Supporting Information For:

“Twelfth-Position Deuteration of Nevirapine Reduces 12-Hydroxy-Nevirapine Formation and Nevirapine-Induced Hepatocyte Death”

Carley J.S. Heck[a], Herana Kamal Seneviratne[b], Namandjé N. Bumpus[a,b,*]

[a] Department of Pharmacology & Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[b] Department of Medicine – Division of Clinical Pharmacology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

* Corresponding author

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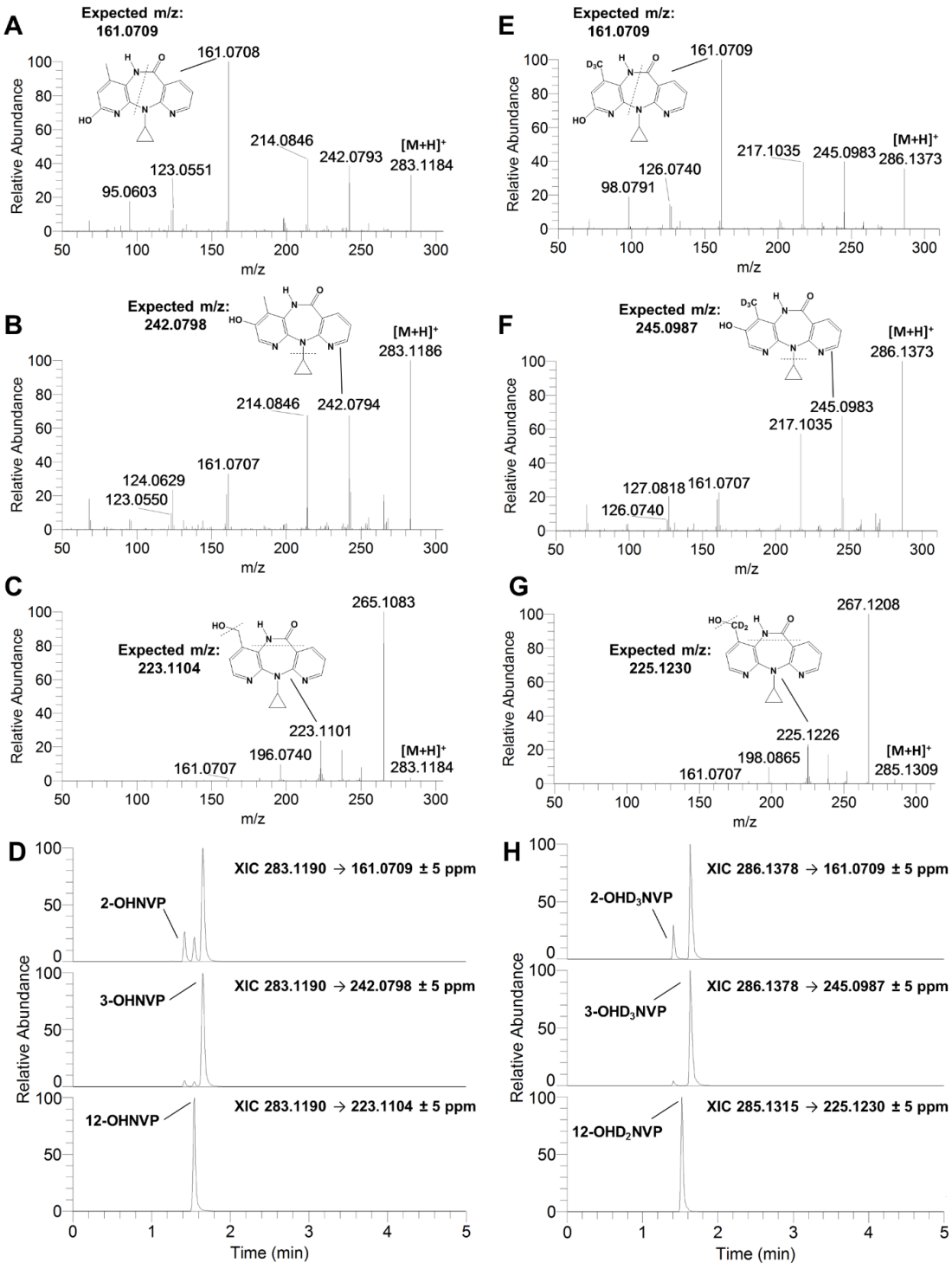
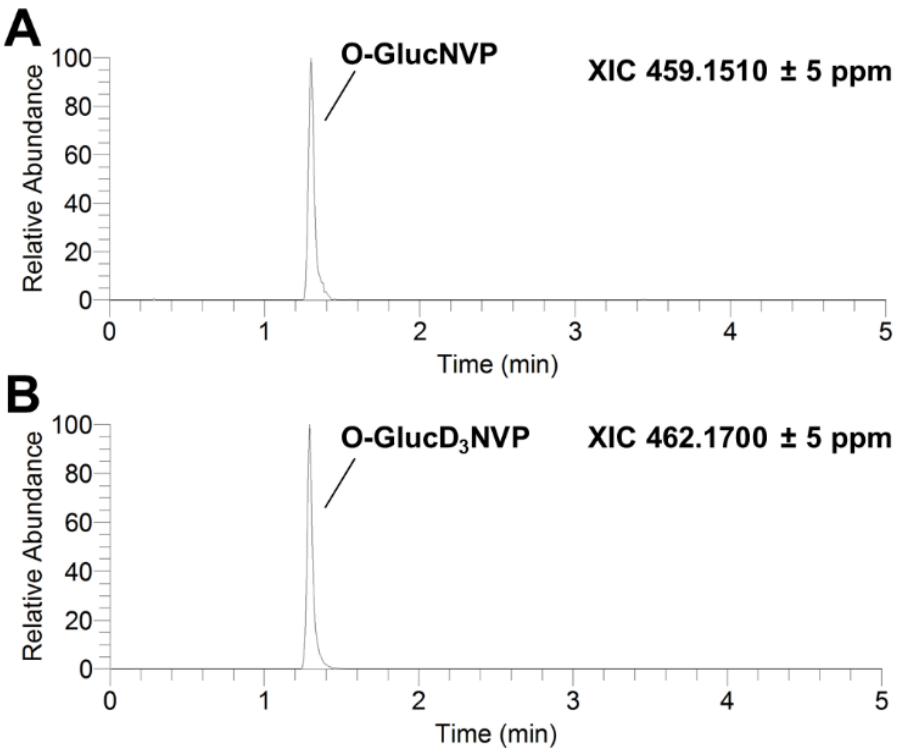
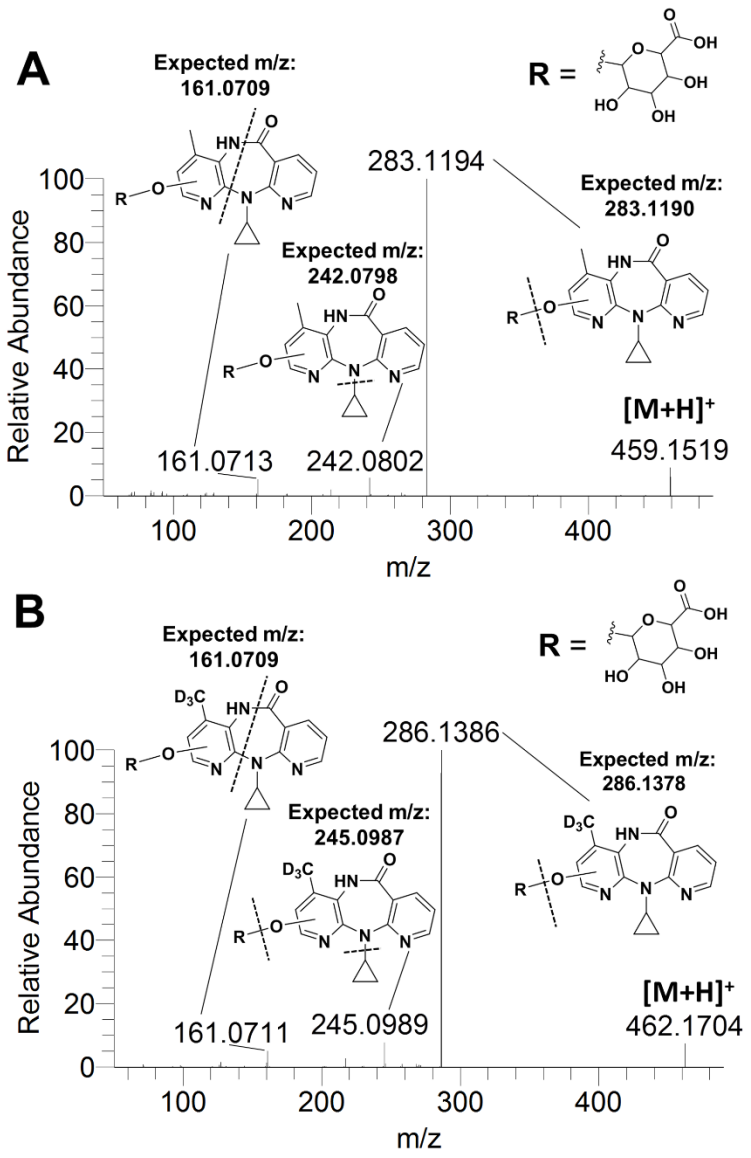


Supplemental Figure 1. Fragment selection for uHPLC-MS/MS based quantitation of monooxygenated NVP and 12-D$_3$NVP metabolites. Fresh primary mouse hepatocytes from male C57BL/6J mice were cultured and treated with 10 µM NVP or 12-D$_3$NVP for 24 hours. Metabolites were extracted from the medium from these treatments and subjected to uHPLC-MS/MS (orbitrap)

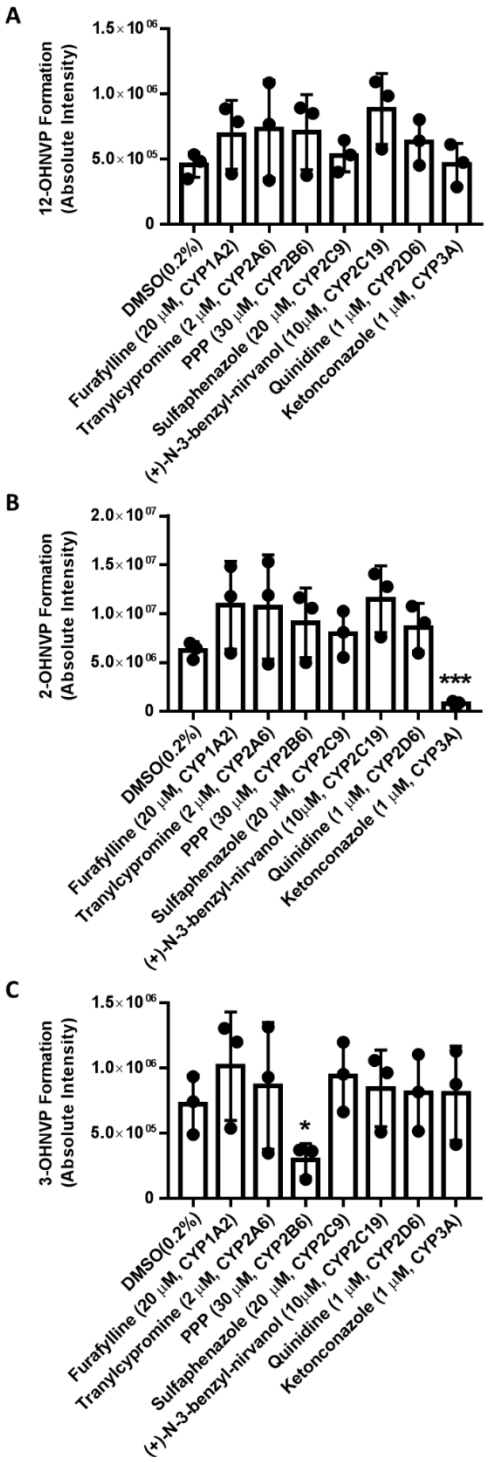
analysis. Representative fragmentation spectra are shown for the following metabolites from NVP incubations: 2-OHNVP (A), 3-OHNVP (B), and 12-OHNVP (C), with the selected fragmentation ions used for quantitation and their proposed sights of fragmentation highlighted. The high resolution parent mass is indicated as $[M+H^+]$. The extracted ion chromatograms (XICs) of these fragments from incubations with NVP are shown, with the metabolite highlighted that is quantified with each mass transition (D). Representative fragmentation spectra are also shown for the following metabolites from 12-D_3NVP incubations: 2-OHD_3NVP (E), 3-OHD_3NVP (F), and 12-OHD_2NVP (G), with the selected fragmentation ions used for quantitation and their proposed sights of fragmentation highlighted. The extracted ion chromatograms (XICs) of these fragments from incubations with 12-D_3NVP are shown (H). Results are representations of four experimental replicates.



Supplemental Figure 2. Extracted ion chromatograms of an O-glucuronidated NVP and 12-D_3NVP metabolites formed in primary mouse hepatocytes. Fresh primary mouse hepatocytes from male C57BL/6J mice were cultured and treated with 10 µM NVP or 12-D_3NVP for 24 hours. Metabolites were extracted from the cell culture medium and subjected to uHPLC-MS (orbitrap) analysis. The following high resolution ions were observed to assay for the presence of P450 metabolites: 459.1510 ± 5 ppm for O-glucuronidated, undeuterated NVP (O-GlucNVP) and 462.1700 ± 5 ppm for O-glucuronidated, trideuterated NVP (O-GlucD_3NVP). Representative extracted ion chromatograms (XICs) from this analysis are shown for O-GlucNVP formed during incubations with NVP (A) and O-GlucD_3NVP formed during incubations with 12-D_3NVP (B). Results are representations of four experimental replicates.

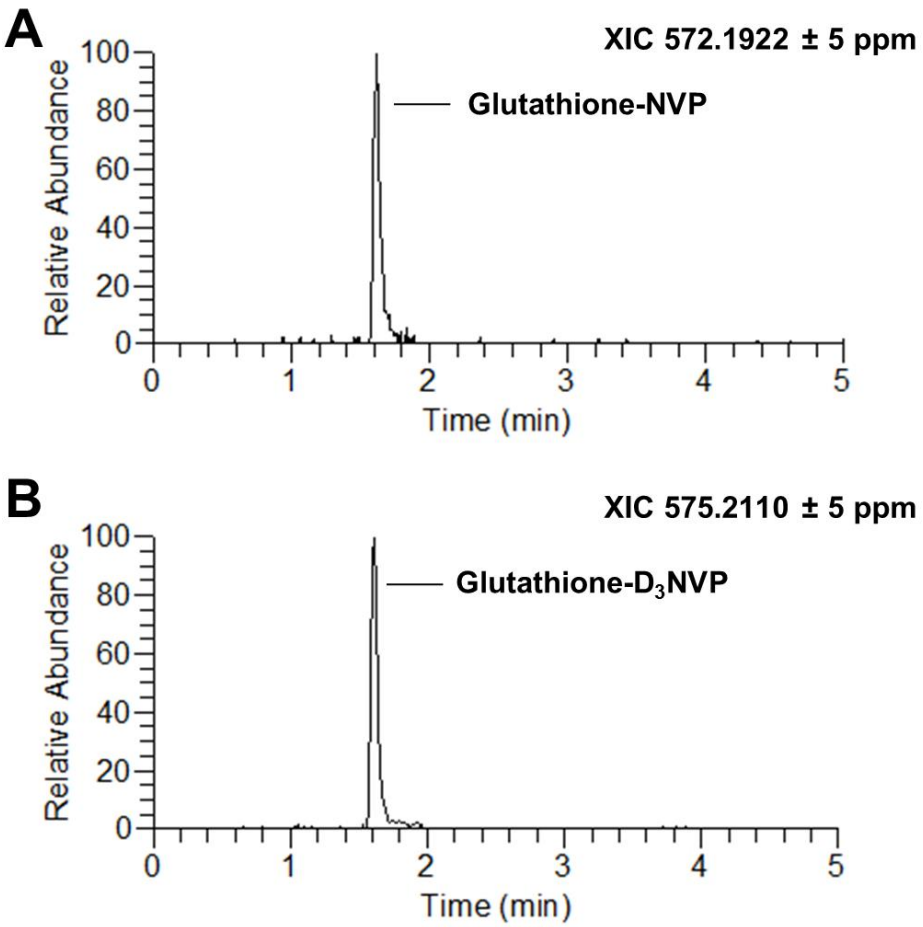


Supplemental Figure 3. Representative fragmentation spectra for an O-glucuronidated NVP and 12-D$_3$NVP metabolite formed in primary mouse hepatocytes. Fresh primary mouse hepatocytes from male C57BL/6J mice were cultured and treated with 10 µM NVP or 12-D$_3$NVP for 24 hours. Metabolites were extracted from the cell culture medium and subjected to uHPLC-MS/MS (Orbitrap) analysis. Fragmentation scans for the following ions were performed: 459.1510 ± 5 ppm for O-glucuronidated, undeuterated NVP (O-GlucNVP) and 462.1700 ± 5 ppm for O-glucuronidated, trideuterated NVP (O-GlucD$_3$NVP). Representative fragmentation spectra for these ions are shown are shown for O-GlucNVP formed during incubations with NVP (A) and O-GlucD$_3$NVP formed during incubations with 12-D$_3$NVP (B). The high resolution parent mass is indicated as [M+H$^+$], and the high resolution fragments used for structural confirmation are indicated, along with their predicted sites of fragmentation. Results are representations of four experimental replicates.

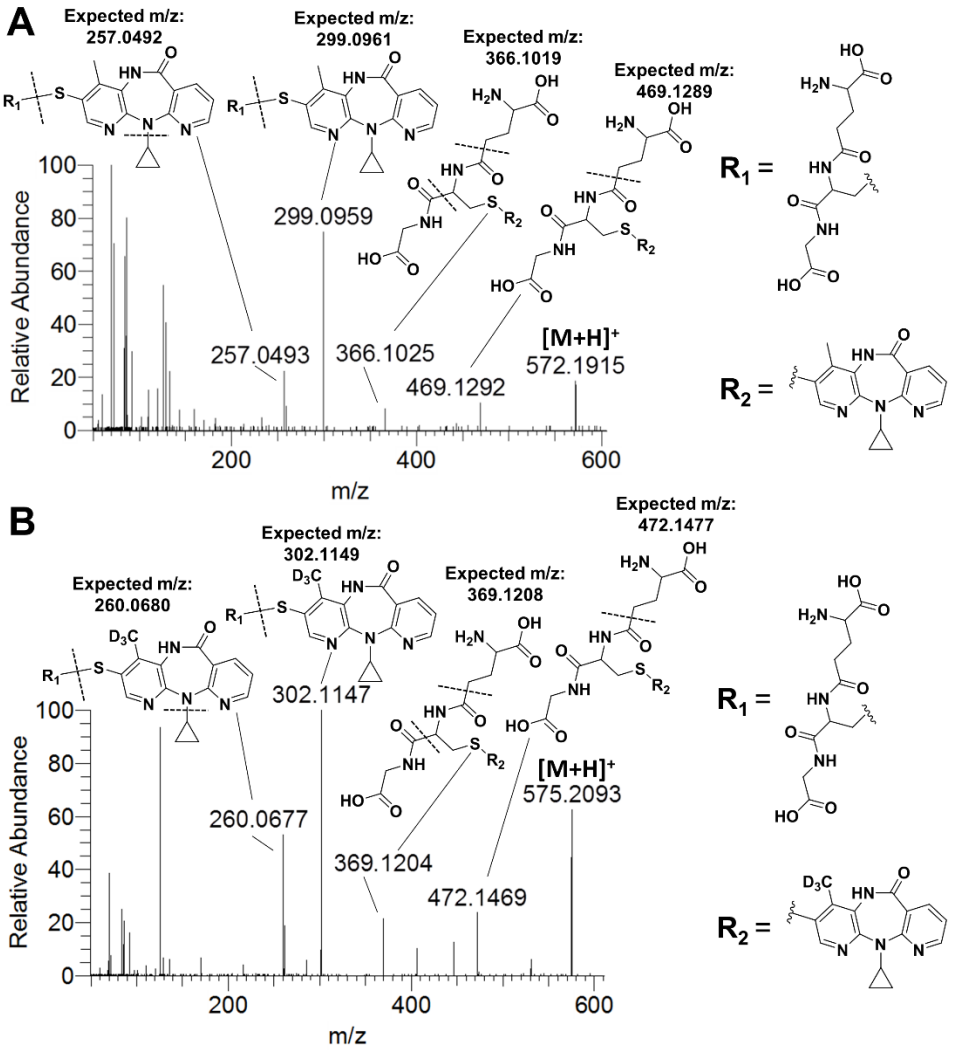


Supplemental Figure 4. NVP cytochrome P450-dependent metabolism in human liver microsomes during co-incubations with cytochrome P450 inhibitors. Liver microsomes (0.5 mg/mL) prepared from human liver were incubated with NVP (10 µM) and NADPH regenerating reagents for one hour either with additional vehicle as a control (0.2% DMSO) or with the following Cytochrome P450 inhibitors: 20 µM furafylline (CYP1A2 inhibitor), 2 µM tranylcypromine (CYP2A6 inhibitor), 30 µM PPP (CYP2B6 inhibitor), 20 µM

sulfaphenazole (CYP2C9 inhibitor), 10 µM (+)-N-3-benzyl-nirvanol (CYP2C19 inhibitor), 1 µM quinidine (CYP2D6 inhibitor), or 1 µM ketoconazole (CYP3A inhibitor). Metabolites were measured using uHPLC-MS/MS (Orbitrap) detection. 12-OHNVP (A), 2-OHNVP (B), and 3-OHNVP (C) were monitored using MS/MS scans for the following transitions: 283.1190 → 223.1104 *m/z* (12-OHNVP), 283.1190 → 161.0709 *m/z* (2-OHNVP), and 283.1190 → 242.0798 *m/z* (3-OHNVP). Data are representative of the mean ± standard deviation of three experimental replicates. Significant differences in inhibitor containing incubations to vehicle control incubations were determined using an unpaired t-test generating two-tailed P values (* $P < 0.05$ and *** $P < 0.001$).



Supplemental Figure 5. Extracted ion chromatograms of a glutathione conjugated NVP and 12-D$_3$NVP metabolites formed in primary mouse hepatocytes. Fresh primary mouse hepatocytes from male C57BL/6J mice were cultured and treated with 10 µM NVP or 12-D$_3$NVP for 24 hours. Metabolites were extracted from isolated cell pellets and subjected to uHPLC-MS (Orbitrap) analysis. The following high resolution ions were observed to assay for the presence of P450 metabolites: 572.1922 ± 5 ppm for glutathione conjugated, undeuterated NVP (Glutathione-NVP) and 575.2110 ± 5 ppm for glutathione conjugated, trideuterated NVP (Glutathione-D$_3$NVP). Representative extracted ion chromatograms (XICs) from this analysis are shown for glutathione conjugates formed during incubations with NVP (A) and with 12-D$_3$NVP (B). Results are representations of four experimental replicates.



Supplemental Figure 6. Representative fragmentation spectra for a glutathione conjugated NVP and 12-D_3NVP metabolites formed in primary mouse hepatocytes. Fresh primary mouse hepatocytes from male C57BL/6J mice were cultured and treated with 10 µM NVP or 12-D_3NVP for 24 hours. Metabolites were extracted from isolated cell pellets and subjected to uHPLC-MS/MS (Orbitrap) analysis. Fragmentation scans for the following ions were performed: 572.1922 ± 5 ppm for glutathione conjugated, undeuterated NVP (Glutathione-NVP) and 575.2110 ± 5 ppm for glutathione conjugated, trideuterated NVP (Glutathione-D_3NVP). Representative fragmentation spectra for these ions are shown are shown for glutathione conjugates formed during incubations with NVP (A) and 12-D_3NVP (B). The high resolution parent mass is indicated as [M+H^+], and the high resolution fragments used for structural confirmation are indicated, along with their predicted sites of fragmentation. Results are representations of four experimental replicates.

Protein Changes with NVP Treatment (in comparison to Vehicle)				
UniProt Accession	**UniProt Entry**	**Gene Name**	**Fold Change**	**P-Value**
P70404	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	*Idh3g*	1.8 ± 0.36	0.0071
P60710	Actin, cytoplasmic 1	*Actb*	1.15 ± 0.64	0.8847
A0A0A0MQF6	Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	-1.01 ± 0.33	0.8643
Q9DBG7	Signal recognition particle receptor subunit alpha	*Srpra*	-1.27 ± 0.1	0.0408
Q9D710	Thioredoxin-related transmembrane protein 2	*Tmx2*	-1.39 ± 0.1	0.0310
Q569Z6	Thyroid hormone receptor-associated protein 3	*Thrap3*	-1.46 ± 0.09	0.0064
Q9DB05	Alpha-soluble NSF attachment protein	*Napa*	-1.48 ± 0.09	0.0108
Q8C2T9	Putative uncharacterized protein	*Ik*	-1.51 ± 0.19	0.0484
A1L2Z3	C230096C10Rik protein	*Emc1*	-1.52 ± 0.13	0.0404
Q3UMF0	Cordon-bleu protein-like 1	*Cobll1*	-1.58 ± 0.18	0.0477
Q8BFP9	Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1, mitochondrial	*Pdk1*	-1.66 ± 0.09	0.0394
Q3TKM9	Actin-related protein 2/3 complex subunit 5	*Arpc5*	-1.69 ± 0.1	0.0061
Q542U7	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	*Lsm6*	-1.72 ± 0.22	0.0292
Q91VC3	Eukaryotic initiation factor 4A-III	*Eif4a3*	-2.02 ± 0.12	0.0047
Q9Z1N5	Spliceosome RNA helicase Ddx39b	*Ddx39b*	-2.15 ± 0.16	0.0381
Q91W92	Cdc42 effector protein 1	*Cdc42ep1*	-2.54 ± 0.05	0.0039
P13597	Intercellular adhesion molecule 1	*Icam1*	-2.85 ± 0.01	0.0001
Q9DC13	Lysosomal membrane glycoprotein 1, isoform CRA_a	*Lamp1*	-3.24 ± 0.19	0.0336
P70318	Nucleolysin TIAR	*Tial1*	Not found with NVP	0.0047
D3Z5N6	Zinc finger protein ubi-d4	*Dpf2*	Not found with NVP	0.0038

Supplemental Table 1. Relative-quantitation proteomics analysis of protein expression changes with NVP treatment of primary mouse hepatocytes. Freshly isolated primary mouse hepatocytes were treated for 8 hours with vehicle (0.2% DMSO) or 400 µM NVP. Cell lysate was then prepared for and subjected to Top10 nanoLC-MS based proteomics, with data analysis performed in Proteome Discoverer 2.1, using Sequest HT for peptide spectral matching/protein identification and precursor ion area detection for relative quantitation. Up to 10 unique or razor peptides were used in protein precursor ion-based quantitation. Statistically significant relative-quantitation changes are shown for treatment with NVP as compared to vehicle, as well as the results for housekeeping proteins actin and GAPDH. UniProt proteome *Mus musculus* 10090 was used for protein identification profiling, with UniProt accession numbers, protein entry (protein name), and gene name's provided. P-values were generated using un-paired t-test and effect size calculated using Cohen's d equation. Data are representative of four biological replicates.

Protein Changes with 12-D_3NVP Treatment (in comparison to Vehicle)				
UniProt Accession	**UniProt Entry**	**Gene Name**	**Fold Change**	**P-Value**
Q8BKT7	THO complex subunit 5 homolog	*Thoc5*	Not found with DMSO	0.0072
A2AIL4	NADH dehydrogenase (ubiquinone) complex I, assembly factor 6	*Ndufaf6*	Not found with DMSO	0.0089
P47876	Insulin-like growth factor-binding protein 1	*Igfbp1*	4.18 ± 1.16	0.0179
O35405	Phospholipase D3	*Pld3*	3.36 ± 0.98	0.0250
Q9DAS9	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	*Gng12*	2.41 ± 0.98	0.0408
Q3TJ01	tRNA-splicing ligase RtcB homolog	*Rtcb*	1.65 ± 0.47	0.0340
Q3TND1	Peptidyl-prolyl cis-trans isomerase	*Fkbp2*	1.57 ± 0.31	0.0295
P60710	Actin, cytoplasmic 1	*Actb*	1.19 ± 0.68	0.7786
A0A0A0MQF6	Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	1.11 ± 0.53	0.6387
P13597	Intercellular adhesion molecule 1	*Icam1*	-1.36 ± 0.03	0.0015
Q3TEX7	Putative uncharacterized protein	*Mfn2*	-1.45 ± 0.09	0.0266
P11352	Glutathione peroxidase 1	*Gpx1*	-1.5 ± 0.03	0.0028
Q9DBG7	Signal recognition particle receptor subunit alpha	*Srpra*	-1.58 ± 0.17	0.0225
Q14C24	MCG14259, isoform CRA_a	*U2af1*	-1.95 ± 0.13	0.0042
B9EKL6	MCG114106	*Ptp4a1*	-2.16 ± 0.2	0.0179
Q921I1	Serotransferrin	*Tf*	-2.22 ± 0.07	0.0057
Q3UUI3	Acyl-coenzyme A thioesterase THEM4	*Them4*	-3.18 ± 0.16	0.0460
P15116	Cadherin-2	*Cdh2*	Not found with 12-D_3NVP	0.0385

Supplemental Table 2. Relative-quantitation proteomics analysis of protein expression changes with 12-D_3NVP treatment of primary mouse hepatocytes. Freshly isolated primary mouse hepatocytes were treated for 8 hours with vehicle (0.2% DMSO) or 400 µM 12-D_3NVP. Cell lysate was then prepared for and subjected to Top10 nanoLC-MS based proteomics, with data analysis performed in Proteome Discoverer 2.1, using Sequest HT for peptide spectral matching/protein identification and precursor ion area detection for relative quantitation. Up to 10 unique or razor peptides were used in protein precursor ion-based quantitation. Statistically significant relative-quantitation changes are shown for treatment with 12-D_3NVP as compared to vehicle, as well as the results for housekeeping proteins actin and GAPDH. UniProt proteome *Mus musculus* 10090 was used for protein identification profiling, with UniProt accession numbers, protein entry (protein name), and gene name's provided. P-values were generated using un-paired t-test and effect size calculated using Cohen's d equation. Data are representative of four biological replicates.

Protein Changes with NVP Treatment (in comparison to Vehicle)				
UniProt Accession	**UniProt Entry**	**Gene Name**	**Fold Change**	**P-Value**
Q13098	Isoform 3 of COP9 signalosome complex subunit 1	*GPS1*	2.06 ± 0.26	0.0289
Q13190	Syntaxin-5	*STX5*	2.02 ± 0.39	0.0183
O95298	NADH dehydrogenase [ubiquinone] 1 subunit C2	*NDUFC2*	1.91 ± 0.23	0.0135
Q96I59	Pprobable asparagine--tRNA ligase, mitochondrial	*NARS2*	1.88 ± 0.06	0.0263
Q9UQ53	Isoform 3 of Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase B	*MGAT4B*	1.87 ± 0.45	0.0339
Q14320	Protein FAM50A	*FAM50A*	1.82 ± 0.12	0.0387
Q14117	Dihydropyrimidinase	*DPYS*	1.73 ± 0.15	0.0359
P15907	Beta-galactoside alpha-2,6-sialyltransferase 1	*ST6GAL1*	1.65 ± 0.33	0.0371
O95197	Isoform 3 of Reticulon-3	*RTN3*	1.62 ± 0.04	0.0111
P63208	S-phase kinase-associated protein 1	*SKP1*	1.59 ± 0.15	0.0298
Q9Y3B2	Exosome complex component csl4	*EXOSC1*	1.55 ± 0.17	0.0181
Q9BVV7	Mitochondrial import inner membrane translocase subunit TIM21	*TIMM21*	1.52 ± 0.18	0.0368
P28072	Proteasome subunit beta type-6	*PSMB6*	1.44 ± 0.09	0.0241
Q9ULA0	Aspartyl aminopeptidase	*DNPEP*	1.42 ± 0.06	0.0075
Q9HD45	Transmembrane 9 superfamily member 3	*TM9SF3*	1.41 ± 0.1	0.0258
Q9H6R3	Acyl-CoA synthetase short-chain family member 3, mitochondrial	*ACSS3*	1.31 ± 0.11	0.0214
O15382	Branched-chain-amino-acid aminotransferase, mitochondrial	*BCAT2*	1.3 ± 0.09	0.0270
Q9NX47	E3 ubiquitin-protein ligase MARCH5	*MARCH5*	1.3 ± 0.13	0.0489
Q5JTZ9	Alanine--tRNA ligase, mitochondrial	*AARS2*	1.21 ± 0.06	0.0202
Q6P1M0	Long-chain fatty acid transport protein 4	*SLC27A4*	1.17 ± 0.01	0.0019
P04406	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	1.18 ± 0.13	0.2236
P60709	Actin, cytoplasmic 1	*ACTB*	1.03 ± 0.3	0.9894
P56182	Ribosomal RNA processing protein 1 homolog A	*RRP1*	-1.32 ± 0.07	0.0402
P36543	V-type proton ATPase subunit E 1	*ATP6V1E1*	-2.16 ± 0.25	0.0460

Supplemental Table 3. Relative-quantitation proteomics analysis of protein expression changes with NVP treatment of cryopreserved human hepatocytes. Cryopreserved pooled-donor (10, mixed-sex) primary human hepatocytes were treated for 48 hours with vehicle (0.1% DMSO) or 10 µM NVP. Cell lysate was then prepared for and subjected to Top10 nanoLC-MS based proteomics, with data analysis performed in Proteome Discoverer 2.1, using Sequest HT for peptide spectral matching/protein identification and precursor ion area detection for relative quantitation. Up to 10 unique or razor peptides were used in protein precursor ion-based quantitation. Statistically significant relative quantitation changes are shown for treatment with NVP as compared to vehicle, as well as the results for housekeeping proteins actin and GAPDH. UniProt proteome *Homo sapiens* 9606 was used for protein identification profiling, with UniProt accession numbers, protein entry (protein name), and gene name's provided. P-values were generated using un-paired t-test and effect size calculated using Cohen's d equation. Data are representative of experiments with three different 10-donor pools of hepatocytes.

Protein Changes with 12-D_3NVP Treatment (in comparison to Vehicle)				
UniProt Accession	**UniProt Entry**	**Gene Name**	**Fold Change**	**P-Value**
Q9Y5U8	Mitochondrial pyruvate carrier 1	*MPC1*	15.96 ± 11.14	0.0015
P60059	Protein transport protein Sec61 subunit gamma	*SEC61G*	1.96 ± 0.21	0.0285
Q96CU9	FAD-dependent oxidoreductase domain-containing protein 1	*FOXRED1*	1.95 ± 0.27	0.0343
Q15397	Pumilio homolog 3	*PUM3*	1.94 ± 0.37	0.0320
P33261	Cytochrome P450 2C19	*CYP2C19*	1.79 ± 0.12	0.0339
Q8WUK0	Phosphatidylglycerophosphatase and protein-tyrosine phosphatase 1	*PTPMT1*	1.74 ± 0.01	0.0225
P60709	Actin, cytoplasmic 1	*ACTB*	-1.13 ± 0.06	0.1272
P04406	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	-1.17 ± 0.11	0.1835
P16152	Carbonyl reductase [NADPH] 1	*CBR1*	-1.25 ± 0.04	0.0082
O94973	AP-2 complex subunit alpha-2	*AP2A2*	-1.3 ± 0.07	0.0373
Q02252	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	*ALDH6A1*	-1.32 ± 0.06	0.0229
P42765	3-ketoacyl-CoA thiolase, mitochondrial	*ACAA2*	-1.33 ± 0.02	0.0244
Q9ULC5	Isoform 2 of Long-chain-fatty-acid--CoA ligase 5	*ACSL5*	-1.36 ± 0.09	0.0371
P61604	10 kDa heat shock protein, mitochondrial	*HSPE1*	-1.38 ± 0.06	0.0291
P26440	Isovaleryl-CoA dehydrogenase, mitochondrial	*IVD*	-1.39 ± 0.09	0.0349
Q13243	Serine/arginine-rich splicing factor 5	*SRSF5*	-1.45 ± 0.06	0.0035
Q6YN16	Hydroxysteroid dehydrogenase-like protein 2	*HSDL2*	-1.46 ± 0.05	0.0052
Q96I99	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	*SUCLG2*	-1.48 ± 0.1	0.0302
Q9UJZ1	Stomatin-like protein 2, mitochondrial	*STOML2*	-1.53 ± 0.1	0.0441
Q9BYG3	MKI67 FHA domain-interacting nucleolar phosphoprotein	*NIFK*	-1.54 ± 0.08	0.0188
Q9BYT8	Neurolysin, mitochondrial	*NLN*	-1.57 ± 0.13	0.0332
P51398	28S ribosomal protein S29, mitochondrial	*DAP3*	-1.62 ± 0.03	0.0109
O95297	Myelin protein zero-like protein 1	*MPZL1*	-1.64 ± 0.03	0.0039
O00515	Ladinin-1	*LAD1*	-1.66 ± 0.09	0.0068
O14561	Acyl carrier protein, mitochondrial	*NDUFAB1*	-1.7 ± 0.15	0.0456
Q07020	60S ribosomal protein L18	*RPL18*	-1.77 ± 0.15	0.0336
Q15392	Delta(24)-sterol reductase	*DHCR24*	-1.85 ± 0.15	0.0397
Q92734	Protein TFG	*TFG*	-1.87 ± 0.04	0.0497
P62995	Transformer-2 protein homolog beta	*TRA2B*	-1.94 ± 0.08	0.0067
P62241	40S ribosomal protein S8	*RPS8*	-1.98 ± 0.16	0.0067
Q9UL25	Ras-related protein Rab-21	*RAB21*	-1.99 ± 0.03	0.0482
Q16718	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	*NDUFA5*	-2.14 ± 0.1	0.0247
Q92552	Isoform 2 of 28S ribosomal protein S27, mitochondrial	*MRPS27*	-2.4 ± 0.17	0.0490
P61457	Pterin-4-alpha-carbinolamine dehydratase	*PCBD1*	-2.63 ± 0.17	0.0042
O15127	Secretory carrier-associated membrane protein 2	*SCAMP2*	-2.68 ± 0.05	0.0380
Q7L8L6	FAST kinase domain-containing protein 5, mitochondrial	*FASTKD5*	-4.73 ± 0.06	0.0456
Q96K37	Solute carrier family 35 member E1	*SLC35E1*	Not Found in 12-D3NVP	0.0151

Supplemental Table 4. Relative-quantitation proteomics analysis of protein expression changes with 12-D_3NVP treatment of cryopreserved human hepatocytes. Cryopreserved pooled-donor (10, mixed-sex) primary human hepatocytes were treated for 48 hours with vehicle (0.1% DMSO) or 10 µM 12-D_3NVP. Cell lysate was then prepared for and subjected to Top10 nanoLC-MS based proteomics, with data analysis performed in Proteome Discoverer 2.1, using Sequest HT for peptide spectral matching/protein identification and precursor ion area detection for relative quantitation. Up to 10 unique or razor peptides were used in protein precursor ion-based quantitation. Statistically significant relative quantitation changes are shown for treatment with 12-D_3NVP as compared to vehicle, as well as the results for housekeeping proteins actin and GAPDH. UniProt proteome *Homo sapiens* 9606 was used for protein identification profiling, with UniProt accession numbers, protein entry (protein name), and gene name's provided. P-values were generated using un-paired t-test and effect size calculated using Cohen's d equation. Data are representative of experiments with three different 10-donor pools of hepatocytes.