Lisofylline is a novel anti-inflammatory compound that inhibits stress-activated lipid metabolic pathways. In clinical studies administration of lisofylline produced a dose-dependent reduction in circulating levels of oxidized fatty acids (1). Lisofylline is being developed to decrease morbidity and mortality associated with dose-intensive cancer treatment and for the prevention of acute lung injury after severe trauma. In preclinical models it is highly protective against reperfusion injury (2, 3). Pentoxifylline was introduced as a hemorheologic agent for the treatment of intermittent claudication, putatively by decreasing blood viscosity through enhanced deformability of erythrocytes (4). Pentoxifylline has since been claimed to be useful for the treatment and prophylaxis of chemotherapy-induced organ toxicity (5, 6), treatment of the wasting syndrome of AIDS (7, 8), the complications of diabetes (9), enhancing platelet recovery after bone marrow transplantation (5), and increasing sperm motility (10, 11). It has also been reported that pentoxifylline decreases portal pressure in an experimental model of cirrhosis (12) and prevents the renal toxicity of cyclosporine in heart transplantation (13). All these later claims are controversial.

Pentoxifylline is reduced to stereoisomeric alcohol metabolites by carbonyl reductase (14–16). The less favored R enantiomer, accounting for 5–10% the total alcohol in blood, is lisofylline (6). The interconversion of lisofylline, the S-alcohol, and pentoxifylline has been studied in human liver microsomes and cytosol (17). In these studies the highly stereoselective formation of the S-alcohol from pentoxifylline was confirmed, while both lisofylline and the S-alcohol could be converted back to pentoxifylline in a cytochrome P450-catalyzed reaction. Pentoxifylline was a major metabolite of both chiral alcohols. The specific P450 isoforms involved in the oxidations of lisofylline to pentoxifylline have not been identified.

The purpose of this paper is to describe the kinetics of pentoxifylline formation from lisofylline in human liver microsomes and to identify the individual P450 isoforms involved using selective chemical inhibitors, correlation with specific P450 activities, and cDNA expressed isoforms.

Materials and Methods

Chemicals. Lisofylline and CT-2410 R (internal standard, a compound structurally similar to lisofylline) were obtained from Cell Therapeutics (Seattle, WA). Furafylline was a gift from Dr. Kent Kunze of the Department of Medicinal Chemistry, University of Washington (Seattle, WA). Pentoxifylline, tolbutamide, diethylthiocarbamate, sulfaphenazole, orphenadrine, quinidine, and troleandomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were Optima grade (Fisher Chemical Co., Santa Clara, CA). All other chemicals were reagent grade.

Microsomes and Incubation Conditions. Human liver microsomes were prepared and protein determined as previously described (17). Micromosomal incubation conditions were optimized to provide linearity of pentoxifylline formation. The incubation mixture (0.5 ml) consisted of 0.5–1 mg microsomal protein and 0.1–100 μM lisofylline (peak plasma concentrations in clinical trials are ca. 5–20 μM) in 100 mM potassium phosphate buffer, pH 7.4. The mixture was preincubated briefly (15 sec) at 37°C in a shaking water bath, and pentoxifylline formation was initiated by adding NADPH to achieve a final

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chemical inhibition studies. Studies to determine the effect of cytochrome P450 isoform-selective inhibitors on the human microsomal oxidation of lisofylline to pentoxifylline were undertaken with 5 μM lisofylline in five human livers. The inhibitors used and the isozymes inhibited were: furafylline (CYP1A2) (18–20), coumarin (CYP2A6) (21), orphenadrine (CYP2B6) (22), tolbutamide (CYP2C8/9/10) (23, 24), sulfaphenazole (CYP2C9/10) (20, 25, 26), S-mephenytoin (CYP2C19, 500 μM) (27, 28), quinidine (CYP2D6) (29, 30), and diethylthiocarbamate (CYP2E1) and minor effects on CYP2A6 and CYP1A2 (20, 31–33), and troleandomycin (CYP3A) (33, 34). Concentrations used in the incubation were selected to inhibit 80% of the respective isoform activity while retaining specificity (20, 35). Poorly water soluble inhibitors (troleandomycin, tolbutamide, and sulfaphenazole) were initially dissolved in acetone, the appropriate amount of inhibitor was transferred to the incubation tube, and the acetone was evaporated to dryness under N₂. Mechanism-based inhibitors (furafylline, troleandomycin, diethylthiocarbamate, and orphenadrine) were preincubated with microsomes and NADPH for 15 min at 37°C. In these incubations, pentoxifylline formation was initiated by adding lisofylline and NADPH. To examine the general participation of P450, 1-aminobenzotriazole (1 mM) and orphenadrine) were preincubated with microsomes and water soluble inhibitors (troleandomycin, tolbutamide, and sulfaphenazole) were initially dissolved in acetone, the appropriate amount of inhibitor was transferred to the incubation tube, and the acetone was evaporated to dryness under N₂. Mechanism-based inhibitors (furafylline, troleandomycin, diethylthiocarbamate, and orphenadrine) were preincubated with microsomes and NADPH for 15 min at 37°C. In these incubations, pentoxifylline formation was initiated by adding lisofylline and NADPH. To examine the general participation of P450, 1-aminobenzotriazole (1 mM) and N-octamylamine (3 mM) were used. Aminobenzotriazole was dissolved in acetone, the acetone was evaporated as above, and N-octamylamine was diluted in methanol. Final methanol concentration in incubations was 2%. All incubations with inhibitors were performed in triplicate.

HPLC Analysis. Pentoxifylline formed in incubations was assayed by adding 100 μl (1 μg in H₂O) of internal standard (CT-2410 R) to the incubation mixture which had been stopped by the addition of 6 ml ice-cold methylene chloride. The tubes were shaken on a reciprocal shaker for 10 min and centrifuged at 3,000 rpm at 4°C. The aqueous phase was aspirated to waste and the organic phase was transferred to a second tube and evaporated to dryness at 50°C under N₂. The residue was reconstituted in 100 μl mobile phase and the 30–60 μl supernatant of pentoxifylline was pentoxifylline injected onto the HPLC.

The HPLC system consisted of a Beckman pump (114M solvent Delivery Module), Hewlett-Packard 1050 auto sampler (Hewlett-Packard Co., Wilmington, DE), Spectroflow 757 UV detector (ABI Analytical Kratos Division), and an IonPac AS11-HC analytical column (3 mm, 4.6 mm × 100 mm, Rainin Instrument Co., Woburn, MA) preceded by an Opti-Guard guard column (Optimize Technologies Inc., Portland, OR). The mobile phase was 25 mM ammonium phosphate buffer containing 0.25% acetic acid (pH 4.5 adjusted with ammonium hydroxide) : methanol, 65:35, delivered at a flow rate of 0.7 ml/min. The eluate was monitored at 273 nm. The retention times for pentoxifylline, lisofylline, and internal standard were 9.3, 13.5, and 20 min, respectively. The limit of detection for pentoxifylline was 0.01 μM.

Correlation Studies. The velocity of pentoxifylline formation at 5 μM lisofylline was determined in duplicate in microsomes prepared from 14 human livers for comparison with R-warfarin 6-hydroxylation (36) at 1 mM R-warfarin (a marker of CYP1A2 activity) and chlorozoxazone 4-hydroxylation (37) at 500 μM chlorozoxazone (a marker of CYP2E1 activity).

Incubations with cDNA Expressed CYP1A2 and CYP2E1. cDNA-expressed human CYP1A2 (Supersomes) and CYP2E1 were purchased from Gentest Corporation (Woburn, MA). Both isoforms were co-expressed with human cytochrome P450 NADPH reductase and cytochrome b₅. CYP1A2 (20 pmol) or CYP2E1 (92 pmol) were incubated for 30 min with 5 and 30 μM lisofylline and 1 mM NADPH in 0.5 ml (final volume) 100 mM potassium phosphate buffer at 37°C. The effect of 150 μM diethylthiocarbamate was also determined in cDNA-expressed CYP1A2 at 5 μM lisofylline.

Results and Discussion

Metabolism of Lisofylline to Pentoxifylline in Human Liver Microsomes. Pentoxifylline formation by human liver microsomes was linear with respect to time of incubation (up to 10 min) and microsomal protein concentration (up to 2 mg/ml) (data not shown). In vitro kinetic parameters of microsomal lisofylline oxidation to pentoxifylline were determined using substrate concentrations ranging from 0.1 to 100 μM in microsomes prepared from four human livers. Results of a representative microsomal preparation, HL-142, are

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Michaelis-Menten parameters for pentoxifylline formation in human liver microsomes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Kₘ1 (μM)</td>
</tr>
<tr>
<td>HL-127</td>
<td>0.220</td>
</tr>
<tr>
<td>HL-135</td>
<td>0.270</td>
</tr>
<tr>
<td>HL-142</td>
<td>0.501</td>
</tr>
<tr>
<td>HL-146</td>
<td>0.138</td>
</tr>
<tr>
<td>Mean</td>
<td>0.282</td>
</tr>
<tr>
<td>SD</td>
<td>0.135</td>
</tr>
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</table>
shown in fig. 1. The Eadie-Hofstee plot for pentoxifylline formation was biphasic, indicating the involvement of at least two isoforms of P450. Values of $K_m$ and $V_{max}$ determined in four human liver microsomes are listed in table 1. The mean ($\pm$ SD) values for $K_m$ and $V_{max}$ were 0.282 $\pm$ 0.013 $\mu$M and 0.003 $\pm$ 0.001 nmol/min/mg protein, and for $K_m$ and $V_{max}$ were 158 $\pm$ 4.6 $\mu$M and 0.928 $\pm$ 0.308 nmol/min/mg protein, respectively.

Chemical Inhibition Studies. To identify the low apparent $K_m$ enzyme, a range of cytochrome P450 isozyme-selective inhibitors were incubated with 5 $\mu$M lisofylline in five human liver microsome preparations (HL 127, 135, 139, 142, and 146). The results are shown in table 2. Furafylline and DDC inhibited pentoxifylline formation by 59% and 22%, respectively. No other compound caused significant inhibition of pentoxifylline formation. The combination of furafylline and DDC inhibited formation of pentoxifylline by 71% in the same five livers (table 2), which was not significantly different from the inhibition caused by furafylline alone.

The identity of the high apparent $K_m$ enzyme was probed in the same manner as the low apparent $K_m$ enzyme with 200 $\mu$M lisofylline in HL-127 and HL-146 (data not shown). The maximum inhibition with any of the chemical inhibitors was 16%, observed with furafylline. Increasing the concentration of competitive inhibitors by $>10$-fold did not result in inhibition. ABT inhibited pentoxifylline formation by only 10%, and N-octylamine by 22%. There was no pentoxifylline formed when NADPH was omitted and none was formed by boiled microsomes in the presence of NADPH.

Activity Correlation Studies. The correlation between pentoxifylline formation (5 $\mu$M) with R-warfarin 6-hydroxylation (1 mM R-warfarin) as a probe of CYP1A2 activity and with chlorzoxazone 4-hydroxylation (500 $\mu$M chlorzoxazone) as a probe of CYP2E1 activity.

![Figure 2](image_url)

**TABLE 2**

Effect of CYP isoform inhibitors on lisofylline metabolism to pentoxifylline in microsomes from five human livers at 5 $\mu$M lisofylline

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration ($\mu$M)</th>
<th>HL-127 % of Control Activity</th>
<th>HL-135 % of Control Activity</th>
<th>HL-139 % of Control Activity</th>
<th>HL-142 % of Control Activity</th>
<th>HL-146 % of Control Activity</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furafylline</td>
<td>30</td>
<td>43.9</td>
<td>51</td>
<td>28.7</td>
<td>45.2</td>
<td>37.2</td>
<td>41.2 ± 8.5</td>
</tr>
<tr>
<td>Coumarin</td>
<td>8</td>
<td>87.8</td>
<td>94.1</td>
<td>92.5</td>
<td>92.4</td>
<td>98.8</td>
<td>93.1 ± 4.0</td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>100</td>
<td>122</td>
<td>129</td>
<td>110</td>
<td>143</td>
<td>106</td>
<td>122 ± 15</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>400</td>
<td>114</td>
<td>99.8</td>
<td>1</td>
<td>103</td>
<td>101</td>
<td>104 ± 5.90</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>20</td>
<td>109</td>
<td>84.6</td>
<td>93</td>
<td>90.2</td>
<td>85.6</td>
<td>92.5 ± 9.9</td>
</tr>
<tr>
<td>5-mephenytoin</td>
<td>500</td>
<td>118</td>
<td>92.6</td>
<td>91.3</td>
<td>88.9</td>
<td>86.8</td>
<td>95.5 ± 11.4</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1</td>
<td>95.8</td>
<td>92.1</td>
<td>104</td>
<td>95.3</td>
<td>105</td>
<td>98.4 ± 5.7</td>
</tr>
<tr>
<td>DDC</td>
<td>150</td>
<td>76.7</td>
<td>78.9</td>
<td>76.3</td>
<td>81.3</td>
<td>78.3</td>
<td>78.3 ± 2</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>40</td>
<td>114</td>
<td>115</td>
<td>109</td>
<td>144</td>
<td>106</td>
<td>117 ± 15.6</td>
</tr>
<tr>
<td>Furafylline + DDC</td>
<td>30/150</td>
<td>28.0</td>
<td>39.2</td>
<td>20.1</td>
<td>32.0</td>
<td>23.5</td>
<td>28.6 ± 7.46</td>
</tr>
</tbody>
</table>

Values are mean of triplicate incubations.

DDC: Diethyldithiocarbamate.

$^a$ Not significantly different from furafylline alone, paired t-test, $p > 0.05$. 
P450 OXIDIZING LISOFYLLINE TO PENTOXIFYLLINE

activity was assessed in microsomes prepared from 14 human livers (fig. 2). The pentoxifylline formation varied approximately 10-fold, from 0.0152 to 0.141 nmol/min/mg protein, among the 14 livers. A strong correlation ($r^2 = 0.870, p < 0.001$) was observed between pentoxifylline formation and R-warfarin 6-hydroxylation. There was no correlation between pentoxifylline formation and chlorzoxazone 4-hydroxylation ($r^2 = 0.143, p = 0.181$) or between R-warfarin 6-hydroxylation and chlorzoxazone 4-hydroxylation ($r^2 = 0.141, p = 0.183$).

Metabolism in Human cDNA Expressed CYP1A2 and 2E1. The formation of pentoxifylline from lisofylline in cDNA expressed CYP1A2 and CYP2E1 was determined to assess directly the formation of pentoxifylline by these enzymes. The velocities for pentoxifylline formation and chlorzoxazone 4-hydroxylation ($r = 0.0152$ to $0.141$ nmol/min/mg protein, among the 14 livers. A high apparent $K_m$ for chlorzoxazone 4-hydroxylation ($r^2 = 0.183$).

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References


