Supplementary Materials and Methods

Generation and molecular characterization of ES cell clones targeted at the Cyp2c55 and Cyp2c70 gene loci. *Cyp2c55*: The targeting vector was linearized with *Not*I and electroporated into a C57BL/6 mouse ES cell line. Of 384 G418 resistant ES cell colonies screened by standard Southern blot analyses, six correctly targeted clones were identified, expanded and further analysed by Southern blot analyses with 5' and 3' external probes and an internal neomycin probe. Five of the six clones were confirmed as correctly targeted at both homology arms. None of the five clones carried additional random integrations (data not shown). *Cyp2c70*: The targeting vector was linearized using *Not*I and electroporated into the correctly targeted *Cyp2c55* ES clone described above. Of 684 hygromycin resistant ES cell colonies screened by standard Southern blot analyses six correctly targeted clones were identified, expanded and further analysed by Southern blot analyses as described above. Five of these six clones were confirmed as correctly targeted at both homology arms without additional random integrations (data not shown).

Targeting of *Cyp2c55* and *Cyp2c70* has to be on the same allele in order to allow Cre-mediated deletion of the mouse *Cyp2c* Cluster. Therefore, four of the correctly double targeted ES cell clones described above were further analysed by in vitro deletion with Cre, followed by Gancyclovir selection and PCR analysis to detect deletion of the *Cyp2c* cluster. For *Cre*-mediated deletion of the *Cyp2c* Cluster in the double targeted ES cells, 1×10^7 ES cells derived from each of the finally validated clones were electroporated with the Cre-expression plasmid pCAGGScrepA as described previously (Seibler et al., 2005) and plated on 10 cm dishes. The Cre-transfected ES cells were grown in the presence of Gancyclovir, which is toxic in the presence of thymidine kinase (TK). Because Cre-mediated deletion of the mouse *Cyp2c* cluster will also lead to a loss of the TK selection cassette (Fig. 1E), only ES

cell clones with targeting of *Cyp2c55* and *Cyp2c70* on the same allele survived the Gancyclovir selection. Approximately 200-300 cells survived this selection in two of the four clones tested. Gancyclovir resistant cells from one of the two clones (A-C10) was transferred to individual wells of a 96-well plate, expanded and further analysed by PCR and Southern Blot analysis, confirming the successful deletion of the *Cyp2c* cluster in these cells (data not shown). *Cyp2c* deleted ES cell clones were used to generate Cyp2c KO mice and were further modified by the insertion of a CYP2C9 expression cassette (see below).

In order to generate ES cell clones with a replacement of the mouse *Cyp2c* cluster with a CYP2C9 expression cassette, a targeting vector was generated in which a cDNA of *CYP2C9* containing the original human *CYP2C9* intron 4 was linked to a fusion of the 8.5-10.4 kb BamHI/NheI enhancer and 0.3 kb promoter fragment of the mouse albumin upstream regulatory region (Pinkert et al., 1987). This targeting vector also contained *loxP*, *lox5171* and *f3* sites, polyadenylation and splice acceptor polyadenylation motifs and a 5' deficient neomycin selection cassette as depicted in Fig. 1F. This allowed the insertion of the CYP2C9 expression cassette via Cre-mediated recombination at the corresponding *lox* sites in the prepared *Cyp2c* deleted ES cell clones (see above) and selection of correctly targeted clones with high stringency by the complementation of the deficient neomycin cassette with the promoter and ATG remaining at the deleted *Cyp2c* locus (Fig. 1G). For this purpose, $1x10^7$ cells were electroporated under standard conditions with supercoiled targeting vector and the Cre-expression plasmid pCAGGScrepA as previously described (Seibler et al., 2005) and selected with G418. 11 G418 resistant ES cell clones were obtained after the electroporation procedure. Six of the clones were expanded and further analysed by PCR and Southern blot with different suitable restriction enzymes, 5′ and 3′ external probes, and an internal neomycin probe. Five of the six clones were confirmed as correctly recombined at both *lox* sites, were shown to carry a single copy of the integrated CYP2C9 expression cassette

and didn't carry additional random integrations. One of these clones was used to generate the hCYP2C9 mice. The neomycin selection cassette was subsequently deleted in vivo by flipase recombinase (Flp) mediated recombination at the *f3* recognition sites (Fig. 1H).

Molecular characterization of CYP2C9 humanized and Cyp2c KO mice. Offspring from chimeras derived from *Cyp2* cluster deleted ES cells or ES cells with an insertion of the CYP2C9 expression cassette were analysed by PCR in order to determine the genotype of these mice. The following PCR primer pairs were used for the detection of the different alleles: (1) WT mouse *Cyp2c* cluster allele: 5'-CTACAATGCTCTGCCTACCC-3' and 5'-AAATCTGACTCCCTCTTCTGG-3', resulting in a 307 bp PCR fragment; (2) Deleted *Cyp2c* cluster allele: 5'-GACATTGACATCCACTTTGCC-3' and 5'-GATGGATGTGTGGAATGAAGAG-3', resulting in a 559 bp PCR fragment and (3) Allele with CYP2C9 expression cassette: 5'-GCAGGCCAGAGTCCATTCAG-3' and 5'-CTGGAGTGGCAACTTCCAG-3', resulting in a 712 bp PCR fragment. Mice heterozygous for the deletion of the mouse *Cyp2c* cluster or carrying the CYP2C9 expression cassette were crossed to generate homozygous Cyp2c KO and hCYP2C9 mice, respectively. Homozygosity was determined by the presence of the corresponding modified allele and absence of the 307 bp WT PCR fragment.

Preparation of tissues. Terminal blood samples were mixed on a roller for 5 minutes at room temperature. Erythrocytes were removed by centrifugation (2,000 – 3,000 rpm for 10 min at 8 - 10°C) and the resulting plasma was stored on ice prior to clinical chemistry analysis. The gall bladder was removed and the liver removed and weighed. Two samples of liver (30 mg +/- 10%) were cut into smaller pieces and placed into two separate cryovials prior to addition of RNAlater (1.8 ml). Samples were incubated at +4^{o}C overnight and then stored at approximately -70^{o}C before TaqMan analysis. The remaining liver was weighed and used

immediately for subcellular fractionation by differential centrifugation according to standard methods. Mouse liver microsomes were prepared and stored as described recently (Scheer et al., 2008).

Immunoblot analysis of Cyp2c and CYP2C9 protein expression. The expression of Cyp2c and CYP2C9 apoproteins was visualized in liver microsome samples by immunoblot analysis. A human CYP2C9/19-specific antibody (a rabbit polyclonal anti-CYP2C9/19 developed in house by CXR Biosciences) was used to detect the human CYP2C9 and a polyclonal rabbit anti-Cyp2c1 antibody (kindly provided by Dr. Colin Henderson, University of Dundee, UK) was used to detect mouse Cyp2c apoproteins. 10 μg of microsomal protein were loaded for the immunoblots. The positive controls were pooled human liver microsomes (BD Biosciences, #452161), and recombinant human CYP2C9 (0.1 pmol) (Lifetechnologies, #P2378, Grand Island, NY).

Quantitative Reverse Transcriptase-PCR (qRT-PCR). qRT-PCR and analysis was carried out as described previously (Hasegawa et al., 2011). Primers used were from the following Assay-On-Demand Kits (Applied Biosystems, Foster City, CA): Hs00426397_m1 (*CYP2C9*), Mm00656110_gH (*Cyp2c39*), Mm01197188_m1 (*Cyp2c44*), Mm00472168_m1 (*Cyp2c55*), Mm00521058_m1 (*Cyp2c70*), Mm00731567_m1 (*Cyp3a11*), Mm00484110_m1 (*Cyp3a13*), Mm00651731_m1 (*Cyp2d9*), Mm00530542_m1 (*Cyp2d22*), Mm01307122_g1 (*Cyp2d26*), Mm01967851_s1 (*Ugt1a6*), Mm01623253_s1 (*Ugt2b5*) and Mm00655373_m1 (*Ugt2b34*).

In vitro determination of CYP2C-dependent activities. A tolbutamide (100 μM) microsome (0.5 mg protein/ml) mixture in phosphate buffer (100 mM KH_2PO4 pH7.4, 3.3 mM $MgCl_2$) was incubated in a water bath for 5 min at 37^{o}C prior to the start of the reaction by addition of NADPH regenerating system (final concentrations: 1.3 mM NADPH, 4 mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase). After 30 min, the reaction

was stopped by addition of an equal volume of ice-cold acetonitrile containing dextrorphan (1 μM) as internal standard. Samples were centrifuged for approximately 15 minutes at approximately 3,500 g on a Legend RT centrifuge (Sorvall, Newton, CT) and concentration of hydroxymethyltolbutamide in the supernatant determined by LC-MS/MS. Calibration standards were prepared in the phosphate buffer/acetonitrile with the internal standard (1:1 v/v; centrifuged (10 min, ~16000g) on a Biofuge Fresco centrifuge (Sorvall, Newton, CT)) by adding an appropriate amount of hydroxymethyltolbutamide. Chromatographic separation was performed on a Luna, C18 column (5μm, 150 x 2.0mm) (Phenomenex, Macclesfield, UK) using an injection volume of 10 μl and a run time of 4.51 minutes. The detector used was a Micromass Quattro Micro mass spectrometer (Micromass UK Ltd, Manchester, UK) run in electrospray positive ion mode. The multiple reaction monitoring parameters for hydroxymethyltolbutamide and dextrorphan were 287.31 and 258.2 (parent ions) and 171.25 and 199.3 (collision ions), respectively.

A diclofenac (4 μM) microsome (0.25 mg protein/ml) mixture in phosphate buffer (100 mM KH_2PO4 pH7.4, 3.3 mM $MgCl_2$) was incubated in a water bath for 5 min at 37^{o}C prior to the start of the reaction by addition of NADPH (1 mM final concentration). After 5 min, the reaction was stopped by addition of an equal volume of ice-cold acetonitrile containing dextrorphan (1 μM) as internal standard. Samples were centrifuged for approximately 15 minutes at approximately 3,500 g on a Legend RT centrifuge (Sorvall, Newton, CT) and 4-hydroxydiclofenac concentrations in the supernatant were determined by LC-MS/MS. Calibration standards were prepared in the phosphate buffer/acetonitrile with the internal standard (1:1 v/v; centrifuged (10 min, ~16000g) on a Biofuge Fresco centrifuge (Sorvall, Newton, CT)) by adding an appropriate amount of 4-hydroxydiclofenac. Chromatographic separation was performed on a Luna, C18 column (5μm, 150 x 2.0mm) (Phenomenex, Macclesfield, UK) using an injection volume of 10 μl and a run time of 4.51 minutes. The

detector used was a Micromass Quattro Micro mass spectrometer (Micromass UK Ltd, Manchester, UK) run in electrospray positive ion mode. The multiple reaction monitoring parameters for 4-hydroxydiclofenac and dextrorphan were 312.04 and 258.2 (parent ions) and 230.06 and 199.3 (collision ions), respectively.

Pharmacokinetic analysis for tolbutamide. Tolbutamide solution in ethanol (5 mg/ml) was diluted 10 times by PEG200 solution in PBS (2:1; v/v) and administered intraperitoneally (5 mg/kg body weight) to all animals. The volume of dosing solution was 10 ml/kg bodyweight. For the analysis of tolbutamide pharmacokinetics, blood (10 µl aliquots) samples were collected at 0.083, 0.25, 0.5, 1h, 2h, 4h, 8h after dosing, placed immediately into microfuge tubes containing 10 µl MilliQ water, snap frozen in liquid nitrogen and stored at approximately -70ºC prior to analysis. Concentrations of tolbutamide in whole blood were measured by LC/MS/MS. Calibration standards were prepared in whole blood water (1:1 v/v; 95 µl) by adding an appropriate amount of tolbutamide in acetonitrile (5 µl). 20 µl of the test samples and of the calibration standards were extracted in 80µL of acetonitrile containing 500ng/ml dextrorphan as an internal standard. The mixture was vortexed for approximately 30 sec, sonicated in an ultrasonic water-bath for 30 sec and centrifuged at approximately 13,000 g for 5 minutes. The supernatant was transferred to a 96-well plate and the concentrations tolbutamide and dextrorphan were measured by LC/MS/MS. Chromatographic separation was performed on a Luna, C18(2) column (5µm, 150 x 2 mm) (Phenomenex, Macclesfield, UK) using an injection volume of 10 µl and a run time of 5.5 minutes. The detector used was a Micromass Quattro Micro mass spectrometer (Micromass UK Ltd, Manchester, UK) run in electrospray positive ion mode. The multiple reaction monitoring parameters for tolbutamide and dextrorphan were 271.15 and 258.2 (parent ions) and 73.64 and 199.2 (collision ions), respectively. Pharmacokinetic parameters were calculated using WinNonlin Professional version 5.2 (Pharsight, Mountain View, CA).

In vitro CYP2C inhibition studies. A tolbutamide (100 µM) microsome (0.5 mg protein/ml) mixture in phosphate buffer (100 mM KH_2PO4 pH7.4, 3.3 mM $MgCl_2$) was incubated with and without an inhibitor (final inhibitor concentrations: 2 µM fluvoxamine; 15 µM fluoxetine; 5 µM fluconazole; 50 µM sulfaphenazole and 0.25 µMbenzbromarone) in a water bath for 5 min at 37^{o}C prior to the start of the reaction by addition of NADPH regenerating system (final concentrations: 1 mM NADPH, 4 mM glucose-6-phosphate and 2 Units/ml glucose-6-phosphate dehydrogenase). After 20 min, the reaction was stopped by addition of an equal volume of ice-cold acetonitrile containing bupropion (10 ng/ml) as an internal standard. Samples were centrifuged for approximately 15 minutes at approximately 3,500 g on a Legend RT centrifuge (Sorvall,Newton, CT) and hydroxymethyltolbutamide concentrations in the supernatant were determined by LC-MS/MS. Calibration standards were prepared in the phosphate buffer/acetonitrile with the internal standard (1:1 v/v; centrifuged (10 min, ~16000g) on a Biofuge Fresco centrifuge (Sorvall, Newton, CT)) by adding an appropriate amount of hydroxymethyltolbutamide. Chromatographic separation was performed on a Luna, C18(2) column (5µm, 150 x 2.0mm) (Phenomenex, Macclesfield, UK) using an injection volume of 10 µl and a run time of 4.51 minutes. The detector used was a Micromass Quattro Micro mass spectrometer (Micromass UK Ltd, Manchester, UK) run in electrospray positive ion mode. The multiple reaction monitoring parameters for hydroxymethyltolbutamide and bupropion were 287.33 and 240.31 (parent ions) and 171.11 and 184.17 (collision ions), respectively.

Supplementary Methods References

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