CHEMOPROTECTIVE PROPERTIES OF NATURALLY OCCURRING METHYLENEDIOXYBENZENES AND CATECHOLIC METABOLITES

by

Ziwei Sylvia Zhao

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
Faculty of Pharmacy
University of Toronto

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Chemoprotective Properties of Naturally Occurring Methylene-dioxybenzenes and Catecholic Metabolites

Ziwei Sylvia Zhao

Graduate Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Toronto

Naturally occurring methylenedioxybenzenes (MDBs) are found in a wide variety of human food, essential oils and flavors. MDBs have been widely accepted as cytochrome P450 inhibitors. The chemoprotective effects of this group of dietary compounds are, however, still unknown. In the present work, the dietary MDBs, in particular isosafrole, have been found to: 1) prevent CCl4-induced liver necrosis in vivo and BrCCl3-induced hepatocyte injury in vitro; 2) protect against carcinogens N-dimethylnitrosamine and chloroform hepatotoxicity in vivo; 3) prevent phenetidine (a deleterious metabolite of phenacetin)-induced methemoglobinemia in mice. Isosafrole was highly protective even at dosages as low as 10 mg/kg, and was found to be a potent inhibitor of P450 isozymes CYP2E1, CYP2B1/2 and CYP1A2 that are involved in the bioactivation of the toxicants both in vivo and in vitro. The protective effects of MDBs were therefore predominantly attributed to the formation of inhibitory MDB metabolic intermediate-ferrocytochrome P450 complexes, which resulted in inactivation of P450 isozymes and thus prevented the metabolic activation of the xenobiotics.
It was also discovered for the first time that these MDBs and their catecholic metabolites markedly inhibited reactive oxygen species (ROS)-mediated ferric nitrilotriacetate-induced renal necrosis in mice as well as lipid peroxidation with rat hepatocytes and liver microsomes. The antioxidative effect of isosafrole was however decreased if the hepatocytes were pre-incubated with other P450 inhibitors. This suggested that MDBs are potential antioxidants that could prevent ROS-mediated cytotoxicity mainly through their catecholic metabolites, which are direct antioxidants and metal chelators.

Catechol and its derivatives were also found to form stable catecholic ferric complexes at physiological pH, which possess greater superoxide dismutase mimic activity than uncomplexed catechols. These catecholic iron complexes were also much more effective than uncomplexed catechols at protecting hepatocytes against hypoxic cell injury.

The chemoprotective properties of this group of compounds suggested that MDBs, particularly the non-genotoxic isosafrole, could protect against in vivo chemical carcinogenesis catalyzed by cytochrome P450-dependent monooxygenases as well as ROS-mediated oxidative injury catalyzed by the presence of iron.
Dedicated to

My dear husband YI REN
mother XUN-ER CHEN
father CHANGJIU ZHAO
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<tr>
<td>3MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>AHH</td>
<td>Benzo[a]pyrene 3-hydroxylase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AminoMDB</td>
<td>Methyleneoxyaniline</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BrMDB</td>
<td>4-Bromo-1,2-(methyleneoxybenzene)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>PUN</td>
<td>Plasma urea nitrogen</td>
</tr>
<tr>
<td>ButylMDB</td>
<td>4-t-Butyl-1,2-(methyleneoxybenzene)</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Cytochrome P4501A2</td>
</tr>
<tr>
<td>CYP2B1/2</td>
<td>Cytochrome P4502B1/2</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Cytochrome P4502E1</td>
</tr>
<tr>
<td>DMN</td>
<td>N-Dimethylnitrosamine</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin O-deethylase</td>
</tr>
<tr>
<td>FeNTA</td>
<td>Ferric nitrilotriacetate</td>
</tr>
<tr>
<td>HC</td>
<td>Hydroxychavicol</td>
</tr>
<tr>
<td>hr</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HX</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal(ly)</td>
</tr>
<tr>
<td>Iso-HC</td>
<td>Iso-hydroxychavicol</td>
</tr>
<tr>
<td>I.U.</td>
<td>International unit</td>
</tr>
<tr>
<td>Ka</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>Kf</td>
<td>Formation/Stability constant of a complex</td>
</tr>
<tr>
<td>MDB(s)</td>
<td>Methyleneoxybenzene(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MeMDB</td>
<td>3,4-(Methylenedioxy)toluene</td>
</tr>
<tr>
<td>Metapyrone</td>
<td>2-Methyl-1,2-di-3-pyridyl-1-propanone</td>
</tr>
<tr>
<td>MI</td>
<td>Metabolic intermediate</td>
</tr>
<tr>
<td>MROD</td>
<td>Methoxyresorufin O-demethylase</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium</td>
</tr>
<tr>
<td>NHE</td>
<td>Normal hydrogen electrode</td>
</tr>
<tr>
<td>NitroMDB</td>
<td>1,2-(Methylenedioxy)-4-nitrobenzene</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>PBO</td>
<td>Piperonyl butoxide</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>PNP</td>
<td>( p )-Nitrophenol</td>
</tr>
<tr>
<td>p.o.</td>
<td>per os</td>
</tr>
<tr>
<td>PROD</td>
<td>Pentoxysorufin O-dealkylase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase(s)</td>
</tr>
<tr>
<td>TBARS</td>
<td>2-Thiobarbituric acid-reactive substances</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
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Chapter 1

GENERAL INTRODUCTION

1.1 Naturally Occurring Methylene dioxybenzenes

1.1.1 Sources

Naturally occurring methylenedioxybenzenes (MDBs) are found in a wide variety of plants, including those used as flavors, spices and seasonings, such as parsnips, carrots, parsley, nutmeg, sesame seeds, pepper, and sassafras (Hodgson and Philpot, 1974; Ashurst, 1991). For example, both nutmeg and mace are widely used as spices. Nutmeg is the seed, and mace the outer seed coat, of Myristica fragrans Houtt. They contain a large number of organic compounds, among which myristicin and safrole are two important components. The commercially available nutmeg oil contains 10% myristicin and 2% safrole (Ashurst, 1991). Naturally occurring MDBs are also principal physiologically active constituents in many folk medicines. The medicinal use can be traced back to the early Middle Age of Asia and Middle East, or probably even earlier. Since then, these spices have gained high esteem as a treatment for a variety of conditions including toothache, dysentery, cholera, rheumatism, halitosis, and skin diseases. The chemical structures of methylenedioxybenzenes and structurally related compounds that were investigated in the present work are listed in Table 1.1. The chemistry, biotransformation and pharmacological properties of safrole and piperonyl butoxide (PBO) have been reviewed by Ioannides et al. (1981) and Haley (1978), respectively. The following four MDBs investigated in the present work are described in more detail.
Table 1.1  Chemical Structures of Methyleneedioxybenzenes and Structurally Related Compounds

<table>
<thead>
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<tr>
<td><img src="image" alt="Benzodioxole" /></td>
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<tr>
<td><img src="image" alt="Methylenedioxyaniline" /></td>
<td>Methylenedioxyaniline</td>
</tr>
<tr>
<td><img src="image" alt="Sesamol" /></td>
<td>Sesamol</td>
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<tr>
<td><img src="image" alt="Safrole" /></td>
<td>Safrole</td>
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<tr>
<td><img src="image" alt="Isosafrole" /></td>
<td>Isosafrole</td>
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<tr>
<td><img src="image" alt="Dihydrosafrole" /></td>
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</tr>
<tr>
<td><img src="image" alt="Myristicin" /></td>
<td>Myristicin</td>
</tr>
<tr>
<td><img src="image" alt="MethylMDB" /></td>
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</tr>
<tr>
<td><img src="image" alt="ButylMDB" /></td>
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(Continued)

Piperonyl Butoxide

BrMDB

NitroMDB

Hydroxychavicol

Iso-hydroxychavicol

Eugenol

Isoeugenol

Curcumin
a) Safrole and Isosafrole. A major source of human exposure to safrole is through consumption of spices, such as nutmeg, mace, cinnamon and black pepper, in which safrole is a constituent (Ioannides et al., 1981). It forms 95% of micranthum oil, from Cinnamomum micranthum Hayata. It also occurs in several related species and varieties, such as Japanese wild ginger and California bay laurel. In addition, safrole is the major constituent of many essential oils. The oil of sassafras, from root bark of Sassafras albidum (Nutt.) Nees., contains as high as 85% of safrole. Isosafrole is present in the essence of Ylang-Ylang and Illicium religiosum (Curò et al., 1987). Safrole and isosafrole are also present in root beer, other beverages and foodstuffs, and have been used as an additive in chewing gum, toothpaste, soaps and certain pharmaceutical preparations (Ioannides et al., 1981; Merck Index). In Europe, for alcoholic beverages, the recommended limits for safrole and isosafrole are 1 mg/kg in drinks with less than 25% of alcohol by volume, and 5 mg/kg in drinks with more than 25% of alcohol by volume (Curò et al., 1987).

b) Myristicin. Myristicin is a volatile aroma constituent of parsley leaf oil used as food flavors and seasonings, and present in perfumery and pharmaceutical preparations (Formacek and Kubeczka, 1982). Although myristicin is present in small quantities in parsley (0.007% in fresh parsley, 14% in parsley leaf oil) (Shaath et al., 1986), it is widespread in a number of other members of the carrot family (Umbelliferae), including celery (MacLeod et al., 1988), and dill (Huopalahti and Linko, 1983). Myristicin is also found in culinary food flavors and seasonings such as mace and nutmeg which are regularly consumed by humans (Salzer, 1977). Nutmeg and mace contain 8-15% of a volatile oil
(i.e., Myristica oil). Myristicin (about 4% of the oil) is its only physiologically active ingredient (Salzer, 1977).

c) **Sesamol**  Sesamol is a constituent of sesame seed oil. Sesame oil is widely used as an edible oil and as a solvent for injections. Sesamol is formed from sesamolin during the processing of sesame oil, and serves as an antioxidant of the oil.

d) **Piperonyl butoxide**  Piperonyl butoxide (PBO), a synthetic methylenedioxy-benzene, is widely used as a commercial pesticide synergist with pyrethroid and carbamate insecticides on a variety of fruit, vegetable, forage, and grain crops (Haley, 1978). It is also used against fleas on livestock and household cats or dogs. PBO has been allowed as a food contaminant in Japan since 1955, with maximum approved use level of 0.024 g/kg in raw cereals (Takahashi *et al.*, 1994).

### 1.1.2 Effects on Cytochrome P450

1.1.2.1 **Cytochrome P450**

Cytochrome P450 (P450)-dependent monooxygenases comprise a large and diverse family of enzymes responsible for the initial oxidation of lipophilic compounds including many drugs, pesticides and environmental carcinogens, as well as many endogenous substrates. P450s have been found in bacterial, yeast, higher plants, insects, and a large number of vertebrate species. In vertebrates, the liver contains the highest levels of P450s with lower concentrations in kidney, lung, skin, brain, adrenal gland, testes and ovaries, placenta, and most other tissues examined (Lewis, 1996; Nebert *et al.*, 1991; Ortiz de Montellano, 1995).
Inhibitors of cytochrome P450 isozymes have been classified into three categories based on their inhibitory mechanisms: (1) reversible inhibitors, (2) quasi-irreversible inhibitors that form complexes with the heme iron ion, and (3) irreversible inhibitors that bind to the protein or the prosthetic heme group, or that accelerate degradation of the prosthetic heme group (Ortiz de Montellano and Correia, 1995). Some inhibitors such as methylenedioxybenzenes are mechanism-based (catalysis-dependent), in that they are catalytically activated by the enzymes to reactive transient metabolic intermediates that irreversibly or quasi-irreversibly inhibit the enzymes. These inhibitors are further divided into four categories: (1) intermediates that bind covalently to the protein, (2) intermediates that quasi-irreversibly coordinate to the prosthetic heme iron, (3) intermediates that alkylate or arylate the prosthetic heme group, and (4) intermediates that degrade the prosthetic heme group, or modify the protein (Ortiz de Montellano and Correia, 1995).

Cytochrome P450s occur as both constitutive and inducible enzymes. Many of the constitutive P450 isozymes are also inducible. Some cytochrome P450 enzymes are able to be induced by their substrates. Induced isozymes usually metabolize the compounds which induce them, thus facilitating metabolism of endogenous and exogenous chemicals to more polar, excretable compounds (Lewis, 1996; Nebert et al., 1991).

The structure, function, mechanism and biochemistry of cytochrome P450s, and inhibition and induction of the enzymes have been extensively studied and reviewed (Lewis, 1996; Nebert et al., 1991; Ortiz de Montellano, 1995).
1.1.2.2 Inhibition of cytochrome P450 by MDBs

It is now well established that the activity of MDBs as insecticide synergists and drug potentiatators resides in their ability to inhibit P450-dependent monooxygenases that play a critical role in the primary metabolism of insecticides and other xenobiotics (Haley, 1978; Hodgson and Philpot, 1974; Franklin, 1972; Wilkinson, 1967). An apparent decrease in cytochrome P450 levels was observed in microsomes isolated from animals treated in vivo with PBO (Matthews et al., 1970). A similar decrease in detectable cytochrome P450 was also observed in microsomal suspensions incubated in vitro with PBO and NADPH (Franklin, 1972; Philpot and Hodgson, 1971). Dalvi and Dalvi (1991) suggested that a decrease in the activity of P450 and other components of the mixed-function oxidase system may have occurred through several mechanisms, including direct binding of MDBs to P450, formation of metabolic intermediate (MI)-P450 complexes, decrease in hepatic heme by the inhibition of δ-aminolevulinic acid synthetase or less probably by liver damage. Till present, the formation of a stable complex of MDB metabolic intermediates with cytochrome P450 has been generally accepted to account for the observed decrease in P450 levels (Franklin, 1972; Philpot and Hodgson, 1972).

The mechanisms of inhibition of other xenobiotic metabolism by MDBs have been suggested to involve both initial competitive inhibition and subsequent non-competitive inhibition by MDBs (Franklin, 1972). In a competitive inhibition, MDBs act as alternate substrates to compete for occupancy of the active site of the enzymes in order to undergo metabolic activation (Casida et al., 1966; Franklin, 1972; Kamienski and Casida, 1970; Wilkinson and Hicks, 1969; Marcus et al., 1985). MDBs have been identified in vitro as type I substrates in insects and mammals (Lewis, 1996; Matthews et al., 1970; Philpot and
Hodgson, 1971), based on the classification of substrate binding spectra with cytochrome P450 devised by Schenkman et al. (1967). In a non-competitive inhibition, metabolic intermediates of MDBs are generated. The reactive metabolic intermediate, possibly a carbene (Dahl and Hodgson, 1979; Mansuy et al., 1979), subsequently binds to the heme of ferrous cytochrome P450 and thereby blocks carbon monoxide binding to the enzyme and inhibits oxygen activation and further metabolism of P450 substrates (Franklin, 1972; Philpot and Hodgson, 1971). In addition to forming complexes that bind tightly with P450s, MDB metabolism also produces CO and thereby artificially lowering apparent cytochrome P450 concentrations (Yu et al., 1980).

Some selectivity was observed in the capacity of MDBs to inhibit several monooxygenase reactions in reconstituted systems (Marcus et al., 1985). Isosafrole has been shown to selectively inhibit metabolism of androst-4-ene-3,17-dione at different positions of the steroid (Murray and Reidy, 1989). Moreover, MDBs exhibited some degree of selectivity in their interactions with microsomal fractions from differently induced animals (Bornheim and Franklin, 1982; Murray et al., 1983b) and with different forms of purified P450 (Ryan et al., 1980).

As potent P450 inhibitors, PBO and related MDBs are able to influence the toxicity and pharmacological action of a variety of other chemicals. PBO has been demonstrated to inhibit the in vitro metabolism of parathion, carbamate, ethylmorphine, p-nitroanisole and aniline (Haley, 1978). MDBs have also been shown in vivo to enhance the toxicity and co-carcinogenesis of fluorocarbons and other drugs (Epstein et al., 1967), and to prolong barbiturate sleeping time (Anders, 1968) and zoxazolamine paralysis time (Fujii et al., 1968, 1970).
In vivo administration of high dosages of PBO (400-1200 mg/kg) has been demonstrated to prevent various xenobiotic toxicity. It was reported that PBO protected against cyclophosphamide-induced bladder damage by preventing P450-catalyzed production of acrolein and/or related compounds (Fraser and Kehrer, 1993). PBO also prevented acetaminophen-induced GSH depletion, covalent binding and hepatotoxicity, which was associated with a decrease in acetaminophen reactive metabolite formation (Brady et al., 1988). Post-treatment of PBO 2 hr after acetaminophen was still effective at reducing acetaminophen cytotoxicity (Brady et al., 1988). PBO also successfully prevented the formation of methemoglobinemia by dapsone for over 24 hr (Coleman et al., 1990), and lowered both renal binding and kidney necrosis caused by bromobenzene (Reid, 1973). Moreover, PBO partially reduced N-diethylnitrosamine-induced pulmonary carcinogenesis (Schuller and McMahon, 1985), although a high concentration of PBO (K_i ≥ 1 mM) was required to inhibit N-diethylnitrosamine deethylase in vitro (Puccini et al., 1989). Furthermore, Kluwe and Hook (1981) reported that PBO treatment partially reduced CHCl_3-induced nephrotoxicity and hepatotoxicity in mice, in agreement with the observations by Ilett et al. (1973).

However, pre- and post-treatment with PBO (400 mg/kg) had no appreciable protective effect on the nephrotoxic, hepatotoxic and gonadotoxic potency of 1,2-dibromo-3-chloropropane (Kluwe, 1983). In addition, PBO did not prevent the destruction of hepatic P450 in rats given CCl_4 orally (Reiner et al., 1972). Neither did it significantly affect very low density lipoprotein (VLDL) secretion by CCl_4 (Pencil et al., 1984).
1.1.2.3 MDB MI-cytochrome P450 complexes

The well-known inhibitory activity of many MDBs towards microsomal P450-mediated monooxygenase activity has been attributed to the formation of reactive intermediates that coordinate tightly to the prosthetic heme iron of P450 (Philpot and Hodgson, 1972; Hodgson and Philpot, 1974). The metabolism-dependent reactive intermediate formation has been confirmed by the important role of NADPH, oxygen, or cumene hydroperoxide as well as the time and concentration dependence of the process (Hodgson and Philpot, 1974; Wilkinson et al., 1984; Kulkarni and Hodgson, 1978; Elcombe et al., 1975b).

A carbene that forms a stable complex with P450 has been suggested to be the possible reactive intermediate (Dahl and Hodgson, 1979; Mansuy et al., 1979), and intermediates of benzodioxolium ion (Hennessy, 1965), free radical (Hansch, 1968), and carbanion (Ullrich and Schnabel, 1973) have also been suggested. The metabolic generation of the reactive intermediates has been studied, and mechanisms of MI-complex formation have been proposed (Scheme 1.1), although the actual structure of the hemoprotein complex has yet to be established (Ortiz de Montellano and Correia, 1995). As shown in Scheme 1.1, oxidation of the methylene bridge to the putative carbene involved in complex formation could occur by P450-catalyzed hydroxylation of the methylenedioxy carbon (path a), followed by elimination of a molecule of water (Anders et al., 1984). Alternatively, the hydroxylation of the methylenedioxy carbon could also result from a radical formed by hydrogen abstraction in the presence of P450 or a hydroxyl radical (path b) (Kumagai et al., 1991, Ortiz de Montellano and Correia, 1995).
Scheme 1.1 Proposed Mechanisms for the Formation of MI-Complexes of Methylenedioxybenzenes with P450 Enzymes. (Testa, 1995)
Decrease in P450 activities by MDBs was found to be associated with the *in vitro* formation of the MI-P450 complex, which is characterized by an isocyanide-like difference absorption spectrum with dual Soret peak maximum at about 427 and 455 nm (Elcombe *et al.*, 1975a, 1975b; Franklin, 1971; Philpot and Hodgson, 1971; Skrinjaric-Spoljar *et al.*, 1971; Wilkinson *et al.*, 1984). This spectrum is termed a "type III" optical difference spectrum, the relative intensity of which depends on the pH of the media (Philpot and Hodgson, 1971). The MI-P450 complexes have also been isolated intact from animals treated with MDBs (Dickins *et al.*, 1979; Elcombe *et al.*, 1975a; Philpot and Hodgson, 1971/72). The precise nature of the MDB MI-P450 complex is not yet established but appears to involve binding of MI to both the heme iron as well as to neighboring sites on the apoprotein of the cytochrome (Wilkinson *et al.*, 1984).

The characteristic type III optical difference spectrum of MDBs is, however, observed only under reducing conditions, and usually in NADPH-fortified microsomal incubations *in vitro*. A similar difference spectrum could appear in hepatic microsomes from animals treated *in vivo* with PBO and other MDBs, provided that reducing agents, such as NADPH, NADH or dithionite, were added to the microsomes prepared (Elcombe *et al.*, 1975a; Philpot and Hodgson, 1971/72). Under oxidized conditions, the dual Soret peaks are replaced by a single peak at about 437 nm that represents the MDB MI-ferricytochrome P450 complex (Elcombe *et al.*, 1975b; Philpot and Hodgson, 1971/72; Wilkinson *et al.*, 1984). The oxidized spectrum can also be observed in hepatic microsomes from animals treated *in vivo* with MDBs prior to reducing agents (Elcombe *et al.*, 1975a; Philpot and Hodgson, 1971/72), or by addition of cumene hydroperoxide to microsomal suspensions (Dahl and Hodgson, 1979; Elcombe *et al.*, 1975b; Kulkarni and
Hodgson, 1978) due to the peroxidase nature of cytochrome P450 (Hrycay and O'Brien, 1972).

The spectra of MDB MI-P450 complexes and the ability of MDBs to form type III spectral complexes also depend on both the structure of the MDBs and the nature of the P450 isozyme employed (Marcus et al., 1985; Murray et al., 1983b; Ryan et al., 1980; Wilkinson et al., 1984; Yu et al., 1980). Instead of forming characteristic type III complexes, addition of MDBs with electron withdrawing aromatic substituents, or using reconstituted systems containing CYP2B or CYP1A1, produced optical difference spectra with a single transient absorbance maximum at 456-458 nm (Marcus et al., 1985; Wilkinson et al., 1984; Yu et al., 1980). Similar spectrum with peak absorption at 456 nm was also observed for dihydrosafrole MI-P450 complex (Kao and Wilkinson, 1987).

The MDB MI-P450 complexes are very stable as witnessed by their preservation through the preparation procedure for microsomes or after dialysis or detergent treatment (Dickins et al., 1979; Elcombe et al., 1975a; Philpot and Hodgson, 1971/72). Moreover, under reduced conditions, the type III complex is remarkably stable and is not displaced by CO, type II compounds, such as pyridine, or type I compounds such as hexobarbital (Philpot and Hodgson, 1971, 1971/72). It has been suggested that the high stability of the complex between a MDB carbene and ferrocytochrome P450 results from the strong Fe(II)-carbene bond and back-donation of electrons from Fe(II) to carbene (Dickins et al., 1979). In contrast, the MDB MI-P450 complex in its oxidized form is far less stable and the MDB carbene moiety can be displaced by a variety of lipophilic type I and reverse type I monooxygenase substrates and inhibitors with the correct steric configuration. The ferric form of the complex has been dissociated by treatment of microsomes in vitro with 2-n-
alkylbenzimidazoles, n-alkylcarbamates, alkanes and even MDB compounds (Dickins et al., 1979; Elcombe et al., 1975a, 1976), although it was considered quite stable in the absence of highly lipophilic compounds (Wilkinson et al., 1984). Dissociation of the MDB-ferricytochrome P450 complex resulted in an increase in detectable cytochrome P450 levels and a concomitant recovery of monooxygenase activity (Elcombe et al., 1976).

1.1.2.4 Induction of cytochrome P450 by MDBs

The inductive effects of MDBs on P450 activities were first described by Fujii et al. (1968) and Wagstaff and Short (1971), who proposed that MDBs have a biphasic effect on hepatic P450 levels and associated monooxygenase activities. That is, the initial inhibition of P450 activity over the short-term is followed by induction of several forms of P450. This biphasic effect has been confirmed later by the in vivo administration of many MDBs including PBO and isosafrole to laboratory animals, indicating that MDBs are potent inducers of hepatic drug-metabolizing enzyme system (Goldstein et al., 1973; Kamienski and Murphy, 1971; Murray et al., 1985; Parke and Rahman, 1971; Philpot and Hodgson, 1971/72; Ryan et al., 1980; Skrinjaric-Spoljar et al., 1971; Snyder and Remmer, 1979). The induction effect was demonstrated by an increase in P450-catalyzed activity as well as total P450 level, which was associated with changes in the intensity of the cytochrome P450 difference spectra produced by adding carbon monoxide (Wagstaff and Short, 1971; Kamienski and Murphy, 1971). The induction phase of various MDBs has variable magnitude and duration, which may depend on the polarity of the ring substituents. It has been shown that substitution with various functional groups on either the MDB ring or the
methylene carbon affected their ability to induce as well as to inhibit hepatic P450 (Wilkinson et al., 1984; Marcus et al., 1985).

Isosafrole and safrole have been shown to exhibit a pattern of induction that is similar to that obtained with 3MC. The new P450 isozyme induced by isosafrole was later classified as CYP1A2 (Dickins et al., 1978; Ryan et al., 1980). Treatment of rats with dihydrosafrole resulted in similar time profiles for the induction of P450 and other mixed-function oxidases (Murray et al., 1983a). In addition to inducing the novel hemoprotein CYP1A2, MDBs have been shown to induce several other forms of cytochrome P450 including CYP1A1 and CYP2B. A similar pattern for the induction of P450 activities in mouse liver was also obtained by MDB treatment (Fennel et al., 1980). An Ah receptor-independent regulation of CYP1A2 induction by isosafrole, PBO and some other MDBs has been reported using Ah receptor knock-out mice (Cook and Hodgson, 1985; 1986; Lewandowski et al., 1990; Ryu et al., 1996). However, it was reported that the induction of AHH activity by MDBs probably resulted from interaction with the Ah receptor and induction of CYP1A1 (Wilkinson et al., 1984).

An increase in liver weight and total microsomal protein was observed when rats were treated with PBO (Philpot and Hodgson, 1971/72; Skrinjaric-Spoljar et al., 1971; Wagstaff and Short, 1971), and was associated with significant elevation of the levels of P450. Increase in the activities of NADPH-cytochrome c reductase, benzphetamine N-demethylase and aniline hydroxylase, as well as the induction of hexobarbital metabolism, and the hydroxylation of biphenyl and benzo[a]pyrene have also been observed (Dalvi and Dalvi, 1991; Goldstein et al., 1973; Jaffe et al., 1969; Kamienski and Murphy, 1971). In contrast to the 3MC pattern induction by isosafrole, early studies on the characterization of
the pattern of induction by PBO showed that it most closely resembled PB induction (Goldstein et al., 1973).

In a study with several MDBs of varying structure, Fennel and Bridges (1979) observed that the inductive capacity of MDBs was closely associated with the ability of MDBs to form a MI-P450 complex that absorbed at 455 nm. The formation of a complex between a MDB metabolite and P450 was also observed during the induction phase by other laboratories (Elcombe et al., 1975a; Murray et al., 1986). Fennel et al. (1980) therefore proposed that the formation of a MDB MI-P450 complex might be the initiating event for induction by MDBs. Cook and Hodgson (1983) showed that substitution by methyl groups on the methylene carbon prevented the induction of P450 in mice. Thus, an unsubstituted methylene bridge resulting in a complex formation appears to be a prerequisite for induction, as well as inhibition, of P450 by MDBs. The elevation of P450 levels following treatment with MDBs was also ascribed to the porphyrogenic-like property of MDBs, which may enhance the activity of δ-aminolevulinic acid synthetase that normally is the rate-limiting enzyme in the biosynthesis of porphyrins and heme and is essential to the induction response of the hepatic monooxygenase system (Skrinjaric-Spoljar et al., 1971).

1.1.3 Metabolism of MDBs

MDBs are metabolized by the microsomal mixed-function oxygenase system both in vivo and in vitro (Wilkinson and Hicks, 1969). The major metabolic pathway of MDBs is demethylation of the methylenedioxy moiety to yield catechols, formic acid, carbon dioxide and carbon monoxide (Anders et al., 1984; Casida et al., 1966; Hodgson and
Mechanisms consistent with these observations have been proposed (Anders et al., 1984). As shown in Scheme 1.2, it has been proposed that the initial step in the demethylolation reaction sequence is the oxidation of the methylene carbon to form an unstable hydroxy-MDB by P450-dependent monooxygenases (Anders et al., 1984) or hydroxyl radicals (Kumagai et al., 1991). The resulting hydroxylated MDB then rearranges to an intermediate 2-hydroxyphenylformate that is subsequently hydrolyzed to a catechol and formate or CO (path a). Alternatively, it may yield a carbene via elimination of one molecule of water (path b). Subsequent addition of a hydroxyl to the iron-coordinated carbene would yield an iron-coordinated anion that could readily fragment into a catechol and CO.

The in vivo metabolism of safrole is now reasonably well understood. Safrole undergoes extensive metabolism through cleavage of the methylenedioxy moiety, which leads to the formation of hydroxychavicol (i.e., 4-allyl catechol, the catecholic metabolite of safrole) and its conjugates (Kamienski and Casida, 1970; Klungsøyr and Scheline, 1983; Scheline, 1991; Stillwell et al., 1974). Treatment of mice with safrole containing radiolabeled methylenedioxy carbon showed that about 61% of the radioactivity was recovered as CO₂ in the expired air during 48 hr (mostly during the first 12 hr). The hypothesis of extensive demethylenation of safrole was further confirmed by Klungsøyr and Scheline (1983), who found that hydroxychavicol excreted in the rat urine was 72% of an oral dose. Moreover, the hydroxychavicol, mainly in conjugated form, was found to be a major urinary metabolite of safrole in rats and guinea pigs (Stillwell et al., 1974). Similar hydroxychavicol conjugates, as the main urinary metabolites of safrole, have also been reported in rats and man (Benedetti et al., 1977). Other important metabolic routes of
Scheme 1.2 Proposed Metabolic Pathways of Catecholic Metabolite Formation from Methylenedioxybenzenes. (Modified from Anders et al., 1984)
safrole observed were side chain allylic hydroxylation and the epoxide-diol pathway (Klungsøyr and Scheline, 1983), which contributed to the carcinogenic effect of safrole (Boberg et al., 1983; Miller and Miller, 1983).

The major metabolic route of isosafrole in the rat was also found to be demethylenation, with side chain hydroxylation and epoxide-diol formation as minor metabolic pathways (Klungsøyr and Scheline, 1982). The demethylenated metabolites recovered accounted for 92% of the identified material, and the metabolite iso-hydroxychavicol (i.e., 4-propenylcatechol, the catecholic metabolite of isosafrole) comprised about 96% of the demethylenated material (78.5% of the dose). In addition, dihydrosafrole was similarly extensively demethylenated in mice and rats, and the demethylenated derivatives accounted for 92-95% of the urinary metabolites (Kamienski and Casida, 1970; Klungsøyr and Scheline, 1982).

The biotransformation of other MDBs including PBO and myristicin has also been studied in plants, insects and animals (Haley, 1978; Kamienski and Casida, 1970), suggesting that cleavage of the methylenedioxy moiety and expiration of the methylene carbon as carbon dioxide is the prominent metabolic reaction. Catecholic metabolites were also obtained in the liver microsomal NADPH-catalyzed metabolism of 4-nitromethylenedioxybenzene and 3,4,5,6,-tetrachloromethylenedioxybenzene (Casida et al., 1966; Wilkinson, 1967). In contrast, oxidation and/or conjugation of the side chain is the major metabolic pathway for Tropital, piperonal, piperonyl alcohol and piperonylic acid (Casida et al., 1966; Wilkinson, 1967). This suggests that MDB metabolism also depends on the nature of the side chain and the ease with which they are oxidized, hydrolyzed or conjugated.
1.2 CATECHOLS

1.2.1 Sources

Catechols (i.e., o-dihydroxybenzenes) occur ubiquitously in nature. They are found in vegetables such as onions, citrus fruits such as grapefruit, tea, coffee, and in industrial products such as film developers or oxidative types of hair dyes (Ribéreau-Gayon, 1972; Ito and Hirose, 1989). Catechol also exists in naturally occurring antioxidants including gallic acids, flavonoids such as quercetin and myricetin, and catechins; and is a constituent nucleus of 3,4-dihydroxyphenethylamine (dopamine), 3,4-dihydroxyphenylalanine (dopa), ardenaline (epinephrine), vitamin K, and some important iron-chelating compounds (e.g., enterobactins) found in bacteria (Avdeef et al., 1978; Waite and Tanzer, 1981).

Catechol-containing compounds are of widespread biological importance as well. They function as precursors of melanins, lignins and insect scleroproteins. For example, dopa contributes to molluscan scleroprotein formation (Waite and Tanzer, 1981). Catechols also serve as neurotransmitters and hormones (Waite and Tanzer, 1981). Catecholamines, a major class of catechol, function as neurotransmitters and are of pharmacological use in such diverse areas as the treatment of Parkinson's disease, hypertension, and breast cancer (Avdeef et al., 1978). Catechols may act as reductants and electron donors in either their monoanionic or dianionic forms as well (Saleem and Wilson, 1982). In addition, catechols are major metabolites of other compounds such as methylenedioxybenzenes.
1.2.2 Antioxidative Properties of Catechols

1.2.2.1 Oxidative stress and antioxidants

The superoxide anion radicals are generated through single electron reduction of O2 by mitochondrial and other cellular electron transport systems, as well as other endogenous sources such as xanthine oxidase, NADPH oxidase in phagocytic cells (neutrophils and monocytes), and D-amino acid oxidases, etc (Scheme 1.3). In addition, they are produced via redox cycling of epinephrine and quinoid substrates, such as coenzyme Q10 and vitamin K, or in the cytochrome P450-dependent monooxygenase system (Hennekens, 1994; Sies, 1991). The superoxide radicals generated may be dismutated to H2O2 and O2 by superoxide dismutases (SOD) or SOD mimics. The hydrogen peroxide and superoxide radical, in the presence of catalytic amounts of metal ions such as iron and copper ions, produce •OH, a process termed "Haber-Weiss reaction". Finally the resulting reactive oxygen species (ROS) will react readily with neighboring biological molecules at the site of formation. As a result, as shown in Scheme 1.3, the initial superoxide radical can give rise to other ROS as well as cause oxidative damage to various biological macromolecules, such as cellular membranes and lipoproteins, proteins as enzymes, DNA and carbohydrates (Halliwell, 1991; Frei, 1994). There are also some exogenous sources, such as cigarette smoke, ozone, diet-derived quinoid substances and quinoid drugs, that contribute to the total load of ROS to which we are exposed daily (Halliwell, 1991; Frei, 1994).

Antioxidants are compounds that can scavenge ROS before they cause damage to various biomolecules, or compounds that prevent oxidative damage from spreading out, e.g., by interrupting the radical chain reactions of lipid peroxidation (Sies, 1991). There are many naturally occurring and synthetic antioxidants in our environment, which are present,
Sources of $O_2^{--}$

- Electron Transport Systems
- Oxidases (Xanthine, NADPH, amino acid, etc.)
- Redox Cycling of Quinones
- Cytochrome P450

ROS Formation

- $2O_2^{--} + 2H^+ \rightarrow O_2 + H_2O_2$
- $H_2O_2 \rightarrow H^+ + O_2^{--}$
- $Fe^{2+}$ or $Cu^+$

Oxidative Damage to

- Lipids
- Proteins
- Nucleic Acids
- Carbohydrates

Scheme 1.3 Endogenous ROS Formation and Oxidative Damage. (modified from Hennekens, 1994)

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for example, in foodstuffs, plants, oils, cosmetics, and medicines. Some antioxidants may even be synthesized by human beings in vivo. They prevent ROS-mediated damage through reduction of the rate of initiation by inactivating catalytic metals (e.g., citric acid, phytic acid), or terminate one or more propagation steps by interfering with chain reactions (e.g., phenolic antioxidants, tertiary amines, flavonoids) or by decomposing peroxides or radicals (e.g., sulfur compounds, selenium, enzymatic antioxidants, vitamins). In addition, they may function through synergism with other antioxidants (e.g., vitamin E and ascorbic acid) (Ito and Hirose, 1989). These antioxidants have long been considered to have potential application as protective agents against aging, atherosclerosis, shock or ischemic tissue damage and chemical carcinogenesis (Ito and Hirose, 1989; Kahl, 1991; O'Brien, 1994).

Superoxide anion radicals have been implicated in the pathogenesis of inflammation, carcinogenesis, ischemia-reperfusion injury and aging (Halliwell and Gutteridge, 1984, 1986). Superoxide dismutases are a family of metalloenzymes that catalyze the conversion of superoxide radicals to $\text{H}_2\text{O}_2$ plus $\text{O}_2$ (McCord and Fridovich, 1969), and thereby provide a defense system under conditions where superoxide appears to play an important role (Halliwell and Gutteridge, 1989). SOD and SOD mimics have been viewed as promising therapeutic drugs for superoxide radical involved diseases. Low-molecular-weight complexes of Mn, Cu and Fe have been prepared and extensively investigated as SOD mimics (Beyer and Fridovich, 1989; Faulkner and Fridovich, 1997; Iuliano et al., 1992; Minotti and Aust, 1987; Nagano et al., 1989).
1.2.2.2 Free radical scavenging by catechols

Catechols are known as prototypic chain-breaking antioxidants owing to their ability to scavenge oxygen-derived radicals such as superoxide, hydroxyl and peroxyl radicals, which enables them to act as additives for the protection of oils and fats (Kahl, 1991; Kitagawa et al., 1992; Ribéreau-Gayon, 1972). When encountered with reactive oxygen species, catechols are able to donate hydrogen atoms or electrons from the neutral molecule or anionic forms, respectively. A mechanism involving formation of a semiquinone radical (a singly-bound oxygen atom carrying an unpaired electron) via one-electron oxidation of catechol by oxidizing agents (e.g., free radicals) has been proposed (Saleem and Wilson, 1982). As shown in Scheme 1.4, the semiquinone formation is followed by radical delocalization which enables dimerization or polymerization of catecholic compounds, a process that usually involves the side chain of the catechols (Cilliers and Singleton, 1991). Alternatively, two molecules of the resulting semiquinone radicals may undergo disproportionation to yield one molecule of the parent catechol and one molecule of o-quinone, the oxidized end product.

Similarly, flavonoids containing the catechol motif such as quercetin, rutin, and catechin have been shown to possess superoxide dismutase activities (Cotelle et al., 1992; Robak and Gryglewski, 1988), through the known one-electron transfer mechanism or a "concerted" mechanism (that is, superoxide may abstract simultaneously a hydrogen atom and a proton from the hydroxy groups of the flavonoids) (Afanas'ev et al., 1989a). Moreover, flavonoids have been shown to exhibit chain-breaking potency by interacting with free hydroxyl and peroxyl radicals (Afanas'ev et al., 1989a; De Whalley et al., 1990; Torel et al., 1986).
Scheme 1.4 Mechanisms of Free Radical Scavenging by Catecholic Compounds

\[
\begin{align*}
R-\begin{array}{c}
\text{OH} \\
\text{OH}
\end{array} & \xrightarrow{H^+ + O_2^- + H_2O/LH} R-\begin{array}{c}
\text{O} \\
\text{O}^-
\end{array} + H^+ \quad (1) \\
\begin{array}{c}
\text{R-} \\
\text{O}^-
\end{array} & \xrightarrow{\text{(possible radical delocalization)}} \text{Dimer} \\
& \quad \text{Trimer} \\
& \quad \text{Polymer} \quad (2) \\
2 \begin{array}{c}
\text{R-} \\
\text{O}^-
\end{array} & \xrightarrow{2H^+} \begin{array}{c}
\text{R-} \\
\text{OH}
\end{array} + \begin{array}{c}
\text{R-} \\
\text{C}
\end{array} \quad (3)
\end{align*}
\]
The cytoprotective effects of catechol and polyphenolic compounds have also been ascribed to the inhibition of enzymes involved in ROS generation and the induction of enzymes involved in detoxification of ROS once formed (Kahl, 1991; O’Brien, 1994). It has been reported that catechol derivatives are potent inhibitors of lipoxygenase that catalyzes dioxygenation of polyunsaturated fatty acids (Kemal et al., 1987; Naito et al., 1991). Catecholic flavonoids are also inhibitors of xanthine oxidase and neutrophil NADPH oxidase (Kahl, 1991; Robak and Gryglewski, 1988). Furthermore, polyphenolic compounds are able to selectively induce detoxification enzymes such as glutathione transferase, epoxide hydrolase and quinone reductase (Kahl, 1997; O’Brien, 1994).

The antioxidant activity of catechols and catecholic flavonoids have enabled them to play a role in food preservation and to suppress lipid peroxidation in biological material. In addition, they have been considered as promising potential drugs for combating free radical-mediated pathogenesis such as inflammatory disease, ischemia-reperfusion injury, atherosclerosis, anemia, arthritis, asbestosis, diabetes, cancer, aging and so on (Halliwell, 1989; Haramaki et al., 1997).

1.2.2.3 Iron chelation by catechols

Catechol and its derivatives have long been known to form thermodynamically stable complexes with a variety of transition metals such as iron and copper, and non-transition metals such as aluminum. Catechols have a high affinity towards ferric ion and are able to form a number of stable complexes with iron ions (log$K_f \geq 40$) (Hider et al., 1981). Similar to hydroxamates (the other iron chelating group in siderophores), catechol is a bidentate ligand that coordinates through two oxygen atoms to form a chelate ring (Isied
et al., 1976), and is able to form an octahedral complex with ferric ion at an alkaline pH (Raymond et al., 1976). The complex equilibrium normally depends on the nature of the metal ion and the catechol ligand, as well as the pH of the media. At a physiological pH, most of the catechols form 2:1 (catechol to iron) complexes with iron, whereas catechols having low pH values such as tiron form 3:1 complexes with iron ions (Avdeef et al., 1978; Kawabata et al., 1996; Krishna et al., 1992; Marcocci et al., 1994). A proposed structure of an iron complex of a catechol derivative under physiological conditions is shown in Figure 1.1.

The salts of tris(catecholato) complex of ferric iron (i.e., the \([\text{Fe(catechol)}_3]^{3-}\) complex) have been prepared in alkaline solution and examined using X-ray crystallography by Raymond et al. (1976), indicating the occupation of all six coordination sites of ferric ion by the catechol ligand at high pH. Iron complexes with catechol-containing ligands such as catechol, tiron, 4-nitrocatechol, 2,3-dihydroxybenzaldehyde, 3,4-dihydroxyphenylacetic acid, and 2,3-dihydroxybenzoic acid have also been previously investigated and the stability constants of various catecholic iron complexes have been reported (Avdeef et al., 1978; Hider et al., 1981; McBryde, 1964). Coordination of iron changes iron redox potential as well as its solubility. The reduction potentials for ferric-enterobactin and ferric-tiron have been estimated to be -750 mV and -950 mV (vs. NHS) at pH 7, respectively, well below the range of physiological reducing agents (Cooper et al., 1978; Krishna et al., 1992).

The iron chelating capability of catechols has also been widely applied in nature. One important example is its application as a common ligand functional group in siderophores. Siderophores (or siderochromes) are a class of low molecular weight
Figure 1.1 Proposed Structure of a Catecholic Iron Complex at Physiological pH
chelating agents which are synthesized by microorganisms in response to an iron deficiency. They are able to sequester iron from the environment and transport it into the organisms, via their catechol moiety (Avdeef et al., 1978). Enterobactin, a well studied siderophore, is the iron sequestering agent for enteric bacteria such as Escherichia coli. It is a hexadentate ligand containing three catechol groups that are covalently linked together via a cyclic triester of serine. Enterobactin forms an extremely stable complex with iron with a formation constant logKf > 45 (Avdeef et al., 1978; Hider et al., 1981).

The metal-chelating property of catechols contributes to their antioxidative activity by sequestering and inactivating the catalysts required for the formation of highly reactive oxygen species, in particular by depressing the superoxide-driven Fenton reaction which is currently considered as the most important route to generate reactive oxygen radicals (Kozlov et al., 1994). As a result, the protection against lipid peroxidation and other oxidative cell damage has also been attributed to the suppression of iron-catalyzed free radical generation by catecholic compounds in the literature (Afanas'ev et al., 1989a; Kawabata et al., 1996; Krishna et al., 1992). In addition, if iron can be kept in the ferric form by a chelator under physiological conditions and has a low redox potential as a complexed form, the catalytic activity of iron in free radical reactions will be significantly decreased.

In a word, catechol and its derivatives could inhibit free radical processes at three different stages: the initiation (by scavenging superoxide anion radicals), the formation of hydroxyl radicals (by chelating iron ions and other metal ions), and the propagation of lipid peroxidation (by interacting with lipid peroxyl radicals).
1.3 Hypotheses and Objectives of This Thesis

As discussed above, methylenedioxybenzenes are potent inhibitors of cytochrome P450-dependent monooxygenase systems and are extensively metabolized to catecholic metabolites that are possible potent antioxidants and iron chelators. Therefore, the Hypotheses of this thesis were "Naturally occurring methylenedioxybenzenes, in particular the non-carcinogenic isosafrole, are chemoprotective agents that may prevent cytochrome P450-mediated xenobiotic toxicity by inhibiting P450 isozymes, and may prevent oxidative injury through their antioxidative catecholic metabolites. Moreover, the catecholic compounds may form stable complexes with iron which are also capable of interacting with reactive oxygen species especially superoxide radicals".

In Chapters 3 and 4, the chemoprotective properties of methylenedioxybenzenes have been investigated against carbon tetrachloride, bromotrichloromethane, chloroform and N-dimethylnitrosamine-induced liver cell injury as well as phenetidine-induced methemoglobinemia in mice. The protective capability of MDBs has also been compared with that of structurally related non-MDB compounds. This protection was ascribed to the potent inhibitory effects of MDBs on cytochrome P450 isozyme activities in particular CYP2E1, CYP2B1/2 and CYP1A2 activities.

In Chapter 5, the antioxidative properties of various methylenedioxybenzenes have been compared at preventing reactive oxygen species-mediated ferric nitrilotriacetate-induced hepatocyte lipid peroxidation in vitro and nephrotoxicity in vivo. The crucial role of the catecholic metabolites in the antioxidant effects of MDBs has also been confirmed.
In Chapter 6, the antioxidant activity of catecholic compounds was further ascribed to their ability to chelate iron ions and the effectiveness of catecholic iron complexes at reacting with oxygen-derived free radicals, in particular superoxide anion radicals. A comparison of the superoxide dismutase mimic and ROS-scavenging activity of various catecholic iron complexes has been implemented.
Chapter 2

MATERIALS AND METHODS

2.1 CHEMICALS

Benzodioxole, 4-bromo-1,2-(methylenedioxybenzene), 4-t-butylcatechol, 4-t-butyl-1,2-(methylenedioxybenzene), caffeic acid, catechol, eugenol, isoeugenol, isosafrole, 3-methoxycatechol, methylenedioxyaniline, 1,2-(methylenedioxy)-4-nitrobenzene, 3,4-(methylenedioxy)-toluene, 4-nitrocatechol, piperonyl butoxide, safrole, tetrachlorocatechol, aminobenzotriazole, 1-benzylimidazole, bromotrichloromethane, ferric chloride, para-nitrophenol, phenazine methosulfate, phenetidine and 1-phenylimidazole were purchased from Aldrich Chemical CO. (Milwaukee, WI). Catechin, curcumin, epicatechin, 4-methylcatechol, myristicin, protocatechuic acid, quercetin, resorcinol, sesamol, ascorbic acid, benzo[a]pyrene, N-dimethylnitrosamine, ethoxyresorufin, ferric ammonium citrate, hypoxanthine, metyrapone, 3-methylcholanthrene, nitrilotriacetic acid, nitro blue tetrazolium, pentoxyresorufin, Cu,Zn-SOD (from bovine erythrocytes), xanthine oxidase, 2-thiobarbituric acid (TBA), Triton X-100, Trizma® base and trypan blue were obtained from Sigma Chemical CO. (St. Louis, MO).

Bovine serum albumin (BSA), collagenase (from Clostridium histolyticum), HEPES, NADH and NADPH were obtained from Boehringer Mannheim (Montréal, Québec). Tiron, acetone and hydroquinone were purchased from J. T. Baker Chemical Co. (Phillipsberg, NJ). Methoxyresorufin was obtained from Molecular Probes, Inc. (Eugene, OR). Other chemicals including carbon tetrachloride (analytical grade), phenobarbitone sodium and isopropanol (analytical grade) were purchased from BDH (Toronto, ON).
Trichloroacetic acid (TCA) was obtained from Anachemia (Montréal, Québec). Dihydrosafrole and iso-hydroxychavicol were gifts from Dr. Yi Ren (Chemistry Department, University of Michigan). Hydroxychavicol was a gift from Dr. Judy L. Bolton (Department of Medical Chemistry and Pharmacognosy, University of Illinois at Chicago).

Ferric nitrilotriacetate (FeNTA) solution was prepared by mixing 470 mg nitrilotriacetic acid with 200 mg ferric ammonium citrate in 62.5 ml distilled water as reported by Morel et al. (1990). The final solution was adjusted to pH 7 with sodium bicarbonate to give a solution containing 10 mM of ferric iron with nitrilotriacetic acid in a molar ratio of 1:2. Stock solutions of iron complexes of catechols and related compounds were prepared freshly before use, by mixing 5 mM ferric chloride solution with 10 or 15 mM solutions of catechols.

2.2 Animals

Male CD-1 mice, weight 25-30 g, were purchased from Charles River (St-Constant, Québec), and fed with a normal standard chow diet (Rodent Laboratory Chow 5001, Ralston Purina International, Strathroy, ON) and tap water ad libitum. The animals were housed 5 per cage in ventilated plastic cages over PWI 8-16 hardwood bedding (Sawdust Industries P.W.I. Inc., St.-Hyacinthe, Québec) in our central facilities (12 air changes per hour) at 22 ± 1 °C, 50-60% relative humidity and a 12-h light-dark cycle (light: 7:00-19:00). The animals were maintained in these facilities for 1-2 weeks prior to the experiments. Two groups of animals with 3 per group were used as stated for each experiment. More groups were used for normal and toxicant-treated control.
Male Sprague-Dawley rats (275-300 g) purchased from Charles River (St-Constant, Québec) were fed with a normal standard chow diet and water *ad libitum*. The animals were maintained 2 per cage under the conditions stated above for 3-7 days prior to hepatocyte or microsome preparation.

2.3 **In vivo Studies**

2.3.1 Treatments for the protection against CCl₄, DMN and phenetidine toxicity

MDBs and other alkenylbenzenes were administered intraperitoneally in 0.1 ml of corn oil at 200 mg/kg or as otherwise specified, except for sesamol which was given in 40 μl 50% DMSO. Control animals received the equivalent amount of vehicles only. CCl₄, DMN or phenetidine were given ip 1 hr later (or as specified) in 0.1 ml of corn oil, at dosages of 0.4 ml/kg, 100 mg/kg and 200 mg/kg, respectively. To determine if the MDBs could interfere with CCl₄ absorption, isosafrole, as an example of the tested compounds, was given *per os*, i.e., by a different route of administration used for CCl₄. In another set of experiments, mice received a single dose of isopropanol (2.4 ml/kg) in normal saline solution (0.2 ml) by gavage. Sixteen to eighteen hours later, CCl₄ (10 μl/kg) or CHCl₃ (0.5 ml/kg) in 0.1 ml of corn oil was given intraperitoneally. Isosafrole (100 mg/kg) or PBO (200 mg/kg) was also administered ip to one group of animals 1 hr before the toxicant.
2.3.2 Treatments for the protection against FeNTA toxicity

Male CD-1 mice (25-30 g) fasted overnight were given freshly prepared FeNTA ip at a dosage of 2.6 mg Fe(III)/kg. Isosafrole (200 mg/kg or 50 mg/kg in corn oil) was given ip 18 or 3 hr before FeNTA treatment. In one set of experiments, acetone (5 ml/kg) was given p.o. for two consecutive days before FeNTA treatment.

2.3.3 Determination of plasma transaminase activities

Twenty-four hours after CCl₄, DMN or CHCl₃ administration, the animals were kept under light ether anesthesia and blood samples were collected by cardiac puncture with a heparinized syringe. The animals were then killed by exsanguination. Plasma AST and ALT activities were measured according to Reitman and Frankel (1957), using the assay kit purchased from Sigma Chemical CO. (St. Louis, MO).

2.3.4 Determination of methemoglobin content

The methemoglobin content in the blood of the animals treated with phenetidine was determined using a modified method of Dubowski (1966). Briefly, 25 µl of blood sample was collected from the tail of the animal using a heparinized capillary tube, and then transferred to 1975 µl of the phosphate buffer solution (0.05 mM, pH 6.6) containing 1 or 2 drops of Triton X-100. The 2 ml mixture was then centrifuged at 5,000 g for 2 minutes. 900 µl of the solution was then pipetted into a cuvette and the absorbance at 630 nm was read against the phosphate buffer using a Beckman DU-7 spectrophotometer, and recorded as A1. To the same sample, 34 µl of potassium cyanide solution (0.1 M) was added and the absorbance at 630 nm was recorded as A2. To another 900 µl of the original solution,
34 μl of potassium ferricyanide (0.1 M) was added and the absorbance was recorded as A3. To this sample, 34 μl of potassium cyanide (0.1 M) was then added and the absorbance was read as A4. The methemoglobin content of the blood sample was then calculated using the following equation

\[
\text{methemoglobin content (\% of total hemoglobin)} = \frac{A_1 - A_2}{A_3 - A_4} \times 100
\]

### 2.3.5 Determination of kidney damage

Twenty-four hours after FeNTA administration, the animals were kept under light ether anesthesia and blood samples were collected by cardiac puncture with a heparinized syringe. The animals were then killed by exsanguination. Plasma urea nitrogen activity (PUN) was measured using a Sigma assay kit (Catalog No. 640-A). The kidneys were removed, and excised in cold 1.15% (w/v) KCl. Tissue samples were then prepared as 25% (w/v) homogenates in KCl containing 0.01% butylated hydroxytoluene (BHT). The formation of TBARS in kidney homogenates was measured as described in Section 2.4.2. BHT was added to prevent additional chromophore formation during the assay.

### 2.4 In vitro Studies

#### 2.4.1 Preparation of rat hepatocytes and determination of cytotoxicity

The rat liver was perfused with collagenase as described previously by Moldéus et al. (1978). Briefly, the rats were anesthetized with sodium pentobarbital (65 mg/kg). The livers were then perfused with Hank's balanced salt solution (pH 7.4) with HEPES 12.5 mM and an anticoagulant EGTA. The livers were removed and further perfused using collagenase. Isolated cells (10⁶ cell/ml) were suspended in Krebs-Henseleit buffer [NaCl
(13.9 g), KCl (0.71 g), KH₂PO₄ (0.32 g), MgSO₄•7H₂O (0.59 g), and CaCl₂•2H₂O (0.76 g), NaHCO₃ (4.2 g) and HEPES (6.0 g) dissolved in 1 L of deionized water and adjusted to pH 7.4] containing 12.5 mM HEPES in continuously rotating round bottomed 50 ml flasks, and incubated in a water bath of 37 °C under an atmosphere specified for each experiment. The cells were preincubated for 30 min prior to the addition of any other chemicals. For experiments under hypoxia, isolated cells incubated under an atmosphere of 95% O₂ and 5% CO₂ for 30 min. were further incubated under an atmosphere of 0.1% O₂, 5% CO₂ and 95% N₂. For other hepatocyte experiments, O₂ content used in the incubation atmosphere as specified in corresponding chapters was based on the literature, or previous studies from the same laboratory, or the author's own preliminary experiments. Cell cytotoxicity was determined by trypan blue (0.2%) inclusion (Moldéus et al., 1978), and expressed as the percentage of cells taking up trypan blue.

2.4.2 Determination of lipid peroxidation.

Lipid peroxidation was determined as the formation of thiobarbituric acid-reactive substances (TBARS) according to Buege and Aust (1978). Samples were taken from hepatocyte or liver microsomal or kidney homogenate incubation at times of analysis. TCA (15%) was added to precipitate the proteins in the samples, followed by adding 0.8% TBA. The samples were stored overnight and then measured at 535 nm using a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Irvine, CA). The amount of TBARS formed was then calculated using a molar extinction coefficient of 1.56 x10⁵ M⁻¹ cm⁻¹ (Buege and Aust, 1978).
2.4.3 Determination of ROS formation under hypoxia

The formation of reactive oxygen species (ROS) in hepatocyte incubation under hypoxia was determined using 2',7'-dichlorofluorescin diacetate which is hydrolyzed by intracellular esterases and oxidized by ROS to highly fluorescent dichlorofluorescein (LeBel et al., 1992). Samples of hepatocytes were taken at time of interest and fluorescence was determined at an excitation wavelength of 500 nm and an emission wavelength of 520 nm using a Shimadzu RF-5000 recording spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan).

2.4.4 Preparation of liver microsomes

To prepare rat liver microsomes, the animals were anesthetized with sodium pentobarbital. Livers were perfused with cold 1.15% (w/v) KCl and were rapidly removed, minced and homogenized in 3 volumes of KCl. Homogenates were centrifuged at 1,000 g for 10 min and then 9,000 g for another 10 min. The supernatants were collected and centrifuged again at 105,000 g for 60 min. The cytosol was discarded, whereas the pellets were rinsed with 0.1M phosphate buffer (pH 7.4) several times before suspension in phosphate buffer and storage at -75 °C until use. Microsomal protein content was determined using Bio-Rad protein assay (Bio-Rad Laboratories, CA) and applying the BIO-RAD micro assay provided by the company using BSA as a standard. To prepare mouse liver microsomes for experiments in Chapter 4, mice following various treatments as specified in the chapter were sacrificed by cervical dislocation. The livers were then removed, rinsed using cold 1.15% (w/v) KCl and the liver microsomes were prepared as described above. To prepare cytochrome P450 induced mouse hepatic microsomes,
animals were treated with phenobarbitone sodium (PB, 80 mg/kg, ip) in saline for 3 consecutive days (to induce CYP2B1/2), or 3-methylcholanthrene (3MC, 25 mg/kg, ip) in corn oil for 3 consecutive days (to induce mainly CYP1A1), or 1% acetone in drinking water for 8 days (to induce CYP2E1 and CYP2B1/2). Liver microsomes were then prepared as described above.

2.4.5 Enzyme assays

The activity of hepatic microsomal CYP2E1 was assayed by determining the rate of p-nitrophenol (PNP) hydroxylation to 4-nitrocatechol (Koop, 1986; 1990). Liver microsomes (1 mg protein/ml) isolated from normal, CCl4-, isosafrole- or PBO-, or CCl4 and isosafrole-treated mice were incubated in 0.1 M phosphate buffer (pH 6.8) with PNP (250 μM) and NADPH (1 mM) under 37 °C. In another set of experiments, acetone-induced mouse liver microsomes were used, and MDBs (20 μM) were preincubated in vitro with NADPH (1 mM) 5 min prior to PNP (250 μM). The reaction was stopped by adding perchloric acid (0.6 N). After centrifugation, the supernatant was mixed with 10 N NaOH and the amount of 4-nitrocatechol formed was determined at 546 nm using a Beckman DU-7 spectrophotometer.

The rate of pentoxyresorufin and methoxyresorufin O-dealkylation was used to measure CYP2B1/2 and CYP1A2 activities respectively (Lubet et al., 1985; Nerurkar et al., 1993). A Shimadzu RF-5000 recording spectrofluorophotometer was utilized with an excitation wavelength at 530 nm and an emission wavelength at 580 nm. The quartz cuvette was embedded in a temperature-controlled holder, which enables all the reactions to take place at 37 °C. The incubation mixtures consisted of phosphate buffer (0.1 M, pH 7.4) that
contained PB-induced mouse liver microsomes (60 μg protein/ml), or normal or treated mouse liver microsomes (100 μg protein/ml), NADPH (100 μM) and compounds of interest. Reactions were initiated by the addition of pentoxyresorufin (250 nM) or methoxyresorufin (500 nM) 5 min after preincubation. The fluorescence formed was recorded with an X-Y recorder so as to determine the rate of production of resorufin. CYP1A1 activity was also determined spectrofluorometrically by ethoxyresorufin O-deethylation assay (Prough et al., 1978) and 3-hydroxy benzo[a]pyrene formation method (Nebert and Gelboin, 1968) modified by Yang et al. (1978).

2.4.6 Liver histology

Mice were given isosafrole (100 mg/kg, ip) or corn oil 1 hr before CCl4 (0.4 ml/kg, ip). The animals were sacrificed, and livers were removed at 6 or 24 hr after the toxicant. The liver slices were made from left and central lobes, and immediately fixed in 10% buffered formalin phosphate solution (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin and stained with hematoxylin and eosin.

2.4.7 Determination of nitro blue tetrazolium reduction

Nitro blue tetrazolium (NBT) was reduced by superoxide generated by a non-enzymatic phenazine methosulfate (PMS)/NADH system as described by Robak and Gryglewski (1988). The reaction mixture contained 10 μM PMS, 78 μM NADH, 25 μM NBT, and various concentrations of catechols or catecholic iron complexes in 0.1 M Tris buffer (pH 7.4). PMS was added last to initiate the reaction. The absorbance at 560 nm was recorded against blank samples containing no PMS.
Nitro blue tetrazolium reduction was also conducted in an enzymatic system of superoxide radical generation by hypoxanthine (HX)/xanthine oxidase (XO) according to Younes and Weser (1976). The reaction mixture was prepared with 0.1 M Tris buffer (pH 7.4) containing 200 μM NBT, 35 μM HX, 25 mU/ml XO, and various concentrations of catechols or catecholic compounds. Xanthine oxidase was added last to start the reaction, and the reduction of NBT was measured by following the absorbance at 560 nm.

The SOD mimic activities for catechols and catecholic ferric complexes were expressed as IC\textsubscript{50} values that were obtained by determining the concentration of the catechol or the catecholic iron complex required to inhibit 50% of the initial NBT reduction rate of control. The IC\textsubscript{50} values were calculated from regression lines where: \( x \) was the log of the catechol concentration and \( y \) was the percentage of the initial rate of NBT reduction in the presence of the catecholic inhibitors over that of the control (Younes and Weser, 1976).

### 2.5 Statistical Analysis

Statistical significance of the difference between pooled mean values was determined by ANOVA followed by a Dunnett's \( t \) test. The acceptable level of significance was \( P \leq 0.05 \). The results presented are as means ± SEM for at least three separate experiments or using six animals (or more for normal and toxicant treated control groups).
Chapter 3

THE PREVENTION OF CCl₄, PHENETIDINE AND N-DIMETHYLNITROSAMINE TOXICITY IN MICE BY NATURALLY OCCURRING METHYLENEDIOXYBENZENES.

PART I. THE PROTECTION.

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3.1 ABSTRACT

Methylenedioxybenzenes (MDBs) and structurally related compounds were compared for their effectiveness at preventing carbon tetrachloride (CCl₄) induced liver necrosis in mice. Pretreatment with isosafrole, saffrole, dihydrosafrole or benzodioxole at dosages as low as 10 mg/kg significantly prevented the increase in plasma transaminase levels and histochemical changes associated with CCl₄-induced liver necrosis, whereas piperonyl butoxide, eugenol, isoeugenol, sesamol and curcumin did not prevent CCl₄ hepatotoxicity even at 200 mg/kg. However, isosafrole was only partly hepatoprotective if administered 10 min after the toxicant. Isosafrole and some other MDBs also prevented in vitro BrCCl₃-induced hepatocyte death and lipid peroxidation. Furthermore, pretreatment of mice with isosafrole significantly protected against N-dimethylnitrosamine-induced liver necrosis as well as phenetidine-induced massive methemoglobin formation. Isosafrole was still effective at preventing phenetidine-induced methemoglobinemia at a dosage of 10 mg/kg, or given even 30 min after or 24 hr prior to phenetidine administration.
3.2 INTRODUCTION

Carbon tetrachloride (CCl4) intoxication always leads to hepatic injury in experimental animals as well as in human exposure (Williams and Burk, 1990). The characteristic lesion is centrilobular hepatic necrosis associated with transaminase activity elevation. It has been now widely accepted that the centrilobular hepatic necrosis caused by CCl4 and another halogenated hydrocarbon BrCCl3 involves bioactivation of the toxicants by the microsomal cytochrome P450 (P45O)-dependent monooxygenase system, resulting in the formation of reactive free radical metabolites that can bind covalently to macromolecules as well as initiate lipid peroxidation (Albrecht et al., 1978; Johansson and Ingelman-Sundberg, 1985; Sipes et al., 1977). N-DimethylNitrosamine (DMN) is also metabolized by mixed-function oxidases to hepatotoxic intermediates that alkylate macromolecules in various target tissues and produce acute liver necrosis (Magee, 1989; Magee and Barnes, 1967; Schut and Castonguay, 1984). Similarly, phenetidine, a metabolite of the aromatic amine drug phenacetin, induces methemoglobin formation in vivo in experimental animals as well as in human beings as a consequence of reactive intermediate formation mediated by the P450-dependent monooxygenase system (Kiese, 1966; Weisburger and Weisburger, 1973).

Naturally occurring methylenedioxybenzenes (MDBs), such as safrole, isosafrole and myristicin, are found in a wide variety of human food, essential oils and flavors. They are extracted from plants, such as sassafras, nutmeg, parsnips, carrots, parsley, pepper, and sesame seeds (Hodgson and Philpot, 1974). A synthetic methylenedioxybenzene, piperonyl butoxide (PBO), is a well-known commercial pesticide synergist with pyrethroid and carbamate insecticides. MDBs are potent inhibitors of xenobiotic metabolism mediated
by mixed-function monooxygenases, both *in vivo* and *in vitro* (Fujii et al., 1970; Jaffe et al., 1968). The mechanisms of their inhibitory effects were suggested to involve both competitive and non-competitive inhibition (Wilkinson et al., 1984). The inhibition by MDBs is initially competitive since MDBs compete with the cytochrome P450 substrates for metabolism, whereas it subsequently becomes non-competitive as so called "metabolic intermediate" (MI) complexes are formed between the reactive intermediate (a carbanion or carbene formed from the methylene carbon), and the reduced cytochrome P450 isozymes (Dahl and Hodgson, 1979; Philpot and Hodgson, 1972; Ullrich and Schnabel, 1973).

High dosages of PBO have been used to partly prevent hepatotoxicity induced by acetaminophen (Brady et al., 1988; Mitchell et al., 1973), bromobenzene (Reid et al., 1971), as well as chloroform (Illett et al., 1973; Kluwe and Hook, 1981), probably by preventing metabolic activation of the hepatotoxicants catalyzed by cytochrome P450 (Haley, 1978). However, little is known about the protective effects of naturally occurring MDBs. In the following it has been found some naturally occurring and synthetic MDBs were highly effective at preventing CCl4 or DMN induced liver necrosis and phenetidine-induced methemoglobin formation in mice, as well as BrCCl3-induced rat hepatocyte toxicity.

3.3 RESULTS

3.3.1 *In vivo* Prevention of CCl4- or DMN-Induced Liver Necrosis

As shown in Table 3.1, twenty four hours after administration of CCl4, the plasma transaminase ALT activity was increased 700 fold whereas AST was increased 70 fold. However, the CCl4-induced increase in plasma transaminase activities was completely
prevented if the mice were treated intraperitoneally with isosafrole, safrole, dihydrosafrole, benzodioxole or myristicin 1 hr before CCl₄ administration. Similarly, isosafrole given by gavage also exerted complete protection against CCl₄ hepatotoxicity (Table 3.1). By contrast, pretreatment with sesamol, or with PBO 1 hr or 3 hr prior to CCl₄ did not show any protection at 200 mg/kg (Table 3.1). No hepatoprotection was found for structurally related alkenylbenzene antioxidants such as eugenol, isoeugenol and curcumin as well as the safrole metabolite hydroxychavicol. Only partial protection was obtained when PBO was given at a dosage as high as 1200 mg/kg. No hepatotoxicity occurred when animals were treated with PBO, sesamol, eugenol or isoeugenol alone (Table 3.1).

The hepatoprotection by isosafrole was also examined at times longer than 24 hr. Normal plasma AST and ALT activities were still maintained 48 hr or 80 hr after CCl₄ administration (30±9 and 6±1 I.U./ml respectively at 48 hr, and 35±7 and 3±1 I.U./ml respectively at 80 hr) provided that the animals were pretreated with isosafrole. However, for animals treated with CCl₄ alone, AST and ALT activities were 203±38 and 832±75 I.U./ml respectively at 48 hr, and 54±4 and 38±10 I.U./ml respectively at 80 hr.

Protection by lower dosages of effective MDBs was also investigated. Isosafrole and safrole at 10 mg/kg demonstrated marked reduction of CCl₄-induced increase in transaminase activities, whereas partial protection was obtained with dihydrosafrole or benzodioxole at the same dosage used (Table 3.2). Isosafrole was still highly protective at dosages of 5 mg/kg or 2.5 mg/kg, whereas safrole was only partially effective at 5 mg/kg and was ineffective at the lower dosage. Therefore, isosafrole was more hepatoprotective than safrole when low dosage treatments were applied (Table 3.2). Furthermore, as shown in Table 3.3, treatment with isosafrole at the same time as CCl₄ administration also
Table 3.1 *In vivo* Prevention of CCl₄-Induced Liver Necrosis by Methyleneedioxybenzenes*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (I.U./ml)</th>
<th>ALT (I.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Corn oil)</td>
<td>48 ± 7</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Control (DMSO)</td>
<td>40 ± 5</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>CCl₄</td>
<td>3264 ± 96ᵃ</td>
<td>6394 ± 770ᵃ</td>
</tr>
<tr>
<td>+ Isosafrole</td>
<td>40 ± 2ᵇ</td>
<td>7 ± 1ᵇ</td>
</tr>
<tr>
<td>+ Safrole</td>
<td>32 ± 2ᵇ</td>
<td>5 ± 1ᵇ</td>
</tr>
<tr>
<td>+ Dihydrosafrole</td>
<td>41 ± 15ᵇ</td>
<td>6 ± 2ᵇ</td>
</tr>
<tr>
<td>+ Benzodioxole</td>
<td>33 ± 3ᵇ</td>
<td>4 ± 1ᵇ</td>
</tr>
<tr>
<td>+ Myristicin</td>
<td>25 ± 4ᵇ</td>
<td>5 ± 1ᵇ</td>
</tr>
<tr>
<td>+ Piperonyl butoxide</td>
<td>2669 ± 158ᵃ</td>
<td>5789 ± 374ᵃ</td>
</tr>
<tr>
<td>+ Sesamol (in DMSO)</td>
<td>1901 ± 691ᵃᵇ</td>
<td>5904 ± 374ᵃ</td>
</tr>
<tr>
<td>+ Isoeugenol</td>
<td>2392 ± 222ᵃ</td>
<td>6485 ± 715ᵃ</td>
</tr>
<tr>
<td>+ Eugenol</td>
<td>2869 ± 635ᵃ</td>
<td>7085 ± 115ᵃ</td>
</tr>
<tr>
<td>+ Curcumin</td>
<td>2992 ± 399ᵃ</td>
<td>5344 ± 528ᵃ</td>
</tr>
<tr>
<td>+ Hydroxychavicol</td>
<td>3076 ± 443ᵃ</td>
<td>5945 ± 647ᵃ</td>
</tr>
<tr>
<td>+ Isosafrole (po)</td>
<td>51 ± 6ᵇ</td>
<td>9 ± 3ᵇ</td>
</tr>
<tr>
<td>+ PBO (3 hr)</td>
<td>3327 ± 226ᵃ</td>
<td>6164 ± 753ᵃ</td>
</tr>
<tr>
<td>+ PBO (1200 mg/kg)</td>
<td>314 ± 46ᵃᵇ</td>
<td>1478 ± 139ᵃᵇ</td>
</tr>
</tbody>
</table>

MDBs and structurally related compounds (200 mg/kg) were given ip 1 hr before mice received CCl₄ (0.4 ml/kg, ip). Control mice received the equivalent amount of vehicles only. Mice were sacrificed at 24 hr after the toxicant administration, plasma AST and ALT activities were measured. Results are means ± SEM for 6 mice (or more for control and CCl₄ treated alone).

ᵃ Significantly different from control (P < 0.05).
b Significantly different from the animal groups treated with CCl₄ alone (P < 0.05).

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Table 3.2  Prevention of CCl₄-Induced Liver Necrosis by Low Dosages of Metylenedioxybenzenes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (LU/ml)</th>
<th>ALT (LU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>CCl₄</td>
<td>2835 ± 136ᵃ</td>
<td>6029 ± 355ᵃ</td>
</tr>
<tr>
<td>+ Isosafrole (10 mg/kg)</td>
<td>37 ± 4ᵇ</td>
<td>8 ± 2ᵇ</td>
</tr>
<tr>
<td>+ Isosafrole (5 mg/kg)</td>
<td>186 ± 16ᵃᵇ</td>
<td>211 ± 36ᵃᵇ</td>
</tr>
<tr>
<td>+ Isosafrole (2.5 mg/kg)</td>
<td>641 ± 70ᵃᵇ</td>
<td>1545 ± 86ᵃᵇ</td>
</tr>
<tr>
<td>+ Safrole (10 mg/kg)</td>
<td>91 ± 3ᵃᵇ</td>
<td>67 ± 5ᵃᵇ</td>
</tr>
<tr>
<td>+ Safrole (5 mg/kg)</td>
<td>465 ± 54ᵃᵇ</td>
<td>1579 ± 43ᵃᵇ</td>
</tr>
<tr>
<td>+ Safrole (2.5 mg/kg)</td>
<td>2189 ± 96ᵃᵇ</td>
<td>5256 ± 216ᵃᵇ</td>
</tr>
<tr>
<td>+ Dihydrosafrole (10 mg/kg)</td>
<td>830 ± 62ᵃᵇ</td>
<td>2213 ± 139ᵃᵇ</td>
</tr>
<tr>
<td>+ Benzodioxole (10 mg/kg)</td>
<td>1335 ± 164ᵃᵇ</td>
<td>2736 ± 102ᵃᵇ</td>
</tr>
</tbody>
</table>

MDBs were given ip 1 hr before mice received CCl₄ (0.4 ml/kg, ip). Control mice received the equivalent amount of vehicles only. The animals were sacrificed at 24 hr after the toxicant administration, plasma AST and ALT activities were measured. Results are means ± SEM for 6 mice (or more for control and CCl₄ treated alone).

ᵃ Significantly different from control (P < 0.05).
ᵇ Significantly different from the animals treated with CCl₄ alone (P < 0.05).
completely prevented CCl₄ induced liver injury. However, only partial hepatoprotection was observed if isosafrole was administered 10 min after CCl₄, whereas no hepatoprotective effect was observed if the mice received isosafrole 1 or 3 hr after CCl₄.

Significant prevention of another hepatotoxican DMN induced liver injury was also obtained by isosafrole pretreatment. As shown in Table 3.4, treatment of mice with DMN caused 30 fold elevation in AST level and 225 fold elevation in ALT level, associated with observable liver necrosis. However, pretreatment with isosafrole markedly reduced the elevation of transaminase activities as well as histological change in liver caused by DMN.

3.3.2 Histological Studies

Liver sections from the mice received various treatments were examined under a light microscope for signs of injury. Histological examination of the livers showed that a change in normal architectural pattern occurred within 6 hr of the intraperitoneal administration of CCl₄ (Figure 3.1a) even though plasma AST and ALT levels were not increased at this time, which were 45±12 and 8±2 I.U./ml respectively. Intense centrilobular necrosis typical of massive CCl₄ hepatotoxicity and dead cell debris scattered over a significant portion of the hepatic lobule were observed 24 hr after CCl₄ treatment (Figure 3.1c), at which time there was also a drastic increase in plasma transaminas activities. On the contrary, no histological changes were observed at either 6 or 24 hr after the administration of CCl₄ provided that the animals were pretreated with isosafrole (Figures 3.1b and 3.1d). Administration with isosafrole alone did not modify the architectural pattern of the liver.
Table 3.3* Effects of Administration Time of Isosafrole on Plasma Transaminase Activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Injection Time</th>
<th>AST (I.U./ml)</th>
<th>ALT (I.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>/</td>
<td>47 ± 12</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>CCl4</td>
<td>/</td>
<td>3110 ± 58(^a)</td>
<td>7472 ± 1352(^a)</td>
</tr>
<tr>
<td>+ Isosafrole</td>
<td>- 1 hr</td>
<td>38 ± 2(^b)</td>
<td>8 ± 1(^b)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>69 ± 29(^b)</td>
<td>59 ± 15(^ab)</td>
</tr>
<tr>
<td></td>
<td>+ 10 min</td>
<td>1610 ± 19(^ab)</td>
<td>4578 ± 50(^ab)</td>
</tr>
<tr>
<td></td>
<td>+ 1 hr</td>
<td>3258 ± 232(^a)</td>
<td>7416 ± 978(^a)</td>
</tr>
<tr>
<td></td>
<td>+ 3 hr</td>
<td>3038 ± 1080(^a)</td>
<td>7188 ± 1812(^a)</td>
</tr>
</tbody>
</table>

Isosafrole was given ip at 200 mg/kg at different times with respect to CCl4 (0.4 ml/kg, ip). Mice were sacrificed 24 hr after the toxicant administration, plasma AST and ALT activities were measured. Results are means ± SEM for 6 mice.

\(^a\) Significantly different from control (P < 0.05).

\(^b\) Significantly different from CCl4 alone (P < 0.05).

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Table 3.4 Prevention of N-Dimethylnitrosamine-Induced Liver Injury by Isosafrole Pretreatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (I.U./ml)</th>
<th>ALT (I.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36 ± 4</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>N-Dimethylnitrosamine</td>
<td>1080 ± 194&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2022 ± 462&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Isosafrole</td>
<td>159 ± 17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>112 ± 16&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Isosafrole (200 mg/kg) was given ip 1 hr before mice received N-dimethylnitrosamine (100 mg/kg, ip). Control mice received the equivalent amount of vehicles only. Mice were sacrificed at 24 hr after the toxicant administration, plasma AST and ALT activities were measured. Results are means ± SEM for 6 mice.

<sup>a</sup> Significantly different from control (P < 0.05).

<sup>b</sup> Significantly different from the animals treated with DMN alone (P < 0.05).
Figure 3.1* Histological Studies on the Protection against CCl4-Induced Liver Necrosis by Isosafrole Pretreatment. (a) Liver section taken from a mouse 6 hr after CCl4 administration. Note the abnormal structure of liver cells. (b) Liver section taken from a mouse that received isosafrole 1 hr before CCl4 administration. Samples were taken 6 hr after CCl4 treatment.

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Figure 3.1(Continued)  (c) Liver section taken from a mouse 24 hr after CCl₄ administration. Note the intense centrilobular necrosis. (d) Liver section taken from a mouse that received isosafrole 1 hr before CCl₄ administration. Samples were taken 24 hr after CCl₄ treatment. The liver structure is essentially normal. All liver slices shown in Figure 3.1 were taken immediately after mice were sacrificed, fixed in 10% buffered formalin solution, embedded in paraffin and stained with hematoxylin and eosin (x 120). All photographs represent those seen in all livers examined (n = 6). Bar = 100 μm.
3.3.3 *In vitro* Prevention of BrCCl3-Induced Cytotoxicity and Lipid Peroxidation

Figure 3.2 shows that addition of BrCCl3 to hepatocytes caused severe cell injury at 2 hr of incubation. However, BrCCl3 cytotoxicity towards isolated hepatocytes was markedly decreased if the hepatocytes were preincubated with isosafrole, safrole or dihydrosafrole. The structurally related alkenylbenzene antioxidants isoeugenol and curcumin also prevented BrCCl3 cytotoxicity. On the contrary, PBO, sesamol and eugenol were not as effective as those compounds (Figure 3.2). Lipid peroxidation determined as 2-thiobarbituric acid-reactive substances (TBARS) formed was also observed when the cells were treated with BrCCl3 alone, and however was significantly inhibited by isosafrole pretreatment (Figure 3.3.). Addition of known antioxidants sesamol, isoeugenol, eugenol and curcumin was also effective at preventing BrCCl3-induced TBARS formation. By contrast, other MDBs, PBO, safrole and dihydrosafrole were ineffective (Figure 3.3).

3.3.4 Prevention of Phenetidine-Induced Methemoglobinemia

As shown in Figure 3.4, administration of phenetidine in mice caused abrupt massive methemoglobin formation, which decayed with time and returned to the normal level at 6 hr after injection. However, the phenetidine-induced methemoglobinemia was completely prevented if mice were given isosafrole ip at 50 mg/kg prior to phenetidine, which maintained a normal methemoglobin content at 2.1(± 0.4)% of total hemoglobin at all time intervals tested. Pretreatment with lower dosages of isosafrole was still protective (Figure 3.4). Fifteen minutes after phenetidine administration, isosafrole demonstrated ca.
Figure 3.2* Prevention of BrCCl3-Induced Cytotoxicity to Isolated Hepatocytes. Hepatocytes (10^6 cells/ml) were preincubated in Krebs-Henseleit buffer, pH 7.4 at 37 °C. Cells were maintained under an atmosphere of 1% O₂. MDBs and other compounds at final concentrations of 100 μM were preincubated for 10 min prior to BrCCl3 (600 μM) addition. Cell toxicity was determined after 2 hr incubation with BrCCl3 and expressed as the percentage of cells taken up trypan blue. Results are means ± SEM for 3 separate experiments. (*Significantly different from the hepatocytes treated with BrCCl3 only.)

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Figure 3.3 Prevention of BrCCl3-Induced Hepatocyte Lipid Peroxidation. Hepatocytes (10^6 cells/ml) were preincubated in Krebs-Henseleit buffer, pH 7.4 at 37 °C. Cells were maintained under an atmosphere of 1% O₂. MDBs and other compounds at final concentrations of 100 μM were preincubated for 10 min prior to BrCCl3 (600 μM) addition. Lipid peroxidation was determined after 2 hr incubation with BrCCl3 and expressed as TBARS formed. Results are means ± SEM for 3 separate experiments.

(* Significantly different from the hepatocytes treated with BrCCl3 only.)
Figure 3.4 Dosage Dependent Prevention of Phentidine-Induced Methemoglobinemia by Isosafrole Pretreatment. Isosafrole was given ip 1 hr before mice received phentidine (200 mg/kg, ip). Control mice received the equivalent amount of vehicles only. Blood samples were taken at time of interest, and methemoglobin content was determined. Results are means ± SEM for 6 mice (or more for phentidine treated alone). (* Significantly different from animals treated with phentidine only.)
Isosafrole (30 min after) + Isosafrole (10 min after) + Isosafrole (5 min after) + Isosafrole (24 h before) (200 mg/kg)

The Figure 3.5 Antidotal Effect and Early Treatment of Isosafrole on Phenetidine-Induced Methemoglobinemia. Isosafrole was given ip at 50 mg/kg after phenetidine (200 mg/kg, ip) administration, or at 200 mg/kg 24 hr prior to phenetidine. Control mice received the equivalent amount of vehicles only. Blood samples were taken at time of interest, and methemoglobin content was determined. Results are means ± SEM for 6 mice (or more for phenetidine treated alone). (* Significantly different from animals treated with phenetidine only.)
46%, 91% and 97% inhibition of phenetidine-induced methemoglobin formation at dosages 10, 12.5 and 25 mg/kg respectively (Figure 3.4).

It is also noteworthy to point out that a complete protection by isosafrole was observed even if it was given 24 hr prior to phenetidine, provided that a dosage of 200 mg/kg was used (Figure 3.5). No protection was obtained for isosafrole treatment at 50 mg/kg for 24 hr. Moreover, the antidotal effect of isosafrole on phenetidine toxicity was investigated. Post-treatment with isosafrole (50 mg/kg) 5, 10 or 30 min after phenetidine administration also significantly reduced methemoglobin content in experimental animals, as shown in Figure 3.5. The effectiveness of isosafrole as an antidote of phenetidine toxicity highly depended on the time of administration. The methemoglobin content essentially returned to the normal level at 4 hr after phenetidine administration (Figure 3.5).

3.4 DISCUSSION

It is now generally accepted that the hepatotoxicity of CCl4 results from reductive dehalogenation catalyzed by reduced cytochrome P450 to form a highly reactive trichloromethyl free radical (CCl3•), which interacts readily with molecular oxygen to form a trichloromethyl peroxy radical (CCl3OO•) (McCay et al., 1984; Williams and Burk, 1990). Both radicals are capable of binding covalently to proteins and lipids, or abstracting a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and liver cell injury (Lee et al., 1982; McCay et al., 1984; Recknagel and Ghoshal, 1966; Williams and Burk, 1990). Administration of cytochrome P450 inhibitors and/or antioxidants, such as SKF 525-A (Castro et al., 1968; 1974), pyrazole (D'Acosta et al., 1972), methoxsalen (Labbe et al., 1987), silymarin (Lettéron et al., 1990) and butylated hydroxyanisole
(Ansher et al., 1983), has been shown to either delay or partially prevent hepatocellular necrosis caused by CCl₄ (Masuda and Nakayama, 1982; Wolfgang et al., 1990).

In the present work, a group of dietary methylenedioxyphenyl compounds, namely, isosafrole, safrole, dihydrosafrole, benzodioxole and myristicin have been shown to be the first agents that can completely prevent CCl₄-induced hepatotoxicity in mice. Full protection was observed by isosafrole at dosages as low as 10 mg/kg, provided that isosafrole was administered 1 hr prior to CCl₄. The hepatoprotection by isosafrole pretreatment was maintained for at least 3 days. Post-treatment with isosafrole 10 min after CCl₄ administration, even at 200 mg/kg, only partially prevented CCl₄-induced liver injury. However other MDBs and structurally related known antioxidants such as PBO, sesamol, eugenol, isoeugenol and curcumin showed little protection in mice against CCl₄ hepatotoxicity at 200 mg/kg. BrCCl₃ is more hepatotoxic than CCl₄ (Sipes et al., 1977), and is much more cytotoxic and more effective at inducing lipid peroxidation with isolated hepatocytes (McGirr et al., 1990). It also has a similar mechanism of metabolic activation to that of CCl₄ (Sipes et al., 1977). Isosafrole, safrole and dihydrosafrole again prevented the in vitro hepatocyte cytotoxicity caused by BrCCl₃, and isosafrole and the known antioxidants also reduced BrCCl₃-induced lipid peroxidation.

DMN is a nitrosamine found in the environment and in food, cigarette smoke and alcoholic beverages (Goff and Fine, 1979; Tricker, 1997). High dosages of DMN lead to acute in vivo toxicity, including centrilobular liver necrosis in rodents and fatal liver cirrhosis in humans as well as malignant liver tumors (Lai and Arcos, 1980; Magee, 1989; Magee and Swann, 1969; Schut and Castonguay, 1984). Both cell death and liver neoplasm depend on DMN metabolism. It has been suggested that α-carbon hydroxylation,
a process that is mediated by cytochrome P450-dependent mixed-function oxidases, is a crucial step in the bioactivation of DMN to a toxic reactive methyl diazonium intermediate that can react directly with cellular nucleophiles (Kawanishi et al., 1984; Lijinsky et al., 1968; Magee and Farber, 1962; Magee and Hultin, 1962; Schut and Castonguay, 1984). DMN-induced acute liver toxicity in mice was, however, significantly prevented by isosafrole pretreatment in the present work.

Similarly, cytochrome P450-dependent monooxygenase-catalyzed reactive metabolic intermediate formation is also implicated in the toxic effects of the aromatic amine phenetidine (Kiese, 1966; Weisburger and Weisburger, 1973). It has been reported that methemoglobinemia was observed in vivo in laboratory animals as well as in human beings as a consequence of overdose and chronic abuse. The methemoglobin formation has been ascribed to the generation of $N$-hydroxylated phenetidine, which readily participates in a coupled oxidation with oxyhemoglobin in the erythrocyte, yielding methemoglobin and a nitrosobenzene (Jensen and Jollow, 1991; Kiese, 1966; Weisburger and Weisburger, 1973). A few cytochrome P450 inhibitors, including PBO, have been shown to decrease arylamine drug-induced methemoglobinemia (Coleman et al., 1990; Nakayama and Masuda, 1985). Pretreatment with isosafrole at dosages as low as 10 mg/kg, as shown in this work, was highly effective at preventing phenetidine-induced extensive formation of methemoglobin. In addition, isosafrole also exerted an antidotal effects and significantly inhibited methemoglobinemia even given 30 min after phenetidine administration.

The chemoprotective effects of this group of dietary MDBs against CCl$_4$, DMN and phenetidine toxicity could be attributed to their ability to form MI complexes with the ferrous form of cytochrome P450. As discussed in Chapter 1, the metabolic intermediate
involved in the complex formation is probably a carbene or a carbanion formed from the hydroxylation and demethylenation of the methylene carbon catalyzed by cytochrome P450. The formation of stable MI complexes would result in inhibition of the activities of various cytochrome P450 isozymes, and thus prevent the monooxygenase-mediated bioactivation of the toxicants. Curcumin, eugenol and isoeugenol lack the methylene bridge carbon required to form MI complexes with cytochrome P450, which could explain their inability to prevent CCl₄-induced liver necrosis even though they are effective antioxidants (Kumaravelu et al., 1995; Rajakumar and Rao, 1993; Reddy and Lokesh, 1992; Soudamini et al., 1992). The importance of MI complex formation for hepatoprotection would also explain why isosafrole was not hepatoprotective if administered after CCl₄. These MI complexes can be displaced in vitro by alternative cytochrome P450 substrates, e.g., 2-methylbenzimidazole (Dickins et al., 1979; Murray et al., 1983a). Studies of the inhibitory effects of MDBs on various P450 isozymes that are involved in CCl₄, DMN and phenetidine biotransformation have been implemented and discussed in Chapter 4.

In addition, lipid peroxidation also contributes to CCl₄-induced hepatotoxicity (McCay et al., 1984; Recknagel, 1967). The reported partial prevention of CCl₄-induced liver cholestasis by sesamol was attributed to its antioxidant properties (Ohta et al., 1994). Curcumin, eugenol and isoeugenol were also reported to inhibit CCl₄-initiated lipid peroxidation both in vivo and in vitro, possibly by their antioxidant action (Kumaravelu et al., 1995; Rajakumar and Rao, 1993; Reddy and Lokesh, 1992; Soudamini et al., 1992) or by their ability to induce phase 2 drug-detoxifying enzymes (Yokota et al., 1988). In this work, BrCCl₃-induced lipid peroxidation in rat hepatocytes was also decreased by isosafrole and the known antioxidants. Furthermore, it has been suggested that a peroxide
or a free radical which originates from the oxygen of oxyhemoglobin could also be the reactive intermediate causing the oxidation of hemoglobin to methemoglobin and contribute to arylamine toxicity (Kiese, 1966; Weisburger and Weisburger, 1973). Therefore, the protection by MDBs against CCl4 and phenetidine toxicity could also partly result from the antioxidant activity of MDBs, mainly through their demethylenated catecholic metabolites. However, pretreatment of mice with hydroxychavicol (a major metabolite of safrole) did not prevent CCl4-induced liver necrosis in vivo. Similarly, sesamol, curcumin, eugenol and isoeugenol which are also antioxidants were ineffective at preventing CCl4-induced hepatotoxicity in vivo, suggesting that the cytochrome P450 inhibitory effects through MDB MI-P450 complex formation should account mainly for their hepatoprotective properties.

In summary, the pretreatment of mice with some dietary MDBs (i.e., isosafrole, safrole, dihydrosafrole, benzodioxole and myristicin) completely prevented CCl4-induced liver necrosis. A completely protection by isosafrole pretreatment in vivo was obtained at a dosage as low as 10 mg/kg, suggesting that isosafrole is a highly effective protecting agent known at preventing CCl4 hepatotoxicity (Table 3.5). Pretreatment with isosafrole also prevented DMN-induced liver injury and phenetidine-induced methemoglobinemia. Isosafrole was also an antidote of phenetidine intoxication. The protective effects of MDBs against various aspects of CCl4, DMN and phenetidine toxicity were hypothesized to be due mainly to their ability to inactivate the cytochrome P450 isozymes that are involved in the toxicant bioactivation. The inhibitory effects of dietary MDBs may enable them to prevent hepatic injury produced by other hepatotoxicants activated by cytochrome P450-dependent monooxygenases. Isosafrole, unlike safrole, is not genotoxic (Boberg et al.,
1986; Tsai et al., 1994), and does not bind to DNA (Randerath et al., 1984), and could prove useful as a chemopreventive agent against chemical carcinogenesis in vivo.
Table 3.5  Examples of Protection against **CCl₄**-induced Hepatotoxicity

*in vivo*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg/kg)</th>
<th>CCl₄ dose (ml/kg)</th>
<th>Animals</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Literature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKF 525-A*</td>
<td>50</td>
<td>2.5</td>
<td>rats</td>
<td>Castro <em>et al.</em>, 1968</td>
</tr>
<tr>
<td>Methoxsalen†</td>
<td>55</td>
<td>0.1</td>
<td>mice</td>
<td>Labbe <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>Pyrazole*</td>
<td>150</td>
<td>5</td>
<td>rats</td>
<td>D'Acosta <em>et al.</em>, 1972</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>1000</td>
<td>1</td>
<td>rats</td>
<td>Brady <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Diallyl sulfide*</td>
<td>200</td>
<td>1</td>
<td>rats</td>
<td>Brady <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>Silymarin*</td>
<td>800</td>
<td>0.018</td>
<td>mice</td>
<td>Lettérion <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>60</td>
<td>0.16</td>
<td>mice</td>
<td>Kohno <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>N-Acetyl cysteine</td>
<td>2000</td>
<td>5</td>
<td>rats</td>
<td>Valles <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>This work</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isosafrole</td>
<td>10</td>
<td>0.4</td>
<td>mice</td>
<td>Zhao and O'Brien, 1996</td>
</tr>
</tbody>
</table>

Note.
Protective agents were given i.p. 30 - 60 min. before CCl₄ (i.p.), except that disulfiram and diallyl sulfide were given p.o. 18 hr before CCl₄ administration.

* partial protection.
† Delay of CCl₄-induced hepatotoxicity
Chapter 4

THE PREVENTION OF CCl₄, PHENETIDINE AND N-DIMETHYLNITROSAMINE TOXICITY IN MICE BY NATURALLY OCCURRING METHYLENEDIOXYBENZENES.

PART II. THE MECHANISMS.

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4.1 ABSTRACT

The chemoprotective effects of isosafrole and other MDBs on CCl₄, DMN and phenetidine toxicity could be attributed to the inhibition of P450 isozymes involved in the toxicant metabolic activation. Liver microsomes isolated 1 hr after isosafrole but not after PBO administration had a markedly decreased cytochrome P4502E1 activity. Isosafrole, safrole, dihydrosafrole and benzodioxole in vitro also inhibited CYP2E1-dependent metabolism more effectively than eugenol and isoeugenol, whereas PBO did not inhibit CYP2E1 activity. The protection by isosafrole, safrole and benzodioxole against CCl₄ toxicity was therefore predominantly attributed to their ability to inactivate CYP2E1, the major isozyme involved in CCl₄ bioactivation. The marked potentiation of CCl₄ hepatotoxicity in CYP2E1-induced mice was also completely prevented by isosafrole but not PBO pretreatment, supporting the hypothesis that CYP2E1 inhibition by isosafrole contributes to its hepatoprotective effect against CCl₄. Moreover, isosafrole was a potent CYP1A2 inhibitor in vivo, even after 24 hr of administration, which contributed to its protection against phenetidine-induced methemoglobinemia in mice.
4.2 INTRODUCTION

It has been widely accepted that CCl₄ is biotransformed to reactive radical metabolites catalyzed by P450-dependent monooxygenases. In addition to initiating cytotoxicity, the radicals may also interact with the heme of P450, thereby destroying the isozymes responsible for their formation. This has been demonstrated by a decrease in total hepatic P450 content and a loss of microsomal protein in the 52,000-58,000 molecular weight range on SDS-PAGE as well as a loss of the enzyme catalytic activity after in vivo CCl₄ administration (Head et al., 1981; Noguchi et al., 1982a; Sasame et al., 1968; Sesardic et al., 1989). Therefore, CCl₄ acts as a suicide inhibitor of hepatic P450. The mechanisms by which the P450s are destroyed have been investigated and ascribed to the destruction of the heme moiety of cytochrome P450 (Guzelian and Swisher, 1979; Levin et al., 1972; Manno et al., 1988).

Bioactivation of CCl₄ is catalyzed by several specific forms of P450 isozymes. The involvement of CYP2B1/2, the major PB-inducible forms (Noguchi et al., 1982b; Davies et al., 1985, 1986), and in particular CYP2E1, the ethanol-inducible form (English and Anders, 1985) has been reported. Moreover, it has been demonstrated that a 3MC-inducible form, CYP1A2, was selectively destroyed by the treatment of rats with CCl₄ (Degawa et al., 1993), suggesting the involvement of this isozyme in CCl₄ metabolism.

The hepatotoxicity of CCl₄ is also susceptible to potentiation by aliphatic alcohols administered acutely or chronically (Cornish and Adefuin, 1967; Ray and Mehendale, 1990; Traiger and Plaa, 1971; Ueng et al., 1983), which has been attributed to the induction of CYP2E1 that is involved in the bioactivation of halogenated compounds (Johansson and Ingelman-Sundberg, 1985; Raucy et al., 1993; Yang et al., 1990; 1991).
It has been shown in Chapter 3 that some naturally occurring MDBs prevented CCl₄, DMN and phenetidine toxicity in experimental animals. This seemed to correlate with inactivation of cytochrome P450 isozymes that are involved in the metabolic activation of halomethanes, nitrosamines and arylamines (English and Anders, 1985; Johansson and Ingelman-Sundberg, 1985; Noguchi et al., 1982b; Sesardic et al., 1989; Yang et al., 1991). Therefore, the inhibitory effects of MDBs on the P450 isozymes, such as CYP2E1, CYP1A2 and CYP2B1/2 that are important to the toxicant metabolism have been determined in this chapter. It was demonstrated that the hepatoprotective MDBs such as isosafrole are better inhibitors of CYP2E1 than those less effective MDBs such as PBO both in vivo and in vitro. Moreover, isosafrole has also been shown to be a potent inhibitor of CYP1A2 in vivo.

4.3 RESULTS
4.3.1 Prevention of Isopropanol-Potentiated CCl₄ and CHCl₃ Hepatotoxicity in Mice by Isosafrole Pretreatment

The administration of isopropanol to rats induced CYP2E1 and CYP2B1/2 activity 16-18 hr later, and much lower dosages of CCl₄ were needed to induce liver necrosis (Raucy et al., 1993). The protective effects of isosafrole and PBO on this potentiated hepatotoxicity were therefore investigated. As shown in Table 4.1, administration of a very low dosage of CCl₄ (10 μl/kg) to isopropanol-induced mice caused a 100-fold increase in plasma AST level, and in particular, a 900-fold increase in plasma ALT activity, compared with that of control. CCl₄ at the low dosage or isopropanol administration alone did not affect plasma AST and ALT levels. However, the elevation of plasma AST and ALT
Table 4.1 Prevention of Isopropanol Potentiated CCl₄ and CHCl₃ Hepatotoxicity by Isosafrole or PBO Pretreatment *in vivo*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (I.U./ml)</th>
<th>ALT (I.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31 ± 4</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>CCl₄</td>
<td>32 ± 6</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>75 ± 19ᵃ</td>
<td>99 ± 38ᵃ</td>
</tr>
<tr>
<td>Isopropanol Pretreatment</td>
<td>31 ± 1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>+ CCl₄</td>
<td>3075 ± 696ᵃ</td>
<td>5530 ± 1358ᵃ</td>
</tr>
<tr>
<td>+ Isosafrole + CCl₄</td>
<td>39 ± 5ᵇ</td>
<td>7 ± 1ᵇ</td>
</tr>
<tr>
<td>+ PBO + CCl₄</td>
<td>3249 ± 420ᵃ</td>
<td>5741 ± 765ᵃ</td>
</tr>
<tr>
<td>+ CHCl₃</td>
<td>2503 ± 313ᵃ</td>
<td>4056 ± 712ᵃ</td>
</tr>
<tr>
<td>+ Isosafrole + CHCl₃</td>
<td>46 ± 5ᶜ</td>
<td>10 ± 4ᶜ</td>
</tr>
<tr>
<td>+ PBO + CHCl₃</td>
<td>1445 ± 216ᵃᶜ</td>
<td>3016 ± 432ᵃ</td>
</tr>
</tbody>
</table>

Mice received a single dose of isopropanol (2.4 ml/kg, po) 16-18 hr before being given CCl₄ (10 µl/kg, ip) or CHCl₃ (0.5 ml/kg). To some groups of animals, isosafrole (100 mg/kg, ip) or PBO (200 mg/kg, ip) were administered 1 hr before the hepatotoxicants respectively. Mice were sacrificed 24 hr after CCl₄ or CHCl₃ administration and plasma transaminase activities were determined. Results are means ± SEM for 6 mice (or more for isopropanol treated alone).

ᵃ Significantly different from control (P < 0.05).
b Significantly different from the group treated with isopropanol and CCl₄ (P < 0.05).
c Significantly different from the group treated with isopropanol and CHCl₃ (P < 0.05).
activities by CCl₄ in isopropanol-pretreated mice was completely prevented if the mice received isosafrole 1 hr prior to the hepatotoxicant exposure. On the contrary, pretreatment with PBO was not hepatoprotective (Table 4.1).

In addition to increasing CCl₄ hepatotoxicity, isopropanol appeared to potentiate CHCl₃-induced liver injury in mice as well. As shown in Table 4.1, 0.5 ml/kg of CHCl₃ caused only 2-fold increase in AST level and 16-fold increase in ALT level. These transaminase activities were however enhanced 80- and 675-fold respectively, provided that the animals were pretreated with a single dose of isopropanol, indicating severe liver damage. Likewise, the isopropanol-potentiated CHCl₃ hepatotoxicity was prevented if isosafrole was given 1 hr prior to CHCl₃. The protection by PBO pretreatment, on the contrary, was insignificant if there was any (Table 4.1).

4.3.2 Prevention of CCl₄-Induced in vivo Cytochrome P450 Isozyme Destruction by Isosafrole

Figure 4.1 shows that treatment of mice with a single dose of CCl₄ caused a drastic suppression of mouse liver microsomal p-nitrophenol (PNP) hydroxylase activity, which was decreased to only 40% of control at 1 hr of the treatment and reached a minimum (5% of the control) 12 hr later. By contrast, isosafrole given 1 hr prior to CCl₄ significantly blocked CYP2E1 destruction by CCl₄ administration. Although the PNP hydroxylase activity was still decreased at the first hour of CCl₄ dosing, it was correlated with the inhibition of the isozyme activity by isosafrole itself. Moreover, the CYP2E1-catalyzed PNP hydroxylation gradually returned to the level comparable to that of microsomes treated with isosafrole alone (Figure 4.1).
Figure 4.1 Prevention of CCl₄-Caused CYP2E1 Destruction in Mice. Liver microsomes were prepared from mice treated with CCl₄ (0.4 ml/kg, ip), or isosafrole (200 mg/kg, ip), or isosafrole and CCl₄. For the third group, isosafrole was given 1 hr prior to CCl₄, and the animals were sacrificed at various time intervals with respect to CCl₄ administration. PNP hydroxylation was performed in a reaction mixture containing microsomes (1 mg protein/ml) and PNP (250 μM) in phosphate buffer (0.1 M, pH 6.8) under 37 °C. Reaction was initiated by adding NADPH (1 mM) and stopped 20 min after incubation. Results are means ± SEM for 3 determinations. (* Significantly different from microsomes prepared from animals treated with CCl₄ alone.)
Unlike PNP hydroxylase, pentoxyresorufin O-dealkylase (PROD) activity was still 85% of control 1 hr after CCl\textsubscript{4} administration, as shown in Figure 4.2. However, it was rapidly reduced to ca. 5% of control in CCl\textsubscript{4}-treated animals within 6 hr of exposure. Isosafrole pretreatment also prevented the complete inactivation of PROD activity by CCl\textsubscript{4}. The isozyme activity rebounded to greater than control levels 12 hr after CCl\textsubscript{4} administration provided that the animals were pretreated with isosafrole. Moreover, this profile of pentoxyresorufin O-dealkylation exerted a pattern similar to that of isosafrole treatment alone (Figure 4.2).

Similarly, the activity of CYP1A2-mediated methoxyresorufin O-demethylase (MROD) was also reduced in a time-dependent manner by CCl\textsubscript{4} injection (Figure 4.3). At 3 hr after CCl\textsubscript{4} administration the isozyme activity was about 30% of control, and decreased to less than 4% of control at 6 hr exposure and remained approximately the same until 24 hr. On the contrary, if the microsomes were prepared from the animals treated with isosafrole prior to CCl\textsubscript{4}, the activities of MROD were essentially the same as those of microsomes prepared from the animals treated with isosafrole alone, indicating that CCl\textsubscript{4} did not cause degradation of this isozyme as it did in the absence of isosafrole pretreatment. Furthermore, it was observed that CYP1A2-mediated MROD activity was still only 45-50% that of control at 24 hr after isosafrole treatment (Figure 4.3), suggesting that isosafrole is a potent inhibitor of CYP1A2 activity \textit{in vivo}. 

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Figure 4.2 Prevention of CCl₄-Caused PROD Destruction in Mice. Liver microsomes were prepared from mice treated with CCl₄ (0.4 ml/kg, ip), or isosafrole (200 mg/kg, ip), or isosafrole and CCl₄. For the third group, isosafrole was given 1 hr prior to CCl₄, and the animals were sacrificed at various time intervals with respect to CCl₄ administration. Pentoxyresorufin $O$-dealkylation was performed in a reaction mixture containing microsomes (0.1 mg protein/ml), pentoxyresorufin (250 nM) and NADPH (0.1 mM) in phosphate buffer (0.1 M, pH 7.4) under 37 °C. Results are means ± SEM for 3 determinations. (* Significantly different from microsomes prepared from animals treated with CCl₄ alone.)
Figure 4.3 Prevention of CCl4-Caused CYP1A2 Destruction in Mice. Liver microsomes were prepared from mice treated with CCl4 (0.4 ml/kg, ip), or isosafrole (200 mg/kg, ip), or isosafrole and CCl4. For the third group, isosafrole was given 1 hr prior to CCl4, and the animals were sacrificed at various time intervals with respect to CCl4 administration. Methoxyresorufin O-demethylation was performed in a reaction mixture containing microsomes (0.1 mg protein/ml), methoxyresorufin (500 nM) and NADPH (0.1 mM) in phosphate buffer (0.1 M, pH 7.4) under 37 °C. Results are means ± SEM for 3 determinations. (* Significantly different from microsomes prepared from animals treated with CCl4 alone.)
4.3.3 Comparison of the in vivo Inhibitory Effects of Isosafrole and PBO on CYP2E1 Activity

In order to investigate the hepatoprotective mechanisms of MDBs against CCl4-induced liver necrosis as well as isopropanol-potentiated CCl4 and CHCl3 hepatotoxicity, the inhibitory effects of MDBs, in particular isosafrole and PBO, on CYP2E1, believed to be the major isozyme responsible for the metabolic activation of the toxicants (Johansson and Ingelman-Sundberg, 1985; Koop, 1992), were determined. As shown in Figure 4.4, microsomal CYP2E1-catalyzed p-nitrophenol hydroxylation was inhibited 65% when the liver microsomes were prepared 1 hr after isosafrole administration. However, PBO-pretreated mouse liver microsomes did not show any decrease in the isozyme activity (Figure 4.4).

Figure 4.4 also shows that microsomes isolated from mice treated with isopropanol for 17-18 hr had about two fold higher p-nitrophenol hydroxylase activities compared with that of non-induced microsomes, thereby confirming that CYP2E1 activity had been induced by isopropanol treatment. Furthermore, the induced CYP2E1 activity was inhibited by 70% if isosafrole was administered to the mice 1 hr before the microsomes were prepared. However, no inhibition of the isozyme activity was observed if PBO was administered similarly (Figure 4.4).

4.3.4 In vitro Inhibition of Hepatic Microsomal Cytochrome P450 Activities by MDBs

Table 4.2 compares the in vitro inhibitory effects of different MDBs on the CYP2E1 activity of liver microsomes isolated from CYP2E1-induced acetone-pretreated
Figure 4.4* Effect of Isosafrole and PBO Pretreatment on CYP2E1-Catalyzed $p$-Nitrophenol Hydroxylation. Mouse hepatic microsomes were prepared from non-induced (dotted bars) or isopropanol-induced (hatched bars) control and 1 hr isosafrole- or PBO-treated mice. PNP hydroxylation was performed in a reaction mixture containing microsomes (1 mg protein/ml) and PNP (250 μM) in phosphate buffer (0.1 M, pH 6.8) under 37 °C. Reaction was initiated by adding NADPH (1 mM) and stopped 20 min after incubation. Results are means ± SEM for 3 determinations. (*Significantly different from control or normal groups; $^a$ significantly different from the animal group treated with isopropanol alone.)

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mice. Isosafrole, safrole and dihydrosafrole were found to be the most effective inhibitors. The Ki for isosafrole was determined to be 10 μM (for 1 mg protein/ml), whereas the Ki for benzodioxole was estimated to be 100 μM from Dixon plots using two substrate concentrations. However, pre-incubation with either eugenol or isoeugenol at even 100 μM exerted less than 20% inhibition of CYP2E1-mediated PNP hydroxylation. Surprisingly, PBO, the most well known cytochrome P450 inhibitor of the MDB family, had little effect on CYP2E1 activity even at 100 μM. A PNP hydroxylase activity of 2.14±0.08 nmol 4-nitrocatechol formed/mg protein/min was obtained with PBO at 100 μM.

The susceptibilities of other cytochrome P450s to different MDBs were also measured. As shown in Table 4.2, the inhibitory effects of different MDBs on phenobarbital-inducible isozymes were determined by assaying PROD activity of microsomes isolated from phenobarbital-pretreated mice. Pentoxyresorufin O-dealkylase activity was decreased to ca. 80% of control when these microsomes were preincubated with 10 μM of isoeugenol, eugenol or benzodioxole. However, isosafrole, safrole, dihydrosafrole or PBO exerted complete inhibition at the 10 μM concentration used, all of which resulted in PROD activities less than 100 pmol resorufin formed/mg protein/min. When 2 μM of these compounds were used as shown in the table, safrole, dihydrosafrole and PBO had higher inhibitory effects than isosafrole and benzodioxole which inhibited 50% of the isozyme activity. The estimated Ki for isosafrole was 2 μM (for 0.1 mg protein/ml), and for safrole or dihydrosafrole was 1 μM.

As also shown in Table 4.2, the susceptibility of CYP1A2 to different MDBs was determined in control microsomes by assaying MROD activity. Isosafrole was much more effective than other MDBs with an estimated Ki of < 1 μM (for 0.1 mg protein/ml). Other
Table 4.2* In Vitro Inhibition of Hepatic Microsomal Cytochrome P450 Activities by Methyleneedioxybenzenes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PNP Hydroxylase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PROD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MROD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>EROD&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.73 ± 0.10</td>
<td>70 ± 4</td>
<td>3326 ± 130</td>
<td>175 ± 27</td>
</tr>
<tr>
<td>Induced</td>
<td>2.08 ± 0.14</td>
<td>1145 ± 31</td>
<td>NA</td>
<td>4147 ± 147</td>
</tr>
<tr>
<td>+ Isosafrole</td>
<td>0.32 ± 0.04</td>
<td>509 ± 43</td>
<td>260 ± 45</td>
<td>1032 ± 53</td>
</tr>
<tr>
<td>+ Safrole</td>
<td>0.35 ± 0.04</td>
<td>294 ± 37</td>
<td>1200 ± 72</td>
<td>3726 ± 101</td>
</tr>
<tr>
<td>+ Dihydrosafrole</td>
<td>0.30 ± 0.03</td>
<td>206 ± 31</td>
<td>505 ± 33</td>
<td>1895 ± 73</td>
</tr>
<tr>
<td>+ Benzodioxole</td>
<td>1.53 ± 0.11</td>
<td>698 ± 14</td>
<td>627 ± 71</td>
<td>4505 ± 203</td>
</tr>
<tr>
<td>+ PBO</td>
<td>2.24 ± 0.15</td>
<td>367 ± 26</td>
<td>379 ± 35</td>
<td>1200 ± 46</td>
</tr>
<tr>
<td>+ Isoeugenol</td>
<td>1.81 ± 0.08</td>
<td>1018 ± 29</td>
<td>1032 ± 37</td>
<td>2905 ± 53</td>
</tr>
<tr>
<td>+ Eugenol</td>
<td>1.77 ± 0.11</td>
<td>913 ± 35</td>
<td>1621 ± 35</td>
<td>2695 ± 78</td>
</tr>
</tbody>
</table>

<sup>a</sup> Acetone-induced mouse hepatic microsomes were incubated in phosphate buffer (0.1 M, pH 6.8) at a protein concentration of 1 mg/ml under 37 °C. NADPH (1 mM) and MDBs (20 μM) were pre-incubated 5 min before adding PNP (250 μM). Isozyme activity is expressed as nmol 4-nitrocatechol formed/mg protein/min.

<sup>b</sup> PB-induced mouse hepatic microsomes (60 μg protein/ml) were incubated in phosphate buffer (0.1 M, pH 7.4) under 37 °C. Isosafrole, safrole, dihydrosafrole and PBO at 2 μM, or isoeugenol, eugenol and benzodioxole at 10 μM were pre-incubated in the presence of NADPH (0.1 mM) 5 min before adding pentoxyresorufin (250 nM). Isozyme activity is expressed as pmol resorufin formed/mg protein/min.

<sup>c</sup> Normal mouse hepatic microsomes were incubated in phosphate buffer (0.1 M, pH 7.4) at a protein concentration of 100 μg/ml under 37 °C. NADPH (0.1 mM) and MDBs (10 μM) were pre-incubated 5 min before adding methoxyresorufin (500 nM). Isozyme activity is expressed as pmol resorufin formed/mg protein/min.

<sup>d</sup> 3-MC-induced mouse hepatic microsomes (50 μg protein/ml) were incubated in phosphate buffer (0.1 M, pH 7.4) under 37 °C. Compounds tested at 10 μM were pre-incubated in the presence of NADPH (0.1 mM) 5 min before adding ethoxyresorufin (500 nM). Isozyme activity is expressed as pmol resorufin formed/mg protein/min.

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methylenedioxybenzenes such as dihydrosafrole, benzodioxole and PBO were better inhibitors than eugenol and isoeugenol which were partly effective at 10 µM. Similarly, CYP1A1 activity was determined in 3MC-induced microsomes by measuring ethoxyresorufin O-deethylase activity (EROD, Table 4.2) and benzo[a]pyrene 3-hydroxylase (AHH) activity. Using AHH assay, the inhibition by isosafrole, safrole, dihydrosafrole, benzodioxole, PBO, isoeugenol and eugenol was ca. 68%, 26%, 37%, 1%, 74%, 33% and 53%, respectively. Isosafrole and PBO were found to be much better inhibitors of CYP1A1 than other MDBs.

4.4 DISCUSSION

It has been shown that CCl4 is a suicide inhibitor of several cytochrome P450 isozymes (Davies et al., 1985, 1986; Gadeholt, 1984; Manno et al., 1988). The inactivation of P450 is one of the earliest aspects of damage by CCl4. It has been observed that cytochrome P450 was rapidly inactivated during anaerobic metabolism of CCl4 in NADPH-fortified liver microsomes as well as in liver microsomes prepared from CCl4 treated animals, which was accompanied with a loss of protoheme, the prosthetic group of P450 (de Groot and Haas, 1980, 1981; Head et al., 1981; Masuda, 1981; Noguchi et al., 1982a; Sesardic et al., 1989; Yamazoe et al., 1979). It has been proposed that free radical metabolites such as •CCl3 formed during the reductive microsomal activation of CCl4 could attack and irreversibly modify the prosthetic group (Fernandez et al., 1982; Guzelian and Swisher, 1979) or the apoprotein (Noguchi et al., 1982a) of cytochrome P450, thereby destroying the P450 isozymes required for xenobiotic activation. Therefore, heme is both the site of and the target for the suicidal activation of CCl4 by P450. It has also been
postulated that the alkylated heme products could bind covalently to the P450 apoprotein and contribute to a loss of the isozymes of P450 (Manno et al., 1988). However, the post-translational reduction of CYP2E1, the major isozyme involved in CCl4 biotransformation, was attributed to the specific destruction of the CYP2E1 protein by the substrate rather than the alkylated heme moiety (Sohn et al., 1991).

In the present work, it was observed that pretreatment of animals with isosafrole completely blocked CCl4-induced destruction of P450 isozymes such as CYP2E1, PROD and CYP1A2, which may contribute to the prevention of CCl4-induced liver necrosis by isosafrole. The inhibitory effects of isosafrole and other dietary MDBs could be attributed to their ability to form stable MI complexes and thereby inactivating various cytochrome P450 isozymes. By studying the selective reactivation of androstenedione hydroxylases following dissociation of the isosafrole MI-P450 complexes, it was concluded that isosafrole forms MI complexes with CYP2B1/2, CYP1A2 and CYP3A but not CYP1A1, CYP2C11 or CYP2A1 (Murray et al., 1986). Isosafrole, safrole and myristicin administered to mice also induced CYP1A2 and CYP2B10 mRNA and protein (Adams et al., 1993) perhaps initiated by the MI complexes. The rapid decrease in the activities of cytochrome P450 isozymes by isosafrole treatment observed in this study suggested that isosafrole was readily absorbed and the formation of isosafrole MI complexes with cytochrome P450 was fast. However, cytochrome P450 isozyme activities subsequently recovered in a time dependent manner in isosafrole-treated mice. This could be attributed to the isosafrole metabolite formation through the MI-P450 complexes and the clearance of the parent compound. In the case of CYP1A2 inhibition by isosafrole, the recovery of this isozyme activity was much slower compared with CYP2E1 and PROD, indicating that
isosafrole metabolic intermediates form more stable complexes with CYP1A2 than with other cytochrome P450 isozymes.

CYP2E1 is involved in the bioactivation of many small hepatotoxic and carcinogenic compounds (Koop, 1992; Coon and Koop, 1987), such as N-dimethylnitrosamine (English and Anders, 1985; Yang et al., 1990), acetaminophen (Anundi et al., 1993; Patten et al., 1993), CCl₄ and CHCl₃ (English and Anders, 1985; Guengerich et al., 1991; Johansson and Ingelman-Sundberg, 1985; Raucy et al., 1993). In this work, treatment of mice with CCl₄ resulted in a complete loss of CYP2E1-mediated p-nitrophenol hydroxylation within 1 hr of administration. Similar observation was also reported by other authors, and was associated with loss of immunochemically detectable CYP2E1 protein from the endoplasmic reticulum membrane and formation of high molecular weight microsomal ubiquitin conjugates (Tierney et al., 1992). The inhibition of CYP2E1 catalytic activity by CCl₄ has been attributed to a post-translational destruction of CYP2E1 protein by its substrate via metabolism to reactive free radicals (McCay et al., 1984; Sohn et al., 1991). DMN also undergoes oxidative demethylation by cytochrome P4502E1 to a reactive methyl diazonium intermediate which was implicated as the primary toxic metabolite in the liver (Kawanishi et al., 1984; Lijinsky et al., 1968; Yang et al., 1985a,b). The protection by isosafrole and other MDBs against CCl₄ and DMN toxicity could therefore be attributed to their inhibitory effects on CYP2E1 activity.

Hepatic CYP2E1 activity in rats is inducible by ketones and alcohols, as well as by fasting and diabetes (Yang et al., 1991). Studies have shown that this alcohol-inducible isozyme is localized preferentially in the centrilobular regions of liver lobules (Forkert et al., 1991). Alcohol administration, usually 16-18 hr prior to the halogenated solvent, has
been demonstrated to potentiate the hepatotoxicity and sometimes the lethality of CCl4, although the extent of potentiation is structure dependent (Cornish and Adefuin, 1967; Ray and Mehendale, 1990). Among various alcohols, isopropanol was very effective at enhancing CCl4-induced hepatotoxicity (Ray and Mehendale, 1990; Traiger and Plaa, 1971; Ueng et al., 1983). The potentiating effect of isopropanol could result from metabolism to acetone in vivo, which induces CYP2E1 activity (Forkert et al., 1991; Yang et al., 1990; 1991). As shown above the administration of isosafrole before CCl4 or CHCl3 completely prevented isopropanol-potentiated liver necrosis, as evidenced by increased plasma AST and ALT levels. Furthermore the CYP2E1 activity of liver microsomes isolated from non-induced and isopropanol-induced mice following isosafrole treatment was markedly inhibited showing that isosafrole is a potent in vivo CYP2E1 inhibitor. Moreover, other hepatoprotective MDBs, such as safrole and dihydrosafrole, were as effective as isosafrole at inhibiting CYP2E1 activity in vitro, whereas PBO, eugenol and isoeugenol, which were not hepatoprotective against CCl4 toxicity, were poor inhibitors of CYP2E1 in vitro and in vivo (in the case of PBO). Although benzodioxole was less effective than isosafrole at inhibiting CYP2E1 activity in vitro, its inhibitory effect was much greater than that of PBO, eugenol and isoeugenol and thus it was more hepatoprotective.

PBO is used extensively as an insecticide synergist and is a well-known cytochrome P450 inhibitor, which prevented hepatotoxicity in mice induced by acetaminophen (Brady et al., 1988) probably by inactivating CYP1A2 or CHCl3 probably by inactivating CYP2B1/2 (Ilett et al., 1973; Kluwe and Hook, 1981). As shown in Chapter 3, PBO pretreatment for 1 or 3 hr, however, failed to prevent CCl4-induced liver
necrosis in vivo, and did not inhibit BrCCl3-induced in vitro hepatocyte cytotoxicity. This was attributed to its ineffectiveness at inhibiting CYP2E1 activity in vivo or in vitro, presumably steric hindrance by the large side chain of PBO prevents it from forming MI complex with CYP2E1 whose substrate specificity is restricted to small molecules. Puccini et al. (1989) also reported that CYP2E1-dependent diethylnitrosamine deethylase activity of acetone-induced rat microsomal preparations required 1 mM PBO for 50% inhibition. Furthermore, PBO neither suppressed the alkylation of nucleic acids or proteins by DMN in mouse liver nor changed the LD50 of DMN (Friedman and Sanders, 1976), although covalent binding of radioactive diethylnitrosamine to total cellular macromolecules of liver was found decreased by PBO treatment by Schuller and McMahon (1985).

The rat phenobarbital-inducible isozyme CYP2B1/2 is also involved in the metabolism of CCl4 to its reactive trichloromethyl radical (CCl3•) (Davies et al., 1985, 1986; Noguchi et al., 1982b), and is susceptible to CCl4-dependent destruction (Gadeholt, 1984; Noguchi et al., 1982b). Isosafrole, safrole and dihydrosafrole were also found to be inhibitory towards phenobarbital-inducible isozyme activities in vitro. Interestingly, PBO also showed significant inhibition comparable to that of isosafrole. Benzodioxole was less effective, whereas eugenol and isoeugenol were not effective at the concentrations used. A time-dependent inactivation of mouse PROD by CCl4 administration was also observed in this work. The destruction of PROD by CCl4 was, however, prevented by isosafrole pretreatment, although isosafrole itself is a potent inhibitor of PROD in vivo.

Another isozyme CYP1A2, unlike CYP1A1, was also reported to be preferentially degraded by CCl4 administration (Sesardic et al., 1989; Degawa et al., 1993), as CYP1A2, but not CYP1A1, is involved in CCl4 metabolism (Masuda and Yasoshima, 1987). The
catalytic activity of CYP1A2-mediated phenacetin O-deethylase was reported to be decreased in hepatic microsomes from CCl₄-treated 3MC-induced rats with concomitant loss of CYP1A2 assessed with an inhibitory monoclonal antibody (Sesardic et al., 1989). In this work, a complete destruction of CYP1A2-dependent methoxyresorufin O-demethylase activity was also observed, and was however prevented if the animals received isosafrole prior to CCl₄. Furthermore, it has been shown that CYP1A2 but not PB-inducible forms of P450 is the major isozyme that catalyzes the metabolic activation of N-hydroxylation of phenetidine to toxic metabolites that initiate oxyhemoglobin oxidation (Nohmi et al., 1987). In this work, phenetidine-induced methemoglobinemia was completely prevented by isosafrole treatment either prior to or after the toxicant, which was consistent with the inhibition of CYP1A2 activity by isosafrole in vivo. It was also observed that isosafrole still significantly suppressed CYP1A2 activity 24 hr later, which could explain the protection by isosafrole when it was given at 200 mg/kg even 24 hr prior to phenetidine administration (Chapter 3). No protection was observed when 50 mg/kg of isosafrole was administered for 24 hr. This could be attributed to the metabolism and clearance of isosafrole, which consequently resulted in lower level of isosafrole MI-P450 complexes and thus less inhibitory effects. Our in vitro studies also showed that isosafrole and PBO significantly inhibited CYP1A2 activity at 1 μM, and were more effective at inhibiting CYP1A1 activity than other MDBs.

The protection against CCl₄, DMN and phenetidine toxicity by isosafrole and other MDBs could, therefore, be mainly attributed to the inhibitory effects of the MDBs on P450 isozymes, in particular CYP2E1 and CYP1A2, that are involved in the generation of reactive metabolic intermediates that may lead to cytotoxicity as well as lipid peroxidation.
(Scheme 4.1). Even though PBO, eugenol and isoeugenol were effective at inhibiting PROD and CYP1A2 activities \textit{in vitro}, they did not prevent CCl₄-induced liver necrosis and were not effective as CYP2E1 inhibitors. This suggests that CYP2E1 rather than PROD or CYP1A2 is the major catalytic isozyme for the reductive bioactivation of CCl₄. Johansson and Ingelman-Sundberg (1985) also reported that reconstituted rabbit CYP2E1 was 100-fold more active at metabolizing CCl₄ than CYP1A2 or CYP2B4.
Scheme 4.1 Proposed Mechanism of Protection against Halomethanes, DMN and Phenetidine Toxicity by Isosafrole Pretreatment.
Chapter 5
THE PREVENTION OF FERRIC NITRILOTRIACETATE TOXICITY BY METHYLENEDIOXYBENZENE ANTIOXIDANTS

Materials in this chapter have been accepted for publication in Chemico-Biological Interactions. Reprinted with permission.

5.1 ABSTRACT

Previously we showed that methylenedioxybenzenes (MDBs), particularly isosafrole, were highly effective at preventing CCl4-induced liver necrosis in vivo (Zhao and O'Brien, 1996), probably as a result of forming metabolic intermediate complexes with cytochrome P450. In the following it is shown that pretreatment of mice with isosafrole also completely prevents ferric nitrilotriacetate (FeNTA)-induced renal necrosis and lipid peroxidation even though metabolic activation by cytochrome P450 is not involved in FeNTA toxicity. The MDBs, such as isosafrole, that prevented CCl4 hepatotoxicity also prevented hepatocyte lipid peroxidation induced by FeNTA, but other cytochrome P450 inhibitors were ineffective. These compounds in decreasing order of antioxidant effectiveness were sesamol, 4-t-butyl-methylenedioxybenzene, isosafrole, piperonyl butoxide, 4-bromo-methylenedioxybenzene and safrole, whereas benzodioxole, 3,4-(methylenedioxy)-toluene and 1,2-(methylenedioxy)-4-nitrobenzene were ineffective. Preincubating the hepatocytes with P450 inhibitors decreased the protective effects of isosafrole, suggesting that the catecholic metabolites of MDBs were responsible for the antioxidant activity. A greater inhibition of FeNTA-induced lipid peroxidation by catecholic...
metabolites of MDBs was observed. Since cytochrome P450 did not participate in FeNTA-induced hepatocyte or microsomal lipid peroxidation, it is likely that the antioxidant properties of MDBs or their catecholic metabolites mainly contribute to their in vivo protection against FeNTA-induced nephrotoxicity.

5.2 INTRODUCTION

The administration of the ferric complex of nitrilotriacetic acid to animals has been used as an experimental model for iron overload or idiopathic hemochromatosis as iron is readily deposited in the parenchymal cells of the liver, kidney, pancreas and adrenal glands. Ferric nitrilotriacetate (FeNTA) was found to be most effective among the iron chelates investigated at generating reactive oxygen species and initiating lipid peroxidation in vitro, which causes the accumulation of thiobarbituric acid-reactive substances (TBARS), DNA strand breakage, and oxidative DNA base modification in the liver and kidney (Goddard and Sweeney, 1983; Hamazaki et al., 1985; 1989; Umemura et al., 1990; Preece et al., 1988; 1989; Aruoma et al., 1989). This results in FeNTA-mediated hepatocellular injury, diabetes, proximal tubular necrosis and renal adenocarcinomas.

Methylenedioxybenzenes (MDBs), such as safrole, isosafrole, sesamol and myristicin, occur in nature and are constituents of human food, essential oils and flavors. Methylenedioxybenzenes especially the synthetic MDB piperonyl butoxide are well known potential inhibitors of cytochrome P450-mediated monooxygenases (Franklin, 1972; Hodgson and Philpot, 1974; Wilkinson et al., 1984). We reported recently that naturally occurring MDBs, particularly isosafrole, at very low dosages fully prevented carbon tetrachloride induced centrilobular necrosis in mice in vivo and bromotrichloromethane-
induced hepatocyte toxicity in vitro (Zhao and O'Brien, 1996). Isosafrole and safrole also prevented the in vivo hepatotoxicity of N-dimethylnitrosamine, and methemoglobinemia induced by phenetidine (Chapter 3). This protection by MDB pretreatment was attributed to the formation of stable inhibitory metabolic intermediate-ferrocytochrome P450 complexes, which inactivated the P450 isozymes involved in the toxicant bioactivation and thereby prevented the formation of toxic metabolic intermediates of the toxicants (Chapter 4).

MDBs are extensively metabolized by the microsomal mixed-function oxygenase system both in vivo and in vitro. It has been shown that the major metabolic pathway with MDBs is the demethylation of the benzodioxole moiety to a catechol (Casida et al., 1966; Kamienski and Casida, 1970; Klungsøyr and Scheline, 1982; 1983; Wilkinson, 1967). Derivatives of catechol are potential antioxidants and metal chelators (Sies, 1991; Zhao et al., 1997). Therefore, we hypothesized that the MDBs may also possess antioxidant activities in addition to their inhibitory effects on P450. In the following, the administration of isosafrole was found to prevent reactive oxygen species-involved FeNTA-induced kidney damage in mice. Moreover, administration of isosafrole and some other MDBs prevented hepatocyte and liver microsomal lipid peroxidation induced by FeNTA, even though other cytochrome P450 inhibitors were ineffective. This supports our hypothesis that the chemoprotective properties of isosafrole and other MDBs may involve their antioxidant properties as well.
5.3 RESULTS

5.3.1 Prevention of FeNTA-Induced Lipid Peroxidation in vitro by MDBs

Incubation of FeNTA with isolated rat hepatocytes generated marked lipid peroxidation as shown by the massive formation of TBARS. After a 30 min incubation, the amount of TBARS formed was increased by more than 10-fold that of control hepatocytes (Table 5.1). However, preincubation of the hepatocytes with methylenedioxybenzenes significantly inhibited TBARS production. The order of inhibitory effectiveness found was sesamol > 4-tert-butyl-1,2-(methylenedioxybenzene) (ButylMDB), isosafrole, piperonyl butoxide (PBO) > 4-bromo-1,2-(methylenedioxybenzene) (BrMDB). Isosafrole inhibited lipid peroxidation by ca. 50% at 30 min incubation of FeNTA. Other MDBs, such as safrole, benzodioxole, 3,4-(methylenedioxy)-toluene (MeMDB) and 1,2-(methylenedioxy)-4-nitrobenzene (NitroMDB) were ineffective at this concentration (50 μM). Safrole inhibited hepatocyte lipid peroxidation by 40% at 100 μM. The cytochrome P450 inhibitors metyrapone and 1-benzylimidazole did not prevent lipid peroxidation. Similar results were obtained with rat liver microsomal lipid peroxidation induced by FeNTA in the presence of ascorbic acid. As shown in Table 5.1, the order of prevention of TBARS formation obtained was sesamol, methylenedioxyaniline (AminoMDB) > isosafrole > PBO > ButylMDB, whereas safrole, benzodioxole, BrMDB, MeMDB and NitroMDB were ineffective. In the case of isosafrole, microsomal lipid peroxidation was inhibited by ca. 80%.

89
<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatocytes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(nmol/10&lt;sup&gt;6&lt;/sup&gt; cells/30 min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>FeNTA</td>
<td>3.41 ± 0.4</td>
</tr>
<tr>
<td>+ Sesamol</td>
<td>0.80 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ AminoMDB</td>
<td>—</td>
</tr>
<tr>
<td>+ Isosafrole</td>
<td>1.77 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ PBO</td>
<td>1.90 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ ButylMDB</td>
<td>1.62 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ BrMDB</td>
<td>2.19 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Safrole</td>
<td>3.41 ± 0.2</td>
</tr>
<tr>
<td>+ MeMDB</td>
<td>2.89 ± 0.2</td>
</tr>
<tr>
<td>+ Benzodioxole</td>
<td>3.24 ± 0.2</td>
</tr>
<tr>
<td>+ NitroMDB</td>
<td>4.01 ± 0.4</td>
</tr>
<tr>
<td>+ Safrole (100 µM)</td>
<td>2.18 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Metyrapone</td>
<td>3.32 ± 0.5</td>
</tr>
<tr>
<td>+ Benzylimidazole</td>
<td>3.14 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hepatocytes were preincubated with MDBs (50 µM), metyrapone (1 mM), or 1-benzylimidazole (100 µM) for 15 min before FeNTA (120 µM) was added. Thirty minutes later, samples were taken for TBARS analysis. Results are means ± SEM for 3 separate experiments.

<sup>b</sup> FeNTA (25 µM), MDBs (50 µM) and ascorbic acid (0.5 mM) were added to the liver microsomes in this order. Samples for lipid peroxidation measurements were taken 20 min after ascorbic acid addition. Results are means ± SEM for 3 separate experiments.

<sup>c</sup> Significantly different from samples treated with FeNTA only (P < 0.05).

<sup>d</sup> Significantly different from samples treated with FeNTA and ascorbic acid (P < 0.05).
5.3.2 Prevention of FeNTA-Induced Lipid Peroxidation by Catechols

Catecholic metabolites of MDBs formed by cytochrome P450-catalyzed oxidative demethylenation were proposed to be responsible for the antioxidant properties of MDBs. The effects of these catechol derivatives on FeNTA-induced lipid peroxidation in hepatocytes were therefore studied. As shown in Figure 5.1, the decreasing order of effectiveness at preventing hepatocyte lipid peroxidation was 4-t-butylcatechol (a ButylMDB metabolite), hydroxychavicol (HC, a safrole metabolite), catechol (a benzodioxole metabolite), 4-methylcatechol (a MeMDB metabolite) and caffeic acid, at 2 hr after FeNTA addition. No significant difference in the effectiveness of protection was observed among the catechols at short time of incubation and at the concentration used. The safrole metabolite hydroxychavicol exerted 51%, 73%, and 84% inhibition of TBARS formation at 15, 30 and 60 min of FeNTA incubation, respectively. 4-Nitrocatechol (a Nitro-oMDB metabolite) and guaiacol (a phenolic antioxidant) were, however, ineffective at 50 μM, giving TBARS content of 9.28±0.63 and 9.05±0.56 nmol/10^6 cells at 2 hr of FeNTA addition respectively.

Lower concentrations (25 μM) of hydroxychavicol and iso-hydroxychavicol (Iso-HC, an isosafrole metabolite) were also tested. As shown in Figure 5.2, hydroxychavicol was still very effective at preventing FeNTA-induced hepatocyte lipid peroxidation at the lower concentration. Compared with the safrole metabolite HC, the isosafrole metabolite iso-HC was even more effective. After 2 hr of FeNTA incubation, iso-HC demonstrated ca. 60% inhibition of TBARS formation, whereas HC at 25 μM showed ca. 36% inhibition. However, the parent catechol was not effective at the concentration applied (Figure 5.2).
Figure 5.1 Inhibition of FeNTA-Induced Hepatocyte Lipid Peroxidation by Catecholic Metabolites. Hepatocytes (10^6 cells/ml) suspended in Krebs-Henseleit buffer (pH 7.4) were incubated with FeNTA (120 μM) and catechol derivatives (50 μM) at 37 °C under an atmosphere of 20% O₂, 5% CO₂ and 75% N₂. Data points were the means ± SEM of results obtained from three separate experiments.
Figure 5.2*  Inhibition of FeNTA-Induced Hepatocyte Lipid Peroxidation by Lower Concentration of Catechols. Hepatocytes (10^6 cells/ml) suspended in Krebs-Henseleit buffer (pH 7.4) were incubated with FeNTA (120 μM) and catechol derivatives (25 μM) at 37 °C under an atmosphere of 20% O₂, 5% CO₂ and 75% N₂. Data points were the means ± SEM of results obtained from three separate experiments.

* Not included in the paper.
5.3.3 Effects of P450 Inhibitors on the Antioxidative Property of Isosafrole

The important role of MDB metabolism in the protection against FeNTA-induced TBARS formation was further confirmed when hepatocytes were pre-incubated with 1-phenylimidazole or other P450 inhibitors to block the P450 catalyzed O-demethylation of MDBs to catecholic metabolites. As shown in Figure 5.3, ca. 65% inhibition of TBARS formation by isosafrole was observed at 60 min in the absence of 1-phenylimidazole. However, only ca. 20% inhibition of TBARS formation by isosafrole was observed if hepatocytes were preincubated with the P450 inhibitor 1-phenylimidazole. Addition of 1-phenylimidazole alone did not affect FeNTA-induced lipid peroxidation. Similarly, addition of other P450 inhibitors such as aminobenzotriazole (100 μM), 1-benzylimidazole (100 μM), metyrapone (1 mM) or isopropanol (2 mM) also decreased the protective effects of isosafrole.

5.3.4 Prevention of FeNTA Nephrotoxicity by Isosafrole Pretreatment

The protective and antioxidant properties of MDBs against FeNTA toxicity were further studied in vivo. The administration of FeNTA to mice caused severe kidney damage, as demonstrated by a three fold increase in plasma urea nitrogen (PUN) levels compared with control mice (Table 5.2). Both kidneys of the treated animals were discolored and slightly larger in size, with noticeable abnormality of the structure. Pretreatment of the mice with 200 or 50 mg/kg isosafrole 18 hr or 3 hr before the toxicant resulted in complete protection against FeNTA induced nephrotoxicity, as shown by the inhibition of FeNTA-induced PUN level elevation. The kidneys of isosafrole-pretreated
Figure 5.3 Decrease in the Antioxidant Activity of Isosafrole by Inactivating Hepatocyte Cytochrome P450. Hepatocytes were pre-incubated for 30 min with 1-phenylimidazole (Phen, 300 μM) to inactivate cytochrome P450 prior to the addition of isosafrole (50 μM) and/or FeNTA (100 μM). Data points were the means ± SEM of results obtained from three separate experiments.
Table 5.2 Prevention of FeNTA-Induced Kidney Necrosis and Lipid Peroxidation in Mice by Isosafrole Pretreatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PUN (mg/dl)</th>
<th>TBARS formed (nmol/mg homogenate/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.4 ± 3.7</td>
<td>1.54 ± 0.12</td>
</tr>
<tr>
<td>NTA</td>
<td>23.1 ± 3.0</td>
<td>—</td>
</tr>
<tr>
<td>Isosafrole&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.5 ± 3.0</td>
<td>1.36 ± 0.08</td>
</tr>
<tr>
<td>FeNTA</td>
<td>58.2 ± 10.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.97 ± 0.32&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isosafrole (&lt;span&gt;-18±2h&lt;/span&gt;)&lt;sup&gt;a&lt;/sup&gt; + FeNTA</td>
<td>20.1 ± 2.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.25 ± 0.19&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isosafrole (&lt;span&gt;-3±0.5h&lt;/span&gt;)&lt;sup&gt;a&lt;/sup&gt; + FeNTA</td>
<td>18.8 ± 1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Isosafrole (&lt;span&gt;-18±2h&lt;/span&gt;)&lt;sup&gt;b&lt;/sup&gt; + FeNTA</td>
<td>22.5 ± 3.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Acetone&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.5 ± 4.0</td>
<td>—</td>
</tr>
<tr>
<td>Acetone&lt;sup&gt;c&lt;/sup&gt; + FeNTA</td>
<td>65.5 ± 8.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>

Isosafrole was given ip at 3 or 18 hr before the mice received FeNTA (2.6 mg Fe(III)/kg, ip). Isosafrole- or NTA- treated mice received the same dosages. Control mice received the equivalent amount of vehicles only. Mice were sacrificed 24±2 hr after the FeNTA was administered and PUN activity was measured. Kidney homogenates (25% w/v) were also prepared 24±2 hr after FeNTA injection and the amount of TBARS formed was determined. Results are means ± SEM for 6 mice (or more for FeNTA treated alone).

-<sup>a</sup> Isosafrole was administered at a dosage of 200 mg/kg.

-<sup>b</sup> Isosafrole was administered at a dosage of 50 mg/kg.

-<sup>c</sup> Mice were given acetone (5 ml/kg, po) for two consecutive days before further treatment.

-<sup>d</sup> Significantly different from control (P<0.05).

-<sup>e</sup> Significantly different from FeNTA group in different sample preparations (P<0.05).
mice were essentially normal. Treatment with nitrilotriacetic acid or isosafrole alone at the same dosages did not show any renal toxicity. Also as shown in Table 5.2, compared with control, a six fold increase in TBARS formed in kidney homogenates was observed 24 hr after FeNTA administration. Pretreating the mice with isosafrole inhibited FeNTA-elevated TBARS production by 55%, although the amount of TBARS formed was still 276% of that of control. Moreover, it was observed that pretreatment of the mice with acetone to induce kidney cytochrome P4502E1 (CYP2E1) (Longo and Ingelman-Sundberg, 1993) did not affect the susceptibility of the mice to FeNTA. This suggested that kidney CYP2E1 did not contribute to FeNTA-induced nephrotoxicity.

5.4 DISCUSSION

Nitrilotriacetic acid (NTA) is a synthetic chelating agent that has been used as a substitute for polyphosphates in detergents for household and hospital use in various countries. The uncomplexed NTA had a low toxicity in experimental animals (Hamazaki et al., 1985; Okada et al., 1987). Its iron complex, ferric nitrilotriacetate (FeNTA), was, however, found to be the most effective autoxidizing agent among several different iron chelates, and was the only one that initiated lipid peroxidation in linoleate micelles, lipid lysosomes and rat liver microsomes (Hamazaki et al., 1985). FeNTA is capable of generating superoxide radicals that subsequently potentiate the iron-catalyzed Haber-Weiss reaction to produce hydroxyl radicals, the prime initiator of lipid peroxidation (Aruoma et al., 1989; Umemura et al., 1990). FeNTA has also been shown to be more efficient than all potential biological chelators of iron at converting superoxide radical to the hydroxyl radical (Preece et al., 1988). Antioxidants vitamin E and butylated hydroxyanisole as well as the
iron chelator desferrioxamine have been reported to prevent the toxic effects of FeNTA (Goddard et al., 1986; Okada et al., 1987). Now we are reporting for the first time that some methylenedioxybenzenes, such as isosafrole, piperonyl butoxide, 4-t-butyl-methylenedioxybenzene and 4-bromo-methylenedioxybenzene as well as their catecholic metabolites prevented FeNTA-stimulated lipid peroxidation in rat hepatocytes and liver microsomes.

We have also found that pretreatment of mice with isosafrole, a naturally occurring MDB, markedly prevented FeNTA-induced kidney damage and in vivo renal lipid peroxidation. Kidney is the organ most affected by FeNTA, as demonstrated by the elevation of lipid peroxidation and irreversible morphological changes, resulting in renal cell carcinoma (Okada et al., 1987; Preece et al., 1988). The iron-promoted production of free radicals leading to lipid peroxidation has been proposed as a major cause of FeNTA-induced renal toxicity. The low molecular weight FeNTA is easily filtered through the glomeruli and excreted into the lumen of the renal proximal tubules, where it initially acts on the brush border membrane from the luminal side, and initiates peroxidation of the membrane lipids (Hamazaki et al., 1985; 1989; Okada et al., 1987; Preece et al., 1988; Umemura et al., 1990). Administration of the iron chelating agent desferrioxamine decreased FeNTA-induced ethane exhalation and malondialdehyde production in kidney (Preece et al., 1990).

The protection against FeNTA toxicity by some MDBs both in vivo and in vitro suggests that they could act as potent antioxidants. In this work, it was shown that safrole, 4-t-butyl-1,2-(methylenedioxybenzene) and 3,4-(methylenedioxy)-toluene were much less effective at inhibiting FeNTA-initiated TBARS formation in rat hepatocyte incubation than
their respective catecholic metabolites hydroxychavicol, 4-t-butylicatechol and 4-methylcatechol. Moreover, isosafrole was less inhibitory if hepatocytes were pre-incubated with P450 inhibitors. As a result, the antioxidant activities could be mainly attributed to the catecholic metabolites formed by the action of cytochrome P450, as catecholic compounds are phenolic antioxidants that inhibit radical formation through chain reactions, and are strong iron chelators (Sies, 1991; Zhao et al., 1997). Demethylation of the MDB moiety by hepatic cytochrome P450 dependent mixed function oxidases to the corresponding O-dihydroxybenzene derivatives (i.e., catecholic metabolites) accounts for 73-80% of MDB metabolism with side chain oxidation as a minor metabolic pathway (Anders et al., 1984; Casida et al., 1966; Kamienski et al., 1970; Klungsøyr and Scheline, 1982; 1983; Wilkinson, 1967). We have also observed that 1,2-(methylenedioxy)-4-nitrobenzene or its metabolite 4-nitrocatechol were ineffective at inhibiting FeNTA-stimulated lipid peroxidation, indicating that the antioxidant property of MDBs depends on the side chain substituted to the benzene ring as well. The correlation between the redox potential (Horner and Geyer, 1965), \( E^o \) (V vs. NHE), and the corresponding antioxidant activity expressed as the percentage of inhibition of FeNTA-induced TBARS formation by various catechols is shown in Figure 5.4. A good linear correlation with an \( r^2 \) (r: correlation coefficient) value of 0.956 was obtained, indicating that the inhibitory capacity correlated with the redox potential of the catechols. The antioxidative property of catechols in the presence of iron has been further studied and discussed in Chapter 6.

It has been demonstrated that hydroxyl radicals generated from two different systems are capable of abstracting a hydrogen atom from the methylene-bridge carbon to
Figure 5.4 Correlation between the Redox Potential and the Antioxidant Activity of Catechols. Redox potentials of various catechols were obtained from Horner and Geyer (1965), except for hydroxychavicol which was calculated according to this reference. The antioxidant activity of catechols was expressed as percentage of inhibition of FeNTA-induced TBARS formation by catechols at 30 min of hepatocyte incubation. BC: 4-t-butylcatechol; C: catechol; CA: caffeic acid; HC: hydroxychavicol; MC: 4-methylcatechol; NC: 4-nitrocatechol.
form a methylenedioxybenzene radical prior to catechol formation (Kumagai et al., 1991). In our work, it was observed that isosafrole, PBO and 4-t-butyl-methylenedioxybenzene significantly blocked FeNTA/ascorbic acid-induced lipid peroxidation with rat liver microsomes in the absence of NADPH, suggesting that these MDBs may react directly with hydroxyl radicals in a NADPH-independent pathway and form catecholic metabolites that are better antioxidants. However, the reaction between MDBs and hydroxyl radicals unlikely takes place in vivo or at low concentrations of the hydroxyl radicals because hydroxyl radicals are so reactive that they may react with the environment (for example, proteins) before reacting with MDBs.

The prevention of FeNTA toxicity by MDBs could suggest that cytochrome P450 may contribute to the FeNTA-induced nephrotoxicity. MDBs are well accepted as inhibitors of cytochrome P450-dependent xenobiotic metabolism. Our previous studies showed the prevention of CCl4-induced liver injury in mice as well as BrCCl3-induced rat hepatocyte injury in vitro by naturally occurring MDBs, which could be attributed to their ability to form metabolic intermediate complexes with the ferrous form of cytochrome P450, particularly cytochrome P4502E1 (CYP2E1) (Zhao and O'Brien, 1996). Kukielka and Cederbaum (1996) observed a potentiation of ferritin-stimulated lipid peroxidation by CYP2E1-induced microsomes after chronic ethanol treatment, and thereby proposed that CYP2E1-derived superoxide radical may play a role in the development of oxidative stress and thus iron-involved lipid peroxidation. Others have speculated that reduced cytochrome P450 could contribute to the reductive activation of ADP-Fe3+ in microsomal lipid peroxidation (Sevanian et al., 1990). However, Tampo and Yonaha (1995) suggested that it was a heat-labile microsomal membrane component but not cytochrome P450 that was
associated with iron-mediated lipid peroxidation in microsomes. In support of this we have shown that cytochrome P450 inhibitors, including CYP2E1 inhibitors other than MDBs, did not inhibit FeNTA-induced microsomal or hepatocyte lipid peroxidation. Furthermore, the in vivo induction of liver and kidney CYP2E1 in mice with acetone did not increase their susceptibility to FeNTA-induced kidney damage.

The above studies, therefore, showed that MDBs, and especially their catecholic metabolites, prevented FeNTA-induced renal necrosis as well as in vitro hepatocyte lipid peroxidation, probably as a result of the antioxidant properties of the MDBs or their catecholic metabolites. Naturally occurring MDBs could prove therapeutically useful in preventing iron overload toxicity, for example, hemochromatosis.
Chapter 6

CATECHOLIC IRON COMPLEXES AS CYTOPROTECTIVE SUPEROXIDE DISMUTASE MIMICS

Materials in this chapter have been submitted for publication. Dr. Sumsullah Khan contributed to the hypoxia experiments.

6.1 ABSTRACT

Reactive oxygen species (ROS) including superoxide radicals have been implicated in the pathogenesis of radiotherapy, ischemia-reperfusion injury, aging and inflammatory diseases. In the present work, it was found that 2:1 catecholic iron complexes were not only much more effective than uncomplexed catechols at protecting hepatocytes against hypoxic cell injury but also significantly decreased the level of ROS formed before cytotoxicity ensued. These catecholic iron complexes were also much more effective than uncomplexed catechols at scavenging superoxide radicals generated both enzymatically and non-enzymatically. The superoxide dismutase activity of catecholic iron complexes seemed to be correlated with the redox potential of the catechols. High redox potential and low pKa catechols, such as tiron, which form 3:1 complexes with iron at physiological pH were much less effective at scavenging superoxide radicals and preventing hypoxia cytotoxicity.

6.2 INTRODUCTION

The superoxide radical (\( O_2^- \)), generated by a one-electron reduction of molecular oxygen both enzymatically and nonenzymatically, plays a deleterious role in the pathogenesis of ischemia-reperfusion injury, aging, cancer and other metabolic,
degenerative, and inflammatory diseases (Kuzuya et al., 1990; Poston and Parenteau, 1992; Zweier, 1988). The pharmacological administration of superoxide dismutase (SOD) or cell permeable SOD mimics has been proposed as a therapy to alleviate the damage mediated by superoxide or superoxide-derived reactive radicals via removing excessively generated $O_2^*$ (Michelson, 1982). Various low-molecular-weight SOD-like complexes of transition metals especially Mn and Cu have been reported to have SOD mimic activity (Archibald and Fridovich, 1982; Benov and Fridovich, 1996; Darr et al., 1988; Iuliano et al., 1992; Wada et al., 1994a, 1994b; Weiss et al., 1993). The manganic porphyrin or manganic desferrioxamine have recently been shown to prevent cell growth inhibition caused by superoxide radical-generating agents (Gardner et al., 1996; Hahn et al., 1991). A few iron complexes have also been suggested to mimic SOD by some authors (Minotti and Aust, 1987; Nagano et al., 1989).

At a physiological pH, catechol (i.e., o-dihydroxybenzene) readily forms a thermodynamically stable complex with ferric iron as a bidentate ligand. The complex has been prepared in alkaline solution, crystallized from organic solvents and demonstrated by X-ray analysis to be an octahedral chelate tris(catecholato)ferrate(III) (Raymond et al., 1976). Catecholic compounds are also synthesized and utilized as siderophores, e.g., enterobactin, by micro-organisms to sequester and transport iron from the external environment into the organism in response to an iron deficiency (Avdeef et al., 1978; Neilands, 1973). These catechol-type chelators have large stability constants for ferric iron ($\log K_f \geq 40$) and low reduction potentials (Cooper et al., 1978; O'Brien et al., 1971).

Previously we showed that hepatocyte cytotoxicity induced by hypoxia or respiratory inhibitors could be attributed to reductive stress which resulted in intracellular
iron release and cytotoxic oxygen activation. The cytotoxicity and oxygen activation under hypoxia were prevented by catecholic antioxidants presumably as a result of their superoxide radical scavenging activity (Khan and O'Brien, 1995; Niknahad et al., 1995).

In the present work, we have found that the catecholic iron chelates were highly potent as SOD mimics at inhibiting nitro blue tetrazolium reduction in both enzymatic and nonenzymatic superoxide-generating assay systems, and were remarkably more effective than uncomplexed catechols at preventing hypoxic hepatocyte injury caused by reactive oxygen species. The chemical structures of the catecholic compounds studied in this Chapter are listed in Table 6.1, and the known first acid dissociation constants and formal reduction potentials of some catechols are listed in Table 6.2.

6.3 Results

6.3.1 Protection against ROS-Mediated Hypoxic Hepatocyte Injury

Previously we showed that maintaining hepatocytes under nitrogen containing 0.1% O2 resulted in hepatocyte cell death, which was prevented by addition of oxypurinol, a xanthine oxidase inhibitor, or quercetin, a catecholic flavonoid (Khan and O'Brien, 1995; Niknahad et al., 1995). The formation of reactive oxygen species (ROS) as determined by luminol chemiluminescence was also prevented (Niknahad et al., 1995). Similarly, hepatocyte toxicity obtained when hepatocytes under aerobic conditions were treated with respiratory inhibitors was also prevented by oxypurinol or quercetin (Khan and O'Brien, 1995; Niknahad et al., 1995). In the present work, as shown in Table 6.3, iron complexes of the catecholic compounds caffeic acid, protocatechuic acid, 4-t-butylcatechol, catechin and quercetin significantly protected hepatocytes against ROS-mediated hypoxic cell injury,
**Table 6.1** Chemical Structures of Catechols and Related Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R</th>
<th>Catechols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>—H</td>
<td>3-Methoxycatechol</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>—CH₃</td>
<td>Tiron</td>
</tr>
<tr>
<td>4-t-Butylcatechol</td>
<td>—C(CH₃)₃</td>
<td>Hydroxychavicol</td>
</tr>
<tr>
<td>Hydroxychavicol</td>
<td>—CH₂CH=CH₂</td>
<td>Iso-hydroxylchavicol</td>
</tr>
<tr>
<td>Iso-hydroxylchavicol</td>
<td>—CH=CHCH₃</td>
<td>Caffeic acid</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>—CH=CHCOOH</td>
<td>Protocatechuic acid</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>—COOH</td>
<td>4-Nitrocatechol</td>
</tr>
<tr>
<td>4-Nitrocatechol</td>
<td>—NO₂</td>
<td></td>
</tr>
</tbody>
</table>

**Others**

- Resorcinol
- Hydroquinone

**Flavonoids**

- Catechin
- Epicatechin
- Quercetin

*Not included in the manuscript*
Table 6.2*  First Acid Dissociation Constants and Formal Reduction Potentials of Catecholic Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>pKα₁</th>
<th>E° (V vs. NHE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylcatechol</td>
<td>9.56&lt;sup&gt;a&lt;/sup&gt;; 9.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.753&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-t-Butylcatechol</td>
<td>9.53&lt;sup&gt;a&lt;/sup&gt;; 9.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.732&lt;sup&gt;d&lt;/sup&gt;; 0.739&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catechol</td>
<td>9.37&lt;sup&gt;a,c&lt;/sup&gt;; 9.22 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.795&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-Methoxycatechol</td>
<td>9.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.741&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>9.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.793&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protocatechuic Acid</td>
<td>8.82&lt;sup&gt;a&lt;/sup&gt;; 8.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.833&lt;sup&gt;d&lt;/sup&gt;; 0.883&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tiron</td>
<td>7.70 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.955&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-Nitrocatechol</td>
<td>6.84&lt;sup&gt;a&lt;/sup&gt;; 6.65 ± 0.01&lt;sup&gt;b&lt;/sup&gt;; 6.78&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.891&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tetrachlorocatechol</td>
<td>5.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.830&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>9.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.700&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the constants are cited from<sup>a</sup> Slabbert, 1977; <sup>b</sup> Avdeef <i>et al.</i>, 1978; <sup>c</sup> Saleem and Wilson, 1982; <sup>d</sup> Horner and Geyer, 1965; <sup>e</sup> Mentasti <i>et al.</i>, 1976.

* Not included in the manuscript.
Table 6.3 Prevention of Hypoxia Cytotoxicity in Hepatocytes by Iron Chelates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity (% of Trypan blue uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>+ Caffeic acid (150 μM)</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>+ Caffeic acid (20 μM)</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>+ Caffeic acid-Fe$^{3+}$ (20 μM:10 μM)</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>+ Protocatechuic acid (20 μM)</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>+ Protocatechuic acid-Fe$^{3+}$ (20 μM:10 μM)</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>+ 4-t-Butylcatechol (20 μM)</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>+ 4-t-Butylcatechol-Fe$^{3+}$ (20 μM:10 μM)</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>+ Catechin (20 μM)</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>+ Catechin-Fe$^{3+}$ (20 μM:10 μM)</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>+ Quercetin (20 μM)</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>+ Quercetin-Fe$^{3+}$ (20 μM:10 μM)</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>+ Tiron (30 μM)</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>+ Tiron-Fe$^{3+}$ (30 μM:10 μM)</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>+ Fe$^{3+}$ (10 μM)</td>
<td>47 ± 6</td>
</tr>
</tbody>
</table>

Rat hepatocytes (10⁶ cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) under an atmosphere of 95% N₂, 0.1% O₂ and 5% CO₂. Samples were taken at times of interest for viability assessment, which was determined as the percentage of cells taking up trypan blue. Results are means of three separate experiments (± SEM).
although the uncomplexed catechols were not hepatoprotective at the concentrations applied. Much higher concentration of uncomplexed caffeic acid was required to give the same degree of protection as the ferric complex of caffeic acid. By contrast, the tiron-Fe complex and uncomplexed tiron were ineffective at the concentrations used (Table 6.3). Furthermore, as shown in Figure 6.1, the iron complexes of the catecholic compounds caffeic acid and 4-\(t\)-butylcatechol were more effective than uncomplexed catechols at decreasing the levels of ROS formed under hypoxia as determined by dichlorofluorescin fluorescence.

6.3.2 Inhibition of NBT Reduction in PMS/NADH Mediated Superoxide Generating System

As shown in Table 6.4, the catecholic ferric complexes were far better inhibitors of 
\(O_2^\cdot\) -mediated nitro blue tetrazolium (NBT) reduction than their corresponding uncomplexed catechols in the phenazine methosulfate (PMS)/NADH-mediated superoxide generating system. Catecholic iron chelates had IC50 values 23 fold lower for the catechol-Fe complex than that for catechol, up to 118 fold lower for the caffeic acid-Fe complex than that for caffeic acid. Iron chelation also decreased the IC50 values of the flavonoids by 25-, 10-, and 14- fold for epicatechin, catechin and quercetin, respectively. The order of SOD mimic activity for the catecholic iron complexes was caffeic acid > epicatechin > 3-methoxycatechol, 4-\(t\)-butylcatechol, 4-methylcatechol, protocatechuic acid, catechin > catechol > quercetin. The order of superoxide scavenging activity for uncomplexed catechols was catechin, epicatechin > 3-methoxycatechol, 4-methylcatechol, 4-\(t\)-butylcatechol > quercetin > catechol >> protocatechuic acid.
Figure 6.1 Inhibition of ROS-Mediated Dichlorofluorescin Fluorescence by Catecholic Iron Complexes. Rat hepatocytes (10⁶ cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) with 2',7'-dichlorofluorescin diacetate (8 μM) and catecholic iron complexes (20 μM catechols : 10 μM Fe³⁺) under an atmosphere of 95% N₂, 0.1% O₂ and 5% CO₂. Hepatocytes incubated with 2',7'-dichlorofluorescin diacetate only were used as control. Samples were taken at times of interest for fluorescent intensity measurement (λₓ = 500 nm, λₑm = 520 nm). Results are means (± SEM) of three separate experiments.
Table 6.4  Inhibition of Phenazine Methosulfate/NADH Mediated Nitro Blue Tetrazolium Reduction

<table>
<thead>
<tr>
<th>Fe³⁺ Chelates</th>
<th>IC₅₀ (µM)</th>
<th>Catechols</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic Acid-Fe</td>
<td>1.15</td>
<td>Caffeic Acid</td>
<td>136.3</td>
</tr>
<tr>
<td>3-Methoxycatechol-Fe</td>
<td>3.45</td>
<td>3-Methoxycatechol</td>
<td>107.9</td>
</tr>
<tr>
<td>4-t-Butylcatechol-Fe</td>
<td>3.53</td>
<td>4-t-Butylcatechol</td>
<td>129.4</td>
</tr>
<tr>
<td>4-Methylcatechol-Fe</td>
<td>3.65</td>
<td>4-Methylcatechol</td>
<td>127.5</td>
</tr>
<tr>
<td>Protocatechuic Acid-Fe</td>
<td>4.15</td>
<td>Protocatechuic Acid</td>
<td>444.4</td>
</tr>
<tr>
<td>Catechol-Fe</td>
<td>7.72</td>
<td>Catechol</td>
<td>184.2</td>
</tr>
<tr>
<td>Tiron-Fe (3:1)</td>
<td>28.5</td>
<td>Tiron</td>
<td>588.4</td>
</tr>
<tr>
<td>Tetrachlorocatechol-Fe(3:1)</td>
<td>&gt; 37.5*</td>
<td>Tetrachlorocatechol</td>
<td>&gt; 150*</td>
</tr>
<tr>
<td>4-Nitrocatechol-Fe (3:1)</td>
<td>&gt; 37.5*</td>
<td>4-Nitrocatechol</td>
<td>&gt; 150*</td>
</tr>
<tr>
<td>Epicatechin-Fe</td>
<td>2.26</td>
<td>Epicatechin</td>
<td>56.4</td>
</tr>
<tr>
<td>Catechin-Fe</td>
<td>4.50</td>
<td>Catechin</td>
<td>47.6</td>
</tr>
<tr>
<td>Quercetin-Fe</td>
<td>9.94</td>
<td>Quercetin</td>
<td>141.6</td>
</tr>
</tbody>
</table>

The reaction mixture contained 10 µM phenazine methosulfate (PMS), 78 µM NADH, 25 µM nitro blue tetrazolium (NBT), and various concentrations of iron complexes in 0.1 M Tris buffer (pH 7.4). PMS was added last to initiate the reaction. The absorbance at 560 nm was recorded against blank samples containing no PMS. IC₅₀ values were calculated from regression lines where: \( x \) was log of the concentration of the tested catechol and \( y \) was the percentage of the initial rate of NBT reduction in the presence of catecholic compounds over that of the control. Seven different concentrations were used for the calculation of IC₅₀ of each compound.

* Precipitation in the reaction systems at higher concentrations of these catechols interfered with the assay.
It was also observed that the ferrous complex of caffeic acid had a similar SOD-mimic activity as caffeic acid-Fe$^{3+}$, giving an IC50 value of 1.21 μM. Iron complexes of hydroxychavicol and iso-hydroxychavicol (the metabolites of safrole and isosafrole respectively) were also potent SOD mimics with IC50 values of 2.74 μM and 2.07 μM respectively, whereas the IC50 values of these two uncomplexed catechols were 129.6 μM and 115.4 μM respectively. A 50% inhibition of NBT reduction was obtained using 1.35 units/ml of Cu,Zn-SOD. Free ferric ion at the concentrations used above (1-10 μM) had no SOD mimic activity. The maximum inhibition of NBT reduction by superoxide dismutase (100 units/ml) was 82%, which was not further increased by the catecholic iron complexes, indicating that NBT radical intermediates were not re-oxidized by the complexes. The cytochrome c assay method for determining superoxide dismutase activity (Benov and Fridovich, 1996) could not be used as cytochrome c was observed to be reduced by the catecholic iron complexes.

At a physiological pH most of the catechols form 2:1 complexes with ferric ions, whereas catechols with low pKa values (Table 6.2) such as tiron, 4-nitrocatechol and tetrachlorocatechol form 3:1 complexes with ferric ion (Avdeef et al., 1978; Kawabata et al., 1996; Krishna et al., 1992). As shown in Table 6.4, the tiron-Fe, 4-nitrocatechol-Fe and tetrachlorocatechol-Fe complexes were much less effective as SOD mimics than other catecholic ferric complexes. For example, the tiron-Fe complex had an IC50 value ca. 25-fold less than that of caffeic acid-Fe. It however was still more effective than uncomplexed tiron with an increase of 20-fold in SOD mimic activity. Due to solubility problem at higher concentrations, the IC50 concentrations of the 4-nitrocatechol-Fe and tetrachlorocatechol-Fe complexes could not be determined.
6.3.3 Inhibition of NBT Reduction in HX/XO Mediated Superoxide Generating System

The SOD-mimic activity of catecholic iron complexes was also demonstrated using the superoxide generating system hypoxanthine (HX)/xanthine oxidase (XO). As shown in Table 6.5, complexation of the catechol with iron increased the SOD mimic activity of the catechols several fold. Again, the tiron-Fe complex was the least effective among the catecholic iron chelates tested, having an IC50 more than 6 fold that of caffeic acid-Fe. It was however still 7 fold more effective than uncomplexed tiron. Inhibition of XO activity by the catecholic iron complexes was not observed even at 100 μM.

The superoxide scavenging activity of catechol-Fe chelates was also compared with iron complexes of catechol isomers resorcinol and hydroquinone (Table 6.5). Using the HX/XO mediated superoxide generating system, the rate of NBT reduction was significantly decreased by dihydroxybenzene-Fe3+ complexes, and the order of inhibition for the complexes was catechol-Fe >> hydroquinone-Fe > resorcinol-Fe. Also as shown in Figure 6.2, at 50 μM, catechol-Fe, hydroquinone-Fe and resorcinol-Fe inhibited NBT reduction by ca. 80%, 40% and 20%, respectively.

6.4 DISCUSSION

Catechols occur ubiquitously in nature, functioning as precursors of melanins, lignins, and insect scleroproteins (Higuchi, 1971; Mason, 1966). Catechol is also a constituent nucleus of neurotransmitters and hormones such as dopamine (3,4-dihydroxyphenylethylamine), dopa (3,4-dihydroxyphenylalanine) and epinephrine (adrenaline) (Nagastu, 1973). Vitamin K and some important bacterial iron-sequestrating
**Table 6.5 Inhibition of Hypoxanthine/Xanthine Oxidase Mediated Nitro Blue Tetrazolium Reduction**

<table>
<thead>
<tr>
<th>Fe$^{3+}$ Chelates</th>
<th>IC50 (μM)</th>
<th>Catechols</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic Acid-Fe</td>
<td>26</td>
<td>Caffeic Acid</td>
<td>80</td>
</tr>
<tr>
<td>4-Methylcatechol-Fe</td>
<td>29</td>
<td>4-Methylcatechol</td>
<td>46</td>
</tr>
<tr>
<td>Catechol-Fe</td>
<td>36</td>
<td>Catechol</td>
<td>119</td>
</tr>
<tr>
<td>Tiron-Fe (3:1)</td>
<td>240</td>
<td>Tiron</td>
<td>1850</td>
</tr>
<tr>
<td>Tetrachlorocatechol-Fe (3:1)</td>
<td>&gt; 225*</td>
<td>Tetrachlorocatechol</td>
<td>&gt; 150*</td>
</tr>
<tr>
<td>4-Nitrocatechol-Fe (3:1)</td>
<td>&gt; 225*</td>
<td>4-Nitrocatechol</td>
<td>&gt; 150*</td>
</tr>
<tr>
<td>Resorcinol-Fe</td>
<td>192</td>
<td>Resorcinol</td>
<td>676</td>
</tr>
<tr>
<td>Hydroquinone-Fe</td>
<td>113</td>
<td>Hydroquinone</td>
<td>280</td>
</tr>
</tbody>
</table>

The reaction mixture contained 200 μM nitro blue tetrazolium (NBT), 35 μM hypoxanthine, 25 mU/ml xanthine oxidase (XO), and various concentrations of 2:1 Fe$^{3+}$ complexes in 0.1 M Tris buffer (pH 7.4). XO solution was added last to start the reaction, and the reduction of NBT was measured by following the absorbance at 560 nm. IC50 values for the inhibition of the reduction of NBT by the investigated compounds were calculated as described in Table 6.4.

* Precipitation in the reaction systems at higher concentrations of these catechols interfered with the assay.
Figure 6.2*  Inhibition of Nitro Blue Tetrazolium Reduction in HX/XO System by Dihydroxybenzenes. The reaction mixture contained 200 µM nitro blue tetrazolium (NBT), 35 µM hypoxanthine, 25 mU/ml xanthine oxidase and iron complexes (50 µM of catechol, resorcinol, or hydroquinone with 25 µM Fe$^{3+}$) in 0.1 M Tris buffer (pH 7.4). Xanthine oxidase solution was added last to start the reaction, and the reduction of NBT was measured by following the absorbance at 560 nm.

* Not included in the manuscript.
siderophores contain the catechol motif as well (Ong et al., 1979; Tait, 1975). Enterobactin, the catechol based siderophile from enteric bacteria such as E. coli or Salmonella typhimurium, binds iron especially ferric ion from the environment. Its three bidentate catechol groups form a hexacoordinate ferric complex with a low reduction potential (Cooper et al., 1978), and facilitate the uptake of iron into the aerobic microorganisms (Neilands, 1952; 1973).

In the present work, complexation of the catechols with ferric ion was found to markedly increase their SOD mimic activity. In both enzymatic (i.e., HX/XO) and non-enzymatic (i.e., PMS/NADH) systems, catecholic compounds were weak superoxide scavengers in inhibiting superoxide radicals-involved nitro blue tetrazolium reduction. However, by complexation with iron, the SOD-mimic activity was greatly increased as evidenced by a significant decrease in IC50 values required for inhibiting NBT reduction. This suggests that the catecholic compounds are capable of forming thermodynamically stable iron complexes, which in turn are potent free radical scavengers.

Complexes of catechols with iron have also been observed to protect hepatocytes against ROS-dependent hypoxic cell injury. Previous studies by our research group suggested that reactive oxygen species are produced under hypoxic conditions as measured by luminol chemiluminescence, which contributes to the hypoxic cell injury in hepatocytes (Khan and O'Brien, 1995; Niknahad et al., 1995). Under hypoxia, it was proposed that large amounts of AMP formed from massive ATP consumption yields hypoxanthine and xanthine, which produce superoxide radicals as well as other ROS in the presence of xanthine oxidase (Corte and Stirpe, 1972). Polyphenolic antioxidants caffeic acid, purpurogallin and quercetin at concentrations higher than 100 µM significantly prevented
hepatocyte cytotoxicity under hypoxia (Khan and O'Brien, 1995; Niknahad et al., 1995). Flavonoids have also previously been shown to protect mammalian and bacterial cells from cytotoxicity induced by ROS, which was attributed to the catechol moiety of the B-ring (Nakayama, 1994; Nakayama et al., 1993). However, in this work, iron complexes of catechols and catecholic flavonoids catechin and quercetin exerted their protective effects at concentrations as low as 20 µM, further confirming that the iron complexes possess greater free radical scavenging activity than the uncomplexed catechols. Moreover, the iron complexes of catechols were much more effective than the uncomplexed catecholic compounds at inhibiting ROS generation as determined by dichlorofluorescin fluorescence.

The SOD mimic activity of catechols and catecholic iron complexes, expressed as the logarithm values of 1/IC50 of phenazine methosulfate/NADH system seemed to correlate with the redox potential of catechols (Horner and Geyer, 1965; Mentasti et al., 1976), E° (V vs. NHE). As shown in Figure 6.3, a good linear correlation with an r² (r: correlation coefficient) value of 0.95 for catechols and 0.98 for catecholic iron complexes was obtained between the reduction potentials and the SOD mimic activities. However, the iron complexes of caffeic acid and protocatechuic acid did not fit this linear correlation and were more active than expected, suggesting that the acid side chain may also contribute to iron chelation and thus superoxide scavenging properties. The SOD mimic activity may relate to the reduction potentials of iron as various catecholic iron complexes. This correlation was however not determined, due to lack of reduction potentials for the iron complexes of catechols. Also, the reduction potentials of the iron center are expected to be very low and therefore ferric ion in the catecholic complexes is less reactive than in other complexes of chelators like ADP or EDTA.
Figure 6.3 Correlation between the Redox Potentials and the SOD Mimic Activities. The open circles stand for catechols, and the filled circles stand for catecholic iron complexes. B: 4-‐t-buty1catechol; C: catechol; CA: caffeic acid; M: 4-methylcatechol; MO: 3-methoxycatechol; P: protocatechuic acid; T: Tiron.
The iron complexes of the high redox potential catechols tiron, tetrachlorocatechol and 4-nitrocatechol were much less effective SOD mimics than those of other catecholic compounds. The low activity of these complexes could also be partly explained by the stereochemistry of the stable 3:1 catecholic iron complexes that are formed at a physiological pH. Graf et al. (1984) suggested that the availability of an iron coordination site that is open or occupied by a readily displaceable ligand such as water facilitates the reaction of iron chelates with superoxide radicals, although it is not a stringent requirement. In the 3:1 complexes, all the ligand sites are occupied by the hydroxyl groups of catechols, which consequently hinders a free electron transfer between the complex and a superoxide radical.

The dismutation of superoxide radicals by catecholic iron complexes could be explained by the redox cycling of the metal and the ligand as shown in Scheme 6.1, in which a 1:1 catechol-Fe complex is shown as an example for simplicity. Catechols bind tightly with ferric ion to form a complex (Raymond et al., 1976), which has been hypothesized to undergo internal redox reaction to generate a semiquinone radical intermediate of catechol chelated to the ferrous iron (Mentasti and Pelizzetti, 1973; Hider et al., 1981), a process that is favored at low pH or by low redox potential catechols. The resulting semiquinone ferrous complex is then oxidized by $O_2^\cdot$ to form a semiquinone ferric complex, which may be easily reduced back to the catecholic iron complex in the presence of $O_2^\cdot$. The semiquinone iron complexes may also undergo disproportionation to give the parent catechol and the o-quinone, the latter of which could also be reduced by $O_2^\cdot$ (Brunmark and Cadenas, 1989). Alternatively, the radical character in the semiquinones may be delocalized to the ring carbon atoms and form catecholic dimers or trimers. It is
Scheme 6.1 Proposed Mechanisms of Superoxide Scavenging by Catecholic Iron Complexes
possible that the initial ferric complexes could also be reduced by $O_2^-$ or intracellular reducing agents to form catecholic ferrous complexes. The catecholic ferrous complexes formed may be readily oxidized by $O_2^-$ due to their much lower redox potentials compared with that of Fe$^{3+}$/Fe$^{2+}$, and resulting in SOD mimic activity.

The catechol moiety is important to iron chelation and thus the increased SOD mimic activity of the catecholic iron complexes. Among dihydroxybenzenes, catechol was the most effective, and hydroquinone was more effective than resorcinol. This is attributed to the two conjugated hydroxyl groups on the ring system, so that radicals may readily dismutate to give the semiquinone or quinone products. However, the quinone structure is not formed in resorcinol where the conjugation is not present. Similarly, it was reported by others that hydroquinone but not resorcinol reduced both mammalian and bacterial cytochrome c (Saleem and Wilson, 1982).

Caffeic acid has been reported to be genotoxic in bacterial and mammalian in vitro genotoxicity tests, which was ascribed to H$_2$O$_2$ formed during autoxidation, but millimolar concentrations of caffeic acid were used and stored for days (Hanham et al., 1983; Stich, 1991). However, the caffeic acid-Fe complex at 1 mM was not cytotoxic when incubated with hepatocytes even under an atmosphere of 95% O$_2$.

Our studies demonstrated that iron complexes of catecholic compounds were potent SOD mimics which may manifest their therapeutic function by scavenging superoxide radicals. They also suggested that iron complexes of catecholic compounds are able to cross the cell membrane, and a fraction that does not bind to cellular proteins is available to scavenge intracellular superoxide radicals. In the absence of iron, higher concentrations of catecholic compounds are required to reach the same level of cytoprotection against hypoxic
injury (Khan and O'Brien, 1995; Niknahad et al., 1995). Therefore, part of the
cytoprotective mechanism of catecholic compounds could involve chelation with
intracellular iron to form complexes with powerful SOD mimic activity. The
characterization of these metallocomplexes would provide us with a physiological model to
pursue a successful pharmacological approach to oxygen-mediated diseases.
SUMMARY AND FUTURE WORK

SUMMARY

Naturally occurring methylenedioxybenzenes (MDBs) including the non-genotoxic isosafrole widely occur in nature and are present in spices, seasonings, essential oils and folk medicines. The synthetic MDB piperonyl butoxide (PBO) has been used as a pesticide synergist for years and has been shown to partially protect experimental animals against various hepatotoxins activated by cytochrome P450 (P450)-dependent monooxygenase systems. In the present work, this group of compounds has been discovered to possess potential chemoprotective properties which enable them to prevent both P450-catalyzed and ROS-mediated toxic cellular injury. The chemoprotective properties of MDBs have been attributed to their ability to inactivate P450 isozymes that are involved in xenobiotic metabolism and to the formation of MDB catecholic metabolites that are powerful iron chelators and free radical scavengers.

In Chapters 3 and 4, the chemoprotective activity of MDBs, especially naturally occurring MDBs were tested on the hepatotoxicity induced by various xenobiotics both in vivo and in vitro. It was found that this group of dietary compounds particularly isosafrole was highly effective at preventing CCl4 or N-dimethylnitrosamine-induced centrilobular liver necrosis in mice and BrCCl3-induced hepatocyte cytotoxicity and lipid peroxidation in vitro. Pre- or post-treatment with isosafrole in mice also prevented phenetidine-induced methemoglobinemia. Isosafrole was still significantly protective even at dosages as low as 10 mg/kg. However, pretreatment was required for the protection against CCl4 hepatotoxicity by isosafrole. The chemoprotective properties of isosafrole and other MDBs
were attributed to the inhibition of P450 isozyme activity by forming stable MDB metabolic intermediate complexes with the ferrous form of P450, which prevented the toxic metabolic activation of the toxicants. This hypothesis was supported by evidence showing that isosafrole was a potent *in vivo* and *in vitro* inhibitor of CYP2E1, CYP1A2 and PROD that are involved in the toxicant bioactivation. Moreover, isosafrole also prevented isopropanol-potentiated CCl4 and CHCl3 hepatotoxicity as a consequence of the induction of CYP2E1 by isopropanol. However, PBO did not affect CYP2E1 activity either *in vivo* or *in vitro*, and thus was ineffective at preventing CCl4 toxicity.

Chapter 5 further demonstrated that MDBs, naturally occurring or synthetic, also possessed antioxidative properties. It was observed that isosafrole and other MDBs prevented ROS-mediated ferric nitrilotriacetate (FeNTA)-induced lipid peroxidation in hepatocytes and liver microsomal incubates. Isosafrole also prevented FeNTA-induced kidney damage *in vivo* and significantly decreased lipid peroxidation with kidney homogenates prepared from treated animals. This antioxidative activity of MDBs was mainly attributed to their catecholic metabolites, as catechols are well known chain-breaking antioxidants that are capable of reacting with oxygen-derived free radicals. Moreover, preincubating hepatocytes with other P450 inhibitors significantly decreased the protective capacity of isosafrole, confirming the important role of bioactivation in the antioxidative activity of MDBs.

Further studies on the ROS-scavenging activity of catechols in the presence of iron have been implemented as discussed in Chapter 6. Complexes of catechols with iron were found to be more effective than uncomplexed catechols at protecting hepatocytes against ROS including superoxide radical mediated hypoxic cell injury. Catecholic iron complexes
also significantly prevented the ROS generation as evidenced by the formation of fluorescent product of 2',7'-dichlorofluorescin by ROS. Moreover, catecholic iron complexes were much better superoxide dismutase (SOD) mimics than uncomplexed catechols in preventing superoxide-catalyzed nitro blue tetrazolium reduction. The SOD mimic activities of catecholic iron complexes and the uncomplexed catechols were found to be correlated with the redox potential of catechols.

It is well accepted that most chemical carcinogens require metabolic activation before exerting their carcinogenic effects, which are mainly catalyzed by cytochrome P450-dependent monooxygenases (Guengerich, 1991). Therefore, blocking of the metabolic bioactivation process by drugs, especially dietary compounds, may help to prevent chemical carcinogenesis. The chemoprotective properties of MDBs discovered in the present work suggested that some MDBs, in particular the non-genotoxic isosafrole, could find application in preventing chemical carcinogenesis and xenobiotic toxicity that are catalyzed by cytochrome P450-dependent monooxygenase systems. Furthermore, the antioxidative activity of MDBs and catechols and particularly the SOD mimic activity of catecholic iron complexes suggested that they might find application in preventing iron-overload oxidative injury.

Nevertheless, cytochrome P450-dependent monooxygenases are also required for the biotransformation of many important endogenous compounds and the detoxification of various xenobiotics. Therefore, inhibiting P450 isozyme activities may not be a practical approach for the prevention of chemical carcinogenesis and xenobiotic toxicity. Also, the adverse effects of chemoprotective agents need to be considered. For example, catechols have been shown as both pro-oxidative and antioxidative agents, which highly depends on
the redox environment of tested systems. Moreover, caution has to be applied in extrapolating laboratory results to possible application to humans. The effective dosages of protective agents found using animal models may not be in the range of those encountered by humans from dietary sources. The large variety of components in human diet also complicates the chemoprotective effects of tested compounds. Furthermore, species difference in cytochrome P450 isozymes and their modulations by dietary chemicals are also a factor of concern. The observed protective effects of compounds in this work may be different from those in humans. Therefore, the therapeutic use of chemoprotective agents that are cytochrome P450 inhibitors and/or antioxidants in preventing chemical carcinogens and xenobiotic toxicity may only be found in certain special occasions. Compounds that are able to selectively prevent target carcinogens, but do not significantly affect physiological function, may find application for those who are known to be exposed to the carcinogens. Chemopreventive clinical trials are therefore absolutely necessary to the therapeutic advantage of isosafrole. In the meantime, fruit and vegetable diets should be encouraged as epidemiological evidence indicates that this is associated with a decreased cancer risk (Taylor et al., 1997).

**Future Work**

Future research will focus on the chemoprotective effects of flavonoids, including dietary flavonoids, coffee catechols and tea polyphenols, on liver/kidney injury induced by various toxicants including metal ions in vivo. Flavonoids containing the catechol moiety such as quercetin, myricetin and rutin, are found ubiquitously in nature and are generally components of human diet (Havsteen, 1983; Kandaswami and Middleton, 1994).
Flavonoids have also been shown to possess metal chelating properties and particularly have a strong affinity towards iron ions (Korkuc, 1974; Afanas'ev et al., 1989a). Afanas'ev et al. (1989b) also reported that a rutin-iron complex was more effective than rutin at preventing rat erythrocyte hemolysis or rat peritoneal macrophage ROS formation induced by asbestos fibers. Therefore, in future studies, the ROS-scavenging properties of iron complexes with various structures of flavonoids will be determined and compared with their uncomplexed flavonoids.

Low-molecular-weight SOD mimics, in particular complexes of Cu, Mn and Fe, have been shown to prevent ROS-mediated CCl4-induced liver injury in rats (Joyave et al., 1985), paraquat toxicity towards Escherichia coli growth and survival (Nagano et al., 1989), and paraquat-caused light-dependent bleaching in Dunaliella salina cells (Rabinowitch et al., 1987). Similarly, in vivo models will also be utilized to test the effectiveness of flavonoid iron complexes at preventing ROS-mediated toxicity. In addition to the superoxide dismutase mimic activity, the ability of flavonoid iron complexes to react with other ROS such as hydroxyl and peroxyl radicals and H2O2 will also be determined. A study on the structure-activity relationship of these flavonoid iron complexes will be implemented as well.

The long term goal of this research is to explore the possible application of dietary components to prevent liver/kidney toxicity induced by various hepato- or nephrotoxicants and heart injury in iron overload diseases. The dietary components may also help to increase people's resistance to toxicants and could find therapeutic application for cancer patients before they receive chemotherapy. This could help to prevent the adverse effects of the chemotherapeutic agents.
REFERENCES


Corte, E. D. and Stirpe, F. (1972) The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. *Biochem. J.*, **126**, 739-745.


\[ N,N,N',N' \text{-tetrakis(2-pyridylmethyl)ethylenediamine. Arch. Biochem. Biophys., 293, 153-157.} \]


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*Biochem. Pharmacol.*, 37, 837-841.


IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"

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