BIOACTIVATION OF TRIMETHOPRIM, FLUPERLAPINE AND NEVIRAPINE: ROLES OF IMINOQUINONE-TYPE MICHAEL ACCEPTORS IN IDIOSYNCRATIC DRUG REACTIONS

by

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A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy
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Abstract

Idiosyncratic drug reactions are significant medical problems for both modern drug therapy and drug development. Although their mechanisms are poorly understood, the involvement of drug reactive metabolites has been generally proposed. Michael acceptors such as quinones, iminoquinones and quinone methides, are common reactive metabolites of many drugs and drug candidates.

Trimethoprim is associated with idiosyncratic adverse reactions including liver toxicity and agranulocytosis. We demonstrated that trimethoprim was oxidized by activated human neutrophils, as well as myeloperoxidase (MPO), or HOCl to a reactive iminoquinone methide intermediate with a MH\(^+\) ion of m/z 289. In the presence of N-acetyl-L-cysteine (NAC), the reactive intermediate could be trapped as three NAC adducts. Incubation of trimethoprim with isolated hepatic microsomes in presence of NAC gave the same set of TMP-NAC adducts. Immunoblotting studies detected covalent binding of trimethoprim to proteins both \textit{in vitro} and \textit{in vivo}.

Fluperlapine has been associated with idiosyncratic agranulocytosis in humans. We demonstrated that 7-hydroxyfluperlapine, the major metabolite of fluperlapine, is oxidized by HOCl or by MPO system to a reactive iminoquinone species with a MH\(^+\) ion at m/z 324. Using an
antibody developed against the drug's analogue, clozapine, we demonstrated that 7-hydroxyfluperlapine could covalently modify human MPO. Furthermore, we demonstrated that 7-hydroxyfluperlapine is metabolized by activated neutrophils to the reactive metabolite that covalently binds to neutrophils. In the presence of NAC or glutathione (GSH), such covalent binding could be inhibited and the NAC or GSH adducts were formed.

Nevirapine is associated a high incidence of skin rash, hepatitis and granulocytopenia. We demonstrated that radiolabeled nevirapine covalently bound to activated human neutrophils. Protein binding was also detected when the drug was incubated with MPO in the presence of bovine serum albumin. Oxidized by HOCl, nevirapine formed a reactive intermediate with a molecular ion of m/z 281. This is presumably a reactive quinoneiminium ion and it could be trapped by NAC. The NAC adduct was also detected when 3-hydroxynevirapine, the major metabolite of nevirapine, was metabolized by rat hepatic microsomes in the presence of NAC. Immunoblotting studies detected modified polypeptides in the hepatic cytosol fraction and the skin homogenate from nevirapine-treated rats.
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List of Abbreviations

BSA  bovine serum albumin;
CID  collision-induced dissociation;
COSY  correlated spectroscopy;
DMSO  dimethyl sulfoxide;
EDC  1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide;
EDTA  ethylenediaminetetraacetic acid;
FLUP  fluperlapine;
HBSS  Hanks’ balanced salt solution;
HF  hydroxyfluperlapine;
HNVP  hydroxynevirapine;
HPLC  high performance liquid chromatography;
KLH  keyhole limpet hemocyanin;
LC/MS  liquid chromatography interfaced with mass spectrometry;
LC/MS/MS  liquid chromatography interfaced with tandem mass spectrometry;
MALDI  matrix-assisted laser desorption/ionization;
MH'  molecular ion;
MHC  major histocompatibility complex;
MPO  myeloperoxidase;
MS  mass spectrometry;
m/z  mass over charge ratio;
NAC  N-acetyl-L-cysteine;
NADP* β-nicotinamide adenine dinucleotide phosphate;
NADPH NADP* reduced form;
NMR nuclear magnetic resonance;
NVP nevirapine;
PAGE polyacrylamide gel electrophoresis;
PMA phorbol 12-myristate-13-acetate;
RSA rabbit serum albumin;
SDS sodium dodecyl sulfate;
SIM selective ion monitoring;
TLC thin-layer chromatography;
TMP trimethoprim;
TOF time-of-flight.
List of Publications


Lai WG, Zahid N and Uetrecht JP (manuscript) Bioactivation of nevirapine and 3-hydroxynevirapine by neutrophils and hepatic microsomes: implications for nevirapine-induced adverse drug reactions. (Chapter 4 of the thesis).
Chapter 1

General Introduction.
1.1 Overview of problems, rationale and research objectives.

Adverse drug reactions are common clinical problems and may account for about 2 - 6% of all hospital admissions (Bates et al., 1995; Classen et al., 1997; Einarson, 1993). The majority of adverse drug reactions are due to the drug’s normal pharmacological effects. This type of adverse drug reactions would occur in most patients at a sufficiently high dose of the drug. The observed differences in pharmacological adverse drug reactions among different groups of the population may be governed by genetic, environmental or physiological factors (Lu, 1998). At least in principle, this type of adverse drug reaction can be predicted and prevented. The unpredictable adverse drug reactions are referred to as idiosyncratic drug reactions. Although this type of reaction is less common than the predictable ones, they are often more severe and sometimes life-threatening. Since idiosyncratic drug reactions may account for about 10% of all adverse drug reactions, they also represent a significant health problem (Goldstein & Patterson, 1984; Jick, 1984). Furthermore, due to their unpredictable nature, idiosyncratic drug reactions are one of the major obstacles in the course of drug discovery. Since the problems are not often detectable by in vitro toxicological or in vivo animal tests during the pre-clinical stage, many new drugs or drug candidates have been removed from the market or withdrawn during late stage of their clinical trials due to an unacceptable risk of idiosyncratic drug reactions (Bakke et al., 1984). Therefore, they also represent a significant cost to innovative pharmaceutical companies.
The mechanisms of most idiosyncratic drug reactions are still unknown. However, accumulated evidence suggests that reactive metabolites play a central role in many drug-induced idiosyncratic reactions (Park et al., 1992; Pirmohamed et al., 1994; Uetrecht, 1990; Uetrecht, 1992). Reactive metabolites can be generated from both phase I and phase II metabolic pathways, and these processes are often referred to as bioactivation pathways. Reactive metabolites generated by bioactivation may cause either direct toxic effects such as cytotoxicity, cellular oxidative stress or genotoxicity, or they may cause covalent modification of cellular macromolecules, such as proteins, DNA or RNA. The reactive metabolite-modified cellular macromolecules (e.g. proteins) may also be involved in drug-induced auto-immune responses, which can escalate the cellular damage and cause irreversible idiosyncratic drug reactions.

The liver is quantitatively the most important organ involved in drug metabolism. The liver contains a "super-family" of hemoprotein enzymes, cytochrome P450s, which are dominant in the role of phase I biotransformation as well as converting xenobiotics to their reactive metabolites (Guengerich, 1987; Waterman & Johnson, 1991). Therefore, the liver is also a primary target of many drug-induced adverse reactions (Zimmerman, 1993). The highest concentration of cytochrome P450 enzymes involved in biotransformation are found in hepatic endoplasmic reticulum (microsomes). Due to their high reactivities and short biological half-lives, the reactive metabolites formed in the liver may not easily reach other target tissues to cause adverse drug reactions there. Neutrophils have also been shown to be actively involved in the bioactivation of many xenobiotics to
their reactive metabolites (Liu & Uetrecht, 1995; Uetrecht, 1992; Uetrecht et al., 1994). Upon activation, neutrophils release myeloperoxidase (MPO) and produce superoxide anion. Hydrogen peroxide ($H_2O_2$) can be generated either enzymatically or spontaneously from the superoxide, and MPO can use endogenous $H_2O_2$ and chloride ion to generate many strong oxidants including hypochlorous acid (HOCl). The cells use this mechanism to kill invaders, such as bacteria. However, the resultant oxidants can also oxidize drugs or stable drug metabolites present in the blood stream. Hence, the formation of reactive drug metabolites by neutrophils is speculated to be responsible for many drug-induced blood dyscrasias, including agranulocytosis and lupus, which are also common idiosyncratic drug reactions (Uetrecht, 1990; Uetrecht, 1992; Uetrecht, 1997).

Skin is another major target for idiosyncratic drug reactions. Being the largest organ of the body, skin also contains many enzyme systems that may potentially be involved in drug bioactivation. The possible role of cutaneous cytochrome P450 enzymes in drug metabolism has been studied by many researchers (Mukhtar & Khan, 1989; Wolkenstein et al., 1998). However, the generally low enzymatic concentration as well as the physical toughness of the skin make it a difficult system for the study of drug metabolism.

Trimethoprim and nevirapine have been associated with drug-induced idiosyncratic agranulocytosis, hepatitis and skin rashes (Carr et al., 1996; Das et al., 1988; Gleckman et al., 1979; Haaverstad & Kannelonning, 1984; Havlir et al., 1995; Hawkins et al., 1993; Nwokolo et al., 1988; Pollard et al., 1998; Williamson & Crowe, 1972). A potential replacement for clozapine, the drug candidate fluperlapine was
withdrawn during a late stage of its clinical trials due to an apparent unacceptable incidence of agranulocytosis (Woggon et al., 1984).

The objectives of this thesis are:

• To determine if trimethoprim and nevirapine, or their major in vivo metabolites, can be oxidized in vitro by HOCl, MPO and activated neutrophils, as well as by hepatic microsomes;

• To determine if fluperlapine's major in vivo metabolite, 7-hydroxyfluperlapine, can be oxidized in vitro by HOCl, MPO and activated neutrophils;

• To identify the reactive metabolites generated by these oxidations and to characterize their reactivity using nucleophilic trapping agents, such as N-acetyl-L-cysteine (NAC), glutathione (GSH), etc.;

• To study covalent modification of cellular proteins by these reactive metabolites, both in vitro and in vivo.

The results obtained from the experiments described in this thesis are expected to contribute to the understanding of mechanisms of the idiosyncratic reactions associated with the drugs. Furthermore, the general principles and strategies used in these projects may also find applications in the development of safer therapeutic agents and reduction of idiosyncratic drug reactions.
1.2 General hypothesis.

Reactive iminoquinone-type metabolites of trimethoprim and hydroxylated metabolites of fluperlapine and nevirapine are generated by the target tissues involved in the idiosyncratic reactions associated with these drugs. The resultant iminoquinone-type Michael acceptors are electrophilic species that are capable of covalent binding to physiological nucleophiles, such as GSH, NAC and cellular proteins. The covalent modification of cellular proteins by the reactive metabolites plays an important role in the idiosyncratic reactions associated with these drugs.
1.3 Adverse drug reactions.

An adverse drug reaction can be defined as any action of a drug that is not of intended therapeutic, diagnostic or prophylactic benefit to the patient (Pirmohamed et al., 1998). The exact frequency of adverse drug reactions is difficult to determine. However, many studies have shown that they are a major clinical problem, and may account for approximately 5% of all hospitalizations (Classen et al., 1997; Jick, 1984; Lazarou et al., 1998). There are various ways to classify adverse drug reactions, mostly based on whether or not the effects are predictable (Rawlins & Thompson, 1991; Rieder, 1994). Clinically, the adverse drug reactions can be divided into two general categories: Type A (pharmacological) and Type B (idiosyncratic) (Rawlins & Thompson, 1991).

Type A adverse drug reactions are related to the known pharmacological effects of a therapeutic agent or its stable metabolites. Therefore, they are theoretically predictable and normally reproducible in experimental animals. This type of reaction is usually dose-dependent and should occur in all patients or experimental animals if they were treated with a sufficiently high dose. Approximately 80% of adverse drug reactions can be included in this general category (Pirmohamed & Park, 1999). Type A adverse drug reactions have a high rate of occurrence and can be severe, but they are rarely associated with drug-induced fatality. However, due to interindividual differences in pharmacodynamics and pharmacokinetics, this type of reaction will continue to be an important clinical problem.
Type B adverse reactions are unpredictable and are often referred to as idiosyncratic, hypersensitivity or allergic reactions. Throughout this thesis, the term idiosyncratic drug reactions will be used to refer to this particular category of adverse drug reactions. This type of drug-induced adverse reaction cannot be explained on the basis of the known pharmacological effects of the drug; hence they are unpredictable and rarely reproducible in experimental animals. For idiosyncratic drug reactions, there is usually no simple relationship between the dose and occurrence of toxicity. The reactions do not occur in most people or experimental animals even when they have been treated with a high dose of the drug. Clinically, idiosyncratic drug reactions are often more severe than the predictable reactions and frequently they are life-threatening. The rate of occurrence of this type of drug reactions are lower than that of Type A reactions, and they account for approximately 10 - 20% of all adverse drug reactions (Goldstein & Patterson, 1984; Pirmohamed & Park, 1999). However, they are not rare considering the extensive use of drugs in the general population. Furthermore, due to their severity, idiosyncratic drug reactions are a major threat to people’s health.
Table 1-1 Classification of adverse drug reactions.

(Pirmohamed et al., 1994; Pirmohamed & Park, 1999, partially)

<table>
<thead>
<tr>
<th>Type A (Pharmacological)</th>
<th>Type B (Idiosyncratic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Dose-dependent</td>
<td>• No simple dose-reaction relationship</td>
</tr>
<tr>
<td>• Predictable</td>
<td>• Unpredictable</td>
</tr>
<tr>
<td>• Less host-dependent</td>
<td>• Host-dependent</td>
</tr>
<tr>
<td>• Common</td>
<td>• Uncommon</td>
</tr>
<tr>
<td>• Usually mild but can be severe</td>
<td>• Can be severe</td>
</tr>
<tr>
<td>• Low mortality</td>
<td>• High mortality</td>
</tr>
<tr>
<td>• Reproducible in animals</td>
<td>• Few animal models</td>
</tr>
</tbody>
</table>

Clinical Example

- Zidovudine-induced bone marrow depression
- Clozapine-induced agranulocytosis

1.3.1 Pharmacological adverse drug reactions.

Pharmacological adverse drug reactions are characterized by their predictable nature. They are often consequences of known pharmacological effects of the therapeutic agents. Therefore, by proper planning and monitoring during the treatment, this type of adverse drug reaction, at least theoretically, can be avoided.
1.3.1.1 Toxicity.

Toxicity is a simple form of predictable adverse drug reactions. It refers to the untoward effects produced on a particular organ or to the patient in general when high therapeutic dosages are utilized. Many commonly used drugs are capable of producing toxicity when taken in supratherapeutic dosages. As an example, the anti-tumor agent, 5-fluorouracil, is expected to cause bone marrow suppression, leukopenia and gastrointestinal damage at high doses (Donowitz & Quesenberry, 1986; Biran et al., 1989; MacDonald, 1999). The mechanism of injury can be traced to the drug's intended physiological effects to the target organs. However, by using an optimized dosing regimen and extensive therapeutic drug monitoring, the toxic effects can at least be minimized.

1.3.1.2 Side effects.

Side effects are expected but not desired biological response produced by a medication. Their mechanisms are often closely related to their intended physiological effects. Side effects are also very common with many drugs. As illustrations, a cholinergic antagonist, atropine, at a small dose causes impaired salivary secretion. The patient may experience a dry mouth and difficulty swallowing (Spero, 1989). Being a β agonist, isoproterenol may produce signs of coronary insufficiency due to tachycardia and myocardial stimulation (Flattery, 1989). Similar to the case of toxicity, side effects can also be minimized by optimizing dosing regimen and intensifying drug monitoring.
1.3.1.3 Interactions.

Interactions are often of a pharmacokinetic nature, which occur due to the effects of medications or diseases on the disposition and effects of other medications taken concurrently. Interactions can be further classified into drug-drug interactions and drug-disease interactions.

Drug-drug interactions can often be predicted by knowing the major pathways of metabolism and disposition of the drug involved. As an example, the fact that ketoconazole is a potent inhibitor of cytochrome P450 3A4 suggests that drugs predominantly metabolized by this cytochrome P450 isozyme, such as terfenadine, will have exaggerated effects if taken concurrently with ketoconazole (Honig et al., 1993; Jurima-Romet et al., 1994).

Interaction can also occur between certain disease processes and medications. As an illustration, patients with infectious mononucleosis have a high incidence of maculopapular rashes if they are given amoxicillin (Van Arsdel Jr, 1988). The mechanism(s) of many drug-disease interactions are not known. Therefore, they are also not easily predicted.

Drug interactions can also be pharmacodynamic. Physiological actions of drugs may have net effects that augment or offset each other. As an example, barbiturates and other sedatives, such as phenothiazines, rauwolfia and benzodiazepines show additive or potentiating effects with ethanol (Sellers, 1989; Kalant & Khanna, 1989).
1.3.1.4 Secondary Effects.

Adverse effects that are predictable and avoidable consequences of the biological activity of a drug are often referred to as secondary effects (Patterson et al., 1986). An example of this type of adverse drug reaction, antibiotics, such as clindamycin or aureomycin, may suppress the bowel flora and cause overgrowth of an endotoxin-producing strain of Clostridium difficile in the bowel of patients. This effect may lead to colitis and inflammatory diarrhea (Patterson et al., 1986). The mechanism(s) of secondary adverse drug reactions are also associated with the anticipated pharmacological activity of the drug.

1.3.2 Idiosyncratic adverse drug reactions.

Idiosyncratic adverse drug reactions are characterized by their unpredictable nature. They are unrelated to any known pharmacological effects of the therapeutic agents, and they do not occur in most patients, even with high doses. The majority of serious and life-threatening adverse drug reactions are included in this general category and they usually are not detected until thousands of patients have been treated. Therefore, idiosyncratic drug reactions have become one of the major concerns of both clinical practitioners and innovative pharmaceutical firms (Pohl et al., 1988; Uetrecht, 1992).

1.3.2.1 Intolerance.

When medications are given in usual doses some individuals may exhibit exaggerated adverse responses which can be referred to as intolerance to the drugs.
The adverse reactions may often be considered as an extreme form of those reactions that may occur, but only at extremely high doses, in the majority of patients. As an example, most patients treated with erythromycin may develop mild gastrointestinal discomfort. However, 3 - 5% of the patients may develop serious nausea and vomiting (Patterson et al., 1986).

The mechanism(s) responsible for drug intolerance are not well known. However, genetic variations in drug targets, transporters and metabolic enzymes may have the potential to contribute significantly to the individual differences in drug-induced adverse responses (Lu, 1998). Genetic variations in the drug receptor genes have been shown to cause "hyporesponsiveness" to therapeutic agents (Green et al., 1993; Malloy et al., 1997). It is also a reasonable speculation that certain mutations in known or unknown drug receptors may be responsible for "hyperresponsiveness" to therapeutics in certain individuals. Genetic variations of drug transporters, which have been shown to be able to significantly alter drug absorption, distribution and excretion, are also likely to lead to interindividual differences in responses to drugs (Lin & Lu, 1997). However, in spite of a large body of published work, the importance of genetic factors in determining pharmacodynamics and pharmacokinetics have yet to be properly defined. On the other hand, the genetic polymorphism of enzymes involved in drug metabolism has been well studied and shown to play an important role in some rare and unpredictable adverse drug reactions. As an example, it has been reported that a 33-year-old female patient experienced severe abdominal pain after a normal dose of codeine. Genotyping and phenotyping studies showed that the patient is an
ultrarapid metabolizer with a high capacity to metabolize codeine to morphine by cytochrome P450 2D6. The quick onset and severity of the adverse effects are presumably due to the rapid formation of morphine in liver, leading to a high concentration in the biliary tract (Dalen et al., 1997). This example clearly illustrates the importance of interindividual metabolism variability in drug safety.

1.3.2.2 Hypersensitivity reactions.

The idiosyncratic nature of some adverse drug reactions has been associated with immune-mediated reactions (Anderson, 1992; Pohl et al., 1988; Uetrecht, 1997). As a matter of fact, the terms idiosyncratic drug reactions, drug hypersensitivity reactions, and allergic drug reactions are often used interchangeably. The involvement of the immune system provides an attractive explanation for interindividual differences observed in unpredictable drug reactions. However, it is generally difficult to prove the role of the immune system in most cases of idiosyncratic reactions. It is also quite possible that hypersensitivity reactions do not cover all drug-induced idiosyncratic reactions (Uetrecht, 1992). Drug hypersensitivity reactions are often described as extreme immune responses to drug-modified proteins. These reactions have been classified into the following four categories, which represent four different mechanisms leading to tissue damage (Coombs & Gell, 1963).

Type I reactions are referred to as immediate hypersensitivity. Sensitization occurs as the result of first exposure to antigens through various routes, which initiates production of immunoglobulin IgE. Both mast cells and blood basophils
bind IgE molecules via their Fc receptors. During re-exposure, adjacent bound immunoglobulin molecules become cross-linked by binding to the specific antigens and this results in degranulation of the mast cells and the release of pharmacological mediators, such as histamine, prostaglandins and leukotrienes. These mediators promote vasodilation, bronchial constriction and inflammation. Clinical manifestations may vary from mild urticarial skin reactions to more serious drug-induced asthma, to life-threatening anaphylaxis. These responses may start within minutes after re-exposure to the antigen, and penicillin-induced anaphylaxis is a typical example for this type of drug hypersensitivity reaction (Weiss & Adkinson, 1988).

Type II reactions are referred to as antibody-dependent cytotoxic hypersensitivity. The reaction is initiated when antibodies bind to specific tissue antigens, followed by complement-independent or complement-dependent effector cytotoxic processes. The immunoglobulin involved can be IgG or IgM class. For complement-independent cytotoxic processes, tissue damage may result from the direct action of cytotoxic cells, such as macrophages, neutrophils or eosinophils, linked to immunoglobulin-coated target cells through the Fc receptor on the antibody. Alternatively, it may result from antibody activation of the classical complement pathway. For complement-dependent cytotoxic processes, complement mediator C3b or C3d binds to the target cell surface, which can be recognized by effector cells. Alternatively, the C5b-9 membrane attack complex may be bound to the target cell surface resulting in cell lysis. Hemolytic anemia associated with high-dose penicillin or quinine is due to this type of hypersensitivity. The drug
molecules are expressed on the surface of red blood cells. The red blood cells are subsequently coated with drug-specific antibodies, which lead to complement activation and red blood cell lysis (Dunagin & Millikan, 1980).

Type III reactions are referred to as immune-complex-mediated hypersensitivity. Immune complexes can be formed whenever antibodies meet a multi-valent antigen. The immune complexes formed at discrete locations can be deposited in tissues, or onto the surfaces of cells, which may bind and activate complement, leading to cell lysis in a similar manner as in type II reactions. The classical example of type III hypersensitivity is the serum-sickness-like syndrome, which may present clinically with rash, fever and lymphadenopathy. Many therapeutics, such as penicillins, cephalosporins, barbiturates, isoniazid, phenytoin and non-steroidal anti-inflammatory drugs may induce a serum-sickness-like syndrome via this immunological mechanism (Anderson, 1992; Rieder, 1994).

Type IV reactions are referred to as cell-mediated hypersensitivity or delayed-type hypersensitivity (DTH), which is a consequence of the interaction of antigens with specific lymphocytes and not with specific antibodies. The target cell presents the antigen in combination with a class I major histocompatibility complex (MHC I) molecule which is recognized by a specific T cell, such as a CD8+ T cell. The activated T cell then secretes cytokines that bring about further proliferation of T cells and induce the expression of adhesion molecules on the surfaces of target cells. The specific effector T cells, known as T_c (cytotoxic class) and T_DH (delayed hypersensitivity class) cells, may cause cellular damage through two different mechanisms. T_c cells can kill the target cells by direct lysis. In contrast, the T_DH cells
cause damage indirectly through the release of proinflammatory cytokines, which cause influx and activation of inflammatory cells, such as monocytes and macrophages, to the target tissue. Drug-induced contact dermatitis and other organ-specific cytotoxicities are thought to be mediated by this immunological mechanism (Pohl et al., 1988).

The Coombs and Gell's classification is a very useful tool in illustrating the mechanisms involved in hypersensitivity. However, not enough is known about the mechanism of most idiosyncratic reactions to place them in this scheme. In addition, a drug-induced hypersensitivity reaction can be the result of a combination of these mechanisms. To complicate the situation even more, adverse drug reactions of both immune and non-immune mechanisms may resemble each other. Therefore, it is usually difficult to fit all the different types of immunologically-based adverse drug reactions in this over simplified framework. Clinically, a more practical classification is often adopted (Table 1-2), and this classification is based on predominant clinical features of drug reaction, the usual anatomical organ involved, as well as recognized specific drug reaction syndromes (Anderson, 1992).
Table 1-2 Classification of immune-based adverse drug reactions.

(Anderson, 1992)

<table>
<thead>
<tr>
<th>Organ-Specific Reactions</th>
<th>Generalized Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Skin</td>
<td>• Mast-cell-mediator or -associated reactions</td>
</tr>
<tr>
<td>• Blood</td>
<td>Systemic anaphylaxis</td>
</tr>
<tr>
<td>• Lung</td>
<td>Anaphylactoid reactions</td>
</tr>
<tr>
<td>• Liver</td>
<td>Generalized urticaria and angioedema</td>
</tr>
<tr>
<td>• Heart</td>
<td>• Serum-sickness-like reactions</td>
</tr>
</tbody>
</table>

|                                | • Isolated drug fever |
|                                | • Autoimmune drug reactions |
|                                | • Drug-induced vasculitis |

<table>
<thead>
<tr>
<th>Pseudoallergic Reactions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Drug reactions associated with specific chemical mediator release or activators</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histamine-releasing agents</td>
</tr>
<tr>
<td></td>
<td>Complement-activating agents</td>
</tr>
<tr>
<td></td>
<td>Autonomic drugs</td>
</tr>
<tr>
<td></td>
<td>Enzyme inhibitors</td>
</tr>
<tr>
<td>• Generalized pseudoallergic drug reactions that involve mediator release but for which the cause and effect etiology is unclear</td>
<td></td>
</tr>
<tr>
<td>• Radio contrast media reactions</td>
<td></td>
</tr>
<tr>
<td>• Aspirin/nonsteroidal anti-inflammatory reactions</td>
<td></td>
</tr>
</tbody>
</table>
1.3.3 Clinical manifestations of adverse drug reactions.

Any organ may be involved in drug-induced toxicity, either individually or in combination with other organs. Adverse drug reactions involving multiple organs can affect each organ directly, or they can affect mainly one organ and cause problems in other organs as secondary effects. For those immune-mediated reactions, generalized hypersensitivity manifestations may also be observed. Among all adverse drug reactions, idiosyncratic drug reactions or type B reactions are the most prominent ones. Although rare, idiosyncratic drug reactions are generally severe and frequently life-threatening. Therefore, this particular type of adverse drug reaction has become a major concern to both physicians and patients (Table 1-3). Furthermore, since idiosyncratic drug reactions are rarely detected during the pre-clinical stage of drug development, they are also the major reason for the withdrawal of potentially useful drugs or drug candidates from the market or during a late stage of clinical trials (Table 1-4). Although adverse drug reactions can occur in any organ system, the focus of this thesis is on the major three affected systems, liver, blood and skin.
Table 1-3. Examples of organs affected by severe idiosyncratic drug reactions.

(Pirmohamed & Park, 1999, partially)

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Type of reaction</th>
<th>Drug examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generalized reaction</td>
<td>Anaphylaxis</td>
<td>Penicillins</td>
</tr>
<tr>
<td></td>
<td>Hypersensitivity</td>
<td>Temafloxacin</td>
</tr>
<tr>
<td>Skin</td>
<td>Toxic epidermal necrolysis</td>
<td>Trimethoprim-sulfamethoxazole</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatitis</td>
<td>Halothane</td>
</tr>
<tr>
<td>Hematological system</td>
<td>Aplastic anemia</td>
<td>Remoxipride</td>
</tr>
<tr>
<td></td>
<td>Agranulocytosis</td>
<td>Clozapine</td>
</tr>
<tr>
<td></td>
<td>Hemolysis</td>
<td>Nomifensine</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Guillain-Barré syndrome</td>
<td>Zimeldine</td>
</tr>
<tr>
<td>Kidney</td>
<td>Interstitial nephritis</td>
<td>Penicillins</td>
</tr>
<tr>
<td>Lung</td>
<td>Pneumonitis</td>
<td>Nitrofurantoin</td>
</tr>
<tr>
<td>Heart</td>
<td>Cardiomyopathy</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>Reproductive toxicity</td>
<td>Various fetal abnormalities</td>
<td>Etretinate</td>
</tr>
</tbody>
</table>
Table 1-4 Drugs and drug candidates withdrawn due to idiosyncratic drug reactions.

(Park et al., 1992, partially)

<table>
<thead>
<tr>
<th>Drugs or drug candidates</th>
<th>Adverse reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcofenac</td>
<td>Hypersensitivity reactions</td>
</tr>
<tr>
<td>Althesin</td>
<td>Anaphylaxis</td>
</tr>
<tr>
<td>Benoxaprofen</td>
<td>Hepatotoxicity, nephrotoxicity</td>
</tr>
<tr>
<td>Fenclofenac</td>
<td>Toxic epidermal necrolysis</td>
</tr>
<tr>
<td>Fluperlapine</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Glafenine</td>
<td>Anaphylaxis</td>
</tr>
<tr>
<td>Ibufenac</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Nomifensine</td>
<td>Hemolytic anemia</td>
</tr>
<tr>
<td>Practolol</td>
<td>Oculo-mucocutaneous syndrome</td>
</tr>
<tr>
<td>Prinomide</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Temafloxacin</td>
<td>Hepatotoxicity, hemolysis</td>
</tr>
<tr>
<td>Tienilic acid</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Zimeldine</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td>Zomepirac</td>
<td>Anaphylaxis</td>
</tr>
</tbody>
</table>
1.3.3.1 Hepatotoxicity.

Being the major site of drug metabolism, the liver is also a frequent target for drug-induced adverse reactions. It has been reported that more than 600 drugs cause hepatotoxicity (Park et al., 1992). Drug-induced liver damage (hepatitis) can vary in severity from mild and transient increases in serum transaminases to fulminant and irreversible hepatic failure. From a clinical point of view, the manifestations can be categorized in two general groups: acute injury and chronic damage. Acute injury can be cytotoxic, cholestatic or combined. Cytotoxic injury includes necrosis and steatosis, which both elevate aminotransferase levels and lead to hepatocellular jaundice. Cholestatic injury is characterized by arrested bile flow and may be associated with portal inflammation, which can also lead to jaundice and elevation of aminotransferase levels. Chronic liver damage may include chronic hepatitis, steatosis, phospholipidosis, pseudoalcoholic liver disease, chronic cholestasis, granulomatous disease, several different vascular lesions, several forms of cirrhosis, noncirrhotic portal hypertension and various hepatic tumors (Zimmerman, 1993).

Drug-induced hepatitis can also be classified into two general categories based on their mechanisms: the hepatitis due to direct toxicity of a drug or its metabolites and the hepatitis due to a drug-triggered adverse immune response against the liver (Beaune & Lecoeur, 1997). However, clinically it is difficult to determine whether the toxicity is directly chemical or immune-mediated in nature because it is often difficult to access the damaged tissue, especially at the time of the initial tissue injury. Similarly, drugs causing hepatotoxicity can be classified into intrinsic hepatotoxins and idiosyncratic hepatotoxins (Zimmerman, 1993). The liver damage
induced by intrinsic hepatotoxins is usually dose dependent and host independent. Intrinsic hepatotoxins, such as carbon tetrachloride, acetaminophen and alcohol, are metabolized to reactive metabolites that are cytotoxic. Idiosyncratic hepatotoxins also require further metabolism in order to be cytotoxic. However, the hepatotoxicities induced by these compounds are host dependent and there is no simple dose-reaction relationship for these toxic effects. It is generally believed that the idiosyncratic characteristics related to liver damage induced by the compounds in this category are due to immunologic or/and metabolic factors (Zimmerman, 1993). Potentially hepatotoxic drugs falling into this category include halothane, ticrynafen (tienilic acid), dihydralazine, diclofenac, isoniazid, valproic acid, carbamazepine, phenytoin, phenobarbital, and more recently troglitazone (Beaune & Lecoeur, 1997; Neuschwander-Tetri et al., 1998; Zimmerman, 1993).

The role of immune system in drug-induced idiosyncratic liver injury is suggested by some key clinical features, such as the low frequency, the delayed onset of symptoms, the shortening of the delay upon rechallenge and more importantly the presence of autoantibodies in the serum of the patients (Beaune & Lecoeur, 1997). These features are observed in hepatitis induced by drugs such as halothane, ticrynafen, dihydralazine and troglitazone. Halothane-induced hepatitis has been considered as a classic model for immune-mediated hepatotoxicity. Only about one in every 40,000 patients exposed to halothane develops hepatic necrosis (National Halothane Study, 1966). Drug-related antibodies and autoantibodies can be detected in the affected patients but not in the controls (Kenna et al., 1988; Kitteringham et al., 1995). These antibodies recognize several proteins of the endoplasmic reticulum.
Ticrynafen was withdrawn from the market due to its hepatotoxicity. The incidence of hepatitis associated with this drug is between 0.1 to 0.7% (Park et al., 1998; Zimmerman et al., 1984). Further studies show that the ticrynafen-hypersensitive patients have developed antibodies against the hepatic cytochrome P450 2C9 (Beaune et al., 1987). Dihydralazine also causes a low incident of hepatitis. Biopsies of drug-affected liver samples show centrolobular necrosis accompanied by an inflammatory infiltration of immune cells (Beaune & Lecoeur, 1997). Autoantibodies against cytochrome P450 1A2 are also found in the sera of the patients who are hypersensitive to dihydralazine (Bourdi et al., 1990). Although the evidence strongly supports the involvement of an immune response in the drug-induced hepatotoxicity, it is still difficult to prove if the cytotoxicity is directly induced by the autoimmune response or the immune response is the result of direct toxicity of the reactive drug metabolite. Even if the problem is immune-mediated, it is possible that the toxicity is due to cytotoxic T-cells, and the antibodies are just a marker for the immune responses. It is also possible that the autoimmune reaction is a response to abnormal folding of proteins caused by covalent modification of the drug (Uetrecht, 1997).

1.3.3.2 Hematological toxicity.

The hematological system is also one of the most commonly affected sites of adverse drug reactions. Drug-induced hematological toxicity can affect either a certain element of the blood, such as erythrocytes, leukocytes or platelets, or it can affect all the cellular elements of the bone marrow. If all of the blood cell lines are
depleted, it is known as aplastic anemia, which is associated with a high mortality rate. The destruction of peripheral erythrocytes is called hemolytic anemia; if the destruction involves erythrocyte precursors, it is known as pure red cell aplasia. The depletion of blood platelets is referred to as thrombocytopenia. The term leukopenia is used to describe a decrease in the number of peripheral leukocytes. One frequent type of serious idiosyncratic drug reaction involves depletion of granulocytic leukocytes, including neutrophils, eosinophils and basophils. This manifestation is referred to as agranulocytosis, which is characterized by extremely low peripheral granulocyte count (less than 500 cells per microlitre of blood). If the depletion only involves neutrophils, the most abundant granulocyte, it is usually called neutropenia (Uetrecht, 1990; Young & Alter, 1994). Agranulocytosis and neutropenia can lead to very grave consequences, since the patients are at very high risk of uncontrolled infections. The infection symptoms associated with drug-induced agranulocytosis can present in the form of fever; sore throat; abscesses of the head, neck, perineum and rectum; cellulitis; pneumonia; pseudomembranous enterocolitis; and bacteremic shock (Young & Alter, 1994). Many commonly used therapeutic agents cause various hematological toxicities (Table 1-5). However, agranulocytosis and neutropenia are more frequent manifestations than any other blood dyscrasias. Several drugs that cause agranulocytosis are also associated with aplastic anemia.

Agranulocytosis can be classified into two simplified categories: peripheral destruction of mature cells and the bone marrow injury (Uetrecht, 1990). Many examples of drug-induced destruction of granulocytes in peripheral blood appear to
be mediated by antibodies. However, direct evidence for such a mechanism is often inadequate. The clearest example of this type is aminopyrine-induced agranulocytosis. Aminopyrine causes a high incidence (1%) of agranulocytosis, which is characterized by a rapid onset of symptoms. By co-incubating acute serum from a patient with neutrophils from a normal donor, agglutination of neutrophils can be induced. Furthermore, infusion of serum from a sensitive patient after drug exposure into a normal subject leads to a precipitous decrease in granulocyte count (Magis et al., 1968; Moeschlin & Wagner, 1952). Bone marrow injury can be either due to immune-mediated damage or direct toxic damage. Although some drugs, such as phenothiazine derivatives, are believed to be caused by non-immune-mediated bone marrow suppression, it is generally difficult to clearly differentiate the two mechanisms for most drugs (Uetrecht, 1990; Uetrecht, 1992; Young & Alter, 1994).
Table 1-5. Drugs commonly associated with hematological toxicity.

(Park et al., 1998, partially)

<table>
<thead>
<tr>
<th>Agranulocytosis</th>
<th>Hemolytic anemia</th>
<th>Thrombocytopenia</th>
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<tbody>
<tr>
<td>Aminopyrine</td>
<td>Aminopyrine</td>
<td>α-Methyldopa</td>
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<tr>
<td>Amodiaquine</td>
<td>α-Methyldopa</td>
<td>Carbamazepine</td>
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<tr>
<td>Captopril</td>
<td>Cephalosporins</td>
<td>Cephalosporins</td>
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<tr>
<td>Clozapine</td>
<td>Chlorpromazine</td>
<td>Co-trimoxazole</td>
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<tr>
<td>Gold</td>
<td>Indinavir</td>
<td>Gold</td>
</tr>
<tr>
<td>Levamisole</td>
<td>Nomifensine</td>
<td>Heparin</td>
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<tr>
<td>Mianserin</td>
<td>Penicillins</td>
<td>L-Dopa</td>
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<tr>
<td>Penicillin-G</td>
<td>Penicillamine</td>
<td>Penicillins</td>
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<tr>
<td>Propylthiouracil</td>
<td>Quinidine</td>
<td>Sulfasalazine</td>
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<tr>
<td>Sulfamethoxazole</td>
<td>Sulfasalazine</td>
<td>Valproate</td>
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<td>Sulfasalazine</td>
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<tr>
<td>Trimethoprim</td>
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1.3.3.3 Cutaneous toxicity.

The skin is the most common target for adverse drug reactions. It has been reported that drug-associated cutaneous reactions affect 2 - 3% of hospitalized patients (Bigby et al., 1986). Most adverse cutaneous reactions are not severe, but some are fatal.
Drug-induced skin eruptions are most often morbilliform or exanthematous, which clinically may not affect the course of the drug treatment. However, some morbilliform rashes that at first appear benign are harbingers of more serious reactions, including erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis (Roujeau & Stern, 1994). Erythema multiforme is associated with individual target lesions (typical or atypical) with no or minimal epidermal separation. The two most severe skin reactions are Stevens-Johnson syndrome and toxic epidermal necrolysis. Stevens-Johnson syndrome is associated with small blisters on dusky purpuric macules or atypical targets, rare areas of confluence and less than 10% of detachment of body surface. Similar to Stevens-Johnson syndrome, but more severe, toxic epidermal necrolysis is characterized by confluent erythema with extensive loss of epidermis (more than 30% of body surface) leaving the skin surface with the appearance of being scalded. Steven-Johnson syndrome and toxic epidermal necrolysis are life-threatening skin reactions, with mortality rates of 5% and 30%, respectively (Roujeau & Stern, 1994). The incidence of erythema multiforme, Stevens-Johnson syndrome or toxic epidermal necrolysis associated with drug use is 7.0, 1.8, and 9.0 per million patients, respectively.

Almost every drug causes adverse cutaneous reactions to a certain extent. However, some commonly used drugs are associated with a fairly high incidence. Among drugs usually used for short periods, trimethoprim-sulfamethoxazole and other sulfonamide antibiotics have the highest incidence of Stevens-Johnson syndrome and toxic epidermal necrolysis, and are followed by chlormezanone, aminopenicillins, quinolones, cephalosporins and acetaminophen. Among drugs
used for long-term therapy, carbamazepine has the highest incidence, and it is followed by phenobarbital, phenytoin, valproic acid, oxicam non-steroidal anti-inflammatory drugs, allopurinol and corticosteroids (Roujeau et al., 1995). Drug-induced severe cutaneous reactions occur more frequently in patients with human immunodeficiency virus than normal objects (Saiag et al., 1992).

Most severe drug cutaneous reactions are idiosyncratic and thought to be immune-mediated. The skin exhibits high immunological activity. Specialized cells in the skin, such as the Langerhan’s cells and the dendritic cells, are capable of processing and presenting agents for immune responses (Stingl et al., 1981). Being the largest organ in the body and the outer-most barrier against the outside world, the skin is also metabolically active, containing many enzyme systems that may be involved in production of reactive drug metabolites (Mukhtar & Khan, 1989; Pannatier et al., 1978). In patients with drug-induced erythema multiforme and toxic epidermal necrolysis, inflammatory infiltration of CD8+ T cells has been observed in the epidermal layer. Furthermore, positive lymphocyte stimulation tests against drugs or their metabolites have been demonstrated in these cases (Friedmann et al., 1994; Hertl & Merk, 1995). This evidence suggests that these reactions are T cell-mediated. However, for many cutaneous adverse reactions, the possibility of direct toxic effects induced by reactive drug metabolites cannot be excluded.

1.3.4 Trimethoprim-induced idiosyncratic drug reactions.
The trimethoprim-sulfamethoxazole combination (co-trimoxazole) is commonly used against mostly gram-positive bacteria. The combination has been approved for therapy of established recurrent bacterial urinary tract infections, *Pneumocystis carinii* pneumonia, otitis media in children and shigellosis (Gleckman et al., 1979).

Cutaneous reactions are the most common adverse reaction associated with the use of co-trimoxazole with an incidence of 5.9% (Arndt & Jick, 1976). Furthermore, co-trimoxazole has a higher incidence of Stevens-Johnson syndrome and toxic epidermal necrolysis than any other drug used on a short-term basis (Chan et al., 1990; Roujeau et al., 1995). Low incident drug-induced liver damage is also a manifestation of co-trimoxazole toxicity (Frisch, 1973; Haaverstad & Kannelonning, 1984). Idiosyncratic life-threatening hematological toxicity is also associated with the use of co-trimoxazole. These reactions include anemia (hemolytic and aplastic), thrombocytopenia and fatal agranulocytosis (Inman, 1977; Keisu et al., 1990; Lawson & Henry, 1977; Myers & Jick, 1997).

Generally, the sulfonamide component of co-trimoxazole has been thought to be responsible for these adverse drug reactions. After reports that trimethoprim is as effective and has fewer side effects when used alone as when combined with sulfamethoxazole, trimethoprim alone has increasingly been used to treat some infections, such as urinary tract infections (Anonymous, 1986). However, when used alone, trimethoprim has also been reported to induce severe idiosyncratic drug reactions, including toxic epidermal necrolysis (Das et al., 1988; Nwokolo et al., 1988), bilateral anterior uveitis, retinal hemorrhages (Kristinsson et al., 1997), as well as
life-threatening neutropenia and thrombocytopenia (Hawkins et al., 1993). The clinical evidence suggests that the trimethoprim component of co-trimoxazole may also contribute to the overall idiosyncratic reactions associated with the combination of trimethoprim and sulfamethoxazole. Furthermore, by rechallenging patients with a prior history of hypersensitivity to co-trimoxazole with trimethoprim alone, Carr et al. demonstrated that about 20% of the adverse reactions were due to trimethoprim (Carr et al., 1993).

1.3.5 Clozapine- and Fluperlapine-induced idiosyncratic reactions.

Clozapine is an atypical antipsychotic agent that has been shown to be effective in the treatment of refractory schizophrenia. Clinically, the use of clozapine is limited because it has been shown to cause idiosyncratic agranulocytosis and fatal acute fulminant liver failure (Alvir et al., 1993; Krupp & Barnes, 1989; Macfarlane et al., 1997; Panagiotis, 1999). The incidence of clozapine-induced serious liver toxicity has been estimated to be 0.001 - 0.06% (Macfarlane et al., 1997). The incidence of agranulocytosis has been reported to be about 2% (Alvir et al., 1993; Lieberman et al., 1988), although it appears to be lower in some studies (Anderman & Griffith, 1977; Idanpaan-Heikkila et al., 1977). In most cases, clozapine-induced agranulocytosis is associated with a complete absence of neutrophil precursors in the bone marrow (Idanpaan-Heikkila et al., 1977; Lieberman et al., 1988). Fluperlapine is a structural analogue to clozapine, and it was introduced as a potential replacement for clozapine. Early clinical trials found that fluperlapine has sedative, muscle-relaxing and anticholinergic properties similar to clozapine, but it appeared to have
much fewer undesirable side effects than clozapine (Fischer-Cornellssen, 1984). However, during further clinical trials, fluperlapine induced 2 cases of reversible agranulocytosis among more than 800 drug-treated patients. As a result, the drug was withdrawn from development (Mann et al., 1987; Scholz & Dichgans, 1985). Like clozapine, fluperlapine caused moderate to severe liver toxicity in rats, and it may potentially cause similar toxicity in humans (Dain & Jaffe, 1988).

1.3.6 Nevirapine-induced idiosyncratic reactions.

Nevirapine is a non-nucleoside, reverse transcriptase inhibitor approved for use in the treatment of the human immunodeficiency virus infections (Cheeseman et al., 1995). Clinically, nevirapine demonstrated a significant efficiency in reducing virus load and increasing CD4 cell count when it is used in combination with other reverse transcriptase inhibitors and protease inhibitors (D’Aquila et al., 1996; Havlir & Lange, 1998). Nevirapine does not cause bone marrow suppression, which is the major toxicity associated with nucleoside analogue reverse transcriptase inhibitors, such as zidovudine or didanosine (Richman et al., 1987). In pre-clinical toxicology tests, nevirapine was shown to induce moderate to severe skin rash and liver injury in experimental animals, including rats and dogs. During early clinical trials, high-dose nevirapine was shown to induce a high incidence of skin rash (32 - 48%) in patients (Carr et al., 1996; Havlir et al., 1995). However, in more extensive clinical trials, the rate of nevirapine-associated skin rash dropped to 16%, presumably due to an optimized dosing program. The same study found that the incidence of nevirapine-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in a
large number of patients is about 0.3% (Pollard et al., 1998). The therapeutic use of nevirapine is also associated with a high incidence of drug-induced hepatitis in adult patients (1.0%) as well as granulocytopenia in children (Beach, 1998; Leitze et al., 1998; Pollard et al., 1998).
1.4 Possible mechanisms for idiosyncratic drug reactions.

In general, the mechanisms of idiosyncratic drug reactions are poorly understood. However, for all of them the mechanisms can be hypothesized to involve either a direct or an immune-mediated toxicity. It is unlikely that many idiosyncratic drug reactions are due to direct toxicity of the drug or its metabolites. If such were the case, the problem would be more predictable and be detected in preclinical animal tests using high doses. However, these characteristics are generally not associated with most idiosyncratic drug reactions. Clinically, many adverse drug reactions classified as idiosyncratic exhibit many features similar to those often associated with typical immune responses. These clinical features include the low incidence, the delayed onset, the shortening of delay upon rechallenge and the frequent presence of autoantibodies in the sera of the patients. Such signs can often be found in idiosyncratic reactions triggered by various drugs such as halothane, ticrynafen, dihydralazine and many anticonvulsants (Anderson, 1992; Anderson & Adkinson Jr, 1987; Witte & West, 1982). The clinical features of these reactions lead many researchers to believe that most idiosyncratic drug reactions are immune-mediated in spite of the general lack of direct proof (Park et al., 1992; Pohl et al., 1988; Uetrecht, 1990; Uetrecht, 1992). However, it would be dangerous to claim that all idiosyncratic reactions are immune-mediated. The possibility of direct toxicity triggered by the drug or its metabolites can not be ruled out completely. Genetic variations in terms of drug targets, transporters and metabolic enzymes may also play important roles in the presentation of interindividual and interspecies
differences in drug toxicity. Nevertheless, the involvement of the immune system provides an attractive explanation for manifestations of many idiosyncratic reactions.

1.4.1 Hapten hypothesis.

The current understanding of idiosyncratic drug reactions is mainly based on the hapten hypothesis, which is derived from model studies with very defined molecules that are intrinsically immunogenic. In general, small molecules with molecular masses less than 1,000 daltons do not directly induce an autoimmune response (Pohl et al., 1988). Therefore, in order for a compound of small molecular mass, such as a drug, to induce an immune response, it has to be covalently linked to a larger molecule called a carrier. The carriers are often cellular macromolecules such as a "self-protein". The smaller molecule is referred to as a hapten. The hapten-carrier conjugate can then be recognized by the immune system as "non-self", and an immune response can be subsequently initiated against the "non-self" hapten-modified immunogen.

The immune response can be a specific humoral (antibody) response, a specific cellular (T cell) response or both responses. In specific humoral responses, the hapten-carrier conjugate is taken up by an antigen-presenting cell, such as a macrophage, and processed. The processing usually involves partial hydrolysis. The processed hapten-carrier conjugate is then combined with the class II major histocompatibility complex (MHC II) and together expressed on the surface of the antigen-presenting cell. If the combination of processed immunogen and MHC II is
recognized by specific CD4+ T cells (known as helper T cells, or T\textsubscript{H} cells), these T cells can be activated and stimulate the clones of B cells that recognize the original hapten-carrier conjugate. Upon activation by the helper T cells, B cells can proliferate and differentiate into mature plasma cells that are capable of producing antibodies against the immunogen. The antibodies generated in such a way may show specificity against the hapten, the carrier or both. In a specific cellular response, the antigen-presenting cell can combine the processed hapten-carrier conjugate with either the MHC II or MHC I and express them on its surface. Two types of specific T cells, T\textsubscript{C} (cytotoxic class) and T\textsubscript{DH} (delayed hypersensitivity class), become activated by recognition of these MHC-bound antigens. Upon activation, T\textsubscript{C} cells can directly kill target cells by lysis, and the T\textsubscript{DH} cells produce lymphokines that cause a localized inflammatory response with infiltration and activation of macrophages and natural killer cells (Pohl \textit{et al.}, 1988; Abbas \textit{et al.}, 1991). Since the MHCs are highly polymorphic in higher vertebrates, individuals who have MHCs facilitating more efficient recognition of a certain antigen may produce a more vigorous immune response to the presence of a certain hapten-carrier conjugate. Therefore, the polymorphism in human MHCs provides a plausible explanation for the individual differences on drug-induced idiosyncratic reactions (Park \textit{et al.}, 1992; Yunis \textit{et al.}, 1992). However, studies have not shown strong associations between specific idiosyncratic reactions and specific MHC alleles (Tournade \textit{et al.}, 1990; Yunis \textit{et al.}, 1995).

To initiate autoimmune responses, drugs associated with idiosyncratic reactions may covalently bind to cellular proteins, either directly or by forming
reactive metabolites. Penicillin is chemically reactive due to its β-lactam ring, and both itself and some of its metabolites may covalently bind to "self-proteins" to form hapten-carrier conjugates. IgE antibodies against those hapten-carrier conjugates have been detected in patients with penicillin-induced anaphylaxis (Park et al., 1998). In contrast, most drugs causing idiosyncratic reactions do not covalently bind to cellular proteins by themselves. They require metabolic activation prior to protein conjugation. The classic example is halothane-induced hepatitis. Halothane is metabolized by liver cytochrome P450 2E1 to reactive trifluoroacetyl chloride, which covalently binds to cellular proteins through their lysine residues. Trifluoroacetyl-specific antibodies of IgG class have been found in the sera of patients who developed halothane-induced hepatitis. These antibodies were found in patients who were treated with halothane but did not develop any symptoms of hepatitis (Kenna et al., 1988a; Kenna et al., 1988b; Vergani et al., 1980).

1.4.2 Danger hypothesis.

The hapten hypothesis provides a plausible mechanism for the idiosyncratic nature of drug-induced adverse reactions, but some observations associated with these reactions remain unexplained. Although anti-hapten antibodies have been found in some patients with idiosyncratic drug reactions (Kenna et al., 1988a; Kenna et al., 1988b; Park et al., 1998; Vergani et al., 1980), it is not clear if the antibodies are the cause of these reactions or are simply a consequence (marker) of the immune response. Furthermore such antibodies are not found in most cases of drug-induced idiosyncratic reactions (Pirmohamed et al., 1998). If adverse reactions are T-cell-
mediated, theoretically one would expect a positive lymphocyte stimulation test in all of these reactions; however, the positive tests are not always observed (Uetrecht, 1999). Rechallenging patients who are hypersensitive to a certain drug with that drug does not always shorten the onset of adverse symptoms (Guest et al., 1998; Safferman et al., 1992).

The danger hypothesis is a newly developed immunological concept. This hypothesis claims that the immune system does not differentiate "self" from "non-self". What the immune system senses is the presence of a "danger signal" rather than the foreignness of the antigen. The immune system responds to most antigens with tolerance as a default, and only becomes activated when it detects the presence of "danger". Cellular damage and stress may provide such "danger signals". The danger hypothesis is associated with several fundamental concepts. The first concept is that a lymphocyte will die if it recognizes an antigen without the presence of a danger signal. The second concept is that a lymphocyte will accept a danger signal only from antigen-presenting cells. Some additional statements include: 1) Inexperienced T cells that have not encountered any antigen can accept a danger signal only from "professional" antigen-presenting cells, such as dendritic cells, while experienced T cells that have encountered antigen at least once can accept danger signals from other antigen-presenting cells, such as B cells or macrophages; 2) Resting B cells can only accept danger signals from experienced or activated T cells; 3) Effector B cells or T cells respond to the recognition of antigen without the presence of a danger signal; 4) After a short period, the effector cells either revert
back to a resting state or die, so that the response is always under control (Matzinger, 1998; Matzinger, 1994; Uetrecht, 1999).

Applying the danger hypothesis to idiosyncratic drug reactions, the central statement is that the immune response is carrying out its normal functions in these reactions, and what provokes the immune system is the way in which a cell dies and activates local antigen-presenting cells. According to this hypothesis, local antigen-presenting cells are activated by cellular necrosis or stress but not activated by cellular apoptosis. The role of a danger signal provides a plausible explanation for many observations associated with many drug-induced idiosyncratic reactions. For example, it has been well known that patients with viral infections exhibit a much higher risk of idiosyncratic reactions to many drugs, such as co-trimoxazole and nevirapine (Medina et al., 1990; Pollard et al., 1998). An influenza vaccination appears to increase the risk of agranulocytosis induced by vesnarinone (Uetrecht et al., 1994), and open-heart surgery appears to increase the risk of agranulocytosis induced by procainamide (Ellrodt et al., 1984; Meyers et al., 1985). In these cases, the viral infections, the influenza vaccine and the tissue injury from an operation may all supply sufficient cell stress or necrosis to generate enough danger signal, to trigger immune responses.

Using the danger hypothesis, reactive metabolites generated by drug metabolism remain as the central character in idiosyncratic drug reactions. The reactive drug metabolites, depending on their intrinsic reactivity, may cause sufficient cell stress or necrosis to result in a danger signal without causing overt direct cellular damage. In addition to acting as a hapten, a reactive drug metabolite
may provide a strong danger signal in certain individuals who have cells that are more susceptible to the reactive metabolite-induced damage or stress. Such danger signals may provoke on the immune response against target tissues that are damaged by the reactive metabolite and generate adverse reactions to the drug. As an illustration, some researchers have already shown that the cells obtained from a patient who has had an idiosyncratic drug reaction are more sensitive to the toxic effects of the reactive metabolite of the drug than cells obtained from normal subjects (Gardner et al., 1998b; Spielberg, 1984; Rieder et al., 1989; Uetrecht, 1999).

1.4.3 Roles of reactive metabolites.

In general, the process of xenobiotic metabolism in humans is considered to be a detoxification mechanism that facilitates the excretion of xenobiotics by converting them from lipophilic and nonpolar compounds to hydrophilic and polar compounds. However, this process may also lead to the formation of chemically reactive species, i.e. reactive metabolites. Reactive metabolites are usually electron deficient molecules, and they are referred to as electrophiles. If inadequately detoxified, the electrophilic reactive metabolites can readily form covalent adducts with electron-rich cellular macromolecules (nucleophiles), such as peptides, proteins and nucleic acids, and result in various types of toxicity, including cellular necrosis, hypersensitivity, carcinogenicity and teratogenicity (Nelson, 1982; Pirmohamed et al., 1994). Although cellular enzyme systems involved in phase I metabolism are mainly responsible for the formation of reactive metabolites, reactive species can also be generated by phase II metabolic enzymes. In this section,
several common types of reactive metabolites are discussed with regard to their reactivity and toxicity. Although some example compounds are not therapeutic agents per se, their bioactivation pathways illustrate the formation of some toxicologically important reactive metabolites.

1.4.3.1 Quinones.

Quinones are generated from many xenobiotics and represent a class of toxicological intermediate that can cause a variety of hazardous effects in vivo, including acute cytotoxicity, immunotoxicity and carcinogenesis. Quinones are electrophilic Michael acceptors and can generate cellular damage through direct alkylation of crucial cellular proteins and/or DNA (Peter, 1989). On the other hand, quinones can be actively involved in cellular redox cycling with their semiquinone radicals leading to the formation of reactive oxygen species including superoxide anion, hydrogen peroxide and the more reactive hydroxy free radical (Figure 1-1). Formation of reactive oxygen species can cause severe oxidative stress within cells through the peroxidation of important cellular macromolecules, such as lipids, proteins and nucleic acids (O'Brien, 1991).
Figure 1-1. Schematic diagram of quinone-induced oxidative stress. SOD, superoxide dismutase:

P450 R, cytochrome P450 reductase; Q, quinone; QH', semiquinone; QH2, hydroquinone.

Benzene induces toxicity to various bone marrow cell populations, which may be manifested as pancytopenia, aplastic anemia, leukemia and immunotoxicity (Zhu et al., 1995). Peroxidases appear to play an important role in the bone marrow suppression generated by chronic exposure to benzene. Liver cytochrome P450 2E1 converts benzene to phenol and hydroquinone. The hydroquinone can subsequently be oxidized to the electrophilic benzoquinone (Figure 1-2) by myeloperoxidase in bone marrow leukocytes (Li, 1993; Li & Trush, 1993). Benzoquinone has a high reactivity toward sulphhydryl-containing molecules and can cause rapid depletion of cellular glutathione (Rossi et al., 1986).
Polycyclic aromatic hydrocarbons are a group of classic DNA-damaging carcinogens and teratogens (Phillips, 1983; Shum et al., 1979). The parent compounds, such as benzo[a]pyrene, are relatively non-toxic themselves, but they are bioactivated to toxic reactive metabolites. Recent studies show a novel bioactivation pathway of polycyclic aromatic hydrocarbons involving the formation of reactive and redox active ortho-quinone metabolites. In this pathway, non-K-region trans-dihydrodiols are generated by cytochrome P450 1A1 and subsequently yield the corresponding ortho-quinones catalyzed by dihydrodiol dehydrogenase in a NADP⁺-dependent oxidation (Figure 1-3; Smithgall et al., 1988). The generated ortho-quinone reactive metabolites can form stable and depurinating adducts with
DNA bases as well as conjugate with L-cysteine, mercapturic acid and glutathione (McCoull et al., 1999; Murty & Penning, 1992). The ortho-quinone metabolites of polycyclic aromatic hydrocarbons are also involved in cellular redox cycling and generation of reactive oxygen species, which lead to DNA damage (Flowers et al., 1997; Penning et al., 1996).

Figure 1-3. Formation of reactive ortho-quinone metabolites from benzo[a]pyrene.

Diethylstilbestrol has been used as a synthetic estrogen. However, its use has been associated with breast cancer and transplacental induction of tumors in the female offspring. Diethylstilbestrol and the steroidal estrogen, 2-hydroxy-estradiol, can both undergo a uterine peroxidase-catalyzed oxidation to quinones (Figure 1-4), which can covalently bind to the protein sulfhydryl groups of the protein tubulin, the major component of microtubules. This interrupts the formation of
microtubules and induces abnormal mitotic spindle formation. The mechanism is unusual since covalent binding to cellular DNA is not involved (O'Brien, 1991).

Figure 1-4. Bioactivation of diethylstilbestrol and estrogen.

Prinomide was an anti-inflammatory drug candidate; however, it was withdrawn in late-phase clinical trials due to a low incidence (< 0.3%) of agranulocytosis. In vivo, one of the major metabolites of prinomide is the para-hydroxyprinomide, which is presumptively generated by hepatic cytochrome P450 enzymes. In vitro, the para-hydroxyprinomide can be rapidly oxidized by human
myeloperoxidase in the presence of hydrogen peroxide and chloride. The reactive metabolites generated in this metabolic system include 1,4-benzoquinone, which can irreversibly bind to both cellular protein and cysteine (Figure 1-5). Since myeloperoxidase is one of the major enzymes present in neutrophils and leukocyte progenitor cells, it was proposed that this pathway was responsible for the idiosyncratic agranulocytosis associated with the parent drug (Parrish et al., 1997).

Figure 1-5. Bioactivation pathway of stable hydroxy metabolite of prinomide.
1.4.3.2 Iminoquinones.

Iminoquinones, or quinoneimines, are structurally related to quinones by substituting one or both oxygens on quinones with nitrogens. Iminoquinones are also electrophilic Michael acceptors and can alkylate nucleophilic cellular peptides and proteins. The iminoquinone-modified proteins may subsequently generate cellular damage through either a direct or an immune-mediated mechanism. Unlike quinones, there is very little evidence showing the involvement of iminoquinones in production of reactive oxygen species. However, by affecting the cellular glutathione balance, iminoquinones have been shown to induce redox-cycling-mediated oxidative stress without oxygen activation (Sood et al., 1997). Iminoquinones represent a class of reactive metabolites that are commonly associated with drugs. Consequently, they are speculated to be responsible for various drug-induced idiosyncratic reactions.

Acetaminophen is a widely used analgesic and antipyretic agent. In overdose, it can cause severe hepatotoxicity with centrilobular necrosis and sometimes acute renal tubular necrosis in the kidney cortex. In a minor metabolic pathway, acetaminophen is converted by cytochrome P450 enzymes (2E1, 1A2 and 3A4) to an iminoquinone metabolite, N-acetyl-benzoquinoneimine (Figure 1-6). This reactive metabolite is a cytotoxic electrophile that binds covalently to cellular glutathione and proteins. The formation of this reactive metabolite in the liver is believed to be responsible for the drug-induced hepatotoxicity. In the kidney, which contains less cytochrome P450, acetaminophen can be converted by prostaglandin H synthase to
N-acetyl-benzoquinoneimine via a one-electron oxidation product, N-acetylbenzosemiquinoneimine radical. The formation of this semiquinoneimine radical by renal prostaglandin H synthase also likely contributes to the nephrotoxicity of acetaminophen (Parkinson, 1996).

![Diagram of acetaminophen bioactivation](image)

**Figure 1-6.** Bioactivation of acetaminophen by cytochrome P450 and prostaglandin H synthase.

Amodiaquine is an antimalarial drug associated with a relatively high incidence (about 0.05%) of agranulocytosis (Hatton *et al.*, 1986). The drug was also
found to inhibit bone marrow colony growth using cells from the patients but not using cells from normal controls (Rhodes et al., 1986). However, drug-dependent circulating neutrophil IgG antibodies have also been detected in patients who developed amodiaquine-induced agranulocytosis (Rouveix et al., 1989). In vitro, amodiaquine is rapidly oxidized to a reactive iminoquinone metabolite by activated human neutrophils (Tingle et al., 1995). This reactive metabolite readily forms adducts with glutathione, and it is thought to be responsible for covalent binding of the drug to neutrophils (Figure 1-7) (Ruscoe et al., 1996; Thompson et al., 1997). Recent studies also show that the bioactivation of amodiaquine causes glutathione depletion and cell apoptosis (Park et al., 1998).
Vesnarinone is a potential inotropic agent for the treatment of severe congestive heart failure. There were no significant adverse drug reactions observed during early clinical trials conducted in the Japanese population. However, further clinical trials in the United States showed that 4 out of the first 28 patients developed potentially fatal agranulocytosis (Uetrecht et al., 1989). In vitro,
vesnarinone is metabolized to a reactive quinoneiminium ion by activated human neutrophils. The same reactive metabolite can also be obtained by a combination of myeloperoxidase/hydrogen peroxide/chloride, or by hypochlorous acid, which is the major oxidant generated by this system. The quinoneiminium ion is further hydrolyzed to another iminoquinone metabolite. Both reactive metabolites readily form adducts with glutathione and covalently bind to neutrophil proteins (Figure 1-8). These iminoquinone reactive metabolites are thought to be responsible for vesnarinone-induced agranulocytosis (Uetrecht et al., 1994).
Carbamazepine is one of the most commonly used anticonvulsants. However, the drug induces various idiosyncratic adverse reactions, including skin
rashes, hematological toxicity and liver injury. *In vivo*, one of the major metabolites of the drug is 2-hydroxycarbamazepine. However, a recent study showed that 2-hydroxyiminostilbene, a further metabolite of 2-hydroxycarbamazepine, may be present in higher concentrations. The same study also demonstrated that 2-hydroxyiminostilbene can be easily further oxidized to an iminoquinone metabolite, which covalently binds to sulfhydryl-containing nucleophiles such as glutathione and N-acetylcysteine (Figure 1-9). This reactive iminoquinone metabolite is speculated to play a role in carbamazepine-induced idiosyncratic reactions (Ju & Uetrecht, 1999).

![Proposed bioactivation pathway of carbamazepine](image)

**Figure 1-9.** Proposed bioactivation pathway of carbamazepine.

1.4.3.3 Epoxides and arene oxides.

Cytochrome P450 isozymes (e.g. CYP2E1) can catalyze direct insertion of oxygen into a double bond of an alkene generating an epoxide. The same process
applied to an aromatic compound leads to the formation of an arene oxide. Both epoxides and arene oxides are electrophilic species and, therefore, potentially toxic metabolites. Many epoxides and arene oxides are considered to be carcinogens due to their ability to alkylate cellular nucleic acid molecules. Many common food contaminants and drugs can be metabolized to epoxides and arene oxides. The major in vivo detoxification pathways for these reactive metabolites involve enzymes such as epoxide hydrolase and glutathione S-transferase (Parkinson, 1996).

Ethyl carbamate, or urethan, is a genotoxic compound found in fermented food and beverages. It can be bioactivated to reactive ethyl carbamate epoxide in two sequential steps catalyzed by cytochrome P450 (Figure 1-10). The ethyl carbamate epoxide is a strong electrophile and has been shown to covalently bind to cellular glutathione and nucleic acids (Forkert & Lee, 1997; Kemper et al., 1995; Park et al., 1993; Parkinson, 1996).

\[
\begin{align*}
\text{CH}_3\text{-CH}_2\text{-O-C-NH}_2 & \xrightarrow{\text{P450 2E1}} \text{CH}_2\text{-CH-O-C-NH}_2 \\
\text{Ethyl carbamate} & \xrightarrow{\text{Vinyl carbamate epoxide}} \text{Vinyl carbamate}
\end{align*}
\]

**Figure 1-10.** Bioactivation of ethyl carbamate (urethane).

The classic mechanism for polycyclic aromatic hydrocarbon-induced carcinogenesis involves the formation of reactive epoxides (Figure 1-11). One of the major epoxide metabolites of benzo[a]pyrene is benzo[a]pyrene-7,8-dihydrodiol-9,10-oxide, which is often referred to as the bay-region dioleopoxide. This reactive epoxide
is considered to be highly carcinogenic since it cannot be detoxified easily by mammalian epoxide hydrolase (Parkinson, 1996).

![Diagram of the classic bioactivation pathway of benzo[a]pyrene.](image)

**Figure 1-11.** The classic bioactivation pathway of benzo[a]pyrene.

An alternative mechanism for carbamazepine-induced idiosyncratic reactions involves the formation of a reactive arene oxide, carbamazepine-2,3-oxide (Figure 1-12). Although there was no direct evidence, this reactive arene oxide metabolite was speculated to covalently bind to cellular proteins and to be responsible for the autoantibody formation in drug-treated patients (Park et al., 1998).
1.4.3.4 Other reactive metabolites.

Some drugs are converted to reactive metabolites with good leaving groups. These reactive metabolites may alkylate cellular proteins in nucleophilic substitution reactions. Halothane is a volatile anesthetic and associated with idiosyncratic hepatotoxicity. Halothane is metabolized by cytochrome P450 2E1 to a reactive intermediate, trifluoroacetyl chloride, which may undergo a nucleophilic substitution with the chloride as leaving group to covalently bind to the lysine residues of proteins (Figure 1-13). The trifluoroacetylated proteins are thought to be responsible for elicitation of an autoimmune response and drug-induced hepatitis (Pohl et al., 1988).

Glucuronidation is normally associated with rapid clearance and detoxification of xenobiotics. However, in the case of diclofenac, the carboxylic acid
forms an acyl (ester) glucuronide. Since glucuronic acid is also a moderately good leaving group, the glucuronide reacts slowly with protein amino groups. The acyl glucuronide may also undergo a rearrangement that opens the glucopyranoside ring. The resultant aldehyde form of the sugar may react with a protein amino group to form an imine which may undergo an Amadori rearrangement to generate an irreversible protein adduct (Figure 1-14) (Parkinson, 1996).

![Diagram of glucuronidation in bioactivation of carboxylic acids]

**Figure 1-14.** Role of glucuronidation in bioactivation of carboxylic acids.

Clozapine is an atypical antipsychotic agent that is known to induce idiosyncratic agranulocytosis. *In vitro*, the drug can be bioactivated to a nitrenium
ion by activated human neutrophils, myeloperoxidase/hydrogen peroxide/chloride, or hypochlorous acid (Figure 1-15). The nitrenium ion can be trapped by glutathione and was shown to cause cytotoxicity and to covalently bind to neutrophil proteins (Gardner et al., 1998a; Gardner et al., 1998b; Liu & Uetrecht, 1995).

![Bioactivation pathway for clozapine](image)

*Figure 1-15. A possible bioactivation pathway for clozapine.*

The sulfonamides are among the oldest antibiotics. Their uses have been associated with a high incidence of idiosyncratic reactions, including skin rash, hepatitis, renal and hematological toxicity. The most commonly used sulfonamide,
sulfamethoxazole, is metabolized by hepatic cytochrome P450 2C9 to the corresponding hydroxylamine, which is further oxidized to the nitroso metabolite (Figure 1-16). The nitroso metabolite may covalently bind to sulfhydryl-containing proteins by forming sulfinamides, and this may cause either direct or immunemediated toxicity (Cribb et al., 1991; Cribb et al., 1996; Rieder et al., 1988).

\[ \text{Sulfamethoxazole} \rightarrow \text{Hydroxylamine} \rightarrow \text{Nitroso} \rightarrow \text{Protein sulfinamide} \]

**Figure 1-16.** Bioactivation pathway for sulfamethoxazole.

Free radicals can also be considered as reactive metabolites. They are generally electron deficient species and may covalently bind to proteins. However, \textit{in vivo} the major reaction is hydrogen atom extraction leading to free radicals and lipid peroxidation, which can lead to cell membrane damage (Parkinson, 1996). An alternative mechanism for halothane-induced liver toxicity involves a free radical pathway (Figure 1-17).
Figure 1-17. Activation of halothane in a free radical pathway.
1.5 General metabolism of trimethoprim, fluperlapine and nevirapine.

The metabolism and pharmacokinetics of trimethoprim, fluperlapine and nevirapine have been studied by previous researchers. This section provides a brief summary of those studies.

1.5.1 General metabolism of trimethoprim.

Trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine, is a selective inhibitor of bacterial dihydrofolate reductase and is commonly used as an antibacterial agent. Trimethoprim is rapidly absorbed after oral administration. After a single dose of 160 mg, the plasma drug concentration reaches 2 - 6 μM within 4 hours. Trimethoprim exists in the plasma primarily as the intact drug, 45% bound to protein. Its apparent volume of distribution is estimated to be 47 to 133 liters, suggesting reasonably extensive tissue distribution. The elimination half-life for trimethoprim in patients with normal renal function is 8.6 - 11.6 hours (Gleckman et al., 1979).

Known metabolic pathways of trimethoprim include O-demethylation, N-oxidation and α-hydroxylation. Giving radio-labeled trimethoprim to rats, 3-desmethyl-trimethoprim is the major metabolite which accounts for more than 30% of total radioactivity excreted in the first 8 hours. 4-Desmethyl-trimethoprim is excreted mainly as its glucuronide which accounts for about 19% of the dose. Trimethoprim N-oxide and α-hydroxy-trimethoprim are excreted in amounts of 7%
and 5% of the dose, respectively (Meshi & Sato, 1972). A similar metabolic pattern of trimethoprim is also observed in vitro using rat hepatic microsomes and hepatocytes, and the pathways are thought to be cytochrome P-450 dependent (van't Klooster et al., 1992; Watkins & Gorrod, 1987).

1.5.2 General metabolism of fluperlapine.

Fluperlapine, 3-fluoro-6-(4-methyl-1-piperazinyl)-11H-dibenzo[b,e]-azepine, is an atypical antipsychotic agent that shows a similar pharmacological profile as its predecessor, clozapine. It has sedative, muscle-relaxing and anticholinergic effects, and it can be used for the treatment of refractory schizophrenia (Mann et al., 1987; Woggon et al., 1984).

Fluperlapine is extensively metabolized in vivo. In rats, after multiple oral doses in the diet, 95% of the identified products were hydroxylated metabolites, and only a minor amount of parent drug can be detected. The major metabolic pathways include N-oxidation, N-demethylation, 7-hydroxylation and the subsequent glucuronidation and sulfation (Dain & Jaffe, 1988). The metabolic pattern can be reproduced in vitro with human hepatocyte cultures, and the metabolism is shown to be mediated by cytochrome P450-dependent and flavin-dependent monooxygenases (Fischer & Wiebel, 1990; Guillouzo et al., 1988). In humans, the major metabolite formed from fluperlapine in vivo is reported to be 7-hydroxy-fluperlapine (Fischer et al., 1992).

1.5.3 General metabolism of nevirapine.
Nevirapine is a potent and selective non-nucleoside inhibitor of the human immunodeficiency virus reverse transcriptase. It blocks the RNA- and DNA-dependent DNA polymerase activities of the enzyme by causing a disruption of the catalytic site (Merluzzi et al., 1990; Richman et al., 1991). Nevirapine is rapidly absorbed after oral administration. After a single dose of 200 mg, the plasma drug concentration reaches about 7.5 μM within 4 hours (Lamson et al., 1995). The intrinsic elimination half-life after a single dose of nevirapine is about 45 hours. However, because nevirapine is an autoinducer of cytochrome P450 enzymes, the mean plasma elimination half-lives in multiple dosing experiments decreased to about 20 hours (Riska et al., 1999).

Nevirapine is extensively metabolized in the liver, and the metabolites are mainly excreted in urine (81.3%). The major metabolites identified in urine include: 2-hydroxynevirapine glucuronide (18.6%), 3-hydroxynevirapine glucuronide (25.7%), 12-hydroxynevirapine glucuronide (23.7%), 8-hydroxynevirapine glucuronide (1.3%), 3-hydroxynevirapine (1.2%), 12-hydroxynevirapine (0.6%) and 4-carboxynevirapine (2.4%). Only a small fraction of the parent drug (2.7%) is detected in urine. In vitro, using human hepatic microsomes containing cDNA-expressed cytochrome P450 isozymes, it has been shown that the formation of 2- and 3-hydroxynevirapine are mediated exclusively by cytochrome P450 3A and cytochrome P450 2B6, respectively; and the formation of 12- and 8-hydroxynevirapine are mediated by multiple enzymes including cytochrome P450 3A and cytochrome P450 2D6. Furthermore, the study also suggests
that both cytochrome P450 3A4 and cytochrome P450 2B6 pathways can be autoinduced by nevirapine (Riska et al., 1999).
Chapter 2

Metabolism of Trimethoprim to A Reactive Iminoquinone Methide by Activated Human Neutrophils and Hepatic Microsomes.
2.1 Abstract.

The antibacterial agent, trimethoprim, is normally used synergistically with sulfonamides. Its use is associated with idiosyncratic reactions including liver toxicity and agranulocytosis. In this study, we demonstrated that trimethoprim was oxidized by activated human neutrophils, as well as a combination of myeloperoxidase/hydrogen peroxide/chloride, or hypochlorous acid to a reactive pyrimidine iminoquinone methide intermediate with a MH⁺ ion of m/z 289 as detected by mass spectrometry. In the presence of N-acetyl-L-cysteine (NAC), the pyrimidine iminoquinone methide could be trapped as three NAC adducts. The three NAC adducts were separable on HPLC but showed the same molecular ion of m/z 452. The proton NMR spectrum of the major NAC adduct showed that the NAC group was at the 6 position of the pyrimidine ring. LC/MS/MS of the two minor NAC adducts indicated that they were the two diastereomers in which NAC was attached to the exo-cyclic prechiral carbon of the pyrimidine iminoquinone methide. Incubation of trimethoprim with isolated hepatic microsomes, both human and rat, in presence of NAC gave the same set of TMP-NAC adducts. Immunoblotting studies with an anti-trimethoprim antiserum detected covalent binding of trimethoprim to neutrophil proteins in vitro when trimethoprim was incubated with activated human neutrophils (major trimethoprim adducts of 46, 49, and 58 kDa). Similar trimethoprim-modified polypeptides were also found in bone marrow cells of trimethoprim-treated rats. We propose that the formation of this pyrimidine iminoquinone methide by both hepatic microsomes and neutrophils
may be responsible for trimethoprim-induced idiosyncratic hepatotoxicity and agranulocytosis.
2.2 Introduction.

Trimethoprim is widely used in combination with sulfonamides as an effective antibacterial agent against many bacterial species. However, the use of this combination has been associated with various adverse reactions, such as skin rashes, liver toxicity, blood dyscrasias and generalized hypersensitivity reactions (Frisch, 1973; Haaverstad & Kannelonning, 1984; Myers & Jick, 1997). Skin disorders that are related with trimethoprim/sulfonamide therapy range from mild drug rashes and urticaria to toxic epidermal necrolysis (TEN) (Roujeau et al., 1995; Roujeau & Stern, 1994). Although the incidence of trimethoprim/sulfonamide-associated blood dyscrasias, including agranulocytosis, thrombocytopenia, leukopenia and aplastic anemia is low (1 case per 18,000 prescriptions), they are potentially fatal (Anonymous, 1989; Keisu et al., 1992; Keisu et al., 1990; Williamson & Crowe, 1972). The incidence of idiosyncratic reactions appears to be increased dramatically by some viral infections. For example, when patients with AIDS were treated with trimethoprim-sulfamethoxazole for pneumocystis carinii pneumonia, the incidence of adverse reactions increased to almost 50% (Medina et al., 1990). Evidence suggests that most adverse reactions associated with the combination of trimethoprim-sulfamethoxazole are due to the sulfamethoxazole component (Cribb et al., 1996; Leeder et al., 1991; Rieder et al., 1988). The use of trimethoprim as a single agent is increasing because of the high incidence of adverse reactions associated with trimethoprim/sulfamethoxazole. However, when trimethoprim was used alone, several idiosyncratic reactions, including skin rashes (e.g. toxic epidermal necrolysis)
and neutropenia, were also reported (Das et al., 1988; Hawkins et al., 1993; Nwokolo et al., 1988). Furthermore, using trimethoprim to rechallenge AIDS patients with a history of hypersensitivity to trimethoprim-sulfamethoxazole, Carr et al. were able to demonstrate that about 20% of the hypersensitivity cases were due to trimethoprim (Carr et al., 1993). The mechanism of trimethoprim-induced adverse drug reactions is still unknown. However, it has been proposed that the hematologic, hepatic and cutaneous reactions associated with trimethoprim are based on an immunologic rather than a directly toxic mechanism and the characteristics of these reactions are consistent with this hypothesis (Frisch, 1973).

Although idiosyncratic reactions are a serious clinical problem associated with many drugs, there is no preclinical model to predict such reactions in humans. It has been demonstrated that many marketed medicines associated with idiosyncratic agranulocytosis can be bioactivated by neutrophils to Michael acceptors that bind covalently to nucleophilic proteins (Uetrecht, 1992; Uetrecht et al., 1994). Many other drugs, such as amodiaquine and acetaminophen that cause chemical-induced hepatic toxicity were found to be oxidized to quinone imines by liver microsomes and bind irreversibly to liver proteins (Maggs et al., 1988; Parkinson, 1996; Tingle et al., 1995). In the following study, we used an in vitro approach to investigate the oxidation of trimethoprim to reactive intermediates by both human neutrophils and hepatic microsomes.
2.3 Materials and Methods.

2.3.1 Materials.

Trimethoprim, NAC, PMA, Casein, rabbit serum albumin (RSA), thimerosal, methylumbelliferyl phosphate, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hypochlorite and GSH were purchased from Aldrich Chemical Company (Milwaukee, WI). Hydrogen peroxide (H$_2$O$_2$) was obtained from Mallinckrodt Canada Inc. (Pointe-Claire, Quebec). The concentration of sodium hypochlorite was determined by a spectrophotometric method (Hussarin et al., 1970). All solvents used for HPLC and LC/MS analyses were HPLC grade. MPO was obtained from Cortex Biochemical (San Leandrow, CA). One unit of MPO activity was defined as the amount of enzyme that decomposed 1 µmol of H$_2$O$_2$ per min at 25°C and pH 6. Stock acrylamide solution (40%) was purchased from BioRad (Mississauga, Ontario). Nitrocellulose and enhanced chemiluminescence (ECL) films were purchased from Amersham Canada (Oakville, Ontario). Supersignal ECL detection reagents and keyhole limpet hemocyanin were purchased from Pierce Chemical (Rockford, IL). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chains) was purchased from Zymed (San Francisco, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The neutrophils were isolated from venous blood collected from normal subjects.

2.3.2 Analytical.
The HPLC analyses were carried out on a Shimadzu HPLC system containing a LC-600 pump, a SPD-6A UV detector set at 254 nm and a C-R6A integrator (Shimadzu Corporation, Kyoto, Japan). The chromatography columns, packed with 5 μm Ultracarb ODS 30, were supplied by Phenomenex (Torrance, CA). The column used for all analytical work was 2 x 100 mm with a 2 x 30 mm guard column. The column used for isolation of the NAC adduct was 10 x 150 mm with a 10 x 60 mm guard column. A mobile phase of water:acetonitrile:acetic acid (90:10:1, v/v) with 2 mM ammonium acetate was used unless otherwise specified.

LC/MS and LC/MS/MS were performed on a Sciex API III mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario) with an IonSpray interface. Analyses were carried out with an ionizing voltage of 5 kV and an orifice voltage of 65 V. 1H NMR spectra were recorded at 500 MHz with a Varian Unity Plus 500 spectrometer with D2O as the solvent.

2.3.3 Neutrophil isolation.

Blood (60 mL), collected from normal subjects, was mixed with 3% dextran (MW 500 kD, Sigma) in 0.9% NaCl at a ratio of 4:1 (v/v). The erythrocytes were allowed to settle for 30 min. The supernatant was carefully drawn off and underlaid with Ficoll-Paque (Parmacia LKB Biotechnology Inc., Piscataway, NJ) at a 5:2 (v/v) ratio. After centrifugation at 500 x g for 25 min, neutrophils were collected as a pellet and the supernatant was discarded. Contaminating erythrocytes were lysed by a 0.2% NaCl solution (10 mL). Isotonicity was restored after 1 min by adding an equal volume of 1.6% NaCl solution. A second hypotonic lysis was performed if the
cell pellet was still red. The cells were then centrifuged at 350 x g for 5 min, and the cell pellet was washed twice with Hanks' balanced salt solution (HBSS) without phenol red (Media Services, University of Toronto) before finally suspending the cells in HBSS (10 mL). The neutrophils were stained with 0.1% trypan blue and counted with a hemocytometer. Trypan blue exclusion showed the cell viability to be more than 95% in all isolations.

2.3.4 Metabolism of trimethoprim by activated neutrophils in the presence of NAC.

To 1.7 x 10^7 neutrophils suspended in HBSS (3 mL) was added an ethanolic solution of trimethoprim (1.5 µL, final concentration 5 µM), an aqueous solution of NAC (75 µL, final concentration 5 mM) and PMA (120 ng in 1.2 µL of DMSO). The suspension was incubated at 37°C for 60 min. After incubation, the suspension was centrifuged at 500 x g for 10 min, the supernatant was collected and the solvent was removed with a stream of nitrogen at 25°C. The samples were redissolved in water and analyzed by LC/MS using SIM at m/z 291 (trimethoprim) and m/z 452 (TMP-NAC). In the control experiments DMSO replaced the DMSO solution of PMA. For immunoblotting studies, the neutrophil pellets were resuspended in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100; 400 µL). An aliquot of the sample was taken for protein concentration measurement. An equal volume of sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing dithiothreitol (6 mg/ml) was added to the remainder of the lysed cell sample. The samples were then boiled at 100 °C for 10 min prior to loading on the gel.
2.3.5 Metabolism of trimethoprim by rat and human liver microsomes in the presence of NAC.

Rat-liver microsomes were prepared from male Sprague Dawley rats (average weight 300 g). The animals were sacrificed by cervical dislocation, and their livers were removed and minced in ice cold sucrose buffer (0.25 M sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 6.8). The liver homogenates were prepared using a homogenizer (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany). The liver homogenates were filtered through a piece of cheese-cloth and centrifuged at 1,000 x g for 11 min at 4°C. The pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined supernatants were centrifuged at 10,000 x g for 30 min at 4°C, and the pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined 10,000 x g supernatants were further centrifuged at 100,000 x g for 90 min at 4°C. The microsome pellets were finally resuspended in a storage buffer (100 mM potassium phosphate, 10% sucrose, pH 7.5) and stored at -80°C before use. Human liver microsomes were a gift from Prof. Tadanobu Inaba (Department of Pharmacology, University of Toronto) and obtained from accident victims.

Microsomal cytochrome P450 was qualitatively demonstrated by the reduced carbon monoxide difference spectrum method of Omura and Sato (Omura & Sato, 1964). To phosphate buffer (0.1 M, pH 6.8, 747.4 μL with 5 mM MgCl₂) was added a microsome suspension (82.6 μL, final concentration 2 mg protein/mL), an ethanolic solution of trimethoprim (20 μL, final concentration 0.4 mM), an aqueous solution of NAC (50 μL, final concentration 5 mM) and a NADPH-generating system (100 μL,
final concentration 0.5 mM NADP*, 5 mM glucose-6-phosphate and 0.25 unit glucose-6-phosphate dehydrogenase). The suspension was incubated at 37°C for 3 hrs after which an equal volume of acetonitrile was added to precipitate the proteins in the mixture. After centrifugation at 500 x g for 10 min, the supernatant was isolated and dried with a stream of nitrogen. The residue was redissolved in water and analyzed by LC/MS using SIM at m/z 291 (trimethoprim) and m/z 452 (TMP-NAC). In the control experiments the NADPH-generating system was omitted.

2.3.6 Oxidation of trimethoprim by hypochlorous acid.

An ethanolic solution of trimethoprim (10 μL, 10 mM) and an aqueous solution of sodium hypochlorite (NaOCl) (2 μL, 100 mM) were added to an aqueous solution of 60% (v/v) ethanol with 0.2% (v/v) acetic acid (88 μL). The reaction mixture (5 μL) was immediately injected into the mass spectrometer though a HPLC injector. The solvent (methanol) flow rate was set at 200 μL/min and decreased to 20 μL/min with a splitter.

The oxidation of trimethoprim by hypochlorous acid was also monitored by a Hewlett Packard diode-array spectrophotometer (HP8452A, Hewlett Packard Company, Palo Alto, CA). An ethanolic solution of trimethoprim (100 μL, 10 mM) and an aqueous solution of NaOCl (20 μL, 100 mM) were added to phosphate buffer (0.1 M, pH 6, 1880 μL) in a quartz cuvette with rapid stirring by a micro magnetic stirring bar. The reaction mixture was immediately scanned by the spectrophotometer at 5-sec intervals for 180 sec over a wavelength range of 200 to 600 nm.
2.3.7 Trapping the HOCl oxidation product of trimethoprim with NAC.

Ethanolic solutions of trimethoprim (10 mM, 10 μL) were added to phosphate buffers (0.1 M, 83 μL) with pHs of 5.8, 6.0, 6.4, 6.8, 7.0, 7.2, 7.6, respectively. The oxidation was initiated by adding aqueous solution of NaOCl (100 mM, 2 μL). Immediately after addition of oxidant, a phosphate-buffered (0.5 M, pH 8.5) NAC solution (200 mM, 5 μL) was added to each reaction mixture. The resulting mixtures were analyzed by LC/MS using SIM at m/z 452 for TMP-NAC adducts.

2.3.8 Preparation of the major NAC adduct of the reactive intermediate of trimethoprim for NMR study.

Trimethoprim (100 mg, 0.34 mmol), acetonitrile (15 mL) and phosphate buffer (30 mL, 0.1 M, pH 6) were added to a 150 mL Erlenmeyer flask. An aqueous solution of NaOCl (0.971 mL, 700 mM, 0.68 mmol) was rapidly added to the mixture with vigorous stirring. The solution immediately became bright yellow. After 5-10 sec, NAC (222 mg, 1.36 mmol) in phosphate buffer (13.6 mL, 0.5 M, pH 8) was rapidly added to the mixture. The reaction mixture was stirred at room temperature for 1 hr before it was extracted three times with an equal volume of chloroform. The aqueous phase was separated and then washed with a small amount of pentane to remove any chloroform residue. Methanol was then added to the aqueous phase, and the solution was concentrated by rota-evaporation. After the solution was reduced to about 10 mL, the supernatant was decanted from the sticky residue, which was washed by methanol a few times. The combined methanol solutions
were further concentrated by rota-evaporation and finally applied to a 20 x 20 cm TLC plate (Aldrich, Milwaukee, MI). The TLC plate was developed with a solvent of ethyl acetate:methanol (6:4, v/v). A broad band with a \( R_f = 0.2 \) was scraped from the TLC plate and the silica gel was washed with methanol to recover the product. The crude product obtained was finally purified by HPLC using a mobile phase of water:acetonitrile:acetic acid (90:10:1, v/v) at a flow rate of 5 mL/min, and the fraction with a retention time of 23 min was collected. HPLC analysis showed that the purity of the final product was more than 95%. The typical yield for the preparation was about 10%. Separation of the other two isomers was not successful.

2.3.9 Oxidation of trimethoprim by the MPO enzyme system.

An ethanolic solution of trimethoprim (10 \( \mu \)L, 10 mM) and MPO (1 \( \mu \)L, 1 unit/\( \mu \)L) was added to phosphate-buffered saline (81.5 \( \mu \)L, 0.1 M, pH 7), and the reaction was initiated by addition of hydrogen peroxide (2.5 \( \mu \)L, 80 mM). After 30 sec at 25°C, an aqueous solution of NAC (5 \( \mu \)L, 200 mM) was added. The reaction mixture was then incubated at 25°C for 1 hr and analyzed by LC/MS using SIM at m/z 291 (trimethoprim) and m/z 452 (TMP-NAC). In the control experiments, hydrogen peroxide was replaced by water.

2.3.10 Synthesis of trimethoprim-NAC-modified KLH and RSA.

The major trimethoprim-NAC adduct (1.0 mg, 2.22 \( \mu \)mol for RSA; 4.0 mg, 8.88 \( \mu \)mol for KLH) in N,N-dimethylformamide (100 \( \mu \)L) was added to a 10 mL test tube. Under vigorous stirring, a dichloromethane solution of 2 equivalent EDC was
slowly added. The reaction mixture was stirred at room temperature for 1 hr, before dichloromethane was evaporated by a stream of nitrogen. Under stirring, either RSA (1.0 mg) or KLH (10.0 mg) in phosphate buffer (0.1 M, pH 8.5, 50 μL for RSA and 200 μL for KLH) was subsequently added. The mixture was stirred at room temperature for another 5 hrs. The modified proteins were purified by dialysis against water (molecular weight cut-off 6 - 8 kDa) for 48 hrs. The resultant protein solution was then freeze-dried overnight and stored at -20°C before use.

2.3.11 Production of anti-trimethoprim-NAC-KLH antiserum.

Polyclonal anti-trimethoprim-NAC-KLH antibodies were raised in a 2-kg, male, pathogen-free New Zealand White rabbit (Charles River, Montreal, Quebec) housed in the animal care facility at the Hospital for Sick Children, Toronto. After preimmune serum was obtained, the animal was immunized with the trimethoprim-NAC-KLH conjugate (1 mg in 1 mL of PBS emulsified with an equal volume of Freund’s complete adjuvant) subcutaneously at multiple sites. Injections with 0.5 mg trimethoprim-NAC-KLH conjugate in Freund’s incomplete adjuvant divided into six to eight subcutaneous sites was repeated 4, 6, 8, and 12 weeks after the initial immunization. Exsanguination of the animal while it was under pentobarbital anesthesia was conducted 10 days after the final immunization. Blood was allowed to clot overnight at 4°C and then centrifuged at 400 x g. The serum was recovered and heat-inactivated at 56°C for 30 min before being placed in aliquots and stored at -20°C.
2.3.12 Enzyme-linked immunosorbent assay (ELISA) for specificity of anti-
trimethoprim antiserum.

Trimethoprim-NAC-RSA or native RSA (100 μL of a 15 μg/mL solution) was incubated overnight in flat-bottom 96-well plates (Costar, Cambridge, MA) at 4 °C. The plates were emptied and washed with ELISA wash buffer (10 mM Tris-HCl, pH7.5, 154 mM NaCl, 0.5% (w/v) casein, and 0.002% (w/v) thimerosal) for four times. After the last wash, the plates were tapped dry, and various dilutions of the anti-trimethoprim-NAC-KLH antiserum (100 μL PBS) were added to the plates. The ELISA plates were incubated at room temperature for 3 hr. Plates were then washed four times with ELISA wash buffer and tapped dry. Alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:5000 in PBS) was subsequently added to each well of the plate (100 μL/well). The plates were incubated at room temperature for 2 hr, before they were washed four times with ELISA wash buffer and two times with PBS. A stock solution of methyl umbelliferyl phosphate (10 mg/mL in dimethylsulfoxide, kept in -20 °C) was diluted 1:100 in PBS, and this solution was added to the ELISA plates (100 μL/well). Plates were incubated at room temperature for 10 min before fluorescence was measured with a Fluorescence Concentration Analyzer (Pandex, Mundelein, IL) set at 365/450 nm (excitation/emission).

2.3.13 SDS-PAGE and Western Blotting.

Samples for analysis by SDS-PAGE were solubilized by boiling for 10 min in sample buffer (8% (w/v) SDS, 20% (v/v) glycerol, 0.002% bromophenol blue, 125 mM Tris-HCl (pH 6.8)) containing dithiothreitol (6 mg/ml). SDS-PAGE was
performed using a mini-gel system (Mini-PROTEAN II, Bio-Rad, Mississauga, ON) and the discontinuous buffer system described by Laemmli (Laemmli, 1970). Stacking and resolving gels were 4 and 10% acrylamide, respectively. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (~45 min). Electrophoretic transfer of resolved proteins to nitrocellulose was carried out, using a buffer of 15.7 mM Tris, 120 mM glycine (pH 8.3) containing 20% (v/v) methanol, for 60 min at 100 V using a mini Trans-Blot transfer cell (Bio-Rad, Mississauga, ON). Nitrocellulose was either stained for protein for 5 min using 0.1% amido black 10B in 45% (v/v) methanol, 10% (v/v) acetic acid and then destained using 70% (v/v) methanol, 2% (v/v) acetic acid or used for antibody development.

The subsequent steps were conducted at room temperature with constant shaking. Before exposure to antiserum, the nitrocellulose was blocked by the blocking buffer (10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 2.5% (w/v) casein, and 0.02% (w/v) thimerosal) for 2 hr. The blocked nitrocellulose was incubated overnight with anti-trimethoprim-NAC-KLH antiserum diluted in the ELISA wash buffer. Unbound antibodies were removed by washing the nitrocellulose in the ELISA wash buffer (3 x 10 min). The nitrocellulose was subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chain) antiserum (diluted 1:5,000 in the ELISA wash buffer). The nitrocellulose was washed with Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 154 mM NaCl, 3 x 5 min). The nitrocellulose sheets was then incubated in Super Signal ECL reagents for 5 min, and the bound antibodies were visualized by exposing the nitrocellulose to ECL film under safe-light condition.
2.3.14 Dosing of rats with trimethoprim \textit{in vivo}.

Female Sprague Dawley rats (about 200 g) were obtained from Charles River Canada and housed in standard cages with free access to water and powdered rodent laboratory chow 5001 (Agribrands Purina, Woodstock, Ontario). After a one-week acclimation period, during which food consumption was monitored (~10g/rat/day), the rats were either continued on the powdered lab chow diet (controls) or switched to a diet in which trimethoprim was mixed with the powdered lab chow such that the rats had an intake of 100 mg of trimethoprim/kg/day (treated). Rats were fed with control or trimethoprim-containing diet continuously for a period of 8 weeks. At the end of the study, rats were killed by i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). The femurs were removed, and the bone marrow was collected into RPMI 1640 culture medium (Media Services, University of Toronto). After centrifugation at 500 x g for 25 min, bone marrow cells were collected as a pellet and the supernatant was discarded. Contaminating erythrocytes were lysed by a 0.2% NaCl solution (10 mL). Isotonicity was restored after 1 min by adding an equal volume of 1.6% NaCl solution. The cells were then centrifuged at 350 x g for 5 min, and the cell pellet was washed twice with HBSS without phenol red. Bone marrow cells were finally lysed in cell lysis buffer, and protein concentration was determined, and the samples were diluted with cell lysis buffer to give a protein concentration of 3 mg/mL. An equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/mL) was then added. The sample was boiled at 100 °C for 10 min before loading on SDS-PAGE.
2.3.15. Protein concentration measurement.

Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.
2.4 Results.

2.4.1 Metabolism of trimethoprim by neutrophils and microsomes in the presence of NAC.

Trimethoprim was metabolized by PMA-activated human neutrophils at pH 7.4. In the presence of NAC, three TMP-NAC adducts with protonated molecular ions at m/z 452 were detected using LC/MS in the SIM mode. The major TMP-NAC adduct had a HPLC retention time of 23 min, while the two minor adducts had retention times of 7.5 min and 8.5 min, respectively (Figure 2-1). No significant metabolism was detected in the control experiment in which the cells were not activated. The approximate ratio of the three TMP-NAC adducts was 17:1:1.

Upon incubation of trimethoprim with human-liver microsomes in the presence of NAC and a NADPH generating system at pH 6.8, three TMP-NAC adducts with protonated molecular ions of m/z 452 were again observed using LC/MS in the SIM mode (Figure 2-2a). The three TMP-NAC adducts showed the same HPLC retention times as those obtained using activated neutrophils. Similar results were obtained when rat-liver microsomes were used (Figure 2-2b). While the peak with a retention time of 23 min remained dominant, the approximate ratio of the three TMP-NAC adducts generated in human liver microsome system was 5:1:1, while the ratio in the rat liver microsome system was 4:1:1.

With CID mass spectrometry, the two minor TMP-NAC adducts with equal intensities gave identical patterns of molecular ion fragmentation (Figure 2-3a). The fragment ions were at m/z 258 (15%; MH⁺ - C₅H₈O₂NS - 2CH₃), 289 (100%; MH⁺ -
C₅H₅O₃NS - H), 308 (15%; MH⁺ - C₅H₆O₂N - CH₃ + H) and the parent ion at m/z 452 (20%; MH⁺). Under similar CID conditions the major TMP-NAC adduct with a retention time of 23 min showed fragmentation ions at m/z 155 (80%; MH⁺ - C₅H₅O₂N - C₉H₁₁O₂), m/z 181 (25%; MH⁺ - C₅H₆O₃NS - C₄H₄N₄ - H), m/z 290 (20%; MH⁺ - C₅H₆O₃NS), m/z 323 (100%; MH⁺ - C₅H₆O₃N + H) and the parent ion at m/z 452 (50%; MH⁺; Figure 2-3b). The major fragment ions of the two minor TMP-NAC adducts (m/z 289) were generated from losing the whole NAC moiety while the major fragment ion of the major TMP-NAC adduct (m/z 323) was generated from losing the NAC moiety with sulfur still attached to trimethoprim. This suggested that in the major TMP-NAC adduct the NAC was bound to the pyrimidine ring, and the minor adducts were diastereoisomers of TMP-NAC with NAC attached to the methylene carbon bridging the pyrimidine and phenyl rings.

2.4.2 Oxidation of trimethoprim by hypochlorous acid.

Trimethoprim was readily oxidized by hypochlorous acid. The data from diode array spectrophotometry showed an increased absorption at 320 nm immediately after addition of hypochlorite. The absorption increased to a maximum 40 sec after adding hypochlorite and then decreased with a half-life of ~1.5 min (Figure 2-4). When the reaction mixture of trimethoprim and hypochlorite was directly analyzed by mass spectrometry, a small but significant iminoquinone methide ion peak (MH⁺ 289) was observed (Figure 2-5). When the reaction mixture was analyzed by LC/MS with a mobile phase of water:acetonitrile:acetic acid (90:10:1, v/v), stable products were found with
protonated molecular ions of m/z 307 (TMP + OH), MH⁺ 325 (TMP + Cl), MH⁺ 343 (TMP + OH + Cl), MH⁺ 377 (TMP + OH + 2Cl), which were presumably due to reactions of the pyrimidine iminoquinone methide with chloride ion and/or water (Figure 2-6). In the presence of NAC, the reactive iminoquinone methide could be trapped as the same set of three NAC adducts of m/z 452 and the ratio was pH dependent. At pH 7.0 the ratio of the NAC adducts with retention times of 23 min, 8.5 min, and 7.5 min was approximately 1.1:1:1 (Figure 2-7a). At low pH, the major NAC adducts were those with retention times of 8.5 min and 7.5 min, while at high pH, the NAC adduct with a retention time of 23 min became dominant (Figure 2-8).

2.4.3 ¹H NMR of the major TMP-NAC adduct.

The major TMP-NAC adduct with a HPLC retention time of 23 min was purified as described above. The proton NMR spectrum of this major NAC adduct consisted of peaks with δ 1.81 ppm (3H, s), δ 3.34 ppm (1H, dd, J₁=14.4Hz, J₂=9.1Hz), δ 3.70 ppm (1H, dd, J₁=14.4Hz, J₂=3.9Hz), δ 3.73 ppm (3H, s), δ 3.79 ppm (6H, s), δ 3.83 ppm (1H, s), δ 3.85 ppm (1H, s), δ 4.38 ppm (1H, dd, J₁=9.1Hz, J₂=3.9Hz), δ 6.53 ppm (2H, s) (Figure 2-9). The three doublet of doublet peaks were attributed to the two diastereotopic protons and the chiral proton of NAC. Compared with the spectrum of trimethoprim, the proton on the heterocyclic ring of the major NAC adduct is missing. In addition, the two previously equivalent protons of the exo-cyclic methylene group in trimethoprim became diastereotopic due to the presence of chiral NAC, and they gave two close, but separated peaks at δ 3.83 ppm and δ 3.85
ppm, respectively. This supported the assignment of the major NAC adduct's structure in which the NAC was attached to the pyrimidine ring of trimethoprim shown as adduct C in Figure 2-13.

2.4.4 Oxidation of trimethoprim by the MPO enzyme system.

Trimethoprim was oxidized by MPO/H₂O₂/Cl⁻ in a similar manner as by HOCl. In the presence of NAC, the same three NAC adducts were also observed by LC/MS in the SIM mode. The ratio of the NAC adducts was about 0.4:1:1 (Figure 2-7b). In the absence of NAC, one of the stable chlorinated metabolites with a MH⁺ ion of m/z 325 (TMP+Cl⁻) was also observed as the major product by LC/MS with a mobile phase of water:acetonitrile:acetic acid (90:10:1, v/v) (data not shown). The NAC adducts were not detected in the control experiments when hydrogen peroxide was replaced by water.

2.4.5 Induction of anti-trimethoprim-NAC-KLH antiserum.

As the trimethoprim-NAC adduct formed, NAC introduced a carboxyl group on trimethoprim. Using a routine EDC coupling reaction, the trimethoprim-NAC adduct was coupled onto the carrier proteins (KLH and RSA) via the amino residues on the proteins (Figure 2-10). After the trimethoprim-NAC-modified KLH was injected to a rabbit, antibodies were raised in the rabbit against the immunogen. Trimethoprim-NAC-RSA adduct was used as an artificial antigen to determine the specificity of the resultant antiserum. The specificity of the antibodies against the trimethoprim epitope was demonstrated by ELISA, which showed that the
antiserum recognized the trimethoprim-NAC-RSA adduct but not the native RSA (Figure 2-11).

2.4.6 Covalent binding of trimethoprim to human neutrophils in vitro and to rat bone marrow in vivo.

Using the anti-trimethoprim-NAC-KLH antiserum, immunoblot detected covalent binding of trimethoprim (17 µM) to PMA-activated human neutrophils in vitro. The covalent binding was not detected in the controls, which contained no trimethoprim or no PMA. The trimethoprim-bound polypeptides showed molecular mass of 46, 49, and 58 kDa (Figure 2-12a). Using the antiserum, it was possible to show that trimethoprim becomes covalently bound to the bone marrow of the drug-treated (100 mg/kg/day) rats in vivo. The major modified polypeptides showed similar molecular mass as in vitro with human neutrophils. It was also possible to inhibit binding of the antiserum to the trimethoprim-modified polypeptides by preincubating it with the major trimethoprim-NAC adduct (50 µM) before adding the antiserum to the nitrocellulose (Figure 2-12b).
2.5 Discussion.

Trimethoprim was metabolized by both activated human neutrophils and hepatic microsomes to a reactive intermediate. The intermediate proved to be electrophilic and reacted with sulfhydryl-containing nucleophiles, such NAC, to give three adducts with protonated molecular ions of m/z 452. The CID mass spectra of the NAC adducts and the NMR spectrum of the major adduct provide strong evidence of the assigned structures shown in Figure 2-13.

The two trimethoprim-NAC adducts (NAC adducts A and B) with shorter retention times on HPLC (7.5 min and 8.5 min) show an identical CID product ion spectrum (Figure 2-3a). The major fragment is at m/z 289, which corresponds to the cleavage of the exo-cyclic methylene carbon-sulfur bond and the loss of the attached NAC group along with its sulfur atom. The LC/MS peaks of these two trimethoprim-NAC adducts are of equal intensity. Together, these data strongly support the assertion that the two trimethoprim-NAC adducts with shorter HPLC retention times are diastereomers generated by the attack of chiral NAC on the prechiral exo-cyclic carbon of an iminoquinone methide intermediate.

The major trimethoprim-NAC adduct formed in both the neutrophil and microsome systems with a longer retention time on HPLC (23 min, NAC adduct C) gives very different CID molecular ion fragments (Figure 2-3b). The major MS/MS fragment is at a m/z of 323, which is generated by losing the NAC moiety except for the sulfur atom. This major fragment is able to further lose the trimethoxyphenyl moiety to give the second most abundant fragment at m/z 155. The proton NMR
spectrum of the trimethoprim-NAC adduct C (Figure 2-9) confirms the proposed structure. Compared with the proton NMR spectrum of trimethoprim itself (data not shown), the two protons of *exo*-cyclic methylene group become diastereotopic due to the presence of chiral NAC, and they now give two peaks with slightly different chemical shifts (δ 3.83 ppm and δ 3.85 ppm, respectively), and the proton on the heterocyclic ring is missing.

The formation of this major NAC adduct (NAC adduct C) produced by the neutrophil and liver microsome oxidation systems was also attributed to the iminoquinone methide intermediate. Unlike quinone methides generated from phenolic compounds, which preferentially react with nucleophiles at the *exo*-cyclic methylene carbon (Bolton *et al.*, 1997), the major site of reaction for the iminoquinone methide of trimethoprim was on the ring. However, the ratio of cyclic to *exo*-cyclic products was pH dependent. At low pH, the protonated iminoquinone methide may exist partially as the *exo*-cyclic carbocation leading to more *exo*-cyclic adduct. At high pH, the iminoquinone methide of trimethoprim predominantly reacts with nucleophiles (i.e. NAC) on the pyrimidine ring. This phenomenon is consistent with the reactivities of heterocyclic aromatic compounds in which nucleophilic additions often occur preferentially in the 2 and 4 positions relative to the hetero atom because of its electron-withdrawing effect. Figure 2-13 summarizes the proposed pathway for the bioactivation of trimethoprim and the formation of NAC adducts.

Trimethoprim is also oxidized by HOCl and MPO/H₂O₂/Cl⁻ in a similar manner as by neutrophils and liver microsomes. The production of the same set of
NAC adducts implies that the same iminoquinone methide intermediate is involved. The pyrimidine iminoquinone methide is presumably formed from one of the possible chloramines. Upon addition of HOCl, trimethoprim gives a new UV absorption peak with a λ_{max} at approximately 320 nm. This new peak increases to maximum intensity in 40 seconds, which presumably represents the formation of the iminoquinone methide intermediate. The half-life of the iminoquinone methide, as determined by UV spectrometry, is about 1.5 min, which is considerably shorter than many quinone methides generated from phenolic compounds; thus, it may be much more reactive than most quinone methides in vivo. In the absence of nucleophilic trapping agents, the pyrimidine iminoquinone methide reacts with water and/or chloride to produce several relatively stable products including α-hydroxytrimethoprim (m/z 307) (Figure 2-6). This α-hydroxytrimethoprim has been identified as one of the major metabolites of trimethoprim both in vivo (Meshi & Sato, 1972) and in vitro (van't Klooster et al., 1992). It could be formed by the direct oxidation of trimethoprim by cytochrome P450, or it could come from the reaction of the quinone methide metabolite with water.

The short-lived pyrimidine iminoquinone methide intermediate can also be detected by MS in the flow system (Figure 2-5). The combination of evidence from the UV spectrum, mass spectrum and the structures of the NAC adducts formed by trapping the reactive intermediate strongly support the formation of a pyrimidine iminoquinone methide from trimethoprim as proposed.

The reactivity of the pyrimidine iminoquinone methide toward sulfhydryl nucleophiles suggests binding of this reactive metabolite to sulfhydryl-containing
cellular proteins. Using antiserum developed against trimethoprim-NAC-KLH, we are able to show that trimethoprim can covalently modify PMA-activated human neutrophils in vitro. Since trimethoprim-modified polypeptides are not detected in the controls when the drug is absent or neutrophils are not activated, it is conceivable that such a modification in activated neutrophils is due to the reactive iminoquinone methide metabolite. The modified polypeptides include those with molecular mass of 49 and 58 kDa, which is consistent with the result obtained when human neutrophils are covalently modified by the reactive nitrenium metabolite of clozapine (Gardner et al., 1998a). Using the immunochemical techniques, we also demonstrate the covalent binding of trimethoprim to bone marrow cells of the drug-treated rats (Figure 2-12). MPO is a covalently linked homodimer of 140 kD. Each half of the dimer consists of a 58-kD and a 13-kD subunit. There is one free cysteine residue on the 58-kD subunit of MPO (Andrews & Krinski, 1981; Zeng & Fenna, 1992). Although formation of reactive metabolite is responsible for such a covalent binding, at the current stage it is still speculative that MPO is covalently modified by trimethoprim in vivo.

It has been demonstrated that cytochrome P-450 enzymes or peroxidases can oxidize phenolic compounds with appropriate alkyl substituents in the para-position to quinone methides (Peter, 1989; Thompson et al., 1993). The formation of quinone methides has been linked to many adverse reactions, including hepatotoxicity, pulmonary toxicity and carcinogenicity (Guyton et al., 1993; Mayalarp et al., 1996; Mizutani et al., 1983; Takahashi, 1988; Thompson et al., 1998). We have demonstrated the formation of a pyrimidine iminoquinone methide from
trimethoprim, a commonly used antibacterial agent, which is associated with infrequent, but sometimes serious, idiosyncratic agranulocytosis and hepatotoxicity. Although the mechanism responsible for drug-induced agranulocytosis is not well known, it has been shown that most drugs associated with a high incidence of agranulocytosis are also oxidized to reactive intermediates by activated neutrophils (Uetrecht, 1992; Uetrecht et al., 1994). The formation of the trimethoprim iminoquinone methide by activated neutrophils is consistent with this pattern. Therefore, we propose that the iminoquinone methide formed by neutrophils, or neutrophil precursors in the bone marrow that contain myeloperoxidase, is responsible for trimethoprim-induced agranulocytosis. Likewise, the formation of the pyrimidine iminoquinone methide by hepatic cytochrome P450 is likely responsible for trimethoprim-induced hepatotoxicity. Although, the pyrimidine quinone methide may also be responsible for other trimethoprim-induced idiosyncratic drug reactions, the relevant site of formation for these reactions is more speculative.
Figure 2-1. Selective ion monitoring of TMP-NAC adducts generated by PMA-activated human neutrophils at pH 7.4. The mass spectrum was obtained in the IonSpray mode using LC/MS at a flow rate of 200 μL/min. The concentrations for substrates were 5 μM for TMP and 5 mM for NAC.
Figure 2-2. Selective ion monitoring of TMP-NAC adducts generated by a) isolated human-liver microsomes and b) rat-liver microsomes in presence of a NADPH generating system at pH 6.8. The mass spectrum was obtained in the IonSpray mode using LC/MS at a flow rate of 200 μL/min. The concentration of TMP was 0.4 mM and that of NAC was 5 mM.
Figure 2-3. a) CID spectra of TMP-NAC adduct A, with a HPLC retention time of 7.5 min. b) CID spectrum of TMP-NAC adduct C with HPLC retention time of 23 min.
Figure 2-4. Repetitive absorption spectra from the reaction of trimethoprim with hypochlorous acid at pH 6. The dotted curve is the UV spectrum of trimethoprim, and the dashed curve is the first UV spectrum of the reaction. Integration time and total run time were 0.5 and 180 sec, respectively. The spectra were plotted with a 20-sec interval. The concentration of trimethoprim was 0.5 mM and that of hypochlorous acid was 1 mM.
Figure 2-5. Mass spectra of the reactive pyrimidine iminoquinone methide intermediate produced by the reaction of trimethoprim with HOCl obtained in the IonSpray mode. a) ES/MS spectrum of the iminoquinone methide obtained with computer extraction of the ion at m/z 289. b) Mass spectrum of the reaction mixture containing both the iminoquinone methide (m/z 289) and unreacted trimethoprim (m/z 291).
Figure 2-6. LC/MS spectra of the stable products of trimethoprim oxidation by HOCl when the reaction mixture was allowed to stand at 25°C without addition of trapping agents. The spectra were obtained with computer extraction of the ions at m/z 307, m/z 325, m/z 343 and m/z 377. The reaction was carried out in 0.1 M pH 6 phosphate buffer at 25°C, and the concentrations of trimethoprim and HOCl were 1 mM and 2 mM, respectively. The HPLC mobile phase was water:acetonitrile:acetic acid (90:10:1) with 2 mM ammonium acetate. The number in the upper right hand corner of each trace is the total ion current for that ion.
Figure 2-7. Selective ion monitoring of TMP-NAC adducts generated by: a) HOCl in 0.1M pH 7.0 phosphate buffer at 25°C; b) MPO in presence of H₂O₂ in 0.1 M pH 7.0 phosphate-buffered saline. The concentrations for substrates were 1 mM for TMP, 2 mM for HOCl, 1 unit for MPO, 2 mM for H₂O₂ and 10 mM for NAC. The mass spectra were obtained in the IonSpray mode using LC/MS at a flow rate of 200 μL/min.
Figure 2-8. LC/MS-SIM ion currents of TMP-NAC adducts when TMP was oxidized by HOCl in presence of NAC at different pHs. The concentration for reactants were 1 mM for TMP, 2 mM for NaOCl and 10 mM for NAC.
Figure 2-9. $^1$H NMR spectrum of the major TMP-NAC adduct (NAC adduct C), which has a retention time of 23 min on HPLC.
Figure 2-10. Formation of the trimethoprim-NAC-protein adduct.
Figure 2-11. ELISA analysis showing binding of the anti-trimethoprim-NAC-KLH antiserum to wells of microtiter plates coated with the trimethoprim-NAC-RSA adduct (circles) or native RSA (squares).
Figure 2-12. (a) Immunochemical detection of covalent binding of trimethoprim (TMP) to PMA-activated human neutrophils in vitro. Neutrophils were incubated with (+) or without (-) the drug in the presence (+) or absence (-) of PMA under the condition described in the Methods section. (b) Immunochemical detection of covalent binding of trimethoprim (TMP) to bone marrow cells of the drug-treated rats (+) in comparison with the controls (-). The blots were incubated with the anti-trimethoprim-NAC-KLH antiserum alone (CONT) or with antiserum that was preincubated with the trimethoprim-NAC adduct (TMP-NAC, 50 μM). The anti-trimethoprim-NAC-KLH antiserum dilution was at 1:3,000 and the secondary goat anti-rabbit antiserum dilution was at 1:5,000. Protein loading was 30 μg/lane.
Figure 2-13. Proposed pathway for the bioactivation of trimethoprim to a reactive pyrimidine iminoquinone methide intermediate and its reactions with NAC and protein.
Chapter 3

Bioactivation and Covalent Binding of Hydroxyfluperlapine in Human Neutrophils: Implications for Fluperlapine-induced Agranulocytosis.
3.1 Abstract.

The use of fluperlapine and the structurally related clozapine have been associated with the induction of agranulocytosis in humans. Unlike clozapine, fluperlapine is relatively resistant to chemical and biochemical oxidations. In this study we demonstrated that 7-hydroxyfluperlapine, the major metabolite of fluperlapine in humans, is oxidized to a reactive intermediate by hypochlorous acid (HOCI) and by myeloperoxidase (MPO) in the presence of hydrogen peroxide (H₂O₂) and chloride (Cl⁻). This reactive intermediate was identified as an iminoquinone species with a M+1 ion at m/z 324 by mass spectrometry. The iminoquinone intermediate was trapped by N-acetyl-L-cysteine (NAC) as well as glutathione (GSH). Nuclear magnetic resonance (NMR) spectra of the NAC adducts indicated that the NAC was bound to the 6 and 9 positions of the aromatic ring. This is the same orientation as the binding of nucleophiles to the reactive metabolite of clozapine. We were able to use an antibody against clozapine to demonstrate that 7-hydroxyfluperlapine, but not fluperlapine itself, covalently modifies human MPO. Furthermore, we demonstrated that 7-hydroxyfluperlapine is metabolized by activated neutrophils to a reactive intermediate that covalently binds to neutrophils. In the presence of NAC or GSH, such covalent binding was inhibited and the NAC or GSH adducts were formed. Thus, the reactivity and even the orientation of the binding of the reactive metabolite of 7-hydroxyfluperlapine is very similar to that of clozapine. These results provide a mechanism for the
formation of a reactive metabolite of fluperlapine similar to clozapine that may explain its ability to induce agranulocytosis.
3.2 Introduction.

Clozapine (Figure 3-1) is an atypical antipsychotic agent that has been shown to be effective in the treatment of refractory schizophrenia. Therapeutically the use of clozapine is limited because it causes agranulocytosis in approximately 0.8 - 1.0 % of patients treated with the drug (Alvir et al., 1993; Krupp & Barnes, 1989). The mechanism underlying clozapine-induced agranulocytosis is at present undefined, but toxic (Veys et al., 1992; Williams et al., 1997) and immunological mechanisms (Pisciotta et al., 1992) have been proposed. We favor an innate immune mechanism (Guest et al., 1998; Uetrecht, 1999) but the formation of a reactive metabolite is likely to be the initial step in all of these proposed mechanisms.

Clozapine is oxidized to a reactive nitrenium ion by HOCI (the major oxidant of activated human neutrophils; Uetrecht, 1992), by MPO/H2O2/Cl- and by activated human neutrophils (Liu & Uetrecht, 1995) and bone marrow cells (Maggs et al., 1995). This nitrenium ion of clozapine covalently binds to human neutrophils (Gardner et al., 1998a; Liu & Uetrecht, 1995) and bone marrow cells (Maggs et al., 1995), and it is cytotoxic to human neutrophils in vitro (Gardner et al., 1998b; Williams et al., 1997). In fact, the ability to form reactive intermediates in the presence of HOCl, MPO/H2O2/Cl- and activated human neutrophils is one property shared by most drugs that cause a high incidence of idiosyncratic agranulocytosis (Uetrecht, 1992).

Due to the problems associated with the clinical use of clozapine a number of pharmaceutical companies have tried to develop antipsychotic agents that have a
similar therapeutic profile as clozapine but do not cause agranulocytosis. One compound that showed early promise in this regard was the structural analogue, fluperlapine (Figure 3-1). Unlike clozapine, fluperlapine is relatively resistant to electrochemical and peroxidase-catalyzed oxidation (Liegeois et al., 1995). Furthermore the carbon for nitrogen substitution at position 5 means that fluperlapine cannot form a reactive nitrenium ion analogous to that seen with clozapine. Thus, from a chemical oxidation standpoint fluperlapine would appear to be a good choice for development as a drug candidate as it would not be expected to form reactive metabolites. However, in clinical trials fluperlapine appeared to induce agranulocytosis in humans and development of the drug was stopped, although the cases were not well documented in the literature (Mann et al., 1987; Muller-Oerlinghausen, 1984). Even though fluperlapine is resistant to chemical and peroxidase oxidation it is extensively metabolized by the cytochromes P450 system of both rats and humans (Dain & Jaffe, 1988; Fischer et al., 1992; Fischer & Wiebel, 1990; Guillouzo et al., 1988; Figure 3-2). In humans, the major metabolite formed is 7-hydroxyfluperlapine. In this work we show that 7-hydroxyfluperlapine, unlike the parent fluperlapine, is readily oxidized by HOCl, MPO/H₂O₂/Cl⁻ or by activated human neutrophils to form a reactive iminoquinone species which leads to the formation of NAC or GSH adducts and binds to human neutrophils in vitro. Thus, bioactivation of 7-hydroxyfluperlapine may be involved in the mechanism of fluperlapine induced-agranulocytosis.
3.3 Materials and Methods.

3.3.1 Materials.

Clozapine, fluperlapine and 7-hydroxylfluperlapine were generous gifts from Novartis Pharmaceuticals. H$_2$O$_2$ was purchased from ACP Chemicals (Montreal, QC). Sodium hypochlorite (NaOCl) was purchased from Aldrich Chemical Company (Milwaukee, WI). MPO were obtained from Cortex Biochemical (San Leandrow, CA). NAC, GSH, casein and thimerosal were purchased from Sigma Chemical Co. (St Louis, MO). Nitrocellulose and stock acrylamide solution (40%) were purchased from Bio-Rad (Mississauga, ON). Enhanced chemiluminescence film was purchased from Amersham Canada (Oakville, ON) and the enhanced chemiluminescence detection reagents were purchased from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase-conjugated goat-anti-rabbit IgG (H+L chains) was purchased from Zymed (San Francisco, CA). The concentration of NaOCl was determined spectrophotometrically (Hussarin et al., 1970). One unit of MPO activity was defined as the amount of enzyme that decomposed 1 μmol of H$_2$O$_2$ per min at 25°C and pH 6.

3.3.2 Analytical.

HPLC analyses were carried out on a Shimadzu HPLC system including a SPD-6A UV detector set at 254 nm (Shimadzu Corporation, Kyoto, Japan). The chromatography columns were supplied by Phenomenex (Torrance, CA). The column used for analytical work was 2 x 100 mm with a 2 x 30 mm guard column
and packed with 5 µm Prodigy ODS(3). The column used for isolation of the NAC adducts was 10 x 150 mm with a 10 x 60 mm guard column and packed with 5 µm Ultracarb ODS 30. A mobile phase of water:acetonitrile (80:20, v/v) with 5 mM ammonium acetate was used unless otherwise stated.

Liquid chromatography interfaced with mass spectrometry (LC/MS) and tandem mass spectrometry (LC/MS/MS) were performed on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Thornhill, ON) with an IonSpray interface. Analyses were carried out with an ionizing voltage of 5000 V and orifice voltage of 65 V. ¹H and ¹⁹F NMR spectra were recorded with a Varian Unity Plus 500 spectrometer (Varian Associates, Palo Alto, CA) using deuterium-substituted dimethyl sulfoxide (DMSO-d₆) as the solvent. The ultraviolet and visible spectrophotometry was carried out on a Hewlett Packard diode-array instrument (HP8452A, Hewlett Packard Company, Palo Alto, CA).

3.3.3 Oxidation of 7-hydroxyfluperlapine by HOCl.

7-Hydroxyfluperlapine (1mM in 60:40 ethanol water containing 0.2% v/v acetic acid, 99 ml) was reacted with an aqueous solution of NaOCl (100 mM, 1 µl). This reaction mixture (5 µl) was immediately injected into the mass spectrometer. Methanol was used as the mobile phase, and the flow rate was 200 µl/min and decreased to 20 µl/min with a splitter.

The oxidation of 7-hydroxyfluperlapine was also monitored by the ultraviolet (UV) spectrophotometer. An ethanolic solution of 7-hydroxyfluperlapine (10 mM, 20 µl) and an aqueous solution of NaOCl (10 mM, 20 µl) were added to phosphate
buffer (0.1 M, pH 6.0, 1960 µl) in a quartz cuvette with rapid stirring by a micro
magnetic stirring bar. The reaction mixture was immediately scanned by the
spectrophotometer at 2-s intervals for 30 s over a wavelength range of 200 to 600 nm.

3.3.4 Oxidation of 7-hydroxyfluperlapine by the MPO enzyme system.

7-Hydroxyfluperlapine in ethanol (10 mM, 20 µl) was added to phosphate-
buffered saline (PBS) (0.1 M, pH 7.0, 150 mM NaCl, 115 µl). MPO (5 units) and NAC
in 0.5 M pH 8.5 phosphate buffer (100 mM, 40 µl) were also added. The reaction was
initiated by addition of H₂O₂ (80 mM, 20 µl). The mixture was incubated at room
temperature for 30 min, before being analyzed using HPLC and LC/MS. In the
control experiments H₂O₂ was replaced by water.

3.3.5 Preparation of the NAC adducts of the reactive intermediate for NMR studies.

7-Hydroxyfluperlapine (10 mg, 0.031 mmol) was dissolved in ethanol (3.1 ml)
and added to PBS (0.1 M, pH 6.5, 17.6 ml) in a 50-ml Erlenmeyer flask. MPO (50
units) and NAC (100 mg, 0.62 mmol) in phosphate buffer (0.5 M, pH 8.5, 17.6 ml)
were then added. The reaction was started by addition of H₂O₂ (80 mM, 3.1 ml) and
stirred at room temperature for 5 hours. The solvents were then removed by rota-
evaporation, and the residue was redissolved in water. The aqueous sample was
applied to a Supelco C-18 cartridge (Supelco Inc., Bellefonte, PA), then rinsed with
water, and eluted using methanol. The methanol fraction of the extraction was
collected and concentrated. The products were further purified by HPLC with a
mobile phase of water:acetonitrile (85:15, v/v) with 5 mM ammonium acetate and a
flow rate of 5 ml/min. The fractions with retention times of 17 and 40 min were collected separately. HPLC analysis showed the purity of the final products was more than 95%.

3.3.6 Human leukocyte isolation.

Neutrophils and peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers as described previously in Chapter 2 (section 2.3.3). Trypan blue exclusion showed the initial viability to be more than 98% for all preparations. Cytospin slides were prepared and stained with Wright’s stain. Light microscopy, confirmed that > 90% of the cells had characteristic neutrophil morphology.

3.3.7 Incubation of neutrophils with fluperlapine, 7-hydroxyfluperlapine and clozapine.

Neutrophils, 5 x 10⁶ in 1 ml Hanks' balanced salt solution (HBSS), were incubated with various concentrations of fluperlapine (0 - 50 µM), 7-hydroxyfluperlapine (0 - 50 µM) and clozapine (0 - 8 µM) at 37°C for 1 hr in a shaking water bath in the presence or absence of phorbol myristate acetate (PMA; 40 ng/ml). PMA was dissolved in dimethyl sulfoxide (DMSO) and control incubations received an equal volume of DMSO (5 µl/ml). In some experiments the ability of NAC, GSH or N-acetyl-L-lysine (5 mM) to inhibit covalent binding of reactive metabolites to neutrophils was examined. At the end of the incubation period, cells were pelleted by centrifugation (500 g, 5 min) and resuspended in cell lysis buffer (10
mM tris(hydroxymethyl)aminomethane partially neutralized with HCl (Tris-HCl), pH 7.4; 1 mM ethylenediaminetetraacetic acid; 0.2% Triton X-100; 100 µl). A portion of the lysed sample was used for the determination of protein concentrations and the remainder of the sample was diluted with an equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml). The samples were then boiled at 100°C for 10 min prior to loading on the gel.

After the incubation of 7-hydroxyfluperlapine (50 µM) and neutrophils (5 x 10⁶) in presence of NAC or GSH (5mM), the supernatants were collected and solvents were removed by a stream of nitrogen at 25°C. The samples were redissolved in water and analyzed by LC/MS using selective ion monitoring (SIM) at m/z 487 for NAC adducts and at m/z 631 for GSH adducts. LC/MS/MS spectra of the NAC and GSH adducts were also obtained under appropriate collision-activated dissociation (CID) conditions.

3.3.8 Covalent binding of fluperlapine and 7-hydroxyfluperlapine to human MPO.

Fluperlapine or 7-hydroxyfluperlapine (0 - 50 µM; 0.1 ml) was incubated with MPO (1 or 5 units) in the presence of H₂O₂ (100 µM). After incubation for 30 min at 37°C the reaction was stopped by cooling the sample on ice and an equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was added. The samples were then boiled at 100°C for 10 min before analysis by SDS-PAGE.

3.3.9 Production of anti-clozapine-NAC-KLH antiserum.
The polyclonal anti-clozapine-NAC-KLH antiserum used in these studies was raised as described previously (Gardner et al., 1998a). As well as recognizing clozapine this antiserum has been shown by immunoblot inhibition experiments to recognize structural analogues of clozapine such as olanzapine. Similar hapten inhibition experiments (see below for details) were performed with fluperlapine and hydroxyfluperlapine to ensure that the antiserum would also recognize these two structural analogues.

3.3.10 Hapten inhibition experiments.

To produce clozapine-modified neutrophils for use in the hapten inhibition experiments the following method was used. Clozapine (20 μM in PBS pH 6.0) was reacted with NaOCl (18 μM) to generate clozapine reactive metabolite and human neutrophils (5 x 10⁶ in 1ml HBSS) were added immediately to the reactive metabolite solution as described (Gardner et al., 1998a). After incubation at 37°C for 15 minutes the neutrophils were collected by centrifugation and lysed in cell lysis buffer. A portion of the lysed sample was used for the determination of protein concentrations and the remainder of the sample was diluted with an equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml). The samples were then boiled at 100°C for 10 min prior to loading on the gel.

3.3.11 SDS-PAGE and immunoblotting.

Samples for analysis by SDS-PAGE were solubilized by boiling for 10 min in sample buffer (8% (w/v) SDS, 20% (v/v) glycerol, 0.002% bromophenol blue, 125
mM Tris-HCl (pH 6.8)) containing dithiothreitol (6 mg/ml). SDS-PAGE was performed using a mini-gel system (Mini-PROTEAN II, Bio-Rad, Mississauga, ON) and the discontinuous buffer system described by Laemmli (Laemmli, 1970). Stacking and resolving gels were 4 and 10% acrylamide, respectively. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (~45 min). Electrophoretic transfer of resolved proteins to nitrocellulose was carried out, using a buffer of 15.7 mM Tris, 120 mM glycine (pH 8.3) containing 20% (v/v) methanol, for 60 min at 100 V using a mini Trans-Blot transfer cell (Bio-Rad, Mississauga, ON). Nitrocellulose was either stained for protein for 5 min using 0.1% amido black 10B in 45% (v/v) methanol, 10% (v/v) acetic acid and then destained using 70% (v/v) methanol, 2% (v/v) acetic acid or used for antibody development. Comprehensive details of the immunoblotting methods used have been described previously in Chapter 2 (section 2.3.13).

In experiments to determine the specificity of the anti-clozapine-NAC-KLH antiserum, the antiserum (1:4,000 final dilution) was incubated with various concentrations (0 - 20 μM) of clozapine, fluperlapine, 7-hydroxyfluperlapine, mianserin and procainamide (see Figure 3-1 for structures) for 30 min prior to addition of the primary antiserum to the nitrocellulose membrane. Clozapine, fluperlapine and procainamide were dissolved in PBS; mianserin was dissolved in H₂O and 7-hydroxyfluperlapine was dissolved in ethanol. Control samples received an equivalent volume of ethanol. The washing and subsequent development of the immunoblots were then performed as outlined previously in Chapter 2 (section 2.3.13).
3.3.12 Protein concentration measurement.

Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.
3.4 Results.

3.4.1 Oxidation of 7-hydroxyfluperlapine by HOCl.

7-Hydroxyfluperlapine was easily oxidized by HOCl. Upon oxidation, a major reactive intermediate of 7-hydroxyfluperlapine was detected by mass spectrometry with a M+1 ion at m/z 324. This is 2 mass units less than the M+1 ion of the parent drug and is presumed to be the iminoquinone shown in Figure 3-3A. The mass spectrum of the iminoquinone species of 7-hydroxyfluperlapine obtained under collision-activated dissociation (CID) condition gave fragment ions at m/z 58 (85%; (CH₂)₂NHCH₃⁺), m/z 84 (70%; CH₂CHN(CH₃)CH₂CH₂⁺), m/z 101 (100%; CH₃N(CH²CH₂)₂NH₂⁺), m/z 226 (50%; MH⁺ - methylpiperazine), m/z 252 (50%; MH⁺ - (CH₂)₂N(CH²CH₂)), and m/z 267 (75%; MH⁺ - (CH₂)₂NCH₃) as well as the MH⁺ ion of the iminoquinone at m/z 324 (75%) (Figure 3-3B).

Upon addition of an equal equivalent of HOCl, 7-hydroxyfluperlapine there was an increase in absorptions at 308 and 420 nm, which then decreased rapidly (Figure 3-4). Analyzed by LC/MS, the resultant iminoquinone species showed reactivity toward both water and chloride ion forming stable products with molecular ions of m/z 342 and m/z 360, respectively. The stable product with m/z 360 contained the characteristic chlorine isotope peak at m/z 362.
3.4.2 Oxidation of 7-hydroxyfluperafine by MPO enzyme system and formation of 7-hydroxyfluperafine-NAC adducts.

7-Hydroxyfluperafine was extensively oxidized by human MPO in presence of H₂O₂ and Cl⁻. When NAC was also present in the reaction mixture, the reactive iminoquinone intermediate preferentially reacted with NAC rather than water or chloride, and subsequently it could be detected as 7-hydroxyfluperafine-NAC adducts. The LC/MS analysis of the reaction mixture showed two major peaks of the NAC adducts with the molecular ions of m/z 487 and retention times of 5.0 min and 6.0 min, respectively, whereas the stable water or chloride adducts were not detected (Figure 3-5). Each HPLC peak of NAC adduct contained a pair of diastereomers of that NAC adduct, and they were not separable by HPLC. In the control experiments, when H₂O₂ was replaced by water, the NAC adducts were not detected. The LC/MS/MS of the diastereomeric NAC adducts with a retention time of 5.0 min showed major CID fragments at m/z 84 (20%; CH₂CHN(CH₃)CH₂CH₂⁺), m/z 301 (60%; MH⁺ - C₅H₄O₂N - (CH₂)₂NCH₃ + H), m/z 358 (100%, MH⁺ - C₅H₄O₂N + H), and the parent ion at m/z 487 (50%; MH⁺). The LC/MS/MS of the diastereomeric NAC adducts with a retention time of 6.0 min showed major CID fragments at m/z 84 (25%; CH₂CHN(CH₃)CH₂CH₂⁺), m/z 301 (75%; MH⁺ - C₅H₆O₂N - (CH₂)₂NCH₃ + H), m/z 358 (100%, MH⁺ - C₅H₆O₂N + H) and the parent ion at m/z 487 (40%; MH⁺) (Figure 3-6A).

Each pair of the diastereomeric NAC adducts were isolated successfully using the procedure described in the methods section. The positions where NAC covalently modified hydroxyfluperafine were demonstrated by NMR spectroscopy.
In $^1$H NMR, the aromatic region of the diastereomeric NAC adducts with a retention time of 5.0 min showed peaks at $\delta$ 7.28 - 7.46 ppm (4H, m), and two doublet peaks at $\delta$ 6.77 ppm and $\delta$ 6.83 ppm that had the same coupling constant of $J = 9.5$ Hz, and together they integrated to 1H. The aromatic region of the $^1$H NMR of the diastereomeric NAC adducts with a retention time of 6.0 min consisted of peaks at $\delta$ 7.28 - 7.46 ppm (4H, m), and one doublet peak at $\delta$ 6.79 ppm (1H, d, $J = 12.5$ Hz). By comparison, the aromatic region of the $^1$H NMR of 7-hydroxyfluperlapine showed peaks at $\delta$ 7.26 - 7.44 ppm (4H, m), $\delta$ 6.76 ppm (1H, d, $J = 9.5$ Hz), $\delta$ 6.72 ppm (1H, d, $J = 12.5$ Hz) (Figure 3-7). For 7-hydroxyfluperlapine, the doublet peak at 6.76 ppm was due to H-6, whose coupling with $^{19}$F on its meta position was $J_{H,F} = 9.5$ Hz; the doublet peak at 6.72 ppm was due to H-9, whose coupling with the same $^{19}$F on its ortho position gave the coupling constant of $J_{H,F} = 12.5$ Hz. The two doublet peaks with the same coupling constants of 9.5 Hz given by the two diastereomers of NAC adduct with a retention time of 5.0 indicated that the NAC was bound to position 9 of 7-hydroxyfluperlapine. Similarly, the overlapping doublet peaks with the coupling constant of 12.5 Hz given by the two diastereomers of NAC adduct with a retention time of 6.0 min indicated that the NAC was bound to position 6 of 7-hydroxyfluperlapine. The assignment of binding positions of NAC in the NAC adducts was further supported by $^{19}$F NMR. The $^{19}$F in 7-hydroxyfluperlapine had a doublet of doublets at $\delta$ -139.35 ppm with $J' = 12.5$ Hz and $J'' = 9.5$ Hz, which was due to the coupling with H-9 and H-6, respectively. However, the $^{19}$F in the diastereomeric NAC adducts with a retention time of 5.0 min contained two doublet
peaks having the same coupling constant of $J_{H,F} = 9.5$ Hz at $\delta$ -130.75 ppm and $\delta$ -130.96 ppm, respectively; the $^{19}$F in the diastereomeric NAC adducts with a retention time of 6.0 min contained two doublet peaks having the same coupling constant of $J_{H,F} = 12.5$ Hz at $\delta$ -135.79 ppm and $\delta$ - 135.90 ppm, respectively. From $^{19}$F NMR, the loss of coupling with H-6 in the pair of diastereomers with a retention time of 5.0 min and the loss of coupling with H-9 in the diastereomers with a retention time of 6.0 min confirmed that NAC was bound to positions 6 and 9.

3.4.3 Metabolism of 7-hydroxyfluperlapine by human neutrophils in presence of NAC or GSH.

7-Hydroxyfluperlapine was metabolized by PMA-activated human neutrophils at pH 7.4. In the presence of NAC, two pairs of 7-hydroxyfluperlapine-NAC adducts with molecular ions of m/z 487 were also detected using LC/MS in the SIM mode (Figure 3-8A). Their LC/MS/MS spectra and HPLC retention times indicated that they were the same as those adducts obtained in MPO enzyme system. The NAC adducts showed retention times at 5.0 min and 6.0 min, with the approximate ratio of 1:2. The two diastereomeric adducts with NAC attached to position 6 remained dominant. When GSH was used instead of NAC, 7-hydroxyfluperlapine-GSH adducts with molecular ions of m/z 631 were detected using LC/MS in the SIM mode (Figure 3-8B). The GSH adducts also showed two major peaks with retention times of 3.2 min and 4.0 min, respectively. It is probable that each peak consisted of a pair of diastereomers, and the dominant pair had a retention time of 4.0 min and GSH substitution at position 6. However, the ratio of
the two pairs of diastereomers of GSH adducts was 1:3 in favor of the substitution on position 6. The LC/MS/MS of the adducts showed similar CID fragments with major peaks at m/z 301 (40%; 7-hydroxyfluperlapine + S - (CH₂)₂NCH₃ + H), m/z 358 (100%; 7-hydroxyfluperlapine + S + H), m/z 502 (20%; MH⁻ - C₅H₈O₃N), and the parent ion at m/z 631 (MH⁺; Figure 3-6B)

3.4.4 Recognition of fluperlapine by the anti-clozapine-NAC-KLH antiserum.

Hapten inhibition studies demonstrated that, in immunoblot experiments, the recognition of the clozapine reactive metabolite-modified neutrophil proteins by the antiserum could be completely inhibited by preincubation of the antiserum with clozapine, fluperlapine and 7-hydroxyfluperlapine at concentrations of 2 (Figure 3-9B) or 20 μM (Figure 3-9A). Mianserin, in which the piperazine is fused to the dibenzazapine ring, inhibited the antiserum binding completely at a concentration of 20 μM but only partially at the lower concentration (Figure 3-9). Procainamide, which lacks the piperazine ring but still has a tertiary amine group, was a much weaker inhibitor of antibody recognition. Using a wider range of fluperlapine concentrations, the IC₅₀ for inhibition of the antiserum binding was determined to be approximately 10 nM (data not shown). This is similar to that reported previously for olanzapine (Gardner et al., 1998a).
3.4.5 Immunoblotting of neutrophils exposed to fluperlapine, 7-hydroxyfluperlapine and clozapine in vitro.

When neutrophils were incubated with 7-hydroxyfluperlapine (0 - 50 μM) in the presence of PMA the metabolite became covalently bound to a number of neutrophil polypeptides (Figure 3-10). At concentrations of 2, 5 or 10 μM, a number of 7-hydroxyfluperlapine-modified polypeptides between 45 and 116 kDa were observed. At the highest concentration of 7-hydroxyfluperlapine used (20 μM), a wide range of neutrophil polypeptides became covalently modified. However, in the absence of PMA this binding was not observed. When clozapine (0.5 or 5 μM) was incubated with human neutrophils in the presence of PMA, the major polypeptides modified also had molecular masses of between 45 and 116 kDa but the pattern was somewhat different (Figure 3-10). Covalent binding of fluperlapine to human neutrophil polypeptides was not observed either in the presence or absence of PMA (Figure 3-10). The recognition of clozapine and 7-hydroxyfluperlapine-modified polypeptides by the antiserum could be inhibited by pre-incubating the antiserum with clozapine or hydroxyfluperlapine (Figure 3-11) indicating that this binding represented specific recognition of drug-modified polypeptides by the antiserum. Covalent binding of reactive metabolites of clozapine (Figure 3-12A) and hydroxyfluperlapine (Figure 3-12B) to PMA-activated human neutrophils in vitro could be inhibited by including the nucleophiles NAC or GSH in the incubation. No such inhibition was observed when N-acetyl-L-lysine was included in the incubation. In fact N-acetyl-L-lysine appeared to increase the binding which is difficult to explain.
3.4.6 Covalent binding of fluperlapine and 7-hydroxyfluperlapine to human MPO in vitro.

When human MPO was incubated with 7-hydroxyfluperlapine (0 - 50 μM) in the presence of H₂O₂ (100 μM), the metabolite became covalently bound to the protein in a concentration dependent manner and was detected as a 58 kDa adduct by immunoblotting (Figure 3-13). In addition, some high molecular weight aggregates of 7-hydroxyfluperlapine-modified MPO were observed. No covalent binding was observed in the absence of drug or H₂O₂. No such binding was observed when parent fluperlapine was incubated under identical conditions (data not shown).
3.5 Discussion.

As a potential replacement for the antipsychotic drug, fluperlapine shares many structural similarities with clozapine (Figure 3-1). However, replacing the secondary amine at position 5 with a methylene carbon prevents fluperlapine from forming a nitrenium ion analogous to clozapine. The results from the current study demonstrate that although fluperlapine is unreactive, its major metabolite, 7-hydroxyfluperlapine, is readily oxidized to a reactive intermediate by HOCl, which is the major oxidant produced by activated neutrophils. The mass spectrum, UV spectrum and the identity of the adducts formed on addition of nucleophiles provide substantive evidence that the reactive intermediate is the iminoquinone shown in Figure 3-14. Another possible structure for the reactive intermediate is a quinoneiminium ion involving the piperazine ring. Such a quinoneiminium ion would be expected to readily hydrolyze to give a quinone analogue and the free piperazine. The hydrolysis of a similar intermediate of vesnarinone has been reported previously (Uetrecht et al., 1994). However, we failed to find any hydrolytic products of the proposed quinoneiminium ion or any GSH or NAC adduct of the hydrolytic product.

The tricyclic backbone of 7-hydroxyfluperlapine is not a co-planar structure. Due to the sp³ hybridization on C-5 and the non-twisting π bond between N-10 and C-11, the 7-membered ring is bent and forces the two aromatic rings to the same side of the molecule. This configuration creates a chiral center. The two enantiomers of 7-hydroxyfluperlapine are probably not interchangeable because a completely planar
and high-energy transition state would be required for such an interchange. The chirality of 7-hydroxyfluperlapine is demonstrated by its proton NMR spectrum in which the two diastereotopic protons on C-5 near the chiral center are split into a doublet of doublets (δ 3.4 - 3.6 ppm, J' = 48.3 Hz, J'' = 13.0 Hz).

The iminoquinone species generated from 7-hydroxyfluperlapine is electrophilic and preferentially reactive toward sulfhydryl-containing nucleophiles. In the presence of NAC or GSH, the pre-chiral iminoquinone intermediate generates two pairs of diastereomeric NAC or GSH adducts. The CID mass spectra of both NAC and GSH adducts show the common major fragment at m/z 358, which is generated from the loss of the NAC or GSH moiety with sulfur still attaching to 7-hydroxyfluperlapine. This suggests that NAC and GSH are bound to the aromatic ring (Figure 3-6). The proton NMR spectra of the NAC adducts show that the binding of the substituents are to positions 6 and 9 (Figure 3-7). The same set of NAC and GSH adducts were also obtained with MPO/H₂O₂/Cl⁻ enzyme system (Figure 3-5A) and with PMA-activated human neutrophils (Figure 3-8). Hence, we presume that the same iminoquinone intermediate is formed by activated neutrophils. The positions to which NAC and GSH bind to the reactive metabolite of 7-hydroxyfluperlapine are the same as when they bind to the reactive metabolite of clozapine and its other analogues (Liu & Uetrecht, 1995). Also analogous to the reactive metabolite of clozapine, the iminoquinone was shown to be unreactive toward the "harder" nucleophile, N-acetyl-L-lysine. Therefore, it is reasonable to speculate that the iminoquinone of 7-hydroxyfluperlapine will modify sulfhydryl-containing proteins in a manner similar to that of the nitrenium ion generated
from clozapine and olanzapine. These results also provide the justification for the attempt to use the anti-clozapine antibodies for the detection of 7-hydroxyfluperlapine-modified proteins.

Using the clozapine-NAC-KLH adduct as an immunogen, a polyclonal antiserum had previously been generated. This antiserum also recognized fluperlapine and its 7-hydroxyl metabolite, with high affinity. With less structural similarities to clozapine, mianserin and procainamide had lower affinities for the antiserum (Figure 3-9). It seems that the presence and orientation of the methylpiperazine ring is important for high affinity binding of the antibodies. Therefore, we were able to use this antiserum to investigate covalent binding of the reactive intermediate generated by 7-hydroxyfluperlapine to human neutrophil proteins.

At various concentrations (2 - 20 μM), 7-hydroxyfluperlapine covalently bound to PMA-activated human neutrophils. Western blotting shows that both clozapine and 7-hydroxyfluperlapine modify a neutrophil polypeptide with the molecular weight of 58 kDa, which we suspect is MPO. However, when compared to clozapine, 7-hydroxyfluperlapine tends to modify neutrophil polypeptides with higher molecular weights (> 66 kDa; Figure 3-10). When fluperlapine was incubated with PMA-activated neutrophils, no fluperlapine-modified protein was detected even when higher concentrations were used. Fluperlapine is not easily oxidized chemically or biochemically, while its 7-hydroxyl metabolite is readily bioactivated to the reactive iminoquinone, which forms covalent adducts with sulfhydryl containing nucleophiles. Hence, it is probable that the iminoquinone generated by
activated neutrophils is also responsible for the covalent binding of 7-hydroxyfluperlapine to neutrophil proteins.

In presence of GSH or NAC (5 mM), the neutrophil protein modification by the iminoquinone intermediate of 7-hydroxyfluperlapine was effectively inhibited (Figure 3-12B). In these experiments, the 7-hydroxyfluperlapine-GSH or 7-hydroxyfluperlapine-NAC adducts were subsequently detected in the incubation supernatant by LC/MS in SIM mode. These results indicate that most of the reactive iminoquinone species formed by activated neutrophils is generated extracellularly and it is trapped by added GSH or NAC before it can bind to cellular proteins. This observation is in accord with the previous studies on neutrophil activation, which show the release of MPO enzyme by neutrophils upon external stimulation (Bentwood & Henson, 1980; Chatham et al., 1994). Hence, much of the binding may occur to surface proteins; however, this does not exclude the possibility that some of the extracellularly-formed reactive species may bind to intracellular proteins. N-Acetyl-L-lysine did not inhibit the binding of neutrophil proteins by the iminoquinone and there was no N-acetyl-L-lysine adduct detected in the supernatant. This implies that the iminoquinone intermediate selectively reacts with sulfhydryl nucleophiles and only binds to sulfhydryl-containing proteins. This is similar to the reactivity of the nitrenium ion of clozapine.

The covalent binding of the iminoquinone to MPO was demonstrated using purified human MPO in presence of H₂O₂ and Cl⁻. With 7-hydroxyfluperlapine at a concentration as low as 2 μM, the MPO was modified by the bioactivated reactive intermediate. The modified MPO polypeptide has a molecular mass of 58 kDa
(Figure 3-13), which is the heavy subunit of the enzyme (Andrews & Krinski, 1981). The MPO is presumably bound by the iminoquinone intermediate via its cysteine residues. A proposed bioactivation pathway for fluperlapine is summarized in Figure 3-14.

It has been widely believed that the bioactivated reactive intermediates are probably responsible for most drug-induced idiosyncratic reactions. Iminoquinones are reactive Michael acceptors and their formation from drugs has been associated with several idiosyncratic drug reactions. For example, the iminoquinone intermediates of acetaminophen and amodiaquine generated by liver cytochrome P450 enzymes have been implicated in the idiosyncratic hepatitis caused by these drugs (Chen et al., 1998; Jewell et al., 1995; Parkinson, 1996). The same iminoquinone metabolite of amodiaquine is also formed by activated human neutrophils and may be responsible for the drug-induced agranulocytosis (Tingle et al., 1995; Winstanley et al., 1990). It has also been shown that many other drugs associated with high incidence of agranulocytosis can be oxidized to iminoquinone-type intermediates (Ju & Uetrecht, 1999; Tingle et al., 1995; Uetrecht, 1992; Uetrecht et al., 1994; Winstanley et al., 1990). Even though nominally the reactive metabolite of fluperlapine is different than that of clozapine, i.e. iminoquinone rather than nitrenium ion, the reactivity, orientation of binding and physical structure of the two are very similar. We propose that the subsequent covalent binding and/or redox cycling of this reactive intermediate is responsible for fluperlapine-induced agranulocytosis and subsequent steps in the mechanism may be very similar to those involved in clozapine-induced agranulocytosis. The current study also
suggests that during studies of the mechanism of an idiosyncratic drug reaction, in addition to testing the ability of the drug to form a reactive metabolite, the further metabolism of the major stable metabolites should also be investigated.
Figure 3-1. Chemical structures of (A) clozapine, (B) fluperlapine, (C) 7-hydroxy fluperlapine, (D) mianserin and (E) procainamide.
Figure 3-2. Major metabolic pathways of fluperlapine *in vivo*. 
Figure 3-3. Mass spectra of the iminoquinone intermediate produced by reacting 7-hydroxyfluperlapine with HOCl: (A) Mass spectrum containing the iminoquinone (m/z 324), unreacted 7-hydroxyfluperlapine (m/z 326) and stable further hydroxylated product(s) (m/z 342); (B) Mass spectrum of the iminoquinone intermediate obtained under CAD condition.
Figure 3-4. Repetitive absorption spectra from the reaction of 7-hydroxyfluperlapine with HOCl at pH 6. The dashed curve is the UV spectrum of 7-hydroxyfluperlapine, and the dotted curve is the UV spectrum obtained immediately after addition of HOCl. Times for integration, cycle and total run were 0.5 s, 2 s and 30 s, respectively. The concentrations of 7-hydroxyfluperlapine and HOCl were 100 μM each.
Figure 3-5. (A) LC/MS spectrum of reaction mixture when 7-hydroxyfluperlapine was oxidized by MPO/H₂O₂/Cl⁻ in presence of NAC; and (B) the computer extraction of ions at m/z 487 (for the NAC adducts), m/z 342 (for stable hydroxylated products, not observed) and m/z 360 (for stable chlorinated products, not observed).
Figure 3-6. (A) LC/MS/MS spectrum of the major 7-hydroxyfluperlapine-NAC adducts with a HPLC retention time of 6.0 min; (B) LC/MS/MS spectrum of the major 7-hydroxyfluperlapine-GSH adducts with a HPLC retention time of 4.2 min.
Figure 3-7. Proton NMR spectra of the aromatic regions of (A) 7-hydroxyfluperlapine; (B) the pair of diastereomeric NAC adducts with a HPLC retention time of 5.0 min; and (C) the pair of diastereomeric NAC adducts with a HPLC retention time of 6.0 min.
Figure 3-8. LC/MS selective ion monitoring (SIM) spectra of (A) 7-hydroxyfluperlapine-NAC adducts and (B) 7-hydroxyfluperlapine-GSH adducts generated by PMA-activated human neutrophils at pH 7.4. The concentration of 7-hydroxyfluperlapine was 50 μM and the concentration of NAC or GSH was 5 mM.
Figure 3-9. Inhibition of the binding of the anti-clozapine-NAC-KLH antiserum to clozapine modified neutrophils by fluperlapine and other drugs. The ability of clozapine (CLOZ), fluperlapine (FLU), hydroxyfluperlapine (HF), mianserin (MIAN) and procainamide to inhibit the binding of anti-clozapine-NC-KLH antiserum to neutrophils incubated with NaOCl (-) or NaOCl and clozapine (+) was examined. The concentration of the inhibitors was 20 μM in panel (A) and 2 μM in (B). The antiserum (1:4,000 dilution) was incubated with the drugs for 30 minutes prior to addition to the nitrocellulose membrane. Protein loading was 10 μg/lane and 10% resolving gels were used. This Figure is representative of two independent experiments.
Figure 3-10. Immunochemical detection of covalent binding of clozapine (CLOZ), 7-hydroxyfluperlapine (HFLU) and fluperlapine to PMA-stimulated human neutrophils *in vitro*. Neutrophils were incubated with varying concentrations of drugs in the presence (+) or absence (-) of PMA at 37°C for 1 hour and then processed as described in the Methods section. Protein from 0.5 x 10⁶ PMA-activated neutrophils (15 µg) was loaded in each lane. For immunoblotting primary antiserum (anti-clozapine-NAC-KLH) was used at a dilution of 1:3,000. This Figure is representative of 3 individual experiments.
Figure 3-11. Hapten inhibition experiments. Human neutrophils were incubated with clozapine (8 μM; C) or hydroxyfluperlapine (50 μM; H) in the presence of PMA (40 ng/ml) for 60 minutes at 37°C. Control incubations (-) contained PMA alone but no drug. The blots were incubated with the anti-clozapine-NAC-KLH antiserum alone (dilution 1:5,000) or with antiserum that was pre-incubated with clozapine (CLOZ; 50 μM) or fluperlapine (FLUP; 50 μM) for 30 minutes before addition to the blot. Protein loading was 8.7 μg/lane.
Figure 3-12. Effect of nucleophiles on covalent binding of clozapine and 7-hydroxyfluperlapine to PMA activated human neutrophils in vitro. Human neutrophils were incubated with (A) clozapine (8 μM) or (B) hydroxyfluperlapine (50 μM) and neutrophils were activated with PMA (40 ng/ml). The neutrophils were then incubated at 37°C for 1 hour and processed as described in the methods section. The effect of including 5 mM GSH, NAC or N-acetyl-L-lysine (NAL) in the incubations was examined. Anti-clozapine-NAC-KLH antiserum was used at a dilution of 1:5,000 and protein loading was 18.6 μg/lane for clozapine samples and 6.3 μg/lane for hydroxyfluperlapine samples.
Figure 3-13. Immunochemical detection of the covalent binding of hydroxyfluperlapine (0-50 μM) to MPO (5 units) in the presence of H₂O₂ (100 μM). Samples were treated as described in the methods section. Protein loading was 0.65 μg/lane. For immunoblotting, primary antiserum (anti-clozapine-NAC) was used at a dilution of 1:3,000 and secondary antiserum (goat anti-rabbit IgG) was used at a dilution of 1:10,000.
Figure 3-14. Proposed bioactivation pathway of fluperlapine.
Chapter 4

Bioactivation of Nevirapine and 3-Hydroxynevirapine by Neutrophils and Hepatic Microsomes: Implication for Nevirapine-Induced Adverse Drug Reactions.
4.1 Abstract.

Nevirapine is a potent non-nucleoside inhibitor of the reverse transcriptase enzyme of type 1 human immunodeficiency virus (HIV). Clinically the use of nevirapine is limited because it causes a high incidence of adverse drug reactions including skin rash, hepatitis and granulocytopenia. In this study, we demonstrated that radiolabeled nevirapine covalently bound to phorbol 12-myristate-13-acetate (PMA)-activated human neutrophils. Covalent protein binding was also detected when the drug was incubated with a combination of myeloperoxidase/hydrogen peroxidase/chloride in the presence of bovine serum albumin. Upon oxidation by hypochlorous acid, the major oxidant produced by activated neutrophils, nevirapine formed a reactive intermediate with a molecular ion of m/z 281. This intermediate could be trapped by N-acetyl-L-cysteine (NAC) to an adduct with a molecular ion of m/z 444. The same NAC adduct was also detected when 3-hydroxynevirapine, the major hepatic metabolite of nevirapine, was metabolized by rat hepatic microsomes in the presence of NAC. An anti-hydroxynevirapine antiserum was raised using keyhole limpet hemocyanin (KLH) modified by this NAC adduct. Immunoblotting studies with this antiserum detected hydroxynevirapine-modified polypeptides in the hepatic cytosol fraction and the skin homogenate of nevirapine-treated rats. The bioactivation of nevirapine and its 3-hydroxy metabolite could involve a reactive quinoneiminium metabolite that covalently modified cellular proteins and induced adverse reactions.
4.2 Introduction.

The human toll caused by acquired immunodeficiency syndrome (AIDS) is well known. The HIV virus responsible for AIDS is a retrovirus, and the principal therapy has been based on inhibition of reverse transcriptase. Although in the past the efficacy of these agents has been limited by the development of resistance, recent trials, in which they were combined with protease inhibitors, have shown real promise for significantly altering the course of AIDS (Cheeseman et al., 1995; D'Aquila et al., 1996; Harris et al., 1998; Havlir & Lange, 1998; Maass et al., 1993).

In general, the agents used to inhibit reverse transcriptase have been nucleosides; however, a new nonnucleoside inhibitor, nevirapine, has been developed. Nevirapine (Figure 4-1) is noncompetitive with respect to deoxyguanosine triphosphate with no effect on mammalian DNA polymerases, and in cytotoxicity studies, it had a high therapeutic index (>8000; Merluzzi et al., 1990). The major drawback is that, in contrast to initial trials in normal subjects who had a relatively low incidence of adverse reactions, during clinical trials in patients with AIDS, 10 - 40% of those receiving the drug treatment developed idiosyncratic cutaneous reactions. This included a 0.3% incidence of life-threatening Stevens-Johnson syndrome and toxic epidermal necrolysis (Anton et al., 1999; Beach, 1998; Havlir et al., 1995; Pollard et al., 1998). Nevirapine was also associated with a high incidence of drug-induced hepatitis (1%) and an indeterminant incidence of granulocytopenia in children (Pollard et al., 1998). Patients with AIDS are known to have a much higher incidence of adverse reactions to other medication such as
sulfonamides (Carr et al., 1993; Medina et al., 1990). If the mechanism of these adverse reactions were understood, it might make it possible to prevent them or to develop analogs that are safer.

Although definitive proof is generally lacking, there is a large amount of circumstantial evidence which suggests that most idiosyncratic reactions are caused by chemically reactive metabolites of the drugs rather than the drug itself (Nelson, 1982; Park et al., 1992; Uetrecht, 1992). There is also evidence that many such reactions are immune-mediated and the characteristics of the reactions to nevirapine are consistent with an immune mechanism. The simplest hypothesis that would link the formation of a reactive metabolite and the induction of an immune-mediated reaction is that the reactive metabolite could bind to specific proteins and, by altering them, induce an immune response to the altered protein (Pirmohamed et al., 1996; Pohl et al., 1988; Uetrecht, 1997). Although it is somewhat ironic that a disease that is characterized by immune deficiency should be associated with an increased incidence of immune-mediated adverse reactions, there are parts of the immune system that appear to be overactive in an apparent attempt to compensate (Ahmad & Menezes, 1996; Rodman et al., 1999; Zangerle et al., 1994). For example, urinary neopterin, an indication of monocyte/macrophage activation, is elevated in AIDS patients (Fuchs et al., 1989; Hara et al., 1995). Two possible reasons for the increased incidence of adverse drug reactions in patients with AIDS is an increased formation of reactive metabolites by activated monocytes or decreased levels of glutathione in the leukocytes of patients with AIDS. However, treatment with N-acetylcysteine, a glutathione precursor, does not decrease the
incidence of idiosyncratic drug reactions in HIV positive patients (Akerlund et al., 1997; Walmsley et al., 1998).

In vivo, the major hepatic metabolites of nevirapine include its 2-hydroxyl, 3-hydroxyl, 8-hydroxyl, 12-hydroxyl and 4-carboxyl derivatives, with 3-hydroxyl as the predominant metabolite in humans (Figure 4-1; Riska et al., 1999). Although most drug metabolism occurs in the liver, the reactive nature of most reactive metabolites makes it unlikely that they would reach sites, such as the skin, distant from where they are formed. Therefore, it is logical to study drug metabolism by the cells at the site of the adverse reaction. It has been shown that some P450 isoforms are present in the skin (Mukhtar & Khan, 1989). In addition to keratinocytes, the skin contains cells of the immune system. If the adverse reactions involve the immune system, it is likely that reactive metabolites formed by these cells could play a role in the mechanism. Previously we have found that many drugs that are commonly associated with serious idiosyncratic drug reactions are oxidized to reactive metabolites by myeloperoxidase (MPO), which is released by activated neutrophils and monocytes (Lai et al., 1999; Liu & Uetrecht, 1995; Uetrecht et al., 1994). In order to address nevirapine-induced adverse drug reactions, we studied nevirapine metabolism by neutrophils, the myeloperoxidase (MPO) system as well as by hypochlorous acid, which is the major oxidant produced by neutrophils (Weiss, 1989). It is also possible that a reactive metabolite could be formed from a stable hepatic metabolite. Therefore, we investigated bioactivation by rat-liver microsomes of 3-hydroxynevirapine, the major hepatic metabolite of nevirapine.
4.3 Materials and Methods.

4.3.1 Materials.

Nevirapine \((11\text{-}\text{cyclopropyl}-5,11\text{-dihydro}-4\text{-methyl}-6\text{H}\text{-dipyrido}(3,2\text{-b}:2',3'\text{-e})(1,4)\text{diazepin}-6\text{-one})\) was provided by Boehringer Ingelheim Pharmaceutical Inc. (Ridgefield, CT). N-Acetyl-L-cysteine (NAC), phorbol 12-myristate-13-acetate (PMA), casein, rabbit serum albumin (RSA), thimerosal, methylumbelliferyl phosphate and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hypochlorite and GSH were purchased from Aldrich Chemical Company (Milwaukee, WI). Hydrogen peroxide \((\text{H}_2\text{O}_2)\) was obtained from Mallinckrodt Canada Inc. (Pointe-Claire, Quebec). The concentration of sodium hypochlorite was determined by a spectrophotometric method with its absorption at \(292.5\) nm and extinction coefficient of \(360 \text{ M}^{-1}\text{cm}^{-1}\) (Hussarin et al., 1970). All solvents used for HPLC and LC/MS analyses were HPLC grade. MPO was obtained from Cortex Biochemical (San Leandrow, CA). One unit of MPO activity was defined as the amount of enzyme that decomposed \(1 \mu\text{mol}\) of \(\text{H}_2\text{O}_2\) per min at \(25^\circ\text{C}\) and pH 6. Stock acrylamide solution (40%) was purchased from BioRad (Mississauga, Ontario). Nitrocellulose and enhanced chemiluminescence (ECL) films were purchased from Amersham Canada (Oakville, Ontario). Supersignal ECL detection reagents and keyhole limpet hemocyanin were purchased from Pierce Chemical (Rockford, IL). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chains) was purchased from Zymed (San Francisco, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch.
Laboratories (West Grove, PA). The neutrophils were isolated from venous blood collected from normal subjects.

4.3.2 Analytical.

Most of HPLC analyses were carried out on a Shimadzu HPLC system containing a LC-600 pump, a SPD-6A UV detector set at 254 nm and a C-R6A integrator. The chromatography columns, packed with 5 μm Ultracarb ODS 30, were obtained from Phenomenex. The column used for all analytical work was 2 x 100 mm with a 2 x 30 mm guard column. The column used for isolation of NAC conjugates was 10 x 150 mm with a 10 x 60 mm guard column. The mobile phase consisted of water, acetonitrile and acetic acid (variable ratio as indicated) with 2 mM ammonium acetate.

LC/MS and LC-MS/MS were performed on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer with an IonSpray interface. \textsuperscript{1}H NMR spectra were recorded at 200 MHz with a Varian Gemini 200 spectrometer and at 500 MHz with a Varian Unity Plus 500 spectrometer. The solvents were C\textsubscript{2}HCl\textsubscript{3}, C\textsubscript{2}H\textsubscript{5}OH, or D\textsubscript{2}O depending on solubilities of the compounds.

4.3.3 Isolation of human neutrophils.

Blood (30 mL) was collected from normal subjects and mixed with 3% dextran in 0.9% sodium chloride at a 4:1 ratio. The erythrocytes were allowed to settle for 30 min. The supernatant was carefully withdrawn and underlaid with Ficoll-Paque (Parmacia LKB Biotechnology Inc., Piscataway, NJ) at a 5:2 ratio. After centrifugation
at 1,200 rpm for 25 min, neutrophils were collected as a pellet and the supernatant was discarded. A small amount of contaminating erythrocytes were lysed by 0.2% NaCl solution (5 mL). The isotonicity was restored after 1 min by adding an equal volume of 1.6% NaCl solution. A second hypotonic lysis was performed if the cell pellet was still red. The cells were centrifuged at 1,000 rpm for 5 min, and the cell pellet was washed twice with Hanks’ balanced salt solution (HBSS) before finally suspending it in HBSS (5 mL). The neutrophils were stained with 0.1% trypan blue and counted with a hemocytometer. Trypan blue exclusion showed the cell viability to be more than 96% in all isolations.

4.3.4 Preparation of rat-liver microsomes.

Rat-liver microsomes were prepared from male Sprague Dawley rats (average weight 300 g). The animals were sacrificed by cervical dislocation, and their livers were removed and minced in ice cold sucrose buffer (0.25 M sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 6.8). The liver homogenates were prepared using a homogenizer (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany). The liver homogenates were filtered through a piece of cheese cloth and centrifuged at 1,000 x g for 11 min at 4°C. The pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined supernatants were centrifuged at 10,000 x g for 30 min at 4°C, and the pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined 10,000 x g supernatants were further centrifuged at 100,000 x g for 90 min at 4°C. The microsome pellets were finally resuspended in a storage buffer (100 mM potassium phosphate, 10% sucrose, pH 7.5) and stored at -80°C before use.
4.3.5 Covalent protein binding of nevirapine in MPO enzyme system.

\(^{14}\)C-labeled nevirapine (specific radioactivity: 90.9 \(\mu\)Ci/mg, Boehringer Ingelheim Pharmaceutical Inc., Ridgefield, CT) was first purified and concentrated by solid phase extraction with a Supelco C-18 cartridge (Supelco Inc., Bellefonte, PA). The recovered \(^{14}\)C-labeled nevirapine was redissolved in acetonitrile to make a stock solution of about 5 mM. \(^{14}\)C-labeled nevirapine (20 \(\mu\)L, in various concentrations) was incubated with MPO (20 units, 20 \(\mu\)L), \(\text{H}_2\text{O}_2\) (10 \(\mu\)L, final concentration 4 mM) in phosphate-buffered saline (PBS; 130 \(\mu\)L, 0.1 M, pH 6.0) at 25\(^\circ\)C for 1/2 hr, after which an aqueous solution of bovine serum albumin (BSA; 20 \(\mu\)L, final concentration 2 mg/mL) was added. The total incubation time was 3 hrs. After incubation, the protein was precipitated by adding 1 mL of acetone and then it was redissolved and reprecipitated 3 times with acetone and finally redissolved in water. The radioactivity and protein concentration of this solution was determined by scintillation counting. The final concentration of the drug was also determined by measuring its radioactivity and then calculated using its specific radioactivity. In the control experiments, \(\text{H}_2\text{O}_2\) was replaced by water.

4.3.6 Covalent binding of nevirapine to human neutrophils.

Neutrophils (2 x10\(^7\) cells) were suspended in HBSS (2 mL) and \(^{14}\)C-nevirapine (0.72 \(\mu\)Ci, final concentration 14 \(\mu\)M) was added followed by 5 \(\mu\)g of PMA in DMSO to activate the cells. The cells were incubated for 3 hours at 37\(^\circ\)C and then the cells were collected on filter paper (GF/F, 2.5 cm, Whatman International Ltd.,
Maidstone, UK) and washed several times with hot acetone. The radioactivity on the filters was then determined by scintillation counting. In one set of control experiments, PMA was replaced with DMSO, and in the another set of control experiments, the neutrophils were pre-heated at 100°C for 15 min before use.

4.3.7 Oxidation of nevirapine by HOCl.

Nevirapine (10 μL, 10 mM in acetonitrile) was added to 0.1 M potassium phosphate buffer solution (80 μL, pH 6). Sodium hypochlorite (NaOCl) solution (10 μL, 20 mM) was then added to give a final concentration of 1 mM for nevirapine and 2 mM for NaOCl. The mixture was allowed to stand at room temperature for 5 min before samples were taken for HPLC and LC/MS analysis using a mobile phase of water:acetonitrile:acetic acid (65:35:1, v/v) with 2 mM ammonium acetate. The HPLC fraction containing the reactive intermediate with a retention time of 11 min was collected in a quartz cuvette and immediately scanned using a Hewlett Packard diode-array spectrophotometer (HP8452A, Hewlett Packard Company, Palo Alto, CA). The sample was scanned at 60-s intervals for 15 min over a wavelength range of 200 to 600 nm. To trap the reactive intermediate, a phosphate-buffered NAC solution (10 μL, 20 mM, pH 8) was added. Aliquots were analyzed by HPLC and LC/MS.

4.3.8 Preparation of hydroxynevirapine-NAC adduct for NMR studies.

Nevirapine (150 mg, 0.56 mmol) was dissolved in methanol (10 mL), to which had been added 0.1 M phosphate buffer (150 mL, pH 6). The reaction was
started by adding a NaOCl solution (1.0 mL, 0.7 M, final concentration) with a syringe at a rapid rate. The reaction mixture rapidly turned pale yellow. Immediately after adding NaOCl (2-5 sec), NAC (408 mg, 2.5 mmol, dissolved in phosphate buffer, 25 mL, pH 8) was added with rapid stirring. The mixture turned bright yellow. The reaction mixture was stirred at room temperature for another 30 min before extracting three times with an equal volume of chloroform. The aqueous phase was then washed with 25 mL hexane and concentrated by rotoevaporation. Methanol was then added to precipitate inorganic salts from the buffer solution. After filtration, the methanol solution was concentrated by rotoevaporation. The crude products were further purified by preparative HPLC using a mobile phase of water:acetonitrile:acetic acid (85:15:1, v/v) with 2 mM ammonium acetate at a flow rate of 5 mL/min. The fraction with a retention time of 25 min was collected. The NAC conjugate was separated from buffers by solid phase extraction with a Supelco C-18 cartridge (Supelco Inc., Bellefonte, PA).

The NAC conjugate was analyzed by LC/MS and its \(^1\)H NMR and \(^1\)H COSY spectra were recorded at 500 MHz in \(^2\)H\(_2\)O.

4.3.9 Metabolism of 3-hydroxynevirapine by rat-liver microsomes in presence of NAC.

Microsomal cytochrome P450 content was quantified by the reduced carbon monoxide difference spectrum method of Omura and Sato (Omura and Sato, 1964). To phosphate buffer (0.1 M, pH 6.8, 1.74 mL with 5 mM MgCl\(_2\)) was added a microsome suspension (69 \(\mu\)L, final concentration 2 mg protein/mL), an ethanolic
solution of 3-hydroxynevirapine (40 μL, final concentration 0.2 mM), an aqueous solution of NAC (50 μL, final concentration 5 mM) and a NADPH-generating system (100 μL, final concentration 0.5 mM NADP+, 5 mM glucose-6-phosphate and 0.25 unit glucose-6-phosphate dehydrogenase). The suspension was incubated at 37°C for 3 hrs after which an equal volume of acetonitrile was added to precipitate the proteins in the mixture. After centrifugation at 500 x g for 10 min, the supernatant was isolated and dried with a stream of nitrogen. The residue was redissolved in water and analyzed by LC/MS using SIM at m/z 444 for 3-hydroxynevirpirapine-NAC conjugates. In the control experiments, the NADPH-generating system was replaced by the phosphate buffer.

4.3.10 Synthesis of 3-hydroxynevirapine-NAC coupled RSA and KLH.

The 3-hydroxynevirapine-NAC adduct (1.0 mg, 2.25 μmol for RSA; 4.0 mg, 9.01 μmol for KLH) in N,N-dimethylformamide (100 μL) was added to a 10 mL test tube. Under vigorous stirring, a dichloromethane solution of 2 equivalents of EDC was slowly added. The reaction mixture was stirred at room temperature for 1 hr, and then the dichloromethane was evaporated with a stream of nitrogen. Either RSA (1.0 mg) or KLH (10.0 mg) in phosphate buffer (0.1 M, pH 8.5, 50 μL for RSA and 200 μL for KLH) was subsequently added with stirring. The mixture was stirred at room temperature for another 5 hrs. The modified proteins were purified by dialysis against water (molecular weight cut-off 6 - 8 kDa) for 48 hrs. The resultant protein solution was then freeze-dried overnight and stored at -20°C before use.
4.3.11 Production of anti-hydroxynevirapine-NAC-KLH antiserum.

Polyclonal anti-hydroxynevirapine-NAC-KLH antibodies were raised in a 2-kg, male, pathogen-free New Zealand White rabbit (Charles River, Montreal, Quebec). After preimmune serum was obtained, the animal was immunized with the hydroxynevirapine-NAC-KLH conjugate (1 mg in 1 mL of PBS emulsified with an equal volume of Freund’s complete adjuvant) subcutaneously at multiple sites. Injections with 0.5 mg hydroxynevirapine-NAC-KLH conjugate in Freund’s incomplete adjuvant divided into six to eight subcutaneous sites were repeated 4, 6, 8 and 12 weeks after the initial immunization. Exsanguination of the animal while it was under pentobarbital anesthesia was conducted 10 days after the final immunization. Blood was allowed to clot overnight at 4°C and then centrifuged at 400 x g. The serum was recovered and heat-inactivated at 56°C for 30 min before being placed in aliquots and stored at -20°C.

4.3.12 Specificity of the anti-hydroxynevirapine antiserum.

3-Hydroxynevirapine-NAC-RSA or native RSA (100 µL of a 15 µg/mL solution) was incubated overnight in flat-bottom 96-well plates (Costar, Cambridge, MA) at 4°C. The plates were emptied and washed with enzyme-linked immunosorbent assay (ELISA) wash buffer (10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.5% (w/v) casein, and 0.002% (w/v) thimerosal) four times. After the last wash, the plates were tapped dry, and various dilutions of the anti-trimethoprim-NAC-KLH antiserum (100 µL PBS) were added to the plates. The ELISA plates were incubated at room temperature for 3 hr. Plates were then washed four times with ELISA wash
buffer and tapped dry. Alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:5000 in PBS) was subsequently added to each well of the plate (100 μL/well). The plates were incubated at room temperature for 2 hr and then washed four times with ELISA wash buffer and two times with PBS. A stock solution of methyl umbelliferyl phosphate (10 mg/mL in demethylsulfoxide, kept at -20°C) was diluted 1:100 in PBS, and this solution was added to the ELISA plates (100 μL/well). Plates were incubated at room temperature for 10 min before fluorescence was measured with a Fluorescence Concentration Analyzer (Pandex, Mundelein, IL) set at 365/450 nm (excitation/emission).

4.3.13 SDS-PAGE and Western Blotting.

Samples for analysis by SDS-PAGE were solubilized by boiling for 10 min in sample buffer (8% (w/v) SDS, 20% (v/v) glycerol, 0.002% bromophenol blue, 125 mM Tris-HCl (pH 6.8)) containing dithiothreitol (6 mg/ml). SDS-PAGE was performed using a mini-gel system (Mini-PROTEAN II, Bio-Rad, Mississauga, Ontario, Canada) and the discontinuous buffer system described by Laemmli (Laemmli, 1970). Stacking and resolving gels were 4 and 10% acrylamide, respectively. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (~45 min). Electrophoretic transfer of resolved proteins to nitrocellulose was carried out using a buffer of 15.7 mM Tris, 120 mM glycine (pH 8.3) containing 20% (v/v) methanol, for 60 min at 100 V using a mini Trans-Blot transfer cell (Bio-Rad, Mississauga, Ontario). Nitrocellulose was either stained for protein for 5 min using 0.1% amido black 10B in 45% (v/v) methanol, 10% (v/v)
acetic acid and then destained using 70% (v/v) methanol, 2% (v/v) acetic acid or used for antibody development.

The subsequent steps were conducted at room temperature with constant shaking. Before exposure to antiserum, the nitrocellulose was blocked by the blocking buffer (10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 2.5% (w/v) casein and 0.02% (w/v) thimerosal) for 2 hr. The blocked nitrocellulose was incubated overnight with anti-trimethoprim-NAC-KLH antiserum diluted in the ELISA wash buffer. Unbound antibodies were removed by washing the nitrocellulose in the ELISA wash buffer (3 x 10 min). The nitrocellulose was subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chain) antiserum (diluted 1:5,000 in the ELISA wash buffer). The nitrocellulose was washed with Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 154 mM NaCl, 3 x 5 min). The nitrocellulose sheets were then incubated in Super Signal ECL reagents for 5 min, and the bound antibodies were visualized by exposing the nitrocellulose to ECL film under safe-light conditions.

4.3.14 Dosing of rats with nevirapine in vivo.

Female Sprague Dawley rats (about 200 g) were obtained from Charles River Canada and housed in standard cages with free access to water and powdered rodent laboratory chow 5001 (Agribrands Purina, Woodstock, Ontario). After a one-week acclimation period, during which food consumption was monitored (~10 g/rat/day), the rats were either continued on the powdered lab chow diet (controls) or switched to a diet in which nevirapine was mixed with the powdered lab chow such that the
rats had an intake of 100 mg of nevirapine/kg/day (treated). Rats were fed with control or nevirapine-containing diet continuously for a period of 4 to 8 weeks. At the end of the study, rats were killed by i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Liver samples were cut from the whole liver and immediately immersed in 4% formalin for pathological diagnosis. A hepatic microsomal fraction of rat liver was prepared from the remaining freshly isolated livers by differential centrifugation following the procedure described in section 4.3.4. The liver homogenate sample was taken after the step of cheesecloth-filtration, and the cytosolic fraction was obtained from the supernatant after 100,000g centrifugation. A piece of shaved dorsal skin of rat (4 x 4 cm) was cut into small pieces and homogenized in ice-cold sucrose buffer before being filtered through two layers of cheesecloth. The protein concentration of each sample was determined, and the samples were diluted to give a protein concentration of 3 mg/mL. An equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/mL) was then added. The sample was boiled at 100 °C for 10 min before loading on SDS-PAGE.

4.3.15 Protein concentration measurement.

Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.
4.4 Results.

4.4.1 Covalent Binding of nevirapine to proteins.

The amount of covalent binding due to reactive metabolites generated by the MPO system was calculated (Table 4-1). The difference between binding in the presence and absence of \( \text{H}_2\text{O}_2 \) was significant and dependent on the concentration of nevirapine. However, the amount of covalent binding to albumin at a therapeutic concentration (17 \( \mu \text{M} \)) was low (21 pmole/mg albumin).

<table>
<thead>
<tr>
<th>Nevirapine conc. (( \mu \text{M} ))</th>
<th>Binding of (^{14}\text{C}) labeled nevirapine (pmol/mg BSA)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nev+MPO+Cl(^-) (control)</td>
<td>Nev+MPO+Cl(^-) +H(_2)O(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>410</td>
<td>14.8 ± 0.5</td>
<td>194 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>5.7 ± 0.3</td>
<td>103 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>6.2 ± 0.4</td>
<td>21 ± 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* \(^{14}\text{C}\) labeled nevirapine at various concentrations was incubated with MPO (0.1 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (4 mM) in 0.1M PBS (pH 6) at 25°C for 1/2 hr before addition of BSA (2 mg/mL). The total incubation time was 3 hrs.
After $^{14}$C-labeled nevirapine at a therapeutic concentration (14 µM) was incubated with PMA-activated neutrophils (+ PMA), the value calculated for the degree of covalent binding was 0.53 pmole/million cells. The covalent binding was statistically significant compared with controls in which the neutrophils were either not activated (- PMA) or denatured by heating (D/N) at 100°C for 15 min (Figure 4-2).

4.4.2 Oxidation of Nevirapine by HOCl and the MPO system.

Nevirapine was oxidized by hypochlorous acid at pH 6. The subsequent products from oxidation included a significant amount of 2-hydroxynevirapine and 3-hydroxynevirapine with molecular ions of m/z 283 (Figure 4-3). One reactive intermediate generated in this process with a molecular ion of m/z 317 contained an additional oxygen and chlorine, and another reactive species contained an additional oxygen (m/z 281; Figure 4-4). Both intermediates disappeared upon addition of GSH or NAC. Similar results were obtained with myeloperoxidase in the presence of hydrogen peroxide and chloride ion, although the extent of oxidation was much less than with HOCl. The intermediate of nevirapine with a molecular ion of m/z 317 spontaneously converted to the reactive intermediate with a molecular ion of m/z 281. After collecting the HPLC fraction containing the chlorinated intermediate, this conversion could be monitored by UV spectrophotometry (Figure 4-5). The conversion was complete after 15 mins, and the resultant intermediate, with a molecular ion of m/z 281, reacted rapidly with NAC to give a hydroxynevirapine-NAC conjugate with a molecular ion of m/z 444.
The $^1$H NMR (200 MHz) spectrum of nevirapine in C$_2$HCl$_3$ (Figure 4-6) showed peaks at δ 0.50 ppm (2H, m); 0.98 ppm (2H, m); 2.39 ppm (3H, s); 3.75 ppm (1H, m); 6.94 ppm (1H, d, J = 4.80 Hz); 7.06 ppm (1H, dd, J = 7.61 Hz, 4.76 Hz); 8.13 ppm (1H, dd, J = 7.65 Hz, 2.15 Hz); 8.16 ppm (1H, d, J = 4.88 Hz); 8.55 ppm (2H, m, J = 4.76 Hz; 1.86 Hz). The NAC conjugate with a MH$^+$ ion at m/z 444 was isolated and purified. Its LC/MS/MS (Figure 4-7A) showed fragments at m/z 274 (40%, MH$^+$ - C$_2$H$_4$NO$_3$ - cyclopropane), m/z 315 (100%, MH$^+$ - C$_2$H$_4$NO$_3$) and m/z 444 (20%, MH$^+$).

The $^1$H NMR (500 MHz) spectrum of this NAC adduct (Figure 4-8) consisted of peaks at δ 0.66 ppm (2H, m); 1.18 ppm (2H, m); 2.02 ppm (3H, s); 2.44 ppm (3H, s); 2.86 ppm (1H, dd, J = 8.97 Hz, 6.96 Hz); 3.18 ppm (1H, dd, J = 13.92 Hz, 4.02 Hz); 3.68 ppm (1H, m); 4.18 ppm (1H, dd, J = 5.13 Hz, 4.03 Hz); 7.58 ppm (1H, dd, J = 7.88 Hz, 4.76 Hz); 8.45 ppm (1H, dd, J = 7.87 Hz, 1.83 Hz); 8.80 ppm (1H, dd, J = 4.76 Hz, 1.83 Hz). The $^1$H COSY spectrum of this NAC adduct (Figure 4-9) demonstrated the couplings among three aromatic protons with chemical shifts of 7.58, 8.45 and 8.80 ppm.

4.4.3 Oxidation of 3-hydroxynevirapine by HOCl and activated Neutrophils.

3-Hydroxynevirapine was oxidized by HOCl more rapidly than nevirapine. Upon treatment with HOCl, LC/MS analysis indicated that a stable chlorinated product with a MH$^+$ ion at m/z of 317 was generated. The stable chlorinated species did not disappear after addition of GSH or NAC, nor were any GSH or NAC conjugates observed. Similar results were also obtained by oxidation of 3-hydroxynevirapine with both the MPO/H$_2$O$_2$/Cl$^-$ system and PMA-activated human neutrophils.
The stable chlorinated product with a molecular ion of m/z 317 generated from 3-hydroxynevirapine was isolated. Ethyl acetate was used to extract the product from the aqueous reaction mixture. The crude sample in ethyl acetate was concentrated and applied onto a TLC plate (Silica Gel 60, Aldrich, Milwaukee, WI) and developed by 100% ethyl acetate. The product with a R<sub>f</sub> = 0.5 was subsequently recovered. Its <sup>1</sup>H NMR (200 MHz) spectrum taken in CD<sub>3</sub>Cl<sub>3</sub> (Figure 4-10) consisted of peaks at δ 0.46 ppm (2H, m); 0.94 ppm (2H, m); 2.28 ppm (3H, s); 3.68 ppm (1H, m); 5.40 ppm (1H, bs); 7.06 ppm (1H, dd, J=7.69 Hz, 4.84 Hz); 7.35 ppm (1H, bs); 8.12 ppm (1H, dd, J=7.65 Hz, 2.03 Hz); 8.54 ppm (1H, dd, J=4.85 Hz, 1.96 Hz).

4.4.4 Metabolism of 3-hydroxynevirapine by rat-liver microsomes in the presence of NAC.

Upon incubation of 3-hydroxynevirapine with rat-liver microsomes in the presence of NAC and a NADPH-generating system at pH 6.8, a hydroxynevirapine-NAC conjugate with a molecular ion of m/z 444 was also observed using LC/MS in the SIM mode (Figure 4-11). The NAC conjugate showed the same HPLC retention time as that obtained by oxidation of nevirapine with HOCl followed by addition of NAC. In CID mass spectrometry, the NAC conjugates produced by both nevirapine and its 3-hydroxy metabolite gave identical molecular ion fragmentation (Figure 4-7B).

4.4.5 Production and characterization of Anti-hydroxynevirapine-NAC-KLH antiserum.
3-Hydroxynevirapine-NAC was successfully conjugated with N-acetyl-L-lysine through EDC coupling reaction. The resultant conjugate had a MH⁺ ion at m/z 614 and a MH₂⁺ ion at m/z 307, which is characteristic for a di-peptide species. Applying the same conditions, the 3-hydroxynevirapine-NAC conjugate was also coupled to rabbit serum albumin (RSA) and keyhole limpet hemocyanin (KLH; Figure 4-12). Compared with the native RSA with an average molecular weight of 66.3 kDa, the 3-hydroxynevirapine-modified RSA had a significant increase in molecular weight with the average of 68.9 kDa as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Attempts to determine the modification on KLH were not successful because KLH is too large to obtain a mass spectrum. However, it is likely that, by following the same coupling procedure as that used for RSA, a modified KLH conjugate should be obtained.

Polyclonal antibodies were subsequently raised by immunizing rabbits with the hydroxynevirapine-NAC-KLH conjugate. The specificity of the antibodies against the hydroxynevirapine epitope was demonstrated by ELISA, which showed that the anti-hydroxynevirapine-NAC-KLH antiserum recognized hydroxynevirapine-NAC-RSA conjugate but not native RSA alone (Figure 4-13).

4.4.6 Dosing of rats with nevirapine and detection of covalent binding by immunoblotting.

Among 4 nevirapine treated-rats, 2 rats developed erythema at 4 and 6 weeks, respectively. The animals demonstrated excessive scratching around the
nose/mouth area, loss of body weight and dehydration. Using anti-
hydroxynevirapine-NAC-KLH antiserum, it was possible to show that
hydroxynevirapine covalently bound to polypeptides in the liver homogenate of
rats dosed with nevirapine (100 mg/kg/day) but not the controls that were given a
regular diet. The major hydroxynevirapine-polypeptide adduct had a molecular
mass of 29 kDa, which was mostly concentrated in the cytosolic fraction (Figure 4-
14A). It was also possible to inhibit binding of the antiserum to this band by
preincubating it with the 3-hydroxynevirapine-NAC conjugate (50 μM) before
adding the antiserum to the nitrocellulose (Figure 4-14B). With this antiserum,
bands due to non-specific binding appeared in the controls, but those bands could
not be inhibited by preincubating the antiserum with 3-hydroxynevirapine-NAC
conjugate (Figure 4-14). Using this antiserum, the covalent binding of
hydroxynevirapine to polypeptides in skin homogenate of drug-treated rats could
also be demonstrated (Figure 4-15). There were even more antibodies that bound to
endogenous skin proteins. The drug metabolite-modified cellular polypeptides in
the skin had molecular masses of 54, 58, and 65 kDa. Furthermore, liver necrosis of
the drug-treated rats was demonstrated histologically (Figure 4-16).
4.5 Discussion.

It is generally accepted that bioactivation of drugs to their reactive metabolites and subsequent covalent binding to cellular proteins are necessary steps in the induction of idiosyncratic drug reactions. The idiosyncratic nature of nevirapine-induced cutaneous reactions, hepatotoxicity and granulocytopenia suggests the involvement of reactive metabolites in these adverse reactions. In this study, we demonstrated that nevirapine can be bioactivated by, and subsequently bound to, PMA-activated human neutrophils (Figure 4-2). Furthermore, covalent protein binding of nevirapine to protein was also observed upon activation by MPO, the major enzyme in neutrophils, in the presence of hydrogen peroxide and chloride (Table 4-1). However, at a therapeutic concentration, the covalent binding to either cells or proteins was much lower than we see for many drugs associated with agranulocytosis (Liu & Uetrecht, 1995; Uetrecht et al., 1994).

Nevirapine was also oxidized by HOCl, the major oxidant released by activated neutrophils and MPO enzyme system, to hydroxylated products including 2- and 3-hydroxynevirapine (Figure 4-3). Their identities were confirmed by comparison with authentic standards. The formation of these hydroxy metabolites has been reported as part of the major metabolic pathway of nevirapine in vivo (Riska et al., 1999). Upon oxidation by HOCl, the major reactive intermediate of nevirapine had a molecular ion of m/z 317, which corresponds to further chlorination of hydroxynevirapine (Figure 4-4). Apparently, this chlorinated reactive intermediate gradually lost the chlorine and spontaneously converted to
another reactive species with a molecular ion of m/z 281, which is 2 mass units less than that of hydroxynevirapine (m/z 283) and may represent an oxidative form of hydroxynevirapine. This conversion could also be directly monitored by scanning the collected HPLC fraction containing the reactive intermediates with a diode array spectrophotometer. This experiment showed that the $\lambda_{\text{max}}$ changed from 290 nm to 320 nm (Figure 4-5). Such a red-shift in UV spectrum is consistent with the formation of a species with a more highly conjugated system. We think that this observation can be considered as evidence for the formation of an iminoquinone species (Figure 4-17). The estimated half-life for the chlorinated reactive intermediate is about 4 - 5 min, and at the end of the experiment only the reactive species with a molecular ion of m/z 281 could be detected by mass spectrometry.

In the absence of other nucleophiles, the reactive intermediate of nevirapine reacted with either water or chloride to form several more stable products (data not shown). However, it more preferentially reacted with sulfhydryl-containing nucleophiles, such as NAC or GSH. Upon addition of NAC, the reactive intermediate could be trapped as a bright yellow hydroxynevirapine-NAC conjugate with a molecular ion of m/z 444. This observation is consistent with the reactivity of other iminoquinone species, which are generally "soft" electrophiles and selectively react with "soft" nucleophiles in preference to water and chloride (Lai et al., 1999; Liu & Uetrecht, 1995). The major fragment in the LC/MS/MS spectrum of this hydroxynevirapine-NAC conjugate was m/z 315 (Figure 4-7), which corresponds to the loss of the portion of the NAC moiety without sulfur. This MS/MS fragmentation pattern is characteristic for a NAC adduct with NAC attached
to an aromatic ring. Furthermore, the proton NMR spectrum (Figure 4-8) clearly indicates that all aromatic protons are present on the same pyridine ring. This is based on the interpretation that the peak at δ 7.58 ppm represents H-8, which is split by H-9 with a coupling constant of 7.88 Hz and by H-7 with a smaller coupling constant of 4.76 Hz. The coupling constant between H-9 and H-7 is the smallest, 1.83 Hz. This assignment was confirmed by the proton-proton correlation NMR experiment (Figure 4-9). This leads to the conclusion that both the OH and NAC groups must be on the same pyridine ring as the methyl group, although this does not determine whether the NAC is on the 2 or 3 position.

Our experimental results suggest that bioactivation of nevirapine itself may involve two steps. Nevirapine is first oxidized to hydroxylated metabolites, which can be subsequently activated to reactive iminoquinone intermediates (Figure 4-17). This would explain the low, but significant, protein binding of radiolabeled nevirapine to activated neutrophils and human MPO. However, our attempts to obtain the same NAC conjugate starting from either 2- or 3-hydroxynevirapine directly after HOCl oxidation were not successful. Upon oxidation by PMA-activated neutrophils, the MPO enzyme system or HOCl, 3-hydroxynevirapine produced a stable chlorinated product with a molecular ion of m/z 317, which was unreactive toward both "hard" and "soft" nucleophiles. Compared with the proton NMR of 3-hydroxynevirapine, the stable chlorinated species lacked the lone aromatic proton signal at δ 7.8 ppm. This observation, as well as the mutual doublet-of-doublet coupling among the three remaining protons on the other aromatic ring, indicates that the chloro group must be attached to the 2 position on the same pyridine ring as
the hydroxyl and the methyl groups. Apparently, when 3-hydroxynevirapine is oxidized by HOCl, chloride ion can compete more efficiently for the reactive intermediates than NAC or GSH.

However, the hydroxynevirapine-NAC conjugate can be obtained by incubating 3-hydroxynevirapine with rat-liver microsomes in the presence of NAC. The NAC conjugate obtained in this way had the same HPLC retention time and identical MS/MS fragmentation pattern as the one obtained by oxidizing nevirapine directly with HOCl (Figure 4-11). Furthermore, co-injecting the two conjugates gave a single peak on HPLC. These results indicate that they are the same compound. The generation of this hydroxynevirapine-NAC conjugate from 3-hydroxynevirapine indicates that the NAC is bound to position 2 (Figure 4-7). This result also implies the formation of the proposed reactive quinoneiminium ion in the liver. Therefore, covalent binding of bioactivated 3-hydroxynevirapine to hepatic proteins is speculated to be responsible for the drug-induced idiosyncratic hepatotoxicity.

By trapping the reactive metabolite of 3-hydroxynevirapine with NAC and then coupling this conjugate to KLH, it was possible to produce an immunogen that subsequently was used to raise a polyclonal antiserum. The resultant antiserum displays significant antibody titers against the hydroxynevirapine epitope as shown by ELISA using hydroxynevirapine-NAC-modified RSA against native RSA (Figure 4-13). However, the antigen-specific antibody titers are relatively low and there are several antibodies that bind to endogenous proteins.
Treating female Sprague Dawley rats with a high dose of nevirapine (100 mg/kg/day), 50% of drug-treated animals (2/4) developed significant adverse drug reactions including skin rash and hepatotoxicity. For those rats showing nevirapine-induced hepatotoxicity, the liver histology demonstrated diffuse hepatocellular cytomegaly and peribiliary fibrosis as well as significant proliferation of peroxisomes (Figure 4-16). Immunoblots, using the anti-hydroxynevirapine-NAC-KLH antiserum that we developed, demonstrated that hydroxynevirapine became covalently bound to the hepatic protein of drug-treated rats. The major hydroxynevirapine-polypeptide adduct of approximately 29 kDa was demonstrated to be mostly concentrated in the liver cytosolic fractions and little can be detected in the liver microsomal fractions (Figure 4-14A). This result was unexpected because the bioactivation of 3-hydroxynevirapine in vitro is mediated by hepatic microsomes in a NADPH-dependent manner. However, our in vitro experiment does not exclude the possibility that either 3-hydroxynevirapine or nevirapine itself may be bioactivated by cellular enzyme systems other than cytochrome P450s in hepatic microsomes. Even if the bioactivation is predominantly mediated by cytochrome P450s, the proposed quinoneiminium ion, with a reasonably long half-life, may diffuse into cytosol and bind to various protein targets there.

Glutathione S-transferases (GSTs) are a group of hepatic enzymes that are abundant in liver cytosolic fraction. The major GST sub-classes located in the cytosol include both homo- and hetero-dimeric complexes formed from GST subunits that range in molecular masses from 23 to 28 kDa (Salinas & Wong, 1999). GSTs are reported to be sensitive to irreversible inactivation by quinones and
quinone methides as a result of covalent modification. As an example, eugenol can be oxidized by tyrosinase to a quinone methide, which was shown to covalently bind and inactivate liver cytosolic GSTs in humans, rats and mice (Rompelberg et al., 1996). Furthermore, it has been reported that tetrachloro-1,4-hydroquinone can be oxidized by NADPH-dependent liver microsomal enzymes to form tetrachloro-1,4-benzoquinone, which covalently modify and irreversibly inhibit cytosolic GSTs (van Ommen et al., 1988a; van Ommen et al., 1988b). It is also conceivable that the proposed quinoneiminium reactive metabolite generated by liver cytochrome P450s from 3-hydroxynevirapine can covalently bind and inactivate cytosolic GSTs. Since GSTs are a group of major hepatic enzymes involved in detoxification of reactive metabolites of xenobiotics (Parkinson, 1996), their inactivation may potentially cause accumulation of reactive metabolites in the liver and subsequently result in hepatotoxicity.

Using the anti-hydroxynevirapine-NAC-KLH antiserum, we also demonstrated binding of a nevirapine-derived metabolite to rat skin. The major skin polypeptides affected by nevirapine treatment have molecular masses from 54 to 65 kDa. However, due to non-specific binding similar, but less dense bands, were also observed in the controls. In order to obtain clearer results, we plan to purify the antiserum by affinity chromatography.

We have shown that nevirapine can be bioactivated through one of its major hepatic metabolites, 3-hydroxynevirapine. The proposed reactive metabolite, a quinoneiminium ion, may be responsible for covalent modification of proteins, both in vitro and in vivo. The relationship between this covalent protein binding
and drug-induced idiosyncratic reactions is still not clear. However, generation of reactive metabolites by a certain tissue or organ, such as neutrophils or liver, has been implicated in the etiology of the adverse drug reactions associated with that tissue or organ (Cribb et al., 1996; Gardner et al., 1998a; Liu & Uetrecht, 1995; Uetrecht, 1990; Uetrecht et al., 1994). Therefore, we speculate that the bioactivation of 3-hydroxynevirapine in neutrophils, or its precursors in the bone marrow, is responsible for nevirapine-induced granulocytopenia. Likewise, such bioactivation in the liver is likely to be responsible for drug-induced hepatitis. Although low in concentration, the skin contains many metabolic enzymes, including cytochrome P450s and peroxidases (Kao & Carver, 1990; Mukhtar & Khan, 1989; Pannatier et al., 1978). Hence, it is possible that 3-hydroxynevirapine can be bioactivated in the skin and bind locally. Alternatively, it is even possible that the quinoneiminium metabolite formed in the liver may have a sufficiently long half life in the circulation to reach the skin and covalently bind to proteins. In either case, the reactive metabolite-modified proteins may cause cutaneous reactions in some individuals.
Figure 4-1. Major *in vivo* metabolic pathways of nevirapine (NVP).
Figure 4-2. Covalent binding of $^{14}$C-labeled nevirapine to neutrophils without activation (- PMA), with activation (+ PMA) or with activation but using heat-denatured cells (D/N). P < 0.05 for + PMA vs - PMA; P < 0.09 for + PMA vs D/N.
Figure 4-3. LC/MS spectrum of the reaction mixture obtained from HOCI oxidation of nevirapine (1 mM, 1:1) in 0.1 M phosphate buffer (pH 6). With computer extraction for ions of hydroxynevirapines (m/z 283) and of unreacted nevirapine (m/z 267). The HPLC mobile phase was water:acetonitrile:acetic acid (65:35:1, v/v) with 2 mM ammonium acetate.
Figure 4-4. LC/MS spectrum of the reaction mixture obtained from HOCl oxidation of nevirapine (1 mM, 1:1) in 0.1 M phosphate buffer (pH 6). The ions of m/z 281 and m/z 317 were computer extracted from data. The HPLC mobile phase was water:acetonitrile:acetic acid (65:35:1, v/v) with 2 mM ammonium acetate.
Figure 4-5. Repetitive UV-VIS absorption spectra of reactive intermediates of nevirapine formed by its HOCl oxidation. The chlorinated intermediate (m/z = 317) was collected from HPLC with a mobile phase of H$_2$O:CH$_3$CN:HOAc=65:35:1 with 2 mM NH$_4$OAc and flow rate of 1 mL/min resulting in a retention time of 10.4 min. Its UV-VIS spectra were immediately obtained at 60-s intervals. Its conversion to another reactive intermediate (m/z = 281) was accompanied by a change of $\lambda_{\text{max}}$ from 290 nm to 320 nm. The dashed line represents the UV spectrum of nevirapine in the same solvent.
Figure 4-6. Proton NMR (200 MHz) spectrum of nevirapine. The solvent is $\text{C}_2\text{HCl}_3$. 
Figure 4-7. MS/MS spectrum and proposed structure of the hydroxynevirapine-NAC conjugate (m/z 444) generated by oxidation of nevirapine with HOCl (A) and by oxidation of 3-hydroxynevirapine with hepatic microsomes (B).
Figure 4-8. Proton NMR (500 MHz) spectrum of the hydroxynevirapine-NAC conjugate (m/z 444). The solvent is $^2$H$_2$O.
Figure 4-9. Proton COSY (500 MHz) spectrum of the hydroxynevirapine-NAC conjugate (m/z 444). The solvent is $^2$H$_2$O.
Figure 4-10. Proton NMR (200 MHz) spectrum of the stable chlorinated product (m/z 317) generated by oxidation of 3-hydroxynevirapine with HOCl and its proposed structure. The solvent is C₂HCl₃.
Figure 4-11. LC/MS-SIM spectra of hydroxynevirapine-NAC (m/z 444) generated from: A) incubation of 3-hydroxynevirapine with rat-liver microsomes in presence of a NADPH-generating system and NAC; and B) oxidation of nevirapine by HOCl and then addition of NAC.
3-Hydroxynevirapine-NAC adduct

1) EDC
2) H$_2$N-Protein

3-Hydroxynevirapine-NAC-protein adduct

Protein: KLH or RSA

**Figure 4-12.** Formation of the hydroxynevirapine-NAC-protein adduct.
Figure 4-13. ELISA analysis showing binding of the anti-hydroxynevirapine-NAC-KLH antiserum to wells of microtiter plates coated with the hydroxynevirapine-NAC-RSA adduct (circles) or native RSA (squares).
Figure 4-14. Immunochemical detection of covalent binding of 3-hydroxynevirapine to the complete hepatic homogenate (homo), or the cytosol (cyto), or the microsome (micro) fractions of the drug-treated rats (3 and 4; 100 mg/kg/day, 6 and 4 weeks) compared with the control rats (1 and 2). The blots were incubated with the anti-hydroxynevirapine-NAC-KLH antiserum alone (A) or with antiserum that was preincubated with the 3-hydroxynevirapine-NAC adduct (B, 50 μM). The anti-hydroxynevirapine-NAC-KLH antiserum dilution was at 1:2,000 and the secondary goat anti-rabbit antiserum dilution was at 1:5,000. Protein loading was 30 μg/lane.
Figure 4-15. Immunochemical detection of covalent binding of 3-hydroxynevirapine to skin homogenate of the drug-treated rats (3 and 4; 100 mg/kg/day, 6 and 4 weeks) compared with the control rats (1 and 2). The anti-hydroxynevirapine-NAC-KLH antiserum dilution was at 1:2,000 and the secondary goat anti-rabbit antiserum dilution was at 1:5,000. Protein loading was 30 µg/lane.
Figure 4-16. Histological examination of liver samples from the nevirapine-treated rats (B and C, 100mg/kg/day, 6 and 8 weeks) in comparison with the control rats (A).
**Figure 4-17.** Proposed bioactivation pathway for nevirapine and 3-hydroxynevirapine.
Chapter 5

Summary, Future Studies and Discussion.
5.1 Summary.

Using mainly an *in vitro* approach, the studies described in this thesis investigated the bioactivation pathways for trimethoprim, fluperlapine and nevirapine. These pathways, which lead to the formation of reactive metabolites, may contribute to the occurrence of idiosyncratic reactions associated with these drugs. In order to address these adverse drug reactions involving with a specific tissue or organ, metabolism of the drugs by the target tissue or organ was investigated.

We have demonstrated that trimethoprim, a commonly used antibiotic, is metabolized by activated human neutrophils or human and rat hepatic microsomes to a reactive pyrimidine iminoquinone methide metabolite. This metabolite can be trapped by NAC, a sulfhydryl-containing nucleophile. Using various spectroscopic methods, we determined the structures of the trimethoprim-NAC adducts, and the results support the proposed structure of the reactive intermediate. Using the major trimethoprim-NAC adduct, we also prepared an immunogen which was subsequently used to develop an anti-trimethoprim antiserum. Immunoblotting studies using this antiserum implies that the proposed reactive metabolite of trimethoprim can covalently modify neutrophil proteins. Using the immunochemical techniques that we developed, it was also possible to detect similar trimethoprim-modified proteins in the bone marrow cells of rats treated with trimethoprim.
We have demonstrated that 7-hydroxyfluperlapine, the major hepatic metabolite of the antipsychotic fluperlapine, can be bioactivated by neutrophils to a reactive iminoquinone species. Our experiments further demonstrate that the major enzyme in neutrophils, MPO, as well as the major oxidant produced by this enzymatic system, HOCl, are responsible for the formation of the proposed reactive metabolite. Using antiserum previously developed against clozapine, a structural analogue of fluperlapine, we have shown that the iminoquinone reactive metabolite of hydroxyfluperlapine can covalently bind to neutrophil proteins \textit{in vitro}. Furthermore, this reactive metabolite is highly reactive toward sulphydryl-containing nucleophiles, such as NAC or GSH, which effectively inhibits the \textit{in vitro} protein binding at a physiological concentration. The results suggest the interindividual differences in protein binding and GSH detoxification of the proposed reactive metabolite could be a risk factor for the development of fluperlapine-induced idiosyncratic agranulocytosis. This study also suggests that during investigation of the mechanism of an idiosyncratic drug reaction, in addition to testing the ability of the drug to form a reactive metabolite, attention should also be paid to the further metabolism of major stable metabolites of the drug.

For nevirapine, we have followed a similar research strategy and demonstrated that the radiolabeled drug can be bioactivated by neutrophils or MPO, presumably in two steps, to an iminoquinone reactive metabolite, which can covalently bind to neutrophils or proteins such as BSA. Using HOCl, the major oxidant produced by activated neutrophils, the reactive metabolite generated by nevirapine was trapped by NAC to form a hydroxynevirapine-NAC adduct. The
same NAC adduct was also detected when 3-hydroxynevirapine, the major hepatic metabolite of nevirapine, was metabolized by rat hepatic microsomes in the presence of NAC. Using this 3-hydroxynevirapine-NAC adduct, we also prepared an immunogen which was subsequently used to produce an anti-hydroxynevirapine antiserum. Immunoblotting studies using this antiserum demonstrated nevirapine-modified hepatic and skin proteins in nevirapine-treated rats, presumably involving the proposed iminoquinone reactive metabolite.

In general, the studies included in this thesis demonstrate that three structurally and pharmacologically unrelated drugs all can be bioactivated to iminoquinone-type reactive metabolites. These metabolites are Michael acceptors, which are reactive due to their electron-deficiency. Therefore, many electron-rich biological macromolecules, such as proteins and nucleic acids, may readily form covalent bonds with these reactive metabolites. The iminoquinone-type Michael acceptors generated by the three mentioned drugs are shown to be "soft" electrophiles, which tend to react with "soft" nucleophiles, such as NAC, GSH or sulfhydryl-containing proteins. The iminoquinone-type Michael acceptors are common reactive metabolites of drugs, since many drugs have the structural features which can potentially generate them by either enzymatic or non-enzymatic oxidation. These potential structural features include an aromatic ring with amino- or hydroxy- groups located at appropriate positions (para- or ortho-), which is very common among drugs and drug candidates. Furthermore, one of the major functions of hepatic cytochrome P450 enzymes, the enzymes predominantly involved with drug metabolism, is hydroxylation of an aromatic carbon. This
function makes many major hepatic metabolites of relatively "inert" drugs acquire the structural features that may potentially generate iminoquinone- and quinone-type reactive metabolites by further bioactivation. In addition to further oxidation in the liver, as the hepatic metabolites get into the circulation, further bioactivation may also occur in other tissues or organs, such as the blood or the skin. Therefore, this particular type of reactive metabolite may play important roles in many drug-induced idiosyncratic reactions.
5.2 Future studies.

The current studies have demonstrated the bioactivation of drugs or drug candidates to reactive metabolites, which is often considered as the first step in the initiation of idiosyncratic drug reactions. The reactive metabolites, such as the iminoquinones, can covalently bind to cellular macromolecules such as protein and nucleic acids, resulting in direct cellular damage. Autoimmune responses may also be elicited against the reactive metabolite-altered macromolecules, resulting in immune-mediated cellular damage. Therefore, it would be important for the future studies to identify the reactive metabolite-modified proteins in humans and relate their functions to the observed idiosyncratic reactions associated with trimethoprim, fluperlapine and nevirapine. For this purpose, further studies may involve affinity purification of the antisera and investigation of binding of the reactive metabolites in patients who developed adverse reactions against the drugs.

The current in vivo experiments on nevirapine also demonstrated the possibility of developing an animal model for the major adverse reactions associated with nevirapine, including skin rash and hepatitis. It will be important for further studies to identify the reactive metabolite-modified proteins in the liver and skin of the drug-treated animals and/or humans. It will also be essential to exploit the potential animal model to address the enzyme system(s) that may be responsible for bioactivation of nevirapine and 3-hydroxynevirapine in skin. The animal model can also be used to investigate the relevance of the immune system in nevirapine-induced toxicity.
5.3 Discussion.

Although idiosyncratic drug reactions only account for a small portion of all adverse drug reactions, they have become a major concern in both clinical practice and drug development due to their high mortality and unpredictable nature (Park et al., 1992; Rieder, 1994; Uetrecht, 1992). The mechanisms of many idiosyncratic drug reactions are poorly understood. However, many researchers have pointed out the important roles that reactive metabolites appear to play in these severe drug reactions (Pirmohamed et al., 1994; Pohl et al., 1988; Rieder, 1993; Uetrecht, 1990).

Generally, drug metabolism is considered as a detoxification process. However, during the course of being converted to harmless hydrophilic compounds, most drugs undergo some degree of bioactivation to reactive metabolites. The reactive metabolites generated by bioactivation are capable of covalent binding to cellular macromolecules as demonstrated in this thesis. Such covalent binding may result in either direct or immune-mediated toxicity. With direct toxicity, the reactive metabolite-modified cellular protein may interfere with the essential physiological function of the cell and cause cellular necrosis. Alternatively, the reactive metabolite-conjugated protein may initiate an autoimmune response that leads to tissue damage. However, applying the principles of the danger hypothesis, the boundary between direct and immune-mediated toxicity becomes blurred (Matzinger, 1998; Park et al., 1998; Pirmohamed et al., 1996; Uetrecht, 1999). Nevertheless, reactive metabolites retain a central role in the hypotheses of the mechanisms of most idiosyncratic drug reactions. Although
one can presume that reactive metabolites are generated, at least to some extent, in all the patients, only a faction of them will develop significant adverse drug reactions. The idiosyncratic characteristics of these reactions may have their basis on the individual differences in drug metabolism, system response to the reactive metabolite-induced stress and induction of an immune response. Therefore, in order to develop a safer therapeutical agent, it would be beneficial to address all these issues.

In the pharmaceutical industry, drug metabolism has undergone a realignment of its traditional role, that of drug development support, to that of facilitation of drug discovery. High throughput screening (HTS) technologies and combinatorial chemistry have been able to provide a tremendous number of candidates for therapeutic agents. Hence, to innovative pharmaceutical manufacturers, it is an essential task for drug metabolism studies to identify the candidates that are capable of generating potentially problematic reactive metabolites. At the current stage, successfully screening against the potentially dangerous leading compounds and preventing them from further development should not only reduce the overall clinical incidence of idiosyncratic drug reactions but also save the manufacturer's resources for the development of more promising and safer leading compounds. For example, it has been demonstrated previously, and again in this thesis, that many drugs associated with a high incidence of agranulocytosis are also oxidized to reactive metabolites by activated neutrophils (Lai et al., 1999; Liu & Uetrecht, 1995; Uetrecht, 1990; Uetrecht, 1992; Uetrecht et al., 1994). Therefore, it is conceivable to screen drug candidates for the formation of reactive metabolites using neutrophils,
myeloperoxidase, or hypochlorous acid in order to pick out those that may cause idiosyncratic agranulocytosis in the future.

However, not all drugs that generate reactive metabolites in vitro are associated with a high incidence of idiosyncratic drug reactions. Therefore, by screening drug candidates based solely on the formation of reactive metabolites, the process would eliminate many good candidates, which would have been valuable drugs. In addition, some reactive metabolites are impossible to design out of a lead compound because they are required for drug efficacy. Furthermore, it is always difficult to directly apply in vitro drug metabolism data to the in vivo situation. A drug candidate may form many reactive metabolites in vitro but none of them may be the major pathway in vivo, or vice versa. A more difficult problem would be to identify the individual difference in the response toward the same reactive metabolite in vivo in terms of bioactivation, detoxification, cell stress and immunology. With the advances of proteomics and genomics, it may now be possible to address these in vivo factors that govern the individual response to a drug candidate that shows evidence of reactive metabolite formation in vitro. Proteomics can directly detect changes in the expression of protein, while genomics detects changes in the expression of mRNA for the protein. If a specific pattern of protein and/or mRNA expression can be found in correlation with idiosyncratic drug reactions in the drug-treated experimental animals or humans, this would be an important complement to the in vitro screening of drug metabolism (Uetrecht, unpublished manuscript).
An understanding of drug metabolism is also important for the determination of the chemical mechanisms of toxicity of a drug that may lead to various ways to predict or even prevent the idiosyncratic reactions associated with a currently marketed drug. The knowledge obtained from the studies of the bioactivation of drugs can be used to help the design of safer therapeutic agents. As an example, the bioactivation of halothane gives the reactive trifluoroacetyl chloride, which binds covalently to proteins (Pohl et al., 1988). By replacing bromide with difluoromethoxy group, isoflurane forms the same reactive metabolite but the extent is much less (~100 folds less than halothane). Presumably because of this, isoflurane, although equally potent as an anesthetic, is associated with a significantly lower incidence of hepatitis (Njoku et al., 1997). Identification of major bioactivation pathways that are associated with certain idiosyncratic drug reactions may also provide a way to reduce these reactions by inhibiting the responsible pathways. For instance, if one of a drug’s hydroxy metabolites, generated by hepatic cytochrome P450 3A4, were shown to be further bioactivated to problematic reactive metabolites, by giving the patients grapefruit juice, which is a potent inhibitor of the enzyme (He et al., 1998), it may be possible to avoid the idiosyncratic drug reactions induced by this metabolic pathway.

Reactive metabolites are normally transient and readily detoxified by conjugating to more polar species, such as glutathione, glucuronate, sulfate or amino acids. In the majority of the population, balance can be fairly well maintained between bioactivation and detoxification of a certain drug, so that the amount of reactive metabolite does not buildup to a “toxic level” and cause adverse
reactions. However, in certain individuals, an imbalance between drug bioactivation and detoxification may occur due to some unforeseen factors, and the factors may be genetic or environmental. In these individuals, the inadequate detoxification or abnormally rapid bioactivation may cause the accumulation of the reactive metabolite in excess of the "toxic level" and induce adverse drug reactions that are not normally seen in the general population (Pirmohamed et al., 1996). Studies have shown that certain genetic defects in detoxification enzymes may enhance the incidence of some adverse drug reactions (De Morais et al., 1992; De Morais et al., 1992). From the standpoint of a patient, in order to avoid idiosyncratic drug reactions, it would be helpful for drug metabolism studies to identify the individuals who are genetically susceptible to these reactions. This would not only reduce the risk associated with drug treatment, but it also might allow the re-introduction of some unique drugs or drug candidates that have been withdrawn due to their toxicity to a certain sub-group of the population. With the rapid progress in the understanding of genetic polymorphism and the development of genechip technology, it is feasible to genotype individuals with respect to critical genes that may be associated with the induction of idiosyncratic drug reactions. These genes may control the expressions of the factors that are associated with the individual pharmacokinetic and/or pharmacodynamic profile of the medication. These factors can include drug receptors or transporters, drug bioactivation or detoxification enzymes, cellular stress proteins or proteins essential for the induction of auto-immune responses. In the future, each individual could carry a "gene-card" with these vital genetic information that may govern the occurrence of
adverse drug reactions. The identification of the key genetic variations would have
tremendous impact on the way we use medications as well as drug safety (Lu, 1998).
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