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OXIDATIVE BIOACTIVATION OF THE LACTOL PRODRUG OF A LACTONE CYCLOOXYGENASE-2 INHIBITOR

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ABSTRACT:

The lactol derivative of a lactone cyclooxygenase-2 inhibitor (DFU) was evaluated in vivo and in vitro for its potential suitability as a prodrug. DFU-lactol was found to be 10 to 20 times more soluble than DFU in a variety of aqueous vehicles. After administration of DFU-lactol at 20 mg kg^{-1} p.o. in rats, a C_{max} of 7.5 μM DFU was reached in the plasma. After oral administration, the ED_{50}s of DFU-lactol in the carrageenan-induced paw edema and lipopolysaccharide-induced pyresis assays in rats are comparable with the ED_{50}s observed when dosing with DFU. Incubations of DFU-lactol with rat and human hepatocytes demonstrated that the oxidation of DFU-lactol can be mediated by liver enzymes and that a competing pathway is direct glucuronidation of the DFU-lactol hydroxyl group. Assays with subcellular fractions from rat liver indicated that most of the oxidation of DFU-lactol occurs in the cytosolic fraction and requires NAD(P)$^+$. Human liver cytosol can also support the oxidation of DFU-lactol to DFU when NAD(P)$^+$ is added to the incubations. Fractionation of human liver cytosolic proteins showed that at least three enzymes are capable of efficiently effecting the oxidation of DFU-lactol to DFU. Incubations with commercially available dehydrogenases suggest that alcohol and hydroxysteroid dehydrogenases are involved in this oxidative process. These data together suggest that lactols may represent useful prodrugs for lactone-containing drugs.

Cyclooxygenase catalyzes the first step of the conversion of arachidonic acid into a cascade of prostaglandins, prostacyclins, and thromboxanes. Cyclooxygenase-2 (COX-2)[2] inhibitors, which are selective for COX-2 over COX-1, are currently being evaluated as anti-inflammatory and analgesic agents. COX-2-selective inhibitors are expected to provide improved therapy over less selective nonsteroidal anti-inflammatory drug (NSAID) inhibitors in terms of better gastrointestinal (GI) tolerability (Griswold and Adams, 1996, Wallace 1994).

5,5-Dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2-(5H)-furanone (DFU; Fig. 1) is a potent and selective COX-2 inhibitor that shows a 1000-fold selectivity for the inhibition of COX-2 over COX-1 in vitro and no sign of GI ulceration at >200 times the dose for anti-inflammatory, analgesic, and antipyretic effects (Riendeau et al., 1997). DFU binds selectively to the active site of COX-2 in a manner that appears to be competitive with arachidonic acid. Like many drugs that bind to hydrophobic active sites, DFU has a limited solubility in aqueous media. A polar prodrug form of the COX-2 inhibitor with increased solubility was considered because it might be amenable to an improved i.v. formulation.

Few examples of polar prodrugs of lactones have been reported. Two published accounts describe open hydroxy-carboxylates that lactonize in vivo (Chauret et al., 1995, Rivory et al., 1994). In this article we propose another strategy, namely, using a lactol that is oxidized to the corresponding lactone. To our knowledge, the prodrug concept involving lactol oxidation to a biologically active lactone drug has not been described (Balant and Doelker, 1994, Bundgaard, 1991). Therefore, the lactol analog of DFU shown in Fig. 1 [5,5-dimethyl-3-(3-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-2,5-dihydro-2-furanol (DFU-lactol)] was prepared for evaluation as a prodrug for DFU. In contrast to DFU, DFU-lactol was found to be inactive as a COX-2 inhibitor. It had no measurable inhibitory effect on purified recombinant COX-2 or microsomal human COX-1 (IC_{50} > 100 μM, our unpublished observation), whereas DFU has an IC_{50} value of 0.3 μM for the inhibition of COX-2 (Riendeau et al., 1997). The inactivity of DFU-lactol as a COX inhibitor affords the additional advantage that nonabsorbed prodrug following p.o. dosing will not result in COX inhibition in the GI tract.

To be an effective prodrug, conversion to the drug needs to be efficient and occur with minimal interindividual variability. Failure or variation of the prodrug activation may happen if the enzymes responsible are polymorphic or if coadministration of other drugs in-

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[2] Abbreviations used are: COX, cyclooxygenase; CYP, cytochrome P-450; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; ED_{50}, effective dose required to inhibit 50% of response; HPLC/CF-LSIMS: HPLC/continuous-flow liquid secondary ion mass spectrometry; DFU-lactol, 5,5-dimethyl-3-(3-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-2,5-dihydro-2-furanol; GI, gastrointestinal; Methocel, methocellulose 400; IC_{50}, concentration required to yield 50% inhibition; NSAID: nonsteroidal anti-inflammatory drug; Tris, tris[hydroxymethyl]aminomethane; PEG, polyethyleneglycol.

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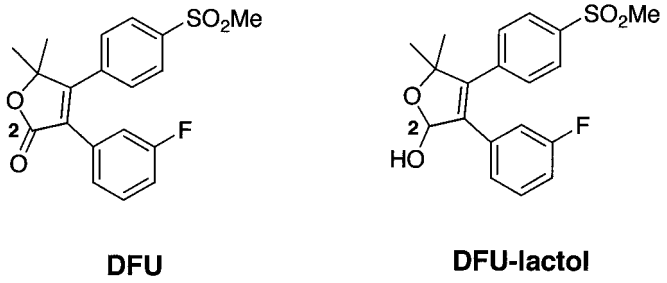

FIG. 1. *Structures of DFU-lactol and DFU.*

terferes with activation. Two examples of oxidative prodrug activation for which clinically relevant interindividual variability has been observed are codeine (Desmeules et al., 1991) and cyclophosphamide (Yule et al., 1995). Codeine is activated by a cytochrome P-450 (CYP) 2D6-mediated demethylation to yield morphine. Approximately 10% of the population is deficient in CYP2D6 and receive little analgesic effect with codeine due to poor bioactivation (Desmeules et al., 1991). Cyclophosphamide is bioactivated by CYP3A4. Induction or inhibition of CYP3A4 is known to affect the blood levels of the relevant active metabolites (Yule et al., 1995). One approach to avoid variability is to ensure that a given prodrug is bioactivated by multiple enzymes. Therefore, if one of the responsible enzymes is impaired for any reason, others are available to compensate, lessening the likelihood of significant interindividual variability.

In this article, we report on in vivo studies in the rat model demonstrating that DFU-lactol can be used as an effective prodrug for DFU. Studies with hepatocytes and hepatic subcellular fractions establish that several rat and human liver enzymes are capable of catalyzing the oxidation of the lactol to the lactone in vitro. Incubations of DFU-lactol with model dehydrogenases suggest that alcohol and hydroxysteroid dehydrogenases are involved in this process. Overall, the observations on the oxidation of DFU-lactol to DFU suggest that lactols may represent useful prodrugs for lactone drugs.

Materials and Methods

Chemicals and Reagents. DFU and DFU-lactol were synthesized at Merck Frosst (Ducharme et al., 1995; Black et al., 1997). Stock solutions were prepared in DMSO. Frozen human liver tissue was obtained from P.F. Guengerich (Vanderbilt School of Medicine, Nashville, TN) and IIAM (Exton, PA), and fresh tissue was obtained from a consenting donor undergoing a partial hepatectomy (St. Luc Hospital, Montreal). All dehydrogenases were purchased from Sigma (St. Louis, MO), except malate dehydrogenase, which was obtained from Worthington Biochem (Freehold, NJ). Molecusol and methocellulose 400 (Methocel) were obtained from Pharmtec Inc. (Alachua, FL) and Sigma, respectively.

Solubility of DFU and DFU-Lactol. Unless otherwise specified, the solubilities of DFU and DFU-lactol were determined by stirring excess solid in either deionized water (Milli-Q, Waters, Milford, MA), saline, or 5% ethanol/10% polyethylene glycol (PEG) 400/saline at room temperature in absence of light for at least 3 h. The samples were either filtered through a 0.45-μm filter or centrifuged for 15 min at 14,000 rpm using an Eppendorf 8415C centrifuge. The clear supernatant of the DFU samples was analyzed directly by UV spectroscopy using a HP8451 UV spectrophotometer and an absorptivity of 30.3 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} at 262 nm (methanol). However, because of the increased solubility of the DFU-lactol, the clear supernatant was diluted with the solvent to an appropriate concentration and analyzed by UV spectroscopy using an absorptivity of 53.3 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} at 227 nm (methanol). The solubility of DFU and DFU-lactol in polyethylene glycol (mw = 400) was determined optically by continuous addition of solid followed by shaking using a vortex mixer.

Pharmacokinetic Studies. Male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, Quebec) and were housed and fed as previously described (Nicoll-Griffith et al., 1995). Two rats were dosed for each regimen. Solutions for oral administration by gavage (20 mg kg^{-1}) were prepared from micronized material (<10 μm) as suspensions in 1% Methocel. Solutions for i.v. administration (5 mg kg^{-1}) were prepared in 25% Molecusol and were injected into the jugular vein. Plasma samples were mixed with an equal volume of acetonitrile to precipitate the proteins. After centrifugation (14,000 rpm, Eppendorf model 5415 C), the supernatant was analyzed by HPLC (system A), as described below.

In Vivo Rat Efficacy Studies. Assays for carrageenan-induced paw edema and lipopolysaccharide-induced pyresis were conducted as previously described (Riendeau et al., 1997). The ED_{50} values are derived from a four-point titration curve (0.3, 1, 3, 10 mg kg^{-1}, n = 5 rats/dose).

HPLC Analysis. For all systems, the column was a Waters Nova-Pak C_{18} (3.9 $\times$ 150 mm, 60A, 4 μm) and the mobile phase flow rate was 1 ml min^{-1}. DFU quantification was determined from external calibration curves of DFU, using peak areas. System A (isocratic): The sample volume was 50 μl and the mobile phase was methanol and 0.1% w/v sodium phosphate (pH 7.0) (42:58). UV detection was monitored at 270 nm. The retention times were 7.2 and 10 min for DFU-lactol and DFU, respectively. System B (isocratic): Samples (50 or 100 μl) were analyzed using a mobile phase consisting of methanol and 20 mM ammonium acetate (pH 7.0) (50:50). UV detection was monitored at 275 nm. The retention times were approximately 4.5 and 5.5 min for DFU-lactol and DFU, respectively. System C (gradient): Samples (25 or 50 μl) were analyzed using the mobile phase components and UV detection described in system B with a linear increase in the methanol from 20 to 90% over 25 min. The retention times were approximately 15.6 and 16.3 min for DFU-lactol and DFU, respectively. The amount of DFU-lactol glucuronide was estimated by comparing the peak areas to an external calibration curve of DFU-lactol, which assumes the lactol and its glucuronide have similar extinction coefficients. The total amount of DFU in the samples was estimated by summing the areas of DFU and the three related metabolites (**M1-M3**) and comparing these with an external standard curve of DFU, again assuming that the extinction coefficients would be similar.

Incubations with Hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats or from human tissue by standard procedures (Moldeus et al., 1978, Li et al., 1992; Silva et al., 1998). The cell viability (>90%) was assessed by trypan blue exclusion, using light microscopy. The cells were incubated in 1.0 or 0.5 ml Krebs Henseleit buffer (2 $\times$ 10^6 cells ml^{-1}) containing 12.5 mM HEPES (pH 7.4) at 37°C under O_2/CO_2 (95%/5%) in continuously shaking 24- or 48-well plates. After a 20-min preincubation, 50 μM DFU-lactol or DFU was added to the cells (final DMSO concentration ~0.6%) and the atmosphere was changed to CO_2/air (5%/95%). Boiled cells were used as controls. For time-course studies, 75-μl aliquots were removed as a function of time, and quenched with 75 μl acetonitrile to lyse the cells and precipitate the proteins. After centrifugation, the supernatant was diluted 1:1 with 20 mM ammonium acetate (pH 7.0) and was analyzed by reverse-phase HPLC (system C) and/or by capillary HPLC/ chromatography/continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS).

Incubations with Subcellular Fractions. Subcellular fractions were prepared as described below and all were stored at −80°C before use. Protein concentrations were determined by the Bio-Rad dye binding assay (Bio-Rad, Hercules, CA) using BSA as the standard. Rat liver fractions were prepared from fresh tissue and human cytosol from frozen tissue. All operations were performed at 0 to 4°C. Mitochondria were isolated as described by Cain and Skilleter (1987) and were resuspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4. Cytosolic and microsomal fractions were prepared according to Lu and Levin (1972). The cytosolic fraction was obtained in 50 mM Tris-HCl buffer, pH 7.4, containing 1.15% KCl. The microsomal pellet was resuspended in 0.25 M sucrose. Incubations were conducted in triplicate with the subcellular fractions and 200 μM DFU-lactol or DFU (2% final DMSO concentration) in a 100 mM phosphate buffer, pH 7.4, in the presence of 1 mM NAD^+, $NADP^+$, or both, in a total volume of 0.5 ml. To have less than 20% conversion of DFU-lactol to DFU in 15 min, the following amounts of protein were used in the incubations: rat microsomes 0.1 mg, rat cytosol 0.01 to 0.03 mg, rat mitochondria 0.1 mg, and human cytosol 0.25 mg. Some incubations were also conducted using 1 mM NADH or NADPH. Control incubations were conducted with boiled protein or no $NAD(P)^+$. Reactions were stopped by quenching with 500 μl of acetonitrile to precipitate the proteins. After vortex

and centrifugation, analysis of the supernatant was conducted with HPLC (system B) to determine the amount of DFU produced from DFU-lactol. To confirm that the DMSO present in the incubations did not inhibit the oxidation, incubations were conducted with human cytosol (Human A) using the methanol evaporation technique described previously (Chauret et al., 1998). For kinetic studies in human cytosol (Human A), the concentration range of 5 to 600 μM DFU-lactol was used. A Lineweaver-Burk kinetic analysis was conducted using the "Enzyme Kinetics" software (Trinity Software. Campton, NH).

Preparation of Glucuronide Derivative of DFU-Lactol. To obtain the glucuronide for characterization, an incubation was conducted for 2 h using 200 μM DFU-lactol (2% final DMSO concentration) and 30 mg of human microsomal protein in 15 ml of 50 mM phosphate buffer, pH 6.6, containing 10 mM $MgCl_2$, 10 mM UDP-glucuronic acid and 16 mM D-saccharic acid-1,4-lactone. The incubation mixture was quenched with 15 ml acetonitrile. After centrifugation, the product in the supernatant (78% yield) was isolated by solid-phase extraction and preparative HPLC as previously described (Nicoll-Griffith et al., 1992) with the following modification. The incubation supernatant was diluted 10-fold with 20 mM ammonium acetate. Solid-phase extraction was conducted on a 6 ml BondElut C18 cartridge (Varian, Palo Alto, Ca). Approximately 1.25 mg of the glucuronide was obtained.

NMR Characterization of DFU-Lactol Glucuronide. NMR spectra were obtained on a Bruker AMX 500 as previously reported (Nicoll-Griffith et al., 1995) except for the following changes. Signals in the spectrum of the metabolite were assigned by inverse proton-carbon and long-range proton-carbon correlation experiments.

Mass Spectral Characterization. HPLC/CF-LSIMS spectra were obtained as previously described (Li et al., 1995) except for the following changes. The column was a KAPPA BDS Hypersil column (Keystone Scientific Inc., Bellefonte, PA). A linear HPLC gradient was used from 20 to 60% methanol and 80 to 40% 20 mM ammonium acetate over 40 min. Both components contained 1.5% glycerol.

Fractionation of Human Liver Cytosol. An aliquot of human liver cytosol extract (Human A, 60 mg protein ml^{-1}, 100 μl) was injected onto a Protein-Pak 300 SW column (Waters) pre-equilibrated with 0.1 M sodium phosphate, pH 7.3 and the column developed at 0.5 ml min^{-1}. Fractions (0.5 ml) were collected and the protein determined by a Bio-Rad dye binding assay. Aliquots (10 μl) of each fraction were assayed for their capacity to catalyze the oxidation of the prodrug with NAD^+ or $NADP^+$ using conditions analogous to those described below. The following standard proteins were used for molecular mass calibration: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), ovalbumin (43 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1.3 kDa).

Oxidation of DFU-Lactol by Dehydrogenases. Nineteen commercial NAD^+- or $NADP^+$-dependent enzymes having aldehydes or alcohols as natural substrates were tested. Each enzyme was resuspended in 0.1 M sodium phosphate, pH 7.3, at a concentrations of 1 to 10 unit μl^{-1}. DFU-lactol (200 μM) was incubated at 37°C for 1 h with 1 unit of enzyme in the presence 1 mM NAD^+ or $NADP^+$ in 500 μl 0.1 M phosphate buffer, pH 7.3. One unit is defined as 1 μmol of the optimal substrate consumed per minute, according to the manufacturer's specifications. After quenching with 500 μl of acetonitrile, the formation of DFU was determined by isocratic HPLC as described above (system B).

Results

The solubility of DFU and DFU-lactol in a variety of vehicles is reported in Table 1. The lactol was at least one order of magnitude more soluble than DFU. In aqueous solutions of pure water or saline, the concentration of DFU-lactol exceeded 0.2 mM. The solubility could be further enhanced with the additional use of ethanol and PEG.

Drug concentrations in rat plasma following administration of DFU and DFU-lactol are shown in Fig. 2. At doses of 20 mg kg^{-1} p.o. of DFU and DFU-lactol, the C_{max} levels of DFU were determined to be ~5 and 7.5 μM, respectively (Fig. 2, a and b). The areas under the curve (AUCs) (0–6 h) of DFU after dosing with DFU or DFU-lactol p.o. were 14.7 and 28.2 μM · h, respectively. Dosing DFU-lactol by the i.v. route (Fig. 2c) showed that efficient oxidation of DFU-lactol occurred in vivo. After oral dosing, the ED_{50} of DFU-lactol in carrageenan-induced paw edema assay in rats was 1.2 mg kg^{-1} (versus 1.1 mg kg^{-1} for DFU). In the lipopolysaccharide-induced pyresis assay in rats, the ED_{50} was 0.5 mg kg^{-1} (versus 0.8 mg kg^{-1} for DFU).

TABLE 1

Solubility of DFU and DFU-lactol in different vehicles

Vehicle	DFU	DFU-lactol
	mM	*mM*
Water	0.023	0.26
0.9% NaCl	0.014	0.24
5% Ethanol/10% PEG/0.9% NaCl	0.034	0.66
PEG400	22–28	>69

HPLC/CF-LSIMS analysis of DFU-lactol indicated that it readily dehydrated in the ion source. The major ion observed was $m/z = 345$ and a much weaker ion was observed for the MH^+ at $m/z = 363$. DFU gave an MH^+ ion at $m/z = 361$. HPLC/UV analysis indicated that two major metabolites were formed after incubation of DFU-lactol with rat hepatocytes (Fig. 3). One peak had the same retention time and mass spectral characteristics as DFU. The other had the same retention time as the biosynthetic glucuronide described below (Fig. 4). It exhibited an MNH_4^+ ion at $m/z = 556$ and the dehydrated aglycone (DFU-lactol-H_2O) at $m/z = 345$. In rat hepatocytes, approximately equal amounts of DFU and the DFU-lactol glucuronide were formed (Figs. 3 and 5). In human hepatocytes, the ratio of DFU to DFU-lactol glucuronide produced was approximately 2:1 throughout the time course (Fig. 5). Incubations of DFU gave rise only to metabolites **M1-M3** (the structures of which will be reported elsewhere): no reduction to DFU-lactol was observed (Fig. 3). Boiled cells did not metabolize either DFU-lactol or DFU.

In human liver microsomes supplemented with UDP-glucuronic acid, DFU-lactol was efficiently converted to the glucuronide. NMR analysis of the isolated material indicated that the product was a mixture of the two possible ether-linked diastereomers in a ratio of approximately 10:1. An anomeric coupling constant of 7.9 Hz was observed for both the major and minor components indicating that their glucuronide linkages are β. The glucuronide linkage in the major isomer was confirmed by the observation of long-range correlations between the anomeric protons and carbon 2 of DFU-lactol and between the 2-proton of DFU-lactol and the anomeric glucuronide carbon (see Fig. 1 for numbering). Unfortunately, there was insufficient material to characterize the glucuronide linkage in the minor isomer. However, it is likely that this component is the diastereomer of the major isomer in which the stereochemistry at carbon 2 is inverted.

Studies with rat liver subcellular fractions showed that the oxidation of DFU-lactol to DFU could be catalyzed by cytosolic, mitochondrial, or microsomal fractions using NAD^+ or $NADP^+$ as the cofactor, with cytosol giving the highest rate of oxidation (Fig. 6). Only traces of DFU oxidation were observed when the incubations were not supplemented with NAD^+ or $NADP^+$ and no oxidation was observed in control incubations with boiled enzyme. In rat microsomal incubations supplemented with 1 mM NADPH, the rate of oxidation of DFU-lactol to DFU was less than 20% of that obtained when using 1 mM $NADP^+$ (data not shown).

The time course of DFU-lactol oxidation in incubations with a human liver cytosol is shown in Fig. 7. DMSO present in the incubations caused no inhibition of enzyme activity, compared with incubations containing no organic solvent. Experiments using NADH or NADPH gave less than 5% of the rate observed with NAD^+ or

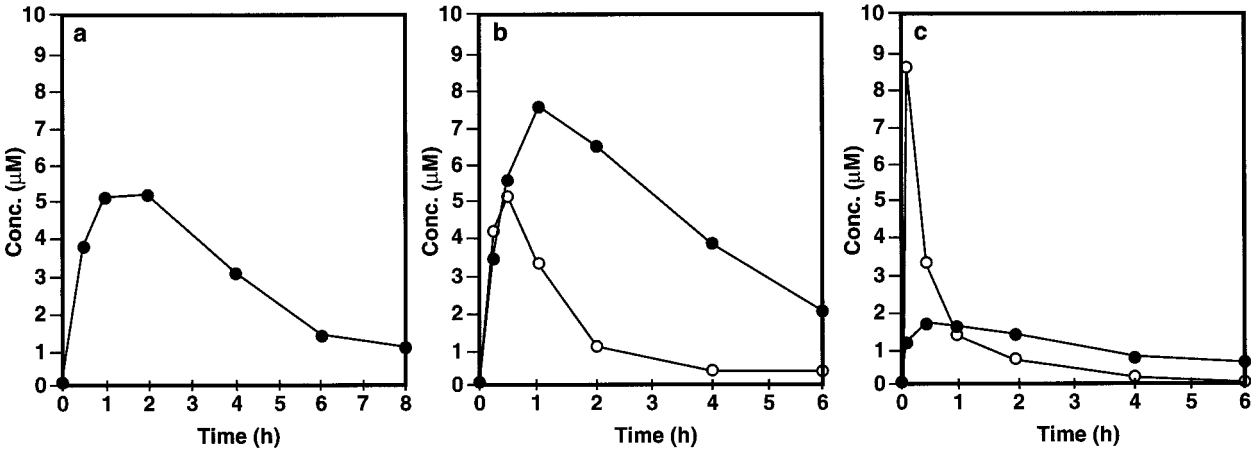

FIG. 2. *Plasma concentrations of DFU and DFU-lactol in rat.*

A, 20 mg kg^{-1} p.o. dosing of DFU in 1% Methocel. B, 20 mg kg^{-1} p.o. dosing of DFU-lactol in 1% Methocel. C, 5 mg kg^{-1} i.v. DFU-lactol in 25% Molecusol. ●, DFU; ○, DFU-lactol.

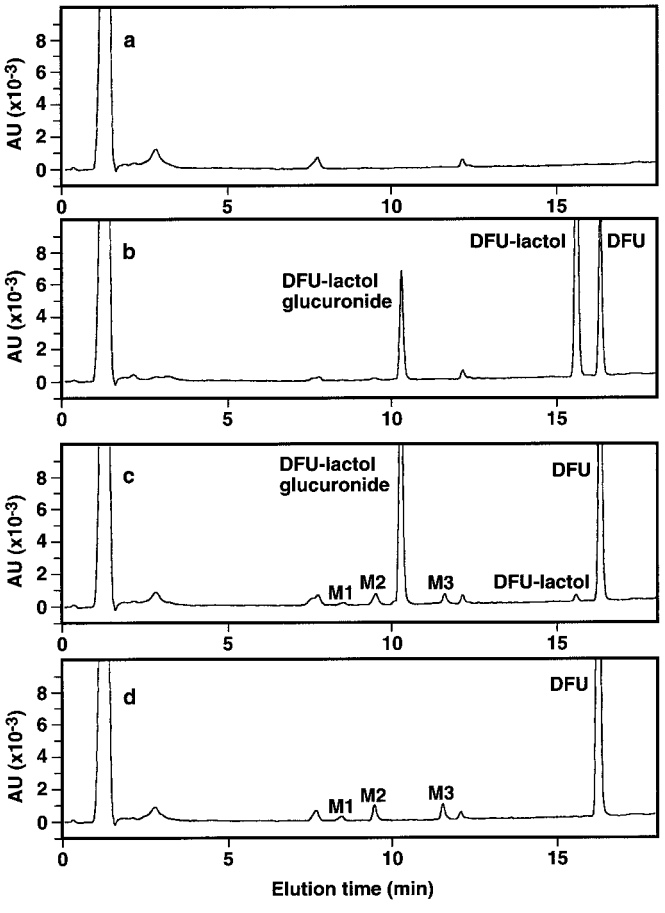

FIG. 3. *HPLC chromatograms from incubations of rat hepatocytes with DFU-lactol and DFU.*

a, 2-h blank incubation; b, 15-min incubation of DFU-lactol; c, 2-h incubation of DFU-lactol; and d, 2-h incubation of DFU. Gradient HPLC (system C) was used with UV detection at 275 nm (see *Materials and Methods*).

$NADP^+$. Studies with cytosol from human A using NAD^+, indicated a V_{max} for the oxidation of DFU-lactol of 9.4 ± 1.3 nmol/(mg protein · min) and half-maximal activity at 189 ± 37 μM, which may reflect the limited solubility of DFU-lactol.

Studies with cytosol from four individual humans, using 200 μM DFU-lactol, showed a relatively low amount of variability between individuals when NAD^+, $NADP^+$, or both, were used as the cofactor, as reported in Table 2. The activity profile from fractionated human liver cytosol showed three discrete bands of DFU oxidizing activity (Fig. 8) eluting at apparent molecular masses of 160 kDa (NAD^+ dependent), 47 kDa (NAD^+ dependent), and 14 kDa (NAD^+ or $NADP^+$ dependent). The relative NAD^+ dependent activity of the peaks was approximately 1:2:2.7.

Six of 19 commercially available dehydrogenases were found to catalyze the oxidation of DFU-lactol and had an activity similar to, or greater than, one nmol hr^{-1} per unit of enzyme activity (Table 3). Apart from a trace amount of activity observed for α-ketoglutarate dehydrogenase, all of the enzymes were alcohol dehydrogenases (EC 1.1.1). Enzymes that showed no detectable activity under these assay conditions were aldehyde dehydrogenase (Baker's yeast), formate dehydrogenase (yeast), formaldehyde dehydrogenase (*Pseudomona pudita*), and pyruvate oxidase (bacterial), glucose 6-phosphate dehydrogenase (Baker's yeast), L-lactic dehydrogenase (human erythrocytes), L-lactic acid dehydrogenase (human liver), malate dehydrogenase (pig heart mitochondria), cholesterol oxidase (*Pseudomonas fluorescens*), β-hydroxyacyl-CoA dehydrogenase (porcine heart), and 3α-20β-hydroxysteroid dehydrogenase (streptomyces hydrogenase).

Discussion

DFU-lactol was prepared as a prodrug of DFU to increase solubility of DFU and yield a compound that would be more amenable to i.v. formulation. DFU-lactol was significantly more soluble than DFU in several vehicles and the increased solubility allowed an i.v. formulation of DFU-lactol to be prepared in Molecusol. In the i.v. studies in the rat, it was clearly shown that DFU-lactol was bioactivated to DFU. This proves that the conversion can occur in the blood and/or tissues and is not dependent on intestinal microflora. DFU-lactol was an effective prodrug in vivo in the rat, whether administered p.o. or i.v. The C_{max} and area under the curve (AUC) (0–6 h) of DFU after p.o. dosing was comparable, or greater, in the DFU-lactol dosed rats, as compared with the DFU dosed rats. The pharmacological activity observed in the in vivo edema and pyresis rat assays confirms that the COX-2 enzyme is inhibited in vivo after oral dosing of DFU-lactol.

The proposed metabolic pathways for DFU-lactol are shown in Fig. 4. From the hepatocyte incubations it is clear that the oxidation of DFU-lactol to DFU is in competition with glucuronidation of DFU-

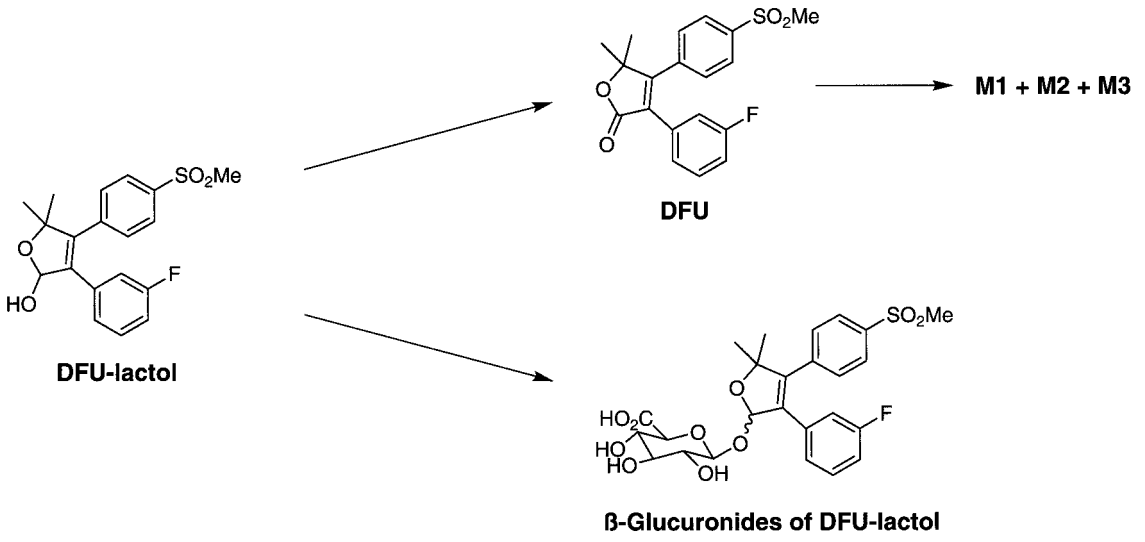

FIG. 4. *Proposed metabolic pathways of DFU-lactol in hepatocytes.*

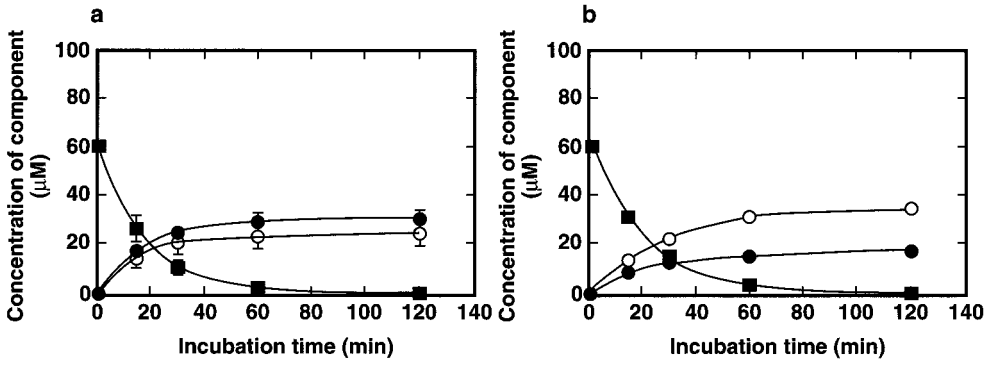

FIG. 5. *Conversion of DFU-lactol to the DFU-lactol glucuronide and DFU in rat hepatocytes* (n = *3) (a) and human hepatocytes* (n = *1) (b).*

■, DFU-lactol; ●, DFU-lactol glucuronide; ○, DFU.

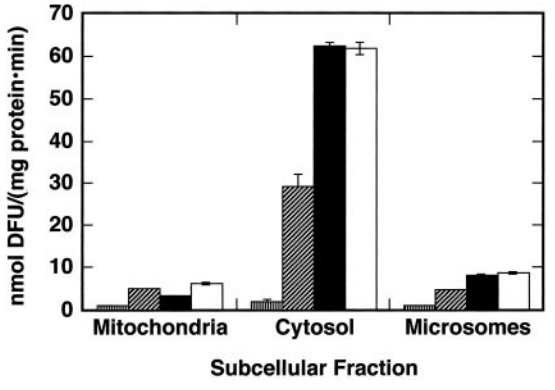

FIG. 6. *Conversion of DFU-lactol to DFU by subcellular fractions from rat liver.*

Vertical-hatch, no added NAD^+ or $NADP^+$; right-hatched columns, 1 mM $NADP^+$; black columns, 1 mM NAD^+ ; and white columns, 1 mM each NAD^+ and $NADP^+$. *See Materials and Methods* for assay conditions. Incubations (15 min) were performed in triplicate (± S.D.).

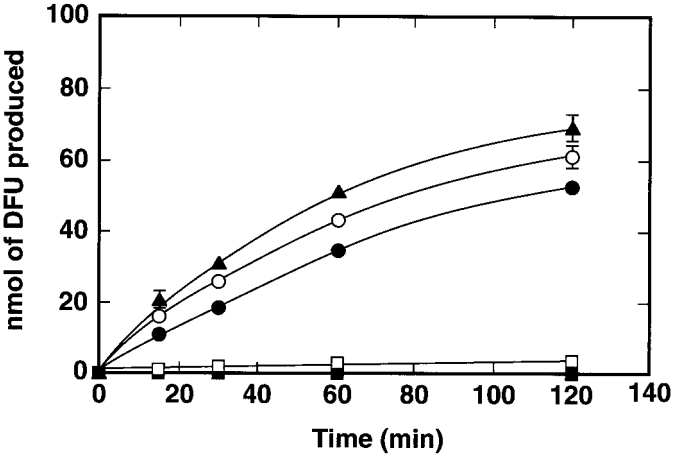

FIG. 7. *Conversion of DFU-lactol to DFU with human liver cytosol from a single donor.*

■, boiled cytosol; □, no added NAD^+ or $NADP^+$; ●, 1 mM $NADP^+$; ○, 1 mM NAD^+; and ▲, 1 mM each NAD^+ and $NADP^+$. See *Materials and Methods* for assay conditions (triplicate incubations (± S.D.)).

lactol. Studies in rat hepatocytes give approximately equal rates for the two pathways at 50 μM DFU-lactol, whereas the corresponding study with human hepatocytes indicated that the rate of oxidation to DFU is faster than glucuronidation. However, despite the glucuronidation pathway, in vivo studies in the rat indicate that oxidation of DFU-lactol occurs to yield blood levels of DFU similar to those obtained with direct administration of DFU. It is possible that although rat hepatocyte-mediated metabolism does not favor oxidation,

TABLE 2

Rates of conversion of DFU-lactol to DFU in cytosols from four human livers

Tissue Code	No NAD^+ or $NADP^+$	NAD^+	$NADP^+$	NAD^+ and $NADP^+$
Human A	0.32 ± 0.11	4.35 ± 0.19	2.91 ± 0.05	5.25 ± 0.40
Human B	0.51 ± 0.11	3.39 ± 0.21	3.52 ± 0.08	5.25 ± 0.16
Human C	0.32 ± 0.04	2.91 ± 0.29	4.00 ± 0.35	4.56 ± 0.27
Human D	0.35 ± 0.05	3.20 ± 0.05	3.39 ± 0.21	5.44 ± 0.16
Average (n = 4)	0.38 ± 0.09	3.46 ± 0.62	3.46 ± 0.45	5.13 ± 0.39

Rates are expressed as nmol DFU produced · mg $protein^{-1}$ · min^{-1} (average ± S.D., n = 3).

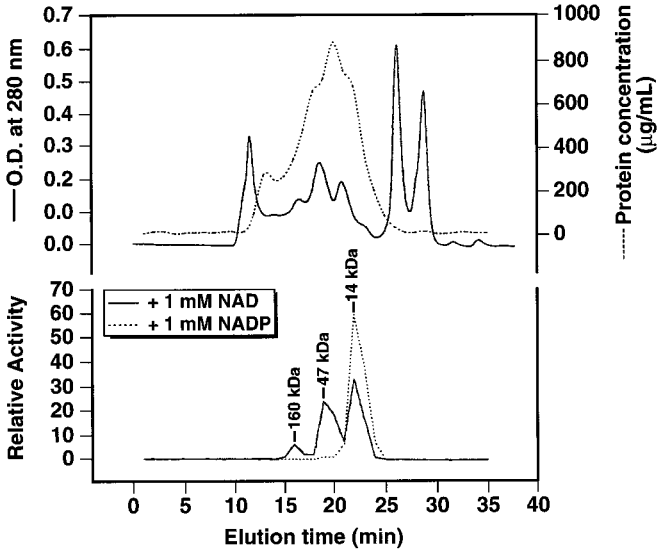

FIG. 8. *Fractionation of human liver cytosol.*

Top, optical density and protein concentrations of fractions eluted from ProteinPAK 300 SW column as a function of elution time. Bottom, relative activity of eluted fractions for oxidation of DFU-lactol to DFU in the presence of either 1 mM NAD^+ or $NADP^+$. *See Materials and Methods* for chromatography and assay conditions.

TABLE 3

Enzymes with ability to oxidize DFU-lactol to DFU in vitro

Enzyme (EC classification)	Source	DFU Produced (nmol h^{-1})[a]	
		NAD^+	$NADP^+$
Alcohol dehydrogenase (1.1.1.1)	Equine liver	15	6.8
3α-Hydroxysteroid dehydrogenase (1.1.1.50)	*Pseudomonas testosteroni*	43	ND
7α-Hydroxysteroid dehydrogenase (1.1.1.159)	*Pseudomonas* sp	1.2	57
β-Hydroxysteroid dehydrogenase (1.1.1.51)	*Pseudomonas testosteroni*	1.2	ND
Isocitrate dehydrogenase (1.1.1.42)	Porcine heart	1.9	3.8
α-Ketoglutarate dehydrogenase (1.2.4.2)	Porcine heart	ND	0.9
L-Lactic dehydrogenase (LDH1,2) (1.1.1.27)	Human erythrocytes	ND	0.7
Sorbitol dehydrogenase (1.1.1.14)	Sheep liver	9.3	ND

[a] Conversion of DFU-lactol to DFU is expressed as nmole DFU produced per hour in the presence of 1 unit of enzyme as defined by manufacturer's specifications (see *Materials and Methods*). ND, not detected.

the wide distribution of dehydrogenase enzymes in tissues favors the conversion to DFU in vivo.

It is known that bioactivation of a prodrug by a single enzyme can lead to significant interindividual differences in blood levels of the active therapeutic agent. For the examples of codeine and cyclophosphamide described in the *Introduction*, bioactivation by individual CYP enzymes is believed to cause the differences observed in vivo. In vitro studies were key to identifying the specific CYP enzymes that played a role (Dayer et al., 1988; Chang et al., 1993) and further support the use of in vitro systems for predicting in vivo metabolic processes.

Following the hypothesis that in vivo interindividual variability will be reduced or avoided if a prodrug is bioactivated by multiple enzymes, an objective for the studies with DFU-lactol was to establish whether numerous enzymes could effect the bioactivation to DFU. In rat liver, cytosol was the most effective subcellular fraction for oxidizing DFU-lactol to DFU in a reaction that was stimulated by $NAD(P)^+$. Fresh rat liver tissue was used to prepare the subcellular fractions to minimize cross contamination, however, the low levels of activity observed in mitochondria and microsomes may be due to slight contamination with cytosolic proteins (Cribb et al., 1994).

Human liver cytosolic enzymes could catalyze the oxidation with either NAD^+ or $NADP^+$ acting as the cofactor. The variability observed for DFU-lactol oxidation with the various human liver cytosols (Table 2) is very low when one considers the variability typically observed for microsomal CYP-mediated oxidations. Depending on the CYP involved, greater than 10-fold differences are commonly observed (Wrighton et al., 1993; Chauret et al., 1997). The data for DFU-lactol oxidation shows less than a 10% coefficient of variation when both NAD^+ and $NADP^+$ are used (5.13 ±0.39 nmol · mg^{-1} · min^{-1}, n = 4) and suggests that human in vivo interindividual variability of DFU-lactol oxidation would also be low. It is not clear why the rate of oxidation in human cytosol was lower than that of rat (5 versus 60 nmol · mg $protein^{-1}$ · min^{-1}, respectively at 200 μM) (Fig. 6 and Table 2), considering that the oxidation rates in human and rat hepatocytes appeared to be similar (Fig. 5).

When human liver cytosol was fractionated, three peaks of enzymatic activity were observed that could oxidize DFU-lactol (Fig. 8). Two peaks were NAD^+ dependent, whereas the third (and most active) was both NAD^+ and $NADP^+$ dependent. These data are consistent with experiments conducted with whole cytosol that indicated that NAD^+ and $NADP^+$ could be cofactors for the oxidation. The use of both NAD^+ and $NADP^+$ together gave an oxidation rate that was slightly greater than when either cofactor was used alone (Table 2 and Fig. 7). Together these in vitro experiments support the conclusion that multiple enzymes are involved in the oxidation of DFU-lactol.

Two general mechanisms potentially exist for the dehydrogenation of DFU-lactol to DFU. The first involves direct dehydrogenation of the closed lactol form: an alcohol to carbonyl oxidation. The second is via a pathway involving opening to the hydroxy aldehyde intermediate, oxidation to the hydroxy acid, and subsequent lactonization.

There is precedent that thromboxane B_2, a lactol, is oxidized from its open hydroxy aldehyde form (Westlund et al., 1994).

Enzyme superfamilies that could be involved in the oxidation of DFU-lactol to DFU are the CYPs, aldo-keto reductases, and alcohol or aldehyde dehydrogenases. The CYP enzymes are not believed to play a major role in the oxidation of DFU-lactol to DFU, because the rate of NADPH-supported oxidation in the rat liver microsomal fraction was less than 3% of the rate of $NADP^+$-supported oxidation in the cytosolic fraction (data not shown). Preliminary studies with commercial enzymes suggest that several alcohol dehydrogenases could play a role. Further experiments using recombinant or purified human enzymes and inhibition studies using human cytosol supplemented with appropriate enzyme inhibitors, such as methylpyrazole for alcohol dehydrogenase (Tolf et al., 1985) and NSAIDs for the hydroxysteroid dehydrogenases (Penning et al., 1984; Miyabe et al., 1995) are needed to evaluate the contribution of these enzymes to DFU-lactol oxidation. The role of the aldehyde dehydrogenases could be clarified with appropriate enzymes and with inhibitors such as disulfiram and its metabolites (Lam et al., 1997).

In the present study, alcohol and several hydroxysteroid dehydrogenases were identified as enzymes that could catalyze the oxidation of DFU-lactol. The implication of alcohol dehydrogenase, as shown with the horse liver alcohol dehydrogenase, raises the possibility that there could be interindividual differences in metabolism, because human alcohol dehydrogenase is polymorphic (Testa, 1995). The implication of the hydroxysteroid dehydrogenases is interesting because the 3α-hydroxysteroid dehydrogenase enzyme comprises up to 3% of protein in rat liver cytosol (Smithgall and Penning, 1988), may use both NAD^+ or $NADP^+$ as a cofactor, and is known to metabolize hydroxyprostaglandins (Penning and Sharp, 1987) and other xenobiotics (Penning et al., 1984). If it is confirmed that human alcohol and hydroxysteroid dehydrogenases play major roles in the DFU-lactol oxidation, drug-drug interaction studies would need to be conducted, in particular, with ethanol and NSAIDs. However, the involvement of multiple enzymes in the bioactivation of DFU-lactol would, hopefully, minimize these potential drug-drug interactions.

The present data with DFU-lactol suggest that the use of a lactol could be a viable approach for the delivery of a lactone drug. In vitro studies with rat and human hepatocytes, subcellular fractions, and model dehydrogenases demonstrate that multiple enzymes can catalyze the oxidation, suggesting a low likelihood for in vivo interindividual variability and for competing substrate drug-drug interactions.

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