IDENTIFICATION OF CRITICAL AMINO ACID RESIDUES OF HUMAN CYP2A13 FOR THE METABOLIC ACTIVATION OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE, A TOBACCO-SPECIFIC CARCINOGEN

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ABSTRACT:

Among all the known human cytochrome P450 enzymes, CYP2A13 has the highest efficiency in catalyzing the metabolic activation (keto aldehyde and keto alcohol formation) of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), a potent lung carcinogen in animals and a suspected human lung carcinogen. As part of the structure-activity relationship (SAR) study, the present work was done to identify the key amino acid residues in CYP2A13 that are responsible for this high catalytic efficiency by using a series of mutants (Ala117Val, His164Gly, Ser208Ile, His372Arg, and Pro465Ser). In these CYP2A13 mutants, the amino acid residues were substituted by the residues at the corresponding positions of CYP2A6, which shares 93.5% amino acid sequence identity with CYP2A13 but is significantly less active (<5%) than CYP2A13 in NNK α-hydroxylation. We demonstrated that, except for the His164Gly mutant, all the CYP2A13 mutant proteins showed a significant decrease in the catalytic efficiency (V_max/K_m) for NNK α-hydroxylation. The His372Arg to Arg substitution resulted in a 20-fold increase in the K_m value and a 7-fold decrease in the V_max value for keto aldehyde formation as well as a total loss of detectable keto alcohol formation. The Ala117Val and His372Arg mutants showed a 9-fold increase in the catalytic efficiency for coumarin 7-hydroxylation. Together with the computational substrate docking, our study provides new SAR information of human CYP2A13.

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) is a potent lung carcinogen in animals and a suspected human lung carcinogen (Hoffmann et al., 1981; Hecht et al., 1983, 1989, 1993b; Hecht, 1998, 2002). Metabolic activation (α-hydroxylation) of NNK is required for its carcinogenicity. During the metabolic activation, both the methyl and methylene carbons of NNK can be hydroxylated, leading to the formation of 4-oxo-4-(3-pyridyl)-1-butane (keto alcohol) and 4-oxo-1-(3-pyridyl)-1-butane (keto aldehyde), respectively, as well as the DNA-methylating and -pyridyloxobutylating species (Hecht et al., 1993a; Wang et al., 2003b). Several cytochrome P450 (P450) enzymes have been reported to be involved in the metabolic activation of NNK (Hecht et al., 1996; Patten et al., 1996; Felicia et al., 2000; Schrader et al., 2000; Su et al., 2000; Fujita and Kamataki, 2001; Jalas et al., 2003). However, there is a significant difference in the catalytic efficiency for NNK α-hydroxylation among these P450 enzymes.

Among all the known human P450 enzymes, CYP2A13 has the highest activity and the lowest K_m value for NNK α-hydroxylation (Su et al., 2000; Jalas et al., 2003). Although CYP2A6, another member of the human CYP2A subfamily, is also active in catalyzing NNK α-hydroxylation, we have previously determined its activity to be less than 5% of the CYP2A13 activity (Su et al., 2000). Both CYP2A13 and CYP2A6 proteins are composed of 494 amino acids and share as high as a 93.5% identity in the amino acid sequence. Whereas CYP2A6 is mainly expressed in the liver, CYP2A13 is predominantly expressed in the respiratory tract (Koskela et al., 1999; Su et al., 2000). Since lung is the major target organ in NNK-induced carcinogenesis in laboratory animals and in smoking-related human cancers (Hecht, 1998), the high activity of CYP2A13 in NNK α-hydroxylation and its predominant expression in human respiratory tissues suggest that CYP2A13 plays an important role in NNK-induced carcinogenesis. This suggestion appears to be supported by a recent molecular epidemiological study in which a CYP2A13/Arg257Cys polymorphism was found to be associated with a substantially reduced risk for lung adenocarcinoma (odds ratio = 0.41, 95% confidence interval 0.23–0.71; Wang et al., 2003a). This is consistent with the finding that heterologously expressed CYP2A13/Arg257Cys variant protein has a lower efficiency in catalyzing NNK α-hydroxylation (Zhang et al., 2002).

As part of our study to elucidate the structure-activity relationship of CYP2A13, the present work was done to identify the key amino acid residues in CYP2A13 that are responsible for its high catalytic

ABBREVIATIONS: NNK, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butane; P450, cytochrome P450; EM, energy minimization.
efficiency in NNK α-hydroxylation. Recently, we have generated a series of CYP2A13 mutant proteins in which the amino acid residues in CYP2A13 were substituted with the amino acid residues at the corresponding positions of CYP2A6 (He et al., 2004). In the present study, the activity and the kinetic parameters of these CYP2A13 mutants for NNK α-hydroxylation were determined and compared with the wild-type CYP2A13 and CYP2A6. We have also docked NNK into an established CYP2A13 model to explore the molecular insights for the observed alterations in enzyme kinetics.

Materials and Methods

Materials. [5-3H]NNK with a specific activity of 14 Ci/mmole and a radioactive purity of 98.7% was purchased from Moravek Biochemicals (Brea, CA). Unlabeled NNK was obtained from ChemSyn (Lenexa, KS). The UV standards for NNK metabolites were a gift of Dr. Stephen Hecht (University of Minnesota Cancer Center, Minneapolis, MN). Hemin, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and phenylmethylsulfonyl fluoride were purchased from Sigma-Aldrich (St. Louis, MO). Bac-to-Bac baculovirus expression system (including an expression vector, pFastbac1) and cell culture media were purchased from Invitrogen (Carlsbad, CA). NADPH-P450 oxidoreductase was purified from rat liver microsomes as described previously (Yasukochi and Masters, 1976). The GeneAmp XL PCR kit was obtained from Applied Biosystems (Branchburg, NJ). A monoclonal antibody against human CYP2A6 antigen that cross-reacts with human CYP2A13 was obtained from BD Genetest (Woburn, MA). Goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate was obtained from Bio-Rad (Hercules, CA). The ECL Western blotting detection reagents were obtained from Amersham Biosciences Inc. (Piscataway, NJ). Emulgen 911 was purchased from Kao Corp. (Tokyo, Japan).

Site-Directed Mutagenesis and Heterologous Expression. All the approaches and the methods used to produce the CYP2A13 mutant proteins were described in detail previously (He et al., 2004). In brief, the wild-type CYP2A13 cDNA was used as a template to generate the mutant cDNAs through site-directed mutagenesis. After confirming that the full-length cDNAs contained only the desired mutations, each mutant CYP2A13 cDNA was subcloned into the expression vector pFastbac1 and expressed in the Bac-to-Bac baculovirus/Sf9 insect cell system. The wild-type CYP2A13 and CYP2A6 cDNAs were expressed in the same system.

Microsome Preparation, Immunoblot Analysis, and P450 Content Determination. After 72 h of incubation, the S9 cells infected with the recombinant baculovirus were harvested, washed with phosphate-buffered saline (100 mM potassium phosphate, pH 7.4), and resuspended in the phosphate-buffered saline containing 20% glycerol and 1 mM freshly added phenylmethylsulfonyl fluoride. Microsomes were prepared by sonication and differential centrifugation as previously described (Hong et al., 1999). The microsome preparations were stored at −80°C before use. Protein concentration was determined by Bio-Rad protein assay reagent.

Micromodal proteins were separated by SDS-polyacrylamide gradient (4–20%) gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblot analysis was conducted with the anti-CYP2A6 monoclonal antibody that cross-reacts with human CYP2A13, followed by incubation with goat anti-mouse IgG-conjugated horseradish peroxidase and detection by enhanced chemiluminescence reagents.

The P450 content was determined as described by Omura and Sato (1964). Micromosomes were solubilized in a buffer (pH 7.4) containing 50 mM KH2PO4, 1 mM EDTA, 25% glycerol, and 0.5% Emulgen 911 and were diluted to a total protein concentration of 2 mg/ml. The reduced CO-difference spectra were recorded using a UV/visible spectrophotometer (Shimadzu UV 160U; Shimadzu, Kyoto, Japan).

NNK Metabolism and Kinetic Analysis. The NNK-metabolizing activity was determined as previously described (Hong et al., 1992). The incubation mixture consisted of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3 mM MgCl2, 5 mM sodium bisulfite, an NADPH generating system (5 mM glucose 6-phosphate, 1 mM NADP+, 1.5 units of glucose 6-phosphate dehydrogenase), 10 μM NNK (containing 1 μCi [5-3H]-NNK), 10 or 20 pmol of P450 enzymes, and NADPH-P450 oxidoreductase (reductase/P450 = 5:1 in molar ratio), in a total volume of 400 μl. Reaction was carried out at 37°C for 15 min (for wild-type CYP2A13 and its Ala117Val, His120Gly, Ser208Ile, and Pro209Ser mutants) or 40 min (for the His372Arg mutant and wild-type CYP2A6). The reaction was terminated with 5 μl of NNK metabolite standards onto a reverse-phase HPLC system equipped with a radioflow detector (Radiomatic, Tampa, FL). For enzyme kinetics studies, at least seven different NNK concentrations, ranging from 10 μM to 500 μM, were used for the incubation.

Computer Modeling. We have recently developed the models of P450A13 and CYP2A6 with the focus on the enzyme active sites (He et al., 2004). These models were constructed based on the X-ray structure (pdb1b6d) of modified rabbit CYP2C5 (Williams et al., 2000a,b). Notably, the amino acid sequence identity of active sites in these enzymes is very high. Five of the six amino acid residues (Ala117, Ala201, Thr203, Leu296, and Leu297) are located at the active site near heme are identical in both CYP2C5 and CYP2A13. In addition, a key residue, His372, is also conserved in these two enzymes. Therefore, it is feasible to use the modified CYP2C5 X-ray structure to model the shape and short-range interactions of the CYP2A13 substrate-binding site. The conformations of mutated residues were carefully searched with local energy minimization (EM). The substrate NNK was docked into the homology model with an incremental build-up procedure (Shen and Borchardt, 2003). A methyl group (CH3) representing the hydroxylation site of NNK was placed into the active site. To prevent this “seed” and the subsequent conformers from escaping from the pocket during EM, a harmonic force constraint (F = 100(ε − ε0)^2 kcal/mol) was applied to the hydroxylation carbon and the heme iron throughout this work. After an EM, each proton around the carbon was replaced with a fragment, such as an amine. The resulting four conformers of methylamine were subjected to another EM. The procedure was repeated until the complete NNK was constructed in the active site.

To eliminate the unfit conformers during the build-up process, to select the best conformer at the end, and to compare different binding modes, a number of docking energies including interaction energy were calculated. The interaction energy here is the nonbonded molecular mechanics energy between a ligand and P450 model, and it is expressed as \( E_{\text{int}} = E_{\text{vdw}} + E_{\text{elec}} + E_{\text{at}} \), where \( E_{\text{vdw}} \), \( E_{\text{elec}} \), and \( E_{\text{at}} \) are electrostatic, van der Waals, and harmonic components, respectively. Although the last one is intended to restrict a ligand inside the pocket, it also provides a measure on the ease of approach of the ligand to the reaction center.

The 300-step Maximin2 with energy convergence at 0.1 kcal/mol was a standard EM in this work. The protein atoms within a 5Å shell surrounding the substrate were allowed to relax during the EM. Various kinds of energy including interaction energy and their components were calculated at the end of the EM to determine a preferred binding mode. A distance-dependent dielectric, 1/\( r^2 \), and the cutoffs of 8 Å and 11 Å for steric and electrostatic interactions were used in all energy calculations according to a similar protocol described previously (Shen et al., 1999). The model building and NNK docking were also carried out using the software SYBYL 6.7 and 6.8 (Tripos, Inc., St. Louis, MO) with MFF94 force field (Halgren, 1996).

Results

Heterologous Expression of the P450 Proteins. All the wild-type and mutant CYP2A13 cDNAs were successfully expressed in S9 insect cells. Immunoblot analysis detected a single protein band with the expected molecular weight in the microsomes prepared from the S9 cells that were infected with the expression vectors containing CYP2A13 cDNAs, but not in the control S9 cells that were infected with the vector alone (containing no CYP2A13 cDNA) (Fig. 1). There was no significant difference in the protein expression level between the wild-type CYP2A13 and its missense mutants. The wild-type CYP2A6 protein was also obtained by the same expression system. All the microsome samples expressing the recombinant CYP2A6 and CYP2A13 proteins displayed the characteristic absorption peak at 450 nm, as determined by Co-difference spectrum analysis (Fig. 1).

NNK Metabolism and Enzyme Kinetics. Consistent with our previous report (Su et al., 2000), there was no detectable keto alcohol
formation in CYP2A6-catalyzed NNK α-hydroxylation under our assay conditions, and the specific activity of CYP2A13 in keto aldehyde formation was 120-fold higher than that of CYP2A6 (Fig. 2). Whereas the activity of NNK α-hydroxylation was not changed in the His164Gly mutant, it was significantly decreased in the Ala117Val, Ser208Ile, His372Arg, and Pro465Ser mutants. The His372Arg mutant showed a complete loss of keto alcohol formation and a 100-fold reduction in keto aldehyde formation (Fig. 2). This activity pattern is very similar to CYP2A6-catalyzed NNK α-hydroxylation. The activity ratio of keto aldehyde and keto alcohol formation was also altered in NNK α-hydroxylation catalyzed by the Ala117Val mutant. The ratio was approximately 3:1 for the wild-type CYP2A13 and other mutants. However, it was changed to approximately 1:2 in the Ala117Val mutant due to a selective reduction in keto aldehyde formation.

These activity changes were confirmed by further enzyme kinetics study (Fig. 3). Except for the His164Gly mutant, the catalytic efficiency (Vmax/Km) for NNK α-hydroxylation in all the other CYP2A13 mutants was reduced (Table 1). However, the enzymatic mechanisms involved in the reduction are apparently different. For the Pro465Ser mutant, both the Km values for keto aldehyde and keto alcohol formation were both significantly increased, but there was no change in the Vmax values. For the His372Arg mutant, both the Km and Vmax values for keto aldehyde and keto alcohol formation were significantly changed. In fact, the kinetic behavior of the His372Arg mutant is very similar to that of CYP2A6 in both keto aldehyde formation (Km, 260 versus 328 μM; Vmax, 1.23 versus 1.03 nmol/min/nmol) and keto alcohol formation (ndetectable). The Ser208Ile mutant also had a change pattern similar to that of the His372Arg mutant, i.e., an increase in the Km values for keto aldehyde and keto alcohol formation as well as a decrease in the Vmax values for both reactions. All these changes were statistically significant (P < 0.05), except for the Vmax of keto alcohol formation (P = 0.057). For the Ala117Val mutant, neither the Km value for the formation of keto aldehyde and keto alcohol nor the Vmax value for keto alcohol formation was changed. However, there was a significant decrease in the Vmax value for keto alcohol formation.

**Computer Modeling.** Our CYP2A13 protein model showed that the amino acid residues His164 and Pro465 are all some distance away from the active site, whereas Ala117, Ser208, and His372 are either within or in close proximity to the active site. Two active NNK binding modes, methylene binding for keto aldehyde formation and methyl binding for keto alcohol formation, were generated with the CYP2A13 model shown in Fig. 4. Both binding modes exhibit an extended conformation with the nitroso group (head) toward the proximal pocket of heme and the pyridine group (tail) pointing away from the heme. The long axis passing the head and tail forms an ~30° angle with the heme plane. The distances from the heme iron to the reactive methylene carbon and methyl carbon are 4.3 Å and 4.1 Å, respectively. Among the studied residues, only Ala117 is in direct contact with the docked NNK (<3 Å). These two binding modes were also generated with the model of CYP2A13/Ala117Val mutant protein. They all have favorable binding interaction in terms of a negative interaction energy shown in Fig. 5. We observed a significant interaction energy drop for the methylene-binding mode but not for the methyl-binding mode in the Ala117Val mutant (CH/Val in Fig. 5), which correlates with the reduced keto aldehyde formation. Compared with the energy of the methyl-binding mode in the same protein model, the primary cause of this drop is due to the reduction of the van der Waals energy. Obviously, the addition of two methyl groups to residue 117, due to the replacement of Ala with Val, reduces the needed space for this binding mode.

**Discussion**

This study investigated the role of several amino acid residues in the high catalytic efficiency of CYP2A13 in NNK α-hydroxylation. The residues for substitution include those at the substrate binding site such as Ala117 and the one located 30 Å away from heme iron such as Pro465. The substitutions cause the changes in hydrophobicity, size of the side chains, and rigidity. Together with the enzyme kinetics data and the knowledge of three-dimensional structure of P450 obtained from our computational modeling, these substitutions provide a good opportunity to understand the functional role of each residue in the catalysis.

Our results clearly demonstrate that Ala117, Ser208, His372, and Pro465 are all important amino acid residues for the high activity of...
CYP2A13 in NNK α-hydroxylation. The CYP2A13/His372Arg mutant behaves like CYP2A6, showing a 20-fold increase in the $K_m$ value and a 7-fold decrease in the $V_{\text{max}}$ value for keto aldehyde formation as well as a total loss of activity in keto alcohol formation. These are clear indications that both substrate binding and catalysis were affected by this amino acid substitution. Since our computer model shows that His372 in CYP2A13 is not in direct contact with NNK, the observed alterations in substrate binding and catalysis occur most probably through the change in interaction between residue 372 and its neighboring residues, which may lead NNK to bind in a less optimal orientation. The mechanisms involved remain to be determined and should be consistent with the presence of Arg372 in mouse CYP2A5, which is still highly efficient in NNK α-hydroxylation (Felicia et al., 2000).

Both the methyl and methylene carbons of NNK can be oxidized by α-hydroxylation, leading to the formation of keto alcohol and keto aldehyde, respectively. It is interesting to observe that the Ala117 to Val substitution did not change the kinetics of the methyl hydroxylation (keto alcohol formation) but caused a significant decrease in the $V_{\text{max}}$ for the methylene hydroxylation (keto aldehyde formation). The selective effect of Ala117 to Val substitution appears to be associated with the reduced volume of the binding pocket. Although NNK can still fit into the model of the Ala117Val mutant, a significant reduction of interaction energy is observed for the methylene-binding mode due to an unfavorable repulsive interaction. The resulting structural rearrangement of substrate-mutant complex could be less optimal for efficient hydroxylation.

For keto aldehyde and keto alcohol formation, the His164Gly mutant showed no changes in enzyme kinetics, but the Ser208Ile mutant displayed a significant change in both $K_m$ and $V_{\text{max}}$ values. These kinetics data are consistent with the localization of these two residues in the CYP2A13 model, in which His164 is far from the active site and the substrate entrance channel, whereas Ser208 appears to gate the entrance of the active site. Located near the binding region for the pyridine tail of NNK, Ser208 is not in direct contact with docked NNK. The closest distance between Ser208 and NNK is 5 Å in a allowable conformation. However, if the Ser208 is substituted with a larger isoleucine, the distance can be shortened to 4 Å, well within the carbon-carbon contact distance. This change in distance may disturb the optimal binding mode. In both methylene- and methyl-binding modes, the residue 208 is similarly in close contact with the tail moiety of NNK. This is probably why the substitution of Ser208 with Ile causes the similar effects on the kinetics of both hydroxylations. In contrast, Val117 interacts with the NNK moiety at the region between

![Graph](image_url)

**FIG. 3.** Kinetics of NNK α-hydroxylation catalyzed by heterologously expressed CYP2A6, CYP2A13, and the CYP2A13 mutants. A, keto aldehyde formation; B, keto alcohol formation. Values for CYP2A13 wild-type and the variants are expressed as mean ± S.D. of three experiments. For CYP2A6 wild-type, the values are expressed as the average of duplicated experiments with less than 15% variation.

**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Keto Aldehyde</th>
<th>Keto Alcohol</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{\text{max}}$ (nmol/min/nmol)</td>
</tr>
<tr>
<td>CYP2A13 WT</td>
<td>14.0 ± 1.55</td>
<td>9.04 ± 1.77</td>
</tr>
<tr>
<td>CYP2A13 mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala117Val</td>
<td>18.5 ± 2.80</td>
<td>1.89 ± 0.37*</td>
</tr>
<tr>
<td>His164Gly</td>
<td>8.98 ± 2.45</td>
<td>8.89 ± 2.13</td>
</tr>
<tr>
<td>Ser208Ile</td>
<td>2.0 ± 0.81**</td>
<td>3.59 ± 0.82*</td>
</tr>
<tr>
<td>His372Arg</td>
<td>260 ± 22.2**</td>
<td>1.23 ± 0.60*</td>
</tr>
<tr>
<td>Pro465Ser</td>
<td>44.6 ± 4.89**</td>
<td>7.12 ± 2.13</td>
</tr>
<tr>
<td>CYP2A6 WT</td>
<td>328**</td>
<td>1.03*</td>
</tr>
</tbody>
</table>

For CYP2A6 wild-type (WT), the values are the average of duplicate experiments with less than 15% variation.

N.D., nondetectable.

* $P < 0.05$, ** $P < 0.01$, compared with CYP2A13 WT. The values are mean ± S.D. of three experiments.
the head and tail. The two binding modes show distinct conformations, and only methylene hydroxylation is affected.

Although Pro\(^{465}\) is also 30 Å away from the active site iron and definitely cannot contact the bound NNK, its substitution with Ser caused an increase in \(K_m\) value. Because of its spatial position, it seems unlikely that this residue directly affects the transport of NNK to the active site. We speculate that Ser\(^{465}\), along with other neighboring amino acid residues, may form a secondary binding site for NNK, which interferes with the transport of NNK to the active site. This can cause a slowdown in the formation of catalytic complex. The hydrophilic nature of Ser\(^{465}\) implies that the binding of a more hydrophilic substrate such as NNK will be affected to a greater degree.

Whereas the catalytic efficiency of CYP2A13 is much higher than that of CYP2A6 in NNK \(\alpha\)-hydroxylation, it is only about one-tenth of CYP2A6 in coumarin 7-hydroxylation. The CYP2A13 mutants in the present study were recently examined for their coumarin 7-hydroxylation activities (He et al., 2004), in which none of the mutants showed significant changes in \(K_m\) values. In addition, there was a 9-fold increase in \(V_{\text{max}}\) in the Ala\(^{117}\)Val and His\(^{372}\)Arg mutants. Therefore, it is clear that the role of these amino acid residues in CYP2A13-mediated catalysis is substrate-dependent. This can be attributed to the fact that the NNK molecule, with its larger size and greater polarity, is more sensitive to the size and polarity changes of its interacting amino acid residues during the catalysis process, which includes initial encounter, diffusion within the active site channel, and binding at the active site.

The function of individual amino acid residues in P450 catalysis has been subjected to extensive studies, but our understanding of this subject is still very limited. Although computer modeling may provide some mechanistic explanations, there are a number of uncertainties that limit its extensive application. The foremost limitation is the reliability of the homology-based P450 models, which is largely determined by the degree of amino acid sequence identity. Another important limitation factor at the current level of modeling is the lack of information on the specific location of all involved reaction species such as water and proton. For these reasons, we did not pursue more sophisticated simulations such as molecular dynamics. There should also be an awareness that the P450-catalyzed hydroxylation is believed to be a multistep reaction with multireactants. Before the kinetic parameters for individual steps are captured, an elucidation of the overall reaction mechanism remains a great challenge.

In summary, we have demonstrated that Ala\(^{117}\), Ser\(^{208}\), His\(^{372}\), and Pro\(^{465}\) are critical amino acid residues in CYP2A13 for NNK \(\alpha\)-hydroxylation. Together with the comparison of their differential role in CYP2A13-catalyzed coumarin 7-hydroxylation and the mechanistic explanations from substrate docking analysis, our results provide new information on the structure-activity relationship of human CYP2A13, which is believed to play a significant role in tobacco-related tumorigenesis in human respiratory tract. Further study on the structure-activity relationship of CYP2A13 should be conducted, which may...
help us to assess the functional significance of the missense genetic polymorphisms of CYP2A13.

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References


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