The Chemical Synthesis of Sulfonamide Metabolites and their role in Drug Hypersensitivity

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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DECLARATION

I declare that the work presented in this thesis is all of my ow
not been submitted for any other degree.

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CONTENTS

ABBREVIATIONS

ABT 1-aminobenzotriazole

ADR adverse drug reaction

APC antigen presenting cell

B-LCL B-lymphoblastic cell line

BCR B-cell receptor

BSA bovine serum albumin

CBZ carbamazepine

CDR complementarity-determining region

CFA complete freund's adjuvant

COX cyclooxygenase

cpm counts per minute

CRAC calcium release activated calcium channels

cSMAC central supramolecular activation cluster

CTL cytotoxic lymphocyte

CTLA-4 cytotoxic lymophocyte-associated antigen-4

CYP cytochrome P450

DC dendritic cell

DCE dichloro ethane

DMF dimethil formamide

DMSO dimetyl sulfoxide

DNA deoxyribonucleic acid

DNCB 2,4-dinitrochlorobenzene

DNFB 2,4-dinitrofluorobenzene

EBV Epstein-Barr virus

ELISA enzyme-linked immunosorbent assay

ER endoplasmic reticulum

ERK receptor-activated kinase

FBS fetal bovine serum

FITC fluorescein isothiocyanate

FMO flavin-containing monooxygenase

GSH glutathione

GSSG reduced glutathione

HBSS Hank's balanced solution

HEPES N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid

HHV human herpes virus

HIV human immunodeficiency virus

HLA human leukocyte antigen

HMGB1 high mobility group box-1

HPLC high pressure liquid chromatography

HSA human serum albumin

IFN interferons

Ig immunoglobulin

$\mathbf{I_i}$ invariant chain

IL interleukin

ITAM immunoreceptor tyrosine based activation motifs

KC Kupffer cell

J joule

KLH keyhole limpet hemocyanin

LAT linker for activation of T-cells

LPS lypopolysaccharide

LGT lamotrigine

LTT lymphocyte transformation test

MAPK mitogen-activated protein kinase

MEOH methanol

MHC major histocompability complex

MMR macrophage mannose receptor

MPO myeloperoxidase

MS mass spectrometry

NADP oxidised nicotinamide adenine dinucleotide phosphate

NADPH reduced nicotinamide adenine dinucleotide phosphate

NFAT nuclear factor of activated T-cells

NF-κB nuclear factor κB

NK natural killer cell

NMR nuclear magnetic resonance

PAMP pathogen-associated molecular patterns

PBMC peripherial blood mononuclear cell

PCR polymerase chain reaction

PGE prostaglandin

PHT phenytoin

PKC protein kinase C

PPR pathogen pattern recognition

pSMAC peripherial supramolecular activation cluster

RAG recombination activating gene

RNA ribonucleic acid

ROS reactive oxygen species

RPMI Roswell Park Memorial Institute

RT-PCR real time prolymerase chain reaction

SDZ sulfadiazine

SDZ-NHOH sulfadiazine hydroxylamine

SDZ-NO sulfadiazine nitroso

SI stimulation index

SJS Stevens-Johnson sydrome

SMX sulfamethoxazole

SMX-NHOH sulfamethoxazole hydroxylamine

SMX-NO sulfamethoxazole nitroso

SP sulfapyridine

SP-NHOH sulfapyridine hydroxylamine

SP-NO sulfapyridine nitroso

STAT signal transducers and activators of transcription

STIM stromal interaction molecule

TAP transporter associated with antigen processing

TCR T-cell receptor

TEN toxic epidermal necrolysis

THF tetrahydrofurane

TLC thin-layer chromatography

TLR toll-like receptors

UV ultraviolet

PUBLICATIONS

- **Submitted papers**

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- **Published abstracts**

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Elsheikh A., Castrejon JL., Lavergne S., Park BK & Naisbitt DJ. Sulfamethoxazole metabolism by mouse splenocytes creates T-cell stimulatory Hapten proteins complexes (2009). *Br J Clin Pharmacol 68(2), 288.*

Castrejon J.L., Farrell J., C., Maggs JL., O'neill P., Park BK., & Naisbitt DJ. (2008) Stimulation of nitroso sulfamethxazole-specific T cells with structurally-related hydroxylamine and nitroso metabolites. Br J Clin Pharmacol 65(6), 977.

- **Manuscripts in preparation**

Elsheikh A., Lavergne S.N., Castrejon J.L., Wang H., Pichler W., Park BK., & Naisbitt DJ. The immunological consequences of metabolic activation in antigen presenting cells. Implications for drug hypersensitivity. *Manuscript in preparation.*

ABSTRACT

The incidence of sulfonamide allergy in human patients is approximately 3%. Moreover, patients with sulfonamide allergy are at risk of re-activation when using others sulfonamides. Although these reactions appear to be immune mediated, the mechanisms underlying T-cell activation and cross-reactivity are not well resolved. Therefore, the aim of this thesis was to define the mechanism(s) by which sulfonamides and their metabolites interact with and stimulate immune cells, focussing particularly on potential T-cell receptor cross-reactivity with closely related drug structures. T-cell cross-reactivity with sulfonamide metabolites has not been studied previously due the lack of synthetic drug metabolites. Three model sulfonamides (sulfamethoxazole [SMX], sulfapyridine [SP] and sulfadiazine [SDZ]) were selected since (1) they are metabolized to hydroxylamine and nitroso metabolites, (2) human exposure is associated with the development of suspected immune-mediated hypersensitivity reactions and (3) T-cell responses to the parent compounds has been shown previously.

In initial experiments, novel synthetic procedures were developed to synthesize hydroxylamine and nitroso metabolites of SMX, SP and SDZ. The nitroso metabolites were synthesized following a three-step procedure that involved synthesis of nitro derivatives of each compound, catalytic reduction and the liberation of hydroxylamine intermediates and finally, direct oxidation of the hydroxylamine compounds to yield the nitroso metabolites. The compounds were characterized (nuclear magnetic resonance spectroscopy, LC-mass spectrometry, electrospray mass spectrometry) and found to be at least 90% pure.

The immunogenicity of the synthetic hydroxylamine and nitroso metabolites was explored using lymphocytes from hypersensitive human patients and animal models.

Exposure of Balb/C strain mice to repeated i.p. injections of nitroso SMX, SP and SDZ over a two week immunization protocol induced an antigen-specific cellular immune response. Splenocytes from nitroso sulfonamide immunized mice proliferated *in vitro* in the presence of hydroxylamine (5 - 50 μM) and nitroso (1 – 25 μM) sulfonamide metabolites. Cross-reactivity studies revealed that splenocytes from immunized mice were stimulated with nitroso SMX, SP and SDZ. Splenocytes were not stimulated to proliferate with SMX, SP or SDZ. The anti-oxidant glutathione, which is known to prevent protein adduct formation, blocked the nitroso sulfonamide-specific proliferative response. Splenocyte proliferation was associated with the secretion of a mixed panel of Th1/Th2 cytokines.

Lymphocytes from three SMX hypersensitive patients were stimulated to proliferate in the presence of SMX, SP and SDZ and their respective hydroxylamine and nitroso metabolites. Over four hundred SMX and SMX metabolite-responsive T-cell clones were generated from the hypersensitive patients. Three patterns of drug(metabolite)-specific stimulation were seen:

44% were SMX metabolite-specific; 43% were stimulated with SMX metabolites and SMX, 14% were stimulated with SMX alone. Most metabolite-responsive T-cells were stimulated with nitroso SMX-modified protein via a hapten mechanism involving processing. In contrast to SMX-specific clones, that were highly specific, greater than 50% of nitroso SMX responsive clones were additionally stimulated with nitroso metabolites of SP and SDZ, but not nitrosobenzene. Pharmacophore modeling illustrated that the summation of available binding energies for protein interactions and the preferred spatial arrangement of atoms in each molecule, determine a drug's potential to stimulate specific T-cells.

To explore the role of metabolism in antigen presenting cells in the stimulation of antigen-specific T-cells, SMX was incubated with mouse splenocytes for 16 hr and adduct formation measured by ELISA using an anti-SMX antibody. SMX metabolism was concentration dependent with high levels of adducts detected with increasing SMX concentrations. Splenocytes incubated with SMX for 16 h, the time needed to detect adduct formation, stimulated the proliferation of splenocytes from nitroso SMX immunized mice.

In conclusion, nitroso SMX was found to be an important antigenic determinant in experimental animals and hypersensitive human patients. T-cell receptor cross-reactivity with nitroso sulfonamides displaying different sulfonamide side chains show the clear potential for hypersensitivity reactions to develop to different drug structures within the same chemical class through metabolite formation and targeting of identical binding sites on protein. Identification of antigenic SMX metabolite protein adducts when SMX the parent compound was incubated with antigen presenting cells suggests that the ultimate antigen in hypersensitive patients might be generated locally at the site of the adverse drug reaction.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

The introduction will be divided in two main sections. The first section will cover the definition, clinical manifestations and the pathogenesis of drug hypersensitivity reactions. Moreover, the mechanism of antigen presentation and T-cell activation will be reviewed as well. The second section will cover drug metabolism and its role in drug hypersensitivity. Finally, the mechanism of T-cell activation by drugs will be reviewed and specific examples of T-cell activation by drugs will be discussed.

Section I

1.1 Adverse drug reactions

Every drug exhibits a therapeutical effect, through a complimentary binding interaction with a specific agent, and an adverse effect (Edwards and Aronson, 2000). The dose administered in general determines whether drug exposure results in a pharmacological effect or toxicity. Adverse drug reactions (ADRs) range in severity from minor readily reversible unpleasant effects to life threating effect leading to fatalities in some cases. The mechanisms of different forms of drug toxicity have been studied in detail in order to develop effective treatment strategies and prevent future reactions.

1.1.1 Definition

Adverse drug reactions are defined according to Edwards and Aronson as follows:

"An appreciable harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration in the dosage regimen, or withdrawal of the product."

This definition is one of the most complete and accepted, and it also clarifies some of the ambiguities found in previous definitions. For example, the change of the word *drug* for *medicinal product* to take into account possible adverse effect caused by the excipients but not the drug itself, and the use the word *unpleasant* instead of *noxious* to take into account minor side effects. This definition is widely accepted and has been used as a reference for the correct classification in different epidemiologic studies in the UK (Pirmohamed et al., 2004; Davies et al., 2006).

1.1.2 Classification

ADRs are commonly classified into Type A or Type B reactions (Park et al., 1998); however, additional types exist C – D (Edwards and Aronson, 2000).

Type A or augmented reaction. They are consequences of the pharmacological action of the drug, so they can be prevented and dose-dependent. These reactions are commonly reversible upon withdrawal of the causative drug. An example of a Type A reaction is postural hypotension induced by β-blockers.

Type B or Bizarre reactions. They cannot be predicted by the basic knowledge of the drug's pharmacology, thus they cannot be prevented. Host factors, immunological or metabolic, seem to play an important role, but this has not been fully demonstrated. A clear example is Penicillin anaphylaxis or clozepine induced agranulocytosis.

Type C or chemical reactions. These reactions can be explained by the chemical structure of the drugs or their metabolites. Paracetamol hepatoxicity is a well-defined example.

Type D or delayed. Includes carcinogenic or teratogenic reactions that often appear after drug treatment.

Type E or end of treatment reactions. These reactions occur after removal of the drug, and especially following sudden withdrawal. A clear example is the adverse effect seen after withdrawal of anxiolytics.

During my thesis I will focus on the mechanism associated with the development of Type B reactions commonly known as *bizarre* reactions. These reactions are uncommon, not related to the pharmacology of the drug and they are unpredictable.

Many type B reactions are believed to be immunological and these reactions can be further classified based on the cells and effectors molecules into four types (I-IV) according to the Coombs and Gell classification.

Many drugs are associated with the development of type B reactions; however the same drug can activate different types of immunological responses in different individuals. Therefore, they cannot be completely classified into one of the different sub-divisions proposed by Coombs and Gell. Our knowledge of this type of reaction has increased during the past twenty years, and now we have a better understanding of the immune components involved (phenotype of cells and cytokines secreted). Thus, the classification of the Type IV reactions, which are cell –mediated, have been divided into four variants (a – d) based on the phenotype of the T-cells involved, the release of effector molecules and clinical symptoms (Pichler, 2003). The revised Gell and Coombs classification is described in table 1.1.

Certain adverse drug reactions cannot be explained by the immunological mechanism described in table 1.1. or any other mechanism. These reactions are known as off-target pharmacological reactions. Off-target reactions are seen at low dose, are unpredictable and are not related with the primary pharmacological properties of the drug. Some drugs can interact with different target to which they were designed to act. Interestingly, this can contribute to the pharmacological action of the drug, but it also might lead to adverse drug reactions. These reactions have found in anorectic drugs (Peters et al., 2009),

antiepileptic drugs (Zaccara et al., 2007) and anticancer therapy (Fernandez and Sessel, 2009).

Type	Type of immune Response	Pathologic Characteristics	Clinical Symptoms	Type of drug Binding	Cell type
Type I	IgE	Mast-cell degranulation	Urticaria and anaphylaxis	Covalent	B-cells
Type II	IgG and FcR	FcR-dependent destruction	Blood cell dyscracia	Covalent	B-cells
Type III	IgG and FcR or complement	Immuno-complex deposition	Vasculitis	Covalent	B-cells
Type IV a	Th1 (INF-γ)	Monocyte activation	Eczema	None and covalent	T-cells
Type IV b	Th2 (IL-5 and IL-4)	Eosinophilic inflammation	ME, BE	None and covalent	T-cells
Type IV c	CTL (perforin and granzyme)	$CD4^+$ or $CD8^+$ cytotoxicity	ME, eczema, BE and PE	None and covalent	T-cells
Type IV d	T-cells (IL-8)	Neutrophil recruitment and activation	PE	None and covalent	T-cells

ME = Maculopapular exhantema, BE = Bullous exhantema, PE = Pustular exhantema

Table 1.1 Extended classification of Gell and Coombs describing the type of immune response, pathology, clinical symptoms, binding and cells involved in these types of reactions (adjusted from Pichler, W., 2003).

1.1.3 Epidemiology

Several studies estimate the percentage of patients hospitalized due to the development of an ADR. These are not conclusive because of the different criteria used to define the ADR, populations of the study, and the methodology for the analysis (Gomes and Demoly, 2005).

A study by Lazarous et al in 1998 in the USA based on the analysis of four electronic databases between 1966 – 1996 showed that the incidence of severe ADRs in patients admitted to Hospital, or those who suffer an ADR during hospitalization was 6.7% with an incidence of fatal reactions of 0.37%; the total incidence of ADR (serious and non-serious) was 15.5%. Based on the incidence estimated by meta-analysis, the authors calculated that the fatalities caused by ADR in 1994 were 76,000, being in that year the fourth cause of death in the USA (Lazarou et al., 1998). This study has been disputed because the estimation of the fatalities in 1994, when ADRs were the fourth cause of death in the USA, was calculated by extrapolating results of incidence of ADRs obtained from years before (< 1988).

More recently, Pirmohamed calculated the incidence of ADR based on the record of admission in two large hospitals in the UK. The study was performed in patients above 16 years of age. During a period of sixth months, a total of 18,829 admissions were recorded and 1225 of the total were classified as ADR related using the Edwards and Aroson definition. The overall incidence of ADR was 6.25% with 0.15% of the total admissions leading to death. The vast majority of reactions were classified as Type A (95%), being women and the elderly people particularly susceptible to the development of ADRs. It was also estimated that the total ADR related cost for NHS was £ 466 m based on the cost of the number of days that the patient stayed in the hospital, which in these two hospitals was 8 days. Recently, an additional UK based study in 2006 found that 24 out 125 (19.2 %) patients experienced one or multiples ADR. These data seems contradictory with previous reports that estimated that the percentage of ADR ranges between 3.5 – 7.4 % (Davies et al., 2006).

The high variability of the results obtained in the estimation of the incidence of ADRs creates an unclear picture of what is the real impact of these reactions is in the General Health System. The high variability may be attributed to the different methodologies used in the analysis, the criteria used to determinate the ADRs and the possible miss-clasification. Nevertheless, besides the variability obtained in the different studies, it is generally accepted that 6 - 10% of the total

admission to hospitals are caused by ADRs and that the majority of them (85 – 90%) were labeled as Type A.

Type B reactions with an immunological involvement are normally referred in the literature as idiosyncratic drug reactions or hypersensitivity drug reactions. However, according with the immune mechanism involved they can be categorized as either immediate (IgE) or delayed (T-cell) hypersensitivity complicating the definition.

Unfortunately, even though the mechanism of these reactions has been studied, it is not possible to have a clear understanding of the pathogenesis, this in turn complicates even further its clear definition. Nevertheless, during this thesis the term drug hypersensitivity will be used to avoid misunderstandings. In this context, drug hypersensitivity reactions are immunological mediated by T-cells, are not related with the known pharmacology of the drug and do not occur in the majority of patients.

1.1.4 Clinical manifestations in drug hypersensitivity

For the correct diagnosis of drug hypersensitivity reactions the clinical manifestations are important. In the following section, I will describe the clinical manifestation normally seen and in a further section the different lesions in the skin will be described. Finally, the drug hypersensitivity syndrome will be discussed in a separate section.

It is estimated that symptoms develop from 2 to 6 weeks after taking the causative drug. In most cases of drug hypersensitivities, the symptoms disappeared when the drug was withdrawn. Patients present mild-grade to high-grade fever from 38 - 40° C. Skin eruptions are present in almost all the patients (> 87%) (Knowles et al., 2000).

The vast heterogenecity of the observed skin eruptions represent an important challenge for the accurate diagnosis of drug hypersensitivity reactions. The most common eruptions affecting the skin are of the exhanthematous or maculo-

papular type (Yawalkar, 2005), but in some cases more severe and life threating skin disorders such as Stevens-Johnson's Syndrome (SJS) and toxic epidermal necrolysis (TEN) have been reported (Roujeau and Stern, 1994; Sullivan and Shear, 2001; Roujeau, 2005).

Recently, it has been suggested that a relationship exists between reactivation of different viruses e.g. (HHV6) and drug hypersensitivity. However convincing evidence relating viral-specific T-cell activation to the clinical manifestations of drug hypersensitivity is yet to be established (Suzuki et al., 1998; Shiohara and Kano, 2007). The clinical manifestations of drug hypersensitivity are diverse and the involvement of many organs in addition to the heterogeneity of the skin lesions makes it difficult to diagnose. Although the skin is the primary organ affected during drug hypersensitivity (Merk et al., 2007; Merk, 2009), other systemic organs are involved such as the liver, kidney, central nervous system and the lungs (Knowles et al., 2000).

1.2 Cutaneous drug induced hypersensitivity reaction

1.2.1 Maculopapular eruptions

Maculopapular eruptions are the most common drug induced skin reaction accounting for approximately 90% of all cutaneous drug eruptions (Yawalkar, 2005). They appear between 4 – 14 days after taking the suspected drug and are normally localized into the trunk and upper extremities with no detectable mucosal membrane involvement (Roujeau, 2005).

Histological studies show an accumulation of lymphocytes at the dermoepidermial junction. Immunohistological characterization revealed that the infiltrating cells were $CD3^+$ with the majority expressing the $CD4^+$ receptor. Up to 20% of $CD4^+$ cells have a cytotoxic capacity mediated mainly by drug-specific release of perforin and granzyme granules (Yawalkar et al., 2000a; Bronnimann and Yawalkar, 2005). Moreover, T-cells liberate high amounts of IL-5 and eotaxin responsible for the recruitment, growth and differentiation of eosinophils (Yawalkar et al., 2000c; Yawalkar and Pichler, 2001; Friedmann et al., 2003; Naisbitt, 2004).

1.2.2 Acute generalized exanthematous pustulosis

Acute generalized exanthematous pustulosis (AGEP), a disease caused mainly by drugs (90%), is characterized by numerous dermis and subcorneal sterile pustules, fever (> 38° C) and high neutrophils count. Lesions appear normally 2 days after the initiation of the treatment (Roujeau, 2005). Histological and immunophenotyping findings show that pustular cells secrete neuthropil elastase. The infiltrating cells were T-cells of which 60-70% were $CD4^+$ and 30-40% $CD8^+$ phenotype. Immunohistochemistry demonstrated the presence of high amounts of IL-8 and RANTES in patients with AGEP when compared with healthy volunteers, and additionally T-cell lines generated from these patients secreted high amounts of the same cytokine (IL-8) and GM-CSF with low levels of IL-5 (Britschgi et al., 2001). IL-8 is an important and potent neutrophil attractor, and seems to play a key role in AGEP because the cytokine, which is secreted by keratinocytes and infiltrating T-cell in AGEP patients, is not detected in any other drug induced skin disorder (Pichler, 2003).

1.2.3 Steven- Johnson syndrome and toxical epidermal necrolysis

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are the most feared skin disorders caused by drugs due to their high mortatility rate (10% and 30% respectively). The incidence is low (6 in 10^6 and 2 in 10^6 respectively) (Roujeau and Stern, 1994; Roujeau, 2005); both reactions are characterized by the formation of small blisters and skin detachment that in SJS is less that 10% of the skin coverage. In TEN at least 30% skin detachment is seen. Mucosal membranes are damaged in both cases (Borchers et al., 2008). Recently, it has been found that patients with a particular HLA phenotype (HLA-B*1502), being predominantly expressed within the Han Chinese population, are predisposed to the development of carbamazepine induced SJS (Chung et al., 2004; Pirmohamed, 2006).

Skin biopsy studies demonstrate the presence of lymphocytes with higher count of activated $CD8^+$, compared with the $CD4^+$ phenotype, expressing skin homing

receptors including cutaneous lymphocyte antigen (CLA). Additionally, blister fluid cells from TEN patients were cytotoxic against autologus activated (with INF-γ) keratinocytes or EBV-derived B-cell lines and the drug or metabolite responsible for the reaction (Nassif et al., 2002a; Nassif et al., 2004). The presence of apoptotic keratinocytes in drug induced SJS/TEN blisters seems to be mediated by perforin/granzyme, but Fas-mediated apoptosis might contribute to the extension of keratinocytes apoptosis (Murata et al., 2008).

The mechanism of massive keratinocyte death, a hallmark in these diseases, remains unclear. In a recent study (Chung Wen-Hung 2008), blister cells from SJS-TEN patients were screened genetically for the presence of soluble markers that might be responsible for the dissemination of necrosis in SJS-TEN. Gene expression from blisters cells compared with PMBCs both from patients showed that expression of granulysin, a cationic cytolitic protein of 15-kDa, in blisters was 20 fold higher than in PBMCs; the levels of granzyme B/perforin and FasL were 8 and 2 fold high respectively. Similar results were seen by RT-PCR. The expressed protein (granulysin) was detected by immunohistochemistry and ELISA analysis with similar results. Finally, granulysin cytotoxicity was corroborated by in-vitro and in-vivo. The authors suggest that granulysin might be responsible for the massive keratinocytes apoptosis in SJS-TEN.

1.3 Drug hypersensitivity syndrome

The drug hypersensitivity syndrome is a disease caused by drugs and was first found in patients taking anticonvulsants. Therefore, it was called anti-convulsant hypersensitivity syndrome (Shear and Spielberg, 1988). Nevertheless, other drugs (e.g. sulfonamides) have been associated with the same syndrome (Roujeau et al., 1995). It is characterized by fever, skin eruptions and internal organ involvement along with hematological abnormalities (hypereosinophilia and lymphocytosis) (Roujeau et al., 1995; Knowles et al., 2000; Kardaun et al., 2007). It has been estimated that the prevalence for this syndrome caused by

anti-convulsants and sulfonamides is 1 in 1000 and 1 in 10000 exposures respectively (Knowles et al., 2000).

Eosinophilia is a common clinical manifestation found in some patients with drug hypersensitivity syndrome. Different studies have demonstrated high eosinophil counts and high levels of the cytokines involved in the recruitment and maturation of eosinophils (IL-5 and eotaxin) in patients with a drug related skin eruption (Mauri-Hellweg et al., 1995; Choquet-Kastylevsky et al., 1998; Yawalkar et al., 2000c). It was demonstrated that the cells responsible for the secretion of IL-5 were mainly lymphocytes. This in agreement with previous studies that have found that T-cell clones generated from drug hypersensitive patients secrete high amount of IL-5 when were re-challenged with the causative drug (Mauri-Hellweg et al., 1995; Pichler et al., 1997). Multi-organ involvement is another important characteristic in the diagnosis of drug hypersensitivity syndrome and is not present in other drug inducing disease such as drug-induced-lupus or serum-sicknesss like reaction.

Recently, new acronyms were proposed to define more accurately this hypersensitivity syndrome: DIDDM-OHS for 'drug induced delayed multiorgan hypersensitivity syndrome' or DRESS for ' drug reaction with eosinophilia and systemic symptoms' (Bocquet et al., 1996). The use of the acronym DRESS was introduced to highlight the finding of high count of esoinophils in patients diagnostic with the hypersensitivity drug syndrome (Roujeau, 2005). In the case of DIDDM-OHS the authors believed that the characteristic delayed setting of the syndrome was not properly mentioned in the definition, so they proposed the DIDDM-OHS acronym.

1.4 Pathogenesis of hypersensitivity reaction to drugs

Clinical manifestations, the presence of antibodies found in the peripherial circulation that recognize different drugs, and the generation and characterization of T-cell clones (TCC) from patients hypersensitive to different

drugs e.g. (Penicillin G, sulfamethoxazone, lidocaine and carbamazepine) should be used in combination for the diagnostic of drug hypersensitivity. The following section describes the three main hypotheses that have been formulated to try to explain the pathogenesis drug hypersensitivity.

1.4.1 The hapten hypothesis

The immune system recognizes peptides that have a molecular weight around 1000 Da; however, many chemicals and drugs have molecular weights below any immunogenic peptide (Park et al., 1987; Pohl et al., 1988; Park et al., 2001). Thus, according with this restriction a drug or chemical would need to be attached to a carrier molecule to become immunogenic. Jacobs and Landstainer proposed in 1935 that small chemical could not induce an immunogenic response unless it was reactive and attached to a carrier protein setting direct evidence for the Hapten Hypothesis (Landsteiner and Jacobs, 1935b).

Penicillins are a clear example to illustrate that the hapten hypothesis can be applied directly to drug allergy. In this case, the drug, which is reactive under normal conditions, binds with free lysine residues found in proteins forming a protein – drug adduct that elicits a immune response characterized by the formation of specific antibodies and T-cells. Nevertheless, the majority of drugs are not reactive under normal conditions, so they need to be metabolized to conjugate with proteins. During metabolism (as explained in detail in the following sections) many drugs are activated by the addition of functional groups that lead to the formation of reactive metabolite which can bind irreversible to proteins. Halothane is a classical example of a drug that requires metabolic activation to induce an immunological response. In this case, the drug un-reactive under normal conditions is metabolized in the liver to form a trifluroacetyl chloride metabolite that is highly reactive and covalently binds with several proteins giving rise to the formation of antibodies to either the drug-protein conjugates or self-proteins.

The hapten hypothesis explains successfully how small drugs can become immunogenic by binding with carrier proteins, but recent evidence suggest that no reactive drugs also induce an immune response without previous biotransformation. These findings will be discussed in detail later section.

1.4.2 The danger hypothesis

During lymphocyte maturation, immature T-cells that strongly recognize self-proteins presented by DC receive the signal to die. This process called negative selection prevents the circulation of highly auto-reactive T-cells, so just T-cells that recognize other molecules (foreign) would be resting waiting for antigen encounter and activation (Germain, 2002; Palmer, 2003; Kyewski and Klein, 2006). The self – non-self hypothesis has successfully explained the mechanism by which the immune system is selectively activated. However several facts such as the presence of foreign entities (gut bacteria) in the body that not induce an immune response or the necessity of use adjuvants together with proteins to activate the immune system challenge this theory. These and other not fully applicable inconsistencies to the self – non-self hypothesis led Polly Matzinger to propose a new hypothesis called the Danger Hypothesis. Matzinger states that the immune system is not focused in the nature of the antigen (self or non-self), but whether or not the antigen is presented in the presence of dead or stressed cells. If an antigen is presented in the presence of danger signals, the immune system would respond by the activation of DC and a positive T-cell interaction. In contrast, if the antigen is present in the absence of danger, the DC would not be activated and T-cells would not receive co-stimulatory signals (Matzinger, 2002a; Matzinger, 2002b). Experimental data have shown that these potential danger signals, which are normally confined within the cell, are DNA-associated HMGB1 protein, uric acid or heat shock proteins (Pirmohamed et al., 2002; Kono and Rock, 2008). Nevertheless, some authors questioned the origin of these these signals arguing that they come from trace of undetectable contaminating endotoxin (Vance, 2000).

Drugs, especially their metabolites, are toxic and can cause stress to cells. Therefore, it has been suggested that drug reactive metabolites could induce the

release of danger signals that would activate DC providing the co-stimulatory signals for T-cell activation (Pirmohamed et al., 2002; Seguin and Uetrecht, 2003; Uetrecht, 2007). Recently it has been demonstrated that nitroso sulfamethoxazole, a highly reactive and toxic metabolite, induces up-regulation of the CD40 co-stimulatory receptor possibly by the generation of stress in the cell (Sanderson et al., 2007). More research is needed, and the bridge between drug reactive metabolites, viral infection and the release of danger signals needs to be established.

Matzinger proposed that tissues rather than cells would deliver signals to activate the immune system (Matzinger, 2007). If this hypothesis is true and tissues give different signals to T-cell to initiate a response or be tolerant this could explain why the skin and not other organs such as the liver, which is responsible of generation of reactive metabolites and in consequence target for toxicity, is the organ most affected by drug hypersensitivity. It is possible that the skin is more "sensible" and therefore is constantly releasing danger or helping signals. Skin does not have the same protective mechanism that other organs have (live) and it is also constantly in contact with external making it more sensible than others organs. A recent study (Martin et al., 2008) suggests that the delivery of endogenous signals by the skin play an important role in the induction of allergic contact hypersensitivity to 2,4,6-trinitro-1-chlorobenzene (TNCB). The authors found that TLR-4 and IL-12 double knockout mice did not develop allergic reaction after being sensitized and re-challenge with TNCB. Additionally, when the experiment was repeated with germ-free double or single knockout mice using endotoxin-free TNCB under germ free conditions, ear-swelling responses were seen in knockout IL-12 (positive TLR-4), but not in double knockout mice. It was concluded that an endogenous rather than an exogenous adjuvant such as bacteria flora, which can be found in the skin, is responsible for the activation of TLR-4 enhancing the allergic contact reaction. Finally, the authors suggested using inhibition assays that hyaluronic acid (HA), a natural polysccharide, was the endogenous molecule responsible for the activation of TLR-4.

1.4.3 The P-I concept

The latest hypothesis formulated to explain the pathogenesis of drug hypersensitivity reactions is the "Pharmacological – Interaction" of drugs with immune receptors (P-I concept). Professor Pichler proposed the P-I concept almost 10 years ago based on studies on T-cell activation with different drugs but especially with sulfamethozaxole. SMX specific T-cell clones were found to proliferate only in the presence of the soluble drug (Mauri-Hellweg et al., 1995), and APC pre-pulsed with the parent drug not induce a response (Schnyder et al., 2000). The fast kinetic in T-cell activation measured by the release of intra-cellular calcium, $CD3^+$ down regulation and ERK protein phosporylation can only be explained by an immediate activation of the T-cell without previous antigen processing (Zanni et al., 1998a; Depta et al., 2004). Moreover, fixed APC, which have lost their capacity for processing antigens but are still functional, present the drug to T-cells (Schnyder et al., 1997). Finally, the addition of glutathione to PMBCs and T-cell clones enhances instead of inhibits the proliferation when the cells were incubated with the nitroso sulfamethoxazole (Engler et al., 2004). The number of T-cell clones specific for the soluble form of the drug was higher that the number that recognized the metabolite (Schnyder et al., 2000). All this evidence lead to the conclusion that certain drugs interact specifically and reversibly with the highly diverse T-cell receptors found in the host and that the inert drug per se can activate the immune system without metabolism or processing (Pichler, 2003; Gerber and Pichler, 2006).

Drugs are chemically diverse, and this diversity could be responsible for the different mechanism involved in the pathogenesis of the drug hypersensitivity reactions. The three hypotheses together may help us to understand the complex mechanism involved in the pathogenesis of hypersensitivity reactions (see figure 1.1); still many questions remain unanswered.

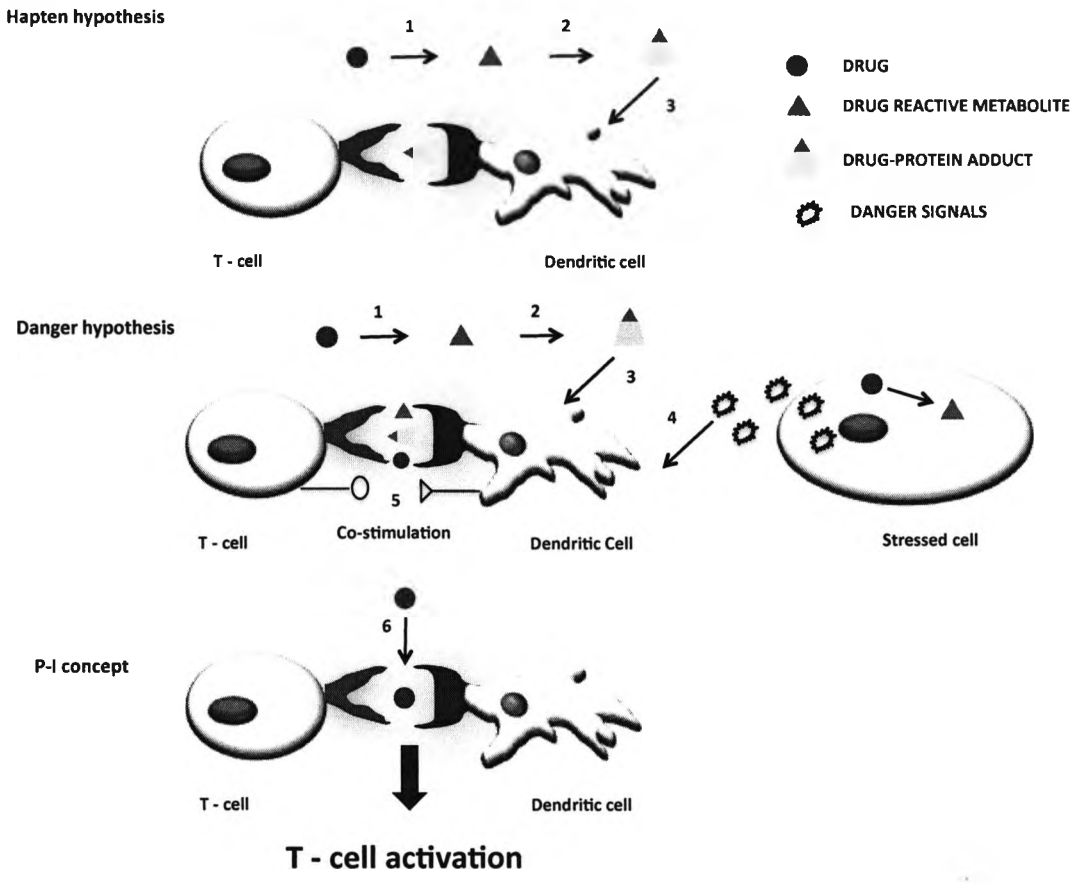


Figure 1.1 Three main hypotheses explain the pathogenesis of drug hypersensitivity. In the hapten hypothesis, the drug is metabolized (1) to a reactive compound that binds with proteins (2) and the drug-protein adduct is internalized, process and presented to T-cells by APC (3). In the danger hypothesis, the drug-protein adduct is presented to specific T-cells (1-3) and the reactive metabolite induces cell damage (4) generating danger signals which induce up-regulation of co-stimulatory receptors (5). In P-I concept, the drug without metabolism or processing interacts directly by a labile weak interaction with the MHC-TCR complex (6) (Adjusted from Uetrecht, J., 2007).

1.5 The immune system and drug hypersensitivity

1.5.1 Innate immunity

The innate immune system is the first line of defense of the body against infections and the response is shared with plant and animals (Janeway and Medzhitov, 2002). Different types of cells compose the innate immune system: neutrophils, macrophages, eosinophils, NK cells, mast cells, and dendritic cells. These cells can be activated during inflammation and the secretion of effector molecules aids the elimination of infections agents (Janeway and Medzhitov, 2002). If the innate immune system cannot eliminate the infectious agent, then the adaptative arm of the immune system is activated. This is achieved by the presentation of the antigen by mature antigen presenting cells, a cell component of the innate immune system, to naïve T-cells.

Dendritic cells, which are the only APC capable to induce primary immune responses, have the function to take up, process proteins, and present peptides to naïve T cells in the lymphoid organs (Banchereau et al., 2000; Lanzavecchia and Sallusto, 2001a). It is generally accepted that DC exist in two states (mature or immature), although a third stated called semi-immature has been proposed (Lanzavecchia and Sallusto, 2001b; Lutz and Schuler, 2002).

Inmmature DCs (imDC) are found in most tissues in a resting state; however, they have high mobilizing capabilities and are continuously travelling through the body looking for antigens and signs of damage (Sallusto and Lanzavecchia, 1999; Randolph et al., 2005). They have high enodocytotic activity constantly taking up antigens, a process that occurs without full activation keeping them in these organs without migrating to secondary lymphoid organs (Guermonprez et al., 2002). Phenotypically, imDCs express low levels of co-stimulatory receptors such as MHC-II, CD40, CD80 or CD86 (Lutz and Schuler, 2002)(Figure 1.2). Presentation of antigens to T-cell found in peripheral organs such as the skin does occurs (Kupper and Fuhlbrigge, 2004). However, T-cells are not activated, because the absence of co-stimulatory signals provide by the DC leading to T-cell anergy (Schwartz, 2003).

imDC can take up antigens by different methods such as receptor-mediated endocytosis or phagocytosis and macropinocitosis (Guermonprez et al., 2002; Kapsenberg, 2003; Steinman et al., 2003) (Figure 1.2). In receptor-mediated endocytosis, different molecules are recognized and internalized by different number of membrane receptors such as FcR, heat shock protein receptors, scavenger receptors, macropaghe mannose receptor (MMR), DEC205 and ICAM-3. The last three belonging to the C-Type Lectin family, which are found in different populations of imDC. During phagocytosis and macropinocitosis antigens are captured forming intracellular vacuoles. Macropinocitosis is an unspecific method of internalization of antigens in which large amounts of liquid is taken up with the soluble antigen (Sallusto et al., 1995), whereas phagocytosis is receptor mediated and more specific (Bell et al., 1999). In this case, after interaction with specialized receptors the DC changes and engulfs the soluble antigen. This process accounts for the internalization of several gram +/- bacteria (d'Ostiani et al., 2000; Rescigno et al., 2001) and apoptotic or necrotic bodies (Gallucci et al., 1999; Subklewe et al., 2001) of different types of cell.
Once these cells are activated, they will migrate to the lymph nodes to present the antigen and during this journey the DC changes to a mature phenotype (Itano and Jenkins, 2003; Randolph et al., 2005). The process where DC become mature is discussed in the following paragraphs after describing the phenotype and functionality of mature dendritic cells.

Mature DCs (mDC) lose phagocytic activity and up-regulation of different receptors vital for co-stimulation (MHC-II, CD40, CD80 and CD86) is evident adding to morphological changes (Guermonprez et al., 2002; Trombetta and Mellman, 2005)(Figure 1.2). A failure in the activation of DCs would lead to an inappropriate response from the immune system to eliminate the host or induce tolerance (Lutz and Schuler, 2002; Moser, 2003). After being activated mDC will travel to the lymphoid organs (spleen and lymph nodes) (Randolph et al., 2005) where they will prime T-cells that would undergo clonal expansion. For complete activation of the immune system, co-stimulatory receptors signals (CD80 and CD86) interact with CD28 or CTLA-4 receptors expressed on T-cells (Appleman and Boussiotis, 2003; Greenwald et al., 2005).

DC maturation is triggered when imDCs are stimulated by different methods such as: interaction with pathogen related molecules (LPS or bacterial RNA and DNA), inflammatory signals (TNF, IL-1, IL-6 or PGs) or T-cell associated signals (CD40L) (Janeway and Medzhitov, 2002; Kapsenberg, 2003)(Figure 1.2).

Pathogen-related molecules are recognized by so-called pathogen-associated molecular patterns (PAMP). These are molecules found in the membrane surface of all cells from the innate immune system that recognize structures conserved in microorganisms (LPS, peptidoglycan, bacterial DNA and RNA, glucans, etc) (Medzhitov and Janeway, 1997b; Medzhitov and Janeway, 1997a; Janeway and Medzhitov, 2002). Toll-like receptors (TLR) are the most important group of PAMP responsible for the transduction of these signals in mammals. The Toll receptor was found first in drosophila and plays a vital role of immune defense in drosophila against fungi and gram positive infections (Lemaitre et al., 1996). Drosophilas lacking this receptor were unable to fight different infections. The Toll receptor shares structural similarities with the IL-1 receptor found in mammals. IL-1 is responsible the activation of the transcriptional factor NFκ-b, the factor responsible for various inflammatory and immune responses (Medzhitov et al., 1997). After the discovery of these receptors, similar receptors were found in humans. Ten (TLR 1- 10) different types of these receptors have been found in man and can be classified according to their amino-acid sequences (Rock et al., 1998; Takeda and Akira, 2005). These receptors have different affinity for different compounds. The first TLR found was the TLR-4. This receptor is involved in the activation of genes responsible in the production inflammatory cytokines. It was further discovered that this receptor recognized lypopolysaccharide (LPS) a major component cell wall component in gramm-negative bacteria. (Takeda et al., 2003). The specifities of the different families are: *TLR-2* binds lipopeptides, *TLR-3* binds double strain RNA, *TLR-5* binds flagellin, *TLR-6-9* bind nucleic acids and heme motifs (Table 1.2) (Takeda and Akira, 2005; Pancer and Cooper, 2006).

Toll-like receptor	Known ligand
TLR-1	Lipoproteins (with TLR-2)
TLR-2	Lipoproteins, peptydoglycan and lypoteichoic acid
TLR-3	Double-stranded DNA
TLR-4	Lyopopolysccharide
TLR-5	Flagellin
TLR-6	Dyacil lipopeptides (with TLR-2)
TLR-7	Single-stranded RNA (mice)
TLR-8	Single-stranded RNA
TLR-9	Unmethylated CpG DNA

Table 1.2 Specificity of TLR towards different ligands (Taked and Akira, 2005)

DC activation is also achieved by different inflammation cytokines such as TNF-α, IL-1β or PGE2. Moreover, $CD4^+$ cells can induce direct activation of DC by the interaction of the CD40L with the CD40 receptor found in DC.

Additionally, maturation of DC can also be induced by molecules (endogenous adjuvants) secreted from either necrotic or apoptotic cells *in-vitro* or *in-vivo* (Kono and Rock, 2008).

In-vitro activation has been widely studied. It has been demonstrated that bone-marrow derived immature DC up-regulate MHC-II, CD80, CD86 and CD40 when they were under stress (caused by mechanical manipulation). Additionally, when resting DCs were incubated with healthy, necrotic or apoptotic synergetic fibroblasts, maturation (measured by up-regulation of different markers) was only achieved in the co-incubation with necrotic cells (Gallucci 1999). However, recently it has been also found that apoptotic cells induce dendritic cell maturation (Janssen et al., 2006).

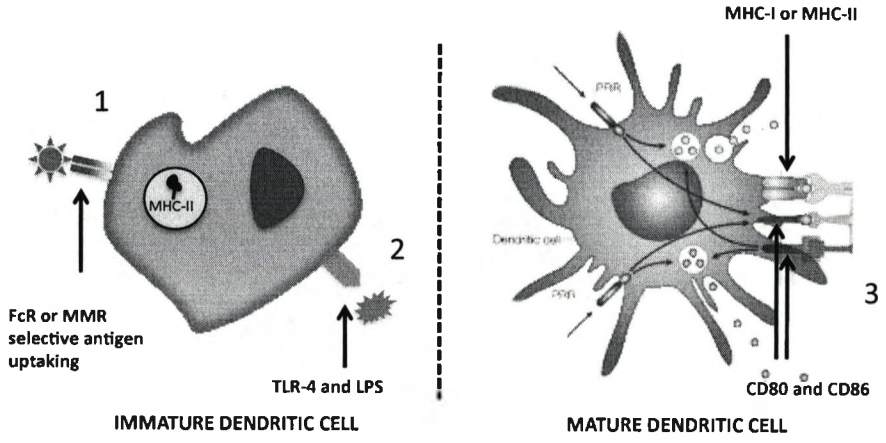
Shi demonstrated that the activity of CTL against mice cells expressing OVA-specific T-cell receptors was increased when naïve Balb-c mice were primed with OVA and injured cells (UV or olygomicyn treated). The injured cells increased the cytotoxic activity of CTL cells by possibly increasing the

concentration of endogenous adjuvants released by the injured cells (Shi et al., 2003).

The maturation of bone-marrow derived DC induced by haptens such as nickel chloride ($NiCl_2$), nickel sulfate ($NiSO_4$), di-nitrochlorobenzene (DNCB) or di-nitrofluorubenzene (DFNB) has been described in detail. These molecules, being strong contact sensitizers, can induce DC maturation measured by the up-regulation of co-stimulation molecules (CD80, 83, 86 and MHC-II) (Aiba et al., 1997). It has been shown that these haptens induce phosphorylation of p-38 MAPK and NF-κb leading to the activation of maturation markers CD80, 83, 86 and HLA-DR. Co-incubation with a p-38 inhibitor blocked the up-regulation of some, but not all maturation markers indicating that other signaling pathways might also be involved in DC maturation (Arrighi JF et al., 2001; Aiba et al., 2003).

In the case of drug hypersensitivity the majority of the research has focused on the interaction between the drug and the T-cells. Until recently, the early events in the initiation of the immune response (activation of the innate immune system), and its possible consequences in the pathology of drug hypersensitivity had not been elucidated. Rodriguez-Pena et al (2006) have shown that drug specific maturation of monocyte-derived DC from patients with a history of maculopapular exanthema associated with by amoxicillin exposure. Incubation with amoxicillin was associated with significant increases in HLA-DR, CD80 and CD86 expression in hypersensitivity patients but not healthy volunteers. The treated DC were able to induce T-activation skewed to a $CD4^+$ phenotype showing a full functional activity (Rodriguez-Pena et al., 2006). The authors concluded that amoxicillin induces a semi-mature DC phenotype, which is characterized by DC with up-regulation of co-stimulatory receptors but no secretion of pro-inflammatory cytokines. An additional study by Sanderson et al (2007) focused on the effect of the maturation of monocyte-derived DC when incubated with sulfamethoxazole and its major oxidative metabolite (SMX-NO). In this study, sulfamethoxazole (200-500 μM) and SMX-NO (10-20 μM), which

did not cause cell death, up-regulated the expression of CD40, but not other surface markers such as CD80, CD83, or CD86 in monocyte-derived DC.



Immature dendritic cells	Activation	Mature dendritic cells
Tissue resident	LPS or bacterial DNA	Migratory
High intracellular MHC-II	Cytokines	Cell shape: dendrites
Expression of CD1a	Viral dsDNA	High MHC class I and II
Active endocytosis and phagocytosis	Danger signals (Apoptotic/necrotic cells)	High co-stimulatory molecules (CD40/80/86)
Low/absent cos-stimulatory molecules (CD 40/54/80/86)	T-cell ligation with CD40L	Secretion of pro-inflammatory cytokines (IL-12, IL-1β and TNF-α)
T-cell anergy	Chemicals and drugs	T-cell activation

Figure 1.2 (1) Immature dendritic cells can internalize antigens using different receptors (FcR or MMR) which are further processed and presented. (2) Immature DC become mature in the presence of pathogen related molecules (LPS or bacterial RNA and DNA) recognized by pathogen-associated receptors (TLR). (3) Mature dendritic cell present the antigen in association with MHC – I or – II molecules. Additionally, mDC present high concentration of co-stimulatory molecules contributing to T-cell activation. The differences between imDC and mDC and the factors responsible for DC maturation are shown (Adjusted from Banchereau et al., 2000, Lutz et al., 2002 and Kapsenberg et al., 2003).

1.5.2 Adaptive immunity

Due to bacterial challenge during evolution, mammals need an effective way of protecting against different infectious agents (Cooper and Alder, 2006). This was achieved by the diversification in the recognition of the different antigens. As described previously, the innate immune system is capable of recognizing antigens through recognition of highly conserved patterns (PAMPs) found in bacteria using PAMPs receptors. However, this recognition is limited, and the pathogens have evolved and found different ways to avoid recognition. To overcome this pathogen versus host race, the immune system evolved to meet the levels of defense needed by the improvement of the system of defense to a more complex system, which is called the adaptive immune system (Pancer and Cooper, 2006).

The adaptive immune system has a wide variety of receptors formed randomly by re-arragements of the immunoglobulin V, D, J gene segments by recombination activating gene (RAG 1-2) enzymes leading to the expression of a unique and specific receptor in the membrane of their cells (Jung et al., 2006). The triggering of these receptors initiates the adaptive response characterized by the involvement of B-cells (humoral immunity) or T-cells (cell-mediated immunity) that are the most important effector cells of the adaptive immune system. They differ from the cells from the innate immune system (neutrophils, macropaghes, dendritic cells) in their life span. Additionally, memory T-cells, a subset of the T-cell repertoire, have long life span and divide rapidly on re-challenge (Krammer et al., 2007).

Adaptive immunity allows the host to fight against the constant threat of bacterial infection by the generation of cells (B or T cells) with a highly diverse and unique receptor. These receptors can bind with soluble or membrane associated antigens leading to the activation of the immune system. They have a specific arrangement of amino acids that allows them to interact with different antigens. This system has been divided according to the cells (B or T-cells) involved and the type of highly diverse receptors expressed (B-cell receptor or T-cell receptor (TCR)). The following section covers briefly the humoral immune

system. Subsequently, the cell-mediated acquired immunity system, which is the main topic of this thesis, is discussed in detail.

1.5.2.1 Humoral immunity

Humoral immunity is orchestrated by B-cells that in the presence of foreign antigens become antigen-specific secreting effector cells (Calame et al., 2003). This recognition is mediated by the B-cell receptor (BCR) that is formed of two immunoglobulin chains (light or heavy) generated after genetic re-arrangements of the different section genes (V-D-J) (Melchers, 2005). The results of this process leads to the formation of unique and specific BCR. The BCR can bind with soluble or membrane associated antigens (Carrasco and Batista, 2006). Soluble antigens activate B-cells by accessing lymph nodes through the afferent lymph vessels. It has been suggested that soluble antigens gain access to B-cells, which are primarily found in the follicle, by passing through small pores (0.1 - 1 μm diameter) found in the subcapsular sinus in the lymph node (Harwood and Batista, 2009). Antigens can also be transported by other cells to B-cell rich zones for presentation. Macrophages can capture antigens using different receptors found on the membrane for further presentation of the antigens to B-cells. Whether the antigen is presented unprocessed or processed remains unclear (Carrasco and Batista, 2007). Additionally, dendritic cells can also present antigens directly to the B-cells. These cells present the antigen in the paracortex section of the lymph node where migrating B-cells survey for antigens. Finally, it is generally accepted that a specialized type of antigen presenting cells confined with B-cells in the follicular zone is responsible for the presentation and further activation of B-cells. Follicular Dendritic Cells (FDC) retain antigens in the follicular zone by two mechanism (complement system and IgG binding with Fc receptors) for further presentation and activation of naïve B-cells (Catalfamo and Henkart, 2003; Kosco-Vilbois, 2003). Nevertheless, how these antigens gain access to the follicular zone where B-cell and FDCs are found remains under investigation (Harwood and Batista, 2009).

Different antigens (soluble or membrane associated) stimulate B-cells through the BCR that provides the signals to determinate the fate of B-cells. The B-cells

activated when a particular antigen, specific for a BCR, brings together or cross-linked two or more receptors gathering together many adapter proteins and signaling molecules forming the immunological synapse.

B-cells that bind with the appropriate antigen would receive signals to survive by different signaling pathway (PI3K and NF-κB). Additionally, the BCR associated with the antigen is internalized and processed for further presentation to Helper T-cells (Lanzavecchia, 1985; Rodriguez-Pinto, 2005; Lanzavecchia, 2007). Activated B-cells up-regulate co-stimulatory molecules (MHC-II, CD86 and CD80), receptors specific for T-cell cytokines, and chemokine receptors (CCR7) that enable them to reach T-cell zones for further interactions. Helper T-cells provide the necessary signals to the activated B-cells by an interaction between the TCR, MHC-II – antigen associated complex (B-cells), and co-stimulatory molecules in both types of cells (CD40 and CD40L). The interaction between the co-stimulatory molecules (mainly CD40 – CD40L) and the cytokines (IL-2, IL-4 and IFN-Ɣ) secreted by helper T-cells leads to the induction of isotype-switching (Snapper and Paul, 1987; Banchereau et al., 1994; Quezada et al., 2004). These activated B-cells migrate again to the deep zone of the follicle and start to proliferate rapidly in a zone called the germinal center. During this rapid proliferation, the BCR mutates in a process called somatic hypermutation (Berek et al., 1991; Jacob et al., 1991). This process allows the formation of highly specific antibodies for a particular antigen. Thus, each B-cell displays an antibody that would differ slightly to the antibody found in other B-cells. Follicular DCs present the antigen to the different B-cell with mutated antibodies, and only the B-cells that bear a high affinity antibody would receive the signals to survive and leave the germinal centers (McHeyzer-Williams and McHeyzer-Williams, 2005). The final outcome of B-cell activation is the production of antibody-secreting-cells and B-cell memory cells.

1.5.2.2 Humoral immunity and drug hypersensitivity

Antibodies of different isotypes have been detected in patients with adverse drug reactions to several drugs such as halothane, penicillin or sulfamethoxazole (Vergani et al., 1980; Carrington et al., 1987; Baldo, 1999). It is believed that these drugs would become immunogenic only when they associated with a

carrier protein. The adduct would be then internalized by receptors found in B-cells for further presentation to helper T-cells that also recognizes the hapten. Subsequently, the B-cell-T-cell interaction would provide the necessary signals for B-cells to become antibody-secreting cells.

Halothane, a drug associated with a high incidence of hepatitis, causes transient increases in transaminase levels in most patients and fatal hepatic necrosis in 1 in 35000 patients (Neuberger, 1990). Sera obtained from patients who suffered severe hepatic failure due to halothane exposure contained antibodies of the immunoglobulin G class that recognized neo-antigens formed in hepatocytes of halothane treated rabbits (Vergani et al., 1980; Kenna et al., 1984). The drug is activated by the enzyme CYP 2E1 to a chemical reactive trifluroacetyl metabolite (TFA) that covalently binds to different proteins, (170 kDa, 100 kDa, 76 kDa 59 kDa 57 kDa and 52 kDa) which have been identified by western blot using sera from different patients or an anti-TFA anti-serum (Kenna et al., 1988). Interestingly, nearly half of the patients with hepatic failure showed the presence of auto-antibodies against the purified native human CYP 2E1 (Bourdi et al., 1996). Even though antibodies against different proteins have been found in these patients, there is no evidence to support a role for these in the pathogenesis of the disease (Park et al., 2005).

Penicillins are associated with severe adverse drug reactions including anaphylaxis, which is immunoglobulin mediated. It was calculated in a retrospective study that the incidence of anaphylactic shock caused by intramuscular injection of penicillin used to prevent rheumatic fever was approximately 0.2%. Despite this, penicillin anaphylactic shock accounts for almost 75% of the anaphylaxis cases in the United States (Gruchalla, 2003). Therefore, the presence and specificity of the different circulating antibodies in the sera of the patients has been widely studied. Penicillins are reactive in nature. The β-lactam ring opens under physiological conditions to form protein adducts with lysine residues. The majority of the IgE antibodies (90%) found in patients with penicillin allergy recognize the haptenic structure formed between the β-lactam ring and the lysine groups. This haptenic structure is known as the

major penicilloyl determinant. However, antibodies found in different patients recognize other haptenic structures (Baldo, 1999). For example, antibodies also recognized adducts formed by the linkage between the carboxyl group in the thiazolidine ring with lysine residues forming penicillyl minor determinats. Additionally, some IgE antibodies discriminate between different side chains making still more diverse the number of antibodies presented in patient sera with allergies to penicillin.

IgE antibodies with specificity for sulfamethoxazole were found in the sera of sulfonamide hypersensitivity patients but not in control patients (Carrington et al., 1987; Gruchalla and Sullivan, 1991). In these studies serum from hypersensitive patients were incubated for 6 – 48 hrs with disks to which SMX, BSA or HSA was covalently bound. Then, a radiolabeled IgE human antibody was added for binding quantification. The majority of the patients who suffered from hypersensitivity reactions to sulfamethoxazole showed high binding ratios.
The formation of adducts between different sulfonamides and protein is a pre-requisite for antibody formation. Therefore, further experiments searched the presence of circulating SMX adducts in patients taking sulfonamides (Meekins et al., 1994). In this study, an anti-SMX antibody raised in rabbits was used for the detection of SMX-haptenated serum proteins in volunteers administered with 500 mg of SMX orally twice per day for 5 days. Western blot analysis using the anti-SMX antibody demonstrated that SMX selectively bind proteins with a molecular weight ranging between 32 – 45 KDa.

These examples demonstrated that low molecular weight drugs could induce the formation of specific antibodies for drug – protein complexes highlighting the importance of humoral immunity in the pathogenesis of hypersensitivity reactions. However, for a complete B-cell activation, the cooperation of helper T-cells are vital. In the following section, the mechanisms involved in the activation of the cell-mediated immune system and the consequences of this activation process are described. Cell - mediated immunity together with humoral immunity complete the adaptative immune response.

1.5.2.3 Cell mediated immunity

During cell-mediated immunity, naïve T-cells, the principal cell in cell-mediated immunity, are activated and become effectors cells that either help activate other cells to remove the threatening pathogens or directly kill cells that have been compromised. Precursor T-cells from the bone marrow migrate to the thymus where they finish their maturation process through positive and negative selection. Then, mature naïve T-cells circulate into and through secondary lymphoid organs (lymph nodes and spleen) looking for specific antigens for activation (Burbach et al., 2007; Krammer et al., 2007).

Naïve T-cells can be found in special zones on the lymphoid organs; the periarteriolar lymphoid sheath, which is a special compartment within the white pulp in the spleen (Mebius and Kraal, 2005). In this rich T-cell zone, T-cells interact with APC or passing B-cells. The T-cells stay in this zone by secreting CCL19 and CCL21, chemokines responsible for the attraction and retention of T-cells. They loose mobility as a consequence of the interaction with APC (Burbach et al., 2007). Here, T-cells are constantly exposed to antigens carried by antigen presenting cells that collect them from different organs in the body, process them and enter to the rich T-cell zones (white pulp in the spleen) for T-cell activation. The activation of the T-cells is mediated by specific receptors found in the cells, a complex is formed between MHC I or II molecules with the antigen on the membrane surface of mature antigen presenting cells, and the co-stimulatory signals provided by CD80 and CD86 receptors from the APC.

1.5.2.3.1 Antigen processing by antigen presenting cells

Antigen processing by APCs is biased to the origin of the antigen (endogenous or exogenous) and is restricted to the association with different carrier molecules for presentation to different sub-sets of T-cells.

Exogenous antigens taken up by APC are processed, degraded and associated with MHC-II molecules for presentation to $CD4^+$ T-cells (MHC-II-restricted pathway). In contrast, endogenous antigens are processed by different mechanisms and associated with MHC-I molecules that present the antigen to

$CD8^+$ T-cells (MHC-I-restricted pathway). Interestingly, even though these restriction pathways are followed in most of the occasions, certain flexibility has been found. In this context, exogenous antigens can activate $CD8^+$ T-cells in a MHC-I restricted pathway in a process called cross-presentation (Banchereau et al., 2000; Guermonprez et al., 2002; Trombetta and Mellman, 2005).

During MHC-II-restricted pathway soluble antigens are captured by different mechanism *(see 1.5.1)* and contained in membrane - bound vesicles called endosomes. These vesicles contain protease enzymes in an acidic medium for the promotion of proteolytical degradation and liberation of antigenic peptides that associate according with their specificity to MHC-II molecules. MHC-II molecules are synthesized in the endoplasmatic reticulum (ER) and associate with transport vesicles for further fusion with endosomes containing the degraded protein. The binding site of the MHC-II molecules, the most polymorphic region of the molecule, is protected by the association with a protein called the invariant chain protein (I_i). This association prevents that newly synthesized MHC-II molecules bind with peptides found in the ER. Newly synthesized MHC-II molecules associated with I_i protein, the components required for degradation and association of the peptide with the MHC-II complex and HLA-DM molecules, which mediate the binding of the antigenic peptides in the MHC-II, molecules are known as MHC class II-rich compartments (MIICs). Endosomes and MIICs vesicles fuse and the proteases found in the endosome remove the I_i form the MHC-II molecules leaving a peptide called class II-associated invariant chain peptide (CLIP). Finally, the HLA-DM molecule, a molecule structurally related to MHC-II but not polymorphic, helps to exchange the CLIP peptide and upload the antigenic peptides for $CD4^+$ T-cells restricted presentation.

In the MHC-I restricted pathway the antigen is degraded in the cytosol by the actions of a multiprotein enzyme complex called proteasome. For antigen degradation by the proteasome system, the antigen has to be targeted by the addition of a small peptide called ubiquitin in which the carboxyl group conjugates with the amino group of lysine residues on the antigen (Rock and Goldberg, 2003). The ubiquitinylated protein is degraded by the proteasome

system and the antigenic peptides are liberated. Like MHC-II molecules, MHC-I molecules are synthesized in the ER but the MHC-I molecules are not transported for antigen assembly. Therefore, the degraded peptides are associated with the transporter associated with antigen processing (TAP) protein that is located in the ER membrane. TAP proteins are associated directly with MHC-I. Once the peptide enters the ER is enzymatically trimmed and then binds with the newly synthesized MHC-I molecule. Finally, the peptide MHC-I complex is transported to cell surface by exocytic vesicles for further presentation to $CD8^+$ T-cells.

Interestingly, the majority of drug specific T-cell clones have a $CD4^+$ phenotype with some $CD8^+$ detected rarely. This might be explained by the formation of exogenous drug-protein adducts that are internalized and presented via an MHC-class-II restricted pathway. However, it is also possible that a drug metabolite formed within the cell binds with intracellular proteins forming neo-antigens that are presented in a MHC-I restricted pathway.

1.5.2.3.2 The structure and diversity of the T-cell receptor

Naïve T-cells express a highly variable receptor called the T-cell receptor (TCR) that is formed of two di-sulphide linked α and β chains that consist in two membrane-distal variable (Vα or Vβ) sections and two constant trans-membrane regions (Cα or Cβ) with short cytoplasmatic segments (Call and Wucherpfennig, 2005; Kuhns et al., 2006). The membrane distal section of the receptor is responsible for the recognition of different antigens. The antigen is recognized primarily by the interaction of the MHC-peptide complex with complementary-determinating regions, which are the more polymorphic section of the TCR, (CDR) found in both variable chains (Vα and Vβ) (van der Merwe and Davis, 2003). The TCR is responsible for the recognition of antigens, although other associated proteins with longer cytoplasmatic tails (CD3) are vital for the complete T-cell activation and signaling transduction (Kuhns et al., 2006). These proteins are non-covalently linked with the TCR, and four different types have been characterized (CD3γ CD3ε CD3δ and the di-sulphide linked ζ). The CD3

proteins in association with TCR appear as dimers formed by the combination of one CD3ε protein and either one CD3δ or CD3γ protein (CD3δε or CD3γε). The simplest functional TCR complex is formed of at least one TCRαβ molecule and one copy of each CD3 heterodimer (CD3δε, CD3γε and CD3ζζ; the monovalent αβ TCR model). However, other authors have proposed that two or more TCRαβ molecules are found in the complex together with the different CD3 dimers (the multivalent αβ TCR model) (Call and Wucherpfennig, 2005).

The variable region in the TCR is formed by different molecular mechanism. These molecular re-combination processes are mediated primarily by RAG1 and RAG2 enzymes and with the collaboration of the mobility group proteins -1 and -2 (HMG 1/2). The HMG1/2 proteins, a protein responsible for the DNA-bending and binding, are believed to bend the DNA facilitating the interaction of the RAG1/2 with the appropriate recombination sections. However, it has been showed that RAG1/2 proteins have the ability of bending, so it has been proposed that HMG1/2 stabilize the interaction through the stabilization of the bend (Fugmann et al., 2000; Schatz, 2004; Jung et al., 2006; Spicuglia et al., 2006). The high variable sections in the receptor are formed by genetic re-arrangement on the VDJ gene sections. The VDJ sections are separated in the gene, so they need to be brought together to form a continuous section. First the V (variable) section is joined together with a J (join) section, and in some cases a D (diverse) section is also added to form a continue encoding segment VDJ that would give rise to the different TCRs. This exon would transcribe the constant parts of the receptor, but also contain the instructions necessary for the generation of the highly variable region (CDR) characteristic of these molecules. This high variability is obtained by the re-arrangement of the VDJ sections by two process called combinatorial and junctional diversity (Fugmann et al., 2000). The combinatorial diversity process is the consequence of the different number of V, D and J sections existing in the VDJ gene. Thus, when a specific VDJ section is re-united this would give rise to a specific TCR; however when a different VDJ section is gathered a different TCR would be formed. In the junctional diversity process, during the VDJ re-arrangement new nucleotides are randomly added to the encoding section. Therefore, these newly added nucleotides would be give

rise to different TCRs. These sites of recombination are specifically found in the encoding gene and are recognized by the recombination signal sequences (RSS) (Fugmann et al., 2000) that are formed of by two highly conserved sequences of different lengths, a 7 bp sequence (heptamer; 5'-CACAGTG) or a 9 bp sequence (nonamer; 5'-ACAAAAACC). These RSSs signals are separated by non-specific bases of a length of 12 or 23 bp. Thus, the recombination only occurs every 12 or 23 bp where the recombination signals are found (12/23 rule) (Schatz, 2004). The RAG1/2 proteins interact with the both RSS sequences and the DNA is cleaved precisely generating four free ends. These endings are joined with the loss or addition of small number of nucleotides giving rise to encoding sequences.

1.5.2.3.3 T-cell activation

Once assembled, the TCR complex either monovalent (one TCR-CD3 complex) or multivalent (two or more TCR associated with CD3) interact with the antigen, associated with MHC in the surface of APC, for the delivery of signals that decide the fate of the naïve T-cells (Figure 1.3)(Davis and van der Merwe, 2003; Gascoigne and Zal, 2004; Call and Wucherpfennig, 2005; Rudolph et al., 2006; Smith-Garvin et al., 2009). The process in which the TCR – Peptide – MHC interact is known as the Immunological Synapse and is one of the most studied cell – cell interactions (Call and Wucherpfennig, 2005; Dustin, 2005; Mossman et al., 2005). During this process two main zones have been described, by imaging technologies, the central supramolecular activation cluster (cSMAC) and the peripheral supramolecular activation cluster (pSMAC) (Huppa and Davis, 2003). The cSMAC region is rich in T-cell complexes together with other signaling molecules such as proteins kinase C-Θ (PKC- Θ), whereas the pSMAC region has more large receptors such as the leukocyte funcion – associated antigen (LFA-1) and the cytoskeletical linker Talin (Huppa and Davis, 2003; emerski and Shaw, 2006). It is believed that receptors at the pSMAC region, which have long extra-cellular domains (LAF-1), would initiate the synapsis, but then smaller molecules with higher binding energy would squeezed out these bulky receptors for continuing the T-cell signaling and further degradation of the TCR complex.

Crystallography studies have been performed in TCR – MHC – Peptide complex and the structure of this complex has been elucidated for several peptides. Although, variability exists in these structures, some similar features have been found. For example, it was found that the Vα region in TCR is oriented to the C-terminal in the peptide sequence, whereas Vβ chains interact with N-terminal residues of the peptide (Rudolph et al., 2006).

MHC molecules according with is spatial configuration can present peptides with different lengths. MHC-II molecules have an open ending allowing the presentation of longer peptides, whereas the end in the MHC-I molecules is constrained allowing the presentation of smaller peptides (8-10 amino-acids). The kinetics of this interaction (peptide - MHC) has been studied and it was found that the affinity in this complex is not too high when compared with other types of cell – cell interactions such as CD8/MHC–I or CD4/MHC-II (van der Merwe and Davis, 2003). Moreover, the kinetic of the association and dissociation of the complex is slow when compared with other interactions.

Once the TCR is engaged with the MHC – protein complex, a series of biochemical signals are transmitted in the cytoplasm of the activated T-cells and the naïve T-cell becomes an effector cell (Figure 1.4). The early step in signaling after TCR ligation is the Src (LcK and Fyn) PTKs activation leading to the phosporylation of the immunoreceptor tyrosine based activation motifs (ITAMs) residues found in the cytoplasmatic domains of the CD3 co-receptor (*see* figure 1.4 [1])(Smith-Garvin et al., 2009). The ITAM section of the CD3 region is a recruitment site for the ZAP-70, a Syn kinase member family. ZAP-70 mediates phosphorylation of several adaptor proteins LAT (linker for the activation of T-cell) and SLP-76 (a domain-containing phosphoprotein of the Src homology 2) (Zhang et al., 1998; Zhang et al., 1999). These adaptor proteins would diversify signaling by the activation several proteins joined by tyrosine (*see* figure 1.4 [2]). The LAT protein has several tyrosines that bind to other proteins such as PLCY1, PI3K and the two adapters proteins GRB2 and Gads. An important protein found in this adaptor complex is the PLCY1. The protein is phosphorylated and activates

second messengers IP_3 and DAG formed after the hydrolazation mediated by PLCϒ1 of the membrane lipid $PI(4,5)P_2$ (*see* figure 1.4 [3]).

The DAG and IP_3 messengers activate several different important pathways, which are described briefly below.

DAG activates two proteins involved in important signaling pathways, RAS and PKCΘ. RAS protein leads to the activation, by different intermediary proteins, of mitogen – associated proteins kinase (MAPK) that leads to the phosporylation of extracellular signal – regulated kinase 1 (Erk 1) and Erk2. These kinases activate different transcriptional factors such as activator protein 1 (AP – 1) or the signal transducer and activator of transcription 3 (STAT3) that would lead to the transcriptional changes in the nucleus (*see* figure 1.4 [3a]). The second major signaling pathway is mediated by PKCΘ. This protein leads to the activation of NF-κB a transcriptional factor consisting of five members (NF-κB1, NF-κB2, RelA, c-Rel and RelB, which shared) that is a vital regulator during innate and adaptative immunity (*see* figure 1.4 [3b]). The five members are characterized by the presence of a N-terminal Rel homology domain (RHD) responsible for sequence-specific DNA binding (Vallabhapurapu and Karin, 2009). When T-cells are resting, the action of NF-κB is inhibited by the IκB family members that keep it outside the nucleus, but as a consequence of T-cell activation the IκB complex is phosphorylated by the IKK proteins leading to degradation of the inhibitory protein and liberation of NF-κB, which migrates to the nucleus and activates genes involved in the survival of T-cells.

Calcium ions (Ca^{2+}) are important messengers during T-cell signaling (Feske, 2007). The IP_3 molecule liberated by the hydrolysis of the membrane lipid $PI(4,5)P_2$ activates permeable ion channel receptors (IP_3R) found in the endoplasmatic reticulum (ER) membrane leading to the release of Ca^{2+} into the cytoplasm (*see* figure 1.4 [4]) (Gallo et al., 2006). Depletion of stored Ca^{2+} in the ER leads to an influx of extra-cellular Ca^{2+} to the cytoplasm by the activation of the plasma membrane Ca^{2+} release –activated Ca^{2+} (CRAC) channels in a process known as store – operated Ca^{2+} entry (*see* figure 1.4 [4]) (Hoth and Penner, 1992). The CRAC channels are opened by the action of the stromal interaction

molecule (STIM) that after Ca^{2+} depletion aggregate in clusters in the membrane of the ER (Zhang et al., 2005). Steps involved of the activation of the CRAC channels by the STIM molecules are an area of on-going investigation.

The increase of intracellular Ca^{2+} has many consequences in the T–cell. Firstly, as the levels of intracellular calcium raise the T - cell lose mobility, which might help for the stabilization and sustaining of the signals delivered in the immunological synapse. Secondly, the sustained influx of intracellular calcium, by the opening of the CRAC channels in the cellular membrane, leads to the activation of several transcriptions factors such as cyclic-AMP-responsive-element-binding protein (CREB), myocyte enhancer factor 2A MEF2 and NAFT nuclear factor of activated T-cells (NAFT). The NAFT transcriptional factor is one of the most widely studied transcriptional factors in cells of the adaptative immunity and is activation process would be briefly described.

Calcium binds calmodulin that activated the calcineurin, which then finally phosphorylated the NAFT protein leading to nuclear translocation and the induction of NAFT – mediated gene transcription (*see* figure 1.4 [5]). Once translocated into the nucleus, NAFT isoforms interact also with other transcriptional factors such as AP-1 leading to the activation of different genes in the T-cells. NAFT proteins are involved in several T-cell process such as activation, T-cell anergy and T-cell differentiation (Macian, 2005).

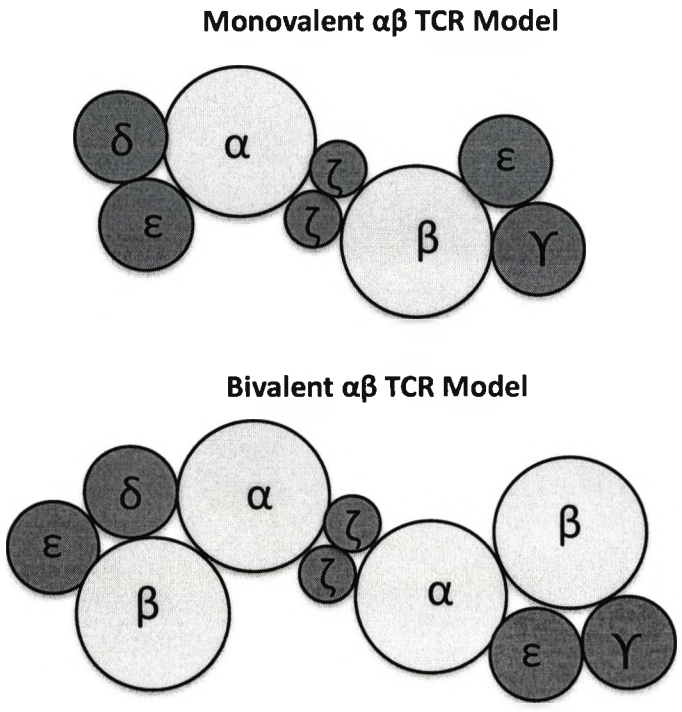


Figure 1.3 Monovalent and multivalent models of the TCR-CDR3 complexes. In the monovalent model one TCR (light gray circles) is associated with the CDR3 receptors (dark gray circles). In the multivalent model one or more TCR are associated with the CDR3 receptors (taken from Call et al., 2005)

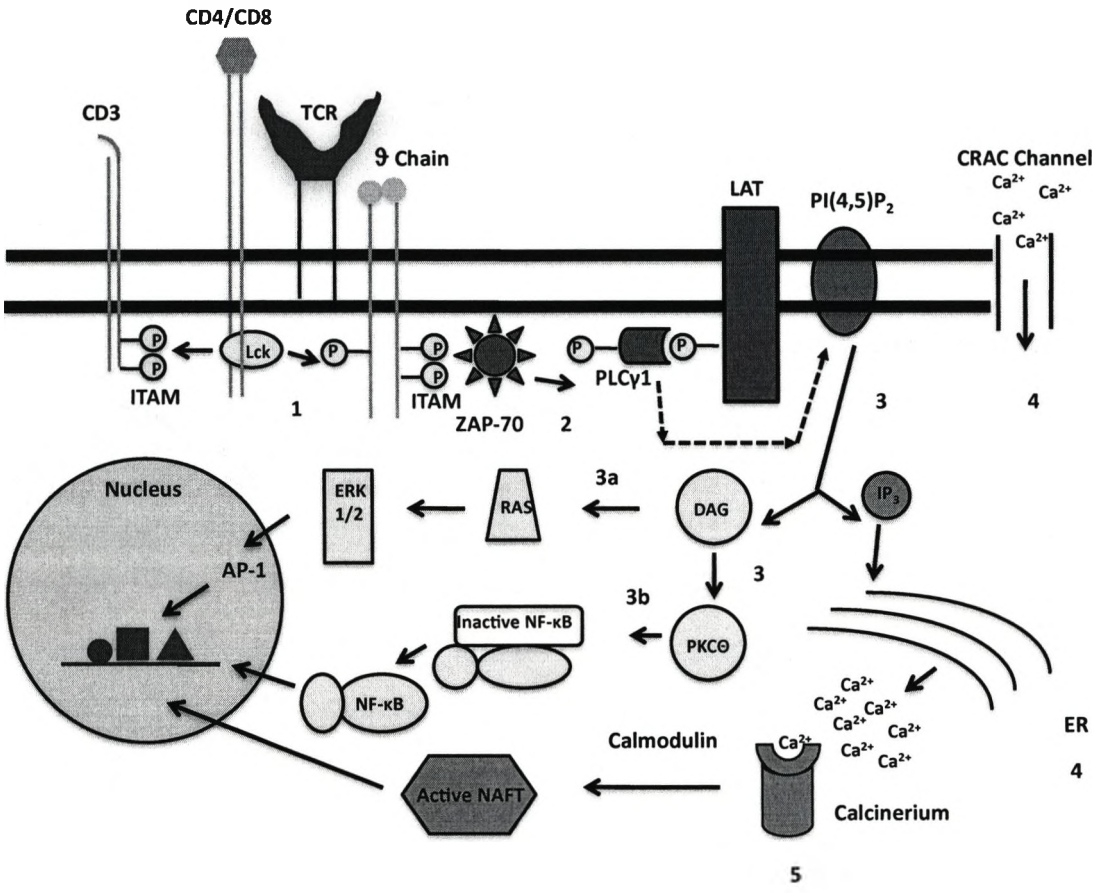


Figure 1.4 After T-cells interaction with MHC-peptides molecules, a complex signaling pathway is activated leading to the phosporylation of several adaptor molecules, which activates second important messengers. (1) Lck proteins after T-cell activation phosphorylates ITAM residues. (2) The ZAP-70 protein mediates phosphorylation of LAT proteins. (3) The PLCγ1 protein, attached to LAT adaptor protein, liberates IP_3 and DAG messengers by the hydrolysis of the membrane lipid $PI(4,5)P_2$. (3a) DAG activates RAS proteins which then further would activate kinases Erk1/2 leading to the activation of transcriptional factor in the nucleus. (3b) Additionally, DAG activates other transcriptional factors in the nucleus mediated by the activation of the NF-κB. (4) The IP_3 molecule activates the release Ca^{2+} to the cytosol from the RE. CRAC channels activate increasing the levels of intracellular Ca^{2+}. (5) Increased levels of Ca^{2+} initiates the activation of transcriptional factors such as the nuclear factor of activated T-cells (NAFT) involved in T-cell activation (Adjusted from Smith-Garvin et al., 2009).

1.5.2.3.4 Effector T-cells

$CD4^+$ T-cells are activated by the interaction of the MHC-II molecules in the APC that would present mainly extra cellular antigens that have been captured by different mechanism and break down into peptides, whereas $CD8^+$ T – cells would be activated by MHC-I molecules that presented intra cellular antigens or extra-cellular antigens that have escape the MHC-II restricted pathway (cross-priming) (Ackerman and Cresswell, 2004; Trombetta and Mellman, 2005).
$CD4^+$ T-cells undergo further differentiation after receiving survival signals. Two subsets of $CD4^+$ helper cells have been characterized in mice and humans (T_H1 and T_H2 helper cells) that play different roles in the response of the adaptative immunity (Farrar et al., 2002). More recently, a new sub-set has been found (T_H17). The primary function of the T_H17 sub-set seems to be the clearance of pathogens that were not appropriate eliminated by the T_H1 or T_H2 sub-sets. Additionally, they are potent inducer of tissue inflammation and have been related with the pathogenesis on many experimental auto-immune induced diseases (Korn et al., 2009). These three sub-types are differentiated by the secretion of specific cytokines. The T_H1-lineage secrete IFN-Υ, T_H2-lineage secrete IL-4, IL-5 and IL-13, and T_H17 IL-17F, IL-21, IL-22 and IL-26.

T-cell polarization lineage is mediated by the secretion of polarizing cytokines from antigen presenting cells that secrete the relevant cytokines depending on the nature or pathway of maturation of the APC (Kapsenberg, 2003).

Several molecules that interact with the PPR which induce DC maturation (Figure 1.2), would lead to the secretion of polarizing proteins that would define the fate of the T-cell. The secretion of polarizing cytokines by the activation of several Toll like receptors has been review recently (Kapsenberg, 2003). It was found that TLR-3 and TLR-4, primarily activated by double-stranded RNA and LPS from bacteria respectively, promote the secretion of IL-12 and type 1 IFNs both related with diversification bias T_H1 type. Interestingly, when the TLR-7 was activated by low molecular weight, compounds from the imidazoquinoline family, different types of antigen presenting cells secreted IL-12 or IFN-α. The

factors involved in PPR signaling and activation in PPR has not been defined in APC leading to T_H2 commitment. However, several parasites (leishmania major) and allergens induce the secretion of IL-4 in APC leading to the T_H2 response.
IL-12, IL-27 and IFN-γ induces T_H1 polarization, whereas IL-4 induced T_H2 polarization (Reiner, 2007). IL-27 and IFN-γ initiate T_H1 commitment by activating the transcriptional factor STAT1 that further activates the T-bet transcriptional factor, a master regulator for T_H1 differentiation. T-bet activates others transcriptional factors (HLX and RUNX3) leading to the IL-12 receptor β2 (IL-12Rβ2) coupling with the IL-12β1 on the surface membrane. Thus, IL-12 secreted by APC further enhances commitment in T_H1 lineage by the activation of complementary transcriptional factors (STAT4). The activation of transcriptional factors for T_H1 polarization additionally inhibits the activation of the transcriptional factors that mediate T_H2 polarization.

IL-4 is a key polarizing cytokine for the development of a T_H2 response. Naïve T-cells have the ability of secrete IL-4 and up-regulate the IL-4-receptor under normal conditions (Ansel et al., 2006). Nevertheless, under certain circumstances such as the absence of IFN-γ or the increased presence of IL-4 secreted by other cells T-cell differentiation is biased towards the T_H2 lineage. IL-4 binds its IL-4R and activates the Janus kinases family leading to the activation of the STAT6 (Mowen and Glimcher, 2004). STAT6 activates the transcriptional factor GATA3 a member of a family of important transcriptional factors with a Zinc motif coordinated (Ho et al., 2009). Similar to the role that the T-bet transcriptional factor plays in T_H1 differentiation, the GATA3 transcriptional factor is vital for T_H2 commitment.

1.5.2.3.5 T-cell cross-reactivity

T-cells have receptors with specificity towards specific antigens that are presented. However, the number of possible p-MHC ligands exceeds the number of TCR that could be generated due to different genetic re-arrangements. Therefore, some TCRs should be able to recognize similar related antigens to deal with the high number of ligands. The process in which TCRs cross-react

with other structure related antigens is called TCR degeneracy (Wilson et al., 2004).

Several studies have shown that the TCR could adapt to recognize related peptides presented by MHC molecules. A series of experiments using the same peptide presented to the same TCR by similar MHC molecules, or similar peptides presented by the same MHC to the same TCR have demonstrated that the CDR3 region, the most polymorphic section of the CDR and related with the interaction with peptides in the TCR, is responsible for these degeneration in the TCR (Ding et al., 1999; Hennecke and Wiley, 2002; Luz et al., 2002; Reiser et al., 2003). Recent findings suggest that TCRs can change their spatial distribution to recognize different peptides presented. TCRs recognize ligands with unfavorable entropy, directly related with a reduction of the protein dynamics or conformational changes. The majority of studies suggest that the CDR3 loop can adapt to these changes (Rudolph and Wilson, 2002). Crystallography studies have shown that the conformational changes in the CDR3 region of the TCR were higher when compared with others regions of the TCR (CDR1 & CDR2). Thus, it has been suggested that TCR cross-reactivity depends primarily in the flexibility that the CDR3 region has to adjust to different amino-acids sequences in the peptides presented.

TCR cross-reactivity is a natural phenomenon occurring that allows the immune system to quickly respond in the presence to the vast arrays of ligands to which the body is exposed. In the other hand, TCR cross-reactivity is also responsible for rejection during organ transplantation. Data presented in this thesis studies the capacity of T-cells generated from patients allergic to sulfonamides to cross-react in the presence of different sulfonamides and their metabolites.

Section II

1.6 Metabolism and drug hypersensitivity

1.6.1 Drug metabolism

The disposition of a drug or a xenobiotic is achieved by a four steps process (absorption, distribution, metabolism and elimination). The way the body handles a drug is know as pharmacokinetic. In this section, the role of metabolism in drug hypersensitivity is discussed.

A drug can be eliminated unchanged; in many cases the drug has to be chemically altered to enhance elimination. The process in which the drug is changed or modified is known as metabolism. The principal aims of metabolic process are to increase molecular weight and water solubility, both of which aid elimination in urine. Drug metabolism is divided into to two types of reactions Phase I or Phase II, which normally occur sequentially. During Phase I reactions, the drug is activated by the addition of a several functional group preparing molecule for Phase II reactions where a polar conjugate is added and finally during phase III the metabolites are eliminated.

1.6.1.1 Phase I reactions

The types, enzymes involved and consequences of Phase I reactions is well-defined. The most important Phase I reaction is oxidation in which an atom or an oxygen molecule is added to the drug. The enzymes mediating this reaction are known as oxygenases. The cytochrome P450 families of enzymes are the main enzymes responsible for this oxidative process.

The cytochrome P450 enzymes catalyse a wide variety of reactions such as hydroxylation, dealkylation or oxidation *(see table 1.3)*

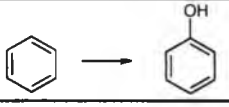
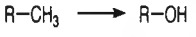
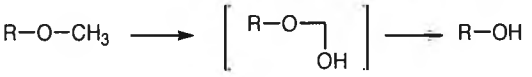
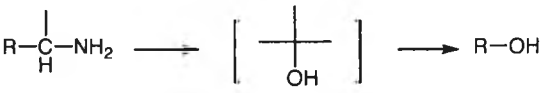
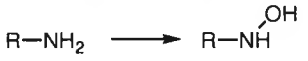
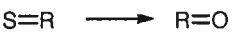
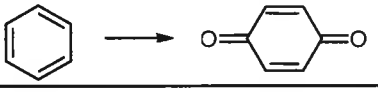
Type of Reaction	Diagram
Aromatic Hydroxylation	
Aliphatic Hydroxylation	
Epoxidation	
O, N, S - dealkylation	
Oxidative deamination	
N-Hydroxilation	
S-Oxidation	
Dehalogenation	
Desulphuration	
Alcohol Oxidation	
Quinone Formation	

Table 1.3 Characteristic reactions performed during Phase 1 metabolism.

Oxidation and hydroxylation occur in atoms of Carbon with different hybridization states, but other atoms are targets (nitrogen or sulfur) of these reactions as well.

The cytochrome P450 super family has more than 7700 distinct CYP's gene sequences across all the organisms, and in humans, the genome encodes for 57 different CYP proteins that are arranged into 18 families and 42 sub-families, where 15 CYPs of this family are involved in the metabolism of drugs and xenobiotics (Pirmohamed and Park, 2003; Brown et al., 2008). The CYP is a haem-thiol protein in which the Ferric center coordinated with thiols groups is the center of the catalytic activity of the enzyme (Danielson 2002). The ferric group first coordinates with the substrate (Fe^{3+} – RH) reducing the ferric group (Fe^{2+} – RH). Then, a molecule of oxygen is coordinated (O_2 – Fe^{2+} – RH) and two electrons are added forming a super oxide anion ($^{2-}O_2$ – Fe^{3+} – RH) that finally would be cleavaged liberating the oxidized product (R-OH) and a molecule of water. The overall reaction is summarized as follows:

$$NADPH + O_2 + RH + H^+ \qquad NADP^+ + ROH + H_2O$$

1.6.1.2 Phase II reactions

Phase II reactions are known as conjugation reactions. In these reactions, a polar motif is added normally by the covalent binding with the functional groups already added during Phase I. The final outcome of Phase II reactions is to form an inactive molecule and to generate a more hydrophilic compound that could be easily excreted in the urine. Major phase II reactions include glucuronidation, sulphation, acetylation and conjugation with glutathione or amino acids.
Quantitatively, glucuronidation is the most important form of conjugation for drugs. In this reaction, the substrate acts as a nucleophil that attacks an electrophilic carbon atom found in the pyranose acid ring of UDPGA (uridine 5' – diphosphate – glucuronic acid) transferring a glucuronic acid moiety mediated UDP – glucurosyl transferase to form the glucuronic conjugated.

Another important Phase II reactions are N-acetylation, which is important in the metabolism of sulfonamides. During this conjugation, aromatic amines and other amines or sulphinamide group are acetylated using the factor acetyl – coenzyme A mediated by the N-acetyltransferases. The general mechanism of acetylation is performed in two steps, the transfer of the acetyl group forming an acetyl – enzyme intermediate and the subsequent acetylation of the aromatic amine with the regeneration of the enzyme.

1.6.1.3 Glutathione conjugation

Glutathione is formed by conjugation of three amino acids (cysteine, glycine and glutamine). It is the most important low molecular intracellular antioxidant (Dickinson and Forman, 2002; Townsend et al., 2003; Forman et al., 2009). Different concentrations of the intracellular thiol are found in different types of cells. For example, in liver cells the concentration of GSH reaches a maximum of 10 mM, whereas others cells have lower concentrations (1-2 mM). The vast majority of the intracellular GSH is found in the cytoplasm, which is the main site for the synthesis of the protective thiol. Cytoplasmatic GSH can be transport to other organs such as the mitochondria where it plays a critical role in reducing reactive oxygen species (ROS) generated during oxidative stress (Lash, 2006).

The protective role of GSH is achieved by two mechanisms (reduction of the reactive species and direct conjugation with soft electrophiles).

Reactive oxygen species (ROS) are highly toxic to the cell; thus, cells require defenses mechanism to remove them. For example, GSH is oxidized in the presence of hydrogen peroxidase (ROS) by the action of the glutathione peroxidase to form glutathione disulfide (GSSG) and water. To maintain normal levels of reduce glutathione, GSSG is reduced by GSH reductase in a process that requires NADPH. Additionally, GSH can conjugate directly with electrophiles forming adducts that are further eliminate out of the cell. The cystein residue found in GSH attack electrophiles forming a covalent bound between the cysteine and the electrophile. The conjugation with GSH results in the detoxification of the

electrophile and preventing their reaction with other nucleophilic molecules such as proteins or nucleic acids.

1.6.1.4 Phase III

Accumulation of the metabolites within the cell can lead to a decrease in the detoxication activity of phase II enzymes. Thus, the newly formed metabolites need to be eliminated. A membrane transport system is responsible for the detoxification of the metabolites. These transporters are known as phase III enzymes (Ieiri et al., 2006; Wakabayashi et al., 2006; Koshiba et al., 2008).

Many of the drugs transporters belong to the ATP-binding cassette (ABC) transporter family, which forms one of the largest protein families encoded in the human genome. A total of 48 ABC proteins genes have been identified and sequenced. Some of them have been demonstrated to be critically involved in the transport of metabolites (ABCB1, ABCB11, ABCC1 and ABCG2).

The transporters are expressed in various human tissues and have different specificities to different substrates. For example, the ABCC2 transporter is found in the liver and kidney and has a specific activity towards diglucoronide, sulfates, glutathione conjugates and indomethacin (Ieiri et al., 2006).

1.6.2 Reactive metabolites and its role in toxicity and drug hypersensitivity

The primary role of drug metabolism is to inactivate and eliminate drugs through a series of chemical modifications. During this process, the drug is activated and becomes more reactive (Park et al., 2005). Thus, a dysregulation between the activation step and elimination step might lead to the presence of protein reactive metabolites that are responsible for toxic reactions in the host. The majority of metabolic transformations are performed in the liver, but other organs such as the kidney or the skin have metabolic activity (Reilly and Ju, 2002; Merk et al., 2007; Merk, 2009). Drug-induced liver injury is the most frequent reason for the withdrawal of approved drugs, and more that 600 drugs are associated with hepatotoxicity (Park et al., 2005; Uetrecht, 2009). Even though the formation of drug reactive metabolites has been associated with hypersensitivity reactions, the liver is not the main target organ for this type of

reactions. Moreover, the involvement of the immune system in drug-induce liver injury has not been well established. Several reasons might contribute to explain why the liver is not the target of hypersensitivity reactions. Firstly, the liver has better protective mechanism that other organs characterized by the presence of high levels of GSH, so reactive metabolite are preventing to form adducts (Park et al., 2005; Sanderson et al., 2006). Secondly, the liver, an immune privileged organ, might show immune tolerance to drug-protein adducts. Previous studies have showed that Kupffer-cell (KC) might be responsible for a toleragenic response to DNCB protein adduct in mice (Ju and Pohl, 2005).

Reactive metabolites in general have very short half-life that in addition with the efficient mechanism of protection found in the liver (high levels of GSH) makes it unlikely that they will migrate to induce a toxic or immune response in other organs (Uetrecht, 2006).

It has been postulated that extra-hepatic drug metabolism might contribute to the pathogenesis of drug hypersensitivity. The skin is the target organ for many drug hypersensitivity reactions, so several studies have been focused on the metabolic activity of keratinocytes (Reilly et al., 2000; Reilly and Ju, 2002; Vyas et al., 2006b). It has been shown that keratinotyces isolated from skin expressed a large number of metabolic enzymes measured by the presence of CYP mRNA or immunochemistry assays using several anti-CYP antibodies (Baron et al., 2001; Baron and Merk, 2001; Oesch et al., 2007). Interestingly, enzymes responsible for drug metabolism (CYP 1A1, 1B1, 2B6, 2C9 and 3A4) have been found in the skin, and they might contribute to the formation of drug-reactive metabolites and further adduct formation and T-cell activation. However, it has also been suggested that other enzymes such as flavinoxygenases (FMO) might be responsible for the metabolic activation of some drugs by keratinocytes (Vyas et al., 2006b).

Additionally, the metabolic capacity of immune cells such as monocytes, neutrophils (Uetrecht et al., 1988; Cribb et al., 1990) and monocyte-derived DC has been investigated (Sanderson et al., 2007). It has been found that neutrophils

and monocytes mediate drug oxidation by the action of myeloperoxidase. Recently, the capacity of monocyte-derived DC has been investigated (Sanderson et al., 2007). It was shown that APCs express CYP mRNA that could be responsible for oxidation of certain drugs.

Drug metabolism in different organs can lead to the formation of reactive drug species, which are believed to be responsible of the initiation of drug hypersensitivity reactions by the activation of T-cells. T-cell activation requires the present of two signals. Signal 1 is delivered by the interaction between the MHC-peptide-TCR complex, and signal 2 provide by the interaction of co-stimulatory receptors (CD80 and CD86) located in the membrane of the APC. Therefore, drug reactive metabolites need to provide these two signals in order to activate naïve T-cells.

Some reactive drug metabolites are highly electrophilic and can bind to proteins forming neo antigens that might break tolerance to self-proteins by exposing new epitopes or by the direct interaction of the drug with the TCR. Several drug – protein adducts have been characterized, but it is not always possible to establish a relationship between adduct formation and drug hypersensitivity (Kenna et al., 1984; Meekins et al., 1994; Lavergne et al., 2006a). Additionally, the nature of the target protein (extra-cellular or intra-celullar) and the degree of binding required for the development of drug hypersensitivity is ill defined (Uetrecht, 2006).

Drug – protein adducts clearly deliver signal 1; however, T-cell activation requires the presence of co-stimulation signals by mature APC. Interestingly, drug reactive metabolites are more toxic than the parent drugs and have been related with oxidative stress or cell death (Rieder et al., 1988; Rieder et al., 1995; Hess et al., 1999; Naisbitt et al., 2002). Therefore, it is possible that reactive metabolites caused cell death, and the release of danger signals from dying cells that induce the up-regulation of co-stimulatory molecules in APC. Interestingly, activation of APC can be consequence of an exogenous stimulus such as viral infection and certain drug hypersensitivity reactions have been related to

reactivation of virus infections (EBV, HHV-6 or HIV) (Suzuki et al., 1998; Pirmohamed and Park, 2001; Pirmohamed et al., 2002; Shiohara and Kano, 2007).

1.7 Cell mediated immunity and drug hypersensitivity

T-cells are activated predominantly by proteins that have been processed and broken down into peptides. Nevertheless, other molecules such as lipids, prepinl-pyrophosphates, sugars, metals and drugs activate T-cells in the presence of MHC molecules (Porcelli et al., 1996; von Greyerz et al., 1998).

Drug hypersensitivity reactions are mediated by the activation of either humoral or cell-mediated immunity. The following section discusses the current understanding of drug-specific T-cell activation.

For over 20 years, lymphocytes from drug hypersensitivity patients have been isolated, expanded and studied in order to further understand how drugs interact with immunological receptors and stimulate a T-cell reponse.
T-cell clones from a diverse range of drugs such as sulfamethoxazole (Schnyder et al., 1997), penicillin-G (Mauri-Hellweg et al., 1996), carbamazepine, lidocaine (Wu et al., 2006), mepivacain (Zanni et al., 1997) and lamotrigine (Wu et al., 2006) have been generated. First, I will discuss the general pathways of drug-specific T-cell activation based on findings originating from these studies. Subsequently, specific issues associated with each drug and the potential for T-cell receptor cross-reactivity is discussed. T-cell activation by SMX is covered in detail in a later section and linked to the aims of the thesis.

1.7.1 Pathways of drug specific T-cell activation

T-cell activation requires the presence of the antigen and antigen presenting cells providing co-stimulatory signals. In this case, the antigen could be the parent drug, a metabolite of the drug or a hapten formed by the conjugation of the reactive drug metabolite with protein.
Drug specific T cell clones are $CD4^+$, $CD8^+$ or double positive $CD4^+CD8^+$ (although only rarely seen). Each type of T-cell has been shown to display

cytotoxic activity following drug-stimulation. The extent of immune-mediated killing is though to relate to the causative drug and the nature of the clinical manifestations (Pichler, 2003).

Drug specific $CD4^+$ T – cell responses are restricted, predominantly to HLA-DR or DP molecules, but not allele restricted (Zanni et al., 1998b). $CD8^+$ T-cells are stimulated by the drug presented by MHC class I or II molecules (Wu et al., 2007). The ability of class I and class II restricted drug antigen to stimulate mediate killing of target cells has not been explored in detail. The TCR repertoire is diverse and a polyclonal response is found in the majority of the cases indicating that different TCR from the same patient recognize the drug. Nevertheless, certain strong associations between a specific TCR Vβ chain and drug are seen (Zanni et al., 1997; Zanni et al., 1998a). The majority of the T-cell clones secrete a heterogeneous profile of cytokines characterized by the presence of IFN-γ and IL-5.

The mechanism of drug-specific T – cell activation has been widely studied, and experimental evidence suggests that the activation of specific T-cell clones can described by two hypothesis, namely: hapten or the pharmacological interaction hypotheses. These two hypotheses were described in previous sections (1.3.1 and 1.3.2), but the experimental evidence supporting these findings are described detail in tables 1.2 and 1.3 and discussed in the following paragraphs.

Drugs or more commonly reactive drug metabolites have the capacity to bind to proteins. The resultant neo-antigens (drug-protein conjugate) can then break tolerance by exposing amino acids that are not tolerated by T-cells triggering T-cell activation. Contact sensitizers (dinitrohalobenzenes) or respiratory allergens (trimellitic anhydride) are good examples of haptens that stimulate T-cell via a hapten mechanism. Certain drugs also bind irreversible to protein. A classical example is β-lactam antibiotics, which are chemically reactive under normal conditions and form adducts that are further recognized by T-cells (Mauri-Hellweg et al., 1996). Even though the majority of drugs are un-reactive, they can gain protein reactivity through metabolic activation.

The formation of drug protein adducts has been widely investigated, and it has been related to the activation of T-cells in animal and human models of immunogenicity (Schnyder et al., 2000; Naisbitt et al., 2001; Manchanda et al., 2002; Naisbitt et al., 2002; Cheng et al., 2008). Reactive metabolites can form protein adducts with either intracellular or extra-cellular membrane proteins in different type of cells (Schnyder et al., 2000; Cheng et al., 2008). Reactive metabolites also bind to proteins outside the cell (e.g. HSA) that could be internalized and presented in a MHC-restricted way. Furthermore, metabolites might bind to peptides within the MHC groove of APC directly. Preincubation of APC with reactive metabolites leads to the formation of immunogenic adducts within the APC that can be further been used as a source of antigen for drug-specific T-cell activation (Schnyder et al., 2000; Burkhart et al., 2001; Naisbitt et al., 2002). The reactive nature of the drug metabolite is vital for the formation of drug-adducts. Thus, if a reactive metabolite is incubated with an excess of an agent that directly competes for binding, the formation of drug-adducts decreases together with the proliferation of specific T-cells (Burkhart et al., 2001).

Despite this knowledge, there are several fundamental questions that need investigation. For example, the nature of the target protein to which the reactive drug metabolites bind, the description of the chemical mechanism in which these adducts are formed and the minimal amount of drug-adduct needed for T-cell activation is not resolved (Uetrecht, 2007). Interestingly, drug-specific T-cells are additionally activated in the presence of the parent drug apparently without the necessity of drug - adduct formation or processing (P-I concept). In this context, drug-specific T-cells only proliferate in the continuous presence of the soluble form of the drug; APC pre-incubated with drugs, washed and presented to specific T-cells fail to induce a proliferative response (Schnyder et al., 2000). Chemically fixed APC present the parent drug to drug-specific T-cells demonstrating that processing is not needed (Schnyder et al., 1997). Finally, drug-specific T-cell activation in the presence of the parent drug seems to be fast due to the lack of antigen up taking and processing. Kinetic measures of several T-cell activation markers demonstrated that drug-specific T-cell activation

occurs as soon as 30 minutes. Normally, antigen uptaking and processing by APC requires several hours (4 – 16 hr) (Zanni et al., 1998a).

Experimental evidence obtained by the use of different laboratory techniques has been gathered supporting both hapten and P-I mechanims. This evidence is discussed in detail in tables 1.4 and 1.5.

Specificity and reactivity of drug-specific T-cells.		
Technique	**Hapten & P-I**	**References**
Proliferation assay	In this assay, specific-drug T-cells are incubated with the drug or drug reactive metabolite with different sets of cells as APC for 48 hr. Proliferation is measured by the addition and incorporation of ^{3}[H]-Thymidine. This assay determinates the specificity and concentration dependency of drug-specific T-cell clones. However, the nature of the antigen (parent drug or drug-peptide conjugate) and the mechanism of antigen presentation cannot be defined.	(Mauri-Hellweg et al., 1995; Mauri-Hellweg et al., 1996; Padovan et al., 1996; Zanni et al., 1997; von Greyerz et al., 1999; Zanni et al., 1999; Schnyder et al., 2000; Yawalkar et al., 2000b; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Depta et al., 2004; Nassif et al., 2004; Wu et al., 2006; Wu et al., 2007)
Cross-reactivity	In this assay, drug-specific T-cells are incubated with structurally related drug or drug metabolites. The degeneracy of the TCR towards drug-specific T-cell clones is determined by at least in part by the density of specific T-cells receptors on a given clone. The mechanism of antigen presentation cannot be established.	(Mauri-Hellweg et al., 1996; Padovan et al., 1996; Zanni et al., 1997; von Greyerz et al., 1999; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Depta et al., 2004; Nassif et al., 2004; Wu et al., 2006)
Cytotoxicity Essay	In this, assay the cytotoxic capacity of drug-specific T-cells is measured by incubating the drug or metabolite with B-LCL or keratinotyces as target cells. Cytotoxicity activity is measured by the release of ^{51}Cr from target cells, and the pathway of killing can be established using different inhibitors.	(Schnyder et al., 1997; Schnyder et al., 1998a; Yawalkar et al., 2000b; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Engler et al., 2004; Wu et al., 2006)
Pulse assay	In this variation of the proliferation assay, APC are preincubated with drug or metabolite for different time intervals. Then, the pulsed-APCs are used as a source of antigen. The reactivity of the drug or metabolite is determined by the formation of adducts within APC that further activate drug-specific T-cells. The assay gives some insight in the nature of the antigen presented.	(Schnyder et al., 2000; Burkhart et al., 2001; Burkhart et al., 2002; Naisbitt et al., 2003b; Depta et al., 2004; Engler et al., 2004; Wu et al., 2007)
Antioxidant incubation	Drug-specific T-cells are incubate with the drug or reactive metabolite in the presence or absence of glutathione, which binds irreversible with electrophilic metabolites. Drug-protein adducts formation decreased, so proliferation is reduced.	(Schnyder et al., 2000; Burkhart et al., 2001; Naisbitt et al., 2003b; Engler et al., 2004; Wu et al., 2006)

Table 1.4 Experimental techniques use to establish the specificity and reactivity of drug specific T-cells.

Technique	Mechanism of antigen presentation Hapten & P-I	References
APC Fixation	Chemically fixed APC, unable to process antigens, are incubated with the drug or metabolite in the presence of the specific T-cells. Metabolites that bind with carrier proteins need processing for presentation; thus, fixed cells do not induce proliferation. In contrast, parent drugs and metabolites that bind directly to MHC stimulate drug-specific T-cells in the presence of fixed APC (without processing).	(Padovan et al., 1996; Schnyder et al., 1997; Naisbitt et al., 2003b; Depta et al., 2004; Wu et al., 2006; Wu et al., 2007)
$CD3^+$ down-regulation	Following T-cell activation, the $CD3^+$ receptor is down regulated. Drugs and reactive metabolites induce fast (≈ 30 min) $CD3^+$ down-regulation. In contrast, immunogenic peptides need at least 360 min to induce a similar response (e.g. tetanus-specific T-cell clones.)	(Zanni et al., 1998a; Burkhart et al., 2001; Naisbitt et al., 2003b; Wu et al., 2006)
Intra-cellular Ca^{2+} release	Following T-cell activation the levels of intracellular Ca^{2+} increases. Sustained Ca^{2+} is seen within minutes in drug-specific T-cells when incubated in the continuous presence of the soluble drug. These data show that processing is not needed.	(Zanni et al., 1998a)
p-ERK 1 / 2 Phosporylation	The phosphorylated form of ERK proteins was detected immediately after drug incubation. These data show that processing is not needed.	(Depta et al., 2004)

Table 1.5 Experimental techniques use to establish the mechanism of antigen presentation of drug specific T-cells.

1.7.1.1 Activation and cross-reactivity of drug-specific T cells in the presence of drugs or theirs reactive metabolites

One of the most useful assays employed to determinate drug specific T-cell activation is the proliferation assay. In this assay, the generated drug specific T-cells are incubated with different micro molar concentrations of the soluble form of the drug or its reactive metabolite in the presence of autologus APC. Normally, autologous generated irradiated B-lymphoblastoid cell lines (B-LCLs) in a ratio 1:5 are used as APC; however, autologus irradiated PBMCs can be used to. Drug-specific T-cells and APCs are incubated with drug or metabolite for 48 hr. Then, ^{3}[H]-thymidine is added for the last 16 hr, and proliferation is measured by scintillation counting (Mauri-Hellweg et al., 1995; Depta et al., 2004). Proliferation is dose dependent yielding a bell shape curve where sub-optimal, optimal and toxic drug concentrations can be established. Generally, drug metabolites are more toxic, and optimal proliferation is seen at lower concentrations when compared with the parent drug (Schnyder et al., 2000; Burkhart et al., 2001). The higher concentrations of parent drug required for optimal stimulation might imply that the drug is metabolized and drug metabolites formation increased; therefore, proliferation is enhanced (Hertl et al., 1995; Merk, 2005).

The capacity of T-cell receptors from drug-specific T-cells to cross-react with structurally related drugs has been studied using the proliferation assay (Mauri-Hellweg et al., 1996; von Greyerz et al., 1999; Schnyder et al., 2000; Naisbitt et al., 2003b; Depta et al., 2004; Wu et al., 2006). Drug specific T-cells are incubated with different concentrations of related drugs and the capacity for cross-reactivity is determined by the strength in the proliferative response with different drug structurally. Generally, drug-specific T-cells recognize drugs that share a similar core structure; small variations outside the core structural motif are normally tolerated by drug-specific T-cells.

Drug-specific $CD4^+$ T-cells proliferate strongly in the presence of the drug and APC; however, drug-specific $CD8^+$ T-cells tend not to proliferate at the same rate. Therefore, other functional studies are needed to better determine the activity of these cells.

T-cells show cytotoxic activity against autologus cells that present antigen in a MHC restricted way. $CD8^+$ T-cells perform this task by the release of soluble factors (perforin / granzyme) or by direct cell-cell interaction (Fas/FasL) with target cells. Interestingly, $CD4^+$ T-cells that contribute to the activation of other cells in the immune system also display cytotoxic activity. T-cells found in blisters or expanded from systemic circulation from hypersensitive patients with different skin clinical manifestations show cytotoxic activity (Schnyder et al., 1998a; Nassif et al., 2004; Wu et al., 2007). To determine the extent of drug-induced cytotoxicity, target cells are loaded with [^{51}Cr] incubated in the presence of the cytotoxic cells (effector) at different ratios (5, 10, 10, 40 : 1) and different concentrations of the drug or metabolite. Drug specific T-cells directly kill autologous B-lymphocytes cell lines in the presence of an agonist, the incriminating drug, its metabolite or structurally related drugs. In some cases, IL-2 or IFN-γ has to be added to enhance cytotoxicity by increasing the number of responsive lymphocyte and MHC class I expression on target cells, respectively. Drug –specific cytotoxic lymphocytes can be incubated during the assay in the presence of $MgCl_2$, which inhibits perforin/granzyme release, or an antagonist CD95/Fas mAb, which blocks the FasL receptor, to determinate the cytotoxic pathway involved in killing. SJS and TEN are characterized by massive keratinocytes death and in most cases the disease is related with use of certain drugs. Therefore, it is believed that drug-specific cytotoxic lymphocytes play a key role in the pathogenesis of the disease.

Information regarding specificity, cross-reactivity, cytotoxicity and drug dose-dependency can be obtained using the proliferation assay. However, the exact nature of the antigen (parent drug or drug-protein adduct) cannot be elucidated. Moreover, the mechanism in which the drug or metabolite activates the specific

T-cells cannot be defined. Therefore, variations of the proliferation assay are used to determinate the pathway of drug specific T-cell activation.

1.7.1.2 Role of covalent binding and drug metabolism in drug-specific T cell activation.

Pulse and antioxidant incubation assays are variants of the proliferation assay that are used to determinate the impact of covalent binding and drug metabolism in drug-specific T-cell activation.

During pulsing experiments, APC are incubated with different concentrations of drug or drug metabolite for different time intervals. Subsequently, APC are washed to remove any un-bound drug, irradiated and incubated with drug-specific T-cells in the absence of the drug or metabolite. In this context, reactive drug metabolites would bind with extracellular membrane proteins within the APC or with peptides in the surrounding environment that are later internalized. The pulsed APC containing drug-protein adducts are then used as source of antigen. It has been found that reactive metabolites form adducts almost immediately (Callan et al., 2009b), so normally APC are pulsed for 1 hour. When APC are pulsed with reactive metabolites and presented to drug-specific T-cells, the proliferation is almost the same as that found in the presence of the soluble form of the metabolite. Additionally, pulsed APC at concentrations that normally cause cell death induces proliferation of drug metabolite-specific T-cells. In contrast, APC pulsed with the parent drug (normally un-reactive) does not induce a response in the presence of the drug-specific T-cells. In most cases, pulsed APC incubated with the parent drug fail to induce proliferation due to the absence of protein adduct formation. However, it has been shown that APC and other non hepatic cells metabolize drugs (Reilly et al., 2000; Sanderson et al., 2007), so longer incubation times and higher concentrations of the parent drug might be needed for the generation of antigenic drug-protein adducts within the APC that could activate drug-specific T-cells.

In a variation of the proliferation assay, glutathione (GSH) is added in excess (1mM) prior to the drug or metabolite. GSH conjugates with drug reactive

metabolites. Thus, the amount of free reactive metabolite and drug-protein adducts decreases drastically. Drug metabolite - specific T-cell proliferation is completely abolished when GSH is added. Interestingly, GSH incubation stabilizes certain reactive metabolites and in some cases inhibits further oxidation or converts by reductions mechanism the metabolites back to their stable form (parent). Therefore, proliferation is enhanced in drug-specific T-cell (specific for the parent form of the drug) during incubation with some metabolites (Burkhart et al., 2001).

1.7.1.3 Determination of the mechanism and kinetics of antigen presentation

Glutaraldehyde fixation of APC blocks antigen processing. APC are normally fixed by incubating the cells with a 0.05% glutaraldehyde solution for 30 s. The reaction is stopped by adding an excess of L-glycine. Parent drug stimulation of specific T-cells is not abolished by fixation of APC suggesting that antigen processing is not needed and that drugs stimulate directly the TCR (Schnyder et al., 1997). APC fixation alters processing, but fixed cells retain their ability to present processed antigens (Schnyder et al., 1997). Reactive drug metabolites can bind directly with MHC molecules or an embebed peptide in the MHC groove. Thus, drug-specific cells can be activated without the requirement of antigen processing (Padovan et al., 1996; Schnyder et al., 2000). However, if the drug-protein adduct is formed in the medium or in the cell membrane and needs processing, the fixed APC are unable to activate drug-specific T-cells clones (Padovan et al., 1996).

Kinetic studies have been performed to corroborate findings with fixed APC. Generally rapid T-cell activation (5 - 60 minutes) is related to processing independent antigen presentation, whereas longer time period (up to 6 hr) is related to processing dependent mechanism.

The CD3 receptor is vital for T-cell activation. In addition, it has been shown that in T-cells TCR/CD3 complex are internalized and recycled after activation (Valitutti et al., 1995). TCR activation can be measured by the down-regulation of this marker using and anti-CD3 antibody. Drug-specific cells are incubated with

APC and the drug or metabolite for different time periods (1, 4 or 16 hr.). At every time point, down-regulation is measured by flow cytometry using an anti-CD3 antibody and compared with CD3 expression on untreated T-cells (drug-specific T-cells and APC without drug). CD3 down-regulation is dose and time dependent. Parent drugs induce significant CD3 down-regulation after 30 minutes. Similar CD3 down-regulation has been seen with super antigens, pre-processed peptides or previously peptide pulsed (16 h) APC (Zanni et al., 1998a). Interestingly, drug reactive metabolites incubate with specific T-cells and APC show similar results (Zanni et al., 1998a; Schnyder et al., 2000). When the reactive metabolites where incubated with APC, CD3 down-regulation was seen within minutes indicating that processing is not required for antigen presentation.

In the following sections, the activation of specific T-cells by small molecules (chemicals and drugs) is discussed. Classical examples for hapten and P-I like activation are discussed. In some cases (particularly with drugs) both mechanism overlap. In the subsequent section, T-cell activation by sulfamethoxazole, which is the central topic of this investigation, is discussed.

1.7.2 Dinitrohalobenzenes

Dinitrohalobenzenes, 2-4-dinitrochorobenzene (DNCB), 2,4-dinitroflurobenzene (DNFB) and 2,4,6-trinitrochlorobenzene (TNCB), are powerful contact sensitizers and have been normally used as models for the study of immunological mechanisms behind contact sensitivity. These compounds are electrophilic and tend to react with nucleophiles molecules, normally lysine and cysteine groups in proteins. DNCB, at physiological pH, binds predominantly to lysine, but binding with cysteine has been reported (Maggs et al., 1986). Proteins adducts formed with intra-cellular or extra-cellular protein are highly immunogenic and activate T-cells via processing dependent pathway (Martin et al., 1992; Weltzien et al., 1996; Pickard et al., 2007). Studies performed in animals showed that both $CD4^+$ and $CD8^+$ cells are activated and secrete high concentrations of IFN-γ following stimulation (Dearman et al., 1996). Human

Additionally, a sub-set of $CD4^+$ T-cell clones secreting high levels of IL-10 were seen in certain patients (Lecart et al., 2001).

More recently, the phenotype of the lymphocytes, the protein target of DNCB conjugation and the mechanism of antigen presentation of DNCB have been described (Pickard et al., 2007). Lymphocytes proliferate in the presence of DNCB secreting a Th1 cytokine profile little or not secretion of IL-10 detected. Moreover, the majority of T-cell clones generated from two sensitized subjects proliferated in a processing dependent way in the presence of DNCB. APC pulsed with DNCB induced proliferation, but when APC where fixed before pulsing, proliferation was significantly decreased. Confocal microscopy showed that DNCB binds to membrane, cytoplasmatic and nuclear proteins. Nevertheless, the nature of the target protein was not established.

1.7.3 Penicillins

Penicillins are associated with type B reactions ranging from maculopapular and bulluos skin reactions (T-cell mediated) to severe anaphylactic reactions (IgE mediated) (Gruchalla, 2003). Penicillins are reactive under normal conditions. The β-lactam ring is target of nucleophilic attack by free amino groups (lysine) found in proteins. This haptenic structure (penicilloyl) accounts for more that 90% of the haptens seen between proteins and penicillins. Therefore, this particular structure is normally referred as the "major determinant". Interestingly, not only the β-lactam ring is a target of nucleophilic attack, but also the thiazoidine ring can form haptenic structures with lysine residues (penicilloyl), which is known as the "minor determinant".

Lymphocytes from penicillin hypersensitive patients proliferate in the presence of the soluble drug, other penicillin or a conjugate formed between the drug and HSA. T-cell clones can be generated by stimulation with soluble drug or protein conjugates. Each antigen shows a different pattern of antigen presentation (Brander et al., 1995; Mauri-Hellweg et al., 1996; Padovan et al., 1997).

Interestingly, T-cell clones generated show two patters of cross-reactivity. Some T-cell clones were highly specific and recognize the drug to which they were stimulated with other were more cross-reactive. Interestingly, ampicillin-T-cell clones did not recognize cefaclor, a cephalosporin that share the ampicillin side chain and β-lactam motifs, but differs in the structure of the second ring (Padovan et al., 1996). These results suggest that the haptenic structure together with the side chain and the thiazolidine ring contribute to the activation of the specific T-cells.

Fixation assays show two patterns of antigen presentation. Some penicillin-specific T-cells do not proliferate in the presence of the soluble form of the drug or conjugated with HSA when incubated with fixed APC (Brander et al., 1995). This clearly indicates that certain T-cell clones recognize haptens that are formed extracellulary or intracellulary and need processing for presentation. In contrast, fixed APC also present the penicillin to other specific T-cell clones (Padovan et al., 1996). This suggests direct binding with peptides embedded in the MHC-groove. Interestingly, penicillin modification of MHC associated peptides has been studied. The results demonstrated that only peptides containing lysine residues in specific positions are able to stimulate TCR (Padovan et al., 1997).

1.7.4 Anticonvulsants

Anticonvulsants (carbamazepine (CBZ), lamotrigine(LTG) and phenytoin (PHT)) are associated with severe hypersensitivity reactions in certain individuals (Roujeau et al., 1995; Schlienger and Shear, 1998; Zaccara et al., 2007). Recent studies suggest that the incidence of these reactions in patients under treatment with anticonvulsant is between 1 in 3000 (Schlienger and Shear, 1998). Drug–specific T-cells have been obtained and the mechanism of antigen presentation described.

The majority of CBZ, LTG and PHT T-cell clones are $CD4^+$; however some $CD8^+$ or dual positive ($CD4^+$ $CD8^+$) T-cell clones are found (Mauri-Hellweg et al., 1995; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Wu et al., 2006). The number of

The majority of CBZ, LTG and PHT T-cell clones are $CD4^+$; however some $CD8^+$ or dual positive ($CD4^+$ $CD8^+$) T-cell clones are found (Mauri-Hellweg et al., 1995; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Wu et al., 2006). The number of circulating CD4 T-cell clones is higher than $CD8^+$, during serial dilution is not surprising to have a skewed $CD4^+$ phenotype. Nevertheless, the number of $CD8^+$ drug specific T-cells can be increase by removing the $CD4^+$ T-cells using an anti-CD4 antibody and magnetic sorting before serial dilution (Wu et al., 2007).

Specific T-cell clones have been generated to the drugs or their metabolites, and different cross-reactivity patterns were found. Importantly, CBZ-specific T-cell clones do not recognize chemical unrelated anticonvulsants (LTG or PHT) and vice versa. However, proliferation assays show that CBZ and LTG specific T-cell clones can recognize other structurally similar compounds.
Importantly, T-cell clones specific towards major carbamazepine metabolites (carbamazepine 10,11 epoxide and 10-hydroxy carbamazepine) were generated, and approximately 30% of these metabolite specific T-cell clones also proliferated in the presence of the parent drug (Wu et al., 2006). In contrast, LTG specific T-cell clones where less cross-reactive, but the generation of LTG metabolite specific T-cells has not been studied (Naisbitt et al., 2003b).

Experimental evidence suggests that CBZ (or its metabolites), PHT and LTG stimulate specific T-cell clones by a pharmacological interaction in which antigen processing is not required (Mauri-Hellweg et al., 1995; Naisbitt et al., 2003b; Wu et al., 2006). Fixed antigen presenting cells present the drugs (CBZ or metabolite and LTG) indicating that the drug interacts directly with the TCR-MHC complex or in the case of CBZ metabolites by the direct binding with the MHC molecules in APC. However, pulsing and antioxidant assays were both negative indicating that drug-protein adduct formation is not relevant for T-cell activation. Recently a strong MHC genetic association (HLA-B*1502) in patients with SJS induced by carbamazepine in Han Chinese has been found (Chung et al., 2004; Chung et al., 2007). Forty-four patients with carbamazepine induced SJS, one hundred and one tolerant patients and ninety-three healthy individuals were all genetically screen for variations in drug metabolism enzymes and immune molecules such

as the human leukocyte antigen (HLA). The results found a striking association of the HLA-B*1502 molecule in 100% of the carbamazepine-induce SJS. Interestingly, the incidence of this particularly HLA phenotype in tolerant and healthy patients was 3% and 8.6% respectively.

Therefore, the direct interaction or binding with this characteristic phenotype could be crucial for carbamazepine specific drug-activation in SJS in this patient's population.

1.8 Hypersensitivity reactions to sulfonamides

Since sulfonamides were developed for the treatment of systemic bacterial infections diseases, their use has been associated with a high incidence of adverse effects (Domagk, 1957; Lehr, 1957). Early studies reported that sulfonamides caused "drug rash", "drug fever" and other toxic effect including blood dyscracias, hepatitis, and central and peripheral nervous system reactions (Lehr, 1957).

It is now estimated that ADRs to sulfonamides occur in 2-5 % of healthy patients, but the percentage is higher in HIV positive patients (30 - 60 %). A decreased levels of cysteine found in HIV-positive patients and a decreased capacity to reduce nitroso-SMX has been related with the high incidence in HIV-positive patients (Pirmohamed and Park, 2001). ADRs to the sulfonamides are diverse and include maculopapular skin reactions (CD 4^+ mediated), anaphaxylasis (IgG mediated), bulluous skin reactions ($CD8^+$ mediated) and SJS or TEN ($CD8^+$ mediated).

Skin disorders are the most common adverse effects associated with sulfonamides exposure. The severity of the skin lesions varies, as does the immunological mechanism. In the case of maculopapular reactions, immunohistochemical analysis of skin sections of allergic patients found the presence of high number of cells that display cytotoxicity against target cells. In contrast, bulluous skin reactions are associated with the infiltration of cytotoxic $CD8^+$ T-cells (Hari et al., 2001; Yawalkar and Pichler, 2001).

SJS and TEN are life treating skin disorders with a mortality rate of 5 and 30%, respectively (Roujeau et al., 1995). In 70% of the cases, drugs are implicated in the development of the disease. Among the drugs responsible for these reactions, sulfamethoxazole-trimetropim has the highest incidence followed by anticonvulsants and NSAIDs (Roujeau, 2005). Blister fluid lymphocytes obtained from patients with TEN were $CD3^+/TCR\ \alpha\beta^+$ of the subtype $CD8^+$. Moreover, cells from 4 out of 6 were cytotoxic against MHC-matched EBV derived cells, and 2 out of the 6 were cytotoxic against the autologus MHC-activated keratinocytes in the presence of the drug. When the cells were incubated with EGTA and $MnCl_2$ the cytotoxic activity was abolished suggesting a perforin/granzyme-dependent mechanism of killing (Nassif et al., 2004).

1.8.1 Metabolism of sulfonamides and drug hypersensitivity

Sufonamides are formed by benzyl – amine group conjugation at the *para* position of the benzyl ring with a sulfonyl – amine group, which is finally linked to different side chains giving rise to different sulfonamides.

The primary amine in the benzyl – sulfonyl amine structure is the main target for oxidation and conjugation reactions.

N-acetyl and glucuronidate conjugates are the main metabolites found in human and rodents urine. Additionally, the aromatic amine is susceptible to oxidation, generating an amine metabolite (Cribb and Spielberg, 1992; Gill et al., 1996; Gill et al., 1997). Several studies demonstrated the presence of a hydroxylamine metabolite of SMX, sulfadiazine (SDZ) and dapsone (DPS) in urine of exposed patients. Incubation of sulfonamides with microsomes obtained from different species demonstrated that the reaction was catalyzed by the P450 enzymatic system and/or experimental animals (Shear and Spielberg, 1985; Uetrecht et al., 1988; Cribb and Spielberg, 1990). Using specific inhibitors to different CYPs and highly pure recombinant CYP isoforms, CYP2C9 was found to be responsible for the N-Oxidation of SMX in humans, whereas CYP2C6 catalyzes the same reaction

in rats (Cribb et al., 1995). CYP2C9 allelic variants have been found (CYP2C9*2 and CYP2C9*3) and related with a decrease clearance of warfarin and phenytoin. In SMX metabolism, different allelic variants of the CYP2C9 were not associated with an increase of SMX oxidation (Gill et al., 1999; Pirmohamed and Park, 2003).

The hydroxylamine metabolites of SMX, SDZ and DPS are more toxic that the parent drugs and can bind covalently to proteins (Rieder et al., 1988; Vage et al., 1994; Rieder et al., 1995; Hess et al., 1999; Naisbitt et al., 2002). Thus, they have been implicated in the pathogenesis of drug hypersensitivity reaction (Shear and Spielberg, 1985; Rieder et al., 1988). Nevertheless, further studies have shown that the hydroxylamine metabolite was not the ultimate toxic agent. The hydroxylamine metabolite undergoes auto-oxidation to form a highly reactive nitroso sulfonamide metabolite (figure 1.5)(Cribb and Spielberg, 1990; Cribb et al., 1991).

SMX-NO is highly electrophilic and is thought to be the final toxic agent in SMX metabolism. The capacity of SMX-NO to form drug-protein adducts have been widely studied (Cribb et al., 1996; Manchanda et al., 2002; Naisbitt et al., 2002; Vyas et al., 2006b; Sanderson et al., 2007; Cheng et al., 2008; Callan et al., 2009b). It has been found that SMX-NO binds covalently with either intracellular or extracellular proteins in different type of cells; however, the nature of the target protein and the chemical mechanism behind this reaction are not well understood. Previous studies have demonstrated that SMX-NO binds with serum protein, but the nature serum protein target of modification has not been described (Meekins et al., 1994).

The adduct formation between SMX-NO and mouse or human serum proteins (albumin) has been investigated recently (Cheng et al., 2008; Callan et al., 2009b). The drug-protein adduct formation has been related with the exposure of hidden epitopes that might be responsible for T-cell activation. The immunobloting assay showed that SMX-NO binds with native mouse serum albumin, but the adduct was not fully characterized by mass spectrometry. Additionally, the adduct failed to induce and immunogenic response in animals

models. However, when the same animal was immunized with SMX-NO conjugated with keyhole limpet hemocyanin (SMX-NO-KLH), isolated sensitized splenocytes proliferate vigorously in the presence of SMX-NO-KLH adduct but not KLH alone (Cheng et al., 2008).

Modification of human serum albumin (HSA) and other proteins with free cysteine by SMX-NO was detected by mass spectrometry, immunobloting and ELISA techniques (Callan et al., 2009b). The results showed that SMX-NO binds with HSA at dose dependent concentrations (> 10 μM), and that the binding occurs in less than a minute and remain constant during time (120 minutes). Mass spectrometry studies showed that SMX-NO bind covalently with the free cystein residue in HSA. These results showed that SMX-NO bind covalently with cystein residues found in different proteins that might give raise to multiple antigenic determinats for T-cells. Further studies are needed to demonstrate that these drug modified proteins can induced a proliferative response in naïve or drug – specific T-cells. The possible mechanism involved in T-cell activation by SMX is shown in figure *1.6.*

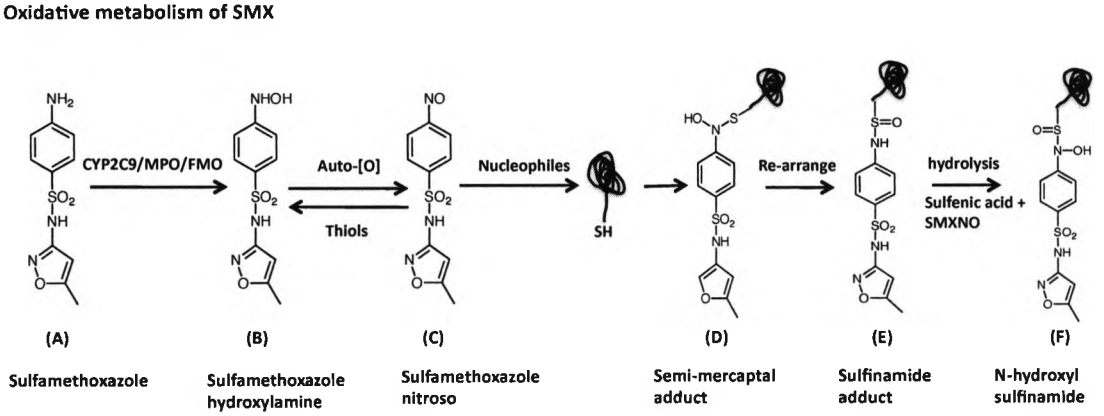


Figure 1.5 (A) SMX is oxidized by CYP2C9 or MPO to SMX-NHOH (B) that auto-oxidizes to form SMX-NO (C). SMX-NO binds irreversible with cysteine residues to form the semimercaptal adduct (D) that re-arranges to form sulfinamide adduct (E). The sulfinamide adduct can further be hydrolyzed to yield sulfenic acid that reacts with SMX-NO again forming N-hydroxy sulfinamide (F)(adjusted from Callan, et al., 2009).

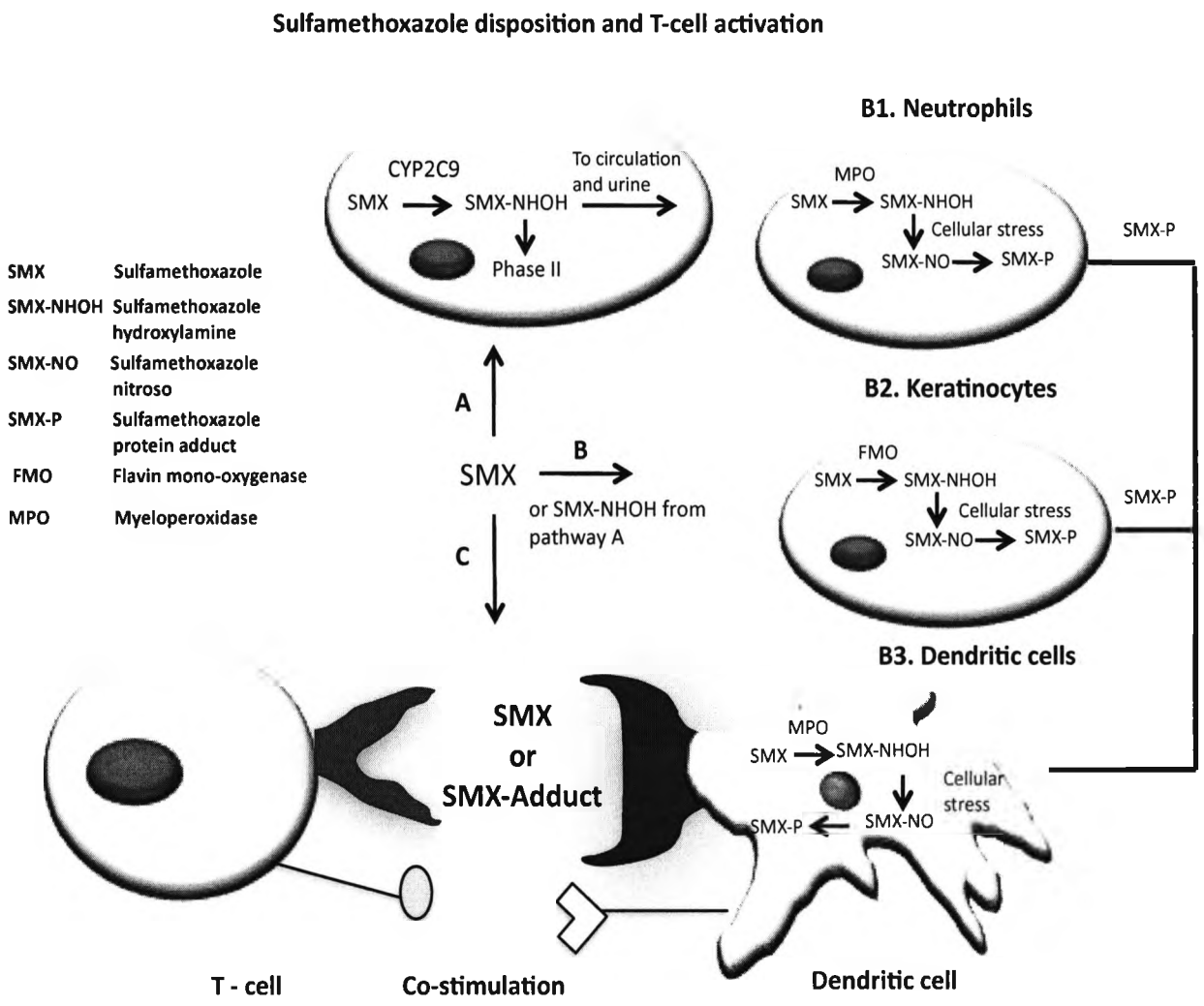


Figure 1.6 (A) SMX is oxidized in the liver by the action of the CYP2C9 enzyme. The hydroxylamine is then processed by phase II enzymes and both metabolites (N-acetyl or SMXNHOH) are excreted in the urine. (B) SMX or SMXNHOH (from the liver) can reach other organs where it can be metabolized intracellularly by different cells and enzymes. SMX is metabolized by myeloperoxidase (MPO) in neutrophils (B1), by flavin mono-oxygenase (FMO) in keratinocytes in the skin (B2) or MPO by monocyte derived dendritic cells (B3). After SMX oxidation, the hydroxylamine auto-oxidises to form SMX-NO, which is toxic and can generated cellular stress. Additionally, SMX-NO reacts with nucleophiles to form SMX-protein adducts. The adducts are finally presented by APC to T-cells. (C) SMX that reaches other organs interacts non-covalently with the MHC and TCR complexes stimulating T-cell activation.

1.8.2 T-cell activation by sulfonamides

Lymphocytes from hypersensitive patients were found to proliferate or exhibit cytotoxic activity in the presence of the parent drug or its metabolite in vitro. SMX-specific T – cell clones obtained by limiting dilution were predominantly $CD4^+$, they secreted high levels of IL-5 and displayed cytotoxic activity against autologus B-LCL and pre-activated keratinocytes. Moreover, the majority of T-cells recognized the parent drug with limited numbers of metabolite specific T-cells detected (Mauri-Hellweg et al., 1995; Schnyder et al., 1998a; Schnyder et al., 2000; Nassif et al., 2002a; Nassif et al., 2004).

Studies into mechanisms of antigen presentation have shown that the parent drug-specific T-cell response is HLA-DR restricted, fixed APC present the antigen and T-cell activation occurred rapidly. $CD3^+$ expression was down regulated in 20 – 30 minutes compared with 6 – 12 hr for classical proteins antigen. Furthermore, Ca^{2+} secretion occurred within seconds following drug stimulation (Schnyder et al., 1997; Zanni et al., 1998a). These data support the P-I concept; however, the role of reactive metabolites of SMX in antigen-specific T-cell activation has only been addressed in a limited cohort of patients and as such requires further investigation.

It is possible that the SMX-NO is responsible for the initiation of the immune response by the formation of proteins adducts or by the direct interaction between the TCR and the drug covalently bound to the MHC molecules. This is supported by several findings in animal models in which the nitroso metabolite but not the parent drug primed naïve lymphocytes. Moreover, PBMCs from naïve healthy volunteers (9 out of1 0) were stimulated with the nitroso metabolite, whereas only 3 out of 10 patients respond with SMX (Engler et al., 2004).

1.8.3 Cross-reactivity reactions between sulfonamides

Patients with a previous hypersentivity reactions to sulfonamides are at risk of a further reaction when talking other structurally related sulfonamides (Strom et al., 2003).

Sulfonamides share a common sulfonyl core structure R_1-SO_2-NH_2-R_2 with an amine-benzyl substitution at the R_1 for antibacterial sulfonamides (see figure 1.5) and a high range of substituent functional groups for non-antibacterial sulfonamides. Cross-reactivity between antibacterial sulfonamides has been described (von Greyerz et al., 1999; Depta et al., 2004); however, the immune mechanism is not clearly defined. In the case of the antibiotic sulfonamides, the structure of the molecules differs at the R_2 position, so similarities in the structure could be vital for the cross-reactivity of these compounds. Additionally, the primary amine attached to the benzyl group at position R_1, which is not present in non-antibacterial sulfonamides, share similar metabolic pathways of detoxication or activation leading to the formation of similar non-toxic or toxic metabolites. The impact of similar reactive metabolites of different sulfonamides in cross-reactivity reactions has not yet been defined due to the lack of synthetic metabolites.

It is generally accepted that T-cells play an important role in hypersensitivity reactions to sulfonamides. T-cell clones (TCC) obtained from patients hypersensitive to sulfamethoxazole have been generated, phenotyped, and the mechanism of antigen presentation has been fully explained (Mauri-Hellweg et al., 1995; Schnyder et al., 1998a; Schnyder et al., 2000).

The stimulation of SMX specific T-cell clones with structurally related sulfonamides has been studied in detail. 13 TCC (9 $CD4^+$ and 2 $CD8^+$) were incubated with 13 different sulfonamides with a sulfonylamine core structure but different side chains. The TCC analyzed were grouped in six different categories according to the different specificities found by analysis of antigen-specific T-cell stimulation. The authors concluded that the different sulfonamides structures have an effect on receptor affinity, possibly by hydrogen interactions with nitrogen and oxygen atoms found on specific T-cell receptors (von Greyerz et al., 1999). It is important to note that these data derive from specific T-cell clones obtained from 1 patient and there were not tested against any sulfonamide metabolite. In more recent studies blister fluid cells from patients with TEN were shown to be stimulated (measured by cytotoxic assays) with

three different sulfonamides (sulfamethizole, sulfapyridine and sulfadiazine) (Nassif et al., 2004).

Furthermore, when the TCRs from a SMX hypersensitive patient were transfected to a TCR negative murine T-cell hybridoma and cross-reactivity with different sulfonamides analyzed, cross-reactivity was found to be dependent of the affinity of the TCR-MHC-peptide-drug interaction and also the density of TCR found in the cell surface (Depta et al., 2004). These data clearly show that T-cell specific to SMX can recognize other sulfonamdies bearing a sulfonylamine, none of the transfectants recognized celecoxib, glibenclamide or furosemide, core with slight changes in the side chains.

Recently, the use of celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor with a sulfonyl core, but not benzyl amine structure, has been associated with ADR, and warnings have been raised for patients with known sulfa allergy. Nevertheless, in vitro studies show that TCC of patients with hypersensitivity to antibiotic sulfonamides do not cross-react with other structures that do not have the sulfanilamide core structure (Britschgi et al., 2001; Depta et al., 2004). Additionally, patients with reported hypersensitivity to sulfamethoxazole were challenged orally to celecoxib reaching a dose of 200 mg without detectable adverse effects (Figueroa et al., 2007). Figure 1.7 depicts the possible mechanisms involved in the recognition of three different sulfonamides by SMX-specific T-cells.

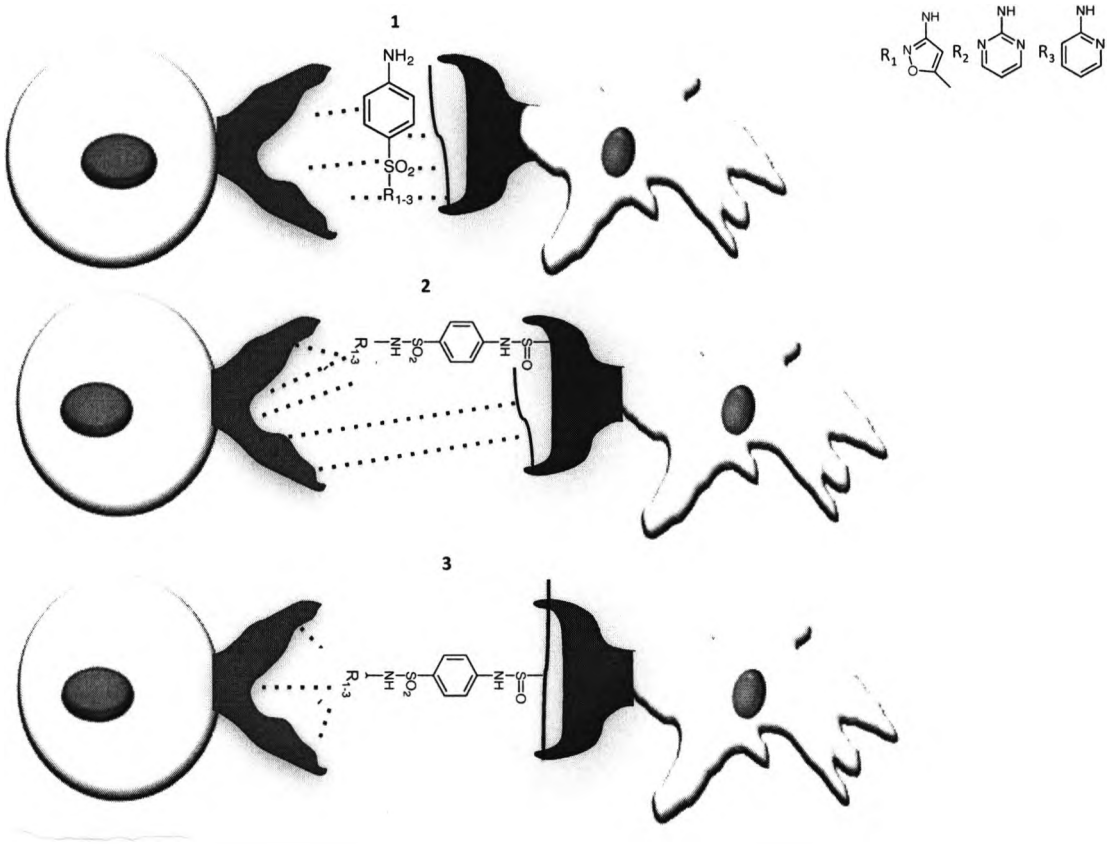


Figure 1.7 Proposed model for T-cell recognition of sulfonamide derivatives. (1) The sulfonamides interact within the TCR – MHC – peptide complex in a weak labile form. (2) The different nitroso sulfonamide metabolites bind the same target amino acids in the MHC molecule and the side chain interacts with the specific TCR. (3) The nitroso sulfonamide metabolites irreversible bind to the same extracellular proteins (e.g. HSA); the drug-protein adduct is processed and presented to specific T-cells and the side chain interacts with the TCR.

1.9 Aims of the thesis

The aim of this thesis is to define the mechanism(s) by which sulfonamides and their metabolites interact and stimulate immune cells, focussing particularly on potential T-cell receptor cross-reactivity with closely related drug structures. Sulfamethoxazole, sulfapyridine and sulfadiazine were selected for these studies for three main reasons. Firstly, the three sulfonamides have been associated with hypersensitivity reactions. Secondly, they share a similar core structure (sulfonyl-benzyl-amine), necessary for T-cell recognition. Thirdly, they are metabolized to hydroxylamine and nitroso metabolites.

To demonstrate that cross-reactivity between sulfonamide metabolites may occur. In chapter 2, the procedure for the synthesis of the hydroxylamine and nitroso metabolites will be described. The immunogenicity of the sulfonamides metabolites in addition with the cross-reactivity patterns will be addressed in chapter 3 using an animal model. T-cell activation and cross-reactivity studies in human models will be investigated in chapter 4. To achieve this, SMX specific T-cell clones will be generated from PBMCs from sulfonamide hypersensitive patients, and the degree of cross-reactivity will be evaluated using the different sulfonamides metabolites. Finally once investigated the role that sulfonamide reactive metabolites play in the activation and cross-reactivity of T-cells, in chapter 5 the capacity of mouse splenocytes to oxidise SMX, the formation of SMX-protein adducts and the capacity of these adduct to activate cells from SMX-NO immunized mice will be studied.

CHAPTER 2

THE CHEMICAL SYNTHESIS OF SULFONAMIDE METABOLITES

2.1 Introduction

2.1.1 Sulfonamides

The antibacterial properties of sulfonamides were discovered in 1936 by the studies of Trefouel (Gray et al., 1937). The authors probed the antibacterial activity of *p*-amino benzene sulfonamides in mice infected with *Haemolityc Streptococci* (Buttle et al., 1938). Further studies focused on the use of synthetic sulfonamide derivatives to improve efficacy and most importantly to reduce the toxic effects associated with drug exposure (Buttle et al., 1938). Results from these studies demonstrated that the sulfonilamide core structure was responsible for its antibacterial activity. The sulfonylamine structure competitively inhibits the binding of the *p*-aminobenzoate, the precursor in the biosynthesis of folic acid compounds in bacteria. Specifically sulfonamides inhibit the enzymatic step where *p*-amino benzoate reacts with 2-amino-4 hydroxy-6-hydroxy methyl dihydropteridine to yield dihydropteorate (Brown, 1962).

Sulfamethoxazole (SMX) has been widely used in the treatment of urinary and gastrointestinal infections, nocardiosis, and more recently for opportunistic infections found in HIV positive patients (Stamm et al., 1980; Smego et al., 1983; Fischl, 1988). Other sulfonamides in therapeutic use include sulfadiazine and sulfapyridine. Both were introduced around 1940 and together with SMX have been widely used. Sulfadiazine in combination with pyrimethamine is commonly used and is effective in the treatment of Toxoplasmosis, which is caused by *Toxoplasma gondii* an intracellular protozoan (Montoya and Liesenfeld, 2004).

In addition to its anti-bacterial properties, sulfapyridine, a moiety derived by the cleavage of the azo bond of sulfasalazine by bacterial azoreductases in the large intestine, is used in the treatment of rheumatoid arthritis and Crohn's disease (Plosker and Croom, 2005). The mechanism and site of action of the drug in these diseases remains unclear.

The three sulfonamides share a common metabolic pathway and have been highlighted as causative agents responsible for type B reactions including hypersensitivity reactions (Shear and Spielberg, 1985; Haverkos, 1987; Leport C

Fau - Raffi et al., 1988; Rieder et al., 1988; Roujeau and Stern, 1994; Uetrecht, 2002).

2.1.2 Sulfonamides metabolism

Sulfonamides are N-acetylated by different isoforms of the human N-acetyl transferase (NAT_{1-3}); SMX N-acetylation is mainly catalyzed by NAT_1 (Cribb et al., 1993). Additionally, oxidation of the primary aromatic amine by human P450 in the liver to form the hydroxylamine metabolite is an important metabolic pathway for sulfonamides (Uehleke, 1973; Hammons et al., 1985; Cribb and Spielberg, 1992; Hlavica, 2002b). SMX is N-oxidised to a hydroxylamine by CYP2C9 in human hepatic microsomes (Cribb et al., 1995). Outside of the liver, keratinocytes also catalyze the conversion of SMX to SMX hydroxylamine by the enzymatic action of the flavin mono-oxygenase (FMO) enzyme, although the levels of SMX-NHOH detected were very low (Reilly et al., 2000). Other enzymes, MPO, found in neutrophils and monocytes, and cyclooxygenase (COX) can oxidize SMX (Cribb et al., 1990; Sanderson et al., 2007).

Metabolism has not been studied in as much detail for SDZ as it has been for SMX. Previous studies report the presence of the hydroxylamine metabolite and hydroxylated SDZ (Schoondermark-van de Ven et al., 1995). Nevertheless, the enzymes responsible for the N-acetylation and hydroxylation have not been defined. A recent report using different P450 human isoforms and NAT enzymes with different inhibitors shows that oxidation of SDZ to SDZ-NHOH may be mediated by CYP2C9 and CYP2C8 and N-acetylation is catalyzed by the action of NAT_2 (Winter and Unadkat, 2005). Extra hepatic metabolism for SDZ has not been investigated.

Commercial formulations contain sulfapyridine attached to mesalazine by an azo bond in sulfasalazine. (Plosker and Croom, 2005). Sulfasalazine concentrations that reach the large intestine are cleaved at the azo bond liberating sulfapyridine and mesalazine (Peppercorn and Goldman, 1972). Almost 90% of sulfapyridine is absorbed from the large intestine and reaches peripheral circulation (Houston

et al., 1982). Sulfapyridine is N-acetylated by NAT_2 to N-acetyl-sulfapyridine (Tanigawara et al., 2002) which can be found in the urine along with glucuronide conjugates. Like SMX and SDZ, a hydroxylated metabolite (5-hydroxypyridine) has been detected. The formation of SP-NHOH by oxidation of SP is also an important metabolic pathway and it may be responsible for the toxicity and immunogenic activity of SP.

2.1.3 Chemical synthesis of sulfonamides and their metabolites

Sulfonamides have been typically prepared by the reaction of the corresponding amine with sulfonyl-chlorides. This method is highly efficient in small-scale laboratories. Moreover, several synthetic methods are available (Shi et al., 2009). More recently, environmental friendly syntheses based on C – N bonding catalyzed with immobilized nano - Ru catalyst has been performed successfully (Shi et al., 2009).

Oxidation of aryl amines is important for the synthesis of many products such as azo, azoxy, anil and hydroxylamines, which have many industrial and pharmaceutical applications. (Lu and Xi, 2008) The primary amine of sulfonamides, as stated above, can be enzymatically transformed to a hydroxylamine intermediate, following oxidation is thought to be responsible for the toxicity associated with this class of compounds (Hammons et al., 1985; Reilly et al., 1998; Reilly et al., 1999; Guengerich, 2002; Hlavica, 2002a). Thus, the chemical synthesis of these hydroxylamine metabolites is vital to have a better understanding of the interactions between the metabolite and different biological agents.

Hydroxylamines were detected by thin layer chromatography (TLC) as an intermediary products during the hydrogen transfer reduction of nitro benzene to aniline (Entwistle et al., 1978). The reduction of nitro compounds has been traditionally performed using zinc as catalyst and ammonium chloride as a hydrogen donor in an ethanol and water solvent system (Gowenlock and Richter-Addo, 2004). Since these reduction reactions were introduced, many

catalysts, hydrogen donors and solvent systems have been used for a more selective synthesis of hydroxylamines (Entwistle et al., 1975; Entwistle et al., 1977; Rondestvedt and Johnson, 1977; Entwistle et al., 1978; Ayyangar et al., 1981; Ayyangar et al., 1984). For example, the reduction of *m*-dinitrobenzene using cyclohexene and palladium-charcoal occurs so rapidly that the hydroxylamine intermediate could not be isolated due to further reduction to the *m*-nitroaniline. Nevertheless, when the catalyst and hydrogen donor where changed to rhodium-charcoal and hydrazine respectively, the rate of reduction was slowed down, and the hydroxylamine compound, N-3-nitrophenylhydroxylamine was isolated. Other reduction systems using raney-nickel, di-chloroethane and hydrazine have proved to also be highly effective for reductions of a variety of nitro compounds (Rondestvedt and Johnson, 1977; Ayyangar et al., 1981; Ayyangar et al., 1984).

The poor solubility of sulfonamide compounds in the common solvents used in these reductions represents an important challenge in the synthesis of the hydroxylamine derivatives. The complete reduction of the highly insoluble 3,3-dinitrodiphenylsulphone and 4,4-dinitrodiphenylsulphone to its di-amine derivative was achieved with high purity and yield (Ayyangar et al., 1981). However, the formation of the hydroxylamine intermediates was not reported. In a following study by the same authors the synthesis of different hydroxylamines under similar conditions was reported (Ayyangar et al., 1984).

In the case of the synthesis of sulfonamide metabolites, different methods of reduction of the nitro compound have been used to form hydroxylamine metabolites. Dapsone hydroxylamine (DPS-NHOH), SDZ-NHOH and SMX-NHOH were reduced using platinum oxide with triethyl phosphite dissolved in ethanol under pressured conditions (30-40 psi) (Rieder et al., 1988; Uetrecht et al., 1988). More recently, SMX-NHOH was synthesised with high yield and purity under milder drastic conditions using a variation of the hydrogen - transfer reduction system employed by Josthone (Naisbitt et al., 1996a).

Aryl-nitroso compounds have been widely studied due to their toxic, allergic, carcinogenic, and mutagenic effects (Gowenlock and Richter-Addo, 2004).

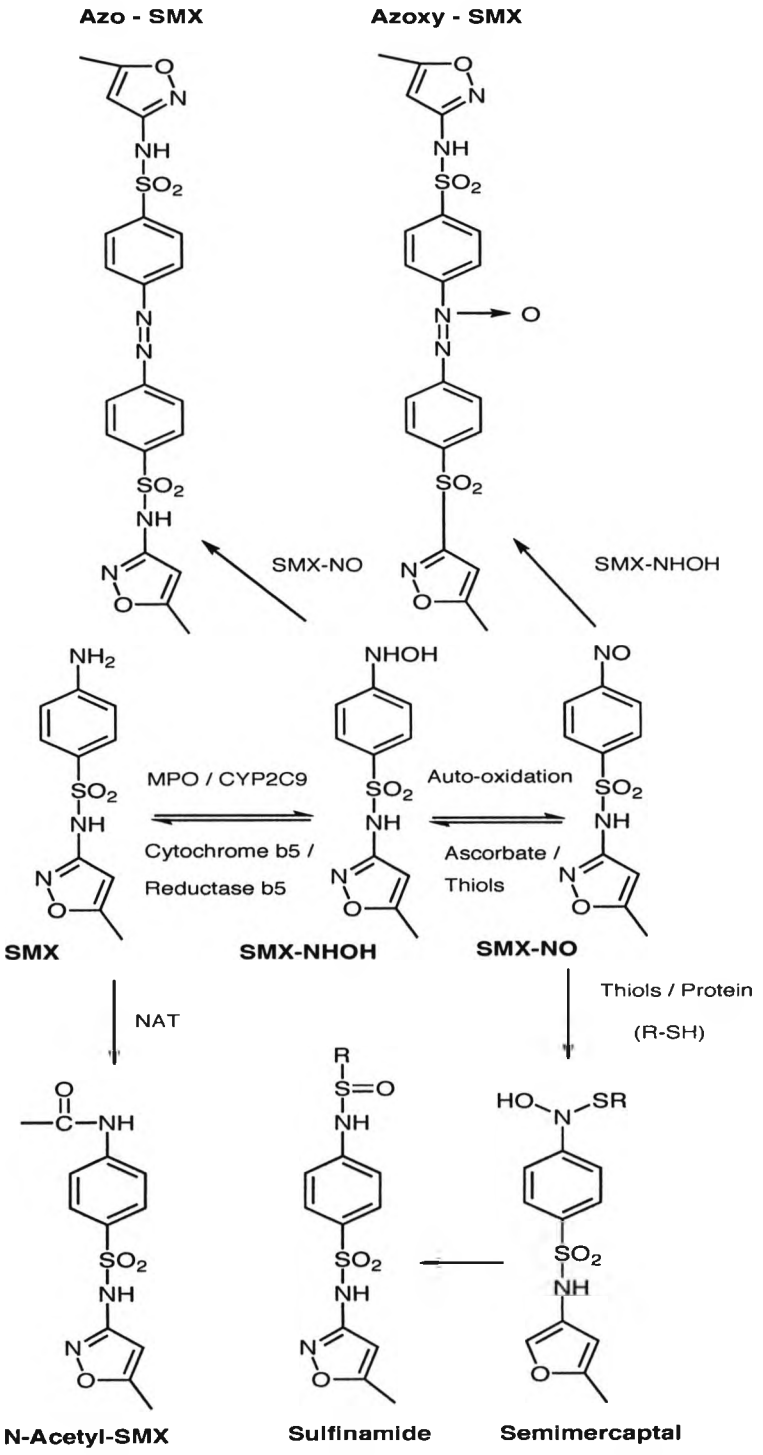
These compounds are highly electrophilic, so they easily react with nucleophiles found within the environment (Cribb et al., 1991; Riley et al., 1991; Ellis et al., 1992). Chemically C-nitroso compounds can be prepared by wide variety of different synthetic routes (Gowenlock and Richter-Addo, 2004). Nevertheless, direct oxidation of the precursor hydroxylamine has been traditionally used in the synthesis (Davey et al., 1999; Gowenlock and Richter-Addo, 2004).

Different oxidizing agents can be used in the oxidation of hydroxylamines to form their nitroso derivative (ferric chloride, sodium dichromate and sulfuric acid, silver carbonate). Among these oxidizing agents, ferric chloride is one of the oldest agents used to obtain a wide variety of nitroso compounds (Gowenlock and Richter-Addo, 2004).

The heterogeneous oxidation with ferric chloride can in some cases be slow (Davey et al., 1999). The slow rate of oxidation can lead to the formation of azo and azoxy dimers by the coupling between newly formed nitroso and remaining hydroxylamine or the degradation of the hydroxylamine precursors, which are not particulary stable in solutions. Oxidation using silver carbonate occurs within minutes, and can prevent the formation of azo and azoxy dimers and the degradation of the hydroxylamine in some nitroso synthesis (Davey et al., 1999).

The synthesis of nitroso sulfonamides has helped mechanistic investigation into the adverse reactions associated with this class of compounds since they are directly protein reactive. Furthermore, *in situ* metabolite generating systems are not available for *in vitro* assays (Uetrecht, 1985; Cribb et al., 1991; Naisbitt et al., 1999; Schnyder et al., 2000; Farrell et al., 2003; Sanderson et al., 2007). The synthesis of these compounds has helped us to better understand the mechanism behind these reactions.

In other studies SMX-NO was obtained by spontaneous oxidation of the hydroxylamine metabolite at 37° C in buffered solution (Cribb et al., 1991). In 1996, SMX-NO was synthesized by direct oxidation with ferric chloride (Naisbitt et al., 1996a). The product was isolated and fully characterized with yield and purity of 85% and 99% respectively. Since then, this synthesis has been widely used for the synthesis of SMXNO, but is has not been applied to the synthesis of other nitroso sulfonamides.



Scheme 2.1 Different metabolic pathways of SMX. SMX is metabolized by CYP2C9 or MPO enzymes to SMX-NHOH, or is acetylated to form N-Acetyl-SMX. SMX-NHOH auto-oxidizes to SMX-NO that binds with cysteine residues found in proteins forming the semimercaptal and sulfinamide. SMX-NO and SMX-NHOH can conjugate to form the dimers compounds (azo or azoxy).

2.2 Aim of the investigation

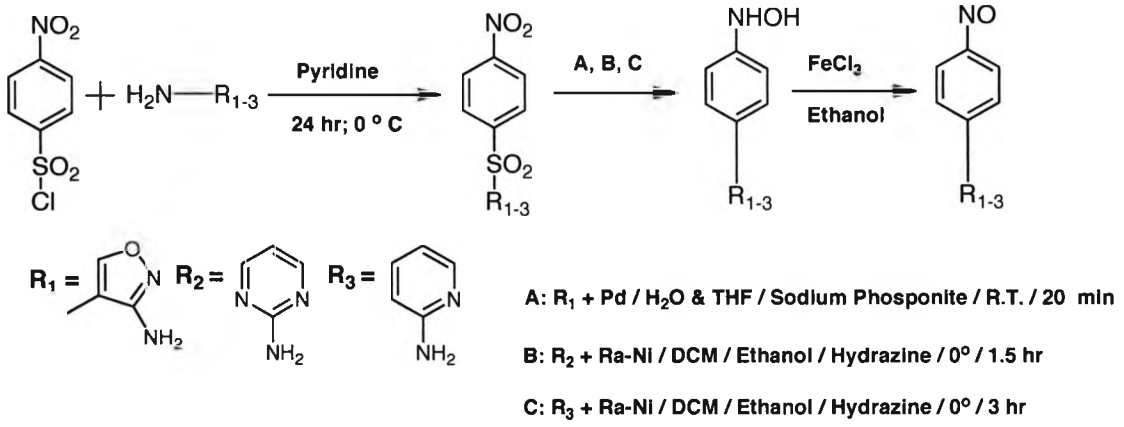
The objective of this chapter was to synthesize the hydroxylamine and nitroso metabolites of common sulfonamides SMX, SP and SDZ. The synthesis was based on a three-step procedure involving the formation of the nitro compounds of the three sulfonamides, carefull reduction of these compounds to form the hydroxylamine metabolites, and finally oxidation of the hydroxylamine to yield the nitroso metabolites.

2.3 Results and discussion

Sulfonamide metabolites have an important role in the pathogenesis of drug hypersensitivity reactions. In order better understand of the mechanism involved in this disease, access to the metabolites is vital. SMX-NO has been synthesized previously and used *in vitro* and *in vivo* systems to study models of disposition and the relationship between direct toxicity and the induction of the immune response. This chapter discussed the steps involved in the chemical synthesis of sulfonamides metabolites. In following chapters, the immunogenicity of the synthetic compounds is assessed in animal and humans

The chemical synthesis was achieved by a three-step procedure. Firstly, the nitro compounds from the three different sulfonamdies (SMX, SDZ and SP) were synthesized using the procedure **2.4.4.** Secondly, these newly synthesized compounds were chemically reduced to form the hydroxylamine metabolites using procedure **2.4.5.** Finally, the hydroxylamine metabolites were oxidized as described in procedure **2.4.6.** The description of the chemical synthesis of these compounds is covered in this section **(Result and discussion section)**, while the experimental procedures, yield, purity, and characterization of the compounds can be found in the **Experimental section 2.4.**

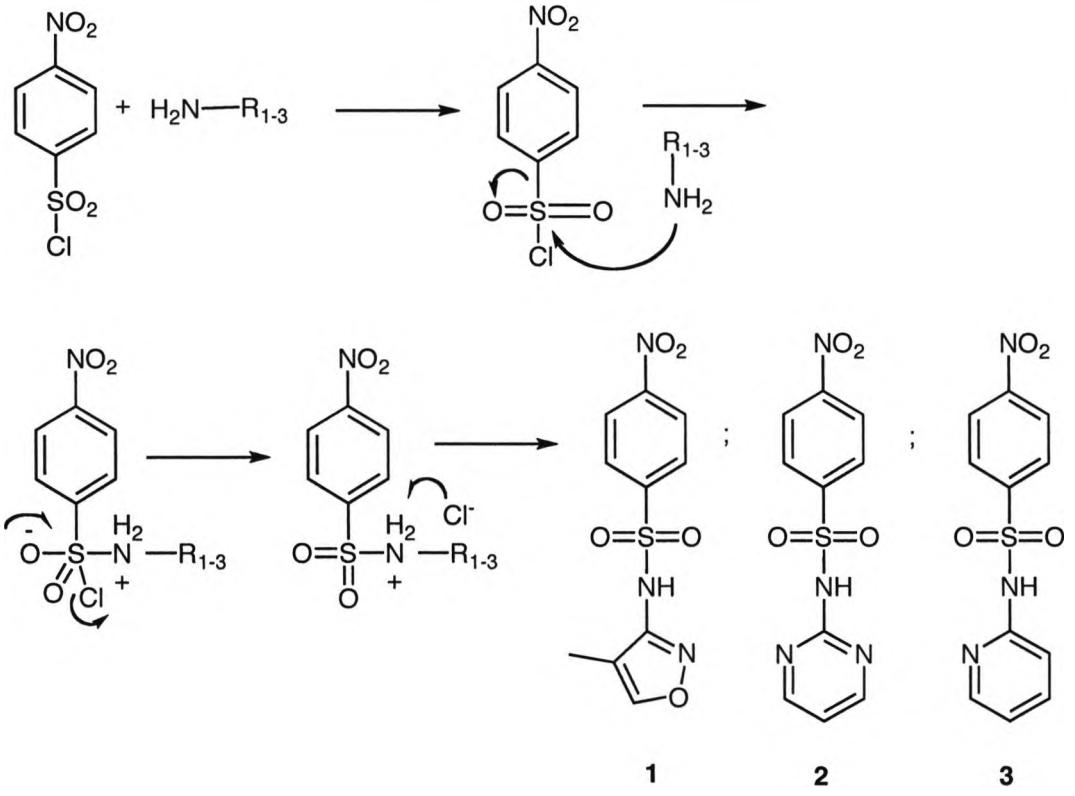
The following diagram shows the three-step procedure involved in the chemical synthesis of the different nitro, hydroxylamine and nitroso compounds from the different sulfonamides (Scheme 2.2).



Scheme 2.2 Chemical route for the synthesis of hydroxylamine and nitroso sulfonamide metabolites.

2.3.1 Synthesis of nitro sulfonamides

A common method for the synthesis of aromatic nitro compounds is the formation of the nitro sulfonamide derivative using 4-nitro-benzyl-sulfonyl chloride coupled with different side chains. The nucleophilic substitution reaction starts with the nucleophilic attack of a lone pair of electrons from the primary amine to the sulfonyl chloride compound followed by the expulsion of chlorine atom which is a good leaving group (Scheme 2.3).

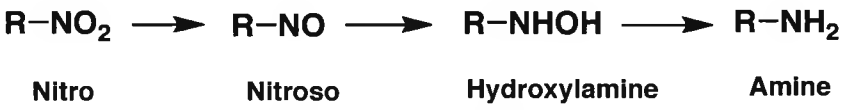


Scheme 2.3 Proposed mechanisms for the formation of the different nitro sulfonamides compounds.

Nitro sulfonamides (SMX, SDZ and SP) were synthesized **(1-3)** with a yield and purity higher than 50% and 97%, respectively. The nitro compounds **(1-3)** were fully characterized by nuclear magnetic resonance (NMR) and liquid chromatography - mass spectrometry (LC-MS); the analysis is described in the experimental section **2.4.4**

2.3.2 Synthesis of sulfonamide hydroxylamines

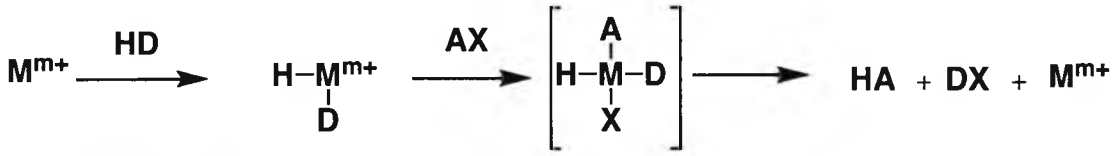
Catalytic transfer hydrogenation is a common method used in the reduction of nitro compounds to form amines (Ayyangar et al., 1984; Johnstone et al., 1985). During the reduction process several intermediates have been characterized, when the reaction was followed by thin layer chromatography (Entwistle et al., 1978).



Scheme 2.4 Intermediaries found during catalytic transfer hydrogenation of nitro-benzyl to aniline.

The intermediate compounds found correspond to nitroso and hydroxylamine compounds. The reduction is complicated, and in most cases it is extremely difficult to stop the reaction at a single intermediate. Different catalysts, hydrogen donors, and solvents used in the reduction have been reported in the literature; all of them affected the velocity, selectivity and yield of the reaction (Rondestvedt and Johnson, 1977; Entwistle et al., 1978; Ayyangar et al., 1981). The most widely used reduction system consists of zinc as catalyst and ammonium chloride as the hydrogen donor in an aqueous or alcoholic suspension (Entwistle et al., 1978).

The following diagram illustrates the general mechanism for the hydrogenolysis reaction, which is similar to the process occurring during the catalytic reduction of unsaturated molecules.



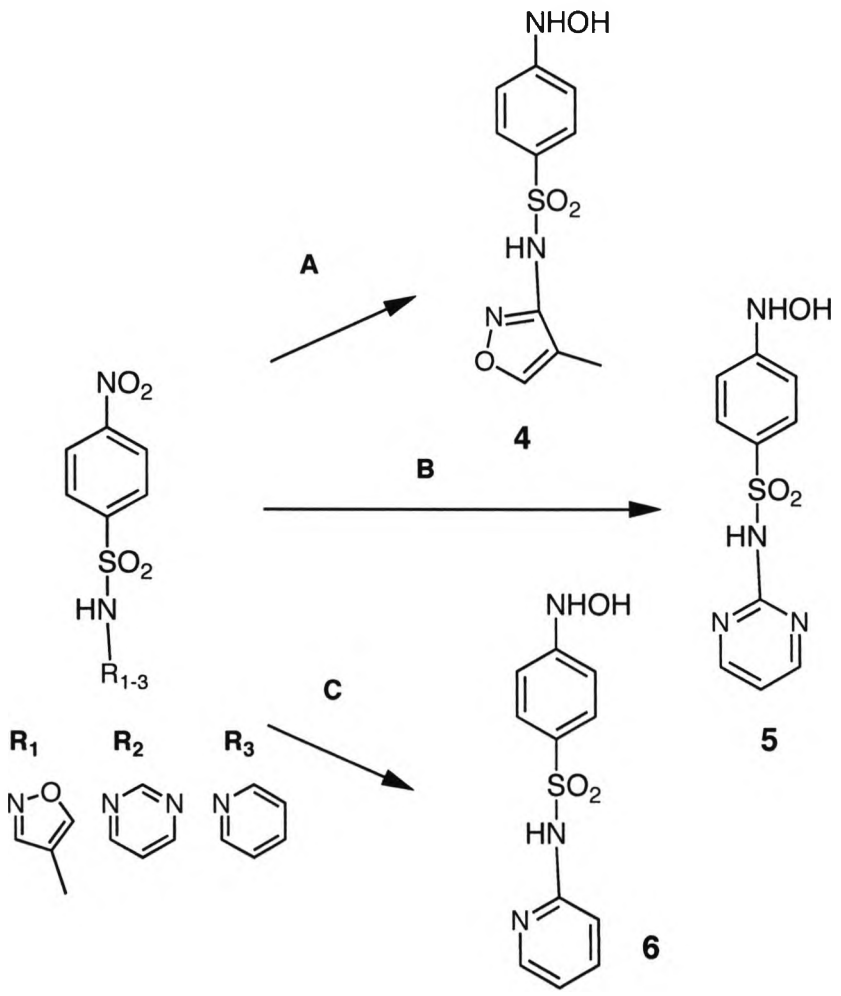
M = Catalytic metal

HD = Hydrogen Donor

AX = Hydrogen Acceptor

Scheme 2.5 General mechanism for the hydrogenolysis reaction.

The catalyst transfer reduction hydrogenation has been successfully used in the reduction of several nitro sulfonamide compounds to form hydroxylamine sulfonamide metabolites (Rieder et al., 1988; Cribb et al., 1991; Naisbitt et al., 1996a). During this reduction process, the hydrogens are released by the hydrogen donors species, which then co-ordinate with the catalyst which in turn transfers the hydrogen to the respective acceptor molecule to form the hydroxylamine. Over-reduction may occur yielding the primary amine. The catalyst transfer reduction hydrogenation is a method for the synthesis of different sulfonamide hydroxylamines as showed in scheme 1 and 2. Several commonly used catalysts (palladium, zinc and Raney-nickel) and hydrogen donors (sodium phosponite, ammonium chloride and hydrazine) were used in different solvents systems (THF/Water, DMF/Ethanol/Water and Dichloroethane; Scheme 2.6).



A: R_1 + Pd / H_2O & THF / Sodium Phosponite / R.T. / 20 min

B: R_2 + Ra-Ni / DCM / Ethanol / Hydrazine / 0° / 1.5 hr

C: R_3 + Ra-Ni / DCM / Ethanol / Hydrazine / 0° / 3 hr

Scheme 2.6 Chemical routes for the synthesis of SMX, SDZ and SP hydroxylamine metabolites.

A variation of the Johnstone's method for reduction of substituted nitro-benzenes was used for reduction of SMX-NO_2. This method has been used previously with a reported yield and purity greater than 60% and 97%, respectively (Naisbitt et al., 1996a). We successfully synthesized SMX-NHOH with similar yield and purity.

The synthesis of the other two hydroxylamines (SDZNHOH [**5**] and SPNHOH [**6**]) was more challenging. Different catalyst, hydrogen donors, and solvent systems were employed to obtain the desired products. The results are summarized in table 2.1

Conditions	*Catalyst*	*H donor*	*Solvents and Temperature*	*Time*	*Results*
A	Palladium	Sodium Phosponite	THF / H2O / R.T.	< 7 min	Hydroxylamine Amine Nitro
B	Zinc	NH4+Cl-	DMF / H2O / Ethanol / R.T.	≈ 30 min	Hydroxylamine Amine Nitro
C	Palladium	Hydrazine	DMF / H2O / Ethanol / THF / R.T.	24 hrs	No reaction
D	Zinc	$NH_4^+Cl^-$	DMF / H2O / Ethanol / R.T.	20 min	Hydroxylamine Nitro
E	Raney-Nickel	Hydrazine	Dichloroethane / Ethanol / 0° C	3 hr	SPNHOH (95%)
F	Raney-Nickel	Hydrazine	Dichloroethane / Ethanol / 0° C	1.5 hr	SDZNHOH (95%)

Table 2.1 Conditions use during the reduction $SPNO_2$ and $SDZNO_2$ to their respective hydroxylamine metabolites

During catalytic hydrogen reduction four variables need to be considered carefully; the catalyst, the hydrogen donor molecule, the solvent system and the temperature. The time of the reaction is just a consequence of the four variables.

In the synthesis of compounds **5** and **6** many reaction conditions were used to obtain the desired products (see, Table 2.1). The following paragraphs discusses the effect of different catalysts on the reduction process.

In initial attempts to synthesize SDZ-NHOH and SP-NHOH, the system described for the synthesis of SMX-NHOH was used (see **A** in table 2.1*)*. The reaction occurred quickly using this system, and the presence of hydroxylamines was immediately detected by TLC (< 5 min). Palladium is one the most widely used catalyst in nitro reduction. Studies comparing the catalytic activity of the catalyst have shown that in certain reactions under the same conditions of temperature and hydrogen donor palladium is the most active catalyst (Palladium > Rhodium > Nickel > Platinum (Johnstone et al., 1985).

The use of other active catalysts (zinc and Raney-Nickel; **B-F**) had a positive effect. During the zinc reduction, the time for the formation of the hydroxylamine was relatively longer than the one observed with palladium (20 and 5 minutes respectively). When a less active catalysts was used (Raney-Nickel; **E & F**), the time for hydroxylamine formation was considerable longer (1½ - 3 hrs) than the observed with Pd and Zn (5 or 20 minutes respectively). The results showed that the use of less active catalyst such as Raney-Nickel allowed a mild hydrogen reduction in which the hydroxylamine intermediary is formed without rapid over reduction to form the amine.

Sodium phosponite, ammonia, and hydrazine are commonly used hydrogen donors. We employed each of these hydrogen donors in the reduction of nitro SDZ & SP. These hydrogen donors successfully transferred hydrogen to the acceptor molecules under the different reactions conditions. In all cases, except for condition **C**, hydrogen transfer was measured by the formation of the different hydroxylamine or amine derivatives by TLC. The hydrogen donor molecules were used in combination with the catalyst which showed the best reduction catalysis.

Finally, the effect of the different solvent systems and temperature on the hydrogenation reduction was explored. One of the major problems during the

reduction of the nitro sulfonamide was the extremely poor solubility of the nitro compounds. The two nitro sulfonamides (**2** and **3**) unlike compound **1** were insoluble in THF and other commonly used non-polar solvents. The low solubility directly affects the course of the reaction, and just a small fraction of the nitro compound was reduced and over-reduction products were consistently detected. Additionally, the solvents can often *poison* the reduction. In this case, the affinity of the solvent for the catalyst is higher than the affinity of the hydrogen donor to the catalyst reducing considerably the rate of reduction or in some cases completely blocks the transference of hydrogens (Johnstone et al., 1985).

As observed in table 2.1, different solvent systems were used in the reduction of nitro SDZ and SP. As mentioned above, the low solubility of the compounds had a negative effect on the reduction process. Therefore, to solve the problem, DMF, a strong polar solvent, was used to dissolve the starting materials and improve the synthesis. The use of DMF in the reduction hydrogenation has been reported before (Balaban et al., 1998; McGill et al., 2000). In our results (*see table 2.1, **B***) DMF completely dissolves both nitro SDZ and SP (**2** and **3**) and allowed hydrogen transfer to these compounds. Unfortunately, the use of DMF is not always recommended, mainly because the solvent is difficult to remove from the reaction, and additional steps together with high temperatures are often needed to remove it, which in some cases can affect the stability of the compounds. In conditions **C** and **D** the volume of DMF was reduced to the minimum amount needed to dissolve the nitro compounds facilitating the purification process. The combination of Pd with DMF (**C**) did not yield any hydroxylamine after 24 hrs. In this case, it is possible that the affinity of DMF for the catalyst was higher than the affinity of hydrogen donors.

When DMF was used with zinc the reaction occurred at the same rate as **B** in which the volume of DMF was considerably higher. This demonstrated that the addition of DMF did not have an effect on the reduction using Zn and ammonium chloride. In the reductions of nitro SDZ and SP, the hydroxylamine product was seen after 20 - 30 minutes.

The catalytic reduction of low solubility nitro compounds, 3,3-dinitrodiphenylsulphone and 4-4-dinitrodiphenylsulphone, in common solvent systems (ethanol, methanol, dichloromethne and THF) has been studied previously (Ayyangar et al., 1981). These compounds were effectively reduced to the corresponding amines and the optimal conditions were determined. The use of Raney-Nickel as a catalyst, hydrazine as a hydrogen donor and a solvent mixture of DCE/Ethanol, which are commonly used and easily remove, and temperatures ranging 30-60°C proved to be the most appropriate method transfer-hydrogenation. In a further report by the same author, conditions for the hydrogen-transfer were used to obtain aryl-hydroxylamines using soluble nitro precursors (Ayyangar et al., 1984). The author showed a selective reduction of a wide range of aryl-nitro compounds using raney-nickel, DCE / ethanol (1:1), two molar equivalents of hydrazine and a strict control of the temperature (0°C during the entire reaction). A similar protocol was used in an attempt to improve the yield of the hydroxylamine compounds (see **E** and **F**). It has been mentioned before that Raney-Nickel (a less active catalyst) allows mild reduction (Ayyangar et al., 1981).

The mixture DCE / Ethanol used in **E** and **F** has previously been shown to be the best for the rapid and selective synthesis of nitro compounds with low solubility (Ayyangar et al., 1981; Ayyangar et al., 1984). In this case the solvent system used on **E** and **F** compared with the solvents used in **A-D** was the most effective to use, not only because it allowed the formation of the different hydroxylamines, but also because these solvents are common, not expensive, and more importantly can be easily removed from the reaction mixture.

Finally, the temperature used in the different conditions also has an important effect in the synthesis of the aromatic hydroxylamines. Normally, an increase in temperature results in increased hydrogen transfer (Johnstone et al., 1985). Most of the reductions (**A-D**) were performed at room temperature as described in previous protocols. Although in some cases temperatures higher than 50 °C has been reported, we decided not to increase the temperature to avoid

decomposition of the hydroxylamine intermediates. Temperature had a crucial effect in conditions **E** and **F**. In these cases, it was extremely important to keep the temperature at 0 - 10 °C during the entire course of the reaction to increase the yield of the hydroxylamine product. Decreasing the temperature had a direct effect on the reduction catalyst allowing formation of intermediary hydroxylamine products (Hydroxylamine) that are further reduced much more slowly. Of all the methods used for the reduction of the different nitro compounds (table 1), Raney-Nickel, hydrazine, and DCE / methanol at temperatures between 0 - 10° C (**E** and **F**) proved to best the method for the several reasons.

The optimal time for the hydroxylamine formation ranged between 1-3 hr depending of the starting sulfonamide nitro compound, and the formation of the amine was observed after 10 - 20 min after the hydroxylamine was detected giving enough time to stop the reaction and purify the hydroxylamine. In addition to the mild reduction provided by conditions **E** and **F,** the temperature used 0 - 10° C prevented the further degradation of the hydroxylamines which in some cases are unstable at higher temperatures. Finally, the use of DCE and methanol, used as a solvent system, can be easily removed at room temperature from the reaction mixture.

During all the different methods (**A - F**) a common problem was the appearance of over-reduction products along with the desired (hydroxylamine). In initial attempts, the reaction was stopped at appearance of the hydroxylamine and prior to the formation of over reduction products. Still in many cases the yield of the pure hydroxylamine was low (< 10%; **D-F**). Thus, reduction was allowed to proceed until the nitro starting material was consumed and just the hydroxylamine and amine were present in the solution. The products were purified by re-crystallization or flash chromatography; however the results were not always were satisfactory. The similar polarity of the hydroxylamine and the amine in addition to the instability of the hydroxylamine were obstacles for the purification process. The two products (amine and hydroxylamine) dissolved in similar solvents making difficult to find the right combination of solvents for the re-crystallization. Therefore, flash chromatography was used to purify the

compounds. The hydroxylamine was affected by the silica decreasing considerably the yield of the desired product.

When the desired product has a polarity higher than the impurity sometimes it is recommended to use reverse phase chromatography. In this system the stationary phase has a higher affinity for polar compounds changing the elution order. In this case, the polar product (hydroxylamine) was eluted more quickly than the amine. This method was successfully used for the purification of small quantities of the mixture, but not when higher amounts of the hydroxylamine were desired.

In later experiments the reaction was stopped as soon as the hydroxylamine was formed. The polarity between the nitro compounds and hydroxylamine was higher compared with the amine. Thus, the mixture of the nitro and hydroxylamine was purified by re-crystallization. The experimental procedure, yield, and characterization of sulfadiazine and sulfapyridine hydroxylamines (**5** and **6** respectively) are described in **2.4.5.**

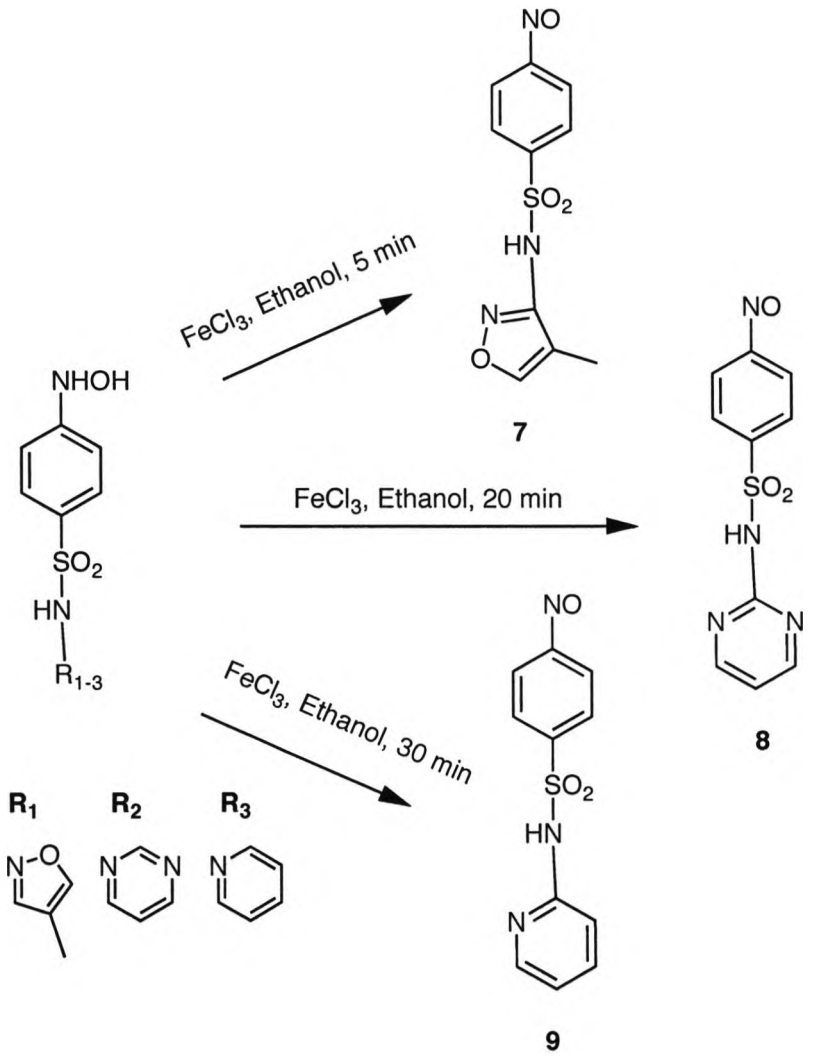
2.3.3 Synthesis of nitroso sulfonamides

Aromatic nitroso compounds play an important role in toxicity associated with human exposure to aromatic amines (Benigni, 2005). Benzyl nitroso adducts of hemoglobin have been found in nitroso benzene poisoning (Gowenlock and Richter-Addo, 2004). Additionally many aromatic nitroso compounds cause carcinogenic effects (Benigni and Passerini, 2002; Helguera et al., 2007). Therefore, the full characterization of these compounds is extremely important to have a better understanding of their interaction with different biological matrices.

In the case of sulfonamide hypersensitivity, it has been postulated that nitroso compounds and their hydroxylamine precursors play an important role in the hypersensitivity reaction through protein binding to nucleophilic residues and generation of novel antigenic determinants for T-cells. (Cribb et al., 1991; Naisbitt et al., 1999; Schnyder et al., 2000; Naisbitt et al., 2002). In addition, several reports have also shown that SMX-NO is toxic when it was incubated *in vitro* with lymphocytes from either healthy or hypersentivity donors (Rieder et

al., 1995; Schnyder et al., 2000). These reports found that concentrations above 100 μM are directly toxic to cells inducing apoptosis. SMX-NO is the final metabolite in a series of oxidations that start with the formation of an unstable, hydroxylamine metabolite, by different enzymes systems (Ayyangar et al., 1984; Cribb and Spielberg, 1990)

Nitroso molecules can be chemically synthesized by different methods (Gowenlock and Richter-Addo, 2004), nevertheless, in the case of the SMX-NO, the method of synthesis employed has been the direct oxidation of the precursor hydroxylamine (Rieder et al., 1988; Cribb et al., 1991; Naisbitt et al., 1996a). Direct heterogeneous oxidation of hydroxylamine precursors to obtain the nitroso compunds is a common method. Heterogeneous oxidation reactions are in some cases slow taking hours and in some cases leading to the formation of azoxy derivatives caused by the coupling of unconverted hydroxylamine with newly formed nitroso. Additionally, the slow oxidation process may cause the degradation of unstable hydroxylamines in solution (Davey et al., 1999).
The chemical synthesis of nitroso compounds is complicated for many reasons. Firstly, the highly reactive nature of these compounds makes it in some cases impossible to isolate the pure product. Secondly, the yields in these reactions tend to be low due to the instability of the newly formed compound. In fact, in many instances the determination of the yield of these compounds is measured indirectly by the coupling reactions with other compounds to form more stable products. Finally, the chemical reactivity of these compounds drastically limits the methods of purification available. The following diagram (scheme 2.7) shows the experimental procedure followed for the synthesis of the different nitroso metabolites.



Scheme 2.7 Chemical routes for the synthesis of SMX, SDZ and SP nitroso metabolite.

SMX-NO was successfully synthesized by direct oxidation of the hydroxylamine precursor with relative good yields and purity (Naisbitt et al., 1996a).

The oxidation occured quickly within 5 - 10 minutes compared with other reported oxidations that last hours (Davey et al., 1999). The reaction mixture turned immediately yellow when the oxidizing agent was added, and the presence of an insoluble bright yellow solid was detected. The reaction mixture was filtered and the solid collected, dried and analyzed.

In a similar procedure **5** and **6** were dissolved in ethanol and oxidized with $FeCl_3$. The time of the reaction was longer when compared with SMX-NO. In the case of **5**, the reaction mixture was left for about 10 - 20 minutes. The reaction mixture

was filtered and an insoluble bright green product was analyzed. The nitroso product was obtained (8) with trace of the over oxidation product 2 (< 10%). Finally, the oxidation of **6** was conducted under the same conditions. The reaction yielded, after 30 minutes, a pale yellow solid that was dried and characterized. Analysis demonstrated that the predominant product was **9** with trace quantities of the azoxy dimer also detected (< 10%). The characterization of the nitroso compounds and experimental procedures for the synthesis can been found in the experimental section **(2.4.6)**. The nitro, hydroxylamine and nitroso sulfonamides compounds were analyzed by different techniques (NMR. IR, HPLC and MS). The results are summarized in table 2.2.

COMPOUND	ANALYTIC DATA					
	Formula	$[m/z]^+$	R.T (minutes)	Acc. Mass $[M+Na]^+$	Yield (%)	Purity (%)
SMX-NO_2 (1)	$C_9H_9O_5N_3S$	N.T.	N.T.	284.0341	60	> 95
SDZ-NO_2 (2)	$C_{11}H_9O_4N_4S$	N.T.	N.T.	303.0164	55	> 95
SP-NO_2 (3)	$C_{11}H_9O_4N_3S$	N.T.	N.T.	302.0211	65	> 95
SMX-NHOH (4)	$C_9H_{11}O_4N_3S$	270	8.36	292.0368	50	> 90
SDZ-NHOH (5)	$C_9H_{11}O_3N_3S$	267	7.93	289.0371	30	> 90
SP-NHOH (6)	$C_{11}H_{11}O_3N_3S$	265	9.56	288.0419	20	> 90
SMX-NO (7)	$C_9H_9O_3N_3S$	268	18.30	290.0211	40	> 90
SDZ-NO (8)	$C_{11}H_9O_3N_3S$	268	18.30	289.0371	20	> 90
SP-NO (9)	$C_{11}H_9O_3N_3S$	264	16.69	286.0262	20	> 85

Table 2.2 The nitro (1-3), hydroxylamine (4-6) and nitroso (6-9) sulfonamide compounds were synthesized according with the procedures detailed previously. The compounds were analyzed by different analytic techniques (HPLC and MS) and characteristic information for each compound (retention time and accurate mass) was obtained. The last two columns show the yield and purity obtained during the synthesis of the different sulfonamide derivatives (N.T. not tested).

2.3.4 Characterization of the chemical oxidation of sulfapyridine and sulfadiazine hydroxylamine metabolites by liquid chromatography and mass spectrometry

Due to the difficulties encountered synthesizing SDZ-NO and SP-NO, the oxidation of SDZ-NHOH (**5**) and SP-NHOH (**6**) with $FeCl_3$ was followed by LC-MS according with procedure **2.4.3** in an attempt to try to detect the presence and time of formation of the respective nitroso products. The HPLC system allows characterization the different products formed during the reaction. Briefly, the reaction mixture containing the different compounds was processed through a column and separated based on the polarity and the affinities between the compounds and the material in the column. To further characterize the compounds the HPLC system was coupled with a Quatro II mass spectrometer.
In mass spectrometry analysis, the molecules to be analyzed are converted to gas-phase ions that are further separated by the mass/ion ratio. In the system used during our experiments, the ions were generated by electrospray ionisation. In this process, the eluent from the liquid chromatography is dissolved in a polar solvent and pumped through a stainless steel capillary, which carries high voltage. The liquid is aerosolized and ionized, before the droplets of ions flow to the mass spectrometer. Then, the ions are separated, detected and measured according to their mass-to-charge-ratio (m/z). Small molecules typically exhibit only a single charge. Therefore, the mass is divided by 1, which represents the proton added during the ionization process. It is represented as $[M+1]^+$ if a proton is added or $[M-1]^-$ if a proton is loss.

Compound **5** was incubated with $FeCl_3$, mixed, and injected to the LC-MS system. Four peaks were detected but just two of them were fully characterized by mass charge and retention time. The first two peaks had retention times (R_t = 7.54 and 8.39, respectively; Figure 2.1 (A)) similar to the ones found for the sulfadiazine and its hydroxylamine metabolite. The last two peaks were fully characterized by retention time and ion mass by the selective ion region (SIR). The first signal (R_t = 15.19; *m/z* $[263]^-$; Figure 2.1 (B)) corresponds to the nitroso metabolite (**8**) and the second signal (R_t = 17.20; *m/z* $[511]^-$; Figure 2.1 (C)) to the azoxy dimer.

In the case of the oxidation of compound **6** four signals were detected in the UV chromatogram. These peaks were fully characterized based on the retention time and molecular weight detected by SIR. The peak number one with at R_t = 8.12 minutes is SPNHOH (*m/z* $[266]^+$; Figure 2.2 A). The second peak (R_t = 9.47 minutes) matches with the retention time found for the parent drug (SP). However, the mass found in the analysis did not correspond to the molecular weight of the SP [m/z 248]$^-$ or any other know derivative (amine, hydroxylamine, azo or azoxy). Analysis by LC-MS demonstrated that SPNO (**9**)(R_t = 16.18 min; Figure 2.2 A, [*m/z* 262]$^-$; Figure 2.2 B) is formed immediately under the oxidation conditions, and that the metabolite is highly reactive probed by the immediate appearance of the azoxy product generated by the coupling between hydroxylamine and nitroso metabolites.

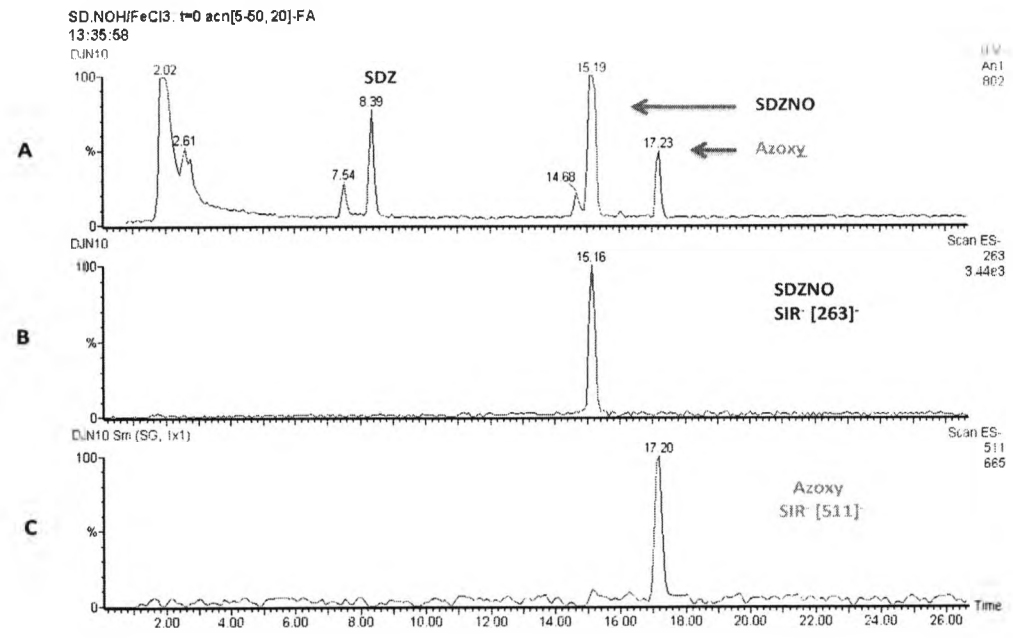


Figure 2.1 UV Chromatogram and LC-MS mass chromatograms showing the oxidation of SDZ-NHOH with $FeCl_3$ (1:6; t = 0; DMSO; R.T.). (A) UV Chromatogram (254 nm). (B) Mass chromatogram for $[M+H]^-$ (*m/z* 263) of SDZ-NO. (C) Mass chromatogram for $[M+H]^-$ (*m/z* 511) of azoxy – sulfadiazine.

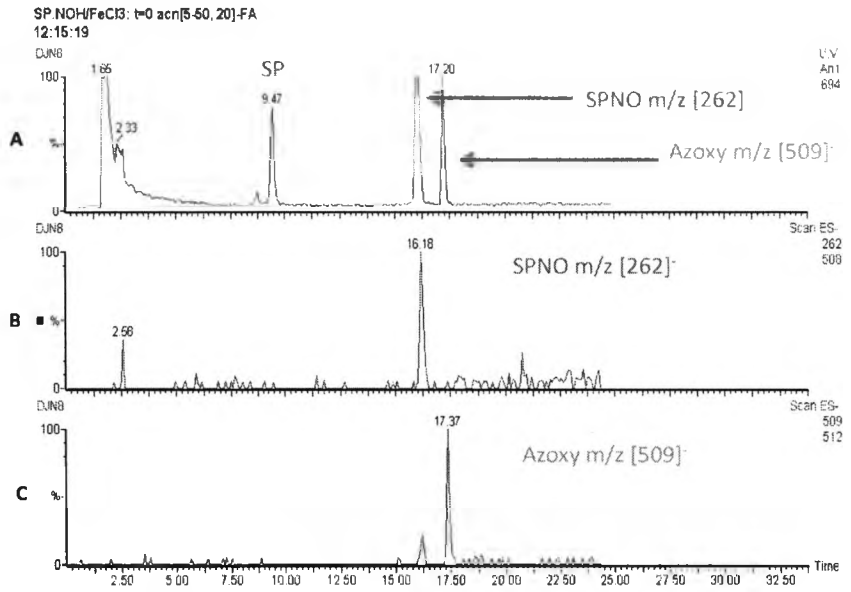


Figure 2.2 UV Chromatogram and LC-MS mass chromatograms showing the oxidation of SP-NHOH with $FeCl_3$ (1:6; t = 0; DMSO; R.T.). (A) UV Chromatogram (254 nm). (B) Mass chromatogram for $[M+H]^-$ (*m/z* 262) of SP-NO. (C) Mass chromatogram for $[M+H]^-$ (*m/z* 509) of azoxy – sulfapyridine.

In both cases, the presence of nitroso metabolites were detected within minutes and were still detectable after two hours. Additionally, the presence of azoxy dimers, formed by the reaction with one molecule of nitroso with and a molecule of hydroxylamine, were found in both oxidation reactions showing that these nitroso metabolites are highly reactive and immediately react with surrounding molecules. After 24 hr, the analysis showed just the presence of the azoxy compound of both sulfonamides (SDZ and SP).

2.3.5 Analysis of the stability of sulfonamide metabolites by liquid chromatography and mass spectrometry

Assays were performed by LC-MS to assess the stability of the sulfonamides and their hydroxylamine metabolites. Incubation of the parent drugs (SMX, SP and SDZ; 100 μM) during a period of time of 48 hrs showed that the drugs are stable in medium containing mouse splenocytes. At different time - points (0, 0.1, 1, 4, 24, 48 hr) the signal at the UV chromatogram for the different amines (SMX, R_t = 9.32 minutes; SP, R_t = 9.52 minutes; SDZ R_t = 9.21 minutes) was detected with no change in signal strength or appearance of other signals generated by

degradation or metabolism of the original drug. It is possible that metabolites were generated by the metabolic activity of splenocytes. However, the concentration could be under the levels of detection of the instruments.

Incubation of SMXNHOH (**4**) in the same conditions led to a more complex series of reaction products. During the first 10 minutes, compound **4** (R_t = 8.30 minutes; *m/z* $[270]^+$; Figure 2.3 A-B) remained stable. Analysis in the SIR^+ for SMXNO (*m/z* $[268]^+$) did not showed any evidence of the formation of the nitroso metabolite. Other signals were present (SMX; R_t = 9.51 minutes; *m/z* $[254]^+$; Azoxy R_t = 24.02 minutes; *m/z* $[519]^+$) although the signals were detected in low quantities. After 1 hr, the signal for SMX-NHOH (R_t = 8.30; *m/z* $[270]^+$; Figure 2.3 C) started to decrease as well as the resolution of the peak. A small peak assigned to SMXNO (R_t = 18.16 minutes; *m/z* $[268]^+$) was detected. Other signals, azoxy-SMX (R_t = 24.02 minutes; *m/z* $[519]^+$) and SMX (R_t = 9.51 minutes; *m/z* $[254]^+$) were more evident and increased in intensity when compared with earlier time points (Figure 2.4 and 2.4.1). At the 4 hr time point, many changes were evident. Firstly, the signal for the SMXNHOH was no longer present (Figure 2.3 D) indicating the full consumption of the metabolite. Secondly, the signals corresponding to SMX and azoxy-SMX increased (Figure 2.4 A-F and figure 2.4.1 A-E) and were better defined. The increase in the peak area in the SMX-azoxy (table 2.3) showed indirect evidence of the formation of SMX-NO. The presence of the SMX-azoxy only can be explained by the reaction between the newly formed SMX-NO with SMX-NHOH. The peak area in the SMX-azoxy ion region increases with time indicating that SMX-NO is formed during time (Table 2.3). Even though the increase in the peak area is directly proportional with the concentration of the SMX-azoxy ion, the results have to be taken with precaution. The experiments were performed in different days, resulting in variations in the peak area reading. Moreover, the exact concentration of SMX-azoxy was not obtained, because the lack of a synthetic standard for SMX-azoxy or any other azoxy. Thus, it was not possible to do a calibration curve for these compounds. Despite these limitations, an increase in the peak area was seen during the incubation time.

Finally, SMXNO was detected, although the signal remained weak as previously.

Time point (hr)	SMX-Azoxy $[519]^+$	SDZ-Azoxy $[513]^+$
	Peak area	Peak Area
0.1	1938	776
1	14698	3680
4	57246	3792
24	22596	6111
48	58747	8648

Table 2.3 The peak area was calculated for SMX- and SDZ-azoxy at different time points.

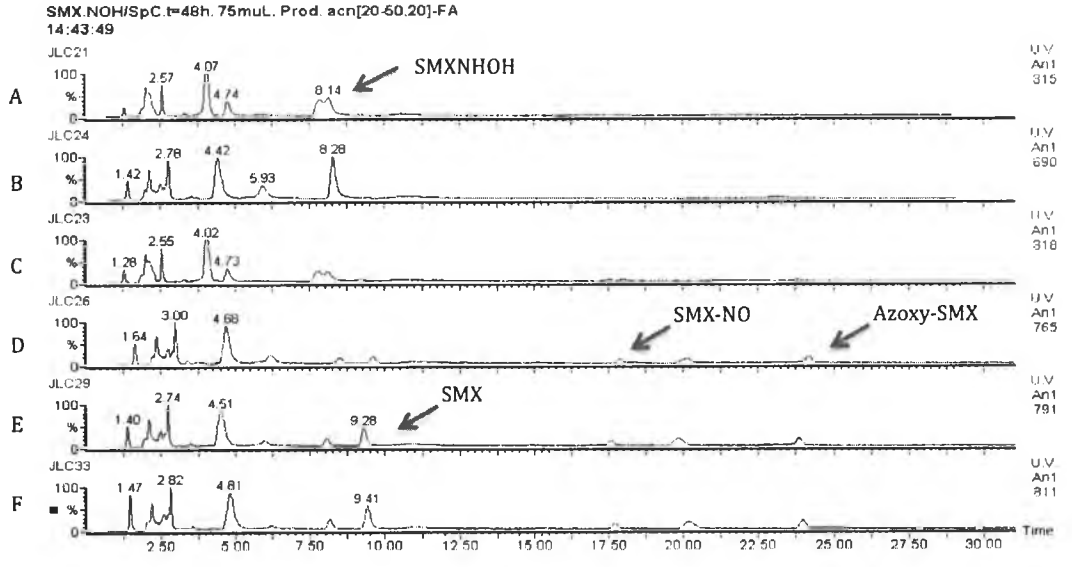


Figure 2.3 UV Chromatograms showing the chemical fate of SMX-NHOH (100 μM) incubated with splenocytes (0.6×10^5) at different time points (A 0, B 0.1, C 1, D 4, E 24 and F 48 hr)

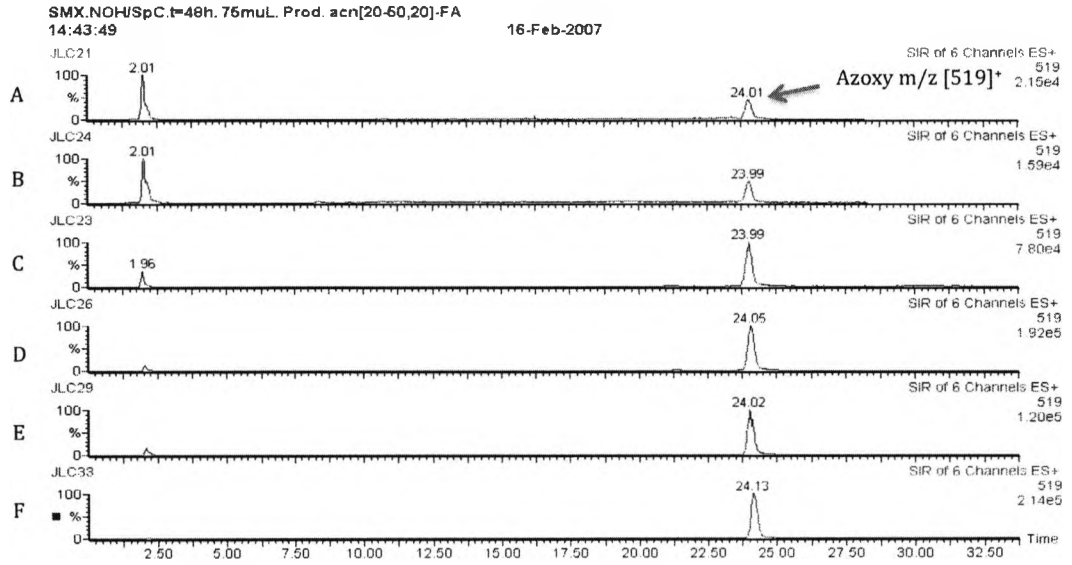


Figure 2.4 Mass chromatogram for $[M+H]^+$ (*m/z* 519) of SMX-Azoxy formed during SMX-NHOH decomposition in culture medium with splenocytes during time (A 0, B 0.1, C 1, D 4, E 24 and F 48 hr).

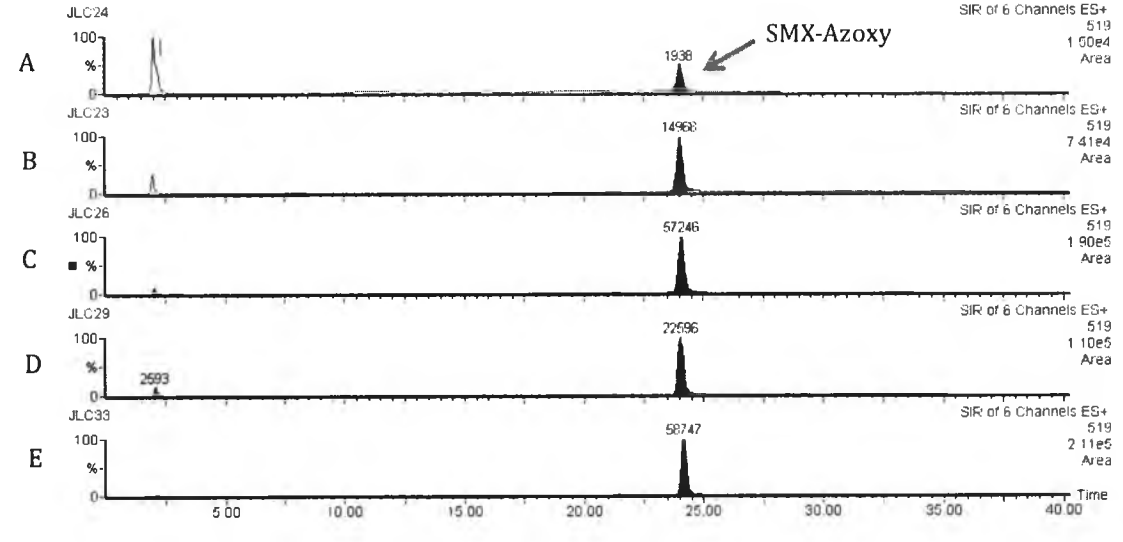


Figure 2.4.1 Mass chromatogram showing the increase on the peak area for $[M+H]^+$ (*m/z* 519) of SMX-Azoxy formed during SMX-NHOH decomposition in culture medium with splenocytes during time (A 0.1, B 1, C 4, D 24, and E 48 hr).

Two principal pathways consumed the hydroxylamine. The first involved the direct oxidation of the hydroxylamine forming SMX-NO, which is very unstable and immediately reacts with any hydroxylamine left to form the azoxy compound. The second mechanism involved a reduction process detected by the increasing formation of the parent drug. The two mechanisms contribute to the degradation of the SMX-NHOH and after 4 hr practically none of the metabolite is left.

Incubation of SDZ-NHOH with splenocytes in cell culture medium yielded similar results to that seen with SMX-NHOH. The hydroxylamine was detected (R_t = 7.79 minutes; *m/z* $[267]^+$; Figure 2.5 A-C) in the first hour of the incubation period. Similar to SMXNHOH, the presence of SDZ-NHOH was not detectable after 4 hrs. The oxidation product (SDZ-NO, R_t = 15.41; *m/z* $[267]^+$) was not detected at any time point. SDZ was not formed through the incubation. The presence of SDZ-NO was not detected directly during the analysis. However, its transitory presence was shown by the formation of the azoxy dimer. Azoxy dimers are formed when molecules of newly formed nitroso, which are highly unstable, react directly with a hydroxylamine molecule in the medium (see figure 2.1). The UV chromatogram showed a weak signal (t=0; R_t = 17.47; m/z $[513]^+$; Figure 2.5 C-F; Figure 2.6 A-F; that increased during the time of the incubation. The increased strength of this signal, azoxy derivative, can be explained by the formation of SDZ-NO, which reacts with SDZ-NHOH yielding the azoxy dimer (Figure 2.6.1 A-E azoxy; Table 2.3).

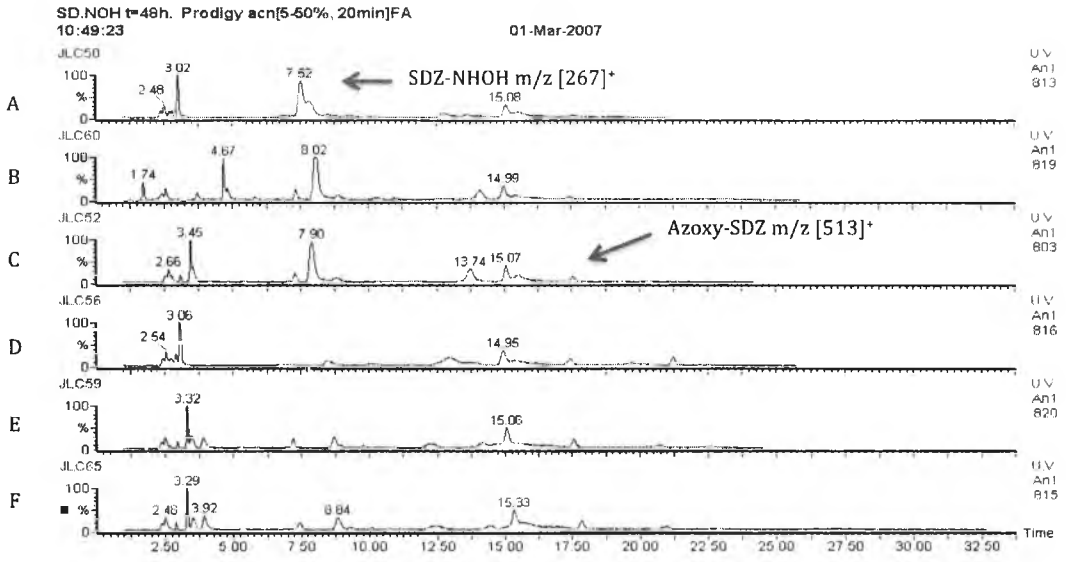


Figure 2.5 UV Chromatograms showing chemical fate of SDZ-NHOH (100 μM) incubated with splenocytes (0.6 x 10^5) at different time points (A 0, B 0.1, C 1, D 4, E 24 and F 48 hr)

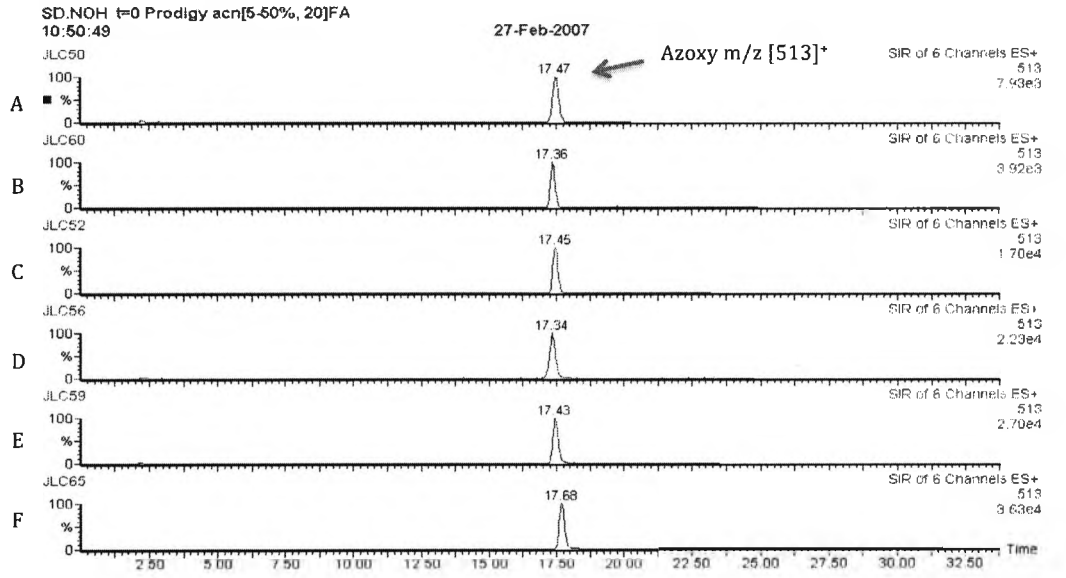


Figure 2.6 Mass chromatogram for $[M+H]^+$ (*m/z* 513) of SDZ-Azoxy formed during SDZ-NHOH decomposition in culture medium with splenocytes during time (A 0, B 0.1, C 1, D 4, E 24 and F 48 hr).

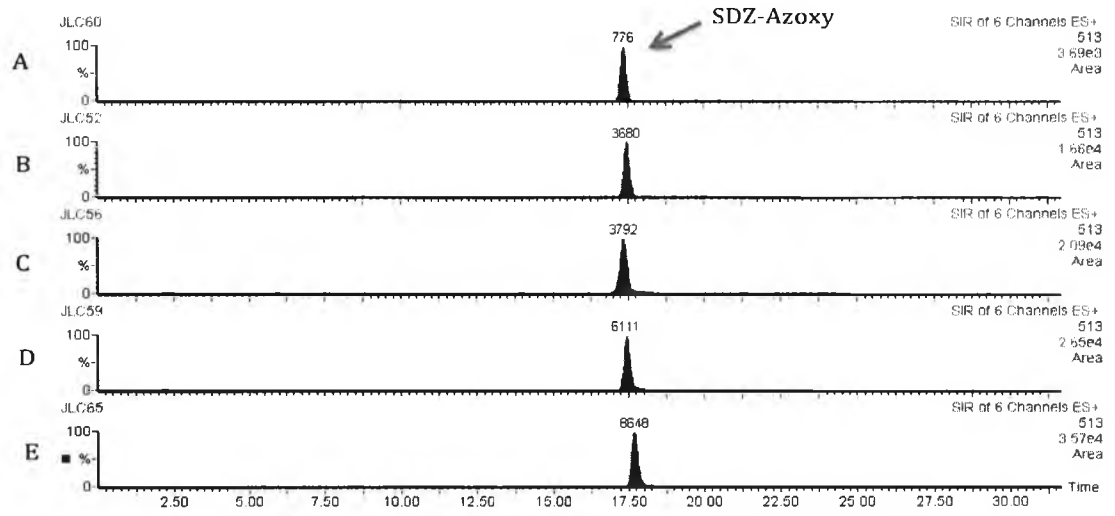


Figure 2.6.1 Mass chromatogram showing the increase on the peak area for $[M+H]^+$ (*m/z* 513) of SDZ-Azoxy formed during SDZ-NHOH decomposition in culture medium with splenocytes during time (A 0.1, B 1, C 4, D 14, and E 48 hr).

Finally, in incubations containing SP-NHOH (R_t = 8.67; *m/z* $[266]^+$; Figure 2.7 A-C), the metabolite was just detectable during the first hour and then the signal disappeared. A signal of the reduction product (SP; R_t = 9.52; *m/z* $[250]^+$) did not have major changes during the incubation, so the reduction process seems not to be predominant. In this case, the UV chromatograms did not show an increase in either the signal for SP (R_t = 9.48; *m/z* $[250]^+$) or azoxy (R_t = 17.08; *m/z* $[511]^+$). At t = 0 the predominant signal corresponds to the hydroxylamine; however, small traces of the amine and azoxy were detected. Further analysis of the UV chromatograms did not show an increase in these peaks during the entire incubation period in contrast with the changes observed with SMX-NHOH and SDZ-NHOH.

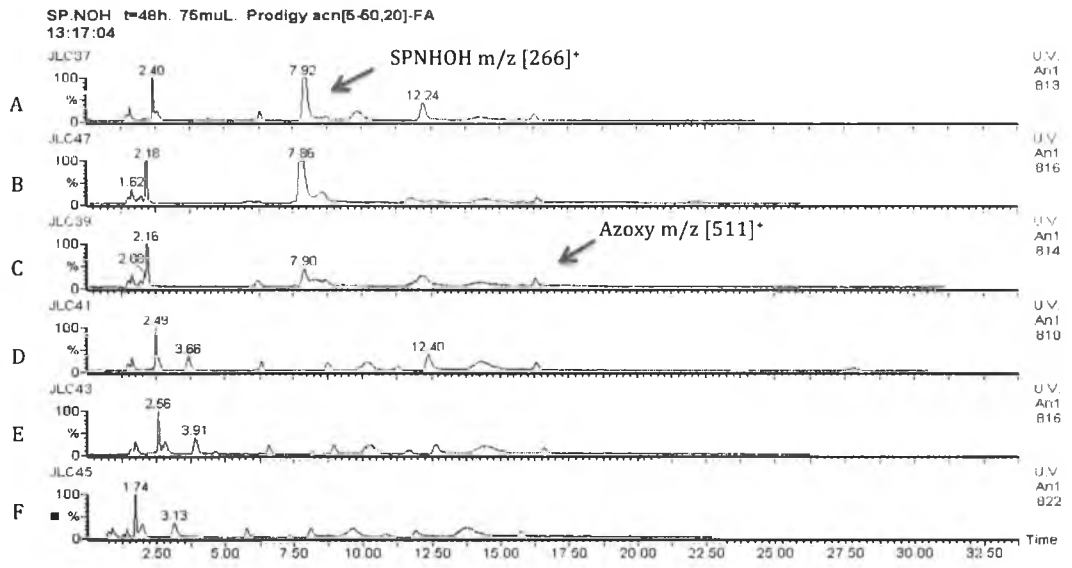


Figure 2.9 UV Chromatograms showing chemical fate of SP-NHOH (100 μM) incubated with splenocytes (0.6 x 10^5) at different time points (A 0, B 0.1, C 1, D 4, E 24 and F 48 hr)

In summary, each hydroxylamine (SMX-NHOH, SD-NHOH and SP-NHOH) was found to degrade rapidly in culture and no more than trace amounts were detected after 1 hr. During SMX-NHOH incubation, low levels of SMX-NO were detected directly after 1 – 4 h. For SD-NHOH and SP-NHOH, nitroso metabolite formation was confirmed indirectly through the characterization of azoxy dimers, which are formed by the conjugation between the newly formed nitroso sulfonamides with the hydroxylamine compound remaining. Interestingly, SP-NHOH appeared to be particularly unstable in culture. A weak signal corresponding to SP-NHOH was only detected by mass spectrometry at the earliest time-point tested (0.1 h), which is in agreement with the previously described short half-life of SP-NHOH (Pirmohamed et al., 1991). Furthermore, unlike SMX-NHOH and SD-NHOH, products of reduction and dimerization were only detected at low levels throughout the incubation period. These results indicate that SP-NO protein adduct formation may be favored over other potential reactions. To investigate this possibility further, specific anti-drug antibodies are being generated in on-going experiments.

The data confirm the high instability of sulfonamide hydroxylamines in cell culture media. The analysis was performed in medium containing a high concentration of proteins (10% fetal bovine serum), used for different experiments in the following chapters. Thus, we assessed the stability of the compounds in the culture medium where the cells will be exposed to the sulfonamide metabolites. The differences observed with each compound highlight the importance of continuing cellular immunological investigations with analysis of compounds disposition.

2.4 Experimental

Organic extracts were washed with satd. aq. NaCl and dried over anhydrous Na_2SO_4 prior to rotary evaporation at < 30°C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Unless otherwise stated, ^1H NMR spectra were recorded on DMSO solutions using either Bruker 250 or 400 MHz instruments with tetramethylsilane as internal standard.

2.4.1 Chemicals and reagents

The following chemicals were obtained from sigma-aldrich (Gillingham, UK): 4-Nitrobenzylsulfonylchloride, 2-aminopyrimidine, pyridine anhydrous, 2-aminopyridine, 3-amino-5-methylisoxazole, dichloroethane, sodium hypophosphite, palladium, tetrahydrofurane, sulfate magnesium, ion chloride, dichloromethane, ethyl acetate, dimethyl sulfoxide HPLC and sterile grade, raney-nickel 2800, and L-glutathione reduced 98%. Hydrazine monohydrate (98%) was obtained by Alfa Aesar (Lancashire, UK) and methanol, 2-propanol and chloroform HLPC grade by Fisher Scientific (Leicestershire, UK).

2.4.2 Liquid chromatography and mass spectrometry analysis

LC-MS analysis was performed using the following conditions: Aliquots (75 μL) of the different solutions to be analyzed were chromatographed at room temperature on a Prodigy 5-μm ODS-2 (C-18) column (150 × 4.6 mm i.d.; Phenomenex, Macclesfield, Cheshire, UK) using linear gradients of acetonitrile in 0.1% (v/v) formic acid: 20-50% over 20 min for SMX and their metabolites, 5-50% over 20 min for SD and SP and their metabolites. The eluent flow rate was 0.9 mL/min. The LC system consisted of two Jasco PU980 pumps (Jasco UK, Great Dunmow, Essex, UK) and a Jasco HG-980-30 mixing module. Eluted compounds were monitored at 254 nm with a Jasco UV-975 spectrophotometer. Eluate split-flow to the LC-MS interface was ca. 40 μL/min. A Quattro II mass spectrometer (Waters Corp, Manchester, UK) fitted with the standard co-axial

electrospray source was operated using nitrogen as the nebulizing and drying gas. The interface temperature was 80 °C; electrospray capillary voltage, 3.8 kV; cone voltage, 40 V. The instrument was set up for selected ion monitoring (see Table 2.2) in the positive-ion or mode as follows: channel dwell time, 200 ms; cycle time, 230 ms; span, 0.5 amu.

Analyte	*m/z* ([M+1]$^+$)		
	SMX	SD	SP
Amine	254	251	250
Nitroso	268	265	264
Hydroxylamine	270	267	266
Azoxy	519	513	511
Nitro	284	281	280

Table 2.4 Molecular weight and identity of the different analytes detected by mass spectrometry in positive ion mode.

2.4.3 Chemical oxidation of hydroxylamine sulfapyridine and sulfadizine analyzed by liquid chromatography and mass spectrometry

10 mg (0.03mM) of SP-NHOH and SDZ-NHOH were dissolved in 900 µL of DMSO and mixed separately with 6 eq of $FeCl_3$ (0.18 mmol) dissolved in 100 µL of distilled water. The reaction mixture was vigorously stirred and aliquots of (70 µL) were analyzed according with the conditions described at **2.4.2** but ions were analyzed in negative-ion mode.

2.4.4 General procedure for the synthesis of the nitro compounds

Nitro compounds were synthesized by nucleophilic addition. Briefly, 4.5 g (53 mmol) of 3-amino-5-methylisoxazole, 4.30 g (46 mmol) of 2-amino pyrimidine, or 4.30 g (45 mmol) of 2-amino pyridine was dissolved in 20 mL of anhydrous pyridine at 0°C with constant stirring until the product dissolved forming a clear solution. Then, 10 g (45 mmol) of 4-nitrobenzylsulfonylchloride was added slowly to the reaction mixture maintaining the temperature at 0°C. Constant and vigorous stirring was continued during the reaction since the reaction process forms a brown insoluble paste. The reaction was left for 24 hr at room temperature.

After 24 hr, the brown solid was filtered and washed with an excess of water to remove the remaining pyridine from the solid. The different formed compounds were purified by different methods. SMX-NO_2 was re-crystallized with a mixture of ethyl acetate and toluene (1:3). The hot solution was filtered to remove the activated charcoal and the filtrate left at 0° C overnight to crystallize. SP-NO_2 and SDZ-NO_2 are insoluble in most of the common solvents used in re-cristallization. However, the starting materials are broadly soluble in many common polar solvents. Therefore, both products were washed with boiling ethyl acetate. The hot ethyl acetate successfully removed the starting material leaving a pale yellow product. The product was left to dry under high vacuum.

2.4.5 General procedure for the synthesis of hydroxylamine metabolites

Hydroxylamine sulfonamides were prepared by a carefully controlled reduction of the nitro sulfonamides (SMX-NO_2, SDZ-NO_2 and SP-NO_2).

SMX-NHOH: 1 g (3.71 mmol) of SMX-NO_2 was dissolved at room temperature in 100 mL tetrahydrofurane with constant stirring to form a clear solution. 1.12 g (12.73 mmol) of sodium hypophosponite is dissolved in 10 mL of distilled H_2O and added to the reaction mixture. Then, 0.1 g of palladium catalyst was added slowly to the reaction mixture containing nitro sulfamethoxazole and sodium

hypophosponite. After 20 minutes the reaction was stopped, and the mixture filtered through a celite bed to remove the catalyst. The product was extracted using 100-150 mL diethyl ether and the organic phase was collected and dried using magnesium sulfate ($MgSO_4$). The final solution was then concentrated to form and oily yellow product, which after dried under high vacuum formed a yellow solid paste with crystals on the surface. The yellow solid was then recrystallized using chloroform. Finally, the crystals were collected and dried under high vacuum to remove the solvent.

SP-NHOH and SDZ-NHOH: 2.5 g (8.92 mmol) nitro sulfadiazine or nitro sulfapyridine were suspended in 100 mls of a solvent mixture containing, 50% ethanol and 50% dichloroethane; the compounds, which are highly insoluble, did not dissolve in the mixture. The reaction mixture was then cooled in an ice bath (0° C), and drops of Raney Nickel (≈ 0.1 g) catalyst were slowly added to the reaction mixture. After adding the catalyst, 0.9 g (17.97 mmol) of hydrazine was added during constant and vigorous stirring. Hydrazine is extremely toxic and has to be added slowly to the solution in the reaction mixture. The addition of hydrazine raised the temperature of the solution and the starting materials dissolved to form a bright green solution. The reaction was followed by TLC using (10:90 of MeOH and DCM). In the synthesis of SPNHOH the reaction mixture was left for 3 hours and for the synthesis of SDZHOH was completed after 1.5 hr. It was extremely important to maintain the temperature between 0-10° C during the entire course of the reaction. The reaction was stopped by filtering the mixture through a celite bed. The solid was washed several times with acetone to extract any hydroxylamine. The synthesized compounds were purified by the different methods. SP-NHOH was re-crystallized using a mixture of chloroform and methanol (9:1). SDZ-NHOH was re-crystallized using 2-propanol.

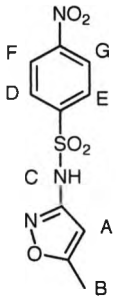
2.4.6 General procedure for the synthesis of nitroso metabolites

The final nitroso metabolites were obtained by the oxidation of the synthetic hydroxylamines.

SMX-NO: 1 g (3.71 mmol) of SMX-NHOH was completely dissolved in 70 mL ethanol, and 3.61 g (22.26 mmol) of $FeCl_3$ previously dissolved in 50 mL of distilled water was added at room temperature to the ethanol mixture. The reaction mixture turned immediately into a bright yellow solution with some precipitated solid. After five minutes, the reaction was stopped by filtering. The bright yellow solid was collected and dried under high vacuum.

SDZ-NO and SP-NO: 500 mg (1.87 mmol) of SDZ-NHOH and 500 mg (1.88 mmol) of SP-NHOH were dissolved in 90 mL of ethanol. Then, 1.79 g (11.22 mmol) of Fe(III)Cl, previously dissolved in 80 mL of distilled H_2O, was added slowly to the reaction mixture at room temperature. After 30 minutes for the synthesis of SDZ-NHOH and 40 minutes for SP-NHOH half of the reaction volume was reduced under vacuum and the precipitated solids filtered. The yellow solid was dried under high vacuum and analyzed by HPLC, MS and NMR.

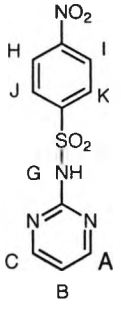
_N_-(5-methylisoxazol-3-yl)-4-nitrobenzenesulfonamide ($SMXNO_2$)



1

The compound was obtained by procedure **2.4.5**. White solid (7.80 g, 97%), m.p. = 190 – 200 °C ^{1}H NMR (400 MHz, d-DMSO): δ 2.40 (s, 3H, B), δ 6.2 (s, 1H, A), δ 8.2 (d, J + 9.29 Hz, 2H, F + G), δ 8.5 (d, J = 9.36 Hz, 2H, D + E) and δ 12.10 – 19.80 (br, 1H, C). Kbr Ir Absorption: 2923 cm^{-1} (NH, Sulfonamide), 1616 cm^{-1} 1461 cm^{-1} 1403 cm^{-1} (C=C, Phenyl), 1535 (NO_2), 1349 cm^{-1} 1180 cm^{-1} (aS=O, sS=O), 1002 cm^{-1} (N-S), 740 cm^{-1} (C-S) and 609 cm^{-1} (SO_2). $[M+Na]^+$ requires 284.0351 found 284.0341

4-nitro-_N_-(pyrimidin-2-yl)benzenesulfonamide ($SDZNO_2$)

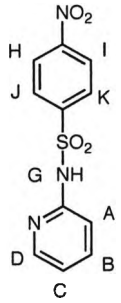


2

The compound was obtained by procedure **2.4.5**. Yellow compound (8.37 g, 97 %), m.p. = 270 – 280 °C ^{1}H NMR (400 MHz, d-DMSO): δ 7.01 (t, J = 4.9 Hz, 1H, B), δ 8.2 (d, J = 9.1 Hz, 2H, J + K), δ 8.4 (d, J = 9.1 Hz, 2H, H + I) and δ 8.5 (d, J = 4.9 Hz, 2H, A + C). Kbr Ir Absorption: 2969 cm^{-1} (NH, Sulfonamide), 1581 cm^{-1} 1442 cm^{-1} 1407 cm^{-1} (C=C, Phenyl, and C=N), 1519 (NO_2), 1346 cm^{-1} 1180 cm^{-1} (a S=O, NO_2;

s S=O), 956 cm^{-1} (N-S), 740 cm^{-1} (C-S) 860 cm^{-1} (Ph-NO_2) 609 cm^{-1} (SO_2). $[M+Na]^+$ requires 303.0173 found 303.0164

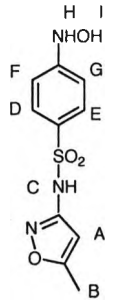
4-nitro-*N*-(pyridin-2-yl)benzenesulfonamide ($SPNO_2$).



3

The compound was obtained by procedure **2.4.5**. White compound (7.70 g, 97%), m.p. = 160 – 170 °C ^1H NMR (400 MHz, d-DMSO): δ 6.8 (t, J=6.8 Hz, 1H, C), δ 7.3 (d, J = 8.8 Hz, 1H, A), δ 7.01 (t, J = 4.9 Hz, 1H, B), δ 8.2 (d, J = 9.1 Hz, 2H, J + K), δ 8.4 (d, J = 9.1 Hz, 2H, H + I), δ 8.5 (d, J = 4.9 Hz, 2H, A + C) and δ 13 - 13.50 (br, 1H, G). Kbr Ir Absorption: 2969 cm^{-1} (NH, Sulfonamide), 1631 cm^{-1} 1349 cm^{-1} (C=C, Phenyl, aS=O, NO_2), 1141 cm^{-1} (sS=O), 964 cm^{-1} (N-S), 732 cm^{-1} (C-S), 613 cm^{-1} (SO_2). $[M+Na]^+$ requires 302.0206 found 302.0211.

4-(hydroxyamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (SMXNHOH).

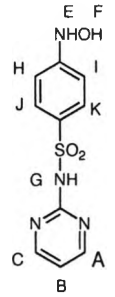


4

The compound was obtained by procedure **2.4.6.** White solid (0.71 g , 97%), m.p. = 160 – 180 °C ^1H NMR (400 MHz, d-DMSO): δ 2.29 (s, 3H, B), δ 6.10 (s, 1H, A), δ

6.86 (d, J = 8.79 Hz, 2H, F + G), δ 7.63 (d, 2H, J = 8.8, D + E), δ 8.72 (s, 1H, H), δ 9.07 (s, 1H, I), and δ 11.10 – 12.80 (s, 1H, C). Kbr Ir Absorption: 3243 cm^{-1} (OH-), 2923 cm^{-1} (NH, Sulfonamide), 1612 cm^{-1} 1457cm^{-1} (C=C, Phenyl), 1334 cm^{-1} 1160 cm^{-1} (aS=O, sS=O), 902 cm^{-1} (S-N), 779 cm^{-1} (C-S) and 613 cm^{-1} (SO_2). $[M+Na]^+$ requires 292.0361 found 292.0368. LC-MS, Prodigy/ACN [5-50%, 20 minutes] – FA, R_t = 8.36 minutes; m/z (ES +ve mode): 270 $[M + 1]^+$.

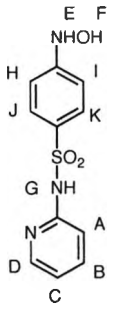
4-(hydroxyamino)-*N*-(pyrimidin-2-yl)benzenesulfonamide (SDZNHOH).



5

The compound was obtained by procedure **2.4.6.** Orange solid (0.701 g, 95%), m.p. = 220 – 250 °C. ^1H NMR (400 MHz, d-DMSO): δ 6.84 (d, J = 8.9 Hz, 2H, H+I), δ 7.01 (t, J = 4.9 Hz, 1H, B), δ 7.75 (d, J = 8.7 Hz, 2H, J + K), δ 8.47 (d, J = 4.7 Hz, 2H, A + C), δ 8.64 (s, 1H, E), δ 8.97 (s, 1H, F) and δ 11.38 (br, 1H, G). Kbr Ir Absorption: 3320 cm^{-1} (OH-), 2973 cm^{-1} (NH, Sulfonamide), 1577 cm^{-1} - 1492cm^{-1}, 1442 cm^{-1} - 1403cm^{-1} (C=C, Phenyl and C=N), 1315 cm^{-1} 1145 cm^{-1} (aS=O, sS=O), 944 cm^{-1} (S-N). $[M+Na]^+$ requires 289.0359 found 289.0371. LC-MS, Prodigy/CAN [5-50%, 20 minutes] – FA, R_t = 7.93 minutes; m/z (ES +ve mode): 267 $[M + 1]^+$.

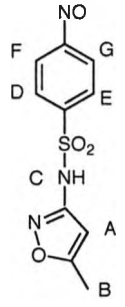
4-(hydroxyamino)-*N*-(pyridin-2-yl)benzenesulfonamide (SPNHOH).



6

The compound was obtained by procedure **2.4.6**. White solid (0.473 g, 95%), m.p. = 160 – 180 °C ^{1}H NMR (400 MHz, d-DMSO): δ 6.80 (d, J = 8.7 Hz, 2H, H + I), δ 6.90 (t, J = 6 Hz, 1H, C), δ 7.10 (d, J = 8.5 Hz, 1H, A), δ 7.65 (m, 3H, B + J + K), δ 8.0 (d, J = 4.0 Hz, 1H, D), δ 8.60 (s, 1H, E), δ 8.90 (s, 1H, F) and δ 11.10 – 11.30 (br, 1H, G). KBr Ir Absorption: 2969 cm^{-1} (NH, Sulfonamide), 1631 cm^{-1} (C=C, Phenyl; C=N), 1388 cm^{-1} 1130 cm^{-1} (aS=O, sS=O), 1079 cm^{-1} (S-N), 775 cm^{-1} (C-S) and 613 cm^{-1} (SO_2). $[M+Na]^+$ requires 288.0418 found 288.0419. LC-MS, Prodigy/CAN [5-50%, 20 minutes] – FA, R_t = 9.56 minutes; m/z (ES +ve mode): 265 $[M + 1]^+$.

***N*-(5-methylisoxazol-3-yl)-4-nitrosobenzenesulfonamide (SMXNO).**

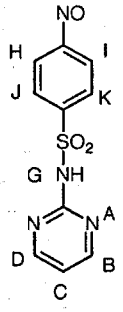


7

The compound was obtained by procedure **2.4.7**. Yellow solid (0.59 g 97 %), m.p. = 160 – 170 °C (discompose) ^{1}H NMR (400 MHz, d-DMSO): δ 2.26 (s, 3H, B), δ 6.21 (s, 1H, A) and δ 8.14 – 7.82 (m, 4H, D-G). Kbr Ir Absorption: 2923 cm^{-1} (NH, Sulfonamide), 1616 cm^{-1} 1461 cm^{-1} 1403 cm^{-1} (C=C, Phenyl; N=O), 1349 cm^{-1} 1180 cm^{-1} (aS=O, sS=O), 1261 cm^{-1} (N=O dimers), 1110 cm^{-1}, 856 cm^{-1} (Ph-NO)

902 cm^{-1} (S-N), 779 cm^{-1} (C-S) and 617 cm^{-1} (SO_2). $[M+Na]^+$ requires 290.0210 found 290.0211. LC-MS, Prodigy/CAN [5-50%, 20 minutes] – FA, R_t = 18.30 minutes; m/z (ES +ve mode): 268 $[M + 1]^+$.

4-nitroso-*N*-(pyrimidin-2-yl)benzenesulfonamide (SDZNO).

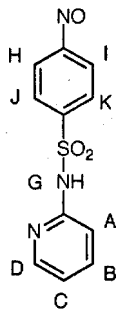


8

The compound was obtained by procedure **2.4.7**. Brown compound, (0.05 g, 90%), m.p. = 230 – 240 °C (discompose). ^1H NMR (400 MHz, d-DMSO): δ 2.26 (s, 3H, B), δ 6.21 (s, 1H, A) and δ 8.14 – 7.82 (m, 4H, D-G). Kbr Ir Absorption: 3320 cm^{-1} (OH-), 2973 cm^{-1} (NH, Sulfonamide), 1577 cm^{-1} - 1492cm^{-1}, 1442 cm^{-1} - 1403cm^{-1} (C=C, Phenyl and C=N), 1315 cm^{-1} 1145 cm^{-1} (aS=O, sS=O), 944 cm^{-1} (S-N). $[M+Na]^+$ requires 289.0359 found 289.0371.

LC-MS, Prodigy/CAN [5-50%, 20 minutes] – FA, R_t = 18.30 minutes; m/z (ES +ve mode): 268 $[M + 1]^+$.

4-nitroso-*N*-(pyridin-2-yl)benzenesulfonamide (SPNO).



9

The compound was obtained by procedure **2.4.7.** Green compound (0.074 g, 90%), m.p. = 150 – 160 °C (discompose). ^1H NMR (400 MHz, d-DMSO): δ 6.85 (t,

J = 6.58 Hz, B), δ 7.30 (d, J = 9.06 Hz, 1H, A), δ 7.81 (t, J = 7.14 Hz, 1H, C), δ 7.94 (d, 1H, J = 4.96, D), δ 8.06 (d, J = 8.50 HZ, H + I), δ 8.18 (d, J = 8.52 Hz, J + K). Kbr Ir Absorption: 2969 cm^{-1} (NH, Sulfonamide), 1581 cm^{-1} 1442 cm^{-1} 1411 cm^{-1} (C=C, Phenyl; C=N), 1500 cm^{-1} (NO), 1346 cm^{-1} 1168 cm^{-1} (aS=O, sS=O), 952 cm^{-1} (S-N), 794 cm^{-1} (C-S) and 617 cm^{-1} (SO_2). $[M+Na]^+$ requires 286.0238 found 286.0262. LC-MS, Prodigy/CAN [5-50%, 20 minutes] – FA, R_t = 16.69 minutes; m/z (ES +ve mode): 264 $[M + 1]^+$.

CHAPTER 3

THE IMMUNOGENICITY OF NITROSO SULFONAMIDES IN MICE

3.1 Introduction

Landsteiner and Jacobs in 1935 demonstrated that many chemicals sensitize animals following repeated intracutaneous injections (Landsteiner and Jacobs, 1935b). The authors suggested that the induction of an immune response was dependent of the chemical electrophilicity and their propensity to conjugate with nucleophilic residues on carrier proteins. The Hapten Hypothesis was formulated from these seminal observations. Many directly protein-reactive chemicals have now been shown to induce an immune response. The majority of drugs that activate the immune system leading to un-wanted reactions ranging from simple rash to life threating diseases such as TEN or SJS. Several chemical classes including antibiotics, anticonvulsants and anti-retro viral drugs are associated with a high incidence of hypersensitivity reactions in patients and have been widely studied in order to understand the pathogenesis of these peculiar reactions (Shear and Spielberg, 1988; Roujeau et al., 1995; Gruchalla, 2003). Importantly, almost all drugs associated with a high incidence of hypersensitivity have been shown to form reactive metabolites that bind covalently with proteins. Drugs are enzymatically activated mainly by phase I oxidizing enzymes that generate reactive species that can be easily conjugated with different amino acids residues on carrier proteins for excretion.

Sulfamethozaxole is oxidized by the P450 enzymatic system, CYP2C9 in humans and CYP2C6 in rats, to a hydroxylamine, which can be excreted in urine in man and rats (Cribb et al., 1995; Gill et al., 1997). The hydroxylamine is unstable in solution and auto-oxidizes to form a highly reactive nitroso metabolite. SMX-NO is highly reactive and can bind covalently to cysteine residues and cysteinyl sulfoxy acids expressed in serum proteins (Cheng et al., 2008), liver (Cribb et al., 1996) and the surface of immune cells (Naisbitt et al., 2001; Manchanda et al., 2002; Sanderson et al., 2007). SMX-NO is immunogenic when administered to mice, rats and rabbits (Choquet-Kastylevsky et al., 2001; Naisbitt et al., 2001; Farrell et al., 2003; Cheng et al., 2008). T-cells are stimulated with SMX-NO via a

classical hapten mechanism involving protein-complex formation, processing and the presentation of derived peptides in the context of MHC molecules (Naisbitt et al., 2002). SMX-NO also stimulates T-cells isolated from blood and blister fluid of hypersensitive human patients. In contrast to animal models, incubation of the parent compound SMX with PBMC and T-cell clones from allergic patients also stimulates a proliferative response (Schnyder et al., 2000; Burkhart et al., 2001; Farrell et al., 2003; Nassif et al., 2004).

As mentioned previously, the immunogenicity of sulfamethoxazole metabolites has been successfully demonstrated in different animals models (mouse, rat and rabbit). The animals were immunized with SMX-NO, and the isolated splenocytes from each animal were re-challenged in vitro with SMX-NO or SMX. The results showed that splenocytes from the three different species only proliferate in the presence of SMX-NO, but not SMX. However, clinical manifestations of hypersensitivity associated with drug or drug metabolite exposure in these models have not been detected. Thus, as yet undefined patients factors must contribute towards the conversion of an immune response into tissue pathology. Recently, Matzinger (2007) suggested that tissues could be the responsible for the activation of the immune system during antigen challenge. Dogs are the only species to develop hypersensitivity reactions to sulfonamides with a range of clinical manifestations including fever, skin eruptions, and liver toxicity (with a low incidence; < 0.25%). Dogs could be a suitable animal model for studying drug hypersensitivity caused by sulfonamides, but the low incidence and ethical issues involved in the use of large mammals complicates potentials investigations (Lavergne et al., 2006a; Uetrecht, 2007; Lavergne et al., 2008).

The aim of this chapter was to explore the immunogenicity of nitroso metabolites from three different sulfonamides (SMX, SDZ and SP), the synthesis of which was described and discussed in chapter 2, using an animal model of immunogenicity. Furthermore, we studied the cross-reactivity patterns of splenocytes from primed animals tested against different hydroxylamine and nitroso metabolites of the sulfonamides used. Finally the role of drug metabolism and the cytokine profile of the splenocytes immunized with the different drugs were analyzed.

3.2 Materials and methods

3.2.1 Materials and culture medium

[^{3}H]-methyl thymidine was obtained from Moravek (California, USA), and Lympoprep was obtained from Axis-Shield (Oslo, Norway). All other reagents, chemicals and components were supplied by Sigma-Aldrich (Gillingham, UK).

The culture medium used contains: RPMI medium, supplemented with 10% fetal bovine serum (FBS), HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL).

3.2.2 Sulfonamides and their metabolites

Nitroso and hydroxylamine metabolites from sulfamethoxazole, sulfapyridine and sulfadiazine were chemically synthesized as detailed before in chapter 2, and stock solutions of 10 mM (10% DMSO) were prepared. Identity and purity of the metabolites were assessed by NMR and LC-MS. Parent drugs were obtained from Sigma-Aldrich and stock solutions of 5 mg/mL (10% DMSO) were prepared. The final concentration of DMSO at the final incubation was lower than < 0.2%

3.2.3 Animals and immunizing protocols

Female balb/c (40 – 42 weeks; 16 – 20 g) and C57BL/6 (6 - 9 weeks; 20 – 30 g) mice were purchased from Charles River U.K. Ltd (Kent U.K.). Mice were dose with SMX-NO (5 mg/kg; n = 8), SDZ-NO (5 mg/kg; n = 3) and SP-NO (5 mg/kg; n = 3) in DMSO (100 μL) i.p. four times weekly for two weeks. On a completion of the dosing regime, the mice were humanely killed and the spleens were collected under aseptic conditions.

3.2.4 Isolation of splenocytes

Splenocytes were isolated from the removed spleens of the different strains of mice by centrifugation on a density gradient of lymphoprep. Homogenized spleens in 10 mL of cold HBSS were filtered under a cell strainer to remove connective tissue, carefully layered on 7 mL of Lympoprep, and centrifuged (18 min; 750g rpm). The middle cloudy layer (containing the splenocytes) formed between the plasma and the lymphoprep layers was carefully aspirated with a sterile pasteur pipette. The aspirated layer was then washed twice with HBSS solution to remove the excess of lymphoprep. Finally the cells were re-suspended in 10 mL of HBSS for counting and assessment of the viability using a Neubauer haemo-cytometer (Sigma) under a Wilovert microscope (Will Wertzlar, Germany) with trypan blue staining. Briefly, 40 µL of the cell suspension was mixed with trypan blue solution (10 µL; 0.2%), and 10 µL of the solution was placed on the haemo-cytometer and counted. The percentage of viability was calculated as follows.

% Viable Cell = [(total number of viable cells) / (total number of cells)] * 100

Cell viability was consisting greater than 95%. Splenocytes were re-suspended in culture medium and the concentration was adjusted to 1.5 x 10^6 cells/mL.

3.2.5 Immunogenicity of nitroso sulfamethoxazole in two different strains of mouse (C57BL/6 and Balb/c)

Splenocytes from the Balb/c or C57BL/6 strain mice were obtained as mentioned above **3.2.4**, re-suspended and adjusted to a concentration of 1.5 x 10^6 cells/mL in culture medium. Splenocytes were then incubated (1.5 x 10^5 cells/well; 200 µL final volume) with SMX (0 - 787 µM; 0 – 200 µg/mL), SMX-NHOH (5 - 100 µM), or SMX-NO (1 - 100 µM) in 96-well U-bottomed tissue culture plate for 3 days (37 °C; 5% CO_2). To measure proliferation, [^{3}H]-thymidine (0.5 µCi) was added to the cultures for the last 16 h of the incubation.

Cells were harvested and [^{3}H]-thymidine incorporation was measured as counts per min on a β-counter (PerkinElmer Life Sciences, Cambridge, U.K.)

3.2.6 Immunogenicity of different nitroso sulfonamide metabolites in Balb/c mice

Splenocytes from treated Balb/c (SMX-NO, SDZ-NO, and SP-NO; 5 mg/kg i.p.) mice were re-suspended in culture medium (1.5 x 10^5 cells / well; 200 µL final volume). Splenocytes then were incubated with different concentrations of the sulfonamides and their metabolites. The concentrations used in the assay were: SMX (0 – 787 µM; 0 – 200 µg), SDZ and SP (0 – 800 µM; 0 – 400 µg/mL), SMX-NO, SP-NO, SDZ-NO (1-25 µM), SMX-NHOH, SDZ-NHOH and SP-NHOH (5-50 µM) in U-bottomed 96-well plates for 72 h (37ºC, 5% CO_2). To measure proliferation, [^{3}H]-thymidine (0.5 µCi) was added to the cultures during the last 16 h. Cells were harvested and [^{3}H]-thymidine incorporation was measured as counts per min on a β-counter (PerkinElmer Life Sciences, Cambridge, U.K.).

3.2.7 Cross-reactivity of splenocytes from nitroso sulfonamide immunized Balb/c mice with different sulfonamide metabolites

Splenocytes (1.5 x 10^5 cells/well; 200 µL final volume) immunized with the different nitroso (SMX-NO, SDZ-NO or SP-NO) were re-challenged in the presence of different nitroso (1 – 25 µM) or hydroxylamine (5 – 50 µM) metabolites from different sulfonamides to which they have never been exposed before.

3.2.8 Role of drug metabolism in the Immunogenicity of sulfonamides metabolites

Splenocytes from nitroso SMX, SD and SP immunized Balb/c (1.5 x 10^5 cells/well; 200 µL final volumen) mice were incubated with the nitroso or hydroxylamine metabolites of the compound that was used during immunization

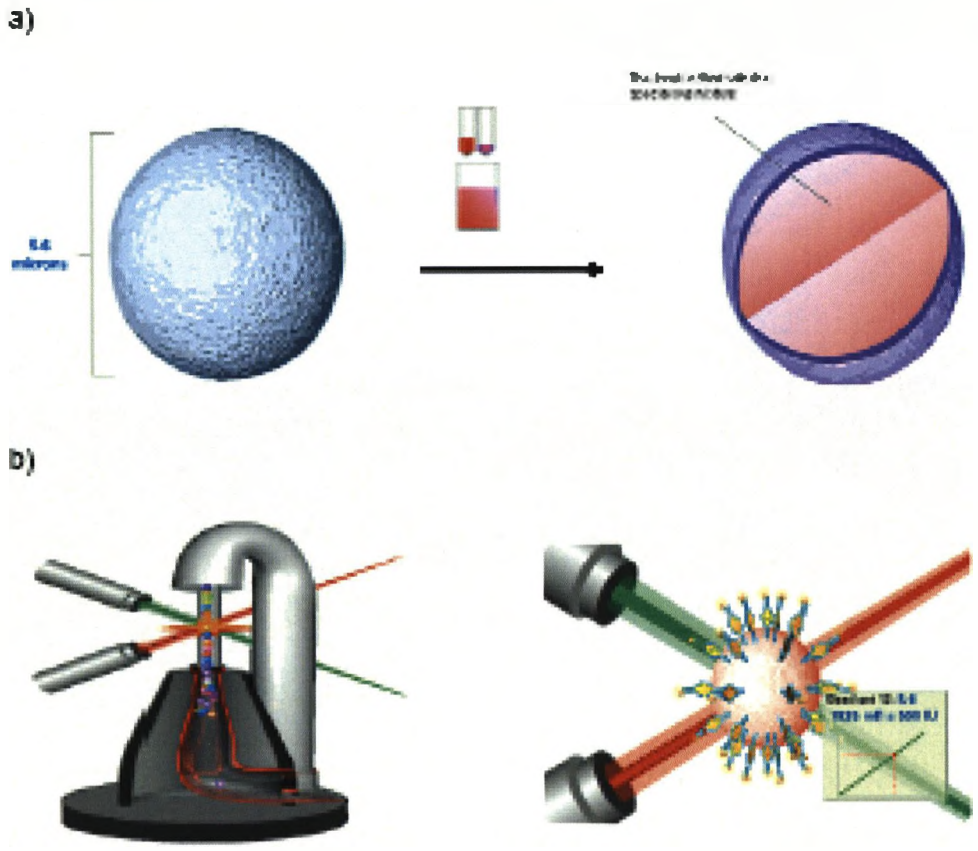
at the concentration associated with maximal proliferative response seen in the presence or absence of GSH (1 mM). GSH was added just before the metabolites in 96-well plates during 72 hr (37°C, 5% CO_2), and proliferation was measured by [^{3}H]- thymidine incorporation.

3.2.9 Cytokine analysis

Supernatant from immunized splenocytes stimulated with SMX-NO, SDZ-NO, SP-NO (all 25μM), were collected after 72 h and stored at -70°C for analysis of cytokine secretion. Concentrations of IL-2, IL-4, IL-5, TNF-α, GMCSF and IFN-γ were measured in the supernatant using a mouse cytokine/chemokine LINCOplex multiplex assay kit (LINCO Research Inc., Hampshire, UK). Cytokine content was measured using a Liquichip 100 workstation (Qiagen Ltd., West Sussex, UK) with LiquiChip IS 2.3 software.

3.2.10 LINCOplex multi-analyte detection assay procedure

The LINCOplex assay was conducted according to the manufacturer's instructions (Linco Research, Inc.). The assay is based on conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to the coated Luminex microspheres allowing capture and detection of specific analytes in supernatant samples. Each antibody is coupled to a different microsphere uniquely labelled with a fluorescent dye mixture (Scheme 3.1 a). Assays are carried out in a 96- well plate format with up to 100 tests per well. High-tech fluidics based on the principles of flow cytometry causes the stream of suspended microspheres to line up in a single file prior to passing through the detection chamber of the Luminex 100 compact analyzer which allows the particles to be measured discretely. Within the analyzer, lasers excite the internal dyes that identify each microsphere particle; a red laser excites both the internal red and infrared dyes, allowing the proper classification of the microsphere to one of the 100 sets (i.e. classification channel reading), whereas the green laser excites any orange fluorescence associated with the binding of your analyte (i.e. reporter channel reading) (Scheme 3.1 b).



Scheme 3.1. Luminex® system color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. By using different intensities of the two dyes each microsphere has a unique spectral signature (i.e. determined by the internal red/infrared fluorophore mixture) (a), which when passed through the detection chamber (b) allows the particles to be measured discretely. A red laser excites both the internal red and infrared dyes, allowing the proper classification of the microsphere to one of the 100 sets (i.e. classification channel reading) and a green laser excites any orange fluorescence associated with the binding of the analyte (i.e. reporterchannel reading). (Figures taken from the Luminex website: www.luminexcorp.com/)

All reagent dilutions (beads, cytokine standards, cytokine controls, biotinylated detection antibody, etc.) were prepared according to the manufacturer's instructions using a LINCOplex kit. Cytokine standard provided as a lyophilized cocktail was reconstituted with 250 µl de-ionized water to give a stock concentration of 10,000 pg/ml of each cytokine. Standard concentrations of

concentration of 10,000 pg/ml of each cytokine. Standard concentrations of 5000, 2000, 666, 222, 75, 25, 8.3 and 2.76 pg/ml were prepared by diluting the 10,000 pg/ml stock standard with assay buffer. The 0 pg/ml standard was buffer alone. Each of the two vials of lyophilized cytokine controls provided in the kit was reconstituted with 250 µl of de-ionized water. Firstly, the filter plate was blocked by adding 200 µl assay buffer (50 mM PBS with 25 mM EDTA, 0.08% Sodium Azide, 0.05% Tween 20, and 1% BSA, pH 7.4) into each well of the microtiter plate. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature. The assay buffer was removed by vacuum, and 25 µl of assay buffer was added to the background wells, sample wells and the quality control wells. Then cell culture media was added to the background and control wells, followed by 25 µl of samples into the appropriate wells. To each well, 25 µL of the mixed colour coded beads was added ensuring that the bead mix was thoroughly shaken to avoid the beads settling. The plate was sealed and covered with aluminium foil then incubated with agitation on a plate shaker for 1 hour at room temperature. After the incubation period, the fluid was removed by vacuum, and the plate washed twice with 200 µL/well of wash buffer (10X Wash Buffer 1:10 dilution required with deionized water to give 10 mM PBS with 0.05% Proclin, and 0.05% Tween 20, pH 7.4), removing wash buffer by vacuum filtration between each wash to which 25 µL of detection antibody cocktail was added to each well. The plate was then sealed, covered and incubated with agitation on a plate shaker for 30 minutes at room temperature. After incubation, 25 µL Streptavidin-Phycoerythrin was added to each well containing the 25 µL of detection antibody cocktail, the plate was resealed, covered, and incubated with agitation on a plate shaker for a further 30 minutes at room temperature. The contents of the plate were again removed by vacuum, the plate was washed twice with 200 µL/well wash buffer, removing wash buffer by vacuum filtration between each wash. Finally, 150 µL sheath fluid was added to all wells, the plate was covered and the bead mix re-suspend on a plate shaker for 5 minutes. Cytokine content was measured using a Liquichip 100 workstation (Qiagen Ltd, West Sussex, UK) with LiquiChip IS 2.3 software. Samples were analyzed per well using a minimum of 50 beads per region. The raw data (mean fluorescence intensity, MFI) were captured using LiquiChip IS 2.3 software; for data analysis, a

5-parameter logistic (5-PL) or curve-fitting method for calculating cytokine/chemokine concentrations in samples was applied to each standard curve and sample concentrations were interpolated from the standard curve. The gating specifications / settings ranged from 8,060 to 13,000 with the limit of detection (LOD), defined as the lowest concentration of analyte that can be detected, was 3.2 pg/ml. A standard curve (range of detectability between 3 and 10,000 pg/ml) was generated from a single mixed standard and the concentration of each analyte in the sample was determined automatically and data is presented as pg/ml.

3.2.11 Statistical analysis

Statistical analysis was performed using SPSS 16.0 using a Macintosh plataform. Average data is presented as mean ± standard error of the mean. The Shapiro-Wilks test was used to determine normality. Data was not normally distributed; therefore, the Mann-Whitney test (non-parametric test) was used to compare data points, accepting $p \leq 0.05$ as significant.

3.3 Results

3.3.1 Immunogenicity of nitroso sulfamethoxazole in two different strains of mice

Preliminary experiments were designed to explore the immunogenicity of SMX-NO (5mg/mL; 4 x weekly for 2 weeks) in BALB/c and C57BL/6 strain mice. Splenocytes from BALB/c and C57BL/6 strain mice exposed to SMX-NO were stimulated to proliferate *ex-vivo* with SMX (40 – 787 μM; 10 – 200 μg/mL), SMXNHOH (5 – 100 μM) and SMX-NO (1 – 100 μM) (Figure 3.1 A-B). The parent drug did not induce proliferation at any of the concentrations tested in both strains; however, the SMX metabolites induced dose-dependent proliferation. In the C57BL/6 strain, maximal proliferation (SMXNHOH and SMXNO) was detected at 25 μM and 10 μM, respectively (SMXNHOH [0 μM, 480 ± 136; 25 μM, 6335 ± 1527; Figure 3.1 A] and SMXNO [0 μM, 480 ± 136; 10 μM, 4174 ± 685; Figure 3.1 A] whereas in the Balb-c strain, maximal proliferation with the metabolites was seen at 50 μM and 25 μM (SMXNHOH [0 μM, 1454 ± 1068; 50 μM, 21436 ± 5534; Figure 3.1 B] and SMXNO [0 μM, 1454 ± 1068; 25 μM, 13706 ± 7671; Figure 3.1 B]. Proliferation was abolished at 100 μM for both metabolites in the two strains.

Additionally, the proliferative response induced by the two SMX metabolites in both strains was compared (Figure 3.2 A – B). The ability of primed splenocytes to proliferate in the presence of the two sulfamethoxazole metabolites was different between the different strains. SMXNO at 25 μM and SMXNHOH at 50 μM induced a significantly stronger response in splenocytes from Balb-c immunized mice than C57BL/6 strain splenocytes (Balb-c [SMX-NO 25 μM; 13706 ± 7671] C57BL/6 [SMX-NO 25 μM; 3039 ± 549] and [SMX-NHOH 50 μM; 21436 ± 5534] C57BL/6 [SMX-NHOO 50 μM; 6130 ± 3317], Figure 3.2 A-B). The proliferation in the controls (without drug) in the two strains was similar.

As strength of the response with SMX-NO and SMXNHOH was stronger with splenocytes from Balb/c strain mice, this strain was selected for all subsequent investigations.

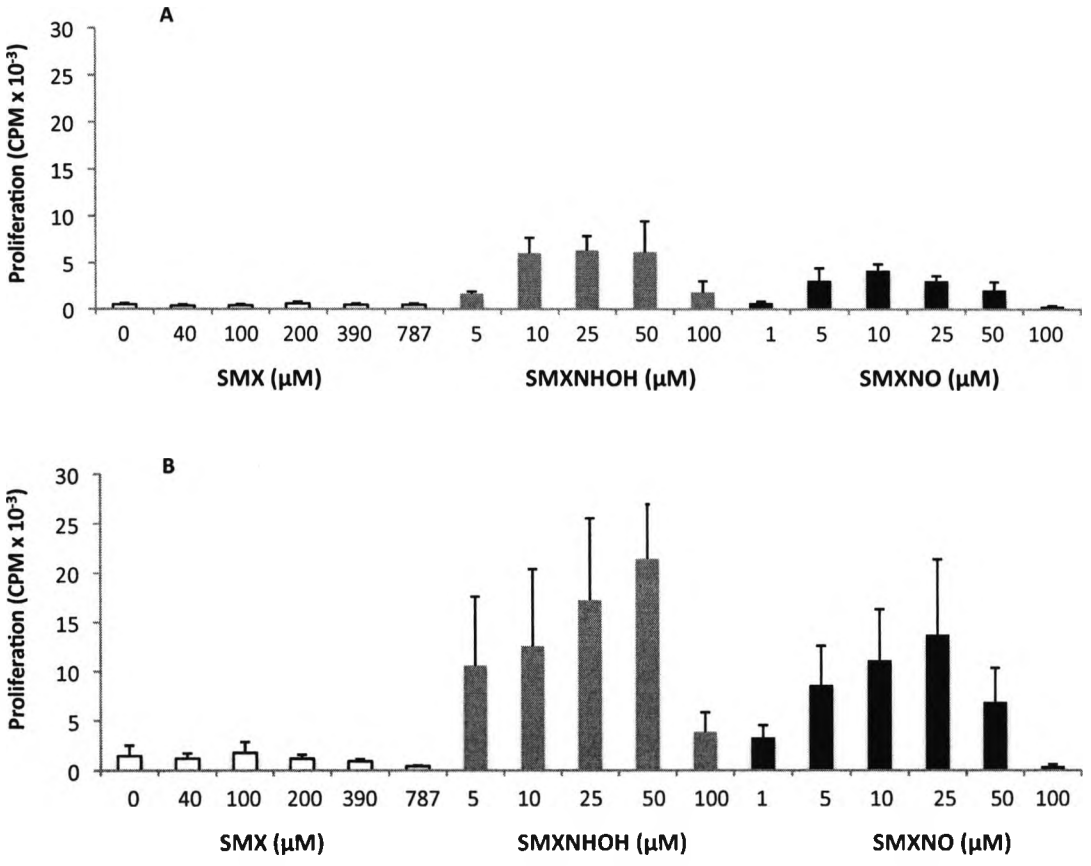


Figure 3.1 Proliferative response of (A) C57BL/6 and (B) Balb/c strain mice immunized with SMX-NO (5 mg/kg) and in vitro cultured with SMX, SMX-NHOH or SMX-NO for 72 h. Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control cultures did not exceeded 1500 cpm. Results represent the mean ± S.D from two animals with incubations carried out in triplicate..

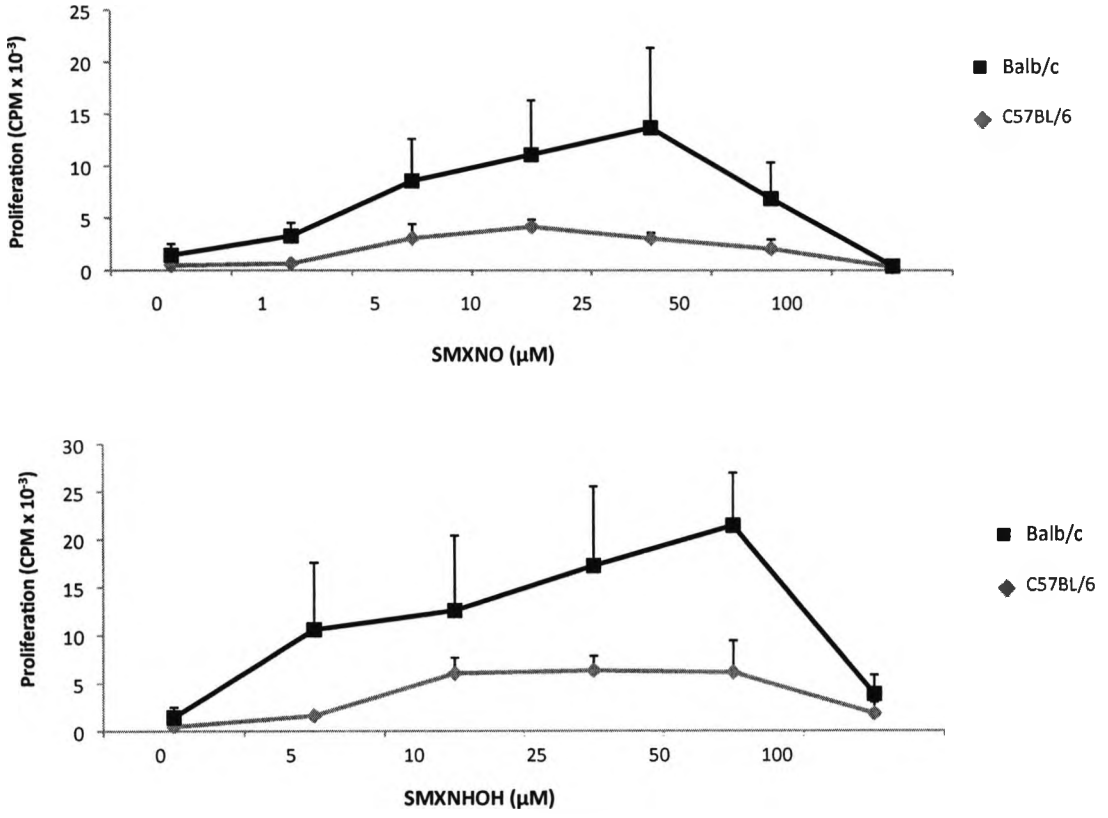


Figure 3.2 Comparative proliferative response of two different strains of mice (Balb-c & C57BL/6) immunized with SMX-NO (5 mg/kg) cultured with different concentrations of (A) SMX-NO or (B) SMX-NHOH for 72 h. Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control cultures did not exceed 1500 cpm. Results represent the mean ± S.D from two animals with incubations carried out in triplicate.

3.3.2 Nitroso sulfonamide metabolites are immunogenic in Balb/c strain mice

As described previously (Farrell et al., 2003), splenocytes from mice exposed to SMX-NO, (5mg ml^{-1}; 4 x weekly for 2 weeks) proliferated following stimulation with SMX-NO and SMX-NHOH, but not the parent compound SMX (Figure 3.3 A). Proliferation was dose dependent for both metabolites and maximal proliferation was observed at 25 μM and 50 μM respectively ([0 μM, 2967 ± 985; SMX-NO 25 μM, 26120.41 ± 11900; $p \leq 0.05$] and [0 μM, 2967 ± 985; SMX-NHOH 50 μM, 25019.41 ± 8068; $p \leq 0.05$]; Figure 3.3 A). Individual proliferative response of each mouse in the presence of SMX metabolites is shown in figure 3.3.1. (A). SMX-NHOH is less toxic than SMX-NO (Rieder et al., 1995; Hess et al., 1999). Therefore higher concentrations of SMX-NHOH induced proliferation whereas a similar concentration of SMX-NO inhibited splenocytes proliferation.
Similar results were obtained with SDZ-NO and SP-NO immunized mice. Splenocytes were stimulated to proliferate with nitroso and hydroxylamine metabolites, but not the parent compounds (Figures 3.3 B & C). In case of mice immunized with SDZ-NO maximal proliferation in the presence of SDZ-NO was seen at 5 μM and at 10 μM for SDZ-NHOH ([0 μM, 158 ± 72; SDZ-NO 5 μM, 4120 ± 4340; $p \leq 0.05$] and [0 μM, 158 ± 72; SDZ-NHOH 10 μM, 6760 ± 4816; $p \leq 0.05$]; Figure 3.3 B). Individual proliferative response of each mouse in the presence of SDZ metabolites is shown in figure 3.3.1. (B). Incubation with 25 μM of both metabolites resulted in a decrease in the response. Splenocytes from SP-NO immunized mice proliferate strongly when re-challenged with the different SP metabolites. Maximal proliferation was seen at 25 μM and 10 μM (SP-NO and SP-NHOH respectively) ([0 μM, 3009 ± 3589; SP-NO 25 μM, 35592 ± 13837; $p \leq 0.05$] and [0 μM, 3009 ± 2614; SP-NHOH 10 μM, 46498 ± 21676; $p \leq 0.05$]; Figure 3.3 C). The proliferation with SP-NO was dosed dependent (maximal response detected at 25 μM) whereas for SP-NHOH the proliferation remained constant with a range of concentrations of the metabolite (10 – 50 μM). Individual proliferative response of each mouse in the presence of SP metabolites is shown in figure 3.3.1. (C).

In summary, hydroxylamine and nitroso metabolite-specific proliferation was concentration dependent up to 25 μM. Metabolite concentrations above 25 μM inhibited splenocyte proliferation. The strength of the maximum proliferative response of splenocytes from SDZ-NO immunized mice with SDZ-NO was significantly lower than that observed with SMX-NO-stimulated and SP-NO-stimulated splenocytes from SMX-NO and SP-NO immunized mice, respectively (SMX-NO immunized [0 μM, 2967 ± 985; SMX-NO 25 μM, 26120.41 ± 11900; $p \leq 0.05$]; SP-NO immunized [0 μM, 3009 ± 3589; SP-NO 25 μM, 35592 ± 13837; $p \leq 0.05$]; SDZ-NO immunized [0 μM, 158 ± 72; SDZ-NO 5 μM, 4120 ± 4340; $p \leq 0.05$]; Figure 3.3 A-C).

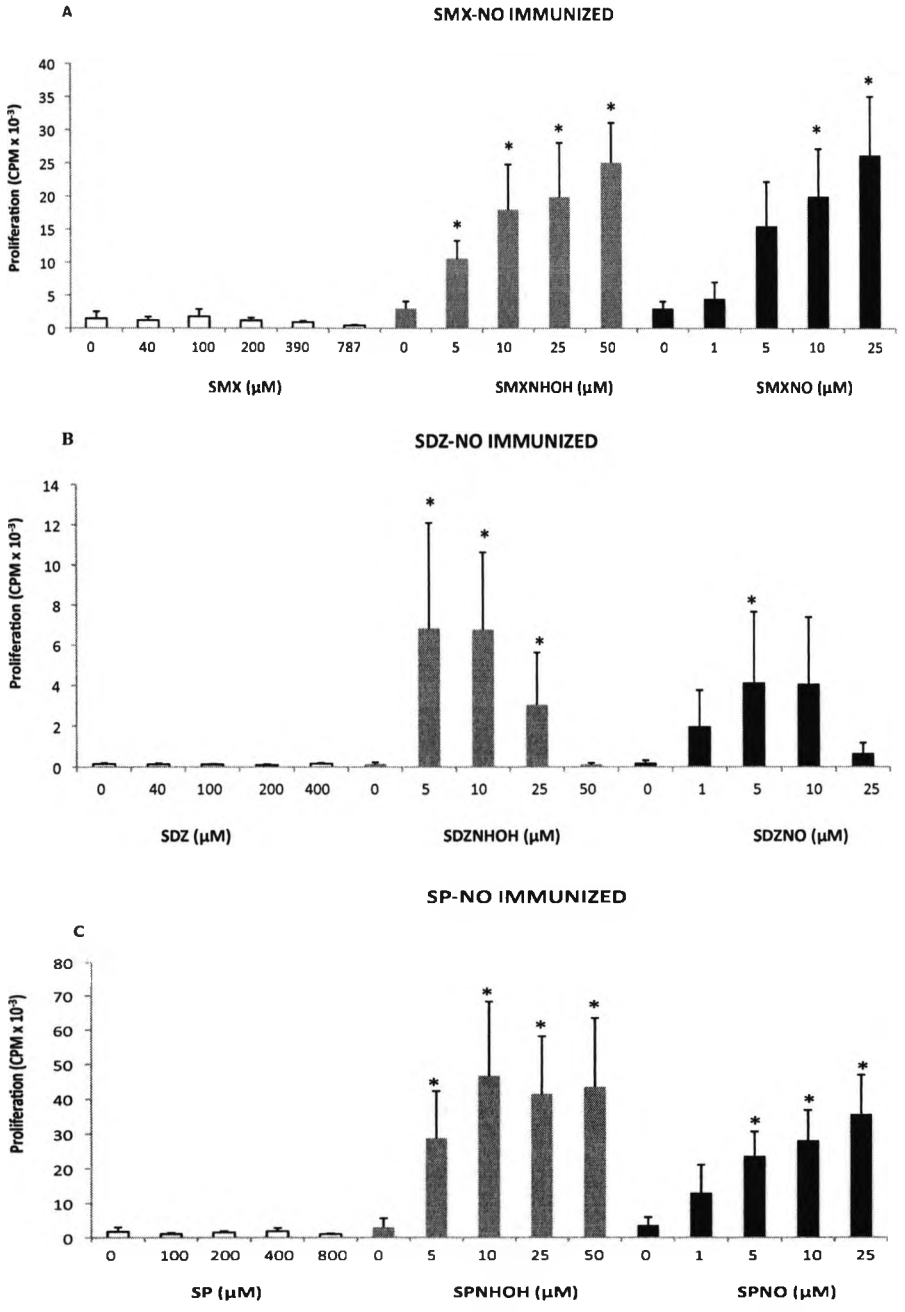


Figure 3.3 Proliferative response of splenocytes from Balb-c strain mice immunized with of (A) SMX-NO, (B) SDZ-NO or (C) SP-NO (5 mg/kg) and cultured with the metabolites to which the cell were immunized against for 72 h. Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control culture ranged between 200 – 3000 cpm. Results represent the mean ± S.D from three animals with incubations carried out in triplicate. Statistical analysis compares drug treated splenocytes with incubations containing DMSO alone (*, $p \leq 0.05$, n = 3).

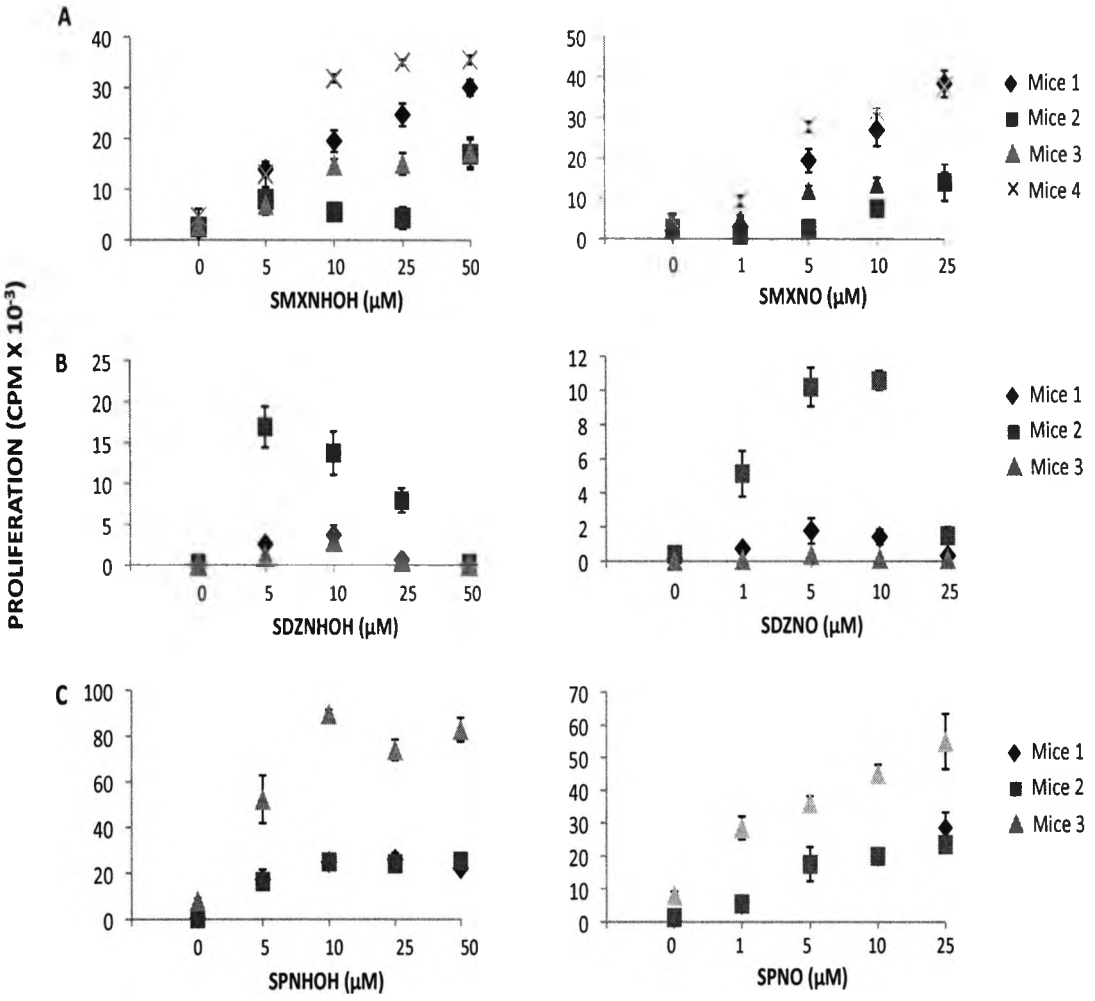


Figure 3.3.1. Individual proliferative response of splenocytes from different mice immunized with SMX-NO (A; n=4), SDZ-NO (B; n=3) or SP-N0 (C; n=3) in the presence of the hydroxylamine (0 – 50 μM) or nitroso (0 – 25 μM) metabolite of the compound used during immunization. Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control culture ranged between 200 – 3000 cpm. Results represent the mean ± S.D from each mouse with incubations carried out in triplicate

3.3.3 Cross-reactivity between sulfonamide metabolites

Splenocytes from mice immunized with SMX-NO were additionally stimulated to proliferate in the presence of SDZ-NHOH (0 μM, 2967 ± 985.34; 25 μM, 13832 ± 4653; $p \leq 0.05$, Figure 3.4 A) and SDZ-NO (0 μM, 2967 ± 1073.14; 10 μM, 9905 ± 3933; $p \leq 0.05$, Figure 3.4 A). Hydroxylamine and nitroso metabolites of SP stimulated a weak response and only at the highest concentration tested (figure 3.4 A). A proliferative response of splenocytes from SDZ-NO and SP-NO immunized mice was detected with hydroxylamine and nitroso metabolite of all 3 sulfonamides (Figures 3.4 B & C). The strength of the response in SDZ-NO immunized splenocytes, measured by proliferation, was in the following order SDZ-NO > SMX-NO > SP-NO and SDZ-NHOH > SMX-NHOH > SP-NHOH. Metabolites concentrations above of 25 μM of both SDZ metabolites decreased the proliferative response. Likewise SDZ-NO primed splenocytes, splenocytes primed with SP-NO showed high cross-reactivity (Figure 3.4 C). The strength of the response seen for nitroso and hydroxylamine metabolites was SP-NO > SMX-NO > SDZ-NO and SP-NHOH > SMX-NHOH > SDZ-NHOH respectively.

The proliferative response was significantly stronger when splenocytes from SMX-NO, SP-NO and SDZ-NO treated mice were stimulated with the nitroso and hydroxylamine of the compound to which animals were originally immunized against.

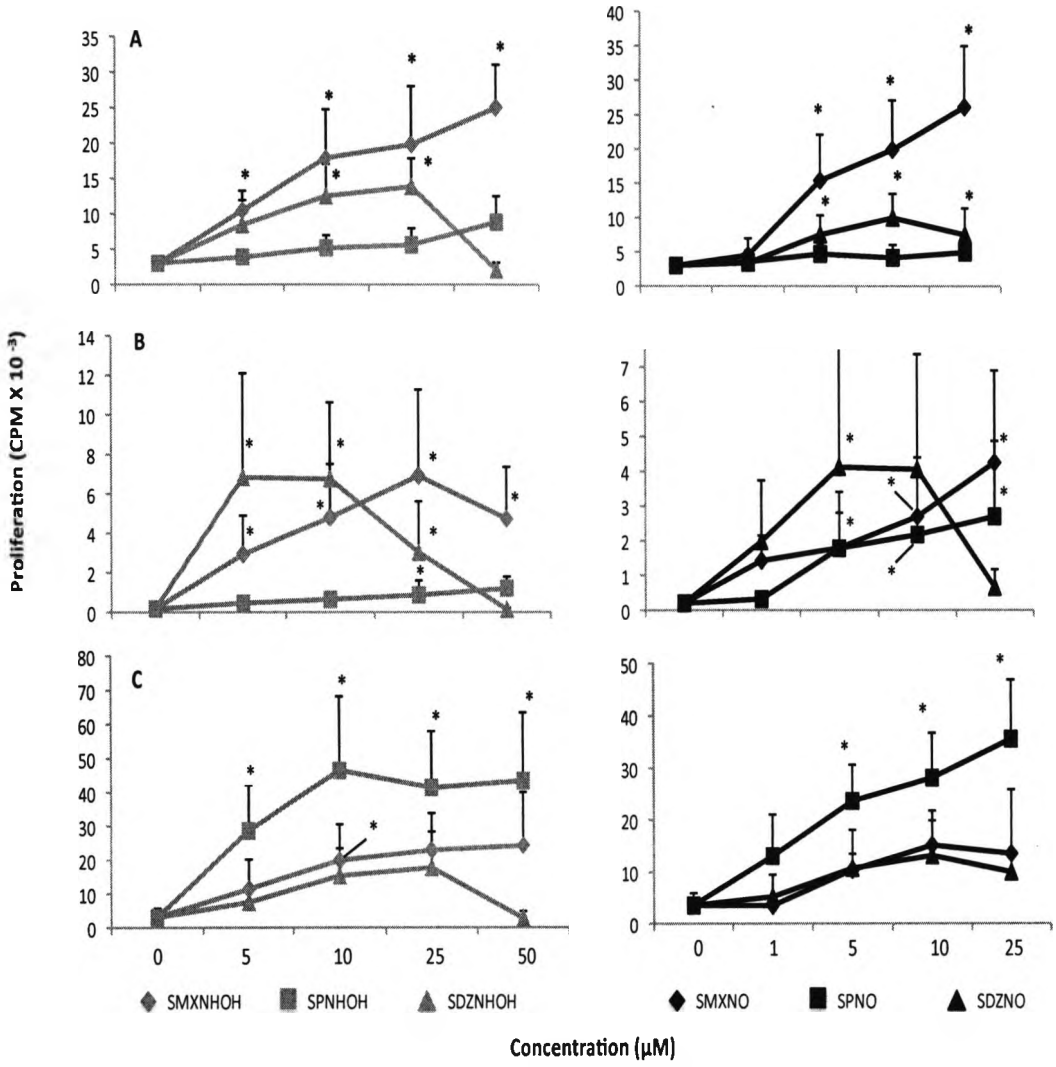


Figure 3.4 Proliferative response of splenocytes from Balb-c strain mice immunized with (A) SMX-NO, (B) SDZ-NO or (C) SP-NO (5 mg/kg) and cultured with the metabolites compounds to which the mice were immunized. Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control cultures ranged between 200 – 3000 cpm. Results represent the mean ± S.D from three animals with incubations carried out in triplicate. Statistical analysis compares drug treated splenocytes with incubations containing DMSO alone (*, $p \leq 0.05$, n = 3).

3.3.4 Glutathione prevents the specific stimulation of splenocytes with hydroxylamine and nitroso metabolites of sulfamethoxazole, sulfadiazine and sulfapyridine

Glutathione prevents the conversion of hydroxylamine metabolites to nitroso compounds and rapidly reduces synthetic nitroso compounds (Cribb et al., 1991; Naisbitt et al., 1996b; Naisbitt et al., 2001; Trepanier et al., 2004). Furthermore, glutathione inhibits SMX-NO-specific stimulation of lymphocytes from allergic patients and animal models of sulfamethoxazole immunogenicity (Burkhart et al., 2001; Naisbitt et al., 2001).

SMX-NO primed splenocytes proliferate strongly to SMX metabolites in the absence of GSH. However, when glutathione was added prior to the respective metabolite, the proliferative response was significantly decreased (no GSH [25 μM; 6685 ± 4541] + GSH [25 μM; 568 ± 192] $p \leq 0.05$; Table 3.1 and figures 3.5 A and 3.5.1 A). Similar results were obtained with splenocytes from immunized mice with SDZ-NO and SP-NO re-challenged with their respective nitroso metabolite {no GSH [10 μM; 5368 ± 5812] + GSH [10 μM; 481 ± 288] ; Table 3.1 and figures 3.5 B and 3.5.1 B} and SP-NO immunized {no GSH [25 μM; 30442 ± 13880] + GSH [25 μM; 4843 ± 1932;] $p \leq 0.05$; Table 3.1 and figures 3.5 C and 3.5.1 C})

In a similar context when immunized splenocytes were exposed to the different hydroxylamine metabolites, cells proliferate vigorously. The hydroxylamine metabolites auto-oxidaze under normal conditions to the different nitroso metabolites. These are believed to be the ultimate antigens responsible for stimulating of a proliferative response of previously nitroso-primed cells. To further demonstrate that auto-oxidation of the hydroxylamine to the nitroso is responsible for the response observed ex vivo, immunized splenocytes were incubated with the different sulfonamides hydroxylamines in the presence or absence of glutathione. The proliferative response of splenocytes to hydroxylamines was reduced to control levels when glutathione was added to the cell medium (SMX-NO immunized {none GSH [50 μM; 4771 ± 2316]; + GSH [50 μM; 782 ± 334] $p \leq 0.05$}, SDZ-NO immunized {none GSH [25 μM; 2732 ± 2912]; + GSH [25 μM; 577 ± 385]} and SP-NO immunized {none GSH [25 μM;

29424 ± 12281]; + GSH [25 μM; 2713 ± 1132]; $p \leq 0.05$}, Table 3.1 and figures 3.5 A-C and 3.5.1 A-C).

Immunized	Re-challenge	(μM)	- GSH	+ GSH
SMX-NO	SMX-NHOH	50	4711 ± 2316	782 ± 334*
	SMX-NO	25	6685 ± 4541	568 ± 192*
SDZ-NO	SDZ-NHOH	25	2732 ± 2912	577 ± 385
	SDZ-NO	10	5386 ± 5812	481 ± 288
SP-NO	SP-NHOH	25	28635 ± 12281	2713 ± 1132*
	SP-NO	25	30442 ± 13880	4843 ± 1932*

Table 3.2 Proliferative response of splenocytes from Balb-c strain mice immunized with SMX-NO, SDZ-NO or SP-NO (5 mg/kg) and cultured with the metabolites to which the mice were immunized against and in absence (column 4) or presence (colum 5) of GSH (1 mM). Proliferation was measured by incorporation of [^{3}H]-Thymidine. Results represent the mean ± S.D from three animals with incubations carried out in triplicate. * $P \leq 0.05$ when proliferation in the presence or absence of an excess of GSH were compared.

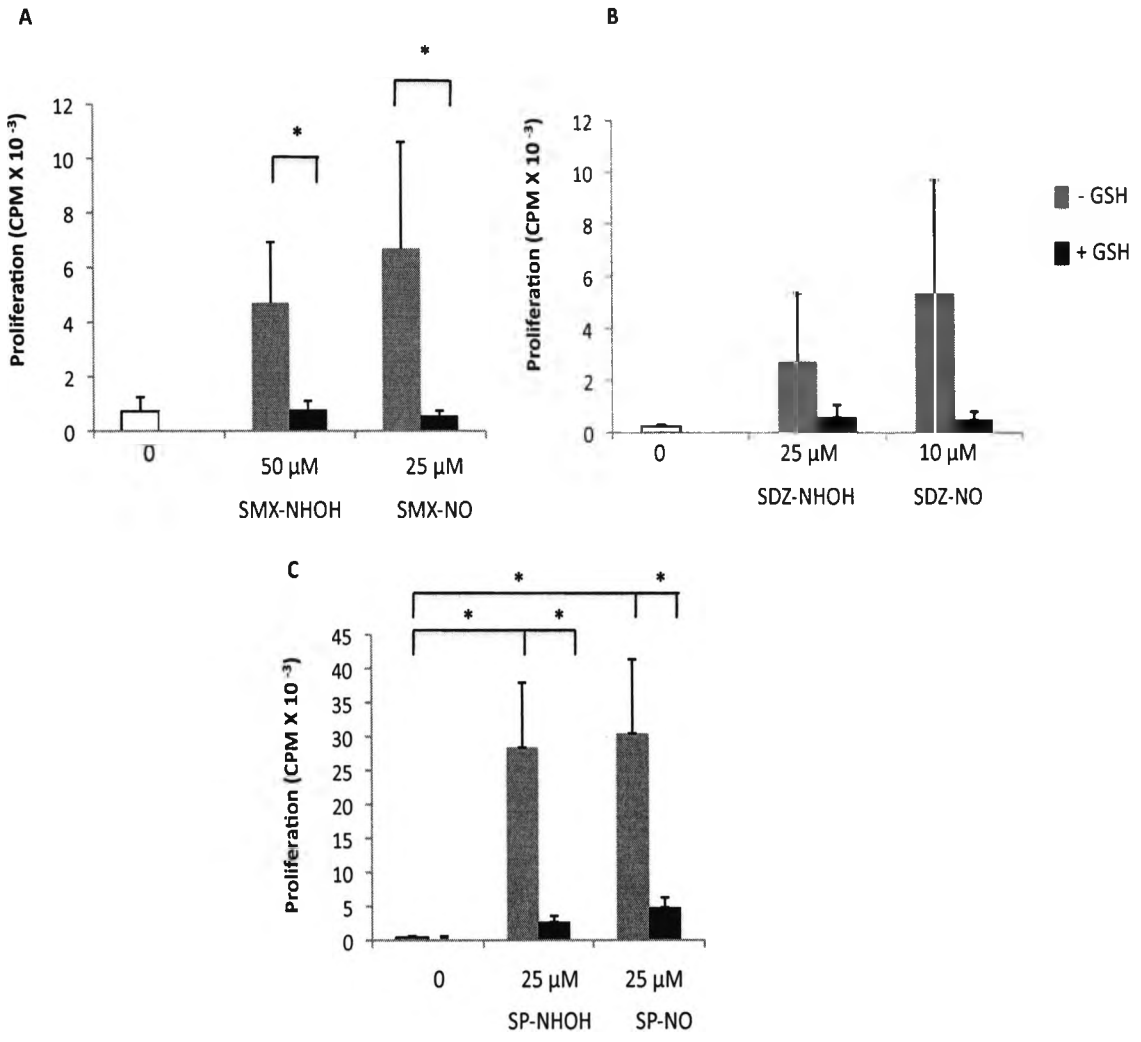


Figure 3.5 Proliferative response of splenocytes from Balb-c strain mice immunized with (A) SMX-NO, (B) SDZ-NO or (C) SP-NO (5 mg/kg) and cultured with the metabolites to which the mice were immunized against and in absence or presence of GSH (1 mM). Proliferation was measured by incorporation of [^{3}H]-Thymidine. Results represent the mean ± S.D from three animals with incubations carried out in triplicate. Statistical analysis compares drug treated splenocytes with incubations containing DMSO alone (*, $p \leq 0.05$, $n = 3$).

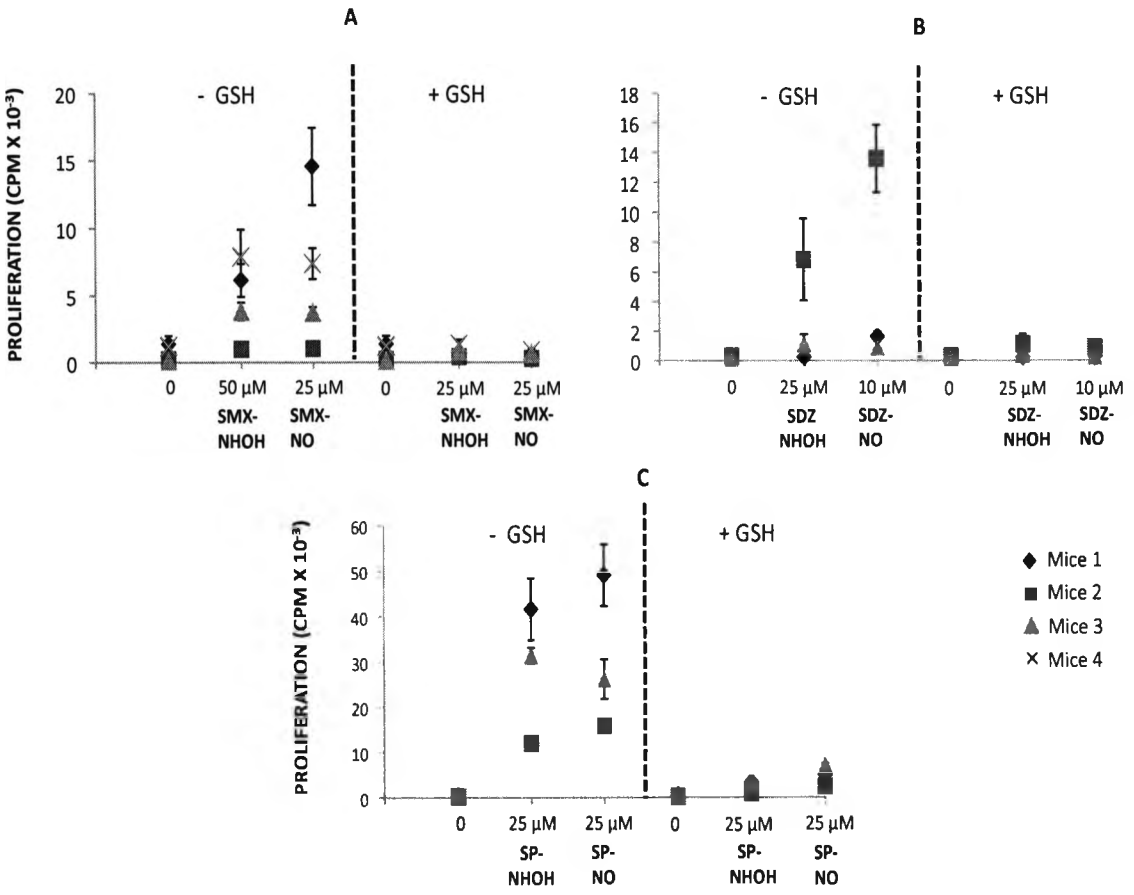


Figure 3.5.1. Individual proliferative response of splenocytes from different mice (Balb-c strain) immunized with SMX-NO (A; n=4), SDZ-NO (B; n=3) or SP-N0 (C; n=3) in the presence of the metabolites to which the mice were immunized against and in the absence or presence of GSH (1mM). Proliferation was measured by incorporation of [^{3}H]-Thymidine. The cpm in control culture ranged between 200 – 3000 cpm. Results represent the mean ± S.D from each mouse with incubations carried out in triplicate

3.3.5 Cytokine secretion from nitroso sulfamethoxazole, nitroso sulfadiazine and nitroso sulfapyridine-stimulated splenocytes

Cytokines secreted by antigen-stimulated splenocytes from nitroso sulfonamide immunized mice were analyzed using a mouse cytokine LINCOplex multiplex assay kit (see 3.2.10). SMX-NO stimulated splenocytes from SMX-NO immunized mice secreted the Th2 cytokines IL-4 and IL-5, IL-2 and GM-CSF (Table 3.2). Levels of secreted IFN-γ and TNF-α did not differ when supernatants from SMX-NO stimulated and control splenocytes were compared. SP-NO and SD-NO stimulation of splenocytes from SMX-NO immunized mice was associated with secretion of the same cytokines, albeit at lower levels (Table 3.2).

The cytokines secreted from SMX-NO, SD-NO and SP-NO stimulated splenocytes from SP-NO immunized were similar to those detected from SMX-NO immunized mice. In contrast, splenocytes from SD-NO immunized mice secreted IL-2 and IL-4 alone, and only when stimulated with SD-NO or SMX-NO (Table 3.2).

Immunogen	Antigen	Cytokine (pg ml^{-1})					
		IL-2	IL-4	IL-5	GMCSF	IFN-γ	TNF-α
SMX-NO	0	7.8±0.41	nd[1]	Nd	Nd	25.4±4.8	nd
	SMX-NO	75.6±1.55	18.5±0.9	39.1±3.5	82.6±6.5	39.4±8.5	nd
	SP-NO	44.3±1.24	9.6±0.3	8.2±0.71	32.2±8.7	16.9±4.7	nd
	SDZ-NO	67.3±1.27	10.2±0.3	9.2±0.05	31.2±18.6	16.6±4.7	nd
SP-NO	0	27.9±3.9	10.0±1.2	Nd	nd	6.6	nd
	SMX-NO	60.4±9.24	23.7±1.8	18.1±2.7	105.5±32.1	2.6	nd
	SP-NO	122.4±15.6	94.2±7.9	48.2±5.7	202.9±28.7	25.8±8.9	nd
	SDZ-NO	104.7±12.7	21.0±2.9	11.7±2.1	45.0±7.9	13.0±14.03	nd
SD-NO	0	10.0±0.80	0.5±0.2	Nd	Nd	12.3±0.2	nd
	SMX-NO	68.8±1.3	16.7±0.2	7.2±0.8	Nd	1.6	nd
	SP-NO	6.1±0.4	4.4±0.3	Nd	Nd	16.0±2.0	nd
	SDZ-NO	54.3±3.4	12.3±0.8	Nd	Nd	21.0±10.5	nd

Table 3.2 Cytokine secretion from antigen-stimulated splenocytes from nitroso SMX, SDZ and SP immunized mice. The sample was pooled from three mice (Balb/c strain) immunized with the different sulfonamide nitroso metabolites. Results represent the mean ± S.D from three animals with incubations carried out in triplicate

3.4 Discussion

Over 70 years ago, Landsteiner and Jacobs (Landsteiner and Jacobs, 1935a) described electrophillic chemicals as incomplete allergens since covalent adduction of specific nucleophillic residues on protein was deemed a prerequisite for immune activation. Using SMX as a model drug allergen, it has been shown that the electrophilic, protein reactive metabolite SMX-NO (Naisbitt et al., 2001; Manchanda et al., 2002; Summan and Cribb, 2002; Callan et al., 2009c) is at least 3 orders of magnitude more immunogenic in different species such as humans, mice, rat or rabbit than the parent compound (Choquet-Kastylevsky et al., 2001; Naisbitt et al., 2001; Farrell et al., 2003; Cheng et al., 2008), stimulates blood and skin-derived T-cells from allergic human patients (Schnyder et al., 2000; Burkhart et al., 2001; Nassif et al., 2004) and activates naïve T-cells from healthy volunteers (Engler et al., 2004). Furthermore, it has been recently shown that intracellular SMX-NO formation in antigen presenting cells is associated with dendritic cell co-stimulatory signaling (Sanderson et al., 2007) and the presentation of a distinct antigenic determinant to T-cells from allergic patients (unpublished observation). Additionally to have a complete immune response, T cells from hypersensitive patients secrete cytokines in the presence of the drug, this complements the activation of different immunological mechanisms. $CD4^+$ T cells can be divided in Th1 or Th2 cells according with the cytokines that produce (O'Garra, 1998). A Th1 profile is seen in contact allergy compounds including 2,4-dinitrochlorobenzene, whereas Th2 profile is associated with respiratory allergy (Dearman et al., 2002). In the case of hypersensitivity reactions caused by SMX in humans, T cell clones secrete preferentially high amount of IL-5, which is involved in eosinophilia, and INF-γ in a lesser extent (Mauri-Hellweg et al., 1995; Pichler et al., 1997). In animal models, $CD4^+$ positive T-cells secrete high amounts of IL-5; still, a clear distinction between Th1 or Th2 cytokines was not detected (Hopkins et al., 2005).

The aim of this chapter was to evaluate whether nitroso sulfonamide-specific T-cells can be stimulated with structurally related nitroso sulfonamides through T-cell receptor cross-reactivity in an animal model. T-cell activation was determinated by proliferation assays complemented with cytokine secretion.
To accomplish this objective, hydroxylamine and nitroso metabolites of SMX, SD and SP were synthesized as detailed in chapter 2. SDZ and SP were selected since they are for the most part ineffective at stimulating SMX (parent drug)-specific T-cells from allergic human patients (von Greyerz et al., 1999), and because the immunogenicity of these compounds and their metabolites has not been studied in detail before. The pyrimidine derivative SDZ stimulated a weak response in 10-15% of SMX-specific T-cell clones, while only 1 out of 13 clones were activated with the pyridine-derivative SP. Furthermore each molecule has a very similar structure each which may permit the formation of similar antigen determinants with the MHC-peptide TCR complex.
SMX-NO was immunogenic in both mice strains. Proliferative response was seen when the splenocytes from each strain were re-challenged with SMX-NHOH or SMX-NO, but failed to response with SMX. This suggests that the specificity of the immunized splenocytes with SMX-NO is against a SMX-NO modified cellular proteins. SMX-NO and SMX-NHOH (after auto-oxidation) can form SMX-protein adducts, whereas SMX is none reactive. The strength of the response was higher in the Balb-c strain mice. Inhibition of splenocytes proliferation, which was observed at metabolite concentrations above 100 μM, is related to the previously described *in vitro* toxicity of hydroxylamine and nitroso sulfonamide metabolites. Several studies have demonstrated that concentrations above 100 μM of both sulfamethoxazole metabolites were associated with cell death by apoptosis followed by secondary necrosis (Rieder et al., 1988; Pirmohamed et al., 1991; Reilly et al., 1998; Hess et al., 1999; Naisbitt et al., 2002). Thus, proliferation at these concentrations decreased drastically in the both strains.

SMX-NO, SDZ-NO and SP-NO induced an immune response in the mouse at the same dose (5mg/kg); higher concentrations were not used due to overt toxicity following repeated dosing. Spleen cells isolated from sensitized mice proliferated following *in vitro* stimulation with the nitroso compound to which the animals

were originally immunized against. The proliferative response of splenocytes was concentration dependent; a significant response was detected at metabolite concentrations between 1 – 50 μM (figure 3.3 A – C). Nitroso sulfonamides are known to degrade readily in solution and are not detectable after 5 min in *in vitro* cell systems (Naisbitt et al., 2002). Their disappearance is associated with the appearance of an azoxy dimer (Naisbitt et al., 2002), irreversible modification of cysteine and cysteinyl sulfoxy acids residues on protein that presumably represent important antigenic determinants for T-cells (Naisbitt et al., 1999; Manchanda et al., 2002; Summan and Cribb, 2002; Callan et al., 2009a) and nitro SMX and SMX-NHOH, products of auto oxidation and reduction by glutathione and anti-oxidants, respectively (Cribb et al., 1991; Naisbitt et al., 1996b; Naisbitt et al., 2002; Lavergne et al., 2006b). Thus, nitroso metabolite exposure to splenocytes in the proliferation assay is low and likely to be several orders of magnitude lower than the concentration stated.

Hydroxylamine derivatives of sulfonamides, including those used in this study, are well-characterized human metabolites; approximately 2% of an oral dose of SMX is excreted as the hydroxylamine in urine (Gill et al., 1996; Mitra et al., 1996). Despite this, hydroxylamine metabolites of sulfonamides are susceptible to oxidation, reduction and self-conjugation reactions (Cribb et al., 1991; Pirmohamed et al., 1991; Naisbitt et al., 1996b; Farrell et al., 2003) and thus when exploring the T-cell stimulatory of hydroxylamine metabolites, it is important to relate proliferation of splenocyte cultures to analysis of compound distribution. SMX-NHOH, SDZ-NHOH and SP-NHOH stimulated the proliferation of splenocytes isolated from mice immunized against the respective nitroso metabolites (figure 3.2 A – C). As described in the previous chapter, each hydroxylamine degraded rapidly in culture and no more than trace amounts were detected after 1 h. Formation of the nitroso derivative was found directly in the case of the SMX-NHOH or indirectly for SDZ-NHOH and SP-NHOH by the formation of azoxy dimers compounds deriving from the conjugation of hydroxylamine and nitroso metabolites (Naisbitt et al., 2002)

In agreement with previous studies focusing on the immunogenicity of SMX (Choquet-Kastylevsky et al., 2001; Naisbitt et al., 2001), SMX, SD and SP did not stimulate splenocytes from nitroso sulfonamide immunized mice. The highly-specific nature of sulfonamide metabolite T-cell responses was significant, since it permitted analysis of the T-cell stimulatory capacity of different protein-reactive nitroso sulfonamide metabolites through cross reactivity. Splenocytes from SMX-NO, SDZ-NO and SP-NO immunized mice displayed a broad cross-reactivity profile with responses detected to all 3 nitroso and hydroxylamine sulfonamides *in vitro* (figure 3.3 A - C). Although the splenocytes from the mice immunized against the three different were broadly cross-reactive, mice immunized with SDZ-NO or SP-NO showed higher cross-reactivity than SMX-NO immunized mice. In all cases, the nitroso metabolite that the mice originally were immunized against displayed the strongest stimulatory properties. Glutathione, which prevents the spontaneous oxidation of sulfonamide hydroxylamine metabolites (Cribb et al., 1991) and the protein reactivity of nitroso sulfonamides (Cribb et al., 1996; Farrell et al., 2003) effectively inhibited drug-metabolite-specific splenocyte proliferation, indicating that protein adduct formation is needed to the stimulation of specific T-cells.

Collectively, these results imply that although the drug structure *per se* may contribute towards the fine specificity of the antigen-T-cell receptor binding interaction, the protein (or the derived peptide) covalently modified at specific cysteine residues provides the principal signal that determines whether or not a specific cell ought to respond. Interestingly, the different cross-reactive splenocytes secreted a diverse range of cytokines when they were tested ex-vivo with the different nitroso sulfonamide metabolites. IL-2 promotes the proliferation of T-cells and was found to be higher than control in all conditions tested proving evidence that the different nitroso metabolites activate splenocytes immunized with different sulfonamide nitroso metabolites. The high levels of IL-2 is in agreement with the reported proliferation measured by [^{3}H]-thymidine both method proved the activation of the different immunized splenocytes. Additionally, the cytokine screening showed that the mice immunized with the different nitroso metabolites secreted a mixed panel of

Th1/Th2 cytokines. Levels of IL-4 and IL-5 were higher than controls, whereas concentration of INF-Y, a hallmark cytokine for the Th1 response, remained closer to the control levels. IL-4, which is clinical related with allergy and atopic manifestations, was found in all the nitroso concentrations. IL-4 skew naïve T cells to the Th2 phenotype at the beginning of the polarization; therefore the levels of the cytokine increase considerably (O'Garra, 1998). Moreover, IL-5, which is associated with the mobilization, localization and activation of eosinophils, is secreted by T-cells of hypersensitivity patients and SMX-NO immunized mice (Mauri-Hellweg et al., 1995; Pichler, 2002; Hopkins et al., 2005). In agreement with these previous results, IL-5 concentration was higher than control in immunized mice re-challenge not only with SMX-NO, but also the other nitroso sulfonamide metabolites (SDZ-NO and SP-NO).

To conclude, our data showing T-cell cross-reactivity through nitroso metabolite formation in an animal model of immunogenicity provides mechanistic explanation as to why structurally related antimicrobial sulfonamides are contraindicated in so-called sulfa allergic patients.

CHAPTER 4

T-CELL ACTIVATION BY SULFONAMIDES AND THEIR METABOLITES

4.1 Introduction

Hypersensitivity reactions to SMX occur in 3-8% of patients. In patients with HIV infection, the incidence rises to up to 50% when the drug is used for prophylaxis (Bigby et al., 1986; Masters et al., 2003), which may relate to an altered co-stimulatory threshold. MHC polymorphisms are not major predisposing factors for SMX hypersensitivity (Alfirevic et al., 2009).

Drug-specific T-cells have been isolated and characterized in terms of their phenotype and function from blood and skin of hypersensitive patients, but not drug-exposed controls (Schnyder et al., 1997; Schnyder et al., 1998b; von Greyerz et al., 1999; Schnyder et al., 2000; Burkhart et al., 2001; Nassif et al., 2002b; Farrell et al., 2003; Depta et al., 2004), indicating that they play an important role in the development of tissue pathology. The nature of the drug interaction with specific T-cells has not been fully elucidated. Studies using T-cell clones obtained from hypersensitivity patients have demonstrated that the parent drug can activate specific T-cells by interacting with, and presumably cross-linking, MHC molecules and specific T-cell receptors through a series of non-covalent binding interactions (Mauri-Hellweg et al., 1995; Schnyder et al., 1997; Schnyder et al., 2000; Burkhart et al., 2001; Depta et al., 2004). This phenomenon, often referred to as the "PI (pharmacological interaction of drugs with immunological receptors) hypothesis" (Pichler, 2003), is based on several experimental observations that are incompatible with the widely accepted "hapten hypothesis" (Landsteiner and Jacobs, 1935a; Park et al., 1998), which states that formation of a drug(metabolite) protein complex is a prerequisite for immune stimulation. SMX was found to stimulate T-cells rapidly even if antigen presenting cells were fixed with glutaraldehyde, preventing in this way processing (Zanni et al., 1998a). Furthermore, antigen-presenting cells pulsed with SMX for short time-periods; followed by repeated washing steps to remove unbound material, did not stimulate T-cells (Schnyder et al., 2000). These data are difficult to reconcile with the formation of T-cell stimulatory levels of SMX metabolites in antigen presenting cells.

SMX-NO is a potent immunogen in experimental models and intracellular generation in dendritic cells is associated with co-stimulatory signalling (Choquet-Kastylevsky et al., 2001; Naisbitt et al., 2001; Naisbitt et al., 2002; Farrell et al., 2003; Hopkins et al., 2005; Sanderson et al., 2007; Cheng et al., 2008). SMX-NO activates T-lymphocytes from 90% of drug-naive volunteers after 14-35 days of *in vitro* culture. Furthermore, T-cells from SMX-NO immunized animals proliferate in the presence of SMX-NO-modified protein via a classical hapten mechanism involving antigen processing. Additionally as shown in chapter 3, other nitroso sulfonamides (SP-NO and SDZ-NO) were as immunogenic as SMX-NO, and primed splenocytes proliferate *ex-vivo* in the presence of sulfonamide metabolites.

The T-cell stimulatory capacity of SMX-NO has only been explored in a limited cohort of hypersensitive patients. Nevertheless, both skin and blood-derived lymphocytes can be stimulated with SMX-NO through a direct covalent interaction with MHC molecules, avoiding antigen presenting cells processing machinery (Schnyder et al., 2000; Burkhart et al., 2001; Farrell et al., 2003; Nassif et al., 2004). In contrast to these findings, Schnyder et al. (2000) found that the vast majority of T-cells from hypersensitive patients were stimulated with SMX and since they did not cross-react with SMX-NO, he suggested that the primary antigenic determinant may be a non-covalently associated drug MHC complex.

The aim of this chapter was to use synthetic nitroso sulfonamide metabolites to explore the T-cell stimulatory capacity of SMX and SMX metabolites and showed for the first time T-cell receptor cross reactivity through the formation of stable drug-metabolite protein conjugates that require processing.

4.2 Materials and methods

4.2.1 Materials and culture medium

[^{3}H]-methyl thymidine was obtained from Moravek (California, USA), and Lympoprep was obtained from Axis-Shield (Oslo, Norway). Human AB serum was obtained from Innovative Research (Michigan, USA). Anti-CD4 and Anti-CD8 were obtained from Serotec (Oxford, UK). Recombinant human IL-2 was obtained from Peprotech (London, UK). All other reagents, chemicals and components were supplied by Sigma-Aldrich (Gillingham, UK).
Two different types of grow medium were used for the different types of cells employed (T-cells and APC [B-LCLs]).

T-cell medium: RPMI medium, supplemented with 10% pooled heath inactivated AB serum, HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and transferrin (25 μg/mL).

APC (B-LCLs) medium: RPMI medium, supplemented with 10% fetal bovine serum (FBS), HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL).

4.2.2 Donors characteristics

Professor Werner Pichler kindly donated PBMCs from three patients with history of hypersensitivity to sulfamethoxazole. Table 4.1 shows clinical information of the patients and the results of the lymphocyte transformation test.

4.2.3 Sulfonamides and their metabolites

Nitroso and hydroxylamine metabolites from sulfamethoxazole, sulfapyridine and sulfadiazine were chemically synthesized as detailed before in chapter 2, and stock solutions of 10 mM (10% DMSO) were prepared. The identity and purity of the metabolites were assessed by NMR and LC-MS. Parent drugs were obtained from Sigma-Aldrich and stock solutions of 5 mg/mL (10% DMSO) were prepared. The final concentration of DMSO at the final incubation was lower than < 0.2%.

4.2.4 Isolation of peripheral blood mononuclear cells

PMBCs were isolated from heparanised blood of drug hypersensitivity patients by centrifugation on a density gradient of Lymphoprep. The blood (15 mL) was carefully layered on 10 mL of Lymphoprep and centrifuged (20 min; 75 g). The middle cloudy layer (containing PBMCs) formed between plasma and the Lymphoprep was carefully aspirated with a sterile pasteur pipette. The aspirated layer was then washed twice with HBSS solution to remove the excess of Lymphoprep. Finally, the cells were re-suspended in 10 mL of HBSS and the viability of the cells was assessed using a Neubauer haemo-cytometer (Sigma) under a Wilovert Microscope (Will Wertzlar, Germany) with trypan blue staining. Briefly, 40 µL of the cell suspension was mixed with trypan blue solution (10 µL; 0.2%), and 10 µL of the solution was placed on the haemo-cytometer and counted. The percentage of viability was calculated as follows.

% Viable Cells = [(total number of viable cells) / (total number of cells)] * 100
Normally the viability was higher than 95%.

The PBMCs were re-suspended in T cell culture medium, and the concentration was adjusted to 1.5 x 10^6 cells/mL.

4.2.5 Generation of EBV-transformed B-lymphoblastic cells lines

Epstein-Barr Virus transformed B-lymphoblastic cell lines (B-LCLs), used as antigen-presenting cells, were generated by incubation with EBV virus supernatant obtained from B9-58 producing cells with freshly isolated PBMCs from the hypersensitivity patients. B9-58 cells were centrifuged, and the supernatant was taken and filtered (0.2 μm) to remove any remaining cells. Then, 5 x 10^6 cells PBMCs were incubated with the B9-58 supernatant (5 mL) and cyclosporin A (1μg/mL) to suppress T-cell response against the virus. After 24 hrs, the cells were incubated (0.25 – 1 x 10^6 cells) in 24 well tissue culture plates and the medium was changed every three days with new APC medium and cyclosporin A (1 μg/mL) for three weeks. During that time the B-LCLs were grown and transferred to culture flasks when medium alone was added and changed every three days.

4.2.6 Lymphocyte transformation test

T-cells response to the drug was measured using the lymphocyte transformation test (LTT). Freshly isolated PBMCs (1.5 x 10^5 cells in 200 μL) were incubated with sulfonamides (SMX, [197 – 3150 μM] SP [201 – 3212 μM] and SDZ [200 – 3200 μM] all 50 – 800 μg/mL) and their hydroxylamine (10 – 80 μM) and nitroso (10 – 80 μM) metabolites in sterile 96-well U-bottomed tissue culture plates for 6 days (37°C; 5% CO_2). Proliferation was measured by adding [^{3}H]- thymidine (5 μCi) for the final 16 hrs of the incubation. Cells were harvested and counted as cpm on a β-liquid scintillation and luminescence counter (PerkinElmer, Life Sciences, Boston, MA). The proliferative response was calculated as S.I. (mean cpm with drug / mean cpm without drug) considering S.I. > 2 as positive.

4.2.7 Generation of T cell lines and T cell clones

T-cell lines were generated as follows. Freshly isolated PBMCs (1 x 10^6 cells; 330 μL) from the hypersensitive patients were cultured in sterile 48 well tissue

culture plates with different drugs or metabolites; SMX (200 – 800 μM / 50 – 200 μg/mL; 330 μL), SMX-NHOH (20 – 40 μM; 330 μL) and SMX-NO (20 – 40 μM; 330 μL). Half of the medium was replaced on days 6 and 9 with fresh medium containing IL-2 (60 U/mL) to maintain T-cell proliferation. On day fourteen, T-cell clones were obtained by serial dilution. Briefly, T-cells were seeded in triplicate in 96 U-bottomed well plates at different cell concentrations (0.3, 1 and 3 cells/well; in a total volumen 100μL) with allogenic irradiated (3000 rad) PBMCs (5×10^4), 5 μg/mL erythroagglutinin (PHA), and 400 U/mL of IL-2. Then, on day 5 T-cells were fed with medium supplemented with IL-2 (60 U/mL) and then feed every two-days with medium plus IL-2. Every 14 days, T-cells were restimulated with allogenic irradiated (3000 rad) PBMCs and 5 μg/mL PHA. Well-growing T cells were split into new 96 U-bottom plates and grown for further analysis.

4.2.8 Specificity of T cell clones

Well-growing T-cells generated by serial dilution were tested for specificity against the antigen used during the first stimulation to select antigen specific T-cell clones. Briefly, SMX, SMX-NHOH and SMX-NO stimulated TCC (0.5×10^5) were incubated with irradiated (4500 rad) APC (0.1×10^5) and the drug to which they were first exposed to (SMX, 787 μM or 200 μg/mL; SMX-NHOH, 80 μM; SMX-NO, 80 μM) in a final volume of 200 μL. The T-cells were incubated for 48 hr (37 °C, 5% CO_2) and [^{3}H]- thymidine was added for the last 16 hrs of the incubation. T-cell proliferation was measured as S.I. considering S.I. > 2 positive. Specific T-cells clones were selected, restimulated (0.5×10^6 allogenic PBMCs, PHA and IL-2), grown expanded in 48 well tissue culture plates.

4.2.9 Cross-reactivity of T cell clones with sulfonamides and their metabolites

T-cell clones with specificity for SMX, SMX-NHOH and SMX-NO were incubated with different sulfonamides and their metabolites to analyze the different cross-reactivity patterns. Briefly, SMX TCCs (0.5×10^5 cells) were incubated with APC

(0.1 x 10^5 cells) and different concentrations of the three sulfonamides (SMX [397 – 787 μM], SP [401 – 1606 μM] and SDZ [400 – 1600 μM]; all 100 – 400 μg/mL), SMX-NHOH (20 – 80 μM) and SMX-NO (20 – 80 μM) in a final volume of 200 μL of T-cell culture medium. In a similar way, SMX-NHOH TCCs were incubated with the different hydroxylamine metabolites (20 – 80 μM), SMX (397 – 787 μM), and SMXNO (20 – 80 μM). Finally, SMX-NO TCCs were incubated with the different nitroso metabolites (20 – 80 μM), SMX (397 – 787 μM), and SMXNHOH (20 – 80 μM). T-cell proliferation was given as cpm.

4.2.10 Phenotypic analyses of T-cell clones

T-cells were phenotyped based on the expression of surface markers CD4 and CD8. T-cells (0.1 x 10^5) were double stained with fluorescein isothiocyanate-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 (in dark; 20 min; 4°C) and then extensively washed to remove the excess of Ab. T-cells were re-suspended in 500 μL of HBSS and analyzed by flow cytometry (Coulter Epics XL software; Beckman Coulter Inc.). A minimum of 10000 events per sample were analyzed. The fluorescence intensity of T-cells with the different dyes was compared with non-stained cells.

Data was analyzed by generating a gate around the cell population based on side scatter / forward scatter as follows. Forward and side scatter were measured simultaneously and the forward threshold was raised to exclude cellular debris; cells were gather for analysis and fluorescence was measured on the FL1 channel for fluorescein isothiocyanate-labeled or FL2 for phycoerythrin-labeled cells. At least 10000 events were analyzed for each sample. All data on the forward and side scatter and the fluorescence signals for the events was stored and analysed by WinMDI 2.8 software.

4.2.11 Role of covalent binding in T-cell activation

The role of covalent binding in T-cell drug activation was demonstrated by incubating the T-cell with an excess of glutathione (1mM) and by pulsing antigen presenting cell with SMX and its metabolites.

4.2.11.1 T-cell proliferation in the presence of pulsed antigen-presenting cells

APC were pulsed with SMX (787 μM; 200 μg/mL), SMX-NHOH (80 μM) or SMX-NO (80 μM) for 1 hr to allow cellular haptenation. Cells were then extensively washed to remove unbound drug. APC were irradiated (4000 rad), adjusted to a concentration of (0.2 x 10^5 cells/mL), and incubated them in fresh medium with the different sets of TCCs (0.5 x 10^5) in a final volume of 200 μL. T-cell incubated with non-pulsed irradiated APC and the soluble form of the drug were used as a positive control. Proliferation was given as cpm.

4.2.11.2 T-cell proliferation in the presence of glutathione

T-cell clones (0.5 x 10^5 cells) were incubated with APC (0.1 x 10^5 cells) in the presence or absence of an excess of glutathione (1 mM) and different drug or metabolite antigen: SMX (787 μM; 200 μg/mL), SMX-NHOH (80 μM) and SMX-NO (80 μM). Glutathione was added prior to the addition of the different drugs or drug metabolites. Proliferation was measured by [^{3}H]-thymidine incorporation as described previously. T-cell proliferation was given as cpm

4.2.12 T-cell Proliferation in the presence of fixed antigen- presenting cells

Gluteraldehyde fixation of APC was performed to explore the role of antigen processing in the presentation of SMX and their metabolites to T-cells. APC (2.0 x 10^6 cells in 500 μL) were fixed with (0.05%) gluteraldehyde for 30 sec at room

temperature. The reaction was stopped by the addition of glycine (0.2 M) for 45 sec. Fixed APCs were extensively washed, incubated with the different set of TCC (0.5×10^6 cells) and SMX (787 μM; 200 μg/mL) or its metabolites (SMX-NHOH and SMX-NO; 80 μM). TCC incubated with non-fixed APC and the soluble drugs were used as positive control. T-cell proliferation was given as cpm

4.2.13 Estimation of the summation of individual binding energies associated with nitroso benzene, nitroso sulfamethoxazole and the drugs sulfamethoxazole, sulfadiazine and sulfapyridine

Low-lying energy conformations of nitroso benzene, SMX-NO and the drugs SMX, SD and SP were located using the MMFF94aq forcefield as implemented in Spartan08 using default settings (http://www.wavefun.com). Pharmacophores were identified using the Liquid algorithm using cut-offs of 2 Å for all bins (Tanrikulu et al., 2007). Estimation of the binding energies associated with nitroso benzene, SMX-NO SMX, SD and SP was achieved using simple summation of the typical binding values that non-covalent interactions can provide. The values associated with the following features were, hydrogen bond ~20 kJ/mol, hydrophobic interaction ~10 kJ/mol, a methyl hydrophobic interaction ~3 kJ/mol and van der Waals ~ 5 kJ/mol. Molecular structures were displayed using PyMol (http://www.pymol.org) with molecular surface rendered as dots, pharmacophores as spheres, molecule depicted in sticks with carbon in grey, hydrogen in white, nitrogen in blue and oxygen in red.

4.2.14 Statistical analysis

Statistical analysis was performed using SPSS 16.0 using a Macintosh plataform. Average data is presented as mean ± standard error of the mean. The Shapiro-Wilks test was used to determine normality. Data was not normally distributed; therefore, the Mann-Whitney test (non-parametric test) was used to compare data points, accepting $p \leq 0.05$ as significant.

4.3 Results

4.3.1 Lymphocytes from sulfamethoxazole hypersensitive patients are stimulated with structurally-related sulfonamides and sulfonamide metabolites

Lymphocytes from 3 SMX hypersensitive patients were tested for their proliferative activity against SMX, SP and SDZ and their respective hydroxylamine and nitroso metabolites.

Lymphocytes from all 3 hypersensitive patients, but not controls, were found to proliferate in the presence of SMX (787 μ M or 100 μg/ml; SI 8.7, 15.3 and 11.2), SMX-NHOH (80 μM; SI 6.1, 3.5 and 16.0) and SMX-NO (80 μM; SI 4.9, 2.1 and 24.8) (Table 4.1). Lymphocytes from each patient were also stimulated with SP (400 μM or 100 μg/ml; SI UNO 8.1, WZ 3.9 and BB 3.3), while SDZ stimulated lymphocytes from UNO (100 μg/ml; SI 12.2) and WZ (400 μ M or 100 μg/ml; SI 8.3), but not BB (SI < 2). Lymphocytes from patients UNO and BB were also stimulated with hydroxylamine and nitroso metabolites of SP and SD (SP-NHOH [40 μM; SI UNO 5.3 and BB 4.2]; SP-NO [40 μM; SI UNO 3.2 and BB 2.3]; SD-NHOH [40 μM; SI UNO 4.6 and BB 5.2]; SD-NO [40 μM; SI UNO 2.1 and BB 2.4]). Specific stimulation of lymphocytes from WZ was not detected with SP-NHOH, SP-NO, SDZ-NHOH or SDZ-NO (SI < 2).

	Patients details		Lymphocyte transformation test (S.I.)								
	Details of the reaction	Culprit drug	Parent drug			Hydroxylamine			Nitroso		
			SMX	SDZ	SP	SMX	SDZ	SP	SMX	SDZ	SP
UNO	Exhantema & malaise	SMX	8.7	12.2	8.1	6.1	4.6	5.3	4.9	2.1	3.2
WZ	DRESS & erythrodema	SMX	15.3	8.3	3.9	3.5	< 2	< 2	2.1	< 2	< 2
BB	SJS (exhantema, conjunctivitis & bullae)	SMX & SP	11.2	< 2	3.3	16.0	5.2	4.2	24.8	2.4	2.3

Table 4.1 Clinical details and results from the lymphocyte transformation test (LTT) performed to three sulfonamide hypersensitive patients. The LTT results are presented as stimulation index. (SI; count per minute in drug-treated culture / counts per minute in culture with DMSO alone). S.I ≥ 2 values are considered as positive.

4.3.2 Generation of T-cell clones following stimulation of lymphocytes with sulfamethoxazole and sulfamethoxazole metabolites

A total of 480 antigen-specific T-cell clones were generated from the hypersensitive patients. Of these, 128 were identified from SMX-stimulated lymphocytes (0, 1703 ± 3150 cpm; SMX 787 μ M or 200 μg/ml, 14418 ± 16881 cpm), and 352 from SMX metabolite stimulated lymphocytes (0, 2439 ± 3713 cpm; SMX-NHOH 80 μM, 16962 ± 17248 cpm: 0, 2122 ± 3128 cpm; SMX-NO 80 μM, 13490 ± 14324 cpm) (Table 4.2). One hundred and one well-growing CD4+ and CD8+ clones were subsequently characterized in terms of cross-reactivity, additional reactivity against structurally-related sulfonamides and mechanism of antigen presentation.

4.3.3 Antigen specificity of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell clones

To define the antigen specificity of T-cell clones, T-cells were incubated with antigen presenting cells in the continuous presence of SMX or SMX metabolites. Three patterns of reactivity were seen with clones from each hypersensitive patient (table 4.2 and figure 4.1). First, 44 metabolite-specific T-cell clones were detected. These clones were stimulated with SMX-NHOH and SMX-NO, but not the parent compound. Concentrations of SMX metabolites used (20 – 80 μM) have previously been shown to be associated with a maximum stimulatory response. Concentrations of 100 μM and above inhibited T-cell proliferation. Secondly, 14 clones were stimulated with the parent compound alone. Finally, 43 clones were defined as cross-reactive since they were stimulated to proliferate following incubation with both SMX and SMX metabolites. Figure 4.1 shows a panel of representative T-cell clones from each patient.

	Initial drug exposure	(n)	Proliferation (cpm) Control / Antigen		Clones analyzed (n)	Phenotype (%)		Reactivity (%)	
						$CD4^+$	$CD8^+$	SMX	SMX-NO
UNO	SMX	18	7703 ± 4301	30386 ± 20189	5	100	0	80	20
	SMX-NO	26	7557 ± 4584	29648 ± 21096	12	100	0	75	25
	SMX-NHOH	19	10102 ± 5946	41185 ± 26021	12	100	0	58	42
WZ	SMX	96	371 ± 319	1044 ± 9160	10	90	10	60	40
	SMX-NO	75	324 ± 314	6915 ± 9151	8	100	0	38	62
	SMX NHOH	54	511 ± 394	12168 ± 15314	15	94	6	60	40
BB	SMX	14	3123 ± 2628	21101 ± 33472	7	72	28	57	43
	SMX-NO	92	2111 ± 2033	14459 ± 11699	16	87	13	25	75
	SMX NHOH	86	1957 ± 1526	14621 ± 10588	16	87	13	25	75

Table 4.2. Phenotype and specificity of T-cell clones from sulfamethoxazole hypersensitive patients. T-cell clones (TCC) from the three hypersensitive patients (UNO, WZ and BB) were generated as described under *Materials and Methods.* The number of TCC responding to the antigen used for initial stimulation and the mean values and ± SD of the proliferative response (control and drug) are shown in columns 3 & 4. Well-growing TCC with different specificities (column 5) were phenotype. The percentage of TCC showing additional reactivity to SMX or SMX-NO is shown in column 7.

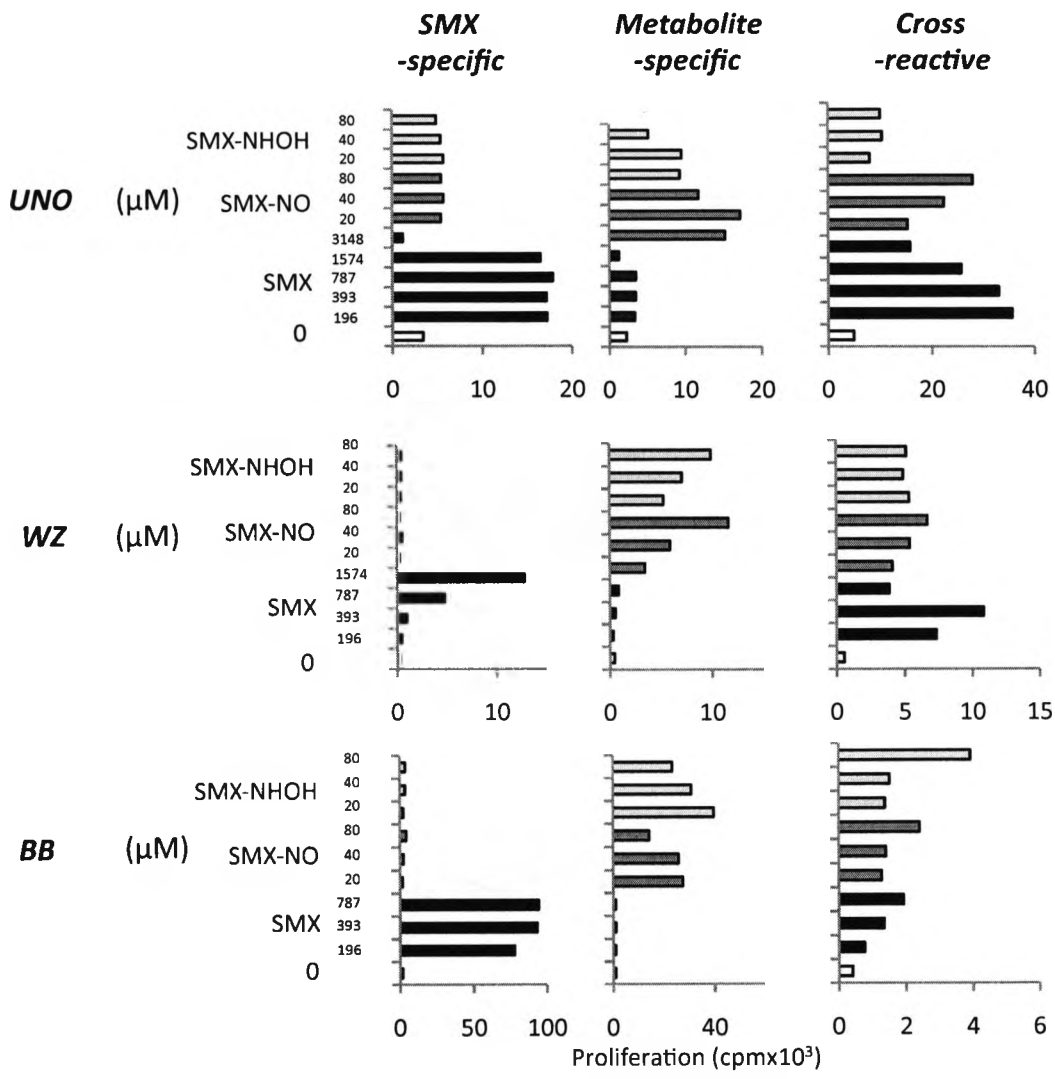


Figure 4.1. Stimulation of representative SMX-specific, SMX metabolite-specific and cross-reactive T-cell clones with SMX and SMX metabolites. To measure proliferation, T cell clones (0.5×10^5/well) were incubated with SMX, SMX-NHOH or SMX-NO and irradiated (60 Gy) autologous B-LCL (0.1×10^5) for 48 h as described under *Materials and Methods*. Results are given as mean [^{3}H]-thymidine incorporation of duplicate cultures.

4.3.4 Response of drug- and drug metabolite-specific T-cell clones towards sulfamethoxazole or sulfamethoxazole metabolite-pulsed antigen presenting cells

To investigate the relationship between protein complex formation and T-cell activation, SMX and SMX-NO were incubated with antigen presenting cells for 1 h prior to washing and the addition of pulsed antigen presenting cells to the proliferation assay as a source of antigen. Furthermore, clones were incubated with antigen presenting cells and SMX or SMX-NO in the presence or absence of glutathione, reducing protein binding (Naisbitt et al., 2001; Callan et al., 2009c). Glutathione treatment significantly reduced the proliferative response of metabolite-specific specific T-cell clones (Figure 4.2 a). However, stimulation of SMX-specific T-cell clones was not affected by the addition of glutathione.

SMX metabolite specific T-cell clones were stimulated to proliferate with both SMX-NHOH and SMX-NO pulsed antigen presenting cells. The strength of the induced proliferative response did not differ when soluble SMX metabolites and SMX metabolite-pulsed antigen presenting cells were used as a source of antigen (Figure 4.2 b). In contrast, SMX-specific clones that do not cross react with SMX metabolites were not stimulated with SMX or SMX metabolite-pulsed antigen presenting cells.

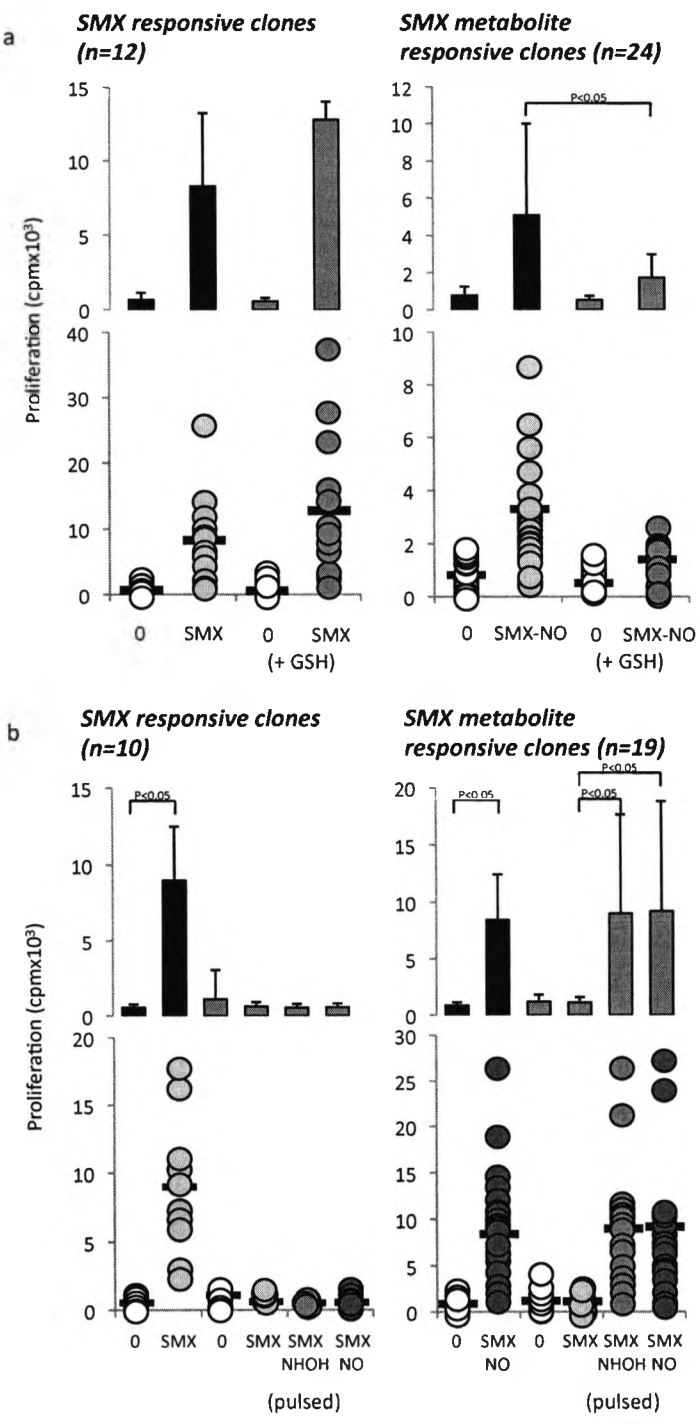


Figure 4.2. The response of SMX and SMX metabolite-specific T-cell clones towards antigen pulsed antigen presenting cells and soluble antigen in the presence and absence of glutathione. (a) Glutathione was added to incubations shown in columns 3 & 4. (b) Pulsing antigen presenting cells with or without SMX and SMX metabolites. Results are given as mean ± SD [^{3}H]-thymidine incorporation of all clones with that specificity. Circles show results of individual clones. Co-efficient of variation for individual data points was consistently less than 20%.

4.3.5 The involvement of processing in the presentation of sulfamethoxazole and sulfamethoxazole metabolites to T-cells

To explore processing requirements for the presentation of SMX and SMX metabolites to T-cells, antigen-presenting cells were fixed with glutaraldehyde. Glutaraldehyde prevents antigen processing, but not the presentation of pre-processed antigen or antigens that bind directly to MHC molecules. The majority (16/23) of SMX-metabolite-specific clones were stimulated to proliferate with SMX-NO via a classical pathway involving antigen processing (i.e., fixation of antigen presenting cells prevented SMX-NO-specific T-cell proliferation). In contrast, the response of SMX-specific T-cell clones was not altered by fixation of antigen presenting cells (Figure 4.3).

4.3.6 Response of cross-reactive T-cell clones towards antigen-pulsed and glutaraldehyde-fixed antigen presenting cells

The fixation experiments show that SMX stimulates cross-reactive clones by a direct interaction with MHC, while SMX-NO stimulates a proliferative response via both processing dependent and independent pathways (Figure 4.4 a).

In agreement with previous studies (Burkhart et al., 2001), the response of cross-reactive T-cell clones to SMX and SMX metabolites was not blocked with glutathione (Figure 4.4 b). These clones were stimulated to proliferate with SMX-NHOH and SMX-NO, but not SMX, pulsed antigen presenting cells (Figure 4.4 c).

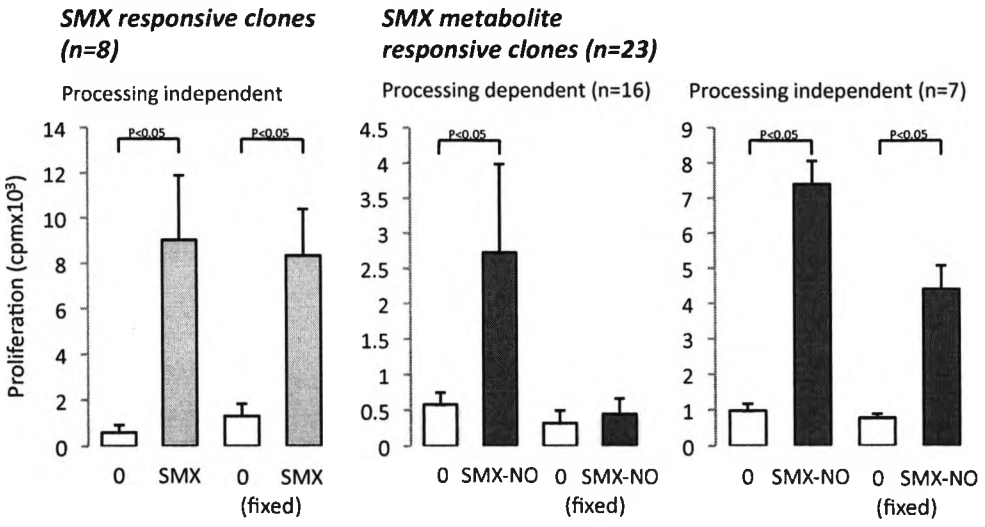


Figure 4.3. Presentation of SMX and SMX metabolites to T-cells in the context of glutaraldehyde fixed antigen-presenting cells (columns 3 and 4). Results are given as mean±SD [^{3}H]-thymidine incorporation of all clones with that specificity. Co-efficient of variation for individual clones was consistently less than 20%

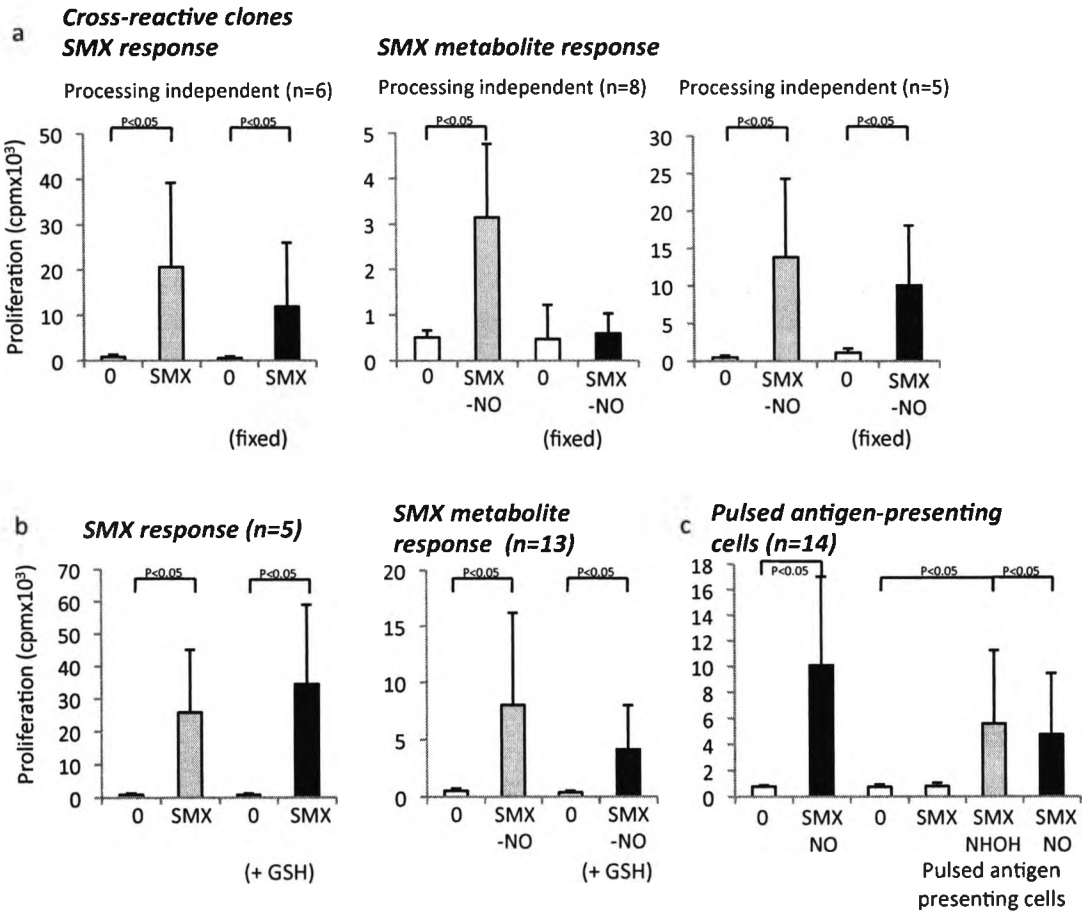


Figure 4.4. The response of cross-reactive T-cell clones towards glutaraldehyde fixed and antigen pulsed antigen presenting cells in the presence and absence of glutathione. (a) Cross-reactive T-cell clones presented the antigen in the presence of fixed and normal APC. (b) Glutathione was added to certain incubations *(columns 3 & 4)*. Antigen presenting cells were pulsed with or without SMX and SMX metabolites. Results are given as mean±SD [^{3}H]-thymidine incorporation of all clones with that specificity.

4.3.7 Additional reactivity of T-cell clones with sulfadiazine, sulfapyridine and their hydroxylamine and nitroso metabolites

The stimulation of SMX-specific T-cell clones with structurally-related sulfonamides has been described previously (von Greyerz et al., 1999; von Greyerz et al., 2001). For the purpose of this investigation 2 sulfonamides, SDZ and SP were chosen. Five out of 14 SMX-specific T-cell clones displayed additional reactivity with SDZ. Two of these clones were also stimulated with SP.

Recently established methods were used to synthesize SDZ and SP hydroxylamine and nitroso metabolites (chapter 2), these were subsequently incubated with SMX metabolite-specific T-cell clones. Greater than 50 % of SMX metabolite-specific T-cell clones were stimulated with SP and/or SD metabolites. The majority of cross-reactive clones proliferated in the presence of SMX, SP and SD metabolites. However, certain clones displayed reactivity towards SMX and SP, but not SD metabolites or SMX and SD, but not SP. Figure 4.5 shows a panel of clones with different cross-reactivity patterns and the number of clones responding in that particular way. Nitroso benzene did not stimulate SMX metabolite responsive T-cell clones (results not shown).

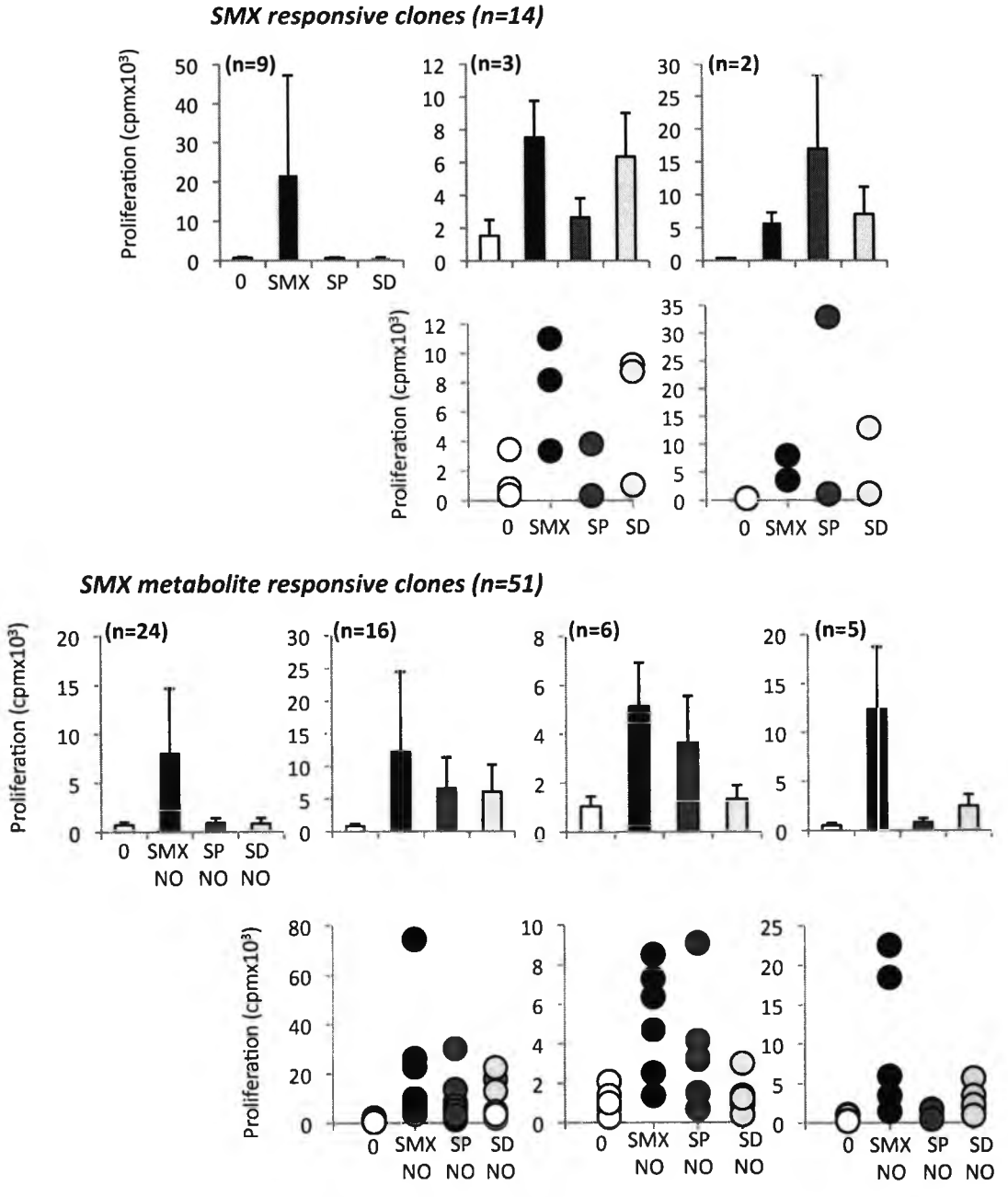


Figure 4.5. Stimulation of T cell clones by SMX, SD and SP and their hydroxylamine and nitroso metabolites. Proliferation was measured after 48 h by [^{3}H]-thymidine incorporation. Results are given as mean [^{3}H]-thymidine incorporation of representative T-cell clones. Numbers refer to the number of clones with that specificity. The coefficient of variation for individual clones was consistently less than 20%.

4.3.8 Estimation of the binding energy associated with nitroso sulfamethoxazole, nitrosobenzene and the drugs sulfamethoxazole, sulfadiazine and sulfapyridine

T-cell activation requires the molecular interaction between the peptide and MHC with a specific TCR. In the case of drug hypersensitivity, it is believed that a drug or a drug covalently bound with proteins interacts with the TCR triggering T-cell activation. Thus, pharmacophore models were calculated for a low energy conformer, which is the preferred energy state for any molecule, of SMX-NO, nitrosobenzene and the drugs SMX, SD and SP. The colored spheres indicate the chemical centers in the molecule that could provide binding energies. Three types of interactions together with their estimated binding energy are depicted in the diagram, the H bond donor (red), acceptor H bond (blue) and hydrophobic areas (green).

The summation of available binding energies for drug protein interactions and the preferred spatial arrangement of atoms in each molecule can determine a compound's potential to stimulate specific T-cell receptors (Figure 4.7).

Comparison of the preferred low energy 3-dimensional spatial arrangement of SMX-NO with SMX, SD and SP revealed that the arrangement of atoms within each compound differed and therefore cross reacting drug antigens must adopt more strained confirmations to interact with and stimulate specific T-cells. Adding together the energy values associated with each moiety provided an estimated total binding energy for each compound. The summation of available energies for SMX, SD and SP MHC – T-cell receptor binding was found to be 121, 145 and 115 kj / mole, respectively. Assuming that the nitroso group of SMX-NO associated covalently with MHC or a peptide embedded in the MHC binding cleft, the binding energy associated with the remainder of the structure is available for T-cell receptor binding. The binding energy associated with nitroso benzene, which does not stimulate SMX-NO-specific clones, was 35 kj / mole.

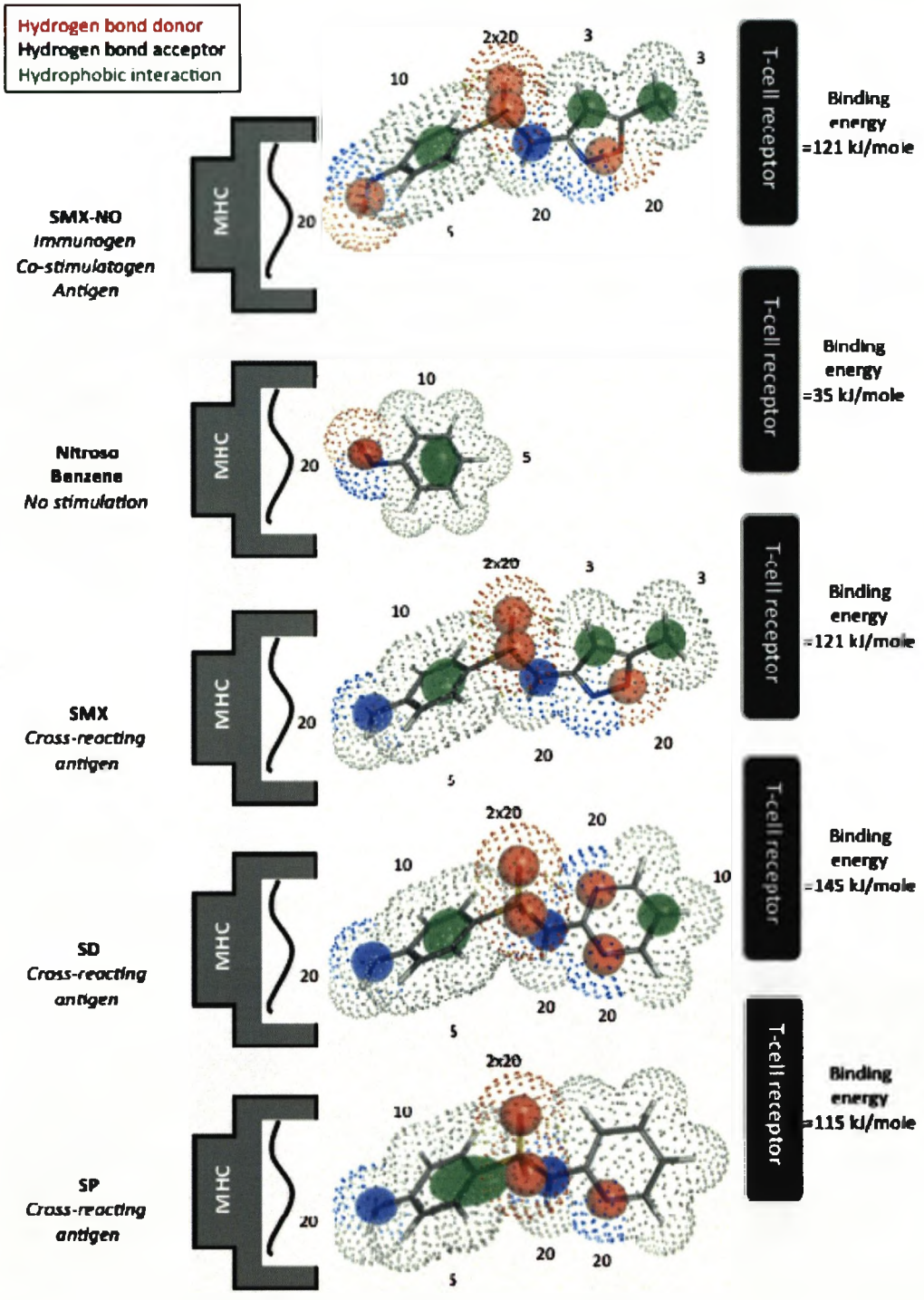


Figure 4.7. Model proposed for the interaction between different sulfonamides and TCR. The low energy conformation was calculated using the Spartan08 software. The estimation of the different binding energies was obtained by the summation of the typical binding values than non-covalent interactions can provide. The binding energy of the different sulfonamides was similar (121-141 kJ/mL). However, the spatial distribution of the molecules (dots) differs slightly between SMX and SP or SDZ.

4.4 Discussion

T-cell mediated drug hypersensitivity reactions are an important health issue, a cause of drug discontinuation, and morbidity and mortality in patients (Pirmohamed et al., 2004). Antibacterial sulfonamides are a class of drug associated with hypersensitivity reactions and represent the model most widely studied to explore the chemical basis of immune activation as well as cellular phenotypes associated with the development of different tissues pathologies (Gruchalla and Pirmohamed, 2006). T-cells responsive towards the parent compound and the protein reactive metabolite SMX-NO associated covalently with MHC or a peptides embedded within MHC have been isolated from patients with both mild and severe forms of hypersensitivity (Schnyder et al., 2000; Burkhart et al., 2001; Farrell et al., 2003; Nassif et al., 2004). Importantly, in contrast to animal models of SMX immunogenicity (Choquet-Kastylevsky et al., 2001; Naisbitt et al., 2002; Cheng et al., 2008), T-cell responses directed against peptides derived from SMX-NO protein adducts have not been well characterized using human cells.

In initial experiments, lymphocytes from 3 hypersensitive patients were found to proliferate *ex vivo* with SMX, SMX-NHOH and SMX-NO (Table 4.1). By generating T-cell clones from lymphocyte cultures stimulated with SMX and SMX metabolites for 14 days, it was possible to explore the frequency of drug and drug metabolite-specific T-cells residing in the peripheral circulation, delineate the specificity of individual clones (i.e., parent drug, drug metabolite or cross-reactive), define mechanisms of antigen presentation and explore the extent of T-cell cross-reactivity with structurally-related sulfonamides and sulfonamide metabolites. It is important to note that SMX metabolite concentrations used for T-cell stimulation (20 to 80 μM) were optimized to detect a proliferative response. Our previous collaborative studies with Pichler and colleagues (Schnyder et al., 2000; Burkhart et al., 2001), which characterized only low numbers of metabolite specific T-cells from hypersensitive patients, were performed using logarithmic increases in metabolite concentration (1, 10, 100, 1000 μM). It is now recognized that SMX-NO concentrations of 10 μM and below

are suboptimal and stimulate only a small number of T-cells, while concentrations of 100 μM have an inhibitory effect on T-cell proliferation. Thus, previous reports may have underestimated the importance of metabolite-specific T-cell responses in SMX hypersensitivity and hence the reason for revisiting this issue.

A panel of 480, primarily CD4+ T-cell clones were generated from lymphocyte cultures stimulated with SMX, SMX-NHOH or SMX-NO. The response of 78 % (373 clones) of clones was directed against SMX metabolites (either SMX-NHOH or SMX-NO) (Table 4.2). To investigate the nature of the antigenic determinant that stimulated specific T-cells in greater detail, cross-reactivity experiments were performed on 101 well-growing clones. From these investigations, three patterns of antigen-specific T-cell stimulation were discernable. First, the 44 clones were beyond doubt metabolite-specific, being stimulated with varying concentrations of SMX-NO and SMX-NHOH, but not SMX. Second, 14 clones were stimulated with the parent compound SMX alone. Finally, 43 clones were defined as cross-reactive, since proliferative responses were detected with SMX and both SMX metabolites. Our previous reports assessing T-cell responses to SMX and SMX metabolites (Schnyder et al., 2000; Burkhart et al., 2001) demonstrated that less than 2 % of clones were metabolite-specific, while 4.5 % were stimulated with SMX and SMX-metabolites.

To define mechanisms of drug (metabolite)-specific T-cell activation, clones were divided into three groups: SMX-specific, SMX metabolite-specific and cross-reactive (as described above), and T-cell responses were investigated with drug (metabolite)-pulsed antigen presenting cells, glutaraldehyde-fixed antigen presenting cells and antigen-presenting cells incubated with drug (metabolite) in the presence or absence of glutathione. Pulsing involves incubation of antigen presenting cells with SMX or SMX metabolites for 1 h, prior to repeated washing to remove unbound antigen. Pulsed antigen presenting cells were then added to the proliferation assay in the absence of soluble drug (metabolite). Protein modification with SMX-NO is known to occurs almost instantaneously and levels of binding remain constant for 2 h (Callan et al., 2009c). In contrast, detection of

protein adducts though metabolism following incubation of SMX with antigen presenting cells is time-dependent and T-cell stimulatory levels of adduct formation requires an overnight incubation (Lavergne, unpublished observation). Thus, a 1 h pulse was used in these experiments to discriminate between T-cell responses to irreversibly bound drug metabolite and weakly associated parent compound (Schnyder et al., 2000; Naisbitt et al., 2002). Glutaraldehyde fixation of antigen presenting cells inhibits antigen processing and therefore blocks the presentation of protein antigens and antigenic drug (metabolite) protein conjugates, but not the presentation of small peptides or drugs that associate directly with MHC (Zanni et al., 1998a). Glutathione, an abundant intracellular thiol, prevents SMX-NO protein adduct formation predominantly by reduction and the liberation of SMX-NHOH and SMX (Cribb et al., 1991; Naisbitt et al., 1996b; Callan et al., 2009c).

SMX-NHOH and SMX-NO pulsed antigen presenting cells were found to stimulate SMX-metabolite-specific T-cell clones. The strength of the proliferative response was similar to that seen with soluble SMX-NO (Figure 4.2 b). Glutathione pre-treatment significantly reduced the strength of the proliferative response (Figure 4.2 a). Furthermore, stimulation of 16 out of 23 clones was blocked when T-cells were incubated with SMX metabolites and fixed antigen presenting cells (Figure 4.3). Thus, processed peptides derived from SMX-NO conjugated protein stimulate the majority of metabolite-specific clones. To generate a protein adduct, the nitroso moiety of SMX-NO binds covalently to cysteine residues on protein. Following processing, linear peptides derived from the protein adduct associate with MHC molecules and in union with the SMX metabolite, provide a binding interaction with specific T-cell receptors. SMX-NO-specific T-cells were not stimulated to proliferate in the presence of nitroso benzene, the simplest aromatic nitroso compound, which binds covalently to cysteine residues on protein (Di Girolamo et al., 2009). Thus, the intermolecular forces associated with the sulfonamide group and the 5-methylisoxazole side chain of SMX (Figure 4.6; totalling 90 kj/mole), are involved in the binding interaction with specific T-cell receptors and stimulation of a T-cell response. Processing-dependent presentation of the directly protein-reactive chemical allergen 2,4-

dinitrochlorobenzene has been demonstrated previously (Pickard et al., 2007). However, data presented herein are the first to show the stimulation of large numbers of T-cells from drug hypersensitive patients via a pathway dependent on covalent binding and processing. Immunological dogma dictates that these CD4+ T-cells are stimulated by MHC class II associated peptides derived from extracellular protein (Kalish, 1995; Kalish and Askenase, 1999). In this respect, SMX-NO associates covalently with (1) cysteine sulfenic acid residues on human serum albumin generating an extracellular adduct (Callan et al., 2009c) and (2) cell surface proteins generating an adduct that is internalized and processed (Naisbitt et al., 2001; Manchanda et al., 2002; Roychowdhury et al., 2007; Sanderson et al., 2007).

Low numbers of T-cell clones with specificity for non-covalently associated SMX were identified from all three patients. Since a binding interaction is critical for pharmacological and presumably immunological activity, *in silico* modelling was also used to estimate the summation of available binding energies for SMX protein complex formation. The binding energy associated with the SMX structure (121 kj / mole), equates to the strength of a weak covalent bond or several co-ordinate linkages, which are involved that have been implicated in the stimulation of nickel-specific T-cells from patients with contact allergic dermatitis (Gamerdinger et al., 2003; Lu et al., 2003; Moulon et al., 2003). Thus, assuming MHC molecules express complimentary binding sites in the correct spatial arrangement, it is feasible chemically for SMX to interact directly with MHC and activate specific T-cells.

Several T-cell clones were stimulated with SMX and SMX metabolites (Figure 4.1; Table 4.2), presumably via different mechanisms. Discriminating between different pathways of drug stimulation with these clones was somewhat difficult for several reasons. First, as described previously (Burkhart et al., 2001), glutathione, which prevents metabolite specific responses, does not inhibit SMX metabolite-specific stimulation of cross-reactive clones. Glutathione treatment generates SMX-NHOH and SMX, themselves are stimulatory through direct MHC binding. Secondly, fixation experiments illustrated that SMX stimulates cross-

reactive clones through a direct interaction with MHC, while SMX-NO activates clones via both processing dependent and independent pathways through covalent adduct formation (Figure 4.4 a).

Collectively, these data raise the intriguing possibility that peptides derived from SMX-NO protein conjugates represent high affinity antigens for T-cells. Metabolite-specific T-cells presumably receive stimulatory signals from MHC-associated peptides and SMX-NO. In *in vitro* experiments, only a small percentage of SMX-NO binds covalently to protein. The majority is consumed by competing reduction, oxidation and self-conjugation reactions(Naisbitt et al., 2001; Naisbitt et al., 2002; Farrell et al., 2003). Furthermore, following protein binding and processing, the concentration of MHC-associated antigenic peptides will be several orders of magnitude lower than soluble SMX-NO. Thus, metabolite-specific T-cells are likely to be responsive towards incredibly low antigen concentrations. In contrast, SMX is present at mM concentrations for the duration of the experiment and will ligate and saturate all available MHC associated peptide binding sites. These conditions produce large numbers of densely coated, but weakly associated SMX MHC peptide complexes with somewhat lower affinity for SMX-NO-specific T-cell receptors. In this scenario, SMX may stimulate hapten-specific (cross-reactive) T-cells through molecular or structural mimicry of hapten peptide complexes and/or epitope spreading. It is also possible that SMX-specific clones display activity towards SMX-hapten protein complexes, but the specific high affinity hapten-peptide epitope for these clones is not generated in stimulatory concentrations in the *in vitro* cell culture system. To address this issue, in on-going experiments, MHC-restricted designer peptides with SMX-metabolite binding sites are being synthesized.

To investigate whether cross-reactivity through protein complex formation can explain the so called multiple drug hypersensitivity syndrome observed in certain hypersensitive patients, hydroxylamine and nitroso metabolites of the sulfonamides SDZ and SP were synthesized and tested for their ability to stimulate lymphocytes and T-cell clones from SMX hypersensitive patients. In initial experiments using the lymphocyte transformation test, the parent

compounds and metabolites of all 3 sulfonamides were shown to stimulate lymphocyte proliferation. The pyrimidine derivative SDZ has previously been shown to stimulate a weak response in approximately 10 % of SMX-specific T-cell clones, whereas only 1 out of 21 clones displayed activity towards the pyridine containing compound SP (von Greyerz et al., 1999; von Greyerz et al., 2001; Depta et al., 2004; Schmid et al., 2006). Our data pertaining to SMX-specific clones show largely the same effect; many clones were highly specific and stimulated with only one compound SMX. In contrast, 53% (27 / 51 clones) of SMX metabolite-specific clones displayed additional reactivity towards hydroxylamine and/or nitroso metabolites of SDZ and SP (Figure 4.6 b-c). Pharmacophore modelling of preferred low energy conformations of SMX, SDZ and SP illustrated that although the summation of intermolecular forces for each compound is similar, cross-reacting drug antigens must adopt twisted higher energy structures to mimic the binding interaction of SMX at specific T-cell receptors. These data demonstrating high levels of T-cell receptor cross-reactivity with nitroso sulfonamides displaying different sulfonamide side chains have important clinical ramifications. They show the clear potential for hypersensitivity reactions to develop to different drug structures within the same chemical class through metabolite formation and targeting of identical binding sites on protein.

In conclusion, SMX-NO was found to be a potent high affinity antigenic determinant for T-cells from hypersensitive patients. T-cell responses against SMX were detected infrequently and may be dependent on the formation of densely coated, but weakly associated SMX MHC peptide complexes that cross-react with the haptenic immunogen. SMX-NO-specific T-cells were also stimulated with different antimicrobial sulfonamide metabolites.

CHAPTER 5

THE IMMUNOGENICITY OF SMX-PROTEIN ADDUCTS GENERATED IN MICE

5.1 Introduction

In previous chapters (3 & 4) the immunogenicity and stimulatory capacity of nitroso sulfonamides was studied in animal and human models respectively. The results suggest that sulfonamide metabolites play an important role in the activation of T-cells in animals and humans. Therefore, the capacity of cells in immune system (APC) to metabolize SMX, and the stimulatory capacity of SMX-protein adducts is studied in this chapter.

The primary task of drug metabolism is to convert (Phase I) drugs into more reactive compounds that can be conjugated (Phase II) with different carrier-molecules which are finally eliminate by transporters molecules (Phase III). The balance between phase 1 and phase 2 enzymes determine the metabolic fate of a particular drug.

Even though the final outcome of drug metabolism is to generate hydrophilic and less reactive compounds that are easily eliminate, in some cases drug metabolites, formed predominantly by Phase I enzymes, become more toxic or immunogenic than the parent drug leading to un-wanted reactions (Brown et al., 2008).

Different enzymes of the P450 system are encoded by more than 57 CYP genes. However, hepatic CYP1, CYP2 and CYP3 families are responsible for the metabolism of most therapeutic drugs (Pirmohamed and Park, 2003; Brown et al., 2008). They are also found in extra-hepatic tissues such as the kidney, lungs, the intestine and the skin.

The metabolic capacity of the skin, the largest organ in the body, has not been well studied. Different CYP's and non-CYP enzymes have been found in the skin of different species (human, rats and pig) (Oesch et al., 2007). In humans, biopsy samples, reconstructed epidermal models and keratinocytes several CYP's have been detected at low levels. Additionally, mRNA coding for different CYP's have been detected in skin samples from donors or keratinocytes cultures.

In normal human epidermal keratinocytes cells (NHRK) several CYP and carrier proteins, responsible for the influx and efflux of drugs into the cells, were detected using PCR, immunoblotting and intracellular staining (Baron and Merk, 2001). High levels of CYP 1A1, 1B1, 2B6, 2E1 and 3A5 were found when compared with other extra-hepatic cells such as monocytes and lymphocytes (Merk et al., 2007; Pavek and Dvorak, 2008).

Among all the different CYP enzymes found in the skin or cultured keratinocytes, the presence of the CYP2C sub-family, responsible for many drug metabolism conversions, is our particular interest. CYP2C enzymes were found in the epidermis by immunocytochemistry analysis, and mRNA of several CYP2C has also been found in differentiated keratinocytes. Specifically, the CYP2C9 isotype, the major CYP isoform involved in oxidative metabolism of SMX, was found in 27 skin samples from male volunteers by quantitative RT-PCR studies (Yengi et al., 2003; Du et al., 2004).

NHEK incubated in a high Ca^{2+} medium metabolized SMX (Reilly et al., 2000). N-acetyl SMX was the main metabolite found in the supernatant demonstrating the metabolic capacity of this cell line. More interestingly, low amounts of the oxidative metabolite SMX-NHOH was also found during the first 10 hrs of the incubation. The enzymes responsible for this conversion have been investigated, and although CYP2C9 have been detected in the skin previously, the enzyme was not present in the NHEK cell line (Vyas et al., 2006a). In further studies using the same cell line, it was shown that Flavin Mono-oxygenase (FMO) enzymes, in particular the FMO3, could be responsible for the oxidation of sulfamethoxazole and dapsone (Vyas et al., 2006b).

Skin contains high numbers of resident dendritic cells and Langerhans cells that recognize and respond to foreign antigen, stimulating a localized immune response. Dendritic cells also express specific drug metabolizing enzymes. In a recent study (Sanderson et al., 2007) monocyte-derived dendritic cells were incubated with SMX-NO and SMX, adduct formation was detected with both cell surface and intra-cellular proteins respectively. Moreover, mRNA for MPO,

CYP1B1 and into a lesser extent CYP2C9 was detected in dendritic cells by RT-PCR obtained from healthy volunteers. Incubation with ABT, an inhibitor for of hemooxygenases, inhibited completely the activity of the CYPs and MPO enzymes and SMX-specific dendritic cell signaling. Therefore, the authors concluded that MPO expressed in antigen presenting cells could be responsible for the oxidation of sulfamethoxazole.

The aim of this chapter was to demonstrate the oxidative capacity of cells obtained from the spleen of naïve Balb/c mice using an anti-SMX antibody to detect protein adducts. Furthermore, the T-cell stimulating capacity of SMX-derived protein adducts was explored in an established mouse model of SMX immunogenicity.

5.2 Materials and methods

5.2.1 Materials and culture medium

[^{3}H]-methyl thymidine was obtained from Moravek (California, USA), and Lympoprep was obtained from Axis-Shield (Oslo, Norway). Human AB serum was obtained from Innovative Research (Michigan, USA). Anti-CD4 and Anti-CD8 were obtained from Serotec (Oxford, UK). Recombinant human IL-2 was obtained from Peprotech (London, UK). All other reagents, chemicals and components were supplied by Sigma-Aldrich (Gillingham, UK). Culture medium was prepared according with 3.2.1

5.2.1 Mice immunization protocols

Female Balb/c (6-9 weeks; 20-30 g) mice were purchased from Charles River U.K. Ltd. (Kent U.K.). Mice were administered with either 1 mg/kg or 5 mg/kg of SMX-NO in DMSO via *i.p.* injection four times weekly for two weeks to induce an immune response. On completion of this dosing regime, animals were killed and the spleen was removed for analysis of T-cell proliferation.

5.2.2 Isolation and viability of Balb/c mice splenocytes

Spleens were obtained under aseptic conditions, homogenized and the viability was assessed as detailed in previously (3.2.4). Splenocytes were finally adjusted to a concentration of 3 x 10^6 cells/mL and re-suspended in culture medium. (RPMI 1640 supplemented with fetal bovine heat-inactivated serum [10%], HEPES [25 mM], L-glutamine [2mM], streptomycin [100 µg/ml] and penicillin [100 U.mL^{-1}]). A similar procedure was used to isolate splenocytes from naïve Balb/c mice (non- immunized).

5.2.3 Incubation of naïve splenocytes with sulfamethoxazole and nitroso sulfamethoxazole.

Naïve splenocytes were obtained as described previously 5.2.2. Cells were re-suspended in culture medium with different concentrations of SMX (100 – 2000 μM) and SMX-NO (10 - 100 μM) for 16 hr to allow adducts formation (37°C; 5% CO_2). After 16 hr, the splenocytes were extensively washed with HBSS solution to remove unbound drug. Pulsed splenocytes were counted, adjusted to a concentration of (1x 10^6 cells/mL), and irradiated (3000 rad).

5.2.4 Proliferation assay

A proliferative response was measured by incubating splenocytes (1.5 x 10^5; total volume 200 μL) in a sterile U-bottom 96-well tissue culture plate (Falcon) with a range of concentrations of SMX (100 – 2000 μM) and SMX-NO (10 – 100 μM) for three days (37°C; 5% CO_2). Proliferation was measured by ^{3}[H]-thymidine (0.5 μCi/well) incorporation during the last 16 hrs of the incubation. Cells were harvested and measured as counts per minute (cpm) on a β-liquid scintillation and luminescence counter (Perkin Elmer, Life Sciences, Boston, MA).

5.2.5 Proliferation assay using naïve drug-treated syngeneic splenocytes as source of antigen

Splenocytes (1.5 x 10^5; total volume 200 μL) obtained from immunized mice were incubated in a sterile U-bottom 96-well tissue culture plate with naïve pulsed splenocytes (0.5 x 10^5; total volume 200 μL) in a ratio 3:1 respectively. Proliferation was measured by ^{3}[H]-thymidine (0.5 μCi/well) incorporation during the last 16 hrs of the incubation and responses were represented as count per minute (cpm). See scheme 5.1 for more details.

5.2.6 Determination of protein quantity

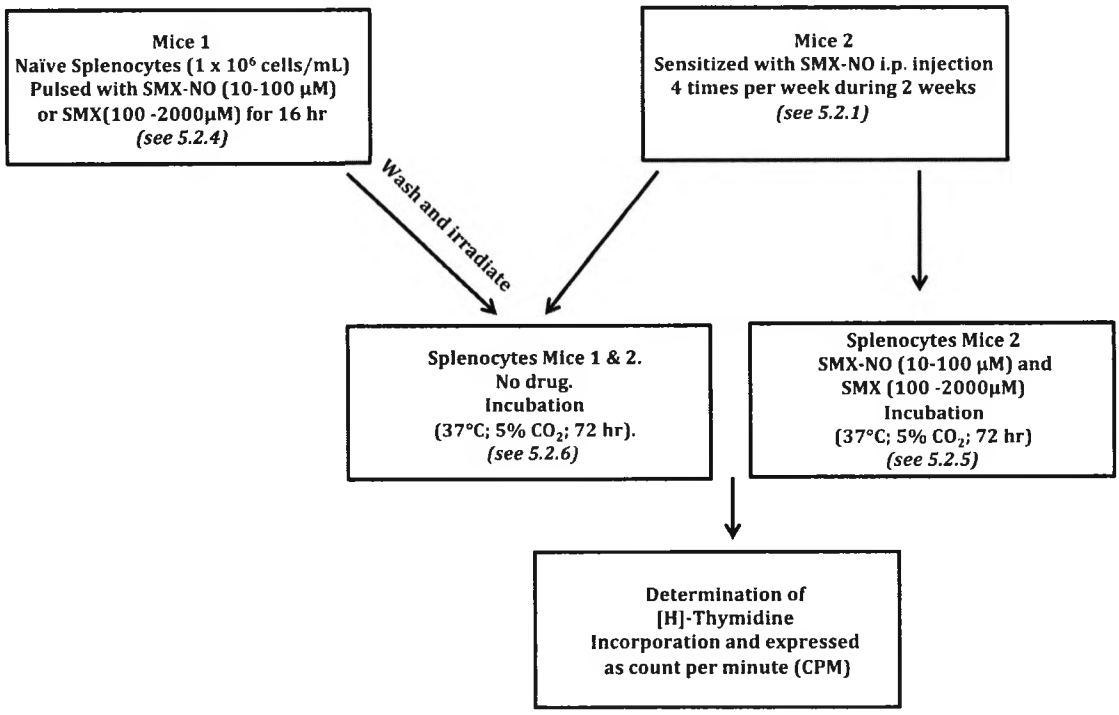
Protein levels in samples were determined by the method of Bradford (1976). A standard curve of bovine serum albumin (BSA; 0.25-2000 μg/mL) was prepared using a stock solution of 2 mg/mL BSA in dH_2O. Samples were diluted as required and 10 μL added to each well of a flat-bottomed 96-well plate. Bradford reagent was diluted 1:5 with dH_2O and 200 μL added to each well immediately prior to spectrophotometric analysis (MRX microplate reader running Revelation version 3.04 software, Dynex Technologies, Billinghurst, W. Sussex) at 570 nm. Blank wells were subtracted from all values, and linear standard curves were derived to allow quantification of sample protein concentrations.

5.2.7 ELISA for determination of sulfamethoxazole irreversible binding to cellular protein

Splenocytes were incubated with SMX-NO (1 – 250 μM) and SMX (100 – 2000 μM) for 16 h for analysis of protein adduct formation. In subsequent experiments the time dependence of adducts formation was determined by incubation of splenocytes with SMX and SMX-NO (0.1, 2, 6 and 16 hr). Quantification of adducts formation was performed in wells coated with 100 μL (250 μg/ml) of cell lysate (4°C; 16 hr). After extensive washing with PBS-Tween (0.001%), the plate was blocked with milk (2.5%). Samples were then incubated at 4°C with a rabbit anti-SMX antiserum (1:2000 in PBS-Tween) for 16 h. Plates were washed extensively and incubated with goat anti-rabbit alkaline phosphatase-linked antibody (1:1000) at room temperature for 2 hours. Then, the plates were washed and incubated for 30 min with alkaline phosphatase yellow liquid substrate in the dark at room temperature. Finally, the plate was read at 450 nm using a microplate reader (MRX; Dynatech Laboratories Inc. Chantilly, VA, USA). Results are expressed as "ΔOD = sample OD – vehicle OD".

5.2.8 Statistical analysis

Statistical analysis was performed using SPSS 16.0 using a Macintosh plataform. Average data is presented as mean ± standard error of the mean. The Shapiro-Wilks test was used to determine normality. Data was not normally distributed; therefore, the Mann-Whitney test (non-parametric test) was used to compare data points, accepting $p \leq 0.05$ as significant.



Scheme 5.1 The methods and procedures employed during the proliferation assay.

5.3 Results

5.3.1 Splenocytes from nitroso sulfamethoxazole immunized mice proliferate in the presence of nitroso sulfamethoxazole

Maximal proliferative response of splenocytes was detected with SMX-NO at 20 μM (0 μM, 413 ± 298; 20 μM, 6691 ± 2295 $p < 0.05$; Figure 5.1). Proliferation was also seen at the lowest concentration tested (10 μM), but concentrations above 50 μM a decrease in the proliferative response was observed.
Sulfamethoxazole did not induce a proliferative response at any of the concentrations tested (100 – 2000 μM) in splenocytes obtained from SMX-NO immunized mice as previous results have shown (Naisbitt et al., 2001; Farrell et al., 2003).

5.3.2 Nitroso sulfamethoxazole and sulfamethoxazole form detectable adducts with splenocyte protein

SMX-protein adducts were measured by ELISA using an anti-SMX antibody. Adduct formation was assessed at a ranging concentration's of SMX-NO (1 – 250 μM) or SMX (100 – 2000 μM) incubated for 16 hr with splenocytes from naïve mice. Adduct formation with SMX-NO was detected at either low (1 μM) or high concentrations of SMX-NO (250 μM) (Figure 5.2 B). During SMX incubation, SMX-protein adducts were detected at SMX concentrations above 200 μM and increased directly with higher concentration of SMX (figure 5.2 A). In contrast, during SMX-NO incubations the levels of SMX-protein adducts remained constant at concentrations above 5 μM.

In order to study the role that drug metabolism plays in the formation of SMX-protein adducts, SMX-protein binding was measured at different time points (0.1 – 16 h). Different levels of SMX-protein adducts were detected at different time points during SMX incubation, but the levels remained constant with time during SMX-NO incubation. SMX-proteins adducts were detectable at the earliest time (0.1 h) with SMX (only at 1000 μM) and SMX-NO (all concentrations)(figure 5.2 C & D).

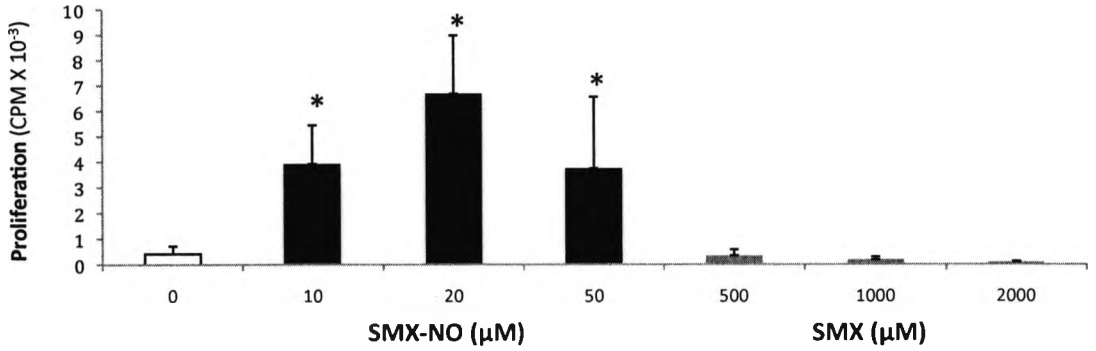


Figure 5.1 Proliferative response of splenocytes from Balb/c strain mice immunized with SMX-NO and cultured with SMX-NO (black bars) or SMX (gray bars). Proliferation was mesaured by the [^{3}H]-thymidine incorporation. Results are given as mean cpm by triplicate cultures. Statistical analysis compares drug treated splenocytes with incubations containing DMSO alone (n = 3; * $P < 0.05$)

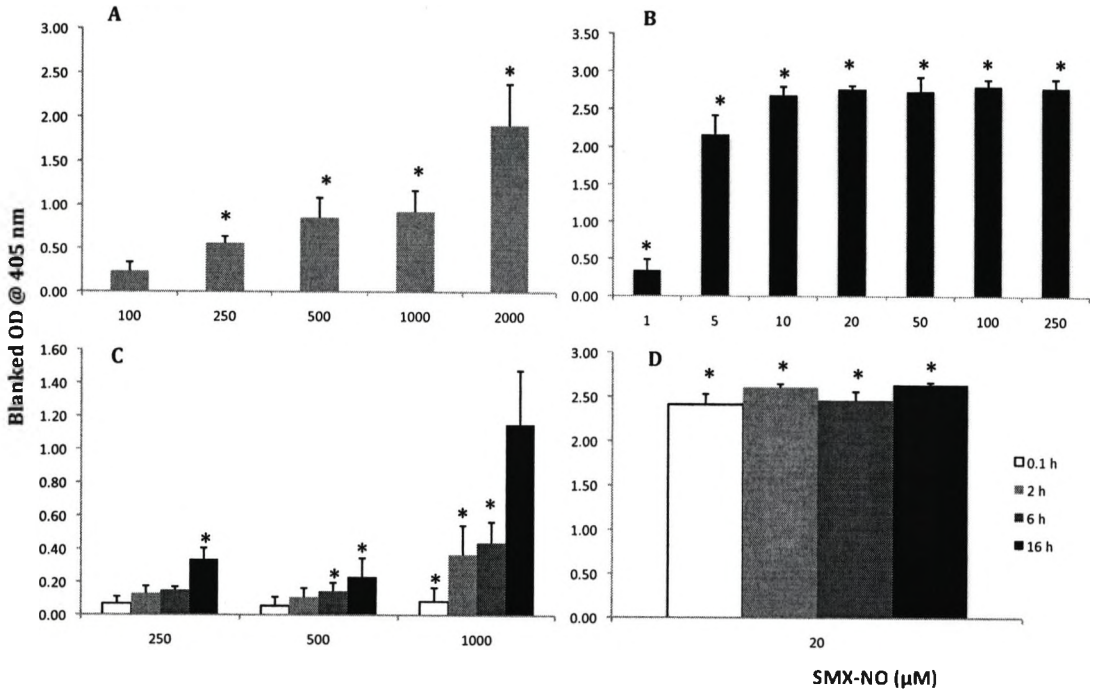


Figure 5.2 Hapten – protein complex formation in splenocytes exposed to SMX (A) or SMX-NO (B) for 16 h or at different time points (0.1, 2, 6, 16 h) with SMX (C) or SMX-NO (D) was measured by ELISA using an anti-SMX-antibody. Statistical analysis compares drug-treated splenocytes with incubations containing DMSO alone (n = 3; * $P < 0.05$).

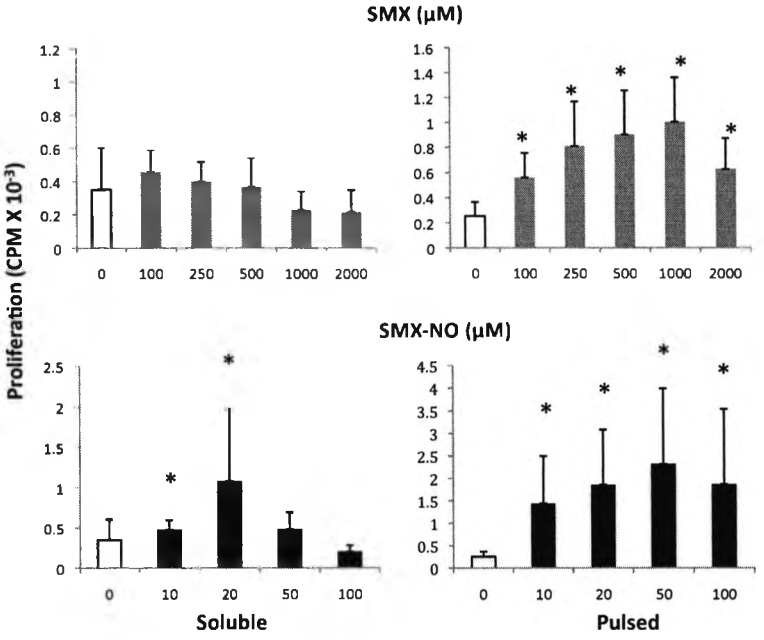
5.3.3 Nitroso sulfamethoxazole immunized splenocytes proliferate in the presence of sulfamethoxazole protein adducts

Splenocytes immunized with SMX-NO proliferated *in vitro* with SMX-NO, but not to SMX. To investigate the functional consequences of SMX adduct formation in spleen cells, SMX was incubated with cells from naïve mice for 16 hr. The newly formed SMX-cellular protein adducts were then used as an antigen to address whether SMX-NO generated intra-cellularly through metabolism could stimulate specific SMX-NO splenocytes in vitro.

In initial experiments, naïve splenocytes were incubated with increasing concentrations of SMX-NO (10 – 100 μM) for 16 hr. This procedure was followed by intensive washing steps to remove un-bound drug. The treated cells were then irradiated and incubated with SMX-NO primed splenocytes to measure proliferation. Primed splenocytes proliferated in a dose-dependent manner in the presence of SMX-NO-pulsed naïve splenocytes, and at concentrations that were toxic with the soluble drug metabolite. Proliferation was observed at a concentration as low as 10 μM (0 μM, 253 ± 109; 10 μM, 1435 ± 1058; $p < 0.05$), and maximal proliferation responses were observed at 50 μM (0 μM, 253 ± 109; 50 μM, 2322 ± 1675; $p < 0.05$; Figure 5.3 A).

Interestingly, naïve splenocytes incubated during the same period of time, but with different concentrations of SMX (100 – 2000 μM) induced proliferation in SMX-NO splenocytes in a dose-dependent manner. Maximal proliferation was detected at 1000 μM (0 μM, 253 ± 109; 1000 μM, 1003 ± 357) (Figure 5.3 A); similar concentrations of the soluble drug did not induce proliferation in SMX-NO-specific splenocytes directly (Figure 5.3 A).

A



B

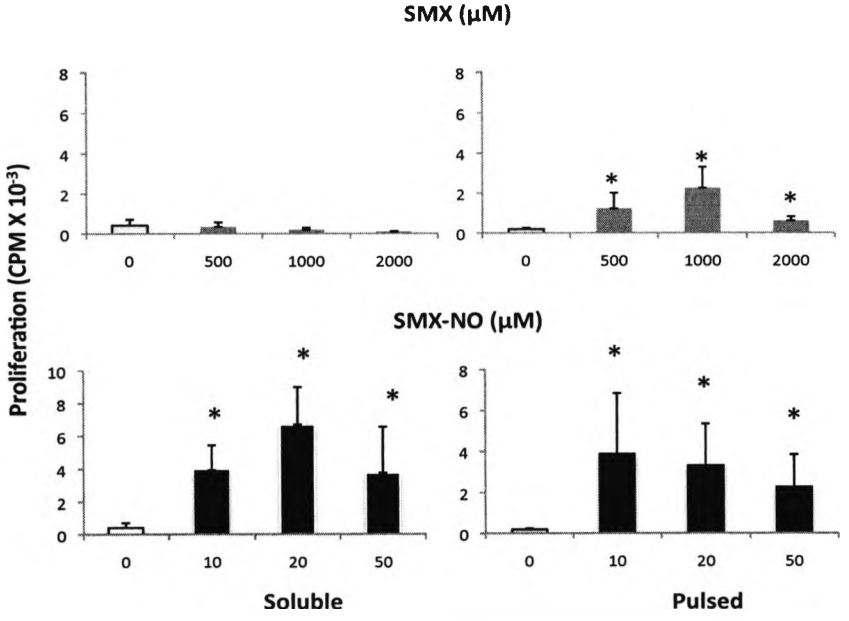


Figure 5.3 Proliferative response of splenocytes from Balb-c strain mice immunized with of SMX-NO (1 mg/kg, A or 5 m/kg, B) in the presence of soluble SMX (Left gray bars graph) or SMX-NO (Left black bars graph), or in the presence of naïve synergic mice pulsed with SMX (Right gray bars graph) or SMX-NO (Right black bars graph) for 16 hrs. Proliferation was mesaured by the incorporation of [^{3}H]-thymidine. Results are given as mean cpm by triplicate. Statistical analysis compares drug treated splenocytes with incubations containing DMSO alone (n = 3; * $P < 0.05$).

5.4 Discussion

Sulfamethoxazole is oxidized in humans by the CYP 2C9 and myeloperoxidase (MPO) enzymes to an unstable hydroxylamine metabolite that further auto-oxidize to the highly reactive nitroso metabolite. SMX-NO binds covalently with cysteine residues in proteins (Uetrecht et al., 1988; Cribb et al., 1990; Cribb et al., 1991; Cribb and Spielberg, 1992; Cribb et al., 1995; Naisbitt et al., 1999; Cheng et al., 2008). Additionally, it has been demonstrated using different animals models that SMX-NO is more immunogenic than SMX-NHOH or SMX (Naisbitt et al., 2001; Farrell et al., 2003). Splenocytes from mice immunized with SMX did not respond *in-vitro* in the presence of SMX or its metabolites. In contrast, splenocytes from animals immunized with SMX-NO (1 mg/kg) proliferate when incubated with SMX-NO or SMX-NHOH but not SMX. Importantly as shown in chapter 3, SP-NO and SDZ-NO were as immunogenic as SMX-NO. When sulfamethoxazole (10 - 250 mg/kg) was administered by a single i.p. dose in rats together with CFA (complete Freunds adjuvant), which is known to increase and provide signals two and three during T-cell priming, the splenocytes were found to proliferate *ex-vivo* in the presence of SMX-NO. These data suggested that SMX-NO is formed in mice at levels sufficient to generate antigenic signals; however, the quantity formed is insufficient to generate the additional co-stimulatory signals needed to induce a primary T-cell response.

Interestingly, different results have been found in humans. PBMCs from sulfamethoxazole hypersensitive patients proliferate not only in the presence of the SMX metabolites, but also with the parent drug (Schnyder et al., 2000). It is important to clarify that in these cases the patients have already been sensitized and the nature of the first antigen responsible for the reaction is not yet known. Further studies have shown that PBMCs obtained from patients who have never been exposed to sulfamethoxazole could be stimulated to develop a proliferative response against SMX and SMX-NO. The results demonstrated that SMX-NO induced a proliferative response or cytotoxic activity when incubated for 4 weeks in 9 of the 10 of patients tested, whereas SMX induced response in 3 out of 10 of the same patients (Engler et al., 2004). All this evidence suggests that the

reactive metabolite plays a key role in the initiation of the hypersensitivity response caused by sulfonamides.

Metabolism is vital for the formation of these reactive metabolites. Current evidence suggested that sulfamethoxazole oxidation occurs not only in the liver, but also in different organs. This may explain why the skin is one of the principal targets in drug hypersensitivity reactions. Different types of extra hepatic cells including neutrophils, keratinocytes or monocyte- derived antigen-presenting cells in humans have been found to oxidize SMX. Consequently, the newly formed reactive metabolites outside the liver could bind with proteins forming new antigenic structures that could be responsible for the activation of the immune system.

The aim of this chapter was to explore whether splenocytes from naïve mice metabolize SMX *in vitro* to a metabolite with the capacity to form adducts with proteins and stimulate primed splenocytes from SMX-NO immunized mice.

Previous studies indicate that SMX-NO administrated (10 mg/kg i.p) into rats undergoes an extensive reduction process to form the parent amine (18.4 ± 4.6%), N_4-acetyl SMX (27.6 ± 6.7%) and a hydroxylamine (13.6 ± 5.5%) (Gill et al., 1996; Gill et al., 1997). In our dosing regime, SMX-NO has been shown to form adducts in keratinocytes and isolated splenocytes from SMX-NO immunized mice proliferate vigorously in response to SMX-NO in vitro.

Our results using an anti-SMX antibody and an established ELISA method indicates that SMX-NO was capable of forming adducts with protein rapidly at low concentrations. Additionally, adduct formation was detected in splenocytes incubated with SMX demonstrating that these cells metabolize SMX to more reactive product. Adduct formation with SMX was dose- and time-dependent; increasing concentrations and time of incubation was associated with higher levels of adduct formation. These data permitted us to investigate whether intracellular adduct formation in antigen-presenting cells is associated with stimulation of SMX-NO-specific T-cells.

Several extra-hepatic cells have been shown to express enzymes involved in metabolic activation of sulfamethoxazole. For example, neutrophils have been shown to oxidize SMX to its hydroxylamine metabolite, a reaction catalyzed by MPO enzymes. Moreover, keratinocytes metabolize SMX to SMX-NHOH possibly by the enzymatic action of cytochrome P450. However, mRNA for CYP2C9, which is known to be the primary enzyme involved in SMX oxidation, was not detected in the different cell lines tested (Reilly et al., 2000; Vyas et al., 2006a). More recently, monocyte-derived antigen presenting cells were found to oxidize SMX by different enzymes such as MPO, CYP2C9 and CYP2C8 all of them involved in the oxidation of primary amines (Sanderson et al., 2007).
Our results suggested that this metabolic activation is not exclusively performed only by hepatic cells in the Balb/c mice, but also by other cells outside the liver. It is possible that antigen-presenting cells located in the spleen together with macrophages and leukocytes, similar to other cells, have P450 or MPO enzymes that could oxidize sulfamethozaxole. Nevertheless, more research such as enzyme inhibition assays or RT-PCR techniques would help to elucidate which enzymes or particular isoforms of the P450 enzyme family is involved in the SMX oxidation by Balb/c splenocytes.

It has been demonstrated that sulfamethoxazole metabolites are more toxic than sulfamethoxazole itself (Rieder et al., 1995; Hess et al., 1999; Naisbitt et al., 2002). Apoptosis in PBMCs was seen with SMX-NHOH at concentrations of 100 μM and above. Moreover, similar concentrations of SMX-NO (100 – 200 μM) also induce cell death (Naisbitt et al., 2002). Interestingly, the level of haptenation in this study was correlated with cell death. It was found that although maximal cell death was correlated with an increase in haptenation, SMX-NO membrane adducts were also seen at non-toxic concentrations (10-50 μM) indicating that SMX-NO adduct formation can occur in viable cells.

Protein adducts were seen at both non-toxic and toxic concentrations. At non-toxic concentrations of SMX-NO (10 – 50 μM), the SMX-protein adduct could be internalized, processed and further presented to provide the immunological signal. On the other hand, at toxic concentrations of SMX-NO (100 μM), the

apoptotic or necrotic cell could be taken up by adjacent cells, that would process and further present the antigen to SMX-NO primed splenocytes.

In our experiments, the reactive metabolite was formed independently by the metabolic activity of naïve splenocytes, and after being covalently bound was presented to specific SMX-NO splenocytes obtained by previous immunization procedures.

Sulfamethoxazole incubated for 16 hr with naïve splenocytes resulted in the formation of reactive metabolite in sufficient quantity to stimulate splenocytes from sensitized mice. Proliferation was dose-dependent with SMX levels of adduct formation. The threshold concentration of drug metabolite for T-cell activation is not known, but it is believed to be low. T cells are extremely sensitive and can be activated in the presence of just one MHC-peptide ligand; however it is estimated that at least 30 ligands are required for full activation (Huppa and Davis, 2003). In this respect, metabolites formed by metabolic activation of the parent drug in splenocytes are not detected using currently available analytic techniques, but could be sufficient for splenocyte proliferation.

In conclusion, data presented in this chapter demonstrate that SMX is metabolized to a reactive intermediate in antigen-presenting cells within splenocytes. The newly formed protein adducts stimulate splenocytes from SMX-NO immunized mice, possibly via a classical pathway involving processing and presentation of derived peptides in the context of MHC molecules.

CHAPTER 6

FINAL DISCUSSION

Drugs have had an indisputably beneficial effect for humanity. Diseases that decades ago were deadly can now be effectively treated or cured increasing the quality and length of life. Nevertheless, the use of drugs has always been associated with adverse effects, and therefore exploration of mechanisms of drug toxicity is an important field of pharmacological research.

Generally, adverse drug reactions have been divided into several groups of which type A or augmented accounts for almost 80 - 90% of the total. Type B or bizarre reactions (10 – 20 % of the total) are also of significance, because the mechanisms involved are not fully understood, and they are associated with high levels of morbidity and mortality in patients. Many type B reactions are believed to be immune-mediated since specific T-cells and/or antibodies for the implicated drug have been identified in patients. Furthermore, the delayed onset of the symptoms suggests that there is an initiation period where the immune system is responding to a new antigenic determinant. Human exposure to several drug classes including carbamazepine, penicillins, sulfonamides or anti viral drugs is associated with a high incidence of type B reactions. Fortunately, in the majority of cases the symptoms are mild and reversible, but in some cases the outcome can be life treating.

Sulfonamide hypersensitivity has been widely studied for several reasons. Firstly, they have been linked with a high occurrence of hypersensitivity reactions (Gruchalla and Pirmohamed, 2006; Brackett, 2007). Secondly, their metabolites, which seem to play an important role in hypersensitivity reactions, are available (Naisbitt et al., 1996a), and their reactivity towards cysteine residues which allows the formation of adducts has been fully characterized (Callan et al., 2009b). Although several aspects of the activation of T-cells by SMX are now understood, there are still certain issues that require further investigation or revision. Specifically, the aim of this thesis was to focus on the

ability of hydroxylamine and the nitroso metabolites to stimulate T-cells, which may have been underestimated because T-cells from hypersensitive patients in all cases proliferate in the presence of SMX metabolites, and the importance of metabolism of sulfonamides in the T-cell receptor cross-reactivity, which has been observed in several patients. Patterns of drug metabolite-specific T-cell activation have not been investigated previously due to the lack of synthetic sulfonamide metabolites other than SMX.

In the first experimental section (chapter 2), hydroxylamine and nitroso metabolites of three different sulfonamides (SMX, SP and SDZ) were synthesized. The synthesis followed a three-step procedure. Firstly, the nitro sulfonamide compounds were synthesized by nucleophilic addition of the different side chains (3-amino 5-methyl isoxazole, 4-amino pyridine and 4-amino pyrimidine) to 4-nitro-benzene sulfonyl chloride, a commonly used method for the synthesis of aromatic nitro compounds. Secondly, the nitro derivatives were reduced by catalytic transfer hydrogenation, used in the reduction of nitro compounds, to form the sulfonamide hydroxylamines derivatives. Thirdly, the hydroxylamine metabolites were oxidized with iron (III) chloride to form the nitroso sulfonamide metabolites.

This approach has been adopted previously for the synthesis of nitroso SMX (Naisbitt et al., 1996a). However, the synthesis of SP & SDZ metabolites was more challenging due to the poor solubility of the nitro compounds. Using a modification of a previously described procedure (Entwistle et al., 1977; Ayyangar et al., 1981; Ayyangar et al., 1984) developed to assist the synthesis of compounds with low solubility, nitro SP and SDZ were successfully reduced to their respective hydroxylamine. For the nitro reduction, Raney-Nickel and hydrazine were used as a catalyst and hydrogen donor respectively in a solvent system formed of di-chloroethane and ethanol. This procedure allowed a mild reduction in which the hydroxylamine intermediate was isolated without over-reduction and formation of the primary amine.

The sulfonamide hydroxylamine derivatives were then oxidized to the corresponding nitroso compound using iron (III) chloride and then used to explore the antigenic determinant that stimulates T-cells from hypersensitive patients and animal models of sulfonamide immunogenicity. Furthermore, stability studies were performed by LC-MS to determine the fate of hydroxylamine and nitroso metabolites in culture. The results obtained demonstrated that the hydroxylamine but particularly the nitroso sulfonamide metabolites were detectable in solution for a period of time of less than 4 hr or just minutes respectively. Additionally, the formation of dimers such as azoxy was observed during the incubation of the sulfonamide hydroxylamine and nitroso metabolites demonstrating the high reactivity of these compounds.

Most drugs associated with a high incidence of hypersensitivity reactions have been shown to form protein-reactive metabolites in humans, and because of this causal relationship, reactive metabolites are thought to play a crucial role in the initiation of drug hypersensitivity reactions (Park et al., 1987; Park et al., 2001). It is possible that these compounds could bind with self-proteins breaking tolerance and initiating a response against the self-proteins (Hapten hypothesis) or damage the integrity of the cell (Danger hypothesis) leading to the release of danger signals that promote the activation of the immune system (Pirmohamed et al., 2002; Uetrecht, 2007).

Several studies have shown that reactive metabolites of sulfonamides are more toxic that the parent drug (Rieder et al., 1988; Rieder et al., 1995), have the ability to form adducts with cysteine residues in proteins (Cribb et al., 1996; Naisbitt et al., 1999; Callan et al., 2009b) and these adducts are at least three orders of magnitude more immunogenic that the parent compound (Manchanda et al., 2002; Cheng et al., 2008). Therefore, it is possible that structurally similar sulfonamide metabolites induced T-cell activation through binding to similar proteins. Recognition of similar structural motifs found may result in cross-reactivity at immunological receptors.

In chapter three, nitroso metabolites from the three different sulfonamides were shown to prime T-cells within the spleen of two different mouse strains. An antigen-specific cellular immune response was shown by analysis of *ex vivo* splenocyte proliferation following incubation of the cells with the different sulfonamide metabolites. These data are in accordance with previous studies focusing specifically on the nitroso metabolite of SMX, where it was found that the nitroso compound, but not SMX, primed T-cells in naïve animals of different species (Farrell et al., 2003).

Our studies expand the current knowledge in SMX-NO immunogenicity. Similar to what has been described with SMX-NO, SP-NO- and SDZ-NO-primed splenocytes responded strongly *ex-vivo* at non-toxic concentrations of the nitroso metabolites (1 - 25 μM) used during immunization. Interestingly, the hydroxylamine metabolite, which undergoes auto-oxidation in culture (Cribb and Spielberg, 1990; Naisbitt et al., 1996a), also induced a T-cell proliferative response. The parent sulfonamides did not induce a response at any of the concentrations tested. These results clearly show that the reactive nitroso metabolites are involved in the interaction with specific immunological receptors leading to the activation of specific T-cells. These data were confirmed by pre-incubating splenocytes with glutathione (GSH), preventing the spontaneous auto-oxidation of hydroxylamine metabolites (Cribb et al., 1991) and the protein reactivity of nitroso sulfonamides (Naisbitt et al., 1999; Lavergne et al., 2006a). GSH incubation effectively inhibited drug-metabolite-specific splenocyte proliferation to control levels during nitroso or hydroxylamine exposure.

Splenocytes immunized with nitroso metabolites proliferate in the presence of the different nitroso and hydroxylamine sulfonamides to which the cells had never been exposed. It is possible that the different nitroso metabolites bind covalently with the same cysteine residues on proteins, forming similar antigenic determinants. Moreover, protein adduct formation might modify the protein structure, generating neo-antigens and breaking tolerance by exposing epitopes that were previously hidden. It is also possible that the side chain of the

sulfonamide nitroso metabolites once bound with proteins interacts directly with the T-cell receptor that showed certain flexibility and can recognize the different sulfonamides.

T-cells secrete cytokines after antigen challenge, activating different immune mechanisms of defense. They can be classified according to the cytokines secreted in two main phenotypes (Th1 or Th2). Contact sensitizers such as DNCB skew cytokine secretion to a Th1 profile characterized by high levels of INF-γ and IL-12. In contrast, respiratory allergens such as TMA induce a Th2 response characterized by the secretion of IL-4, IL-5, IL-10 and IL-13. Previous studies have demonstrated that SMX-NO induces a mixed Th1/Th2 response with high secretion of IL-5, involving in the differentiation and recruitment of eosinophils and is a hallmark of sulfonamide hypersensitivity. Our results expand these findings and demonstrate that nitroso sulfonamide primed splenocytes secrete a mixed Th1/Th2 cytokine profile when re-challenged with the different nitroso sulfonamide metabolites.

Obviously, animal models of immunogenicity do not reflect completely the situation in humans since animals do not present tissue damage and an immune response is detectable in 100% of animals. The only described animal model with clinical manifestations of a drug hypersensitivity reaction (skin rash) is nevapirine. Long-term oral exposure of nevirapine to brown norway rats is associated with the development of cutaneous eruptions (Shenton et al., 2003; Uetrecht, 2007). In this animal model, the skin rash was detected approximately two weeks after the first contact with the drug. Moreover, on re-exposure to the drug, a skin rash develops within days.

Even though our studies were conducted in a model of immunogenicity (not hypersensitivity), the system allows a detailed investigation of the primary antigenic determinant as well as analysis of T-cell antigenicity and cross-reactivity. How the data relates to the clinical situation in patients was studied in chapter 4 and is discussed in detail below.

Cells from hypersensitive human patients cannot be used to explore the nature of the primary antigenic determinant involved in the initiation of an immune response. However, detailed analysis of individual T-cell clones provides important information regarding the nature of the antigen(s) that stimulate circulating memory T-cells. Furthermore, they provide an invaluable tool to explore mechanisms of drug (metabolite)-specific T-cell activation. Thus, in chapter 4 T-cells from three SMX hypersensitive patients were used to study antigen specificity, mechanisms of antigen presentation and the cross-reactivity patterns with structurally related drugs and drug metabolites.

Drugs are thought to stimulate T-cells via two pathways; drug-protein adduct formation or labile weak interaction with immune receptors. Immunological dogma dictates that a T cell antigen must have a molecular weight in excess of 1000 Da. These molecules are normally peptides originating from processed proteins, which interact with immunological receptors (MHC and the T-cell receptor) through a series of complimentary non-covalent binding interactions. In the presence of co-stimulatory signals provided by activated antigen presenting cells, the delivered peptide interaction with specific T-cell receptors may stimulate a T-cell response. Therefore, it is assumed that drugs would need to bind with a carrier protein to become immunogenic (Signal 1) and the drug itself or its metabolite could be responsible for the release of danger signals (Signal 2) that would induce the maturation of antigen presenting cells, as has been reported recently (Rodriguez-Pena et al., 2006; Sanderson et al., 2007).

However, Pichler has questioned the necessity of a hapten carrier protein for drug T-cell activation. Data derived from experiments using SMX as model test compound has shown that T-cell activation is possible without apparent metabolism, covalent drug protein binding or antigen processing. The authors postulated that T-cell activation by drugs is consequence of a labile and weak interaction between the MHC molecules and the specific T-cell receptors. Since these seminal observations, several drugs have been shown to activate T-cells via a direct interaction with MHC and the T-cell receptor. However, drug metabolites also activate T-cells and metabolite-specific T-celnes have been

expanded in patients suffering from SMX hypersensitivity reactions (Schnyder et al., 2000; Nassif et al., 2004; Wu et al., 2006). Moreover, drug and drug metabolite-specific T-cells have been identified from blood and skin of majority patient studied to date.

It has been suggested that patients with history of sulfonamide hypersensitivity are at risk of suffering an unwanted reaction when taking another sulfonamide that they have never been exposed to (Strom et al., 2003). All previous studies did not take into account the possibility of cross-reactivity between the metabolites of the sulfonamides tested (von Greyerz et al., 1999; Depta and Pichler, 2003; Depta et al., 2004). Interestingly, when cross-reactivity was seen in sulfonamides, they all contained the benzyl-sulfonyl amine motif, which it is target of bio-activation by the P450 oxidizing system. Celecoxib and glibenciamide, a non-antibiotic sulfonamide, that does not share this structural moiety, do not stimulate T-cell clones from antibiotic sulfonamide hypersensitive patients (Britschgi et al., 2001; Strom et al., 2003).

To explore these issues, T-cell clones were generated by incubating blood lymphocytes with SMX or its metabolites with optimal antigen concentrations Four hundred and forty T-cell clones were generated of which the vast majority of them, in contrast to previous reports (Schnyder et al., 2000), proliferated in the presence of SMX metabolites (78%). Detailed antigen presentation and cross reactivity studies were subsequently conducted on 101 well growing T-cell clones expanded from lymphocyte cultures exposed to SMX and nitroso SMX. Forty four T-cell clones (43%) responded in the presence of SMX-NO or SMX-NHOH and did not proliferate with SMX, whereas 43 T-cell clones (42%) were cross-reactive and responded in the presence of the parent drug or its metabolites. Only 15% of clones were stimulated with the parent compound alone. Thus, three patterns of specificity were seen; T-cell clones responsive to the parent compound, T-cell clones responsive toward drug metabolites and cross-reactive T-cell clones.

Functional studies performed to investigate the mechanism of antigen presentation in the different sets of T-cell clones demonstrated that SMX stimulates a proliferative response via a direct interaction with MHC and the T-cell receptor, avoiding the antigen-presenting cell's processing machinery. However, these clones were only rarely detected and their scarcity indicates that they might not be involved in the initial hypersensitivity reaction and simply represent an *in vitro* epiphenomena. In metabolite specific T-cell clones protein adduct formation was crucial for T-cell activation and were stimulated by both processing independent and dependent pathways via formation of an irreversible linkage with protein, as incubation with GSH significantly inhibited the response. Finally, the mechanism of antigen presentation in cross-reactive T-cell clones was complicated, because of the different overlapping pathways. The same clones were stimulated by the parent drug and the nitroso metabolite via processing-dependent and -independent pathways.

T-cell clones were not only cross-reactive between SMX and its metabolites, but they also recognized other structurally-related sulfonamide metabolites (SP and SDZ). The incidence of cross-reactivity was higher in metabolite specific T-cell clones incubated with the different hydroxylamine and nitroso metabolites (53%), when compared with SMX-specific T-cell clones (15%).

Nitroso sulfonamide metabolites can bind directly with cysteine residues found in extracellular proteins (e.g. HSA) or with peptides embedded in MHC molecules. The nitroso motif of the metabolite binds with the cysteine residues, and the side chain of the compound, which is different in every sulfonamide, is free and could directly interact with the SMX-specific T-cell receptor. Therefore, pharmacophore modelling of preferred low energy conformations of SMX, SD and SP was investigated. The results illustrate that although the summation of intermolecular forces for each compound was similar, cross-reacting drug antigens must adopt twisted higher energy structures to mimic the binding interaction of SMX at specific T-cell receptors. These data indicate that susceptibility to hypersensitivity reactions to closely related drugs might in part be due to metabolism and protein adduct formation.

Sulfonamides, in their parent form, like the majority of drugs are un-reactive and cannot form drug proteins adduct. Thus, metabolic activation is necessary for the formation of drug reactive metabolites (nitroso sulfonamides) that are highly immunogenic and could initiate hypersensitivity reactions by probably forming Sulfonamide-protein adducts and generating cellular stress. The capacity of APC within splenocytes to oxidize SMX and form SMX-protein adducts that could be used as a source of antigen to SMX-NO primed splenocytes was studied in chapter 5.

Drug metabolism takes place primarily in the liver. However, the liver is not the principal organ affected in drug hypersensitivity reactions. Skin is also metabolically active and as such cutaneous metabolism and adduct formation may explain the high incidence of skin lesions found in drug hypersensitive patients. Keratinocytes, which are the most abundant cells in the epidermal zone of the skin, express several cytochrome P450 isoforms (Baron and Merk, 2001; Merk et al., 2007; Oesch et al., 2007). In 2000 Reilly showed that neonatal normal human epidermal keratinocytes cells (NHEK) oxidized SMX and dapsone to hydroxylamine metabolites. Although the levels formed were extremely low (≈ 1nM), they may be sufficient for the initiation of an immune response. Keratinocyte cell lines, blood monocytes and neutrophils have also been shown to oxidize sulfonamides (Cribb et al., 1990). Furthermore, adducts were recently detected in monocyte-derived dendritic cells incubated with SMX for 24 hrs, indicating that SMX-metabolite protein adducts may be formed directly in antigen-presenting cells (Sanderson et al., 2007).

Evidence gathered from different studies suggest that other cells outside the liver have metabolic activity and could play an important role in sulfonamide hypersensitivity reactions by the generation of reactive drug metabolites. The capacity of APC within splenocytes and its consequence in T-cell activation was studied in chapter 5 and the findings are described below.

Naïve splenocytes were found to metabolize SMX as shown by detection of protein SMX (metabolite) adducts. Adduct formation was dose- and time-dependent. To determine whether these haptenic structures were formed in sufficient levels to induce splenocyte proliferation, SMX-NO primed splenocytes were incubated in the presence of irradiated splenocytes from naïve animals pre-treated with SMX for 16 hr followed by intensive washes to remove the unbound drug or in the presence of the soluble form of SMX-NO. Intracellular adducts formed through metabolism were found to stimulate a T-cell response. These data may have important ramifications for the study of drug hypersensitivity since the enzymes involved in drug metabolism in antigen presenting cells may be very different to that seen in liver. In on-going investigations, the T-cell stimulatory capacity of SMX adducts, generated in human antigen presenting cells incubated with SMX, is being investigated.

In conclusion, these data show that the number of metabolite specific T-cells circulating in hypersensitive patients has been underestimated. Furthermore, adduct formation represents an important process in the stimulation of T-cells from human patients and experimental animal models of drug immunogenicity. The degree of T-cell cross-reactivity is high when structurally-related protein-reactive drug metabolites are used as antigens.

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