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Microsomal Metabolism of the 5-Lipoxygenase Inhibitor L-739,010: Evidence for Furan Bioactivation†

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The novel 5-lipoxygenase inhibitor [1*S*,5*R*]-3-cyano-1-(3-furyl)-6-{6-[3-(3α-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridin-2-yl-methoxyl}naphthalene (L-739,010), when administered to rats and rhesus monkeys, was found to produce metabolites which appeared to be covalently bound to plasma proteins. Incubation of [^{14}C]L-739,010 with rat liver microsomes did not yield appreciable amounts of soluble metabolites but resulted in covalent binding to microsomal proteins. The covalent binding was NADPH-dependent and was enhanced by 1.5- and 2-fold in liver microsomes from rats, pretreated with phenobarbital and dexamethasone, respectively. Addition of triacetyloleandomycin and diethyldithiocarbamate to the incubation mixture inhibited the covalent binding by 60% and 46%, respectively. These findings suggest that the cytochrome P450 3A family of enzymes play an important role in the bioactivation of L-739,010. The presence of GSH attenuated the covalent binding by 50%, while methoxylamine, an aldehyde trapping agent, blocked the covalent binding completely and, concurrently, produced several soluble metabolic adducts. Subsequently, major methoxylamine adducts were identified by LC-MS/MS and NMR as *O*-methyloximes of the ring-opened furan moiety of L-739,010. Incubation of L-739,010 with methoxylamine and hepatic microsomes from dog, rhesus monkey, and human produced similar metabolic adducts as those formed by rat liver microsomes. Therefore, under these experimental conditions, the furan moiety, which undergoes oxidative cleavage to the highly reactive 2-butene-1,4-dialdehyde, represents the major site of L-739,010 biotransformation. This putative reactive intermediate could react with microsomal proteins *in vitro* and physiological proteins *in vivo*. Since furan bioactivation is believed to be responsible for the toxicity of many furan-containing compounds, the furan moiety of L-739,010 may be regarded as undesirable.

Introduction

5-Lipoxygenase catalyzes the initial two-step reactions in the biosynthesis of leukotrienes from arachidonic acid (*1*). Inhibition of this enzyme should, in theory, block leukotriene-mediated respiratory smooth muscle contraction and inflammatory responses (*2, 3*). This class of agents could provide a novel therapy for the treatment of asthma and inflammatory bowel disease (*4*).

[1*S*,5*R*]-3-Cyano-1-(3-furyl)-6-{6-[3-(3α-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridin-2-yl-methoxyl}-naphthalene (L-739,010,[1] Figure 1) is a non-redox, direct inhibitor of 5-lipoxygenase, discovered at Merck Frosst (*5*). During preclinical disposition studies, it was found that following iv administration of [^{14}C]L-739,010, the plasma half-lives of radioactivity were 2.7 and 4 days in the rat and rhesus monkey, respectively, while those for the parent drug were only about 3 h (*6*). Furthermore, plasma radioactivity at later times could not be extracted by organic solvent. These findings suggested that L-739,010, either chemically or metabolically, produced reactive metabolite(s) that became covalently bound to plasma proteins *in vivo*. In the safety assessment of L-739,010, the compound was found to moderately elevate alanine transaminase (ALT) levels in dogs in a dose-dependent fashion, suggesting that L-739,010 causes mild hepatotoxicity in this species.[2]

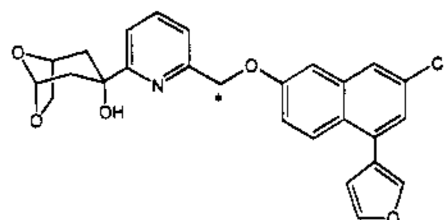

Figure 1. Structure of L-739,010. *Denotes position of ^{14}C label.

Although the relationship between covalent binding to proteins and chemically-induced toxicity has not been established firmly (*7*), two general concepts have been widely accepted, *viz.*, that covalent modification of proteins responsible for critical cellular functions may lead to acute cytotoxicity (*7, 8*), and that the covalent modification of certain proteins to form immunogens may lead to hypersensitivity reactions (*7–9*).

This study was aimed to investigate the nature of the putative reactive intermediate(s), generated from L-739,010, in order to gain insight into the mechanism by which this compound binds covalently to plasma proteins and induces hepatotoxicity. This information could be used to facilitate the selection of a new and safer

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[1] Abbreviations: L-739,010, [1*S*,5*R*]-3-cyano-1-(3-furyl)-6-{6-[3-(3α-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridin-2-yl-methoxyl}-naphthalene; ALT, alanine transaminase; ANF, α-naphthoflavone; BNF, β-naphthoflavone; PB, phenobarbital; DEX, dexamethasone; DDC, diethyldithiocarbamate; TAO, triacetyloleandomycin; DMSO, dimethyl sulfoxide; TFA, trifluroacetic acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NOE, nuclear Overhauser effect; ADME, absorption, disposition, metabolism, and excretion.

[2] Unpublished data, Merck Safety Assessment.

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candidate among analogs of L-739,010.

Materials and Methods

Materials. L-739,010 was synthesized by the Process Research Department, Merck Research Laboratories (Rahway, NJ). [^{14}C]L-739,010 (19.3 mCi/mmol, 98.4% pure by HPLC) was synthesized by the Labeled Compound Synthesis Group, Drug Metabolism Department, Merck Research Laboratories (Rahway, NJ). GSH, NADP$^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). α-Naphthoflavone (ANF), β-naphthoflavone (BNF), diethyldithiocarbamate (DDC), triacetyloleandomycin (TAO), methoxylamine, and dimethyl sulfoxide (DMSO) were purchased from Aldrich (Milwaukee, WI). Methanol, acetonitrile, and trifluroacetic acid (TFA) were purchased from Fisher Scientific (Pittsburgh, PA).

Preparation of Microsomes. Hepatic microsomes were prepared from Sprague-Dawley rats, beagle dogs, rhesus monkeys, and humans according the method of Lu *et al.* (*10*). Rat liver microsomes were prepared from uninduced animals as well as rats that were pretreated with phenobarbital (PB, 50 mg/kg, ip, for 3 days), BNF (50 mg/kg, ip, for 3 days), dexamethasone (DEX, 50 mg/kg, ip, for 3 days) or acetone (5% in drinking water for 10 days). Human liver samples were obtained from Keystone Skin Bank (IIAM, Exton, PA), and microsomes were pooled from three individuals. Protein concentrations were determined according to the method described by Bradford (*11*).

Microsomal Incubations. Incubations (1 mL) contained [^{14}C]L-739,010 (50 νM), microsomal proteins (1 mg/mL), and an NADPH-generating system, which included NAPD$^+$ (1 mM), glucose 6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (2 units/mL), and were carried out at 37 °C for 1 h in phosphate buffer (0.1 M, pH 7.4, containing 3 mM $MgCl_2$). In some cases, microsomes were preincubated for 10 min with an NADPH-generating system and P450 inhibitors (ANF, DDC, TAO; each at 0.1 mM), or incubations were carried out in the presence of nucleophiles (GSH or methoxylamine; each at 5 mM). The reaction was stopped by adding 3 mL of methanol to precipitate microsomal proteins.

Covalent Binding to Microsomal Proteins. After 1 h incubation, microsomal proteins were precipitated and washed extensively with methanol and acetonitrile until no further radioactivity could be extracted. The protein pellets were treated with 1 mL of Beckman tissue solubilizer-450 (Beckman Instruments, Fullerton, CA) and heated at 60 °C until completely dissolved. An aliquot of the alkaline protein solution was neutralized with HCl (0.1 mL, 6 M), mixed with scintillation cocktail (Ready Safe, Beckman Instruments, Fullerton, CA), and counted for total radioactivity. Another aliquot was used to measure protein concentration by the method of Bradford (*11*). Covalent binding of L-739,010 to microsomal proteins was expressed as nmol equiv/(mg of protein·h) or percentage of total radioactivity.

Metabolite Identification by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). The supernatant and methanol extracts of the microsomal incubations were combined and dried under vacuum, and the residues were reconstituted in methanol/water (20/80, v/v) and analyzed by LC-MS/MS equipped with an on-line radiochemical detector. To obtain molecular weight information on metabolic adducts, the mass spectrometer was set in the MS (Q1) mode, and to obtain structural information, the instrument was operated in the MS/MS mode. The relative abundance of each metabolic adduct was determined by an on-line radiochemical detector. The LC-MS/MS system included a Hewlett-Packard Model 1050 HPLC system and a Sciex API III mass spectrometer. The chromatography was performed on a Spherisorb C_8 (3 νm, 2 × 100 mm) narrow bore column (Thomson liquid chromatography, Springfield, VA). Analytes were eluted with a gradient mobile phase at a flow rate of 0.2 mL/min, programmed at 2%/min from 40% to 100% A (A = 90% acetonitrile, 10% water containing 0.1% TFA; B = 10% acetonitrile, 90% water containing 0.1% TFA). The HPLC effluent was split between the mass spectrometer (25%) and an IN/US β-RAM radiochemical detector (75%). Samples were introduced into the mass spectrometer *via* an IonSpray interface operating at ambient temperature in the positive ion mode. The ion source was maintained at 4.8 kV, and the orifice potential was set at 60 V. High-purity air served as the nebulizing gas and was maintained at an operating pressure of 40 psi. Product ion spectra were obtained upon collision-induced dissociation of $[M + H]^+$ parent ions. Ions entering the collisional region were accelerated with an energy of 60 eV and collided with argon gas at a thickness of 3.5×10^{15} molecules/cm^2. The third quadrupole (Q3) was operated in R3 auto mode. The radiochemical detector employed a 100 νL liquid flow cell, and the HPLC effluent and scintillation cocktail (Ready Flow III, Beckman Instruments, Fullerton, CA) were mixed in a ratio of 1:2.

Metabolite Purification and Identification by NMR. To isolate metabolic adducts for NMR characterization, large scale incubations were carried out with rat liver microsomes in the presence of methoxylamine in ten 10 mL reaction mixtures. Incubation conditions were the same as described above. After precipitation of proteins with methanol, the supernatant from each incubation was dried under vacuum and the residue was reconstituted in 2 mL of methanol/water (20/80 v/v) which was injected onto a semipreparative HPLC system. The latter consisted of a Nova-Pak C_{18} column (6 νm, 7.8 × 300 mm, Waters Chromatography, Milford, MA), Waters 600E pump, 996 UV diode array detector, and a Foxy 200 fraction collector. The fraction collector was programmed to collect those UV-absorbing components (μ = 244 nm) which exceeded 10% of the full-scale deflection (100 mV). All instruments were controlled by Millennium 2010 Chromatography Manager software. Metabolic adducts were eluted with a gradient mobile phase at 3 mL/min, programmed at 2%/min from 20% to 80% methanol in water. All major adducts were repurified by the same semipreparative HPLC system, and the dried extracts were dissolved in deuterochloroform and analyzed by NMR. ^{1}H-NMR spectra were recorded on a Varian Unity 400 MHz spectrometer at 25 °C. Chemical shifts are given in the ppm scale referenced to the chloroform peak set at 7.26 ppm.

Results

Covalent Binding to Microsomal Proteins. Incubation of [^{14}C]L-739,010 with rat liver microsomes did not produce appreciable amounts of soluble metabolites (Figure 2), but resulted in the formation of metabolites covalently bound to microsomal proteins. The rate of covalent binding was approximately 5.7 nmol equiv of [^{14}C]L-739,010/(mg of protein·h), such that after 1 h incubation, about 7% of the total radioactivity was bound to microsomal proteins. The remaining radioactivity was accounted for by the parent drug. The covalent binding was NADPH-dependent and was enhanced by 1.5- and 2-fold in liver microsomes from rats pretreated with PB and DEX, respectively, whereas pretreatment with BNF decreased the covalent binding and acetone had no effect (Table 1). Among the three P450 inhibitors used in this experiment, TAO and DDC inhibited the covalent binding by 60% and 46%, respectively (Table 2). Both PB and DEX are known inducers of P450 3A, whereas TAO is an irreversible inhibitor of P450 3A. These results implicate the involvement of cytochrome P450 3A family of enzymes in the metabolic activation process. The physiological nucleophile GSH attenuated the binding by about 50%, while methoxylamine, an aldehyde trapping agent, inhibited the covalent binding to near background level (Table 2).

Identification of Metabolites. The presence of methoxylamine, besides inhibiting the covalent binding,

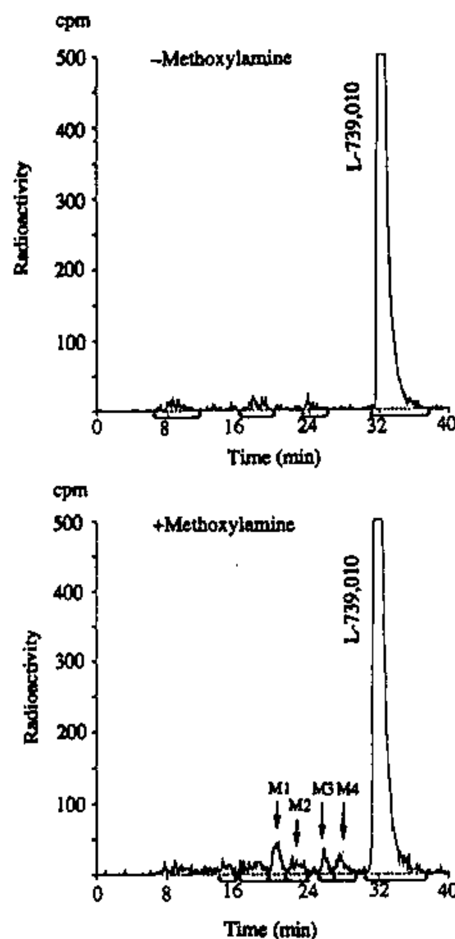

Figure 2. Radiochromatograms of rat liver microsomal incubates of L-739,010 in the absence (top) and presence (bottom) of methoxylamine (5 mM).

Table 1. Covalent Binding of L-739,010 to Rat Liver Microsomes[a]

liver microsomes	NADPH	covalent binding [nmol equiv/(mg of protein·h)]
control	−	0.4 ± 0.0
control	+	5.7 ± 0.6
BNF	+	3.1 ± 0.3[b]
PB	+	8.1 ± 0.6[b]
DEX	+	11.2 ± 1.5[b]
acetone	+	5.6 ± 0.4

[a] Conditions for microsomal incubation and assessment of covalent binding are described in Materials and Methods. Results are expressed as the mean ± SD from three incubations. [b] Significantly different from "+NADPH" control (Dunnett's comparison, $p < 0.05$).

also generated several soluble metabolic adducts. These methoxylamine adducts were designated M1 to M4 in order of elution from the HPLC column (Figure 2). The formation of these metabolic adducts was NADPH-dependent (data not shown), indicating that an intermediate metabolite(s) of L-739,010, rather than the parent drug itself, formed adducts with methoxylamine. The relative abundance of these adducts, estimated from an average of two injections, was 2.8%, 1.6%, 1.7%, and 1.4% of the total radioactivity for M1, M2, M3, and M4, respectively. These metabolic adducts were detected simultaneously by IonSpray LC-MS (Figure 3). The protonated molecular ions ($[M + H]^+$) for both M1 and M2 were observed at m/z 502, whereas for both M3 and M4 they were observed at m/z 500. An additional adduct (M5) was detected by LC-MS with an $[M + H]^+$ ion at m/z 529 (Figure 3).

Table 2. Effects of P450 Inhibitors and Trapping Agents on the Covalent Binding of L-739,010[a]

additions	concn (mM)	covalent binding [nmol equiv/(mg of protein·h)]
no inhibitor	−	5.6 ± 0.9
ANF	0.1	3.8 ± 1.8
DDC	0.1	3.0 ± 0.1[b]
TAO	0.1	2.2 ± 0.1[b]
GSH	5	2.7 ± 0.8[b]
methoxylamine	5	1.0 ± 0.1[b]

[a] Conditions for microsomal incubation and assessment of covalent binding are described in Materials and Methods. Results are expressed as the mean ± SD from three incubations. [b] Significantly different from "no inhibitor" control (Dunnett's comparison, $p < 0.05$).

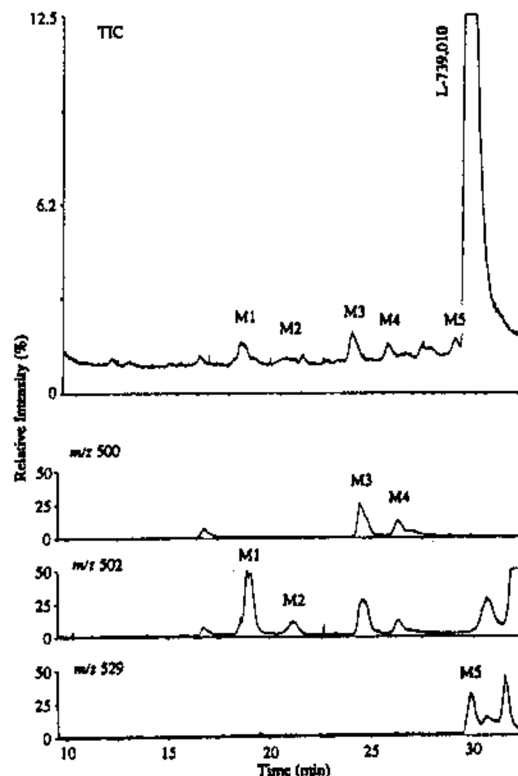

Figure 3. Ion current chromatograms obtained from LC-MS analysis of rat liver microsomal incubates of L-739,010 containing methoxylamine (5 mM) and an NADPH-generating system. The upper trace represents the total ion current (TIC) chromatogram, and the lower traces represent extracted ion chromatograms for m/z 500, 502, and 529.

To obtain structural information on these derivatized metabolites, product ion mass spectra were recorded and compared to that of L-739,010. As shown in Figure 4, the most prominent fragment ion in the spectrum of L-739,010 is at m/z 203, which resulted from cleavage of the ether bond between the two aromatic moieties, accompanied by the loss of water, and charge retention on the pyridine-containing moiety.

Figure 5 shows the product ion mass spectra of all five methoxylamine adducts of L-739,010. Similar to the parent drug, the most prominent fragment ion was observed at m/z 203 in the spectra of M1, M2, M3, and M5. These data indicate that the pyridylbicyclooctane structure of L-739,010 was intact, consistent with the structures shown in Figure 5 which represent *O*-methyloxime derivatives of the ring-opened furan structure. In contrast, the spectrum of M4 showed a completely different fragmentation pattern, in which the presence of ion at m/z 248 suggested that the naphthyl furan

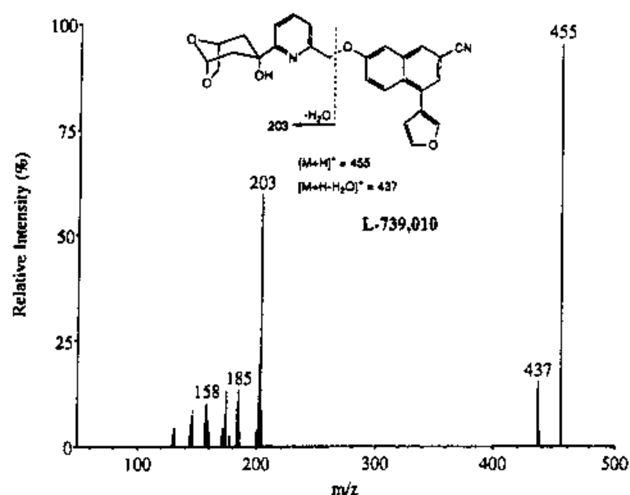

Figure 4. Product ion mass spectrum of L-739,010 obtained by collision-induced dissociation of the protonated molecular ion at m/z 455.

structure was intact and that the *O*-methyloxime was formed by reaction on the bicyclooctane moiety. This latter metabolic pathway was demonstrated in a separate study with L-739,010 and a structural analog thereof, using semicarbazide as the trapping agent (*12*). In the present study, simultaneous quantitation by an on-line radiochemical detector suggested that M4 represents a minor metabolic adduct.

NMR analyses of the isolated metabolic adducts confirmed the mass spectral findings for M1, M2, M3, and M5. The NMR spectra of the parent drug and a representative metabolite (M1) are shown in Figure 6. Proton assignments in the spectrum of L-739,010 were deduced by a combination of inspection, double irradiation, and nuclear Overhauser effect (NOE) experiments. The spectrum of M1 revealed that the characteristic furan signals at 6.67, 7.61, and 7.67 ppm were absent. It was reasonable to infer that two novel vinyl proton signals at 7.22 and 6.76 ppm, together with a methylene resonance at 4.47 ppm and a methoxyl peak at 3.81 ppm, represented the *E* or *Z* isomers of the *O*-methyloxime formed by the reaction of methoxylamine with the putative aldehyde of the ring-opened furan. Aside from modest upfield displacements of the naphthyl H2 and H8 protons, all of the remaining signals were essentially unchanged from those of the parent drug.

Table 3 summarizes the NMR spectral properties of L-739,010 and the methyloxime derivatives of metabolites M1, M2, M3, and M5. The spectra of M1 and M2 were very similar and showed differences in the chemical shifts of one vinyl proton (H_a) and the CH_3O protons. Hence, M1 and M2 were identified as the *Z* and *E* isomers of the 1-*O*-methyloxime-2-butene-4-alcohol derivative of L-739,010. The spectrum of M3, in comparison to that of M1, showed the characteristic aldehyde proton signal (H_c) at 9.89 ppm and notably downfield shifts for the two vinyl protons. This metabolite was identified as the 1-*O*-methyloxime-2-butene-4-aldehyde derivative of L-739,-010. The structure of M4 could not be assigned fully by NMR due to the availability of only a limited quantity of this isolate. M5 was confirmed by NMR to be the bis-*O*-methyloxime derivative of the ring-opened furan, based on the presence of two methoxy groups and a one-proton singlet at 8.06 ppm (H_c).

Species Comparison. When [^{14}C]L-739,010 was incubated with hepatic microsomes from dog, rhesus monkey, and human, fortified with methoxylamine and an NADPH-generating system, the LC-MS profiles were found to be qualitatively similar to that from rat (Figure 7). M1 was formed in all species as the most prominent adduct. The relative abundance of these metabolic adducts, quantified by an on-line radiochemical detector, were M1 > M3 ≈ M4 > M2 (chromatograms not shown). M5 did not elute as a distinct radioactive peak due to overlap with the large parent drug peak, but its presence was revealed by LC-MS, probably at a level slightly lower than that of M3 or M4. Species difference was observed as to the total amounts of adducts formed, which followed the rank order: monkey > rat > human > dog. A new metabolite was detected in incubations with monkey and human liver microsomes, which eluted at 20.5 min and showed an $[M + H]^+$ ion at m/z 471. The product ion mass spectrum of this metabolite suggested that it was a monohydroxylated derivative of L-739,010, in which the hydroxyl group was on the pyridylbicyclooctane side of the molecule.

Discussion

The 5-lipoxygenase inhibitor L-739,010 was found to form reactive metabolite(s) which appeared to be bound covalently to plasma proteins *in vivo* in the rat and rhesus monkey (*6*). In the present study, metabolism of L-739,010 was examined in hepatic microsomes in order to investigate possible mechanisms by which L-739,010 undergoes metabolic activation to a reactive intermediate.

In rat liver microsomes, L-739,010 did not form any metabolite that could be detected in the supernatant of the incubation mixture. Similar observations were made during the discovery phase, from which it was concluded that the metabolism of this compound was very limited. An unusual observation was made in the earlier study using unlabeled L-739,010 that the total recovery of the parent drug was particularly low in monkey liver microsomes. With the help of the radiolabeled drug, however, it could be demonstrated that L-739,010 underwent metabolism by microsomes primarily to reactive species which became bound covalently to microsomal proteins. Thus, the earlier observation of low recovery could be rationalized and could become informative in the absence of radiolabeled drug (*12*). In rat liver microsomes, the covalent binding was NADPH-dependent and was modulated by inducers and inhibitors of cytochrome P450, implicating the involvement of P450 enzymes. Specifically, PB and DEX, both of which are P450 3A inducers (*13, 14*), increased the covalent binding, whereas the P450 3A inhibitor TAO (*15*) decreased the covalent binding. These observations suggested that L-739,010 is metabolically activated, to a significant degree, by P450 3A. The P450 1A inducer BNF (*13*) decreased the covalent binding of L-739,010, which suggested that P450 1A enzymes are poor catalysts for the bioactivation of L-739,010. While inducing P450 1A, BNF concurrently suppresses P450 3A (*16*), or it may induce alternative metabolic pathways of L-739,010. The covalent binding of L-739,010 was not affected by pretreatment with acetone, a P450 2E1 inducer (*17*), but appeared to be inhibited by DDC. Although DDC primarily inactivates P450 2E1, at high concentrations, it has been found to inhibit other P450 isoforms including the 3A family of enzymes (*18, 19*). It is possible that the inhibition of covalent binding by DDC is due to its nonspecificity on P450 isoforms other than 2E1. Thus,

Figure 5. Product ion mass spectra of *O*-methyloxime derivatives of five microsomal metabolic adducts of L-739,010, obtained by collision-induced dissociation of the respective protonated molecular ions.

P450 2E1 does not appear to play a significant role in the bioactivation of L-739,010.

Evidence for the formation of reactive intermediate from L-739,010 became more apparent when it was found that the covalent binding was decreased by GSH and was blocked almost completely by methoxylamine, an aldehyde trapping agent (*20*). These findings implicated an aldehyde as the most likely candidate for the reactive species. More importantly, the reactive species was trapped as *O*-methyloxime derivatives, and these metabolic adducts were amenable to analysis by LC-MS/MS and NMR techniques to provide definitive structural information.

In rat liver microsomal preparations, five methyloxime adducts were detected by LC-MS, four of which were identified by MS/MS and NMR as the mono- and bis-adducts on the ring-opened furan moiety of L-739,010. Thus, in the presence of methoxylamine, metabolic adducts accounted for 7.5% of the total radioactivity, covalent binding was about 1%, and the remainder was due to the parent drug. Collectively, the four furan-derived adducts accounted for more than 6% of the total radioactivity in the incubation and, therefore, represented the majority of sequestered reactive intermediate that could bind to microsomal proteins in the absence of methoxylamine (*ca.* 7% covalent binding). Among the three mono-adducts, one was identified as the 1-*O*-methyloxime-2-butene-4-aldehyde derivative of L-739,010 (M3), and the other two were identified as the *E* and *Z* isomers of the 1-*O*-methyloxime-2-butene-4-alcohol (M1

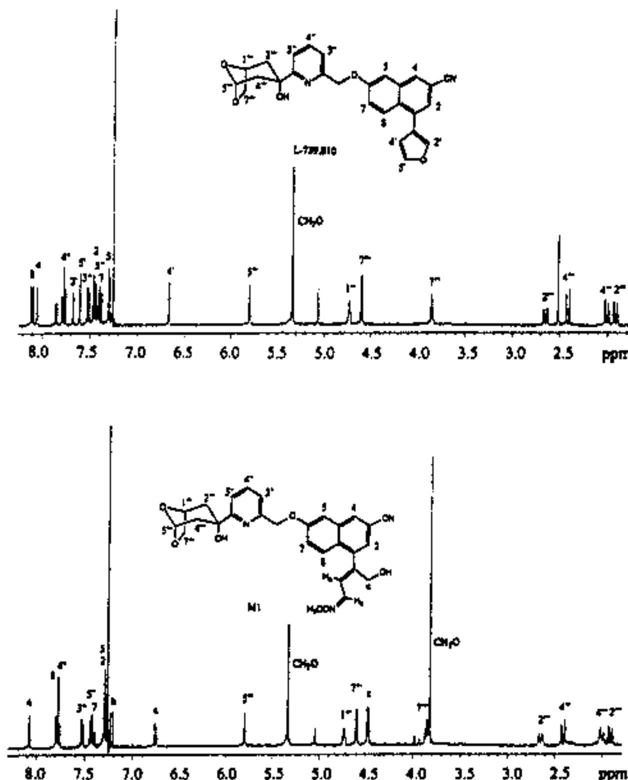

Figure 6. ^{1}H-NMR spectra of L-739,010 and the *O*-methyloxime derivative of a major metabolic adduct (M1).

Table 3. NMR Spectral Properties of L-739,010 and Its *O*-Methyloxime Derivatives of Metabolites M1, M2, M3, and M5

protons	parent drug L-739,010	mono-adduct M1	mono-adduct M2	mono-adduct M3	bis-adduct M5
H2	7.46, d, $J = 1.7$	7.30, m[a]	7.27–7.30, m[a]	7.31, m[a]	7.30, d, $J = 1.8$
H4	8.05, br s	8.08, s	8.09, s	8.14, s	8.10, s
H5	7.30, d, $J = 3.2$	7.30, m[a]	7.27–7.30, m[a]	7.31, m[a]	7.28, d, $J = 3.0$
H7	7.39, dd, $J = 9.3, 2.7$	7.41, dd, $J = 9.1, 3.0$	7.42, dd, $J = 10.0, 2.8$	7.38, dd, $J = 9.1, 2.8$	7.37, dd, $J = 9.3, 2.4$
H8	8.10, d, $J = 9.3$	7.79, d, $J = 9.1$	7.76, d, $J = 9.1$	7.49, d, $J = 9.1$	7.67, d, $J = 9.3$
H2′	7.67, dd, $J = 1.5, 0.9$				
H4′	6.67, dd, $J = 1.9, 0.9$				
H5′	7.61, t, $J = 1.7$				
H_a		6.76, dt, $J = 10.3$	6.56, d, $J = 10.0$	7.46, d, $J = 10.1$	*ca.* 7.29[b]
H_b		7.22, d, $J = 10.1$	7.27–7.30[a]	7.52, d, $J = 10.1$	6.77, d, $J = 10.3$
H_c		4.47, dd, $J \approx 6, 1.6$, 2H	4.48, dd, $J = 6.1, 1.6$, 2H	9.89, s	8.06, s
CH_3O		3.81, s, 3H	3.94, s, 3H	3.98, s, 3H	3.86, s, 3H 3.70, s, 3H
CH_2O	5.34, s	5.33, s	5.34, s	5.34, s	5.33, s
H3″	7.52, dd, $J = 7.3, 0.9$	7.53, d, $J = 7.9$	7.53, d, $J = 7.9$	7.53, d, $J = 7.7$	7.53, d, $J = 7.7$
H4″	7.77, t, $J = 7.8$	7.78, t, $J = 7.9$	7.77, t, $J = 7.7$	7.77, t, $J = 7.7$	7.78, t, $J = 7.8$
H5″	7.45, dd, $J = 7.8, 0.9$	7.44, d, $J = 8.0$	7.44, d, $J = 8.0$	7.42, d, $J = 8.0$	7.45, d, $J = 7.5$
H1‴	4.73, t, $J = 4.2$	4.73, br t, $J \approx 3$	4.74, t, $J \approx 3$	4.74, t, $J \approx 4$	4.74, t, $J \approx 4.5$
H2‴	2.64, ddd, $J = 14.3, 4.1, 1.4$ 1.92, dt, $J = 14.6, 1.4$	2.64, dt, $J = 14.7$ 1.92, d, $J = 14.5$	2.64, d, $J = 14.5$ 1.92, d, $J = 14.2$	2.65, dd, $J = 14.8, 4.0$ 1.92, d, $J = 14.3$	2.65, dd, $J = 14.3, 3.8$ 1.92, d, $J = 14.5$
H4‴	2.41, dd, $J = 11.5, 1.7$ 2.01, dt, $J = 14.4, 1.7$	2.41, d, $J = 14.3$ 2.01, d, $J = 14.7$	2.41, d, $J = 14.7$ 2.01, d, $J = 14.3$	2.41, d, $J = 14.5$ 2.01, d, $J = 14.5$	2.42, d, $J = 14.5$ 2.02, d, $J = 14.5$
H5‴	5.80, t, $J \approx 2$	5.80, br s	5.80, s	5.80, s	5.80, s
H7‴	4.60, d, $J = 6.7$ 3.85, ddd, $J = 6.5, 5.2, 1.3$	4.60, d, $J = 6.8$ 3.85, t, $J = 6.1$	4.60, d, $J = 6.6$ 3.85, t, $J = 5.4$	4.60, d, $J = 6.8$ 3.86, t, $J = 5.9$	4.60, d, $J = 6.6$ 3.86, t, $J \approx 6$

[a] Doublet pattern obscured due to overlapping signals. [b] Overlaid by $CHCl_3$ signal.

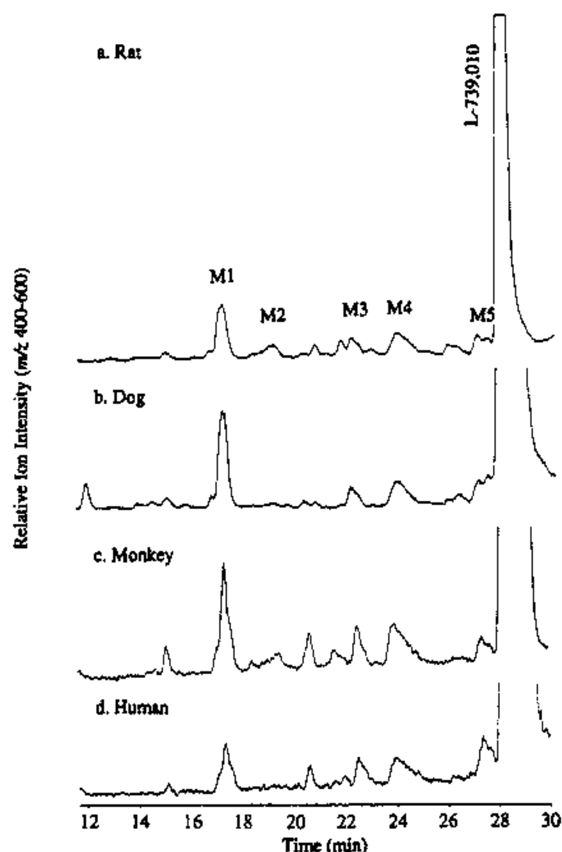

Figure 7. Total ion current chromatograms obtained from LC-MS analysis of L-739,010 incubates with methoxylamine and hepatic microsomes from rat (a), dog (b), monkey (c), and human (d).

and M2), which presumably resulted from reduction of M3. Reduction of an aldehyde to alcohol group also has been observed following the metabolic ring opening of the furan moiety of AT-1801, a hypolipidemic agent (*21*). M5 was identified as the 1,4-bis-methoxylamine adduct of furan ring-opened L-739,010. It is interesting that M5 was not the sole or predominant product, suggesting that, in the furan ring-opened dialdehyde structure, one aldehyde group may be more reactive than the other. To summarize the above findings, one can rationalize all four methoxylamine adducts as being derived from a common reactive intermediate, *i.e.*, the 2-butene-1,4-dialdehyde derivative of L-739,010 which resulted from metabolic ring opening of the furan moiety. In the absence of methoxylamine, this reactive species presumably binds to microsomal proteins *via* nucleophilic addition to the aldehyde group(s) or Michael addition to the C═C double bond. *In vivo*, if this process were to take place in the liver, the reactive 2-butene-1,4-dialdehyde could bind to liver proteins. In the absence of data from tissue distribution studies, we have observed that L-739,010-related materials were bound to plasma proteins, which perhaps were formed during protein synthesis in the liver (*22*), or were formed in the systemic circulation after the reactive species escaped from the liver. Covalent binding to plasma proteins have been reported with a number of other drugs that form reactive metabolite(s) (*23, 24*). Therefore, if prolonged plasma half-life for the total radioactivity was observed with a drug candidate during absorption, disposition, metabolism, and excretion (ADME) studies, one explanation could be covalent binding of reactive metabolite(s) to physiological macromolecules, and therefore, further studies are warranted.

In addition to the furan-derived adducts, a minor metabolite (M4), accounting for 1.4% of the total radioactivity, was identified tentatively by MS/MS as a methoxylamine adduct on the bicyclooctane moiety. More detailed structural characterization of this metabolite was carried out by N. Chauret *et al.* (*12*).

When L-739,010 was incubated with methoxylamine and hepatic microsomes from the dog, rhesus monkey, and human, similar metabolic profiles were observed, in that all rat metabolic adducts except M2 were present, with M1 being the most abundant product. Thus, under these experimental conditions, furan bioactivation also is the major metabolic pathway in these species. In monkey and human liver microsomes, one additional minor metabolite was identified tentatively as a product of hydroxylation on the bicyclooctane or pyridine rings.

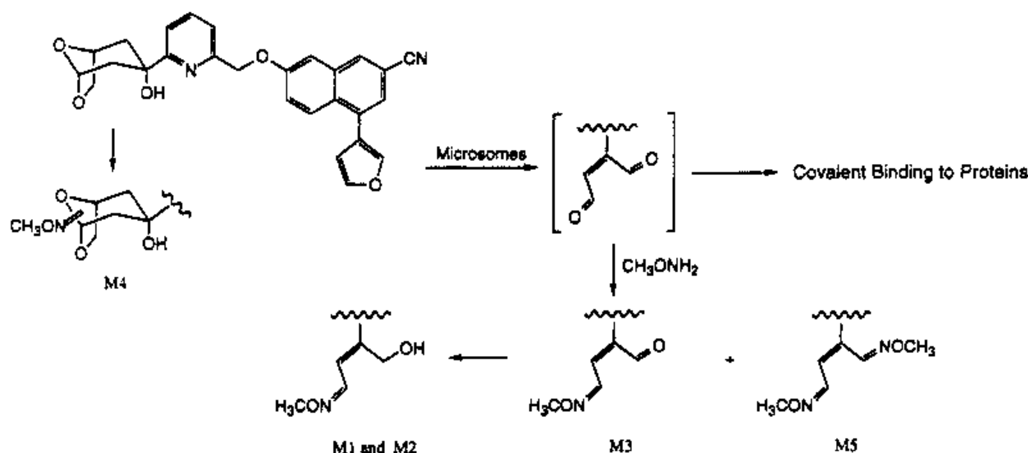

Figure 8. Proposed scheme for the metabolic activation of L-739,010 in hepatic microsomes.

The presence of M4 and this metabolite indicates that the bicyclooctane ring structure is an alternative site for metabolic attack, in agreement with previously reported results (*12*).

Many furan-containing compounds have been found to be toxic or carcinogenic to various organs including the liver, lung, and kidney (*25–28*). Furan toxicity is believed to be the result of bioactivation to reactive metabolite(s) which are capable of binding covalently to physiological macromolecules, preferentially proteins (*25, 29, 30*). P450 2E1 has been shown to play an important role in the metabolic activation and toxicity of furan itself (*31, 32*). Exposure of furan to animals results in decreased P450 activities in a nonselective fashion (*33*). Tienilic acid, which contains a thiophene structure, has been shown to induce antibodies against the cytochrome P450 isoform that metabolizes the compound (*34*).

Thus, furan-derived reactive species can inactivate the enzymes that are responsible for their formation or react with other cellular proteins distant from the site of biotransformation. Although it is not clear which process is important for furan cytotoxicity, preliminary findings suggest that impairment of mitochondrial functions represents an early event during furan exposure to hepatocytes (*35*).

Several reactive species have been proposed for the metabolic activation of furan (*25, 36, 37*). The process may be initiated by a one-electron oxidation to form a 1,2 or a 1,4 radical cation species, which can either recombine with an activated oxygen from P450 or inactivate the P450 enzyme. Between the two radical cation species, usually the more stable one will prevail. Nonetheless, oxygenation of the 1,2 radical cation will eventually lead to an epoxide, whereas the 1,4 radical cation will generate a 2-butene-1,4-dialdehyde. The epoxide can also undergo intramolecular rearrangement to form the 2-butene-1,4-dialdehyde. To date, only the dialdehyde has been observed indirectly in trapping experiments or in the form of further metabolites (*21, 36, 37*). Other intermediates either are too reactive to be observed or are never formed. Studies using stable isotope labeling with AT-1801 support the direct formation of 1,4-dialdehyde without the involvement of an epoxide intermediate (*21*).

In the present study, the furan moiety of L-739,010 also was found to be the major site of metabolism in liver microsomes from rat, dog, monkey, and human. As shown in Figure 8, ring opening of the furan generated a 2-butene-1,4-dialdehyde reactive intermediate, which could form covalently linked adducts with microsomal proteins *in vitro* and physiological proteins *in vivo*. These results demonstrated that when an unhindered furan structure is incorporated in a large molecule, it is also subject to metabolic attack, in common with smaller furan-containing compounds. Therefore, L-739,010 may share some of the toxicological properties associated with this class of compounds, and furan bioactivation may have been the underlying mechanism for the observed hepatotoxicity of this compound in dogs. Unlike furan itself, which is metabolized by P450 2E1 (*31, 33*), the bioactivation of L-739,010 was catalyzed, to a large extent, by P450 3A. One possible explanation is that P450 3A favors to metabolize large molecules (*15*), whereas P450 2E1 favors small ones (*38*). Finally, the findings of this study argue against the incorporation of an unhindered furan substituent in the design of potential drug candidates.

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