

Western  Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

12-7-2015 12:00 AM

Pathophysiologic Mechanisms of Immune-mediated Drug Hypersensitivity Reactions to Sulfonamides

Elham A. Sultan
The University of Western Ontario

Supervisor
Dr. Michael Rieder
The University of Western Ontario

Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
© Elham A. Sultan 2015

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>

 Part of the [Medical Pharmacology Commons](#)

Recommended Citation

Sultan, Elham A., "Pathophysiologic Mechanisms of Immune-mediated Drug Hypersensitivity Reactions to Sulfonamides" (2015). *Electronic Thesis and Dissertation Repository*. 3430.
<https://ir.lib.uwo.ca/etd/3430>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

Pathophysiologic Mechanisms of Immune-mediated Drug Hypersensitivity Reactions to
Sulfonamides

(Thesis format: Monograph)

By

Elham Sultan

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Elham Sultan 2015

ABSTRACT

As sulfonamide hypersensitivity reactions are serious clinical problem, it is necessary to determine which patients tolerate therapy and which patients are at risk. Although the exact pathogenesis of these reactions remains unclear, the imbalance in the production and detoxification of reactive sulfamethoxazole (SMX) metabolites appears to be important in the propagation of these reactions. It is known that these reactive metabolites can cause lymphocytes toxicity and produce reactive oxygen species (ROS) which can damage proteins, lipids, and DNA. The hypothesis of this research is that there are differences in cytotoxicity and expression of oxidative stress to reactive SMX metabolites in the cells of patients who have sustained sulfonamide hypersensitivity reactions versus the cells of controls or sulfonamide tolerant patients. Sulfa hypersensitive patients were found to express high degrees of cell death and more ROS accumulation. This finding indicates that the biotransformation of SMX to its reactive metabolites could be the reason for cytotoxicity, and the oxidative Stress can be a mediator for cell death and inducing allergy reactions.

Keywords: drug hypersensitivity reactions, sulfamethoxazole, cytotoxicity, Reactive oxygen species, oxidative stress, Glutathione.

ACKNOWLEDGEMENTS

First of all, I would like to thank Almighty Allah for the countless blessings given to me, and the knowledge to complete this thesis.

I would like to extend many thanks to my supervisor Dr. Michael Rieder for his acceptance to investigate science in his lab, and his guidance which has made the completion of this dissertation possible. I would never have succeeded without your scientific support and encouragement. Thank you.

I would like to express my deep gratitude to my advisors, Drs. Jorge Mazza, Brad Urquhart and Rommel Tirona. The scientific knowledge and the advices from all of you helped me move my ideas forward and solve many problems.

No thesis is possible without skilled technical support and as such a special thanks to Anda Marcu and Dr. Abdelbaset Elzagallaai. I greatly appreciate your cooperation, teaching the methodology and providing help while in the lab.

I wish to thank Dr. Michael Miller for his assistance in the statistical analysis. Also, thanks to my lab mates, Yadira Tejada and Thu Chau, who have helped me through so much.

A very special thanks to Fatma Etwel for being a great friend throughout graduate school and for your advice and expertise. Our coffee breaks and enjoyable conversations will be missed.

Cheers to all the participants in this study, the staff at department of Physiology and Pharmacology and Robart's Research Institute, and for my sponsor: Ministry of Higher Education, Libyan Government.

Last but certainly not least, thanks to my family who has always been a great source of inspiration, encouragement and mental support. To my lovely husband Emhemed, my kids Muawia and Joud, my parents and my siblings, thank you for everything and being there all the time.

TABLE OF CONTENTS

<u>Contents</u>	Pages
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iii
TABLE OF CONTENTS.....	v
LIST OF FIGURES.....	vii
LIST OF TABLES.....	ix
LIST OF ABBREVIATIONS.....	x
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.....	1
1.1 ADVERSE DRUG REACTIONS (ADRs).....	1
1.1.1 Classification of ADRs.....	2
1.1.2 Drug Hypersensitivity Reactions (DHRs).....	3
1.1.3 Immune Involvement and Mechanistic Hypothesis of Drug Hypersensitivity...8	
1.1.3.1 Hapten Hypothesis.....	10
1.1.3.2 Danger Hypothesis.....	11
1.1.3.3 Pharmaceutical Interaction (PI) concept.....	14
1.2 SULFONAMIDES.....	19
1.2.1 Sulfonamide Mechanism of Action.....	19
1.2.2 Metabolism of Sulfamethoxazole.....	20
1.2.3 Hypersensitivity Reactions Toward Sulfamethoxazole.....	24
1.3 OXIDATIVE STRESS.....	29
1.3.1 Reactive Oxygen Species (ROS).....	29
1.3.2 Drugs induced Oxidative Stress.....	30
1.3.3 Triggering of Apoptosis by Reactive Oxygen Species.....	32
1.4 CONCLUSION.....	35
CHAPTER 2: RESEARCH HYPOTHESIS AND OBJECTIVES.....	37
CHAPTER 3: MATERIALS AND METHODS.....	39

3.1	Materials and Chemicals.....	39
3.2	Study Participants.....	39
3.3	Blood Collection and Cells Isolation.....	40
3.4	Assessment of Cells Viability and Immune Sensitization.....	42
3.4.1	Lymphocyte Toxicity Assay (LTA).....	42
3.4.2	<i>In vitro</i> Platelets Toxicity Assay (iPTA).....	46
3.5	Assessment of Oxidative stress.....	46
3.5.1	Measurement of Reactive Oxygen Species Formation (ROS).....	46
3.5.2	Measurement of Protein Carbonyl Content and Lipid Peroxide levels.....	47
3.5.2.1	Protein Carbonyl Content.....	48
3.5.2.2	Lipid Peroxidation.....	50
3.6	Determination of Reduced Glutathione Content.....	53
3.7	Statistical Analysis.....	55
	CHAPTER 4: RESULTS.....	56
4.1	Assessment of Cell Viability and Immune Sensitization by the Lymphocyte Toxicity Assay (LTA) and <i>in vitro</i> Platelets Toxicity Assay (iPTA).....	56
4.2	Measurement of Oxidative Stress Parameters.....	60
4.2.1	Quantification of Reactive Oxygen Species (ROS).....	60
4.2.2	Protein Carbonyl Content.....	65
4.2.3	Lipid Peroxidation.....	71
4.3	Estimation of Reduced Glutathione (GSH) Content.....	71
	CHAPTER 5: DISCUSSION.....	79
	CHAPTER 6: FUTURE DIRECTIONS.....	90
	REFERENCES.....	92
	APPENDIX I: Permission for re-print figures.....	108
	APPENDIX II: Research Ethics Board (REB) approval.....	110
	CURRICULUM VITAE.....	111

LIST OF FIGURES

<u>FIGURES</u>	<u>PAGE</u>
Figure 1: Classification of hypersensitivity reactions according to Gell and Coombs.....	6
Figure 2: Hapten Hypothesis	12
Figure 3: Danger Hypothesis	15
Figure 4: Pharmacological Interaction Hypothesis.....	17
Figure 5: Basic chemical structures of sulfonamides and of sulfa moiety containing molecules.	21
Figure 6: Schematic representation of the metabolic pathways of SMX and its proposed immunogenicity.	25
Figure 7: Schematic representation for ROS generation and increase in oxidative stress by SMX-HA.....	33
Figure 8: Procedures of the lymphocytes toxicity assay (LTA).	44
Figure 9: Assessment of cell viability by LTA and iPTA.	57
Figure 10: Quantification of ROS produced by lymphocytes upon their incubation with varying concentrations of SMX-HA.....	61
Figure 11: Correlation between the amount of ROS and the percent of cell death for allergic patients.	67
Figure 12: Protein carbonyl content of lymphocytes treated with SMX (200 μ M) or SMX-HA (400 μ M).	69
Figure 13: Lipid peroxide content of lymphocytes treated with SMX (200 μ M) or SMX-HA (400 μ M).	73

Figure 14: Determination of GSH level and GSH/GSSG ratio in PBMCs treated with SMX and SMX-HA.	75
Figure 15: Correlation between the amount of ROS and the GSH level for hypersensitive patients.	77

LIST OF TABLES

<u>TABLES</u>	<u>PAGE</u>
Table 1: Characteristics of patients with SMX hypersensitivity reactions and the assays that used for each one.	41
Table 2: LC ₅₀ values of SMX-HA for study participants in both assays (LTA and <i>i</i> PTA).	59
Table 3: Area under the curve (AUC) of ROS over time (5-120 min) for study participants upon incubation of their lymphocytes with SMX-HA 200 or 400 μM.	66

LIST OF ABBREVIATIONS

ADRs	Adverse drug reactions
ANOVA	Analysis of variance
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
B-cells	Bone marrow derived cell
CA	Corrected absorbance
CAT	Catalase
CYP	Cytochrome P450
DCF	2', 7'-dichlorfluorescein
DCFH-DA	2', 7'-dichlorfluorescein diacetate
DDS	Dapson
DHRs	Drug hypersensitivity reactions
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNBH	4-dinitrophenylhydrazin
DTH	Delayed type hypersensitivity
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
FBS	Fetal bovine serum
Fc	Fragment crystallizable
G-6-PDD	Glucose-6-phosphate dehydrogenase deficiency

GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSH _t	Total glutathione
GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HS	Hypersensitivity
HSRs	Hypersensitivity reactions
HSPs	Heat-shock proteins
IDRs	Idiosyncratic drug reactions
IFN	Interferon gamma
IG	Immunoglobulin
IL	Interleukin
<i>i</i> PTA	<i>in vitro</i> platelet toxicity assay
LTA	Lymphocyte toxicity assay
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MPA	Metaphosphoric acid
MTT	3-(4, 5-dimethylthiazol-2-yl) 2,5- diphenyl-tetrazolium bromide
NAC	N-acetylcysteine

NADPH	Nicotinamide adenine dinucleotide phosphase hydrolase
NAT	N-acetyle transferase
NFT	Nitrofurantoin
NHEK	Normal human epidermal keratinocytes
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
O ₂	Molecular oxygen
O ₂ ^{•-}	Superoxide anion radical
OH [•]	Hydroxyl radical
PABA	Para-amino benzoic acid
P/S	Penicillin / streptomycin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline solution
PCP	Pneumocystis pneumonia
PI concept	Pharmacological interaction of drugs with immune receptors
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SE	Standard error
SJS	Stevens-Johnson Syndrome
SMX	Sulfamethoxazole
SMX-HA	Sulfamethoxazole hydroxylamine
SMX-NO	N-nitroso- sulfamethoxazole
SMX-TMP	Sulfamethoxazole-trimethoprim

SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
T-cell	Thymus derived cell
TCR	T-cell receptor
TEN	Toxic Epidermal Necrolysis
TMP	Trimethoprim
TNF	Tumor necrosis factor
TNB	5-thio-2-nitrobenzoic acid
VPA	Valproic acid
WHO	World Health Organization

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 ADVERSE DRUG REACTIONS (ADRs)

Adverse drug reactions (ADRs) are considered to be one of the important complications of modern drug therapy in health care. Many of these reactions are mild, not life threatening and may be unnoticed. However, other ADRs are more severe and sometimes major causes of sickness and death and highly costly to the health care system (Pirmohamed et al., 2004; Kongkaew, 2008). The World Health Organization (WHO) defines ADR as a physiological response to a drug that is deleterious and undesired occurring at the doses typically used in humans for diagnosis, prophylaxis, therapy or for the modification of physiologic function (World Health Organization: Technical Report Series No. 498, 1972). ADRs affect as many as 25% of all hospitalized patients, and complicate 5-15% of therapeutic drug courses (Gruchalla, 2003). In the United State, ADRs represent the sixth leading cause of death after heart disease, cancer, stroke, pulmonary disease and accidents (Lazarou et al., 1998). Additionally, these reactions are highly costly to the health care system since has estimated that the annual cost of drug-related morbidity and mortality was more than \$177 billion in the United States in 2000 (Ernst and Grizzle, 2001). Unfortunately, some of these reactions can cause the discontinuation of a drug that was otherwise effective in the treatment of the primary disease.

Although ADRs are common, it has been challenging to estimate their exact frequency because accurate statistics do not exist. Moreover, the clinical symptoms of

ADRs commonly overlap with symptoms of the underlying disease and this makes the diagnostic approach to these reactions difficult (Rieder, 1997).

1.1.1 Classification of ADRs

ADRs can manifest in many different ways and they can affect different organ systems. ADRs have been classified by Rawlins and Thompson in the late 1970s into two major types, known as Type A and Type B reactions (Rawlins and Thompson, 1977).

Type A (augmented) reactions are predictable from the pharmacological action of the drug. Also, these reactions are dose dependent and usually due to an excessive drug's therapeutic effect. Therefore, Type A reactions are reversible with reduction of the dose or withdrawal of the drug (Rawlins and Thompson, 1977). This type is common, accounting for about 80% of ADRs and includes toxic effects, side effects, secondary effects and drug interactions (Rieder, 1994; Naisbitt et al., 2003). An example of Type A pharmacological reaction is peptic ulceration and hemorrhage with nonsteroidal anti-inflammatory drugs (NSAIDs).

In contrast to Type A reactions, Type B (idiosyncratic or bizarre) reactions are not predictable from pharmacological properties of the drug, and not dose dependent. Thus, there is no simple dose-response relationship, and the drug must be withdrawn upon development of these reactions. This type comprises 10-15% of all ADRs (Rieder, 1994; Naisbitt et al., 2003). The term of idiosyncratic drug reactions (IDRs) applies for the adverse events that do not occur in most people (i.e. specific to an individual) within the range of doses used clinically. Although this type affects a small percentage of the population, it is very serious and accounts for many drug induced deaths due to unpredictability and dose independency (Pirmohamed et al., 2002). Thus, identification

of patients at risk for this type of reactions for a specific class of medication is difficult. In addition, Type B reactions can be divided into immune-mediated (hypersensitivity reactions) and non-immune mediated (metabolic idiosyncrasy) reactions (Pirmohamed et al., 2002; Naisbitt et al., 2003; Shear et al., 1986). An example of immune-mediated Type B reactions is allopurinol induced skin rash. An example of non-immune mediated type B reaction would be hepatic failure and teratogenicity observed in rare patients treated with valproic acid (VPA). This idiosyncratic reaction is thought to be triggered by an excessive quantity of toxic VPA metabolites, and referred as metabolic idiosyncrasy based on a lack of fever and rash and/or lack of immediate onset on rechallenge (Jeavons, 1984; Sadeque et al., 1997). This thesis will focus on immune-mediated Type B reactions, and more specifically on delayed hypersensitivity to sulfamethoxazole (SMX).

It should be noted that there are supplementary categories which were successively added and include types C (Continuous; dose and time-related), D (Delayed; time-related), E (End-of-use; withdrawal) and F (Failure; unexpected failure of therapy) (Rieder et al., 2009; Zuniga et al., 2012).

1.1.2 Drug Hypersensitivity Reactions (DHRs)

For type B reactions, there are many different mechanisms which are not fully understood. These reactions usually trigger the immune system and are called allergic reactions. Hypersensitivity reactions (HSRs) are defined as immunologically-based adverse reactions that occur in susceptible patients upon exposure to drugs, chemicals or other antigens (Kanji and Chant, 2010). Most of DHRs are thought to be immune-mediated based on the delay between starting a drug and the onset of the reactions which are more severe at re-exposure, and the presence of some immunological markers in

patients with a history of drug hypersensitivity reactions (Park et al., 1992). These reactions cause problems that range from nuisance (e.g, itchy skin) to life threatening (e.g, Stevens - Johnson syndrome, SJS, and Toxic Epidermal Necrolysis, TEN). Any drug is potentially able to evoke hypersensitivity reactions; however, the prevalence differs challenging as well. To most clinicians the term “drug hypersensitivity” is synonymous with the symptoms produced by type I hypersensitivity which is characterized by the production of IgE. However, when this term is used by pharmacologists, this would usually include undesired drug-induced effects mediated by an immune response that produces tissue damage (Naisbitt et al., 2003; Pirmohammed, 2005; Rieder, 2009).

Tilles (2001) has reported that drug induced allergic reactions affect about 5% of the population, and these reactions are responsible for 6% to 10% of the ADRs. Allergic reactions can be classified according to the Coombs and Gell classification system (1968) into four distinct pathophysiological types (Type I- Type IV), which can be applied to those adverse drug reactions with a known immunologic component: immediate type hypersensitivity reactions (Type I), antibody-mediated cytotoxic reactions (Type II), immune complex-mediated reactions (Type III), and delayed-type hypersensitivity reactions (Type IV) (Figure 1).

Type I (IgE- mediated hypersensitivity) reactions: These reactions manifest almost immediately upon allergen exposure (usually within 30 minutes). This type activates B-cells to release IgE antibodies which are directed against soluble protein antigens (such as pollen, animal dander, and foods) or some drugs. This type is triggered by the presence of mast cells armed with high affinity Fc-IgE (fragment- crystallizable) receptors to which allergen/drug-specific IgE is bound. The result of linking antigens with IgE molecules on

the surface of mast cells or basophiles causes degranulation of these cells and the release of inflammatory and vasodilatory mediators such as histamine, leukotriens and prostaglandins. Clinical symptoms of this type may manifest as anaphylaxis, allergic rhinitis, urticaria, or bronchospasm. An example of drugs that have been implicated in causing this type is β -lactam antibiotics (Vega et al., 1994; Rocken et al., 2010).

Type II (antibody-mediated cytotoxic) reaction: This type involves binding of an antibody to a cell with subsequent binding of complement causing cell death and tissue damage. These reactions are caused by cytotoxic antibodies, which are primarily IgM or IgG. The essence of this type is that an antigen bound to the surface of a cell combines with IgG antibodies. This bound antibody can then activate and direct killer cells or phagocytic cells against the antigen presenting host cells. The most common clinical manifestations of type-II hypersensitivity reactions to drugs are agranulocytosis, thrombocytopenia and immunoallergic haemolytic anemia (Giner et al., 2003; Akamizu et al., 2002). Penicillin causing hemolytic anemia is an example of this type.

Type III (immune-complex mediated) reactions: These reactions involve the creation of antigen-antibodies complexes (immune complexes either antigen-IgM or antigen- IgG) that can deposit in tissues and small blood vessels causing a local inflammatory response. This response causes the release of inflammatory mediators such as tumor necrosis factor (TNF) and interleukin. Slow removal of these complexes by phagocytes leads to their deposition in the skin, glomeruli, joints, and gastrointestinal system. These immune complexes activate the complement cascade. Then granulocytes and macrophages are attracted to the site of activation and release of lytic enzymes from

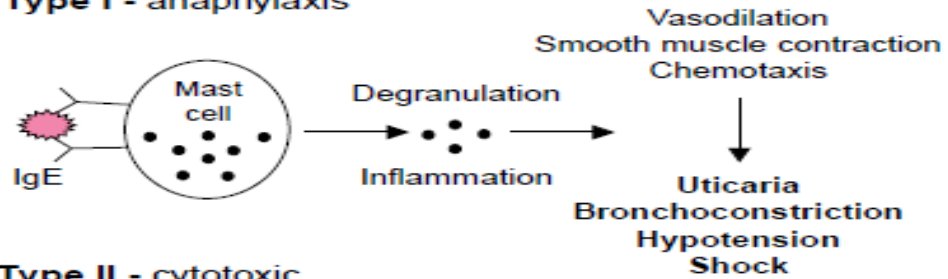
Figure 1: Classification of hypersensitivity reactions according to Gell and Coombs

Refer to pages 4, 5, and 8 for details about each of the four classification types

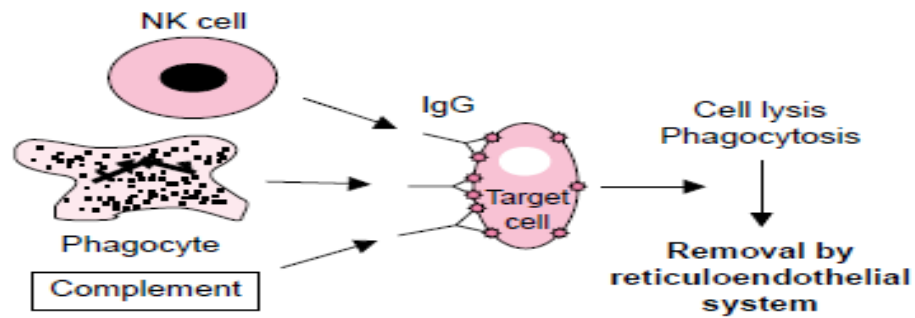
(Ig = immunoglobulin; NK = natural killer)

Figure reprinted from Naisbitt et al. (2000), with permission

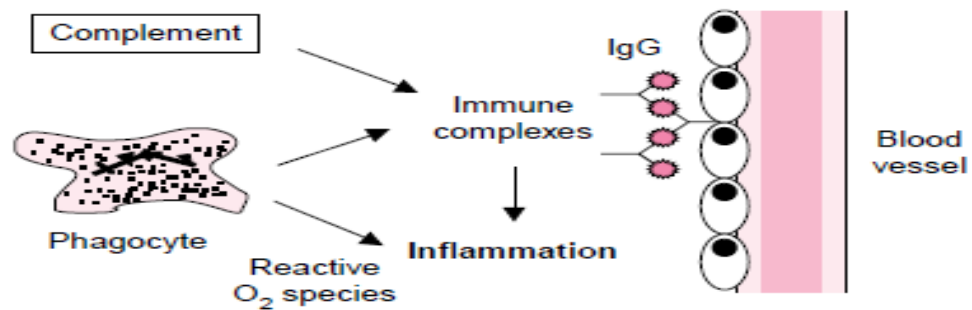
Type I - anaphylaxis



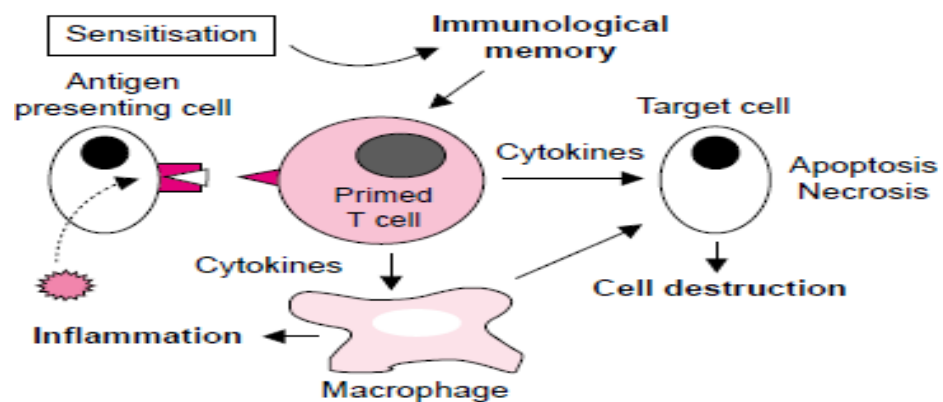
Type II - cytotoxic



Type III - complex-mediated



Type IV - cell-mediated



- Y Antibody
- Antigen
- Major histocompatibility complex (MHC)
- ◀ T cell receptor

their granules causing damage. The clinical symptoms of type III reaction include serum sickness or vasculitis. There are many drugs that cause serum sickness or vasculitis-like reactions such as β -lactam antibiotics (Kanji and Chant, 2010; Pichler 2007).

Type IV (T-cell mediated) reactions: This type is also called delayed type hypersensitivity (DTH) due to the delay in the immune response compared to antibody-mediated reactions. All forms of DTH reactions require several days or even weeks to reach their optimum strength (Rocken et al., 2010). This type involves the activation, proliferation, and mobilization of antigen-specific T cells. These activated T cells will release large amounts of cytokines causing damage to host cells and tissues. Also, this will initiate the activity of other mononuclear cells, such as monocytes and macrophages, causing harmful outcomes. The clinical symptoms for this type commonly manifest as skin eruptions such as morbilliform rash or contact dermatitis. Sulfonamides are an example of the drugs that can develop these reactions (Naisbitt et al., 2000; Choquet-Kastylevsky et al., 2001).

It should be noted that ADRs may be caused by several immunological and toxic mechanisms and some of these reactions may not fit into any category of the Gell and Coombs classification.

1.1.3 Immune Involvement and Mechanistic Hypothesis of Drug Hypersensitivity

Understanding the mechanisms of many ADRs has remained a big challenge to clinical and basic science researchers either due to the variation of effects elicited from one individual to another or the lack of good animal models which have hindered detailed experimental investigations. It has been estimated that the immune system is involved in 10-50% of all idiosyncratic ADRs (Rieder, 1994). However, evidence of immune

involvement such as the presence of antibodies against the drug or drug modified proteins or immune cells that recognize the offending drug are required to diagnose these responses as true hypersensitivity (Park et al., 1992). The immune system includes the innate (natural) and adaptive (acquired) immune systems. The innate immune system consists of cells and proteins that are always present and ready to fight microbes at the site of infection. The main components of this system are physical epithelial barriers such as skin and mucus membrane, phagocytic leukocytes, dendritic cells, and natural killer (NK) cell. The adaptive immune system is called into action against specific foreign substances that are able to evade or overcome innate immune defenses. This system includes B-cells and T-cells (Caamano and Hunter, 2002). Thus; both the innate and adaptive immune systems play a crucial part in the removal of pathogens from the body.

The immune response can be either a cellular (cell-mediated) response or a humoral (antibody-mediated) response. Cell-mediated immunity can be induced by cytotoxic T-lymphocytes that mediate apoptosis of antigen presenting target cells. Alternatively, humoral immunity can be elicited by B-cell production of antigen-specific antibodies through the interaction of B-cells with T-cells activated by antigen presenting cells (APCs) (Zaccara et al., 2007). In this thesis we will focus on the cellular (T-cell mediated) response of the immune system.

In general, the immune system can recognize the foreign molecules (antigens) and attack them, and the immune response may be generated against the altered macromolecules (Rieder, 1994). For the immune system to recognize these antigens, they should be internalized, fragmented and displayed as antigenic determinants, or epitopes, on the surface of the cell bound to major histocompatibility complex (MHC) molecules

(Cammano and Hunter, 2002). Extracellular antigens are expected to be presented via MHC class II to CD4⁺ T-cells, whereas intracellular antigens appear to be presented via MHC class I to CD8⁺ T-cells (Robinson and Delvig, 2002)

The proposed mechanisms for initiation or eliciting an immune response to a drug involve the metabolism of a parent drug to its reactive metabolites which can bind covalently to intracellular or extracellular proteins of target cells. This drug-protein complex is then processed and bound within the antigen binding cleft of a MHC molecule and displayed on the cell surface for recognition by T-cells. Once this occurs, T-cells can ultimately act either in a cytotoxic manner to kill the antigen-displaying target cell or can secrete cytokines that attract or activate other immune cells to the local area causing cell destruction (Naisbitt et al., 2001).

It is known that a drug or a drug metabolite can induce an immune response by involving one or more processes. However, the precise mechanisms involved are unclear and the proposed mechanisms for many of these drugs are based on several hypotheses (hapten hypothesis, danger hypothesis and pharmacological interaction concept).

1.1.3.1 Hapten Hypothesis

It is the oldest and the most widely accepted theory advanced by several investigators, notably Park et al., 1987. A "hapten" is chemically reactive, small molecule, with a molecular weight less than 1000 daltons, which able to form covalent bonds with proteins or peptides (Pichler et al., 2010). The hapten hypothesis (Figure 2) proposes that drug and/ or drug metabolites are too small to elicit an immune response, and therefore must act as haptens which can covalently bind and modify macromolecules (such as proteins or DNA) to become immunogenic. This drug-protein complex will be

recognized, processed by antigen presenting cells (APCs) and presented via MHC-II to helper T-cells. Consequently, this will stimulate the immune response (Landsteiner and Jacobs, 1936; Park et al., 1998).

A few drugs, such as penicillins and cephalosporins, are spontaneously reactive, can directly bind proteins and act as a hapten which evokes an immune response (Parker, 1982). However, other drugs are not chemically reactive, but may undergo chemical or enzymatic modification (by Phase I or Phase II metabolic enzymes) to form reactive metabolites that can bind macromolecules or be directly cytotoxic. Halothane-induced hepatotoxicity, in which halothane is metabolized by cytochrome P450 to the reactive metabolite trifluoroacetylchloride, is a good example of drugs that must be metabolized to reactive metabolites that can trigger an immune response (Njoku et al., 1997).

1.1.3.2 Danger Hypothesis

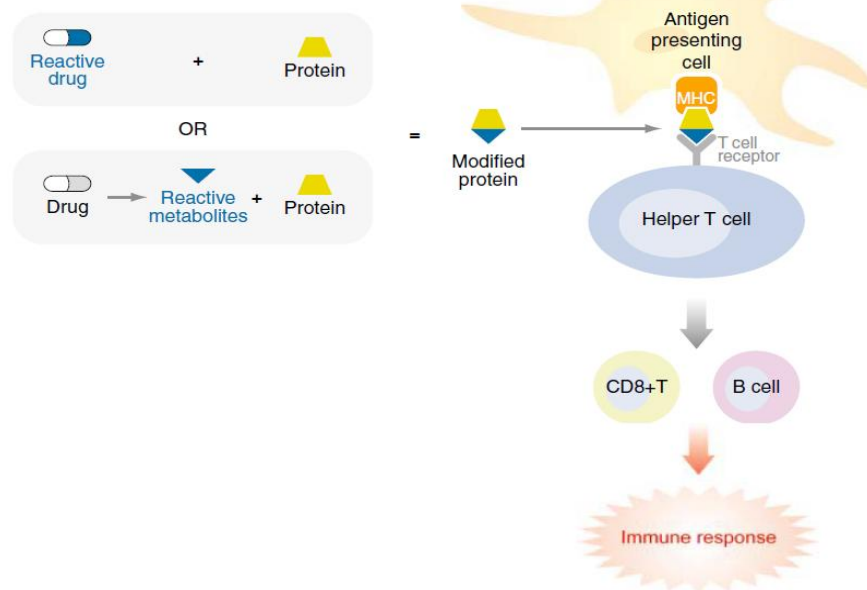
The Danger Hypothesis, as proposed by Matzinger in 1994, states that the antigen can trigger an immune response in the presence of danger signals that are necessary for APCs to generate a full or active immune response rather than tolerance (Figure 3). According to this theory, it is insufficient for reactive metabolites alone to initiate the immune response. This is supported by the observations that drug-serum adducts can also be found in tolerant patients that do not develop hypersensitivity after drug exposure (Gruchalla et al., 1998).

In terms of idiosyncratic drug reactions (IDRs), these danger signals could be generated as a result of proteins' alteration due to drug haptentation, drug-associated oxidative stress, and drug-induced cell maturation or cell death (Matzinger, 1994; Pirmohamed et al., 2002; Lavergne et al., 2009). These signals may also result from

Figure 2: Hapten Hypothesis

The drug or reactive metabolite binds covalently to proteins and modifying them. This drug-protein complex will be recognized as foreign and processed by antigen presenting cells (APC), which eventually stimulate the immune response.

Figure reprinted from Uetrecht (2007), with permission.



disease-induced oxidative stress, inflammation or bacterial and viral infections (Gallucci and Matzinger, 2001). In fact, HIV-infected patients have high levels of chemokines and oxidative stress that may act as danger signals (Lavergne et al., 2009; Sandler et al., 2011). Thus, this may explain the higher incidence of sulfonamide hypersensitivity in AIDS patients (50-60%) compared to HIV negative subjects (1-3%) (Fischi et al., 1988). It is possible that the drug reactive metabolites can develop these reactions because of their ability to act as haptens to provide signal 1 for recognition by specific T cells, or as danger signals (signal 2) by the activation of signaling pathways linked to oxidative stress or protein damage (Utrecht, 2007).

1.1.3.3 Pharmacological Interaction (PI) concept

The PI concept postulates that the chemically inert drugs are unable to covalently bind to proteins as well as associate with the MHC on APCs. Therefore, these drugs may interact in non covalent manner with T- cell receptor (TCR) or with human leukocyte antigen (HLA) molecules without the antigen processing pathway involved (Figure 4). This concept has been demonstrated for some drugs such as sulfamethoxazole, carbamazepin and lidocaine (Schnyder et al., 1997; Naisbitt, 2003; Zanni, 1997). Pichler and colleagues were able to show that the clones of lymphocytes from SMX hypersensitive patients proliferated when exposed to soluble SMX. This implies that the parent drug itself may act as a pharmacological agent that can stimulate an immune response (Pichler, 2002; Pichler, 2008). Furthermore, to date, there is no evidence for the contribution of P-I model *in vivo* drug toxicity.

It should be noted that not all drugs hypersensitivity reactions can be elucidated by one individual theory. Therefore, it is probable that many drugs can develop these

Figure 3: Danger Hypothesis

The stressed or damaged cells release danger signals that can activate APCs and stimulate an immune response.

Figure reprinted from Uetrecht (2007), with permission

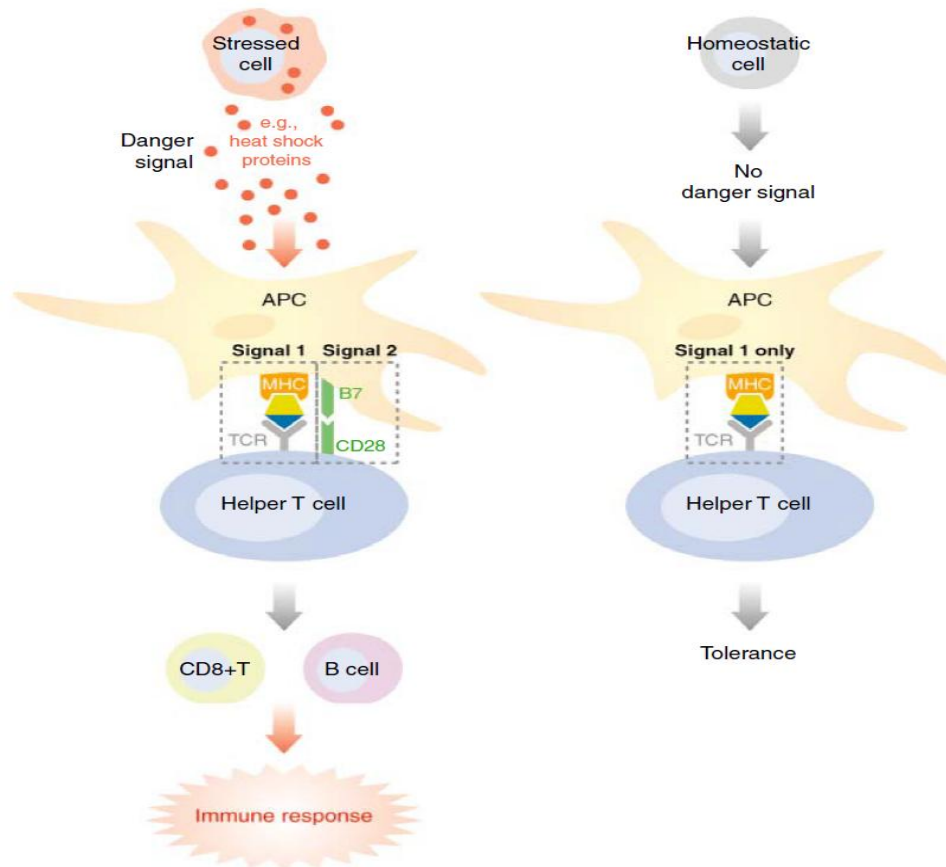
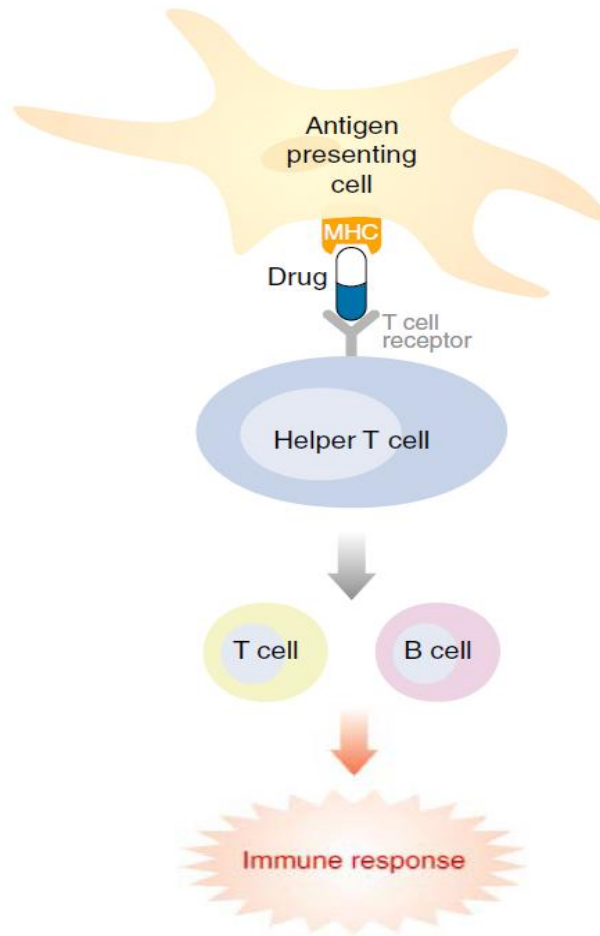


Figure 4: Pharmacological Interaction Hypothesis

The drug binds directly to the MHC-TCR complex and induces an immune response to the parent drug.

Figure reprinted from Uetrecht (2007), with permission



reactions depending on different or complex mechanisms that vary from one person to another.

1.2 SULFONAMIDES

1.2.1 Sulfonamide Mechanism of Action

Many of new antibiotics are not a good choice either due to their high cost or because of the development of resistant organisms. The sulfonamides, the drugs of study in this thesis, are considered to be one of the oldest, inexpensive antibacterial agents, with broad spectrum of action and a wide tissue distribution. They have been used effectively in treating many infectious diseases such as bronchitis, urinary tract and gastrointestinal infections (Marchant and Shurin, 1983). These drugs are structural analogues of para-aminobenzoic acid (PABA). PABA is a substrate in the formation of folic acid which is essential for synthesis of precursors for RNA and DNA synthesis in both bacteria and mammals. However, mammalian cells can take their needs of folic acid from exogenous sources. Sulfonamides compete with PABA for the enzyme dihydropteroate synthase, resulting in the reducing folic acid in the bacteria cells and ultimately inhibiting the growth of bacteria (Hitchings, 1973; Flotzer and Reese, 1987). The most frequently encountered antimicrobial sulfonamide is sulfamethoxazole (SMX) in combination with trimethoprim (TMP), together known as Co-trimoxazole. TMP was found to add a synergistic bactericidal therapeutic benefit when used with SMX, as a selective inhibitor of microbial dihydrofolate reductase, another enzyme which is essential for folic acid synthesis (Flotzer and Reese, 1987). Indeed, since both SMX and TMP act on enzymes in the same pathway, resistance towards one drug can be overcome by the action of the other.

Chemically, Sulfonamides are compounds that contain SO_2NH_2 moiety. The bacterial sulfonamides contain an arylamine moiety (an amine linked to benzene ring) at N4 position as well as 5 or 6 membered nitrogen containing ring attached at N1 nitrogen of sulfonamide (Figure 5). Of importance, it has been established that the sulfonamide containing drugs with both the N1 and N4 substituents, the antibiotics, were most strongly associated with hypersensitivity reactions compared to other sulfonamide-containing medications. This might be because of that the other sulfonamide-containing medications (as diuretic, antihypertensive or hypoglycemic drugs) lack the primary arylamine group within their chemical structure, and they cannot produce the reactive intermediate (i.e. nitroso) which is absolutely essential to cause hypersensitivity (Cribb et al., 1996; Tilles, 2001; Slatore and Tilles, 2004; Brackett et al., 2004). Thus, this would support that the cross allergenicity can exist among sulfonamide antimicrobials, but not with other sulfonamide containing medications (Hemstreet and Page, 2006; Dibbern and Montanaro, 2008).

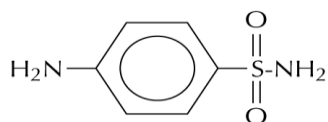
1.2.2 Metabolism of Sulfamethoxazole

It is important to note that numerous therapeutic agents cause severe IDRs, but the mechanism behind these reactions is still uncertain. It is believed that bioactivation of certain drugs to their reactive metabolites plays a crucial role in the initiation of these reactions. For example, lamotrigine, phenytoin and carbamezipine have been reported to bioactivate to highly reactive arene oxide metabolites that can form the protein-hapten conjugate, which is then capable of generating an immune response and inducing allergic reactions (Madden et al., 1996; Maggs et al., 2000).

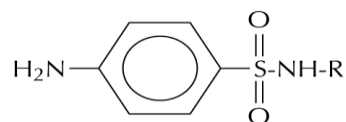
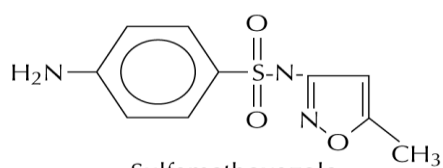
The pathogenesis of SMX hypersensitivity (SMX HS) reactions has not yet been

Figure 5: Basic chemical structures of sulfonamides and of sulfa moiety containing molecules.

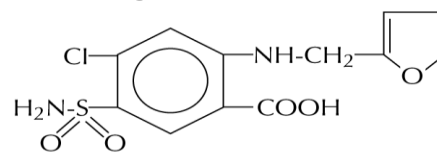
(Sulfonamides are compounds that contain SO_2NH_2 moiety)



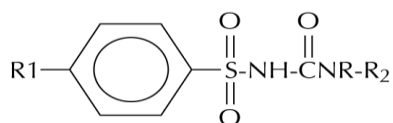
Sulfanilamide

Antimicrobial sulfonamides:
general structure

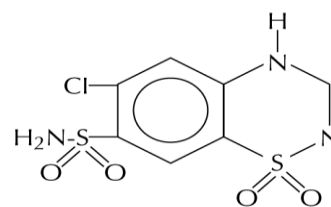
Sulfamethoxazole



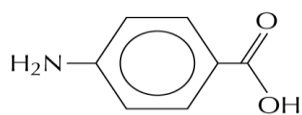
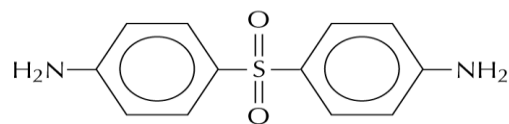
Furosemide



Basic sulfonyleurea structure



Hydrochlorothiazide

Para-aminobenzoic
acid (PABA)

Dapsone

completely elucidated. However, it has been proposed that the metabolism of the parent drug, SMX, to its reactive metabolites, sulfamethoxazole hydroxylamine (SMX-HA) and N-nitroso sulfamethoxazole (SMX-NO) play a central role in the pathogenesis of these reactions (Rieder et al., 1988; Utrecht, 1989) (Figure 7). SMX is rapidly absorbed after oral administration and detectable levels are found in the plasma within 5 min. The peak plasma SMX concentration is up to 2.5 mM, which can be reached within 2-4 hr, and half life of SMX is 9-11 hr. SMX can be metabolized by two separate pathways. The major pathway (70-95 %) is acetylation by N-acetyltransferase (NAT) to form an inactive and non-immunogenic acetylated metabolite (Acetyl-SMX) that is directly filtered by the kidneys and excreted in the urine (Cribb and Spielberg, 1992; Naisbitt et al., 1999). Acetylation occurs by the activity of phase II enzymes, and represents direct detoxification processes. The second pathway involves the metabolism of SMX (about 5-10 %) by the cytochrome P450 isozyme, CYP 2C9, to its unstable, electrophilic reactive metabolite, SMX-HA. The latter can spontaneously auto-oxidize to form the more reactive metabolite, SMX-NO. CYP2C9 is expressed in the liver, skin, macrophages and keratinocytes; however, SMX-HA also may be produced in monocytes and neutrophils via the myeloperoxidase pathway (Cribb and Spielberg, 1992). Thus, this may explain the diversity in the target tissues for ADRs to individual drugs.

SMX-HA can be reduced back to the parent drug by CYP3A4 (Cribb et al., 1995). It is relatively stable *in vivo* compared to SMX-NO. The evidence for this is based on the ability to detect the SMX-HA, while SMX-NO cannot, in the urine of patients receiving this drug. SMX-NO can bind covalently to cysteine residues of proteins, and haptenate them resulting in cellular apoptosis.

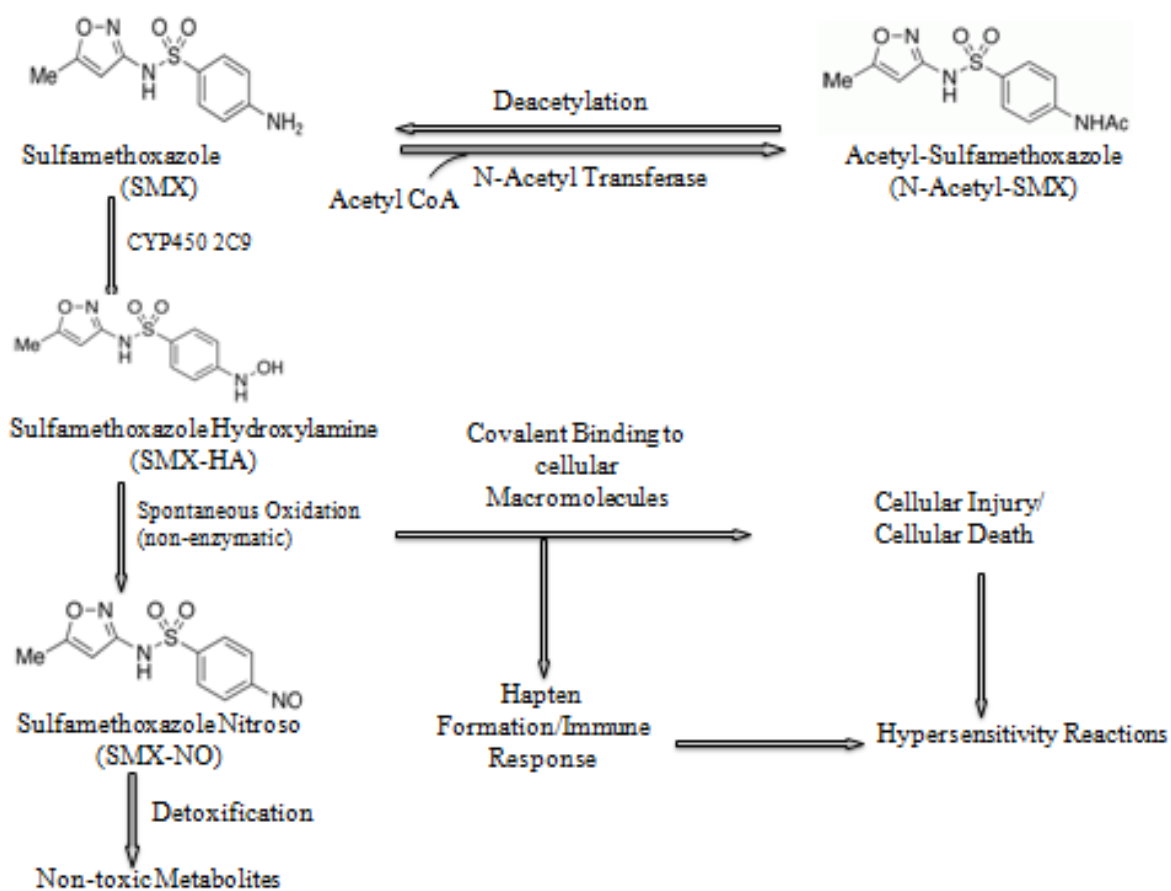
SMX-NO can be reduced back to the SMX-HA by a non-enzymatic pathway using antioxidants, such as ascorbic acid, cysteine and glutathione. It should be also noted that SMX-HA does not always undergo auto-oxidation to SMX-NO under biological conditions. Cribb et al. (1991) have demonstrated that the presence of glutathione (GSH) can prevent the auto-oxidation of SMX-HA to SMX-NO. Furthermore, GSH can effect reduction of SMX-NO to both SMX and SMX-HA and thereby prevents the formation of drug-protein adducts and reduces toxicity (Summan and Cribb, 2002; Trepanier and Miller, 2000). Thus, increased production or decreased elimination of these reactive metabolites is considered to be a key component in the initiation of metabolite-induced hypersensitivity reactions. SMX-HA can also be detoxified enzymatically by the enzymes cytochrome b5 (b5) and cytochrome b5 reductase (b5R) through a detoxification pathway that is 10 times more efficient than forward oxidation to SMX-HA (Kurian et al., 2004; Cribb et al., 1996).

1.2.3 Hypersensitivity Reactions Toward Sulfamethoxazole

SMX-TMP is an effective agent for treatment of many infectious diseases. In HIV patients, this drug is used in prophylaxis and treatment of *Pneumocystis pneumonia* (PCP). However, recently the clinical uses of SMX were restricted due to the fear of developing severe reactions that occur in 2-4% of general users of the drug and 50-60% of AIDS patients (Gruchalla, 2003). Indeed, the skin is considered to be the most common target for drug-induced allergic reactions. This may be because of that the skin is immunologically very active, and has the capacity of drug metabolism and defense system. SMX rarely causes IgE-mediated reactions such as urticaria and anaphylaxis, and more commonly develops delayed hypersensitivity(T-cell mediated) reactions that appear

Figure 6: Schematic representation of the metabolic pathways of SMX and its proposed immunogenicity.

SMX can be metabolized along two separate pathways. The first pathway involves the enzyme N-acetyltransferase to yield a stable non- reactive N- acetye SMX. The second pathway involves the CYP450 2C9 to form the reactive metabolite, SMX-HA, which can spontaneously oxidize to form more reactive SMX-NO.



after 1-2 weeks from the time of drug initiation, and become much shorter with re-exposure (within hours). SMX-specific T-cells, a marker of drug-specific cellular immunity, as well as sulfonamide-specific CD4+ and CD8+ T cells have been detected in sulfa sensitive patients (Mauri-Hellweg et al., 1995).

The clinical manifestations SMX reactions are consisting of fever, skin rash, blood dyscrasias, hepatotoxicity, renal dysfunction, hemolytic anemia, or severe dermatopathies (Cribb et al, 1996). A pediatric study has demonstrated that SMX-TMP was among the most frequently associated antimicrobial agents induced serum sickness (Heckbert et al., 1990). Also, Stevens - Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN) are two of the more serious reactions that can be caused by SMX (Tilles, 2001). SJS/TEN are severe cutaneous reactions characterized by extensive necrosis and detachment of epidermis, and can affect the function of many organs. A retrospective study between 2000 and 2010 from four sub Saharan African countries including 177 adults and children, of which 54.8% were HIV positive, with SJS/TEN revealed that antimicrobial sulfonamides were the most commonly (38.4%) associated drugs (Saka et al., 2013). Subsequent to their formation, the SMX reactive metabolites can bind covalently to cellular proteins to form a hapten-conjugate or an antigen, which is then capable of inducing an immune response (Park et al., 2000). Thus, it has been accepted that SMX hypersensitivity follows the hapten hypothesis. Recently, some studies have shown that SMX hypersensitivity may also involve mechanisms of danger hypothesis by generating danger signals (such as chemokines and oxidative stress) required to initiate an immune response (Lavergne et al., 2009; Sanderson et al., 2007). Also, the toxic effects of these metabolites in combination with other disease related factors, such as decreased GSH

level as well as other genetic factors, have contributed in the high incidence of SMX hypersensitivity in HIV patients (Carr et al., 1991; Rieder et al., 1997).

Hydroxylamine metabolites of sulfonamides can induce cytotoxicity in the peripheral blood mononuclear cells (PBMCs) of SMX-hypersensitive patients when incubated *in vitro* (Shear and Spielberg 1985). In fact, the *in vitro* cytotoxicity of SMX-HA in PBMCs has been used as a predictive tool to diagnose delayed-type hypersensitivity reactions associated with SMX (Rieder et al., 1989; Carr et al., 1993; Reilly et al., 1999; Manchanda et al., 2002; Neuman et al., 2000). Rieder and colleagues demonstrated that *in vitro* incubation of human lymphocytes with SMX-HA caused concentration-dependent toxicity, whereas the parent drug, SMX, incubated at equal concentrations exhibited no toxicity.

Yet, it has been hypothesized that this enhanced *in vitro* cytotoxicity is due to a defect in a sulfonamide metabolic or detoxification pathway. This defect could be related to disease, environmental or genetic factors. The lymphocyte toxicity assay (used to predict SMX hypersensitivity) had been shown to be abnormal in the first-degree relatives of SMX-HS patients. This suggests a genetic contribution to SMX HS risk (Neuman et al., 2000; Wolkenstein et al., 1995). In fact, the possible genetic variations may be occurring either in genes related to bioactivation or detoxification pathways. Rieder and colleagues demonstrated that the slow acetylator (NAT2 gene mutation) phenotype is considered to be a risk factor for SMX hypersensitivity (Rieder et al., 1991). However, these genotypes alone are not sufficient to lead to SMX HS because they make up 50% of the United States population, and only 3 % of patients develop these reactions (Carr et al., 1994; Relling, 1989; Eliaszewicz et al., 2002).

In a recent study by Sacco et al. (2012), they found no difference in NAT2 genotype as well as CYB5A (encoding cytochrome b(5)) or CYB5R3 (encoding cytochrome b(5) reductase) between sulfa hypersensitive and tolerant patients. Furthermore, the balance between CYP2C9 (the enzyme oxidizes SMX to SMX-HA) and CYP3A4 (the enzyme reduces SMX-HA to SMX) isoform expression may contribute to SMX HS. Since drug hypersensitivity reactions are thought to be immune mediated ADRs, it is likely there would be association with certain HLA alleles. Examples for strong associations were found between the HLA-B*5701 and abacavir hypersensitivity, as well as HLA-B*1502 and carbamazepine-induced SJS/TEN in Han Chinese patients (Mallal et al., 2002; chung et al., 2004; Teraki et al., 2010; Elzagallaai et al., 2011). Also, another research group has reported a possible link between HLA-B22 haplotype and SMX-TMP-induced fixed drug eruption in Turkey (Ozkaya-Bayazit and Akar, 2001). To date, there is no research that has investigated polymorphisms of TCR as a risk factor in T-cell mediated hypersensitivity.

1.3 OXIDATIVE STRESS

1.3.1 Reactive Oxygen Species (ROS)

Oxidative stress is a condition defined by an imbalance between increased levels of reactive oxygen species (ROS) and a low activity of antioxidant mechanisms (Sies, 1991). ROS, which are continuously produced during aerobic metabolism, are needed for adequate cell function, including the production of energy by the mitochondria and functioning as essential signaling molecules to maintain the cellular redox balance (Filomeni and Ciriolo, 2006). ROS are generated both from endogenous sources, such as enzymes activity, and external sources, such as ultraviolet radiation. Many physiological

conditions, such as host defense or aging, and pathological conditions, such as neurodegenerative diseases and diabetes, are associated with the accumulation of high levels of ROS (Kohen and Nyska, 2002; Rahimi et al., 2005). Furthermore, a number of skin diseases have been associated with oxidative stress, including psoriasis, skin cancer and allergic contact dermatitis (Dimon-Gadal et al., 2000; Matés et al., 2000).

ROS are formed in biological system by the reduction of molecular oxygen (O_2). The generation of ROS, such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{\cdot}), is associated with respiration. $O_2^{\cdot-}$ is the primary metabolite of oxygen and undergoes dismutation to generate H_2O_2 either spontaneously or when catalyzed by superoxide dismutase (SOD). After that, H_2O_2 can undergo the Fenton reaction to form the highly cytotoxic OH^{\cdot} (Schenkman, and Greim, 1993). Increased concentrations of H_2O_2 are harmful for cells; however, these cells are equipped with defense systems that detoxify or remove ROS. These systems include scavenger enzymes, such as Cu/Zn-superoxide dismutase (SOD), catalase (CAT) and cellular glutathione peroxidase (GPX), and non-enzymatic antioxidants, such as ascorbate (vitamin C) and glutathione (GSH) (Matés et al., 1999).

1.3.2 Drugs induced Oxidative Stress

Drugs are also considered to be a main source of ROS (kohen and Nyska, 2002). There are many drugs whose mechanism of action is associated with production of ROS, and those that produce ROS indirectly. It has been established that CYP450 enzyme-mediated metabolism of many drugs accounts for the major production of ROS and inducing oxidative stress (Zangar et al., 2004). For example, acetaminophen, upon overdose, is metabolized by cytochrome P450 isozymes, producing N-acetyl-p-

benzoquinone imine (NAPQI). The latter is conjugated with and depleting GSH resulting in oxidative stress and subsequent necrosis of hepatocytes. This can result in hepatic failure and death of the patient (James et al., 2003; Rousar et al., 2012). Indeed, N-acetylcysteine (NAC), a non-toxic synthetic thiol antioxidant, has been used clinically as an antidote for acetaminophen overdose.

Other types of medicinal drugs are redox-cycling substances that undergo a redox cycle reactions forming deleterious ROS. They have an original oxidative form and are considered to be direct generators of ROS. These drugs are able to induce toxicity/adverse effects via transformation to highly-active radical intermediates, usually through reducing half-action. An example of these drugs is nitrofurantoin (NFT) which is an antibiotic used for treatment of urinary tract infection. However, its uses have been restricted because of the fear of inducing pulmonary and hepatic toxicity. It is believed that NFT can induce toxicity by undergoing single-electron reduction on its nitro group. The highly reactive intermediate derived from NFT could cause damage to macromolecules, either by direct attack or via generation of superoxide anions (Pourahmad et al., 2001; Wang et al., 2008).

With sulfonamides, such as SMX, the metabolism by cytochrome P450 isozyme, CYP 2C9, generates the reactive metabolite, (SMX-HA) which is spontaneously auto oxidized to form the highly toxic metabolite, (SMX-NO). Indeed, the exposure of the cells to these electrophilic metabolites causes significant depletion of GSH, producing cellular oxidative stress (Cribb et al., 1991; Carr, 1994). In the process of auto-oxidation, SMX-HA also has the potential to form ROS by the reduction of a molecule of oxygen to form the reactive $O_2^{\cdot-}$, which can undergo spontaneous dismutation by SOD to generate

H₂O₂. The latter, in turn, may be fully reduced to water by catalase, or partially reduced by Fenton reaction to form the highly cytotoxic OH[•] (Zangar et al., 2004; Vyas et al., 2005). Thus, the metabolism of SMX may potentially lead to oxidative stress (Figure 7).

Importantly, hydroxylamine metabolites of both SMX and dapson (DDS), a sulfone antibiotic, were found to induce concentration- and time-dependent cytotoxicity in different types of cells tested. However; DDS hydroxylamine metabolites are proven to be more toxic and more ROS generators. This is due the fact that DDS have two N4 substituents that can generate two equivalents of hydroxylamines upon metabolism (Vyas et al., 2005; Khan et al., 2006).

Another rare but life-threatening toxic effect of antibiotic sulfonamides is hemolytic anemia, which is categorized as Type II hypersensitivity reaction. SMX is capable of inducing hemolytic anemia via a distinct mechanism that involves oxidative stress. This typically occurs in individuals with certain genetic disorders, among which the most common is glucose-6-phosphate dehydrogenase deficiency (G-6-PDD) (Beutler, 1991; Pichler, 2007). In this case the mechanism involves the depletion of NADPH, which is the source of reducing power to detoxify free radicals. NFT is also contraindicated in those patients with G-6-PDD or related genetic disorders due to the risk of life-threatening hemolytic anemia (Wang et al., 2008).

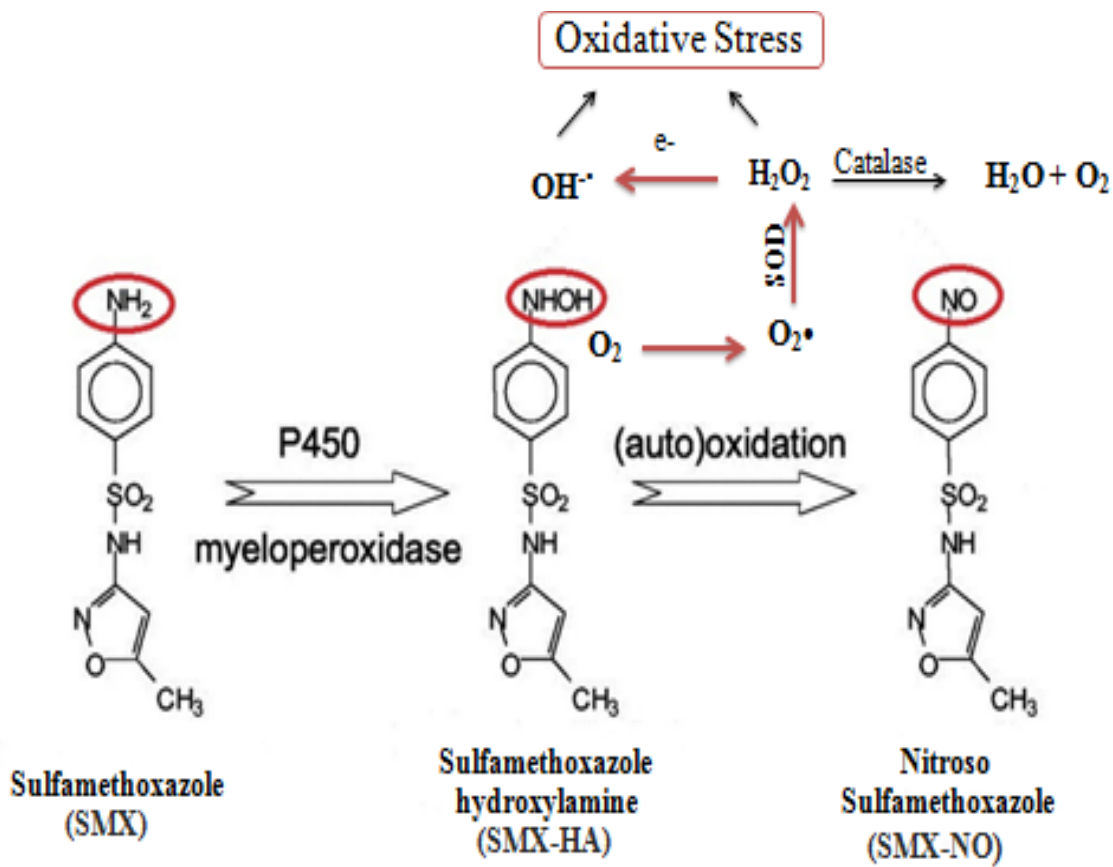
1.3.3 Triggering of Apoptosis by Reactive Oxygen Species

Apoptosis (cell death) is characterized by distinct morphological features such as shrinkage of the cell, cell surface blebbing, condensation and fragmentation of the nucleus. Also, there are distinct biochemical features such as an increase in cell density, proteolytic cleavage of proteins and nuclear DNA and exposure of phosphatidylserin on

Figure 7: Schematic representation for ROS generation and increase in oxidative stress by SMX-HA

For SMX, the process of auto-oxidation of SMX-HA to SMX-NO has the potential to generate ROS by the reduction of a molecule of oxygen to form the reactive superoxide anion radical ($O_2^{\cdot-}$) which undergoes dismutation to generate H_2O_2 . The latter can undergo a Fenton reaction to form the highly cytotoxic OH^{\cdot} . Thus, the metabolism of SMX may potentially lead to oxidative stress due to ROS formation.

Figure adapted from Vyas et al. (2005)



the cell surface (Desagher and Martinou, 2000). ROS are the most potent death stimuli faced by cells, and can induce apoptosis by different mechanisms. A longer half life of ROS can diffuse and reach a sensitive location of the cell causing selective toxicity or damaging the signaling pathway (kohen and Nyska, 2002).

There are several mechanisms proposed for the induction of apoptosis by formation of ROS. One of these mechanisms implies the ability of H_2O_2 to diffuse the mitochondria resulting in the loss of the mitochondrial membrane potential due to the opening of the mitochondrial permeability transitions pore .Subsequently, this allows the influx and efflux of important proteins, such as cytochrome c. Therefore, the increased permeability of the mitochondria leads to the apoptosis (Chandra et al., 2000; Chiaramonte et al., 2001). The other mechanism involves the upregulation of the Fas/ FasL system. The binding of FasL to its receptor triggers the initiation of the mitochondrial apoptotic death pathway by releasing of pro-apoptotic proteins (Regula and Kirshenbaum, 2005). Lastly, the formation of ROS can cause apoptosis by nuclear translocation and activation of transcription factors and the p53 tumor suppressor protein. Thus, this may drive the transcription of pro-apoptotic genes or suppress the expression of anti-apoptotic proteins.

1.4 CONCLUSION

Drug hypersensitivity reactions are a wide range of morphological reaction patterns, caused by different drugs. The physiopathology of these reactions is largely obscure. They may be immunological or non-immunological. Evidence support that these reactions are immune-mediated is largely based on the role of T cells in the pathogenesis of skin rashes and the other manifestations of the hypersensitivity reaction, as well as the presence of anti drug antibodies in some patients. For SMX, it is known that its

disposition and metabolism are related to the clinical manifestation of hypersensitivity. Under normal conditions, tissues are protected from the damaging effects of ROS by antioxidant defense systems. However, the high ROS accumulation can alter the normal levels of these antioxidant systems, and a state of oxidative stress will be reached. It is known that with SMX, ROS may be related to the toxicity seen *in vitro* in PBMCs of patients who develop hypersensitivity reactions. This is probably through the metabolism of SMX leading to increased generation of reactive intermediates which can disturb the anti-oxidant capacity of GSH. As such the imbalance between the drug bioactivation and detoxification can lead to oxidative damage of proteins and lipids, as well as consumption of GSH level. Vyas et al. (2005) have concluded that both the generation of SMX highly reactive metabolites and the inducing of oxidative stress may play an important role in inducing cell death even though they may not be directly correlated. Thus, more studies are needed to determine the exact role of ROS in inducing cell toxicity as well as their relationship with the immunological response. In addition, estimation of oxidative stress parameters may give unique and valid markers of adverse drug reactions.

CHAPTER 2: RESEARCH HYPOTHESIS AND OBJECTIVES

Drug hypersensitivity reactions have been implicated as a major problem for patients, clinicians and health care systems. The pathogenesis of these reactions is still unclear. Studies have shown that the imbalance between detoxification and metabolic activation is thought to be a key component of individual susceptibility at least for the sulfonamides. SMX, a drug which is an antimicrobial agent, is known to develop this type of reactions. The *in vitro* cytotoxicity of SMX-HA in peripheral blood mononuclear cells (PBMCs) has been used as a predictive tool to diagnose delayed-type hypersensitivity reactions associated with these drugs. Additionally, the reactive metabolites of this drug increase production of ROS which may affect the natural antioxidant defense system of the cell, such as a depletion of glutathione (GSH) (Rieder et al., 1995). The increased level of ROS leads to oxidative stress, which may cause oxidative modifications to lipids, nucleic acids and proteins. This can definitely cause cell death and develop SMX allergy.

Research Hypothesis

The hypothesis of this research is that there are differences in cytotoxicity and expression of oxidative stress to reactive SMX metabolites in the cells of patients who have sustained sulfonamide hypersensitivity reactions versus the cells of controls or sulfonamide tolerant patients.

Research Objectives

1. Perform *in vitro* toxicity testing to determine the cells viability for a number of sulfamethoxazole hypersensitive patients, sulfamethoxazole tolerant individuals,

as well as healthy volunteers, by using two *in vitro* diagnostic tests called lymphocyte toxicity assay (LTA) and *in vitro* platelet toxicity assay (iPTA).

2. Investigate the role of differential ROS induced by SMX and SMX-HA in PBMCs, and to study the state of oxidative stress in allergy.
3. Quantify protein carbonyl content and lipid peroxide levels as complementary assays for oxidative stress
4. Measure the reduced GSH level in PBMCs treated with SMX and SMX-HA, and to show whether the variability in GSH could be associated with individual differences.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials and Chemicals

Sulfamethoxazole (SMX), tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl) 2, 5 diphényl-tetrazolium bromide (MTT), Hydrogen peroxide (H₂O₂), 2',7'-Dichlorofluorescein diacetate (DCFH-DA), Histopaque[®] -1077 (ficoll), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St, Louis, MO, United states). Sulfamethoxazole -hydroxylamine (SMX-HA) was synthesized in Professor M. Kerr's Lab, Department of Chemistry, University of Western Ontario, London, Ontario, Canada. The methanol was obtained from Caledon Lab Chemicals (Georgetown, ON). RPMI 1640 and trypan blue were purchased from Invitrogen™, Life Technologies Inc (Burlington, ON, Canada. Protein Carbonyl Assay kit and Lipid Peroxidation Assay Kit were purchased from Cayman Chemical Company (Ann Arbor, MI). Reduced/Oxidized Glutathione (GSH/GSSG) Assay Kit was purchased from Oxford Biomedical Research Inc. All other buffers were prepared in the lab.

3.2 Study Participants

There were three groups included in our research. The first group was sulfa hypersensitive patients (patient group) who were recruited by Dr. Rieder at the Drug Allergy Clinic at London Health Sciences Centre in London, Ontario, Canada. Referral letters were received in the lab and patients were contacted to set up an appointment for blood drawing. These patients were having a medical and clinical history of DHS because of SMX and their LTA test were positive to SMX. Overall, 26 hypersensitive patients between ages 7 and 77 were recruited. Two hypersensitive patients were male but the rest

were female (Table 1). The second group was healthy volunteers (control group) who have denied having any history or hypersensitivity reactions or have not been exposed to SMX (overall 13 controls). The third group was 6 sulfa tolerant patients (tolerant group) who were taking SMX and who have not developed hypersensitivity reactions. Informed consent was obtained verbally from each patient by Dr. Rieder during their clinic visit.

3.3 Blood Collection and Cells Isolation

Venous blood samples from all participants were collected into heparinized syringes and separated immediately. To isolate the cells, blood was diluted 1:1 with phosphate-buffered saline (PBS, 10mM NaH₂PO₄, 2mM KH₂PO₄, 137mM NaCl, 2.7mM KCl; pH 7.2). PBMCs were isolated by layering ≤ 30 ml of blood over 15 ml of Ficoll-Paque density gradient, which was then centrifuged at 500 g for 20 min. The interface layer (grayish layer that contains the lymphocytes) between dilute plasma and Ficoll was collected and added to a clean tube. Cells were washed twice with PBS and adjusted, by using the trypan blue exclusion method, to 1×10^6 cell/mL in HEPES buffered saline (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (15mM HEPES, 125mM NaCl, 6mM KCl, 1.2mM MgSO₄, 1.0 mM NaHCO₃, 1.0mM CaCl₂, 10mM glucose; pH 7.4). Trypan blue is a vital stain used to colour dead tissue or cells blue to estimate the proportion of living cells in a population. The basis of this assay is that this dye does not react with the cell unless the membrane is damaged.

To collect platelets, blood samples were centrifuged at 200 x g for 15 min, and then the platelet rich plasma was centrifuged at 900 x g for 15 min to pellet platelets. Platelets were then washed twice with modified calcium-free Locke's solution (154mM NaCl, 2.6mM KCl, 2.14mM K₂HPO₄, 0.85m M KH₂PO₄, 1.2mM MgCl₂, 10mM glucose, and

Table 1: Characteristics of patients with SMX hypersensitivity reactions and the assays that used for each one.

Patient	Age	Sex	Manifestation	LTA	iPTA	ROS	PC	LP	GSH
1	61	F	Pruritus, swollen throat	+	+	-	-	-	-
2	30	F	Rash	+	+	-	-	-	-
3	6	F	Rash, alteration in urinary pattern	+	+	+	-	-	-
4	17	M	Mild skin rash	+	+	+	-	-	-
5	31	F	Rash, sever urticaria and pruritus	+	+	+	-	-	-
6	6	F	Full- body rash, hive like rash and pruritic	+	+	+	-	-	-
7	60	F	Rash, asthma	+	+	+	-	-	-
8	40	F	Urticaria	+	+	+	-	-	-
9	43	F	No clinical data found	+	+	+	-	+	-
10	29	F	Urticaria , swelling of the lips	+	+	+	-	+	-
11	53	F	Vasculitic rash with swelling of hands and feet	+	+	+	+	+	-
12	59	F	Rash	+	+	+	+	+	-
13	58	F	Pruritic rash	+	+	+	+	+	-
14	69	F	Rash	+	+	+	+	+	-
15	76	F	not remember the adverse events	+	+	+	+	+	-
16	53	F	Rash	+	+	+	+	+	-
17	57	F	Erythematous skin rash	+	+	+	+	+	-
18	29	F	Rash	+	+	+	+	+	-
19	50	F	No clinical data found	+	+	+	+	+	+
20	66	F	Itchy rash	+	+	+	+	+	+
21	38	F	Itchy rash	+	+	+	+	+	+
22	14	M	No clinical data found	+	+	+	+	+	+
23	45	F	Rash, difficulty with breathing and chest congestion	+	+	+	+	+	+
24	60	F	Erythematous skin rash	+	+	+	+	+	+
25	18	F	Itchy rash, organ involvement.	+	+	+	+	+	+
26	13	F	No clinical data found	+	+	+	+	+	+

(+) = if test done; (-) = if test not done

Abbreviations: **LTA**= Lymphocyte toxicity assay, **iPTA**= *in vitro* platelets toxicity assay, **ROS** (reactive oxygen species), **PC** (protein carbonyl content), **LP**(lipid peroxide level), **GSH** (glutathione).

2.0mM EGTA; pH 7.2) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells density was adjusted to 1×10^8 cell/mL in calcium free Locke's solution. All procedures were performed in an aseptic condition in a laminar flow hood at room temperature. All solutions were also brought up to room temperature before use unless stated otherwise.

3.4 Assessment of Cells Viability and Immune sensitization

Two *in vitro* toxicity tests (lymphocyte toxicity assay and *in vitro* platelets toxicity assay) were used to measure and assess the cytotoxicity that can be caused by SMX metabolites.

3.4.1 Lymphocyte Toxicity Assay (LTA)

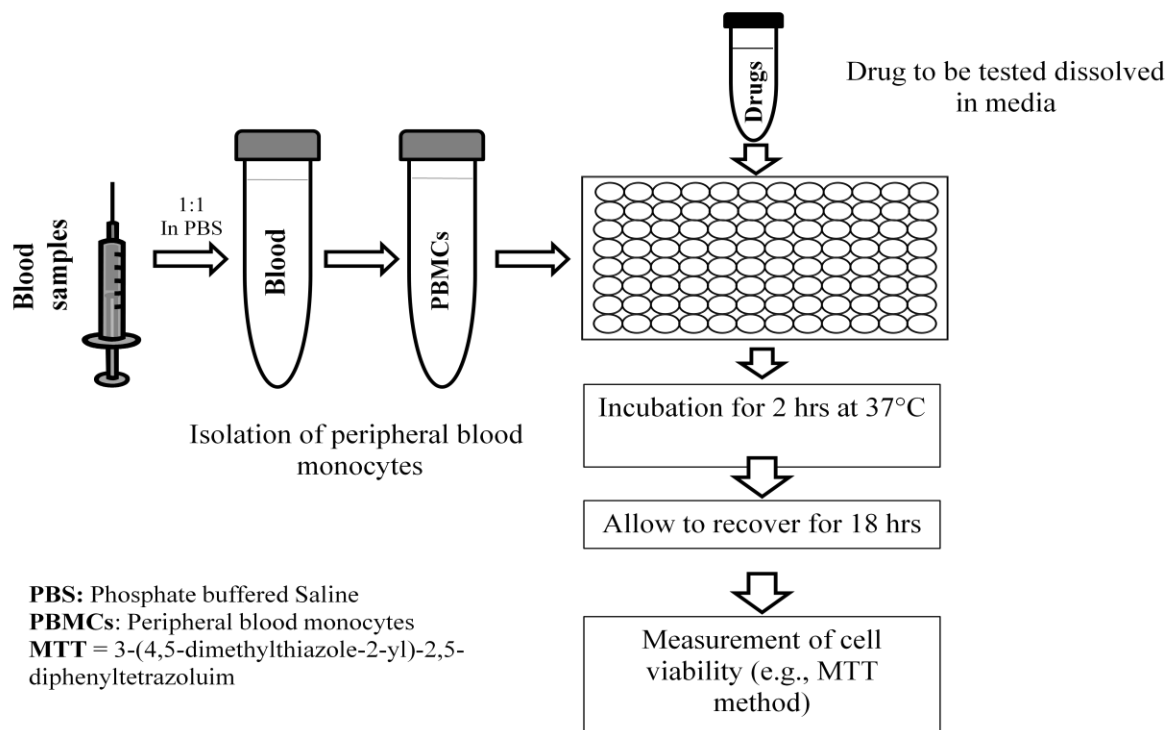
The LTA is a research tool that has been used to investigate the metabolic pathogenesis of HSRs to SMX for many years (Rieder et al., 1989; Carr et al., 1993; Neuman et al., 2000). The principle of this test is based on the reactive metabolite hypothesis which states the imbalance between metabolic activation and detoxification which leads to accumulation of toxic reactive metabolites correlates with drug hypersensitivity reactions. This diagnostic test also is based on the observation that the lymphocytes (PBMCs) from hypersensitive patients express a higher degree of cell death when exposed to the culprit drug metabolites (SMX-HA) than cells from healthy volunteers or drug naïve patients. Our goal here is to identify the percentage of cell death for each subject, to show that the incubation of their cells with the reactive metabolites, SMX-HA, cause more cell death than with the parent drug, SMX, and to assess their sulfa allergy reactions.

The conventional LTA was performed as described previously (Shear and Spielberg, 1988). Briefly, lymphocytes were treated at 1×10^5 per well in quadruplicate with final well concentrations of SMX at 200 μM and varying concentration of SMX-HA of 25, 50, 100, 200, 400, or 800 μM in a Falcon[®] flat-bottom tissue culture 96- well plate for 2 hr at 37°C and 5% CO₂ humidified environment. SMX and SMX-HA were freshly prepared in 3% dimethyl sulphoxide (DMSO) to obtain the final well concentration of 1% DMSO. A standard curve was generated by seeding the cells at 25%, 50%, and 100% of cell concentration in HEPES buffer in quadruplicate. After incubation, drugs were removed by centrifugation at 500 x g for 10 min, and cells were suspended in 100 μL RPMI1640 media supplemented with 10% FBS, 100U/mL penicillin G sodium and 100 $\mu\text{g}/\text{mL}$ streptomycin sulfate (P/S), and incubated for 18 h in an atmosphere of 5% CO₂ at 37°C to allow for recovery of the cells. Cell viability was quantified using MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium] staining. The MTT was diluted to a concentration of 5mg/mL in HEPES, and 25 μL was added to each well. Plates were then incubated for 4 h and the reaction was stopped by adding 100 μL of stop solution [50% N, N-dimethylformamide (DMF) and 20% sodium dodecyl sulphate (SDS)]. After that the plates were left overnight at room temperature, and protected from light. The MTT Assay is a colorimetric assay based on the ability of mitochondria of viable cells to reduce the soluble yellow tetrazolium salt to purple formazan crystals by mitochondria succinic dehydrogenase; however, non viable cells do not cause this conversion (Bellamy, 1992; Berridge and Tan, 1993). Thus, the number of viable cells is dependent on the activity of mitochondrial succinic dehydrogenase that converts the yellow dye to purple formazan. The absorbance of the plates was measured at 590nm

Figure 8: Procedures of the lymphocytes toxicity assay (LTA).

This assay was used to assess the cytotoxicity that can be caused by SMX and SMX-HA, and to confirm the sulfa hypersensitivity reactions. Briefly, the procedures for this test involve the incubation of lymphocytes with SMX and SMX-HA at different concentrations. The percentage of cell death is then determined using the MTT technique.

Figure adapted from Elzagallaai et al. (2009)



using a Molecular Devices spectrophotometer (Vmax Kinetic Microplate Reader) (Figure 8).

3.4.2 *In vitro* platelets Toxicity Assay (iPTA)

This is a novel assay which has been introduced recently in our lab in an attempt to simplify the LTA procedures. It is based on use of platelets as a surrogate cell model to assess the *in vitro* cytotoxicity. Elzagallaai et al (2011) has demonstrated that platelets are similar to PBMCs in the way they respond to SMX. The *iPTA* was also performed as described previously (Elzagallaai et al., 2011). This test has similar procedures to LTA except for the use of platelets instead of lymphocytes as a cell model, and calcium free Locke's solution was used as a medium in the experiments. Also, platelets were treated at 1×10^7 cell/well, and plates were spun at 900 x g.

3.5 Assessment of Oxidative stress

3.5.1 Measurement of Reactive Oxygen Species Formation (ROS)

It has been shown that many drugs can be cytotoxic due to their ability to generate high levels of ROS which can cause cellular damage. To investigate the differential oxidative stress induced by SMX metabolites and to study its role in allergy, ROS formation was determined and quantified in PBMCs by using the DCFH-DA (2', 7'-dichlorofluorescein diacetate) fluorescence Assay. The principle of this assay is that this dye is transported in to the cell where DCFH-DA deacetylated, forming the non-fluorescent DCFH which is oxidized and yields a highly fluorescent DCF. An increase in the oxidation of DCFH to DCF results in an increase in DCF fluorescence which is suggestive of ROS generation primarily H_2O_2 and OH^\bullet (Gomes et al., 2005).

PBMCs were harvested and washed with PBS by centrifugation at 500 x g for 10 min. Cells were then re-suspended in Hank's balanced salt solution (HBSS), and adjusted to 1×10^6 cell/ml of HBSS using the trypan blue dye exclusion assay. After that, cells were plated at 1×10^5 cell per well in quadruplicate in a Falcon[®] flat-bottom tissue culture 96- well plate which then centrifuged at 500 x g for 7 minutes to pellet the cells and decant the HBSS solution. DCFH-DA dye was dissolved in sterilized methanol at a concentration of 0.97mg/mL, and then diluted to 1% volume/volume (10 μ M) in HBSS. Cells were re-suspended with 100 μ L of 10 μ M DCFH-DA/HBSS solution, and 100 μ L was also added to the blank (containing no cells) wells. Cells were incubated in an atmosphere of 5% CO₂ at 37°C for 1 hour. After incubation, DCFH-DA- HBSS was removed by centrifugation of the plates at 500 x g for 7 min, and 100 μ L of pure HBSS was added to each cells containing well, and the blank wells received 150 μ L of pure HBSS. A baseline reading of fluorescence (excitation 485nm; emission 527nm) was taken using a multiwell fluorescence plate reader (Fluoroskan Ascent FL, Thermo Labsystems). SMX (200 μ M) or SMX-HA at varying concentrations(50, 100, 200, 400, or 800 μ M) were added in quadruplicate. A time zero fluorescence reading was taken. Wells were incubated for 2 h at 37°C with fluorescence readings taken every 30 min. H₂O₂ (50 μ M, 100 μ M, 200 μ M, or 400 μ M) was used as a positive control and 1% DMSO as vehicle control. The data were analyzed using Microsoft Excel[™] 2007 software.

3.5.2 Measurement of Protein Carbonyl Content and Lipid Peroxide levels.

It has previously been shown that the generation of ROS in excess can lead to oxidative stress which can cause cellular damage by lipid peroxidation and by disrupting the structure of nucleic acids and proteins (Matés et al., 2000). Thus, we determined the

markers of lipid peroxidation [thiobarbituric acid reactive substances (TBARS)] and of protein oxidation (carbonyl content) in PBMCs as complementary assays for oxidative stress.

3.5.2.1 Protein Carbonyl Content

A general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content. Several approaches have been taken to detect and quantify protein carbonyl content. The Cayman's Protein Carbonyl Colorimetric Assay Kit (10005020) utilizes the 2,4-dinitrophenylhydrazin (DNPH) reaction to measure the protein carbonyl content. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically (Reznick and Packer, 1994). The amount of protein-hydrazone produced is quantified at an absorbance of 360 nm.

PBMCs were harvested and washed 2 times with PBS by centrifugation at 500 x g for 10 min. Cells were then re-suspended in HEPES, and adjusted to 2×10^6 cell/ml of HEPES using the trypan blue dye exclusion assay. Cells were then treated at 6×10^6 cells per well with 1% DMSO (vehicle control), 400 μ M H₂O₂ (positive control), 200 μ M SMX or 400 μ M SMX-HA in a Falcon[®] flat-bottom tissue culture 6 well plate for 2 h at 37°C and 5% CO₂ humidified environment. Drugs were freshly prepared in 3% DMSO to obtain the final well concentration of 1% DMSO. After incubation, drugs were removed through collection of supernatant in 5 mL polypropylene round bottom tubes and centrifugation at 500 x g for 10 min. Cells were then re-suspended in 1 mL of RPMI 1640 media supplemented with FBS and P/S and incubated for 18 h in an atmosphere of 5% CO₂ at 37°C.

After that, cells were collected by centrifugation at 500 x g for 10 min, and re-suspended in 250 μ L of homogenization buffer (50 mM potassium phosphate containing 1 mM EDTA; pH 6.7). The cells were then sonicated on ice in 1.5 mL microcentrifuge tubes. The homogenate was then spun at 10,000 x g for 10 min at 4°C, and the supernatant was transferred to clean microcentrifuge tubes and stored at -80 °C until assaying. To determine if there were contaminating nucleic acids present in the sample, the absorbance of the supernatant was taken at 280 nm and 260 nm using the homogenization buffer as a blank. If the ratio 280/260 was less than 1, a further step to remove nucleic acids with 1 % streptomycin sulfate was needed.

All reagents were equilibrated to room temperature before beginning the assay, and each sample was analyzed according to the procedure provided in the kit. Samples were thawed from -80 °C and well vortexed, and 100 μ L of the sample were then transferred to two 1.5 mL microcentrifuge tubes (sample tube and control tube). Subsequently, 400 μ L of DNPH reagent were added to the sample tubes, while 400 μ L of 2.5 M HCl were added to the control tubes. Both sets of tubes were incubated in the dark at room temperature for 1 h, and briefly vortexed every 15 min during the incubation. After incubation, 500 μ L of 20 % trichloroacetic acid (TCA) solution was added to each tube, vortexed and incubated on ice for 5 min. The tubes were then centrifuged at 10,000 x g for 10 min at 4°C in a microcentrifuge, and the supernatant was discarded. The protein pellet was then re-suspended in 500 μ L of 10 % TCA solution and incubated on ice for 5 min. The tubes were again centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was discarded. The pellet was then re-suspended in 500 μ L of ethanol/ethyl acetate (1:1) mixture, vortexed thoroughly and centrifuged at 10,000 x g for 10 min at

4°C. The washing with ethanol/ethyl acetate mixture was repeated two more times, and after the final wash, the protein pellets were re-suspended in 500 µL of guanidine hydrochloride by vortexing. The tubes were then centrifuged at 10,000 x g for 10 min at 4°C to remove any leftover debris. After that, 220 µL of the supernatant from the sample tubes and control tubes were transferred to two wells of the 96- well plate. The absorbance was measured at a wavelength 360 nm using a multi mode microplate reader (Synergy™ H4 Software Version 2.01.14).

To measure the protein carbonyl content, the average absorbance of each sample and control was calculated, and the corrected absorbance (CA) was calculated by subtracting the average absorbance of the controls from the average absorbance of the samples. The concentration of the carbonyls was determined by inserting the CA in to the following equation:

$$\text{Protein Carbonyl (nmol/ml)} = [(CA) / (*0.011 \mu\text{M}^{-1})] (500 \mu\text{L} / 200 \mu\text{L})$$

To determine the protein content in final pellets after the washes, 100 µL of the sample control (C#) were transferred from one of the wells to a 1mL quartz cuvette, and then 900 µL of guanidine hydrochloride were added. The absorbance at 280 nm of each diluted sample control was determined. The amount of protein was calculated from a bovine serum albumin (BSA) standard curve (0.25-2.0 mg/mL) dissolved in guanidine hydrochloride and read at 280 nm.

$$\text{Protein concentration (mg/mL)} = [(A_{280} - y\text{-intercept}) / \text{slope}] \times *2.5 \times 10 \text{ (dilution factor)}$$

*(*2.5 is the correction factor that adjusts the results back to the original sample).*

$$\text{Carbonyl content (nmol/mg)} = (\text{carbonyl nmol/ mL}) / (\text{protein mg/mL})$$

3.5.2.2 Lipid Peroxidation

Lipid peroxidation is a well established mechanism of cellular damage and is used as a marker of oxidative stress. Highly reactive free radical species can oxidize lipids and cause overproduction of malondialdehyde (MDA) which is a naturally occurring product of polyunsaturated fatty acids' peroxidation in the cells. The measurement of thiobarbituric acid reactive substances (TBARS) is a well established approach for detecting and verifying lipid peroxidation (Yagi, 1998). The Cayman Chemical Company's TBARS assay kit (10009055) provides a convenient tool for sensitive detection of MDA present in a variety of samples. The principle for this assay is that the MDA in the sample reacts with thiobarbituric acid (TBA), under high temperature and acidic conditions, to generate MDA-TBA adducts. The MDA-TBA adduct can be easily quantified colorimetrically at 530-540 nm, or fluorometrically (excitation 530 nm; emission 550 nm).

PBMCs were harvested and washed 2 times with PBS by centrifugation at 500 x g for 10 min. Cells were then re-suspended in HEPES, and adjusted to 2×10^6 cell/mL of HEPES using the trypan blue dye exclusion assay. Then, the cells were treated at 6×10^6 cells per well with 1% DMSO (vehicle control), 400 μ M H₂O₂ (positive control), 200 μ M SMX and 400 μ M SMX-HA in a Falcon[®] flat-bottom tissue culture 6 well plate for 2 h in an atmosphere of 5% CO₂ at 37°C. Drugs were freshly prepared in 3% DMSO to obtain the final well concentration of 1% DMSO. After incubation, drugs were removed through collection of supernatant in 5 mL polypropylene round bottom tubes and centrifugation at 500 x g for 10 min. Cells were then re-suspended in 1 mL of RPMI 1640 media supplemented with FBS and P/S and incubated for 18 hr in an atmosphere of 5% CO₂ at 37°C.

After that, cells were collected by centrifugation at 500 x g for 10 min, re-suspended in 150 μ L of PBS, and transferred to 1.5 mL microcentrifuge tubes. The cells were then sonicated 3 times for 5 second intervals over ice. The whole homogenate was then stored at -80 °C until assaying.

On the day of the experiment, all reagents were equilibrated to room temperature before beginning the assay. The standard was prepared by diluting 25 μ L of the MDA Standard with 975 μ L of distilled water to obtain a stock solution of 12.5 μ M. Then, different concentrations of MDA standards, labeled A-H, were obtained (0, 0.0625, 0.125, 0.25, 0.5, 1, 2.5 or 5 μ M MDA). Subsequently, 100 μ L of the sample or standard were added to 5 mL polypropylene round bottom tubes that were labeled with a standard number or sample identification number. Then, 100 μ L of sodium dodecyl sulfate (SDS) solution were added to each tube and vortexed. Also, 4 mL of the Color Reagent [containing 530 mg of thiobarbituric acid (TBA), 50 mL of diluted acetic acid and 50 mL of diluted sodium hydroxide) were added to each tube. Tubes were then placed in a holder, added to vigorously boiling water, and boiled for 1 hr. After boiling, tubes were removed and incubated on ice for 10 min. After 10 min, tubes were centrifuged at 1600 x g for 10 min at 4°C. To complete this assay, 150 μ L from each sample or standard were loaded in triplicate to a 96-well plate, and PBS was used as a sample blank. The fluorescence (excitation 530nm; emission 550nm) was taken using a multi mode microplate reader (Synergy™ H4 Software Version 2.01.14).

To determine the MDA level, the average fluorescence of each standard and sample was determined, and the corrected fluorescence was calculated by subtracting the fluorescence value of the standard A (0 μ M) from itself and all other values (both

standards and samples). The value of MDA for each sample was calculated from the standard curve.

$$\text{MDA } (\mu\text{M}) = [(\text{corrected fluorescence}) - (\text{y-intercept}) / \text{slope}]$$

3.6 Determination of Reduced Glutathione Content

It is known that reduced glutathione (GSH) appears important in protecting cells against toxicity of reactive metabolites, and conjugation with GSH is considered as an essential detoxification pathway as well (Cribb et al, 1991). Therefore, we determined the level of reduced GSH in the isolated PBMCs using Reduced/Oxidized Glutathione (GSH/GSSG) Assay Kit, GT-40, with modifications. This assay depends on the reaction of glutathione with DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)) forming a yellow colored product [5-thio-2-nitrobenzoic acid, (TNB)] that can be quantified spectrophotometrically at 405 or 412 nm. This assay measures total amount of glutathione (GSH + GSSG) and GSSG concentration. The quantity of GSH can be determined by subtracting the amount of GSSG from the total amount of glutathione (GSH_t). Also, this assay employs a pyridine derivative (1-methyl-2-vinyl-pyridium trifluoromethane sulfonate) as a thiol-scavenging reagent to prevent the oxidation of GSH to GSSG during sample preparation (Tietze, 1969).

PBMCs were harvested and washed 2 times with PBS by centrifugation at 500 x g for 10 min. Cells were then re-suspended in HEPES, and adjusted to 2×10^6 cell/ml of HEPES using the trypan blue dye exclusion assay. Cells were then plated at the concentration of 2×10^6 cells per well, and incubated with 1% DMSO (vehicle control), 200 μM SMX, 200 μM SMX-HA or 400 μM SMX-HA in a Falcon® flat-bottom tissue culture 12 well plate for 2 h in an atmosphere of 5% CO₂ at 37°C. Drugs were freshly

prepared in 3% DMSO to obtain the final well concentration of 1% DMSO. Subsequently, drugs and HEPES were removed by centrifugation at 500 x g for 10 min. Cells were then allowed to recover in 500 μ L of RPMI media for 18 h in an atmosphere of 5% CO₂ at 37°C. After that, cells were collected in microcentrifuge tubes and washed with cold PBS. For GSH sample, cells were lysed by sonication in 200 μ L of cold buffer (containing 50mM potassium phosphate, 1mM EDTA, pH=7). Tubes were then centrifuged at 10,000g for 10 min at 4°C, and the supernatant was transferred to a clean tube. The same procedures were followed for preparing GSSG samples except 20 μ L of Scavenger were added to the buffer for cell lysis. Because almost all biological samples used for GSH quantitation may contain large amounts of proteins, it was essential to remove those proteins from the sample to avoid the interference due to sulfhydryl groups of proteins in the assay. Therefore, the supernatant was deproteinated by adding an equal volume of 5 % metaphosphoric acid (MPA) to the sample, mix it on a vortex mixer, and allowed to stand at room temperature for 5 min. Subsequently, tubes were centrifuged at 2000 x g for 5 min, and the supernatant was collected without disturbing the precipitate. Samples were stored at – 80°C if not assaying on the same day.

On the day of the experiment, 100 μ L of both sets of samples were added to 500 μ L of Assay Buffer in a new microcentrifuge tube. All chemical solutions were prepared according to the procedures in the kit. Briefly, DTNB and NADPH were reconstituted with 500 μ L of Assay Buffer, and then diluted with 5.5 mL of Assay Buffer and vortexed. Also, 30 μ L of glutathione reductase were diluted with 6 mL of Assay Buffer, and left on ice until using. Standards were prepared within a concentration range from 0 to 3 μ M of GSH and 0 to 1.5 μ M of GSSG in Assay Buffer. After that, 50 μ L of

standards, samples, or blank (Assay Buffer) were added to the corresponding wells, in triplicate, on a 96 -well plate. To each well, 50 μ L of DTNB and glutathione reductase were added using a multiple-channel pipettor. Plates were then placed on an orbital shaker for mixing, and incubated at room temperature for 5 min. Subsequently, 50 μ L of NADPH solution were added to each well. The absorbance at 405 nm was taken every minute for 10 min using a kinetic plate reader (Multiskan Ascent, Thermo Labsystems).

To calculate the concentration of GSSG and GSH in the sample, the average absorbance of each standard and sample was plotted as a function of time. The slope of each curve, which is equal to the rate, was determined. The calibration curves were then determined by plotting the Net Rate as a function of the concentration of GSSG or GSH. Net Rate is the difference between the sample or standard rate and the blank rate. The concentration of the total GSH (GSH_t) or GSSG in the sample as well as the reduced GSH was determined using the following equations:

$$GSH_t \text{ or } GSSG = (\text{Net Rate} - \text{Intercept})/\text{Slope} \times \text{Dilution factor}$$

$$\text{Reduced GSH} = GSH_t - 2GSSG$$

3.7 Statistical Analysis

Statistical analysis was performed using the SPSS Statistics program, version 23. Data are expressed as percentages of vehicle control cells except for the glutathione assay. The area under the curve (AUC) of ROS formed over time (5-120 min) was calculated. Significant differences were determined by either a t-test or repeated measures analysis of variance (rmANOVA) where appropriate. A probability of more than 95% ($P < 0.05$) was considered statistically significant. Correlations were made using Pearson correlation analyses. Values are presented as mean \pm standard error (SE).

CHAPTER 4: RESULTS

4.1 Assessment of Cell Viability by the Lymphocyte Toxicity Assay (LTA) and the *in vitro* Platelets Toxicity Assay (iPTA)

To confirm the patient's susceptibility to SMX, and to determine the cell viability for each SMX-HS patient, tolerant patient or healthy subject, two *in vitro* diagnostic tests (LTA and iPTA) were employed. Briefly, cells (PBMCs or platelets) were incubated with SMX (200 μ M) or varying concentrations of SMX-HA (25 to 800 μ M). After incubation, drugs were discarded and cells were resuspended in RBMI 1640 media for 18 hr to allow for the recovery of the cells. Viability was determined spectrophotometrically using MTT method.

The exposure of lymphocytes or platelets to the parent drug, SMX, did not induce a harmful effect (no toxicity). However, the reactive metabolites, SMX-HA, caused a dose-dependent cytotoxicity (for all groups). With both assays, hypersensitive patients had significantly less cell viability (i.e. higher cytotoxicity) than the controls or tolerant group at SMX-HA 100,200, 400 or 800 μ M concentrations (Figure 9A and 9B). The LC_{50} values of SMX-HA were also calculated in case of both assays. As expected, SMX-HS patients had significantly the lower LC_{50} than controls ($P < 0.001$) and SMX tolerant patients ($P < 0.0001$) (Table 2). Thus, our data had approved that these *in vitro* cytotoxicity assays, LTA and iPTA, have predictive value in diagnosing SMX hypersensitivity in which total cytotoxicity is the standard reported outcome.

Figure 9: Assessment of cell viability by LTA and *i*PTA.

LTA (A) and *i*PTA (B) for sulfa hypersensitive patients (n=26), controls (n=19) and sulfa tolerant patients (n=6). PBMCs or platelets were isolated and incubated with increasing concentration of SMX-HA or 200 μ M SMX for 2 hr. The experiments were conducted in quadruplicate, and cell death was then determined using the MTT method and expressed as percentages of control (vehicle without drug). Results are presented as mean \pm S.E. (*P < 0.05, **P < 0.01, ***P < 0.001) as compared to the patients group.

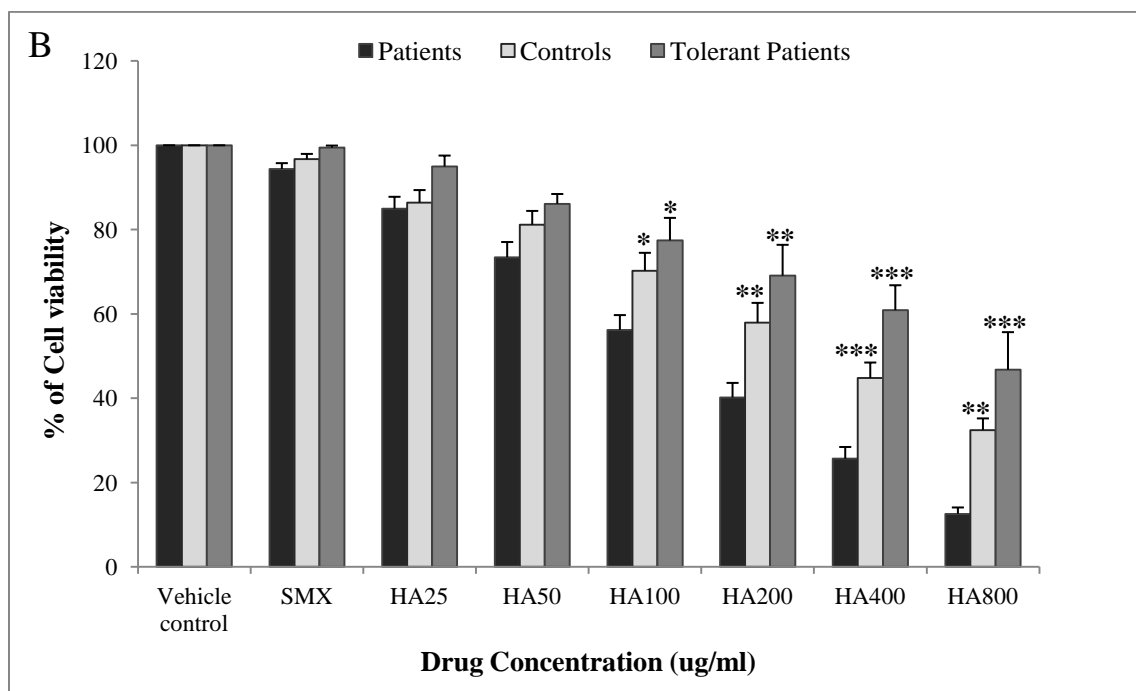
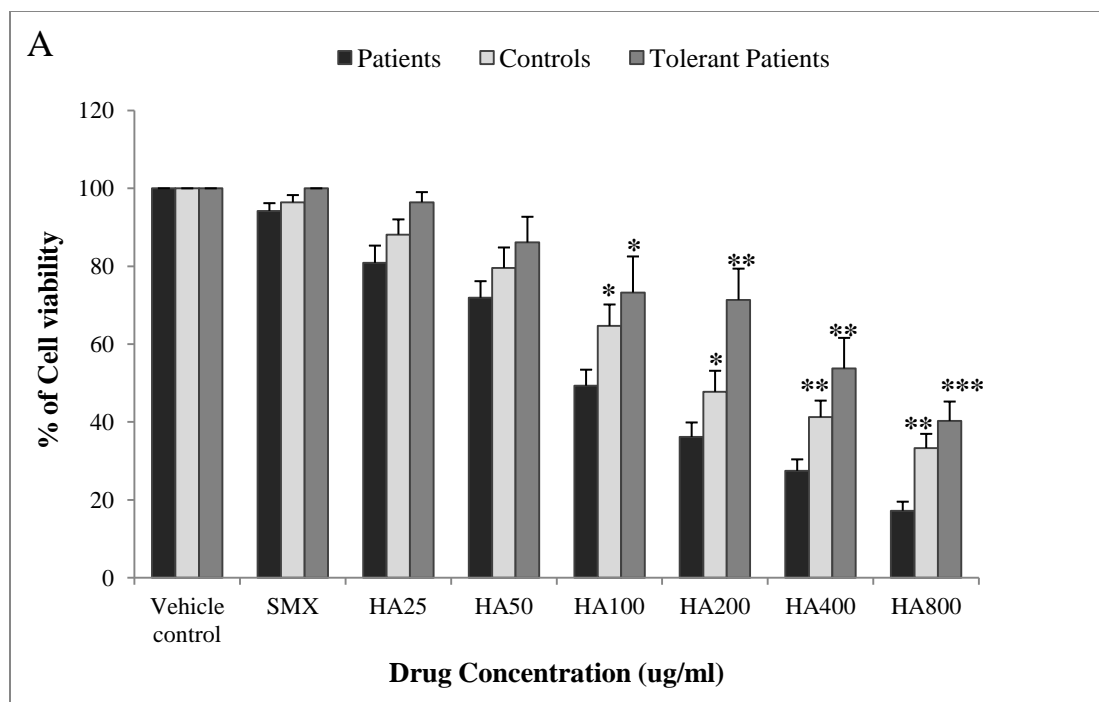


Table 2: LC₅₀ values of SMX-HA for study participants in both assays (LTA and *i*PTA).

Data are presented as mean \pm S.D, and expressed as a percentage of vehicle control cells.

*P < 0.001, **P < 0.0001 as compared to patients group.

LC₅₀	Patients (n = 26)	Controls (n=19)	Tolerant (n=6)
LTA	121.8 \pm 6.11	243.9 \pm 5.38*	513.8 \pm 7.84**
<i>i</i>PTA	133.9 \pm 5.13	300.9 \pm 9.01*	610.9 \pm 8.91**

4.2 Measurement of Oxidative Stress Parameters

4.2.1 Quantification of Reactive Oxygen Species (ROS)

To determine the contribution of ROS in SMX-HA induced cytotoxicity, and to show whether hypersensitive patients exhibit higher ROS levels than other groups, the 2', 7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence assay was employed. PBMCs were loaded with DCFH-DA dye for 1 h, and then the dye was removed. The baseline reading was taken using a fluorometer set to excitation at 485nm and emission at 527nm. Cells were incubated with SMX (200 μ M) or varying concentrations of SMX-HA (25 to 800 μ M) and H₂O₂ (50, 100, 200, or 400 μ M) was used as a positive control. Fluorescence was measured every 30min for 2 h; however, due to the difficulty to read the plates immediately after adding the drugs, a 5 min reading was considered instead of a 0 min reading. The amount of ROS formed was expressed as a percentage of control (vehicle without drug).

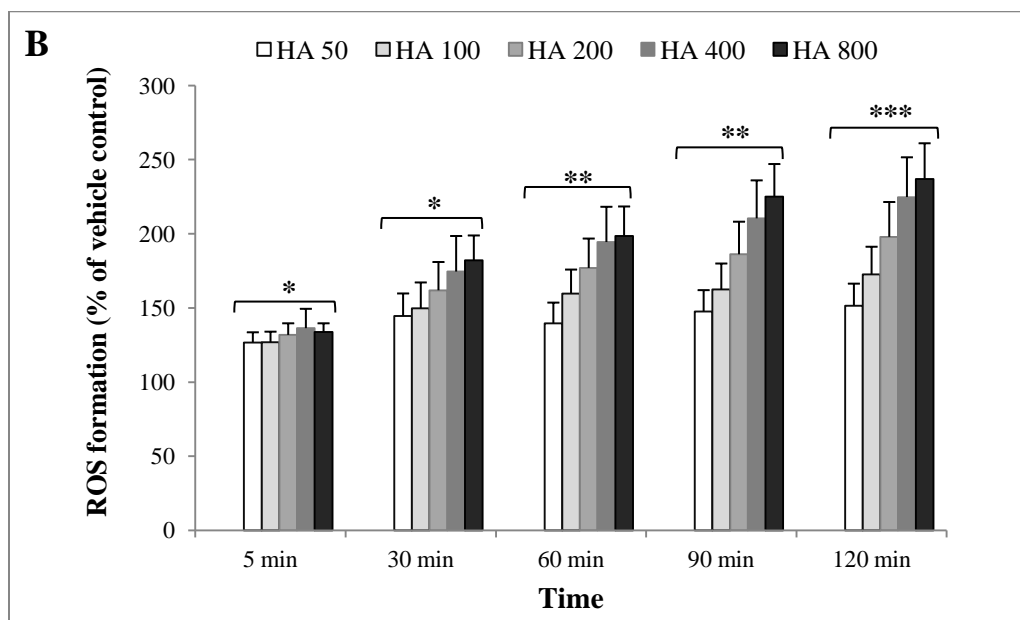
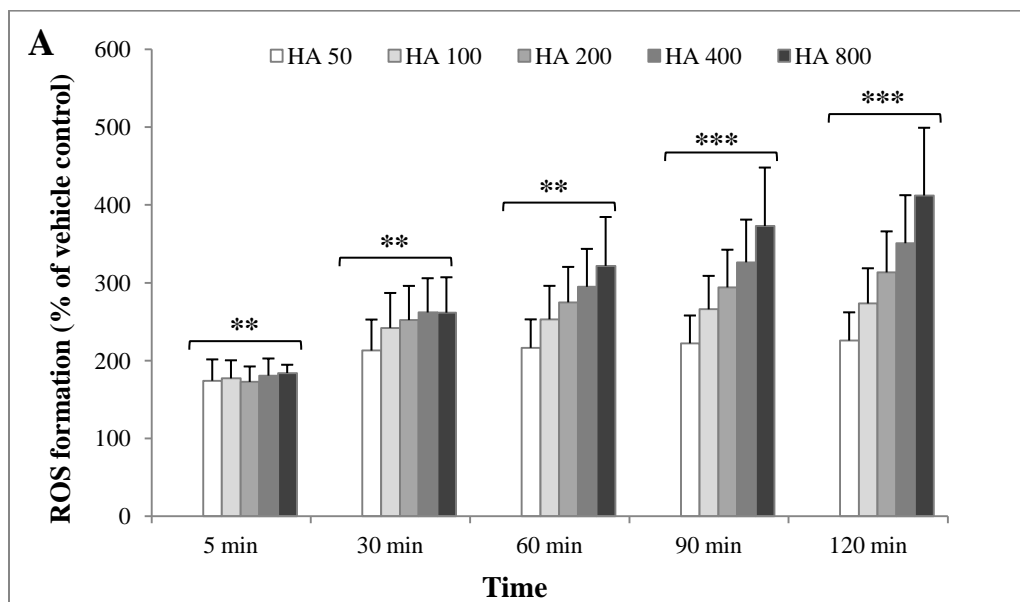
The treatment of PBMCs with SMX did not alter the amount of ROS formed during incubation times (data not shown). However, with the experimental concentrations of SMX-HA of 50 to 800 μ M, there was a significant increase in the ROS formation for all study groups at each time point as compared to the corresponding vehicle control (Figure 10A, 10B and 10C). Also, the amount of ROS continued to increase as incubation times increased, this is an indicative of a time dependency in ROS formation associated with SMX-HA treatment.

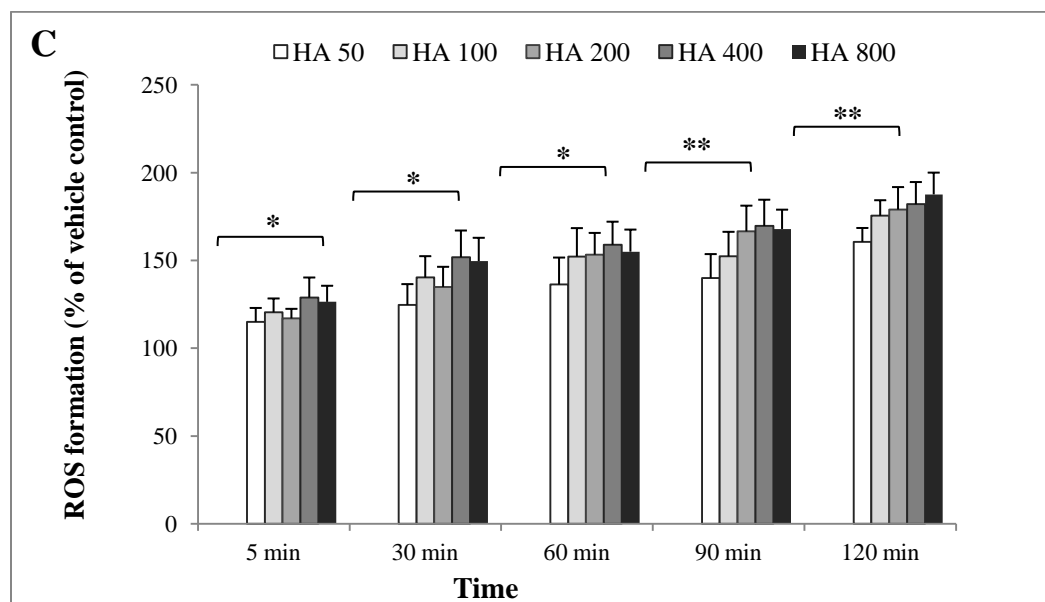
The area under the curve (AUC) was used to quantify the amount of ROS that formed over time (5 to 120 min) by exposure to SMX-HA metabolites. As expected, lymphocytes from sulfa sensitive patients accumulated higher levels of ROS at the more

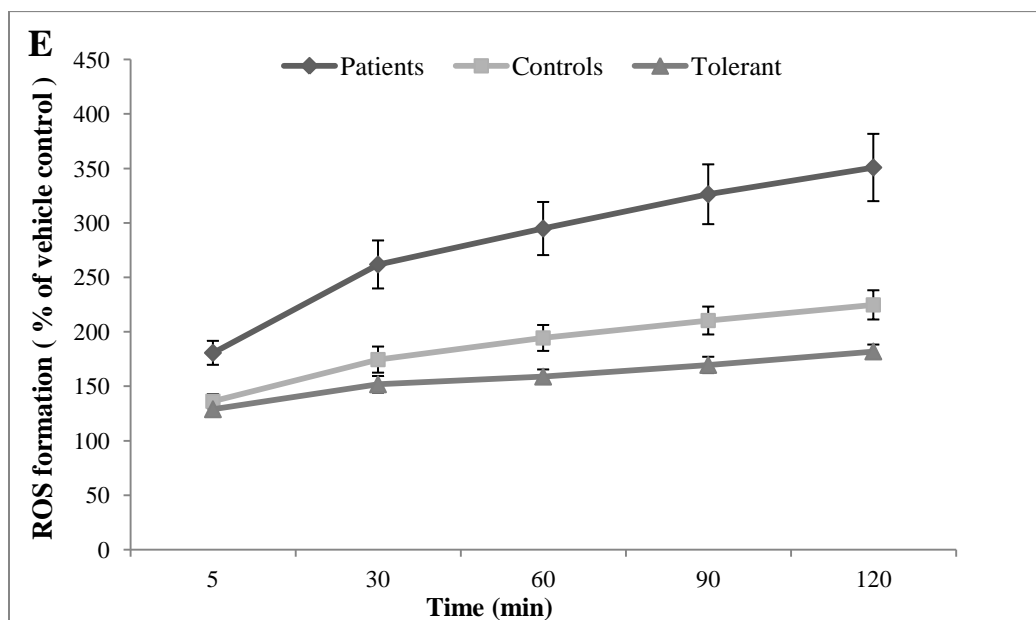
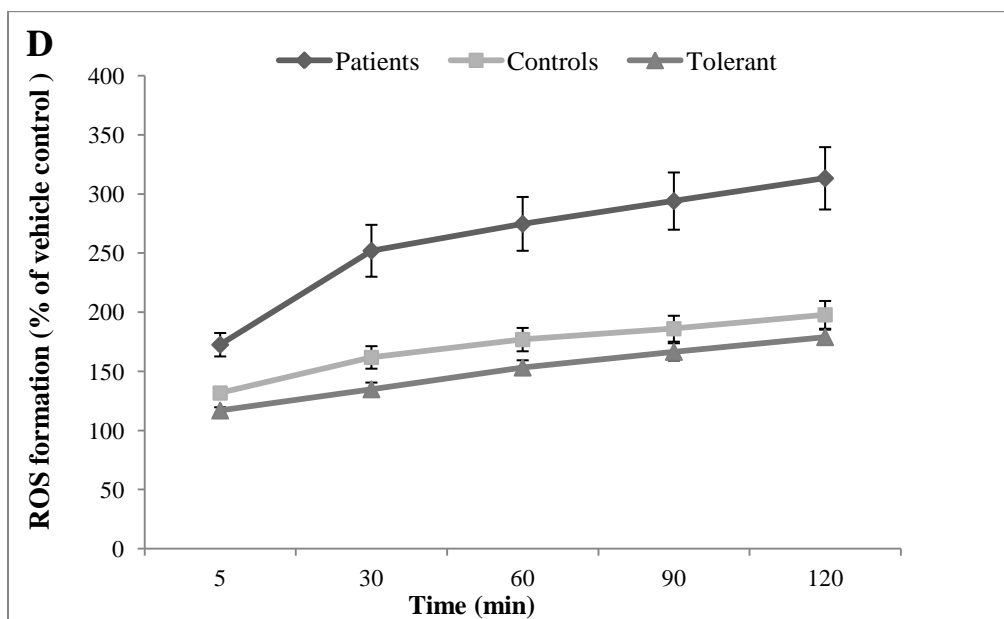
Figure 10: Quantification of ROS produced by lymphocytes upon their incubation with varying concentrations of SMX-HA.

Using the DCFH-DA fluorescence Assay (2', 7'-dichlorofluorescein diacetate), DCFH is oxidized and yields a highly fluorescent DCF (excitation 485nm; emission 527nm) which is used as indicator for ROS formation.

- The amount of ROS formed over incubation times by lymphocytes of SMX hypersensitive patients (n=24) [A], healthy volunteers (n=16) [B], and SMX tolerant patients (n=6) [C] when treated with SMX-HA (50 to 800 μ M). Values are presented as mean \pm S.E. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the corresponding vehicle control.
- The amount of ROS formed by the PBMCs of all study participants upon incubation with 200 μ M SMX-HA [D] or 400 μ M SMX-HA [E]. Data were expressed as a percentage of vehicle control. The area under the curve (AUC) over time (5-120 min) was calculated and the differences between patients and controls, or patients and the tolerant group were determined by independent t-tests. Values are presented as mean \pm S.E.







relevant physiological SMX- HA concentrations (i.e. 200 and 400 μM) compared with other groups (Figure 10D and 10E). AUC_{5→120} of ROS that formed upon incubation of cells with SMX-HA 200 or 400 μM for the patient group (AUC HA200=527.8112%; AUC HA400=638.0%) was significantly greater than for the control (AUC HA200=248.1%; AUC HA400=331.6%; $P=0.0001$), and tolerant group (AUC HA200=232.55%; AUC HA400=198.9%; $P<0.001$) (Table 3). No significant correlation was shown between the amount of ROS formed and percentage of cell death when lymphocytes from allergic patients were incubated with 200 μM SMX-HA ($p=0.99$, $r=0.002$) and 400 μM SMX-HA ($p=0.17$, $r=-0.29$) (Figure 11A and 11B).

4.2.2 Measurement of Protein Carbonyl Content

The carbonyl content, which is the index of protein oxidative damage, was determined in the lymphocytes of the study participants. Cells were treated for 2 h with 200 μM SMX or 400 μM SMX-HA using H_2O_2 as a positive control and DMSO (1 %) as a vehicle control. Protein carbonyl content was determined through DNPH reaction with protein carbonyl groups, and expressed as the percentage of vehicle control.

There was no significant difference in protein carbonyl content over incubation of PBMCs with SMX (parent drug) as compared to the corresponding vehicle control. However, all groups' cells that were exposed to 400 μM SMX- HA showed significantly ($P<0.05$) higher carbonyl content. This observation explains the role of drug metabolism in inducing oxidative stress through the damaging of cellular proteins. Interestingly, hypersensitive patients significantly ($P<0.05$) expressed higher protein carbonyl content as compared to tolerant patients upon incubation their cells with 400 μM SMX- HA (Figure 12).

Table 3: Area under the curve (AUC) of ROS over time (5-120 min) for study participants upon incubation of their lymphocytes with SMX-HA 200 or 400 μ M.

Data are presented as mean \pm S.D, and expressed as a percentage of vehicle control cells.

*P < 0.001, **P < 0.0001 as compared to patients group.

AUC_{5→120}	Patients (n = 24)	Control (n = 16)	Tolerant (n = 6)
SMX-HA 200 μ M	527.8 \pm 213.2	248.0 \pm 78.8**	232.5 \pm 26.5*
SMX-HA 400 μ M	638.0 \pm 232.1	331.6 \pm 96.1**	198.9 \pm 30.9*

Figure 11: Correlation between the amount of ROS and the percent of cell death for allergic patients.

The amount of ROS formed and the cytotoxicity of SMX-HA 200 μM (A) or 400 μM (B) measured after a 2 hr of incubation. [(p = 0.99, r = 0.002) ; (p = 0.17, r = -0.29); respectively].

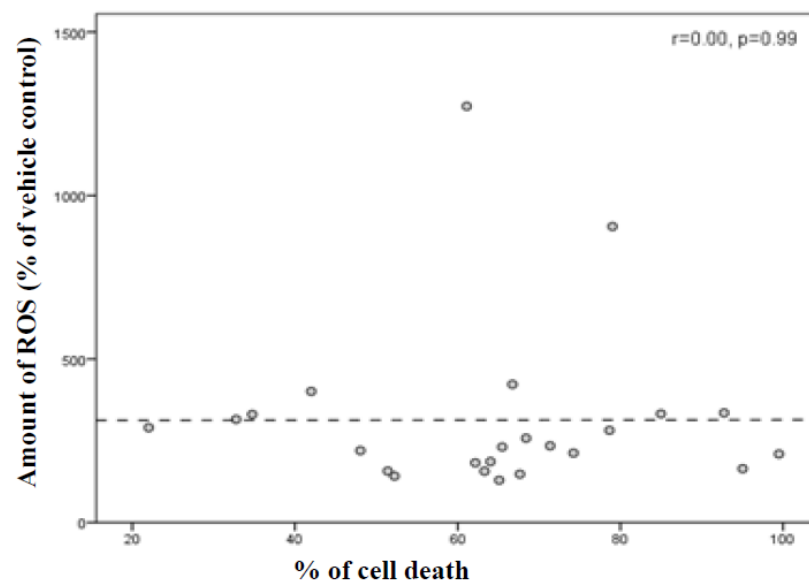
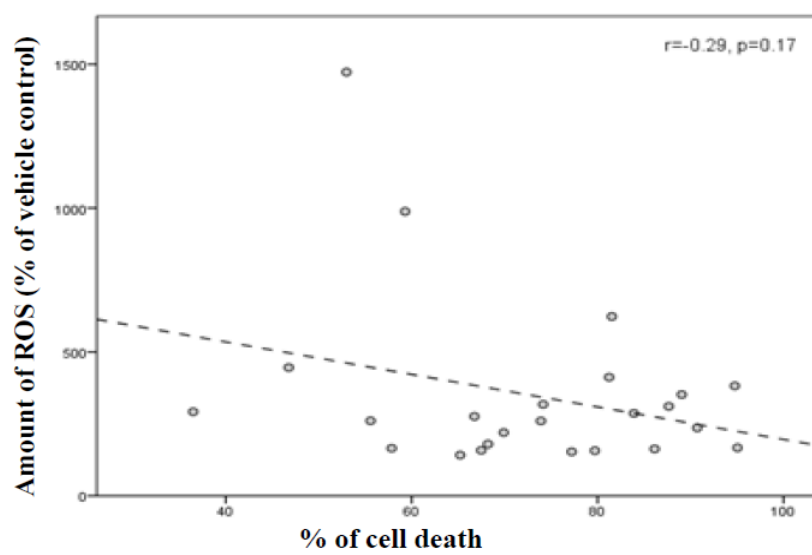
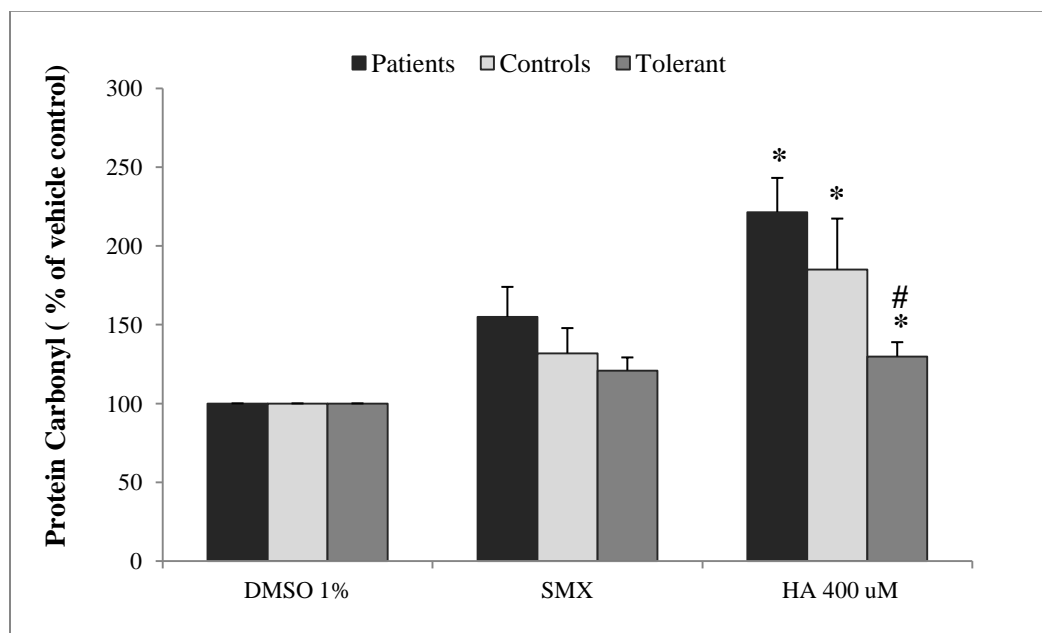
A**B**

Figure 12: Protein carbonyl content of lymphocytes treated with SMX (200 μ M) or SMX-HA (400 μ M).

Lymphocytes of participants were treated with 200 μ M SMX or 400 μ M SMX-HA for 2 h. Drugs were then removed and cells were incubated in RPMI media for 18 h. Cell lysates were used for this assay. Protein carbonyl content was determined through DNPH reaction with carbonyl groups, and expressed as a percentage of vehicle control. Data are presented as mean \pm S.E (n= 16 sulfa sensitive patients, 14 controls and 6 sulfa tolerant patients). *P < 0.05 compared with the corresponding vehicle control. # P < 0.05 compared with hypersensitive patients group for the same treatment.



4.2.3 Lipid Peroxidation

The lipid peroxides level was also determined as a complementary assay (to protein oxidation) for oxidative stress. The TBARS level, which is the index of lipid peroxidation, was measured in PBMCs to show whether SMX metabolites cause increasing in lipid peroxide concentration or whether lymphocytes of allergic patients exhibit higher degrees of membrane lipid peroxidation than others. Cells were treated for 2 hr with 200 μ M SMX or 400 μ M SMX-HA. Lipid peroxidation was assessed through the reaction of MDA with TBA forming MDA-TBA adduct.

The lipid peroxide content was expressed as a percentage of vehicle control. For all groups, cells treated with SMX showed no trends towards significant difference in lipid peroxide concentration as compared with the corresponding vehicle control. However, incubation of patient or control cells with SMX-HA exhibited significantly higher TBARS levels. In comparing allergic patients with other groups, no significant difference was obtained (Figure 13).

4.2 Estimation of Reduced Glutathione (GSH) Content

GSH, in its reduced form, is the most powerful intracellular antioxidant, and the ratio of reduced to oxidized glutathione (GSH/GSSG) is representative of the oxidative status of the cell. GSH and GSSG levels were measured in PBMCs to show whether the SMX itself may have some effect on the protective role of GSH, probably because of its metabolic pathways. As well, it is worth knowing if there is a relationship between the allergic pathologies and the glutathione defense system. GSH/GSSG assay is based on the development of yellow color with DTNB as outlined in Materials and Methods. Cells

were treated for 2 hr with 200 μ M SMX, 200 μ M SMX-HA, or 400 μ M SMX-HA. GSH was taken to be the difference between total glutathione content and GSSG.

All groups' cells that were exposed to 400 μ M SMX-HA revealed a significant depletion in GSH content compared with the corresponding vehicle control, but a more pronounced decrease was observed within allergic patients cells ($P < 0.001$). Treatment of patients' cells with 200 μ M SMX-HA also showed a significant decrease in GSH content. No significant changes in GSH values were observed in comparing allergic patients with others (Figure 14A). Similar trends were revealed with the GSH/GSSG ratio (i.e. significant reductions in GSH/GSSG ratio upon incubation of cells with SMX-HA 400 μ M) (Figure 14B).

Interestingly, there was a negative correlation between ROS and GSH content for patients group at SMX-HA 200 μ M ($p = 0.01$, $r = -0.8$) and 400 μ M ($p = 0.002$, $r = -0.91$) (Figure 15A and 15B). As such, this indicates the functioning of the antioxidant defense systems to detoxify free radicals produced through the SMX allergy.

Figure 13: Lipid peroxide content of lymphocytes treated with SMX (200 μ M) or SMX-HA (400 μ M).

Lymphocytes from participants were incubated with 200 μ M SMX or 400 μ M SMX-HA for 2 h. Media containing drugs was removed and cells were then incubated for 18 h in RBMI media. Lipid peroxidation was detected through the reaction of MDA with TBA as described in the methods, and expressed as a percentage of vehicle control. Data are presented as mean \pm S.E (n= 18 sulfa sensitive patients, 15 controls and 6 sulfa tolerant patients). *P < 0.05 compared with the corresponding vehicle control.

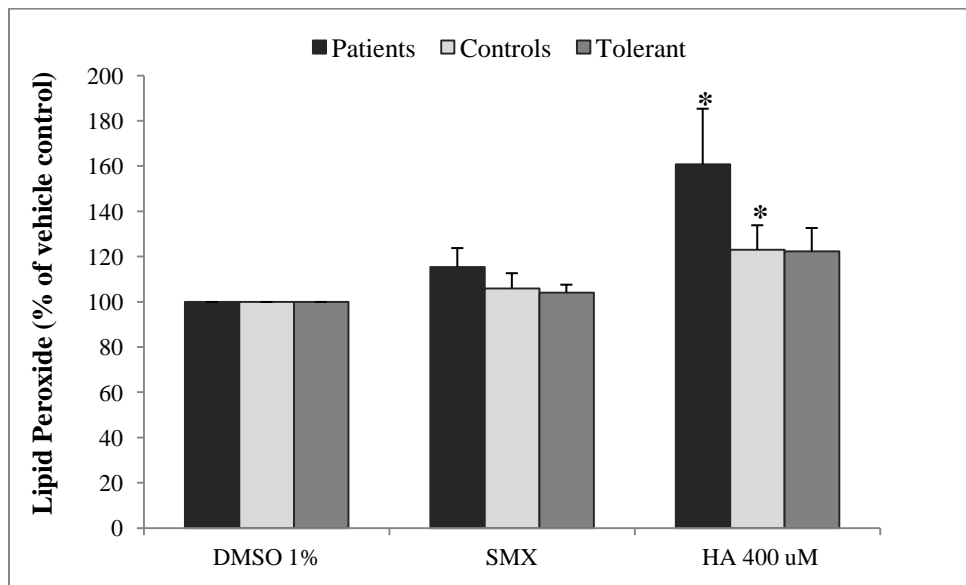


Figure 14: Determination of GSH level and GSH/GSSG ratio in PBMCs treated with SMX and SMX-HA.

Lymphocytes were treated with 200 μ M SMX or SMX-HA (200 or 400 μ M) for 2 h. Media containing drugs was removed and cells were then incubated for 18 h in RBMI media. GSH and GSSG was detected through the reaction with DTNB. GSH (A) and GSH/GSSG ratio (B) were calculated. Data are expressed as mean \pm S.E, (n= 8 allergic patients, 8 controls and 6 tolerant patients). *P < 0.05, **P < 0.01 compared with the corresponding vehicle control group.

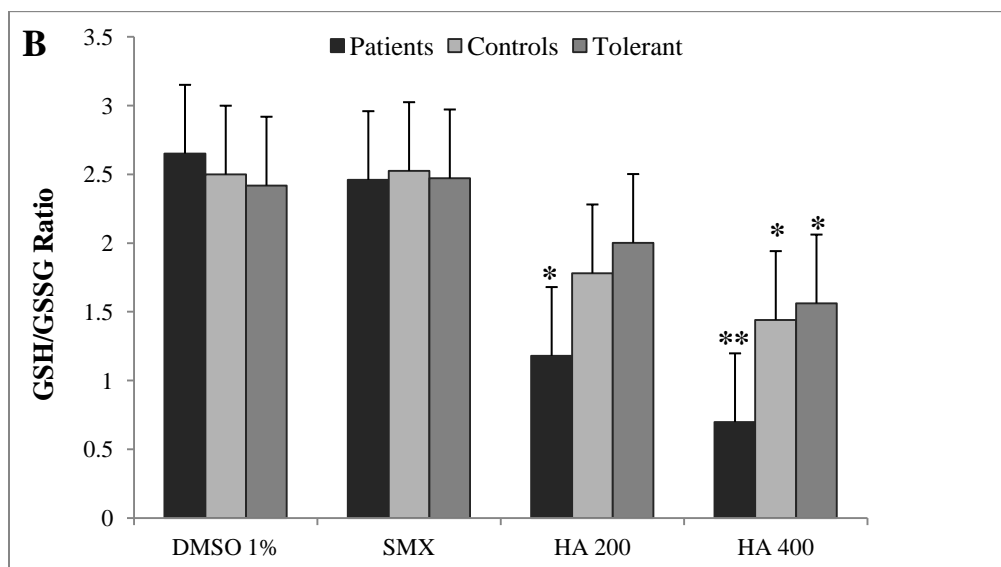
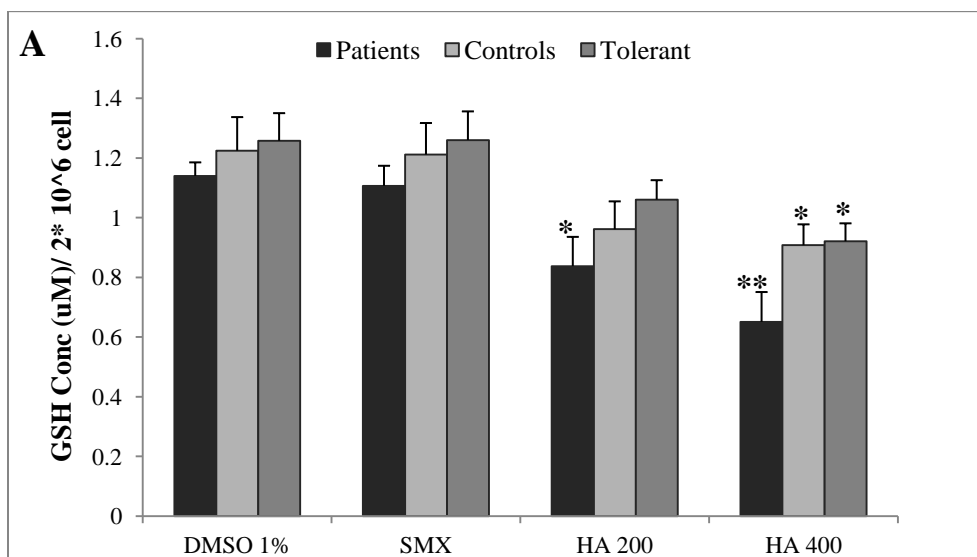
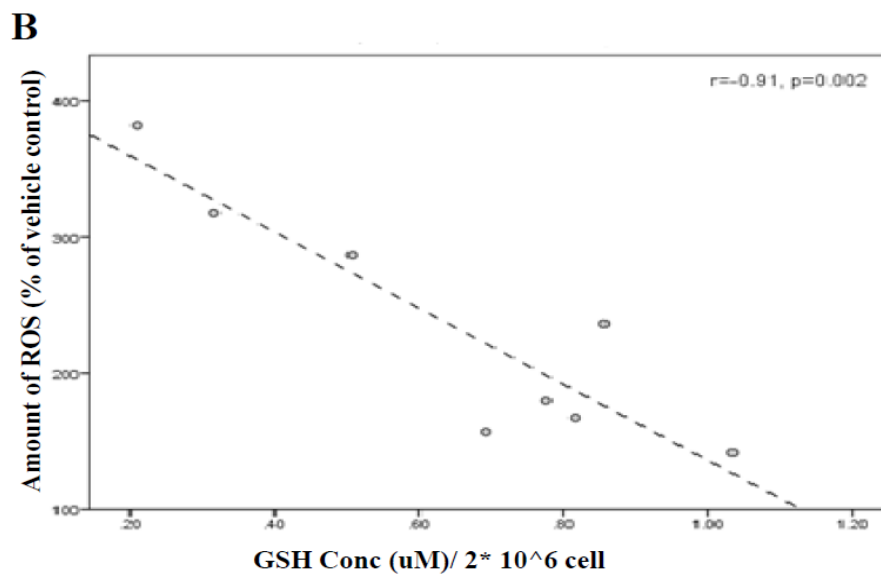
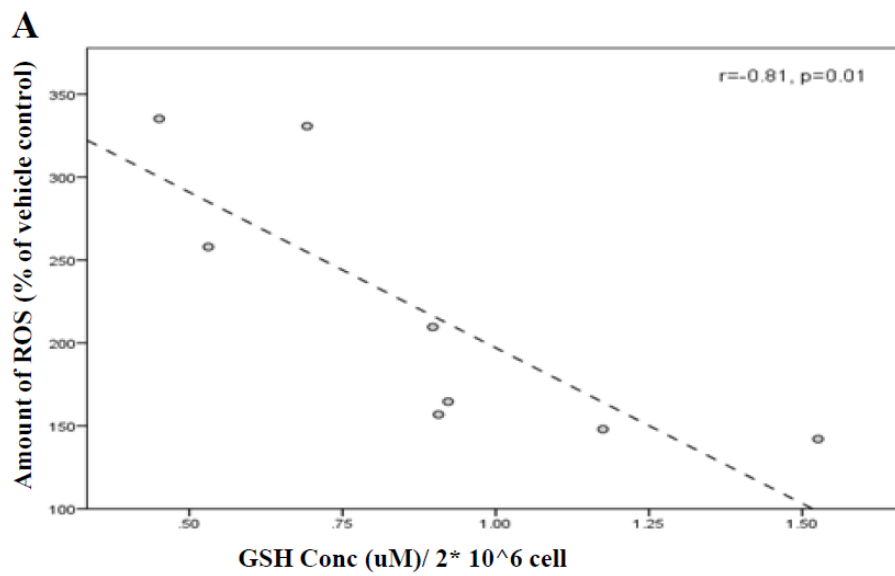


Figure 15: Correlation between the amount of ROS and the GSH level for hypersensitive patients.

The amount of ROS formed and the level of reduced glutathione (GSH) were measured after incubation the lymphocytes for 2 hr with SMX-HA 200 μ M (A) or 400 μ M (B). [(p = 0.01, r = -0.81) ;(p = 0.002, r = -0.91,); respectively].



CHAPTER 5: DISCUSSION

Since hypersensitivity reactions can cause high morbidity and mortality rates, it is very important to determine which patients tolerate therapy and which patients are at risk for ADRs. The pathophysiology underlying drug hypersensitivity is not well understood, however; it is believed that complex events of metabolic, toxic, and immunologic factors play key roles. In addition, there are other factors which may predispose an individual's sensitivity towards a specific agent including species differences, genetic makeup, concomitant dietary supplements or medications, and health state (Zuniga et al., 2012; Rogers et al., 2002). A strong genetic association have recently been suggested to contribute the HLA in the pathogenesis of drug hypersensitivity such as the presence of HLA-B*5701 and abacavir hypersensitivity, and HLA-B*1502 and carbamazepine-induced SJS/TEN in Han-Chinese patients (Mallal et al., 2002; chung et al., 2004; Teraki et al., 2010)

Sulfonamides, such as sulfamethoxazole (SMX), are inexpensive antimicrobials with a wide spectrum of action, and are commonly used in the therapy of infectious diseases and for AIDS-related complications. However, their uses have been limited because of the high incidence of potentially life threatening reactions. Sulfonamide hypersensitivity reactions frequently present with skin rash, fever and internal organ involvement, usually after 10 to 14 days of therapy and are resolved by withdrawing the offending drug. Moreover, the occurrence of SMX allergy has increased in some disease states, such as viral infections like HIV (Slatore and Tilles, 2004). The toxicity of SMX has been widely studied in a variety of cell models in *in vitro* studies, as well as in *in vivo*

studies. Also, a lot of research has been focused on understanding the mechanisms involved in SMX induced hypersensitivity due to the availability and the known protein reactivity of SMX metabolites, as well as much is known about its disposition in the body. It has been established that the genetic factors, such as HLA polymorphisms and drug metabolizing enzyme gene polymorphisms, are not major predisposing factors for SMX hypersensitivity (Alfirevic et al., 2009; Alfirevic et al., 2003; Pirmohamed et al., 2000).

Studies, by various investigators, have shown that oxidative bioactivation of SMX to its reactive metabolites and protein haptentation appear to be essential steps in the precipitation of immune reactions to SMX (Rieder et al., 1992; Reilly et al., 2000; Svensson et al., 2001; Reilly and Ju, 2002). However, many of the mechanistic details of how these toxic metabolites initiate immune activation remain unclear. Indeed, these reactive metabolites can associate with macromolecules forming adducts that may disturb cell function by damaging DNA or alterations in key signal transduction pathways, such as disruption of ionic gradients and intracellular calcium stores, which ultimately cause apoptosis (Hess et al., 1999). It has been also illustrated that SMX upon its metabolism produces ROS which can impair the antioxidant system and modify immune responses causing immunological tissue damage (Cornejo-Garcia et al., 2006). High ROS, particularly $O_2^{\cdot-}$ and H_2O_2 , concentration also disturbs cellular macromolecules, including proteins and nucleic acids (Siems et al., 2005).

The purpose of our study was to investigate the role of SMX reactive metabolites, as cytotoxic agents, and the oxidative stress on purified PBMCs subpopulations *in vitro* in order to better understand the mechanisms and pathways that lead to ADRs toward

SMX. As such, this knowledge will be very useful in preventing the incidence of these critical reactions.

The first objective of our research was to assess and confirm the metabolic and immunologic basis of SMX hypersensitivity reactions. *In vitro* cell death associated with reactive electrophilic drug metabolites has been used as a marker to diagnose sulfonamide hypersensitivity in a number of studies (Rieder et al., 1989; Reilly et al., 1999; Neuman et al., 2000); however, it should be noted that the diagnosis of drug allergy continues to represent a challenge until now. This is due to the lack of a safe and reliable test and because of the variable clinical pictures.

We examined the total cytotoxicity in lymphocytes and platelets following drug exposure and overnight incubation. Lymphocyte toxicity assay (LTA) has been used for many years in our lab and has proven to be a very useful diagnostic tool for idiosyncratic reactions to sulfonamides. However, *in vitro* platelets toxicity assay (*i*PTA) was introduced recently as a novel diagnostic tool which focused on the use of platelets as a surrogate cell model. All of the participants in this study underwent *in vitro* LTA and *i*PTA testing with SMX and its metabolites. Our data demonstrated that cytotoxicity due to SMX-HA varies between hypersensitive patients and other groups at higher SMX-HA (100, 200, 400 or 800 μ M) concentrations, with cell viability being lower in allergic patients than others (Figure 9A and 9B). This is consistent with the previous findings which established that these *in vitro* toxicity assays are good diagnostic tools to detect sulfa hypersensitivity reactions (Neuman et al., 2007; Elzagallaai et al., 2010; Elzagallaai et al., 2011). Also, we were able to report that the bioactivation of SMX to its

arylhydroxylamine metabolites appears to be a critical step in the development of hypersensitivity reactions.

These differences in the cytotoxicity could be explained by the fact that there are cellular, molecular or biochemical variation in human tissues, cells or fluid which make some patients susceptible to specific drugs. Those patients may have pharmacokinetic issues related to excessive quantities of reactive metabolites, or may have pharmacodynamic difference in the ability of their cells to deal with the metabolites, or may be due to tissue-specific difference in the drug metabolism.

Our second objective was to study the state of oxidative stress in allergy, and to determine whether ROS accumulation following metabolite exposure may play a causal role in the cytotoxicity observed with these compounds. Indeed, the releasing of oxygen radicals and the imbalance in ROS production has been established to cause tissue damage contributing in the clinical manifestations of asthma and other allergic diseases (Bibi, et al., 1998; Boluda et al., 1998). Upon uptake, SMX can be oxidized by CYP 2C9 to corresponding hydroxylamine (SMX-HA) which automatically oxidized to the corresponding nitroso (SMX-NO). The latter is a highly electrophilic metabolite which can conjugate with proteins forming haptens, or deplete GSH which can directly cause oxidative stress. Also, the reaction of the auto-oxidation of SMX-HA can be linked with a partial reduction of molecular oxygen to superoxide anion, which is another source of sulfonamide-induced oxidative stress.

We focused the study on PBMCs, because these cells are metabolically active and expressing most of the enzymes that are required for drug detoxification, more closely related to the immune system, and play an important role in allergic diseases. ROS

formation in PBMCs was quantified by DCFH-DA fluorescence Assay over 2 h. Overall, the amount of ROS significantly increased over time upon incubation with SMX-HA but not with SMX, which supports the role of the reactive metabolites of a drug in inducing oxidative stress and developing ADRs rather than the parent drug. This finding was in consonance with previously published results by Vyas et al., (2005) which illustrated that ROS generation in normal human epidermal keratinocytes (NHEK) was increased by all arylhydroxylamines of SMX and DDS as compared to vehicle control, and the metabolite of DDS produced a larger amount of ROS.

In general, to stimulate a full and efficient immune response, two definite signals are required: the first from the interaction between a MHC restricted antigen and the T cell receptor, and the second from the co-stimulation of the T-cell by the antigen presenting cells (APCs) (Naisbitt et al., 2000; Maemura et al., 2005). Without the generation of the second signal, this would lead to the tolerance. ROS may be an essential mediator in the initiation and amplification of MHC class II and co-stimulatory molecule expression (Maemura et al., 2005), in which ROS enhance MCH II expression by increasing the DNA binding of the transcription factor NY-F (Harari and Liao, 2004). Lavergne et al. (2009) have illustrated that danger signals, such as cytokines, H₂O₂ and bacterial or viral pathogenic factors, enhance the formation of intracellular SMX-protein adducts in human APCs. Also, they reported that oxidative stress within APCs may be a risk factor for SMX hypersensitivity even though it does not directly affect cell viability (Lavergne et al., 2009).

Indeed, it is known that the danger signals, such as ROS, which cause cell death may be related to the drug (or chemically reactive metabolite) itself, or to other factors

such as bacterial or viral infection. However, other studies showed that danger can also be received in the absence of foreign substances from virally infected cells or dead cells (Gallucci et al., 1999; Shi et al., 2000). When ROS were measured for all participants in our study, cells from allergic patients treated with SMX-HA (200 or 400 μ M) appeared to accumulate higher ROS content than others (Figure 10D and 10E). This indicates the pivotal role of oxidative stress as one of the mechanisms of hypersensitivity although it is still unclear whether oxidative stress is the cause or the consequence of the allergic reactions to the drugs. Our data supporting the fact that these serious reactions can occur along with enhanced ROS production, either due to instability of ROS scavenging systems or to enhanced oxidative stress production in cells of sensitive patients. Also, the difference in relative potencies for ROS generation observed between study groups may be related to the genetic variation in antioxidant defense enzymes or other important oxidative metabolism. Collectively, these data add a new dimension to our current understanding of the pathogenesis of drug allergy.

Even though our data did not show a simple correlation (like Vyas et al., 2005 finding with NHEK) between ROS formed and the percent of cell death, this should not be interpreted to suggest that the increase in ROS generation in hypersensitive patients is unimportant. Manchanda et al. (2002) have demonstrated that SMX metabolites haptenate cellular proteins at concentrations below that associated with toxicity and without loss of viability. Thus, haptentation alone does not cause enough direct cellular damage to lead to toxicity. In reality, although reactive oxygen species can cause oxidative stress, they are not essential for the apoptotic processes to occur. This establishes that ROS can induce some apoptotic events, such as damaging the signaling

pathway, releasing or surface expression of further danger signals (as heat-shock proteins (HSPs), TNF and cytokines). Additionally, ROS are able to regulate the recruitment of T-cells by expression of adhesion molecules, such as E-selectin, or by up-regulate cell surface markers on APCs (Khan et al., 2006). This will ultimately cause cytotoxicity and elicit an immune response.

SMX-protein adducts are thought to play a role in the pathogenesis of SMX hypersensitivity (Naisbitt et al., 2002; Cheng et al., 2008). However, it has been established that the haptentation of the proteins by SMX-NO can occur without loss of cell membrane integrity, depletion of GSH or detectable disruption of redox-sensitive factors (Naisbitt et al., 1999; Naisbitt et al., 2000). Binding of SMX-HA to proteins is postulated to imply the auto-oxidation to SMX-NO. However, whether the SMX-HA itself or SMX-HA generated ROS conjugates or interacts with lipid membranes is unknown.

In order to examine the oxidative status, we went on to assay the oxidative stress parameters that include lipid and protein oxidation. Lipid peroxidation is believed to be an important cause of damage to cell membranes, and occur as a result of the increased ROS production and decreased antioxidant capacity (Reiter et al., 2001). We determined lipid peroxidation from cell lysates by the TBARS method which is a standardized tool for assaying malondialdehyde (MDA). The latter is one of the stable products of degradation of polyunsaturated fatty acid by radicals. Also, it is a highly reactive and potentially mutagenic compound which can form covalent protein adducts and DNA adducts (Farmer and Davoine, 2007; Marnett, 1999). Therefore, MDA must be maintained at a low intracellular level. When lipid peroxide content was measured in our

study, there was a significant increase in lipid peroxide content in cells of hypersensitive patients and healthy controls when treated with 400 μ M SMX-HA compared to the corresponding vehicle control treated cells. Thus, this suggests that the SMX metabolites can produce oxidative effects on the lipid cell membrane (Figure 13).

In a study by Neuman et al. (2007), they found that there is a correlation between the severity of symptoms observed in affected patients by sulfonamide induced-hypersensitivity and chemokine levels, with these mediators becoming higher in the reactions that presented the organ involvement when compared with the individuals that presented less important symptoms such as skin rash and fever. Also, data from Pérez-Gómez et al. (2000), and Verma et al. (2012) showed that there is higher TBARs within severe symptomatic patients. In our research, we failed to find a significant difference between allergic patients and other groups in terms of lipid peroxide content, and this may be due to the fact that the majority of our sulfa allergic patients developed relatively minor manifestations including only mild cutaneous reactions, and none of them were clinically ill at the time of sampling. It has also demonstrated that the metabolic risk factors for SMX hypersensitivity are likely to differ over time with HIV infections (Eliaszewicz et al., 2002). Therefore, the severity of adverse reaction and the time elapsed since hypersensitivity events may also play key roles.

Protein carbonyl content, which is used as an index of the oxidative damage to proteins, was also quantified from cell lysates of all study subjects to assess the relationship between oxidative stress and different allergic reactions. Higher carbonyl contents were seen in cells treated with 400 μ M SMX-HA compared to the corresponding vehicle control treated cells. Also, PBMCs from allergic patients express higher protein

carbonyl levels in comparison with values from sulfa tolerant patients (Figure 12). Although a deeper and larger study with more defined clinical cases is needed before definitive conclusions can be drawn, we can suggest using the protein carbonyl as a suitable recovering marker for oxidative stress. This discrepancy in the oxidative stress parameter has also been established at high-altitude training conditions in animal models within an increase in the reactive carbonyl derivatives but not lipid peroxidation (Radak et al., 1997). Another probable reason for the higher carbonyl content is the fact that proteins seem to be more sensitive to oxidative modification and the higher relative stability of oxidized proteins over time than lipids (Pantke et al., 1999). Also, protein carbonyl groups can be induced by almost all types of ROS, and this may have some advantages in comparison with lipid peroxidation products (Dalle-Donne et al., 2003).

The other goal of this study was to see if an allergic reaction to drugs induces a response to antioxidant activities. Cells need appropriate redox balance for surviving and to function well. GSH is the major endogenous antioxidant that is important for reducing reactive metabolites to inactive compounds and scavenging of free radicals (Cribb et al., 1996). Importantly, the electrophilic reactive intermediates of SMX-TMP are detoxified by conjugation with glutathione, and subsequently this conjugate is metabolized and excreted in the urine. SMX-induced hepatotoxicity is rarely observed although the liver is the major site for the formation of SMX reactive metabolites, and this may be related to the higher levels of GSH in hepatocytes than other cells.

Under conditions of inadequate intracellular glutathione, the reactive metabolites of this drug can bind covalently to cellular macromolecules, initiating immune-mediated hypersensitivity reactions, and hepatic and renal cellular necrosis (Walmsley et al., 1997;

Rieder et al., 1989). Lymphocytes are affected by the alteration in the redox balance due to their needs for a reducing medium for ideal proliferation and activation (Gelderman et al., 2006). Further evidence for the induction of oxidative stress because of the SMX metabolic pathways is provided by the ability of SMX metabolites to reduce GSH content, but not with the SMX itself, as well as the significant reduction in GSH/GSSG ratio (Figure 14). Importantly, the significant negative correlation found between the GSH content and ROS formation for the allergic patients may demonstrate the benefit of complementary antioxidant therapy in alleviating the oxidative alteration and the consequently pathophysiological complications of sulfa toxicity (Figure 15).

The Glutathione deficiency has been hypothesized to be a risk factor for ADRs to sulfonamides in AIDS patients (Van der Ven et al., 1996; Witschi et al., 1995; Walmsley et al., 1997); however, this finding was not confirmed by other studies (Pirmohamed et al., 1996; Wolkenstein et al., 2000; Eliaszewicz et al., 2002). These discrepancies between the studies might be because of the instability of the GSH which require a careful and prompt sample analysis for its determination. It is known that the low GSH levels observed in HIV patients could contribute to increased cells haptentation, drug immunogenicity and intracellular accumulation of highly reactive species, thus providing an explanation for the high prevalence of sulfa allergy within those patients.

Indeed, if GSH depletion plays a critical role in the pathogenesis of these reactions, as has been suggested for HIV-positive patients, it is possible that more significant or long-standing deficiencies are necessary. When GSH was evaluated in our study groups, we did not find a significant effect on GSH content between hypersensitive patients in comparing with other groups. This is probably because of the very small number of

subjects whose their GSH content was determined since our research limitations were the size of blood sample and the need of having more cells to work in all assays. This observation establishes that GSH may not play a major role, but could still make a contribution to sulfonamide-induced oxidative stress or sulfa toxicity. In a study done recently by Verma et al., (2012) to assess the oxidative stress status in the blood of 33 cutaneous drug reaction patients and 33 drug naïve patients by assaying for GSH and MDA levels, they found that the allergic patients had significantly raised MDA levels and reduced GSH content as compared to the other group.

Although not all *in vitro* results can be translated to clinical data, and since a deeper study is required before definitive conclusions can be drawn, our explanations can report estimation of cell death, oxidative stress parameters, and GSH content, may give unique and valid markers of ADRs associated with SMX. As oxidative stress is considered to be at least partially involved in a variety of pathogenesis and drugs toxicity, it will be of interest to assess the antioxidant potential against oxidative stress induced by sulfonamides in *in vivo* studies.

CHAPTER 6: FUTURE DIRECTIONS

The present study offered insight on the pathogenesis of sulfonamide induced hypersensitivity, and we were interested to know why certain patients develop these serious reactions while others do not. Significant progress toward a better understanding of the mechanisms involved in drug hypersensitivity would be worthy and have a powerful impact. Also, further investigations in this area will not only aid the development of new therapeutic strategies, but may allow us to use current drugs more safely.

Data have been presented suggesting the formation of both highly reactive metabolites and reactive oxygen species may be important in generation of further danger signals that can induce cytotoxicity even though they may not be directly correlated. However, more and large clinical studies should be done to evaluate whether ROS are a major predisposing determinant of individual susceptibility leading to clinical hypersensitivity.

More exploration, including better animal models and innovative *in vitro*, pharmacogenetic, and clinical studies, will be beneficial to explain the mechanisms involved in oxidative stress, as well as their relationship with the immunological response. A much larger study with more carefully defined clinical and symptomatic cases is also needed to determine if the reactive oxygen species, that were observed *in vitro*, are observed in patients receiving these drugs at the time of the reaction.

GSH is a primary intracellular anti-oxidant for scavenging free radicals and detoxification of xenobiotics. Decreased GSH content have been shown to play a pivotal

role in the modulation of immune functions and promote HIV expression (Arp et al., 2005; Choi et al., 2000). It would be interesting to determine how the external danger signals, such as HIV infection, increase the risk of hypersensitivity reaction and this may help in designing safer and more effective therapy for those patients. Our findings detail that the GSH defense is out of balance upon incubation of lymphocytes with SMX metabolites. More clinical trials are required, however, to show whether there is a significant inhibition of allergic reactions by treatment with different antioxidants, and to determine to what extent is antioxidant supplementation beneficial for the purpose of alleviating oxidative stress. Moreover, studies investigating the role of genetic variations in genes related to oxidative stress (i.e. polymorphisms in antioxidant defense system genes) could be fruitful for better understanding the pathogenesis of drug hypersensitivity reactions and their metabolic complications.

REFERENCES

- Akamizu, T., Ozaki, S., Hiratani, H., Uesugi, H., Sobajima, J., Hataya, Y., Kanamoto, N., Saijo, M., Hattori, Y., Moriyama, K., Ohmori, K., and Nakao, K. (2002) Drug-induced neutropenia associated with anti-neutrophil cytoplasmic antibodies (ANCA): possible involvement of complement in granulocyte cytotoxicity. *Clin Exp Immunol*, 127(1):92-98.
- Alfirevic, A., Stalford, A. C., Vilar, F. J., Wilkins, E. G., Park, B. K., and Pirmohamed M. (2003). Slow acetylator phenotype and genotype in HIV-positive patients with sulphamethoxazole hypersensitivity. *Br J Clin Pharmacol*, 55:158-65.
- Alfirevic, A., Vilar, F. J., Alsbou, M., Jawaid, A., Thomson, W., Ollier, W. E, Bowman, C. E., Delrieu, O., Park, B. K., and Pirmohamed, M. (2009). TNF, LTA, HSPA1L and HLA-DR gene polymorphisms in HIV-positive patients with hypersensitivity to cotrimoxazole. *Pharmacogenomics* ,10:531-40.
- Arp, J., Rieder, M.J., Urquhart, B., Freeman, D., Tucker, M. J., Krizova, A., Lehmann, D., and Dekaban, G. A. (2005). Hypersensitivity of HIV-1-infected cells to reactive sulfonamide metabolites correlated to expression of the HIV-1 viral protein tat. *J Pharmacol Exp Ther*, 314(3):1218-25.
- Bates, D. W., Spell, N., Cullen, D. J., Burdick, E., Laird, N., Petersen, L.A., Small, S.D., Sweitzer, B. J., and Leape, L. L. (1997). The costs of adverse drug events in hospitalized patients. *JAMA*, 277 (4): 307-311.
- Bellamy, W. T. (1992). Prediction of response to drug therapy of cancer. A review of in vitro assays. *Drugs*, 44(5):690-708.
- Berridge, M.V., and Tan, A. S. (1993). Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys*, 303(2):474-82.
- Bibi, H., Schlesinger, M., Tabachnik, E., Schwartz, Y., Iscovitz, H. and Iaina, A. (1998). Erythrocyte glutathione peroxidase activity in asthmatic children. *Ann. Allergy*, 61: 339-340.

Boluda, L., Alonso, C. and Fernandez-Caldas, E. (1998). Purification, characterization, and partial sequencing of two new allergens of *Olea europaea*. *J Allergy Clin Immunol*, 101:210-216.

Brackett, C. C., Singh, H., and Block, J. H. (2004). Likelihood and mechanisms of crossallergenicity between sulfonamide antibiotics and other drugs containing a sulfonamide functional group. *Pharmacotherapy*, 24: 856–70.

Caamaño, J., and Hunter, C. A. (2002). NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. *Clin Microbiol Rev*, 15(3):414-29.

Carr, A., Cooper, D.A., and Penny, R. (1991). Allergic manifestations of human immunodeficiency virus (HIV) infection. *J.Clin. Immunol*, 11: 55-64.

Carr, A., Gross, A. S., Hoskins, J. M., Penny, R., and Cooper, D. A. (1994). Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulfamethoxazole in HIV-infected patients. *Aid*, 8:333-337.

Carr, A., Tindal, B., Penny, R., and Cooper, D. A. (1993). In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol*, 94:21-25.

Chandra, J., Samali, A., and Orrenius, S. (2000). Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med*, 29:323-333.

Cheng, L., Stewart, B. J., You, Q., Petersen, D. R., Ware, J. A., Piccotti, J. R., Kawabata, T. T., and Ju, C.(2008). Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol*, 73:1769–1775.

Chiamonte, R., Bartolini, E., Riso, P., Calzavara, E., Erba, D., Testolin, G., Comi, P., and Sherbet, G. V. (2001). Oxidative stress signaling in the apoptosis of Jurkat T-lymphocytes. *J Cell Biochem*, 82:437- 444.

Choi, J., Liu, R. M., Kundu, R. K., Sangiorgi, F., Wu, W., and Maxson, R., Forman, H, J. (2000). Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J Biol Chem*, 275(5):3693-8.

Choquet-Kastylevsky, G., Vial, T., and Descotes, J. (2001) Drug allergy diagnosis in humans: possibilities and pitfalls. *Toxicology*, 158(1-2):1-10.

Chung, W. H., Hung, S. I., Hong, H. S., Hsieh, M. S., Yang, L. C., Ho, H.C., Wu, J. Y., and Chen, Y. T. (2004). Medical genetics: a marker for Stevens-Johnson syndrome. *Nature*, 428:486.

Coombs, P. R., and Gell, P. G. (1968). Classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Gell RR, editor, *Clinical aspects of immunology*. Oxford University Press, Oxford, p. 575-96.

Cornejo-Garcia, J. A., Mayorga, C., Torres, M. J., Fernandez, T. D., R-Pena, R., Bravo, I., Mates, J. M., and Blanca, M.(2006). Anti-oxidant enzyme activities and expression and oxidative damage in patients with non-immediate reactions to drugs. *Clin Exp Immunol*, 145(2):287-95.

Cribb, A. E., Lee, B. L., Trepanier, L. A., and Spielberg, S. P.(1996). Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev*, 15(1):9-50.

Cribb, A. E., and Spielberg, S. P. (1992). Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clin Pharmacol Ther*, 51(5):522-6.

Cribb, A. E., Spielberg, S. P., and Griffin, G. P. (1995). N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos*, 23:406.

Cribb, A. E., Miller, M., Leeder, J. S., Hill, J., and Spielberg, S. P. (1991). Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity. *Drug Metab Dispos*, 19(5):900-6.

Cribb, A., Miller, M., Leeder, J., Hill, H., and Spielberg, S. (1991). Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. *Drug Metab Dispos*, 19: 900 –6.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta*, 329(1-2):23-38.

Desagher, S., and Martinou, J. C. (2000). Mitochondria as the central control point of apoptosis. *Trends Cell Biol*, 10:369-377.

Dibbern, DA. Jr., and Montanaro, A. (2008). Allergies to sulfonamide antibiotics and sulfur containing drugs. *Ann Allergy Asthma Immunol*, 100: 91–100.

Dimon-Gadal, S., Gerbaud, P., Therond, P., Guibourdenche, J., Anderson, W. B., Evain-Brion, D., and Raynaud, F. (2000). Increased oxidative damage to fibroblasts in skin with and without lesions in psoriasis. *J Invest Dermatol*, 114:984–989.

Eliaszewicz, M., Flahault, A., Roujeau, J., Fillet, A., Challine, D., Mansouri, S., Wolkenstein, P., Aractingi, S., Penso-Assathiany, D., Maslo, C., Bourgault-Villada, I., Chosidow, O., Caumes, E., and Group, E.S. (2002). Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol*, 47: 40–46.

Elzagallaai, A. A., Jahedmotlagh, Z., Del Pozzo-Magaa, B. R., Knowles, S. R., Prasad, A. N., Shear, N.H., Rieder, M. J., and Koren, G. (2010). Predictive Value of the Lymphocyte Toxicity Assay in the Diagnosis of Drug Hypersensitivity Syndrome. *Mol Diagnosis Ther*, 14:317–322.

Elzagallaai, A. A., Knowles, S. R., Rieder, M. J., Bend, J. R., Shear, N. H., and Koren, G. (2009). In vitro testing for the diagnosis of anticonvulsant hypersensitivity syndrome: a systematic review. *Mol Diagn Ther*, 13(5):313-30

Elzagallaai, A. A., Rieder, M. J., and Koren, G. (2011). The in vitro platelet toxicity assay (iPTA): a novel approach for assessment of drug hypersensitivity syndrome. *J Clin Pharmacol*, 51(3):428-35.

Elzagallaai, A. A., Garcia-Bournissen, F., Finkelstein, Y., Bend, J. R., Rieder, M. J., and Koren, G. J. (2011). Severe bullous hypersensitivity reactions after exposure to carbamazepine in a Han-Chinese child with a positive HLA-B*1502 and negative in vitro toxicity assays: evidence for different pathophysiological mechanisms. *Popul Ther Clin Pharmacol*, 18(1):e1-9.

Ernst, F. R., Grizzle, A. J. (2001). Drug-related morbidity and mortality: updating the cost-of-illness model. *JAPHA*, 41:192-199.

Farmer, E. E., and Davoine, C. (2007). Reactive electrophile species. *Curr. Opin. Plant Biol.*, 10 (4): 380–6.

Filomeni, G., and Ciriolo, M. R. (2006). Redox control of apoptosis: an update. *Antioxid. Redox Signal*, 8:2187-2192.

Fischi, M. A., Dickinson, GM., and Vole, L. L. (1988). Safety and efficacy of sulfamethoxazole and trimethoprim chemoprophylaxis for *Pneumocystis carinii* pneumonia in AIDS. *JAMA*, 259:1185.

Foltzer, M. A., and Reese, R. E. (1987). Trimethoprim-sulfamethoxazole and other sulfonamides. *Med Clin North Am*, 71(6):1177-94.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.*, 5:1249 -1255.

Gallucci, S., and Matzinger, P. (2001). Danger signals: SOS to the immune system. *Curr Opin Immunol*, 13:114–119.

Gelderman, K. A., Hultqvist, M., Holmberg, J., Olofsson, P., and Holmdahl, R. (2006). T cell surface redox levels determine T cell reactivity and arthritis susceptibility. *Proc Natl Acad Sci U S A*, 103(34):12831-6.

Gillette, J. R., Lau, S.S., and Monks, T.J. (1984) Intra- and extra-formation of metabolites from chemically reactive species. *Biochem.Soc.Trans*, 12:4-7.

Giner, V., Rueda, D., Salvador, A., Hernández, J. C., Esteban, M. J., and Redon, J. (2003) Thrombocytopenia associated with levodopa treatment. *Arch Intern Med*, 163(6):735-6.

Gruchalla, R. S. Drug allergy. (2003). *J Allergy Clin Immunol*, 111(2 Suppl): S548–S55.

Gruchalla, R., Pesenko, R., Do, T., and Skiest, D. J. (1998). Sulfonamide-induced reactions in desensitized patients with AIDS - the role of covalent protein haptentation by sulfamethoxazole. *J Allergy Clin Immunol*, 101:371-378.

Gomes, A., Fernandes, E., and Lima, J. L. (2005). Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods*, 65:45-80.

Heckbert, S.R., Stryker, W. S., Coltin, K. L., Manson, J. E., and Platt, R. (1990). Serum sickness in children after antibiotic exposure: estimates of occurrence and morbidity in a health maintenance organization population. *Am J Epidemiol*, 132: 336–42.

Hemstreet, B. A., and Page, R. L. (2006). Sulfonamide allergies and outcomes related to use of potentially cross-reactive drugs in hospitalized patients. *Pharmacotherapy*, 26(4):551-7.

Hess, D. A., Sisson, M. E., Suria, H., Wijsman, J., Puvanesasingham, R., Madrenas, J., and Rieder, M. J. (1999). Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8 (+) cells by the hydroxylamine of sulfamethoxazole. *FASEB J*, 13(13):1688-98.

Hitchings, G. H. (1973). Mechanism of action of trimethoprim-sulfamethoxazole. *J Infect Dis*, 128: Suppl:433-6 p.

James, L. P., Mayeux, P. R., and Hinson, J. A. (2003). Acetaminophen-induced hepatotoxicity, *Drug Metabolism and Disposition*, 31:1499-1506

Jeavons PM. (1984). Non-dose-related side effects of valproate. *Epilepsia*, 25 Suppl LS50-55.

Jick H. Adverse drug reactions: the magnitude of the problem. (1984). *J Allergy Clin Immunol* 74: 555-557.

Kanji, S., and Chant, C. (2010). Allergic and hypersensitivity reactions in the intensive care unit. *Critical Care Medicin*, 38(6):S162-S168.

Khan, F.D., Roychodhury, S., Nemes, R., Vyas, P., Woster, P. M., and Svensson, C. K. (2006). Effect of pro-inflammatory cytokines on the toxicity of the arylhydroxylamine metabolites of sulphamethoxazole and dapsone in normal human keratinocytes. *Toxicology*, (218):90–99.

Kohen, R., and Nyska, A. (2002). Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol*, 30(6):620-50.

Kongkaew, C., Noyce, P.R., and Ashcroft, D.M. (2008). Hospital admissions associated with adverse drug reactions: a systematic review of prospective observational studies. *Ann Pharmacother*, 42(7):1017–1025.

Kurian, J. R., Bajad, S. U., Miller, J. L., Chin, N. A., and Trepanier, L. A. (2004). NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *Journal of Pharmacology and Experimental Therapeutics*, 311:1171.

Landsteiner, K., and Jacobs, J. (1936). Studies on the sensitization of animals with simple chemical compounds. II. *J Exp Med*, 64(4):625-639.

Lavergne, S. N., Wang, H., Callan, H. E., Park, B. K., and Naisbitt, D. J. (2009). Danger conditions increase sulfamethoxazole- protein adduct formation in human antigen-presenting cells. *J Pharmacol Exp Ther*, 331(2):372-81.

- Lazarou, J., Pomeranz, B. H., and Corey, P. N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA*, 279(15): 1200-5.
- Madden, S., Maggs, J. L., Park, B. K. (1996). Bioactivation of carbamazepine in the rat in vivo: evidence for the formation of reactive arene oxide(s). *Drug Metab Dispos*, 24, 469 - 479.
- Maemura, K., Zheng, Q., Wada, T., Ozaki, M., Takao, S., Aikou, T., Bulkley, G. B., Klein, A.S., and Sun, Z. (2005). Reactive oxygen species are essential mediators in antigen presentation by Kupffer cells. *Immunol Cell Biol*, 83(4):336-43.
- Maggs, J.L., Naisbitt, D. J., Tettey, J. N., Pirmohamed, M., and Park, B. K. (2000). Metabolism of lamotrigine to a reactive arene oxide intermediate. *Chem Res Toxicol*, 13,1075–1081.
- Mallal, S., Nolan, D., and Witt, C., Sayer, D., Castley, A., Mamotte, C., Maxwell, D., James, I., and Christiansen, F. T. (2002). Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet*, 359:727-32.
- Manchanda, T., Hess, D., Dale, L., Ferguson, S. G., and Rieder, M. J. (2002). Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol*, 62(5):1011-26.
- Marchant, C. D., and Shurin, P. A. (1983). Therapy of otitis media. *Pediatr Clin North Am*, 30: 281-296.
- Marnett, L. J. (1999). Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res.*, 424 (1–2): 83–95.
- Matés, J. M., Pérez-Gómez, C., Olalla, L., Segura, J. M., and Blanca, M. (2000). Allergy to drugs: antioxidant enzymatic activities, lipid peroxidation and protein oxidative damage in human blood. *Cell Biochem Funct*, 18: 77–84.
- Matés, J. M., Segura, J. M., Pérez-Gómez, C., Rosado, R., Olalla, L., Blanca, M., and Sánchez-Jiménez, F. M. (1999). Anti-oxidant enzymatic activities in human blood cells after an allergic reaction to pollen or house dust mite. *Blood Cells Mol Dis*, 25:103–9.
- Matzinger, P. (1994). Tolerance danger and the extended family. *Annual review of immunology*, 12:991-1045.

Mauri-Hellweg, D., Bettens, F., Mauri, D., Brander, C., Hunziker, T., and Pichler, W. J. (1995). Activation of drug-specific CD4⁺ and CD8⁺ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol*, 155:462-472.

Naisbitt, D. J., Britschgi, M., Wong, G., Farrell, J., Depta, J. P., Chadwick, D.W., Pichler, W. J., Pirmohamed, M., and Park, B. K. (2003). Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. *Mol Pharmacol*, 63:732.

Naisbitt, D.J., Farrell, J., Gordon, S. F, Maggs, J. L., Burkhart, C., Pichler, W. J., Pirmohamed, M., and Park, B. K. (2002). Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol*, 62:628–637.

Naisbitt, D. J., Gordon, S. F., Pirmohamed, M., and Park, B. K. (2000). Immunological principles of adverse drug reactions: the initiation and propagation of immune responses elicited by drug treatment. *Drug Saf*, 23(6):483-507.

Naisbitt, D. J., Hough, S. J., Gill, H.J., Pirmohamed, M., Kitteringham, N. R., and Park, B. K. (1999). Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. *Br J Pharmacol*, 126(6):1393-407.

Naisbitt, D. J., Pirmohamed, M., and Park, B. K. (2003) Immunopharmacology of hypersensitivity reactions to drugs. *Curr Allergy Asthma Rep*3:22-9.

Naisbitt D, J., Williams, D. P., Pirmohamed, M., Kitteringham, N. R., and Park, B. K. (2001). Reactive metabolites and their role in drug reactions. *Curr Opin Allergy Clin Immunol*, 1(4):317-25.

Neuman, M. G, Malkiewicz, I., and Shear, N. H. (2000). A Novel Lymphocyte Toxicity Assay to Assess Drug Hypersensitivity Syndromes. *Clinl Biochem*, 33 (7):517–524.

Neuman, M. G., Shear, N. H., Malkiewicz, I. M., Taeri, M., Shapiro, L. E., Krivoy, N., Haber, J., Gomez, M., Fish, J., Cartotto, R., and Cohen, L. (2007). Immunopathogenesis of hypersensitivity syndrome reactions to sulfonamides. *Transl Res*, 149(5):243-53.

Njoku, D., Laster, M. J., Gong, D. H., Eger, E. I., Reed, G. F., and Martin, J. L. (1997). Biotransformation of halothane, enflurane, isoflurane, and desflurane to trifluoroacetylated liver proteins: association between protein acylation and hepatic injury. *Anesth Analg*, 84:173.

O'Neil, M., Drobitch, R. K., MacArthur, R. D., Farrough, M. J., Doll, M. A., Fretland, A. J., Hein, D. W., Crane, L. R., and Svensson, C. K. (2000). Acetylator phenotype and genotype in patients infected with HIV: discordance between methods for phenotype determination and genotype. *Pharmacogenetics*, 10(2):171-82.

Ozkaya-Bayazit, E., and Akar, U. (2001). Fixed drug eruption induced by trimethoprim-sulfamethoxazole: evidence for a link to HLA-A30 B13 Cw6 haplotype. *J Am Acad Dermatol*, 45(5):712-7.

Pantke, U., Volk, T., Schmutzler, M., Kox, W. J., Sitte, N., and Grune, N. (1999). Oxidized proteins as a marker of oxidative stress during coronary heart surgery. *Free Radic Biol Med*, 27:1080-6.

Park, B. K., Coleman, J. W., and Kitteringham, N. R. (1987). Drug disposition and drug hypersensitivity. *Biochem Pharmacol*, 36:581-90.

Park, B., Kitteringham, N., Powell, H., and Pirmohamed, M. (2000). Advances in molecular toxicology-towards understanding idiosyncratic drug toxicity. *Toxicology*, 153:39-60.

Park, B. K., Pirmohamed, M., and Kitteringham, N. R. (1992). Idiosyncratic drug reactions: a mechanistic evaluation of risk factors. *Br J Clin Pharmacol*, 34:377-395.

Park, B. K., Pirmohamed, M., and Kitteringham, N. R. (1998). Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. *Chemical research in toxicology*, 11:969-988.

Parker, C. W. (1982). Allergic reactions in man. *Pharmacological reviews*, 34:85.

Pérez-Gómez, C., Segura, J. M., Blanca, M., Asenjo, M., and Mates, J. M. (2000). Antioxidant activity levels and oxidative stress as blood markers of allergic response to drugs. *Biochem Cell Biol*, 78(6):691-8.

Pichler, W. (2007). Drug hypersensitivity reactions: Classification and relationship to T-cell activation. In: *Drug Hypersensitivity*. (ed. Pichler, W.) 168-189 (Karger, Basel, Switzerland).

Pichler, W. J. (2002). Pharmacological interaction of drugs with antigen-specific immune receptors: the pi concept. *Curr Opin Allergy Clin Immunol*, 2(4):301-5.

Pichler, W.J. (2008). The p-i Concept: Pharmacological Interaction of Drugs With Immune Receptors. *World Allergy Organ J*, 1(6):96-102.

Pichler, W.J., Adam, J., Daubner, B., Gentinetta, T., Keller, M., and Yerly, D. (2010). Drug hypersensitivity reactions: pathomechanism and clinical symptoms. *Medical Clinics of North America*, 94:645-664.

Pirmohammed M. (2005). Anticipating, investigating and managing the adverse effects of drugs. *Clin Med*, 5(1):23-6

Pirmohamed, M., Alfirevic, A., Vilar, J., Stalford, A., Wilkins, E. G., Sim, E., and Park, B. K. (2000). Association analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. *Pharmacogenetics*, 10:705-13.

Pirmohamed, M., Clarke, S. E., and Park, B. K. (2007). Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol*, 178:5533-5542.

Pirmohamed, M., James, S., Meakin, S., Green, C., Scott, A.K., Walley, T.J., Farrar, K., Park ,B.K.,and Breckenridge ,A. M. (2004). Adverse drug reactions as cause of admission to hospital: prospective analysis of 18820 patients. *BMJ*, 329(7456):15–9.

Pirmohamed, M., Naisbitt, D. J., Gordon, F., and Park, B. K. (2002). The danger hypothesis—potential role in idiosyncratic drug reactions. *Toxicology*, 181:55-63.

Pirmohamed, M., Williams, D., Tingle, M. D., Barry, M., Khoo, S. H., O'Mahony, C., Wilkins, E. G., Breckenridge, A. M., and Park, B.K. (1996). Intracellular glutathione in the peripheral blood cells of HIV-infected patients: failure to show a deficiency. *AIDS*, 10(5):501-7.

Pourahmad, J., Khan, S., and O'Brien, P.J. (2001). Lysosomal oxidative stress cytotoxicity induced by nitrofurantoin redox cycling in hepatocytes. *Advances in Experimental Medicine and Biology*, 500:261-5.

Radak, Z., Asano, K., Lee, K. C., Ohno, H., Nakamura, A., Nakamoto, H., and Goto, S. (1997). High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. *Free Radic Biol Med*, 22:1109–14.

Rahimi, R., Nikfar, S., Larijani, B., and Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomed Pharmacother*, 59(7):365-73.

Rawlins, M. D., and Thompson, J. W. (1977). Pathogenesis of adverse drug reactions. In: Davies DM, editor, Textbook of adverse drug reactions. Oxford: Oxford University Press, p. 10.

Reilly, T. P., and Ju, C. (2002). Mechanistic perspectives on sulfonamide-induced cutaneous drug reactions. *Curr. Opin. Allergy Clin. Immunol*, 2:307–315.

Reilly, T.P., Lash, L.H., Doll, M.A., Hein, D.W., Woster, P.M., Svensson, C.K., (2000). A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J. Invest. Dermatol*,114:1164–1173.

Reilly, T.P., MacArthur, R.D., Farrough, M.J., Crane, L.R., Woster, P.M., and Svensson, C.K. (1999). Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? *J.Pharmacol. Exp Ther*, 291:1356–1364.

Reiter, R. J., Tan, D. X., Manchester, L. C., and Tamura, H.(2001). Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. A review of the evidence. *Cell Biochem Biophys*, 34:237-56.

Regula, K. M, and Kirshenbaum, L. A. (2005). Apoptosis of ventricular myocytes: a means to an end. *J Mol Cell Cardio*, 38:3-13.

Relling M. (1989). Polymorphic drug metabolism. *Clin Pharm* ,8:852-863.

Reznick, A. Z., and Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol*, 233:357-63.

Rieder, M. J. (1997). In vivo and in vitro testing for adverse drug reactions. *Pediatr Clin North Am*, 4:93-111

Rieder, M. J. (1994). Mechanisms of unpredictable adverse drug reactions. *Drug Safety*, 11:196-212.

Rieder, M. J., King, S. M., and Read, S. (1997). Adverse reactions to trimethoprim-sulfamethoxazole among children with human immunodeficiency virus infection. *Ped Infect Dis J*,16: 1028-1 031.

Rieder, M. J., Sisson, E., Bird, I. A., and Almawi, W. Y. (1992). Suppression of T-lymphocyte proliferation by sulphonamide hydroxylamines. *Int J Immunopharmacol*, 14(7):1175-80.

Rieder, M. J., Uetrecht, J., Shear, N. H., Cannon, M., Miller, M., and Spielberg, S. P. (1989). Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. *Ann Intern Med*, 110(4):286-9.

Rieder, M. J., Uetrecht, J., Shear, N. H., and Spielberg, S. (1988).P. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther*, 244(2):724-8.

Robinson, J. H., and Delvig, A. A. (2002). Diversity in MHC class II antigen presentation. *Immunology*, 105(3):252-62.

Rocken, M., Schaller, M., Sattler, E., and Burgdorf, W. (2010). Intolerance reactions. In: *Colors atlas of dermatology*, 34-43 (Theim Stuttgart, New York,).

Rogers, J. F., Nafziger, A. N., and Bertino, J. S. Jr. (2002). Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs. *Am J Med*, 113(9):746-50.

Rousar, T., Nydlova, E., Cesla, P., Stankova, P., Kucera, O., Parik, P., and Cervinkova, Z. (2012). Purified Acetaminophen-Glutathione conjugate is able to induce oxidative stress in rat liver mitochondria, *Physiological Research*, 61:103-109.

Sacco, J. C., Abouraya, M., Motsinger-Reif, A., Yale, S. H., McCarty, C. A., and Trepanier, L. A. (2012). Evaluation of polymorphisms in the sulfonamide detoxification genes NAT2, CYB5A, and CYB5R3 in patients with sulfonamide hypersensitivity. *Pharmacogenet Genomics*, 22(10):733-40.

Sadeque, A. J., Fisher, M. B., Korzekwa, K. R., Gonzalez, F. J., and Rettie, A. E. (1997). Human CYP2C9 and CYP2A6 mediate formation of the hepatotoxin 4-ene-valproic acid. *J Pharmacol Exp Ther*, 283:698-703.

Saka, B., Barro-Traore, F., Atadokpede, F. A., Kobangue L, Niamba, P. A., Adégbidi, H., Yedomon, H. G., Traoré, A., and Pitché, V. P. (2013). Stevens-Johnson syndrome and toxic epidermal necrolysis in sub-Saharan Africa: a multicentric study in four countries. *Int J Dermatol*, 52: 575–9.

Slatore, C. G., and Tilles, S. A. (2004). Sulfonamide hypersensitivity. *Immunol Allergy Clin North Am*, 24: 477–90.

Sanderson, J. P., Naisbitt, D.J., Farrell, J., Ashby, C.A., Tucker, M. J., Rieder, M. J., Shear, N. H., and Spielberg, S. P. (1985). In vitro evaluation of a toxic metabolite of sulfadiazine. *Can J Physiol Pharmacol*, 63:1370-1372.

Sandler, N. G., Wand, H., Roque, A., Law, M., Nason, M. C., Nixon, D. E., Pedersen, C., Ruxrungtham, K., Lewin, S. R., Emery, S., Neaton, J. D., Brechley, J. M., Deeks, S. G., Sereti, I., and Douek, D. C. (2011). Plasma levels of soluble CD 14 independently predict mortality in HIV infection. *J Infect Dis*, 203:780-790.

Schenkman, J. L, and Greim, H. (1993). *Cytochrome P450*, vol. 105. Springer-Verlag, Berlin; NewYork.

Schnyder, B., Mauri-Hellweg, D., Zanni, M., Bettens, F., and Pichler, W.J. (1997). Direct, MHC-dependent presentation of the drug sulfamethoxazole to human T cell clones. *J Clin Invest* ,100:136-141.

Shear, N. H., and Spielberg, S. P. (1988). Anticonvulsant hypersensitivity syndrome. In vitro assessment of risk. *J Clin Invest*, 82: 1826-32.

Shear, N. H., Spielberg, S.P., Grant, D.M., Tang, B. K., and Kalow, W. (1986). Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. *Ann Intern Med*, 105:179-84.

Shi, Y., Zheng, W., and Rock, K.L. (2000). Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. *PNAS*, 97: 14590 -14595

Siems, W., Wiswedel, I., Salerno, C., Crifò, C., Augustin, W., Schild, L., Langhans, C. D., and Sommerburg, O. (2005). Beta-carotene breakdown products may impair mitochondrial functions--potential side effects of high-dose beta-carotene supplementation. *J Nutr Biochem*, 16(7):385-97.

Sies, H. (1991). Oxidative stress: Introduction. In: Sies H (ed) *Oxidative stress*. Vol. xv-xxii. Academic Press, San Diego.

Slatore, C. G and Tilles, S. A. (2004). Sulfonamide hypersensitivity. *Immunol Allergy Clin North Am*, 24:477–490.

Summan, M., and Cribb, A. E. (2002). Novel non-labile covalent binding of sulfamethoxazole reactive metabolites to cultured human lymphoid cells. *Chem Biol Interact*, 142:155-173.

Svensson, C.K., Cowen, E.W., Gaspari, A.A. (2001). Cutaneous drug reactions. *Pharmacol. Rev*, 53:357–379.

Teraki, Y., Shibuya, M., and Izaki, S. (2010). Stevens-Johnson syndrome and toxic epidermal necrolysis due to anticonvulsants share certain clinical and laboratory features with drug-induced hypersensitivity syndrome, despite differences in cutaneous presentations. *Clin Exp Dermatol* ,35(7):723-8.

Tietze, F., (1969). *Analytical Chemistry*, 27:502-520.

Tilles, S. A. (2001). Practical issues in the management of hypersensitivity reactions: Sulfonamides. *South Med J*, 94(8):817-824.

Trepanier, L. A., and Miller, J. L. (2000). NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. *Xenobiotica*, 30:1111-1121.

Utrecht, J. (2007). Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol*, 47:513-39.

Utrecht, J. P. (1989). Idiosyncratic drug reactions: possible role of reactive metabolites generated by leukocytes. *Pharm Res*, 6(4):265-73.

Van der Ven A, J., Vree, T. B., Koopmans, P. P., and van der Meer J, W. (1996). Adverse reactions to co-trimoxazole in HIV infection: a reappraisal of the glutathione-hydroxylamine hypothesis. *J Antimicrob Chemother*, 37 Suppl B:55-60.

Vega, J. M., Blanca, M., Garica, J. J., Carmona, M. J., Miranda, A., Perez- Estrada, M., Fernandez, S., Acebes, J. M., and Terrados, S. (1994) Immediate allergic reactions to amoxicillin. *Allergy*, 49: 317-322.

Verma, P., Bhattacharya, S, N., Banerjee, B. D., and Khanna, N. (2012). Oxidative stress and leukocyte migration inhibition response in cutaneous adverse drug reactions. *Indian J Dermatol Venereol Leprol*, 78(5):664.

Vyas, P. M., Roychowdhury, S., Woster, P. M, and Svensson, C. K. (2005). Reactive oxygen species generation and its role in the differential cytotoxicity of the

arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes. *Biochem Pharmacol*, 70:275–286.

Walmsley, S.L., Winn, L. M., Harrison, M. L., Uetrecht, J. P., and Wells, P. G. (1997). Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *AIDS*, 11(14):1689-97.

Wang, Y., Gray, J. P., Mishin, V., Heck, D. E., Laskin, D. L., and Laskin, J. D.(2008). Role of cytochrome P450 reductase in nitrofurantoin-induced redox cycling and cytotoxicity. *Free Radical Biology and Medicine*, 44(6):1169-79.

Witschi, A., Junker, E., Schranz, C., Speck, R. F., and Lauterburg, B. H. (1995). Supplementation of N-acetylcysteine fails to increase glutathione in lymphocytes and plasma of patients with AIDS. *AIDS Res Hum Retroviruses*, 11:141-143.

Wolkenstein, P., Charue, D., Laurent, P., Revuz, J., Roujeau, J. C., and Bagot, M. (1995). Metabolic predisposition to cutaneous adverse drug reactions: role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. *Arch Dermatol*, 131:544-551.

Wolkenstein, P., Lorient, M. A., Aractingi, S., Cabelguenne, A., Beaune, P., Chosidow, O. (2000). Prospective evaluation of detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. *Pharmacogenetics*, 10(9):821-8.

World Health Organization. International Drug Monitoring: The Role of National Centres. Geneva, Switzerland: World Health Organization; 1972:9. Tech Rep Ser WHO No. 498.

Yagi, K. (1998). Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol Biol*, 108:101-6.

Zaccara, G., Franciotta, D., and Perucca, E. (2007). Critical review: idiosyncratic adverse reactions to antiepileptic drugs. *Epilepsia*, 48(7):1223-44.

Zangar, R. C., Davydov, D. R., and Verma, S. (2004). Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharmacol*, 199: 316- 331.

Zanni, M.P., Mauri-Hellweg, D., Brander, C., Wendland, T., Schnyder, B., Frei, E., von Greyerz, S., Bircher, A., and Pichler, W. J. (1997). Characterization of lidocaine-specific T cells. *J Immunol*, 158:1139.

Zuniga, F. I., Loi, D., Ling, K. H., and Tang-Liu, D. D. (2012). Idiosyncratic reactions and metabolism of sulfur-containing drugs. *Expert Opin Drug Metab Toxicol*, 8(4):467-85.



PERMISSION LETTER

July 16, 2015

Springer reference

Drug Safety

December 2000, Volume 23, Issue 6, pp. 483-507

Date: 19 Oct 2012

Immunological Principles of Adverse Drug Reactions

Authors: Dr Dean J. Naisbitt, S. Fraser Gordon, Munir Pirmohamed, B. Kevin Park

© Adis International Limited 2000

Material to be used: Figure 2

DOI 10.2165/00002018-200023060-00002

Print ISSN 0114-5916

Online ISSN 1179-1942

Journal no. 40264

Your project

Requestor: Elham Sultan
MSc Candidate

University: University of Western Ontario
Department of Physiology & Pharmacology

Purpose: Dissertation/Thesis

With reference to your request to reuse material in which **Springer Science+Business Media** controls the copyright, our permission is granted free of charge under the following conditions:

Springer material

- represents original material which does not carry references to other sources (if material in question refers with a credit to another source, authorization from that source is required as well);
- requires full credit (Springer and the original publisher, book/journal title, chapter/article title, volume, year of publication, page, name(s) of author(s), original copyright notice) to the publication in which the material was originally published by adding: "With permission of Springer Science+Business Media";
- figures, illustrations, and tables may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author and/or Springer Science+Business Media;
- **Springer does not supply original artwork or content.**

This permission

- is non-exclusive;
- is valid for one-time use only for the purpose of defending your thesis limited to university-use only and with a maximum of 100 extra copies in paper. If the thesis is going to be published, permission needs to be reobtained.
- includes use in an electronic form, provided it is an author-created version of the thesis on his/her own website and his/her university's repository, including UMI (according to the definition on the Sherpa website: <http://www.sherpa.ac.uk/romeo/>);
- is subject to courtesy information to the author (address is given in the publication);
- is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer's written permission;
- is only valid if no personal rights, trademarks, or competitive products are infringed.

This license is valid only when the conditions noted above are met.

Permission free of charge does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Rights and Permissions
Springer Science+Business Media

To whom it may concern,

My name is Elham Sultan. I am a MSC student in the department of Physiology & Pharmacology at the University of Western Ontario, London, Ontario-Canada. I am currently preparing my thesis and I am writing to you to ask permission to reprint the figure 1, 2 and 3 of the following paper: Jack Uetrecht. 2007. Idiosyncratic Drug Reactions: Current Understanding. Annu. Rev. Pharmacol. Toxicol. 47:513–39.

The current title of my thesis is: Pathophysiologic Mechanisms of Immune-mediated Drug Hypersensitivity Reactions to Sulfonamides.

If there are any other questions or concerns I would be happy to answer them.

Thanks in advance,

Elham Sultan

MSc Candidate

Physiology & Pharmacology

University of Western Ontario

From: customercare@copyright.com

Sent: Thu 2015-07-16 3:37 PM

To: Elham Sultan;

Dear Elham Sultan,

Thank you for contacting Copyright Clearance Center's RightsLink service. RightsLink is a licensing service that provides permission for the reproduction and distribution of copyrighted materials in print and electronic formats on behalf of Rightsholders who list their titles with us. RightsLink is accessed directly through a publisher's website. Please be aware that the types of licenses offered are established by each individual Rightsholder, and will vary.

Getting Started:

Please visit the article at the participating publisher's website here:

<http://www.annualreviews.org/doi/abs/10.1146/annurev.pharmtox.47.120505.105150>

Click on "Rights & Permissions" link , and you will be directed to the RightsLink order page. Next, select your permission options from the drop down menus. You can obtain a price quote in advance by using the "quick price" button. Please note that any fees are set by the publisher, and not by RightsLink, which is a service that acts on behalf of the publisher. Before you can place a license order, kindly create an account with RightsLink (click "Create Account"). After you complete the 2-page account creation, you will be able to submit your order.

Title: Idiosyncratic Drug Reactions: Current Understanding

Author: Jack Uetrecht

Publication: Annual Review of Pharmacology and Toxicology

Publisher: Annual Reviews

Date: Feb 1, 2007

Copyright © 2007, Annual Reviews

Permission Not Required

Material may be republished in a thesis / dissertation without obtaining additional permission from Annual Reviews, providing that the author and the original source of publication are fully acknowledged.



Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1

Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. M.J. Rieder

Review Number: 11883E

Revision Number: 4

Review Date: January 05, 2009

Review Level: Expedited

Protocol Title: GATC: Genotype Specific Approaches to Therapy in Childhood.

Department and Institution: Paediatrics, London Health Sciences Centre

Sponsor: Genome

Ethics Approval Date: January 22, 2009

Expiry Date: December 31, 2013

Documents Reviewed and Approved: Revised study end date.

Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

- changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
- all adverse and unexpected experiences or events that are both serious and unexpected;
- new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information			
<input type="checkbox"/> Janice Sutherland	<input type="checkbox"/> Elizabeth Wambolt	<input checked="" type="checkbox"/> Grace Kelly	<input type="checkbox"/> Denise Grafton

This is an official document. Please retain the original in your files.

Curriculum Vitae

Elham Sultan

Master of Science Candidate
Department of Physiology and Pharmacology
University of Western Ontario

EDUCATION:

Master of Science

Jan. 2014- Present

Department of Physiology & Pharmacology

University of Western Ontario, London, Ontario, Canada

Supervisor: Dr. Michael Rieder M.D., Ph.D., FRCPC, FAAP, FRCP (Glasgow)

Bachelor of Pharmacy

2005-2009

Faculty of Pharmacy

Al-Mergeb University, Al-Khoms, Libya

AWARDS:

The Libyan Ministry of Higher Education MSc Scholarship award

2013-Present.

RELATED WORK EXPERIENCE

Teaching Assistant

2015

University of Western Ontario, London, ON

Pharmacology 2060b

PRESENTATIONS:

Poster Presentation; London Health research day 2015

April 2015

Poster Presentation; Roberts Research Retreat 2015

June 2015