総合討論

鎌滝　それでは総合討論を始めさせていただきます。

最初に，私から２つ質問をさせていただきます。１つは佐藤先生に，もう１つは仁平先生にお願いしたいのですが，最初の佐藤先生への質問は，おそらく製薬会社の方々が一番気になっているのではないかと思っている質問です。その次の質問をお聞きしてから，また佐藤先生に戻ってお考えいただく必要があるのではないかと思います。

最初に佐藤先生にお聞きしたいのは，例えば今，津谷先生がスライドに出されていましたが，非常に患者数が少なくなった。例えばこういう遺伝子を持っている人に，あるいは遺伝子の変異を持っている人にこの薬を使うという，ハーセプチン®の場合もそうですが，患者数が大変限られてしまいます。すると，市場の規模は小さくなる，患者数が少なくなり，薬価が今までと同じならば製薬会社はやっていけないのではないか，という懸念です。そのあたりの基本的な厚労省の考え方というのをお聞きしたい。

もう１つは，仁平先生にお聞きしたいのですが，いわばハーセプチン®の開発した結果をお聞きしましたが，実はその前のdecision makingを，誰がどのようにしたのか，責任体系がどうで，多分これはかなり大きなdecisionだったと思うのです。そのあたり，裏話がもしお分かりでしたらお聞きしたいと思っています。まず佐藤先生にお願いします。

患者が限られてしまうことによる影響

佐藤　最初のご質問ということで承りましたが，患者数が少ない，しかし，場合によっては開発コストが結構高いものに対して，そこをどのように保険上評価していくかということです。非常に難しいご質問で，その難しさには２つポイントがあります。１つは，私の今いる立場が，医薬食品局という審査をやる側でして，保険を担当しているサイドではないということ。もう１つは，実際にそのような医薬品として，ハーセプチン®以外のものはまだ上市されていない状況という中で，保険的な部分で希望的なことが申し上げられるかというと，なかなか難しいだろうということです。

ただ，審査と保険の世界でも共通する部分は当然あるわけで，そういうところから一言お答えとして言えるとすれば，やはりそのもの自体がどれだけ画期的なものかというところが１つポイントになるだろうということです。当然保険の世界も今，非常に財政が厳しい折ですし，そこでどれだけ医療経済的なものが取り上げられるか分かりませんが，こういった開発を行うことによってどれだけ画期的なものが出るかということを，ぜひ企業の方にも考えていただきたい，というお答えでいかがでございましょうか。

鎌滝　仕方がないでしょうね。それでは仁平先生，お願いします。

仁平　本剤は，皆さんご存じのように米国のジェネンテク社，米国以外ではロシュが開発をしております。日本では日本ロシュが担当しまして，現在は中外製薬となっております。そういう経過の中で，どこの段階でというよりも，いろいろなステップでかなりcriticalなdecisionがされてきたのだろうと，私は想像しております。

ただ，本剤の場合，非常に幸せだったというのは，１つは本剤が「抗体」であったということで，当然のことながら非常に特異性の高いものであることがはじめから明らかに分かっていた，そしてHER2に対して非常に特異性が高いということから，そこに患者集団を限定しようという発想が自然の流れとして出ていたという意味では，幸せな薬だったのではないかと想像します。

実際問題として，これがもし一般的な乳癌という対象で臨床試験をしますと，第Ⅱ相試験で

second，third line等の患者さんを対象としますと，単剤では15％くらいの奏効率しか示しておりません。乳癌全体のpopulationで見ますと，これはおそらく５％で，通常，開発の対象にはならなかったという意味では，逆に絞ったが故に生き残ってこれたという部分があったかと思っています。

もう１点，decision makingですが，日本においてこれを開発，そして上市するという一連の作業の中で，やはりHER2の検査をきちんとやって，positiveな患者にのみ限定するというところを，どこまでcriticalにやるかというのが，我々も含めて経験がなかったので，そこのところをどの程度厳密にやるかというのが，行政当局からは審査の過程で厳密さを求められていました。社内でもどのようにやったらいいかということも含めて，いろいろ議論がありましたが，可能な限りやはりやりましょうと。確かに限定はされるけれども，それでHER2 positiveの転移性乳癌という，難しい予後をたどる患者が，延命として３ヵ月，５ヵ月延びるかもしれないという意味では，人によってはたった３ヵ月とおっしゃいますが，患者さんにとっては例えば「子供の入学式を見ることができる」などということもありました。きちんとした形で，この薬を日本の市場に生み出していったらよいのではないかというところでは，私は開発サイドにいる人間ですか，営業サイドからは歓迎されてはいなかったかもしれないですが，トラスツズマブ部会とか，あるいはそういうものも含めてきちんとしたものを導入したいというdecisionはあったかと思います。

鎌滝　ありがとうございました。それではフロアから質問を受けたいと思います。

どこまでインセンティブに結びつけるか

別府宏圀（TIP）　津谷先生に質問です。養老孟司流に言えば，「ああすればこうなる」式に原因と結果を余りに単純化した議論は非常に危険です。ここでお話しになった事柄も，いろいろな要素が入ってきて，必ずしも非常にきれいな答えがすぐに出るとは限らない，そういう前提を皆さん踏まえた上でお話しになっていますが，現実の話になると，どこでインセンティブを与えるかはなかなか難しい問題です。

現実にはゲフィチニブの話でもありましたように，一度情報が動き出しますと，益のある部分だけ非常に拡大され，害の部分は無視されて，結局思わぬ方向へ行くという危険性があります。そういう意味での配慮というのが，こういう先端の技術に関しては非常に大事だと思います。その点についての注意がやはり必要なのではないかという気がいたしますが，いかがでしょうか。

津谷　先ほどのCIOMS Reportのトピック中に，「コミュニケーション＆エデュケーション」とありましたが，やはり消費者も含めて，エンドユーザーに近いところの人が合理的な判断ができるというのが理想なのだと思います。

先ほど私は，コミュニケーションにあたって，同じ言葉を使うべきだと言いました。またそのトピックでディスカッションされたことの１つはメディアに対する教育です。こうした技術においては，どうしても光の面だけ見て，それがメディアに乗り伝わります。情報をcriticalに見るだけの力を持った人というのはそれほどいないのです。確かに別府先生のお話を聞くと，レポートには情報伝達がうまくいかなかった例なども入れておくというのも，１つの手かもしれません。

私はリスクマネージメントという言い方もしましたが，このリスクマネージメントにインセンティブを与えるということも考えられます。なんでもかんでもインセンティブに結びつけることはないのかもしれませんが，こうした　新技術の合理的使用に関係する人々の自覚だけでは不充分なのではないかとも思われます。

鎌滝　他にフロアの方からご質問ありませんでしょうか。

遺伝子評価とTDM

津谷　こういうセッションだと行政の人に多く質問がいって大変申し訳ないのですが，佐藤さんに質問があります。TDMをPGx testで代替できるとすると，その価格もTDMと同じ程度になる可能性がある。類似薬効方式に似たシステムです。本日，野中さんが話されたような，経済評価を行った上で，その価格を決める方向にそろそろ進むべきだと思います。

これまで，検査と薬物治療のペアの経済評価というのはあまりなされてきませんでした。ただ考えてみますと，薬剤の経済評価もさほどなされていない。その問題の１つとして，その結果が行政で使われていないという現状があります。そうなるとPGx testを含めた経済評価を用いての価格づけは，現在の保険制度の中ではずいぶん先かと，悲観的な感じもしますが，その辺の見通しをお聞かせ願います。これも所轄外の質問で申し訳ありません。

佐藤　医療経済的な見通しを，もう少し医薬品の評価の中に入れるべきではないか，検査手法の評価の中に入れるべきではないかという，そういうご質問だと私は理解をしました。大体想定外の質問をいただいた時には，役所の見解というものはないわけで，あくまで個人的にどういうことを私が答えるかということになってくるかと思います。

そういうお断りのもとでお話をすると，先ほどのTDMの話は期待ということであって，必ずしも本当に，遺伝子検査というものはTDMに置き変わるということを断言したわけではなく，そういうことも将来的に起こり得るだろうということを言ったまでです。もしそういうことが起こるのだとすると，まさしくそれは津谷先生のおっしゃるように，経済評価的に見て，費用対効果というところから見て，TDMに優るということが前提としてあるのだろうということになってくるのだと思います。

ただ，TDMで価格が高いからといってそれは駄目だということではなく，そこに出てくるサービス，そういうものの質が向上すれば，当然そのマーケットというか，ユーザー側から求められてくることにもなってきますし，そういうファクターを考えていくのは，非常に難しいところだと思います。それは我々頭の堅い審査当局の発想では，なかなかまだ十分に追いつけない，そういう部分かと思っております。

鎌滝　私は研究の方をやっていますが，厚生労働省の方に知っていていただきたいと思うのですが，現状では，確かにTDMが一番確実な方法だし，よいと思いますが，将来を考えると，１回遺伝子診断をしておけばそれは一生使えるわけです。ですから，そういう具体例が出てくるか，出てこないかの問題ですが，もし具体例が出てくるとすると，そのコストは明らかに安くなるはずです。もう１つは，TDMの場合は，薬を投与しなくてはわからない。ところが，遺伝子の場合は，投与する前にわかってしまう，ある程度予測ができるということがあります。ですから，今すぐという意味ではなくて，長い将来を言えば，そちらの方が明らかにコストは安くなるはずだと私は思います。その都度やるというのと，１回やっておけば一生使えるということになります。

津谷　他にご質問はありませんか。

加藤隆一（慶應大）　一番重要なことは，企業が遺伝的多型に強く影響される，特に代謝の面では，そういうような薬はできるだけ開発の早い時期に，eliminationするという考え方を持つということです。ただし，化学構造などを変換しても，遺伝的多型をさけることは非常に難しいことも事実です。ですから，遺伝的多型の影響の大きいものの開発は難しいということではなくて，そういうものは例外的に新規性とか，効力によっては市場に出してもかまわないと思います。その場合には二次的に必ず遺伝子検査ということがついてくるということになるのではないかと思います。医療経済から見ても，やはり遺伝子検査が臨床で必要なものというのはできるだけ少なくしていくというのが今後の重要な姿勢ではないかと思います。

津谷　どうもありがとうございました。

検査を行わない場合の倫理的問題

津谷　野中さんにお聞きします。例えば，すでにあるPGx testが開発されている，その際，PGx testをせず，投薬後，有害反応が出てしまうとか，あるいは無効の人をtestで除外できるのにそのまま使ってしまう。そうすると，倫理的な問題が出てきますね。野中さん，その辺はどう考え，あるいは世界の現状はどうなっているのですか。

野中　例えば，検査を行わずにハーセプチン®を投与してしまうような例の場合では，それは副作用の影響がありますので，少し倫理的問題があると思われます。またIHCのような感度があまりよくない検査で，HER 2検査を行って，その結果に従ってトラスツズマブを投与されてしまったような場合には，もしその患者さんがFISH法で検査していたら陽性と出たような場合に，もしかしたらハーセプチン®を投与すれば助かったかもしれない，何ヵ月間はmedian TTPの延長があったかもしれなかった，そういうことがリスクになりまして，例えばIHCだけでしか検査を行わなかった患者さんが訴訟を起こしてしまうとか，そういう可能性も生じると思います。

津谷　そろそろまとめに入りたいと思います。実はこのセッションのタイトルは，当初「国民皆保険の中のファーマコジェネティクス」にしようかと思ったのですが，あまりにも生々しすぎるということで，それで「ヘルスサービスシステムの中のファーマコジェネティクス」というタイトルにしました。全部調べたわけではありませんが，日本臨床薬理学会の年会として経済のことを含めたセッションはこれまでなかったと思います。それがファーマコジェネティクスという先端的な領域と重なって初めてなされたということになります。日本臨床薬理学会の兄弟学会として日本薬剤疫学会があります。市販後におけるファーマコジェネティクスの研究のセッションはそこでもあってもいいのかなという気もします。

今回，イントロの鎌滝先生の教育的な講演から始まって，仁平さんの企業の立場での開発を中心にした話，野中さんには先駆け的な経済評価の実際を話していただきました。佐藤さんからは行政の考え方が述べられました。いろいろなプレイヤーがいる中でこの領域では，行政の持つ意味合いは特に大きいと思います。正しくガイドをするというのは言い過ぎかもしれませんが，やはり明確な方向性を出していただきたいと思っております。私はインセンティブについて話しました。

このセッションを終わりますが，これを機会にファーマコジェネティクスの領域でのナチュラルサイエンスだけではなくて，ソーシャルサイエンスとしてのディスカッションが今後ますます盛んになってもらいたいと思っております。皆様どうも長時間ありがとうございました。

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Pharmacokinetic Evaluation of Anticonvulsants in a Patient with Porphyria

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The aim of this study was to establish the appropriate regimen of anticonvulsants for a female patient with porphyria by pharmacokinetic evaluation of the influence of anticonvulsants on porphyria. The pharmacokinetics of phenytoin, carbamazepine, clonazepam, and clobazam were estimated by the Bayesian method. The urinary 6β-hydroxycortisol/cortisol (6β-OHF/F) ratio was also evaluated as an index of hepatic CYP3A4 induction.

The phenytoin concentrations in the toxic area fitted the predicted value for *CYP2C9*1/*3* better than that for *CYP2C9*1/*1* (her genotype). The concomitant phenytoin altered the clearance of carbamazepine considerably. The clearances of clonazepam and clobazam were not altered, although hepatic CYP3A4 induction was implied from the value of the urinary 6β-OHF/F ratio.

From the pharmacokinetic evaluations, the following were concluded: (1) phenytoin was not the proper medication for this patient, (2) carbamazepine can be used safely within a relatively small dose, 500 mg/day, (3) the combination of clonazepam and carbamazepine can be used, and (4) a concomitant small dose of clobazam with carbamazepine can also be used.

Key words: anticonvulsant, porphyria, cytochrome P450, Bayesian method, urinary 6β-hydroxycortisol/cortisol ratio

Introduction

Although many anticonvulsants are known to deteriorate porphyria, some patients with porphyria have seizures and require anticonvulsant therapy. Part of the deteriorating mechanism has been conjectured to cause an imbalance of heme protein biosynthesis, which is due to the induction of cytochrome P450 (CYP) by anticonvulsants. We previously demonstrated that the measurement of the human urinary 6β-hydroxycortisol/cortisol (6β-OHF/F) ratio is a useful indicator of safe medication in a patient with hereditary coproporphyria[1]. The previous results implied that hepatic CYP induction was profoundly related to her condition of porphyria.

In the present paper, the pharmacokinetic parameters of the anticonvulsants used in the patient during two hospitalizations (phenytoin, carbamazepine, clonazepam, and clobazam) were retrospectively evaluated by the Bayesian method[2,3]. We established the effective and safe dose of anticonvulsants for her seizures based on the alteration of the estimated pharmacokinetic parameters and the measurement of the urinary 6β-OHF/F ratio.

Case

A female in her twenties was diagnosed with rare dual porphyria involving partial δ-aminolevulinate dehydratase deficiency with epilepsy. She had been treated with sodium valproate, phenytoin and carbamazepine when she was admitted to our hospital on June 24, 1998. Sodium valproate was discontinued because of abdominal side effects. For

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better control of the epilepsy, phenytoin was increased from 100 to 125 mg/day and carbamazepine was gradually increased from 800 to 1200 mg/day. However, her condition deteriorated, and the serum concentrations of phenytoin were within the toxic range. The frequency of her seizures decreased eventually by discontinuing phenytoin, gradually reducing carbamazepine, and adding clonazepam. She was discharged from our hospital after four months.

She was re-hospitalized 2 years later (March 22, 2001) for reevaluation of the medication for epilepsy. She had been treated with carbamazepine, clobazam, and zonisamide. Zonisamide was discontinued. The clobazam dose, 15 mg/day, was not changed. The carbamazepine dose was gradually increased from 450 to 500 mg/day. The frequency of her seizures decreased 2 weeks after receiving modified treatment. The urinary 6β-OHF/F ratio gathered over 24 hours was measured and compared with the value from before she left our hospital the first time[1].

Methods

The patient's genotype of the metabolic enzymes of phenytoin had been judged to be *CYP2C9*1/*1* and *CYP2C19*1/*2*[1]. The pharmacokinetic parameters of phenytoin were estimated from serum phenytoin concentrations in the toxic area, with the use of subpopulation parameters of *CYP2C9*1/*1* and *CYP2C9*1/*3*[4]. The typical predicted dose of phenytoin was calculated from equation (1) (below), based on these estimated parameters. The percentage of deviation between the predicted dose and the administered dose was calculated from equation (2) (below).

Each alteration of carbamazepine and clonazepam clearance was estimated to evaluate the pharmacokinetic influence of these agents on porphyria. In the second hospitalization, the alteration of carbamazepine and clobazam clearances was estimated. The alteration of the N-desmethylclobazam/clobazam ratio was also evaluated, because clobazam is metabolized to N-desmethylclobazam by CYP3A4. The original data used for this study were obtained as described previously[1].

Her pharmacokinetic parameters of anticonvulsants were estimated by the software PEDA (parameter estimation and dosage adjustment)[5] incorporating the Bayesian method, using the population pharmacokinetic parameters described in the literature[4,6-8]. The phenytoin dose was calculated by the following equation from the observed concentration of phenytoin:

$$Dose_{,pre} = V_{max}C_{ss}/(K_m + C_{ss}) \quad (1)$$

where V_{max}, K_m, $Dose_{,pre}$, and C_{ss} are the maximal elimination rate of the Michaelis-Menten equation (mg/day), the Michaelis-Menten constant (μg/mL), the serum concentration of phenytoin at a steady state (μg/mL), and the predicted dose (mg/day), respectively. The deviation between the dose predicted from equation (1) and the actual dose was calculated by the following equation:

$$Dev = (Dose_{,act} - Dose_{,pre})/Dose_{,act} \quad (2)$$

where Dev, $Dose_{,act}$, and $Dose_{,pre}$ are the deviation, the actual dose, and the predicted dose, respectively. The plasma concentrations of carbamazepine, clonazepam, and clobazam were calculated by the following equations:

$$C = D \cdot K_a/Vd/(K_a - K_e) \cdot \{Exp(-K_e t) - Exp(-K_a t)\} \quad (3)$$

$$C_{ss} = D/CL \quad (4)$$

where D, C, and C_{ss} are the dosage (mg), carbamazepine or clobazam concentration in plasma (μg/mL), and plasma clonazepam concentration at a steady state (μg/mL), respectively; and K_e, K_a, Vd, t, and CL are the elimination rate constant (hr^{-1}), absorption rate constant (hr^{-1}), apparent volume of distribution normalized with bioavailability (L), time from initial administration (hr), and apparent total body clearance normalized with bioavailability (L/hr), respectively. From this point on, the term "clearance" will be used for "apparent total body clearance normalized with bioavailability". Equation (3) was used for carbamazepine and clobazam, and equation (4) was used for clonazepam.

Results

The pharmacokinetic parameters of phenytoin in *CYP2C9*1/*1* were estimated by the Bayesian method, as follows: $V_{max} = 3.8$ mg/kg/day and $K_m = 5.6$ μg/mL. The typical predicted dose of phenytoin was calculated to be approximately 141 mg/day. The percentage of deviation was approxi-

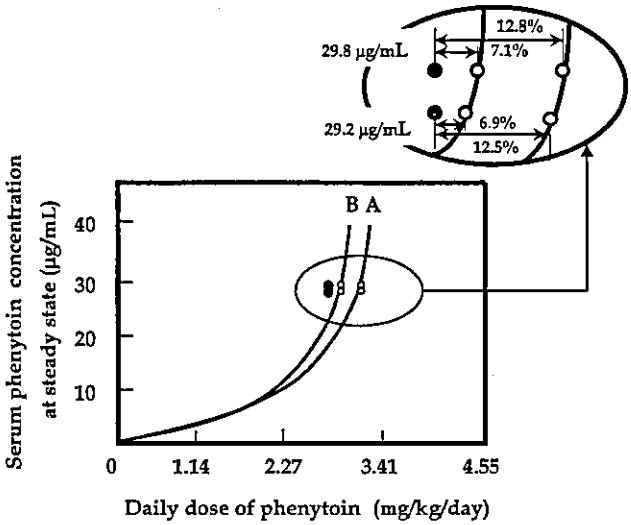

Fig. 1 Profile between serum concentration and dose of phenytoin

Curves A and B were the estimated curves from the subpopulation parameters[6] of *CYP2C9*1/*1* with *CYP2C19*1/*2* and *CYP2C9*1/*3* with *CYP2C19*1/*2*, respectively, by the Bayesian method. Closed circles (29.8, 29.2 μg/mL) represent serum phenytoin concentrations on July 31 and August 3, below 125 mg/day, respectively. Open circles are the predicted dose of phenytoin.

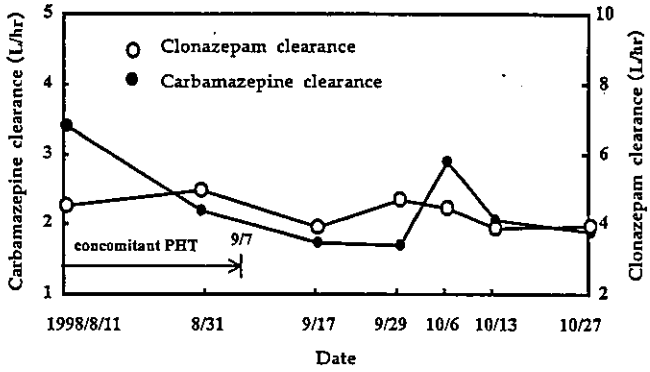

Fig. 2 Alteration of carbamazepine and clonazepam clearances

Open and closed circles represent carbamazepine and clonazepam clearances, respectively.

Table Alteration of the N-desmethylclobazam/clobazam ratio and the clearance of clobazam and carbamazepine

	April 3, 2001	May 11, 2001
Carbamazepine clearance (L/hr)	2.54	2.22
Clobazam clearance (L/hr)	2.25	2.25
Clobazam (μg/mL)	0.26	0.26
N-desmethylclobazam (μg/mL)	3.52	3.95
N-desmethylclobazam/ clobazam ratio	13.8	15.49

mately 11%. The estimated parameters in *CYP2C9*1/*3* were also calculated, as follows: V_{max}=3.3 mg/kg/day and K_m=4.1 μg/mL. The typical estimated dose was approximately 129 mg/day. The percentage of deviation was about 3%. Therefore, the predicted dose for *CYP2C9*1/*3* better fits the model used [equation(1)] than that for *CYP2C9*1/*1* (Fig. 1).

Carbamazepine clearance was altered between 1.7 and 3.4 L/hr, whereas clonazepam clearance was almost unaltered (Fig. 2). Although the carbamazepine dose was decreased by 80%, from 1200 to 1000 mg/day, the plasma concentration increased by 1.2 times when carbamazepine was combined with phenytoin, in the relationship between the daily dose and plasma concentration of carbamazepine. The clearance decreased by 50% based on the alteration. However, clonazepam clearance did not show a remarkable alteration even when clonazepam was combined with carbamazepine and phenytoin.

The alteration of carbamazepine and clobazam clearances and the N-desmethylclobazam/clobazam ratio after re-hospitalization are shown in Table. The carbamazepine and clobazam clearances remained almost unaltered. The N-desmethylclobazam/clobazam ratio was unaltered. The measured value of the urinary 6β-OHF/F ratio was 20.2.

Discussion

1. Discrepancy between the patient's genotype and phenotype of phenytoin metabolism

The patient was an extensive metabolizer of CYP2C9, which is the main metabolizing enzyme of phenytoin[1]. Her genotype was *CYP2C9*1/*1*, whereas her phenotype as indicated by the behavior of her serum phenytoin concentration was identical to that of *CYP2C9*1/*3*. *CYP2C9*3* is generally known to be a genetic polymorphism that decreases the enzyme activity. In addition, she had no inhibitor of CYP2C9 in her medications. The reason underlying the discrepancy between her genotype and phenotype of phenytoin metabolism remains an

area of considerable interest. One proposal is that phenytoin as an inducing agent of CYP3A4 might have destroyed the equilibrium of her abnormal heme pathway and depleted CYPs. Her metabolism of phenytoin might have been saturated by the reduction of CYP synthesis.

2. Alteration of the clearance of carbamazepine and clonazepam

The decrease in carbamazepine clearance (Fig. 2) leads us to conjecture that the suppression of CYP biosynthesis was brought on by a mechanism similar to that of the metabolic saturation of phenytoin by the concomitant phenytoin. In addition, an increase in carbamazepine clearance was transiently observed (Fig. 2), which might have been due to a high blood concentration of carbamazepine resulting from the improvement of the equilibrium of CYP biosynthesis, because a month had passed after the discontinuation of phenytoin. The results suggest that phenytoin markedly affected CYP biosynthesis in this patient with porphyria. The influence on CYP might have caused saturation of phenytoin metabolism and an excessive decrease in carbamazepine clearance.

The clonazepam clearance (CL/F) was estimated to be approximately 4.3 L/hr as the mean value (Fig. 2). The total body clearance (CL_{tot}) is calculated to be 4.4 L/hr because the bioavailability of clonazepam is reported to be approximately 0.98[9]. The product of the value of unbound fraction and the hepatic intrinsic clearance was calculated to be 4.4 L/hr from equation (5) when the absorption ratio was regarded to be 1.0 according to the high bioavailability (see "Appendix"). The general value of hepatic blood flow is about 90 L/hr. Therefore, the hepatic extraction ratio of clonazepam was calculated to be approximately 0.05 from equation (7) (see "Appendix"). Clonazepam is considered to be a metabolic capacity-limited agent, according to the calculated value of the hepatic extraction ratio. Carbamazepine is a similar type agent. Carbamazepine clearance seems to be excessively affected by the alteration of hepatic CYP3A4 activity, whereas the clonazepam clearance was not altered in spite of concomitant phenytoin.

The metabolic pathway of clonazepam proceeds by nitroreduction, acetylation, and hydroxylation. The acetylation is reported to be affected by the polymorphic N-acetyltransferase (NAT) that determines the acetylation phenotype of the individual[10]. This patient was considered to be an intermediate acetylator since her genotype was *NAT2*4/*6*. There has been no report of a relationship between the NAT2 genotype and a phenotype of clonazepam pharmacokinetics. The influence of the NAT2 genotype on the alteration of clonazepam clearance was unclear.

Seree et al, suggested that the nitroreduction of clonazepam is catalyzed by *CYP3A4*[11]. Therefore, clonazepam metabolism might also be affected by the suppression of CYP biosynthesis (Fig. 2). However, the alteration of clonazepam and carbamazepine clearances was different. Binding plasma proteins of phenytoin, clonazepam, and carbamazepine is 90-95%, 80-90%, and 70-80%, respectively[12,13]. This result implies that the difference in the affinity ratio of binding to plasma proteins affects the alteration of these clearances.

3. Pharmacokinetic evaluation of anticonvulsants after the second hospitalization

The patient's medication after the second hospitalization was evaluated according to the alteration of carbamazepine clearance, clobazam clearance, and the N-desmethylclobazam/clobazam ratio (Table). The carbamazepine and clobazam clearances and N-desmethylclobazam/clobazam ratio were unaltered after re-hospitalization. However, the measured value of the urinary 6β-OHF/F ratio was slightly high compared with the value (15.4) before the patient left our hospital the first time[1].

Her condition had not improved with a carbamazepine dose of 450 mg/day (see "Case"), whereas the frequency of seizures decreased by increasing the carbamazepine dose to 500 mg/day. Carbamazepine was considered to be effective and safe at a dose of 500 mg/day. The dosage regimen after the second hospitalization was considered to have been appropriately modified as a result.

4. Conclusions

From the pharmacokinetic evaluations for this patient, the following conclusion was established : phenytoin was not a proper medication. Carbamazepine was effective and safe, within a moderate

dose. In addition, the clearance of clonazepam was not affected by carbamazepine. Therefore, the concomitant use of carbamazepine and clonazepam was also considered effective. Clobazam and carbamazepine might be used safely for controlling seizures of porphyria within relatively small doses, at 15 mg/day for the former and at 500 mg/day for the latter.

The influence of anticonvulsants on porphyria was pharmacokinetically evaluated by estimating the clearance of anticonvulsants, in addition to the measurement of urinary 6β-OHF/F. We obtained information on effective and safe medications for porphyria on the basis of estimation of pharmacokinetic parameters.

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Appendix

The total body clearance (CL_{tot}) for a drug is generally expressed as follows, when a drug is mostly eliminated in the liver :

$$CL_{tot}=f_{ub}\cdot CL_{int,h}/F_a \quad (5)$$

where F_a is the absorption ratio of the drug ; f_{ub} is the unbound fraction in the blood ; and $CL_{int,h}$ is the hepatic intrinsic clearance. Furthermore, the hepatic clearance (CL_h) and hepatic extraction ratio (ER_h) of drugs are expressed as follows :

$$CL_h=Q_h\cdot f_{ub}\cdot CL_{int,h}/(Q_h+f_{ub}\cdot CL_{int,h}) \quad (6)$$
$$ER_h=f_{ub}\cdot CL_{int,h}/(Q_h+f_{ub}\cdot CL_{int,h}) \quad (7)$$

where Q_h is the hepatic blood flow ; f_{ub} is the unbound fraction in the blood ; and $CL_{int,h}$ is the hepatic intrinsic clearance. A drug with an ER_h of more than 0.8 is classified as an agent of the hepatic flow-limited type[14,15]. A drug with an ER_h of less than 0.2 is classified as an agent of the metabolic capacity-limited type.

An oral drug absorbed in the gut passes the liver via the portal vein. Only a drug which passes to the liver flows in circulating blood. Therefore, such a drug can be described by the following equation :

$$AUC_{po}=F_h\cdot F_a\cdot AUC_{iv} \quad (8)$$

where F_h is the hepatic availability ; F_a is the fraction absorbed into the portal vein from the gut ; and AUC_{iv} and AUC_{po} are the areas under the blood concentration-time curve after intravenous bolus and oral dosing, respectively.

AUC_{po} is expressed as a function of dosage (D) and total body clearance after oral dosing ($CL_{tot,po}$) as follows :

$$AUC_{po}=D/CL_{tot,po} \quad (9)$$

The following equation is obtained from equations (8) and (9) :

$$CL_{tot,po}=CL_{tot,iv}/(F_h\cdot F_a) \quad (10)$$

F_h is described by the following equation according to the literature[14,15] :

$$F_h=Q_h/(Q_h\cdot f_{ub}\cdot CL_{int,h}) \quad (11)$$

$CL_{tot,iv}$ assumes $CL_{,h}$ as the hepatic clearance, and equation (5) is obtained from equations (6), (10), and (11).

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Research report

Effect of genetic polymorphism on the metabolism of endogenous neuroactive substances, progesterone and *p*-tyramine, catalyzed by CYP2D6

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Abstract

Metabolic activities toward endogenous substrates in the brain, progesterone and *p*-tyramine, by cytochrome P450 2D6.2 (CYP2D6.2), CYP2D6.10A, CYP2D6.10C, and P34S, G42R, R296C, and S486T mutants expressed in recombinant *Saccharomyces cerevisiae* were compared with those by CYP2D6.1 (wild-type) in order to clarify the effects of genetic polymorphism of CYP2D6 on the metabolism of neuroactive steroids and amines in the brain. For the 6β-hydroxylation of progesterone, the V_{max} values for CYP2D6.2, CYP2D6.10A, and the P34S and G42R mutants, were less than half of those for CYP2D6.1, and CYP2D6.10C had a higher K_m and a lower V_{max} than the wild-type. The V_{max}/K_m values for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were 12–31% of that for CYP2D6. The 16α-hydroxylation and 21-hydroxylation of progesterone by CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were not detected, and the R296C mutant had a higher K_m for the 16α-hydroxylation and a lower V_{max} for the 21-hydroxylation than those for CYP2D6.1. For dopamine formation from *p*-tyramine, the K_m values for CYP2D6.2 and the R296C mutant were higher than those for CYP2D6.1, CYP2D6.10A, and CYP2D6.10C had a higher K_m and a lower V_{max} than the wild-type. The V_{max}/K_m values for CYP2D6.2, CYP2D6.10A, CYP2D6.10C and the P34S, G42R and R296C mutants were less than 45% of those for the wild-type. These results suggest the possibility that the polymorphism of CYP2D6, including *CYP2D6*2, CYP2D6*10* and *CYP2D6*12*, might affect an individual behavior and the central nervous system through endogenous compounds, such as neuroactive steroids and tyramine, in the brain.

Theme: Other systems of the CNS
Topic: Brain metabolism and blood flow

Keywords: CYP2D6; Progesterone hydroxylation; Dopamine formation from *p*-tyramine; Human brain; Polymorphism

1. Introduction

Cytochrome P450s (P450 or CYP) comprise a superfamily of enzymes that catalyze the oxidation of a wide variety of xenobiotic chemicals including drugs, carcinogens, and steroids [10,12,35]. In spite of the fact that CYP2D6 constitutes only 2–9% of constitutively expressed hepatic P450s among humans [17,36], it plays important roles in the metabolism of a wide range of therapeutic agents including drugs affecting the central nervous system [5,9,30,35]. Interestingly, CYP2D6 is expressed in the brain, especially the midbrain [27], as well as in the liver. The reverse transcriptase-polymerase chain reaction (RT-PCR)

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product from CYP2D4, the predominant CYP2D isoform in rat brain, is more abundant in cerebellum, striatum, pons, and medulla oblongata [21]. However, the physiological and pharmacological functions of CYP2D isoforms in the brain are still unknown.

Progesterone not only is one of the female steroid hormones secreted from the placenta and corpus luteum but also has various functions in the central nervous system as a neurosteroid in the brain [2,19]. For example, progesterone has the ability to increase myelin-specific protein levels and to enhance γ-aminobutyric acid (GABA)-induced chloride current [19,39], and the progesterone metabolites, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α,5α-tetrahydrodeoxycorticosterone, act as positive allosteric modulators of GABA type A receptors, and thereby reduce brain excitability and elicit sedative-hypnotic, anxiolytic, and anticonvulsant effects [32]. Recently, we have shown that CYP2D6 catalyzes the 2β-, 6β-, 16α-, and 21-hydroxylation of progesterone [15,29], and that progesterone 2β- and 21-hydroxylation activities in rat brain microsomes are completely inhibited by CYP2D antibodies, suggesting that CYP2D may be involved in the regulation (metabolism and/or synthesis) of endogenous neuroactive steroids, such as progesterone and its derivatives, in the brain [15]. Additionally, we have reported that the 21-hydroxylation of allopregnanolone as well as progesterone and 17α-progesterone is catalyzed by CYP2D isoforms in the brain [9,20].

Tyramine is not only an exogenous compound, which is found in fermented foods such as cheese and wine, but also an endogenous compound, which exists in the brain. Tyramine is especially present in the basal ganglia or limbic systems, which are thought to be related to an individual behavior and emotion [33], and dopamine is a neurotransmitter and a precursor of norepinephrine and epinephrine [14]. Previous studies conducted in this laboratory demonstrated that dopamine is formed from *p*-tyramine as well as *m*-tyramine by CYP2D6 [14].

CYP2D6 is one of the most extensively characterized polymorphic drug-metabolizing enzymes; the CYP2D6 gene is highly polymorphic, with more than 70 allelic variants [4,6,26]. Interestingly, it has been shown that CYP2D6 polymorphism has some relationship with an individual behavior [3,24]. Five to ten percent of Caucasians [1] and less than 1% of Japanese and Chinese [16] lack in vivo metabolic activity toward CYP2D6 substrates estimated by use of the urinary metabolic ratio, and are referred to as poor metabolizers. The CYP2D6*12 allele, which is associated with a deficient activity and consequently with the poor metabolizer phenotype, carries three functional mutations, G42R, R296C, and S486T [6,26]. On the other hand, in spite of the very low prevalence of CYP2D6 poor metabolizers in Asians, these groups display less CYP2D6 activity, and this has been attributed to the high frequency of the CYP2D6.10 enzyme [40]. That is, the *CYP2D6*10* allele, including both *CYP2D6*10A* and *CYP2D6*10B* variants, is widely observed in Japanese (31–38%) [22,28] and Chinese (51%) [18], and has two amino acid substitutions, P34S and S486T [6,26]. Additionally, *CYP2D6*10C* has the gene conversion in exon 9 derived from CYP2D7 and has 13 base substitutions more than *CYP2D6*10B* [18]. Fukuda et al. [8] reported that the K_m values of CYP2D6.10A and CYP2D6.10C for bufuralol 1′-hydroxylation and venlafaxine *O*-demethylation were higher than those of CYP2D6.1, and Tsuzuki et al. [37] reported that the substitution G42R increased the K_m and decreased the V_{max} for debrisoquine 4-hydroxylation, whereas it increased both V_{max} and K_m for bunitrolol 4-hydroxylation. Recent studies have shown that CYP2D6.10A had a higher K_m and/or a lower V_{max} than CYP2D6.1 for various exogenous substrates, including dextromethorphan, methamphetamine, and amitriptyline [34]. On the other hand, it has been reported that the R296C and S486T substitution (CYP2D6.2) affected only minimally the metabolim of dextromethorphan, bufuralol, and debrisoquine [25]. Furthermore, a number of investigators have proposed the key residues of CYP2D6 for exogenous substrates containing a basic nitrogen [7,13,38]. However, the key residues of CYP2D6 for the metabolism of other substrates, including the endogenous chemicals, which exist in the brain, and non-nitrogen containing compounds, are still unknown.

The present study was designed to elucidate the effects of CYP2D6 polymorphism, especially P34S, G42R, R296C, and S486T substitutions such as CYP2D6.2, CYP2D6.10A, and CYP2D6.10C, on CYP2D6 activities toward endogenous substrates in the brain, progesterone and *p*-tyramine.

2. Materials and Methods

2.1. Materials

Progesterone and 16α- and 21-hydroxyprogesterone were obtained from Sigma-Aldrich (St. Louis, MO, USA). 6β-Hydroxyprogesterone, dopamine hydrochloride, and NADPH were purchased from Steraloids (Newport, RI, USA), Research Biochemicals International (Natick, MA, USA), and Oriental Yeast (Tokyo, Japan), respectively. *p*-Tyramine and other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Microsomal fraction specifically expressing human P450

Cloning of human CYP2D6 cDNA, site-directed mutagenesis, the expression of mutated cDNA in recombinant *Saccharomyces cerevisiae* and the preparation of microsomal fractions from the cells were carried out according to methods described previously [8,37]. With these methods, we prepared CYP2D6.2 (R296C/S486T), CYP2D6.10A (P34S/S486T), CYP2D6.10C, and four mutant proteins

with single amino acid substitutions of P34S, G42R, R296C, and S486T.

2.3. Assay of progesterone hydroxylase activity

Progesterone hydroxylase activity was measured by the method described previously [15] with a minor modification. The incubation mixture consisted of microsomes from cells containing recombinant P450s (10–20 pmol/ml), 5, 10, 20, 50, 100, or 200 μM progesterone, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. After a 3-min preincubation at 37 °C, the reaction was started by adding NADPH. Incubation was carried out at 37 °C for 10 min and the reaction was terminated by the addition of 2 ml of ethyl acetate. The mixture was shaken and centrifuged at 1900×*g* for 5 min. The organic phase (1.5 ml) was evaporated under reduced pressure, and residue was dissolved immediately in 250 μl of 50% methanol. The HPLC system consisted of a Tosoh model DP-8020 pump (Tosoh, Tokyo, Japan), Tosoh model CO-8020 column heater, Tosoh model AS-8021 autosampler, an SPD-6-AV UV-detector (Shimazdu Corporation, Kyoto, Japan) set at 240 nm, and an analytical column TSK-gel ODS-80Ts (5 μm, 2.0×150 mm; Tosoh). The column temperature was set at 40 °C. The mobile phase was eluted at a flow rate of 0.3 ml/min as follows. The mobile phase was water as eluent A and methanol as eluent B, and the initial eluent profile was 50% B and then the eluent B was linearly increased to 65% over 20 min.

2.4. Assay of dopamine formation from p-tyramine

Dopamine formation from *p*-tyramine was measured by the method described previously [14] with a minor modification. The incubation mixture consisted of microsomes from cells containing recombinant P450s (10–20 pmol/ml), 0.05, 0.1, 0.2, 0.5, 1, or 2 mM, 0.05, 0.1, 0.2, 0.5, 1, 2, or 4 mM (for the G42R mutant), or 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 10, or 20 mM (for CYP2D6.10C) *p*-tyramine, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. After a 3-min preincubation at 37 °C, the reaction was started by adding NADPH. Incubation was carried out at 37 °C for 10 min and the reaction was terminated by the addition of 20 μl of 60% perchloric acid. After the mixtures were shaken and centrifuged at 1900×*g* for 10 min, dopamine in the supernatant was determined by HPLC. The HPLC system described above was used except that a Tosoh model FS-8011 fluorometric detector, and an analytical column TSK-gel ODS-120T (5 μm, 4.6×250 mm; Tosoh) were employed. The fluorescence intensity was determined at an excitation wavelength of 280 nm and emission wavelength of 340 nm. The column temperature was set at 40 °C, and flow rate was 0.7 ml/min. The mobile phase was a 6.8:93.2 (v/v) mixture of acetonitrile and an aqueous solution containing 160 mM ammonium dihydrogen phos-

Table 1
Kinetic parameters for the progesterone hydroxylation by CYP2D6 and its variants

P450	6β-Hydroxylation			16α-Hydroxylation			21-Hydroxylation		
	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)	K_m (μM)	V_{max}/K_m (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)
CYP2D6.1	23±5	0.39±0.03	17.3±4.2	16±8	0.079±0.013	5.0±2.7	34±23	0.12±0.03	3.5±2.5
CYP2D6.2	19±8	0.19±0.02	9.8±4.3	17±8	0.068±0.010	4.0±1.8	49±21	0.091±0.018	1.8±0.9
(R296C/S486T) CYP2D6.10A (P34S/S486T)	11±7	0.061±0.010	5.4±3.3	–	<0.02[a]	–	–	<0.02[a]	–
CYP2D6.10C	50±37	0.10±0.03	2.0±1.6	–	<0.02[a]	–	–	<0.02[a]	–
P34S	36±15	0.19±0.03	5.3±2.4	–	<0.02[a]	–	–	<0.02[a]	–
G42R	35±21	0.068±0.017	2.0±1.3	–	<0.02[a]	–	–	<0.02[a]	–
R296C	44±6	0.38±0.02	8.6±1.2	53±21	0.11±0.02	2.1±0.9	15±8	0.053±0.008	3.6±2.0
S486T	21±6	0.29±0.02	13.8±3.7	22±11	0.089±0.015	4.1±2.2	21±9	0.073±0.011	3.6±1.6

Progesterone at 5–200 μM was incubated with CYP2D6 and its variants (10–20 pmol/ml) and 1 mM NADPH at 37 °C for 10 min after a 3-min preincubation. Values are the means±S.D. of the data set using a nonlinear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

[a] The activity at 5–500 μM as a substrate concentration was <0.02 nmol/min/nmol P450.

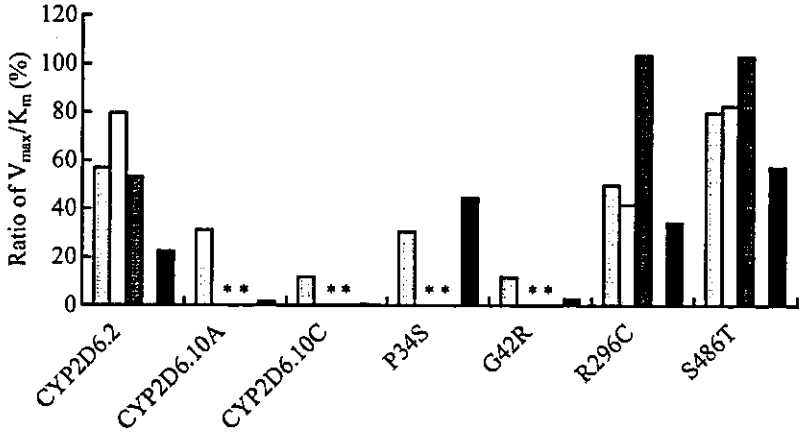

Fig. 1. Ratio of V_{max}/K_m for the metabolism of progesterone and *p*-tyramine by CYP2D6 variants. Ratios of V_{max}/K_m for 6β-hydroxylation (shaded column), 16α-hydroxylation (open column), 21-hydroxylation (striped column) of progesterone and dopamine formation from *p*-tyramine (closed column) were calculated by dividing the V_{max}/K_m for the variant by that for CYP2D6.1. *The 16α-hydroxylated and 21-hydroxylated metabolites for CYP2D6.10A, CYP2D6.10C and the P34S and G42R mutants were not detected (less than 0.02 nmol/min/nmol P450) even using 500 μM progesterone.

phate, 60 mM citric acid, 150 mM disodium EDTA, 10 mM dibutylamine, and 6 mM sodium 1-octanesulfonate.

2.5. Data analysis

In preliminary experiments, the linearity of the reaction with the protein concentration and incubation time was confirmed for each set of assay conditions. All data were analyzed using the mean of duplicate determinations. V_{max} and K_m values for progesterone hydroxylation and dopamine formation from *p*-tyramine were determined by fitting to Michaelis-Menten kinetics by nonlinear regression analysis (Microcal Origin, version 5.0J, Origin LabCorp, Northamptom, MA, USA).

3. Results

3.1. Progesterone hydroxylation by CYP2D6 and its variants

Kinetic parameters for progesterone hydroxylase activities of CYP2D6 and its variants are summarized in Table 1. The V_{max} value of CYP2D6.1 was highest for the 6β-hydroxylation followed by 21-hydroxylation and 16α-hydroxylation, whereas there were no marked differences between the K_m values for the three reactions. Although the K_m values for the 6β-hydroxylation by the CYP2D6 variants except for CYP2D6.10C were similar to those of CYP2D6.1, the V_{max} values for CYP2D6.2, CYP2D6.10A, and the P34S and G42R mutants, were less than half of those for CYP2D6.1. CYP2D6.10C had a higher K_m and a lower V_{max} than CYP2D6.1, whereas the V_{max} values as well as the K_m values for the R296C and S486T mutants were similar to those for the wild-type. The V_{max}/K_m values for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were 12–31% of that for CYP2D6.1 (Fig. 1).

The 16α-hydroxylated and 21-hydroxylated metabolites for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were not detected (less than 0.02 nmol/min/nmol P450) even using 500 μM progesterone. In addition, the K_m value for the 16α-hydroxylation and the V_{max} value for the 21-hydroxylation by the R296C mutant were 333% and 45%, respectively, of those for CYP2D6.1.

Table 2
Kinetic parameters for the dopamine formation from *p*-tyramine by CYP2D6 and its variants

P450	K_m (mM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)
CYP2D6.1	0.13±0.02	8.0±0.3	61±9
CYP2D6.2 (R296C/S486T)	0.33±0.07	4.5±0.3	14±3
CYP2D6.10A (P34S/S486T)	1.0±0.4	1.1±0.2	1.1±0.5
CYP2D6.10C	16.2±0.5	3.9±0.1	0.24±0.01
P34S	0.91±0.13	25.0±1.6	27±4
G42R	1.6±0.3	2.6±0.2	1.6±0.4
R296C	0.28±0.01	5.9±0.1	21±1
S486T	0.21±0.05	7.4±0.5	35±8

p-Tyramine at 50–2000 μM, 50–4000 μM (for G42R mutant) or 50–40000 μM (for CYP2D6.10C) was incubated with CYP2D6 and its variants (10–20 pmol/ml) and 1 mM NADPH at 37 °C for 10 min after a 3-min preincubation. Values are the means±S.D. of the data set using a nonlinear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

3.2. Dopamine formation from p-tyramine by CYP2D6 and its variants

Kinetic parameters for dopamine formation from *p*-tyramine by CYP2D6 and its variants are shown in Table 2. Although a mutation at 486 (S486T) had no marked effect on the K_m and V_{max} values, the K_m values for CYP2D6.2 and the R296C mutant were 2.1–2.5 times higher than those for CYP2D6.1 without affecting the V_{max} values. CYP2D6.10A had an 8-fold higher K_m and a 7-fold lower V_{max} than CYP2D6.1, and CYP2D6.10C exhibited an 124-fold higher K_m and a 51% reduction in V_{max} relative to the wild type. The P34S mutant had a 7-fold higher K_m and a 3-fold higher V_{max} than CYP2D6.1, and the G42R mutant had a 12-fold higher K_m and a 3-fold lower V_{max} than the wild type. Therefore, the V_{max}/K_m for CYP2D6.2 and the P34S and R296C mutants were 23–45% of those for CYP2D6.1, and the values for CYP2D6.10A, CYP2D6.10C and the G42R mutant, were 0.3–2.6% of those for the wild-type (Fig. 1).

4. Discussion

Progesterone exists in the brain and has various functions in the nervous system as a neurosteroid [2,19]. Although it is well known that CYP3A4 is one of the major metabolizing enzymes for progesterone hydroxylation in human liver [35], we have reported that progesterone 2β- and 21-hydroxylation in rat brain microsomes are catalyzed by CYP2D [15] and that the 21-hydroxylation of allopregnanolone as well as progesterone and 17α-progesterone is catalyzed by CYP2D isoforms in the brain [9,20], suggesting that CYP2D is involved in the regulation of endogenous neuroactive steroids in brain tissues. In addition, tyramine, one of the trace amines, is present in the brain, especially in the basal ganglia or limbic systems, which are thought to be related to an individual behavior and emotion [33], and CYP2D6 polymorphism has some relationship with an individual behavior [3,24]. In this study, we have demonstrated that the V_{max} and/or K_m values for the metabolism of progesterone and *p*-tyramine by CYP2D6.2, CYP2D6.10A, and CYP2D6.10C were different from those for CYP2D6.1, and that the G42R, P34S, and R296C substitutions affected these metabolic activities (Tables 1 and 2). Additionally, the V_{max}/K_m values for all of the variants except for progesterone 16α-hydroxylation by CYP2D6.2 and progesterone hydroxylations by the S486T mutant were less than 57% of those for CYP2D6.1 (Fig. 1). The G42R substitution is found in a CYP2D6*12 allele in combination with R296C and S486T [6,26]. Furthermore, it has been shown that, when an individual behavior was compared between extensive and poor metabolizers of debrisoquine, a typical probe substrate of CYP2D6, using the Eysenck personality questionnaire and the Karolinska Scales of personality inventory in 769 healthy Swedes, poor metabolizers had significantly lower scores in the Karolinska psychasthenia scales and a higher frequency of extreme responses than extensive metabolizer [3]. Comparison of the debrisoquine hydroxylation capacity and the Karolinska scales of personality in 225 healthy subjects in Spain indicated that poor metabolizers of debrisoquine are more anxiety-prone and less successfully socialized than extensive metabolizers [24]. These studies suggest that there may be a relationship between an individual behavior and the activity of the enzyme hydroxylating debrisoquine (CYP2D6). Although the patients are phenotyped but not genotyped in these papers, it has been reported that the study to assess the relationship between CYP2D6 genotype (including *CYP2D6*10* allele) and debrisoquine phenotype in African-Americans and Caucasians in Los Angels shows the positive identification of 88% of phenotypic poor metabolizers by genotyping [23]. Therefore, the present results suggest that the polymorphism of CYP2D6, including *CYP2D6*2, CYP2D6*10* and *CYP2D6*12*, might affect not only the metabolic activities toward exogenous compounds in the liver [25,34,37] but also an individual behavior and the nervous system through endogenous compounds, such as neuroactive steroids and tyramine, in the brain.

For all of the metabolic activities investigated, the V_{max} values for the G42R mutant were lower than those for CYP2D6.1 (wild-type), and the K_m values for the mutant were higher than those for the wild-type except for progesterone 6β-hydroxylation. On the other hand, the substitution at Pro34 decreased the V_{max} value for progesterone 6β-hydroxylation and increased both the V_{max} and K_m values for dopamine formation from *p*-tyramine. Tsuzuki et al. [37] reported that the G42R substitution but not the P34S substitution increased K_m and decreased V_{max} for debrisoquine 4-hydroxylation, whereas the G42R substitution increased both V_{max} and K_m and the P34S substitution gave only an increased K_m for bunitrolol 4-hydroxylation. Therefore, the present findings suggest that Gly42 is essential for the metabolic activities toward not only exogenous substrate but also endogenous compounds such as progesterone, a non-nitrogen containing compounds, and *p*-tyramine, and that the P34S substitution also affects the metabolism of progesterone and *p*-tyramine.

Gotoh [11] predicted six potential substrate recognition sites (SRS) in the CYP2 family, and the SRSs span residues 100–125, 211–218, 239–247, 294–312, 367–377 and 477–484 for CYP2D6.1. In this study, although the metabolic activities were affected only minimally by the S486T substitution, a mutation of 296 (R296C) of CYP2D6 decreased the V_{max}/K_m for progesterone hydroxylations and dopamine formation from *p*-tyramine. Although it has been reported that the R296C mutation is of little importance for debrisoquine 4-hydroxylation and bunitrolol 4-hydroxylation [37], it is possible to speculate that Arg296, which is included in SRS 4, also might be important to some extent to the metabolism of progesterone and tyramine.

Similarly, V_{max}, K_m and V_{max}/K_m of debrisoquine 4-hydroxylation and bunitrolol 4-hydroxylation by CYP2D6.2 (R296C/S486T) are similar to those by CYP2D6.1 [37], whereas consistent changes in the kinetic characterizing dextromethorphan, bufuralol, and debrisoquine biotransformation by CYP2D6.2 relative to CYP2D6.1 are observed for all three substrates, with an increase in K_m and V_{max} such that V_{max}/K_m values are the same or slightly greater for CYP2D6.2 [34]. In addition, it has been reported that the V_{max}/K_m of CYP2D6.2 toward dextromethorphan, fluoxetine, and codeine decreased levels to less than 35% that of CYP2D6.1 [41], and that the V_{max} for codeine *O*-demethylation catalyzed by CYP2D6.2 are significantly higher than for CYP2D6.1 [31]. In the present study, the V_{max}/K_m values for dopamine formation from *p*-tyramine by CYP2D6.2 were 23% of those for CYP2D6.1, whereas the K_m and V_{max} values for the 21-hydroxylation and 16α-hydroxylation by CYP2D6.2 were comparable with those for CYP2D6.1. Therefore, it is possible to speculate that the effect of the R296C/S486T variant is substrate-dependent.

In summary, our results suggest that the polymorphism of CYP2D6 might influence an individual behavior and the nervous system through endogenous compounds, including neuroactive steroids and tyramine, in the brain.

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ORIGINAL ARTICLE

CYP3A5 genotype did not impact on nifedipine disposition in healthy volunteers

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ABSTRACT

CYP3A5 expression is regulated by single-nucleotide polymorphisms (SNPs). The CYP3A5 genotype might contribute to a marked interindividual variation in CYP3A-mediated metabolism of drugs. Nifedipine is a typical substrate of CYP3A4 and CYP3A5 *in vitro*. The aim of this study was to elucidate the influence of the CYP3A5 genotype on nifedipine disposition in healthy subjects. A single capsule containing 10 mg of nifedipine was administered to 16 healthy male Japanese subjects (eight subjects: *CYP3A5*1/*3*; eight subjects: *CYP3A5*3/*3*). Blood samples were collected to analyze the pharmacokinetics of serum nifedipine and nitropyridine metabolite (M-I). The area under the plasma concentration–time curve (AUC), the peak plasma concentration (C_{max}) and the terminal half-life ($t_{1/2}$) of nifedipine, and the ratio of the nifedipine AUC to M-I AUC showed large intragroup variations, but no significant differences between the two genotypes. Based on the present findings, the functional relevance of CYP3A5 polymorphism should be re-evaluated in clinical trials.
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Keywords: CYP3A5; nifedipine; polymorphism; clinical; pharmacokinetics

INTRODUCTION

Cytochrome *P*450 3A (CYP3A) is abundantly expressed in human liver and small intestine, [1,2] and contributes to the metabolism of 50% of prescribed drugs. The activities of CYP3A in the general population show interindividual variations in CYP3A-mediated metabolism of drugs.[2] Recently, single-nucleotide polymorphisms (SNPs) were identified in intron 3 (*A–G*: *CYP3A5*3*) and exon 7 (*G–A*: *CYP3A5*6*) of the *CYP3A5* gene.[3] In addition, *CYP3A5*5* and *CYP3A5*7* were reported as a defective allele of CYP3A5, which gave a substantial impact on CYP3A5 expression.[4,5] These SNPs cause a frame-shift mutation or alternative splicing and protein truncation, and result in the absence of CYP3A5, suggesting that only people with at least one *CYP3A5*1* allele express large amounts of CYP3A5 protein. Therefore, these findings suggest that polymorphic CYP3A5 expression might be one factor contributing to the marked interindividual variation observed in CYP3A-mediated metabolism of drugs.

We previously reported the frequencies of CYP3A5-related SNPs in 200 healthy Japanese subjects.[6] As a result, the allele frequency of *CYP3A5*3* was approximately 70%, but *CYP3A5*6* was not detected in the Japanese population. Accordingly, these findings suggested that about 40% of Japanese express relatively high levels of metabolically active CYP3A5 protein.

Table 1 Enzyme kinetic analyses of the conversion of M-I using baculovirus-expressed human =P450s

	Km (μM)	*V_{max} (pmol/min/pmol P450)*	*V_{max}/Km (μl/min/pmol P450)*
CYP3A4	3.0	3.1	1.0
CYP3A5	6.5	3.3	0.5

To obtain clinical evidence of CYP3A5 polymorphism, we focused on nifedipine, a typical substrate of CYP3A4 and CYP3A5,[7–9] because a large individual difference was observed in nifedipine disposition,[10] which was thought to be regulated by a genetic background rather than environment.[11] Consistently, using baculovirus-expressed human CYP3A5 and CYP3A4, we confirmed their contribution to the metabolism of nifedipine (Table 1). These findings suggested that CYP3A5 contributes to the metabolism of nifedipine with kinetics similar to CYP3A4, implying that the interindividual differences in nifedipine disposition might be explained in part by CYP3A5 polymorphism. Thus, in the present study, we evaluated the influence of the CYP3A5 genotype on nifedipine disposition in healthy subjects to examine the polymorphic activities of CYP3A5 *in vivo*.

RESULTS

The subjects were genotyped and divided into two groups, *CYP3A5*1/*3* and *CYP3A5*3/*3* (Table 2). No subject had the other CYP3A5 alleles, *CYP3A5*5*, *CYP3A5*6* and *CYP3A5*7*. First, the plasma concentration profiles of nifedipine and M-I were compared between **1/*3* and **3/*3* groups. Unexpectedly, the time profiles of both plasma nifedipine and M-I were not significantly different between the two genotypes (Figure 1). Moreover, plasma nifedipine and M-I showed a large intragroup variation. Next, the typical pharmacokinetic parameters of nifedipine, such as the area under the plasma concentration–time curve from 0 to 12 h after administration (AUC_{0-12h}), the peak plasma concentration (C_{max}), terminal half-life ($t_{1/2}$) and clearance (CL/F) were calculated (Table 3). The AUC_{0-12h} values showed large intragroup variations without significant differences between the two genotypes (218.8±80.9 ng h/ml in *CYP3A5*1/*3* subjects, 178.7±92.8 ng h/ml in *CYP3A5*3/*3* subjects; mean±SD). Furthermore, the ratio of the nifedipine AUC_{0-12h} to the M-I AUC(4.77 in *CYP3A5*1/*3* subjects, 3.62 in *CYP3A5*3/*3* subjects; mean) also showed large intragroup variations with no significant differences between the two genotypes (Figure 2). The differences in the C_{max}, $t_{1/2}$ and CL/F of nifedipine between the two groups were not significant.

Finally, we measured systolic and diastolic blood pressure and pulse rate to estimate the significance of CYP3A5 polymorphism in the pharmacodynamics of nifedipine. Consistent with the pharmacokinetics, there were no significant differences in the pharmacodynamics between the two genotypes (Figure 3 and Table 4).

Table 2 Characteristics of the subjects in the study

Subject no.	*Age (years)*	*Height (cm)*	*Weight (kg)*	*BMI (%)*
*CYP3A5*1/*3 (n=8)*				
1	23	174.2	65.0	97.3
2	21	171.2	65.4	102.1
3	22	169.0	57.5	92.6
4	23	177.7	58.9	84.2
9	22	185.3	64.9	84.5
10	21	180.1	72.3	100.3
11	23	177.7	62.6	89.5
12	23	163.3	49.8	87.4
Mean	22.3	174.8	62.1	92.2
SD	0.9	6.9	6.7	7.0
*CYP3A5*3/*3 (n=8)*				
5	22	170.8	56.1	88.0
6	32	182.4	70.9	95.6
7	21	166.7	60.9	101.4
8	20	179.5	66.1	92.4
13	22	179.4	61.2	85.6
14	21	175.0	62.9	93.2
15	22	173.5	57.7	87.2
16	25	171.8	71.6	110.8
Mean	23.1	174.9	63.4	94.3
SD	3.9	5.3	5.7	8.4

DISCUSSION

In the present study, we examined the effects of CYP3A5 genotype on nifedipine pharmacokinetics, and demonstrated that an interindividual variation of plasma nifedipine concentration was not over-ridden by the CYP3A5 genotype. The interindividual variation was not beyond our conception and was almost similar to that described in the previous report following the administration of a 10-mg capsule.[12] The present finding suggests that CYP3A5 polymorphism is unlikely to be responsible for interindividual variation in the plasma level of nifedipine because the remaining CYP3A5 alleles, *CYP3A5*5*, *CYP3A5*6* and *CYP3A5*7*, were not found in the present subjects.

With respect to nifedipine metabolism, nifedipine disposition is slightly affected by the expression of intestinal CYP3As because grapefruit juice influences nifedipine disposition significantly but to a lesser extent than felodipine or nisoldipine,[10,13,14] suggesting that nifedipine is mainly metabolized not in the intestine but in the liver. In addition, it is hypothesized that P-glycoprotein (P-gp) is responsible for the large interindividual difference in

CYP3A-mediated drug disposition, since P-gp exists in the similar tissue to CYP3A4. However, this hypothesis is not the case with nifedipine disposition because nifedipine is not a substrate of P-gp.[15,16] Therefore, nifedipine pharmacokinetics must be crucially determined by the total liver CYP3A activities.

It was reported that nifedipine, as well as midazolam, were not only metabolized by CYP3A4 but also by CYP3A5 *in vitro*.[7–9,17] Prior to clinical study, we conducted kinetic study on the formation of M-I using recombinant microsomes (CYP3A4 and CYP3A5) because previous reports provided the oxidation activity at a single high concentration of nifedipine. We confirmed the contribution of CYP3A5 toward the metabolism of nifedipine at relatively low concentrations.

We, however, observed a discrepancy between the *in vitro* and *in vivo* contribution of CYP3A5 to nifedipine metabolism in the present study. Interestingly, a similar result has been obtained in the case of midazolam, a typical CYP3A5 substrate. Namely, midazolam pharmacokinetics was also hardly influenced *in vivo* by the genotype of CYP3A5,[18] although midazolam is metabolized *in vitro* by CYP3A5 rather than CYP3A4.[3,17,19] Several possibilities can be proposed to explain these discrepancies between the *in vitro* and *in vivo* data.

It was previously reported that total CYP3A activity showed an interindividual variation,[2] and the ratio of CYP3A4 to CYP3A5 might also vary interindividually in the liver.[20] Recently, Westlind-Johnsson *et al*[21] reported that CYP3A5 did not contribute to total CYP3A activity using

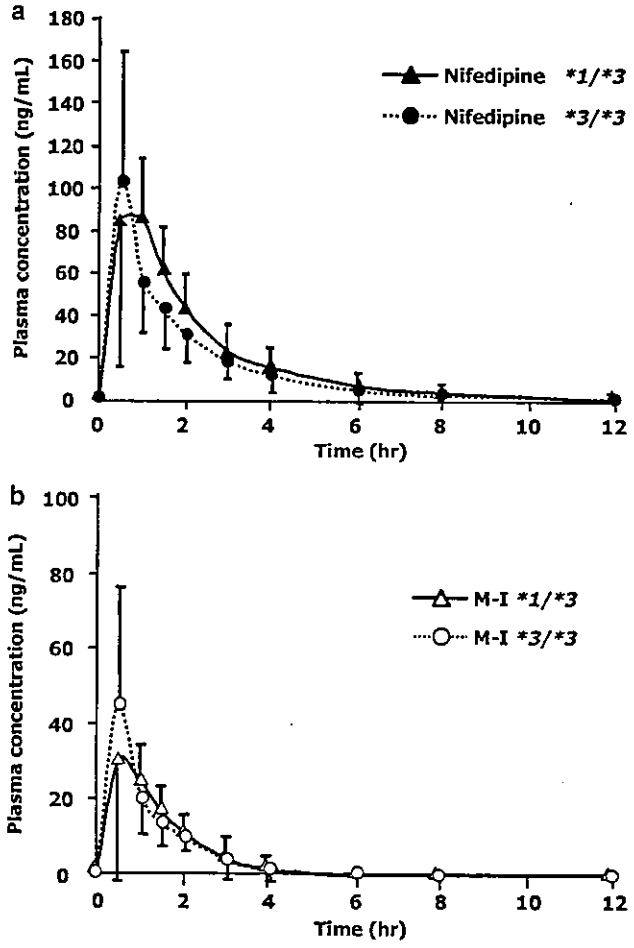

Figure 1 Plasma concentration–time curves of nifedipine (a) and M-I (b) in the *CYP3A5*1/*3* and *CYP3A5*3/*3* subjects. Values represent the means with SD ($n=8$).

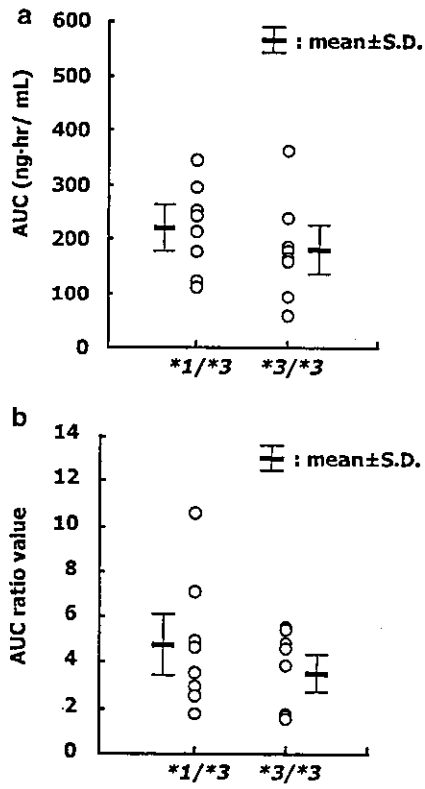

Figure 2 Individual (dot) and mean (line) values with SD of the $=AUC_{0-12h}$ of nifedipine (a) and the ratio of the nifedipine $=AUC_{0-12h}$ to the M-I $=AUC_{0-12h}$ (b) in the two genotypes.

Table 3 Pharmacokinetic parameters of nifedipine after oral administration to subject with *CYP3A5*1/*3* and *CYP3A5*3/*3* genotypes (mean ± SD)

Genotype	C_{max} *(ng/ml)*	$t_{1/2}$ *(h)*	AUC_{0-12} *(ng h/ml)*	*CL/F (ml/min)*
*CYP3A5*1/*3* ($n=8$)	116±43.3	1.77±0.66	219±80.9	877±375
*CYP3A5*3/*3* ($n=8$)	111±53.8	1.81±1.09	178±92.8	1246±837