THE ALKALOID RUTAECARPINE IS A SELECTIVE INHIBITOR OF CYTOCHROME P450 1A IN MOUSE AND HUMAN LIVER MICROSOMES

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ABSTRACT:

Rutaecarpine, evodiamine, and dehydroevodiamine are quinazolonicarboline alkaloids isolated from a traditional Chinese medicine, Evodia rutaecarpa. In the in vitro effects of these alkaloids on cytochrome P450 (P450)-catalyzed oxidations were studied using mouse and human liver microsomes. Among these alkaloids, rutaecarpine showed the most potent and selective inhibitory effect on CYP1A1-catalyzed 7-methoxyresorufin O-demethylation (MROD) and 7-ethoxyresorufin O-deethylation (EROD) activities in untreated mouse liver microsomes. The IC_{50} ratio of EROD to MROD was 6. For MROD activity, rutaecarpine was a noncompetitive inhibitor with a K_i value of 39 ± 2 nM. In contrast, rutaecarpine had no effects on benzo[a]pyrene hydroxylation (AHH), aniline hydroxylation, and nifedipine oxidation (NFO) activities. In human liver microsomes, 1 μM rutaecarpine caused 98, 91, and 77% decreases of EROD, MROD, and phenacetin O-deethylation activities, respectively. In contrast, less than 15% inhibition of AHH, tolbutamide hydroxylation, chlorzoxazone hydroxylation, and NFO activities were observed in the presence of 1 μM rutaecarpine. To understand the selectivity of inhibition of CYP1A1 and CYP1A2, inhibitory effects of rutaecarpine were studied using liver microsomes of 3-methylcholanthrene (3-MC)-treated mice and Escherichia coli membrane expressing bicistronic human CYP1A1 and CYP1A2. Similar to the CYP1A2 inhibitor furafylline, rutaecarpine preferentially inhibited MROD more than EROD and had no effect on AHH in 3-MC-treated mouse liver microsomes. For bicistronic human P450s, the IC_{50} value of rutaecarpine for EROD activity of CYP1A1 was 15 times higher than the value of CYP1A2. These results indicated that rutaecarpine was a potent inhibitor of CYP1A2 in both mouse and human liver microsomes.

Cytochrome P450 (P450)-dependent monooxygenase is the primary enzyme responsible for the oxidoreductive metabolism of a variety of endogenous and exogenous compounds including steroids, drugs, and chemical carcinogens. Oxidations catalyzed by monooxygenase require P450, NADPH-P450 reductase, and phospholipids. The P450 enzymes consist of a family of related hemoproteins that show broad substrate specificity (Guengerich, 1995). Medicinal and herbal drug-dependent inhibition and induction of P450s are a major cause of drug interactions (Guengerich, 1997; Lin and Lu, 1998); therefore, it is important to determine the effects of xenobiotics on P450s in vivo and in vitro. In vitro studies of the interactions help the assessment of drug interaction and explaination of toxicity or lack of efficacy. On the other hand, selective inhibitors of P450 forms are also powerful tools for the identification of P450s involved in the metabolism of drugs. Identification of the role of individual P450s involved in the biotransformation of a therapeutic agent can be useful in the interpretation and prediction of its pharmacological and toxicological actions.

Rutaecarpine, evodiamine, and dehydroevodiamine are quinazolonicarboline alkaloids isolated from Evodia rutaecarpa, which has been used in traditional Chinese medicine for the treatment of gastrointestinal disorder, headache, and hypertension (Tang and Eisenbrand, 1992). These alkaloids had many pharmacological effects including vasorelaxation, antithrombotic, and ertoroton effects (Tsai et al., 1995; Sheu, 1999). However, the influences of these alkaloids on P450-catalyzed oxidations were not reported. Among P450s, the CYP1A subfamily shows overlapping substrate specificity and plays a key role in the activation and detoxication of many therapeutic agents and environmental pollutants. CYP1A1 is mainly localized in extrahaletic tissues and plays an important role in the oxidative activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene (Guengerich, 1995). CYP1A2 is the main hepatic CYP1A member and is involved in the activation of arylamines and the metabolism of drugs such as theophylline, phenacetin, and tamoxifen. Furafylline is a potent and selective CYP1A2 inhibitor in mouse and human (Kunz and Trager, 1993; Tsyrlov et al., 1993; Racha et al.,...
The inhibition by furafylline requires P450-catalyzed hydroxylation to form the protein adduct and inactivate CYP1A2 irreversibly. In the present study, we demonstrated that the naturally occurring and pharmacologically active alkaloid rutecarpine was a selective inhibitor of CYP1A in vitro. The inhibitory effect of rutecarpine on monoxygenase activity was compared with the effect of furafylline. The inhibition selectivity and parameters of rutecarpine were studied using mouse and human liver microsomes and bicistronic human CYP1A1 and CYP1A2.

Materials and Methods

Chemicals and Enzymes. Rutecarpine, evodiamine, and dehydroevodiamine were isolated and purified from the unripe fruits of *E. rutecarpus* (Lin et al., 1991). The purity of these alkaloids was >98% as judged by HPLC and NMR. Acetaminophen, aniline, benzo[a]pyrene, chlorozoxazine, cytosome c, 7-ethoxyresorufin, 7-methoxyresorufin, NADH, NADPH, nifedipine, and phenacetin were purchased from Sigma-Aldrich (St. Louis, MO). Furafylline, tolbutamide, 4-hydroxytolbutamide, and 6-hydroxychlorozoxazine were purchased from Sigma/RBI (Natick, MA).

Microsomal Preparations and Bicistronic P450 Expression. C57BL/J6 mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Mice were allowed a 1-week acclimation in the animal center with air conditioning and an automatically controlled photoperiod of 12 h light daily. Mice were treated with a single injection of 80 mg of 3-methylcholanthrene (3-MC)/kg intraperitoneally. After 48 h, mouse liver microsomes were prepared by differential centrifugation (Alvares and Manning, 1970). Caucasian liver samples (denoted as Cal) were obtained through Tennessee Donor Services (Nashville, TN). Chinese liver samples (denoted as ChL) were obtained from patients who underwent liver resection in National Taiwan University Hospital (Taipei, Taiwan). Human liver microsomes were prepared following the method of Guengerich (1994). Bicistronic human CYP1A1 and CYP1A2 constructs consisting of the coding sequence of *Ca108L* and *Rafael, CA*) and *human CYP1A* were coexpressed from a P450 vector and transformed to *Escherichia coli* DH5α by electroporation. Bacterial membrane fractions were prepared as described previously (Parikh et al., 1997). Microsomes and bacterial membrane fractions were stored at −75°C until use.

Enzyme Assays. P450 content was determined by the spectrophotometric method of Omura and Sato (1964). NADPH-P450 reductase activity was determined following the method of Phillips and Langdon (1962) using cytosome c as a substrate. Benzo[a]pyrene hydroxylation (AHH) was determined by measuring the fluorescence of phenolic metabolites (Nebert and Gelboin, 1968). *O*-Dealkylations of 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD) were determined by measuring the fluorescence of resorufin (Pohl and Fouts, 1980). Aniline hydroxylation was determined by measuring the formation of p-aminophenol (Imai et al., 1966). Nifedipine oxidation (NFO) was determined following the method of Guengerich et al. (1986). Tolbutamide hydroxylation was determined by HPLC analysis (Yamazaki et al., 1998). Chlorozoxazine hydroxylation was assayed following the method of Peter et al. (1990). Phenacetin O-deethylation activity was determined using 25 μM phenacetin, and the acetaminophen formation was analyzed by HPLC (Butler et al., 1989; von Moltke et al., 1996). Concentrations of substrates used in assays were 100 μM AHH, 2 μM EROD, 20 μM MROD, 6 mM aniline hydroxylation, 200 μM NFO, 2.5 mM tolbutamide hydroxylation, 500 μM chlorozoxazine hydroxylation, and 25 μM phenacetin O-deethylation. Reactions of membranes expressing bicistronic P450s contained 0.1 μM P450, an NADPH-generating system, and 2 μM 7-ethoxyresorufin in 0.1 M potassium phosphate buffer, pH 7.4. Rutecarpine, evodiamine, and dehydroevodiamine were dissolved in DMSO and added to the incubation mixture of microsomes and bacterial membranes. The same volume of DMSO was added to the control, and the final concentration of DMSO was <0.5%. Microsomal protein concentration was determined by the method of Lowry et al. (1951).

Data and Statistical Analysis. The concentrations of alkaloids required for 50% inhibition of catalytic activities (*IC*₅₀) were calculated by curve fitting (Grafit, Erithacus Software Ltd., Staines, UK). Kinetic analysis of MROD activity was done following Michaelis-Menten kinetic property. Values of velocity (*v*) at various substrate concentrations (*S*) were fitted by nonlinear least-squares regression without weight due to the equation, consistent with noncompetitive inhibition according to the Michaelis-Menten equation: 

\[ v = \frac{V_m \times S}{K_m + S} \times \left[ 1 + \left( \frac{I}{K_i} \right) \right] \]

where *Vₘₐₓ* and *I* are the maximal velocity and the inhibitor concentration, respectively. Sigma Plot, Jandel Scientific, San Rafael, CA). The statistical significance of differences between control and treated animals was evaluated by Student’s *t* test. A *p* value of <0.05 was considered statistically significant.

Results

Effects of Alkaloids on Monoxygenase Activities in Mouse Liver Microsomes. In liver microsomes of untreated mice, addition of rutecarpine in the enzyme reaction mixture caused a concentration-dependent inhibition on CYP1A-catalyzed MROD and EROD activities. The IC₅₀ values of rutecarpine for MROD and EROD were 0.08 ± 0.01 μM and 0.51 ± 0.01 μM, respectively (Table 1). In the presence of 1 μM rutecarpine, MROD and EROD activities were decreased to 10 and 43% of the control values, respectively (Table 2). In contrast, >80% of AHH and NFO activities remained in the presence of rutecarpine at concentrations up to 100 μM (Fig. 1B). The IC₅₀ values of dehydroevodiamine for MROD and EROD activities were 28 ± 5 and 106 ± 10 μM, respectively (Table 1). The addition of 100 μM dehydroevodiamine resulted in 43 and 67% decreases of AHH and NFO activities, respectively (Fig. 1B).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ M</th>
<th>IC₅₀ Ratio*&lt;sub&gt;EROD/MROD&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furafylline</td>
<td>4.6 ± 1.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rutecarpine</td>
<td>0.08 ± 0.01</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>Evodiamine</td>
<td>62 ± 6</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>Dehydroevodiamine</td>
<td>38 ± 5</td>
<td>120 ± 7</td>
</tr>
<tr>
<td>3-Methylcholanthrene-treated mouse</td>
<td>3.6 ± 0.7</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Rutaecarpine</td>
<td>0.32 ± 0.04</td>
<td>1.42 ± 0.07</td>
</tr>
<tr>
<td>Ca108L</td>
<td>0.05 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

N.D., not determined.

*IC*₅₀ ratio, the ratio of IC₅₀ value for 7-ethoxyresorufin O-deethylation to the value for 7-methoxyresorufin O-demethylation.

The IC₅₀ values of rutecarpine for MROD and EROD were 0.08 ± 0.01 μM and 0.51 ± 0.01 μM, respectively (Table 1). In the presence of 1 μM rutecarpine, MROD and EROD activities were decreased to 10 and 43% of the control values, respectively (Table 2). In contrast, >80% of AHH and NFO activities remained in the presence of rutecarpine at concentrations up to 100 μM (Fig. 1A; Table 1). The IC₅₀ values of evodiamine for MROD and EROD activities were 62 ± 6 and 106 ± 10 μM, respectively (Table 1). The addition of 100 μM evodiamine resulted in 43 and 67% decreases of AHH and NFO activities, respectively (Fig. 1B). The IC₅₀ values of dehydroevodiamine for MROD and EROD activities were 38 ± 5 and 120 ± 7 μM, respectively (Table 1). The addition of 100 μM dehydroevodiamine caused 41% inhibition of MROD and EROD activities without affecting AHH activity (Fig. 1C). Aniline hydroxylation activity was not affected by these alkaloids. Among these alkaloids, rutecarpine showed the most potent and selective inhibition of MROD and EROD activities (Fig. 1; Table 1).

To better understand the selectivity of inhibition of CYP1A1 and CYP1A2 by rutecarpine, mice were treated with 3-MC, which induced both CYP1A1 and CYP1A2 in C57BL/J6 mice (Decy et al., 1999). 3-MC treatment resulted in 8-, 12-, and 6-fold increases of AHH, EROD, and MROD activities, respectively (Table 2). However, aniline hydroxylation and NFO activities were not affected. Consistent with the inhibition observed in untreated mice, rutecarpine also strongly inhibited EROD and MROD activities in 3-MC–treated mouse liver microsomes (Table 2). In the presence of 1 μM rutecarpine, MROD activity was decreased to 16% of the control activity, whereas 69% of
TABLE 2
Effects of rutaecarpine on monooxygenase activities of liver microsomes from control and 3-methylcholanthrene-treated mice

<table>
<thead>
<tr>
<th>Assay (nmol/min/mg protein)</th>
<th>Control</th>
<th>3-Methylcholanthrene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>Rutaecarpine</td>
</tr>
<tr>
<td>7-Ethoxyresorufin O-deethylation</td>
<td>0.28 ± 0.08</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>7-Methoxyresorufin O-demethylation</td>
<td>1.42 ± 0.17</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Benzo[a]pyrene hydroxylation</td>
<td>0.58 ± 0.14</td>
<td>0.59 ± 0.10</td>
</tr>
<tr>
<td>Aniline hydroxylation</td>
<td>1.09 ± 0.05</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Nifedipine oxidation</td>
<td>0.57 ± 0.02</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

* Values of 3-MC-treated group significantly different from the control group, \( p < 0.05 \). The percentage represents the percentage of activity in the presence of rutaecarpine to the control.

Results are presented as means ± S.E. of determinations of benzo[a]pyrene hydroxylation (●), nifedipine oxidation (○), and aniline hydroxylation (□) activities of three mice.

Inhibition of EROD and MROD Activities by Furafylline and Rutaecarpine in Mouse Liver Microsomes. Furafylline, a metabolism-dependent CYP1A2-selective inhibitor, showed NADPH dependence in the inhibition of MROD activity (results not shown). Preincubation of microsomes and furafylline with an NADPH-generating system resulted in strong inhibition of MROD activity. The IC\(_{50}\) value was 4.6 ± 1.1 \( \mu \)M in untreated mouse microsomes (Table 1). Furafylline had a stronger inhibitory effect on MROD than on EROD in 3-MC-treated mouse microsomes (Table 1). The IC\(_{50}\) values for furafylline for EROD and MROD activities were 28 ± 5 \( \mu \)M and 3.6 ± 0.7 \( \mu \)M, respectively. In contrast to furafylline, preincubation of microsomes and rutaecarpine with an NADPH-generating system had no effect on the potent inhibition of MROD activity by rutaecarpine (results not shown). Rutaecarpine inhibited MROD with an IC\(_{50}\) value lower than the value for EROD in both untreated and 3-MC-treated mouse liver microsomes (Table 1). The IC\(_{50}\) ratio of EROD to MROD was 6 and 4 in untreated and 3-MC-treated mouse microsomes, respectively. This preference of inhibition of MROD by rutaecarpine was similar to the inhibition by furafylline. The IC\(_{50}\) value of rutaecarpine for MROD was smaller than the value of furafylline in both untreated and 3-MC-treated mouse microsomes.

Mechanism of Inhibition of 7-Methoxyresorufin O-Demethylation by Rutaecarpine in Mouse Liver Microsomes. Kinetic analysis of MROD was performed using untreated mouse liver microsomes. The velocity (\( v \)) versus 7-methoxyresorufin concentration (\( S \)) plot is shown in Fig. 2A. Kinetic analysis generated a \( V_\text{m} \) of 845 ± 97 pmol/min/mg of protein and a \( K_s \) of 0.58 ± 0.18 \( \mu \)M in the absence of rutaecarpine. Analysis of the Lineweaver-Burk and Dixon plots (Fig. 2, B and C) indicated that rutaecarpine was a noncompetitive inhibitor. The \( K_i \) value was calculated by a nonlinear regression analysis after initial estimates were obtained from the Dixon plots (27 ± 1 nM). Rutaecarpine showed potent inhibition on MROD with a \( K_i \) of 39 ± 2 nM. To examine the possible destruction of the heme of the protein by rutaecarpine and interference on NADPH-P450 reductase reduction activity, the effect of rutaecarpine on microsomal CO difference spectra and NADPH-P450 reductase activity were determined in untreated mouse liver microsomes. Microsomes were preincubated with 10 \( \mu \)M rutaecarpine, and the microsomal CO difference spectra were determined. Rutaecarpine had no effect on spectrally detectable P450 (results not shown). NADPH-P450 reductase activity was not affected by the addition of rutaecarpine at the concentration up to 50 \( \mu \)M.

The Inhibition of Rutaecarpine on Monooxygenase Activities of Human Liver Microsomes and Bicistronic Human CYP1A1 and CYP1A2. To examine the selectivity of inhibition of P450 forms by rutaecarpine in human liver microsomes, we expanded the number of substrates used in activity determination. Microsomal oxidations of 7-ethoxyresorufin, 7-methoxyresorufin, and phenacetin were determined for characterizing CYP1A2. Tolbutamide, chlorzoxazone, and nifedipine were used as selective substrates of CYP2C9, CYP2E1, and CYP3A4, respectively (Guentherich, 1995). The IC\(_{50}\) values of rutaecarpine in human liver microsomes were determined using human liver sample CaI08L. The IC\(_{50}\) values for MROD and EROD activities were 0.05 ± 0.01 and 0.03 ± 0.01 \( \mu \)M, respectively. Addition of 1 \( \mu \)M rutaecarpine caused 98, 91, and 77% decreases of EROD, MROD, and phenacetin...
O-deethylase activities, respectively, of human liver microsomes using six human liver samples (Table 3). In contrast, >85% of AHH, chlorozoxazone hydroxylation, tolbutamide hydroxylation, and NFO activities remained in the presence of rutaecarpine (Table 3). There were no interindividual variations in the inhibition selectivity of rutaecarpine on the human liver microsomal P450-catalyzed oxidations (results not shown). To elucidate the selectivity of rutaecarpine inhibition on CYP1A1 and CYP1A2, EROD activity catalyzed by bicistronic human CYP1A1 and CYP1A2 was determined in the absence and presence of rutaecarpine. Rutaecarpine decreased CYP1A1- and CYP1A2-catalyzed EROD activity with IC_{50} values of 0.90 ± 0.09 and 0.06 ± 0.00 μM, respectively (Fig. 3).

Discussion

A previous report indicated that rutaecarpine reduced the mutagenic effect of benzo[a]pyrene 7.8-dihydrodiol in XEM2 cells expressing CYP1A1 (Ramug et al., 1992). Our results showed that rutaecarpine caused the most potent and selective inhibition of CYP1A-catalyzed EROD and MROD activities without affecting CYP3A-catalyzed NFO activity among three alkaloids isolated from E. rutaecarpa (Table 1; Fig. 1A). Microsomal AHH activity was not affected by rutaecarpine. Evidamine decreased EROD, MROD, NFO, and AHH activities (Table 1; Fig. 1B). Dehydroevidamine decreased EROD, MROD, and NFO activities (Table 1; Fig. 1C). These results suggest that evidamine and dehydroevidamine are inhibitors of CYP1A1 and CYP3A. All of these alkaloids had no effect on the hydroxylation of aniline. This result suggested that CYP2E1-catalyzed oxidations were not affected by these alkaloids. The structural differences among these alkaloids are the N14-methyl group and the double bond of C3-N14. The structural differences might have affected the selective binding of inhibitor to mouse CYP1A1 and resulted in the differential potency and selectivity of inhibition by alkaloids.

Addition of rutaecarpine had no effect on microsomal CO difference spectra. This result indicated that rutaecarpine did not cause the destruction of the heme moiety of P450. Rutaecarpine did not decrease the activity of NADPH-P450 reductase. Preincubation of microsomes and rutaecarpine with an NADPH-generating system had no effect on the inhibition of MROD by rutaecarpine. This result indicates that NADPH-dependent microsomal metabolism is not required for the inhibitory effect. The results of our kinetic analyses indicate that rutaecarpine is a noncompetitive inhibitor (Fig. 2); this result suggests that rutaecarpine inhibits the CYP1A1-catalyzed oxidations without affecting substrate binding.

CYP1A1 and CYP1A2 have overlapped substrate specificity. Hamm et al. (1998) reported that 7-methoxyresorufin might not be an appropriate substrate for CYP1A2 in mice, as judged by an induction study in CYP1A2 knockout mice. However, 7-methoxyresorufin was preferentially O-dealkylated by CYP1A2 in 2,3,7,8-tetrachlordibenzo-p-dioxin-treated mice, and 7-ethoxyresorufin was preferentially O-dealkylated by CYP1A1 in 3-MC-treated mice (Gradelet et al., 1997). CYP1A1 had the highest activity in the oxidation of benzo[a]pyrene and was highly induced by 3-MC. Thus, oxidations of 7-ethoxyresorufin, 7-methoxyresorufin, and benzo[a]pyrene were studied in 3-MC-treated mice to help understand the selectivity of the inhibitory effect of rutaecarpine on CYP1A1 and CYP1A2. α-Naphthoflavone is a known inhibitor of both CYP1A1 and CYP1A2. In the presence of α-naphthoflavone, all EROD, MROD, and AHH activities were inhibited in liver microsomes of benzo[a]pyrene-treated mice (Tsyrov et al., 1993). Furafylline is known as a CYP1A2-selective inhibitor and preferentially inhibits MROD activity (Tsyrov et al., 1993). To a lesser extent, EROD was also inhibited by furafylline. However, AHH activity was not affected by furafylline in benzo[a]pyrene-treated mice. In the present study, our results also showed that furafylline preferentially inhibited MROD activity in 3-MC-
Rutaecarpine was added into the reaction mixture, and reaction was started with the addition of an NADPH-generating system. Activity was determined as described under Materials and Methods. Results are presented as means of two experiments.

Acknowledgments. We thank Hsiao-Chi Peng for help with the microsomal preparation and activity determination.

References


Furafylline inhibited MROD activity with a potency much higher than for rutaecarpine. Similar to furafylline, rutaecarpine had an IC50 value for the MROD activity was close to 1 in mouse liver microsomes, the IC 50 ratio was close to 1 in human liver microsomes. The IC50 value of rutaecarpine for the MROD activity was 1:1 adduct to protein and evidence for the formation of a novel imidazothione intermediate. Biochemistry 37:7407–7419.

Rutaecarpine preferentially inhibited CYP1A2-catalyzed oxidations in human liver microsomes in the presence of rutaecarpine (Table 3). These results demonstrate that rutaecarpine is a selective inhibitor of human hepatic CYP1A2 and shows no effect on CYP2C, CYP2E1, and CYP3A4. Together with the mouse study, our results indicated that rutaecarpine preferentially inhibited CYP1A2-catalyzed oxidations in both mouse and human liver microsomes. Our results with bicistronum human CYP1A1 and CYP1A2 further demonstrated that rutaecarpine preferentially inhibited EROD activity of CYP1A2 more than the activity of CYP1A1. The IC50 value of inhibition of CYP1A1 was 15 times higher than that of CYP1A2 by rutaecarpine (Fig. 3). Thus, in future investigations of drug metabolism and interactions in drug discovery, rutaecarpine can be used as a potent CYP1A2 inhibitor. Further studies on the in vivo effect of rutaecarpine are planned to generate information in the assessment of drug interactions.


