Supplimentary Methods:

Method for the preparation and analysis of AZD8055, AZD6244 and BEZ235 by LC-MS/MS

Calibration standards

Calibration and quality control solutions were prepared in DMSO from individual 2mM stocks for AZD8055, AZD6244 and BEZ2235. Calibration standards used for spiking were prepared over the dynamic range 20-100000 nM by serial dilution of the calibration stock solution with DMSO to give final concentrations of 2-10000 nM. Quality Control (QC) standards were also prepared at 250, 2500, 7500 and 25000 nM by serial dilution of the quality control stock solution with DMSO to give a final concentration of 25, 250, 750 and 2500nM. A stock solution of Olomoucine internal standard (IS) was prepared in DMSO at a concentration of 1 mM. A working I.S. solution was prepared at a concentration of 250 nM in methanol.

Plasma and tissues sample preparation

Plasma and tissue samples were thawed on ice. All tissues were homogenised in 3 mL/g PBS and kept on ice. 100 µl aliquots of untreated mouse plasma or tissue homogenates were spiked with 10 µl of the appropriate calibration or QC standard solutions. 100 µl aliquots of the unknown samples were spiked with 10 µl DMSO. Where appropriate, unknown plasma samples were diluted with untreated plasma.

Protein precipitation

Spiked calibration standards, QCs and unknown samples were protein precipitated with 300 µL methanol containing 250 nM IS. Blank samples were prepared by spiking 100 µl untreated plasma/tissue with 10 µl DMSO and protein precipitated with 300 µl methanol. All samples were centrifuged at 3700 rpm for 20 minutes at 4°C. Supernatants were transferred into a new 96 well plate and subsequently analysed by LC/MS/MS.

Dried Blood Spots

Blood is taken from dosed mice (IV and PO) at 15mins, 40mins, 1hr, 2hr and 6hrs. 20 µL blood is spiked onto Whatman FTA DMPK-B cards (GE Healthcare, Bucks, UK) and samples are left to dry for at least 6hrs. Standard curve and QC stocks solutions are made up in 50:50 DMSO/water. In a 96 well greiner plate standards/QC's were diluted by the addition of 50 µL blank mouse blood to 2.5 µL stock solution. The plate was mixed and left for 30 minutes. 20 µL standard curve and QC blood was then spiked onto Whatman FTA DMPK-B card and left to dry for at least 6hours. Samples, standard curves and QC's are then punched from the Whatman cards with Harris unicore 6mm punch (GE Healthcare, Bucks, UK), and placed in appropriate wells in a 1mL 96 well plate. 200 µL methanol containing internal standard is added before vortex mixing for 10 minutes. The plate is then centrifuged at 3700rpm at 4°C for 5mins. 40 µL of this is then transferred to a clean 96 well plate with the addition of 40 µL water to improve chromatographic peak shape.

LC/MS/MS Method

Extracted plasma and tissue samples were analysed by LC-MS/MS for the quantification of AZD6244, AZD8055, BEZ235 and Olomoucine (Sigma, Dorset, UK) as internal standard using an Agilent 1290 LC system coupled with a 6410 triple mass spectrometer (Agilent, Berkshire, UK). Compounds were separated on a Kinetex C18, 2.6µ 50x2.1mm analytical column (Phenomenex, Macclesfield, UK) with a 10µL injection volume. The mobile phase consisted of Methanol and 0.1% Formic Acid with a constant flow rate of 0.5mL/min at 55ºC. A linear gradient was initiated from the 10% methanol/90% formic acid starting conditions over 5 minutes before sustaining at 100% organic for 1 minute. The column was then re-equilibrated with the original mobile phase for a further 2 minutes.

Analytes were ionised by electrospray interface (ESI) in positive mode and detected by multiple reaction monitoring (MRM). The characteristic ion transitions monitored were m/z 466.6-450.3 for AZD8055, 314.2-116.9 for AZD6244, 470.2-454.1 for BEZ235 and 299.1-176.9 for Olomoucine. Ionisation was optimised with a gas temperature of 300°C and flow rate of 12 L/min. The nebulizer pressure was set to 40 psi with the capillary charge at 4000 Volts. Fragmentation in the presence of Argon was achieved using 240, 147, 200 and 140 Volts for AZD6244, AZD8055, BEZ235 and Olomoucine respectively with a collision energy of 50, 17, 50 and 30V. Data acquisition was performed using MassHunter software version B.01.03. The assay was linear over the range 2-10,000nM.

Drug-drug interaction assay

One of the major causes of Drug-Drug Interactions is the inhibition of the liver Cytochromes P450 (CYP). To measure CYP inhibition the rate of formation of metabolites from probe substrates, selectively catalysed by individual CYP isozymes in a microsomal incubation, are monitored. Any change in the rate of reaction on introduction of a compound into the incubation indicates the inhibitory potential of that compound. Compounds with known selective CYP inhibition identified from literature reviews (referred to as 'known inhibitors') are used as positive controls in the assay.

Compounds were incubated with 0.5mg.ml^{-1} human liver microsomes (Tebu-bio, Cambs., UK) at 1µM, 10µM and 50µM. Inhibition of CYP isozymes (Sigma, Dorset, UK) was determined using a mixture of probe substrates consisting of Phenacetin (CYP1A2), Coumarin (CYP2A6), Tolbutamide (CYP2C9), Mephenytoin (CYP2C19), Bufuralol (CYP2D6) and Midazolam (CYP3A4). The samples were incubated for 10 minutes followed by protein precipitation with methanol. The substrate metabolites in each sample were measured by LC/MS using an Acquity UPLC (Waters, Herts., UK) coupled with a QTRAP 4000 ion trap quadrupole mass spectrometer (AB Sciex, Cheshire, UK). 10µL of reconstituted extract was injected and the compounds were seperated on an Acquity BEH C18 column 1.7µ, 50 x 2.1mm (Waters, herts,. UK). The mobile phase consisted of 0.1% formic acid and methanol with a flow rate of 0.6 mL/min at 50°C. A linear gradient to 100% organic over 2 minutes was achieved from an initial 100% formic acid phase. This was sustained for one minute before returning to the starting conditions by 4 minutes and re-equilibriating with the original mobile phase for a further minute. Analyte detection was achieved with electrospray ionisation in positive mode by multiple reaction monitoring mode (MRM) for all six analytes. The source temperature was set at 550°C with an ion spray voltage of 3000 V. Medium collision gas and optimal curtain gas pressure was maintained at

25 psi. The exit potential was optimised at 10 V. Data acquisition was performed using Analyst 1.5.1 software.

1. Moreno-Farre J, Workman P, Raynaud FI. Analysis of potential drug-drug interactions for anticancer agents in human liver microsomes by high throughput liquid chromatography/mass spectrometry assay. Recent Advances and Research Updates 7,207-224 (2006)