Mechanism-Based Inactivation of Cytochrome P450 3A4 by 4-Ipomeanol

Teresa M. Alvarez-Diez and Jiang Zheng*

Department of Pharmaceutical Sciences, School of Pharmacy, Bouvé College of Health Sciences, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115

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Earlier phase I and II clinical studies showed that 4-ipomeanol produced selective hepatotoxicity. To investigate the mechanism of bioactivation of 4-ipomeanol, we thoroughly studied the interaction of 4-ipomeanol with human cytochrome P450 3A4 (EC 1.14.14.1). 4-Ipomeanol produced a time- and concentration-dependent inactivation of P450 3A4. More than 80% of the P450 3A4 activity was lost after its incubation with 4-ipomeanol at the concentration of 75 μM in 12 min. The inactivation was characterized by a rate of inactivation (k_{inact}) of 0.15 min^{-1} and by an inactivation potency (K_I) of 20 μM. In addition, the inhibition of P450 3A4 by 4-ipomeanol was NADPH-dependent and irreversible. Glutathione, catalase, and superoxide dismutase failed to protect P450 3A4 from inactivation by 4-ipomeanol. The presence of testosterone, a substrate of P450 3A4, protected the enzyme from inactivation. The estimated partition ratio of the inactivation was approximately 257. Covalent binding studies demonstrated that reactive metabolites of 4-ipomeanol modified P450 3A4 but not P450 reductase (EC 1.6.2.4). The stoichiometry of binding between reactive metabolites of radiolabeled 4-ipomeanol and P450 3A4 was approximately 1.5:1. In addition to P450 3A4, reactive metabolites of 4-ipomeanol were found to covalently bind to other proteins. 4-Ipomeanol failed to inactivate P450 1A2 in human liver microsomes. In conclusion, 4-ipomeanol irreversibly inhibited P450 3A4, and it was characterized as a mechanism-based inactivator of P450 3A4. This finding facilitates the understanding of the mechanism of bioactivation of 4-ipomeanol by human hepatic enzymes.

Introduction

4-Ipomeanol (**1**), 1-(3-furyl)-4-hydroxypentanone, is a natural cytotoxin first isolated by Boyd et al. in 1972 from *Ipomoea batatas* (sweet potatoes) infected with *Fusarium solani* (*1*, *2*). Extensive investigations have demonstrated that 4-ipomeanol is a lung specific toxin in animals and that its toxicity is initiated through bioactivation catalyzed by cytochrome P450 enzymes (*3*−*9*). Pulmonary P450 4B1 of rats and rabbits was shown to bioactivate 4-ipomeanol (*10*, *11*). It was also suggested that covalent binding of reactive metabolites derived from 4-ipomeanol to cellular proteins results in lung cytotoxicity, since preclusion of bioactivation resulted in absence of toxicity (*6*, *12*−*15*).

Animal studies showed that 4-ipomeanol was bioactivated in the lung. Promising anticancer effects observed in cell culture and animal models (*16*−*18*) led to phase I clinical trials using 4-ipomeanol as a potential chemotherapeutic agent (*19*, *20*). Surprisingly, these studies have shown unexpected hepatotoxicity, which prevented further development of 4-ipomeanol in lung cancer therapy. A phase II clinical study of 4-ipomeanol in patients with hepatocellular carcinoma also reported selective liver toxicity (*21*). Currently, 4-ipomeanol is being investigated as a potential prodrug for P450-directed gene therapy of liver and brain cancers by other groups (*22*, *23*). Successful targeting of brain tumors transfected with P450 4B1 has been reported in animal studies (*23*).

The mechanisms by which 4-ipomeanol produces human liver toxicity remain unknown. It has been speculated that the difference in the target organ specificity of 4-ipomeanol between humans and other mammal species studied could be due to the differences in their P450 enzymes (*19*). A study has shown that 11 human P450 enzymes are relatively less efficient in biotransforming 4-ipomeanol into reactive metabolites covalently bound to DNA when compared to animal P450 enzymes (*24*). Among human P450 enzymes, the most active isoforms in biotransforming 4-ipomeanol were P450 1A2, P450 3A3, and P450 3A4,[1] which are hepatic enzymes. This study helps to explain in part the differences in organ selectivity between animals and humans. However, activity tests of human P450 enzymes with 4-ipomeanol have not yet been reported. Characterization of the interaction between 4-ipomeanol and human hepatic P450 enzymes is a critical step in understanding the bioactivation of 4-ipomeanol in liver.

A number of furan-containing compounds were found to be mechanism-based inactivators of P450 enzymes (*25*−*29*). We reasoned that 4-ipomeanol, a furan com-

* To whom correspondence should be addressed. Tel: + 1-617-373-5258. Fax: +1-617-373-8886. E-mail: J.Zheng@neu.edu.

[1] Abbreviations: DBF, dibenzylfluorescein; dpm, disintegrations per minute; GSH, glutathione; 3-MC, 3-methylcholanthrene; NADPH, β-nicotinamide adenine dinucleotide phosphate; P, partition ratio; P450 3A4, cytochrome P450 3A4; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TIC, total ion chromatogram.

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pound, could be an inactivator of human P450 enzymes. Preliminary screening tests in human microsomes, aimed to test the potential inhibitory effect of 4-ipomeanol on four major P450 enzymes of liver, showed that P450 1A2, P450 2D6, and P450 2C9 were not inactivated by 4-ipomeanol metabolism and that by contrast P450 3A4 was significantly inactivated in a time-dependent manner. These observations led us to further investigate in depth the P450 3A4 inactivation produced by 4-ipomeanol metabolism using the expressed human enzyme. This study is the focus of our report. Results from the activity test of human P450 1A2 are also presented here, since this enzyme has been shown to be important in 4-ipomeanol's bioactivation. Screening tests of the other human hepatic P450 isoforms tested are available in the Supporting Information.

P450 1A2 has been postulated to be involved in the bioactivation of 4-ipomeanol in rats pretreated with 3-MC prior to the administration of 4-ipomeanol, which resulted in liver toxicity (*10*, *30*). In liver, 3-MC induces P450 1A2, which is predominantly a hepatic enzyme in rats. Increased covalent binding of reactive species of 4-ipomeanol to P450 proteins was also observed using liver microsomes of rats induced with 3-MC (*13*, *30*−*31*). In humans, P450 1A2 is specific for liver and has been reported to have similar substrate specificities to rat P450 1A2 with some exceptions (*32*). Human P450 1A2 has been shown to be a metabolizing enzyme of 4-ipomeanol since it metabolizes it into reactive metabolites that bind to DNA (*24*). However, to our knowledge, no inactivation tests of this P450 isoform with 4-ipomeanol have been previously reported. In our study, we did not observe inactivation of human P450 1A2.

We found that 4-ipomeanol irreversibly inhibits P450 3A4 (EC 1.14.14.1). 4-Ipomeanol is characterized as a mechanism-based inactivator of this isoform. P450 3A4 is the most abundant P450 isoform in human liver and is responsible for biotransformation of over 60% of clinically relevant drugs and biosynthesis of several endogenous compounds (*33*−*40*). The finding of mechanism-based inactivation of P450 3A4 by 4-ipomeanol may provide insights into the mechanism of its toxic action.

Materials and Methods

Chemicals. Ethyl-β-oxo-3-furan-propionate, propylene oxide, anhydrous ethanol, and hyamine (benzethonium) hydroxide were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). 7-Benzyloxy-4-(trifluoromethyl)coumarin and DBF were obtained from Gentest Co. (Woburn, MA). Methoxyresorufin, β-NADPH reduced form, and GSH were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H(G)]-4-ipomeanol, with a specific activity at the time of use of 8 μCi/mg and a radiochemical purity >99%, was generously provided by Dr. Alan L. Buckpitt, University of California Davis (Davis, CA). Novex 10% tris-glycine SDS gels were obtained from Invitrogen (Carlsbad, CA). Precision Plus unstained protein standards (10−250 kDa), Laemmli sample buffer, and Bio-Safe Coomassie blue stain were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Scintiverse liquid scintillation counting cocktail was from Fisher Scientific (Atlanta, GA).

Enzymes. Pooled human hepatic microsomes were purchased from Gentest Co. (St. Louis, MO). Human recombinant P450 3A4 expressed in *Escherichia coli* bacterial cultures according to previously described procedures (*41*) was a generous gift of Dr. Magang Shou, Merck Research Laboratories (West Point, PA). The membrane fractions expressing P450 3A4 were isolated from the bacterial cell pellets by differential centrifugation (*41*). Rat recombinant NADPH−P450 reductase (EC 1.6.2.4) expressed in *E. coli* bacterial membranes following published procedures (*42*, *43*), also a gift from Merck, was supplementally added to expressed human P450 3A4, and the mixture was used in the P450 3A4 inactivation assays. These enzymes were mixed in a 1:1 molar ratio. Expressed human P450 3A4 in insect cell membranes with supplemental c-DNA expressed human NADPH−P450 reductase (P450 3A4 Supersomes) from Gentest Co. was used to study covalent modification of P450 3A4 by [^{3}H]-4-ipomeanol. In this system, the P450 3A4/reductase molar ratio was 1:10. Catalase (from bovine liver) and SOD (from bovine liver) were obtained from Sigma Chemical Co.

Instruments. A 300 MHz NMR spectrometer (Variant Mercury-300, Variant, Palo Alto, CA) was utilized for structure identification. An API 2000 triple−quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) coupled inline with an Agilent LC system and equipped with a heated electrospray source was used for structure analysis. SpectraMAX Gemini XS (Molecular Devices Co., Sunnyvale, CA), a microplate dual-scanning fluorescent spectrophotometer, was used for enzyme assays and enzyme kinetics.

Synthesis of 4-Ipomeanol. 4-Ipomeanol was synthesized under anhydrous conditions following a two step synthetic method previously described (*2*, *44*) with some modification. Briefly, ethyl-β-oxo-3-furan-propionate (18 mmols) was mixed with a 12 M excess of propylene oxide in EtONa solution followed by overnight stirring at room temperature. The mixture was brought to pH 7.0 with diluted HCl and extracted with chloroform (3 × 50 mL). A furyl-γ-lactone intermediate was obtained in 51% yield after purification by flash chromatography on silica gel. In the second step, the resulting lactone was hydrolyzed and spontaneously decarboxylated at room temperature by treatment with 10.0 N sulfuric acid for 20 h, giving 4-ipomeanol in 80% yield, after purification by silica gel flash chromatography. The structure of 4-ipomeanol was confirmed by ^{1}H NMR. The purity of 4-ipomeanol was ≥ 99% as observed by reverse phase HPLC analysis using a mass spectrometer detector. In the TIC acquired, the area under the peak, which had the expected mass of 4-ipomeanol, accounted for ≥99% of the total area (Supporting Information). 4-Ipomeanol was obtained as a racemic mixture.

P450 1A2 Assay. Human hepatic microsomal samples (1 mg of protein) in 0.1 M potassium phosphate buffer (pH 7.4) were incubated with 4-ipomeanol in 10 μL of methanol or vehicle (control) and 10 mM NADPH at 37 °C for 0, 6, 13, and 20 min. The volume of the reaction mixtures was 0.5 mL. At these times, 40 μL aliquots of the primary reaction mixtures in triplicate were transferred to a 96 well microplate containing a secondary assay mixture consisting of 0.25 mM NADPH and 7.0 μM 7-methoxyresorufin substrate in 0.1 M potassium phosphate buffer (pH 7.4) with traces of acetonitrile. The final concentration of 4-ipomeanol was 45 μM. The final volume of the incubation was 200 μL/well. *O*-demethylation of 7-methoxyresorufin was measured spectrophotometrically during a 2 min reading interval using an excitation wavelength of 530 nm and a wavelength of emission of 585 nm.

Time- and Concentration-Dependent Inactivation of P450 3A4. A 1 mL sample of a stock mixture containing human P450 3A4 (2.0 μM) expressed in *E. coli* membranes with supplemental NADPH−P450 reductase (2.0 μM), also expressed in *E. coli* membranes, was thawed at room temperature and diluted (1:4) with 0.25 M potassium phosphate buffer (pH 7.4). Primary reaction mixtures, which contained 0.1 μM P450 3A4, 0.1 μM NADPH−P450 reductase, increasing concentrations of 4-ipomeanol dissolved in ethanol, and 0.2 M potassium phosphate buffer (pH 7.4) were prepared. The total content of alcohol was 0.6%. These reaction mixtures were kept on ice until the reactions were initiated by adding NADPH at a final concentration of 0.45 mM (primary reaction). NADPH was excluded in negative control incubations. After incubation at 37 °C in a water bath shaker for 0, 4, 8, and 12 min, 45 μL aliquots (5 pmols of P450 3A4) were withdrawn from the primary reaction

mixtures and transferred to secondary reaction mixtures placed in a 96 well microplate. Secondary reaction mixtures consisted of 0.32 mM NADPH and 13.5 μM DBF in 0.2 M potassium phosphate buffer (pH 7.4) with a trace of acetonitrile. The final concentrations of 4-ipomeanol used were as follows: 0 (control), 10, 25, 50, 75, and 100 μM. The final volume of the incubation in the 96 well microplate was 200/μL well. Fluorescence was measured at 37 °C for 2 min at intervals of 10 s (kinetic mode) with excitation at 485 nm and emission at 538 nm. All wells contained the same amount of reagent alcohol (approximately 0.1%), and the assays were performed in triplicate.

The semilogarithmic plots of the remaining P450 3A4 activity vs the preincubation time were prepared. Observed rate constants (k_{obs}) for the inactivation of P450 3A4 by 4-ipomeanol were calculated from the slopes of the linear regression lines of these plots. The double-reciprocal plot of k_{obs} and 4-ipomeanol concentrations was prepared in order to calculate kinetic constants K_I and k_{inact}, which are the concentration of inhibitor that produces a half-maximal rate of inactivation and the maximal rate constant of inactivation, respectively (*45*).

Substrate Protection. Substrate protection from 4-ipomeanol-dependent inactivation of P450 3A4 was studied by including 2.6 M excess testosterone (130 μM) together with 50 μM 4-ipomeanol (final concentrations) in the primary reaction mixtures. After incubation at 37 °C for 0, 4, and 10 min, aliquots (45 μL) in triplicate were taken from the primary mixtures and transferred into a secondary mixture containing 13.5 μM DBF and 0.32 mM NADPH in 0.2 M potassium phosphate buffer. The final volume was 200 μL/well. Control incubations (lacking both 4-ipomeanol and testosterone), incubations with testosterone but without 4-ipomeanol, and incubations containing a 0:1 molar ratio of testosterone/4-ipomeanol were also prepared. The fluorescence was monitored for 2 min in the kinetic mode as previously described.

Effects of GSH and Catalase/SOD on the Inactivation of P450 3A4. GSH (2 mM) was included in the primary reaction mixture together with P450 3A4 (0.11 μM), NADPH−P450 reductase (0.11 μM), and 4-ipomeanol (50 μM). Aliquots in triplicate were taken for determining the remaining enzyme activity using the kinetic assay previously described. In control samples, vehicle was added in the place of GSH. In a separate study, P450 3A4 was incubated with 4-ipomeanol and NADPH in the presence or absence of a mixture of catalase and SOD. The concentrations of SOD and catalase used were 800 units of each enzyme per milliliter.

Partition Ratio of Inactivation. Primary reaction and secondary reaction mixtures were prepared similarly to those reported in the section on time and concentration dependence. In this experiment, the concentrations of 4-ipomeanol tested were as follows: 0 (control), 1, 1.5, 5, 10, 25, 50, 75, and 100 μM. The primary reactions were initiated by adding NADPH at a final concentration of 0.45 mM. Negative control incubations lacked NADPH. After incubation at 37 °C in a water bath shaker for 12 min, 45 μL aliquots (5 pmols of P450 3A4) in triplicate were withdrawn from the primary reaction mixtures and transferred to secondary reaction mixtures for measurement of fluorescence in the kinetic mode as reported above. P was estimated graphically using the titration method (*45*).

Irreversibility of Inactivation. Expressed human P450 3A4 and NADPH−P450 reductase samples were thawed at room temperature. Inactivation of P450 3A4 was performed by addition of 4-ipomeanol (to a final concentration of 50 μM) in the presence of NADPH (0.45 mM) at 37 °C. This reaction mixture contained equimolar amounts of each enzymes (0.1 μM). Control incubations lacked 4-ipomeanol. Aliquots (45 μL) in triplicate were withdrawn at 0 and 10 min and dialyzed using Slide-A-lyzer membranes (molecular mass cut off, 3500 Da; Pierce, Rockford, IL) against 0.1 M potassium phosphate buffer (pH 7.4) overnight at 4 °C. The dialyzed samples were brought to room temperature, and the enzymatic activity of dialyzed and nondialyzed samples was measured in kinetic mode as described above.

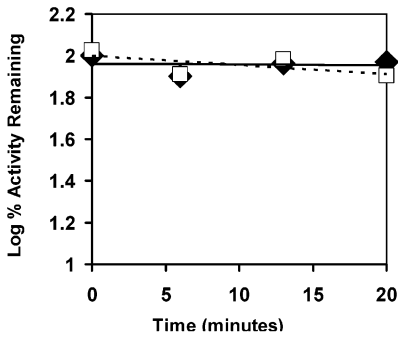

Figure 1. Lack of effect of 4-ipomeanol metabolism in P450 1A2 activity in human hepatic microsomes. Microsomal samples were incubated with (□; 45 μM) or without (←) 4-ipomeanol and NADPH for 0, 6, 13, and 20 min. The P450 1A2 activities were assayed using the methoxyresorufin/resorufin reaction. Results are the means of three determinations. SDs were ≤10%.

Covalent Binding to Proteins. P450 3A4 Supersomes (120 pmols) were mixed with [^{3}H]-4-ipomeanol (72 nmols) in 0.2 M potassium phosphate buffer. The reactions were initiated by the addition of NADPH (8 mM final concentration, pH 7.4) or vehicle (phosphate buffer) as controls and allowed to proceed for 45 min at 37 °C. The reactions were terminated by the addition of equal volumes of Laemmli sample buffer. The resulting mixture was boiled for 5 min, and the proteins were separated by SDS−PAGE (10%). The protein bands were stained by Coomassie Blue. The gel lanes of interest were sliced into 3 mm sections, and the proteins were extracted into 50% hyamine hydroxide at 65 °C for 12 h. Longer extraction times are not recommended because quenching of radioactivity may occur due to color formation. The samples were cooled to room temperature, and their radioactivity was measured in triplicate by liquid scintillation counting for 5 min. The counting efficiency for ^{3}H was 92%. Measured counts per minute (cpm) values were background-subtracted (natural radioactivity background was 20 cpm), corrected for counting efficiency, and converted to dpm.

Results

Lack of Inactivation of P450 1A2 by 4-Ipomeanol's Metabolism in Human Hepatic Microsomes. The methoxyresorufin *O*-deethylase reaction (*32*) was used to measure P450 1A2's activity in human hepatic microsomal samples. Figure 1 shows that 4-ipomeanol (45 μM) does not inactivate P450 1A2 and that the time of incubation of 4-ipomeanol with the microsomes (from 0 to 20 min) has no effect in P450 1A2 activity. No important loss of P450 1A2 activity was observed in control samples, which lacked 4-ipomeanol, over the time course of the experiment.

P450 3A4 Assays. P450 3A4 activity was measured in the kinetic mode using the DBF/fluorescein assay (*46*) in a 96 well plate format. The dilution factor for the transfer of aliquots from the primary reaction mixture to the secondary mixture was 4.4. The concentration of DBF substrate used in our study (13.5 μM) produced saturation of P450 3A4 in the secondary assay and quenching of the inactivation. Saturation and quenching, which are desirable in these studies (*45*), were determined in preliminary experiments. The measured K_m of DBF was 6 μM. The ionic strength of potassium phosphate buffer in the reaction was 0.2 M as recommended by Gentest, the manufacturer of this assay, for optimum fluorescence (*46*). However, we did not observe any differences in activity when more diluted potassium phosphate buffer, i.e., 0.1 M, was used. The fluorescence signal/noise ratio observed was 25/1 or higher.

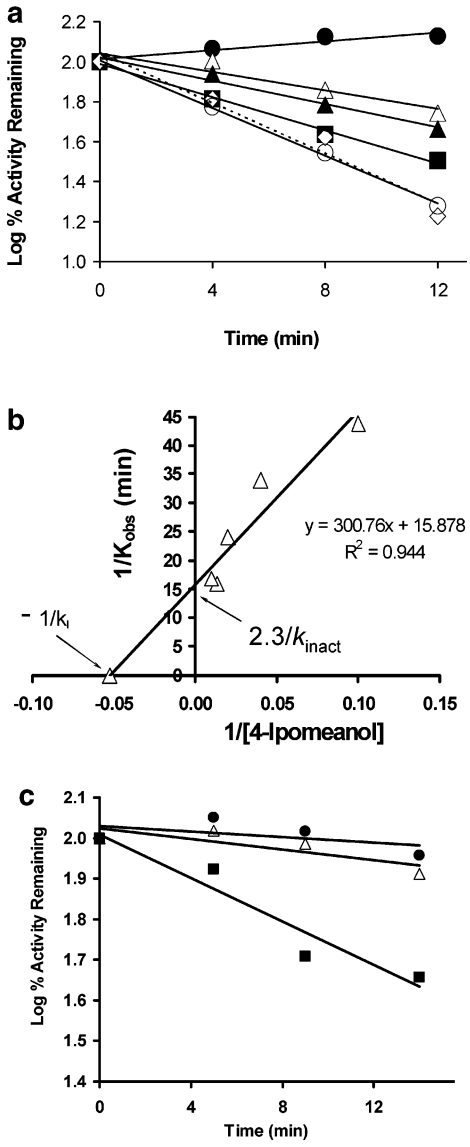

Figure 2. (a) Time- and concentration-dependent inactivation of P450 3A4 by 4-ipomenol. Human P450 3A4 and NADPH−P450 reductase were incubated with 4-ipomeanol in the presence of NADPH. At the indicated time points, the residual P450 3A4 activity was monitored, using the DBF assay. The concentrations of 4-ipomeanol used were as follows: 0, ●; 10, △; 25, ▲; 50, ■; 75, ○; and 100, ◇ μM. Each point represents the mean of triplicate values. SDs were <14%, and most were between 3 and 6%. (b) Wilson's plot. The observed inactivation rate constant k_{obs} was calculated from the slope of the regression lines shown in Figure 1a. K_I and k_{inact} were 20 μM and 0.15 min^{-1}, respectively. These are apparent constants. (c) NADPH-dependent inactivation of P450 3A4 by 4-ipomeanol. Human P450 3A4 and NADPH−P450 reductase were incubated with vehicle (●) and 4-ipomeanol (50 μM) in the presence (■) or absence (△) of NADPH.

Time- and Concentration-Dependent Inactivation. Time-dependent enzyme inhibition is one of the important criterion to determine irreversible inhibitors (*45*, *47*). The kinetics of inactivation of P450 3A4 by 4-ipomeanol (concentrations range, 10−100 μM) were studied by monitoring the loss in the DBF debenzylation activity. The residual P450 3A4 activity relative to control was measured by using the fluorescent assay at the indicated times. As shown in Figure 2a, 4-ipomeanol produced time- and concentration-dependent inactivation of human P450 3A4. In this figure, the residual enzymatic activities at time 0 were normalized to 100% to facilitate visual comparison of the rates of inactivation at each concentration. The inactivation of P450 3A4 progressed rapidly without a lag time. The loss in P450 3A4 activity increased progressively with increasing concentrations of 4-ipomeanol. More than 80% of debenzylation activity of P450 3A4 was suppressed after its incubation with 75 μM 4-ipomeanol for 12 min at 37 °C. No loss of enzyme activity was observed in the absence of 4-ipomeanol.

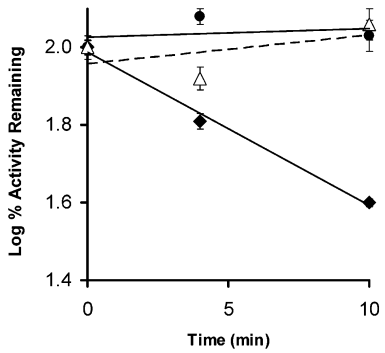

Figure 3. Testosterone protection against inactivation of P450 3A4 by 4-ipomeanol. Human P450 3A4, NADPH−P450 reductase, and NADPH were incubated with vehicle (●) and 4-ipomeanol (50 μM) in the presence of testosterone (△, 130 μM) or in the absence of testosterone (◆). Data shown represent the mean ± SD of three measurements. In some cases, the SDs were smaller than the font of the markers used.

The Wilson's plot (*45*, *48*), a double-reciprocal plot of the observed rates of inactivation (k_{obs}) and 4-ipomeanol concentrations, was used to calculate the kinetic constants K_I and k_{inact} (Figure 2b). The K_I was determined from the intercept of the *x*-axis. The k_{inact} was obtained from the reciprocal of the *y*-axis intercept, which is equal to $k_{inact}/2.303$. K_I was equal to 20 μM, and k_{inact} was 0.15 min^{-1}. Therefore, the half-life of inactivation ($t_{1/2} = 0.693/k_{inact}$) was 4.6 min.

NADPH-Dependent Inactivation. To determine whether biotransformation of 4-ipomeanol is required for enzyme inactivation, time course inactivation of P450 3A4 was investigated in the presence or absence of NADPH. No significant differences in the percentage of P450 3A4 remaining activity were observed between control incubations (without 4-ipomeanol) and incubations with 4-ipomeanol (50 μM) lacking NADPH (Figure 2c) for all time points tested. As expected, 4-ipomeanol produced a time course P450 3A4 inhibition in the presence of NADPH.

Substrate Protection. To study whether inactivation occurred in the active site of the enzyme, P450 3A4 was incubated with 4-ipomeanol in the presence or the absence of testosterone, a substrate of P450 3A4 ($K_m = 50$ μM). Remaining enzymatic activities were measured using the DBF/fluorescein assay and normalized to control activity at time 0 (100% activity). Testosterone reduced the rate of P450 3A4 inhibition produced by 4-ipomeanol (Figure 3). Remaining P450 3A4 activities in samples coincubated with testosterone and 4-ipomeanol were 23 ± 7% and 60 ± 14% higher at 4 and 10 min, respectively, as compared with samples containing 4-ipomeanol that lacked testosterone. These data are reported after having taken into account the inhibitory effect of testosterone on fluorescein formation (52 ± 14%).

Effects of GSH and Catalase/SOD. To determine the protective effect of the nucleophile GSH against the enzymatic inactivation produced by 4-ipomeanol, the

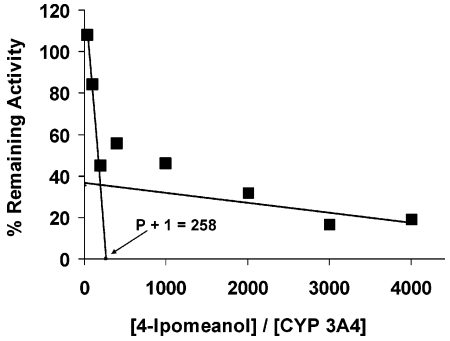

Figure 4. Loss of P450 3A4 activity as a function of the [4-ipomeanol]/[P450 3A4] molar ratio. Human P450 3A4, NADPH−P450 reductase, and NADPH were incubated with increasing concentrations of 4-ipomeanol for 12 min. Kinetic fluorescence was measured in a secondary mixture (excitation, 485 nm; emission, 538 nm). The extrapolated P was determined from the *X*-axis intercept of regression line of the lower ratios (refer to Material and Methods). SDs were ≤10%.

P450 3A4 inactivation incubation was performed in the presence or the absence of GSH (2 mM). This agent produced only minor protection of the enzyme from inactivation. The presence of GSH resulted in only a 9.7% increase in the P450 3A4 activity remaining (42.7 ± 2.9) vs the activity of GSH-free incubation (33.0 ± 2.7). A similar lack of protective effect (8.7%) was observed when a mixture of catalase and SOD, scavengers of reactive oxygen species, was included in the primary incubation mixture together with 4-ipomeanol.

Partition Ratio. The P (*45*, *47*, *49*), the number of molecules of 4-ipomeanol metabolized per molecule of P450 3A4 inactivated, was estimated graphically using the titration method (*45*) as shown in Figure 4. This figure shows a plot of the percentage remaining activity vs the 4-ipomeanol/P450 3A4 molar ratio. The turnover number (P + 1) is by definition the *x*-intercept of the extrapolated regression line observed (which indicates 0% activity or complete inactivation) for the lower ratios (*45*). Because the turnover number was 258, therefore P = 257. This number is approximate and gives an idea of the number of cycles of metabolism per cycle of inactivation. Using this P value, we estimated that large amounts of 4-ipomeanol were consumed in the concentration-dependent assay when P450 3A4 was incubated with the lower concentrations of this compound, but relatively lower amounts were consumed with the higher concentrations. This indicates that since steady state was not reached, the observed kinetic constants (K_I and k_{inact}) are apparent constants.

Irreversibility of Inactivation. To study whether the inactivation of P450 3A4 produced by 4-ipomeanol is irreversible, P450 3A4 was incubated with 4-ipomeanol (50 μM) in the presence of NADPH, followed by overnight dialysis against phosphate buffer. Table 1 shows percentage remaining P450 3A4 activity vs control (100% activity) of the samples measured before and after dialysis. In this table, the percent remaining P450 3A4 activities at time 0 were not normalized to 100% activity but represent direct measurements. Only minor P450 3A4 activity (<5%) was recovered after dialysis. Furthermore, addition of fresh NADPH−P450 reductase to the dialyzed samples did not lead to significant recovery of the P450 3A4 activity (less than 4% recovery). This indicates that the reactive metabolites of 4-ipomeanol inactivated P450 3A4 rather than the NADPH−P450 reductase.

Table 1. Irreversibility of Inactivation of P450 3A4 by 4-Ipomeanol[a]

	before dialysis		
	0 min	10 min	after dialysis
control	100 ± 8	96.6 ± 7	100 ± 0.2[b]
4-ipomeanol (50 μM)	62 ± 9	4.5 ± 7	9 ± 1.5

[a] P450 3A4 was exposed to 4-ipomeanol as described in the Materials and Methods. The remaining enzyme activity was monitored before and after overnight dialysis. The activities represent the mean and SD of four samples. [b] Normalized to 100% to adjust for the volume change due to dialysis.

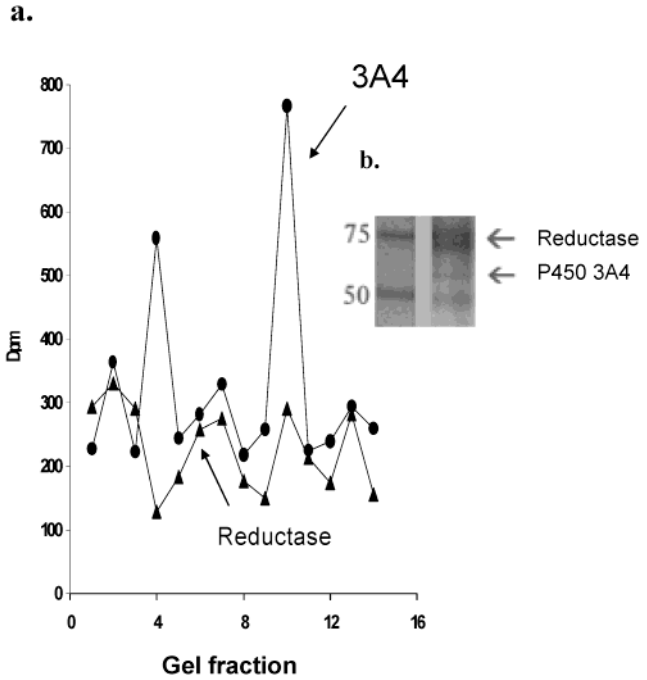

Figure 5. (a) Covalent binding of radioactivity to proteins. Proteins obtained from incubations containing human P450 3A4, NADPH−P450 reductase, and [^{3}H]-4-ipomeanol in the presence (●) or the absence (▲) of NADPH were separated by SDS−PAGE. The gel was sliced into 3 mm sections, followed by measuring the radioactivity of the resulting slices by liquid scintillation counting. The average dpm of duplicate experiments is shown. Each sample was measured in triplicate for 5 min. SDs were ≤20%. (b) SDS−PAGE of proteins obtained from P450 3A4 inactivation incubations. Enzyme incubations were performed as described in panel a. The resulting protein samples were separated by SDS−PAGE, followed by staining with Coomassie Blue.

Covalent Binding. To confirm covalent binding of reactive metabolites of 4-ipomeanol to P450 3A4, a sample of the P450 3A4 Supersomes was incubated with [^{3}H]-4-ipomeanol in the presence or absence of NADPH followed by separation of the proteins by SDS−PAGE. P450 3A4 was completely separated from NADPH−P450 reductase (Figure 5b). A mean of 770 and 290 dpm was associated with the band corresponding to the electrophoretic mobility of P450 3A4 (molecular mass of 57 kDa) in samples containing and lacking NADPH, respectively (Figure 5a). The difference between these values (480 dpm) was used to calculate the binding stoichiometry between 4-ipomeanol's reactive metabolites and P450 3A4. This stoichiometry was approximately 1.5:1. No significant differences were observed between the amount of radioactivity bound to the reductase (molecular mass = 78 Da) in the presence (282 dpm) and absence (270 dpm) of NADPH. These numbers fell in the range of the baseline or ^{3}H background radioactivity observed in the

gel fractions without protein bands (130−330 dpm). Interestingly, we also observed significant radioactivity (435 dpm) bound onto a protein responsible for a band with electrophoretic mobility of approximately 100 kDa. This protein belongs to the host cells used for P450 3A4 expression. These dpm data indicate the mean of triplicate reading from two experiments, which varied ≤20%.

Discussion

The mechanism of cytotoxicity produced by 4-ipomeanol is not well understood. Several furan-containing agents were found as mechanism-based inactivators of P450 enzymes (*25*−*29*). P450 1A2 has also been reported to involve the metabolism of 4-ipomeanol (*24*). In our study, we did not observe P450 1A2 inactivation by 4-ipomeanol (45 μM) in human hepatic microsomes in incubations lasting 20 min. One explanation to our observations could be that although human P450 1A2 is a metabolizing enzyme of 4-ipomeanol, this enzyme may lack some of the nucleophilic amino acids in its active site for binding these metabolites. We also postulate that human P450 1A2 will probably not be inactivated by 4-ipomeanol's metabolism in human liver. Further studies would need to be conducted to determine, for example, modulation of P450 1A2 in human hepatocytes and whether the cytotoxic effect of 4-ipomeanol is due to metabolism or modulation of this isoform. However, in this report, we focus instead in studying the inactivation of P450 3A4 by 4-ipomeanol. We concentrated in studying P450 3A4 using the expressed enzyme only after preliminary experiments in human microsomes failed to show significant inactivation of other important hepatic P450 enzymes (P450 1A2, P450 2D6, and P450 2C9) and by contrast showed important time-dependent inactivation of P450 3A4. Time-dependent inactivation of an enzyme is an important criterion accomplished by mechanism-based inactivators.

Our kinetic study clearly confirms that 4-ipomeanol produced a time-dependent inhibition of P450 3A4. Inactivation of human P450 3A4 by 4-ipomeanol was also found to be concentration-dependent, and it reached saturation at a concentration of 75 μM. 4-Ipomeanol-induced P450 3A4 inactivation was not observed unless NADPH was present in the incubation system. This indicates that 4-ipomeanol itself is not an inactivator of P450 3A4 and that the enzyme inactivation by 4-ipomeanol needs to be initiated through biotransformation with assistance of the P450 cofactor NADPH. Taken together, these results provided strong evidence for mechanism-based inactivation of P450 3A4 by 4-ipomeanol.

Minor protection of enzyme inactivation from 4-ipomeanol by catalase and SOD was observed. Catalase and SOD are known as enzymes quenching hydrogen peroxide, superoxide anion, and other reactive oxygen species, possible agents to inactivate enzymes (*32*). The observed lacking protection effect by catalase and SOD indicates that reactive oxygen species may not play a significant role in P450 3A4 inactivation induced by 4-ipomeanol. In addition, GSH, a nucleophile, was not found to significantly protect P450 3A4 inactivation against 4-ipomeanol. This implies in part that P450 3A4 is covalently modified by electrophilic metabolites of 4-ipomeanol before escaping from the active site. The protective effect of testosterone on P450 3A4 inactivation observed in the substrate competition experiment indicates that testosterone competed with 4-ipomeanol reducing the rate of generation of its reactive metabolites. This critical finding of substrate protection provides further evidence that bioactivation of 4-ipomeanol occurs in the active site of P450 3A4.

Mechanism-based enzyme inactivators are often irreversible inhibitors. Extensive dialysis of P450 3A4 after exposure to 4-ipomeanol did not reverse the loss in enzyme activity, suggesting that the reactive species of 4-ipomeanol covalently modified the enzymes participating in reactions. Biochemical oxidation/reduction reactions catalyzed by P450 enzymes require two enzyme components including the P450 enzyme itself and P450 reductase. Covalent modification of either P450 3A4 or the reductase may inactivate and destroy the reaction system (*47*). To examine whether P450 3A4 or P450 reductase are attacked by the reactive metabolites of 4-ipomeanol, P450 3A4 along with P450 reductase were incubated with radioactive 4-ipomeanol. We used for this experiment the enzymatic Gentest system since it has been shown to be superior to other enzymatic P450 systems for SDS−PAGE analysis of covalently modified proteins (*29*). We also reasoned that this enzymatic system would be more robust than the P450 3A4 system from Merck to ensure that inactivation is not due to significant binding to the reductase because it contains a relatively higher concentration of NADPH−P450 reductase vs P450 3A4 (10:1), while the Merck system contains equimolar amounts of these enzymes. Inactivation of P450 3A4 was observed with both systems. A significant amount of radioactivity was found to bind to the protein corresponding to P450 3A4 (57 kDa) (Figure 5a). The calculated binding stoichiometry between reactive metabolites of 4-ipomeanol and P450 3A4 was 1.5:1. Potential instability of the adducted protein and loss of the radiolabeled reactive metabolites, which is inherent to the conditions of SDS−PAGE, could indicate that the actual stoichiometry value may be higher. This limitation has also been reported by other groups (*29*, *50*). Little radioactivity (background level) was observed in the protein corresponding to P450 reductase (78 kDa). Furthermore, the adding of fresh P450 reductase into P450 3A4/P450 reductase/NADPH system pretreated with 4-ipomeanol did not recover the P450 activity (data not shown). This further confirmed that the loss in enzymatic activity resulted from covalent modification of P450 3A4 and not by covalent modification of the P450 reductase. In addition, little radioactivity was observed in the protein samples obtained from the 4-ipomeanol/P450 3A4 incubations lacking NADPH (Figure 5b). This indicates that the protein modification is NADPH-dependent and biotransformation is required for the protein covalent binding. The enzyme kinetic work along with the covalent binding studies clearly showed that 4-ipomeanol inhibited P450 3A4 by mechanism-based inactivation.

The P gives an idea of the efficiency of the inactivator. Reported Ps in the literature for mechanism-based inactivators of P450 enzymes vary from 3 (very highly efficient inactivators) to >1000 (inefficient) (*47*). For example, L-754,394, a furan-containing compound that is a very potent mechanism-based inactivator of P450 3A4 (K_I = 7.5 mM), has a P of 4 (*26*). Reported P values for potent mechanism-based inactivators of P450 2A6 among the furanocumarin group ranged between 11 and 215, when measured using the expressed enzyme, and from 24 to 840 when the purified enzyme was used (*29*).

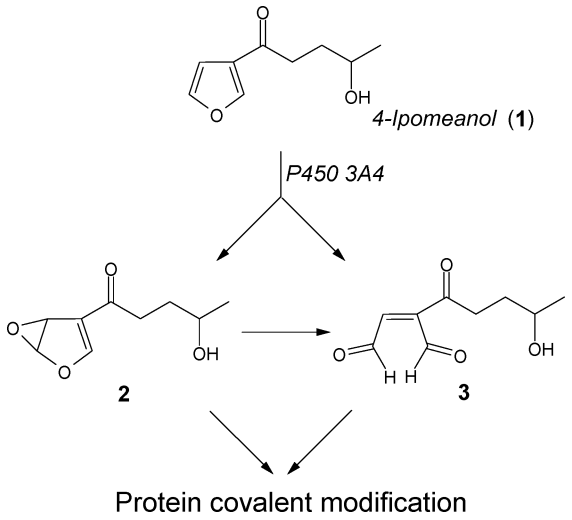

Figure 6. Proposed mechanism of inactivation of P450 3A4 by 4-ipomeanol.

4-Ipomeanol with an estimated P of 257 is a moderately efficient inactivator of P450 3A4.

The major pathway reported for the bioactivation of furan and other furan-containing compounds by P450 enzymes involves furan ring opening leading to the formation of unsaturated α,β-aldehyde/ketone of dialdehyde reactive metabolites (*51*−*55*). A review article can be found in ref *56*. Earlier structure−activity relationship studies of 4-ipomeanol showed that replacement of its furan ring by a phenyl ring diminished the potency in lung toxicity (*12*). This indicates that the furan ring is critical for the cytotoxic effects. It was previously postulated that metabolism of 4-ipomeanol by P450 enzymes may occur through oxidation of its furan ring, following a mechanism similar to the metabolism of other furans (*5*, *56*); however, this has not yet been confirmed. We propose the mechanism by which 4-ipomeanol inactivates P450 3A4 as follows (Figure 6). P450 3A4 inactivation may be initiated by epoxidation of the furan ring to epoxide metabolite 2. The resulting furan oxide may be further rearranged to butenedial 3, an α,β-unsaturated dialdehyde. The unsaturated dialdehyde metabolite may also be generated by direct oxidation of the furan ring without first generating a furan epoxide intermediate. A detailed mechanism of this pathway can be found in ref *51*. Both furan epoxide (2) and butenedial (3) intermediates are electrophiles, which may potentially attack in situ nucleophilic sites of the enzyme. The covalent modification may be responsible for the enzyme inactivation.

In addition to covalent binding to P450 3A4, significant covalent binding to an insect protein impurity present in the P450 3A4 system was also observed. Covalent binding of radioactivity to this protein occurred only through P450 3A4-mediated metabolism of 4-ipomeanol (with NADPH). This suggests the possibility that if a similar process takes place in vivo, it may play a role in human liver toxicity since covalent modification of cellular proteins has been suggested to be one of the major biochemical mechanisms of cytotoxicity (*57*). P450 3A4 is the most abundant P450 enzyme and participates in the biosynthesis of a number of endogenous substances required for cell life, as well as in the detoxification of many xenobiotics (*33*−*40*). Interferences on the biochemical function of P450 3A4 may induce cellular instability, possibly leading to cell injury and death. The previously observed covalent modification of DNA by 4-ipomeanol metabolism may also contribute to cytotoxicity. However, reported binding to DNA was relatively low (2%) when compared to binding to microsomal proteins (>90%) (*5*, *13*). In addition to P450 3A4, other metabolizing enzymes of 4-ipomeanol, P450 1A2, and P450 3A3 may also contribute to bioactivation and liver toxicity (*24*).

In conclusion, we have shown that 4-ipomeanol is enzymatically activated by P450 3A4 and is characterized as a mechanism-based inactivator of human P450 3A4. In addition, reactive metabolites of 4-ipomeanol generated by P450 3A4 may possibly attack other cellular proteins. This work opens new avenues for understanding the mechanism of bioactivation and cytotoxicity induced by 4-ipomeanol.

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Supporting Information Available: TIC of a sample of 4-ipomeanol synthesized in house. Inactivation assays of P450 3A4, P450 2D6, and P450 2C9 in human liver microsomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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