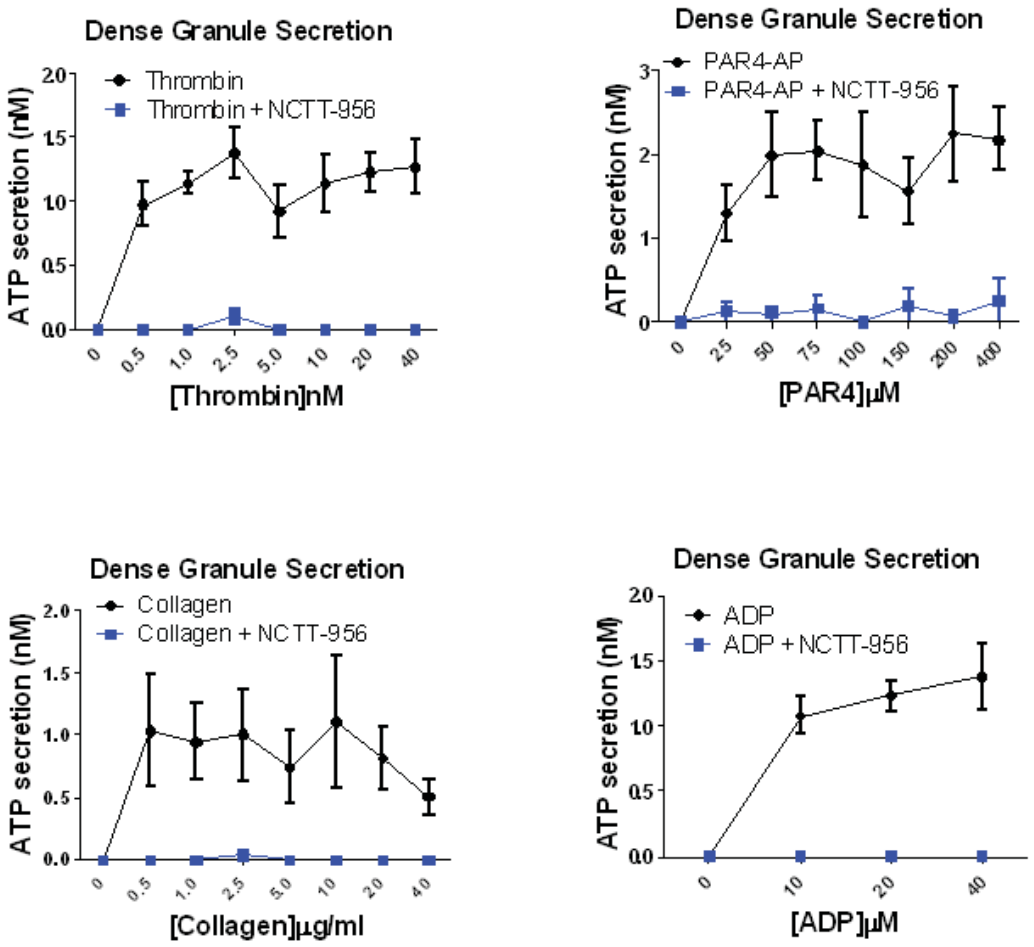
Supplemental Figure 1: 12-LOX inhibitor selectivity towards 12-LOX. To confirm substrate selectivity for NCTT-956, platelets were stimulated in the absence or presence of 25 μM NCTT-956 and the levels of TxB_2 and 12-HETE formation were assessed by mass spectrometry (N=2-3). While NCTT-956 did not significantly affect TxB_2 formation (an observation similar to what was previously observed with the addition of deuterated arachidonic acid as a substrate, Reference 16), the level of 12-HETE produced in the presence of 25 μM NCTT-596 was substantially reduced.

Supplemental Figure 2:



Supplemental Figure 2: 12-LOX inhibitors do not interfere with ATP luciferase assay. To confirm the positive and negative analogs for the 12-LOX inhibitors did not directly interact with the luciferase assay, washed platelets were incubated with chronolume for 2 minutes in the absence or presence of NCTT-956 or NCTT-694. Following incubation, the platelets were spiked with 2 nM ATP and the level of ATP was confirmed in the lumi-aggregometer. Neither NCTT-956 nor NCTT-694 significantly inhibited the interaction of the chronolume reagent with ATP, confirming the observed inhibition of ATP secretion in Figure 2 is not due to inhibition of the reaction due to the addition of the small molecule inhibitors.

Supplemental Figure 3:



Supplemental Figure 3: NCTT-956 inhibition of dense granule secretion. The presence of 25 µM NCTT-956 inhibits ATP secretion (dense granule secretion) following stimulation with a number of agonists including thrombin, PAR4-AP, collagen, and ADP.