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An investigation into the biotransformation and enzymology of a methyl-imidazole containing cyclin dependent kinase inhibitor

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Abstract

The cyclin dependent kinase inhibitor AZD5438 was in development by AstraZeneca for treatment of solid tumours where it showed a pattern of emerging toxicity loosely correlated with exposure during clinical trials in patients. Metabolites identified from clinical and preclinical studies recognised the possibility of reactive metabolite formation from AZD5438, possibly from an identified Phase II sulfate metabolite which could react via a carbocation intermediate. This research project used an array of *in vitro* techniques to investigate the reactive metabolite formation potential of AZD5438. The sulfate metabolite of interest and its precursor, a hydroxyl metabolite, were detected in clinical plasma samples. Both metabolites were also produced by *in vitro* incubations with human hepatocytes and active uptake of [^{14}C]-AZD5438 into hepatocytes was observed. Optimisation of an *in vitro* system to selectively produce the sulfate metabolite of interest was unsuccessful. *In vitro* assays with human liver microsomes and human hepatocytes indicated that CYP2C9 and CYP3A4/5 were responsible for the production of the covalently bound metabolite of AZD5438. Binding values showed a negative trend as the complexity of the metabolic system increased, resulting in binding of 124.95, 57.10, 29.26, 5.14 pmol equivalents per mg protein for incubation of [^{14}C]-AZD5438 (10 μM) to human liver microsomes, human liver microsomes with uridine diphosphate glucuronic acid (UDPGA), human liver S9 fraction and fresh human hepatocytes, respectively. The sulfate metabolite of interest was therefore, unlikely to be the source of reactive metabolite formation. *In vitro* toxicity assays with an endothelial cell line (THLE-CYP) showed parent AZD5438 could cause cell death, although whether further toxicity due to reactive metabolite formation occurred was not determined. Extrapolation of the microsomal covalent binding data using literature calculations could not conclusively evaluate risk of drug induced liver injury. However, the same calculation using values from the hepatocyte incubations predicted a lower comparative risk.

Acknowledgements

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Contents

Introduction

Adverse drug reactions (ADRs) are a considerable cause of hospital admissions in developed countries and are a common cause for drug withdrawals post-registration and black box warnings printed on prescription drug packaging (required by regulatory authorities where serious or clinically significant adverse events have been identified) (Food and Drug Administration, 2006; Lassar, 2002; Pirmohamed, 2004). ADRs also play a major part in the attrition of new chemical entities (NCEs) during pharmaceutical development and due to their nature, are often only observed towards the latter stages of the development process where many millions of research pounds have already been invested (Evans, 2004). The causes of ADRs are multiple and may be due to the formation of so-called reactive metabolites – a metabolic product that is itself chemically reactive and is often unstable and capable of forming covalent bonds with molecules present in a biological system.

The metabolism of xenobiotics within an organism is a vital process to promote detoxification and/or excretion of potentially harmful substances (Maurel, 1996), with compounds undergoing biotransformation to more polar or easily excretable entities to be expelled via gaseous exchange, urine or faeces (Maurel, 1996). In mammalian enzyme catalysed systems these processes are roughly grouped into two categories; Phase I 'functionalisation' reactions (including oxidation, reduction, hydrolysis, cyclisation, decyclisation) and Phase II 'conjugation' reactions (including glucuronidation, sulfation, acetylation, glucosylation, glutathionation) (Maurel, 1996).

The most important metabolic enzymes responsible for biotransformation of xenobiotics reside in their highest concentrations within the various organelles and cellular compartments of the hepatocyte (Eddershaw & Dickens, 1999; Guengerich, 1989). Cytochromes P450 (CYPs), flavin containing monooxygenases (FMOs), epoxide hydroxylases and urindine diphosphate (UDP) glucuronosyltransferases are all present in hepatic microsomal fractions (vesicular compartments of smooth

endoplasmic reticulum purified from liver tissue by mechanical homogenation and fractional centrifugation) (Sharer, 2003). The cytosolic fraction of hepatocytes contains important biotransformation enzymes such as alcohol and aldehyde dehydrogenases, sulfotransferases and glutathione-s-transferases. All of these metabolic enzymes are present in whole fresh or primary hepatocytes along with the co-factors needed to allow a good model of *in vivo* hepatic metabolism to take place. Many of these enzymes are also available off-the-shelf as purified recombinant protein produced using an insect cell line transfected with a baculovirus which codes for a specific human protein. Incubation of a test compound with any of these systems in the appropriate conditions (temperature, co-factor, pH) can provide fundamental information about its metabolism (Evans, 2004).

The fundamental purpose of detoxification by this system can backfire, however with metabolic enzymes producing metabolites that pose a greater toxicological risk to the organism than the parent substance itself. This risk can be due to a pharmacological response (similar or differing to that of the parent compound) or by bioactivation which results in a chemical interaction between the metabolite and cellular macromolecules (Knowles, 2000). This latter reaction can result in decreased function of cellular macromolecules (e.g. proteins and nucleic acids) and a possible immune mediated adverse event (Uetrecht, 2008). Reactions such as this cannot be predicted from the pharmacology of the compound and so are known as idiosyncratic drug reactions (Uetrecht, 2007).

Paracetamol, diclofenac and tamoxifen (Figure 1, Figure 2, Figure 3) are well known examples of drugs that produce reactive metabolites (Hargus, 1994; Kim S. , 2000; Kretz-Rommel & Boelsterli, 1993; Mitchell J. , 1973). The analgesic paracetamol undergoes metabolism via cytochromes P450 (CYP) 2E1 and 3A4 to form N-acetyl-*p*-benzoquinoneimine (NAPQI), an electrophilic product which, at low concentrations is ‘mopped up’ by cellular glutathione (GSH) and excreted as NAPQI-glutathione. In cases of overdose however, GSH is rapidly depleted as NAPQI is formed leaving the

excess metabolite to react with proteins and nucleic acids ultimately resulting in cell death (Mitchell J. , 1973).

Diclofenac is an anti-inflammatory that is metabolised to an acyl glucuronide conjugate (diclofenac-1-*O*-acyl glucuronide) by uridine glucuronyltransferase (UGT) 2B7. Acyl glucuronides can react with cellular macromolecules either by a transacylation reaction with the metabolite or via a glycation reaction following acyl migration on the metabolite (Hargus, 1994; Kretz-Rommel & Boelsterli, 1993).

The breast cancer treatment tamoxifen is predominantly hydroxylated to α-hydroxy-tamoxifen and then glucuronidated prior to excretion. An alternative sulfotransferase (SULT) mediated pathway is also present where α-hydroxy-tamoxifen undergoes conjugation to form tamoxifen *O*-sulfate, the sulfate moiety of which can act as a leaving group with the resulting electrophilic cation binding covalently to nucleic acids (Kim S. , 2000).

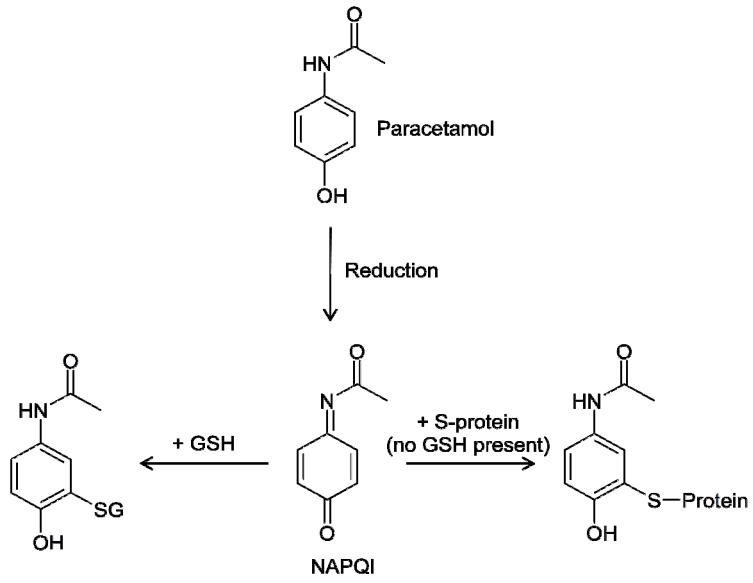


Figure 1: Production of a paracetamol reactive metabolite

N-acetyl-*p*-benzoqunioneimine (NAPQI), formed via metabolism of paracetamol, can covalently bind to protein in the absence of cellular glutathione (GSH).

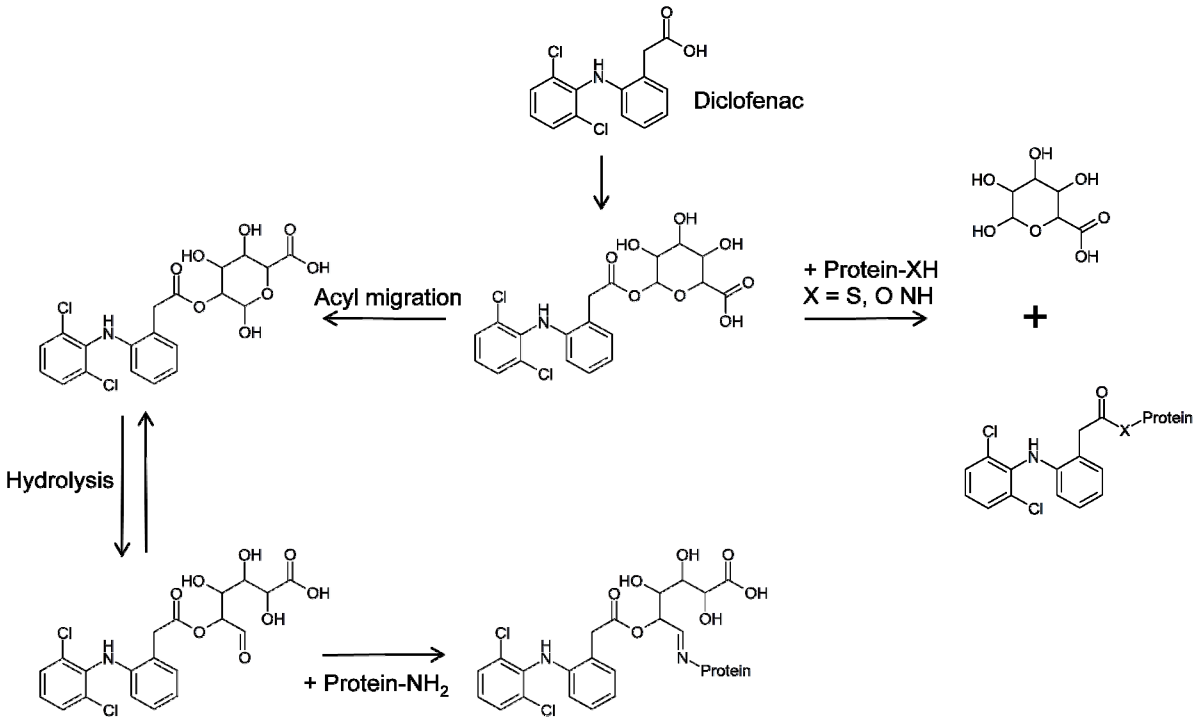


Figure 2: Production of diclofenac reactive metabolites

Conjugation of diclofenac with a glucuronide moiety via Phase II metabolism, can lead to two types of reactive metabolite formation. Firstly the ester group can be attacked by a nucleophilic protein group to reform glucuronic acid and covalently bind diclofenac to the protein. Alternatively, the glucuronide moiety can undergo acyl migration followed by ring-opening hydrolysis and subsequent attack by the lone pair on the primary amine group of an amino acid to form a covalently bound species.

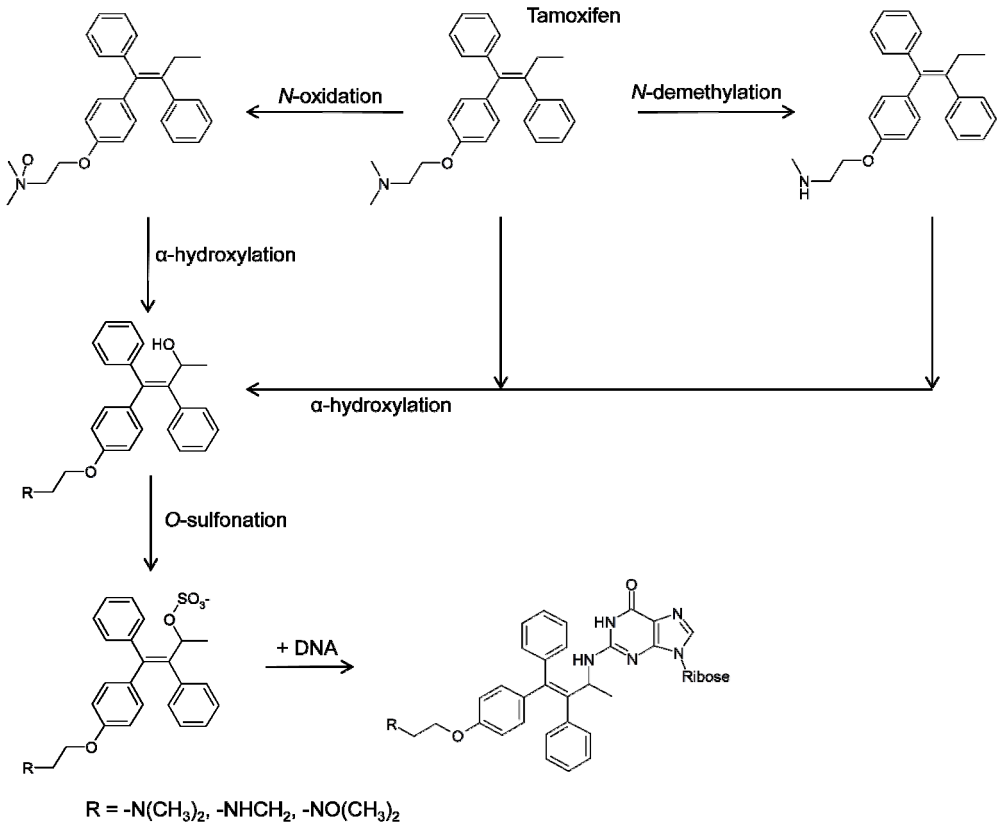


Figure 3: Production of a tamoxifen reactive metabolite

Hydroxylation of tamoxifen (or two of its primary metabolites) form a metabolite which then becomes a substrate for Phase II enzymes resulting in a conjugated sulfate form. The sulfate moiety then acts as a leaving group when attacked by a guanine residue, and tamoxifen becomes covalently bound to the DNA strand. Unlike many examples of reactive metabolite formation, this is the desired outcome of the drug.

AZD5438 (Figure 4) is an AstraZeneca discovered compound that entered the development phase of the drug production process with the aim of treating several late stage cancer types through the means of cyclin dependent kinase (CDK) inhibition.

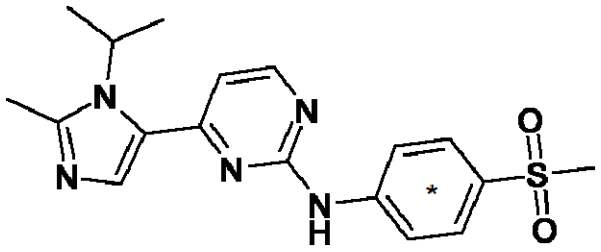


Figure 4: Structure of [^{14}C]-AZD5438

Kinases are a large family of enzymes which catalyse the transfer of a phosphate group from a co-factor donor molecule (for example adenosine triphosphate (ATP)) to a specific substrate, thus altering the latter's function, location or activity. The human genome contains greater than 500 protein kinase genes which are split into numerous groups and have various levels of positive and negative control over a vast range of cellular processes (Cicenas, 2011).

Cyclin-dependent kinases (CDKs) are one such subgroup (21 genes encoding CDKs have been identified in the human genome) of small molecular weight proteins (34 – 40 kDa) which are heavily involved in the mitotic cell cycle as well as other cellular functions such as transcription, DNA repair and post-translational protein modification (Cicenas, 2011, Harper & Adams, 2001). In order to fulfill their duties, CDKs must associate with certain proteins, cyclins, to form active complexes. The monomeric form of the CDK is inactive and binding to a specific cyclin will alter the conformation of the CDK structure to yield a specific dimer complex capable of performing a particular function (Morris, 2002). The level of CDKs in their monomeric form is fairly constant within the cell and so it tends to be regulation of the cyclins (by synthesis and degradation) which controls the activity of the complex (Pines, 1991). A single CDK may have multiple potential cyclin partner types with which it can form complexes to exert different effects, with the reverse also true.

In the mitotic cell cycle, active CDKs act as regulators for progression between the various stages (Figure 5).

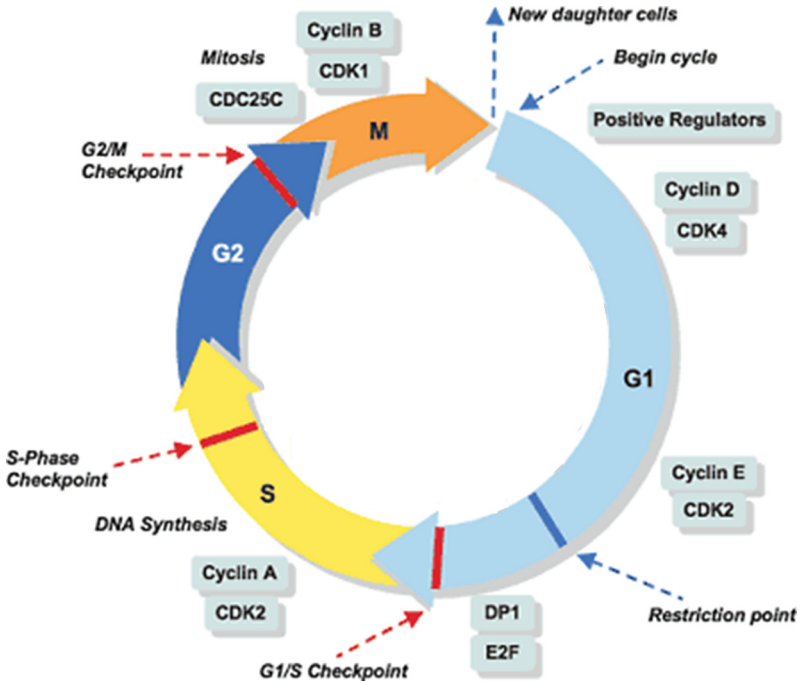


Figure 5: Cell cycle of a mitotically dividing cell with examples of active CDK-cyclin complexes acting as promoters at various stages of the mitotic cell cycle.

The G1 (Gap 1) Phase involves protein synthesis and cell growth prior to DNA synthesis. The S (Synthesis) Phase encompasses the replication of DNA. The G2 (Gap 2) Phase involves further protein synthesis and cell growth, prior to mitosis. The M (Mitosis) Phase is the division of the cell into the daughter cells. The various restriction points and checkpoints throughout the cycle are policed by CDK complexes, the phosphorylation activity of which is required to proceed to the next Phase. CDK inhibition by signal proteins released for example due to DNA damage, would result in immediate halting of the cycle and possible downstream apoptosis of the cell. Cartoon is a partial and altered figure from Kong, 2003.

The 'gatekeeping' role that CDKs inhabit between the various stages of cell division means that should deregulation of these proteins occur, cells will proliferate in an uncontrolled manner and this has been repeatedly associated with cancer growth. For example, CDK2 levels have been used in prognosis for breast cancer (Kim, 2008) and CDK5 in lung cancer (Liu, 2010).

The increased activity of CDKs in cancer cells, therefore suggests a possible target for cancer treatments. Small molecule or protein based inhibitors of CDKs should halt the cell cycle and therefore decrease cell proliferation and / or cause apoptotic death in target cells.

Sequencing of human CDKs has shown an homology of residues around the ATP binding domain and so the design or discovery of clinically useful inhibitors has centred on molecular structures capable of reversibly blocking the binding of ATP to one or more isoforms of CDK enzyme (Hardcastle, 2002). Prior to the discovery of AZD5438 by AstraZeneca, the first CDK inhibitor to enter clinical trials was Flavopiridol (Figure 6).

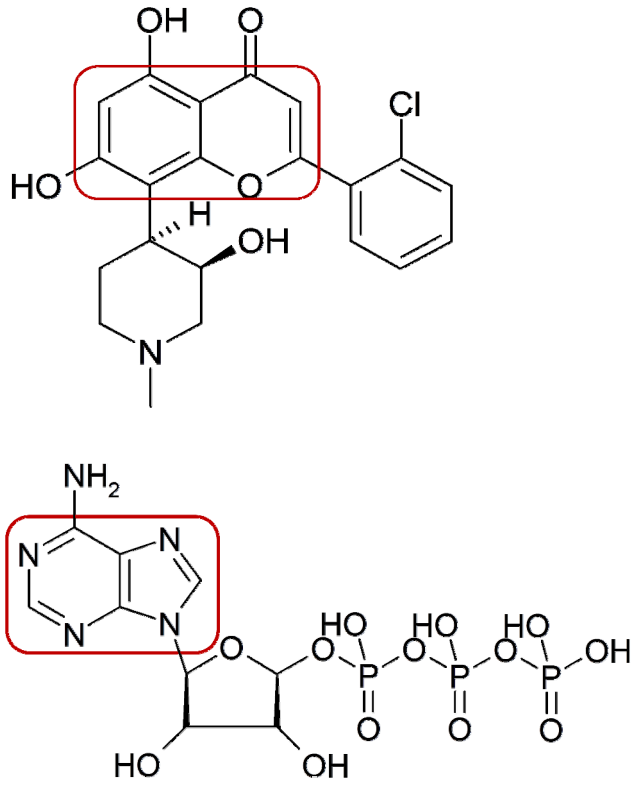


Figure 6: Structure of Flavopiridol and adenosine triphosphate (ATP)

The highlighted ring-structures are the respective pharmacophorse for binding to the ATP binding site of CDKs.

Originally identified as a potential cancer therapy acting as an epidermal growth factor receptor (EGFR) inhibitor, it was later shown to be a sub-μM inhibitor of CDK1, CDK2, CDK4 and CDK7 (Senderowicz, 1999). X-ray crystallographic analysis of the drug-enzyme complex indicated that Flavopiridol did bind to the ATP binding site of CDK2, with the benzopyran moiety mimicking the adenine of the natural substrate (Figure 6; De Azevedo, 1995). The most recent trials of this compound have shown clinical activity in a Phase II study in patients with chronic lymphocytic leukemia. The

adverse effects most commonly suffered by patients in this trial were nausea, vomiting, diarrhea, and blood disturbances such as neutropenia, anemia and thrombocytopenia (Lin, 2009). These were unsurprising for a compound known to inhibit cell proliferation, acting most agressively on quickly dividing cell types.

AZD5438 is an orally active CDK inhibitor, with a greatest potency against the CDK2-cyclin A complex. It also inhibits CDK1 and CDK5. It was postulated that due to its increased target specificity over compounds such as Flavopiridol, AZD5438 may have had an advantageous toxicity profile. However, Phase I clinical studies investigating AZD5438 in human cancer patients did reveal a serious emerging toxicity pattern in certain individuals that loosely correlated with increasing exposure. This, combined with variable clinical exposure that even at tolerated doses did not approach the levels required pre-clinically for efficacy, resulted in the decision to stop clinical development of this compound. It was theorised that metabolism of AZD5438 may play a part in the toxicity profile observed.

A range of Phase I and Phase II metabolites of AZD5438 were identified in *in vitro* studies performed using fresh hepatocytes in suspension from human and preclinical species. *In vivo* metabolism studies undertaken on AZD5438 in rat and dog hepatocytes have revealed both glutathione and mercapturic acid (a downstream breakdown product of GSH conjugation) conjugate metabolites, as well as a similar variety of metabolites to those observed in *in vitro* experiments. The position of the conjugation with mercapturic acid gave a possible clue to the form of the potential reactive metabolite.

The major Phase I metabolite formed *in vitro* in humans is hydroxy-AZD5438 (labelled P19 in previous studies), on where the site of hydroxylation is the same as that where the mercapturic acid motif is seen. Some conjugation of P19 with a sulfate moiety (via addition to the –OH of P19, Figure 7) was observed in preclinical studies and inspection of the structure of this sulfate metabolite suggests conjugation of the ring system could allow the sulfate moiety to act as a leaving group (Figure 8).

This sulfate metabolite would be expected to be present in most *in vitro* and *in vivo* studies, both preclinical and clinical, in which the appropriate metabolising enzymes were present. This was not the case. However, if this metabolite is reactive and as a result binds covalently to incubation or physiological protein, then detection of the metabolite subsequent to protein removal during sample preparation would be expectedly reduced.

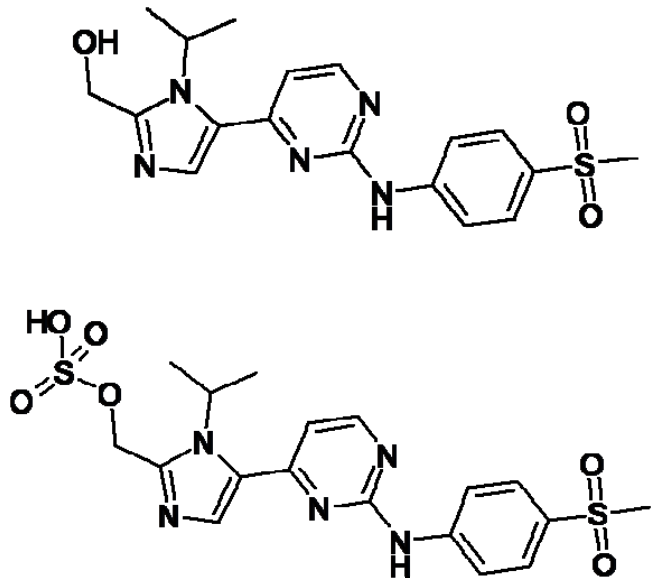


Figure 7: The major Phase I metabolite of AZD5438 (P19) and the subsequent sulfonation product of interest.

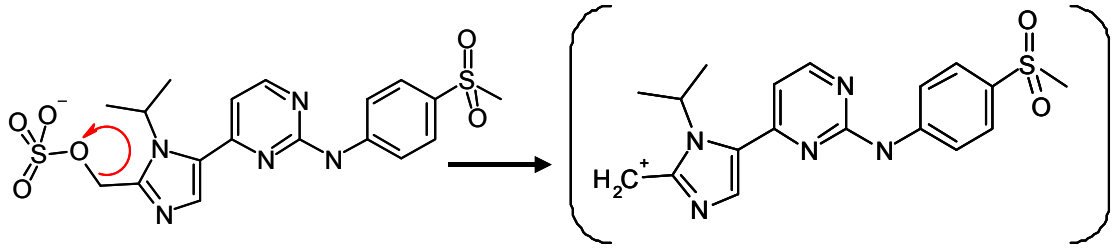


Figure 8: The postulated loss of the sulfate group to produce the reactive carbocation metabolite.

Loss of the SO_4^{2-} to produce the carbocation as shown is an unlikely even compared to the alternative of a nucleophilic attack by, for example, the lone pair of electrons of an SH from a cysteine residue of a cell protein. This would result in the irreversible covalent binding of AZD5438 to the protein via a SN2 type reaction

There is a known disconnect between current *in vitro* assessment of reactive metabolites and the situation in the clinic, with drugs shown to produce reactive metabolites that bind to proteins in the test tube having no observable effect in patients (Evans, 2004; Kretz-Rommel & Boelsterli, 1993). This may be due to the vast complexity of the whole body system when compared to the *in vitro* assay (which will have a greater range of detoxifying and metabolic processes available). Several useful *in vitro* assays have been developed to uncover any reactive metabolite formation and to quantify the possible reactivity of those produced. These include covalent binding and metabolite trapping assays.

A covalent binding assay involves using radiolabelled test compound to assess the total amount of reactive metabolite formation with no regard as to what species are being formed. The quantitative nature of this assay allows scaling to an *in vivo* situation (Evans, 2004; Day, 2005; Obach, 2009; Obach, 2008; Usui, 2009).

A metabolite trapping assay uses high concentrations of electrophile trapping agents (e.g. glutathione, cyanide) added to an incubation that promotes the capture of any reactive metabolites which can then be separated and the structure investigated by techniques such as high performance liquid chromatography coupled with mass spectrometry (HPLC-MS^n) (Baille, 1993).

Metabolic toxicology assays are also available for assessing more definite toxic effects of test drugs. Simple measures such as hepatocyte viability (live hepatocytes divided by total hepatocytes as assessed by trypan blue exclusion), adenosine triphosphate (ATP) depletion (Ponsoda, 1991), a lactate dehydrogenase (LDH) leakage (Bort, 1999) or an MTS assay (containing (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) measuring reductase activity as a surrogate of cell viability) (Dambach, 2005) can give useful information linking data from a covalent binding assay for example, to an *in vivo* study. One alternative cell based assay utilises T antigen immortalised human liver epithelial cells (THLE) which have been transfected with a gene for specific drug metabolising enzymes. This allows cell toxicity of a compound to be assessed while maintaining control of metabolic pathways. THLE-CYP cells have been used to study metabolism (Molden, 2000) and identified as useful tools for metabolic toxicity assessment (Greer, 2010; Gebhardt, 2003). Test compound is incubated with cultured THLE-CYP cells and cell viability can be measured using a standard method (as listed above). Each type of cell contains activity for only one CYP isoform (CYP1A2, CYP2C9, CYP2D6 and CYP3A4 are available). This allows CYP mediated metabolism to be evaluated using a toxicity endpoint, with either higher cell death (due to metabolic production of a more potent toxin than parent) or lower cell death (due to metabolic detoxification of a toxic parent) occurring in cells with metabolic capability when compared to null cells.

The present work aims to answer the following:

- Are the P19 and the subsequent sulfate metabolite present in both clinical plasma and *in vitro* incubation samples?
- Can these metabolites of interest be preferentially formed by optimising *in vitro* techniques?
- Can a metabolic scheme for [^{14}C]-AZD5438, detailing both chemical species and method of formation, be established using a range of *in vitro* metabolic systems and covalent binding assays?
- Can the reactive metabolite data realised be extrapolated to assess possible clinical impact?

1. Materials and methods

1.1. Radio-labelled chemicals

[^{14}C]-AZD5438 (test compound) and [^{14}C]-clozapine (covalent binding positive control) were synthesised by Isotope Chemistry, AstraZeneca, Alderley Park, UK. [^{14}C]-AZD5438 was dissolved in ethanol to a concentration of 1.5 mM or 2 mM and a specific activity of 57.5 mCi/mmol. Radiochemical purity of the compound was >99%. [^{14}C]-clozapine (covalent binding positive control) was provided in ethanol and further diluted to a concentration of 2 mM and a specific activity of 10 mCi/mmol. [^{14}C]-7-ethoxycoumarin (metabolic viability positive control) was obtained from GE Healthcare UK Ltd and prepared in ethanol at a concentration of 6 mM and a specific activity of 15.8 mCi/mmol. All compounds were stored at -20°C until use.

1.2. Other chemicals

AZD5438 and P19 were synthesised by Pharmaceutical & Analytical R&D, AstraZeneca, Macclesfield, UK. All other compounds were of highest purity available from Sigma Aldrich, UK.

1.3. Experimental

1.3.1. *In vivo* samples

Samples of varying quality and quantity from a total of seven clinical studies were available for analysis. These were predominantly plasma samples with some urine samples also. The study selected for use was entitled 'A Phase I, Open-label, Multicentre Study to Assess the Safety, Tolerability, Pharmacodynamics and Pharmacokinetics of AZD5438 Given Orally, in 2 Dosing Schedules (4 x Daily Dosing for 28 days or 4 x Daily Dosing for 21 Consecutive Days in a 28 Day Period) in Patients with Advanced Solid Malignancies.' A total of 28 patients (18 male, 10 female) were recruited into the study, all aged 18 years or over (mean 58.3 years)

with histological or cytological confirmation of malignant tumour, for whom there was no available standard therapy. Most common primary tumours locations were colorectal (n=7), lung (n=3) and skin (n=3). Other locations of primary tumour growth included uterus, adrenal gland and head and neck.

Samples for analysis by HPLC-MSn could not be selected purely using the clinical outcome of the patients who provided the plasma, as remaining samples stored by AstraZeneca were varied in volume and quality. Consequently, nine plasma samples from Patient 21 were selected based on coverage of a range of time points and volume of sample available, along with known levels of AZD5438 present (as assessed by AstraZeneca bioanalysis reports). Patient 21 was male, Caucasian, aged 57 years and was suffering from colorectal metastases with secondary liver and lung tumours. This patient was concomitantly taking atenolol, nexium, darbepoetin and lovenox, none of which would be expected to act as precipitants for metabolic drug-drug interactions (search for 'atenolol', 'esomeprazole', 'darbepoetin' and 'heparin' as precipitant for inhibiton, induction or activation using DTDI Database, www.druginteractioninfo.org), i.e. these drugs were not expected to have an impact on the metabolism of AZD5438 by means of inhibition or induction of drug metabolising enzymes. Patient 21 was treated with a 20 mg dose on day one of the study followed by 80 mg on days 2 to 21. This regime was conducted three times in total, until the study was terminated due to the emerging safety data in this and one other study. Patient 21 was in the study for 57 days before termination. Seven patients in this study suffered one or more of the following serious adverse events; dyspnoea, vomiting, anorexia, myocardial infarction, hyponatraemia, hypoxia, pericarditis, laryngeal obstruction, vocal chord paralysis and fatigue. Two patients died following myocardial infarction and pericarditis, respectively, which were recorded by the Principal Investigator as being related to the treatment. One further patient died during the study period due to advancement of disease. Patient 21 was

not one of the patients who died in this study but specifically suffered from fever and gastrointestinal complaints.

Nine plasma samples from Patient 21 were thawed (schedule day 8; 1.5 hr, schedule day 15; 1.5 hr, schedule day 29; 0.5, 1.0, 1.5, 2, 3, 4 and 5 hr). Plasma aliquots (100 µL per sample) were extracted with three volumes of acetonitrile, blown to dryness under nitrogen, reconstituted in initial mobile phase (45 µL) and pooled. The reconstituted extract was then analysed by HPLC-MS^n (Appendix A1).

1.3.2. *In vitro* samples

General incubation conditions for all *in vitro* assays can be found in Appendix A2.

1.3.2.1. Whole hepatocyte incubations

[^{14}C]-AZD5438 was incubated with primary human hepatocytes in both suspension and culture. Human hepatocytes in suspension ($1x10^6$ viable cells /mL; 3 hours) were incubated with [^{14}C]-AZD5438 (10 µM) and terminal samples (500 µL) taken into Eppendorf tubes containing ethanol (500 µL) . Primary human hepatocytes were plated onto 12 well collagen pre-coated plates at a cell density of $1.25x10^5$ viable cells /cm^2, allowed to attached for at least 24 hours then the wells aspirated and media containing Matrigel™ (1 mL per well, 0.25 mg/mL) added. Cells were aspirated after a further 24 hours and fresh media containing [^{14}C]-AZD5438 (5 µM) added (t = 0). Sampling of cells at 0, 24 and 49 hours was conducted by addition of ethanol (500 µL per well, to lyse the cells and terminate any further metabolism) and collection into Eppendorf tubes. Hepatocyte incubation samples (suspension and culture) were centrifuged to remove cell debris prior to injection onto the HPLC system.

Analysis of all incubation samples was performed by HPLC with radiological detection and mass spec (HPLC-RAD-MS^n), (Appendix A1).

1.3.3. *In vitro* optimisation

1.3.3.1. CYP450 supersomes

[^{14}C]-AZD5438 was incubated (10 µM, 60 minutes) in the presence of heterologously expressed human CYP isoforms (100 pmol/mL). Samples were taken into an equal volume of ethanol and stored at -20°C until analysis. For analysis, samples were allowed to reach room temperature and centrifuged to remove particulates prior to analysis.

1.3.3.2. Sulfotranferases and Cytosolic extract

[^{14}C]-AZD5438 (10 µM) was incubated (60 minutes) with SULTs 1A1*2, 1A2*1, 1A3, 1E and 2A1 (50 µg/mL; 20 µM 3'-phosphoadenosine-5'-phosphosulphate (PAPS)) with each incubation vessel also containing CYP2D6 (100 pmol/mL; 1mM nicotinamide adenine dinucleotide phosphate (NADPH)). The positive control compound for sulfonation 7-hydroxycoumarin (7-HC; 100 µM) was also incubated under the same conditions.

Human cytosolic extract was also incubated in series and in parallel with CYP2D6 and with the unlabelled P19 synthetic standard. For the series incubation [^{14}C]-AZD5438 (10 µM) was incubated with CYP2D6 (100 pmol/mL; 1mM NADPH; 60 minutes) and the reaction terminated via the addition of an equal volume of ethanol. An aliquot of the sample was removed and checked for metabolism via HPLC-RAD to ensure P19 had been formed. The remainder of the sample was then blown to dryness under nitrogen, reconstituted in incubation buffer (1 mL) and the level of radioactivity checked by (liquid scintillation counting) LSC resulted in a 17.7 µM equivalent solution of which 20% of the radioactivity was assigned to [^{14}C]-P19 (as calculated from HPLC-RAD analysis) and the remainder to parent [^{14}C]-AZD5438. The reconstituted sample was then incubated (5 µM [^{14}C]-AZD5438 equivalents) with human cytosolic extract (1 mg/mL; 100 µM PAPS; 60 minutes).

Non-radiolabelled P19 and 7-HC were also incubated with cytosol under the same conditions. For the parallel incubation [^{14}C]-AZD5438 (10 µM) was incubated (60 minutes) with CYP2D6 (100 pmol/mL; 1mM NADPH) and human cytosolic extract (1 mg/mL; 100 µM PAPS) in the same incubation vessel. 7-HC (100 µM) was also incubated under the same conditions.

1.3.4. Covalent binding to proteins and metabolite trapping

Incubations with [^{14}C]-AZD5438 were carried out with human liver microsomes (HLM), human S9 (HS9) and human hepatocytes to determine the level of covalent binding.

All incubations with hepatocytes as well as some incubations with HLM were conducted by hand. All other incubations were carried out using a Hamilton Star liquid handler. Incubation volumes when using the robotic method were lower and samples taken into 96-deep well plates, but otherwise incubation conditions were comparable and by control compound binding.

[^{14}C]-AZD5438 (10 µM) was incubated with HLM (1 mg/mL; ±2 mM NADPH; ± 4 mM uridine 5'-diphospho-glucuronic acid (UDPGA) and 50 µg/mg protein alamethecin; ± 100 µM GSH; ± 100 µM KCN; 60 minutes). Incubation with HLM in the presence of inhibitory antibodies specific for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5. Antibodies were diluted by 25 mM tris(hydroxymethyl)aminomethane (TRIS) buffer prior to use, using volumes detailed in **Table 1**.

[^{14}C]-AZD5438 (10 µM) was also incubated with HS9 (1 mg/mL; ±2 mM NADPH; 60 minutes).

CYP isoform	Source	Volume of inhibitory antibody as supplied (µL)	Volume of TRIS buffer added (µL)	Volume of 20 mg/mL HLM (µL)
Control	BD	0	120	60
1A2	BD	12	108	60
2A6	BD	120	0	60
2B6	BD	12	108	60
2C8	BD	120	0	60
2C9	Xenotech	120	0	60
2C19	BD	24	96	60
2D6	BD	120	0	60
2E1	BD	24	96	60
3A4/5	Xenotech	120	0	60

Table 1: Volume of inhibitory antibody and HLM required for specific inhibition of CYP enzymes. Antibodies acquired from BD (BD Biosciences) or Xenotech llc.

[^{14}C]-AZD5438 (10 µM) was also incubated with fresh human hepatocytes in suspension (1x10^6 viable cells /mL; ± 1mM 1-aminobenzotriazole (ABT); ± 10 and 100 µM pentachlorophenol (PCP); 180 minutes) and fresh rat hepatocytes in suspension (1x10^6 viable cells /mL; ± 100 µM GSH; ± 100 µM KCN; 180 minutes). [^{14}C]-clozapine (10 µM), known to form a covalently binding reactive metabolite, was also incubated with both HLM and hepatocytes under the same conditions as a positive control.

Samples taken from all incubations (into four volumes of acidified acetone to precipitate the protein) were processed using a Brandel™ cell harvester which deposits the protein of the sample onto filter paper before washing it extensively with a solvent (80% methanol) to remove any non-covalently bound radiolabelled compound. Protein samples were then solubilised (5% sodium dodecyl sulfate; 18 hours; 55°C), and aliquots subsequently removed and analysed using a Pierce® BCA Protein Assay Kit to determine the mass of protein in the sample. The remaining solubilised protein solution was then counted by LSC to determine the mass of radiolabelled compound covalently bound to the protein. A value in pmol equivalents/mg protein was then calculated.

1.3.5. Incubation with THLE cells

Incubation of AZD5438 (0 – 500 μM) with THLE cells was completed by Mhairi Greer of Global Safety Assessment, Alderley Park, AstraZeneca. The cells were supplied by Nestec, Switzerland. Cells were plated at 15,000 cells/well (24 well plates), which were left for 24 hours to attach. AZD5438 was added up to 72 hours with repeat daily dosing. Cell viability was measured using Promega™ CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS) and displayed as percentage survival based on no compound controls. Data are mean of triplicate samples.

2. Results

2.1. Metabolite identification and confirmation

Initially it was necessary to verify the presence of the metabolite of interest (AZD5438-sulfate metabolite, Figure 7, Figure 8) in both human plasma samples and in *in vitro* incubation samples produced from incubation with primary human hepatocytes. This was to ensure that metabolites produced in *in vitro* systems were those present in the clinic. Parent AZD5438, P19 and the AZD5438-sulfate metabolite were all observed via HPLC-MS analysis of both *in vivo* and *in vitro* samples.

2.1.1. *In vivo* samples

Parent AZD5438 and metabolites P19 and the AZD5438-sulfate metabolite were observed in unquantifiable amounts, confirming the presence of the compounds of interest in pooled human plasma samples analysed (Figure 9). The proposed fragmentation patterns of AZD5438, P19 and the AZD5438-sulfate metabolite in negative ion mode are shown in Figure 10, Figure 11 and Figure 12, respectively.

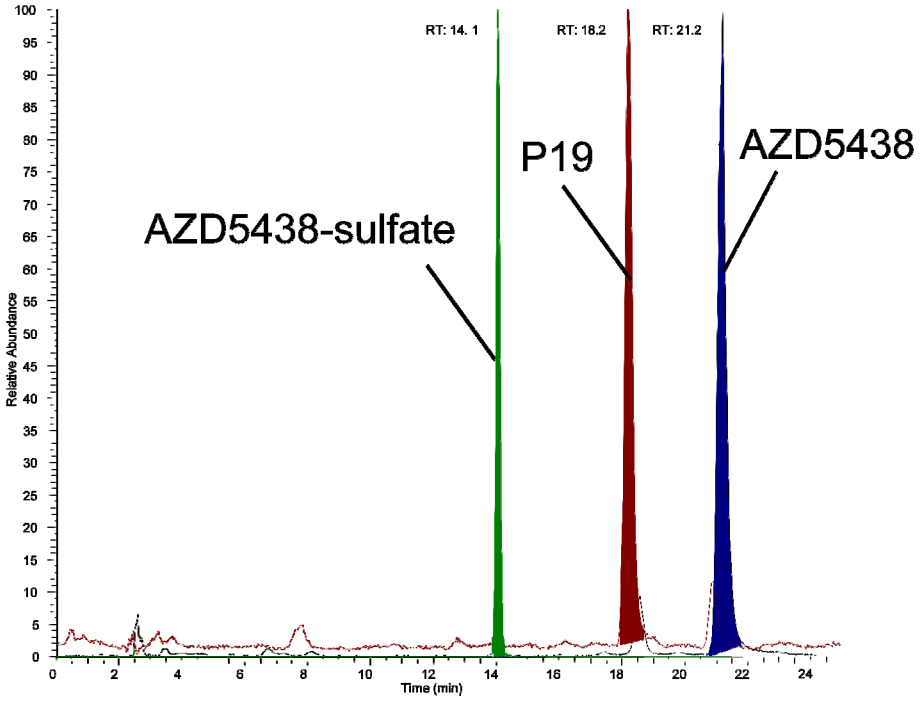


Figure 9: Mass chromatogram of pooled human plasma sample

Three overlaid HPLC-MS^n chromatograms produced from a pooled human plasma sample. Plasma samples were pooled and extracted by simple protein crash using acetonitrile, before being diluted with an initial mobile phase of organic and aqueous prior to injection on the HPLC-MS^n system. The compounds were separated by an HPLC gradient and identified using a full scan in negative ion mode, the three compounds of interest were identified in the pooled plasma using a targeted search for respective parent mass ($[M\text{-}H^+]^-$ being one mass unit lower than neutral parent mass). Masses as follows; AZD5438 m/z 370 (blue peak), P19 m/z 386 (red) and AZD5438-sulfate m/z 466 (green). This mass chromatogram is not quantitative and so amount of compound (absolute or relative) present cannot be determined from peak area.

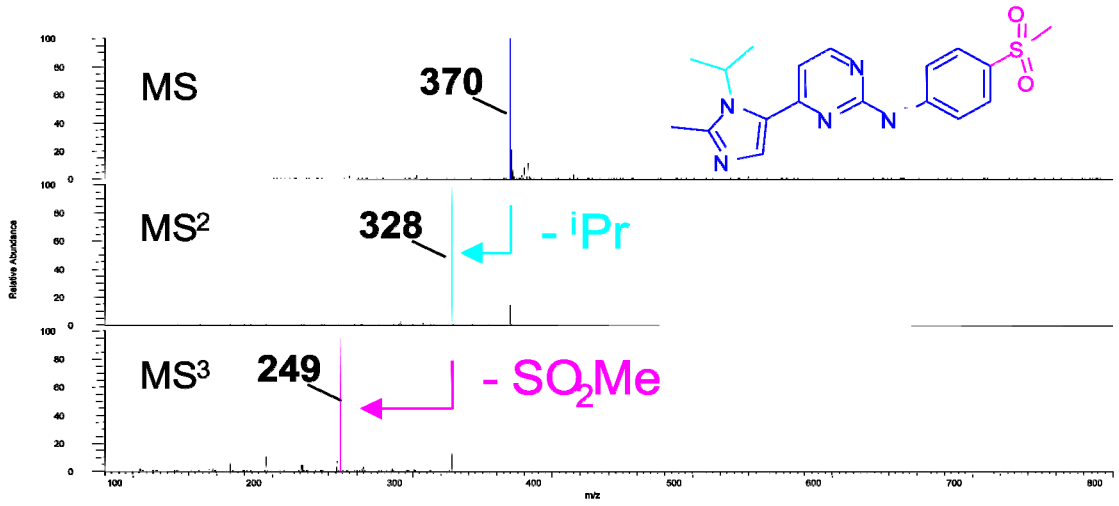


Figure 10: Ion spectrum showing fragmentation of AZD5438 in negative ion mode.

Identification of AZD5438 by mass spectrometry using consecutive targeted mass scans. Following isolation of parent ion AZD5438 (m/z 370) by MS, fragmentation by MS^2 results in the loss of the isopropyl group (light blue). Further fragmentation by MS^3 results in the loss of the methyl-sulphone moiety (pink). These data are produced using an AZD5438 standard at 10 µM in initial mobile phase.

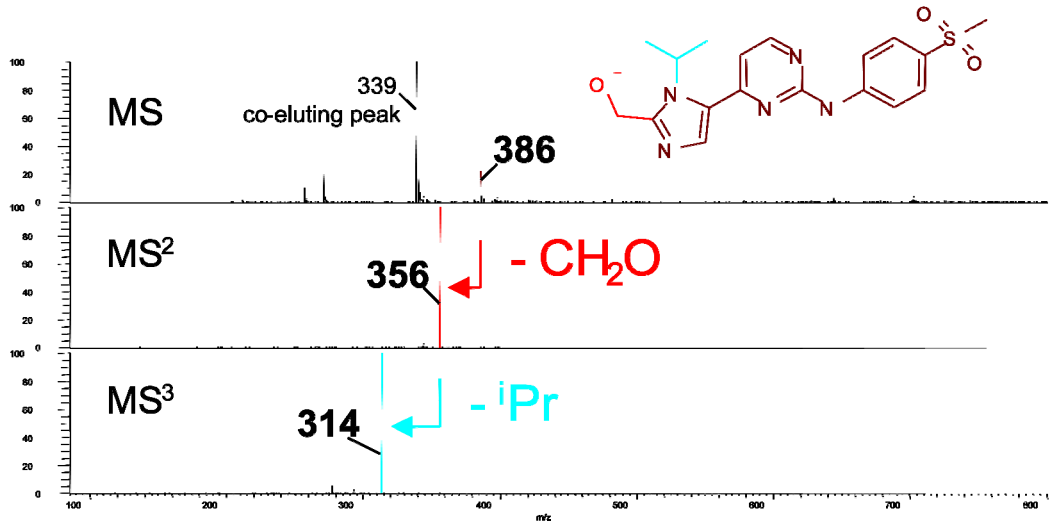


Figure 11: Ion spectrum showing fragmentation of P19 in negative ion mode.

Identification of P19 by mass spectrometry using consecutive targeted mass scans. Following isolation of parent ion P19 (m/z 386) by MS, fragmentation by MS^2 results in the loss of the CH_2O moiety (red). Further fragmentation by MS^3 results in the loss of the isopropyl group (light blue). These data are produced using a P19 standard at 10 µM in initial mobile phase.

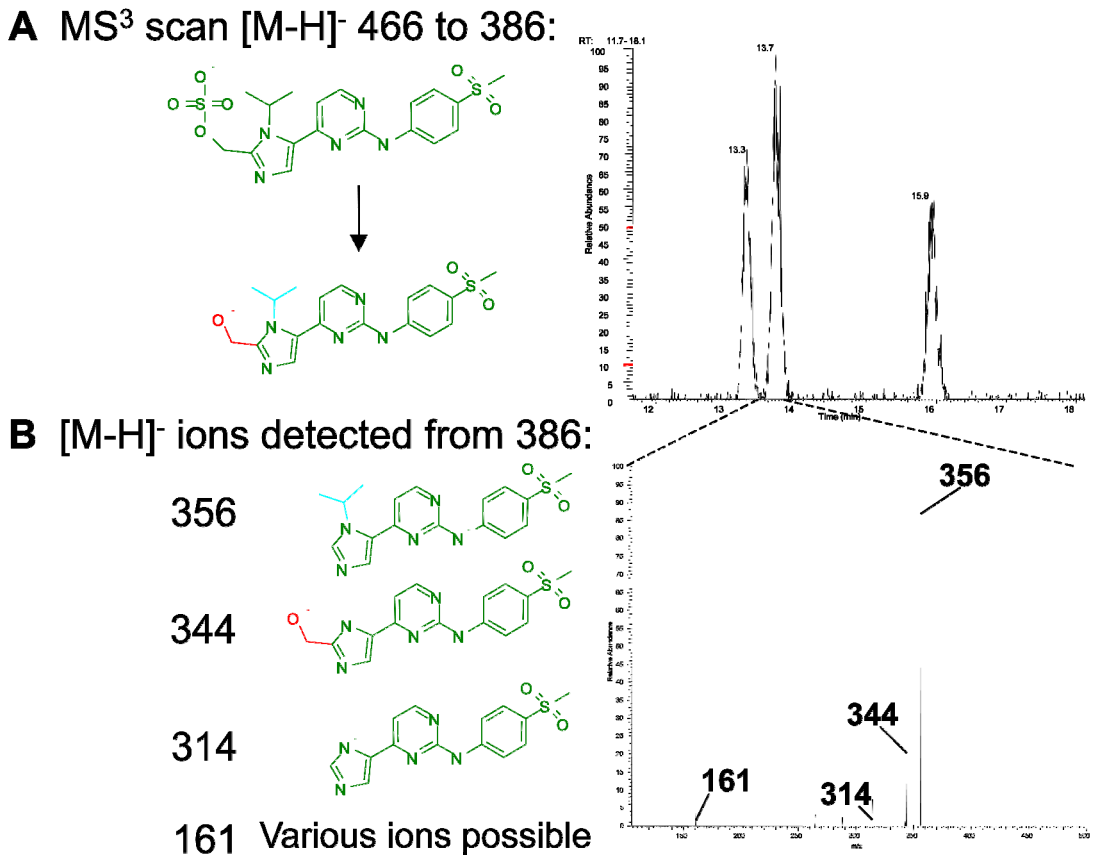


Figure 12: Mass chromatogram and ion spectrum for AZD5438-sulfate metabolite

Mass chromatogram and ion spectrum produced in negative ion mode from a pooled human plasma sample. Plasma samples were pooled and extracted by simple protein crash using acetonitrile, before being diluted with an initial mobile phase of organic and aqueous prior to injection on the HPLC-MS^n system. A: MS^3 mass chromatogram with three peaks each showing a transition from parent ion AZD5438-sulfate (m/z 466) to hydroxyl-AZD5438 (m/z 386) due to loss of the SO_3 moiety. Peaks indicate three isomeric sulfate metabolites with identical chemical formulae but different structures with different chromatographic properties. The fragmentation scheme depicted in B is representative of the peak with elution time 13.4 minutes and shows an ion spectrum. Major ions present are m/z 356 (loss of the CH_2O moiety (red)), m/z 344 (loss of the isopropyl group (light blue)), m/z 314 loss of both the CH_2O moiety and the isopropyl group an m/z 161 (various ions possible).

2.1.2. *In vitro* samples and optimisation of incubation conditions

Several *in vitro* systems were used in an attempt to produce the metabolites of interest identified in the clinical plasma samples. An optimised test tube system to produce these metabolites would be useful in further toxicity testing.

As with the *in vivo* samples, parent compound and both metabolites of interest were present in late time point samples from hepatocyte incubations when analysed by mass spectrometry (Figure 13), but HPLC-RAD data showed no quantifiable AZD5438-sulfate metabolite (data not shown) indicating turnover to these metabolites was low.

2.1.2.1. Recombinant cytochrome P450 incubations

The production of P19 from [^{14}C]-AZD5438 was observed in previous work completed during the drug development process at AstraZeneca, and indicated that both CYP2D6 and CYP3A4 were capable of producing the metabolite. Using heterologously expressed CYP2D6 and CYP3A4 these tests were repeated in order confirm production of P19 and to select which of these isoforms would be used in further optimisation experiments.

Radiochromatograms indicating formation of the P19 metabolite by CYP2D6 and 3A4 following incubation with [^{14}C]-AZD5438 are shown in Figure 14.

CYP3A4 produced a greater amount of P19 than CYP2D6 under the reaction conditions used, but the latter isoform converted AZD5438 to the P19 metabolite more selectively, with no other metabolites detected that accounted for greater than 1% of total compound related material. CYP3A4 however, produced at least two other detectable metabolites. In order to optimise an *in vitro* metabolic system to produce the desired [^{14}C]-AZD5438-sulfate, selectivity is as important as the quantity

produced and so a decision was made to use expressed CYP2D6 protein to catalyse the Phase I reaction and produce P19.

2.1.2.2. Recombinant SULT and cytosol incubations

The production of the desired [^{14}C]-AZD5438-sulfate from P19 was attempted in *in vitro* systems using heterologously produced SULT enzymes and separately using human cytosol.

Analysis of samples from all CYP2D6 and SULT or cytosol incubations by HPLC-RAD-MS showed conversion of [^{14}C]-AZD5438 to P19 but no [^{14}C]-AZD5438 sulfate metabolite was observed. The positive control, 7-hydroxycoumarin was seen to be sulfonated by several of the SULT isoforms following analysis by HPLC with ultraviolet detection (HPLC-UV), chromatograms for which are not included in this report due to electronic raw data loss.

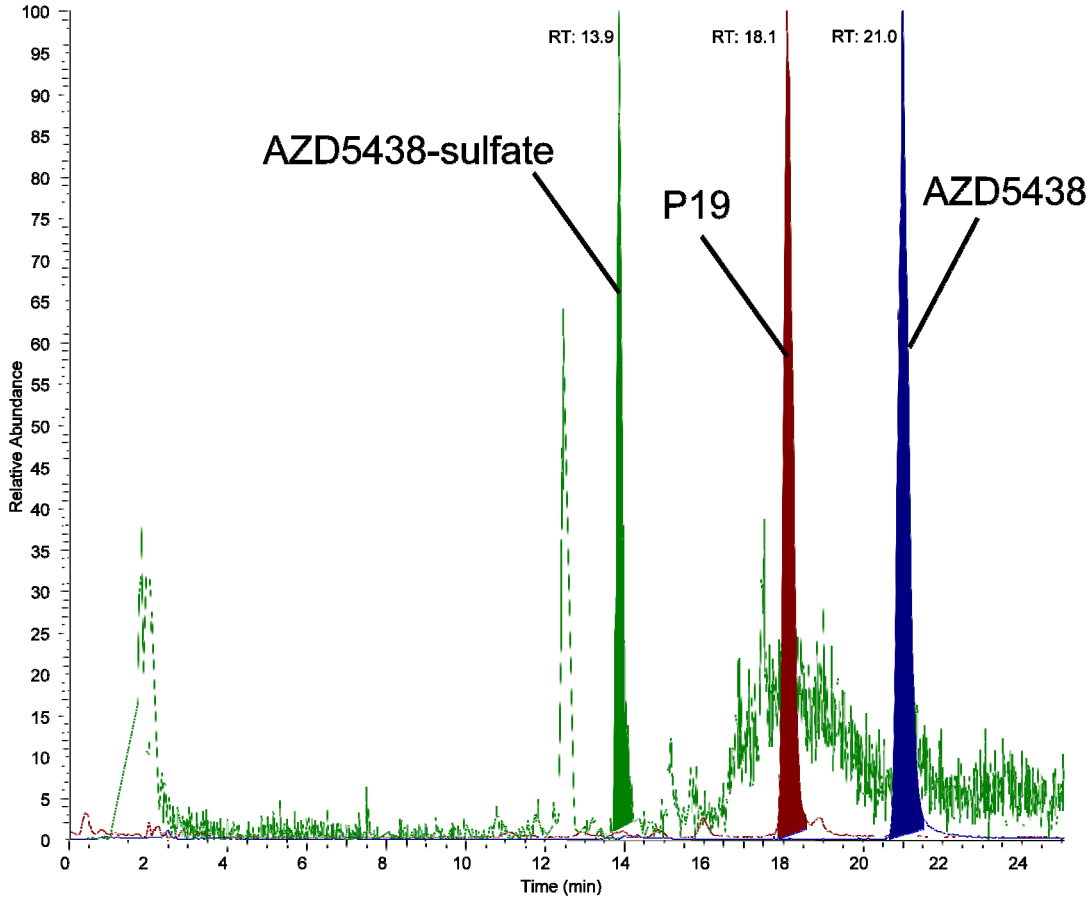


Figure 13: Constructed mass chromatogram of *in vitro* human hepatocyte incubation sample

Three overlaid chromatograms following incubation of AZD5438 (10 µM) for 72 hours with cultured primary human hepatocytes. The compounds were separated by an HPLC gradient and identified using a full scan in negative ion mode, the three compounds of interest were identified in the sample using a targeted search for respective parent mass ($[M\text{-}H^{+}]^{-}$ being one mass unit lower than neutral parent mass). Masses as follows; AZD5438 m/z 370 (blue peak), P19 m/z 386 (red) and AZD5438-sulfate m/z 466 (green). This mass chromatogram is not quantitative and so amount of compound (absolute or relative) present cannot be determined from peak area.

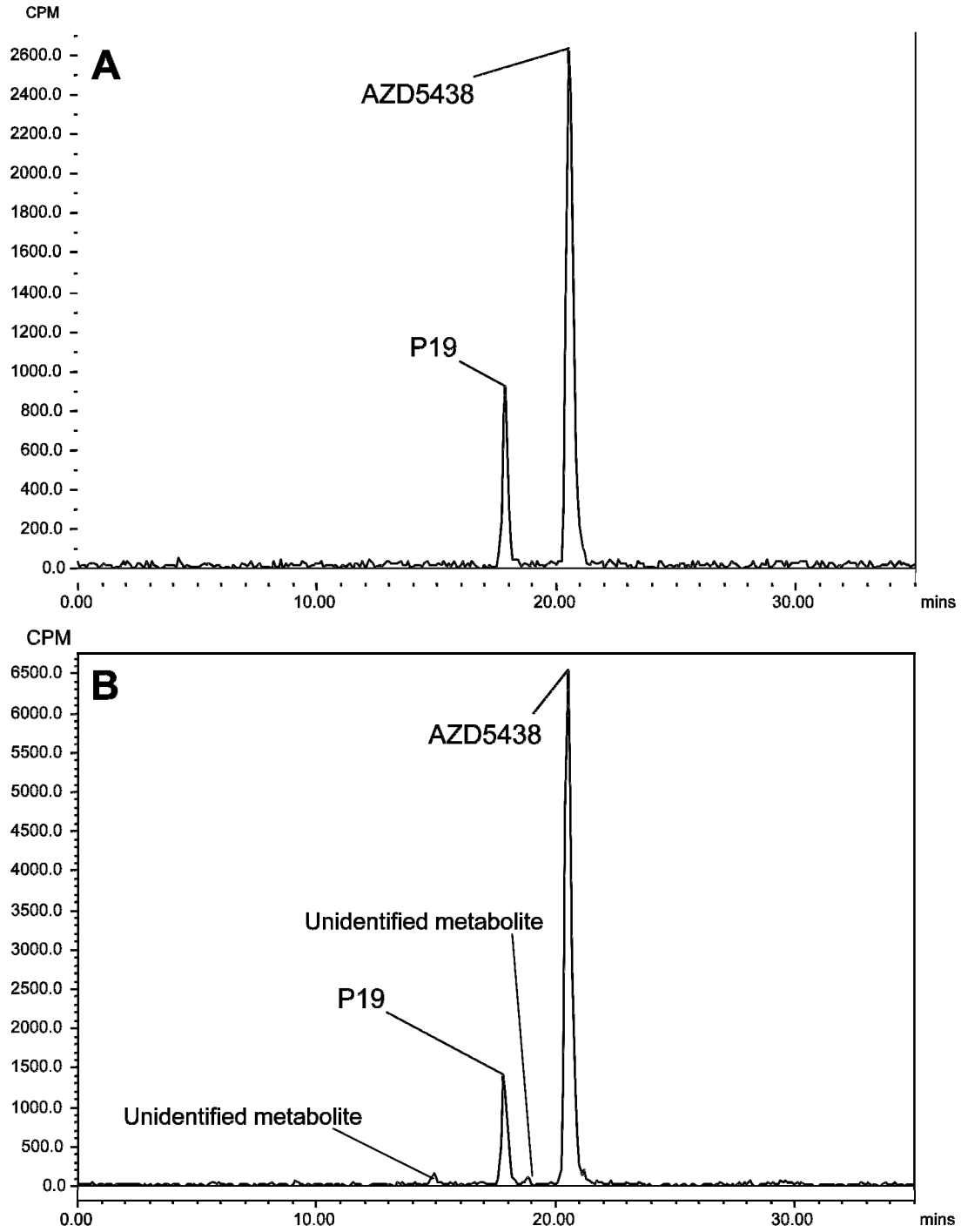


Figure 14: HPLC radiochromatogram of [^{14}C]-AZD5438 and metabolites subsequent to incubation with CYP supersomes

HPLC-RAD chromatograms showing metabolism of [^{14}C]-AZD5438 following incubation with A: CYP2D6 (1 mg/mL) or B: CYP3A4 (1 mg/mL). Incubation time was 60 minutes in the presence of NADPH (2 mM). Metabolism by heterologously expressed CYP2D6 shows selective metabolism to P19 whereas CYP3A4 produces to further quantifiable metabolites which were not identifiable by mass spectrometric analysis. Due to the superior selectivity, the use of CYP2D6 to form P19 in subsequent incubations was therefore selected.

2.2. Covalent binding

Utilising radiolabelled substrate and the quantitative end point of covalent binding experiments, various *in vitro* test systems can be manipulated (using the presence and absence of co-factors or inhibitors) to provide information on which metabolic pathways produced metabolites capable of covalent binding to incubation protein.

2.2.1. Human primary hepatocytes

Hepatocytes contain the whole panel of metabolic enzymes (with appropriate co-factors) and so are capable of forming a reactive metabolite and of detoxifying any reactive metabolites formed before covalent binding occurs.

The extent of covalent binding after 180 minutes of [^{14}C]-AZD5438 (10 μM) in the presence and absence of ABT and PCP are shown in Figure 15. Low levels of time dependent covalent binding were observed, with a significant decrease in binding in the presence of the CYP inhibitor ABT (1 mM) (5.15 and 3.02 pmol/mg protein, respectively, $P<0.05$). Incubation in the presence of the SULT inhibitor PCP at 10 or 100 μM showed no significant change (4.62 and 7.98 pmol/mg protein, respectively).

2.2.2. Human microsomes and S9 fraction

Human liver microsomes and human S9 fraction are simpler systems than whole hepatocytes and so can give a clearer view of which enzymes are responsible for the production of covalently binding reactive metabolites.

The extent of covalent binding after 60 minutes of [^{14}C]-AZD5438 (10 μM) subsequent to incubation with HLM (±NADPH, ±UDPGA) and HS9 (±NADPH) is shown in **Figure 16**. Binding was shown to be time and NADPH dependent in HLM and to a much greater extent (124.95 pmol/mg protein) than with hepatocytes. In the presence of UDPGA binding was still evident (57.10 pmol/mg protein) but to a significantly lesser extent ($P<0.02$) than where HLM can perform Phase I metabolism

alone. HS9 incubations, also showed time and NAPDH dependent binding, but again overall binding (29.26 pmol/mg protein) was less than with HLM.

2.2.2.1. In the presence of known trapping agents

Trapping agents such as GSH and KCN can lead to a decrease in covalent binding as may react with the reactive species before the latter has a chance to interact with incubation protein.

The extent of covalent binding after 60 minutes of [^{14}C]-AZD5438 (10 μM) with HLM in the presence of known trapping agents KCN (100 μM) or GSH (100 μM) is shown in Figure 17. Time and NADPH dependent covalent binding was apparent but no significant change in binding was observed in the presence of either of the trapping agents (123.29, 153.32 and 127.23 pmol/mg protein for no trapping agent, KCN and GSH, respectively).

2.2.2.2. In the presence of specific inhibitory antibodies

A decrease in covalent binding in the presence of an antibody inhibitory to a specific CYP isoform would implicate that CYP as being involved in the production of a reactive metabolite.

The extent of covalent binding after 60 minutes of [^{14}C]-AZD5438 (10 μM) with HLM in the presence of antibodies inhibitory to specific CYP isoforms is shown in Figure 18 and binding values after 60 minutes incubation shown in Table 2. Significantly decreased covalent binding to protein was seen in the presence of the inhibitor of CYP2C9 ($P < 0.0005$) and to a greater extent, the inhibitor of CYP3A4/5 ($P<0.0001$). The specificity of these inhibitory antibodies allows an estimation of the proportion of metabolism attributable to each isoform to be made from the percentage decrease in binding. It therefore seemed that ~35% and ~65% of the reactive metabolites bound to protein were formed by CYP2C9 and CYP3A4/5, respectively.

CYP isoform	Covalent binding of [^{14}C]-AZD5438 to incubation protein (pmol equivalents/mg protein) ± SD
Control	42.63 ± 6.19
1A2	38.57 ± 4.95
2A6	40.91 ± 3.42
2B6	41.07 ± 5.38
2C8	42.30 ± 3.42
2C9	25.83 ± 2.14
2C19	40.39 ± 4.93
2D6	42.34 ± 5.82
2E1	42.93 ± 5.93
3A4/5	13.46 ± 0.27

Table 2: Covalent binding of [^{14}C]-AZD5438 (10 µM) after 60 minutes, in the presence of inhibitory antibodies specific for various CYP isoforms

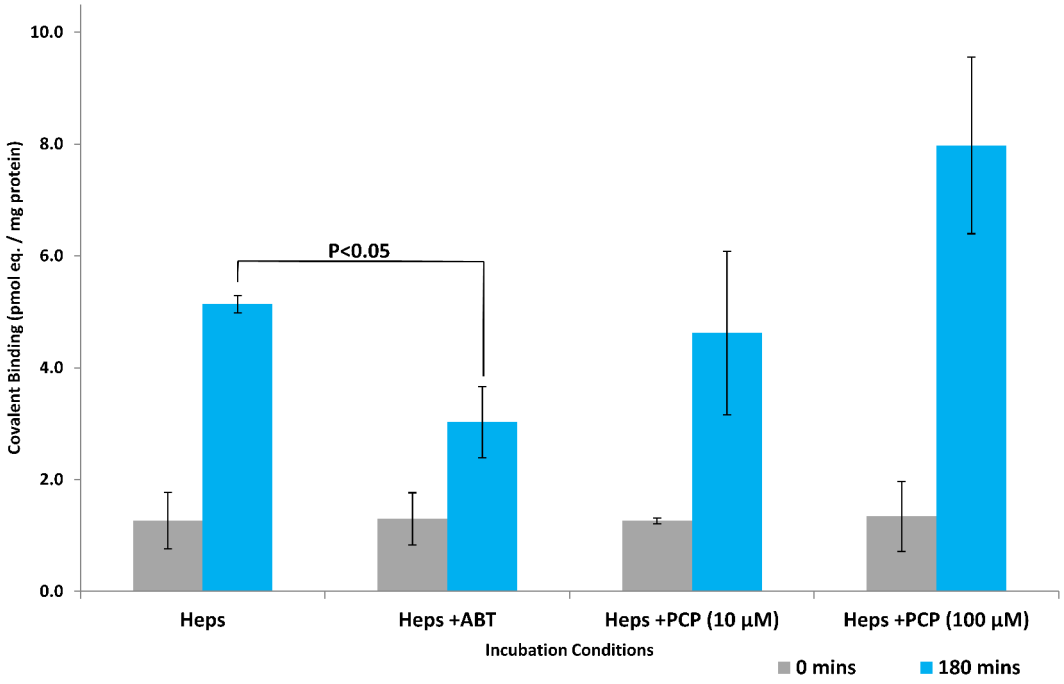


Figure 15: Covalent binding to protein of [^{14}C]-AZD5438 subsequent to incubation with fresh human hepatocytes.

Levels of covalent binding to protein of [^{14}C]-AZD5438 (10 µM) following incubation for 180 minutes with fresh human hepatocytes in suspension significantly decreased (P<0.05) in the presence of the nonspecific CYP inactivator 1-aminobenzotriazole (ABT, 1mM) indicating that CYP mediated metabolism of [^{14}C]-AZD5438 was implicated in covalent binding in this system. Incubation in the presence of pentachlorophenol (PCP, 10 and 100 µM), an inhibitor of SULT enzymes, showed no significant difference in covalent binding. Positive control incubations under the same conditions with [^{14}C]-clozapine resulted in binding of 15.3±12.0 and 59.0±11.0 pmol eq. / mg protein at 0 and 180 minutes, respectively in the absence of ABT and 5.9±1.0 and 15.5±1.3 pmol eq. / mg protein at 0 and 180 minutes, respectively in the presence of ABT. The decrease in binding of [^{14}C]-clozapine at 180 minutes between the absence to the presence of ABT in this system was significant (P<0.05). N=3 in all incubations. Error bars show ± 1 SD. P values calculated using a double-tailed students T-test.

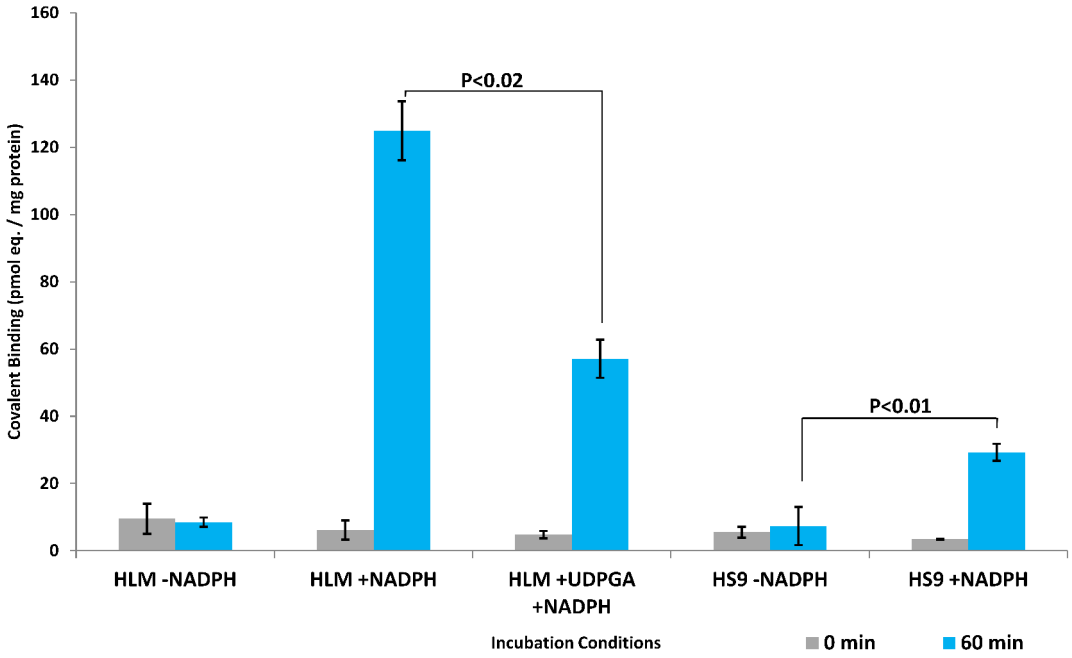


Figure 16: Covalent binding to protein of [^{14}C]-AZD5438 subsequent to incubation with human liver microsomes or human S9 mix.

Levels of covalent binding to protein of [^{14}C]-AZD5438 (10 µM) following incubation for 60 minutes with human liver microsomes significantly decreased (P<0.02) in the presence of NADPH (1 mM), UDPGA (4 mM) and alamethecin (50 µg/mg protein) when compared to incubation with NADPH alone, indicating that the ability of a metabolic system to produce glucuronide conjugates resulted in a decrease in [^{14}C]-AZD5438 covalent binding. Incubation of [^{14}C]-AZD5438 with human S9 mix showed a significant increase (P<0.01) in covalent binding in the presence of NADPH. Positive control incubations with HLM under the same conditions with [^{14}C]-clozapine resulted in binding of 34.5±0.7 and 390.5±103.5 pmol eq. / mg protein at 0 and 60 minutes, respectively in the absence of UDPGA and alamethecin and 21.3±6.6 and 445.6±70.0 pmol eq. / mg protein at 0 and 60 minutes, respectively in the presence of UDPGA and alamethecin. There was no significant difference in [^{14}C]-clozapine binding in the presence of UDPGA and alamethecin. N=3 in all incubations. Error bars show ± 1 SD. P values calculated using a double-tailed students T-test.

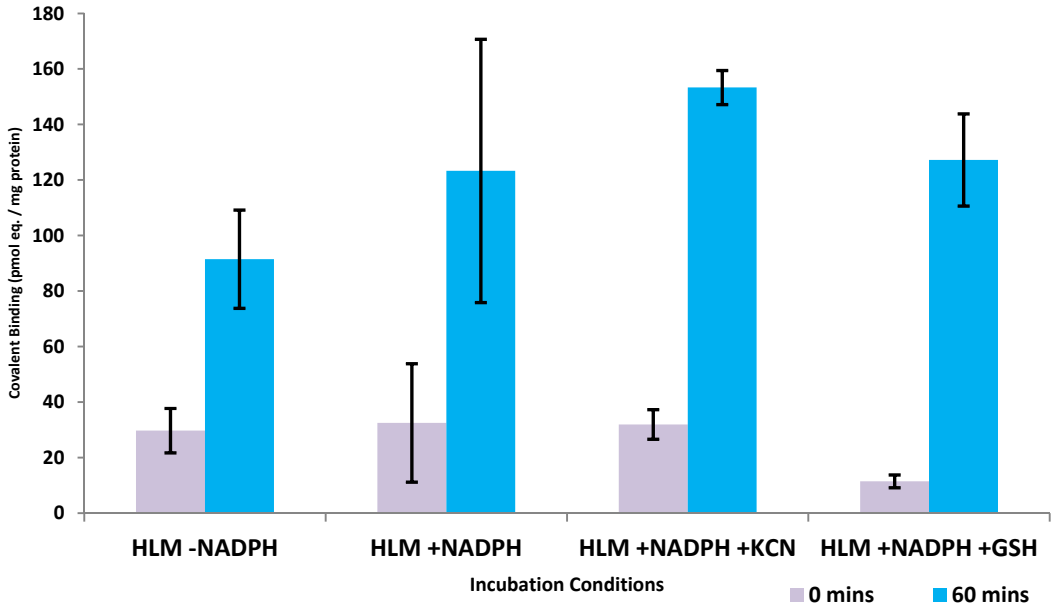


Figure 17: Covalent binding to protein of [^{14}C]-AZD5438 subsequent to incubation with human liver microsomes in the absence and presence of known reactive metabolite trapping agents.

Levels of covalent binding to protein of [^{14}C]-AZD5438 (10 µM) following incubation for 60 minutes with human liver microsomes were unchanged in the presence of KCN (100 µM) or GSH (100 µM). Positive control incubations with HLM under the same conditions with [^{14}C]-clozapine resulted in binding of 99.3±21.7 and 833.3±80.1 pmol eq. / mg protein at 0 and 60 minutes, respectively in the absence of trapping agents, 79.0±11.0 and 578.3±177.7 pmol eq. / mg protein at 0 and 60 minutes, respectively in the presence of KCN and 52.8±17.5 and 588.4±23.7 pmol eq. / mg protein at 0 and 60 minutes, respectively in the presence of GSH. Covalent binding of [^{14}C]-clozapine to HLM significantly decreased in the presence of KCN ($P<0.07$) and GSH ($P<0.01$). N=3 in all incubations. Error bars show ± 1 SD. Significance values assessed using a double-tailed students T-test.

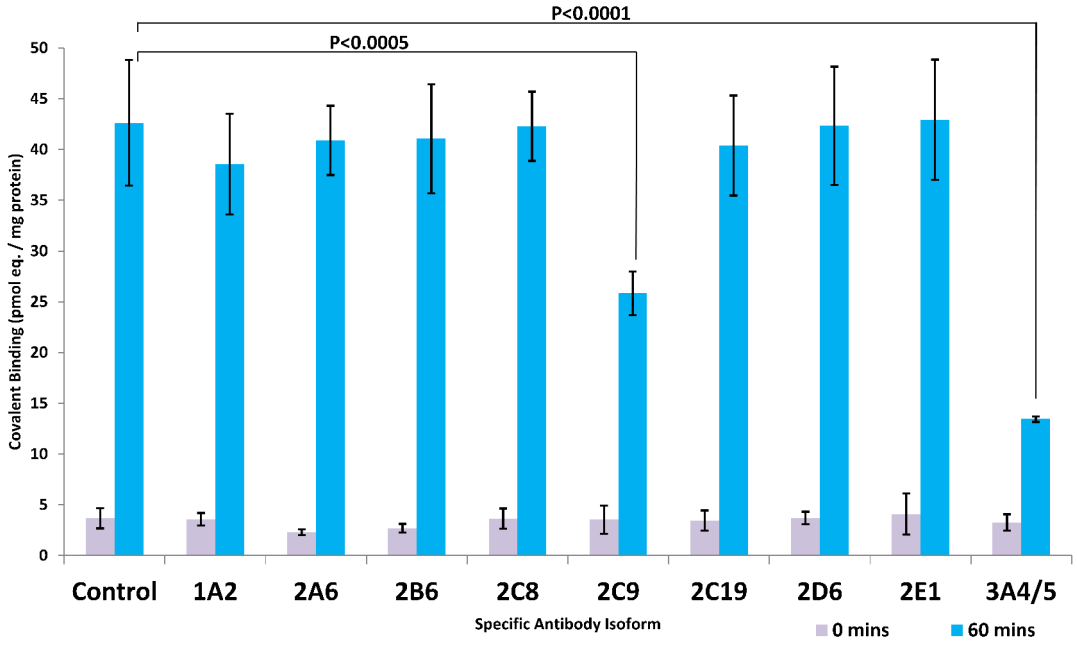


Figure 18: Covalent binding to protein of [^{14}C]-AZD5438 subsequent to incubation with human liver microsomes in the presence of antibodies inhibitory to specific CYP isoforms.

Levels of covalent binding to protein of [^{14}C]-AZD5438 (10 µM) following incubation for 60 minutes with human liver microsomes were significantly decreased in the presence of inhibitory antibodies specific for CYP2C9 (P<0.0005). Binding in the presence of all other inhibitory antibodies was unchanged from control value. All incubations performed in the presence of NADPH (2 mM). N=6 in control incubations and N=4 in all other incubations. Error bars show ± 1 SD. P values calculated using a double-tailed students T-test.

2.3. Extrapolation of binding results

The quantitative degree of covalent binding in *in vitro* test systems has been investigated by several groups (Obach, 2009; Obach, 2008; Usui, 2009) as a potential predictor of drug induced liver injury (DILI) in patients. Overall exposure of the liver to a reactive metabolite forming compound is an important factor in calculation of risk and so dose can be used to scale for this factor. Usui et al. attempt to differentiate between drugs known to cause DILI and drugs known not to cause DILI by multiplying covalent binding in *in vitro* systems (pmol/mg protein) by daily dose (µmols), with some success (Figure 19). A range of drugs that could be easily separated into those which clinically cause DILI and those which do not were

incubated in *in vitro* systems (HLMs and human hepatocytes) and covalent binding values in each system calculated. Using the maximum daily doses of these compounds, and the formula above, the compounds were then ranked. It can be seen from the results that the two groups could not be fully separated.

Using this method and the predicted dose of AZD5438 obtained from development work at AstraZeneca (70 – 360 mg/day), a prediction can be made as to which group AZD5438 may fall into (Usui, 2009). Note that *in vitro* assays were carried out in a similar way here to those in Usui, 2009.

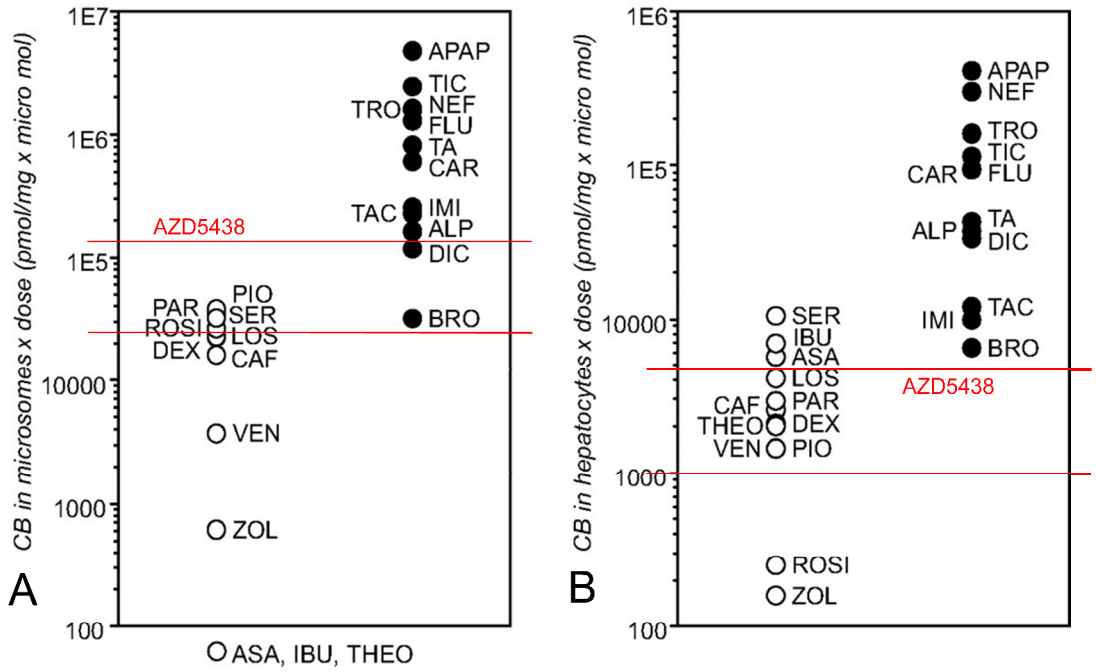


Figure 19: Prediction of potential drug induced liver injury by AZD5438 as calculated by covalent binding.

Partial and altered reproduction from (Usui, 2009). Scaling of *in vitro* covalent binding values by dose, separated by compounds which cause DILI (closed circles) and those which do not (open circles). Predictions for DILI are shown using covalent binding values in HLM (A) or human hepatocytes (B). Predictions for AZD5438 based on covalent binding values realised in the present study combined with maximum and minimum predicted daily dose are superimposed on the relevant scale. The predicted values for AZD5438 with HLM fall on the overlap between the two groups whereas values calculated from human hepatocytes seem to indicate that AZD5438 would fall into the low risk category for possible DILI. A key detailing full identity of drugs from presented abbreviations can be seen in (Usui, 2009).

2.4. THLE cell toxicity

Cell lines containing heterologously expressed CYP isoforms can be assessed as a whole cell system in terms of toxicity but in a similar way to much simpler systems, such as CYP supersomes, in terms of metabolic capability. Control null cells with no CYP metabolic apparatus give an indication of the toxicity of the parent compound and comparison to other cell lines which do contain metabolic enzymes can then be considered. This assay uses an endpoint of mitochondrial reductase activity (via an MTS assay) to assess cell viability and as a result any inhibition of this enzyme by the test compound may result in a false positive result of cell death. It would be expected that a CDK inhibitor would cause cell death (including via

pathways associated with mitochondrial function) and so decrease in mitochondrial reductase activity in a THLE cell containing a single CYP isoform is not enough to implicate that isoform in toxicity. Comparison with the null cell line is therefore required.

IC_{50} calculations for incubation of THLE-CYP cells in the presence of AZD5438 (1 – 500 μM) subsequent to 48 hour incubation is shown in Figure 20. Non-metabolic toxicity is evident in the null cells (IC_{50} = 241 μM) which is understandable considering the mechanism of action for which AZD5438 was designed. THLE-CYP2D6 and –CYP3A4 cells appeared to be more susceptible to incubation with AZD5438 producing IC_{50} values of 116 and 43 μM, respectively. Conversely, THLE-CYP1A2 cells survived in greater numbers at the same concentrations, indicated with an IC50 of 307 μM.

Clearer data indicating the toxicity of the parent compound can be seen when comparing reductase activity of all cell lines over 72 hours incubation from 0 – 500 μM AZD5438 (Figure 21). Cells remain viable over the 72 hour period where no AZD5438 is present, but even at time 0, a concentration dependent decrease in

viability is apparent. All incubations containing AZD5438 indicate time dependent decrease in cell viability.

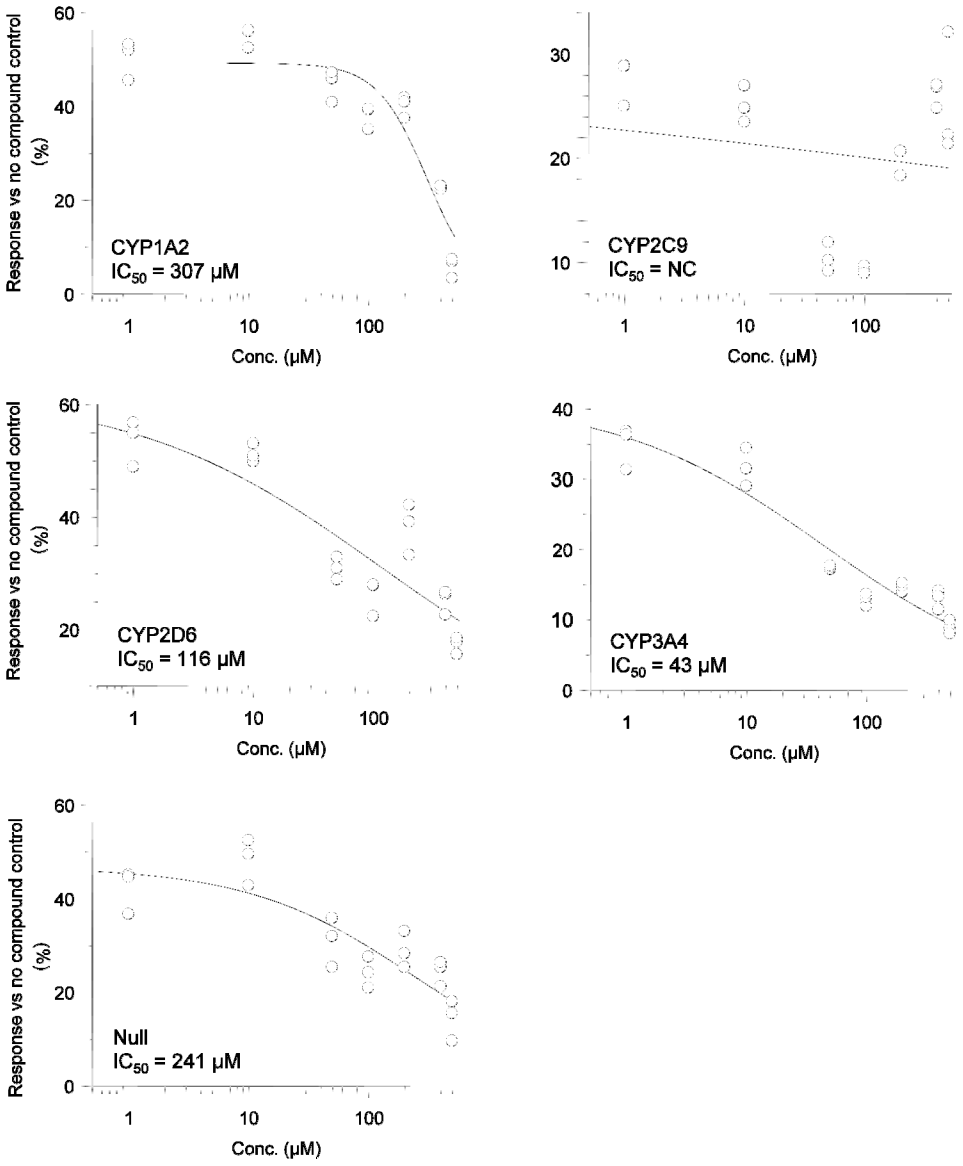


Figure 20: Reductase activity in specific CYP expressing THLE cells subsequent to incubation with AZD5438 (0 – 500 µM) (48 hours).

Response is as a percentage of reductase activity (as measured by a ProMega MTS assay kit) compared to respective cell line incubated over 48 hours with no compound present. Calculated IC_{50} values following incubation of AZD5438 (0 – 500 µM) for 48 hours with THLE cells expressing specific CYP isoforms (CYP1A2, CYP2C9, CYP2D6, CYP3A4) and null cells expressing no CYP enzyme. All cell lines show concentration dependent toxicity to AZD5438, even at 1 µM where % response ranges from 25 – 60% of no compound controls. When comparing cell lines, these data suggest that the toxicity of AZD5438 (as measured via MTS reduction by microsomal reductase enzymes) is enhanced in the presence of CYP2D6 or CYP3A4 when compared to null cells which contain no metabolic activity. Cells containing CYP1A2 activity appear to have a protective effect when compared to null cells. An IC_{50} for 2C9 was not calculated (NC) due to unexpected assay response at high AZD5438 concentrations. N=3 for all incubations. IC_{50} calculations performed by Grafit version 6.0.5.

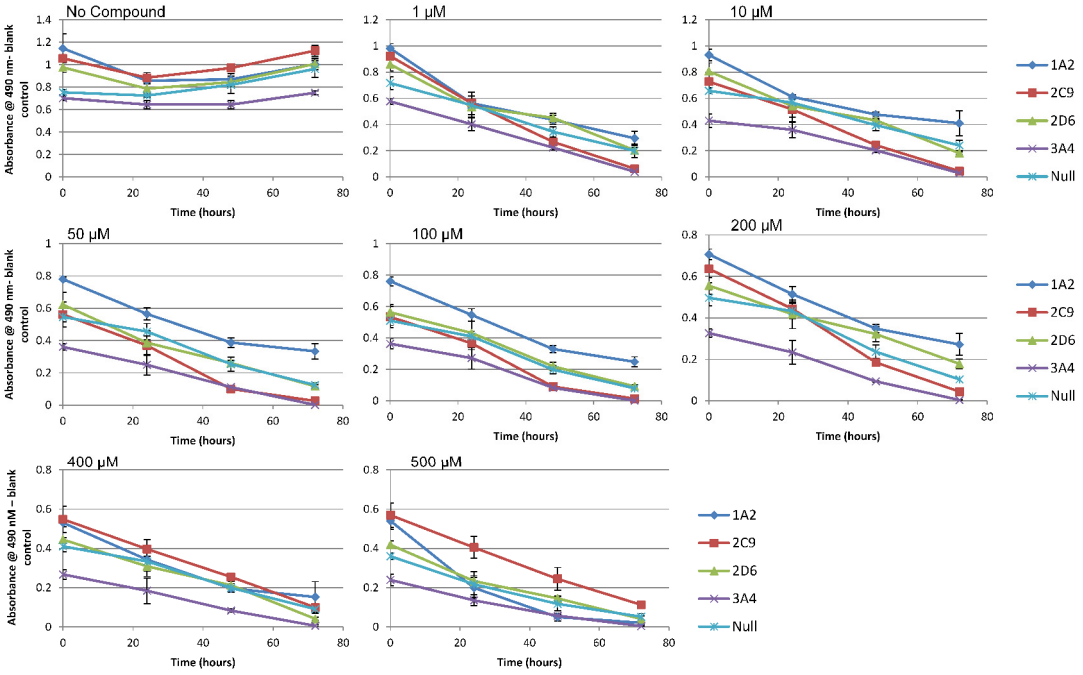


Figure 21: Reductase activity in specific CYP expressing THLE cells subsequent to incubation with AZD5438 (0 – 500 µM) over a 72 hour time course.

Reductase activity is presented as absorbance values at 490 nm minus blank background control (as determined by a ProMega MTS assay kit). The No Compound controls show no apparent decrease in reductase activity over the 72 hour incubation period for any cell line. A decrease in activity over 72 hours was observed in incubations with all cell lines at all concentrations of AZD5438. A decrease in activity at the 0 hour time point was apparent as concentration of AZD5438 increased, possibly due to an effect on the cells that occurred in the time between spiking and sampling. N=3 for all incubations. Error bars show ± 1 SD.

3. Discussion

3.1. Metabolism of AZD5438

The sulfate metabolite of AZD5438 was seen to be present in both *in vivo* and *in vitro* samples analysed, indicating that the hepatocyte assays tested were suitable as models for investigation into this metabolite. Attempts to determine the mechanism of formation of the sulfate metabolite via the use of simpler metabolic systems were more difficult. The precursor of the sulfate metabolite, the hydroxyl metabolite named P19, was formed readily using either human liver microsomes (HLM) or expressed CYP supersomes (both CYP2D6 and CYP3A4). Conjugating P19 (either using the synthesised compound or first initiating hydroxylation of AZD5438) with a sulfate group using an incubation of expressed sulfotransferases or human liver cytosol (which contains a range of sulfotransferase enzymes) were unsuccessful, suggesting that the conditions in the incubations were not optimised for conversion of P19 to the desired sulfate metabolite. The co-factor, adenosine 3'-phosphate 5'-phosphosulfate (PAPS), required to supply the sulfonate group during enzymatic sulfonation is known to be unstable and break down to 3'-adenosine 5'-phosphate (PAP) (Maurel, 1996). This degradation product is also the by-product of the sulfonation reaction catalysed by SULT enzymes and acts as a SULT inhibitor (Maurel, 1996). Therefore, an impure sample of PAPS containing PAP could have an inhibitory effect on that which is desired. PAPS obtained from Sigma UK claims to be >60% pure with PAP being listed as one of the major impurities. Samples with greater purity are available commercially, but over time they too will break down forming the inhibitory PAP molecule. This may partly explain the lack of sulfonation of either [^{14}C]-AZD5438 or P19, if the concentration of PAPS present was not in significant excess.

3.2. Reactive metabolite formation

The greatest insight into the reactive metabolite formation came from the panel of covalent binding assays completed with [^{14}C]-AZD5438. Binding in HLM (after 60 minutes) was over 20-fold greater than that in human hepatocytes (over 180 minutes) and so, it can be postulated that the hepatocyte either doesn't form reactive metabolites in the same quantities or that a detoxification mechanism is in place. It can be seen that, as the complexity of the metabolic apparatus increased, the binding value decreased (comparative binding at terminal time points is summarised in Table 3), which suggests that it is the presence of alternative metabolic pathways (or those additional to Phase I CYP mediated pathways, at least) that affords protection from covalent binding rather than a detoxification mechanism (e.g. trapping).

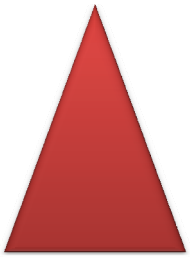
Complexity of metabolic system	**Test system**	**Covalent binding of [^{14}C]-AZD5438 to incubation protein ± SD (pmol equivalents/mg protein)**
Low	Human liver microsomes[a]	124.95 ± 8.73
	Human liver microsomes +UDPGA	57.10 ± 5.67
	Human liver S9	29.26 ± 2.55
	Human hepatocytes	5.14 ± ND
High		

Table 3: Covalent binding of [^{14}C]-AZD5438 (10 µM) at the terminal time point in each of four test systems of varying metabolic complexity. [a] value from microsomal incubation carried out by Hamiltonrobot

The lack of change in binding to microsomes in the presence of trapping agents KCN and GSH (Figure 17) is consistent with this. Data from binding experiments where metabolic inhibitors were present with hepatocytes (Figure 15) were also consistent

with this theory as the CYP inhibitor ABT significantly decreased binding, whereas SULT inhibitor PCP had no apparent effect.

These data are strongly supported by the extent of binding in the presence of inhibitory antibodies specific for various CYP isoforms (Figure 18). Due to the specificity of the antibodies, the covalent binding values in this experiment are additive showing that all of the binding in HLM can be attributed to either CYP2C9 or CYP3A4/5 (accounting for ~33% and ~67% of binding, respectively).

An increase in the complexity of the systems used in the incubation resulting in a decrease in covalent binding is an expected result in terms of cell and organism survival – a more complex system will have evolved in order to defend against such chemical attack. However, it should be noted that the end point of these assays was a measure of binding per mass of protein and so a comparison of this nature assumes equal activity of drug metabolism enzymes per μM of compound and an excess of macromolecules suitable for reactive metabolites to bind to.

Extrapolation of the covalent binding values using the method outlined in Usui, 2009, allows a comparison of the covalent binding values between different systems by using the quantitative measures as an attempt to separate known DILI causing compounds from those which do not. Overlap of the two groups of compounds following assessment of covalent binding and normalisation by dose indicates that the utility of this method at this level of simplicity is limited. As the compounds were chosen in order to create as large a gap as possible between the two groups, it would suggest that most compounds assessed for possible DILI using this method would fall into the middle area. It is therefore unsurprising that calculated values for AZD5438 occupied this space. A further refinement of the calculation may result in a more predictive model, for example if intracellular concentrations could be more accurately determined.

The implication of the sulfate metabolite of AZD5438 as being involved with reactive metabolite formation and resultant covalent binding was not confirmed by covalent

binding. Should the formation the sulfate conjugate result in the formation of a covalently binding reactive metabolite then it was in low amounts that were indistinguishable from the Phase I mediated covalent binding.

The structure of the reactive metabolite bound to protein could not be identified from these experiments, however it should be noted that a Phase I metabolite may be a primary, secondary, etc. metabolite and not just be one enzyme catalysed reaction away from the parent molecule. For example, one or more further Phase I biotransformations of P19 may be what is required to form the reactive species.

3.3. Toxicity in THLE-CYP cells

Incubation of AZD5438 with THLE-CYP cells showed that the test compound itself caused a decrease in mitochondrial reductase activity, even at the 0 hour time point. It can be expected that the zero time point is not in fact 0 hours but practically a short period of time is required for the samples to be taken subsequent to dosing. AZD5438 seemed to cause time and dose dependent decrease in cell viability (based on mitochondrial reductase activity) when compared to cells from all lines incubated in the absence of test compound.

This response would be expected due to the cytotoxic mechanism of AZD5438 and so comparison of metabolism related viability had to be done by comparison to the null cell controls. The transfected lines were also not normalised for CYP protein level or activity and so each THLE-CYP line can be compared to the null cell but should not be ranked against each other. After 48 hours incubation, THLE-CYP2D6 and -CYP3A4 both had IC_{50} values lower than that of the null cells (116, 43 and 241 μM, respectively) whereas THLE-CYP1A2 had a higher value (307 μM). An IC50 could not be calculated for THLE-CYP2C9 cells at this time point.

These data are interesting but require further investigation in order to both separate metabolism related response from parent compound effect and to ensure

mitochondrial reductase activity is directly proportional to cell viability in the presence of this compound.

3.4. Further research

It is clear that a Phase I reactive metabolite is formed by CYP2C9 and CYP3A4/5. Elucidation of the identity of this metabolite could be investigated further using a number of techniques. By incubating the structural analogues of AZD5438 (which were originally synthesised as part of the AstraZeneca CDK project), with the same range of *in vitro* systems, small changes in structure can be assessed for impact on covalent binding and cell toxicity. This would give valuable information into which moieties on AZD5438 are instrumental for reactive metabolite formation.

It was seen that neither of the prospective trapping agents (GSH or KCN) decreased the amount of covalent binding in microsomes. Other agents that are preferable to GSH or KCN for the trapping of certain types of reactive metabolite are available. Methoxylamine or semicarbazide can be used to trap aldehyde metabolites (Evans, 2004) and other agents based on peptides have successfully been used to trap a range of both 'soft' and 'hard' electrophiles (Yan, 2007; Mitchell M. , 2008). Performing an incubation of [^{14}C]-AZD5438 with HLM in the presence of one or more of these trapping agents, followed by HPLC-RAD-MS analysis (and possibly nuclear magnetic resonance spectroscopy if required) of any sample where binding has decreased should allow identification of the trapped species and thus an idea of the structure of the reactive species itself.

Synthesising radiolabelled structures of known primary metabolites of AZD5438 followed by their incubation in the covalent binding test systems used here could help elucidate the reaction steps needed for AZD5438 to become a reactive metabolite.

Further exploration of the effects of AZD5438 on THLE-CYP cells would be required to help show the link between AZD5438 reactive metabolite formation and cell toxicity. A covalent binding experiment with THLE-CYP cells would determine if cells

containing specific CYP isoforms actually produce reactive metabolites similar to those quantified in other systems of if the decrease in MTS assay response is independent from metabolism.

4. Conclusions

The potential of AZD5438 to form reactive metabolites under certain *in vitro* conditions has been readily shown. Systems that contained only Phase I metabolic enzymes, predominantly cytochrome P450 (CYP) 2C9 or CYP3A4, activated [^{14}C]-AZD5438 and caused covalent binding to incubation protein. A toxicity assay using cells that selectively express CYP enzymes showed toxicity of the parent compound to cells but failed to confirm whether or how much metabolism played a part.

A difference in metabolism between simple systems and more complex metabolic apparatus was apparent. When compared to binding after incubation with human liver microsomes (HLMs) alone, covalent binding of [^{14}C]-AZD5438 decreased when incubated in HLMs with glucuronidation capacity or with human liver S9 fraction and decreased by an even greater extent when incubated with whole human hepatocytes. The presence of alternative metabolic pathways seemed to offer protection against covalent binding of AZD5438, rather than detoxification by scavenging molecules (addition of trapping agents caused no change in binding values) or lack of access to the cell (hepatocyte uptake was observed).

Scaling from one system to another to truly compare reactive metabolite formation between systems is limited however and other data such as full metabolic pathway data should be taken into account.

Extrapolation of covalent binding values using simple literature calculations to predict drug induced liver injury (DILI) in patients showed how a more complex metabolic system affords protection from toxicity. HLM predictions showed a greater comparative risk of DILI than hepatocyte predictions.

The Phase II sulfate metabolite of AZD5438 which was initially identified as a structural alert for reactive metabolite formation was shown not to be significantly involved in the covalent binding of [^{14}C]-AZD5438.

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Appendix A1

HPLC-MS method for analysis of *in vivo* and *in vitro* incubation samples

Column type: Phenomenex; Synergi; 4µm; Polar RP80A; 150 x 4.6 mm

Mobile phase A: 0.1% formic acid in deionised water

Mobile phase B: Acetonitrile

HPLC gradient:

	Proportion of solvent (%)		
Cumulative time (minutes)	**Mobile phase A**	**Mobile phase B**	**Gradient**
Initial	90	10	Isocratic
0	90	10	Isocratic
15	80	20	Linear
25	80	20	Isocratic
30	10	90	Step
35	90	10	Step
40	90	10	Isocratic

Flow rate: 1.0 mL/min

Column temperature: 50°C

Radio flow-cell type: Liquid; 500 µL

Scintillant: Ultima Flo M

Scintillant/eluent ratio: 3:1

Mass spectrometer: Thermo LTQ Linear Ion Trap

Ionisation source/mode: Electrospray; positive and negative ion modes

HPLC-MS^n:

Full scan: 100-900 mu

Data dependent scan (-ve mode):

MS: 466.1 mu

MS^2: 386.1 mu

MS^3: 356.1 mu

MS^4: Most intense ion from MS^3

MS^5: Most intense ion from MS^4

Appendix A2

Buffer for *in vitro* assay incubations

Assay	System	Buffer	Additives
Metabolism	Hepatocytes in suspension	Leibovitz L-15	None
	Hepatocytes in culture	Williams's e w/o phenol red	10% FCS 1% penn/strep/neomycin 1% ITS 100 µM dexamethasone
	CYPs	0.1 M phosphate buffer	None
	SULTs	0.1 M phosphate buffer	None
	Cytosol	0.1 M phosphate buffer	None
Covalent binding	Hepatocytes in suspension	Leibovitz L-15	None
	Microsomes and S9 fraction	0.1 M phosphate buffer	10 mM magnesium chloride
Uptake	Hepatocytes in suspension	Krebs-Henseleit	25 mM sodium bicarbonate

All buffers at pH 7.4
CYPs; heterogeneously expressed human CYP450s
SULTs; heterogeneously expressed human sulfotransferases
FCS; foetal calf serum
penn/strep/neomycin; penicillin, streptomycin, neomycin
ITS; insulin, transferring, sodium selenide