**Cell culture**

Osteosarcoma U2OS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone) supplemented with L-Glutamine (Gibco ), penicillin (100 U/mL), streptomycin (0.1 mg/mL) (PAA), and 10% fetal bovine serum (Gibco). MDCK, HeLa.S-Fucci, MiaPaCa-2, and PANC-1 cell lines were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), which was supplemented with penicillin (100 U/mL), streptomycin (0.1 mg/mL) (PAA), and 10% fetal bovine serum (PAA). BPH-1 and BPH-1 CAFTD04 cells were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen), which was supplemented with 5% FBS, streptomycin (0.1 mg/mL), and penicillin (100 U/mL). A2780, A2780cis, H441, MDA-MB-231, and DU 145 cells were cultivated in RPMI-1640 medium, which was supplemented with 10% FBS, streptomycin (0.1 mg/mL), and penicillin (100 U/mL). A549 and PC3 cells were cultivated in F-12 medium (Invitrogen), which was supplemented with 10% FBS, streptomycin (0.1 mg/mL), and penicillin (100 U/mL). SKOV-3, CAKI-2 and Sk-Br-3 cells were maintained in McCoy’s 5A modified medium containing 1.5 mmol/L L-glutamine (Sigma-Aldrich), penicillin (100 U/mL), streptomycin (0.1 mg/mL), sodium bicarbonate (1.5 g/L; Serva), and 10% FBS. HCT-116 p53-/-, HCT 116 p53+/+, HCT 116 PTEN-/-, HCT 116 PTEN+/+, SW620, SW480, and HT-29 human colon adenocarcinoma cells were maintained in McCoy’s 5A modified medium containing 1.5 mmol/L L-glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/mL), sodium bicarbonate (1.5 g/L), and 10% heat-inactivated FBS (PAA). The Caco-2 cells were cultured in E-MEM (Invitrogen) supplemented with 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were harvested after a brief incubation in 0.05% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), followed by trypsinization (0.25% w/v trypsin/0.53 mmol/L EDTA in PBS). They were then counted by using a CASY TT automatic cell counter (Roche Diagnostics), diluted in the appropriate volume and seeded for experimental procedure. SW480, SW620, HT-29, DU 145, and H441 cell lines were obtained from American Type Culture Collection (LGC Standards). The AmpFLSTR® Identifiler® PCR Amplification Kit (Life Technologies) was used to verify the origin of cell lines.

**Drug treatments**

Attached cells were treated with Hydroxyurea (Sigma) or gemcitabine (Sigma) overnight, for 24 hours followed by addition of CHK1 inhibitors either SCH900776 or MU380 for 2 hours. Thereafter the cells were refurbished with fresh medium and harvested for appropriate assay at indicated time points.

**Cell proliferation assays**

For the determination of IC50 values in U2OS cells, WST-1 (Roche) survival assay was performed. Cells (5000-6000 cells/well, 3 wells per concentration range 0.3 µmol/L to 64 µmol/L) were seeded in 96 well plates and allowed to attach overnight. Next day cells were treated as mentioned earlier and 72 hours later they were assayed according to manufacturer's protocol. For cytotoxicity screening the cells were seeded at a density of 20000, 30000 or 40000 cells/cm2. On the next day, cells were treated with HU (4 wells per concentration, range 0.037 mmol/L to 27 mmol/L using 6 points) or GEM (4 wells per concentration, range 0.781 nmol/L to 400 nmol/L using 10 points). CHK1 inhibitors (range 0.25 µmol/L to 4 µmol/L using 6 points) or vehicle were added after 24 hour incubation with hydroxyurea or gemcitabine for next 2 hours. Treatments by drugs were realized using automated pipetting system epMotion 5075® according the procedure described earlier (Suchankova et al. 2014, PMID: 25369341). Then the media was completely exchanged and cells were allowed to proliferate for next 48 hours, harvested and analyzed using CyQuant assay. The CyQuant cell proliferation assay (Invitrogen) was performed, according to the manufacturer’s recommendations, and the results were analyzed using a Fluostar Galaxy reader (BMG Labtech).

**Immunoblotting**

Cells were washed twice in PBS and harvested in RIPA buffer (50 mmol/L Tris–HCl Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl or Sigma Aldrich, protease inhibitors (Serva or Roche) and phosphatase inhibitor cocktail (Merck) or PhosSTOP (Roche). The protein concentration was determined using a detergent-compatible protein assay (Bio-Rad) or bradford (Applichem). The cell lysates were diluted to the same concentrations and mixed with loading buffer (150 mmol/L Tris–HCl pH 6.8, 3% SDS, 0.03% bromphenol blue, 30% glycerol, 3% β-mercaptoethanol). Equivalent protein quantities (30-50 µg) were separated by SDS–PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked in TBS (20 mmol/L Tris–HCl pH 7.2, 140 mmol/L NaCl) containing 0.1% Tween-20 and 5% nonfat dry milk for 1 hour. The membranes were washed with TBS– Tween and incubated with specific primary antibodies overnight at 4 °C. The following primary antibodies were used: CHK1 (# sc-8408 Santa Cruz Biotechnologies or #ADI-KAM-CC111 Enzo), pS296 CHK1 (#2349 Cell Signaling), pS345 CHK1 (#2341 Cell Signaling) and β - actin (#A5441, Sigma-Aldrich). The membranes were washed and then incubated with secondary antibodies anti-mouse IgG (#NA931 GE Healthcare, #A0168 Sigma Aldrich) or anti- rabbit IgG (#NA934 GE Healthcare, #A6154 Sigma Aldrich) for 1 hour. Detection of antibody reactivity was performed using Immobilon Western HRP Substrate (#WBKLS0500 Millipore) and visualized on X-ray films (Agfa) or detected by Image reader LAS-4000.

**Flow cytometry**

Samples for γH2AX staining were done as described previously (Huang and Darzynkiewicz, 2006). Briefly, cells were harvested at 48 hours after treatment, washed and fixed in ice-cold 1% methanol-free formaldehyde solution and incubated in primary Anti-phospho-Histone H2A.X (Ser139) antibody (#05-636, Merck Millipore) overnight followed by FITC conjugated secondary antibody (AlexaFluor 488 goat anti mouse #A-11001, Life technologies). Cells were simultaneously stained with Propidium Iodide and then analyzed by flow cytometer (BD FACS Diva). The data was analyzed by Flow (vX.0.7). Only singlets were chosen for analysis. For γH2AX quantification, the cells were plotted γH2AX vs DNA content (scatterplots). An arbitrary threshold was determined between the untreated cells (negative control) and HU treated cells (positive control) to assess the γH2AX positive population and applied to all samples. For apoptotic assay, Annexin V/PI staining (PI, #556463, Annexin V-FITC #556420, BD Biosciences) was done according to manufacturer's protocol. Singlets were quantified by scatter plots (PI vs Annexin V) gated into arbitrary quadrants using untreated cells (negative control) and HU treated cells (positive control). Single stained cells, either PI or γH2AX antibody or Annexin V alone were used for compensation purposes.

**Kinase assays**

The Eurofins Kinase Profiler service was used to obtain general selectivity data for SCH 900776, MU378, MU379, and MU380 at 1 μmol/L concentration against a panel of 207 human serine/threonine and tyrosine kinases. The data were provided as percent activity remaining, relative to uninhibited controls.

**High content microscopy**

DU 145 cells were seeded at 8000 cells/well in a 96 well plate. Cells were treated with 12.5 nmol/L GEM for 24 hours alone or 4 µmol/L of CHK1 inhibitors alone for 2 hours or combination where gemcitabine was followed after 24 hours by 2 hours incubation with CHK1 inhibitors. Thereafter the cells were let to recover in drug free medium over 0, 6, 24, and 48 hours. The experiment was terminated by fixing the cells in 4% formaldehyde for 10 mins. Briefly, after fixation, cells were washed with 1X PBS, permeabilized in 0.1% Triton-X for 10 minutes, washed in 1X PBS again and blocked in 3% BSA-PBS solution for 30 minutes. Thereafter, cells were incubated overnight at 4 ºC in primary γH2AX antibody (1:1000, Anti-phospho-Histone H2A.X (Ser139) antibody (#05-636, Merck Millipore), washed and incubated in secondary antibody 1:1000 AlexaFluor 488 goat anti mouse (#A-11001 Life technologies) for 1 hour at RT. Finally after washing PBS containing DAPI (1:2000) was added for nuclear staining. All the preparations for staining of the cells in 96 well plates were done using multidrop combi (Thermo Scientific). The plates were scanned at 40X magnification for FITC and DAPI by ImageXpress Micro System (Molecular Devices). Thereafter the data was analyzed using CellProfiler 2.1.1 for nuclear detection and foci detection. Total number of cells and total intensity of γH2AX staining was measured. An arbitrary threshold for total intensity was set by comparing untreated and positively stained cells based on which the data was represented as percentage of γH2AX positive cells above the predefined thresholds.

**Pharmacokinetics**

5-6 weeks old male NMOL/L RI/CD1 mice (Medical Faculty, Masaryk University, Brno, Czech Republic) of 25 g average weight were used as experimental subjects. Single dose of SCH900776 and MU380 (30 mpk, i.p.) in 20% (w/v) sterile Kolliphor ELP (Sigma) was injected and blood was collected by cardiac puncture in deep anesthesia using ketamine/xylazine in time intervals 0, 30 min, 1, 2, 4, 8, 16, 24 h (n = 5 for each time point). The whole blood in plastic blood collection tubes with K2EDTA was centrifuged (3000g/15min) and plasma was collected and stored at -80 °C for further analysis. 50 μL of plasma sample was mixed with 950 μL of acetonitrile, the mixture was sonicated for 5 minutes, then centrifuged at 10 000 rpm for 5 minutes, and 100 μL of the supernatant was transferred to a vial for direct HPLC/MS analysis. HPLC analysis was performed under isocratic elution on a Gemini-NX C18 (Phenomenex, 4.5 x 150 mm, 3 μm) chromatographic column using mobile phase which consisted of 25 mmol/L NH4COOH and methanol (45/55, v/v). Flow rate was set to 0.55 mL/min, injected volume was 15 μl. MS spectra were recorded with an Agilent 6224 Accurate-Mass TOF mass spectrometer (Agilent Technologies). The technique of atmospheric pressure chemical ionization in positive mode was chosen for analyte ions formation. The parameters of the ion source and the mass analyzer were set as follows: nitrogen flow 8 l/min at 325°C, vaporizer 200°C, nebulizer 45 psig, corona 4 μA, fragmentor voltage of 60 V and skimmer voltage of 65 V. The MS spectra were recorded from m/z 50 to 500 and extracted ion chromatograms were based on a 10 ppm mass window.

**Mouse xenograft experiments**

Mice survival experiments: A colony of NSG mice (NSG, NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ) was obtained from the Jackson Laboratory (Bar Harbor, Maine), maintained under specific-pathogen-free conditions with 12/12 light cycles, and fed ad libitum. For xenograft experiments, 9- to 12-week old mice were subcutaneously injected with 1.0 × 106 A2780 cells in 100 mL of sterile DMEM mixed with HC Matrigel (Corning) 1:1 in the left dorsal flank. After one week, mice were randomly divided into 6 cohorts and treated with either vehicle (Kolliphor ELP, Sigma) gemcitabine (150 mg/kg) or SCH900776 (25 mg/kg) or MU380 (25 mg/kg) or combinations of gemcitabine and SCH900776 or MU380. The treatment was repeated after two weeks. The survival analysis was performed for a period of 66 days and represented as the Kaplan-Meier survival plot. The study was performed in two independent repetitions (n=9 for each cohort). A colony of hairless SCID (Crl:SHO-PrkdcscidHrhr) mice was obtained from the Charles River, maintained under specific-pathogen-free conditions with 12/12 light cycles, and fed ad libitum. For xenograft experiments, 4–6 week old mice were subcutaneously injected with 0.25×106 MiaPaCa2 luc cells in sterile 1x PBS and HC Matrigel (2:1). After one week, mice were randomly divided into 6 cohorts and treated 3 times every 5 or 7 days with either vehicle (Kolliphor, Sigma) gemcitabine (150 mg/kg) or SCH900776 (25 mg/kg) or OH209EN1 (25 mg/kg) or combinations of gemcitabine and SCH900776 or OH209EN1. CHK1i were administered 4 hrs or 24 hrs after gemcitabine. Animals were checked daily and MiaPaCa2 luc tumor size was analyzed under isoflurane anesthesia using optical imaging system IVIS Lumina XR (1 min exposure, binnig 4, f/2, Perkin Elmer) after i.p. luciferin injection (150 mg/kg, Goldbio). For pharmacodynamic marker analysis, 3 weeks after MiaPaCa2 luc cell injection, mice were randomly divided and injected i.p. either with gemcitabine or Kolliphor which was followed by Kolliphor, SCH900776 or MU380 injection 4 hrs later. Mice were sacrificed by cervical dislocation 24 hrs after the initial injection of gemcitabine/Kolliphor and tumors were dissected. Immediately after the excision tumors were fixed and processed as described below. Animal experiment was approved by the MEYS of the Czech Republic (MSMT-2981/2016-5) and REKOZ CAS (79/2016, 65/2016), supervised by the local ethical committee of the Faculty of Medicine, Masaryk University and Institute of Biophysics CAS; and performed by certified individuals (KK, PV, KS, LB, HK).

**Immunohistochemistry**

Mouse skin fixed samples were processed and stained with CHK1 pS345 (clone 133D3; Cell Signaling) as described in Guzi et al, 2011 (DOI: 10.1158/1535-7163.MCT-10-0928). MiaPaCa2 luc xenograft slices were stained with γH2A.X (Ser139, clone 20E3; Cell Signaling), anti-rabbit Alexa Fluor 568 antibody (ThermoFisher Scientific, A-11036) and counterstained with DAPI. Bright field images of skin tissue and fluorescent images of tumors were captured on TissueFAXS scanning system (TissueGnostics) using 20x objective, composite image of whole slice was exported and areas of hair follicles were manually cropped from the image. Haematoxylin and DAB channel was color deconvolved by specialized module of CellProfiler (ver. 2.2.0, Carpenter AE, 20116, DOI: 10.1186/gb-2006-7-10-r100). Pipeline for bright field image quantification further consisted of nuclei segmentation based on haematoxylin channel, unspecific signal of DAB from hair root was removed before this step and DAB intensity was measured on segmented area of nuclei. Data are presented as % of highly positive nuclei (≥0.75 maximal DAB intensity). As for the CellProfiler analysis of fluorescent images, first we removed areas of unspecific signal from both Alexa Fluor 568 and DAPI channel, which were larger than the area of nuclei, then nuclei were segmented from processed DAPI images and the intensity of Alexa Fluor 568 signal was measured on segmented area of nuclei. For threshold adjustment of nuclei positivity pair tissue slices stained with DAPI and secondary antibody only were acquired and analyzed in the same manner.Threshold of positivity, total numbers of positive and all segmented nuclei were then calculated using RStudio (ver. 1.0.136, RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL <http://www.rstudio.com/>).

**Statistical analysis**

For cell line screening, data were standardized as % of control. A non-linear regression was applied to generate curves that best fitted the data. A four-parameter dose-response model with a sigmoidal shape was used: Y=Bottom + (Top-Bottom)/(1+10^five[(LogEC50-X)\*HillSlope]), where EC50 denoted the concentration of the agonist that gave a response that was halfway between Bottom and Top. HillSlope described the steepness of the curve, and the Top and Bottom denoted plateaus in the units of the Y-axis. Lower and upper bound of a 95% confidence interval for EC50 was calculated.

Concentration of HU or GEM in combination HU+CHK1i or GEM+CHK1i with the same effect (EC50) as HU or GEM alone and EC50 ratio (EC50 of HU or GEM alone / EIC50 of HU or GEM in combination) were calculated for all available concentrations (see supplementary tables S1 and S2). Comparison of the best-fit model for HU+MU380 with the model for HU+SCH900776 was also assessed. The most effective concentration were selected and shown including the P-value from a comparison of the best-fit model for HU+CHK1i or GEM+CHK1i with the model with constraining the EC50 to equal the EC50 of the model for HU or GEM alone.

For microscopy, recovery time in different time points (0, 6, 24 and 48 hours) and for different treatments (DMSO, GEM, MU380, SCH900776, GEM+MU380 , GEM+SCH900776) were summarized using mean and SD. Comparison of recovery time among different time points or treatments were assessed with two independent one-way ANOVA tests followed by Tukey HSD post-hoc tests. One-way ANOVA was preferred against repeated measures ANOVA, since measurements were independent (values were not measured in the same sample in time). Results of post-hoc tests were described using homogeneous groups, where different letter means statistically significant difference between groups.

The level of statistical significance in all analyses was α=0.05. All alternative hypotheses were two-sided. Analyses were performed using IBM SPSS Statistics 23 (IBM Corporation, Armonk, NY, USA), Statistica for Windows 12 (StatSoft, Dell Inc., USA) or GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

**Caco-2 permeability drug assay**

For the transport experiments, the Caco-2 cell monolayers was cultured on filter inserts in 24-well plates (1 μm pore size, PET filter, translucent, Falcon or Millipore / Merck). 0.8 mL of culture medium was added to the basolateral compartments and complemented with cells seeded on the inserts at a density of 3 × 105 cm2 in volume of 0.3 mL. The cell monolayers were cultured for 3 weeks, and the medium was refreshed in both compartments three times a week. On the day of experiment, the monolayers were washed twice with the transport buffer (HBSS + 25 mM HEPES buffer, pH 7.4, warmed to 37 °C). Tested compounds (MU380 and SCH900776 ) were dissolved at 4 μmol/L in transport buffer and at the time 0 applied either in apical compartments (in volume 0.3 mL) or in basolateral compartments (in volume 0.8 mL). The blank transport medium was added to complementary inserts / wells. Plates were covered and incubated for 120 min at 37°C in humidified incubators. After withdrawal of samples, the integrities of the monolayers were checked by monitoring the transport of Lucifer Yellow (Sigma-Aldrich, Prague, Czech Republic). All monolayers were washed with transport buffer and again transport buffer containing 100 μmol/L LY solution was added to the apical side. After 1 h, samples were taken from both compartments, and fluorescence was measured using a Fluostar Galaxy reader at 480 / 540 nm (BMG Labtech, Ortenberg, Germany). Lucifer yellow rejection was calculated and only monolayers with rejection between 97 and 100% were used for transport evaluation. Samples for tested solutions were analysed by liquid chromatography with tandem mass spectrometry (LC/MS).

Calculations

The apparent permeabilities Papp (cm/s) for transport from apical to basolateral compartment (Papp (A🡪B)) and from basolateral to apical compartment (Papp (B🡪A))were calculated with the equation:

where S is membrane surface area (cm2), C0 is donor concentration at the beginning of the experiment (μmol/L), dQ/dt is amount of drug transported per time (mol/s), where dQ = c receiver (μmol/L) x V receiver (cm3). Data are presented as the average Papp (cm/s) x 10-6 ± S.D. from three monolayers and three independent experiments. Efflux ratio values were calculated as (Papp (B🡪A)) / (Papp (A🡪B)). Mass balance was calculated with the following equation.

,

where C0 is the initial concentration in the donor solution, Vr is the volume of the receiver compartment, cr is the concentration detected in receiver compartment, Vd is the volume of the donor compartment, Cd,final is the donor concentration at the end of the incubation.

**Real – time cell analysis**

Acea 96-well E-plates® and an xCELLigence real-time cell analysis (RTCA) SP system including RTCA Software v1.2 (both Roche) were used to monitor dynamics of cytotoxic effects of drugs and combinations by measuring the electrical impedance across microelectrodes integrated on the bottom of its special tissue culture plates. First, a standard background measurement was performed using complete culture medium. Then, cells were seeded directly into the wells in volume 150 μL (in multiplicates of 3 wells for each cell line). After 24 hours HU was added. The CHK1 inhibitors were added 48 hours after seeding and subsequently washed out after 2 hours. All drugs were used in 10 μL volume per well. The cell index (normalized electrical impedance) was monitored continually to obtain maximal value (plateau) of the control. HeLa.S-Fucci cells were seeded on IBIDI µ-Slide and treated with vehicle (DMSO), GEM (0,5 μmol/L), CHK1 inhibitor (MU380 and SCH900776, 0,5 μmol/L) or their combination. Movies (3 per group) were acquired at frame rate of one image every 15 minutes for total length of 96 hours using Olympus CM FV10i- LIV (37°C, 5% CO2). TrackMate plugin inside FIJI open source software package (Schindelin et al., 2012, DOI: 10.1016/j.ymeth.2016.09.016; Tinevez et al., 2016, DOI: 10.1016/j.ymeth.2016.09.016) was used to determine time of division and progression of individual cells through different stages of cell cycle. We used semi-automated mode of object detection and linking. Times between cell divisions were extracted from obtained tracks and median value for each treatment was calculated. Intensity in eGFP/mKusabira-Orange channel for each nuclei was measured. Mean values were exported and used for further analysis in open source language for statistical computing R (https://cran.r-project.org/). Length of cell phases G1 and S-G2 were calculated for individual cells (Marcus et al., 2015, DOI: 10.1038/srep14391). G1 phase was considered to start right after division and ends at the peak of mKusabira-Orange intensity. S-G2 phase ends with next cell division/or cell death. Final graphs were plotted using ggplot2 package (Wickham, 2009, DOI: 10.1007/978-0-387-98141-3).

**CHK1 knockdown**

We established stable CHK1 knockdown U2OS cells. shRNA from Sigma-Aldrich (Sigma MISSION shRNA DNA Clone, TRCN0000000500, CCGGCGCAGTGAAGATTGTAGATATCTCGAGATATCTACAATCTTCACTGCGTTTTT) cloned in PLKO.1-PURO plasmid targeting CHK1 was transfected together with lentiviral packaging plasmids pMD2.G and psPAX2 into HEK 293T cells using PEI (1:3 DNA: PEI) and 5% NaCl. Virus supernatant were collected each 24hours for 3 days and concentrated. Concentrated lentiviruses were mixed with polybrene to infect U2OS cells. 72 hours after post transfection the cells were put on puromycin (1ug/mL) selection for 15 days. Thereafter, cells were harvested and confirmed for knockdown by western blot and RT-PCR.

**Mutation status of tumor suppressor genes in cells (Table S1)**

Available data for tumor suppressor status of cell lines used in the screen was extracted from Sonkin et al., 2013 based on Cancer Cell Line Encyclopedia (CCLE) data. Sonkin et al., made a comprehensive list of systematically derived status of 69 known or putative tumor suppressors, across 799 samples of the Cancer Cell Line Encyclopedia ( Sonkin et al. 2013, NIHMS469553-supplement-04). The tumor suppressor status was divided into 10 categories (See for detailed description Sonkin et al. 2013):

|  |
| --- |
| G-M - Homozygous nonsense, frame shift, known loss of function missense mutation or dominant negative mutation. |
| G-D - Deletion of both alleles { CN ratio < 0.25 } |
| E-G-D - Deletion of one allele. {CN ratio < 0.6} |
| E-G-M - Heterozygous nonsense, frame shift or known loss of function missense mutation. |
| E - No expression. |
| E-LOH - No expression and LOH. |
| { (expression < 32) AND (average expression > 100) AND (median expression > 100) } |
| WT-E - No non-synonymous mutations, no CN loss (CN ratio > 0.9), no LOH and expression >= 300 |
| WT - No non-synonymous mutations, no CN loss (CN ratio > 0.9), no LOH |
| Category 0 ( catch-all ) is defined for cases which do not qualify for categories: |
| 0-M - Heterozygous nonsense, frame shift or known loss of function missense mutation. |
| 0-D - Deletion of one allele {CN ratio < 0.6} |

We categorized these into fewer categories, wild type- WT, no category - 0, mutation / deletion - no loss of mRNA expression - 0-M/D, homozygous mutation / deletion - M/D, loss of mRNA expression - no mutation / deletion - E, loss of mRNA expression / heterozygous mutation or deletion - E-M/D.

|  |  |
| --- | --- |
| Our categories | Sonkin et al |
| WT | WT, WT-E |
| 0 | Category 0 |
| 0-M/D | 0-M, 0-D |
| M/D | G-D, G-M |
| E | E, E-LOH |
| E-M/D | E-G-D, E-G-M |

**Fluorescent drug retention assay and index determination**

Fluorescent probes JC-1 (Thermo Fisher Scientific, T3168) and mitoxantrone (MTX, Sigma-Aldrich, M6545) were used as a substrates for flow cytometric determination of drug retention indexes (DRI). Fumitremorgin C (FTC, Sigma-Aldrich, F9054) was used for ABCG2 inhibition. Tested cell lines (600 000 cells/sample) were incubated in their complete culture medium with JC-1 (0,1 μmol/L) or MTX (15 μmol/L) in absence or presence of FTC (10 μmol/L) for 1 hour at 37 °C. After incubation, cells were washed by PBS and fed with substrate-free media, and cultured 30 minutes at 37 °C, again in absence or presence of FTC, to evaluate its effect on substrate retention. The samples were analysed by flow cytometer BD FACS Verse (JC-1: laser 488 nm, BP filter 586/42 nm, MTX: laser 640 nm, BP filter 660/10 nm) and evaluated by FlowJo (v7.6.5, Tree Star). Analysis of cell autofluorescence was performed for appropriate setting of the gates. Cell doublets, aggregates and debris were excluded from analysis based on a dual-parameter dot plot in which the pulse ratio (signal area/signal high; y-axis) versus signal area (x-axis) was displayed. All analyses were performed in duplicate in three separate experiments, and the results were expressed as the medians of fluorescence intensity (MFI). DRIs were calculated as DRI = MFI (without FTC) / MFI (with FTC). Cells with no ABCG2-dependent efflux of the substrate have DRI=1, on the other hand cells with high ABCG2-dependent efflux have DRI close to 0.5.

**Thermodynamic Solubility**

The thermodynamic solubility of MU380 and SCH900776 was determined by Cyprotex services. Briefly, 2.5 mg of compounds are weighed and dissolved in 0.5 mL PBS (pH 7.4) by overnight agitation. Samples are then filtered and quantified using gradient HPLC-UV.

**Cytochrome P450 inhibition (standard 5 isoform IC50 determination**

The cytochrome P450 inhibition profile was done by Cyprotex services. Briefly, the compounds were incubated with human liver microsomes and NADPH in presence of cytochrome P450 isoform-specific (CYP1A, CYP2C9, CYP2C19, CYP2D6, CYP3A4) probe substrate. The metabolites were monitored by LC-MS/MS or fluorescence and a decrease in the formation of metabolite compared to the vehicle control was used to calculate IC50 value.

**Microsomal metabolic stability**

The microsomal metabolic stability profile of MU380 and SCH900776 was determined by Cyprotex services. Briefly, 3 μmol/L of each compound was incubated with pooled liver microsomes at 5 time points (0,5,15,30,45 min) and the compounds were analysed by LC-MS/MS.

**Plasma protein binding**

Mouse plasma protein binding of MU380 and SCH900776 was profiled by Cyprotex services. Briefly, 5μmol/L of compounds were prepared in 100% species-specific plasma and buffer (pH 7.4) and each added to either side of the equilibrium dialysis system. After the system reached equilibrium, compound concentration on both sides of the membrane was measured by LC-MS/MS and the fraction of unbound (fu) compound was calculated.