IMMUNOLOGICAL INVESTIGATIONS INTO NAPROXEN – INDUCED IDIOSYNCRATIC HEPATOTOXICITY

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"A thesis (Θέσις) is a supposition of some eminent philosopher that conflicts with the general opinion...for to take notice when any ordinary person expresses views contrary to men's usual opinions would be silly" – **Aristotle**

"Logic will take you from A to B. Imagination will take you everywhere" – **Albert Einstein**

"Success is not final, failure is not fatal: it is the courage to continue that counts" – **Winston Churchill**

CONTENTS

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ABBREVIATIONS

ACN	Acetonitrile
ACS	Acyl-CoA synthetase
ADR	Adverse drug reaction
AE	Adverse event
AG	Acyl glucuronide
AGE	Advanced glycation end product
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMAP	N-acetyl-m-aminophenol
APAP	N-acetyl-para-aminophenol
APC	Antigen-presenting cell
APCN	Allophycocyanin
AQ	Amodiaquine
AST	Aspartate transaminase
ATP	adenosine triphosphate
ATPase	Adenosine triphosphatase
AUC	Area under the curve
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CCK-8	Cell counting kit - 8
CCL-17	Chemokine ligand 17
CD	Cluster of differentiation
CoA	Coenzyme A
COX	Cyclooxygenase
cpm	Counts per minute
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CYP	Cytochrome P450
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DHR	Drug hypersensitivity reaction
DILI	Drug-induced liver injury

DMSO	Dimethyl sulfoxide
DPPIV	Dipeptidyl peptidase IV
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EMA	European Medicines Agency
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell scanning
FBS	Foetal bovine serum
FcεR	IgE receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate dehydrogenase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
GWAS	Genome wide association study
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigen
HMGB1	High mobility group box protein 1
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HSP	Heat shock protein
iDILI	Idiosyncratic drug-induced liver injury
IDR	Idiosyncratic drug reaction
IFNγ	Interferon γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IS	Internal standard
KC	Kupffer cell

LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal epithelial cell
LTT	Lymphocyte transformation test
MACS	Magnetic-activated cell sorting
MALT	Mucosa-associated lymphoid tissues
MDH	Multiple drug hypersensitivity
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence index
MHC	Major histocompatibility complex
MIST	Metabolites in safety testing
Mo-DCs	Monocyte-derived dendritic cells
MRM	Multiple reaction monitoring
MRP	Multidrug resistance associated protein
MS	Mass spectrometer
m/z	Mass-to-charge ratio
NAD	Nicotinamide adenine dinucleotide
NAPQI	N-acetyl-p-benzoquinonimine
NGS	Next generation sequencing
NHS	National Healthcare System
NK	Natural killer
NKT	Natural killer T
NLRP3	NOD-like receptor 3
NSAID	Non-steroidal anti-inflammatory drug
OATP	Organic-anion-transporting polypeptide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PD-1	Programmed cell death protein-1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PHA	Phytohaemagglutinin

PTGS	Prostaglandin synthase
QC	Quality control
S/N	Signal to noise ratio
SEC	Size exclusion chromatography
SFU	Spot forming unit
SI	Stimulation index
SITA	Stress-induced transcriptional attenuation
SMX	Sulfamethoxazole
SMX-NO	Sulfamethoxazole-nitroso
SPE	Solid phase extraction
(S)-DNAP	(S)-6-O-desmethylnaproxen
(S)-NAG	(S)-naproxen-1β-acyl glucuronide
(S)-NAP	(S)-naproxen
TB	Total bilirubin
TCR	T-cell receptor
Tc	T-cytotoxic
Th	T-helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TR-/-	Transport deficient
Treg	Regulatory T-cell
TT	Tetanus toxoid
UDP	Uridine diphosphate
UDPG	UDP-D-glucose
UDPGA	UDP-glucuronic acid
UGT	UDP-glucuronosyl transferase
UK	United Kingdom
ULN	Upper limit of normality
ULOQ	Upper limit of quantification
UPR	Unfolded protein response
USA	United States of America
WHO	World Health Organization
WST-8	Dojindo's highly water-soluble tetrazolium salt

ABSTRACT

Background: Naproxen is a member of the aryl acetic acid class of non-steroidal anti-inflammatory drugs that has anti-inflammatory, antipyretic and analgesic properties. Naproxen is extensively metabolized in the liver by P450 enzymes to 6-O-desmethylnaproxen. Both parent drug and desmethyl metabolite undergo phase II biotransformation to their respective acyl glucuronide conjugated metabolites. Naproxen-induced idiosyncratic hepatotoxicity is a rare event. The mechanism of the tissue injury and the nature of the chemical entity involved in the adverse reaction have not been studied.

Aims: To explore and characterise naproxen (metabolite)-specific immune responses from patients with or without liver injury.

Methods: The chemical instability and protein reactivity of naproxen acyl glucuronide were investigated *in vitro* and an adduct with human serum albumin was generated. Peripheral blood mononuclear cells were isolated from patients and healthy volunteers. Lymphocyte proliferation and IFNγ secretion against naproxen, its major metabolites and the serum albumin adduct were examined. T-cell cloning by limiting dilution was undertaken in order to generate drug-specific T-cell clones and appropriate immune functional assays were used to characterize responses. Next generation sequencing was used in order to interrogate the transcriptional signature of peripheral blood mononuclear cells in response to naproxen and its major metabolites.

Results: Naproxen acyl glucuronide is chemically unstable with an identified degradation half-life of 2.72 hrs in 0.1 M phosphate buffer at 37°C (pH 7.4). Furthermore, irreversible binding was measured at 247.37 ± 57.64 nmol of drug per μmol serum albumin under 50 : 1 metabolite : protein molar ratio incubation at 37°C (pH 7.4). 6-O-desmethylnaproxen, but not naproxen or naproxen acyl glucuronide, exhibited lymphocyte stimulation in a patient with naproxen-induced liver injury. A $CD4^+CD8^+$ Th17 desmethylnaproxen – responsive T-cell clone was identified. The clone was found to be MHC-class II restricted and become activated via a pharmacological-interaction mechanism. 6-O-desmethylnaproxen exerts significant differential gene expression in peripheral blood mononuclear cells isolated from patients and healthy volunteers and an overall down-regulatory pattern was revealed.

Conclusion: Results show lymphocyte responses were directed to the primary desmethyl metabolite of naproxen, but not the parent drug or acyl glucuronide metabolite. In addition, the desmethyl metabolite shows consistent differential gene expression in human peripheral blood mononuclear cells. Taken together, this evidence suggests an overall immune-mediated basis for naproxen-induced idiosyncratic hepatotoxicity with a role for the inert oxidative metabolite. Whilst the FDA considers acyl glucuronide adduction of protein to be responsible for the liver reactions associated with carboxylic acid drugs such as naproxen, no evidence of this was found herein.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND OF THE THESIS

Safety risk assessment of novel compounds, in particular carboxylic acid drugs, some of which are found to be extensively metabolized to reactive acyl glucuronide (AG) drug metabolites, continues to represent a major challenge for pharmaceutical drug development. The ability of AGs to covalently bind to proteins in conjunction with epidemiological associations of carboxylic acid drugs with idiosyncratic drug-induced liver injury (iDILI) continues to raise anxiety regarding their safety. However, the mechanism of iDILI secondary to AG forming drug treatment remains poorly defined and covalent binding to proteins – though not favoured – does not necessarily equate to toxicity. It is believed that idiosyncratic drug reactions (IDRs) are predominantly driven by the immune system and several mechanistic hypotheses are in place to describe how an inadvertent activation of the immune system can bring about liver injury. To date, no *in vivo* human evidence exploring the immunological basis for the reaction in relation to AG drug metabolites has been published. Until the mechanisms behind carboxylic acid drug-induced liver injury are fully elucidated, any risk-mitigation strategies will remain challenging. The work in this Thesis is designed to explore unanswered questions regarding the involvement of AG drug metabolites in iDILI as well as the role of the adaptive immune system. The rare occurrence of serious cases of iDILI in few susceptible individuals suggests a multifactorial aetiology and pathogenesis and makes it a clinically important, yet challenging subject for investigation.

Given the multidisciplinary nature of this field of research, this introduction will guide the interested reader through different relevant areas of science and summarize the current concepts in immunology, drug metabolism, specifically AG metabolism, and adverse reactions. Naproxen is introduced as a model carboxylic acid drug to investigate the role of the adaptive immune system in a rare case of naproxen-induced idiosyncratic hepatotoxicity.

1.2 ADVERSE DRUG REACTIONS

When xenobiotics interact with an organism, biochemical reactions take place that may lead to pathophysiological changes. In the case of drugs, those reactions are normally meant to result in a beneficial change against a disease or health-related problem, but quite often harmful and unintended responses may arise from their use. It is important to distinguish an adverse drug reaction (ADR) from an adverse event (AE), which can be considered as a broader term including any adverse outcome that may appear during treatment with a certain drug but does not necessarily have a causal relationship with the drug (Rawlins, 1981; Edwards & Aronson, 2000).

1.2.1 Definition and burden of adverse drug reactions

An ADR is defined according to the *World Health Organization (WHO)* as "a response to a drug that is noxious and unintended and which occurs in doses normally used for the treatment, prophylaxis, or diagnosis of disease, or the modification of physiological function". According to the *European Medicines Agency (EMA),* the definition has been adapted to include adverse responses that may arise from the use of the product outside its marketing authorisation (overdose, misuse, abuse and medication errors). Finally, the term side-effect is mistakenly taken to be synonymous with an ADR since the former is defined as any effect other than the expected therapeutic effect that may be beneficial, neutral or harmful (Ferner & McGettigan, 2018).

ADRs have a serious impact on patients' lives, and according to Pirmohamed et al., (2004), account for 1 in 16 hospital admissions in the United Kingdom (UK), while Lazarou, Pomeranz, & Corey, (1998) suggested that in the United States of America (USA) the number

of deaths caused by ADRs lists fourth after heart disease, cancer and stroke. In fact, in the USA alone, it is estimated that approximately 100,000 patients die on a yearly basis due to serious ADRs (Wilke et al., 2007). Aside from the impact on patients, ADRs pose a financial burden on healthcare systems as illustrated by cost estimations of hospital admissions in England accounting for 5 – 9 % of total in-patient costs equating to a £637 million annual burden on the National Healthcare System (NHS) (Kongkaew, Noyce, & Ashcroft, 2008; Davies et al., 2009).

1.2.2 Classification and terminology

Aronson & Ferner, (2003) described one of the best framework methods for classifying ADRs, termed DoTS (**Do**se-relatedness, **T**ime-course and **S**usceptibility). DoTS classification focuses on three main features that characterise clinical manifestations: Dose-relatedness of the drug, Time-course of the adverse reaction and Susceptibility of the patient (Ferner & Aronson, 2010). ***Table 1.1*** summarises the types of ADRs according to the DoTS classification system.

Dose-relatedness: The main criterion that is used to distinguish between them is the relative position of the dose-response curve. Therefore, the occurrence of ADRs at doses higher than those meant to exert therapeutic effect corresponds to an intrinsic dose-related toxic effect (e.g. acetaminophen hepatotoxicity).

Time-relatedness: Concerning their relationship with time, ADRs may be either time-related or time-independent. Thalidomide was responsible for causing teratogenesis and the results of its use became apparent long after exposure (Woollam, 1965).

Susceptibility: Some individuals are more prone to developing rare ADRs than others. Patient risk factors, such as a unique genetic profile, account for this difference. Age and gender may also contribute to susceptibility reactions.

Table 1.1: Types of ADRs, features and examples, adapted from Edwards & Aronson, (2000).

Type of Reaction	Mnemonic	Features	Examples
A: Dose-related	**A**ugmented	Common, Predictable, Related to drug's pharmacology	Toxic effects (e.g. digoxin toxicity), Side effects (e.g. anticholinergic effects of TCAs [1])
B: Non – Dose-related	**B**izarre	Uncommon, Unpredictable, Not related to drug's pharmacology	Immunological reactions (e.g. penicillin hypersensitivity), Idiosyncratic reactions (e.g. acute porphyria)
C: Dose-related & time-related	**C**hronic	Uncommon, Related to cumulative dose	Hypothalamic pituitary-adrenal axis suppression by corticosteroids
D: Time-related	**D**elayed	Uncommon, Occurs or becomes apparent sometime after the use of the drug	Teratogenesis (e.g. Thalidomide), Carcinogenesis
E: Withdrawal	**E**nd of use	Uncommon, Occurs soon after withdrawal of the drug	Opiate withdrawal syndrome, Myocardial ischemia (e.g. β-blocker withdrawal)
F: Unexpected failure of therapy	**F**ailure	Common, Dose-related, often caused by drug interactions	Inadequate dosage of an oral contraceptive, particularly when used with specific enzyme inducers

[1] TCAs: Tricyclic antidepressants

Whilst type A reactions are predictable and account for the majority of ADRs (~ 80%), type B reactions are bizarre, unpredictable, occur infrequently (rare) and therefore called idiosyncratic (Edwards & Aronson, 2000; Iasella, Johnson, & Dunn, 2017). IDRs, usually identified after a drug has been marketed, represent a main safety issue due to their low frequency that cannot easily be detected in clinical trials as well as their severity potential which can sometimes lead to organ failure and death (e.g. idiosyncratic hepatotoxicity) (Park, Kitteringham, Powell, &

Pirmohamed, 2000). IDRs are discussed in more detail in section **1.6**. Reactive drug metabolites and genetic polymorphisms alongside additional risk factors have been postulated to account for the initiation of a multistep process that involves non-immune and / or immune-mediated mechanisms leading to the pathogenesis of idiosyncratic toxicity (Park, Pirmohamed, & Kitteringham, 1998; Ju & Uetrecht, 2002; Cho & Uetrecht, 2017; Roth & Lee, 2017). Notwithstanding correlations between reactive drug metabolite formation and IDR risk (Obach, Kalgutkar, Ryder, & Walker, 2008; Usui, Mise, Hashizume, Yabuki, & Komuro, 2009), there are drugs such as ximelagatran that cause IDRs without an apparent formation of reactive metabolites (Kenne et al., 2008). Clinical evidence suggests that the majority of IDRs involves the activation of the immune system and it has been proposed that formation of drug-protein adducts initiates an immune response that under special circumstances leads to tissue injury (see section **1.6.2**). However, newer concepts have emerged supporting an immunological basis even in the absence of drug / reactive metabolite covalent binding to a protein (see section **1.6.3**). Individual genetic variation, concurrent diseases and other patient risk factors (see section **1.6.4**) promote the random clinical appearance of IDRs (Ulrich, 2007; Uetrecht & Naisbitt, 2013; Chen, Suzuki, Borlak, Andrade, & Lucena, 2015).

Type B reactions which are the result of inadvertent activation of the immune system by the parent drug or reactive metabolites are termed drug hypersensitivity reactions (DHRs) (Naisbitt, Gordon, Pirmohamed, & Park, 2000; Roujeau, 2005). DHRs are traditionally classified based on the definitions of the four known hypersensitivity sub-types introduced by Gell & Coombs, (1963) and summarised in ***Table 1.2***.

Table 1.2: Drug Hypersensitivity Reactions, adapted from Tharpe, (2011).

Reaction Type	Hypersensitivity Reactions	Timing of Reaction	Mechanism of Reaction	Reaction Mediators
Type I: Immediate hypersensitivity (allergy)	Anaphylaxis, urticaria, pruritus bronchospasm	Minutes to hours (< 6 hrs) upon challenge	Mast cell activation releases histamine & inflammatory molecules	IgE
Type II: antibody-mediated cytotoxicity	Hemolytic anemia, thrombocytopenia, neutropenia, agranulocytosis	Several days after initial exposure	IgM and IgG mark drug-hapten complexes on cell for destruction by complement and / or innate cells	IgG and IgM
Type III: immune complex reaction	Glomerulonephritis, serum sickness, drug-induced lupus, vasculitis	Several days to months after initial exposure	Drug-antibody complexes in tissues cause complement activation	IgG and IgM
Type IV: cell-mediated reaction (delayed-type hypersensitivity)	Contact dermatitis, maculopapular rash, SJS/TEN [1], DRESS [2], DILI [3]	24-72 hours upon challenge	Sensitized T-cells lysing target cells (Tc) or releasing cytokines (Th) activating and / or recruiting inflammatory cells	T-cells and cytokines

[1] SJS / TEN: Stevens-Johnson syndrome / Toxic epidermal necrolysis, [2] DRESS: Drug rash with eosinophilia and systemic symptoms, [3] DILI: Drug-induced liver injury

As this classification of drug hypersensitivity was not specifically incepted upon the drug mode of action but rather accommodated in the existing immunological framework of hypersensitivity, an adapted classification was recently introduced that incorporates novel mechanisms of immune activation by drugs (Posadas & Pichler, 2007; Pichler et al., 2010; Pichler & Hausmann, 2016). According to this new classification, most type B reactions should be referred to as drug hypersensitivity reactions and sub-divided into i) allergic / immune (involving neo-antigen formation and mechanistically leading to either one of the four classical hypersensitivity reactions), ii) pharmacological – interaction (involving direct non-covalent

binding of drug onto T-cell receptor (TCR) and / or human leukocyte antigen (HLA)) and iii) pseudo-allergic (involving a range of heterogeneous non-immune mediated reactions resulting from “off-target” drug binding to receptors of effector cells of inflammation) (Pichler, 2019). Last, it should be noted that drug hypersensitivity reactions are phenotypically diverse and present with significant clinical heterogeneity affecting single or multiple organs and symptoms ranging from mild to severe (Roujeau, 2005). Occasionally, stimulation to one drug can initiate a cascade of reactions to other different drugs. This long-lasting hyper-responsiveness to chemically distinct entities with diverse clinical symptoms has been termed as multiple drug hypersensitivity (MDH) syndrome (Pichler, Srinoulprasert, Yun, & Hausmann, 2017).

1.3 DRUG METABOLISM AND THE IMMUNE SYSTEM

As previously discussed, ADRs are often the result of complex interactions between drugs and biotransformation and / or immune systems. Both systems, enabling finely regulated and balanced metabolism and immune responses, naturally exist to detoxify and protect an organism from xenobiotics and foreign pathogens, respectively. However, under special circumstances, the natural homeostatic role of these physiological elements may become dysregulated leading to insufficiently controlled effects which eventually may harm the organism. This section will present the basic principles of drug metabolism and the immune system as well as introducing situations that reveal their role in pathophysiological conditions. This will facilitate a better understanding of concepts and information that are subsequently discussed in this chapter.

1.3.1 Detoxification versus reactive drug metabolites and covalent binding to macromolecules

Generally, the metabolic system utilizes a range of different enzymes with diverse substrate capacity in order to render small molecules from food and environment (including drugs and chemicals) more water-soluble and thus facilitate elimination from the tissues of the body (Gibson & Skett, 2001). Several variables such as the structure of the drug, physicochemical properties as well as tissue-specific enzymes determine the type of ensuing biotransformation reactions, which are generally divided in 2 pathways (phase I and II). In general, phase I reactions aim to alter the structure of the parent compound in a way that the resulting molecule is capable of accepting a chemical group that may subsequently be conjugated during phase II reactions. Enzymes catalysing phase I metabolism are predominantly localized in the endoplasmic reticulum (microsomes), the majority of which belongs in the Cytochrome P450 (CYP) family. Phase II enzymes reside in the cytosol, except for UDP-glucuronosyl transferase (UGT) that is localized in the endoplasmic reticulum too (Gibson & Skett, 2001).

Whilst drug metabolism is considered predominantly a detoxification process, biotransformation reactions may occasionally lead to reactive metabolites (bioactivation) with a toxic potential. Generation of reactive metabolites largely depends on the structure of the parent compound as well as the enzyme profile and availability at the site of biotransformation. Various chemical motifs and groups (ex. aniline, hydrazines, thiazoles, aryl acetic acids) have been identified in parent inert compounds which enable formation of reactive metabolites (Kalgutkar et al., 2005). The liver, being significantly exposed to orally absorbed drugs (first tissue to be encountered after gastrointestinal tract uptake by drug absorption into the portal vein and the hepatic-portal blood system) and representing a rich enzyme-containing tissue, is

responsible for the bioactivation of many drugs via phase I and / or II metabolism (Kalgutkar & Soglia, 2005; Srivastava et al., 2010).

Reactive metabolites are divided in four main groups: electrophiles, nucleophiles, free radicals and redox reagents. Electrophiles are by far the most common type of reactive metabolites and may be hard (e.g. benzylic carbonium ions, aldehydes), usually reacting with nucleophilic sites on nucleic acids, or soft (e.g. quinones and quinone imines, nitroso intermediates, acyl glucuronides), usually reacting with nucleophilic -SH groups in glutathione (GSH) or proteins (Attia, 2010; Leung, Kalgutkar, & Obach, 2012). Thus, proteins carrying strong nucleophilic sites (ex. lysine amines, cysteine thiols) are more susceptible for attack by electrophiles (Attia, 2010).

Electrophilic reactive metabolites can irreversibly modify macromolecules, an attribute that has been associated with toxicity (Zhou, Chan, Duan, Huang, & Chen, 2005; Stachulski et al., 2013). The intensity of chemical reactivity may vary amongst chemical compounds and it is worth emphasizing that reactive metabolites do not react indiscriminately with all macromolecules. Consequently, the profile of target macromolecules and extent of covalent binding may differ significantly as a function of the compound's chemistry and overall disposition mechanisms (Nelson & Pearson, 1990; Hinson & Roberts, 1992). Nucleophilic substitution and Schiff's base mechanism constitute the principal proposed mechanisms by which reactive metabolites bind covalently on proteins (Pumford, Halmes, & Hinson, 1997). Reactive metabolites, covalent binding and formation of drug-protein adducts is an area of evolving research and advances in proteomics have enabled the identification and quantification of target proteins for certain drugs (Tailor, Waddington, Meng, & Park, 2016). It is generally thought that covalent protein modification by drugs and / or reactive metabolites can cause cellular dysfunction or death by altering protein structure / function or by generating a neo-antigen and activating the immune system against self (Park et al., 2011; Stachulski et

al., 2013). However, not all covalent protein modifications are critical (in fact the efficacy of certain drugs relies on covalent binding, e.g. acetylsalicylic acid) and the link between reactive metabolite formation, covalent protein binding and toxicological insult is in most occasions obscure and dependent on several variables (Thompson et al., 2011). Acetaminophen (N-acetyl-para-aminophenol (APAP)) and halothane are two examples of how reactive drug metabolite formation is associated with toxicity due to covalent binding to endogenous macromolecules.

Acetaminophen at therapeutic doses is metabolized predominantly via glucuronidation and sulfation whilst a smaller amount undergoes oxidation via CYP enzymes forming the electrophilic metabolite N-acetyl-p-benzoquinonimine (NAPQI) (Raucy, Lasker, Lieber, & Black, 1989; Thummel, Lee, Kunze, Nelson, & Slattery, 1993). Unless APAP is administered at higher doses, NAPQI is naturally detoxified by GSH thereby exemplifying that bioactivation does not always equate with toxicity. However, during overdose the sulfation pathway becomes saturated thereby directing higher amounts towards oxidation metabolism (Mitchell, Jollow, Potter, Gillette, & Brodie, 1973). The increased NAPQI formation depletes GSH levels, leading to covalent binding of the available reactive metabolite to critical hepatic proteins such as γ-glutamylcysteine synthetase, glyceraldehyde-3-phosphate dehydrogenase (GADPH) and Ca^{+2} / Mg^{+2} adenosine triphosphatase (ATPase) (Nelson, 1990; Landin, Cohen, & Khairallah, 1996; Dietze, Schafer, Omichinski, & Nelson, 1997). This results in a number of parallel events like oxidative stress reactions, mitochondrial dysfunction and Ca^{+2} homeostasis disruption that eventually culminate to cell death (Kitteringham et al., 2000). The importance of targeting critical proteins and extent of covalent binding is illustrated by comparing APAP to its regioisomer N-acetyl-m-aminophenol (AMAP), which despite a similar level of overall covalent binding does not lead to hepatotoxicity (Tirmenstein & Nelson, 1989).

Halothane, forms a chemically reactive acyl halide (trifluoroacetyl chloride) following bioactivation by CYP2E1 that subsequently modifies liver microsomal proteins by trifluoroacetylation of lysine residues (Gandolfi, White, Sipes, & Pohl, 1980; Hayden, Ichimura, McCann, Pohl, & Stevens, 1991). A small number of patients develop liver injury (halothane hepatitis), and immunoglobulin G (IgG) antigen-specific antibodies (against CYP enzymes) have been detected in the sera of injured patients (Kenna, Satoh, Christ, & Pohl, 1988; Knight, Scatchard, Van Pelt, & Kenna, 1994; Kitteringham, Kenna, & Park, 1995). Moreover, isolated hepatocytes expressing the antigens have shown susceptibility to antibody-dependent cytotoxic killing *in vitro* (Mieli-Vergani et al., 1980). In addition, experimental autoimmune hepatitis animal models have shown the involvement of T-cells in cell death (Lohse, Manns, Dienes, Meyer zum Buschenfelde, & Cohen, 1990). Thus, whilst the entire mechanisms of halothane hepatitis are not completely elucidated, it is likely that drug-protein adducts formed by the acyl halide metabolite mediate activation of the immune system that might explain the severe idiosyncratic nature of the reaction.

1.3.2 Protection versus inflammation, hypersensitivity, allergy and autoimmunity

The immune system is a complex interplay between several different organs, molecules, cells and pathways. It evolved to protect multicellular organisms such as vertebrates from pathogens such as bacteria, fungi, viruses and parasites (Parkin & Cohen, 2001). Immune responses can be triggered by non-infectious foreign substances such as proteins, peptides, polysaccharides, and small molecules) (Landsteiner & Jacobs, 1935; Neefjes & Ovaa, 2013). Moreover, aside from foreign entities, even self-molecules can under certain occasions lead to an immune response, a concept known as autoimmunity. The main properties of host defence mechanisms

are divided into innate (natural) and adaptive (acquired) immunity. Immune cells are predominantly found in lymphoid organs (bone marrow, thymus, spleen, lymph nodes and gastrointestinal mucosa-associated lymphoid tissues (MALT)) (Krishnamurty & Turley, 2020), and importantly also in organs such as the liver, which is positioned at the crossroad between the gut and the systemic circulation (Thomson & Knolle, 2010). Immunocompetent cells are capable of migrating throughout the body via the bloodstream and lymphatic system (Yatim & Lakkis, 2015). Because of the immune system's complexity and power, natural homeostatic properties exist to control and regulate immunity. Defects in immunity can lead to an abnormally reduced / absent (immunodeficiency) or aberrant (hypersensitivity) immune response (Warrington, Watson, Kim, & Antonetti, 2011). ***Table 1.3*** summarizes the main characteristics of the innate and adaptive immune responses.

Table 1.3. Summary of differences between innate and adaptive immune responses. The innate immune response is faster but lacks the specificity and memory function characteristic of the adaptive immune system.

	INNATE RESPONSE	ADAPTIVE RESPONSE
REACTION TIME	Fast (minutes to hours)	Slow (takes days)
EFFECTORS	Phagocytes/granulocytes Inflammatory mediators Complement Natural killer cells	B-cells → Antibodies T-cells
SPECIFICITY	Pattern recognition	Yes
MEMORY	No	Yes

Under certain circumstances, notwithstanding the natural defensive role of the immune system, inadvertent immune activation can be the cause of tissue injury and disease. Separate terms exist to describe specific immune phenomena. However, given the complex and intertwined nature of the immune system, it is not surprising that different immune-mediated pathophysiological processes may involve overlapping mechanisms.

Inflammation is generally a physiologic immune process forming part of the defence mechanisms and aims to restore tissue injury and homeostasis. The cardinal signs of inflammation are pain, oedema, erythema and heat as a result of increased blood flow, vascular permeability and infiltration of immuno-inflammatory cells (initially neutrophils during the acute phase but subsequently also other phagocytes, macrophages and lymphocytes) to the site of injury (Chaplin, 2010). Pro-inflammatory cytokines (e.g. interleukins (ILs)) as well as chemokines are responsible for inducing and mediating the inflammatory response. Cytokines and chemokines can have multiple actions (pleiotropic) such as instructing cells about effector functions, migration from one location to another as well as survival and death. They may exert these actions in an autocrine, paracrine or endocrine manner but in all cases binding onto a corresponding expressed receptor on the surface of the target cell is a prerequisite for effects to take place (Warrington et al., 2011). Once bound onto their receptor, a signalling cascade is initiated that activates transcription factors and gene expression, ultimately leading to the biologic effect. Despite its natural homeostatic role, inflammation may become abnormally increased and cause unintentional tissue damage alongside the targeted antigen or become chronic, leading to a number of known inflammatory diseases (Schett, Elewaut, McInnes, Dayer, & Neurath, 2013).

Hypersensitivity is defined as an inappropriately exaggerated reaction of the immune system (innate and / or adaptive) to an antigen that would otherwise pose little or no threat to the host. These reactions are unpleasant, can lead to tissue injury and occasionally may be fatal (Parkin

& Cohen, 2001). They are classified according to time of onset and mechanism into four different types (Gell & Coombs Type I – IV classification) as detailed in section **1.2.2**. Hypersensitivity type I - III reactions are mediated by antibodies and involve activation of complement or inflammatory cells while hypersensitivity type IV reactions are mediated by antigen-specific T-cells (hence also termed as delayed-type hypersensitivity). Hypersensitivity typically involves a sensitization (symptom free primary immune response) and an effector (harmful secondary response) phase and most often occurs after long-term exposure which need not be continuous necessarily (Chaplin, 2010).

Allergy is a Type I hypersensitivity reaction mediated by antigen-specific immunoglobulin E (IgE) binding onto corresponding IgE receptors (FcεRs) expressed on the surface of mast cells and basophils. Allergic reactions generally consist of a sensitization phase (priming of immune system upon first exposure to the antigen and production of antigen-specific IgE antibody), an activation phase (IgE-mediated mast cell degranulation upon antigen re-exposure) and an effector phase (inflammatory symptoms secondary to release of mediators) (Yatim & Lakkis, 2015). The main characteristic of allergic reactions, which can be local or systemic (i.e. anaphylaxis), is the extremely rapid occurrence of symptoms upon antigen challenge (within minutes, i.e. immediate hypersensitivity). Allergy (that has an immunological specific causality) differs from anaphylactoid or pseudo-allergic reactions that result from a direct action of a substance on mast cells or inflammatory mediators' production (e.g. non-steroidal anti-inflammatory drugs (NSAIDs) shifting arachidonic acid metabolism towards leukotriene production) (Chaplin, 2010).

Autoimmunity occurs when the host mounts an immune response against its own components (self-antigens). The reasons behind this immune abnormality are complex but generally may be the result of failing self-tolerance mechanisms and / or sequestration of autoantigen, central and peripheral tolerance and / or process and presentation of self-molecules (Parkin & Cohen,

2001). Autoimmune responses do not necessarily imply tissue damage and / or disease and the presence of specific autoantibodies is not always a definitive proof for a causal role in the pathogenesis of a disease (Warrington et al., 2011).

1.4 CARBOXYLIC ACID DRUGS

Carboxylic acids constitute a large group of endogenous and xenobiotic compounds that vary in terms of physicochemical properties and biological actions. In particular, carboxylic acid – containing drugs are represented in several therapeutic classes such as antibiotics, anticonvulsants, diuretics with perhaps the most prominent being that of NSAIDs. The -COOH moiety found on NSAIDs is indispensable for their mechanism of action. NSAIDs are widely used against a number of inflammatory conditions such as osteoarthritis and rheumatoid arthritis (Knodel, Roush, & Barton, 1992). Their mechanism of action relies on the inhibition of the prostaglandin endoperoxide synthase (cyclooxygenase isoforms; COX-1 and / or COX-2) that normally catalyses the conversion of arachidonic acid to prostaglandins, prostacyclins and thromboxanes, which are mediators of pain and inflammation (Rowlinson et al., 2003; Waterbury, Silliman, & Jolas, 2006). Thus, the carboxylic acid group enables the xenobiotic to mimic the arachidonic acid, resulting in competitive antagonism of its binding onto COX receptors (Rowlinson et al., 2003). In addition, -COOH facilitates non-covalent interactions with receptors through hydrogen bonds with polypeptide side chains and electrostatic interactions (Istvan & Deisenhofer, 2001; Ballatore, Huryn, & Smith, 2013). Aside from acting as a pharmacophore, the carboxylate plays an important role in pharmacokinetic properties. The small intestine's pH generally favours dissolution of carboxylic acids to a greater extent due to their ionized nature (Bocker, Bonneau, Hucke, Jakalian, & Edwards, 2010). An increase in polarity and hydrophilicity enhances passive absorption from the gastrointestinal (GI) tract

to the bloodstream, although this property may render intracellular distribution difficult without the action of uptake transporters. However, membrane penetration can be improved through the compound's zwitterion species by matching the -COOH group with a complementary basic centre in the molecule (Cavet, West, & Simmons, 1997; Bocker et al., 2010). Finally, carboxylic acid groups are sometimes inserted into aromatic ring side-chains to protect against CYP metabolism given their increased polarity and electronegativity thus introducing non-CYP mediated metabolism and biliary clearance (Smith, 2010; Bocker et al., 2010).

Metabolic activation of carboxylic acid drugs varies but primarily involves glucuronidation in the liver through the action of the uridine diphosphate (UDP) - glucuronosyl transferase (UGT) enzyme family leading to the formation of reactive AGs (Zia-Amirhosseini, Spahn-Langguth, & Benet, 1994; Ritter, 2000; Skonberg, Olsen, Madsen, Hansen, & Grillo, 2008; Fujiwara, Yoda, & Tukey, 2018). In addition, carboxylic acid drugs may form conjugates with coenzyme A (CoA), catalysed by acyl-CoA synthetases (ACSs), and these drug acyl-CoA thioesters have been shown to exhibit a significant degree of chemical reactivity and protein binding as well as interfering with endogenous lipid metabolism (Olsen, Bjornsdottir, Tjornelund, & Honore, 2002; Grillo, 2011; Darnell & Weidolf, 2013). It is noteworthy that even if a parent compound does not possess a carboxylic acid group within its original structure, appropriate metabolic reactions may be responsible for unmasking a carboxylate and enabling formation of secondary AGs following metabolism by UGTs (Balani et al., 1997; Paulson et al., 2000; Mutlib et al., 2002).

Carboxylic acid – containing drugs have been associated with rare but severe IDRs during their post-marketing phase (Fung et al., 2001). Specifically, NSAIDs have been associated with rare cases of liver injury with hospitalization rates ranging from 3.1 to 23.4 per 100,000 patient years and responsible for about 10 % of total iDILI cases (Jick, Derby, Garcia Rodriguez, Jick, & Dean, 1992; Rubenstein & Laine, 2004; Bessone, 2010; Jessurun & van, 2015). While

jaundice incidence may be as low as 0.01 – 0.1 %, alanine transaminase (ALT) and aspartate transaminase (AST) plasma levels may be abnormal in as much as 5 – 15 % of patients exposed to a particular NSAID (Boelsterli, Zimmerman, & Kretz-Rommel, 1995; Schmeltzer et al., 2016). The pharmacology of the parent compound itself could be contributing to some of these reactions seen in the clinic with COX inhibition potentially dysregulating protective pathways and / or accounting for pseudo-allergic cases, although this is not yet sufficiently explored (Pessayre et al., 1979; Hagmann, Steffan, Kirn, & Keppler, 1987; Reilly et al., 2001; Berkes, 2003; Pham, Kim, Trinh, & Park, 2016). Moreover, the role of underlying disease as a risk factor for NSAID-induced liver injury has also been highlighted with rheumatoid arthritis and osteoarthritis associated with an increased rate of drug hepatotoxicity (Garcia Rodriguez, Williams, Derby, Dean, & Jick, 1994; Banks, Zimmerman, Ishak, & Harter, 1995). While the mechanism of idiosyncratic hepatotoxicity from NSAIDs is generally not well understood, it is believed that bioactivation to reactive metabolites may initiate a cascade of toxicological and immunological events that lead to tissue injury in susceptible individuals (Boelsterli, 2003; Aithal & Day, 2007; Aithal, 2011; Agundez et al., 2011).

1.5 ACYL GLUCURONIDE DRUG METABOLITES

AG drug metabolites are products of phase II biotransformation (glucuronidation), known to be chemically reactive (electrophiles) and able to form protein covalent adducts in human (Smith, Benet, & McDonagh, 1990; Sallustio, Fairchild, & Pannall, 1997; Qiu, Burlingame, & Benet, 1998; Stachulski et al., 2006; Hammond et al., 2014). Furthermore, the instability of AGs in aqueous conditions has been successfully correlated with the amount of irreversible binding to protein (Benet et al., 1993; Bailey & Dickinson, 1996; Ebner, Heinzel, Prox, Beschke, & Wachsmuth, 1999; Wang et al., 2004; Shimada et al., 2018). It is speculated that

AGs may be responsible for some IDRs (including iDILI) seen in the clinic following treatment with certain carboxylic acid drugs and it is furthermore hypothesized that they may be doing so via an immune-mediated mechanism, which involves modification of self-protein and subsequent activation of the immune system (Faed, 1984; Spahn-Langguth, Dahms, & Hermening, 1996; Sallustio, Sabordo, Evans, & Nation, 2000; Bailey & Dickinson, 2003; Stachulski, 2007; Boelsterli, 2011; Iwamura et al., 2015). The following sections summarize existing knowledge on AG drug metabolites and discuss concerns regarding their formation.

1.5.1 Glucuronidation and acyl glucuronides

Conjugation reactions involve the addition of an endogenous chemical group to the molecule resulting in increased hydrophilicity (with the exception of methylation and acetylation) thus facilitating excretion in bile or urine (depending on size of parent compound) and reducing the potential for toxicity (Gibson & Skett, 2001). Phase II reactions are catalysed by transferases, which work by forming a covalent bond between the acceptor group on the foreign compound and the endogenous conjugating group. Glucuronide metabolites are formed through the coupling of nucleophilic acceptor groups (such as hydroxyl and carboxylate) of aglycones with D-glucuronic acid, an oxidative product of D-glucose (Ritter, 2000). Glucose 1-phosphate and uridine triphosphate form UDP-D-glucose (UDPG), which in the presence of nicotinamide adenine dinucleotide (NAD) and UDPG-dehydrogenase generates UDP-D-glucuronic acid (Spahn-Langguth & Benet, 1992). The reaction leading to the formation of glucuronide metabolites is catalyzed by the enzyme family of UDP-glucuronosyltransferases (Kuehl, Lampe, Potter, & Bigler, 2005; Skonberg et al., 2008). There are 2 distinct UGT families (UGT1 & UGT2) that include 19 enzyme isoforms residing in the endoplasmic reticulum and predominantly expressed in the liver (Tukey & Strassburg, 2000). Depending on the atom to

which the glucuronic acid is linked, glucuronide metabolites are categorized in O-, C-, N-, S-glucuronides of which the most frequently encountered are the O-glucuronides. Most glucuronide conjugates are stable however the conjugation of D-glucuronic acid with carboxylic acids leads to the formation of electrophilic, chemically reactive AG metabolites (Shipkova, Armstrong, Oellerich, & Wieland, 2003).

AGs are not only formed by the glucuronidation of carboxylate moieties of drugs. Endogenous substances carrying a -COOH group such as bilirubin, bile and fatty acids are also subject to glucuronidation (Pellock & Redinbo, 2017). Bilirubin, a breakdown product of heme, is one of the most heavily glucuronidated endogenous compounds. The monoglucuronide and diglucuronide conjugates of bilirubin reflect approximately 16 and 80 % of total bilirubin, respectively (Fevery, Blanckaert, Leroy, Michiels, & Heirwegh, 1983). AG metabolites of bilirubin can react with human serum albumin (HSA) *in vivo* and form covalent adducts (Van Breemen, Fenselau, Mogilevsky, & Odell, 1986).

It has been determined that drug AGs are intrinsically reactive chemical entities (electrophiles) both *in vitro* and *in vivo*, capable of undergoing a number of reactions including hydrolysis (re-formation of parent drug), rearrangement and covalent binding to proteins (Williams, Worrall, de Jersey, & Dickinson, 1992; Hayball, 1995; Stachulski et al., 2006; Regan et al., 2010; Camilleri, Buch, Soldo, & Hutt, 2018). During rearrangement, 1β-conjugates undergo intramolecular acyl migration thus forming 2-, 3- and 4- isomers which are equally or even more potent electrophilic species and in addition, unlike 1β-conjugates, resistant to enzymatic hydrolysis by β-glucuronidase (Dickinson, 2011; Stachulski, 2011). In aqueous solution at physiological pH and temperature, 1β-conjugates spontaneously degrade to form either the parent drug (hydrolysis) or isomers (acyl migration). The physicochemical properties and inherent chemical instability of drug AGs is discussed in more detail in ***Chapter 2*** (see ***Figure 2.1***).

1.5.2 Physiological and toxicological implications of drug acyl glucuronide formation

As previously discussed, glucuronidation represents a natural detoxification mechanism which enables excretion of carboxylic acid drugs through two ways. By attaching a glucuronic acid onto the aglycone, the molecules polarity and water solubility increases thereby facilitating renal excretion (Silva et al., 2003). Secondly, glucuronide metabolites are good substrates for biliary transporters (e.g. diclofenac-AG and multidrug resistance associated protein 2 (MRP-2)) and hepato-canalicular drug transporters facilitate excretion in the bile (Lagas, Sparidans, Wagenaar, Beijnen, & Schinkel, 2010; Tetsuka, Gerst, Tamura, & Masters, 2014). The role of glucuronidation as a detoxification pathway is highlighted by a study evaluating *in vitro* the hepatocyte cytotoxicity of certain NSAIDs when borneol was used to inhibit the enzymatic reaction (Siraki, Chevaldina, & O'Brien, 2005). Cytotoxicity was generally increased by 2 to 5-fold, although 5 NSAIDs showed no difference. Similarly, negative results from *in vitro* toxicological evaluation of drug AGs have been presented by other investigators (Koga, Fujiwara, Nakajima, & Yokoi, 2011; Surendradoss, Chang, & Abbott, 2014).

The toxicological concern regarding the formation of drug AGs derives from their covalent binding propensity as a result of their inherent chemical instability alongside the association of AG forming drugs (such as NSAIDs) with IDRs. Drug AGs have been shown to form covalent adducts *in vitro* and *in vivo* with plasma and hepatic proteins, two of which have been identified as HSA and Dipeptidyl Peptidase IV (DPPIV), respectively (Ding, Zia-Amirhosseini, McDonagh, Burlingame, & Benet, 1995; Bailey & Dickinson, 1996; Wang, Gorrell, McGaughan, & Dickinson, 2001; Shipkova et al., 2004; Aithal et al., 2004; Hammond et al., 2014). However, it should be noted that *in vivo* data for DPPIV covalent binding are not conclusive and apart from serum albumin, all other hepatic protein adducts detected and

identified thus far are derived from animal experimentation (Sallustio et al., 2000). Notably, clinically there appears to be an association between a number of AG forming drugs, whose *in vivo* hepatic protein covalent binding has been shown, and hepato-biliary toxicity thus suggesting a mechanistic role of the protein adduction (Bakke, Wardell, & Lasagna, 1984; Powell-Jackson, Tredger, & Williams, 1984; Zimmerman, 1990). Nonetheless, whether these are simply "bystander" proteins and their covalent modification, albeit an interesting finding, is of no actual immunotoxicological concern is currently unknown. Moreover, formation of AGs alone cannot directly imply induction of tissue-specific toxicity. As mentioned in section ***1.5.1***, other endogenous compounds, such as bile acids, may be converted to AGs in rats and humans (Goto, Murao, Oohashi, & Ikegawa, 1998) and it has been shown that they can also form covalent adducts *in vitro* and *in vivo* (Van Breemen et al., 1986; Ikegawa et al., 1999).

Benet et al. first supported the idea of a correlation between different AG structure, protein covalent binding and possible toxicity, while more recently, Sawamura et al. introduced a risk classification system according to the chemical stability of AGs (Benet et al., 1993; Sawamura et al., 2010). Different reactive metabolites might have different binding patterns and within the class of AGs, different half-lives and degrees of substitution at the α-carboxy carbon of the aglycone have been demonstrated to influence the profile of covalent binding (Benet et al., 1993; Ebner et al., 1999). Covalent adduction is achieved by two main mechanisms, namely transacylation and glycation. During transacylation, the AG can directly attack nucleophiles such as GSH or nucleophilic targets (-SH, -OH and $–NH_2$) on proteins and form covalently bound aglycone protein-adducts whilst liberating glucuronic acid (Hyneck, Smith, Munafo, McDonagh, & Benet, 1988; Brunelle & Verbeeck, 1997; Kenny et al., 2005). Conversely, the glycation mechanism involves the AG rearrangement initially, which exposes the inherent reactivity of the sugar moiety. This allows for a non-enzymatic reaction with protein amino groups via the exposed aldehyde group therefore retaining the glucuronic acid in the formed

adduct (Ding et al., 1995; Nicholls et al., 1996; Sallustio et al., 1997; Qiu et al., 1998). This protein adduct can then undergo further non-enzymatic reactions which can lead to advanced glycation end products (AGEs) (Smith & Wang, 1992; Chiou, Tomer, & Smith, 1999). These mechanisms are discussed in more detail in ***Chapter 2*** (see ***Figure 2.2***).

Aside from the intrinsic chemical properties of AGs, their disposition and tissue exposure may play a critical role with regards to their questionable toxicological significance. Indeed, whilst the metabolic turnover to an AG may indicate formation of an increased amount of metabolite, the distribution in various tissues as well as clearance mechanisms will have a direct implication in the overall metabolite burden (Regan et al., 2010). The exposure levels to AGs may differ between tissues and an exemplary case is the situation occurring in the liver / bile canaliculi region. Even in the absence of detectable plasma AG concentrations, significant intra-hepatic protein adduct formation can occur as a result of the concentrative effect of carrier-mediated hepatic membrane transport (Evans, 1996). MRP-2, MRP-3 and organic-anion-transporting polypeptide (OATP) play an important role in the hepatic distribution of AGs leading to a large gradient of metabolite concentration (Sabordo, Sallustio, Evans, & Nation, 1999). Most importantly, MRP2 is responsible for secretion of drug AGs into the bile, while MRP3 and OATP represent basolateral efflux and uptake transporters, respectively (Lagas et al., 2010). Due to active uptake / efflux transportation of AGs, the concentration ratio between blood, hepatocyte and bile may reach the scale of up to 1:50:5000 (Sallustio et al., 2000). This presumably explains the selective targeting of proteins facing the biliary tree (e.g. DPPIV) by various drug AGs (Hargus, Martin, George, & Pohl, 1995; Seitz, Kretz-Rommel, Oude Elferink, & Boelsterli, 1998; Wang et al., 2001; Aithal et al., 2004; Shipkova et al., 2004). To this end, the competing glucuronidation and hydrolysis processes, as well as systemic and entero-hepatic cycling of AGs, in conjunction with clearance rates need also to be taken into consideration. In humans, the predominant route of AG secretion is renal as opposed to biliary

in rats, possibly due to a difference in the size threshold for clearance (500-600 Da in human vs 300-400 Da in rat) (Hirom, Millburn, & Smith, 1976; Klaassen & Watkins, 1984). Nonetheless, it is clearly a variable process with various AGs exhibiting different elimination routes and rates (Verbeeck, Dickinson, & Pond, 1988; Balani et al., 1997; Wang et al., 2006). A rapid AG *in vivo* clearance may render a highly reactive metabolite less concerning with regards to covalent protein binding, although overall tissue exposure is determined by additional factors such as UGTs, β-glucuronidase, esterases, drug transporters as well as underlying disease state. In particular, bacterial β-glucuronidase and intestinal carboxylesterases facilitate de-conjugation of drug AGs (but not iso-glucuronides) that are excreted via the biliary tree into the gut (Brunelle & Verbeeck, 1997). This leads to a rapid re-uptake of the aglycone followed by entero-hepatic circulation which favours prolonged exposure to the hepatic tissue as well as retention of the metabolites in the hepatocytes due to competition at the hepato-canalicular transporter site (Sallustio et al., 2000).

Limited research presenting *in vivo* efforts to link specific AG – protein adducts with a stimulation of the adaptive immune system has been published, mainly via the use of animal models. Worrall & Dickinson, (1995) presented data to support that diflunisal-AG – rat serum albumin adduct was immunogenic (production of antibodies) in treated rats and similar work was carried out by Zia-Amirhosseini, Harris, Brodsky, & Benet, (1995) for tolmetin-AG. It has to be noted, however, that their experimentation setting included the use of adjuvants in order to provoke a systemic immune response. Another interesting experiment by Kretz-Rommel & Boelsterli, (1995) demonstrated an immune-based mechanism secondary to diclofenac-mediated protein conjugates, although it also relied on the presence of an adjuvant and the direct involvement of diclofenac-AG cannot be ascertained. On the other hand, Naisbitt et al., (2007) used an animal model to test the immunogenicity of diclofenac and its metabolites. They concluded that diclofenac-AG did not stimulate an immune response in that test system but

instead the oxidative metabolite, 5'-hydroxy diclofenac, did. ***Chapter 3***, describes in more detail the hypotheses and current knowledge between AG formation and immunogenicity.

1.6 IDIOSYNCRATIC DRUG TOXICITY

The term idiosyncrasy derives from Greek "ιδιοσυγκρασία" denoting a peculiar temperament of the body unique to an individual. Idiosyncratic drug toxicity is a rare phenomenon affecting a very small group of patients (between 1/1,000 – 1/200,000) (Guengerich, 2011; Iasella et al., 2017) and the causality is usually a function of the chemistry of the drug and biology of the individual (Pichler, Naisbitt, & Park, 2011). Despite the low incidence rate, idiosyncratic toxicity associated with widely used drugs, for which millions of prescriptions may be written over the course of the marketed life, can lead to hundreds of patients becoming affected. Whilst it is suggested that idiosyncratic drug toxicity is not necessarily dose-dependent, there is an element of dose-relatedness associated with drug exposure as illustrated by Lammert et al., who indicate that doses of 10 mg / day or less are less likely to elicit idiosyncratic hepatotoxicity for a range of examined medications (Lammert et al., 2008). Mechanistically, a simplified view distinguishes idiosyncratic drug toxicity into immune-mediated (immune idiosyncrasy) or non-immune-mediated (metabolic idiosyncrasy). However, the molecular basis of most IDRs is not fully deciphered and it is likely that some incidents may be a combination of both metabolic and immune idiosyncrasies (Waring & Anderson, 2005; Uetrecht, 2007; Uetrecht, 2009; Zhang, Liu, Chen, Zhu, & Uetrecht, 2011). Metabolic idiosyncrasy typically lacks clinical features of an immunological reaction (e.g. fever, rash, eosinophilia and anti-drug antibodies) and is most often linked with genetic polymorphisms of biotransformation enzymes or enzymes affecting other biochemical pathways (such as energy pathways or mitochondrial function). A classic example of metabolic idiosyncrasy is drug-

induced haemolysis as a result of glucose-6-phosphate dehydrogenase (G6PD) deficiency (Frank, 2005). Conversely, immune idiosyncrasy involves DHRs as well as organ-specific reactions, sometimes with autoimmune features, and whilst it is difficult to estimate the frequency between the two types of idiosyncratic drug toxicity as definitive evidence is lacking in most cases, it is believed that the majority of IDRs are driven by the activation of the immune system (Zhang et al., 2011; Uetrecht & Naisbitt, 2013; Uetrecht, 2019a). Halothane hepatitis, as described in section **1.3.1**, is an example of immune-mediated IDR. IDRs can affect several organs but the most common targets are the skin (e.g. maculopapular rash, urticaria, fixed drug eruption, acute generalized exanthematous pustulosis, drug reaction with eosinophilia and systemic symptoms (DRESS), Stevens-Johnson syndrome), liver (e.g. hepatocellular and / or cholestatic liver injury) and hematopoietic system (e.g. agranulocytosis, thrombocytopenia, anaemia) (Uetrecht & Naisbitt, 2013). IDRs can exhibit their symptoms locally or systemically with varying severity and may affect more than one organ at a time (e.g. DRESS). Finally, a drug that is associated with a high incidence of idiosyncratic toxicity may be responsible for causing a wide variety of IDR types amongst different patients (Neftel, Woodtly, Schmid, Frick, & Fehr, 1986; Jain, 1991; Syn, Naisbitt, Holt, Pirmohamed, & Mutimer, 2005).

1.6.1 The liver as a target organ for idiosyncratic drug toxicity

Drug-induced liver injury (DILI) represents the most common reason for acute liver failure in the USA and Europe (Larrey & Pageaux, 2005; Fontana et al., 2014), while it accounts for up to 14.7 % of fatal ADRs (Friis & Andreasen, 1992). DILI can be the result of intrinsic toxicity which is predictable (e.g. acetaminophen overdose) or idiosyncratic toxicity mediated by immune and / or non-immune mechanisms (Kaplowitz, 2005; Russmann, Kullak-Ublick, & Grattagliano, 2009; Tujios & Fontana, 2011; Fontana, 2014; Mosedale & Watkins, 2017;

Andrade et al., 2019). Idiosyncratic hepatotoxicity, despite being a rare phenomenon, accounts for about 10 – 15 % of acute liver failure in the USA (Ostapowicz et al., 2002; Reuben, Koch, & Lee, 2010) and is the single most important cause for drug attrition in the pharmaceutical industry as well as being implicated in numerous post-marketing drug withdrawals (Guengerich, 2011; Regev, 2014; Yamashita et al., 2017). Epidemiological studies reveal the crude annual incidence of iDILI as 13.9 and 19.1 cases per 100,000 inhabitants in France and Iceland, respectively, with nearly 30 % exhibiting jaundice (Sgro et al., 2002; Bjornsson & Jonasson, 2013; Bjornsson, 2014). Given the liver's anatomical position and vascularized condition, receiving blood via the hepatic artery and portal vein (30 % of total blood volume passes through the liver each minute), as well as functional properties as a major organ for biotransformation reactions, it is not surprising that it is a prominent target for drug toxicity (Sheth & Bankey, 2001; Racanelli & Rehermann, 2006; Amacher, 2012). Furthermore, the liver is a highly immunocompetent organ containing several types of innate and adaptive immune cells thus maximizing immune surveillance for all incoming pathogens (Jenne & Kubes, 2013). Aside from hepatocytes (~ 2/3 of total liver cell population), the liver comprises approximately 10^{10} resident lymphocytes including T-cells ($CD8^+$ enriched), natural killer (NK) cells (between 30 – 50 % of total resident lymphocytes) and natural killer T (NKT) cells as well as various resident antigen-presenting cells (APCs) such as Kupffer cells (KCs), liver sinusoidal epithelial cells (LSECs) (~ 50 % of non-parenchymal cells in the liver), dendritic cells (DCs) and stellate cells (Parker & Picut, 2005; Crispe, 2011). Hepatocytes express major histocompatibility complex (MHC) class I constitutively and can engage with and prime $CD8^+$ T-cells that are found in higher proportion in the liver vasculature. Moreover, peripheral blood leukocytes can be carried into the liver either as part of the normal blood circulation (~ 10^8 peripheral lymphocytes per day) or migrate in response to the activation of immune sentinel cells (Wick, Leithauser, & Reimann, 2002; Racanelli & Rehermann, 2006). Although the liver

is an immune privileged organ with high capacity for immune tolerance, situations exist where this may be overcome (Knolle & Gerken, 2000) (see section **1.6.3**). Thus, the co-existence of an immunologic surveillance environment in the liver alongside increased reactive drug metabolite formation provides a platform for idiosyncratic drug toxicity risk.

Clinically, iDILI usually presents like acute hepatitis and may resemble forms of other hepatic injuries with diverse biochemical and histological features (Abboud & Kaplowitz, 2007; Licata, 2016; Watkins, 2019). Therefore, liver biopsy is of limited value and there are no definitive pathologic features to enable diagnosis based on biopsy information alone (Kullak-Ublick et al., 2017; Hassan & Fontana, 2019). Clinical diagnosis of iDILI relies on a clinical index of suspicion, the chronological relationship between drug intake and onset of symptoms, biochemical liver injury tests as well as exclusion of alternative explanations for the clinical manifestations (Fontana, 2014; Ortega-Alonso, Stephens, Lucena, & Andrade, 2016; Marrone et al., 2017). The suggested liver test abnormality thresholds are: an ALT value of ≥ 5 times the upper limit of normality (ULN) or an alkaline phosphatase (ALP) value of ≥ 2 times the ULN or an ALT value of ≥ 3 times the ULN and total bilirubin (TB) of ≥ 2 times the ULN (Aithal et al., 2011). Even though elevations in serum AST and ALT do not reliably correlate with the degree of hepatic impairment and perhaps the prothrombin time test can better indicate loss of hepatic functional mass, the former have been associated with development of acute liver failure in iDILI patients (Zimmerman, 1993; Goldkind & Laine, 2006). Based on the biochemical signature and the ratio of liver function abnormalities (R ratio = (ALT/ULN) / (ALP/ULN)), the pattern of liver damage may be classified in 3 types: hepatocellular (ALT $\geq$ 3x ULN and R ≥ 5), cholestatic (ALP $\geq$ 2x ULN and R ≤ 2) or mixed (ALT $\geq$ 3x ULN and ALP $\geq$ 2x ULN with $2 < R < 5$) (Danan & Benichou, 1993). Severity of the injury may range from mild asymptomatic (elevated ALT or ALP and TB < 2x ULN), to jaundice and to acute liver failure (Ortega-Alonso et al., 2016). Patients who present with acute hepatitis (i.e. serum

ALT ≥ 3x ULN) and jaundice (i.e. total bilirubin > 2.5 mg/dl) have at least 10 % risk of mortality, otherwise known as “Hy’s law” (Zimmerman, 1968). Depending on the duration of biochemical liver abnormalities, iDILI may be characterized as acute or chronic (lasting ≥ 3 months after iDILI onset) (Hassan & Fontana, 2019). Although the mechanisms behind iDILI are still not entirely understood and the involvement of the immune system in this organ-specific IDR is more controversial than others, the translational research evidence presented in the following sections strengthens the case for an immunological component in the pathogenesis.

1.6.2 Role of the immune system in idiosyncratic drug hepatotoxicity

There exist direct and indirect clinical characteristics to indicate an involvement of the immune system in most iDILI. Almost universally, there is a delay between first drug intake and onset of iDILI (typically 1 – 3 months); however the time to onset can vary significantly between different incidents (Graham, Green, Senior, & Nourjah, 2003; Clay et al., 2006; Orman et al., 2011; Uetrecht & Naisbitt, 2013). This lag between drug exposure and onset of reaction is suggestive of a delayed-type hypersensitivity immune response, justified by the time required for the adaptive immune cells to become activated and proliferate in numbers sufficient to bring about a clinical response. Regardless of the histopathological type of the injury (hepatocellular, cholestatic, mixed), the latency period has been reported to range from a couple of days or weeks to several months from first intake and in some cases the reaction may occur even after the drug is withdrawn presumably due to a combination of slow immune response development and prolonged retention of the drug in the body (Devereaux, Crawford, Purcell, Powell, & Roeser, 1995; Fontana, 2014; Kuzu et al., 2016; Lu et al., 2016). Specifically, in autoimmune-like iDILI, the onset of injury features may not arise for several years after first drug exposure

in some cases (Lawrenson, Seaman, Sundstrom, Williams, & Farmer, 2000; Weiler-Normann & Schramm, 2011; deLemos et al., 2014). Despite the scarcity of data, reports indicate that iDILI reoccurs upon drug re-challenge (a sign of immune memory) (Greaves et al., 2001; Hunt, Papay, Stanulovic, & Regev, 2017), however exceptions to this have also been reported (Maddrey & Boitnott, 1973; Papay et al., 2009; Keisu & Andersson, 2010). Occasionally, additional clinical manifestations of iDILI point towards a hypersensitivity-type reaction such as the presence of skin rashes, urticaria and fever, however, the absence of these symptoms does not dismiss the potential involvement of immune mechanisms in the pathogenesis of the reaction (Maddrey & Boitnott, 1973; Ibanez, Perez, Vidal, & Laporte, 2002; Andrade et al., 2005; Devarbhavi & Raj, 2019). Certain histology features of the injured liver commonly including the presence of a mononuclear immune cell infiltrate (e.g. T-cells) are suggestive of activated immune responses although it has to be acknowledged that the presence of these cells at the site of injury may be a response to the injury itself rather than the cause of it (Fukano et al., 2000; Mennicke et al., 2009; Wuillemin et al., 2014; Kleiner et al., 2014; Foureau et al., 2015). iDILI has sometimes been associated with the presence of circulating autoantibodies and / or anti-drug or anti-drug protein adduct antibodies indicating the presence of an active humoral immunity but, similarly to the histological findings, it's unclear whether these antibodies mediate the injury especially since they can also be found in drug-exposed patients without clinically obvious liver injury (Kenna et al., 1988; Satoh et al., 1989; Pirmohamed, Kitteringham, Breckenridge, & Park, 1992; Aithal et al., 2004; Metushi, Sanders, Lee, & Uetrecht, 2014). As described in **1.2.2**, hypersensitivity-type reactions may involve multiple mechanisms and are usually classified in four sub-types. Unlike type I drug hypersensitivity, which is IgE-mediated and exhibits immediate clinical symptomatology upon challenge (i.e. anaphylaxis), the majority of iDILI is considered to be type IV drug hypersensitivity given its delayed onset of symptoms. It is furthermore noteworthy that, although the aforementioned

clinical features provide insights for an involvement of the immune system in iDILI, it is still possible that an immunological basis can exist in the absence of a clear clinical signature of hypersensitivity reactions (Bjornsson, Kalaitzakis, Av, V, Alem, & Olsson, 2007; Kindmark et al., 2008).

Human immunological investigations for certain drugs implicated in iDILI cases have enabled researchers to enrich clinical and histopathological observations with mechanistic findings pointing towards a functional relationship between the adaptive immune system and iDILI. Warrington et al. conducted a Lymphocyte Transformation Test (LTT) in patients with a history of isoniazid-induced idiosyncratic hepatotoxicity and found that 95% of patients with elevated ALTs during isoniazid treatment correlated with positive test responses (Warrington, Tse, Gorski, Schwenk, & Sehon, 1978). Further expanding on this, an assessment of 95 cases of drug-induced liver injury (various medications) using the LTT demonstrated positive responses in over 50% of included patients (Maria & Victorino, 1997). Genome Wide Association Studies (GWAS) have revealed associations between iDILI incidents and various HLA genes aside from metabolizing enzymes (Berson et al., 1994; Daly & Day, 2012; Alfirevic & Pirmohamed, 2012; Aithal & Grove, 2015; Usui & Naisbitt, 2017; Nicoletti et al., 2017). HLA associations with iDILI are exemplified by drugs such as flucloxacillin (HLA-B*5701), amoxicillin / clavulanic acid (HLA-A*0201, HLA-DRB1*1501), isoniazid (HLA-DQB1*0201), lumiracoxib (HLA-DQA1*0102, HLA-DRB1*1501), lapatinib (DQA1*0201, HLA-DRB1*0701), ticlodipine (HLA-A*3301) and ximelagatran (HLA-DRB1*0701) (Sharma, Balamurugan, Saha, Pandey, & Mehra, 2002; Kindmark et al., 2008; Daly et al., 2009; Singer et al., 2010; Lucena et al., 2011; Spraggs et al., 2011; Nicoletti et al., 2017). Further in-depth characterization of immune responses for some of these drugs has strengthened the immunological basis in the development of iDILI. In patients with trimethoprim-induced liver injury, $CD4^+$ and $CD8^+$ T-cells were found to proliferate against

trimethoprim and secrete interferon γ (IFNγ) and IL-13 (El-Ghaiesh et al., 2011). Flucloxacillin-responsive T-cell clones secreting IFNγ, Th2 cytokines and cytotoxic mediators (granzyme B, perforin, FasL) were identified using blood samples isolated from flucloxacillin-induced liver injury patients and the response was shown to be processing-dependant and HLA-B*5701 restricted (Monshi et al., 2013; Wuillemin et al., 2014; Yaseen et al., 2015). Moreover, isoniazid-specific $CD4^+$ T-cell clones that secreted IFNγ as well as cytotoxic mediators (granzyme B, perforin, FasL) were discovered in patients with anti-tuberculosis drug-related liver injury (Usui et al., 2017). Kim et al. generated amoxicillin and clavulanic $CD4^+$ and $CD8^+$ T-cell clones from amoxicillin-clavulanic-induced liver injury patients and showed an MHC class II (in particular a HLA-DR) restricted proliferative response of the amoxicillin $CD4^+$ clones (Kim et al., 2015).

Despite these findings across a range of different drugs involved in iDILI, it's worth highlighting that not every investigation has yielded positive results and Faulkner et al. describe the absence of evidence of drug-specific activation of circulating T-cells in blood isolated from lapatinib-induced liver injury expressing HLA-DRB1*0701 (Faulkner, Meng, Naisbitt, Spraggs, & Park, 2016). Although the knowledge around the involvement of the adaptive immune system in iDILI is increasing, many aspects along the cascade of haptenicity, antigenicity, immunogenicity and tissue injury are still unclear (Pirmohamed, Ostrov, & Park, 2015; White, Chung, Hung, Mallal, & Phillips, 2015). Notably, Metushi et al. investigated peripheral blood mononuclear cells (PMBCs) isolated from isoniazid-treated patients using flow cytometry and observed an increase in $CD4^+IL17^+$ T-cells (Th17) and $CD3^+IL10^+$ in those patients that were experiencing a mild liver injury based on increased ALT levels as opposed to patients with stable ALTs (Metushi, Zhu, Chen, Gardam, & Uetrecht, 2014). A possible involvement of Th17 in iDILI is attracting attention due to the role of IL-17 in immune-mediated diseases (Bettelli, Korn, & Kuchroo, 2007; Korn, Bettelli, Oukka, & Kuchroo, 2009;

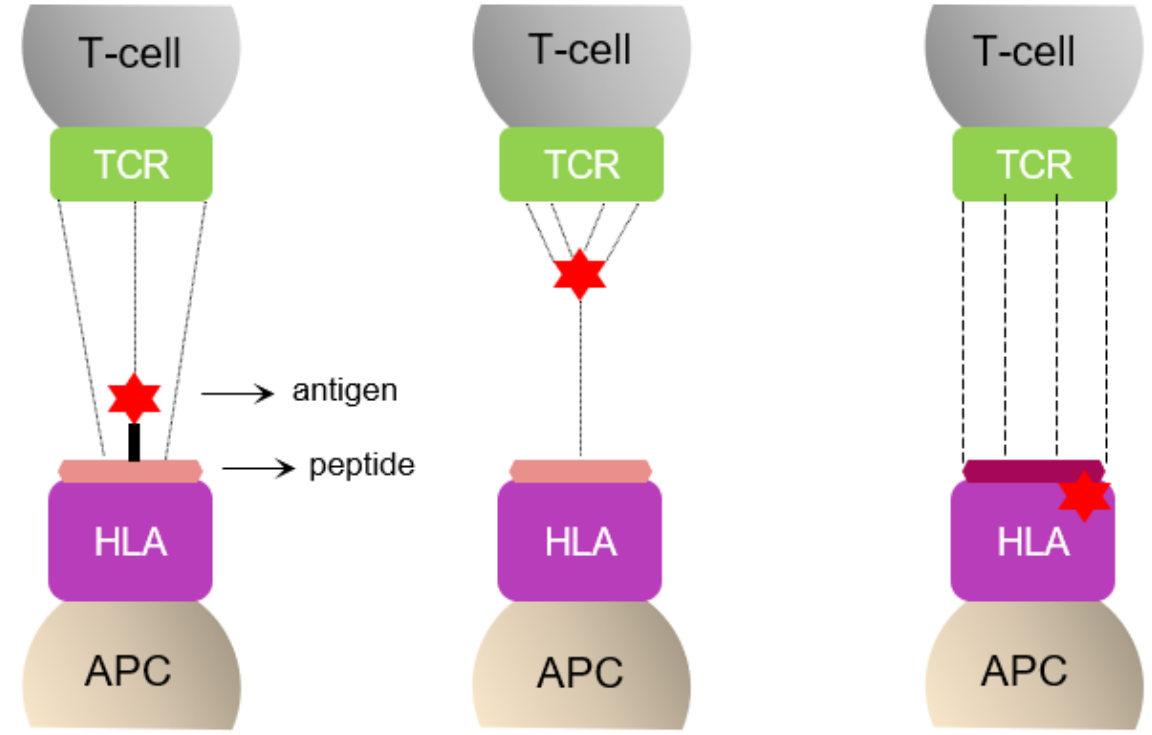
Ye, Li, Zheng, & Chen, 2011; Noack & Miossec, 2014). These data also suggest that induction of immune tolerance may control the injury progression. Consequently, it is possible that immune tolerogenic cytokines, such as IL-10, facilitate the induction of immune tolerance in sensitized patients, which abrogates injury, while a small fraction of patients fails to clinically adapt and thus progresses onto severe liver injury (Aithal et al., 2004; Dara, Liu, & Kaplowitz, 2016).

IDRs in animals are as idiosyncratic as in humans and differences in drug exposure between animal and human as well as the lack of relevant HLA alleles hinder the establishment of valid animal models to investigate the mechanistic nature of the reaction (Ng et al., 2012). However, novel approaches with few drugs in animals have recently enabled simulation of the delayed onset and other typical characteristics of the reaction seen in human and may shed additional light into the role of immune responses and subsequent liver injury. As mentioned before, halothane hepatitis is thought to be an immune-mediated IDR. Multiple attempts from various research groups to replicate the reaction in animal were unsuccessful, albeit revealing an initial innate immune response (McLain, Sipes, & Brown, Jr., 1979; Furst & Gandolfi, 1997; Cheng, You, Yin, Holt, & Ju, 2010; Proctor et al., 2014). Recently, Chakraborty et al. observed that intraperitoneal treatment of BALB/cJ mice with 30 mmol / kg halothane displayed self-limiting ALT increases similar to humans as well as a marked increase in myeloid-derived suppressor cells (MDSCs; $CD11b^{+}Gr1$ high) infiltrating the liver (Chakraborty et al., 2015). MDSCs exhibit immunosuppressive properties and when the investigators depleted this cell population with anti-Gr1 prior to initial halothane treatment, a liver injury reaction occurred with delayed onset (9 days after drug re-challenge) characterized by eosinophilia and increases in IL-4, $CD4^{+}$ and $CD8^{+}$ T-cell infiltration. Notably, further characterization revealed that $CD4^{+}$ T-cell depletion protected the animals from liver injury. Typical of an idiosyncratic reaction, not all animals exhibited the same delayed onset reaction. Even though the injury eventually resolved

with continued treatment, the findings from this animal investigation may help to explain why most patients adapt to halothane treatment whilst some progress onto severe iDILI by breaking immune tolerance.

1.6.3 Mechanisms of immune activation by drugs and reactive metabolites

The immune system may become activated during idiosyncratic hepatotoxicity via several diverse mechanisms. However, it should be emphasized that they are not mutually exclusive and thus can be occurring concomitantly (Adams, Ju, Ramaiah, Uetrecht, & Jaeschke, 2010; Bell et al., 2013; Wuillemin et al., 2013; Mak & Uetrecht, 2017). In addition, non-immune elements may work synergistically to initiate and / or propagate an immune response as will be described later in this section. Notably, the immune activation mechanisms of the same iDILI phenotype caused by different drugs may vary and the mechanisms of a specific iDILI phenotype associated with a specific drug may differ amongst affected patients. ***Figure 1.1*** depicts the currently known main mechanisms of T-cell activation by drugs / metabolites. ***Figure 1.2*** illustrates the complex theoretical interplay between immune and non-immune elements in the context of iDILI. Given the nature and scope of this Thesis, emphasis will be placed in the mechanisms of T-cell activation by drugs / metabolites while non-immune aspects will only be discussed in the context of synergy with immune activation.



A. Hapten model **B. p-i model** **C. Altered peptide repertoire model**

Figure 1.1. Models of drug / metabolite-specific T-cell activation, adapted from Bharadwaj et al., (2012). A) Hapten model: drugs / metabolites act as haptens and modify certain self-proteins in the host that lead to immune recognition of the resulting hapten-self-peptide complexes as *de novo* antigens. **B)** Pharmacological – interaction (p-i) model: drugs / metabolites can induce the formation of HLA or TCR - drug / metabolite complexes that can activate T-cell immune responses directly without requiring a specific peptide ligand. **C)** Altered peptide repertoire model: drugs / metabolites bind to the MHC in a noncovalent manner thus altering the shape and chemistry of the antigen binding cleft and leading to the "loading" of an altered array of self-peptides. The red star represents an antigen (drug / metabolite).

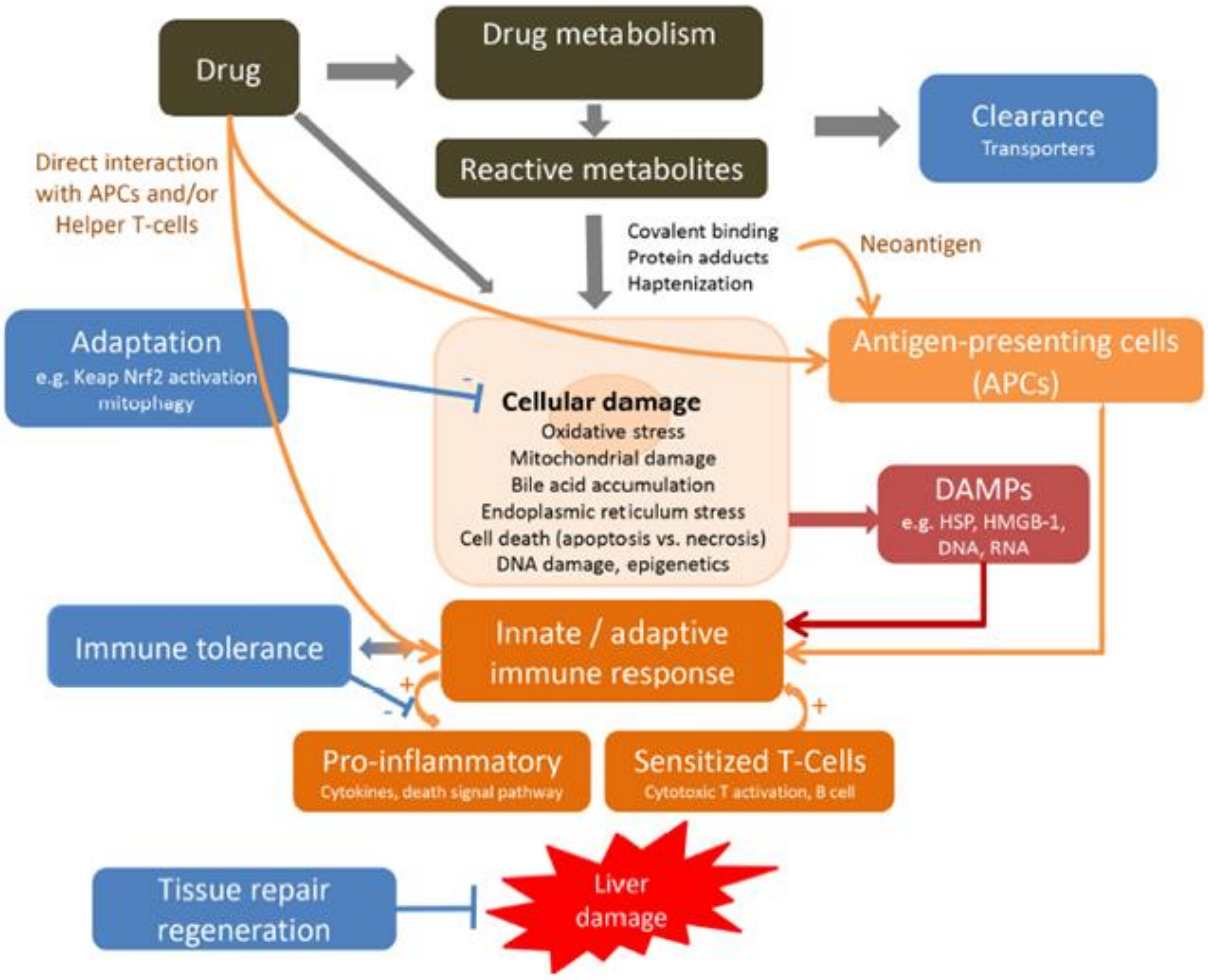


Figure 1.2. Illustration of theoretical interplay between non-immune and immune mediated events relevant to idiosyncratic hepatotoxicity, adapted from Chen et al., (2015). The immune system can become activated directly by inert chemical compounds or following drug metabolism to a reactive metabolite. The latter may bind covalently onto macromolecules forming neo-antigens that can be recognized as foreign by the immune system. In addition, reactive metabolites may cause a series of cellular events which ultimately can act synergistically and facilitate immune activation (adaptive system) and / or propagate an inflammatory response (sterile inflammation). Alongside the cascade, natural homeostatic mechanisms exist such as drug elimination and clearance, adaptation to cellular stress, immune tolerance and liver repair regeneration. In few susceptible individuals, an imbalance in any of these mechanisms may mean that the threshold to injury is impacted.

- *Hapten theory*

The traditional theory behind xenobiotic-induced immune responses originates from the initial observations that chemical sensitization potential *in vivo* correlated with protein reactivity *in vitro* (Landsteiner & Jacobs, 1935). This formed the basis of the theory postulating that a small (low-molecular weight) chemical entity is unable to directly activate the adaptive immune system, however if it is chemically reactive it may be able to irreversibly bind onto endogenous proteins (cellular or circulating) and form an adducted protein complex which is subsequently recognized as non-self by the immune system (Park et al., 1998; Uetrecht, 2000). This hypothesis was termed as the hapten theory ("άπτειν": Ancient Greek word meaning "to fasten") and involves the uptake of the resultant drug-protein complex by APCs, such as DCs, processing and release of drug-modified peptides that bind directly onto HLA molecules and are presented to T-cells (Merk, Baron, Hertl, Niederau, & Rubben, 1997; Steinman, Inaba, Turley, Pierre, & Mellman, 1999). Aside from cell-mediated immunity, the hapten theory also applies in humoral immune responses and it has been shown that the variable regions of the antibody's antigen binding part form a hydrophobic pocket that facilitates hapten-protein complex recognition upon contact (Anglister, Frey, & McConnell, 1985). Some drugs, like β-lactams, are intrinsically reactive and can irreversibly bind onto proteins directly, whereas others, such as sulfamethoxazole (SMX), are chemically inert and require metabolism to a reactive metabolite first (usually termed pro-haptens) (Yvon, Anglade, & Wal, 1990; Cheng et al., 2008; Jenkins et al., 2009). Investigations on β-lactam-induced hypersensitivity reactions have formed the cornerstone of the involvement of haptenated proteins in the activation of the adaptive immune system. Cloned T-cells from hypersensitive patients were shown to respond against β-lactams as well as synthetically made drug – HSA conjugates with the responses inhibited when the antigen-processing pathway was blocked (Brander et al., 1995; Padovan, Bauer, Tongio, Kalbacher, & Weltzien, 1997; El-Ghaiesh et al., 2012; Jenkins et al., 2013;

Meng et al., 2016; Meng et al., 2017). In addition, several studies have delineated the significance of SMX's oxidative metabolism into the reactive sulfamethoxazole-nitroso (SMX-NO) and subsequent covalent protein binding leading to protein-processing-dependant T-cell responses and presentation of modified peptides to specific TCRs in an MHC-restricted or unrestricted manner (Schnyder et al., 2000; Naisbitt et al., 2001; Farrell et al., 2003; Castrejon et al., 2010; Ogese et al., 2015). Moreover, halothane-induced hepatitis is another example that incorporates the hapten concept. Halothane is metabolized via P450 into trifluoroacetyl chloride and antibodies against trifluoroacetyl chloride-modified protein have been found in the sera of hepatotoxicity patients, however, as mentioned earlier, it is not yet clear whether these antibodies mediate halothane-induced liver injury (Vergani et al., 1980; Satoh et al., 1989; Njoku et al., 1997). Nonetheless, haptenated proteins are also found in patients not exhibiting hypersensitivity reactions (Jenkins et al., 2009; Hammond et al., 2014) and recently it was shown that the threshold levels of piperacillin – HSA modification sufficient to activate T-cells are achieved in hypersensitive as well as non-hypersensitive patients thus indicating that additional factors such as the presence of unique T-cells in the individual's repertoire and / or immune balance deregulation may play a role (Meng et al., 2017).

- *Danger hypothesis*

Although irreversible binding of a hapten onto a protein can form an antigen, it is evident that mounting an immune response may require additional "signalling". The simple immunogenic model of self-nonself as first proposed by Burnet, (1959) and further elaborated by Bretscher & Cohn, (1970) does not explain why exposure to every single foreign antigen does not necessarily trigger an immune response. Janeway, (1989) refined this simple model by introducing the infectious-nonself model where the gate-keeping step to enable an immune reaction is activation of APCs by recognition of pathogen-associated molecular patterns (PAMPs) found on pathogens. Thus, in the context of drug hypersensitivity, Matzinger, (1994)

reasoned that alongside hapten-protein complex uptake, processing and presentation, an additional signal is required to activate the APCs; a concept known as the danger hypothesis. These danger signals, referred to as damage-associated molecular patterns (DAMPs), analogous to PAMPs, may be released from damaged cells during tissue injury or be drug irrelevant and associated, for example, with a viral infection, physical injury or other inflammatory conditions (Levy, 1997; Gallucci & Matzinger, 2001; Seguin & Uetrecht, 2003; Harris & Raucci, 2006; Weston & Uetrecht, 2014). Far from a complete list, extracellular high mobility group box protein 1 (HMGB1), adenosine triphosphate (ATP), heat shock proteins (HSPs), S100 proteins, mitochondrial DNA, uric acid and N-formyl peptides are some of the molecules that have been described as DAMPs (Magna & Pisetsky, 2014; Nakahira, Hisata, & Choi, 2015; Venereau, Ceriotti, & Bianchi, 2015). According to this framework, danger signals facilitate the activation of APCs leading to co-stimulatory molecule upregulation and acting as on-off switch for the immune response (Anderson & Matzinger, 2000; Matzinger, 2007; Pallardy & Bechara, 2017). Consequently, it has been shown that metabolism of SMX and formation of protein adducts above a certain threshold stimulates cell injury thus providing a strong activation signal to DCs (Elsheikh et al., 2010). However, SMX and SMX-NO can also directly activate DCs (Sanderson et al., 2007). Other drugs have also been shown to activate APCs directly and various mechanisms have been proposed including reactive metabolite formation by the myeloperoxidase system in several APCs, binding to Toll-like receptors (TLRs) or irreversible binding to aldehyde containing signalling molecules (Uetrecht, 1989; Li & Uetrecht, 2009).

- *Pharmacological – interaction (p-i) model*

The hapten theory and its complementary concepts, such as danger signalling, have dominated the mechanistic basis of adaptive immune system activation for decades, however newer concepts of drug hypersensitivity point towards alternative pathways of immune stimulation

and T-cell activation, excluding the necessity of chemical reactivity and irreversible protein binding (Pichler, 2008; White et al., 2015; Pavlos et al., 2015; Sullivan, Watkinson, Waddington, Park, & Naisbitt, 2018). Zanni et al. first observed that T-cells isolated from sulphonamide-induced hypersensitivity patients were capable of proliferating against sulfamethoxazole and in the absence of metabolism (Zanni et al., 1998). This led to the pharmacological-interaction (p-i) model put forth by Pichler, (2008), which stipulates that a drug or drug metabolite can directly bind onto HLA or TCR molecules through a non-covalent "pharmacological interaction" and thus initiate an immune response without haptenation. In other words, this model resembles an off-target adverse reaction where a drug "docks" onto HLA or TCR out of chance and bypasses the need for danger signalling and innate system involvement. Notably, several drug hypersensitivity studies (including but not limited to iDILI) exemplified by sulfamethoxazole, carbamazepine, lamotrigine, abacavir, penicillin and dapsone, have shown that it is possible to activate T-cells in an MHC-restricted manner even when APCs are fixated (i.e. blocking the protein processing pathway) while removal of soluble drug through extensive washing of drug-treated APCs prevents T-cell activation (Schnyder et al., 2000; Naisbitt et al., 2003b; Engler, Strasser, Naisbitt, Cerny, & Pichler, 2004; Wu et al., 2006; Alzahrani et al., 2017; Zhao et al., 2019). The p-i model is divided into two main categories depending on HLA or TCR non-covalent drug binding (Pichler, 2019): a) the drug may interact preferentially with the TCR (p-i TCR) eliciting a T-cell response directly (Depta et al., 2004; Watkins & Pichler, 2013) or b) the drug may interact preferentially with the binding groove of HLA resulting in the formation of an allo-HLA (p-i HLA) or with the peptide presented in the binding groove of HLA resulting in a foreign peptide-HLA complex (von Greyerz et al., 2001; Mallal et al., 2002; Yun et al., 2014).

- *Altered self-peptide repertoire concept*

A recent concept, termed as altered self-peptide repertoire, proposes that drugs may form Van der Waals contacts with the binding groove of HLA thus altering the shape and chemistry of the cleft, which results in presentation of self-peptides different to those normally bound (i.e. the endogenous peptide is not new to the cell but is has never been presented in this way before) and thus ultimately elicit a T-cell response (Adam et al., 2012; Illing et al., 2012; Norcross et al., 2012; Ostrov et al., 2012; Naisbitt et al., 2015). This concept shares some similarities with the p-i HLA model in that drug is binding non-covalently to the HLA pocket. However, the p-i HLA model states that activation of T-cells is possible through the drug-HLA interaction alone without presentation of a novel peptide ligand. The altered self-peptide repertoire mechanism could potentially explain in part the diverse phenotypes of IDRs, but it should be emphasized that this concept has thus far been proven conclusively only for one drug (abacavir; HLA-B*5701) and not in the context of iDILI. In particular, flucloxacillin was not capable of modifying the repertoire of HLA-B*5701 binding peptides, thus highlighting the differences in stimulation mechanisms (Norcross et al., 2012).

- *Sterile inflammation and innate immune response*

The liver has been described as an organ of predominant innate immunity due to its selective enrichment in macrophages (KCs), NK cells and NKT cells (Racanelli & Rehermann, 2006). Aside from the resident innate cells, other immune cells, such as neutrophils and monocytes, can be recruited into the liver following appropriate inflammatory stimuli (Liu & Kaplowitz, 2006; Holt, Salmon, Buckley, & Adams, 2008; Mak & Uetrecht, 2019). Most of the knowledge to date around involvement of the innate immune system in DILI derives from studies on acetaminophen hepatotoxicity whereby drug covalent binding to macromolecules initiates a cascade of molecular and cellular events that lead to induction of sterile inflammation which

propagates tissue damage (Laskin, Gardner, Price, & Jollow, 1995; Yohe et al., 2006; Imaeda et al., 2009; Antoniades et al., 2012; Mossanen et al., 2016). As discussed in section **1.3.2**, inflammation is a natural homeostatic protective process that aims to repair injury, but an excessive response can be detrimental for the organism. Normally, KCs exhibit a tolerogenic phenotype however under certain circumstances they can shift to a pathologically activated state and there is evidence to suggest a role for macrophages in iDILI, possibly through activation of the inflammasome by danger signals or drug-modified proteins (Holt et al., 2008; Dragomir, Laskin, & Laskin, 2011; Kato & Uetrecht, 2017; Kato, Ijiri, Hayashi, & Uetrecht, 2019). The inflammasomes, such as NOD-like receptor 3 (NLRP3), are multi-protein complexes that recognize PAMPs and DAMPs and reside in the cell cytoplasm. Activation leads to caspase-1–mediated production of IL-1β, IL-18 and IL-33 and although it is an important innate immune property to fight off infection, over-activation has been shown to result in hepatocyte pyroptosis and severe liver inflammation thereby offering an attractive hypothesis for iDILI pathogenesis (Krishnaswamy, Chu, & Eisenbarth, 2013; Wree et al., 2014; Weston & Uetrecht, 2014). Non-immune intrinsic toxicity mechanisms, such as mitochondrial injury, oxidative stress, endoplasmic reticulum (ER) stress / unfolded protein response (UPR) and bile salt export pump (BSEP) inhibition have gained appreciation as mediators of hepatocyte damage and death during DILI and potentially implicated in iDILI as prodromal events (Krahenbuhl, Brandner, Kleinle, Liechti, & Straumann, 2000; Jaeschke, Knight, & Bajt, 2003; Boelsterli & Lim, 2007; Dara, Ji, & Kaplowitz, 2011; Pessayre et al., 2012; Li, Cai, & Boyer, 2017). These mechanisms are complex, and it is possible that individual susceptibility to pharmacological doses could exist at either a cellular stress or adaptive response level. A detailed description is out of the scope of this Thesis, however, even though challenging to account alone for the characteristics and delayed onset of iDILI, it is likely that these early drug- and / or reactive metabolite – induced events are not mutually exclusive with the role of

the immune system in iDILI and may promote release of DAMPs that activate innate immunity and propagate the immune response and damage to the liver (Shaw et al., 2009a; Roth & Ganey, 2010; Cho, Wang, & Uetrecht, 2019; Iorga & Dara, 2019). iDILI is not thought to be driven exclusively by the innate immune system but rather by a subclinical innate response is synergistic and facilitates stimulation of the adaptive system and / or progression of the tissue injury alongside a pro-inflammatory cytokine milieu (Fredriksson et al., 2011; Brenner, Galluzzi, Kepp, & Kroemer, 2013; Maiuri et al., 2015; Roth, Maiuri, & Ganey, 2017).

- *Immune tolerance*

There exists a critical interplay between immune activation and immune tolerance. Moreover, as briefly discussed in section **1.6.1**, the liver's microenvironment and cell composition promotes immune tolerogenic properties, which are crucial given the organ's continuous exposure to antigenic challenges (e.g. food, gut bacteria) (Knolle & Gerken, 2000; Crispe, 2009). However, the liver can mount an effective immune response to combat incoming pathogens when necessary. The immune response is a balance between a response strong enough to clear pathogens but potentially cause tissue damage and immune tolerance that decreases tissue damage but may reduce effective pathogen clearance (Kubes & Jenne, 2018). A typical feature of iDILI is that mild injury with transient elevation of liver enzymes, which later subsides despite drug continuation, is much more frequent than severe hepatotoxicity (Mitchell, Long, Thorgeirsson, & Jollow, 1975; Black, Mitchell, Zimmerman, Ishak, & Epler, 1975; Chalasani et al., 2014). This phenomenon is attributed to clinical adaptation (not to be confused with homeostatic adaptive signalling pathways to cell stress) and in the context of immune-mediated iDILI, it is reasonable to suggest that defective adaptation may be the result of abrogated immune tolerance (Uetrecht & Kaplowitz, 2015; Dara et al., 2016). Activation of the immune system outside the liver or a strong inflammatory stimulus in the liver (e.g. sudden increase in PAMPs, viral infection) have been suggested as mechanisms that may overcome

the liver's tolerogenic response (Bowen, McCaughan, & Bertolino, 2005; Heymann et al., 2015). In addition, an individual's immune memory profile is shaped by the continuous exposure to various pathogens and it is known that a response can be mounted to an antigen that bears a similar epitope to another antigen (molecular mimicry) or even structurally unrelated antigen (heterologous immunity) (Welsh & Selin, 2002; Depta & Pichler, 2003). Therefore, this cross-reactivity with a drug or drug-modified protein could perhaps lead to stronger immune responses overcoming immune tolerance.

Various molecules and cells have been found to regulate immune responses whilst impairment of immune tolerance in animals has been shown to lead to iDILI for certain drugs. Programmed cell death protein-1 knockout (PD-$1^{-/-}$) mice treated with amodiaquine (AQ) and anti-cytotoxic T-lymphocyte-associated protein-4 (anti-CTLA-4) developed delayed onset DILI that sustained during continuous drug treatment and exhibited significant infiltration of lymphocytes and piecemeal necrosis similar to what is seen in human (Metushi, Hayes, & Uetrecht, 2015; Foureau et al., 2015). Moreover, further characterization revealed $CD8^+$ T-cells as the responsible effector cells for the tissue injury (Mak & Uetrecht, 2015a). The same approach unmasked the iDILI potential of isoniazid and nevirapine whilst it effectively differentiated similarly structured drugs that are iDILI clinically positive or negative (Mak & Uetrecht, 2015b; Mak, Kato, Weston, Hayes, & Uetrecht, 2018). It was previously discussed that depletion of MDSCs in mice treated with halothane led to a $CD4^+$ T-cell mediated liver injury (see section **1.6.2**). However, when this approach was combined with PD-1 and CTLA-4 blockade alongside AQ treatment, the liver injury was less than expected thus highlighting the complexities of immune tolerance regulation (Mak, Cho, & Uetrecht, 2018). To date, very limited human evidence exists to conclusively link defective adaptation with severe iDILI; however, a few clinical studies point towards an association with factors implicated in immune tolerance (Aithal et al., 2004; Metushi et al., 2014). The mild liver injury profile and subsequent

resolution has also been observed in iDILI cases from individuals with specific HLA alleles denoting that although involvement of HLA-restricted immune responses are key in some cases, these are not sufficient on their own to induce severe hepatotoxicity (Singer et al., 2010; Aithal & Grove, 2015). Thus, the concept of deficient immune tolerance, although not a mechanism of immune activation by drugs and / or metabolites per se, is an immunological hypothesis that reasonably explains the low frequency of ensuing severe liver injury in susceptible individuals.

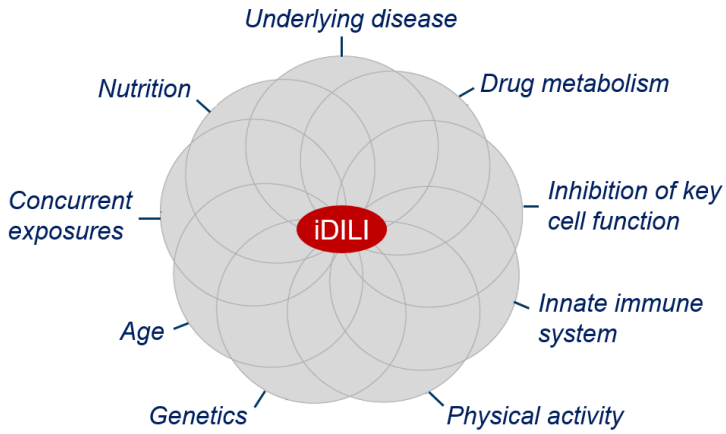
1.6.4 Patient risk factors

Despite significant progress being made in understanding the mechanisms of iDILI pathogenesis, knowledge gaps concerning patients at risk still remain. The HLA-B*5701 genetic association with flucloxacillin iDILI has been a remarkable discovery; however, it is estimated that only 1 in every 500 – 1000 carriers of HLA-B*5701 will develop hepatotoxicity upon treatment (Daly et al., 2009). HLA polymorphisms and variations in metabolizing enzymes and / or drug transporters are not sufficient susceptibility factors on their own to determine which patients will develop iDILI. Most patients who will be treated with iDILI clinically positive drugs will not suffer severe hepatotoxicity, therefore additional host factors must be in place (Ulrich, 2007; Chalasani & Bjornsson, 2010). It is suggested that female and elderly individuals are more susceptible to liver injury compared to the overall population (Fountain, Tolley, Chrisman, & Self, 2005; Mitchell & Hilmer, 2010; Chalasani et al., 2015), while diet and physical activity might also be influential (Seeff, Cuccherini, Zimmerman, Adler, & Benjamin, 1986; Pande, Singh, Khilnani, Khilnani, & Tandon, 1996). More importantly, underlying disease relevant to conditions of systemic infection and inflammation has been theorized to account for an increased risk of iDILI (Roth, Luyendyk, Maddox, &

Ganey, 2003). According to this inflammagen hypothesis, sporadic and modest inflammatory episodes may augment the liver's sensitivity to certain drugs, which in turn interact with the pro-inflammatory cytokine environment and activated innate immune system to bring about hepatocyte damage (Cosgrove et al., 2009; Roth & Ganey, 2010; Maiuri et al., 2015). The liver is "trained" to tolerate gut bacteria and in response to physiological lipopolysaccharide (LPS) concentrations, KCs maintain a tolerogenic status. However, a transient inflammatory imbalance in the gut – liver axis (such as one deriving from temporal intestine damage) could lead to LPS concentrations sufficient enough to overcome immune tolerance. Notably, LPS co-administration in drug-treated animals led to hepatotoxicity; however, the characteristics of liver injury was different from iDILI seen in human in terms of onset and histology (Buchweitz, Ganey, Bursian, & Roth, 2002; Deng et al., 2008; Shaw et al., 2009b; Lu, Jones, Harkema, Roth, & Ganey, 2012). Despite the inconsistencies with iDILI, it is still possible that a coincidental inflammatory superimposition could decrease the threshold of danger-mediated innate system activation and thus increase the risk of immune-mediated liver injury. Viral infection and re-activation could also increase the risk for iDILI (Fujita et al., 2015; Pavlos, White, Wanjalla, Mallal, & Phillips, 2017), while a misbalanced microbiome (Thaiss, Zmora, Levy, & Elinav, 2016) and epigenetic effects (Kacevska, Ivanov, & Ingelman-Sundberg, 2011) might have a role to play in iDILI development. Perhaps a missing patient risk factor may lie in the patient's unique memory T-cell pool. Previously, it was discussed how T-cells recognize drugs and / or metabolites directly though interaction with the TCR (p-i model) and considering the diversity of the TCR repertoire, it is possible that polymorphisms in TCR genes may account for specific immune responses. In addition, whilst the number of TCRs formed by gene recombination is almost limitless, the number of T-cells is not and hence heterologous immunity could be responsible for equipping an individual with primed T-cells (from prior exposure to a pathogen) leading to an augmented immune response against a drug and / or

drug-modified protein (Uetrecht, 2019b). Whilst drug-related risk factors might be easier to determine (chemistry of the drug), patient risk factors (biology of the individual) are much more complex to decipher and further exploration is required in understanding their contribution to iDILI pathogenesis and thus enable patient risk stratification (Kaplowitz, 2013; Chen et al., 2015; Naisbitt et al., 2019). ***Figure 1.3*** illustrates the multifactorial basis of iDILI.

A)



B)

$$\text{Frequency / Severity of iDILI} = f_1(\text{Chemistry of the drug}) + f_2(\text{Biology of the individual})$$

Figure 1.3. Convergence of patient risk factors in idiosyncratic hepatotoxicity, adapted from Ulrich, (2007) and Pichler et al., (2011). A) Pathogenesis of iDILI is considered multifactorial and a convergence of risk factors might be required. **B)** Ultimately, the frequency and severity of iDILI is a function of the chemistry of the drug and the biology of the individual.

1.7 NAPROXEN

In the context of exploring the role of AG in triggering drug-specific immune responses in the pathogenesis of iDILI, as outlined above, naproxen has been chosen as a model carboxylic acid

drug, because it mainly metabolizes into AGs. We were able to study aspects of the role of naproxen metabolites in a rare case of naproxen-associated idiosyncratic hepatotoxicity.

Naproxen (S-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid) is a NSAID that has been on the market since 1976 and approved as a prescription product or over-the-counter (OTC) medication in many countries. Naproxen, belonging to the group of aryl-propionic acids, is indicated for painful inflammatory rheumatic as well as non-rheumatic conditions and prescribed dosing is usually 500 mg two to three times daily without exceeding 1500 mg in total per day. Maximum plasma concentration (C_{max}) is achieved at 1 – 2 hrs post-inception with a half-life (t½) of 12 – 17 hrs (Vree, Biggelaar-Martea, & Verwey-van Wissen, 1992). Naproxen is marketed as the single S (+)-enantiomer and is the only NSAID manufactured as the pure active (S)-enantiomer. Drugs are usually marketed as single enantiomers as they are less complex than the racemic mixture with more selective pharmacodynamic effects, exhibit a better safety profile and pose less metabolic burden and chances of drug interactions (Chhabra, Aseri, & Padmanabhan, 2013). Only (S)-naproxen ((S)-NAP) will bind onto cyclooxygenase enzymes (COX-1 and COX-2) and inhibit their action thereby decreasing the production of prostaglandins and exerting its pharmacological effect in reducing pain and inflammation. Although naproxen is not considered to be COX-selective, inhibition of COX-1 enzyme is 5-fold greater than COX-2 (Rao & Knaus, 2008).

Human elimination of (S)-NAP is predominantly through hepatic metabolism with only a small amount of drug excreted in urine unchanged (Runkel, Chaplin, Boost, Segre, & Forchielli, 1972). Major metabolic products are via glucuronidation (mainly by UGT2B7) yielding (S)-naproxen-1β-acyl glucuronide ((S)-NAG) (~ 45 – 60 % of recovered dose in urine) and by O-dealkylation (CYP1A2, CYP2C9) to form the oxidative metabolite (S)-6-O-desmethylnaproxen ((S)-DNAP) (~ 1 – 5 % of recovered dose in urine) (Segre, 1975; Vree et al., 1992; Bowalgaha et al., 2005). The rate of acyl glucuronidation of naproxen is much higher

than that of O-dealkylation (Vree, Biggelaar-Martea, Verwey-van Wissen, Vree, & Guelen, 1993). (S)-DNAP is also excreted in a conjugated form (~ 15 – 30 % of recovered dose in urine) (Segre, 1975; Vree et al., 1993). Thus, (S)-DNAP can be further glucuronidated (both phenolic and acyl glucuronidation) and sulphated, although exposure to sulphation conjugates is far less than those of the AG (Falany, Strom, & Swedmark, 2005). Moreover, Jaggi et al. identified in rats a novel AG – sulfate diconjugate of (S)-DNAP (sulfation of the phenolic group and acyl glucuronidation of the carboxy group) as a major biliary metabolite (Jaggi, Addison, King, Suthers, & Dickinson, 2002). ***Figure 1.4*** illustrates the currently known metabolic pathways of (S)-naproxen in human.

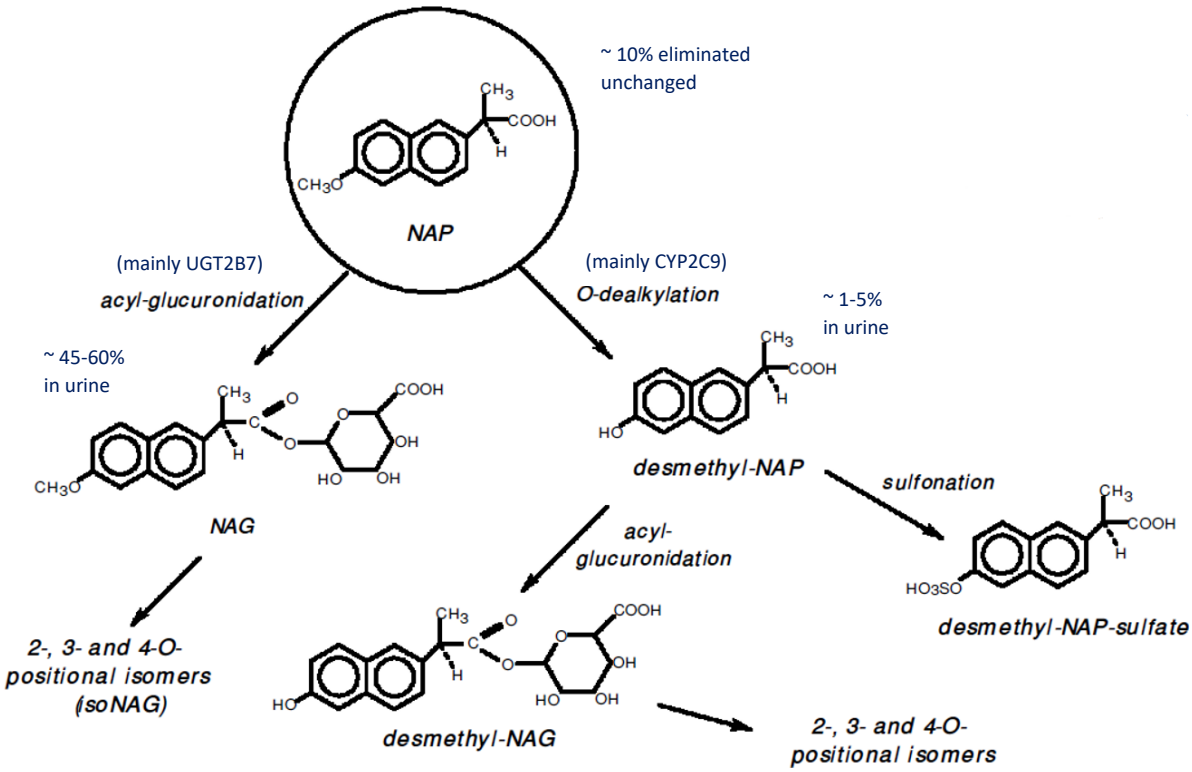


Figure 1.4. Currently known metabolic profile of naproxen in human, adapted from Lo, Addison, Hooper, & Dickinson, (2001). Naproxen is primarily metabolized to naproxen-1β-acyl glucuronide (mainly by UGT2B7) and 6-O-desmethylnaproxen (mainly by CYP2C9). The latter is subject to further glucuronidation, forming 6-O-desmethylnaproxen-1β-acyl glucuronide, and sulphation.

Human pharmacokinetic characteristics have been reported in the literature for naproxen and its major metabolites (although data is scarce for the metabolites). A C_{max} of 300 μM was reported for naproxen after administration of 500 mg in healthy volunteers (Niazi, Alam, & Ahmad, 1996). Following a single oral dose of 250 mg naproxen in a male healthy volunteer, C_{max} was measured at 199 μM, 2.9 μM and 1 μM for (S)-NAP, (S)-NAG and (S)-DNAP, respectively (Spahn-Langguth & Benet, 1992). The area under the concentration time curve (AUC) was 2,553 μmol h/L and 30.5 μmol h/L for (S)-NAP and (S)-NAG, respectively, while the apparent renal clearance (CL/F) was significantly higher for (S)-NAG (279 ml/min) than (S)-NAP (0.04 ml/min) (Spahn-Langguth & Benet, 1992). In 0.1 M phosphate buffer at 37°C (pH 7.4), (S)-NAG is chemically unstable with a t½ that ranges between 1.4 and 3.1 hrs, depending on the study (Bischer, Zia-Amirhosseini, Iwaki, McDonagh, & Benet, 1995; Sawamura et al., 2010; Jinno, Ohashi, Tagashira, Kohira, & Yamada, 2013). Thus, despite the single dose, an active renal elimination of the glucuronide conjugate and its chemical instability, significant (S)-NAG concentration could be detected in plasma. Moreover, (S)-NAG has been shown to form covalent adducts with HSA *in vitro* (Bischer et al., 1995; Iwaki, Ogiso, Inagawa, & Kakehi, 1999) and with animal protein in perfused rat livers (Lo et al., 2001).

Naproxen is considered by many as a safer alternative to other anti-inflammatory drugs, such as diclofenac, especially due to lower cardiovascular risks. However, IDRs have occasionally been reported following naproxen treatment, ranging from anaphylaxis and hypersensitivity (Bridges, Marshall, & Diaz-Arias, 1990; Klote & Smith, 2005) to cutaneous reactions (fixed drug eruption) (Bandino, Wohltmann, Bray, & Hoover, 2009; Akyazi, Baltaci, Mungan, & Kara, 2011) and liver injury (Victorino, Silveira, Baptista, & de Moura, 1980; Demirag et al., 2007; Ali, Pimentel, & Ma, 2011). Overall, the exact pathomechanism of these naproxen-induced IDRs remains largely unknown.

1.8 SPECIFIC AIMS OF THE THESIS

Safety risk assessment of novel drug candidates found to be extensively metabolized to a reactive AG drug metabolite continues to represent a major challenge for the pharmaceutical industry. The *in vitro* and *in vivo* confirmed capacity of AGs to bind covalently onto proteins in conjunction with epidemiological associations of carboxylic acid drugs with iDILI, raises anxiety over their immunotoxicological implications. However, the mechanism of iDILI secondary to AG-forming drug treatment remains poorly defined and, as was previously discussed, protein covalent binding does not necessarily equate to toxicity. It is believed that IDRs are predominantly driven by the immune system and several mechanistic hypotheses are in place to describe how an inadvertent activation of the immune system can bring about liver injury. To date, no *in vivo* human evidence exploring the immunological basis for the reaction in relation to AG drug metabolites has been published. Until the mechanisms behind carboxylic acid drug-induced liver injury are fully elucidated, any risk-mitigation strategies will remain challenging. The work in this Thesis is designed to explore unanswered questions regarding the involvement of AG drug metabolites in iDILI in relation to the role of AG-triggered immune responses, by utilizing peripheral blood mononuclear cells (PBMCs) obtained from naproxen-treated patients and healthy drug-naïve volunteers.

To achieve this, the following experimental aims are undertaken:

a. **Define and confirm the chemical instability and protein reactivity of (S)-NAG through analysis of its degradation in aqueous and protein solutions as well as its covalent binding capacity with HSA, thus confirming naproxen as a model AG-forming drug for further investigations throughout this Thesis.** This work is described in ***Chapter 2***. AGs are known to be chemically unstable and react with proteins. A lower degradation t½ of AG in aqueous solution has been associated with

an increased IDR risk (Sawamura et al., 2010). A method is applied to measure the degradation of (S)-NAG in phosphate buffer 0.1 M (pH 7.4) at 37°C and covalent adduction to HSA.

b. **Investigate the role of the adaptive immune system in a case of naproxen – induced idiosyncratic hepatotoxicity and interrogate the involvement of (S)-NAP, its major metabolites and the model (S)-NAG – HSA adduct.** This work is described in ***Chapter 3***. Reactivity of AGs and modification of self-proteins has been hypothesized to lead to activation of the immune system as outlined by the hapten theory. Various immunological techniques are used to assess the activation of the immune system using cells from relevant patients and healthy volunteers.

c. **Explore gene expression profiles of PBMCs isolated from naproxen-treated patients and healthy drug-naïve volunteers following *in vitro* exposure to (S)-NAP and its major metabolites.** This work is described in ***Chapter 4***. Limited *ex vivo* human evidence exists interrogating the gene expression pattern of PBMCs in relation to AG drug metabolites. Moreover, there is a role for the innate immune system and inflammation in iDILI and some researchers have published data to suggest a correlation between immuno-inflammatory responses and AG reactivity, however the data are not conclusive.

CHAPTER 2

CHEMICAL INSTABILITY OF (S)-NAPROXEN-1β-ACYL GLUCURONIDE IN AQUEOUS AND PROTEIN SOLUTIONS

2.1 INTRODUCTION

The carboxylic acid structure remains an important functional group for medicinal chemists for a wide range of different purposes as described in ***Chapter 1***. Consequently, the carboxylic acid structure has been incorporated into over 450 marketed medicinal products, intended to address a wide range of therapeutic needs (e.g. NSAIDs, hypolipidemics, diuretics, anticonvulsants) (Smith, 2010). Furthermore, carboxylic acid structures can also be revealed through metabolism. Some of the most consumed pharmaceutical products contain carboxylic acid groups. The carboxylate-containing NSAIDs represent a good example of this, where 6 % of the adult USA population are reported to have used a prescription NSAID in a month and 24 % using non-prescription ibuprofen (Paulose-Ram et al., 2003). In the vast majority of patients these drugs provide safe and efficacious therapy.

However, a number of carboxylic acid drugs have been withdrawn from the market due to safety concerns. Fung et al. reported that 17 of 121 drugs (14 %) withdrawn from the market between 1960 and 1999 contained a carboxylic acid functional group (Fung et al., 2001). Consequently, concern has been raised over the potential of the carboxylic acid group to represent a potential toxicophore. Withdrawals of carboxylic acid – containing drugs are exemplified by cases such as ibufenac and benoxaprofen, which were withdrawn due to associations with liver injury, and zomepirac, withdrawn due to its association with eliciting anaphylactic reactions (Levy & Vasilomanolakis, 1984; Zimmerman, 1993) . However, to date, it is not clear whether or not a carboxylic acid functional group is causally involved in these serious ADRs, and if so, why only certain drugs would carry this liability.

Phase II conjugation reactions are usually considered to result in the pharmacological deactivation of a molecule, whilst also enhancing the molecule's hydrophilicity and affinity for export transporters and therefore elimination. Consequently, phase II metabolism is generally

considered to represent a detoxification mechanism. Direct phase II conjugation of carboxylic acid drugs via their UGT-mediated glucuronidation at the carboxylate functional group represents a major metabolic pathway for this class of drugs, resulting in the formation of AG metabolites. Clinical exposure to AG metabolites is high, and in some cases the circulating AUC of the AG metabolite(s) can exceed that of the parent drug (Smith, Langendijk, Bosso, & Benet, 1985). The extent of glucuronidation of carboxylate drugs has been reported to be highly variable with reports of the fraction of a parent drug glucuronidated varying from 2.5% for lumiracoxib (Mangold et al., 2004) and 10 % for salicylic acid (Levy, Tsuchiya, & Amsel, 1972) to 90 % for zomepirac (O'Neill, Yorgey, Renzi, Jr., Williams, & Benet, 1982).

AG metabolites have consistently been shown to be chemically unstable and protein reactive in aqueous solution. Furthermore, serum exposure towards AG metabolites of carboxylic acid drugs including zomepirac, tolmetin and ibuprofen following parent drug administration to human, has been observed to correlate with irreversible binding of these drugs to plasma proteins (Smith, McDonagh, & Benet, 1986; Hyneck et al., 1988; Benet et al., 1993; Castillo, Lam, Dooley, Stahl, & Smith, 1995). It is this suspected *in vivo* protein reactivity of AGs which has led to their conjectured involvement in eliciting the adverse reactions of their parent aglycones (Smith et al., 1986). Finding a new compound or metabolite to be protein-reactive can result in concerns over the compound's potential safety. One of these concerns arises when the compound behaves as a hapten or pro-hapten. Examples for this come from the β-lactam antibiotics, such as penicillin, where the drug-derived protein adducts have been shown to elicit immune-mediated / hypersensitivity reactions (Park, Coleman, & Kitteringham, 1987; Brander et al., 1995; Padovan et al., 1997; Ariza et al., 2015; Meng et al., 2017). Further to acyl glucuronidation, other bioactivation can occur at the carboxylic acid resulting in the formation of further reactive metabolites including acyl CoA thioesters (and acyl GSH thioesters) which, whilst much less abundant, exhibit far increased intrinsic reactivity when compared to AGs.

This is exemplified by the CoA-conjugates of mefenamic acid and naproxen which were found to be 80 and 100 times more reactive towards GSH than their AG counterparts, respectively (Olsen et al., 2002; Grillo, Tadano, & Wait, 2012). Due to their very high intrinsic reactivity, acyl CoAs are unlikely to be eliminated from the cell in any great quantity. It should also be noted however that systemic exposure of carboxylate CoA or GSH conjugate-mediated protein adducts could hypothetically occur via intra-hepatocyte mediated metabolism prior to subsequent transport / elimination of the adducted protein into the blood (Sidenius, Skonberg, Olsen, & Hansen, 2004; Darnell, Breitholtz, Isin, Jurva, & Weidolf, 2015; Lassila et al., 2015). Furthermore, bioactivation of carboxylate molecules into protein-reactive metabolites could feasibly also occur at sites distant to the carboxylate group. A potential example of this is the CYP-mediated oxidation of the NSAID diclofenac. Both oxidative metabolites of diclofenac (5'-hydroxy and 4'-hydroxy diclofenac) can undergo spontaneous oxidation into reactive quinone-imine intermediates (Bort et al., 1999; Poon et al., 2001; Kumar et al., 2002).

As detailed in ***Chapter 1***, the hypothesized link between AG formation and delayed (Type IV) T-cell mediated immune reactions associated with the idiosyncratic hepatotoxicity seen with certain carboxylic acid drugs has been speculated for decades, however no definitive *in vivo* human causality evidence has been shown to date. Despite the ongoing controversy over whether AGs represent a metabolite of toxicological relevance, the Food and Drug Administration (FDA) has labelled AGs as 'toxic' in their Metabolites in Safety Testing (MIST) regulatory guidance document, stating:

"…if a conjugate forms a toxic compound such as acyl glucuronide, additional safety assessment may be needed…" (FDA, 2008).

Notably this statement has been retained in the updated 2016 version, indicating that the FDA's opinion over AGs remains unchanged. Strictly according to MIST guidance, metabolites

expected or found to be formed in human at exposures greater than 10 % of parent drug related material should be considered for additional safety assessment. Clearly, most AG metabolites of carboxylic acid drugs would fall into this category (Regan et al., 2010). MIST guidance stipulates such metabolites undergo general toxicity (*in vivo*), genotoxicity, embryo-fetal development toxicity and carcinogenicity studies. However, it is extremely difficult to chemically synthesize stable AGs at yields sufficient for toxicology studies. Furthermore, the predominant *in vivo* consequence of administration of an AG is efficient and rapid renal (Smith et al., 1985; Castillo et al., 1995), biliary (Verbeeck et al., 1988; Balani et al., 1997; Wang et al., 2004) and enzymatic (Williams et al., 1992; Akira, Uchijima, & Hashimoto, 2002; Karlsson et al., 2010) clearance. Consequently, other strategies predominantly aimed at investigating protein reactivity of generated AGs are used as a means to convince regulators of the mitigation of potential risk.

The most commonly used assay to assess AG protein reactivity is the investigation of its stability in 0.1 M phosphate buffer (pH 7.4) at 37 °C. The reason for this is that the degradation t½ of AG metabolites under these conditions has been shown to correlate almost perfectly with their irreversible binding to HSA following *in vitro* incubation (Benet et al., 1993). ***Figure 2.1*** shows the chemical pathways of drug AG degradation. The electrophilicity of the ester carbonyl carbon is the foundation of the AG's intrinsic reactivity (Baba & Yoshioka, 2009). During hydrolysis, the ester carbonyl carbon is subject to nucleophilic attack by -OH ions (hydrolysis) or enzymatic (β-glucuronidase or esterases, including serum albumin) cleavage leading to the displacement of the glucuronic acid and regeneration of the parent aglycone (Hyneck, Munafo, & Benet, 1988; Smith, McDonagh, & Benet, 1990; Dubois-Presle et al., 1995; Brunelle & Verbeeck, 1997). Alternatively, acyl migration is an intramolecular process involving relocation of the aglycone moiety from the 1-O-β-position to the 2-, 3- and 4-positions of the glucuronic acid ring forming the respective positional isomers (Hasegawa,

Smith, & Benet, 1982). This intramolecular process occurs due to nucleophilic attack of the ester carbonyl carbon by the adjacent hydroxyl group causing electron displacement to form an intermediate ortho-acid ester which finally stabilizes to a positional isomer as described above. Although acyl migration among the C2-, C3- and C4- isomers is reversible, degradation profiles indicate migration back to the original 1-O-β-AG does not occur to any great extent. Interestingly, when the aglycone is at the C2-, C3- or C4- position, the hydroxyl group at 1-position fluctuates from the β to α positions resulting in transient sugar-ring opening.

Irreversible binding to proteins has been shown to occur via two main mechanisms, namely transacylation and glycation as depicted in ***Figure 2.2*** (Ruelius, Kirkman, Young, & Janssen, 1986; Wells, Janssen, & Ruelius, 1987; Smith et al., 1990; Williams et al., 1992; Williams & Dickinson, 1994; Kretz-Rommel & Boelsterli, 1994). Similar to hydrolysis, transacylation is the result of direct nucleophilic attack by amino, guanidine and hydroxyl groups found on proteins displacing the glucuronic acid and eventually leading to the aglycone covalently binding onto the protein via amide and ester links (Ding et al., 1995; Qiu et al., 1998). On the other hand, for glycation adductions the aldehyde group on the open sugar ring (formed during β to a anomerisations) is subject to nucleophilic attack by amino groups on proteins eventually resulting in formation of imine adducts (Schiff bases) (Smith et al., 1990) which can be further stabilized through an Amadori rearrangement (Neglia, Cohen, Garber, Thorpe, & Baynes, 1985; Smith et al., 1990).

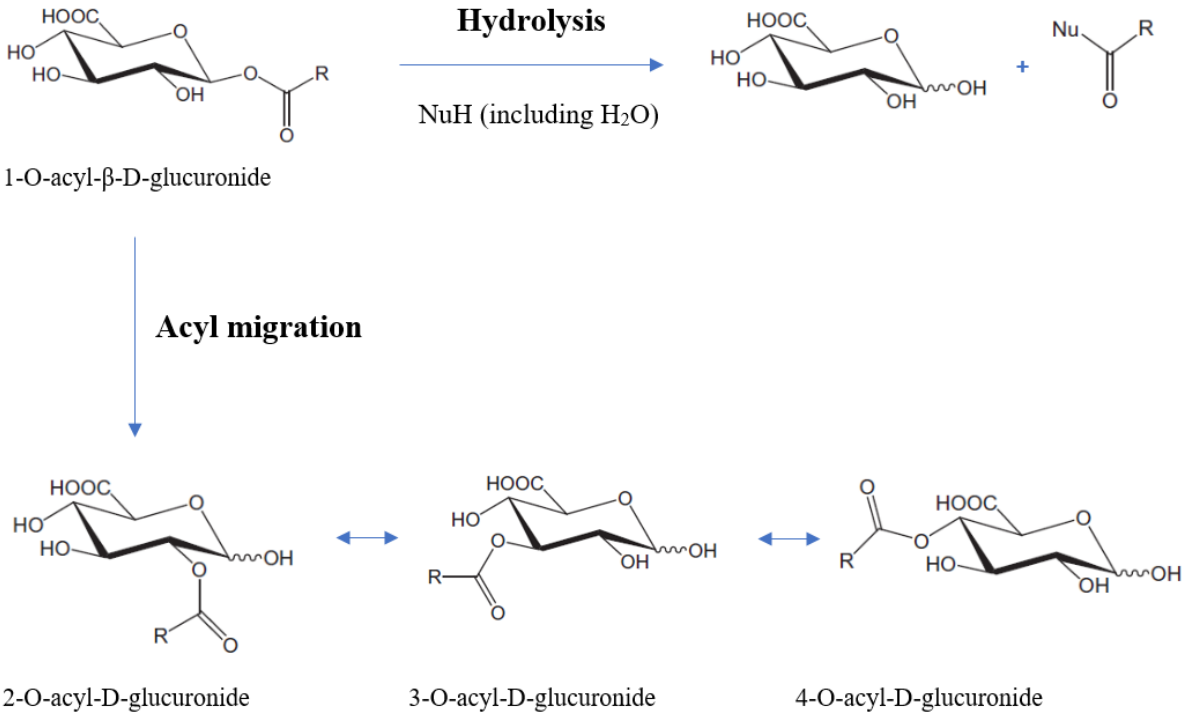


Figure 2.1. Chemical pathways of 1β-acyl glucuronide degradation in aqueous and / or protein solutions, adapted from Camilleri et al., (2018). The AG can degrade either via hydrolysis or acyl migration. Both α- and β- anomers of the positional isomers are formed due to mutarotation. R: functional group of the carboxylic acid, NuH: Nucleophile.

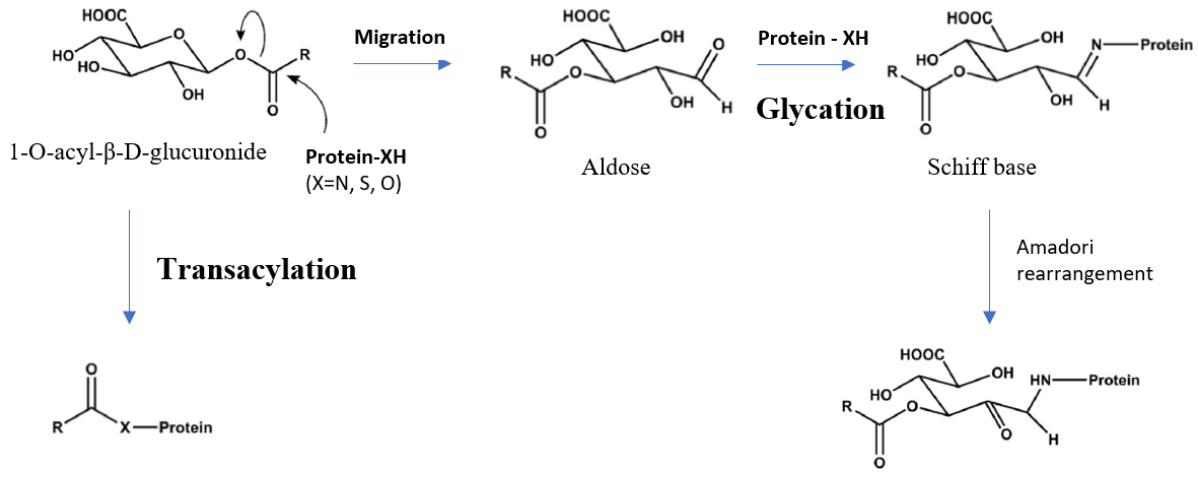


Figure 2.2. Mechanisms of 1β-acyl glucuronide irreversible binding to protein, adapted from Iwamura, Nakajima, Oda, & Yokoi, (2017). Transacylation and glycation represent the two different pathways of AG irreversible binding to protein. Following acyl migration, any positional isomer can be glycated. As an example, the C-3 is depicted here.

The chemical instability and protein reactivity of different AGs is highly variable. Their degradation rate in 0.1 M phosphate buffer (pH 7.4) at 37 °C is reported to range from 0.26 hrs for tolmetin-AG to 79 hrs for valproic acid-AG (Williams et al., 1992; Ding et al., 1995). The steric and electronic properties of the parent drug are considered to represent the primary determinants of resulting AG reactivity. It is hypothesized that the degree of substitution of the carbon alpha to the carboxylic acid represents the most important structural determination of the resulting AG reactivity. This is exemplified by the simple carboxylic acid structure-activity relationship for reactivity of their resulting AGs: acetic acid > propionic acid > benzoic acid derivatives (Benet et al., 1993). Furthermore, the differing stability of the AGs of the 2-arylproprionic acid derivatives can be attributed to this role of steric hindrance, where the α-methyl group is in close proximity to the carboxylate group (Smith, Hammond, & Baillie, 2018). Whilst the degree of substitution around the carbon alpha to the carboxylic acid is the primary determinant of AG reactivity, modifications to the molecule at sites distant to the carboxylate group can also affect the stability of the resulting AG, as exemplified by (S)-ibuprofen-AG (AG degradation t½ of 3.68 hrs) and (S)-2-OH-ibuprofen-AG (AG degradation t½ of 5.03 hrs) (Johnson et al., 2007).

In vivo evidence for the irreversible binding of AGs was originally determined through the correlation of their serum exposure (AUC) following the administration of parent drug with the total irreversible binding of drug to serum proteins (Smith et al., 1986; Hyneck et al., 1988; Volland, Sun, Dammeyer, & Benet, 1991; Benet et al., 1993; Castillo et al., 1995). More recently, HSA has been used as a surrogate circulating protein to investigate drug or metabolite reactivity in greater detail. HSA is a soluble protein of approximately 67 kDa size (609 aminoacids), synthesized almost exclusively within the hepatocytes of the liver and represents the most abundant protein in human blood (approx. 43 mg/ml or 0.6 mM concentration) (Peters, 1996; Nicholson, Wolmarans, & Park, 2000). In the blood, HSA circulates with a long t½ of

elimination of approximately 19 – 25 days (Peters, 1996; Anderson & Anderson, 2002; Colombo et al., 2012). The long t½ of albumin in the blood alongside its high circulating concentration and technically feasible isolation for detailed study, means HSA represents a useful model protein to investigate drug / metabolite interactions with protein *in vivo.* Antibody methods have confirmed the covalent adduction of carboxylic acid drugs or their metabolic derivatives towards protein *in vivo* (Bailey & Dickinson, 1996; Aithal et al., 2004). The strong correlation between plasma AG exposure and parent drug irreversible plasma protein binding provides convincing evidence for the potential of AGs to adduct circulating proteins (Benet et al., 1993). However, it was not until the development of high-purity albumin isolation strategies alongside modern mass-spectrometric techniques that the protein reactivity of AGs *in vivo* was confirmed (Hammond et al, 2014). However, it must be understood that the link between albumin adduction *in vivo* and toxicological / immunological consequences is not assured.

A number of drug AG degradation and covalent binding assays have been reported lately in an effort to assess the risk posed by AG formation. The degradation of an AG in 0.1M phosphate buffer (pH 7.4) at 37°C is the most commonly used assessment of AG reactivity as it represents a simple assay, and the *in vitro* irreversible binding of different AGs was shown to correlate almost perfectly to their degradation in phosphate buffer (Benet et al., 1993). Using the t½ of AG degradation in 0.1 M phosphate buffer (pH 7.4) at 37°C, a group of investigators recently proposed a risk classification system (Sawamura et al., 2010). In this work, authors reported a separation of drugs classified as "withdrawn" (zomepirac, ibufenac, fenclofenac and benoxaprofen) from those classified as warning or safe by defining a degradation t½ threshold of 3.6 hrs. This has resulted in medicinal chemists deriving confidence when identifying that AG metabolites of their compounds under development exhibit a degradation t½ greater than this 3.6 hrs threshold. It should however be understood that within the warning group (and

exhibiting a degradation t ½ less than this 3.6 hrs threshold) remain marketed over-the-counter drugs providing safe therapy to vast numbers of patients, including diclofenac and ibuprofen.

For the work entailed in this thesis, (S)-NAP (propionic acid, mono-a-substituted carboxy group) was selected for investigation as a model AG forming drug. The single S (+)-enantiomer selection was based on naproxen's marketed profile, as the drug is sold only as its (S)-enantiomer rather than racemic mixture. The resulting (S)-NAG has been shown to degrade in 0.1 M phosphate buffer (pH 7.4) at 37°C with a t½ of 2.2 ± 0.1 hrs to 2.89 ± 0.09 hrs (Sawamura et al., 2010; Jinno et al., 2013), placing (S)-NAG within the warning range as proposed by Sawamura et al., (2010). The protein reactivity of (S)-NAG has also been confirmed via observation of its irreversible binding with HSA following *in vitro* incubation (Bischer et al., 1995; Iwaki et al., 1999). (S)-NAP is usually administered at a cumulative daily dose of up to 1.5 grams, and has been associated with idiosyncratic iDILI reactions in human (9 – 56 cases per 100,000 patient years) (Walker, 1997), suggesting (S)-NAP to represent a useful model compound to investigate the potential association of AG metabolites with immune-mediated off-target reactions in human.

The main aim of this chapter was to confirm naproxen as a suitable model drug to investigate the association of AG metabolites with iDILI and hypersensitivity reactions associated with parent carboxylate compounds. The *in vitro* data generated here, aim to confirm the available literature on the reactivity and covalent binding profile of (S)-NAG. Moreover, experiments described in this chapter seek to validate the employed bioanalytical assay and demonstrate the binding of (S)-NAG to HSA.

2.2 QUESTION AND AIMS

The key question is:

"Can the presumed chemical instability and protein reactivity of (S)-NAG be repeated, therefore confirming the utility of (S)-NAP as a model AG forming drug for further investigations throughout this thesis?"

To address this question, the aims were to:

- Establish and validate a bioanalytical method to measure (S)-NAG's chemical instability and protein binding propensity.
- Characterize (S)-NAG degradation in 0.1 M potassium phosphate buffer (pH 7.4) and HSA solution at 37°C.
- Assess the *in vitro* irreversible protein binding of (S)-NAG to HSA at 37°C using a solid phase extraction / alkaline hydrolysis technique.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Acetonitrile (ACN) (LC-MS grade), ammonium acetate (LC-MS grade), DU-lactic acid 70%, acetic acid (LC-MS grade), ammonium hydrogen carbonate (LC-MS grade), Dimethyl Sulfoxide (DMSO), HSA (approx. 99% pure, essentially globin free and fatty acid free) were purchased from Sigma-Aldrich, Dorset, UK. Bio Rad Bradford reagent was purchased from

Bio Rad, Hertfordshire, UK. Oasis Solid Phase Extraction (SPE) (1 cc, 30 mg sorbent) cartridges were purchased from Waters, Macclesfield, UK. (S)-NAP and (S)-NAG were purchased from Sigma-Aldrich and Toronto Research Chemicals, respectively. Internal Standard (IS) was kindly provided by Novartis Pharma AG. 0.1 M phosphate buffer (pH 7.4) was made using 0.3117 % monosodium phosphate monohydrate, 2.0747 % disodium phosphate, heptahydrate w/v in distilled water. All other reagents were purchased from Sigma-Aldrich, Dorset, UK unless otherwise stated.

2.3.2 (S)-naproxen-1β-acyl glucuronide aliquots and storage

Purchased (S)-NAG was dissolved in DMSO at a final concentration of 10 mM before aliquoting and stored at -20°C until use. HPLC-UV analysis (at 254 nm) of an aliquot of stored (S)-NAG prior to use in each experiment confirmed that no degradation of the analyte had occurred, based on the failure to detect liberated aglycone or (S)-NAG positional isomers. For the purposes of (S)-NAG degradation in HSA and irreversible binding, incubates were carried out using freshly solubilized AG in 0.1 M phosphate buffer (pH 7.4).

2.3.3 HPLC-MS apparatus and conditions

The LC-MS system consisted of a 1290 HPLC system (Agilent Technologies, Palo Alto, California) connected to a LTQ-Orbitrap XLmass spectrometer (Thermo Scientific, USA) via electrospray ionization interface. Chromatographic separation was carried out on an Acquity UPLC HSS T3 C18 column (2.1 × 150 mm, 1.8 µm) (Waters, Milford, MA, USA) at a flow rate of 1 mL/min. The column temperature was maintained at 50°C throughout all analyses. The solvents used for chromatographic separation were buffer A (10 mM ammonium acetate

pH 5.0 containing 5 % ACN (v/v)) and buffer B (10 mM ammonium acetate pH 5.0 containing 95 % ACN (v/v)). Chromatographic separation of analytes was achieved by using an initial isocratic phase of 10 % buffer B for 1 minute. A gradient elution from 10 % to 20 % buffer B was achieved over the following 2 minutes, before the gradient was steepened from 20 % to 35 % buffer B over the following minute. A final steeping of the gradient from 35 % to 90 % buffer B was achieved over the following 0.6 minutes before the column was re-equilibrated to starting gradient over the next 0.4 minutes. Chromatographic separation was controlled using Chemstation software (Version B.04.04, Agilent Technologies, Palo Alto, CA software). High resolution mass spectrometric detection was performed with only 1st quadrupole detection employed. The instrument was operated in negative ion mode. The source parameters were as follows: spray voltage, 3 kV; sheath gas pressure 20 bar; auxiliary gas pressure 15 bar; source temperature 275°C; cone voltage -44V; tube lens -115V. Data were acquired from 160-900 Da with MS^1 mode at a resolution of 30,000. All operations were controlled by Xcalibur software (version 2.1, Thermo Scientific, USA).

2.3.4 Assessment of the degradation of (S)-naproxen-1β-acyl glucuronide in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C

Prior to the experiment, 0.1 M potassium phosphate buffer (pH 7.4) was warmed to 37°C. Following this, (S)-NAG was added to the phosphate buffer to achieve a final concentration of 10 µM (final DMSO solvent concentration in the reaction mixture was 0.01 %) and incubated at 37°C under agitation (1000 rpm). Aliquots (50 µl) were removed at desired time points (5 mins, 30 mins, 1 hr, 2 hrs, 4 hrs, 8 hrs and 24 hrs), followed by immediate addition of the IS to a final concentration of 0.5 µM and stabilisation of the AG through acidification of the sample via addition of lactic acid yielding a final solution (150 µl) of 10 % ACN containing 1 % lactic

acid (v/v). All samples were immediately placed in the HPLC-MS autosampler which was maintained at 4°C for subsequent analysis using the analytical method described in section **2.3.3**. (S)-NAG in the reaction mixture was estimated using a seven-point calibration curve (0.01, 0.04, 0.1, 0.4, 1, 4 and 10 µM) generated through the addition of stock (S)-NAG into phosphate buffer yielding final solution (150 µl) of 10 % ACN containing 1 % lactic acid (v/v) and IS at a final concentration of 0.5 µM. In each run, two separate sets of standard curves were generated. To confirm / monitor absence of sample degradation in the autosampler, one curve was injected before samples' analysis and the second afterwards. Additionally, quality control (QC) standards at 0.01, 2 and 4 µM were prepared in triplicate in order to assess the quantifiable range of the assay. For method acceptance, the accuracy must be 100 ± 15 % at all points tested, excepting the lower limit of quantification set within 100 ± 20 %. Similarly, the precision (coefficient of variance) must be ± 15 %, excepting the lower limit of quantification set within ± 20 %.

2.3.5 Assessment of the degradation of (S)-naproxen-1β-acyl glucuronide in human serum albumin solution (pH 7.4) at 37°C

Prior to the experiment, 0.1 M phosphate buffer (pH 7.4) containing 40 µM HSA was warmed to 37°C. Following this, (S)-NAG was immediately solubilized in 0.1 M phosphate buffer (pH 7.4) before immediately being spiked into the reaction mixture to a final concentration of 1 mM or 200 µM alongside volume adjustment using phosphate buffer to ensure the concentration of HSA was 20 µM (consequently the (S)-NAG : HSA molar ratio in the incubation was at 50:1 and 10:1, respectively). The reaction mixture was subsequently incubated at 37°C and 900 rpm agitation using a thermoshaker. At desired time points (0 min, 30 mins, 2 hrs, 4 hrs, 8 hrs and 24 hrs) 150 µl of the incubation mixture was removed and

stabilised via addition of 1% lactic acid (v/v). All samples were prepared in triplicates and stored at -80°C until analysis. A SPE technique was used to separate protein in the incubation mixture from unbound analyte. Following thawing of samples to room temperature, Oasis HLB SPE (1 cc, 30 mg sorbent) cartridges (Waters, Macclesfield, UK) were primed through the passing of 1 ml of ACN through the column before equilibration by passing 1 ml of 1 % lactic acid (v/v) through the column using vacuum pressure. Sample (300 µl) or 20 µM HSA solution (300 µl), used subsequently for calibration control or assay QC, was added to the SPE cartridge, with the unbound fraction collected, frozen at -20°C and subsequently subjected to alkaline hydrolysis assessment of covalent binding as described in section **2.3.6**. Analytes remaining bound to the column were washed with 1 ml of 5 % ACN containing 1 % lactic acid (v/v) before elution by 1 ml 95 % ACN containing 1 % lactic acid (v/v) and 75 nM IS, followed by 0.5 ml 100 % ACN containing 1 % lactic acid (v/v). Resulting extracts were evaporated to dryness under a constant stream of N_2 gas, before reconstitution in 150 µl of 10 % ACN containing 1 % lactic acid (v/v).

In order to allow quantification of remaining (S)-NAG in the reaction mixture and its hydrolysis into the parent drug, increasing amounts of (S)-NAG and (S)-NAP (0.01, 0.04, 0.1, 0.4, 1 and 4 µM final concentration) were spiked into 20 µM HSA in 0.1 M phosphate buffer (pH 7.4) containing 1 % lactic acid (v/v). In each run, two separate sets of standard curves were generated (standards injected before and after samples run). Additionally, QC standards at 0.01, 2 and 4 µM were prepared in triplicate in order to assess the quantifiable range of the assay. All of these standards were subjected to SPE as described above, and resulting eluates containing the IS were similarly evaporated to dryness under a constant stream of N_2 gas, before reconstitution in 150 µl of 10 % ACN containing 1 % lactic acid (v/v). All prepared calibration and quality control standards were stored at -20°C until use. For method acceptance, the accuracy must be 100 ± 15 % at all points tested, excepting the lower limit of quantification

set within 100 ± 20 %. Similarly, the precision (coefficient of variance) must be ± 15 %, excepting the lower limit of quantification set within ± 20 %.

To assess analyte recovery from the SPE columns, reference calibration standards of (S)-NAG and (S)-NAP at 0.01, 2 and 4 µM were prepared (n=6 for each concentration) in 0.1 M phosphate buffer (pH 7.4) and subjected to SPE as described above. All washes and eluate were collected and evaporated to dryness under a constant stream of N_2 gas, before storage at -20°C. Similarly, the same reference calibration standards were prepared in 95 % ACN containing 1% lactic acid (v/v) were immediately evaporated to dryness under a constant stream of N2 gas and storage at -20°C. All samples were subsequently reconstituted in 150 µl of 10% ACN containing 1 % lactic acid (v/v) and 0.5 µM IS before analysis by LC-MS as described in section **2.3.3**. Recovery of the analyte was calculated by the following equation:

$$Recovery\ (\%) = \frac{AUC\ Analyte\ Peak\ Following\ SPE\ /\ AUC\ IS}{AUC\ Analyte\ Peak\ Without\ SPE\ /\ AUC\ IS} \times 100$$

2.3.6 Assessment of the irreversible binding of (S)-naproxen-1β-acyl glucuronide to human serum albumin solution (pH 7.4) following incubation at 37°C

From the experiment above investigating AG degradation in the presence of HSA, frozen protein containing initial eluates from the first SPE were thawed at room temperature before the alkaline hydrolysis procedure was performed. Ice-cold ACN (1 ml) was added to the 300 µl thawed sample and mixed by vortex for 30 seconds before centrifugation at 18000 rpm for 10 mins at 4°C to precipitate the protein and leave any non-covalently bound drug in the supernatant. This supernatant was aspirated and discarded before addition of a further 500 µl

ice-cold ACN before vortex mixing and centrifugation at 18000 rpm for 10 mins at 4°C with the supernatant again discarded. All samples were reconstituted in 800 µl of 0.25 M NaOH. Calibration and QC standards were generated through spiking (S)-NAP (0.01, 0.04, 0.1, 0.4, 1 and 4 µM final concentration) in duplicate for calibration standards, and 0.01, 2 and 4 µM in triplicate for QC standards in a final volume of 800 µl of 0.25 M NaOH. All samples, calibration and QC standards were incubated at 80^{o}C for 90 mins and 1400 rpm agitation. After this they were left to cool to room temperature before addition of 100 µl of 2 M HCL in order to neutralize the pH. Subsequently, a 20 µl aliquot of the sample was taken for protein determination by the Bradford assay (Bradford, 1976). The remaining hydrolysate was subjected to SPE using Oasis HLB extraction cartridges (1 cc, 30 mg sorbent) (Waters, Macclesfield, UK). Cartridges were primed through addition of 1 ml of ACN before equilibration using 1 ml of 1 % lactic acid (v/v). Sample (880 µl), calibration and QC standards were subsequently added to the SPE cartridge. Analyte bound to the column was washed through addition of 5 % ACN containing 1 % lactic acid (v/v). Bound analytes were subsequently eluted through addition of 1 ml of 95 % ACN containing 1 % lactic acid (v/v) before addition of 0.5 ml ACN containing 1 % lactic acid (v/v). Resulting extracts were evaporated to dryness under a constant stream of N2 gas, before reconstitution in 150 µl of 10 % ACN containing 1 % lactic acid (v/v) and introduction to the mass-spectrometer. For method acceptance, the accuracy must be 100 ± 15 % at all points tested, excepting the lower limit of quantification set within 100 ± 20 %. Similarly, the precision (coefficient of variance) must be ± 15 %, excepting the lower limit of quantification set within ± 20 %.

To assess HSA recovery from the SPE columns, reference samples of 10 µM HSA in 0.1 M phosphate buffer (pH 7.4) at 37°C were prepared (n=3) and subjected to SPE. All washes and elute were collected and protein was quantified via Bradford assay (Bradford, 1976). Recovery of human serum albumin was calculated by the following equation:

$$Recovery\ (\%) = \frac{[HSA]\ Following\ SPE}{Initial\ [HSA]} \times 100$$

The chemical structure of the analytes is shown in ***Figure 2.3***. A summary of the experimental process assessing the degradation of (S)-NAG in HSA solution (pH 7.4) at 37°C and irreversible binding to protein is illustrated in ***Figure 2.4***.

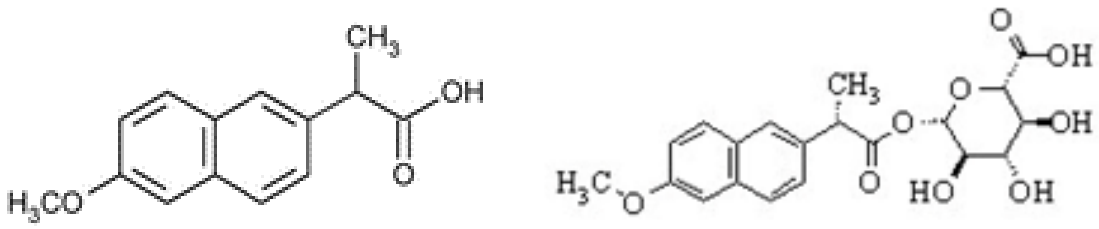


Figure 2.3. Chemical structures of analytes. (S)-NAP (left) and (S)-NAG (right). A D-glucuronic acid moiety attaches to the aglycone ((S)-NAP) to form the AG metabolite ((S)-NAG). Molecular weight for (S)-NAP and (S)-NAG is 230.259 g/mol and 406.38 g/mol, respectively.

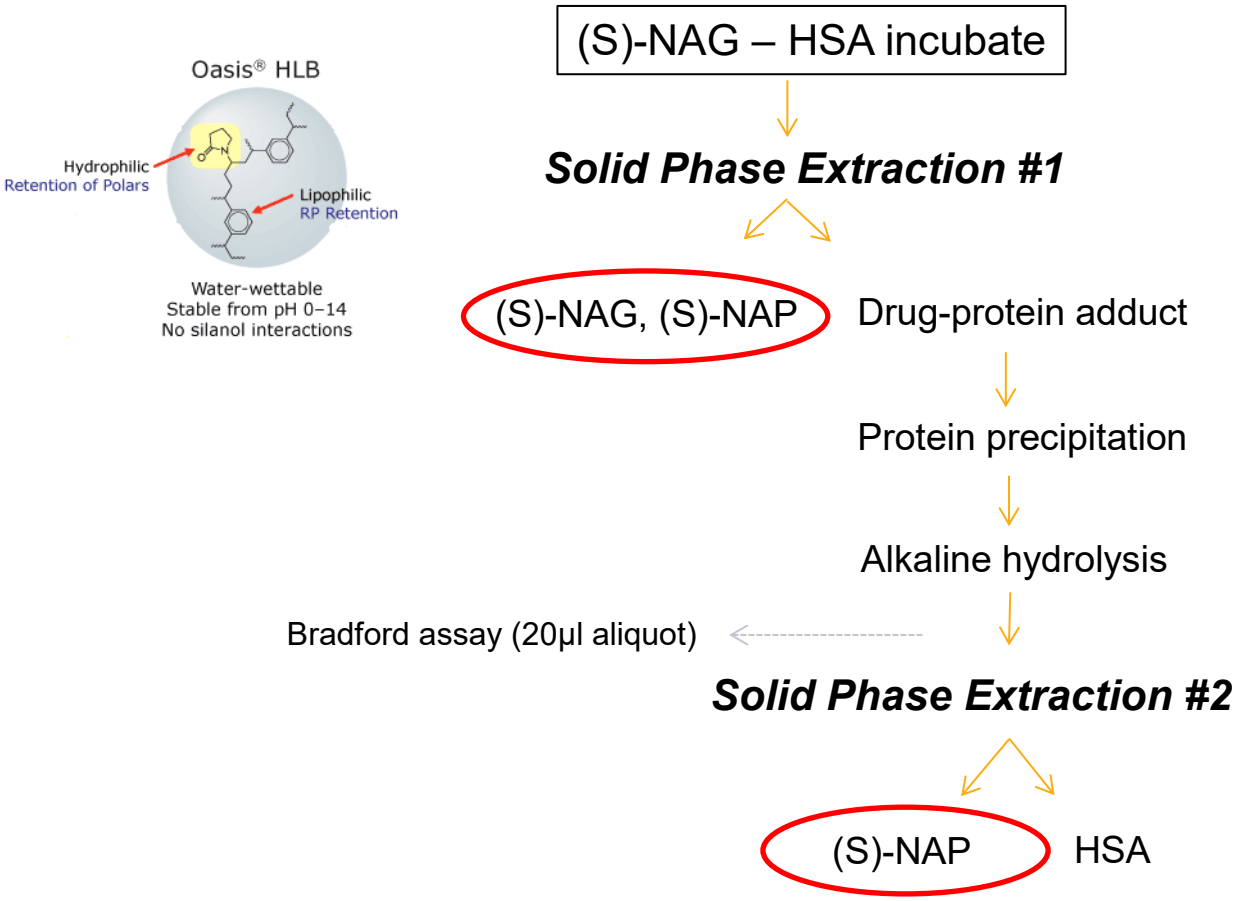


Figure 2.4. Summary of HPLC-MS assay of (S)-naproxen-1β-acyl glucuronide degradation in human serum albumin solution (pH 7.4) at 37ºC and of irreversible binding to human serum albumin using a solid phase extraction / alkaline hydrolysis technique. The initial (S)-NAG – HSA incubate is subjected to a first step of SPE in order to separate unbound drug species from the (S)-NAG – HSA adduct. Quantification of the species via HPLC-MS enable calculation of the rates of AG degradation and hydrolysis (spontaneous & enzymatic). The protein adduct is then precipitated and hydrolysed in alkaline conditions which release the drug from its irreversible binding. A small aliquot (20 µl) is collected for the purposes of protein quantification and the protein adduct is then subjected to a second step of solid phase extraction in order to separate liberated parent drug from the protein. Quantification of (S)-NAP and normalization over the amount of protein enable calculation of the level of irreversible binding.

2.4 RESULTS

2.4.1 Validation of the HPLC-MS assay

- **ANALYTE CHROMATOGRAPHY & MASS SPECTROMETRY**

In order to detect and quantify the analytes investigated in this project, they were separated by high performance liquid chromatography (HPLC) followed by detection using a high-resolution mass spectrometer (MS). Traditionally, mass-spectrometric quantification of low-molecular weight analytes is performed using selected or multiple-reaction-monitoring (SRM or MRM, respectively) protocols run on triple-quadrupole MS. In these systems the MS is programmed to select precursor ions of interest in the first quadrupole (Q1) for subsequent fragmentation in the collision cell (Q2). Resulting fragment ions of interest are then selected in the third quadrupole (Q3) and delivered to the detector resulting in the provision of the analyte signal. Whilst often automated and directed by software provided with the MS, occasionally selecting optimal transitions and instrument settings for analyte quantification can be time consuming. In the work described in this chapter, a high-resolution MS was used for the quantification of (S)-NAP, (S)-NAG and the IS. In the approach used in this work, a high-resolution ThermoFisher Orbitrap-LTQ-XL MS was used with full precursor scan data (160-900 m/z) applied to quantify analytes at resolution set to 30,000. Masses were extracted using a mass-accuracy filter of 5 ppm. For (S)-NAP, 3 masses were used due to in-source fragmentation: $[M\text{-}H]^-$, $[M\text{-}CO_2\text{-}H]^-$, $[M\text{-}CO_2\text{-}Me\text{-}H]^-$ (***Figure 2.5***). Only negligible in-source fragmentation was observed for (S)-NAG and no in-source fragmentation was detectable for the IS.

(S)-NAP and (S)-NAG were eluted at the retention times of 2.99 mins and 2.13 mins, respectively (see ***Table 2.1***). Mass spectra (only MS^1 spectrum) for the two analytes are presented in ***Figure 2.5***. In positive ion mode the analytes showed no mass signal. By contrast, in negative ion mode, (S)-NAP showed mass response with deprotonated molecule ion $[M-H]^-$ at mass-to-charge ratio (*m/z*) 229.0870 (-0.1189 ppm, elemental composition $C_{14}H_{13}O_3$) and in-source fragments at *m/z* 185.0972 (0.3019 ppm, elemental composition $C_{13}H_{13}O$) and 169.0659 (-0.0048 ppm, elemental composition $C_{12}H_{19}O$). The fragment *m/z* 185.0972 was generated by the loss of CO_2 (-43.9898 Da). Subsequent cleavage of CH4 (-16.0313 Da) resulted in the second fragment *m/z* 169.0659. As far as (S)-NAG is concerned, a strong mass response was identified in negative ion mode with the deprotonated molecule ion $[M-H]^-$ at *m/z* 405.1191 (0.0869 ppm, elemental composition $C_{20}H_{21}O_9$). This increased mass of 176.0321 Da compared to (S)-NAP represents the glucuronic acid conjugated to the aglycone. As the composition of analytes in the solution was known already and all matrices were simple, MS^2 spectrum analysis was not deemed necessary for the purposes of detection & quantification. The retention times, observed and calculated masses, mass errors and in-source fragments of the analytes are summarized in ***Table 2.1***.

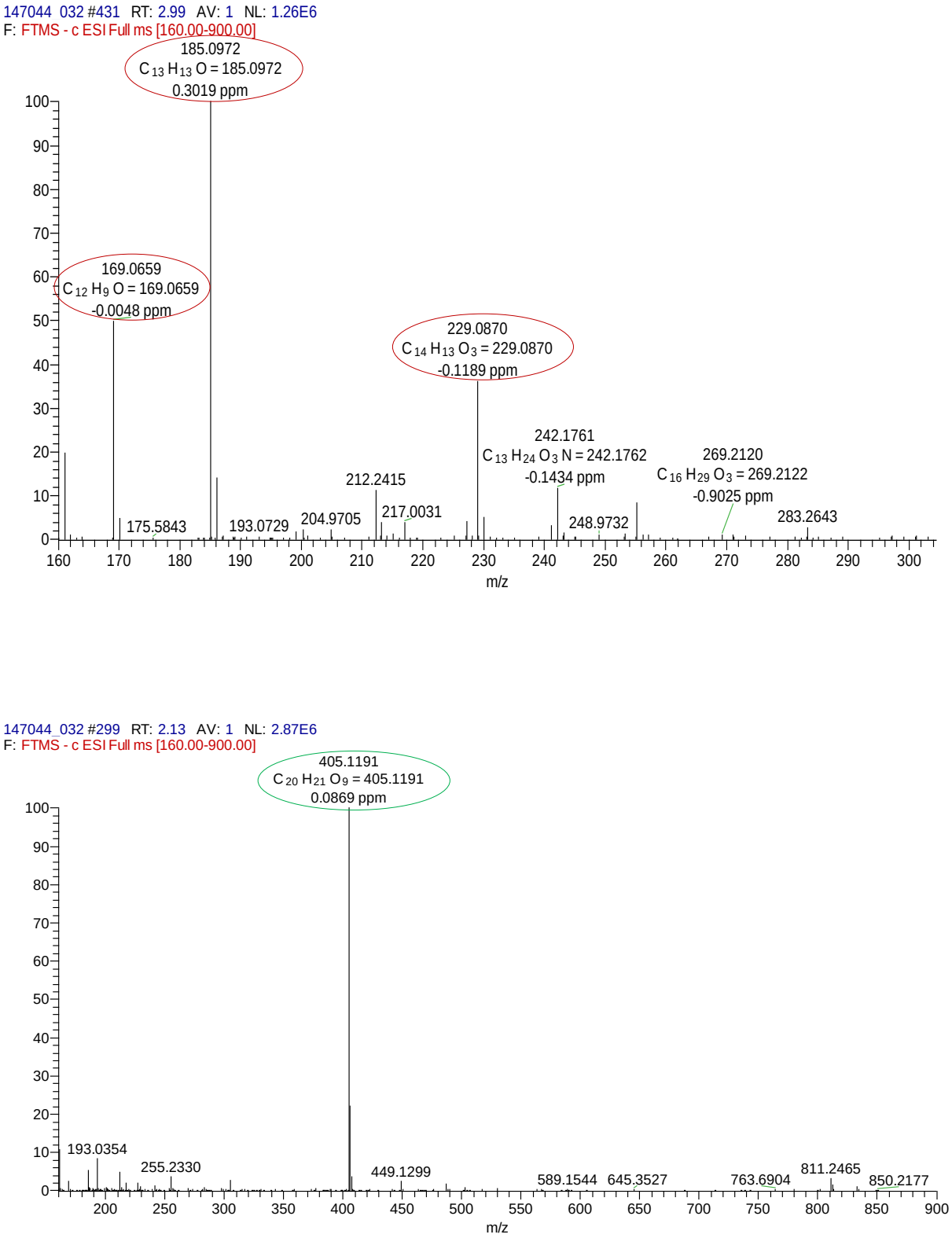


Figure 2.5. MS^1 spectra of (S)-naproxen (upper) and (S)-naproxen-1β-acyl glucuronide (lower). In-source fragmentation was observed for (S)-NAP with 3 fragmentation ions determined (circled in red) whilst in-source fragmentation for (S)-NAG was negligible and 1 m/z was used for quantification (circled in green).

Table 2.1. Characterization of the analytes by HPLC – LTQ Orbitrap XL - MS.

Molecule	RT (min)	Formula	Meas. *m/z*	Error (ppm)	In-source fragments
(S)-NAP (parent)	2.99	$C_{14}H_{13}O_3$	229.0870	-0.12	185.0972, 169.0659
(S)-NAG (metabolite)	2.13	$C_{20}H_{21}O_9$	405.1191	0.08	-
(IS)	2.39	$C_{13}H_{15}O_3$	219.1026	0.05	-

In degradation studies, (S)-NAG was quantified using the mass-spectrometric settings described above. From the extracted (S)-NAG from the calibration samples, a calibration curve was drawn and fitted in accordance to the following equation:

$Y = 0.0038779+2.34775*X-0.151183*X^2$, ($R^2 = 0.9938$, W: 1/X).

The formation of (S)-NAP following hydrolysis was similarly quantified, with a calibration curve fitted according to the below equation:

$Y = -0.00609727+1.04534*X$, ($R^2 = 0.9913$, W: $1/X^2$).

Finally, liberated (S)-NAP from HSA following alkaline hydrolysis was quantified with a calibration curve fitted according to the below equation:

$Y = -0.00623057+0.885531*X$, ($R^2 = 0.9884$, W: $1/X^2$).

The lower limit of detection (LLOD) was determined at 0.003 μM (Signal to Noise ratio (S/N) > 3) and lower limit of quantification (LLOQ) at 0.01 μM (S/N > 10).

- **RECOVERY OF ANALYTES THROUGH SOLID PHASE EXTRACTION**

(S)-NAP and (S)-NAG were prepared in 0.1 M phosphate buffer (pH 7.4) 37°C at the validated LLOQ and upper limit of quantification (ULOQ) of the developed analytical assay (0.01 and 4 µM, respectively) as well as in the middle of the quantifiable range of the assay (2 µM). These analytes were subsequently subjected to the SPE protocol described in the experimental methods. Wash and elution fractions were collected, evaporated to dryness and reconstituted in 150 µl of 10 % ACN containing 1 % lactic acid (v/v) and 0.5 µM IS. Similarly, the same QC concentrations of (S)-NAP and (S)-NAG were prepared in 95 % ACN containing 1 % lactic acid (v/v) before immediate evaporation to dryness and the same reconstitution in 150 µl of 10% ACN containing 1% lactic acid (v/v) and 0.5 µM IS. Resulting concentrations of (S)-NAP and (S)-NAG were below the LLOD (0.003 µM) in all wash fractions tested, indicating efficient analyte binding to the column. In elution fractions, the recovery of the tested analytes was found to be > 85 % at all concentrations tested and > 90 % for concentrations used during the sample preparation (1 mM and 200 µM AG) (***Table 2.2***), indicating efficient elution of analytes from the column for subsequent quantification under the tested protocol. Consequently, with respect to enrichment of the tested (S)-NAP and (S)-NAG analytes, the tested SPE protocol was deemed appropriate for subsequent experiments described in this chapter.

Table 2.2. Recovery of eluted analytes following solid phase extraction (n=6). Analyte recovery was assessed in 6 separate incubations (in the presence of 20 μM HSA) as described in Materials and Methods. Three different concentrations were used (20 μM, 200 μM and 1 mM).

Analyte	1 mM Recovery ± SD (%)	1 mM CV (%)	200 μM Recovery ± SD (%)	200 μM CV (%)	20 μM Recovery ± SD (%)	20 μM CV (%)
(S)-NAG	92.1 ± 2.0	2.31	90.2 ± 3.4	3.46	89.5 ± 4.9	6.81
(S)-NAP	93.1 ± 6.7	7.22	91.1 ± 3.0	3.29	86.9 ± 4.3	4.93

- **STABILITY OF (S)-NAPROXEN-1β-ACYL GLUCURONIDE THROUGH SOLID PHASE EXTRACTION AND IN AUTOSAMPLER**

It is well established that AG drug metabolites undergo spontaneous degradation when in aqueous conditions. Consequently, without tight control it is perceivable for AG degradation to occur through the SPE process. Acidifying aqueous samples to pH between 3 and 5 has been shown to slow this degradation to almost negligible amounts. Using litmus paper, addition of 1 % lactic acid to all SPE buffers was shown to reduce pH to this range. In order to confirm that no AG degradation was occurring following the SPE protocol a 1 mM solution of (S)-NAG was subjected to SPE, evaporated to dryness and reconstituted in the reconstitution buffer (10 % ACN containing 1 % lactic acid (v/v)) before immediate injection into the MS. Resulting signal for the known degradation products, namely the parent drug and positional isomers remained below the limit of detection of the instrument, confirming undetectable degradation of the (S)-NAG throughout the extraction process. A further plausible source of AG degradation was in the autosampler of the instrument, whilst batch-analysis of samples was performed. In the autosampler, samples were reconstituted in reconstitution buffer (10 % ACN

containing 1 % lactic acid containing 0.5 µM of the IS) and maintained at 4°C. No batch runs throughout the work undertaken in this thesis exceeded 8 hours, therefore AG stability over 8 hours under these conditions was investigated. (S)-NAG was reconstituted to a final concentration of 1 mM in reconstitution buffer containing 0.5 µM IS before immediate injection into the LC-MS (n=3). The samples were then kept in the autosampler for 8 hours before re-injection to the LC-MS. The ratio of the AUC of the peak attributable to (S)-NAG to the AUC of the peak attributable to the IS was then compared. Over the 8-hour time course, a 5.4 ± 0.8 % loss in (S)-NAG was observed. This minor AG degradation provides confidence of the suitability of the reconstitution buffer to not be susceptible to (S)-NAG degradation throughout LC-MS analysis of batches of samples in the work undertaken in this chapter.

- **ASSAY (WITHIN-RUN) ACCURACY & PRECISION**

In order to estimate the quantifiable range of the developed degradation and irreversible binding assays, several standards and QC samples were prepared as detailed in the materials and methods. All tested QC points fell within range as shown in ***Table 2.3*** for the degradation assays and ***Table 2.4*** for the irreversible binding assay. Overall, the degradation assay in HSA exhibited an accuracy and precision of 100 % and 10.27 %, respectively for (S)-NAG whereas for (S)-NAP accuracy was determined as 113.17 % with a 15 % precision. For the irreversible binding assay (quantification of liberated (S)-NAP) accuracy was identified as 107.90 % and precision 5.61 %. Consistent with the above, accuracy and precision for the phosphate buffer degradation assay was measured as 100.1 % and 8.83 %, respectively.

Table 2.3. Within-run accuracy & precision for assessing (S)-naproxen-1β-acyl glucuronide degradation in 0.1 M phosphate buffer (pH 7.4) and human serum albumin solution at 37°C. Values were calculated for the LLOQ (0.01 μM), medium (2 μM) and ULOQ (4 μM). All calculated accuracies and precisions fell within the acceptable ranges.

Analyte (Degradation)	LLOQ Accuracy (%)	LLOQ Precision (%)	Med QC Accuracy (%)	Med QC Precision (%)	ULOQ Accuracy (%)	ULOQ Precision (%)
(S)-NAG (HSA solution)	105.75	13.71	99.25	5.66	93.33	5.90
(S)-NAP (HSA solution)	119.57	18.57	114.75	3.88	102.00	14.60
(S)-NAG (Phosphate buffer)	80.12	6.61	101.34	5.21	98.76	3.43

Table 2.4. Within-run accuracy & precision for assessing (S)-naproxen-1β-acyl glucuronide irreversible binding to human serum albumin at 37°C. Values were calculated for the LLOQ (0.01 μM), medium (2 μM) and ULOQ (4 μM). All calculated accuracies and precisions fell within the acceptable ranges.

Analyte (Liberated)	LLOQ Accuracy (%)	LLOQ Precision (%)	Med QC Accuracy (%)	Med QC Precision (%)	ULOQ Accuracy (%)	ULOQ Precision (%)
(S)-NAP	108.8	2.55	102.4	5.92	112.2	4.95

2.4.2 Assessment of the degradation of (S)-naproxen-1β-acyl glucuronide in 0.1 M potassium phosphate buffer (pH 7.4) and human serum albumin at 37ºC

Synthetic (S)-NAG spontaneously degraded in both 0.1 M phosphate buffer (pH 7.4) at 37°C (10 µM NAG concentration) or 20 µM HSA in 0.1 M phosphate buffer (pH 7.4) at 37°C (1 mM or 200 µM (S)-NAG concentration; 50:1 or 10:1 AG to HSA molar ratios, respectively) (***Figures 2.6*** and ***2.7***). In both conditions, the 1β-AG degraded by first-order kinetics, meaning non-linear regression analysis could be performed. A curve was fitted to the data following the equation:

$C=C_0 exp^{(-kdeg)(time)}$

C represents the amount of (S)-NAG quantified in the mixture at specific time points and k_{deg} represents the degradation rate constant (***Table 2.5***). All regression curves fitted to the experimental data with r^2 greater than 0.98. Using these regression curves, the degradation t½ of (S)-NAG in phosphate buffer and HSA solution could be calculated.

Figure 2.6 shows this degradation of (S)-NAG in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. (S)-NAG was found to degrade with a calculated t½ of 2.72 hours. More than 80 % of initial metabolite concentration disappeared after 8 hours of incubation while negligible amounts where quantified at 24 hours. In presence of HSA, the rate of (S)-NAG degradation is enhanced (***Figure 2.7***) as is further reflected by the shorter t½ of the AG degradation (***Table 2.5***). The rate of (S)-NAG degradation reflects the ratio of AG to HSA, where the reduced amount of AG : HSA (and therefore an increased amount of HSA in relation to the AG) results in a faster rate of AG degradation. Similarly, the extent of AG hydrolysis as measured by the liberation of the aglycone from the glucuronide is increased as the ratio of HSA to the AG is increased.

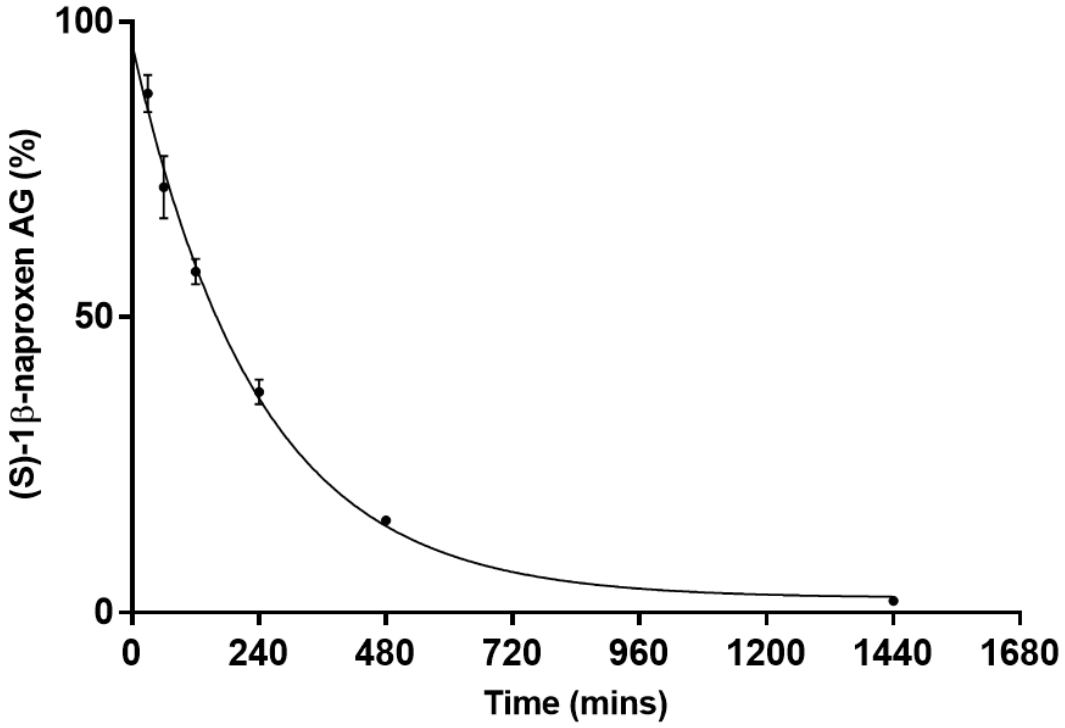


Figure 2.6. Degradation of (S)-naproxen-1β-acyl glucuronide in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. Fitted regression curve presenting the degradation of (S)-NAG during incubation in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. All incubations were performed in triplicate. Data are presented as means (± standard deviation; n=3) of % (S)-NAG remaining as quantified from initial amount of AG (10 µM) that was added to the incubation mixture at t = 0 mins. Data points are fitted with exponential decay equation (r^2 = 0.9905) and results of curve fitting are presented in Table 2.5.

Table 2.5. Half-lives of degradation and parameters for the fitted degradation curves shown in Figures 2.6 and 2.7. Data are fitted to the first-order degradation rate equation $C=C_0exp^{(-kdeg)(time)}$.

Matrix	t ½ (hour)	t ½ (min)	K_{deg} (min^{-1})	r^2
Phosphate Buffer	2.72	163.30	0.0042	0.9905
1mM (S)-NAG in 20µM HSA	1.60	96.45	0.0071	0.9859
200µM (S)-NAG in 20µM HSA	0.97	58.34	0.0118	0.9951

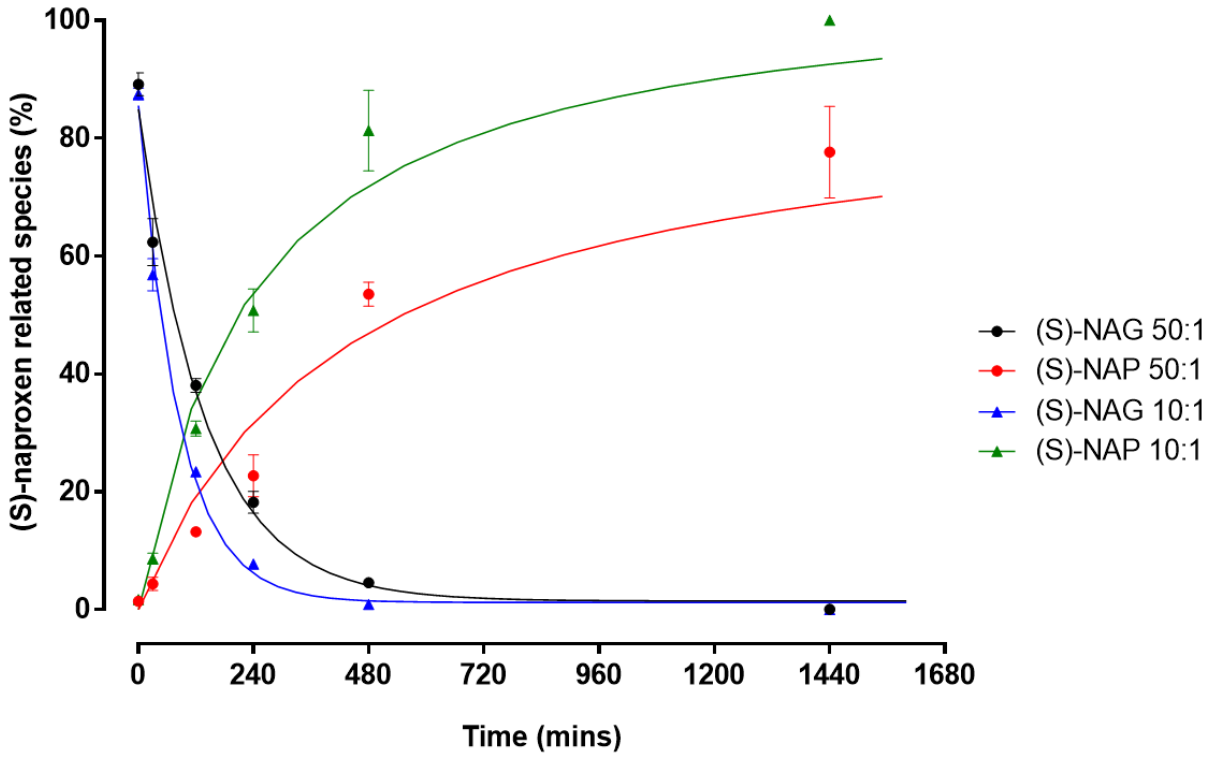


Figure 2.7. Degradation of (S)-naproxen-1β-acyl glucuronide in human serum albumin solution (pH 7.4) at 37°C. Fitted regression curves presenting the degradation and hydrolysis of (S)-NAG during incubation with 20 µM HSA at 1 mM (50:1) or 200 µM (10:1) in pH 7.4 solution at 37°C. All incubations were performed in triplicate. Data are presented as means (± standard deviation; n=3) of % (S)-NAG remaining or % (S)-NAP formed as quantified from initial amount of AG (1 mM or 200 µM) that was added to the incubation mixture at t = 0 mins. (S)-NAG at 50:1 and 10:1 incubation ratio is represented by blacked filled circles & blue filled triangles, respectively. (S)-NAP at 50:1 and 10:1 is represented by red filled circles and green filled triangles, respectively. Data points representing the degradation of (S)-NAG are fitted with exponential decay equation (50:1; $r^2 = 0.9859$, 10:1; $r^2 = 0.9951$) while data points representing the formation of (S)-NAP (i.e. hydrolysis of (S)-NAG) are fitted with hyperbola equation (50:1; $r^2 = 0.9372$, 10:1; $r^2 = 0.9741$) and results of curve fitting are presented in Table 2.5.

2.4.3 Assessment of the irreversible binding of (S)-naproxen-1β-acyl glucuronide to human serum albumin (pH 7.4) at 37ºC using a solid phase extraction / alkaline hydrolysis technique

Synthetic (S)-NAG was found to irreversibly bind to HSA in 0.1 M phosphate buffer (pH 7.4) when incubated at 37ºC (1 mM (S)-NAG or 200 µM (S)-NAG in 20 µM HSA, i.e. molar ratio of AG to HSA 50:1 or 10:1) in a concentration and time-dependent manner (***Figure 2.8***). At both AG incubation concentrations, maximal irreversible binding was observed at 24 hours of incubation (10:1; 36.76 ± 5.68 nmol naproxen/µmol HSA and 50:1; 247.37 ± 57.64 nmol naproxen / µmol HSA). However, a significant increase in irreversible binding to albumin was observed for 50:1 AG : HSA incubations when compared to 10:1 AG : HSA incubations at 8-hour and 24-hour incubation time points (5.28-fold; p=0.02 and 6.73-fold; p=0.003 increase in mean irreversible binding, respectively). Whilst the same trend was observed for the 2-hour incubation time point (10:1; 13.95 ± 5.67 nmol/µmol and 50:1; 23.55 ± 15.31 nmol/µmol), this was not statistically significant (p=0.37).

A statistically significant time-dependent increase in irreversible binding was observed across all time points for the 50:1 incubation. A 4.5-fold increase (p=0.05) in mean irreversible binding was observed between 2 and 8 hours, and a 1.9-fold (p=0.006) increase was observed between 8 and 24 hours. The difference was significant between 2 and 24 hours reflecting a 10.5-fold increase ($p < 0.001$). A similar time-dependent increase in irreversible binding was also observed for the 10:1 incubation, excepting that the 1.45-fold increase in mean irreversible binding between 2 and 8-hour incubations was not statistically significant (p=0.34). However, the increase in mean irreversible binding between 2 and 24 hours (2.6-fold; p=0.01) or 8 and 24 hours (1.81-fold; p=0.04) was statistically significant.

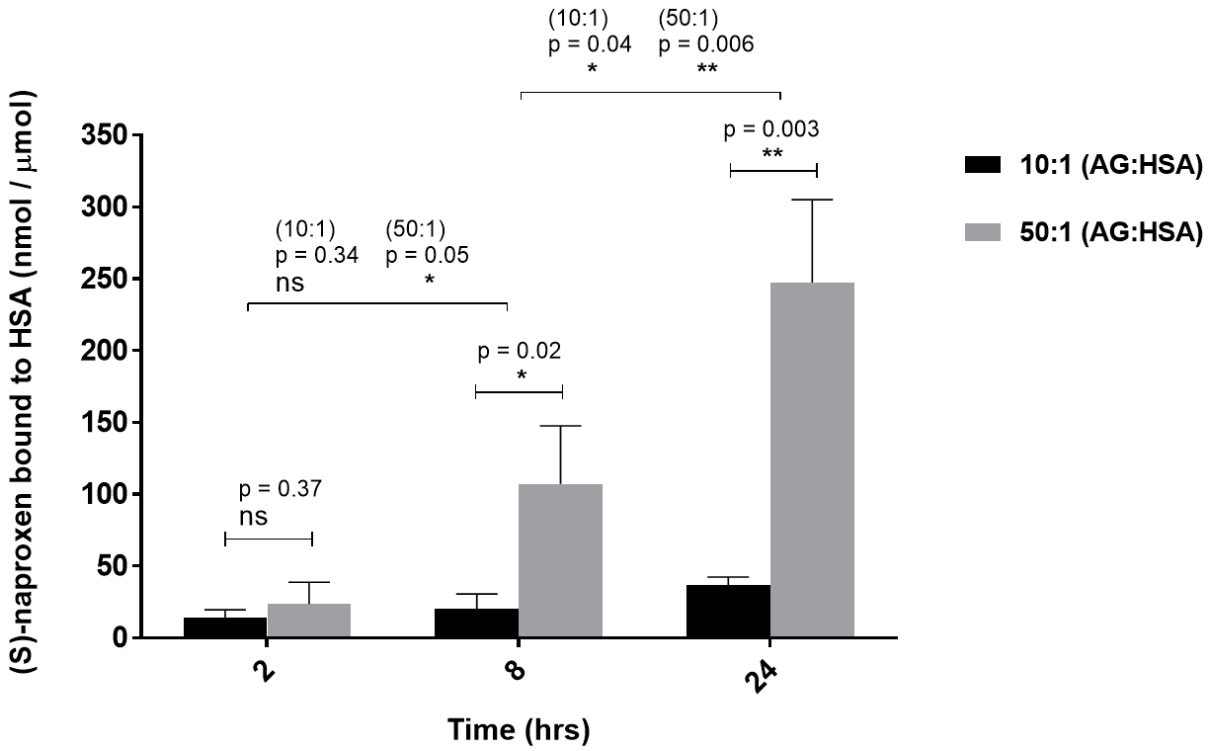


Figure 2.8. Irreversible binding of (S)-naproxen-1β-acyl glucuronide to human serum albumin (pH 7.4) at 37ºC. (S)-NAG (1 mM or 200 µM) was incubated *in vitro* with 20 µM HSA (pH 7.4) at 37°C and irreversible binding was measured with a SPE / alkaline hydrolysis technique at 2, 8 and 24 hrs of incubation (120, 480 and 1440 mins). All incubations were performed in triplicate. Data are presented as means (± standard deviation; n=3) of drug bound to HSA (nmol / µmol). Statistical comparisons of experimental groups (50:1 vs 10:1) within the same time point were made using multiple t-tests while comparisons between different time points were made using one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

2.4.4 Investigation of poor irreversible binding kinetics assessment following use of solid phase extraction

Through the work entailed in this chapter, SPE was used as a strategy to isolate HSA from non-covalently bound analytes. During irreversible bindings investigations, it was not possible to assess total binding in early time points of incubation (< 2 hrs) as the liberated analyte ((S)-NAP) was below the limit of quantification. Although the amount of drug bound onto albumin was expected to be lower in those early incubation time-points as has been shown with other

AGs (Smith et al., 1986; Dubois et al., 1993; Hammond et al., 2014), the protein recovery from SPE was investigated in more detail.

The Oasis HLB SPE columns used in this chapter are routinely used internally within the lab for isolation of polar analytes from a range of matrices including human plasma. Consequently, it was assumed that HSA would not exhibit a strong interaction with the SPE column under the experimental conditions used in this Thesis and would primarily elute in the load fraction, whereas non-covalently bound drug would exhibit a stronger interaction and elute in the elution fraction (as shown previously). In order to confirm this and the low affinity of HSA towards the Oasis HLB packing material, we investigated the recovery of HSA from the columns in load, wash and elute fractions. As all experiments involved the loading of 10 µM HSA in 300 µl of 0.1 M phosphate buffer (pH 7.4) at 37°C, it was this concentration and volume that HSA recovery was investigated. As shown in ***Figure 2.9***, following administration of HSA to the Oasis HLB column, only 23.8 ± 3.1 % of the loaded protein was recovered. This indicates that HSA does exhibit an interaction with the Oasis HLB packing material. In the wash fraction, no recovery of HSA was observed indicating that all non-bound HSA is either eluted in the load fraction or very strongly bound to the column. In a separate recovery experiment, where the same experiment was performed but two additional wash steps were included, this finding was confirmed where again only negligible HSA was recovered (0.8 ± 0.3 %) in the second wash only. In the elute fraction intended to liberate the low-molecular weight analytes interacting with the SPE column, 14.3 ± 5.9 % of initially loaded HSA was recovered. This observation, in combination with approximately 61 % of HSA not being recovered from the column provides a good indication that HSA in fact does interact strongly with Oasis HLB columns. However, the main question raised by this finding is whether this interaction of HSA with Oasis HLB columns would be predicted to interfere with subsequent degradation or irreversible binding measurements. For irreversible binding measurements, the amount of irreveresibly

bound drug liberated by alkaline hydrolysis is normalised to the amount of HSA recovered. Consequently, the low recovery of HSA would not affect any irreversible binding results when normalised in this manner. The low recovery however may mean that whilst the amount of drug's irreversible binding normalised to amount of recovered protein is unchanged, the reduced amount of protein recovered would result in a reduced total amount of liberated drug. Consequently, capture of data where irreversible binding is low proves challenging as liberated drug can be lower than the defined lower limits of quantification of this assay. Hence, capture of good kinetic profiles at these earlier time-points was not be possible. However, whilst detailed kinetics of (S)-NAG's irreversible binding could not be achieved by this work, the later time-points, were irreversible binding could be quantified, confirm the protein reactivity of (S)-NAG. The second question is whether the co-elution of HSA with (S)-NAP and (S)-NAG could affect the results in degradation studies. As the amount of irreversibly bound drug attached to the eluted HSA is very low (approx. 1.5 % for the highest (S)-NAG concentration by extrapolation from the findings in section **2.4.3**), and no liberating conditions such as alkaline hydrolysis were used, it is very unlikely that the irreversibly bound drug would affect quantification of non-bound (S)-NAP or (S)-NAG. Furthermore, as organic solvents (ACN) were used in the elution step, any HSA would be denatured and lose esterase / non-covalent binding capacity it may possess. Finally, the eluate was centrifuged before the supernatant was extracted and subjected to evaporation under nitrogen. This centrifugation step is also expected to remove much of the eluted contaminant.

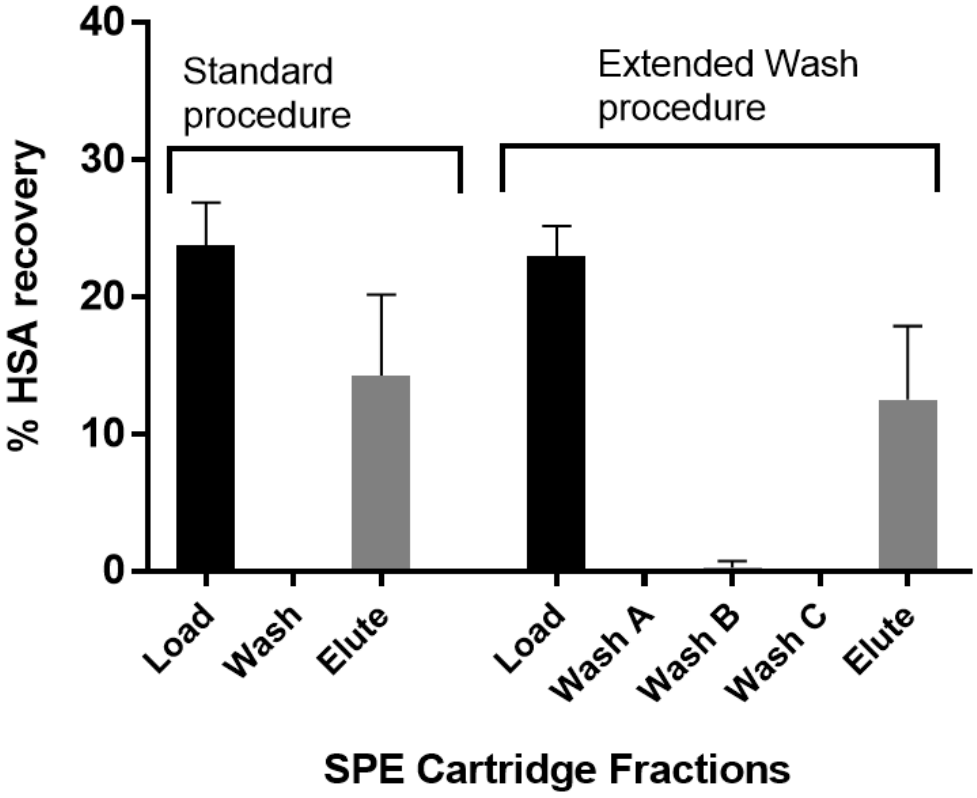


Figure 2.9. Recovery of human serum albumin across fractions following solid phase extraction method (n=3). Samples of 10 µM HSA in 0.1 M phosphate buffer (pH 7.4) were loaded onto SPE cartridges (1 cc; 30 mg sorbent). The flow through fraction (load), wash fraction(s) and elute were collected and protein was quantified via Bradford assay (Bradford, 1976). Recovery of HSA over the initial amount was calculated. All incubations were performed in triplicate. Data are presented as means (± standard deviation; n = 3).

2.5 DISCUSSION

The work described in this chapter confirms the premise that (S)-NAG is chemically unstable in aqueous conditions (potassium phosphate buffer 0.1 M (pH 7.4) at 37°C). The calculated degradation t½ of 2.72 hrs is below the 3.6 hrs threshold proposed by Sawamura et al. separating "safe" drugs from "withdrawn" and "warning" (Sawamura et al., 2010). Furthermore, the protein reactivity of (S)-NAG was confirmed through identifying its irreversible binding to HSA following *in vitro* incubation. (S)-NAP undergoes significant

glucuronidation in human as revealed by clinical metabolism studies and metabolism studies using human liver microsomes supplemented with UDP-glucuronic acid (UDPGA) (Segre, 1975; Sugawara, Fujihara, Miura, Hayashida, & Takahashi, 1978; Vree et al., 1993; Bowalgaha et al., 2005). The degradation t½ data of (S)-NAG combined with (S)-NAP's known extensive glucuronidation indicate that if (S)-NAP, which was introduced in 1976 as a novel NSAID for chronic administration, was developed as a novel therapeutic today, safety concerns would likely be raised over the potential of the molecule as a consequence of its metabolism to its AG. In hindsight, however, naproxen-induced serious ADRs (such as iDILI) are very rare, especially when considering the vast numbers of patients exposed to the drug. In view of the above, this may actually lower the perceived concern that AG should be classified as a toxicophore associated with severe immune-mediated adverse reactions, and highlights the value of investigating naproxen and (S)-NAG in this context.

Through the work described in this thesis (S)-NAG degraded in 0.1 M phosphate buffer at (pH 7.4) at 37°C with a calculated t½ of 2.72 hrs. This finding is in the same range as the (S)-NAG degradation half-lives reported by Sawamura et al. and Jinno et al. (2.2 ± 0.2 hrs and 2.89 ± 0.09 hrs, respectively) (Sawamura et al., 2010; Jinno et al., 2013). Notably, an earlier study by Bischer et al. reported a shorter t½ of (S)-NAG degradation in phosphate buffer 0.1 M (pH 7.4), of 1.75 ± 0.07 hrs, resulting in almost 90 % of the starting (S)-NAG concentration being degraded by 5.5 hrs of incubation (Bischer et al., 1995). Whilst in all of the reported studies the degradation t½ of (S)-NAG is below the proposed 3.6 hrs risk-threshold (Sawamura et al., 2010), there is a clear inter-lab variation. The reason for this slight inconsistency is unclear. One reason could be the source of the AG used in the experiments. AG metabolites have traditionally been challenging for medicinal chemists to synthesize at yield and purities sufficient for experimentation. Consequently, for these degradation studies AGs have been isolated and purified from biological matrices (such as bile or urine) of animals or human

administered the parent drug. Alternatively, *in vitro* AG biosynthesis systems such as liver microsomes supplemented with UDPGA have also been used, with potential for contaminants remaining after AG isolation which may slightly effect degradation data.

The irreversible binding of AG metabolites of different drugs towards HSA has been shown to correlate almost perfectly with their rate of degradation in 0.1 M phosphate buffer (pH 7.4) at 37°C (Benet et al., 1993). Consequently, due to its simplicity and requirement for only low amounts of AG, the degradation assay has been used as a surrogate to predict the AG protein reactivity. However, it was not until much more recently that the association of AG reactivity and the marketable fate of the parent drugs was explored (Sawamura et al., 2010). A t½ of (S)-NAG degradation in 0.1 M phosphate buffer (pH 7.4) of 3.6 hours was reported to separate withdrawn drugs (identified half-lives ≤ 3.6 hrs) from those that were classified as 'safe' or those remaining on the market but associated with warnings (identified half-lives ≥ 3.6 hrs). Consequently, it has been proposed that identification of AG t½ under these conditions of ≥ 3.6 hrs would provide confidence in the safety of the parent molecule. However, in investigating the drugs within the classifications more deeply, it should be noted that of the four drugs in the withdrawn group, zomepirac and benoxaprofen are included. Zomepirac was withdrawn due to associations with anaphylaxis reactions (Guerrero, 1983; Levy & Vasilomanolakis, 1984) which have a different pathogenesis than the delayed hepatotoxicity reactions associated with most of the carboxylic acid drug class. Whilst benoxaprofen was associated with eliciting fatal cholestatic jaundice in elderly patients (Duthie, Nicholls, Freeth, Moorhead, & Triger, 1982; Taggart & Alderdice, 1982), it was also phototoxicity which has been reported to represent a major reason for the drug's withdrawal (Halsey & Cardoe, 1982). Nevertheless, our experiments reveal (S)-NAG to have a degradation t½ of 2.72 hrs in 0.1 M phosphate buffer (pH 7.4). This falls below the 3.6 hrs proposed by Sawamura et al. and consequently would place naproxen into the warning and withdrawn categories (Sawamura et

al., 2010). Ultimately, the chemical reactivity of (S)-NAG is sufficient for anxiety to be raised over the potential of the metabolite to haptenise endogenous macromolecules and elicit hypersensitivity or hepatotoxic ADRs.

Addition of HSA to the (S)-NAG incubation mixture resulted in a faster rate of AG degradation. In the presence of HSA solution (0.13 %; 20 µM), 1 mM (S)-NAG (50:1 AG : HSA molar ratio) and 200 µM S-NAG (10:1 AG : HSA molar ratio) degraded with a t½ of 1.6 hrs and 0.97 hrs, respectively. Whilst the isomerization of the (S)-NAG was not measured in these studies, extensive hydrolysis of the conjugate was observed in the presence of HSA. Esterase-like activity of HSA has been previously reported (Ma et al., 2005) and its catalysis of the hydrolysis of AGs has already been described (Mizuma, Benet, & Lin, 1999; Karlsson et al., 2010; Hammond et al., 2014). As development of chromatographic methods separating each of the acyl migration isomers in order to allow acyl migration kinetics is very challenging and time-consuming, this was deemed beyond the scope of our work. Consequently, the effect of HSA on (S)-NAG acyl migration could not be ascertained in our experiments. Moreover, the extent of spontaneous AG hydrolysis was not measured when (S)-NAG was incubated in 0.1 M phosphate buffer (pH 7.4) at 37°C, meaning the extent of increased hydrolysis purely through the addition of the HSA to the incubation mixture could not be ascertained. However, it is noticeable that increased hydrolysis occurred when the ratio of HSA to AG in the incubation mixture is increased. Whilst extensive (S)-NAG hydrolysis was observed when it was incubated in the presence of HSA at a 50:1 molar ratio, the extent and rate of (S)-NAG hydrolysis was increased for the 10:1 incubation. This phenomenon has been reported for other AGs such as ketoprofen (Dubois, Lapicque, Abiteboul, & Netter, 1993) and diclofenac (Hammond et al., 2014).

The degradation of (S)-NAG in the presence of HSA has been investigated before, albeit with variations in experimental conditions and techniques when compared to this Thesis. Bischer et

al. calculated the degradation t½ of (S)-NAG when incubated with 0.3 % HSA ($t_{1/2}$ = 0.78 hrs) and 3 % HSA ($t_{1/2}$ = 0.45 hrs) solution (Bischer et al., 1995). These half-lives are shorter when compared to the ones identified in this Chapter's experiments but in line with the authors' reported shorter degradation t½ in phosphate buffer 0.1 M of 1.75 ± 0.07 hrs. It is important to acknowledge the molar ratio of AG : HSA incubation that was used in Bischer et al.; the authors describe an incubation of 50 μM (S)-NAG with 45 μM HSA which reflects an almost 1:1 incubation ratio (Bischer et al., 1995). The results of the experiments presented in this Chapter show that (S)-NAG's degradation rate constant (K_{deg}) increased from the high (50:1; 0.0071 min^{-1}) to lower (10:1; 0.0118 min^{-1}) AG : HSA incubation ratio. Therefore, it is plausible that a faster rate of AG degradation and exacerbated hydrolysis could be expected for a 1:1 incubation. Indeed, although Bischer et al. used a single (S)-NAG concentration, they measured degradation in the presence of different HSA concentrations (Bischer et al., 1995). The authors highlighted this AG : HSA response through reporting increasing degradation rate constants (K_{deg}) as the AG : HSA ratio in the incubation mixture decreased. For a 1.1:1 AG : HSA molar ratio the K_{deg} was 0.89 ± 0.02 hr^{-1}, but increased to 1.55 ± 0.08 hr^{-1} when the AG : HSA molar ratio decreased to 0.11:1. Further studies have also investigated the rate of degradation of (S)-NAG in the presence of HSA. Sawamura et al. incubated 10 μM of (S)-NAG with 4 % HSA solution and calculated a degradation t½ of 0.3 hrs (0.015:1 AG : HSA incubation ratio) (Sawamura et al., 2010). This much lower t½ in comparison to the half-lives measured in this Thesis' experiments can be explained by the significantly decreased incubation ratio used by the investigators. Furthermore, Iwaki et al. calculated an increase in hydrolysis rate for the lower molar ratio in their AG : HSA incubations of 0.08:1 (35 μM (S)-NAG : 435 μM HSA) and 0.3:1 (135 μM (S)-NAG : 435 μM HSA) as reflected by K_{hyd} 1.01 ± 0.10 hr^{-1} and K_{hyd} 0.860 ± 0.199 hr^{-1}, respectively (Iwaki et al., 1999).

In section **2.4.2**, it was also observed that not only did the rate of hydrolysis increase in the 10:1 AG : HSA incubation, but hydrolysis also represented an important degradation pathway as seen by the appearance of aglycone naproxen. Furthermore, whilst to a lesser extent, formation of the aglycone was also observed in the 50:1 incubation. Although hydrolysis during the incubation of (S)-NAG in phosphate buffer 0.1 M was not assessed in our experiments, several authors independently report acyl migration being the dominant chemical pathway of (S)-NAG degradation *in vitro* in phosphate buffer 0.1 M (pH 7.4) at 37°C (Bischer et al., 1995; Iwaki et al., 1999; Mortensen, Sidelmann, Tjornelund, & Hansen, 2002). As described above, hydrolysis represented a quantitatively more important degradation pathway when HSA was added to the incubation mixture. Although quantification of AG isomerization was out of the scope of this Chapter's investigational aims, the literature provides further insights into the degradation pathways of (S)-NAG in phosphate buffer 0.1 M. In these reports, even though hydrolysis was not the primary pathway of degradation for (S)-NAG in phosphate buffer, a significant amount of hydrolysed aglycone was still detected (Iwaki et al., 1999; Mortensen et al., 2002) as opposed to the negligible amount in the case of diclofenac-AG, for example, (Hammond et al., 2014) thus highlighting how structurally different AGs may present with different rates and levels of acyl migration and hydrolysis. Hydrolysis has been shown to be a major pathway of degradation for several AGs when incubated with HSA as opposed to phosphate buffer alone (Watt & Dickinson, 1990; Sallustio et al., 1997; Mizuma et al., 1999; Iwaki et al., 1999; Akira et al., 2002). However, similarly to data derived from phosphate buffer incubations, levels of hydrolysis and the effect of different molar ratio of incubation appears to vary between AGs. Increased molar ratio of incubation (AG : HSA) led to higher hydrolysis for ketoprofen-AG (Dubois, Lapicque, Magdalou, Abiteboul, & Netter, 1994) however the hydrolysis of diflunisal-AG remained relatively unchanged between 117 μM and 23 μM incubations (Watt & Dickinson, 1990). Hammond et al. determined negligible hydrolysis of

diclofenac-AG at a 50:1 AG : HSA molar ratio, similar to the levels of hydrolysis of diclofenac-AG in phosphate buffer 0.1 M (pH 7.4) at 37°C (Hammond et al., 2014). However, in the 10:1 AG : HSA molar ratio, hydrolysis represented a much more important pathway of degradation. In this Thesis' experiments, the kinetics of hydrolysis for (S)-NAG during incubation with HSA (10:1 and 50:1 AG : HSA incubation ratio) were reflected by an initial steep rise of the liberated aglycone which over time slowed down, gradually reaching a plateau towards the later time points alongside negligible amounts of (S)-NAG, indicating that the 1β-AG is primarily undergoing HSA-mediated hydrolysis . Indeed, in experiments described by Iwaki et al., during incubation of 35 μM (S)-NAG with 435 μM HSA (0.08:1 AG : HSA), the AG was rapidly hydrolysed within the first couple of hours and after 8 hours of incubation the majority of the initial 1β-AG concentration was identified mainly as (S)-NAP (Iwaki et al., 1999). During phosphate buffer incubation, hydrolysis of (S)-NAG was much slower compared to isomerization, however, a significant amount of hydrolyzed (S)-NAP was measured at 24 hours, at which point the 1β-AG had completely degraded and only (S)-NAP and positional isomers of (S)-NAG were present in the mixture. The kinetics of positional isomers were not investigated in the experiments of this Thesis as this was not reflecting the primary aims (the current analytical technique would not be the most appropriate for this), however literature offers insights for several AGs including naproxen. It is apparent that the balance between hydrolysis and acyl migration is affected by the matrix, pH, temperature as well as structure of the AG (Hyneck et al., 1988; Spahn-Langguth & Benet, 1992; Stachulski et al., 2006). Most AGs undergo preferential acyl migration, rather than hydrolysis, during incubation in phosphate buffer at physiological conditions (Watt & Dickinson, 1990; Iwaki et al., 1999; Corcoran, Mortensen, Hansen, Troke, & Nicholson, 2001; Akira et al., 2002; Berry et al., 2009; Johnson et al., 2010; Karlsson et al., 2010; Hammond et al., 2014). Positional isomers (2-, 3-, 4-) appear sequentially following degradation of the 1β-conjugate (Ebner et al., 1999; Iwaki et

al., 1999; Stachulski et al., 2006) and level off towards the later time-points of the incubation. This is explained by the movement of the aglycone structure around the ring, across one adjacent ring-carbon at a time until an equilibrium is reached (Bradow, Kan, & Fenselau, 1989; Berry et al., 2009). In the case of (S)-NAP, during phosphate buffer 0.1 M incubation, the major 2-isomer reached a maximum concentration as early as 6 hours, and after 24 – 48 hours of incubation all identified positional isomers equilibrated to almost the same concentration (Iwaki et al., 1999; Mortensen et al., 2001; Mortensen et al., 2002). In the presence of HSA (35 μM (S)-NAG in 435 μM HSA), the maximum concentration of the 2-isomer was achieved at approximately 2 hours whilst only minor further acyl migration to form the 3- and 4- isomers was reported (Iwaki et al., 1999).

A further main aim of this Chapter was to confirm the translation of AG reactivity in phosphate buffer 0.1 M to the reported protein reactivity of the AG metabolite. In our experiments, alongside the vast majority of studies investigating AG protein reactivity, (S)-NAG irreversible binding towards HSA was investigated using an alkaline hydrolysis technique. This assay allows the liberation of the aglycone from HSA thus measuring collectively the transacylation and glycation pathways of covalent modification (Hyneck et al., 1988; Smith et al., 1990; Munafo, McDonagh, Smith, & Benet, 1990; Dubois et al., 1993; Mayer, Mutschler, Benet, & Sphahn-Langguth, 1993; Castillo et al., 1995; McGurk, Remmel, Hosagrahara, Tosh, & Burchell, 1996; Presle, Lapicque, Fournel-Gigleux, Magdalou, & Netter, 1996; Sallustio et al., 1997). Maximal irreversible binding (measured as nmol of naproxen bound per μmol of HSA) was observed at 24 hrs of incubation (10:1; 36.76 ± 5.68 nmol/μmol and 50:1; 247.37 ± 57.64 nmol/μmol) with the mean level of irreversible binding at the 50:1 molar ratio being 6.7-fold and significantly higher ($p = 0.003$). Limited data are available on the irreversible binding of (S)-NAG with HSA. Bischer et al. identified the maximal covalent binding yield at 1.03 ± 0.19 μmol / mmol protein when incubating 50 μM of (S)-NAG with 450 μM HSA (3 % solution)

and being achieved at approximately 2 hrs while remaining relatively constant thereafter (Bischer et al., 1995). This incubation, reflecting 0.11:1 AG : HSA molar ratio, does not permit direct comparison with the data generated herein, but it does highlight the expected lower irreversible binding yield. In our experiments, a yield of 13.95 ± 5.67 nmol/μmol protein was measured for the 10:1 ratio (lowest) at 2 hrs. In addition, although the three time-points (2, 8, 24 hours) do not enable detailed kinetic characterisation and comparison with existing literature, it was evident that there was an increase in the irreversible binding yield over time. This trend is in line with published evidence for various AGs (Smith et al., 1986; Munafo et al., 1990; Watt & Dickinson, 1990; Mayer et al., 1993; Dubois et al., 1994; Castillo et al., 1995; McGurk et al., 1996; Sallustio et al., 1997; Hammond et al., 2014). While it cannot be determined whether the measured yield of (S)-NAG – HSA at 24 hours was the maximum during the incubation period, the findings are nonetheless in striking contrast with the kinetic evidence from Bischer et al. who identified maximum binding after 2 hrs of incubation (Bischer et al., 1995). This could be explained by the much lower molar ratio of incubation used in their investigations as opposed to the 10:1 or 50:1 used in this Thesis. As an example, a maximum irreversible binding yield of diclofenac-AG to HSA has been observed at an earlier time point during the 10:1 incubation as opposed to the 50:1 (Hammond et al., 2014). Modification of protein generally occurs up to the point where C_{max} is reached. Thereafter, covalent binding either plateaus or slowly decreases (thus suggesting that AG adduction of protein may not be fully irreversible) based on observations for various AGs such as zomepirac (Smith et al., 1986), tolmetin (Munafo et al., 1990), ketoprofen (Presle et al., 1996) and diclofenac (Hammond et al., 2014). Iwaki et al. provided some additional interesting data around the irreversible binding of (S)-NAG to HSA (Iwaki et al., 1999). According to their results, the lower drug concentration incubate (0.08:1 AG : HSA) generated a maximal yield of approximately 0.5 mmol / mol protein a little after 1 hour of incubation while the higher (0.3:1

AG : HSA) produced a maximal of about 1.6 mmol / mol protein at approximately 6 hours (Iwaki et al., 1999). Thus, not only is it evident that the binding yield is proportional to the initial concentration of AG but that, perhaps, the kinetics of irreversible binding may be impacted by such changes as well, leading to maximum yields at a faster rate. Furthermore, increased clearance of AG by hydrolysis will normally lead to less AG available for irreversible binding thus affecting the maximum levels of binding. Not surprisingly, herein, the 10:1 (S)-NAG : HSA incubation exhibited significantly higher hydrolysis and was associated with significantly less total irreversible binding when compared to the 50:1 (S)-NAG : HSA incubation.

Several publications provide information on the irreversible binding levels of AGs to HSA and it has been suggested that the maximum percentage of AG irreversibly bound to HSA can represent a tool to rank the AG protein reactivity *in vitro* (Castillo et al., 1995). Quantifiable data exist for ibuprofen (1.5%), zomepirac (2.3%), ketoprofen (3.2%), etodolac (3.3%) and diclofenac (1.8%) (Smith et al., 1990; Smith, Song, & Rodriguez, 1992; Dubois et al., 1993; Castillo et al., 1995; Hammond et al., 2014). Considering the calculated mean values described earlier for naproxen (0.36 % for 10:1 and 2.47 % for 50:1), it's fairly evident that (S)-NAG represents a chemically unstable and protein reactive metabolite. It should be noted though that these values are dependent on the molar ratio of incubation (AG : HSA) rather than just the intrinsic reactivity of the metabolite (Dubois et al., 1993; Hammond et al., 2014). As previously mentioned, the current analytical technique employed for the assessment of irreversible binding (alkaline hydrolysis) cannot distinguish between modification via transacylation or glycation and it represents a crude method of investigating protein binding. However, combination of tryptic peptide HPLC, MRM survey scanning and product ion scanning has unveiled site and structure of modification and hence the chemical route of modification for a few compounds (Grigoryan et al., 2009; Jenkins et al., 2009; Frolov & Hoffmann, 2010; Meng et al., 2011;

Whitaker et al., 2011; Deng, Zhong, & Chen, 2012; Hammond et al., 2014). No robust evidence exists for the in-depth modification profile of HSA following incubation with (S)-NAG, however it is anticipated that both transacylation and glycation mechanisms take place due to the presence of acyl migration (Iwaki et al., 1999) as is the case for all AGs. This however will largely depend on the matrix and *in vitro* results may differ significantly compared to *in vivo* situation (Ding et al., 1995; Qiu et al., 1998; Hammond et al., 2014).

As detailed in Materials and Methods, SPE was used throughout the experimentation involving HSA for determining the degradation of (S)-NAG while a combination of SPE with alkaline hydrolysis enabled the assessment of irreversible binding to HSA. The principal advantage of this extraction method for drug separation lies in the speed at which multiple samples can be processed alongside high analyte recovery and reproducibility. Indeed, using a 12-cartridge vacuum it has been possible to separate unbound from bound AG very quickly without the need for sequential washes with organic solutions. The latter is also increasing the burden of organic waste as opposed to SPE. It was apparent however, that SPE comes with a big limitation, namely the amount of protein recovered as detailed in section **2.4.4**. Due to the nature of the sorbent and the size of HSA, a proportion of the protein is held in the cartridge as is evident from the protein recovery measured in the elute fraction. Bolze et al. did use in their experiments SPE for the quantification of AG degradation in protein solution however they did not utilize this extraction method for their irreversible binding assessments (Bolze et al., 2002). Considering the extent of irreversible binding of AGs to HSA, it became very difficult to measure binding below 2 hours due to the small amount of protein recovered, as the amount of liberated (S)-NAP was below the limit of detection. Therefore, this method should be avoided if the main purpose of experimentation is kinetic characterization of irreversible binding and / or the analyte and protein concentrations used are such that require increased sensitivity for quantification.

In summary, the work undertaken in this chapter has confirmed the existing literature. (S)-NAG degrades in phosphate buffer 0.1 M (pH 7.3) at 37°C with a degradation t½ of 2.72 hrs. Sawamura et al. have suggested a degradation t½ in phosphate buffer of above 3.6 hrs to indicate drugs without potential associations towards liver and hypersensitivity adverse reactions, whereas the lower degradation t½ of 2.72 hrs for (S)-NAG would fall in the withdrawn / warning group and therefore would be considered a potential safety concern (Sawamura et al., 2010). The primary concern relates to the theoretical potential of AGs to haptenate endogenous macromolecules, and subsequently elicit delayed hypersensitivity and hepatotoxicity reactions in susceptible individuals. *In vitro* protein reactivity of (S)-NAG has also been confirmed in this work by showing irreversible binding to HSA.

These data combined with the known extensive glucuronidation of naproxen indicate that naproxen, which was introduced in 1976 as a novel NSAID for chronic administration, would by today's standards during drug development possibly be classified in a risk group associated with serious immune-mediated ADRs according to Sawamura et al., (2010). In hindsight, however, naproxen-induced serious ADRs (such as iDILI) are very rare: hypersensitivity (3 cases per 54,038 patient years) and hepatotoxicity (9 – 56 cases per 100,000 patient years) (Walker, 1997; McMahon, Evans, & MacDonald, 2001). Tailored investigations can generate evidence with regards to the chemical entities involved in these reactions and the mechanism of the injury. ***Chapter 3*** describes *ex vivo* investigations characterizing immune responses to naproxen and its metabolites (S)-DNAP, (S)-NAG and its protein adduct (S)-NAG – HSA, using blood samples from a patient with naproxen-associated liver injury in comparison with appropriate controls.

CHAPTER 3

NAPROXEN – INDUCED IDIOSYNCRATIC HEPATOTOXICITY: EVIDENCE OF IMMUNOLOGICAL MEMORY TO THE OXIDATIVE METABOLITE (S)-O-DESMETHYLNAPROXEN, BUT NOT TO THE ACYL GLUCURONIDE

3.1 INTRODUCTION

Naproxen – induced serious ADRs, such as iDILI, are very rare: hypersensitivity (3 cases per 54,038 patient years) and hepatotoxicity (9-56 cases per 100,000 patient years) (Walker, 1997; McMahon et al., 2001). Carboxylic acid drugs, such as naproxen, can be metabolized to reactive AGs (see ***Chapter 1***), and the concern for delayed hypersensitivity and hepatotoxicity reactions in susceptible individuals relates to the potential of AG metabolites to haptenize endogenous macromolecules. In ***Chapter 2***, we have shown that chemical instability of (S)-NAG leads to irreversible binding to HSA *in vitro*. In this chapter, we describe *ex vivo* investigations characterizing immune responses to (S)-NAP, its metabolites (S)-DNAP and (S)-NAG as well as the protein adduct (S)-NAG – HSA, using blood samples from a patient with naproxen-associated liver injury in comparison with appropriate controls.

The predominant toxicological concern over AG drug metabolites centres around their established capacity to form covalent adducts with endogenous macromolecules (Benet et al., 1993; Bailey, Worrall, de Jersey, & Dickinson, 1998; Wang et al., 2001; Hammond et al., 2014). It is postulated that these metabolites act as immunogenic haptens, with their formation representing a critical step in the pathogenesis of the delayed (Type IV) T-cell mediated immune reactions associated with the idiosyncratic hepatotoxicity seen with certain carboxylic acid drugs (Naisbitt et al., 2000; Bailey & Dickinson, 2003; Shipkova et al., 2003; Faulkner, Meng, Park, & Naisbitt, 2014; Iwamura et al., 2017). The chemical instability of AG drug metabolites has been extensively shown *in vitro* (Stachulski et al., 2006; Regan et al., 2010; Sawamura et al., 2010) and their covalent adduction has been proven using tandem mass-spectrometry in patients receiving therapeutic doses of diclofenac (Hammond et al., 2014). However, to date, no convincing data has yet proven acyl glucuronidation to represent a metabolic pathway with potential immunological consequences. Through the work entailed in

this chapter, a patient who has previously experienced an iDILI reaction to naproxen has been recruited to the study. *Ex vivo* assays were used in order to assess the immunological basis of the adverse reaction that the patient has experienced, and to identify to which of the naproxen species (parent drug or metabolites) the patient exhibits immunological memory, if any. This approach might establish a bridge between a clinical case of iDILI and *ex vivo* immune responses to the drug or its major metabolites, and importantly add understanding to whether AG formation represents a toxicological hazard.

Whilst the reported incidence of iDILI reactions is generally low (e.g. 1 case per 10,000 patient years of NSAID use) (Walker, 1997), the large numbers of patients that are exposed to certain culprit drugs means that iDILI reactions can represent a heavy burden to healthcare systems. Furthermore, with iDILI being the cause for several drug withdrawals including ximelagatran, lumiracoxib, ibufenac, benoxaprofen, troglitazone, trovofloxacin and tienilic acid, these reactions clearly pose an important financial risk for drug development organizations (Lasser et al., 2002; Smith & Schmid, 2006; Regev, 2014; Kullak-Ublick et al., 2017; Weaver et al., 2020). Liver, bone marrow and skin are the major organs affected during idiosyncratic toxicity incidents (Zhang et al., 2011; Uetrecht & Naisbitt, 2013). The liver ranks high in prevalence possibly due to its place as the predominant site of metabolism for most xenobiotics (Kalgutkar & Soglia, 2005), its vascularization (Sheth & Bankey, 2001) and its importance as an immunological organ downstream of the intestine (Jenne & Kubes, 2013). Thorough characterization of clinical cases of iDILI are critical to provide mechanistic understanding of the pathogenesis of the disorder and associated host (patient) risk-factors. Upon this understanding, development of preclinical assays allowing improved identification of compound hazards and risk mitigation strategies, such as designing out structural alerts in pharmaceutical discovery and identification of at-risk patient populations, may become feasible.

The carboxylic acid constitutes an important structure in drug design, representing the pharmacophore for a number of drugs whilst allowing improved absorption and metabolic profiles for others. However, whilst the carboxylate functional group is a very important tool for medicinal chemists in drug design, it also has been suggested to represent a toxicophore. A significant number (14 %) of drugs withdrawn between 1960 and 1999 contained a carboxylic acid functional group and most of these drug withdrawals were due to association with iDILI (Fung et al., 2001). Furthermore, many of the carboxylic acid – containing drugs remaining on the market do so with concerns over their association with eliciting iDILI reactions in susceptible patients. Based on their clinical presentation and time-to-onset, these events resemble delayed T-cell mediated (Gell and Coombs Type-IV) hypersensitivity reactions. The observed delayed onset of the adverse reaction after initial treatment (several days to months), early flu-like symptoms, rapid onset upon re-challenge, inflammatory infiltrates seen upon liver biopsy examination as well as specific HLA associations, all point to the involvement of the adaptive immune system in the pathogenesis of iDILI seen with certain carboxylic acid – containing drugs (Lewis, 1984; Ouellette, Slitzky, Gates, Lagarde, & West, 1991; Berson et al., 1994; Greaves et al., 2001; Goldkind & Laine, 2006).

There are a number of naproxen liver injury cases described in the literature, the clinical phenotype of which resembles that of a delayed hypersensitivity reaction. Jaundice and fulminant hepatitis associated with naproxen have been reported extensively (Bass, 1974; Law & Knight, 1976; Giarelli, Falconieri, & Delendi, 1986), as well as liver biopsy findings revealing signs of drug-induced hepatitis (Victorino et al., 1980). Histopathological examination usually showed a hepatocellular or mixed cholestatic / hepatocellular type of injury. Moreover, the presence of a relatively fixed sensitization period (1 – 6 weeks) alongside the sudden onset of fever and the presence of eosinophils in the infiltrate as well as positive antinuclear antibody screening tests are all circumstantial evidence supporting the

hypersensitive nature of the reaction (Victorino et al., 1980; Demirag et al., 2007; Ali et al., 2011). Symptoms and elevated hepatocellular enzymes all resolved upon treatment discontinuation, however prolonged recovery has also been reported with a return of lasting normal liver function achieved after three years (Ali et al., 2011).

Critical in the pathogenesis of these delayed hypersensitivity immune reactions is the presentation of an immunological epitope to T-cells (Naisbitt, Pirmohamed, & Park, 2003; Pichler, 2003; Hammond, Thomson, Ogese, & Naisbitt, 2020). Small molecules such as carboxylic acid drugs are generally considered to be below the 1000Da approximate threshold for detection by APCs for subsequent antigen presentation (de Weck, 1986). Nevertheless, several small molecule drug-classes such as β-lactam antibiotics and other anti-infectives (e.g. SMX and isoniazid), NSAIDs, anesthetics, anticonvulsants and antiretrovirals are associated with eliciting off-target ADRs (Gomes & Demoly, 2005; Uetrecht & Naisbitt, 2013). A common feature of these drugs is that the parent molecule or their metabolites have been proven to be chemically reactive and exhibit the capacity to adduct macromolecules found *in vivo*, such as proteins. These drug-protein adducts then may constitute neo-antigens able to elicit an immune response, a critical step in the pathogenesis of the delayed T-cell mediated hypersensitivity reactions according to the hapten theory (Landsteiner & Jacobs, 1935). Convincing evidence of the hapten theory has been achieved for a few different classes of drugs, most notably for the β-lactam antibiotics, which has contributed to clarifying the nature of T-cell responses against β-lactam – HSA conjugates (Brander et al., 1995; Naisbitt et al., 2001; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Bell et al., 2013; Kim et al., 2015; Usui et al., 2017; Meng et al., 2017)**.** However, it must also be understood that identification of the innate chemical instability or protein reactivity of a drug or its metabolite does not necessarily equate to a clinically relevant toxicological outcome. A good example of this is acetaminophen (paracetamol), which following oxidative metabolism forms the reactive metabolite NAPQI

(Raucy et al., 1989; Thummel et al., 1993). At therapeutic doses, NAPQI is naturally detoxified by GSH thereby exemplifying that bioactivation does not imply toxicity per se. Moreover, even at overdose, where intrinsic hepatotoxicity may occur, the importance of critical protein target and subsequent toxicological outcome is illustrated by comparing APAP (i.e. acetaminophen) to its regioisomer AMAP which despite a similar level of overall protein covalent binding observed, does not lead to hepatotoxicity (Tirmenstein & Nelson, 1989; Holme, Hongslo, Bjorge, & Nelson, 1991).

A common major metabolic pathway of carboxylic acid drugs is their direct conjugation with glucuronic acid, resulting in the formation of hydrophilic AG drug metabolites. These metabolites have consistently been shown to be chemically unstable in aqueous conditions and to irreversibly bind to protein (Smith et al., 1986; Hyneck et al., 1988; Williams et al., 1992; Benet et al., 1993; Sallustio et al., 1997; Qiu et al., 1998; Iwaki et al., 1999) - findings verified with (S)-NAG in this thesis (see ***Chapter 2***). Furthermore, the protein reactivity of AG metabolites has been confirmed in patients taking therapeutic doses of diclofenac (Hammond et al., 2014). This proven chemical instability and protein reactivity of AG metabolites has led to debate over their association with the off-target ADRs associated with their parent carboxylate drugs (Faed, 1984; Regan et al., 2010; Van Vleet, Liu, Lee, & Blomme, 2017) and has led the FDA to stipulate AG metabolites as 'toxic' in their MIST guidance (FDA, 2008). As a result, attempts to understand the potential risk of the AG of a new compound are commonplace in preclinical drug design, usually through ascertaining its chemical instability in 0.1 M phosphate buffer (pH 7.4) at 37°C or their irreversible binding to protein *in vitro* (Benet et al., 1993; Bolze et al., 2002; Wang et al., 2004; Sawamura et al., 2010; Jinno et al., 2013; Gunduz, Argikar, Cirello, & Dumouchel, 2018). However, newer strategies such as assessment of cytokine secretion by PBMCs exposed to AG metabolites have also been described (Wieland et al., 2000; Miyashita, Kimura, Fukami, Nakajima, & Yokoi, 2014;

Iwamura et al., 2015). Furthermore, examples in the literature have been described where medicinal chemists have modified chemical series with the intention of reducing compound metabolism into reactive AG metabolites (Scott et al., 2012), increasing the stability of AG metabolites generated from parent carboxylate compounds (Nicholls et al., 1996; Baba & Yoshioka, 2009; Yoshioka & Baba, 2009) and even designing out the carboxylic acid functional group from chemical series (Kalgutkar, 2019). All of these efforts preformed with the intention of mitigating the hypothesised risk of AG drug metabolites. Despite the proven capacity of AG metabolites to covalently modify endogenous proteins in patients (Hammond et al., 2014), currently no convincing (experimental) data has yet provided a compelling association between AG formation and immunotoxicological outcomes. Moreover, it is infrequently considered that compared to other reactive metabolites (e.g. quinone imines), AG metabolites may be perceived as 'low reactivity' and can be bio-inactivated through both spontaneous and enzyme driven hydrolysis, further to their rapid and efficient renal and biliary elimination (Spahn-Langguth & Benet, 1992; Shipkova et al., 2003; Stachulski et al., 2006; Regan et al., 2010; Smith et al., 2018).

The association between AG formation and immunological outcomes has been investigated by very few studies and mainly via exploring humoral responses. To date, there has been only one published *in vivo* human research work looking into potential immune involvement in carboxylic acid drug-induced hepatotoxicity. Aithal et al. investigated diclofenac hepatotoxicity in human in a multidisciplinary manner by examining hepatic adduct formation, circulating antibodies and cytokine polymorphisms (Aithal et al., 2004). The identification of several diclofenac adducts in the liver of a patient with diclofenac-induced liver injury as opposed to absence in a normal human donor liver provided *in vivo* human evidence for drug-modified liver protein formation. The detection of antibodies to diclofenac metabolite-modified liver protein adducts in the sera of all (seven) patients with diclofenac hepatotoxicity,

60 % of diclofenac-treated patients without hepatotoxicity and none of the heathy donors, suggested the engagement of the humoral immune system. Although the humoral system is likely to represent an important contributor to iDILI, further factors are clearly required (Aithal, 2011). The identified association of genetic polymorphisms, encoding IL-10 and IL-4 in these patients, points to a role for cell-mediated immune responses. Although, the exact nature of the liver protein adducts was not determined, their location along the canalicular membrane suggests possible involvement of AGs, as extensive AG hepatic extraction in isolated perfused livers has previously been shown; 50 – 5,000 times greater AG concentration across the canalicular membrane (Sallustio et al., 2000). This tissue-specific exposure is mediated by transporters (Lagas et al., 2010), while these adducts are absent in transport deficient (TR-/-) rats not expressing functional MRP2 (Seitz et al., 1998). The association between AG formation and humoral immune system engagement has also been explored by Williams et al., who identified antibodies against valproic acid-AG – HSA adducts in the plasma of 9 out of 57 patients on chronic valproic acid therapy (Williams et al., 1992). However, none of the patients recruited in the study had experienced an ADR and the antibody titres in those 9 patients were very low, suggesting very little immunogenicity. Moreover, circulating antibodies could detect only the parent drug but not sugar-ring (present in AGs), therefore making it likely that adducts were derived from carboxylate phase I bioactivation, and possibly oxidative / quinone mediated too. This in fact coincides with other evidence postulating that hepatotoxicity secondary to valproic acid treatment is due to intrinsic (drug metabolite) toxicity rather than hypersensitivity (Zimmerman & Ishak, 1982; Eadie, Hooper, & Dickinson, 1988). Some studies in animals have revealed circulating antibodies against metabolite-modified protein, such as with diflunisal-AG – rat serum albumin (Worrall & Dickinson, 1995) and tolmetin-AG – mouse serum albumin (Zia-Amirhosseini et al., 1995); however it is important to emphasize the use of adjuvants for provoking an immune response in all these animal

models. Lastly, unlike the aforementioned humoral investigations, Naisbitt et al. examined cell-mediated immune responses of diclofenac using an established mouse model based on the local lymph node assay (Naisbitt et al., 2007). The antigenic potential of diclofenac and its main metabolites (diclofenac-AG, 4'-hydroxy diclofenac, 5'-hydroxy diclofenac as well as their respective quinone-imine derivatives) was explored and in these experiments, lymph node cells proliferated against 5'-hydroxy diclofenac and the quinone-imine derivatives of both oxidative metabolites but not against diclofenac-AG. In a different study, (Kretz-Rommel & Boelsterli, 1993) showed that although diclofenac protein binding was attributed to some extent to AG formation, this was inversely proportional to acute cell injury which was dependent on oxidative metabolism. Furthermore, cytotoxic T-cells and non T-cells derived from diclofenac / keyhole limpet haemocyanin – immunized mice recognized antigenic determinants on syngeneic murine hepatocytes pre-exposed to diclofenac, the nature of which was not determined however, and could perhaps be the result of quinone-imine mediated adducts (Kretz-Rommel & Boelsterli, 1995).

In summary, currently available clinical evidence indicates idiosyncratic hepatotoxicity towards carboxylic acid – containing drugs to exhibit characteristics consistent with delayed hypersensitivity. The defined protein reactivity of the AG metabolites of carboxylic acids has led to assumptions that AGs can form haptens (neo-antigens), thus playing a critical role in propagating these adverse immune-mediated drug reactions. However, further bioactivation at both the carboxylic acid and at further non-carboxylate sites of many carboxylate drug molecules into protein-reactive metabolites have also been characterised. Currently, the immunological consequences of all these bioactivation products remains poorly explored *in vivo* in human and hence uncertain. Here, we asked whether immunological memory exists against naproxen or one of its metabolites in a patient with naproxen-associated iDILI. The work in this chapter describes for the first time, to our knowledge, data using LTTs and

subsequently derived T-cell lines to investigate immune responses to naproxen and its metabolites using PBMCs from a patient with naproxen-associated iDILI, in comparison with several other individuals with or without exposure to naproxen.

3.2 QUESTION AND AIMS

The question is:

"Does immunological memory exist towards (S)-NAP or its major metabolites (S)-NAG and (S)-DNAP in a patient previously experiencing an idiosyncratic liver injury following exposure to naproxen?"

To address this question, the aims were:

- Recruit a patient that has experienced an idiosyncratic liver injury secondary to naproxen alongside appropriate controls (patients receiving naproxen therapy without evidence of an idiosyncratic liver injury and healthy volunteers never knowingly exposed to naproxen) to enable experiments allowing thorough characterisation of the immunological memory of the naproxen iDILI patient.
- Investigate whether PBMCs, isolated from the above described individuals, exhibit lymphocyte stimulation and proliferative response following exposure to (S)-NAP, (S)-DNAP, (S)-NAG or (S)-NAG – HSA.
- Identify any responsive T-cell clones with the intent to further characterize and understand immune responses to (S)-NAP and its metabolites in the naproxen-associated iDILI patient by functional and phenotyping experiments.

- Investigate whether a T-cell priming assay using PBMCs from naproxen-naïve donors represents a useful tool to model immune responses as characterised in the naproxen iDILI patient.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Lymphoprep was purchased from Axis-Shield (Dundee, UK). [^{3}H]-methyl tritiated thymidine was purchased from Moravek (California, USA). Recombinant human GM-CSF, IL-2 and IL-4 were purchased from Peprotech (London, UK). Cyclosporin-A was purchased from Fluka Analytical (Dorset, UK). Pooled human serum and foetal bovine serum (FBS) were obtained from Innovative Research (Michigan, USA) and Invitrogen (Paisley, UK), respectively. Tetanus toxoid (TT) (pure) was purchased from Statens Serum Institute (Copenhagen, Denmark). Cell Counting Kit-8 (CCK-8) was purchased from Sigma-Aldrich (Dorset, UK). All antibody conjugated magnetic beads were obtained from Miltenyi Biotec (Surrey, UK). All conjugated fluorochromes, monoclonal MHC blocking antibodies and isotypes were obtained from BD Biosciences (Oxford, UK). CCL17 was purchased from eBioscience (Ireland, UK). ELISpot multiscreen filter plates and ELISpot kits containing coating and detection antibodies as well as BCIP/NBT Plus were purchased from Millipore (Watford, UK) and Mabtech (Nacka Strand, Sweden), respectively. PD-10 desalting columns were purchased from GE Healthcare (Pittsburgh, USA). HSA (approx. 99% pure, essentially globin free and fatty acid free) was purchased from Sigma-Aldrich (Dorset, UK). (S)-NAP was purchased from Sigma-Aldrich (Dorset, UK). (S)-NAG and (S)-DNAP were purchased from Toronto Research Chemicals

(North York, Canada). All other solvents, reagents and supplies were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise mentioned.

3.3.2 Panel of instruments

Cell harvester (TomTec, USA), Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK), AID ELISpot Reader (Cadama Medical, Stourbridge, UK), MRX plate reader (Dynex, Lincoln, UK), BD FACS Canto II flow cytometer (BD Biosciences, Oxford, UK), autoMACS Pro separator (Miltenyi Biotec, Germany), autoMACS columns (Miltenyi Biotec, UK), 1290 HPLC system (Agilent Technologies, Palo Alto, California), LTQ-Orbitrap XLmass spectrometer (Thermo Scientific, USA).

3.3.3 Human subject recruitment and sample storage

One iDILI patient, four patients receiving chronic naproxen therapy without evidence of an ADR and four healthy volunteers not knowingly previously exposed to naproxen were recruited at the MRC Centre for Drug Safety Science (University of Liverpool, Liverpool, UK) and donated PBMCs for phenotypic and functional immunological assessments and next generation sequencing (NGS) studies. Further healthy volunteers not knowingly exposed to naproxen were recruited in Basel, Switzerland for investigations using T-cell priming experiments. Written and informed consent was obtained from each patient included in these studies and the study protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committees. Up to 120 ml of human peripheral blood was collected in heparinized vacutainer tubes and transferred immediately to the lab at room temperature.

3.3.4 Drug aliquots and storage

Purchased (S)-NAP, (S)-DNAP and (S)-NAG were dissolved in culture medium before immediate dilution to desired concentrations and administration to cells. Due to concerns over reactive metabolite (specifically AG) instability, in each experiment compounds were immediately administered to cell assays following solubilisation and appropriate dilution. No aliquots of solubilised compounds were stored for future use. All compounds were reconstituted at concentrations of 1 mg/ml, with no optical evidence of compound insolubility at this concentration, but (S)-NAP and (S)-DNAP required sonication for 30 seconds using a water bath sonicator. HSA adducted by (S)-NAG was generated via incubation of 2.5 mM (S)-NAG with 50 µM HSA in 0.1 M phosphate buffer (pH 7.4) (2 ml reaction volume) at 37°C for 24 hours under rotation at 900 rpm. At this 24-hour time-point, lactic acid was added to the incubation mixture to a final concentration of 1 % (v/v) to prevent further AG protein reactivity. HSA was subsequently separated from non-bound (S)-NAG or (S)-NAP by size exclusion chromatography (SEC) (also known as gel filtration) as described below. A non-adducted HSA control was also generated for this experiment via incubation of 50 µM HSA in 0.1M phosphate buffer (pH 7.4) at 37°C for 24 hours before also being processed through SEC.

3.3.5 Cell culture media and buffers

Cell culture medium: 500 ml RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10 % (v/v) Human AB Serum, 25 mM HEPES buffer, 2 mM L-glutamine.

Epstein-Barr virus-transformed B-cells culture medium: 500 ml RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 10 % (v/v) FBS, 25 mM HEPES buffer, 2 mM L-glutamine.

Cell freezing mix: 80 % (v/v) FBS, 20 % DMSO. (1 volume of freezing mix was mixed with 1 volume of cell culture medium prior to cell freezing).

Chemotaxis buffer: 500 ml RPMI 1640, 0.5 % (v/v) Bovine Serum Albumin (BSA)

Fluorescence-Activated Cell Scanning (FACS) buffer: 500 ml Hanks Balanced Salt Solution (HBSS), 10 % (v/v) FBS, 0.2 mg/ml sodium azide.

Magnetic-Activated Cell Sorting (MACS) buffer: 50 ml HBSS, 50 µg/ml BSA, 20 mM EDTA.

3.3.6 Size Exclusion Chromatography (SEC)

Following incubation as described in section **3.3.4**, HSA was separated from non-bound low molecular weight entities via SEC by gravitational force using PD-10 desalting columns (GE Healthcare) containing Sephadex G-25 medium. In this procedure, PD-10 columns were equilibrated via passing 25 ml of 0.1 M phosphate buffer (pH 7.4) through the column. After column equilibration, HSA samples were administered to the columns with the full sample volume allowed to reach the column before continuous addition of 0.1 M phosphate buffer (pH 7.4). Resulting eluate was collected in 500 µl fractions. Fractions were assessed for protein concentration using the Bradford assay (Bradford, 1976), and unbound drug ((S)-NAP or (S)-NAG) via absorbance using a cuvette assay and spectrophotometer set to measure optical density at 254 nm. For cell experiments, HSA – containing fractions were pooled before determination of protein concentration using the Bradford assay and HSA concentrations were adjusted to 30 µM (2 mg/ml) using 0.1 M phosphate buffer (pH 7.4). Irreversible drug-protein binding of pooled HSA fractions was quantified using the alkaline hydrolysis method as described in section **2.3.6**. Resulting adducted and non-adducted HSA were aliquoted at 350 µl

and stored at -80 °C before immediate thawing and use in *in vitro* experiments. All procedures were performed under sterile conditions as the final eluted product is intended for biological use (*in vitro* human immune assays).

3.3.7 Peripheral blood mononuclear cell isolation

PBMCs were isolated from samples of peripheral blood by density gradient separation using Lymphoprep (Axis-shield, Dundee) according to manufacturer's instructions and as illustrated in ***Figure 3.1***. Following collection of blood in heparinised vacutainer tubes, approximately 25 ml of undiluted blood was layered carefully using a syringe on top of 25 ml Lymphoprep in 50 ml tubes (an average of 100 ml was collected per donor hence around 4 tubes were used at a time). The tubes were centrifuged at 2,000 rpm for 25 mins at room temperature without engaging the brake in order to avoid disruption to the density layers. Subsequently, the PBMC – containing buffy coat fraction was isolated using a sterile Pasteur pipette and diluted 1 in 4 using HBSS. This diluted buffy coat solution was centrifuged at 1,800 rpm for 15 mins at 4°C with brake engaged. Resulting supernatant was discarded and the cell pellet was reconstituted in HBSS before re-centrifugation under the same conditions. This was repeated one further time to ensure thorough washing of PBMCs. Following this, PBMCs were reconstituted in cell culture medium and cells counted manually under the microscope with viability assessed using trypan blue exclusion. Cells were used in subsequent assays if viabilities were ≥ 95 %. For all primary LTT and ELISpot assays, PBMCs were used immediately after and plated in 96-well U-bottomed plates containing cell culture medium. If PBMCs were frozen before use in experiments, they were aliquoted into 5-10 x 10^6 cells per 1.8 ml cryovial in cell culture medium and cell freezing mix before being placed in Mr. Frosty containers and in -80°C freezers for 24 hrs. Cryovials were subsequently transferred to -150°C for longer term storage.

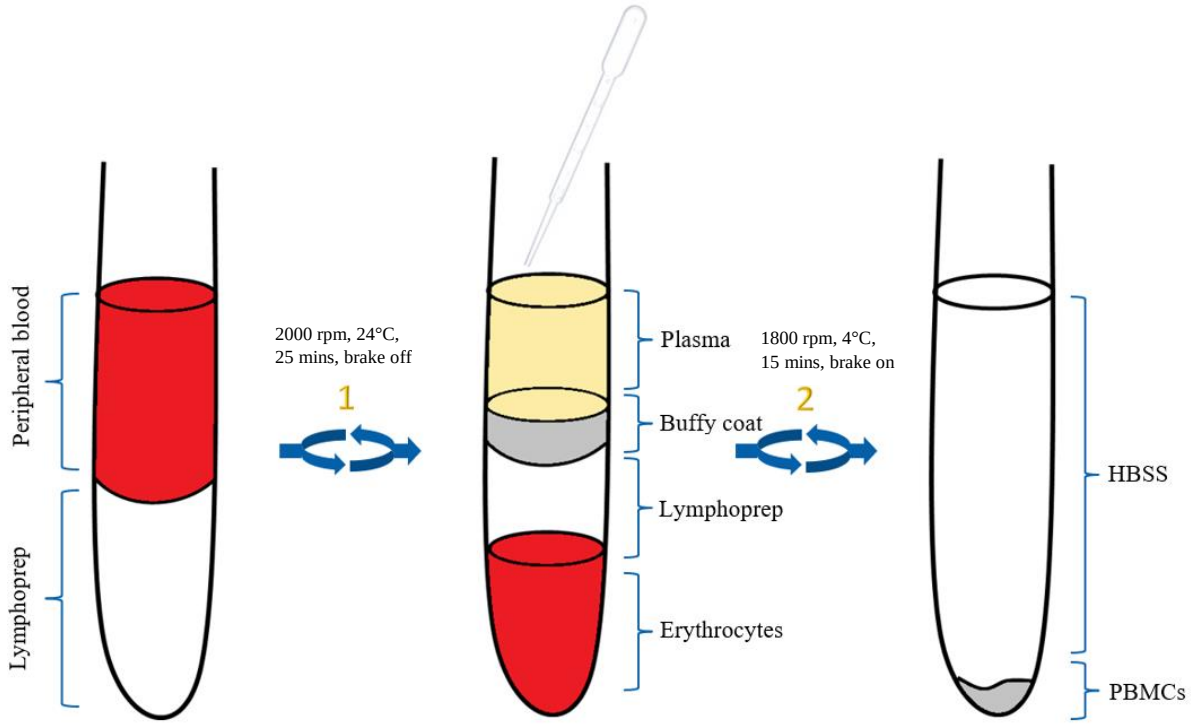


Figure 3.1. Isolation of human peripheral blood mononuclear cells from peripheral blood by density gradient separation. Following careful layering of blood onto Lymphoprep, tubes are centrifuged (1: 2,000 rpm, 24°C, 25 mins, brake off) and then buffy coat (PBMCs) collected via a sterile Pasteur pipette. PBMCs are washed twice with HBSS and centrifugation (2: 1,800 rpm, +4°C, 15 mins, brake on). PBMCs are re-suspended and counted.

3.3.8 Phytohaemagglutinin-induced cell proliferation assay

To establish appropriate ranges of drug or metabolite concentrations to be used in subsequent functional experiments, a threshold of 20 % of inhibition of phytohaemagglutinin (PHA) – induced lymphocyte proliferation was used to identify the maximum suggested concentration for the dose-response curve in accordance to recommendations by Pichler & Tilch, (2004). Herein, two assays were performed to examine inhibition of PBMC proliferation using ^{3}H-thymidine incorporation or Dojindo's highly water-soluble tetrazolium salt (WST-8) reduction.

For the ^{3}H-thymidine incorporation assay, PBMCs ($0.15x10^{6}$ cells/well) were cultured with increasing concentrations of the corresponding drug or metabolite (final well concentrations

ranging from 0 to 600 µM drug or metabolite) in 96-well U-bottomed plates containing 200 µl culture medium. Cells were incubated for a period of 3 days inside a humidified incubator of 5 % CO_2 at 37°C before addition of PHA (final well concentration 5 µg/ml; 10 µl/well) in order to stimulate proliferation and incubation was continued for another 2 days. On day 5, ^{3}H-thymidine was added (0.5 µCi/well) into the culture and incubated under the same conditions for 16 hrs. Cells were subsequently harvested (Cell harvester; TomTec, USA) and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK).

For the WST-8 reduction assay, PBMCs ($0.15x10^{6}$ cells/well) were cultured with increasing concentrations of the corresponding drug or metabolite (final well concentration 0 – 600 µM) in 96-well transparent flat-bottomed plates containing 200 µl culture medium. PHA (5 µg/ml; 10 µl/well) was added immediately in every well and incubated for 24 hrs inside a humidified incubator of 5 % CO2 at 37°C. Following this period, WST-8 (20 µl/well) was added and incubation was continued for another 4 hrs. Resulting formation of the formazan dye by viable cells was assessed via measuring absorbance of the plate at 450 nm using a microplate reader.

In both assays the extent of proliferation / assessment of cell count per well containing drugs or metabolites was related to control wells containing culture media alone. All incubations were performed in triplicate and carried out using PBMCs isolated from 2 healthy volunteers.

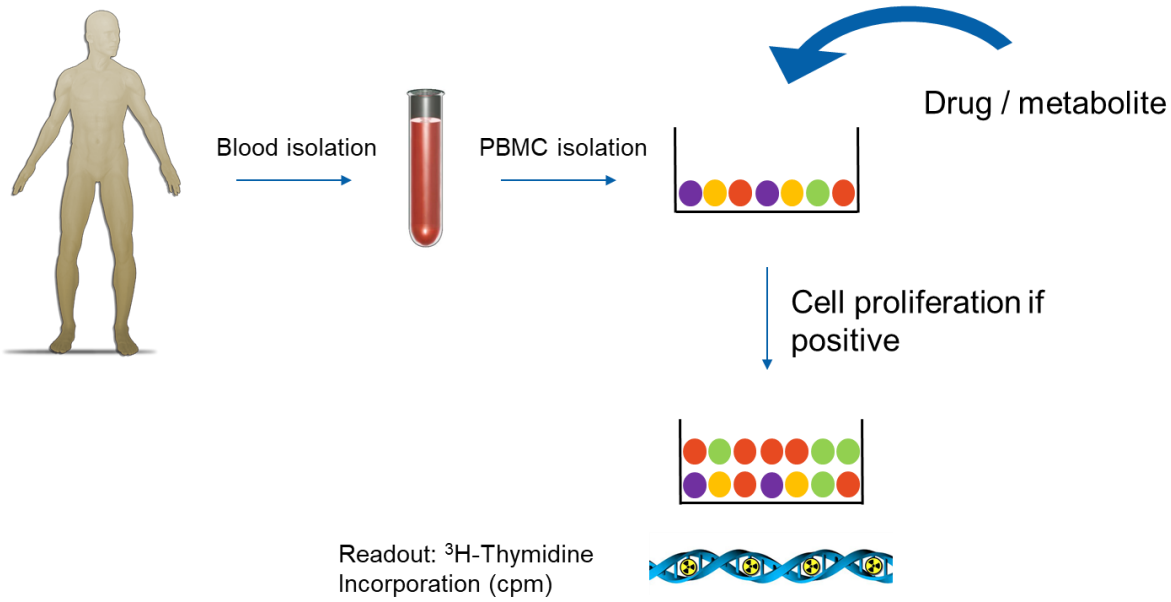
3.3.9 Lymphocyte Transformation Test (LTT)

As depicted in ***Figure 3.2***, upon isolation of PBMCs from blood, cells are *in vitro* exposed to the suspected antigen and after 5 days of incubation cell proliferation is measured via ^{3}H-thymidine incorporation. An increase in cell counts is observed if memory T-cells are present.

Herein, PBMCs were isolated (< 6 hrs following blood collection) as described in **3.3.7** and incubated fresh ($0.15x10^6$ cells/well) with the test compounds added at titrated concentrations (final well concentrations 0 – 600 μM) or the drug – protein adduct (final well concentration 1 mg/ml) in 96-well U-bottomed plates containing 200 μl culture medium for a period of 5 days inside a humidified incubator of 5 % CO_2 at 37°C. PHA (final well concentration 5 μg/ml) and TT (final well concentration 10 μg/ml) were used to non-specifically activate lymphocytes and thus act as positive controls. Culture medium (200 μl) and HSA (final well concentration 1 mg/ml; 200 μl) served as negative controls for the drug / metabolites and drug - protein adduct, respectively. After this period, ^{3}H-thymidine was added (0.5 μCi/well) into the culture and incubated under the same conditions for 16 hrs. Finally, cells were harvested (Cell harvester; TomTec, USA) and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK). All incubations were performed in triplicate. A proliferative response greater than 2-fold increase over negative control (Stimulation Index (SI) > 2) indicates immune memory, however, responses > 1.6 are also considered meaningful and positive, albeit weakly (Pichler & Tilch, 2004; Naisbitt, Nattrass, & Ogese, 2014). The SI was calculated using the following equation:

$$SI = \frac{Mean\ counts\ per\ minute\ (cpm) of\ stimulated\ cells}{Mean\ counts\ per\ minute\ (cpm) of\ negative\ control}$$

A)



B)

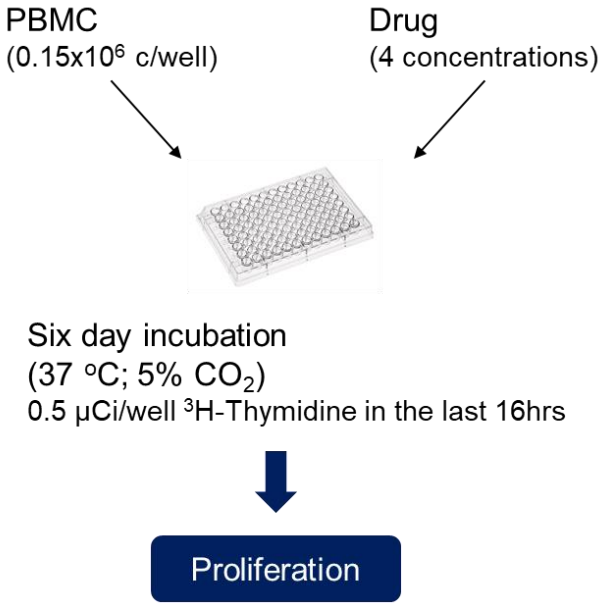


Figure 3.2. Lymphocyte Transformation Test (LTT). A) Simple illustration of the assay from blood withdrawal to cell proliferation readout, **B)** Isolated PBMCs are incubated fresh ($0.15x10^6$ cells/well) with the test compounds at titrated concentrations (optimal concentrations) or the drug – protein adduct (1 mg/ml) in a total volume of 200 µl culture medium for a period of 5 days. After this period, ^{3}H-thymidine is added for 16 hours and cell proliferation is measured with a beta-counter.

3.3.10 Enzyme-Linked ImmunoSpot (ELISpot) assay

ELISpot may be used for cytokine response monitoring either at a mixed (PBMCs) or single (T-lymphocytes) cell level. ***Figure 3.3*** illustrates the basic principles of ELISpot assays. Herein, ELISpot plates were initially prepared by pre-wetting with 35 % ethanol (15 µl) and washing 5 times with distilled water (5 x 200 µl). Capture antibodies were diluted in HBSS (10 µg/ml IL-5, IL-13, IL-17A; 15 µg/ml IFNγ, GranzymeB; 20 µg/ml IL-22, FasLigand; 30 µg/ml Perforin), added (100 µl) to respective wells on the ELISpot plate and incubated overnight at 4°C. Next, excess capture antibody was removed by washing 5 times with sterile PBS (200 µl) and plates were blocked for > 30 mins at room temperature with cell culture medium (200 µl). Following removal of blocking solution, PBMCs were incubated ($0.15x10^6$ cells/well) with the test compounds added at titrated concentrations (final well concentrations 0 – 600 µM) or the drug – protein adduct (final well concentration 1 mg/ml) in a total volume of 200 µl culture medium for a period of 48 hours inside a humidified incubator of 5 % CO2 at 37°C. Where ELISpot was used for T-cell clone cytokine profiling, T-lymphocytes were cultured ($5x10^4$ cells/well; 50µl) with autologous irradiated Epstein-Barr virus (EBV)-transformed B-cells ($1x10^4$ cells/well; 50µl) and incubated with the test compounds (final well concentration 200 µM) in a total volume of 200 µl culture medium for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. PHA (final well concentration 5 µg/ml) was used to non-specifically activate lymphocytes and thus act as positive control. Culture medium (200 µl) and HSA (final well concentration 1mg/ml) served as negative controls for the drug / metabolites and drug-protein adduct, respectively. After incubation, cells were discarded, and ELISpot plates were washed 5 times with HBSS (200 µl). Biotinylated detection antibodies were diluted in 0.5 % FBS solution, added to the corresponding wells (1 µg/ml; 100 µl) and following a 2-hour incubation at room temperature, plates were washed another 5 times with HBSS (200 µl). Streptavidin-ALP was diluted in 0.5 % FBS solution, added to each well (1 µg/ml; 100 µl) and plates

incubated for 1 hour at room temperature. Following a final 5 times washing with HBSS (200 µl), BCIP-NBT-plus substrate was sterile filtered (0.45 µm) and added (100 µl/well) for approximately 15 mins incubation in the dark to allow spot formation. Reaction was stopped by rinsing carefully with water and following overnight drying, spots were counted using an AID ELISpot reader. Results were recorded as Spot Forming Units (SFUs) per $0.5x10^6$ cells.

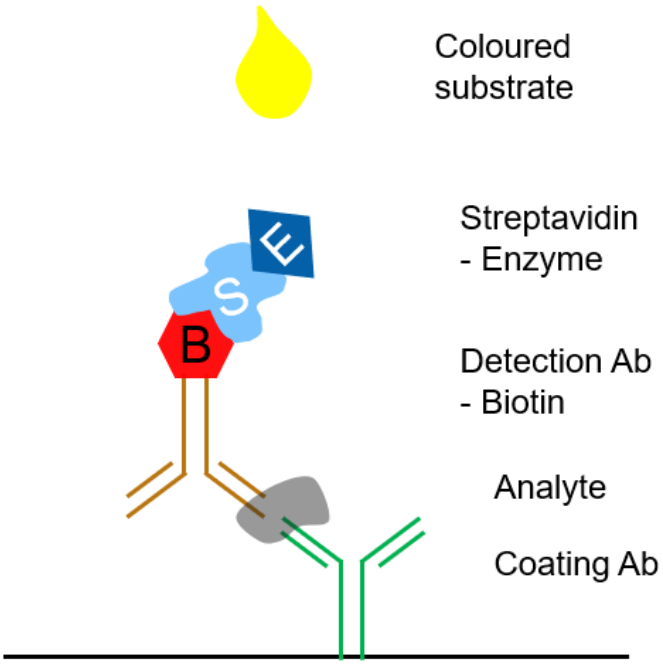


Figure 3.3. Basic principles of the ELISpot assay. A specific coating antibody bound on the surface of an ELISpot plate initially captures the intended analyte (cytokine) and is subsequently detected via a biotinylated specific antibody followed by a streptavidin-enzyme conjugate. Finally, a precipitating coloured substrate leads to visible spots on the surface with each spot corresponding to an individual cytokine-secreting cell.

3.3.11 Magnetic-activated cell sorting

Magnetic-activated cell sorting was used in order to separate specific cell subsets out of the overall PBMC population. Cell subsets can be separated either via positive selection (magnetic bead-bound) or negative selection (magnetic bead-unbound). Herein, this method was used in

order to isolate $CD14^+$ (monocytes) and naïve $CD3^+$ (T-cells) subsets. $CD14^+$ cells were isolated via positive selection (binding of antibody to CD14 does not lead to signal transduction due to the absence of cytoplasmic domain). PBMCs were centrifuged at 1400 rpm for 10 mins at 4°C and after discarding supernatant, the cell pellet was resuspended in 80 µl MACS buffer / 10^7 total cells. Human CD14 microbeads (Miltenyi; 130-050-201) were added (20 µl / 10^7 total cells), mixed well and incubated for 15 mins in the refrigerator (2 - 8°C). Cells were then washed by adding 2 ml MACS buffer / 10^7 total cells and centrifuged at 1,400 rpm for 10 mins at 4°C. After discarding supernatant, the cell pellet was resuspended in 500 µl MACS buffer. Magnetic separation was achieved with the autoMACS Pro© separator. After preparing and priming the instrument, the labelled cell fraction ($CD14^+$) was collected via 'Possel' mode whereas the flow through was used for naïve $CD3^+$ cell separation. Naïve $CD3^+$ cells were isolated via negative selection. The cell flow through was centrifuged at 1,400 rpm for 10 mins at 4°C and after discarding supernatant, the cell pellet was resuspended in 40 µl MACS buffer / 10^7 total cells. Human naïve pan T-cell biotin-antibody cocktail (Miltenyi; 130-097-095) was added (10 µl / 10^7 total cells), mixed well and incubated for 5 mins in the refrigerator (2 - 8°C). Following this, 30 µl MACS buffer / 10^7 total cells and 20 µl naïve pan T-cell microbead cocktail / 10^7 total cells were added, mixed well and incubated for 10 mins in the refrigerator (2 - 8°C). Finally, the mixture was topped up to 500 µl with MACS buffer and magnetic separation was achieved with the autoMACS Pro© separator. After preparing and priming the instrument, the non-labelled cell fraction (naïve $CD3^+$) was collected via 'DepleteS' mode. All cell subsets where used either fresh or frozen at $5\text{-}10x10^6$ cells/cryovial.

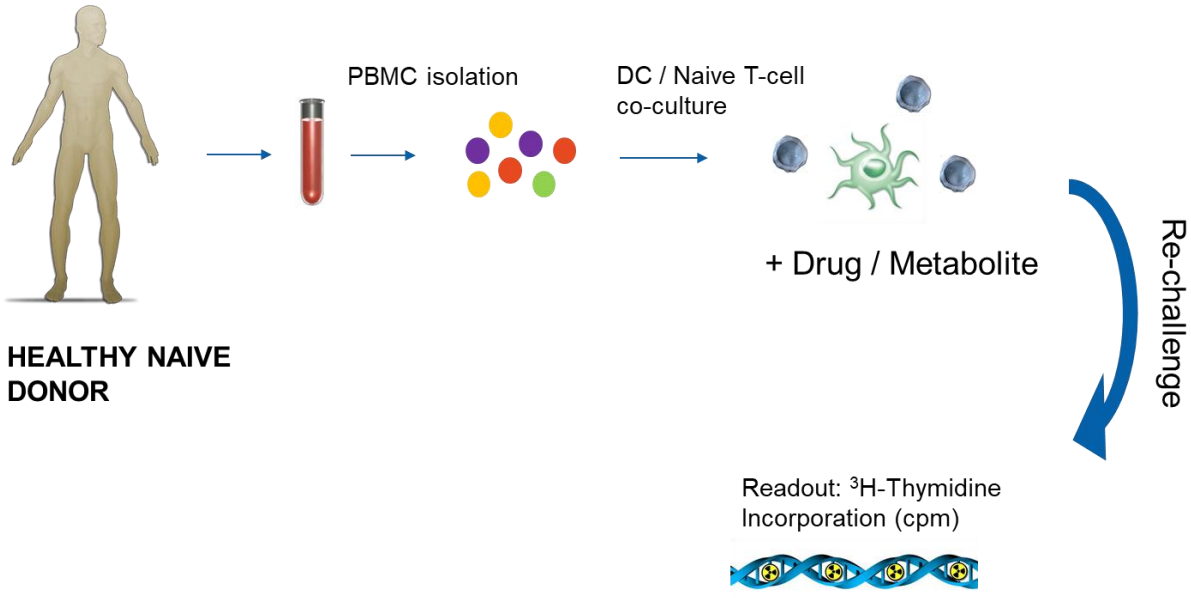
3.3.12 *In vitro* T-cell priming assay using peripheral blood mononuclear cells from healthy naïve subjects

Herein, the protocol developed by (Faulkner et al., 2012) was used. ***Figure 3.4*** illustrates the assay scheme. On day 0, isolated $CD14^+$ cells were aliquoted ($5x10^6$ cells at 3 ml / well) in 6-well plates using cell culture medium supplemented with 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 to induce differentiation of monocytes into immature monocyte-derived dendritic cells (Mo-DCs). On day 2, cells were fed with 3 ml of culture medium supplemented with 800 U/ml GM-CSF and IL-4. This was repeated twice (in 2-day intervals) and on the last day (day 6), 25 ng/ml tumour necrosis factor α (TNFα) and 1 μg/ml LPS were added to the culture for 24 hours in order to mature the Mo-DCs. The following day, mature DCs were scrapped carefully, washed, and resuspended in cell culture medium. DCs were aliquoted ($1.6x10^5$ cells at 0.5 ml / well) in 24-well plates. Frozen naïve $CD3^+$ cells were thawed and after being resuspended in cell culture medium were added ($2.5x10^5$ cells at 1 ml / well) to the mature DCs. Finally, (S)-NAP and its metabolites were added into the mixed cell culture at 200 μM (optimal concentration). In addition, SMX – NO was incorporated in the assay as a positive control (Faulkner et al., 2012; Gibson et al., 2014; Faulkner et al., 2016; Gibson, Faulkner, Wood, Park, & Naisbitt, 2017) and was added in the mixed cell culture at 50 μM (optimal concentration). Drugs and cells were co-incubated for 7 days inside a humidified incubator of 5 % CO_2 at 37°C. In the meantime, on day 10, the procedure for Mo-DC generation from $CD14^+$ cells was repeated as described earlier. On day 17, mature DCs were scrapped, washed, re-suspended and aliquoted ($0.8x10^5$ cells at 50 μl / well) in 96-well U-bottomed plates. Naïve $CD3^+$ cells were harvested and added ($1x10^6$ cells at 100 μl / well) into the mature DCs. Finally, for the purposes of dose-response assessment, (S)-NAP and its metabolites were added at 50 μM, 100 μM and 200 μM while SMX – NO was used at 12.5 μM, 25 μM and 50 μM. Culture medium (200 μl) served as negative control. After

48 hrs of incubation, ^{3}H-thymidine was added (0.5 µCi/well) into the culture and incubated under the same conditions for 16 hrs. Finally, cells were harvested (Cell harvester; TomTec, USA) and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK). All incubations were performed in triplicate. A proliferative response greater than 2-fold increase over the negative control (SI > 2) indicates successful priming potential, however, responses > 1.6 are also considered meaningful and positive, albeit weakly (Faulkner et al., 2012; Naisbitt et al., 2014). The SI was calculated using the following equation:

$$SI = \frac{Mean\ counts\ per\ minute\ (cpm) of\ stimulated\ cells}{Mean\ counts\ per\ minute\ (cpm) of\ negative\ control}$$

A)



B)

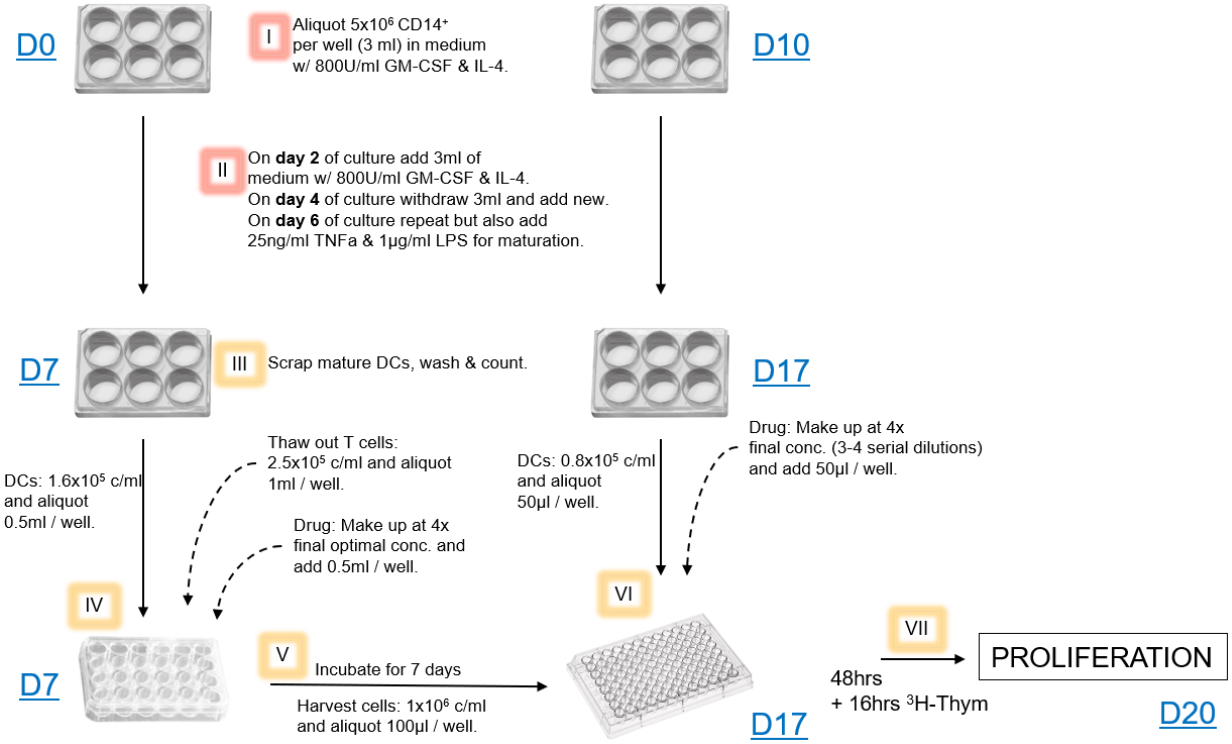


Figure 3.4. ***In vitro*** **naïve human T-cell priming assay. A)** Simple illustration of the assay from blood withdrawal to cell proliferation readout (DC: Dendritic Cells), **B)** Summary of independent assay steps: I. Plating isolated $CD14^+$ in 6-well plates, II. Process of generating mature DCs, III. Collection of matured DCs, IV. Co-incubation of autologous matured DCs, naïve T-cells and drugs, V. Harvesting challenged T-cells, VI: Co-incubation of autologous matured DCs, challenged T-cells and drugs, VII: Measuring proliferation via ^{3}H-thymidine incorporation.

3.3.13 Epstein-Barr virus-transformed B-cell generation

B-lymphocytes were transformed with EBV in order to immortalize them and thus provide readily available autologous APCs for the purposes of multiple functional experiments. The supernatant from a B95.8 cell line was initially filtered (0.2 µm) and subsequently used to suspend donor PBMCs ($5x10^6$ cells). Following addition of 1 µg/ml Cyclosporin A (impedes T-cell survival), the PBMCs were incubated for 24 hours inside a humidified incubator of 5 % CO_2 at 37°C. Subsequently, cells were washed, resuspended in EBV-transformed B-cell culture

medium supplemented with Cyclosporin A (1 µg/ml) and plated in 24-well plates (2 ml / well). Cells were incubated for 3 weeks inside a humidified incubator of 5 % CO_2 at 37°C with their medium renewed every 3 days. After 3 weeks, Cyclosporin A was removed from the cell culture, and cells were allowed to expand (fed bi-weekly with EBV transformed B-cell medium). Finally, when sufficient expansion had occurred, the EBV transformed B-cells were transferred to 25 ml culture flasks and maintained inside a humidified incubator of 5 % CO_2 at 37°C (fed bi-weekly with EBV-transformed B-cell medium). A small number of EBV-transformed B-cells from each donor was frozen at – 150°C.

3.3.14 T-cell cloning by limiting dilution

Figure 3.5 shows a simple scheme of the rationale behind this procedure.

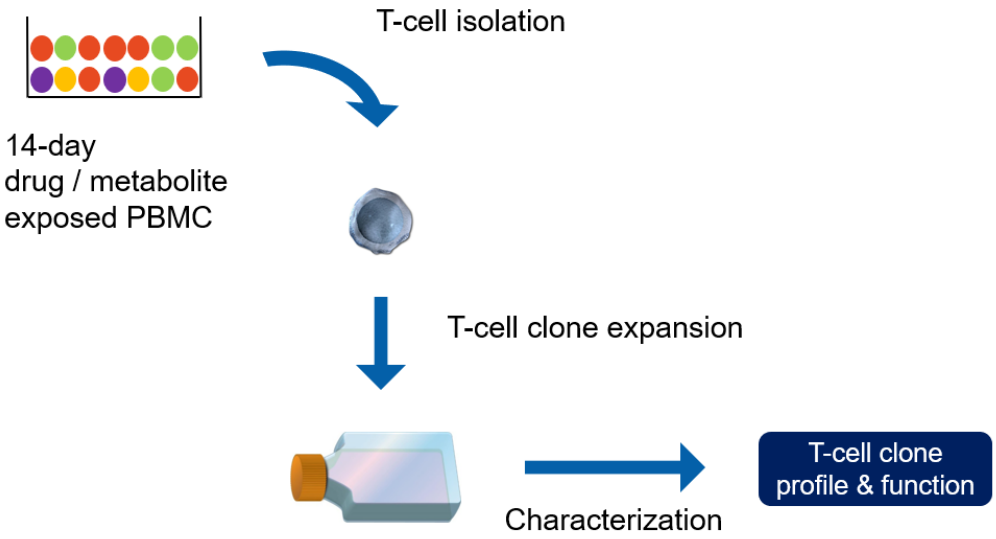


Figure 3.5. Simple scheme illustrating the rationale of T-cell cloning by limiting dilution. A combination of limiting dilution and antigen stimulation steps enables the growth of a single T-cell into a clone which if deemed antigen-specific can be further expanded to facilitate experiments identifying the T-cell clone's phenotype and function.

Herein, PBMCs were plated fresh ($1x10^6$ cells / well; 350 µl) in 48-well plates and suspected antigen was added (350 µl) at the optimal concentration. A final concentration of 200 µM was used for (S)-NAP, (S)-DNAP and (S)-NAG while (S)-NAG – HSA was added at final concentration of 1 mg/ml. Separate plates were used for the different antigens to avoid contamination and placed inside a humidified incubator of 5 % CO_2 at 37°C for 14 days. On days 6 and 9 the cell cultures were fed with 200 U/ml (350 µl) of IL-2 supplemented medium to enable continued growth of antigen specific T-cells. Following this period, cells were harvested, pooled and serially diluted as shown in ***Figure 3.6***.

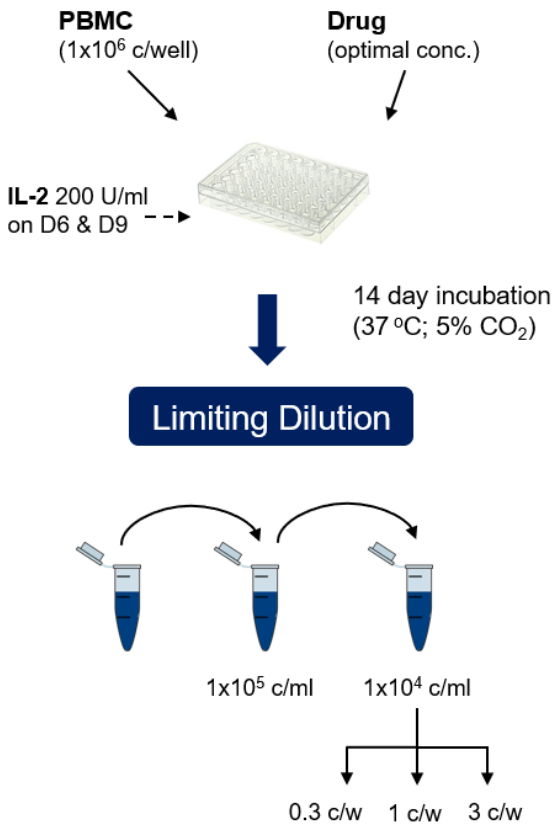


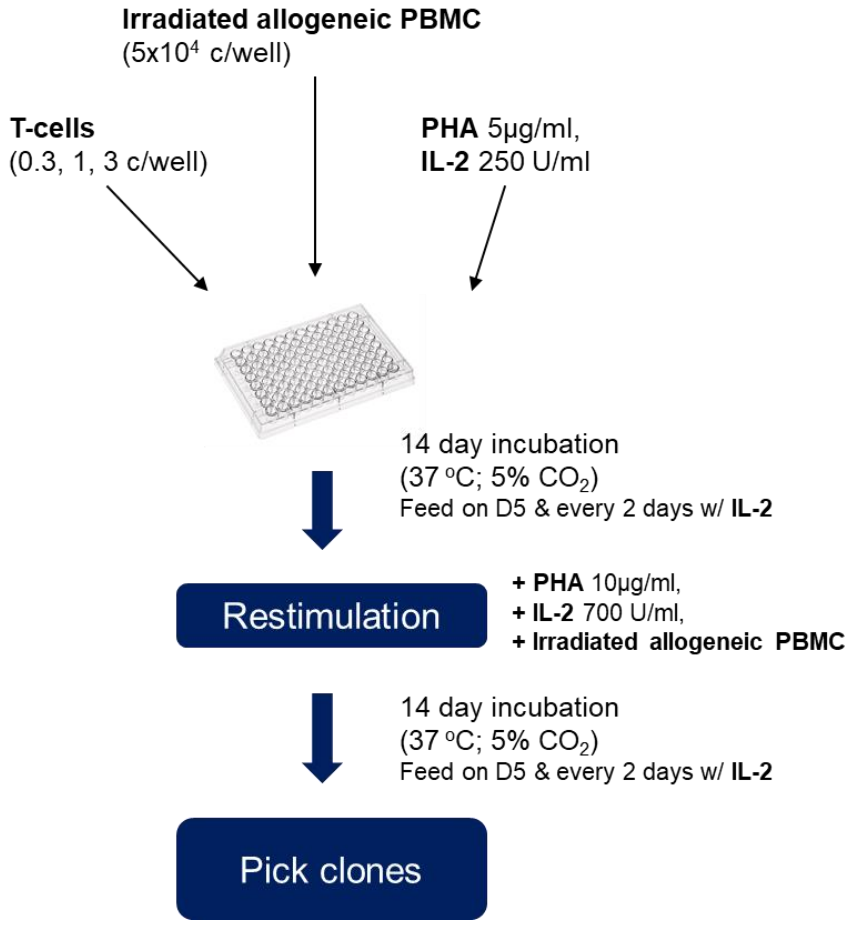
Figure 3.6. Limiting dilution of peripheral blood mononuclear cells. PBMCs are initially plated ($1x10^6$ cells / well) in 48-well plates with the drug at the optimal concentration and cultured together for 14 days inside a humidified incubator of 5 % CO_2 at 37°C. Cells are fed with 200 U/ml of IL-2 supplemented medium on days 6 and 9. Finally cells are serially diluted to enable single cell cultures.

Thus, cells were plated at 3 different concentrations (0.3, 1 and 3 cells / well; 50 µl) using 96-well-U-bottomed plates. Cell counting can rarely be accurate to a single cell level hence the different concentrations aimed to address this deviation. Mitogen-driven expansion was initiated by adding 50 µl of "stimulation cocktail" consisting of irradiated allogeneic PBMCs ($5x10^4$ cells / well), PHA (5 µg/ml) and IL-2 (250 U/ml) and culturing for 14 days inside a humidified incubator of 5 % CO_2 at 37°C while feeding cells with 200 U/ml (25 µl) of IL-2 supplemented medium on day 5 and every other day thereafter.

As shown in ***Figure 3.7***, repetitive expansion is achieved via adding 50 µl of "re-stimulation cocktail" consisting of irradiated allogeneic PBMCs ($5x10^4$ cells / well), PHA (10 µg/ml) and IL-2 (700 U/ml) and culturing for 14 days inside a humidified incubator of 5 % CO_2 at 37°C while feeding cells with 200 U/ml (25 µl) of IL-2 supplemented medium on day 5 and every other day thereafter. Once cells have formed a visible pellet in their respective wells, they are picked one by one and seeded (80 µl) in a new 96-well-U-bottomed plate. IL-2 supplemented medium is added (200 U/ml; 20 µl) and cells are fed every other day until they have grown sufficiently for splitting and eventually antigen-specificity testing. Antigen-specificity is assessed by measuring proliferation via ^{3}H-thymidine incorporation following exposure to the corresponding drug. More specifically, after reducing the culture volume to 50 µl, irradiated autologous EBV-transformed B-cells were used as APCs and added (50 µl) at $1x10^4$ cells / well. Then, corresponding antigen was added (50 µl) at the optimal concentration. A final concentration of 200 µM was used for (S)-NAP, (S)-DNAP and (S)-NAG while (S)-NAG – HSA was added at final concentration of 1 mg/ml. Culture medium (50 µl) served as negative control. After 48 hrs of incubation inside a humidified incubator of 5 % CO_2 at 37°C, ^{3}H-thymidine was added (0.5 µCi/well) into the culture and incubated under the same conditions for 16 hrs. Finally, cells were harvested (Cell harvester; TomTec, USA) and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK).

All incubations were performed in duplicate. A proliferative response greater than 2-fold increase over the negative control (SI > 2) indicates antigen-specificity, however, responses > 1.6 are also considered meaningful and positive, albeit weakly. Positive T-cell clones were then transferred into a 48-well plate and expanded via addition of "re-stimulation cocktail" as described above. T-cell clones were cultured inside a humidified incubator of 5 % CO_2 at 37°C and fed every other day with 200 U/ml (25 µl) of IL-2 supplemented medium.

A)



B)

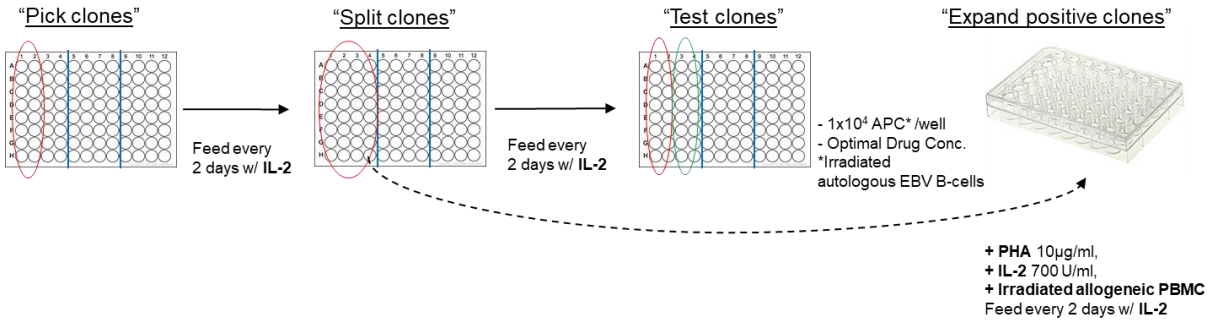


Figure 3.7. T-cell cloning and expansion process. A) Single T-cell cultures are co-incubated with irradiated allogeneic PBMCs (5x10^4 cells / well), PHA (5 µg/ml) and IL-2 (250 U/ml) for 14 days inside a humidified incubator of 5 % CO2 at 37°C to enable mitogen driven expansion. **B)** Following re-stimulation and sufficient growth, T-cell clones are picked, split and tested for antigen-specificity. Positive clones are then transferred into a 48-well plate and expanded for subsequent investigations.

3.3.15 T-cell clone functional assays

The unique characteristics of a T-cell clone can be determined with a series of functional assays. Assays using cell proliferation as their primary endpoint utilized the ^{3}H-thymidine incorporation method. Following appropriate incubations for 48 hrs inside a humidified incubator of 5 % CO_2 at 37°C, ^{3}H-thymidine was added (0.5 µCi/well) into the culture and incubated under the same conditions for 16 hrs. Finally, cells were harvested (Cell harvester; TomTec, USA) and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK). All incubations were performed in duplicate apart from the dose-response assay that was performed in triplicate. Where cytokine secretion was assessed, the ELISpot assay was employed as described in **3.3.10**. ELISpot assay incubations were performed in singlicate. Culture medium (100 µl) served as negative control in both proliferation and cytokine secretion assays. Cell proliferation and cytokine secretion assays used in T-cell clone assessment are summarized in ***Figure 3.8***.

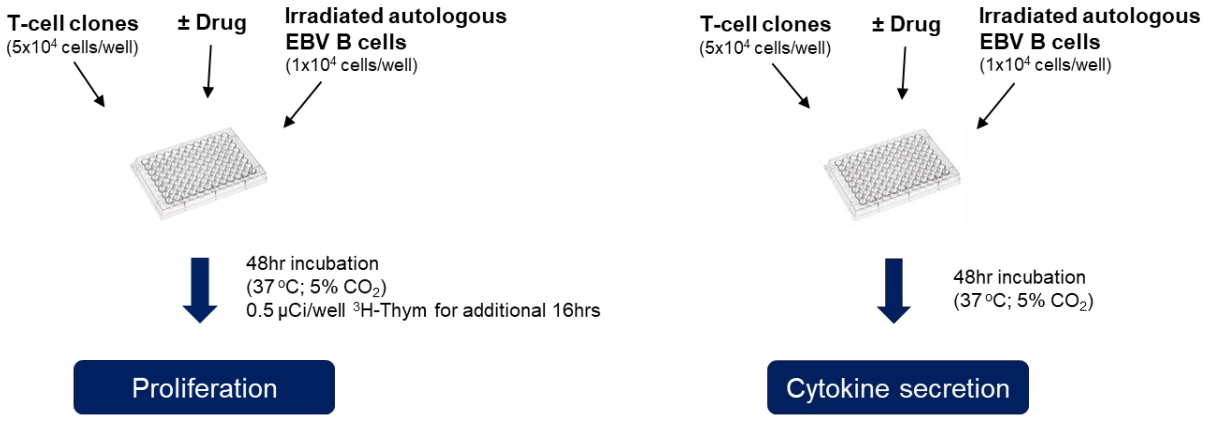


Figure 3.8. Principal assays to enumerate and study functional responses of T-cell clones. Assays using cell proliferation as primary endpoint utilized ^{3}H-thymidine incorporation method. Cytokine secretion was measured via ELISpot assay.

- *Dose-response assay*

Suspected positive T-cell clones were cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and exposed to differing concentrations of test compound (final well concentration 0-400 µM; (S)-NAP and (S)-NAG or 0-600 µM; (S)-DNAP) or the drug – AG protein adduct (1 mg/ml)) in a total volume of 200 µl culture medium. Only clones exhibiting clear dose-response relationship are deemed true positives and progress to subsequent investigations.

- *Cross reactivity assay*

The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and exposed to differing concentrations of structurally diverse compounds (final well concentration 0 – 200 µM; (S)-NAP and (S)-NAG or 0 – 50 µM; SMX-NO or 0 – 100 µM; zomepirac) in a total volume of 200 µl culture medium.

- *Antigen-presenting cell dependency assay*

The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with or without irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and exposed to final well concentrations of (S)-DNAP (100 µM & 200 µM) or (S)-NAP (200 µM) or (S)-NAG (200 µM) in a total volume of 200 µl culture medium.

- *MHC restriction assay*

The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) previously treated for 30 mins with 5 µg/ml of anti-MHC Class I & II (including specific -DP, -DQ, -DR). The cell culture was subsequently exposed to (S)-DNAP (final well concentration 100 µM) in a total volume of 200 µl culture medium. EBV-transformed B-cells treated with corresponding antibody isotypes (100 µM) in a total volume of 200 µl culture medium were used to normalize the T-cell clone's positive response to (S)-DNAP.

- *Antigen-presenting cell fixation assay*

Irradiated autologous EBV-transformed B-cells ($2x10^6$ cells) were initially suspended in 1 ml of HBSS and fixed via addition of 25 % glutaraldehyde (1 µl). Following 30 secs incubation at room temperature, reaction was stopped via addition of glycine (0.2 mM; 1 µl). After 45 secs cells were washed 3 times with culture medium and suspended. The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with the glutaraldehyde - fixed irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and exposed to (S)-DNAP or (S)-NAP or (S)-NAG, all at 200 µM (final well concentration) in a total volume of 200 µl culture medium.

- *Antigen-presenting cell pulsing assay*

Irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) were initially pulsed for 16 hours with the test compounds (final well concentration 200 µM; (S)-NAP, (S)-NAG, (S)-DNAP) or cultured without pulsing in a total volume of 200 µl culture medium inside a humidified incubator of 5 % CO_2 at 37°C. Following this, cells were washed 3 times with HBSS and the responsive (S)-DNAP T-cell clone ($5x10^4$ cells/well; 50 µl) was cultured with pulsed or non-pulsed APCs ($1x10^4$ cells / well; 50 µl). (S)-DNAP (final well concentration 200 µM) was only added to the non-pulsed cell culture in a total volume of 200 µl culture medium serving as positive control.

3.3.16 Fluorescence-activated cell scanning

Figure 3.9 depicts typical graphs of PBMC flow cytometry gating and T-lymphocyte profiling based on fluorescence-activated cell scanning. Herein, T-lymphocytes ($5x10^3$; 200 µl) were incubated with specific fluorescently tagged antibodies or their isotypes for 20 mins at 4°C in darkness. The following were used: CD8-APCN, CD4-FITC, CCR1-PE, CCR2-APCN, CCR3-FITC, CCR4-PE, CCR5-FITC, CCR6-APCN, CCR8-PE, CCR9-APCN, CXCR3-APCN, CXCR6-PE, CXCR10-PE, CLA-FITC and CD69-FITC. Cells were then washed with 1 ml FACS buffer and following centrifugation (1,400 rpm, 10 mins, 4°C) and supernatant discarding, were re-suspended in 200 µl FACS buffer and analysed using FACS Canto II flow cytometer. Results are presented as Mean Fluorescence Index (MFI), calculated by the following equation:

$$MFI = \frac{mean\ fluorescence\ intensity\ of\ test\ stained\ cells}{mean\ fluorescence\ intensity\ of\ isotype\ stained\ cells}$$

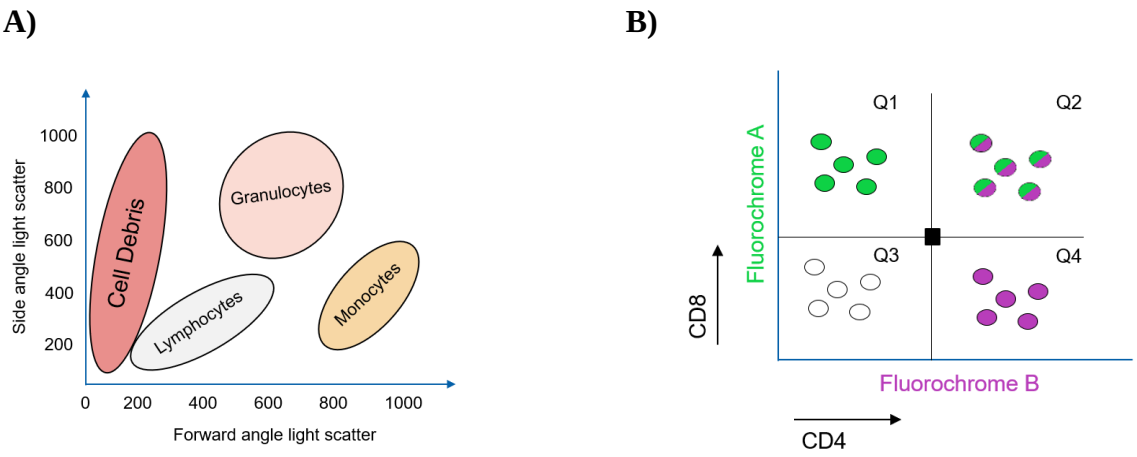


Figure 3.9. Peripheral blood mononuclear cell gating and T-lymphocyte profiling based on Fluorescence-activated cell scanning. A) Typical side vs forward scatter flow cytometry graph allowing PBMC subsets to be distinguished from each other and from subcellular debris on the basis of their physical properties, **B)** Fluorescence-activated cell scanning analysis of T-lymphocyte subsets following initial gating. Depending on the expression of markers on their surface a subset may have bound an antibody to CD8 (conjugated with fluorochrome A) [Q1: $CD8^+$], or to CD4 (conjugated with fluorochrome B) [Q4: $CD4^+$] or to both [Q2: $CD4^+CD8^+$], or neither [Q3: $CD4^-CD^-$].

3.3.17 Chemotaxis assay

The chemotaxis assay was utilized in order to further assess the functionality of T-cell clones on the basis of their migration capacity through a semi-permeable membrane. T-lymphocytes are known to express a range of different profile-specific chemokine receptors on their surface which facilitate cell migration towards the corresponding chemokine ligands. Herein, chemotaxis was assessed by using 24-well plates consisting of trans-well chambers with 5 µm pores. T-lymphocytes were suspended in chemotaxis buffer and placed ($0.1x10^5$; 100 µl) on the upper chamber of the trans-well. Then, 600 µl of chemotaxis buffer containing 100 ng/ml chemokine ligand 17 (CCL-17), were added into the lower chamber of the trans-well. Cells were incubated inside a humidified incubator of 5 % CO_2 at 37°C and at desired time-points (0.5, 1, 2, 4, 8, 16, 24 hrs) 10 µl of sample were withdrawn from the lower chamber to count

migrating cells with a haemocytometer. Incubations were performed in duplicate and results are presented as % cell migration, calculated using the following equation:

$$\%\ Cell\ migration = \frac{Mean\ cell\ count\ at\ lower\ chamber}{100{,}000}\ x\ 100\ \%$$

3.4 RESULTS

3.4.1 Isolation of (S)-naproxen-1β-acyl glucuronide – human serum albumin adduct using size exclusion chromatography

SEC as described in **3.3.6** was used in order to isolate and purify (S)-NAG – HSA adduct. Following SEC, the retrieved (S)-NAG – HSA product was adjusted to 30 μM (2 mg/ml) with potassium phosphate buffer 0.1 M (pH 7.4). ***Figure 3.10*** demonstrates the elution profile of the drug - protein adduct and unbound drug species. During this profiling (no organic solvent used) (S)-NAG – HSA eluted between fractions 2.5 – 7.5 ml whereas unbound naproxen species between 10 – 15 ml. No cross-over elution between the two different fractions was observed. Moreover, it is evident that this is a reproducible method with high consistency exhibiting a mean of 95 ± 2.9 % of protein adduct recovery and a 100 % separation capacity as displayed by three independent isolations, thereby offering optimal protein recovery and purity. The stability of (S)-NAG – HSA adduct when incorporated in cell culture medium was assessed using the previously described HPLC-MS assay (see ***Chapter 2***). For the purpose of quantification, serum free cell culture medium (RPMI 1640) was used and (S)-NAG – HSA was incubated for a total of 16 hrs inside a humidified incubator of 5 % CO_2 at 37°C, following SEC. Quantification of irreversible binding was conducted as described previously (section

2.3.6) using incubation aliquots at t = 0 hrs and t = 16 hrs. ***Figure 3.11*** shows that most of the (S)-NAG – HSA adduct remained relatively intact with 25.9 % of bound naproxen lost during this incubation period (241.35 ± 84.77 nmol / μmol; t = 0 hrs, 182.90 ± 87.32 nmol / μmol; t = 16 hrs).

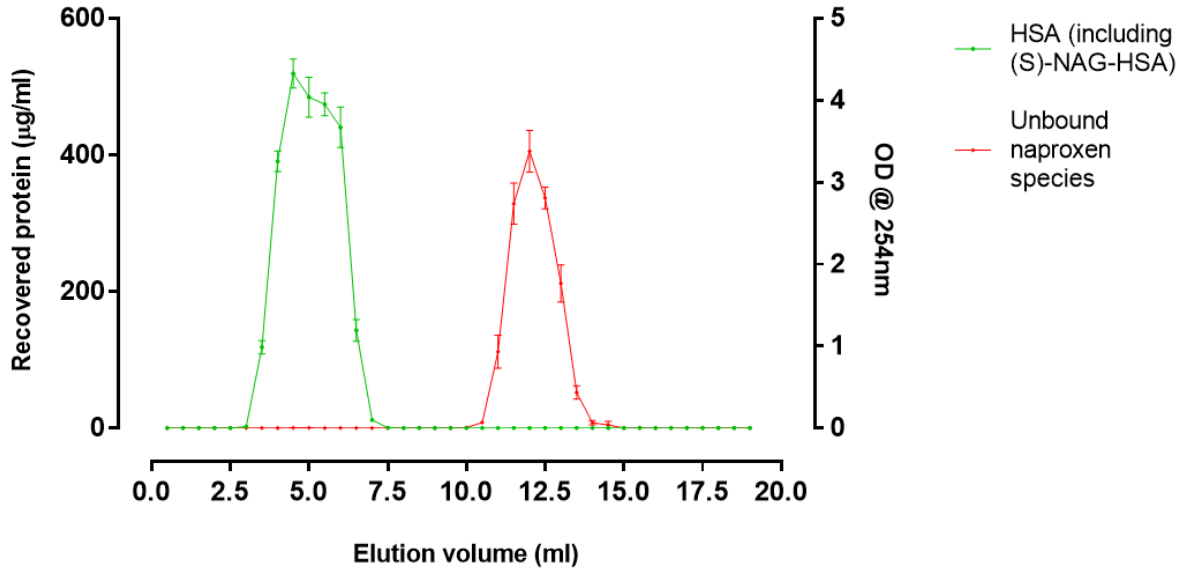


Figure 3.10. Size exclusion separation of unbound naproxen species from protein using PD-10 desalting column (n=3). Elution was performed with 0.1 M potassium phosphate buffer (pH 7.4) and fractions of 0.5 ml were kept for measurement. Recovered protein was quantified with Bradford assay and unbound drug was detected spectrophotometrically at 254 nm. All incubations were performed in triplicate. HSA (including (S)-NAG – HSA) elutes in volume fractions between 2.5 – 7.5 ml whereas unbound naproxen species between 10 – 15 ml. The protein recovery was 95 ± 2.9 % and separation capacity 100 %.

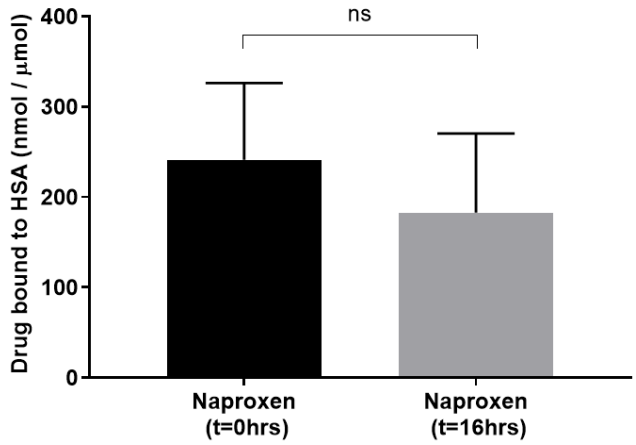


Figure 3.11. ***In vitro*** **assessment of the stability of (S)-naproxen-1β-acyl glucuronide – human serum albumin upon 16 hrs of culture in serum free medium at 37°C (n=3).** (S)-NAG – HSA was constructed at 50 : 1 molar ratio (2.5 mM of (S)-NAG : 50 µM HSA) and following size exclusion chromatography was incubated in serum free cell culture medium for 16 hrs inside a humidified incubator of 5 % CO_2 at 37°C. Irreversibly bound naproxen was quantified as described in Chapter 2 (section 2.3.6). Data are presented as means (± standard deviation; n=3 donors) of irreversibly bound drug. Statistical comparison was made using simple t-test. A value of $p < 0.05$ was considered to be statistically significant.

3.4.2 Dose-dependent inhibition of phytohaemagglutinin-induced peripheral blood mononuclear cell proliferation by (S)-naproxen and its major metabolites

(S)-NAP, (S)-DNAP and (S)-NAG were incubated under the same conditions as described in **3.3.8** and on the same plate / donor to minimize technical variability during the determination of the concentration range. ***Figure 3.12*** shows the cell inhibition profile for the 3 compounds as determined by the 5-day ^{3}H-thymidine incorporation assay whereas the 24-hour profile as measured by WST-8 reduction is depicted in ***Figure 3.13***.

Based on the WST-8 reduction method (***Figure 3.13***), at 24 hours of PBMC incubation with the test compounds, concentrations greater than 400 µM for (S)-NAP and (S)-NAG and 600 µM for (S)-DNAP exert more that 20 % inhibition of PHA-induced PBMC proliferation.

However, the concentration range decreases when cells are incubated with the compounds for 5 days as assessed by ^{3}H-thymidine incorporation (***Figure 3.12***). On this occasion, the corresponding threshold (> 20 % inhibition) is seen at concentrations greater than 330 μM for (S)-NAP, 270 μM for (S)-NAG and 400 μM for (S)-DNAP. Moreover, while higher concentrations of (S)-NAP and (S)-NAG show a steep decrease in the percentage of PHA-induced PBMC proliferation, (S)-DNAP does not follow the same pattern and exhibits a less drastic change. Notably, (S)-DNAP's profile differs from the other two compounds consistently in both experiments and affects cells to a lesser extent.

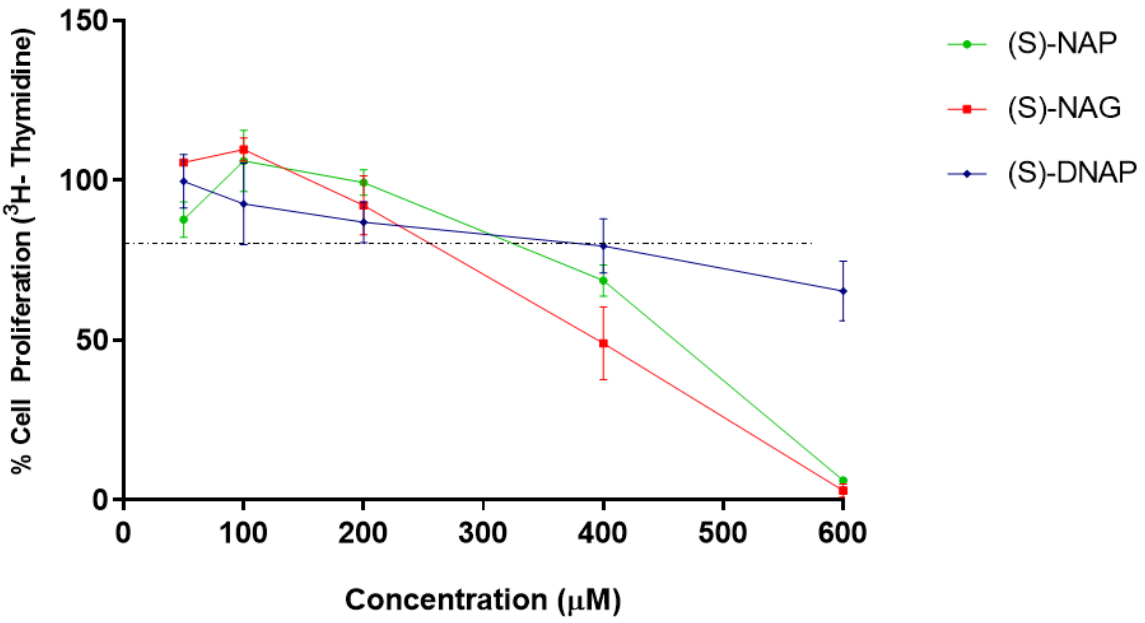


Figure 3.12. Inhibition of phytohaemagglutinin-induced PBMC proliferation following incubation with (S)-naproxen and its metabolites for 5 days as measured by ^{3}H-thymidine incorporation (n=2). PBMCs ($0.15x10^{6}$ /well) were cultured with increasing concentrations of the corresponding drug or metabolite in a total volume of 200 μl inside a humidified incubator of 5 % CO_2 at 37°C for a period of 5 days. On day 3, PHA (5 μg/ml; 10 μl/well) was added in order to stimulate proliferation. Following the 5-day incubation, ^{3}H-thymidine (0.5 μCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hrs. Cells were harvested and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter. All incubations were performed in triplicate. Cell proliferation (%) was calculated as the ratio over the positive control (PHA) x 100. Data are presented as means (± standard deviation; n=2 donors) of % cell proliferation.

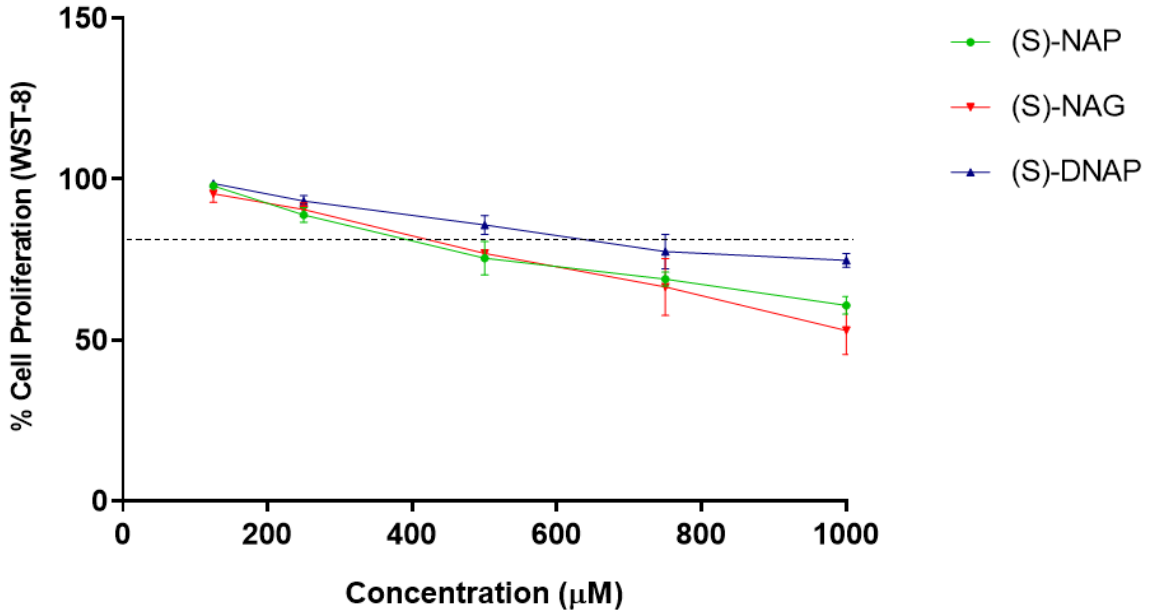


Figure 3.13. Inhibition of phytohaemagglutinin-induced PBMC proliferation following incubation with (S)-naproxen and its metabolites for 24 hrs as measured by Dojindo's highly water-soluble tetrazolium salt reduction (n=2). PBMCs ($0.15x10^6$ /well) were cultured with increasing concentrations of the corresponding drug or metabolite in a total volume of 200 µl and PHA (5 µg/ml; 10 µl/well) as an inducer of proliferation inside a humidified incubator of 5 % CO_2 at 37°C for a period of 24 hrs. Following this period, WST-8 (20 µl/well) was added and incubation was continued inside a humidified incubator of 5 % CO_2 at 37°C for another 4 hrs. Finally, absorbance at 450 nm was measured with a microplate reader. All incubations were performed in triplicate. Cell proliferation (%) was calculated as the ratio over the positive control (PHA) x 100. Data are presented as means (± standard deviation; n=2 donors) of % cell proliferation.

3.4.3 Human peripheral blood mononuclear cells isolated from an idiosyncratic hepatotoxicity patient proliferate and secrete IFNγ in the presence of (S)-O-desmethylnaproxen

LTT and ELISpot IFNγ assays were run as described in sections **3.3.9** and **3.3.10**, using PBMCs from a patient that had received naproxen and suffered an idiosyncratic liver injury reaction, as diagnosed by the treating physician. In addition, control patient samples were used and divided in two groups: patients receiving naproxen on a long-term basis (defined here as a period of more than 3 months of consecutive medication use) and subjects that were otherwise healthy and naïve in terms of exposure to the medication in question. Further details regarding these patients and healthy subjects are shown in ***Table 3.1*** (page 149).

Figures 3.14 and ***3.15*** illustrate the proliferative responses and cytokine secretion levels secondary to corresponding antigen exposure. PBMCs from the iDILI patient proliferated and secreted IFNγ in the presence of (S)-DNAP but not (S)-NAP or (S)-NAG or (S)-NAG – HSA. More specifically, with the LTT assay a maximal SI of 2 (control: 330 ± 29 cpm; (S)-DNAP: 675 ± 157 cpm) was observed at 200 μM although a concentration of 100 μM exerted a meaningful proliferative response as well, albeit weaker (SI = 1.6). PHA (5 μg/ml) induced strong proliferation (SI = 98), thus verifying assay functionality. Similar results were obtained with the ELISpot IFNγ assay, where (S)-DNAP induced IFNγ secretion from PBMCs with the highest responses seen at 200 μM (89 SFUs / 0.5 x 10^6 cells) and 400 μM (82 SFUs / 0.5 x 10^6 cells) over control (22 SFUs / 0.5 x 10^6 cells). PHA (5 μg/ml) induced strong IFNγ secretion (296 SFUs / 0.5 x 10^6 cells) thus verifying the robustness of the assay. This clear dose-dependent response is also illustrated in ***Figure 3.15B***, where IFNγ-secreting cells are visualized following stimulation with increasing concentrations of (S)-DNAP or positive control (PHA 5 μg/ml). As was the case with the LTT assay, no response was observed for (S)-NAP or (S)-NAG or (S)-NAG – HSA. To ensure the validity of both LTT and ELISpot IFNγ

results, a second blood acquisition was performed from the same iDILI patient and the entire experimental procedure was repeated as described above. Apart from a small variability, the response pattern remained consistent. On this occasion, the LTT assay yielded a maximal SI of 2.2 (control: 523 ± 55 cpm; (S)-DNAP: 1,148 ± 31 cpm) at 100 μM and a weaker response (SI = 1.7) at 200 μM whereas the ELISpot IFNγ assay recorded the highest IFNγ secretion at 200 μM (82 SFUs / 0.5 x 10^6 cells) and 400 μM (79 SFUs / 0.5 x 10^6 cells) for (S)-DNAP only over control (32 SFUs / 0.5 x 10^6 cells). Consistently with the first experiment, no responses were observed for the other test compounds or the AG – HSA adduct.

The same experiments were performed for all four long-term naproxen-treated patients (not having suffered adverse reaction) as well as the four healthy naïve subjects. No positive responses were observed in any of these individuals, thus reinforcing the finding of (S)-DNAP's specific lymphocyte proliferation and IFNγ secretion from the isolated PBMCs of the iDILI patient. ***Figures 3.16*** and ***3.17*** show the data collectively across the different test compounds and sample cohorts. The positive response for (S)-DNAP alongside the lack of a response for (S)-NAP, (S)-NAG and (S)-NAG – HSA from the isolated PBMCs of the iDILI patient in conjunction with the complete lack of responses for all test compounds from the isolated PBMCs of the two control subject groups, prompted further investigative characterization as described in the next section.

Table 3.1. Clinical details of patients and healthy subjects. Liver function tests and details of reaction are described for the iDILI subject.

ID	Subject	Age	Sex	Underlying Disease	Co-medication	Peak LFTs	Details of Reaction
HCA RLH 041	iDILI	51	F	Osteoarthritis	Evorel, Glucosamine	ALT: 596 IU/L *Ref: [2-53 IU/L]* ALP: 268 IU/L *Ref: [40-130 IU/L]* Bilirubin: 47 Umol/L *Ref: [3-17 Umol/L]*	Onset: 24/9/10 Resolution: 01/11/10 Unbearable pain, jaundice whilst on Naproxen. Additionally, the patient was diagnosed with cholelithiasis & underwent cholecystectomy. It is possible that the LFTs reflect both occurrences.
HC1 RLH 004	Long-term treated	64	F	Osteoarthritis, Hip replacement	Omeprazole, Ibuprofen, Co-codamol		
HC1 RLH 005	Long-term treated	65	F	Musculoskeletal	Sildenopril, Ambrosentin, Quinine, Aspirin, Levothyroxine, Omeprazole,		
HC1 RLH 007	Long-term treated	45	M	Gout	Febuxostat		
HC1 RLH 008	Long-term treated	64	F	Rheumatoid Arthritis	Methotrexate, TNFα, Omeprazole, Folic Acid, Sulfasalazine		
HC2 RLH 001	Healthy naïve	28	M	N/A	N/A		
HVN 108	Healthy naïve	24	F				
HVN 061	Healthy naïve	59	F				
HVG 050	Healthy naïve	27	M				

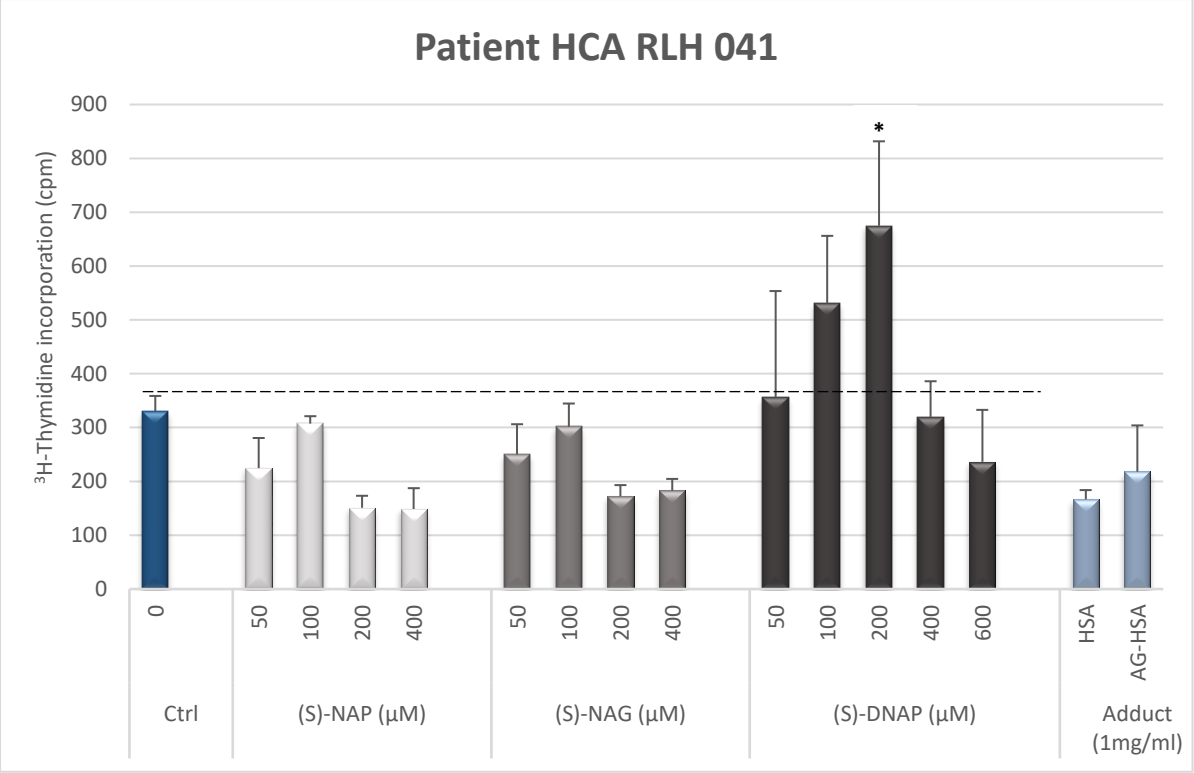
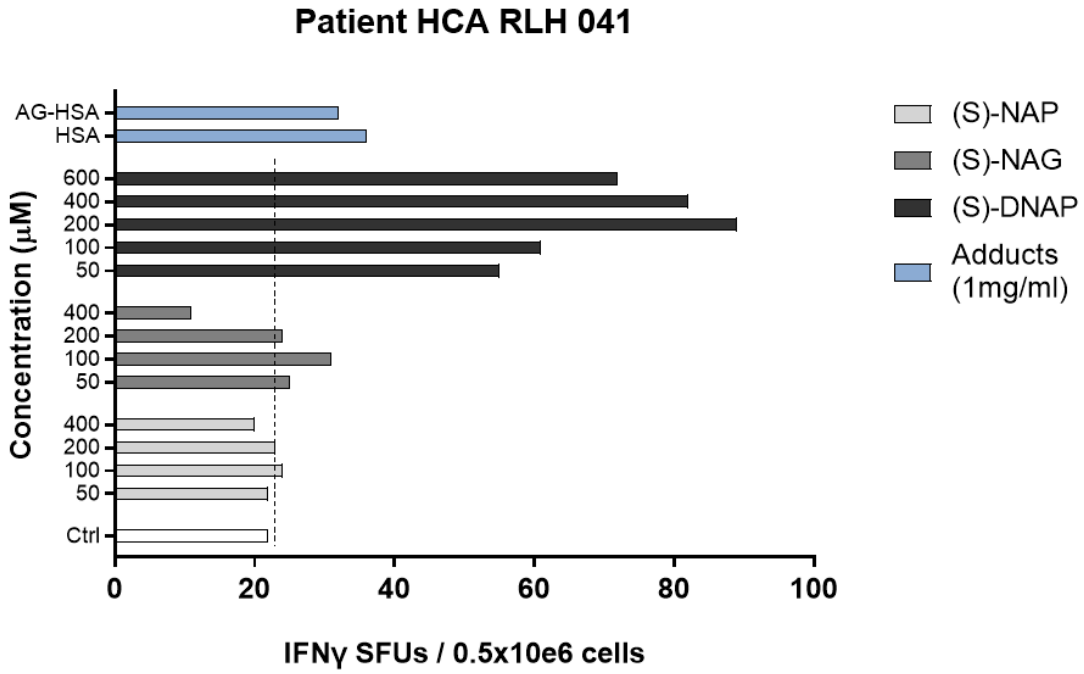


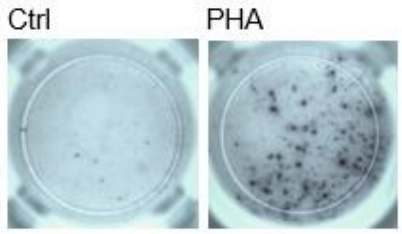
Figure 3.14. Lymphocyte Transformation Test using peripheral blood mononuclear cells from a patient having suffered idiosyncratic hepatotoxicity secondary to naproxen (n=1). PBMCs ($0.15x10^6$ /well) were cultured with relevant concentrations of the corresponding drug or metabolite or protein adduct in a total volume of 200 µl cell culture medium inside a humidified incubator of 5 % CO_2 at 37°C for a period of 5 days. Following the 5-day incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hrs. Cells were harvested and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter. All incubations were performed in triplicate and data are presented as means (± standard error of mean) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

A)



B)

CONTROL (Medium) & PHA 5µg/ml



(S)-O-DESMETHYLNAPROXEN

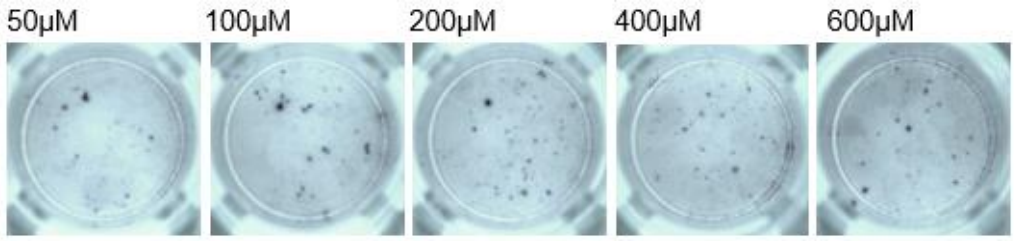


Figure 3.15. ELISpot IFNγ assay using peripheral blood mononuclear cells from a patient having suffered idiosyncratic hepatotoxicity secondary to naproxen (n=1). PBMCs ($0.15x10^6$ /well) were cultured with relevant concentrations of the corresponding drug or metabolite or protein adduct in a total volume of 200 µl cell culture media inside a humidified incubator of 5 % CO_2 at 37°C for a period of 48 hours. After incubation, cells were discarded and ELISpot plates were developed as described in section 3.3.10. **A)** Results were recorded as SFUs per $0.5x10^6$ cells. **B)** Spots were visualized using a AID ELISpot reader.

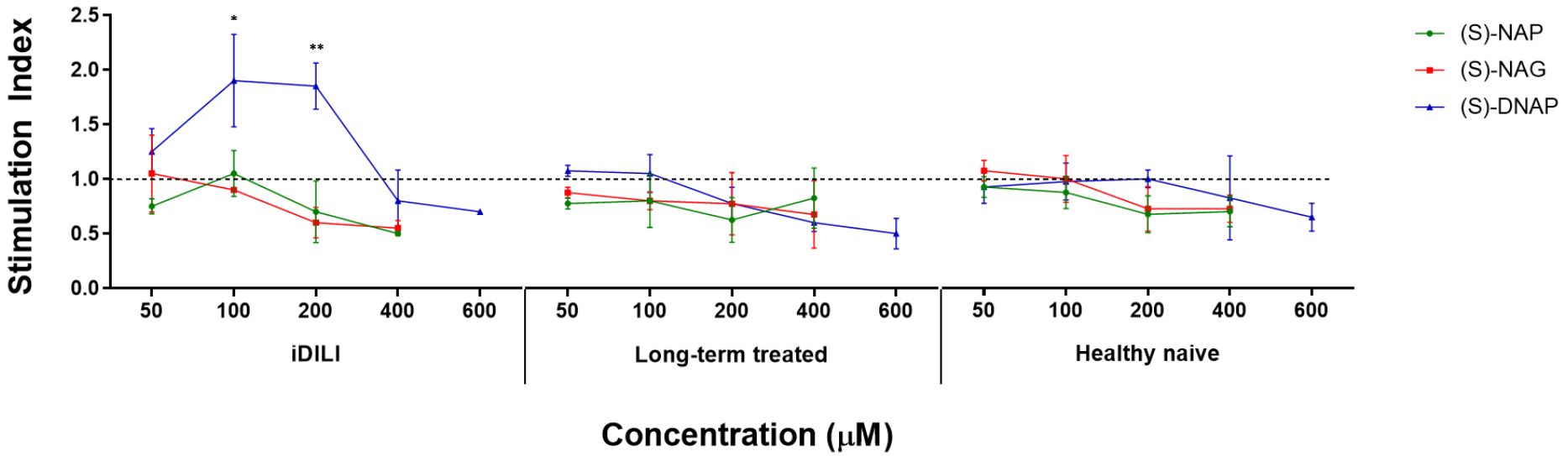


Figure 3.16. Lymphocyte Transformation Test using peripheral blood mononuclear cells from a patient having suffered idiosyncratic hepatotoxicity secondary to naproxen (n=1) and control group subjects (n=4 long-term treated and n=4 healthy naïve volunteers). PBMCs ($0.15x10^6$ /well) were cultured with relevant concentrations of the corresponding drug or metabolite or protein adduct in a total volume of 200 µl cell culture medium inside a humidified incubator of 5 % CO_2 at 37°C for a period of 5 days. Following the 5-day incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hrs. Cells were harvested and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter. For the iDILI patient, data are presented as means (± standard deviation; n=2 separate blood donations from one iDILI patient) of SI. For the control group subjects, data are presented as means (± standard deviation; n=4 donors) of SI. The SI was calculated as mean cpm of stimulated cells over mean cpm of negative control. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

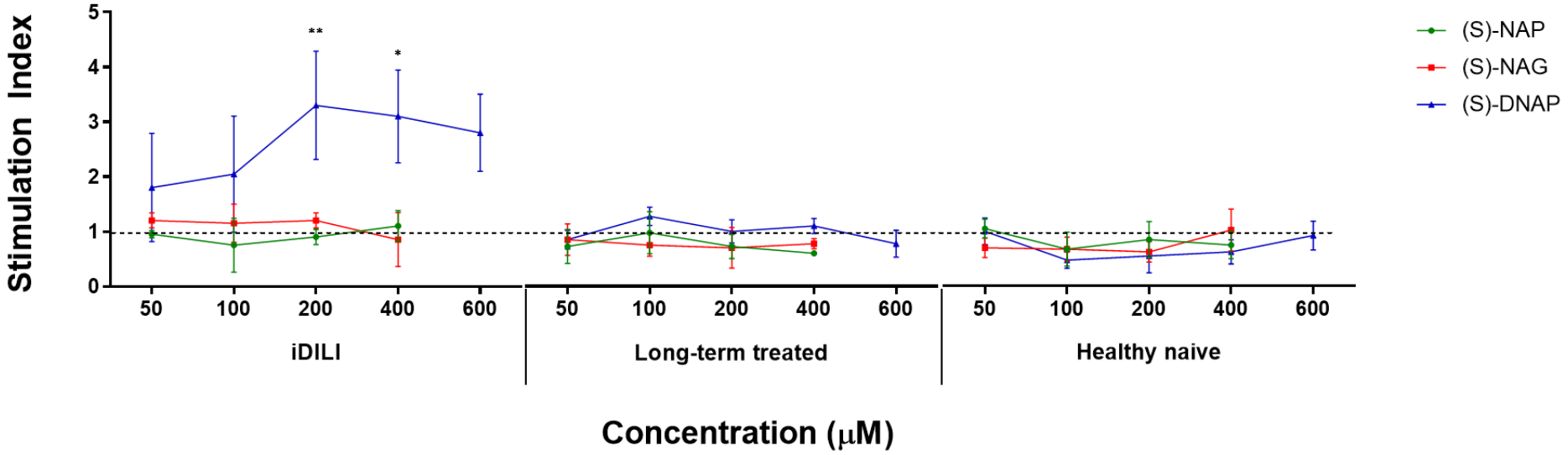
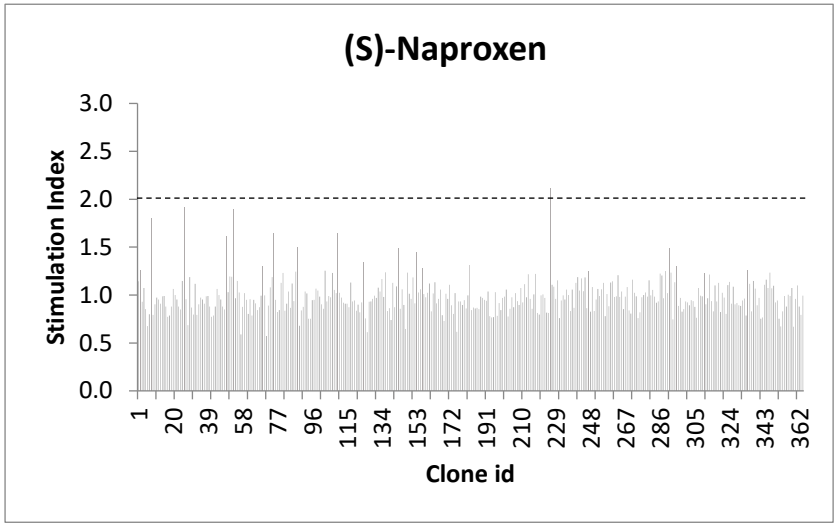


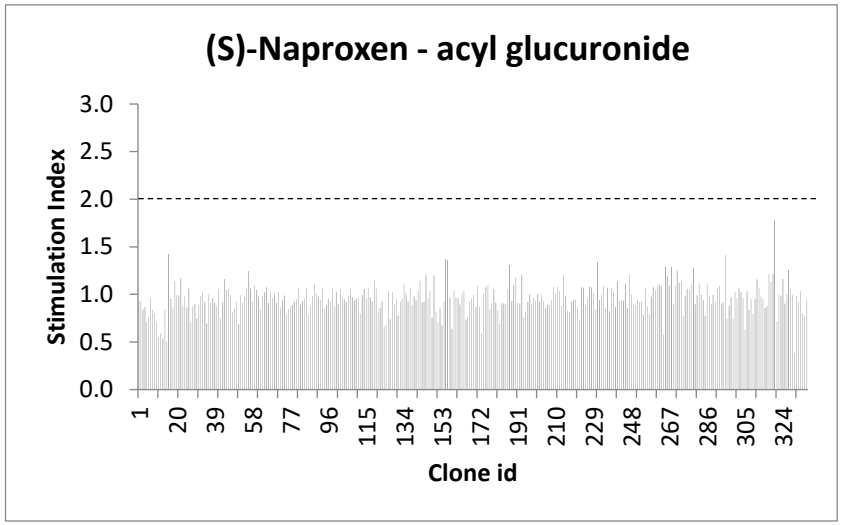
Figure 3.17. ELISpot IFNγ assay using peripheral blood mononuclear cells from a patient having suffered idiosyncratic hepatotoxicity secondary to naproxen (n=1) and control group subjects (n=4 long-term treated and n=4 healthy naive volunteers). PBMCs ($0.15x10^6$ /well) were cultured with relevant concentrations of the corresponding drug or metabolite or protein adduct in a total volume of 200 µl cell culture medium inside a humidified incubator of 5 % CO_2 at 37°C for a period of 48 hours. After incubation, cells were discarded and ELISpot plates were developed as described in section 3.3.10. Results were recorded as SFUs per $0.5x10^6$ cells. For the iDILI patient, data are presented as means (± standard deviation; n=2 separate blood donations from one iDILI patient) of SI. For the control group subjects, data are presented as means (± standard deviation; n=4 donors) of SI. The SI was calculated as mean SFU of stimulated cells over mean SFU of negative control. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

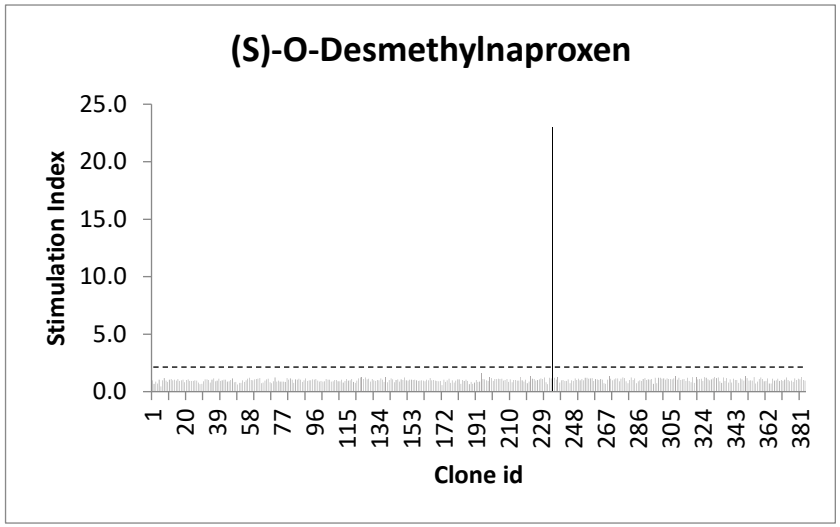
3.4.4 (S)-O-desmethylnaproxen is the only compound capable of yielding a drug-responsive T-cell clone from the idiosyncratic hepatotoxicity patient

To further investigate and characterize T-cell responses to (S)-DNAP, PBMCs from the iDILI patient were subjected to T-cell cloning by limiting dilution as described in section **3.3.14**. Overall, 1,204 T-cell clones were generated. More specifically, 364 T-cell clones for (S)-NAP, 336 for (S)-NAG, 384 for (S)-DNAP and 120 for (S)-NAG – HSA were screened for antigen-specificity using the assays described in section **3.3.15**. ***Figure 3.18*** shows the T-cell clone screening panels for the respective antigens used.

Several clones across the different screen panels achieved a SI above 1.6 but only one showed a pronounced proliferation. (S)-DNAP T-cell clone 236 yielded a SI = 23.1 (control: 2,045 ± 720 cpm; (S)-DNAP: 47,138 ± 1,353 cpm) thus indicating a very strong positive response. To further validate these responses, all clones exhibiting an initial screening SI > 1.6 were subjected to a dose-dependent cell proliferation experiment upon antigen stimulation. (S)-DNAP T-cell clone 236 was the only clone capable of exhibiting a dose-dependent response as seen in ***Figure 3.19***. The highest stimulation was observed between concentrations of 50 μM – 400 μM and the maximal SI = 2.5 (control: 1,265 ± 45 cpm; (S)-DNAP: 3,186 ± 557 cpm) was recorded at 200 μM. Although the clone's initial screening SI was 23.1 at 200 μM, the observed decrease potentially reflects a loss of activity due to long-term culture. None of the other T-cell clones achieved dose-dependent responses. In addition, (S)-DNAP T-cell clone 236 was examined for its IFNγ secretion potential using the ELISpot IFNγ assay. Results are illustrated in ***Figure 3.20***. Strong IFNγ secretion was observed at concentrations between 100 μM and 600 μM with the maximal response (control: 2 SFUs / $0.5x10^6$ cells; (S)-DNAP: 67 SFUs / $0.5x10^6$ cells) recorded at 400μM.







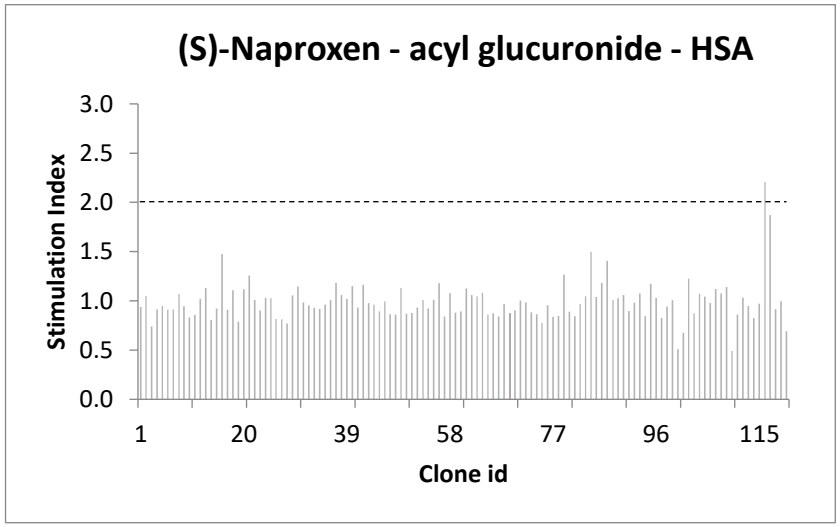


Figure 3.18. Panels of T-cell clones screening for (S)-NAP, (S)-NAG, (S)-DNAP and (S)-NAG – HSA. T-cell clones were generated as described in section 3.3.14. The SI was calculated as mean cpm of stimulated cells over mean cpm of negative control**.**

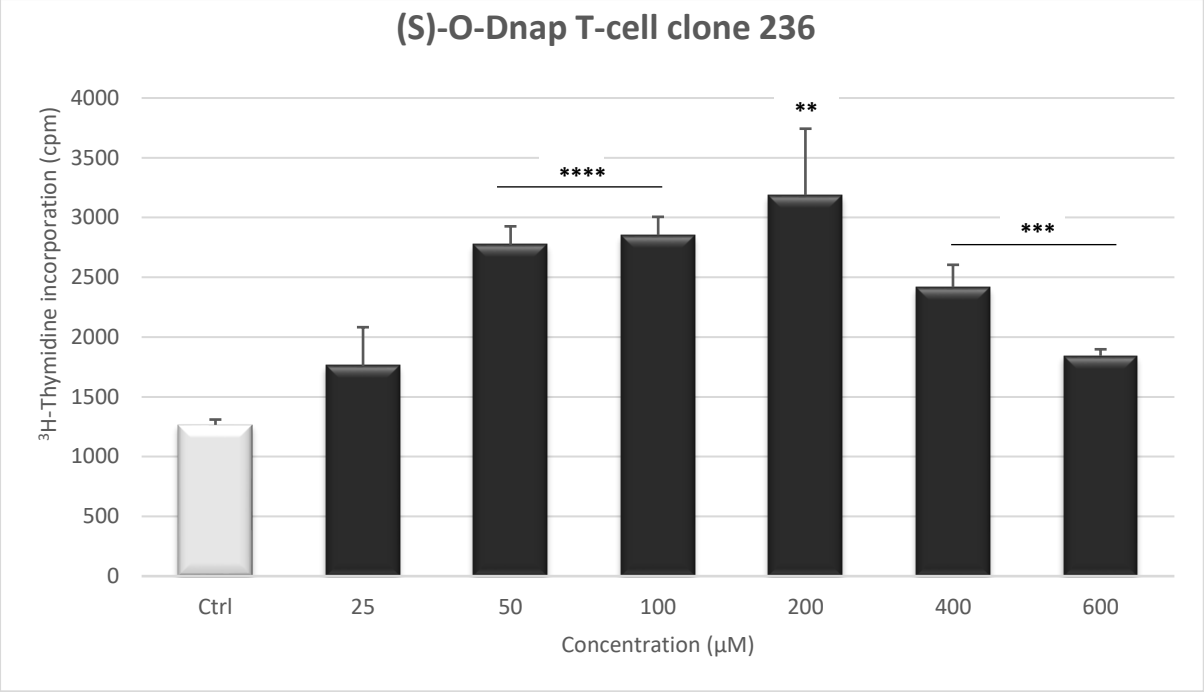
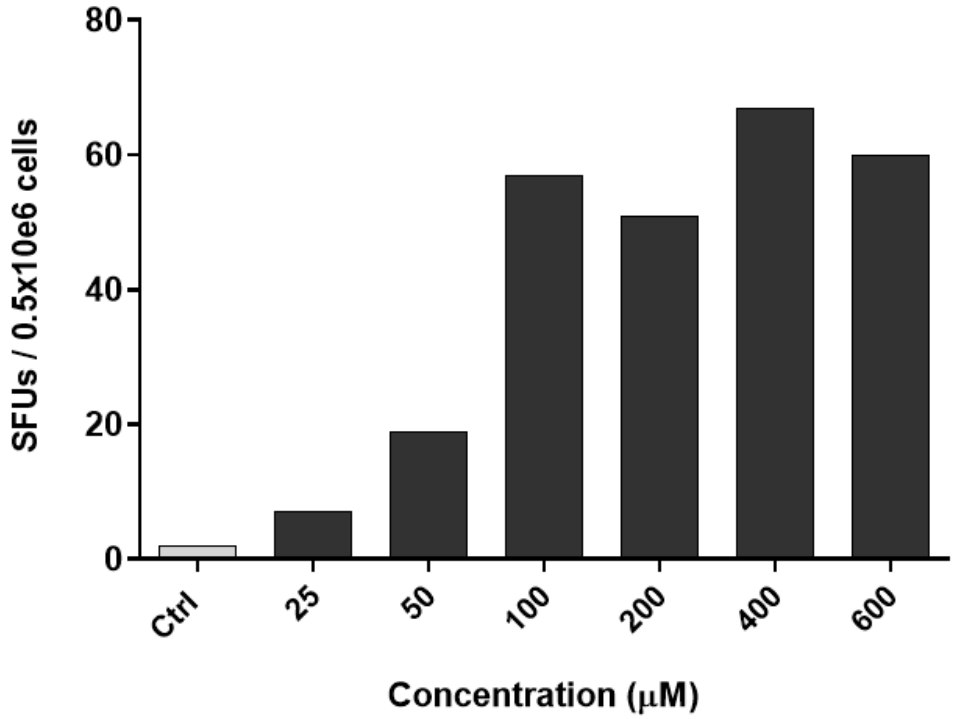


Figure 3.19. Dose-response proliferation assay for (S)-DNAP T-cell clone 236. T-lymphocytes were cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and incubated with differing concentrations of (S)-DNAP for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. Following the 48-hour incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5% CO_2 at 37°C for 16 hours. Cells were harvested and ^{3}H-thymidine incorporation was measured with a Micro Beta Trilux Counter. All incubations were performed in triplicate. Data are presented as means (± standard deviation; n=3) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

A)



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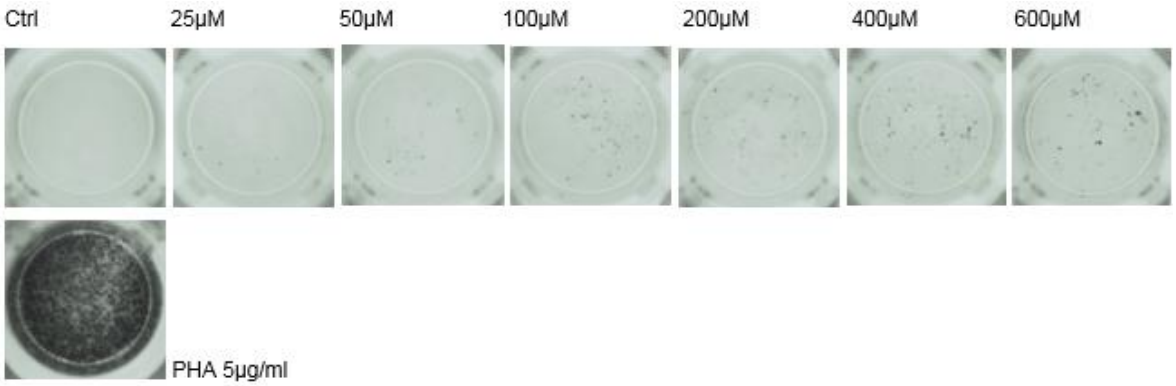


Figure 3.20. Dose-response ELISpot IFNγ assay for (S)-DNAP T-cell clone 236. T-lymphocytes were cultured ($5x10^4$ cells/well; 50µl) with autologous irradiated EBV-transformed B-cells ($1x10^4$ cells/well; 50µl) and incubated with differing concentrations of (S)-DNAP for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. After incubation, cells were discarded and ELISpot plates were developed as described in section 3.3.10. **A)** Data were recorded as SFUs per $0.5x10^6$ cells. **B)** Spots were visualized using an AID ELISpot reader.

Finally, the phenotype of (S)-DNAP T-cell clone 236 was determined using flow cytometry as detailed in section **3.3.16**. Surprisingly, two separate populations were identified within the same well, namely $CD4^+CD8^+$ and $CD8^+$ as can be seen in ***Figure 3.21***. To ensure this result, cells were re-analysed and stained with a different set of $CD4^+$ and $CD8^+$ antibodies and the outcome was the same.

Additional efforts were conducted to identify more drug-responsive T-cell clones for (S)-DNAP, if possible. However, two separate repeats of T-cell cloning by limiting dilution generating 430 clones overall (280 and 150 clones in the first and second trial, respectively) were unsuccessful in producing more drug – responsive (S)-DNAP clones.

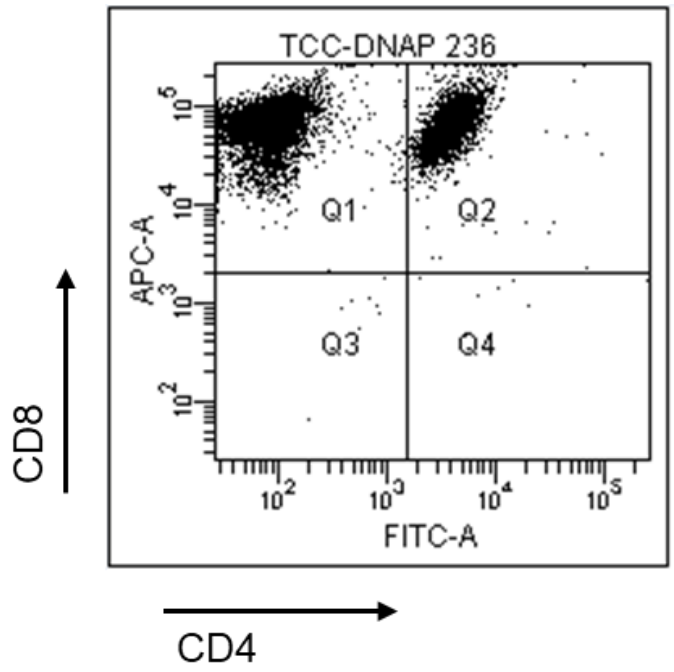


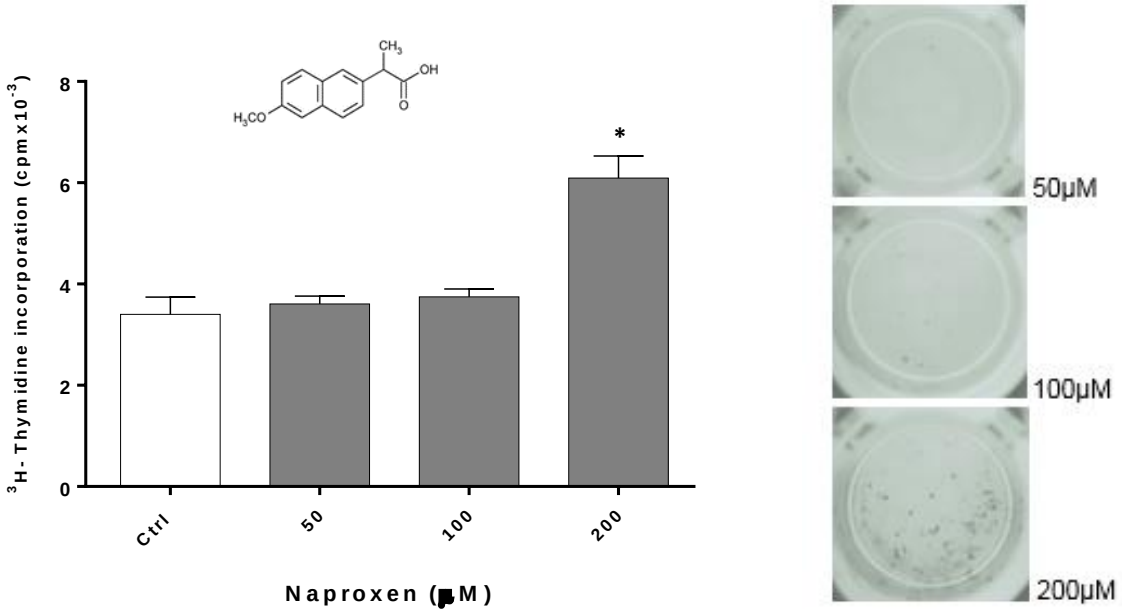
Figure 3.21. Flow cytometry dot plot of (S)-DNAP T-cell clone 236. T-lymphocytes ($5x10^3$; 200 µl) were incubated with anti-CD4-FITC and anti-CD8-APCN for 20 mins at 4°C in darkness. Cells were then washed with 1 ml FACS buffer and following centrifugation (1,400 rpm, 10 mins, 4°C) and supernatant discarding, were re-suspended in 200 µl FACS buffer and analysed using FACS Canto II flow cytometer.

3.4.5 (S)-O-desmethylnaproxen T-cell clone 236 does not cross-react with structurally unrelated compounds

Antigen-specificity and cross-reactivity of the (S)-DNAP clone 236 was examined against the related test compounds (S)-NAP and (S)-NAG as well as two structurally unrelated compounds, namely SMX-NO and zomepirac. SMX-NO is a reactive metabolite of SMX and has been widely studied for its hypersensitivity as well as T-cell priming potential (Schnyder et al., 2000; Naisbitt et al., 2001; Faulkner et al., 2012). Zomepirac is a NSAID which was withdrawn from the market due to anaphylaxis and IDR incidents (Guerrero, 1983).

The cross-reactivity assay (section **3.3.15**) was used for this investigation. Concentration range for SMX-NO was determined based on literature whereas for zomepirac concentrations above 100 μM appeared to significantly inhibit cell proliferation during these experiments. ***Figure 3.22*** shows the results for (S)-NAP, (S)-NAG, SMX-NO and zomepirac. Cells were capable of showing a significant proliferative response but only for the highest concentration tested (200 μM). This was also verified by the capacity of the cells to secrete IFNγ at the same concentration. (S)-NAP yielded a maximal SI of 1.8 (control: 3,403 ± 241 cpm; (S)-NAP: 6,095 ± 306 cpm) at 200 μM while 8 and 90 IFNγ SFUs / $0.5x10^6$ cells were recorded at 100 and 200 μM, respectively. (S)-NAG yielded a maximal SI of 1.5 (control: 3,403 ± 241 cpm; (S)-NAP: 5,163 ± 128 cpm) at 200 μM while 19 and 38 IFNγ SFUs / $0.5x10^6$ cells were recorded at 100 and 200 μM, respectively. No relevant cell proliferation or IFNγ secretion was observed when the (S)-DNAP T-cell clone 236 was exposed to SMX-NO or zomepirac. Based on these findings it was decided to include (S)-NAP and (S)-NAG alongside (S)-DNAP in subsequent functional experiments.

A.


^{3}H- Thymidine incorporation (cpm x10^{-3})
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Naproxen (μM)
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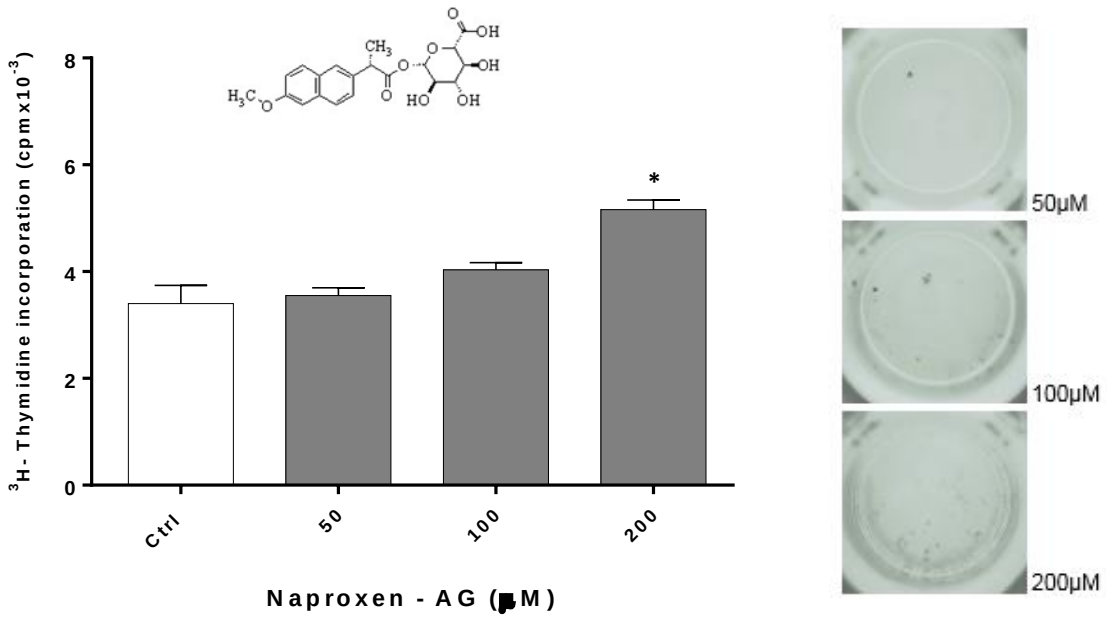
200μM

B.


^{3}H- Thymidine incorporation (cpm x10^{-3})
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Naproxen - AG (μM)
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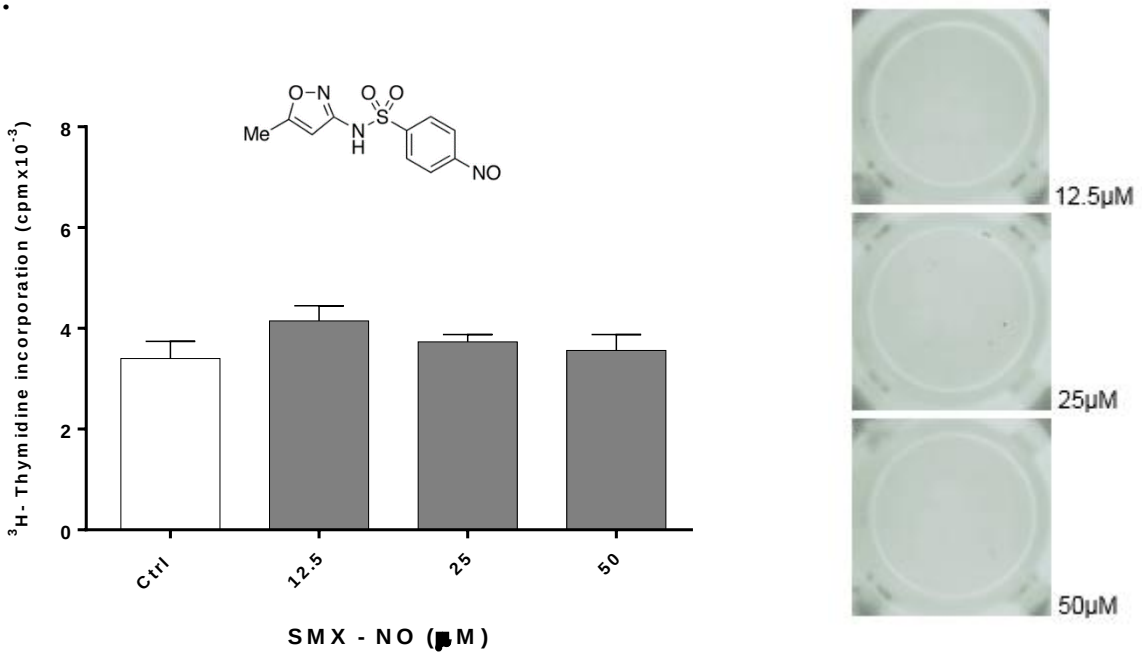
C.



D.

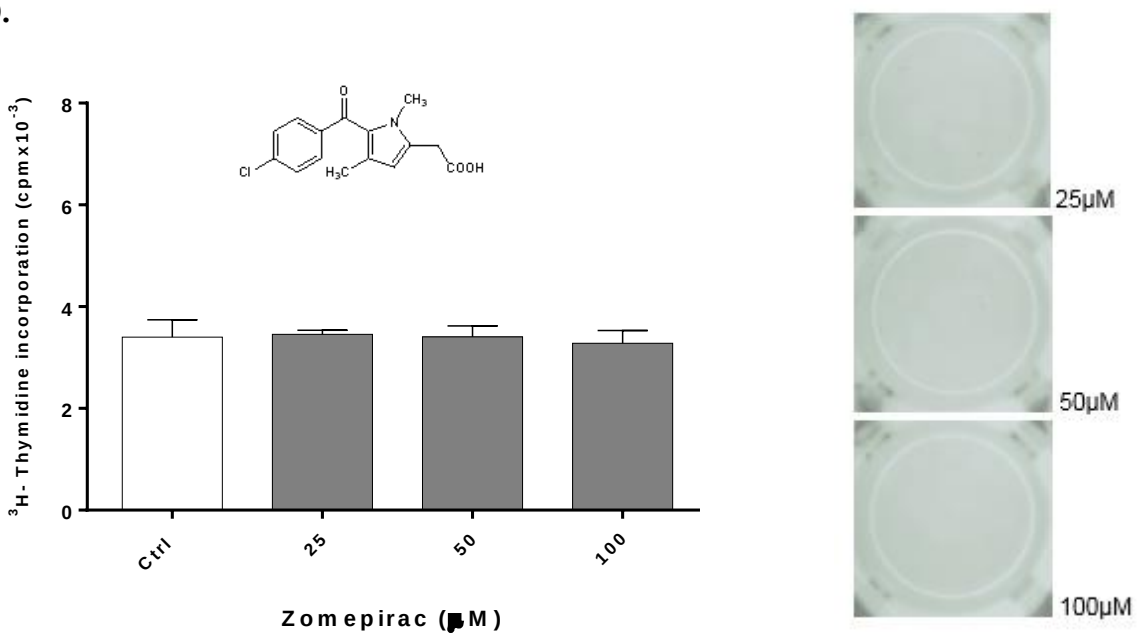


Figure 3.22. Cross-reactivity proliferation and ELISpot IFNγ assays for (S)-DNAP T-cell clone 236. The responsive (S)-DNAP T-cell clone 236 was cultured (5x10^4 cells/well; 50 µl) with irradiated autologous EBV transformed B-cells (1x10^4 cells/well; 50 µl) and incubated with differing concentrations of structurally diverse compounds: **A)** (S)-NAP (0 – 200 µM; 100 µl), **B)** (S)-NAG (0 – 200 µM; 100 µl), **C)** SMX-NO 0 – 50 µM; 100 µl) and **D)** Zomepirac (0 – 100 µM; 100 µl) for 48 hours

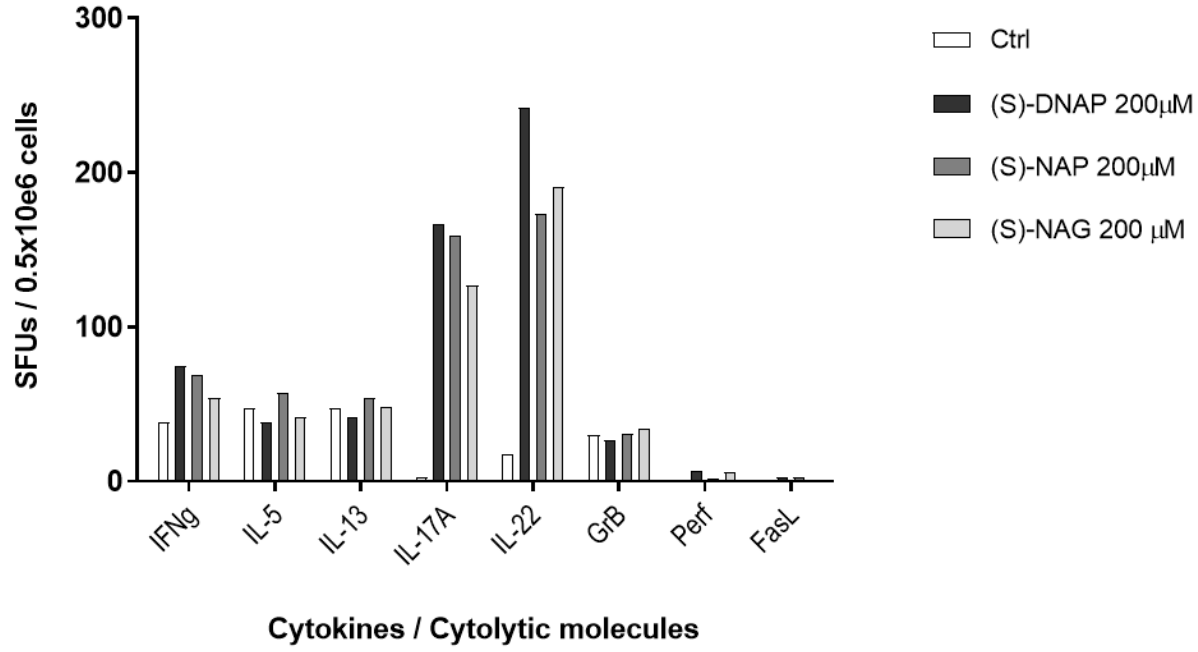
inside a humidified incubator of 5 % CO_2 at 37°C. For the cell proliferation assay (left), following the 48-hour incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hours. All incubations were performed in duplicate. Data are presented as means (± standard deviation; n=2) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant. For the ELISpot IFNγ assay (right), after incubation, cells were discarded and ELISpot plates were developed as described in section 3.3.10. Spots were visualized using a AID ELISpot reader.

3.4.6 (S)-O-desmethylnaproxen T-cell clone 236 secretes IL-17A and IL-22 alongside IFNγ

As T-cells can secrete a number of different cytokines depending on their phenotype, a basic cytokine screen panel was used in order to further query the phenotypic and functional profile of (S)-DNAP T-cell clone 236. The panel included the cytokines IL-5, IL-13, IL-17A, IL-22, Granzyme B, Perforin, Fas Ligand alongside IFNγ and the experiment was developed as described in section **3.3.15**. To ensure consistency of results, all cytokines and test compounds were screened simultaneously on the same plate.

Results are illustrated in ***Figure 3.23***. Upon exposure to the respective compounds, significant secretion was observed for cytokines IL-17A (control: 3 SFUs / 0.5x10^6 cells; (S)-DNAP: 167 SFUs / 0.5x10^6 cells; (S)-NAP: 159 SFUs / 0.5x10^6 cells; (S)-NAG: 127 SFUs / 0.5x10^6 cells) and IL-22 (control: 18 SFUs / 0.5x10^6 cells; (S)-DNAP: 242 SFUs / 0.5x10^6 cells; (S)-NAP: 173 SFUs / 0.5x10^6 cells; (S)-NAG: 191 SFUs / 0.5x10^6 cells) which surpassed the IFNγ secretion (control: 38 SFUs / 0.5x10^6 cells; (S)-DNAP: 75 SFUs / 0.5x10^6 cells; (S)-NAP: 69 SFUs / 0.5x10^6 cells; (S)-NAG: 54 SFUs / 0.5x10^6 cells). Secretion levels for the other tested cytokines were comparable to control. Whilst all test compounds were capable of inducing the positive Th17 response from the clone, a slightly increased cytokine induction was observed following exposure to (S)-DNAP when compared to (S)-NAP or (S)-NAG.

A)



B)

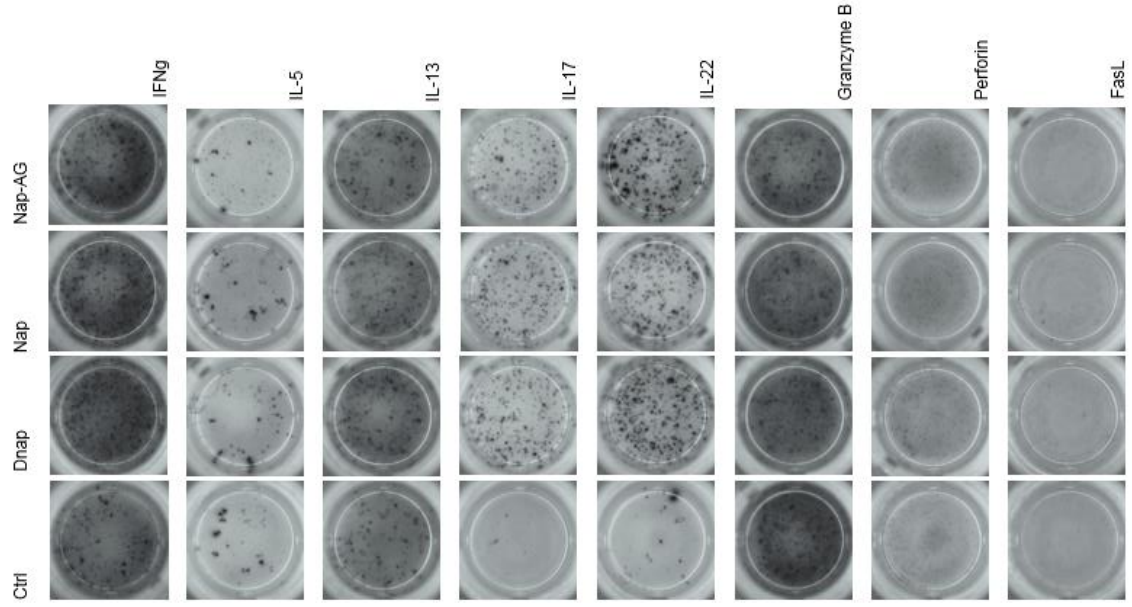


Figure 3.23. ELISpot cytokine secretion assay for (S)-DNAP T-cell clone 236. The responsive (S)-DNAP T-cell clone 236 was cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and incubated with (S)-DNAP or (S)-NAP or (S)-NAG (200 µM; 100 µl) for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. After incubation, cells were discarded and ELISpot plates were developed as described in section 3.3.10. **A)** Data were recorded as Spot Forming Units (SFUs) per $0.5x10^6$ cells. **B)** Spots were visualized using a AID ELISpot reader.

3.4.7 (S)-O-desmethylnaproxen T-cell clone 236 expresses several functional tissue homing receptors

Activated T-cells are known to express specific tissue homing receptors that facilitate their migration between tissues. Flow cytometry (section **3.3.16**) was used to examine and ascertain the expression of several cell surface receptors. As shown in ***Figure 3.24***, several chemokine receptors displayed increased expression alongside CD69 (MFI = 7.83), a marker of cell activation. In particular, CCR2 (MFI = 5.47), CCR4 (MFI = 9) and CXCR3 (MFI = 7.28) showed increased expression, while CXCR10 (MFI = 2.93), CCR9 (MFI = 3.04) and CCR5 (MFI = 2.75) demonstrated a significant, albeit lower degree of expression.

To further determine the functionality of the tissue homing receptor expression, a T-cell migration (chemotaxis) assay was performed as described in section **3.3.17**. As CCR4 was the chemokine receptor with the highest expression, the assay was performed using CCL17, a chemokine known to elicit its effects by interacting with CCR4 and inducing chemotaxis in T-cells. Results depicted in ***Figure 3.25*** show a steep increase in the percentage of cells migrating during the first 8 hours and peaking at 16 hours with 43.5 ± 1.5 % of T-cells having migrated to CCL17-containing medium. No further increase in cell migration was seen between 16 and 24 hours.

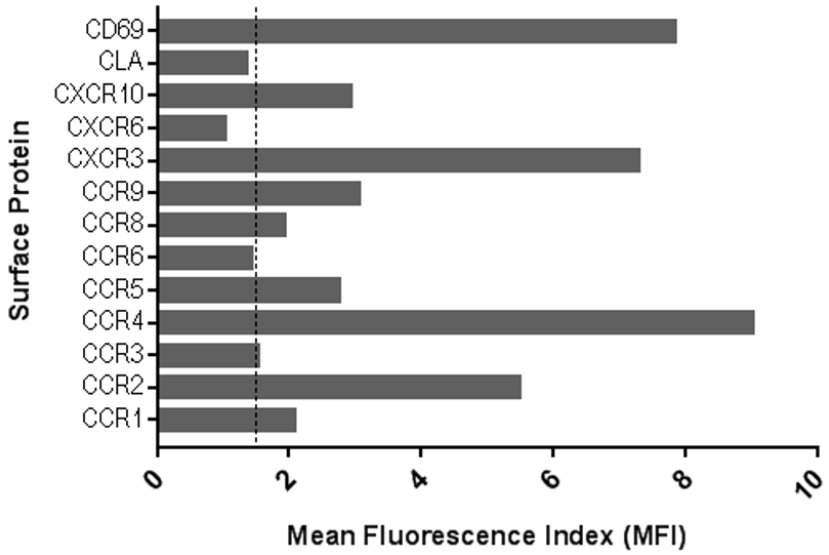


Figure 3.24. Expression of tissue homing receptors on the surface of (S)-DNAP T-cell clone 236. T-lymphocytes ($5x10^3$; 200 µl) were incubated with specific fluorescently tagged antibodies or their isotypes (see 3.3.16) for 20 mins at 4°C in darkness. Following preparation as described in 3.3.16, cells were analysed using FACS Canto II flow cytometer. Data are presented as Mean Fluorescence Index.

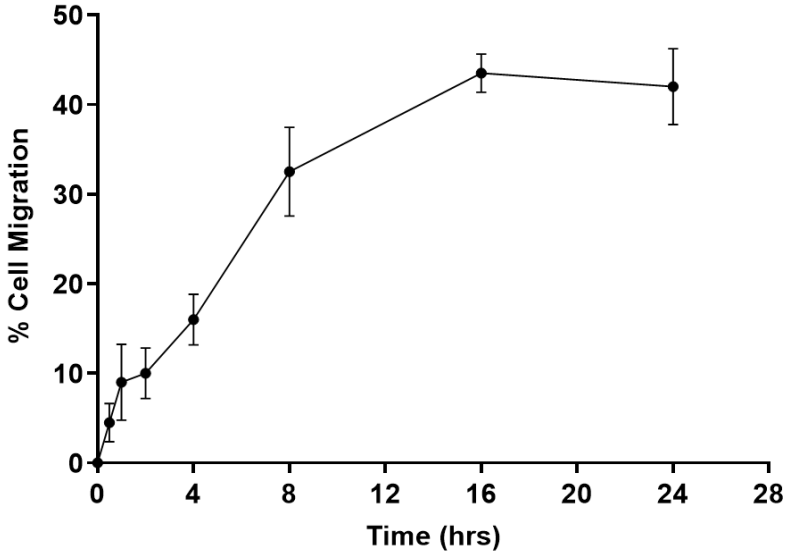


Figure 3.25. Chemotaxis of (S)-DNAP T-cell clone 236 to CCL17. T-lymphocytes were suspended in chemotaxis buffer and placed ($0.1x10^5$; 100 µl) on the upper chamber of the trans-well. Then, 600 µl of chemotaxis buffer containing 100 ng / ml chemokine ligand 17 (CCL-17), were added into the lower chamber of the trans-well. Cells were incubated inside a humidified incubator of 5 % CO_2 at 37°C and at desired time-points 10 µl of sample were withdrawn from the lower chamber to count migrating cells with a haemocytometer. Data are presented as means (± standard deviation; n=2) of % cell migration.

3.4.8 (S)-O-desmethylnaproxen T-cell clone 236 requires antigen-presenting cells in order to proliferate and is major histocompatibility complex class II-restricted

While the typical dogma of T-cell activation requires the presence of APCs, evidence exists to suggest that on certain occasions T-cells can become activated even in their absence (Rao, Ko, Faas, & Cantor, 1984). Therefore, the APC dependency assay (section **3.3.15**) sought to investigate this aspect. As can be seen in ***Figure 3.26***, proliferation was demonstrated by all test compounds in the presence of APCs with the highest response (and statistically significant) of SI = 1.64 (control: 5,874 ± 589 cpm; (S)-DNAP: 9,657 ± 569 cpm) observed with the highest concentration (200 μM) of (S)-DNAP. This is consistent with the responses recorded previously (section **3.4.4**; ***Figure 3.19***). Interestingly, in the absence of APCs, only (S)-DNAP at 200 μM caused a small statistically significant proliferative response of SI = 1.4 (control: 4,999 ± 286 cpm; (S)-DNAP: 6,926 ± 128 cpm), albeit weaker than what was measured in their presence and below the threshold of meaningful proliferation (< 1.6).

The effect of APC MHC blockade was also investigated using the MHC restriction assay (section **3.3.15**). (S)-DNAP was used at 100 μM. This concentration was chosen to avoid "bypassing" the requirement of APCs as seen previously with the higher concentration of 200 μM. Proliferative responses are shown in ***Figure 3.27***, where the lack of a response shows the effect of MHC blockade, pointing to a MHC-restricted T-cell response directed to (S)-DNAP. Significant inhibition of cell proliferation was observed when APCs where treated with an anti-MHC class II antibody. A slight effect was also seen during MHC class I blockade, albeit not significant (control: 11,190 ± 930 cpm; (S)-DNAP: 18,746 ± 1,594 cpm; anti-Class I: 16,390 ± 807 cpm). Finally, inhibition of proliferation was more profound in the presence of HLA-DP and HLA-DR blockade.

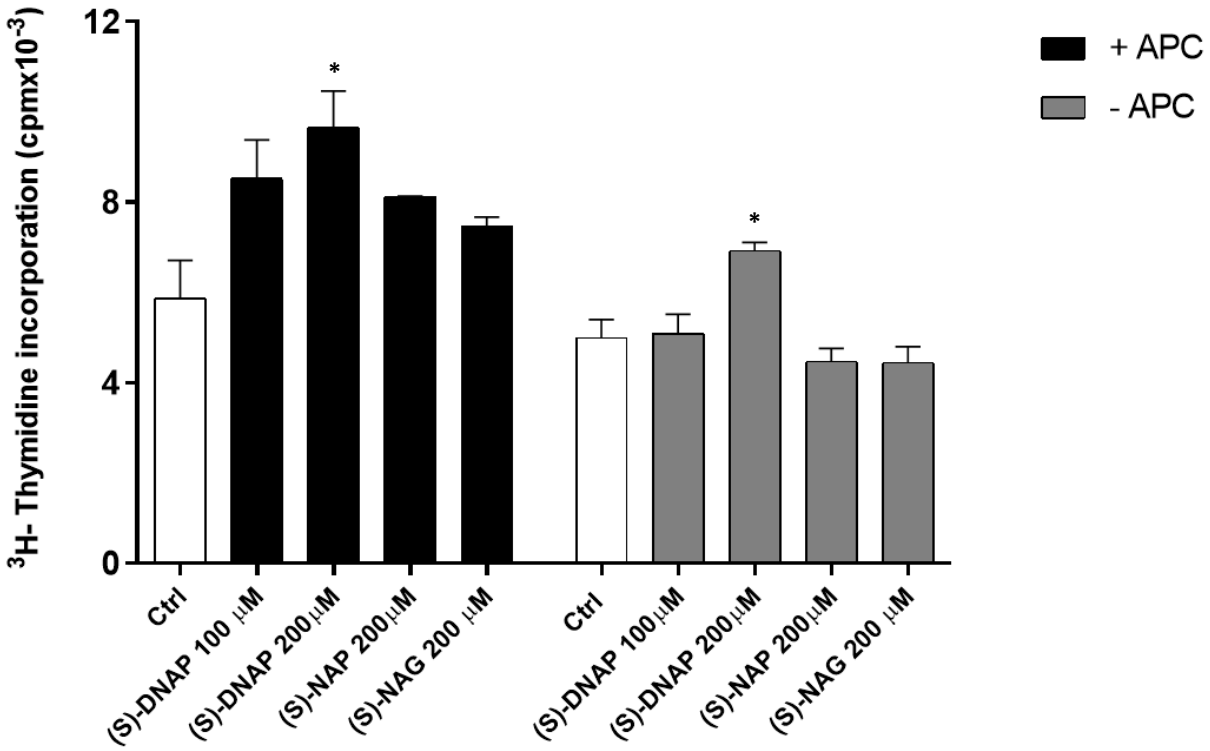


Figure 3.26. Drug-specific proliferation of (S)-DNAP T-cell clone 236 in the presence or absence of antigen-presenting cells. The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 μl) with or without irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 μl) and incubated with (S)-DNAP (100 μM & 200 μM; 100 μl) or (S)-NAP (200 μM; 100 μl) or (S)-NAG (200 μM; 100 μl) for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. Following the 48-hour incubation, ^{3}H-thymidine (0.5 μCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hours. All incubations were performed in duplicate. Data are presented as means (± standard deviation; n=2) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

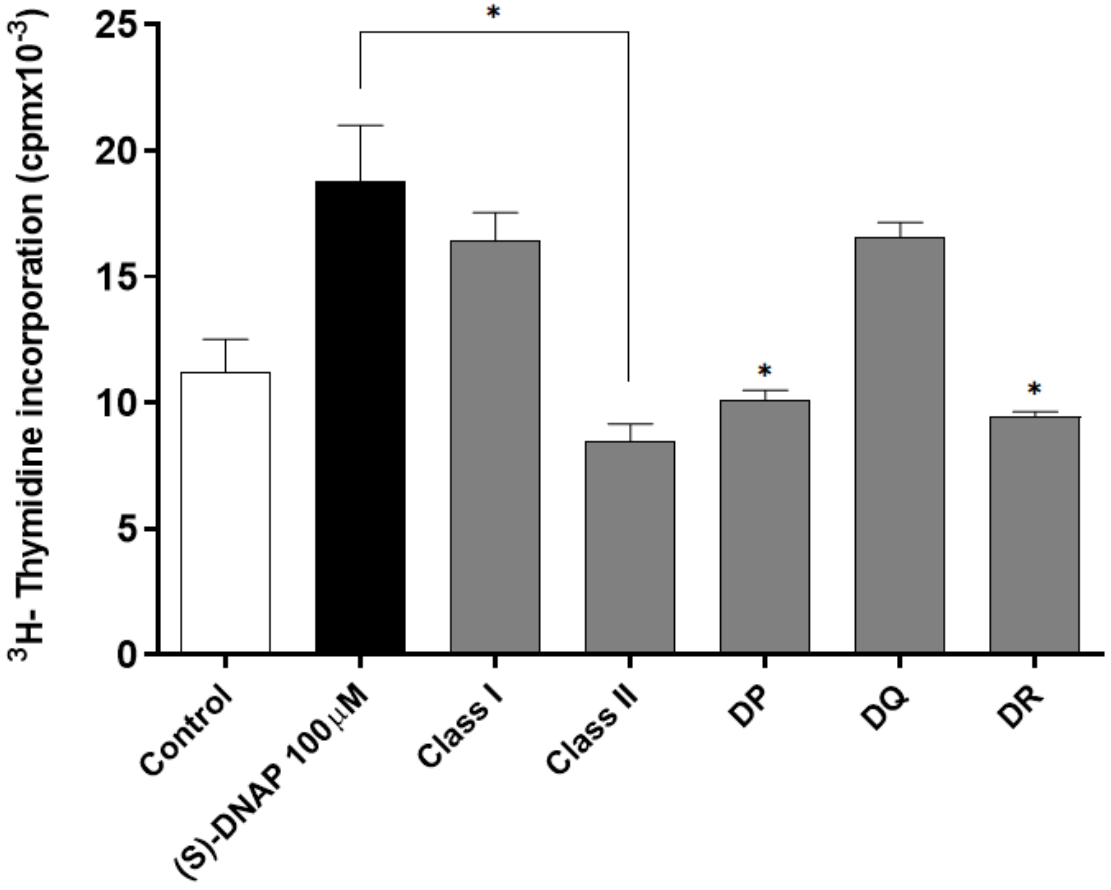


Figure 3.27. Major histocompatibility complex-restricted proliferation of (S)-DNAP T-cell clone 236. The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) previously treated for 30 mins with 5 µg/ml of anti-MHC Class I & II antibodies (including specific HLA-DP, -DQ, -DR antibodies). The cell culture was subsequently incubated with (S)-DNAP (100 µM; 100 µl) for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. EBV-transformed B-cells treated with corresponding antibody isotype controls (100 µM; 100 µl) were used to normalize the T-cell clone's positive response to (S)-DNAP. Following the 48-hour incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hours. All incubations were performed in duplicate. Data are presented as means (± standard deviation; n=2) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

3.4.9 (S)-O-desmethylnaproxen T-cell clone 236 becomes activated via a pharmacological – interaction mechanism

Two experiments were conducted to ascertain mechanisms of (S)-DNAP T-cell clone 236 activation. The first aimed at examining the proliferative potential of the clone when APCs are fixed with glutaraldehyde, thus neutralizing their antigen-processing mechanisms (APC fixation assay; section **3.3.15**). The second experiment looked at the effect of proliferation when APCs are pre-treated with the test compounds and then washed prior to addition into the T-cell culture (APC pulsing assay; section **3.3.15**).

Figures 3.28 presents the results from the first experiment. It is evident that the clone responds positively in the presence of 200 μM (S)-DNAP or (S)-NAP or (S)-NAG and is capable of proliferating significantly despite APC fixation. The maximal proliferative response was recorded upon exposure to (S)-DNAP yielding a SI of 1.81 (control: 9,076 ± 271 cpm; (S)-DNAP 16,428 ± 120 cpm) while (S)-NAP and (S)-NAG where also above the threshold of meaningful proliferation (> 1.6). ***Figure 3.29*** presents the results from the second experiment. When non-pulsed APCs where used and (S)-DNAP added into the T-cell culture, a proliferative response of SI = 1.82 (control: 3,903 ± 573 cpm; (S)-DNAP 7,110 ± 166 cpm) was determined, however, the (S)-DNAP clone 236 failed to respond positively in the presence of drug-pulsed (with (S)-DNAP or (S)-NAP or (S)-NAG) APCs that had been washed off prior to addition in the culture.

Taken together, these two experiments indicate that T-cell clone 236 does not dependent on APC processing mechanisms to become activated. Rather, the presence of free drug presented in the context of HLA-DP and HLA-DR molecules (see 3.4.8) to activate T-cells is indicative of a pharmacological – interaction concept.

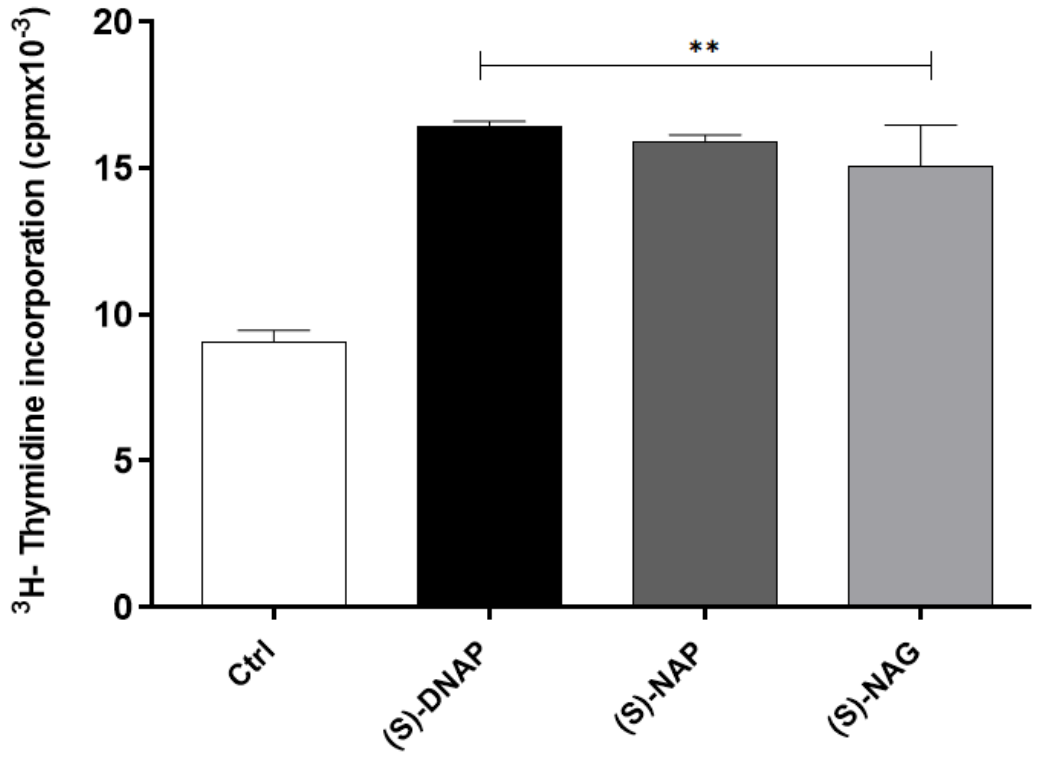


Figure 3.28. Drug-specific proliferation of (S)-DNAP T-cell clone 236 upon antigen-presenting cell fixation. Irradiated autologous EBV-transformed B-cells ($2x10^6$ cells) were initially suspended in 1 ml of HBSS and fixed via addition of 25 % glutaraldehyde (1 µl). Following 30 secs incubation at room temperature, reaction was stopped via addition of glycine (0.2 mM; 1 µl). After 45 secs cells were washed 3 times with culture medium and suspended. The responsive (S)-DNAP T-cell clone was cultured ($5x10^4$ cells/well; 50 µl) with the glutaraldehyde-fixed irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) and incubated with (S)-DNAP or (S)-NAP or (S)-NAG (200µM; 100µl) for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. Following the 48-hour incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hours. All incubations were performed in duplicate. Data are presented as means (± standard deviation; n=2) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

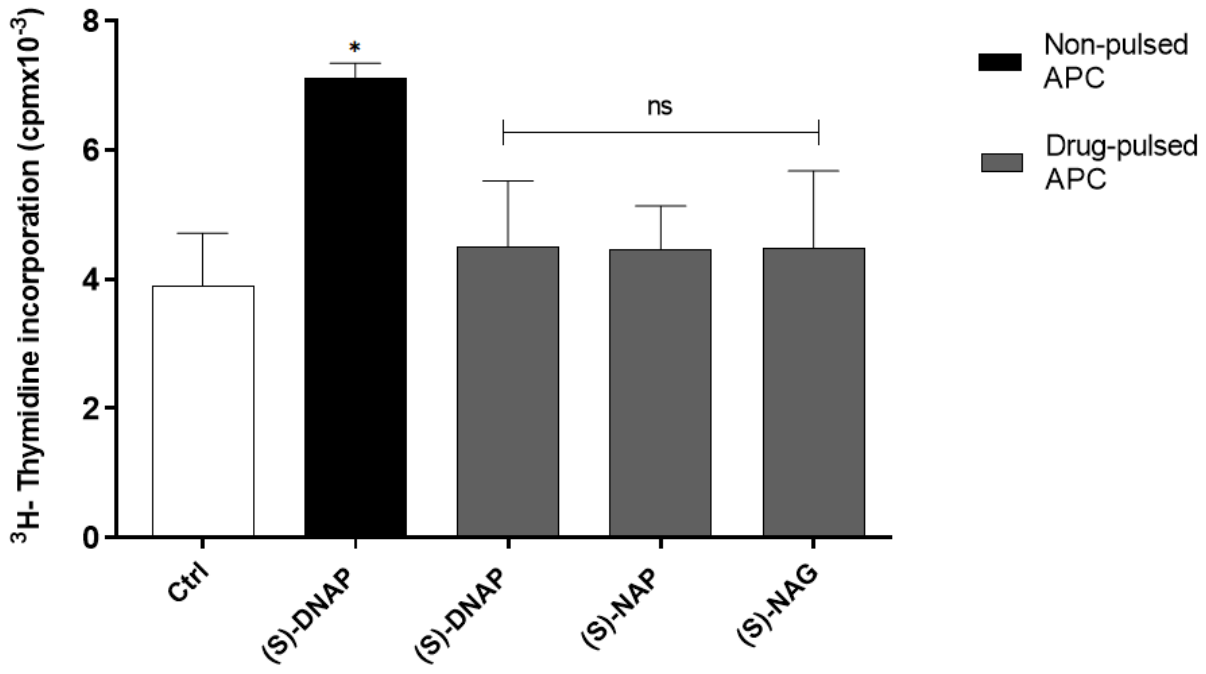


Figure 3.29. Drug-specific proliferation of (S)-DNAP T-cell clone 236 upon antigen-presenting cell pulsing. Irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well; 50 µl) were initially pulsed for 16 hours with the test compounds (200 µM; 100 µl of (S)-NAP, (S)-NAG, (S)-DNAP) or cultured without pulsing inside a humidified incubator of 5 % CO_2 at 37°C. Following this, cells were washed 3 times with HBSS and the responsive (S)-DNAP T-cell clone ($5x10^4$ cells/well; 50 µl) was incubated with pulsed or non-pulsed APCs ($1x10^4$ cells / well; 50 µl) for 48 hours inside a humidified incubator of 5 % CO_2 at 37°C. (S)-DNAP (200 µM; 100 µl) was only added to the non-pulsed cell culture serving as positive control. Following the 48-hour incubation, ^{3}H-thymidine (0.5 µCi / well) was added and incubation continued inside a humidified incubator of 5 % CO_2 at 37°C for 16 hours. All incubations were performed in duplicate. Data are presented as means (± standard deviation; n=2) of counts per minute. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

3.4.10 (S)-naproxen and its major metabolites cannot prime T-cells from healthy volunteers *in vitro*

The potential of (S)-NAP or its metabolites to prime T-cells obtained from healthy naproxen-naïve donors was investigated using the T-cell priming assay as described in **3.3.12**. ***Figure 3.30*** illustrates the findings of this experiment. The isolated naïve T-cells from 3 separate donors were successfully primed with SMX-NO (as positive control) and proliferated significantly in a clear dose-response manner upon re-exposure to the compound with a maximal response of SI = 2.4 observed at 50 μM. However, no proliferation was observed upon testing with (S)-NAP, (S)-DNAP or (S)-NAG.

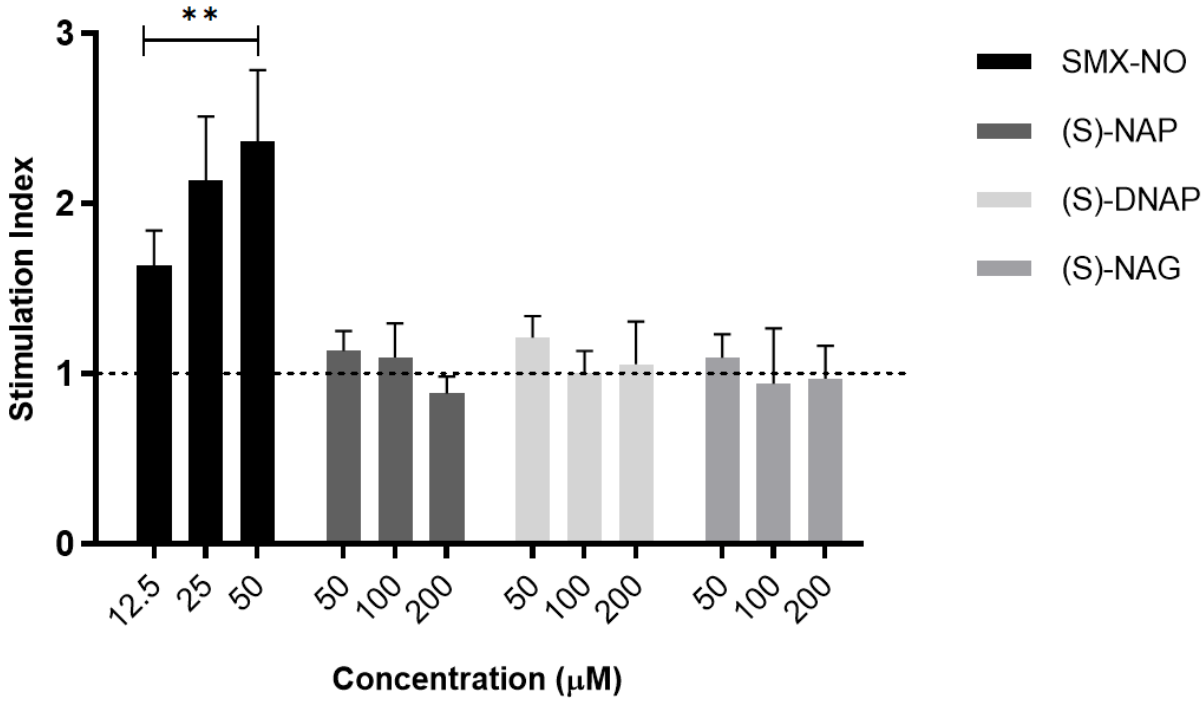


Figure 3.30. ***In vitro*** **T-cell priming assay using cells from healthy naïve donors (n=3).** The T-cell priming assay was carried out as described in 3.3.12 for SMX-NO, (S)-NAP, (S)-DNAP and (S)-NAG. Cells from 3 healthy naproxen-naïve donors were used and for each donor drug incubations were performed in triplicate. Data are presented as means (± standard deviation; n=3 donors) of SI. Statistical comparisons were made using multiple t-tests. A value of $p < 0.05$ was considered to be statistically significant.

3.5 DISCUSSION

In this chapter, we describe *ex vivo* investigations characterizing immune responses to naproxen and its metabolites (S)-DNAP and (S)-NAG as well as the protein adduct (S)-NAG – HSA, using blood samples from a patient with naproxen-associated liver injury (HCA RLH 041; see ***Table 3.1***) in comparison with appropriate controls. We asked whether immunological memory exists against naproxen or one of its metabolites. The work in this chapter describes – to our knowledge for the first time – data indicating that in a patient with naproxen-associated iDILI, immunological memory exists to the major oxidative metabolite, (S)-DNAP, but not to (S)-NAG. This finding conflicts with the prevailing perception that AG drug metabolites may be responsible for eliciting immune responses following binding to endogenous proteins. None of the patients on chronic naproxen treatment (defined here as more than 3 months of continuous use) or healthy treatment-naïve subjects exhibited a similar response.

We used the LTT and IFNγ-ELISpot assay to assess immune sensitization to (S)-NAP or one of its metabolites in PBMCs obtained from the patient and control subjects. The LTT is an assay designed to detect the presence of sensitized (memory) T-cells within a population of PBMCs. The assay has a reported specificity and sensitivity of 85 % and 74 %, respectively and has been used in both immediate and delayed hypersensitivity reaction cases, however, higher sensitivity is associated with the latter (Nyfeler & Pichler, 1997; Romano et al., 2004; Porebski, Gschwend-Zawodniak, & Pichler, 2011). The ELISpot assay is utilized for the detection and characterization of cytokine secretion in a cell population. More specifically, it exhibits increased sensitivity over the conventional enzyme-linked immunosorbent assay (ELISA) and enables the detection and quantification of low-frequency cytokine-secreting cells upon recognition of a specific antigen (Czerkinsky, Nilsson, Nygren, Ouchterlony, & Tarkowski, 1983). Even though the LTT is generally an acceptable and sufficient assay for

measuring and detecting memory T-cells, ELISpot IFNγ provides an independent readout, thus increasing the sensitivity of the overall experimental approach. Moreover, Monshi et al., (2013) confirmed sensitization to flucloxacillin in iDILI patients through ELISpot IFNγ analysis despite obtaining negative LTT results. Based on the inhibition of cell proliferation (see section **3.4.2**), immunostimulation assays were performed with (S)-NAP and (S)-NAG in a concentration range of 0 – 400 μM and of 0 – 600 μM for (S)-DNAP. The rationale behind this selection was to ensure comparable concentration ranges between the different test compounds whilst taking into consideration variability in donor sensitivity that may have not been captured with the number of donors (n = 2) used in the aforementioned experiments. (S)-NAG – HSA adduct was constructed as described in **3.3.6** and used in the assays at a total concentration of 1 mg/ml. This decision was based on available literature on the concentration of HSA used as a conjugate in similar investigations with other drugs (Palm & Medzhitov, 2009; Jenkinson et al., 2010; Whitaker et al., 2011; Meng et al., 2011; El-Ghaiesh et al., 2012; Usui et al., 2017). The limitation of this approach however was that without detailed mass-spectrometric characterisation, it is impossible to know the exact concentration of modified epitope used in the *in vitro* immune cell culture.

Figures 3.16 and ***3.17*** illustrate the LTT and ELISpot IFNγ responses of (S)-NAP and its major metabolites (S)-NAG and (S)-DNAP collectively for all human subjects tested. Overall, both assays independently were indicative of a memory T-cell response to the major oxidative metabolite (S)-DNAP, but not to (S)-NAG. Results from the two assays correlated in terms of response and were consistent between two independent repeats conducted with cells obtained from two separate blood acquisitions from iDILI patient HCA RLH 041 (see ***Table 3.1***). Data from the two LTT experiments using PBMCs from the iDILI patient revealed a mean SI of 1.9 ± 0.42 and 1.85 ± 0.21 for the 100 μM and 200 μM (S)-DNAP, respectively. A SI > 1.6 is indicative of a positive but weak to moderate drug-specific T-cell response (Pichler & Tilch,

2004; Naisbitt et al., 2014). The response was slightly more pronounced with the ELISpot IFNγ assay, again showing a response only for (S)-DNAP with a maximal SI of 3.3 ± 0.99 observed at 200 μM. The small differences of SI responses between LTT and ELISpot IFNγ are most likely explained by the nature of readout (cell proliferation vs cytokine secretion) and the difference of culture duration between the assays (5 days vs 48 hours) which can be reflective of a different cell state.

Most evidence supporting the utility of LTT and ELISpot IFNγ assays for the investigation of immune sensitization to a drug or metabolite derives from skin hypersensitivity research where strong responses have been observed against a range of drug classes including β-lactams, anticonvulsants and sulphonamides (Brander et al., 1995; Padovan et al., 1997; Schnyder et al., 2000; Naisbitt et al., 2003a; Castrejon et al., 2010; Zawodniak et al., 2010; Whitaker et al., 2011). The approach has been adopted for examining the immuno-allergic component of iDILI. Positive LTT responses have been described for a number of cases ranging from 12 % to 56 % for various drugs (however only parent compounds used) while exhibiting particularly high sensitivity (> 90 %) for isoniazid (Mizoguchi, Yamada, Monna, Yamamoto, & Morisawa, 1975; Warrington et al., 1978; Yoshimura, Kurita, Yamazaki, Nakano, & Nagai, 1994; Maria & Victorino, 1997; Murata, Shimizu, Okada, Higuchi, & Watanabe, 2003; Klein, 2009). Even though the LTT assay has also been used with modifications (Maria & Victorino, 1997; Whritenour et al., 2017), it is rather difficult to overcome certain challenges and therefore assay limitations persist. In the aforementioned iDILI studies, weak stimulation indexes were not an uncommon phenomenon and this may be attributed to one or a combination of the following factors: 1) delay between blood acquisition and PBMC isolation (note: not the case for the samples used in this Thesis); 2) timing of PBMC collection relative to the onset of the liver injury which is particularly important as the frequency of drug specific T-cells circulating in the blood may decrease over time (note: the iDILI patient investigated in this Thesis had

suffered the adverse liver reaction several years before blood samples were acquired); 3) use of fresh vs frozen PBMCs (note: these LTT and ELISpot IFNγ experiments were performed with fresh PBMC only); 4) the iDILI reaction may be driven by an adaptive immune mechanism only in part; 5) antigen-specific T-cells may reside in tissues during the acute phase of the reaction (such as liver) and may not be easily detectable in the circulation (PBMC-derived T-cells); and 6) the sensitization *in vivo* may have occurred against an alternative antigen such as a metabolite or drug-protein conjugate (note: in our work we have examined both parent drug and major metabolites as well as a model drug-protein adduct). Since both assays independently showed memory T-cell responses to the major oxidative metabolite (S)-DNAP, but no responses to (S)-NAP, (S)-NAG and (S)-NAG – HSA, even the weak to moderate proliferative response observed with (S)-DNAP warranted subsequent phenotypic and functional investigations of (S)-DNAP – specific T-cells.

It is stipulated that modification of protein through irreversible reactive metabolite binding renders the former antigenic with a potential to mount an immune response. This is exemplified by various studies with β-lactams showing that irreversible binding to HSA leads to hapten-specific immune cell proliferation (Meng et al., 2011; Whitaker et al., 2011; Jenkins et al., 2013; Meng et al., 2016; Meng et al., 2017). In ***Chapter 2***, a procedure for generating and quantifying (S)-NAG – HSA adducts is outlined. Here, a slightly modified approach as described in sections **3.3.6** and **3.4.1** was undertaken in order to create a (S)-NAG – HSA adduct for incorporation in the immune assays, producing similar levels of irreversibly bound (S)-NAG to HSA at a 50:1 molar ratio used and allowing significantly higher protein recovery. Previously, it was determined that SPE does not represent an efficient method for maximal HSA recovery (see section **2.4.4**). In addition, the method requires steps of washing and elution using organic solvents which are known to cause protein denaturation. Since a non-denatured protein will better resemble the *in vivo* situation in terms of hapten-carrier introduction to

APCs, a method to ensure this aspect was sought via the use of SEC. SEC, otherwise known as gel filtration, is the simplest and mildest of all chromatography techniques and allows separation of molecules on the basis of their size. The flow through of smaller entities will be hindered by the pores of the column's medium whereas large molecules will elute faster. However, this raises a concern of artificially influencing experiment responses based on non-covalently bound AG and aglycone by HSA in the Sudlow sites (Iwakawa, Spahn, Benet, & Lin, 1990; Williams & Dickinson, 1994; Ghuman et al., 2005). To this end, (S)-NAP and (S)-NAG served as indirect controls in the assay aside from the HSA alone control. Moreover, the concentrations of non-covalently bound drug are expected to be very low as elucidated in ***Chapter 2***. Following incorporation in the LTT and ELISpot IFNγ assays and subsequent data analysis, no positive reaction was observed for the (S)-NAG – HSA adduct. (S)-NAG alone was also incapable of mounting a positive response in either assay. For those cases where a drug – HSA adduct has been determined as immunogenic (β-lactams), a positive response has been seen for the soluble drug component as well (Jenkinson et al., 2010; Whitaker et al., 2011; Meng et al., 2011; El-Ghaiesh et al., 2012; Meng et al., 2017). The consistent lack of responses between (S)-NAG and (S)-NAG – HSA is in line with this correlation.

An important element when considering introduction of compounds / antigens into *in vitro* cell immune assays is the concentration range. The concentration used for (S)-NAG – HSA (1 mg/ml) was in accordance to what has been applied previously for several other drugs as already explained. The *in vivo* C_{max} for (S)-NAP, (S)-DNAP and (S)-NAG have been measured at 199 μM, 2.9 μM and 1 μM, respectively, following a single oral dose of 250 mg naproxen in a healthy male volunteer (Spahn-Langguth & Benet, 1992). The concentration range used in these *ex vivo* experiments was based on cell toxicity and inhibition of cell proliferation, thus deviating from *in vivo* expected values. *In vivo* human drug exposure to specific tissues / cells follows a complex path which depends on a balance between absorption, metabolism,

distribution and excretion that cannot be entirely mimicked in the *ex vivo* and *in vitro* immune assays described herein. In particular, this holds true when considering the investigation of major metabolites. Depending on drug metabolism and drug transporter enzymes, the metabolites' concentrations may vary significantly among microenvironments. Thus, when considering the target cell population (PBMCs), it would become increasingly difficult to predict the exact *in vivo* human molar ratio of drug / metabolite to lymphocytes, and even if that was achievable, any direct *in vitro* translation would be artificial. Perhaps more important than this, is the ability of AG metabolites to penetrate the cell membrane and enter the cells during *in vitro* incubation. This is of particular relevance for reactive metabolites such as (S)-NAG, as it may enable the adduction with proteins of cellular origin aside from soluble proteins. One study published by Gensburger, Picard, & Marquet, (2009) using LC-MS/MS to measure intracellular concentrations of mycophenolic acid and its AG metabolite in Jurkat cells 6 hours after *in vitro* introduction in the cell culture, indicated that the mycophenolic acid-AG was capable of crossing the cell membrane, although the intracellular concentration measured was very low. Decreased permeability might be an explanation, but intracellular instability of the metabolite and degradation into its parent form could also play a role. It is not known if the lipophilicity and instability of (S)-NAG would allow for the presence of significant intracellular AG concentration and whether this would reach the threshold for irreversible modification of intracellular proteins.

To further analyse the positive immune response identified with (S)-DNAP, T-cell cloning by limiting dilution was performed. PBMCs are a very heterogeneous population of cells consisting of several different subsets of adaptive and innate immune cells. Moreover, each T-lymphocyte derives from a certain precursor cell which has matured in a way leading to a unique phenotype and function. T-cell cloning by limiting dilution is a useful method for generating and enriching a population of T-lymphocytes (T-cell clones) deriving from an

antigen-specific progenitor, thus facilitating mechanistic investigations of T-cell responses (Beeler & Pichler, 2006). A limitation of this procedure however is the susceptibility of the T-cell clone to infection and "exhaustion" due to long-term culture. Even though (S)-DNAP was the only compound for which memory was detected during the primary experiments (LTT and ELISPOT IFNγ assays), the experimental procedure as described in **3.3.14** was undertaken for (S)-NAP, (S)-DNAP, (S)-NAG and (S)-NAG – HSA to ensure consistency and test for false negatives. In line with the negative results obtained with the LTT and ELISPOT IFNγ assays, no drug-responsive T-cell clones were detected for (S)-NAP, (S)-NAG and (S)-NAG – HSA. However, (S)-DNAP stimulation yielded one responsive T-cell clone (clone 236) with a pronounced proliferation (SI = 23.1), which was subsequently validated as true positive (***Figures 3.19*** and ***3.20***) exhibiting a dose-response relationship with a maximal proliferative response seen at 200 μM (SI = 2.5). It is worth noting that during the validation experiment, the clone demonstrated much lower stimulation than the originally identified index during screening. This most likely relates to cell culture extension and cellular exhaustion which over time weakens the clone's immuno-proliferative potential.

Further work aimed to characterize the profile of clone 236 as well as mechanisms of T-cell activation. *First*, the clone was found to exhibit a small degree of intra-structural cross-reactivity but no cross-reactivity with other non-related carboxylic acid – containing drugs (zomepirac) or drugs / metabolites from other chemical classes (SMX-NO). At the molecular level, pharmacological cross-reactivity can be observed for the class of NSAID hypersensitivity when mediated by COX inhibition, however, may be drug-specific when it involves activation of antibodies or T-cells (Pham et al., 2016). The level of intra-structural cross-reactivity for (S)-NAP and (S)-NAG was significant only for the higher concentrations (200 μM), indicating that recognition of the structure at lower concentrations was rather weak. When considering the structural differences between (S)-NAP, (S)-NAG and (S)-DNAP, it

becomes apparent that they reflect small functional group changes. Such cross-reactivity may enable the immune system to respond appropriately to all relevant antigens, thus strengthening the physiological defences (Mason, 1998).

Secondly, the T-cell clone's phenotypic characterization revealed a cytokine profile characterized by high IL-17A, IL-22 and low IFNγ secretion. However, analysis by flow cytometry showed the presence of two distinct populations consisting of $CD4^+CD8^+$ and $CD8^+$ T-cells (***Figure 3.21***). This finding is rather peculiar, as normally T-cell cloning enables the growth of a single T-cell yielding a clone of specific origins. Nonetheless, it's possible that during limiting dilution more than one T-cells were placed in the same well and cultured. With the recent emergence of the new Tc17 cell phenotype (IL-17A producing $CD8^+$ T-cells) (Kondo, Takata, Matsuki, & Takiguchi, 2009; Oo et al., 2012; Srenathan, Steel, & Taams, 2016) alongside the known Th17 cells, it is difficult to ascertain cell specificity based on the cytokine secretion profile alone. However, the MHC class restriction experiments (section **3.4.8**) provide the results most indicative of a $CD4^+CD8^+$ specificity as evident by an MHC class II restriction. APCs were shown to be necessary for the activation of the clone and when they were blocked with HLA-DP or -DR antibodies, the proliferation was abrogated. The small effect seen during MHC class I blockade may be a result of the double CD-positive profile ($CD8^+$ component) of the cell phenotype. $CD4^+CD8^+$ responsive T-cell clones, albeit uncommon, have been observed in hypersensitivity reactions to drugs, such as carbamazepine (Wu, Farrell, Pirmohamed, Park, & Naisbitt, 2007), as well as *in vitro* primary immune responses to SMX using blood from healthy volunteers (Engler et al., 2004). In addition, IFNγ secretion of T-cell clone 236 was observed to decrease from the original ELISpot assays after several rounds of re-stimulation to a point where late-stage characterization experiments were carried out only with ^{3}H-thymidine incorporation. Unfortunately, by the time phenotypic characterization (flow cytometry) was conducted most of the available T-cells had already been

used up. An effort to magnetically separate the two populations using a sample aliquot that was kept frozen for future potential analyses failed as when these cells were thawed out and cultured, they became infected and had to be discarded.

Thirdly, the functionality of the clone was investigated by migration experiments whereby migration was shown towards CCL17 (ligand for CCR4; the tissue homing receptor showing most increased expression on the clone surface). Enhanced cell surface expression of CXCR3, CXCR10, CCR9 and CCR5 has been shown in other iDILI cases with flucloxacillin and amoxicillin (Monshi et al., 2013; Kim et al., 2015). These tissue homing receptors alongside the migration capacity of the clone underpin the relevance of the observed antigen-specificity with mounting a targeted tissue immune reaction.

Finally, the results of the experiments described in section **3.4.9** point towards a pharmacological – interaction model of MHC-dependent T-cell activation to the (S)-DNAP metabolite (Pichler, 2002). It is perhaps this mechanism of the T-cell clone activation that best explains (a) the phenomenon of weak cross-reactivity, (b) the lack of APC intracellular antigen-processing requirement, and (c) the reduced importance of MHC restriction at the higher (S)-DNAP concentration tested (200 μM).

Despite multiple efforts to generate additional (S)-DNAP responsive T-cell clones from frozen cells or fresh blood acquisition (round 2: 380 clones, round 3: 229 clones), no further positive clones were identified for this iDILI patient. The single (S)-DNAP responsive T-cell clone detected raises a significant point for discussion. Although published results for other hypersensitivity positive drugs have shown the potential for multiple clones to be detected from a single patient, it is clear that patient variability is high and it is not uncommon for limited identification of drug-responsive T-cell clones to occur. Consequently, the identification of a sole (S)-DNAP responsive T-cell clone could potentially be attributed to several factors. A loss

of immunological memory over time may have occurred, considering the extended period between blood acquisition and occurrence of the adverse liver reaction in this patient. This finding in connection with weak positive LTT positive results has been discussed in the literature (Kano, Hirahara, Mitsuyama, Takahashi, & Shiohara, 2007). However, the temporal disconnection does not always affect reactivity and / or frequency of drug responsive cells (Beeler, Engler, Gerber, & Pichler, 2006). Moreover, previous investigations are suggesting that it might generally be harder to identify metabolite-responsive T-cell clones, when metabolites are implicated in adverse immune responses (Naisbitt et al., 2003a; Nassif et al., 2004; Wu et al., 2006).

Various currently known mechanisms by which drugs can cause idiosyncratic liver injury have been detailed in ***Chapter 1***. In this Thesis, we have asked whether immunological memory exists against naproxen or one of its metabolites in the context of a case of naproxen-associated iDILI. Our work describes - to our knowledge for the first time – that immunological memory exists to the major oxidative metabolite (S)-DNAP, but not to (S)-NAG or the parent compound. Although the results from this work are based on one iDILI patient and one metabolite-responsive T-cell clone, it's still important that they are discussed against existing knowledge. In fact, this novel finding should be further substantiated by investigating additional samples from naproxen-treated patient with iDILI and might even be extended to include cases treated with other carboxylic acid – containing drugs. Here, we have started to explore immune responses based on peripheral blood, however, future studies should try to correlate antigen-specific PBMC responses with those found in the liver, as much as possible. As discussed before (***Chapter 1***), the liver is a key immunological organ positioned at the crossroad between gastrointestinal and systemic circulation, favouring induction and maintenance of immunological tolerance over immunity, by avoiding immune responses to harmless dietary antigens. Reversal of liver immune tolerance enables efficient beneficial

immune responses to pathogens for example, but it may also contribute to adverse hepatic immune reactions. The unique liver anatomy allows immunosurveillance of parenchymal cells, such as hepatocytes, by lymphocytes. Inadvertent activation of T-cells could lead to direct cell-mediated hepatotoxicity or create a localized inflammatory environment via pro-inflammatory cytokine secretion and inflammatory cell recruitment. The positive LTT and ELISpot IFNγ assays suggest that (S)-DNAP-specific memory T-cells circulate in the patient's blood. Moreover, one (S)-DNAP-specific clone provided evidence of a $CD4^+CD8^+$, MHC class II-restricted, p-i – activated Th17 clone. Wu et al. reported that carbamazepine-specific CD4+, $CD8^+$, and $CD4^+CD8^+$ T-cells display different effector functions and homing characteristics which persist in carbamazepine-hypersensitive patients' blood for many years after resolution of clinical symptoms (Wu et al., 2007). Peripheral $CD4^+CD8^+$ double positive T cells have been reported to play a role in several autoimmune diseases (Quandt, Rothe, Scholz, Baerwald, & Wagner, 2014), viral infections and cancer (Desfransois et al., 2010), but their role in drug hypersensitivity remains to further explored and substantiated. Little is known about the role of Th17 cells in hepatotoxicity, but it is speculated that they may be involved in driving an inflammatory / autoimmune component of the tissue injury (Wang, Zhang, & Jiang, 2015). Notably, elevated levels of plasma IL-17 were detected in 60 % of patients with DILI (Li et al., 2010), Th17 cells were identified in the serum of patients with mild isoniazid hepatotoxicity (Metushi et al., 2014) and most of IL-17 was found to be produced by Th17 cells in a mouse model of APAP-liver injury (Zhu & Uetrecht, 2013). Perhaps more importantly and in the context of NSAIDs, COX inhibition is being thought to shift metabolism of arachidonic acid to the lipoxygenase pathway. Overproduction of leukotrienes has been associated with Th17 differentiation and activation (Lee, Su, & Lee, 2015). However, the (S)-DNAP Th17 clone did not proliferate when exposed to zomepirac (another COX inhibitor) in the cross-reactivity assay indicating that our *ex vivo* finding is unlikely to be pharmacologically induced and that

it truly reflects immunological memory to (S)-DNAP. Moreover, no gene expression changes were revealed in the leukotriene pathway when PBMCs were cultured with (S)-DNAP for 24 hours as will be described in ***Chapter 4***.

The rarity and peculiarity of iDILI may be the result of a combination of different parameters such as the structure of the parent drug, formation of different metabolites, differences in age, sex and ethnicity, drug-metabolizing enzymes and immune polymorphisms, underlying disease, genetic susceptibility, concomitant medication and environmental co-factors (Kaplowitz, 2005; Ulrich, 2007; Chalasani & Bjornsson, 2010; Tujios & Fontana, 2011; Chen et al., 2015). Investigating and understanding the pathogenesis of iDILI in human, may enable the translation of these findings into well-characterized preclinical assays which could address the significant gap in hazard identification and risk assessment (Weaver et al., 2020). Since we were able to show immunological memory responses in the naproxen iDILI patient, we asked whether it would be possible to sensitize T-cells obtained from healthy, naproxen-untreated (naproxen-naïve) donors to naproxen and its metabolites *in vitro* (section **3.4.10**). The human T-cell priming assay is a mechanistically useful tool to study T-cell sensitization to drugs *in vitro* (Faulkner et al., 2012; Gibson et al., 2014; Sullivan et al., 2018; Ahmed et al., 2019). Using PBMCs from healthy naïve donors, the T-cell priming assay has generated positive responses in multiple drugs associated with drug hypersensitivity reactions such as carbamazepine, amoxicillin, piperacillin, flucloxacillin, SMX-NO (metabolite of SMX) and oxypurinol (metabolite of allopurinol) (Faulkner et al., 2012; Monshi et al., 2013; Faulkner et al., 2016). Even though the assay performed well in our hands with SMX-NO as a positive control, (S)-NAP, (S)-NAG and (S)-DNAP all failed to generate a response in a T-cell priming assay in three independent assay biological repeats (section **3.4.10**). It is not the first time that a seemingly positive drug based on *ex vivo* LTT and T-cell cloning investigations yields negative results when tested *in vitro* using the T-cell priming assay. Drug hypersensitivity

positive drugs like carbamazepine, flucloxacillin and oxypurinol (metabolite) have all exhibited positive responses in T-cell priming assays (Monshi et al., 2013; Faulkner et al., 2016). Interestingly, a common aspect between the aforementioned drugs is that they are clinically associated with HLA-class I restriction. Conversely, no responses were observed when drugs that have been associated with HLA-class II restriction (ximelagatran, lapatinib, lumiracoxib) were tested in T-cell priming assays (Faulkner et al., 2016). In section **3.4.8**, it was found that MHC class II restriction abrogated the activation of the single (S)-DNAP responsive clone. No GWAS data are available for naproxen iDILI, but if it is associated with HLA-class II polymorphisms it could mean that application of a T-cell priming assay could have its limitations. Nonetheless, HLA-restriction is not a prerequisite for *in vitro* T-cell priming as has been shown with amoxicillin, piperacillin and SMX-NO (metabolite of SMX) for which no clinical association with specific HLA alleles has been reported. These chemical entities were capable of inducing T-cell proliferation using cells from healthy naïve donors (Faulkner et al., 2012; Faulkner et al., 2016). Another factor that may be contributing to the negative result of the assay is the mechanism of T-cell activation. Evidence for p-i – activating drugs capable of priming naïve T-cells is scarce and limited to drugs that have the potential to activate T-cells via a hapten mechanism as well (Faulkner et al., 2012; Gibson et al., 2017; Alzahrani et al., 2017; Alhilali et al., 2019). In addition, the p-i concept proposes that drug-responsive T-cells derive predominantly from the memory pool (Pichler, 2005) and although switching from a naïve to a memory phenotype has been observed during *in vitro* T-cell priming (Faulkner et al., 2012), it may be argued that this could pose a limitation in the assay's predictive power. Finally, tolerance mechanisms during *in vitro* T-cell priming may be in place for distinct TCR subtypes in addition to dysregulation of co-inhibitory signalling pathways and dysfunctional regulatory T-cells (Tregs) (Gibson et al., 2017). More recently, the importance of immune tolerance in iDILI pathogenesis has been highlighted through animal

experimentation concerning the tolerogenic nature of the liver including the use of immune checkpoint (PD-1 and CTLA-4) inhibition. Dysregulated or imbalanced immunological tolerance may perhaps explain predisposition to hypersensitivity and why certain individuals with initially mild reaction characteristics progress to severe iDILI (Chakraborty et al., 2015; Metushi et al., 2015; Uetrecht & Kaplowitz, 2015; Mak & Uetrecht, 2015b). This was approached in the context of T-cell priming assays by Gibson et al., (2014), where it was shown that PD-L1/PD-1 binding regulates negatively the priming of drug-specific T-cells. Consequently, a multi-factorial nature of events enabling naïve T-cell priming may limit the transferability of the assay across multiple compounds.

The aim of this chapter was to examine immune responses to (S)-NAP and its metabolites (S)-DNAP, (S)-NAG and its protein adduct (S)-NAG – HSA, using blood samples from a patient with naproxen-associated liver injury in comparison with appropriate controls. Earlier in ***Chapter 2***, it was shown that (S)-NAG is unstable in aqueous and protein solutions and capable of binding irreversibly onto HSA. Using established assays to test for immune memory against naproxen, its major metabolites as well as a model (S)-NAG – HSA adduct, positive response was identified only for the chemically inert metabolite (S)-DNAP. This response was further validated by T-cell cloning experiments, suggesting a p-i model of T-cell activation in the sole $CD4^+CD8^+$ MHC-class II-restricted Th17 identified clone. Efforts to recapitulate the events based on an *in vitro* T-cell priming assay via the use of healthy naïve PBMCs were unsuccessful. Overall, this evidence points towards an immunological basis for the observed adverse reaction in the naproxen iDILI patient. More importantly, to our knowledge, this is the first time that an investigation of this type is being described for an AG-forming drug and yielding negative responses for its AG metabolite whilst exhibiting a positive signal for its oxidative metabolite via a p-i model of activation. Certainly, this evidence derives from one single iDILI patient and one single T-cell clone and although that represents a significant

limitation for the extrapolation of results towards the mechanism of naproxen – induced idiosyncratic hepatotoxicity, it does pave the way for similar investigations with additional samples from more naproxen iDILI cases. Perhaps more importantly, the results of this work emphasize the lack of mechanistic evidence behind the hypothesized immunogenic potential of AGs by highlighting that other chemical entities and / or mechanisms may be responsible for driving the iatrogenic basis of iDILI associated with AG-forming drugs. Furthermore, the finding of a Th17 signature is of importance in the field of idiosyncratic hepatotoxicity and warrants deeper interrogation. Ideally, future research should focus on repeating the same workflow in more naproxen – induced idiosyncratic hepatotoxicity cases and identifying additional drug responsive T-cell clones to characterize. Equally, the inability of the T-cell priming assay to model the *in vivo* finding suggests that alternative translational assays should be explored for their capacity to identify immunotoxicological hazards as basis for risk assessments.

Even though there are no published results of the T-cell priming assay using AG drug metabolites, a group of researchers have used *in vitro* human PBMCs to assess immuno-inflammatory responses subject to AG exposure (Miyashita et al., 2014; Iwamura et al., 2015). Following a 24-hour incubation of PBMCs with several AGs, a correlation was observed between those AGs whose parent drugs have been withdrawn from the market due to IDRs and high mRNA expression levels of selected pro-inflammatory cytokines (Iwamura et al., 2015). Therefore, characterizing gene expression of human PBMCs deriving from patients and control subjects could potentially not only strengthen existing findings but also uncover novel mechanistic details enabling a better understanding of occurring events. Thus, the next and final experimental chapter (***Chapter 4***) will concentrate on interrogating the transcriptome of PBMCs derived from the blood of the aforementioned naproxen iDILI case as well as selected control subjects.

CHAPTER 4

GENE EXPRESSION PROFILE ANALYSIS OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS IN RESPONSE TO (S)-NAPROXEN AND ITS MAJOR METABOLITES

4.1 INTRODUCTION

Carboxylic acid – containing drugs have been associated with rare, but severe idiosyncratic drug reactions during their post-marketing phase (Fung et al., 2001). On most occasions, little is known about the mechanism of toxicity, but the clinical manifestations of many of these reactions, including iDILI, resemble that of delayed hypersensitivity. The carboxylate moiety present in these drugs subjects the parent molecule to various metabolic pathways including UGT-mediated glucuronidation leading to AG formation (Skonberg et al., 2008). AGs possess an inherent chemical instability *in vitro* (Stachulski et al., 2006) and their protein reactivity has been proven *in vivo* (Hammond et al., 2014). This has led to assumptions over their toxicological implications based on the capacity of certain drugs that act as haptens, such as β-lactams, to inadvertently stimulate the immune system (Padovan et al., 1997; Meng et al., 2017). However, to date, no data has provided a confident and definitive link between AG protein reactivity and activation of the adaptive immune system. In the previous Chapter, (S)-DNAP, the oxidative metabolite of (S)-NAP, but not (S)-NAG or (S)-NAG – HSA, was shown to induce lymphocyte proliferation in PBMCs isolated from a patient having suffered idiosyncratic hepatotoxicity following naproxen treatment. This showed for the first time – to our knowledge – in *ex vivo* human investigations that immune memory can be seen against the oxidative metabolite of a carboxylate drug but not the AG and that alternative mechanisms of immune activation might be in place (on this occasion, the p-i concept). To further interrogate these findings, but also to explore potential AG-mediated inflammatory effects using a different experimental setting, the work described in this final experimental Chapter aimed to examine gene expression changes in human PBMCs from the same patients that were recruited for the T-cell investigations presented in ***Chapter 3***.

A few research groups have previously explored mRNA expression levels in healthy human PBMCs in order to assess immuno-inflammatory responses following AG treatment *in vitro*. Following a 24-hour incubation of human PBMCs with several different AGs, a correlation was observed between those AGs whose parent drugs have been withdrawn from the market due to IDRs and high mRNA expression levels of selected pro-inflammatory cytokines such as IL-8, IL-1α, IL-6, MT2A, NAMPT (Iwamura et al., 2015). However, the use of a standardized concentration (100 μM) across all AGs in these experiments raises questions in terms of varied cell viability as the authors do not report viability data. PBMCs are a composite of different cell populations and total mRNA gene expression changes may be skewed by drug effects on specific cells. Diclofenac-AG, probenecid-AG and tolmetin-AG have been shown to activate the inflammatory response *in vitro* in healthy human PBMCs via up-regulation of IL-8 and MCP-1 and the metabolite concentration used (100 μM) was also linked with reduced viability for $CD14^+$ cells (Miyashita et al., 2014). Despite the limitations, this evidence does introduce a potential for new risk assessment approaches supplementing the existing degradation t½ assays in phosphate buffer 0.1 M (pH 7.4) (Iwamura et al., 2017). A link between clinically relevant inflammation and *in vitro* induction of pro-inflammatory cytokines by AG in human PBMCs was described by Wieland et al., who identified IL-6 and TNFα up-regulation after treating cells with mycophenolic acid-AG but not with the parent drug or its phenolic glucuronide (Wieland et al., 2000).

Gene expression investigations on the inflammatory potential of AGs in human PBMCs stem mainly from the danger hypothesis and inadvertent activation of the innate immune system, which stipulate that early inflammatory events can initiate and / or amplify a wider immunotoxicological reaction (Matzinger, 1994; Matzinger, 2002; Li & Uetrecht, 2010). Release of DAMPs can facilitate activation of the adaptive immune system by acting as a synergistic signal (section **1.6.3**). This concept has been approached with animal

experimentation on hepatic gene expression changes using drugs that have been associated with iDILI (Pacitto, Uetrecht, Boutros, & Popovic, 2007), including carboxylates such as diclofenac (Yano et al., 2012). Even though over-expression of pro-inflammatory genes such *IL1B*, *IL-6*, *CXCL8* (encoding IL-8), *IL-17A*, *CCL2*, *CCL3*, *IFNγ* and *TNFα* has been observed, it's difficult to ascertain whether these changes are AG-mediated or driven by oxidative metabolites / parent drug. It should be borne in mind that glucuronidation is aimed as a detoxification pathway *in vivo*, and carboxylic acid – containing drugs such as naproxen, diclofenac, ketoprofen and ibuprofen have shown increased cytotoxic potential in human hepatocytes when their AG formation was inhibited by borneol (Koga et al., 2011). Oda et al. specifically examined diclofenac-AG's relationship with *in vivo* animal hepatic immuno-inflammatory gene expression changes (Oda et al., 2017). mRNA expression levels of *CXCL1*, *CXCL2* and *CD11b* were decreased in the livers of mice that had been pre-treated with borneol prior to diclofenac administration as opposed to mice treated with diclofenac alone (Oda et al., 2017). Although the diclofenac treated animals exhibited increased plasma ALT levels alongside the innate immunity and neutrophil expression markers, this animal model represents an acute liver injury state rather than idiosyncratic hepatotoxicity. Taken together, this evidence shows that any *in vivo* link between inflammatory events and AG formation is speculative. Moreover, in ***Chapter 3***, no evidence of immune activation was found against the AG of naproxen. However, immune responses were directed to the oxidative metabolite, (S)-DNAP, using blood sample for a patient previously experiencing an idiosyncratic liver injury reaction secondary to naproxen treatment.

Drug specific-signalling pathways between hepatocytes and immune cells have been identified (Ogese et al., 2017; Ogese et al., 2019) and it is thought that cytotoxic synergy between cytokines and drugs might be linked with inflammation-associated idiosyncratic hepatotoxicity (Cosgrove et al., 2009; Maiuri et al., 2015). Hence, various assays have been developed to

assess the immune / inflammatory – related factors associated with iDILI using human hepatic and other cell lines (Yano, Oda, Fukami, Nakajima, & Yokoi, 2014; Oda, Matsuo, Nakajima, & Yokoi, 2016), identifying genes such as *S100A8* and *IL1B* as high expression genes for drugs associated with increased iDILI risk. Furthermore, analysis of intracytoplasmic cytokines in PBMCs from patients with DILI has leveraged the possibility for use in exploring immune mechanisms underlying the toxic reaction (Murata et al., 2003), while a distinct blood transcript signature involving Th2-mediated and innate immune responses has been demonstrated in clinical subjects with elevated serum ALT following acetaminophen treatment (Fannin et al., 2016). Ultimately, gene expression profiles in hepatic cells reflect more closely the differential changes occurring in the liver, but examining PBMCs could represent a readily accessible and less invasive approach for *ex vivo* human genome investigations. Moreover, the clinical phenotype of most iDILI cases points towards a delayed Type IV drug hypersensitivity reaction profile and are therefore thought to be T-cell mediated, further justifying the use of PBMCs for tailored investigations.

The work presented in this Chapter intends to investigate the effects of (S)-NAP and its major metabolites, (S)-NAG and (S)-DNAP on gene expression profiles in human PBMCs. Unlike similar work described in the literature (Wieland et al., 2000; Miyashita et al., 2014; Iwamura et al., 2015) that was conducted solely in healthy human PBMCs, here the PBMCs under examination derive from two healthy drug-naive volunteers as well as one long-term naproxen treated patient and one patient having experienced an idiosyncratic liver injury reaction due to naproxen treatment. Findings will supplement the lymphocyte investigations described in ***Chapter 3*** as well as further explore immune / inflammatory – related factors potentially implicated in naproxen - induced idiosyncratic hepatotoxicity.

4.2 QUESTION AND AIMS

The key question is:

"Do (S)-NAP or its major metabolites (S)-NAG and (S)-DNAP induce gene expression changes in PBMCs isolated from a patient previously experiencing an idiosyncratic liver injury following exposure to naproxen and appropriate control subjects?"

To address this question, the aims were to:

- Use human PBMCs isolated from well characterized patients previously investigated for immunological memory against (S)-NAP and its major metabolites (see ***Chapter 3***).
- Extract high quality RNA following exposure of PBMCs to (S)-NAP, (S)-NAG and (S)-DNAP at 24 hours.
- Use NGS to interrogate the human PBMC genome in an unsupervised manner for differential gene expression.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Direct-zol RNA MiniPrep kit (Cat# R2053) was purchased from Zymo Research (Freiburg, Germany). TruSeq Stranded mRNA LT Sample Prep Kit (Cat# RS-122-2101) and NextSeq 500 High Output Kit 75-cycles (Cat# FC-404-1005) were obtained from Illumina (San Diego, CA, USA). RNA 6000 Pico Chip (Cat# 5067-1513) was purchased from Agilent (Santa Clara,

CA, USA). Standard Sensitivity NGS Fragment Analysis Kit (Cat# DNF-473) was bought from Advanced Analytical Technologies Inc. (Ankeny, IA, USA). (S)-NAP was purchased from Sigma-Aldrich (Dorset, UK). (S)-NAG and (S)-DNAP were purchased from Toronto Research Chemicals (North York, Canada). All other solvents, reagents and supplies were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise mentioned.

4.3.2 Human peripheral blood mononuclear cells and drug aliquots

Frozen human PBMCs (stored at -150°C) were used from the donors described in ***Chapter 3*** (see ***Table 3.1***). More specifically, PBMCs from donors HCA RLH 041 (naproxen - induced idiosyncratic hepatotoxicity patient), HC1 RLH 004 (long-term treated naproxen patient without evidence of an ADR), HVN 061 and HC2 RLH 001 (healthy volunteers not knowingly previously exposed to naproxen) were used for the purposes of this Chapter's investigations. Herein, HCA RLH 041 and HC1 RLH 004 are labelled as "iDILI" and "long-term treated" while HVN 061 and HC2 RLH 001 as "Healthy A" and "Healthy B", respectively. (S)-NAP, (S)-NAG and (S)-DNAP were dissolved in culture medium and appropriately diluted (see section **3.3.4**) prior to immediate administration to cells.

4.3.3 Cell treatment and RNA extraction

Frozen human PBMCs were thawed by gently swirling the cryotube vials in a 37°C water bath and subsequently suspended in cell culture medium (500 ml RPMI 1640, 100 μg/ml penicillin, 100 U/ml streptomycin, 25 μg/ml transferrin, 10% (v/v) Human AB Serum, 25 mM HEPES buffer, 2 mM L-glutamine). PBMCs were seeded at 0.15×10^6 cells / well (100 μl) in 96-well U-bottom plates and after a 15-min rest period, (S)-NAP, (S)-NAG and (S)-DNAP were added

all at a final concentration of 150 μM (total well volume 200 μl). Culture medium alone served as negative control. Each condition was added in 3 replicates and each replicate consisted of 3 different wells. A separate 96-well U-bottom plate was used for each donor. Treated PBMCs were then incubated for a period of 24 hours inside a humidified incubator of 5 % CO2 at 37°C. Cell viability was assessed upon cell thawing and after drug treatment using the TC20 automated cell counter (Cat# 145-0101, Bio-Rad Laboratories Inc., Watford, UK) with initial viability exceeding 90 % and final cell viability in range of < 5 % difference.

Following incubation, cells for every replicate were pooled together (yield ~ $0.45x10^6$ cells) and total RNA was extracted for each replicate / condition using Direct-zol RNA MiniPrep kit (Cat# R2053, Zymo Research, Freiburg, Germany) according to the supplier's protocol. Total RNA was re-suspended in 25 μl RNase-free water and assessed spectrophotometrically for yield and purity with Nanodrop ND1000 (Thermo Fisher Scientific, Basel, Switzerland) (acceptable A260/A280 ratio > 1.8). Total RNA was aliquoted at 200 ng in 50 μl RNase-free water using 0.2 ml PCR tubes and kept frozen at -80°C until NGS. ***Figure 4.1*** summarizes the process of cell treatment and RNA extraction.

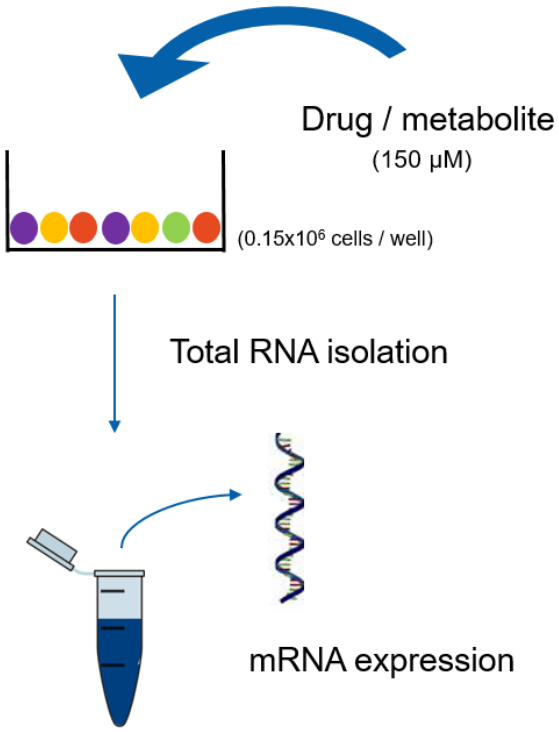


Figure 4.1. Extraction of total RNA from human peripheral blood mononuclear cells following treatment with (S)-NAP, (S)-NAG or (S)-DNAP. Human PBMCs are seeded at 0.15x10^6 cells / well and treated with (S)-NAP, (S)-NAG or (S)-DNAP at a final concentration of 150 µM in a total volume of 200 µl cell culture media. Following incubation for a period of 24 hours inside a humidified incubator of 5 % CO2 at 37°C, total RNA is extracted as described in Materials and Methods (section **4.3.3**).

4.3.4 Next Generation Sequencing (NGS)

RNA quality control and quantification

Total RNA was quality-checked on the 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA) using the RNA 6000 Pico Chip (Agilent, Cat# 5067-1513) and quantified by Fluorometry using the QuantiFluor RNA System (Cat# E3310, Promega, Madison, WI, USA). All samples exhibited a RNA Integrity Number (RIN) > 7. ***Figure 4.2*** shows a representative electropherogram for one of the samples.

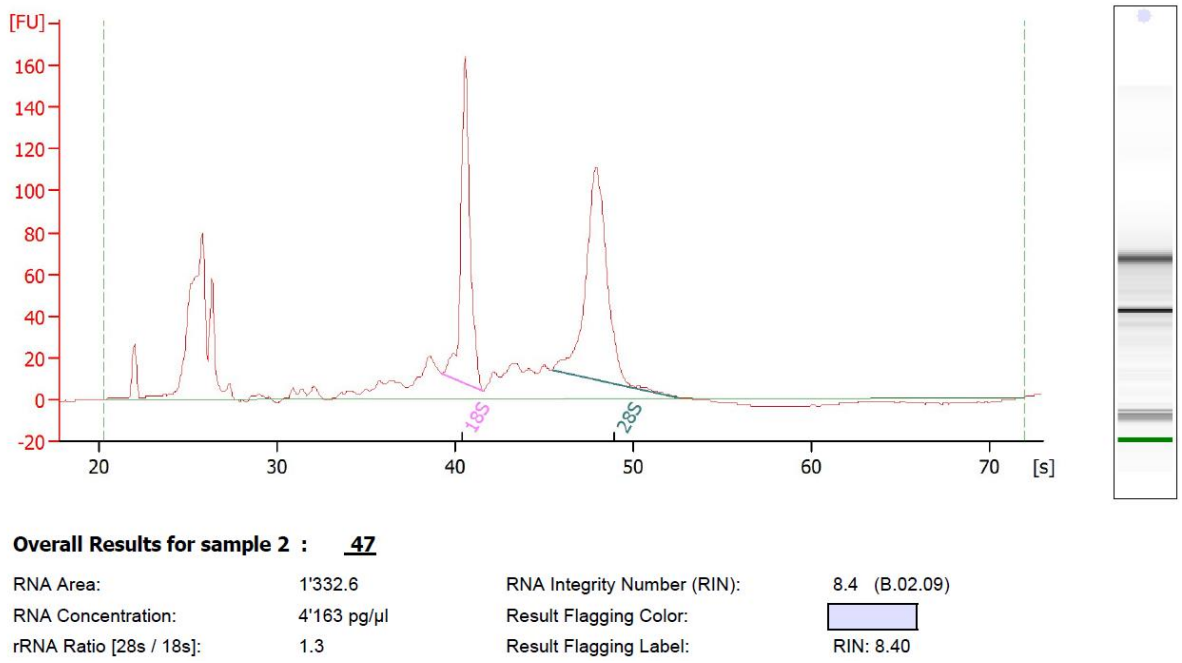


Figure 4.2. Representative electropherogram demonstrating RNA integrity analysis. The distinct ribosomal RNA bands are being shown (18S and 28S). On this occasion a RIN = 8.4 was achieved as determined using the 2100 Bioanalyzer.

Library preparation

Library preparation was performed with 200 ng total RNA using the TruSeq Stranded mRNA LT Sample Prep Kit (Cat# RS-122-2101, Illumina, San Diego, CA, USA) in 2 sets of 24 samples. Libraries were quality-checked on the Fragment Analyzer (Advanced Analytical, Ames, IA, USA) using the Standard Sensitivity NGS Fragment Analysis Kit (Cat# DNF-473, Advanced Analytical) revealing excellent quality of libraries (average concentration was 104 ± 16 nmol/L and average library size was 334 ± 7 base pairs). Samples were pooled to equal molarity. Each pool was quantified by PicoGreen Fluorometric measurement in order to be sequenced on the NextSeq 500 instrument (Illumina) at a final concentration at 1.8 to 2 pM.

Clustering and Sequencing

Samples were sequenced Single-reads 81 bases using the NextSeq 500 High Output Kit 75-cycles (Illumina, Cat# FC-404-1005) and primary data analysis was performed with the Illumina RTA version 2.4.11 and bcl2fastq-2.19.1.403.

Identification of differentially expressed genes

The alignment tool, Salmon (Patro, Duggal, Love, Irizarry, & Kingsford, 2017),was used to count the number of reads aligning to gene annotations (cDNA and ncRNA) of Homo Sapiens from Gencode version 29. R/Bioconductor was used primarily for the entire analysis of this study. DESeq2 (Love, Huber, & Anders, 2014), was used to normalize read counts and calculate the fold-changes of genes in control compared to (S)-NAP or metabolite-treated samples.

Fold change and log2 fold change values were calculated as the geometric mean of values computed from DESeq2 of all genes within the signature per group. Transcripts per kilobase million (TPM) was calculated using the Bioconductor package tximport (Soneson, Love, & Robinson, 2015). A 2-fold increase or decrease in differential gene expression was selected as threshold to indicate meaningful gene expression changes.

4.4 RESULTS

4.4.1 Principal component analysis to investigate global differential gene expression of human peripheral blood mononuclear cells in response to (S)-naproxen and its metabolites

The principal component analysis (PCA) for all subjects and treatment conditions at 24 hours is illustrated in ***Figure 4.3***.

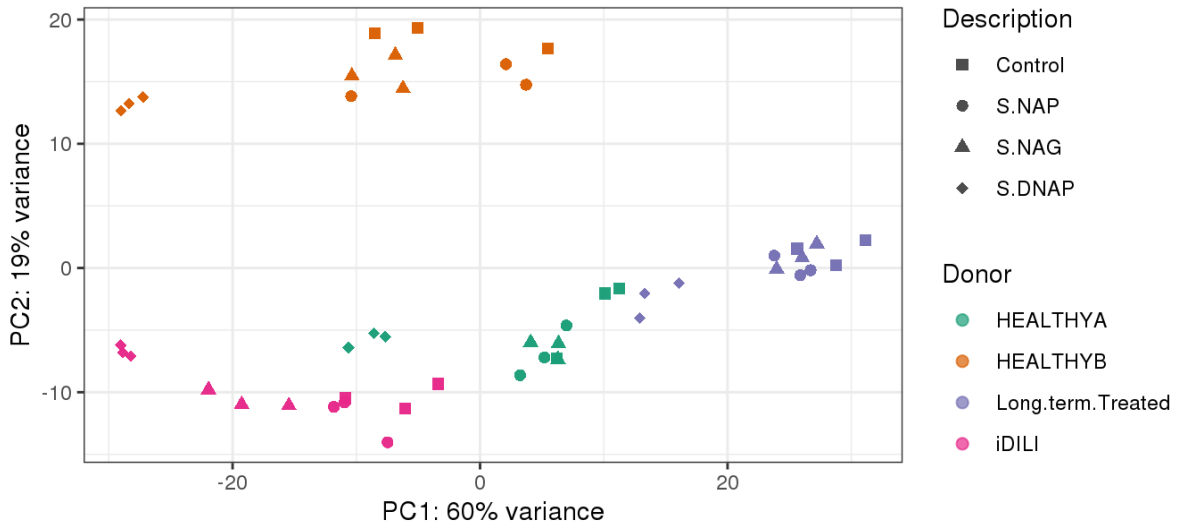


Figure 4.3. Principal component analysis (PCA) of human peripheral blood mononuclear cells exposed to (S)-NAP, (S)-NAG and (S)-DNAP for 24 hours. Total RNA was extracted from human PBMCs of two healthy drug-naïve donors, one long-term naproxen treated subject and one naproxen iDILI patient following exposure to (S)-NAP and its metabolites for 24 hours as described in Methods and Materials (see section **4.3.3**). Each one of the colour-filled squares, circles, triangles and diamonds represents one technical repeat of vehicle control, (S)-NAP, (S)-NAG and (S)-DNAP, respectively.

A high inherent patient variation is observed, thus dictating gene expression amongst different subjects. As a result, investigation of differential gene expression changes induced by compounds as relative to vehicle control for each patient is deemed to be more appropriate.

The PCA indicates that (S)-DNAP induces reproducible (three technical repeats with consistent results) gene expression changes across all subjects as determined by the left shift on the PC1 axis. Notably, (S)-NAG appears to be exerting some changes for the iDILI patient only, but not as drastic as (S)-DNAP's effect. In all subjects, (S)-NAP's gene expression changes do not show clear differentiation (close to vehicle control), and in all but the iDILI patient, (S)-NAG's effect is also remarkably low.

4.4.2 Differential gene expression of human peripheral blood mononuclear cells isolated from the idiosyncratic hepatotoxicity patient in response to (S)-naproxen and its metabolites

Figure 4.4 illustrates the global differential gene expression observed in human PBMCs isolated from the iDILI patient following exposure to (S)-DNAP, (S)-NAG and (S)-NAP for 24 hours. Overall, in the iDILI patient, most differentially expressed genes (orange dots) relative to control are seen as downregulated while only a few are upregulated. A similar response of downregulation, albeit apparently dampened, is also seen for PBMCs exposed to (S)-NAG but only minimal changes following exposure to (S)-NAP, as also verified through the PCA. Whilst similar genes are seen downregulated following (S)-NAG treatment (genes found as differentially expressed from vehicle control but not in the 'iDILI_S.DNAP' are shown as blue, while genes differentially expressed in the relevant panel but also in the 'iDILI_S.DNAP' are shown as orange), these changes are not as large as observed for the (S)-DNAP. Most genes seen as differentially expressed in response to (S)-NAP or (S)-NAG are also seen as differentially expressed in response to (S)-DNAP (hence orange).

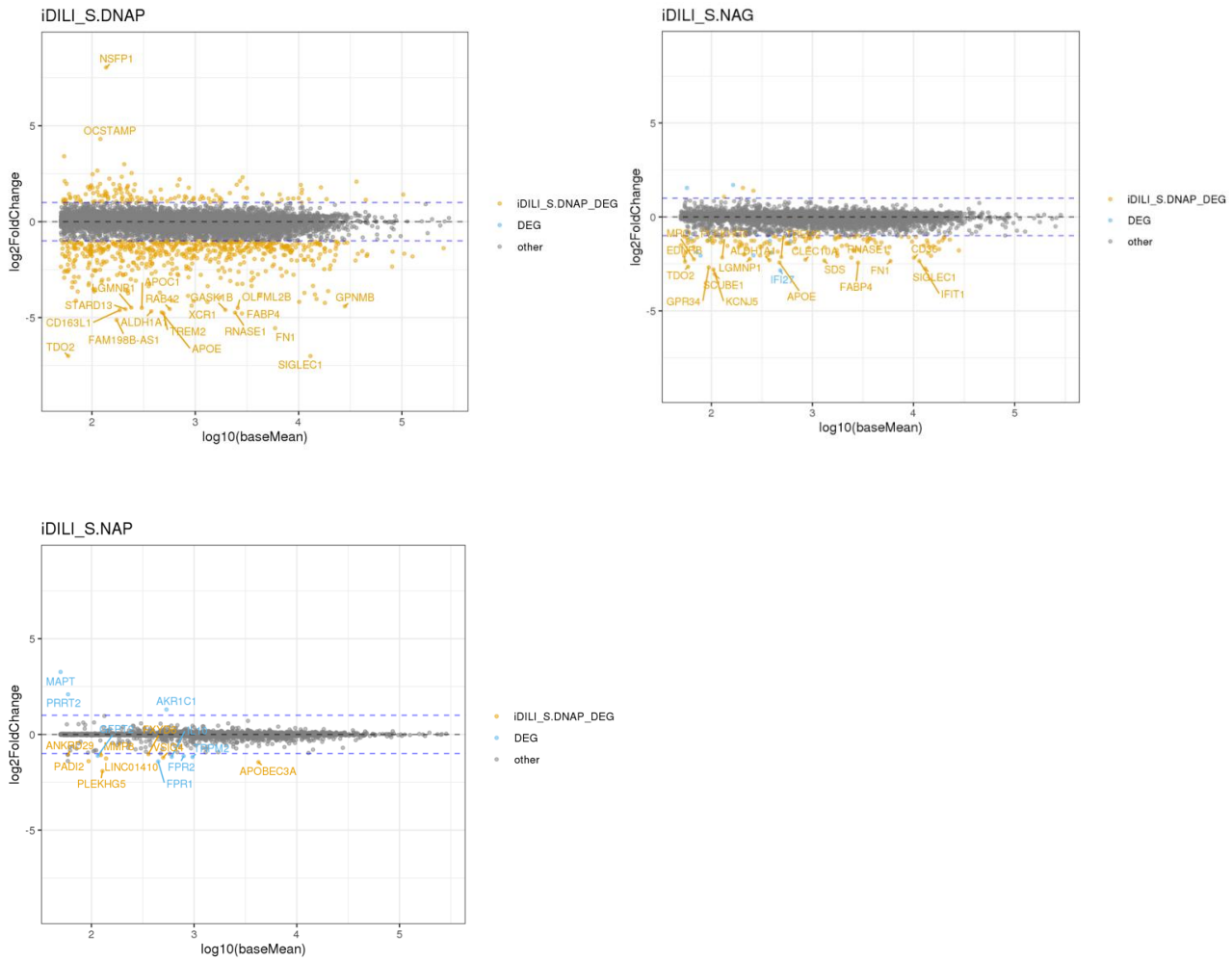


Figure 4.4. Differential gene expression in peripheral blood mononuclear cells isolated from the idiosyncratic hepatotoxicity patient in response to (S)-DNAP, (S)-NAG and (S)-NAP at 24 hours. Every gene is illustrated by a single dot on the plots. Grey, blue and orange dots represent non-differentially expressed genes (other) (< 1 log2), differentially expressed genes (DEG) (> 1 log2) and genes differentially expressed (> 1 log2) and in common with 'iDILI_S.DNAP' (iDILI_S.DNAP_DEG), respectively.

Figure 4.5 is cluster heat map of the top 50 genes found to be most differentially expressed in the iDILI patient (left-hand side: most downregulated, right-hand side: most upregulated). The differential gene changes induced following exposure to (S)-DNAP are presented and compared to other conditions ((S)-NAG, (S)-NAP and vehicle control). Even though the changes shown herein are not relative to control, the effects of the vehicle control on gene

expression are also included as reference. Aside from identification of the top 50 genes most differentially expressed, the cluster heat map also confirms that responses in association to (S)-NAG exposure are dampened responses of (S)-DNAP.

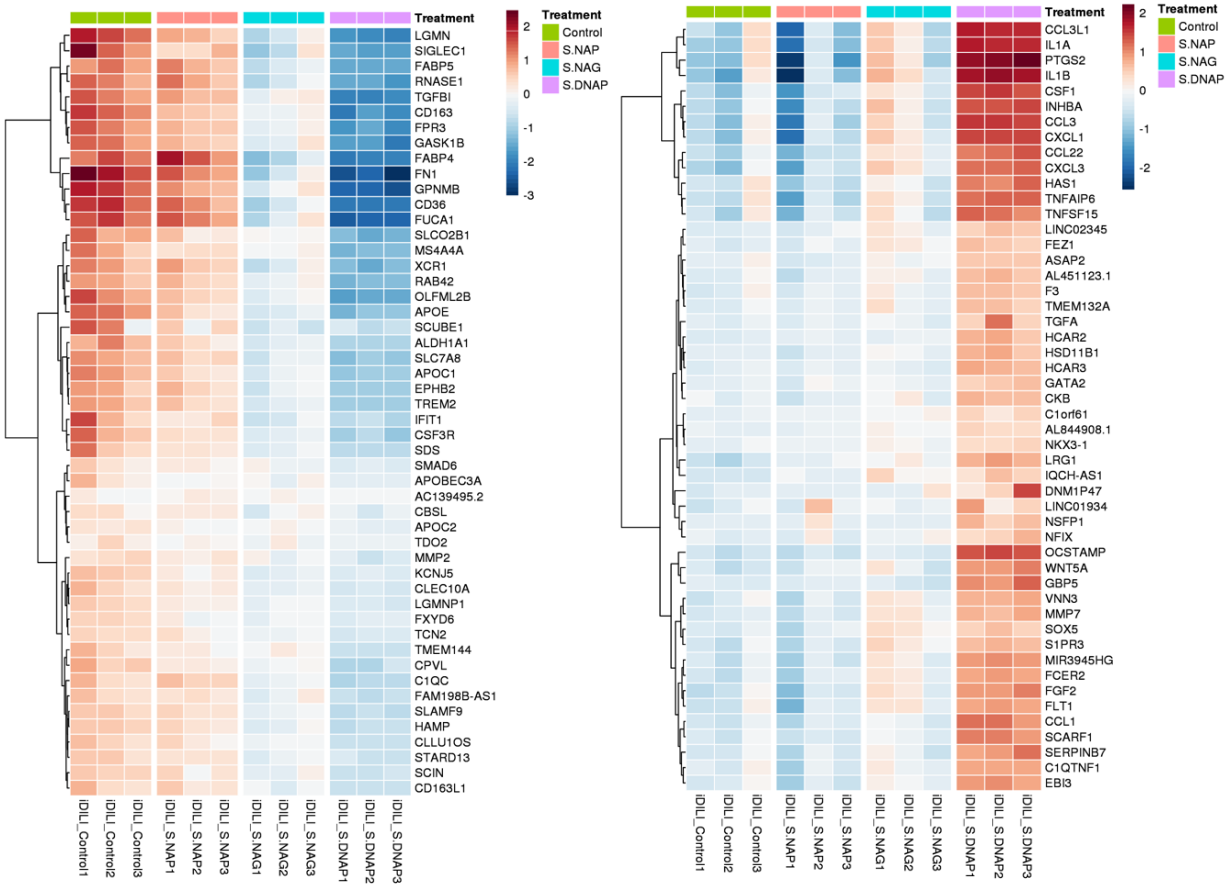


Figure 4.5. Top 50 differentially expressed genes in peripheral blood mononuclear cells isolated from the idiosyncratic hepatotoxicity patient in response to (S)-DNAP, (S)-NAG and (S)-NAP at 24 hours. Top 50 most downregulated genes and upregulated genes are shown on the left-hand side and right-hand side, respectively. Each column represents one technical repeat while each row one single gene.

4.4.3 Consistent gene expression changes in association to (S)-O-desmethylnaproxen exposure are seen across all investigated subjects

The differential gene expression in human PBMCs following exposure to (S)-DNAP for 24 hours was compared across all four investigated subjects and the results are illustrated in ***Figure 4.6***.

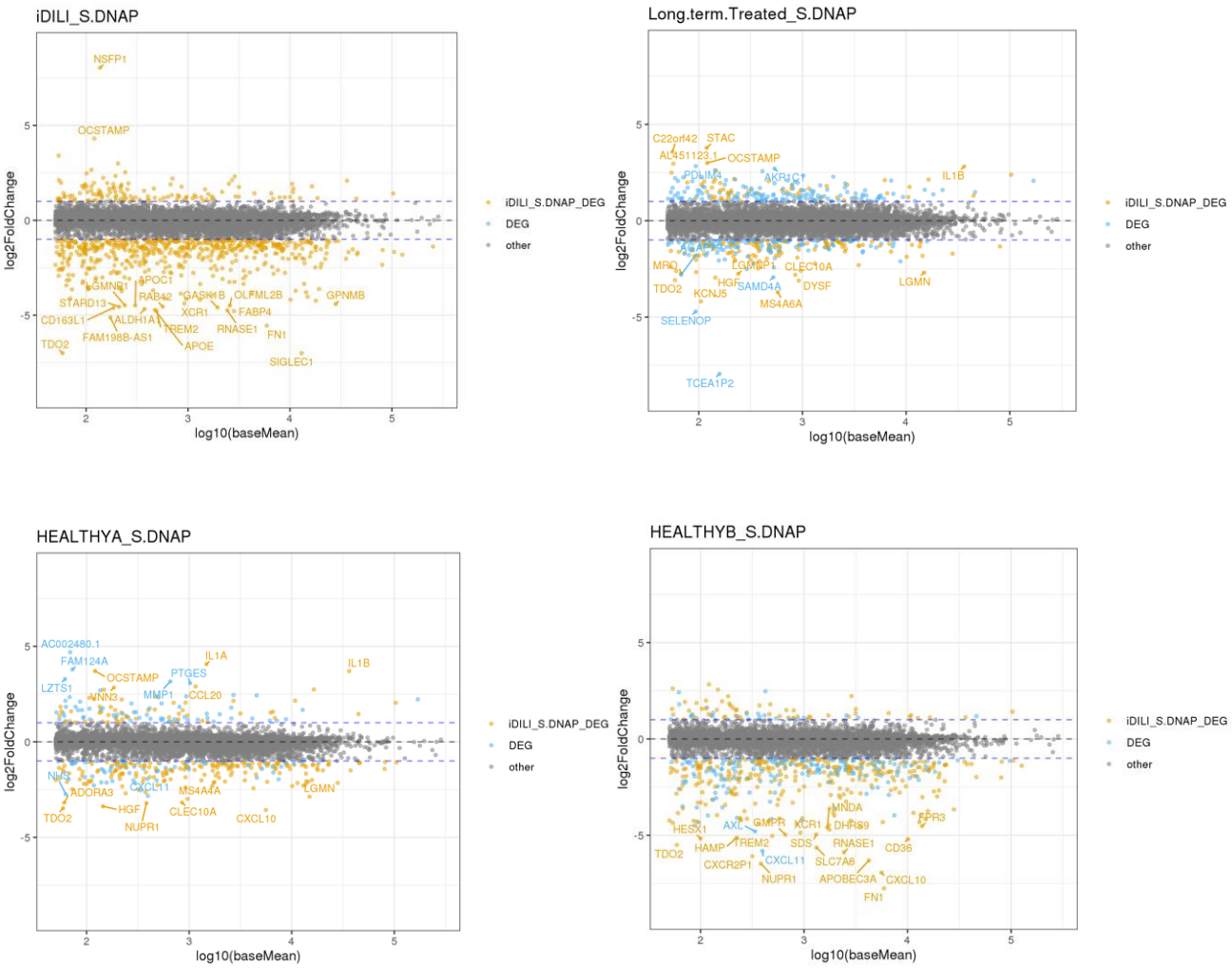


Figure 4.6. Differential gene expression in human peripheral blood mononuclear cells in association to (S)-DNAP exposure for 24 hours. The global transcriptome is plotted for each subject investigated in response to (S)-DNAP exposure. Grey, blue and orange dots represent non-differentially expressed genes (other) (< 1 log2), differentially expressed genes (DEG) (> 1 log2) and genes differentially expressed (> 1 log2) and in common with 'iDILI_S.DNAP' (iDILI_S.DNAP_DEG), respectively.

The PCA described earlier showed that (S)-DNAP exerts gene changes across all subjects. It is evident through this comparison that most genes seen as differentially expressed in control subjects (two healthy drug-naive and one long-term naproxen treated) are also seen as changed (mainly downregulated) in the iDILI patient thus suggesting a universal response to exposure to the oxidative metabolite. Upon closer investigation, it is observed that there are similar genes differentially expressed across the different subjects. Although the "Healthy B" and "iDILI" subjects both show a general downregulation of gene transcription, "Healthy B" shows some distinct genes differentially expressed while iDILI exhibits significantly more altered gene transcription therefore suggesting an individual phenotype.

4.4.4 (S)-O-desmethylnaproxen exhibits a differing effect on COX enzymes but no effect in the leukotriene or prostaglandin pathway in human peripheral blood mononuclear cells

The effects of (S)-NAP, (S)-NAG and (S)-DNAP human PBMC treatment for 24 hours on COX enzymes as well as the leukotriene / prostaglandin pathway gene expression are shown in ***Figures 4.7*** and ***4.8***. Notably, (S)-DNAP exerts a consistent downregulation and upregulation of gene expression for COX-1 (prostaglandin synthase 1 (*PTGS-1*)) and COX-2 (prostaglandin synthase 2 (*PTGS-2*)) enzymes, respectively across all subjects. Minimal changes were observed for the long-term naproxen treated patient. There are no clear and significant drug-induced gene transcription changes associated with the leukotriene or prostaglandin pathway in any subject, with the exception of the upregulation of COX-2.

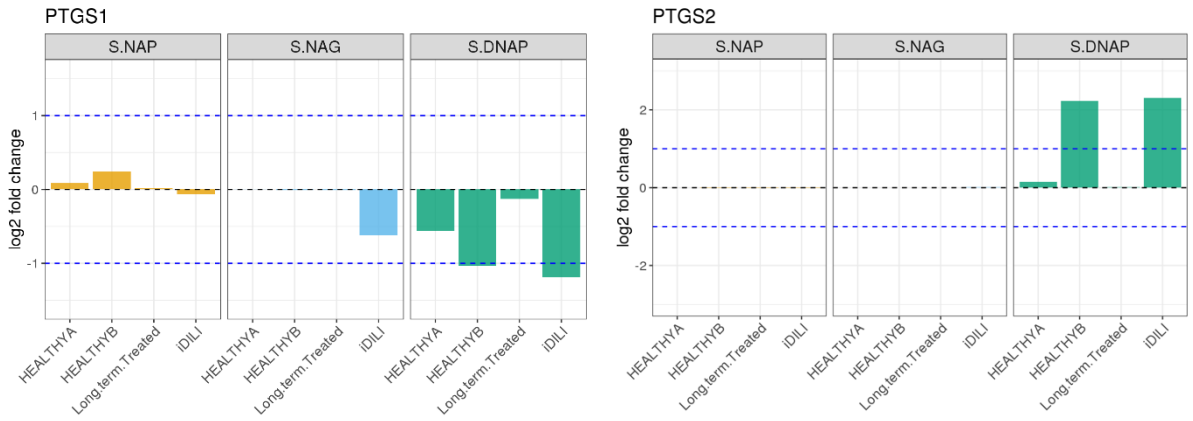


Figure 4.7. Effects on COX-1 and COX-2 enzymes expression in human peripheral blood mononuclear cells in response to (S)-NAP, (S)-NAG and (S)-DNAP at 24 hours. Gene expression changes are depicted for COX-1 (PTGS1) and COX-2 (PTGS2) enzymes on the left-hand and right-hand side, respectively. Orange, blue and green bars represent gene changes induced by (S)-NAP, (S)-NAG and (S)-DNAP, respectively for each specific subject. A > 1 log2 change is considered meaningful and significant.

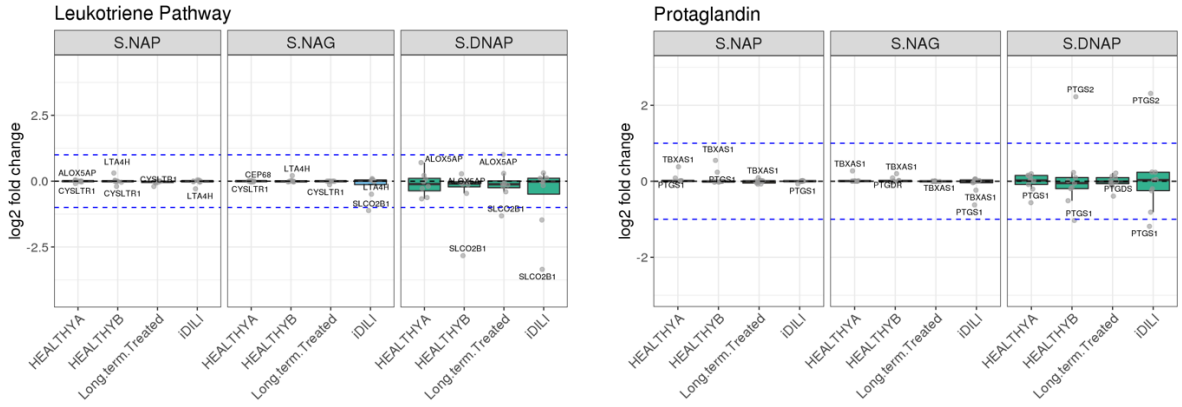


Figure 4.8. Effects on leukotriene and prostaglandin pathway gene expression in human peripheral blood mononuclear cells in response to (S)-NAP, (S)-NAG and (S)-DNAP at 24 hours. Gene expression changes are depicted for the leukotriene and prostaglandin pathway on the left-hand and right-hand side, respectively. Orange, blue and green bars represent gene changes induced by (S)-NAP, (S)-NAG and (S)-DNAP, respectively for each specific subject. A > 1 log2 change is considered meaningful and significant. The leukotriene pathway genes consist of *CYSLTR1*, *CYSLTR2*, *LTB4R2*, *ALOX5AP*, *CEP68*, *LTC4S*, *LTA4H*, and *SLCO2B1*. The prostaglandin pathway genes include *PTGS1*, *PTGS2*, *TBXAS1*, *TBXA2R*, *PTGIS*, *PTGES2*, *PTGDS*, *PTGDR* and *PTGER4*.

4.4.5 Assessment of drug - induced changes on *IL1A, IL6, CXCL8, MT2A* and *NAMPT* genes in human peripheral blood mononuclear cells

Figure 4.9 illustrates the effects of (S)-NAP, (S)-NAG and (S)-DNAP on the expression of five specific genes (*IL1A*, *IL6*, *CXCL8*, *MT2A* and *NAMPT*) in human PBMCs (all subjects) following 24-hour exposure. The responses are not normalized to control but can be compared to the effects of vehicle control on gene expression as shown. A significant overexpression (> 2-fold increase) was observed only for *CXCL8* (which encodes IL-8) and only for (S)-DNAP across all subjects investigated. No unique gene changes for the iDILI patient versus the other subjects was determined.

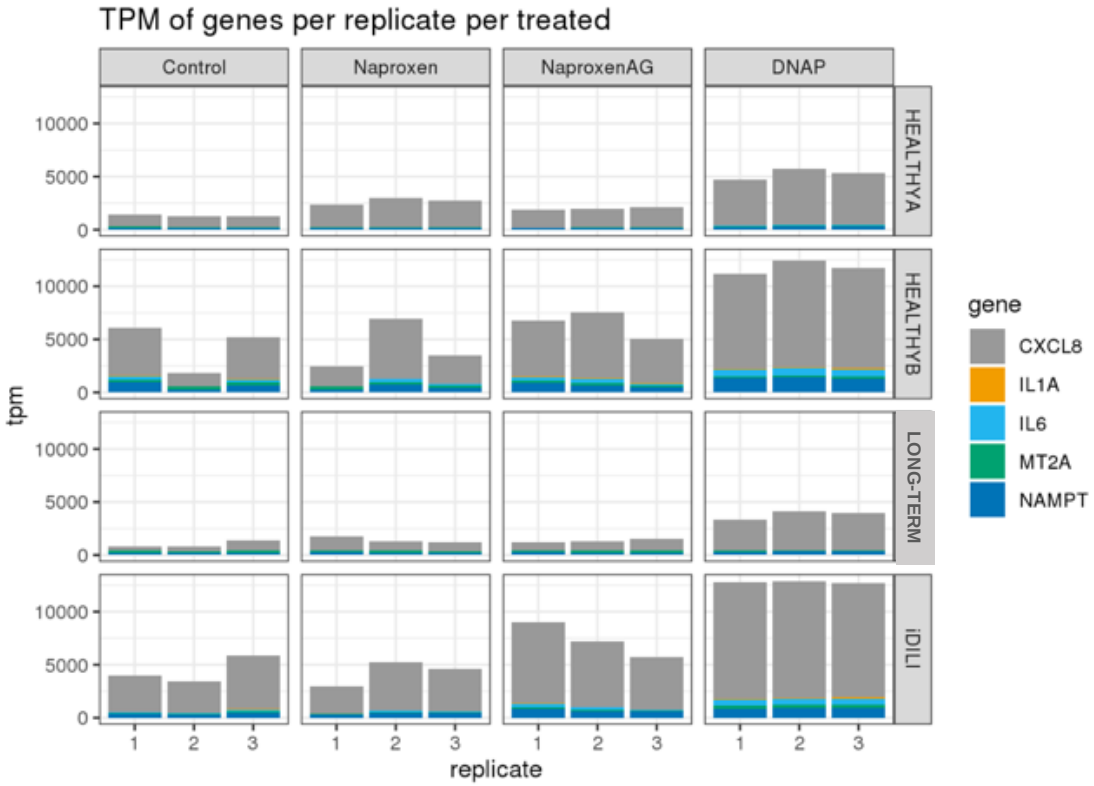


Figure 4.9. Drug-induced changes on *IL1A, IL6, CXCL8, MT2A* and *NAMPT* gene expression in human peripheral blood mononuclear cells in response to (S)-NAP, (S)-NAG and (S)-DNAP at 24 hours. Gene changes for each specific gene and every subject investigated in response to each drug condition and vehicle control is plotted. Results are shown as transcripts per kilobase million (TPM).

4.5 DISCUSSION

The work described in this final experimental Chapter, sought to investigate the effects of (S)-NAP and its major metabolites (S)-NAG and (S)-DNAP in the total transcriptome of human PBMCs isolated from two healthy volunteers, one long-term naproxen treated patient and one patient having suffered an idiosyncratic hepatotoxicity reaction secondary to naproxen use. Previously, it has been shown (see ***Chapter 3***) that immunological memory was detected only against (S)-DNAP in this naproxen iDILI patient but none of the control subjects. Moreover, evidence that describes upregulation of immuno-inflammatory genes following *in vitro* treatment of healthy drug-naive PBMCs with certain AGs for 24 hours exists in the literature (Wieland et al., 2000; Miyashita et al., 2014; Iwamura et al., 2015). Consequently, the opportunity to investigate the effects of naproxen, its AG metabolite and also its major oxidative metabolite (S)-DNAP not only in healthy human PBMCs but also in a naproxen iDILI well-characterized patient was considered crucial towards generating further evidence on the risk assessment of AGs.

Collectively, the differential gene expression data generated herein indicate that 24-hour (S)-DNAP *in vitro* exposure is capable of inducing significant changes in the total transcriptome of human PBMCs isolated from the iDILI patient with only a low effect exhibited by (S)-NAG and none by (S)-NAP *in vitro* exposure for the same period of time. Several genes identified as differentially expressed by (S)-DNAP in the iDILI patient were also changed by (S)-NAG albeit to a lesser effect. This is further corroborated by the top 50 differentially expressed genes (***Figure 4.5***) where the (S)-DNAP responses were amplified when compared to (S)-NAG. In ***Chapter 3***, it was shown that (S)-NAG exerted a level of cross-reactivity for the generated (S)-DNAP specific T-cell clone from the iDILI patient. Whether the transcriptomic changes observed herein reflect this type of cross-reactivity or are mediated to some extent by common

pharmacology mechanisms could not be verified in this work, but is plausible. Moreover, unlike the immunological memory investigations, a similar shift in gene expression induced by (S)-DNAP was also observed for the controlled subjects, although in subsequent analysis (see section **4.4.3**), it is shown that much more genes are significantly changed (> 2-fold) in the iDILI patient than any other subject with the exception of Healthy B. Notably, the small gene changes induced by (S)-NAG in the iDILI patient were not observed in other subjects. This is consistent with data generated in ***Chapter 3*** showing a degree of cross-reactivity to (S)-NAG for the (S)-DNAP – responsive T-cell clone 236 identified in the iDILI patient.

An interesting finding during these investigations has been the overall pattern of differential gene expression observed. Across all subjects, the significant total transcriptional change observed at 24 hours was reflected by a general gene downregulation. Although this was not an expected outcome, it is not necessarily unexplained. Very recently, a group of researchers have described how cells on certain occasions following a response to stressful environment proceed to a rapid downregulation of many normally used genes in a process called stress-induced transcriptional attenuation (SITA) (Vihervaara et al., 2017; Aprile-Garcia, Tomar, Hummel, Khavaran, & Sawarkar, 2019). Nonetheless, when the top 50 differentially expressed genes are summarized (***Figure 4.5***), a number of immuno-inflammatory genes are shown to be upregulated in response to (S)-DNAP at 24 hours specifically for the iDILI patient. Amongst these genes, *IL1B*, *CXCL2*, and *CXCL8* (encoding for IL-8) are of particular importance. Activation of the inflammasome as measured by production of IL-1β could represent an important determinant in idiosyncratic toxicity (Weston & Uetrecht, 2014) while CXLC2 is recently being investigated for its role in drug hepatotoxicity including AGs (Mitsugi et al., 2016; Oda et al., 2017; Mak & Uetrecht, 2019).

The effects of (S)-NAP and its metabolites were also investigated against the COX enzymes as well as the leukotriene and prostaglandin pathway. A differing effect of (S)-DNAP on these

two enzymes was observed (COX-1: downregulation and COX-2: upregulation). The exact reasons behind this observation are not fully elucidated, but it is likely that structural changes can reflect preferential activity towards a COX isoform since, for example, substitution of CH2 for the O atom of the p-methoxy group has yielded analogs with increased COX-2 selectivity relative to naproxen (Duggan et al., 2010) while desmethyl derivatives of indomethacin are extremely poor inhibitors of COX enzymes (Kalgutkar, Marnett, Crews, Remmel, & Marnett, 2000). It should be recognized that naproxen itself is not regarded as an isotype-specific COX inhibitor. Perhaps more importantly, no significant changes were observed in the overall leukotriene pathway (***Figure 4.8***). Overexpression of leukotrienes has been linked with a pro-inflammatory pharmacology and Th17 differentiation and migration (Lee et al., 2015). In ***Chapter 3***, a Th17 (S)-DNAP – specific clone was identified in the iDILI patient. COX inhibition is expected to lead to reduced prostaglandin production and potentially shift arachidonic acid metabolism into the lipoxygenase pathway. This shunt from the cyclooxygenase to the lipoxygenase pathway would therefore mean increased leukotriene abundance (Docherty & Wilson, 1991; Smith et al., 2018). Moreover, Lucena et al. recently discovered *PTGS* gene variations in patients who had experienced NSAID-induced liver injury suggesting that a risk could be related to genetic alteration in the arachidonic acid metabolism (Lucena et al., 2019). However, in our transcriptomics experiment in human PBMCs at 24 hours no changes were observed in the leukotriene pathway, meaning data generated in our work could not add further support to this hypothesis.

Targeted research efforts by groups investigating AG-mediated transcriptional changes in human PBMCs prompted detailed assessment of drug-induced effects on *IL1A*, *IL6*, *CXCL8*, *MT2A* and *NAMPT* gene expression (Miyashita et al., 2014; Iwamura et al., 2015). Through the experimentation conducted herein, it is evident that (S)-NAG does not exert significant changes of any of the aforementioned genes. This in fact somewhat coincides with the findings

of Iwamura et al. showing limited changes (< 2-fold) induced by (S)-NAG on these genes compared to other AGs (Iwamura et al., 2015). However, (S)-DNAP was able to upregulate *CXCL8* gene transcription by a little over than 2-fold across all subjects, perhaps suggesting a role in driving immuno-inflammatory responses. There are limitations in the research described by Miyashita et al. and Iwamura et al., as there was not a clear rationale behind the selection of these specific genes, a fixed dose (100 μM) was used across all investigated AGs and effects were only investigated in healthy human PBMCs, but not iDILI characterized patients (Miyashita et al., 2014; Iwamura et al., 2015). Moreover, it should be borne in mind that all responses are only assessed at one time-point (24 hours) and the gene expression snapshot may be very different at other time-points (Fan, Nishanian, Breen, McDonald, & Fahey, 1998).

Collectively, the results presented in this Chapter further promote a role for (S)-DNAP in activating the immune system and driving drug-specific early changes. In particular, (S)-DNAP exhibited very strong differential expression in the iDILI patient compared to (S)-NAP and (S)-NAG. Moreover, the pattern of gene expression was predominantly down-regulatory, potentially reflecting cellular response to increased stress. The dampened responses of (S)-NAG in the iDILI patient resemble findings in ***Chapter 3*** and could perhaps reflect cross-reactivity. However, a shift in the transcriptome following (S)-DNAP exposure was also observed for the control subjects, albeit to a lesser extent, with Healthy B exhibiting the most profound changes. Several genes of immuno-inflammatory nature were identified to be amongst the top 50 upregulated in the iDILI patient in response to (S)-DNAP. It remains unknown whether there are specific genes that may be more responsible for the distinct iDILI phenotype or if a combination of drug-induced changes represents a higher mechanistic risk. Future work should be directed towards further exploring gene expression changes from more naproxen iDILI patients as well as different drugs forming AG metabolites and investigate their relationship to iDILI pathogenesis.

CHAPTER 5

GENERAL DISCUSSION

This Thesis was designed and carried out under the overarching perception that AG drug metabolite formation is involved in idiosyncratic toxicity through immune-mediated mechanisms. The evidence behind this hypothesis is equivocal. Formation of drug – protein adducts is known to represent a critical mechanism in the pathogenesis of some delayed Type IV drug hypersensitivity reactions, such as β-lactam hypersensitivity. AG metabolites have been shown to be capable of forming adducts with macromolecules *in vitro* and in patients, as described previously (***Chapter 2***). However, covalent binding of drugs onto proteins does not necessarily equate to toxicity. To date, no evidence has conclusively associated AG metabolite formation with clear toxicological endpoints for IDRs. It is this uncertainty, however, that creates anxiety in the pharmaceutical industry during early drug development stages. Most, if not all, marketed carboxylic acid drugs exhibit a good safety profile despite acyl glucuronidation and have transformed many patient lives through efficacious management of chronic conditions. Hence, an immediate question arises: How should the pharmaceutical industry respond when identifying an AG as a major drug metabolite during early human studies and what should the position of the regulators be? In the vast majority of patients, carboxylic acid drugs provide safe and efficacious therapy solutions. However, despite IDRs being rare phenomena, they can have a detrimental impact either for the pharmaceutical industry (drug withdrawals) or the patient (severe life-threatening reactions for few susceptible individuals or withdrawals of otherwise efficacious drugs). Current development strategies for drugs forming AG metabolites imply that additional regulatory hurdles delay process and

access to market. It is therefore critical to explore the involvement of AG metabolites in IDRs by conducting tailored investigations in humans treated with carboxylic acid drugs with or without associated ADRs (such as iDILI). Data and knowledge generated from such experiments will add weight to existing evidence and assist the relevant stakeholders in appropriate decision making.

Naproxen was selected as a model drug to investigate the aforementioned and for reasons explained elsewhere in the Thesis (***Chapter 1***; section **1.7**). One of the major metabolites of naproxen identified in human studies is an AG. Moreover, in ***Chapter 2***, it was shown that (S)-NAG is chemically unstable with an *in vitro* degradation t½ of 2.72 hrs in 0.1 M phosphate buffer at 37°C (pH 7.4) as well as protein reactive with 247.37 ± 57.64 nmol of drug irreversibly bound per μmol HSA when the AG is incubated with HSA in a 50 : 1 molar ratio at 37°C (pH 7.4). What these data show is that if naproxen was a new chemical entity under investigation nowadays, anxiety would be raised over its future development based on existing AG risk assessments (Sawamura et al., 2010) as well as regulatory views (FDA, 2008). However, naproxen has been in the market since 1976 benefiting many patients that suffer from chronic diseases (e.g. osteoarthritis). Despite an overall good safety profile, naproxen has been associated with IDRs, in particular with idiosyncratic hepatotoxicity. The possibility to acquire clinical samples from few individuals treated with naproxen – including one patient with associated liver abnormalities – provided a unique opportunity to investigate immunological responses towards the parent drug or its metabolites in these subjects, allowing to translationally link any potential immunological responses to these inadvertent liver findings.

The experiments conducted in ***Chapter 3*** are unique since, to the best of our knowledge, no similar immunological *ex vivo* human investigations for a carboxylic acid drug and its major metabolites (including an AG) have been published to date. This set of experiments carried out on well-characterized clinical samples yielded a novel finding, challenging assumptions over

AG toxicity. Immunological memory in a patient having suffered idiosyncratic hepatotoxicity due to naproxen was found to be directed to the oxidative metabolite of naproxen, (S)-DNAP, but not the AG metabolite or the model protein adduct used ((S)-NAG – HSA adduct). None of the control subjects exhibited a similar response, thus verifying the validity of the finding in the naproxen iDILI patient. Moreover, T-cell cloning investigations revealed a Th17 (S)-DNAP – responsive T-cell clone which, following further characterization, was found to become activated via a p-i mechanism. Notably, *in vitro* human T-cell priming using drug-naive healthy PBMCs failed to replicate a similar proliferative response. This highlights the importance of mechanistic translational *ex vivo* investigations utilizing patient sample to enhance understanding of clinical adverse responses, which may enable further optimization of appropriate preclinical *in vitro* assays allowing drug profiling during pharmaceutical development (Kenna & Uetrecht, 2018). The gene signature in human PBMCs following exposure to naproxen and its major metabolites was investigated in ***Chapter 4***. (S)-DNAP was shown to be responsible for inducing differential gene expression relative to control vehicle with a profound effect in PBMCs derived from the naproxen iDILI patient. However, on this occasion, (S)-DNAP also exerted differential gene expression in PBMCs deriving from the long-term treated naproxen patient as well as the healthy drug-naive volunteers. Moreover, (S)-NAG induced slight gene expression changes in PBMCs from the iDILI patient. Notably, despite an overall down-regulation pattern, certain immuno-inflammatory genes such as *IL1B*, *CXCL8* and *CXCL2* were found to be amongst the top 50 upregulated genes in response to (S)-DNAP exposure. However, their relationship to the drug reaction remains to be determined. How the immunological memory findings relate to the differential gene expression observed in human PBMCs is not entirely clear and would require further investigation, however, the consistency of responses to (S)-DNAP exposure further reinforces its immunological involvement in this rare case of naproxen – induced hepatotoxicity.

Despite novel findings described in this Thesis, it is very important to acknowledge certain limitations. Identifying well-characterized and rare human clinical samples has been a challenging task and due to the novelty of this project and time constraints, only one naproxen iDILI patient could be sourced. Furthermore, advanced T-cell characterization data were derived from only one (S)-DNAP responsive T-cell clone, despite multiple efforts to identify additional (S)-DNAP T-cell clones. Finally, during gene expression investigations, high innate patient variability meant that responses could only be reliably interpreted on an individual patient level relative to vehicle control.

Prior to this Thesis, very little was known about the immunological consequences of AG formation in human and most research was centred on animal investigations exhibiting notable limitations, particularly the requirement for the use of adjuvants to artificially stimulate immune responses. It is the first time that immunological memory was detected against (S)-DNAP and not (S)-NAP or (S)-NAG in a case of naproxen – induced idiosyncratic hepatotoxicity. Moreover, the differential gene expression signature in patient and control PBMCs further suggests that (S)-DNAP is involved in immune responses associated with the investigated case. Moving forward, there are several questions that remain unanswered and warrant further exploration:

- Are these findings reproducible with other naproxen – induced idiosyncratic hepatotoxicity cases?
- Are these findings reproducible with other drugs known to form AG metabolites?
- Can these findings be further substantiated with human liver investigations to identify immunopathological evidence and what is the role of inflammatory cytokines?

Reproducibility and consistency of these data would mean that the scientific perception of acyl glucuronidation as a safety liability could change. Throughout this Thesis, results have consistently shown that (S)-DNAP, the oxidative metabolite of (S)-NAP, and not (S)-NAG has been immunologically involved in the examined idiosyncratic hepatotoxicity case under the experimental approaches used. Should this be replicated with more patients and more drugs, then it could mean that, perhaps, it is metabolic pathways or elements other than acyl glucuronidation which are in need of further interrogation for appropriate de-risking strategies. Further supporting this notion, Smith et al., (2018) discuss in their commentary alternative mechanisms (irrelevant to AG formation) by which carboxylic acid drugs may cause serious ADRs.

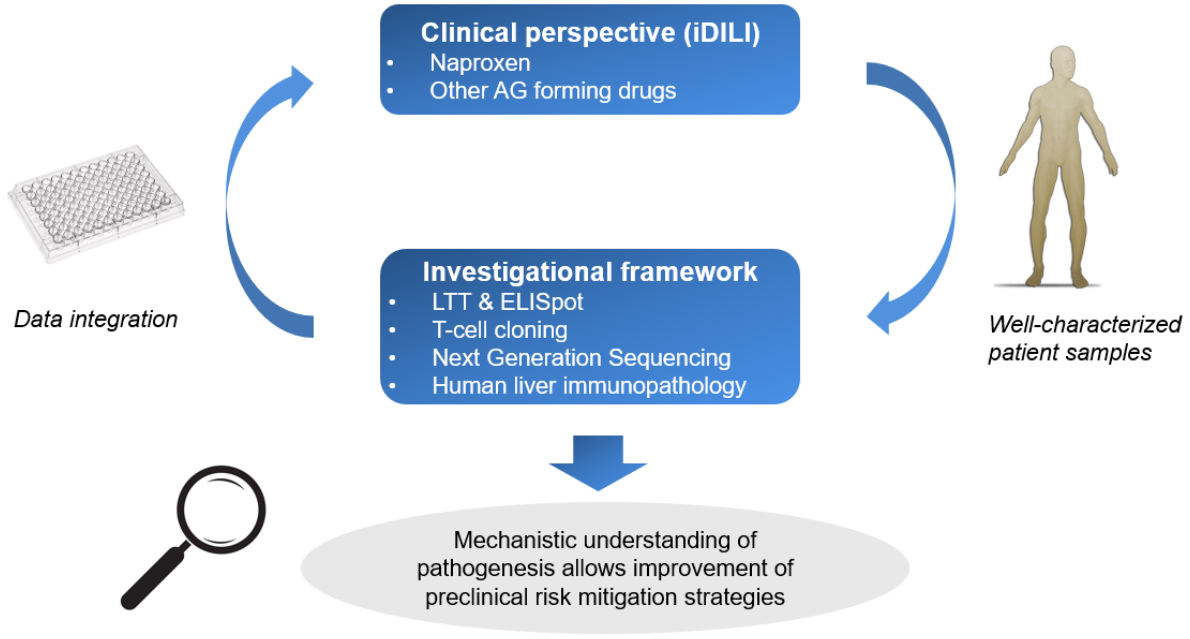


Figure 5.1. Investigational workflow for future research. Additional patients having previously experienced idiosyncratic hepatotoxicity due to naproxen and other drugs known to form AGs as major metabolites need to be recruited and investigated under a common investigational immune-related framework such as the one described throughout this Thesis.

More recently, the FDA issued its latest MIST guidance (FDA, 2020) where AG metabolites are now referred to as ‘potentially toxic’ from previously ‘toxic’ (FDA, 2008). The changed wording presents a shift in mind-set from the original guidance. It is imperative for the pharmaceutical industry and academia to collaborate and push the field further with additional research. Ultimately, this scientific puzzle can only be solved through empowered collaborations and multidisciplinary translational safety investigations. Identifying well-characterized human clinical samples and working across a set of different drugs known to form AG metabolites under a common framework will be key towards generating consistent and insightful evidence. Back-translation and elucidation of clinical IRDs by *ex vivo* investigations can open up the door for a better understanding of structural alerts and improve de-risking strategies, preclinically. Overall, this could help and guide researchers in the early stages of drug development, as well as safeguard patients through introducing the right drug for the right individual in need.

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