Beneficial Effects of Bempedoic Acid Treatment in Polycystic Kidney Disease Cells and Mice

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ABSTRACT

Background: ADPKD has few therapeutic options. Tolvaptan slows disease but has side effects limiting its tolerability. Bempedoic acid (BA), an ATP citrate-lyase (ACLY) inhibitor FDA-approved for hypercholesterolemia, catalyzes a key step in fatty acid/sterol synthesis important for cell growth and proliferation. BA also activates AMPK in mice. We hypothesized that BA could be a novel ADPKD therapy by inhibiting cyst growth, proliferation, injury, and metabolic dysregulation via ACLY inhibition and AMPK activation.

Methods: ACLY gene and protein expression levels were measured in murine *Pkd1*-null kidney cell lines derived from proximal tubule (PT) and collecting duct (IMCD), grown in 3D Matrigel cultures and treated ± BA before cyst analysis. Mitochondria were also characterized using MitoSox and MitoTracker staining. *Pkd1*$^{fl/fl}$*;Pax8-rtTA;Tet-O-Cre* C57BL/6J mice were induced with IP doxycycline injection on postnatal days 10 and 11 (P10 and P11) and then treated ±BA (30 mg/kg/d) ±tolvaptan (30-100 mg/kg/d) by oral gavage from P12-21. To assess disease severity, %total-kidney-weight-to-bodyweight (%TKW/BW) and BUN levels at euthanasia (P22) were measured. Kidney and liver homogenates were immunoblotted for expression of key disease and other cellular signaling biomarkers.

Results: ACLY protein expression and activity were upregulated in ADPKD kidney tissues relative to wild-type. BA inhibited cystic growth in both PT and IMCD-derived *Pkd1*-null kidney cells. BA also inhibited mitochondrial superoxide production and promoted mitochondrial elongation in these cells, suggesting improved mitochondrial function. In ADPKD mice, BA reduced %TKW/BW and BUN to a similar extent as tolvaptan relative to untreated controls. Addition of BA to tolvaptan caused a further reduction in %TKW/BW and BUN vs. tolvaptan alone. BA reduced ACLY and stimulated AMPK activity in kidneys vs. controls. BA also inhibited mTOR and ERK signaling and sharply reduced the injury marker KIM-1 and, to a lesser extent, NGAL. In liver, BA increased mitochondrial biogenesis while inhibiting apoptosis. These BA effects occurred alone and together with tolvaptan.

Conclusion: BA inhibited cyst growth in vitro and ADPKD severity in vivo. Combining BA with tolvaptan further improved various ADPKD disease parameters. Repurposing BA may be a promising new ADPKD therapeutic strategy, having beneficial effects when used alone and along with tolvaptan in mice.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD), the most common genetic cause of end-stage kidney disease (ESKD), affects every ethnicity with a prevalence of ~1:500-1000 and ~600,000 patients in the U.S. alone (Gabow, 1993, Chebib and Torres, 2016). Patients with ADPKD present with enlarging cystic lesions in the kidney and often the liver as well, leading to a progressive decline in kidney function that is associated with ESKD in half of ADPKD patients by age 50-60 (Chebib and Torres, 2016). Most ADPKD patients have loss-of-function mutations in the multifunctional proteins polycystin-1 or -2 (PC1 and PC2, encoded by the genes *PKD1* and *PKD2*) (Chebib and Torres, 2016). ADPKD therapeutic options to specifically address the decline in glomerular filtration rate (GFR) are very limited. The only current FDA-approved drug for ADPKD is tolvaptan, a vasopressin 2 receptor (V2R) antagonist. This drug slows disease progression in patients at risk for rapid progression towards end-stage kidney disease (ESKD) (Torres et al., 2012). However, tolvaptan has the dose-dependent side effect of polyuria and a risk of hepatotoxicity that requires monthly monitoring of liver function tests (Blair, 2019, Woodhead et al., 2017). Thus, there is a clear need for additional ADPKD therapies targeting different cellular pathways dysregulated in ADPKD that could potentially be used alone or in combination with tolvaptan.

There is growing recognition that ADPKD cyst-forming PC1-deficient cells have major metabolic derangements that likely contribute to kidney tubular epithelial cyst formation and expansion. Specifically, compared to control kidney tubular epithelial cells, ADPKD cells display increased aerobic glycolysis (the Warburg effect), impaired fatty acid oxidation, increased cellular proliferation, and reduced AMP-activated protein kinase (AMPK) activity (Rowe et al., 2013, Menezes et al., 2016). Earlier, we helped pioneer the use of the AMPK activator metformin to inhibit ADPKD kidney cyst growth in mouse models of *Pkd1* knockout (Takiar et al., 2011).

AMPK is a ubiquitous metabolic sensor that regulates many cellular processes (Hallows, 2005, Steinberg and Kemp, 2009, Steinberg and Carling, 2019). The role of AMPK in the protection of kidney function has been studied in many models of acute and chronic kidney disease (Rajani et al., 2017). Of note, kidney AMPK activity is generally decreased in both humans and mice with chronic kidney disease (CKD) (Dugan et al., 2013, Li et al., 2015). The renoprotective role of AMPK in CKD is thought to occur through activation and induction of several effector pathways including autophagy, fatty acid oxidation, antioxidant pathways (Decleves et al., 2011) and via inhibition of the inflammatory cascade (Peairs et al., 2009). In response to metabolic and other cellular stresses, AMPK activation helps maintain cellular energy balance by restoring ATP levels through regulation of metabolic enzymes, promoting cellular energy efficiency, and inhibiting pro-growth anabolic pathways.

Our group recently demonstrated that the AMPK activator metformin ameliorates relevant disease parameters in a hypomorphic PKD mouse model that closely mimics human ADPKD (Pastor-Soler et al., 2022). We have also been involved in the TAME-PKD study where metformin was found to be safe and tolerable in ADPKD patients (Perrone et al., 2021). Of note, metformin doses that inhibit cyst growth in pre-clinical ADPKD models may not be as tolerable or clearly efficacious in ADPKD patients (Brosnahan et al., 2022, Perrone et al., 2021). Moreover, along with the various beneficial effects of metformin, including inhibition of cyst fluid secretion, cell proliferation and cAMP production (Takiar et al., 2011, Miller et al., 2013), metformin inhibits Complex I of the mitochondrial respiratory chain (Owen et al., 2000), which may hamper the promotion of defective mitochondrial oxidative metabolism in ADPKD. Thus, novel drugs targeting complementary pathways in ADPKD that could potentially synergize with tolvaptan or metformin may afford lower

effective drug dosing and have better efficacy against the disease when used in combination in patients.

Here we explored targeting and inhibiting the enzyme ATP-citrate lyase (ACLY) to determine its effects on relevant disease parameters in vitro and in a conditional *Pkd1* knockout mouse model of ADPKD. ACLY is a key metabolic enzyme that promotes lipid and cholesterol biosynthesis by generating acetyl-CoA from cytosolic citrate and has been recently identified as a new therapeutic target for lowering cholesterol in patients with atherosclerotic cardiovascular disease (Huynh, 2019). In addition, ACLY inhibition has emerged as a new therapeutic strategy for cancer, where ACLY inhibition blocks lipid synthesis and cellular proliferation (Zhao et al., 2016). As with tumor growth, cystic growth in ADPKD relies on such mechanisms that support enhanced cellular proliferation. Importantly, ACLY has also been reported to bind to and inhibit the AMPK-β_1 subunit (Lee et al., 2015), suggesting a mutual antagonism between ACLY and AMPK.

Bempedoic acid (BA; also known as ETC-1002) inhibits ACLY and is approved by the FDA as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with atherosclerotic cardiovascular disease who require additional lowering of LDL-C. BA also activates AMPK in mice (Pinkosky et al., 2016). BA exists as a pro-drug that gets converted to its active form by an enzyme (Very long-chain acyl-CoA synthetase; ACSVL1 or FATP2) whose tissue expression is primarily limited to kidney and liver (Pinkosky et al., 2016), the two principal organs affected in ADPKD. We thus hypothesized that BA treatment could be beneficial in ADPKD by inhibiting kidney cyst growth, inflammation, injury, and metabolic dysregulation via simultaneous ACLY inhibition and AMPK activation with limited off-target effects. The rationale for the use of BA in ADPKD involves correcting the dysregulated metabolism and excessive cell proliferation in ADPKD and is summarized schematically in **Figure 1**. Herein, we tested the potential beneficial effects of BA alone and in combination with tolvaptan on 3D cyst growth and mitochondrial function in *Pkd1*-null kidney cells in vitro, and on key parameters of disease severity in kidneys and liver in conditional *Pkd1* knockout mice.

MATERIALS AND METHODS

Reagents and chemicals. All reagents and chemicals used were purchased from Sigma (St. Louis, MO, USA) or Thermo Fisher (Pittsburgh, PA, USA) unless otherwise stated. Pharmaceutical grade bempedoic acid (BA) and tolvaptan were obtained from Esperion Therapeutics, Inc. (Ann Arbor, MI, USA) and Otsuka Pharmaceuticals (Japan), respectively. Please see **Table 1** for detailed information on the antibodies and conditions that were used for immunoblotting.

Cell culture models. *Pkd1*-Null (PN24) and *Pkd1*-Het (PH2) cells were a kind gift of Dr. Stefan Somlo and were originally derived by microscopically dissecting and then dissociating proximal tubules (PTs) from *Pkd1*$^{flox/-}$:TSLargeT (ImmortoMouse) mice. Parental *Pkd1*$^{flox/-}$ cells from a clone showing epithelial properties were then transiently transfected with a plasmid encoding Cre recombinase and cloned again by limiting dilution, giving rise to daughter cells that either expressed Cre and therefore had undergone transformation to *Pkd1*$^{-/-}$ or that had not expressed Cre and retained the parental *Pkd1*$^{flox/-}$ genotype, as described previously (Joly et al., 2006). These cells were cultured in medium composed of equal volumes of DMEM and Ham's F-12 plus 7.5 nM sodium selenate, 5 µg/ml transferrin, 2 mM glutamine, 2 nM triiodothyronine, 5 µg/ml insulin, and 2% (vol/vol) FBS. Cells were maintained under permissive conditions (33°C with 10 U/ml γ-interferon) in a humidified 5% CO_2-95% air incubator with medium changes every other day and passaged at least twice weekly. To induce differentiation, cells were kept under non-permissive conditions (37°C without γ-interferon) for 5-7 days prior to use in experiments. Mouse inner medullary collecting duct (IMCD) cell lines were also generously provided by Dr. Stefan Somlo. Wild-type (Wt)-IMCD3 (Cas9) is a control cell line for IMCD3-CRISPR-knockout cells (ID1-3E5 *Pkd1*$^{-/-}$) (Decuypere et al., 2021). Both cell lines were cultured in regular DMEM/F12 medium containing 5% FBS in a 37°C humidified 5% CO_2-95% air incubator with medium changes every other day and passaged approximately twice weekly.

3D culture. Matrigel™ from BD Biosciences (BD #356234) was thawed overnight at 4°C prior to adding 50 µl to each well of an 8-well glass chamber slide (Lab-Tek #155409) and spreading evenly in the well using a P-200 tip. The slides were then placed in a cell culture incubator to allow the Matrigel™ to solidify for at least 15 minutes. During this time, *Pkd1*$^{-/-}$ cells were trypsinized and mixed into a stock of Assay Medium containing 2% Matrigel™ at a density of 6,000 cells per 400 µl of this medium. 400 µl of this mixture was then plated on top of the solidified Matrigel™ in each well of the chamber slide on day 0. Cells were then allowed to grow in a 5% CO_2 humidified incubator at 37°C and fed with Assay Medium containing 2% Matrigel™ every other day. By day 5-6, the cells formed 3-D clusters and subsequently started forming cysts. On day 6, we began treatment with 10 µM forskolin plus 100 µM IBMX, in the absence or presence of 100 µM BA (ETC-1002) for the last 3 days. The vehicle control was dimethylsulfoxide (DMSO). Cell structures were thresholded from background, and the cross-sectional area of each cell cluster grown in 3D Matrigel culture was calculated using the ImageJ Analyze Particles plug-in software (NIH), with a minimum size cutoff of 50 pixels to filter out isolated cells and debris.

Mitochondrial morphology and superoxide quantification. To analyze mitochondrial morphology and superoxide production, PT- and IMCD-derived *Pkd1*$^{-/-}$ cell lines were stained with MitoTracker™ Deep Red FM (M22426) or MitoSOX™ Red mitochondrial superoxide indicator (M36008), along with the nuclear stain Hoechst 33342 (H3570; Thermo Fisher Scientific Inc. Pittsburgh, PA, USA). Briefly, PN24 or ID1-3E5 cells were seeded onto 4-well chamber slides in the above-described media prewarmed to 37°C. Staining and washes were performed 2 days after plating and 24 h after the indicated treatments according to the manufacturer's protocols. Cells were imaged using a Leica

DMi8 live cell imaging fluorescence microscope using a 40X oil objective at zoom 1.6. Automated quantification of mitochondrial morphology was done on MitoTracker-stained cells using ImageJ software. The aspect ratio (length/width) was used as a measurement of mitochondrial elongation. We compiled the mean aspect ratio of >1,000 mitochondria in each of the selected cells and then compared the mean values (± SEM) from each of the cells analyzed across the two conditions (± BA treatment). MitoSOX Red fluorescence was used to assess mitochondrial superoxide production. The fluorescence intensity of each randomly selected cell was quantified using ImageJ software. Results are reported as compiled mean cellular values ± SEM from at least three independent experiments with the indicated number of cells analyzed (n = 26 to 39 cells).

ADPKD mouse models. All animal procedures followed NIH guidelines for the care and use of laboratory animals and were approved by the University of Southern California's Institutional Animal Care and Use Committee. Male and female *Pkd1*$^{fl/fl}$*;Pax8-rtTA;Tet-O-Cre* transgenic mice in the C57BL/6J background were obtained as a generous gift from the Baltimore PKD Core Center and were used as an ADPKD model for in vivo studies, as described previously (Ma et al., 2013). Genotyping was confirmed between postnatal days 5 and 7 (P5-P7) and *Pkd1* inactivation was induced with IP doxycycline injection (50 mg/kg) on P10 and P11 to induce rapidly progressive cyst development. Mice were then treated in the absence or presence of BA (30 mg/kg/d) with or without cotreatment with tolvaptan (30-100 mg/kg/d) by daily oral gavage from P12-21. These drugs (or vehicle) were formulated into micelle suspensions comprised of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-poly(ethylene glycol 2000) (DSPE-PEG(2000)-maleimide, Avanti Polar Lipids, Alabaster, AL, USA) to improve solubility prior to daily gavage injection by syringe into the mouse pups. Drug-loaded micelles were self-assembled via thin film evaporation using previously described methods (Huang et al., 2020). Briefly, 18 mg of BA (and/or up to 66 mg of tolvaptan) and 100 µM of DSPE-PEG(2000)-methoxy were dissolved in methanol and evaporated with nitrogen gas to form thin films. The resulting thin films were dried overnight under vacuum and hydrated in PBS at 80 °C for 30 min, and drug-loaded micelles were stored at 4°C and used within 3 days of formulation. Mice were euthanized at the end of the experiment at P22 to harvest kidneys and livers and obtain blood to assess various measures of disease severity.

Whole blood chemistry measurements. We used the Abbott i-STAT handheld blood analyzer equipped with Chem 8+ cartridges for measurements blood urea nitrogen (BUN) from mixed venous blood at the time of euthanasia at P22 (Tinkey et al., 2006). Briefly, mice were anesthetized with isoflurane, and approximately 100 µl of blood was obtained from the submandibular venous plexus using a single-use lancet (Golde et al., 2005). The blood was collected in a heparinized vial and quickly added to the i-STAT cartridge, and results were obtained within two minutes.

Kidney weight measurements, tissue preparation and microscopy. After clamping the renal pedicle at P22 the left kidney was quickly removed, rapidly weighed, placed in a microcentrifuge tube and frozen in liquid nitrogen. The right kidney was also removed after clamping of the renal pedicle, quickly weighed, and rapidly cut coronally. One half of this right kidney was fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS, and the other half was placed in RNAlater (Qiagen, Waltham, MA) for future preparation of cDNA.

After overnight fixation at 4°C, the kidney tissues were washed in PBS, quenched in NH_4Cl and further washed in PBS. The samples were then placed in 10% neutral buffered formalin (VWR, Radnor, PA, USA) at 4°C for 16-18 hours. The fixed kidney samples were then dehydrated in serial alcohols, then cleared with xylene, embedded in paraffin, and cut into 4-µm sections on a rotary microtome at the Keck School of Medicine of USC Norris Pathology Core. The fixed tissues were

stained with hematoxylin and eosin (H&E) for histological evaluation. We then obtained images using an Olympus IX73 inverted microscope using a Plan Achromat 2X objective for a total magnification of 20X (numerical aperture of 0.06 and working distance of 5.8mm).

Electrophoresis and immunoblotting analysis. Kidney and liver lysates were prepared from frozen tissues, homogenized, centrifuged, proteins quantitated, and samples electrophoresed and transferred to nitrocellulose membranes, as described previously (Pastor-Soler et al., 2022). The membrane was first stained with Revert 700 total protein stain solution (LI-COR) and then washed prior to imaging at 700 nm for total protein quantitation per lane, as per the manufacturer's recommendations. After destaining, the membrane was then blocked and probed with primary and secondary antibodies of interest. Quantification of immunoblot images was performed by densitometry and analyzed using Image Studio Lite Ver 5.2 software (LI-COR, USA).

Statistics. Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA, USA) to obtain the mean values and SEM for each treatment group. In most experiments, significance was determined using two-tailed, unpaired Student's t-tests assuming unequal variances for the groups, or one-way ANOVA with post-hoc Tukey corrections for multiple comparisons. Individual data points are shown in the bar graphs of figures, along with the mean (± S.E.) for each treatment condition. P values <0.05 were considered significant.

RESULTS

As outlined above, the purpose of this initial study was to test as a proof of concept the potential role of the ACLY inhibitor and AMPK activator bempedoic acid (BA) as a novel therapeutic for ADPKD using in vitro and in vivo ADPKD model systems. A particular feature of BA that may lend itself specifically to ADPKD therapy is its expected activity limitation primarily to kidney and liver, the two organs predominantly affected in the disease, where the BA pro-drug can get converted to its active metabolite via local expression of very long-chain acyl-CoA synthetase (FATP2 or ACSVL1; Cf. **Figure 1**, step 1), as described below.

Very long-chain acyl-CoA synthetase is detected in mouse kidney, liver, and in key model cell lines for the study of ADPKD. To determine whether our kidney cell lines and conditional *Pkd1* KO mouse model would be useful tools to evaluate the potential beneficial effects of BA in reducing cyst size or number in ADPKD, we first evaluated FATP2 protein expression in cells and tissues by immunoblot. Specifically, two bands were detected using an anti-FATP2 primary antibody that detects both a shorter FATP2 splice variant (FATP2b) and the long form of FATP2a at ~70kDa (Melton et al., 2011). The FATP2b variant lacks the protein domain required for the conversion of BA to its active metabolite (BA-CoA) and was expressed in all mouse tissues tested (**Figure 2A**, *lower band*). However, the FATP2a form was previously reported to have significant expression only in liver and kidney tissue, thus providing specificity of BA pro-drug conversion to its active form only in these organs (Pinkosky et al., 2016, Steinberg et al., 1999). Consistent with these earlier studies, we detected the full-length FATP2a primarily in liver and kidney tissue homogenates, with minimal expression in other tissues (**Figure 2A**, *upper band*). Importantly, FATP2 immunoblotting of representative *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mouse kidney homogenates treated with or without doxycycline to induce tubule-specific *Pkd1* gene inactivation revealed comparable expression levels of both FATP2 isoforms (**Figure 2B**). We also detected both FATP2a and FTAP2b isoforms in immortalized mouse kidney epithelial cells derived from both PT (**Figure 2C**) and IMCD (**Figure 2D**). Altogether, these results confirm that the ADPKD cell lines and ADPKD mouse model used in our studies express the enzyme required to convert the BA pro-drug to its active compound and help support a rationale to evaluate the effects of BA on cyst growth in cell culture and in vivo.

Pkd1 knockout in kidney cell lines is associated with an increase in active ATP-citrate lyase when compared to control parental cell lines. Due to the abundance of ACLY expression in non-cystic PT cells, it was challenging to evaluate differences in ACLY expression and activity by immunoblot of total kidney homogenates. To assess whether cell lines that recapitulate ADPKD cystic disease ex vivo express ACLY, one of the targets of BA, we tested the expression of this enzyme by immunoblotting PT- and IMCD-derived model cell lines. All four cell lines tested, parental PT-derived *Pkd1*$^{flox/-}$ cells, PT-derived *Pkd1*$^{-/-}$ cells (Joly et al., 2006) and IMCD-derived WT-IMCD3 and ID1-3E5 *Pkd1*$^{-/-}$ cells (Decuypere et al., 2021) express ACLY (total ACLY or tACLY; **Figure 3A** and **B**). In addition, these cell lines also expressed an active form of ACLY phosphorylated at a target site of Akt (also known as Protein Kinase B; PKB) at Ser-455 (pS455 ACLY). We then normalized the levels of active pS455 ACLY to tACLY in the same samples. We found a significant increase of active ACLY in the *Pkd1*$^{-/-}$ cells from both PT and CD origin, compared to parental controls (**Figure 3C** and **D**). These results suggest that ACLY activity is increased in ADPKD epithelial cells, potentially contributing to cystic growth and disease progression, and thus that targeting this enzyme for inhibition by BA in vivo may be beneficial in reducing cyst size.

Bempedoic acid treatment and the ATP-citrate lyase inhibitor SB-204990 dramatically inhibited cystic growth in Pkd1-null kidney cells lines grown under 3D cyst-forming conditions. We generated

cysts from PT-derived *Pkd1*$^{-/-}$ kidney epithelial cell lines by growing the cells in Matrigel for a total of 12 days and treating these cells with forskolin plus a phosphodiesterase inhibitor from day 2-12. This treatment enhances cyst formation in these commonly used cell culture models (Cabrita et al., 2020). Once cysts appeared by day 9, we exposed these cultures to either vehicle control (DMSO) or the AMPK activator and ACLY inhibitor BA for the last three days of the experiment. Under light microscopy, we observed a substantial decrease in cyst size in the cultures treated with BA and another ACLY inhibitor SB-204990 (Chu et al., 2010) compared to vehicle control (**Figure 4Ai**). Using Image J software, we quantified the cystic area from those micrographs. Our summary of these data (**Figure 4Aii)** confirmed a significant reduction in cyst area in the BA- and SB-204990-treated cultures as compared to those exposed to vehicle control.

We then proceeded to test whether these two ACLY inhibitors could also reduce cyst area in an IMCD-derived *Pkd1*$^{-/-}$ kidney epithelial cell line (**Figure 4B**). Cysts developed when these cells were grown in Matrigel for a total of 7 days and treated with forskolin and IBMX after the first day. For the final 3 days in Matrigel culture when cysts were observed, the cultures were then treated with either vehicle control, BA or SB-204990. By visual inspection of the cultures and micrographs, we confirmed that the ACLY-inhibiting compounds (**Figure 4Bi**) reduced cyst size compared to vehicle. Cyst size quantification showed a statistically significant cyst reduction of IMCD-derived ADPKD cysts by BA and SB-204990 versus DMSO (**Figure 4Bii**). These experiments further confirmed our hypothesis that ACLY inhibitors have the promise of a therapeutic effect in vivo by inducing a significant reduction in cyst size in ADPKD in vitro 3D cell culture models.

Bempedoic acid inhibits mitochondrial superoxide production and promotes mitochondrial elongation in ADPKD kidney-derived cell lines. Normal mitochondrial function is regulated in part by the interplay of two targets of BA, AMPK and ACLY (Cf. **Figure 1**). These key metabolic enzymes appear to be dysregulated in ADPKD, where there is upregulation of ACLY activity (**Figure 3**) and downregulation of AMPK activity (Rowe et al., 2013, Song et al., 2020). Moreover, when mitochondria are unable to efficiently utilize fatty acids for oxidative metabolism (Cf. **Figure 1 - step 6**) with decreased electron transfer in the respiratory chain, there is increased reactive oxide species (ROS) production leading to accumulation of superoxide (Lenaz, 2001). Mitochondrial dysfunction with increased oxidative stress is a key feature of ADPKD (Chang and Ong, 2018). Moreover, it has been shown that AMPK activation may improve mitochondrial function in ADPKD and overall disease severity (Song et al., 2020). BA would thus appear to be a good candidate drug to induce simultaneous ACLY inhibition and AMPK activation as regulators of mitochondrial function.

To examine whether BA-mediated cyst reduction is associated with improved mitochondrial function in ADPKD, we first tested the effects of BA on mitochondrial superoxide production in both PT- and IMCD-derived *Pkd1*$^{-/-}$ cells (**Figure 5A**). These cells were loaded with MitoSOX™, a mitochondrial superoxide indicator, and incubated in the presence of BA or vehicle for 24 h. Our results supported the hypothesis that BA improves mitochondrial function, as demonstrated by reduced fluorescence signal from MitoSOX^{TM} in cells treated with BA compared to the vehicle control. These findings indicated that there is reduced mitochondrial superoxide production with BA treatment relative to controls in both PT-derived *Pkd1*$^{-/-}$ (PN24) or IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) cells.

Mitochondrial morphology is another important indicator of mitochondrial oxidative function. Specifically, mitochondrial elongation facilitates cristae formation and assembly of respiratory complexes to enhance oxidative phosphorylation in cells (Li et al., 2017). Germino and colleagues demonstrated that mitochondrial elongation is defective in ADPKD model cell lines with decreased elongation in *Pkd1*-null cells compared to wild-type controls (Lin et al., 2018), as reviewed in

(Padovano et al., 2018). Here we tested the effects of BA treatment on mitochondrial elongation (or aspect ratio) using MitoTracker™ Deep Red staining in both PT-derived PN24 and IMCD-derived ID1-3E5 cells (**Figure 5Bi**). Quantification of mitochondrial length divided by width was performed on the IF images using Image J. We found that BA treatment significantly increased mitochondrial elongation of both *Pkd1*-null cell lines (**Figure 5Bii**). These findings are consistent with the hypothesis that BA promotes mitochondrial oxidative function in *Pkd1*-deficient cells.

In vivo studies testing the effects of bempedoic acid and tolvaptan on PKD disease severity in an early/rapid induced Pkd1 gene inactivation ADPKD mouse model. The use of tolvaptan, the only FDA-approved drug currently to treat ADPKD progression in patients with certain characteristics, is limited by its side effects, such as polyuria and thirst, potential hepatotoxicity, and its availability and cost (Blair, 2019). Future ADPKD treatment strategies may involve combination therapies including tolvaptan and other drugs that target complementary dysregulated cellular signaling pathways, potentially conferring synergistic or additive benefits, and allowing lower drug doses than those used in ADPKD monotherapy. To evaluate the effects of BA and tolvaptan in vivo we used an early and rapid model of ADPKD progression, the *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mouse. The disease was induced via doxycycline administration at P10-P11 to inactivate *Pkd1*. Subsequently the mice were treated with either vehicle, BA, BA plus tolvaptan or tolvaptan alone P21 days prior to euthanasia at P22, a timeline summarized in **Figure 6A**. These mice progress to ESKD at around the time of euthanasia (Ma et al., 2013). We first measured changes in the % total kidney weight/body weight (%TKW/BW) and blood urea nitrogen (BUN) levels at the time of euthanasia as markers of disease severity in this ADPKD model. Representative kidney section micrographs stained by H&E under the different treatment conditions are shown in **Figure 6B**. As shown in **Figure 6C**, there were graded reductions of the %TKW/BW that occurred with the BA and/or tolvaptan treatments. Specifically, BA (30 mg/kg/d) reduced %TKW/BW vs. vehicle at euthanasia (6.9 vs. 11.9%; $P<0.01$). Similarly, tolvaptan (100 mg/kg/d) reduced %TKW/BW to 7.8% vs. vehicle ($P<0.05$). Addition of BA (30 mg/kg/d) to tolvaptan (100 mg/kg/d) caused a further reduction in %TKW/BW (4.9%; $P<0.05$) vs. tolvaptan alone. As shown in **Figure 6D**, there were also graded reductions of BUN levels at euthanasia with BA and/or tolvaptan treatments. BA treatment was associated with a reduced levels of BUN vs. vehicle (59 vs. 107 mg/dL; $P<0.05$). Tolvaptan treatment is associated with lower levels of BUN relative to vehicle at 30 (68 mg/dL; $P<0.05$) and 100 mg/kg/d (35 mg/dL; $P<0.01$). Again, addition of BA to tolvaptan at 30 mg/kg/d led to further significant reduction in BUN (38 mg/dL; $P<0.05$). In summary, these findings indicate that both BA and tolvaptan reduce kidney growth and improve kidney function in this early onset ADPKD mouse model, and there are additive benefits with combination therapy.

Effects of bempedoic acid and tolvaptan treatment in PKD mice on protein expression of key signaling and injury markers in kidney homogenates. Kidneys from the conditional *Pkd1* knockout mice were harvested at the time of euthanasia to analyze the effects of treatment with BA and tolvaptan on relevant target proteins (**Figure 7**). As expected, BA treatment reduced ACLY activity (pS455 ACLY; **Figure 7A**) and stimulated AMPK activity (pT172 AMPK; **Figure 7B**) in kidney tissue homogenates relative to vehicle controls or treatment with tolvaptan alone. BA also inhibited mTOR and ERK pathway signaling, which are upregulated in ADPKD (Saigusa and Bell, 2015), as evidenced by decreased phosphorylation of P70S6K (**Figure 7C**) and ERK (**Figure 7D**), respectively, relative to paired controls in the absence of BA. Finally, BA also sharply reduced expression of the PT kidney injury marker KIM-1 (**Figure 7E**) and, to a lesser extent, the distal kidney injury marker NGAL (**Figure 7F**) relative to controls. These BA effects occurred both alone and in combination with tolvaptan.

Effects of bempedoic acid and tolvaptan treatment in ADPKD mice on protein expression of key signaling markers in liver homogenates. The liver is a main target for BA in its inhibition of sterol synthesis and its other metabolic effects (Pinkosky et al., 2016). As idiosyncratic hepatotoxicity is a concern in ADPKD patients treated with tolvaptan (Blair, 2019), we also examined protein expression of several markers in liver homogenates from the conditional *Pkd1* knockout mice to analyze the effects of treatment with vehicle control, BA (30 mg/kg/d) in the presence or absence of high-dose tolvaptan (**Figure 8**). BA treatment resulted in the expected decrease in ACLY activity (pACLY) in liver tissue (**Figure 8A**) while increasing AMPK activity, as evidenced by the increase in pT172 AMPK (**Figure 8B**). BA also increased phosphorylation of acetyl-CoA carboxylase (pACC), a canonical target of AMPK (**Figure 8C**). Interestingly, BA treatment caused a significant enhancement in the expression in liver of FATP2, the fatty acid transporter that is required for activation of the BA pro-drug (Pinkosky et al., 2016), both alone and in combination with tolvaptan (**Figure 8D**). Of note, BA also appears to promote mitochondrial biogenesis as measured by the expression of PGC-1α in the ADPKD livers, while high-dose tolvaptan may decrease it (**Figure 8E**). Finally, BA treatment dramatically decreased cleaved caspase-3, a key marker for apoptosis, in ADPKD mice livers, both alone and in combination with tolvaptan (**Figure 8F**). This finding suggests that BA may serve a protective role in preventing apoptosis, a marker of hepatocyte injury and death, in the setting of high-dose tolvaptan treatment.

DISCUSSION

There are few therapeutic options for people living with ADPKD to arrest disease progression. As it has become recognized that ADPKD is a metabolic disease with dysregulated mitochondrial function (Padovano et al., 2018), identifying new therapies that target dysregulated metabolism are especially attractive. In searching for new metabolic targets to ameliorate the disease and prevent its progression, we found that ACLY activity was increased in PT- and IMCD-derived *Pkd1* KO cells relative to controls (**Figure 3**). As BA was recently FDA-approved for treatment of hypercholesterolemia, we considered that repurposing this drug could be compelling to test as a novel ADPKD therapeutic. Consistent with previous work (Pinkosky et al., 2016, Steinberg et al., 1999), we confirmed that mouse kidney and liver tissue, along with ADPKD cells, express the FATP2 (ACSVL1) enzyme that mediates the activation of BA (**Figure 2**). Thus, BA should only be converted to its active form in kidney and liver, the two organs primarily affected with cysts in ADPKD. This targeting specificity would be advantageous in ADPKD to reduce potential side effects in other organs, as ADPKD patients may need to be treated for several decades. Indeed, off-target side effects have historically limited the use of certain promising experimental drugs that would appear to have therapeutic benefits in ADPKD (e.g., rapamycin and analogues) (Serra et al., 2010).

For this initial study to explore the potential utility and efficacy of BA as a new therapy for ADPKD, we opted to study its effects first in PKD cell lines and in an inducible ADPKD mouse model that develops early, severe kidney disease. We found that both BA and a distinct ACLY inhibitor SB-204990 inhibited 3D cystic growth in PT- and IMCD-derived *Pkd1*-null epithelial cells (**Figure 4**). In these *Pkd1*-null cell lines, BA treatment also reduced mitochondrial superoxide production, a marker of cellular oxidative stress, and increased mitochondrial elongation, a marker of mitochondrial oxidative function (**Figure 5**). To test whether BA is effective in ADPKD to reduce cyst size in vivo, we used an early *Pkd1* conditional KO mouse model of rapidly progressive kidney cystic disease, similar to that described previously (Ma et al., 2013). In this mouse model, we found that BA treatment reduced kidney size and function (BUN) to a similar extent as tolvaptan relative to untreated controls, and addition of BA to tolvaptan caused a further reduction in both markers of cystic disease severity and kidney function when compared with tolvaptan alone (**Figure 6**). The apparent additive benefits of combination BA plus tolvaptan therapy could have important clinical implications if confirmed in human clinical ADPKD trials as it may afford lower dosing to achieve efficacy and with fewer side effects.

Treatment with BA also inhibited key cellular signaling pathways associated with ADPKD cellular proliferation (mTOR and ERK) (Saigusa and Bell, 2015) and sharply reduced the kidney injury marker KIM-1 and, to a lesser extent, NGAL (**Figure 7**). Finally, BA therapy in combination with tolvaptan in the *Pkd1* conditional KO mouse model dramatically inhibited apoptosis (cleaved caspase-3) and tended to increase mitochondrial biogenesis (PGC-1α) in liver tissue relative to tolvaptan treatment alone (**Figure 8**), which suggests that BA combination therapy with higher dose tolvaptan could help mitigate tolvaptan-associated hepatotoxicity. Taken altogether, the findings of this study suggest that BA may be considered a promising new therapeutic candidate for ADPKD, which deserves additional exploration.

Further studies will be important to explore the potential benefits of BA in more relevant mouse and/or other animal models of ADPKD (e.g., slower onset disease models, including the hypomorphic *Pkd1*$^{RC/RC}$ mouse model (Hopp et al., 2012), and in other inducible and *Pkd2* mutant animal models). Employing animal models with slower disease trajectories that encompass the animals' reproductive ages will also allow exploration of potential sex differences with respect to

disease parameters and BA treatment effects in the setting of ADPKD. In the ADPKD model presented here, mice develop early disease and are treated before having reproductive capability, with euthanasia occurring at P22, so sex differences were likely not relevant. It is also currently unclear what are the specific downstream targets or relevant ADPKD cellular signaling pathways of BA. Administration of this drug to mice with more chronic cystic disease will allow a more careful and comprehensive exploration of its effects on cellular pathways and its role in regulating disease progression.

Of note, as BA is already FDA-approved and is generally well-tolerated even in patients that lack hypercholesterolemia (Banach et al., 2020, Powell and Piszczatoski, 2021), initiating human clinical trials for ADPKD may become feasible relatively soon. Importantly, as BA targets different cellular signaling pathways than tolvaptan and other emerging therapies, it will be important to test for benefits of BA both alone and in combination with tolvaptan and other drugs that target complementary cellular signaling pathways in future clinical trials.

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AUTHOR CONTRIBUTIONS

K.R.H., H.L., B.S., E.J.C., S.L.P., and N.M.P-S. conceived and designed research. H.L., B.S., S.S., P.H., J.P., J.W., and V.M. performed experiments. K.R.H., H.L., B.S., S.S., P.H., J.P., V.M. and N.M.P.-S. analyzed data. K.R.H., H.L., B.S., S.S., P.H., J.P., V.M., S.L.P., and N.M.P.-S. interpreted results of experiments. K.R.H., H.L., B.S., and N.P.S. prepared figures. K.R.H. H.L., and N.M.P.-S. drafted manuscript. K.R.H., H.L., B.S., S.L.P. and N.M.P.-S. edited and revised the manuscript.

CONFLICTS OF INTEREST

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Table 1 - Antibodies Used for Western Blot

Antigen	Manufacturer	Cat. #	Host	Dilution	Incubation	
					Time	Temperature
p-ACC(Ser79)	Cell Signaling	3661	Rabbit polyclonal	1:1,000	O/N	4˚C
p-ACLY(Ser455)	Cell Signaling	4331	Rabbit polyclonal	1:1,000	O/N	4˚C
t-ACLY	Cell Signaling	4332	Rabbit polyclonal	1:1,000	O/N	4˚C
p-AMPKα(Thr172)	Cell Signaling	2531	Rabbit polyclonal	1:1,000	O/N	4˚C
p-ERK(Thr202/Tyr204)	Cell Signaling	9101	Rabbit polyclonal	1:1,000	O/N	4˚C
Cleaved Cas3(Asp175)	Cell Signaling	9661	Rabbit polyclonal	1:1,000	O/N	4˚C
PGC-1α	Santa Cruz	SC-517380	Mouse monoclonal	1:500	O/N	4˚C
pP70S6K(Thr389)	Santa Cruz	SC-11759R	Rabbit polyclonal	1:500	O/N	4˚C
NGAL	Abcam	ab63929	Mouse polyclonal	1:1,000	O/N	4˚C
KIM-1	R&D systems	AF1817	Goat polyclonal	1:800	O/N	4˚C
FATP2	Proteintech	14048-1-AP	Rabbit polyclonal	1:1000	O/N	4˚C
Revert 700 total protein stain kit	LI-COR	926-11010			5 min	RT

O/N – Overnight; p – Phosphorylated or phosphor-; RT – Room Temperature; T – Total.

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FIGURE LEGENDS

Figure 1 – Potential roles of bempedoic acid in ADPKD. Schematic flow diagram of the effects of bempedoic acid (BA; a.k.a. ETC-1002) on various metabolic pathways and cellular proliferation via inhibition of ATP-citrate lyase (ACLY) and activation of AMP-activated protein kinase (AMPK). 1. The pro-drug BA gets converted to its active form (BA-CoA) via Very Long-Chain Acyl-CoA Synthetase (ACSVL1; a.k.a. FATP2), whose long isoform is only substantially expressed in liver and kidney tissues (Pinkosky et al., 2016, Steinberg et al., 1999). BA-CoA simultaneously inhibits ACLY (2a) and activates AMPK (2b) in cells. Inhibition of ACLY, which converts citrate to acetyl-CoA and oxaloacetate, results in decreased cytosolic acetyl-CoA production (3a). Activated AMPK inhibits HMG-CoA reductase (HMGR) and acetyl-CoA carboxylase (ACC) (3b), which along with decreased acetyl-CoA production, inhibits the formation of malonyl-CoA (4) and HMG-CoA and thus inhibits both sterol and fatty acid (FA) synthesis. Decreased levels of malonyl-CoA, which is an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), promotes FA uptake into mitochondria via CPT-1 (5) and thus promotes FA beta oxidation in mitochondria via the tricarboxylic acid (TCA) cycle (6). Decreased sterol and FA synthesis inhibits the synthesis of cellular membranes (7), which along with AMPK-dependent inhibition of various cellular pathways (incl. the mammalian target of rapamycin (mTOR) pathway) (8), causes inhibition of cellular proliferation and thus inhibition of cyst growth and expansion in ADPKD (9).

Figure 2 – The bempedoic acid-activating enzyme ACSVL1 (FATP2) is expressed in different mouse tissues and PKD kidneys and kidney cell lines. A. Immunoblotting of various mouse tissues reveals expression of two distinct FATP2 isoforms. The short splice variant (FATP2b), which lacks the acyl-CoA synthetase domain required for conversion of BA to its active metabolite BA-CoA, is expressed in all tissues tested. However, the full-length long form (FATP2a at ~70kDa) has significant expression only in liver and kidney tissue, thus providing specificity of BA pro-drug conversion to its active form only in these tissues. **B**. FATP2 immunoblotting of representative *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mouse kidneys treated with or without doxycycline to induce tubule-specific *Pkd1* gene inactivation at P10-P11 revealed comparable expression levels of both FATP2 isoforms at the time of euthanasia (P22). Both FATP2a and FTAP2b isoforms are also expressed in immortalized mouse kidney epithelial cells that were derived from both proximal tubule (**C**) and inner medullary collecting duct (**D**) cells, with generally lower FATP2a expression in *Pkd1* KO cells (*right*) than in controls (*left*). Three representative lysate immunoblots are shown for each condition.

Figure 3 – ACLY activity is increased in *Pkd1*$^{-/-}$ kidney epithelial cells as compared to controls. A. Immunoblotting of proximal tubule (PT)-derived mouse epithelial cells probed for total ACLY expression (tACLY) and activated ACLY, as detected using a phospho-specific antibody directed against the Akt phosphorylation site Ser455 (pS455 ACLY), in PN24 cells with *Pkd1* expression knocked out at both alleles as compared with PH2 cells heterozygous for *Pkd1* deletion. **B.** Densitometric quantitation of the pS455 ACLY/tACLY ratio shows a significant ~250% increase in ACLY activation in the *Pkd1*-null cells as compared to *Pkd1*-het controls (***P*<0.01; unpaired t-test; n = 3). **C.** Immunoblotting of inner medullary collecting duct (IMCD)-derived mouse epithelial cells for expression of tACLY and pS455 ACLY in *Pkd1*-null (ID1-3E5) cells as compared with WT (IMCD3) **D**. Densitometric quantitation revealed a significant ~50% increase in the pS455 ACLY/tACLY ratio in the ID1-3E5 cells relative to control IMCD3 cells (***P* < 0.01; unpaired t-test; n = 3).

Figure 4 – ACLY inhibitors dramatically inhibited cystic growth in 3D cultures in both PT- and IMCD-derived *Pkd1*$^{-/-}$ kidney cells. A. ACLY inhibitors (bempedoic acid (BA) and SB-

204990) inhibit cyst growth of PT-derived *Pkd1*$^{-/-}$ (PN24) kidney epithelial cells in 3D culture. Cells were cultured for a total of 12 days in Matrigel supplemented with forskolin + IBMX after 1 day, and then treated with either vehicle (DMSO; Con), 100 µM BA, or 30 µM SB-204990 for the last 3 days of culture before imaging and cyst size analysis. **i.** Representative images of cystic structures under the different treatment conditions (scale bar = 100 µm). **ii.** Summary data reveal that treatment with either BA or SB-204990 dramatically reduced cyst area relative to Con (n = 9-10, $P < 0.0001$ for the indicated comparisons by one-way ANOVA and Tukey's multiple comparison test). **B.** BA and SB-204990 also inhibit cyst growth of IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) kidney epithelial cells in 3D cultures. Cells were grown in Matrigel for a total of 7 days and then treated forskolin + IBMX for after 1 day and then treated with either vehicle (DMSO), BA or SB-204990 for the last 3 days. **i.** Representative images of cystic structures under the different treatment conditions (scale bar = 100 µm). **ii.** Summary data reveal that treatment with either BA or SB-204990 significantly reduced cyst area relative to Con (n = 21-28, $P < 0.0001$ for the indicated comparisons by one-way ANOVA and Tukey's multiple comparison test).

Figure 5 – Bempedoic acid inhibits mitochondrial superoxide production and promotes mitochondrial elongation in two different ADPKD cell lines. A. To analyze the effect of BA on mitochondrial superoxide production, either PT-derived *Pkd1*$^{-/-}$ (PN24) or IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) cells were stained with MitoSOX™ mitochondrial superoxide indicator (*red*) and Hoechst 33342 nuclear stain (*blue*). **i.** Representative epifluorescence micrograph images are shown of PN24 (*left*) and ID1-3E5 (*right*) cells in the absence (*top*) or presence (*bottom*) of BA treatment (100 µM) for 24 h. **ii.** Summary data reveal that BA treatment dramatically decreased mitochondrial superoxide production in both PN24 (*top*) and ID1-3E5 (*bottom*) cells (n = 26-39 cells analyzed; ****$P<0.0001$, unpaired t-tests). **B.** BA treatment significantly increased mitochondrial elongation of *Pkd1* null cells. **i.** Representative images of MitoTracker Deep Red-stained PN24 (*left*) and ID1-3E5 (*right*) cells in the absence (*top*) or presence (*bottom*) of BA treatment (100 µM) for 24 h. **ii.** Summary data reveal that BA treatment significantly increased mean cellular mitochondrial elongation in both PN24 (*top*) and ID1-3E5 (*bottom*) cells (mean cellular mitochondrial elongation values on n = 26-39 cells analyzed, as described in *Materials and Methods*; *$P<0.05$, unpaired t-tests).

Figure 6 – Bempedoic acid treatment alone and in combination with tolvaptan reduced PKD disease severity in an early/rapid induced *Pkd1* gene inactivation ADPKD mouse model. As measures of disease severity in vivo, *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mice were induced with doxycycline to inactivate *Pkd1* and then treated ± BA ± tolvaptan for 10 days prior to euthanasia, as shown schematically (**A**), and then total kidney weight/body weight ratio (TKW/BW) and blood urea nitrogen (BUN) levels by iStat were evaluated as described in *Materials and Methods*. **B.** Representative H&E-stained sagittal kidney sections under different treatment conditions are shown. BA (30 mg/kg/d) reduced mean (± SE) TKW/BW (**C**) and BUN (**D**) to a similar extent as tolvaptan (30 and 100 mg/kg/d). Addition of BA to tolvaptan caused further reductions in TKW/BW and BUN vs. tolvaptan alone (*$P < 0.05$, **$P < 0.01$, and $^{\#}0.05 < P < 0.10$ for the indicated comparisons; two-tailed, unpaired t-tests). $^{\$}$Significantly different from all other treatment conditions.

Figure 7 – Effects of bempedoic acid and tolvaptan treatment in PKD mice on protein expression of key signaling and injury markers in kidney homogenates. Immunoblots were performed on kidney tissue homogenates from *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mice with early/rapid induced *Pkd1* gene inactivation with or without concurrent treatment with BA (30 mg/kg/d) and/or tolvaptan (30 or 100 mg/kg/d). Densitometric intensities of immunoblot bands from each lane were normalized to the total protein signal from that lane as a loading control. Kidney homogenates were probed for ACLY activity (pS455 ACLY; **A**), AMPK activity (pT172 AMPK; **B**), mTOR activity

(pP70S6K; **C**), ERK activity (pERK; **D**), in kidney, and the kidney injury markers, KIM-1 (**E**), and NGAL (**F**) (**P* < 0.05, ***P* < 0.01 for the indicated comparisons; two-tailed, unpaired t-tests). Representative immunoblots are shown at top and summary quantitation of mean (± SE) relative protein expression levels are shown at bottom of each panel.

Figure 8 – Effects of bempedoic acid and tolvaptan treatment in PKD mice on protein expression of key signaling markers in liver homogenates. Immunoblots were performed on liver tissue homogenates from *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mice with early/rapid induced *Pkd1* gene inactivation with or without concurrent treatment with BA (30 mg/kg/d) and/or tolvaptan (100 mg/kg/d). Densitometric intensities of immunoblot bands from each lane were normalized to the total protein signal from that lane as a loading control. Liver homogenates were probed for ACLY activity (pS455 ACLY; **A**), AMPK activity (pT172 AMPK (**B**) and pACC (**C**)), FATP2 expression (**D**), the mitochondrial biogenesis marker PGC-1α (**E**), and the apoptosis marker cleaved caspase 3 (**F**) (**P* < 0.05, ***P* < 0.01, ****P* < 0.001 or as stated for the indicated comparisons; two-tailed, unpaired t-tests). Representative immunoblots are shown at top and summary quantitation of mean (± SE) relative protein expression levels are shown at bottom of each panel.

Figure 1

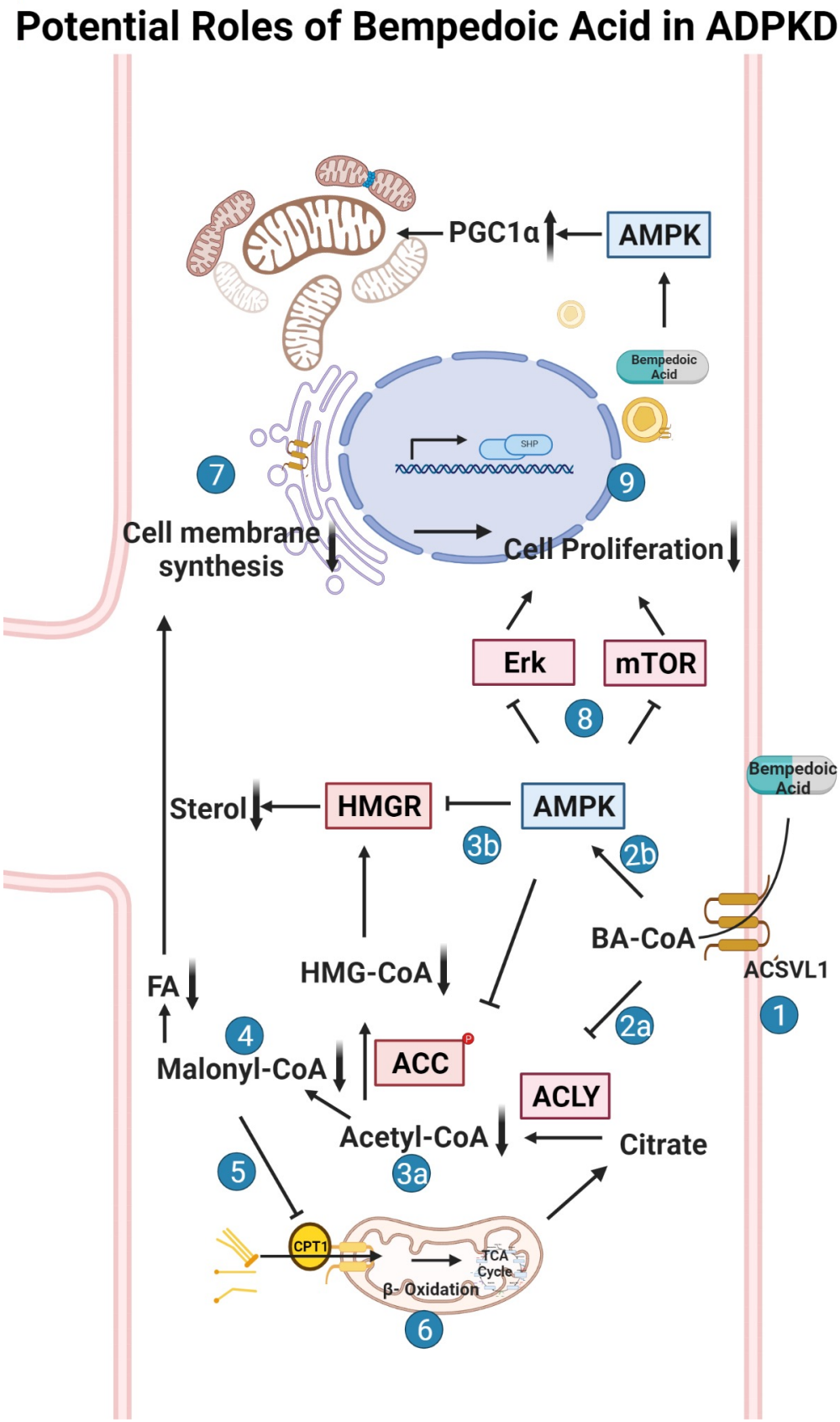

Potential Roles of Bempedoic Acid in ADPKD
PGC1α
AMPK
Bempedoic Acid
SHP
7
9
Cell membrane synthesis
Cell Proliferation
Erk
mTOR
8
Bempedoic Acid
Sterol
HMGR
AMPK
3b
2b
BA-CoA
ACSVL1
1
FA
HMG-CoA
2a
4
Malonyl-CoA
ACC
ACLY
Acetyl-CoA
Citrate
5
3a
CPT1
TCA Cycle
β- Oxidation
6

Figure 2

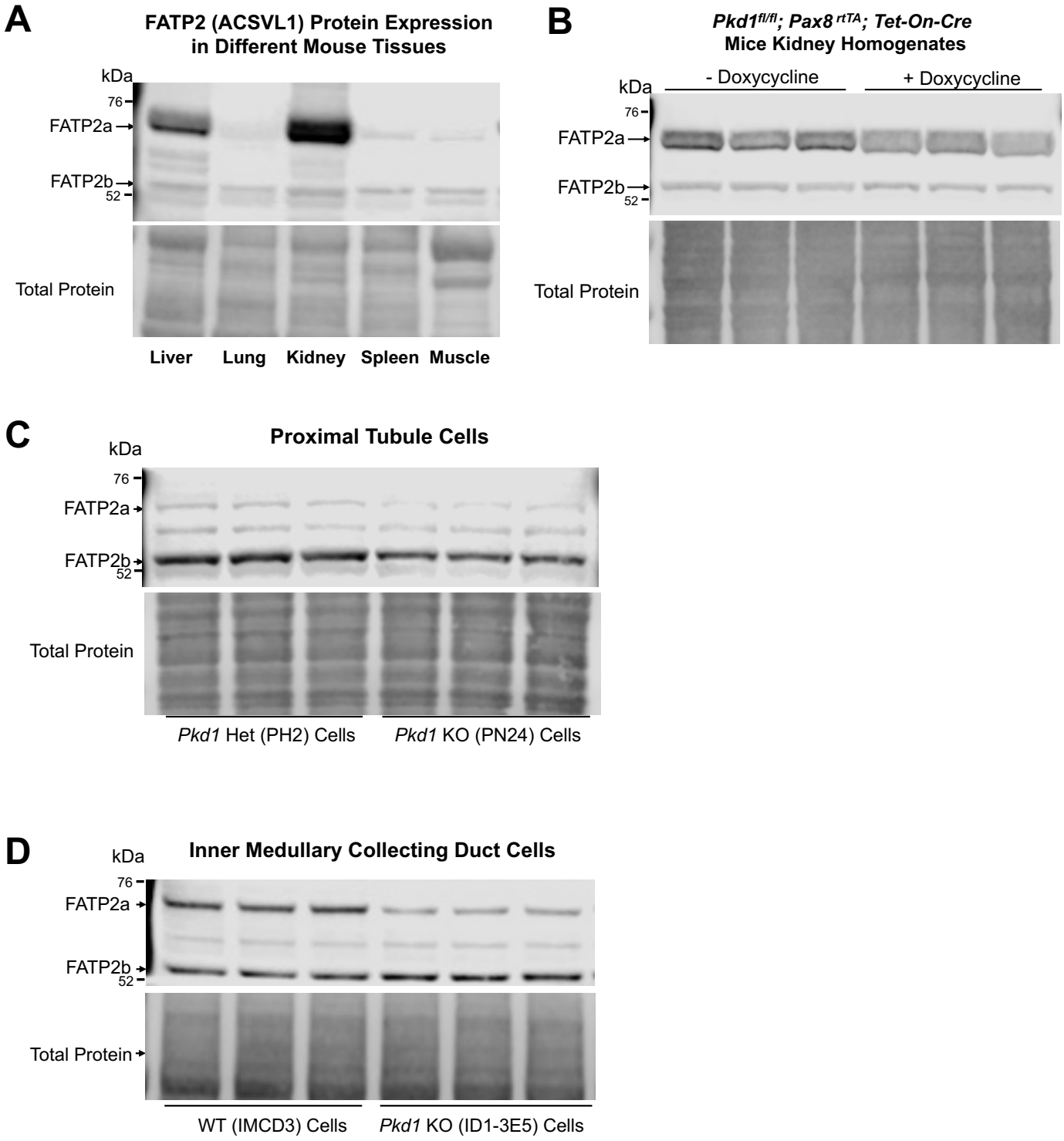

A
FATP2 (ACSVL1) Protein Expression in Different Mouse Tissues
kDa
76
FATP2a
FATP2b
52
Total Protein
Liver
Lung
Kidney
Spleen
Muscle
B
Pkd1fl/fl; Pax8 rtTA; Tet-On-Cre Mice Kidney Homogenates
- Doxycycline
+ Doxycycline
kDa
76
FATP2a
FATP2b
52
Total Protein
C
Proximal Tubule Cells
kDa
76
FATP2a
FATP2b
52
Total Protein
Pkd1 Het (PH2) Cells
Pkd1 KO (PN24) Cells
D
Inner Medullary Collecting Duct Cells
kDa
76
FATP2a
FATP2b
52
Total Protein
WT (IMCD3) Cells
Pkd1 KO (ID1-3E5) Cells

Figure 3

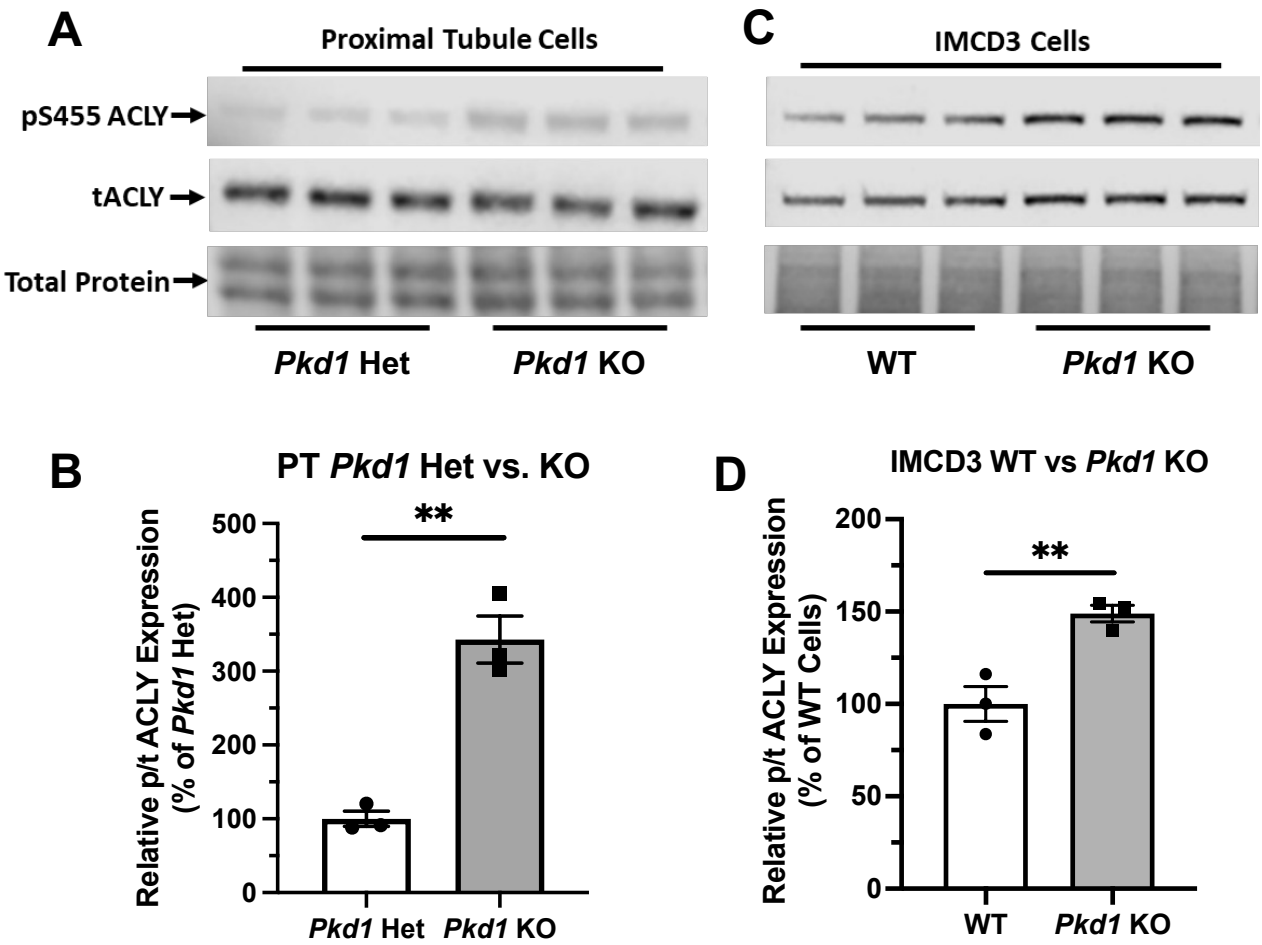
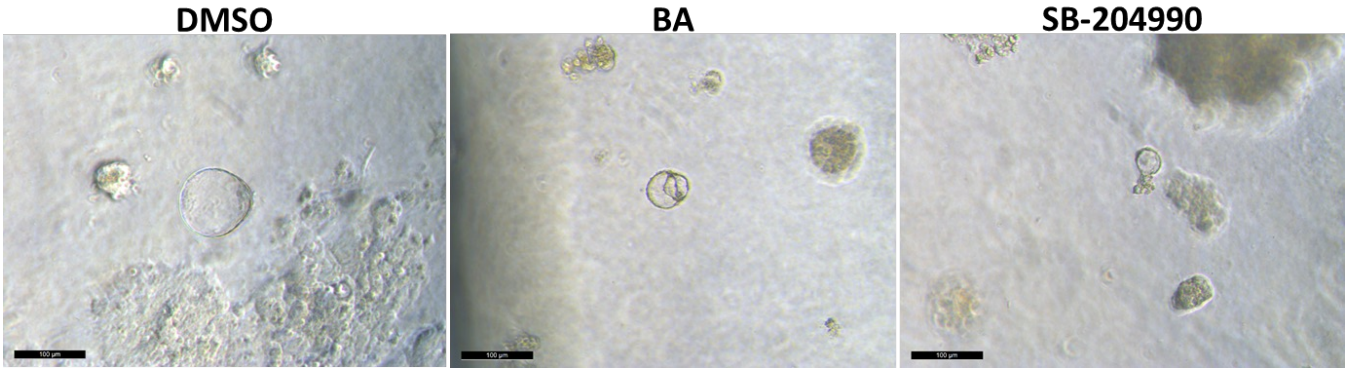

A
Proximal Tubule Cells
pS455 ACLY
tACLY
Total Protein
Pkd1 Het
Pkd1 KO
C
IMCD3 Cells
WT
Pkd1 KO
B
PT Pkd1 Het vs. KO
Relative p/t ACLY Expression (% of Pkd1 Het)
500
400
300
200
100
0
**
Pkd1 Het
Pkd1 KO
D
IMCD3 WT vs Pkd1 KO
Relative p/t ACLY Expression (% of WT Cells)
200
150
100
50
0
**
WT
Pkd1 KO

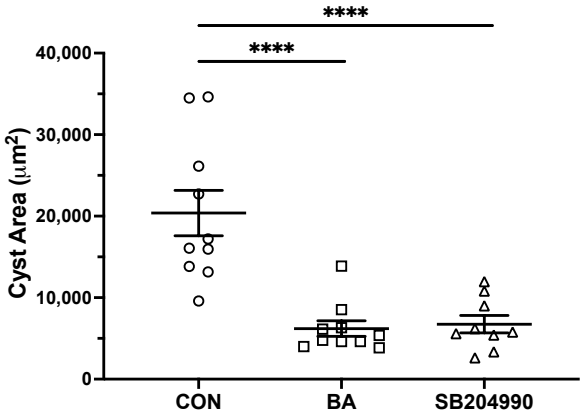
Figure 4

A PT-derived *Pkd1*$^{-/-}$ Cell (PN24) 3D Cultures

i

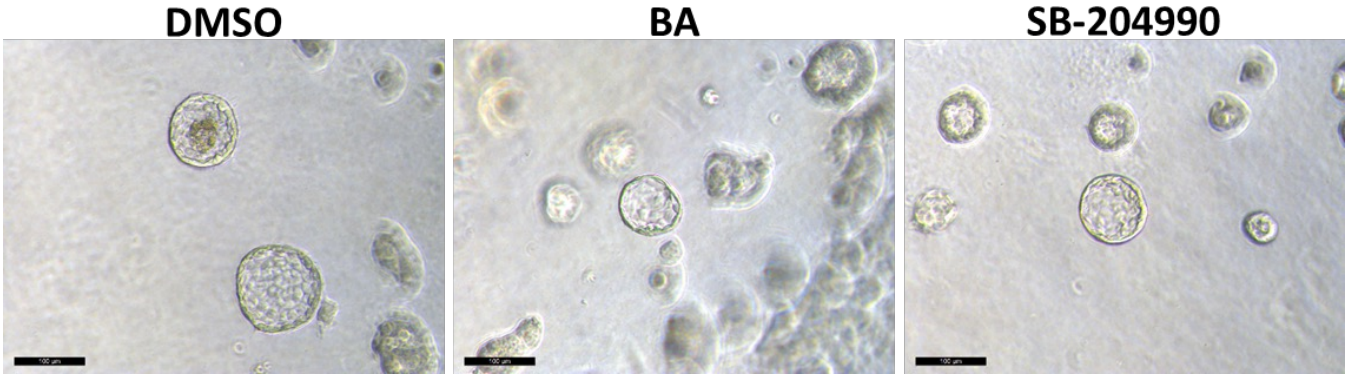


ii



B IMCD-derived *Pkd1*$^{-/-}$ Cell (ID1-3E5) 3D Cultures

i



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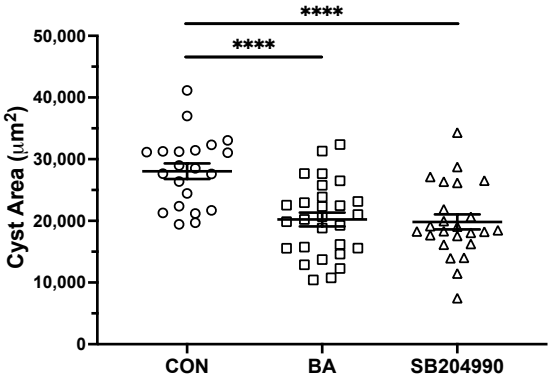
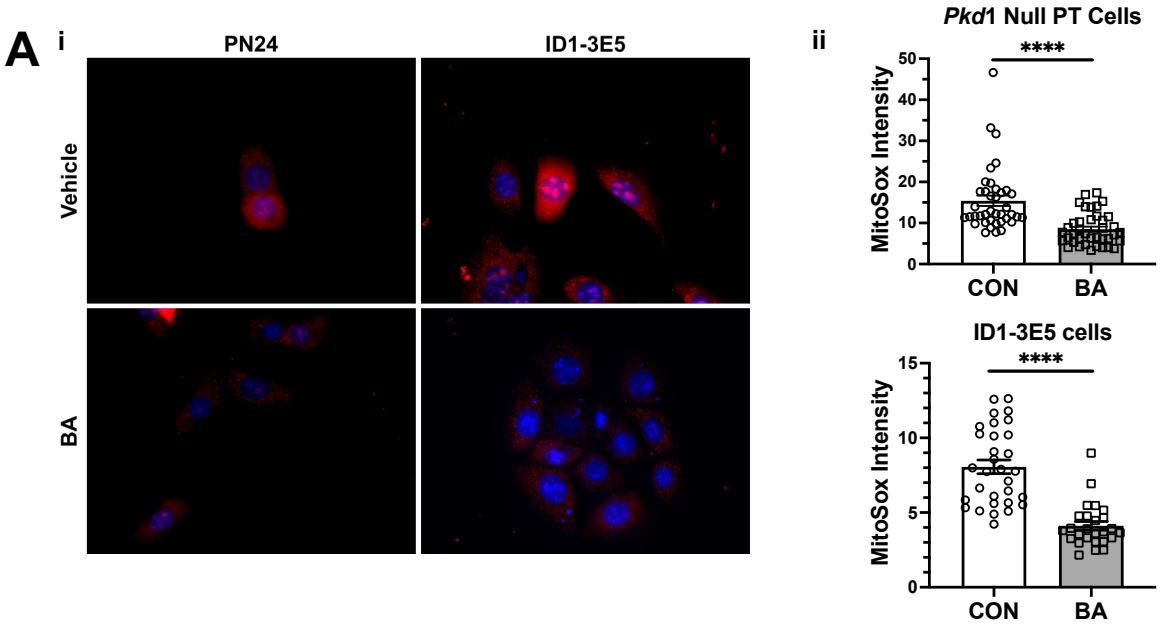


Figure 5


A
i
PN24
ID1-3E5
Vehicle
BA
ii
*Pkd*1 Null PT Cells
****
MitoSox Intensity
50
40
30
20
10
0
CON
BA
ID1-3E5 cells
****
MitoSox Intensity
15
10
5
0
CON
BA

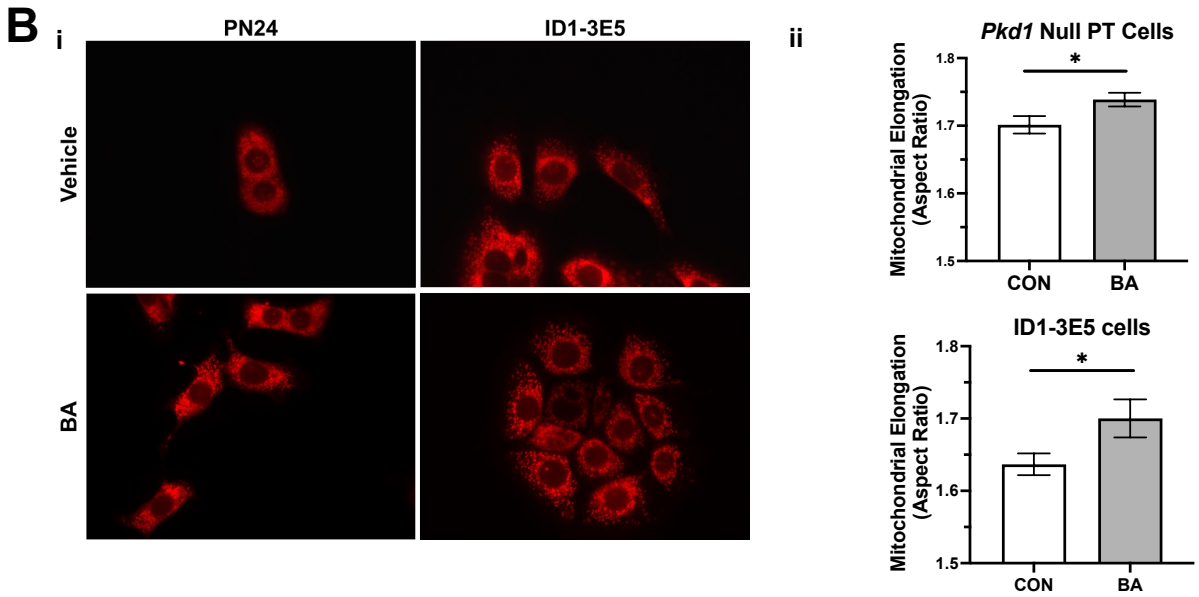
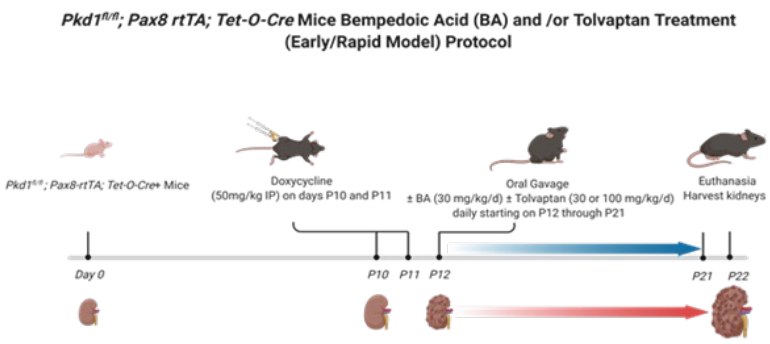
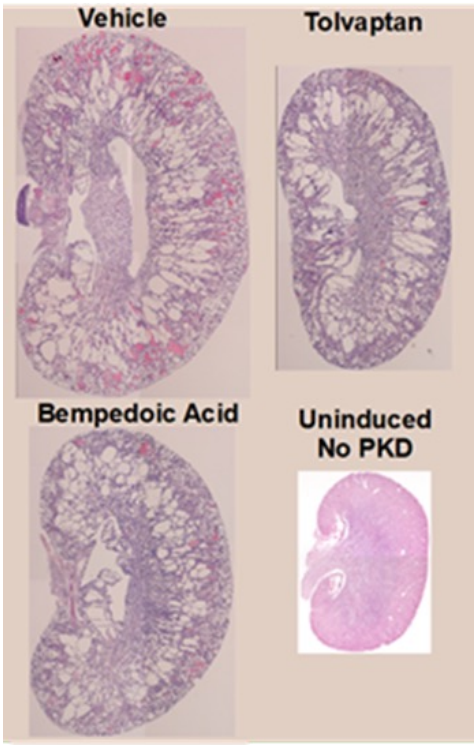

B
i
PN24
ID1-3E5
Vehicle
BA
ii
Pkd1 Null PT Cells
*
Mitochondrial Elongation (Aspect Ratio)
1.8
1.7
1.6
1.5
CON
BA
ID1-3E5 cells
*
Mitochondrial Elongation (Aspect Ratio)
1.8
1.7
1.6
1.5
CON
BA

Figure 6

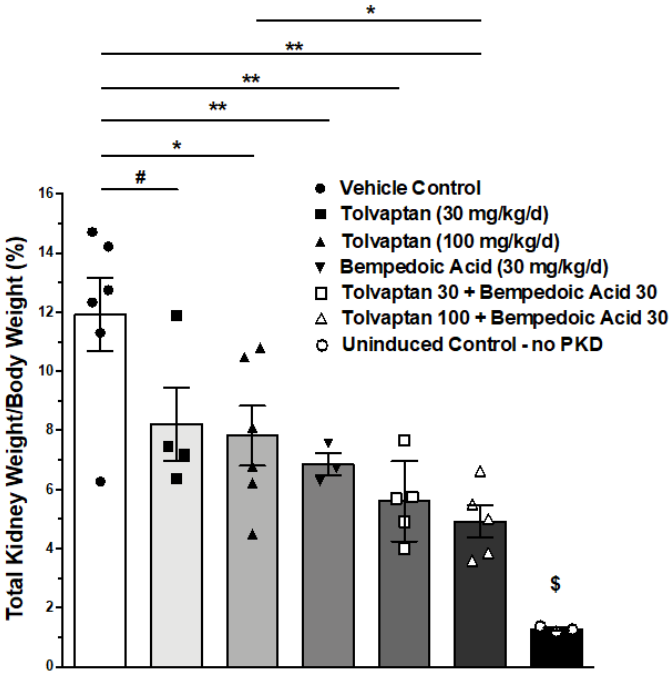
A



B



C



D

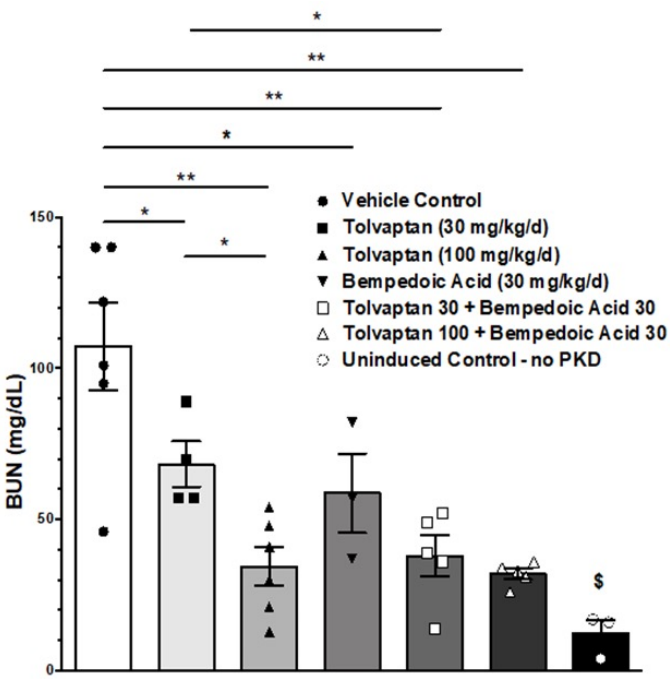


Figure 7

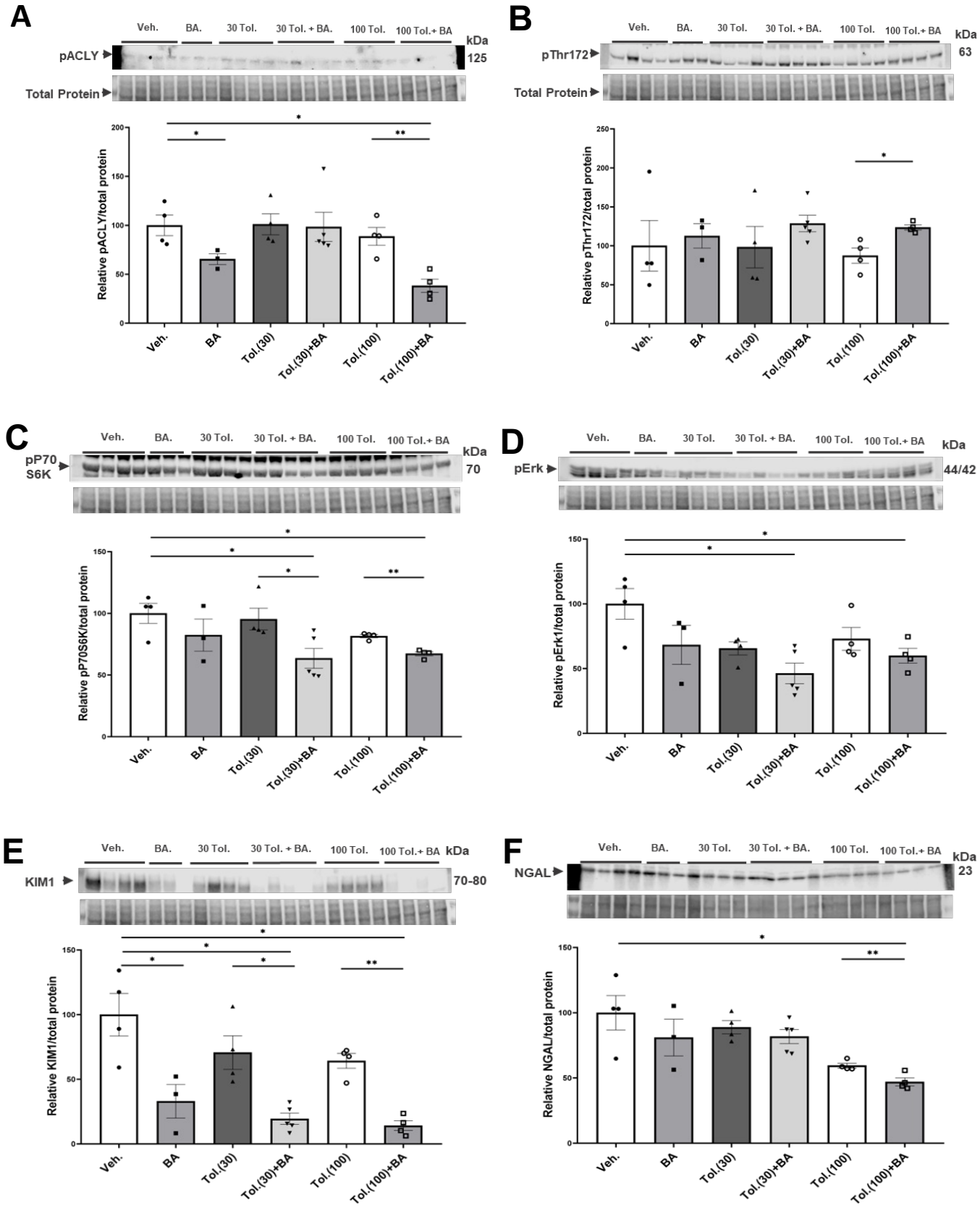
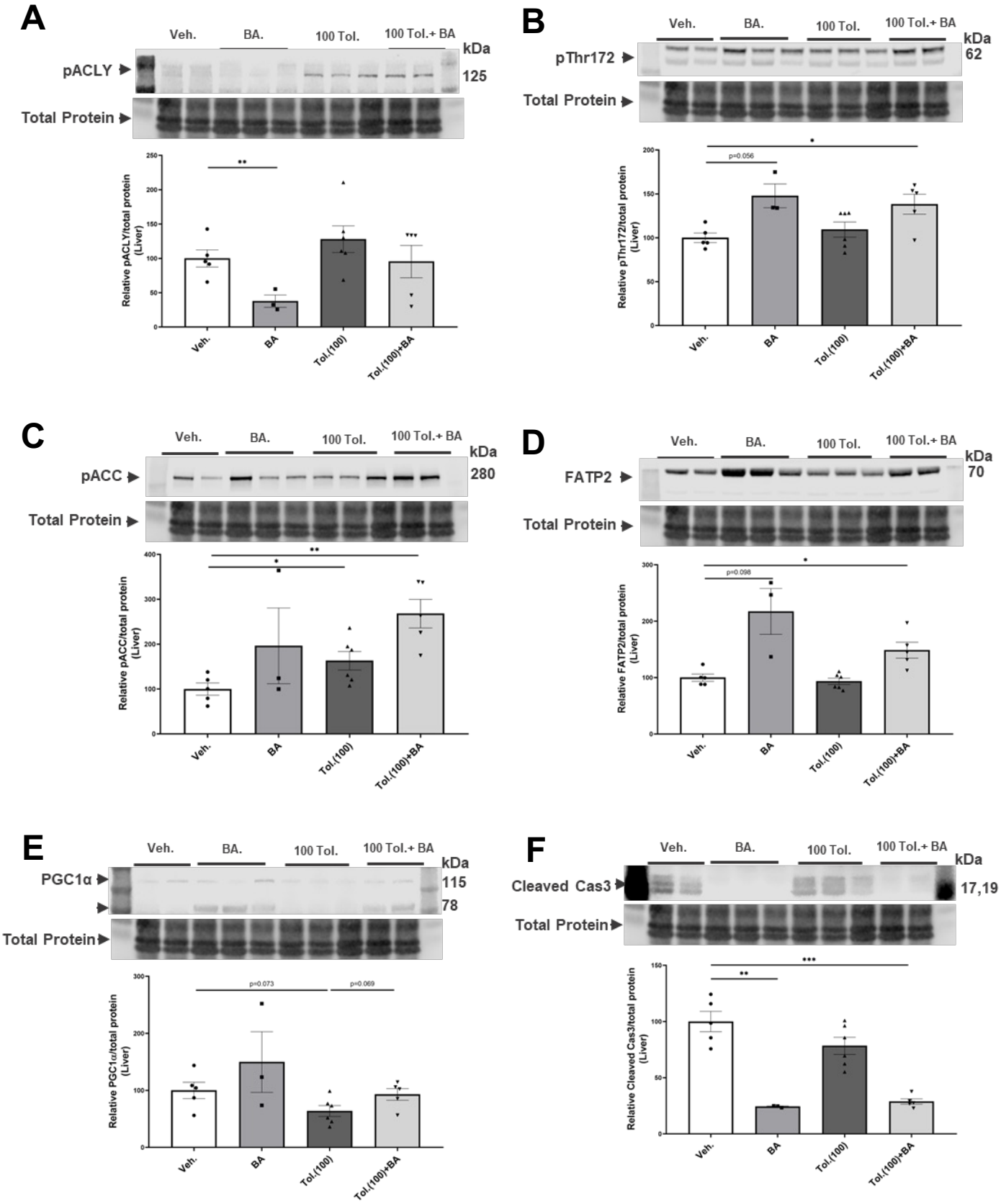

A
Veh.
BA.
30 Tol.
30 Tol. + BA.
100 Tol.
100 Tol.+ BA
kDa
pACLY
125
Total Protein
Relative pACLY/total protein
Veh.
BA
Tol.(30)
Tol.(30)+BA
Tol.(100)
Tol.(100)+BA
B
pThr172
63
Relative pThr172/total protein
C
pP70 S6K
70
Relative pP70S6K/total protein
D
pErk
44/42
Relative pErk1/total protein
E
KIM1
70-80
Relative KIM1/total protein
F
NGAL
23
Relative NGAL/total protein

Figure 8


A
Veh.
BA.
100 Tol.
100 Tol.+ BA
kDa
pACLY
125
Total Protein
Relative pACLY/total protein (Liver)
Veh.
BA
Tol.(100)
Tol.(100)+BA
B
pThr172
62
Total Protein
p=0.056
Relative pThr172/total protein (Liver)
C
pACC
280
Relative pACC/total protein (Liver)
D
FATP2
70
p=0.098
Relative FATP2/total protein (Liver)
E
PGC1α
115
78
p=0.073
p=0.069
Relative PGC1α/total protein (Liver)
F
Cleaved Cas3
17,19
Relative Cleaved Cas3/total protein (Liver)