THE ROLE OF NEUTROPHILS IN THE PATHOGENESIS OF IDIOSYNCRATIC DRUG TOXICITY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

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This thesis is the result of my own work. The material contained within the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

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To my mam, dad and Rachel

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Table of Contents

Abbreviations

ACMA	9-amino 6-chloro 2-methoxyacridine
ADR	adverse drug reaction
AIDS	acquired immunodeficiency syndrome
AQ	amodiaquine
ASC	ascorbic acid
Ci	curie
Cl^-	chloride ion
CLZ	clozapine
CQ	chloroquine
CYC	cycloquine
CYS	cysteine
CYSSYC	cystine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
Em	emission
eV	electron volts
Ex	excitation
FAD	flavin adenine dinucleotide
GSH	glutathione (reduced)
GSSG	oxidised glutathione (oxidised)
H^+	hydrogen ion
$[^3H]$	tritium
HIV	human immunodeficiency virus
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
IC_{50}	concentration required to cause 50% inhibition
ICE	interleukin 1-β converting enzyme
LC-MS	liquid chromatography-mass spectrometry
M	molar
MHC	major histocompatibility complex
MNL	mononeuclear leucocytes / lymphocytes
MPO	myeloperoxidase
m/z	mass to charge ratio
n	sample population size
NADPH	nicotinamide adenine nucleotide phosphate (reduced)
NBT	nitroblue tetrazolium
NMR	nuclear magnetic resonance
O_2	oxygen

$O_2^{\cdot-}$	superoxide
$OH^{\cdot}$	hydroxyl radical
OH^{-}	hydroxyl ion
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leucocytes / neutrophils
ppm	parts per million
PYRO	pyronaridine
RBC	red blood cells / erythrocytes
S.D.	standard deviation
S.E.M.	standard error of mean
SMX	sulphamethoxazole
SMX-NHOH	sulphamethoxazole hydroxylamine
SMX-NO	nitroso sulphamethoxazole
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEB	tebuquine
TLC	thin layer chromatography
TNFR	tumour necrosis factor receptor
UDP	uridine diphosphate
vs	versus
v/v	volume per volume
w/v	weight per volume
WHO	world health organisation

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Williams, D.P., Naisbitt, D.J., Pirmohamed, M., and Park, B.K. (submitted for publication) Clozapine induces neutrophil apoptosis by two concentration-dependent pathways: Implications for agranulocytosis. *Br. J. Pharmacol.*

PhD Abstract

Metabolism of drugs to chemically reactive metabolites by neutrophils has been implicated in the pathogenesis of idiosyncratic agranulocytosis. Three drugs (amodiaquine [AQ], sulphamethoxazole [SMX], clozapine[CLZ]) which are thought to cause toxicity by the formation of chemically reactive metabolites have been investigated. The work described was performed to investigate the chemical and biochemical mechanisms of neutrophil toxicity, and in particular to understand the relationship between drug disposition and drug toxicity.

Phorbol ester-stimulated neutrophils bioactivated AQ to a protein-reactive and cytotoxic quinoneimine metabolite which caused concentration-dependent depletion of intracellular glutathione (GSH; IC_{50}; 18μM). Structure disconnection showed that the *p*-aminophenol group was necessary for bioactivation. In addition, AQ was taken up more avidly by neutrophils (34%) than by lymphocytes (23%) and red blood cells (4%). Compounds which are structurally related to AQ were examined in order to determine other structural features involved in agranulocytosis. Derivatives of AQ that contain hydrophilic substituents in the 5'-position (cycloquine [CYC], pyronaridine [PYRO]) had little effect on neutrophil function or viability (IC_{50}; > 100μM). Additionally, they did not deplete GSH (IC_{50}; > 100μM) and accumulated within the lysosome to a similar extent as AQ (AQ, 29 ± 4%; CYC, 11 ± 1%; PYRO, 30 ± 4%). The data suggest that these highly effective antimalarial agents are less likely than AQ to cause life-threatening agranulocytosis. However, these findings should be interpreted with caution since further studies which utilised horseradish peroxidase and hydrogen peroxide (as a more potent oxidising system) to generate the quinoneimine metabolite of CYC and PYRO *in situ*, demonstrated significant neutrophil toxicity (IC_{50}; CYC, 10μM; PYRO, 32μM; AQ, 16μM) and GSH depletion to an extent similar to that seen with AQ (IC_{50}; CYC, 3μM; PYRO, 3μM; AQ, 1μM).

Sulphamethoxazole (SMX) is associated with agranulocytosis as part of a generalised hypersensitivity reaction. It has been proposed that products of drug metabolism (sulphamethoxazole hydroxylamine [SMX-NHOH] or nitroso sulphamethoxazole [SMX-NO]) are responsible for the idiosyncratic toxicity associated with SMX therapy. SMX-NHOH and SMX-NO were synthesised in order to investigate the role of drug bioactivation and drug detoxification in SMX-induced hypersensitivity. The role of sulphydryl containing compounds has been characterised at a chemical level. Interestingly, neutrophils and lymphocytes were resistant to cytotoxicity (IC_{50}; > 100μM) and GSH depletion (IC_{50}; > 100μM). Reduction of SMX-NO to SMX-NHOH and SMX, and SMX-NHOH to SMX was observed when these compounds were incubated individually with red blood cells, neutrophils, lymphocytes and plasma. The red blood cells were more potent at reducing SMX-NHOH back to SMX (12 ± 2%). In contrast, plasma was more potent than the other blood components at reducing the nitroso metabolite (SMX-NO→NHOH, 45 ± 10%; SMX-NO→SMX, 16 ± 4%). Plasma cysteine (CYS) may be of particular importance in the reduction since a deficiency in patients with HIV-infection has been postulated to predispose to both disease progression and drug hypersensitivity reactions. The study described in the thesis confirms that HIV-infected patients (n =27) had lower reduced plasma CYS levels (HIV-positive patients, 13 ± 3μM; healthy controls, 17 ± 3μM), and this resulted in decreased detoxification of SMX-NO (HIV-

positive patients, SMX-NO→NHOH, 40 ± 6%; SMX-NO→SMX, 14 ± 2%; healthy controls, SMX-NO→NHOH, 55 ± 4%; SMX-NO→SMX, 13 ± 1%).

Metabolism of clozapine (CLZ) to a chemically reactive intermediate(s) has been implicated in the pathogenesis of agranulocytosis. No studies have thus far been performed with regard to the functional toxicity of the reactive metabolite(s). To address this issue, a novel *in vitro* assay was developed in which the *in situ* generation of the reactive metabolite(s) was coupled to an assessment of neutrophil viability. CLZ was bioactivated to a metabolite which caused depletion of intracellular GSH (IC_{50}; 2μM) and neutrophil toxicity (IC_{50}; 30μM) at therapeutic concentrations.

In these studies cell death was used as a toxicological end-point. It is important to note that these techniques do not distinguish between apoptosis and necrosis, the two major forms of cell death. Therefore, flow cytometry was used to investigate the mechanism of drug-induced cell death. Incubation of AQ (30-300μM) and CLZ (300μM) with neutrophils resulted in an increase in spontaneous apoptosis. In contrast, SMX had no effect (1-300μM). In the presence of horseradish peroxidase and hydrogen peroxide, AQ and CLZ induced neutrophil apoptosis at the lowest concentrations studied (1-3μM), while apoptosis was only observed at concentrations of 100μM and above when SMX-NHOH and SMX-NO were incubated individually with neutrophils. These findings may provide a novel insight into the pathogenesis of drug-induced agranulocytosis.

In conclusion, the studies described in this thesis have utilised a variety of *in vitro* systems. Novel insights of the relationship between the chemical, the way it is handled by the cell, and how this may ultimately lead to agranulocytosis have been elucidated.

Chapter 1:

General Introduction

CONTENTS

1.1 Introduction

Metabolism is a process that the body utilises to chemically modify foreign components, with the overall aim of increasing either their biliary or urinary excretion rate. In the context of pharmacology, drug metabolism increases a compounds molecular weight and water solubility by two kinds of biochemical reaction, which may occur sequentially. The majority of drugs undergo functionalisation by the introduction of a reactive group into the molecule, prior to conjugation with a large water-soluble substituent. Such enzymatic modification usually abolishes a drug's pharmacological activity.

In general, drug metabolism is regarded as a process of detoxification, although occasionally an imbalance in the normal metabolic process may produce a reactive and potentially toxic metabolite (Park, 1986; Park *et al.*, 1987). If inadequately detoxified, these metabolites can combine with or damage cellular macromolecules such as proteins and nucleic acids and result in various forms of toxicity, including teratogenicity, carcinogenicity, cellular necrosis / apoptosis and hypersensitivity (Gillette, 1974; Park, 1986).

Specific drugs can be metabolised by individual organs, especially compounds which possess an easily oxidisable functional group. Most reactive metabolites have a short biological half-life and would not be expected to reach significant concentrations at a site distant from their site of formation. Consequently, organ-specific adverse drug reactions (ADRs) are often determined by the site of formation and the stability of the chemically reactive intermediate. Organs also differ in their immune responsiveness to toxic events, and their ability

to accumulate specific drugs. Thus, it is rational to investigate the ability of the target organ of a specific ADR to form reactive drug metabolites.

The liver is quantitatively the most important site of drug metabolism. Its therefore not surprising that in some instances drug bioactivation can lead to both mild and severe hepatic toxicity (Stricker and Spoelstra, 1985). Other tissues such as the skin, lungs, and kidneys have considerable metabolic activity and can also be affected either in isolation or combination with other organs by ADRs (Devereux *et al.,* 1989; Bickers, 1991; Pirmohamed *et al.*, 1994).

The haematological system is one of the most commonly affected sites of drug toxicity. Drug toxicity may affect either the pluripotential stem cell, resulting in aplastic anaemia (Vincent, 1986), neutrophils (polymorphonuclear leucocytes; PMN), resulting in agranulocytosis (Pisciotta, 1990) or erythrocytes resulting in various forms of red cell dyscrasias including methaemoglobinaemia, haemolysis and megaloblastic anaemia (Ammus and Yunis, 1989). Chemically reactive metabolites play an important role in haematological toxicity. The metabolites may be produced by hepatic enzymes, or alternatively in the bone marrow or peripheral blood cells by the cytochrome P450 enzymes, prostaglandin synthetase, NADPH oxidase and myeloperoxidase (MPO) (Hurst, 1987; Murray *et al.,* 1988; Cribb *et al.,* 1990). In some cases, intermediary bacterial metabolism has also been implicated in the production of haemotoxic metabolites (fig. 1.1).

The haematological system only plays a minor role in the overall scheme of drug metabolism; nevertheless, neutrophil / bone marrow bioactivation, with

resultant reactive metabolite formation, may be responsible for the agranulocytosis observed with a wide variety of compounds (Pohl *et al.*, 1988).

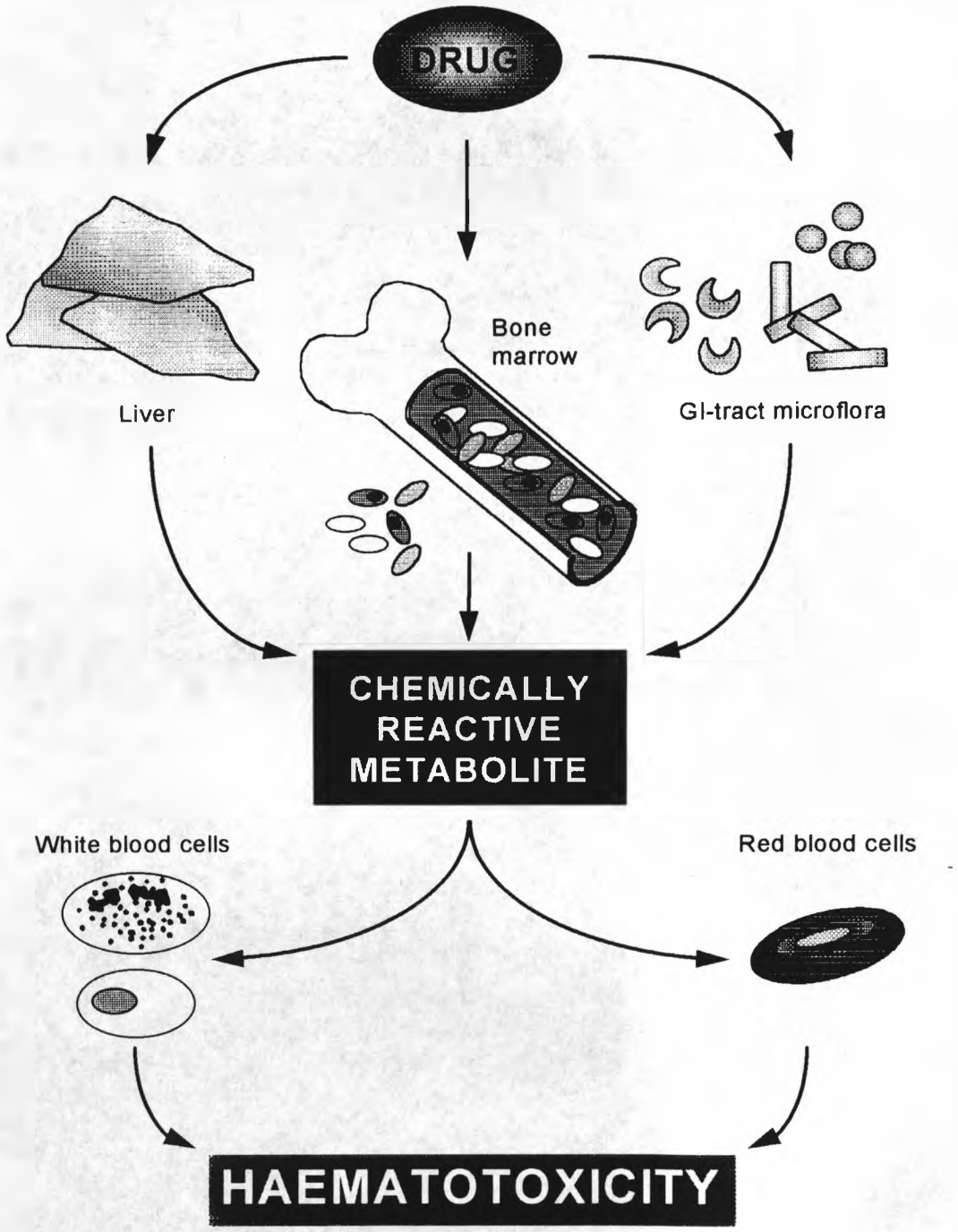


Figure 1.1 Mechanisms of drug-induced haematotoxicity.

1.2 Adverse Drug Reactions

ADRs are a major complication of drug therapy and account for a great deal of patient morbidity and significant patient mortality. The occurrence of an ADR represents an unnecessary burden on hospital resources, often resulting in further hospital treatment. It is estimated that 2-3% of hospital admissions are as a direct result of an ADR, while 10-20% of patients develop some form of drug toxicity prolonging their stay (D'Arcy, 1986).

ADRs take many different forms varying from a mild skin reactions to multiple organ failure. Such reactions have been classified clinically according to the following scheme (Rawlins and Thompson, 1977; Park *et al.*, 1994 and 1995).

Type A: Dose dependent reactions which are usually predicted from the known primary or secondary pharmacology of the drug (e.g. hypotension with anti-hypertensives and bleeding with anticoagulants). Such reactions are usually prevented by a dose reduction.

Type B: Idiosyncratic reactions (hypersensitivity reactions), the mechanisms of which are not fully understood (e.g. aplastic anaemia with chloramphenicol). They show no simple dose-response relationship and cannot be predicted from the known pharmacology of the drug. They occur in only a small percent of the population, but are often serious and are responsible for many drug induced deaths. Such reactions are not detected by preclinical toxicology testing,

cannot usually be reproduced in animal models and show marked interindividual variation.

Type C: Reactions associated with the long term effects of drug therapy, where prolonged exposure of the drug results in an altered pharmacological response (e.g. benzodiazepine dependence and analgesic nephropathy). These reactions are well described and can normally be anticipated.

Type D: Delayed effects such as carcinogenicity and teratogenicity (e.g. polyaromatic hydrocarbons). Such toxicities are generally avoided by extensive preclinical mutagenicity and carcinogenicity programmes that a new chemical entity must undergo before a product is granted a licence.

Type E: Drug overload by either overdose or excessive accumulation of drug or metabolite (e.g. paracetamol hepatotoxicity).

Type F: Pharmacodynamic or pharmacokinetic drug interactions.

The classes are not mutually exclusive, the majority of ADRs being described by more than one characteristic. Such a classification is useful in the clinical diagnosis of ADRs although it does not provide an understanding of the underlying mechanism of toxicity.

The mechanism of toxicity can be broadly described as either pharmacological or chemical (Park *et al.*, 1994). Almost all adverse reactions have a pharmacological basis and are therefore dose dependent, being described in terms of the primary and secondary pharmacology of the drug. Such reactions are readily reversible by drug withdrawal or a simple dose-reduction (Rawlins and

Thompson, 1977). ADRs with a chemical basis are generally more serious and although only occurring in a minority of individuals, they are often life-threatening. Such reactions can be defined in terms of the physicochemical properties or chemical reactivity of the drug or more often drug metabolite (Park *et al.*, 1994). They are irreversible and dependent on the relative rates of accumulation and clearance of the toxic species. The ability of such a small amount of toxic species to produce such extensive tissue damage suggests that some form of biological amplification may be involved.

1.3 Drug Metabolism and Disposition

1.3.1 Distribution

Following administration of a drug (either orally or parenterally), distribution and metabolism are the main contributing factors towards its duration of action. Drugs are distributed around the body by diffusion through aqueous compartments (eg. plasma, interstitial fluid, transcellular fluid and intracellular fluid). Each compartment is separated by various non-aqueous barriers. The rate of diffusion depends largely on particle size and since the majority of drugs fall within the region of 200-1000 atomic mass units, the rate of diffusion has only a small affect on the overall pharmacokinetic behaviour of the molecule. Thus, the movement of molecules between non-aqueous and lipophilic compartments is what generally determines where and for how long a drug will remain in the body (Gilman *et al.*, 1985; Rang and Dale, 1991).

1.3.2 Movement of Drugs Across Cell Membranes

A single layer of membrane separates intracellular from extracellular compartments. In general, drugs pass through cell membranes in two ways:

i) Diffusion through the lipid membrane; and

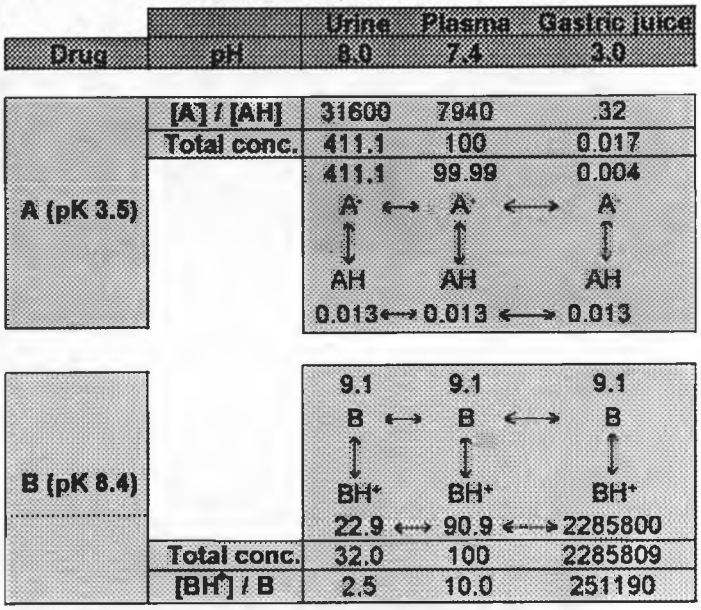
ii) By combination with a protein which carries the drug across the membrane.

Non-ionised substances dissolve in lipids and can pass freely through cell membranes, while ionised substances are normally impeded. One complicating factor regarding membrane permeability is that many drugs are weak acids / bases which may exist in either protonated or unprotonated forms. The ratio of the two forms is governed by the pKa and pH of the specific intracellular and extracellular compartments. Fig. 1.2 represents the theoretical accumulation of aspirin (A, pKa 3.5) and quinidine (B, pKa 8.4) into urine, plasma and gastric juice. Upon accumulation, a process of ion-trapping occurs where the unionised form of the drug regains a charge effectively removing it from the equilibrium. Theoretically, the process is capable of driving large concentration gradients between compartments if there is a large difference in pH.

1.3.3 Drug Partition into Body Fat

Fat is a large, non-polar compartment of the body (around 15% of body weight). Lipid soluble drug molecules exist largely in body fat where they remain inactive and act as a reserve prolonging the duration of action of the drug. In practise, this is important for only a few drugs for two reasons; first, many drugs

exist largely in an ionised form, and secondly, lipid solubility is limited by the presence of hydrophilic groups on the molecule.



Taken from Rang and Dale, (1991)

Figure 1.2 Differential accumulation of aspirin and quinine.

1.3.4 Drug-Plasma Protein Binding

At normal therapeutic concentrations, many drugs show a high degree of plasma protein binding (e.g. phenylbutazone and phenytoin show greater than 90% binding). Only drugs circulating in the aqueous layer are pharmacologically active. Thus when a drug becomes protein bound, the circulating concentrations of the drug is drastically reduced. Albumin is the most abundant plasma protein, with β-globulin and acid glycoprotein being present in smaller amounts.

1.3.5 Drug Metabolism

Metabolism is an important determinant of the duration of action of a drug, its pharmacological efficacy and its toxicity. Metabolism is generally regarded as a process of detoxification in that it facilitates the excretion of drugs and foreign compounds from the body. The reactions are catalysed by a wide range of enzymes found in various sub-cellular compartments, including the mitochondria, cytosol and endoplasmic reticulum (table 1.1). In certain circumstances, normal metabolic processes are required for the formation of biologically active molecules, which account in whole or in part, for the pharmacological and toxicological actions of the parent compound (e.g. diazepam and levodopa) (Park *et al.*, 1987).

1.3.6 Phase I and Phase II Metabolism

Phase I reactions usually consist of oxidation, reduction or hydrolysis, and the products may be more toxic than the parent drug. Phase II reactions involve conjugation of the drug to biological co-factors, which normally results in inactivation of the compound. Drugs may undergo sequential phase I and then phase II reactions prior to excretion, although alternatively they may be conjugated without functionalisation, if a functional group is available on the parent compound.

Endoplasmic reticulum	Cytochrome P450 NADPH-cytochrome P450 reductase UDP glucuronosyl transferases Glutathione S-transferases Epoxide hydrolase Flavin-containing monooxygenase Xanthine and aldehyde oxidase Carboxyl esterase Aldehyde dehydrogenase
Cytosol	Glutathione S-transferases Epoxide hydrolase Aldehyde and ketone reductase Catechol O-methyl transferase Aldehyde dehydrogenase N-acetyl transferases Sulphotransferases
Mitochondria	Monoamine oxidase Glutathione peroxidase Aldehyde dehydrogenase Cytochrome P450

Adapted from Jakoby, (1980)

Table 1.1 The subcellular location of the major drug metabolising enzymes.

The cytochrome P450 mixed function oxidase system is responsible for around 95% of phase I metabolism (Burchell *et al.*, 1991). Reactions occur in parallel or in series, and bioactivation can be catalysed by more than one enzyme. The enzymes are located in the smooth endoplasmic reticulum of most mammalian tissues, and are able to catalyse the oxidative biotransformation of many chemically and biologically unrelated substrates (Gonzalez *et al.*, 1991). Catalysis is driven by activation of oxygen rather than binding of a substrate. This enables the system to metabolise a limitless amount of low molecular weight

lipophilic molecules. There are many different P450 enzymes, with more than 200 isozymes having been identified in terms of their gene sequence (Nelson *et al.*, 1993). Characteristics that individual enzymes possess include a strongly conserved haem binding site, a powerful oxidising capacity and relatively low substrate affinity (table 1.2).

Phase II metabolic pathways include glucuronidation, amino acid conjugation, glutathione (GSH) conjugation, sulphation and acetylation (table 1.3). Quantitatively, the most important phase II pathways are glucuronidation and sulphation (Dutton and Burchell, 1977). Glucuronidation is catalysed by uridine diphosphate (UDP) glucuronyltransferases, which like P450 enzymes form a superfamily of enzymes encoded by multiple genes (Burchell *et al.*, 1991). To date 20 different enzymes are known to exist. Conjugation involves the transfer of glucuronic acid from the high energy UDP-glucuronic acid to an electron rich atom from the substrate (alcohols, hydroxylamines, carboxylic acids, amides, quinones and thiols). Sulphation also requires an energy rich donor, in this case 3'-phosphoadenosine-5'-phosphosulphate. The reaction is catalysed by non-specific sulphonyl transferase enzymes, and major substrates include phenols, alcohols and amines (Gibson and Skett, 1994).

Acetylation and amino acid conjugation are metabolic pathways for many aromatic amines. Acetylation is catalysed by the *N*-acetyl transferases and requires activation of the cofactor acetyl CoA which is obtained either via glycolysis or direct interaction of acetate and coenzyme A. Amino acid conjugation is similar although the drug and not the endogenous cofactor is activated.

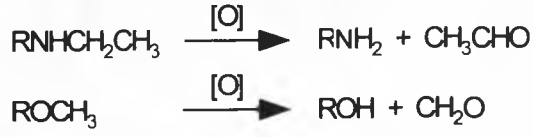
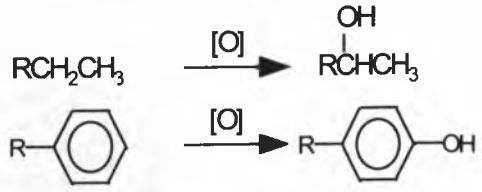
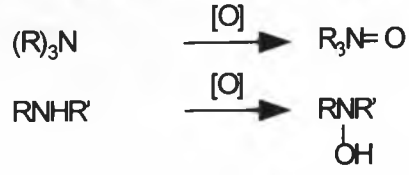
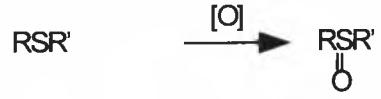
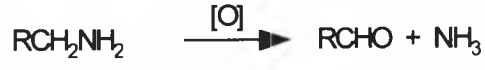
Conjugation with the tripeptide GSH (γ-glutamyl-cysteinylglycine) is a major endogenous protective system for the removal of potentially toxic species from the body (Reed, 1986). GSH is the most abundant intracellular thiol (Larsson *et al.*, 1983), with concentrations in the millimolar range in most cells. Conjugation can either occur spontaneously or may be catalysed by the GSH transferases (Deleve and Kaplowitz, 1990), a family of isozymes with unique but overlapping substrate specificity (Jackoby, 1978; Boyer, 1989).

The spontaneous nucleophilic addition reaction is particularly likely to occur with so-called soft electrophiles, while enzyme catalysis is more common with the hard electrophiles. Enzyme catalysis assumes dominance when GSH concentrations are low, even with soft electrophiles (Coles, 1985; Coles *et al.*, 1988). Compounds which conjugate with GSH include epoxides, aliphatic and aromatic halo- and nitro compounds, alkenes and quinones (Gibson and Skett, 1994).

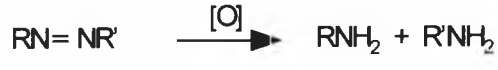
1.4 The Relationship Between Drug Metabolism and Drug Toxicity

In general chemically reactive agents tend to be toxic (Guengerich, 1990). Most drugs are not chemically reactive, although in certain circumstances, by the normal process of drug metabolism a chemically reactive and potentially toxic metabolite may be produced (Park *et al.*, 1992). If the chemically reactive metabolite is inadequately detoxified due to an imbalance in drug metabolism it may bind irreversibly to cellular macromolecules, resulting in various forms of

1. Oxidative reactions

1. N- and O- Dealkylations	
2. Aliphatic and Aromatic Hydroxylations	
3. N-Oxidation and N-Hydroxylation	
4. Sulphoxide Formation	
5. Deamination of Amines	
6. Desulphuration	

2. Reductive reactions

1. Azo Reduction	
2. Nitro Reduction	

3. Hydrolysis

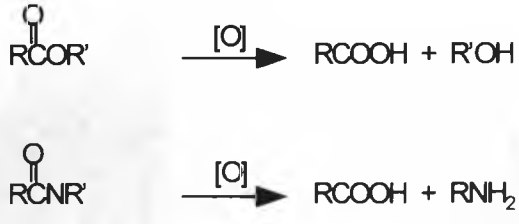
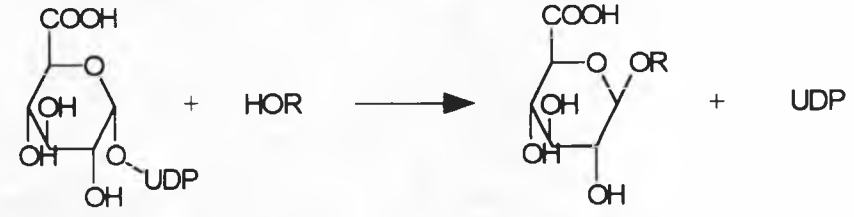


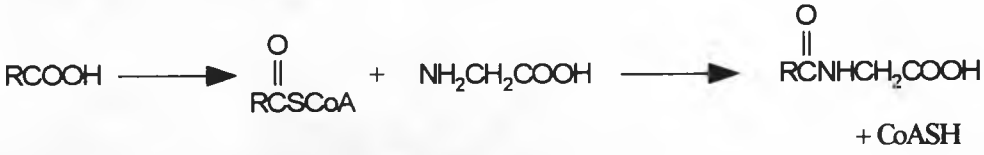
Table 1.2 Phase I reactions.

1. Glucuronidation



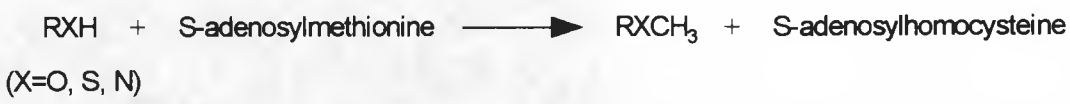
2. Acetylation

3. Amino acid conjugation



4. Conjugation with Sulphide

5. O-, S-, N-methylation



6. Glutathione conjugation

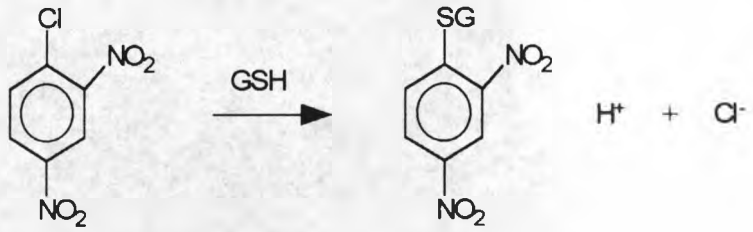


Table 1.3 Phase II reactions.

toxicity, including cellular necrosis, apoptosis, hypersensitivity, teratogenicity and carcinogenicity (fig. 1.3) (Park *et al.*, 1994; Pirmohamed *et al.*, 1994). Important factors in determining the site and frequency of drug toxicity include the rate of metabolism (both bioactivation and detoxification), which will influence the amount of drug / metabolite present at its site of action, the route of metabolism, which will determine the nature of the chemical entities which are present in the body, the half life of the toxic metabolite and the nature of the macromolecule to which this metabolite binds.

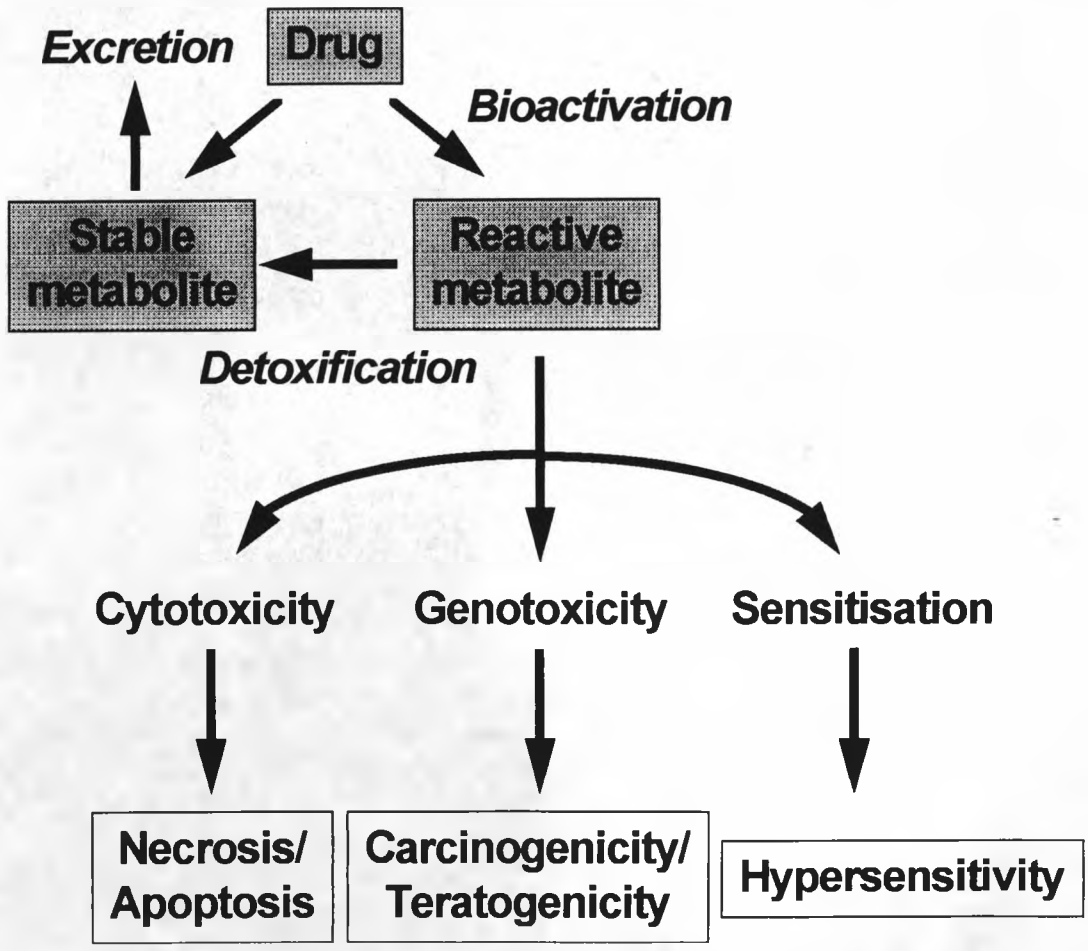
Population studies have shown that there are large differences between individuals in their capacity to detoxify drugs. Such differences are dependent upon an individuals genetic constitution (which varies according to age), environment, disease and drug interactions (Vesell, 1984). All these factors determine an individuals susceptibility to drug toxicity.

1.4.1 Idiosyncratic Drug Toxicity

Although idiosyncratic drug reactions are less common than predictable dose dependent reactions, they are not rare, accounting for approximately 10% of all ADRs (Goldstein and Patterson, 1984). The clinical characteristics of idiosyncratic drug reactions are diverse, and can vary considerably between different drugs and also for the same drug between different individuals (Pohl *et al.*, 1988; Pirmohamed *et al.*, 1994). Despite this diversity, certain characteristics are common to most ADRs (section 1.2).

1.4.2 Organ Directed Toxicity

Any organ can be affected by drug toxicity, either in combination or isolation. When multiple organs are affected, the effect on each organ may primarily be due to the drug, or one organ may be predominantly involved with secondary effects on the other organs (Pirmohamed *et al.*, 1994). The liver, haematological system and the skin are the three most common organs affected, while the kidneys, nervous system and lungs are to a lesser extent.



Taken from Park *et al.*, (1994)

Figure 1.3 Scheme illustrating the relationship between drug metabolism and toxicity.

The reactivity of a drug or drug metabolite may determine the site of toxicity. A compound of relatively low reactivity can be selectively detoxified prior to binding to an essential macromolecule, while a compound may be so reactive that it reacts with water or the enzyme which formed it. The majority of reactive metabolites have a short biological half life and are not expected to reach significant concentrations at a site distant from where they were formed. Thus, with the exception of long-lived intermediates, most toxicity should be limited to the organ that formed the reactive intermediate (Uetrecht, 1992).

This reasoning leads to the prediction that most idiosyncratic drug reactions would involve the liver, since the liver is the major site of drug metabolism. Although the liver is the major organ affected by ADRs (Stricker and Spoelstra, 1985), the skin and possibly the bone marrow are involved more frequently (Uetrecht, 1992). One likely explanation is that different tissues in the body have different complements of drug activation and drug detoxification enzymes (Pirmohamed *et al.*, 1994); in particular, the liver has a high concentration of GSH, GSH transferase and epoxide hydrolase enzymes which by the inactivation of reactive metabolites, protect it from drug toxicity. A combination of the metabolic activity (Mukhtar and Khan, 1989; Bickers, 1991) and immunological responsiveness (Longley *et al.*, 1988) of the skin may partly explain why it is so often affected by ADRs. Drug-induced skin eruptions can be caused by almost any drug in clinical practice (Raviglione *et al.*, 1990).

The classes of drug which most frequently affect the haemopoietic stem cell are the cytotoxic chemotherapeutic agents. Bone marrow suppression

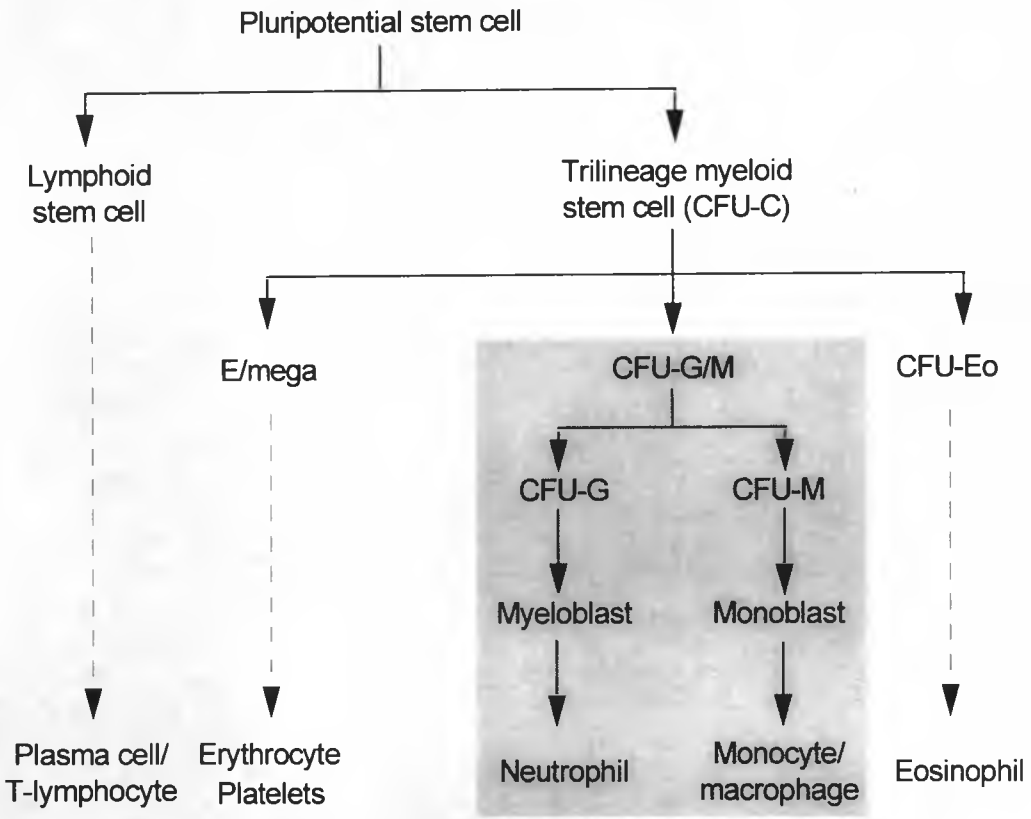
represents a direct extension of their known pharmacological effect. Drugs reported to cause metabolism-dependent aplastic anaemia include butazones, chloramphenicol, anticonvulsants, sulphonamides, anti-thyroid drugs and phenothiazines (Uetrecht, 1992; Hofstra and Uetrecht, 1993).

1.5 Neutrophils - Structure and Function

Neutrophils are commonly called polymorphonuclear leucocytes or granulocytes, because of their multi-lobed nucleus and prominent vesicles (granules). Like all blood cells, neutrophils are derived from a pluripotential haematopoietic cell line (fig. 1.4). This stem line gives rise to committed precursors that ultimately result in the production of mature cells by a process of differentiation and commitment. They have a mean survival time of 9 hours, prior to dying by the process of apoptosis (Savill *et al.*, 1989) (section 1.5.6).

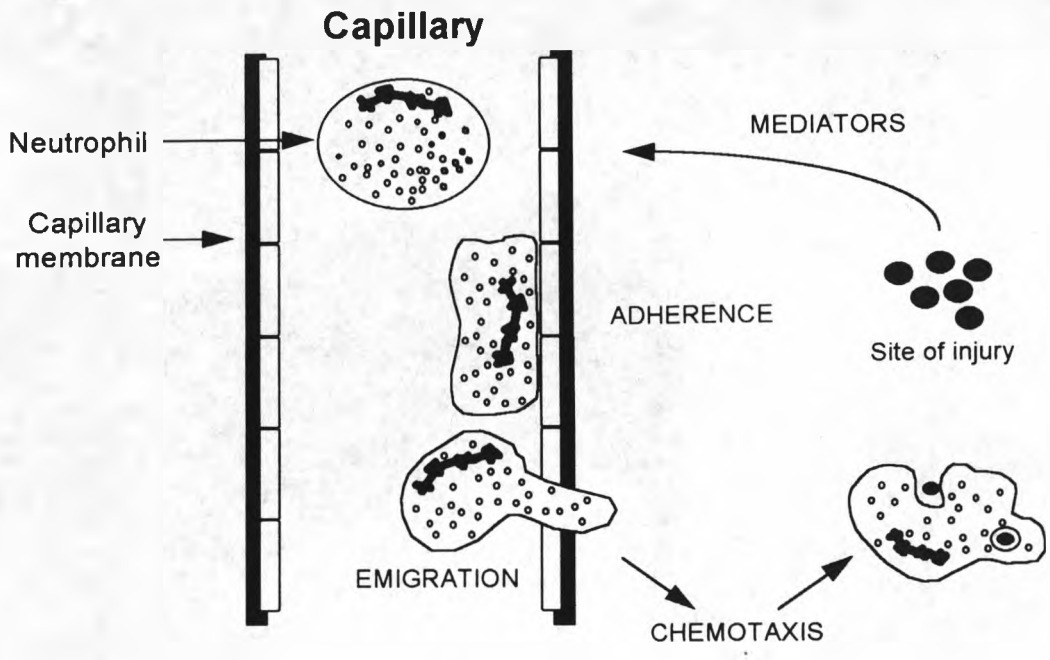
Neutrophils are the major constituent (40-75%) of the total white blood cell pool (4.0-11.0 x 10^9/l). Cytoplasmic organelles are rare, with the exception of primary (azurophilic) and secondary (specific) cytoplasmic granules, that were originally identified by Cohn and Hirsch (1960). Primary granules are similar in structure to lysosomes, but in addition they contain antibacterial and digestive substances, most notably MPO. Secondary granules are smaller and almost twice as numerous. They release substances into the extracellular environment, initiating inflammation and complement activation. Tertiary granules have also been identified (Baggiolini, 1980). They are released extracellularly and are thought to promote cell adhesion.

Neutrophils are highly motile, secretory cells. Their most important function is the ingestion and destruction of invading micro-organisms (phagocytosis). To fulfil this role, the cell must penetrate the capillary wall and migrate to the site of bacterial replication, prior to recognition and destruction of the foreign material (fig. 1.5).



Taken from Pirmohamed and Park (1997).

Figure 1.4 A schematic representation of the normal process of haematopoiesis.



Taken from Hellewell and Henson (1991).

Figure 1.5 Scheme illustrating the various processes involved in neutrophil phagocytosis.

1.5.1 Drug Induced Neutropenia and Agranulocytosis

Drug-induced neutropenia is an asymptomatic disorder characterised by a moderate decline in neutrophil number (0.5-1.0 x 10^9/l). Cell levels are still sufficient to protect the body against infection; the cell count may drop further to cause agranulocytosis, or it may recover despite drug continuation.

Agranulocytosis is characterised by a depletion in neutrophil number below 0.5 x 10^9/l (Pisciotta, 1990). It may initially be asymptomatic although sooner or later symptoms of sepsis will appear and progress, resulting in death if administration of the drug is continued (Pisciotta, 1990). The incidence of agranulocytosis is hard to define accurately due to difficulty in obtaining reliable data. A literature search carried out for the period 1969-1979 (Young and

Vincent, 1980), found 714 papers reporting drug-induced agranulocytosis, while in Sweden, Bottinger *et al.* (1979) found an annual incidence of agranulocytosis of 2.6 per million. These data represent only a small portion of the total number of cases of drug-induced agranulocytosis. Most other cases will not be submitted for publication, or wil not have been recognised. Symptoms accompanying agranulocytosis relate to lysis of peripheral neutrophils, and release of their pyrogenic contents. They often include violent chills, sore throat, pneumonia, fever, malaise, bone pain and physiological collapse. Fig. 1.6 shows a patient with neutropenia who later died from bronchopneumonia. The ulcers on both sides of the neck persisted for 6 months until death.

1.5.2 Mechanisms of Drug-Induced Agranulocytosis

As with other forms of idiosyncratic toxicity, the clinical presentation can be variable and diagnosis is difficult. The chemical mechanism of agranulocytosis differs from drug to drug, but with some overlap may fit into two categories.

1. Non immunogenic;

    a) Direct chemical suppression by the drug with compensatory marrow failure in the host.

    b) Suppression by accumulation of inadequately detoxified chemically reactive drug metabolites.

2. Immunogenic.

    Neutrophil destruction by a drug-related immune response.

Figure 1.6 Chronic ulceration of the neck associated with neutropenia.

Chlorpromazine is one of a few chemically reactive drugs. It combines with cellular proteins or nucleic acids by electron donation and the formation of an ionic bond (Hoffman *et al.*, 1963; Pisciotta, 1974). There are many examples of chemically reactive drug metabolites interacting with cellular macromolecules (Mitchell *et al.*, 1973; Yunis, 1989; Park *et al.*, 1994; Pirmohamed *et al.*, 1994). The resultant drug (metabolite)-macromolecule complex leads to denaturation and precipitation of the protein or nucleic acid, which could either disrupt enzyme activity or stimulate cellular proliferation. Cell death may result by either necrosis or apoptosis (section 1.5.6).

Idiosyncratic drug reactions often require pre-exposure of the drug, or a delay of more than a week between starting the drug and onset of toxicity. Consequently, Parker (1982) and others (Kenna *et al.*, 1988; Park and Kitteringham, 1990) have suggested the immune system may be involved in such ADRs.

The mechanism by which a drug (or more commonly drug metabolite) can lead to immune-mediated idiosyncratic toxicity is based on the hapten hypothesis. The term hapten describes a foreign substance of low molecular weight that is only immunogenic when conjugated to a macromolecule carrier (Park *et al.*, 1987). Drugs induce an immune response when covalently bound to a macromolecule such as protein or DNA. The immune system recognises the drug-macromolecule complex (immunogen) and initiates the immune response, which is characterised by either specifically committed T-lymphocytes and / or antibodies directed against the drug (haptenic epitopes), the carrier protein (autoantigenic determinant) or the neoantigen created by the combination of the drug and the protein (new antigenic determinant) (Pohl *et al.*, 1988) (fig. 1.7).

A drug-macromolecule complex (antigen) requires "processing" by macrophages (antigen presenting cells) prior to the induction of an antibody / T-lymphocyte response. The way in which antigens are processed determines the nature of such a response. T-lymphocytes (either $CD4^+$ or $CD8^+$) recognise antigens presented by major histocompatibility complex (MHC) molecules. $CD4^+$ cells recognise antigens presented by MHC II and $CD8^+$ cells recognise antigens presented by MHC I molecules. The majority of drug-macromolecule complexes

are thought to become internalised by macrophages and presented to $CD8^+$ cells on MHC I molecules. However, some drug related antigens are presented by either MHC II, or a combination of MHC I and MHC II molecules (Kalish, 1995).

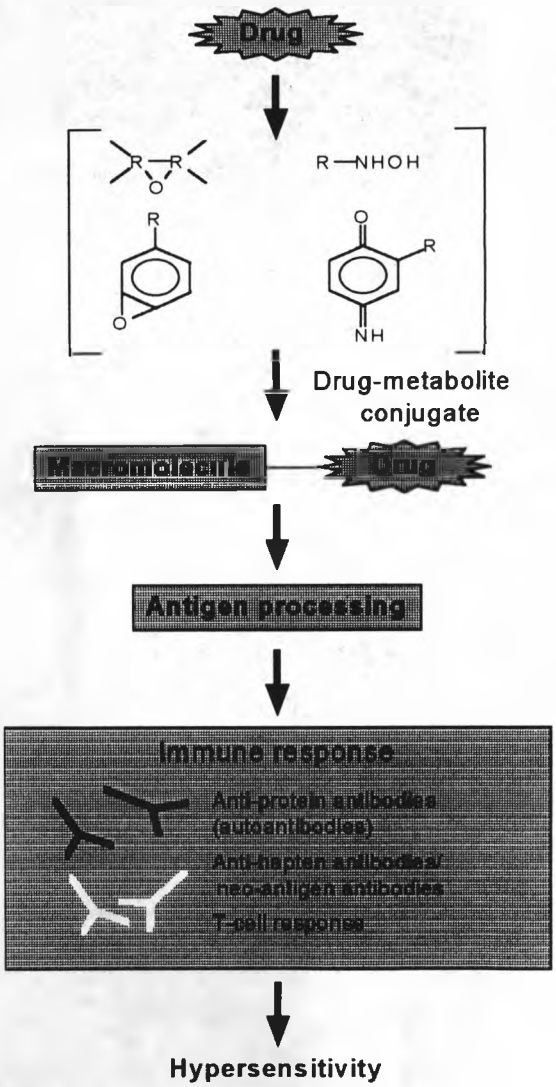


Figure 1.7 Mechanism of immune-mediated idiosyncratic drug toxicity.

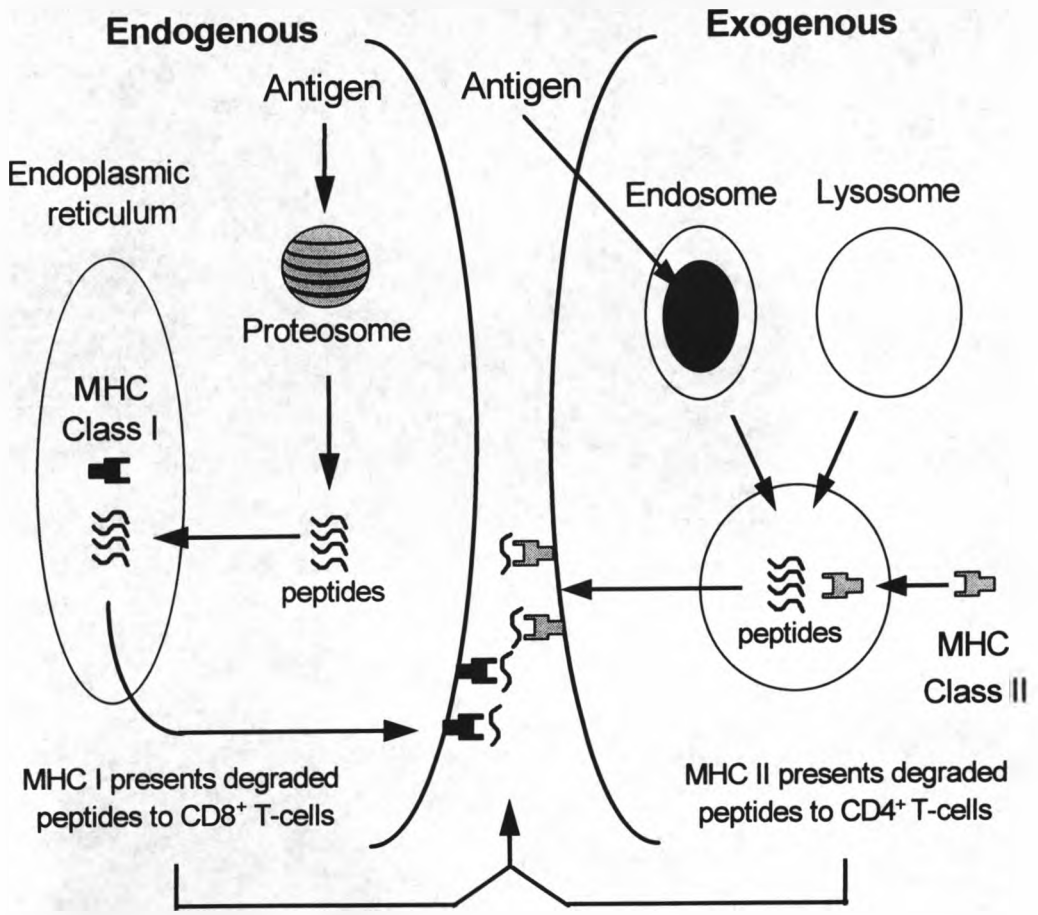


Figure 1.8 Processing of endogenous and exogenous antigens.
(a) Endogenous antigens are degraded by proteosome complexes. The resultant peptides are transported into the endoplasmic reticulum were they associate with MHC class I molecules. (b) Exogenous antigens are internalised in endosomes, which fuse with lysosomes. Within this complex proteins are degraded into peptides, which bind to MHC class II molecules, prior to transportation to the cell surface (Taken from Kalish, 1995).

Exogenous and endogenous antigen processing differs considerably (fig. 1.8), although both pathways involve the proteolytic hydrolysis of the antigen. The peptide fragments are transported to the cell surface and presented to $CD4^+$ or $CD8^+$ cells, resulting in either an antibody-mediated or a cell-mediated response (Kammuller *et al.*, 1989).

Once the immune system has been activated, tissue damage occurs through four general mechanisms (Coombs and Gell, 1968) (table 1.4). The types of hypersensitivity are not mutually exclusive since a particular drug may involve more than one mechanism. This classification does provide a useful framework with which to begin to investigate the immunological basis of adverse reactions.

Hypersensitivity	**Mechanism**
Type I (immediate or anaphylactic)	Specific IgE antibodies bind to mast cells via their F_c receptors. Binding of multivalent antigen to adjacent IgE induces degranulation and release of mediators, such as histamine and leukotrienes.
Type II (cytotoxic)	Antibodies (IgG and IgM) are directed against an individuals own cells. This may lead to cell destruction by killer T-cells or complement-mediated lysis. Alternatively the cell may be removed by phagocytosis.
Type III (immune complex)	Immune complexes are deposited in tissues such as the blood vessels and glomerular basement membrane. Activation of complement leads to a recruitment of polymorphs and a local inflammatory response.
Type IV (delayed)	Specific T-cells bind to a fixed antigen. Lymphokines are released which induce an inflammatory reaction and attract and activate macrophages, which release mediators.

Table 1.4 Classification of drug hypersensitivity reactions.

1.5.3 The Role of Neutrophil Metabolising Enzymes

Idiosyncratic drug toxicity is thought to occur via an immunological

reaction or as a direct association between drug and essential cellular macromolecules (section 1.5.2). In either case, the initial process often involves an imbalance between bioactivation and detoxification, resulting in the formation of a chemically reactive intermediate (Park *et al.*, 1992). Of particular importance here is the site of drug metabolism. With regards to agranulocytosis, it is difficult to imagine how a chemically reactive intermediate (often with a half life of less than one minute) can be formed in the liver and travel through the circulation to the bone marrow or peripheral blood cells. Thus, the ability of neutrophils to metabolise drugs has major implications in the study of ADRs (Uetrecht, 1992).

Neutrophils contain a low concentration of cytochrome P450 enzymes, prostaglandin synthase and nitric oxide synthetase, all of which are known to metabolise a wide variety of xenobiotics (Hofstra and Uetrecht, 1993). In addition, neutrophils possess a powerful oxidising system consisting of MPO and NADPH oxidase. The overall characteristics of this system include increased oxygen consumption, induction of glycolysis and generation of superoxide ($O_2^{\cdot-}$) and other oxy radicals. When activated, a process known as the respiratory burst, the two enzymes are capable of metabolising almost any drug with an easily oxidisable functional group (Hurst and Barrette, 1989).

A variety of physiological agents and artificial stimuli initiate the respiratory burst (Hurst, 1987) (table 1.5). The molecular activation of the respiratory burst is complex; the final steps comprise one common pathway which involves phosphorylation of a component of the cell surface NADPH oxidase enzyme (Hurst and Barrette, 1989) (fig. 1.9). NADPH oxidase contains

Receptor Independent	**Membrane Receptor Dependent**
Fluoride ion	Soluble Stimuli
Arachidonic acid	FMLP
Calcium ionophore	Platlet activating factor
Digitonin	Compliment 5a
	Leukotrine B_4
Cytoplasmic Receptor Dependent	Particle Stimuli
	Opsonised bacteria
Phorbol esters	Yeast
	Viruses

Table 1.5 Activators of the respiratory burst.

an unusual *b*-type cytochrome (Cross *et al.*, 1981) and a flavoprotein containing flavin adenine dinucleotide (FAD) (Cross and Jones, 1986). A complex transport system passes electrons from NADPH to the cytochrome via the FAD-containing flavoprotein. This aids the rapid reduction of molecular oxygen to $O_2^{\cdot-}$. The $O_2^{\cdot-}$ ion then dismutates either spontaneously or enzymatically (catalysed by superoxide dismutase), to form hydrogen peroxide (Fee and Valentine, 1977). Hydrogen peroxide and $O_2^{\cdot-}$ are potential oxidising agents although in the neutrophil their reactivity can be increased by two mechanisms:

(1) The reaction of hydrogen peroxide with iron. Both hydroxyl radicals ($OH^{\cdot}$) and ions (OH^-) are produced (fig. 1.9).

(2) The reaction of hydrogen peroxide-activated MPO with chloride ions (Cl^-) and hydrogen ions (H^+). Powerful halide oxidation products are produced (Hurst and Barrette, 1989) (fig. 1.10).

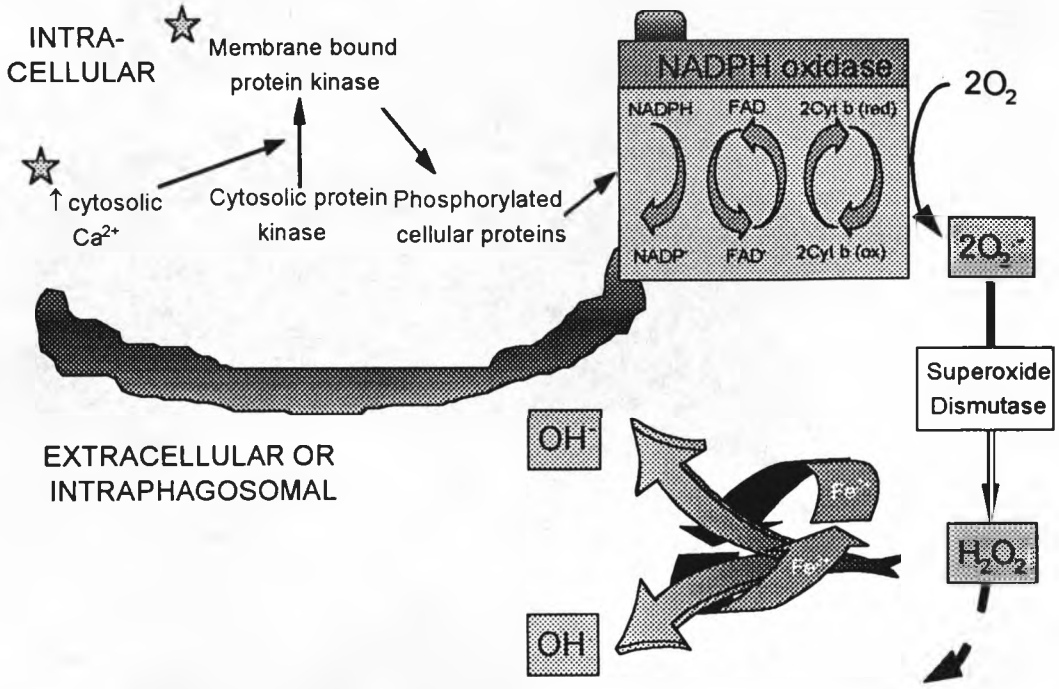


Figure 1.9 The role of NADPH oxidase and iron in the production of reactive oxygen species.
The respiratory burst is stimulated by a variety of particles (☆, table 1.5). Phosphorylation of cellular proteins activates NADPH oxidase, which converts molecular oxygen to the more reactive $O_2^{\cdot-}$. $O_2^{\cdot-}$ then dismutates to hydrogen peroxide either spontaneously or catalytically (superoxide dismutase). Finally, hydrogen peroxide can be reduced by iron or taken up by MPO.

MPO is green in colour and consists of two fundamental elements; a heat-labile peroxidase component and heat-stable thiocyanate ions (Dogon *et al.*, 1962; Klebanoff and Luebke, 1965). Neutrophils possess high concentrations of MPO and reports suggest that it constitutes between 1-5% of the cells dry weight (Schultz and Kaminker, 1962). In resting neutrophils, MPO is inactive and stored in the primary granules. Upon stimulation of the respiratory burst, MPO is

activated and released either extracellularly or directly into cytoplasmic phagasomes.

MPO reacts with either cell surface or internalised hydrogen peroxide producing the active enzyme (compound I) (Odajima and Yamazaki, 1970) (fig. 1.10). Compound I is capable of oxidising drugs directly (Harrison, 1976), although in the presence of Cl^-, the major reaction is the formation of hypochlorous acid (HOCl). HOCl is a potent oxidising agent which is responsible for the majority of drug metabolism in neutrophils (Uetrecht, 1992).

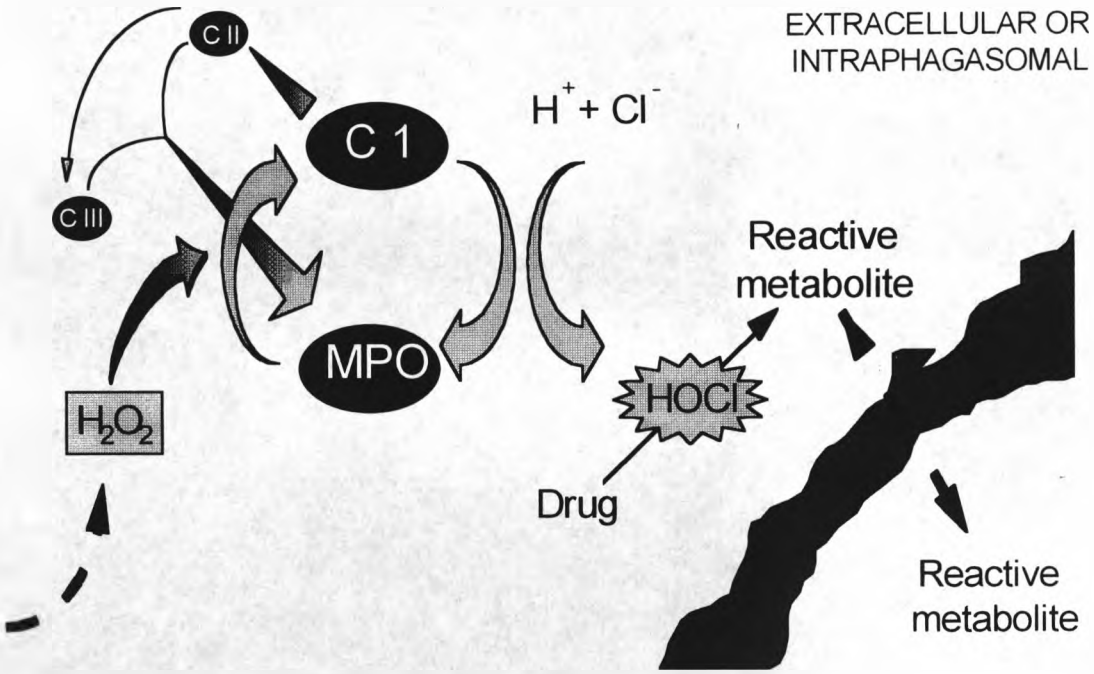


Figure 1.10 The role of MPO in the production of HOCl.
Hydrogen peroxide converts MPO to the active enzyme (compound I). In the presence of both H^+ and Cl^-, compound I catalyses the formation of HOCl.

In the absence of Cl^- and in the presence of excess hydrogen peroxide, compound I is converted to compound II. Compound II is catalytically inactive; however, its reduction to MPO by ascorbic acid and O_2^- restores catalytic activity

(Winterbourn and Vissers, 1983; Kettle and Winterburn, 1988). A third complex, compound III, is formed by the reaction of compound II with hydrogen peroxide. Compound III does not oxidise drugs directly, but reacts with compound II to reform the native enzyme (Winterbourn, 1985) (fig. 1.10).

Neutrophil metabolism is unlikely to make a significant contribution to the overall metabolism of a drug; nevertheless it is thought to contribute towards a variety of ADRs (Uetrecht, 1989; Keetle and Winterbourn, 1991). Oxidation products of drug metabolism and reactive oxygen species produced by the respiratory burst enter the cytoplasm where damage to cellular components may occur. Cytoplasmic targets are protected by a number of reactive oxygen species scavengers. Superoxide dismutase limits the lifespan of $O_2^{\cdot-}$ and more distal oxidants such as $OH^{\cdot}$, while hydrogen peroxide is reduced to water by two molecules of GSH. Taurine reacts with HOCl generated by the MPO / NADPH oxidase system to form a less toxic taurine-chloramine complex (fig. 1.11).

Other metabolite scavenging systems include catalase, ascorbic acid and GSH. GSH is the most abundant intracellular thiol, constituting 90% of non-protein thiols. (Larsson *et al.*, 1983). It exists largely in a reduced form (GSH), although GSH disulphide (GSSG) and mixed disulphides to a lesser extent, contribute to the total cellular pool (Mills and Lang, 1996). A large number of reactive metabolites are detoxified by GSH. It can serve either as a nucleophile in conjugation reactions or as a reductant. The route of metabolism depends on the chemical nature of the reactive intermediate. Arene oxides and quinones are detoxified by conjugation to GSH, while hydroxylamines and free radicals

undergo reduction (Moldeus and QuanGuan, 1987) (fig. 1.11). Metabolite detoxification by intracellular GSH (and ascorbic acid) in neutrophils is displayed by an increase in oxidised levels within the cytoplasm (Voetman *et al.*, 1980; Winterbourn and Vissers, 1983).

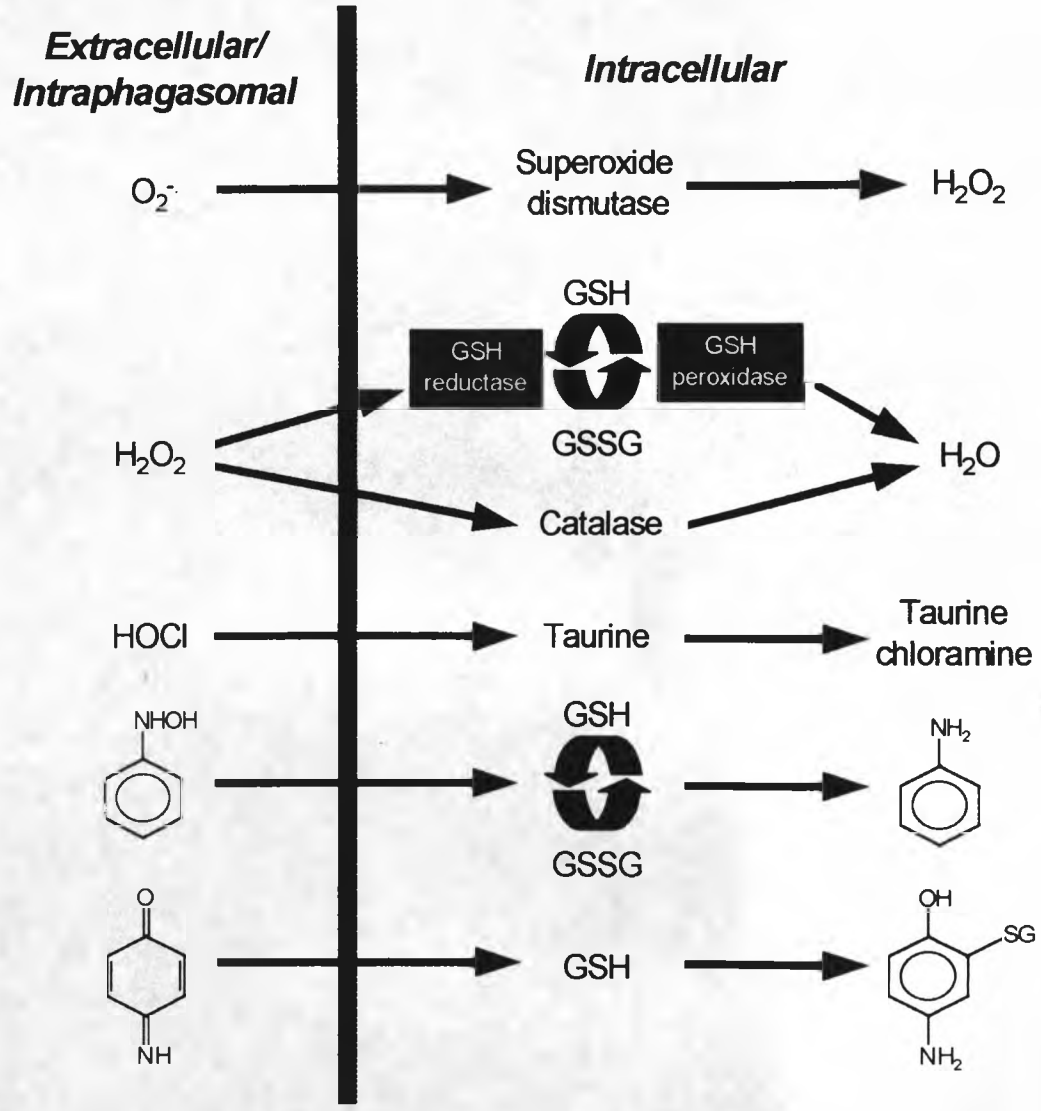


Figure 1.11 Cytoplasmic detoxification of reactive oxygen species and drug metabolites.

1.5.4 Neutrophil Drug Metabolism

Activated neutrophils, MPO and other peroxidase enzymes are known to metabolise a wide range of chemically unrelated compounds to reactive intermediates. The products of drug metabolism are often implicated in the idiosyncratic toxicity associated with these drugs. Table 1.6 provides a brief overview of some of the reactions involved.

Group	Example	Metabolite	Reference
Aromatic amines	Sulphonamides Procainamide Dapsone	Hydroxylamaine / Nitroso	Uetrecht *et al.*, 1988 Uetrecht and Zahid, 1991
Other aromatic nitrogens	Amodiaquine Paracetamol	Quinoneimine	Maggs *et al.*, 1988 Coles *et. al.*, 1988 Tingle *et al.*, 1995
Other nitrogen compounds	Aminopyrine Clozapine	Free radicals / Iminium and Nitrenium ions	Sayo and Saito, 1990 Fischer *et al.*, 1991 Uetrecht, 1992
Sulphur oxidations	Propylthiouracil	Sulphenyl chloride / Sulphonic acid intermediates	Lee *et al.*, 1988
Carbon oxidations	Phenylbutazone Diclofenac	Hydroxy- / Hydroperoxy intermediates	Ichihara *et al.*, 1986 Shen *et al.*, 1997

Table 1.6 Peroxidase-mediated drug metabolism.

1.5.5 Distribution of Drugs, Enzymes and Enzyme Co-factors in Human Blood

The chemical characteristics of a drug determines its mode of action; however, the chemical nature of a drug also determines its kinetics. Most drugs

bind to plasma proteins (mainly albumin, section 1.3.4), and/or accumulate within specific neutrophil compartments. Therefore, the peripheral distribution of drugs, drug metabolising enzymes and biological cofactors may be an important determinant of drug-induced agranulocytosis.

Amphiphilic drugs (e.g. chloroquine) consist of a basic amine group attached over a short chain to a hydrophobic moiety. The tissue:plasma ratio of such compounds may amount to greater than 100 fold that expected (De Duve *et al.*, 1974). Penetration of the plasma membrane depends upon; firstly, the pK_a of the amino group, and secondly, the degree of hydrophobicity of the entire molecule (Lullmann *et al.*, 1975). Intracellularly drugs are not evenly distributed, with lysosomes being particular sites of accumulation. Lysosomes are membrane limited vesicles distinguished by an unusually high proton concentration (pH 4.5-5.0; De Duve *et al.*, 1974). Amphiphilic drugs penetrate the lysosome, where they become protonated and trapped. Within the lysosome a second event takes place; the amphiphilic drug forms a complex with polar lipids, which are subsequently removed from the diffusion equilibria (fig. 1.12).

HOCl and other strong oxidants can metabolise drugs in either intra- or extracellular compartments (section 1.5.3). Detoxification reactions depend upon whether the reactive metabolite is generated in plasma or the cytoplasm. GSH exists almost exclusively intracellularly, with less than 1% in the plasma (section 1.3.6). In contrast, cysteine (CYS), a precursor in GSH synthesis (Meister and Anderson, 1983) is present in the plasma (Mills and Lang, 1996). Disulphides of both CYS and GSH are believed to play an important regulatory role in drug

detoxification. They act as a buffer system protecting the cell from drug-induced oxidative damage (Isaacs and Blinkley, 1977; Cotgreave *et al.*, 1990).

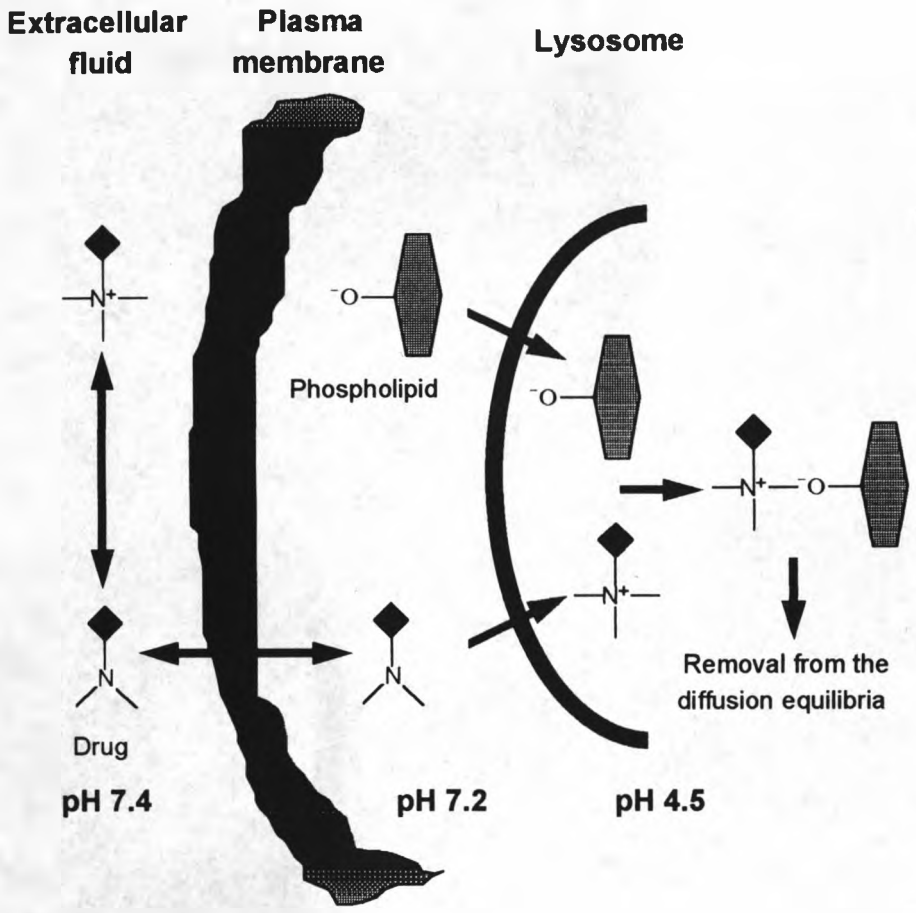


Figure 1.12 Diagrammatic representation of cellular uptake and lysosomal accumulation of an amphiphilic drug.

1.5.6 Mechanisms of Drug-Induced Cell Death

Toxic chemicals can induce cell death by either necrosis (pathological cell death) or apoptosis (programmed / physiological cell death), two morphologically and biochemically distinct modes of cell death (Kerr *et al.*, 1972; Searle *et al.*, 1982).

Necrosis is a degenerative process characterised by a marked increase in plasma and mitochondrial membrane permeability after direct injury by a variety of non-physiological agents (including toxic chemicals). Groups of contiguous cells are usually involved. It is an uncontrolled destructive phenomenon that disturbs energy producing pathways, resulting in an osmotic imbalance in the cell, swelling of the cytoplasm and irreversible failure of cell structures (Farber, 1971; Popper *et al.*, 1984). Apoptosis, on the other hand, plays an important role in the controlled depletion of cells during metamorphosis, differentiation and normal cell turnover. It is an active process capable of destroying isolated cells. Regulation of apoptosis occurs via signal transduction-coupled events which are characterised by cytoplasmic and chromatin shrinkage, with endonuclease-induced DNA fragmentation into nucleosome sized pieces (Wyllie, 1980; Searle *et al.*, 1982) (fig. 1.13).

Foreign chemicals induce necrotic cell death by direct alkylation of proteins, DNA and other essential macromolecules. Hence, necrosis is typically unplanned and may result in organ failure and death. Several studies have examined whether the loss of calcium regulation, upon exposure to toxic chemicals, correlate with the appearance of DNA fragmentation (prominent features of apoptosis) in cells undergoing acute cellular necrosis (Wyllie, 1980; Cohen and Duke, 1984; Corcoran *et al.*, 1994). Results from these studies lead to the proposal of the calcium hypothesis of cell death, which defines DNA as a vital target in necrosis, and suggests that uncontrolled calcium uptake may be lethal.

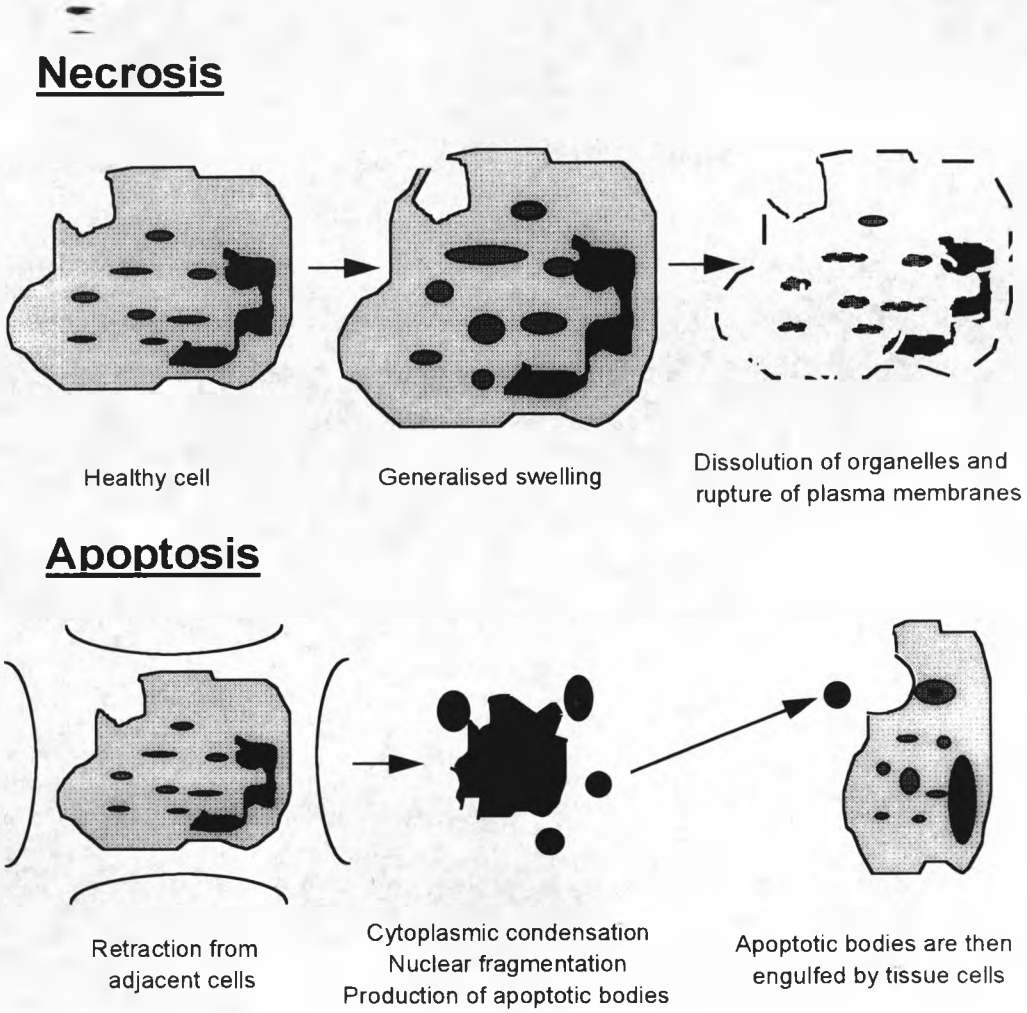


Figure 1.13 Morphological characteristics of necrosis and apoptosis.

The induction of apoptosis by a wide range of toxic agents has been extensively described in the literature (Lennon *et al.*, 1991; Offen *et al.*, 1995; Manaster *et al.*, 1996; Ross *et al.*, 1996; Watson *et al.*, 1996c); the biochemical mechanism permitting cells to die in this fashion is now of great interest. Raised intracellular calcium, oxidative stress, disruption of the mitochondrial transmembrane potential, breakdown in the organisation of DNA and activation of cysteinyl-endonuclease enzymes which digest chromatin into oligonucleosome

length fragments are all integral steps in apoptotic cell death (Searle *et al.*, 1982; Arends *et al.*, 1990; Buttke and Sandstrom, 1994). Additionally, protein synthesis often mediates calcium influx in cells undergoing apoptosis (Nicotera *et al.*, 1992).

A family of protein cutting enzymes, of which interleukin 1-β converting enzyme (ICE) is the most abundant, mediate apoptosis. The inactive enzyme is cleaved into amino acid subunits when activated by a variety of stimuli. The cleaved amino acid chains self associate to form an active tetrameric enzyme which is responsible for apoptotic cell death (Wilson *et al.*, 1994).

Three molecular mechanisms regulate apoptosis. Firstly, binding of TNF-α or the Fas ligand to their respective cell surface receptors (Fas-R, TNF-R 1 and 2) stimulates apoptosis. The Fas-R and TNF-R 1 trigger apoptosis by interacting with a series of proteins that contain a conserved amino acid chain known as a death domain (Feinstein *et al.*, 1995). In contrast, the interaction of TNF-α with TNF-R 2 may inhibit apoptosis by binding to the receptor associated proteins TRAF 1 and TRAF 2 (Rothe *et al.*, 1995). Secondly, the protein product of *Bcl-2* inhibits apoptosis (Hockenbery *et al.*, 1990). Other *Bcl-2* homologues have been identified, some of these act like *Bcl-2* to protect the cell, whereas others act to promote cell death (Oltvai *et al.*, 1993). Regulation of cell death by the *Bcl-2* gene family is a function of their various interactions with each other (Yang *et al.*, 1995). In cells where inducers predominate, apoptosis is accelerated. Finally, *p53*, a tumour suppressor gene, also regulates apoptotic cell death. It encodes a protein whose function has been likened to a molecular policeman. When DNA

becomes damaged, p53 accumulates and switches off cell replication to allow time for repair. If DNA is damaged or mutated beyond repair, p53 may stimulate apoptosis (Haffner and Oren, 1995). In addition, over-expression of c-myc, a protein which binds to DNA and functions as a transcriptional factor, can lead to apoptosis. The induction of apoptosis by c-myc requires the presence of a protein partner (max), as well as a functional p53 protein (Evan *et al.*, 1992). Fig. 1.14 describes how the individual processes interact to determine cell survival or apoptosis following a toxic insult.

Neutrophils are the shortest lived of all leucocytes. A mature cell usually migrates to a site of infection within 9 hours of entering the blood stream, after which time it dies by apoptosis. Regulation of neutophil apoptosis remains unclear; however it has been demonstrated that neutrophils undergo apoptosis at the site of inflammation and are phagocytosed by surrounding cells (Savill *et al.*, 1989).

Generation of reactive oxygen species from the respiratory burst is the principal mechanism by which neutrophils kill invading pathogens. Since many chemotherapeutic agents and physical treatments that induce apoptosis initially evoke oxidative stress, it is now believed that oxidative stress may mediate apoptosis (Buttke and Sandstrom, 1994). Whether oxidative stress is the primary trigger of apoptosis, or just a contributory factor has yet to be elucidated. However, oxidative stress seems to be crucial to the process for several reasons; first, the add-on of reactive oxygen species or depletion of cellular antioxidants such as GSH can induce apoptosis (Verhaegen *et al.*, 1995); and secondly,

apoptosis can be blocked by the protective effects of antioxidants (Iwata *et al.*, 1992).

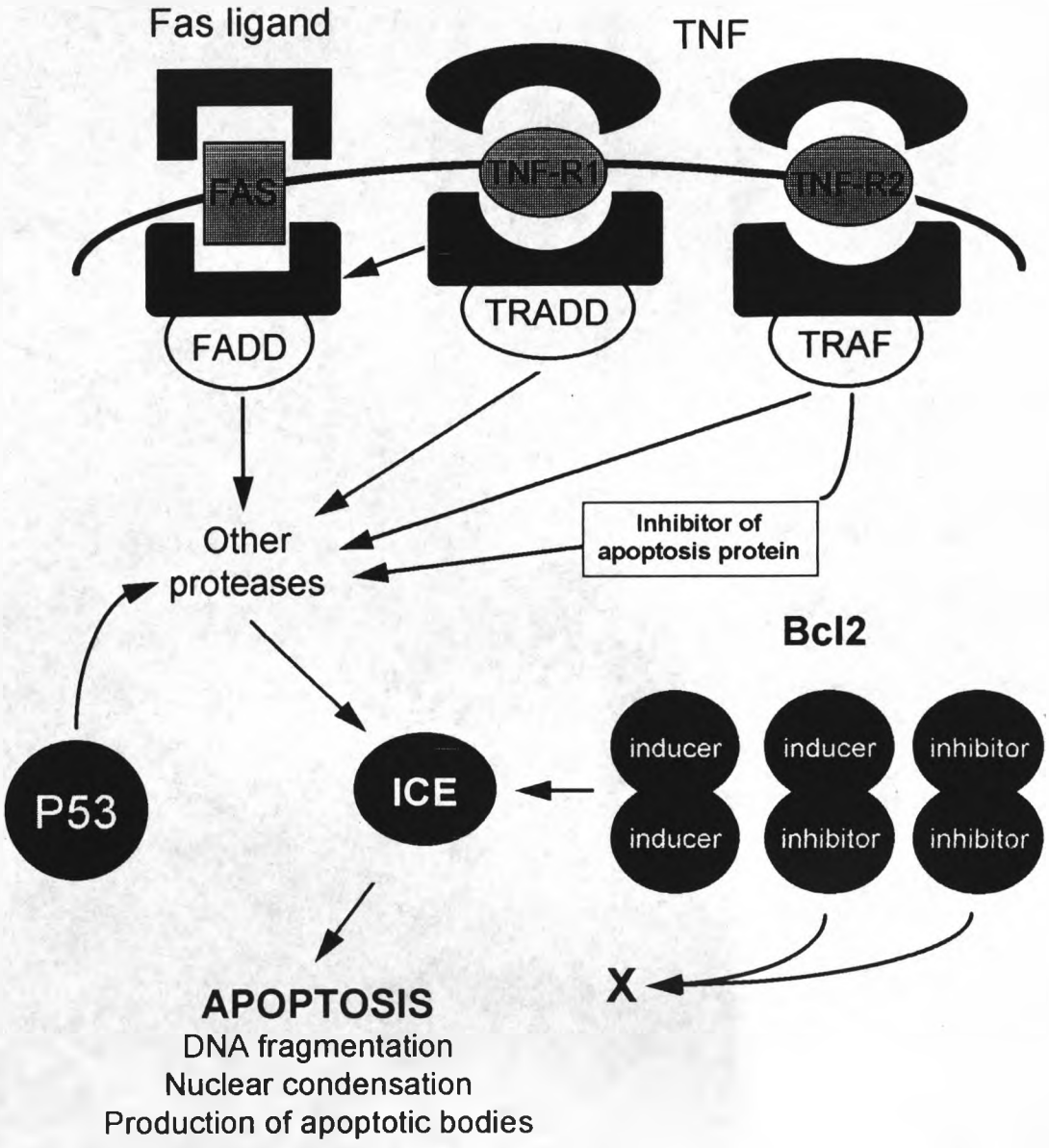
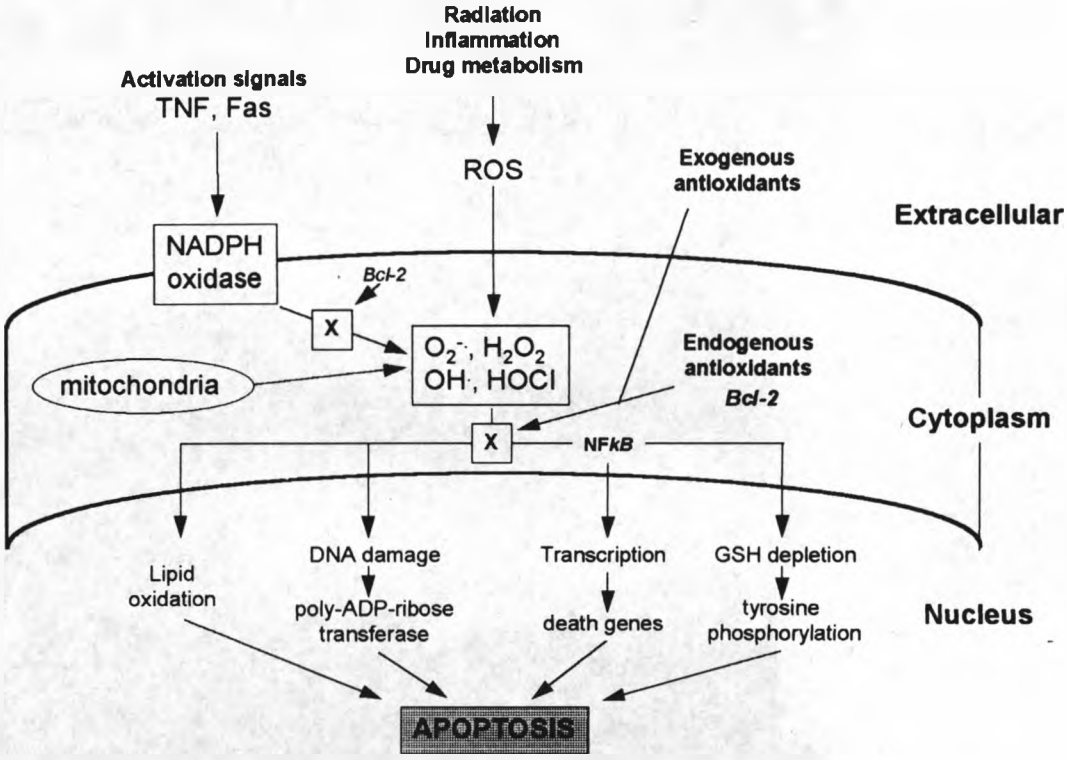


Figure 1.14 Genetic control of apoptosis.

A wide variety of reactive oxygen species (including $O_2^{\cdot-}$, hydrogen peroxide, HOCl and $OH^{\cdot}$) are thought to mediate apoptosis. Intracellular sources of reactive oxygen species include mitochondrial oxidation, the microsomal cytochrome P450 system and plasma membrane NADPH oxidases. The primary reactive oxygen species generating system in activated neutrophils is the plasma membrane NADPH oxidases (section 1.5.3), while the mitochondria serve as the primary source in dormant cells (Buttke and Sandstrom, 1994). $O_2^{\cdot-}$ is not very reactive and must be converted to a more reactive compound to cause intracellular damage (section 1.5.3). Additionally, Narayanan *et al.* (1997) demonstrated that hydrogen peroxide levels are down-regulated in ageing neutrophils. Production of $OH^{\cdot}$ from the metal catalysed Haber-Weiss reaction results in lipid peroxidation by the abstraction of a hydrogen atom from the carbon chain of unsaturated fatty acids. Lipid peroxidation initiates a radical chain reaction, resulting in protein modification, activation of endonucleases and oxidative damage to DNA (Hedley and Chow, 1992; Sandstrom *et al.*, 1995). DNA damage mediated by reactive oxygen species activates poly-ADP-ribose polymerase and p53, both of which induce apoptotic cell death.

Reactive oxygen species are also thought to mediate apoptotic cell death by various other mechanisms. First, ROS induce apoptosis through the release of NF-κB from its inhibitory subunit I-κB (Schreck *et al.*, 1992). When cells are under a state of oxidative stress, amplification of cytoplasmic TNF-α rapidly dissociates NF-κB from its inhibitory subunit. The active complex translocates to the nucleus and subsequent binding to DNA signals apoptosis (Westendorp *et al.*, 1995). Secondly, *Bcl-2* protects cells from apoptosis induced by hydrogen

peroxide and menadione, a quinone compound which undergoes redox cycling and generates $O_2^{\cdot-}$ (Hockenbury *et al.*, 1990). This suggests that *Bcl-2* contributes to the regulation of cellular levels of reactive oxygen species. Furthermore, intracellular sites of *Bcl-2* include the mitochondria and nuclear membrane, the major sites where reactive oxygen species are formed. Similarly, the E1B protein, a functional homologue of *Bcl-2* was found to prevent TNF-, Fas-



Taken from Buttke and Sandstrom (1994)

Figure 1.15 Proposed role of oxidative stress as a mediator of apoptosis.

and *p53*-mediated apoptosis (Hockenbery *et al.*, 1990; Itoh *et al.*, 1993). Finally, Watson *et al.* (1996a) demonstrated that a reduction of intracellular GSH induced neutrophil apoptosis through a process of tyrosine phosphorylation. The effect was blocked by raising intracellular GSH but not by the direct addition of

antioxidants. These results suggest that apoptosis was not mediated through an oxidative pathway, but more likely through a direct thiol mediated process. A scheme describing the role of reactive oxygen species as a mediator of apoptosis is shown in fig. 1.15.

1.6 Amodiaquine

1.6.1 History and Development

Amodiaquine (AQ) is a cheap and effective antimalarial that has been in use for over 40 years. Early studies did not reveal any major toxicity problems, although in the mid-1980s, reports of fatal agranulocytosis and hepatotoxicity were described in travellers taking AQ for prophylaxis (Hatton *et al.*, 1986; Neftel *et al.*, 1986). As a consequence, Parke-Davis withdrew the drug and the World Health Organisation (WHO) stopped using it in malaria control programmes.

Although AQ is no longer used for prophylaxis, it can be used for treatment if the risk of infection outweighs the potential for an ADR (WHO, 1993). Moreover, a recent overview of 40 randomised trials also makes a good case for the reconsideration of AQ for the treatment of uncomplicated malaria (Olliaro *et al.*, 1996).

1.6.2 Metabolism of Amodiaquine

Desethyl AQ (Churchill *et al.*, 1986), bis desethyl AQ (Laurent *et al.*, 1993) and hydroxydesethyl AQ (Churchill *et al.*, 1985) are the major plasma

metabolites in man. Moreover, animal studies have identified a carboxylic acid derivative (Jewell *et al.*, 1995). Subsequent *in vitro* studies have suggested that AQ can form an electrophilic intermediate (AQ quinoneimine), that conjugates with thiols such as GSH and *N*-acetyl CYS (Maggs *et al.*, 1988) (fig 1.16). More recently, Jewell *et al.* (1995) and Tingle *et al.* (1995) have characterised the metabolism of AQ to the same conjugate in the liver and peripheral blood cells. Phase II conjugation of AQ with GSH *in vivo* provided direct evidence that AQ undergoes bioactivation to form a chemically reactive intermediate (Jewell *et al.*, 1995).

1.6.3 Distribution of Amodiaquine

AQ is absorbed rapidly and has a half-life of 5 hours after oral administration (Winstanley *et al.*, 1987). In comparison, chloroquine, a closely related 4-aminoquinoline antimalarial has a half-life estimated to be between 30 and 60 days (Frisk-Holmberg *et al.*, 1979). Thus, AQ appears to undergo substantial first pass metabolism and has a low bioavailability. The plasma concentration of AQ rarely exceeds 3ng/ml (0.01μM), which would suggest that desethyl AQ is responsible for antimalarial activity associated with AQ therapy (Winstanley *et al.*, 1987). Recent work by Laurent *et al.* (1993) confirmed this early study. Desethyl AQ has a plasma half-life of 98 hours, and peak plasma concentrations (50ng/ml, 0.15μM) were reached approximately 3 hours after drug administration.

1.6.4 Amodiaquine-Induced Agranulocytosis

Adverse reactions to AQ have been reported for many years (Love *et al.*, 1953); however, many of these reports involved patients taking multiple drugs. It was not until much later that AQ-induced agranulocytosis was observed (Hatton *et al.*, 1986; Rouvieux *et al.*, 1989). Twenty-two cases of agranulocytosis associated with AQ therapy were reported to the CSM between December 1984 and 1986 (Philips-Howard and West, 1990). The incidence of blood dyscrasias was thought to be as high as 1 in 2000, with a fatality rate of 1 in 31000 users of the drug. Dose, number and duration of drug challenges were thought to be important in the appearance of adverse reactions to AQ; however, they do not account for the idiosyncratic nature of the adverse reaction alone.

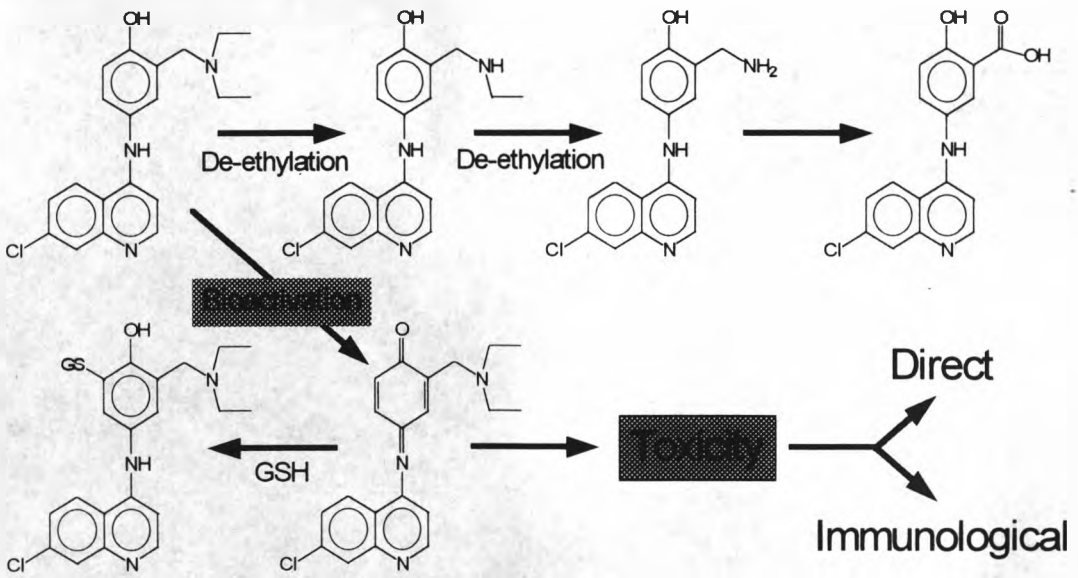


Figure 1.16 Major pathways of AQ metabolism.

1.6.5 Other Adverse Effects Associated with Amodiaquine Therapy

AQ is also associated with idiosyncratic hepatitis which has been reported in 1 in 15600 users (Neftel *et al.*, 1986; Larry *et al.*, 1986). Other side effects associated with AQ therapy include nausea, vomiting and diarrhoea (Love *et al.*, 1953).

1.7 Sulphamethoxazole

1.7.1 History and Development

Sulphamethoxazole (SMX) belongs to a class of drugs known as sulphonamides. They are among the oldest antibacterial agents in current clinical use. In addition to their clinical utility, they are associated with a variety of well characterised ADRs, which has severely restricted their use (Mandell and Sande, 1985).

In the last thirty years, two significant events have led to the increased use of SMX. Initially, co-trimoxazole (a combination product of trimethoprim and SMX) was introduced in the late 1960's, for the treatment of urinary tract infections. Subsequently, the emergence of acquired immunodeficiency syndrome (AIDS) led to an increased use of co-trimoxazole for the treatment of *Pneumocystis carinii* pneumonia. Its use in AIDS patients has been hampered by a much higher incidence of idiosyncratic drug reactions, ranging from 44-83% in different studies (Pirmohamed and Park, 1995).

1.7.2 Metabolism of Sulphamethoxazole

SMX undergoes extensive acetylation *in vivo*. Other metabolic pathways

include *C*-hydroxylation and glucuronidation (Vree *et al.*, 1995; Gill *et al.*, 1996). *N*-hydroxylamine and nitroso metabolites are produced by P-450 (Cribb and Spielberg, 1990) and peroxidase enzymes systems (Cribb *et al.*, 1990) (fig. 1.17). These metabolites are protein reactive and more toxic to cells from patients susceptible to sulphonamide toxicity than cells from healthy volunteers (Shear *et al.*, 1986; Rieder et al., 1988). No thiol conjugates of either SMX hydroxylamine (SMX-NHOH) or nitroso SMX (SMX-NO) have been identified *in vivo* (Gill *et al.*, 1996); however, various groups have described the role of GSH, ascorbic acid and various enzymes in the reduction of both metabolites to the less toxic parent compound (Cribb *et al.*, 1991; Ellis *et al.*, 1992; Cribb *et al.*, 1995).

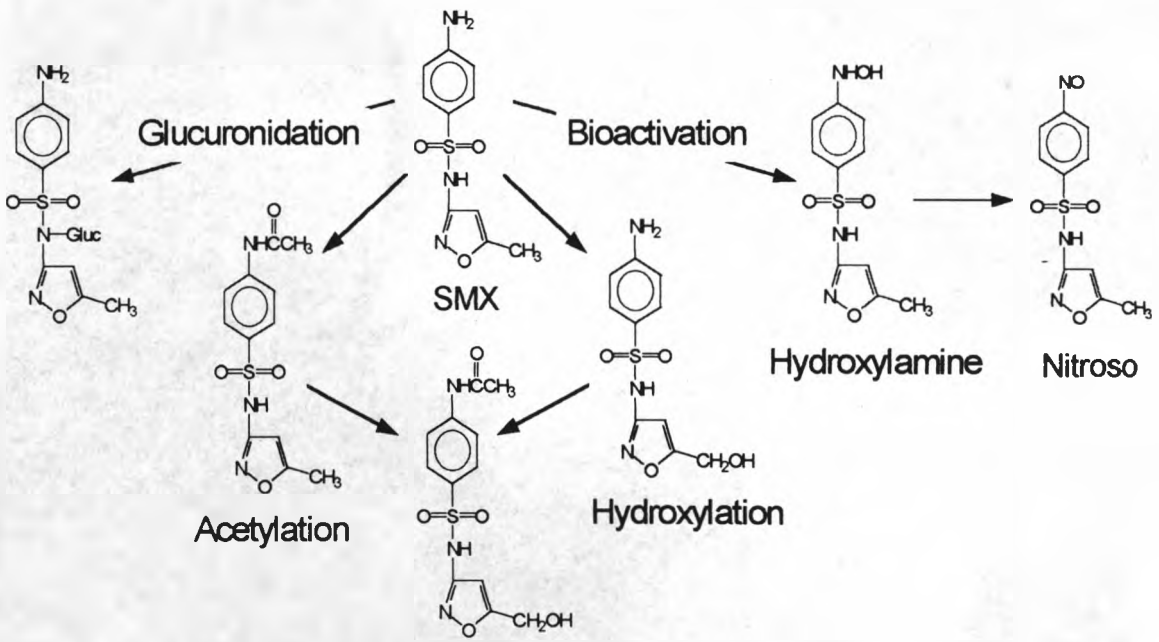


Figure 1.17 Major pathways of SMX metabolism.

1.7.3 Distribution of Sulphamethoxazole

After a single dose of SMX, typical plasma levels reach 50-80μg/ml (200-300μM) (Vree *et al.*, 1995). Under high dose regimes, similar to those prescribed for the treatment of *Pneumocystis carinii* pneumonia in HIV-positive individuals, plasma concentrations of SMX can reach 350μg/ml (1.5mM) (Stevens *et al.*, 1993).

The level of SMX-NHOH in human urine has been estimated to be between 2 and 5% of the total dose (Gill *et al.*, 1996). This may be an underestimation of the tissue exposure to the reactive metabolites since they undergo rapid reduction by GSH and other reactive thiols.

1.7.4 Sulphamethoxazole-Induced Agranulocytosis

Idiosyncratic toxicity associated with SMX therapy is a systemic reaction involving one or more than one internal organ in the same patient. Although skin rashes are the most frequent ADR, occurring in about 1-3% of exposed individuals (Koch-Weser *et al.*, 1971; Bigby *et al.*, 1986), agranulocytosis is observed in approximately 0.1% of individuals (Cribb *et al.*, 1996b). The reactions usually occur ten days after therapy and may be preceded by mild leucopenia (Mandell and Sande, 1985). In certain cases weeks or months may elapse before clinical onset (Heimpel and Raghavacher, 1987).

1.7.5 Other Adverse Effects Associated with Sulphamethoxazole Therapy

SMX inhibits folate synthesis in bacteria. Since mammalian cells use folate

but do not synthesise it, they are resistant to adverse reactions associated with the primary function of the drug. SMX has relatively few pharmacological side-effects, but a number of toxicities not related to the therapeutic target exist. These include renal tubular acidosis, nausea and vomiting, headache, hypoglycemia and goitrogenic effects.

1.8 Clozapine

1.8.1 History and Development

Clozapine (CLZ) is a dibenzazapine derivative (fig. 1.18) which was first investigated by German and Austrian clinicians, who described it as a potent antipsychotic drug (Hippus, 1989). It is effective in treating patients with chronic refractory schizophrenia who are unresponsive to standard neuroleptic drugs (Kane *et al.*, 1988). Approximately 30-40% of previously treatment-resistant patients demonstrate significant improvement after CLZ treatment.

There is a 0.8% risk of developing life-threatening agranulocytosis for patients taking CLZ, approximately ten times greater risk than with conventional neuroleptics (Lieberman and Safferman, 1992; Alvir and Lieberman, 1994). This led to the withdrawal of the drug in the 1970's, although it was reintroduced in the 1980's due to the lack of an effective alternative for the treatment of schizophrenia. The use of CLZ is dependent upon the patient being registered under the Clozaril Patient Management System in which weekly, followed by bi-weekly white blood cell counts are performed.

1.8.2 Metabolism of Clozapine

The major metabolic pathways for CLZ in humans are the formation of CLZ *N*-oxide and demethyl CLZ (Gauch and Michaelis, 1971). More detailed studies of the metabolism of CLZ have reported the formation of aromatic dechloro hydroxy- and methyl-thio compounds (Stock *et al.*, 1977). Further metabolites include the *N*-demethyl derivatives of these compounds and a metabolite postulated as having an oxidised piperazine ring (Fitton and Heel, 1990). In addition to the major stable metabolites, the formation of either a radical cation or the di-imine of CLZ have been identified from secondary radical production, UV absorption and inspection of molecular models (Fischer *et al.*, 1991). Addition of GSH to incubations containing CLZ and various metabolising systems resulted in the formation of two stable adducts, suggesting that GSH may protect against bioactivation *in vivo* (Fischer *et al.*, 1991; Williams, 1996) (fig. 1.18).

The liver is thought to be the major site of CLZ metabolism (Jann *et al.*, 1993). The role of hepatic metabolism in CLZ agranulocytosis depends upon the stability of the reactive species (Gillette *et al.*, 1984) and therefore its ability to translocate from the liver to the target cells, which may be peripheral blood or bone marrow precursors. Studies by Williams (1996) show that similar metabolites are also produced by neutrophils. From a clinical viewpoint, the bioactivation of CLZ is consistent with the depletion of both neutrophil precursors and peripheral neutrophils in cases of CLZ-induced agranulocytosis (Uetrecht, 1992).

1.8.3 Distribution of Clozapine

Since clinical studies in schizophrenic and psychotic patients are very difficult to conduct, there are only a small number of controlled studies examining the pharmacokinetics of CLZ (Gauch and Michaelis, 1971; Choc *et al.*, 1987; Cheng *et al.*, 1988; Lin *et al.*, 1994). Each study reported wide inter-patient variability in the plasma levels of CLZ and its two major metabolites. Patient gender, age, body weight and smoking behaviour all contribute to the variation (Haring *et al.*, 1989). After an oral dose of 100mg, the circulating concentration of CLZ rarely exceeds 150ng/ml (0.5μM) (Sayers and Amsler, 1977).

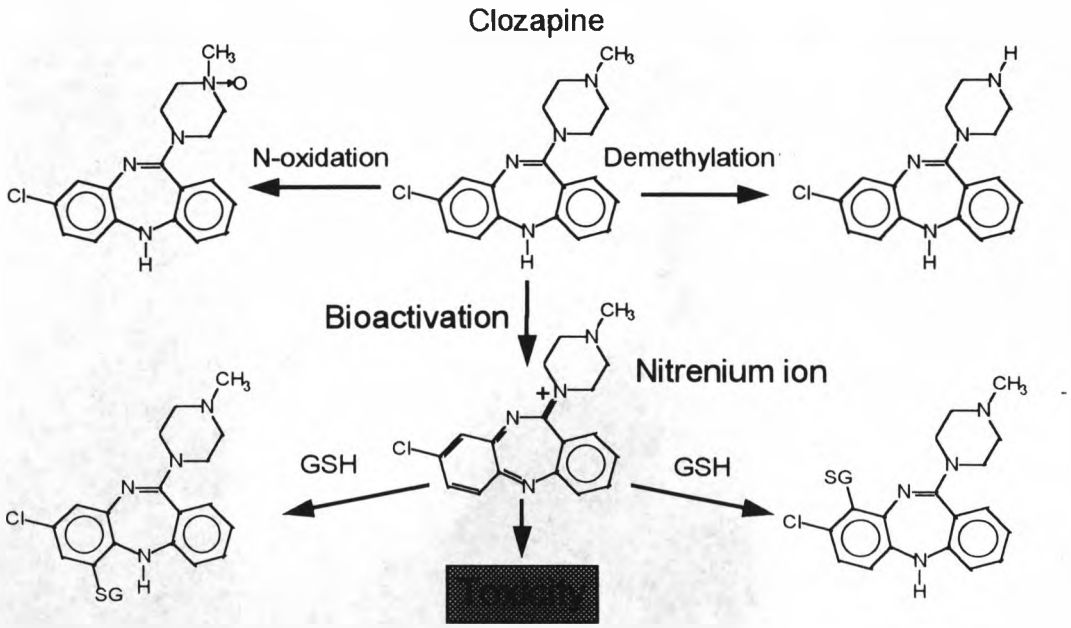


Figure 1.18 Major pathways of CLZ metabolism.

1.8.4 Clozapine-Induced Agranulocytosis

The onset of CLZ-induced agranulocytosis is usually gradual, the period

of maximum risk occurring in the first 18 weeks of therapy (Krupp and Barnes, 1989). Toxicity is characterised by an absence or suppression of myelopoiesis in the bone marrow (Gerson and Meltzer, 1992). Moreover, when medication is stopped (because of a low neutrophil count), patients often go on to develop agranulocytosis lasting between 14 and 24 days. Taken together, these factors suggest that an early myeloid precursor is affected by CLZ treatment and it is not simply peripheral destruction of neutrophils.

1.8.5 Other Adverse Effects Associated with Clozapine Therapy

CLZ is associated with central, autonomic, cardiovascular, gastrointestinal and haematological side-effects (Fitton and Heel, 1990). In a study of 959 patients exposed to CLZ, 8.1% suffered an ADR which led to withdrawal of the drug. 3.9% of these reactions were considered to be severe and potentially life-threatening disorders, such as toxic delirium (Grohmann *et al.*, 1989). CLZ has also been reported to cause a transient and moderate asymptomatic increase in liver enzymes in about 50% of patients; this may progress to severe hepatic injury, although this seems rare (Hovens *et al.*, 1994).

Other frequently reported adverse effects of CLZ include sedation, hypersalivation, tachycardia, dizziness, constipation, nausea/vomiting, postural hypotension, xerostomia and hypothermia. Generalised seizures have been reported although these appear to be dose related (Fitton and Heel, 1990).

1.9 Aims of Thesis

Idiosyncratic agranulocytosis resulting from either direct toxicity of

inadequately detoxified drugs (or more commonly their reactive metabolites) or an immunological mechanism is a highly individualised reaction. The work described in this thesis was performed to investigate the metabolism and disposition of drugs in the peripheral circulation, with the overall aim of further understanding the chemical and biochemical mechanisms of neutrophil toxicity.

Three drugs (CLZ; SMX; AQ) have been studied. Each compound is well tolerated in most individuals; however, their use has been restricted, or the drug has been withdrawn, due to the occurrence of life-threatening idiosyncratic toxicity (agranulocytosis for CLZ, agranulocytosis and hepatotoxicity for AQ and generalised hypersensitivity, including agranulocytosis for SMX). Although dissimilar in structure, bioactivation of the parent drug by the neutrophil NADPH oxidase / MPO system is thought to initiate toxicity. A variety of *in vitro* test systems have been utilised to investigate the relationship between neutrophil metabolism, cellular disposition of the drug, the chemistry of the drug and the mechanism by which these factors induce cell death. The chemical basis of any effect has been rationalised in terms of the physico-chemical properties of the drug, or the chemical biotransformation of particular functional groups. The ultimate aim of all the studies performed is to develop strategies for toxicity prevention, either by identifying individual risk factors or by modifying the drug so that efficacy is retained while toxicity is abolished.

Chapter 2:

Disposition and Toxicity of Amodiaquine and Related Antimalarials in Human Peripheral Blood

Contents **Page**

2.1 Introduction

Malaria remains one of the most prevalent diseases in the developing world, and with the increase in world travel, many more people are coming into contact with the disease. The development and rapid spread of multidrug resistance, especially against chloroquine (CQ; fig. 2.1), for many years the first drug of choice, is of major concern (Peters, 1970). Amodiaquine (AQ; section 1.6), a 4-aminoquinoline antimalarial agent, was introduced because of its effectiveness against both resistant and sensitive strains of *Plasmodium falciparum*, the most pathogenic of all the different malarial parasites (Watkins *et al.*, 1984). However, a number of cases of fatal agranulocytosis, as well as hepatotoxicity in patients during prophylactic administration, led to its withdrawal from use (Neftel *et al.*, 1986; Hatton *et al.*, 1986; Larry *et al.*, 1986).

Drug administration is now considered to be the major cause of agranulocytosis, and indeed amongst the blood dyscrasias, agranulocytosis is the commonest cause of death (section 1.5.1). The mechanisms of drug-induced agranulocytosis are poorly understood, with the exception of the direct antimitotic effects of chemotherapeutic agents. In general, drug disposition is thought to play a major role in many forms of cell-selective toxicity (Pirmohamed *et al.*, 1994). AQ has been reported to cause direct bone marrow stem cell toxicity (Rhodes *et al.*, 1986); however, other studies have detected little direct toxicity to peripheral cells at therapeutic concentrations (Ellis *et al.*, 1987; Aymard *et al.*, 1992). Neutrophil toxicity has been observed in the presence of AQ-specific serum components, indicative of an indirect immunological

mechanism for the agranulocytosis (Douer *et al.*, 1985). Subsequent work demonstrated that AQ was immunogenic in the rat, and that antibodies against AQ were detected. (Christie *et al.*, 1989). In addition, antibodies were identified in patients taking AQ prophylactically who developed an adverse drug reaction (Clarke *et al.*, 1991).

CQ is known to be lysosomotropic; its weak base properties allow it to accumulate down a pH gradient into the acidic lysosomes of the cell, where it becomes protonated and trapped, causing a rise in intralysosomal pH (Klempner and Styrt, 1983) (section 1.3.2). This is thought to be important in the development of phospholipidosis, a disturbance of lipid metabolism within lysosomes, which is manifested as foamy macrophages in the livers of patients administered CQ and other amphiphilic drugs (Lullmann *et al.*, 1975; Yamamoto *et al.*, 1976). The development of tebuquine (TEB), a substituted biphenyl aminoquinoline with greater antimalarial potency than AQ *in vivo* (Werbel *et al.*, 1986) and *in vitro* (Hawley *et al.*, 1996; O'Neill *et al.*, 1996), was halted because of chronic toxicity in animals, including the presence of foamy macrophages (L. M. Werbel and H. Chung, Walter Reed Army Institute of Research, personal communication). However, the mechanism of action of the 4-aminoquinoline antimalarial agents, which involves inhibition of haem polymerisation, is partly dependent on drug accumulation down a pH gradient into the acidic vacuoles of the parasite (Slater, 1993). Thus, it may be difficult to dissociate the therapeutic effects of antimalarial drugs from their toxic effects.

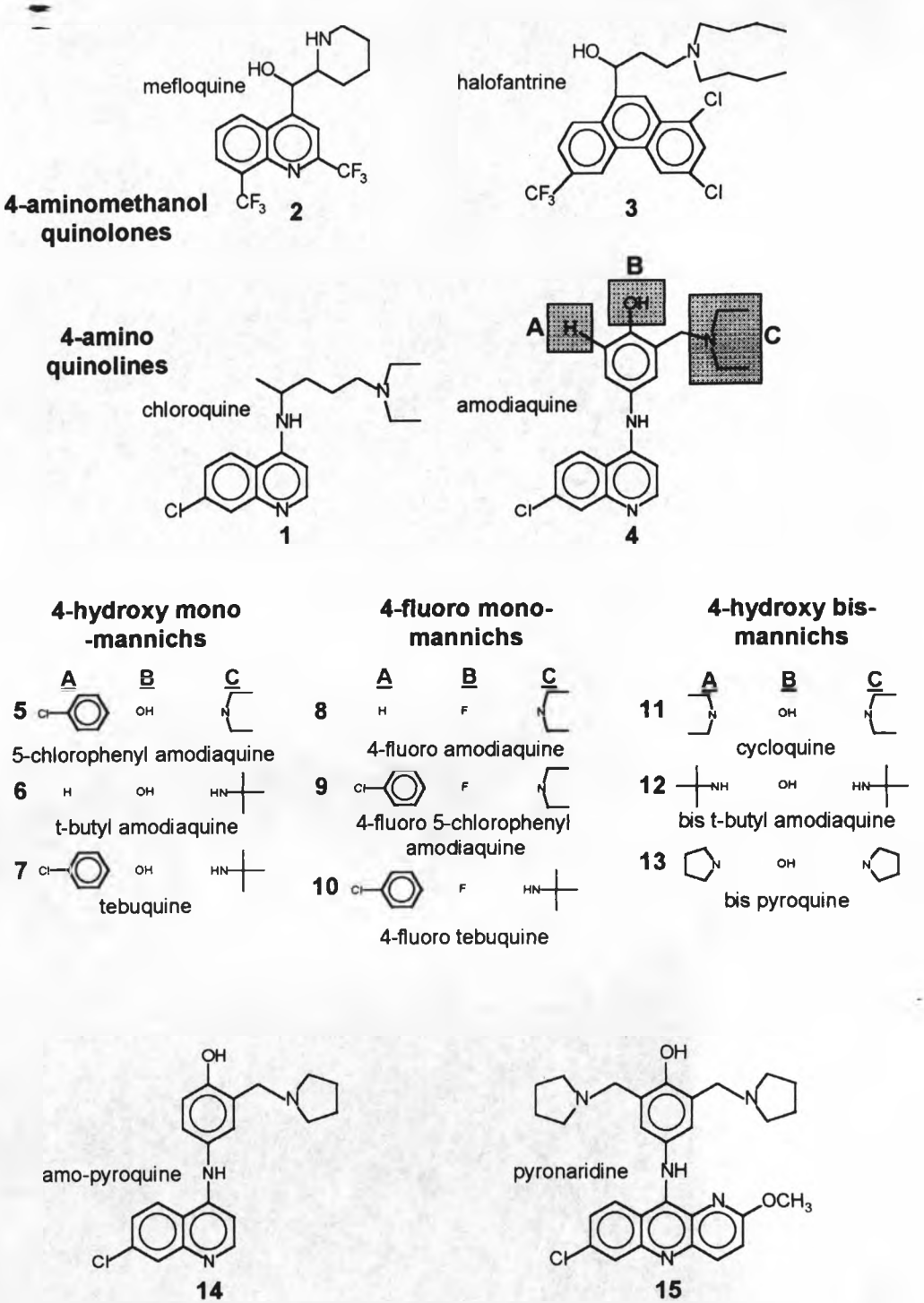


Figure 2.1 Chemical structures of antimalarial drugs, synthetic analogues and new compounds currently undergoing clinical trials.

Neutrophils contain a high concentration of myeloperoxidase (MPO) which is able to metabolise a wide range of unrelated compounds to reactive intermediates (Clarke *et al.*, 1990; Hofstra and Uetrecht, 1993; Maggs *et al.*, 1995) (section 1.5.3). AQ undergoes bioactivation in the presence of activated neutrophils, to a protein-reactive quinoneimine metabolite (Maggs *et al.*, 1988; Tingle *et al.*, 1995). Therefore, drug bioactivation may provide an alternative mechanism through which drugs can cause agranulocytosis (fig. 2.2). Drug bioactivation can also occur in the liver (section 1.3-1.4), although this would require the translocation of the chemically reactive metabolite from the liver to the bone marrow or peripheral blood, which, given that the quinoneimine metabolite is relatively short-lived, seems unlikely.

In order to protect themselves, neutrophils have a wide range of defence mechanisms, one of the most important of which is glutathione (GSH). GSH is the most abundant thiol present in cells, constituting 90% of non-protein thiols (Larsson *et al.*, 1983). It has several important regulatory functions in the body (Kosower and Kosower, 1978; Meister, 1989) (section 1.5.3); however, its main role in drug metabolism is to protect the cell from oxidative damage by conjugating with chemically reactive metabolites formed from drugs and chemicals. AQ has previously been shown to deplete intracellular GSH in neutrophils (Tingle *et al.*, 1995). Therefore, a relative deficiency of GSH may predispose to white cell toxicity by reducing cellular detoxification capacity.

AQ remains one of only a few drugs effective against both CQ-resistant and -sensitive isolates of *Plasmodium falciparum*. Since its discovery numerous

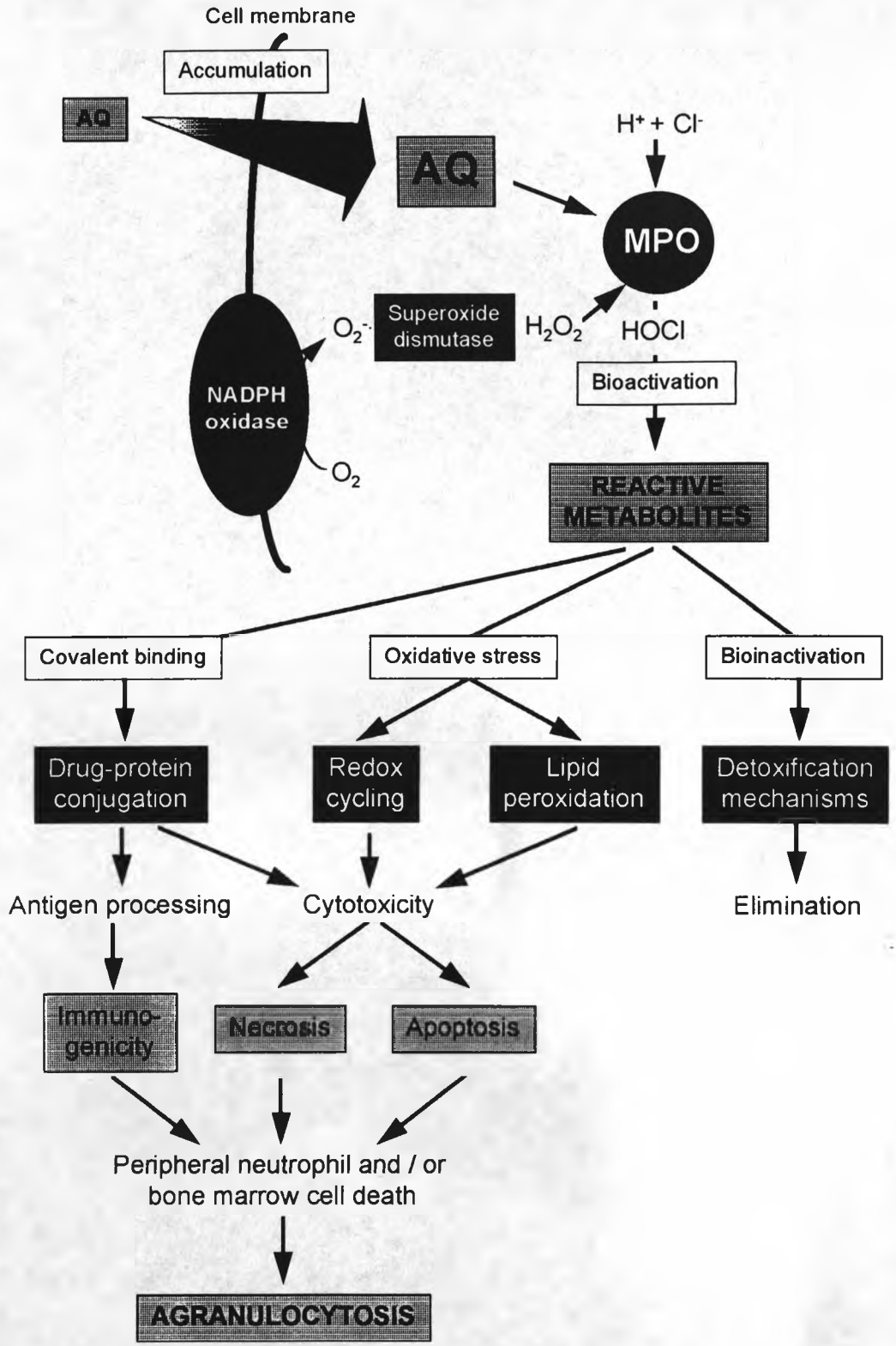


Figure 2.2 Scheme depicting the proposed pathway for the formation of idiosyncratic agranulocytosis from MPO catalysed metabolism of AQ.

attempts have been made at developing a safer and more effective antimalarial drug. These studies have shown that the 7-chloroquinoline nucleus and the diamino side chain separated by a 4-carbon chain are essential for antimalarial potency (Wiselogle, 1946; Thompson and Werbel, 1972; Werbel *et al.*, 1993). This leaves the diethyl amino side chain and *p*-aminophenol ring open to structural modification. Replacement of the terminal hydroxyl group with fluorine and alkyl chain substitutions at the C-3' and C-5' positions have been partly successful in preventing bioactivation of AQ (Harrison *et al.*, 1992; O'Neill *et al.*, 1994; Tingle *et al.*, 1995; Ruscoe *et al.*, 1995; O'Neill *et al.*, 1996).

This chapter examines the chemical basis of AQ-induced agranulocytosis, utilising both synthesised analogues and new drugs currently being investigated for the treatment of acute malaria [amopyroquine, cycloquine (CYC) and pyronaridine (PYRO)] in order to determine the chemical features involved in drug accumulation, bioactivation, neutrophil toxicity and inhibition of cellular function. These criteria have also been applied to other related antimalarial agents [including the 4-(substituted aminomethanol)quinolones, mefloquine and halofantrine] (fig. 2.1). Such investigations of structure-toxicity relationship may aid the design of safer antimalarial agents.

2.2 Materials and Methods

2.2.1 Chemicals

9-Amino-6-chloro-2-methoxyacridine (ACMA), *p*-aminophenol, 5-amino salicylic acid, AQ, bromobimane, 7-chloro-4-aminoquinoline, CQ, eugenol,

Giemsa stain, halofantrine, May-Grunwald stain, mefloquine, *N*-ethylmorpholine, nitroblue tetrazolium (NBT), paracetamol, phorbol 12-myristate 13-acetate (PMA), GSH and trichloroacetic acid were all obtained from Sigma Chemical Co. (Poole, UK). Monopoly® resolving medium (Ficoll Hypaque; density 1.114g/ml) was obtained from ICN Biomedicals (Bucks, UK) and Lymphoprep® (density 1.077g/ml) was obtained from Nycomed (Oslo, Norway). Desethyl AQ, bis desethyl AQ and TEB were all gifts from Parke Davis (Ann Arbor, MI, USA). PYRO was a gift from Dr. D. Warhurst (London School of Hygiene and Tropical Medicine, UK). *t*-Butyl AQ, [*quinoline*-^{3}H(G)] AQ (specific activity, 106.5μCi/μmol), AQ quinoneimine, amopyroquine, 5'-chlorophenyl AQ, deshydroxy-4'-fluoro AQ, deshydroxy-4'-fluoro-5'-chlorophenyl AQ, deshydroxy-4'-fluoro TEB, bis *t*-butyl AQ, bis pyroquine, CYC and AQ (-diethylamino side chain) were synthesised by Dr. P.M. O'Neill (Department of Pharmacology, The University of Liverpool, UK) using the methods of Burckhalter *et al.* (1948), Dahlin and Nelson (1982), Barlin and Tan (1985), Ruscoe *et al.* (1995) and O'Neill *et al.* (1996). All solvents were of HPLC grade and were purchased from Fischer PLC (Loughborough, UK).

2.2.2 Isolation of Human Peripheral Blood Cells

Polymorphonuclear leucocytes (neutrophils; PMN) and mononeuclear leucocytes (lymphocytes / monocytes; MNL) were isolated from the venous blood of 20 healthy male volunteers (age range 21-50 years). Micro-organism contamination was prevented by isolating the cells in a Class II Biohazard Cabinet

with a vertical laminar air flow (Gelaire BSB 4A, Flow Laboratories, Italy). The freshly drawn blood was layered onto a dual density gradient of Lymphoprep (4ml) and Monopoly resolving medium (8ml) and centrifuged at 800*g* for 0.5-2.0h in a Centaur 2 centrifuge (MSE, Sussex, UK) (fig. 2.3).

The bands containing PMN and MNL were removed from the resolving medium using a sterile Pasteur pipette and washed with previously filter sterilised (0.22μM pore size disposable membrane filter; Nohon Millipore, Kogyo K.K., Japan) phosphate buffered saline (PBS; sodium chloride, 8g/l; di-sodium hydrogen orthophosphate, 1.15g/l; potassium chloride, 0.2g/l; potassium di-hydrogen orthophosphate, 0.04g/l; pH 7.4). Red blood cell (RBC) contamination was removed by re-layering the cell suspensions on either Monopoly resolving medium (PMN) or Lymphoprep (MNL) and centrifuged for a further 10min (800*g*). The cells were washed twice with PBS and then resuspended in Dulbeccos PBS (calcium chloride, 0.13g/l; potassium chloride, 0.2g/l; potassium di-hydrogen orthophosphate, 0.2g/l; magnesium chloride, 0.1g/l; sodium chloride, 8g/l; di-sodium hydrogen orthophosphate, 1.15g/l; glucose, 0.9g/l; pH 7.4). The yield of cells was assessed using an improved Neubauer haemocytometer (Weber Scientific Int., U.K.) under a Wilovert microscope (Will Wertzlar, Germany).

2.2.3 Determination of Cell Purity

MNL and PMN were diluted in Dulbeccos PBS to yield a preparation of $1x10^6$ cells/ml. 100μl of each solution was then centrifuged onto a glass slide at 90*g* for 10min using a Shandon-Elliot cyto-centrifuge (London, UK). The slides were air dried for 30min prior to staining with May Grunwald (0.25%, w/v; in

methanol) for 8min, a procedure which also fixed the cells. The slides were then washed with distilled water and stained with Giemsa (0.03%, w/v) for 18min. The purity of each preparation was characterised morphologically by counting 200 randomly selected cells under a Wilovert microscope.

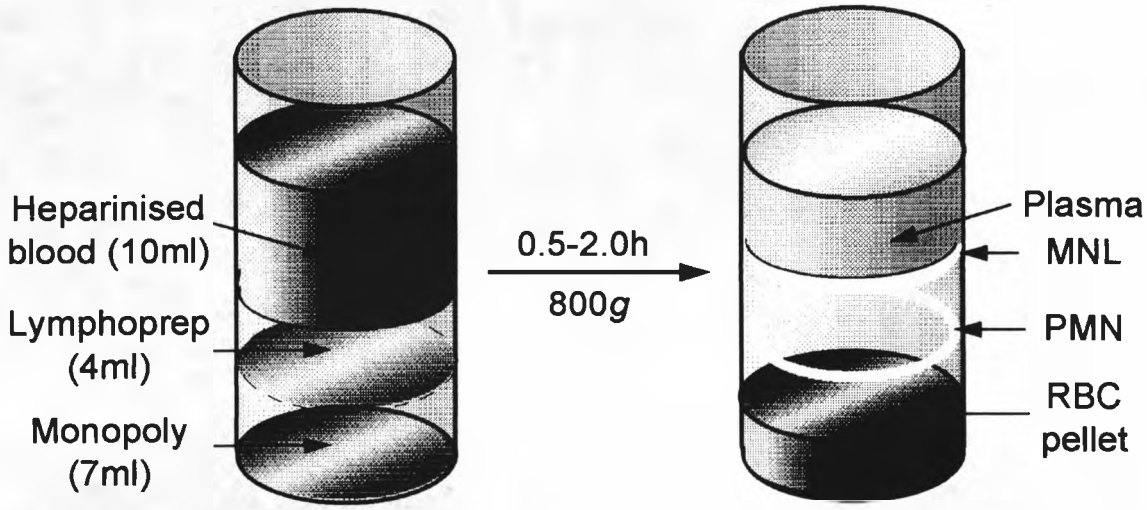


Figure 2.3 Separation of peripheral blood.

2.2.4 Determination of Cell Viability

The viability of both MNL and PMN were determined by trypan blue dye exclusion. This method is based on the principle that intact cells do not take up the dye, while non-viable cells (i.e., cells with a damaged plasma membrane) take up the dye and are stained blue. Trypan blue (20µl; 0.2%, w/v) was added to a suspension of cells (100µl; $1x10^6$/ml) in Dulbeccos PBS. 20µl of the trypan blue-cell suspension was transferred to both chambers of a Neubauer haemocytometer and 200 cells from each suspension were counted. % cell viability was calculated as,

$$\frac{\text{the total number of viable cells (unstained cells)}}{\text{the total number of cells (stained and unstained)}} \times 100$$

2.2.5 Determination of [^{3}H]-Amodiaquine Accumulation in Isolated Human Blood Cells

MNL, PMN and RBC were incubated separately with [^{3}H]AQ (0.1μCi, 1-100μM; 1-24h) at 37°C in either Dulbeccos PBS or platelet poor plasma. The incubation was terminated by centrifugation (750*g*, 5min) and the supernatant was assayed for radioactivity in scintillant (4ml). Liquid scintillation counting was carried out using a Packard Tri-carb 1500 liquid scintillation analyser (Canberra-Packard, Berks, UK). Disintegrrations per minute calculations were automatically carried out using external standards and all samples were automatically corrected for quenching. The pelleted cells were washed with PBS, lysed and the amount of radioactivity was determined.

2.2.6 Determination of Intralysosomal pH Changes

Changes in intralysosomal pH, which can be used as a measure of drug accumulation (Klempner and Styrt, 1983; Styrt and Klempner, 1985), were monitored by determining the distribution of the fluorescent weak base ACMA. ACMA was preferred over the more commonly used 9-aminoacridine or a combination of the fluorescent probes (Rottenberg and Moreno-Sanchez, 1993), because (1) the test compounds had an effect on 9-aminoacridine fluorescence, which was not observed with ACMA (fig. 2.4), and (2) the enhanced sensitivity of ACMA to small pH changes allowed the analysis of low concentrations of test compound that were non-toxic to PMN.

Fluorescence intensity was measured using a modification of the method of Klempner and Stryt (1983). ACMA (2μM) in ethanol (final concentration,

0.5% v/v) was used for pH monitoring. All aqueous solutions were adjusted to pH 7.4 except where indicated. A spectrofluorophotometer (excitation at 415nm, emission at 480nm, RF-5001 PC; Shimadzu, Duisburg, Germany) with a slit width of 5nm was used in the analysis. The cuvette compartment was maintained at 37°C using a Howe thermocell controller (Howe, Oxon, UK). After the initial fluorescence was recorded, PMN (8 x 10^6/ml) were added and the suspension was mixed thoroughly. Fluorescence was monitored until steady state was reached. All readings were corrected for the volumes added, and for the effects of added agents on background fluorescence.

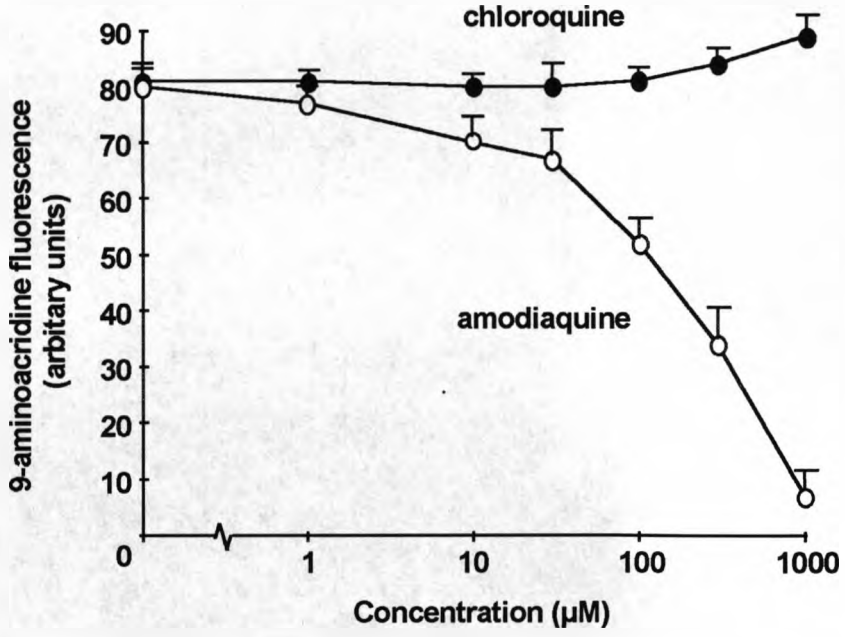


Figure 2.4 The effect of antimalarial drugs on 9-aminoacridine fluorescence. 9-aminoacridine (0.05μM) was made up in Dulbeccos PBS (pH 7.4). Fluorescence was measured with excitation at 400nm and emission at 456nm.

2.2.6.1 Characteristics of 9-amino-6-chloro-2-methoxyacridine Fluorescence

Initial experiments were performed to ensure that ACMA could be used successfully as a weakly basic probe to monitor pH changes within lysosomes. The concentration dependence of ACMA fluorescence in Dulbeccos PBS (pH 7.4) was examined. Solutions containing ACMA (0.1-3μM) were prepared and the fluorescence intensity was measured as described above. The effect of pH changes (pH 5.0-8.0) on the fluorescence of ACMA (2μM) was also determined. Similarly, the effect of external pH changes on the quenching of fluorescence by PMN was determined over a pH range of 5.0-8.0.

2.2.6.2 Determination of Antimalarial Lysosomal Accumulation by Changes in Intralysosomal pH

Alkalinisation of lysosomal pH induced by the accumulation of antimalarial drugs (1-100μM) was calculated from the reversal of the initial fluorescence quenching, *i.e.*, in the absence of drug. All drugs were dissolved in dimethyl sulphoxide (DMSO; 0.5%, v/v), which by itself did not affect fluorescence. Fluorescence was monitored continuously until steady state was reached (fig. 2.5). CQ was used as a positive control for each experiment. Results are expressed as the mean ± S.D. of three experiments using cells from different individuals.

2.2.7 Determination of Depletion of Intracellular Glutathione

Initial experiments were designed to evaluate the optimum number of PMN required to demonstrate depletion of GSH. This was performed with AQ

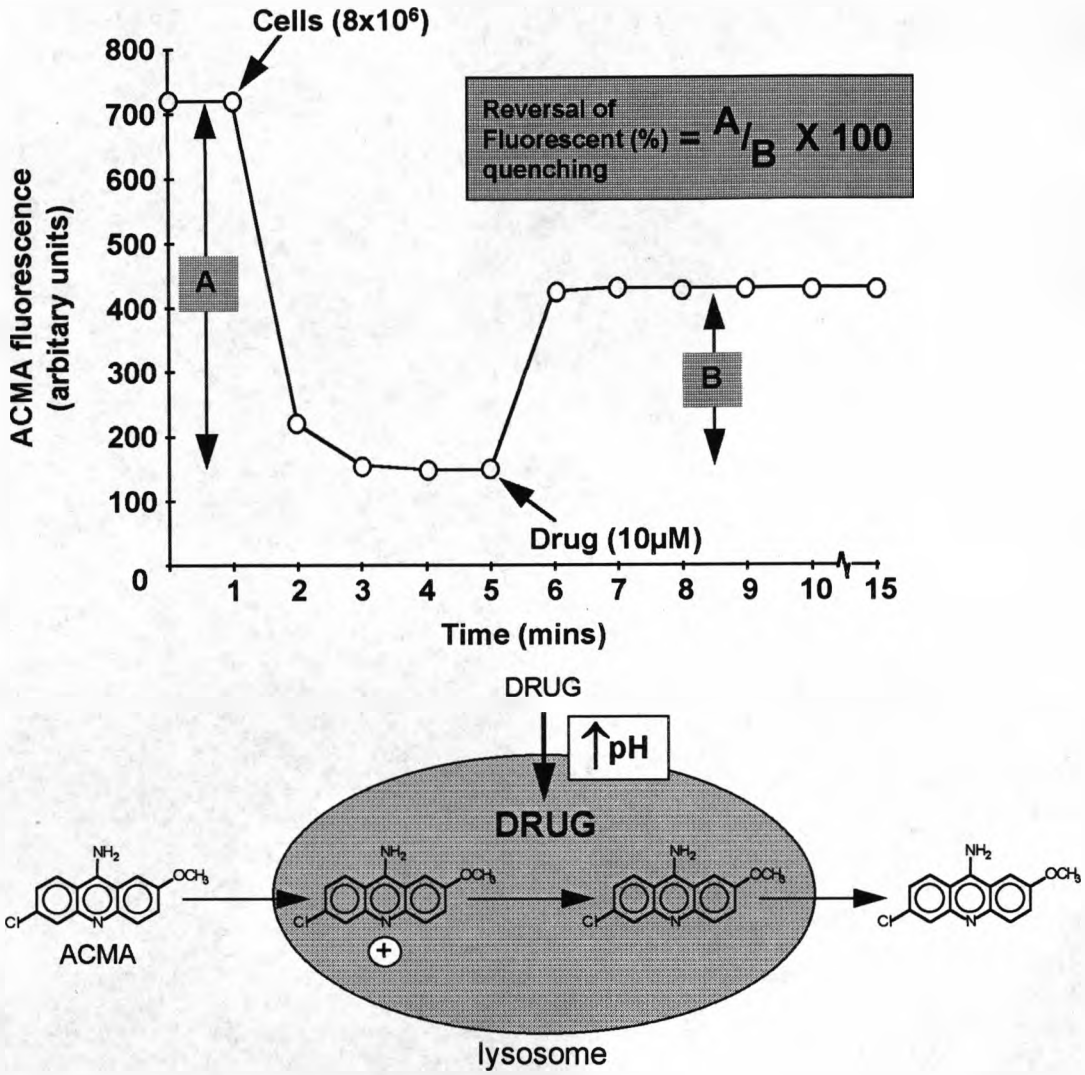


Figure 2.5 Scheme depicting the reversal of fluorescent quenching of ACMA after the addition of antimalarial drugs.

(100μM) which has previously been shown to deplete GSH in PMN (Tingle *et al.*, 1995). Isolated PMN (0.5-5 x 10^6 cells) were incubated in Dulbeccos PBS at 37°C for 1h with AQ, in the presence or absence of PMA (10ng/ml). The total incubation volume was 1ml. GSH levels were determined by the method of Cotgreave and Moldeus (1986). Bromobimane (3mM, in *N*-ethylmorpholine; pH

8.0; 100µl) was added to the incubations, which were then left in the dark for 5min. Reduced sulphydryl groups react with bromobimane via an electrophilic substitution reaction to yield a highly fluorescent bimane-thiol conjugate (fig. 2.6). Protein was precipitated with 100% trichloroacetic acid (10µl) and sedimented by centrifugation (850*g*, 3min). An aliquot of the supernatant was injected onto an HPLC column (5-µm Hypersil BDS C_{18} column, 4.5 x 150mm). GSH adducts were eluted with mobile phase (0.25% acetic acid / 9% aqueous acetonitrile, pH 3.7) for 7min, followed by 75% aqueous acetonitrile for 4min, with a re-equilibrium period of an additional 5min with the mobile phase. The flow rate was 1ml/min throughout. Detection of eluent was monitored using a fluorescence detector (Hitachi 1080) set for excitation at 394nm and emission at 480nm. A GSH standard curve was constructed between 0 and 100nmol of GSH.

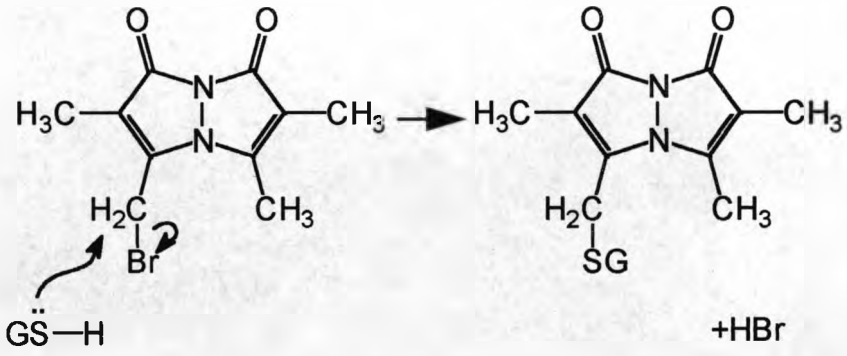


Figure 2.6 Structure of bromobimane and mechanism of binding to GSH.

A cell concentration of 0.5 x 10^6/ml was found to be optimum to demonstrate GSH depletion, and thus all subsequent experiments were performed

using 0.5 x 10^6 PMN/incubation. Varying concentrations (1-300μM) of all compounds were used to determine their ability to deplete GSH using the protocol described above. The compounds were dissolved in either methanol or DMSO (final concentration, 1%, v/v); the relevant vehicle was used for the control incubations. With every experiment, eugenol (4-allyl-2-methoxyphenol, 1-300μM), which has been shown to deplete intracellular GSH (Thompson *et al.*, 1989), was used as a positive control.

2.2.8 Determination of Direct Cytotoxicity to Neutrophils and Lymphocytes

Direct cytotoxicity of antimalarial drugs and their analogues was determined by incubating either PMN (1 x 10^6/incubation) or MNL (1 x 10^6/incubation) in Dulbeccos PBS (1ml) at 37°C for 1h, in a shaking water bath (Pirmohamed *et al.*, 1991). The drugs were added in either methanol or DMSO, which, as a 1% solution (v/v) was non-toxic to the cells. After 1h, the cells were sedimented and resuspended in drug-free Dulbeccos PBS. Cytotoxicity was determined by measurement of cell loss and cell viability. Cell viability was assessed by trypan blue dye exclusion (0.2%, w/v, trypan blue), as described above, while cell loss was assessed by counting the total number of cells in a fixed area on an improved Neubauer haemocytometer.

2.2.9 Determination of Inhibition of Neutrophil Function

2.2.9.1 Luminol-Enhanced Chemiluminescence

The PMN respiratory (oxidative) burst was measured by luminol-

enhanced chemiluminescence using a method similar to that described previously (Labro *et al.*, 1987). To determine whether our test compounds (AQ, CQ, TEB; 1-100μM, in methanol; 1%, v/v) inhibited the respiratory burst, PMN (0.5×10^6) were incubated with drug at 37°C in Dulbeccos PBS. The total incubation volume was 949μl. After 30min, 1μl of luminol (20mM) and 50μl of PMA (1μg/ml) were added to the incubations. Luminol-enhanced chemiluminescence was measured using a Packard luminometer. Light emitted was recorded continuously for a period of 50min. The results represent the mean ± S.D. of three separate experiments (cells from different individuals) and are expressed as a percentage (drug treated PMN / control PMN) for the peak value of luminescence. Control incubations containing either PMN or drug alone were set up in each experiment.

2.2.9.2 Nitroblue Tetrazolium Slide Test

NBT is a pale yellow dye that is reduced in the presence of superoxide to a dark blue insoluble product called formazan (Lace *et al.*, 1975). Inhibition of this process, which occurs with inhibition of PMN function forms the basis of the NBT slide test, as described by Meerhof and Roos (1986) (fig. 2.7). PMN (0.5×10^6) were preincubated in Dulbeccos PBS for 5min at 37°C in a shaking water bath in the presence of drug (1-300μM, made up in 1% DMSO or methanol, which did not affect superoxide formation). The cells were mixed with 10μl of NBT solution (5mg/ml in Dulbeccos PBS) and 10μl of PMA (1μg/ml). The final incubation volume was 750 μl. The resultant solution was incubated for an additional 30min in a shaking water bath at 37°C. The incubations were then fixed

with paraformaldehyde (4%, w/v 250µl), and 200µl of each solution was centrifuged (90*g*, 10min) onto a glass slide using a Shandon Elliot cyto-centrifuge (Shandon Elliot, London, UK). The slides were dried and 200 cells from each incubation were counted. The percentage of cells stained blue in the control incubations (>95%) were taken as the base line.

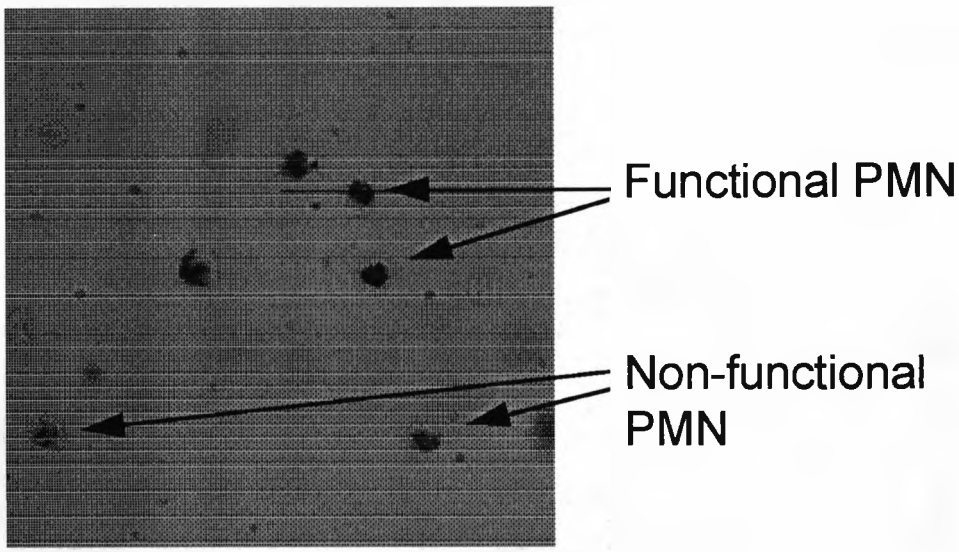


Figure 2.7 Slide showing functional (stained with blue formazan deposits) and non-functional PMN.

2.2.9.3 Inhibition of Eugenol-Induced Glutathione Depletion

To determine whether any of the test compounds inhibited the depletion of intracellular GSH in PMN, we assessed the effect of coincubating the test compound (10 or 100µM) with eugenol (1-300µM). Essentially the same protocol was used as described above, except that the test compound was also present in the incubation and the final concentration of solvent was 2% (v/v).

2.2.10 Statistical Analysis

The results are presented as the mean ± S.D., with the exception of the IC_{50} values which are presented as IC_{50} ± S.E.M. The IC_{50} values were calculated using the four-parameter logistic method (Grafit program; Erithacus Software, Staines, UK) by interpolation of the logarithmic concentration curve. The S.E.M. was calculated from the error associated with the sigmoidal fit of the curve. Statistical analysis was performed with the Mann-Whitney test, accepting $P < 0.05$ as significant.

2.3 Results

2.3.1 Cell Purity and Viability

Freshly isolated PMN and MNL were greater than 98% pure and 95% viable as assessed by Wright's stain and trypan blue dye exclusion. After a 1h incubation containing cells in Dulbeccos PBS at 37^0C there was no significant reduction in cell viability.

2.3.2 Accumulation of Amodiaquine into Peripheral Blood Cells

Incubation of peripheral blood cells with [^{3}H]AQ revealed that accumulation was linear over the concentration range (1-100μM) studied and was independent of time. [^{3}H]AQ selectively accumulated in PMN and MNL (fig. 2.8), with little accumulation in RBC. PMN accumulation was significantly decreased on incubation with plasma, suggesting that plasma protein binding may play a major part in the distribution of AQ in blood.

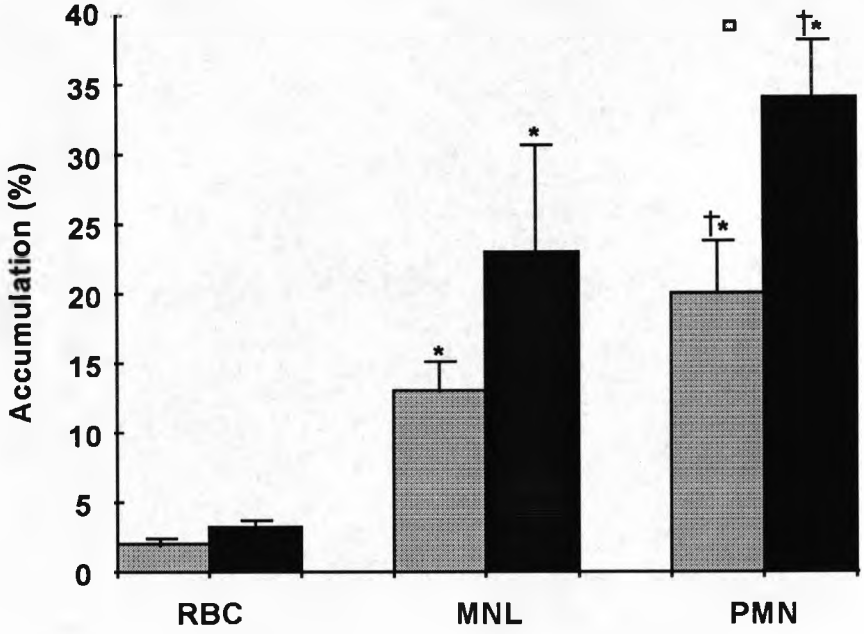


Figure 2.8 Accumulation of [^{3}H]AQ (10μM, 0.1μCi) in RBC, MNL and PMN after 1h in the presence of either platelet-poor plasma (▣) or Dulbeccos PBS (■). Statistical analysis was performed by comparing the accumulation in RBC with that seen in MNL or PMN (*P < 0.05) and that in MNL with that in PMN (†P < 0.05).

2.3.3 Monitoring Intralysosomal pH with 9-amino-6-chloro-2-methoxy-acridine

ACMA showed all the characteristics necessary for analysis of intralysosomal pH changes (Klempner and Styrt, 1983). The fluorescence intensity was found to be dependent on the concentration of ACMA (0.1-3.0μM). For subsequent studies with PMN, the concentration of ACMA incubated with the cells was 2μM. The absolute fluorescence of ACMA was not affected by changes in the external pH (5.0-8.3). In contrast, fluorescence quenching by PMN (8×10^6/ml) was dependent on the external pH. In an acidic extracellular environment (pH 5.0) there was little lysosomal accumulation of ACMA, whereas

an increase in the pH (5.0-8.0) resulted in a significant ($P < 0.05$) increase in fluorescence quenching by PMN (fig. 2.9).

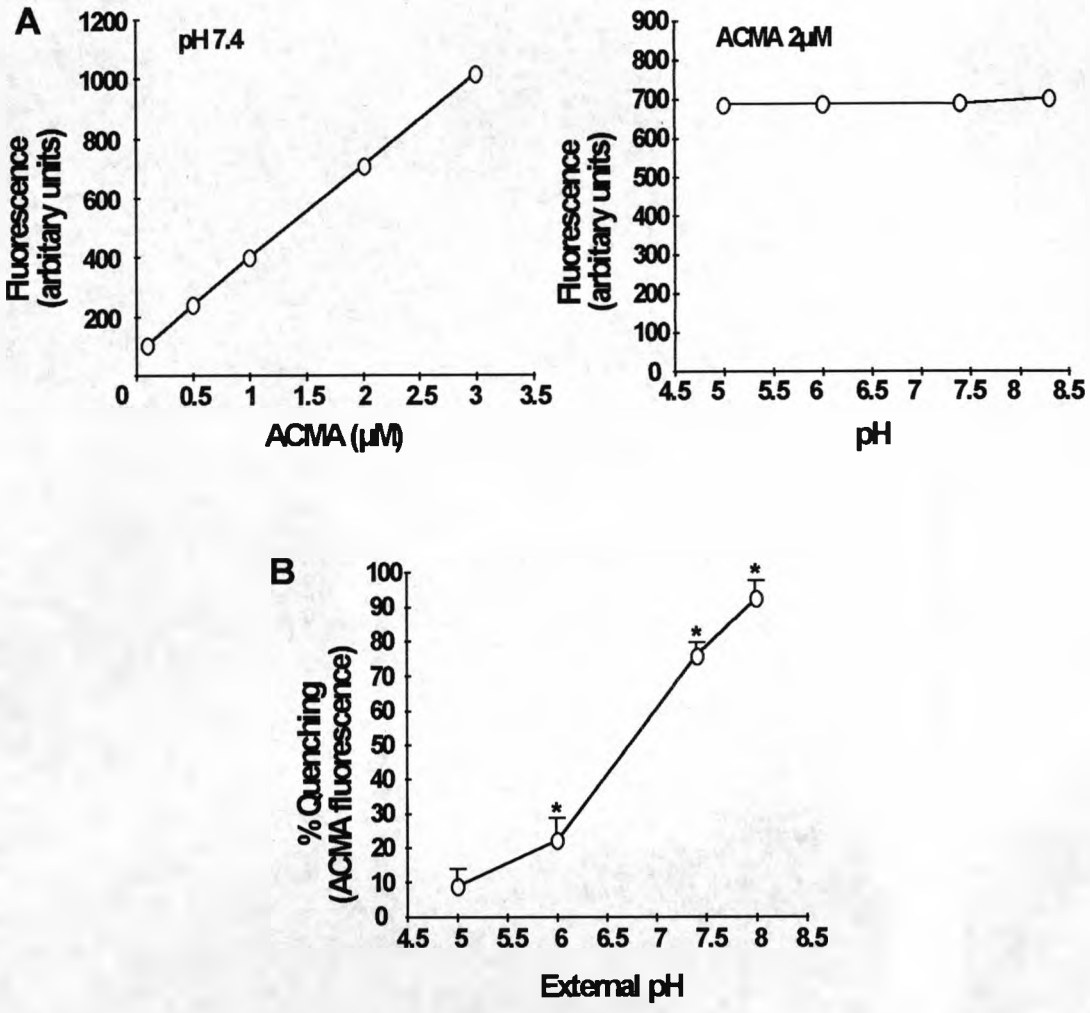


Figure 2.9 A) Concentration dependence and pH independence of ACMA fluorescence. Solutions of ACMA were made up in Dulbeccos PBS.

B) Effect of external pH on ACMA fluorescence with intact PMN. PMN (8×10^6) were incubated with ACMA (2μM) at various pH and the quenching of fluorescence was measured when steady state was reached. Results represent the mean of three separate experiments. Statistical analysis was performed by comparing fluorescence at pH 5.0 with that seen at pH 6.0, 7.4 and 8.0 (*$P < 0.05$).

2.3.4 Accumulation of Antimalarial Drugs in Lysosomes

The lysosomal accumulation of CQ, AQ and TEB was concentration dependent (1-100μM), with the rank order of accumulation (TEB > AQ > CQ) being maintained with the change in concentration (fig. 2.10). All of the other compounds were tested at 10μM, a concentration that was not cytotoxic during the time-course of the experiment, *i.e.*, 15min.

Accumulation of all the compounds occurred rapidly, with steady-state fluorescence being reached in < 5min, with the exception of halofantrine, which required 20min to reach steady state. There was no significant difference in the lysosomal accumulation of AQ (28.6 ± 4.2%) and AQ quinoneimine (25.7 ± 1.6%). However, both compounds accumulated to a significantly ($P < 0.05$) greater extent than CQ and mefloquine (fig. 2.11).

To determine the structural features responsible for accumulation of AQ, the effect of chemical substitution was determined. Alteration of the diethylamino side chain of AQ (*t*-butyl AQ and amopyroquine) caused a significant increase in the intralysosomal accumulation ($P < 0.05$). Conversely, removal of the side-chain decreased accumulation to 11.3 ± 2.1% ($P < 0.05$). Addition of the lipophilic 5'-chlorophenyl group to AQ resulted in a slight decrease in lysosomal accumulation (5'-chlorophenyl AQ; $P < 0.05$), whereas the same structural modification to *t*-butyl AQ (TEB) had no significant effect. Chemical substitution of the 4'-hydroxyl group of AQ , 5'-chlorophenyl AQ and TEB with fluorine increased lysosomal accumulation. In fact, deshydroxy-4'-fluoro TEB caused the greatest reversal of fluorescence quenching (fig. 2.11).

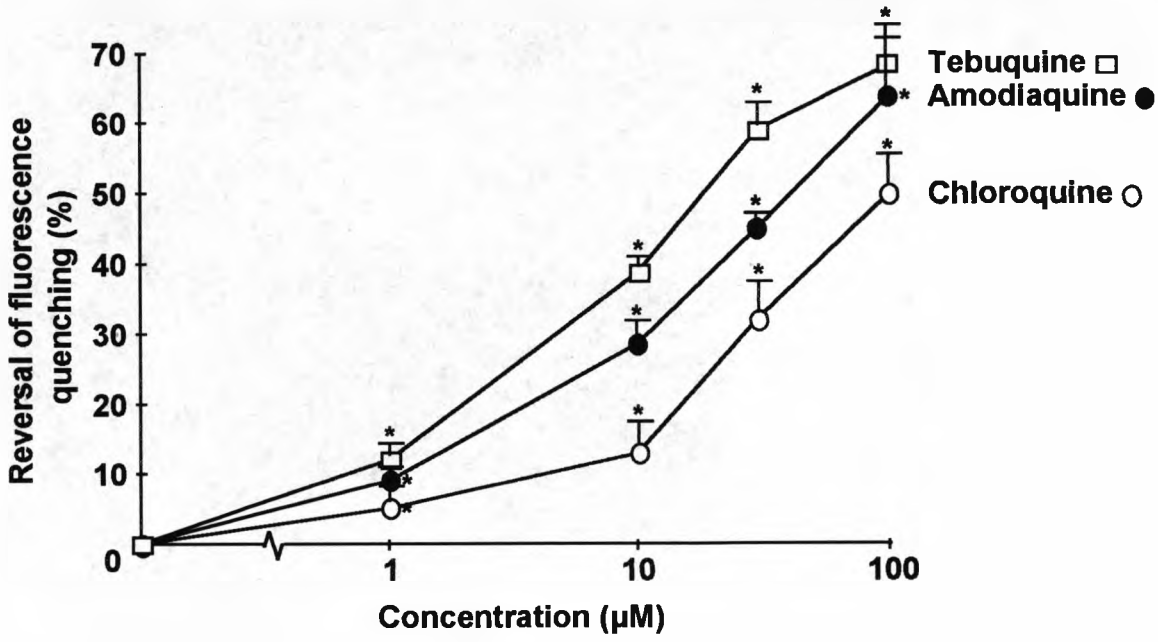


Figure 2.10 Lysosomal accumulation of AQ, CQ and TEB (10-100μM), monitored by reversal of fluorescence quenching of ACMA (2μM), after pre-incubation of the probe with 8 x 10^6 PMN for 5min. Each point represents the mean ± S.D. from three separate incubations. Statistical analysis was performed by comparing the accumulation at different concentrations of compound with that of solvent alone (*P < 0.05).

The bis-mannich compounds (CYC, bis *t*-butyl AQ and bis pyroquine), which all contain three charges at physiological pH accumulated to a significantly ($P < 0.05$) lesser extent than did their mono-mannich derivatives. The new antimalarial PYRO, an aza-acridine analogue of bis pyroquine, was also tested. This compound interfered with the fluorescence causing it to decrease significantly in the absence of PMN (19.1 ± 0.9%; $P < 0.05$). However, if this effect was taken into account, intralysosomal accumulation was found to be increased significantly ($P < 0.05$) when compared to bis pyroquine (fig. 2.11).

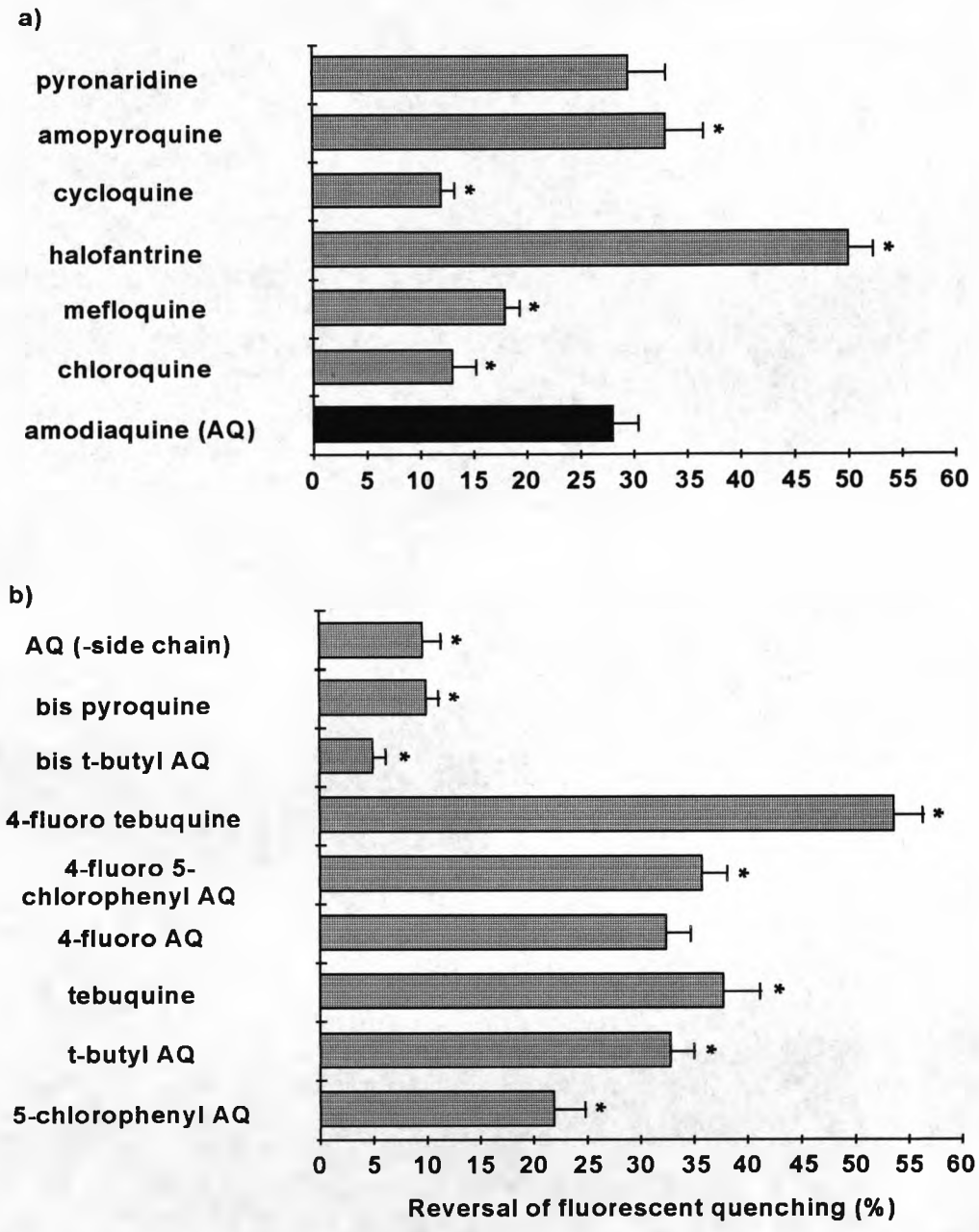


Figure 2.11 (a) Lysosomal accumulation of antimalarial drugs and compounds proposed for clinical use (10μM), monitored by reversal of fluorescence quenching of ACMA, after preincubation of the probe with 8 x 10^6 PMN for 5min. (b) Lysosomal accumulation of synthetic analogues of AQ (10μM), measured under the same conditions. Each bar represents the mean ± S.D. from three separate incubations. Statistical analysis was performed by comparing the accumulation of AQ with that of other compounds studied ($^*P < 0.05$).

2.3.5 Depletion of Intracellular Glutathione

The ability of drugs to deplete intracellular GSH in PMN was determined in both the presence and absence of PMA, a compound known to activate such cells. PMA by itself, in the absence of any drug, caused a significant ($P < 0.05$) depletion of GSH (28.5 ± 9.3%; n = 6 experiments). The results from subsequent experiments with various compounds thus take into account any depletion by PMA itself.

AQ has previously been shown to deplete GSH (Tingle *et al.*, 1995). Therefore AQ was used as a test compound to determine the optimum number of cells required to demonstrate GSH depletion in PMN. The depletion by AQ was dependent on the presence of PMA and the cell number per incubation. In the absence of PMA, there was a significant reduction in intracellular GSH (32.0 ± 4.0%; $P < 0.05$) with AQ (300μM), although this was markedly less than that observed in the presence of PMA (83.1 ± 4.2%; $P < 0.05$). Maximum percentage depletion was observed at a cell concentration of 0.5×10^6 cells / incubation, with <45% depletion being observed at a cell concentration above 2.5×10^6 PMN / incubation (fig. 2.12). Therefore for subsequent experiments, 0.5×10^6 PMN / incubation were used to determine the potential of other drugs to deplete intracellular GSH.

Eugenol was used as a positive control in each experiment. It was previously shown to deplete intracellular GSH in PMA-stimulated PMN (Thompson *et al.*, 1989). A maximal depletion of 95.0 ± 3.0% (n = 20 experiments in triplicate) at a concentration of 300μM and an IC_{50} value

(concentration required to deplete GSH by 50%) of 15.4 ± 3.2μM were observed.

All the antimalarial compounds tested apart from AQ and amopyroquine did not deplete GSH (1-300μM), in either the presence or absence of PMA (fig. 2.13). These two compounds were equipotent at depleting GSH, with IC_{50} values of about 20μM.

AQ quinoneimine, which is known to be the chemically reactive metabolite of AQ (Maggs *et al.*, 1988), produced 92.7 ± 2.2% depletion of GSH at 300μM, with an IC_{50} value of 2.6μM even in the absence of PMA. Two stable metabolites of AQ, *i.e.*, desethyl-AQ and bis desethyl-AQ, also caused a significant depletion of GSH, although this was significantly less than that observed with AQ and AQ quinoneimine ($P < 0.05$; fig. 2.14).

Disconnection of AQ identified the structural requirements for GSH conjugation (fig. 2.14). 7-Chloro-4-aminoquinoline produced no GSH depletion; in contrast, removal of the diethylamino side chain from the AQ molecule and the *p*-aminophenol nucleus caused significant depletion of GSH in the presence of PMA ($P < 0.05$), which was, in fact, greater than that observed with AQ itself.

Paracetamol and 5-amino salicylic acid, both of which contain the *p*-aminophenol nucleus were also tested for their ability to deplete GSH. Paracetamol (300μM) resulted in 42.0 ± 2.8% depletion in the presence of PMA-stimulated PMN, whereas 5-amino salicylic acid caused no depletion (fig. 2.14).

Analogues of AQ with different substitutions at the 3'-, 4'- and 5'-positions on the benzene ring were also investigated. Alteration of these positions produced compounds that either did not deplete GSH or depleted GSH to a

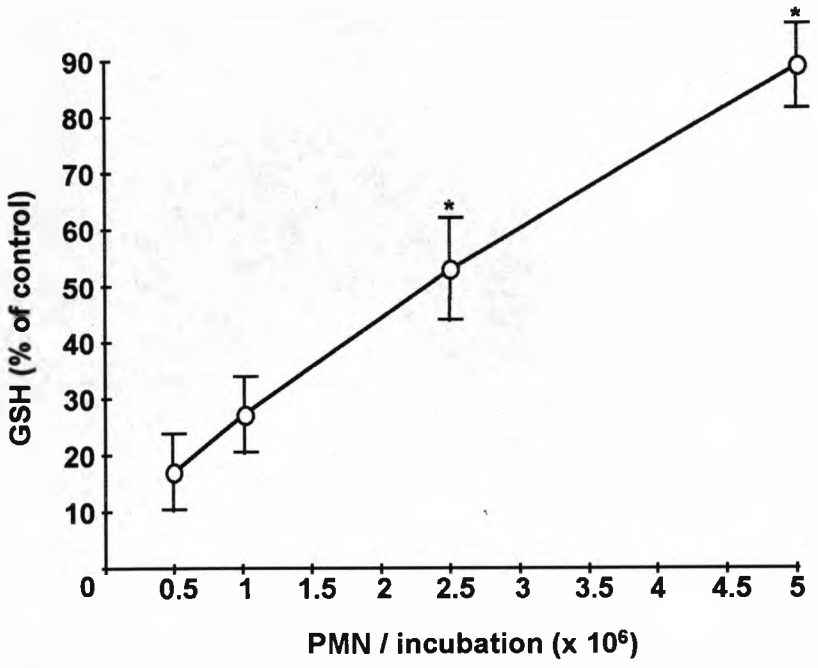


Figure 2.12 GSH depletion in PMA-stimulated PMN (0.5-5 x 10^6 PMN / ml) by AQ (100μM). GSH levels were measured using bromobimane, by fluorescence HPLC. Results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing the ability of AQ to deplete GSH at different concentrations of PMN with that observed at 0.5 x 10^6 PMN / ml ($^*P < 0.05$).

lesser extent than AQ ($P < 0.05$). 5'-Chlorophenyl AQ (300μM) caused 45.0 ± 4.8% depletion of GSH in the presence of PMA ($P < 0.05$), whereas the closely related compound TEB did not cause any depletion. The 4'-fluorinated analogues of AQ, 5'-chlorophenyl AQ and TEB also did not deplete GSH. As expected, *t*-butyl AQ, which is similar to AQ, was no more potent at depleting GSH than AQ. The bis-mannich 4-aminoquinoline compounds CYC, bis *t*-butyl AQ, bis pyroquine and PYRO did not deplete GSH.

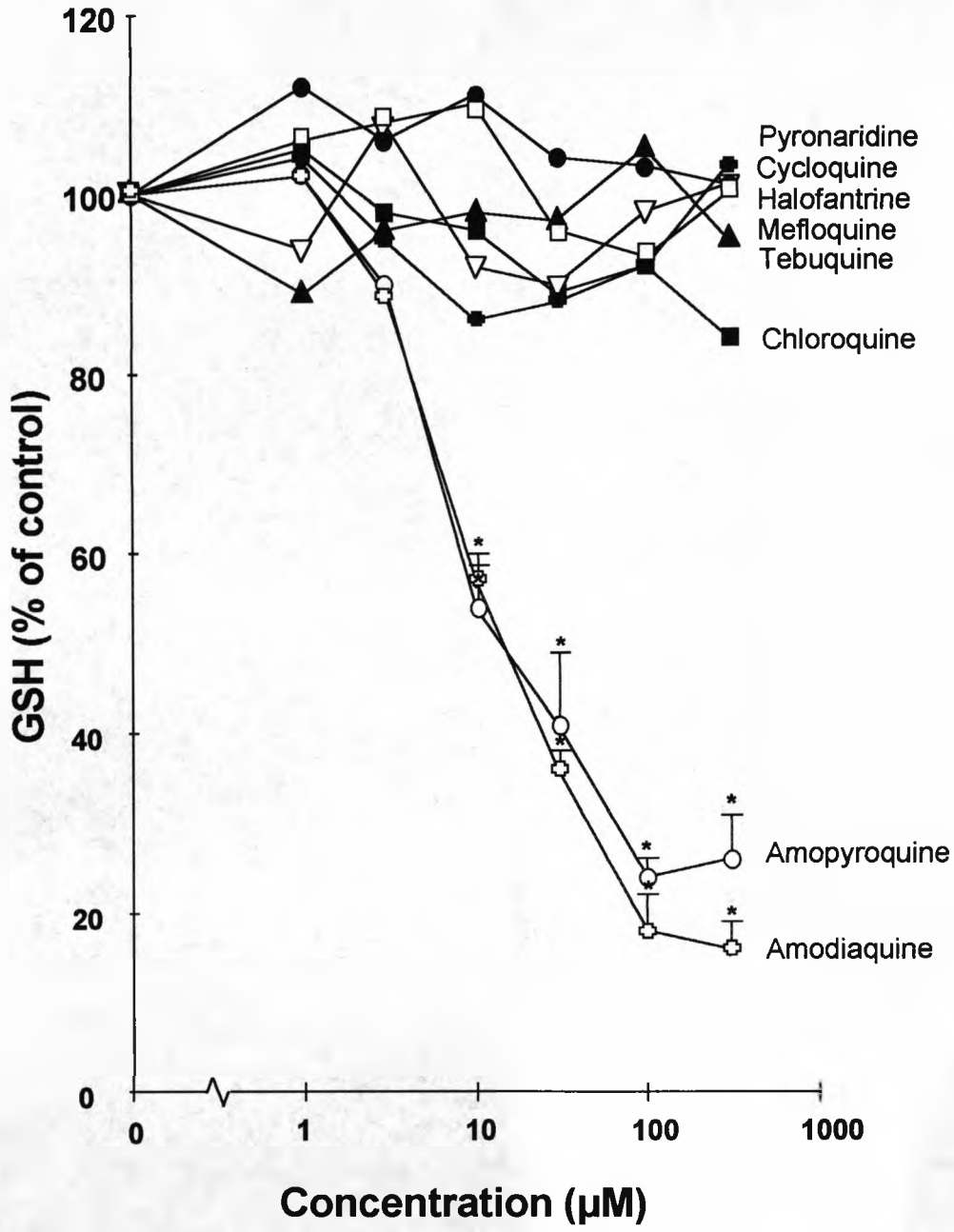


Figure 2.13 GSH depletion in PMA-stimulated PMN (0.5 x 10^6 / ml) by the antimalarial compounds (1-300µM). The results represent the mean ± S.D. of triplicate incubations. Error bars have been omitted for some of the compounds for the sake of clarity. Statistical analysis was performed by comparing the ability of different concentrations of compounds to deplete GSH with that of solvent alone (*P < 0.05).

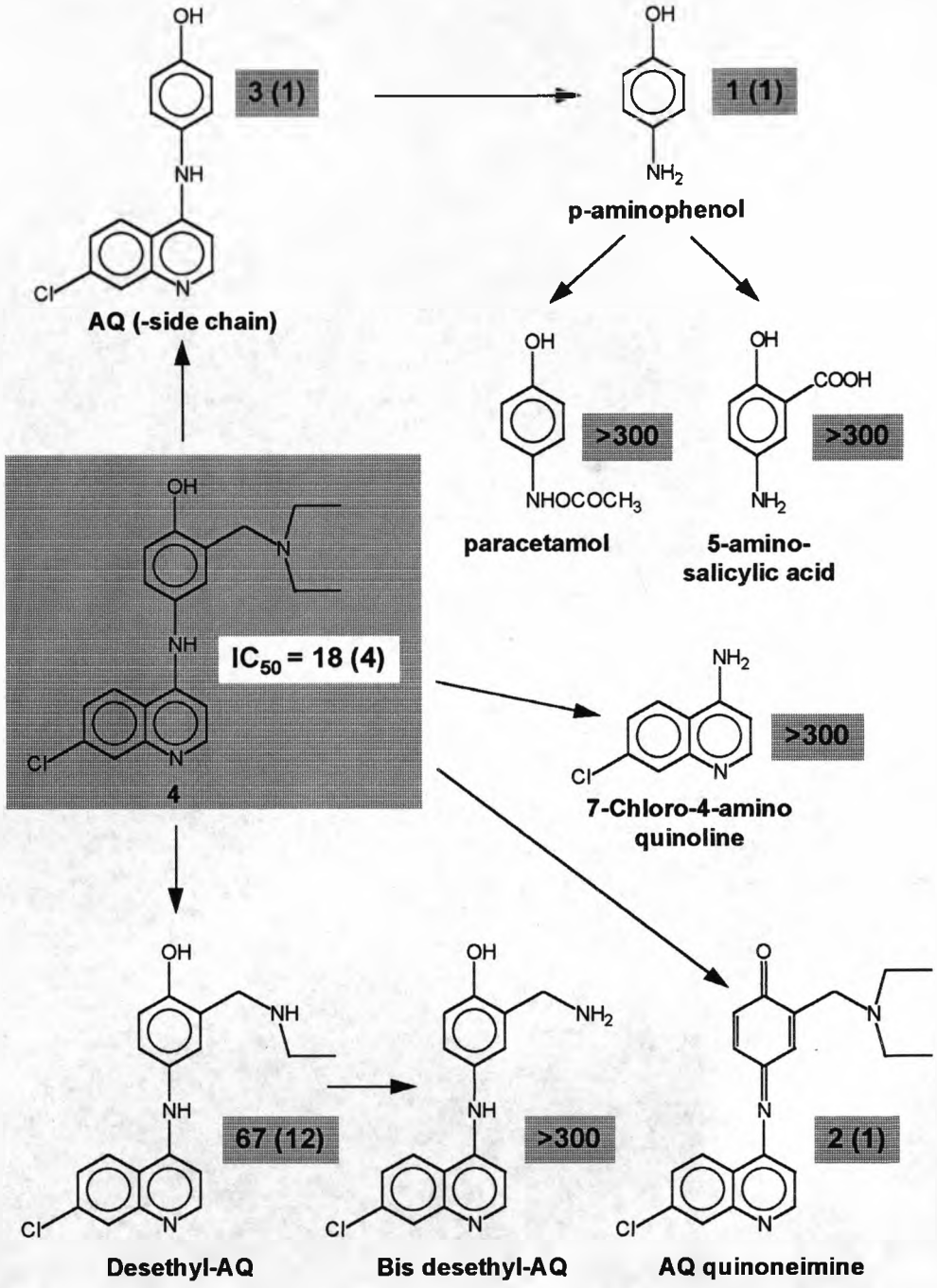


Figure 2.14 Scheme showing the structure disconnection of AQ into its major plasma metabolites and disconnection products. Results show GSH depletion from PMA-stimulated PMN (0.5×10^6 / ml) and are expressed as IC_{50} values (mean ± S.E.M.; μM). The IC_{50} values were calculated using the four-parameter logistic method (Grafit program; Erithacus Software), as described in section 2.2.10.

2.3.6 Direct Cytotoxicity of Antimalarial Compounds to Neutrophils and Lymphocytes

All compounds tested except for CQ, PYRO and paracetamol caused a concentration-dependent decrease in PMN recovery ($P < 0.05$), with only TEB, deshydroxy-4'-fluoro-5'-chlorophenyl-AQ and deshydroxy-4'-fluoro-TEB causing cell loss below 30μM (fig. 2.15; table 2.1). Only mefloquine, *t*-butyl AQ, TEB, deshydroxy-4'-fluoro-5'-chlorophenyl-AQ and CYC resulted in a concentration-dependent increase in cell death at 100μM or below, as assessed by trypan blue bye exclusion, but none was significantly toxic below 30μM. Cell death could not be assessed for deshydroxy-4'-fluoro-TEB because of a marked decrease in cell recovery. With MNL, the pattern of cytotoxicity was similar to that observed with PMN (fig. 2.15; table 2.1).

2.3.7 Inhibition of Neutrophil Function

2.3.7.1 Luminol-Enhanced Chemiluminescence

The respiratory burst of PMA-stimulated PMN can be measured using luminol-enhanced chemiluminescence. Preincubation of the cells with AQ (1-100μM) for 30min prior to PMA-stimulation caused a concentration-dependent depression of the PMN response (fig. 2.16). Labro and Babin-Chevaye (1988) have previously demonstrated that AQ inhibits the PMN respiratory burst, but only at concentrations of 100μg/ml. In this study, the depressive effect was also observed with CQ at concentrations of 100μM, while TEB severely inhibited the PMN response. Chemiluminescence was completely abolished at a concentration of 10μM (table 2.2).

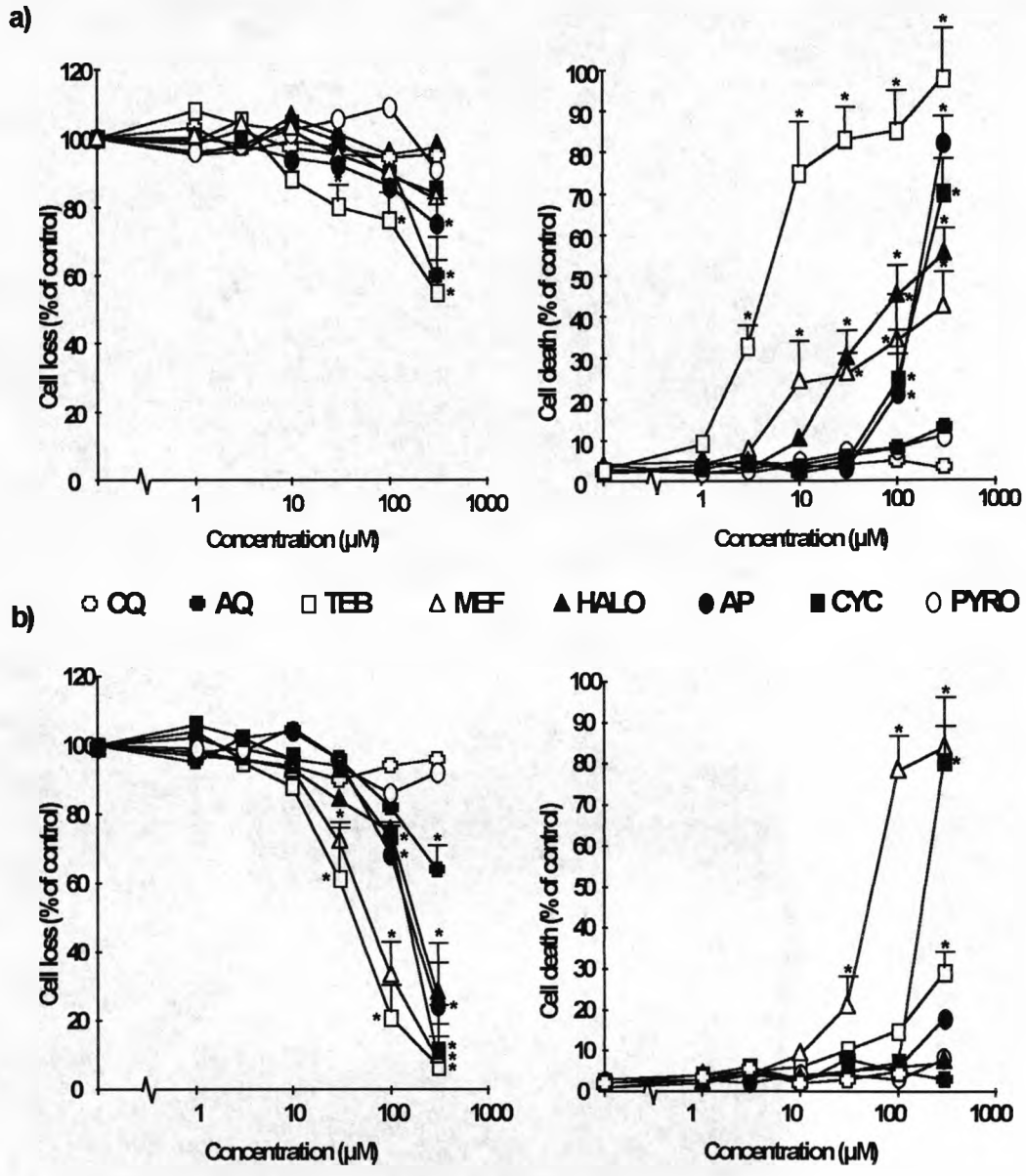


Figure 2.15 Cytotoxicity in a) MNL and b) PMN (1×10^6) by antimalarial drugs and compounds proposed for clinical use (1-300μM). Cytotoxicity was assessed by measuring cell recovery (cell loss) and trypan blue dye exclusion (cell death). The results represent the mean ± S.D. of triplicate incubations. Error bars have been omitted for some of the compounds for the sake of clarity. Statistical analysis was performed by comparing cell loss and cell death at different concentrations of compound with that of solvent alone (*$P < 0.05$).

Drug	Cytotoxicity (IC_{50}[a])			
	MNL		PMN	
	Cell loss	Cell death	Cell loss	Cell death
	μM		μM	
p-aminophenol	>300	>300	>300	>300
paracetamol	>300	>300	>300	>300
7-chloro 4-aminoquinoline	>300	>300	148.6 ±4.6	>300
AQ (-side chain)	>300	>300	>300	>300
desethyl AQ	>300	>300	244.3 ± 4.3	>300
bis desethyl AQ	>300	>300	>300	>300
5'-chlorophenyl AQ	>300	224.5 ±23.8	179.8 ±31.7	>300
t-butyl AQ	>300	221 ± 9.4	88.0 ± 3.5	122.9 ± 6.1
4'-fluoro AQ	>300	>300	159.3 ±22.4	>300
4'-fluoro-5-chlorophenyl AQ	186.4 ±14.1	102.6 ±18.4	26.2 ± 2.4	75.3 ± 3.5
4'-fluoro TEB	7.2 ± 1.6	4.4 ± 0.8	3.0 ± 0.3	n.a.[b]
bis *t*-butyl AQ	>300	144 ± 11.8	>300	>300
bis pyroquine	>300	>300	>300	>300

Table 2.1 Effect of analogues, disconnection products and metabolites of AQ on PMN and MNL toxicity.
[a] IC_{50} represents the concentration of test compound required to cause 50% cell loss / death, taking control values as 100%. Results are expressed as IC_{50} values ± S.E.M., calculated using the four parameter logistic method (Grafit program; Erithacus Software).
[b] not assessed because of the marked decrease in cell recovery.

2.3.7.2 Nitroblue Tetrazolium Slide Test

Neutrophil function was significantly inhibited by all the antimalarial drugs and compounds proposed for clinical use ($P < 0.05$), with the exception of CQ (fig. 2.17). However, only 5'-chlorophenyl AQ, TEB, deshydroxy-4'-fluoro-5'-chlorophenyl AQ and deshydroxy-4'-fluoro-TEB inhibited function at therapeutic concentrations (fig. 2.17; table 2.3). IC_{50} (concentration required to inhibit formazan formation by 50%) values are shown in table 2.3.

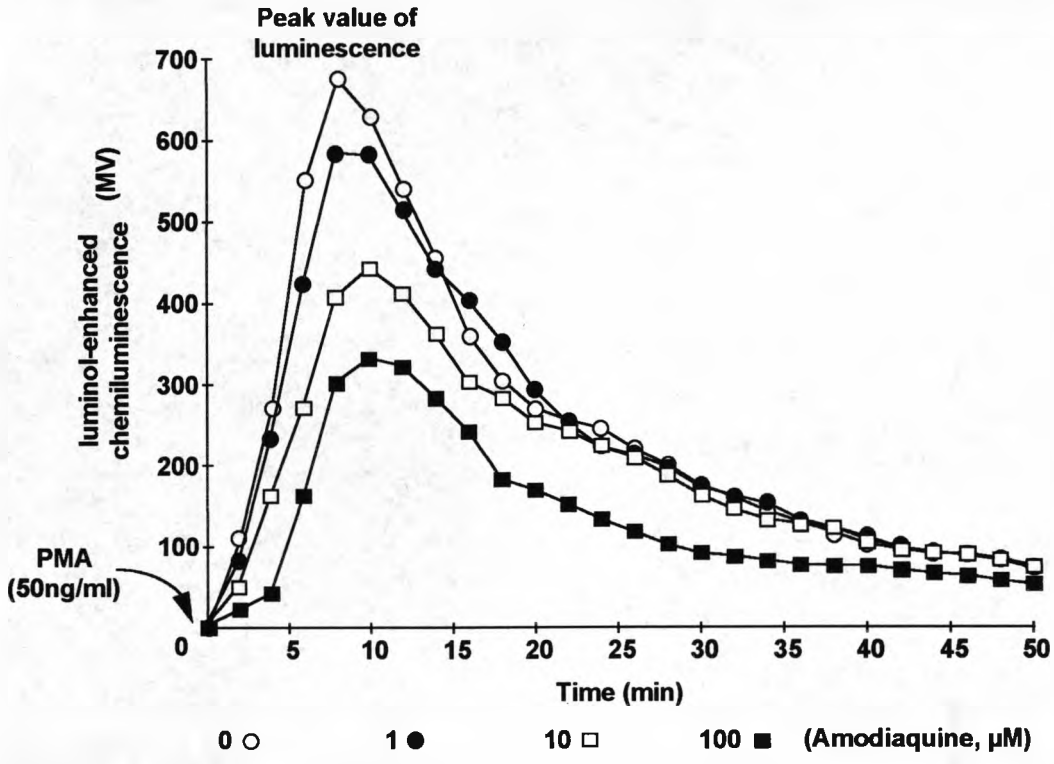


Figure 2.16 Effect of AQ (1-100μM) on luminol-enhanced chemiluminescence of PMA-stimulated PMN (0.5 x 10^6). The results represent one of three separate experiments.

	% of control PMN response[a]		
Concentration (μM)	CQ	AQ	TEB
1	96.8 ± 5.4	94.5 ± 8.2	59.3 ± 11.2*
10	95.0 ± 3.2	74.2 ± 10.5*	1.4 ± 0.5*
100	76.5 ±11.8*	45.5 ± 4.8*	0.3 ± 0.1*

Table 2.2 Effect of antimalarial drugs on luminol-enhanced chemiluminescence of PMA-stimulated PMN.
[a] Compounds were preincubated with cells (30min) prior to the addition of PMA. Results represent the mean ± S.D. of three separate experiments using cells from different individuals. Statistical analysis was performed by comparing the ability of different concentrations of compound to inhibit chemiluminescence with that of solvent alone (*P < 0.05).

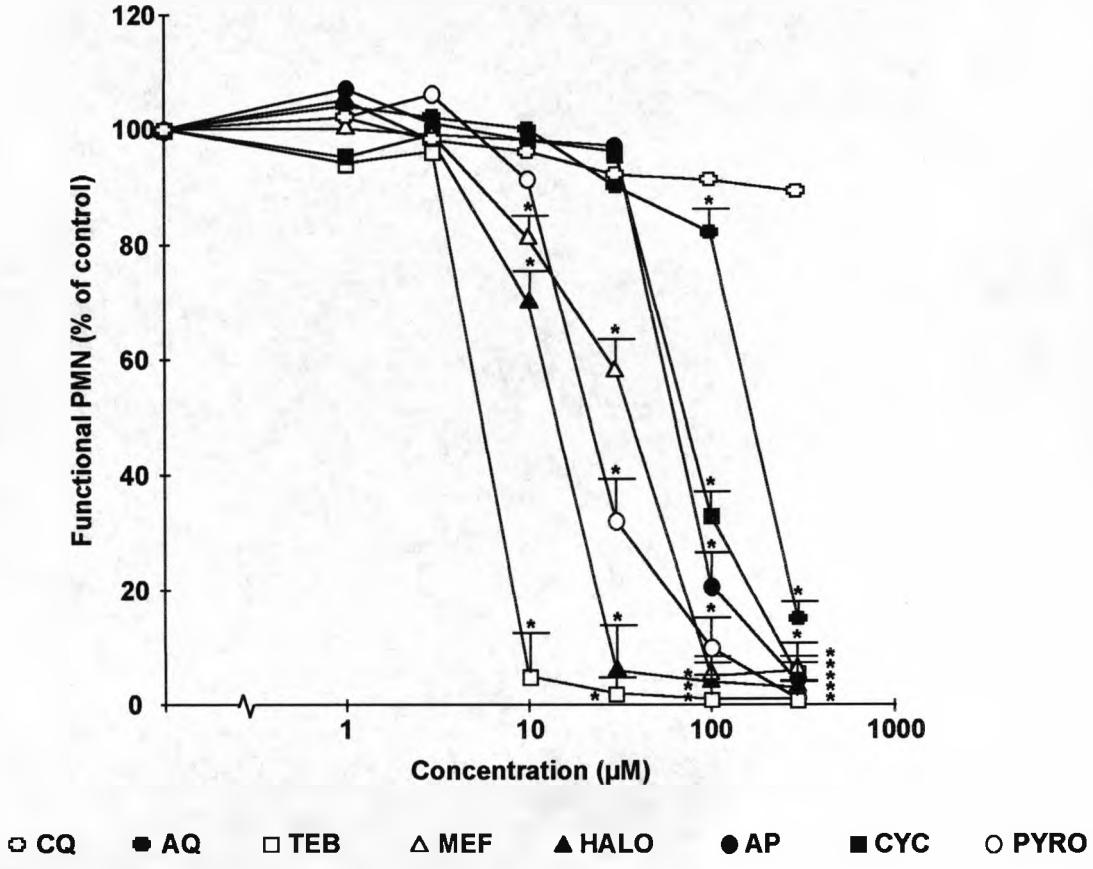


Figure 2.17 Inhibition of PMN (1 x10^6/ml) oxidative metabolism in PMA stimulated cells, by antimalarial compounds (1-300μM). The PMN respiratory burst was measured using the NBT slide test. The results represent the mean ± S.D. of triplicate incubations. Error bars have been omitted for some of the data points for the sake of clarity. Statistical analysis was performed by comparing the different concentrations of compounds required to inhibit the respiratory burst when compared with that of solvent alone (*P < 0.05).

2.3.7.3 Inhibition of Eugenol-Induced Glutathione Depletion

Certain antimalarial compounds have been shown to inhibit neutrophil function (Labro and Babin-Chevaye, 1988). Depletion of GSH by eugenol in PMN depends on their activation by PMA.

	Inhibition of Neutrophil Function		
	Inhibition of Eugenol-Induced GSH depletion[a]		NBT Assay[b]
Drug	10μM	100μM	
	%		μM
desethyl AQ	1.8 ± 1.9	4.2 ± 2.2	135.4 ± 12.1
bis desethyl AQ	3.6 ± 0.5	9.4 ± 2.1	126.0 ± 11.5
5'-chlorophenyl AQ	0 ± 3.0	5.8 ± 1.3	6.8 ± 1.5
t-butyl AQ	3.3 ± 3.9	22.8 ±4.0	48.9 ± 4.3
4'-fluoro AQ	1.6 ± 0.3	26.8 ± 3.6	286.8 ± 10.5
4'-fluoro-5-chlorophenyl AQ	49.7 ± 5.8	70.9 ± 3.1	4.9 ± 0.9
4'-fluoro TEB	55.2 ± 5.2	68.4 ± 6.1	1.5 ± 0.1
bis *t*-butyl AQ	4.5 ± 2.1	23.5 ± 4.8	108.1 ± 5.5
bis pyroquine	0 ± 3.4	29.3 ± 2.0	46.4 ± 6.2

Table 2.3 Effect of metabolites and analogues of AQ on PMN function.
[a] Compounds were incubated (10 and 100μM) in the presence of the model toxin eugenol (1-300μM), which has previously been shown to deplete intracellular GSH. Results are expressed as mean ± S.D.
[b] IC_{50} represents the concentration of test compound required to inhibit by 50% formazan formation, taking control values as 100%. Results are expressed as IC_{50} values ± S.E.M., calculated using the four parameter logistic method (Grafit program, Erithacus Software).

To determine whether the compounds tested would also affect eugenol-induced GSH depletion by inhibiting PMN function, the test compounds were coincubated with eugenol (1-300μM) in the presence of PMA-stimulated PMN. Mefloquine, halofantrine, TEB, deshydroxy-4'-fluoro-5'-chlorophenyl AQ and deshydroxy-4'-fluoro TEB caused significant ($P < 0.05$) inhibition of eugenol-induced GSH depletion at 10μM, whereas *t*-butyl AQ, deshydroxy-4'-fluoro AQ, CYC, bis-*t*-butyl AQ, bis-pyroquine and PYRO inhibited eugenol-induced GSH depletion at 100μM (fig. 2.18; table 2.3). There was no inhibition of eugenol-induced GSH depletion with CQ, AQ, 5'-chlorophenyl AQ, amopyroquine, AQ (-

side chain), *p*-aminophenol, paracetamol, 5-aminosalicylic acid, 7-chloro 4-aminoquinoline, desethyl AQ or bis desethyl AQ.

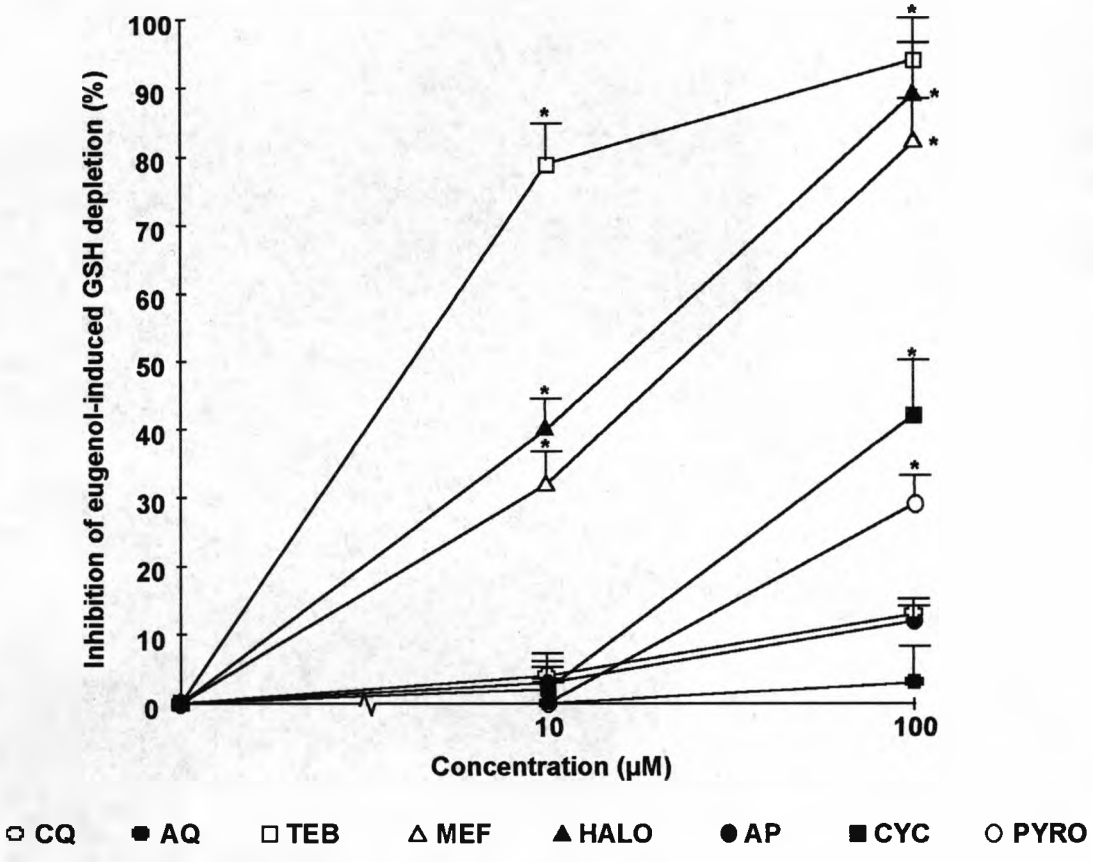


Figure 2.18 Inhibition of eugenol-induced GSH depletion in PMA-stimulated PMN (1 x 10^6) by antimalarial compounds. GSH levels were measured using bromobimane, by fluorescence HPLC. The results represent the mean ± S.D. of triplicate incubations. Statistical analysis was performed by comparing the ability of different compounds (10μM and 100μM) to inhibit eugenol (300μM) induced GSH depletion with that of solvent alone (*P < 0.05).

2.4 Discussion

Despite its clinical effectiveness against CQ-resistant malaria, AQ was withdrawn from general use because of reports of severe and often fatal agranulocytosis and hepatotoxicity after prophylactic administration (Hatton *et*

al., 1986; Neftel *et al.*, 1986). Although mechanisms have still to be elucidated, the involvement of the reactive metabolite of AQ is thought to be central. This chapter explores two aspects of AQ disposition in human neutrophils that may contribute to the cell-selective nature of the toxicity, i.e., lysosomal accumulation and bioactivation by the MPO system.

Radiometric analysis showed that AQ is taken up more avidly by lymphocytes and neutrophils than red blood cells from either buffer or plasma (fig. 2.8). This is in accordance with results from other groups showing that under physiological conditions, AQ and its major plasma metabolite, desethyl AQ, reside in the white cell fraction rather than red blood cell (Laurent *et al.*, 1993). It also corresponds to results seen with CQ, which reaches its highest concentrations within white cells and platelets (Bergqvist and Domeij-Nyberg, 1983). Indeed, after oral administration, the whole blood / plasma concentration ratio of desethyl AQ was found to be 3:1 (Winstanley *et al.*, 1987). This reflects the amphiphilic nature of AQ which, like CQ (Klempner and Styrt, 1983), accumulates within the lysosomes, resulting in a rise in lysosomal pH, as detected by the displacement of the fluorescent probe ACMA (figs. 2.10 and 2.11). At physiological pH, 98% of AQ exists in the monoprotonated form; in this form, its lipophilic quinoline ring allows it to pass through the lipid bilayer into the cell, where it accumulates in the acidic lysosome. It exists as a di-cation at lysosomal pH (pH 5-6; Styrt and Klempner, 1985), with protonation occurring on the side chain and quinoline nitrogens. In the diprotonated form, it is unable to pass back through the lipid layer and thus becomes trapped (section 1.3.2). Accordingly,

removal of the basic diethylamino side chain reduced lysosomal trapping and thus accumulation. However, the enhanced accumulation of AQ, compared with CQ, cannot be explained solely by the weak base properties of the drugs. If this was the case, CQ, which is more basic than AQ, would undergo greater lysosomal accumulation. A recent report relating drug accumulation to antimalarial potency has suggested that other physico-chemical parameters may be important, particularly the additional phenol ring in AQ, which increases lipophilicity (Hawley *et al.*, 1996a).

Accumulation of AQ and CQ in neutrophils does not lead to a decrease in cell viability or cell function at therapeutic concentrations. This agrees with the results of Labro and Babin-Chevaye (1988) who also observed that AQ did not alter cellular functions, including phagocytosis and chemotaxis. Mefloquine, in contrast, decreased cell viability and affected cellular function, despite accumulating to a similar extent to that of AQ and CQ. This suggests that factors other than cellular accumulation are involved in the pathogenesis of AQ-induced agranulocytosis.

In marked contrast to the other established antimalarial drugs (which have not been associated with such a high frequency of agranulocytosis), in particular the structurally related compound CQ, AQ depleted intracellular GSH in activated neutrophils (IC_{50} = 18μM) (fig. 2.14). This is a consequence of the ability of the MPO in neutrophils to biactivate (section 1.5.3) AQ to a chemically reactive quinoneimine metabolite (Tingle *et al.*, 1995). This metabolite binds covalently to protein or can be detoxified by GSH conjugation via a Michael

addition reaction (Maggs *et al.*, 1988) (fig. 2.19). The same metabolite is also generated by cytochrome P450 enzymes in the liver (Harrison *et al.*, 1992; Jewell *et al.*, 1995). Addition of AQ quinoneimine itself to nonactivated neutrophils resulted in total GSH depletion at much lower concentrations (2μM). Of the two stable hepatic metabolites, i.e., desethyl AQ and bis desethyl AQ, only the former produced significant depletion of GSH below 100μM in activated neutrophils. This confirms *in vivo* data, where only the GSH conjugates of AQ and desethyl AQ were identified in the bile of mice administered AQ (Ruscoe *et al.*, 1995).

Structure disconnection (fig. 2.14) of the AQ molecule showed that the *p*-aminophenol group was necessary for GSH depletion. This explains why CQ and other established antimalarial drugs do not deplete GSH, but suggests that other drugs that contain this function may have adverse consequences. The importance of the 4'-hydroxyl group in this regard is confirmed by the fact that biosteric substitution with fluorine (deshydroxy-4'-fluoro AQ) can block hepatic and neutrophil bioactivation (Ruscoe *et al.*, 1995; Tingle *et al.*, 1995) and, in this study, the depletion of GSH. Importantly, this structural alteration did not cause loss of pharmacological activity (O'Neill *et al.*, 1994). Interestingly, slight chemical modification by blocking the amine group of *p*-aminophenol, as found in paracetamol reduced the degree of GSH depletion. Furthermore, bioactivation was also blocked by the addition of a carboxylic acid group ortho to the hydroxyl group as in 5-amino salicylic acid. An explanation for this may be that 5-amino salicylic acid does not accumulate in the acid media of the lysosomes. Alternatively, the formation of intramolecular hydrogen bonds and hence an

increased stability of the molecule may lead to a reduction in quinoneimine formation.

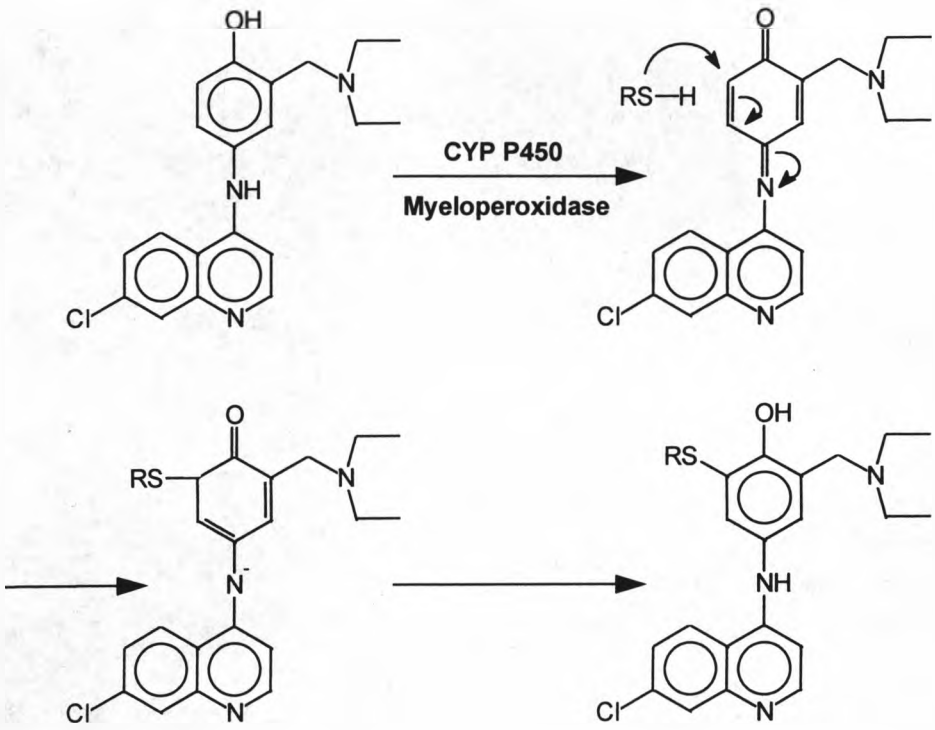


Figure 2.19 Michael-addition of a protein or glutathionyl sulphydryl group to AQ.

AQ quinoneimine is directly toxic to neutrophils, but only at concentrations higher than those that might be expected *in vivo*. The time course of these reactions, the rapid recurrence of the toxicity upon readministration of AQ (1-3 days) (Larry *et al.*, 1986) and lack of dose dependency (Hatton *et al.*, 1986) all suggest that an indirect, immune-mediated hypersensitivity mechanism may be responsible, rather than direct toxicity to peripheral neutrophils or bone marrow stem cells. Previous studies have shown that AQ quinoneimine is highly

immunogenic in animal models, causing specific IgG and T cell responses, whereas AQ was only weakly immunogenic (Clark *et al.*, 1990; Hough *et al.*, 1996). Furthermore, a recent study in our laboratory (Ruscoe, 1997) demonstrated cell-surface haptenation of AQ quinoneimine, using an *in vitro* neutrophil system. These data are in accordance with other investigators and they provide strong evidence that a type II hypersensitivity reaction is responsible for AQ-induced agranulocytosis (Larrey *et al.*, 1986; Rouveix *et al.*, 1989). Intracellular GSH depletion by AQ was metabolism- and concentration-dependent; how does this relate to the occurrence and nature of the toxicity? Clinical studies have shown that agranulocytosis mostly occurred in patients who were given AQ prophylactically rather than for acute treatment (Hatton *et al.*, 1986), suggesting that the total dose of drug received may be important in the occurrence of the toxicity. Thus, it can be hypothesised that large doses of AQ, by depleting intracellular GSH may reduce the detoxication capacity of the neutrophil, allowing the reactive quinoneimine metabolite to act as a hapten and initiate an immune response.

It is clear that lysosomal accumulation and bioactivation are two undesirable features of 4-aminoquinoline antimalarial agents that should be eliminated or at least minimised, provided that this does not lead to a loss of pharmacological activity. With these considerations in mind, other antimalarial agents were investigated to determine how chemical modification of AQ would affect the parameters used in this study. The two side-chain nitrogens separated by a four-carbon bridge, along with the 7-chloro group, are necessary for

antimalarial activity (Werbel *et al.*, 1986). All compounds studied thus retained these features. *t*-Butyl AQ and amopyroquine are simple *N*-alkyl variants of AQ, with greater *in vitro* antimalarial activity (Hawley *et al.*, 1996b). They were found to accumulate to a greater extent than AQ, without causing significant inhibition of neutrophil function or direct cytotoxicity at concentrations expected to be reached *in vivo*. Both compounds depleted GSH to a similar extent to that of AQ (fig. 2.13). Amopyroquine is used in the treatment of acute malaria in Western Africa, but thus far there have been no reports of idiosyncratic toxicity. However, our results do tend to suggest that amopyroquine has the potential to cause toxicity similar to that produced by AQ if given chronically (*i.e.*, for prophylaxis).

Introduction of a 5'-aryl group into AQ produced a series of compounds that showed increased potency and thus appeared to be a promising new group of therapeutic agents (Werbel *et al.*, 1986). However, the development of one such compound, TEB, was halted because of neutrophil toxicity in animals, including rats and monkeys, which was characterised histologically by foamy macrophages (L.M. Werbel and H. Chung, Walter Reed Army Institute of Research, personal communication). Interestingly, TEB did not deplete intracellular GSH, despite containing a *p*-aminophenol function. There are two possible reasons for this; first, AQ undergoes bioactivation in the 5'-position, which is chemically blocked in TEB. Secondly, TEB showed enhanced lysosomotropism, leading to inhibition of neutrophil function and direct cytotoxicity. These effects may offer an explanation for the *in vivo* toxicity of TEB. It is notable that the deshydroxy-4'-fluoro analogue of TEB, a more basic and lipophilic compound, was more

lysosomotropic and cytotoxic to neutrophils *in vitro*. Importantly, this compound shows reduced *in vitro* pharmacological activity (O'Neill *et al.*, 1996; Hawley *et al.*, 1996a), thus illustrating that parasite toxicity and host cell toxicity are not inextricably linked.

The enhanced cytotoxicity of analogues of AQ with different substituents in the 3'-, 4'- and 5'- positions (table 2.1) is difficult to explain. There was no relationship between cell death and the degree of GSH depletion. Indeed, it seems the converse of what has been observed for anti-cancer agents such as cisplatin where an increase in intracellular GSH leads to a decrease in cytotoxicity (Godwin *et al.*, 1992). The analogues of AQ were more potent at inhibiting the respiratory burst of neutrophils, which in part may be a result of their enhanced lipophilicity (Hawley *et al.*, 1996a) and hence lysosomotropism. The inhibition of neutrophil function may also have been exaggerated by the increase in cytotoxicity observed with these compounds. However, this is unlikely to be the whole explanation, since with drugs such as deshydroxy-4'-fluoro-5'-chlorophenyl AQ, TEB and deshydroxy-4'-fluoro TEB, inhibition of neutrophil function was observed at concentrations lower than those which caused cytotoxicity.

Bis-mannich derivatives, which contain a hydrophilic rather than a lipophilic substituent in the 5'- position have been shown to be as effective as AQ against *Plasmodium vinkei vinkei in vivo* (Barlin and Tan, 1985; Peters and Robinson, 1992). Furthermore, four new bis-mannich compounds were found to possess greater and more prolonged antimalarial activity than AQ or CQ against

multi-drug resistant strains of *Plasmodium falciparum* (Kotecka *et al.*, 1997). None of the three bis-mannich compounds studied, had any effect on neutrophil function or viability. Additionally, they did not deplete GSH, and exhibited lower lysosomal accumulation than AQ.

Finally, PYRO, a highly active new antimalarial agent (Basco and Le Bras, 1992) that has been developed in China and is currently undergoing clinical trials was investigated (Chen *et al.*, 1992; Ringwald *et al.*, 1996). To date, little toxicity has been observed (Shao, 1990). This compound contains a *p*-aminophenol function, with pyrolidinomethyl substituents at the 3'- and 5'-positions (fig. 2.1). Additionally, the 7-chloroquinoline nucleus has been replaced with a more lipophilic aza-acridine group. Although PYRO accumulated within lysosomes, the accumulation was not significantly greater than that observed with other antimalarial drugs, and the substituted 5'-position of the drug meant that it did not deplete GSH. No significant cytotoxicity was seen, and at *in vivo* concentrations there was no inhibition of neutrophil function.

In conclusion, this data demonstrates that consideration of the cellular disposition of 4-aminoquinolines can provide an understanding of the neutrophil toxicity which has been reported for this group of compounds. Of particular importance to drug design seems to be the avoidance of bioactivation and excess lysosomotropism. In this respect, bis-mannich derivatives appear to have an advantage over mono-mannichs, and the data suggests that PYRO is less likely than amopyroquine to cause life-threatening agranulocytosis that has been seen

with AQ. Fig. 2.20 illustrates the structural requirements of a safe and effective 4-aminoquinoline antimalarial drug.

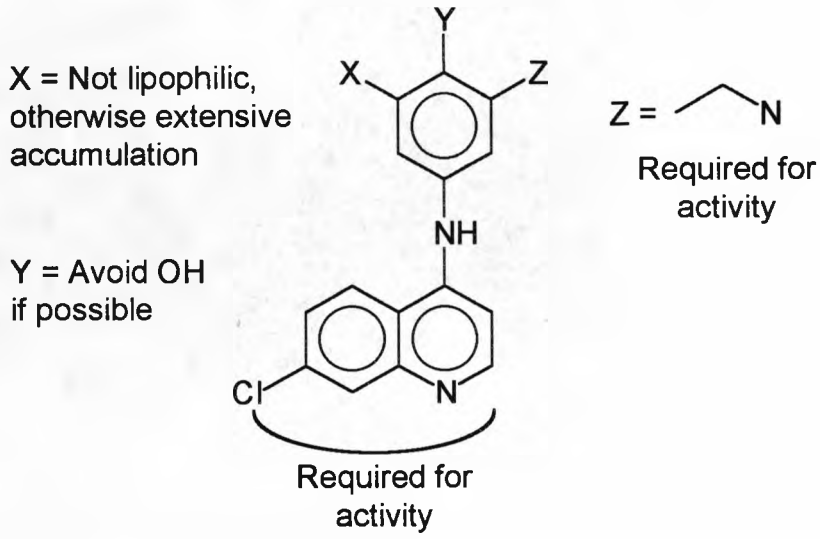


Figure 2.20 Scheme illustrating the structural requirements of novel 4-aminoquinoline antimalarial drugs.

Chapter 3:

Disposition and Toxicity of Sulphamethoxazole in Human Peripheral Blood

Contents **Page**

3.1 Introduction

Sulphonamides are the oldest antimicrobial agents in current clinical use. Since their introduction in the 1930's they have been associated with a variety of unpredictable idiosyncratic drug reactions, including fever, lymphadenopathy, skin rashes, hepatitis, nephritis and blood dyscrasias in about 2-3% of patients. In the UK, this has resulted in restricted indications for drugs such as co-trimoxazole, a combination of sulphamethoxazole (SMX) and trimethoprim (Mandell and Sande, 1985).

Recently, co-trimoxazole has been widely used for the treatment of *Pneumocystis carinii* pneumonia in HIV-infected patients. In comparative studies with other agents such as dapsone and pentamidine, co-trimoxazole has been found to be the most efficacious both for acute treatment and for prophylaxis (Smith, 1994). However, the rate of adverse reactions is significantly higher than in seronegative patients severely limiting its usefulness. For example, hypersensitivity reactions have been reported to occur in 30-80% of patients in different studies (Carr and Cooper, 1995; Koopmans *et al.*, 1995; Pirmohamed and Park, 1995; Tshachler *et al.*, 1996). The types of reactions are similar to those observed in HIV-negative individuals and occur within 5-15 days of the start of therapy, although, in general, they are more severe. Virus-induced changes in drug metabolism and drug detoxification, immune dysregulation, drug-drug interactions and drug dosage have all been postulated to be responsible for the increased risk of hypersensitivity in HIV-infected patients (Carr and Cooper, 1995; Pirmohamed and Park, 1995). However further research is required to

identify the exact mechanism(s) in order to develop methodology to prevent the reactions and improve the tolerability of co-trimoxazole in these patients.

SMX is metabolised not only to stable metabolites, but also to the protein reactive and cytotoxic hydroxylamine metabolite (SMX-NHOH) (Rieder *et al.*, 1988; Riley *et al.*, 1991; Carr, *et al.*, 1993; Rieder *et al.*, 1995a), which is further oxidised to a nitroso metabolite (SMX-NO; section 1.7) (Rieder *et al.*, 1995b). Hepatic metabolism is considered the major source of SMX *N*-oxidation (hydroxylation) (Cribb and Spielberg, 1990 and 1992). However, extra-hepatic metabolism by the NADPH oxidase / myeloperoxidase (MPO) system may be important in the organ specific toxicity of many compounds (Uetrecht, 1992). In particular, SMX and other aromatic amines, including dapsone and procainamide are metabolised to their corresponding hydroxylamines by MPO or peripheral neutrophils (Uetrecht, 1985; Uetrecht *et al.*, 1988; Cribb *et al.*, 1990). Formation of the hydroxylamine requires a two electron oxidation, which occurs through a one electron intermediate. Identification of SMX-NO has proved difficult *in vivo* and *in vitro*. Nevertheless, cell-free experiments have shown autooxidation of SMX-NHOH to occur through a nitroxide radical. Parallel oxidation and reduction reactions eventually lead to an equilibrium between SMX-NHOH and SMX-NO (Cribb *et al.*, 1991) (fig. 3.1).

It has been proposed that SMX-NO is responsible for the idiosyncratic toxicity associated with SMX therapy. Tissue injury may occur via a direct or immune-mediated mechanism (Riley *et al.*, 1988; Rieder *et al.*, 1988 & 1995b; Meekins *et al.*, 1994; Daftarian *et al.*, 1995). In an *in vivo* rat model, both SMX-

NHOH and SMX-NO underwent extensive reduction which serves as a detoxification mechanism (Gill *et al.*, 1997), indicating that measurement of urinary concentrations of SMX-NHOH, estimated to be about 2% of an ingested dose in man (Gill *et al.*, 1996), may underestimate the total level of tissue exposure to the toxic metabolites. Therefore, an imbalance between oxidation of SMX and the reduction of its toxic metabolites may predispose to toxicity.

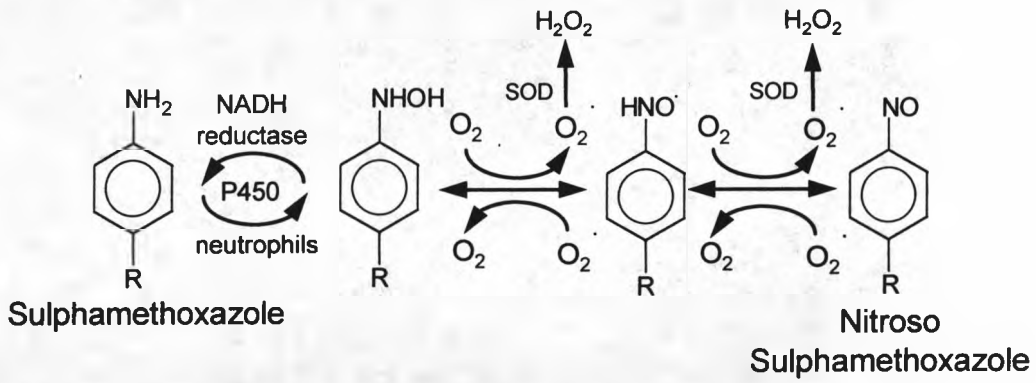


Figure 3.1 Scheme of the proposed reactions involved in bioactivation (oxidation) of SMX to SMX-NHOH and SMX-NO through an intermediate nitroxide radical (SOD; superoxide dismutase) (Cribb *et al.*, 1991).

Glutathione (GSH) (γ-L-glutamyl-L-cysteinyl-glycine) and *N*-acetyl cysteine (CYS) decrease covalent binding and cytotoxicity of synthetic hydroxylamines. GSH is the most abundant intracellular thiol, while CYS is found almost exclusively in the plasma (Mills and Lang, 1996) (section 1.5.5). These two thiols can protect cells from nitrosoaromatics and aryl hydroxylamines via either a conjugation or reduction reaction. Studies with nitronitrosobenzenes and the model compound 1-thioglycerol have shown nucleophilic attack of the thiol on the nitroso-nitrogen forms an unstable semi-mercaptal intermediate (Klehr *et*

al., 1985; Ellis *et al.*, 1992). Once formed, the semi-mercaptal undergoes either reduction, thiolytic addition or molecular rearrangement (Eyer, 1979; Dolle *et al.*, 1980; Ellis *et al.*, 1992) (fig. 3.2).

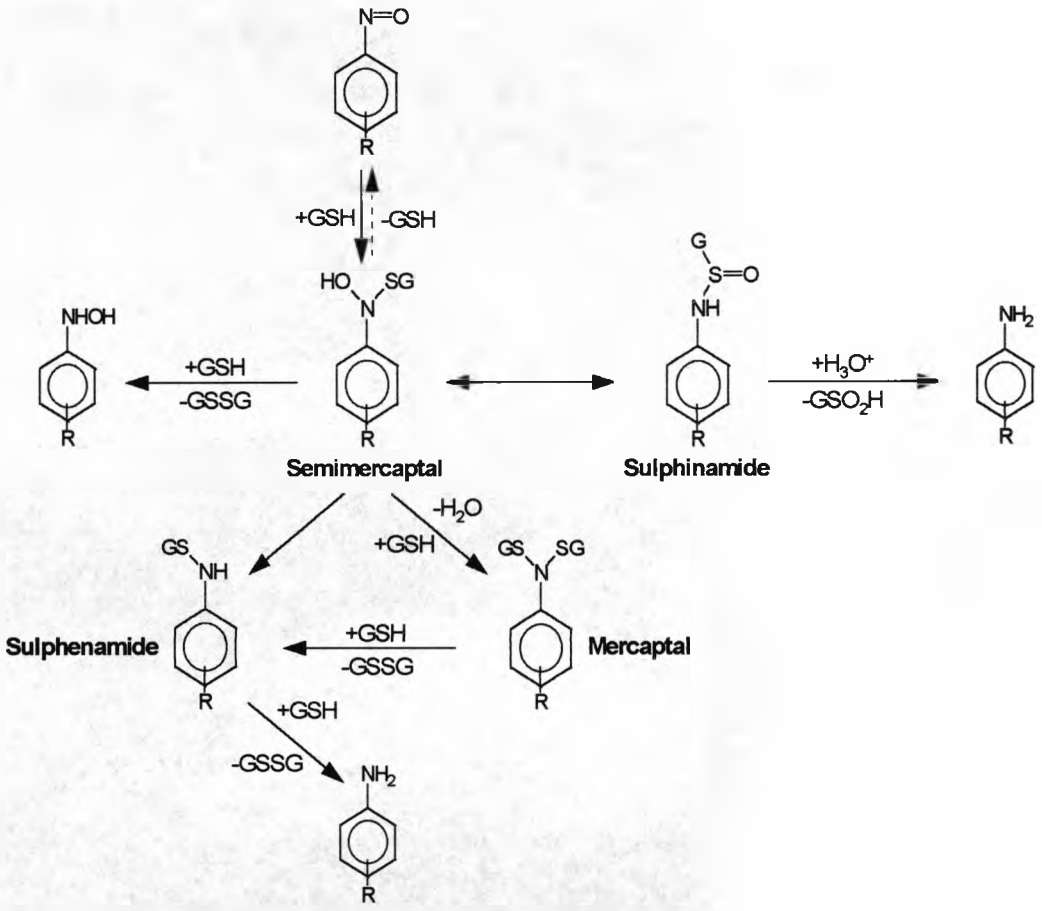


Figure 3.2 Scheme of the proposed reactions involved in the reduction of nitrosoaromatics by GSH (Ellis *et al.*, 1992).

In order to further characterise the role of human tissues in maintaining the balance between oxidation and reduction, the studies in this chapter are related to the disposition and toxicity of SMX *in vitro*. In particular, the synthesis, and the chemical reactivity of SMX-NHOH and SMX-NO towards biological nucleophiles were investigated.

3.2 Materials and Methods

3.2.1 Chemicals

3-Amino-5-methylisoxazole, bromobimane, CYS, d_6-dimethyl sulphoxide (DMSO), dapsone, glacial acetic acid, GSH, human serum albumin (HSA), *N*-ethylmorpholine, 4-nitrobenzenesulphonyl chloride, palladium / carbon catalyst (5%; commercial grade), phorbol 12-myristate 13-acetate (PMA), pyridine, sodium phosphinite, SMX, tetrahydrofuran, trichloroacetic acid, triethylamine and trypan blue were obtained from Sigma Chemical Co. (Poole, UK). Monopoly® resolving medium (Ficcoll Hypaque, 1.114g/ml) and Lymphoprep® (1.077g/ml) were from ICN Biomedicals (Bucks., UK) and Nycomed (Birmingham, UK) respectively. All HPLC-grade solvents were purchased from Fischer Scientific (Loughborough, UK).

3.2.2 Chemical Characterisation

Proton NMR spectra were obtained using a Bruker (200Mhz) NMR spectrometer. Deuterated solvents are indicated in the text and tetramethylsilane was used as an internal standard. Mass spectra were recorded at 70eV using a VG7070E mass spectrometer. The samples were introduced using a direct insertion probe.

The compounds were analysed by HPLC with UV detection. Eluent was injected onto a Spherisorb 5 ODS 2 column (25cm x 4.6mm; HPLC Technology, Macclesfield, UK) and analysed with a mobile phase of distilled water, acetonitrile, glacial acetic acid and triethylamine in a ratio of 80:20:1:0.1 (v/v), with a flow rate of 1.2ml/min using a SP8800 pump (Spectra-Physics, San Jose,

USA). UV absorbance was monitored at 254nm through a SP100 wavelength detector (Spectra-Physics) and peak areas were calculated using a Spectra-Physics Chromojet integrator. Elemental analysis was carried out by the University of Liverpool micro-analysis laboratory.

3.2.3 Synthesis of Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

A modification of the method described by Johnstone *et al.* (1978) was utilised to synthesise SMX-NHOH and SMX-NO (fig. 3.3). Initially, nitro SMX was prepared by mixing 4-nitrobenzenesulphonyl chloride (9g; 41mmole) with 3-amino-5-methylisoxazole (4.0g; 41mmole) in pyridine (15ml) at 0^0C. The reaction mixture was left overnight at room temperature, and then precipitated in 300ml of millipore water yielding a muddy brown precipitate. The product was filtered and recrystallized using the minimum amount of an ethyl acetate : toluene mixture (1:3, v/v). The mixture was left overnight at 0^0C and the crystals were then collected on filter paper. The overall yield was 57%.

Reduction of aromatic nitro compounds to amines proceeds through two intermediate stages involving nitroso compounds and hydroxylamines. Johnstone *et al.* (1978) developed a general method for stopping the reduction at the hydroxylamine stage. This was achieved using sodium phosphinite as a hydrogen donor and a two phase solvent system (tetrahydrofuran / water), with a palladium / carbon catalyst. SMX-NHOH was also prepared using this method. 5% Palladium / carbon catalyst (0.3g) was added to a stirred solution containing sodium phosphinite (2.7g; 30mmole) and nitro SMX (3g; 10mmole) in water

(25ml) / tetrahydrofuran (300ml). The reaction mixture was monitored by thin layer chromatography (TLC) until nearly all the starting material had reacted. Ether extraction (2 x 400ml) produced a crude product in 85% yield. This was then recrystallized from chloroform (500ml).

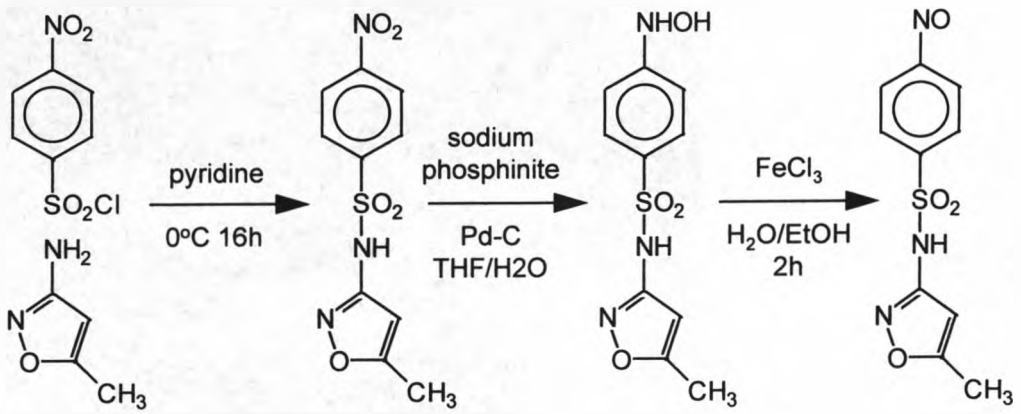


Figure 3.3 Synthesis of SMX-NHOH and SMX-NO.

Initial attempts at synthesising SMX-NO using iron (III) chloride in water proved unsuccessful. Consideration of known syntheses revealed that compounds with electron releasing groups in the *para*-position to the hydroxylamine functionality produced yields in excess of 80%, whereas electron-withdrawing groups generally gave reduced yields. Therefore, the reaction time was increased and progress monitored closely by TLC. SMX-NHOH (1g; 4mmole) in ethanol (150ml) was added to a stirred solution of iron (III) chloride (4.5g; 28mmole) in water (200ml) over a period of 10min. The reaction reached completion two hours later and after diethyl ether extraction (3 x 200ml) and recrystallization from chloroform (500ml), the overall yield was 80%.

3.2.4 Isolation of Human Peripheral Blood Cells

Polymorphonuclear leucocytes (PMN; neutrophils) and mononuclear leucocytes (MNL; lymphocytes / monocytes) were isolated from the venous blood of 20 healthy male volunteers (age range, 21-50 years) on a dual density gradient of Monopoly resolving medium and Lymphoprep, as described in section 2.2.2. Cells were resuspended in Dulbeccos phosphate buffered saline (Dulbeccos PBS; pH 7.4) and diluted to the concentration required for each experiment. Both cell types were greater than 98% pure and 95% viable as assessed by Wright's stain and trypan blue dye exclusion (section 2.3.1). Isolated red blood cells (RBC) were washed twice and resuspended in Dulbeccos PBS to produce a 50% haematocrit. When required, cells were lysed with a sonic probe (Heat Systems, Farmingdale, N.Y, USA; 0°C, 3 x 20sec).

Platelet-free plasma was isolated from ice cold heparinised venous blood by centrifugation at 0°C (1200*g*, 10min). All samples were kept on ice, and the experiments were initiated within 25min of venepuncture.

3.2.5 Reaction of Sulphamethoxazole, Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole with Biological Nucleophiles

To determine the relative importance of intracellular and extracellular thiols in the detoxification of the toxic metabolites of SMX, the reactions of CYS (the most abundant plasma thiol [Eck *et al.*, 1989]) and GSH (an intracellular thiol [Larsson *et al.*, 1983]) were investigated. SMX (50μM) or its metabolites (SMX-NHOH and SMX-NO; 50μM) were incubated with thiols (CYS and GSH;

100μM) in Dulbeccos PBS (1ml) at 37^{0}C for 0, 0.25, 1 or 24h. Aliquots (25μl) of the reaction mixture were injected at each time point onto a Spherisorb 5 ODS 2 column (25cm x 4.6mm) and separated with the mobile phase described previously (section 3.2.2). Chromatography was performed with an isocratic flow at a rate of 1.2ml/min, using two Jasco PU 980 pumps (Jasco Corporation, Tokyo, Japan). Eluent was passed through a UV absorbance detector (254nm; Jasco UV-975), a Valco tee-union steam splitter (Phase Separations, Deeside, Clwyd, UK), and a fused silica capillary (75μM id, 150cm length) to the electrospray probe and interface of a Quattro II tandem quadrapole mass spectrometer (Fisons Biotech MS, Manchester, UK). Nebulizing and drying gas (nitrogen) was delivered at 12 l/h and 280 l/h, respectively. The interface temperature was 60^{0}C, the capillary voltage 4 x 10^{3}V, and the cone voltage 30-80V. Spectra were acquired between m/z 100-850 over a scan duration of 4.91sec. Data were processed via MassLynx 2.1 software. SMX and SMX-NHOH were identified from authentic standards, whereas any reaction intermediates were identified from their parent molecular ion peak (protonated molecule, [M+1]) and retention times quoted previously (Cribb *et al.*, 1991).

Two major difficulties were encountered with the use of LC-MS. Firstly, the concentration of SMX-NO throughout the reaction could not be measured because it only produces a very weak protonated molecule; and second, absolute quantification of the amounts of products formed is not possible. To overcome these problems, the reactions of SMX, SMX-NHOH and SMX-NO (10mM) with CYS and GSH (30mM) in d_6-DMSO were analysed by proton NMR over a

period of 96h. The amount of each intermediate formed was quantified by measurement of the integral peak height from each spectrum throughout the reaction period. SMX, SMX-NHOH, SMX-NO, nitro SMX, CYS, CYSSYC, GSH and GSSG peaks were identified from analysis of standard compounds, thus allowing the analysis of SMX-thiol conjugates by examination of their chemical shifts and previous studies involving the reaction of GSH and substituted nitroso benzenes (Ellis *et al.*, 1992). The assignment of integrals corresponding to the sulphinamide conjugate was difficult because the peaks had similar chemical shifts to GSH and GSSG. In order to assign the signals from the sulphinamide product, an NMR was run on a mixture of SMX-NO (10mM) and mercaptoethanol (30mM). The spectra were easily interpreted as mercaptoethanol has no protons between 7.5 and 8.5ppm.

The reactions of SMX, SMX-NHOH and SMX-NO with CYS and GSH were also determined by fluorescence HPLC. The compounds (1-100μM) were incubated with either CYS or GSH (3μM) in Dulbeccos PBS (1ml) at 37°C for 20min. Free thiol levels were measured as described previously, using the fluorescent probe bromobimane (section 2.2.7; Cotgreave and Moldeus, 1986). The reaction between SMX-NO (30μM) and a mixture of CYS (10μM) and GSH (10μM) in Dulbeccos PBS was analysed by a similar method. Incubations were carried out at 37°C and thiol levels were measured at intervals between 0-60min.

3.2.6 Determination of Thiol Depletion from Plasma and Peripheral White Blood Cells

Intact and lysed peripheral white blood cells (MNL and PMN; 0.5 x 10^6

cells / incubation) were incubated (final volume 1ml) in Dulbeccos PBS at 37°C for 1h with SMX, SMX-NHOH or SMX-NO (1-400μM). The experiments with PMN were performed both in the presence and absence of PMA (10ng/ml), a cell activating factor (Thompson *et al.*, 1989). Protein was precipitated by the addition of 100% trichloroacetic acid (10μl) and thiol levels were measured using bromobimane (section 2.2.7). Plasma thiol depletion was analysed by a similar method except that 1ml of ice-cold plasma was added to each incubation instead of the cells.

The basal levels of the thiols were measured prior to the incubation and in the absence of any of the compounds. Additionally, thiol stability after isolation of the plasma and cells was assessed between 0-5h. The results are presented as the mean of three separate incubations conducted in triplicate. Standard GSH and CYS curves were constructed between 0-400μM.

3.2.7 Determination of the Toxicity of Sulphamethoxazole, Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

3.2.7.1 Trypan Blue Dye Exclusion

PMN and MNL were incubated with either SMX, SMX-NHOH or SMX-NO (1-300μM) in Dulbeccos PBS (1ml, pH 7.4) at 37°C for 1h. Cell viability was then assessed by trypan blue dye exclusion as described previously (section 2.2.4) by taking an aliquot of the cells. In some experiments, the remainder of the cells were washed, resuspended in 1ml of drug-free Dulbeccos PBS containing HSA

(5mg/ml) and gentamycin (0.4mg/ml), and then left at 37^0C for a further 16h, after which cytotoxicity was assessed again.

3.2.7.2 Methaemoglobinemia

RBC toxicity was assessed by measuring the amount of methaemoglobin formed when RBC (50% haematocrit) were incubated with SMX-NHOH (3-100μM) and SMX-NO (30μM). After 1h, PBS containing 0.05% Triton X-100 (pH 7.8; 2ml) was added to the RBC. 1ml of the haemolysed solution was transferred to a tube containing a 50% solution of $K_3Fe(CN)_6$ (20μl). Both sets of tubes (in the presence and absence of $K_3Fe(CN)_6$ were measured spectrophotometrically at 635nm to determine methaemoglobin formation. Subsequently, a 10% solution of KCN (20μl) was added to both sets of tubes, and once again they were measured spectrophotometrically at 635nm. $K_3Fe(CN)_6$ binds to the haemoglobin in the red blood cells whereas the KCN binds to the methaemoglobin. This provides a measure of % methaemoglobin using the following equation.

$$\textbf{\% Meth.} = \frac{OD_1 - OD_2}{OD_3 - OD_4}$$

OD_1, optical density - KCN and $K_3Fe(CN)_6$

OD_2, optical density + KCN

OD_3, optical density + $K_3Fe(CN)_6$

OD_4, optical density + KCN and $K_3Fe(CN)_6$

3.2.8 Determination of Inhibition of Neutrophil Function by Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

3.2.8.1 Inhibition of Eugenol-Induced Glutathione Depletion

To determine whether SMX, SMX-NHOH or SMX-NO inhibited the depletion of intracellular GSH in PMN, the effect of co-incubating the test compounds (10 or 100μM) with eugenol (300μM) was assessed. The same protocol was used as described in section 2.2.9.3.

3.2.8.2 Nitroblue Tetrazolium Slide Test

The ability of SMX, SMX-NHOH and SMX-NO (1-300μM) to inhibit the neutrophil oxidative burst was determined using the nitroblue tetrazolium slide test (Meerof and Roos, 1986). The same protocol was used as described in section 2.2.9.2. 200 cells were counted in each incubation. The percentage of blue-stained cells in the control incubations (>95%) were taken as the baseline.

3.2.9 Determination of the Reduction of Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole by Peripheral Blood Cells and Plasma

Isolated PMN, MNL (5×10^6) and plasma (1ml) were incubated with SMX-NHOH and SMX-NO (30μM) in a shaking water bath at 37°C in Dulbeccos PBS (pH 7.4). The PMN incubations were carried out in the presence or absence of PMA (10ng/ml). The reaction was initiated by the addition of the compounds. After 1h, the reaction was terminated by placing the tubes on ice.

The internal standard dapsone (5µl of 1mM solution) was added. In some experiments, PMN were lysed by repeated freeze thawing. Extraction was performed with ethyl acetate (2 x 3ml). The combined extracts were evaporated to dryness under nitrogen, reconstituted in mobile phase (100µl) and analysed by HPLC using the same system as described earlier (section 3.2.2).

Incubations containing RBC (0.5ml, 50% haematocrit) and SMX-NHOH (30µM) or SMX-NO (30µM) were carried out as above and terminated after 1h by placing the tubes on ice. The internal standard (dapsone, 5µl of 1mM solution) was added, and cells were lysed by the addition of 0.5ml distilled water. Following extraction with ethyl acetate (2 x 3ml), the samples were reconstituted in mobile phase (100µl) and analysed by HPLC.

SMX-NHOH and SMX-NO were also incubated for 1h in Dulbeccos PBS, ethyl acetate and mobile phase (37°C), and analysed by HPLC to measure any spontaneous reduction. All incubations were performed in triplicate from four individuals (age range 22-40 years).

3.2.10 Statistical Analysis

The results are presented as the mean ± S.D., with the exception of the IC_{50} values where they are given as IC_{50} ± S.E.M. The IC_{50} values were calculated using the four-parameter logistic method (Grafit program; Erithacus Software, Staines, UK) by interpolation of the logarithmic concentration curve. The S.E.M. was calculated from the error associated with the sigmoidal fit of the

Statistical analysis was performed using the Mann-Whitney test and results were considered to be significant when the P value was less than 0.05.

3.3 Results

3.3.1 Characterisation of Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

SMX-NHOH was obtained as a white solid. The purified product was >99% pure by HPLC. ^{1}H-NMR (d^6-DMSO, 200MHz) δ 9.08 (s,1H, NHOH), 8.73 (s, 1H, NHOH), 7.61 (d, 2H, J=8.80 Hz, Ar-H), 6.85 (d, 2H, J-8.80 Hz, Ar-H), 6.11 (s, 1H, Ar-H), 2.33 (s, 3H, CH_3). Analysis calculated for $C_{10}H_{11}N_3O_4S$: C, 44.61; H, 4.12; N, 15.61. Found C, 44.55; H, 4.11; N, 15.63. FABMS m/z 270 (MH^+), HRMS calculated for above, 269.04703; found 269.04732. The melting point was 148.5 (literature, 149; Rieder *et al.*, 1987).

SMX-NO was obtained as a yellow / orange solid. ^{1}H NMR (d^6-DMSO, 200MHz) δ 8.19-8.04 (m, 4H, Ar-H), 6.18 (s, 1H, Ar-H), 2.31 (s, 3H, CH_3). Analysis calculated for $C_{10}H_9N_3O_4S$: C, 44.94; H, 3.39; N, 15.73. Found C, 45.20; H, 3.42; N, 15.75. FABMS m/z 268 (MH^+), HRMS calculated for above, 267.03138; found 267.03167. The melting point was 269 (decomposed).

3.3.2 Reaction of Sulphamethoxazole, Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole with Biological Nucleophiles

Fig. 3.4 shows the reaction of SMX-NO (50μM) with GSH (100μM) at 37°C analysed by LC-MS (0-24h; in Dulbeccos PBS). The initial product formed was the semi-mercaptal; it then rearranged to the sulphinamide, SMX-NHOH and

SMX itself. Products of reduction, SMX-NHOH and SMX, and products of thiol addition were detected as parent molecular ions. The concentration of semi-mercaptal declined with time and coincided with increased formation of SMX and sulphinamide. With addition of excess GSH, formation of sulphinamide metabolite was reduced and reduction to SMX occurred at a greater rate. SMX was the only product formed when SMX-NHOH and GSH were incubated in buffer and analysed by LC-MS.

Fig. 3.5 shows the ratio of products formed during the reaction of SMX-NO (10mM) with GSH (30mM) at 37°C when analysed by NMR spectroscopy (0-96h; in d_6-DMSO). Similar products to those described above were obtained in the initial conjugate-rearrangement reaction. However, no reduction of SMX-NHOH was observed because of its enhanced stability in d_6-DMSO. No reaction was obtained when either SMX-NHOH or SMX were incubated with GSH.

The reaction of SMX, SMX-NHOH and SMX-NO with mercaptoethanol produced similar results. The spectra were easily analysed as mercaptoethanol has no protons between 7.5 and 8.5ppm on the NMR spectrum.

During the reaction of SMX-NO (50μM) with CYS (100μM) at 37°C (0-24h; in buffer, LC-MS), the initial product was SMX-NHOH. However after 24h, greater than 95% of this product underwent further reduction to SMX (fig. 3.6). The semi-mercaptal and sulphinamide conjugates formed during the reaction between SMX-NO and GSH were not identified. The results were similar when the reaction was analysed by NMR (0-96h; 37°C; in d_6-DMSO). SMX-NO was completely reduced to SMX-NHOH within 4h without any conjugate formation. Fig. 3.7 shows the ratio of SMX-NHOH formed and an example of a NMR trace

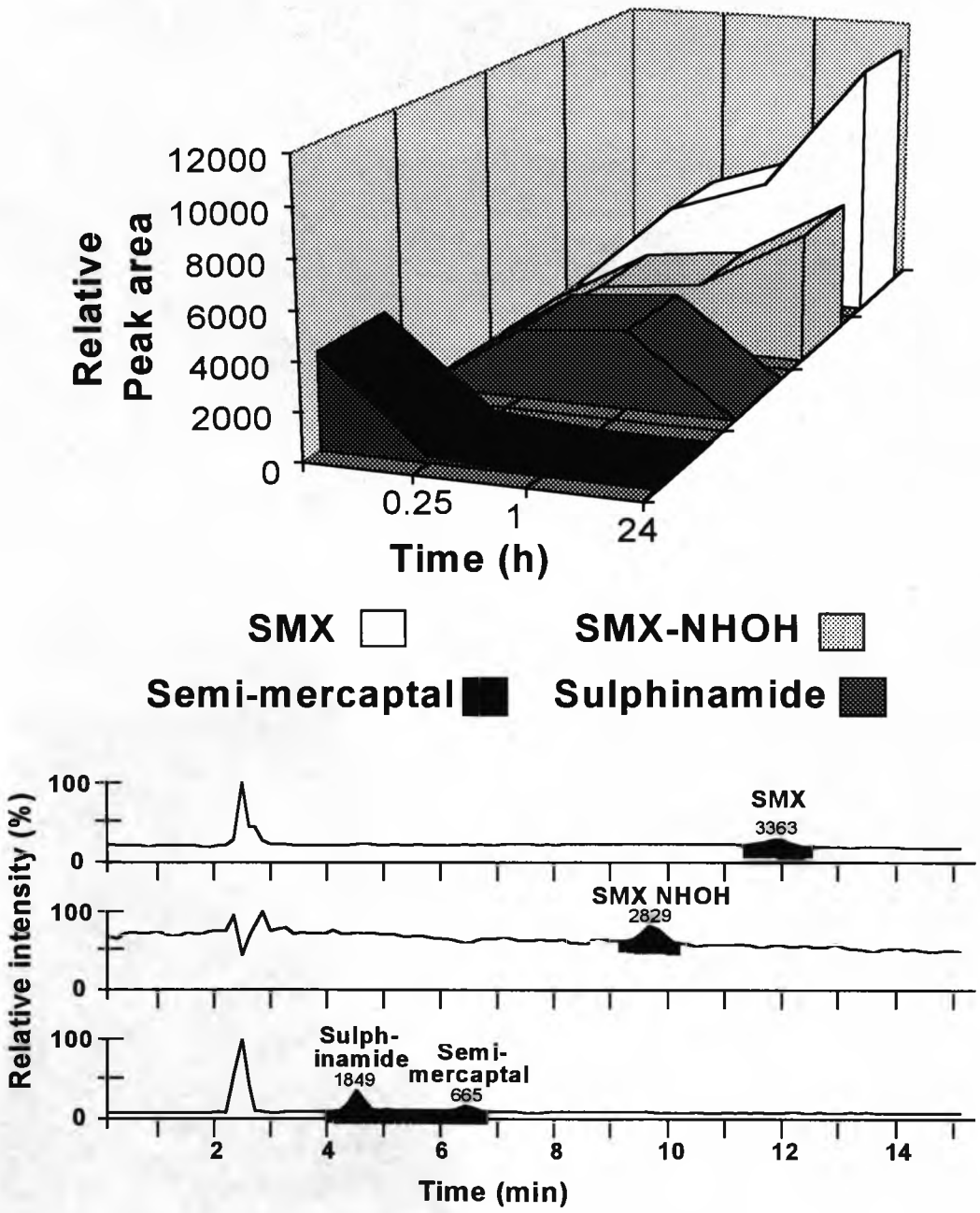


Figure 3.4 Ratio of product formation / disappearance and a molecular ion spectrum showing the retention times of the intermediates in the reaction of SMX-NO (50μM) with GSH (100μM) under aqueous conditions, analysed by LC-MS. SMX and SMX-NHOH were identified from authentic standards, whereas any reaction intermediates were identified from their parent molecular ion peak and retention times quoted previously (Cribb *et al.*, 1991).

obtained during the reaction period. SMX was the only product detected when SMX-NHOH was incubated with CYS in buffer. The reduction of SMX-NHOH by CYS or GSH may proceed via the nitroso derivative as suggested previously (Cribb *et al.*, 1991).

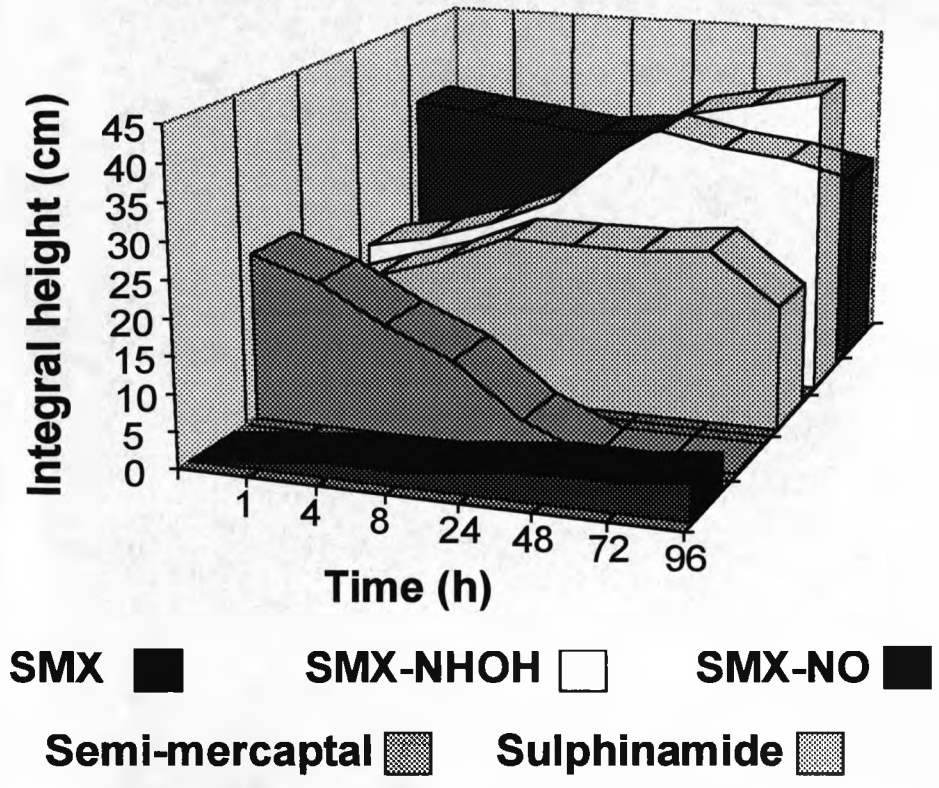
Identical conditions were used to analyse the reaction of *N*-acetyl lysine with SMX-NHOH and SMX-NO. After a period of 96h, NMR analysis revealed no reaction in either case (fig. 3.8). Following this, we studied whether aniline would react with either SMX-NHOH or SMX-NO. Even after heating and under deuterated acid catalysis, no products were observed.

SMX-NHOH and SMX-NO (1-100μM) both caused a concentration-dependent depletion of CYS (3μM) and GSH (3μM) when analysed by fluorescent HPLC (fig. 3.9). Both metabolites were equipotent at depleting the thiols, with greater than 80% depletion being observed at concentrations of 10μM and above. SMX did not deplete either CYS or GSH.

The reaction between SMX-NO (30μM) and CYS (10μM) was rapid, resulting in 92.4 ± 3.2% depletion within 1min. The reaction with GSH was significantly slower than with CYS ($P < 0.05$), with 66.6 ± 8.4% depletion being observed after 1min. Complete depletion of CYS was seen after 10min, while a significant amount of GSH remained for a further 20min (fig. 3.10).

3.3.3 Thiol Depletion from Human Plasma and White Blood Cells

The basal intracellular GSH concentration measured in 20 individuals (3-6μM / 10^6 cells) was similar to that reported previously (Cotgreave and Moldeus,



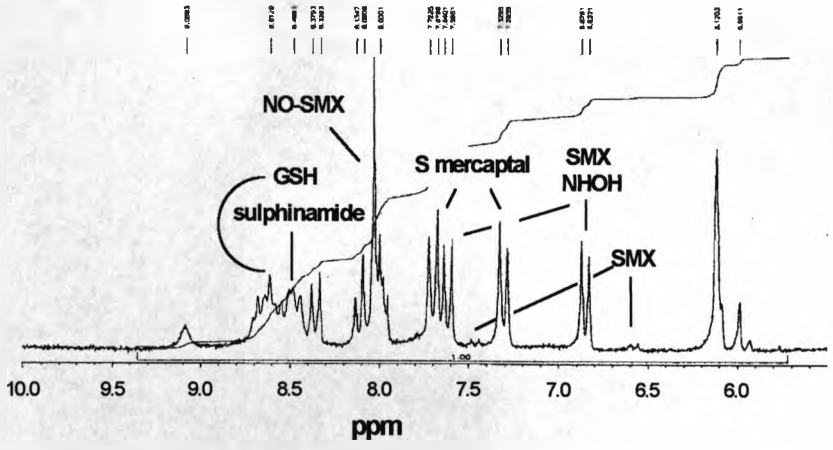


Figure 3.5 Ratio of product formation / disappearance and an example of a spectrum obtained in the reaction of SMX-NO (10mM) with GSH (30mM) in DMSO, analysed by NMR. SMX, SMX-NHOH, SMX-NO and GSH were identified from NMR analysis of standard compounds, thus allowing analysis of the semi-mercaptal and sulphinamide metabolites by examining their chemical shifts and from previous studies involving the reaction of GSH with substituted nitroso benzenes (Ellis *et al.*, 1992). The spectrum corresponds to time 1h.

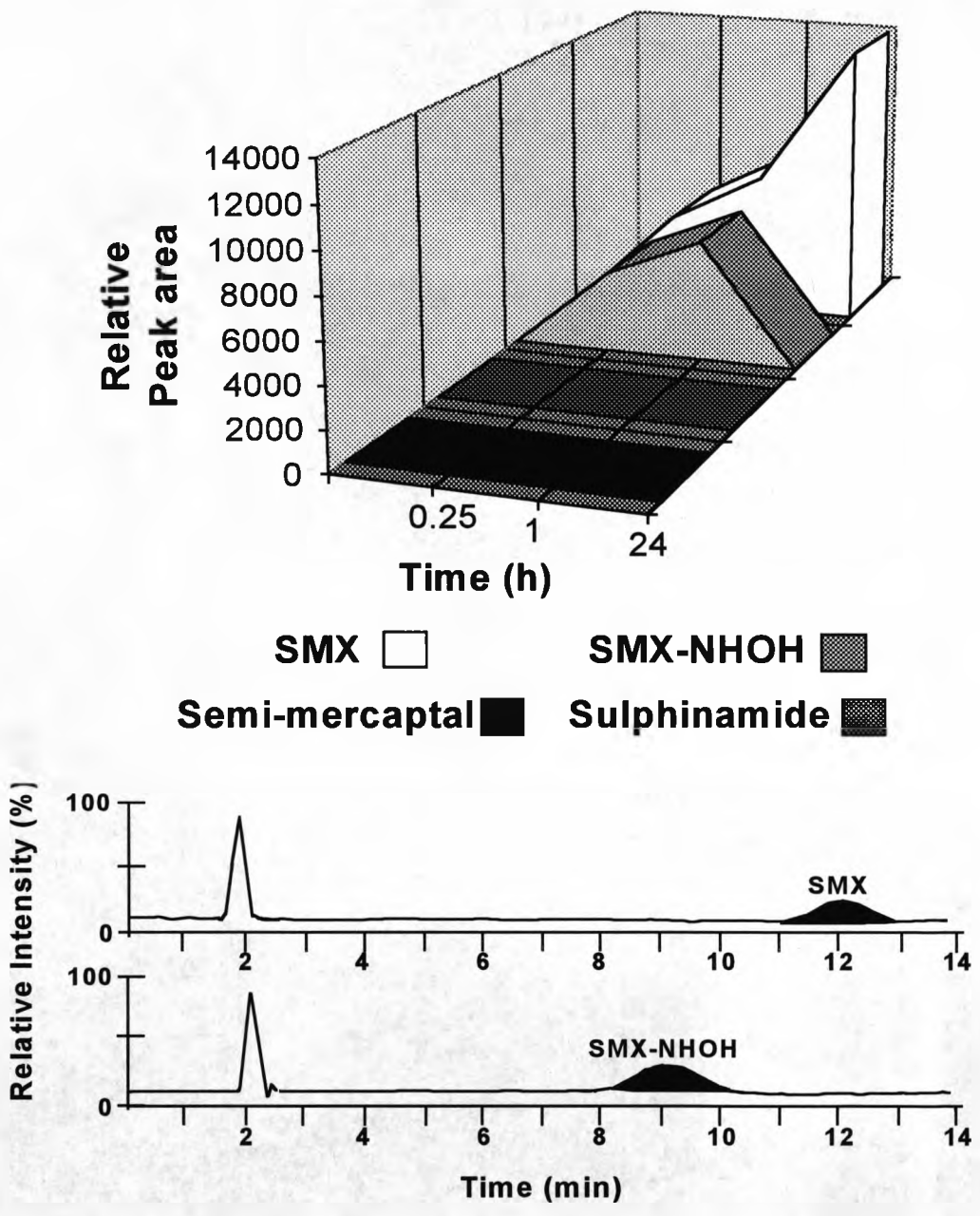


Figure 3.6 Ratio of product formation / disappearance and a molecular ion spectrum showing the retention times of the intermediates in the reaction of SMX-NO (50μM) with CYS (100μM) under aqueous conditions, analysed by LC-MS. SMX and SMX-NHOH were identified from authentic standards, whereas any reaction intermediates were identified from their parent molecular ion peak and retention times quoted previously (Cribb *et al.*, 1991).

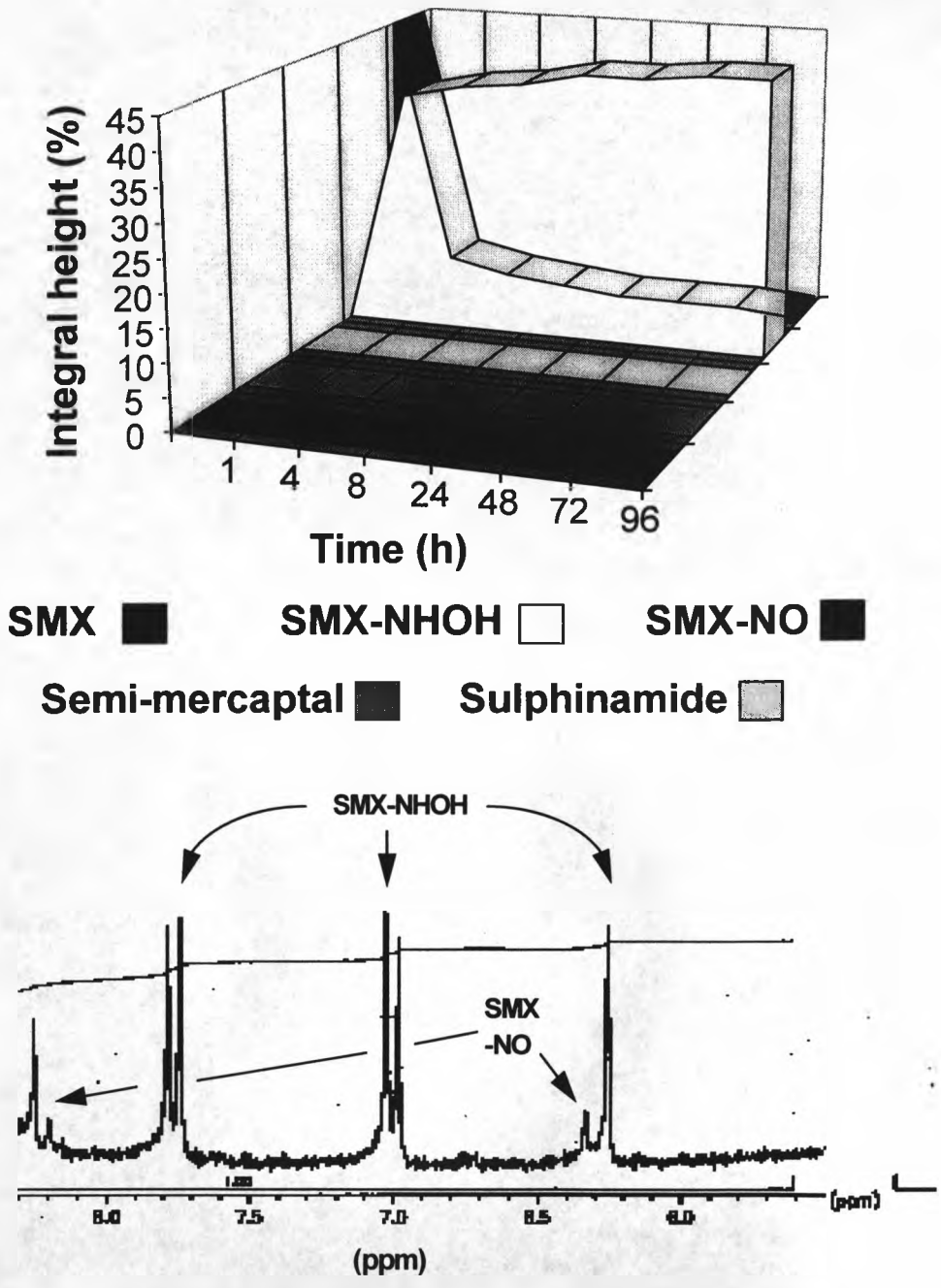


Figure 3.7 Ratio of product formation / disappearance and an example of a spectrum obtained in the reaction of SMX-NO (10mM) with CYS (30mM) in DMSO, analysed by NMR. SMX, SMX-NHOH, SMX-NO and GSH were identified from NMR analysis of standard compounds, thus allowing analysis of the semi-mercaptal and sulphinamide metabolites by examining their chemical shifts and from previous studies involving the reaction of GSH with substituted nitroso benzenes (Ellis *et al.*, 1992). The spectrum corresponds to time 1h.

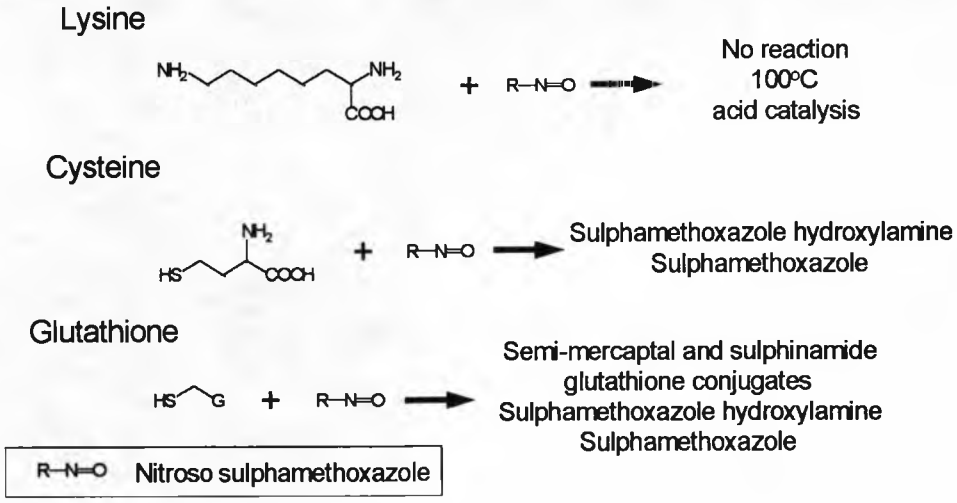


Figure 3.8 Scheme depicting the reaction of SMX-NO with various biological nucleophiles.

1986; Pirmohamed *et al.*, 1996). CYS was present almost exclusively in plasma, with control values (7-15μM, measured in 10 individuals) again being similar to those reported previously (Mills and Lang, 1996). In control incubations there was no significant decreases in either the CYS or GSH levels for the duration of the experiment (1h), although 2.5h after cell isolation, thiol levels were reduced by 24-37%.

There was no significant depletion of GSH in both PMA-stimulated and unstimulated PMN with either SMX-NHOH or SMX-NO up to a concentration of 400μM. In contrast, with MNL, both SMX-NHOH and SMX-NO depleted GSH ($P < 0.05$) at concentrations of 10μM or greater. GSH depletion with SMX-NO (400μM; 39.3 ± 4.2% of the control value) was significantly ($P < 0.05$) greater than with an equivalent concentration of SMX-NHOH (51.1 ± 2.8%). GSH depletion was significantly greater with lysed PMN than with intact cells ($P < 0.05$). The concentration of SMX-NHOH and SMX-NO causing 50% depletion

(IC_{50}) being 11.1 ± 1.4μM and 2.9 ± 0.6μM, respectively, and greater than 90% depletion being observed at 30μM (fig. 3.11).

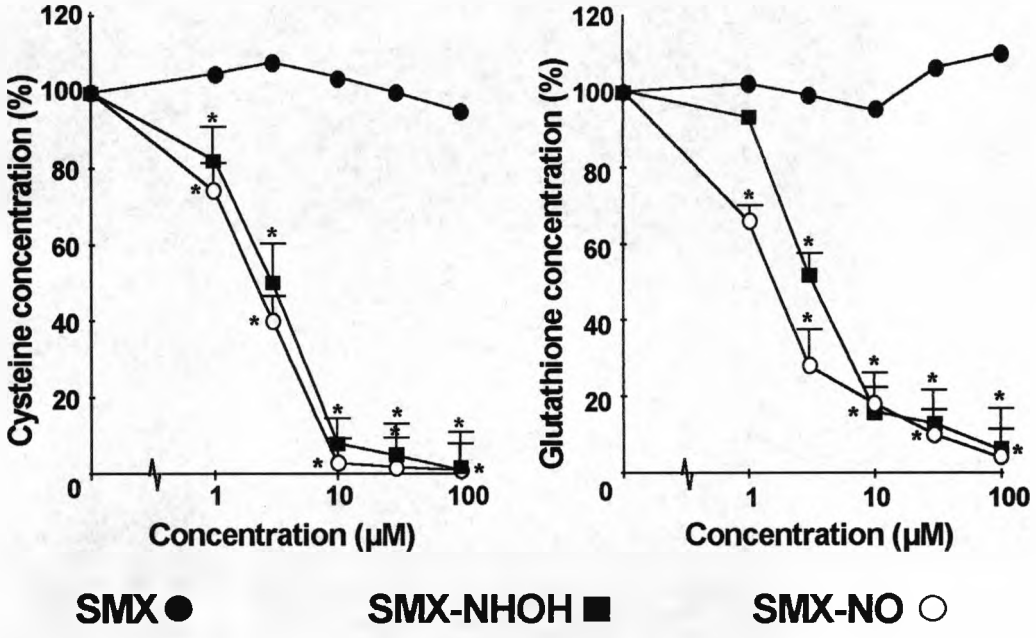


Figure 3.9 Thiol depletion after incubation of free CYS or GSH (3μM) with SMX and its metabolites. The sulphydryl concentration, expressed as the percentage of thiol depletion in the absence of drug, was measured by fluorescent HPLC using bromobimane. The results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing the depletion of thiols in the absence and presence of SMX or its metabolites (*P < 0.05). Error bars have been omitted for clarity when there was no depletion.

In plasma, both SMX-NHOH and SMX-NO caused significant ($P < 0.05$) depletion of CYS at concentrations above 10μM. At the maximal concentration (400μM) tested, SMX-NHOH and SMX-NO caused 82.3 ± 4.7% and 90.3 ± 3.1%, respectively, when compared to the control values.

SMX did not deplete either GSH or CYS in intact cells, lysed cells or plasma.

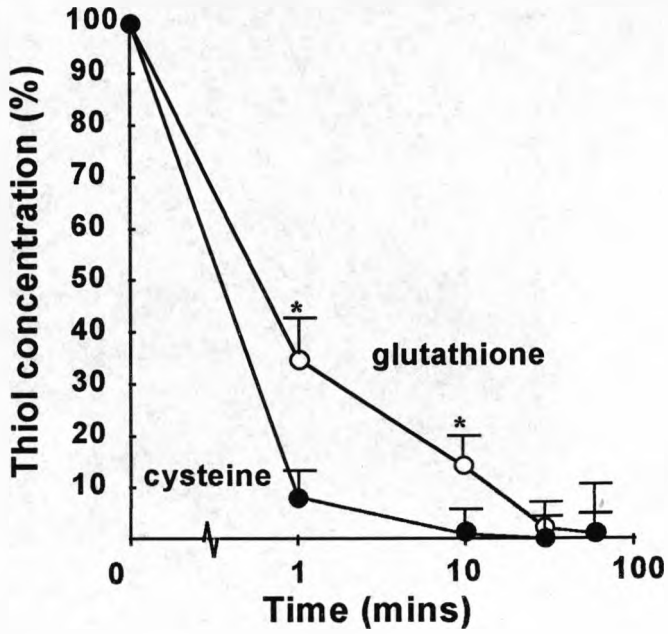


Figure 3.10 Comparison of the depletion of free CYS and GSH (10μM) during incubation with SMX-NO (30μM). The sulphydryl concentration, expressed as percentage of thiol depletion in the absence of drug, was measured by fluorescent HPLC, using bromobimane. The results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing the depletion of cysteinyl or glutathionyl sulphydryl groups at each time point (*P < 0.05).

3.3.4 Toxicity of Sulphamethoxazole, Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

3.3.4.1 Trypan Blue Dye Exclusion

The cytotoxicity of the hydroxylamine and nitroso metabolites of SMX were assessed in both MNL and PMN by determination of cell death as described in chapter 2. There was a significant ($P < 0.05$) increase in cell death (25.2 ± 3.2%) above background values when SMX-NO (300μM) was incubated with MNL for 1h. In contrast, PMN were relatively resistant to cytotoxicity, with no cell death being observed at the concentrations tested (1-300μM). In

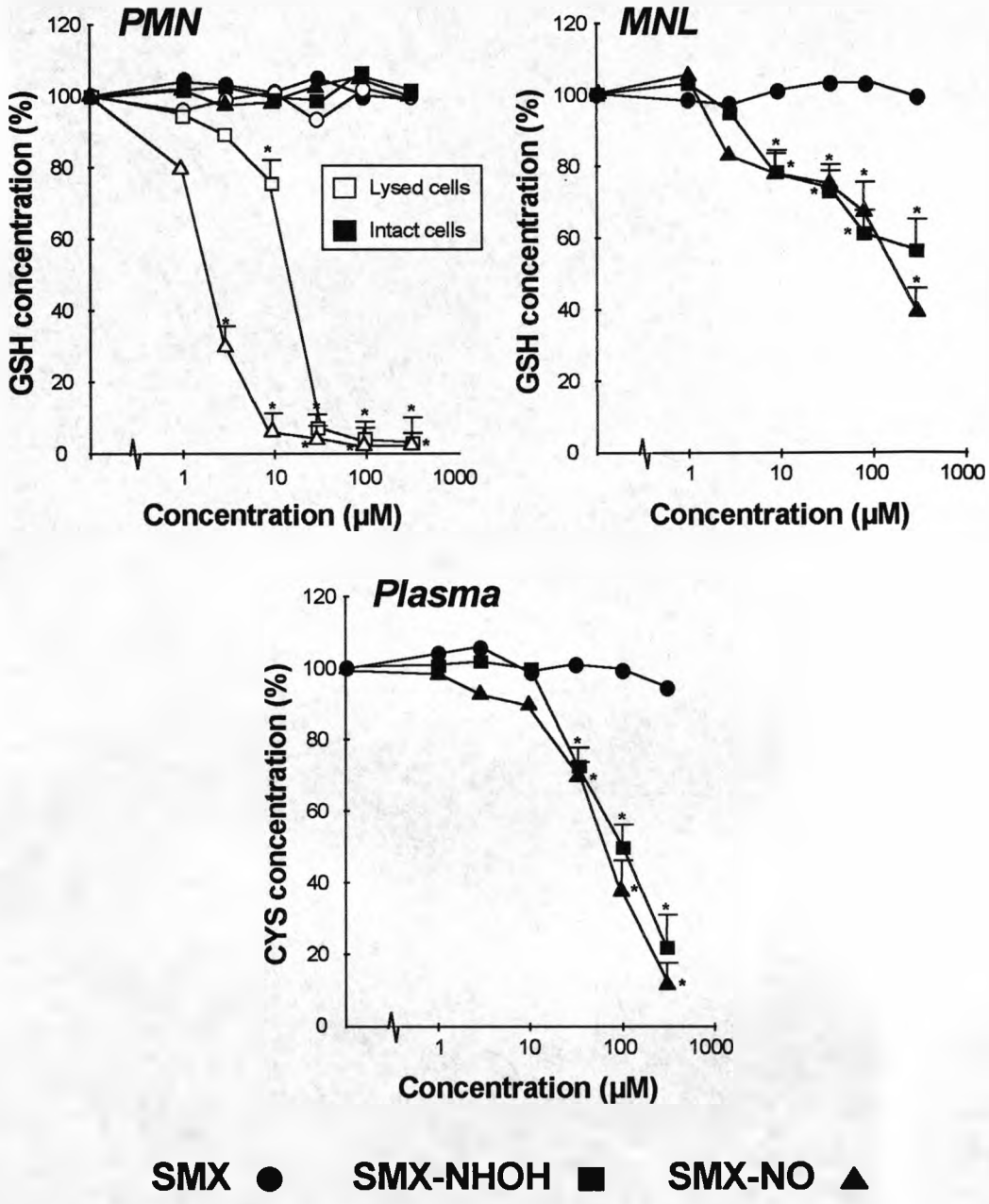


Figure 3.11 Depletion of CYS and GSH from cells and plasma after incubation with SMX and its metabolites. Thiol levels were measured by fluorescent HPLC using bromobimane. Results are expressed as the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing depletion at different concentrations of drug with that of solvent alone (*P < 0.05). Error bars have been omitted for clarity when there was no significant depletion.

addition, there was no significant increase in cell death on incubation of either SMX-NHOH or parent amine with MNL or PMN for 1h. After the cells were incubated overnight in gentamycin / HSA buffer, no cytotoxicity was observed with SMX. The pattern of cytotoxicity with SMX-NHOH and SMX-NO was similar to that described above (fig. 3.12).

3.3.4.2 Methaemoglobinemia

Methaemoglobin formation with SMX-NHOH was concentration-dependent (fig. 3.13). Methaemoglobin formation after incubation of RBC with SMX-NO (30μM; 4.9 ± 0.8%) was lower ($P < 0.05$) than that observed with SMX-NHOH (9.0 ± 2.2%).

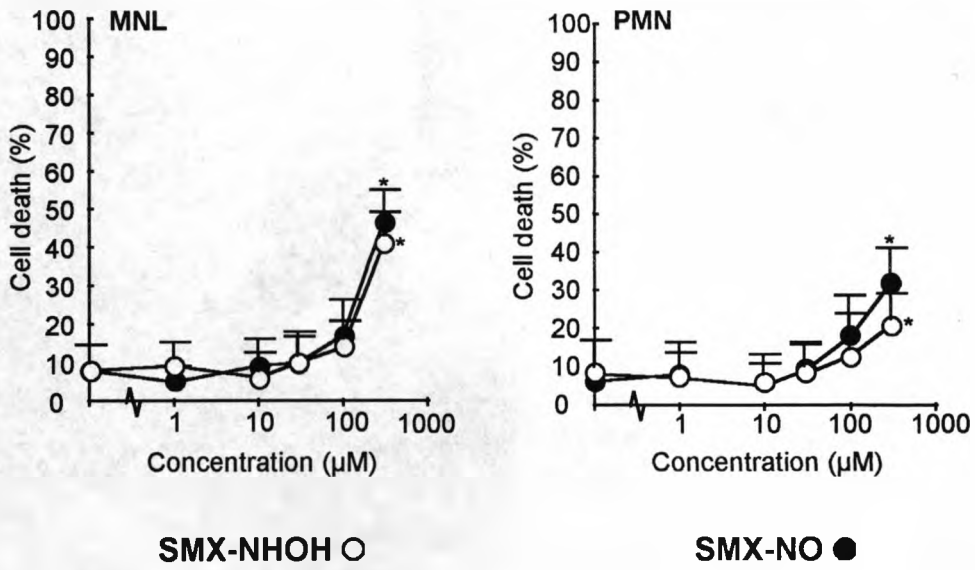


Figure 3.12 Cytotoxicity in MNL and PMN by SMX-NHOH and SMX-NO (1-300μM). Cytotoxicity was assessed by trypan blue dye exclusion. The results represent the mean ± S.D. of three experiments carried out in triplicate. Statistical analysis was performed by comparing cell death at different concentrations of compound with that of solvent alone (*P < 0.05).

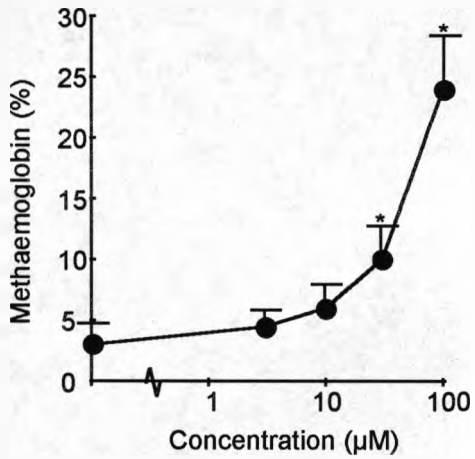


Figure 3.13 Methaemoglobin caused by SMX-NHOH (3-100μM) after a 1h incubation with RBC (50% haematocrit). Results represent the mean of three experiments carried out in triplicate. Statistical analysis was performed by comparing methaemoglobin formation in the presence and absence of SMX-NHOH (*P < 0.05).

3.3.5 Determination of Inhibition of Neutrophil Function by Sulphamethoxazole, Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole

3.3.5.1 Inhibition of Eugenol-Induced Glutathione Depletion

To determine whether SMX, SMX-NHOH or SMX-NO inhibited eugenol-induced GSH depletion, each compound (10 and 100μM) was co-incubated with eugenol (300μM) in the presence of PMA-stimulated PMN. SMX-NHOH and SMX-NO caused a concentration-dependent inhibition of eugenol-induced GSH depletion. However, depletion was not inhibited by SMX (fig. 3.14a).

3.3.5.2 Nitroblue Tetrazolium Slide Test

Neutrophil function was significantly inhibited by SMX-NHOH and SMX-

NO ($P < 0.05$), but not the parent amine. At 300μM, SMX-NHOH and SMX-NO caused 66.2 ± 8.9% and 80.9 ± 10.2% inhibition respectively (fig. 3.14b).

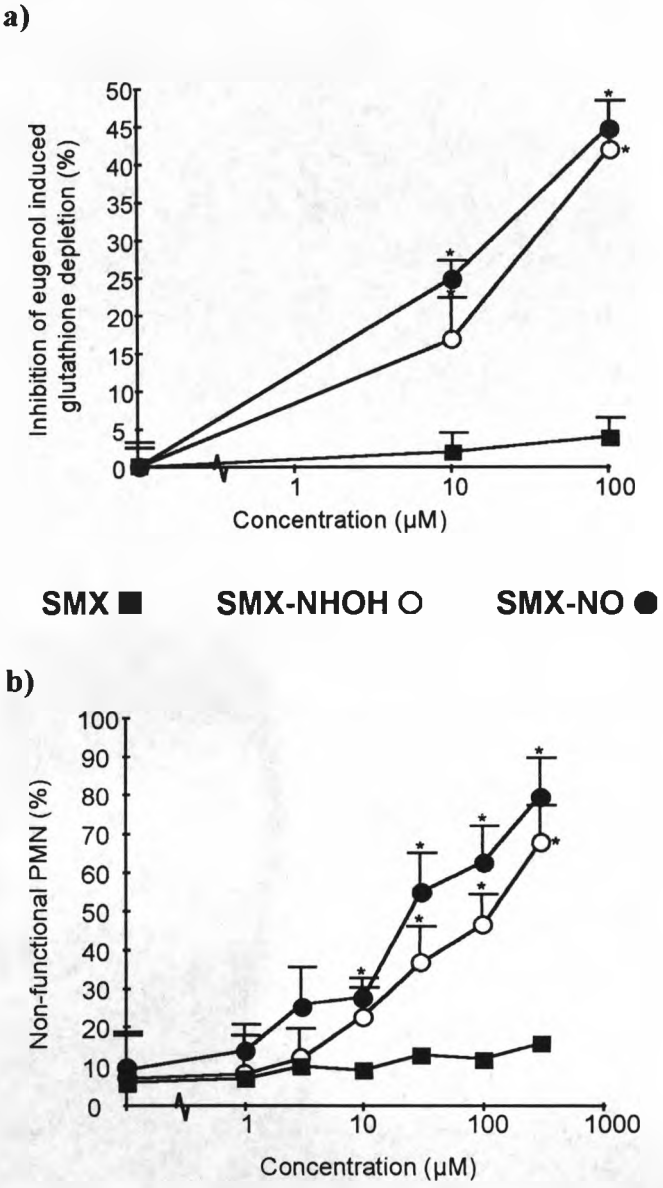


Figure 3.14 a) Inhibition of eugenol-induced GSH depletion in PMA-stimulated PMN. GSH levels were measured using bromobimane, by fluorescence HPLC.

b) Inhibition of PMN oxidative metabolism. The respiratory burst was measured using the NBT slide test.

The results represent the mean ± S.D. of three experiments carried out in triplicate. Statistical analysis was performed by comparing the different concentrations of compounds required to inhibit the respiratory burst with that of solvent alone (*$P < 0.05$).

3.3.6 Reduction of Sulphamethoxazole Hydroxylamine and Nitroso Sulphamethoxazole by Components of Peripheral Blood

There was no spontaneous reduction of SMX-NHOH or SMX-NO after a 1h incubation in either buffer, mobile phase or solvent. Reduction of SMX-NHOH (30μM) to SMX and of SMX-NO (30μM) to the hydroxylamine and SMX was observed when these compounds were incubated individually with RBC, MNL, PMN and plasma (table 3.1). The RBC were more active at reducing the hydroxylamine back to SMX than the other components of blood. In contrast, plasma was more potent than the other blood components at reducing the nitroso metabolite back to the hydroxylamine and parent compound. The PMN and MNL were equipotent at reducing both the hydroxylamine and nitroso metabolites, and PMA-stimulation of PMN did not alter the ability for reduction. Interestingly, lysis of the white cells lessened their ability to reduce SMX-NO back to SMX-NHOH, the reduction being between 12-16 times greater in the intact cells.

		Reduction (%)		
		$NHOH \rightarrow NH_2$	$N=O \rightarrow NH_2$	$N=O \rightarrow NHOH$
PMN	+PMA	3.0 ± 0.8	1.8 ± 0.6	35.3 ± 3.6
	-PMA	2.6 ± 0.2	1.7 ± 0.4	41.0 ± 7.2
Lysed PMN		2.5 ± 0.4	7.8 ± 0.6	3.2 ± 2.6
MNL		2.9 ± 1.1	2.8 ± 0.5	49.3 ± 5.4
Lysed MNL		2.4 ± 0.3	7.9 ± 0.3	3.1 ± 0.3
RBC		12.1 ± 2.4	6.2 ± 1.3	17.2 ± 3.6
Plasma		4.8 ± 1.1	16.3 ± 3.6	45.0 ± 10.3

Table 3.1 Reduction of SMX-NHOH or SMX-NO by the individual components of human blood.

3.4 Discussion

Activation of SMX to its hydroxylamine and nitroso metabolites is believed to be important in the pathogenesis of idiosyncratic toxicity associated with this compound. There is evidence to support both a direct mechanism of toxicity, via interaction with essential cellular macromolecules, and immune-mediated toxicity, via hapten formation and stimulation of the immune response. Current evidence suggests that the likely mechanism of toxicity is immune mediated. For example, SMX-substituted proteins have been detected in patient sera (Meekins *et al.*, 1994), cell-mediated immunity was observed in a patient with co-trimoxazole hypersensitivity (Mauri-Hellweg *et al.*, 1995) and a recent study in HIV-positive hypersensitive patients showed the presence of circulating anti-SMX antibodies, although these were also detected at a lower titre in non-hypersensitive patients (Daftarian *et al.*, 1995). More recently, work in our laboratory (Gill *et al.*, 1997) discovered high titres of specific anti-drug antibodies present in the serum of rats administered SMX-NO. In the present study therefore, human tissues have been used to further characterise how the disposition and metabolism of the toxic metabolites of SMX may predispose to the development of idiosyncratic toxicity.

The balance between bioactivation and detoxification may be an important determinant of individual susceptibility. Thus, the relative rates of oxidation, glucuronidation and acetylation of SMX may influence both the frequency and severity of drug hypersensitivity. In addition, the ability to reduce SMX-NHOH and SMX-NO back to the parent compound, which has been demonstrated *in*

vivo in the rat (Gill *et al.*, 1997) and *in vitro* in human liver microsomes (Cribb *et al.*, 1995), is likely to be another determinant of susceptibility. The results of the present study show that intact neutrophils, lymphocytes and red blood cells are capable of reducing these metabolites back to the parent compound (table 3.1). Interestingly, reduction was also seen with human plasma, indicating not only that other enzymes are capable of catalysing the reduction, but also that the reduction may occur non-enzymatically, and that it may occur in most tissues. With the white cells, the reduction of SMX-NO to SMX-NHOH and SMX may occur in intra- or extra-cellular compartments, and may be dependent on two processes which are not mutually exclusive. First, protein thiols on the plasma membrane and / or free intracellular GSH may reduce SMX-NO. Secondly, the reduction may be catalysed by an enzyme, possibly alcohol dehydrogenase, which has been characterised from liver (Kuwada *et al.*, 1980), and other tissues including human neutrophils (Horie and Ogura, 1980; Gotoh *et al.*, 1989). The ability to reduce SMX-NO to SMX-NHOH was abrogated by lysing the cells, suggesting that reduction occurs by an enzyme which is destroyed, or by a co-factor which is lost on lysis of the cells. In contrast, lysing the cells caused a significant increase in the reduction of SMX-NO to SMX. This process may be thiol-dependent, which would indicate that SMX, SMX-NHOH and SMX-NO are, at least in part, excluded from the cells. To investigate these hypotheses, it may be possible to inhibit the potential for reduction by preincubation of intact cells with inhibitors of alcohol dehydrogenase such as dicumarol or *p*-hydroxymercuribenzoate (Tatsumi *et al.*, 1982).

GSH can act as a detoxification process by the prevention of the oxidation of SMX and SMX-NHOH to the more electrophilic and thus potentially more toxic nitroso metabolite (Rieder *et al.*, 1995b). The reactions of GSH with hydroxylamine and nitroso metabolites have been characterised at a chemical level and found to be dependent on the thiol concentration, pH and the electronic properties of aromatic ring substituents of the drug (Eyer, 1979; Cribb *et al.*, 1991; Ellis *et al.*, 1992). The reaction of SMX-NO with GSH is initiated by nucleophilic attack of GSH on the nitroso moiety, resulting in formation of an unstable semi-mercaptal intermediate. Once formed the semi-mercaptal is converted, depending on conditions, to yield either a sulphinamide, hydroxylamine or the parent amine. The sulphinamide is stable in both aqueous and non-aqueous conditions, although it may undergo hydrolysis to the parent amine under physiological conditions of antigen processing (Ellis *et al.*, 1992; Kalish, 1995).

The ultimate product in the reaction of SMX-NO with GSH is the parent amine (fig 3.4). This result conflicts mechanistic studies of Diepold *et al.* (1982), who showed that strong electron-withdrawing substituents *para-* to the nitroso moiety (i.e., the sulphonamide component of SMX) favour hydroxylamine formation (fig. 3.15). Interestingly, SMX-NHOH is rapidly and non-enzymatically converted back to SMX-NO under aerobic conditions (Cribb *et al.*, 1991). Therefore, formation of a facile redox cycling system may, at least in part explain this discrepancy (fig. 3.16).

Plasma was found to effectively reduce SMX-NO (table 3.1). This may have involved plasma GSH, although it is important to note that greater than 99%

of reduced GSH in the circulation is intracellular, where the concentration is approximately 3-4nmols/10^6 cells (Pirmohamed *et al.*, 1996). From previous studies, the amount of GSH in the plasma would be insufficient to reduce SMX-NO. Therefore, the effect of CYS on the reduction of SMX-NO was also studied. CYS, unlike GSH, is found almost exclusively extracellularly with total levels (both reduced and oxidised) in human plasma reaching 300μM (Mills and Lang, 1996). CYS rapidly and completely reduced SMX-NO, yielding

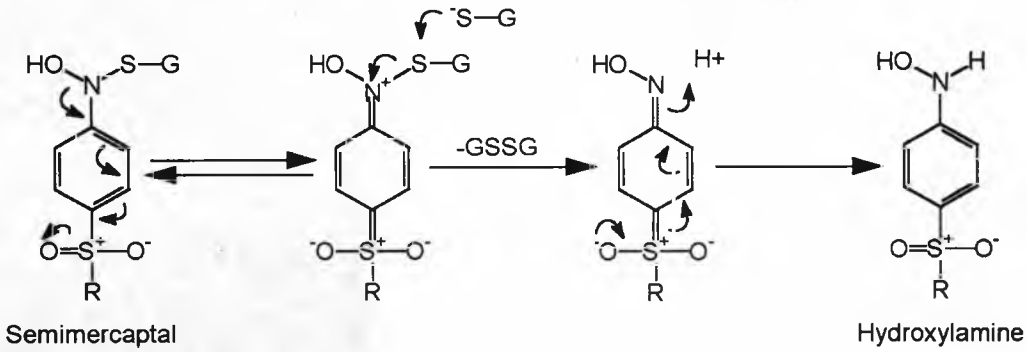


Figure 3.15 Stabilisation of the semi-mercaptal intermediate by electron withdrawing *para-* substituents, leading to the formation of SMX-NHOH.

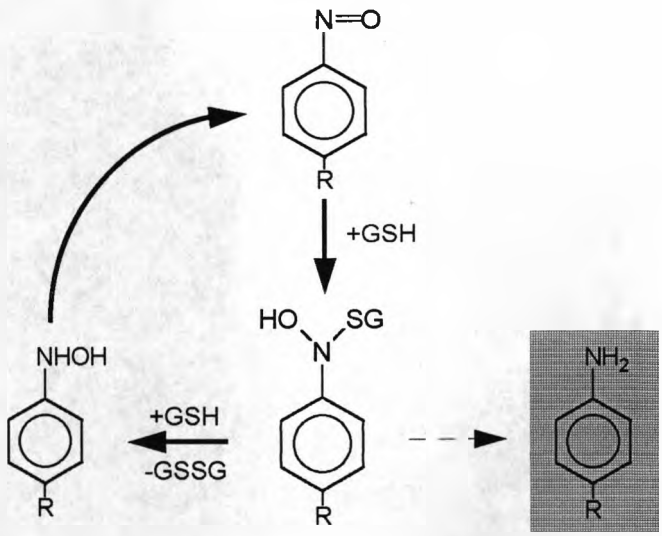


Figure 3.16 The effect of redox cycling on SMX formation.

only the hydroxylamine and parent amine, with the reactions being more rapid than with equivalent concentrations of GSH (fig. 3.10). In contrast to the reaction with GSH which produced a complex series of reaction intermediates, no thiol conjugates were identified (fig. 3.17). However, the mechanism of reduction is likely to be similar to that observed with GSH. A recent study by Weinander *et al.* (1994) demonstrated that GSH and CYS were approximately 1% and 7% charged respectively at physiological pH. These differences may explain the lack of conjugate formation and the enhanced reactivity of CYS towards SNX-NO. Other groups working with reactive electrophiles such as quinones, diethyl maleate and diethyl fumarate have observed an increased reactivity of CYS over GSH (Murty and Penning, 1992; Kubal *et al.*, 1995). Kubal *et al.* (1995) suggested that donation of a proton from the amine group of CYS may enhance product formation, while in turn the amide linkage between CYS and glutamate in GSH, may result in stabilisation of the intermediate products of conjugation.

The reaction of SMX-NHOH and SMX-NO with CYS was accompanied by its depletion. Plasma levels of reduced CYS are in the range of 5-15μM, with the rest existing in either an oxidised or protein bound form (Chawla *et al.*, 1984; Mansoor *et al.*, 1992; Mills and Lang, 1996). Such disulphides are believed to play an important regulatory role in drug detoxification (Issacs and Blinkley, 1977), with Smolin and Benevenga (1989) initially describing the concept that oxidised and reduced thiols exist in an equilibrium and act as buffer system that protects against drug induced oxidative damage (Cotgreave *et al.*, 1990). Interestingly, reduced plasma CYS levels declined only when exposed to greater

than five times the concentration of SMX-NO required to deplete CYS in buffer. This would suggest that oxidised and protein bound CYS in plasma is somehow mobilised to replenish free CYS concentrations during oxidative stress. Although this phenomena has been reported previously, there is no clear chemical rationale for the process.

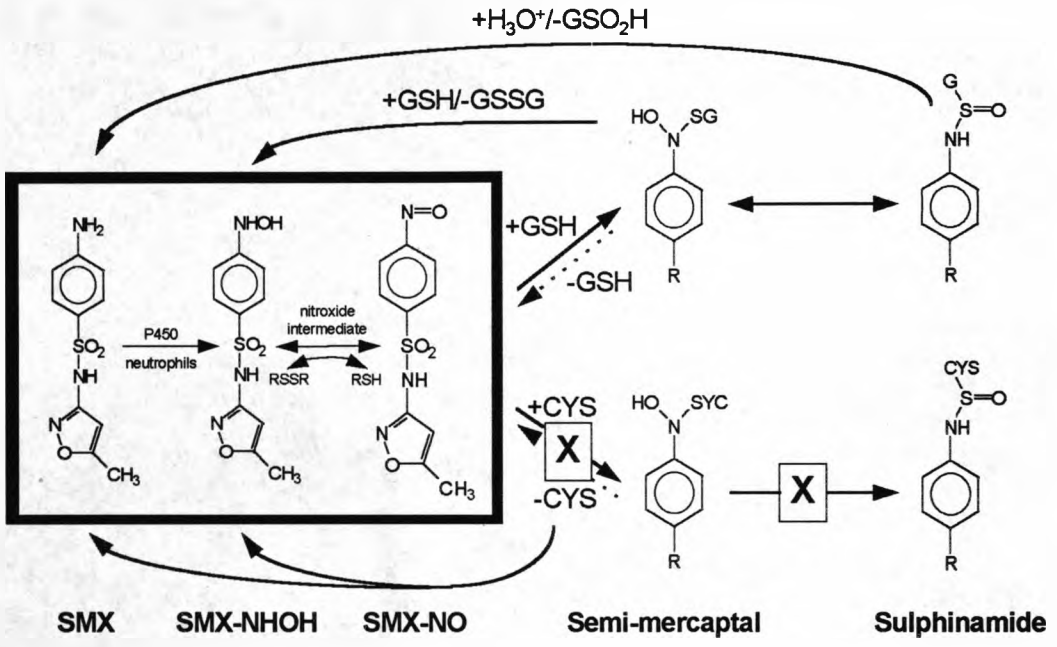


Figure 3.17 The metabolic pathways of SMX bioactivation and subsequent detoxification reactions with GSH and CYS.

SMX-NHOH and SMX-NO caused a concentration dependent depletion of intracellular GSH in lymphocytes, but not neutrophils, at concentrations which were not cytotoxic (10-300nmoles/ml). It is important to note that these drug concentrations are far in excess of the amount of GSH present (2-3nmoles; 0.5 x 10^6 cells/ml). After one hour, neutrophils were also more resistant to the cytotoxic effects of these two metabolites. SMX and other anti-microbial agents

have previously been shown to accumulate to higher concentrations in lymphocytes than neutrophils (Climax *et al.*, 1986). These data may explain the absence of neutrophil toxicity and GSH depletion.

Lysing the cells resulted in a much greater degree of GSH consumption than with intact cells, the extent of reaction being similar to when the metabolites are incubated with GSH in buffer. There are three possible explanations for this; first, cell lysis may destroy an enzyme responsible for the regeneration of GSH, second, reduction by an alternative (enzyme-mediated) pathway may preclude consumption of GSH, and thirdly, the cell membrane may partially exclude the entry of SMX-NHOH and SMX-NO into the intracellular compartment, and thus reduce the ability to deplete GSH.

Red blood cells also reduce SMX-NHOH and SMX-NO back to SMX. The red blood cells showed an apparent decreased capacity to reduce SMX-NO to SMX-NHOH when compared to other cell types and plasma. However, reduction of SMX-NHOH to the parent amine was significantly greater. It must be noted that the hydroxylamine can undergo oxidation by haemoglobin within the red blood cell and the analysis is a net value of all oxidation and reduction processes. Exposure of red blood cells to the hydroxylamine and nitroso metabolites also resulted in methaemoglobin formation. In fact, there was a significant negative correlation between the extent of reduction and degree of methaemoglobin formation. Reduction of the structurally related compound, dapsone hydroxylamine to dapsone, has also been shown previously with red blood cells *in vitro* (Coleman and Jacobus, 1993). However, in contrast to

dapsone, SMX therapy is not associated with methaemoglobinaemia *in vivo* (Cribb *et al.*, 1996). Clearly, the reductive capacity of red blood cells therefore cannot be the only factor which prevents SMX-mediated methaemoglobinaemia *in vivo*. Other possible factors include the relative instability of SMX-NHOH and SMX-NO, and the relative ease with which they cross the red blood cell membrane, when compared to dapsone hydroxylamine.

A lack of detoxification of SMX-NHOH and SMX-NO could, in theory, lead to conjugation with autologous proteins, and initiate an immune response (Meekins *et al.*, 1994; Cribb *et al.*, 1996). To investigate this hypothesis, the structure of HSA was considered to identify other groups on proteins which may be sufficiently nucleophilic to react with SMX-NO. Various amino acids within HSA contain free amine groups, which could react with nitroso compounds forming diazo-products. In this study, lysine which is one of the major constituents of HSA (greater than 10% of the total amino acid content by mass) was utilised (fig. 3.18). Identical conditions were used to study the reaction of lysine with SMX-NHOH and SMX-NO. However after ninety six hours, NMR analysis revealed no reaction in either case, even after heating under deuterated acid catalysis. From the results obtained, the initial step of a SMX-induced hypersensitivity reaction may be the formation of a stable SMX-protein adduct, formed in the reaction of SMX-NO with sulphydryl nucleophiles. Consistent with this hypothesis, covalent binding of SMX-NHOH and SMX-NO with thiolated HSA, but not HSA itself, has also been shown in our laboratory (Hough, 1998). The same study demonstrated that SMX-NHOH and SMX-NO, but not the

parent amine were able to bind irreversibly to the extracellular surface of neutrophils and lymphocytes. These findings indicate that extracellular thiols (in particular CYS) may be more important in determining the degree of reduction of SMX-NHOH and SMX-NO back to SMX than intracellular thiols, and consequently are more important in determining susceptibility to idiosyncratic toxicity with SMX. This is supported by data in this chapter which shows that the rate of reaction of these toxic metabolites with CYS was faster than with GSH.

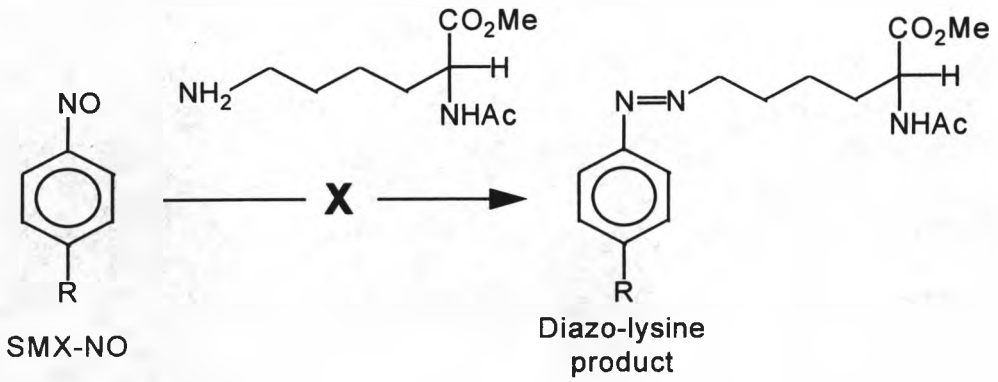


Figure 3.18 Hypothesised conjugate structure from the reaction of SMX-NO and protected lysine.

In theory, SMX hypersensitivity could arise from an indirect effect on essential cellular processes. Changes in intracellular esterase activity, which contributes to the inhibition of cell function have been previously noted with SMX-NHOH at concentrations preceding the loss of membrane integrity (Leeder *et al.*, 1991a and 1991b). Furthermore, aromatic nitroso compounds such as nitrosobenzene have been shown to inhibit the respiratory burst of intact neutrophils by a direct action on the NADPH oxidase system (Nakata *et al.*, 1997).

1997). In this study, inhibition of the neutrophil oxidative burst was observed with SMX-NHOH and SMX-NO, but not SMX. Whether these observations are, first, an early marker of toxicity, and secondly, related to SMX-induced hypersensitivity remains unclear and requires further investigation.

In summary, this chapter shows that the body is equipped with a number of detoxification mechanisms which may prevent the idiosyncratic toxicity observed with SMX. Foremost amongst these processes is the ability of various tissues including plasma, to reduce the toxic metabolites of SMX, the hydroxylamine and nitroso derivatives, back to the parent amine. Reduction may be enzymatic, particularly in the liver and peripheral blood, while in plasma it may be non-enzymatic, and is due to an interaction with CYS, the predominant extracellular thiol. Consequently, a deficiency of plasma CYS, which has been demonstrated in HIV-positive individuals (Eck *et al.*, 1989; Helbling *et al.*, 1996; Walmsley *et al.*, 1997), may allow binding of SMX-NHOH and SMX-NO to various macromolecules and initiate immune-mediated toxicity. Further studies investigating this hypothesis are described in chapter 6.

Chapter 4:

Neutrophil Toxicity of the Chemically Reactive Metabolite(s) of Clozapine : Possible Role in Agranulocytosis

Contents **Page**

4.1 Introduction

Clozapine (CLZ) is an atypical antipsychotic agent which represents a major therapeutic advance over conventional neuroleptics such as chlorpromazine. Its main advantages include the lack of extrapyramidal side-effects such as Parkinsonism (Baldessarini and Frankenberg, 1991) and its effectiveness in about 30% of otherwise "treatment-resistant" patients (Kane *et al.*, 1988). However, administration of CLZ is associated with a high frequency (about 0.8%) of agranulocytosis, which has restricted its general use (Lieberman and Safferman, 1992; Alvir and Lieberman, 1994). Furthermore, patients prescribed CLZ need weekly haematological monitoring for the first 18 weeks of therapy, which is then decreased to fortnightly (Baldessarni and Frankenburg, 1991). This procedure is an inconvenience to the patient who must attend hospital for regular blood sampling.

Neutrophils, as with all blood cells, are derived from a pluripotential haematopoietic stem cell (section 1.5). Patients with CLZ-induced agranulocytosis show an absence of myeloid precursors in the bone marrow (Gerson and Meltzer, 1992). This suggests that an early neutrophil precursor is the ultimate site of toxicity, although the mature neutrophil may also be targeted simultaneously. The mechanism of CLZ-induced agranulocytosis remains unclear. CLZ itself is not directly cytotoxic to neutrophils and does not interfere with the differentiation of bone marrow precursor cells (Veys *et al.*, 1992; Gerson *et al.*, 1994). However, CLZ undergoes extensive metabolism, with only 2 to 5% of the drug being excreted unchanged (Jann *et al.*, 1993). The principal stable

metabolites are demethyl CLZ and CLZ *N*-oxide (Gauch and Michaelis, 1971; section 1.8.2). Gerson *et al.* (1994) showed that demethyl CLZ is more toxic towards myeloid cells, but this was only observed at concentrations higher (>5μM) than those achieved therapeutically (<0.5μM) (Gauch and Michaelis, 1971; Sayers and Amsler, 1977). Additionally, a recent study did not find any relationship between the plasma concentrations of CLZ and demethyl CLZ and the occurrence of toxicity (Hasegawa, 1994). These facts taken together suggest that agranulocytosis is unlikely to be due to a direct toxic effect of CLZ or one of its stable metabolites.

Drugs with arylamine functional groups are known to form free radicals and other reactive intermediates (Hoffman *et al.*, 1985; Ross *et al.*, 1985) (section 1.5.4). In a recent study using rodents, the detection of several glutathione (GSH) conjugates indicated that CLZ undergoes extensive bioactivation *in vivo* (Maggs *et al.*, 1995). In man CLZ can also be metabolised to a reactive intermediate by hepatic P450 enzymes (Pirmohamed *et al.*, 1995), myeloid cells (Maggs *et al.*, 1995) and peripheral neutrophils (Maggs *et al.*, 1995; Liu and Uetrecht, 1995). Myeloperoxidase (MPO), the major enzyme present in neutrophils, can bioactivate CLZ to a radical cation, and then to a nitrenium ion (Fischer *et al.*, 1991; Uetrecht, 1992) (fig. 4.1). The ability of MPO to active CLZ to a free radical stems from the detection of glutathionyl and superoxide radicals as products in the reaction of GSH, or NADPH and oxygen with CLZ. A two electron disproportionation reaction results in the formation of the nitrenium ion.

The nitrenium ion has been implicated in the pathogenesis of the agranulocytosis, although at present there is no clear evidence of the mechanism. As postulated for other types of metabolite (Park *et al.*, 1992; Park *et al.*, 1995), agranulocytosis may arise from an interaction of the nitrenium ion with a cellular macromolecule which leads to either direct or indirect immune-mediated toxicity (Safferman *et al.*, 1992; Maggs *et al.*, 1995; Liu and Uetrecht, 1995).

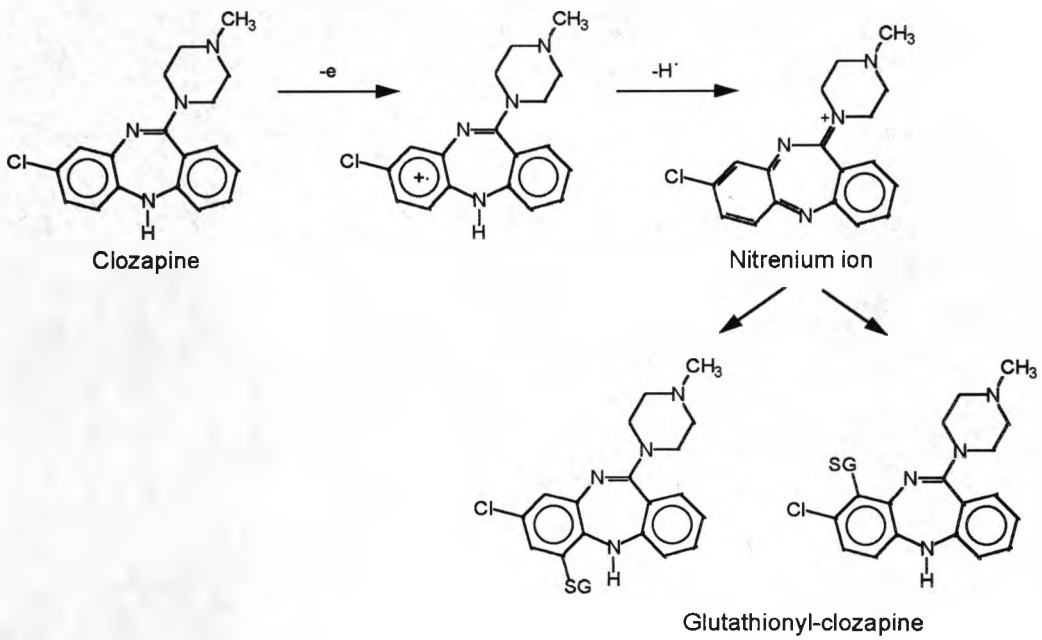


Figure 4.1 Bioactivation of CLZ to a chemically reactive nitrenium ion. The electrophilic metabolite can react with GSH to form either a C6-glutathionyl or a C9-glutathionyl conjugate (Taken from Pirmohamed and Park, 1997).

Given that neutrophil numbers are governed by a delicate balance between their production and their death, any factor decreasing their production or increasing their death rate will lead to neutropenia or agranulocytosis. Although this process has been examined with regard to CLZ and its stable metabolites, no

studies have thus far been performed with regard to the functional toxicity of the putative nitrenium ion. Using a liver microsomal metabolising system, it has been shown that CLZ undergoes bioactivation to a metabolite(s) which is cytotoxic to lymphocytes (Pirmohamed *et al.*, 1995). However studies in our laboratory have shown that this assay cannot reliably be used to assess toxicity to the target cell, i.e. the neutrophil, because microsomes bind to the neutrophil surface (Dr. M. Pirmohamed, The University of Liverpool; unpublished data). Although the chemically reactive metabolite can be synthesised, it has a short half life, estimated to be less than one minute (Liu and Uetrecht, 1995), making it difficult to use in direct cytotoxicity assays.

To address the issue of toxicity to neutrophils, a novel *in vitro* assay has been developed in which the *in situ* generation of the reactive metabolite is coupled to an assessment of neutrophil viability and chemical characterisation of the metabolism of CLZ.

4.2 Materials and Methods

4.2.1 Chemicals

CLZ, demethyl CLZ and CLZ *N*-oxide were all gifts from Novartis Pharmaceuticals (Basle, Switzerland). Ascorbic acid (ASC), bromobimane, gentamycin, GSH, horseradish peroxidase (type VI; HRP), human serum albumin (HSA), MPO, *N*-acetyl cysteine (CYS), *N*-ethylmorpholine, trichloroacetic acid and trypan blue were all obtained from Sigma Chemical Co. (Poole, UK). Hydrogen peroxide was obtained from BDH (Dorset, UK). Monopoly® resolving

medium (Ficoll Hypaque; density, 1.114g/ml) and Lymphoprep® (1.077g/ml) were obtained from ICN Biomedicals (Bucks, UK) and Nycomed (Birmingham, UK) respectively. All solvents were of HPLC grade and were purchased from Fischer Scientific plc (Loughborough, UK).

4.2.2 Isolation of Human Peripheral Blood Cells

Polymorphonuclear leucocytes (neutrophils; PMN) and mononuclear leucocytes (lymphocytes; MNL) were isolated from the venous blood of 10 healthy male volunteers (age range 21-40 years) on a dual density gradient of Monopoly resolving medium and Lymphoprep, as described in section 2.2.2. Cells were resuspended in HEPES buffered balanced salt medium (pH 7.4) and diluted to the concentration required for each experiment. Both cell types were greater than 98% pure and 95% viable as assessed by Wright's stain and trypan blue dye exclusion (section 2.3.1).

4.2.3 Comparison of the Metabolism of Clozapine by Myeloperoxidase and Horseradish Peroxidase

CLZ (30μM) was incubated at 37°C for 2h with either MPO (1unit) or HRP (20 units), and hydrogen peroxide (10μM) in HEPES buffer (pH 7.4; final incubation volume 1ml). The reaction was initiated by the addition of hydrogen peroxide, and GSH (1mM) was added within 30sec. After 2h, methanol (1ml) was added to each tube. The solutions were then evaporated to dryness under a

stream of nitrogen at 37°C, reconstituted in methanol / water (1:1, 300µl), and analysed by HPLC.

4.2.4 Chemical Characterisation of the Metabolites of Clozapine

CLZ and its stable metabolites, CLZ *N*-oxide and demethyl CLZ (30µM), were incubated with HRP (20 units), hydrogen peroxide (10µM) and GSH (1mM) in the presence or absence of PMN ($1x10^6$ cells/ml) in HEPES buffer. CLZ was also incubated with HRP (20 units) and hydrogen peroxide (10µM) in the absence of both cells and GSH. Hydrogen peroxide was added to initiate the reaction and GSH was added no later than 40sec after the hydrogen peroxide. After 2h, the tubes containing the cells were centrifuged (10min, 650*g*) and the supernatant removed. Methanol (1ml) was added to the supernatant, which was evaporated to dryness under a stream of nitrogen at 37°C and reconstituted in 150µl of methanol / water (1:1, v/v) for analysis by HPLC and LC-MS.

4.2.5 Detection of Metabolites by HPLC

The conditions for HPLC and LC-MS have been described previously (Maggs *et al.*, 1995). Samples (20µl) were eluted from a 5µm Nucleosil C_8 column (25 x 0.32cm; Phenomenex, Macclesfield, Cheshire) with gradients of acetonitrile in 6mM ammonium formate, pH 3.5: 20 to 40% over 15min (gradient 1); 10 to 25% over a 15min period and 25 to 55% over a 20min period (gradient 2). The flow rate was 0.75ml/min throughout. The HPLC system was the same as that described in section 3.2.2. CLZ, demethyl CLZ, CLZ *N*-oxide and their

respective GSH conjugates were identified by comparison of their retention times with those of co-injected compounds by UV absorbance at 254nm.

4.2.6 Detection of Metabolites by LC-MS

Incubations containing drug, HRP, hydrogen peroxide and GSH were combined and reconstituted in 150µl methanol / water (1:1, v/v) to provide the mass required for mass spectrometry. Samples (10µl) were eluted from a 5µm Nucleosil C_8 column (25 x 3.2mm; Phenomenex, Macclesfield, UK) with gradients 1 and 2 (section 4.2.5). The flow rate was 0.75ml/min. Mobile phase was delivered to the LCMS interface under the conditions described in section 3.2.8. Data was processed using Masslynx 2.1 software.

4.2.7 Bioactivation of Clozapine and its Metabolites by Horseradish Peroxidase and Hydrogen Peroxide and Determination of Cytotoxicity

The combination of HRP and hydrogen peroxide form hypochlorous acid *in situ* (Uetrecht, 1992). Initial experiments were designed to investigate PMN cytotoxicity of HRP, hydrogen peroxide and hypochlorous acid. HRP (1-50 units), hydrogen peroxide (1-30µM) and hypochlorous acid (1-30µM) were incubated with PMN (1 x 10^6 cells/ml) in 15ml plastic conical tubes in an agitating water bath for 2h at 37°C. After 2h, the tubes were centrifuged (10min, 650*g*) to pellet the cells. The supernatants were discarded and the cells were resuspended in 1ml of HEPES buffer containing HSA (5mg/ml) and gentamycin (50µg/ml). Samples were then placed in an incubator at 37^0C for 16h. Cell death was assessed by trypan blue dye exclusion, as described in section 2.2.4.

Hypochlorous acid resulted in a concentration-dependent increase in PMN death (fig. 4.2), while HRP and hydrogen peroxide were non-toxic. Thus, for all subsequent experiments the combination of HRP (20 units) and hydrogen peroxide (10μM), were used to produce hypochlorous acid *in situ*.

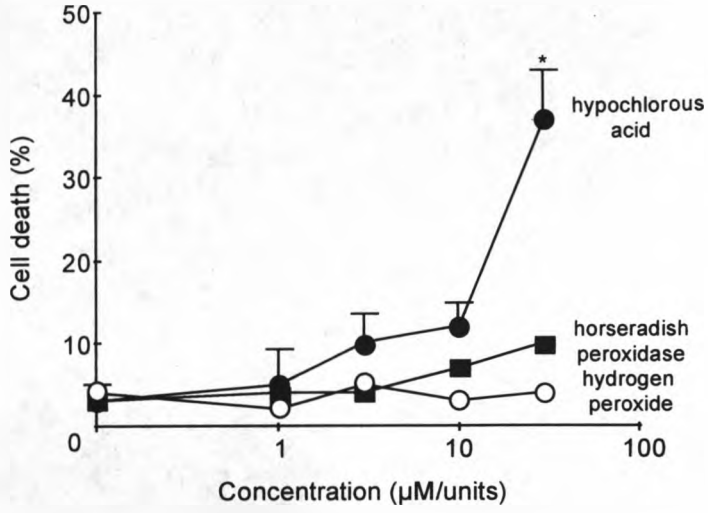


Figure 4.2 Cytotoxicity of HRP (1-30 units), hydrogen peroxide (1-30μM) and hypochlorous acid (1-30μM) towards PMN (1 x 10^6). Each point represents an incubation carried out in triplicate. Statistical analysis was performed by comparing incubations containing either HRP, hydrogen peroxide or hypochlorous acid to that of solvent alone (*P < 0.05).

In subsequent experiments, PMN and MNL were incubated with CLZ, demethyl CLZ and CLZ *N*-oxide in an agitating water bath for 2h at 37°C. Cells (1 x 10^6/ml) were incubated with drug (0-30μM) in the presence or absence of HRP (20 units), hydrogen peroxide (10μM), GSH (1mM), *N*-acetyl CYS (1mM) and ASC (1mM). The reactions were initiated with hydrogen peroxide and after 2h, the tubes were centrifuged (10min, 650*g*) to pellet the cells. The supernatants were discarded and the cells were resuspended in 1ml of drug-free HEPES buffer containing HSA and gentamicin as described above. Samples were incubated for a further 16h (37°C), and cell death was assessed by trypan blue dye exclusion.

4.2.8 Determination of Depletion of Intracellular Glutathione

In order to determine whether CLZ or its metabolites depleted intracellular GSH in PMN and MNL, the fluorescent probe bromobimane was used (Cotgreave and Moldeus, 1986). The cells (0.5×10^6) were incubated with either CLZ, demethyl CLZ or CLZ *N*-oxide (0-30μM) in HEPES buffer at 37^0C for 1h in the presence or absence of HRP (20 units) and hydrogen peroxide (10μM). The total incubation volume was 1ml. Bromobimane (3mM) in *N*-ethylmorpholine was added to the incubations and thiol levels were measured as described in section 2.2.7.

4.2.9 Statistical Analysis

The results are presented as the mean ± S.D. Statistical analysis was performed by the Mann-Whitney test, accepting $P < 0.05$ as significant.

4.3 Results

4.3.1 Direct Cytotoxicity of Clozapine and its Metabolites

CLZ did not exhibit any cytotoxicity towards either MNL or PMN up to a concentration of 30μM (fig. 4.3). Similar results were obtained for demethyl CLZ and CLZ *N*-oxide (fig. 4.4).

4.3.2 Metabolism of Clozapine by Myeloperoxidase and Horseradish Peroxidase

CLZ was metabolised by MPO to C6 glutathionyl CLZ (6.2 ± 0.5%) only, while HRP produced both C6 glutathionyl CLZ (21.0 ± 1.1%) and C9

glutathionyl CLZ (4.0 ± 0.7%). These metabolites were identified by co-elution with glutathionyl CLZ isomers which have been characterised previously (Maggs *et al.*, 1995). Given the higher turnover achieved with HRP, all of the further experiments were performed using an activating system comprising HRP and hydrogen peroxide.

4.3.3 Horseradish Peroxidase / Hydrogen Peroxide-Mediated Metabolism Dependent Cytotoxicity

In the presence of both HRP and hydrogen peroxide, CLZ was bioactivated to a metabolite which showed concentration-dependent cytotoxicity (fig. 4.3) towards both MNL and PMN ($P < 0.05$, at all concentrations studied). There was no difference in the sensitivity of the MNL and PMN up to a concentration of 10μM CLZ, while at 30μM CLZ, PMN were significantly ($P < 0.05$) more sensitive.

Significant cytotoxicity was also observed at concentrations of CLZ achieved therapeutically (3μM; Sayers and Amsler, 1977). When the drug was incubated with an incomplete activating system, i.e., in the absence of either HRP or hydrogen peroxide, no cell death (above background values) was observed. Additionally, in the absence of the drug (i.e., solvent control) but in the presence of a full activating system, again there was no increase in cell death above background values.

In the presence of the full activating system, demethyl CLZ, like CLZ, also showed a concentration-dependent cytotoxicity. However, it was less

cytotoxic than CLZ at all concentrations (fig. 4.4). In contrast, CLZ *N*-oxide did not exhibit any cytotoxicity up to a concentration of 30μM in the presence of a full activating system (fig. 4.4).

4.3.4 Effect of Biological Modifiers on Horseradish Peroxidase / Hydrogen Peroxide-Mediated Clozapine Cytotoxicity

The cytotoxicity of CLZ and demethyl CLZ towards both cell types was reduced to background values when either GSH, *N*-acetyl CYS or ASC was included in the incubation (fig. 4.5 and 4.6).

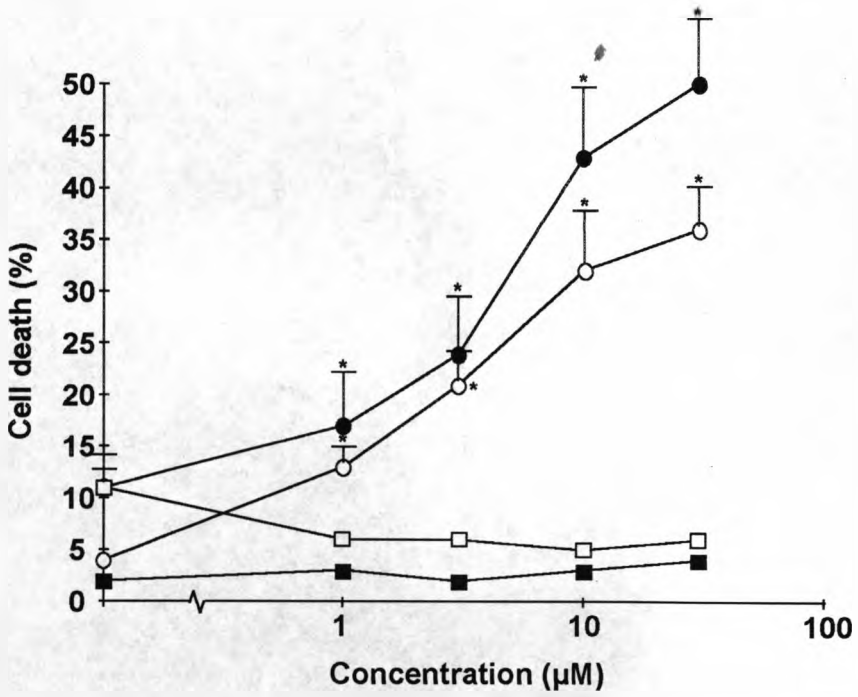


Figure 4.3 Cytotoxicity of CLZ towards MNL (open symbols) and PMN (filled symbols) in the presence (○) and absence (□) of HRP and hydrogen peroxide. The results represent the mean ± S.D. of three separate experiments using cells from different individuals (all incubations performed in triplicate). Statistical analysis was performed by comparing incubations in the presence and absence of activating system at the same drug concentrations (*P < 0.05). Error bars have been omitted from some of the curves for the sake of clarity.

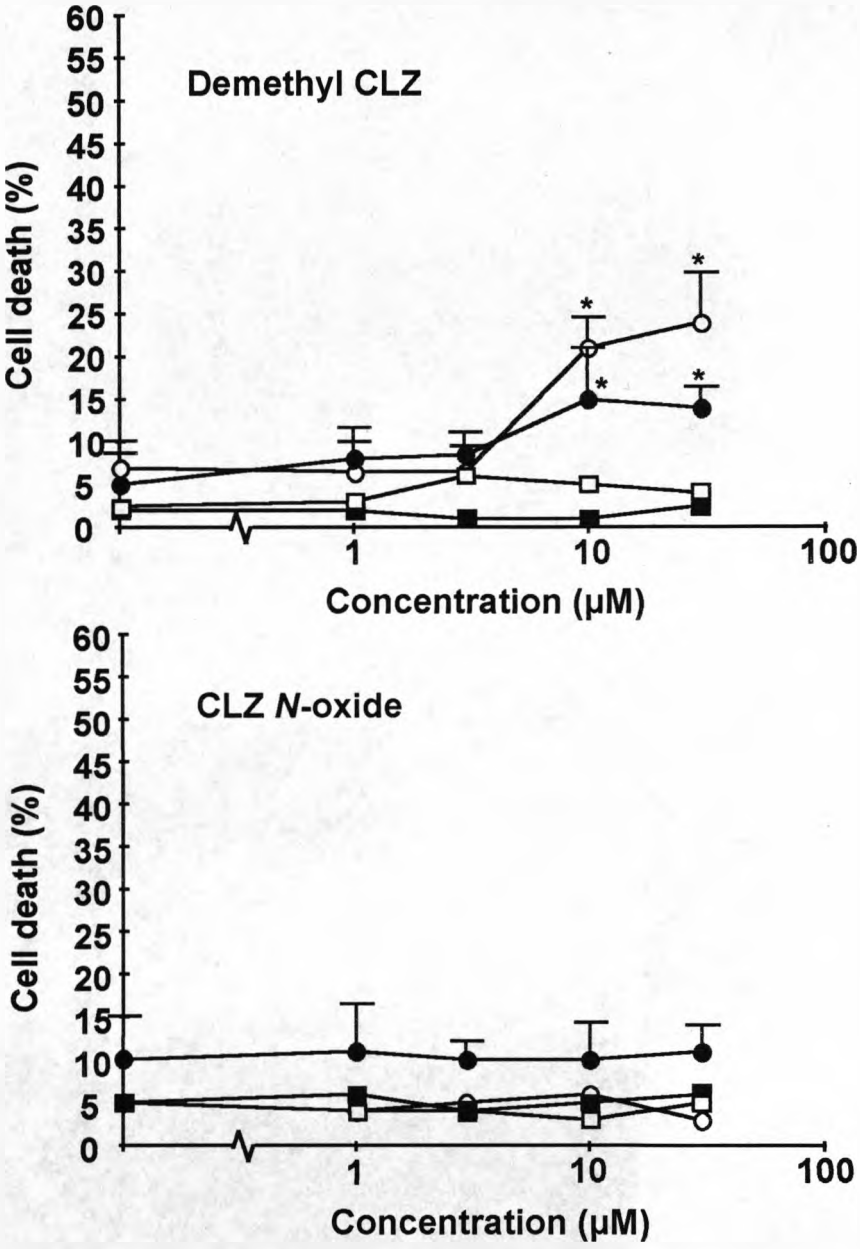


Figure 4.4 Cytotoxicity of demethyl CLZ and CLZ *N*-oxide towards MNL (open symbols) and PMN (filled symbols) in the presence (○) and absence (□) of HRP and hydrogen peroxide. The results represent the mean ± S.D. of three separate experiments using cells from different individuals (all incubations performed in triplicate). Statistical analysis was performed by comparing incubations in the presence and absence of activating system at the same drug concentrations (*$P < 0.05$). Error bars have been omitted from some of the curves for the sake of clarity.

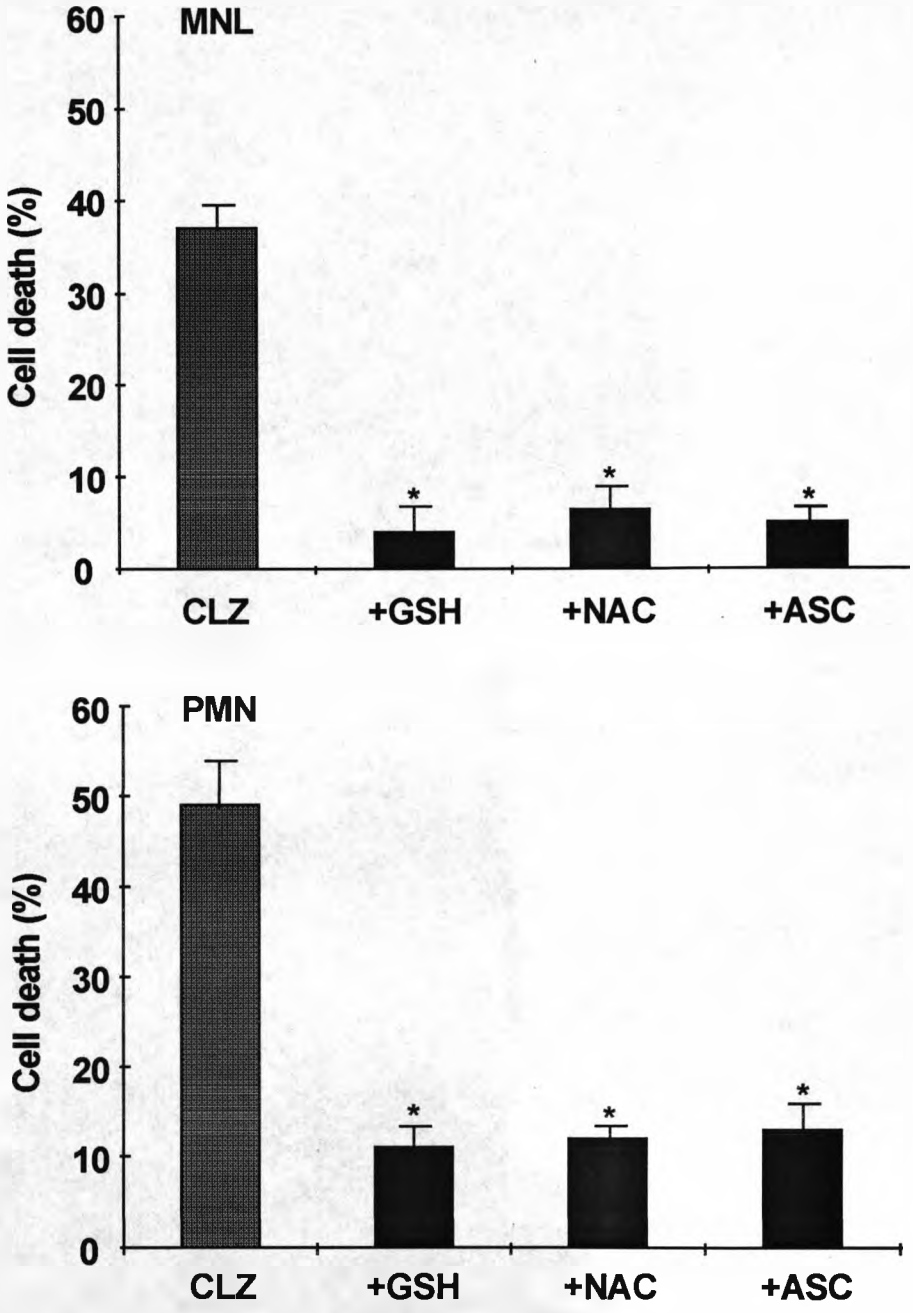


Figure 4.5 Cytotoxicity of CLZ (30μM) towards MNL and PMN, in the presence of GSH (1mM), *N*-acetyl CYS (NAC; 1mM) and ASC (1mM). The results represent the mean ± S.D. of 3 experiments carried out in triplicate. Statistical analysis was performed by comparing incubations with and without the antioxidants (*$P < 0.05$).

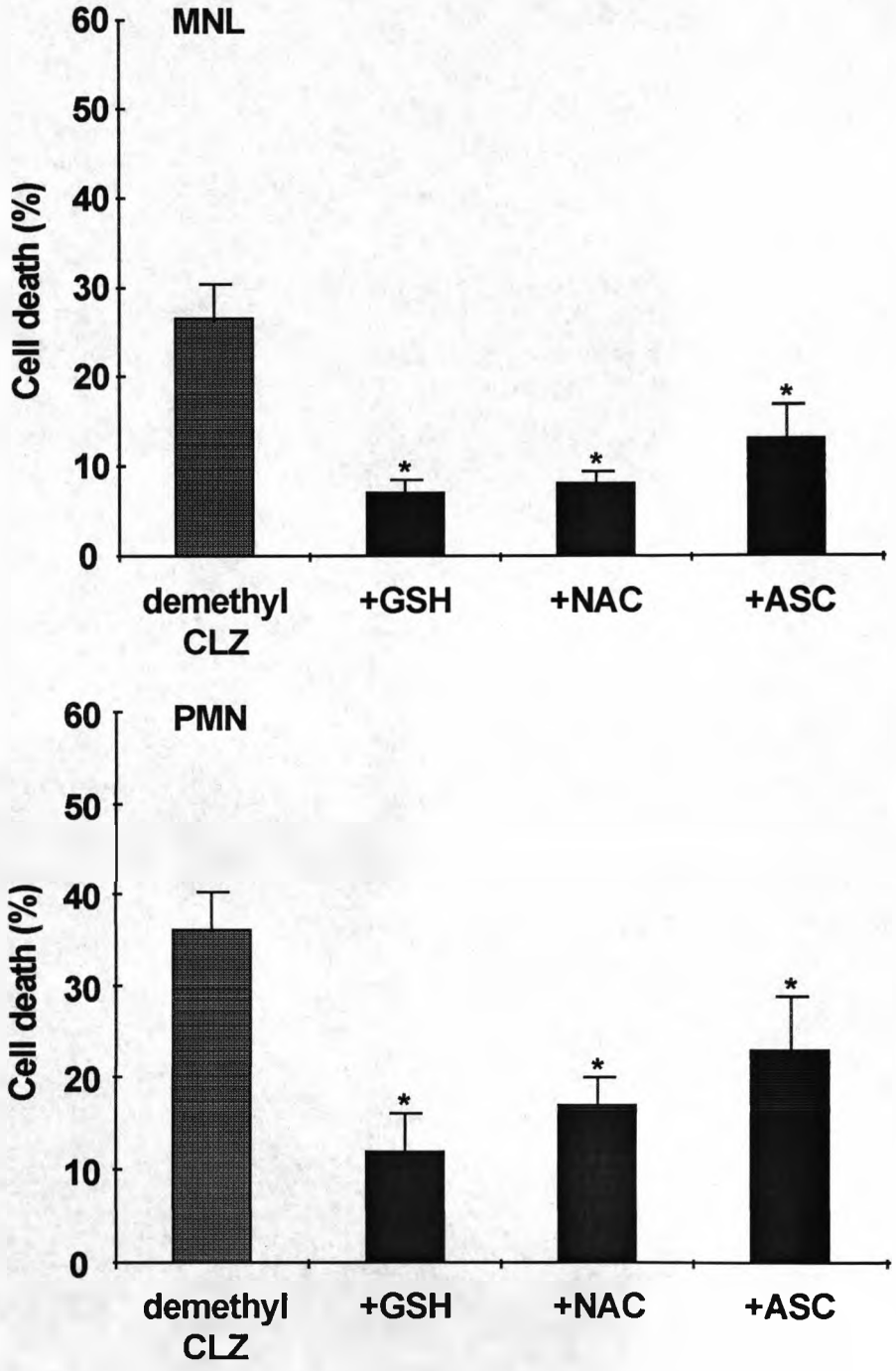


Figure 4.6 Cytotoxicity of demethyl CLZ (30μM) towards MNL and PMN, in the presence of GSH (1mM), *N*-acetyl CYS (NAC; 1mM) and ASC (1mM). The results represent the mean ± S.D. of 1 experiments carried out in quadruplicate. Statistical analysis was performed by comparing incubations with and without the antioxidants (*$P < 0.05$).

4.3.5 Determination of the Extent of Bioactivation

When CLZ, demethyl CLZ and CLZ *N*-oxide (30μM) were incubated individually with HRP, hydrogen peroxide and GSH in the presence and absence of PMN, there was extensive bioactivation to the respective C6-glutathionyl and C9-glutathionyl adducts irrespective of the presence or absence of cells (table 4.1). An ion-current chromatogram (m/z 632) obtained by incubating CLZ with the activating system and GSH showed the formation of C6 and C9 glutathionyl conjugates (fig. 4.7). In the absence of GSH, the ion-current chromatograms (m/z 343) revealed the presence of two peaks, one of which co-eluted with CLZ *N*-oxide, and the other was a more polar metabolite putatively identified as 7-hydroxy CLZ (fig. 4.8). There was no metabolism of CLZ when either HRP or hydrogen peroxide was omitted from the incubations.

	% conversion to C6-glutathionyl CLZ[a]		% conversion to C9-glutathionyl CLZ[a]	
	+PMN	-PMN	+PMN	-PMN
	(%)		(%)	
CLZ	25.5 ± 2.4	26.9 ± 1.0	5.0 ± 1.2	5.9 ± 0.4
Demethyl CLZ	28.7 ± 0.8	18.2 ± 3.3	12.6 ± 0.6	8.9 ± 1.3
CLZ *N*-oxide	23.1 ± 0.1	19.1 ± 3.6	7.7 ± 0.2	6.2 ± 1.0

Table 4.1 Conjugation of CLZ and its stable metabolites with GSH in the presence and absence of PMN.
[a] All incubations contained the activating system, HRP and hydrogen peroxide, together with 30μM of each compound. The results represent the mean ± S.D. of triplicate incubations.

4.3.6 Metabolism-Dependent Depletion of Intracellular Glutathione by Clozapine and its Metabolites

There was no GSH depletion observed when CLZ, demethyl CLZ and CLZ *N*-oxide were incubated alone with either MNL (fig. 4.9) or PMN (fig. 4.10). Similarly, there was no GSH depletion when CLZ was incubated with either HRP or hydrogen peroxide in isolation. In the presence of the full activating system (i.e., HRP and hydrogen peroxide), in contrast, CLZ resulted in a concentration-dependent depletion of intracellular GSH in both MNL (fig. 4.9) and PMN (fig. 4.10) ($P < 0.05$, at all concentrations when compared to incubations without drug). Demethyl CLZ also caused GSH depletion but this was significantly ($P < 0.05$) less than that with CLZ. CLZ *N*-oxide, however, did not cause any depletion of GSH in either PMN or MNL in the presence of both HRP and hydrogen peroxide.

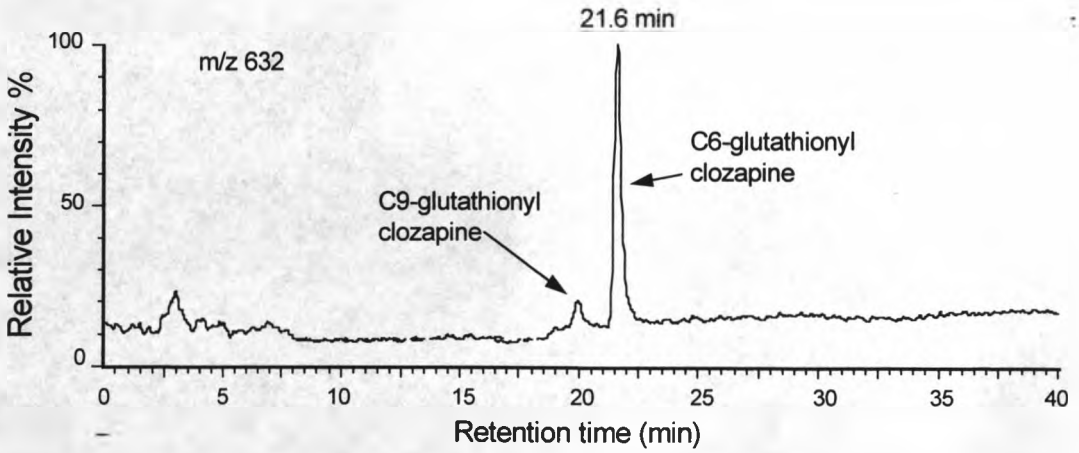


Figure 4.7 Ion-current chromatogram of CLZ (30μM) incubated with HRP (20 units) and hydrogen peroxide (10μM) in the presence of GSH (1mM). The gradient system used was gradient 2. Ions were protonated molecules.

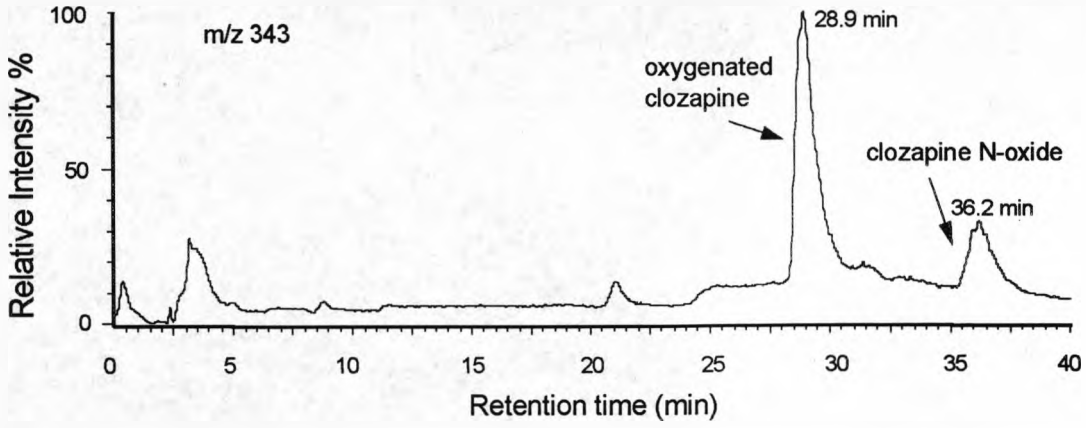


Figure 4.8 Ion-current chromatogram of the metabolites of CLZ (30μM) incubated with HRP (20 units) and hydrogen peroxide (10μM) in the absence of GSH. The gradient system used was gradient 2. Ions were protonated molecules.

4.4 Discussion

The purpose of this study was to investigate the relative abilities of CLZ, its stable metabolites and reactive metabolites, to cause damage to neutrophils at therapeutically relevant concentrations in order to gain an insight into the mechanism(s) of CLZ-induced agranulocytosis.

Assessment of the cytotoxic potential of the parent drug and its stable metabolites was performed by incubating the compounds with lymphocytes and neutrophils. In accordance with a previous study (Gerson *et al.*, 1994), CLZ and its major stable metabolites were not directly toxic even at relatively high concentrations. Assessment of whether chemically reactive metabolites are cytotoxic is not as straightforward largely because these metabolites are, by definition, unstable and therefore cannot be isolated. Thus, to date, it has not been possible to determine whether the nitrenium ion which has been postulated to be the chemically reactive metabolite derived from CLZ is cytotoxic.

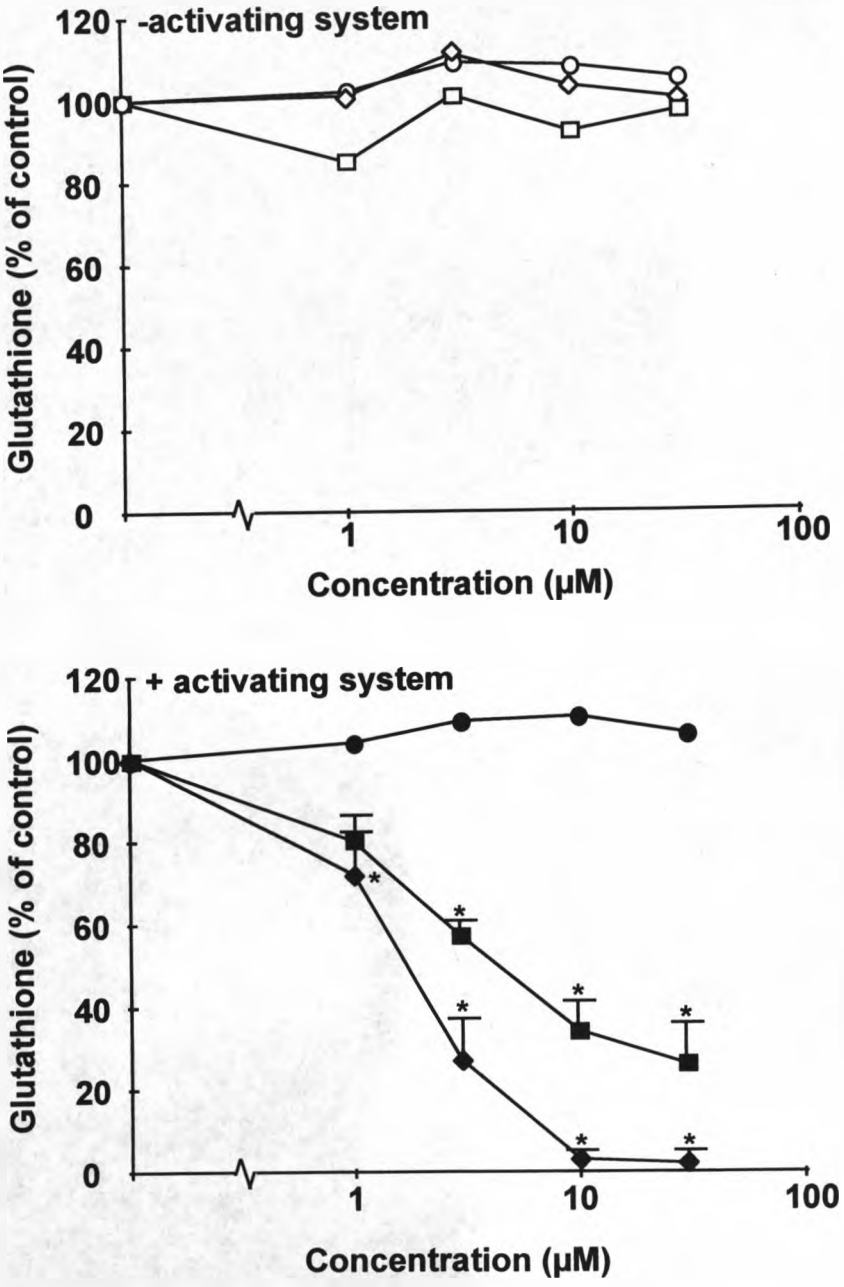


Figure 4.9 **GSH depletion in MNL by CLZ (◆), demethyl CLZ (■) and CLZ *N*-oxide (●) in the presence and absence of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of 3 separate experiments using cells from different individuals (all incubations carried out in triplicate). Statistical analysis was performed by comparing incubations of the compounds in the presence and absence of the activating system at the same concentrations (*P < 0.05). Error bars have been omitted from some of the curves for the sake of clarity.**

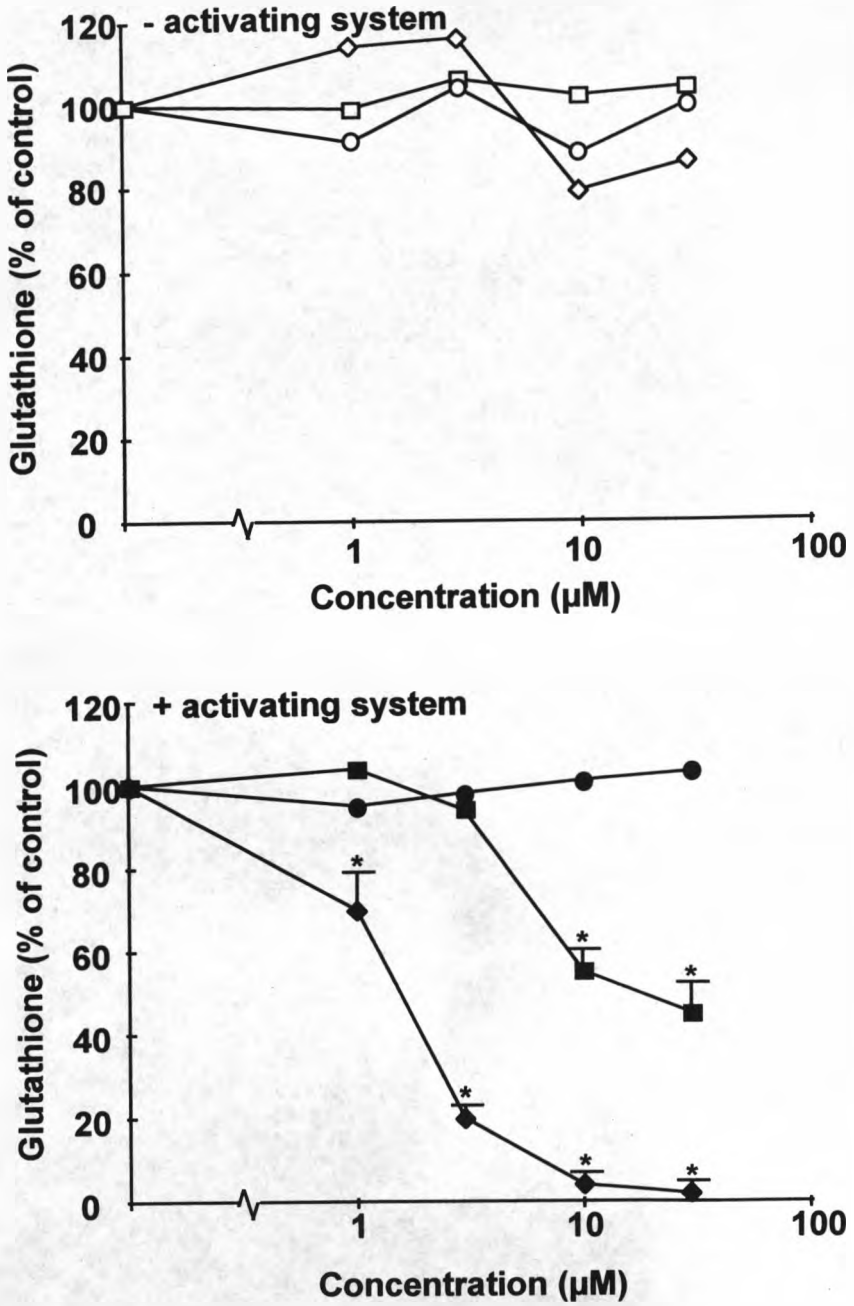


Figure 4.10 GSH depletion in PMN by CLZ (◆), demethyl CLZ (■) and CLZ *N*-oxide (●) in the presence and absence of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of 3 separate experiments using cells from different individuals (all incubations carried out in triplicate). Statistical analysis was performed by comparing incubations of the compounds in the presence and absence of the activating system at the same concentrations (*P < 0.05). Error bars have been omitted from some of the curves for the sake of clarity.

In order to ascertain cytotoxicity of chemically reactive metabolites, an ***in vitro*** cytotoxicity assay in which such metabolites can be generated ***in situ*** by a liver microsomal metabolising system has been devised (Spielberg, 1980; Riley *et al.*, 1988). Using such an assay, CLZ has previously been shown to be bioactivated by human and phenobarbitone-induced mouse microsomes to a metabolite that was cytotoxic toward lymphocytes (Pirmohamed *et al.*, 1992). A similar investigation has also been conducted by Tschen *et al.* (1996) using a rat liver microsomal system. However, studies in our laboratory showed that a microsomal system could not be used to assess neutrophil cytotoxicity because the microsomes bound to the cell surface and caused non-specific cell death (Dr. M. Pirmohamed, The University of Liverpool; unpublished data). Therefore, the major purpose of this study was to devise an assay which could be used to assess cytotoxicity of the nitrenium ion of CLZ towards neutrophils.

The nitrenium ion can be generated by co-incubation with hypochlorous acid (Liu and Uetrecht, 1995). However, use of hypochlorous acid produced unacceptably high background cytotoxicity (fig. 4.2). Both HRP and MPO can also metabolise CLZ to a chemically reactive species (Fischer *et al.*, 1991). Although HRP and MPO are not homologous in structure, the catalytically active amino acids are positioned in a similar manner (Welinder, 1985). Additionally, the metabolites produced by HRP are qualitatively similar to those produced by MPO (Eastmond *et al.*, 1986). With CLZ, it was recently shown that the metabolites produced by HRP are identical to those produced by both neutrophils and neutrophil precursors (Maggs *et al.*, 1995). Furthermore, the metabolic turnover

to the GSH conjugates with HRP (9.8μmols/l) was significantly ($P < 0.05$) higher than with MPO (3μmols/l). Therefore, HRP was used in conjugation with hydrogen peroxide to determine whether the reactive metabolite was cytotoxic.

The results presented in this chapter clearly show the nitrenium metabolite is cytotoxic towards both MNL and PMN, and indeed, cytotoxicity was seen at concentrations much lower than those needed with microsomes (where turnover to the GSH conjugates is comparatively lower, 0.3μmols/l; Pirmohamed *et al.*, 1995). Moreover, cytotoxicity was seen at therapeutic concentrations. Interestingly, cytotoxicity was also accompanied by depletion of GSH. In fact, GSH depletion occurred at concentrations which were lower than that needed for cytotoxicity. This indicates that conjugation with GSH serves as a detoxification mechanism, and when GSH is depleted below a threshold level, binding to neutrophil proteins may initiate cellular death. In support of this, exogenous GSH afforded protection to the cells. The role of GSH in preventing cell death has been examined previously in relation to anti-cancer agents (O'Brien and Tew, 1996). Indeed, an increase in cellular GSH content is one of the major mechanisms by which cancer cells develop resistance to chemotherapeutic agents. In accordance with previous studies (Maggs *et al.*, 1995), chemical analysis of the incubations showed that bioactivation of CLZ in the presence of exogenous GSH was accompanied by the formation of C6- and C9-glutathionyl CLZ (fig. 4.11). Restriction of the electron delocalisation to the diazapine nitrogens and chlorobenzenoid ring, a consequence of the dibenzodiazepine moiety being non-planar, explains the regioselectivity of GSH conjugation. In addition to GSH,

both *N*-acetyl CYS and ASC also protected the cells from CLZ-induced toxicity, by either conjugation or reduction reactions. The cytoprotection offered by ASC is of particular interest. Concentrations of 50-150μM and 1.0-1.4mM have been recorded in plasma and the cytosol of neutrophils, respectively (Washko *et al.*, 1989). ASC functions as a reducing agent and at physiological concentration has been shown to reduce the cation free radical of CLZ (Fischer *et al.*, 1991). Moreover, schizophrenics have decreased ASC concentrations (Suboticanec *et al.*, 1990), and it has thus been suggested that ASC supplementation may prevent CLZ-induced agranulocytosis (Fischer *et al.*, 1991). However, to date, this has not been tested in clinical studies.

In this study, the two stable metabolites of CLZ, demethyl CLZ and CLZ *N*-oxide, were also examined to investigate whether they underwent bioactivation to cytotoxic metabolites. The results suggest that bioactivation of demethyl CLZ may contribute to the pathogenesis of the agranulocytosis. The putative N4-demethylated nitrenium ion also exhibited cytotoxicity but this was less than that observed with the toxic metabolite of CLZ, and was prevented by thiols and ASC. GSH depletion was also observed, but again was less than with CLZ. In contrast, with CLZ *N*-oxide, no cytotoxicity or GSH depletion was observed on incubation with HRP and hydrogen peroxide. It was initially thought that the *N*-oxide did not undergo bioactivation to the respective nitrenium ion. However, HPLC and LC-MS analysis showed the formation of GSH adducts with the *N*-oxide indicating that it was being activated (table 4.1). Toxicity and GSH

depletion may not have been seen with CLZ *N*-oxide because of the increased polarity of the N4-nitrogen which may be preventing the molecule from crossing the cell membrane. If that is the case, then it would dictate that reactive metabolites of CLZ and demethyl CLZ are crossing the membrane and causing toxicity by interacting with intracellular proteins. Additionally, it is possible that the reactive metabolite derived from the *N*-oxide may bind to cell surface proteins on neutrophils, and lead to their depletion.

The cytotoxicity induced by the reactive metabolite of CLZ may not necessarily be due to a direct interaction with essential cellular proteins but may be due to an indirect effect on cellular function. For example, binding to the PMN cell membrane may cause the release of cytokines such as TNF-α (Pollmacher *et al.*, 1996), which then induce cell death in neutrophils or their precursors. The role of TNF-α in particular merits further investigation for three reasons: first, it has recently been shown that CLZ increases plasma levels of TNF-α along with the TNF receptors p55 and p75 (Pollmacher *et al.*, 1996). Second, in hepatocytes, GSH depletion can sensitise the cells to TNF-α mediated cytotoxicity (Xu and Czaja, 1996). Third, CLZ agranulocytosis has recently been shown to be associated with various polymorphisms in the TNF-α gene (Turbay *et al.*, 1996); these polymorphisms are known to modulate TNF-α secretion (Wilson and Duff, 1995). CLZ also causes a dose-dependent decrease in GM-CSF release in bone marrow cultures (Sperner-Unterweger *et al.*, 1993), thus the use of GM-CSF in treating CLZ agranulocytosis is a logical therapeutic manoeuvre (Pirmohamed and Park, 1997). In this study, although cell death was

used as an end-point, it is important to note that the two major forms of cell death, apoptosis and necrosis have not been distinguished (Corcoran *et al.*, 1994). TNF-α is known to induce apoptosis (Takeda *et al.*, 1993) while GM-CSF inhibits apoptosis (Brack *et al.*, 1992). Thus, the possible combination of GSH depletion (as a result of drug bioactivation), reduced GM-CSF production and increased TNF-α production, could predispose to CLZ-induced agranulocytosis by enhancing apoptosis.

Although the data presented in this chapter suggests that the pathogenesis of CLZ-induced agranulocytosis is due to direct effects of its reactive metabolites on neutrophils and their precursors, the involvement of immune mechanisms cannot be completely discounted. It is possible that *in vivo* there is binding of the reactive metabolites of CLZ to neutrophil surfaces which is not enough to cause cell death, but may act as a potent immunogenic stimulus and initiate an immune response. However, to date, there has been no convincing evidence to implicate an immune mechanism in CLZ agranulocytosis (Pirmohamed and Park, 1997).

In summary, this study shows that CLZ and demethyl CLZ are bioactivated *in vitro* to chemically reactive nitrenium metabolites which cause neutrophil cytotoxicity at drug concentrations that can be achieved *in vivo*. Whether bioactivation occurs *in vivo* in man is unknown but is suggested by studies performed in rodents (Maggs *et al.*, 1995). The mechanism by which these metabolites cause neutrophil depletion is unknown and requires further investigation.

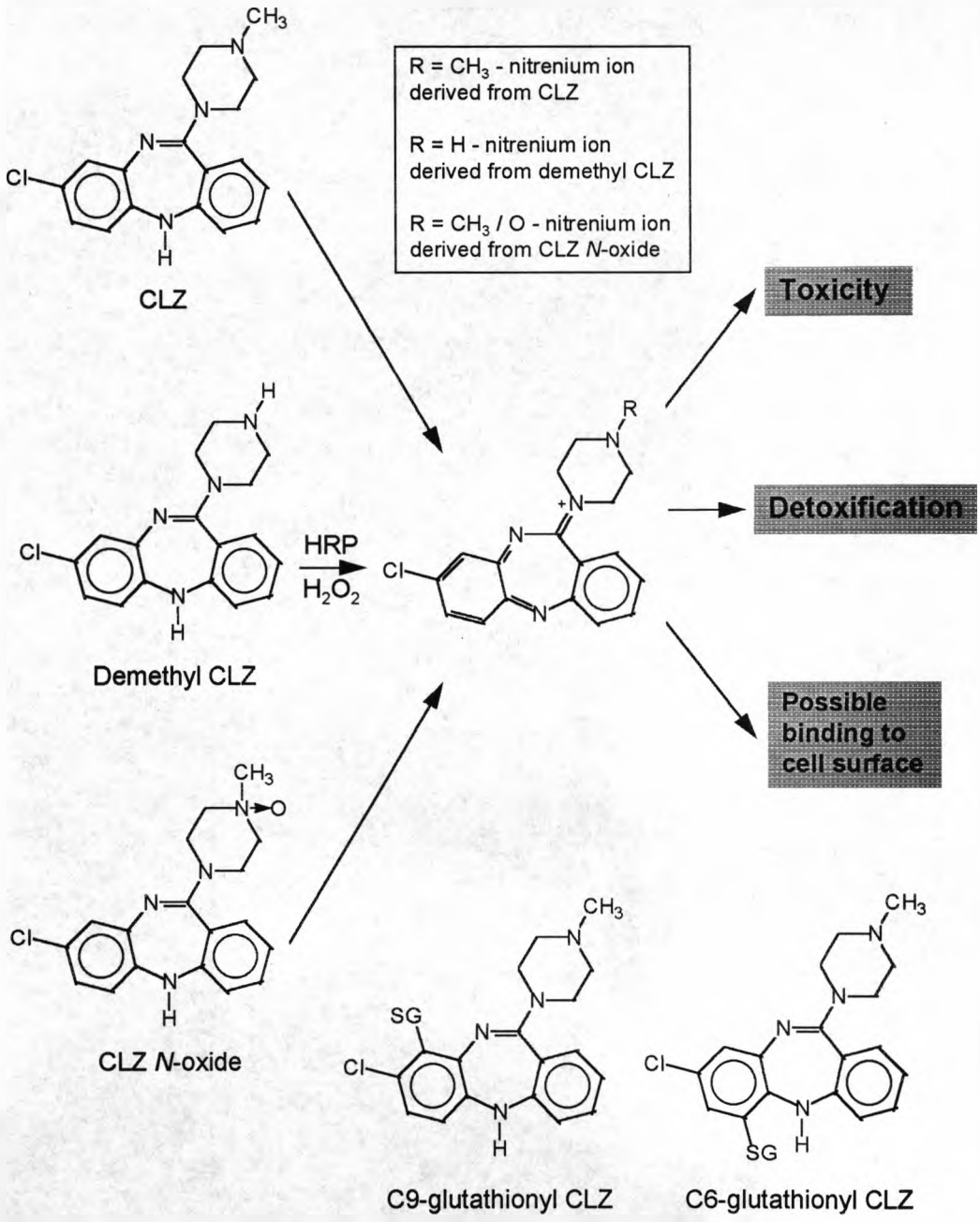


Figure 4.11 Bioactivation of CLZ and its stable metabolites by horseradish peroxidase.

Chapter 5:

Metabolism-Dependent Neutrophil Cytotoxicity of Amodiaquine : A Comparison with Pyronaridine and Related Antimalarial Drugs

Contents **Page**

5.1 Introduction

There are few effective antimalarial drugs; most of Africa still relies on chloroquine (CQ) because of its low cost, widespread availability and good oral tolerance. CQ, however, can no longer be relied upon for the treatment of *Plasmodium falciparium* malaria because of the spread of resistant parasites throughout Africa and most of the developing world (Bjorkman and Philips-Howard, 1990). In Asia, Oceania and South America, resistance has been overcome primarily by the use of mefloquine and derivatives of artimesinin which are unfortunately too expensive for widespread use in Africa.

Amodiaquine (AQ) is a 4-aminoquinoline antimalarial agent synthesised in the late 1940's by Burckhalter *et al.* (1948). It contains a 7-chloroquinoline substituted system connected to a phenolic-butyldiamino side chain (fig. 5.1; section 1.6). In the 1980's, investigators demonstrated that AQ was effective against both CQ-resistant and sensitive isolates of *Plasmodium falciparum* (Watkins *et al.*, 1984). This observation resulted in an increase in its use; however, life-threatening agranulocytosis and hepatotoxicity in about 1 in 2000 patients during prophylactic administration led to its withdrawal (Neftel *et al.*, 1986; Hatton *et al.*, 1986; Larry *et al.*, 1986). Despite its toxicity, AQ is still used for the treatment of acute malaria if the risk of infection outweighs the potential for drug toxicity (WHO, 1993).

The mechanism of AQ-induced agranulocytosis remains unclear, but both direct stem cell toxicity and immune-mediated mechanisms have been implicated (Douer *et al.*, 1985; Rhodes *et al.*, 1986). AQ undergoes extensive bioactivation

to an electrophilic quinoneimine metabolite *in vivo* in the rat (Harrison *et al.*, 1992) and *in vitro* by both hepatic microsomes (Jewell *et al.*, 1995) and phorbol ester-stimulated neutrophils (Tingle *et al.*, 1995). Subsequent oxidative stress or conjugation to cysteinyl sulphydryl groups of proteins are likely to be involved in the induction of toxicity by either cytotoxic or immunological mechanisms (Maggs *et al.*, 1988; Clarke *et al.*, 1990). However, the factors determining individual susceptibility are unknown.

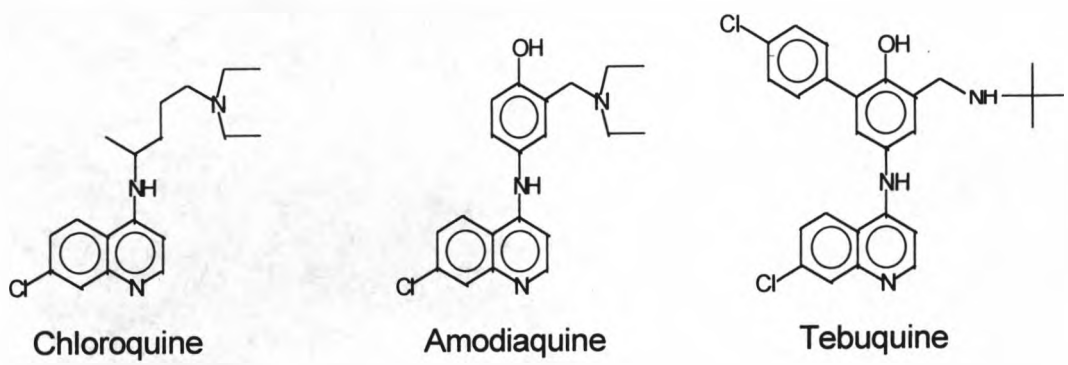


Figure 5.1 Chemical structures of CQ, AQ and TEB.

The toxicity of AQ and lack of a cheap replacement for the treatment of CQ-resistant *Plasmodium falciparum* has prompted a search for alternative antimalarial agents. The preparation of tebuquine (TEB; fig. 5.1) (Werbel *et al.*, 1986) has been one of the major advances in the development of potent 4-aminoquinolines. The introduction of a 5'-chlorophenyl group conferred maximal antimalarial activity (Werbel *et al.*, 1986) and blocked glutathione (GSH) conjugation *in vitro* (chapter 2). On the basis of its potent antimalarial activity, TEB was selected for preclinical toxicology studies prior to evaluation in man. However, development was halted due to neutrophil toxicity, which was

characterised histologically by foamy macrophages (M. Werbel and H. Chung, Walter Reed Army Institute of Research, personal communication).

Pyronaridine (PYRO; fig. 5.2), is another promising candidate for the treatment of CQ-resistant malaria. It contains an azo-acridine group rather than the quinoline group present in AQ. In addition, the 4-aminophenol function contains pyrolidinomethyl substituents at the 3'- and 5'- positions. Clinical trials of PYRO have shown a favourable efficacy profile (Chen *et al.*, 1992; Ringwald *et al.*, 1996), and to date, little toxicity has been observed (Shao, 1990). However, the toxicity of PYRO with long-term use is not known.

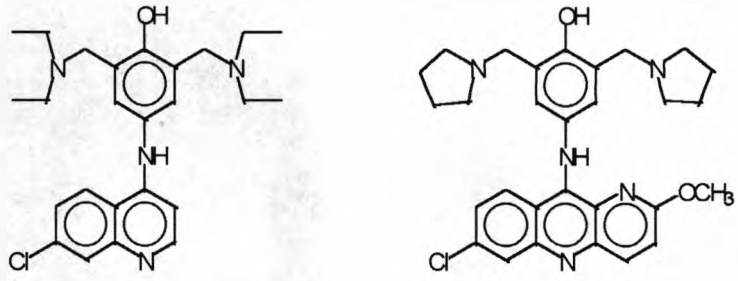


Figure 5.2 Chemical structures of CYC and PYRO.

Chapter 2 compared AQ to other antimalarials including cycloquine (CYC; fig. 5.2), bis pyroquine and PYRO in order to understand the chemical features involved in drug accumulation, bioactivation and cellular function. This study demonstrated that bis-mannich antimalarials including CYC and PYRO appeared to have an advantage over mono-mannichs with respect to the

avoidance of bioactivation and excessive lysosomal accumulation. Furthermore, if PYRO were to be administered for malaria prophylaxis, the results suggest that it may be less likely than AQ to cause life-threatening agranulocytosis. In contrast to these findings, Winstanley (1996) has expressed concern over the structural similarities of AQ and PYRO, and thus the potential for PYRO to cause idiosyncratic toxicity.

The aim of this study therefore, was to further investigate the metabolism and toxicity of AQ, PYRO and related antimalarial agents. The combination of horseradish peroxidase (HRP) and hydrogen peroxide were used to generate the same metabolites as neutrophils *in vitro* (Eastmond *et al.*, 1986; Sadler *et al.*, 1988; chapter 4). Furthermore, the ability of the reactive metabolite scavengers (GSH; N-acetyl cysteine [CYS]; ascorbic acid [ASC]) to inhibit drug-induced cytotoxicity was assessed.

5.2 Materials and Methods

5.2.1 Chemicals

AQ, ASC, bromobimane, CQ, gentamycin, GSH, HRP (type VI), human serum albumin (HSA), *N*-acetyl CYS, *N*-ethylmorpholine, trichloroacetic acid and trypan blue were all obtained from Sigma Chemical Co. (Poole, UK). Hydrogen peroxide was obtained from BDH (Dorest, UK). TEB was a gift from Parke Davis (Ann Arbor, MI), and PYRO was a gift from Dr. D. C. Warhurst (London School of Hygiene and Tropical Medicine). CYC was synthesised using the method of Barlin and Tan (1985), and AQ quinoneimine was synthesised by the

method of Harrison *et al.* (1992). The purity of all compounds ranged from 95-100%, as assessed by HPLC and NMR spectroscopy. Monopoly® resolving medium (Ficoll Hypaque; density, 1.114 g/ml) and Lymphoprep® (1.077 g/ml) were obtained from ICN Biomedicals (Bucks, UK) and Nycomed (Birmingham, UK), respectively. All solvents were of HPLC grade and were purchased from Fischer Scientific plc (Loughborough, UK).

5.2.2 Isolation of Human Peripheral Blood Cells

Neutrophils (polymorphonuclear leucocytes; PMN) and lymphocytes (mononuclear leucocytes; MNL) were isolated from the venous blood of 10 healthy volunteers (age range, 21-40 years) on a dual density gradient of Monopoly resolving medium and Lymphoprep, as described in section 2.2.2. Cells were resuspended in HEPES (pH 7.4) and diluted to the concentration required for each experiment. Both cell types were greater than 98% pure and 95% viable as assessed by Wright's stain and trypan blue dye exclusion (section 2.3.1).

5.2.3 Determination of Depletion of Intracellular Glutathione

AQ has previously been shown to deplete intracellular GSH from PMN in the presence of phorbol 12-myristate 13-acetate (PMA), a cell-activating factor (Tingle *et al.*, 1995; chapter 2). In order to determine whether AQ and chemically related antimalarial drugs cause peroxidase-dependent GSH depletion, the

fluorescent probe, bromobimane, which reacts with intracellular GSH was used (Cotgreave and Moldeus, 1986).

Initial experiments were designed to evaluate the role of individual components of the peroxidase activating system (described in chapter 4) on AQ-induced GSH depletion. GSH (3μM) was incubated with AQ (30μM), AQ / HRP (20 units), AQ / hydrogen peroxide (10μM) and AQ / HRP (20 units) / hydrogen peroxide (10μM) in HEPES buffer at 37°C for 20min. The total incubation volume was 1ml. Bromobimane (3mM) was then added to the incubations, which were left in the dark at 37°C for a further 5min. Aliquots (50μl) were analysed by fluorescence HPLC, as described previously (section 2.2.7).

A cell concentration of 0.5 x 10^6 PMN/ml was used to demonstrate intracellular GSH depletion. The cells were incubated with drugs (1-100μM) in HEPES buffer at 37°C for 1h in the presence and absence of HRP (20 units) and hydrogen peroxide (10μM). GSH levels were determined using the protocol described in section 2.2.7. The compounds were dissolved in dimethyl sulfoxide (DMSO; 1%, v/v), a concentration which did not deplete GSH.

5.2.4 Determination of Direct and Metabolism-Dependent Cytotoxicity of the Antimalarials

The direct and peroxide-dependent cytotoxicity of AQ, AQ quinoneimine and structurally related antimalarial drugs (CQ, TEB, CYC and PYRO, 1-100μM) was determined by incubating either PMN (1 x 10^6/ml) or MNL (1 x 10^6/ml) in 15ml plastic conical tubes using a similar method to that described in

section 4.2.7. The cells were incubated in the presence or absence of HRP (20 units) and hydrogen peroxide (10μM) and cell viability was assessed after 16h by trypan blue dye exclusion (0.2% w/v, trypan blue). Some incubations also contained the antioxidants GSH (1mM), *N*-acetyl CYS (1mM) or ASC (1mM). Drugs were added in DMSO, which as a 1% (v/v) solution, was not toxic to the cells.

5.2.5 Metabolism of the Antimalarials by Horseradish Peroxidase and Hydrogen Peroxide

Drugs (AQ, CYC, PYRO and TEB; 100μM; DMSO, 1% v/v) were incubated at 37^{0}C for 2h in the presence or absence of HRP (20 units), hydrogen peroxide (10μM) and GSH (1mM) as described previously (Potter *et al.*, 1985; Eastmond *et al.*, 1986; Thompson *et al.*, 1988). The reaction was initiated by the addition of hydrogen peroxide, and GSH was added within 30sec. After 2h, the reaction was terminated by the addition of ethanol (1ml). The solutions were evaporated to dryness under a stream of nitrogen and stored at -20^{0}C for no longer than 3 days prior to analysis by HPLC and LC-MS.

5.2.6 Analysis of Metabolites by HPLC

Incubations containing drug, HRP, hydrogen peroxide and GSH were reconstituted in an ethanol-water mixture (1:1; 500μl) prior to HPLC analysis. The HPLC system was the same as that described in section 3.2.2. GSH conjugates were eluted from either a μbondapak C18 (AQ and TEB; flow rate

1ml/min) or Columbus (HPLC Technology, Macclesfield, UK) column (CYC and PYRO; flow rate 0.75ml/min) with a mobile phase consisting of acetonitrile (ACN) and 20mM ammonium formate (pH 2.75). Gradient conditions varied slightly with each drug: AQ, 5-20% ACN over 18min, followed by 20% for 8min, with re-equilibrium at 5% for 5min; TEB, 10-40% ACN over 25min, followed by 40% for 20min, with re-equilibrium at 10% for 5min; CYC, 5-20% ACN over 35min, with re-equilibrium at 5% for 5min; PYRO, 5-12% ACN over 12min, followed by 12% for 18min, with re-equilibrium at 5% for 5min.

5.2.7 Analysis of Metabolites by LC-MS

Triplicate incubations containing drug, HRP, hydrogen peroxide and GSH were reconstituted in ethanol-water (1:1; 100µl) and combined to provide the mass required for LC-MS. Samples were eluted using the HPLC gradients described above. The mobile phase was delivered to the LCMS interface by two Jasco PU980 pumps (Jasco Corporation, Tokyo, Japan) under the conditions described in section 3.2.8. Full-scan spectra were acquired over m/z 50 to 800, and the extent of fragmentation was modulated via the cone voltage. The data was processed by MassLynx 2.1 software.

5.2.8 Statistical Analysis

The results are presented as mean ± S.D. Statistical analysis was performed by the Mann-Whitney test, accepting $P < 0.05$ as significant. IC_{50} values ± S.E.M. were calculated using the four-parameter logistic method (Grafit

program; Erithacus software, Staines, UK) by interpolation of the logarithmic concentration curve. The S.E.M. was calculated from the error associated with the sigmoidal fit of the curve.

5.3 Results

5.3.1 Depletion of Intracellular Glutathione

AQ has previously been shown to deplete intracellular GSH (Tingle *et al.*, 1995; chapter 2). Therefore AQ was used as a test compound to determine the extent of GSH depletion when a drug was incubated with individual components of the HRP / hydrogen peroxide activating system. Depletion was dependent on the presence of both HRP and hydrogen peroxide, being significantly greater (98.6 ± 0.7%) than in the absence of HRP (29.3 ± 4.6%,; $P < 0.05$) (fig. 5.3).

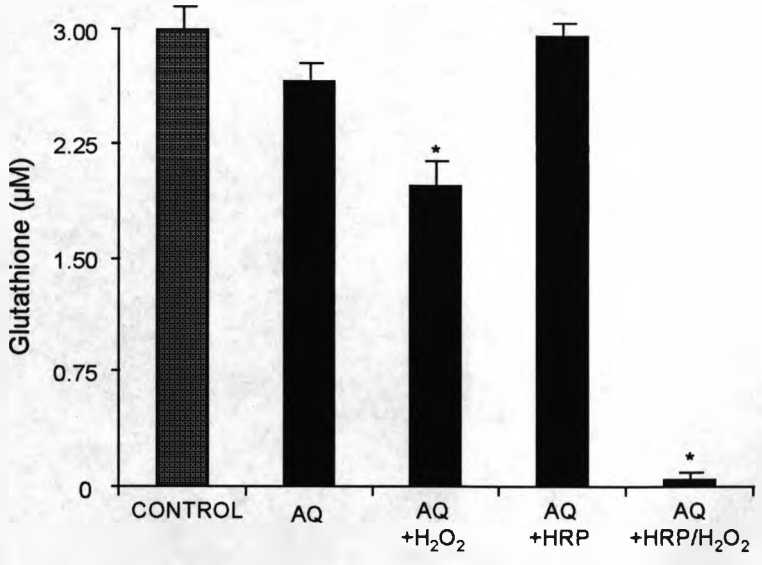


Figure 5.3 GSH (3µM) depletion by AQ (30µM) in the presence and absence of HRP (20 units) and hydrogen peroxide (10µM). Thiol levels were measured using bromobimane. Each bar represents the mean ± S.D. of an incubation carried out in triplicate. Statistical analysis was performed by comparing the ability of AQ to deplete GSH (in the presence and absence of HRP and hydrogen peroxide) with that of solvent alone (*$P < 0.05$).

None of the antimalarial compounds tested (1-100μM) depleted intracellular GSH when incubated alone with PMN (fig. 5.4 and 5.5). In the presence of the full activating system however, AQ, CYC and TEB resulted in a concentration-dependent depletion of GSH (1-100μM; $P < 0.05$). PYRO also produced peroxidase-dependent depletion of GSH, but this was significant only at concentrations of 3μM and above (fig. 5.4 and 5.5; $P < 0.05$). There was no GSH depletion with CQ either in the presence or absence of the activating system.

AQ quinoneimine, which is known to be a reactive metabolite of AQ (Maggs *et al.*, 1988), produced greater than 90% depletion of GSH at 10μM, either in the presence or absence of both HRP and hydrogen peroxide (fig. 5.4).

5.3.2 Direct Cytotoxicity of Antimalarial Drugs Towards Neutrophils

All the compounds (1-100μM) tested apart from CQ caused a concentration-dependent increase in PMN cell death ($P < 0.05$, when compared to solvent alone). Only TEB and AQ quinoneimine caused cell death at concentrations below 10μM (fig. 5.6 and 5.7). MNL cytotoxicity in the presence of the antimalarial compounds was similar to that observed with PMN.

5.3.3 Peroxidase-Mediated Metabolism-Dependent Cytotoxicity

There was no increase in cell death above background values when PMN were incubated with HRP and hydrogen peroxide (i.e., solvent control). In the presence of the full activating system, all compounds tested apart from CQ caused a concentration-dependent increase in cell death ($P < 0.05$, when

compared to solvent alone; fig. 5.6 and 5.7). With the exception of AQ quinoneimine, cell death in the presence of the activating system was significantly greater than with drug alone ($P < 0.05$; fig. 5.6 and 5.7).

5.3.4 Effect of Biological Modifiers on Peroxidase-Mediated Metabolism-Dependent Cytotoxicity

The cytotoxicity of AQ (30μM), TEB (3μM), CYC (30μM) and PYRO (100μM) towards PMN in the presence of the full activating system was reduced to background values when the drugs were co-incubated with either GSH, *N*-acetyl CYS or ASC (fig. 5.8). AQ quinoneimine-mediated cell death was also significantly reduced on co-incubation with the biological modifiers ($P < 0.05$).

5.3.5 Metabolism of Antimalarial Drugs by Horseradish Peroxidase and Hydrogen Peroxide

When AQ was incubated with HRP (20 units), hydrogen peroxide (10μM) and GSH (1mM), LC-MS analysis revealed unchanged AQ and one polar metabolite, identified from its parent ion (m/z 661 $[M+1]^+$) as a GSH adduct; the adduct formed *in vivo* has been identified by co-chromatography and NMR spectroscopy as the C-5' glutathionyl conjugate of AQ (Harrison *et al.*, 1992). Typical absorbance and selected ion (m/z 356 and 661) chromatograms are shown in fig. 5.9. Metabolism of AQ was dependent upon the initial drug concentration, being greatest at 100μM (19.1 ± 3.8%). This concentration was

used for all further studies. TEB was converted to two polar metabolites in the presence of the full activating system and GSH (T1, 6.4 ± 1.3; T2, 33.1 ± 1.7%; fig. 5.10). Unchanged TEB gave a protonated molecule at m/z 466, together with the characteristic fragment (m/z 392), representing loss of the *t*-butyl amino side chain. The principle metabolite (T2) was identified as a glutathionyl conjugate. However, the typical fragment pattern representing loss of the *t*-butyl amino side chain was not observed (fig. 5.10). This result coupled with the identification of a protonated molecule at m/z 686, suggests that TEB may undergo GSH conjugation in an enzyme-mediated substitution reaction involving loss of the nitrogen containing side chain. The minor metabolite T1 gave a protonated molecule at m/z 496.

In contrast to AQ and TEB, only parent ions were identified when either CYC (m/z 441) or PYRO (m/z 518) were incubated with HRP, hydrogen peroxide and GSH.

5.4 Discussion

Given the known toxicity of AQ, it is important to develop congeners which retain efficacy but do not cause agranulocytosis. However, the latter may only become evident after large numbers of patients have been exposed to the drug. Thus, while the structurally related compounds CYC and PYRO are highly effective against CQ-resistant and -sensitive parasites, and although clinical trials have not produced any cases of severe idiosyncratic toxicity (Shao, 1990), it

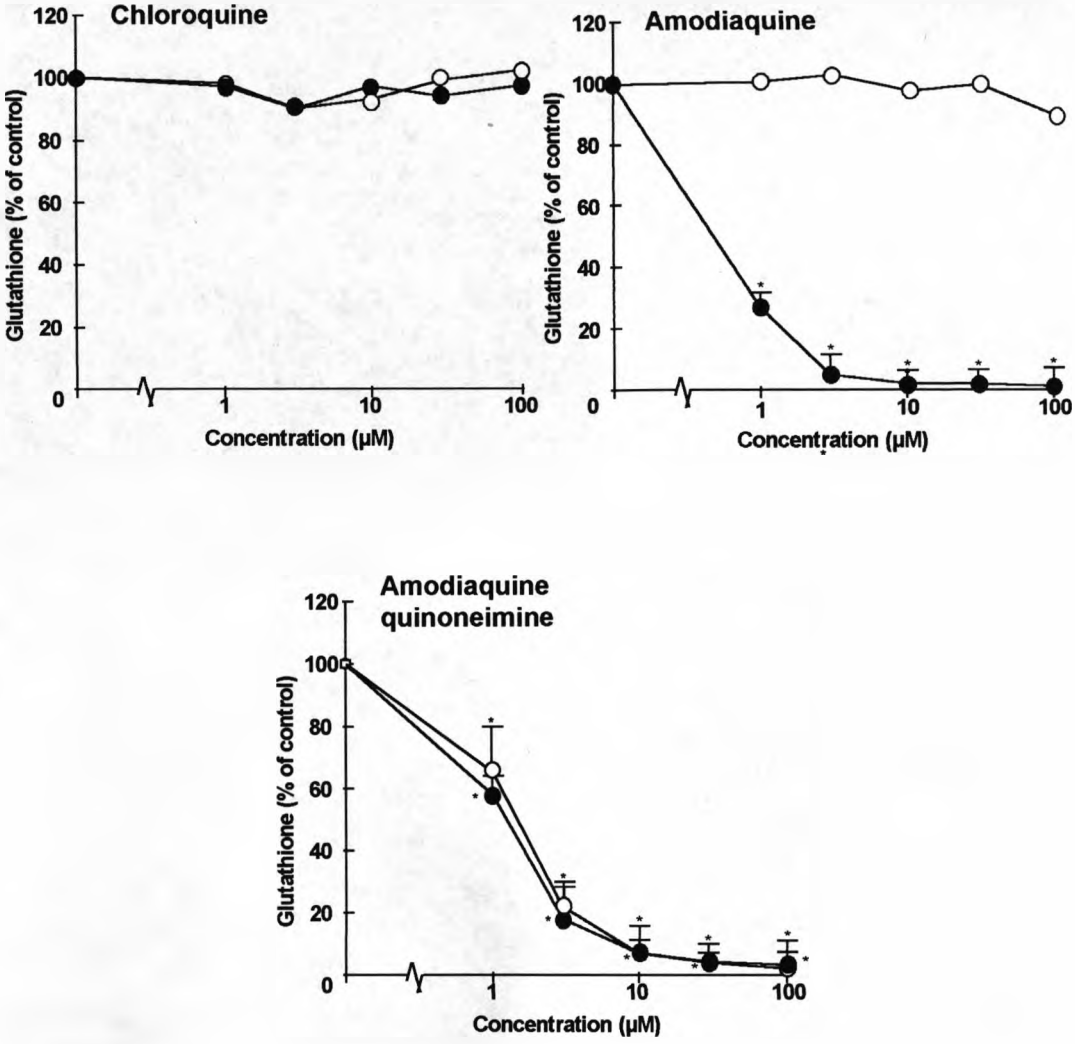


Figure 5.4 GSH depletion in PMN by CQ, AQ and AQ quinoneimine in the presence (●) and absence (○) of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of 3 separate experiments using cells from different individuals (all incubations carried out in triplicate). Statistical analysis was performed by comparing the ability of different concentrations of compounds in depleting GSH with that of solvent alone (*P < 0.05). Error bars have been omitted from some of the data points for the sake of clarity.

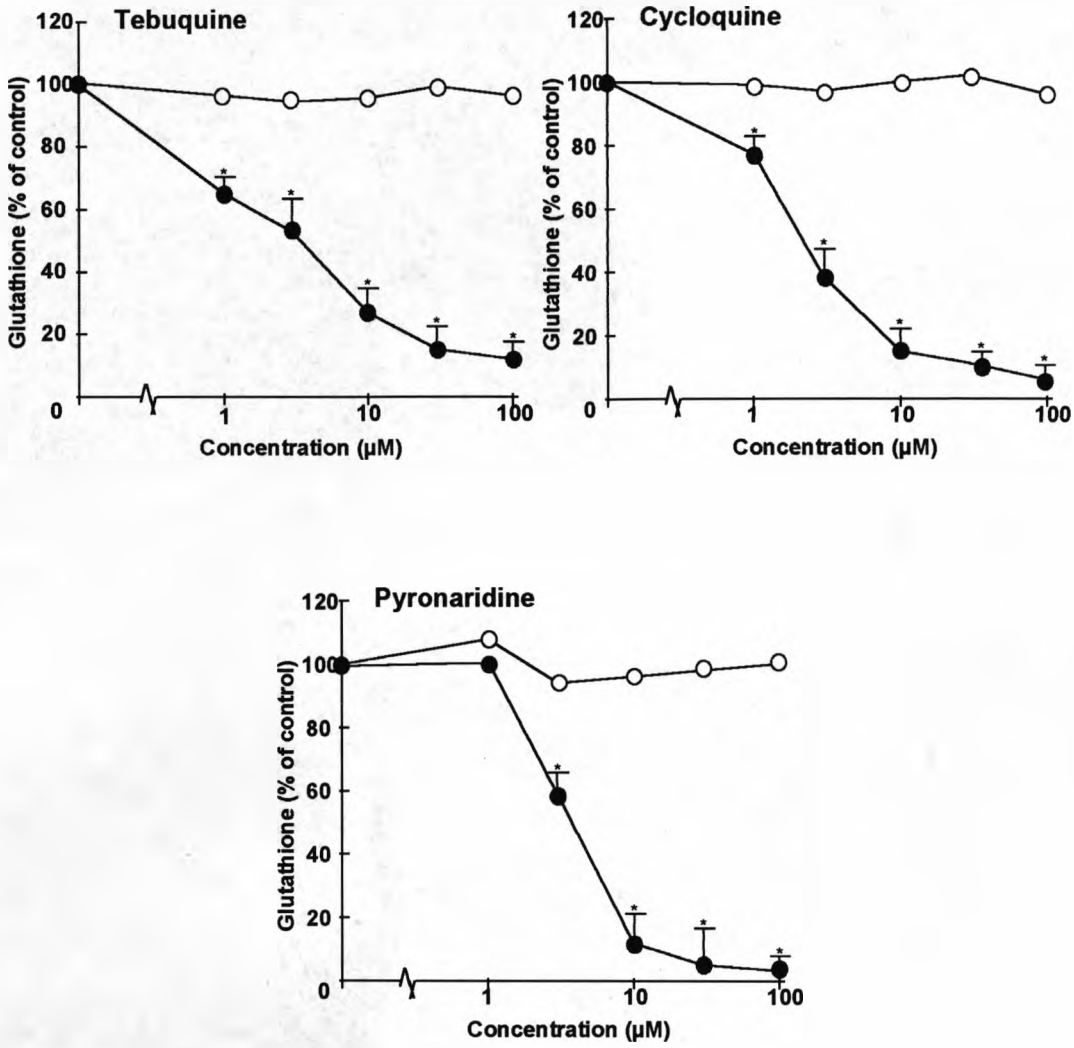


Figure 5.5 GSH depletion in PMN by PYRO, TEB and CYC in the presence (●) and absence (○) of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of 3 separate experiments using cells from different individuals (all incubations carried out in triplicate). Statistical analysis was performed by comparing the ability of different concentrations of compounds in depleting GSH with that of solvent alone (*P < 0.05). Error bars have been omitted from some of the data points for the sake of clarity.

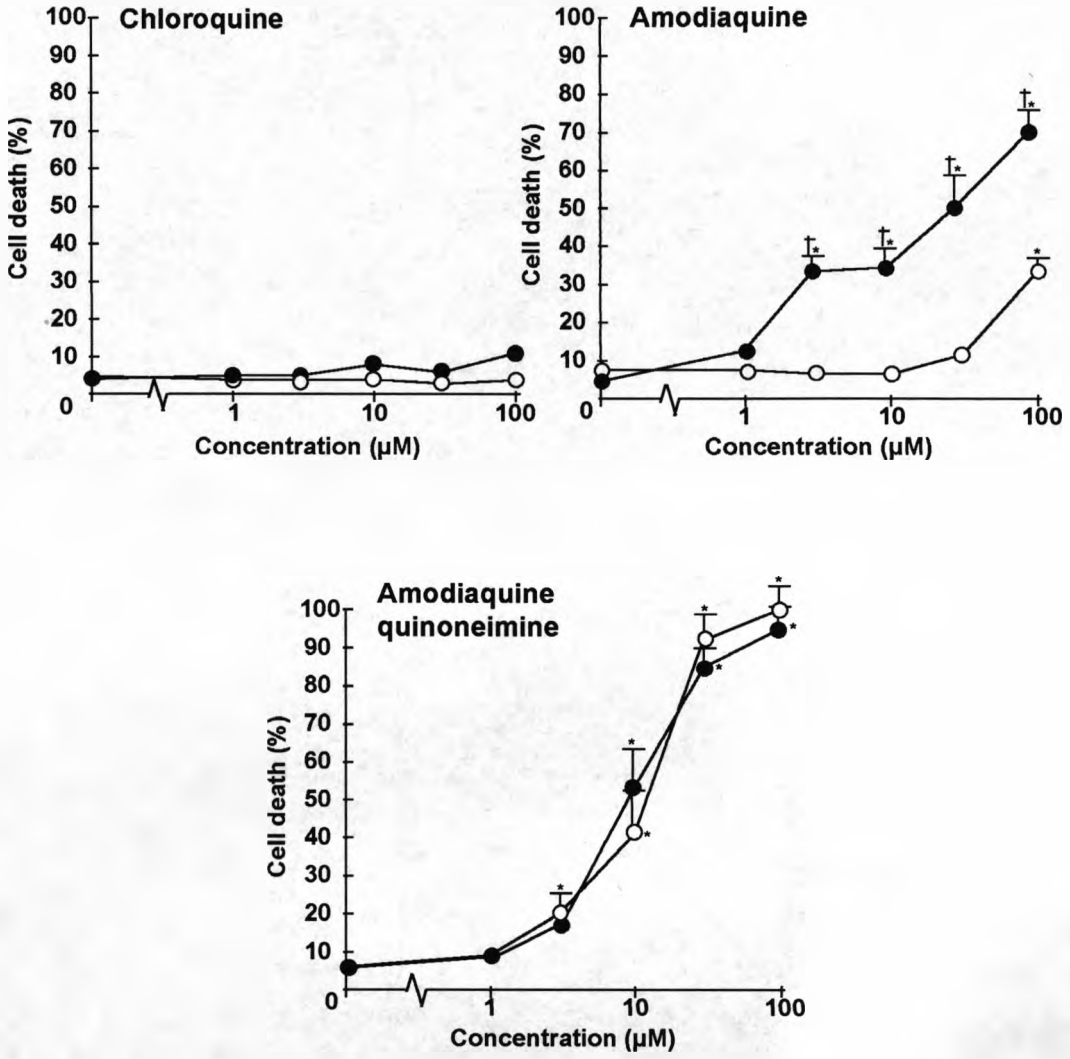


Figure 5.6 Cytotoxicity of CQ, AQ quinoneimine and AQ towards PMN (1×10^6) in the presence (●) and absence (○) of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of three experiments using cells from different individuals (all incubations performed in triplicate). Statistical analysis was performed by comparing the ability of different concentrations of the compounds to cause cell death with that of solvent alone (*$P < 0.05$), and by comparing different concentrations of the same compound in the presence and absence of the activating system (†$P < 0.05$). Error bars have been omitted from some of the data points for the sake of clarity.

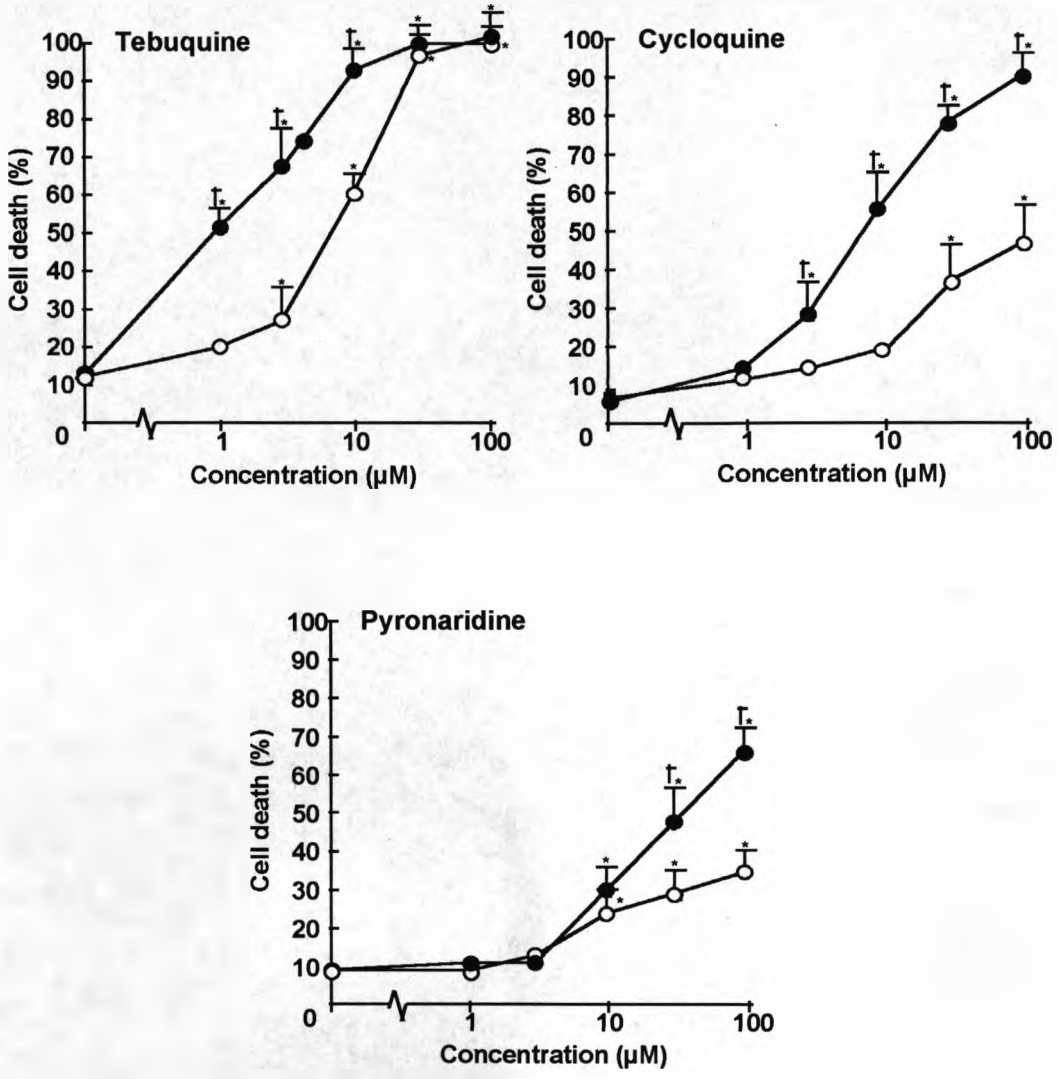


Figure 5.7 Cytotoxicity of PYRO, TEB and CYC towards PMN (1 x 10^6) in the presence (●) and absence (○) of HRP (20 units) and hydrogen peroxide (10μM). The results represent the mean ± S.D. of three experiments using cells from different individuals (all incubations performed in triplicate). Statistical analysis was performed by comparing the ability of different concentrations of the compounds to cause cell death with that of solvent alone (*P < 0.05), and by comparing different concentrations of the same compound in the presence and absence of the activating system (†P < 0.05). Error bars have been omitted from some of the data points for the sake of clarity.

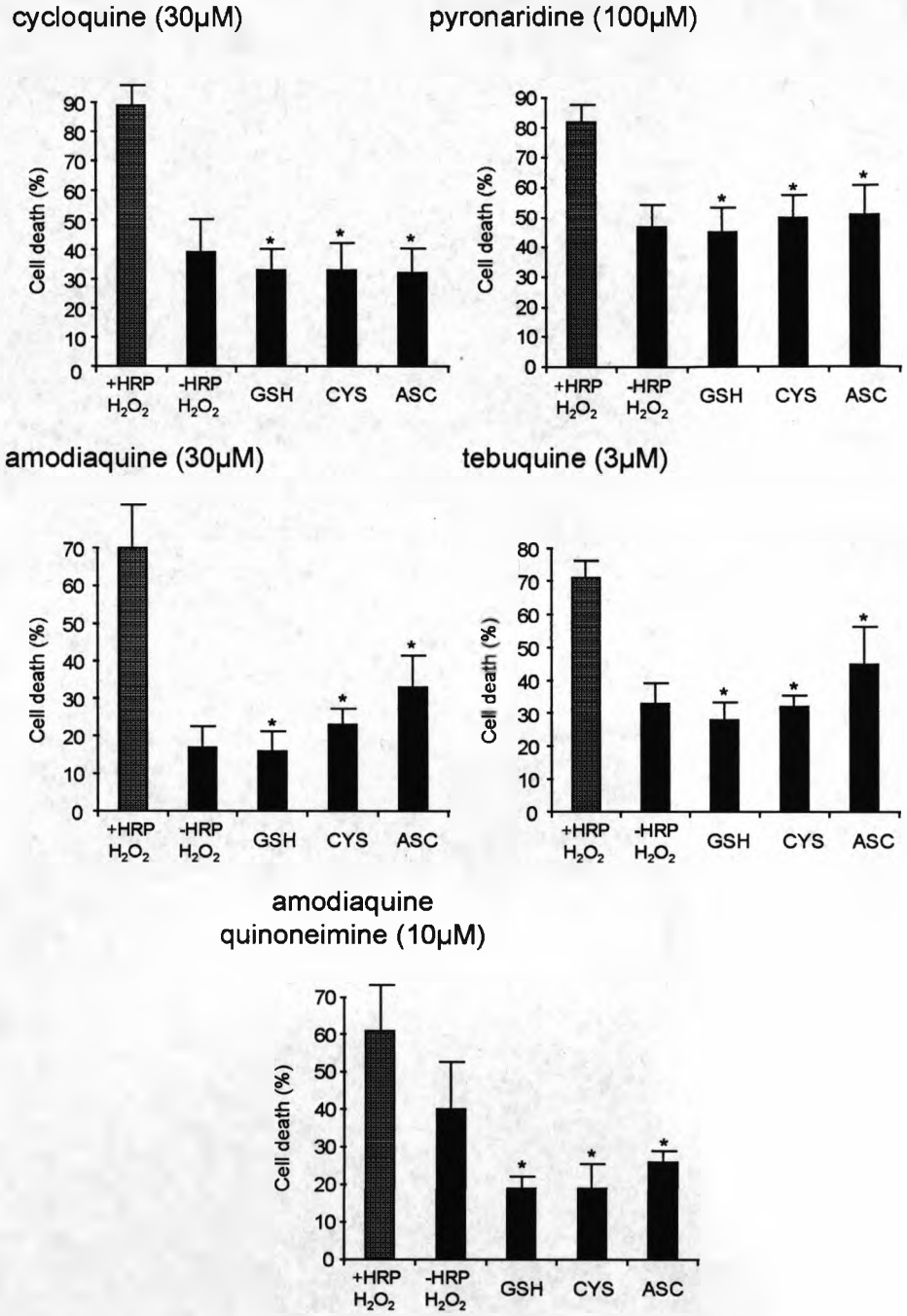


Figure 5.8 Peroxidase-mediated cytotoxicity of antimalarial compounds towards PMN (1 x 10^6) in the absence and presence of GSH (1mM), *N*-acetyl CYS (1mM) and ASC (1mM). Results represent the mean ± S.D. of 3 experiments using cells from different individuals (all incubations performed in triplicate). Statistical analysis was performed by comparing peroxidase-mediated cytotoxicity in the presence and absence of the antioxidants (*P < 0.05).

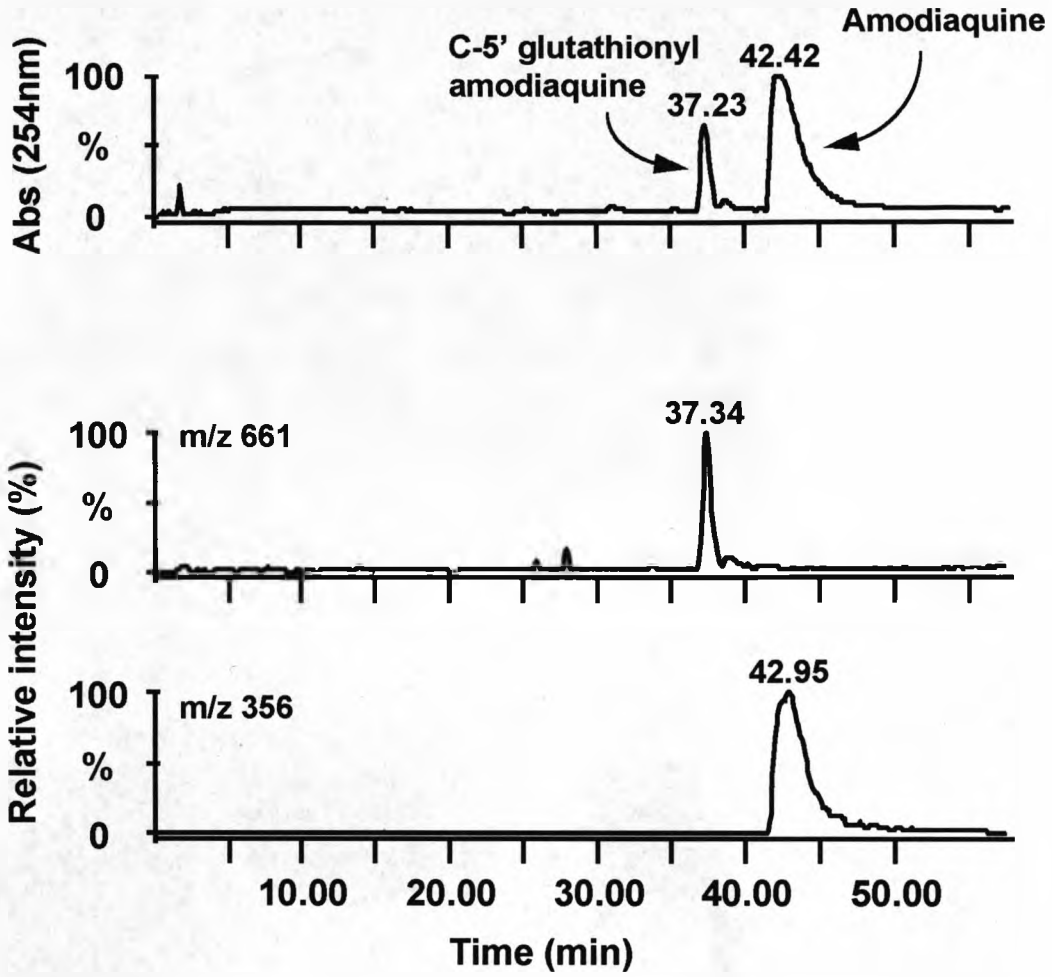


Figure 5.9 Typical absorbance (UV, 254nm) and selected ion chromatograms of AQ (m/z 356) and its glutathionyl conjugate (m/z 661) when AQ (100μM) was incubated in the presence of HRP (20 units), hydrogen peroxide (10μM) and GSH (1mM). Ions were protonated molecules.

should be noted that only a small number of patients have been exposed. The aim of the present study, therefore, was to compare these recently developed antimalarial agents with AQ in respect of their metabolism and their toxicity towards human neutrophils *in vitro*.

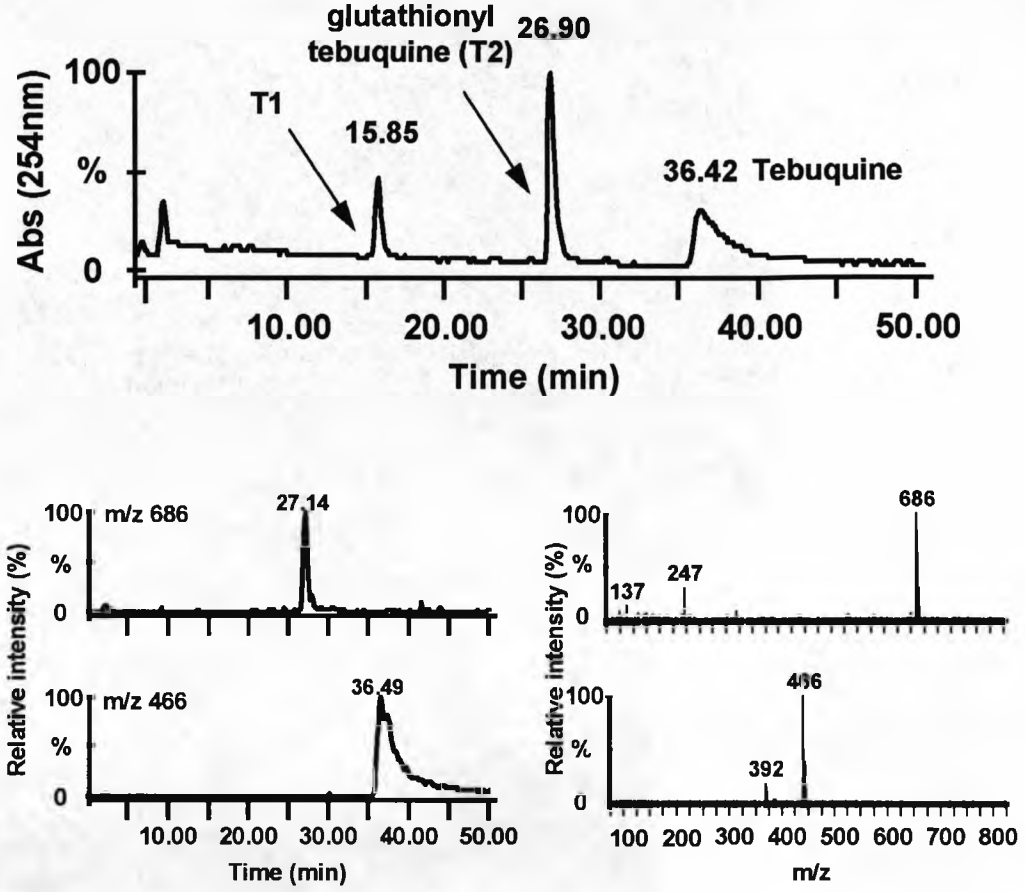


Figure 5.10 Typical absorbance chromatogram (UV, 254nm), selected ion chromatograms and mass spectra of TEB (m/z 466) and its glutathionyl conjugate (m/z 686) when TEB (100μM) was incubated in the presence of HRP (20 units), hydrogen peroxide (10μM) and GSH (1mM). Ions were protonated molecules.

AQ is converted via a semiquinone to the electrophilic and protein-reactive quinoneimine metabolite in activated neutrophils (Clarke *et al.*, 1990). This reaction is thought to be catalysed by myeloperoxidase (MPO) (Tingle *et al.*, 1995). In this study, HRP was used as a surrogate for MPO to determine the bioactivation of the antimalarials for several reasons. Firstly, although HRP and MPO are not homologous in structure, the catalytically active amino acid residues are positioned in a similar manner (Welinder, 1985). Secondly, despite differences

in enzymatic reactivity, the metabolites produced by HRP are qualitatively similar to those produced by MPO (Eastmond *et al.*, 1986). More importantly, the activity of HRP against a range of substrates has the added advantage of being significantly higher (Eastmond *et al.*, 1986; Sadler *et al.*, 1988; chapter 4). The validity of this approach was confirmed by showing that the same GSH conjugate was formed on bioactivation of AQ by both HRP and hydrogen peroxide (in this study; fig. 5.10) and MPO (Tingle *et al.*, 1995).

In the absence of a full metabolising system, AQ was not cytotoxic except at the highest concentration tested. However, in the presence of both HRP and hydrogen peroxide, AQ showed a significantly higher degree of cytotoxicity at lower concentrations (fig. 5.6), which was attenuated by the addition of exogenous GSH and other antioxidants (fig. 5.8). Additionally, bioactivation was accompanied by depletion of intracellular GSH, this also occurred at sub-cytotoxic drug concentrations. Using HPLC and LC-MS analysis, it was found that 19.1 ± 3.8% of AQ underwent conjugation during the reaction with the formation of a single AQ-glutathionyl adduct at the 5'- position as identified previously (Maggs *et al.*, 1988). It is important to note, however, that measurement of GSH conjugation by itself is likely to under-estimate the degree of bioactivation since electrophilic quinoneimine metabolites can also be detoxified by reduction, resulting in formation of oxidised GSH and the parent drug (van de Straat *et al.*, 1986; Monks *et al.*, 1992). In accordance with the hypothesis that AQ is bioactivated to a quinoneimine metabolite, pure AQ

quinoneimine caused both cytotoxicity and GSH depletion, neither of which were enhanced by the presence of a full activating system.

Using the same systems, CQ did not cause either cytotoxicity or GSH depletion, and did not undergo GSH conjugation. This clearly reflects the structural differences between CQ and AQ, since CQ does not contain a *p*-aminophenol moiety. Additionally, CQ has not been associated with agranulocytosis over many years of clinical use.

Werbel *et al.* (1986) synthesised a series of 5'-aryl analogues of AQ that demonstrated higher antimalarial activity against CQ-sensitive and -resistant parasites. TEB (fig. 5.1) was one of the compounds selected for preclinical toxicology studies, but its development was halted prior to evaluation in man because of neutrophil toxicity in animals (L. M. Werbel and H. Chung, Walter Reed Army Institute of Research, personal communication). Studies monitoring intralysosomal pH using the fluorescent probe 9-amino-6-chloro-2-methoxyacridine demonstrated that TEB is more lysosomotropic than AQ, leading to an inhibition of neutrophil function (chapter 2). This data may provide a partial explanation for the toxicity associated with the drug *in vivo*. In the same study, although TEB did not deplete intracellular GSH, it showed a high degree of direct neutrophil toxicity. This has also been shown in the present chapter (fig. 5.7); however, the cytotoxicity was further enhanced by the presence of a metabolising system. Additionally, the metabolism-dependent cytotoxicity was accompanied by depletion of GSH (fig. 5.5), and as with AQ, the cytotoxicity was reduced by antioxidants (fig. 5.8). Characterisation of a stable thiol adduct by

LC-S indicated that TEB was undergoing bioactivation. The identification of a protonated molecule at m/z 686 and the absence of the common fragment ion, resulting from loss of the *t*-butyl amino side chain, suggested the formation of a previously unidentified 3'-glutathionyl conjugate (fig. 5.10). To our knowledge, an enzymic reaction of this type has not been described. A postulated scheme for the reaction is presented in fig. 5.11.

The bis-mannich derivatives CYC and PYRO, which, like AQ, contain a *p*-aminophenol function but have a hydrophilic rather than a lipophilic substituent at the 5'-position, were also investigated. (fig. 5.2). CYC has been shown to be as effective as AQ against *Plasmodium vinkei vinkei in vitro* (Barlin and Tan, 1985), while PYRO is currently undergoing clinical trials and seems to be well tolerated (Chen *et al.*, 1992; Ringwald *et al.*, 1996). Preliminary studies (chapter 2) have shown that these compounds were not directly cytotoxic (unlike TEB) and did not deplete GSH from phorbol ester-stimulated neutrophils. However, in the present study, in the presence of a strong chemical activating system (HRP and hydrogen peroxide), these compounds demonstrated significant cytotoxicity (fig. 5.7), and depleted intracellular GSH to a similar extent to AQ (IC_{50}; AQ, 0.5 ± 0.2μM; PYRO, 3.3 ± 2.4μM; CYC, 3.4 ± 1.0μM). Taken together, the results suggest that these compounds can also undergo bioactivation to electrophilic semiquinone or quinoneimine metabolites despite the fact that the C-5' position is blocked. It is important to note that cytotoxicity is not simply just a function of GSH depletion. Several findings support this hypothesis; firstly, TEB-, CYC- and PYRO-induced cytotoxicity was observed at concentrations that did not deplete

GSH (fig. 5.5 and 5.7). Second, addition of exogenous GSH failed to protect the cells from toxicity caused by incubating drug alone. Finally, no difference in cytotoxicity was observed when PYRO (10μM) was incubated in the presence and absence of peroxidase activation, despite the fact that >80% of the GSH was depleted from cells in the presence of the full activating system.

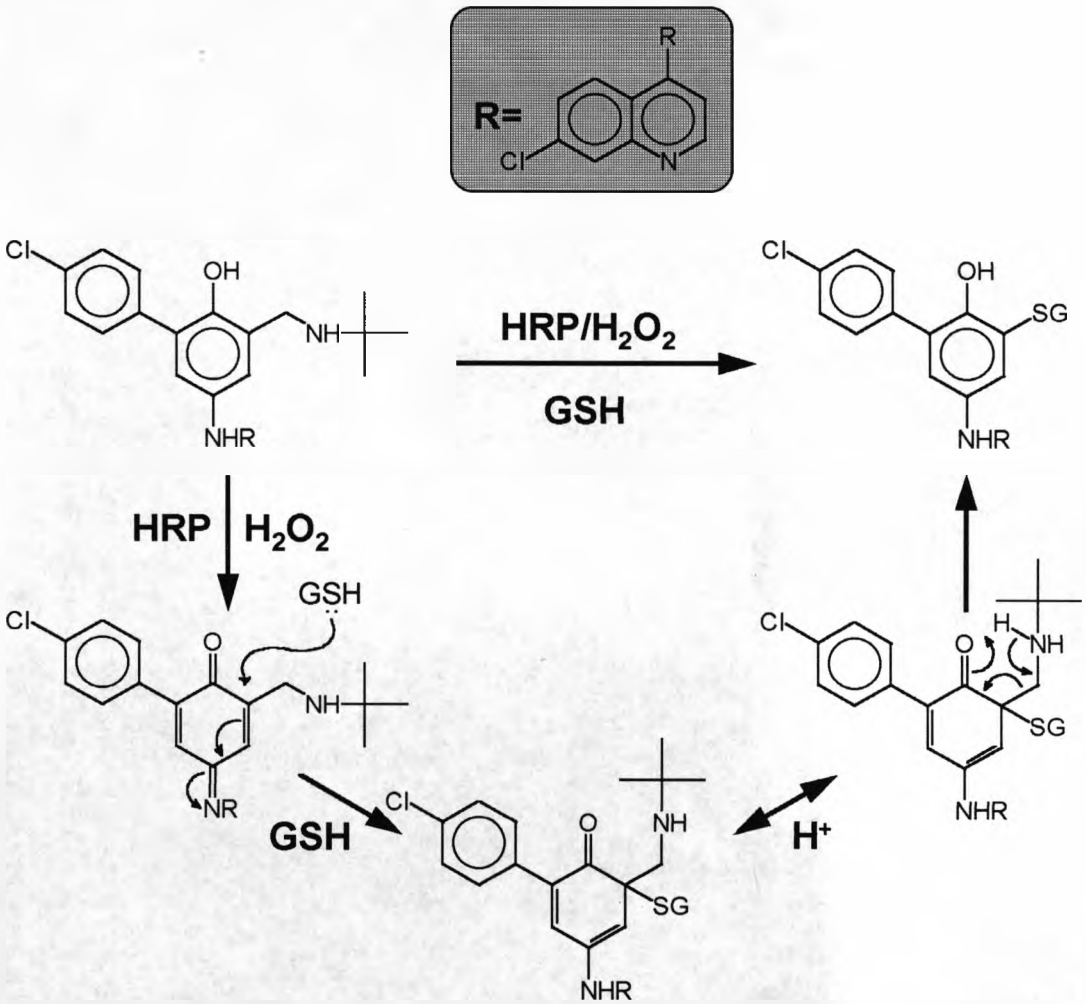
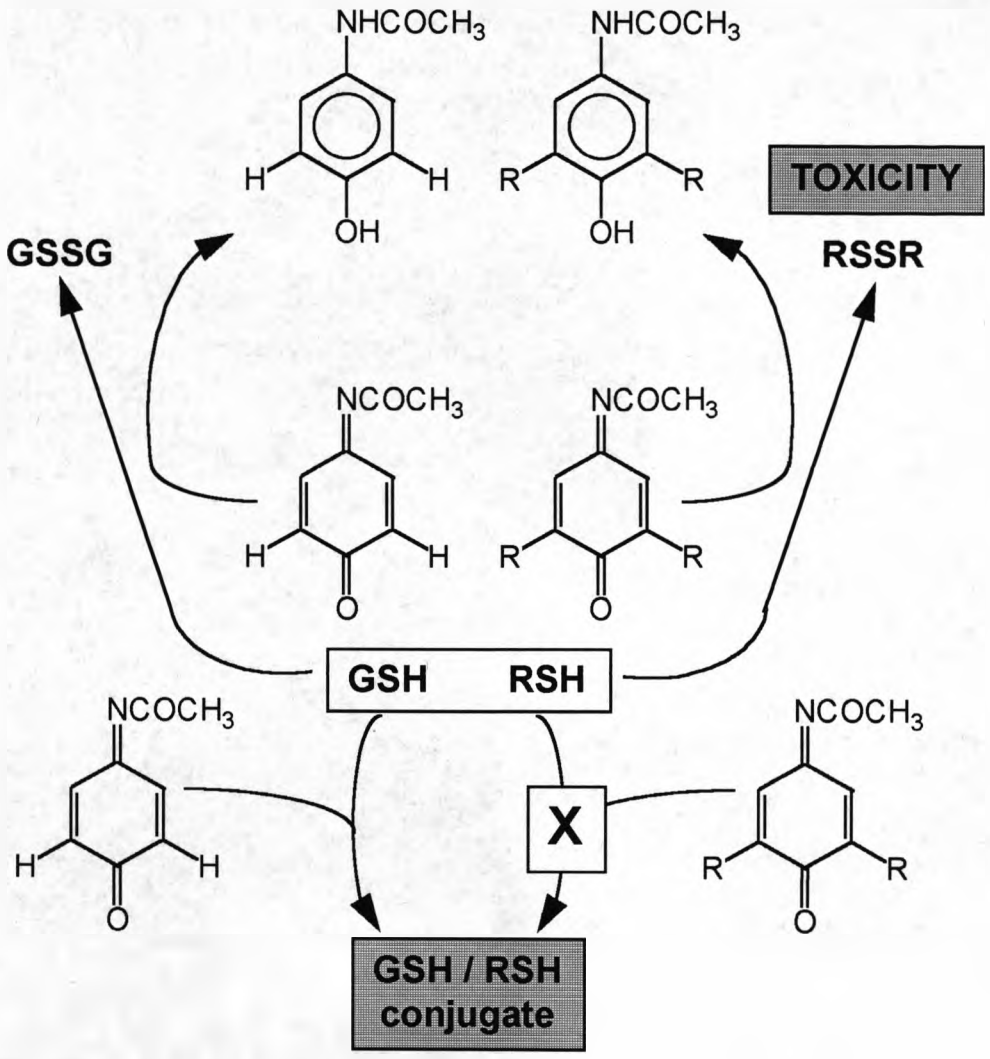


Figure 5.11 Scheme depicting the possible mechanism of TEB bioactivation and GSH conjugation.

LC-MS analysis of incubations containing peroxidase and GSH revealed distinct differences in the reactions of mono- and bis-mannich substituted compounds. GSH depletion by mono-substituted compounds such as AQ was primarily a result of conjugate formation; however, as no conjugates were formed with either CYC and PYRO, GSH depletion was likely to have been due to the oxidation of GSH to GSH disulphide.

The mechanism of neutrophil toxicity associated with mono- and bis-mannich antimalarial agents correlates closely with work carried out on paracetamol and a series of 3,5-diacetylated analogues (van de Straat *et al.*, 1987; Rossi *et al.*, 1988; Rundgren *et al.*, 1988). Paracetamol is widely used as an analgesic and does not cause toxicity except when taken in overdose. The hepatocellular necrosis observed with paracetamol overdosage has been linked to bioactivation of paracetamol to *N*-acetyl-*p*-benzoquinoneimine (Jollow *et al.*, 1974). Although the precise mechanism of the liver toxicity is still widely debated it has been shown that the quinoneimine metabolite causes depletion of intracellular thiol stores, can bind covalently to protein thiol groups, and induce oxidative stress (Potter *et al.*, 1974; Albano *et al.*, 1985; Moore *et al.*, 1985). It was expected that the 3,5-di-acetylated analogues of paracetamol would not bind to thiols and thus would not cause toxicity. However, 3,5-dimethyl paracetamol was found to be as hepatotoxic as paracetamol in rats and mice (Fernando *et al.*, 1980). No GSH conjugates were identified, and therefore, the mechanism of 3,5-dimethyl paracetamol cytotoxicity was attributed to oxidative stress and protein thiol oxidation (fig. 5.12). A recent report by Weis *et al.*, (1996) confirmed these



Taken from van de Straat *et al.* (1987)

Figure 5.12 Proposed effects of quinoneimines formed from paracetamol and 3,5-dialkyl substituted derivatives on the cellular thiol status and cell viability. RSH denotes protein thiol group.

initial findings and furthermore the authors demonstrated that the cytotoxicity of 3,5-dimethyl paracetamol involved oxidative modification of essential cysteinyl proteins. It is important to note that AQ, in addition to causing agranulocytosis, also causes hepatotoxicity (Neftel *et al.*, 1986; Larrey *et al.*, 1986). Bioactivation of AQ to the quinoneimine metabolite by liver P450 enzymes has been

demonstrated (Jewell *et al.*, 1995). Clearly, paracetamol does not cause agranulocytosis, and this may be due to two main reasons: (a) it is rapidly metabolised in the liver, and (b) it does not accumulate within neutrophils, i.e., it is not lysosomotropic. The property of lysosomotropsim is exhibited by most antimalarial agents (chapter 2), and apart from affecting neutrophil function, it may also play an important part in the pathogenesis of the agranulocytosis by allowing a high concentration of the drugs and their reactive metabolites to be present within the target tissue.

In summary, the data presented in this chapter has shown that AQ and related antimalarials containing a *p*-aminophenol moiety can undergo bioactivation *in vitro* to a chemically reactive intermediate(s). In our system, PYRO was also metabolised to a compound which was toxic to neutrophils, and thus the possibility of it causing agranulocytosis in clinical practice cannot be excluded, and will require careful monitoring.

Chapter 6:

Reduced Plasma Cysteine in Patients with HIV Infection : Implications for Sulphamethoxazole Hypersensitivity

Contents **Page**

6.1 Introduction

Reactive oxygen species are produced through the incomplete electron transfer on oxygen or by the activity of various enzymes. Reactive oxygen species include superoxide, hydrogen peroxide and hydroxyl radicals. They are well tolerated in low amounts; however, increased levels can react with biological macromolecules, resulting in lipid peroxidation, inactivation of essential proteins and DNA strand breakage (Halliwell and Gutterridge, 1989). In response, the body is equipped with a variety of defence mechanisms. Antioxidant molecules such as glutathione (GSH) and ascorbic acid act directly, while enzyme systems such as superoxide dismutase, GSH peroxidase and catalase can either block free radical chain reactions and interconvert or eliminate reactive oxygen species (Gutteridge, 1993; section 1.5.5). Oxidative stress results from an imbalance between these processes.

GSH, the most abundant cellular thiol (Reed, 1990), has three important functions in the body. First, it plays a key role in the maintenance of cellular redox state (Meister, 1989). Secondly, it conjugates with electrophilic metabolites and free radicals produced from xenobiotics (Moldeus and Quanguan, 1987; Reed, 1990). Thirdly, GSH enhances immune function; in particular, lymphocyte function is dependent on GSH concentration (Staal *et al.*, 1992). Unlike GSH, cysteine (CYS), a precursor in GSH synthesis is found almost exclusively outside the cell (Mills and Lang, 1996). Oxidation of CYS results in a variety of disulphide forms. The relative amounts of free and protein bound plasma CYS have been reported previously (table 6.1). In addition, reduced plasma CYS is present at

fairly high concentrations (8-15μM; Mansoor *et al.*, 1992; de Quay *et al.*, 1992; Mills and Lang, 1996; Muller *et al*, 1996). At these concentrations it has a protective role, serving as an antioxidant, in conjugation or reduction reactions (Kubal *et al.*, 1995; chapter 3) (fig. 6.1).

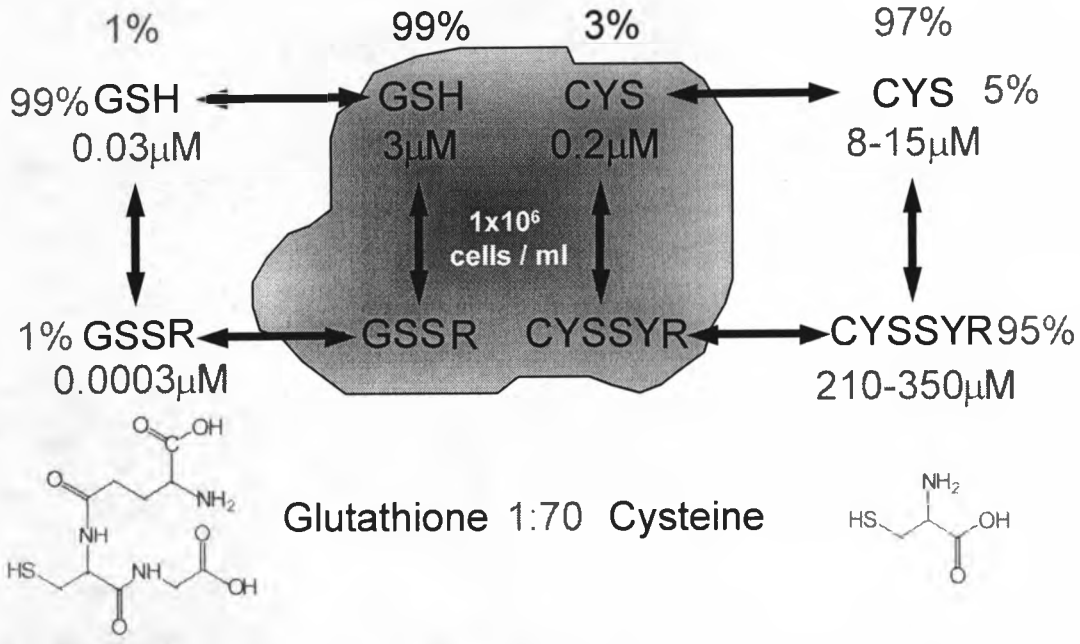


Figure 6.1 Intracellular and extracellular distribution of GSH and CYS.

Several groups have reported that patients with human immunodeficiency virus (HIV) infection have a deficiency of intracellular GSH (table 6.2). The deficiency has been detected in asymptomatic HIV-positive individuals as well as in those with AIDS, and has been postulated to be a consequence of increased consumption or decreased production, or a combination of both (Staal *et al.*, 1992). In addition, Ciriolo *et al.* (1997) demonstrated that thiol depletion in HIV-infected patients was a direct consequence of viral infection. A reduction in plasma CYS has also been reported (table 6.2); it was postulated that CYS deficiency

plays a role in the progressive destruction of the immune system in HIV-infected patients (Droge, 1993).

Free (CYS + CYS-SYC)	**Protein bound**	**Total**	**Protein bound**	**Reference**
µM	µM	µM	%	
143	213	357	60	Mills and Lang, 1996
122	146	268	54	Andersson *et al.*, 1993
152	80	232	34	Fiskerstrand *et al.*, 1993
92	165	250	66	Mansoor *et al.*, 1992
126	103	229	45	Araki *et al.*, 1989
114	161	275	58	Wiley *et al.*, 1988
129	115	244	47	Johansson *et al.*, 1988
102	108	210	51	Smolin *et al.*, 1987
90	151	241	63	Chawla *et al.*, 1984
98	105	215	49	Malloy *et al.*, 1981

Table 6.1 Summary of free, protein bound and total CYS in human plasma.

Studies investigating thiol levels in HIV-positive individuals have used several different analytical techniques and protocols. The validity of one method has recently been questioned (van der Ven *et al.*, 1994). Furthermore, other studies have failed to show a thiol deficiency in HIV-positive individuals (table 6.2). In fact two studies measured an increase in the amount of GSH disulphide instead of a depletion of reduced GSH (Aukrust *et al.*, 1995; van der Ven *et al.*, in press). In addition, they did not confirm a decrease in reduced plasma CYS as reported elsewhere (table 6.2).

A deficiency of GSH and CYS resulting in oxidative stress is believed to play an important pathophysiological role in patients with HIV (Muller, 1992;

Droge, 1993). Prooxidative conditions enhance the activity of the transcriptional factor NF-κB (Staal *et al.*, 1990), thereby enhancing transcription of the HIV virus. Additionally, activation of NF-κB has been associated with a substantial decrease in $CD4^+$ lymphocytes (Meyaard *et al.*, 1992), a process thought to occur by apoptosis (Ameison and Capron, 1991; chapter 7).

Deficiency	Tissue	Failure to show a deficiency	Tissue
GSH		**GSH**	
Eck *et al.*, 1989	lymphocytes/ plasma	Aukrust *et al.*, 1995	lymphocytes
		Pirmohamed *et al.*, 1996	"
Buhl *et al.*, 1989	plasma	van der Ven *et al.*, in press	"
Roederer *et al.*, 1991	lymphocytes		
de Quay *et al.*, 1992	"		
Staal *et al.*, 1992	"		
Helbling *et al.*, 1996	"		
Walmsley *et al.*, 1997	"		
CYS		**CYS**	
Droge *et al.*, 1988	plasma	Jacobsen *et al.*, 1990	plasma
Eck *et al.*, 1989	"	Aukrust *et al.*, 1995	"
de Quay *et al.*, 1992	"	Muller *et al.*, 1996	"
Hortin *et al.*, 1994	"	Lopez-Galera *et al.*, 1996	"
Helbling *et al.*, 1996	"	van der Ven *et al.*, in press	"
Akerlund *et al.*, 1996	"		
Walmsley *et al.*, 1997	"		

Table 6.2 Summary of studies investigating the thiol status of HIV-positive individuals.

HIV-positive patients are more susceptible to idiosyncratic toxicity (Bayard *et al.*, 1992). With regard to sulphamethoxazole (SMX), which is administered for the treatment of *Pneumocystis carinii* pneumonia, toxicity is significantly more common in patients with HIV-infection (30-80%) than in

seronegative patients (less than 3%). It is important to note that HIV-positive patients are on high doses of SMX (up to 120mg/kg/day). However, toxicity is not completely dose-dependent, for example, when SMX is administered for prophylaxis in HIV-infection, at doses lower than those used in HIV-negative individuals, the incidence of adverse reactions is still higher (approx. 25%) (Carr and Cooper, 1995; Koopmans *et al.*, 1995; Pirmohamed and Park, 1995; Tshachler *et al.*, 1996).

Drug metabolism is thought to play an important role in the pathogenesis of idiosyncratic toxicity (Park *et al.*, 1992). SMX is extensively metabolised *in vivo* and *in vitro* (Koopmans *et al.*, 1995). The hydroxylamine of SMX (or its nitroso metabolite) has been postulated to be the toxic metabolite (Shear *et al.*, 1986; Rieder *et al.*, 1988; Carr *et al.*, 1993). The importance of extracellular thiols (in particular CYS) in the detoxification of SMX hydroxylamine (SMX-NHOH) and nitroso SMX (SMX-NO) has been investigated in chapter 3. Several important points were observed in this study. First, incubation of SMX-NO with CYS resulted in its rapid and complete reduction back to SMX. Secondly, plasma CYS was more potent than the other blood components at reducing the nitroso metabolite back to the hydroxylamine and parent compound. Finally, the reaction of both SMX-NHOH and SMX-NO with plasma was accompanied by a depletion of CYS, while there was no significant depletion of GSH in freshly isolated neutrophils. These data and the failure of recent studies to demonstrate thiol deficiency in HIV-positive patients has led to further investigations regarding the role of extracellular thiols in predisposing to idiosyncratic toxicity in HIV disease.

CYS, homo-CYS, CYS-glycine and GSH levels have been measured in freshly isolated plasma taken from HIV-positive patients using a recently developed procedure (Mansoor *et al.*, 1992; Muller *et al.*, 1996). In addition, the plasma reduction of SMX-NHOH and SMX-NO to SMX was also determined as this process is partly dependent on the thiol status.

6.2 Materials and Methods

6.2.1 Chemicals

Bromobimane, CYS, CYS-glycine, dithioerythritol, *N*-ethylmorpholine, GSH, homo-CYS, SMX, sodium borohydride, 5-sulphosalicylic acid, trichloroacetic acid and triethylamine were obtained from Sigma Chemical Co. (Poole, UK). SMX-NHOH and SMX-NO were synthesised using a modification of the method described by Johnstone *et al.* (1978; chapter 3). All HPLC-grade solvents were purchased from Fischer Scientific (Loughborough, UK). Solution B was 65% DMSO and 35% water (v/v) containing 51mM sodium chloride and 140mM hydrogen bromide.

6.2.2 Patients and Controls

The study was approved by the Ethics Committees in Liverpool and Manchester. Thirty three patients (age, 40 ± 8 years; range, 28-52 years) with HIV-infection took part in the study. Four of the patients were known to be hypersensitive to SMX and seven were on trimethoprim-SMX at the time of the blood sampling and had not had any adverse reaction to the drug.

For comparison, thirty three healthy volunteers (age, 29 ± 7 years; range, 22-45 years) were used as a control group. All HIV-positive samples were tested in parallel with an equivalent number of controls.

6.2.3 Sample Collection and Processing

25ml of venous blood was collected into tubes containing heparin as an anti-coagulant. A mixture of bromobimane (75µl, 180mM; in acetonitrile) and phosphate buffered saline (PBS, 425µl; pH 7.4) was added to 5ml of the blood within 20sec of venepuncture. All tubes were placed on ice until centrifugation at 1000*g* (10min) to remove cells and platelets. From the different plasma preparations, aliquots were drawn and treated as described below.

6.2.4 Determination of Reduced, Oxidised, Protein-Bound and Total Homo-cysteine, Cysteine, Cysteinyl-Glycine and Glutathione Concentrations in Human Plasma

A modification of the method utilised by Mansoor *et al.* (1992) was used to determine reduced, oxidised, protein-bound and total thiol levels in human plasma. Reduced thiol concentrations were measured from bromobimane-treated plasma samples. Reduced thiols react with bromobimane and form highly fluorescent adducts which were separated and quantified by fluorescent HPLC. Total and protein-bound thiols were analysed from untreated plasma. Sodium borohydride was utilised to reduce disulphides prior to derivatisation with bromobimane. Oxidised thiol levels were determined by subtracting the reduced

and protein-bound fractions from the total thiol concentrations. CYS, CYS-glycine, homo-CYS and GSH standards (10 and 100μM) were analysed prior to chromatographic analysis of patient samples. In addition, processed samples were stored at -20°C for no longer than 2 weeks and thawed only once. Each sample was analysed in triplicate.

6.2.4.1 Determination of Total Plasma Thiol Components

30μl of 2.0M sodium borohydride (in 0.05M sodium hydroxide) was added to 30μl of untreated plasma. Plasma proteins were then precipitated by the addition of 60μl of a 20% solution of sulphosalicylic acid (in 100μM dithioerythritol). The samples were left uncovered for 30min at 0°C to release the gas. Precipitated protein was then removed by centrifugation (800g; 5min). To the supernatant was added 30μl of 1.4M sodium borohydride (in 0.05M sodium hydroxide), followed by 130μl of solution B, 50μl of 1M *N*-ethyl morpholine (pH 9.0) and 10μl of 20mM bromobimane (in acetonitrile). Samples were then incubated in the dark at 37°C for 5min prior to the addition of 100% trichloroacetic acid (w/v, 10μl).

6.2.4.2 Determination of Protein-Bound Plasma Thiol Components

To 30μl of untreated plasma was added 150μl of 5% sulphosalicylic acid (in 50μM dithioerythritol). Following protein precipitation and centrifugation, 30μl of 2.0M sodium borohydride (in 0.05M sodium hydroxide) was added to the pellet. To the dissolved pellets, 30μl of a 40% solution of sulphosalicylic acid (in

100µM dithioerythritol), 30µl of 1.4M sodium borohydride (in 0.05M sodium hydroxide), 130µl of solution B, 50µl of 1M *N*-ethylmorpholine (pH 9.0) and 10µl of 20mM bromobimane (in acetonitrile) were added. After a 5min incubation at 37°C in the dark, 10µl of 100% trichloroacetic acid (w/v) was added.

6.2.4.3 Determination of Reduced Plasma Thiol Components

55µl of a 50% solution of sulphosalicylic acid (in 500µM dithioerythritol) was added to 500µl of bromobimane-treated plasma. Precipitated protein was removed by centrifugation and 30µl of 5% sulphosalicylic acid (in 50µM dithioerythritol), 160µl of distilled water, 50µl of 1M *N*-ethylmorpholine (pH 9.0) and 10µl of acetonitrile were added to 30µl of the supernatant. After a 5min incubation in the dark at 37^{0}C, 10µl of 100% trichloroacetic acid (w/v) was added.

6.2.5 Fluorescent Chromatographic Analysis of Thiol Conjugates

Samples were centrifuged (1000g; 5min) prior to analysis. Aliquots of the supernatant (10µl) were analysed for thiol conjugates using HPLC (Spectra Physics 880, Stone, UK) with fluorescence detection (Hitachi 1080, Tokyo, Japan) set for excitation at 394nm and emission at 480nm. The peaks were integrated using a Spectra Physics Chromojet Integrator. Separation of the thiol adducts was obtained using a 5µm Hypersil BDS C_{18} column, (4.5 x 150mm), and a mobile phase which consisted of 0.25% acetic acid and 5.5% aqueous acetonitrile (pH 3.7) for 13min, followed by 75% aqueous acetonitrile for 9min,

and a re-equilibrium period for a further 7min with the acetic acid / acetonitrile eluent. The flow was 1ml/min throughout. The retention times of the bimane derivatives of CYS, CYS-glycine, homo-CYS and GSH were 5.8, 7.2, 10.8 and 11.7min, respectively (fig. 6.2).

The precision of the assay and recoveries of the plasma thiol components have been validated previously (Mansoor *et al.*, 1992). The detection limit for thiol conjugates was 0.1μM and the mean intra-assay co-efficent of variation was less than 10% for all samples.

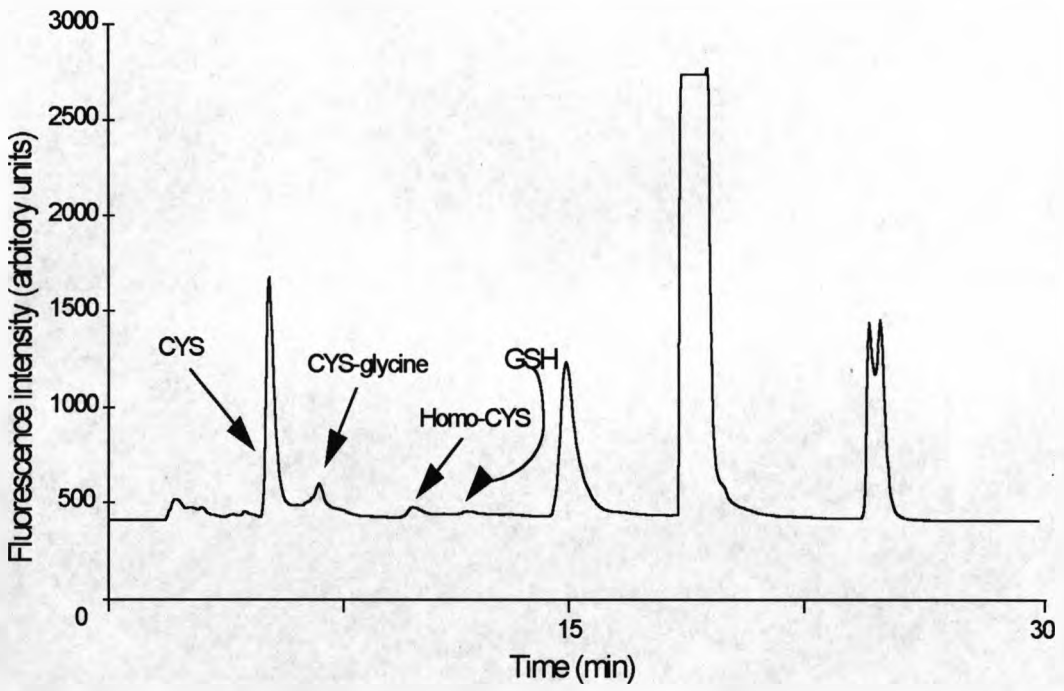


Figure 6.2 HPLC chromatogram of the thiol-bimane conjugates formed from human plasma.

6.2.6 Determination of the Reduction of Sulphamethoxazole Metabolites by Human Plasma

Reduction of SMX-NHOH and SMX-NO by human plasma has been described previously in section 3.2.9. A similar procedure was used to compare the reduction in plasma from HIV-infected individuals. Freshly isolated plasma (1ml) was incubated with SMX-NHOH and SMX-NO (50μM) in a shaking water bath at 37°C. After 4h, the reaction was stopped by the addition of ice cold acetonitrile (1ml). The samples were placed on ice until HPLC analysis using the same system as described earlier (section 3.2.2).

In addition, reduced CYS levels were determined at the time of venepuncture. All incubations were performed in triplicate from six HIV-positive individuals and controls.

6.2.7 Statistical Analysis

The results represent the mean ± S.D. All values to be compared were analysed for non-normality using the Shapiro-Wilk test. Values were often found to be non-normally distributed, and therefore, the Mann-Whitney test was used for comparison of the two groups, accepting $P < 0.05$ as significant. Correlation was determined by the least-squares linear regression analysis.

A power calculation was used to determine the minimum number of individuals needed to detect a true difference in plasma CYS concentrations in HIV-positive individuals and controls. From a previous study (Helbling *et al.*, 1996), the difference between the mean values of reduced CYS was 42.6%

(controls, 13.4μM; HIV-positive, 7.7μM) with a standard deviation of 36.6% in the control group. At a significance level of 5% and a power of 95%, a minimum of 21 individuals should be included from each group to detect a true difference in reduced plasma CYS. All calculations were carried out using the Arcus Pro-Stat 3 statistical software package (Dr. Iain Buchan, The University of Liverpool).

6.3 Results

6.3.1 Determination of Thiol Concentrations

Bromobimane reacts non-enzymatically with all reduced thiols. The highly fluorescent products can be separated and quantified using previously described HPLC methods (Cotgreave and Moldeus, 1986; Muller *et al.*, 1996; Pirmohamed *et al.*, 1996). In this study, CYS, homo-CYS, CYS-glycine and GSH bimane-adducts were identified and quantified by fluorescent HPLC from commercially available standards and retention times quoted previously (Muller *et al.*, 1996; fig. 6.2).

Reduced thiols were derivatised within 20sec of venepuncture by the addition of bromobimane to whole blood. The stability of stored thiol-bimane adducts has been evaluated previously (Cotgreave and Moldeus, 1986; Mansoor *et al.*, 1992). In this study, processed samples were stored at -20^{0}C for no longer than 2 weeks until chromatographic analysis. Additionally, samples were thawed only once, which did not cause a significant reduction in plasma thiol concentrations.

6.3.2 Plasma Cysteine Concentrations

The mean plasma concentration of total CYS was 246.5 ± 27.3μM and 246.2 ± 30.9μM in HIV-negative and HIV-positive individuals respectively (fig. 6.3). There was a significant decrease in reduced plasma CYS concentrations in HIV-positive patients (12.9 ± 3.1μM) when compared to controls (16.9 ± 3.0μM; $P < 0.0001$, 95% CI for the difference between means, -5.5 to -1.9). In addition, patients had a significantly lower ratio of reduced to oxidised ($P < 0.0005$) and reduced to total CYS ($P < 0.0001$) when compared with control subjects (fig. 6.4). No differences in oxidised, protein-bound or total plasma CYS concentrations were found between the patients and controls. Furthermore, there was a large degree of inter-individual variability in CYS levels in both patients and controls (fig. 6.3).

6.3.3 Plasma Homo-Cysteine Concentrations

The concentration of plasma homo-CYS in HIV-positive patients and controls has been recorded previously (Muller *et al.*, 1996). The concentration of total homo-CYS was 11.9 ± 4.7μM and 14.5 ± 5.6μM in HIV-negative and HIV-positive individuals respectively (fig. 6.5). In accordance with Muller *et al.* (1996), no significant difference was observed in total, protein-bound and oxidised levels of homo-CYS between HIV-positive patients and control subjects. However, the concentration of reduced homo-CYS in the patient population was significantly higher than in controls ($P < 0.05$; 95% CI for the difference between means, -0.9 to -0.2; fig. 6.5). In contrast to plasma CYS, there was no significant difference

between the ratio of reduced to oxidised and reduced to total homo-CYS (fig. 6.7a).

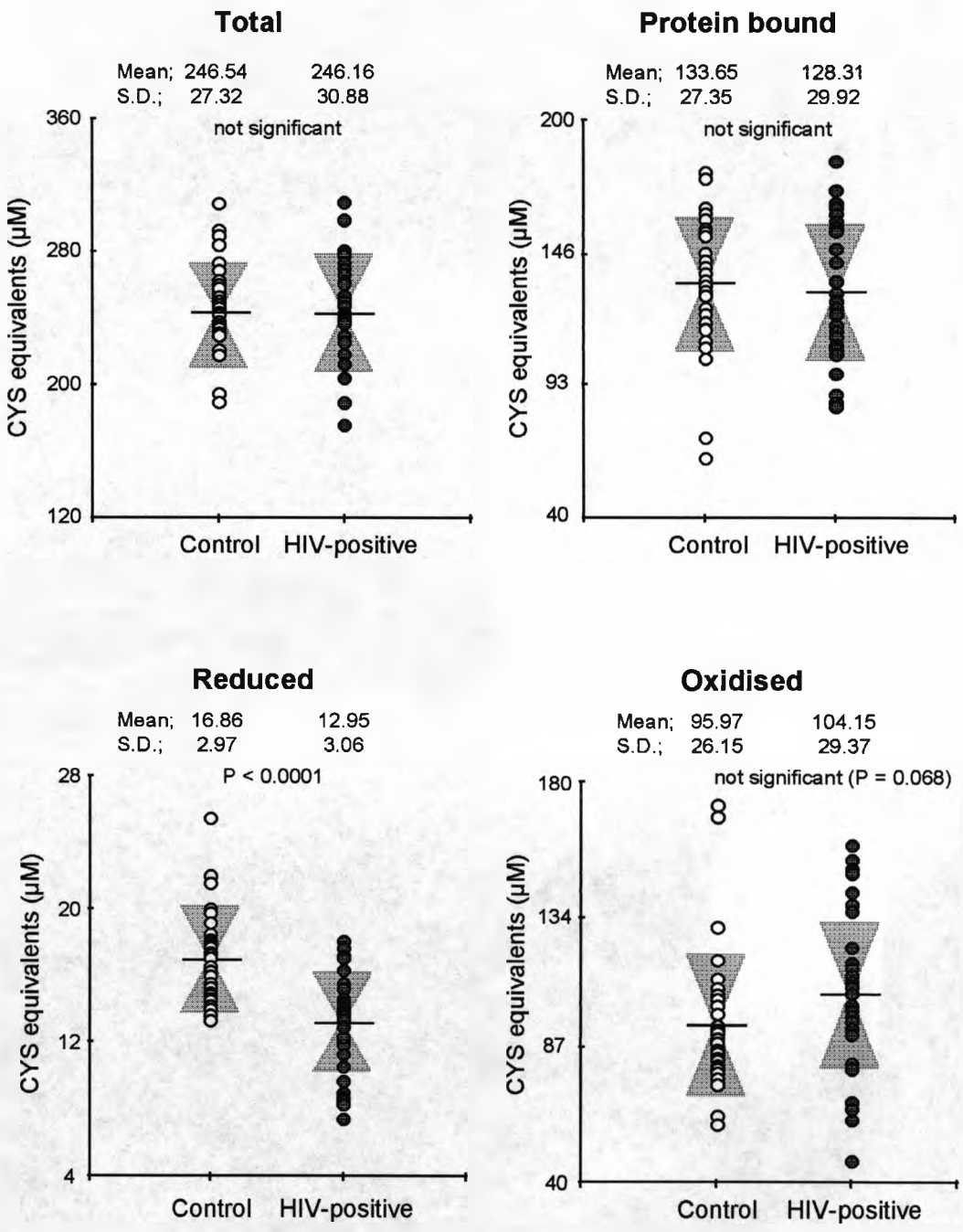


Figure 6.3 Plasma CYS concentrations in HIV-positive patients and controls (n=27). The bars represent the means ± S.D. Statistical analysis was performed by comparing CYS levels in HIV-positive patients to that obtained in controls.

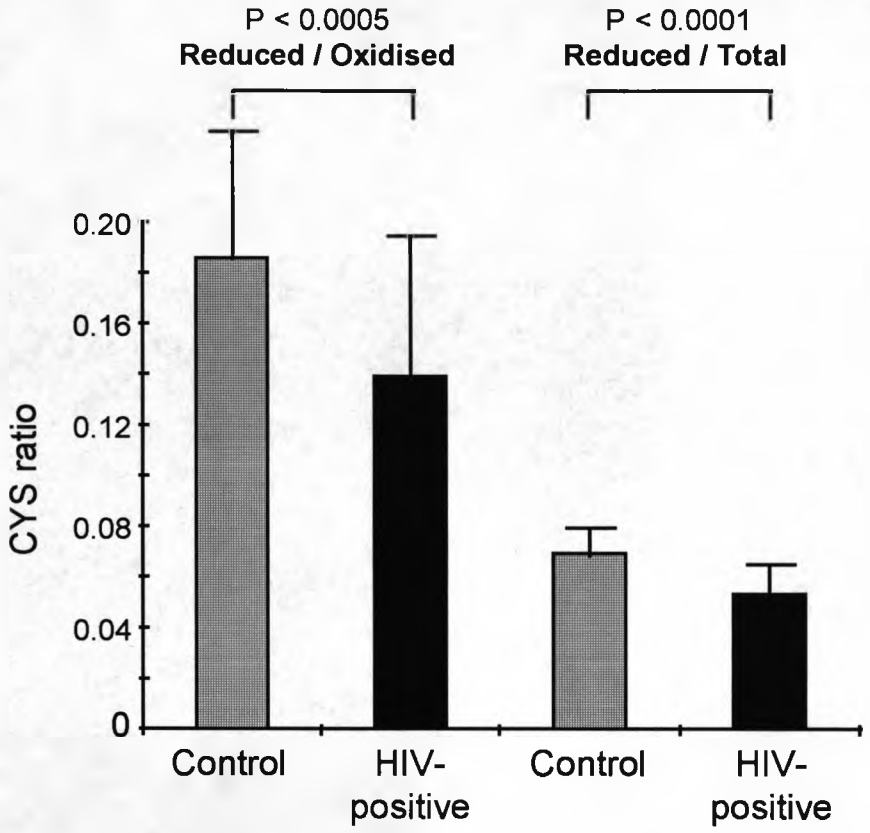


Figure 6.4 Ratios between reduced and oxidised and reduced and total plasma CYS concentrations in 27 HIV-positive individuals and controls. The bars represent the means ± S.D. Statistical analysis was performed by comparing the ratio of CYS levels in HIV-positive patients to that obtained in controls.

6.3.4 Plasma Cysteinyl-Glycine Concentrations

The concentration of total CYS-glycine was 36.2 ± 9.2μM and 38.3 ± 8.1μM in HIV-negative and HIV-positive individuals respectively (fig. 6.6). In HIV-positive patients there were no differences in total, protein-bound, oxidised or reduced forms (fig. 6.6). In addition, there was no difference in the ratio of reduced to oxidised and reduced to total CYS-glycine (fig. 6.7b).

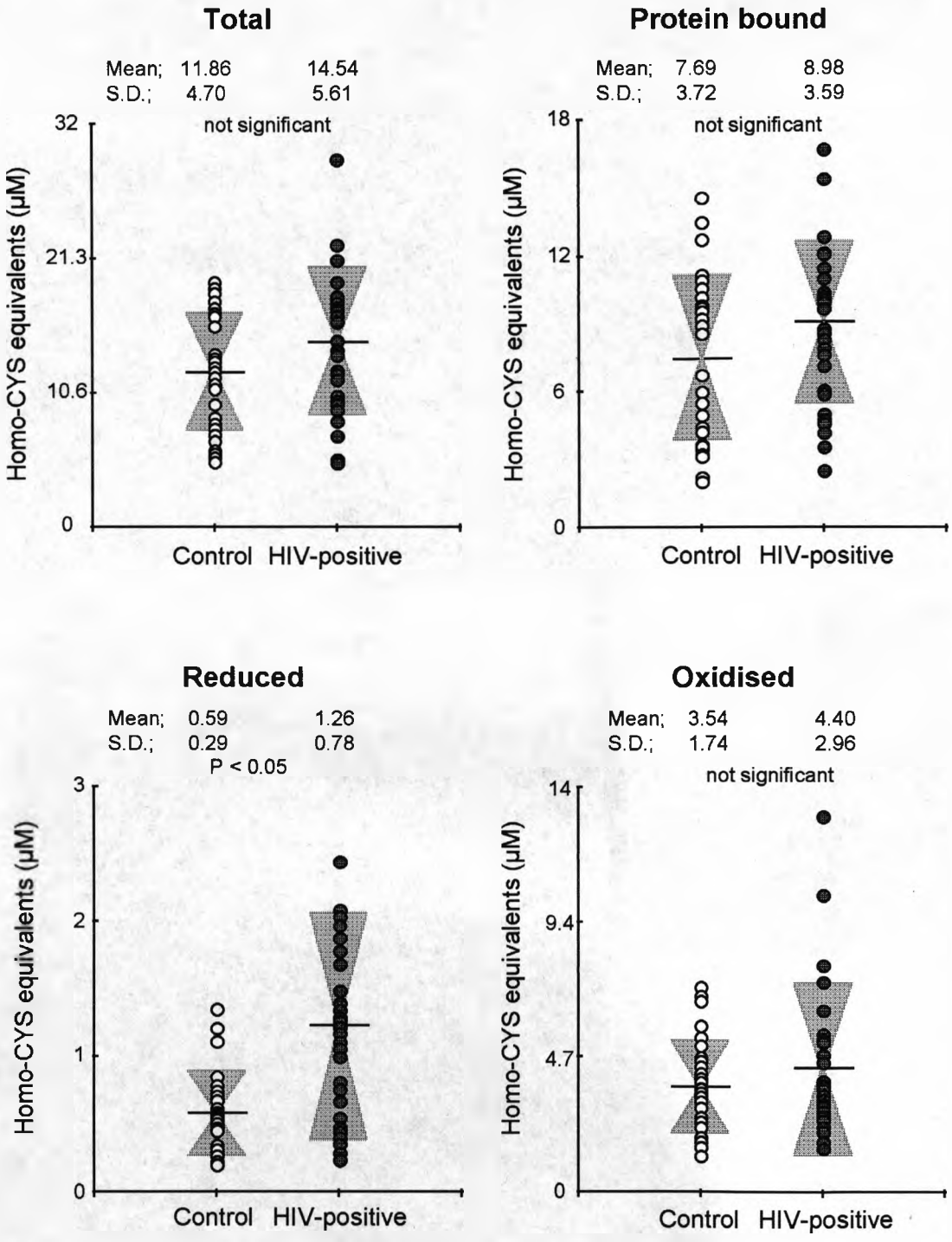


Figure 6.5 Plasma homo-CYS concentrations in HIV-positive patients and controls (n=27). The bars represent the means ± S.D. Statistical analysis was performed by comparing homo-CYS levels in HIV-positive patients to that obtained in controls.

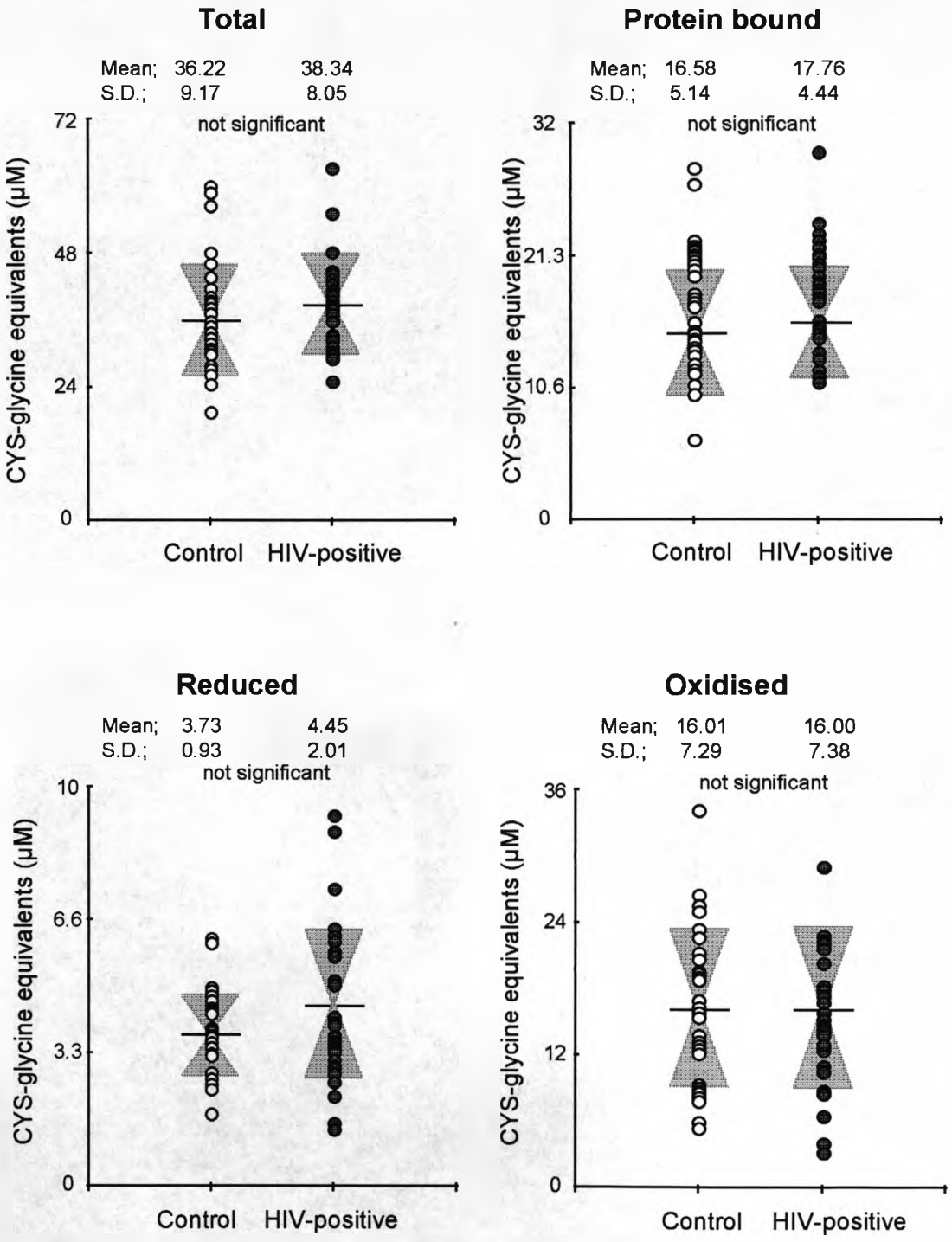


Figure 6.6 Plasma CYS-glycine concentrations in HIV-positive patients and controls (n=27). The bars represent the means ± S.D. Statistical analysis was performed by comparing CYS-glycine levels in HIV-positive patients to that obtained in controls.

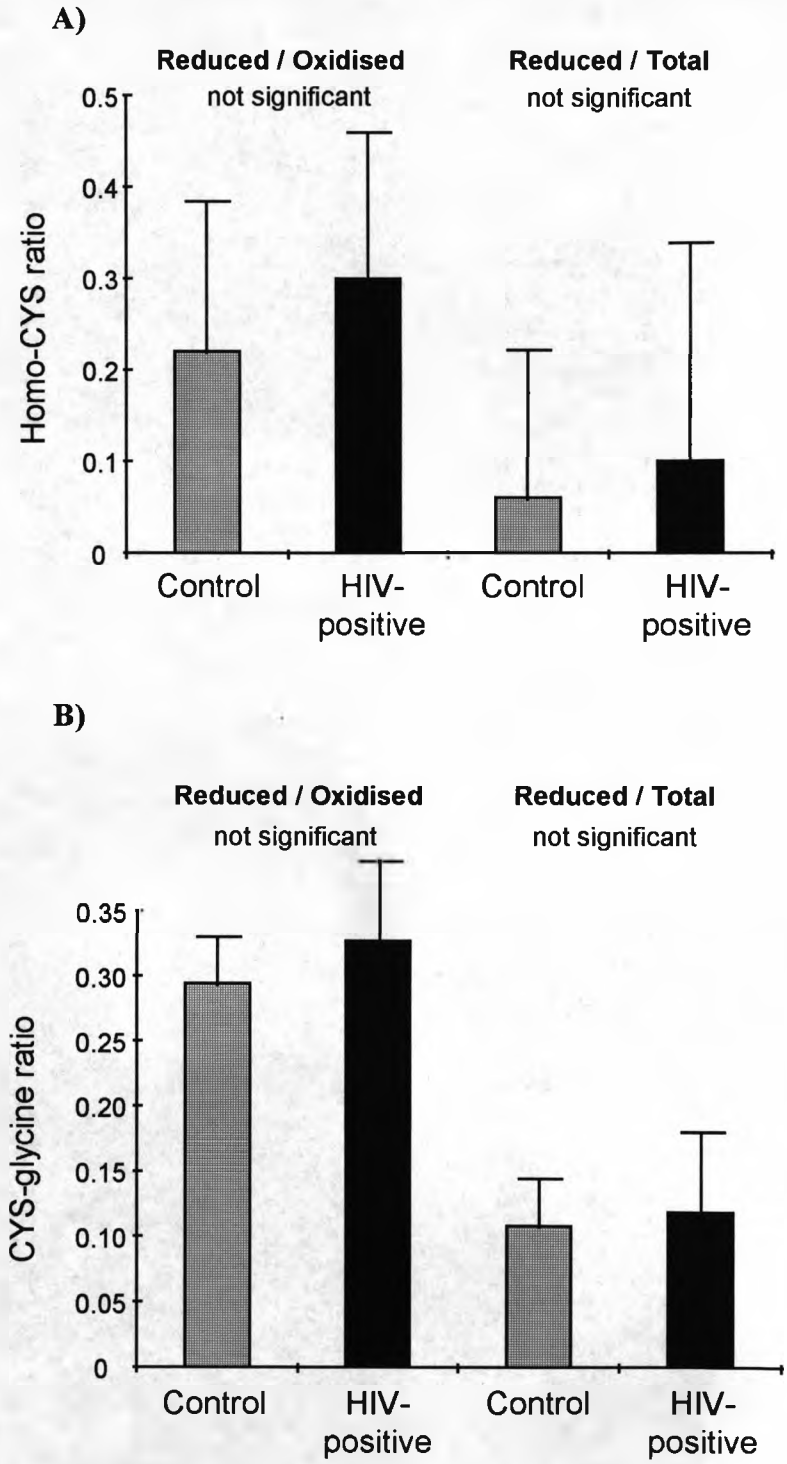


Figure 6.7 Ratios between reduced and oxidised and reduced and total plasma A) homo-CYS and B) CYS-glycine concentrations in 27 HIV-positive individuals and controls. The bars represent the means ± S.D. Statistical analysis was performed by comparing the ratio of thiol levels in HIV-positive patients to that obtained in controls.

6.3.5 Plasma Glutathione Concentrations

Plasma GSH concentrations were measured in 11 HIV-positive patients and 9 healthy controls. GSH levels could not be measured in the other samples due to the presence of interfering fluorescence peaks. There was no significant difference in total (HIV-positive, 10.5 ± 3.3μM; controls, 10.2 ± 2.7μM) and protein-bound (HIV-positive, 3.6 ± 1.8μM; controls, 3.2 ± 1.5μM) levels of GSH between HIV-positive patients and control subjects. Oxidised GSH was observed in 4 HIV-positive patients and 3 healthy controls. In these individuals, oxidised GSH concentrations did not exceed 1μM. Reduced GSH concentrations (HIV-positive, 10.5 ± 5.4μM; controls, 9.9 ± 4.4μM) were higher than those recorded previously (Walmsley *et al.*, 1997).

6.3.6 Relationship Between Thiol Concentration and Disease Progression

There was no relationship between $CD4^+$ count and plasma thiol levels in HIV-positive individuals ($r = -0.1$; 95% CI, -0.3 to 0.5; $P > 0.05$). Furthermore, no relationship was observed between viral load and plasma thiol concentrations ($r = -0.3$; 95% CI, -0.7 to 0.3; $P > 0.05$).

6.3.7 Relationship Between Thiol Concentration and Drug Hypersensitivity Reactions

Several groups have suggested that a thiol deficiency might be involved in the pathogenesis of hypersensitivity to trimethoprim-SMX (van der Ven *et al.*, 1994; Koopmans *et al.*, 1995). In this study, 4 patients had a history of

trimethoprim-SMX hypersensitivity. No difference could be demonstrated between reduced and disulphide concentrations of CYS, homo-CYS, CYS-glycine or GSH in hypersensitive and non-hypersensitive individuals. Of particular importance regarding trimethoprim-SMX hypersensitivity, reduced CYS levels were similar (hypersensitive, 13.9 ± 3.4μM; non-hypersensitive, 12.4 ± 3.0μM; $P > 0.05$). Additionally, no significant difference in reduced plasma CYS was observed in patients currently administered trimethoprim-SMX (patients taking trimethoprim-SMX [n=7], 12.5 ± 2.7μM; patients not taking the drug [n=13], 12.0 ± 2.9μM).

6.3.8 Comparison of the Reduction of Sulphamethoxazole Metabolites by Human Plasma in HIV-Positive Patients and Healthy Controls

Reduction of SMX-NHOH (50μM) to SMX, and SMX-NO (50μM) to the hydroxylamine and SMX were observed when either compound was incubated individually with plasma in patients and controls (table 6.3). Healthy individuals were more active at reducing SMX-NO to SMX-NHOH ($P < 0.01$) when compared with plasma from HIV-positive patients. Concurrently, plasma CYS concentrations were significantly lower in the patient group (table 6.4; $P < 0.05$).

	HIV-positive patients			**Healthy controls**		
	NO→NHOH	NO→NH_2	NHOH→NH_2	NO→NHOH	NO→NH_2	NHOH→NH_2
% Red.	40.2 ± 5.8*	14.4 ± 1.6	2.8 ± 1.0	55.4 ± 3.4	13.2 ± 1.4	2.8 ± 0.6

Table 6.3 Plasma reduction of SMX-NO to SMX-NHOH and SMX and SMX-NHOH to SMX in HIV-positive individuals and healthy controls (*P < 0.01).

Patient No.	Reduced CYS levels	
	HIV-positive	Healthy contols
	(μM)	(μM)
1	9.7 ± 0.6	13.1 ± 1.5
2	11.6 ± 0.4	13.9 ± 1.1
3	8.2 ± 1.0	13.8 ± 0.6
4	10.3 ± 0.8	12.5 ± 0.5
5	12.3 ± 0.8	10.0 ± 1.4
6	5.9 ± 0.8	10.4 ± 1.2
Mean ± S.D.	9.8 ± 2.5*	12.3 ± 1.7

Table 6.4 Reduced plasma CYS levels in HIV-positive patients and healthy controls used in the SMX reduction assay (*P < 0.05).

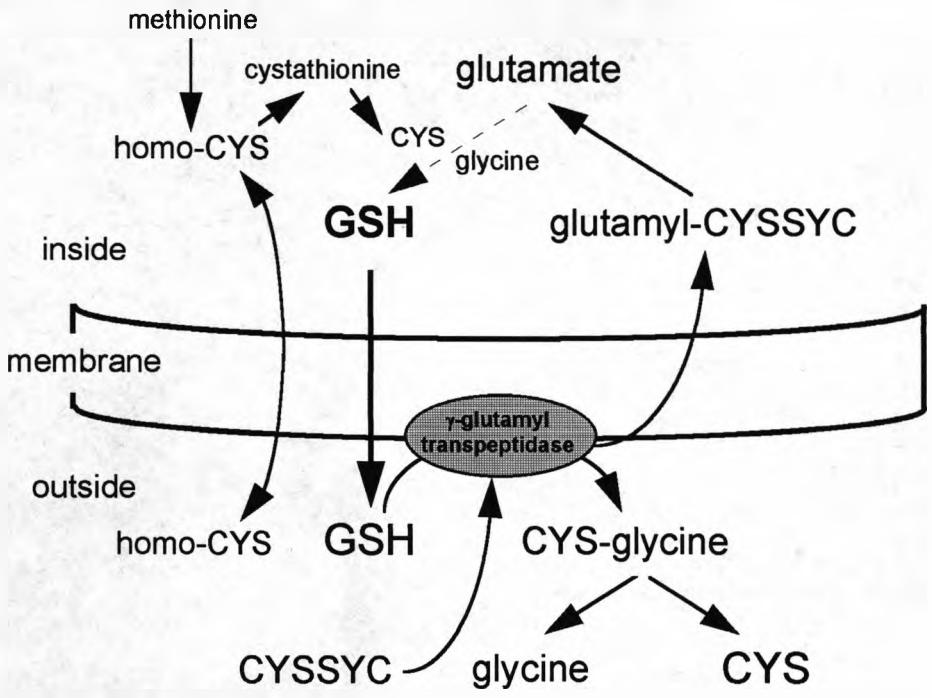
6.4 Discussion

A striking finding of this and previous studies (Lopez- Galera *et al.*, 1996; Muller *et al.*, 1996; Walmsley *et al.*, 1997; Aukrust *et al.*, 1997) was the large inter-individual variation in thiol levels from a given patient population. A variety of independent phenomena are thought to be responsible for the variation. Age, sex, diet, disease status, drug metabolism, environmental toxins and immune phenomena have all been implicated (Meister and Anderson, 1983; references from table 6.2). It is also important to note that intracellular and extracellular thiol concentrations are not static, they are metabolically related and represent a dynamic situation where GSH appears to be the storage form and CYS the transport form. GSH breakdown is catalysed by γ-glutamyl transpeptidase on the

external surface of cell membranes. This reaction results in the formation of CYS-glycine, which is further cleaved to CYS and glycine (Meister, 1981; Tate and Meister, 1981; Meister, 1983). CYS interacts with the enzyme γ-glutamylcysteine synthetase, leading ultimately to the restoration of GSH. Homo-CYS, a product of transmethylation, is exported extracellularly when production exceeds metabolic capacity. Intracellularly, homo-CYS is converted to either cystathionine or to methionine. Cystathionine is further metabolised to CYS (fig. 6.8).

Human thiol levels have been determined using a variety of techniques since the mid 1970s (Brehe and Burch, 1976; Kosower and Kosower, 1978; Akerboom and Sies, 1981). The pathogenic and therapeutic importance of the reported decrease in thiol concentrations in HIV-positive individuals has led to a revaluation of the analytical procedures used (Pirmohamed *et al.*, 1995; Walmsley *et al.*, 1997; van der Ven *et al.*, in press). Previous studies have measured only reduced thiol concentrations, while others have analysed total, protein bound or oxidised levels. Furthermore, to fully evaluate the pathological importance of redox status in HIV-disease, the ratio of reduced to oxidised and reduced to total thiols, which has often been ignored, should be compared from the same individuals using the same experimental protocol. Therefore, in this study, a sensitive HPLC method was used to measure plasma thiol levels in HIV-positive individuals and healthy controls. The method involves derivatisation of plasma thiols with bromobimane to create highly fluorescent adducts that can be quantified by HPLC (Cotgreave and Moldeus, 1986; Mansoor *et al.*, 1992; Muller

et al., 1996). A strict protocol was followed with reference to both blood collection and bromobimane derivatisation. Additionally, all samples were derivatised immediately after venepuncture and one control was analysed in parallel with each patient.



Taken from Meister and Anderson, (1983).

Figure 6.8 Simplified diagram depicting the metabolism and transport of CYS, GSH, homo-CYS and CYS-glycine across cellular membranes.

A significant decrease in reduced plasma CYS was observed in patients infected with the HIV virus (fig. 6.3). These data are in accordance with recent studies that have also reported a deficiency (Akerlund *et al.*, 1996; Helbling *et al.*, 1996; Walmsley *et al.*, 1997). Whether reduced plasma CYS is decreased in HIV-infected patients remains controversial; the failure of certain groups to show a

deficiency (Aukrust *et al.*, 1995; Lopez-Galera *et al.*, 1996; Muller *et al.*, 1996) is probably due to methodological differences, as mentioned above. Interestingly, Muller *et al.* (1996), failed to show a deficiency of reduced plasma CYS in HIV-positive patients, despite using a similar analytical protocol. This discrepancy may be due to the large inter-individual variation observed in this type of analysis. In this respect, genetic and environmental variations may also lead to altered thiol status. Therefore, future studies should standardise for each variable, and secondly, be of a large enough sample size to prevent a type II error.

While no differences in total, oxidised or protein-bound CYS were observed (possibly because reduced CYS in HIV-positive patients only decreased by 3.9μM), HIV-positive patients showed a decrease in the ratio of reduced to oxidised and reduced to total CYS. These findings, which are in contrast with the findings of Walmsley *et al.* (1997), suggest that patients may be subject to enhanced oxidative stress. Several reports have demonstrated that oxidative stress may cause activation of cytokines which play an important role in the progression of HIV and AIDS (Rosenberg and Fauci, 1990; Staal *et al.*, 1990; Westendorp *et al.*, 1995; fig. 6.9). Whether thiol replacement therapy can inhibit replication of the HIV virus is a subject of ongoing research. Preliminary studies have demonstrated an increase in plasma CYS and intracellular GSH after treatment with *N*-acetyl CYS (de Quay *et al.*, 1992; Roederer *et al.*, 1992) and GSH (Helbling *et al.*, 1996); however, these results require confirmation using a strict experimental protocol.

A significant increase in reduced plasma homo-CYS was observed in HIV-positive individuals (fig. 6.5). Oxidised, protein-bound and total concentrations remained the same. These data are in agreement with Muller *et al.* (1996), who postulated that increased levels of reduced homo-CYS enhance HIV replication and disease progression through the activation of NF-κB. Interestingly, patients with various other conditions, including homocystinuria and cobalamin deficiency, have increased levels of reduced homo-CYS (Mansoor *et al.*, 1992 and 1994). Furthermore, a recent study by Aukrust *et al.* (1997) demonstrated elevated concentrations in patients with common variable immunodeficiency.

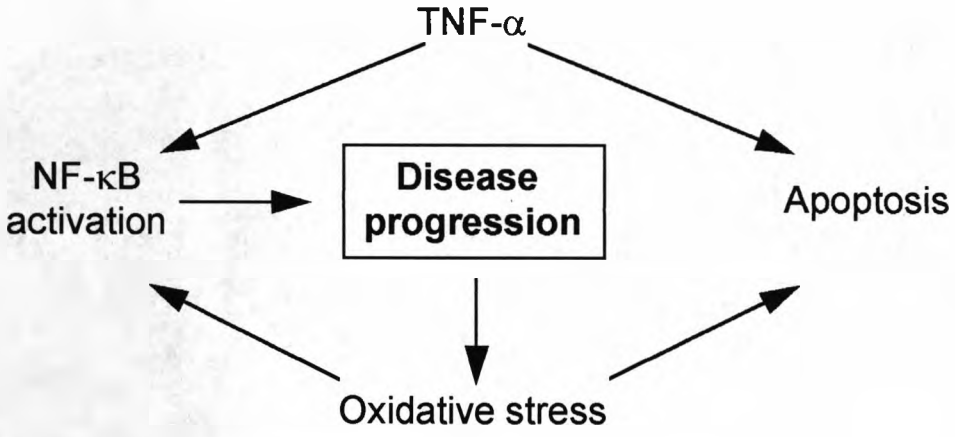


Figure 6.9 Proposed relationship between cytokines, oxidative stress and HIV-infection.

Two major difficulties were encountered when determining plasma GSH concentrations. First, interfering fluorescent peaks prevented the analysis of GSH concentrations in sixteen HIV-positive patients and eighteen healthy controls, and secondly, reduced and protein-bound levels often exceeded total GSH concentrations. Thus, our results should be interpreted with caution, and further

underline the technical problems which can be encountered when measuring thiol concentrations, particularly in patients on multiple drug therapy. No significant differences in total, oxidised, protein-bound or reduced CYS-glycine were noted when HIV-positive patients and controls were compared. These data are in contrast with other groups, that describe CYS as a rate limiting precursor in GSH synthesis (Meister and Anderson, 1983; de Quay *et al.*, 1992; Walmsley *et al.*, 1997).

A relationship between patients with advanced immune failure ($CD4^+$ count < 200 x 10^6 cells/l) and decreased thiol concentrations has been reported by some (de Quay *et al.*, 1992; Kinscherf *et al.*, 1994), but not all investigators (Staal *et al.*, 1992; Muller *et al.*, 1996; Pirmohamed *et al.*, 1996; Walmsley *et al.*, 1997). In this study, there was no discernable relationship between thiol levels and $CD4^+$ count, suggesting that reduced plasma CYS changes occur early in HIV-infection.

Eck *et al.* (1989) demonstrated that treatment of HIV-infected patients with AZT improved plasma CYS concentrations because of retroviral inhibition. It seems logical, therefore, that a high viral load may correlate with low plasma thiol levels. In this study, however, no relationship was observed.

No difference in reduced plasma CYS levels were observed between patients known to be hypersensitive to trimethoprim-SMX and non-hypersensitive patients. This may be a reflection of the fact that these patients were tested when they had recovered from the episode of hypersensitivity, and not actually at the time of the reaction. In addition, no difference in thiol levels were observed with patients currently taking the drug. These data, which have been observed in other

studies (Piromohamed *et al.*, 1996; Walmsley *et al.*, 1997), suggest that trimethoprim-SMX administration is not wholly responsible for the decreased levels of reduced plasma CYS in HIV-positive patients.

A thiol deficiency has been hypothesised to reduce an individuals capacity to detoxify electrophilic drug metabolites (van der Ven *et al.*, 1994). Data presented in chapter 3 and recent reports that failed to show a deficiency of intracellular GSH (Aukrust *et al.*, 1995; Pirmohamed *et al.*, 1996; van der Ven *et al.*, in press) indicate that plasma CYS concentrations may be an important determinant of the degree of reduction of SMX-NHOH and SMX-NO back to SMX, and consequently may determine susceptibility to idiosyncratic toxicity. Furthermore, a pilot study by Lehmann *et al.* (1996) has demonstrated a lower plasma reductive capacity in HIV-positive individuals. These results are supported by this study which has shown that patients with HIV-infection reduce SMX-NO to a lesser extent than healthy individuals (table 6.3).

In conclusion, this study shows that HIV-infected patients have lower plasma CYS levels, and this results in reduced detoxification of SMX-NO, which may partly account for the higher frequency of idiosyncratic toxicity in these patients.

Chapter 7:

The Role of Neutrophil Apoptosis in the Pathogenesis of Drug-Induced Agranulocytosis

7.1 Introduction

Agranulocytosis is characterised by a depletion in neutrophil number below 0.5 x 10^9/l (Pisciotta, 1990). The incidence of drug-induced agranulocytosis is hard to define accurately because of the gross under-reporting of the reactions. However, a literature search carried out for the period 1969-1979 (Young and Vincent, 1980), found 714 papers reporting drug-induced agranulocytosis, while in Sweden, Bottinger *et al.* (1979) found an annual incidence of agranulocytosis of 2.6 per million.

Drug-induced agranulocytosis may be dose-dependent or idiosyncratic (section 1.5.2). Dose-dependent reactions are predictable and can be avoided by a simple dose reduction. Cytotoxic drugs are the most common cause of dose-dependent agranulocytosis, where bone marrow depression is a direct extension of their known pharmacological effect as anti-tumour agents. In contrast, idiosyncratic agranulocytosis is difficult to anticipate because it is often related to an abnormality in drug metabolism that is only expressed in a small proportion of individuals (Park *et al.*, 1992). An imbalance between bioactivation and detoxification leading to the formation of a chemically reactive metabolite has been implicated in the pathogenesis of idiosyncratic agranulocytosis for a variety of unrelated compounds (Park *et al.*, 1987; Pisciotta, 1990; Uetrecht, 1992; Pirmohamed *et al.*, 1994). Toxic metabolites can act directly by binding to essential macromolecules through the formation of a covalent bond. Alternatively, they may act as a hapten, causing tissue damage through an immune-response (Park *et al.*, 1987; Pohl *et al.*, 1988).

Previous studies have used cell membrane permeability as an end-point to measure drug-induced cell death (Riley *et al.*, 1988; Rieder *et al.*, 1988; Winstanley *et al.*, 1990). It is important to note that these techniques do not distinguish between the two major forms of cell death, apoptosis and necrosis (Kerr *et al.*, 1972; Searle *et al.*, 1982). Necrosis is an uncontrolled destructive phenomenon that disturbs energy producing pathways after direct injury by a variety of non-physiological agents. In contrast, apoptosis is a highly regulated form of cell death that plays an important role in the controlled depletion of cells during normal cell turnover (Golstein *et al.*, 1991; Corcoran *et al.*, 1994). The morphological characteristics that distinguish apoptosis from necrosis (section 1.5.6) can be compared at the light microscopic level (fig. 7.1) and are summarised in table 7.1.

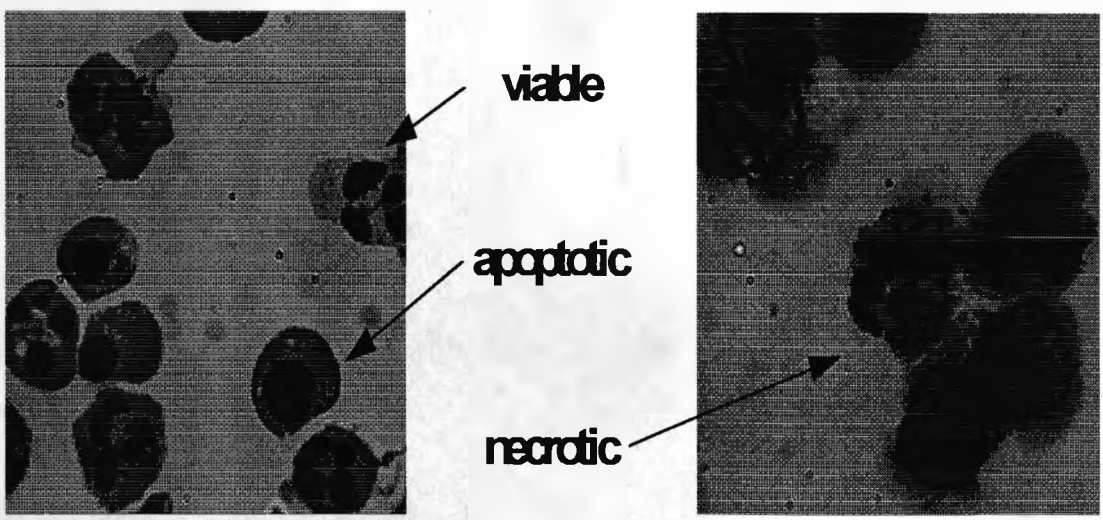


Figure 7.1 Morphological features of viable, apoptotic and necrotic neutrophils.

Feature	Apoptosis	Necrosis
Distribution	Single cells	Clusters of cells
Kinetics	Several hours	Hours to days
DNA cleavage	Early large fragments Late small fragments	Random fragments
Tissue response	No inflammation Phagocytosis by adjacent cells Rapid involution without collapse of overall tissue structure	Acute inflammation Secondary scarring
Circumstance	Physiological Cell-mediated immune killing Hypoxia (mild) Toxicants (low doses)	Pathological Compliment-mediated Hypoxia (severe) Toxicants (high doses)

Taken from Corcoran *et al.* (1994).

Table 7.1 Comparison of the characteristic features of apoptosis and necrosis.

Recent evidence suggests oxidative stress may play an essential role in neutrophil apoptosis. For example, many chemotherapeutic agents and physical treatments that induce apoptosis initially evoke oxidative stress, while the addition of reactive oxygen species or depletion of cellular antioxidants such as glutathione (GSH) can induce apoptosis (Iwata *et al.*, 1992; Buttke and Sandstrom, 1994; Watson *et al.*, 1996a; Narayanan *et al.*, 1997). On the other hand, apoptosis can be blocked by the protective effects of antioxidants or molecules that enhance the antioxidant defence system. Additionally, drugs known to cause direct- (cytotoxic drugs) and metabolism-dependent (e.g. dopamine) toxicity have been shown to induce apoptosis, at least in part by the

production of oxidative stress (Offen *et al.*, 1995; Offen *et al.*, 1996; Gorman *et al.*, 1997).

The compounds used in this chapter (amodiaquine [AQ], clozapine [CLZ] and sulphamethoxazole [SMX]) were chosen because they have all been reported to cause idiosyncratic agranulocytosis (Mandell and Sande, 1985; Neftel *et al.*, 1986; Lieberman and Safferman, 1992). Although chemically unrelated, hepatic metabolism of each compound yields an intermediate(s) which has been implicated in the pathogenesis of toxicity. AQ (chapters 2 and 5) forms a quinoneimine (Harrison *et al.*, 1992; Jewell *et al.*, 1995), CLZ (chapter 4) an unstable nitrenium ion (Maggs *et al.*, 1995; Pirmohamed *et al.*, 1995), while SMX is metabolised to hydroxylamine and nitroso metabolites (Cribb and Spielberg, 1990). The same intermediates can be generated by activated neutrophils *in vitro* (Cribb *et al.*, 1990; Tingle *et al.*, 1995; Maggs *et al.*, 1995). NADPH oxidase and myeloperoxidase (MPO) catalyse the reactions by one and two electron oxidations via one-electron transfer and through the production of hypochlorous acid (Babior, 1984) (fig. 7.2).

Neutrophils possess a powerful defence system, comprising of detoxification enzymes and antioxidants that are capable of protecting the cell from oxidative damage. The reactive metabolites of AQ and CLZ decrease the antioxidant capacity of the cell by the depletion of GSH. This instigated nuclear membrane damage and cell death at concentrations approaching those seen therapeutically (chapters 4 and 5). In contrast, SMX hydroxylamine (SMX-NHOH) and nitroso SMX (SMX-NO) do not deplete intracellular GSH and are not cytotoxic (chapter 3).

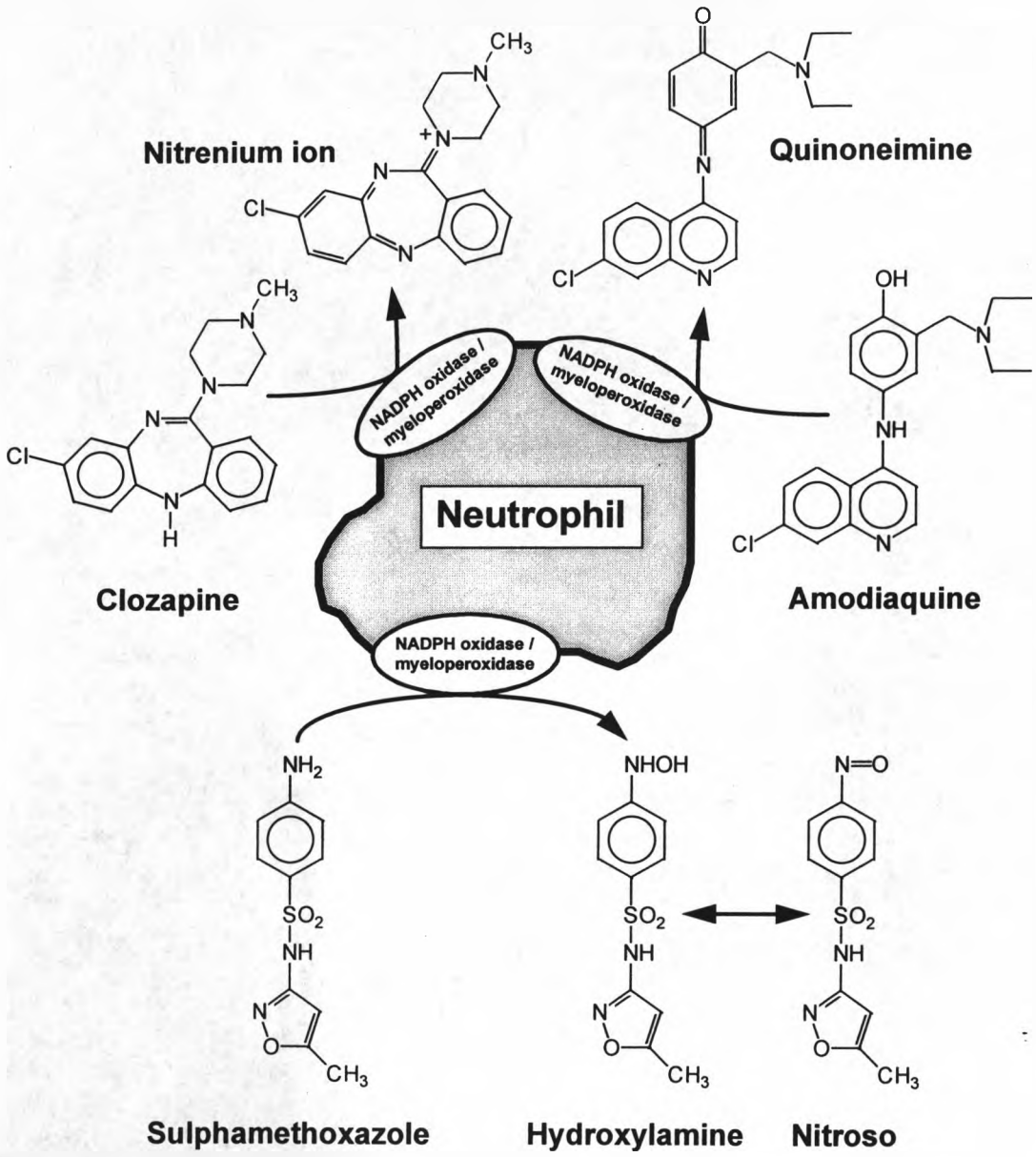


Figure 7.2 Scheme depicting the neutrophil metabolism of CLZ, AQ and SMX.

Therefore, the aims of this study were; first, to study drug-induced neutrophil apoptosis *in vitro*, and secondly, to investigate the role of oxidative stress. SMX-NHOH and SMX-NO were synthesised according to the method of Johnstone *et al.* (1978; chapter 3), while a peroxidase activating system,

previously used to measure neutrophil viability (chapters 4 and 5), was utilised in generating the reactive metabolites of AQ and CLZ *in situ*.

7.2 Materials and Methods

7.2.1 Chemicals

AQ, dimethyl sulphoxide (DMSO), ethylenediaminetetraacetate (EDTA), Giemsa stain, GSH, horseradish peroxidase (HRP; type VI), human serum albumin (HSA), hydrogen peroxide, May-grunwald stain, propidium iodide, RNAse (type I-A), SMX, sodium citrate, Tris and trypan blue were all obtained from Sigma Chemical Co. (Poole, UK). SMX-NHOH and SMX-NO were synthesised using a modification of the method described by Johnstone *et al.* (1978) (chapter 3), while CLZ was a gift from Novartis Pharmaceuticals (Basle, Switzerland). Monopoly® resolving medium (Ficcoll Hypaque, 1.114g/ml) and Lymphoprep® (1.077g/ml) were from ICN Biomedicals (Bucks., UK) and Nycomed (Birmingham, UK) respectively. All HPLC-grade solvents were purchased from Fischer Scientific (Loughborough, UK).

7.2.2 Isolation of Human Neutrophils

Polymorphonuclear leucocytes (neutrophils; PMN) were isolated from the venous blood of 10 healthy male volunteers (age range 21-40 years) on a dual density gradient of Monopoly resolving medium and Lymphoprep, as described in section 2.2.2. The cells were resuspended in HEPES buffered salt solution (pH 7.4) and diluted to the concentration required for each experiment. Cells were

greater than 98% pure and 95% viable as assessed by Wright's stain and trypan blue dye exclusion respectively (section 2.3.1).

7.2.3 Drug Treatment of Neutrophils

Initial experiments were designed to investigate whether DMSO and the individual components of the peroxidase activating system induced PMN apoptosis. PMN ($3x10^6$/incubation) were incubated with either HRP (20 units), hydrogen peroxide (1-100μM), DMSO (1%, v/v) or the combination of HRP (20 units) and hydrogen peroxide (10μM) in HEPES buffered salt solution (3ml; pH 7.4) at 37°C for 2, 4 or 8h. Apoptosis was quantified morphologically and by flow cytometry at each time point (section 7.2.4).

In other experiments, PMN (2×10^6) were incubated with drugs (AQ, CLZ, SMX and metabolites; 1-300μM) in HEPES buffered salt solution (2ml; pH 7.4), in the presence or absence of the peroxidase activating system (HRP [20units], hydrogen peroxide [10μM]) as described previously (chapters 4 and 5). Drugs were added in DMSO, which as a 1% (v/v) solution, did not induce apoptosis. After 2h (37°C), the tubes were centrifuged (650*g*; 10min) to pellet the cells. The supernatants were discarded and the cells were resuspended in 1ml of drug-free HEPES containing HSA (5mg/ml), and incubated for a further 2 or 6h. In all experiments, ethanol (1%, v/v) which has previously been shown to induce apoptosis (Lennon *et al.*, 1991), was used as a positive control.

7.2.4 Determination of Neutrophil Apoptosis

7.2.4.1 Morphologic Evaluation

PMN were assessed for apoptotic changes using Wright's stain. 100µl of each cell solution was spun onto a glass slide at 90*g* for 10min using a Shandon-Elliot cyto-centrifuge (London, UK). The slides were air dried for 30min prior to staining with May-Grunwald solution (0.25% w/v, in methanol; 8min); a procedure which also fixed the cells, and Giemsa solution (0.03% w/v; 18min). The stained cells were viewed under an inverted light microscope (Shandon Elliot, London, UK) for apoptotic features (Watson *et al.*, 1996b; fig. 7.1). A minimum of 200 randomly selected cells in each field were examined.

7.2.4.2 Flow Cytometric Evaluation

Flow cytometric evaluation of apoptotic nuclei was assessed according to the method originally described by Nicoletti *et al.* (1991). After 4 and 8h, PMN ($1x10^6$) were pelleted by centrifugation (200*g*, 8min), resuspended in 1ml of 70% ethanol (v/v) and stored for 30min at 37^0C. The fixed cell suspensions were then centrifuged, washed with HEPES (1ml) and finally suspended in 1ml of hypotonic fluorochrome solution containing 50µg/ml propidium iodide, 3.4mM sodium citrate, 1mM Tris, 100µM EDTA and 500µg/ml RNAse (type I-A). Permabilisation of the cell membrane was required because viable and apoptotic cells have an intact membrane that excludes propidium iodide. The cells were kept overnight in the dark at 4^0C prior to FACScan analysis on a Becton and Dickinson flow cytometer (Becton and Dickinson, Mountain View, CA, USA).

The forward scatter and side scatter of PMN nuclei were measured simultaneously. Propidium iodide fluorescence (FL-2) of individual nuclei were plotted against forward scatter and the data was registered on a logarithmic scale (fig. 7.3). Cell debris was excluded from the analysis by raising the forward threshold. 5000 nuclei were counted and analysed on a Hewlett Packard (HP 9000) computer using Lysis II software (Becton and Dickinson).

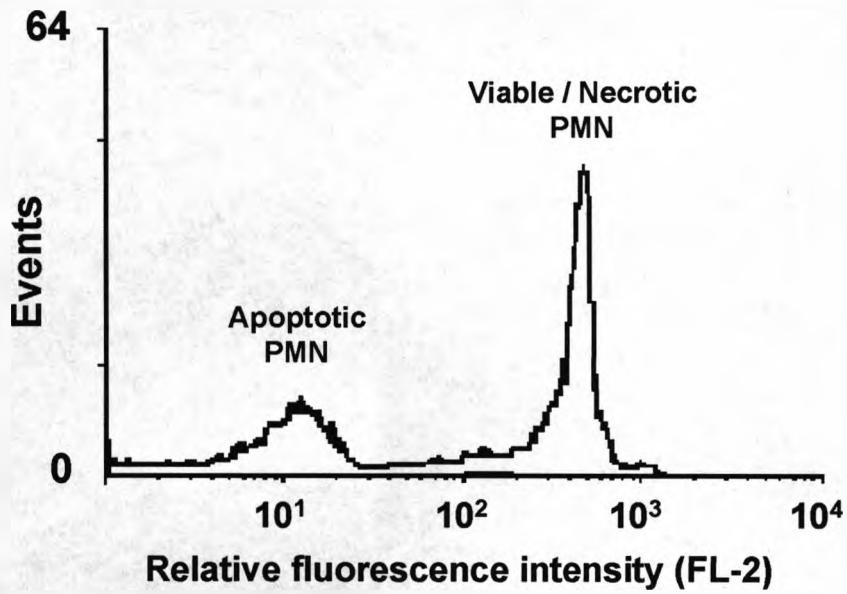


Figure 7.3 DNA distribution analysed by flow cytometry, after PMN membranes were permeabilised, and DNA was stained with propidium iodide. The population of apoptotic PMN show a characteristic decrease in DNA staining.

7.2.5 Determination of Neutrophil Cytotoxicity and Intracellular Glutathione Depletion

PMN cytotoxicity and intracellular GSH levels were measured by trypan blue dye exclusion and fluorescent HPLC, respectively. The experimental procedures have been described previously (chapters 3, 4 and 5).

7.2.6 Statistical analysis

The results are presented as mean ± S.D. of three separate experiments carried out in triplicate. Statistical analysis was performed by the Mann-Whitney test, accepting $P < 0.05$ as significant.

7.3 Results

7.3.1 Spontaneous Neutrophil Apoptosis

PMN have a life span of between 9 and 10h, after which time they die by apoptosis (Savill *et al.*, 1989). Therefore, a time course of 2, 4 and 8h was performed using morphologic and flow cytometric detection to measure spontaneous apoptosis in isolated PMN (fig. 7.4).

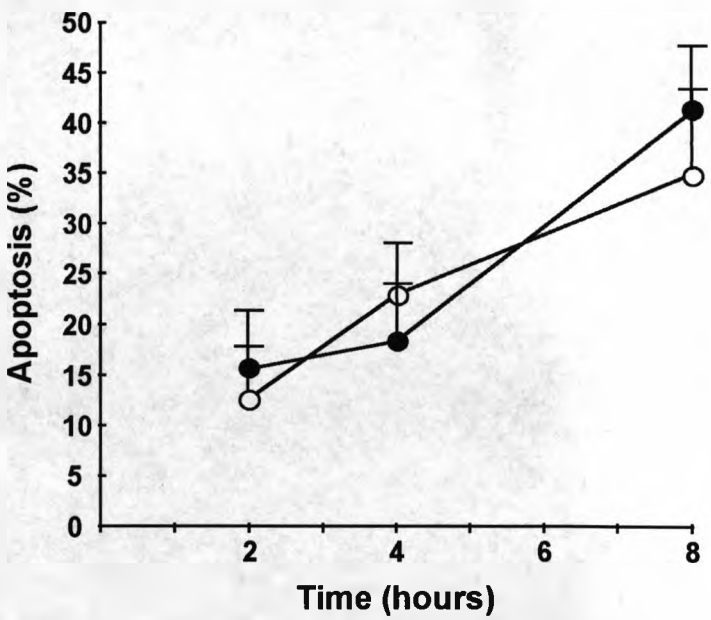


Figure 7.4 Spontaneous PMN apoptosis, assessed by morphologic (○) and flow cytometric detection (●). The results represent the mean ± S.D. of 3 separate experiments using cells from different individuals (all incubations carried out in triplicate).

7.3.2 Effects of Amodiaquine, Clozapine and Sulphamethoxazole on Neutrophil Apoptosis

The ability of AQ, CLZ and SMX (1-300μM) to increase the rate of spontaneous PMN apoptosis was assessed by flow cytometric analysis of DNA content by propidium iodide staining and confirmed morphologically by light microscopy. Incubation of AQ (30-300μM) and CLZ (300μM) with PMN for 8h resulted in an increase in spontaneous apoptosis ($P < 0.05$; fig. 7.5 and 7.6). In contrast, SMX had no effect (fig. 7.7). Similar results were obtained after 4h.

7.3.3 Effects of Amodiaquine, Clozapine and Sulphamethoxazole Metabolites on Neutrophil Apoptosis

The combination of HRP and hydrogen peroxide have previously been used to investigate the metabolism and PMN toxicity of AQ and CLZ (chapters 4 and 5). Initial experiments were designed to evaluate whether a similar system would be suitable to study PMN apoptosis. Neither HRP (20 units) or hydrogen peroxide (1-100μM) increased spontaneous apoptosis when incubated alone or in combination. Additionally, when the drugs were incubated with an incomplete activating system, i.e., in the absence of either HRP or hydrogen peroxide, there was no increase in apoptosis when compared with incubating drug alone. In the presence of the full activating system, AQ and CLZ were metabolised to their respective reactive metabolites (chapters 4 and 5) that induced PMN apoptosis at the lowest concentrations studied (1-3μM; fig. 7.5 and 7.6). At higher concentrations (10-100μM; AQ and CLZ), the mode of cell death switched from

apoptosis to necrosis, as characterised by morphologic and flow cytometric evaluation by a disruption of membrane integrity and an increase in the percentage of high intensity propidium iodide stained nuclei.

In contrast, SMX-NHOH and SMX-NO induced PMN apoptosis only at concentrations of 100μM and above (fig. 7.7). Cells incubated with either metabolite showed no morphological characteristics of necrosis.

7.3.4 Effect of Extracellular Glutathione on Drug-Induced Neutrophil Apoptosis

To investigate whether AQ-, CLZ- and SMX-induced apoptosis followed an oxygen dependent pathway, GSH (1mM) was incubated with the drugs. PMN apoptosis decreased to background levels when GSH was incubated with AQ or CLZ (1-3μM) and the full activating system. However, in the absence of activation, GSH failed to protect the cells (100-300μM; fig. 7.8 and 7.9). Similarly, apoptosis induced by SMX-NHOH and SMX-NO (100-300μM) was inhibited by the presence of GSH (fig. 7.9).

7.3.5 Neutrophil Cytotoxicity and Glutathione Depletion

AQ-, CLZ- and SMX-induced PMN toxicity and GSH depletion has been described previously (AQ, chapter 5; CLZ, chapter 4; SMX, chapter 3). The results are included as inserts to fig. 7.5, 7.6 and 7.7.

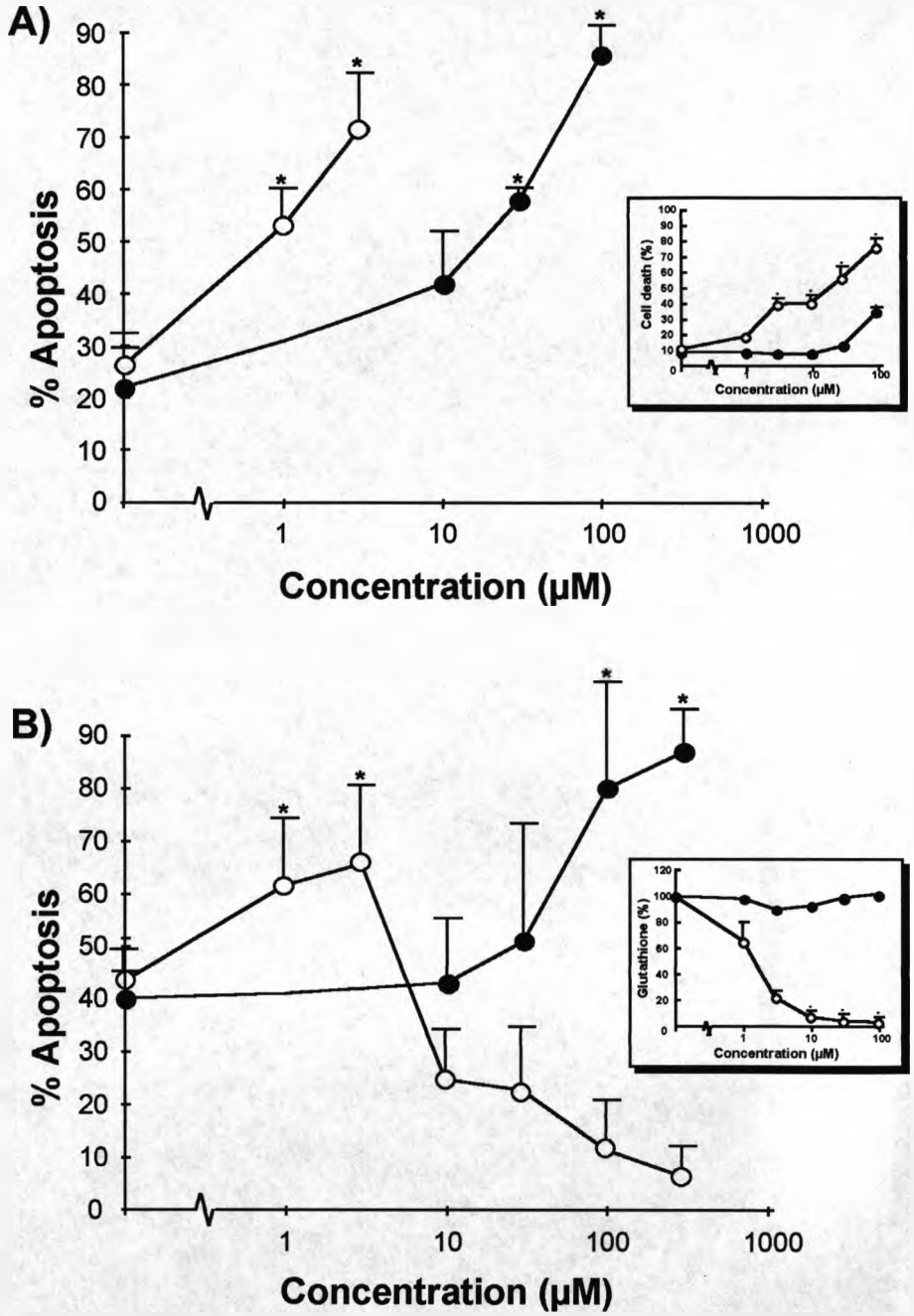


Figure 7.5 AQ-mediated PMN apoptosis in the presence (○) and absence (●) of HRP / hydrogen peroxide (20 units / 10μM). Apoptosis was measured by A) morphologic, and B) flow cytometric evaluation. The results represent the mean ± S.D. of three experiments carried out in triplicate. Statistical analysis was performed by comparing the ability of different concentrations of compound to induce apoptosis with that of solvent alone (*P < 0.05). Insert graphs show AQ-induced PMN cytotoxicity and GSH depletion in the presence (○) and absence (●) of activation. In the presence of peroxidase activation, at high concentrations the mode of cell death switched from apoptosis to necrosis.

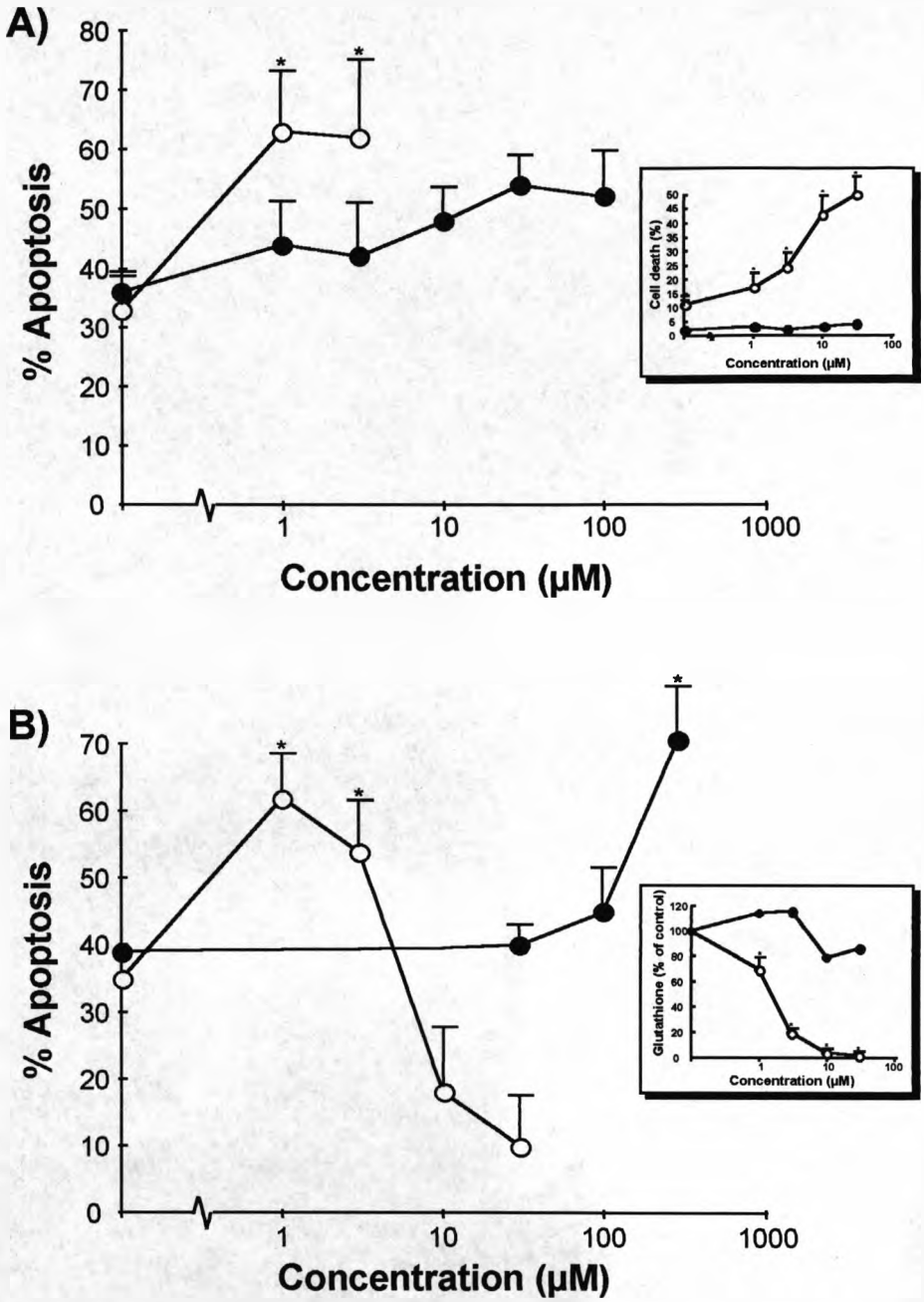


Figure 7.6 CLZ-mediated PMN apoptosis in the presence (○) and absence (●) of HRP / hydrogen peroxide (20 units / 10μM). Apoptosis was measured by A) morphologic, and B) flow cytometric evaluation. The results represent the mean ± S.D. of three experiments carried out in triplicate. Statistical analysis was performed by comparing the ability of different concentrations of compound to induce apoptosis with that of solvent alone (*P < 0.05). Insert graphs show CLZ-induced PMN cytotoxicity and GSH depletion in the presence (○) and absence (●) of activation. In the presence of peroxidase activation, at high concentrations the mode of cell death switched from apoptosis to necrosis.

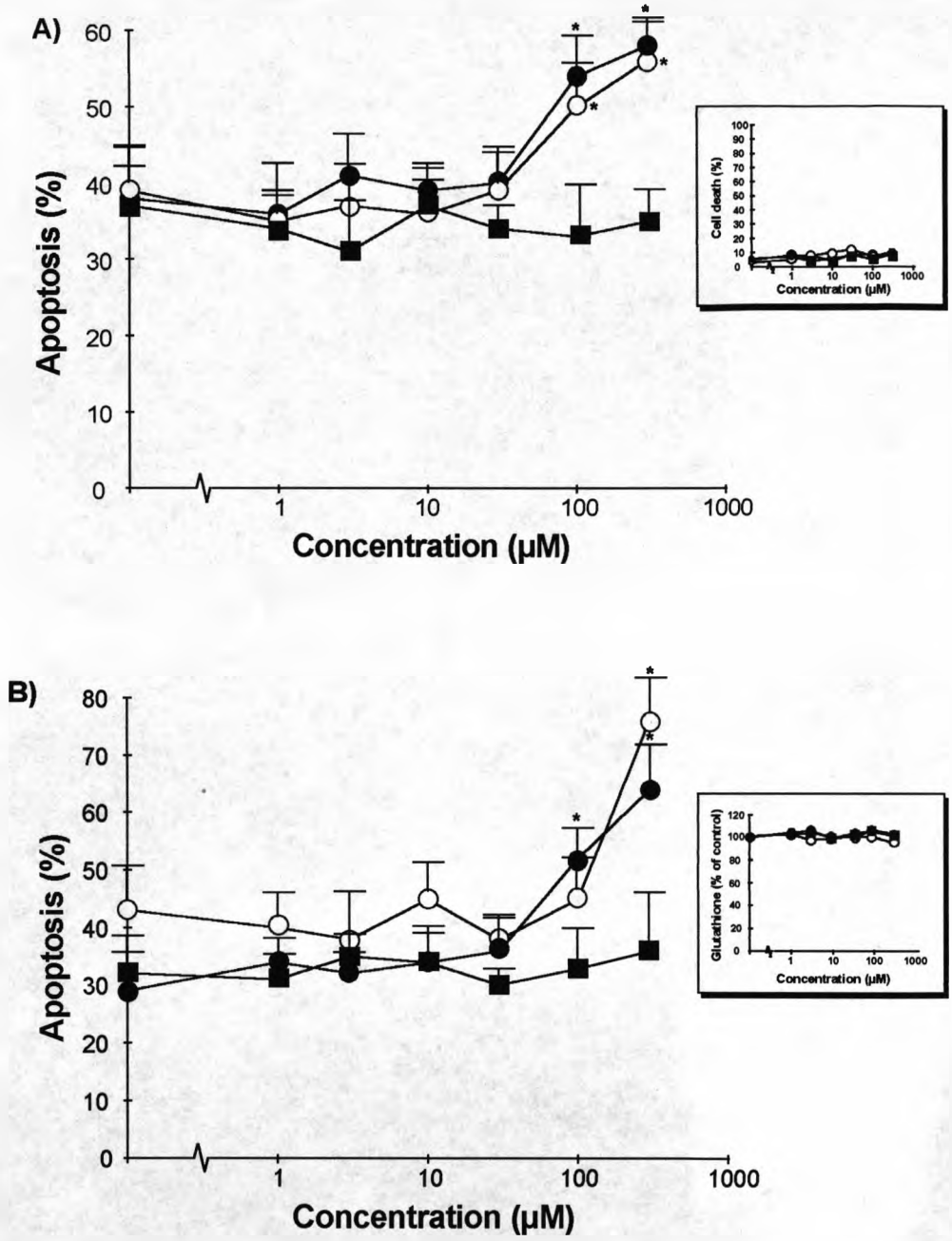


Figure 7.7 PMN apoptosis in the presence of SMX (■), SMX-NHOH (●) and SMX-NO (○,1-300μM). Apoptosis was measured by A) morphologic, and B) flow cytometric evaluation. The results represent the mean ± S.D. of three experiments carried out in triplicate. Statistical analysis was performed by comparing the ability of different concentrations of compound to induce apoptosis with that of solvent alone (*P < 0.05). Insert graphs show SMX, SMX-NHOH and SMX-NO induced PMN cytotoxicity and GSH depletion.

7.4 Discussion

Drug-induced toxicity towards neutrophils or their precursors may lead to neutropenia, which in severe cases can progress to agranulocytosis (Pisciotta, 1990). Cell death may occur via necrosis or apoptosis, two morphologically and biochemically distinct modes of cell death (Kerr *et al.*, 1972; Golstein *et al.*, 1991). Neutrophil apoptosis presents itself in a manner similar to that seen in other cells; i.e., cell condensation, cytoplasmic and chromatin shrinkage and DNA fragmentation into nucleosome sized pieces (Wyllie *et al.*, 1980; Searle *et al.*, 1982). Studies by Lennon *et al.* (1991) demonstrated that altering drug concentrations can influence the mode of cell death. Furthermore, decreasing a cells antioxidant capacity can change the mode of cell death from apoptosis to necrosis (Fernandes and Cotter, 1994). Bioactivation of AQ, CLZ and SMX yields compounds which are toxic to either lymphocytes or neutrophils (Rhodes *et al.*, 1986; Rieder *et al.*, 1988; Winstanley *et al.*, 1990; chapters 3, 4 and 5); however, whether the cells die by apoptosis or necrosis is unknown. The aim of this study therefore was to investigate the ability of these drugs and their reactive metabolites to induce neutrophil apoptosis *in vitro*.

Neutrophils are the major constituent of the total white blood cell pool (section 1.5). They have a half life of 5-6 hours in the peripheral circulation, after which time they die by apoptosis (Savill *et al.*, 1989). In apoptosis, flow cytometry detects cells with low propidium iodide stainability (fig. 7.3). The low stainability of apoptotic nuclei results from a change in DNA accessibility to propidium iodide and / or from endonuclease-catalysed DNA breakdown

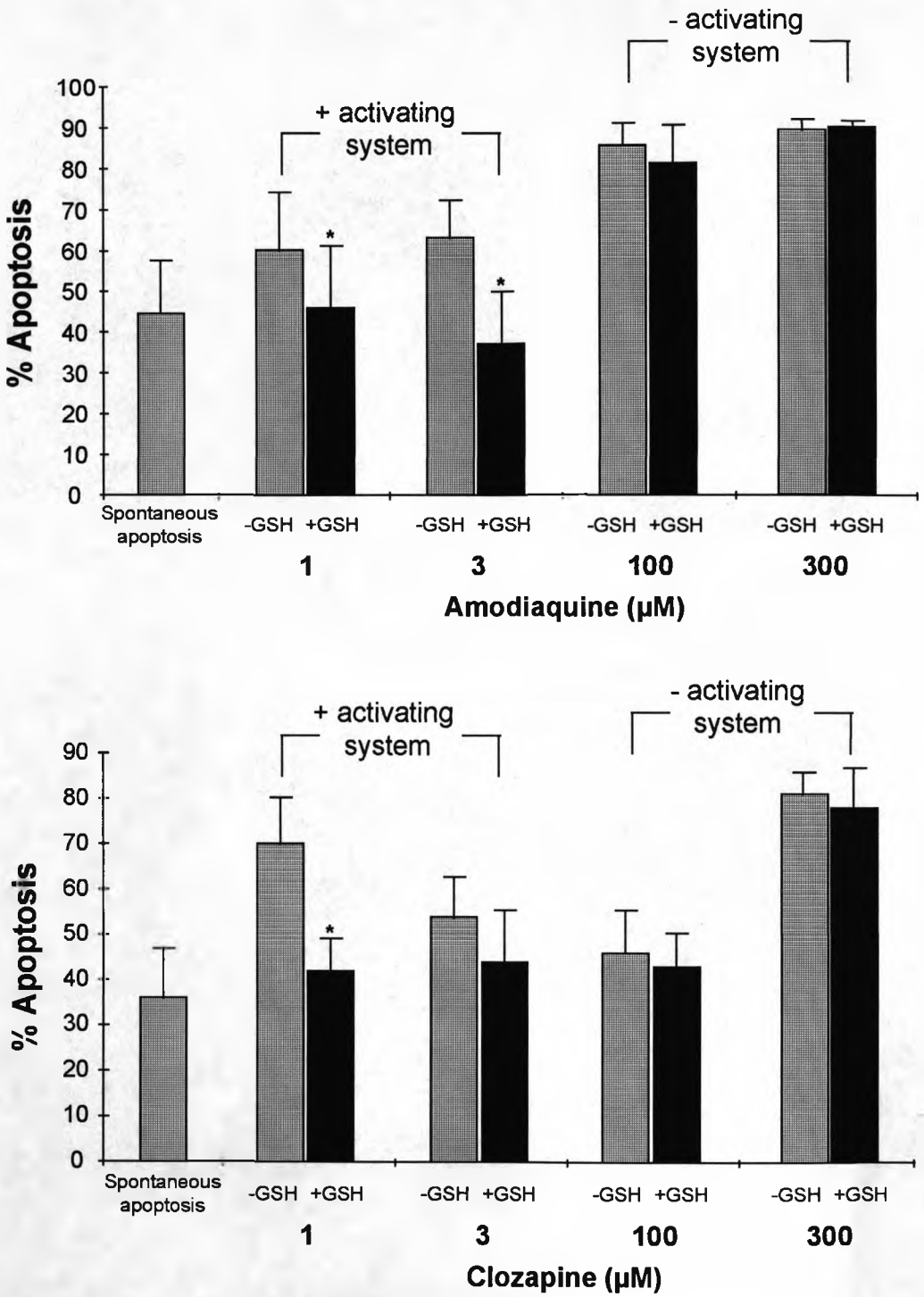


Figure 7.8 The effect of GSH (1mM) on drug-induced PMN apoptosis in the presence and absence of HRP (20 units) and hydrogen peroxide (10μM). Apoptosis was measured using propidium iodide by flow cytometric evaluation. The results represent the mean ± S.D. of three experiments (using cells from different individuals). Statistical analysis was performed by comparing PMN apoptosis with the same concentration of drug, in the presence and absence of GSH (*P < 0.05).

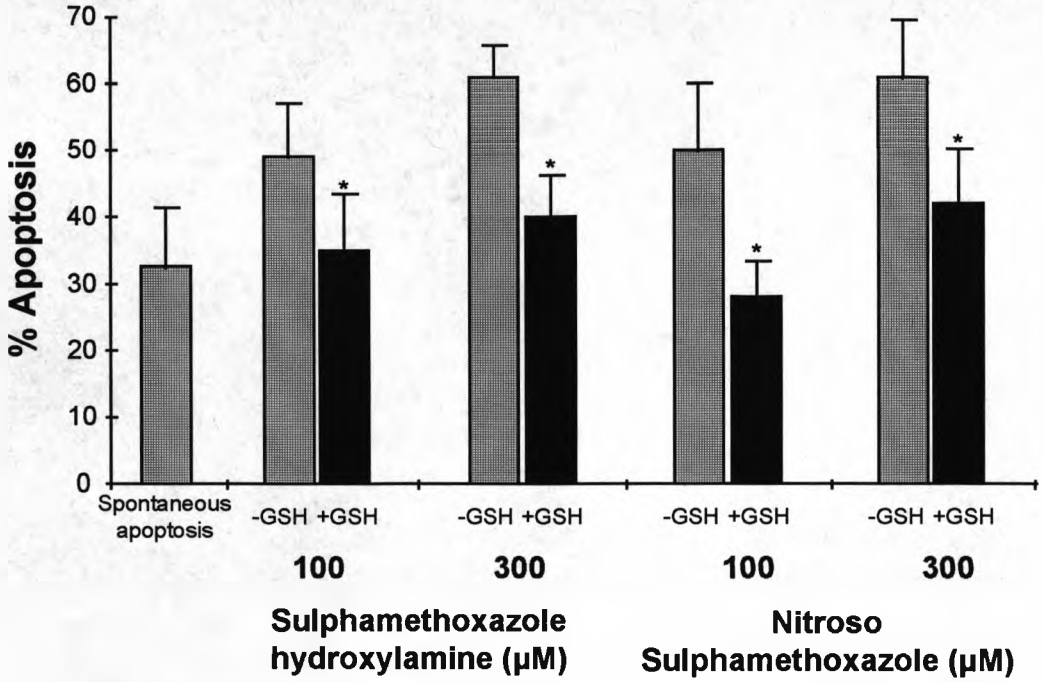


Figure 7.9 The effect of GSH (1mM) on drug-induced PMN apoptosis. Apoptosis was measured using propidium iodide by flow cytometric evaluation. The results represent the mean ± S.D. of three experiments (using cells from different individuals). Statistical analysis was performed by comparing PMN apoptosis with the same concentration of drug, in the presence and absence of GSH (*P < 0.05).

(Darzynkiewicz *et al.*, 1992). To confirm that flow cytometric evaluation was a true measure of apoptosis, typical morphological characteristics were identified by light microscopy (fig. 7.1). Initial experiments were performed to investigate the time-dependent nature of spontaneous neutrophil apoptosis *in vitro*. In accordance with previous studies (Watson *et al.*, 1996a, b and c), a significant increase in spontaneous apoptosis was observed with time (fig. 7.4).

A wide variety of physiological and chemical agents are capable of regulating cell death (Manaster *et al.*, 1996; Watson *et al.*, 1996a and c; Gorman *et al.*, 1997). Treatment of the cells with AQ (30-300μM) and CLZ (300μM)

produced a significant increase in apoptosis, while SMX was non-toxic (fig. 7.5, 7.6 and 7.7). Apoptotic cells excluded trypan blue, demonstrating that membrane permeability only occurred at higher concentrations and / or later time points. Additionally, apoptosis was independent of thiol concentration.

The mechanism of AQ-induced apoptosis may be related to the findings of Potvin *et al.* (1997). They demonstrated that chloroquine (chapter 2), a structurally related antimalarial, induced apoptosis in human endothelial cells by modulating the *Bcl-2* gene balance. Importantly, these effects were observed at similar concentrations to that used in this study.

Several groups have reported that apoptotic cells have a reduction in the mitochondrial transmembrane potential before they exhibit the common signs of nuclear apoptosis. This applies to various cell types and to biochemical pathogens and pharmacological agents (Zamzami *et al.*, 1995; Petit *et al.*, 1995; Castedo *et al.*, 1995). The mechanism of apoptotic disruption involves the voltage-mediated opening of mitochondrial permeability transition pores (Kroemer *et al.*, 1995). Consequently, accumulation of any agent causing depolarisation of the mitrochondrial membrane favours the onset of permeability transition. The amphiphilic nature of cationic compounds such as AQ and CLZ (Klempner and Styrt, 1983) allows passage through the lipid membranes, where they accumulate and become trapped. The resulting rise in pH may lead to pore formation and apoptosis. In accordance with this hypothesis, Petronilli *et al.* (1993) proposed that many inducers of apoptosis modulate the mitochondrial pore voltage;

however, the role of mitochondria in drug-induced toxicity requires further investigation.

Although the mechanism of AQ, CLZ and SMX-induced agranulocytosis remains unclear, metabolism of the parent compound to a protein-reactive metabolite is thought to be central (Maggs *et al.*, 1988; Carr *et al.*, 1993; Maggs *et al.*, 1995). In this study, HRP was used as a surrogate for MPO to produce reactive metabolites of AQ and CLZ *in situ*. The validity of such an approach has been described previously (chapters 4 and 5). In the presence of the full activating system (HRP and hydrogen peroxide), AQ and CLZ were metabolised to intermediates that induced apoptosis at the lowest concentrations studied (1-3μM). In addition, apoptosis was accompanied by GSH depletion and was observed at concentrations below those required to cause cell membrane damage. Importantly, apoptosis was also observed at therapeutic concentrations (Laurent *et al.*, 1993; Lin *et al.*, 1994).

Oxidative stress, reactive oxygen species and GSH depletion have been implicated in neutrophil apoptosis (Buttke and Sandstrom, 1994; Watson *et al.*, 1996a and 1996b; section 1.5.6). To investigate whether the increased rate of AQ- and CLZ-mediated apoptosis followed an oxygen-dependent pathway, the drugs were incubated in the presence of GSH. Neutrophil apoptosis induced by the drugs in the presence of the full activating system was reduced to background levels, while in the absence of activation, GSH failed to protect the cells. The ability of GSH to protect the cells from metabolism-mediated apoptosis suggests

that an oxidant-antioxidant balance may be involved in the maintenance of cell integrity.

Redox cycling is often associated with the toxicity of chemically reactive drug metabolites. The net result of redox cycling is increased oxidative stress and the formation of reactive oxygen species (superoxide, hydrogen peroxide and hydroxyl radicals; Monks *et al.*, 1992). Hydrogen peroxide and hydroxyl radicals have been implicated in the induction of apoptosis by several mechanisms. First, they can cause direct DNA damage, and result in an increase in poly (ADP-ribose) polymerase (Schraufstatter *et al.*, 1986). Second, they can increase the rate of transcription of *c-jun* and the proto-oncogenes; and finally, by amplification of cytoplasmic TNF-α and dissociation of NF-κB from its inhibitory subunit. The active NF-κB complex translocates to the nucleus and subsequent binding to DNA initiates the apoptotic cascade (Westendorp *et al.*, 1995).

A recent report by Watson *et al.* (1996a) demonstrated a redox-sensitive pathway of neutrophil apoptosis, where a reduction of intracellular GSH stimulated cell death. AQ quinoneimine and the nitrenium ion of CLZ conjugate directly with thiols, resulting in a depletion of intracellular GSH (Tingle *et al.*, 1995; chapter 4 and 5). Therefore conjugation of either metabolite, leading to GSH depletion, may cause apoptosis directly.

The ability of SMX-NHOH and SMX-NO to induce neutrophil apoptosis was also investigated. Both compounds caused a significant increase in apoptosis, which was attenuated by the addition of GSH. However, apoptosis was only observed at concentrations greater than those obtained therapeutically (Vree *et*

al., 1995). SMX is widely administered for the treatment of *Pneumocystis carinii* pneumonia in patients with HIV (Hughes *et al.*, 1993). HIV infection is associated with a substantial decrease in $CD4^+$ lymphocytes (Meyaard *et al.*, 1992), a process that is thought to occur by apoptosis (Ameisen, 1992; Muro-Cacho *et al.*, 1993). A steady increase in TNF-α, activation of NF-κB and a decrease in cellular antioxidants is known to stimulate apoptosis and disease progression (Eck *et al.*, 1989; Buttke and Standstrom, 1994). Therefore, SMX-induced neutrophil apoptosis may, at least in part, explain why the drug is associated with rapid disease progression in HIV-infected individuals (Veenstra *et al.*, 1997). Additionally, the ability of GSH to inhibit neutrophil apoptosis highlights an advantage of administering antioxidants such as *N*-acetyl cysteine, which replenish GSH stores (Droge, 1993), to patients with HIV infection.

In conclusion, this study demonstrated that AQ, CLZ, SMX-NHOH and SMX-NO induce neutrophil apoptosis *in vitro.* The pharmacological relevance of these results are questionable, since apoptosis was only observed at supra-therapeutic concentrations. More importantly, bioactivation of AQ and CLZ to their respective metabolites caused apoptosis at therapeutic concentrations. The ability of exogenous GSH to inhibit this effect, and not apoptosis induced by drug alone, suggests apoptosis proceeds via two distinct pathways, one dependent and one independent of oxidative stress. Finally, the mechanism by which these metabolites cause apoptosis, whether apoptosis occurs in man, and its role in drug induced-aganulocytosis, is unknown and requires further investigation.

Chapter 8:

Final Discussion

The aim of the work presented in this study was to investigate the role of metabolism in drug toxicity. Of particular interest in the pathogenesis of toxicity was the formation and cellular disposition of chemically reactive metabolites. Two drugs [amodiaquine (AQ) and clozapine (CLZ)] of different pharmacological and chemical class were studied; these drugs cause similar forms of selective idiosyncratic toxicity, i.e., agranulocytosis and hepatotoxicity (Neftel *et al.*, 1986; Hatton *et al.*, 1986; Gerson, 1992; Alvir *et al.*, 1994). In addition, sulphamethoxazole (SMX), which is associated with agranulocytosis and hepatotoxicity as part of a generalised hypersensitivity reaction, was also investigated (Cribb *et al.*, 1996b). Drugs which cause adverse reactions of this type should be distinguished from those such as cytotoxic chemotherapeutic agents, which cause toxicity by an extension of their normal pharmacological effects.

AQ, CLZ and SMX undergo bioactivation in the liver to form toxic and chemically reactive metabolites. AQ forms a quinoneimine (Harrison *et al.*, 1992; Jewell *et al.*, 1995), CLZ an unstable nitrenium ion (Maggs *et al.*, 1995; Pirmohamed *et al.*, 1995), while SMX is metabolised to hydroxylamine and nitroso metabolites (Cribb and Spielberg, 1990). In each case, several studies have highlighted the role of drug disposition in the cell-selective nature of toxicity (Pohl *et al.*, 1988; Uetrecht, 1989; Park *et al.*, 1992; Uetrecht, 1992; Pirmohamed *et al.*, 1994). Reactive intermediates often have a short half life, and do not reach a site distant from their site of formation. For this reason, toxicity is generally limited to the organ in which they are formed (fig. 8.1).

Neutrophils contain several enzymes which can oxidise drugs (Hofstra and Uetrecht, 1993). The most important of these enzymes is myeloperoxidase (MPO). In the presence of hydrogen peroxide, MPO is converted to an active form (compound I) (Odajima and Yamazaki, 1970). Compound I catalyses the formation of hypochlorous acid, a strong oxidant capable of metabolising almost any drug with an easily oxidisable functional group. AQ quinoneimine (chapters 2 and 5), the nitrenium ion of CLZ (chapter 4), SMX hydroxylamine (SMX-NHOH) and nitroso SMX (SMX-NO) (chapter 3) are generated by activated neutrophils *in vitro* (Cribb *et al.*, 1990; Tingle *et al.*, 1995; Maggs *et al.*, 1995). Although these reactions are unlikely to make a significant contribution to the overall metabolism of a drug, they may have important toxicological consequences (fig. 8.1).

Blood dyscrasias take fifth place after fever, skin, liver and endocrine abnormalities in terms of reported adverse drug reactions (ADRs) (Bottiger *et al.*, 1979; Vincent, 1986). A reduction of the peripheral neutrophil count is referred to as neutropenia. A further decrease in cell count, often caused by a drug, may result in agranulocytosis (a reduction in the peripheral neutrophil count below 0.5 x 10^9/l; Pisciotta, 1990). The overall incidence of agranulocytosis has been estimated to be about 2.6 per million inhabitants (Bottinger *et al.*, 1979). However, the incidence for individual drugs such as AQ, CLZ, aminopyrine, and phenothiazines is higher (Uetrecht, 1992).

The role of drug metabolism in the pathogenesis of agranulocytosis may be illustrated with reference to an example. Procainamide, an anti-arrhythmic drug is associated with a relatively high incidence of agranulocytosis (0.5-4% in various

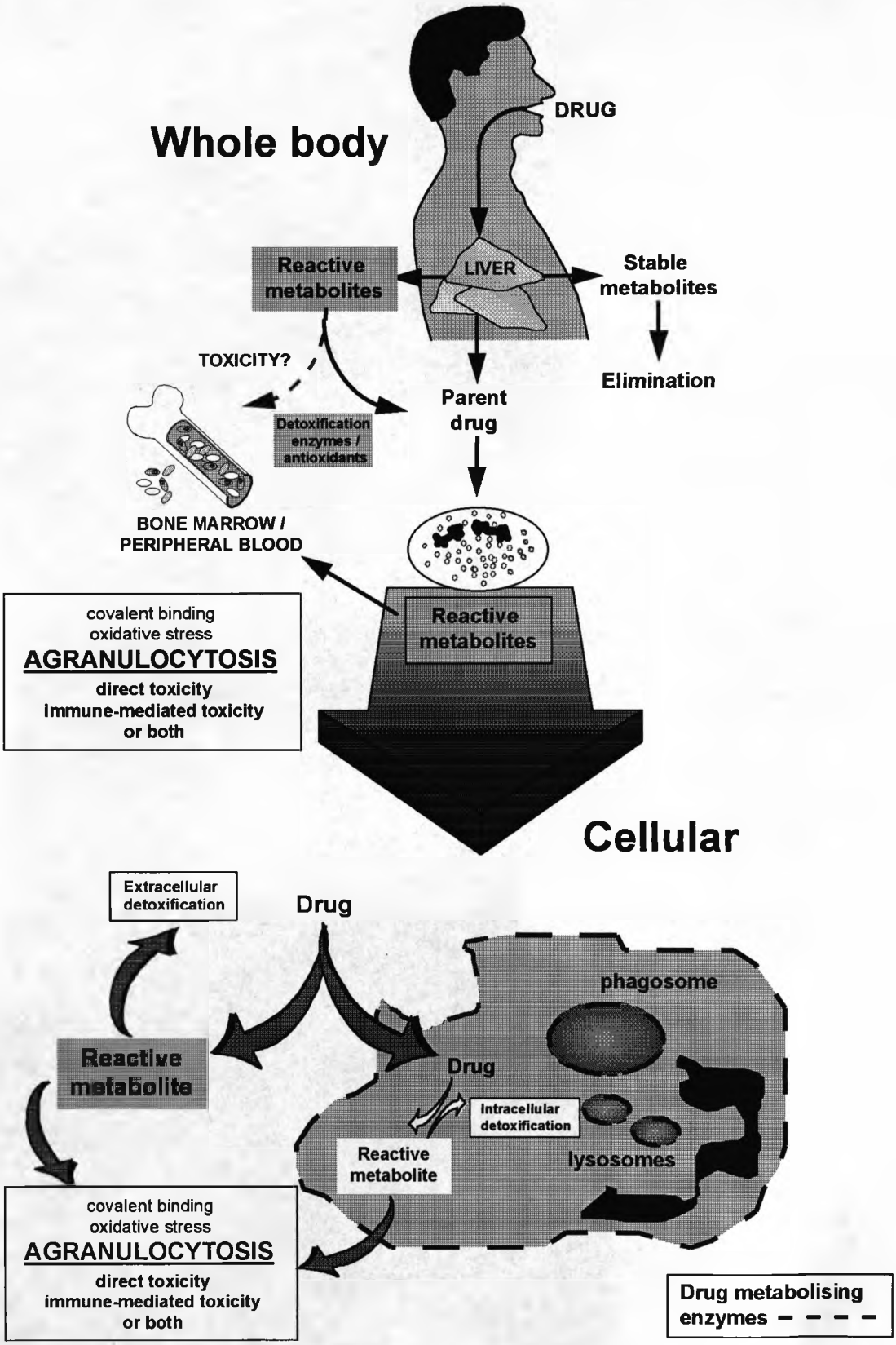


Figure 8.1 Scheme illustrating the role of whole body and cellular disposition in the pathogenesis of idiosyncratic drug-induced agranulocytosis.

studies; Uetrecht, 1992). Activated neutrophils metabolise procainamide to hydroxylamine, nitroso and nitro intermediates (Uetrecht, 1985; Rubin and Curnutte, 1989). Although the hydroxylamine and nitroso metabolites are protein reactive and cause direct neutrophil toxicity (Uetrecht and Zahid, 1991; Jiang *et al.*, 1994), whether agranulocytosis occurs via a direct effect, or is secondary to an immune response remains unknown (Gillette *et al.*, 1974; Park, 1986; Uetrecht, 1992). Procainamide is characteristic of most idiosyncratic ADRs; the ultimate toxin is well defined, but there is little or no direct evidence regarding its role in drug-induced agranulocytosis.

In the following sections, CLZ, AQ and SMX are discussed in turn, in order to illustrate how our knowledge of bioactivation and / or cellular disposition *in vitro* can explain the idiosyncratic toxicity observed in man.

CLZ is associated with a relatively high frequency of agranulocytosis (0.8%; Lieberman and Safferman, 1992; Alvir and Lieberman, 1994). In the UK alone, 51 patients receiving CLZ from January 1990 to July 1994 went on to develop agranulocytosis (Atkin *et al.*, 1996). Toxicity occurs within 3 months and is characterised by an absence or suppression of myelopoiesis in the bone marrow. In order to allow safe clinical use of the drug, a white blood cell monitoring scheme was bought into use by Novartis Pharmaceuticals. All patients are screened weekly for the first eighteen weeks and fortnightly thereafter.

Given that CLZ is not chemically reactive and cannot form stable covalent bonds (Maggs *et al.*, 1995; Pirmohamed *et al.*, 1995; Liu and Uetrecht, 1995), it seems unlikely that it would act as a hapten and induce an immune response. Thus,

if the parent drug were the ultimate cellular toxin, it would appear to act via a direct action on plasma membranes or other essential cellular functions. In these studies, CLZ was not cytotoxic to neutrophils at therapeutic concentrations (chapter 4). These data agree with previous studies, where CLZ has not been shown to be directly cytotoxic and does not interfere with the turnover of bone marrow precursor cells (Veys *et al.*, 1992; Gerson *et al.*, 1994).

CLZ undergoes extensive metabolism, with only 2-5% of the drug being excreted unchanged (Jann *et al.*, 1993). Demethyl CLZ (the major stable metabolite of CLZ [Gauch and Michaelis, 1971]) has been shown to be 4-10 fold more toxic than CLZ towards haemopoietic progenitor cells (Gerson *et al.*, 1994). However, the concentration required to produce toxicity *in vitro* (3-10μM) was much higher than normal serum concentrations (1μM; Gerson *et al.*, 1994). Additionally, data presented in chapter 4 demonstrate that demethyl CLZ and CLZ *N*-oxide had no effect on cell viability at concentrations below 100μM. Taken together, these data suggest that CLZ-induced agranulocytosis is unlikely to be due to a direct effect of the parent compound or its major stable metabolites.

To investigate the functional toxicity of neutrophil generated metabolites, a peroxidase activating system was utilised to generate the nitrenium ion of CLZ *in situ*. Horseradish peroxidase (HRP) was used for this purpose, and thus as a surrogate for the naturally occurring MPO. The validity of such an approach was confirmed by showing that the same products are formed on bioactivation by both HRP (chapter 4) and MPO (Maggs *et al.*, 1995). In the presence of the full activating system, CLZ was found to deplete intracellular glutathione (GSH) and

was toxic towards both neutrophils and lymphocytes. Whether these *in vitro* findings actually occur *in vivo* is a subject of ongoing research. The functional effects of generating the nitrenium ion have been demonstrated by incubating CLZ with lymphocytes and human liver microsomes (Tschen *et al.*, 1996). Furthermore, a recent study by the same group observed that cells from CLZ-hypersensitive patients were more susceptible to drug-induced toxicity (Tschen *et al.*, 1997). These findings, and the susceptibility of neutrophils to oxidative stress tend to suggest a direct mechanism of toxicity. However, an indirect, immune-mediated mechanism has also been suggested. Pisciotta *et al.* (1992) postulated an immunological mechanism on the basis of complement-mediated neutrophil toxicity of acute phase serum from patients with CLZ-induced agranulocytosis. However, direct evidence such as the presence of anti-drug antibodies has not been forthcoming.

Agranulocytosis was observed in 0.05% of individuals administered AQ for chemoprophylaxis (Hatton *et al.*, 1986; Larrey *et al.*, 1986; Neftel *et al.*, 1986). High doses (1.8-6.0g) and a prolonged duration of action (34-63 days) favour the likelihood of these reactions (Hatton *et al.*, 1986). There were fatalities associated with the use of the drug which lead to its withdrawal from use in both the treatment and prevention of malaria, despite the lack of an adequate therapeutic replacement. Patients with AQ-induced agranulocytosis show an absence of circulating neutrophils and a decrease in bone marrow myeloblasts (neutrophil precursors). With cessation of AQ therapy, the neutrophil count returns to normal within 3-45 days (Hatton *et al.*, 1986; Neftel *et al.*, 1986).

Certain *in vitro* data suggest a direct mechanism of AQ-induced cytotoxicity (Rhodes *et al.*, 1986; Winstanley *et al.*, 1990). Cell death is observed in bone marrow cells and peripheral neutrophils incubated with AQ (Winstanley *et al.*, 1990). In addition, Labro and Babin-Chevaye (1988) demonstrated that desethyl AQ (the major plasma metabolite in man [Churchill *et al.*, 1986]) inhibits essential cellular functions, including phagocytosis and chemotaxis, at much lower concentrations than AQ.

Recently, the direct nature of AQ-induced cytotoxicity has been questioned. Concentrations of desethyl AQ (10μM; Labro and Babin-Chevaye, 1988) required to cause toxicity *in vitro* far exceed the maximum dose achieved in clinical practice (<1μM) (Laurent *et al.*, 1993). Furthermore, Aymard *et al.* (1992) were unable to inhibit colony formation in cells taken from normal subjects when incubated with AQ. In this study, AQ and its major plasma metabolites did not inhibit neutrophil function or decrease cell viability at concentrations below 100μM (chapters 2 and 5). The pathogenesis of agranulocytosis is thus unlikely to be due to a direct effect of the parent compound or the stable metabolites.

AQ is a highly lipophilic weak base. These physico-chemical characteristics may account for its lysosomal accumulation (chapter 2), excessive bioactivation by the MPO system (chapter 5), and in part may explain the organ specific nature of idiosyncratic agranulocytosis. This is further supported by the fact that AQ and other 4-aminoquinoline antimalarials are taken up more avidly into neutrophils than into other peripheral blood cells (chapter 2; Bergqvist and Domeij-Nyberg, 1983; Laurent *et al.*, 1993).

HRP was utilised in chapter 5 to investigate the metabolism-dependent cytotoxicity of AQ. As observed with CLZ (chapter 4), bioactivation of the parent compound produced a chemically reactive intermediate (AQ quinoneimine) which caused GSH depletion and direct neutrophil toxicity *in vitro*. Cellular toxicity of AQ quinoneimine has previously been demonstrated by our group (Winstanley *et al.*, 1990). Cytotoxicity was observed towards both peripheral neutrophils and bone marrow cultures from haematologically normal subjects. The ability of intracellular and exogenous GSH to protect neutrophils from AQ-induced cytotoxicity (chapters 4 and 5) suggests that agranulocytosis may occur through an oxygen-dependent pathway. Supporting this hypothesis is the fact that AQ depleted intracellular GSH at lower concentrations than that needed for cytotoxicity. Other established antimalarials that do not possess a *p*-aminophenol moiety (i.e., chloroquine, mefloquine and halofantrine) failed to deplete GSH, and more importantly are not associated with agranulocytosis.

A number of studies and clinical findings point to an immune-mediated mechanism for AQ-induced agranulocytosis. Anti-AQ IgG antibodies have been detected in sera of patients suffering an ADR (Clarke *et al.*, 1991). Moreover, recent *in vitro* studies have identified a drug-protein adduct on the outer neutrophil surface (Ruscoe *et al.*, 1996). Recognition of the adduct by drug-specific antibodies is indicative of an immunological mechanism of idiosyncratic toxicity.

The metabolism of AQ is comparable to that of paracetamol, which is now regarded as a model hepato-toxin. The hepatocellular necrosis observed with

paracetamol overdose is related directly to the formation of a quinoneimine metabolite (Jollow *et al.*, 1974). Generation of the quinoneimine leads to depletion of intracellular thiol stores, oxidative stress and the formation of covalently bound protein adducts (Albano *et al.*, 1985; Moore *et al.*, 1985). A number of animal models have been used to reproduce paracetamol hepatotoxicity (Boyd and Bereczky, 1966; Mitchell *et al.*, 1973), although there is considerable inter-species variation in susceptibility; the rat is resistant, whereas the mouse is susceptible (Gregus *et al.*, 1988). Raised serum transaminases followed by overt necrosis are used as markers of toxicity. In contrast to paracetamol, attempts to develop an animal model of AQ toxicity have failed (Hough, 1998). Direct toxicity in animals, as observed for paracetamol, was precluded by dose-dependent CNS toxicity. Immunisation of animals with AQ quinoneimine (25μM), prior to administration of a large dose of the parent compound (500μM) also failed to induce either hepatotoxicity or agranulocytosis. These studies highlight the general problem associated with the development of animal models of drug-induced hypersensitivity. Thus, even though the metabolism and disposition of AQ is well defined, its role in the pathogenesis of agranulocytosis still requires the development of an appropriate animal model.

The cytotoxicity caused by the reactive metabolites of AQ and CLZ suggest that the pathogenesis of agranulocytosis may be due to a direct effect, particularly in the case of CLZ, as there is little clinical evidence of an immunological reaction. However, an indirect effect on cellular function or the involvement of the immune system cannot be excluded. Reactive metabolites may

bind to cell membranes at concentrations which are not great enough to cause cell death, but may cause the release of cytokines such as TNF-α or act as a hapten and induce an immune response. Many questions remain unanswered and further research is required.

Classical techniques used to measure direct cytotoxic effects of drugs and chemically reactive metabolites do not distinguish between the two major forms of cell death, apoptosis and necrosis. Recent evidence suggests that drugs may induce apoptosis, at least in part, through the production of oxidative stress (Offen, *et al.*, 1996; Gorman *et al.*, 1997). In light of these findings, morphologic and flow cytometric analysis were used to investigate the possible role of apoptosis in AQ- and CLZ-induced neutrophil toxicity (chapter 7). Treatment of the cells with either compound (100-300μM) resulted in an increase in spontaneous apoptosis. However, these concentrations are unlikely to be observed in clinical practice. More importantly, in the presence of peroxidase activation, both compounds were metabolised to intermediates which induced apoptosis at therapeutic concentrations (1μM; Laurent *et al.*, 1993; Gerson *et al.*, 1994). The ability of GSH to inhibit apoptosis caused by the reactive metabolites, and not apoptosis mediated by drugs alone, suggests that apoptosis may proceed via two pathways, one dependent and one independent of oxidative stress. These studies provide a novel insight into the pathogenesis of drug-induced agranulocytosis. However, before apoptosis is accepted as a general mechanism for drug-induced agranulocytosis, at least two problems need to be addressed. Firstly, what is the exact signalling pathway for the apoptosis, and second, can these *in vitro* findings

be rationalised with the clinical characteristics of toxicity. Further studies are underway in our department to investigate these questions.

In contrast to AQ and CLZ, SMX affects a large number of organs either in isolation or as part of a systemic reaction. Serious adverse reactions to SMX occur between 5 and 30 days after a primary course of therapy. On rechallenge, they occur with greater frequency and are more severe. These characteristics suggest a role of the immune system in amplification of the toxic response. Although skin rashes are by far the most frequent adverse reaction (2-3%; Carr and Cooper, 1995; Pirmohamed and Park, 1995), agranulocytosis is observed in approximately 0.1% of individuals (Cribb *et al.*, 1996b).

To investigate the role of distribution in SMX toxicity, it was important to synthesise pure SMX-NHOH and SMX-NO. The method of Johnstone *et al.* (1978) was modified to develop a simple and reproducible synthetic procedure. Considerable attention has focused on the mechanism of SMX-induced idiosyncratic dermatopathies and blood dyscrasias. At the present time, the most widely accepted view is that the formation of SMX-NHOH and SMX-NO, and activation of the immune system ultimately leads to toxicity (Meekins *et al.*, 1994; Cribb *et al.*, 1996; Gill *et al.*, 1997).

In healthy individuals, drug bioactivation is counterbalanced by detoxification mechanisms. An imbalance between these processes, often caused by a depletion of intracellular GSH, may cause toxicity. GSH can serve as either a nucleophile or a reductant in such reactions (Moldeus and QuanGuan, 1987). In susceptible individuals, the balance between bioactivation and detoxification can

be disturbed by either genetic factors or host factors such as age, enzyme induction and disease (Vesell, 1984; Park *et al.*, 1992). The capacity of an individual to reduce SMX-NHOH and SMX-NO back to the parent compound, which has been demonstrated *in vivo* (Gill *et al.*, 1997), is likely to be critical in the pathogenesis of SMX toxicity. The role of GSH in the reduction has been characterised at a chemical level in chapter 3. Interestingly, no intracellular GSH depletion was observed when SMX-NHOH or SMX-NO were incubated with neutrophils *in vitro,* while lysing the cells caused depletion at therapeutic concentrations. These data suggest that SMX may be excluded from cells or undergo rapid intracellular detoxification. If this is the case, neutrophil toxicity may occur via an extracellular mechanism. To investigate this hypothesis, plasma and cellular reduction of SMX-NHOH and SMX-NO was investigated. Plasma cysteine (CYS) was found to effectively reduce SMX-NO (chapter 3) to a greater extent than either erythrocytes, neutrophil or lymphocytes. Further work in our laboratory demonstrated that SMX-NHOH and SMX-NO, but not the parent amine, bound to the extracellular surface of neutrophils and lymphocytes (Hough, 1998). Taken together, these data may provide a further understanding of the immune mediated toxicity observed in patients taking SMX. Specifically, haptenation of cell-surface membranes which are rich in sulphydryl groups, may lead to protein-conjugate formation, internalisation of the drug-protein conjugate, antigen processing and thus stimulation of either a T or B-cell response (Daftarian *et al.*, 1995; Mauri-Hellweg *et al.*, 1995).

A variety of *in vitro* test systems have been utilised to define the chemical basis of white cell toxicity, and to determine which factors are involved. From these data it can be surmised that AQ-, CLZ- and SMX-induced agranulocytosis is mediated by the formation of chemically reactive metabolites, which are likely to be generated in peripheral neutrophils or bone marrow precursors. Further research should aim to identify susceptible individuals and / or develop a structural analogue which retains efficacy but does not cause toxicity.

Susceptibility to agranulocytosis may reside at various levels including bioactivation, detoxification, and factors responsible for inducing cell death. It has been proposed that drug bioactivation could occur *in vivo* in patients with a viral infection (Uetrecht, 1992). Clinical data suggest that influenza may serve as a predisposing factor (Uetrecht *et al.*, 1994), but an explanation would have to be given for why the majority of cases occur within 2-3 months of starting the drug.

Antioxidants and sulphydryl containing compounds such as GSH prevent reactive metabolite-induced neutrophil toxicity *in vitro* (chapters 4 and 5). Thus, co-administration of ascorbic acid or *N*-acetyl CYS may prevent agranulocytosis *in vivo*. To date, however, this remains untested and will require large scale prospective studies.

Administration of SMX is associated with a much higher incidence of toxicity in patients infected with the HIV virus (30-80%; Carr and Cooper, 1995; Pirmohamed and Park, 1995), than in the general population (less than 3%; Mandell and Sande, 1985). Several groups have reported that HIV-infected patients have low thiol levels and are predisposed to develop an idiosyncratic drug

reaction (Eck *et al.*, 1989; de Quay *et al.*, 1992; Walmsley *et al.*, 1997). In chapter 6, HIV-positive patients were found to have significantly lower concentrations of reduced plasma CYS, when compared with healthy controls. Consistent with these data, SMX-NO was less efficiently reduced in patients with HIV infection. Clinical studies have presented preliminary evidence suggesting that oral administration of *N*-acetyl CYS may restore both intracellular GSH and extracellular CYS levels (Burgunder *et al.*, 1989; de Quay *et al.*, 1992), and improve survival of subjects infected with the HIV-virus (Herzenberg *et al.*, 1996). Thus it is possible that *N*-acetyl CYS would decrease the risk of severe idiosyncratic toxicity associated with SMX therapy in HIV-positive individuals. However, a recent study suggest that this may not be the case since multiple doses of *N*-acetyl CYS did not alter the incidence of co-trimoxazole hypersensitivity (Walmsley *et al.*, 1997b). This does suggest that CYS deficiency by itself is not responsible for predisposition to hypersensitivity, but may be one contributory factor among a number of factors (e.g. immune disregulation, dose and genetic predisposition) contributing to the pathogenesis of hypersensitivity.

Given our knowledge of the potential of chemically reactive metabolites to cause agranulocytosis, what lessons do the studies in this thesis provide for the rational design of safer drugs. There are three clear examples of the development of chemical analogues with an improved safety profile in man which are probably related to the avoidance of drug bioactivation (fig. 8.2). First, chloramphenicol, where the nitro group has been replaced by the methylsulphonyl moiety to form thiamphenicol. Thiamphenicol has an antibacterial spectrum identical to that of chloramphenicol, but has not been reported to cause aplastic anaemia associated

with chloramphenicol (Yunis, 1989). Secondly, the agranulocytosis associated with metiamide, a prototype histamine H_2 receptor antagonist, was thought to be due to the thiourea group and was replaced by a cyanoguanidine group to form cimetidine, a drug which has been used safely for many years (Ganellin, 1993). Thirdly, practolol, which can cause the oculomucocutaneous syndrome, was replaced by its geomeric isomer, atenolol, which has not been associated with this syndrome (Main and Tucker, 1993).

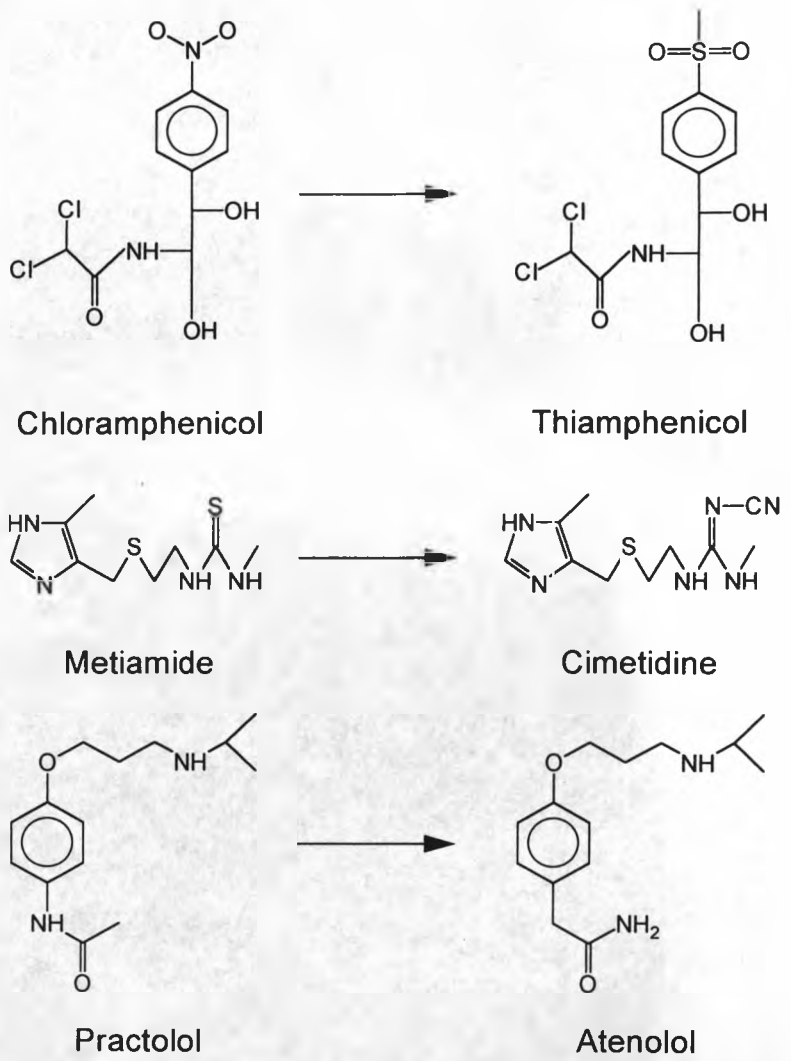


Figure 8.2 Structures of compounds that have been chemically modified to produce congeners to avoid idiosyncratic toxicity.

Several analogues of AQ have been studied (chapter 2), and data presented in this thesis show that the antimalarial effects of AQ can be separated from its bioactivation. Tebuquine and a series of compounds known collectively as bis-mannichs are two of the major advances in the development of potent antimalarial drugs (Werbel *et al.*, 1986; Shao, 1990). Simple chemical modifications block GSH depletion in activated neutrophils without any effect on antimalarial activity. Although these compounds cause oxidative stress, the strong oxidising conditions required suggest a similar reaction would be unlikely to occur in clinical practice (chapter 5). Toxicity of AQ can be prevented using an alternative chemical modification. Substitution of the hydroxyl moiety with a fluorine atom prevents GSH depletion *in vitro* (Tingle *et al.*, 1995), and bioactivation *in vivo* (Ruscoe *et al.*, 1995). Compounds of this class have been patented and are currently under evaluation for drug development.

A similar approach may hold promise in the development of CLZ analogues. A recent report demonstrated that compounds containing an oxygen or sulphur atom rather than a nitrogen bridge were resistant to bioactivation (fig. 8.3) when oxidised by MPO or activated neutrophils (Uetrecht *et al.*, 1997). The metabolism of these and other CLZ analogues are currently under investigation in our laboratory.

The main mechanism of action of SMX is by competing with *p*-aminobenzoic acid, which is essential for bacterial folic acid synthesis. The terminal amine moiety is essential for pharmacological activity (fig. 8.4). Therefore, in contrast to AQ and CLZ, it seems unlikely that a synthetic analogue, which retains efficacy but does not cause toxicity, will be developed.

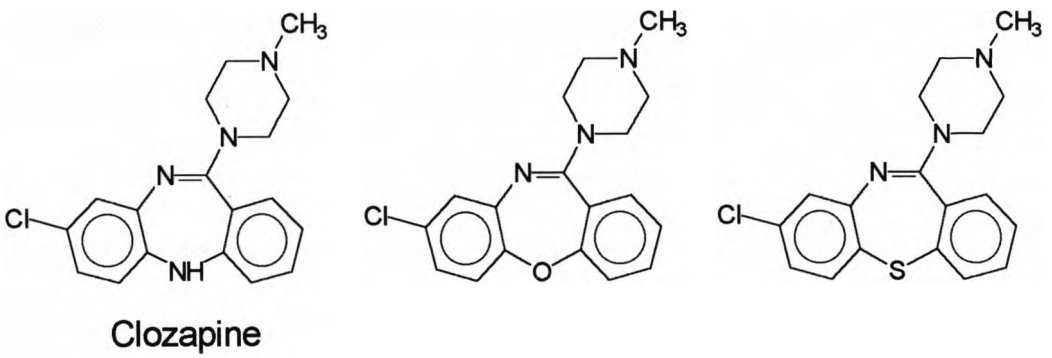


Figure 8.3 Structures of CLZ and its O- and S- derivatives.

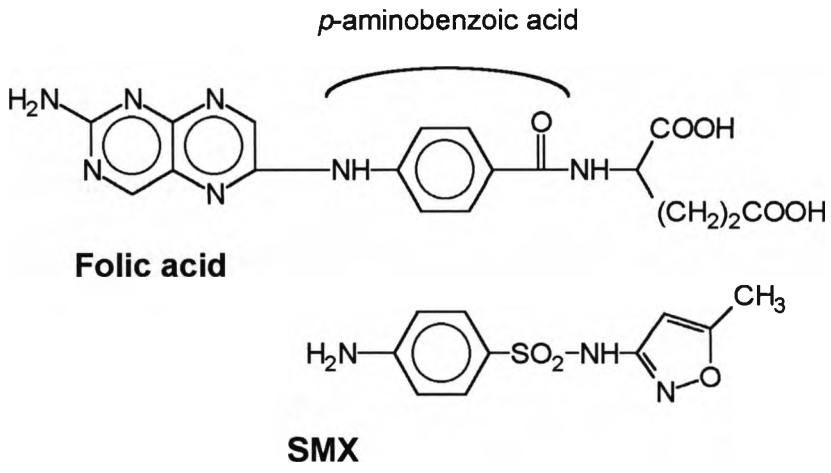


Figure 8.4 The structures and chemical relationship between SMX, *p*-aminobenzoic acid and folic acid.

The lack of an effective alternative to SMX has prompted an investigation into the management and prevention of adverse reactions associated with drug therapy. Options include either discontinuing SMX or continuing therapy, while at the same time treating patients symptomatically with antihistamines, and sometimes corticosteroids. "Treatment through" the adverse reaction is thought to be possible in up to 70% of the patients (Jung *et al.*, 1994). In addition,

rechallenge has been recommended in patients with a history of SMX hypersensitivity. Carr *et al.* (1993) studied 31 hypersensitive patients, and rechallenge with low dose SMX was successful in a third of the patients. An alternative to rechallenge is drug desensitisation. Several groups (Smith *et al.*, 1987; Papakonstantinou *et al.*, 1988) have reported successful desensitisation regimes in 70-80% of patients.

The ultimate aim of this project was to investigate the role of neutrophil metabolism and cellular distribution in the pathogenesis of AQ-, CLZ- and SMX-induced agranulocytosis. Elucidation of the mechanisms of toxicity may provide evidence-based strategies to prevent agranulocytosis, and thus allow the widespread administration of these otherwise effective drugs. The pace of research into agranulocytosis needs to continue to address the questions that have been outlined in this thesis. In particular, there is a need for a greater understanding of the role of cellular disposition and cellular signalling in cell-directed toxicity. Only then may we prevent the occurrence of drug-induced agranulocytosis, one of the most severe forms of idiosyncratic toxicity.

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