Pant A and Kocarek TA. Role of phosphatidic acid phosphatase domain containing 2 (PPAPDC2) in squalestatin 1-mediated activation of the constitutive androstane receptor in primary cultured rat hepatocytes. *Drug Metabolism and Disposition*

Supplemental Methods

PPAPDC2 overexpression by transient transfection in primary cultured rat hepatocytes. A CAR-responsive firefly luciferase reporter plasmid containing ~2.4 Kb of the CYP2B1 5'-flanking region has been previously described (Kocarek et al., 1998). An expression plasmid (pExpress-1) containing the rat PPAPDC2 cDNA sequence (Mammalian Gene Collection Clone ID 7125531) and a cloning plasmid (pBluescript) containing the human PPAPDC2 cDNA (Mammalian Gene Collection Clone ID 5268486) were purchased from GE Healthcare (Pittsburgh, PA). An expression plasmid for human PPAPDC2 was prepared as follows. PCR was performed using the plasmid containing human PPAPDC2 cDNA as template, *Pfu* polymerase (Stratagene Cloning Systems; La Jolla, CA), and the following primer pairs: forward 5'-GCG AGCGGCCGCGCCACCATGCCAAGTCCCCGGAGGA-3' and reverse 5'-GCGAGGATCCTCATCGTTGACTCCACAGT-3' to amplify the coding region of human PPAPDC2 (nt 83 to 970 of NCBI Reference Sequence NM_203453.3). The PCR product was digested with *Not*I and *Bam*HI and ligated into the corresponding sites of the pE1.1 vector (O.D. 260 Inc.; Boise, ID), into which the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences of pcDNA3.1 had been pre-ligated at the *Bgl*II and *Acc*65I/*Kpn*I sites, respectively. The sequence of this clone (and others

described below that were derived by PCR) was verified by the Applied Genomics Technology Center at Wayne State University.

Twenty-four hr after plating, cell culture medium was replaced and hepatocytes were transiently transfected with Williams' Medium E containing 0.2ml of OptiMEM (Life Technologies; Grand Island, NY) and a premixed complex of Lipofectamine 2000 (4 µL), the CYP2B1 reporter plasmid (1.2 µg), rat or human PPAPDC2 expression plasmid (50 ng), pRL-CMV *Renilla* luciferase reporter plasmid (1 ng), and pBlueScript II KS^+ (350 ng) (Agilent Technologies; Santa Clara, CA) to adjust total DNA content to 1.6 µg. Five hr following transfection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun the following day and repeated once after 24 hr. Hepatocytes were harvested 48 hr after initial treatment for measurement of luciferase activities using the Dual Luciferase Reporter Assay System and a GloMax luminometer (Promega Corporation; Madison, WI) according to the manufacturer's instructions. Luciferase data were calculated as firefly/*Renilla* ratios and presented as described in the figure legends. Experiments were repeated in four independent rat hepatocyte preparations.

PPAPDC2 knockdown by transient transfection in primary cultured rat hepatocytes. Four plasmids expressing 29-mer shRNAs targeting rat PPAPDC2 and a non-targeting shRNA plasmid (TR30012) were purchased from Origene Technologies (Rockville, MD). A validation vector (rPPAPDC2-Luc) was additionally prepared to evaluate knockdown of rat PPAPDC2. To prepare this vector, the rat PPAPDC2

expression plasmid described above was used as a template to amplify the rat PPAPDC2 cDNA using the forward primer 5'-GCGAGCGGCCGCGCCACC CGGAGGACTATCGAGGGAC-3' and reverse primer 5'-GCGATAGCGGCCGC CTGGATGGCTCTGGCTTAGG-3' and *Pfu* polymerase to amplify the coding region of rat PPAPDC2 (nt 64 to 1014 of NCBI Reference Sequence NM_001034854.1). The PCR product was digested with *Not*I and ligated into the corresponding site of the pCMV-LUC validation vector (TR30004; Origene Technologies), which is located downstream of the firefly luciferase coding region.

To evaluate the abilities of the different shRNA constructs to knock down PPAPDC2, primary cultured rat hepatocytes were incubated in Williams' Medium E containing 0.2 ml of OptiMEM containing a premixed complex of 4 µl Lipofectamine 2000 with 50 ng PPAPDC2 validation vector, 200 ng of a PPAPDC2-targeting or non-targeting shRNA expression plasmid, 1 ng of pRL-CMV *Renilla* luciferase reporter plasmid, and 1350 ng of pBlueScript II KS^{+}. Five hr following transfection, culture medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. The cells were then harvested 48 hr after transfection, and luciferase activities were measured as described above. The shRNA construct that produced the largest reduction in reporter activity compared to non-targeting control was TI713339, and this construct was used for further experiments.

To evaluate the effect of PPAPDC2 knockdown on SQ1-mediated CAR activation, primary cultured rat hepatocytes were transfected as described above, but using 1.2 µg of CYP2B1 reporter plasmid, 200 ng of shRNA construct (PPAPDC2-targeting or non-

targeting), and 200 ng pBluescript II KS^{+}. After transfection and overnight incubation with Williams' Medium E containing Matrigel, drug treatments were performed as described in the individual figure legends (three wells per treatment group), repeated once after 24 hr. Forty-eight hr following initial treatment, cells were harvested for measurement of luciferase activities.

PPAPDC2 overexpression by adenoviral transduction in primary cultured rat hepatocytes. The generation of a recombinant adenovirus for expression of rat PPAPDC2 (Ad5.CMV-PPAPDC2) was performed as described below. The expression plasmid for rat PPAPDC2 described above was used as template to amplify rat PPAPDC2 cDNA using the following primers: forward primer 5'-GCGAGCGGCCGCGAAGCCTGTCTCCGGTCTG -3' and reverse primer 5'-GCGGGATCCGGATGGCTCTGGCTTAGGT -3' and *Pfu* polymerase to amplify the coding region of rat PPAPDC2 (nt 2 to 1012 of NCBI Reference Sequence NM_001034854.1). The PCR product was ligated into the *Not*I and *Bam*HI sites of the pE1.1 shuttle vector, containing the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences, as described above. This shuttle plasmid was then provided to O.D. 260 Inc., who prepared the recombinant adenovirus expressing rat PPAPDC2 (Ad5.CMV-PPAPDC2). The titer of the recombinant adenovirus as well as of the control adenovirus (Ad-375; O.D. 260 Inc.) was determined using the QuickTiter Adenovirus Titer ELISA Kit (Cell Biolabs, Inc.; San Diego, CA). Twenty-four hr following plating, primary cultured rat hepatocytes were transduced with Ad5.CMV-PPAPDC2 or control adenovirus without insert (Ad-375) at a multiplicity of infection of

5. After 5 hr of infection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun 24 hr after infection (two wells per treatment group), and treatments were repeated once after 24 hr. Forty-eight hr following the initial treatments, cells were harvested for RNA or protein extraction as described below.

Quantitative reverse-transcription polymerase chain reaction analysis. Primary cultured rat hepatocytes were harvested and total RNA was extracted and column purified using the Purelink RNA isolation kit (Ambion; Carlsbad, CA). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's instructions. Primers used to detect CYP2B1 (Forward – 5'-CAACCCTTGATGACCGCAGTA-3' and Reverse – 5'-TTCAGTGTTCTTGGGAAGCAG -3') and primers for the TATA box-binding protein (TBP; Assay ID Rn.PT.51.24118050) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). CYP2B1 mRNA levels were measured using Power SYBR Green Master Mix in the StepOne Plus Real Time PCR System (Applied Biosystems; Foster City, CA). The concentration of each primer was 150 nM, and the real-time cycling conditions were: Initial activation step at 95 ºC (15 min) and 40 cycles of melting (95 ºC, 15 s) and annealing/extension (60 ºC, 1 min). Relative changes in mRNA levels were quantified using the comparative CT (ΔΔCT) method (User bulletin no.2, Applied Biosystems). All assays were performed in duplicate in four independent experiments.

Western blot analysis. After 48 hr of treatment, cultured hepatocytes were incubated with gentle agitation on ice for 60 min with ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA to dissolve the Matrigel. The cells were then scraped into tubes and centrifuged briefly at 1000 rpm for 5 min at 4º C. Supernatants were aspirated and cells washed twice with ice-cold PBS. Following the final wash, cells were lysed in ice-cold RIPA buffer [50 mM Tris, 150 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA] containing Halt Protease Inhibitor Cocktail (ThermoFisher Scientific; Rockford, IL) by passing the suspension through a 24 gauge needle several times. After extensive vortexing, the lysates were centrifuged at 14,000 x *g* for 15 min at 4 °C and the protein concentrations of the supernatants were determined using the bicinchoninic acid assay (Sigma-Aldrich; St. Louis, MO) with bovine serum albumin as the standard. Lysate samples (20 µg protein) were diluted in Laemmli Sample Buffer, denatured at 100º C for 5 min, resolved on SDS-polyacrylamide gels (10% acrylamide), and electrophoretically transferred onto polyvinylidene difluoride membranes (BioRad; Hercules, CA). Following transfer, membranes were incubated in blocking buffer [5% nonfat dry milk diluted in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 (TBST)] for 1 hr at room temperature and then incubated overnight at 4º C with gentle rocking in blocking buffer containing rabbit polyclonal PPAPDC2 antibody (TA306886; Origene Technologies) diluted 1:15,000. The following day, blots were washed 3 times with TBST and then incubated 1 hr at room temperature in blocking buffer containing horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology; Dallas, TX) diluted 1:20,000. Following washing, immunoreactive bands

were visualized using enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare) and a FluorChem E detection system (ProteinSimple, San Jose, CA). Blots were subsequently incubated in stripping buffer (0.1 mM glycine, 1% SDS, 0.05% Tween-20, pH 2.2), blocked, and re-developed with a mouse monoclonal β-actin antibody (Sigma-Aldrich) diluted 1:250 in blocking buffer followed by anti-mouse IgG secondary antibody diluted 1:20,000 (Santa Cruz Biotechnology). Band densities were quantified using ImageJ software (Rasband, 2012).

Reference

Rasband W (2012) ImageJ: Image processing and analysis in Java. *Astrophysics Source Code Library* **1:**06013.