Supplementary Data – Methods

Zebrafish RNA extraction and qRT-PCR

qRT-PCR reactions were performed with the following primers: (scd forward qprimer) 5'-ATCCCGACGCTCCTCAGATA-3', (scd reverse qprimer) 5'-GTGGTAGCCTTCACCTATAGCA-3', (scdb forward qprimer) 5'-CCCTCATATGGACGGGAGTG-3', (scdb reverse qprimer) 5'-ATCCGTAATGGCAGTGAGGC-3', (abcd2 forward qprimer) 5'-TCACCGGAAGGGCTACCT-3', (abcd2 reverse qprimer) 5'-GCTGCTTCATCTCCACCTTGT-3', (mbp forward qprimer) 5'-AGGGAAAGAGACCCCACCAC-3', (mbp reverse qprimer) 5'-GAGGAGAGGACACAAAGCTCC-3', (mpz forward qprimer) 5'-TACCGTCCAGATGGGGCTAA-3', (mpz reverse qprimer) 5'-ACTCCAGGCGGTTTTGGAAT-3', (plp1aforward qprimer) 5'-GGTCTTGGCTCTGCTCACAT-3', (plp1a reverse qprimer) 5'-CCGTGGACTGATCCCTTTCC-3', (elovl1b forward qprimer) 5'-TCAGGACTCAAGAGGAGGCA-3', (elovl1b reverse qprimer) 5'-CTTCAACCGCGGGTCTCTG-3', (elovl6 forward qprimer) 5'-ACTTGCCGCCTTCAGTATATTT-3' and (elovl6 reverse qprimer) 5'-AGCATAAGCCCAGAACTTGCT-3' with the following conditions; 50˚C for 2 min, 95 for 5 min followed by 39 cycles of 95˚C for 15s, 60˚C for 15s, and 72 for 15s, followed by a final melt-curve step ramping in temperature between 60˚C and 95˚C.

Chemicals and Antibodies

Methyl esters of C16:0 (meC16:0) and C18:0 (meC18:0) were obtained from Sigma Aldrich. Methyl esters of C20:0 (meC20:0), C22:0 (meC22:0), C24:0 (meC24:0), C16:1 (meC16:1), C18:1 (meC18:1), C20:1 (meC20:1), C22:1 (meC22:1) and C24:1 (meC24:1) were obtained from Larodan Fine Chemicals. Methyl esters of C26:0 (meC26:0) and C26:1 (meC26:1) were prepared as described previously (23). Tunicamycin was obtained from Sigma Aldrich. Hexadecanoic-16,16,16-D_3 acid (D_3-C16:0) and octadecanoic-17,17,18,18,18-D_5 acid (D_5-C18:0) were obtained from CDN isotopes. TO901317 was obtained from Cayman Chemical. GW3965 and LXR623 were obtained from Sigma Aldrich. SCD1 inhibitor 1716 [4-(2-chlorophenoxy)-N-(3-(3-methylcarbamoyl)phenyl) piperidine-1-carboxamide] was obtained from BioVision. All chemicals were dissolved in DMSO (Sigma Aldrich). Antibodies against SCD1 (M38 #2438) and XBP1s (D2C1F) were obtained from Cell Signaling Technology. Antibody against beta-actin (A5441) was obtained from Sigma Aldrich and IRDye secondary antibodies were obtained from LI-COR Inc.

Analysis of TO901317

Sample preparation was performed essentially as described with minor modifications (73). Briefly, a volume of tissue homogenate corresponding to 1 mg of protein was transferred into a 2 mL tube to which internal standards and 600 µL acetonitrile was added followed by vortex mixing for 3 min and centrifugation at 14,000 RPM for 10 minutes. The supernatant was transferred to a new tube and evaporated under a constant stream of nitrogen. The samples were then reconstituted in 100 µL water/methanol (40/60, v/v) and transferred to a sample vial and capped. Analysis of TO901317 was done using the metabolomics procedure as described by

Molenaars et al. using a Bruker trapped ion mobility-quadrupole time-of-flight mass spectrometer (timsTOF) (84). Identification of TO901317 was based on a combination of accurate mass, (relative) retention time and collisional cross section. Quantification of TO901317 was done by using tissue specific calibration curves. The calibration curve samples consisted of pooled tissue homogenates from untreated mice spiked with TO901317.

Liver histopathology

For histopathological assessment of the liver following TO treatment, two to three cross-sections (1-1.5 mm thick) of the left liver lobe from each mouse were dissected at the treatment endpoint, immersion fixed in phosphate-buffered 4% paraformaldehyde for 48-72 h at 4°C, embedded in paraffin and stained with hematoxylin–eosin and PAS. Biopsies were blinded and semi-quantitatively scored by a pathologist (JYEL) using a scoring system for histopathologic classification of liver lesions (Bedossa et al. (84). Each biopsy was scored for the grade of steatosis (S0: <5%; S1: 5%-33%, mild; S2: 34%-66%, moderate; S3: >67%, marked), ballooning (0: normal; 1: presence of clusters of hepatocytes with a rounded shape and pale cytoplasm usually reticulated; 2: same as grade 1 with some enlarged hepatocytes, at least 2-fold that of normal cells), inflammation (a focus of two or more inflammatory cells within the lobule. Foci were counted at 20 magnification (0: none; 1: 2 foci per 20; 2: >2 foci per 20), and fibrosis (stage 0 (F0) none); stage 1 (F1): 1a or 1b perisinusoidal zone 3 or 1c portal fibrosis, stage 2 (F2): perisinusoidal and periportal fibrosis without bridging, stage 3 (F3): bridging fibrosis and stage 4 (F4): cirrhosis).

Immunoblotting

XBP1s: fibroblasts were incubated with fatty acids as described previously (23). After 4 days of incubation with 60 µM fatty acids or 1 day with 10 µg/ml tunicamycin (positive control for XBP1s induction), cell pellets were prepared. Pellets were resuspended in PBS with protease inhibitor cocktail (Complete mini protease inhibitor cocktail, Roche) and homogenized by sonication with a needle on ice for 12 s at 7-8 Watt. Protein concentration was determined with the BCA assay using human serum albumin as standard. The protein samples were diluted in 5x Laemmli sample buffer and incubated at 95°C for 5 min. Aliquots of 25 µg protein were loaded on a 10% SDS-PAGE running gel. After protein separation, proteins were transferred to a 0.45 µM pore size nitrocellulose membrane. The membrane was incubated for 1 h with 5% (wt/vol) non-fat dried milk powder-TRIS buffered saline + 0.1% Tween-20 (TBST), followed by 2 h of incubation with rabbit anti-human XBP1s (D2C1F, Cell Signaling Technology) 1:1,000 in 5% non-fat dried milk powder-TBST. Next, the membrane was incubated for 1 h with IRDye goat-anti-rabbit 800CW (LI-COR Inc.) 1:10,000 in 5% non-fat dried milk powder-TBST + 0.01% (wt/vol) SDS.

SCD1: control and ALD fibroblasts were seeded at approximately 40% confluence. The next day, culture medium was replaced by culture medium containing LXR agonists or vehicle DMSO. After 3 days of incubation, cell pellets were harvested. Protein samples were prepared as described for the detection of XBP1s. The protein samples were diluted in 2x Laemmli sample buffer with 8 M urea and 40 µg of protein was loaded on a 12.5% SDS-PAGE running gel. After protein separation, proteins were transferred to a nitrocellulose membrane with a pore size of 0.45 µM. The membrane was incubated for 1 h with 5% (wt/vol) BSA in TBST followed by 2 hours of incubation with rabbit anti-human SCD1 (M38 #2438, Cell Signaling Technology) 1:1,000 in 5% BSA in TBST. Next, the membrane was incubated for 1 h with IRDye goat-anti-

rabbit 800CW (LI-COR Inc.) 1:10,000 in 5% BSA-TBST + 0.01% SDS. As a control for equal protein loading, the membranes were incubated with mouse monoclonal anti-beta-actin 1:20,000 (A5441 Sigma-Aldrich) in Odyssey blocking buffer (LI-COR):PBS + 0.1% Tween-20 (PBST) 1:1 for 30 min followed by incubation with IRDye donkey-anti-mouse RD680 1:10,000 (LI-CORin Odyssey blocking buffer (LI-COR):PBST + 0.01% SDS 1:1 for 1 h. All incubations were performed at room temperature. Fluorescently labeled proteins were detected by LI-COR Odyssey.

Supplemental Figure 1. Overview schematics of zebrafish behavior testing, primary compound screen, and secondary screen.

Supplemental Figure 2. Results from zebrafish ALD mutant secondary screen. **A.** Motor behavior results from individual animals, at 5, 6, and 7 dpf. Compound names along x-axis. WT, heterozygote, and sa509/+ indicated. **B**. Heat-map of results in A. **C.** Numbers of animals tested for each compound.

Supplemental Figure 3. Liver histology in WT and Abcd1-deficient mice, at baseline, or following 5 or 10 weeks of TO901317 adminstration. Assessment of histopathological liver changes following TO treatment. The liver biopsy was formalin-fixed and paraffin-embedded. Two to three serial sections stained with hematoxylin and eosin (H&E) and PAS. Biopsies were blinded and semi-quantitatively scored by a pathologist (JYEL) using a scoring system for histopathologic classification of liver lesions (described by Bedossa et al. (84)).

Top image: Each biopsy was scored for the grade of steatosis (**A**) (S0: <5%; S1: 5%-33%, mild; S2: 34%-66%, moderate; S3: >67%, marked), (**B**) ballooning (0: normal; 1: presence of clusters of hepatocytes with a rounded shape and pale cytoplasm usually reticulated. Although shape is different, size is quite similar to that of normal hepatocytes; 2: same as grade 1 with some enlarged hepatocytes, at least 2-fold that of normal cells), (**C**) inflammation (a focus of two or more inflammatory cells within the lobule. Foci were counted at 20 magnification (0: none; 1: 2 foci per 20; 2: >2 foci per 20) and (**D**) fibrosis (stage 0 (F0) none); stage 1 (F1): 1a or 1b perisinusoidal zone 3 or 1c portal fibrosis, stage 2 (F2): perisinusoidal and periportal fibrosis without bridging, stage 3 (F3): bridging fibrosis and stage 4 (F4): cirrhosis).

Bottom image: (x10 magnification) shows an example of a liver with stage 0 steatosis (less than 5% steatosis) and stage 1 inflammation (2 foci of inflammatory cells in x20 magnification, magnified in insert 3). The steatosis (shown by empty fat drops in cytoplasm) and grade 2 ballooning (reticulated cytoplasm with more than 2-fold enlarged cell size) of hepatocytes are magnified in inserts 1 and 2, respectively. PAS stain showed no fibrosis.

Supplemental Table 1.

Zebrafish ALD mutant primary behavior screen compounds and raw behavior results.

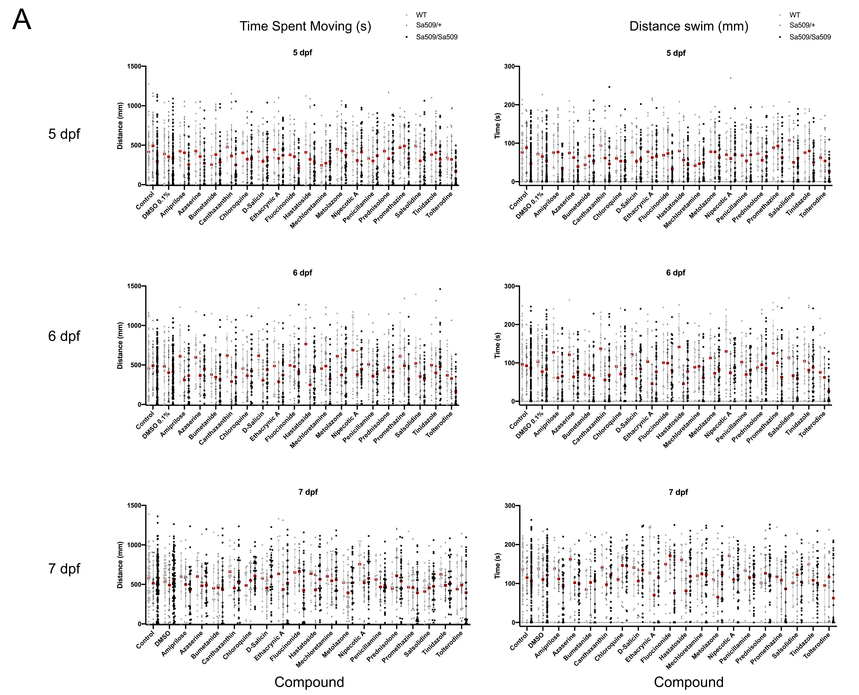
Supplemental Table 2.

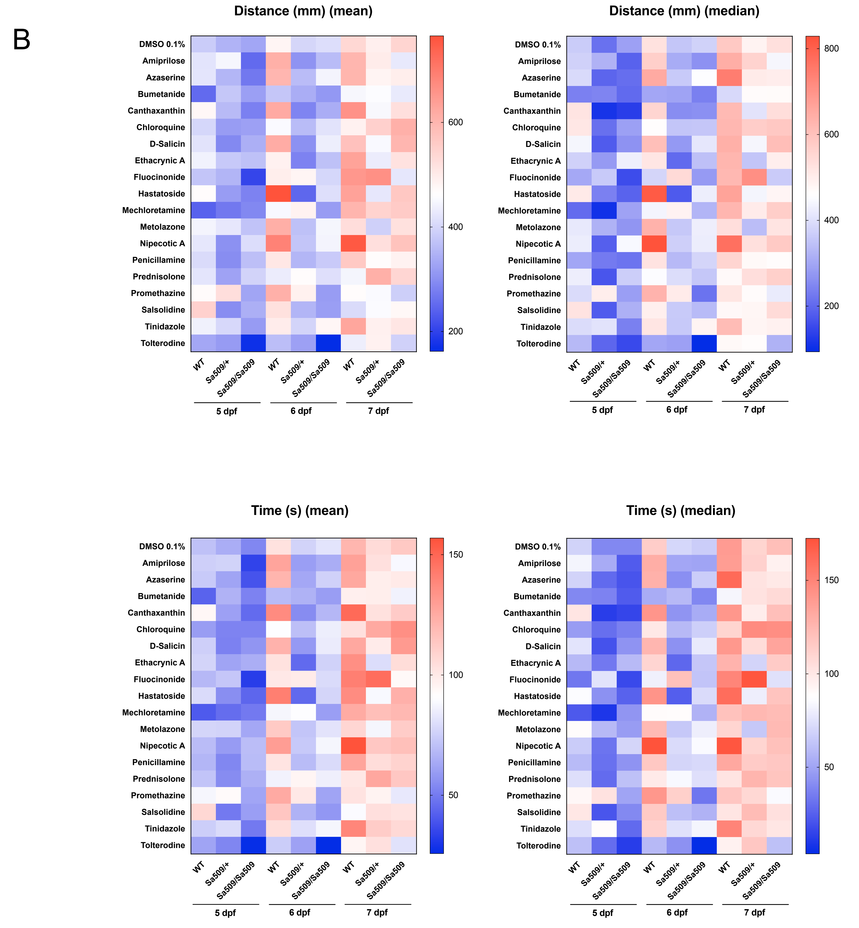
Zebrafish ALD mutant primary behavior screen Z scores for each motor behavior parameter.

Supplemental Table 3.

Zebrafish ALD mutant primary behavior screen; listing of compounds composite Z scores for Z score > 1 standard deviation.

Supplemental Figure 2



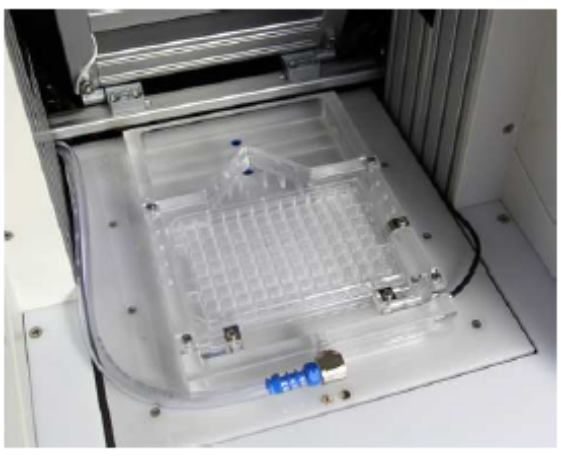


C

	5 dpf			6 dpf			7 dpf			
n	WT	Sa509/+	Sa509/Sa509	WT	Sa509/+	Sa509/Sa509	WT	Sa509/+	Sa509/Sa509	
DMSO 0.1%	44	34	80	44	34	82	44	34	80	476
Amiprilose	14	21	30	14	22	30	14	22	30	197
Azaserine	14	22	30	14	21	29	14	22	30	196
Bumetanide	14	20	30	14	22	29	14	22	29	194
Canthaxanthin	14	22	30	14	22	30	14	20	29	195
Chloroquine	14	22	30	14	22	30	14	19	29	194
D-Salicin	14	22	30	14	22	30	13	22	30	197
Ethacrynic A	14	22	30	14	22	30	14	22	29	197
Fluocinonide	14	22	30	12	22	29	12	21	29	191
Hastatoside	14	22	30	14	21	30	14	21	29	195
Mechloretamine	14	22	29	14	20	30	13	21	28	191
Metolazone	14	22	30	14	22	30	14	22	29	197
Nipecotic A	14	20	30	14	22	29	14	22	29	194
Penicillamine	14	22	30	13	22	29	14	22	30	196
Prednisolone	14	22	30	14	22	30	14	22	30	198
Promethazine	14	21	30	13	20	28	14	18	27	185
Salsolidine	14	22	30	14	22	29	14	22	29	196
Tinidazole	14	21	30	14	22	30	14	22	29	196
Tolterodine	14	22	29	14	21	30	14	21	28	193
	296	423	618	292	423	614	292	417	603	3978

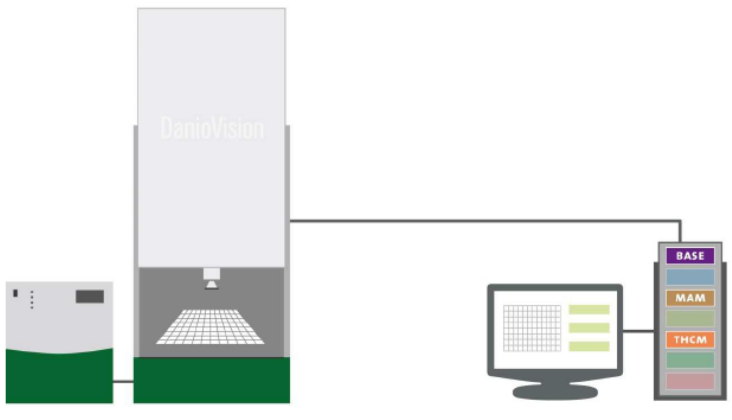
Supplemental Figure 1

A

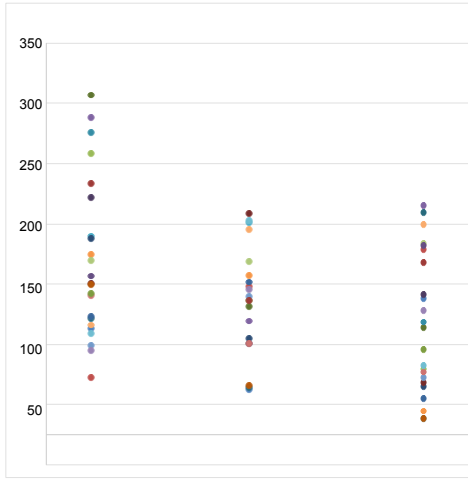


96-well tray holder

Screen hardware



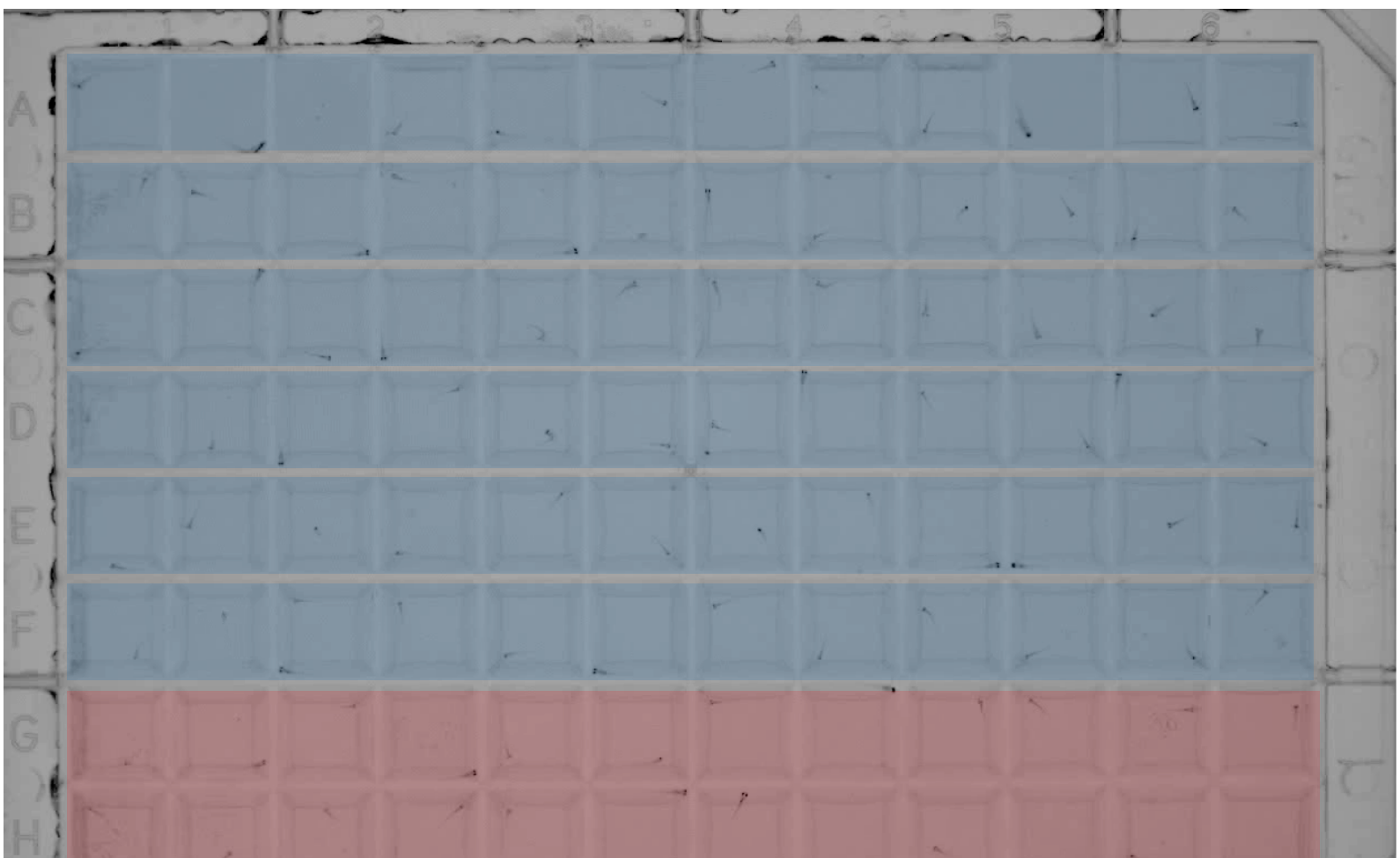
data flow



data output

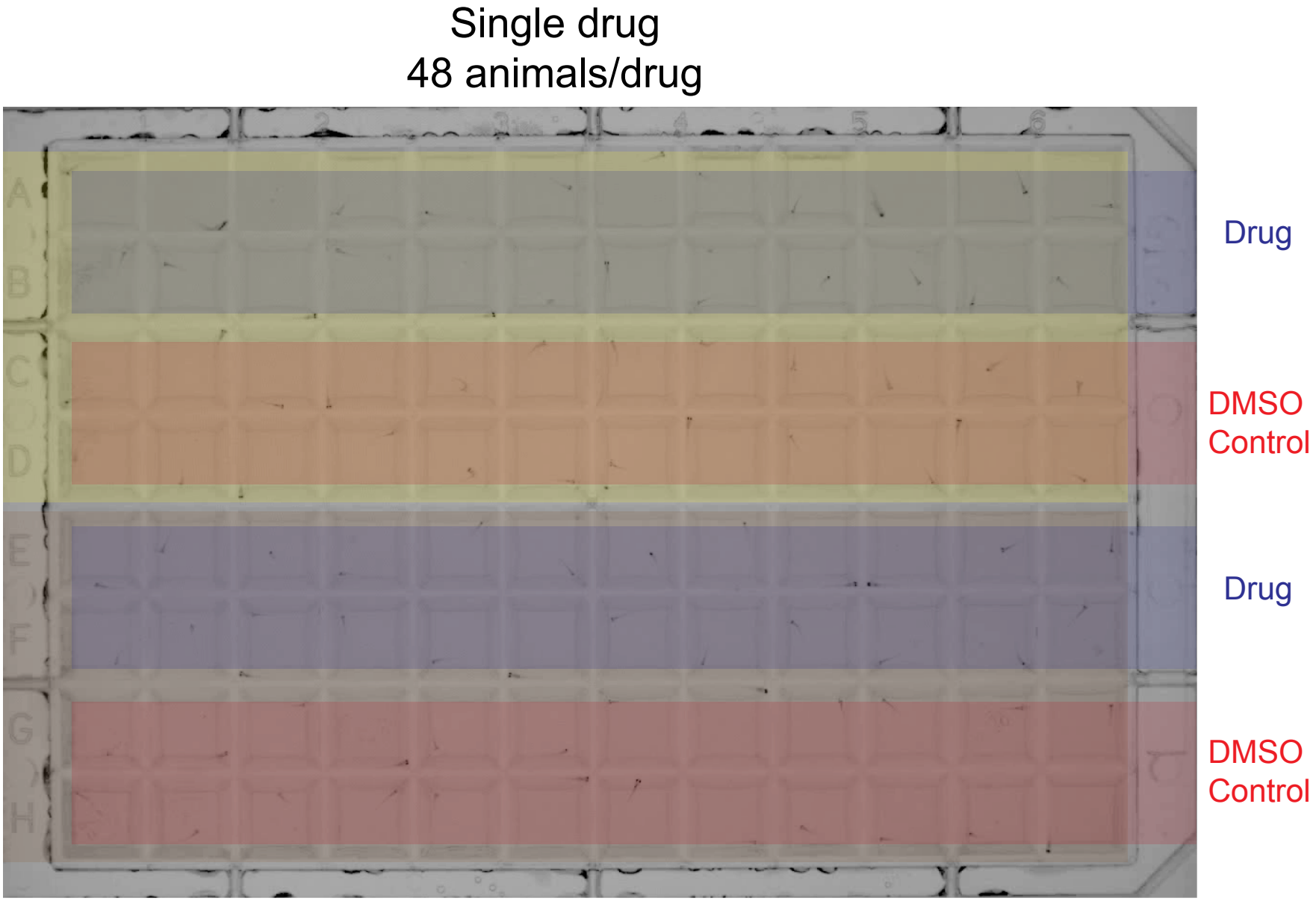
B

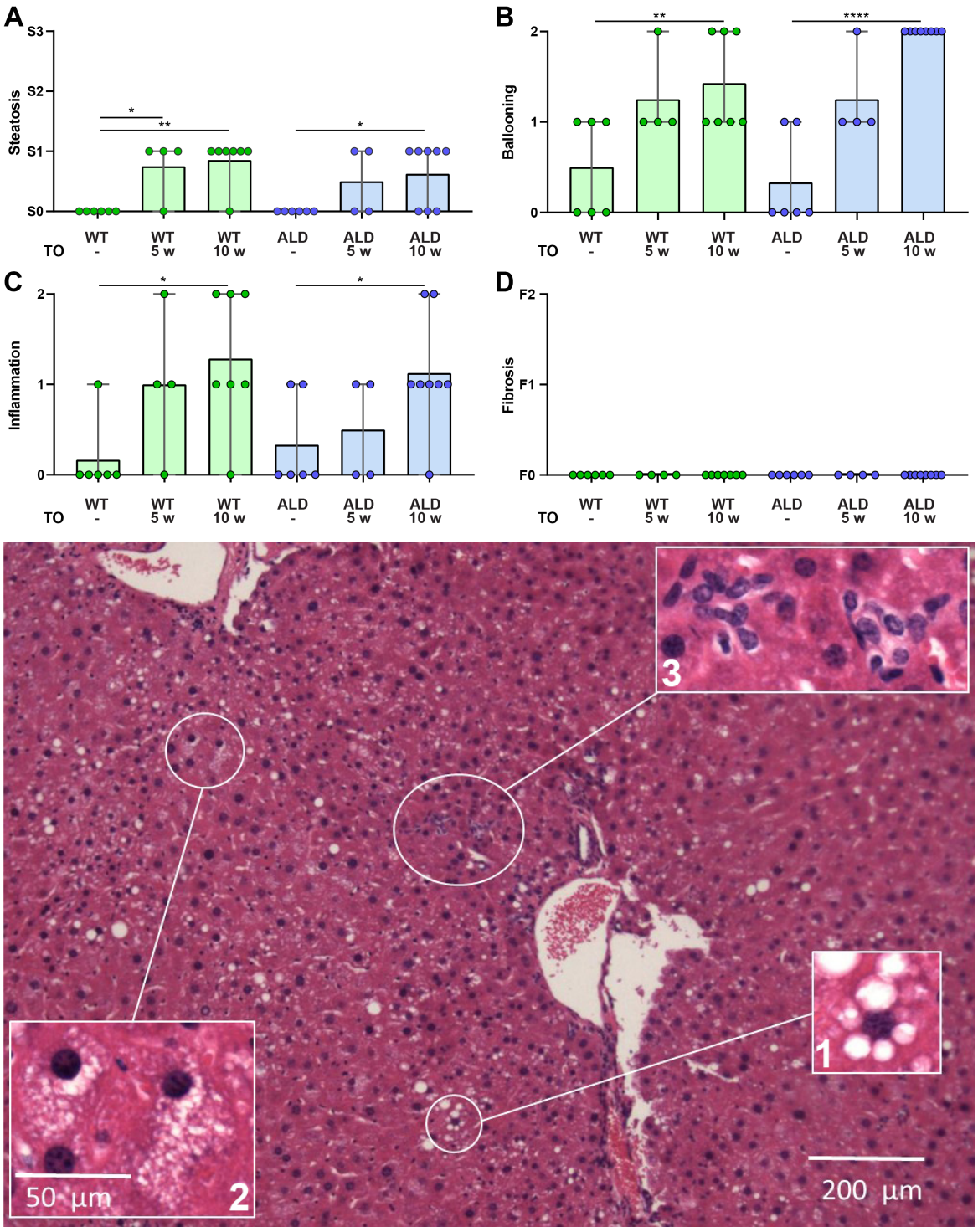
Primary Screen
2538 compounds
Single dose (10μM)
Groups of four drugs
12 animals/drug group



C

Secondary Screen
15 compounds > 1 S.D.
Single dose (2.5μM)
Single drug
48 animals/drug





Supplemental figure 3: Assessment of histopathological liver changes following TO treatment. The liver biopsy was formalin-fixed and paraffin-embedded. Two to three serial sections stained with hematoxylin and eosin (H&E) and PAS. Biopsies were blinded and semi-quantitatively scored by a pathologist (JYEL) using a scoring system for histopathologic classification of liver lesions (Bedossa et al. (2012) Hepatology 56(5):1751-9).

Top image: Each biopsy was scored for the grade of steatosis (**A**) (S0: <5%; S1: 5%-33%, mild; S2: 34%-66%, moderate; S3: >67%, marked), (**B**) ballooning (0: normal; 1: presence of clusters of hepatocytes with a rounded shape and pale cytoplasm usually reticulated. Although shape is different, size is quite similar to that of normal hepatocytes; 2: same as grade 1 with some enlarged hepatocytes, at least 2-fold that of normal cells), (**C**) inflammation (a focus of two or more inflammatory cells within the lobule. Foci were counted at 20 magnification (0: none; 1: 2 foci per 20; 2: >2 foci per 20) and (**D**) fibrosis (stage 0 (F0) none); stage 1 (F1): 1a or 1b perisinusoidal zone 3 or 1c portal fibrosis, stage 2 (F2): perisinusoidal and periportal fibrosis without bridging, stage 3 (F3): bridging fibrosis and stage 4 (F4): cirrhosis).

Bottom image: (x10 magnification) shows an example of a liver with stage 0 steatosis (less than 5% steatosis) and stage 1 inflammation (2 foci of inflammatory cells in x20 magnification, magnified in insert 3). The steatosis (shown by empty fat drops in cytoplasm) and grade 2 ballooning (reticulated cytoplasm with more than 2-fold enlarged cell size) of hepatocytes are magnified in inserts 1 and 2, respectively. PAS stain showed no fibrosis.