Studies on the mechanism of flucloxacillin-induced liver injury

Thomas Campbell Chamberlain, BSc, MSc

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Newcastle University

Faculty of Medical Sciences

Institute of Cellular Medicine

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Abstract

Drug induced liver injury (DILI) due to the isoxazolyl β-lactam antibiotic, flucloxacillin, is a rare idiosyncratic adverse drug reaction. The underlying mechanism remains unclear but a recent association with the human leukocyte antigen class I allele, HLA-B*57:01, indicated a possible T-cell mediated reaction. This study aimed to identify further genetic determinants conferring susceptibility to this form of DILI and to study metabolic and immune mechanisms of this toxicity.

Flucloxacillin DILI cases (n=150) and matched population controls (n=282) were genotyped for HLA-B*57:01, confirming the previous association with disease (OR = 40.1, 95% CI 22.7 – 70.7). Cases negative for HLA-B*57:01 (n=26) were genotyped for HLA-B alleles and this analysis showed a borderline significant association with HLA-B*13:02 (p = 0.0376). Genotyping of all cases for additional immune-related candidate genes such as KIR3DL1/KIR3DS1 and for variants detected in limited exome sequencing studies, performed by others, resulted in confirmation of a significant difference in frequency compared with community controls (n=235) for a Caspase-5 polymorphism (rs45483102) (OR = 2.39 95% CI 1.22 – 4.68).

Reporter gene studies were performed to further investigate the ability of flucloxacillin to act as a ligand for the xenobiotic-sensing nuclear receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR). No flucloxacillin activation of CAR was observed but flucloxacillin was confirmed to be a PXR ligand and studies comparing PXR activation by the isoxazolyl penicillin's cloxacillin and dicloxacillin showed dicloxacillin to be a stronger PXR agonist than flucloxacillin. DILI cases due to cloxacillin (n=3) and dicloxacillin (n=2) were found not to possess the HLA-B*57:01 allele suggesting that the mechanism for DILI due to these drugs is different. Flucloxacillin metabolism pathways were studied using human liver microsomes, recombinant cytochrome P450 isoforms and rat B13/H cells which differentiate to a hepatocyte-like phenotype, with flucloxacillin penicilloic acid the major metabolite detected. Despite previous reports, formation of the metabolite 5'-hydroxymethyl flucloxacillin which was believed to be CYP3A4-mediated, could not be confirmed in any of the systems studied.

Preliminary studies on T-cell mediated responses to flucloxacillin, by exposure of peripheral blood mononuclear cells (PBMCs) from HLA-B*57:01 expressing flucloxacillin-DILI patients and from HLA-B*57:01 positive and negative flucloxacillin-naïve donors to flucloxacillin, indicated increased expression of interferon-γ at the RNA level in 2 out of 3 of the patient samples but not in controls. This finding was generally consistent with reported findings by others.

In summary, a novel HLA-B association involving some flucloxacillin DILI cases has been detected, HLA-B*57:01 does not appear to be an important risk factor for DILI due to other isoxazolyl penicillin's and the ability to act as a PXR agonist appears to be a general feature of these penicillin's so may not be directly relevant to the mechanism for flucloxacillin DILI. The confirmed association with caspase 5 may represent a minor additional risk factor for flucloxacillin DILI.

I hereby declare that all the work presented in this thesis is my own unless stated otherwise within the text or acknowledged accordingly within the references. The data has not been submitted previously for any alternative degrees.

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Table of Contents

List of Figures

List of Tables

List of abbreviations

ABC	ATP binding cassette
ADR	Adverse Drug Reaction
ALF	Acute Liver Failure
AFND	**A**llele Frequency Net Database
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Antigen presenting cells
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BEC	Biliary epithelial cell
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CAR	Constitutive active/androstane receptor
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CI	Confidence interval
CITCO	6-(4-**c**hlorophenyl)**i**midazo[2,1-*b*][1,3]**t**hiazole-5-**c**arbaldehyde *O*-(3,4 dichlorobenzyl)**o**xime
Ct	Threshold cycle
CYP	Cytochrome P450
DAMP	Damage associated molecular pattern
DEPC	Diethyl pyrocarbonate
DILI	Drug induced liver injury
DILIN	Drug-Induced Liver Injury Network
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat

EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot
ER	Everted repeat
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate
GWAS	Genome-wide association studies
HIV-1	Human immunodeficiency virus-1
HLA	Human leukocyte antigen
HLM	Human liver microsome
HPLC	High performance liquid chromatography
iDILIC	international Drug Induced Liver Injury Consortium
IFNγ	Interferon-γ
IL	Interleukin
iSAEC	international Serious Adverse Event consortium
KC	Kupffer cells
KIR	Killer immunoglobulin-like receptors
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MDR	multidrug resistance
MHC	Major histocompatibility complex
MAF	Minor allele frequency
MOPS	3-[N-morpholino] propanesulfonic acid
MRP	Multidrug resistance-associated protein
NK	Natural Killer
NSAID	Non-steroidal anti-inflammatory drugs
NW-EU	North-western European
OR	Odds ratio
p-i	pharmacological interaction

PBMC	Peripheral blood mononuclear cell
PBREM	Phenobarbital-responsive enhancer molecule
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
POPRES	Population Reference Sample
PPD	Protein purified derivative of tuberculin
PXR	Pregnane X receptor
qPCR	Quantitative real-time PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUCAM	Roussel Uclaf Causality Assessment Method
RXR	Retinoid X receptor
SNP	Single nucleotide polymorphism
SSP	Sequence-specific primer
TBE	Tris-borate/EDTA electrophoresis buffer
TBL	Total bilirubin
TCR	T-cell receptor
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
TNF	Tumour necrosis factor
ULN	Upper limit of normal
UGT	Uridine 5' diphosphate-glucuronyltransferase
XREM	Xenobiotic-responsive enhancer module

Chapter 1. Introduction

1. Introduction

1.1 Adverse Drug Reactions

Adverse Drug Reactions (ADRs) are a common and significant cause of morbidity and mortality in healthcare facilities worldwide. In the UK it is estimated ADRs cause up to 10 000 deaths each year and are a large financial burden to the NHS (Pirmohamed *et al.*, 2004). There is no standard definition of an ADR but the most widely used definition remains the 1972 World Health Organisation (WHO) defined ADRs as "any response to a drug that is noxious and unintended and occurs at doses used for the prophylaxis, diagnosis or therapy". A more recent definition was proposed by Edwards and Aronson that aimed to include error as a source of an adverse event as well as taking into account the effect of the additional 'inactive' constituents of drugs and possible contaminants in medicines (Edwards and Aronson, 2000).

ADRs that result in hospitalisation can occur in both outpatients, therefore being the cause of admission, or with in-patients after hospital admission (Davies *et al.*, 2009). A meta-analysis of 39 prospective studies by Lazarou et al put the total incidence of ADR hospitalisation at 6.7% with 2.1% occurring after hospital admission (Lazarou *et al.*, 1998). In the UK, a study of 19000 admissions found that ADRs were estimated to be responsible for 6.5% of hospital admissions in the UK (Pirmohamed *et al.*, 2004). A prospective study of 3695 in-patients estimated the incidence of an ADR occurring to hospitalised patients to be 14.7%, resulting in prolonged hospitalisation and financial strain on the NHS estimated at approximately £637 million per year (Davies *et al.*, 2009). A national time-trend study of ADRs as the cause of admission to UK hospitals, reported that the overall incidence of ADRs increased by 45% in the period 1998 – 2005 (Patel *et al.*, 2007).

Drugs most frequently implicated in causing ADR hospitalisations are non-steroidal anti-inflammatory drugs (NSAIDs), the most common ADR being gastrointestinal bleeding caused by aspirin (Pirmohamed *et al.*, 2004). Drugs most frequently associated with in-patient ADRs are warfarin, opioid analgesics, and loop diuretics (Davies *et al.*, 2009).

ADRs tend to be more common in elderly patients, which may be reflected by a higher prevalence of concomitant chronic conditions like renal, hepatic or cardiac disease and the prescription of multiple drugs for the treatment of chronic conditions.

ADRs can be generally classified as being either type A or B reactions. Type A reactions are common, dose-dependent, predictable in terms of the pharmacology of the drug and usually reproducible in animal models. Type B or idiosyncratic reactions typically do not have a dose-relationship, are unpredictable and affect only a minority of those using the drug. A common feature of idiosyncratic adverse reactions is a delay of onset of toxicity, typically weeks or months after the first exposure to the drug, whereas toxicity usually occurs rapidly with type A reactions (Uetrecht, 2007).

Although less common than type A reactions, idiosyncratic reactions are proportionally more severe and have a higher mortality rate (Pirmohamed and Park, 2001). In most cases, the factors influencing idiosyncratic drug reactions are unknown or seldom characterised, but genetic variations leading to a predisposition to an ADR are likely, along with other host and environmental factors.

Idiosyncratic reactions are difficult to detect during drug development. Unlike Type A reactions, they are difficult to reproduce in animal models and, owing to the small number of individuals affected, they are rarely detected in clinical trials which are limited to a few thousand people. This poses a significant problem to the drug industry as these reactions are nearly always only discovered after the drug is on the market and in use.

1.2 Drug Induced Liver Injury (DILI)

1.2.1 *Introduction*

The liver is considered the most important organ in drug toxicity. This is due to the physiological functions that it performs and its anatomical location, situated at a point where systemic circulation meets the site of absorption from the gastrointestinal tract. 75% of blood reaching the liver arrives via the hepatic portal vein bringing with it xenobiotics absorbed by the gut (Jaeschke *et al.*, 2002). The liver is involved in

numerous physiological functions that are vital for metabolic processes and homeostatic regulation of the body. Examples of these functions include the synthesis, storage and secretion of various biochemicals, metabolic regulation of carbohydrates and lipids, and the biotransformation and excretion of endogenous and exogenous substances. As the main site of xenobiotic metabolism and detoxification, the liver is regularly exposed to toxins making it an important site for ADRs.

Drug-induced liver injury (DILI) is a rare but potentially serious adverse condition and is the most common reason for termination of drug development and post market drug withdrawal (Watkins, 2005). Over 1000 drugs or herbal products have been associated with DILI which manifests predominantly with a hepatocellular, cholestatic or mixed pattern of injury, although it can mimic all forms of acute and chronic liver disease (Abboud and Kaplowitz, 2007). DILI is the leading cause of acute liver failure (ALF) in Western countries accounting for 52% of ALF in the U.S, but the majority of these cases relate to overdose of paracetamol (Ostapowicz *et al.*, 2002; Russmann *et al.*, 2009). The majority of DILI is caused by idiosyncratic drug reactions rather than overt dose-related toxicity as with paracetamol toxicity. One particular reason for this is the improvement in drug screening and testing during development that eliminates many of the compounds that cause dose-related hepatotoxicity. Conversely, idiosyncratic DILI is difficult to detect during both drug development, owing to the low incidence and detection in clinical trials, and in a post-market setting due to limited follow up procedures and challenges that exist in its diagnosis.

Recently the US National Centre for Toxicological Research (NTCR) has assessed prescription drugs, approved for at least ten years, for their potential to cause DILI (Chen *et al.*, 2011). A Liver Toxicity Knowledge Base (LTKB) benchmark dataset was established by searching the DailyMed database using a set of keywords commonly used in FDA labels for DILI. From this, 287 prescription drugs were highlighted, 137 of which were classified as giving the most DILI concern and included drugs that had been withdrawn or given ‘black-box’ warnings highlighting a potential risk of serious injury or death. Examples of drugs that have been withdrawn from use because of idiosyncratic hepatotoxicity include bromfenac, lumiracoxib, troglitazone and ximelagatran while examples of drugs given black-box warnings include amiodarone, didanosine, isoniazid and tolcapone (Chen *et al.*, 2011).

1.2.2 *Epidemiology of DILI*

It is difficult to determine the true incidence of DILI due to under-reporting, a lack of standardised and universally accepted diagnostic criteria and the relatively low frequency of incidence compared to other common types of liver injury (Fontana, 2010). The majority of DILI reactions are idiosyncratic and therefore extremely rare, with the incidence to be estimated between 1 in 10000 and 1 in 100000 individuals exposed to a particular drug (Larrey, 2002). Most studies of DILI incidence are retrospective and suffer from incomplete data registries, highly variable diagnostic criteria and the extent that other forms of liver disease have been excluded (Bjornsson, 2010). As a result, true incidence of DILI is probably underestimated as the majority of cases are typically asymptomatic outpatients whereas severity is probably overestimated due to focus of DILI hospitalisation in studies from tertiary referral centres (Fontana, 2010). Several retrospective studies of DILI have been performed and describe a crude incidence of DILI in the range of 1 – 3 per 100000 inhabitants, although these figures are potentially an underestimation (de Abajo *et al.*, 2004; De Valle *et al.*, 2006; Hussaini *et al.*, 2007). Prospective population-based studies into DILI are rare. Until a recently, there had been only one study of DILI incidence within a general population. Sgro and colleagues estimated a crude incidence of hepatic ADRs in outpatients to be approximately 14 in 100000 people per year, based on data collected by 139 physicians in France over a period of 3 years in a study population of 81301 (Sgro et al., 2002). Recently, a second prospective population-based cohort study into DILI incidence was published by Bjornsson et al (Bjornsson *et al.*, 2013). This study recruited DILI cases nationwide in Iceland over a period of 2 years and, in contrast to the French study, consisted of DILI reports from inpatients as well as outpatients. The crude incidence of DILI, estimated as approximately 19 per 100000 people per year, is similar but slightly higher than that observed in the French study. 82% of the patients developed DILI as outpatients, with approximately a quarter of those hospitalised because of their symptoms, mainly due to jaundice. Incidence was standardised for age and showed a clear trend of increasing DILI incidence with age. Amoxicillin-clavulanate (augmentin) was found to be the most common causative agent as has been reported elsewhere (Andrade *et al.*, 2005; Chalasani and Bjornsson, 2010). However when incidence to individual drugs was quantitated, azathioprine (1 of 133 users) and infliximab (1 of 148

users) where found to have the highest incidence whereas incidence of amoxicillin-clavulanate DILI was 1 per 2350 treated patients.

There have been three prospective registries formed to collect information with the aim to enhance our understanding of DILI. These are the Regional Registry of Hepatotoxicity in Spain, the U.S. Acute Liver Failure Study Group (ALFSG), and the Drug-Induced Liver Injury Network (DILIN) (Bell and Chalasani, 2009). Data from these registries has provided detailed information regarding the incidence and types of DILI, patient characteristics, risk factors and what drugs are implicated in causing disease. Data from the Spanish registry estimated an overall annual incidence of hepatotoxicity to be 34.2 ± 10.2 cases per 10^6 inhabitants per year (Andrade *et al.*, 2005). The ALFSG has provided data regarding drug-induced ALF in the U.S. Two studies have estimated idiosyncratic liver disease is responsible for approximately 11 - 13% of ALF and is the second most common cause of ALF after paracetamol toxicity (Ostapowicz *et al.*, 2002; Reuben *et al.*, 2010). The severity of drug-induced ALF is emphasised by a mortality rate among cases of almost 34%.

1.2.3 *Risk factors for DILI*

Other than genetic risk factors, which are discussed in detail in section 1.4, there are a number of factors that may facilitate the development of DILI. The set-up of various DILI networks and registries that allow an ongoing recruitment of patients have helped facilitate the study of risk factors in DILI including a range of host-related, environmental and compound-specific factors.

There exists conflicting evidence regarding the role that age has as a risk factor for the susceptibility of DILI. The relevance of age as a risk factor may be related to the particular DILI-causing drug and there are a number of drugs where there appears to be a trend of increasing DILI incidence with increasing age (Chalasani and Vuppalanchi, 2013). Examples of these include isoniazid, amoxicillin-clavulanate, halothane and flucloxacillin. A 7-year study in a US tuberculosis centre shows that for isoniazid, incidence of hepatotoxicity rose from 4.4 per 1000 for patients aged 25-34 to 20.8 per 1000 for patients aged 50 and above (Fountain *et al.*, 2005).

The recent population-based study by Bjornsson et al, described above, showed an increasing incidence of DILI when standardised for age. DILI incidence for individuals aged 15-24 was estimated to be 6.5 per 100000 while for individuals aged 80 and above it was found to be 41 per 100000 (Bjornsson *et al.*, 2013). However, findings of a study from the Spanish Registry of Hepatotoxicity did not show a distinct age difference for DILI susceptibility in a prospective cohort of 650 patients (Lucena *et al.*, 2009). However, both of these studies showed an apparent relationship that increasing age is associated with a cholestatic phenotype whereas younger patients are more likely to display hepatocellular damage although no explanations are given for these observations. The reasons for increasing age as a risk factor for DILI are unclear. There doesn't appear to be a significant difference in levels or activity of hepatic metabolising enzymes in the elderly but a decrease in renal function and hepatic blood flow may lead to increased concentrations of drugs in the liver (Chalasani and Bjornsson, 2010). Mean prescription rate has been correlated with age and polypharmacy may play a role in DILI risk but this is also unclear.

Various epidemiological studies have reported a higher prevalence of DILI in females, however a systematic review of multiple studies taking into account DILI from all causes failed to show gender differences for DILI prevalence (Chalasani and Vuppalanchi, 2013). It has been suggested that females may often be over-represented in DILI epidemiological studies due to prescription patterns but for some individual drugs a higher prevalence may have a biological basis (Chalasani and Bjornsson, 2010).

Obesity has been linked to increased DILI due to certain drugs but there appears to be no apparent overall trend as a general risk factor for DILI. However, it has been suggested that obesity is linked to a poorer outcome from severe DILI and underlying fatty liver disease caused by obesity may be a potential factor for increased DILI susceptibility. CYP2E1 activity is increased in obese individuals and may be an additional risk factor where CYP2E1 has a role in the mechanism of DILI as suggested with paracetamol and halothane (Jaeschke *et al.*, 2002).

It is unclear what effect underlying comorbidities have on susceptibility to DILI and again it is likely to depend on the individual causative drug. Underlying diabetes, and chronic liver diseases such as those caused by non-alcoholic fatty liver disease (NAFLD) and hepatitis C infection are potential risk factors for DILI.

With regards to environmental risk factors, the link between alcohol consumption and paracetamol-induced hepatotoxicity has been extensively studied. Alcohol consumption is a criteria used in the RUCAM scoring system widely used to diagnose cases of DILI, however, the effect of alcohol consumption as a risk factor for idiosyncratic DILI has not been shown except for a few drugs that, like paracetamol, are linked to alcohol-induced induction of CYP2E1.

1.2.4 *Prediction and diagnosis of DILI*

There are many challenges regarding the prediction, detection and diagnosis of DILI both during drug development and for clinicians in the post marketing setting. During early drug development many probable hepatotoxic compounds are identified and discarded during the preclinical phase. Drug companies have developed a range of preclinical tests to try and identify potential unpredictable hepatotoxins and reactive metabolites. These include *in silico* assays that identify potentially hazardous toxicophores, *in vitro* covalent binding and glutathione adduct formation assays, bile salt export pump (BSEP) inhibition assays, and studies of mitochondrial function and oxidative stress generation (Park *et al.*, 2011; Przybylak and Cronin, 2012; Kaplowitz, 2013). Other, novel methods and technologies are being developed that look to discover accurate and reproducible DILI-predicting biomarkers. One such area is the 'omic-based technologies such as metabolomics, transcriptomics and proteomics. Animal testing is used to discover and eliminate hepatotoxic compounds during drug development. High drug dosage testing is usually successful at identifying hepatotoxic compounds that are likely to cause intrinsic, predictable liver injury in man, but is far more unsuccessful at identifying compounds that could cause rare, idiosyncratic hepatotoxicity. Identification of idiosyncratic hepatotoxins is also rare during the clinical trial phases of drug development. This is due to the individual nature and rarity of the reactions. Typically, clinical trials involve a limited sample size of approximately 2000 individuals that are treated for a short period of time. Many idiosyncratic DILI reactions have a typical delay of onset and occur at a frequency of 1 in 10000 individuals or less. This means idiosyncratic adverse effects are likely to not be discovered until the drug is given approval and has been on the market several months or years.

Even when potential drug-induced liver reactions are presented to clinicians it is difficult to identify whether a drug is the cause of the reaction with complete certainty. DILI can manifest as a wide range of symptoms that are often not drug specific and may mimic various other types of liver disease. In addition, the clinical signature of any given drug may vary between individuals (Garcia-Cortes *et al.*, 2011). The assessment of a patient that has potential DILI requires a complete, detailed patient and drug-use history. The drug history should include the use of any prescriptions, over-the-counter, herbal or alternative medications (Lee, 2003). A temporal relationship should be sought between the initiation of a drug therapy, onset of DILI symptoms, the course of the reaction and the response to drug withdrawal. The patient history should take into account risk factors for individual susceptibility to DILI such as age, gender, pregnancy, alcohol use or underlying disease (Chalasani and Bjornsson, 2010). Before a DILI diagnosis can be confirmed, it is important to exclude any other liver disease as the cause of injury. This includes viral hepatitis (A, B, C and E), autoimmune liver diseases, biliary diseases (e.g. cholelithiasis), alcohol abuse, non-alcoholic fatty liver disease (NAFLD) or any hereditary conditions (Tajiri and Shimizu, 2008; Verma and Kaplowitz, 2009).

There is still a lack of sensitive, reliable, highly specific biomarkers to detect and diagnose DILI. Biochemical liver parameters are commonly used as biomarkers for its diagnosis. Elevations of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum total bilirubin (TBL) and alkaline phosphatase (ALP) may be indicative of liver injury, although small increases in liver test abnormalities may be transient and resolve even with continuous use of the drug through a process of adaptation (Aithal *et al.*, 2011). To support the diagnosis of DILI, various causality assessment scoring systems have been developed to determine the likelihood that liver injury has been caused by a drug. The most commonly used of these systems, and regarded as generally the most reliable, is the Roussel Uclaf Causality Assessment Method (RUCAM) developed in 1993 (Danan and Benichou, 1993; Aithal *et al.*, 2011). The scale was established by the Council for International Organisations of Medical Sciences (CIOMS) from a panel of experts based on international DILI consensus criteria. The scheme provides an initial semi-quantitative classification of liver injury by dividing DILI into three classes - hepatocellular, cholestatic or mixed pattern of disease, on the basis of ALT and ALP levels at the onset of disease. The categorisation of DILI

is based upon the calculation of the '*R*' ratio of serum ALT to ALP with respect to their upper limits of normal (ULN), so that:

R = (ALT/ULN) / (ALP/ULN)

Generally, an *R* value ≥ 5 defines hepatocellular injury, ≤ 2 defines cholestatic damage and cases with an *R* value between 2 and 5 show a mixed phenotype. When calculating *R* ratio's it is important to consider the time point within the course of the illness that the liver function tests are performed. Occasionally, initial ALT to ALP levels may not coincide with peak levels further into the course of the disease. In some cases there may be a greater initial ALT increase during early disease progression and often cases may be misdiagnosed as hepatocellular when tests at a later time point may indicate that they were actually mixed or cholestatic (Fontana *et al.*, 2010).

A RUCAM score is calculated by placing a score of -3 to 3 on each of the following seven key criteria (the *R*-ratio is used to support scoring in the first three criteria) (Garcia-Cortes *et al.*, 2011):

- Time to DILI onset (calculated from the first day of drug treatment to day of onset of first symptom, or laboratory test abnormality)
- Course of illness - rate of resolution after medication stopped, interpreted as the time taken for a 50% decrease of liver enzyme levels from the peak value
- Risk factors - e.g. age > 55 years, alcohol consumption
- Concomitant drug use
- Exclusion of other aetiologies – e.g. viral hepatitis, biliary obstruction, alcoholic liver disease
- Existing information of the drug's hepatotoxic potential – e.g. if drug has a label-warning for DILI causing potential
- Response to rechallenge – usually inadvertent rechallenge of causative agent

Detailed elements of the scoring system are displayed in Table 1.1. A final score, in a range of -8 to +14, is produced to which the degree of drug association as the cause of

liver injury is interpreted as highly probable (score > 8), probable (score of 6 – 8), possible (3 – 5), unlikely (1 – 2) or excluded (≤ 0).

Table 1.1 – Details of the Roussel Uclaf Causality Assessment Method (RUCAM) scale scoring system for DILI (adapted from (Aithal *et al.*, 2011))

Hepatocellular Injury		**Cholestatic or Mixed Liver Injury**	
1. Temporal relationship of start of drug to ALT>2x ULN	**Score**	**1. Temporal relationship of start of drug to ALP>2x ULN**	**Score**
Initial treatment 5–90 days; subsequent treatment course: 1–15 days	2	Initial treatment 5–90 days; subsequent treatment course: 1–90 days	2
Initial treatment <5 or >90 days; subsequent treatment course: >15 days	1	Initial treatment <5 or >90 days; subsequent treatment course: >90 days	1
From cessation of drug: ≤15 days, or ≤15 days after subsequent treatment	1	From cessation of drug: ≤30 days, or ≤30 days after subsequent treatment	1
Otherwise	0	Otherwise	0
2. After drug cessation- difference between peak ALT and upper limits normal		**2. After drug cessation - difference between peak ALP or total bilirubin and ULN**	
Decreases >50% within 8 days	3	Decreases ≥50% within 180 days	2
Decreases >50% within 30 days	2	Decreases <50% within 180 days	1
No information or decrease >50% after >30 days, or inconclusive	0	Persistence or increase or no information	0
Decrease <50% after 30 days or recurrent increase	-2	If drug is continued – inconclusive	0

3. Risk factors (Scoring is the same for Hepatocellular and Cholestatic/Mixed Injury)	**Score**
No alcohol use	0
Alcohol use	1
Age ≤55 years	0
Age >55 years	1

4. Concomitant drug (Scoring is the same for Hepatocellular and Cholestatic/Mixed Injury)	**Score**
No concomitant drug administered	0
Concomitant drug with suggestive or compatible time of onset	-1
Concomitant known hepatotoxin with suggestive or compatible time of onset	-2
Concomitant drug with positive rechallenge or validated diagnostic test	-3

5. Nondrug causes: Six are primary: recent hepatitis A, B, or C, biliary obstruction, acute alcoholic hepatitis (AST > 2x ALT), recent hypotension Secondary group: Underlying other disease; possible CMV, EBV or HSV infection **(Scoring is the same for Hepatocellular and Cholestatic/Mixed Injury)**	**Score**
All primary and secondary causes reasonably ruled out:	2
All 6 primary causes ruled out	1
4 or 5 primary causes ruled out	0
< 4 primary causes ruled out (*max. negative score for items 4 and 5: –4)*	-2
Nondrug cause highly probable	-3

6. Previous information on hepatotoxicity of the drug in question (Scoring is the same for Hepatocellular and Cholestatic/Mixed Injury)	**Score**
Package insert or labelling mention	2
Published case reports but not in label	1
Reaction unknown	0

7. Rechallenge (Scoring is the same for Hepatocellular and Cholestatic/Mixed Injury)	**Score**
Positive (ALT doubles with drug in question alone)	3
Compatible (ALT doubles with same drugs as given before initial reaction) +1	1
Negative (Increase in ALT but <2x ULN, same conditions as when reaction occurred)	-2
Not done, or indeterminate result	0

Total (range of algebraic sum: –8 to +14)

Score Interpretation: Highly probable >8; Probable 6–8;Possible 3–5; Unlikely 1–2; Excluded <0

1.2.5 *Classification of DILI*

Predictable, high incidence, dose-related hepatotoxic events caused by drugs, e.g. paracetamol, usually occurs rapidly, within a few days of drug administration and result from direct toxicity of the drug or its metabolites. Conversely, idiosyncratic hepatotoxic events occur at low incidence with varying periods of latency depending on the drug in question and type of injury, ranging from a few days to a year.

DILI as a term, describes a wide range of liver injury but is broadly classified as hepatocellular, cholestatic or 'mixed'-pattern. The recent epidemiological study by Bjornsson et al found 42% of DILI-patients to have hepatocellular injury, 32% with cholestatic type injury and 26% with a 'mixed'-pattern (Bjornsson *et al.*, 2013). Although DILI is represented primarily by hepatocellular or cholestatic injury, drug toxicity should be considered when any form of hepatobiliary injury, e.g. fibrosis, granulomas and steatosis, is presented (Abboud and Kaplowitz, 2007).

1.2.5.1 Hepatocellular Injury

A hepatitis pattern of injury indicates hepatocellular damage that consists of cellular degeneration and death through pathways such as necrosis, apoptosis and steatosis. Although the exact mechanism for hepatotoxicity is not fully clear, it seems in many instances a drug is metabolised to a reactive metabolite which then proceeds to interact with various cellular components such as proteins, lipids and nucleic acids. These interactions disrupt important cellular processes such as mitochondrial function, leading to cell death and possible liver failure (Holt and Ju, 2006).

Patients may present as asymptomatic or may experience fatigue, pain in the right upper quadrant, jaundice or in severe cases, show signs of coagulopathy and encephalopathy indicating ALF (Verma and Kaplowitz, 2009). Examples of common drugs that have been known to cause a hepatocellular pattern of DILI include rifampicin, isoniazid, statins and ibuprofen (Devarbhavi, 2012).

Hepatocellular liver damage is characterised by an increase in serum ALT levels, an indication of enzyme release from dying or dead hepatocytes. Hepatocellular damage is

classified as ALT levels greater or equal to three times the ULN, and where the ratio of ALT:ALP ≥ 5. ALP is usually not markedly elevated in cases of hepatocellular injury and as such could be tentatively used to distinguish between hepatocellular and cholestatic/mixed episodes. However, it has been suggested that a better indicator of hepatocellular injury may be to define it through the use of R > 5 (ALT X ULN / ALP X ULN) since increases in ALP are occasionally observed in some serious cases of hepatocellular injury (Kaplowitz, 2013). Care must also be taken when interpreting increased ALT levels as it does not always correlate to the severity of liver disease (Verma and Kaplowitz, 2009). This is because hepatocytes have a regenerative capacity to heal damage and develop an adaptive tolerance to damage. Impaired liver function is a more accurate indicator and can be measured by serum bilirubin levels (Navarro and Senior, 2006). Bilirubin levels are controlled by functions of the liver which remove bilirubin from the blood to bile. If damaged, this control is lost and increased levels of bilirubin can be found in the blood (hyperbilirubinaemia) which presents symptomatically as jaundice. Hyman Zimmerman described that there is a mortality rate of approximately 10% when hepatocellular injury is accompanied by jaundice and bilirubin levels ≥ 3 X ULN. Referred to as 'Hy's Law', it is used by the FDA as a guide to monitoring hepatotoxicity, with recent studies confirming its validation as an indicator for DILI prognosis (Bjornsson and Olsson, 2005).

1.2.5.2 Cholestatic DILI

The cholestatic pattern of injury may arise from a functional defect in bile formation in the hepatocytes or from damage to cholangiocytes resulting in inhibition of drug/bilirubin/bile salt transport and canalicular excretion, causing build-up of bile acids and obstruction of bile ductules or ducts (Zollner and Trauner, 2008). The intracellular build-up of drug metabolites/bile acids leads to further hepatotoxicity (Ansede *et al.*, 2010). In contrast to hepatocellular damage, cholestatic injury is characterised by a more prominent rise in serum levels of ALP not ALT (ALP ≥ 2 X ULN), with an ALT: ALP ratio ≤ 2. The main symptoms of cholestasis are jaundice and pruritus, along with possible acute abdominal pain associated with acute biliary obstruction (Abboud and Kaplowitz, 2007). However, drug-induced cholestasis can mimic other intrahepatic or extrahepatic forms of cholestasis so a detailed drug history is vital for diagnosis and

avoidance of prolonged exposure of the toxic drug (Padda *et al.*, 2011). Cholestasis tends to be less severe than hepatocellular injury with the majority of patients recovering fully after withdrawal of the offending drug. However, the course of disease is often much more prolonged than hepatocellular injury and may take several months to be fully resolved after drug withdrawal. It has been suggested that cholestatic damage is more persistent due to the slower repair and regenerative capacity of cholangiocytes compared to hepatocytes (Abboud and Kaplowitz, 2007). In rare cases, long-term progressive damage to small bile ducts may lead to a condition termed 'vanishing duct syndrome' and ductopenia (Ramachandran and Kakar, 2009). Some examples of drugs that cause acute cholestasis include amoxicillin-clavulanic acid (augmentin), flucloxacillin and erythromycin. Chlorpromazine has been implicated in chronic cholestasis, although numerous other drugs can cause chronic episodes on rare occasions (Kaplowitz, 2013).

1.2.5.3 Mixed pattern of DILI

The mixed pattern of DILI is characterised by the presence of both acute hepatocellular and cholestatic injury. Hepatitis usually resembles an atypical or granulomatous form and there is a moderate elevation of both ALT and ALP with an ALT / ALP ratio between 2 and 5 ($2 < R < 5$) (Kaplowitz, 2013). Drugs that cause cholestatic injury may also present a mixed pattern of injury and vice versa (Verma and Kaplowitz, 2009). Examples include flucloxacillin, amoxicillin-clavulanic acid, diclofenac and carbamazepine (Bjornsson and Olsson, 2005). Fatalities have been recorded in cases of mixed pattern DILI but mortality is generally lower than in cases of purely hepatocellular or cholestatic damage (Bjornsson and Olsson, 2005).

1.3 Pathogenesis of DILI

1.3.1 *Overview of DILI pathogenesis*

One of the challenges preventing the full understanding of the mechanism of DILI is that the pathways that lead to symptomatic disease are usually not just the result of the initial drug toxicity response. Most forms of DILI are multicellular events and involve several mechanisms with complex interactions (Russmann *et al.*, 2009). To gain a greater understanding of the mechanisms of DILI requires an integrated understanding of the chemical, biochemical, molecular, cellular and physiological pathways that are active (Antoine *et al.*, 2008). As much as there is still unknown, the pathogenesis of DILI can broadly be categorised into two major pathways. The first involves the intrinsic hepatotoxicity of a drug or more commonly its metabolites in susceptible individuals. The second mechanism is the involvement of the immune system in adverse immune reactions causing inflammation and hepatic injury (Holt and Ju, 2006). Immune mediated reactions usually have characteristic symptoms of drug hypersensitivity such as fever, rash, eosinophilia and a rapid response to drug rechallenge that are absence in cases of non-immune, 'metabolic'-DILI (Walgren *et al.*, 2005). Both hepatocellular and cholestatic injury can result from immune-mediated reactions or non-immune mediated DILI.

High incidence, dose-related, direct hepatotoxicity is almost always detected in preclinical studies during drug development meaning that the overwhelming majority of available DILI causing drugs are associated with rare, idiosyncratic hepatotoxicity where injury is unrelated to the pharmacology of the drug. Therefore, for the purpose of this report the main focus will be on drugs and the mechanisms that cause idiosyncratic drug hepatotoxicity of which the mechanisms are particularly poorly understood.

1.3.2 *Non-immune mechanisms of drug hepatotoxicity*

1.3.2.1 Drug metabolism and reactive metabolites

One of the liver's major functions is the metabolism and clearance of xenobiotics and consequently it is regularly exposed to high concentrations of potentially toxic drugs. To facilitate this function, the liver has an abundance of metabolising enzymes capable of phase I or phase II metabolism. Through these pathways, lipophilic compounds undergo biotransformation to water-soluble derivatives that can be readily excreted. Although, generally, these pathways are detoxifying, occasionally metabolism can adversely lead to bioactivation of a drug producing a more reactive metabolite than the parent compound (Antoine *et al.*, 2008). Reactive metabolites may cause toxicity by interacting with cellular macromolecules such as proteins, lipids or nucleic acids causing protein dysfunction, DNA damage, mitochondrial dysfunction or oxidative stress (Srivastava *et al.*, 2010). In most instances of immune-mediated DILI, reactive metabolites are also thought to be more important in the initiation of immune pathways than the parent drug itself (Amacher, 2012).

The primary phase I metabolism pathway of xenobiotics, is oxidative biotransformation catalysed by the cytochrome P450 (CYPs) enzyme family. Completion of the human genome project has confirmed that in humans there are 57 functional CYP genes grouped into 18 families and 44 subfamilies (Guengerich, 2008; Zanger and Schwab, 2013). As well as playing a key role in the biotransformation of xenobiotics, CYP enzymes also have many physiological roles and act on endogenous compounds. Important roles include cholesterol metabolism, bile acid biosynthesis and steroid hormone biosynthesis and metabolism (Nebert and Russell, 2002). CYP enzymes are responsible for the primary metabolism of approximately 75% of commercially available drugs. Of this, about 95% are metabolised by five CYP450 enzymes, namely CYP3A4 (including CYP3A5 when expressed), CYP2D6, CYP2C19, CYP2C9 and CYP1A2 (Guengerich, 2007). Examples of DILI-causing drugs that undergo CYP450 metabolism are shown in **Table 1.2**. It has been reported that drugs that undergo significant hepatic metabolism are potentially more hepatotoxic than drugs that undergo lesser degrees of hepatic metabolism (Lammert *et al.*, 2010).

Table 1.2 – Examples of DILI-causing drugs reported in a prospective study by DILIN that are known to undergo significant CYP450 metabolism (Chalasani *et al.*, 2008; Zanger *et al.*, 2008)

Drug	P450
Atorvastatin	3A4/5
Celecoxib	2C9, 3A4/5
Diclofenac	2C9
Duloxetine	1A2
Ibuprofen	2C9, 2C8
Nevirapine	2B6
Phenytoin	2C9, 2C19
Valproate	2C9, 2A6, 2B6

Drug oxidation by CYP450s can lead to the formation of electrophilic intermediate metabolites, free radicals, reactive oxygen species (ROS), epoxides and quinones (Attia, 2010). Such compounds have the ability to undergo various reactions including the covalent binding of reactive species with nucleophilic sites in proteins and nucleic acids, or the depletion of reduced glutathione leading to oxidative stress. These reactions have a direct adverse effect on organelles such as the mitochondria leading to cellular necrosis or the activation of apoptotic pathways (Kaplowitz, 2004). In addition to cytochrome P450 enzymes, reactive metabolites can be produced by other phase I metabolising enzymes including flavin-containing monooxygenases, peroxidases and amine oxidases (Tang and Lu, 2010).

Phase II drug-metabolising enzymes are important for conjugation of electrophilic products of phase I metabolism to aid excretion. They are rarely implicated in producing reactive species themselves unless their function is compromised. An exception to this is the production of acyl glucuronides by uridine 5' diphosphate-glucuronyltransferase (UGT) enzymes, particularly with drugs that contain carboxylic acid functional groups (Russmann *et al.*, 2009). Examples of drugs that produce reactive acyl glucuronide conjugates are NSAIDs, benoxaprofen or bromfenac which were both promptly withdrawn after several cases of fatal hepatotoxicity (Walgren *et al.*, 2005). However, carboxylic acid containing NSAIDs, such as ibuprofen and diclofenac, are still available and have been associated with DILI (Aithal *et al.*, 2004).

The bioactivation of a drug to a reactive metabolite doesn't necessarily lead to toxicity. In balance with bioactivation pathways are detoxifying defence mechanisms involving phase I and phase II metabolising enzymes and drug transporters. Hence, genetic and environmental factors leading to inter-individual differences in the ability to metabolise and clear particular xenobiotics may increase an individual's susceptibility to DILI. Decreased capacity to metabolise or clear a drug may cause an increased build-up and exposure to toxic drugs or metabolites. Variation in CYP450 metabolising capacity can have a significant impact on DILI susceptibility.

1.3.2.2 Mitochondrial disruption and cellular stress responses

Some drugs and reactive drug metabolites have been shown to cause hepatotoxicity because of reactions with hepatocyte mitochondria. Mitochondria are present in high concentrations in hepatocytes where they produce the large amounts of adenosine triphosphate (ATP) required as an energy source for the liver to perform its many roles. Mitochondria perform numerous other roles vital to cell survival such as control of cell apoptosis pathways (Tang and Lu, 2010). It has been suggested that the double lipid bilayer membrane found in mitochondria is a favourable environment for accumulation of lipophilic drugs and metabolites (Tang and Lu, 2010). Accumulation of toxic metabolites can result in binding and damage to mitochondrial components, and alteration of mitochondrial pathways such as an impairment of electron transfer in the respiratory chain and fatty acid oxidation pathways, or ROS production causing oxidative stress (Porceddu *et al.*, 2012). Oxidative stress in mitochondria has numerous adverse effects such as lipid peroxidation, further protein modification and damage via ROS, and activation of cellular apoptotic pathways leading to cell death (Russmann *et al.*, 2009). A number of drugs have been implicated in causing mitochondrial dysfunction including the anti-epilepsy drug valproic acid and the antiretroviral nucleoside reverse transcriptase inhibitors (NRTIs) - zidovudine and didanosine (Begriche *et al.*, 2011). Although mechanisms of how drugs and reactive metabolites may cause mitochondrial dysfunction have been described there is still a significant lack of understanding on what causes the idiosyncratic nature of these reactions.

Mitochondrial stress by a drug or metabolite can lead to alteration of various cellular signalling pathways. Cellular responses may include the up-regulation of cellular defence mechanisms that lead to adaptation and tolerance by the hepatocyte to the drug-induced stress. Conversely, if cellular stress by a drug or metabolite reaches a critical threshold then cell death signalling pathways may dominate leading to apoptosis and necrosis of hepatocytes (Han *et al.*, 2013). Due to the rarity of idiosyncratic DILI and lack of suitable animal models, most of what is known about hepatocellular responses to drug-induced stress has been gained from extensive study of paracetamol which causes dose-related predictable hepatotoxicity.

A minor pathway in the metabolism of paracetamol, primarily by CYP2E1, leads to the production of a highly reactive metabolite *N*-acetyl-*p*-benzo-quinoneimine (NAPQI).

This electrophilic metabolite readily binds covalently to nucleophilic thiol groups in cellular proteins and thiol-containing organic molecules such as glutathione. High doses of paracetamol, as found with overdose, cause extensive binding and subsequent depletion of hepatocellular glutathione. This leads to increased production of ROS, oxidative stress and the activation of cellular apoptotic pathways leading to hepatocyte death and liver injury (Russmann *et al.*, 2009).

Mammalian cells have evolved a highly regulated cellular defence system to protect against cellular stress caused by reactive species through the transcriptional up-regulation of various detoxification and antioxidant enzymes (Copple *et al.*, 2010). These antioxidant response pathways provide the basis of hepatocellular adaptation to drug-induced stressors that in most individuals will protect from drug-induced toxicity.

One of the key regulators of the antioxidant response pathway is the activity of the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf-2). Nrf-2 in unstressed cells is repressed through its binding in the cytoplasm to Kelch-like ECH-associated protein 1 (Keap1). Within Keap1 there are a multitude of cysteine residues that bind to cellular reactive species inducing a conformational change in the dimer leading to nuclear translocation of Nrf-2 and subsequent transcription of multiple anti-oxidative target genes that remove the chemical insult to the cell (Bryan *et al.*, 2013).

It has been shown in mouse liver that toxic and non-toxic doses of paracetamol activate Nrf-2-dependent cellular defences in a dose dependent manner (Goldring *et al.*, 2004). NAPQI has been shown to bind to thiols within Keap1 thus promoting nuclear translocation of Nrf-2 (Copple *et al.*, 2008). In the nucleus, Nrf-2 binds to the antioxidant response element (ARE) regulatory motif leading to the transcription of numerous cytoprotective genes. Through the up regulation of protective mechanisms the cell can adapt to the chemical damage it is exposed to. However, in cases of paracetamol overdose, high doses of the drug lead to these mechanisms becoming overwhelmed. Glutathione synthesis machinery becomes severely depleted leading to hepatocyte death via both necrosis and through the activation of apoptotic pathways such as the JNK pathway (Bryan *et al.*, 2013).

1.3.2.3 BSEP inhibition

Another potential mechanism of non-immune mediated DILI relates to inhibition of the bile-salt export pump (BSEP, encoded by *ABCB11*). BSEP is an ATP-dependent transporter expressed on the apical plasma membrane of hepatocytes between the hepatocyte and bile canaliculus. It acts as an efflux transporter to maintain bile acid secretion required for bile production which aids the absorption of dietary components and for the elimination of endo- and xenobiotics from hepatocytes into bile for excretion (Morgan *et al.*, 2010). Inhibition of BSEP could therefore result in accumulation of inherently toxic bile acids and/or potentially toxic drugs/metabolites in hepatocytes (Andrews *et al.*, 2010; Tang and Lu, 2010). Polymorphisms in the *ABCB11* gene that cause reduced transcription, expression or activity of BSEP result in the hereditary cholestatic conditions such as progressive familial intrahepatic cholestasis type 2 (PFIC2) (Dawson *et al.*, 2012). New-borns with this condition rapidly develop cholestasis that is fatal unless liver transplantation is performed.

There is evidence that a number of drugs can cause functional BSEP inhibition and that this may contribute to the development of cholestatic disease. Examples of drugs where BSEP inhibition has been implicated in liver injury include bosentan, erythromycin, cyclosporine and troglitazone (Morgan *et al.*, 2010; Dawson *et al.*, 2012). *In vitro* BSEP inhibition studies have shown significantly higher incidence and potency of BSEP inhibition in drugs that are known to cause cholestatic/mixed DILI than those implicated in hepatocellular injury or non-DILI causing drugs (Dawson *et al.*, 2012).

1.3.3 *Immune mediated idiosyncratic liver injury*

1.3.3.1 Overview of immune mechanisms

In addition to mechanisms of metabolic idiosyncrasy, there is a large body of evidence that suggests that the immune system plays a key role in the development of most instances of idiosyncratic drug hepatotoxicity. These types of injury are termed ‘allergic’ type reactions and usually present with immune-like clinical features such as symptoms like rash, fever and eosinophilia, a typical delay of onset usually 1-8 weeks,

and a rapid reoccurrence of toxicity upon re-challenge with the culprit drug. But a clear indicator of immune system involvement is the frequent presence of antibodies specific to native or drug-conjugated liver proteins in sensitised patients (Aithal *et al.*, 2004; Holt and Ju, 2006). Clinical characteristics are usually different for different drugs and may be different for the same drug in individual patients. Drug-induced immune mediated injury may be localised to the liver or the reaction may be part of a general systemic drug hypersensitivity (Uetrecht and Naisbitt, 2013). Several hypotheses have been proposed for how the immune system may mediate idiosyncratic drug reactions. Of these, there are three major pathways that can provoke a response from the innate and adaptive immune system – the hapten hypothesis, the danger hypothesis and the pharmacological interaction.

1.3.3.2 The Hapten Hypothesis

It has long been proposed that small, low-molecular-weight organic molecules such as drugs are not immunogenic, i.e. unable to induce an immune response. However if a reactive molecule, or 'hapten', irreversibly binds to a macromolecule such as a protein it may be able to induce an adaptive immune response (Adam *et al.*, 2011). This has become known as the hapten hypothesis (**Figure 1.1**). Most drugs are not chemically reactive but drug metabolism, usually via cytochrome P450 metabolism, can produce reactive electrophilic species that can covalently react and bind to endogenous proteins. The conjugation of a drug modifies the protein producing a drug-protein adduct or neoantigen that may be recognised by the immune system as foreign, inducing a hapten-specific response. Factors such as the extent and nature of protein binding and the mechanism of exposure to immune cells are likely to influence the ability of drug-protein haptens to initiate an immune response (Ju and Reilly, 2012). For an immune response to occur the neoantigen needs to be presented and recognised by circulating T-cells (Uetrecht, 2008). Cellular processing of the drug-modified protein by proteasomes produces peptide chains that are recognised by the major histocompatibility complex [MHC] proteins, which in humans are referred to as the human leukocyte antigen (HLA) proteins. The HLA molecule binds peptide fragments, usually of 8-9 amino acids in length, in the endoplasmic reticulum followed by translocation of the protein complex to the cell membrane. The HLAs can be divided into two main classes: HLA

class I, of which the major genes, HLA-A, HLA-B and HLA-C; and HLA class II, which consists of the groups, HLA-DR, HLA-DP and HLA-DQ. HLA class I are found on all nucleated cells and present intracellular peptides on the cell surface to T-cells via an interaction with the T-cell receptor (TCR). Cytotoxic T-cells express a glycoprotein, CD8 that also reacts with the HLA class I complex strengthening the interaction. Activation of cytotoxic T-cells in the liver via HLA antigen presentation causes the release of cytokines and cytotoxins causing local cellular damage. HLA class II molecules present extracellular antigens to T-cells and are found on specialised antigen presenting cells (APCs) and immune-response cells such as B lymphocytes, dendritic cells and macrophages (Janeway *et al.*, 2001). Antigen presentation on these cells leads to activation of CD4+ T-helper cells, that have no cytotoxic ability but whose proliferation can lead to activate of a wider B-cell and macrophage response. Immune recognition of class II antigens leads to APC migration to the lymph nodes. Here they interact with naive T-cells leading to clonal expansion of long-lived antigen specific memory T-cells. This 'sensitisation' means that on re-exposure to the antigen there is a rapid stimulation of the antigen-specific T-cells leading to an immune response (Naisbitt *et al.*, 2000).

1.3.3.3 Danger Hypothesis

Often in individuals exposed to a reactive drug/metabolite, antibodies are detected that are specific to that particular compound. This is a sign that an immune response has occurred, specifically a B-cell response, against such an antigen. However this doesn't necessarily signify an adverse reaction as commonly antibodies to a drug or metabolite are detected individuals in the absence of any apparent idiosyncratic drug reaction (Aithal *et al.*, 2004). This has led to the hypothesis that haptenisation of a drug/metabolite alone may be insufficient for an immune response to occur and a secondary 'danger' signal is required to act as an adjuvant. This theory is supported by the knowledge that the activation of a T-cell response requires an additional co-stimulatory interaction, in addition to the interaction between the HLA complex and the TCR. Danger signals generated may up regulate the required co-stimulatory molecules leading to APC and T-cell activation. The general understanding is that the immune system response to the majority of antigens is tolerance, and only with the additional

'danger signal' is there a full immune response (Naisbitt *et al.*, 2000). It is suggested that the danger signal may arise from initial cell injury or cell stress and may provide the necessary signals leading to activation of APCs (Uetrecht, 2008). Potential danger signal include cytokines released by innate immune cells or damage associated molecular patterns (DAMPs) in response to cell injury (Williams and Jaeschke, 2012).

1.3.3.4 The pharmacological interaction (p-i) hypothesis

An alternate theory for T-cell activation is without hapten formation, processing and presentation. The pharmacological interaction (p-i) concept hypothesises that some drugs are able to directly bind to either HLA or TCR with sufficient strength to initiate T-cell stimulation (**Figure 1.1**) (Pichler, 2002). This hypothesis can explain why drugs that do not form reactive metabolites can initiate immune responses and was developed after the observation that some T-cells in sulfamethoxazole-hypersensitive patients recognised the parent drug itself rather than the reactive metabolite (Schnyder *et al.*, 1997; Ju and Reilly, 2012). Various experimental procedures have been developed that are reported as providing evidence for this mechanism including: i) the activation of drug-specific T-cells by a parent drug incubated with aldehyde-fixed APCs, that are unable to process or present drug-protein haptens in the classical HLA manner, ii) drug binding to the HLA/TCR is much more labile than covalent hapten binding and can be washed away, and iii) calcium influx in T-cell clones (as a measure of T-cell stimulation) is rapid in response to the addition of the drug, before sufficient time has elapsed for drug uptake, processing and antigen presentation (Pichler *et al.*, 2011). A recent study has reported that flucloxacillin is able to directly activate drug-specific T-cells in a manner that supports the p-i hypothesis. This is explained in detail in section 1.6.2.3 (Wuillemin *et al.*, 2013).

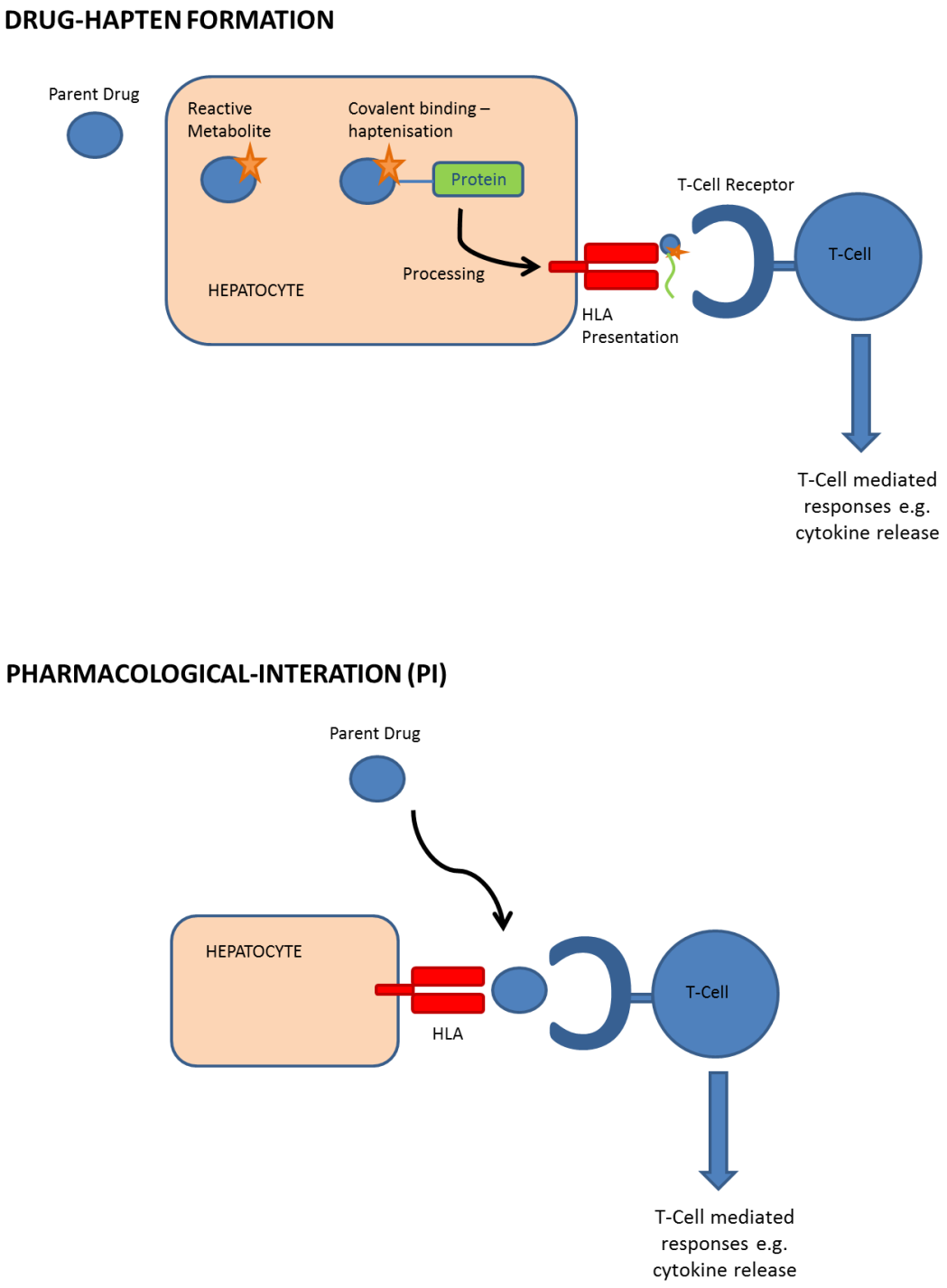

Figure 1.1 – Proposed mechanisms of DILI – The Hapten-Hypothesis and the Pharmacological-interaction (pi) theory.

The Hapten-Hypothesis proposes that the formation of a reactive metabolite in the hepatocyte leads to covalent binding to hepatocellular proteins forming a 'hapten'. Intracellular processing of this hapten via the Human Leukocyte Antigen (HLA) pathway results in extracellular HLA presentation and binding, of the hapten, to the T-cell receptor of circulating T-cells leading to T-cell mediated responses. The Pharmacological-interaction (pi) proposes that a drug can bind directly to expressed HLA molecules or T-cell receptors leading to the initiation of a HLA-T-cell response.

1.3.3.5 Innate immune response in DILI and the inflammatory stress hypothesis

In addition to lymphocytes, there are a number of innate immune cell populations found in the liver including Kupffer cells (KCs) and natural killer (NK) cells (Ju and Reilly, 2012). There are also a number of cell populations that have innate and adaptive immune properties including γδ T-cells and natural killer T (NKT)-cells that are found in the liver in higher concentrations than peripheral blood (Williams and Jaeschke, 2012). The innate immune system provides a rapid, non-specific first line defence against pathogens through the recognition of pathogen-associated molecular patterns (PAMPs). It has been suggested that cellular stress and inflammation caused by underlying infection or disease can increase the risk of hepatotoxicity to a drug by lowering the threshold at which drug toxicity occurs and by activating pathways of the innate immune system. An animal model has been developed where hepatotoxicity can be induced upon the co-administration to rodents of a DILI-causing drug and lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls that can induce inflammatory stress *in vivo*. (Shaw *et al.*, 2010). Such a model has been used to describe a potential mechanism of toxicity to trovafloxacin via cytokine production and leukocyte recruitment leading to further immune pathways and cytotoxicity (Shaw *et al.*, 2007).

1.4 Genetic risk factors of DILI

1.4.1 *Introduction to DILI genetic studies*

The idiosyncratic nature of DILI where upon drug toxicity is unpredictable and occurs only in a few susceptible individuals suggests that the disease has a strong genetic basis. DILI is considered a complex disease that likely involves a multitude of genetic and environmental factors in its pathogenesis. Deciphering a genetic basis of the disease remains a great challenge due to the rarity of disease, the number and range of drugs suspected of causing DILI, and the variety of phenotypes that the disease manifests as (Chalasani and Bjornsson, 2010). Genetic risk factors remain poorly understood and most studies so far have largely been hypothesis driven, focusing on potential candidate-genes for DILI susceptibility. Such studies have had limited success due to a

number of reasons including the relatively small number of patients and selection of inappropriate candidate genes or non-functional polymorphisms (Daly, 2012a). Technological advances over the last decade such as sequencing of the human genome has led to a much more detailed understanding of polymorphisms and haplotype relationships through projects and collaborations such as the Single Nucleotide Polymorphism Consortium and the HapMap project (Daly and Day, 2013). This has helped to revolutionise the study of complex genetic diseases allowing the possibility of wider ranging genome-wide association (GWA) studies that have already achieved some success in finding genetic determinants of DILI. A continuing problem however the recruitment of enough cases for use in GWA studies, which typically require at least 1000 cases and controls. This is especially difficult with regards to DILI where the incidence of disease is particularly low. To aid with this, several consortia groups are now in force to work together in collecting DNA from DILI patients and controls to use in the study of determining a genetic basis of liver injury to various drugs. These groups include the Drug-Induced Liver Injury Network (DILIN), the International Serious Adverse Event consortium (ISAEC) and the DILIGEN project, led by Prof. Ann Daly here at Newcastle University. Collaborative work of these consortia has already achieved some success in finding genetic determinants of DILI, for example in the case of flucloxacillin which will be discussed further below.

As described, there are a number of mechanisms and pathways suspected to be important in the pathogenesis of DILI. The genes that regulate key proteins in these pathways may increase susceptibility of an individual to DILI by a particular drug. Some pathways relate to the pharmacokinetic properties of a drug such as polymorphisms of drug metabolising enzymes or pathways that control a drugs disposition e.g. drug transporter proteins. The pharmacogenetics of immune related genes are of particular interest as there is a growing body of evidence linking DILI reactions to an immune response particularly the highly polymorphic HLA system.

1.4.2 *Xenobiotic metabolism and excretion*

Metabolising enzymes are required for the detoxification and clearance of many drugs but they may also be a cause of bioactivation of drugs to reactive metabolites. This makes drug metabolising enzymes a prime target for investigating genetic susceptibility

to DILI and it has been recently reported that drugs with greater than 50% hepatic metabolism are more likely to cause DILI than drugs that don't undergo significant metabolism (Lammert *et al.*, 2010). Biotransformation of drugs involves several steps including phase I metabolism that introduces a functional moiety to the compound, followed by phase II metabolism which produces hydrophilic conjugated products that facilitates their excretion. Drug transporters are also a plausible target for genetic DILI studies as they are involved in the efflux of drugs and bile acids from hepatocytes. Impairment of transporter function can result in cholestasis as is demonstrated in some inherited cholestatic diseases associated with mutations in drug transporter genes (Daly and Day, 2012)

There is considerable interindividual and interethnic variation in genes that encode drug metabolising enzymes and transporters. This results in a wide range of phenotypes that affects expression and activity that may influence an individual's ability to metabolise and excrete certain drugs. Examples of polymorphisms that have been associated with susceptibility to DILI are described in the following sections.

1.4.2.1 Phase I enzyme polymorphisms

The majority of phase I metabolism is performed by the cytochrome P450 superfamily of enzymes and are often implicated in the formation of toxic reactive metabolites. A number of polymorphisms have been described for these isoforms that lead to phenotypic differences in enzyme catalytic activity. It is proposed that an altered phenotype can result in toxicity via three main outcomes – i) if the parent drug is potentially toxic and the route of elimination is exclusive to a polymorphic enzyme, then impaired metabolism can lead to accumulation of the compound and subsequent toxicity, ii) if a polymorphic enzyme results in impaired metabolism, a secondary metabolising pathway may come into use that results in the formation of a toxic reactive product, iii) if a reactive metabolite is the cause of DILI then polymorphisms resulting in greater enzyme activity will lead to increased formation of the toxic product (Eichelbaum *et al.*, 1992; Amacher, 2012). Despite the polymorphic nature of cytochrome enzymes only a handful of examples exist where a causative link to DILI has been shown.

CYP2C9 and 2C19 are involved in the metabolism of many drugs including NSAIDs, phenytoin, warfarin (by CYP2C9) and proton pump inhibitors, antidepressants and antiepileptics (by CYP2C19) (Chalasani and Bjornsson, 2010). Studies have been performed on both CYP2C9 and CYP2C19 for a potential role in DILI. The two most common variant alleles of CYP2C9 are CYP2C9*2 and CYP2C9*3 which are both associated with impaired enzyme activity (Daly, 2003). CYP2C9 genotype was studied in relation to diclofenac hepatotoxicity (Aithal *et al.*, 2000). Cases of diclofenac DILI were genotyped for CYP2C9*2 and CYP2C9*3 but no differences were observed between cases and controls. These alleles were genotyped in another study with relation to an overall risk factor to DILI from a variety of drugs but again no associations were observed (Pachkoria *et al.*, 2007). A recent publication has identified CYP2C9*2 genotype as a potential risk factor for bosentan-induced liver injury. Liver injury to bosentan is thought to arise from toxicity to the parent drug. CYP2C9*2 is associated with reduced CYP2C9 function which may result in decreased metabolism leading to accumulation of bosentan and subsequent toxicity in individuals carrying this allele (Markova *et al.*, 2013). Polymorphisms in CYP2C19 have been implicated as a risk factor in troglitazone-induced liver injury (Kumashiro *et al.*, 2003). Homozygous mutant alleles were more frequently observed in troglitazone-DILI cases compared to controls, although only 8 cases were studied. Pachkoria et al did not find CYP2C19 genotype to be a risk factor for DILI to drugs overall (Pachkoria *et al.*, 2007).

Perhaps the most significant of the CYP enzymes in relation to drug metabolism is the CYP3A subfamily. These enzymes are the most abundant cytochrome enzymes in the liver comprising between 30 and 50% of total liver CYP content (Plant, 2007). CYP3A enzymes also have a wide range of drug substrates and are responsible for the metabolism of approximately 50% of prescribed medications. The most abundant CYP3A isoform in adult liver is CYP3A4. CYP3A5 has similar substrate specificity to CYP3A4 but is only detected in 10 – 20 % of adult livers (Daly, 2003). A particular feature of CYP3A4 biology is the large inter-individual variability observed in basal expression of the enzyme. There are a substantial number of CYP3A4 variant alleles reported in the coding and 5'flanking regions of the gene but many are thought to exert little biological significance and are observed at low population frequencies and so are thought unlikely to fully account for the large inter-individual variability reported (Plant, 2007; Amacher, 2012). A new CYP3A4 variant has recently been discovered termed CYP3A4*22 and has been associated with decreased CYP3A4 expression

(Wang *et al.*, 2011). Although, rare this variant has been associated with a decrease in metabolism of atorvastatin that could lead to adverse toxicity (Klein *et al.*, 2012).

Full understanding for high inter-individual variability in CYP3A4 expression is unclear but rather than variation in the CYP3A4 gene, it may possibly be explained by genetic variation of factors controlling the transcriptional regulation and activation of the enzyme. CYP3A4 expression is under the control of a combination of transcriptional regulatory mechanisms that include the binding of various transcription factors in regulatory regions upstream of the CYP3A4 promoter. The most important regulator of CYP3A4 expression is the ligand-activated nuclear receptor, pregnane X receptor (PXR). Polymorphisms leading to altered mRNA expression of these transcription factors significantly correlates to basal 3A4 expression (Lamba *et al.*, 2010). A polymorphism in the upstream region of PXR, associated with lower PXR expression, has been linked to flucloxacillin induced liver injury (Andrews *et al.*, 2010).

CYP2E1 is mainly involved in the metabolism of low molecular weight compounds and toxicants such as acetone, ethanol and nitrosamines but also plays a role in the metabolism of certain xenobiotics such as paracetamol, halothane and isoniazid (Daly, 2003; Daly and Day, 2013). The type of oxidative reactions that CYP2E1 performs have been implicated in the toxicity and carcinogenicity of various compounds such as benzene and chloroform (Neafsey *et al.*, 2009). With paracetamol overdose, CYP2E1 plays a role in the formation of toxic quinone intermediates. Functional non-synonymous polymorphisms in CYP2E1 are rare but interindividual variation in adult liver CYP2E1 expression has been shown to exist (Lipscomb *et al.*, 2003). Possession of a wild-type CYP2E1*1A/1A genotype has been reported to carry an increased risk of isoniazid toxicity compared to heterozygous and individuals homozygous for a variant alleles (Huang *et al.*, 2003; Sun *et al.*, 2008). Wild-type CYP2E1 is associated with higher activity than variant alleles and may lead to increased production of reactive metabolites. This appears to be the case with isoniazid which undergoes N-acetyltransferase 2 (NAT2) mediated acetylation in the liver to acetylisoniazid then hydrolysis to form acetylhydrazine, which can then undergo CYP2E1 oxidation to various hepatotoxins (Huang *et al.*, 2003). Some 5'-upstream region polymorphisms have been described and associated with altered enzyme expression (Zanger and Schwab, 2013). Several CYP2E1 variants were genotyped in DILI cases caused by anti-tuberculosis drugs by Ching Soon Ng at Newcastle University but no significant associations were observed (Ng, 2011).

1.4.2.2 Phase II enzyme polymorphisms

N-acetyltransferase 2 (NAT2) is important for the detoxification of isoniazid and sulphonamides through acetylation (Russmann *et al.*, 2010). NAT2 is highly polymorphic and shows interindividual and considerable ethnic variation. Individuals with two copies of a deficient variant allele have reduced NAT2 activity and are termed 'slow acetylators'. Slow acetylators, especially in Asian populations, are thought to be at an increased risk of isoniazid hepatotoxicity (Sun *et al.*, 2008). Acetylhydrazine is a product of isoniazid metabolic pathways and is thought to be responsible for toxicity either inherently or via further oxidation to toxic products by CYP2E1. It is postulated that NAT2 can detoxify acetylhydrazine to diacetylhydrazine but this route is compromised in individuals were NAT2 activity is reduced, resulting in increased levels of acetylhydrazine and toxic CYP2E1 metabolites (Daly and Day, 2013). A number of studies report the association between 'slow acetylator' phenotype and increased risk of isoniazid-DILI in various ethnic populations (Huang *et al.*, 2003; Bozok Cetintas *et al.*, 2008; Lee *et al.*, 2010; Bose *et al.*, 2011). However, there are still unresolved issues regarding this association as a number of studies do not report NAT2 genotype as relevant (Vuilleumier *et al.*, 2006; Yamada *et al.*, 2009). Recent work by Ching Soon Ng at Newcastle University has studied the association of NAT2 genotype with isoniazid-DILI further (Ng, 2011). 26 cases of isoniazid-DILI from Europe (n=13) and South Asia (n=13) were genotyped for NAT2 and grouped for either 'slow' or 'rapid' acetylator phenotype. A significant association was observed for slow acetylators and risk of isoniazid-DILI in the combined European and South Asian cohort. Interestingly, when considered separately, there was significance for this association in the South Asian cohort but not in the European cohort. However, a polymorphism (-9796T>A) in the promoter region of *NAT2*, that has been associated with decreased expression of NAT2, was found to be significant in the European cohort but not the South-Asian cohort. A recent meta-analysis has attempted to confirm the association of NAT2 genotype and susceptibility to isoniazid-DILI (Du *et al.*, 2013). This analysis included 26 case-control studies involving 1198 cases and 2921 controls and found an overall significant association between slow acetylator genotype and risk of disease. Interestingly, when a stratified analysis of ethnicity was performed significance was observed in East Asians, South Asians, Brazilians and Middle Eastern cases but not in

Caucasian cases agreeing with the observation by Ching Ng Soon in South Asian and European cohorts. Since NAT2 and CYP2E1 both play a role in the metabolism of isoniazid, it has been postulated that polymorphisms in CYP2E1 and NAT2 may act synergistically in the development of isoniazid hepatotoxicity (Chalasani and Bjornsson, 2010).

Glutathione S-transferases (GSTs) are conjugation enzymes that play a major role in defence against cellular oxidative stress by detoxifying ROS as well as conjugating reactive products from phase I metabolism. There are various GST isoforms but polymorphisms in the GSTM1 and GSTT1 isoforms that lead to absent enzyme expression (null alleles) has been associated with increased susceptibility to DILI, particularly from antimicrobials and NSAIDs (Lucena *et al.*, 2008).

Glucuronidation catalysed by UGT enzymes is generally a detoxification process but in certain instances can produce reactive acyl glucuronide metabolites that have been attributed to being a factor in DILI. Diclofenac is one such example where glucuronidation by UGT2B7 produces an acyl glucuronide metabolite that has been reported as being able to form adducts leading to hepatotoxicity (Aithal *et al.*, 2004). Possession of the UGT2B7*2 allele is associated with increased UGT activity and a greater susceptibility to diclofenac-DILI presumably via increased acyl glucuronide formation (Daly *et al.*, 2007). Polymorphisms in the enzyme UGT1A6 have been associated with elevated aminotransferase levels in individuals exposed to the catechol-*O*-methyltransferase inhibitor tolcapone (Acuna *et al.*, 2002). It is postulated that such polymorphisms result in reduced UGT1A6 activity leading to slower elimination of the parent drug and subsequent toxicity (Daly and Day, 2012).

1.4.2.3 Phase III transporter polymorphisms

Detoxification of xenobiotics via phase II glucuronide, sulphate or glutathione conjugation forms products that are substrates for efflux hepatobiliary transporters. Transporters of the ATP binding cassette (ABC) family such as the P-glycoprotein (MDR1, *ABCB1*), multidrug resistance-associated protein (MRP2, *ABCC2*) and the bile salt export pump (BSEP, *ABCB11*) play a vital role in the efflux of xenobiotics from the hepatocyte into the canaliculus for excretion into bile (Chalasani and Bjornsson, 2010).

BSEP mediates the efflux of bile acids and xenobiotics into the bile canaliculus and BSEP inhibition has been suggested as a mechanism in the pathogenesis of DILI via accumulation of toxic bile acids leading to cholestasis. BSEP impairment is the cause of a number of cholestatic diseases including intrahepatic cholestasis of pregnancy (ICP) which is associated with a common BSEP polymorphism (c.1331T > C) (Kubitz *et al.*, 2012). In a study of 36 patients who suffered DILI from a range of drugs, an association was also discovered with this polymorphism which is thought to confer to a decreased expression of BSEP (Choi *et al.*, 2007; Lang *et al.*, 2007). MRP2 is important for the efflux of organic anions, plus glucuronide and glutathione conjugates. A study in a Korean population revealed 12 polymorphisms in MRP2 that may play a role in a predisposition to DILI (Choi *et al.*, 2007). Analysis of MRP2 haplotypes showed two major haplotypes responsible for allelic variations. Interestingly, each haplotype was observed to be associated with different types of DILI, one associated with cholestatic/mixed injury (labelled Haplotype 1) and the other with hepatocellular injury (Haplotype 3). A C-24T polymorphism, that exists in the MRP2 haplotype 3, has been associated with diclofenac toxicity where a greater number of cases where found to possess the T variant that confers to lower MRP2 activity (Daly *et al.*, 2007).

1.4.3 *Oxidative Stress*

Mitochondria are the main source of ROS which if allowed to accumulate may lead to oxidative stress and cellular injury and propagation of DILI. To prevent this, mitochondria contain defence mechanisms that reduce ROS including the enzymes, superoxide dismutase (SOD) which reduces superoxide to hydrogen peroxide, and glutathione peroxidase (GPX) and catalase which reduce hydrogen peroxide to water. As such, genetic polymorphisms in these ROS detoxifying enzymes may contribute to DILI susceptibility.

A common SNP exists in mitochondrial manganese-dependent SOD (MnSOD, *SOD2)* where the presence of C/T at position 1183 results in the incorporation of either alanine or valine at position -9 of the mitochondrial targeting sequence (Pessayre *et al.*, 2012). The alanine MnSOD variant (C genotype) leads to a better import of the enzyme into mitochondria and was found to be associated with increased susceptibility to DILI by a range of drugs, particularly anti-tuberculosis medications (Huang *et al.*, 2007). A further

study using 185 cases of DILI from a range of drugs found the homozygous Ala/Ala genotype to be associated with an increased risk to cholestatic/mixed injury (Lucena *et al.*, 2010). In the same study, a glutathione peroxidase I (*GPX1*) genotype associated with decreased enzyme activity was found to be a risk factor for cholestatic DILI. Unexpectedly, the alanine MnSOD variant is associated with greater MnSOD activity. It is suggested that greater MnSOD mitochondrial levels may result in increased production of toxic hydrogen peroxide, overwhelming detoxification mechanisms (Huang *et al.*, 2007). Furthermore, glutathione peroxidase converts hydrogen peroxide to water so impairment of its activity may also serve to increase accumulation of hydrogen peroxide leading to cellular damage.

1.4.4 *Immune related genes*

1.4.4.1 Human Leukocyte Antigen

The strongest genetic associations seen with idiosyncratic drug reactions, including DILI, are found within the human leukocyte antigen (HLA) found in the Major Histocompatibility Complex (MHC) on chromosome 6. Specific associations between certain drugs and HLA alleles support the hypothesis that there is a strong immune component to DILI and that covalent binding of drugs/reactive metabolites to host peptides are an important process in its pathogenesis. The HLA contain the most polymorphic genes in the human genome characterised by high linkage disequilibrium (LD) over large genetic distances (Daly, 2012a). The huge diversity of HLA alleles maximises the peptide binding capacity as allotypic differences predominantly map to residues in the peptide binding groove that come into contact with peptides (Bharadwaj *et al.*, 2012).

Candidate-gene association studies provided the first evidence of HLA association in DILI. An example of which is the finding from two independent small cohort studies that HLA class II allele *DRB1*15:01* is a susceptibility factor for amoxicillin-clavulanate (co-amoxiclav) DILI (Hautekeete *et al.*, 1999; Donaldson *et al.*, 2010). The advancement of our understanding of the human genome and development of genotyping techniques that allow simultaneous genotyping of a large number of SNPs has aided the study of complex diseases such as DILI. GWA studies have been used in

four studies to investigate genetic susceptibility to DILI. Each study, involving a different DILI causing drug, showed a significant association with a particular HLA class I or II allele (summarised in **Table 1.3**).

Table 1.2 – Summary of Genome Wide Association Studies (GWAS) with DILI-causing drugs that show significant HLA associations

Drug	Number of Cases	*P* - value	Odds ratio (95% CI)	HLA nomenclature	Reference
Ximelagatran	74	6.0×10^{-6}	Not calculated	HLA-*DRB1*07:01 – DQA1*02:01*	(Kindmark *et al.*, 2008)
Flucloxacillin	51	8.7×10^{-33}	45 (19.4 – 105)	HLA-*B*57:01*	(Daly *et al.*, 2009)
Lumiracoxib	41	2.8×10^{-10}	5.3 (3.0 – 9.2)	HLA-*DRB1*15:01-DQB1*06:02*	(Singer *et al.*, 2010)
Amoxicillin-clavulanate	201	3.5×10^{-11}	2.8 (2.1 – 3.8)	HLA-*DRB1*15:01-DQB1*06:02* HLA-*A*02:01*	(Lucena *et al.*, 2011)

(table adapted from (Daly, 2012a))

The earliest GWAS relating to a serious adverse reaction was performed on hepatotoxicity cases caused by the anticoagulant drug Ximelagatran. Although, this GWAS was limited, by a relatively low number of single nucleotide polymorphisms (SNPs) covered (266000) compared to later GWAS, a significant association was discovered with *HLA-DRB1*07:01* and *HLA-DQA1*02:01*(Kindmark et al., 2008). A GWAS performed on amoxicillin-clavulanate DILI cases from a Spanish registry confirmed earlier observed associations with HLA-*DRB1*15:01* and discovered further additional HLA associations. A second HLA class II association was reported with an increased frequency of HLA-*DQB1*06:02* in cases of amoxicillin-clavulanate-DILI cases (Lucena *et al.*, 2011). The *DQB1*06:02* allele is found to be part of a DR2 haplotype along with *DRB1*15:01* (Daly and Day, 2013). A novel class I allele HLA-*A*02:01* association was also detected in cases of amoxicillin-clavulanate-DILI (Lucena *et al.*, 2011). A study on 41 cases of DILI caused by the restricted NSAID, lumiracoxib, also found a highly significant association with the HLA DR2 haplotype containing the class II alleles *DQB1*06:02* and *DRB1*15:01* observed in amoxicillin-clavulanate-DILI cases (Singer *et al.*, 2010). This observation is interesting since there are no obvious structural similarities between lumiracoxib and either amoxicillin or clavulanic acid (thought to be the hepatotoxic component) as shown in **Figure 1.2**. The pattern of liver injury is also different with co-amoxiclav-DILI predominantly cholestatic/mixed and lumiracoxib mainly hepatotoxic (Daly and Day, 2013).

Although HLA-class II associations have been more commonly reported in relation to DILI, the strongest HLA association with DILI was discovered for a class I allele in relation to flucloxacillin induced liver injury. A GWAS genotyped 51 cases and matched controls for 900000 SNPs. A number of SNPs in the MHC region were found to be significant with the strongest association in LD with the class I allele HLA-*B*57:01* (Daly *et al.*, 2009). This association is described in further detail in section 1.6.2.1.

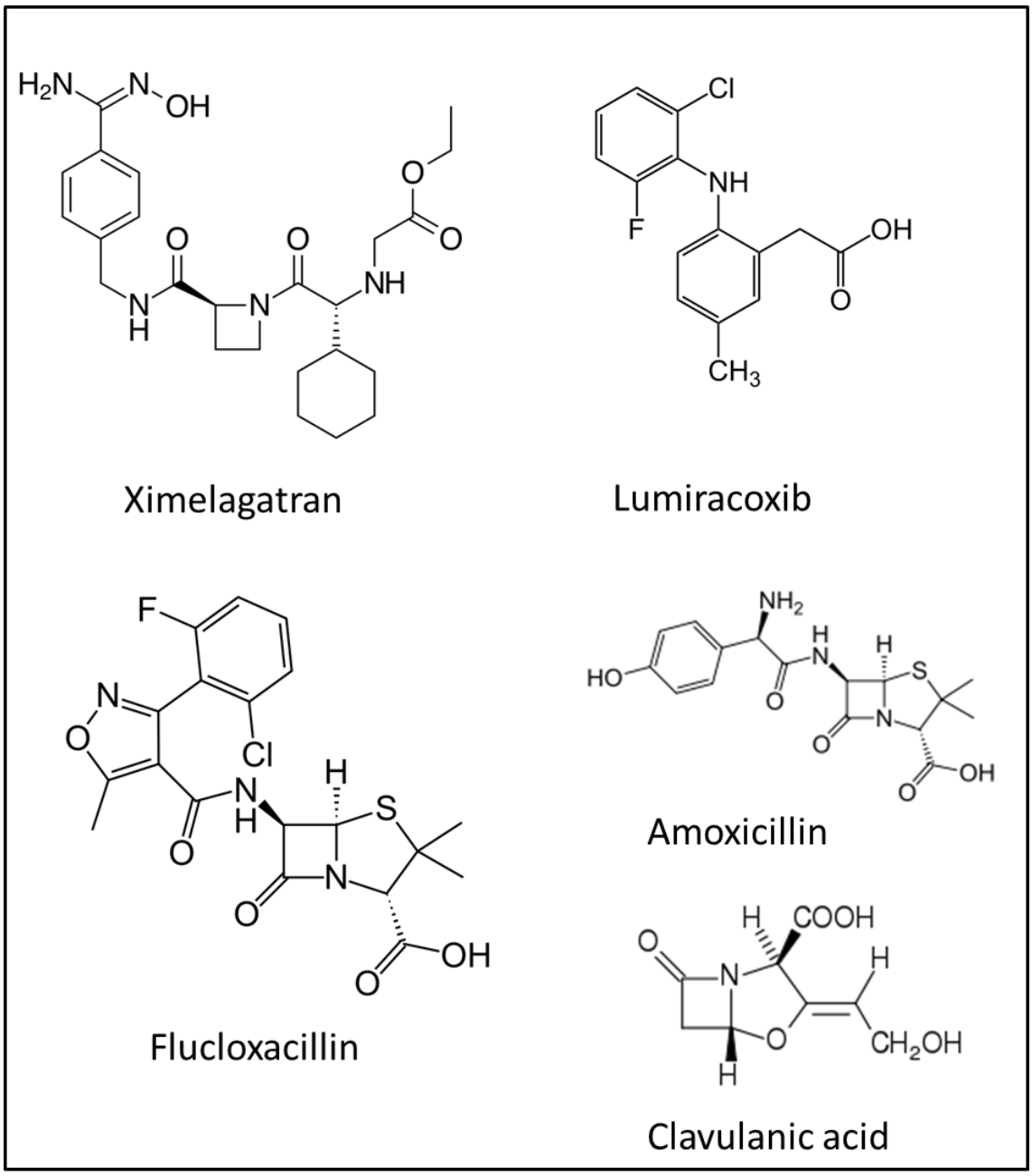

Figure 1.2 – Chemical structures of DILI causing drugs with significant HLA associations

1.4.4.2 Innate immunity

In addition to the established HLA associations there is also evidence that polymorphisms in genes involved with innate immunity may influence susceptibility to DILI. To date, most reports of such genetic polymorphisms focus on pro- and anti-inflammatory cytokine mediators (Daly and Day, 2012). An example of this is the association of interleukin-10 (IL-10) and interleukin-4 (IL-4) genotype with diclofenac-DILI in a small cohort of patients (Aithal *et al.*, 2004). The polymorphisms found frequently in the DILI cases were linked to decreased expression of IL-10 and increased expression of IL-4. IL-10 is a potent anti-inflammatory cytokine that has many protective and immunoregulatory functions (Pachkoria *et al.*, 2008). Contrastingly, IL-4 is a pro-inflammatory cytokine that assists in mediation of Th2 immune responses. A further investigation, with a larger cohort into the relevance of IL-10 and IL-4 genotypes in relation to DILI in a variety of drugs, failed to confirm the association as a general risk factor for DILI. They did observe that low IL-10 was associated with a low eosinophil count that may be linked to DILI progression although this is not well understood (Pachkoria *et al.*, 2008).

1.5 Flucloxacillin-induced Liver Injury

1.5.1 *Background*

Flucloxacillin is a semi-synthetic beta-lactam antibiotic used since the 1970s in the UK, Scandinavia and Australia for the treatment of many gram positive bacterial infections. In the UK, it is the first line of defence for the treatment of soft tissue staphylococcal infections and is particularly effective against penicillinase-producing resistant strains (Carey and van Pelt, 2005; Huwyler *et al.*, 2006). It is typically given orally for skin, soft tissue or respiratory tract infections or may be used intravenously to treat severe infections by methicillin-sensitive *Staphylococcus aureus* (MSSA) (Ritchie *et al.*, 2007).

Flucloxacillin belongs to the isoxazolyl family of β-lactamase resistant penicillin's that also includes the closely related oxacillin, cloxacillin and dicloxacillin. The chemical structures of the isoxazolyl penicillin's differ by the degree and nature of halogenation

at the 2-phenyl and 6-phenyl positions of the isoxazole side chain, where oxacillin has no halogenation, cloxacillin contains a 2-chloro group, dicloxacillin has two chlorinated positions and flucloxacillin has a 2-chloro and 6-fluoro group (**Figure 1.3**).

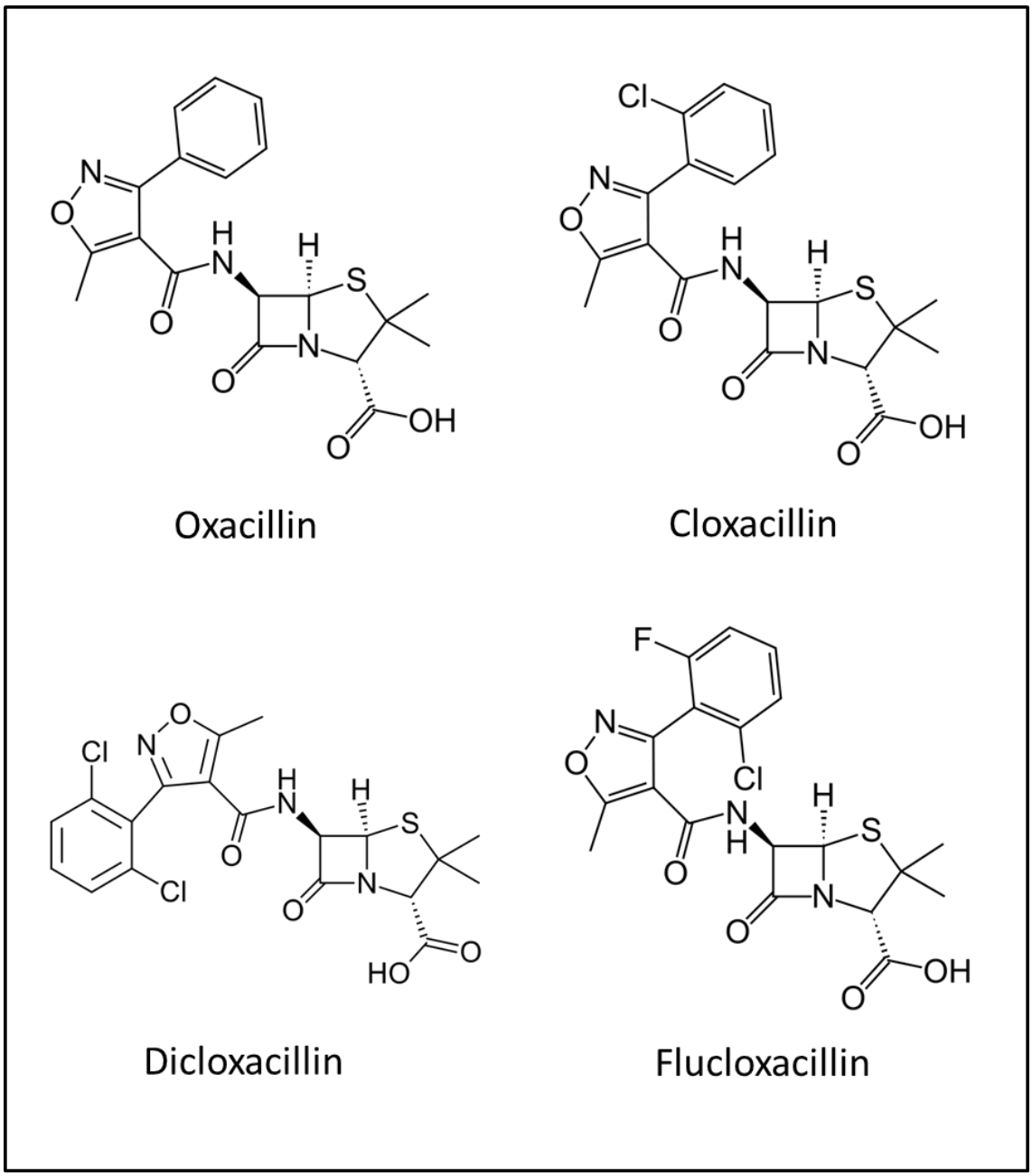

Figure 1.3 Structures of the isoxazolyl penicillins – oxacillin, cloxacillin, dicloxacillin and flucloxacillin

Isoxazolyl penicillin structure differs by the degree and nature of halogenation on the isoxazole side chain where oxacillin has no halogenation, cloxacillin is chlorinated at the 2-phenyl position, dicloxacillin is chlorinated at both the 2-phenyl and 6-phenyl positions and flucloxacillin is chlorinated at the 2-phenyl position and fluorinated at the 6-phenyl position.

A report of penicillin use in Europe showed that isoxazolyl penicillins are widely used in the U.K. and Scandinavian countries including Iceland and Sweden (**Figure 1.4**, blue bars indicate β-lactamase resistant isoxazolyl use) (Ferech *et al.*, 2006). This study showed that 10 of 25 European countries prescribed isoxazolyl penicillins at less than 1% of penicillin use. Flucloxacillin was mostly used in the U.K, Sweden, Ireland and Portugal whereas dicloxacillin was mainly used in Iceland, Denmark and Norway, cloxacillin in Iceland, Spain and France and oxacillin in France (Ferech *et al.*, 2006).

The antibacterial activity of the isoxazolyl penicillin's is similar to that of other β-lactamase resistant penicillin's, nafcillin and methicillin, but with the advantage of being gastric acid stable allowing oral administration. Dicloxacillin and flucloxacillin are better absorbed and more slowly cleared than oxacillin and cloxacillin producing higher and prolonged total serum concentrations. Flucloxacillin is reported as having higher activity than dicloxacillin due to lesser binding to serum albumin resulting in higher free serum levels (Sutherland *et al.*, 1970).

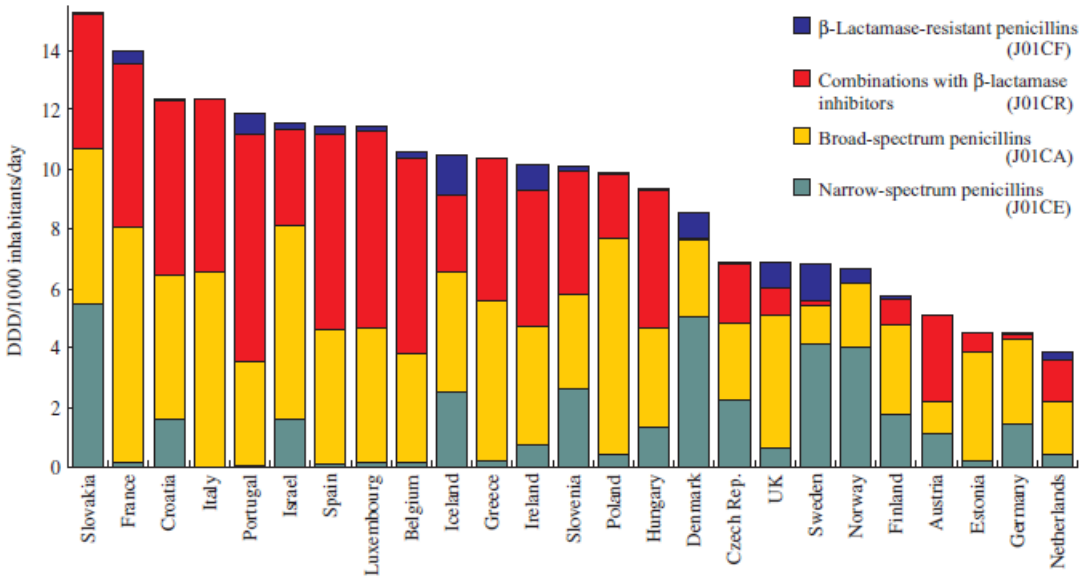

Figure 1.4 – Outpatient penicillin use in 25 European countries in 2003 expressed as defined daily dose (DDD) per 1000 inhabitants per day.

Green bars indicate narrow-spectrum penicillins (e.g. β-lactamase-sensitive penicllins such as benzyl penicillin and phenoxymethylpenicllin), yellow bars indicate broad-spectrum penicillins (e.g. ampicillin, amoxicillin and piperacillin), red bars indicate combinations of penicillins with β-lactamase inhibitors (e.g. amoxicillin-clavulanic acid) and blue bars indicate β-lactamase resistant penicillins (e.g. the isoxazolyl penicillins).(Figure reproduced with permission from (Ferech et al., 2006)).

1.5.2 *Epidemiology of isoxazolyl and flucloxacillin-induced liver injury*

Isolated reports linking hepatotoxic events to the isoxazolyl penicillin's date as far back as the 1960's when an initial report was published describing oxacillin-induced hepatitis (Freedman, 1965). Through the 1980's and early 1990's a growing number of case reports began to emerge from Sweden and Australia describing liver injury specifically related to flucloxacillin use, while reports of liver injury due to the other isoxazolyl penicillin's remained extremely rare (Kleinman and Presberg, 1986; Turner *et al.*, 1989; Miros *et al.*, 1990; Olsson *et al.*, 1992).

In 1992, Olsson and colleagues performed a retrospective study of DILI cases spontaneously reported between 1981 and 1990 to the Swedish Adverse Drug Reaction Advisory Committee (SADRAC) (Olsson *et al.*, 1992). A total of 77 probable and possible DILI cases associated with isoxazolyl penicillin's were reported including 32 probable flucloxacillin liver injury cases, 5 probable cloxacillin and 6 probable dicloxacillin cases. In Australia, two case series were published in 1989 and 1990 describing a total of 11 cases of flucloxacillin induced liver injury (Turner *et al.*, 1989; Miros *et al.*, 1990). This led to an effort by the Australian Adverse Drug Reactions Advisory Committee (ADRAC) to increase awareness of reporting of flucloxacillin induced liver reactions, and by 1994 310 reports of flucloxacillin associated liver disease had been received which included 17 cases with a fatal outcome (Russmann *et al.*, 2005). While awareness and reporting of adverse hepatic events attributed to flucloxacillin had increased, flucloxacillin dispensing rates were also still rising (Roughead *et al.*, 1999). As a result of this concern and because of the frequency and severity of adverse hepatic events attributed to flucloxacillin, regulatory action was taken by the Australian Department of Human Services and Health. Measures introduced included restriction of the use of flucloxacillin to severe staphylococcal infections only, ceasing of all manufacturer advertising and approving the introduction of cephalexin, erythromycin and dicloxacillin as safer alternatives (McNeil *et al.*, 1999; Roughead *et al.*, 1999). The decision to introduce dicloxacillin as a safer alternative was based on the belief that flucloxacillin induced liver injury was substantially more common than dicloxacillin associated toxicity, a hypothesis that was supported by the earlier findings of Olsson and colleagues (Olsson *et al.*, 1992). Additionally, in the USA and Canada it is dicloxacillin, not flucloxacillin, that is widely used yet there is a virtual absence of liver injury reports associated to its use in these countries (Devereaux *et al.*,

1995). This suggests that dicloxacillin poses a lower risk of liver injury than flucloxacillin but due to how adverse events are recognised and reported and the lack of any population based epidemiological studies; this cannot be said with absolute certainty.

Pharmacovigilance of flucloxacillin-induced liver injury was much greater in Sweden and Australia in the early 1990's than in other countries where flucloxacillin was also widely used, such as New Zealand, the Netherlands and the UK. It is thought that this was largely due to Sweden's compulsory regulation for the reporting of serious adverse reactions and Australia's sustained publicity and heightened awareness between health professionals of flucloxacillin's potential risks (Devereaux *et al.*, 1995). Two retrospective cohort studies, published in the Australian literature, were performed at this time using data from the UK General Practice Research Database (GPRD) (Derby *et al.*, 1993; Jick *et al.*, 1994). Combined, these studies examined a cohort of 209 642 individuals who had received flucloxacillin and estimated the incidence of flucloxacillin induced liver injury to be approximately 7 in 100 000 first time users of the drug. In 2005, a follow up study included a further 283 097 patients who had received flucloxacillin in the period from the end of the previous study to 2002. This study yielded a similar estimated risk of flucloxacillin induced liver injury of 8.5 per 100 000 users which equates to approximately 1 in 12 000 patients (Russmann *et al.*, 2005).

Studies into the various drugs responsible for DILI have shown that in countries where it is still commonly prescribed, flucloxacillin is often the most common single agent associated with idiosyncratic drug hepatotoxicity (Bjornsson and Olsson, 2005; Sistanizad and Peterson, 2013). It has also been reported as the leading cause of liver failure and death unless transplantation is performed amongst drugs that cause idiosyncratic DILI (Bjornsson *et al.*, 2005). While these comparative studies do not take into account the relative numbers of prescriptions of each drug it suggests that flucloxacillin is the cause of frequent and potentially serious DILI.

The discontinuation of flucloxacillin in countries such as Australia, in favour of dicloxacillin, and the observation that flucloxacillin remains a common cause of DILI in countries where it is still in use, begs the question as to why flucloxacillin is still preferred. There doesn't appear to be stand-out significant clinical merits of using flucloxacillin over dicloxacillin. The preference for flucloxacillin may result in limited pharmacokinetic and pharmacodynamic data. Although, data suggests that flucloxacillin

and dicloxacillin have superior oral absorption and longer elimination half-lives than oxacillin and cloxacillin there appears to be little difference between flucloxacillin and dicloxacillin themselves. The only slight difference reported that may make flucloxacillin a more effective drug is that it has a slightly lower degree of serum protein binding compared to dicloxacillin, reported as 92-95% compared to 96-97% with dicloxacillin (Turnidge, 2012). Effectively it appears that the two compounds can be used interchangeably to treat the same type of infections and although hepatotoxicity associated with flucloxacillin is an issue that warrants further attention, due to the rarity of the disease it does not appear that this issue is given high enough priority in countries where it is still currently in use.

1.5.3 *Symptoms of flucloxacillin-induced liver injury*

Evidence gathered from episodes of flucloxacillin-induced liver injury and subsequent epidemiological studies have helped to build up some knowledge of the disease. It is established that the type of liver injury caused is predominantly cholestatic in nature though a mixed - hepatocellular and cholestatic phenotype is observed in some cases. Similarly, liver injury caused by the other halogenated isoxazolyl penicillin's – cloxacillin and dicloxacillin, is also predominantly cholestatic, however injury caused by oxacillin is mainly hepatocellular (Olsson *et al.*, 1992; Bjornsson and Olsson, 2005). Prominent symptoms in the majority of flucloxacillin hepatotoxicity cases are painless jaundice and pruritus while there may also be nausea, abdominal pain and fever (Russmann *et al.*, 2005; Andrews and Daly, 2008). Flucloxacillin-induced cholestasis is characterised by abnormalities in liver function tests primarily a rise in serum concentrations of bilirubin, alkaline phosphatase and γ – glutamyl transferase (Devereaux *et al.*, 1995).

As with many idiosyncratic adverse reactions there is a delay of onset of the disease, typically flucloxacillin hepatotoxicity presents between 1 and 45 days after treatment commences and often after the treatment course has ended (Russmann *et al.*, 2005; Andrews and Daly, 2008). Occasionally, liver injury due to flucloxacillin may present itself after 45 days but this is rare. Liver injury caused by flucloxacillin is typically protracted with an average duration of 11 weeks before symptoms are resolved although in a minority of cases cholestasis may exist for periods exceeding six months

(Devereaux *et al.*, 1995). The majority of reports describing flucloxacillin-induced liver injury describe moderate to severe cholestasis with evidence of an inflammatory response including eosinophilia, lymphocyte infiltration and a proliferation of kupffer cells (Bengtsson *et al.*, 1985; Olsson *et al.*, 1992; Devereaux *et al.*, 1995).

1.5.4 *Susceptibility factors for flucloxacillin-induced liver injury*

The precise mechanisms by which flucloxacillin causes liver toxicity remains to be elucidated. However, knowledge built-up from case reports and epidemiological studies has provided some understanding of potential risk factors for susceptibility to the disease. Previously, it was reported that increasing age and a treatment course of over 14 days were risk factors for flucloxacillin-DILI (Fairley *et al.*, 1993). Russmann et al were unable to confirm a link between disease and treatment duration as they found that most cases had a treatment course of less than 14 days. They did however confirm that the disease appears to be age related with patients over the age of 60 estimated to have a six-fold higher risk of disease (Russmann *et al.*, 2005). There is also some evidence to suggest that females may be at a greater risk than males at developing the disease (Russmann *et al.*, 2005; Daly *et al.*, 2009).

1.5.5 *Flucloxacillin metabolism*

There are limited studies on the pharmacokinetics and metabolism of flucloxacillin. It has been reported that flucloxacillin is highly protein bound in serum with values of 92 - 95% reported (Roder *et al.*, 1995). It has been estimated that peak serum concentrations of flucloxacillin are reached approximately 45 minutes after a single oral dose and that approximately 35 – 50 % of flucloxacillin is excreted unchanged in urine (Sutherland *et al.*, 1970; Roder *et al.*, 1995). Full metabolic pathways of flucloxacillin have yet to be fully elucidated but early work by Thijssen and co-workers found flucloxacillin to have three different metabolites: 5`-hydroxymethyl flucloxacillin, and penicilloic acid derivatives of flucloxacillin and the 5'-hydroxymethyl metabolite (Thijssen, 1979) (**Figure 1.5**). 5'-hydroxymethylflucloxacillin is thought to be the main active metabolite and is formed by hydroxylation of the 5-methyl group of the isoxazole ring. The

penicilloic acid metabolites are products of hydrolysis and ring-opening of the β-lactam structure in penicillin's. This may be a spontaneous reaction, as occurs readily at alkaline pH, or may be enzyme-mediated in local physiological conditions that are not alkaline (Kitteringham *et al.*, 1987; Carey and van Pelt, 2005).

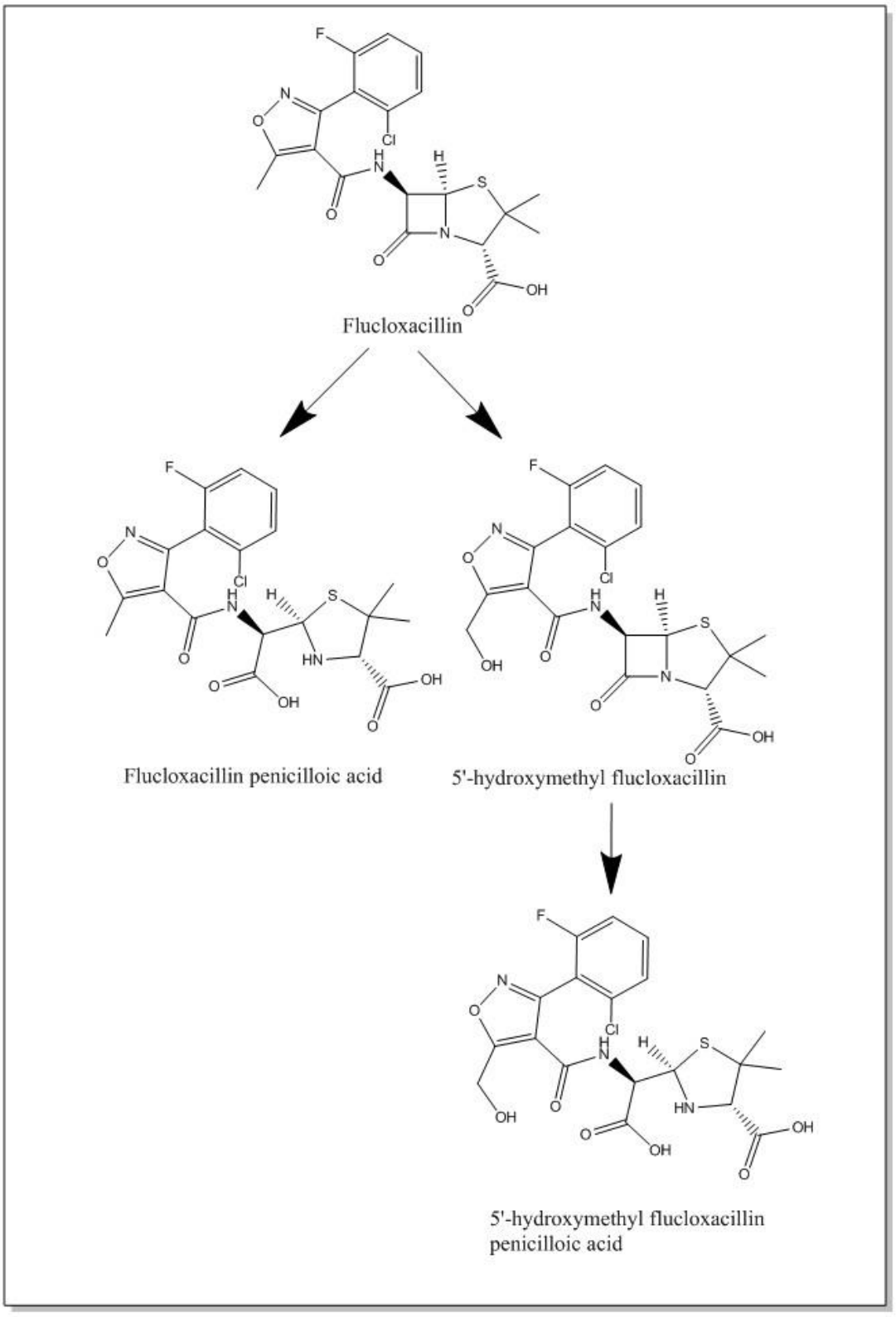

Figure 1.5 – Proposed metabolism pathways for flucloxacillin –

Flucloxacillin has been proposed to proceed via two pathways - formation of a 5'-hydroxymethyl flucloxacillin metabolite by likely enzymatic oxidative metabolism or formation of flucloxacillin-penicilloic acid through spontaneous opening of the β-lactam ring. Additional formation of the penicilloic acid of the 5'-hydroxy methyl flucloxacillin metabolite has also been proposed.

Although, 5'-hydroxymethyl flucloxacillin is the main flucloxacillin metabolite it has been reported that very little of it is produced either *in vivo* or *in vitro*. Thijssen reported peak ratios of 5'hydroxymethyl flucloxacillin to flucloxacillin to be 1:25 in serum after an oral dose (Thijssen, 1980). Similarly, Lakehal et al found that 5'-hydroxymethyl flucloxacillin production in hepatocytes was approximately 1% that of flucloxacillin (Lakehal *et al.*, 2001).

The monooxygenase activity of cytochrome P450 enzymes has been postulated to be important in the formation of 5`-hydroxymethyl flucloxacillin. Lakehal and colleagues reported that the cytochrome isoform CYP3A4 had a specific role through studies in CYP3A-induced rat liver microsomes, human liver microsomes and human recombinant CYP3A4 yeast vectors (Lakehal *et al.*, 2001). They reported that troleandomycin inhibition of CYP3A activity attenuated production of the metabolite, whereas specific inhibitors of CYP1A2 or 2C9 did not. The involvement of cytochrome P450 in flucloxacillin metabolism has been disputed, however, by Huwyler et al, who stated that flucloxacillin was not a substrate for cytochrome P450 enzymes (Huwyler *et al.*, 2006). Further work is needed to better understand how flucloxacillin is metabolised and will be one of the aims of this current study.

1.6 Pathogenesis of flucloxacillin-induced liver injury

1.6.1 *Metabolic basis of flucloxacillin induced liver injury*

1.6.1.1 Toxicity of flucloxacillin and its metabolites

There are relatively few studies that examine the metabolic basis of flucloxacillin-induced liver injury. Lakehal and colleagues reported that flucloxacillin was not intrinsically toxic to cells but a compound produced by hepatocytes, believed to be 5`-hydroxymethyl flucloxacillin, may be toxic to biliary epithelial cells (BECs) (Lakehal et al., 2001). Conditioned media from flucloxacillin treated hepatocytes was incubated with BECs and found to cause toxicity in 7 of 12 preparations. Similarly, media from flucloxacillin treated human liver microsomes incubated with 8 BEC preparations was found to cause toxicity in 50% of the samples suggesting that a toxic metabolite is being produced. It is suggested that cytotoxicity from 5'-hydroxymethyl flucloxacillin may

arise from depletion of glutathione and oxidation of protein thiols leading to oxidative stress. Cytotoxicity in BECs but not hepatocytes has been attributed to the glutathione content with BECs containing over 60% less than hepatocytes (Parola *et al.*, 1990; Lakehal *et al.*, 2001).

It remains unclear whether the penicilloic acid metabolites of flucloxacillin and 5`-hydroxymethylflucloxacillin are also toxic but it is thought that these metabolites are likely to be responsible for adduct formation via the opened β-lactam ring (Carey and van Pelt, 2005).

1.6.1.2 Nuclear Receptors

The studies of Lakehal and colleagues suggest a role for CYP3A4 in the metabolism of flucloxacillin, although this has been disputed and the issue is yet to be fully resolved. The possibility of a role for CYP3A4 metabolism, however, leads to a discussion as to whether inter-individual variation in CYP3A4 activity may affect susceptibility to flucloxacillin-induced liver injury. Studies of CYP3A4 polymorphisms suggest that mutations in the CYP3A4 gene are not likely to exert a significant effect on CYP3A4 metabolism (Lamba et al., 2002). Functionally significant polymorphisms have been shown in the minor CYP3A isoforms, CYP3A5 and CYP3A7 (Lamba et al., 2002). For example, CYP3A5 is only expressed in 10-15% of Caucasians but may account for up to 50% of CYP3A metabolising activity (Andrews and Daly, 2008). A recent study has failed to show a significant difference in CYP3A5 genotype between flucloxacillin-induced DILI cases and controls (Andrews et al., 2010).

Considering CYP3A4, as a risk factor for flucloxacillin-induced toxicity, it may be more important to look at the regulatory factors that control the enzymes induction and expression rather than genetic variation in the enzyme itself. Transcriptional induction of CYP3A4 is mediated by members of the nuclear hormone receptor family. The receptor described as being primarily responsible for CYP3A4 regulation is the pregnane X receptor, PXR (NR1I2) but also the constitutive active/androstane receptor (CAR) (Moore *et al.*, 2000). PXR and CAR are both highly expressed in the liver where they co-ordinately regulate genes involved in all phases of xenobiotic metabolism as well as playing a role in the maintenance of bile acid homeostasis. PXR has been shown to have a large ligand binding domain compared to other nuclear receptors and can be

activated by a wide range of structurally diverse unrelated compounds including antimacrolide antibiotics such as rifampicin, glucocorticoids, statins, environmental pollutants, organic pesticides and endogenous compounds such as steroids and bile acid salts (di Masi *et al.*, 2009; Li *et al.*, 2012). Ligand binding to PXR leads to its nuclear translocation and formation of a heterodimer with the retinoid X receptor (RXR). PXR activates the transcription of CYP3A4 by binding as a heterodimer to various specific response elements in the 5'upstream promoter region: the proximal promoter ER6, the distal DR3 motif in the xenobiotic-responsive enhancer module (XREM), the ER6 in the far distal enhancer module and a recently identified DR4 motif (Ihunnah et al., 2011).

Cholestasis arises as a result of ineffective bile formation in the hepatocyte or impairment of bile secretion leading to accumulation of toxic bile acids. PXR and CAR regulate the expression of enzymes and transporters involved in bile acid synthesis and detoxification. To facilitate excretion, bile acids must undergo phase I and II metabolism. Bile acids are hydroxylated by CYP3A4 which is regulated by PXR and CAR. Bile acids such as lithocholic acid are PXR ligands whereas bilirubin can activate CAR (Staudinger *et al.*, 2001). Phase II metabolism through sulphate and glucuronide conjugation is mediated via enzymes such as SULT2A1 which is mainly CAR regulated and UGT1A enzymes which are CAR and PXR mediated. These mechanisms suggest a protective role for CAR and PXR against cholestasis.

Reporter studies have been used to show that flucloxacillin is a PXR ligand (Andrews et al., 2010). Flucloxacillin has also been shown to be able to induce transcription of CYP3A4 and MDR1, both under control of PXR regulation (Huwyler *et al.*, 2006; Andrews *et al.*, 2010). Furthermore, there is evidence linking a PXR polymorphism, (rs3814055, C-25385T) and flucloxacillin DILI cases, with an increased frequency of CC homozygotes in disease cases compared to drug-treated controls and healthy community controls (Andrews et al., 2010). This allele has previously been associated with decreased CYP3A4 expression when compared to TT homozygotes.

This evidence suggests that flucloxacillin may influence PXR regulation of genes important in drug disposition and bile acid homeostasis. If flucloxacillin is metabolised by CYP3A4, genetic polymorphisms in PXR that result in decreased expression of CYP3A4 may lead to increased accumulation of flucloxacillin in hepatocytes, possibly influencing the toxicity of the drug. There have been no studies assessing whether

flucloxacillin has any impact on other nuclear receptors such as CAR. Like PXR, CAR is also activated by a range of xenobiotics and is involved in the induction of metabolising enzymes including CYP3A4.

1.6.1.3 Transporters

Flucloxacillin is thought to undergo approximately 28% extra-renal or biliary excretion (Nauta and Mattie, 1975). This means that flucloxacillin is a substrate for at least one hepatic drug efflux transporter such as P-glycoprotein (MDR1), MRP2 or BSEP. At present, there is very little knowledge about flucloxacillin's interactions with drug transporters, except for one report that flucloxacillin is not a substrate for P-glycoprotein (MDR1) (Huwyler *et al.*, 2006). As described in section 1.3.2.3, drug transporters play a vital role in bile acid homeostasis as well as the excretion of drugs. Impairment of drug transporter function can contribute to cholestasis with BSEP inhibition in particular associated as a risk factor to some DILI. There is evidence that flucloxacillin inhibits BSEP *in vitro*, although the functional effect of this is unknown (Dawson *et al.*, 2012). The same study reported that two other isoxazolyl penicillin's - cloxacillin and dicloxacillin, were also able to inhibit BSEP. Polymorphisms in drug transporter genes have briefly been studied in relation to flucloxacillin-DILI (Bhatnagar *et al.*, 2008). 36 polymorphisms were genotyped in MDR1, MDR3, BSEP and MRP2 in 56 cases of flucloxacillin-DILI and 199 healthy controls. No significant associations were found except for a modest association in a missense variant in exon 25 of MRP2 (V1188E). However, the authors state that the functional significance of this variant is unknown as is MRP2's relevance in flucloxacillin efflux (Bhatnagar *et al.*, 2008).

1.6.2 *Immune basis of flucloxacillin induced liver injury*

There is now enough evidence to confidently suggest that there is a strong immune basis to the pathogenesis of flucloxacillin induced liver disease. Originally, this stemmed from the presence of immune-like symptoms in episodes of injury, such as rash, fever and eosinophilia, and from an unforeseen incident where such symptoms were caused after an inadvertent re-challenge of flucloxacillin in a patient (Lobatto *et al.*, 1982; Andrews and Daly, 2008).

1.6.2.1 HLA-B*57:01 associations with flucloxacillin-DILI and abacavir hypersensitivity

Genetic associations with DILI in immune-related genes have been described with the strongest associations typically found in the highly polymorphic HLA gene locus. Such an association was discovered in relation to flucloxacillin-induced liver injury as a result of a genome-wide association study (GWAS) in 2009 by Daly et al, under the DILIGEN project (Daly et al., 2009). 51 cases of flucloxacillin DILI and 282 controls were genotyped for 900 000 SNPs and a significant associations were discovered in the MHC region on chromosome 6. The top SNP found was a missense polymorphism, rs2395029, in the HCP5 gene found 100kb centromeric of HLA-B on chromosome 6. This SNP has previously been shown to be in complete linkage disequilibrium with the HLA allele HLA-B*5701 and has been used as a cheaper, less labour intensive method of screening for the B*5701 allele (Colombo et al., 2008). Subsequent genotyping of the flucloxacillin-DILI cases and controls showed a highly significant association between disease and HLA-B*5701 with an 80 fold increased risk of developing disease with this allele present (Daly et al., 2009). Among cases, 85% carried the HLA-B*5701 allele compared to approximately 8% in the general British Caucasian population. Despite this strong association, it has been estimated that the incidence of developing disease with this genotype after flucloxacillin treatment is only 1 in every 500 to 1000 individuals suggesting that other events or genetic factors are also involved.

A similar association between HLA-B*57:01and an ADR has been previously described for a hypersensitivity reaction to the antiretroviral agent, abacavir (Hetherington *et al.*, 2002; Mallal *et al.*, 2002). Unlike flucloxacillin-induced DILI this ADR is a general hypersensitivity reaction and not restricted to the liver. The association between HLA-B*57:01 and abacavir hypersensitivity appears to be stronger than the flucloxacillin association with approximately 50% of individuals possessing HLA-B*57:01developing a reaction when exposed to abacavir compared to less than 1 in 500 exposed to flucloxacillin. Genotyping for HLA-B*57:01 prior to commencing abacavir therapy now takes place on a global scale and has been successful at reducing incidence of abacavir hypersensitivity reactions (Mallal *et al.*, 2008).

The association of the class I allele HLA-B*57:01 and flucloxacillin-DILI and abacavir hypersensitivity suggests a possible role for T-cell mediated pathways in the diseases. This is also supported by the observation that lymphocytes from certain flucloxacillin DILI patients can be stimulated *in vitro* by flucloxacillin (Maria and Victorino, 1997; Spanou *et al.*, 2006). However, this was not observed in all patients and was not replicated subsequently by others (Monshi *et al.*, 2013). Compared to flucloxacillin-DILI, T-cell responses are relatively well characterised with regards to abacavir hypersensitivity. A landmark study by Chessman et al recently defined the role of HLA-B*57:01 in abacavir hypersensitivity. Stimulation was achieved of abacavir specific $CD8^+$ T-cells *in vitro* using peripheral blood mononuclear cells (PBMCs) from abacavir-hypersensitive patients and abacavir-naïve HLA-B*5701 positive donors (Chessman et al., 2008). They demonstrated that the $CD8^+$ T-cells secreted the inflammatory mediators tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) that were cytotoxic to abacavir-loaded antigen presenting cells (APC's). These mechanisms were found to be restricted to individuals expressing HLA-B*57:01 and shown to be dependent on the presence of functional TAP and tapasin molecules suggesting a mechanism of T-cell activation that requires the binding of intracellular processed haptenised-peptides that are presented to HLA-B*57:01 and expressed for T-cell recognition.

1.6.2.2 Flucloxacillin-hapten formation and T-cell mediated responses

Recent work by Monshi et al has uncovered some of the mechanisms of flucloxacillin mediated T-cell activation (Monshi *et al.*, 2013). As with abacavir, drug-specific T-cell responses producing IFN-γ and granzyme B secretion were observed in PBMCs from patients who have suffered from flucloxacillin-induced liver injury but unlike abacavir, this could not be replicated using PBMC's from either flucloxacillin tolerant or naïve HLA-B*57:01 positive donors. However, it was possible to prime flucloxacillin specific CD8+ T-cell clones in naïve individuals that were activated to secrete cytokines and express chemokine receptors following flucloxacillin stimulation in a similar manner to CD8+ clones from patients. Interestingly, flucloxacillin-reactive T-cell clones were generated from a HLA-B*57:01 negative patient but these were found to be CD4+ in nature suggesting that an alternative method of T-cell mediation may exist in B*57:01 negative patients.

Similarly to earlier work with abacavir, Monshi et al described a method of flucloxacillin-mediated T-cell activation that requires the formation of flucloxacillin-peptide haptens followed by intracellular processing and presentation to HLA molecules. Previously, flucloxacillin has been shown to form adducts *in vitro* and *in vivo*. Penicillin's can form adducts *in vitro* with proteins via ring opening of the β-lactam ring structure and it has been demonstrated that flucloxacillin can form adducts with lysine residues in human serum albumin (Kitteringham *et al.*, 1987; Jenkins *et al.*, 2009). With regards to *in* vivo adduct formation, western blot studies using a specific polyclonal antisera to synthetic flucloxacillin-protein conjugates raised by immunisation of rabbits have shown the presence of flucloxacillin adducts in the livers of treated rats (Carey and van Pelt, 2005). Six different adducts were discovered varying in subcellular location and molecular weight. Interestingly, an adduct was found in the microsomal fraction that corresponded in molecular weight to the cytochrome P450 enzymes suggesting that possible adduct formation may occur with the metabolising enzymes. Monshi et al suggest that this could possibly correspond to an flucloxacillin adduct to albumin which they demonstrated was able to stimulate T-cell responses (Monshi *et al.*, 2013).

1.6.2.3 The p-i concept and flucloxacillin-DILI

A recent study by Wuillemin et al has provided evidence that the pharmacological interaction (p-i) concept of T-cell stimulation may play a role in flucloxacillin mediated immune responses (Wuillemin *et al.*, 2013). The group generated cytotoxic, predominantly CD8+, flucloxacillin specific T-cell clones and T-cell lines from both HLA-B*57:01 positive and negative flucloxacillin-naïve healthy donors as reported elsewhere (Spanou *et al.*, 2006; Monshi *et al.*, 2013). The reactivity pattern of T-cell activation was studied by stimulating the T-cell clones with either flucloxacillin-pulsed autologous APCs or with APCs to which flucloxacillin was freshly added to in solution. Using these conditions the group stated that they could differentiate between a stable, covalent association between flucloxacillin and HLA as is produced by an intracellular processed and HLA-presented drug-peptide hapten and a reversible, noncovalent direct pharmacological interaction (p-i) between flucloxacillin and T-cells.

They report that flucloxacillin specific T-cells from all tested HLA-B*57:01 positive individuals reacted immediately with flucloxacillin in solution, independent of proteasomal processing, forming a complex that was not resistant to extensive washing. This suggests a mechanism that involves a direct, reversible bond between flucloxacillin, HLA and the T-cell receptor that is independent of haptenisation and HLA presentation as is described by the p-i concept. Conversely, the group observed that flucloxacillin specific T-cells from HLA-B*57:01 negative individuals did not react in this manner but were found to be activated by flucloxacillin-pulsed APCs. Activation here was time-dependent, required proteasome activity and was resistant to washing suggesting a mechanism that required hapten processing, presentation and covalent binding to the HLA.

Approximately 35% of HLA-B*57:01 positive T-cell lines also reacted in a hapten-like manner suggesting a probable role for both mechanisms in the HLA-B*57:01 response. The authors conclude, however, that because activation by the p-i concept route is specifically restricted to the HLA-B57:01 allele, whereas flucloxacillin reactive T-cells can be activated by haptens presented by various HLA alleles, the dominant pathway for flucloxacillin mediated T-cell activation is that of direct interaction of the drug and T-cell receptor i.e. the p-i concept.

1.6.2.4 Novel mechanisms for peptide binding to HLA-B*57:01

Recently, novel mechanisms have been described with regards to abacavir activation of T-cell responses. These mechanisms describe how abacavir can alter the nature of the peptide repertoire binding to HLA-B*57:01 resulting in the binding of novel self-peptides (Illing *et al.*, 2013). Illing and colleagues demonstrated that abacavir can bind non-covalently to the antigen binding cleft of B*57:01 altering the confirmation of the peptide anchoring site. The authors suggest that this change in the chemistry of the antigen binding cleft alters the repertoire of endogenous peptides that are usually tolerated upon binding to B*57:01, thus creating an ‘altered-self’ repertoire and neoantigens that can activate T-cells (Illing *et al.*, 2012). This was followed by an *in silico* study of randomised peptide libraries that showed that peptides with valine, alanine or isoleucine at the C-terminus had enhanced affinity for B*57:01 in the presence of abacavir (Ostrov *et al.*, 2012). A slightly different mechanism was proposed

by Norcross and colleagues. They demonstrated that abacavir enhances the binding of novel self-peptides to HLA-B*57:01 by binding of abacavir to self-peptides that are not in the constitutive repertoire of HLA-B*57:01 presentation (Norcross *et al.*, 2012). Interestingly, flucloxacillin was also used in this study but was not found to be able to increase binding of the self-peptide to B*57:01 used in the assay. This suggests that flucloxacillin may not bind to HLA-B*57:01 and induce loading of novel self-peptides but further investigation would be required to confirm this.

1.7 Aims of the study

The principal aim of this study was to determine underlying mechanisms of flucloxacillin-induced liver injury. The discovery of a strong association with HLA-B*57:01 and susceptibility to disease will be built upon and genetic polymorphisms studied in a case-control approach to identify novel genetic associations in metabolic and immune pathways. Regulatory responses in target genes are studied including the nuclear receptors PXR and CAR. Building on previous work, the interaction between flucloxacillin and PXR will studied alongside various other isoxazolyl penicillin's. The relevance of CAR in flucloxacillin-DILI is studied through the use of a reporter gene assay to study CAR activation and genotyping of polymorphisms in common CAR haplotypes. Flucloxacillin metabolic pathways are studied in a variety of *in* vitro systems to determining the role of cytochrome P450 in the metabolism of the drug. Mechanisms of flucloxacillin mediated T-cell activation are studied through incubation of patient peripheral blood mononuclear cells (PBMCs) with flucloxacillin and measurement of cytokine gene expression by real-time PCR. Genetic associations within killer-cell immunoglobulin-like receptors (KIR) are investigated using a case-control study approach.

Chapter 2. General Materials and Methods

2.1 Materials

Chemicals and reagents were purchased from the named suppliers listed in **Table 2.1**. As far as possible all reagents were analytical or molecular grade. Aqueous solutions were prepared with reverse osmosis distilled water. Solutions, glassware and other equipment were sterilised by autoclaving at 120 °C, 15 pounds per inch (PSI) pressure for 20 min. Plastic ware was either purchased sterilised or autoclaved where necessary. Solutions used for tissue culture were filter-sterilised using 0.2 mm filters (Millipore). PCR primer dilutions and reaction master mixes were made using pre-purchased sterile water (Fresenius Kabi Limited). For RNA work, diethyl pyrocarbonate (DEPC) treated, nuclease free water (Fisher Scientific) and RNase free plastic ware was used throughout. Compositions of frequently used stock solutions are listed in **Table 2.2**.

Table 2.1 – List of suppliers and addresses

Supplier	Address
Applied Biosystems	Paisley, UK
Bioline	London, UK
Eurofins MWG Operon	London, UK
Fermentas	York, UK
Fisher Scientific	Loughborough, UK
Greiner Bio-One	Stonehouse, UK
Invitrogen	Paisley, UK
Millipore	Watford, UK
New England Biolabs (NEB)	Hitchin, UK
Novagen	Nottingham, UK
Promega	Southampton, UK
QIAGEN	Crawley, UK
Sarstedt	Leicester, UK
Sigma Aldrich	Gillingham, UK
Thermo Scientific	Barnstead, UK

Table 2.2 – Composition of frequently used stock solutions

Solution	Constituents
10 X TBE	0.9 M Tris-Base 0.9 M Boric acid 20 mM EDTA
DNA gel loading buffer	0.25 % (w/v) bromophenol blue 0.25 % (w/v) xylene cyanol 30 % glycerol
Nuclear Lysis Buffer	0.4 M Tris-HCl, pH 8.0 60 mM EDTA 150 mM sodium chloride 1% (w/v) sodium dodecyl sulphate)

2.2 Flucloxacillin-DILI case and control recruitment

2.2.1 *Patient recruitment*

The DILIGEN and iDILIC studies aim to identify genetic determinants susceptibility of DILI through the collection of patient DNA samples retrospectively and prospectively. The DILIGEN study described the study based in the UK which is now part of a larger international study called iDILIC. A number of drugs are under investigation including co-amoxiclav, anti-TB drugs and flucloxacillin. In 2009, a GWA study performed by Daly et al included 51 cases of flucloxacillin-DILI collected from UK regional liver units (Daly *et al.*, 2009). These cases were subsequently used in studies performed by Elise Andrews at Newcastle University as part of her PhD studies. A further 21 DILIGEN cases were subsequently collected and included in further GWAS analysis and in these studies. A further 78 cases that have since been collected, as part of the on-going iDILIC project, have also been included in the present study. Of these additional cases, 21 have been collected from liver units in Sweden while the rest are from liver units across the UK. Clinical and biochemical parameters for the total number of patients (n=150) are listed in **Table 2.3**. Ethical approval for the UK study was provided by the Leeds East Research Ethics committee with the Swedish study obtaining separate approval in Sweden. After consent was given, patient suitability was assessed using the criteria detailed below.

Table 2.3 – Clinical and biochemical variables of DILI patients exposed to flucloxacillin included in the present study

Sex (F/M)	104/46
Age at onset (years)	64.0 ± 13.6
Time to onset (days)	24.0 ± 18.1
Total days on drug	10.4 ± 6.2
Pattern of liver injury	
• Cholestatic	92 (0.61)
• Hepatocellular	12 (0.08)
• Mixed	46 (0.31)
ICC scoring	
• 3 – 5 (possible)	17 (0.11)
• 6 – 8 (probable)	61 (0.41)
• 8 (highly probable)	71 (0.47)
• Not known	1 (0.01)
Peak Bilirubin (µmol/l)	263.7 ± 228.1
Peak ALT (U/l)	404.6 ± 256.5
Peak ALP (U/l)	570.7 ± 677.5
ALT/ALP decreased by ≥ 50% above ULN after drug discontinuation	
• Yes	142 (0.95)
• No	6 (0.04)
• Not known	2 (0.01)
Time taken for ALT/ALP to decrease to ≥ 50% after drug discontinuation (days)	66.9 ± 71.8

2.2.2 *Causality assessment*

A causal relationship of liver injury to flucloxacillin was assessed using the international consensus criteria (ICC) RUCAM method and biochemical parameters. Biochemical criteria used for inclusion of suspected flucloxacillin-DILI cases included (i) clinically apparent jaundice or bilirubin > 40 µmol/l (after exclusion of cases due to hemolysis), (ii) ALT > 5 X ULN or (iii) ALP > 2 X ULN plus any raised bilirubin above ULN. Causality assessment by the RUCAM is outline in section 1.2.3 and cases due to flucloxacillin were scored numerically as 'unlikely', 'possible', 'probable' or 'highly probable'.

2.2.3 *Recruitment of controls*

64 individuals who had been exposed to flucloxacillin in the previous five years without any adverse effects were recruited from UK hospitals and general practices. Controls were made up of 27 males and 37 females, with a mean age of 54.9 years, range 24 – 90 years. A control group consisting of 282 matched Population Reference Sample (POPRES) controls that were used in the Daly et al GWA study, have also been used for PLINK genotyping analysis. These controls were selected by a principal component analysis (PCA) for samples of Northern European origin from a total of 468 controls (Daly *et al.*, 2009).

2.3 Nucleic Acid Extraction Methodology

2.3.1 *Genomic DNA Extraction*

Cells were pelleted by centrifugation at 3000 g for 10 min at 4 °C (Sigma 3-16PK centrifuge) and resuspended in 2 ml nuclear lysis buffer (400 mM tris-HCl pH 8.0, 60 mM EDTA, 150 mM sodium chloride and 1% (w/v) sodium dodecyl sulphate). 0.5 ml 5 M sodium perchlorate was added and the sample mixed at room temperature for 15 minutes on a rotary mixer (Stuart Scientific) followed by incubation at 65 °C for 30 minutes in a Techne Dri-Block. 2.5 ml chloroform was added and the samples rotary

mixed at room temperature for 10 minutes to homogenise the mixture. Samples were centrifuged at 3000 g for 10 min at 4 °C to separate the organic and aqueous phases. The upper DNA-containing aqueous phase was carefully transferred to a clean 15 ml polypropylene centrifuge tube to which 5 ml ethanol was added, and the tube inverted several times to precipitate the DNA out of solution. The DNA was spooled onto a sterile disposable loop and allowed to air-dry for 10 min at room temperature before being dissolved overnight at 60 °C in 200 µl 5 mM tris-HCl buffer (pH 8.0) in a sterile 1.5 ml microfuge tube.

2.3.2 *RNA extraction methodology*

2.3.2.1 RNA isolation

RNA was extracted from cell samples using TRI Reagent solution (Applied Biosystems). 1 ml TRI reagent was added per 5 – 10 x 10^6 cells and the sample homogenised by pipetting. The mixture was transferred to an RNase free 1.5 ml microfuge tube and incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complexes. 200 µl chloroform was added and mixed by vigorous shaking for 15 seconds before incubation at room temperature for 10 minutes. Samples were centrifuged at 13000 rpm, at 4 °C for 15 minutes and the RNA - containing aqueous phase transferred to a fresh tube. 500 µl isopropanol was added to precipitate RNA and the samples vortexed before incubation at room temperature for 10 minutes. The samples were centrifuged at 13000 rpm for 8 minutes at room temperature to pellet the RNA and the supernatant carefully removed and discarded. 1 ml of 75 % ethanol was added to wash the RNA pellet before a final centrifugation at 13000 rpm for 5 minutes at room temperature. The ethanol was carefully removed and the RNA pellet allowed to air dry before resuspension in an appropriate volume of nuclease-free water (typically 30 – 50 µl). RNA samples were stored at – 80 °C.

2.3.2.2 Determining RNA integrity by agarose gel electrophoresis

A 1% agarose gel was prepared by dissolving 1 g agarose in 88 ml water by microwave heating, to which, after cooling to 60 °C, 10 ml 10 X MOPS buffer (0.2 M MOPS (3-

[N-morpholino] propanesulfonic acid), 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0) and 2.7 ml 37% formaldehyde was added. 100 µl RNA loading buffer was prepared containing 10 µl 10 X MOPS buffer, 11.5 µl RNase-free water, 50 µl deionised formamide, 17.5 µl 37% formaldehyde, 10 µl 10 X loading dye (50 % sterile glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and 1 µl ethidium bromide (10 mg/ml). 1 µg RNA was added to 5 µl loading buffer and incubated at 65 °C for 10 min then chilled on ice for 2 min before being loaded onto the gel. Electrophoresis was performed at a constant voltage of 100 V, for 45 min in 1 X MOPS buffer. The integrity of the RNA was determined by gel visualisation. Complete RNA will show two strong bands corresponding to the ribosomal RNA. The large 28S band is approximately of 5 kb size, while the smaller 18S band is approximately 2 kb.

2.3.3 *Evaluation of nucleic acid yield and purity*

DNA and RNA yield and purity was measured using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific). An absorbance unit of 1 at 260 nm is equivalent to 50 µg/ml double stranded DNA and 40 µg/ml RNA. The ratio of the 260 nm measurement to the 280 nm measurement is used to indicate purity. Ratios of 1.8 to 2.0 indicate pure DNA/RNA.

2.3.4 *Storage of nucleic acid samples*

Quantified DNA samples were diluted to concentrations of 50 µg/ml and stored at – 20 °C. RNA samples were stored in 1.5 ml RNase-free screw-cap microfuge tubes and stored at – 80 °C.

2.4 Polymerase Chain Reaction (PCR) Methodology

2.4.1 *Primer design*

Primers for PCR were taken from literature sources, where stated, or otherwise designed using the public tool, Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Primers were designed, where possible, to have a length of approximately 18-20 nucleotides, melting temperatures (T_m) between 52 – 58 °C and a GC content of approximately 50 %. The annealing temperature of each primer pair was determined through PCR optimisation, beginning at 5 °C below the specified T_m. All primers were purchased from Sigma-Aldrich, UK, and resuspended in sterile water to 200 µM. Working stocks (25 µM) were made and stored at 4 °C, with remaining stocks stored at - 20 °C.

2.4.2 *PCR protocol*

Genomic template DNA (50 ng) was amplified in a total reaction volume of 20 µl containing 1 X ThermoPol reaction buffer (NEB), 0.25 µM forward primer, 0.25 µM reverse primer (Sigma-Aldrich, UK), 0.25 mM dNTP's (Bioline), 0.5 U Taq Polymerase (NEB). Reactions were conducted in 0.2 ml sterile thin-walled tubes (Fisher Scientific) and thermocycling performed on an Applied Biosystems 2720 Thermal Cycler. Cycling conditions typically consisted of the following standard programme, with varying primer annealing temperatures optimised specifically to each assay primer set;

DNA denaturation - 5 min - 94 °C

35 cycles of

Denaturation - 30 sec - 94 °C

Annealing – 30 sec – at optimised temperature

Extension – 30 sec – 72 °C

Final extension – 7 min - 72 °C

PCR efficiency was determined by running 10 µl of the product on a 2 % agarose gel as in section 2.5.1.

2.5 Electrophoresis and visualisation of DNA

2.5.1 *Agarose gel electrophoresis*

2 % agarose gels were prepared by dissolving 2 g DNase and RNase free agarose powder in 100 ml 1 X TBE buffer to which ethidium bromide was added (0.5 µg/ml). 1 µl of 6 X gel loading buffer (0.25% bromophenol blue, 0.35% xylene cyanol, 30% glycerol) was mixed with 5 µl DNA product and loaded onto the gel alongside a 100 – 1000 bp molecular weight marker (NEB). Electrophoresis was performed at 80V for ~ 30 min in 1 X TBE buffer followed by gel visualisation as described in section 2.5.3.

2.5.2 *Polyacrylamide gel electrophoresis (PAGE)*

10 % polyacrylamide gels were prepared containing 30 % acrylamide-bis acrylamide 29:1 (Fisher Scientific) in 1 X TBE buffer, 0.4 mg/ml ammonium persulphate (APS) and 0.1 % TEMED. The mixture was poured between two 200 mm x 200 mm sealed glass plates separated by 0.8 mm spacers and the gel allowed to polymerise. 7 µl gel loading buffer was added to 20 µl DNA digestion product and loaded into gel wells. Electrophoresis was performed at 150 V for 3 – 4 hours in 1 X TBE buffer. Following electrophoresis, gels were stained in a solution of 1 X TBE buffer containing 0.5 µg/ml ethidium bromide for 20 min followed by gel visualisation as described in section 2.5.3.

2.5.3 *Gel visualisation*

Gels were visualised on a Syngene GENi v1.1.1.14 gel documentation system (Syngene, Cambridge, UK).

2.6 Quantitative real-time PCR methodology

2.6.1 *cDNA synthesis by Reverse Transcription*

First strand cDNA was synthesised using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (NEB). 1 µg RNA was added to 1 µl random hexamers (0.4 µg/µl) (QIAGEN), 1 µl dNTP's (10 mM) (NEB) and 6 µl DEPC water and incubated at 65 °C for 10 minutes. Samples were placed on ice for 2 minutes before being added to a reverse transcriptase master mix containing 2 µl 10 X RT buffer (75 mM KCl, 50 mM Tris-HCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, pH 8.3 at 25 °C) (NEB), 50 U M-MuLV reverse transcriptase (200 000 U/ml) (NEB), 4 U RNase inhibitor (40 000 U/ml) (NEB) and DEPC water to make a total reaction volume of 20 µl. Samples were incubated on a thermal cycler at 37 °C for 50 minutes to synthesis cDNA followed by 70 °C for 15 minutes to inactivate the reverse transcriptase. cDNA samples were stored at – 20 °C.

2.6.2 *Quantitative real-time PCR (qPCR)*

Quantitative real-time PCR (qPCR) is a powerful tool that allows quantification of gene expression. Quantification of the amplicon is performed during the exponential amplification phase of the reaction based on the continuous collection of fluorescent signals over a range of cycles in 'real-time' (Dorak, 2006). The fluorescent signals are produced by a reporter dye where the increase in fluorescence is directly proportional to the increase of amplified product in the PCR reaction. Quantification of the reaction is based on the cycle at which fluorescence significantly exceeds the baseline background fluorescence during the exponential phase. The cycle at which this occurs is arbitrarily set and termed the threshold cycle (Ct). The Ct value is inversely proportional to the amount of amplicon in the reaction i.e. the lower the Ct, the greater the amount of amplicon (Schmittgen and Livak, 2008). There are generally two different chemistries available for detection of qPCR amplicons including non-specific double stranded DNA binding dyes, e.g. SYBR Green I and sequence specific fluorescent probes e.g. TaqMan assays. Both of these techniques are used and described in these studies.

Gene expression measured by qPCR data can be presented as either absolute or relative expression. Absolute quantification provides the exact copy number of a target determined by a standard curve of known concentrations. Data can also be presented as relative expression (ΔCt) where expression level of a target gene is compared to that of a 'house-keeping' reference gene used to normalise for differences in the amount of initial cDNA added to each reaction. A suitable house-keeping gene should be abundant

and relatively constant in various tissues and cell types. Examples of commonly used control genes include glyceraldehyde-3-phosphate (GAPDH), β-actin and 18S ribosomal RNA (18S rRNA) (Livak and Schmittgen, 2001).

In the present study, relative gene expression in qPCR experiments was measured using the ΔΔCt method of analysis. Expression of the target gene is normalised to expression of the internal control using the equation:

$\Delta Ct = Ct\ (target\ gene) - Ct\ (internal\ control)$

Next, treated samples are calibrated to untreated samples so that:

$\Delta\Delta Ct = \Delta Ct\ (treated) - \Delta Ct\ (untreated)$

Relative fold change in target gene expression is then determined by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.6.2.1 Gene expression by qPCR using SYBR Green I

Primer sequences used for qPCR are displayed in **Table 2.4**. CAR primers were from Lamba et al (Lamba *et al.*, 2004). CYP3A4 primers and primers for the housekeeping control gene GAPDH were taken from the thesis of Elise Andrews, Newcastle University. Reaction conditions were optimised by standard PCR and primers validated for linear amplification to allow for the ΔΔCt method of analysis. cDNA was amplified in a total reaction volume of 20 µl containing 10 µl 2 X SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 300 nM forward primer and 300 nM reverse primer. PCR amplification was performed in MicroAmp® Fast Optical 48-well PCR plates (Applied Biosystems) on an Applied Biosystems StepOne™ Real-Time PCR system.

Table 2.4 – Primers used for SYBR Green I qPCR

Gene Name	**Primer Sequences**
CAR	F - 5'-GGAGAGGCATTCCATACCAG-3' R – 5'- TTCCCACTCCAGTGTATCCAG-3'
CYP3A4	F – 5'-TGTCCTACCATAAGGGCTTTTGTA-3' R – 5'-TTCACTAGCACTGTTTTGATCATG-3'
GAPDH	F – 5'-GGGTGTGAACCATGAGAAGTATGA-3' R – 5'-CATGAGTCCTTCCACGATACCAA-3'

2.6.2.2 qPCR with TaqMan Gene Expression Assay

cDNA was amplified in a total reaction volume of 20 µl containing 2 X TaqMan Universal Master Mix, 20 X TaqMan Gene Expression Assay and DNase free water. Reactions were performed in triplicate and non-template controls were included. PCR amplification was performed in MicroAmp® Fast Optical 48-well PCR plates (Applied Biosystems) on an Applied Biosystems StepOne™ Real-Time PCR system. Thermocycling conditions consisted of an initial hold stage of 10 min at 95 °C to activate the AmpliTaq Gold® polymerase, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min. Gene expression data was calculated by the comparative Ct (ΔΔCt) method and analysed using StepOne Software version 2.1.

2.7 Mammalian cell culture

2.7.1 *Culture of adherent cell lines*

HepG2 cells (human hepatocellular carcinoma cell line) and Caco-2 cells (human colon adenocarcinoma cell line) were supplied by Fiona Fenwick (Institute of Cellular Medicine, Newcastle University). LS180 (human colon adenocarcinoma cell line) were purchased from the European collection of cell cultures (ECACC, Porton Down, UK). Cell culture was performed under aseptic conditions in a Class II laminar flow microbiological safety cabinet. All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 1 % non-essential amino acids (0.1 mM), 2 mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin. All cell lines were cultured as monolayers in 75 cm^2 flasks and incubated at 37 °C with 5 % CO_2 in humidified air. All media were stored at 4°C and warmed to 37°C before use.

2.7.2 *Passage of cells*

Upon reaching approximately 80 % confluency, cell were passaged. Cell medium was aspirated and the cell monolayer washed with sterile 1 x phosphate buffered saline (PBS). Cells were detached from the flasks using 0.25 % trypsin- 0.02 % EDTA in PBS solution at 37 °C. Once detached, 10 ml cell culture medium was added, the suspension transferred to a sterile 50 ml Falcon tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 10 ml fresh complete media. Cells were either then seeded for cell maintenance or counted on a haemocytometer counting chamber then seeded accordingly for experimental protocols as described.

2.8 Reporter gene assay methodology

2.8.1 *E.Coli transformation*

Plasmid DNA was synthesised via transformation of JM109 *E. coli* Competent cells (Promega, UK). Cells were split into working aliquots of 100 µl in polypropylene culture tubes and stored at – 80 °C. For transformation, cells were thawed on ice and 50 ng plasmid DNA added followed by incubation on ice for 30 minutes. The *E. coli* were then heat shocked at precisely 42 °C for 45 seconds before being returned to ice for 5 minutes. 500 µl cold sterile L-broth (1 % tryptone, 0.5 % yeast extract, and 1 % sodium chloride) was added to the transformed cells and the culture incubated in an orbital incubator at 150 rpm, at 37 °C for 1 hour. The cells were pelleted by brief centrifugation, part of the supernatant was removed leaving approximately 100 µl medium in which the cells were resuspended. The resuspended cells were spread onto LB-agar plates contacting the appropriate antibiotic selection marker for the particular plasmid (ampicillin (100 µg/ml) or kanamycin (50 µg/ml)) and incubated overnight at 37 °C.

2.8.2 *Plasmid DNA extraction*

A single, well-defined white colony was picked following overnight incubation and used to inoculate 10 ml of sterile LB medium containing the appropriate antibiotic selection marker. A starter culture was then produced by growing the cells overnight in

an orbital incubator at 150 rpm at 37 °C. The bacterial cells were harvested by centrifugation at 1500 rpm for 10 min at 4 °C to pellet the cells and plasmid DNA was extracted using a QIAGEN miniprep plasmid extraction kit (QIAGEN, UK). DNA was eluted in 50 µl sterile water and DNA concentration determined as described in section 2.3.3.

2.8.3 *Cell seeding*

Cells were seeded into 24-well plates at a density of 5 x 10^4 cells/ml per well in DMEM medium and allowed to adhere until 50 - 80 % confluency was achieved. Medium was removed and replaced with 200 µl fresh DMEM per well.

2.8.4 *Transfection protocol*

Cell transfections were performed using GeneJuice Transfection Reagent (Novagen, UK). For each well, 1.5 µl GeneJuice reagent was added to 100 µl serum-free medium, briefly vortexed and incubated at room temperature for 5 minutes. Experimental reporter plasmid DNA was added to the mixture as required by the procedure being followed and is described in Chapter 4. 0.05 µg of pRL-TK *Renilla* Luciferase reporter vector was added per well as an internal control. The reaction mixture was incubated at room temperature for 15 minutes before adding 100 µl drop wise to the 200 µl of complete medium already in each well. After 4 hours incubation, transfection reagents were replaced with 1 ml fresh growth medium. Experimental treatment procedures are described in chapter 4.

2.8.5 *Luciferase assay*

Reporter activity was assessed using the Dual-Luciferase Reporter Assay system (Promega, UK). This ‘dual-glo’ assay exploits the simultaneous expression of two individual reporter enzymes in one system. The activity of an experimental reporter construct consisting of the firefly luciferase enzyme is correlated to the activity of the co-transfected internal control consisting of the renilla luciferase enzyme. Normalising

the activity of the experimental construct to that of the internal control minimises variability caused by differences in transfection efficiency, cell viability, cell lysis efficiency, etc.

Following treatment, drug-containing medium was removed from cells which were then washed in sterile PBS before the addition of 100 µl 1 X Passive Lysis Buffer per well. Plates were incubated at room temperature, with shaking, for 15 minutes to disrupt the cells. While cells were incubating, the Dual-Luciferase assay reagents were prepared according to the manufacturer's instructions. Luciferase Assay Reagent (100 µl) was added to 20 µl cell lysate in a clear 1.5 ml microfuge tube and mixed thoroughly. Firefly luciferase activity (in relative light units) was then measured using a bench top luminometer (GloMax-Multi Jr, Promega, UK). Firefly luciferase activity was quenched by the addition of Stop and Glo reagent (100 µl). The mixture was briefly vortexed before a second luminescence reading was taken to measure renilla luciferase activity. Luminescence readings were taken as an average of 10 consecutive readings over 10 seconds.

Chapter 3. Genotyping of candidate genes in Flucloxacillin-DILI

3.1 Introduction

There is a strong relationship between carriage of the HLA-B*57:01 allele and risk of flucloxacillin-induced liver injury but this association cannot explain fully the genetic basis of susceptibility to the disease. Most but not all cases of flucloxacillin-DILI are positive for HLA-B*57:01. In this chapter, additional cases of flucloxacillin are genotyped for the HLA-B*57:01 association and a set of HLA-B*57:01 negative cases are genotyped for HLA-B alleles to investigate whether any additional HLA-B associations may exist. Apart from the HLA-B*57:01 association with flucloxacillin-DILI and abacavir hypersensitivity, there are various other HLA-B associations reported as having a role in adverse drug reactions including DILI, with an association reported between the HLA-B*18:01 allele and susceptibility to liver injury by the penicillin antimicrobial co-amoxiclav, though this was detected only in a Spanish population (Stephens *et al.*, 2013). In addition to investigating other possible HLA-B associations, flucloxacillin-DILI cases will also be genotyped for HLA-C allele C*06:02 using a tag SNP approach. HLA-C, and in particular HLA-C*06:02, has been associated in a number of studies as the major susceptibility gene for increased risk of the T-cell mediated inflammatory skin disease, psoriasis (Nair *et al.*, 1997; Helms *et al.*, 2005; Nair *et al.*, 2006; Liu *et al.*, 2008). A number of other MHC associations also exist suggesting an apparent psoriasis susceptibility locus (PSORS1) (Feng *et al.*, 2009). One of these associations is with HLA-B*57:01, as has been shown in a GWAS by Liu and colleagues who identified the HLA-B*57:01 proxy SNP, rs2395029 in HCP5, significant in psoriasis patients with an odds ratio of 4.1 ($p = 2.13 \times 10^{-26}$) (Liu *et al.*, 2008). HLA-B*57:01 and HLA-C*06:02 associations also exist in a subset of human immunodeficiency virus-1 (HIV-1) infected individuals that results in a clinical phenotype whereby these individuals are able to maintain a low level of circulating virus in plasma thus restricting disease progression (Fellay *et al.*, 2007). The HLA-B*57:01 association was described by Migueles and colleagues in a cohort of HIV-1 non-progressors where they found that 11/13 where HLA-B*57:01 positive compared to 19/200 in HIV-1 progressor controls (Migueles *et al.*, 2000). A large number of subsequent studies in various cohorts, including numerous GWA studies, have confirmed this association along with other genetic associations including some involving HLA-C (Carrington and O'Brien, 2003; Fellay *et al.*, 2009; Limou *et al.*, 2009; van Manen *et al.*, 2009). A recent analysis by Chen et al compared HLA region

SNP associations between a cohort of psoriasis patients and controls and a cohort of HIV-1 non-progressors and controls (Chen *et al.*, 2012). They found that there was a pattern of several SNPs that were observed in both psoriasis susceptibility and HIV-1 non-progressor phenotype with the two most significant observations found in HLA-B*57:01 and HLA-C*06:02.

In addition to the role of HLA-B*57:01 in the slow progression of HIV-1 infection, there is also an apparent synergistic relationship with genes that encode receptors involved in innate immunity. Killer immunoglobulin-like receptors (KIRs) are expressed on the surface of natural killer (NK) cells, a subset of lymphocytes that play an important role in innate immunity against pathogens and tumours by targeting infected host cells (Cerwenka and Lanier, 2001). Although they are cells of the innate immune system, NK cell actions share many similarities with those of T-cells. Like $CD8^+$ cytotoxic T-cells, NK cells can also secrete perforin and granzyme as a mechanism to kill infected cells and also produce cytokines such as interferon-γ (Lanier, 2005). NK cell function is controlled by various activating and inhibitory cell surface receptors that regulate their activation, proliferation and effector functions. KIRs recognise HLA class I molecules expressed on host cells and can confer both an activating or inhibitory signal to regulate NK cell responses. Most KIRs are thought to exhibit an inhibitory effect on NK cell function. This means that HLA class I expression on host cells inhibits NK cell activity preventing targeting of host cells. However, if HLA expression is down-regulated, as may occur when cells are infected or transformed into tumour cells, it leads to the generation of a 'missing-self' signal that results in NK cell-mediated cytotoxicity against the cell (Bashirova *et al.*, 2006). *KIR* genes are located on chromosome 19q13.4 in the leukocyte receptor complex (LRC) and, like *HLA* genes, are highly polymorphic (Wende *et al.*, 1999). There are over a dozen *KIR* genes described including *KIR3DL1* and *KIR3DS1* that were previously considered to be two separate genes but have been shown to actually segregate as alleles of the same locus (Bashirova *et al.*, 2006). *KIR3DL1* encodes an inhibitory KIR molecule whereas *KIR3DS1* encodes a receptor molecule thought to activate NK cells. KIR3DL1 specifically recognises HLA-B molecules that contain a Bw4 motif at amino acid positions 77-83 (Bashirova *et al.*, 2011). The Bw4 epitope is found in approximately one third of HLA-B molecules including HLA-B*57:01, therefore cells expressing HLA-B*57:01 are ligands for KIR3DL1 and inhibit NK cell activity. The remaining two thirds of HLA-B molecules contain a Bw6 epitope (Martin *et al.*, 2002). Ligands

for KIR3DS1 have not been determined but it is thought that they are similar to those of KIR3DL1 since the two alleles share 97% sequence homology (Martin *et al.*, 2007). Martin et al described an epistatic synergistic interaction between KIR3DS1 and HLA-B alleles expressing the Bw4 motif with isoleucine at position 80 (Bw4-80Ile) that conferred a protective effect against HIV-1 progression (Martin *et al.*, 2002). This suggests that this activating KIR allele may bind to HLA-B Bw4-80Ile expressing HIV-1 infected host cells targeting them for NK cell destruction. This study also showed that HLA-B*57:01 expresses the Bw4-80Ile motif that interacts with KIR3DS1. Interestingly, the recent study by Chen et al showed that increased risk of psoriasis was associated with HLA-B alleles containing the Bw4-80Ile epitope and KIR3DS1 genotype in patients (Chen *et al.*, 2012). In this chapter, flucloxacillin-DILI cases and controls are genotyped for *KIR3DL1* and *KIR3DS1*. We hypothesise that an association between *KIR3DS1* and flucloxacillin-DILI cases may provide a potential mechanistic pathway of immune-mediated flucloxacillin toxicity in a fashion that is inverse to how *KIR3DS1* and HLA-B*57:01 confer protection in HIV-1 non-progressors. If KIR3DS1 is associated with flucloxacillin-DILI it could indicate that a potential interaction exists with HLA-B*57:01 expressed in host cells in the liver leading to activation of NK cells and cytotoxic responses.

Collaborative studies to determine genetic risk factors for flucloxacillin-DILI are in progress elsewhere. This has included exome sequencing of flucloxacillin-DILI cases which has provided some novel data (Daly, A.K., Goldstein J and Daly, M.J., unpublished). The most significant findings involved variants located on chromosome 6, including variants characteristic of HLA-B*57*01. Two variants not on chromosome 6, rs41475144 in ubiquitin specific peptidase 8 (*USP8*) gene on chromosome 15 ($p = 1.46 \times 10^{-23}$ in exome sequencing analysis) and rs45483102 in caspase 5 (*CASP5*) on chromosome 11 ($p = 0.000163$) were chosen for further analysis in additional flucloxacillin-DILI cases. These variants were chosen based on their location and for their possible roles in apoptosis and inflammatory responses, which seemed biologically relevant to flucloxacillin-DILI.

Table 3.1 – A summary of the variants studied in this chapter

Gene	SNP	Samples genotyped
HCP5 (Tag SNP for HLA-B*57:01)	rs2395029	All additional flucloxacillin-DILI cases
High-resolution HLA-B allele genotyping	N/A	HLA-B*57:01 negative flucloxacillin-DILI cases and cloxacillin/dicloxacillin-DILI cases
KIR3DL1 and KIR3DS1	N/A	All flucloxacillin-DILI cases and controls
HLA-C*06:02	rs12191877	All flucloxacillin-DILI cases and controls
USP8	rs41475144	All flucloxacillin-DILI cases and controls
CASP5	rs45483102	All flucloxacillin-DILI cases and controls

3.2 Methods

3.2.1 *Case and Control recruitment*

150 cases of flucloxacillin-DILI and 63 healthy flucloxacillin-treated controls, as described in section 2.2.1 and 2.2.3, were included for genotyping analysis. 282 POPRES controls were also included for PLINK genotyping analysis of flucloxacillin GWAS data. PLINK is a free, open source software that allows genotype analysis of whole genome association datasets (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.*, 2007).

A subset of POPRES controls consisting of 107 North-western European (NW-EU) individuals that have been previously been genotyped for HLA-B alleles were used for HLA analysis. Where SNP markers were not included in flucloxacillin GWAS data, rendering POPRES controls unavailable for PLINK analysis, an additional cohort of healthy population controls, supplied by Dr Peter Donaldson, were used for genotyping analysis (Donaldson *et al.*, 2006).

For KIR genotyping analysis a control group was selected from the Allele Frequency Net Database (AFND) (http://www.allelefrequencies.net/). The AFND is a free public online repository containing information on the frequencies of several immune genes, including KIR loci, in different worldwide populations (Gonzalez-Galarza *et al.*, 2011). KIR genotype frequencies in this study were compared to an English population cohort containing 483 Caucasian individuals genotyped for KIR3DL1 and KIR3DS1.

3.2.2 *HCP5 (rs2395029) genotyping of flucloxacillin-DILI cases by PCR-RFLP*

Additional flucloxacillin-DILI cases recruited as part of the iDILIC project were genotyped for rs2395029 (T/G) by PCR using the following primers – 5'-ATTGTGTGACAGCAGCCATG-3' and 5'-CACCTGTCGTGGGATTTTGC-3'. PCR was performed as described in section 2.4.2 with standard cycling conditions and an annealing temperature of 55°C. The PCR product (10 µl) was digested overnight at 60°C with 2U of BstNI, with added 1 X bovine serum albumin (BSA). Digested PCR products were separated on a 10% polyacrylamide gel and electrophoresis performed followed by gel visualisation as described in sections 2.5.2 and 2.5.3 respectively.

3.2.3 *HLA genotyping of HLA-B*57:01 negative flucloxacillin-DILI cases*

26 HLA-*B*57:01* negative flucloxacillin-DILI cases were genotyped for HLA-B alleles using an AllSet^{+}™ Gold sequence-specific primer (SSP) HLA-B Locus High Res Kit (Invitrogen) according to the manufacturer's instructions. The SSP method is a PCR based technique that uses sequence-specific primers for genotyping of the HLA-B locus at a high resolution allelic level. The kit consists of a 96 well PCR test plate in which each well contains a lyophilised SSP solution consisting of allele and/or group-specific primers as well as a control primer pair of a non-allelic sequence amplified in all samples. 608 µl water and 7µl Taq Polymerase (5 units/µl) were added to the PCR buffer solution provided and mixed by pipetting. 10 µl of the master mix was added to the negative control well in the 96 well plate. 125 µl of sample DNA (50 ng/µl) was added to the remaining master mix and 10 µl added to each remaining well. The PCR plate was sealed and amplified on an Applied Biosystems 2720 Thermal Cycler set to the cycling conditions specified in the manufacturer's protocol. PCR products from each well were loaded directly onto a 2% agarose gel containing ethidium bromide (0.5µg/ml) and electrophoresis was performed at 100 V for 45 min in 1 X TBE buffer. Gels were visualised as described in section 2.5.3. Positive lane amplifications were identified and HLA-B alleles determined by analysis with UniMatch® PLUS 6.0 SSP software (Invitrogen).

3.2.4 *KIR genotyping of flucloxacillin-DILI cases by SSP-PCR*

KIR genotyping of flucloxacillin-DILI cases was performed using a KIR Typing Kit (MACS molecular, Miltenyi Biotec, UK) according to the manufacturer's instructions. KIR genotype was determined by PCR using sequence-specific primers (SSPs). Briefly, 25 µl resuspension buffer was added to the negative control well. To the remaining 575 µl resuspension buffer, 3 µg genomic DNA was added and the solution mixed well. 25 µl was dispensed onto the lyophilised enzyme mix in each of the remaining 21wells. The plate was sealed and amplified on an Applied Biosystems 2720 Thermal Cycler following cycling conditions according to the manufacturer's instructions. After thermocycling, 10 µl per sample was loaded directly onto a 2 % agarose gel and

electrophoresis was performed as described in section 2.5.1. Following electrophoresis, gels were visualised as described in section 2.5.3 and KIR genotype was interpreted with the evaluation form supplied with the kit.

3.2.5 *KIR3DS1/KIR3DL1 genotyping by multiplex PCR*

Flucloxacillin-DILI cases and controls were genotyped for *KIR3DL1* or *KIR3DS1* genotype by a multiplex SSP-PCR described by Kulkarni et al (Kulkarni *et al.*, 2010). *KIR3DL1* was amplified using primers - 5'- CGCTGTGGTGCCTCGA-3' and 5'-GGTGTGAACCCCGACATG-3' producing a PCR product of 191 bp. *KIR3DS1* was amplified by the primers – 5'-AGCCTGCAGGGAACAGAAG-3' and 5'GCCTGACTGTGGTGCTCG-3' producing a product of 300 bp. All primers were used at a concentration of 5 µM and PCR was performed as described by the protocol in section 2.4.2 with an initial denaturation step of 3 min at 94 °C; 5 cycles of 15 sec at 94 °C, 15 sec at 65 °C and 30 sec at 72 °C; 21 cycles of 15 sec at 94 °C, 15 sec at 60 °C and 30 sec at 72 °C; 4 cycles of 15 sec at 94 °C. 60 sec at 55 °C and 120 sec at 72 °C followed by a final extension step of 7 min at 72 °C. Following amplification, agarose gel electrophoresis was performed as described in section 2.5.1 and gels were visualised as in section 2.5.3.

3.2.6 *HLA-C (rs12191877), USP8 (rs41475144) and CASP5 (rs45483102) genotyping by TaqMan qPCR*

Flucloxacillin-DILI cases and controls were genotyped for three SNPs by qPCR using TaqMan SNP assays. The *CASP5* (rs45483102) SNP was purchased as a readymade 'off-the-shelf' assay whereas assays for *HLA-C* (rs12191877) and *USP8* (rs41475144) were custom designed. Before custom assay design, the SNPs were confirmed to satisfy the requirements needed for successful assay design. For optimal assay design, an approximate 600 nucleotide length of the target SNP flanking sequence was imported from data for each SNP on the 1000 genomes website (http://www.1000genomes.org/) and the SNP nucleotide change highlighted. Sites that contain ambiguous bases were masked and a BLAST query was performed on the target sequence to verify the uniqueness of the sequence (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). TaqMan

SNP assays are designed so that they contain two different TaqMan MGB fluorescent probes, one FAM labelled and one VIC labelled, to distinguish the two different nucleotides present in the SNP. The two labelled probes and an unlabelled primer mix are supplied as an assay mix. Genotyping is performed in a total reaction volume of 20 µl containing 50 ng genomic DNA, 2 X TaqMan Universal Master Mix, 20 X TaqMan SNP assay mix and DNase free water. Non-template controls were included and PCR amplification was performed in MicroAmp® Fast Optical 48-well PCR plates (Applied Biosystems) on an Applied Biosystems StepOne™ Real-Time PCR system. Thermocycling conditions consisted of an initial hold stage of 10 min at 95 °C to activate the AmpliTaq Gold® polymerase, followed by 40 cycles of denaturation at 92 °C for 15 sec and annealing/extension at 60 °C for 1 min. Allelic determination was performed using StepOne Software version 2.1.

3.2.7 *Statistical analysis*

Two-tailed probabilities were calculated for allele and genotype distributions using Fisher's exact test on Prism software (GraphPad). Adherence to the Hardy-Weinberg equilibrium for genotypes was determined by the chi-squared test using a web-based calculator available at http://www.tufts.edu/. Univariate analysis was performed on HLA-B allele genotyping and *P*-values were not corrected for multiple testing.

3.3 Results

3.3.1 *HCP5 (rs2395029) genotyping of flucloxacillin-DILI cases*

In the 2009 GWA study by Daly et al, a missense polymorphism, rs2395029, in *HCP5* was found to be the top SNP associated with flucloxacillin-DILI (Daly *et al.*, 2009). This SNP has been shown to be in complete linkage disequilibrium with *HLA-B*57:01* and direct genotyping for HLA-B*57:01 in the 51 cases of flucloxacillin-DILI included in the GWAS showed a perfect correlation between HLA-B*57:01 and rs2395029 genotypes. Since the GWAS, a further 99 cases of flucloxacillin-DILI have been collected as part of the iDILIC project. These cases have been genotyped, with assistance from Julia Patch, for rs2395029 using a PCR-restriction fragment length polymorphism (RFLP) approach. **Figure 3.1** shows the *BstNI* restriction digest of three samples. This enzyme cuts the wild-type allele (T) producing fragment sizes of 134 bp, 61 bp and 55 bp (Lane 1) and the mutant risk allele (G) producing fragments that have sizes of 72 bp, 62 bp, 61 bp and 55 bp. Individual 1 is TT homozygous and individuals 2 and 3 are TG genotype.

Genotype distributions for rs2395029 for the 51 original cases, the 99 additional cases and POPRES controls are shown in **Table 3.2**. In the original cohort of 51 cases, 43 (84.3%) carried the mutant G allele. This is replicated here, where, among the 99 additional cases, 81 (81.8%) carry the mutant G allele. Carriage of the mutant G allele is associated with HLA-B*57:01 genotype. Direct genotyping by SSP-PCR for HLA-B*57:01 was performed by Julia Patch using an AllSet+™ Gold SSP B17 High Res Kit (Invitrogen) which confirmed HLA-B*57:01 carriage. In the GWA study, carriage of the risk G allele for rs2395029 was significantly associated with flucloxacillin-DILI compared to POPRES controls with an odds ratio of 45.2 (95% CI 19.4 – 105.1). This association is replicated here, in the 99 additional flucloxacillin-DILI cases with a *p*-value of 2.06×10^{-39} and OR of 37.8 (95% CI 20.0 – 71.4). Combining the total number of flucloxacillin-DILI cases gives a *p*-value of 3.67×10^{-52} and an OR of 40.1 (95% CI 22.7 – 70.7).

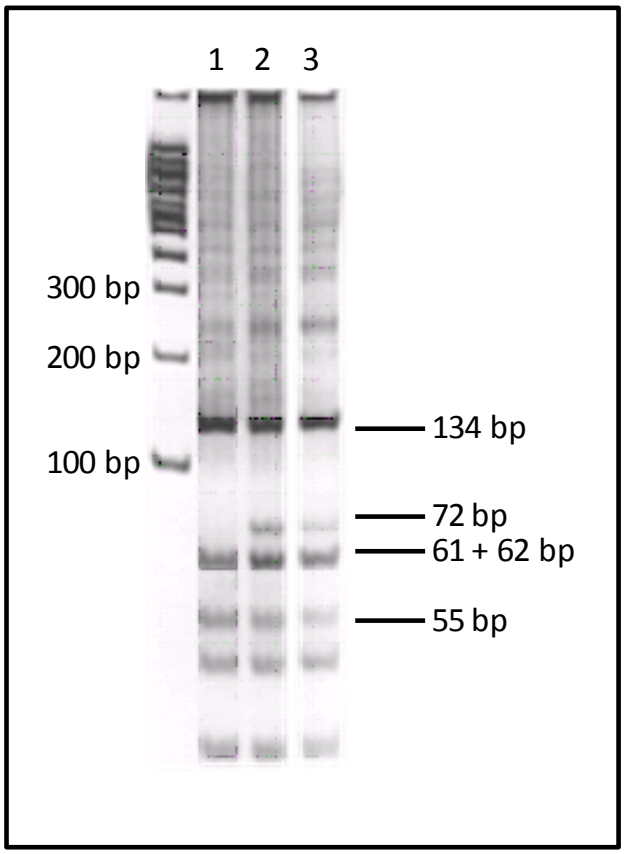

Figure 3.1 – PCR-RFLP analysis of HCP5 (rs2395029)

Restriction analysis of HCP5 (rs2395029) PCR amplicon after digestion by BstNI on a 10% polyacrylamide gel. Lane 1 shows a homozygous wild type (TT) genotype, whereas lanes 2 and 3 show heterozygous GG individuals.

Table 3.2 – Distribution of *HCP5* (rs2395029) genotypes in flucloxacillin-DILI cases and POPRES controls

	GG	GT	TT	P-value	OR (95% CI)
POPRES controls (n=282)	0 (0.0)	30 (10.6)	252 (89.4)		
Original flucloxacillin-DILI case GWAS cohort (n=51)	4 (7.8)	39 (76.5)	8 (15.7)	2.64×10^{-26}	45.2 (19.4 – 105.1)
Additional flucloxacillin-DILI cases (n=99)	0 (0.0)	81 (81.8)	18 (18.2)	2.06×10^{-39}	37.8 (20.0 – 71.4)
Total flucloxacillin-DILI cases (n=150)	4 (2.7)	120 (80.0)	26 (17.3)	3.67×10^{-52}	40.1 (22.7 – 70.7)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval. *P*-values are uncorrected.

3.3.2 *HLA-B allele genotyping of HLA-B*57:01 negative flucloxacillin-DILI cases*

The 26 flucloxacillin-DILI cases that do not possess the HLA-B*57:01 allele were genotyped for HLA-B genotype by SSP-PCR to investigate whether any other HLA-B associations existed. A total of 24 different HLA-B alleles were detected in the 26 samples with 24 of these heterozygous and two homozygous for a single allele. HLA-B genotypes for each case sample are displayed in **Appendix A**. HLA-B alleles and carriage frequencies are displayed in **Table 3.3**. Allele carriage frequencies were calculated as a percentage of the individuals who have a particular allele. The most common alleles detected were HLA-B*08:01 which was found in 7/26 patients (5 heterozygotes, 2 homozygotes) giving a carriage frequency of 34.6% and HLA-B*44:02 in 8/26 patients (8 heterozygotes) giving a carriage frequency of 30.8%.

To investigate whether any of these alleles were associated with flucloxacillin-DILI a suitable control population was required for analysis. A subset of the POPRES control cohort was selected as a suitable control group for comparison. This subset cohort has previously been produced through a principal component analysis of POPRES controls to capture much of the genetic substructure of North-western European individuals. This North-western European (NW-EU) control cohort contains 107 individuals and has previously been used for direct genotyping for HLA allele carriage (Lucena *et al.*, 2011). HLA-B allele carriage frequencies from NW-EU controls are also displayed in **Table 3.3**. The most common alleles reported for this population were HLA-B*07:02 at a carriage frequency of 29.9%, HLA-B*08:01 at 29.0%, HLA-B*44:02 at 15.9% and HLA-B*15:01 at 15.0%.

Allele carriage frequencies overall were similar between flucloxacillin-DILI cases and NW-EU controls. Increased allele carriage frequencies were observed in cases compared to controls for HLA-B*08:01 (34.6% vs. 29.0%), HLA-B*44:02 (30.8% vs. 15.9%) and HLA-B*55:01 (11.5% vs. 6.5%) but these were not found to be significant. A significant association was observed for HLA-B*13:02 which was observed in 7.7% of cases but not observed in the controls giving a *p*-value of 0.0376 and an OR of 21.24 (95% CI 1.00 – 449.6). A decreased frequency of HLA-B*07:02 (11.5% vs. 29.9%) was observed in cases compared to controls but this was not significant. Overall, the only significant differences in HLA-B allele distribution between cases and controls was with HLA-B*13:02. Although the number of cases is small, the increased frequency of HLA-B*13:02 compared to population controls may suggest a possible role in risk of

disease and merits further investigation as more HLA-B*57:01 negative cases become available. Allele carriage frequencies of 13 HLA-B alleles that were not observed in the genotyped flucloxacillin-DILI cases but are present in the NW-EU control cohort are displayed in **Appendix B**. No significant differences between HLA-B distribution between cases and controls was observed.

As part of the iDILIC project, several confirmed cases of DILI caused by isoxazolyl penicillins other than flucloxacillin have been recruited including three cases of cloxacillin-induced liver injury (two from Spain and one from Iceland) and two cases of dicloxacillin-induced liver injury (both from Iceland). To determine whether HLA-B*57:01 genotype was a factor in these cases; they were genotyped for HLA-B allele carriage. **Table 3.4** shows that the HLA-B*57:01 genotype was not found in any of the cases of liver injury to cloxacillin or dicloxacillin suggesting that HLA-B*57:01 may not be associated to liver injury caused by these drugs.

Table 3.3 – HLA-B allele carriage frequencies in 26 HLA-B*57:01 negative flucloxacillin-DILI cases and 107 North-western European (NW-EU) controls

HLA-B allele	Flucloxacillin-DILI cases (n=26)	NW-EU Controls (n=107)	*P* –value
B*07:02	3 (11.5)	32 (29.9)	0.1076
B*08:01	9 (34.6)	31 (29.0)	0.6631
B*08:34	1 (3.8)	0 (0.00)	0.1955
B*13:02	2 (7.7)	0 (0.00)	**0.0376**
B*14:01	1 (3.8)	7 (6.5)	1.0000
B*14:02	1 (3.8)	4 (3.7)	1.0000
B*15:01	1(3.8)	16 (15.0)	0.2085
B*15:57	1 (3.8)	0 (0.00)	0.1955
B*18:01	2 (7.7)	9 (8.4)	1.0000
B*35:01	1(3.8)	7 (6.5)	1.0000
B*37:01	2 (7.7)	4 (3.7)	0.3334
B*38:01	2 (7.7)	2 (1.9)	0.1727
B*39:06	1 (3.8)	1 (0.9)	0.3534
B*40:01	3 (11.5)	13 (12.1)	1.0000
B*40:02	1 (3.8)	3 (2.8)	0.5834
B*41:01	1 (3.8)	0 (0.00)	0.1955
B*44:02	8 (30.8)	17 (15.9)	0.1130
B*44:03	2 (7.7)	11 (10.3)	1.0000
B*44:05	1 (3.8)	0 (0.00)	0.1955
B*44:52N	1 (3.8)	0 (0.00)	0.1955
B*45:01	1 (3.8)	0 (0.00)	0.1955
B*51:01	3 (11.5)	10 (9.3)	0.7229
B*53:01	1 (3.8)	2 (1.9)	0.4807
B*55:01	3 (11.5)	7 (6.5)	0.4161

Number of individuals with a particular allele is shown with the allele carriage frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls. *P*-values are uncorrected.

Table 3.4 – HLA-B genotypes of Cloxacillin and Dicloxacillin-induced liver injury cases

Isoxazolyl penicillin	HLA-B genotype
Cloxacillin	
Spanish Patient A	07:02 + 44:02
Spanish Patient B	08:01 + 35:08
Iceland Patient A	40:01 + 40:01
Dicloxacillin	
Iceland Patient B	08:52 + 51:08
Iceland Patient C	07:02 + 44:02

3.3.3 *HLA-C*06:02 genotyping of flucloxacillin-DILI cases and controls*

To investigate the hypothesis of whether HLA-C*06:02 genotype is associated with flucloxacillin-DILI, a total of 150 flucloxacillin-DILI cases and 60 flucloxacillin-treated healthy controls were genotyped for the SNP rs12191877 (C/T) which has been shown to be in complete linkage disequilibrium (LD) with HLA-C*06:02 (Feng *et al.*, 2009). Carriage of the T-allele associates with possession of the HLA-C*06:02 allele. Genotyping results for rs12191877 in flucloxacillin-DILI cases, flucloxacillin-treated controls and POPRES controls are displayed in **Table 3.5**. The genotype frequencies for rs12191877 in the control groups were in adherence to Hardy-Weinberg equilibrium.

124 of the 150 flucloxacillin-DILI cases (82.7%) were found to carry the T allele compared to 16 out of 60 flucloxacillin-treated controls (26.7%). This was found to be significant with a *p*-value of 2.31×10^{-14} and an OR of 13.1 (95% CI 6.4 – 26.7). 68 out of 282 POPRES controls (24.1%) carried the T-allele giving a significant difference to flucloxacillin-DILI cases with a *p*-value of 1.05×10^{-32} and an OR of 15.0 (95% CI 9.1 – 24.8). This is to be expected due to the tight LD between HLA-B*57:01 and HLA-C*06:02.

To investigate whether HLA-C*06:02 is a risk factor in HLA-B*57:01 negative flucloxacillin-DILI cases, rs12191877 genotype distribution was compared to the POPRES control cohort (**Table 3.6**). No differences in T allele carriage where observed between HLA-B*57:01 negative cases and POPRES controls. 6 out of 26 HLA-B*57:01 negative cases (23.1%) carried the T allele compared to 68 out of 282 POPRES controls (24.1%) giving a *p*-value of 1.0000 and an OR of 0.94 (95% CI 0.36 – 2.45).

Table 3.5 – Distribution of HLA-C*06:02 (rs12191877) genotypes in flucloxacillin-DILI cases and controls

	CC	CT	TT	*P*-value	OR (95% CI)
Flucloxacillin-DILI cases (n=150)	26 (17.3)	110 (73.3)	14 (9.3)		
Flucloxacillin-treated controls (n=60)	44 (73.3)	15 (25.0)	1 (1.7)	2.31×10^{-14}	13.1 (6.4 – 26.7)
POPRES controls (n=282)	214 (75.9)	65 (23.0)	3 (1.1)	1.05×10^{-32}	15.0 (9.1 – 24.8)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance of T-allele carriage between cases and controls; OR = odds ratio, CI = confidence interval

Table 3.6 - rs12191877 genotype distribution in HLA-B*57:01 negative flucloxacillin-DILI cases and POPRES controls

	CC	CT	TT	*P*-value	OR (95% CI)
HLA-B*57:01 negative Flucloxacillin-DILI cases (n=26)	20 (76.9)	4 (15.4)	2 (7.7)		
POPRES controls (n=282)	214 (75.9)	65 (23.0)	3 (1.1)	1.0000	0.94 (0.36 – 2.45)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance of T-allele carriage between cases and controls; OR = odds ratio, CI = confidence interval

3.3.4 *KIR3DL1 and KIR3DS1 genotyping of flucloxacillin-DILI cases*

24 cases of flucloxacillin-DILI were genotyped for *KIR* alleles by a SSP-PCR approach using a commercially available KIR Typing Kit (MACS molecular, Miltenyi Biotec). *KIR* allele genotypes were determined by evaluating primer amplification in the relevant wells. **Figure 3.2** shows a typical result of a *KIR3DL1/KIR3DS1* heterozygous individual (A), a *KIR3DL1* homozygous individual (B) and a *KIR3DS1* homozygous individual (C).

As a cheaper method of genotyping for *KIR3DL1/KIR3DS1* in flucloxacillin-DILI cases and flucloxacillin-treated healthy controls, a multiplex PCR protocol was adapted from Kulkarni et al (Kulkarni *et al.*, 2010). Results from the KIR typing kit served as a positive control to validate the multiplex PCR assay in the remaining cases and controls. *KIR3DL1/KIR3DS1* alleles were determined by gel electrophoresis by positive or negative amplification of the correct bands. **Figure 3.3** shows a typical result with a *KIR3DL1/KIR3DS1* heterozygous individual (lane 1), a *KIR3DL1* homozygous individual (lane 2) and a *KIR3DS1* homozygous individual (lane 3).

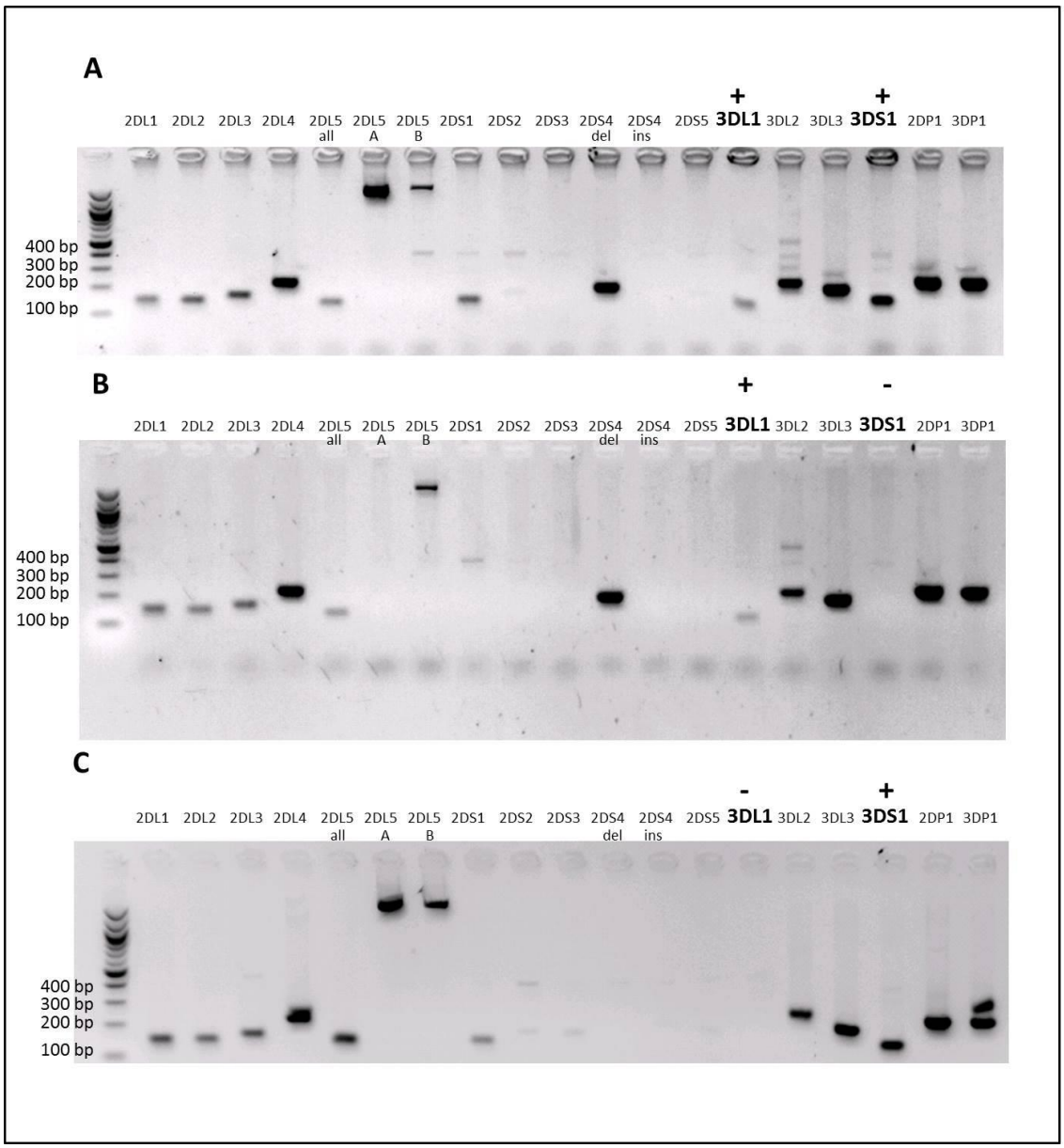

Figure 3.2 – Typical KIR genotyping result by SSP-PCR

2% agarose gel of KIR genotypes of three flucloxacillin-DILI patients, showing A) KIR3DL1/KIR3DS1 heterozygous individual, B) a KIR3DL1 homozygous individual and C) a KIR3DS1 homozygous individual.

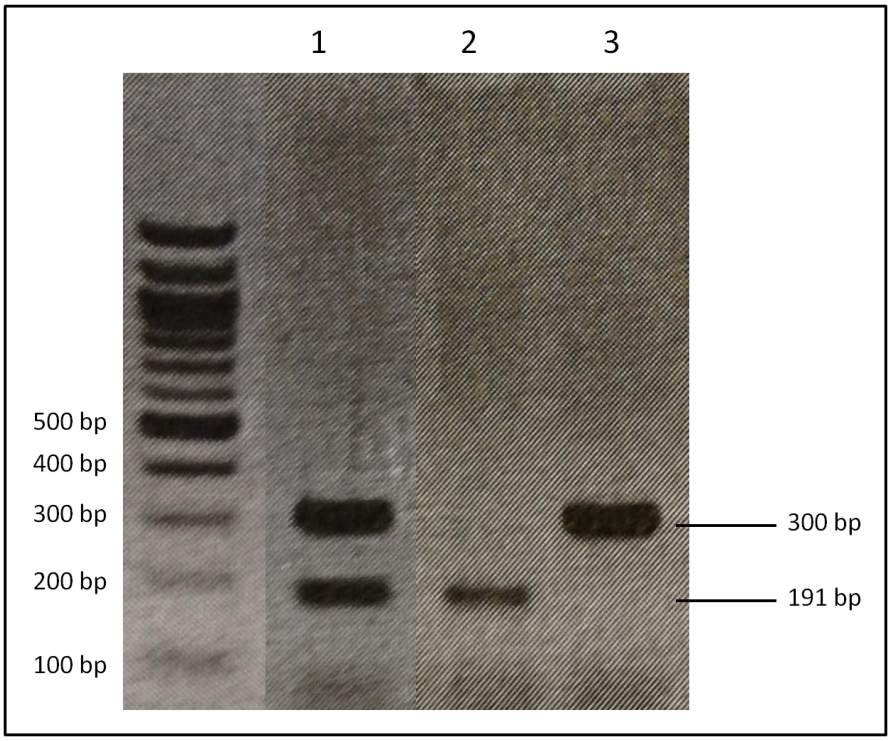

Figure 3.3 – Typical result for KIR3DL1/3DS1 genotyping by multiplex PCR

A 2% agarose gel electrophoresis of KIR3DL11 and KIR3DS1 PCR products, showing lane 1) a KIR3DL1/KIR3DS1 heterozygous individual, lane 2) a KIR3DL1 homozygous individual and lane 3) a KIR3DS1 homozygous individual.

KIR3DL1/KIR3DS1 genotyping results in cases and flucloxacillin-treated controls are displayed in **Table 3.7**. Genotype frequencies in flucloxacillin-treated controls were in adherence of Hardy-Weinberg equilibrium ($X^2 = 1.29$, $p = 0.26$). Although, there appears to be an apparent increase of heterozygous individuals and a decrease of *KIR3DL1* homozygous individuals in flucloxacillin-DILI cases, the association of *KIR3DS1* carriage in cases versus controls was not found to be significant ($p = 0.1166$, OR = 1.72, 95% CI 0.90 – 3.26).

KIR3DL1/KIR3DS1 genotype frequencies were determined in flucloxacillin-DILI cases and flucloxacillin-treated controls by calculating the percentage of individuals that possessed each allele. Genotype frequencies were compared to an English population cohort from the Allele Frequency Net Database (AFND) containing 483 Caucasian individuals (**Table 3.8**). *KIR3DL1* frequency was similar in cases and the two control groups. There was an apparent increase of *KIR3DS1* frequency in flucloxacillin-DILI cases (40.7%) compared to drug-treated controls (28.6%) and population controls (37.0%), however this was not found to be significant.

Whether *KIR3DLI/ KIR3DSI* genotype frequencies were associated with HLA-B*57:01 status of flucloxacillin-DILI cases was investigated (**Table 3.9**). A increased frequency of *KIR3DS1* allele carriage was observed in HLA-B*57:01 positive cases (42.6%) compared to HLA-B*57:01 negative cases (32.0%). Comparison of *KIR3DS1* allele carriage in HLA-B*57:01 positive and negative cases to the population control group showed that observed differences were not significant.

Table 3.7 - *KIR3DL1* and *KIR3DS1* genotype distribution in flucloxacillin-DILI cases and flucloxacillin-treated controls

	KIR3DL1 homozygous	**KIR3DL1/ KIR3DS1**	**KIR3DS1 homozygous**	***P*-value**	**OR (95% CI)**
Flucloxacillin-DILI cases (n=140)	83 (59.3)	52 (37.1)	5 (3.6)		
Flucloxacillin-treated controls (n=63)	45 (71.4)	15 (23.8)	3 (4.8)	0.1166	1.72 (0.90 – 3.26)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance for KIR3DS1 carriage between cases and controls.

Table 3.8 - *KIR3DL1* and *KIR3DS1* genotype frequencies in flucloxacillin-DILI cases, flucloxacillin-treated healthy controls and an English KIR population control cohort

	KIR3DL1	KIR3DS1	*P*-value	OR (95% CI)
Flucloxacillin-DILI cases (n=140)	96.4	40.7		
Flucloxacillin-treated controls (n=63)	95.2	28.6	0.1166	1.72 (0.90 – 3.26)
KIR England population controls (n=483)	91.0	37.0	0.4308	1.17 (0.79 – 1.71)

Genotype Frequency - Percentage of individuals carrying allele (Individuals / n). Two-tailed Fisher's exact test was used to calculate significance of KIR3DS1 frequency between cases and controls; OR = odds ratio, CI = confidence interval

Table 3.9 - *KIR3DL1* and *KIR3DS1* genotype frequencies in HLA-B*57:01 positive and negative flucloxacillin-DILI cases

	Genotype Frequency		*P* - value	OR (95% CI)
	KIR3DL1	**KIR3DS1**		
KIR England population controls (n=483)	91.0	37.0		
HLA-B*57:01 positive (n=115)	96.5	42.6	0.2864	1.26 (0.83 – 1.91)
HLA-B*57:01 negative (n=25)	96.0	32.0	0.6758	0.80 (0.34 – 1.89)

Genotype Frequency - Percentage of individuals carrying allele (Individuals / n). Two-tailed Fisher's exact test was used to calculate significance of KIR3DS1 frequency between cases and population controls; OR = odds ratio, CI = confidence interval

3.3.5 *HLA-Bw4 motif carriage in HLA-B*57:01 negative flucloxacillin-DILI cases*

HLA-B alleles either have a Bw4 or Bw6 motif at amino acid positions 77 – 83. HLA-B molecules expressing Bw4 epitopes have been shown to interact with KIR3DL1 and possibly KIR3DS1. Furthermore, Bw4 motifs that contain an isoleucine amino acid at position 80 (Bw4-80Ile), instead of threonine (Bw4-80Thr), have been previously associated with KIR3DS1 genotype in disease (Korner and Altfeld, 2012). HLA-B*57:01 molecules have been shown to contain the Bw4-80Ile epitope suggesting that interaction with KIRs may occur (Sidney *et al.*, 2008).

To investigate whether an association existed with Bw4 frequency in HLA-B*57:01 negative flucloxacillin-DILI cases, Bw4 and Bw6 motif frequency was determined for the previously identified HLA-B alleles from a published (Sidney *et al.*, 2008). **Table 3.10** shows Bw4/Bw6 epitope frequencies in HLA-B*57:01 negative flucloxacillin-DILI cases and HLA-B*57:01 negative controls from the NW-EU group. 18/26 (69.2%) HLA-B*57:01 negative flucloxacillin-DILI cases were found to carry at least one HLA-B allele that expressed a Bw4 epitope compared to 16/97 (52.6%) of controls, however the difference was not significant ($p = 0.1816$, OR = 2.03 95% CI 0.81 – 5.11). 5/26 (19.2%) of HLA-B*57:01 negative flucloxacillin-DILI cases expressed the specific Bw4-80Ile epitope compared to 16/97 (16.5%) of controls, however the difference was also not significant ($p = 0.7715$, OR = 1.21 95% CI 0.40 – 3.67).

Table 3.10 – Carriage of HLA-Bw4 motif in HLA-B*57:01 negative flucloxacillin-DILI cases and NW-EU controls

Bw4 status	Flucloxacillin-DILI cases (n=26)	NW-EU Controls (n=97)
Bw4-80Ile / Bw4-80Ile	2	1
Bw4-80Ile /Bw4-80Thr	2	1
Bw4-80Ile/Bw6	1	14
Bw4-80Thr/Bw4-80Thr	1	4
Bw4-80Thr/Bw6	12	31
Bw4 positive	**18**	**51**
Bw6/Bw6	**8**	**46**

Number of individuals with a particular Bw4 or Bw6 motif genotype is shown. Two-tailed Fisher's exact test was used to calculate significance between cases and controls

3.3.6 *CASP5 and USP8 genotyping of flucloxacillin-DILI cases*

Two SNPs were chosen for genotyping from exome sequencing results on flucloxacillin-DILI cases and controls based on their significance, chromosomal location and function of the gene they are located in. One of the SNPs is rs45483102 from the *CASP5* gene located on chromosome 11. This gene encodes a member of the cysteine-aspartic acid protease (caspase) family of enzymes. The SNP is described as a non-synonymous missense SNP resulting in the substitution of an adenine (A) base for a thymine (T) base. The genotyping results are displayed in **Table 3.11**. No homozygous AA individuals were detected in cases, flucloxacillin-treated controls or community controls. For analysis, A allele carriage from heterozygous individuals was compared in cases and controls. An increase of A allele carriage was observed in flucloxacillin-DILI cases compared to flucloxacillin-treated controls (15.7% to 11.0%), but this was not found to be significant. As rs45483102 was not present as a marker in the flucloxacillin-DILI GWA study, there is no POPRES control data available for this SNP. As a population control group a cohort of healthy community controls (n=235) donated by Dr Peter Donaldson was also genotyped for rs45483102. Similarly, no AA homozygous genotypes were observed. An increased frequency of heterozygotes was again observed in flucloxacillin-DILI cases compared to this control group (15.7% to 7.2%). A significant difference was observed with carriage of the minor A allele in cases compared to community controls giving a *p*-value of 0.014 with an OR of 2.39 (1.22 – 4.68).

Table 3.11 – Distribution of genotypes for *CASP5* (rs45483102) in flucloxacillin-DILI cases, flucloxacillin-treated controls and community controls

	TT	AT	AA	*P*-value	OR (95% CI)
Flucloxacillin-DILI cases (n=140)	118 (84.3)	22 (15.7)	0 (0.00)		
Flucloxacillin-treated controls (n=63)	56 (88.9)	7 (11.0)	0 (0.00)	0.516	1.49 (0.60 – 3.70)
Community controls (n=235)	218 (92.8)	17 (7.2)	0 (0.00)	**0.014**	2.39 (1.22 – 4.68)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

The second SNP genotyped from the exome sequencing results was rs41475144 located on chromosome 15 in the *USP8* gene encoding a member of the ubiquitin specific protease family of enzymes. Genotyping results for rs41475144 for flucloxacillin-DILI cases and flucloxacillin-treated controls are shown in **Table 3.12**. Genotyping results show a significant association of C allele carriage in flucloxacillin-DILI cases compared to controls giving a *p*-value of 2.17×10^{-14} with and OR of 13.08 (6.44 – 26.58). However, after genotyping was performed it was discovered that this SNP has been previously identified by others as to being found within a pseudogene for *USP8* located upstream of HLA-C within chromosome 6 (Veal *et al.*, 2002). Since, genes of the MHC are located on chromosome 6, it is almost certain that this SNP is in LD with HLA-B*57:01 producing the result in the exome sequencing study. As a result, it was decided not to genotype the community control cohort as with the SNP in *CASP5*.

Table 3.12 – Distribution of genotypes for *USP8* (rs41475144) in flucloxacillin-DILI cases and flucloxacillin-treated controls

	TT	CT	CC	*P*-value	OR (95% CI)
Flucloxacillin-DILI cases (n=140)	24 (17.1)	101 (72.1)	15 (10.7)		
Flucloxacillin-treated controls (n=63)	46 (73.0)	16 (25.4)	1 (1.59)	2.17×10^{-14}	13.08 (6.44 – 26.58)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

3.4 Discussion

Genome-wide association studies have led to the discovery of a strong association between HLA-B*57:01 and flucloxacillin-induced liver injury that has provided some mechanistic insights into what makes certain individuals susceptible to the disease. The original flucloxacillin-DILI GWA study consisted of 51 cases collected between 2004 and 2007, 84% of which were found to carry the risk allele that conferred HLA-B*57:01 genotype (Daly *et al.*, 2009). Direct genotyping of HLA-B*57:01 in flucloxacillin-DILI cases and flucloxacillin-treated controls found that HLA-B*57:01 genotype was associated with increased risk to flucloxacillin-DILI with an odds ratio of 80.6 (Daly *et al.*, 2009). A SNP in the *HCP5* gene, rs2395029, has been found to be in complete linkage disequilibrium with HLA-B*57:01. Genotyping for this SNP therefore provides a cheaper alternative method suitable for processing large number of DNA samples easily by PCR-RFLP (Colombo et al., 2008). In the present study, an additional 99 cases of flucloxacillin-induced liver injury that have been subsequently collected through the on-going DILIGEN and iDILIC projects were genotyped for the rs2395029 SNP. Cases that possessed the G allele associated with HLA-B*57:01 carriage were directly genotyped to confirm *HLA-B*57:01* possession with 100% accuracy. When the 150 cases now available were pooled, the frequency of HLA-B*57:01 genotype remained similar to the 83% observed in the GWAS.

Though the strong association of flucloxacillin-DILI with HLA B*57:01 has now been confirmed and this is the strongest association with a particular HLA allele reported for any type of DILI up to the present, the clinical utility of genotyping for HLA B*57:01 is limited (Daly, 2012b). As discussed previously (Daly *et al.*, 2009), the incidence of flucloxacillin-DILI in the UK is approximately 1 case for 10,000 patients treated which corresponds to a "number needed to harm" of 10,000. Since 5% of the population will be positive for at least one B*57:01 allele and a large majority of flucloxacillin DILI cases are also positive for this allele, we can assume that 1 in every 500 individuals positive for B*57:01 and prescribed flucloxacillin will develop DILI. If genotyping was performed prior to treatment with flucloxacillin, this would mean that 10,000 patients would need to be genotyped and 499 patients would have to be deprived unnecessarily of flucloxacillin treatment and given an alternative antimicrobial to prevent one case of DILI. In contrast, for abacavir hypersensitivity, which is also B*57:01 associated, 50% of those positive for this allele have been reported to develop hypersensitivity so only

approximately 50 patients need to be genotyped and one to two patients deprived unnecessarily of abacavir treatment to prevent one case of hypersensitivity (Mallal *et al.*, 2008). The findings on other genes described in the current chapter do not decrease the number of patients needing to be screened to prevent flucloxacillin-DILI as the effect sizes found for any additional genes or alleles were considerably lower than that for B*57:01. However, the finding that over 80% of all flucloxacillin-DILI cases are positive for B*57:01 may be of relevance to the diagnosis of this form of DILI and help rule out other causes of liver disease.

Although an additional 99 cases of flucloxacillin-DILI have been recruited and included in the present study, it has not been possible to recruit and directly genotype for HLA-B*57:01 additional flucloxacillin-treated healthy controls, to add to the 64 from the GWA study. Since HLA-B*57:01 and rs2395029 genotypes have been found to correlate 100%, previously and, in the present study, it was decided to study HLA-B*57:01 genotype distribution in the new cohort of cases by comparing rs2395029 genotype distribution to the POPRES control cohort containing 282 individuals. It would have been interesting to perform multivariate analysis on data from flucloxacillin-DILI cases and controls for additional variables such as age and gender. However, while such data was available for flucloxacillin-DILI cases and the drug-exposed control cohort, this data was not available for the POPRES control cohort. Due to the small size of the drug-exposed control group, compared with the flucloxacillin DILI case group, performing multivariate analysis was considered inappropriate and instead all comparisons of genotype frequencies involved using univariate analysis involving the cases and larger POPRES control group only. Analysis of rs2395029 genotype in the 99 newer flucloxacillin-DILI cases versus POPRES controls showed that HLA-B*57:01 was associated with increased risk of flucloxacillin-DILI (OR 37.8; $p = 2.06 \times 10^{-39}$), which rose to 40.1 ($p = 3.67 \times 10^{-52}$) when the 150 cases were pooled.

However, there remains a minority of flucloxacillin-DILI cases, 17% or 26 cases, which do not possess the identified risk HLA allele. In this chapter, these cases were genotyped for HLA-B allele carriage to investigate the possibility that other HLA-B associations existed. 24 different HLA-B alleles were detected in the 26 cases. Increased genotype frequencies were observed for HLA-B*08:01, HLA-B*44:02 and HLA-B*55:01 in flucloxacillin-DILI cases, compared to a North-Western European population control cohort. A single significant association was observed with HLA-B*13:02 which was found at frequency of 7.7% in HLA-B*57:01 negative cases while

not at all in the control group (p = 0.0376). This suggests that this allele may also play a role in susceptibility to flucloxacillin-DILI. However, although the association is found to be significant, the presence of this genotype is in only 2 of the 26 cases. As more HLA-B*57:01 negative cases are discovered, it will be interesting to see if this association remains. As this is the only significant HLA association observed in these cases it is likely that mechanisms independent of HLA genotype may be causal factors of DILI in these cases. This seems plausible since, even despite the strong association with HLA-B*57:01, only 1 in every 500 to 1000 individuals with HLA-B*57:01 genotype develop DILI when exposed to flucloxacillin suggesting other undetermined genetic or environmental factors exist for susceptibility to the disease (Daly *et al.*, 2009).

Apart from flucloxacillin, liver injury caused by the other isoxazolyl penicillins is extremely rare. As a result, no large scale genetic studies exist like the GWAS that was performed on flucloxacillin-DILI cases that discovered the association with HLA-B*57:01 carriage and susceptibility to disease. Whereas, flucloxacillin is the preferred isoxazolyl analogue for patient treatment in the UK, in most other European countries, alternative isoxazolyl penicillins such as cloxacillin and dicloxacillin are prescribed. As part of the iDILIC project, DILI cases from other isoxazolyl penicillins have been collected including five in the present study consisting of three cases of cloxacillin-induced liver injury (two from Spain and one from Iceland) and two cases of dicloxacillin-induced liver injury (both from Iceland). To study whether DILI to these isoxazolyl analogues were associated with HLA-B*57:01, these cases were also genotyped for HLA-B allele carriage. None of the cases from either cloxacillin or dicloxacillin were found to be positive for HLA-B*57:01 suggesting different mechanisms may exist in the pathogenesis of DILI caused by other isoxazolyl penicillins.

*HLA-C*06:02* genotype has been identified, along with *HLA-B*57:01* genotype, as a risk factor for psoriasis susceptibility and for a protective role in restricting HIV-1 infection progression (Fellay *et al.*, 2007; Liu *et al.*, 2008). HLA-B*57:01 and HLA-C*06:02 have been reported as part of a relatively common extended haplotype that also contains the HLA-class II alleles, DQB1*03:03 and DRB1*07:01 (Ahmad *et al.*, 2003). This is likely to explain their similar associations in psoriasis susceptibility and HIV protection. In psoriasis susceptibility, this haplotype association is thought to be primarily driven by HLA-C*06:02, whereas in HIV-1 non-progressors it is thought that

HLA-B*57:01 plays the predominant role (Chen *et al.*, 2012). Genotyping of flucloxacillin-DILI cases for HLA-C*06:02 shows a significantly higher frequency compared to flucloxacillin-treated controls, which reflects the haplotype association between HLA-C*06:02 and HLA-B*57:01. This is confirmed when HLA-C*06:02 genotype in HLA-B*57:01 negative cases is compared to POPRES population controls showing no difference in observed genotype distribution

Carriage of the KIR3DS1 allele has been linked with having a role in various human diseases including increased risk of some autoimmune diseases (Lopez-Larrea *et al.*, 2006; Korner and Altfeld, 2012). HLA-B*57:01 and KIR3DS1 genotype are believed to interact synergistically to confer protection from HIV-1 progression in certain individuals (Martin *et al.*, 2002). Some HLA-B molecules, including HLA-B*57:01, display a Bw4 epitope at amino acid positions 77 – 83. HLA-B molecules with this motif present are natural ligands for KIR3DL1 leading to NK cell inhibition. It is still not fully clear if Bw4 expressing HLA-B molecules are ligands for KIR3DS1 but genetic associations have been discovered for Bw4-80Ile expressing HLA-B alleles and KIR3DS1 in protecting from HIV progression and decreased risk of hepatocellular carcinoma (Martin *et al.*, 2002; Lopez-Vazquez *et al.*, 2005). HLA-B molecules expressing a Bw6 motif do not appear to interact with KIRs. Genotyping was first performed to investigate whether there was an association between *KIR3DS1* genotype and flucloxacillin-DILI cases. No significant associations with KIR genotype and flucloxacillin-DILI were observed. An increased frequency of *KIR3DS1* genotype was observed in HLA-B*57:01 positive cases compared to HLA-B*57:01 negative (42.6% vs. 32.0%) but this was also not significant when compared to controls. Due to the known interaction of HLA-Bw4 motifs and KIRs and the fact that HLA-B*57:01 expresses a Bw4 motif, analysis of Bw4 and Bw6 epitopes in HLA-B alleles of the HLA-B*57:01 negative cases was performed. Although there was an increased frequency of Bw4 expressing HLA-B alleles in these cases, compared to controls, it was not found to be significant. The data presented does not show any evidence of genetic associations with KIR3DL1 or KIR3DS1 in flucloxacillin-DILI nor is there significant Bw4 motif associations in cases other than HLA-B*57:01.

Genotyping was performed on two SNPs that were found to be associated with flucloxacillin-DILI in an exome sequencing study. One of the SNPs was identified as being located in the USP8 gene on chromosome 15. However, it was discovered that this association was actually caused by a pseudogene of *USP8* on chromosome 6 so was

likely to be the effect of LD with HLA-B*57:01. This was confirmed by genotyping studies in flucloxacillin-DILI cases and flucloxacillin-treated controls. The second SNP studied, rs45483102, was located in the *CASP5* gene on chromosome 11. Analysis of this SNP showed no significant observation between flucloxacillin-DILI cases and drug-treated controls but a significant difference was observed when a larger community control cohort was compared (p=0.014). The role of this gene in flucloxacillin-DILI may warrant further investigation due to an apparent role for caspase-5 in activation of inflammatory cytokines (Fuentes-Prior and Salvesen, 2004). Interestingly, caspase-5 has been found to be upregulated in psoriatic skin lesions (Salskov-Iversen *et al.*, 2011). As described, HLA-B*57:01 is also thought to have a suspected role in psoriasis.

In summary,

- The strong association of HLA-B*57:01 in flucloxacillin-DILI cases was confirmed in additional cases.
- 17% of flucloxacillin-DILI cases were found to not possess HLA-B*57:01 and genotyping of HLA-B alleles discovered a borderline association with HLA-B*13:02. Genotyping of additional HLA-B*57:01 negative cases, when they become available, will help determine whether this association is important.
- It does not appear that DILI due to cloxacillin and dicloxacillin is related to HLA-B*57:01 but this needs further investigation.
- HLA-C*06:02 is unlikely to be associated with flucloxacillin-DILI independently of HLA-B*57:01 and there appears to be no genetic association between flucloxacillin-DILI and KIR3DL1 or KIR3DS1.
- A significant association was described for the rs45483102 SNP in the gene encoding caspase-5. It would be of interest to investigate this association further to ascertain whether it has a functional impact on the mechanism of flucloxacillin-induced liver injury.

Chapter 4. Interaction of Flucloxacillin and Isoxazolyl Penicillins with the Nuclear Receptors - CAR and PXR

4.1 Introduction

To avoid the accumulation of lipophilic, potentially toxic compounds in the body, there is a requirement for such substances to be metabolised and excreted. Detoxification processes are performed by a variety of phase I and II enzymes and transporters expressed in multiple tissues and organs though primarily the liver. Modulation of these pathways predominantly occurs via the binding of ligands to nuclear receptors that control transcription of genes encoding metabolising enzymes and transporters regulating their levels and activity. This cellular defence mechanism has evolved so that xenobiotic exposure can cause the induction of metabolic proteins to meet the particular requirements of that xenobiotic challenge.

The nuclear receptor, NR1I subfamily members, the pregnane X receptor (PXR) and the constitutive active/androstane receptor (CAR) are the most important nuclear receptors with regards to xenobiotic metabolism and excretion and are both highly expressed in the liver. PXR and CAR regulate the transcription of large number of genes involved in all phases of xenobiotic metabolism and excretion but their function was originally characterised by their transcriptional regulation of the cytochrome P450 isoforms, CYP3A and CYP2B respectively (**Figure 4.1**). When bound and activated by their ligands, both PXR and CAR translocate from the cytoplasm to the nucleus where they form a heterodimer with the retinoid X receptor (RXR). PXR activates the transcription of CYP3A4 by binding as a heterodimer to various specific response elements in the 5'upstream promoter region: the proximal promoter ER6, the distal DR3 motif in the xenobiotic-responsive enhancer module (XREM), the ER6 in the far distal enhancer module and a recently identified DR4 motif (Ihunnah et al., 2011). Similarly CAR mediates CYP2B6 transcriptional activation via two synergistically acting response elements lying upstream of the CYP2B6 promoter, the phenobarbital-responsive enhancer molecule (PBREM) and a distal responsive element, termed the XREM (Wang et al., 2003). Although PXR and CAR were originally shown to regulate CYP3A and CYP2B genes, respectively, it has since been established that there is significant cross talk between the two receptors and considerable overlap in both ligands and target genes (Chai *et al.*, 2013). This crosstalk includes the regulation of CYP2B genes by PXR and CAR regulation of CYP3A (Goodwin *et al.*, 2001; Maglich *et al.*, 2002; Faucette *et al.*, 2006).

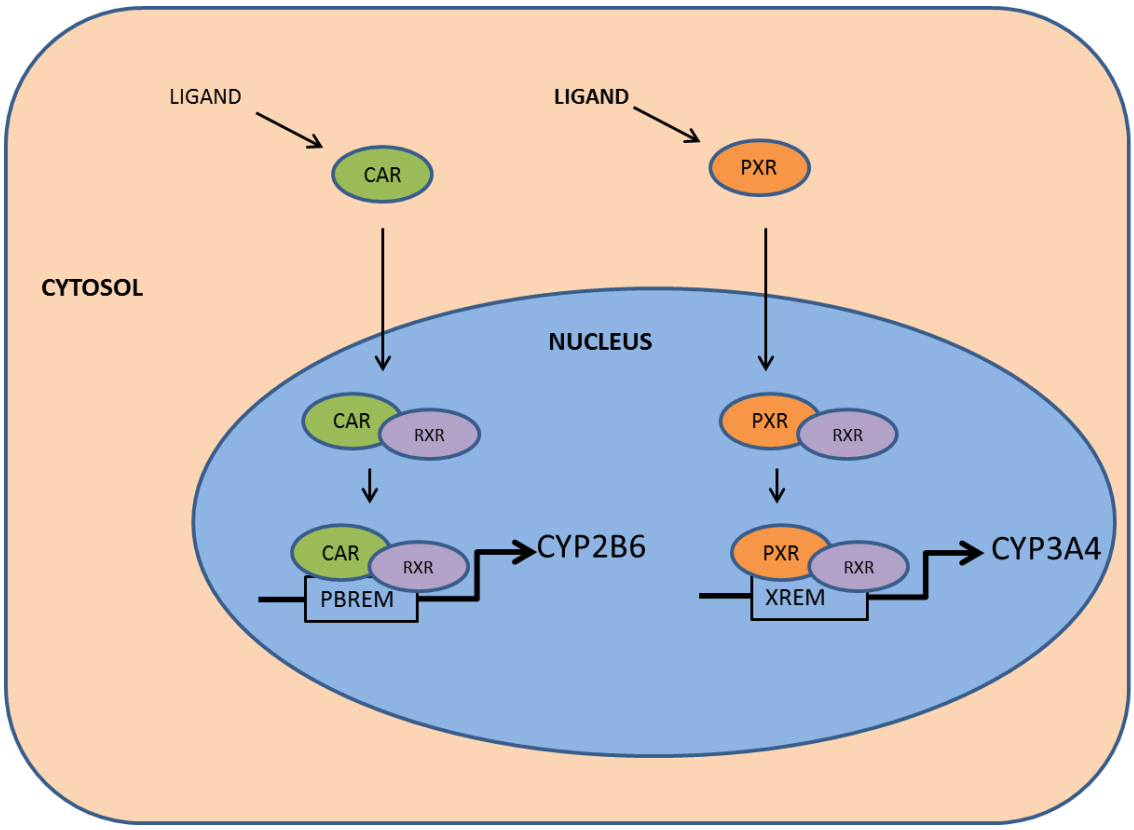

Figure 4.1 – A representation of the hallmark mechanisms of CAR and PXR activation

CAR or PXR are activated by their ligands in the cytosol which is followed by nuclear translocation. In the nucleus, both form heterodimers with RXR and subsequently bind to their corresponding response elements in their target genes leading to transcription characterised by CYP2B6 with CAR and CYP3A4 with PXR.

As described in section 1.6.1.2, PXR and CAR both also play a role in bile acid homeostasis that protects the liver from cholestasis making them interesting targets for understanding the underlying mechanisms of cholestatic DILI. Although PXR and CAR serve to protect the liver, in certain circumstances their activation and induction of metabolising enzymes and transporters can lead to bioactivation and enhanced formation of toxic, reactive metabolites (Tompkins and Wallace, 2007). For example, it has been reported that flucloxacillin may be metabolised via CYP3A4 to a potentially toxic and reactive metabolite, 5'hydroxymethyl flucloxacillin (Lakehal *et al.*, 2001). Flucloxacillin has also been shown to be a PXR ligand which could result in increased CYP3A4 induction and accumulation of reactive metabolites (Andrews *et al.*, 2010). An expression microarray analysis identified a number of PXR regulated genes, including CYP3A4 and MDR1, whose expression changed in response to flucloxacillin treatment (Andrews, 2009). These findings are supported by Huwyler and colleagues who showed that flucloxacillin can induce CYP3A4 and MDR1 gene expression in LS180 cells and human hepatocytes (Huwyler *et al.*, 2006). Flucloxacillin was shown to be a PXR ligand through a reporter gene study. Treatment with flucloxacillin led to increased luciferase activity of the reporter gene construct, containing a concatamer of PXR ER6 response elements, compared to the untreated control suggesting that flucloxacillin can enhance PXR activation. However, this activation was mild compared to that of the classic PXR activator rifampicin suggesting that flucloxacillin is a weak PXR agonist in comparison. It is currently unknown whether flucloxacillin interacts with CAR as it appears to do with PXR. Preliminary findings at Newcastle showed CYP2B6 induction following flucloxacillin treatment of human hepatocytes in a sandwich culture (D. Cowie and M.C.Wright, unpublished). This suggests possible activation of CAR by flucloxacillin. Flucloxacillin activation of CAR will be investigated in this chapter using a reporter gene study. Since CAR can also regulate the transcription of numerous metabolising enzymes and transporters important in drug metabolism and excretion, including CYP3A4, it is important to determine whether, like PXR, flucloxacillin also interacts with this receptor.

Additionally, polymorphisms in the PXR and CAR genes may influence the expression, function and mediated responses leading to decreased drug clearance and liver toxicity. Such a risk has been associated with flucloxacillin induced liver injury. A previously identified polymorphism -25385C/T (rs3814055) in the PXR promoter region suggested that the presence of the T allele was associated with higher CYP3A induction than the C

allele (Zhang *et al.*, 2001). 51 cases of flucloxacillin-induced liver injury were genotyped for the -25385C/T polymorphism and a significant association was discovered between the CC homozygous genotype and flucloxacillin-induced liver injury with an odds ratio of 3.37 compared to drug-treated controls (Andrews *et al.*, 2010). It is therefore hypothesised that individuals with the CC genotype have decreased PXR function and a lower ability to clear flucloxacillin leading to drug accumulation and possible adduct formation. Decreased PXR activity may also have an effect on many other processes such as bile acid detoxification which may also contribute to drug-induced liver injury. In the present study, an additional 99 cases of flucloxacillin-induced liver injury that have been subsequently collected as part of the DILIGEN project were genotyped for the -25385C/T SNP to investigate this association in greater detail. As well as this polymorphism in the PXR gene, this chapter also describes the study of a number of SNPs in the CAR gene to investigate possible associations in flucloxacillin-DILI.

Whereas flucloxacillin is a common cause of idiosyncratic DILI, reports in the literature of liver injury caused by the other isoxazolyl penicillins, including cloxacillin and dicloxacillin, are rare. This is perhaps surprising due to similarity between the structures of the drugs with only a difference the substituted atom at the position 6 of the phenyl ring of the isoxazole side chain. Furthermore, dicloxacillin is the isoxazolyl penicillin commonly prescribed in Denmark and Iceland and has also superseded flucloxacillin as the drug of choice for treating soft tissue infections caused by *Staphylococcus aureus* (*S. aureus*) in Australia and the United States. This suggests that it is not hepatotoxic in susceptible individuals in a manner that flucloxacillin is. However, at present there have not been any population-based studies investigating the risk of liver disease caused by dicloxacillin and so it is possible that differences in reporting of potential dicloxacillin-DILI cases may influence the observed differences. Genetic factors affecting DILI due to dicloxacillin are considered in further in Chapter 3. Here, a study was performed to compare PXR activation by the isoxazolyl penicillins - cloxacillin, dicloxacillin and flucloxacillin using the $(ER6)_3$ PXR reporter construct described above.

4.2 Methods

4.2.1 *Genotyping of CAR in flucloxacillin-DILI cases and controls*

Genotyping for three SNPs in the CAR (NR1I3) gene, rs3003596, rs6686001 (both in Intron 1) and rs2307418 (Intron 8) was performed on 72 flucloxacillin-DILI cases from the flucloxacillin GWA study and 64 drug-treated healthy controls by PCR-RFLP. Primers were designed for each SNP as describe in section 2.4.1 and are shown in **Table 4.1**.

These SNPs were chosen based on their identification as Tag SNPs for the common CAR haplotypes using data from the HapMap database (http://www.hapmap.org) exported into Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview). Haploview was used to generate linkage disequilibrium (LD) plots as shown in **Figure 4.2**. Tag SNPs were selected using the Haploview Tagger function, with a pairwise tagging algorithm set to select SNPs with a minor allele frequency (MAF) ≥ 0.08 and an $r^2 \geq 0.8$. Five additional SNPs from this CAR haplotype were analysed by PLINK analysis using data collected previously from the flucloxacillin GWA study.

Table 4.1 – Primer sequences used for RFLP genotyping of CAR SNPs

SNP	Forward (5'-3')	Reverse (3'-5')	Amplicon Size
rs6686001	TGT GCC CAA AGG TCC CCA CG	TGG ACA CAG CCC ATT AGT CA	161bp
rs3003596	CTG CAA AAG ATC CAA GAT TA	AGT TGT ACA GTC AGT ATT CA	161bp
rs2307418	CAG CTC CCT ATC TTA CAG AC	CTG GTG TGG CCT CCA AGC CC	171 bp

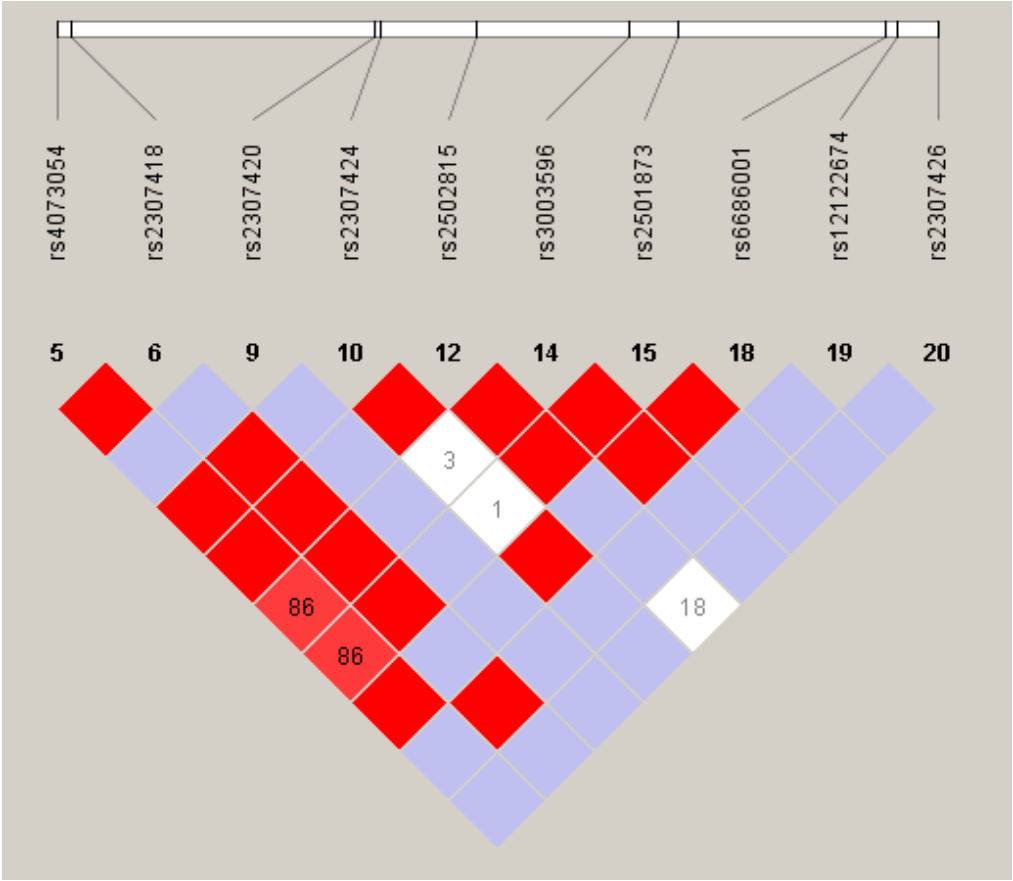

Figure 4.2 – Linkage Disequilibrium (LD) plot of CAR tag SNPs

A total of 9 tag SNPs in the CAR gene were generated using Haploview 4.2 software and displayed in a LD plot with the standard D'-plot colour scheme. Blocks that are bright red/blue define where D'=1 indicating 'complete LD'. D' = 1 unless highlighted by a numerical value e.g. 86 equals a D' value of 0.86. Tag SNPs were selected using the Haploview Tagger function.

4.2.1.1 CAR rs6686001 G → T

Cycling conditions were standard with an annealing temperature of 55 °C and an amplicon of 161 bp was produced. The forward primer was designed so that it contained an engineered site (G → C) that created a restriction site for the enzyme, *BsaAI*, allowing RFLP genotyping of the product. The restriction enzyme cuts in the presence of the T allele producing fragments of 142 bp and 19 bp, while the G allele remains uncut. The PCR product (10 µl) was digested overnight at 37 °C with 2U of *BsaAI* and products separated on a 10 % polyacrylamide gel as described in section 2.5.2. A typical separation of digestion products is displayed in **Figure 4.3**.

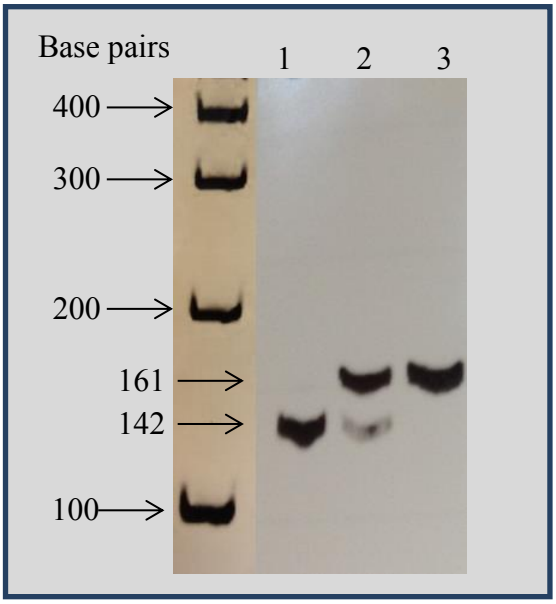

Figure 4.3 – PCR-RFLP analysis of CAR (rs6686001)

A typical gel image for the restriction digest of CAR (rs6686001) with BsaAI; lane 1 shows homozygous TT, lane 2 shows heterozygous GT and lane 3 shows homozygous GG

4.2.1.2 CAR rs3003596 A → G

The annealing temperature of the PCR reaction was 50 °C and all other cycling conditions remained standard. The forward primer was designed containing an engineered site (C → T) creating a restriction site for the enzyme, *MseI*. The PCR product (10 µl) with amplicon size 161 bp was digested overnight at 37 °C with 2U *MseI* and the products separated on a 10 % polyacrylamide gel as described in section 2.5.2. **Figure 4.4** shows a typical separation of digest products. The restriction enzyme cuts in the presence of the A allele producing fragments of 143 bp and 18 bp, while the G allele remains uncut.

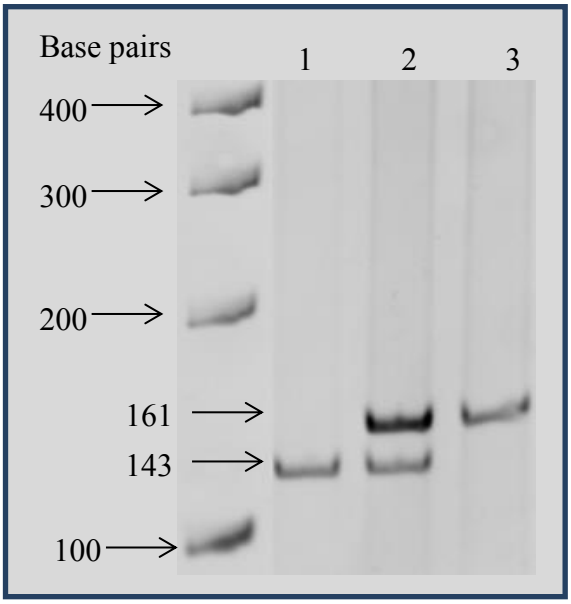

Figure 4.4 – PCR-RFLP analysis of CAR (rs3003596)

A typical gel image for the restriction digest of CAR (rs3003596) with MseI; lane 1 shows homozygous AA, lane 2 shows heterozygous AG and lane 3 shows homozygous GG

4.2.1.3 CAR rs2307418 A → C

Cycling conditions were standard and the annealing temperature was 51°C. The 171 bp PCR product (10 µl) was digested overnight at 37 °C with 2U of *DdeI* and the digestion products separated on a 10 % polyacrylamide gel as described in section 2.5.2. **Figure 4.5** shows a typical separation of digest products. The restriction enzyme cuts the A allele to produce fragments of size 143 bp and 23 bp. The C allele remains uncut.

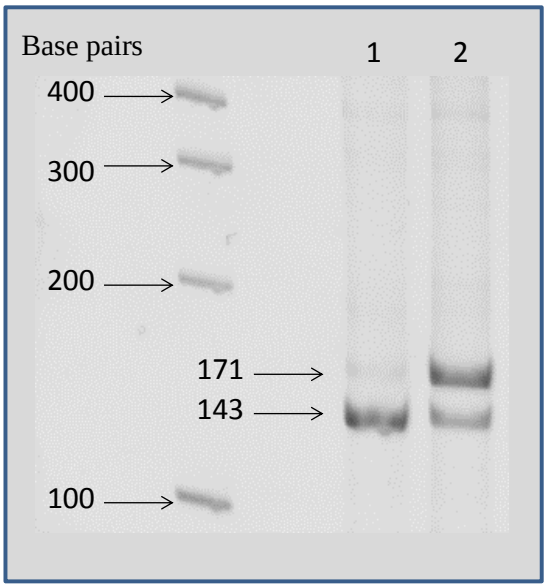

Figure 4.5 – PCR-RFLP analysis of CAR (rs2307418)

A typical gel image for the restriction digest of CAR (rs2307418) with DdeI; lane 1 shows homozygous AA, lane 2 shows heterozygous AC. No homozygous CC genotypes were observed.

4.2.2 *Measurement of endogenous CAR levels in cell lines by SYBR Green I qPCR*

HepG2, LS180 and Caco-2 cells were cultured as described in section 2.7. RNA was extracted from cells as described in section 2.3.2.1 and cDNA synthesised by reverse transcription as described in section 2.6.1. SYBR Green I real-time PCR was performed as described in section 2.6.2.1 with primers for CAR and GAPDH displayed in **Table 2.4** in Chapter 2.

4.2.3 *CAR Reporter Gene Assay*

To investigate whether flucloxacillin is an activator of CAR a luciferase reporter gene system was adopted. A reporter gene construct containing the 1.6kb promoter region of the CYP2B6 gene, PBREM and XREM ligated into a basic p-GL3 vector containing the firefly luciferase gene and a human CAR expression vector was kindly donated by Dr. Masahiko Negishi (National Institutes of Health, North Carolina, USA).

Plasmid DNA was synthesised via transformation of JM109 *E*.coli Competent cells as described in section 2.8.1 and extracted as described in section 2.8.2. For transfections, HepG2 and Caco-2 cells were cultured to approximately 70% confluency then seeded into 24 well plates at a density of 5×10^4 cells/ml per well as described in section 2.8.3. Non-transfected control wells were seeded in triplicate. Transfections were performed as described in section 2.8.4, with the addition of 100 ng CYP2B6/PBREM/XREM reporter construct and 30 ng of pRL-TK *Renilla* Luciferase control vector per well. For co-transfection experiments, 50 ng human CAR expression plasmid was added per well along with the CYP2B6/PBREM/XREM reporter construct and *Renilla* control. After transfection, culture media was replaced and cells treated in triplicate with 0.1% DMSO (for vehicle control), CITCO (1 µM) or flucloxacillin at concentrations of 100 µM, 500 µM and 1 mM for 24 hours. Following treatment, reporter activity was assessed as described in section 2.8.5.

4.2.4 *Genotyping of PXR -25385T (rs3814055) in new flucloxacillin-DILI cases*

Flucloxacillin-DILI cases and controls were genotyped for the PXR SNP rs3184055 by qPCR using a TaqMan SNP assay. The protocol for the genotyping assay is identical to that described in section 3.2.6.

4.2.5 *PXR reporter gene assay*

To investigate PXR activation by flucloxacillin and the isoxazolyl penicillins – cloxacillin and dicloxacillin, a plasmid reporter gene containing a luciferase construct ligated to a concatamer of three ER6 response elements specific to PXR was used (Andrews *et al.*, 2010). Plasmid DNA was synthesised via transformation of JM109 *E*.coli Competent cells as described in section 2.8.1 and extracted as described in section 2.8.2. HepG2 cells were cultured to approximately 70% confluency then seeded into 24 well plates at a density of 5 x 10^4 cells/ml per well, as described in section 2.8.3. Non-transfected control wells were seeded in triplicate. Transfections were performed as described in section 2.8.4, with the addition of 500 ng of p-$(ER6)_3$ reporter construct and 50 ng of pRL-TK *Renilla* Luciferase control vector per well. After transfection, culture media was replaced and cells treated in triplicate with 0.1% DMSO (for vehicle control), rifampicin (20 µM) or flucloxacillin/cloxacillin/dicloxacillin at concentrations of 500 µM, 1 mM and 2 mM for 72 hours. Following treatment, reporter activity was assessed as described in section 2.8.5.

4.2.6 *Measurement of CYP3A4 induction in LS180 cells by SYBR Green I qPCR*

LS180 cells were routinely cultured as described in section 2.7 then seeded into 6 well plates at a density of 2 x 10^5 cells/ml per well. Cells were treated with vehicle (0.1% DMSO), rifampicin (20 µM) or flucloxacillin/dicloxacillin at concentrations of 250 µM, 500 µM, 1 mM or 2 mM for 72 hours. RNA was extracted from cells as described in section 2.3.2.1 and cDNA synthesised by reverse transcription as described in section 2.6.1. SYBR Green I real-time PCR was performed as described in section 2.6.2.1 with primers for CYP3A4 and GAPDH displayed in **Table 2.4** in Chapter 2.

4.2.7 *Statistical analysis*

Genotype distributions between cases and controls were compared with Fisher's exact test on Prism 3.0 software (GraphPad). Adherence to the Hardy-Weinberg equilibrium for genotypes was determined by the chi-squared test using a web-based calculator available at http://www.tufts.edu/.

Gene expression was calculated by the ΔΔCt method of analysis as described in section 2.6.2 and is expressed as relative fold induction as the mean ± S.D. of triplicate experiments. Comparisons of gene expression between individual treatment groups and the untreated control group were analysed by performing unpaired two-tailed students t-tests using GraphPad Prism 3.0 software.

For reporter gene analysis, significance was assessed by student's t-tests and one-way ANOVA.

4.3 Results

4.3.1 *Investigation of CAR genotypes and susceptibility to flucloxacillin DILI*

Three SNPs in the CAR (NR1I3) gene, rs3003596, rs6686001 (both in Intron 1) and rs2307418 (Intron 8) were selected for study. These SNPs were chosen as tag SNPs for the main CAR haplotypes, using European data from the HapMap database and Haploview. Genotyping was performed on 72 cases of flucloxacillin-DILI from the flucloxacillin-GWAS and 64 flucloxacillin-treated controls by PCR-RFLP. An additional comparison was made between flucloxacillin-DILI cases and a freely available online control cohort from the 1000 Genomes Project that contained 379 individuals of European descent. **Table 4.2** shows the genotyping results for the three CAR SNPs. Control groups for all SNPs were found to be in Hardy-Weinberg equilibrium. No significant difference in genotype frequency between cases and controls were observed for any of the SNPs.

In addition to the three CAR tag SNPs genotyped by PCR-RFLP, a further 5 CAR tag SNPs, were analysed by PLINK analysis using data from the GWAS on flucloxacillin (Daly *et al.*, 2009). POPRES control data was used for comparison of flucloxacillin-DILI cases and controls. **Table 4.3** shows the genotype results for these SNPs and POPRES controls. Similarly, no significant associations were found between flucloxacillin-DILI cases and POPRES controls in these five SNPs in the CAR gene.

Table 4.2 – Distribution of CAR genotypes in flucloxacillin-DILI cases, flucloxacillin-treated healthy controls and 1000 Genomes EUR population cohort

CAR	Genotype			*P* – value	OR (95% CI)
rs6686001	**GG**	**GT**	**TT**		
Cases (n=72)	48 (0.67)	23 (0.32)	1 (0.01)		
Controls (n=64)	43 (0.67)	20 (0.31)	1 (0.02)	1.0000	0.98 (0.48 – 2.00)
1000 Genomes EUR (n=379)	254 (0.67)	115 (0.31)	10 (0.02)	1.0000	0.98 (0.58 – 1.68)
rs3003596	**AA**	**AG**	**GG**		
Cases (n=72)	17 (0.24)	39 (0.54)	16 (0.22)		
Controls (n=64)	21(0.33)	32 (0.50)	11 (0.17)	0.5225	1.38 (0.59 – 3.24)
1000 Genomes EUR (n=379)	118 (0.31)	182 (0.48)	79 (0.21)	0.7552	1.09 (0.59 – 1.99)
rs2307418	**AA**	**AC**	**CC**		
Cases (n=72)	56 (0.78)	16 (0.22)	0 (0.00)		
Controls (n=64)	43 (0.67)	21 (0.33)	0 (0.00)	0.1815	1.71 (0.80 – 3.66)
1000 Genomes EUR (n=379)	286 (0.75)	87 (0.23)	6 (0.02)	0.7647	1.14 (0.62 – 2.08)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

Table 4.3 – Distribution of CAR genotypes in flucloxacillin-DILI cases and POPRES controls

CAR	Genotype			*P* – value	OR (95% CI)
rs4073054	**GG**	**GT**	**TT**		
Cases (n=72)	13 (0.18)	37 (0.51)	22 (0.31)		
Controls (n=282)	40 (0.14)	139 (0.49)	103 (0.37)	0.4076	1.31 (0.75 – 2.28)
rs2307420	**GG**	**GA**	**AA**		
Cases (n=72)	0 (0.00)	3 (0.04)	69 (0.96)		
Controls (n=282)	0 (0.00)	15 (0.05)	267 (0.95)	1.0000	0.78 (0.22 – 2.75)
rs2307424	**TT**	**TC**	**CC**		
Cases (n=72)	8 (0.11)	30 (0.42)	34 (0.47)		
Controls (n=282)	35 (0.12)	121 (0.43)	126 (0.45)	0.7909	1.11 (0.66 – 1.86)
rs2502815	**TT**	**TC**	**CC**		
Cases (n=72)	2 (0.03)	29 (0.40)	41 (0.57)		
Controls (n=282)	12 (0.04)	115 (0.41)	155 (0.55)	0.7918	0.92 (0.55 – 1.56)
rs2501873	**AA**	**AG**	**GG**		
Cases (n=72)	14 (0.19)	36 (0.50)	22 (0.31)		
Controls (n=282)	44 (0.16)	142 (0.50)	96 (0.34)	0.6746	1.17 (0.67 – 2.05)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

4.3.2 *Study into the effects of flucloxacillin on CAR activation*

4.3.2.1 Determination of endogenous CAR in LS180, HepG2 and Caco-2 cell lines

Before transfection of the reporter construct into cells for investigation, a suitable cell line that expressed high levels of endogenous CAR needed to be found. This was achieved by assessing endogenous CAR levels by real-time PCR in three different cell lines – the hepatoblastoma cell line, HepG2, and two colorectal cell lines, LS180 and Caco-2. Real-time PCR (qPCR) was performed with SYBR Green I, using GAPDH as a housekeeping gene for normalisation of data. Results from qPCR are normalised to expression levels in LS180 cells and are displayed in **Figure 4.6**. HepG2 cells were found to contain a 1.65 ± 0.55 fold increase in CAR expression relative to LS180 cells although this was not found to be statistically significant (p = 0.1307). Caco-2 cells were found to have 3.25 ± 1.01 fold greater expression of CAR than LS180 (p = 0.0194). From these qPCR results it was decided that Caco-2 and HepG2 cells were to be used for subsequent transfection studies with the CYP2B6/PBREM/XREM reporter plasmid.

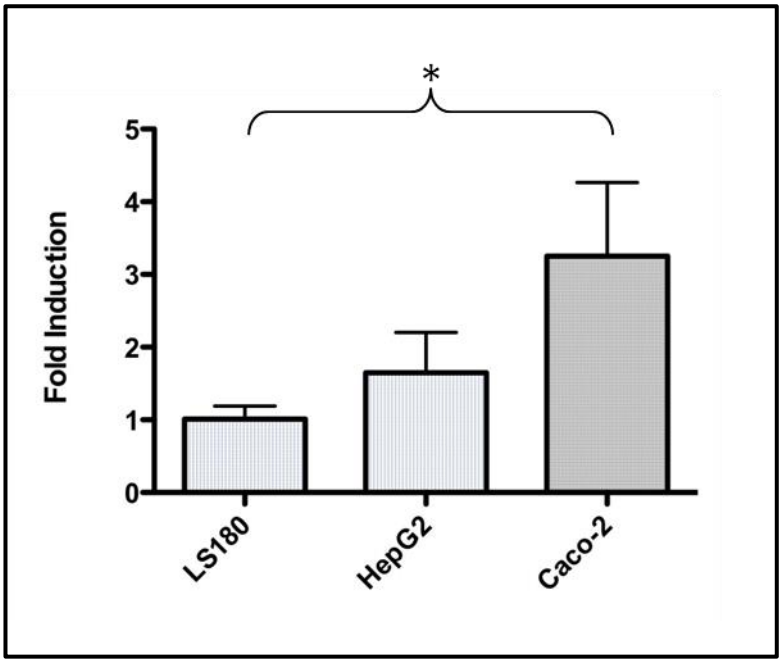

Figure 4.6 – Relative mRNA expression of endogenous CAR in LS180, HepG2 and Caco-2 cell lines

*Expression of endogenous CAR was determined in LS180, HepG2 and Caco-2 cells by qPCR. CAR expression in LS180 cells is normalised to 1 to show comparative expression levels between the different cell lines. Data represents mean ± S.E.M, n = 3, * $p < 0.05$, students t-tests were performed to compare differences between groups. A significant difference in CAR expression was observed between LS180 and Caco-2 cells ($p=0.0194$).*

4.3.2.2 Optimisation of the CAR reporter assay in HepG2 and Caco-2 cells

The transfection protocol was tested by treating cells with the potent CAR agonist, CITCO (1μM). CITCO should activate CAR leading to heterodimer formation with RXR, binding to motifs within PBREM and XREM and transcription of the CYP2B6/PBREM/XREM reporter construct. This leads to the expression and intracellular accumulation of the firefly luciferase enzyme which can be detected by subsequent analysis. For analysis, cells were lysed and reporter firefly luciferase (in relative light units) and control renilla thymidine kinase activity (in relative light units) analysed using the Dual-Glo Luciferase Assay System (Promega, UK). Data was then normalised against renilla luciferase activity and expressed as fold induction versus untreated cells. **Figure 4.7** shows that CITCO treatment had very little effect on reporter luciferase activity in both HepG2 and Caco-2 cells. This suggests that either the protocol doesn't work efficiently or that there are actually low amounts of endogenous CAR present in these cell lines.

To test this hypothesis, HepG2 and Caco-2 cells were co-transfected with the CYP2B6/PBREM/XREM reporter gene construct and a human CAR expression vector (also kindly donated by Dr. M. Negishi). Co-transfecting a CAR expression vector into the cells led to a much greater CYP2B6/PBREM/XREM reporter gene response in both the vehicle (0.5% DMSO) and CITCO treated cells as is also shown in **Figure 4.7**. Reporter activity was significantly increased in vehicle control groups by co-transfection with the CAR expression vector in Caco-2 cells ($p < 0.0001$) and HepG2 cells ($p = 0.0090$). This response in untreated cells can be explained by the constitutively active nature of CAR whereupon it can spontaneously translocate to the nucleus and activate its target genes (Goodwin et al., 2001). However, in CITCO treated cells co-transfected with the CAR expression vector, a significant response is observed in Caco-2 cells ($p = 0.0003$) but not in HepG2 ($p = 0.2262$) when compared to untreated cells transfected with the reporter only.

Reporter activity was compared between untreated and CITCO treated cells that were co-transfected with the reporter construct and CAR expression vector in HepG2 and Caco-2 cells. CITCO treatment gave a significant increase in reporter activity in Caco-2 cells ($p = 0.0150$) but not in HepG2 cells ($p = 0.8180$). This led to the decision to subsequently use Caco-2 cells for further studies.

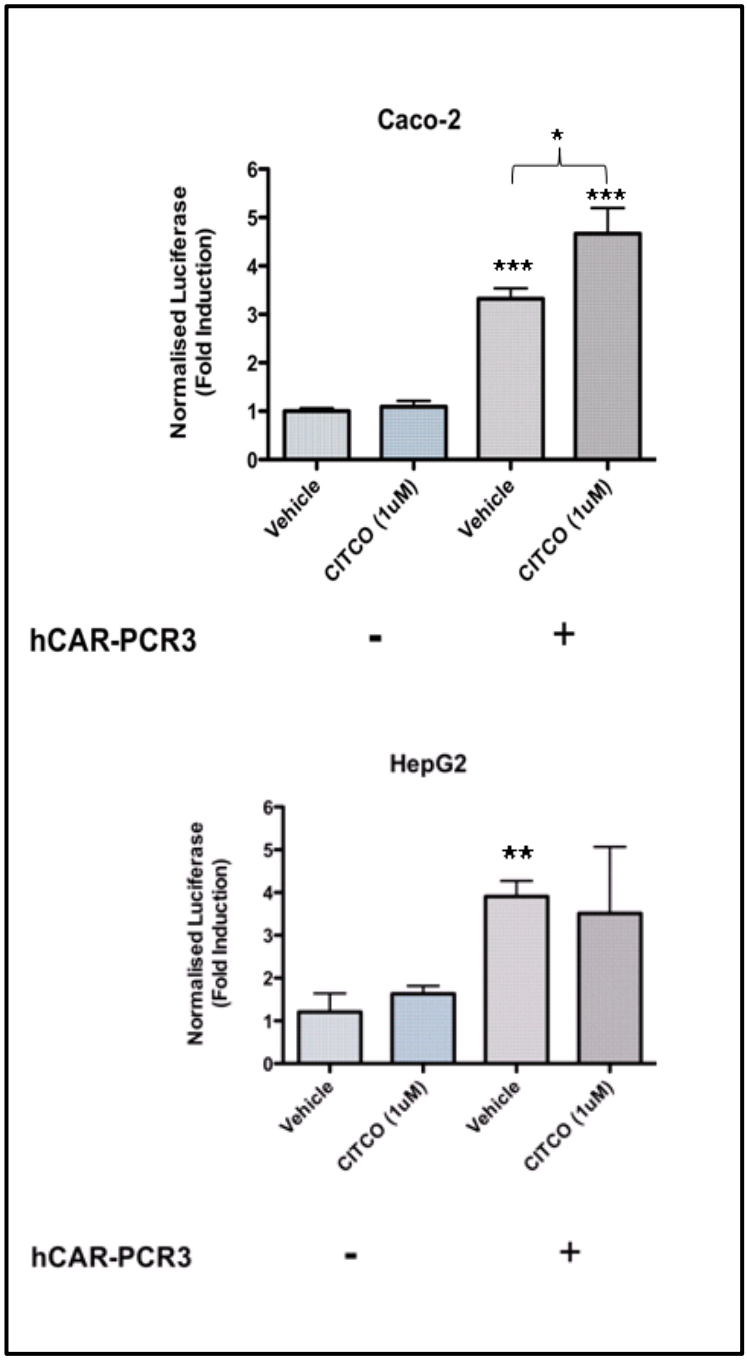

Figure 4.7 – Effect of co-transfection of a human CAR expression vector on luciferase activity of a CYP2B6/PBREM/XREM reporter construct in Caco-2 and HepG2 cells

*Caco-2 and HepG2 cells were either transfected with (+) or without (-) a hCAR expression vector alongside the CYP2B6/PBREM/XREM promoter reporter construct and treated with vehicle (0.1% DMSO) or CITCO (1 µM) for 24 hours. Reporter luciferase activity was normalised for renilla luciferase activity. Data represents mean ± S.E.M, n = 3 of technical replicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ One-Way ANOVA followed by a Bonferroni post-test was performed to compare differences in luciferase activation between all groups. Addition of the hCAR expression vector led to a significant increase of reporter activity in both cell lines. However, only in Caco-2 cells was a significant response observed by treatment with the positive control, CITCO in cells co-transfected with the hCAR vector.*

4.3.2.3 Reporter gene assay to study the effects of flucloxacillin on CAR activation

Caco-2 cells were co-transfected with the CYP2B6/PBREM/XREM reporter gene construct and the human CAR expression vector and treated with CITCO (1 µM) or varying concentrations of flucloxacillin – 100 µM, 500 µM and 1 mM for 24 hours. Luciferase activity of the reporter construct was normalised for renillla activity and results shown as fold increase relative to the vehicle (0.5% DMSO) control group (**Figure 4.8**). CITCO treatment gave a 1.51 ± 0.09 fold increase in reporter activity compared to the vehicle control group ($p < 0.001$). Flucloxacillin was not found to cause reporter gene activation, suggesting that it does not activate CAR leading to CYP2B6 and reporter transcription.

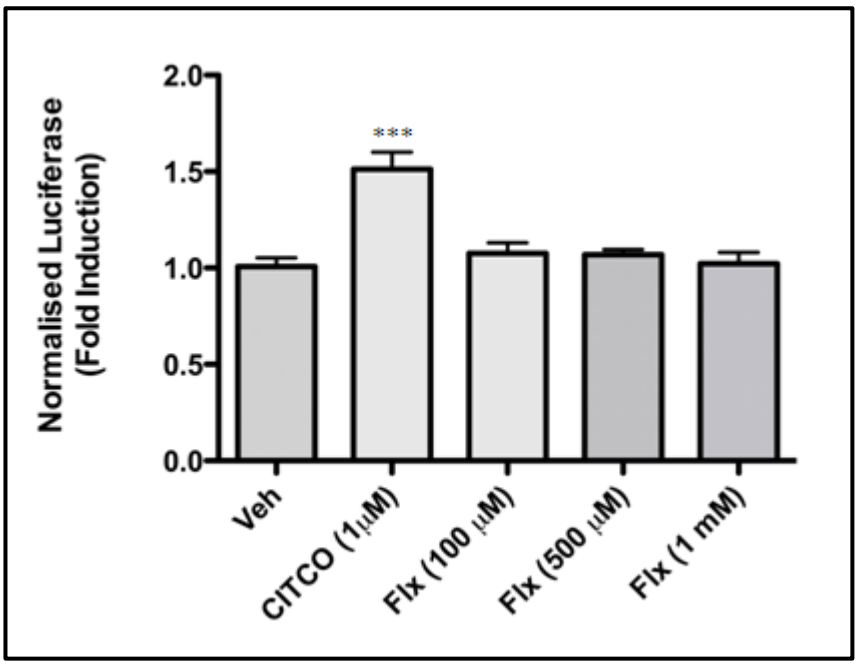

Figure 4.8 – Effect of flucloxacillin treatment on the luciferase activity of a CYP2B6/PBREM/XREM reporter construct in Caco-2 cells

*Reporter luciferase activity was normalised for renilla luciferase activity. Cells were transfected with a hCAR expression vector alongside a CYP2B6/PBREM/XREM promoter reporter construct and treated with vehicle (0.1% DMSO), CITCO (1 µM) or flucloxacillin at 100 µM, 500 µM or 1 mM for 24 hours. Data represents mean ± S.E.M, n = 3 of technical replicates,*** $p < 0.001$ students t-tests were performed to compare treatment groups to vehicle control.*

4.3.3 *Genotyping of additional flucloxacillin-DILI cases for PXR -25385C/T*

Previous studies at Newcastle University showed a significant association between the -25385C/T (rs3814055) polymorphism in PXR and flucloxacillin-DILI (Andrews *et al.*, 2010). In those studies, the original 51 cases of flucloxacillin-DILI from the flucloxacillin-GWAS were genotyped along with drug-treated controls (n=64) and healthy community controls (n=90). In the present study, an additional 99 cases of flucloxacillin-DILI were genotyped for the *PXR* -25385C/T polymorphism (**Table 4.4**). Due to the increased size of the flucloxacillin-DILI case cohort, the POPRES control group (n=282) was used to increase statistical power. The genotype frequencies for rs3814055 in this group were found to be in Hardy-Weinberg equilibrium ($X^2 = 0.20$, $p = 0.6536$).

Analysis of the original 51 cases of flucloxacillin-DILI versus the POPRES controls showed a significant difference in genotype frequency with 57% of these cases having a CC genotype compared to 41% of the controls, giving an odds ratio of 1.89 with a 95% confidence interval of 1.03 – 3.45 ($p = 0.0458$). However, analysis of the additional 99 cases failed to confirm this observation with 36% of these cases found to have the CC genotype compared to 41% of the controls ($p = 0.4743$). Combination of the two flucloxacillin-DILI cohorts showed 43% of the total cases carry the CC genotype which was not significantly different to the POPRES control group ($p = 0.6823$).

The combined effect of -25385CC genotype and HLA-B*57:01 carriage was investigated in the combined cohort of flucloxacillin-DILI cases. Of the 65 PXR -25385CC cases, 56 were also HLA-B*57:01 positive but this frequency was not significantly different to that in POPRES controls ($p = 0.5131$) (**Table 4.5**).

Table 4.4 - PXR SNP genotyping results in flucloxacillin-DILI cases and POPRES population control cohort

PXR -25385 (rs3814055)	Genotype			*P* – value	OR (95% CI)
	CC	CT	TT		
POPRES controls (n=282)	116 (0.41)	127 (0.45)	39 (0.14)		
Original genotyped cases (n=51)	29 (0.57)	14 (0.27)	8 (0.16)	0.0458	1.89 (1.03 – 3.45)
New cases (n=99)	36 (0.36)	42 (0.43)	21 (0.21)	0.4743	0.82 (0.51 – 1.31)
Combined cases (n=150)	65 (0.43)	56 (0.37)	29 (0.19)	0.6823	1.09 (0.73 – 1.63)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

Table 4.5 - Effect of PXR C-25385T genotype in relation to HLA B*57:01 genotype on risk of developing flucloxacillin-DILI in cases compared to POPRES controls

-25385 (rs3814055)	**Genotype**			***P* - value**	**OR (95% CI)**
	CC	**CT**	**TT**		
POPRES controls (n=282)	116 (0.41)	127 (0.45)	39 (0.14)		
HLA-B*57:01 positive (n=124)	56 (0.45)	45 (0.36)	23 (0.19)	0.5131	1.18 (0.77 – 1.80)
HLA-B*57:01 negative (n=26)	9 (0.35)	11 (0.42)	6 (0.23)	0.4043	0.67 (0.28 – 1.61)

Number of individuals with each genotype is shown with the genotype frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls; OR = odds ratio, CI = confidence interval

4.3.4 *Activation of PXR by isoxazolyl penicillins*

To determine PXR activation by various isoxazolyl penicillins, a reporter gene system containing a luciferase construct with a concatamer of the ER6 response element specific to PXR was used. HepG2 cells were transfected with the reporter construct and an internal control renilla-thymidine kinase construct. Cells were exposed to 500µM, 1mM and 2mM concentrations of cloxacillin, dicloxacillin and flucloxacillin for 72 hours, with the classic PXR activator, rifampicin (20 µM), as a positive control. These isoxazolyl penicillin concentrations were chosen based on a previous study of flucloxacillin-PXR activation by Elise Andrews at Newcastle University. In this study, no effect on PXR activation was observed with concentrations below 500 µM, and it is suggested that these concentrations are comparable to peak plasma flucloxacillin concentrations achieved after a 1 g dose (Andrews, 2009; Andrews *et al.*, 2010). Luciferase activity of the reporter construct was normalised for renillla activity and results shown as fold increase relative to the vehicle (0.5% DMSO) control group (**Figure 4.9**)

The findings of this study found that rifampicin produced the greatest PXR activity with a 3.28 ± 0.41 fold increase in luciferase activity of the reporter construct ($p = 0.0054$). Flucloxacillin produced significant dose-dependent increases in luciferase activity at the concentrations tested. A 1.44 ± 0.14 fold increase ($p = 0.0453$) was observed at the lowest concentration of 500 µM, with a 1.99 ± 0.17 fold ($p = 0.0053$) and a 2.11 ± 0.27 fold ($p = 0.0167$) stimulation at 1 mM and 2 mM, respectively.

Dicloxacillin was also found to produce a dose-dependent activation of PXR activity. Dicloxacillin produced a 1.95 ± 0.19 fold increase ($p = 0.0079$) at 500 µM. At 1 mM, a 2.11 ± 0.31 fold increase ($p = 0.0239$) was observed with the greatest increase of luciferase activity seen with 2 mM, with a 2.51 ± 0.12 fold increase ($p = 0.0003$).

In contrast to flucloxacillin and dicloxacillin, cloxacillin was not found to significantly increase PXR activity. A slight increase in report activity was observed at the highest concentration tested but this was not found to be significant ($p = 0.1335$).

The findings of this study suggest that flucloxacillin and dicloxacillin both activate PXR with dicloxacillin being the stronger of the two. Luciferase activities at each concentration for flucloxacillin and dicloxacillin were compared by one-way ANOVA

with a Bonferroni's multiple test comparison. Results show that the reporter activities between the groups at each concentration were not significantly different.

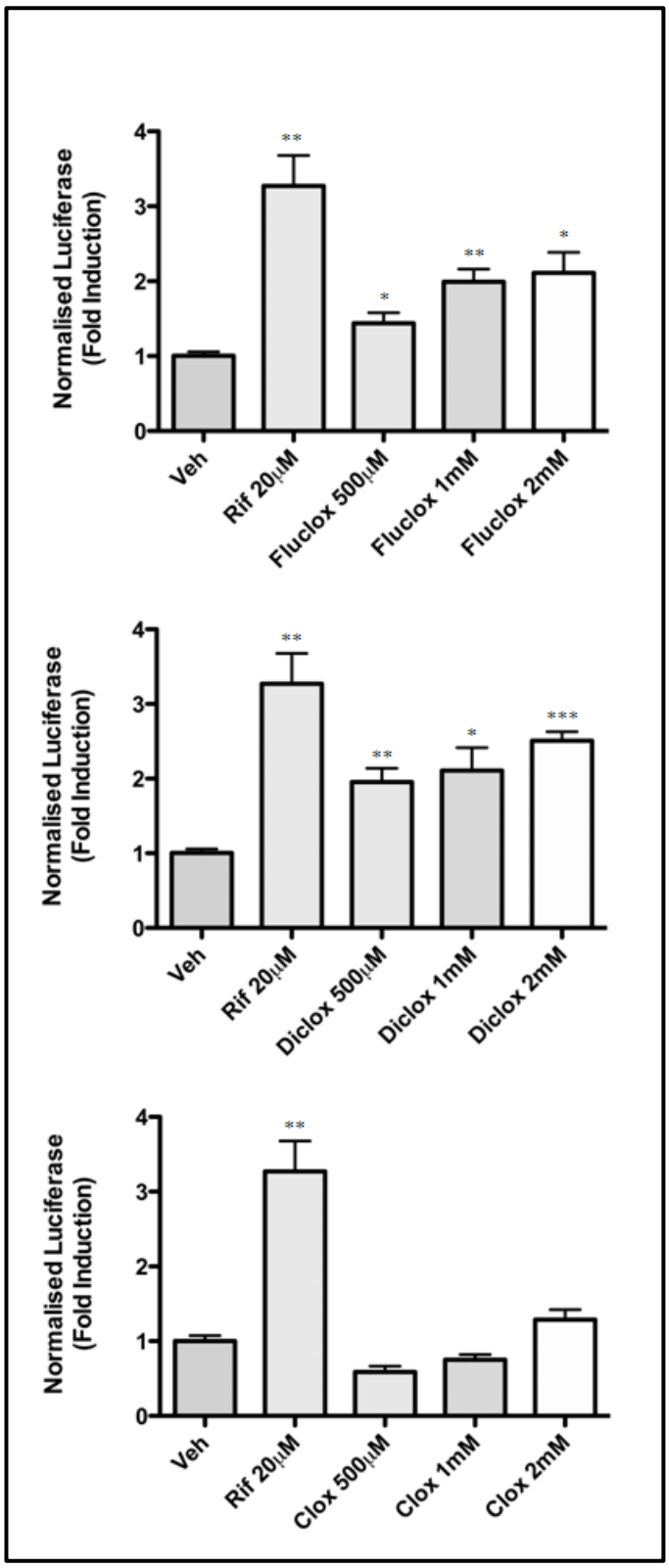

Figure 4.9 - Effect of isoxazolyl penicillin treatment on luciferase activity of PXR-$(ER6)_3$ reporter construct in HepG2 cells

*Reporter luciferase activity was normalised for renilla luciferase activity. HepG2 cells were treated with vehicle (0.1% DMSO), rifampicin (20 µM) or with flucloxacillin (top), dicloxacillin (middle) and cloxacillin (bottom) at 500 µM, 1 mM or 2 mM for 72 hours. Data represents mean ± S.E.M, n = 3 of technical replicates, * p < 0.05, ** p < 0.01, *** p < 0.001 students t-tests were performed to compare treatment groups to vehicle control.*

4.3.5 *CYP3A4 induction in LS180 cells by flucloxacillin and dicloxacillin*

Following the observation that dicloxacillin appears to be a stronger activator of PXR than flucloxacillin, studies were performed to determine whether this observation translated to stronger induction of the PXR regulated enzyme, CYP3A4. For this, LS180 cells were treated with flucloxacillin (250 µM – 2 mM), dicloxacillin (250 µM – 2 mM) or rifampicin (20 µM) as a positive control for 72 hours. CYP3A4 mRNA expression was measured by SYBR Green I real-time PCR. Rifampicin (20 µM) was observed to produce a 10.10 ± 0.62 fold increase (p = 0.0001) in CYP3A4 expression.

Figure 4.10 shows the qPCR analysis of CYP3A4 mRNA expression due to flucloxacillin and dicloxacillin exposure relative to the untreated vehicle control. Flucloxacillin showed a marginal increase in CYP3A4 expression over the concentration ranges which was significant at 1 mM and 2 mM concentrations with 1.64 ± 0.20 (p = 0.0435) and 2.17 ± 0.24 (p = 0.0105) fold increases respectively. CYP3A4 expression was markedly increased in dicloxacillin treated cells in a dose-dependent manner. Treatment with 2 mM produced a highly significant 5.78 ± 0.40 fold increase (p = 0.0003) in CYP3A4 expression.

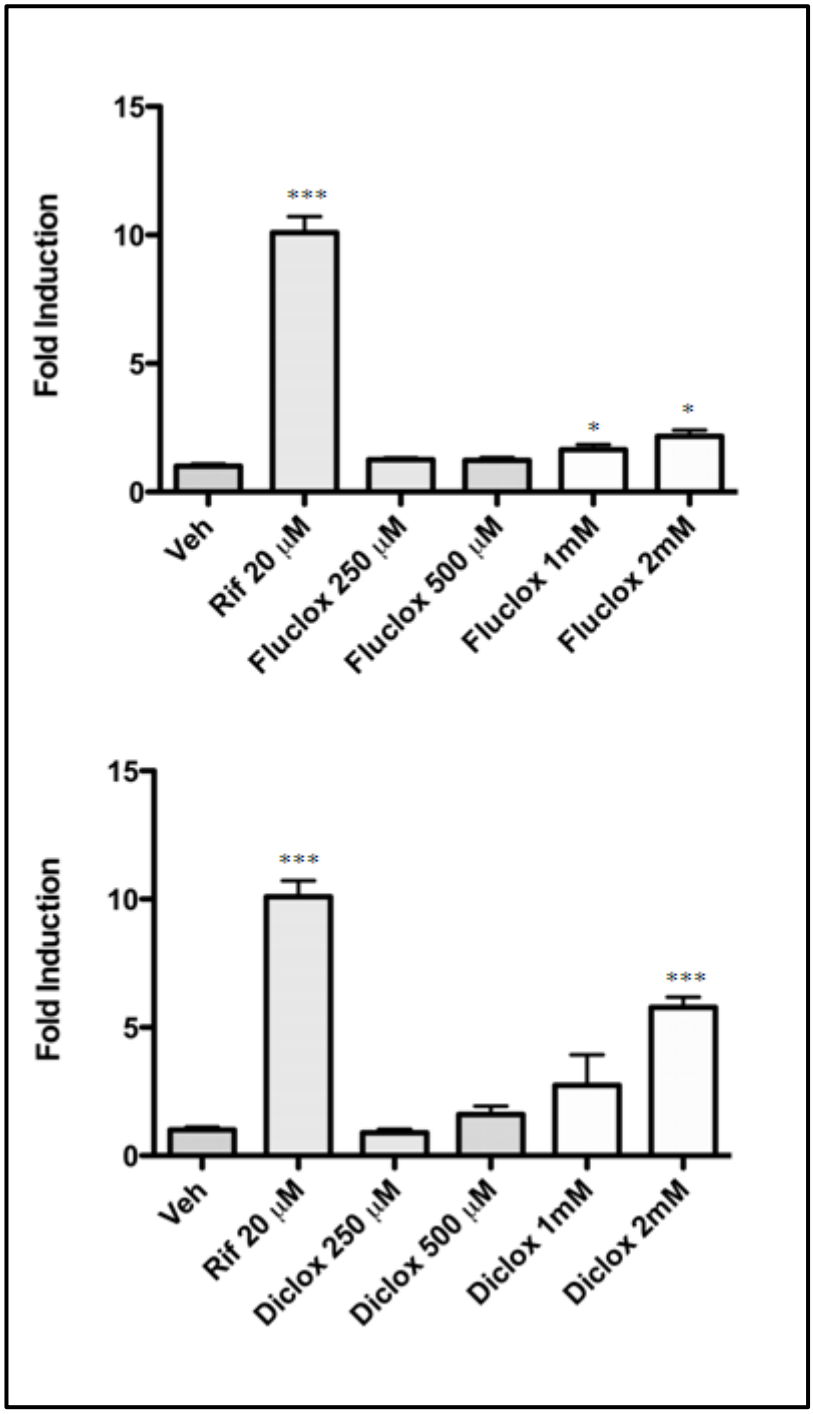

Figure 4.10 – Relative mRNA expression of CYP3A4 in LS180 cells

*Expression of CYP3A4 was determined in LS180 cells by qPCR. Cells were treated with vehicle (0.1% DMSO), rifampicin (20 µM) or with flucloxacillin (top) and dicloxacillin (bottom) at 250 µM, 500 µM, 1 mM or 2 mM for 72 hours. Data represents mean ± S.E.M, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ students t-tests were performed to compare treatment groups to vehicle control.*

4.4 Discussion

The metabolism and excretion of a large number of xenobiotics, as well as key pathways maintaining bile acid homeostasis, are regulated by the nuclear receptors PXR and CAR. These xenosensing receptors regulate the transcription of a large, overlapping set of genes encoding numerous metabolising enzymes and drug transporters that are activated to perform functions that protect the liver from accumulation of toxic compounds.

Flucloxacillin is a common cause of DILI but reports of DILI by the other isoxazolyl penicillins are rare. In this chapter, PXR activation by the isoxazolyl penicillins – cloxacillin and dicloxacillin was compared to flucloxacillin with a reporter gene study using a plasmid containing the (ER6) PXR response element transfected into HepG2 cells. Such a study has previously shown that flucloxacillin is able to activate the (ER6) response element suggesting that PXR plays a role in the disposition of flucloxacillin in the liver (Andrews *et al.*, 2010). The findings of the current study confirmed that flucloxacillin and dicloxacillin can activate the PXR (ER6) response element to a similar extent, but it appears that cloxacillin does not. Previously flucloxacillin and dicloxacillin have both been shown to induce the expression of the PXR-mediated genes, CYP3A4 and the drug efflux transporter MDR1, in cellular assays (Huwyler *et al.*, 2006; Yasuda *et al.*, 2008; Andrews *et al.*, 2010). CYP3A4 and MDR1 mRNA expression in LS180 cells in response to flucloxacillin and dicloxacillin was determined in the current study, confirming that both drugs induce expression of these genes and showing that dicloxacillin perhaps does so to a slightly greater extent. It could be hypothesised that greater activation of PXR by dicloxacillin *in vivo* results in greater clearance from the liver in comparison to flucloxacillin. If accumulation of the drug or reactive metabolites is a factor in DILI caused by dicloxacillin or flucloxacillin, a higher level of PXR activation may be protective against toxicity. However, the overall difference between PXR activation by flucloxacillin and dicloxacillin is debatable suggesting a probable negligible overall effect on flucloxacillin disposition in the liver. Additionally, this hypothesis does not seem to apply to instances of cloxacillin-induced liver injury as no apparent PXR activation was observed.

There is a lack of detailed, thorough investigation of penicillin use in individual countries but a study of 25 European countries found that β-lactamase resistant

isoxazolyl penicillins are not widely prescribed compared to broad spectrum penicillins, narrow-spectrum penicillins and penicillins combined with β-lactamase inhibitors (Ferech *et al.*, 2006). In this report, 40% of the countries studied were reported to prescribe isoxazolyl penicillins at less than 1% of their countries total penicillin prescriptions. Only in four countries - Denmark, Sweden, Iceland and the U.K., did β-lactamase resistant isoxazolyl penicillin prescriptions exceed 10% of total penicillin use. Of these countries, flucloxacillin is the preferred isoxazolyl penicillin for use in the U.K. and Sweden, whereas in Denmark, dicloxacillin is prescribed and in Iceland, cloxacillin and dicloxacillin are both used. The use of flucloxacillin in the U.K. and Sweden is accompanied by frequent reports of flucloxacillin-induced liver injury with the drug commonly the most frequent cause of DILI in these countries (Suzuki *et al.*, 2010). However, in Iceland where dicloxacillin is predominantly prescribed, reports of DILI are extremely rare and in Denmark where dicloxacillin prescriptions have more than doubled in the period 1996 to 2008 there are no reports in the literature of dicloxacillin-induced liver injury (Dalager-Pedersen *et al.*, 2011; Bjornsson *et al.*, 2013). From this, one could assume that dicloxacillin, although widely prescribed within a population, is not as hepatotoxic as flucloxacillin. The differences in PXR activation by the two drugs may potentially be a reason for this.

An upstream polymorphism, -25385C/T in the PXR promoter region has previously been found to be associated with cases of flucloxacillin-DILI where there was an observed increase of CC genotype in 51 cases compared to controls (Andrews *et al.*, 2010). This genotype is associated with decreased PXR expression and CYP3A4 induction suggesting that these individuals have a decreased ability to clear flucloxacillin from the liver which may make them more susceptible to toxicity. In the current study, an additional 99 cases of flucloxacillin-DILI were genotyped for the -25385C/T polymorphism. Although a significant association of CC genotype in the original 51 case-cohort from the flucloxacillin GWA study was found when compared to POPRES population controls, this association was not repeated when the 99 additional cases were included. The more in depth study of this SNP, with a greater number of cases, suggests that the original observation that there is an association between this SNP and flucloxacillin-DILI cases was incorrect due to small numbers of both cases and controls.

The potential for flucloxacillin to interact with CAR in a designed and optimised reporter gene assay was investigated in this chapter. A luciferase reporter construct

containing the CAR response elements - PBREM and XREM and the CYP2B6 promoter region was used for transfection studies. Caco-2 cells were chosen as a suitable cell line for studies due to them being found to have the highest levels of endogenous CAR compared to the other tested cell lines - LS180 cells and HepG2 cells, and from preliminary transfection assays that showed a greater activation of the luciferase reporter in Caco-2 cells compared to HepG2 cells. Another reason for using Caco-2 cells preferentially over HepG2 is due to the relative PXR content of each cell type. PXR has been shown to also be able to bind to motifs in the PBREM and XREM and activate transcription of CYP2B6 (Goodwin et al., 2001). It is thought that there is little active PXR in Caco-2 cells since treatment with the PXR ligand rifampicin is unable to induce CYP3A4 or MDR1 gene expression which are typical of PXR activation (Pfrunder et al., 2003). This is in contrast to HepG2 cells which have been used for study of PXR mechanisms owing to the endogenous levels of the nuclear receptor (Andrews, 2009). Cells were also transfected with a human CAR expression vector to increase cellular CAR levels. Confirmation that the transfection assay was valid was achieved by significant activation of the reporter construct by the human CAR ligand, CITCO. CITCO was chosen as a positive control as it is a selective CAR activator that doesn't interact with PXR, thus minimising potential cross-activation of the CYP2B6 reporter construct through PXR. However, even though CITCO activation of CAR was significant it would have been preferable to seek a positive control that produced a much stronger activation of the reporter construct. CITCO has been used in previous studies by others as a positive control in reporter gene studies of CAR activation (Maglich *et al.*, 2003). It has also been observed that several variants of CAR exist, including a CAR3 variant that is expressed at high levels in the liver along with wild-type CAR1 (Faucette *et al.*, 2006). CAR3 has been shown to have low basal activity but is extensively activated by CITCO (Auerbach *et al.*, 2005). The assay used in the current study, unlike previous groups, is primarily a study of wild-type CAR1 activation as it involved the co-transfection of a CAR1 expression vector. It would be interesting to study CAR activation via the CAR3 variant but due to the unavailability of a CAR3 expression vector this was not possible.

Treatment of transfected cells with various concentrations of flucloxacillin for 24 hours failed to see an increase in luciferase activity of the reporter construct. This leads to the conclusion that flucloxacillin does not bind and activate wild-type CAR1 to bind to its response elements and is unlikely to be a ligand for CAR. However, it would be

interesting to perform additional studies to determine whether flucloxacillin is a ligand for other CAR splicing variants including CAR3. The finding that flucloxacillin does not interact with CAR suggests that CAR does not influence flucloxacillin disposition in the liver and is unlikely to be a factor in flucloxacillin-DILI. This is supported by genotyping analysis of polymorphisms in the CAR gene in cases of flucloxacillin-DILI. Genotyping of 8 Tag SNPs found no significant associations between cases and drug-treated controls or population controls.

In summary,

- This chapter confirms previous observations that flucloxacillin and dicloxacillin are activators of the nuclear receptor PXR and that dicloxacillin appears to be a marginally stronger ligand of the two. Flucloxacillin and dicloxacillin are both widely prescribed in different countries but hepatotoxicity is only frequently reported due to flucloxacillin treatment. Greater PXR activation by dicloxacillin may result in increased clearance by the liver which could be a factor for the differences in observed hepatotoxicity.
- However, a previously reported genetic association in flucloxacillin-DILI that results in decreased PXR expression has not been replicated in the current study.
- In addition to PXR, the interaction of flucloxacillin and CAR was investigated. The findings suggest that flucloxacillin does not activate CAR *in vitro* and no genetic associations were found in flucloxacillin-DILI cases for SNPs in common CAR haplotypes.

Chapter 5. Studies on the Metabolism of Flucloxacillin

5.1 Introduction

The formation of reactive metabolites is thought to be a key mechanism in the pathogenesis of DILI. It has been proposed that bioactivation of drugs leads to the production of toxic compounds that can cause direct cellular damage or lead to adduct formation with cellular proteins. Peptides derived from these adducted proteins may then be presented to T cells by HLA proteins on the surface of antigen presenting cells. The chemically modified adducted peptides are recognised as foreign peptides provoking an inappropriate T cell response.

There is still some dispute over the pathways for flucloxacillin metabolism. Studies by Thijssen in the 1970's discovered that the isoxazolyl penicillins – oxacillin, cloxacillin, dicloxacillin and flucloxacillin, were metabolised *in vivo* to anti-bacterially active 5'hydroxymethyl metabolites (Thijssen and Mattie, 1976; Thijssen, 1979; Thijssen, 1980). As with penicillins in general, hydrolytic cleavage of the β-lactam ring also occurs with the isoxazolyl penicillins producing biologically inactive penicilloic acid products. Murai and colleagues reported that HPLC analysis of urine after flucloxacillin administration in fasted individuals showed that 64.8 % of the dose was excreted unchanged as flucloxacillin while 10.5% was metabolised to 5'-hydroxymethyl flucloxacillin, 3.8% was excreted as the penicilloic acid of flucloxacillin and 1.0% as the penicilloic acid of 5'-hydroxymethyl flucloxacillin (Murai *et al.*, 1983b). Thijssen reported that peak serum levels of 5'-hydroxymethyl flucloxacillin after flucloxacillin administration were 4% of the parent drug suggesting that production of the 5'hydroxymethyl metabolite is low (Thijssen, 1980). Comparison of 5'-hydroxymethyl metabolite formation showed that flucloxacillin yielded the least amount of the metabolite at 14.4%, compared to oxacillin (50.6%), cloxacillin (17.8%) and dicloxacillin (44.8%) (Murai *et al.*, 1983a).

The early studies into flucloxacillin and other isoxazolyl penicillin metabolism suggested that 5'-hydroxymethyl metabolite formation likely occurred in the liver, via hydroxylation of the 5-methyl group on the isoxazole ring, as a result of monooxygenase enzyme metabolism. It has also been suggested that degradation to penicilloic acid derivatives may also occur in the liver but may be spontaneous as degradation can occur slowly in aqueous solutions and, interestingly, rapidly in the presence of β-lactamase (Cole and Hewitt, 1973).

More recent studies into the specific pathways of flucloxacillin metabolism were performed in 2001 when Lakehal and colleagues performed studies using various *in vitro* assays including induced rat liver microsomes, human liver microsomes (HLM) and yeast-microsomes expressing various recombinant human cytochrome P450 isoforms (Lakehal *et al.*, 2001). Lakehal reported that conditioned medium from human hepatocytes treated with flucloxacillin for 24 hours was toxic to approximately 58% biliary epithelial cell (BEC) preparations, whereas flucloxacillin itself was found not to be toxic to any cell preparations. This suggests the formation of a toxic compound with flucloxacillin incubation with hepatocytes. Supernatant from flucloxacillin incubated with HLMs was found to be toxic to 50% of all BEC preparations suggesting that the toxic compound is derived from flucloxacillin metabolism. Supernatant from flucloxacillin incubated with recombinant CYP enzymes showed toxicity when the drug was incubated with CYP3A4 microsomes but not from incubations with CYP1A2, CYP2C9 or CYP3A5 microsomes.

Further studies with rat liver microsomes that were induced with CYP1A, 2B and 3A, followed by analysis by HPLC showed a time and NADPH dependent formation of a metabolite that was greatest in rats treated with dexamethasone to induce CYP3A. This metabolite was identified by using mass spectrometry and ^{1}H nuclear magnetic resonance (NMR), as 5'hydroxymethyl flucloxacillin. Subsequently, these researchers purified this metabolite from CYP3A-induced rat liver microsomes and used it as a standard in further experiments which showed it could be formed in HLMs and recombinant-CYP3A4 microsomes.

The results of the studies by Lakehal and colleagues seemed to strongly suggest that 5'-hydroxymethyl flucloxacillin could be generated *in* vitro and that its production was mediated by CYP3A4. The observations that flucloxacillin can activate PXR and induce expression of CYP3A4, as was confirmed in Chapter 4, suggest a role for CYP3A4 in its metabolism. However, this has been disputed by studies performed by Huwyler and colleagues who investigated possible drug-drug interactions between flucloxacillin and the immunosuppressant drug, cyclosporine (Huwyler *et al.*, 2006). It had been observed that co-treatment with flucloxacillin seemed to lead to decreased plasma levels of cyclosporine causing kidney rejection in transplant patients. They concluded that cyclosporine disposition was significantly affected by CYP3A4 and MDR1 induction by flucloxacillin through PXR-mediated pathways. However, *in vitro* assays performed

with recombinant-CYP isoforms showed that flucloxacillin was not a substrate for cytochrome P450s 3A4, 1A2, 2C9, 2C19 or 2D6.

In this chapter, flucloxacillin metabolism will be further studied using HLMs, recombinant-CYP isoforms and a cell-based assay using a specialised differentiated rat cell line, B13/H. The AR42J-B-13 cell line (simply termed B-13 cell line), is a rat pancreatic acinar adenocarcinoma cell line that was observed to be able to differentiate into a hepatocyte phenotype with glucocorticoid treatment (Shen *et al.*, 2000).Glucocorticoids are potent hormones often used in stem cell differentiation protocols and it has been shown that treatment of B13 cells with the glucocorticoid, dexamethasone, led to the differentiation of the cells to express liver based proteins (Burke *et al.*, 2006; Wallace *et al.*, 2010). Termed B13/H cells, differentiated B13s have also been shown to express metabolically active cytochrome P450 enzymes at levels comparable to freshly isolated hepatocytes (Marek *et al.*, 2003). To perform *in vitro* studies of liver functions such as drug metabolism and biotransformation there are a variety of available liver preparations that can be utilised. The closest to achieving *in vivo* functionality of the liver *in vitro* is through the use of liver preparations either in the form of whole liver perfusion, liver slices of isolation of primary hepatocytes. Whole liver perfusions and liver slices retain specific liver structures and cell types but are difficult to obtain, labour intensive and limited to short term studies. Primary hepatocytes can be isolated from liver and are available commercially and are regularly used to study liver functions. Primary hepatocytes express drug metabolising enzymes comparable to the hepatocyte phenotype *in vivo* but major disadvantages are that the cells are difficult to proliferate in culture meaning and also rapidly undergo de-differentiation losing expression of drug metabolising enzymes after a number of days (Wilkening *et al.*, 2003; Wallace *et al.*, 2010). Hepatoma cancer cell lines such as HepG2 are also widely used to study certain liver functions. Unlike, primary hepatocytes these cells are easily expanded in culture and are cost effective in comparison. However, they have been shown to express low levels of active drug metabolising enzymes which make them unsuitable for the studies described in this chapter. Although, they essentially do not express drug metabolising enzymes they are still useful for other investigations, for example, the studies described in chapter 4, investigating the regulatory mechanisms of drug metabolism via the nuclear receptors CAR and PXR.

The characterisation of the trans-differentiation phenomena in the B-13 cell line showing the cells to express genes and proteins displaying a hepatocellular phenotype including drug metabolising enzymes has allowed them to be used as cost effective, expandable progenitor for functional hepatocytes (Wallace *et al.*, 2010). It is for these reasons that B13/H cells will be used in these studies instead of hepatoma cell lines.

5.2 Methods

5.2.1 *Materials*

5-hydroxymethyl flucloxacillin was purchased from Sarchem Laboratories (New Jersey, USA). Bactosomes prepared from *E.* coli co-expressing human NADPH-cytochrome P450 reductase and individual human cytochrome P450s (CYP3A4, CYP2C8 and CYP2C9) were purchased from CYPEX (Dundee, UK). Human liver microsomes, pooled from 50 donors, were purchased from GIBCO (Invitrogen).

5.2.2 *Flucloxacillin incubations with Human Liver Microsomes and recombinant CYP-expressing Bactosomes*

Human liver microsomes or CYP-expressing bactosomes were incubated in 1.5 ml microcentrifuge tubes in a reaction volume of 100 µl with 500 µM or 1mM flucloxacillin in 0.1 M sodium phosphate buffer (pH 7.4) to give final P450 concentrations of 0.5 nmol/ml and 50 pmol/ml respectively. 5 µl NADPH-regeneration system solution A (BD Biosciences) and 1 µl NADPH-regeneration system solution B (BD Biosciences) was added to initiate the reaction. Tubes were incubated at 37 °C in a water bath with agitation for 60 minutes. 50 µl sodium phosphate:acetonitrile mix (2:1 ratio) was added to the tubes to terminate the reactions. Reaction tubes were centrifuged at 19000 g for 5 min to pellet protein and the supernatant removed to fresh tubes which were stored at - 20°C until HPLC analysis.

5.2.3 *Flucloxacillin incubation with B13/H cells*

5.2.3.1 B13 cell differentiation to B13/H cells and induction of Cyp3a1

B13 cells were routinely cultured in DMEM supplemented with 10 % heat-inactivated FBS, 1 % non-essential amino acids (0.1 mM), 2 mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin in 75 cm^2 flasks and incubated as described in section 2.7.1. When B13 cells reached approximately 70% confluence, cells were seeded into 6-

well plates at a density of 2 x 10^5 cells per well then treated with 10 nM dexamethasone for 14 days to induce differentiation to B13/H cells. B13/H cells were further treated with 10 µM dexamethasone for another 3 days, to induce Cyp3a1.

5.2.3.2 Flucloxacillin treatment of cells and extraction of metabolites

Cyp3a1-induced B13/H cells were treated with 1 mM flucloxacillin for 2 hours or 24 hours. After treatment, culture medium was removed from cells and subjected to solid phase extraction using Sep-Pak Light C_{18} columns (Waters, UK) as described by Lakehal et al (Lakehal *et al.*, 2001). Briefly, samples were applied to the columns and 2 ml water was added to the columns as a wash step. Products were eluted in 3 ml acetonitrile and evaporated to dryness under nitrogen. Samples were resuspended in 200 µl 0.1 M sodium phosphate (pH 7.4):acetonitrile mix (2:1) and centrifuged at 12000 g for 5 minutes to pellet any remaining precipitate. Supernatants were transferred to fresh 1.5. ml microfuge tubes and stored at – 20°C until HPLC analysis.

5.2.4 *HPLC analysis*

Supernatants from HLM, recombinant-CYP bactosomes and cell extracts were analysed by reverse-phase HLPC. Separations were carried out using a C8 MOS-2 Hypersil column (Thermo Scientific) on a Shimadzu HPLC system. The column was equilibrated at a flow rate of 1 ml min^{-1} in solution A (0.1 M ammonium acetate, pH 4.6). Samples were applied in solution A and eluted at a linear gradient of 0 – 70 % solution B (acetonitrile/methanol/water, 70:20:10 v/v/v) in solution A over 30 minutes. Analytes were detected by an online UV spectrophotometer at 260 nm. The limit of detection of 5'hydroxymethyl flucloxacillin was determined to be 1 µM. Peaks were analysed on LCanalysis software v1.21 (LCsolution, Shimadzu).

5.3 Results

5.3.1 *HPLC detection of flucloxacillin and 5'-hydroxymethyl flucloxacillin*

For studies into the metabolism of flucloxacillin, HPLC conditions previously described by Lakehal et al were adopted (Lakehal *et al.*, 2001). **Figure 5.1** displays two overlaid chromatograms showing the UV detection at 260 nm of 1) flucloxacillin and 2) 5'hydroxymethyl flucloxacillin, both in 0.1 M sodium phosphate buffer (pH 7.4). The HPLC method gives a good resolution of the two compounds with the retention time of flucloxacillin and 5'hydroxymethyl flucloxacillin being approximately 18.5 min and 16 min respectively. The HPLC method was subsequently used for the *in vitro* studies into the metabolism of flucloxacillin.

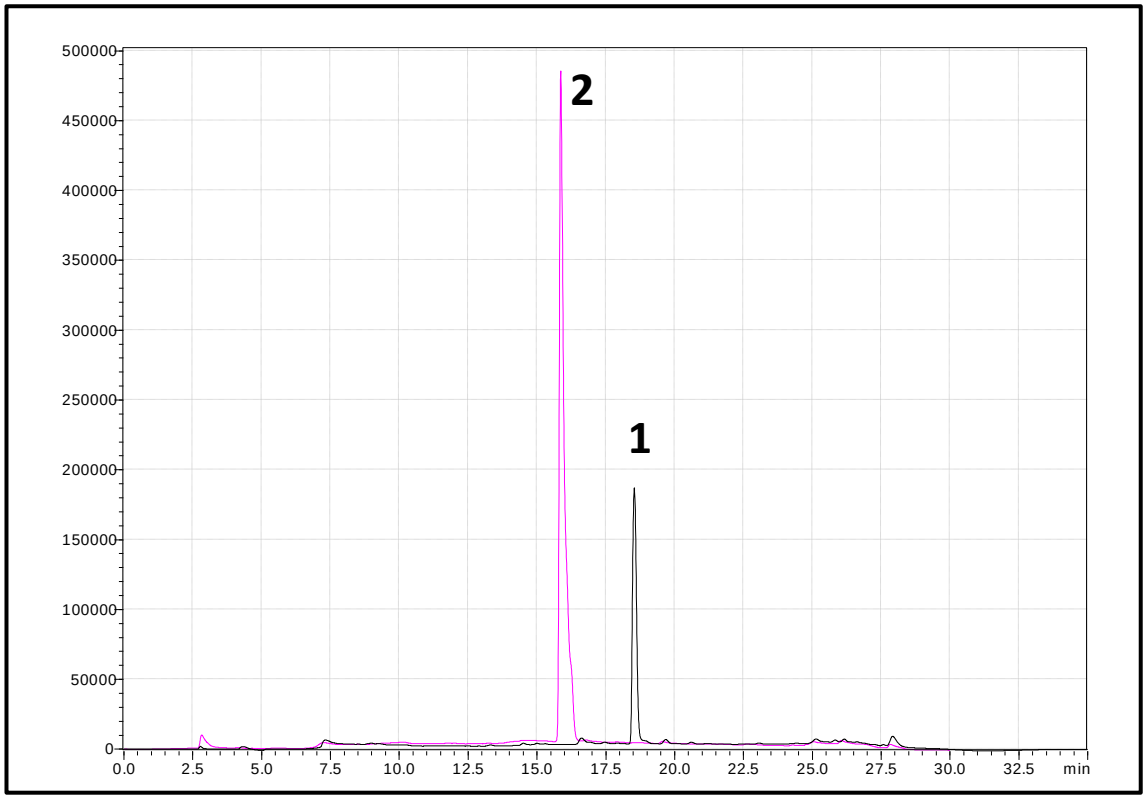

Figure 5.1 – HPLC detection of flucloxacillin and 5'-hydroxymethyl flucloxacillin

HPLC chromatogram showing flucloxacillin (1) and 5'hydroxymethyl flucloxacillin (2) in 0.1 M sodium phosphate buffer (pH 7.4). Flucloxacillin and the 5'-hydroxymethyl flucloxacillin standard have distinctly different retention times of approximately 18.5 minutes and 16 minutes respectively, showing that the two compounds can easily be identified by this HPLC method.

5.3.2 *Incubation of flucloxacillin with Human Liver Microsomes*

Flucloxacillin was incubated with HLM for 60 minutes or 24 hours. **Figure 5.2** shows chromatograms for the incubation of flucloxacillin and HLM at time points 0 minutes and 60 minutes. Overlaying the chromatograms shows that there was no obvious decrease in flucloxacillin concentration or formation of 5'hydroxymethyl flucloxacillin or penicilloic acid derivatives over this time period.

Figure 5.3 shows a chromatogram for the incubation of flucloxacillin with HLM for 24 hours. Again there is no obvious formation of either 5'hydroxymethyl flucloxacillin or penicilloic acid compounds.

These results indicate that flucloxacillin does not appear to be metabolised by HLM to 5'hydroxymethyl flucloxacillin. In contrast to CYP-expressing bactosomes (see below), there was also no indication that flucloxacillin was degraded to penicilloic acid.

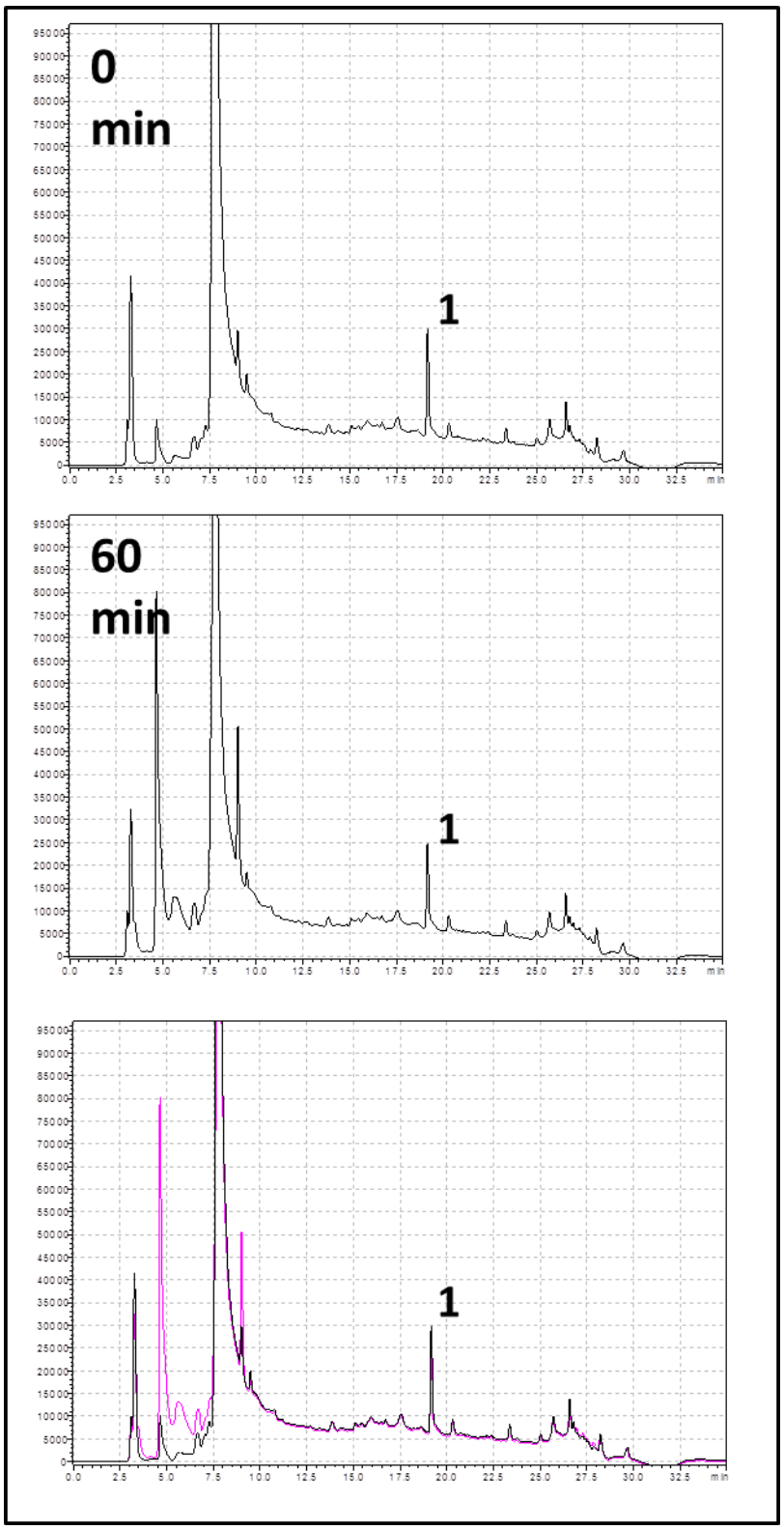

Figure 5.2 – Chromatograms showing the 60 minute incubation of flucloxacillin and Human Liver Microsomes.

Human Liver Microsomes were incubated with 500 µM flucloxacillin (1) in the presence of NADPH regenerating system for 60 minutes. Chromatograms for 0 and 60 minute time points are displayed. Overlaying of the time points show no formation of flucloxacillin metabolites.

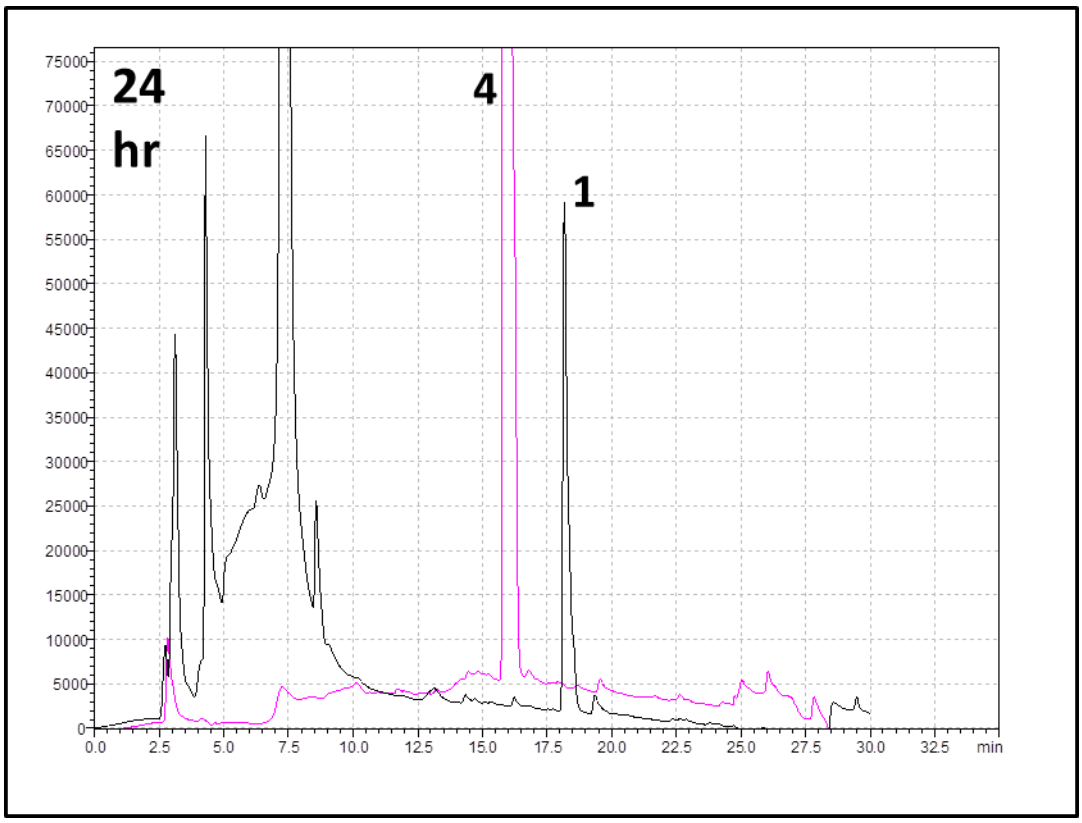

Figure 5.3 – Chromatogram showing the 24 hour incubation of flucloxacillin and Human Liver Microsomes.

Human Liver Microsomes were incubated with 1mM flucloxacillin (1) in the presence of NADPH regenerating system for 24 hours. A chromatogram showing the 24 hour incubation of HLM and flucloxacillin overlaid with a chromatogram (pink trace) for the 5'hydroxymethyl flucloxacillin control(4) shows that there was no formation of the 5'hydroxymethyl metabolite or of the penicilloic acid stereoisomers.

5.3.3 *Incubation of flucloxacillin with recombinant CYP-expressing bactosomes*

Flucloxacillin was incubated for 60 min with recombinant bactosomes each individually expressing the human cytochrome P450s CYP3A4, CYP2C8 and CYP2C9. It has been previously reported that CYP3A4 is responsible for the metabolism of flucloxacillin to its 5'hydroxymethyl metabolite. **Figure 5.4** shows chromatograms for the incubation of flucloxacillin with CYP3A4-expressing bactosomes. **Figure 5.4A** shows a chromatogram for CYP3A4-bactosomes alone. **Figure 5.4B** and **Figure 5.4C** show chromatograms for CYP3A4 bactosomes after the incubation with flucloxacillin for 60 minutes in the absence and presence of NADPH, respectively. In incubations with or without NADPH, there is the apparent production of two new compounds producing peaks at approximately 15.0 minutes (compound 2) and 16.5 minutes (compound 3). **Figure 5.4D** shows **Figure 5.4C** overlaid with a chromatogram of 5'hydroxymethyl flucloxacillin and flucloxacillin in 0.1 M sodium phosphate buffer (pH 7.4) and shows that the two products do not correspond with the retention time of the 5'hydroxymethyl flucloxacillin control.

Figure 5.5 shows chromatograms for flucloxacillin incubations with CYP2C8 and CYP2C9 expressing bactosomes. As with CYP3A4-bactosomes, there is production of two peaks after 60 minute incubations, showing that the production of these peaks isn't restricted to CYP3A4-expressing bactosomes. Similarly, these peaks were also produced in the absence of NADPH (data not shown).

Mass spectrometry was performed by Dr. Jeremy Palmer to aim to identify these two products. Results show that these products were the 5*R* and 5*S* stereoisomers of the ring-opened penicilloic acid derivatives of flucloxacillin. These products have previously been detected in *in vivo* studies of flucloxacillin metabolism (Everett *et al.*, 1989).

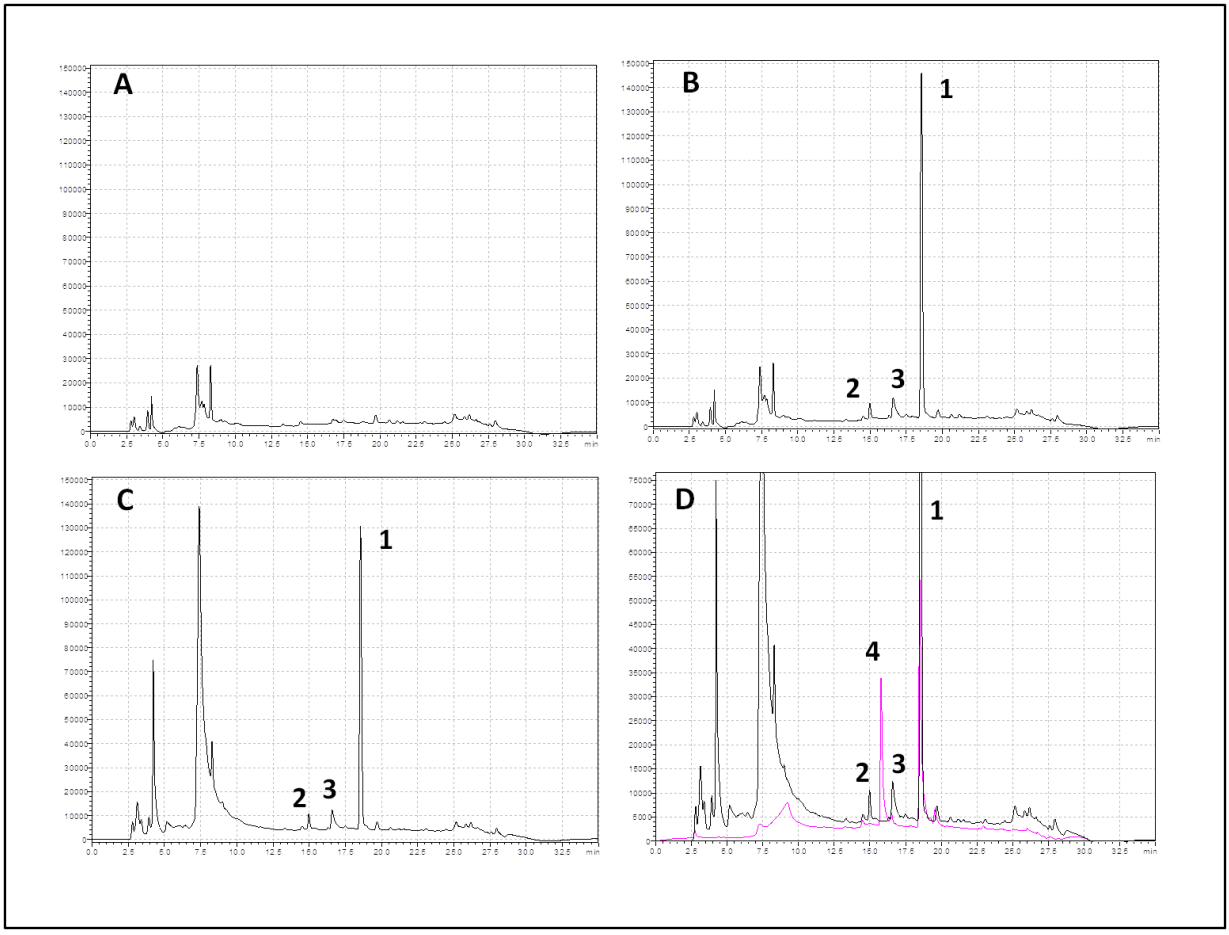

Figure 5.4 – Chromatograms showing the incubation of flucloxacillin and CYP3A4-expressing bactosomes

CYP3A4 bactosomes (A) were incubated with 500 µM flucloxacillin for 60 minutes in the absence (B) or presence (C) of NADPH-regenerating system. Chromatographs show flucloxacillin (1), and the formation of two unidentified products (2 + 3). Comparison with a chromatogram (pink trace) of 5'hydroxymethyl flucloxacillin control (4) show that these products are not the 5'hydroxymethyl metabolite.

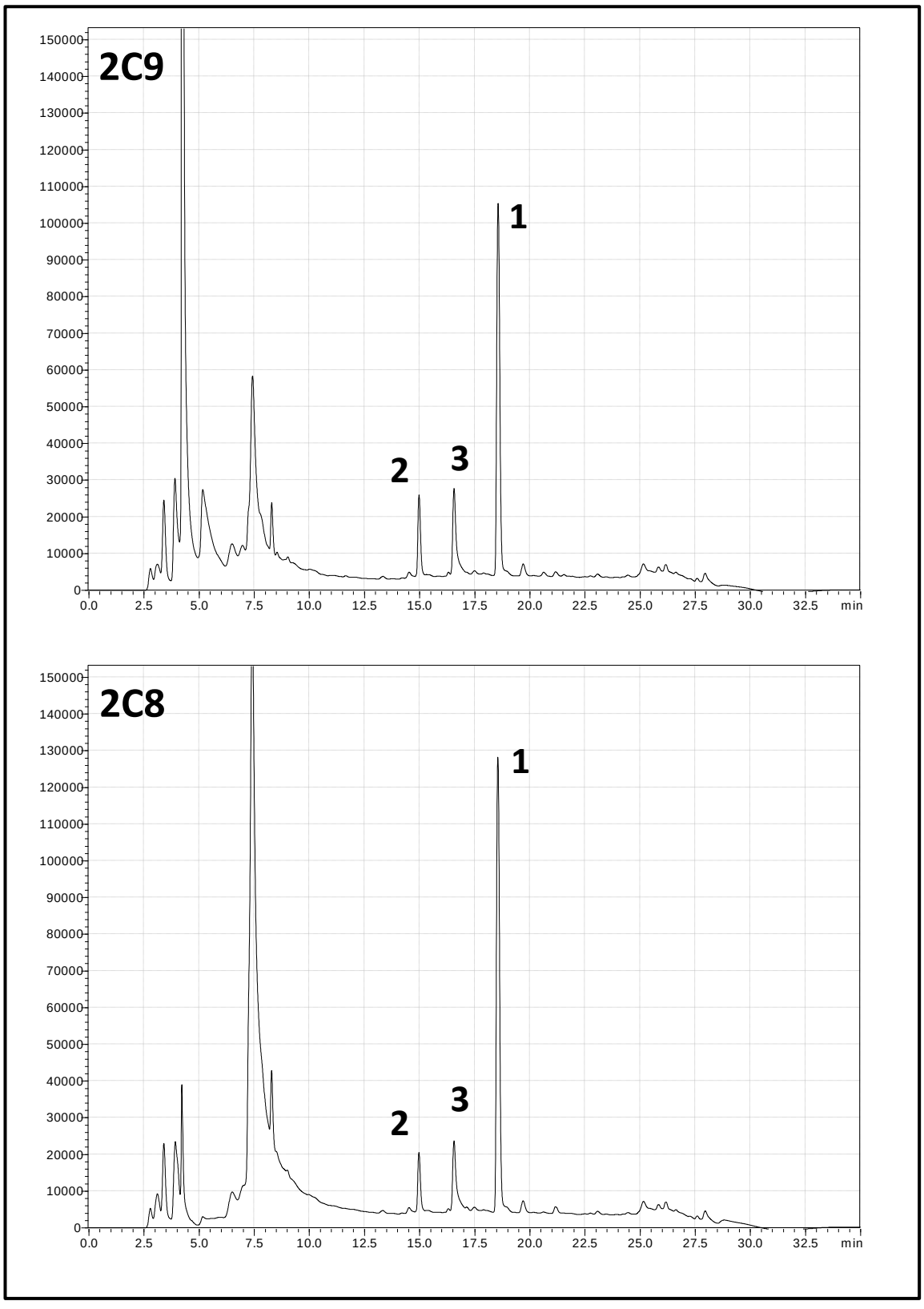

Figure 5.5 - Chromatograms showing the incubations of flucloxacillin and CYP2C9 and CYP2C8-expressing bactosomes

CYP2C9 and CYP2C8 bactosomes were incubated with 500 µM flucloxacillin for 60 minutes in the presence of NADPH-regenerating system. Chromatographs show flucloxacillin (1), and the formation of two unidentified products (2 + 3).

5.3.4 *Incubation of flucloxacillin with cyp3a1-induced B13/H cells*

5.3.4.1 Differentiation of B13 to B13/H cells and induction of cyp3a1

B13 cells were routinely cultured until at approximately 70% confluency then induced to differentiate to a B13/H phenotype with treatment of 10 nM dexamethasone for 14 days. **Figure 5.6** shows a photomicrograph image of the change in observed phenotype. Cells were observed to increase in size and cell proliferation decreased. B13/H cells were treated with 10 µM dexamethasone for 3 days to induce cyp3a enzymes. **Figure 5.7** shows relative cyp3a1 mRNA expression in B13/H cells compared to cyp3a1-B13/H as measured by qPCR. Cyp3a1 expression was undetectable in undifferentiated B13 cells. Treatment of 10 µM dexamethasone led to a highly significant 279.6 ± 11.3 fold induction of cyp3a1 mRNA expression ($p < 0.0001$).

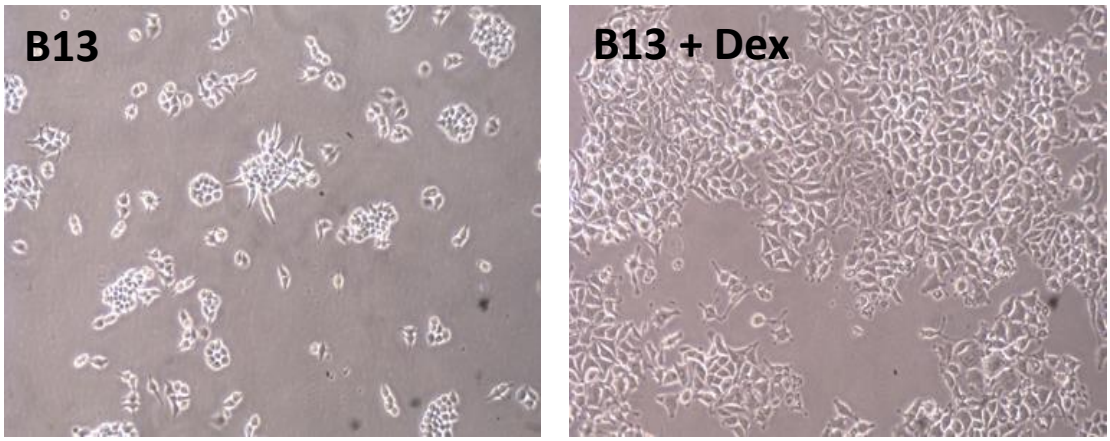

Figure 5.6 – Differentiation of B13 cells to B13/H cells with dexamethasone treatment

10 x magnification photomicrographs of B13 cells and differentiated B13/H cells after treatment with 10 nM dexamethasone treatment for 14 days

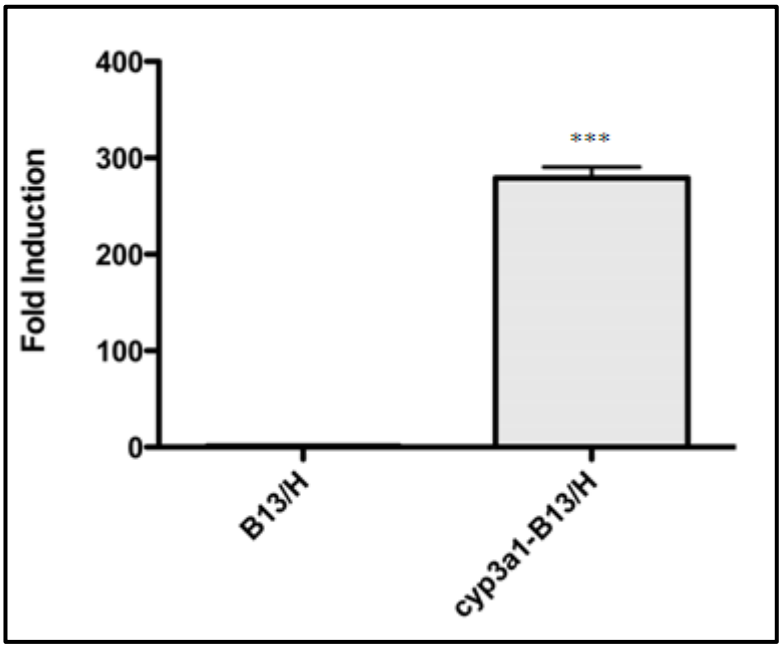

Figure 5.7 – Relative mRNA expression of cyp3a1 in B13/H cells and cyp3a1-induced B13/H cells by qPCR

*Expression of cyp3a1 was determined in B13/H cells and in B13/H cells induced for cyp3a1 following treatment with 10 µM dexamethasone. Expression of cyp3a1 in uninduced B13/H cells is normalised to 1 for comparison. Expression of cyp3a1 was not detected in undifferentiated B13 cells. Data represents mean ± S.E.M, n = 3, *** p < 0.0001, students t-tests were performed to compare differences between groups.*

5.3.4.2 Flucloxacillin incubation with cyp3a1-induced B13/H cells

Cyp3a1-induced B13/H cells were treated with 1 mM flucloxacillin for 2 hours or 24 hours. Cell media was extracted by solid phase extraction and applied to HPLC analysis. **Figure 5.8** shows chromatograms for cell extracts at 0, 2 and 24 hours and a chromatogram for flucloxacillin treated undifferentiated B13 cells for comparison.

As with preceding assays, there was no obvious evidence of 5'hydroxymethyl flucloxacillin production over a period of 24 hours. It appears there is rapid formation of products that are likely to be flucloxacillin penicilloic acid stereoisomers that remain relatively stable over 24 hours. Flucloxacillin appears to steadily decrease over a 24 hour period.

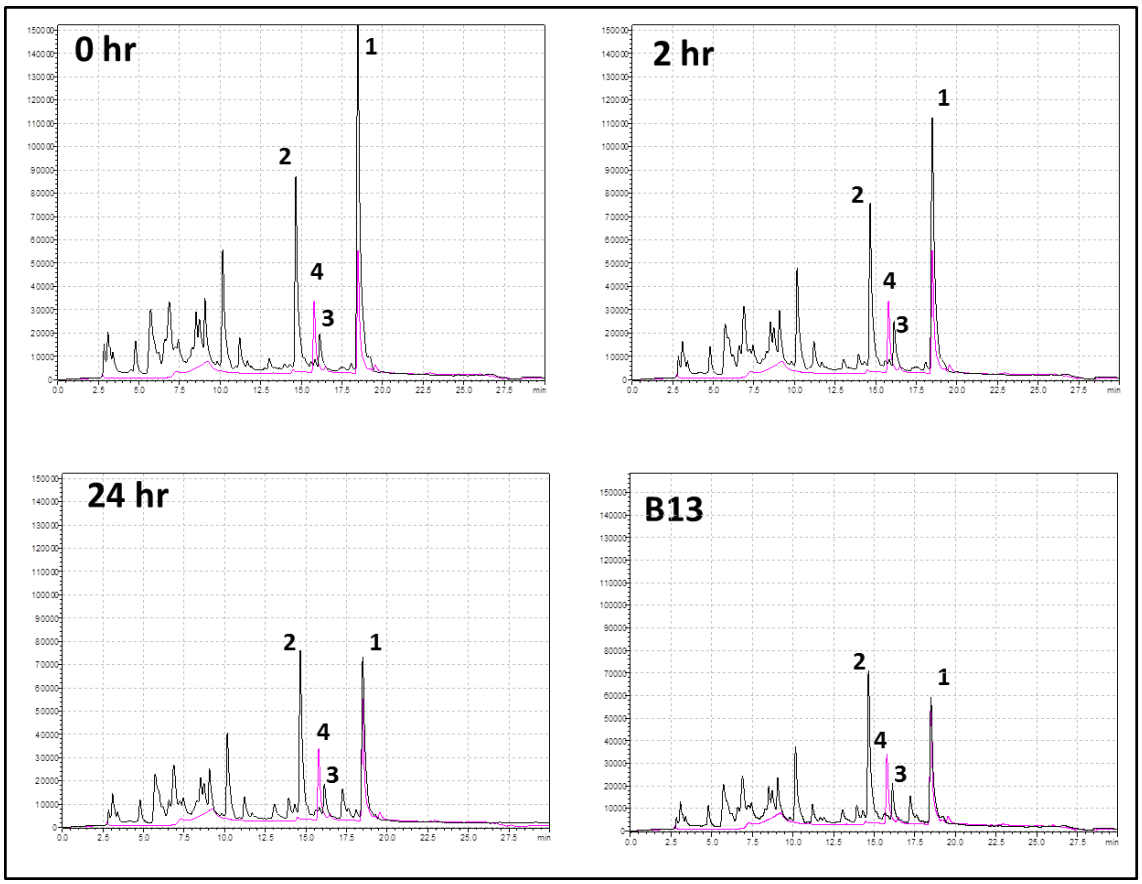

Figure 5.8 – Chromatograms showing the 0, 2 and 24 hour incubation of flucloxacillin and Cyp3a1-induced B13/H cells and a chromatogram for undifferentiated B13 cells after 24 hour incubation of flucloxacillin

Cyp3a1-induced B13/H cells were incubated with 1mM flucloxacillin (1) in the presence of NADPH regenerating system for 24 hours. Chromatograms for 0, 2 and 24 hour time points are displayed overlaid with chromatograms (pink trace) for the 5'hydroxymethyl flucloxacillin control. A chromatogram showing a 24 hour incubation of 1 mM flucloxacillinin undifferentiated B13 cells is also displayed. No evidence was 5'-hydroxymethyl flucloxacillin was apparent.

5.4 Discussion

After dosing, the majority of flucloxacillin is excreted in urine in unchanged form. Approximately 10% of the drug is metabolised *in vivo* by hydroxylation to 5'hydroxymethyl flucloxacillin and undergoes hydrolysis to penicilloic acid derivatives (Thijssen, 1980). Studies by Lakehal et al reported that 5'hydroxymethyl flucloxacillin was produced by human hepatocytes, CYP-induced rat liver microsomes and HLM and stated that this was mediated by CYP3A4 (Lakehal *et al.*, 2001). However, this has since been disputed by Huwyler and colleagues who did not find flucloxacillin to be a substrate for cytochrome P450 enzymes (Huwyler *et al.*, 2006). The aims of this chapter were to determine whether flucloxacillin was metabolised to 5'hydroxymethyl flucloxacillin using *in vitro* assays similar to those used in the previous studies

Flucloxacillin was incubated at various conditions with HLM, bactosomes prepared from *E. coli* expressing CYP3A4, CYP2C8 and CYP2C9, and cyp3a1-induced rat B13/H cells. There was no indication of 5'hydroxymethyl flucloxacillin production by any of these assays using HPLC analysis by the method previously described by Lakehal et al (Lakehal *et al.*, 2001). An advantage of the present studies over those performed by Lakehal et al was the use of a commercially developed 5'hydroxymethyl flucloxacillin standard. HPLC analysis of this standard showed a clear retention time that was sufficiently different to that of the parent drug, flucloxacillin. Unlike the studies performed in this chapter, Lakehal and colleagues reported to produce 5'hydroxymethyl flucloxacillin in large quantities through dexamethasone treated cyp3a-induced rat liver microsomes which was purified by semi-preparative HPLC. This compound was used as 5'hydroxymethyl flucloxacillin in subsequent experiments that showed its formation after flucloxacillin incubations with HLM and human hepatocytes. In the present study, data comparison of the 5'hydroxymethyl flucloxacillin standard to HPLC traces from flucloxacillin incubations with HLM for 60 minutes showed no indication of 5'hydroxymethyl flucloxacillin production as was claimed by Lakehal and colleagues. Incubations were also performed for 24 hours with no apparent flucloxacillin metabolism.

Flucloxacillin metabolism was also studied in recombinant CYP-expressing bactosomes. Bactosomes are bacterial membranes prepared from *E.coli* expressing individual CYP isoforms in large quantities. Since CYP3A4 has been reported as

mediating the production of 5'hydroxymethyl flucloxacillin, a CYP3A4 expressing bactosome was studied along with CYP2C8 and CYP2C9 as controls. No indication of 5'hydroxymethyl flucloxacillin formation was observed in CYP3A4 bactosomes or CYP2C8 and CYP2C9 bactosomes with comparison to the 5'hydroxymethyl flucloxacillin standard. However, two new products were produced that were found not to be dependent on NADPH, and were found in all bactosome preparations. Mass spectrometry performed on these compounds determined that they were 5R and 5S diastereoisomers of flucloxacillin penicilloic acid. These stereoisomers have previously been observed in the urine of rats after high dose administration of flucloxacillin by ^{19}Fluorine NMR techniques (Everett *et al.*, 1989) (**Figure 5.9**).

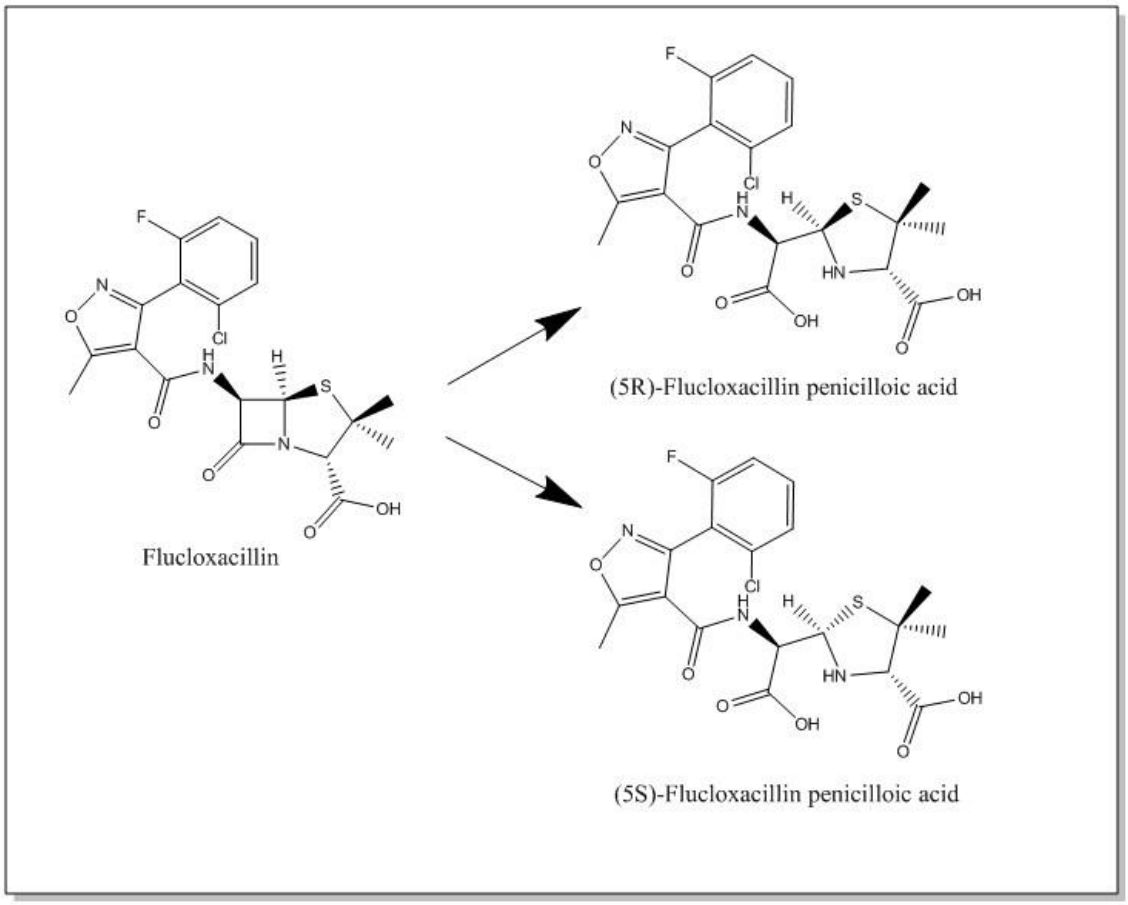

Figure 5.9 – Structures of flucloxacillin and the penicilloic acid diastereoisomers (5R)-flucloxacillin penicilloic acid and (5S)-flucloxacillin penicilloic acid

The two products detected by flucloxacillin incubations with cytochrome-P450 expressing bactosomes were identified by mass spectrometry as 5R and 5S diastereoisomers of penicilloic acid.

Interestingly, Lakehal and colleagues reported additional compounds in the medium of hepatocytes after flucloxacillin treatment that they state could possibly be penicilloic acid derivatives. They also state that they cannot rule out the possibility that these compounds contribute to the toxicity observed when media from flucloxacillin treated hepatocytes was incubated with BEC preparations. An interesting observation also from the Lakehal paper is that they briefly mention that the compound identified as 5'hydroxymethyl flucloxacillin was predominantly produced in dexamethasone cyp3a-induced rat liver microsomes, but was also produced from liver microsomes from methylcholanthrene (cyp1a inducer) and phenobarbital (cyp2b/3a inducer) treated rats. This could of course result from cyp3a activity in rat liver microsomes but may also suggest that production of 5'hydroxymethyl flucloxacillin was not cyp3a-specific.

Flucloxacillin metabolism was also studied in cells to examine the mechanism in an *in vitro* model of a cellular system. The rat pancreatic cell line, B13, that upon simple glucocorticoid treatment differentiates to display a hepatic phenotype, has allowed these cells to be used as a source of hepatocytes acting as a progenitor cell line for expandable functional hepatocytes (Fairhall *et al.*, 2013). B13/H cells have been shown to express functional cytochrome P450 enzymes with activity comparable to hepatocytes (Marek *et al.*, 2003). Key advantages for using B13/H cells over hepatocytes, however, include significant cost-effectiveness, and the characteristic that they remain differentiated and maintain cytochrome P450 activity whereas hepatocytes lost substantial hepatic functions within a few days of culture (Wallace *et al.*, 2010). Like hepatocyte preparations, B13/H cells also respond to treatment with cytochrome P450 inducers. In this study, B13/H cells were induced for cyp3a enzymes by treatment with dexamethasone. Incubation of flucloxacillin with B13/H cells show no obvious formation of 5'hydroxymethyl flucloxacillin. Detection of flucloxacillin penicilloic compounds occurred rapidly suggesting that the hydrolysis of flucloxacillin is a rapid procedure and levels of the penicilloic acid stereoisomers remained relatively steady over 24 hours.

The findings presented in this chapter have indicated the detection of flucloxacillin penicilloic acid in microsomal and cellular assays and not 5'hydroxymethyl flucloxacillin. 5'hydroxymethyl flucloxacillin has previously been suggested as the product of oxidative metabolism and as a potential reactive metabolite that has a role in flucloxacillin-DILI. That no evidence was found of its production *in vitro* in these assays suggests that it is either not a product of oxidative metabolism or that it is

produced in small quantities undetectable by the HPLC analysis performed. Diastereoisomers of flucloxacillin penicilloic acid were detected in the HPLC analysis. These products did not appear to be a result of oxidative metabolism and have previously been suggested to form spontaneously. The opening of the penicillin β-lactam ring creates a penicilloyl moiety that can bind to endogenous proteins forming neoantigens. Such reactions have been suggested as the major determinant of penicillin allergy (Baldo, 1999). Flucloxacillin has been shown to bind to human serum albumin *in vitro* and *in vivo* by ring opening and binding to numerous lysine residues (Jenkins *et al.*, 2009). Flucloxacillin adducts in livers of treated rats have also been detected (Carey and van Pelt, 2005). Findings from this study suggest that penicilloic acid derivatives of flucloxacillin are formed rapidly in rat cells and are relatively stable over time. This was not shown directly for human liver microsomes but it seems likely that the findings for B13 cells will also extrapolate to humans. It seems reasonable to hypothesise that the presence of stable flucloxacillin penicilloic acid may lead to the formation of haptens with cellular proteins in the liver that could contribute to immune activation via HLA presentation and liver toxicity in susceptible individuals. The possibility that 5'hydroxymethyl flucloxacillin is produced at very low levels cannot be discounted and it remains possible that its production could be a factor in DILI. However, the current studies have been unable to confirm that it is a major metabolite as suggested by Lakehal et al. (2001) and are generally consistent with the more recent report from Huwyler et al. (2006). To explore further the possibility of very low levels of 5'hydroxymethylflucloxacillin metabolite production, more sensitive detection using LC-MS would be required.

In summary,

- A HPLC method was employed that allowed the identification of both flucloxacillin, 5’-hydroxymethyl flucloxacillin and flucloxacillin penicilloic acid stereoisomers
- 5’-hydroxymethyl flucloxacillin was not found to be a product of flucloxacillin metabolism in human liver microsomes, CYP3A4 expressing bactosomes or in cyp3a induced B13/H cells
- Formation of flucloxacillin penicilloic acid stereoisomers was observed in cytochrome P450 bactosome preparations, that was independent of NADPH presence indicating that it likely not cytochrome P450 mediated

- A more sensitive LC/MS method of detection could help detect any very low levels of flucloxacillin metabolites produced.

Chapter 6. *Ex vivo* Stimulation of Peripheral Mononuclear Blood Cells by Flucloxacillin

6.1 Introduction

The covalent modification of cellular proteins followed by presentation to circulating T cells by HLA molecules on the cell surface is proposed as a likely mechanism of immune-mediated DILI reactions. Since the discovery of the strong association of HLA-B*57:01 genotype and increased risk to flucloxacillin-DILI, there have been a number of studies that have provided evidence that T-cell mediated mechanisms are involved in the pathogenesis of this disease.

Before the association between HLA-B*57:01 and flucloxacillin-DILI had been discovered by GWAS, an association with HLA-B*57:01 genotype had already been established as a risk factor for a hypersensitivity reaction to the anti-retroviral drug, abacavir (Hetherington *et al.*, 2002; Mallal *et al.*, 2002). The association of HLA-B*57:01 with abacavir hypersensitivity syndrome is one of the strongest HLA-drug associations currently understood, with an estimated 2 – 8 % of those exposed to abacavir developing an adverse reaction (Cutrell *et al.*, 2004). This is in contrast to flucloxacillin-DILI where even with B*57:01 possession, disease manifestation remains rare, with approximately between 1 in 500 and 1 in 1000 individuals with HLA-B*57:01 developing disease when exposed to flucloxacillin (Daly *et al.*, 2009). The reasons why these two structurally distinct compounds should both interact with HLA-B*57:01, to initiate adverse immune responses, as well as why abacavir produces a much more frequent adverse reaction remain poorly understood.

Some insights into the mechanism of abacavir hypersensitivity were provided by Chessman et al who showed that abacavir could stimulate peripheral blood mononuclear cells (PBMCs) from abacavir hypersensitivity patients *in vitro* to secrete inflammatory cytokines including interferon-gamma (IFN-γ). T-cell stimulation was abacavir-specific and only occurred in individuals possessing HLA-B*57:01. This was possible in abacavir naïve HLA-B*57:01 positive volunteers, in addition to abacavir hypersensitivity HLA-B*57:01 positive patients (Chessman *et al.*, 2008). Notable also was the observation that cytokine secretion was mediated by T-cells expressing the CD8 co-receptor, not CD4+ T-cells. CD8+ T-cells recognise antigen peptides presented to HLA class I molecules leading to their activation and proliferation.

CD8+ T-cells are primarily known for their direct cytotoxic activity through the release of cytolytic molecules like perforin, Fas ligand and granzyme B (Uetrecht and Naisbitt,

2013). However, some CD8+ T-cell subsets can also mediate a cytokine secretion pattern similar to that of CD4+ T-helper 1 (Th1) cells such as secretion of IFN-γ (Mosmann *et al.*, 1997). IFN-γ co-ordinates a multitude of cellular immune responses via transcriptional regulation of immune genes and is also produced by Natural Killer (NK) cells, B-cells and dendritic cells (Billiau and Matthys, 2009). Some of IFN-γ's effects include the recruitment and activation of immune cells, stimulation of Th1-like effector mechanisms and the secretion of inflammatory cytokines. IFN-γ also affects the HLA class I antigen presentation pathway, up-regulating cell surface expression of HLA molecules and altering the manner of peptide binding further increasing T-cell recognition of antigens (Schroder *et al.*, 2004).

Similarly to abacavir, recent studies have been reported on PBMCs from flucloxacillin-DILI patients that show evidence for a T-cell mediated reaction. Lymphocyte transformation tests (LTT) can be beneficial for diagnosing whether a patient has developed a T-cell response to a specific drug although its value as a reliable tool has been questioned (Pichler and Tilch, 2004). LTTs measure T-cell proliferation to drug treatment *in vitro* as a means of determining whether an individual is 'sensitised' to a particular drug. LTTs have been performed on PBMCs from flucloxacillin-DILI patients with mixed results (Maria and Victorino, 1997; Monshi *et al.*, 2013). In the study by Maria and Victorino, PBMCs from a flucloxacillin-DILI patient, taken 3 months after disease onset, were found to be proliferate in response to flucloxacillin stimulation, an observation that was repeated a further 3 months from the first study (Maria and Victorino, 1997). More recently, Monshi et al were unable to stimulate PBMCs from flucloxacillin-DILI patients to proliferate by LTT (Monshi *et al.*, 2013). However, they were able to detect PBMC responses by measuring IFN-**γ** secretion by ELISpot assay in flucloxacillin-DILI patients but not in flucloxacillin tolerant or naïve HLA-B*57:01 positive donors. Further characterisation of PBMC responses by generation of flucloxacillin-responsive T-cell clones showed that they were predominantly CD8+ in nature, similar to abacavir-specific T-cell clones. In contrast, Wuillemin et al generated CD8+ flucloxacillin-specific T-cells from both HLA-B*57:01 positive and negative healthy donors, that were IFN-**γ** secreting and cytotoxic, after repeated rounds of stimulation (Wuillemin *et al.*, 2013) (**Figure 6.1**) .

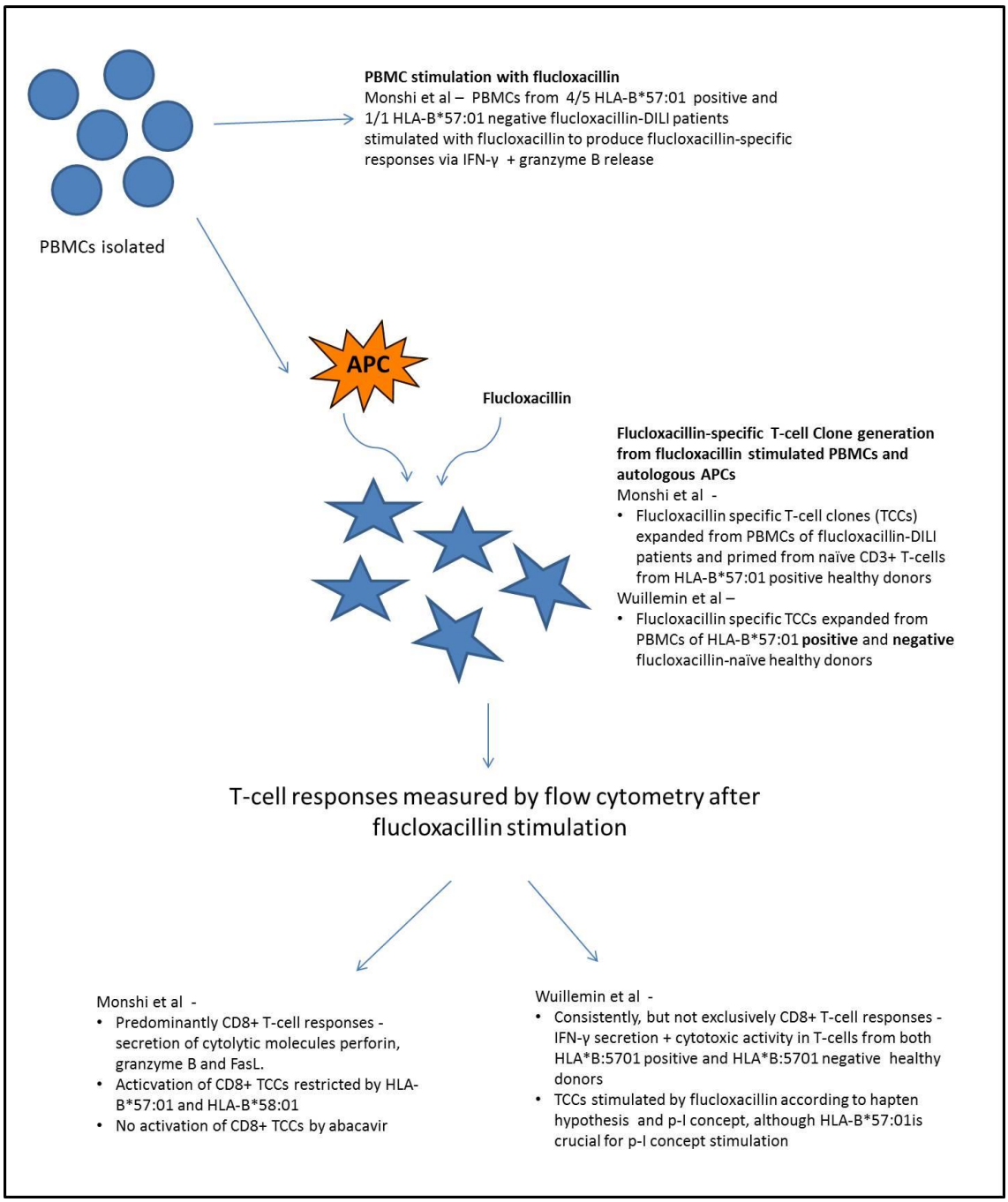

Figure 6.1 – An overview of studies performed into flucloxacillin stimulation of peripheral-blood mononuclear cells (PBMCs) and generation of flucloxacillin-specific T-cell Clones (TCCs)

In studies performed by Monshi et al, PBMCs were directly stimulated with flucloxacillin in 5/6 flucloxacillin-DILI patients but not in PBMCs from healthy donors. Both Monshi and Wuillemin generated flucloxacillin-specific TCCs from PBMC stimulation by flucloxacillin, co-cultured with autologous antigen presenting cells (APCs) and IL-2. Further stimulation of these TCCs by both groups resulted in T-cell responses that were predominantly but not exclusively CD8+ in nature.

In this present chapter, PBMCs were extracted from the blood of flucloxacillin-DILI patients and healthy HLA-B*57:01 positive and negative donors to investigate whether PBMCs can be stimulated by flucloxacillin to up-regulate IFN-γ gene expression. Interleukin-8 (IL-8) expression will also be investigated in flucloxacillin treated PBMCs as a candidate for studying chemokine expression. IL-8 was chosen based on preliminary findings by Matthew Pletcher and colleagues (Pletcher M, personal communication to A. Daly and (Pletcher *et al.*, 2011). These studies involved stimulating donor PBMCs that were positive or negative for various HLA alleles with their corresponding reactive drug (Pletcher *et al.*, 2011). The drug-associations studied included HLA-B*57:01 with abacavir and flucloxacillin and HLA-B*15:02 with carbamazepine. The group studied expression of 27 genes coding for cytokines and chemokines, including IL-8, by stimulation with the particular drug of PBMCs from donors expressing the reactive HLA haplotype. Some changes in gene expression including in IL-8 were reported. IL-8 (also known as CXCL8) is a potent neutrophil attracting chemokine (Baggiolini, 2001). Drug-specific T-cells can produce IL-8 in large amounts to activate neutrophil recruitment and inflammation. In relation to DILI, neutrophils are thought to play a role in halothane-induced liver injury in a mouse model (You *et al.*, 2006). With regard to flucloxacillin-specific T-cells, it is interesting to note that T-cell clones generated from a patient with flucloxacillin-induced interstitial nephritis were able to be stimulated to produce considerable amounts of IL-8 when treated with flucloxacillin (Spanou *et al.*, 2006). More recently, Catherine Bell at Liverpool University, observed that flucloxacillin treatment of PBMCs from HLA-B*57:01 positive and negative healthy volunteers affected the expression of several genes including the upregulation of IL-8 in both HLA-B*57:01 positive and negative donors (Bell, 2012).

The aims of the studies described in the current chapter were to explore flucloxacillin stimulation of cytokine expression in PBMCs to test whether previously reactive T-cells from HLA-B*57:01 positive flucloxacillin-DILI patients can be stimulated and to explore whether PBMCs from HLA-B*57:01 positive flucloxacillin naïve donors can be stimulated compared to a HLA-B*57:01 negative flucloxacillin naïve donor (**Figure 6.2**). In line with the report from Pletcher and colleagues, a simple mRNA based assay of measuring cytokine expression by real-time PCR was used to ascertain its suitability as a method of detecting cytokine stimulation by flucloxacillin.

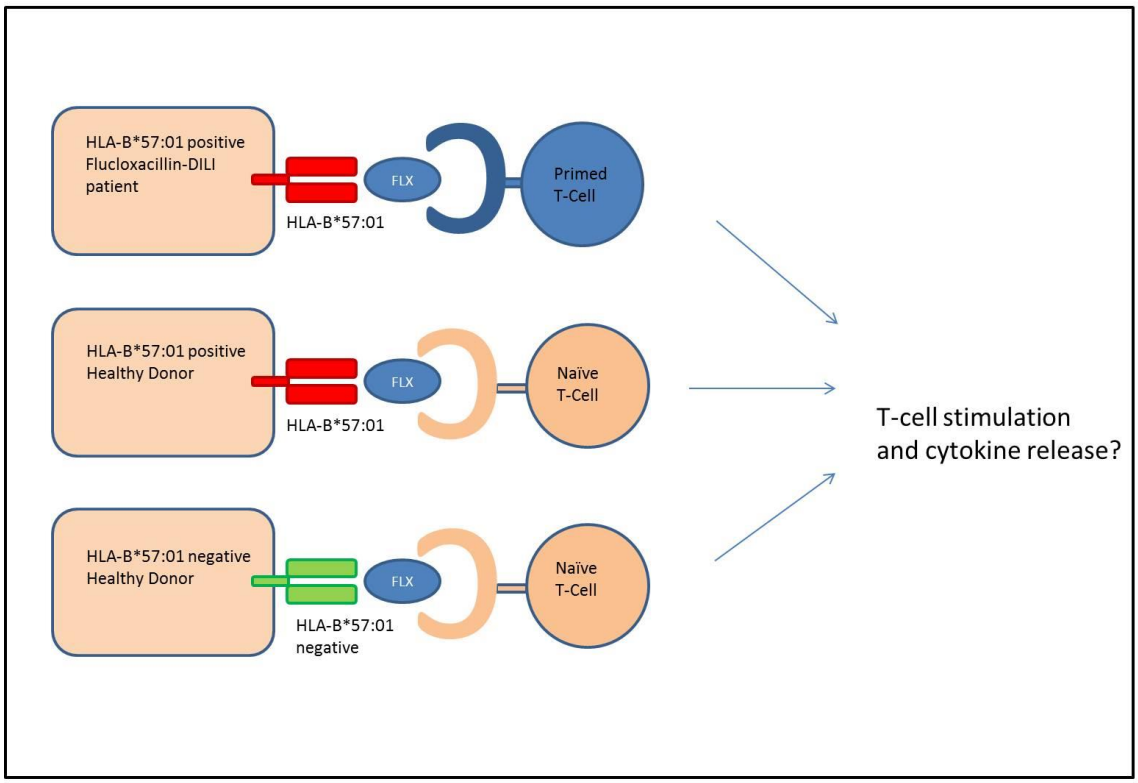

Figure 6.2 – Flucloxacillin stimulation of T-cells from HLA-B*57:01 positive flucloxacillin-DILI patients and HLA-B*57:01 positive and negative flucloxacillin-naïve healthy donors

*Overview of the studies performed in this chapter investigating whether flucloxacillin can stimulate T-cells from HLA-B*57:01 positive flucloxacillin-DILI patients that are hypothesised to be previously sensitised to the drug and whether flucloxacillin can stimulate T-cells from flucloxacillin-naïve healthy individuals with either HLA-B*57:01 positive or negative genotype.*

6.2 Methods

6.2.1 *Flucloxacillin-DILI patients and healthy donors*

20 ml blood samples from three confirmed HLA-B*57:01 positive flucloxacillin-DILI patients were collected as well as samples from one HLA-B*57:01 negative flucloxacillin naïve and two HLA-B*57:01 positive flucloxacillin naïve healthy volunteers. Blood was collected in EDTA-coated blood collection tubes and used immediately for PBMC isolation.

6.2.2 *Isolation of PBMCs from whole blood*

PBMCs were freshly isolated from whole blood using Lymphoprep™ solution (Axis Shield, Norway). Blood samples were transferred to sterile 50 ml centrifuge tubes and diluted 1:1 with sterile PBS (Lonza). In a separate step, 15 ml Lymphoprep™ was added to a sterile 50 ml LeucoSep™ centrifuge tube (Greiner Bio-One) and centrifuged at 400 g for 5 min at room temperature to partition the Lymphoprep™ beneath the porous barrier. The diluted blood was added to the Lymphoprep containing LeucoSep tube on top of the barrier and centrifuged at 800 g for 20 min at room temperature after which lymphocytes form a distinct band at the sample/medium interface. Lymphocytes were removed from the interface using a sterile Pasteur pipette and transferred to a fresh sterile 50 ml centrifuge tube. PBS was added to make a total volume of 10 ml and the cells pelleted by centrifugation at 600 g for 10 min at room temperature. PBS was removed and replaced with 10 ml fresh PBS to wash the pellet before a further centrifugation step at 600 g for 5 min. This wash spin step was repeated and then the cell pellet was resuspended in 10 ml sterile RPMI-1640 medium (Lonza) supplemented with 10% foetal calf serum (FCS), 2 mM L-Glutamine, 20 U/ml penicillin and 20 µg/ml streptomycin.

6.2.3 *Cell Treatments*

PBMCs (2 x 10^6/well) were seeded in a volume of 2 ml sterile complete RPMI-1640 medium in 24 well plates and cultured with flucloxacillin (250 µM or 500 µM), abacavir (25 µM) or protein purified derivative of tuberculin (PPD, 2 µg/ml) as a positive control. Negative control wells contained PBMCs only. After 24 hours, cell suspensions were transferred to sterile 1.5 ml microfuge tubes and the cells pelleted by centrifugation. The supernatant was removed and discarded and the cell pellet washed with fresh sterile PBS. Total RNA was isolated from cells as described in section 2.3.2.1.

6.2.4 *Measurement of IFNγ and IL-8 gene expression by real-time PCR*

cDNA was synthesised from RNA as described in section 2.7.1. TaqMan Gene Expression Assays were purchased from Applied Biosystems, UK for interferon-gamma (assay I.D. - Hs00989291_m1) and interleukin-8 (assay I.D. - Hs00174103_m1). GAPDH (assay I.D. - Hs99999905_m1) was used as a control to normalise for any differences in sample RNA added in each reaction. Assays that spanned exon boundaries were chosen so to prevent amplification of genomic DNA. Each assay contains forward and reverse primers (at final concentrations of 900 nM) and a TaqMan probe (250 nM) containing a fluorescent reporter dye 6-FAM (6-carboxyfluorescein) at the 5' end and a MGB (dihydrocyclopyrroloindole tripeptide minor groove binder) non-fluorescent quencher at the 3' end of the probe. qPCR reactions were performed as outlined in section 2.6.2.2.

6.2.5 *Statistical Analysis*

Gene expression was calculated by the ΔΔCt method of analysis as described in section 2.6.2 and is expressed as fold induction relative to the untreated control. Fold induction of gene expression is displayed as the mean ± S.D. of triplicate experiments. Comparisons of gene expression between individual treatment groups and the untreated control group were analysed by performing unpaired two-tailed students t-tests using GraphPad Prism 3.0 software.

6.3 Results

6.3.1 *Interferon-γ and interleukin-8 expression in flucloxacillin-naïve HLA-B*57:01 negative and HLA-B*57:01 positive healthy donors*

PBMC's from one HLA-B*57:01 negative flucloxacillin-naïve healthy donor and two HLA-B*57:01 positive flucloxacillin-naïve healthy donors were stimulated with two concentrations of flucloxacillin (250 µM and 500 µM) or abacavir (25 µM) for 24 hours. *M. tuberculosis* purified protein derivative (PPD) was used as a positive control. The effect of flucloxacillin and abacavir treatment on mRNA expression of interferon- γ (IFNγ) and interleukin-8 (IL-8) was evaluated by quantitative real-time PCR (qPCR) and is expressed as fold induction relative to an untreated control.

Significant increases in IFNγ expression were observed in response to treatment with the positive control, PPD, in the HLA-B*57:01 negative and HLA-B*57:01 positive flucloxacillin-naïve healthy donors. However, IFNγ expression was not observed to be affected by flucloxacillin or abacavir treatment in either the HLA-B*57:01 negative healthy donor or the two HLA-B*57:01 positive donors (**Figure 6.3**).

Figure 6.3 also shows IL-8 expression in the HLA-B*57:01 negative flucloxacillin-naïve healthy donor and two HLA-B*57:01 positive flucloxacillin-naïve healthy donors. PPD produced a significant stimulation of IL-8 expression in the PBMCs from all healthy volunteers. Flucloxacillin treatment did not affect IL-8 expression in HLA-B*57:01 negative or positive flucloxacillin-naïve healthy donors. Abacavir treatment appeared to increase IL-8 expression in HLA-B*57:01 negative and positive donors although this was only found to be significant in HLA-B*57:01 positive individual A with a 2.64 ± 0.16 (p = 0.0014) fold increase in IL-8 expression.

In summary, flucloxacillin treatment did not appear to affect the mRNA expression of IFNγ or IL-8 in PBMCs from HLA-B*57:01 negative or HLA-B*57:01 positive flucloxacillin-naïve healthy donors. Abacavir treatment did not appear to affect IFNγ expression in healthy donors irrespective of HLA-B*57:01 status, but did cause an increase in IL-8 expression in one of the two HLA-B*57:01 positive healthy donors.

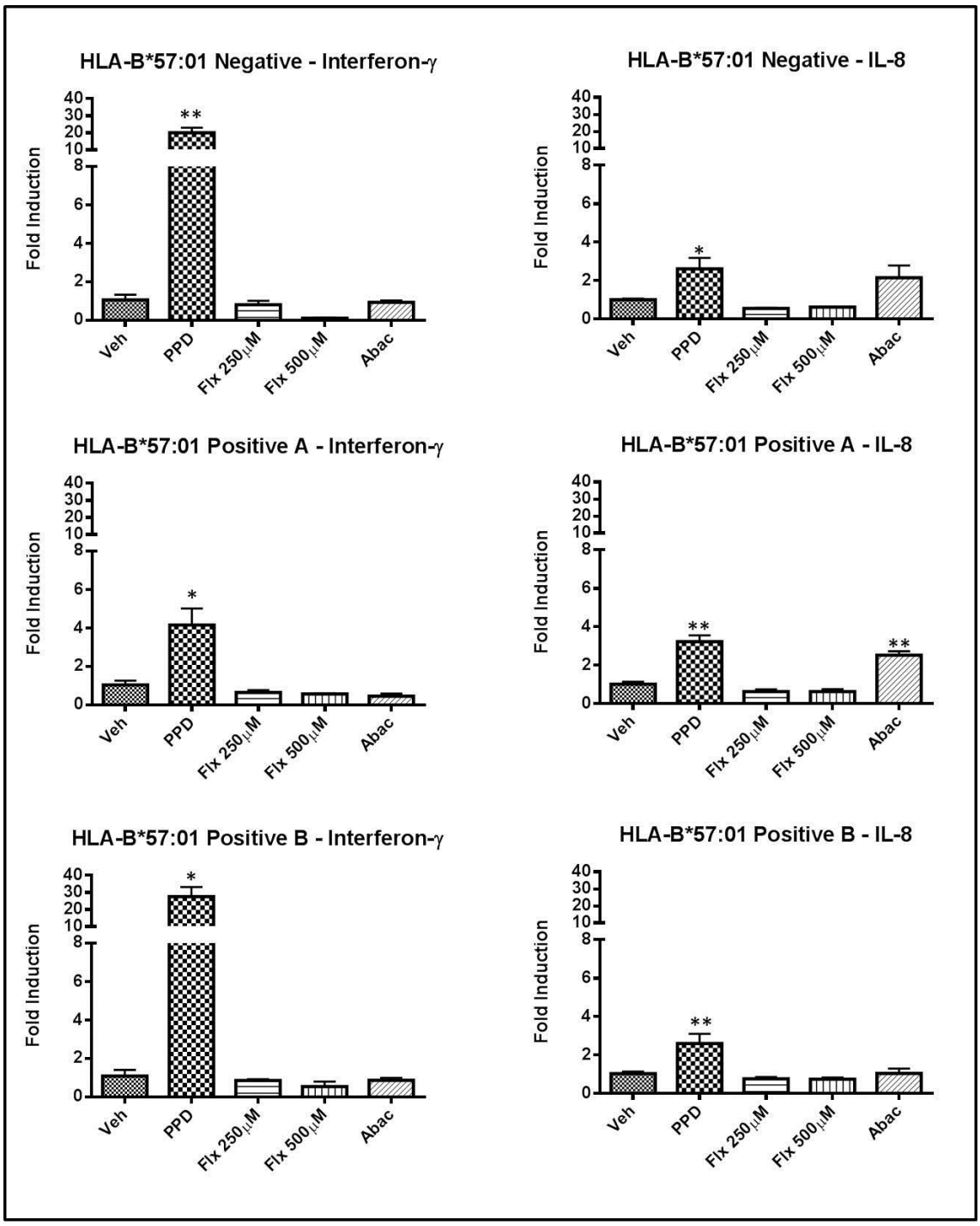

Figure 6.3 - Interferon-γ and IL-8 expression in PBMCs from a HLA-B*57:01 negative flucloxacillin naïve healthy donor and two HLA-B*57:01 positive flucloxacillin naïve healthy donors

*Isolated PBMCs were treated for 24 hours with flucloxacillin (250 µM or 500 µM), abacavir (25 µM) or PPD (2 µg/ml) as a positive control. IFNγ and IL-8 mRNA expression was measured by qPCR and is expressed as fold induction relative to untreated samples. Results are shown as the means ± S.E.M of triplicate experiments. Statistical significance was determined by t-test, * $p < 0.05$, ** $p < 0.01$*

6.3.2 *Interferon-γ and interleukin-8 expression in HLA-B*57:01 positive flucloxacillin-DILI patients*

IFNγ and IL-8 expression was also measured by qPCR in PBMCs from three HLA-B*57:01 positive flucloxacillin-DILI patients stimulated with two concentrations of flucloxacillin (250 µM and 500 µM), abacavir (25 µM) or purified protein derivative (PPD) as a positive control for 24 hours.

Figure 6.4 shows IFNγ expression in response to treatment. Flucloxacillin treatment produced a significant increase in IFNγ expression in two of the three of patients at both tested concentrations. Flucloxacillin produced a significant increase in IFNγ expression in Patient A, at 250 µM with a 3.40 ± 0.39 (p = 0.0039) fold expression increase and at 500 µM, with a 4.78 ± 0.20 (p < 0.0001) fold expression increase. No effect on IFNγ expression was observed by abacavir treatment. In Patient B, flucloxacillin produced a similar increase in IFNγ expression at both 250 µM and 500 µM concentrations. At 250 µM, a 1.84 ± 0.28 (p = 0.0465) fold increase in expression was observed and at 500 µM, a 1.80 ± 0.18 (p = 0.0157) fold increase was observed. As with Patient A, no effect on IFNγ expression was observed with abacavir treatment. In Patient C, there were no significant differences in IFNγ expression due to flucloxacillin treatment at either concentration in Patient C, and as in Patients A and B, abacavir had no effect on IFNγ expression.

Figure 6.4 also shows IL-8 expression in the treated PBMCs from the three HLA-B*57:01 positive flucloxacillin-DILI patients. Flucloxacillin produced an increase in IL-8 expression in the PBMCs of 1/3 flucloxacillin-DILI patients. In Patient A, neither flucloxacillin nor abacavir had any effect on IL-8 expression. In Patient B, flucloxacillin treatment at both 250 µM and 500 µM concentrations produced a significant increase in IL-8 expression with a larger increase observed at 250 µM with a 5.88 ± 0.94 (p = 0.0093) fold increase compared to a 4.78 ± 0.58 (p = 0.0063) fold increase observed at 500 µM. Abacavir treatment appeared to produce an increase in IL-8 expression in Patient B, but this was not found to be statistically significant. In Patient C, no effect on IL-8 expression was observed with flucloxacillin treatment at either concentration although a significant fold increase of 1.55 ± 0.09 (p = 0.0382) was observed with abacavir treatment.

In summary, flucloxacillin treatment led to an increase in IFNγ expression in two of the three of flucloxacillin-DILI patients (Patients A and B) at the two concentrations tested and in one of these patients (Patient B) a significant increase in IL-8 expression was also detected. IL-8 expression was not affected by flucloxacillin treatment in the two other flucloxacillin-DILI patients (Patients A and C). Abacavir treatment did not cause a change in IFNγ expression in any of the flucloxacillin-DILI patients but did appear to lead to an increase in IL-8 expression in two of the patients, although this was only found to be significant in Patient C.

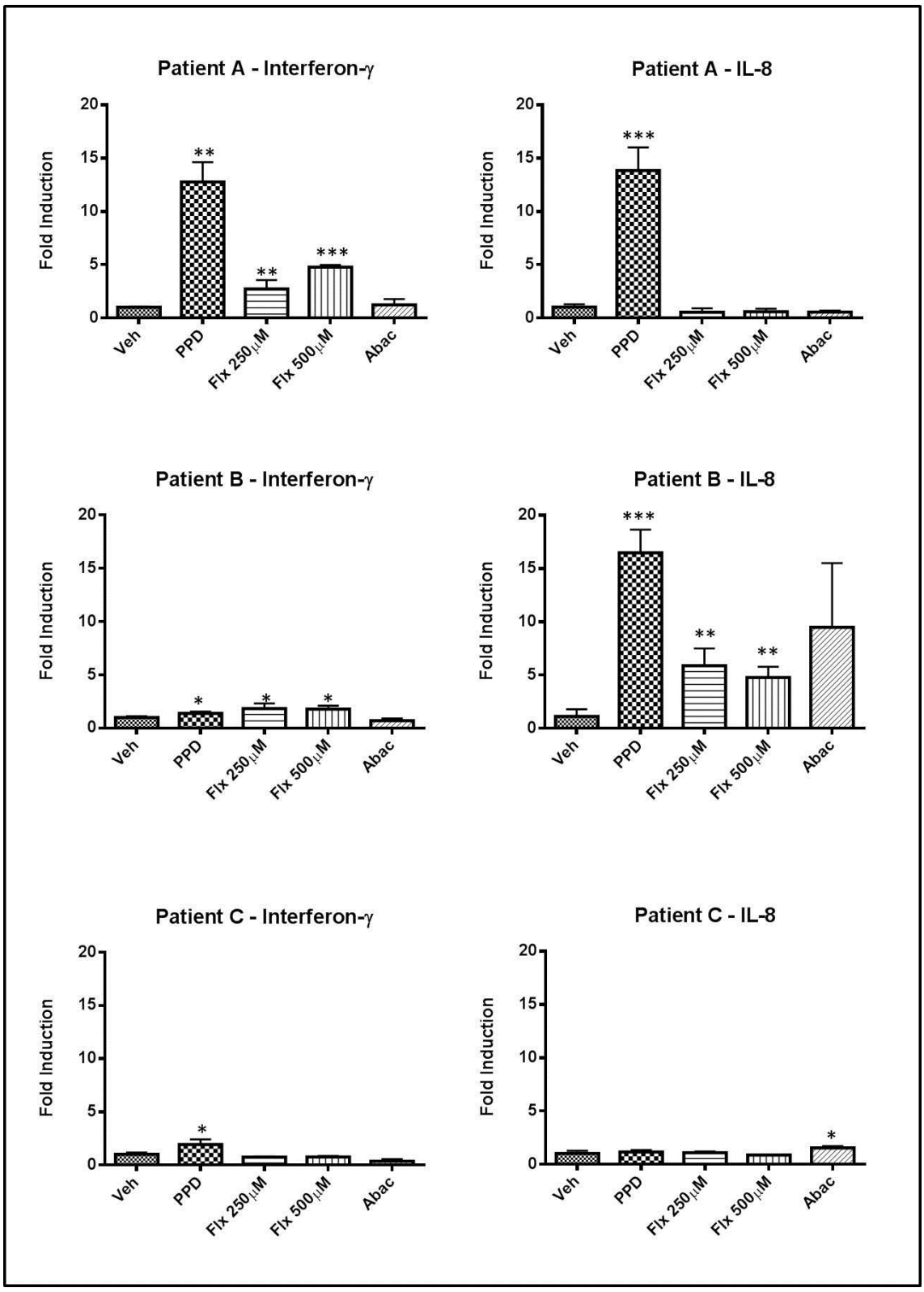

Figure 6.4 – Interferon-γ and Interleukin-8 (IL-8) expression in treated PBMCs from three HLA-B*57:01 positive flucloxacillin-DILI patients.

*Isolated PBMCs were treated for 24 hours with flucloxacillin (250 µM or 500 µM), abacavir (25 µM) or PPD (2 µg/ml) as a positive control. IFNγ and IL-8 mRNA expression was measured by qPCR and is expressed as fold induction relative to untreated samples. Results are shown as the means ± S.E.M of triplicate experiments. Statistical significance was determined by t-test, * $p < 0.05$, ** $p < 0.01$*

6.4 Discussion

Recent studies have provided evidence of a T-cell mediated mechanism in flucloxacillin-induced liver injury. Flucloxacillin-specific T-cell clones have been expanded from the PBMCs of flucloxacillin-DILI patients (Monshi *et al.*, 2013). These were shown to be predominantly CD8+ cytotoxic T-cells capable of secreting IFNγ and cytolytic molecules. Flucloxacillin was also shown to activate naïve T-cells from HLA-B*57:01 positive volunteers after priming and restimulation with flucloxacillin and dendritic cells. Wuillemin et al have also described the expansion of flucloxacillin-specific, predominantly, CD8+ T-cell clones from flucloxacillin-naïve HLA-B*57:01 positive donors after rounds of restimulation with flucloxacillin and autologous PBMCs, but interestingly were also able to generate CD4+ T-cell clones from HLA-B*57:01 negative individuals (Wuillemin *et al.*, 2013).

The characterisation of T-cell responses in flucloxacillin-DILI patients and HLA-B*57:01 positive individuals in the Monshi and Wuillemin studies have been based on the expansion of T-cell clones over a period 14 days, followed by rounds of restimulation with flucloxacillin and autologous PBMCs as antigen-presenting cells. In this chapter, I have described the direct stimulation of PBMCs from flucloxacillin-DILI patients with flucloxacillin to express IFNγ mRNA. IFNγ expression was significantly up-regulated in 2 out of 3 HLA-B*57:01 positive flucloxacillin-DILI patients with flucloxacillin treatment for 24 hours at 250 μM and 500 μM concentrations. PBMCs from a HLA-B*57:01 negative flucloxacillin-naïve healthy donor as well as two HLA-B*57:01 positive flucloxacillin-naïve healthy donors were also treated with flucloxacillin but no effect on IFNγ expression was observed. As well as generating flucloxacillin-specific T-cell clones from patient PBMCs, Monshi et al also directly stimulated PBMCs from 5/6 flucloxacillin-DILI patients, including one that was HLA-B*57:01 negative, to secrete IFNγ (Monshi *et al.*, 2013). Stimulation was performed with flucloxacillin concentrations of 500 μM, 1 mM and 2 mM for 48 hours and IFNγ measured by ELISpot assay. No stimulation of PBMCs was observed in flucloxacillin-tolerant individuals or HLA-B*57:01 positive flucloxacillin-naïve individuals. These findings are in line with what is described in this chapter, though I have shown stimulation at lower concentrations and at a shorter incubation period of 24 hours compared to 48 hours. However, since effects on gene transcription should be measurable sooner than effects on protein levels so use of a 24 hour drug incubation

period followed by mRNA measurement in the current experiment seems comparable to treatment for 48 hours followed by protein measurements.

Activation of T-cells in DILI is proposed to occur via covalent hapten binding or by direct non-covalent binding of the drug to HLA molecules or T-cells. Direct activation of PBMCs by flucloxacillin suggests that metabolism of the drug might not be required for T-cell activation. Flucloxacillin has been shown to bind to, and be presented by, HLA-B*57:01 as a covalently bound hapten but there is also evidence that it can activate T-cells through direct non-covalent binding to HLA-B*57:01 molecules expressed on the surface of cells, as described by the p-i concept (Wuillemin *et al.*, 2013). Activation of flucloxacillin-responsive PBMCs, as described in this chapter, could potentially result from either of these methods as HLA class I molecules are expressed on all nucleated cells, including PBMCs.

In the present work, abacavir was not found to have an effect on IFNγ expression in either patient PBMCs or from HLA-B*57:01 positive volunteers. Abacavir has been shown to stimulate IFNγ expression in PBMCs from HLA-B*57:01 positive abacavir hypersensitive patients but not from HLA-B*57:01 abacavir-naïve positive healthy donors after a 40 hour incubation period (Chessman *et al.*, 2008). Ideally, in the present study, responses should have been studied in PBMCs from an abacavir hypersensitive patient control but this was not feasible. Monshi et al were unable to stimulate flucloxacillin-induced T-cell clones from flucloxacillin-DILI patients or HLA-B*57:01 positive volunteers with abacavir (Monshi *et al.*, 2013). These findings suggest that, although both abacavir and flucloxacillin-induced adverse reactions share a common HLA association with HLA-B*57:01, there appear to be key differences in the mechanisms of drug-HLA association and T-cell activation, with circulating memory T-cells in flucloxacillin-DILI patients and AHS patients being reactive specifically to their causative drugs.

Also in this chapter, IL-8 mRNA expression in the treated PBMCs from flucloxacillin-DILI patients and HLA-B*57:01 positive and negative healthy volunteers was measured. There has been reported evidence of flucloxacillin stimulation of IL-8 expression in PBMCs from HLA-B*57:01 positive volunteers (Pletcher, unpublished data) and in T-cells clones expanded from PBMCs of a flucloxacillin-induced interstitial nephritis patient (Spanou *et al.*, 2006; Pletcher *et al.*, 2011). In the present work, IL-8 expression was increased in response to flucloxacillin treatment in the PBMCs from 1

out of the 3 flucloxacillin-DILI patients. In this patient, IFNγ expression was also upregulated. Interestingly, abacavir treatment was found to increase IL-8 expression in 2 of the 3 flucloxacillin-DILI patients and in a HLA-B*57:01 positive individual. Up-regulation of IL-8 expression has been observed in abacavir treated PBMCs from abacavir hypersensitivity patients but to our knowledge has not been studied in PBMCs from unexposed individuals (Almeida *et al.*, 2008). However, these findings are consistent with report from Chessman et al that PBMCs from abacavir-naïve HLA-B*57:01 negative individuals can be stimulated to proliferate if exposed to abacavir.

The work described in this chapter shows that flucloxacillin can induce cytokine and chemokine responses in PBMCs from some HLA-B*57:01 positive flucloxacillin-DILI patients after a relatively short incubation period of 24 hours. IFNγ and IL-8 were chosen to study cytokine and chemokine responses based on previous observations. Studies performed concurrently to the present work, showed that abacavir and flucloxacillin stimulated IL-8 mRNA expression in PBMCs from both HLA-B*57:01 positive and negative healthy volunteers after 24 hour treatment (Bell, 2012). The current findings support this apparent increase of IL-8 mRNA expression in HLA-B*57:01 positive and negative healthy volunteers in response to abacavir stimulation, as observed by Bell, although this was only found to be statistically significant in one HLA-B*57:01 positive healthy donor. However, unlike in the previous study, I did not observe flucloxacillin stimulation of IL-8 in HLA-B*57:01 positive or negative drug-naïve volunteers. One possible reason for this is the concentrations used in the two studies. In Dr Bell's studies, PBMCs were stimulated with 50 µM abacavir and 1 mM flucloxacillin whereas in my studies, 25 µM abacavir and 250 µM and 500 µM flucloxacillin, were used. These concentrations were chosen based on initial preliminary observations of flucloxacillin and abacavir toxicity in PBMCs but if further studies were to be performed, higher concentrations than those used in the current study could be used. In addition to IFNγ and IL-8, there are numerous other genes that merit study to assess their response to flucloxacillin or abacavir treatment. Studies by Monshi and Wuillemin found that flucloxacillin-specific T-cell responses were generally CD8+ in nature. Both studies showed flucloxacillin stimulation of flucloxacillin-specific T-cell clones to secrete markers of CD8+ responses such as granzyme B, FasL, perforin (Monshi study) and CD107a (Wuillemin study) in addition to IFNγ (both studies). It is unknown whether such responses can be detected by changes in mRNA expression in fresh PBMCs from patients or volunteers, rather than restimulated flucloxacillin-

specific T-cell clones. Its investigation would be of interest to increase the understanding of flucloxacillin-induced T-cell responses, particularly whether they occur rapidly in flucloxacillin-DILI patient PBMCs and whether they can be rapidly induced in drug-naïve healthy donors.

In summary,

- Flucloxacillin was shown to induce cytokine and chemokine responses in PBMC's from some HLA-B*57:01 positive flucloxacillin-DILI patients after a 24 hour stimulation.
- Abacavir was found to stimulate a response in HLA-B*57:01 positive and negative healthy volunteers. However, this couldn't be replicated with flucloxacillin.
- Further studies looking at additional immune candidate genes would be of interest such as the study of specific markers of CD8+ T-cell stimulation.

Chapter 7. General Discussion

7.1 General Discussion

Idiosyncratic drug-reactions remain an important concern for the pharmaceutical industry and in healthcare due to their rare, unpredictable but often severe nature. Drug-induced liver injury (DILI) is a type of idiosyncratic serious adverse reaction associated with a diverse range of drugs. Many of these idiosyncratic reactions are often not detected during drug development due to their rarity. Consequently, many idiosyncratic DILI reactions are only identified when a drug has been in use in wide populations for several months or years, making DILI a leading cause of post-market labelling restrictions and drug withdrawal. The mechanism involved in idiosyncratic DILI, although probably not related to the pharmacological effect of the drug, is often drug-specific and remains poorly understood. It is likely that idiosyncratic DILI involves a combination of genetic and environmental factors that result in an individual having an increased susceptibility to disease on the basis of their genetics. A better understanding of the mechanism of DILI and of predisposing pharmacogenetic factors leading to disease susceptibility could enable better prevention, diagnosis and treatment of DILI in the future.

This aims of this study were to gain further understanding of the mechanism of DILI caused by the isoxazolyl β-lactam antibiotic flucloxacillin. Like several other forms of DILI, it is now known that a contributing factor in flucloxacillin-induced liver injury is the activation of components of the host's immune system, though this was only discovered quite recently. A landmark GWA study published in 2009 reported a strong association between flucloxacillin-DILI and the HLA class I allele, HLA-B*57:01 (Daly *et al.*, 2009). This association has been confirmed in an additional 99 cases of flucloxacillin-DILI in the current study. However, only between 1 in 500 to 1 in 1000 individuals who possess HLA-B*57:01 will develop flucloxacillin-DILI when exposed to the drug, suggesting that the presence of HLA-B*57:01 alone is insufficient to cause disease. Genotyping studies described in the current work have also shown that approximately 20% of confirmed flucloxacillin-DILI cases do not possess the HLA-B*57:01 risk allele. Analysis of HLA-B genotypes in these cases discovered an additional association between HLA-B*13:02 and flucloxacillin-DILI when compared to HLA-B genotypes in a population control cohort. However, due to the small number of HLA-B*57:01 negative cases available at present, there remains a possibility that this association is chance and, therefore, should be studied further with the availability of

additional cases. It is possible that some of the HLA-B*57:01 negative cases were misdiagnosed despite positive adjudication by skilled assessors but this seems unlikely for the majority of the negative cases.

Whole genome sequencing remains the most comprehensive method of detecting rare genetic variants in disease cases, but remains expensive to conduct on a large scale and also the bioinformatic analysis is still challenging (Ng and Kirkness, 2010). An alternative method is to perform sequencing of protein coding sequences i.e. the exome, which constitutes approximately 1% of the human genome (Ng *et al.*, 2009). Following on from the flucloxacillin GWAS, exome sequencing of flucloxacillin-DILI cases has also been performed. A number of SNPs were identified and an association with a polymorphism in the caspase-5 gene on chromosome 11 was confirmed in the present study by direct genotyping of flucloxacillin-DILI cases and controls. Caspase-5 belongs to the caspase-1 subfamily that are activated in cells of the innate immune system where they have been implicated in the activation of inflammatory responses (Martin *et al.*, 2012). It is interesting to note that caspase-5 is upregulated in psoriatic skin and is activated by IFNγ (Salskov-Iversen *et al.*, 2011). As described in chapter 3, an association also exists between HLA-B*57:01 and psoriasis susceptibility. At present, the role of this SNP and whether caspase-5 has a role in the immune mechanism of flucloxacillin-DILI is unknown. In addition, the variant allele frequency, though showing a significantly higher frequency in cases compared with controls, remains low and it seems unlikely that caspase-5 variation is important in most cases of flucloxacillin-DILI. Undiscovered rare variants in other genes could also contribute though. At the time of writing, a second GWAS is in progress as part of the iDILIC project. The GWAS is being performed on 747 DILI cases relating to various drugs including 125 flucloxacillin-DILI cases. The inclusion of a greater number of flucloxacillin-DILI cases could provide significant power to detect additional new genetic risk factors with smaller effect sizes than HLA-B*57:01 such as the caspase-5 variants. The combination of DILI cases from a variety of drugs may also uncover common more general genetic determinants of DILI.

One other genetic risk factor for flucloxacillin DILI has emerged from other ongoing studies in Newcastle. In a recent report, a nonsynonymous polymorphism in PTPN22 (rs2476601), a gene which is involved in the regulation of the T cell response, was found to be a risk factor for DILI due to co-amoxiclav (Lucena et al. 2011). Further studies in the Newcastle flucloxacillin DILI case collection showed that this SNP was

also a risk factor for DILI due to flucloxacillin with a odds ratio of 1.9 (p = 0.02) compared to an odds ratio of 2.9 (p = 0.00033) seen for co-amoxiclav (M. Alshabeeb and A.K.Daly, unpublished). It is possible that susceptibility to DILI due to flucloxacillin, similar to more common complex genetic diseases such as type II diabetes, may involve a relatively large number of SNPs, which impart a slight though significant elevation in risk, in addition to the strong effect from HLA B*5701. These additional risk factors could be a mix of both common and rare variants.

Some understanding of the biological significance of the association of HLA-B*57:01 and flucloxacillin has been recently shown through T-cell studies from HLA-B*57:01 expressing individuals including flucloxacillin-DILI patients. Characterisation of reactive flucloxacillin-specific T-cells has shown that they are predominantly CD8+ and IFNγ secreting providing important insights into potential mechanisms of cellular damage (Monshi *et al.*, 2013; Wuillemin *et al.*, 2013). Findings in this study, showed a rapid T-cell response in HLA-B*57:01 expressing flucloxacillin-DILI patients but not in flucloxacillin-naïve HLA-B*57:01 positive or negative donors. From this it could be suggested that a recall response is occurring with the priming and activation of memory T-cell populations that were generated *in vivo* in previously sensitised patients. This could explain the observation of a rapid immune response in a flucloxacillin-DILI patient inadvertently rechallenged with the drug (Derby *et al.*, 1993).

In addition to the association with flucloxacillin-DILI, HLA-B*57:01 is also strongly associated with hypersensitivity to abacavir (Hetherington *et al.*, 2002; Mallal *et al.*, 2002). Unlike flucloxacillin toxicity, this is a general hypersensitivity reaction and not restricted to the liver. The association with HLA-B*57:01 is also stronger in abacavir hypersensitivity than with flucloxacillin-DILI with 55% of HLA-B*57:01 expressing individuals experiencing an adverse reaction when exposed to abacavir (Mallal *et al.*, 2008). Like flucloxacillin, CD8+ T-cell responses are implicated to play a key role in the aetiology of abacavir hypersensitivity. The differences in strength of the respective HLA-B*57:01 associations, however, suggest that there are differences in the mechanism of T-cell activation. This is supported by evidence presented in the current work and by others that abacavir can stimulate responses in drug-naïve volunteers (Chessman *et al.*, 2008). There is also emerging evidence that abacavir can interact with HLA-B*57:01 in a manner that has not been observed with flucloxacillin. One of these novel mechanisms described how through direct binding to HLA-B*57:01, abacavir, but not flucloxacillin, can alter the structure of HLA-B*57:01 and induce the loading of

novel drug-induced self-peptides to HLA-B*57:01 leading to an auto-immune like response (Norcross *et al.*, 2012).

It is still not known why flucloxacillin specifically causes liver toxicity whereas abacavir causes a general hypersensitivity reaction. Further studies are required to determine how and why flucloxacillin-responsive T-cells attack hepatic cells. Upregulation of chemokine receptors thought to be involved in T-cell migration and accumulation in the liver, in flucloxacillin-specific T-cell clones, may suggest a specific mechanism for hepatic injury but needs further exploration (Monshi *et al.*, 2013). Flucloxacillin metabolism has been implicated as a possible reason for specific toxicity to the liver. The flucloxacillin metabolite 5'hydroxymethyl flucloxacillin has previously been reported as toxic to biliary epithelial cells but not to hepatocytes (Lakehal *et al.*, 2001). Studies performed in the present work, failed to observe 5'hydroxymethyl flucloxacillin production *in vitro*, but did show the formation of penicilloic acid derivatives of flucloxacillin. Drug-adduct formation with cellular peptides is proposed as a key step in flucloxacillin-induced T-cell mediated hepatotoxicity and flucloxacillin-adducts have previously been detected in the livers of treated rats (Carey and van Pelt, 2005). The opening of the β-lactam ring to form penicilloic acids has been hypothesised as a major route for adduct formation. 5'hydroxymethyl flucloxacillin has been detected excreted in urine in higher concentrations than penicilloic acid derivatives however, findings presented here, show greater formation of penicilloic acids *in vitro*. What this means to the *in vivo* situation is unclear but it could be possible that lower urinary concentrations of penicilloic acid may result from slower rates of clearance due to higher protein binding and adduct formation *in vivo*. Further investigations are needed to study adduct formation by flucloxacillin and 5'hydroxymethyl flucloxacillin. In the present study, due to the limited availability of patient PBMCs and 5'hydroxymethyl flucloxacillin, the investigation of 5'hydroxymethyl flucloxacillin stimulation of PBMCs could not be performed.

The observation that flucloxacillin only causes DILI in a small percentage of HLA-B*57:01 expressing individuals exposed to the drug suggest that there are additional mechanisms or 'danger' signals that are required for a full hepatotoxic event. Pathways affecting the disposition of flucloxacillin in the liver are logical targets for factors in flucloxacillin-DILI. Liver injury caused by the isoxazolyl penicillins is predominantly of a cholestatic phenotype characterised by an impairment of bile flow and increase of toxic bile acids (Staudinger *et al.*, 2001). Increased cellular bile acid concentrations

induce changes in expression of various genes that decrease bile acid biosynthesis and increase their efflux (Teng and Piquette-Miller, 2007). PXR is activated by bile acids such as lithocholic acid and classic PXR activators such as rifampicin have been used to treat symptoms of cholestasis (Xie *et al.*, 2001). Previously, flucloxacillin and dicloxacillin have been shown to be activators of the liver expressed nuclear receptor, PXR (Yasuda *et al.*, 2008; Andrews *et al.*, 2010). In this study, a reporter gene assay was used to compare, for the first time, PXR activation by the isoxazolyl penicillins – cloxacillin, dicloxacillin and flucloxacillin, showing that the order of PXR activation was lowest with cloxacillin and greatest with dicloxacillin. A role for PXR in flucloxacillin-DILI has previously been suggested due to the discovery of a genetic association in cases thought to cause decreased PXR expression. However genotyping performed in this study, of an additional 99 cases to the original 51, failed to confirm this association. Flucloxacillin has been shown to be a frequent cause of DILI; however reports of DILI due to the other almost identically structured isoxazolyl penicillins, such as cloxacillin and dicloxacillin, are in comparison extremely rare. There is a lack of data describing the wider population use of cloxacillin and dicloxacillin in countries that commonly prescribe these drugs in preference to flucloxacillin. However, cloxacillin and dicloxacillin are prescribed in a number of countries including Australia, Spain, Denmark and Iceland instead of flucloxacillin. Reports of DILI causes from these countries suggest that both cloxacillin and dicloxacillin are less hepatotoxic than flucloxacillin or DILI caused by these drugs are under-reported. A recent study of DILI incidence in Iceland where both cloxacillin and dicloxacillin are prescribed showed that there was one case of DILI due to each of the drugs indicating an incidence of 1 in 3659 treated patients with cloxacillin and 1 in 22320 patients treated with dicloxacillin but these frequencies need to be treated with caution due to being based on single cases only (Bjornsson *et al.*, 2013). In the current study, 3 cases of cloxacillin-DILI (two from Spain and one from Iceland) and 2 cases of dicloxacillin-DILI (both from Iceland) were found to be negative for HLA-B*57:01 suggesting that at least, in part, there are differences in the mechanism of liver injury to that predominantly observed in flucloxacillin-DILI. Both the non-flucloxacillin isoxazolyl penicillin DILI cases and the HLA-B*57:01-negative flucloxacillin DILI cases could have similarities with DILI due to other penicillins such as amoxicillin. DILI due to amoxicillin alone is rarer than DILI due to flucloxacillin or amoxicillin-clavulanate but cases are occasionally seen. For example, the iDILIC case collection currently being analysed by GWAS includes DNA samples from 16 cases of DILI due to non-isoxazolyl penicillins, mainly amoxicillin.

The possibility that there is a specific HLA risk factor involved will become clearer once the GWAS has been completed as imputation of HLA genotype will be performed on the data.

In summary, the studies described in this thesis have confirmed the major role of HLA-B*57:01 in DILI due to flucloxacillin and have provided some new insights into genetic risk factors for DILI due to related penicillins and in cases of flucloxacillin DILI not related to HLA-B*57:01. The interaction with PXR reported previously for flucloxacillin has been shown to be a more general feature of isoxazolyl penicillins. It has not been possible to confirm findings of others that CYP3A4 converts flucloxacillin to 5'-hydroxymethyl flucloxacillin. This needs follow-up using more sensitive methods for metabolite detection. KIR3DSI genotype did not appear to be a significant risk factor for flucloxacillin DILI but caspase-5 genotype appears to be an interesting minor risk factor.

Appendices

Appendix A – HLA-B genotypes of HLA-B*57:01 negative flucloxacillin-DILI cases

HLA-B genotype	
UK cases	
44:02	55:01
44:05	45:01
44:03	51:01
08:34	13:02
07:02	44:52N
08:01	44:02
18:01	44:02
08:01	14:02
13:02	40:02
07:02	08:01
35:01	39:06
44:02	53:01
44:02	55:01
40:01	44:02
44:02	40:01
18:01	38:01
08:01	08:01
40:01	44:02
08:01	44:03
37:01	55:01
08:01	41:01
07:02	14:01
08:01	08:01
38:01	51:01
Swedish cases	
15:57	51:01
15:01	37:01

Appendix B – HLA-B alleles observed in the NW-EU control cohort that are absent in genotyped HLA-B*57:01 negative flucloxacillin-DILI cases

HLA-B allele	Flucloxacillin-DILI cases (n=26)	NW-EU Controls (n=107)	*P* –value
B*15:17	0 (0.00)	1 (0.9)	1.0000
B*15:18	0 (0.00)	1 (0.9)	1.0000
B*15:24	0 (0.00)	1 (0.9)	1.0000
B*27:05	0 (0.00)	12 (11.2)	0.1313
B*35:03	0 (0.00)	1 (0.9)	1.0000
B*39:01	0 (0.00)	4 (3.7)	1.0000
B*47:01	0 (0.00)	1 (0.9)	1.0000
B*49:01	0 (0.00)	1 (0.9)	1.0000
B*50:01	0 (0.00)	3 (2.8)	1.0000
B*52:01	0 (0.00)	1 (0.9)	1.0000
B*56:01	0 (0.00)	1 (0.9)	1.0000
B*73:01	0 (0.00)	1 (0.9)	1.0000

Number of individuals with a particular allele is shown with the allele carriage frequency shown as a percentage in parenthesis. Two-tailed Fisher's exact test was used to calculate significance between cases and controls

Appendix C – Abstract for a poster presentation at the joint meeting of the International Symposium on Microsomes and Drug Oxidations (MDO) and the European Regional meeting of the International Society of the Study of Xenobiotics (ISSX) in Noordwijk aan Zee, The Netherlands, June 17th – 21st 2012

Nuclear Receptor Interactions with Isoxazolyl Penicillins

Thomas C. Chamberlain, Elise Glen, David Cowie, Matthew C. Wright and Ann K. Daly

Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK

The β-lactamase resistant penicillin, flucloxacillin, is a common cause of idiosyncratic drug-induced liver injury (DILI). Flucloxacillin belongs to a family of semi-synthetic isoxazolyl penicillins that include oxacillin, cloxacillin and dicloxacillin. In contrast to flucloxacillin, reports of liver injury caused by the other isoxazolyl penicillins are extremely rare [1,2]. Previous work has shown that flucloxacillin is an activator of the pregnane X-receptor (PXR) and inducer of CYP3A4 [3]. Further studies on human hepatocytes using sandwich culture have shown induction of CYP2B6 in addition to CYP3A4. In view of the effect of flucloxacillin on CYP2B6, the possibility that flucloxacillin is also a constitutive androstane receptor (CAR) agonist was investigated. CAR shares an overlap in some substrate specificities and transcriptional targets with PXR. Reporter gene studies were performed to establish whether flucloxacillin also interacts with CAR. A CYP2B6 reporter construct containing two distal response elements to which activated CAR binds was transiently transfected into Caco-2 cells along with a human CAR expression vector. Cells were treated for 24 hours with flucloxacillin over a concentration range of 100 μM to 1 mM, with the known hCAR activator CITCO as a positive control. Compared to the untreated control, flucloxacillin had no effect on CAR activation at any of the described concentrations suggesting that it is unlikely that flucloxacillin is a CAR substrate. Three tag SNP's for the main haplotypes in the CAR (NR1I3) gene were also genotyped in a number of flucloxacillin-DILI case samples and controls from flucloxacillin-prescribed healthy individuals. No significant differences in genotype frequency were found between cases and controls.

Reporter gene studies were performed to compare PXR activation by flucloxacillin to that of cloxacillin and dicloxacillin. A luciferase construct for CYP3A4 activation was transfected into HepG2 cells which were treated with flucloxacillin, cloxacillin and dicloxacillin at concentrations of 500 μM to 2 mM for 72 hours. Cloxacillin and dicloxacillin were found to be more potent PXR activators than flucloxacillin. Comparative studies on CYP3A4 induction in LS180 cells by the isoxazolyl penicillins are in progress. We postulate that this greater PXR activation may affect drug metabolism and clearance and be a factor in differences seen in hepatotoxicity between the drugs.

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