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Mechanism-Based Inactivation of Cytochrome P450 2A and 2B Enzymes

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5.1. Introduction

Inhibition of cytochrome P450 enzymes (P450s) contributes significantly to drug–drug interactions (Wienkers and Heath 2005) and recently it has been suggested that mechanism-based inactivation may play a greater role in these interactions than previously recognized (Zhang and Wong 2005). Mechanism-based inactivation of P450s may also be caused by exposure to compounds present in tobacco and food. This inactivation may result in an increase in the toxicity of drugs or other xenobiotics. Alternatively, mechanism-based inactivators (MBIs) may have a positive affect; for example, inactivation of the enzymes that catalyze the metabolic activation of procarcinogens. Mechanism-based inactivators of all human hepatic drug-metabolizing P450 enzymes have been identified (Table 5.1): the majority of these inactivate P450 3A4, the major human drug-metabolizing enzyme. In this chapter we will focus our discussion on the mechanism-based inactivation of two less abundant and less well-studied hepatic enzymes, P450 2B6 and P450 2A6. In addition, some recent studies on the mechanism-based inactivation of P450 2A13, an enzyme believed to play a role in the metabolic activation of the tobacco-specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), will be discussed.

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Table 5.1 Human mechanism-based inactivators.

P450	MBI		References
1A1/1A2	Carbamazepine	1A2 in HLM	Masubuchi et al. (2001)
	Clorgyline	1A2	Polasek et al. (2006)
	Coriandrin	1A1 (purified)	Cai et al. (1996)
	Dihydralazine	HLM, B-lymphoblastoid microsomes	Masubuchi and Horie (1999)
	Furafylline	1A2 in HLM	Kunze and Trager (1993)
	Oltipraz	*E.coli* membranes	Langouet et al. (2000)
	Rhapontigenin	1A1 (bacterial membrane)	Chun et al. (2001)
	trans-Resveratrol	1A2 (Supersomes), HLM	Chang, Chen and Lee (2001)
	Zileuton	1A2 in HLM	Lu et al. (2003)
2A6/2A13	Benzylisothiocyanate (BITC)	2A6 and 2A13 (purified from *E. coli*)	von Weymarn, Chun and Hollenberg (2006b)
	(R)-(+)-Menthofuran	HLM, 2A6 purified from *E. coli*	Khojasteh-Bakht et al. (1998)
	8-Methoxysporalen (8-MOP)	2A6 and 2A13 (purified from *E. coli*)	von Weymarn et al. (2005) Koenigs et al. (1997)
	Nicotine	2A6 and 2A13 (purified from *E. coli*)	von Weymarn, Brown and Murphy (2006a)
	Phenethylisothiocyanate (PEITC)	2A6 and 2A13 (purified from *E. coli*)	von Weymarn, Chun and Hollenberg (2006b)
2B6	Bergamottin	Purified from *E. coli*	Tassaneeyakul et al. (2000)
	Clopidogrel	2B6 Superosomes, HLM	Richter et al. (2004)
	N-(3,5-dichloro-4-pyridyl)-4-methoxy-3-(prop-2-ynyloxy)benzamide	2B6 Superosomes	Fan et al. (2003)
	Efavirenz and 8-hydroxyefavirenz	Purified from *E. coli*	Bumpus, Kent and Hollenberg (2006)
	17α-Ethynylestradiol	Purified from *E. coli*	Kent et al. (2002b)
	Glabridine	Purified from *E. coli*	Kent et al. (2002a)
	Phencyclidine	Purified from *E. coli*	Jushchyshyn, Kent and Hollenberg (2003)
	2-Phenyl-2-(1-piperidinyl)propane	Purified from *E. coli*	Chun et al. (2000)
	n-Propylxanthate	Purified from *E. coli*	Kent, Yanev and Hollenberg (1999)
	Ticlopidine	2B6 Superosomes, HLM	Richter et al. (2004)
	Tamoxifen	Purified from *E. coli*	Sridar et al. (2002)
	N,*N*′,*N*″-triethylenethiophosphoramide tTEPA	Purified from *E. coli*	Harleton et al. (2004) Richter et al. (2005)
2C8	Amiodarone	*E. c*oli membranes	Polasek et al. (2004)
	Fluoxetine		
	Isoniazid		
	Nortriptyline		
	Phenelzine		
	Verapamil		

2C9	Silybin	Purified from *E. coli*	Sridar et al. (2004)
	Suprofen	Recombinant 2C9	O′Donnell et al. (2003)
	Tienilic acid	Yeast microsomes	Jean et al. (1996)
2C19	Phenelzine	*E. coli* membranes, HLM	Polasek et al. (2006)
	Ticlopidine	Yeast microsomes	Ha-Duong et al. (2001)
2D6	5-fluoro-2-[4-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine	Superosomes, HLM	Palamanda et al. (2001)
	Paroxetine	HLM	Bertelsen et al. (2003)
2E1	Trichloroethylene	*E. coli* membranes	Cai and Guengerich (2001)
3A4	Amiodarone		Ohyama et al. (2000)
	Ampenprenavir	Superosomes, HLM	Ernest, Hall and Jones (2005)
	Bergamottin	HLM	Tassaneeyakul et al. (2000)
	Clarithromycin	Superosomes	Mayhew, Jones and Hall (2000)
	Delavirdine	β-lymphoblast microsomes, HLM	Voorman et al. (1998)
	N-desmethyl dilitiazem	Superosomes	Mayhew, Jones and Hall (2000)
	Diclofenac	β-lymphoblast microsomes, HLM	Masubuchi, Ose and Horie (2002)
	Dihydralazine	HLM, B-lymphoblastoid microsomes	Masubuchi and Horie (1999)
	6′,7′-Dihydroxybergamottin	HLM, Purified from *E. coli*	Tassaneeyakul et al. (2000) Schmiedlin-Ren et al. (1997)
	Dilitiazem	Superosomes	Mayhew, Jones and Hall (2000)
	Erythromycin	β-lymphoblast microsomes, HLM	Kanamitsu et al. (2000)
	17α-Ethynylestradiol	Purified from *E. coli*	Lin, Kent and Hollenberg (2002)
	Fluoxetine	Superosomes	Mayhew, Jones and Hall (2000)
	Gestodene	HLM, purified from *E. coli*	Guengerich (1990)
	4-Ipomeanol		Alvarez-Diez and Zheng (2004)
	Irinotecan	Superosomes	Hanioka et al. (2002)
	Limonin	HLM	Iwata et al. (2005)
	Lopinavir	Superosomes, HLM	Ernest, Hall and Jones (2005)
	Mibefradil	HLM	Prueksaritanont et al. (1999)
	Midazolam	Purified from *E. coli*	Khan et al. (2002)
	Mifepristone	Purified from *E. coli*	He, Woolf and Hollenberg (1999)
	Nelfinavir	Superosomes, HLM	Ernest, Hall and Jones (2005)

(*Continued*)

Table 5.1 (*Continued*).

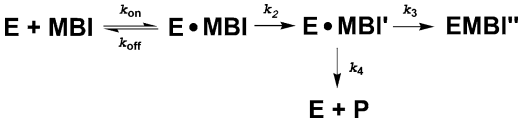
P450	MBI		References
	Nicardipine	HLM, Baculovirus microsomes	Ma, Prueksaritanont and Lin (2000)
	Nor-Verapamil	HLM, Superosomes	Wang, Jones and Hall (2004)
	Phenelzine	*E. coli* membranes, HLM	Polasek et al. (2006)
	Raloxifene	HLM, superosomes	Chen et al. (2002)
	Resveratrol	Baculovirus microsomes	Chan and Delucchi (2000)
	Ritonavir	Superosomes, HLM	Ernest, Hall and Jones (2005)
	Rutaecarpine	HLM	Iwata et al. (2005)
	Saquinavir	Superosomes, HLM	Ernest, Hall and Jones (2005)
	Silybin	Purified from *E.Coli*	Sridar et al. (2004)
	Tamoxifen	HLM, superosomes	Zhao et al. (2002)
	Verapamil	HLM, Superosomes	Wang, Jones and Hall (2004)
	Zafirlukast	Superosomes, HLM	Kassahun et al. (2005)

The mechanism-based inactivators included in this table meet at least two of the criteria for mechanism-based inactivation. The kinetics of inactivation is reported, whenever possible, and in addition at least irreversibility or mechanism of inactivation is also assessed.

5.2. Characterization of Mechanism-Based Inactivation

An enzyme inactivator is any compound that causes the irreversible or quasi-irreversible loss of enzymatic activity. The definition of a mechanism-based or suicide inactivator is more specific. Pratt and Kranz defined an MBI simply as an enzyme inactivator that requires the mechanism of the target enzyme (Krantz 1992; Pratt 1992). The definition that will be used in this chapter is that of Silverman who defines an MBI as a compound that is catalytically transformed by the enzyme to a species that prior to release from the active site renders the enzyme inactive (Silverman 1996). Mechanism-based inactivation, although rare in most enzyme systems, is much more frequent with P450 enzymes (Kent, Juschyshyn and Hollenberg 2001; Zhang and Wong 2005).

The kinetic scheme for an MBI is similar to that of a noninactivating substrate but with an additional product, the modified inactive enzyme (Scheme 5.1). After the MBI associates with the enzyme (E), catalytic turnover generates the inactivating species (MBI′) complexed with the



Scheme 5.1

enzyme (E•MBI′). The inactivating species may then either be released as product (P) or bind irreversibly to the enzyme, rendering it inactive (EMBI″). A more in depth description of Scheme 5.1 and how kinetic constants are derived from it has been reviewed by Silverman (1988). The key parameters which describe mechanism-based inactivation of an enzyme are: k_{inact}, the maximal inactivation rate constant; $K_{I,}$ which can be considered analogous to K_m; and the partition ratio. The partition ratio is a measure of the efficiency of the MBI. It is the ratio of product release to enzyme inactivation, and is described by k_3/k_4. This ratio does not depend on the concentration of inactivator. It depends on the reactivity of MBI′, the rate of diffusion of MBI′ from the active site, and the proximity of an appropriate binding site in the enzyme active site for covalent bond formation. In the case of P450 enzymes binding may occur either with an amino acid side chain or the heme molecule.

There are several criteria that are routinely assessed to determine if a compound is an MBI of a particular P450 enzyme (Silverman 1996). These criteria and a brief rationale are listed below.

- Inactivation is dependent on catalytic turnover; that is, the compound must be metabolized to a reactive species, which then inactivates the enzyme.
- Inactivation is time dependent. Since the inactivation requires catalytic turnover the loss in enzyme activity is usually first-order with respect to time.
- Inactivation exhibits saturation kinetics.
- Inactivation is irreversible or at least quasi-irreversible.
- The enzyme is protected from inactivation through the addition of an alternate substrate. The alternative substrate will compete with the MBI and slow its metabolism and hence enzyme inactivation.
- Only one inactivator molecule is attached to each molecule of inactivated enzyme.
- The inactivation occurs prior to release of the reactive species from the active site of the enzyme. Therefore, the addition of a nucleophile such as glutathione, which will react with electrophilic reactive species released from the active site, should not affect the rate of inactivation.

The protocols that are used to test these criteria are described in detail elsewhere (Silverman 1996) and will only be partially discussed here. The classic method to study an MBI is to characterize enzyme activity in two reactions. In the primary reaction, the P450 of interest is incubated under standard conditions with varying concentrations of the putative MBI in the presence and absence of NADPH. Then at different time intervals, aliquots of the primary reaction are removed and added to a fresh reaction mixture containing NADPH and a saturating concentration of another substrate of this P450. The secondary reaction is carried out so that a minimum 20-fold dilution of the MBI occurs. If the MBI is also a potent inhibitor of the enzyme, the dilution necessary to eliminate inhibition in the secondary reaction might be too great for the accurate quantitation of enzyme activity. In this case, the removal of the MBI may be accomplished through the use of spin-column gel filtration or dialysis. Kinetic

parameters for the MBI reaction may be determined from the enzyme activities measured in these experiments.

Mechanism-based inactivation is usually the result of covalent binding of a reactive intermediate to the active site of the enzyme, although tight-binding noncovalent complexes between the inactivating species and the enzyme also occur. The former are irreversible but the latter can sometimes slowly disassociate over time. Presently, it has not been clearly established when a quasi-irreversible inactivator is no longer classified as an MBI.

There are three pathways by which an MBI may inactivate a P450 enzyme: covalent modification of amino acids residues in the active site, covalent modification of the heme moiety, or destruction of the heme. When the heme is destroyed, fragments of the heme sometimes become bound to the apoprotein. Mechanism-based inactivators may also generate cross-links between the heme and the apoprotein, although this is not common. It is important to note that multiple mechanisms of inactivation may occur for one compound. For example, P450-catalyzed metabolism of the MBI may result in modification of both the heme and the apoprotein. In addition, since several factors influence enzyme inactivation, the relative contribution of different mechanisms of inactivation by an MBI may vary among different enzymes. The factors that will influence the mechanism include the site of MBI oxidation, the structure of the reactive intermediate generated, and the geometry and composition of the active site of the enzyme.

5.2.1. MBI as Tools

Over the years, MBIs have proven to be useful tools with which to study structure–function relationships of individual P450 enzymes (reviewed in Kent, Juschyshyn and Hollenberg 2001). Specifically, the use of MBIs have aided in determining the orientation of the heme in the P450 active site and in identifying regions of the P450 protein involved in substrate binding (Kent et al. 2006; Kunze et al. 1983; Ortiz de Montellano et al. 1992; Ortiz de Montellano 1995). Studies with MBIs by Ortiz de Montellano and co-workers allowed the identification of the correct orientation of the heme molecule in the active site. They concluded that although the general orientation of the heme appears to be the same in different P450s, the extent to which different regions of the heme molecule are accessible to inactivating species varies among P450 enzymes (Ortiz de Montellano 1995). Mechanism-based inactivators that result in covalent modification of the apoprotein are particularly useful for identifying amino acids in the active site that are important in substrate binding (Kent, Juschyshyn and Hollenberg 2001).

Inactivators that affect highly homologous P450 enzymes differently, such as the human P450s 2A6 and 2A13 (94% identical) and 3A4 and 3A5 (84% identical) or the rat P450s 2B1 and 2B2 (97% identical), can also play an important role in the identification of the P450s responsible for the metabolism of a particular compound *in vivo*. The use of inhibitory antibodies are usually sufficient to identify which subfamily of P450 enzymes is involved in the metabolism, but quite often these antibodies cannot distinguish between highly homologous enzymes within that subfamily.

Mechanism-based inactivators that are able to differentially inactivate highly homologous P450 enzymes such as rat P450s 2B1 and 2B2 (Kent et al. 2002b; von Weymarn, Sridar and Hollenberg 2004) and human P450s 3A4 and 3A5 (Khan et al. 2002) could be used to pinpoint which enzyme is responsible for the metabolism of a specific compound *in vivo*.

5.2.2. MBI and Drug Metabolism

Annually about two million serious adverse drug reactions occur in the US (Lazarou, Pomeranz and Corey 1998). Inhibition and/or inactivation of metabolic enzymes such as the P450 enzymes has been recognized as one of the significant causes of adverse drug–drug interactions (Wienkers and Heath 2005). Drug–drug interactions are often caused by one drug inhibiting the metabolism of another and therefore increasing the plasma concentration of the second drug. Since MBIs irreversibly inhibit an enzyme, the risk for serious drug–drug interactions may be greater for these compounds than for competitive inhibitors. Mibefradil is one example where mechanism-based inactivation may have contributed to the serious drug–-drug interactions which resulted in this drug being pulled from the market (Friedman et al. 1999). Mibefradil is a potent MBI of P450 3A4, with a K_i of 2.3 μM and a partition ratio of 1.7 (Prueksaritanont et al. 1999). It was withdrawn from the market due to potentially harmful interactions with 26 identified drugs. Reduced P450 3A4 enzyme activity was believed to play a role in many of these interactions (Friedman et al. 1999). Over the past decade the pharmaceutical industry has invested a great deal of money and effort into screening for P450 inhibition by new drug entities at early stages of the development process.

In addition to MBI-mediated drug–drug interactions, environmental factors may result in inactivation of P450 enzymes and cause adverse drug reactions. Compounds found in the diet, cigarette smoke, and air or water pollution all have the potential to cause mechanism-based inactivation and hence result in dangerous but unexpected drug reactions. Grapefruit juice is the best characterized example of a dietary exposure resulting in the mechanism-based inactivation of P450 enzymes. The influence of grapefruit juice on the pharmacokinetics of a large number of drugs is well documented (reviewed in Saito et al. 2005). Grapefruit juice increases the area under the curve (AUC) and the maximum plasma concentration (C_{max}) of orally co-administered drugs such as simvastatin, triazolam, midazolam, nitrendipine, and a number of others (Saito et al. 2005). The major cause of these effects of grapefruit juice is considered to be mechanism-based inactivation of P450 3A4 (Lown et al. 1997; Schmiedlin-Ren et al. 1997). *In vitro* studies have identified several components of grapefruit juice as inhibitors of P450 enzymes (Guo and Yamazoe 2004; Tassaneeyakul et al. 2000). Among these bergamottin and 6′,7′-dihydroxybergamottin have been well characterized as MBIs of P450 3A4 (Paine, Criss and Watkins 2004; Schmiedlin-Ren et al. 1997; Tassaneeyakul et al. 2000). 6′,7′-Dihydroxybergamottin appears to be the major contributor to grapefruit juice-mediated P450 3A4-mediated inactivation. However in other foods that contain bergamottin but not 6′,7′-dihydroxybergamottin, bergamottin is likely to contribute significantly to the inactivation of

P450 3A4 (Paine, Criss and Watkins 2005). Recently, bergamottin has been identified as a MBI of P450 2B6 (Lin, Kent and Hollenberg 2005). Therefore, grapefruit juice consumption may also cause drug–drug interactions with drugs primarily metabolized by P450 2B6. This will be discussed in the following section.

Another class of naturally occurring compounds that may result in the inactivation of drug-metabolizing P450s are isothiocyanates. These compounds, found in various cruciferous vegetables such as cabbage, watercress, broccoli, and cauliflower as thioglucoside conjugates, have been studied as cancer chemopreventive agents (reviewed in Hecht 2000). The chemopreventive properties of isothiocyanates are believed in part to be due to inhibition and inactivation of P450 enzymes that have been implicated in the bioactivation of chemical carcinogens. Notable, the same mechanisms that result in the chemopreventive properties of the isothiocyanates could also give rise to adverse drug interactions. Mechanism-based inactivation of the rat enzymes P450 2E1 and P450 2B1 by benzyl isothiocyanate has been well characterized (Goosen, Mills and Hollenberg 2001; Moreno et al. 2001). In both cases the mechanism of inactivation was covalent modification of the apoprotein. Some recent work on isothiocyanates as MBIs of P450 2A6 and P450 2A13 is discussed below.

In addition to diet, tobacco smoke is a major source of exposure to a large number of xenobiotics, and smokers clearly metabolize many drugs differently than do nonsmokers. Polycyclic aromatic hydrocarbons present in tobacco smoke are well known to induce drug-metabolizing enzymes, including P450 1A2 and 1A1 (Kroon 2006). Whether or not, mechanism-based inactivation by tobacco smoke constituent influences drug metabolism is unknown. However, we have recently demonstrated that *in vitro*, nicotine, the main addictive agent in tobacco, inactivates two human P450s, 2A6 and 2A13 (von Weymarn, Brown and Murphy 2006a). Considering tobacco smoke contains over 4000 chemicals one might suspect that further studies of the components of tobacco will result in the identification of a number of MBIs of P450 enzymes. Hence smokers may be of potentially higher risk of adverse drug reactions than are nonsmokers. In the following two sections examples of drugs, including nicotine, and dietary constituents that are MBIs of the human P450s 2A6, 2A13, and 2B6 are discussed in detail.

5.3. Inactivation of P450 2B6

The mechanism-based inactivation of P450 2B enzymes, P450 2B1 and 2B2 in rats, P450 2B4 and 2B5 in rabbit, and more recently P450 2B6 in humans, has been well studied (Kent, Juschyshyn and Hollenberg 2001; Kent et al. 2002b, 2004; Lin, Kent and Hollenberg 2005). The main focus of these studies has been on the use of MBIs as structure–function probes (Kent, Juschyshyn and Hollenberg 2001; Lin et al. 2004; von Weymarn et al. 2004). Only recently has mechanism-based inactivation of P450 2B enzymes by pharmacologically relevant drugs and dietary constituents received attention (Bumpus, Kent and Hollenberg 2006; Harleton et al. 2004; Kent et al. 2002b, 2006; Lin, Kent and Hollenberg 2005). P450 2B6 is

not one of the major drug-metabolizing P450 enzymes; it typically accounts for <1% of the total P450 protein in the liver (Guengerich 2003). However, P450 2B6 is inducible and levels as high as 4% of the total P450 protein have been reported (Hanna et al. 2000; Wang et al. 2003). Recently, an increasing number of drugs that are primarily metabolized by P450 2B6 (Chang et al. 1993; Faucette et al. 2000; Ward et al. 2003) have been identified. Therefore, the potential for the inactivation of P450 2B6 to cause drug–drug interactions may be greater than previously appreciated. Mechanism-based inactivation of P450 2B6 has been characterized for a number of pharmaceutical compounds, dietary components, and synthetic compounds (Table 5.1). The discussion in this chapter will focus on five of these: 17α-ethynylestradiol (17EE), phencyclidine, efavirenz, *N*,*N*′,*N*″-triethylenethiophosphoramide (tTEPA), and bergamottin (Figure 5.1). This focus is due to both the extent of characterization of these MBIs and their possible relevance to drug–drug interactions.

P450 2B6 plays an important role in the metabolism of a number of commonly used drugs, such as cyclophosphamide (for cancer chemotherapy and autoimmune disease treatment), bupropion (an antidepressant used in smoking cessation), propofol (an anesthetic agent), and efavirenz (used to treat HIV-1) (Faucette et al. 2000; Roy et al. 1999; Ward et al. 2003). Only one of these compounds, efavirenz, inactivates P450 2B6. However, other compounds that inactivate P450 2B6 may influence the pharmacokinetic parameters of these clinically important drugs. In addition to these pharmaceutical compounds, nicotine- and the tobacco-specific carcinogen, NNK are substrates for P450 2B6 (Dicke, Skrlin and Murphy 2005; Yamazaki et al. 1999). However, while recent studies have shown that P450 2B6, if present in relatively high levels, may play a role in the metabolic activation of NNK in human liver microsomes, it is not likely to contribute significantly to nicotine metabolism (Dicke, Skrlin and Murphy 2005).

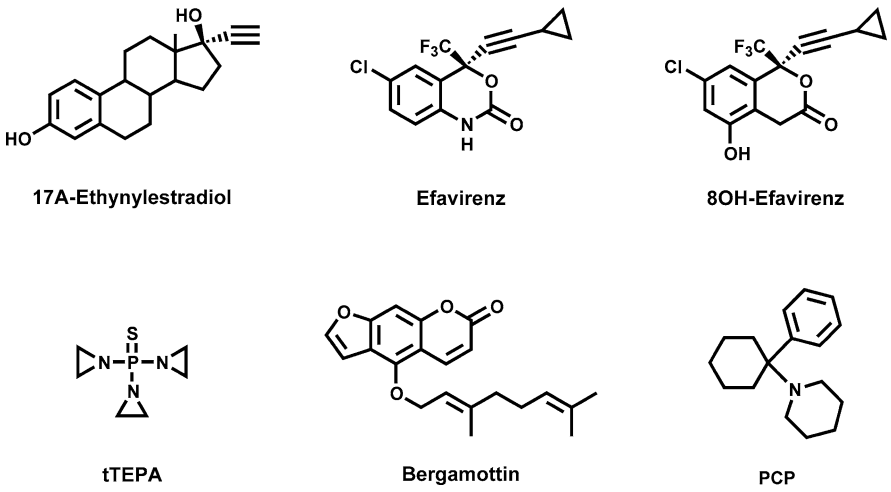


Figure 5.1 Mechanism-based inhibitors of P450 2B6.

5.3.1. 17α-Ethynylestradiol (17EE)

One of the best-characterized MBIs of P450 2B6 is 17EE, the major estrogenic component of oral contraceptives. The ethynyl group at the 17 position was introduced to increase the oral availability of the estradiol; however, it also gave rise to mechanism-based inactivation (Kent et al. 2002b; Lin, Kent and Hollenberg 2002; Lin and Hollenberg 2007). In 2002, Kent et al. reported that 17EE was a potent MBI of rat P450 2B1 and human P450 2B6, but not rat P450 2B2 and rabbit P450 2B4 (Kent et al. 2002b). Inactivation of P450 2B6 was irreversible, and time- and concentration-dependent (Figure 5.2). The apparent K_I for the inactivation of P450 2B6 was quite low, 0.8 μM; $t_{1/2}$ was 28 min. The partition ratio for the inactivation of P450 2B6 by 17EE was 13; that is, 13 molecules of 17EE were metabolized, per molecule of P450 2B6 inactivated. It was also demonstrated that P450 2B6 was protected from inactivation by alternate substrates, but the addition of exogenous nucleophiles had no effect on inactivation. Using [^{3}H]-17EE it was possible to demonstrate the formation of covalently modified P450 2B6 (EMBI″, Scheme 5.1).

The previously identified mechanism of P450 3A4 inactivation by 17EE (K_i, 18 μM; $t_{1/2}$, 25 min) was destruction of the heme (Lin, Kent and Hollenberg 2002). In contrast, inactivation of P450 2B6 by 17EE occurred through the formation of adducts to the apoprotein (Kent et al. 2002b; Kent et al. 2006). However, due to large errors in the mass assignments, it was not possible to detect a 17EE-adducted protein by liquid chromatography-mass spectrometry (LC/MS) analysis (Kent et al. 2006). In general, whole-protein LC/MS analysis of inactivated P450 2B6 has been difficult to perform (Jushchyshyn, Kent and Hollenberg 2003; Lin, Kent and Hollenberg 2005). Inactivated P450 2B6 appears to give rise to very weak signals potentially due to poor ionization or aggregation of the inactivated enzyme; therefore, the deconvolution of the whole-protein mass spectrum gives rise to a relatively large error. P450 2B1 is more easily analyzed by whole-protein LC/MS. The increase in the mass of the P450 2B1 apoprotein upon inactivation with 17EE was consistent with binding of 17EE plus

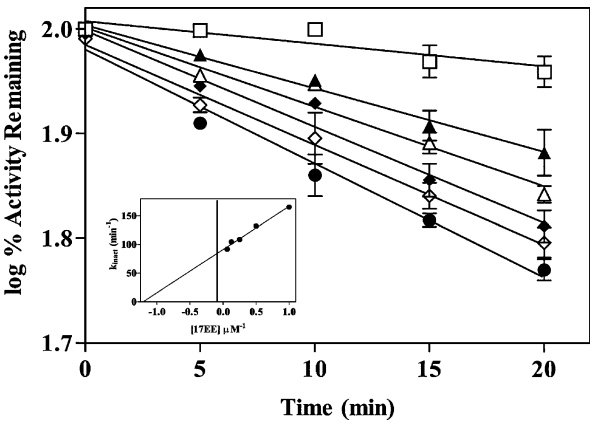


Figure 5.2 Time- and concentration-dependent inactivation of P450 2B6 7-ethoxycoumarin O-deethylation by 17-ethynylestradiol [from Kent et al. 2002b].

one oxygen atom (Kent et al. 2006). Cyanogen bromide digestion of inactivated P450 2B1 and 2B6 followed by Edman sequencing and MS analysis resulted in the identification of one adducted peptide for each protein. The authors proposed that serine 360 within this peptide was the amino acid residue modified since P450s 2B2 and 2B4 do not contain a serine at this position and they are not inactivated by 17EE. The structure of the adduct is hypothesized to be a serine ester generated from the ketene resulting from oxidation of the ethynyl group. However, the formation of this adduct has not been confirmed.

2-Hydroxyethynylestradiol formation is the primary pathway of P450 2B6-catalyzed 17EE metabolism (Kent et al. 2002b). Estriol, estrone, and two unidentified metabolites were also products of P450 2B6-mediated 17EE metabolism (Kent et al. 2002b). These same five metabolites were products of P450 2B1-catalyzed metabolism but all five were not products of P450 2B2- or P450 2B4-catalyzed metabolisms. The fact that the latter two enzymes are not inactivated during 17EE metabolism, led to the suggestion that one or both of the missing metabolites (one identified as estrone and one unidentified) might be the products of a nonbound reactive intermediate; that is, P450 2B6 and P450 2B1, but not P450s 2B2 and 2B4, catalyze the formation of the reactive metabolite responsible for enzyme inactivation. However, whether the lack of 17EE-mediated inactivation of P450s 2B2 and 2B4 is due to differences in metabolism or, as discussed in the preceding paragraph, the lack of binding to the critical residues in the active site of these two enzymes is unknown.

17EE-mediated inactivation on P450 2B6 meets all the criteria for mechanism-based inactivation and it has been shown that inactivation is due to binding of a reactive intermediate to an amino acid in the active site. What remains to be confirmed is whether or not serine 360 is the site of modification, and the structure of the amino acid adduct. The complete characterization of the protein adduct should aid in the identification of the reactive intermediate. This in turn should confirm if the differences in the sensitivity of P450 2B enzymes to 17EE-mediated inactivation are due to differences in the pathways of metabolism or the location and identity of amino acid residues in the active site.

5.3.2. Efavirenz

The reverse transcriptase inhibitor efavirenz is used in combination therapy for the treatment of HIV-1. In humans efavirenz undergoes oxidative hydroxylation to form primarily 8-hydroxyefavirenz, which may be further metabolized to 8,14-dihydroxyefavirenz (Mutlib et al. 1999). P450 2B6 is the principal catalyst of the 8-hydroxylation of efavirenz and the formation of 8,14-dihydroxyefavirenz (Ward et al. 2003). The possibility for drug interactions with efavirenz is high, since efavirenz is always used in combination therapy. In addition, patients receiving efavirenz frequently take herbal and nutritional supplements and/or drugs for the treatment of infections and other HIV-related disorders.

Efavirenz inactivated P450 2B6 in a time-, concentration-, and NADPH-dependent manner with a K_I of 30 μM and a $t_{1/2}$ of 16 min (Bumpus et al. 2005). The inactivation followed pseudo first-order

kinetics. The inactivation of P450 2B6 resulted in a significant loss in both the amount of spectrally measurable enzyme and the amount of native heme. Interestingly, the inactivation was completely reversible upon 24-hour dialysis, indicating that efavirenz is probably not a true MBI of P450 2B6. The most likely cause of reversible inactivation is the formation of a metabolite-intermediate (MI) complex; therefore, the authors looked for the characteristic absorbance maximum at 455 nm that is typical for a MI complex (Chatterjee and Franklin 2003). They did not observe an absorbance maximum at 455 nm, instead they observed what appears to be a type II binding spectra with an absorption maximum of 435 nm and a minimum of 418 nm (Bumpus et al. 2005). The mechanism of the reversible inactivation of P450 2B6 by efavirenz has not been yet characterized.

Interestingly, the primary metabolite of efavirenz, 8-hydroxyefavirenz, inactivated P450 2B6 in a time-, concentration-, and NADPH-dependent manner with kinetic parameters similar to those of efavirenz (Bumpus et al. 2005). However, unlike efavirenz, 8-hydroxyefavirenz gave rise to irreversible inactivation. Further studies on the inactivation of P450 2B6 by both efavirenz and 8-hydroxyefavirenz are currently ongoing (Namandjé Bumpus, personal communication).

5.3.3. N,N′,N″-Triethylenethiophosphoramide (tTEPA)

tTEPA (Figure 5.2) was developed in the 1950s as a nonspecific antineoplastic agent used in the treatment of breast, bladder, and ovarian cancer (Maanen, Smeets and Beijnen 2000). It is also used in high-dose chemotherapy regimens when bone marrow toxicity is not an issue – for example, in a bone marrow transplantation setting. tTEPA is metabolized to its primary metabolite *N*,*N*′,*N*″-triethylenephosphoramide (TEPA) by P450 2B6 and P450 3A4 *in vitro* (Jacobson et al. 2002). TEPA is the active metabolite of tTEPA and is formed by oxidative desulfuration of tTEPA. tTEPA is often co-administered with cyclophosphamide, a chemotherapeutic pro-drug that requires P450-mediated 4-hydroxylation to exert its alkylating activity. It was observed that if tTEPA is administered prior to cyclophosphamide, the levels of 4-hydroxycyclophosphamide are greatly reduced in the plasma (Huitema et al. 2000). Reduced metabolism of cyclophosphamide was also observed in human liver microsomes when tTEPA was co-incubated with cyclophosphamide.

Two laboratories have reported that tTEPA was an MBI of P450 2B6 (Harleton et al. 2004; Richter et al. 2005). Richter et al (2005) demonstrated time-, concentration-, and NADPH-dependent loss in P450 2B6 activity in human liver microsomes and in membranes containing overexpressed P450 2B6. They reported that P450 2B6 was the only P450 enzyme out of a panel of nine that was significantly inhibited by 10 μM tTEPA. Harleton et al. (2004) used purified P450 2B6 expressed in *Escherichia coli* in their studies and also observed time-, concentration-, and NADPH-dependent inactivation. They reported a higher K_I (50 μM) than Richter et al. (3.8 μM). However, the inactivation was not linear over time for the higher tTEPA concentrations used in the study by Richter et al.(2005). Nonlinearity can significantly change the apparent kinetic parameters and could possibly account for the 10-fold difference in K_I

between the two studies. A more important difference between these two studies may be the use of different substrates to determine the extent of inactivation. The substrate used by Harleton and co-workers was 7-ethoxycoumarin, and that used by Richter and co-workers was bupropion. In the characterization of MBIs of the rat P450 2B1 it was reported that different substrates in the secondary reaction resulted in different levels of enzyme inactivation (Kent et al. 2004). A similar phenomenon with P450 2B6 could give rise to the different kinetic parameters that have been reported for tTEPA-mediated inactivation of P450 2B6.

Richter (2005) and Harleton (2004) also reported that different mechanisms were responsible for the inactivation of P450 2B6 by tTEPA. Richter et al did not detect any loss in heme integrity after inactivation and therefore concluded that inactivation was due to binding of a reactive intermediate to the apoprotein. In contrast, Harleton et al concluded that inactivation occurred by heme destruction. They detected a significant loss of intact heme using three different methods: monitoring reduced CO spectrum, quantification by high-performance liquid chromatography (HPLC) with detection at 405 nm, and pyridine hemochrome analysis,. The loss of native heme observed with the latter two methods correlated with the loss of enzyme activity. Considering the differences in analysis and kinetic results between these two studies, it is possible that inactivation of P450 2B6 by tTEPA might occur through both heme and apoprotein modification. One could reasonably propose that due to differences in substrate orientation and size, an apoprotein adduct may not result in a detectable loss in 7-ethoxycoumarin activity (measured by Harleton) but might significantly inhibit bupropion activity (measured by Richter). Therefore, Harleton et al would detect enzyme inactivation only at higher concentration of tTEPA that resulted in significant heme destruction.

These studies should draw attention to the need to be aware of the potential effect of tTEPA-mediated inactivation of P4502B6 on the efficacy of co-administered drugs (such as cyclophosphamide) that are metabolized by P450 2B6. However, based on the differing outcomes of the two studies discussed above, it is critical to determine the affect of tTEPA-mediated inactivation of P450 2B6 on cyclophosphamide 4-hydroxylation.

5.3.4. Grapefruit Juice and Bergamottin

The first clinical study of the effects of grapefruit juice on the pharmacokinetics of drugs was published in 1991 (Bailey et al. 1991). Since then many studies on grapefruit juice–drug interactions have been published including several reviews on the topic (Bailey and Dresser 2004; Dahan and Altman 2004; Greenblatt et al. 2001; Guo and Yamazoe 2004; Saito et al. 2005). Bergamottin, 6′,7′-dihydroxybergamottin, and bergapten are major furanocoumarin components in grapefruit juice and were suggested to play a role in the observed grapefruit juice–drug interactions. All three compounds have been identified as MBIs of P450 3A4 *in vitro* (He et al. 1998; Schmiedlin-Ren et al. 1997; Tassaneeyakul et al. 2000). The major mechanism of grapefruit juice interactions with most drugs is considered to be inactivation of intestinal P450 3A4 by 6′,7′-dihydroxybergamottin (Paine, Criss and Watkins 2005).

Recently, Lin et al. reported that bergamottin was an MBI of P450 2B6 (Lin, Kent and Hollenberg 2005). The inactivation was irreversible, and time-, concentration-, and NADPH-dependent. The K_I was 5 μM, but in the presence of cytochrome b_5 decreased to 0.2 μM. The partition coefficient for the inactivation of P450 2B6 by bergamottin was 2. The low K_I and partition ratio clearly demonstrate that bergamottin is a potent inactivator of P450 2B6; the most potent identified to date. Bergamottin appears to be inactivating P450 2B6 by both heme destruction and apoprotein adduct formation. The ratio of ^{14}C-bergamottin metabolite bound to apoprotein was determined to be 0.5–1. The fact that this value is less than 1:1 is likely due to the contribution of heme destruction to enzyme inactivation. As in other studies of the mechanism-based inactivation of P450 2B6, it was not possible to determine the exact mass of the apoprotein adduct by LC/MS analysis due to large errors in the mass assignment. From the LC/MS data that was obtained, the adduct appeared to contain bergamottin with two or three oxygen atoms attached to it. The potency of inactivation of P450 2B6 by bergamottin is as great, or greater than that of P450 3A4 by either bergamottin or 6′,7′-dihydroxybergamottin (He et al. 1998; Schmiedlin-Ren et al. 1997; Tassaneeyakul et al. 2000). Therefore, grapefruit juice may significantly influence drugs that are primarily metabolized by P450 2B6 as well as those metabolized by P450 3A4.

5.3.5. Phencyclidine

Phencyclidine, PCP or angel dust, became a popular drug of abuse in the 1960s. PCP was developed in the mid-1950s by Parke-Davis as an anesthetic, but due to severe psychotic side effects never made it to the market. Mechanism-based inactivation of P450 enzymes by PCP was first reported in studies with liver microsomes from phenobarbital-treated rabbits (Hoag et al. 1984). More recently, mechanism-based inactivation of human P450 2B6 was reported (Jushchyshyn, Kent and Hollenberg 2003). PCP met all the criteria for a MBI of P450 2B6 and the mechanism of inactivation appeared to be through formation of adducts to the apoprotein. The stoichiometry of binding of a PCP reactive metabolite to the P450 apoprotein was approximately 5.5 nmol PCP per nmol P450. However, when glutathione was present in the reaction mixture the stoichiometry of binding was reduced to 1:1. Neither glutathione nor CN^- was able to protect the enzyme from inactivation. The reactive intermediates responsible for the loss in activity are not trapped by glutathione, giving rise to the 1:1 stoichiometry observed in the presence of glutathione.

The formation of covalent modification of biological macromolecules during the metabolism of PCP was first observed with rabbit liver microsomes (Ward et al. 1982). The PCP iminium ion was proposed as the reactive metabolite responsible. Hoag et al. (1984) investigated the mechanism-based inactivation of rabbit liver microsomal enzymes by PCP and concluded that the iminium ion required further metabolism to inactivate these enzymes. They later showed that the PCP iminium ion was metabolized by rabbit liver microsomes in a P450-dependent manner and that this metabolism resulted in inactivation of one or more P450 enzymes (Hoag et al. 1987). Similar results have been reported with purified rat

P450 2B1 (Crowley and Hollenberg 1995). While the PCP iminium ion was the major PCP metabolite formed by the rodent P450s, P450 2B6 metabolized PCP primarily through oxidation of the cyclohexane ring (Jushchyshyn et al. 2006). The PCP iminium ion was a minor product of P450 2B6-catalyzed PCP metabolism. Interestingly, P450 3A4 and 2D6 were not inactivated by PCP although they metabolize PCP primarily to the iminium ion (Jushchyshyn et al. 2006). These data suggest that the iminium ion might not be the precursor to the reactive intermediate that inactivates P450 2B6, and it has been suggested that the true inactivating metabolite is a product of carbon hydroxylation on the PCP cyclohexane ring (Jushchyshyn et al. 2006). So despite much efforts the mechanisms of phencyclidine-mediated P450 inactivation have not yet been elucidated.

Among the other seven compounds that have been identified as MBIs of P450 2B6 (Table 5.1), three are drugs, clopidogrel, ticlopidine and tamoxifen (Richter et al. 2004; Sridar et al. 2002). These three are relatively potent, but not specific inactivators of P450 2B6; K_i values are less than 1μM. In all cases, the integrity of the heme molecule was unaffected by enzyme inactivation. Therefore, it has been proposed that the mechanism of inactivation is apoprotein modification, but no direct evidence of a protein adduct has been obtained. The mechanism of P450 2B6 inactivation for all the compounds that have been studied appears to be at least in part due to covalent modification of the apoprotein, although heme destruction clearly plays a role for some. However, neither a protein nor a heme adduct of P450 2B6 has yet been characterized.

5.4. Inactivation of Cytochrome P450 2A Enzymes

Relatively few MBIs of P450 2A6 and P450 2A13 have been identified (Table 4.1). This is partially due to the fact that these enzymes play a minor role in drug metabolism (Guengerich 2003). Only a few drugs on the market, such as valporic acid, lisigamone, 3,5-dimethyl-2-(3-pyridyl)-thiazolidin-4-one (SM-12502), and pilcarbine (Endo et al. 2007; Pelkonen et al. 2000) are primarily metabolized by P450 2A6. However, P450 2A6 is the major catalyst of nicotine metabolism (Hukkanen, Jacob III and Benowitz 2005) and both P450 2A6 and P450 2A13 are efficient catalysts of the bioactivation of a number of carcinogens (Camus et al. 1993; He et al. 2006; Jalas, Hecht and Murphy 2005; Wong et al. 2005a; Wong et al. 2005b). Therefore, MBIs of P450 2A6 and P450 2A13 have the potential to be potent cancer chemopreventive agents.

There are three members of the P450 2A subfamily in humans: P450 2A6, P450 2A13, and P450 2A7, an orphan P450 enzyme (Guengerich 2003; Su et al. 2000). P450 2A13 and P450 2A6 are 94.5% identical, differing by only 32 amino acids. P450 2A6 is primarily expressed in the liver, and P450 2A13 is expressed throughout the respiratory tract but not in the liver. These two closely related enzymes catalyze the metabolism of a number of common substrates; for example, both are efficient coumarin 7-hydroxylases. However, with other substrates, the metabolic efficiency of P450 2A6 and P450 2A13 often differs (Brown, von Weymarn and Murphy 2005; Jalas, Hecht and Murphy 2005; Murphy, Raulinaitis and Brown

2005; Su et al. 2000; Wong, Murphy and Hecht 2005). One of the more striking differences was observed for the metabolic activation of the tobacco carcinogen NNK (Su et al. 2000). P450 2A13 is a more than 500-fold more efficient catalyst of NNK metabolism than is P450 2A6. In addition, the site of substrate oxidation sometimes differs between these two enzymes. For example, P450 2A6 catalyzes the metabolism of coumarin exclusively by 7-hydroxylation, whereas P450 2A13 efficiently catalyzes both 7-hydroxylation and 3′, 4′-epoxidation of coumarin (von Weymarn and Murphy 2003).

P450 2A6 catalyzes the conversion of nicotine, the primary addictive agent present in tobacco, to cotinine, a nonaddictive metabolite (Hukkanen, Jacob III and Benowitz 2005). Inactivation of P450 2A6 should lead to slower elimination of nicotine in smokers, and since smokers are believed to titrate their smoking to maintain a minimum plasma nicotine concentration, the treatment with a P450 2A6- specific MBI might lead to a decrease in smoking (Sellers, Tyndale and Fernandes 2003b). The use of 8-methoxysporalen (8-MOP) for this purpose has been investigated and will be discussed below (Sellers, Kaplan and Tyndale 2000). A decrease in smoking, or the use of other tobacco products, would necessarily decrease exposure to tobacco carcinogens and could therefore lower cancer risk. In addition, P450 2A13 is likely the primary catalyst of NNK metabolism in the lungs of smokers (Jalas, Hecht and Murphy 2005; Wong et al. 2005). NNK is a lung carcinogen. Therefore, inactivation of P450 2A13 by a potent and specific MBI might lead to a lower risk of lung cancer, and the development of inactivators of P450s 2A6 and 2A13 may be a valid chemoprevention approach with great potential to lower the lung cancer risk in smokers.

Only a handful of MBIs of either P450 2A6 or P450 2A13 have been studied in any detail: they are listed in Table 5.1. Two additional compounds not listed, isoniazid and valporic acid, have been reported to be MBIs of P450 2A6 based on NADPH- and time-dependent inactivation, but no further characterization has been carried out (Wen et al. 2001; Wen et al. 2002). The most potent MBI of P450 2A6 is (R)-(+)-menthofuran (Figure 5.3), a component of peppermint oil (Bertea et al. 2001) and a toxic

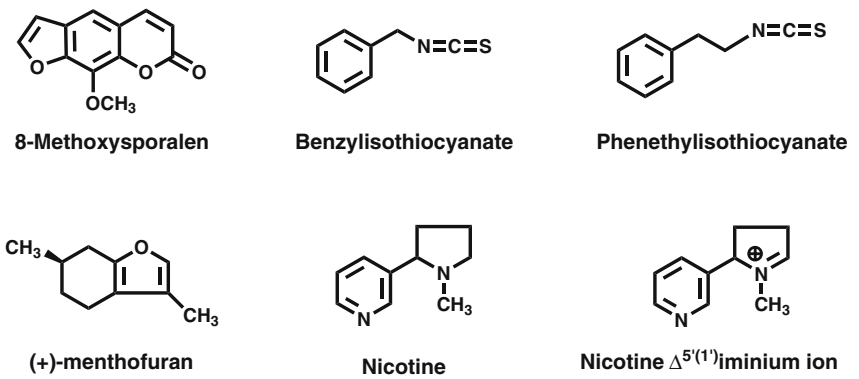


Figure 5.3 Mechanism-based inactivators of P450 2A6 and P450 2A13.

metabolite of the abortifacient (R)-(+)-pulegone. The K_I of inactivation was ~1.0 μM; k_{inact} was 0.2 min^{-1}, the partition ratio was ~3.5, and covalent modification of the apoprotein was detected (Khojasteh-Bakht et al. 1998). Menthofuran was highly selective for P450 2A6; P450s 1A2, 2D6, 2E1, and 3A4 were not inactivated at concentrations significantly higher than those that lead to inactivation of P450 2A6.

5.4.1. 8-Methoxypsoralen

The furanocoumarin 8-MOP is, like menthofuran, a potent MBI of P450 2A6, with a K_I of 0.9μM and $k_{inact.}$ of 1 min^{-1} (Koenigs et al. 1997). The partition ratio was 21, about sixfold greater than that for menthofuran. Inactivation of the enzyme occurred by formation of a covalent adduct to the apoprotein, which was detected using [^{14}C]-8-MOP (Koenigs and Trager 1998). The reactive metabolite responsible for adduct formation was proposed to be a furanoepoxide; however, the structure of the adduct has yet to be identified. More recently, we reported that 8-MOP is both an inhibitor and an MBI of P450 2A13 (von Weymarn et al. 2005). Interference from potent inhibition of P450 2A13 by 8-MOP did not allow us to determine the kinetic parameters of inactivation. However, the irreversible time-dependent inactivation of P4502A13 by 8-MOP was demonstrated by using spin-column gel filtration to remove residual 8-MOP. P450 2A13, like 2A6, was inactivated through the formation of adducts to the apoprotein. Covalently modified P450 2A13 protein was detected by electrospray LC/MS analysis of P450 2A13 following 70% inactivation by 8-MOP. The analysis detected both native P450 2A13 (theoretical mass 56,665) and a co-eluting protein (theoretical mass of 56,888) (Figure 5.4). The 232 mass difference between the proteins corresponds to the addition of one molecule of 8-MOP with an additional oxygen atom. This mass is consistent with the formation of a covalent adduct between P450 2A13 and the furanocoumarin reactive metabolite proposed by Koenigs and Trager (1998).

In experiments with human liver microsomes and enzyme-specific substrates Koenigs et al. (1997) demonstrated that in addition to P450 2A6, P450s 2C19 and P450 2E1 were inactivated by 8-MOP. In comparison to P450 2A6, inactivation of these enzymes was very modest. Therefore, at low concentrations 8-MOP appears to be a specific and potent MBI of both P450 2A6 and 2A13. Sellers et al reported that 8-MOP inhibited P450 2A6-dependent clearance of nicotine *in vivo* in human subjects (Sellers, Kaplan and Tyndale 2000). They also reported that 8-MOP increased the amount of NNK derived from cigarette smoke that was detoxified through NNAL-glucuronidation (Sellers et al. 2003a). The observed increase in NNK detoxification was attributed to a decrease in P450 2A6-mediated NNK bioactivation. However, the interpretation of these data may be more complicated. 8-MOP is a potent inhibitor of P450 1A2 (Zhang et al. 2001), and P450 1A2 may contribute significantly to hepatic NNK metabolism (Jalas, Hecht and Murphy 2005). Therefore, the observed increase in NNAL-glucuronidation could be due to 8-MOP-mediated inhibition of P450 1A2-catalyzed NNK metabolism, not 8-MOP-mediated inactivation of P450 2A6.

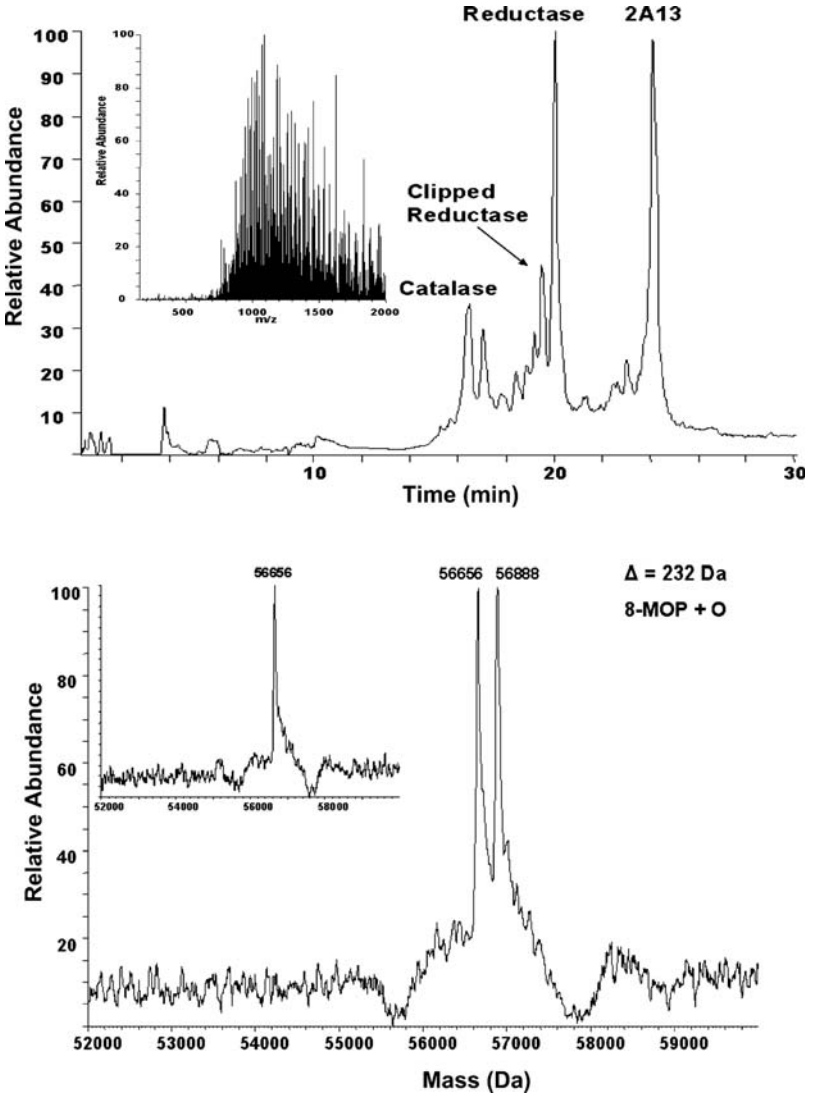


Figure 5.4 Electrospray LC/MS analysis P450 2A13 apoprotein following inactivation by 8-methoxypsoralen. *Top panel*: Total ion chromatograph of inactivated sample. *Bottom panel:* Deconvoluted spectrum of inactivated P450 2A13 (insert is an exposed control sample) (from von Weymarn et al. 2005).

5.4.2. Benzylisothiocyanate and Phenethylisothiocyanate

Isothiocyanates have been studied in some detail as chemopreventive agents of NNK-induced lung carcinogenesis, and one mechanism of action is inhibition of P450s (Hecht 2000). Isothiocyanates can inhibit P450 activity by three different mechanisms: (1) competitive inhibition, (2) inactivation without P450 catalysis, or (3) as a mechanism-based inactivator (Goosen, Mills and Hollenberg 2001; Hecht 2000; Nakajima et al. 2001). These multiple pathways of inhibition complicate the analysis of isothiocyanates as potential MBIs. However, we have demonstrated that

both benzylisothiocyanate (BITC) and phenethylisothiocyanate (PEITC) inactivate P450 2A6 and 2A13 in a time- and NADPH-dependent manner (von Weymarn, Chun and Hollenberg 2006b). This is in contrast to a previous study that reported that PEITC inhibited, but did not inactivated, P450 2A6 (Nakajima et al. 2001). The kinetic parameters for the inactivation of P450 2A6 by BITC were determined. The apparent K_I of 28 μM and a k_{inact} of 0.055 min^{-1} are similar to what has been reported for P450s 2B1 and 2E1 (Goosen, Mills and Hollenberg 2001; Moreno et al. 2001). However, due to the potent inhibition of P450 2A13 by BITC, and inhibition of both P450 2A6 and P450 2A13 by PEITC, the kinetic parameters for the inactivation of P450 2A16 and 2A13 by these isothiocyanates could not be determined.

The inactivation of P450s 2A6 and 2A13 by BITC and PEITC was irreversible and both compounds inactivated the enzymes exclusively through the formation of adducts to the P450 apoprotein (von Weymarn, Chun and Hollenberg 2006b). LC/MS analysis of the inactivated P450s identified a covalently modified enzyme with an adduct that corresponded to the isothiocyanate plus one oxygen atom. Nonspecific binding of the isothiocyanate alone to the enzymes was also observed both in the absence and presence of NADPH. Due to the potent inhibition of P450s 2A6 and 2A13 by BITC and PEITC, not all the criteria of mechanism-based inactivation have been tested. However, the observed time- and NADPH-dependent irreversible activity loss, and the NADPH-dependent formation of covalent adducts to the P450 apoprotein, together with the published data characterizing BITC and PEITC as MBIs of other P450 enzymes lead us to conclude that BITC and PEITC are MBIs of both P450 2A6 and P450 2A13.

5.4.3. Nicotine and Nicotine $\Delta^{5'(1')}$iminium ion

As noted above, P450 2A6 is the primary catalyst of nicotine metabolism. The major pathway of nicotine metabolism in smokers is conversion to cotinine and subsequent metabolism to trans 3′-hydroxycotinine. The first step in the formation of cotinine is P450 2A6-catalyzed 5′-oxidation to generate the nicotine $\Delta^{5'(1')}$iminium ion (Hukkanen, Jacob III and Benowitz 2005). P450 2A6 also catalyzes the 2′ and methyl oxidation of nicotine (Figure 5.5), but these are minor pathways. Conversion of the nicotine $\Delta^{5'(1')}$iminium ion to cotinine may be catalyzed by aldehyde oxidase or P450 2A6 (Hukkanen, Jacob III and Benowitz 2005; von Weymarn, Brown and Murphy 2006b). *In vivo*, cotinine is primarily metabolized to *trans* 3′-hydroxycotinine. However, *in vitro* both P450 2A6 and P450 2A13 also catalyze the formation of 5′-hydroxycotinine and *N*-hydroxymethylnorcotinine (Figure 5.5) (Brown, von Weymarn and Murphy 2005). Surprisingly, both P450 2A6 and P450 2A13 rather quickly catalyzed the oxidation of nicotine all the way to *trans*-3′-hydroxycotinine, 5′-hydroxycotinine, and *N*-hydroxymethylnorcotinine (Murphy, Raulinaitis and Brown 2005; von Weymarn, Brown and Murphy 2006a). We have suggested that sequential oxidation of nicotine may occur without the intermediate products leaving the active site of the enzyme.

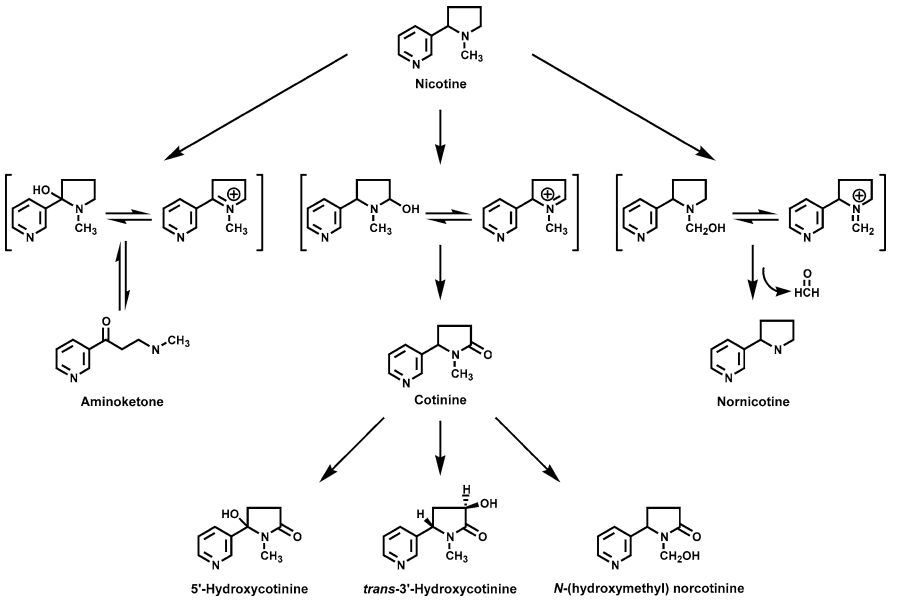


Figure 5.5 P450 2A6 and P450 2A13-catalyzed nicotine metabolism pathways.

We recently reported that nicotine irreversibly inactivates both P450 2A6 and 2A13 (von Weymarn, Brown and Murphy 2006a). The inactivation of P450 2A13 by nicotine was significantly faster than that of P450 2A6; the $t_{1/2}$ values are 7 min and 33 min, respectively. The K_I for the inactivation of P450 2A13 by nicotine is 17 μM. The K_I for the inactivation of P450 2A6 was estimated to be 21 μM. This is an estimate since inactivation was only linear over a very narrow range. The partition ratio for the inactivation of P450 2A13 by nicotine was 33. No partition ratio was determined for P450 2A6, since during the relatively long time required for complete inactivation of P450 2A6 there was significant activity loss in the absence of nicotine. Both enzymes were protected from nicotine-mediated inactivation by alternate substrates. P450 2A13 inactivation was irreversible and the exogenous nucleophile glutathione had no effect on the inactivation. Irreversibility and the effect of exogenous nucleophiles were not tested with P450 2A6. The mechanism of inactivation was investigated using [^{3}H]-nicotine; however, no adducts to either the heme or the apoprotein were observed. It was proposed that the adducts may be unstable to the denaturing acidic conditions of the HPLC analyses.

Interestingly, significant inactivation of both P450 2A6 and P450 2A13 occurred after nicotine metabolism was complete (von Weymarn, Brown and Murphy 2006a). Under conditions where 90% of the nicotine was metabolized in 2 minutes, there was only a 20% loss in activity. However, the extent of P450 2A13 inactivation continued to increase for the next 15 minutes. During those 15 min the primary metabolite, the nicotine $\Delta^{5'(1')}$iminium ion, was further metabolized to cotinine, and cotinine was metabolized to *trans* 3′-hydroxycotinine, 5′-hydroxycotinine, and *N*-hydroxymethylnorcotinine. These data lead us to suggest that the reactive species responsible for nicotine-mediated enzyme inactivation was formed during

the oxidation of the $\Delta^{5'(1')}$iminium ion to cotinine. This would suggest that the nicotine $\Delta^{5'(1')}$iminium ion is an MBI of P450 2A6 and P450 2A13. We have observed time-, concentration-, and NADPH-dependent inactivation of both P450 2A6 and 2A13 by the nicotine $\Delta^{5'(1')}$iminium ion (unpublished data). The nicotine $\Delta^{5'(1')}$iminium ion did not inactivate either P450 2A6 or P450 2A13 in the absence of NADPH. Experiments are ongoing to determine the mechanism of inactivation and to begin to characterize the effects of this inactivation *in vivo* in smokers.

In summary, a number of compounds have been identified as MBIs of P450 2A6 and P450 2A13, and certainly more will be characterized in the future. It is likely that a component of grapefruit juice is an MBI of P450 2A6 (Tassaneeyakul et al. 2000) and that this compound may contribute to the observed affect of grapefruit juice on nicotine clearance (Hukkanen, Jacob III and Benowitz 2006). Mechanism-based inactivators of P450 2A6 and P450 2A13 may be useful compounds for both treating tobacco addiction and as cancer chemopreventive agents. Based on our knowledge MBIs of P450 2A enzymes, nicotine analogs and furan coumarin derivatives appear to be the type of compounds that should be explored for this purpose. It has been demonstrated in a mouse model that NNK-induced lung tumorigenesis was inhibited by 8-MOP (Takeuchi et al. 2003). Presumably the protection from NNK-induced tumors was due to either inhibition or inactivation of P450 2A5, the mouse orthologue of P450 2A6 and 2A13. P450 2A5 is an excellent catalyst of NNK bioactivation (Jalas, Hecht and Murphy 2005). Also, as discussed above, smokers administered 8-MOP decreased the number of cigarettes they smoked (Sellers, Kaplan and Tyndale 2000).

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