Supplementary Methods

Western blot

Samples from cell lysates and tumor homogenates were subjected to SDS-PAGE and transferred to a Hybond-C extra membrane (GE Healthcare). After blocking in Tris-buffered saline (250 mM Tris-HCl, pH 7.4, 200 mM NaCl) containing 0.05 % (v/v) Tween 20 and 5 % (w/v) fat-free milk powder, the membranes were incubated with rabbit anti-CYP2W1 C-terminal peptide antibodies (1) (or rabbit anti-CYP2W1 H-175 antibodies (Santa Cruz, USA)), Phospho-H2AX^{Ser139} (γH2A.X) antibodies (Cell Signaling, USA) or ERp29 antibodies (2) followed by goat anti-rabbit conjugated horseradish peroxidase (HRP) secondary antibodies (Dako Denmark A/S). Filters were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and signals detected by LAS 1000+ gel documentation system (Fujifilm). In order to detect minor amounts of CYP2W1 or γH2A.X in the tumor samples we have applied more sensitive western blot development method using the near-infrared fluorescence detection where the HRP-conjugated secondary antibodies were replaced with the antibodies labeled with IRDye and protein bands visualized by the Odyssey® Infrared Imaging System (LI-COR, USA).

ICT2706 pharmacokinetics

Chemicals and reagents for analysis. Standard solutions of ICT2706 (10 mg/ml) were prepared in DMSO and stored at –20 ^{o}C. High purity HPLC grade solvents (Fisher Scientific, Loughborough, UK), analytical grade chemicals (Sigma-Aldrich) and triple distilled water were used throughout. Heparinized polypropylene tubes were used for collection of blood samples, polypropylene microcentrifuge tubes (Sigma-Aldrich) were used throughout for sample handling

and storage, and polypropylene autosampler vials (Sigma-Aldrich) were used to load samples for HPLC analysis.

Instrumentation and Chromatographic conditions. An LC/MS reverse phase system was used to measure the production of specific metabolites and monitor ICT2706. Chromatographic analysis of ICT2706 used a Waters Alliance 2695 (Milford, MA, USA) quaternary pump chromatography system, which also incorporates the autosampler. The Waters Alliance 2695 was attached to a Waters ZMD quadrupole mass spectrometer (Micromass, Manchester, UK) in series.. A Waters 996 Photodiode Array Detector ($\lambda 1$ = 330nm) with Masslynx 4.0 software (Micromass Ltd) was used for spectral analysis of the peaks of interest. The flow rate was set at 1.0ml / min and a Hichrom RPB column (25cm x 4.6mm id, Hichrom Ltd, UK) was used for the separation. Mobile phase A (MPA) was composed of 90% HPLC grade water, 10% methanol and 0.1% formic acid (FA). Mobile phase B (MPB) was composed of 10% HPLC grade water, 90% methanol and 0.1% FA. A linear gradient separation was used with an initial ratio of 50% MPA and 50% MPB. This ratio was changed over 15 min to 30% MPA and 70% MPB and then to 10% MPA and 90% MPB between 15 and 25 min. This ratio was held until 29 min and returned to initial conditions at 30 min. Mobile phases were degassed by vacuum filtration through a 0.45 μm pore nylon filter membranes.

The quadrupole mass spectrometer was operated in the positive ion electrospray mode with a voltage of +3.00 kV applied to the capillary. A solvent flow of 1.0 ml/min (split 1:10) with a nitrogen gas flow of 400 l/hr and a source temperature of 100 ^{o}C were employed to produce stable spray conditions. The cone voltage was set at 25 V to give clear mass spectra from these samples. To increase sensitivity, single ion recording (SIR) channels were established with

dwell times of 0.5 seconds. SIR channel m/z 384.2: ICT2706; m/z 368.9: ICT2726. Masslynx 4.0 software was used to process the mass spectral data.

Sample preparation (plasma). Mouse plasma samples used in the extraction and calibration procedures were taken by cardiac puncture from drug-free anaesthetized mice. Blank plasma (100 µl) was spiked with ICT2706 to give appropriate final concentrations between 0-10 µg/ml for extraction efficiencies, and calibration purposes. Three volumes of methanol, were added to precipitate protein. After vortex-mixing the samples were centrifuged at 7000 g for 3 min, the supernatant was carefully removed and 10 µl injected onto the HPLC system. The extraction efficiency (n = 6) was calculated as a percentage of non-extracted saline controls. All samples were handled in polypropylene microcentrifuge tubes to avoid potential binding to glass.

Calibration. Plasma calibration standards were prepared by spiking drug-free mouse plasma to give the final concentration range of 0.05-10.0 µg/ml. The calibration curves were determined using the linear regression analysis of the ratios of peak area of the analyte plotted against concentration. The calibration and extraction process was repeated using drug-free tumor tissue homogenate to represent non-plasma samples.

Sample preparation (tissue). Tumor or liver samples were accurately weighed and homogenized in 4 vol of Tris buffer (pH 7.0, 50 mM). Samples were then de-proteinised by adding three volumes of methanol. After vortex-mixing the samples were centrifuged at 7000 x g for 3 min, the supernatant was carefully removed and 10 µl injected onto the HPLC system.

References:

1. Karlgren M, Gomez A, Stark K, Svard J, Rodriguez-Antona C, Oliw E, et al. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. Biochem Biophys Res Commun. 2006;341:451-8.
2. Mkrtchian S, Fang C, Hellman U, Ingelman-Sundberg M. A stress-inducible rat liver endoplasmic reticulum protein, ERp29. Eur J Biochem. 1998;251:304-13.