REACTIVE IMINOQUINONE METABOLITES OF INDOMETHACIN AND CARBAMAZEPINE

Implications for drug-induced idiosyncratic reactions

by

Changqing Ju

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Faculty of Pharmacy
University of Toronto

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ABSTRACT

Indomethacin is a potent nonsteroidal anti-inflammatory drug. Its use has been limited because of a relatively high incidence of adverse reactions including agranulocytosis. Carbamazepine is an anticonvulsant and its use is associated with a range of serious idiosyncratic adverse reactions including blood disorders. We postulated that indomethacin and carbamazepine can be oxidized to reactive intermediates by myeloperoxidase in activated neutrophils, which may be responsible for the adverse effects caused by these two drugs.

We found that desmethyldeschlorobenzoylindomethacin (DMBI), a major metabolite of indomethacin, was oxidized to a reactive iminoquinone intermediate. This iminoquinone intermediate could be trapped with glutathione (GSH) and N-acetylcysteine (NAC) to form conjugates. The same oxidation was observed when DMBI was oxidized by the MPO system or hypochlorous acid. The iminoquinone intermediate with a MH⁺ ion at m/z 204 was directly detected by mass spectrometry in the reaction between DMBI and hypochlorous acid.

In the urine of patients taking carbamazepine, we detected 2-hydroxyiminoestilbene as its glucuronide conjugate and found that it is a major metabolite of carbamazepine. We
have also demonstrated that 2-hydroxyiminostilbene can undergo autoxidation to a reactive iminoquinone intermediate. The iminoquinone intermediate reacts with sulfhydryl-containing nucleophiles, such as GSH and NAC. To further investigate the role of the iminoquinone intermediate in carbamazepine-induced adverse reactions, we developed a polyclonal antibody against the iminoquinone epitope and used it to study covalent binding of the iminoquinone to proteins. The antibodies were able to detect covalent binding in bone marrow cells of rats treated with carbamazepine in vivo. This binding was inhibited by 2-hydroxyiminostilbene but not by carbamazepine or iminostilbene, which suggested that the polypeptides recognized by the antibodies were likely to be covalently modified by the iminoquinone reactive intermediate. Furthermore, we also observed increased protein oxidation in the liver from carbamazepine-treated rats compared to that of control rats. This implied that the iminoquinone, as an oxidizing agent, may undergo redox cycling and lead to protein oxidation in vivo.

Our studies suggest that the iminoquinone intermediates may react with protein sulfhydryl groups in vivo and play an important role in indomethacin- and carbamazepine-induced idiosyncratic reactions.
ACKNOWLEDGMENTS

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<td>ANA</td>
<td>antinuclear antibody</td>
</tr>
<tr>
<td>BP</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>CGS12094</td>
<td>para-hydroxy metabolite of prinomide</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DBI</td>
<td>deschlorobenzoylindomethacin</td>
</tr>
<tr>
<td>DIL</td>
<td>drug-induced lupus</td>
</tr>
<tr>
<td>DMBI</td>
<td>desmethyldeschlorobenzoylindomethacin</td>
</tr>
<tr>
<td>DMI</td>
<td>desmethylinidomethacin</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration of U.S.</td>
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<td>GSH</td>
<td>glutathione</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HSR</td>
<td>hypersensitivity syndrome reactions</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<tr>
<td>LC/MS</td>
<td>liquid chromatography/Mass spectrometry</td>
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<tr>
<td>MH+</td>
<td>protonated molecular ion</td>
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<td>MHC</td>
<td>major histocompatibility genes</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<td>NAC</td>
<td>n-acetylcytsteine</td>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
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<tr>
<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PHS</td>
<td>prostaglandin H synthase</td>
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<td>RSA</td>
<td>rabbit serum albumin</td>
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<td>SJS</td>
<td>Stevens-Johnson syndrome</td>
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<td>SLE</td>
<td>systemic lupus erythematos</td>
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<td>SSLR</td>
<td>serum sickness-like reactions</td>
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<tr>
<td>TCPO</td>
<td>trichloropropene oxide</td>
</tr>
<tr>
<td>TEN</td>
<td>toxic epidermal necrolysis</td>
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<tr>
<td>TIC</td>
<td>total ion current</td>
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Chapter 1

General Introduction
1.1 An overview of research objectives and rationale

Adverse drug reactions represent a significant health problem. In a recent study by Bruce Pomeranz and Jason Lazarou (Lazarou et al., 1998), they estimated that adverse drug reactions kill an average of 100,000 people annually in the U.S., making them the fourth leading cause of death, after heart disease, cancer and stroke (The Financial Post Magazine, June 1998 Page 9). Some of the adverse drug reactions are preventable, in principle, because they are due to known pharmacological effects of the drugs and would be expected to occur in most patients at a sufficiently high dose. Another type of adverse drug reaction is the so-called "idiosyncratic reaction" for which the mechanism is mostly unknown. Idiosyncratic drug reactions, which account for approximately 10% of all adverse drug reactions (Goldstein and Patterson, 1984), are often quite severe and sometimes life threatening. Due to their unpredictable nature, drug-induced idiosyncratic reactions have posed a difficult problem, both in medical practice and for present and future drug development. Although the mechanisms of most drug-induced idiosyncratic reactions are unknown, a large number of studies have suggested that reactive metabolites are involved. Several clinical characteristics of this type of adverse reaction also suggest the involvement of the immune system.

Indomethacin and carbamazepine have been shown to cause idiosyncratic reactions including drug-induced agranulocytosis. The formation of reactive metabolites is thought to be a necessary step in the pathogenesis of these idiosyncratic reactions. Although liver is the major organ responsible for drug metabolism, short-lived reactive metabolites generated in the liver may not be able to escape the liver and induce clinical manifestations in other areas of the body which are involved in idiosyncratic drug reactions. Therefore, it is important to investigate the formation of reactive metabolites in the target organs of drug-induced adverse reactions. In indomethacin- and carbamazepine-induced hematological...
disorders reactive metabolites are more likely to be generated by the oxidative myeloperoxidase system in neutrophils or other cells in the bone marrow than in the liver.

The objectives of this project were: a) to determine if indomethacin and carbamazepine can be oxidized by myeloperoxidase in activated neutrophils; b) to identify the reactive intermediates generated during the oxidation and characterize their chemical reactivity as electrophiles; c) and to further investigate the possible role of the reactive intermediates in the mechanism of idiosyncratic drug reactions.
1.2 Hypothesis

Reactive iminoquinone metabolites are generated during the oxidation of indomethacin and carbamazepine in the targets of drug-induced toxicity. These reactive iminoquinone intermediates play an important role in the mechanism of indomethacin- and carbamazepine-induced idiosyncratic reactions via covalent binding to endogenous proteins that induce an immune reaction.
1.3 Drug-induced idiosyncratic reactions

An adverse drug reaction is defined as any undesirable response to a drug that occurs at therapeutic doses (Park and Kitteringham, 1990). Adverse drug reactions are major complications of modern drug therapy; they account for significant patient morbidity and death (Park et al., 1992). Drug-induced adverse reactions can be classified into predictable reactions and unpredictable reactions (Rieder, 1994). Predictable reactions include toxicity from overdose; drug-drug or drug-disease interactions; and side effects that occur at usual therapeutic doses such as nausea and dizziness. These reactions can often be predicted from the chemical properties and the pharmacology of the drug; they are often host-independent, dose-dependent; and usually reproducible in animals (Pohl et al., 1988). Such reactions should be anticipated and can often be eliminated by dose reduction. Unpredictable reactions include hypersensitivity (or "allergic") reactions and idiosyncratic reactions. The terms "drug hypersensitivity reaction" and "idiosyncratic drug reaction" have been used interchangeably, for example, "drug-induced hypersensitivity reactions" has been used to describe systemic idiosyncratic reactions with fever, rash and multiorgan involvement. Ideally, "hypersensitivity reaction" should be used to describe adverse reactions associated with drug therapy which have a known immunological etiology; however, The term "idiosyncratic drug reactions" will be used, in this thesis, to describe reactions with common characteristics that include:

- Occurrence in a relatively small number of patients. These reactions have been estimated to account for 6% to 10% of all adverse drug reactions caused by a total of 90 million courses of drug therapy per year among medical inpatients (Goldstein and Patterson, 1984)

- An apparent lack of dose-response curve for the risk of toxicity. In principle these reactions must be dose-dependent; however, in many cases the dose that can cause toxicity is much smaller than the dose for desired pharmacological effect.
• An unpredictable nature and lack of animal models. Idiosyncratic reactions occur in humans with a relatively low incidence; therefore it is difficult to reproduce the same reactions in laboratory animals, which are inbred strains, even if a large number of animals are tested.

• Involvement of specific organs, such as the skin, bone marrow and liver. These reactions are often limited to specific organs, although generalized reactions may also occur in which there is fever, skin rash and lymphadenopathy, along with involvement of specific organs.

1.3.1 Clinical manifestations of drug-induced idiosyncratic reactions

The patterns of idiosyncratic reactions are variable depending on both the patients and the drugs. These reactions, although not very common, can be life-threatening and have resulted in removal from the market of many potentially useful drugs. For example, practolol, benoxaprofen, ticrynafen, zomepirac and nomifensine were withdrawn shortly after they were released because of an unacceptable risk of toxicity (Uetrecht, 1990). The most commonly occurring idiosyncratic drug reactions involve the skin, various elements of the blood and the liver. The kidney and nervous system may also be affected and autoimmune syndromes such as drug-induced lupus can also occur.

1.3.1a Systemic reactions

In some cases idiosyncratic reactions show a remarkable degree of organ specificity. In other cases, multiple organs may be affected simultaneously, such as in hypersensitivity syndrome reaction (HSR), serum sickness-like reaction (SSLR) and drug-induced lupus (DIL).
HSRs are serious and possibly life-threatening reactions that usually occur 2 to 4 weeks after the start of therapy. Although this type of reactions is termed "hypersensitivity syndrome", their mechanisms are not fully understood. Drugs that are most often associated with this type of adverse reaction include anticonvulsants, dapsone, sulfonamides and allopurinol (Knowles et al., 1996). The initial sign of HSRs is often fever, which is followed by a rash. The rash may be exanthematous; however, in severe cases it can be erythema multiforme, Stevens-Johnson syndrome (SJS), or toxic epidermal necrolysis (TEN). Internal organ involvement often develops 1 to 2 weeks later. Hepatic injury is a common manifestation, although pulmonary, hematological or renal impairment may also occur.

SSLRs are characterized clinically by fever, rash, arthralgia and occasionally with associated lymphadenopathy (Parker, 1982). Clinical signs and symptoms of SSLRs typically develop 1 to 3 weeks after the start of drug treatment. Unlike classical serum sickness reactions, immune complexes have not been identified in SSLRs although they share many clinical symptoms (Knowles et al., 1996). SSLRs have been shown to occur with a number of drugs including penicillin, cephalosporin, sulfonamides, propylthiouracil, phenytoin, streptomycin, barbiturates, isoniazid, salicylates, captopril, hydralazine and nonsteroidal antiinflammatory drugs (NSAIDs) (Rieder, 1994).

Similar to idiopathic systemic lupus erythematosus (SLE), DIL is an autoimmune disease characterized by antibodies that bind to "self"-antigens. However, in the case of DIL the symptoms are often milder (Anderson, 1992). In particular, the most serious manifestations in SLE, involving the central nervous system and kidney, are uncommon in DIL (Anderson, 1992). Symptoms of DIL are self-limiting once the offending drug has been discontinued. Because it usually requires several months of therapy before lupus is induced, a drug would cause lupus only if it is used for a chronic illness. The drugs commonly associated with DIL are procainamide, isoniazid, hydralazine, chlorpromazine, phenytoin, methyldopa, penicillamine, quinidine and minocycline (Gilliland, 1991;
Knowles et al., 1996). Of the drugs associated with lupus, procainamide induces the highest incidence (10 to 20% of patients on continuous procainamide therapy over 2 years) (Rieder, 1994). The mechanism of DIL is unknown. It has been shown to be associated with antinuclear antibodies (ANA); however, only a minority of patients who develop a positive ANA titer related to drug ingestion develop clinical disease consistent with DIL (Knowles et al., 1996).

1.3.1b Cutaneous Reactions

In comparison with other organs, the skin is quite frequently a target of drug-induced idiosyncratic reactions. The combination of metabolic activity and immunological responsiveness may explain why skin is the organ most frequently affected by adverse drug reactions. In contrast with allergic contact dermatitis, drug-induced skin reactions have a large variability with regard to their pathophysiological pathways, clinical signs and symptoms, severity and the drugs that can elicit these reactions (Merk and Hertl, 1996). Almost any type of skin disease or characteristic symptoms can be mimicked by drug-induced skin reactions. Probably the most common cutaneous manifestations of drug reactions are a variety of maculopapular, morbilliform, scarlatiniform or exanthematous eruptions. Some of the most common offenders being various antibiotics, anticonvulsants, allopurinol, barbiturates, carbamazepine, sulfonamides, gold salts, and antituberculosis agents (Mathews, 1984). The more serious skin reactions due to drugs include erythema multiforme, SJS and TEN. The incidence of SJS, TEN and SJS/TEN overlap has been estimated to be approximately 1.89 cases per one million people per year (Mockenhaupt and Schöpf, 1996). Although SJS and TEN occur very rarely, a mortality rate of more than 40% can be calculated for patients suffering from TEN (Mockenhaupt and Schöpf, 1996). The highest incidence has been implicated to be associated with three groups of
drugs, antibacterials (e.g. sulfonamides, penicillin), NSAIDs and anticonvulsants (Park et al., 1992).

These severe type of skin reactions are thought to be immune-mediated. The evidence for this include: the occurrence of TEN in acute graft-versus-host disease in which the immune system is definitely involved, demonstration of anti-epidermal antibodies and positive lymphocyte transformation tests and demonstration of epidermal infiltration by CD8+ T-cells (Park et al., 1992).

1.3.1c Hepatotoxicity

Adverse drug reactions affecting the liver are common with more than 600 drugs having been reported to cause hepatic injury (Stricker and Spoelstra, 1985). The types of liver injury are variable and include hepatocellular necrosis, steatosis, cholestasis, granuloma, chronic hepatitis and cirrhosis (Pohl, 1990). The fatality rate of drug-induced hepatic injury is often high such that drugs are a major cause of hepatic failure (Zimmerman, 1978). For example, the case fatality rate from halothane-induced hepatic failure may be as high as 50% (Timbrell, 1983).

Drug-induced hepatotoxocities have been classified into two categories: the predictable acute response after an overdose and the idiosyncratic reaction following chronic therapeutic doses. Predictable injury resulting in hepatocellular necrosis is the best understood variety. It is dose-related, host-independent and usually reproducible in animal models. It has been shown to be due to the intrinsic toxicity of a drug or its metabolite produced in the liver. Hepatic necrosis induced by acetaminophen and carbon tetrachloride are typical examples of this type (Kaplowitz et al., 1986). Clinically, idiosyncratic hepatic injury is seen more commonly than predictable toxicity (Kaplowitz et al., 1986). With idiosyncratic hepatotoxicity, there seems to be no dose relationship, usually no animal model and it is often host dependent (Pohl, 1990). Drugs that are associated with this type
of reaction include rifampin, halothane, chlorpromazine, isoniazid, hydantoin and sulfonamides (Mathews, 1984; Timbrell, 1983).

Idiosyncratic hepatotoxicity may be due to direct toxicity of a chemically reactive metabolite, such as isoniazid-induced hepatotoxicity. This is called "metabolic" idiosyncrasy (Zimmerman, 1978). Another type of idiosyncratic hepatotoxicity is secondary to an immune reaction (Pohl, 1990). A diagnosis of immunological liver injury is supported by the association with multiple exposures, the pattern of fever, occasional eosinophilia and skin rash and the reoccurrence of the same pathologic condition upon rechallenging with the culprit drug (Pohl, 1990).

1.3.1d Hematological Reactions

Blood cells are a common target in idiosyncratic drug reactions. Drug-induced hematological toxicity can affect either platelets, red cells, white cells or all formed elements of the bone marrow resulting in thrombocytopenia, hemolytic anemia, agranulocytosis or aplastic anemia, respectively.

Agranulocytosis is characterized by an almost complete lack of granulocytic leukocytes in the circulation - less than 500 cells compared to a normal count of 5,000-10,000 cells per μl of blood. The granulocytic leukocytes are neutrophils, eosinophils, and basophils; the most abundant cell being the neutrophils. Agranulocytosis carries a high mortality rate because a patient without neutrophils has a very high risk of infection that cannot be controlled by antibiotics (Uetrecht, 1992). Drug-induced agranulocytosis is usually reversible with an "overshoot" of the peripheral neutrophils approximately 1 week after the drug is stopped. Depletion of all elements of the bone marrow results in aplastic anemia. The mortality rate of aplastic anemia is much higher than that of agranulocytosis and the time to recovery is usually longer. The estimates of etiologic fractions of agranulocytosis and aplastic anemia due to drug use have been reported in three case-
control studies: the International Agranulocytosis and Aplastic Anemia Study (IAAAS) conducted in Israel and Europe, a study conducted in the northeast U.S. and a study conducted in Thailand (Kaufman et al., 1996). The overall etiologic fractions of agranulocytosis due to drug use were 62% in IAAAS, 72% in the U.S. and 70% in Thailand. Compare to agranulocytosis, the etiologic fractions of aplastic anemia due to drug use were much less and they were 27% in the IAAAS, 17% in the U.S. and 2% in Thailand. Drugs that have been associated with agranulocytosis include: acetaminophen, aminopyrine, amodiaquine, benzene, captopril, carbamazepine, chloramphenicol, chlorpromazine, chlozapine, dapsone, indomethacin, methimazole, mianserin, penicillamine, phenylbutazone, procainamide, propylthiouracil, sulfonamides, trimethoprim, trimethoprim/sulfamethoxazole and vesnarinone (Kaufman et al., 1991; Uetrecht, 1992). Drugs that are often associated with aplastic anemia include allopurinol, captopril, carbamazepine, chloramphenicol, dapsone, indomethacin, mephenytoin, methimazole, phenylbutazone, phenytoin, propylthiouracil. sulfonamides, trimethadione and trimethoprim/sulfamethoxazole (Kaufman et al., 1991).

The mechanism of drug-induced agranulocytosis is unknown; however, either an immunologically-mediated destruction of neutrophils or a direct cytotoxicity to bone marrow has been suggested (Isildar et al., 1988). The clearest example of a drug that causes antibody-mediated peripheral destruction of neutrophils is aminopyrine (Magis et al., 1968; Moeschlin and Wagner, 1952; Uetrecht, 1990). The evidence for an immune-mediated reaction includes: the discovery of a serum factor in patients with aminopyrine-induced agranulocytosis that agglutinates neutrophils (Magis et al., 1968; Moeschlin and Wagner, 1952), a rapid decline in neutrophils when blood from the patient was infused into normal subjects with the same blood type and the rapid onset of agranulocytosis on reexposure of a patient to even a small quantity of aminopyrine. In contrast, chlorpromazine-induced agranulocytosis is considered an example of a direct bone marrow toxicity (Pisciotta et al., 1958). One piece of evident to support this is that the onset is
delayed upon reexposure. In addition, bone marrow examination demonstrated depletion of all elements of the granulocyte series (Uetrecht, 1992). The clinical characteristics of drug-induced agranulocytosis associated with other drugs appear to fall somewhere between those of aminopyrine and chlorpromazine, with some characteristics suggesting more direct toxicity and others suggesting an immune-mediated reaction.

Thrombocytopenia is characterized by bleeding and petechial hemorrhages in the skin that are related directly to the fall in circulating platelet levels. The most commonly proved or strongly suspected causes of immunologically-mediated thrombocytopenia are chlorothiazide, digitoxin, gold, heparin, meprobamate, quinidine, quinine, rifampin, stibophen and sulfonamides (Petz and Fudenberg, 1976). A marked decrease in megakaryocytes in the early phases of a reaction is usually considered to be indicative of a toxic rather than immunologic reaction. However, decreased platelet synthesis and release may also occur to a limited extent in immunologically mediated thrombocytopenia, particularly when the reaction is prolonged. The immunological nature of the reaction has been demonstrated both by passive transfer and in vitro studies in thrombocytopenia produced by apronalide, quinine, and quinidine (Ackroyd, 1975; Ackroyd, 1962; Shulman, 1958). The sera and immunoglobulin (Ig) fraction of affected patients contain antibodies that, in the presence of the drug, produce platelet lysis or agglutination.

A variety of drugs, particularly penicillin and α-methyldopa, have been reported to produce an immune-mediated hemolytic anemia. Other agents include dipyrone, p-aminosalicylic acid, stibophen, quinine, quinidine, phenacetin and nomifensine (Parker, 1982). α-methyldopa produces its effect on red blood cells by inducing an autoantibody. Penicillin-induced hemolytic anemia is an example of an immunodestructive response in which the drug participates as a hapten. It is an occasional complication in individuals receiving prolonged high dose penicillin therapy (Parker, 1982). In about 3% of individuals enough antihapten antibody is produced to give a positive antiglobulin reaction
on the red cells. If enough of the right kind of antihapten antibody is present and the bone marrow cannot compensate, anemia develops.

1.3.2 Carbamazepine-induced idiosyncratic reactions

Carbamazepine has become for many clinicians a drug of choice for the treatment of both partial and generalized convulsive seizures. It has challenged the use of phenytoin because it is considered by many to cause less cognitive impairment than phenytoin (Dodrill and Troupin, 1977; Smith et al., 1987; Thompson and Trimple, 1982), although the two drugs share similar profiles of adverse effects. The incidence of side effects associated with carbamazepine treatment in adults and children ranges from 33 to 50%. Most of the effects are mild, transient and reversible if the dosage is reduced or if initiation of treatment is gradual; however, 5% of the adverse reactions associated with carbamazepine can be classified as idiosyncratic reactions (Durelli et al., 1989). These idiosyncratic adverse reactions include skin rash (Crill, 1973), blood disorders (Gerson et al., 1983), hepatitis (Horowitz et al., 1988) and the carbamazepine hypersensitivity syndrome (Carmela et al., 1995; Shear and Spielberg, 1988). A Swedish survey of 505 reports of 713 idiosyncratic reactions to carbamazepine from 1965 to 1987 reported skin reactions (48%), hematological (12%) and hepatic disorders (10%) to be the most frequent (Askmark and Wiholm, 1990).

Skin reactions were the most frequent adverse reaction associated with carbamazepine treatment that has been reported to Geigy Pharmaceuticals and the Food and Drug Administration (FDA) in the United States. For the years 1975 to 1986 there were a total of 396 cases of rash and nonspecific dermatological reactions reported. These included 23 reported cases of SJS, 2 cases of TEN, 10 cases of exfoliative dermatitis and 12 cases of erythema multiforme (Pellock, 1987). In addition, carbamazepine is one of the drugs most frequently implicated to cause TEN (Park et al., 1992).
In addition to dermatological reactions, hematological adverse reactions to carbamazepine, although rare, are important because of the serious and potentially fatal consequences of protracted bone marrow depression. Of 80 significant cases of carbamazepine-associated hematological reactions reported to Geigy Pharmaceuticals over 12 years, thrombocytopenia ranked first with 31 cases, followed by aplastic anemia (27 cases), agranulocytosis (10 cases), pancytopenia (8 cases) and bone marrow depression (4 cases) (Pellock, 1987).

Most carbamazepine-associated hepatic abnormalities are transient elevations of liver enzymes (Pellock, 1987). However, carbamazepine-induced hepatic injury has been reported to show a cholestatic or mixed pattern as well as a hepatocellular pattern. Cases of granulomatous hepatitis and cholangitis have also been described (Askmark and Wiholm, 1990).

Carbamazepine hypersensitivity syndrome, similar to the hypersensitivity syndrome associated with phenytoin and phenobarbital, is a potentially fatal reaction. In most cases, the reaction involves multiorgan abnormalities accompanied by fever, rash and lymphadenopathy. The onset of the reaction usually occurs within 3 months of the initial treatment, most often within 2 to 4 weeks. It occurs sooner in previously sensitized individuals and occurs within 1 day on rechallenge in those with a history of carbamazepine hypersensitivity syndrome (Carmela et al., 1995).

1.3.3. Indomethacin-induced idiosyncratic reactions

Indomethacin is a very effective NSAID, but its use is limited by a high incidence of adverse reactions, including blood disorders. Adverse effects have been estimated to occur in 30-60% of patients treated with indomethacin, and serious reactions requiring discontinuance of the drug occur in about 10% of patients. Most common side effects,
which appear to be dose-dependent, include gastro-intestinal disturbances, headache and dizziness (Cuthbert, 1974).

Indomethacin also causes idiosyncratic hematological adverse effects in less than 1% of patients. These reactions include hemolytic anemia, bone marrow depression, aplastic anemia, agranulocytosis, leukopenia, thrombocytopenia and thrombocytopenic purpura. Of a total of 1,261 reports of adverse reactions to indomethacin reported to the U.K. Committee on Safety of Medicines between June 1964 and January 1973, blood disorders were recorded in 157 cases (25 fatal) which included thrombocytopenia (35; 5 fatal), aplastic anemia (17; no fatalities), and agranulocytosis or leukopenia (21; 3 fatal) (Cuthbert, 1974). Subsequently, the First Report from the International Agranulocytosis and Aplastic Anemia Study confirmed a significant relationship between the use of indomethacin and agranulocytosis and aplastic anemia. In this population-based case-control study conducted in Europe and Israel, the excess risk estimated for agranulocytosis and aplastic anemia was 0.6 per million for indomethacin exposure in a one-week period (Shapiro, 1986).

1.4 Possible mechanisms of idiosyncratic reactions

1.4.1 Relevance of the immune system to idiosyncratic drug reactions

Adverse drug reactions that involve a specific drug-induced immune response are referred to as drug hypersensitivity reactions and they can be classified according to the general scheme of Gell and Coombs (1963):

- **Type I** (immediate hypersensitivity or anaphylactic) reactions
  
  This type of reactions result from the interaction (cross-linking) of drug antigen with specific IgE antibody on the surface of mast cells and basophils to trigger the release of chemical mediators such as histamine and leukotrienes. The clinical features include
generalized erythema, urticaria, angioedema, gastrointestinal disturbances, bronchospasm, laryngoedema, and hypotension. To most clinicians, the term "allergy" is synonymous with type I hypersensitivity. Anaphylactic reactions to penicillin are an example of a type I reaction.

- **Type II (cytotoxic) reactions**
  This type of reactions are caused by binding of specific IgG or IgM antibody with antigen associated with the surface of a cell, or to a cell surface altered antigenically by a drug. The cell is destroyed by phagocytic cells or by activation of the complement system leading to cell lysis. This may result in thrombocytopenia, anemia or leucopenia, depending on the disposition of the antigen. Hemolytic anemia due to quinine and penicillin are type II reactions.

- **Type III (immune complex) reactions**
  This type of reaction involves the formation of circulating immune complexes, from specific antigen and IgG or IgM antibodies, which can precipitate in the vasculature and renal glomeruli; tissue damage results from activation of complement and recruitment of phagocytes. The classical example of type III hypersensitivity is serum sickness reactions, which may present clinically with lymphadenopathy, arthralgia, fever and rash.

- **Type IV (drug-induced delayed hypersensitivity) reactions**
  This type of reaction usually occurs in the skin. The major cell types involved are T lymphocytes and macrophages. Lymphokines are released following interaction of antigen with specific T-cells; tissue damage is caused by infiltrating mononuclear cells. Topically applied drug becomes attached to a carrier protein in the skin; induces a cell-mediated response and causes contact dermatitis. Such reactions can also occur after either oral or parenteral administration of the drug.

Idiosyncratic drug reactions have often been referred as drug hypersensitivity reactions. However, there is not enough evidence to definitely prove that the immune
system is involved. The most convincing evidence for the involvement of the immune system is demonstration that specific antibodies or sensitized T lymphocytes are reacting with the drug or its metabolites. Drug-related antibodies have been described in some idiosyncratic drug reactions. For example, the evidence for an immune-mediated reaction is strong in penicillin-induced anaphylaxis and aminopyrine-induced agranulocytosis. Penicillin is chemically reactive due to the β-lactam ring and it can act as a hapten. There is strong evidence that most of the antibodies in patients who are allergic to penicillin recognize penicillin bound to lysine (Parker, 1982). In some patients the major type of antibody induced is IgE, and this can result in an anaphylactic reaction. Antibody-mediated peripheral destruction of neutrophils has been suggested to be the mechanism of aminopyrine-induced agranulocytosis by several pieces of evidence. For example, incubation of serum from a patient who has the active disease with normal neutrophils led to agglutination of the neutrophils (Moeschlin and Wagner, 1952). In addition, infusion of blood from a sensitive patient 3 hours after a dose of aminopyrine into a normal subject matched for blood type led to granulocytopenia (Moeschlin and Wagner, 1952). In another study, the serum from a patient with aminopyrine-induced agranulocytosis inhibited colonies cultured from a mononuclear fraction of the patient's blood in the presence of drug, while serum from a normal control did not (Barrett et al., 1976). However, probably due to the complexities of the immune system, in most cases of idiosyncratic reactions there is no direct evidence for the involvement of the immune system, even when effort has been made to search for such evidence. Furthermore, since not all drug-induced antibodies or sensitized T lymphocytes will necessarily cause tissue damage, a definite proof that a toxicity has an allergic basis requires that it be produced in animals, where the immunological mechanisms of cellular damage can be thoroughly studied (Pohl et al., 1988). However, there have been few animal models developed for studying the mechanism of idiosyncratic drug reactions.
Despite this lack of proof that the immune system is involved in the majority of idiosyncratic drug reactions, the idiosyncratic nature of such reactions has led many investigators to believe that most of these reactions are mediated by the immune system (Parker, 1982; Pohl et al., 1988). Some of the clinical characteristics of many idiosyncratic reactions also suggest that the immune system is involved. Such characteristics include:

- A requirement of prior exposures to the drug, or a lag period of more than a week between starting the drug and the development of toxicity.
- A lack of delay in toxicity on reexposure of a patient to the offending drug.
- No apparent correlation between dose and the risk of toxicity.
- The presence of a skin rash and peripheral blood eosinophilia.

However, there is no reason to presume that all idiosyncratic reactions have an immunological mechanism. The role of direct cytotoxicity has not been ruled out and remains a controversial issue. However, it is unlikely that many idiosyncratic drug reactions involve direct cytotoxicity of the drug or its metabolites. If such were true, the reaction would be expected to be more predictable and also to be detected in animal toxicity tests with high doses. Direct cytotoxicity also may not explain the large interindividual and interspecies differences in the toxicity since differences in drug metabolism do not appear to be the sole basis for the idiosyncratic character of these drug reactions.

### 1.4.2 Hapten hypothesis

Unfortunately, not enough is known about the mechanism of many drug-induced idiosyncratic reactions to place them into the Gell and Coombs classification scheme. However, for a drug to cause any immunological reaction, it would probably have to act as a hapten. The term "hapten" has been used to describe a substance which is not immunogenic per se, but becomes immunogenic when conjugated to a macromolecular carrier. In general, in order for a molecule to be recognized as "nonself" and to induce an
immune response, it must have a minimum molecular weight of approximately 1000 daltons (Pohl et al., 1988). Since most drugs are not this large, they must bind to an endogenous macromolecule, such as protein, to form a drug-protein conjugate before they can interact with the immune system. This binding of a drug to a macromolecular carrier must be essentially irreversible, usually covalent, for the adduct to be immunogenic. The reason for the requirement of a covalent interaction is that, for an immune response to be induced, the immunogen must be processed and presented by antigen presenting cells (i.e. macrophages). If the interactions between hapten and macromolecule were reversible, the hapten would diffuse away from the macromolecule before fragments of the adduct could be presented to T-cells. Therefore, central to the hapten hypothesis is the requirement for the ability of a hapten to form a stable adduct with nucleophilic groups on proteins (e.g. lysyl and cysteiny1 residues) in aqueous conditions.

Some drugs are chemically reactive themselves so that they can react directly to proteins, such as penicillin. Penicillin has a reactive structure and can bind covalently to proteins and carbohydrates by reaction with nucleophilic amino, hydroxyl, mercapto and histidine groups. The principal mode of conjugation, referred as the "major" antigenic determinant, is the penicilloyl moiety bound to ε- amino groups of lysine residues of proteins. There are two possible routes for the formation of penicilloyl derivatives. One pathway is the direct aminolysis of penicillin by the protein. An alternative pathway is through rearrangement of penicillin to penicillenic acid (Schwartz, 1969). Penicillenic acid contains a free thiol group that can also form mixed-disulfide bonds with cysteine residues and this is one of the so called "minor determinants" (Figure 1-1). There is also evidence that penicilloic acid and penamaldate are specific antigenic determinants in penicillin allergy and they are included in the "minor determinants" (Schwartz, 1969).
Figure 1-1: The major and minor antigenic determinants of benzylpenicillin (Park and Kitteringham, 1990; Schwartz, 1969).
Some drugs have a free sulfhydryl group that can react with disulfide linkages to form mixed disulfides. For example, both D-penicillamine and captopril have a free sulfhydryl group that can react with cysteine residues on proteins via disulfide linkages. Immunochemical studies have shown that captopril linked by disulfide bonds to proteins can function as a hapten in humans and experimental animals (Park et al., 1987). Besides a free sulfhydryl group, D-penicillamine also possesses a free amino group so that it can react with biological intermediates that contain a free carbonyl group to form thiazolidine derivatives. It has been shown that penicillamine interferes with collagen maturation by chemical reaction with free aldehyde group in pro-collagen (Park et al., 1987).

Unlike penicillin, captopril and penicillamine, most drugs are not chemically reactive nor sufficiently electrophilic to bind to macromolecules. Therefore most drugs must be converted to reactive species within the body in order to act as haptens.

1.4.3 Reactive metabolites involved in idiosyncratic reactions

Although we do not have a complete understanding of the mechanism of drug-induced idiosyncratic reactions, there are a large number of studies to suggest that reactive metabolites are involved (Hinson and Roberts, 1992; Park et al., 1995) and several characteristics suggest the involvement of the immune system (Pohl et al., 1988).

In general, drug metabolism can be considered as a detoxification process in which it converts therapeutically active compounds to inactive, more polar metabolites that can then be removed from the body. However, in certain circumstances, the drug metabolizing enzymes can convert a drug to a chemically reactive metabolite. This process is referred as "bioactivation" (Pirmohamed et al., 1994). Chemically reactive agents can be cytotoxic by reacting with critical biological molecules (Jerina and Dalay, 1974; Stadtman and Oliver, 1991). In most cases, the chemically reactive metabolite will be detoxified before it can initiate tissue damage. If inadequately detoxified, formation of a reactive metabolite as a
result of drug bioactivation is often the first step, although not necessarily the ultimate step, in initiating idiosyncratic drug toxicity. The reactive metabolite may cause direct cellular toxicity; cause cell stress and initiate innate immune responses; or it can act as a hapten and initiate an immune reaction which may be due to a specific humoral (antibody) response, a cellular response (T lymphocytes) or a combination of both (Park et al., 1987; Pohl et al., 1988) (Figure 1-2).
Figure 1-2: The role of drug bioactivation in mediating either direct (metabolic) idiosyncrasy or immune-mediated idiosyncrasy (Pirmohamed et al., 1996).
Reactive metabolites can be formed by most of the enzymes that are involved in drug metabolism, the most important being the enzymes of cytochrome P450 (CYP450) mixed function oxidase system. Other enzymes also play an important role in drug bioactivation. In white blood cells and bone marrow cells, for example, MPO has been shown to bioactivate a wide range of drugs (Pirmohamed et al., 1996; Uetrecht, 1992). In other tissues low in CYP450 activity (e.g. kidney, lung, skin), prostaglandin H synthase (PHS) may also be responsible for bioactivation; e.g. in the kidney acetaminophen toxicity is thought to result from activation via this enzyme (Pirmohamed et al., 1996).

For most drugs, the occurrence of bioactivation and the chemical nature of the reactive metabolite have not been definitely determined. This is largely due to the lack of techniques that allow the detection of in vivo bioactivation and determination of the nature of the metabolites formed. However, a large amount of indirect evidence suggests that many drugs are bioactivated to structurally different reactive metabolites that may play a role in the mechanism of drug-induced idiosyncratic reactions.

In this discussion, drugs will be categorized according to the chemical nature of their reactive metabolites that are generated during the bioactivation of these drugs. Tables 1-1, 1-2, 1-3, 1-4 and 1-5 are lists of drugs and xenobiotics, the types of toxicity that they cause and their reactive metabolites generated during bioactivation by various metabolizing enzymes.

1.4.3a Hydroxylamines

Many drugs have an arylamine moiety or can be metabolized to arylamines (either primary or secondary). Studies by various research groups have demonstrated that arylamine-containing drugs can usually be oxidized to reactive hydroxylamines or further to the closely related nitroso derivatives. These studies have been carried out in various enzyme systems (e.g. CYP450 and MPO), cells (i.e. human neutrophils) or tissue
homogenates (e.g. human and mouse liver microsomes). Typical examples of this class of drugs include procainamide, dapsone and sulfonamides (Table 1-1).

Table 1-1: Formation of the reactive hydroxylamine intermediates during bioactivation of drugs by various oxidizing enzymes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Idiosyncratic Toxicity</th>
<th>Reactive Metabolites</th>
<th>Metabolizing Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procainamide</td>
<td>Agranulocytosis, Hepatotoxicity, Drug-induced lupus</td>
<td>hydroxylamine</td>
<td>CYP450, MPO</td>
</tr>
<tr>
<td>Dapsone</td>
<td>Methemoglobinemia, &quot;Hypersensitivity&quot;, Mononucleosis-like syndrome, Agranulocytosis</td>
<td>hydroxylamine</td>
<td>CYP3A4, CYP2E1, CYP2C9, MPO</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>Hepatoxicity, Skin rash, Blood disorders, &quot;Hypersensitivity&quot;</td>
<td>hydroxylamine</td>
<td>CYP450, MPO</td>
</tr>
</tbody>
</table>
Procainamide is an antiarrhythmic agent, but its chronic use is limited by a high incidence of drug-induced lupus and agranulocytosis (Ellrodt et al., 1984). It has been shown that procainamide was metabolized to a hydroxylamine by both rat and human hepatic microsomes and by activated human neutrophils (Uetrecht et al., 1988a; Uetrecht et al., 1984). In vitro the hydroxylamine was oxidized nonenzymatically by hydrogen peroxide to a nitroso derivative and further to the nitro derivative (Figure 1-3). Of these metabolites, the nitroso-procainamide was the most reactive and accounted for most of the covalent binding to proteins (Uetrecht, 1985). When procainamide was oxidized by the combination of myeloperoxidase and hydrogen peroxide in the presence of chloride ion, a reactive N-chloroprocainamide metabolite was formed (Uetrecht and Zahid, 1991) (Fig 1-3).
Figure 1-3: Metabolism of procainamide by MPO in activated neutrophils (Uetrecht \textit{et al.}, 1988a; Uetrecht and Zahid, 1991).
Dapsone is used in the treatment of leprosy and it is also effective in malarial prophylaxis. Adverse reactions to dapsone include agranulocytosis and severe hemolytic anemia (Coleman et al., 1989). The metabolism of dapsone by activated neutrophils leads to the formation of the hydroxylamine and nitroso metabolites (Uetrecht et al., 1988b). Dapsone has also been shown to undergo bioactivation to a cytotoxic metabolite, in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction catalyzed by microsomes prepared from nine individual human livers (Coleman et al., 1989). The kinetics of dapsone hydroxylamine formation in human liver microsomes were biphasic. In vitro metabolic studies using enzyme inhibitors, inhibitory antibodies, enzyme expression systems and correlations with the levels of individual isozymes have determined that CYP3A4 and CYP2E1 accounted largely for dapsone hydroxylamine formation at high (100 μmol/l) and low (5 μmol/l) substrate concentrations, respectively. These data also suggested that CYP2C isoforms contribute to dapsone hydroxylamine formation at low substrate concentration (Fleming et al., 1992; Gill et al., 1995; Mitra et al., 1995).

Sulfonamides are antimicrobial agents that are associated with various idiosyncratic reactions in humans which include hepatitis, nephritis, blood dyscrasias, drug-induced lupus, serum sickness and the sulfonamide hypersensitivity syndrome. It has been shown that in humans, sulfamethoxazole was metabolized to its hydroxylamine in the liver predominantly by CYP2C9 (Cribb and Spielberg, 1990b; Cribb et al., 1995). Sulfamethoxazole was also shown to be oxidized by activated monocytes and neutrophils (human and canine) to form sulfamethoxazole hydroxylamine and nitrosulfamethoxazole; the latter was presumably formed via the reactive nitroso-sulfamethoxazole intermediate (Cribb et al., 1990a).
1.4.3b Quinones

A large number of drugs or their metabolites contain a phenyl ring with a hydroxyl group in the para (or ortho) position to another hydroxyl group or an amine or methyl group. This structure renders the ability of these drugs (or their metabolites) to be oxidized and converted to reactive quinones, iminoquinones or quinone methides depending on the functional group that is para (or ortho) to the hydroxyl group on the aromatic ring. The drugs (or their metabolites) of this category are listed in Table 1-2.
Table 1-2: Formation of the reactive iminoquinone intermediate during bioactivation of
drugs by various oxidizing enzymes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Idiosyncratic Toxicity</th>
<th>Reactive Metabolites</th>
<th>Metabolizing Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine</td>
<td>Agranulocytosis, Hepatotoxicity</td>
<td>iminoquinone</td>
<td>MPO, HRP</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Hepatotoxicity, Nephrotoxicity</td>
<td>N-acetylbenzo-quinoneimine</td>
<td>CYP1A2, CYP2E1, CYP3A4</td>
</tr>
<tr>
<td>Diclofenac (5-hydroxyl metabolite)</td>
<td>Hepatotoxicity, Blood disorders</td>
<td>iminoquinone</td>
<td>MPO, CYP2C9</td>
</tr>
<tr>
<td>Vesnarinone</td>
<td>Agranulocytosis</td>
<td>imidoiminium ion, iminoquinone</td>
<td>MPO</td>
</tr>
<tr>
<td>Benzene (hydroquinone)</td>
<td>Aplastic anemia</td>
<td>quinone</td>
<td>MPO</td>
</tr>
<tr>
<td>Prinomide (parahydroxy metabolite)</td>
<td>Agranulocytosis</td>
<td>quinone</td>
<td>MPO</td>
</tr>
<tr>
<td>Tacrine</td>
<td>Hepatotoxicity</td>
<td>quinone methide</td>
<td>CYP1A2</td>
</tr>
</tbody>
</table>
Amodiaquine is an antimalarial agent associated with a high incidence of agranulocytosis. A number of cases of fatal agranulocytosis, as well as hepatotoxicity, in patients during prophylactic administration has led to its withdrawal from use (Hatton et al., 1986; Larrey et al., 1986; Neftel et al., 1986). Bioactivation of amodiaquine and covalent binding to various proteins have been demonstrated in several systems: human liver microsomes, horseradish peroxidase (HRP) / H₂O₂ or in the presence of chlorine and activated neutrophils (Jewell et al., 1995; Maggs et al., 1988; Naisbitt et al., 1997). It was shown that a reactive iminoquinone intermediate was formed from amodiaquine by autoxidation at neutral pH, by peroxidases and by silver oxide oxidation, and by activated neutrophils in vitro (Christie et al., 1989; Clarke et al., 1990; Maggs et al., 1987; Maggs et al., 1988) (Figure 1-4).

Figure 1-4: Oxidation of amodiaquine to a reactive iminoquinone intermediate (Park and Kitteringham, 1990).
Acetaminophen is an analgesic agent. Renal toxicity and hepatotoxicity after overdoses have been associated with acetaminophen. There is a large amount of evidence to support the involvement of the reactive N-acetylbenzoquinone imine in this toxicity. At least three forms of CYP450 (CYP1A2, CYP2E1 and CYP3A4), as well as MPO and PHS enzyme systems, have been shown to be involved in acetaminophen bioactivation and the formation of the reactive iminoquinone (Boyd and Eling, 1981; Moldeus and Rahimtula, 1980; Potter and Hinson, 1987; Raucy et al., 1989; Thummel et al., 1993).

Formation of the reactive iminoquinone intermediates during bioactivation of indomethacin and carbamazepine will be discussed in detail in Chapter 2 and Chapter 3, respectively.

Diclofenac is a NSAID, and is widely used in the treatment of rheumatoid arthritis and other inflammatory diseases (Dunk et al., 1982). In rare incidences, diclofenac appears to be associated with hepatitis (Dunk et al., 1982; Helfgott et al., 1990) and hematological toxicity, such as aplastic anemia, neutropenia (Eustace et al., 1989; Kaufman et al., 1991; Kim and Kovacs, 1995; Salama et al., 1989), hemolytic anemia and thrombocytopenia (Epstein et al., 1990; Kim and Kovacs, 1995; Kramer et al., 1986; Salama et al., 1991). It has been shown that when diclofenac is oxidized by MPO or activated neutrophils, the major product was an iminoquinone (Figure 1-5). This iminoquinone reacted with glutathione and it is also likely to react with protein sulfhydryl groups which would contribute to covalent binding (Miyamoto et al., 1997). In addition, the phenolic precursor of the iminoquinone, 5-hydroxydiclofenac, is a significant hepatic metabolite of diclofenac (Sawchuk et al., 1995). It was found that the iminoquinone was also formed by the oxidation of 5-hydroxydiclofenac with rat hepatic microsomes, so it is likely that the iminoquinone is formed both in the liver and in the bone marrow of humans (Miyamoto et al., 1997).
Figure 1-5: Proposed pathway of the oxidation of diclofenac to a reactive iminoquinone (Miyamoto et al., 1997).

Vesnarinone is used in congestive heart failure and its use is associated with agranulocytosis. It was found that vesnarinone was metabolized to a reactive metabolite that covalently bound to activated neutrophils (Uetrecht et al., 1994). The reactive metabolite was identified as an imidoiminium ion (Figure 1-6). This reactive species can either hydrolyze to form veratrylpiperazinamide or react with other nucleophiles, such as
GSH. Hydrolysis also leads to another reactive intermediate, an iminoquinone, which also reacts with GSH (Uetrecht et al., 1994).

![Chemical structure](image)

**Figure 1-6:** Proposed pathway of oxidation of vesnarinone to reactive iminoquinone intermediates (Uetrecht et al., 1994).
Benzene is an environmental toxicant. The most frequently observed toxic effect of benzene in humans and in animal models has been bone marrow depression leading to aplastic anemia (Snyder and Kocsis, 1975). Benzene is initially metabolized in the liver by CYP2E1 to hydroxylated products: phenols, catechols and hydroquinones. The bone marrow has been shown to accumulate hydroquinone and catechol (Greenlee et al., 1981; Rickert et al., 1979). It has been suggested that the reactive intermediates, p-benzoquinone and o-benzoquinone, are formed in the bone marrow during oxidation of hydroquinone and catechol by MPO (Eastmond et al., 1987; Eastmond et al., 1986; Sadler et al., 1988). These quinone reactive intermediates may be responsible for benzene-induced bone marrow toxicity.

Prinomide is an anti-inflammatory drug which was associated with a relatively high incidence of agranulocytosis (< 0.3%) in clinical trials (Parrish et al., 1997). Metabolism of prinomide and its parahydroxy metabolite, CGS12094, by MPO was studied (Parrish et al., 1997). CGS12094 was found to be rapidly metabolized (> 90%; 2 min) and the reaction was dependent on H2O2 and MPO and was inhibited by azide (a MPO inhibitor). During the MPO-catalyzed metabolism of CGS12094, reactive intermediates that irreversibly bound to protein and cysteine were generated. One of the reactive metabolites has been identified as 1,4-benzoquinone which is the same reactive intermediate implicated in benzene toxicity (Figure 1-7).
Figure 1-7: Proposed mechanism for 1,4-benzoquinone formation from the MPO-catalyzed metabolism of CGS12094 (Parrish et al., 1997).

Tacrine is a potent, centrally acting anticholinesterase currently used in the treatment of Alzheimer's disease (Madden et al., 1995a). Tacrine therapy is associated with an unpredictable hepatotoxic reaction in up to 50% of patients (Pirmohamed et al., 1996). Initial in vitro studies demonstrated hepatic microsomal enzyme-dependent bioactivation of tacrine to both cytotoxic and protein-reactive species (Madden et al., 1993). The formation of these metabolites was inhibited by the addition of glutathione and/or ascorbic acid to the incubation medium, indicating that electrophilic species were being formed. The enzyme involved in the generation of chemically reactive metabolites was shown to be CYP1A2 (Madden et al., 1993; Spaldin et al., 1994). A stable thioether conjugate of the reactive
species was formed when tacrine (or its 7-hydroxyl metabolite) was coincubated with microsomes and mercaptoethanol. The reactive intermediate was identified as a quinone methide (Madden et al., 1995a; Madden et al., 1995b) (Figure 1-8).

![Metabolic pathway for the bioactivation of tacrine to its postulated quinone methide reactive metabolite by CYP1A2](Figure 1-8)

1.4.3c Cations and radicals

A large number of compounds, including many drugs can be oxidized under appropriate conditions to potentially toxic free radical (or cation) intermediates. The cations and radicals may cause protein adduct formation or oxidative stress that may further lead to toxicity. Table 1-3 shows some examples of this category.
Aminopyrine is an analgesic agent but its use has been limited by its association with a high incidence of agranulocytosis (Madison and Squier, 1934). Aminopyrine has been shown to be oxidized to a blue cation radical (Figure 2-8) by PHS, MPO and hypochlorous acid (Eling et al., 1985; Sayo and Saito, 1990; Uetrecht et al., 1995). It has been proposed by Sayo and Saito that the mechanism by which hypochlorous acid oxidizes aminopyrine to a cation radical involves N-chlorination followed by the loss of a chlorine radical (Sayo and Saito, 1990). However, a study by Uetrecht et al. (1995) later observed a dication intermediate as the precursor for the cation radical. The dication was formed by the loss of chloride ion from N-chloroaminopyrine and it is very reactive (t1/2 = 15 ms) (Figure 1-9). The same pathway was suggested when aminopyrine was oxidized by the combination of MPO, H₂O₂ and chloride or activated neutrophils (Uetrecht et al., 1995).
Figure 1-9: Proposed scheme of the oxidation of aminopyrine to a cation radical via a dication intermediate (Uetrecht et al., 1995).
Chlorpromazine, an antipsychotic agent, is known to induce agranulocytosis. It was found that chlorpromazine can be oxidized by hypochlorous acid or MPO of activated neutrophils to a free radical (Kalyanaraman and Sohnle, 1985). Covalent binding of $^3$H-labeled chlorpromazine to proteins of stimulated polymorphonuclear leukocytes was also observed (Kelder et al., 1991). Van Zyl et al. (1990) also found that chlorpromazine was oxidized to a relatively stable radical cation by the combination of MPO and H$_2$O$_2$. However, it is not clear whether the reaction involves a direct one-electron oxidation or chlorination to form the two-electron oxidation product followed by coproportionation with the parent drug to form two radical cations.

Clozapine is a unique antipsychotic drug that is more effective than standard neuroleptic drugs in the treatment of refractory schizophrenia. Unfortunately, its use has been restricted due to a 0.8% incidence of drug induced agranulocytosis. Metabolism of clozapine by activated neutrophils, HRP/H$_2$O$_2$, and MPO/H$_2$O$_2$ has been studied (Fischer et al., 1991; Liu and Uetrecht, 1995). Formation of a reactive metabolite was supported by two pieces of evidence: covalent binding to activated neutrophils and formation of glutathione conjugate of the reactive metabolites. The identity of the reactive metabolite has been suggested to be a free radical or a nitrenium ion (Figure 1-10). The nitrenium ion intermediate, which is 2 mass units less than the parent drug, was detected by mass spectrometry (Liu and Uetrecht, 1995). Indirect evidence that peroxidase systems also activate clozapine to free radical intermediates stems from the detection of thiyl and ascorbyl radicals in the presence of glutathione and ascorbate, respectively (Fischer et al., 1991).
Figure 1-10: Proposed pathway for the oxidation of clozapine to a reactive nitrenium ion or a cation radical by HRP and MPO (Fischer et al., 1991; Liu and Uetrecht, 1995).
Studies of the metabolic pathway of vesnarinone by MPO or by HOC1 suggested that a reactive imidoiminium ion was involved (Uetrecht et al., 1994). Formation of the imidoiminium ion could involve a radical cation or chlorination of the nitrogen followed by loss of HCl (Figure 1-11).

A number of studies have suggested that free radical metabolic pathways exist for a wide variety of compounds and free radical metabolites do seem to be implicated in the toxic effects of those xenobiotics which are metabolized to free radicals (Mason, 1979). However, it is much more difficult to obtain direct evidence for free radical formation than that for the formation of electrophilic intermediates. Even if the free radical intermediates do exist, they are generally extremely reactive so that it will be quenched by anything they encounter first before reacting with important molecules (e.g. proteins). In addition, as has been shown in many studies, the major reaction of free radicals with GSH is the abstraction of a hydrogen atom rather than covalent binding. Presumably the same is true of its reaction with other biological molecules. Therefore, the pattern of toxic effects caused by free radical intermediates and by electrophilic intermediates are very likely to be different.
Figure 1-11: Proposed mechanisms of forming the imidoiminium ion intermediate of vesnarinone.
1.4.3d Arene Oxide

A major metabolic pathway for compounds that contain aromatic rings is aromatic hydroxylation. It has been suggested that arene oxides may be intermediates in this reaction. Arene oxides are chemically reactive species and their half-lives are usually very short. For example, the half-life of benzene oxide is less than 2 minutes in water at pH 7 (Jerina and Dalay, 1974). Arene oxides, once formed, rapidly undergo isomerization to phenols or readily react with a variety of nucleophiles including such cellular macromolecules as DNA, RNA and protein. Thus, arene oxides have become prime candidates for the "reactive intermediates" responsible for the binding of aromatic compounds to macromolecules in the cell. Table 1-4 lists some examples of this category.
Table 1-4: Formation of the reactive arene oxide intermediates during bioactivation of xenobiotics by CYP450.

<table>
<thead>
<tr>
<th>Xenobiotics</th>
<th>Toxicity</th>
<th>Reactive Metabolites</th>
<th>Metabolizing Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halobenzenes</td>
<td>Hepatotoxicity, Bone marrow toxicity</td>
<td>Arene oxide</td>
<td>CYP450</td>
</tr>
<tr>
<td>Polycyclic hydrocarbons</td>
<td>Carcinogenicity</td>
<td>Arene oxide</td>
<td>CYP450</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Hepatotoxicity, Blood disorders, “Hypersensitivity”</td>
<td>Arene oxide</td>
<td>CYP3A4</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Teratogenicity, “Hypersensitivity”</td>
<td>Arene oxide</td>
<td>CYP2C9</td>
</tr>
</tbody>
</table>

It has been shown that hepatotoxicities of simple benzene compounds are caused by halobenzenes being metabolized to arene oxides which covalently bind to hepatic protein (Brodie et al., 1971). When radioactive bromobenzene was administered to rats, massive centrolobular necrosis resulted and the necrotic regions contained significant amounts of covalently bound radioactivity (Brodie et al., 1971). Prior treatment of the animals with phenobarbital enhanced both the bromobenzene-induced necrosis and the hepatic binding (Brodie et al., 1971; Reid and Krishna, 1973). Although bioactivation to form an arene oxide intermediate may lead to benzene compound-induced liver damage, it is less likely to be responsible for the bone marrow toxicity associated with these compounds since even if
small amounts can escape the liver, their concentrations at distant sites such as the bone marrow are likely to be very low. In contrast, catechol-type metabolites can be readily oxidised to quinone-type metabolites in the bone marrow. Even in the liver microsomes, it has been demonstrated that for bromobenzene, which does form an arene oxide, most of the covalent binding is due to a quinone intermediate (Hanzlik et al., 1984; Rietjens et al., 1997). This suggests that arene oxide, although it may be formed, may not be the most likely reactive metabolite responsible for toxicity.

Polycyclic hydrocarbons are widespread environmental contaminants. Many of these compounds are known to be carcinogenic in experimental animals (Freudenthal and Jones, 1976; Miller, 1978). It is now well established that metabolic activation is required for the observed biological effects of polycyclic hydrocarbons (Gelboin and Ts'o, 1978; Sims and Grover, 1974). Benzo[a]pyrene (BP), for example, is metabolized to a wide variety of metabolites, such as epoxides, dihydrodiols, quinones and phenols (Gelboin, 1980). Evidence suggests that two of the BP-diol-epoxide metabolites (Figure 1-12), are the ultimate carcinogenic forms of this polycyclic hydrocarbon. The two diastereomers, BP-7, 8-diol-9,10-epoxides, are highly carcinogenic to newborn mice (Kapitulnik et al., 1977; Sims and Grover, 1974); are potential mutagens and cytotoxins to both mammalian and bacterial cells (Huberman et al., 1976; Marnett and Bienkowski, 1980; Wood et al., 1977) and are chemically very reactive in binding to DNA (Jeffrey et al., 1977).
It has been proposed that the reactive metabolite of carbamazepine, which may be responsible for carbamazepine-induced adverse reactions, is an arene oxide. The evidence to support this hypothesis is that carbamazepine can be bioactivated by mouse and human liver microsomes to a cytotoxic and protein-reactive metabolite (Pirmohamed et al., 1992a; Pirmohamed et al., 1992b). The cytotoxicity and covalent binding were enhanced by trichloropropene oxide (TCPO), which is an epoxide hydrolase inhibitor (Riley et al.,
1989). However, TCPO is not a specific inhibitor for epoxide hydrolase, it is also known to deplete glutathione (Pessayre et al., 1979) and inhibit CYP450 (Ivanetich et al., 1982). Furthermore, studies have failed to find a consistent mutation, or pattern of mutations, in the microsomal epoxide hydrolase gene which is common in patients with a history of carbamazepine hypersensitivity reactions (Gaedigk et al., 1994; Green et al., 1995). The only direct evidence for the arene oxide formation in vivo is detection, in the bile, of the GSH conjugate of the postulated arene oxide in rodents (Amore et al., 1997; Madden et al., 1996). However, in humans, the same GSH-conjugate was not detected nor were any of its further metabolized products, such as the NAC, cysteinyI or thiomethyl derivatives (Maggs et al., 1997).

Phenytoin is a commonly used anticonvulsant for the treatment of epilepsy. Phenytoin causes a wide range of adverse reactions including hypersensitivity and it has been shown to be teratogenic in animals and humans (Winn and Wells, 1995). It has been postulated that CYP450 bioactivation to an electrophilic arene oxide intermediate may play an important role in the mechanism of phenytoin-induced toxicities. Evidence for the formation of the arene oxide intermediate is supported by the ability of phenytoin to bind covalently to molecular targets, by the formation of the trans-dihydrodiol metabolite of phenytoin (Oesch, 1973) and by the formation of a catechol or its methylated derivative (Billings, 1985; Billings and Fischer, 1985; Maguire et al., 1979). Nevertheless, none of these observations are direct proof of the formation of an arene oxide intermediate (Brown et al., 1986). It is known that arene oxides generally react readily with GSH to form stable conjugates (Jerina and Dalay, 1974); however, to date neither the GSH adduct of the putative arene oxide, nor its urinary mercapturate metabolite, has been found (Hansen, 1991). Recently, an alternative reactive metabolite, an o-quinone, has been proposed by Gillam and coworkers and it was demonstrated that the phenol and catechol metabolites lead to more covalent binding than the parent drug in vitro (Munns et al., 1997).
1.4.3e Miscellaneous

There are many other drugs that can be bioactivated to chemically reactive metabolites. The chemical nature of these reactive metabolites, however, are different from those discussed earlier in this section. They are classified as miscellaneous drugs (Table 1-5) and will be discussed individually.

Table 1-5: Formation of reactive metabolites during bioactivation of drugs by various oxidizing enzymes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Idiosyncratic Toxicity</th>
<th>Reactive Metabolites</th>
<th>Metabolizing Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tienilic acid</td>
<td>Hepatotoxicity</td>
<td>sulfoxide</td>
<td>CYP2C9</td>
</tr>
<tr>
<td>Halothane</td>
<td>Hepatotoxicity</td>
<td>trifluoroacetyl chloride</td>
<td>CYP2E1</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>Agranulocytosis,</td>
<td>sulfenic chloride, sulfonic acid</td>
<td>MPO</td>
</tr>
<tr>
<td></td>
<td>Hepatotoxicity,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drug-induced lupus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tienilic acid, a diuretic agent, was withdrawn from general use because of a high incidence of hepatitis (Dansette et al., 1991). Bioactivation of tienilic acid has been shown to involve CYP2C9 and to be inhibited by anti-human CYP2C antibodies. The reactive metabolite has been postulated to be the sulfoxide of tienilic acid (Mansuy et al., 1991) (Figure 1-13). It is likely that the bioactivation of tienilic acid to a sulfoxide metabolite by,
and subsequent binding to, the CYP2C proteins causes the antigenic alteration of this CYP450 isoform resulting in antibody production.

\[
\begin{align*}
\text{Tienilic acid} & \quad \text{CYP2C9} \quad \text{Sulfoxide} \\
\end{align*}
\]

Figure 1-13: Metabolism of tienilic acid to the sulfoxide metabolite by CYP2C9 (Mansuy et al., 1991).

Halothane, a widely used anesthetic, is thought to cause two forms of liver damage (Neuberger and Kenna, 1987). The minor form is characterized by slightly raised transaminases and perhaps mild necrosis which occurs in up to 20% of patients. The more severe form of halothane hepatitis (i.e. massive liver cell damage) is observed in 1:35,000 patients. The incidence of this reaction is increased (1:3,700) after multiple exposure to halothane (Park and Kitteringham, 1990). Halothane is metabolized in the liver by cytochrome P450 enzymes by oxidation and reduction to two chemically reactive metabolites, trifluoroacetyl chloride (TFA) and the 1-chloro-2,2,3-trifluorocetyl radical. It has been demonstrated that it is the trifluoroacetyl group which forms the neoantigen on microsomal protein and that sera from the majority of patients with halothane hepatitis contain antibodies that recognize the trifluoroacetyl epitopes (Kenna et al., 1988).

Propylthiouracil is a thioureylene antithyroid drug and its use is associated with several hypersensitivity reactions. The most common serious reaction is agranulocytosis; others include toxic hepatitis and a syndrome similar to SLE (Cooper, 1984).
Propylthiouracil is accumulated not only in the thyroid gland but also in phagocytizing polymorphonuclear leukocytes (Lam and Lindsay, 1979a; Lam and Lindsay, 1979b). Propylthiouracil was found to be degraded by MPO or eosinophil peroxidase, purified from rat bone marrow, in the presence of \( \text{H}_2\text{O}_2 \) and \( \text{Cl}^- \). One of the metabolites was identified as propylthiouracil-sulfonic acid (Lee et al., 1988). A study by Waldhauser and Uetrecht (1991) has found that propylthiouracil was metabolized by activated neutrophils or MPO/\( \text{H}_2\text{O}_2/\text{Cl}^- \) system. The first step is presumably the formation of the sulfenyl chloride. This metabolite was expected to be reactive and was not detected directly. The disulfide metabolite was isolated and it was further oxidized to the sulfonic acid (Figure 1-14). The sulfonic acid is also reactive and reacts with thiols, such as glutathione, to form a thioether.

![Propylthiouracil Metabolism Diagram](image)

**Figure 1-14:** Metabolism of propylthiouracil by MPO to the reactive sulfonic acid (Waldhauser and Uetrecht, 1991).
1.5 Metabolism of indomethacin and carbamazepine

This section summarizes briefly the general pharmacokinetics and metabolism of indomethacin and carbamazepine.

1.5.1 Indomethacin

Indomethacin is rapidly and almost completely absorbed from the gastro-intestinal tract in healthy adults. Following oral administration, bioavailability is virtually 100%, with 90% of a single dose being absorbed within 4 hrs. At therapeutic concentrations, indomethacin is approximately 99% bound to plasma proteins.

In various laboratory animals, indomethacin has been found to undergo extensive biodegradation via O-demethylation, N-deacylation or both (Harman et al., 1964: Hucker et al., 1966). The respective products of these reactions are desmethyindomethacin (DMI), deschlorobenzoylindomethacin (DBI) and DMBI. A study by Duggan et al. (1972) has also shown that in humans demethylation, followed by deacylation, constitutes the major pathway, and direct deacylation is a minor pathway. Demethylation appears to be exclusively mediated by CYP2C9 in humans (Nakajima et al., 1998). An enzyme in pig liver microsomes that catalyzes the hydrolysis of amide-linkage of indomethacin has been purified and characterized (Terashima et al., 1996). Due to the similar activity of indomethacin hydrolysis in pigs and in humans, it is likely that the hydrolysis of amide-linkage of indomethacin in humans would be associated with an enzyme similar to the indomethacin hydrolyzing enzyme found in pig liver microsomes. Indomethacin and its metabolites are excreted in urine and bile both in the free form and as conjugates. Fecal excretion of radioactivity was shown to vary from 21 to 42% of the dose over a period of four days, but it was invariably comprised predominantly of the two demethylated metabolites (> 90%), almost entirely in their unconjugated forms.
1.5.2 Carbamazepine

Carbamazepine is slowly but almost completely absorbed from the gastrointestinal tract. The peak concentration of the drug occurs 4-10 hrs after a single-dose ingestion. In normal healthy volunteers the apparent plasma half-life of carbamazepine ranges from 25-65 hrs (Morselli and Frigerio, 1975). Carbamazepine is extensively metabolized both in human and rat and more than 30 metabolites have been isolated and identified. Following the administration of $^{14}$C-carbamazepine, 72% of the $^{14}$C-activity was recovered in urine; the remaining radioactivity being recovered in feces (Eichelbaum et al., 1984). In vitro and in vivo studies of the bioactivation of carbamazepine in man and rat have demonstrated that epoxidation of the double bond in the 10,11-position and hydration of this 10,11-carbamazepine epoxide to trans-10,11-dihydropdihydroxycarbamazepine is a major route of carbamazepine metabolism (Eichelbaum et al., 1984). Other important pathways include hydroxylation of the aromatic rings, N-glucuronidation of the carbamoyl side chain and substitution of sulfur-containing groups on the aromatic rings (Lertratanangkoon and Horning, 1982; Lynn et al., 1978). One of the major metabolites, which is generated from hydroxylation of the aromatic rings, is 2-hydroxycarbamazepine (Eichelbaum et al., 1984). It can be further metabolized to 2-hydroxyiminoethylbene, either in the liver or in other tissues. Both 2-hydroxycarbamazepine and 2-hydroxyiminoethylbene have been detected in the urine of rats and humans after hydrolyzed from their glucuronide conjugates (Lertratanangkoon and Horning, 1982).

It has been postulated that the reactive metabolite, which may be responsible for carbamazepine-induced toxicity, is an arene oxide (Pirmohamed et al., 1992b; Shear and Spielberg, 1988). The arene oxide is chemically reactive and cannot be characterized by routine analytical methods such as mass spectrometry and HPLC; therefore, its existence can only be implied from the urinary excretion of unquantified phenols and catechols of
carbamazepine (Lertratanangkoon and Horning, 1982; Regnaud et al., 1988). However, the aromatic hydroxyl metabolites are not necessarily derived from an arene oxide intermediate (Hanzlik et al., 1984). Furthermore, although the detection of glutathione (GSH) conjugate of the postulated arene oxide intermediate in rodents has been reported (Amore et al., 1997; Madden et al., 1996), in humans, the same GSH-conjugate was not detected nor were any of its further metabolized products, such as NAC or thiomethyl derivatives (Maggs et al., 1997).
Oxidation of a metabolite of indomethacin (DMBI) to reactive intermediates by activated neutrophils, HOCl and the myeloperoxidase system.
2.1 Abstract

The use of indomethacin is associated with a relatively high incidence of adverse reactions such as agranulocytosis. Many other drugs associated with agranulocytosis are metabolized to reactive metabolites by activated neutrophils. Therefore, we studied the oxidation of indomethacin and its metabolites by activated neutrophils, MPO (the major oxidizing enzyme in neutrophils), and HOCl (the major oxidant produced by activated neutrophils). No significant oxidation of indomethacin by activated neutrophils was observed. However, DMBI, a major metabolite of indomethacin, was oxidized to a reactive iminoquinone which could be trapped with GSH or NAC to form conjugates with MH$^+$ ions at m/z 511 and m/z 367, respectively. No metabolism was detected in neutrophils that had not been activated and the oxidation was inhibited by azide, which inhibits MPO, and by catalase, which catalyzes the breakdown of H$_2$O$_2$. In reactions with HOCl, the same reactive intermediate was formed; its mass spectrum, with a MH$^+$ ion at m/z 204, was obtained by using a flow system in which the reactants were fed into a mixing chamber and the products flowed directly into the mass spectrometer. The same GSH and NAC conjugates were also observed when DMBI was oxidized by HOCl or by the MPO system followed by addition of GSH or NAC. NMR data for the NAC conjugate indicated that the sulfur was substituted in the 4 position on the aromatic ring. The reactive intermediate generated from DMBI by activated neutrophils may be responsible for the indomethacin-induced agranulocytosis.
2.2 Introduction

Indomethacin is a very effective nonsteroidal antiinflammatory drug, but its use is limited by a high incidence of adverse reactions, including blood disorders. Of a total of 1,261 reports of adverse reactions to indomethacin reported to the United Kingdom. Committee on Safety of Medicines between June 1964 and January 1973, blood disorders were recorded in 157 cases (with 25 fatalities) including thrombocytopenia (35 cases, with 5 fatalities), aplastic anemia (17 cases, with no fatalities), and agranulocytosis or leukopenia (21 cases, with 3 fatalities) (Cuthbert, 1974). Subsequently, the First Report from the International Agranulocytosis and Aplastic Anemia Study confirmed a significant relationship between the use of indomethacin and agranulocytosis and aplastic anemia. In this population-based case-control study conducted in Europe and Israel, the excess risk estimated for agranulocytosis and aplastic anemia was 0.6/10^6 for indomethacin exposure in a 1-week period (Shapiro, 1986). The mechanism of drug-induced agranulocytosis is unknown; however, it is speculated that reactive metabolites produced by activated neutrophils or neutrophil precursors may be responsible for agranulocytosis, either by direct toxicity or via an immune mechanism. It has been shown that several drugs that are associated with agranulocytosis are metabolized to reactive intermediates by activated neutrophils and monocytes (Uetrecht, 1990; Uetrecht, 1996; Uetrecht, 1992). When activated, neutrophils release MPO and generate H2O2 (Klebanoff, 1968; Weiss, 1989). MPO is the major oxidizing enzyme in neutrophils: HOCl is generated by the MPO system, which uses H2O2 to oxidize Cl\(^-\) to HOCl.

A study by Duggan et al. (1972) has demonstrated that the dominant pathways of indomethacin metabolism in humans are demethylation and deacylation. The major metabolites are DMI and DMBI (Figure 2-1). The objectives of this work were to determine whether indomethacin and/or its major metabolites can be metabolized to reactive
intermediates by activated neutrophils, HOCl and the MPO system and, if so, to identify the proposed reactive metabolite and to characterize its chemical reactivity.
Figure 2-1: Major metabolic pathway of indomethacin.
2.3 Materials and Methods

2.3.1 Synthesis of DMI.

DMI was synthesized by demethylation of indomethacin using a modification of the method of McOmie et al. (1968). Indomethacin (0.7 g, Sigma Chemical Co., St. Louis, MO) was dissolved in 200 ml of dichloromethane. To this solution, 10 ml of 1 M BBr₃ was added dropwise. The reaction was protected from moisture by bubbling N₂ through the reaction mixture. The reaction was carried out at room temperature for 3 hr. The reaction mixture was then shaken with water to hydrolyze excess reagent and boron complexes. DMI was obtained by extraction into ether. The mass spectrum of the synthesized DMI included of a MH⁺ ion at m/z 344. The synthesized DMI demonstrated the same HPLC retention time as did an authentic sample provided by Merck Research Laboratories, and it was used to synthesize DMBI without further purification.

2.3.2 Synthesis of DMBI.

DMI (15 mg) was hydrolyzed in 3 ml of 1 N sodium hydroxide. The reaction was carried out at room temperature for 5 min. The reaction mixture was made acidic with concentrated hydrochloric acid and then extracted with ethyl acetate. The ethyl acetate layer was dried with magnesium sulfate, and the solvent was evaporated under a stream of N₂. The product was then purified by silica gel TLC, using a solvent of dichloromethane / methanol (9:1, v/v). The yield was approximately 10% [m.p. = 151-152°C; Rf = 0.35; MS: m/z 206 (MH⁺) and 160 [(MH-HCOOH)⁺]; ¹H NMR (dimethylsulfoxide-d₆) δ 10.64 ppm (1H, s); δ 8.50 ppm (1H, bs); H-7, δ 7.00 ppm (1H, d, J=8.54 Hz); H-4, δ 6.72 ppm (1H, d, J=2.20 Hz); H-6, δ 6.48 ppm (1H, dd, J=8.30 Hz, 2.20 Hz); methylene protons, δ 3.42 ppm (2H, s); methyl protons on the indole ring, δ 2.28 ppm (3H, s)].
2.3.3 Oxidation of DMBI by HOCl.

Sodium hypochlorite (various concentrations) was added to DMBI (final concentration, 0.1 mM) in 100 μl of 0.1 M phosphate buffer at room temperature. The pH of the phosphate buffer was varied from 4.0 to 7.0. Aliquots (20 μl) of the solution were analyzed by HPLC.

2.3.4 Oxidation of indomethacin and DMI by HOCl.

Sodium hypochlorite (50 μl of a 4 mM solution) was added to indomethacin or DMI (final concentration, 1 mM) in 150 μl of 0.1 M phosphate buffer (pH = 6) at room temperature. Aliquots (20 μl) of the solution were analyzed by HPLC.

2.3.5 Oxidation of DMBI by the MPO system.

DMBI (5 μl of a 10 mM methanolic solution) was added to 500 μL of phosphate buffer (0.1M, pH 6 or 7) to yield a final concentration of 0.1 mM. MPO (2.5 units / mL) was added, and then the reaction was initiated by the addition of hydrogen peroxide (final concentration, 0.4 mM). Incubations were carried out in the absence or in the presence of added chloride. Aliquots (20 μl) of the solution were analyzed by HPLC.

2.3.6 Oxidation of DMBI, indomethacin and DMI by activated neutrophils.

Blood was drawn from normal volunteers, into heparinized syringes, and the neutrophils were isolated by differential centrifugation on Ficoll-paque (Pharmacia, Uppsala, Sweden) according to a standard procedure (Boyum, 1984). Briefly, whole blood was mixed with equal volumes of 3% dextran (w/v) made up in 0.9% saline. This
mixture was allowed to settle for 30 minutes and the supernatant was then centrifuged (500xg, 5 minutes). The pellet was resuspended and residual erythrocytes were lysed by a brief exposure to 0.2% saline. The neutrophils were suspended in phosphate buffer saline, underlaid with Ficoll-Paque and centrifuged for 25 minutes (500xg). Following centrifugation, the neutrophil pellet was washed twice with Hank's buffer before use. Neutrophils (1 x 10^6 cells/ml) were suspended in phosphate-buffered saline (pH 7.4, 0.5 ml). Phorbol myristate acetate (40 ng/ml; Sigma) was added, followed by DMBI (5 µl of a 10 mM methanolic solution, to a final concentration of 100 µM). The suspension was then incubated at 37°C in a shaking water bath. After incubation for various periods, the cells were centrifuged and 20 µl of the supernatant was analyzed by HPLC. Indomethacin and DMI were also incubated with activated neutrophils under the same conditions, except that their concentrations were twice that of DMBI.

2.3.7 Synthesis of reactive intermediate-NAC adduct.

Sodium hypochlorite (1 ml of a 100 mM aqueous solution) was added to DMBI (50 ml of a 2 mM aqueous solution). The reaction mixture was vortex-mixed for 10 sec, and then NAC (0.5 ml of a 200 mM aqueous solution) was added. The mixture was then concentrated on a rotary evaporator. The NAC adduct was purified by HPLC using a 10-×150-mm preparative column packed with 3-µM Spherisorb ODS 2 material. The solvent consisted of water, acetonitrile, acetic acid, and triethylamine (89:10:1:0.05, v/v). The flow rate was 3 ml/min, and the retention time of the NAC adduct was 9.4 min.

2.3.8 Reaction kinetics determined by spectrophotometry.

A Hewlett-packard diode-array spectrophotometer (HP8452A, Hewlett-Packard, Palo Alto, CA) was used to determine the rate of oxidation of DMBI by HOCl. Scanning
of the reaction mixture was initiated immediately after the addition of sodium hypochlorite (10 μl of a 10 mM aqueous solution) to DMBI (2 ml of a 50 μM aqueous solution), with rapid stirring. The reaction was monitored at 1.0-sec intervals for 15-sec, over a wavelength range of 200-500 nm.

2.3.9 Analytical Methods.

HPLC was performed using a 2 × 100 mm Phenomenex Column (Phenomenex, Torrance, CA) packed with 5-μM Ultracarb ODS 30 material. The solvent used to analyze the products of DMBI oxidation by HOCl, MPO and activated neutrophils consisted of water, acetonitrile and acetic acid (84:15:1, v/v/v) containing 1 mM ammonium acetate. The solvents used to analyze the products of indomethacin and DMI oxidation by HOCl and activated neutrophils consisted of water, acetonitrile and acetic acid (49:50:1 and 59:40:1, v/v/v, respectively). The flow rate was 0.2 ml/min.

A Sciex API III mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) was used for LC/MS. Collisional activation spectra were obtained using the LC/MS/MS mode, with argon as the target gas. A splitter was used to decrease the flow into the mass spectrometer to approximately 10 μl/min.

1H NMR spectra were recorded at 500 MHz with a Brucker AM 500 spectrometer (Brucker Canada; Milton, Ontario). Spectra were obtained in D2O except for that for DMBI, which was obtained in a dimethylsulfoxide-d6. The structure of the NAC conjugate of the iminoquinone intermediate was assigned by the conventional 1H NMR as well as the 1H−1H and 1H−13C correlation experiments.
2.4 Results

2.4.1 Oxidation of DMBI by activated neutrophils.

DMBI was metabolized by neutrophils to a major metabolite with a MH$^+$ ion at m/z 409 and a minor metabolite with a MH$^+$ ion at m/z 407. The retention times of the two products with MH$^+$ ions at m/z 409 and m/z 407 were 5.8 min and 24.6 min, respectively. No significant metabolism occurred in the absence of activation of the cells. The metabolism increased with time, initial DMBI concentration and cell number (Figure 2-2), and it was inhibited by azide and catalase. The amount of the product with a MH$^+$ ion at m/z 409 was decreased by a factor of 2 and 10 when catalase (200 units/ml) or azide (0.2 mM), respectively, was included in the incubation mixture.

The MS/MS spectra of the metabolites with MH$^+$ ions at m/z 409 and m/z 407 are shown in Figure 2-3. To obtain enough material for UV and NMR analysis, manganese dioxide was used to oxidize DMBI; this procedure yielded the same products, except that the amount of the product with a MH$^+$ ion at m/z 407 was greater than that of the product with a MH$^+$ ion at m/z 409. The UV absorption spectrum of the product with a MH$^+$ ion at m/z 409 demonstrated $\lambda_{\text{max}}$ values of 215 and 300 nm. The UV absorption spectrum of the product with a MH$^+$ ion at m/z 407 showed an additional broad peak with a $\lambda_{\text{max}}$ value of approximately 480 nm (Figure 2-4). When the UV absorption spectrum was obtained at a higher concentration, this broad peak was more obvious. The $^1$H NMR spectra of both products were obtained. The NMR of the product with a MH$^+$ ion at m/z 407 showed that the aromatic region consisted of five protons, as follows: H-7, $\delta$ 7.38 ppm (1H, d, J = 9.77 Hz); H-7', $\delta$ 7.29 ppm (1H, d, J = 8.55 Hz); H-6, $\delta$ 6.71 ppm (1H, d, J = 8.55 Hz); H-4', $\delta$ 6.70 ppm (1H, d, J = 1.71 Hz); H-6', $\delta$ 6.65 ppm (1H, dd, J = 9.88 Hz, 1.58 Hz). The loss of the proton signal for the 4-position of the aromatic ring suggested the structure shown in Figure 2-5. A GSH conjugate with a MH$^+$ ion at m/z 714 was formed.
when this product (MH⁺ ion at m/z 407) was reacted with GSH. This result is consistent with the structure of this product having an iminoquinone moiety (Figure 2-5). The simplicity of the NMR of the product with a MH⁺ ion at m/z 409 indicated a highly symmetrical molecule [H-7, δ 7.32 ppm (1H, d, J = 8.55 Hz); H-6, δ 6.78 ppm (1H, d, J = 8.55 Hz); methylene protons of the side chain, δ 2.88 ppm (2H, dd, J = 18.07 Hz); protons of the methyl group, δ 2.22 ppm (3H, s)]. The NMR of the aromatic region suggested that the proton in the 4 position was substituted by another identical molecule. Figure 2-5 shows the postulated pathway of the formation of the two stable metabolites generated in the oxidation by activated neutrophils.

When GSH or NAC was included in the incubation mixture, the GSH or NAC conjugates of a reactive intermediate were observed, with MH⁺ ions at m/z 511 and m/z 367, respectively. At the same time, the amount of the product with a MH⁺ ion at m/z 409 was markedly decreased (data not shown).
Figure 2-2: Metabolism of DMBI by activated neutrophils as a function of time (A), initial DMBI concentration (B), and neutrophil concentration (C), showing decreases in the DMBI concentration (open circle) and the production of the major product (filled square), which has a MH\(^+\) ion at m/z 409 by MS. Standard conditions were as follows: neutrophil concentration, 1x 10\(^6\) cells/ml; DMBI concentration, 40 \(\mu\)M; phorbol myristate acetate concentration, 40 ng/ml; incubation time, 30 min. The peak areas are in arbitrary units, because we did not have enough material to determine a standard curve. The values are the mean ± SD from three experiments.
Figure 2-3: MS/MS spectra of the metabolites with MH$^+$ ions at m/z 409 (A) and m/z 407 (B), formed during DMBI oxidation by activated neutrophils.
Figure 2-4: UV absorption spectrum of the metabolite with a MH⁺ ion at 407.

during DMBI oxidation by activated neutrophils.
Figure 2-5: Proposed pathway of the formation of the two stable metabolites during DMBI oxidation by activated neutrophils.
2.4.2 Oxidation of DMBI by HOCl

DMBI was readily oxidized by HOCl. Scanning of the UV absorption spectrum of this mixture at 1-sec intervals resulted in the pattern shown in Figure 2-6. The absorbance of DMBI ($\lambda_{\text{max}} = 280$ nm) decreased rapidly and a new species was generated, with increased absorption at longer wavelengths.
Figure 2-6: Repetitive absorption spectra from the reaction of DMBI with HOCl.

The concentrations of both DMBI and HOCl were 50 μM.
When the products of the reaction between DMBI and HOCl were analyzed by MS in the flow system, so that the short-lived intermediate could be detected, an intermediate with a MH$^+$ ion at m/z 204 was observed. Using the MS/MS mode, the major fragment ions were at m/z 176 [(MH-CO)$^+$] and 158 [(MH-HCOOH)$^+$].

When GSH or NAC was added immediately to the reaction mixture of DMBI and HOCl, the GSH or NAC conjugate of the intermediate with a MH$^+$ ion at m/z 204 was observed. The GSH and NAC conjugates formed by trapping the reactive intermediate generated during DMBI oxidation by neutrophils and HOCl were found to have the same retention time and molecular weights and identical MS/MS spectra. In addition, a n-butylamine conjugate of this intermediate with a MH$^+$ ion at m/z 277 was observed by LC/MS when n-butylamine, instead of NAC was added to the mixture of DMBI and HOCl. The NAC conjugate was isolated and the NMR analysis was performed (Figure 2-7). The $^1$H NMR data, combined with the finding from $^1$H-$^1$H and $^1$H-$^1$C correlation experiments (Figure 2-9), confirmed the structure of the conjugate where the sulfhydryl group of NAC was substituted in the 4-position on the aromatic ring (Figure 2-8).

When DMBI was oxidized by HOCl, another product was formed, with a MH$^+$ ion at m/z 238 and a chlorine isotope peak for the MH$^+$ ion at m/z 240. The MS/MS spectrum of this product showed a major fragment ion at m/z 202, resulting from the loss of HCl, which suggested that this was a chlorinated species. This product was not observed when the HOCl concentration was lower than one fifth of the DMBI concentration. It was also not observed when GSH or NAC was added immediately to the reaction mixture of DMBI and HOCl. These results suggested that this chlorinated species was formed by further oxidation with excess HOCl.
Figure 2-8: Proposed scheme for DMBI metabolism by activated neutrophils and trapping the reactive intermediate by NAC.
Figure 2-9: Cosy spectrum of the iminoquinone-NAC conjugate.
2.4.3 Oxidation of DMBI by MPO system

Compared with the oxidation by neutrophils, a similar pattern of products was obtained when DMBI was oxidized by the MPO/H₂O₂ system without added Cl⁻. The major product showed a MH⁺ ion at m/z 409. When GSH was included in the incubation mixture, the same GSH conjugate, with a MH⁺ ion at m/z 511, was observed. When 0.15 mM Cl⁻ was added to the phosphate buffer, the reaction was more rapid and the major product observed had a MH⁺ ion at m/z 238. This is the same product as that observed when DMBI was oxidized by higher concentrations of HOCl.

2.4.4 Oxidation of indomethacin by HOCl and activated neutrophils

Indomethacin was oxidized by HOCl to a major product with a molecular weight of 391, corresponding to indomethacin plus chlorine minus hydrogen. However, no GSH conjugates were observed when GSH was added to the reaction mixture of indomethacin and HOCl. When indomethacin was incubated with activated neutrophils under the same conditions as those used for DMBI, no oxidation was observed.

2.4.5 Oxidation of DMI by HOCl and activated neutrophils

DMI was oxidized by HOCl to two major products, with retention times of 16 and 28 min. The molecular weight of the product with a retention time of 16 min was 2 mass units less than that of DMI. The other product, with a retention time of 28 min, showed a molecular weight of 378, corresponding to DMI plus chlorine minus hydrogen. No GSH conjugates were observed when GSH was added to the reaction mixture of DMI and HOCl. When DMI was incubated with activated neutrophils, only the product with a retention time of 16 min was observed. When GSH was included in the incubation mixture, no GSH conjugates were detected.
2.5 Discussion

DMBI was oxidized by neutrophils to a reactive intermediate that could be trapped by glutathione. MPO appears to be the enzyme in activated neutrophils responsible for this oxidation. This is supported by our observations that the MPO/H₂O₂ system generated the same metabolites as did neutrophils, the metabolism by neutrophils required activation of the cells (resulting in release of MPO and the generation of H₂O₂), and that the metabolism by neutrophils was inhibited by azide (which inhibits MPO) and catalase (which catalyzes the breakdown of H₂O₂).

The mechanism presumably involved N-chlorination, followed by the loss of HCl to form an iminoquinone, as shown in Figure 2-8. The N-chloro-DMBI was not directly observed. However, strong evidence for the identity of the reactive iminoquinone intermediate was obtained. The diode-array UV spectrophotometric data showed that DMBI absorbance (λ_max= 280 nm) decreased rapidly upon addition of HOCl and a new species, with absorbance at longer wavelengths (suggesting a quinone-like structure), was formed within seconds. When the products of the reaction between DMBI and HOCl were analyzed by MS in the flow system, the iminoquinone intermediate, with a MH⁺ ion at m/z 204, was observed. This iminoquinone intermediate was reactive with sulfhydryl-containing molecules, such as GSH and NAC. The proton NMR data confirmed the structure of the iminoquinone-NAC conjugate, in which the sulfur was substituted in the ortho-position (relative to the hydroxyl group) on the aromatic ring.

When DMBI was oxidized by activated neutrophils, the final stable metabolites, with MH⁺ ions at m/z 409 and m/z 407, were formed. Both products were generated from the iminoquinone intermediate reacting with another molecule of DMBI. In the case of the product with a MH⁺ ion at m/z 409, the iminoquinone intermediate reacted with another DMBI molecule in the 4-position, which is ortho to the hydroxyl group. The aromatic carbon ortho to the hydroxyl group of DMBI molecule is expected to be electron-rich and
could act as a nucleophile to attack the carbon-4 of the iminoquinone intermediate (Figure 2-5). Another possible mechanism for the formation of the product with a MH$^+$ ion of 409 involves a coproportionation reaction between DMBI and the iminoquinone to form two radicals that could dimerize. However, because of the solvent cage effect, the free radical might not be able to react with other molecules and contribute to the toxicity; even if a free radical is formed, the major reaction with GSH involves the electrophilic attack of the iminoquinone, rather than the abstraction of a hydrogen atom by a free radical. Presumably the same is true of the reaction between the reactive metabolite and other biological molecules. In the case of the minor product with a MH$^+$ ion at m/z 407, the iminoquinone intermediate reacted with another molecule of DMBI, on the nitrogen of the indole ring, to form a dimer. This is consistent with our observation that the iminoquinone intermediate can react with nitrogen-containing nucleophiles (e.g. n-butylamine), in addition to sulphydryl-containing nucleophiles. The initially formed dimer can be further oxidized to yield the product with a MH$^+$ ion at m/z 407 (Figure 2-5).

A product with a MH$^+$ ion at m/z 238 was observed only when DMBI was oxidized by HOCl at high concentrations (presumably yielding an excess of chlorine). When GSH or NAC was added immediately into the reaction mixture of DMBI and HOCl, this product with a MH$^+$ ion at m/z 238 was not observed, which suggested that it was a downstream product where the iminoquinone was the initial reactive metabolite. This product, with MH$^+$ ion at m/z 238, was not observed when DMBI was oxidized by activated neutrophils or by purified MPO in the absence of added Cl$^-$. This product is probably not a significant in vivo metabolite.

The formation of reactive metabolites is central to most mechanistic theories of drug hypersensitivity reactions (Uetrecht, 1990). Studies from our laboratory have found that several drugs that are associated with a relatively high incidence of hypersensitivity reactions (especially agranulocytosis and lupus) are metabolized to reactive metabolites by activated leukocytes. Similarly, we propose that the oxidation of DMBI to the
iminoquinone reactive intermediate by activated neutrophils is responsible for the hypersensitivity reactions to indomethacin (i.e. agranulocytosis). There is evidence to suggest that other iminoquinones generated by the MPO system or activated neutrophils may cause hypersensitivity reactions (Maggs et al., 1987; Miyamoto et al., 1997; Parrish et al., 1997; Smith et al., 1989; Uetrecht et al., 1994).

In summary, DMBI, a major metabolite of indomethacin in the liver, is metabolized by the MPO enzyme system in activated neutrophils, forming a reactive iminoquinone intermediate. We propose that the iminoquinone intermediate is responsible for some of the idiosyncratic reactions associated with indomethacin, especially agranulocytosis.
Chapter 3

Detection of 2-hydroxyimino stilbene in the urine of patients taking carbamazepine and its oxidation to a reactive iminoquinone intermediate.
3.1 Abstract

Carbamazepine is one of the most widely used anticonvulsants in North America; however, its use is associated with a range of serious idiosyncratic adverse reactions. These reactions are thought to result from the formation of chemically reactive metabolites. Carbamazepine is extensively metabolized in the liver and one of the major metabolites is 2-hydroxycarbamazepine, which has previously been detected as a urinary metabolite excreted by rats and humans along with its further metabolized product, 2-hydroxyiminostilbene.

In this study, we found that the urine of patients taking carbamazepine appeared to contain more of the glucuronide of 2-hydroxyiminostilbene than that of 2-hydroxycarbamazepine. We have also demonstrated that 2-hydroxyiminostilbene can be oxidized readily to an iminoquinone species by HOCl, H$_2$O$_2$ or even on exposure to air. The reactivity of this iminoquinone as an electrophile was studied. It was shown to react with sulfhydryl-containing nucleophiles, such as GSH and NAC. We also found a metabolite with the same molecular weight as 4-methylthio-2-hydroxyiminostilbene, but not the corresponding carbamazepine derivative, in the urine of patients taking carbamazepine and this presummably reflects the formation of a GSH conjugate of the reactive iminoquinone in vivo. This iminoquinone intermediate may play a role in carbamazepine-induced idiosyncratic reactions.

3.2 Introduction

Carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide) is an effective drug in the treatment of convulsive disorders (Cereghino et al., 1974; Cereghino et al., 1973) However, carbamazepine-induced adverse reactions have been reported in as many as one-third to one-half of all patients treated with this drug (Durelli et al., 1989; Gram and
Jensen, 1989; Pellock, 1987). Among these adverse reactions, 5% of them can be classified as idiosyncratic reactions (Askmark and Wiholm, 1990). These idiosyncratic adverse reactions include skin rash (Crill, 1973), blood disorders (Gerson et al., 1983) and hepatitis (Horowitz et al., 1988). A Swedish survey of 505 reports of 713 idiosyncratic reactions to carbamazepine from 1965 to 1987 reported skin reactions (48%), hematological (12%) and hepatic disorders (10%) to be the most frequent. (Askmark and Wiholm, 1990) Although the mechanism of carbamazepine-induced adverse reactions is not clear, they are thought to result from the formation of chemically reactive metabolites (Riley et al., 1989; Shear et al., 1988). An arene oxide intermediate has been postulated to be responsible for the idiosyncratic toxicity of carbamazepine. This hypothesis is mostly based on the observation that the metabolism-dependent cytotoxicity of carbamazepine in vitro can be enhanced by an epoxide hydrolase inhibitor, TCPO (Riley et al., 1989). However, this evidence is complicated by the fact that TCPO is also known to deplete glutathione (Pessayre et al., 1979) and inhibit cytochrome P450 (Ivanetich et al., 1982). Furthermore, since the arene oxide is chemically reactive, it may not reach targets, such as the skin and bone marrow, distant from the liver in sufficient concentrations to cause toxicities. It appears that most drugs associated with bone marrow toxicity are metabolized to reactive metabolites by myeloperoxidase or other enzymes present in the bone marrow, such as alcohol dehydrogenase (Uetrecht, 1996). These considerations led us to search for alternative bioactivation pathways of carbamazepine.

Carbamazepine is extensively metabolized and more than 30 metabolites have been identified in urine of patients taking the drug (Lertratanangkoon and Horning, 1982). The major pathways involve N-glucuronidation on the carbamoyl side-chain of carbamazepine, formation of carbamazepine-10,11-epoxide and hydroxylation on the aromatic rings. One of the major metabolites is 2-hydroxycarbamazepine (Eichelbaum et al., 1984), and it can be further metabolized to 2-hydroxyiminostilbene, either in the liver or in other tissues. Both 2-hydroxycarbamazepine and 2-hydroxyiminostilbene have been detected as
glucuronide conjugates in the urine of rats and humans (Lertratanangkoon and Horning, 1982). We hypothesized that 2-hydroxyiminostilbene can be further oxidized to a reactive iminoquinone intermediate (Figure 3-1), which may be responsible for carbamazepine-induced idiosyncratic reactions.
Figure 3-1: Proposed bioactivation pathway of carbamazepine which leads to the formation of a reactive iminoquinone intermediate.
3.3 Materials and Methods

3.3.1 Synthesis of the iminoquinone intermediate

The iminoquinone was synthesized by oxidation of iminostilbene using a modification of the method of Islam et al. (Islam and Skibo, 1991). Fremy’s salt \( (\text{KSO}_3)\text{NO} \), 2.6 g, Aldrich Chemical Co., Milwaukee, WI) was dissolved in 260 ml of phosphate buffer (pH 7.1). To this solution, 1 g of iminostilbene (Aldrich), which was dissolved in 260 ml of acetone, was added. The mixture was stirred at room temperature for 5 min, and the resulting red solution was extracted with 3 x 200 ml portions of chloroform. The combined extracts were washed with 3 x 100 ml portions of water and then dried over magnesium sulfate. The solvent was evaporated by a rotary evaporator. The iminoquinone was then purified by flash chromatography using hexane / ethyl acetate (7:3, v/v) as the eluting solvent. The product was a red solid with a yield of 23% [m.p. = 135-136°C; MS: m/z 208 \( (\text{MH}^+) \) and 180 \( (\text{MH-CO}^+) \); \(^1\text{H}-\text{NMR} \) (chloroform-d): \( \delta \) 7.94 ppm (H6 or H9, d, J = 7.82 Hz); \( \delta \) 7.62 ppm (H7 or H8, dd, J = 7.57 Hz; 1.63 Hz); \( \delta \) 7.59 ppm (H4, d, J = 9.77 Hz); \( \delta \) 7.54 ppm (H8 or H7, dd, J = 7.57 Hz; 1.47 Hz); \( \delta \) 7.48 ppm (H9 or H6, d, J = 7.81 Hz); \( \delta \) 6.98 ppm (H3, dd, J = 9.76 Hz; 2.20 Hz); \( \delta \) 6.96 ppm (H10 or H11, d, J = 11.00 Hz); \( \delta \) 6.78 ppm (H11 or H10, d, J = 11.48 Hz); \( \delta \) 6.58 ppm (H1, d, J = 2.44 Hz)]. This iminoquinone species has been synthesized and the chemical analyses have been reported: m.p. 135-136°C; the NMR spectrum consisted only of a series of multiplets between \( \tau \) 1.8 and 3.6; the base peak in MS was the M-CO ion (Haque and Proctor, 1968).

3.3.2 Synthesis of 2-Hydroxyiminostilbene
2-Hydroxyiminostilbene was synthesized by reduction of the iminoquinone using the method of Chang (1983). A chloroform solution containing 40 mg of iminoquinone was extracted with freshly prepared sodium hydrosulfite solution (in excess, Aldrich) until the color of the organic layer changed from red to yellow. The chloroform layer was washed with water and dried over magnesium sulfate. The solvent was then removed by a rotary evaporator to yield 36 mg (89% yield) of 2-hydroxyiminostilbene as a yellow solid, [m.p. = 225-226°C; MS: m/z 210 (MH+); ¹H NMR(chloroform-d): δ 7.05 ppm (H₇ or H₈, dd, J = 7.50 Hz; 1.41 Hz); δ 6.90 ppm (H₆ or H₉, d, J = 7.57 Hz); δ 6.85 ppm (H₈ or H₇, dd, J = 7.45 Hz; 1.60 Hz); δ 6.55 ppm (H₃, dd, J = 8.3 Hz; 2.93 Hz); δ 6.54 ppm (H₉ or H₆, d, J = 7.81 Hz); δ 6.44 ppm (H₄, d, J = 8.3 Hz); δ 6.42 ppm (H₁₀ or H₁₁, d, J = 12.00 Hz); δ 6.41 ppm (H₁, d, J = 2.68 Hz); δ 6.32 ppm (H₁₁ or H₁₀, d, J = 11.72 Hz) and two broad peaks in the range of δ 4.9 ppm to δ 4.4 ppm due to the proton on the nitrogen and the proton of the hydroxyl group]. The chemical analyses of 2-hydroxyiminostilbene reported in the literature were: m.p. 225-226°C; NMR (chloroform-d/dimethylsulfoxide-d₆): δ 8.27 ppm (s, 1H, -OH), δ 7.5-6.2 ppm (m, 9H, aromatic and olefin), δ 5.41 (s, broad, 1H, -NH); MS: m/e 209 (M⁺) (Chang, 1983).

### 3.3.3 Synthesis of NAC-adduct of the iminoquinone

A methanolic solution (4.8 ml) of the iminoquinone (10 mg) was added to NAC (4 g), which was dissolved in 43.2 ml of phosphate buffer (pH 8). The mixture was stirred for 10 min at room temperature and then concentrated by a rotary evaporator. The NAC adduct of the iminoquinone was purified by silica gel TLC using a solvent of ethyl acetate/methanol (7:3, v/v). The TLC band (R_F = 0.3) that contained the iminoquinone-NAC adduct was scraped off the TLC plate and the adduct was extracted with ethyl acetate. The yield was approximately 33%. The mass spectrum of the iminoquinone-NAC adduct showed a MH⁺ ion at m/z 371.
3.3.4 Synthesis of 2-Hydroxycarbamazepine

The hydroxyl group on 2-hydroxyiminostilbene was first protected using a modification of the method of Kendall et al. (Kendall et al., 1979). Imidazole (12 mg, 0.18 mmol, Aldrich) was added to a solution of 2-hydroxyiminostilbene (18.4 mg, 0.09 mmol) and tert.-butyldimethylchlorosilane (26.4 mg, 0.18 mmol, Aldrich) in dry N, N-dimethylformamide (0.1 ml). The mixture was stirred for 3 hr, followed by dilution with 5% aqueous sodium bicarbonate (0.5 ml). The reaction mixture was then extracted four times with hexane / dichloromethane (9 : 1, v/v). The combined extracts were washed with water and dried, the solvent was evaporated by a rotary evaporator.

Without further purification, the silylated 2-hydroxyiminostilbene (27 mg, 0.083 mmol) was reacted with 4-nitrophenylchloroformate (50 mg, 0.25 mmol, Aldrich) in chloroform (1 ml). Triethylamine (36 μl, 0.026 mmol) was added at the beginning of the reaction to neutralize the hydrochloric acid generated from the reaction. The mixture was stirred for 3 days and water (1 ml) was then added to quench the excess 4-nitrophenylchloroformate. The product was extracted with chloroform three times and the solvent was evaporated by a rotary evaporator. The product was dissolved in acetonitrile (1 ml) and the ammonia solution (in excess) was added. The mixture was stirred for 3 days before it was neutralized by concentrated hydrochloric acid. The product was extracted into ethyl acetate (3 × 10 ml) and the solvent evaporated. 2-hydroxycarbamazepine was purified by TLC using ethyl acetate : chloroform (8 : 2, v/v) as the eluting solvent. The product was a white solid and the yield was approximately 20% [m.p. = 234-237°C; Rf = 0.35; MS: m/z 253 (MH⁺) and 210 ([(MH-HNCO)⁺]; ¹H-NMR (chloroform-d): δ 7.47 ppm (H₆ or H₉, d, J = 7.08 Hz); δ 7.43 ppm (H₇ or H₈, dd, J = 6.83 Hz; 1.71 Hz); δ 7.38 ppm (H₉ or H₆, d, J = 7.08 Hz); δ 7.34 ppm (H₈ or H₇, dd, J
\( \delta = 6.84 \text{ Hz}; 1.56 \text{ Hz}; \delta 7.32 \text{ ppm (H}_4, d, J = 8.54 \text{ Hz}); \delta 6.93 \text{ ppm (H}_10 \text{ or H}_11, d, J = 11.72 \text{ Hz}); \delta 6.84 \text{ ppm (H}_3, d, J = 8.06 \text{ Hz}); \delta 6.83 \text{ ppm (H}_11 \text{ or H}_10, d, J = 11.32 \text{ Hz}); \delta 6.76 \text{ ppm (H}_1, s); \text{ protons on the amide nitrogen, } \delta 4.5 \text{ ppm (2H, s) and a broad peak at } \delta 5.4 \text{ ppm due to the proton on the hydroxyl group]. The chemical analyses of 2-hydroxycarbamazepine reported in the literature were: m.p. 239-242^\circ \text{C}; \text{ NMR (dimethylsulfoxide-d}_6); \delta 8.25 \text{ ppm (s, 1H, -OH), } \delta 7.5-6.6 \text{ (m, 9H, aromatic and olefin), } \delta 5.45 \text{ (s, 2H, -CONH); MS: m/e 252 (M}^+)\), 209 [(M}^+)-\text{HNCO}] (Chang, 1983).

### 3.3.5 Treatment of the urine sample

Random urine samples were obtained from two male patients (ages, 93 and 83, respectively) who had received carbamazepine (250 and 200 mg/day, respectively) for more than 1 year. The urine sample (~100 ml) was concentrated to about 5 ml by lyophilization. The concentrated urine sample was acidified to pH 4.5 and purified by C-18 Prep-Sep solid phase extraction column (Fisher Scientific, Unionville, ON). The sample was loaded onto the column followed by washing with 2 \times 10-ml portions of water. The desired urinary metabolites were then eluted from the column by 2 \times 10-ml portions of methanol. The methanol effluents were combined and concentrated to approximately 1 ml. Aliquots (10 \mu l) were analyzed by LC/MS. Enzymatic hydrolysis was carried out by incubating the urine samples with \( \beta \)-glucuronidase (100 U, Sigma) in 0.2 ml of pH 5.1 buffer for 20 hr at 37^\circ \text{C}. The carbamazepine metabolites were then extracted with ethyl acetate and the solvent was evaporated under a stream of \text{N}_2. The final products were dissolved in methanol and aliquots of 10 \mu l were analyzed by LC/MS.
3.3.6 Analytical Methods

The analyses of carbamazepine urinary metabolites were performed by interfacing an Ultracarb C-18 column (2-x 100-mm, Phenomenex, Torrance, CA) to a Sciex API III mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada). Aliquots of a urine sample (10 µl) were eluted at a flow rate of 0.2 ml/min, with a solvent consisted of water, acetonitrile and acetic acid (49:50:1, v/v) containing 1 mM ammonium acetate. When the metabolites with shorter retention times (e.g. the glucuronide conjugate of 4-methylthio-2-hydroxyiminostilbene) were analyzed, the eluting solvent consisted of water, acetonitrile and acetic acid (74:25:1, v/v) containing 1 mM ammonium acetate. When β-glucuronide conjugates were analyzed, the eluting solvent consisted of water, acetonitrile and acetic acid (84:15:1, v/v) including 1 mM ammonium acetate. A splitter was used to decrease the flow to the LC/MS interface at approximately 10 µl/min. The collisional activation spectra were obtained by using the LC/MS/MS mode, with argon as the target gas.

$^1$HNMR spectra were recorded at 500 MHz with a Brucker AM 500 spectrometer (Brucker Canada, Milton, Ontario, Canada). Spectra were obtained in chloroform-d except that for the iminoquinone-NAC adduct, which was obtained in deuterium oxide (D$_2$O).
3.4 Results

3.4.1 Oxidation of 2-hydroxyiminostilbene

2-hydroxyiminostilbene was readily oxidized to the iminoquinone intermediate by HOCl. When 2-hydroxyiminostilbene was reacted with HOCl at equal concentrations, the color of the reaction mixture changed instantaneously from yellow to red, and a new peak due to the iminoquinone was observed by HPLC. Figure 3-2 shows the UV absorption spectra of 2-hydroxyiminostilbene and that of the reaction mixture after HOCl was added. The UV absorption spectrum of the oxidation product was similar to that of the iminoquinone. H$_2$O$_2$ was also able to oxidize 2-hydroxyiminostilbene to the iminoquinone species; however, at a much slower rate than did HOCl. It was also observed that 2-hydroxyiminostilbene underwent autoxidation to the iminoquinone intermediate by air at room temperature. The half-life of 2-hydroxyiminostilbene was approximately 2 hr in phosphate buffer (pH 7.4).
Figure 3-2: The UV absorption spectra of the synthesized iminoquinone (dashed line) and 2-hydroxyiminostilbene before (0 sec) and after (10 sec) reaction with HOCl. The concentration of both 2-hydroxyiminostilbene and HOCl were 0.1 mM.
3.4.2 Reactivity of the iminoquinone intermediate

The reactivity of the iminoquinone as an electrophile was studied. It was found to react with sulfhydryl-containing nucleophiles, such as GSH and NAC, to form conjugates. The iminoquinone-NAC conjugate was isolated and analyzed by NMR (Figure 3-3). The $^1$H NMR ($D_2O$) consisted of protons as follows: $\delta$ 7.17 ppm (H$_7$ or H$_8$, dd, $J$ = 7.9 Hz; 1.65 Hz); $\delta$ 6.99 ppm (H$_6$ or H$_9$, d, $J$ = 7.48 Hz); $\delta$ 6.97 ppm (H$_8$ or H$_7$, dd, $J$ = 7.80 Hz; 1.70 Hz); $\delta$ 6.91 ppm (H$_3$, d, $J$ = 2.77Hz); $\delta$ 6.89 ppm (H$_9$ or H$_6$, d, $J$ = 7.56 Hz); $\delta$ 6.51 ppm (H$_{10}$ or H$_{11}$, d, $J$=11.72 Hz); $\delta$ 6.44 ppm (H$_{1}$, d, $J$ = 2.78 Hz); $\delta$ 6.40 ppm (H$_{11}$ or H$_{10}$, d, $J$=11.75 Hz); NAC-CH, $\delta$ 4.28 ppm (1H, dd, $J$ = 7.05 Hz, 3.66 Hz); NAC-CH$_2$α, $\delta$ 3.34 ppm (1H, dd, $J$ = 14.11 Hz, 3.84 Hz); NAC-CH$_2$β, $\delta$ 3.13 ppm (1H, dd, $J$ = 14.10 Hz, 7.05 Hz); NAC-CH$_3$, $\delta$ 1.67 ppm (3H, s). Compared with the NMR spectrum of 2-hydroxyiminostilbene, the proton signal on C4 disappeared in the iminoquinone-NAC conjugate and the proton signal on C3 changed from a doublet of doublets to a doublet. This suggested that the sulfur of NAC was substituted in the 4-position on the aromatic ring (Figure 3-4). This assignment was also confirmed with the findings from the $^1$H-$^1$H and $^1$H-$^{13}$C correlation experiments (Figure 3-5).
Figure 3-3: Proton NMR of the iminoquinone-NAC conjugate.
Figure 3-4: Oxidation of 2-hydroxyiminostilbene to the iminoquinone intermediate and its reaction with NAC.
Figure 3-5: Cosy spectrum of the iminoquinone-NAC conjugate.
3.4.3 Analysis of urine samples from patients

When the urine sample was analyzed by LC/MS, neither the GSH nor the NAC conjugate of iminoquinone was detected. However, a metabolite with MH$^+$ ion at m/z 432 ($R_t = 5.7$ min) was detected. This metabolite has the same molecular weight as the glucuronide conjugate of 4-methylthio-2-hydroxyiminostilbene, which is a probable further metabolite of the iminoquinone-GSH conjugate. The MS/MS of the metabolite with MH$^+$ ion at m/z 432 showed a fragment ion at m/z 256, corresponding to the loss of dehydroglucuronic acid moiety ([M+1-176]$^+$) (Figure 3-6). When the urine sample was hydrolyzed by β-glucuronidase, a peak with a MH$^+$ ion at m/z 254 was detected by LC/MS. The molecular weight of this species was 2 mass units less than that of 4-methylthio-2-hydroxyiminostilbene suggesting that it could be a further oxidized product of 4-methylthio-2-hydroxyiminostilbene.

A glucuronide conjugate of 2-hydroxyiminostilbene was also detected, which showed a MH$^+$ ion at m/z 386. Figure 3-7 shows the comparison of the total ion current (TIC) of the 2-hydroxyiminostilbene glucuronide conjugate with that of other metabolites excreted in the urine including the glucuronide conjugate of carbamazepine (with a MH$^+$ ion at m/z 413), the glucuronide conjugates of monohydroxylated carbamazepines, as well as the N-glucuronide of carbamazepine 10.11-epoxide (with MH$^+$ ions at m/z 429), and the 10,11-dihydrodiol-carbamazepine (with a MH$^+$ ion at m/z 271). The ratios of the TIC of 2-hydroxyiminostilbene glucuronide to that of other urinary metabolites are shown in Table 3-1.

MS/MS spectrum of 2-hydroxyiminostilbene glucuronide conjugate demonstrated that it underwent characteristic fragmentation with the major fragment ion at m/z 210 which represents the loss of the dehydroglucuronic acid moiety ([M+1-176]$^+$) (Figure 3-8). Interestingly, the iminoquinone intermediate was also observed by LC/MS and it showed the same retention time ($R_t = 8.4$ min) and MS/MS fragmentation pattern as that of the
synthesized iminoquinone. When NAC was added to the sample, the peak due to the iminoquinone disappeared, suggesting that it reacted with sulfhydryl-containing nucleophiles. When the urine samples were hydrolyzed by β-glucuronidase, the peak of the iminoquinone increased dramatically (Figure 3-9). In addition, when the glucuronide conjugates of monohydroxylated carbamazepines were hydrolyzed, a peak due to 2-hydroxycarbamazepine was observed, which has the same retention time and MS/MS spectrum as that of the synthesized 2-hydroxycarbamazepine.
Figure 3-6: MS/MS spectrum of the metabolite, which has the same molecular weight as that of 4-methylthio-2-hydroxyiminostilbene, in its glucuronide form (with a $\text{MH}^+$ ion at m/z 432).
Figure 3-7: Selected ion current of the urinary metabolites from the LC/MS of a patient's urine: 7-hydroxyiminostilbene-glucuronide (A), carbamazepine-glucuronide (B), monohydroxycarbamazepine-glucuronide (C) and 10,11-dihydrodiol-carbamazepine (D). The ion current for each ion is provided in the upper right corner of each trace.
Figure 3-8: MS/MS spectrum of the glucuronide conjugate of 2-hydroxyiminostilbene.
Figure 3-9: LC/MS ion current at m/z 208 corresponding to the iminoquinone intermediate in a urine sample before (A) and after (B) hydrolysis by β-glucuronidase.
Table 3-1: The ratios of the TIC of 2-hydroxyiminostilbene-glucuronide (m/z 386) to that of carbamazepine-glucuronide (m/z 413); monohydroxycarbamazepine-glucuronide (m/z 429) and 10,11-dihydrodiol-carbamazepine (m/z 271).

<table>
<thead>
<tr>
<th>Samples</th>
<th>TIC Ratios</th>
<th>m/z 386/m/z 413</th>
<th>m/z 386/m/z 429</th>
<th>m/z 386/m/z 271</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient #1</td>
<td></td>
<td>1.76</td>
<td>0.64</td>
<td>0.41</td>
</tr>
<tr>
<td>Patient #2</td>
<td></td>
<td>4.16</td>
<td>1.23</td>
<td>0.47</td>
</tr>
<tr>
<td>*Patient #3</td>
<td></td>
<td>7.60</td>
<td>2.70</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* The third urine sample was collected from a patient in chronic use of carbamazepine. However, detailed information about this patient was not obtained.
3.5 Discussion

The mechanism(s) of the idiosyncratic reactions associated with carbamazepine therapy are poorly understood. It has been postulated that bioactivation to a reactive arene oxide metabolite is a prerequisite to toxicity (Pirmohamed et al., 1992; Shear et al., 1988) and a risk factor for the adverse reactions is a deficiency of epoxide hydrolase (Spielberg et al., 1981). However, two good studies have failed to find a consistent mutation, or pattern of mutations, in the microsomal epoxide hydrolase gene that is common in patients with a history of carbamazepine hypersensitivity reactions (Gaedigk et al., 1994; Green et al., 1995). Carbamazepine has been shown to be bioactivated to a protein-reactive metabolite by human liver microsomes, however the covalent binding of $^{14}$C-carbamazepine to human liver microsomes was relatively low (Pirmohamed et al., 1992). The formation of the arene oxide metabolite has only been inferred from the urinary excretion of unquantified phenols and catechols of carbamazepine (Lertratanangkoon and Horning, 1982; Regnaud et al., 1988). However, the aromatic hydroxyl metabolites are not necessarily derived from an arene oxide intermediate (Hanzlik et al., 1984). A study by Lillibridge et al. (1996) using mouse liver microsomes has shown that a quinone intermediate may also be formed in carbamazepine bioactivation. Furthermore, it has been demonstrated that for bromobenzene (which does form an arene oxide) most of the covalent binding is due to a quinone (Hanzlik et al., 1984; Rietjens et al., 1997). Although the detection of GSH conjugate of the postulated arene oxide intermediate in rodents has been reported (Armour et al., 1997; Madden et al., 1996), in humans, the same GSH-conjugate was not detected nor were any of its further metabolized products, such as the NAC or thiomethyl derivatives (Maggs et al., 1997). In addition, due to their reactivity, arene oxides are short lived and probably would not be able to reach sites (e.g. the bone marrow or skin) distant from the major site of production, i.e. liver, at sufficient concentrations to cause toxicity. Therefore,
the objective of this work was to investigate alternative bioactivation pathways where reactive metabolites might be generated at the targets of toxicity.

One of the major routes of carbamazepine metabolism in the liver is hydroxylation of the aromatic ring to yield one of the major carbamazepine metabolites, 2-hydroxycarbamazepine, which can be further hydrolyzed to 2-hydroxyiminostilbene, either in the liver or in other tissues (Lertratanangkoon and Horning, 1982). In this study, we were able to detect the glucuronide conjugate of the 2-hydroxyiminostilbene by LC/MS. Two isomers of hydroxyiminostilbene (2-OH and 3-OH-iminostilbene) had been detected in human urine during an earlier study in which they were characterized as their trimethylsilyl (TMS) derivatives after enzymatic hydrolysis of the urine sample with Glusulase (Lertratanangkoon and Horning, 1982). One of the TMS derivatives of the hydroxyiminostilbene isomers was observed to have the same GC/MS properties as the TMS derivative of 2-hydroxyiminostilbene (Lertratanangkoon and Horning, 1982). Although the amount of the metabolite was too small for us to identify the structure by NMR, the fact that we also observed a peak due to the iminoquinone in the same sample suggested that 2-hydroxyiminostilbene glucuronide conjugate must be excreted in the urine because other hydroxyiminostilbene isomers would not generate the iminoquinone intermediate upon oxidation. In addition, when the sample was hydrolyzed by β-glucuronidase to liberate 2-hydroxyiminostilbene, a much larger peak due to the iminoquinone was observed instead of a peak due to 2-hydroxyiminostilbene (Figure 3-8). This was not unexpected since we had shown that 2-hydroxyiminostilbene was readily oxidized to the iminoquinone by HOCl, H₂O₂ or even by air. The free 2-hydroxyiminostilbene released from enzymatic hydrolysis of the glucuronide conjugate was presumably oxidized to iminoquinone readily, before it was detected by LC/MS. We also detected a urinary metabolite with the same molecular weight as the glucuronide conjugate of 4-methylthio-2-hydroxyiminostilbene. However, we did not obtain enough material to identify its structure.
In this work, we also demonstrated that the iminoquinone reacted with sulfhydryl-containing nucleophiles, such as GSH and NAC, to form conjugates. The NMR data confirmed that the sulfur was substituted on the aromatic ring in the meta-position to the hydroxyl group. This result suggested that the iminoquinone is a reactive electrophile, and it may bind to macromolecules (i.e. proteins) in vivo to cause direct toxicity or act as a hapten to modulate the immune system. The iminoquinone intermediate could also undergo redox cycling and generate reactive oxygen species, since it is an oxidizing agent.

In summary, we showed that the β-glucuronide conjugate of 2-hydroxyiminostilbene was excreted in the urine of patients taking carbamazepine. We have also demonstrated that 2-hydroxyiminostilbene was readily oxidized to a reactive iminoquinone intermediate that can be trapped by GSH and NAC. In vivo, 2-hydroxyiminostilbene could be readily oxidized in target organs, such as bone marrow and skin, by peroxidases or even by oxygen. The ease of oxidation of 2-hydroxyiminostilbene makes the iminoquinone an attractive candidate for the reactive metabolite of carbamazepine responsible for idiosyncratic reactions in the bone marrow and skin.
Chapter 4

Investigating the role of the reactive iminoquinone metabolite of carbamazepine in the mechanism of carbamazepine-induced idiosyncratic reactions
4.1 Abstract

We have previously shown that 2-hydroxyimino stilbene, a major metabolite of carbamazepine, can undergo autoxidation to a reactive iminoquinone intermediate. The iminoquinone intermediate reacts with sulfhydryl-containing nucleophiles such as GSH and NAC. It is thought that this iminoquinone intermediate may play a role in carbamazepine-induced idiosyncratic reactions. Immunoblotting studies with anti-iminoquinone-NAC-KLH antibodies detected protein binding in the bone marrow of rats treated with carbamazepine for 4 days. The major modified polypeptides had molecular masses of 115 and 133 KDa. The recognition of these polypeptides by the antiserum could be inhibited by preincubation of the antiserum with 2-hydroxyimino stilbene (10μM) but not by the same concentration of carbamazepine or iminostilbene. Protein oxidation possibly caused by reactive oxygen species generated by redox cycling of the iminoquinone intermediate was also investigated. Increased protein oxidation was found in the nuclear and cytosol fractions of the liver from rats treated with carbamazepine comparing to that of the controls. Several polypeptides with molecular masses of 43, 31, 30 and 28 KDa were found to be oxidized in these two subcellular liver fractions isolated from carbamazepine treated rats but not from control rats.

4.2 Introduction

Idiosyncratic drug reactions are frequently life threatening, and they are almost totally unpredictable. Although the mechanism of idiosyncratic drug reactions are mostly unknown, there is a large amount of circumstantial evidence to suggest that reactive metabolites are involved (Hinson and Roberts, 1992; Park et al., 1995) and some of the clinical characteristics also suggested the involvement of the immune system (Pohl et al., 1988). One proposed mechanism for idiosyncratic drug reactions is based on the hapten
hypothesis. The hapten hypothesis postulates that the drug, or a metabolite, becomes covalently bound to a macromolecular carrier, such as a protein, and that the resulting drug-protein conjugate can be recognized as an immunogen (Park et al., 1987). Some drugs, such as penicillin, captopril and penicillamine, are chemically reactive and react directly with proteins. However, most drugs that cause hypersensitivity reactions are not intrinsically reactive and it is, therefore, assumed that bioactivation must occur in vivo. There is now good evidence that bioactivation of drugs is involved in immunogen formation (Park and Kitteringham, 1990).

Acetaldehyde, the primary metabolite of ethanol, was shown to bind covalently to microsomal proteins, and the binding was shown to increase after chronic alcohol consumption (Nomura and Lieber, 1981). It was found that, in man, chronic abuse is associated with the appearance of an increased titer of antibodies against acetaldehyde protein conjugates (Hoerner et al., 1986). The specificity of antibodies against acetaldehyde adducts produced in vitro suggested that in vivo sensitization occurs as a result of acetaldehyde binding to proteins.

Amodiaquine has been associated with two major side effects, agranulocytosis and hepatitis (Hatton et al., 1986; Larrey et al., 1986; Neftel et al., 1986). Studies have shown that amodiaquine is readily oxidized to an electrophilic quinone imine intermediate and that both amodiaquine and the quinone imine metabolite are immunogenic in the rat (Clarke et al., 1990; Maggs et al., 1987; Maggs et al., 1988). The antibodies raised recognize a common antigenic determinant. The clinical relevance of these results obtained in animal studies is shown by the detection of specific anti-amodiaquine IgG antibodies in man (Park and Kitteringham, 1990). The specificity of the antibody binding again indicated an important role of the quinone imine metabolite in drug sensitization.

Tienilic acid is another example of a drug which requires bioactivation to induce idiosyncratic hepatotoxic effects. It was shown that reactive metabolite formed by oxidation of 14C-tienilic acid by human liver microsomes were irreversibly bound to
cytochrome P450-8 (now known as CYP2C9), the liver enzyme responsible for bioactivation of the drug (Dansette et al., 1989). Sera of patients with tienilic acid-induced hepatitis contained antibodies which specifically recognized a protein of human liver microsomes determined to CYP2C9.

Halothane, a widely used anesthetic, is known to cause idiosyncratic liver damage. Halothane is oxidized in the liver by CYP450 enzymes to a reactive trifluoroacetylchloride which forms the neoantigen on microsomal proteins. Immunochemical studies have shown that sera from the majority of halothane hepatitis patients contain antibodies of the IgG isotype that recognize trifluoroacetyl modified proteins expressed in the liver of animals or humans treated with halothane (Pohl et al., 1988). This is the only case where the identity of the bound metabolite and characterization of the endogenous carrier molecules have been defined.

In the sera of nine patients with carbamazepine-induced hypersensitivity syndrome, autoantibodies (IgG) were found to recognize a 53-Kd protein in rat liver microsomes (Leeder et al., 1992; Riley et al., 1993). No such reactivity was observed in sera from controls. The protein target was identified as CYP3A1; however, these antibodies did not recognize related human CYP3A proteins despite the high degree of structural similarity (Leeder et al., 1996).

We have shown that 2-hydroxyiminostilbene, a major metabolite of carbamazepine found in patient's urine, can undergo autoxidation to a reactive iminoquinone intermediate. Furthermore, the iminoquinone intermediate reacts with sulfhydryl-containing nucleophiles such as GSH and NAC. As discussed earlier, covalent modification of critical endogenous proteins by reactive metabolites is thought to be important in drug-induced idiosyncratic toxicity. To further investigate the role of this reactive iminoquinone metabolite in carbamazepine-induced idiosyncratic reactions, we raised an antibody against the iminoquinone epitope and used it to investigate covalent binding of the iminoquinone intermediate to proteins in vivo. We demonstrated that 2-hydroxyiminostilbene can be
readily oxidized to the iminoquinone intermediate and which not only reacted with GSH or NAC to form a GSH or NAC conjugate, but mostly was reduced to form 2-hydroxyiminostilbene. This result suggested that redox cycling may occur in vivo and result in oxygen activation and possible protein oxidation. Protein oxidation in vivo was therefore investigated.

4.3 Materials and Methods

4.3.1 Synthesis of iminoquinone-NAC-conjugated KLH and RSA

The iminoquinone-NAC conjugate (15 mg) was synthesized as described in Chapter 3 and dissolved in 10 ml N,N-dimethylformamide which contained hydroxysuccinamide (230 mg, Sigma). This solution was then slowly added to a 25 ml dichloromethane solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 310 mg, Sigma). Dichloromethane was evaporated under a stream of N₂ after the reaction was stirred for 0.5 hr. The reaction mixture was then added dropwise with stirring to either Keyhole limpet hemocyanin (KLH, 9 mg, Sigma) or to rabbit serum albumin (RSA, 9 mg, Sigma) dissolved in 5 ml phosphate buffer (pH 7.4). The reactions were carried out by stirring at room temperature for 2 hr. The protein conjugates were dialyzed extensively against water, and then lyophilized.

4.3.2 Production of anti-iminoquinone-NAC-KLH antiserum

Polyclonal anti-iminoquinone-NAC-KLH antibodies were raised in 2.0-2.5 kg male, pathogen-free New Zealand white rabbits (Charles River, Quebec, Canada). A pre-immune serum sample was obtained, and the rabbits were immunized with 500 µg of the iminoquinone-NAC-KLH conjugate emulsified with an equal volume of Freund's complete
adjuvant at multiple subcutaneous sites. Injections with 500 μg antigen emulsified with Freund's incomplete adjuvant divided into 6-8 subcutaneous sites were repeated 2, 4, and 6 weeks after the initial immunization. Exsanguination under pentobarbital anaesthesia was conducted 2 weeks after the final injection and the blood was allowed to clot overnight at 4°C and then centrifuged at 400 g. The serum was recovered and heat-inactivated at 56°C for 30 min before being aliquoted and stored at -20°C.

4.3.3 Activity of the anti-iminoquinone-NAC-KLH antiserum

The activity of the anti-iminoquinone-NAC-KLH antibodies against the iminoquinone epitope was determined by an enzyme-linked immunosorbent assay (ELISA). Iminoquinone-NAC-RSA or RSA (100 μl of a 15 μg/ml solution) was incubated in flat-bottom 96-well plates (Costar, Cambridge, MA) at 4°C overnight. The plates were then emptied and washed four times with ELISA wash buffer [10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.5% (w/v) casein, and 0.02% (w/v) thimerosal]. After the last wash, the plates were tapped dry. Various dilutions (1/100 to 1/100,000) of the anti-iminoquinone-NAC-KLH antiserum (100 μl in PBS) were added to the plates and incubated at room temperature for 3 hr. Plates were again washed four times with ELISA wash buffer and tapped dry. Alkaline phosphate-conjugated goat anti-rabbit IgG (diluted 1:5000 in PBS) was added to each well (100 μl/well) and incubated at room temperature for 2 hr. Plates subsequently were washed four times with ELISA wash buffer and twice with PBS. A stock solution of methyl umbelliferyl phosphate (10 mg/ml in dimethylsulfoxide, kept at -20°C) was diluted 1:100 in PBS, and this solution was added to each well of the 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).
4.3.4 Dosing of rats with carbamazepine in vivo

Female Lewis rats (= 200 g) were obtained from Charles River and housed in standard cages with free access to water and powdered lab chow. After one week acclimation period, the rats were either treated daily with carbamazepine (250 mg/kg, gavage) as a suspension in hydroxypropyl cellulose or with hydroxypropyl cellulose alone (control). At the end of the 4-day treatment, the rats were killed by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg).

4.3.5 Isolation of rats bone marrow cells

Rats femurs were removed. The bone marrow was obtained by pushing a needle through the femurs and was collected into RPMI 1640 culture medium (University of Toronto, Media Service). The bone marrow was suspended by passing through a 1-ml automatic pipette tip a few times. After washing the cells twice in RPMI 1640 and once in Hanks' buffer (HBSS), contaminating red blood cells were lysed by incubation in ammonium chloride (0.16 M)/Tris (17 mM; pH 7.2) buffer for 10 min. The bone marrow cells were then washed twice in HBSS and counted using a hemocytometer. Bone marrow cells were lysed in cell lysis buffer [10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100]. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. The samples were diluted to give protein concentrations of 3 mg/ml; then, an equal volume of 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). The samples were boiled at 100°C for 10 min before analysis by SDS-PAGE.

4.3.6 Preparation of subcellular fractions of rat liver
Subcellular fractions of rat liver were prepared from freshly isolated livers by differential centrifugation. Rat livers from control and carbamazepine treated groups were pooled, weighed, and minced in five volumes of sucrose buffer (0.25 M sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 6.8), then homogenized (Janke-Kunkel homogenizer, 1000 rpm, 10 strokes), and filtered through two layers of muslin. Subcellular fractions, nuclear (1000xg pellet, 10 min), mitochondria (10,000xg pellet, 20 min), microsomes (100,000xg pellet, 90 min) and cytosol (100,000xg supernatant, 90 min), were prepared by sequential centrifugation of the crude whole homogenate as described by Wade et al. (1997) Each pellet was washed twice by resuspending in buffer using a Dounce homogenizer (loose fitting pestle) followed by recentrifugation. At each step, the supernatants decanted from the "crude" pellets and those obtained from the subsequent wash steps were combined and then used in the next centrifugation step to obtain the next fraction. The pelleted subcellular fractions were resuspended in one volume of sucrose buffer, aliquoted and stored at -70°C.

4.3.7 Preparation of rat skin homogenates

A piece of dorsal skin of rat (2x2 cm) was shaved and cut into small pieces. The skin samples were homogenized in sucrose buffer and then filtered through two layers of muslin. Protein concentration was determined using a BCA protein assay kit.

4.3.8 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). Protein (30 μg) was loaded on each lane for all samples (i.e. bone marrow cells, skin homogenates and liver subcellular fractions). SDS-PAGE was carried out using a minigel system (Mini-PROTEAN II; BioRad, Mississauga, Ontario). Stacking and resolving gels were 4% and 10% (or 12%) acrylamide, respectively. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (≈ 45 min). Resolved proteins were transferred onto nitrocellulose using a buffer of 15.7 mM Tris, 120 mM glycine, pH 8.3, containing 20% (v/v) methanol, for 75 min at 100 V using a mini Trans-blot transfer cell (BioRad). The subsequent steps were conducted at room temperature with constant shaking. Before exposure to the antiserum, the nitrocellulose was blocked by incubation in a solution containing 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-iminoquinone-NAC-KLH antiserum (1:500) diluted with ELISA wash buffer [10 mM Tris·HCl, pH 7.5, 154 mM NaCl, 0.5% (w/v) casein and 0.02% (w/v) thimerosal]. Unbound antibodies were removed by washing the nitrocellulose in wash buffer (3 x 10 min). The nitrocellulose then was incubated for 2 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L chain) antiserum (diluted 1:10,000 in wash buffer). The nitrocellulose was washed with ELISA wash buffer (3 x 10 min) followed by washing with Tris buffer [50 mM Tris·HCl, pH 7.4 and 154 mM NaCl] (3 x 5 min). The nitrocellulose sheets were incubated in Super Signal ECL reagent for 5 min, and bound antibodies were visualized by exposing the nitrocellulose to ECL film under safe-light conditions.

4.3.9 Determination of oxidatively-modified proteins

Protein oxidation was determined utilizing the method described by Levine et al. (1994). Protein concentrations of subcellular rat liver fractions were determined and
diluted to 10 mg/ml. One volume of 12% sodium dodecyl sulfate (Sigma) was added to the samples with mixing followed by adding 2 volumes of the 2,4-dinitrophenylhydrazine solution [20 mM in 10% (v/v) trifluoroacetic acid]. The mixture was allowed to stand for 5 min at room temperature before it was neutralized by adding 1.5 volume of 2 M Tris containing 30% glycerol and 19% 2-mercaptoethanol. When the solution was neutralized, the color changed from light yellow to orange. For SDS-PAGE analysis, the samples (36 mg/lane) were loaded directly onto the gel without heating. The subsequent steps for immunoblotting were the same as described above. Rabbit polyclonal antibodies against the 2,4-dinitrophenyl moiety (Sigma, diluted 1:1000) were used to recognize oxidatively modified proteins.
4.4 Results

4.4.1 Synthesis of the iminoquinone-NAC-KLH (RSA) conjugate

As shown in Chapter 3, the iminoquinone intermediate reacted with NAC and formed a conjugate. This introduced a carboxyl group on the molecule so that it can be coupled with amino groups on proteins. (Fig 4-1). In this case KLH was used as the carrier protein because it is a large foreign protein and has been commonly used in immunogen synthesis. The iminoquinone-NAC-RSA conjugate was also synthesized in the same way and it was used as an artificial antigen to determine the activity of the antiserum.
Fig 4-1: The structure of the iminoquinone-NAC-Protein conjugate.
4.4.2 Determination of the activity of the antiserum

The polyclonal antibodies were raised by immunizing rabbits with the iminoquinone-NAC-KLH conjugate. The activity of the antibodies against the iminoquinone epitope was determined by ELISA. ELISA analysis (Fig 4-2) demonstrated that the anti-iminoquinone-NAC-KLH antibodies recognized the iminoquinone-NAC-RSA conjugate but not RSA itself. However, the antisera could not detect covalent binding of the iminoquinone intermediate to other proteins, such as reduced lysozymes.

4.4.3 Immunoblotting of bone marrow cells isolated from rats treated with carbamazepine

Using the anti-iminoquinone-NAC-KLH antiserum, it was possible to detect covalent binding in bone marrow cells of rats dosed with carbamazepine (250 mg/kg/day) (Fig 4-3A). The major modified polypeptides had molecular masses of 115 KDa and 133 KDa. The recognition of these polypeptides by the antiserum could be inhibited by preincubation of the antiserum with 2-hydroxyiminostilbene (10 μM). However, the same amount of carbamazepine and iminostilbene (10 μM) were not able to inhibit the binding (Fig 4-3B). Surprisingly, carbamazepine not only did not inhibit the binding but also increased the background signal.

4.4.4 Protein oxidation in subcellular fractions of rat liver

Female Lewis rats were treated with carbamazepine (250 mg/kg/day) and protein oxidation in various subcellular fractions of rats liver was studied. An immunochemical assay (Keller *et al.*, 1993; Levine *et al.*, 1994) was used to detect carbonyl moieties (aldehydes and ketones) that result from oxidative damage to proteins. The carbonyls were
reacted with 2,4-dinitrophenylhydrazine, giving the corresponding hydrazones, which were detected by Western blotting using a polyclonal anti-dinitrophenyl antibodies. In nuclear and cytosol fractions, an increase in protein aldehydes was found in rats treated with carbamazepine comparing to that in the controls (Fig 4-4). Furthermore, in cytosol and nuclear fractions, oxidized polypeptides with molecular masses of 43, 31, 30 and 28 KDa were detected in the samples from rats treated with carbamazepine but not in the samples from control rats (Fig 4-4).

4.4.5 Immunoblotting of skin homogenate and subcellular liver fractions from rats treated with carbamazepine

Proteins of skin homogenates and subcellular liver fractions were separated by SDS-PAGE. Unfortunately, no covalent binding of the iminoquinone to skin and liver proteins was detected using the anti-iminoquinone-NAC-KLH antisera.
Figure 4-2: ELISA analysis showing binding of the anti-iminoquinone-NAC-KLH antiserum to wells of microtiter plates coated with an iminoquinone-NAC-RSA conjugate (square) or RSA (diamond).
Figure 4-3: Immunochemical detection of protein binding in the bone marrow of rats in vivo. A, Bone marrow was isolated from rats treated with carbamazepine (250 mg/kg/day) (T) or hydroxypropyl cellulose (C). B, Effect of preincubation of anti-iminoquinone-NAC-KLH antiserum with various inhibitors on the antibody binding. The inhibitors used were none (CONT), carbamazepine (10 μM, CBZ), iminostilbene (10 μM, IM) and 2-hydroxyiminostilbene (10 μM, 2-OH-IM). Protein (30 μg/lane) was loaded on 10% gel and the primary antiserum was used at a dilution of 1:500.
Figure 4-4: Immunoblotting experiment showing protein oxidation in subcellular fractions of rat liver in vivo. Liver samples were obtained from rats treated with carbamazepine (250 mg/kg/day) (T) or hydroxypropyl cellulose (C). Various subcellular liver fractions were nuclear (NUL), mitochondria (MIT), microsome (MIC) and cytosol (CYT). Protein loading was 36 µg/lane on 10% gel and the primary antiserum was used at a dilution of 1:1000.
Bioactivation of xenobiotics to highly reactive intermediates *in vivo* which can subsequently undergo covalent binding to proteins has been widely implicated as a critical event in target organ toxicity (Boelsterli, 1993; Hinson *et al*., 1994; Nelson and Pearson, 1990; Vermeulen *et al*., 1992). We hypothesized that bioactivation of carbamazepine to a reactive metabolite and the subsequent covalent binding of this reactive intermediate to proteins may play a role in carbamazepine-induced idiosyncratic reactions. We have shown that a reactive iminoquinone intermediate was formed readily from oxidation of 2-hydroxyiminositilbene, a major metabolite of carbamazepine. By trapping the iminoquinone intermediate with NAC and then coupling this conjugate to KLH, it was possible to produce an immunogen that subsequently was used to raise the polyclonal antibodies. The antibodies were shown to recognize the iminoquinone epitope by ELISA experiment. The possible role of the iminoquinone in carbamazepine-induced toxicity was investigated using the raised antiserum. Female Lewis rats were treated with carbamazepine (250 mg/kg/day, gavage) for four days and the liver and bone marrow were isolated at the end of the drug treatment. Using the developed anti-iminoquinone-NAC-KLH antiserum, it was possible for us to detect protein binding in the bone marrow cells of rats treated with carbamazepine but not in that of the control animals. The major modified polypeptides had molecular masses of 115 KDa and 133 KDa. This binding was inhibited by 2-hydroxyiminositilbene (10 μM) but not by carbamazepine or iminositilbene at the same concentration. This result suggested that the polypeptides that were recognized by the antiserum were likely to be covalently modified by the iminoquinone reactive intermediate. Nonspecific binding was detected in the bone marrow isolated from control rats that had not been treated with carbamazepine. This can be explained by two reasons. First, if the rabbits used to raise antibodies were not kept in a properly sterilized environment, they could develop antibodies against various environmental bacteria and pathogens that will cross-react with proteins in
the bone marrow. Second, to detect the iminoquinone-modified proteins, a low dilution (1:500) of the antiserum had to be used, which may also contribute to the background binding.

In a previous study, we have shown that when the iminoquinone intermediate of carbamazepine reacted with GSH or NAC, the GSH or NAC conjugate was formed. However, the major pathway of this reaction was reduction of the iminoquinone to 2-hydroxyiminosilibene. This suggested that the iminoquinone intermediate is both an electrophile and an oxidizing agent; therefore, it may covalently bind to critical proteins or it may initiate oxidative damage. The iminoquinone intermediate, as an oxidizing agent, may deplete the cell of glutathione and cause oxidative stress. Oxidative stress is generally associated with production of reactive oxygen species, such as $H_2O_2$, $O_2^-$ and the highly reactive $-OH$. These species may cause lipid peroxidation, DNA damage and oxidation of protein amine groups. Oxidation of a protein may alter its structure, function and integrity. Oxidative damage to proteins has been shown to result in protein turnover and decreased enzymatic function, and it has been associated with aging and a number of pathological processes including emphysema, atherosclerosis and neurological diseases (Stadtman and Oliver, 1991). Alteration of proteins by oxidation may also be mechanistically important in the toxicity of various chemicals and drugs.

Various functional groups on a protein may be oxidized and form different types of products: cysteine-cysteine disulfide linkages, tyrosine-tyrosine linkages, methionine sulfoxide and aldehydes from lysine, arginine and proline. In this study we used an immunoblotting assay to detect protein aldehyde formation, which may occur in vivo as a result of oxygen activation via redox cycling between the iminoquinone intermediate and its reduced product 2-hydroxyiminosilibene. Increased protein oxidation was found in the nuclear and cytosol fractions of the liver from rats treated with carbamazepine comparing to that of the controls. Furthermore, in these two fractions, several oxidized polypeptides with molecular masses of 43, 31, 30 and 28 KDa were oxidized in the samples from
carbamazepine-treated rats but not in that of control rats. This suggested that carbamazepine treatment caused the oxidation of target proteins in rats in vivo. The pattern of protein oxidation was also different in carbamazepine-treated rats than that of the control rats. However, we do not have direct evidence that the protein oxidation was due to the formation of the iminoquinone intermediate in vivo. It is possible that the parent drug or other unidentified metabolites may result in the protein oxidation. Agents that can prevent the formation of the iminoquinone intermediate may prove whether it plays an important role in protein oxidation and covalent binding. Since inhibition of the hydrolysis of 2-hydroxycarbamazepine to 2-hydroxyiminostilbene would prevent the formation of the iminoquinone in vivo, treating the animals with an acyl amidase inhibitor along with carbamazepine should be able to inhibit both the covalent binding and protein oxidation.

In summary, we have developed an anti-iminoquinone-NAC-KLH antiserum and used the antiserum to investigate the covalent binding of the iminoquinone to the bone marrow cells of rats treated with carbamazepine in vivo. In addition, we have also detected increased protein oxidation in the nuclear and cytosol fractions of the liver from rats treated with carbamazepine compared to the controls.
Chapter 5

Summary and Discussion
5.1 Summary

The aim of the studies reported in this thesis was to investigate whether bioactivation of indomethacin and carbamazepine could lead to the formation of reactive metabolites that may be responsible for the idiosyncratic adverse reactions caused by these two drugs.

The results from the indomethacin study demonstrated that DMBI, a major metabolite of indomethacin, can be further oxidized by the MPO system in activated neutrophils to a reactive iminoquinone intermediate. The identity of this reactive intermediate was supported by several strong pieces of evidence. The diode-array UV spectrophotometric data showed that when DMBI reacted with HOCl, a new species was formed that showed a UV absorbance at longer wavelengths (suggesting a quinone-like structure). The iminoquinone intermediate with a MH+ ion at m/z 204 was detected by MS in a flow system when the products of the reaction between DMBI and HOCl were analyzed. Furthermore, this iminoquinone intermediate reacted with sulfhydryl-containing nucleophiles, such as GSH and NAC, to form conjugates, and the structure of the iminoquinone-NAC conjugate was confirmed by proton NMR analysis. The NAC was bound to the aromatic ring ortho to the hydroxyl group. The reactivity of the iminoquinone intermediate as an electrophile and its formation by MPO in activated neutrophils suggested that this reactive iminoquinone intermediate may be responsible for indomethacin-induced idiosyncratic adverse reactions, especially agranulocytosis.

It has been suggested that idiosyncratic drug reactions associated with carbamazepine are due to bioactivation of carbamazepine by CYP450 to form an arene oxide and deficiency of epoxide hydrolase is a risk factor for these adverse reactions (Shear and Spielberg, 1988). However, in patients with a history of carbamazepine hypersensitivity reaction, the decreased epoxide hydrolase genotype was no more prevalent than in the control group (Gaedigk et al., 1994; Green et al., 1995). The only direct
evidence to support the "arene oxide hypothesis" was the finding of a GSH adduct of carbamazepine in rat bile (Madden et al., 1996). The structure of this GSH adduct is consistent with an arene oxide precursor and the GSH was substituted ortho-to the hydroxyl group (Figure 5-1); however, such an adduct was not found in humans (Maggs et al., 1997). In addition, the carbamazepine hypersensitivity reaction involves many organs, in which CYP450 is not a major oxidizing enzyme, that are unlikely to be able to form significant amounts of arene oxide.

We have discovered an alternative metabolic pathway of carbamazepine leading to a reactive iminoquinone (Figure 3-1). The formation of 2-hydroxycarbamazepine, which is the first step in this pathway, is known to be a major metabolic pathway in humans (Eichelbaum et al., 1984). The second step in the formation of the iminoquinone is hydrolysis of 2-hydroxycarbamazepine to 2-hydroxyiminostilbene, either in the liver or peripheral tissues. We have found that, in the urine of three patients taking carbamazepine, 2-hydroxyiminostilbene is a major metabolite. Furthermore, we also demonstrated that 2-hydroxyiminostilbene is readily oxidized to the reactive iminoquinone and this oxidation even occurs spontaneously in air. This iminoquinone reacts with GSH and NAC and the substitution is meta-to the hydroxyl group, as opposed to the ortho substitution of the putative arene oxide. Another difference between the GSH adduct of the iminoquinone and that of the arene oxide is the presence of the intact urea group in the adduct formed from the arene oxide. In the urine of these three patients, we also found a metabolite that appears to be a thiomethyl derivative of the 2-hydroxyiminostilbene, which presumably came from further gut metabolism of a GSH conjugate, although its structure has not been conclusively demonstrated. However, we did not find any sulfur-containing derivatives of 2-hydroxycarbamazepine, which presumably is the further metabolite of a GSH adduct of the arene oxide.

To further investigate the role of the iminoquinone intermediate in carbamazepine-induced adverse reactions, an antibody (anti-iminoquinone-NAC-KLH) against the
Iminoquinone epitope was developed and used to study the covalent binding of the iminoquinone to endogenous proteins. Immunoblotting studies showed that the antibody was able to detect covalent binding in the bone marrow of rats treated with carbamazepine for four days. Furthermore, the covalent binding was inhibited by 2-hydroxyiminostilbene but not by carbamazepine or iminostilbene. These results suggested that the polypeptides recognized by the antiserum were likely to be covalently modified by the iminoquinone reactive intermediate. Furthermore, although the iminoquinone covalently binds to sulphydryl groups, the dominant reaction is reduction to 2-hydroxyiminostilbene, and we have also detected increased protein oxidation in the nuclear and cytosol fractions from the liver of rats treated with carbamazepine compared to the controls. The protein oxidation is probably due to reactive oxygen species generated in vivo during the redox cycling between the iminoquinone and its reduced product 2-hydroxyiminostilbene (Figure 3-4).

We have demonstrated that the major metabolites of indomethacin (DMBI) and carbamazepine (2-hydroxyiminostilbene) can be oxidized to reactive iminoquinone intermediates in one target of toxicity, specifically neutrophils. We have also shown that these iminoquinone intermediates are reactive electrophiles and that they react with sulphydryl-containing nucleophiles (i.e. GSH and NAC). We were able to detect covalent binding to bone marrow cells in rats treated with carbamazepine using the antiserum raised against the reactive iminoquinone epitope. However, covalent binding was not detected in other target organs, such as the liver or skin, from rats treated with carbamazepine. One possible reason for this discrepancy is that the antiserum was raised against a specific antigen in which the iminoquinone was covalently bound to NAC before binding to protein and this may not be the same way that the iminoquinone binds to proteins in vivo. Therefore, in the future, it will be necessary to develop another antibody against the immunogen in which the iminoquinone directly binds to proteins. In addition, although we have detected covalent binding using the antiserum developed against the iminoquinone epitope, the role of the reactive iminoquinone in the covalent binding needs to be further
examined. If the major reactive metabolite responsible for the binding is the iminoquinone, agents that prevent its formation should inhibit the covalent binding. Since inhibition of the hydrolysis of 2-hydroxycarbamazepine to 2-hydroxyimino stilbene would prevent the formation of the iminoquinone *in vivo*, treating the animals with an acyl amidase inhibitor at the same time as carbamazepine should be able to inhibit the covalent binding. A positive result from this experiment, would suggest that the iminoquinone, not the arene oxide, is responsible for most of the covalent binding. It is important, in the future, to study the covalent binding in humans because metabolism may be different in animals than in humans. The common targets of carbamazepine-induced toxicity are the liver, skin and blood. Although obtaining a liver biopsy is difficult, it should be much easier to obtain blood and skin biopsies from patients who are treated with carbamazepine. If we find binding in these tissues, we should then be able to determine in which cells and where within the cells the binding is the greatest. Furthermore, we can make an affinity column with the antibodies to separate proteins that are modified by the reactive metabolite and to determine their sequences.
5.2 Discussion

Idiosyncratic drug reactions pose a significant complication in modern therapy because of their unpredictable nature and potentially fatal outcome. Although the mechanisms involved are poorly understood, there is a large amount of circumstantial evidence to suggest that chemically reactive metabolites play a pivotal role. Such reactive metabolites may be directly toxic to the cells or they may become covalently bound to a macromolecular carrier, such as a protein, which may initiate an immune-mediated reaction.
Chemically reactive metabolites are, in general, electrophiles that can react with nucleophilic groups on proteins to become haptens. The nucleophilic groups on proteins include lysyl and cysteiny1 residues, and the imidazole and phenol groups present in histidine and tyrosine respectively. For example, halothane is oxidized in the liver to reactive metabolite TFA, which reacts preferentially with lysine residues on hepatic proteins (Kenna et al., 1988). It has also been demonstrated that patients with halothane-induced hepatitis have antibodies against specific TFA-modified hepatic proteins. Therefore, it is essential to identify the reactive metabolite of a drug in order to understand their interactions with cellular macromolecules. Having a clear idea of the chemical nature of the reactive metabolite also may help in developing strategies to prevent drug-induced adverse reactions. For example, drugs that have an aromatic amino group are usually oxidized to highly reactive metabolites, such as hydroxylamine and nitroso derivatives. These reactive metabolites have been associated with adverse reaction caused by drugs with a free amino group, such as sulfonamides, dapsone and procainamide (Coleman et al., 1989; Rieder et al., 1989; Uetrecht et al., 1988b; Uetrecht, 1990). Although the incidence and clinical outcome of adverse reactions caused by these drugs are variable, in each case the initial event in drug toxicity is N-hydroxylation which occurs in most individuals taking the drug. In both carbamazepine and indomethacin studies, we have demonstrated that reactive iminoquinone intermediates were formed during the oxidation of the major metabolite of indomethacin (DMBI) and of carbamazepine (2-hydroxyiminostilbene). The common feature of these two major metabolites (DMBI and 2-hydroxyiminostilbene) is that they both have an amine and a hydroxyl group in para positions to each other on the same aromatic ring. This allows to bioactivation of the molecules to iminoquinone species. As discussed in Chapter 1, it has been shown that a number of drugs or their metabolites with similar structural features as DMBI and 2-hydroxyiminostilbene can be bioactivated to reactive iminoquinone intermediates by various metabolizing enzymes (e.g. CYP450, MPO or PHS). Studies have also suggested that the formation of reactive iminoquinone
intermediates are responsible for the adverse reactions caused by these drugs. For example, bioactivation of amodiaquine to a reactive iminoquinone and its covalent binding to various proteins have been demonstrated in several systems: human liver microsomes, the horseradish peroxidase system and activated neutrophils. Bioactivation of acetaminophen to a reactive N-acetylbenzoquinone imine by CYP450, MPO and PHS systems has also been associated with renal and hepatotoxicity in cases of acetaminophen overdoses. Therefore, a knowledge of the functional groups on a molecule which lead to bioactivation and formation of a reactive metabolite may allow the development of analogs which do not contain such functional groups but still retain their therapeutic properties.

Although it is commonly accepted that reactive metabolites are involved in idiosyncratic drug reactions, in most cases, this has not been rigorously proven. Identification of a reactive metabolite of a drug does not equal proving its role in causing toxicity. It is possible that other undiscovered reactive metabolites are more important; even if the identified reactive metabolite appears to be responsible for the toxicity, there could be other risk factors for idiosyncratic reactions besides formation of a reactive metabolite. Most drugs can be metabolized to chemically reactive metabolites; however, they do not all cause a high incidence of idiosyncratic reactions. This can be explained by several possibilities. First, the reactive metabolites can be detoxified, in many cases, before they can initiate tissue damage. It has been demonstrated that a number of drugs, which are associated with occasional immunological reactions, may undergo bioactivation by human liver microsomes to chemically reactive metabolites in vivo (Park and Coleman, 1988). However, parallel in vivo studies have shown that such bioactivation is normally precluded or severely restricted by competing oxidation and conjugation reactions. When bioactivation does occur, the chemically reactive metabolites are rapidly detoxified by cellular defense mechanisms such as conjugation by glutathione (or glutathione transferase) and hydrolysis by epoxide hydrolase. Therefore, the production of haptens, which could stimulate the immune system, would be prevented (or reduced). However, the normal
balance between the bioactivation and detoxication pathways may be disturbed, in which case the reactive metabolite can escape detoxication.

Second, the chemical reactivity of the reactive metabolite is a major factor in determining the degree and character of toxicity (Gillette, 1974a; Gillette, 1974b; Park and Kitteringham, 1990). If a reactive metabolite has low reactivity, it can be selectively detoxified before binding to some critical molecules. At the other extreme, if a reactive metabolite is too reactive, it may react with anything it encounters, such as water or the enzyme that formed it, and have little chance to bind to other important molecules (Uetrecht, 1992). For example, acetaminophen is associated with a low incidence of idiosyncratic reactions. The reactive metabolite of acetaminophen is the acetylbenzoquinone imine and the low incidence of acetaminophen-associated idiosyncratic reactions is probably due to the selectivity of its reactions. The acetylbenzoquinone imine metabolite is a soft electrophile and preferentially reacts with soft nucleophiles, such as glutathione. However, its reaction with GSH is relatively slow in the absence of glutathione transferase (Coles et al., 1988). Therefore, there is little chance for covalent binding to other important molecules, i.e. proteins. Cysteine and lysine are probably the two most common nucleophilic amino acids. The reactivity of the reactive metabolite and whether it is a "soft" or "hard" electrophile also determine the amino acid residue to which it binds (Uetrecht, 1992). Furthermore, the amount of reactive metabolites binding to proteins is also important. In general, if a molecule is going to induce an immune-mediated reaction by acting as a hapten, the higher the hapten density (number of drug molecules conjugated per molecule of protein), the higher intensity of the immune response.

Third, the site of reactive metabolite formation and the reactivity of the intermediate can also determine the target organ for toxicity. If the reactive metabolite is only formed in one organ and is too reactive to escape that organ, then the toxicity should be confined to that organ. For example, halothane appears to be oxidized exclusively by CYP450 in the liver to form a very reactive metabolite, TFA. As would be expected, the idiosyncratic
reactions associated with halothane are limited to the liver. Since liver is the major site of drug oxidation, it may be considered to be particularly vulnerable to toxic effects of drugs and their metabolites. However, many idiosyncratic reactions do not involve the liver. This can be explained by the high capacity of cellular detoxication systems and the large reserve of glutathione in the liver.

Fourth, the irreversible binding of the reactive metabolite to specific proteins in vivo is presumably important for initiating an immune reaction. For example, captopril can extensively and covalently bind to cysteine residues in plasma proteins. However, disposition studies have shown that the disulfide linkage is biochemically labile and can be readily dissociated by endogenous thiols such as glutathione, which possibly explains why captopril is only very weakly immunogenic in both humans and experimental animals (Coleman et al., 1988).

Fifth, the probability of a reactive metabolite of a drug to cause adverse reactions is dependent on its toxicity to the cells and its efficiency in protein binding. The efficiency in protein binding of a reactive metabolite is increased by the therapeutic dose of the drug, the percentage of the drug that can be bioactivated to the reactive metabolite and the reactivity of this metabolite to specific proteins.

If all idiosyncratic drug reactions involve a reactive metabolite, it might be expected that all drugs that can form a similar reactive metabolite would cause the same type of reactions. However, this is often not the case. For example, it has been suggested that the hydroxylamine metabolite of procainamide, which can be nonenzymatically oxidized to a reactive nitroso-derivative, plays an important role in the mechanism of procainamide-induced lupus. However, dapsone, which is also an arylamine and can be oxidized to a hydroxylamine, causes several different types of hypersensitivity reactions. Specifically they both cause agranulocytosis but procainamide causes lupus and dapsone does not, while dapsone causes methemoglobinemia and procainamide does not. In this thesis, we found bioactivation of carbamazepine and indomethacin lead to the formation of structurally
similar reactive metabolites (i.e. the iminoquinone species); however, the patterns of idiosyncratic drug reaction caused by these two drugs are somewhat different. The major target of indomethacin-induced idiosyncratic drug reactions is the blood; but the most common serious adverse reaction associated with the use of carbamazepine is "hypersensitivity" reaction, which can affect any organ. This difference is probably due to the following reasons. First, indomethacin is given in lower doses (75 mg/day) than carbamazepine (200 mg/day). Second, in contrast to carbamazepine, which is usually used chronically, indomethacin is usually given in acute situations (such as gout) for a short period of time. Third, our studies have shown that 2-hydroxyiminostilbene is more readily oxidized to the iminoquinone intermediate, in fact it undergoes autooxidation when exposed in air. Therefore, presumably the iminoquinone of carbamazepine can be formed in any tissue where 2-hydroxyiminostilbene reaches. Fourth, although both reactive metabolites are iminoquinone species, their chemical reactivities are not identical. We showed that the iminoquinone of indomethacin reacts with both N- and S-containing nucleophiles, but the iminoquinone of carbamazepine appears to only react with S-containing nucleophiles. In addition, we found that the iminoquinone of carbamazepine can also undergo redox cycling which could lead to different toxicological consequences than protein binding in vivo.

It is thought that many drug-induced toxicities are due to the covalent binding of reactive metabolites to tissue proteins. It has also been suggested that protein binding is not random, but rather selective with respect to the proteins targeted (Cohen et al., 1997). The selective binding may influence homeostatic or other cellular responses which in turn contribute to drug toxicity, hypersensitivity or autoimmunity. Modern biochemical, molecular, and immunochemical approaches have made it possible to study covalent binding of chemically reactive metabolites to cellular proteins and to identify the specific protein targets. For example, several protein targets modified by the reactive metabolite of halothane, TFA, have been isolated from liver microsomes of halothane-treated rats and identified. The 57, 58, 59, 63, 80, 82 and 100 KDa proteins have been identified as
protein disulfide isomerase, a disulfide isomerase isoform, a carboxylesterase, calreticulin, 72 KDa endoplasmic reticulum protein, 78 KDa glucose-regulated protein and 94 KDa glucose-regulated protein, respectively (Amouzadeh and Pohl, 1995). Results from other studies indicate that similar proteins are present in the livers of humans (Smith et al., 1993) and they appear to become covalently modified by the TFA moiety after patients have been administered halothane (Pohl et al., 1989). The ability to identify individual proteins which become modified by reactive intermediates may greatly increase our understanding of the role of such binding in drug-induced idiosyncratic reactions. Once these protein targets of reactive metabolites have been purified, they can serve as antigens for identifying sensitized individuals who have experienced a hypersensitivity reaction to the drug. This information can be used to prevent not only an allergic reaction to the drug, but possibly cross-reactions to other drugs that are structurally related. Another important application of these studies is the design of safer alternative drugs that will not produce structurally similar reactive metabolites.

Although protein binding is important, it may not be the only way that a reactive metabolite can modify proteins and initiate an immune response. Chemically reactive metabolites can also cause oxidative stress, which may in turn, initiate an innate immune response. The innate immune system, although primitive, is an essential part of our defense system against infections. An innate immune response involves recognition of certain chemical features shared by groups of microorganisms but not by the host, such as the lipopolysaccharide of Gram-negative bacterial cell walls; and it also appears to be able to detect cells that have been stressed (Fearon, 1997; Fearon and Locksley, 1996; Poccia et al., 1998). There is also evidence that the innate immune response communicates its recognition to the adaptive system by providing the "danger" signal, without which a new antigen usually induces tolerance in the adaptive system (Bendelac and Fearon, 1997). Some reactive metabolites, which are oxidizing agents, can cause oxidative damage of the cells either directly or through reactive endogenous oxygen species. Stressed or injured
cells can be detected by the innate immune system, which can destroy the cells or may provide "danger" signals to the adaptive immune system. This will ultimately lead to tissue damage.

In any discussion of the mechanisms involved in idiosyncratic drug reactions, one question that must be asked is why only certain individuals are exceptionally susceptible to the toxicity. A number of possibilities exist to explain this (Park et al., 1992; Pohl et al., 1996; Uetrecht, 1990). Individual susceptibility to toxicity will depend on the balance between the relative roles of drug bioactivation and detoxication of both parent drug and the metabolite. Some people may have abnormally high levels of an enzyme that bioactivates the drug to a reactive metabolite or an allelic variant of the enzyme that is catalytically more active than the normal enzyme. Alternatively individuals that develop a particular drug reaction may have abnormally low levels or activities of enzymes that detoxify reactive metabolites, such as glutathione S transferases, epoxide hydrolase or N-acetyl transferase.

However, abnormal drug metabolism cannot explain why only a small number of patient are susceptible to idiosyncratic drug reaction. For example, halothane is metabolized to an acylhalide by CYP450 and the reactive metabolite covalently binds to proteins to form neoantigens. Studies have shown that all experimental animals and patients exposed to the drug generate the antigen (Kenna et al., 1988); however, the incidence of halothane hepatitis is between 1 in 35,000 and 1 in 350,000 (Park et al., 1992). Thus it is thought that the difference in susceptible patients is more likely to be in the immune response to the antigen rather than in neoantigen formation (Kenna et al., 1988).

The ability to initiate an immune response on antigen exposure is controlled by the major histocompatibility (MHC) genes, which are the most polymorphic genes known in higher vertebrates (Park et al., 1992). Such a high level of polymorphism could theoretically result in individuals who are more efficient at recognizing certain epitopes than others with the result that only these individuals are likely to mount a vigorous immune
response to drug-related antigens. The relationships between idiosyncratic drug reactions and drug-related antigens associated with the MHC expressed on accessible cells have not been established; however, epidemiological studies have shown that certain polymorphism of MHC genes are weakly associated with increased susceptibilities to certain autoimmune diseases such as insulin-dependent diabetes mellitus (Baisch et al., 1990) and coeliac disease (Bugawan et al., 1989).

Although initiation of a drug-induced immune response is likely the primary event in drug hypersensitivity, it is not a pathological process. Many patients with anti-drug antibodies or drug-induced autoantibodies remain asymptomatic. For example, with chronic procainamide therapy, most patients develop antinuclear antibodies and only about 20% develop symptomatic lupus. Therefore, it is likely that there are individual risk factors associated with the translation of an immune response into tissue damage. The factors in determining when sensitization is translated into tissue damage could be the amount and site of drug antigen formation, the half-life of the antibodies, the magnitude of the immune response and the type of the immune response, i.e. IgE, IgG or cell-mediated. Furthermore, some individuals with certain diseases or infections may increase their susceptibility to drug-induced adverse reactions. For example, AIDS is associated with an increase in the incidence in sulfonamide hypersensitivity reactions (Fischl et al., 1988) and mononucleosis is associated with an increased incidence of ampicillin rash (Pohl et al., 1988). Aspirin hepatotoxicity and other types of idiosyncratic drug reactions appear to be more common in patients with diseases such as lupus (Cameron and Ramsay, 1984; Konttinen and Tuominen, 1971; Uetrecht, 1990).

Each of these factors in itself may not be sufficient to produce toxicity, but a combination of factors acting collectively in a certain individual may lead to serious toxicity. At present, there is no simple screening test with which to predict which drugs are likely to cause adverse reactions and to identify those individuals who may be susceptible to drug-induced hypersensitivity. However, studies to identify possible reactive
metabolites, to identify and purify the protein targets of reactive metabolites and to analyze the genetic polymorphism, both with respect to immune function and drug metabolism, in susceptible individuals may provide some insight into the mechanism of idiosyncratic drug reactions.
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