Small molecule lysyl oxidase-like 2 (LOXL2) inhibitors: the identification of an inhibitor selective for LOXL2 over LOX

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Supporting Data

LOX – lysyl oxidase; protein-lysine 6-oxidase; protein-L-lysine:oxygen 6-oxidoreductase; EC 1.4.3.13

LOXL2 - lysyl oxidase-like 2; lysyl oxidase homolog 2; LOR2, WS9-14; EC 1.4.3.13

LOXL2 is a member of the lysyl oxidase (LOX) gene family.

Generation of concentrated conditioned media. Full length human, mouse and rat LOXL2 and full length human LOX were cloned into the pCDNA5/FRT vector (Thermo Fisher) and stable cell lines generated by Lipofectamine™ 2000 mediated transfection into CHO/FlpIn (LOXL2) or HEK/FlpIn (LOX) cells (Thermo Fisher). The stable cell lines were cultured under normal growth conditions in 15 cm tissue culture plates until cells were ~80% confluent. Cells were then washed with PBS before the addition of 25-30 mL serum-free media. For the generation of LOX/HEK conditioned media, the serum-free media consisted of phenol red-free DMEM/F12 mix w/glutamax containing pen/strep, 10 μM $CuCl_2$ and 0.1% BSA (BSA and 10 μM $CuCl_2$ were determined to be required for measurable LOX activity). For the generation of LOXL2 conditioned media, the serum-free media consisted of phenol red-free DMEM/F12 mix w/glutamax containing pen/strep and 100 μM $CuCl_2$ (this concentration of $CuCl_2$ was determined to be optimal for maximal LOXL2 activity) and ± 0.1% BSA. For primary screening of test compounds, the hLOXL2/CHO CCM lacking BSA was used as the source of enzyme. For head-to-head comparison with LOX, the LOXL2/CHO CCM prepared with BSA was used so that concentrations of albumin, which could affect compound potency, were identical between the two assays. Cells were incubated at 37°C, 5% CO_2 in the serum-free media for 40-48 hours before the conditioned media was removed and centrifuged at 2000 rpm for 5 min at 4°C to pellet cells/debris. The media was concentrated 10-20X using either 30 kDa MWCO or 10 kDa

MWCO centriprep columns for concentrating LOXL2 or LOX, respectively, according to the manufacturer's instructions (EMD Millipore, Billerica, MA). Concentrated conditioned media (CCM) was aliquoted and stored at -80°C until use.

Immunoblot Analysis of LOXL2 protein. Conditioned media or concentrated conditioned media was separated by SDS-PAGE using 4-12% BOLT Bis-Tris gels then the proteins transferred to nitrocellulose using the iBLOT system. The membrane was blocked for 1 hour at room temperature in a 1:1 mix of Odyssey Blocking Buffer (LI-COR Biosciencs):PBS then probed overnight at 4°C with a 1:500 dilution of a polyclonal anti-LOXL2 goat IgG antibody (R&D Systems, #AF2639). Blots were washed, incubated with a Donkey anti-goat-IRDye800CW and imaged using the Odyssey system (LI-COR Biosciences).

LOXL2/LOX Amplex Red Activity Assays using CCM. Test compounds (2 µL diluted in DMSO) or vehicle were added to 73 µL Assay Buffer (50 mM sodium borate, pH 8.0) and either 10 µL human LOXL2/CHO or human LOX/HEK CCM in black-wall, clear bottom 96-well optical plates. The plates were mixed and incubated for 2h at 37°C. After the 2h incubation, 5 µL of Assay Buffer containing 10 mM DAP and 10 µL of Assay Buffer containing 0.5 mM Amplex® Red Reagent (Thermo Fisher Scientific, Waltham, MA) and 50 U/mL HRP were added to each well (final concentrations were 0.5 mM DAP, 50 µM Amplex® Red and 5 U/mL HRP). The plates were mixed quickly and immediately loaded into a Flex Station 3 (Molecular Devices, Sunnyvale, CA). A kinetic top read of fluorescence was performed every 2 minutes for a total of 50 minutes at excitation = 544 nm and emission = 590 nm. The slope from the linear portion of the read was used as rate of the reaction. ***Data Analysis*.** Maximal activity was determined from wells containing CCM incubated with vehicle and this slope value was set to 0% inhibition. Maximal inhibition was determined from wells containing CCM incubated with 100 µM of the pan-LOX family inhibitor, βAPN, and this slope value was set to 100% inhibition. The % inhibition values of each test compound treated well relative to the vehicle and βAPN controls for each well was graphed and the IC_{50} values generated by non-linear regression analysis of the slope data using CDD Vault (Collaborative Drug Discovery, Burlingame, CA). IC_{50} values are presented as the geometric mean x/÷ geometric standard deviation.

Note: βAPN was used as the control in every assay run at PharmAkea hence the high N-numbers

LOXL3 Assay. The LOXL3 Amplex® Red Assay was performed exactly as described for the CCM assay, except that 10 µL of 0.075 µg/µL human purified, recombinant LOXL3 (R&D Systems, Minneapolis, MN) was used as the enzyme source.

Human Whole LOXL2 Blood Assay. Human blood was drawn into heparin vacutainer tubes, kept at room temperature and used within one hour of draw. For the human blood assay, test compounds (2 µL diluted in DMSO) or vehicle (2 µL DMSO) were added to 182 µL blood and 16 µl of 0.25 mg/ml purified, recombinant human LOXL2 (diluted in water) (R&D Systems) in each well of a 0.45 mL, V-bottom, 96-well polypropylene plate. The plates were mixed and incubated for 2h at 37°C, 5% CO_2. After the 2h incubation, plates were centrifuged at 2000 x g for 15 min at room temperature to isolate plasma and 50 µL of plasma was transferred to a black-walled clear-bottom 96-well assay plate. 25 µL of 40 mM DAP (diluted in water) and 25 µL of Assay Buffer (200 mM sodium borate buffer, pH 8.0) containing 20 U/mL HRP and 0.2 mM Amplex® Red Reagent (Thermo Fisher Scientific, Waltham, MA) were added to each well (final concentrations were 10 mM DAP, 5 U/mL HRP and 50 µM Amplex® Red Reagent). The plates were mixed quickly and immediately loaded into a Flex Station 3 (Molecular Devices, Sunnyvale, CA). A kinetic read of fluorescence was performed every 1 minute, 30 sec for a total of 45 minutes at excitation = 544 nm and emission = 590 nm. The slope from the linear portion of the read was used as rate of the reaction. ***Data Analysis*.** Maximal activity was determined from wells containing blood spiked with LOXL2 and incubated with vehicle and this slope value was set to 0% inhibition. Maximal inhibition was determined from wells containing blood not spiked with recombinant LOXL2, and this slope value was set to 100% inhibition. The % inhibition values of each test compound treated well, relative to the vehicle and unspiked control wells, were graphed and the IC_{50} values generated by non-linear regression analysis of the slope data using CDD Vault (Collaborative Drug Discovery, Burlingame, CA). IC_{50} values are presented as the geometric mean x/÷ geometric standard deviation.

MAO-A/B Assay. Amine oxidase activity of the MAO enzymes was evaluated using Amplex® Red reagent and either tyramine (MAO-A) or benzylamine (MAO-B) as a substrate. 2 µL test compound in DMSO (or DMSO control) were added to 88 µL Assay Buffer (50 mM HEPES, pH 7.5) and 0.2 U of human recombinant MAO-A or MAO-B in black-wall, clear bottom 96-well optical plates. The plates were mixed and incubated for 2h at 37°C. After the 2h incubation, 10

μL of Assay Buffer containing 0.5 mM Amplex® Red Reagent and 50 U/mL HRP and either 1 mM tyramine or 2 mM benzylamine were added to each well. The plates were mixed and kinetically read at room temperature on a Flex Station 3 every 30 seconds for a total of 10 minutes at excitation = 544 nm and emission = 590 nm. The slope from the linear portion of the read was used as rate of the reaction. Maximal activity was determined from wells containing MAO incubated with vehicle and this slope value was set to 0% inhibition. Maximal inhibition was determined from wells lacking recombinant MAO and this slope value was set to 100% inhibition. The % inhibition of human MAO was calculated relative to the vehicle and no enzyme controls for each well.

SSAO Assay. Amine oxidase activity of human plasma SSAO was evaluated using Amplex® Red reagent and benzylamine as a substrate. 2 μL test compound diluted in DMSO (or DMSO control) were added to 83 μL Assay Buffer (50 mM HEPES, pH 7.5) and 15 μL of human plasma (prepared from normal donor whole blood collected in sodium heparin vacutainer tubes optical plates. The plates were mixed and incubated for 2h at 37°C. After the 2h incubation, 100 μL of Assay Buffer containing 2 mM benzylamine, 0.1 mM Amplex® Red Reagent and 10 U/mL HRP were added to each well. The plates were mixed and fluorescence read kinetically on a Flex Station 3 every 30 seconds for a total of 15 minutes at excitation = 544 nm and emission = 590 nm. The slope from the linear portion of the read was used as rate of the reaction. Maximal activity was determined from wells containing plasma incubated with vehicle and this slope value was set to 0% inhibition. Maximal inhibition was determined from wells containing plasma incubated with 1 μM of PXS-4681A, a published SSAO inhibitor[1] and this slope value was set to 100% inhibition. The % inhibition of SSAO activity was calculated relative to the vehicle and PXS-4681A controls for each well.

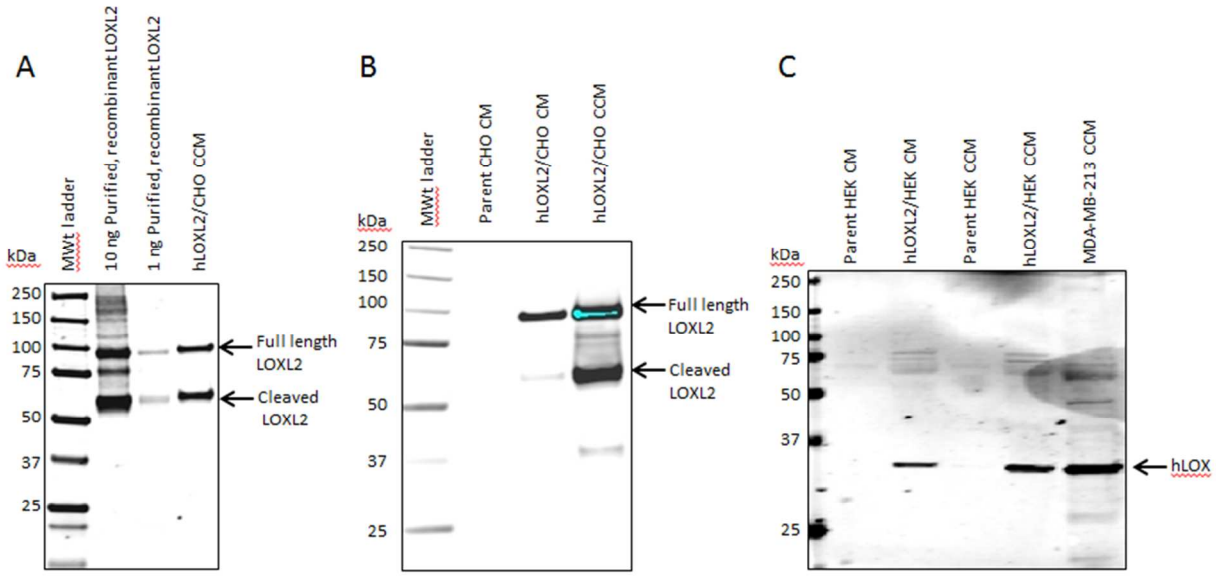
LOXL2 Reversibility Assay. The wells of a 96-well, F-bottom, Fluotrac, polystyrene high binding black plate (Greiner Bio-one, Monroe, NC) were coated overnight at room temperature with 100 μl of 0.005 mg/mL purified recombinant human LOXL2 (diluted in 50 mM PBS, pH 7.4). Replicate wells for each compound to be tested were coated to serve as washed and non-washed controls. Following the overnight coating, the wells were blocked with 150 μl of 1% BSA in 50 mM PBS, pH 7.4 for 1.5 h at room temperature. The wells were rinsed twice with 200 μl of 50 mM PBS, pH 7.4 and the wells emptied by aspirating the contents. The coated plate was

immediately used for the assay to assess reversibility by adding 83 µl of assay buffer (50 mM sodium borate buffer, pH 8.0) into the wells (for both the washed and non-washed samples) of the enzyme coated assay plate. In the washed sample wells only, 1000X the IC_{50} concentration of test co mpound (2 µL diluted in DMSO) or vehicle (2 µL DMSO) was added to each well. The plates were mixed and incubated for 2h at 37°C. After the 2h incubation, the washed sample wells that were incubated with test compound or vehicle were washed 4 times with 200 µl 50 mM PBS, pH 7.4 and left in the fourth wash. In wells to be used as the non-washed controls, 1000X the IC_{50} concentration of test compound (2 µL diluted in DMSO) or vehicle was added to each well still containing 83 µl of assay buffer added in the first step. The plates were mixed and incubated for another 2h at 37°C. After this 2h incubation, the 200 µl of 50 mM PBS, pH 7.4 in the washed sample wells was aspirated and replaced with 85 µl of assay buffer. 5 µL of 10 mM DAP (diluted in Assay Buffer) or 5 µl of Assay Buffer (maximal inhibition control) and 10 µL of Assay Buffer (50 mM sodium borate buffer, pH 8.0) containing 50 U/mL HRP and 0.5 mM Amplex® Red Reagent were added to each well. The plates were mixed quickly and read kinetically at 37°C every 2 minutes for a total of 40 minutes at excitation = 544 nm and emission = 590 nm on a Flex Station . The slope from the linear portion of the read was used as rate of the reaction. Maximal activity for either washed or non-washed wells was determined from enzyme coated wells incubated with vehicle and substrate added, and this slope value was set to 0% inhibition for each respective condition. Maximal inhibition for either washed or non-washed wells was determined from enzyme coated wells incubated with vehicle with no substrate added, and this slope value was set to 0% inhibition, respectively. The % inhibition values of the washed and non-washed PAT-1251 were derived from their respective washed or non-washed maximal activity and inhibition controls and graphed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

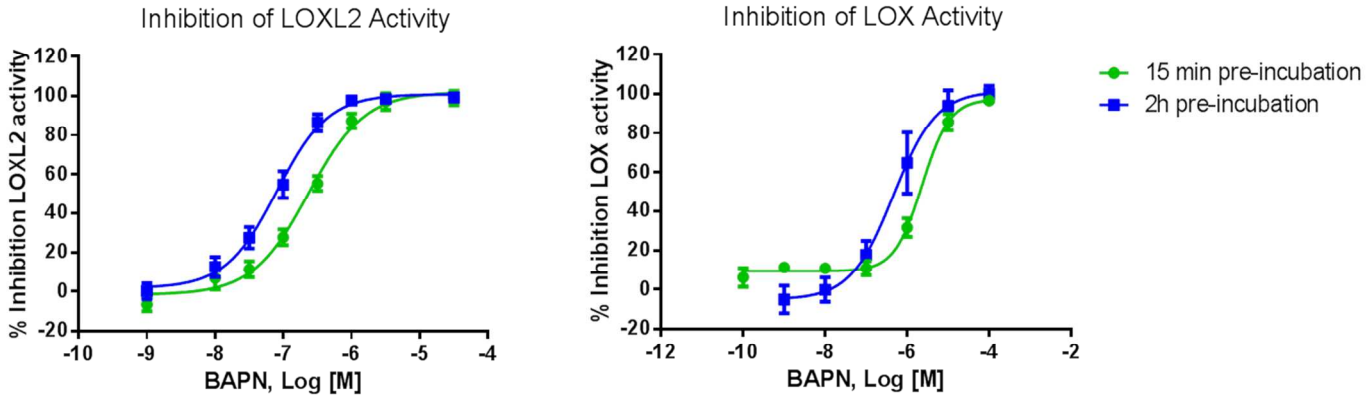
References

1. Foot, J. S.; Yow, T. T.; Schilter, H.; Buson, A.; Deodhar, M.; Findlay, A. D.; Guo, L.; McDonald, I. A.; Turner, C. I.; Zhou, W.; Jarolimek, W. PXS-4681A, a potent and selective mechanism-based inhibitor of SSAO/VAP-1 with anti-inflammatory effects in vivo. *J Pharmacol Exp Ther* **2013,** *347* (2), 365-374.

Supplementary Fig. 1. Western blot analysis of conditioned media (CM) or concentrated conditioned media (CCM) from the human LOXL2/CHO stable cell line (A,B) and the human LOX/HEK stable cell line (C). Both forms of LOXL2, the full length (~100 kDa) band and the processed form of LOXL2 (~60 kDa), are detected in the conditioned media from the hLOXL2/CHO stable cell line as well as in the commercially available (R&D Systems, Inc), purified, recombinant LOXL2 standard shown in (A). (B) No LOXL2 is detected in the parent CHO cell line, and approximately 10-fold more LOXL2 is found in the conditioned media after a 10X concentration with a 30 kDa MWCO centriprep column (CCM). (C) No LOX is detected in the parent HEK cells, whereas the processed, active form of LOX is detectable in the CM and CCM in the hLOX/HEK stable cell line. CCM from the human breast cancer cell line, MDA-MB-231, was used as a positive control for the LOX Western.



Supplementary Fig. 2. Inhibition of LOX and LOXL2 activity by BAPN. Concentration-response-curves for inhibition of LOX and LOXL2 activity by BAPN after either a 15 minute or 2 hour pre-incubation of inhibitor with enzyme. Shown is the average % inhibition ± Std Dev of at least 5 independent experiments.



Supplementary Fig. 3. Analysis of reversible inhibition. Purified, recombinant LOXL2 bound to a 96-well plate was incubated with 1000X the IC_{50} concentration of either bAPN (A) or Cmpd **20** (B) for 2 hours at 37°C. The wells were then either left unwashed (maximum inhibition) or washed extensively and then incubated in assay buffer for 0, 2 or 6 hours prior to addition of substrate and Amplex Red reagent for measurement of catalytic activity. Shown is the average percent inhibition of LOXL2 activity from triplicate wells.