GEFITINIB (IRESSA) INHIBITS THE CYP3A4-MEDIATED FORMATION OF 7-ETHYL-10-(4-AMINO-1-PIPERIDINO)CARBONYLOXYCAMPTOTHECIN BUT ACTIVATES THAT OF 7-ETHYL-10-[4-N-(5-AMINOPENTANOIC ACID)-1-PIPERIDINO]CARBONYLOXYCAMPTOTHECIN FROM IRINOTECAN

Ken-ichi Fujita, Yuichi Ando, Masaru Narabayashi, Toshimichi Miya, Fumio Nagashima, Wataru Yamamoto, Keiji Kodama, Kazuhiro Araki, Hisashi Endo, and Yasutsuna Sasaki

Department of Clinical Oncology, Saitama Medical School, Saitama, Japan

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ABSTRACT:

Gefitinib (Iressa) is an anticancer drug that selectively inhibits tyrosine kinases of epidermal growth factor receptor. Gefitinib might affect CYP3A4-mediated metabolism, since the drug is a substrate of human CYP3A. In this study, we evaluated the effects of gefitinib on drug metabolism catalyzed by human CYP3A4. The effects of gefitinib on the CYP3A4-mediated formation of NPC (7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin) and that of APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) from irinotecan were examined with the use of human liver and small intestinal microsomes. Gefitinib inhibited the formation of NPC in liver and small intestinal microsomes. The apparent intrinsic metabolic clearance (CLint) in the presence of 40 μM gefitinib was equivalent to about 28% of control in liver microsomes and 45% of control in small intestinal microsomes. Gefitinib stimulated the formation of APC by CYP3A4. CLint in the presence of 20 μM gefitinib with human liver microsomes was about 1.9 times higher than control. In human small intestinal microsomes, APC formation was enhanced by the addition of gefitinib at concentrations 20 μM or higher. CLint in the presence of 40 μM gefitinib was 2.8 times higher than control. Thus, we discovered that gefitinib inhibited the formation of NPC but stimulated the formation of APC from irinotecan.

Gefitinib (Iressa) is a member of a new class of oral drugs used to treat locally advanced or metastatic non-small cell lung cancer (NSCLC) (Cohen et al., 2004). This drug is a synthetic anilinoquinoline derivative that selectively inhibits tyrosine kinases of epidermal growth factor receptor. Gefitinib reversibly competes with ATP at a critical ATP-binding site within epidermal growth factor receptor protein (Ward et al., 1994; Barker et al., 2001). Previous studies have shown that gefitinib is metabolized by CYP3A4 (Culy and Faulds, 2002; Cohen et al., 2004; McKillop et al., 2005). Cytochrome P450 is a heme-containing enzyme that catalyzes the oxidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotic chemicals (Nelson et al., 1996). CYP3A4 is the most abundant cytochrome P450 expressed in the human liver as well as in the small intestine. This cytochrome participates in the metabolism of 25% of all therapeutic compounds, including drugs, carcinogens, and other xenobiotic chemicals. (Nelson et al., 1996). CYP3A4 (Culy and Faulds, 2002; Cohen et al., 2004; McKillop et al., 2005) is the most abundant cytochrome P450 expressed in the human liver as well as in the small intestine. This cytochrome participates in the metabolism of 25% of all therapeutic drugs or 50% of therapeutic drugs undergoing biotransformation. In general, modulation of a drug-metabolizing enzyme by a given drug alters the pharmacokinetics of other drug(s) metabolized by the enzyme, leading to drug interaction(s). Various interactions resulting from drug metabolism by CYP3A4 have been reported (Honig et al., 1993a,b,c). Gefitinib may also affect the CYP3A4-mediated metabolism of other substrates such as steroids (Gentile et al., 1996) and antiemetic agents (Villikka et al., 1999), which are simultaneously used with gefitinib to improve treatment- and disease-related symptoms in patients with cancer. Besides affecting the CYP3A4-mediated metabolism of concurrently administered supportive medication, gefitinib may influence the CYP3A4-mediated metabolism of irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) as described below. Irinotecan is a camptothecin analog with potent antitumor activity resulting from inhibition of topoisomerase I. It is widely used for the treatment of colorectal and lung cancers (Negoro et al., 1991; Kudoh et al., 1998; Rougier et al., 1998). Irinotecan is metabolized by carboxylesterases to form SN-38 (7-ethyl-10-hydroxyirinotecan), an active metabolite (Mathijssen et al., 2003), as well as by CYP3A4 to produce the nonactive metabolites NPC (7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin) and APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) (Haaz et al., 1998a,b; Santos et al., 2000) (Fig. 1). Mathijssen et al. (2004) have shown that CYP3A4 phenotype as determined by midazolam clearance significantly correlates with irinotecan clearance, indicating that CYP3A4 has an essential role in the metabolism of irinotecan in humans. Since gefitinib and irinotecan are used for the treatment of NSCLC and have different mechanisms of action, these drugs may act synergistically. Gefitinib and irinotecan

ABBREVIATIONS: NSCLC, non-small cell lung cancer; SN-38, 7-ethyl-10-hydroxyirinotecan; NPC, 7-ethyl-10-(4-amino-1-piperidino)carbonyloxyirinotecan; APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxyirinotecan; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; CLint, metabolic intrinsic clearance.
may thus be used concurrently for the treatment of NSCLC in the future. Stewart et al. (2004) have suggested that gefitinib may affect the CYP3A-mediated metabolism of irinotecan. They investigated the effects of gefitinib on the pharmacokinetics of orally administered irinotecan in mice. When administered concurrently with irinotecan, gefitinib was found to increase the oral bioavailability of irinotecan and to subsequently increase the plasma concentration of SN-38. They concluded that gefitinib might inhibit ABCG2 transporter, prominently expressed on the apical side of enterocytes. ABCG2 transporter is considered to play a crucial role in the efflux of irinotecan, thereby increasing its oral bioavailability. However, an alternative hypothesis proposes that gefitinib, a substrate of CYP3A (Culy and Faulds, 2002; Cohen et al., 2004; McKillop et al., 2005), inhibits irinotecan metabolism catalyzed by mouse CYP3A present in the liver and small intestine (Sakuma et al., 2000; Zhang et al., 2003), thus increasing the bioavailability of orally administered irinotecan and consequently elevating the plasma concentration of SN-38. In humans, low bioavailability of orally administered irinotecan (8–24%) was observed in some phase I trials, suggesting the high first-pass effects probably in the small intestine and the liver (Kuppens et al., 2004).

This study focused on the effects of gefitinib on the CYP3A4-mediated formation of NPC and APC from irinotecan. Human liver and small intestinal microsomes were used in vitro to investigate the effects of gefitinib on the CYP3A4-catalyzed metabolism of irinotecan.

Materials and Methods

Chemicals. 1-β-Hydroxyimidazolam, midazolam hydrochloride, and pooled human liver microsomes [catalog number 452161(H161)] were purchased from Daiichi Pure Chemicals (Tokyo, Japan). The pool comprised 22 specimens (21 white and 1 Hispanic). Microsomes were diluted in 250 mM sucrose. Aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were used in the preparation of the microsomes. No information was described on CYP3A5. Irinotecan, NPC, and APC were gifts from Yakult (Tokyo, Japan). Gefitinib was obtained from AstraZeneca (London, UK). All chemicals and solvents were of the highest grade commercially available.

Assay of NPC and APC Formation Catalyzed by Human CYP3A4. The amounts of NPC and APC formed by reactions catalyzed by human CYP3A4 were assayed as described by Haaz et al. (1998a,b), with minor modifications. Briefly, the incubation mixture consisted of 100 mM sodium potassium phosphate buffer (pH 7.4), 50 μM EDTA disodium salt, an NADPH-generating system (0.5 mM NADP⁺, 5 mM MgCl₂, 5 mM glucose 6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase), and microsomal fractions of human liver or human small intestine in a final volume of 0.25 ml. When human liver microsomes were used, the protein content and reaction time were predetermined with 80 μM irinotecan based on the linearity between the microsomal protein concentration (up to 0.133 mg/ml) and the reaction time (up to 10 min) versus the rate of metabolite formation. On the basis of the results, the protein content and the reaction time were determined to be 0.133 mg/ml and 10 min, respectively. When human small intestinal microsomes were used, linearity was obtained up to a microsomal protein concentration of 0.375 mg/ml and an incubation time of 20 min with 80 μM irinotecan. On the basis of these results, the protein content and reaction time were set at 0.375 mg/ml and 20 min, respectively. The NPC and APC metabolites were analyzed by HPLC using a HPLC system (Hitachi model 7000 series; Hitachi, Tokyo, Japan) equipped with a TSK-gel ODS-120T analytical column (4.6 × 250 mm, 4 μm; Tosoh, Tokyo, Japan). The mobile phase consisted of 75 mM ammonium acetate (pH 4.0) for solvent A and acetonitrile for solvent B. The metabolites were separated using a linear gradient of 85% to 65% solvent A, a time of 0 to 20 min, and a flow rate of 1.0 ml/min. The metabolites were quantified by comparing the HPLC peak area to that of the internal standard. Lower limits of quantification were 0.56 nM for NPC and 0.48 nM for APC, respectively. Effects of Gefitinib on NPC and APC Formation by CYP3A4. The effects of gefitinib on the CYP3A4-mediated metabolism of irinotecan to form the respective metabolites NPC and APC were investigated as follows. After
preincubation of the incubation mixture with gefitinib at 37°C for 5 min, the substrate irinotecan was added (final concentration of 20 μM). Irinotecan was used as lactone (stock diluted in 0.01 M citric acid, pH 3.0). Gefitinib was dissolved in DMSO. The final concentration of the solvent in the reaction mixture was 1%. The reaction was performed as described above. The effects of gefitinib on the CYP3A4-catalyzed metabolism of irinotecan were expressed as a percentage of activity compared with control in the absence of an effector. Control experiments were performed with the representative CYP3A4 inhibitors ketoconazole and erythromycin and a typical stimulator, α-naphthoflavone. Each chemical was dissolved in DMSO. The final concentration of the solvent in the incubation mixture was 1%. Each assay was performed three times in duplicate.

**Enzyme Kinetics.** The concentrations of irinotecan and gefitinib ranged from 5 to 80 μM and from 5 to 40 μM, respectively. Data points were fitted to the Michaelis–Menten equation by nonlinear least-squares regression analysis with the use of Origin 7.5 software (OriginLab Corp., Northampton, MA). The V_{max}/K_{m} value represented the metabolic intrinsic clearance (CL_{int}).

**Assay of Midazolam 1′-Hydroxylase Activity of Human CYP3A4.** Midazolam 1′-hydroxylase activity of human CYP3A4 was assayed by our method (Fujita et al., 2003), with minor modifications. Briefly, a typical incubation mixture consisted of 100 mM sodium potassium phosphate buffer (pH 7.4), 50 μM EDTA disodium salt, an NADPH-generating system (0.5 mM NADP+, 5 mM MgCl2, 5 mM glucose 6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase), and microsomal fraction of human liver in a final volume of 0.25 ml. The protein content and reaction time were predetermined with 10 μM midazolam based on linearity between the microsomal protein concentration (up to 0.16 mg/ml) and the reaction time (up to 4 min) versus the metabolite formation rate. On the basis of the results, the protein content and the reaction time were determined to be 0.16 mg/ml and 4 min, respectively. Reactions were initiated by the addition of midazolam and terminated by the addition of 2.5 ml of ethyl acetate. The 1′-hydroxymidazolam metabolite was analyzed by HPLC as described by us (Fujita et al., 2003).

**Effects of Gefitinib on Midazolam 1′-Hydroxylase Activity of CYP3A4.** The effects of gefitinib on the midazolam 1′-hydroxylase activity of CYP3A4 were investigated as follows. After preincubation of the mixture with gefitinib at 37°C for 5 min, the substrate midazolam was added (final concentration of 10 μM). DMSO was used as a solvent to dissolve gefitinib. The final concentration of the solvent in the reaction mixture was 1%. The reaction was performed as described above. The effects of α-naphthoflavone on the midazolam 1′-hydroxylase activity of CYP3A4 were also examined as control. α-Naphthoflavone was also dissolved in DMSO. The final concentration of the solvent in the incubation mixture was 1%. The effect of gefitinib on midazolam 1′-hydroxylation was expressed as the percentage of activity compared with control in the absence of an effector. Each assay was performed three times in duplicate.

**Statistical Considerations.** Statistical significance in the differences of NPC- and APC-producing activity or midazolam 1′-hydroxylation activity of CYP3A4 determined in the absence or the presence of an inhibitor or an activator was analyzed with a two-sample t test. All test results with p < 0.05 were regarded as statistically significant.

**Results**

**Effects of Gefitinib on Irinotecan Metabolism by Human CYP3A4.** The effects of gefitinib on the metabolism of irinotecan by CYP3A4 were evaluated. Table 1 shows the results obtained with human liver microsomes. Gefitinib had opposing effects on the formation of NPC and that of APC. The addition of gefitinib inhibited the formation of NPC but stimulated the formation of APC. The production of NPC and of APC in the presence of 40 μM gefitinib was 30.8% and 173% of the control value, respectively. No metabolite of gefitinib had a retention time similar to that of APC on the HPLC chromatogram after incubating the chemical alone with human liver microsomes (data not shown). The typical CYP3A4 inhibitors ketoconazole and erythromycin inhibited the formation of both NPC and APC from irinotecan. The representative CYP3A4 activator α-naphthoflavone (Shou et al., 1994; Harlow and Halpert, 1997; Koley et al., 1997) enhanced the production of both NPC and APC.

We also evaluated the effects of gefitinib on irinotecan metabolism in the presence of human small intestinal microsomes (Table 1). The formation of NPC and of APC in the presence of 40 μM gefitinib was 70.4% and 252% as compared with control, respectively. As expected, ketoconazole and erythromycin inhibited the formation of both NPC and APC. In contrast, α-naphthoflavone activated the production of both NPC and APC.

**Kinetik Analysis.** To investigate the effects of gefitinib on kinetic parameters of the CYP3A4-catalyzed formation of NPC and APC from irinotecan, reaction velocity versus substrate concentration was plotted in the presence or absence of gefitinib. The results obtained with human liver microsomes are shown in Fig. 2. Kinetic parameters were determined on the basis of the best fits of the velocity versus substrate concentration plots of Fig. 2 (Table 2).

The apparent K_{m} and V_{max} values for the formation of NPC were 44 μM and 128 pmol/min/mg protein, respectively. As shown in Table 2, CL_{int} for NPC formation decreased as the gefitinib concentration increased. The CL_{int} in the presence of 40 μM gefitinib was about 26% of that in the absence of gefitinib. A decrement in V_{max} contributed to a decrease in CL_{int} at gefitinib concentrations ranging from 0 to 20 μM. In contrast, an increase in the K_{m} value lowered the CL_{int} at a gefitinib concentration of 40 μM.

The K_{m} and V_{max} for the formation of APC were 68 μM and 46 pmol/min/mg protein, respectively. The CL_{int} was increased by the addition of gefitinib. The increment in CL_{int} seen at gefitinib concentration of 5 μM was caused by a decrease in the K_{m} value, whereas the increase at gefitinib concentrations ranging from 10 to 20 μM was by an increase in V_{max}. The maximum CL_{int} was observed in the presence of 20 μM gefitinib. The CL_{int} was about 1.9 times higher than the control value. The potential of gefitinib to increase CL_{int} decreased at gefitinib concentrations higher than 20 μM, since the K_{m} value simultaneously increased with the V_{max} value. K_{i} value of the inhibition of NPC formation by gefitinib was calculated to be 16 μM.

The effects of gefitinib on kinetic parameters for the formation of NPC and APC by CYP3A4 were also examined with human small intestinal microsomes. The reaction velocity versus the substrate concentration was plotted in the presence or absence of gefitinib. The results are shown in Fig. 3. The best fits of the velocity versus substrate concentration plots of Fig. 3 were used to determine the kinetic parameters (Table 3).
with human liver microsomes, \( \text{CL}_{\text{int}} \) for NPC formation decreased as the gefitinib concentration increased (Table 3). The \( \text{CL}_{\text{int}} \) in the presence of \( 40 \) \( \mu M \) gefitinib was about 45% of that in the absence of gefitinib. An increment in the \( \text{CL}_{\text{int}} \) by gefitinib was 48%.

The apparent \( K_m \) and \( V_{\text{max}} \) values for the formation of NPC were 49 \( \mu M \) and 15 pmol/min/mg protein, respectively. Similar to the results with human liver microsomes, \( \text{CL}_{\text{int}} \) for NPC formation decreased as the gefitinib concentration increased (Table 3). The \( \text{CL}_{\text{int}} \) in the presence of \( 40 \) \( \mu M \) gefitinib was about 45% of that in the absence of gefitinib. An increment in the \( K_m \) value was associated with a decrease in the \( \text{CL}_{\text{int}} \). The \( K_m \) value of the inhibition of NPC formation by gefitinib was 48 \( \mu M \).

The \( K_m \) and \( V_{\text{max}} \) values for the formation of APC were 59 \( \mu M \) and 6.7 pmol/min/mg protein, respectively. There was no increase in \( \text{CL}_{\text{int}} \) when 5 or 10 \( \mu M \) gefitinib was added to the reaction mixture, since the \( K_m \) value increased despite a rise in the \( V_{\text{max}} \) value. The \( \text{CL}_{\text{int}} \) value for APC formation was increased by the addition of gefitinib at concentrations of 20 \( \mu M \) or higher. As the gefitinib concentration increased, the \( K_m \) value decreased and the \( V_{\text{max}} \) increased, thereby elevating the \( \text{CL}_{\text{int}} \). The \( \text{CL}_{\text{int}} \) in the presence of 40 \( \mu M \) gefitinib was 2.8 times higher than that in the absence of the gefitinib.

These findings indicated that similar results were obtained with human liver microsomes and human small intestinal microsomes. With both systems, gefitinib had opposing effects on the formation of NPC and that of APC from irinotecan by CYP3A4. Gefitinib inhibited the formation of NPC but stimulated the formation of APC.

**Effects of Gefitinib on Midazolam 1'-Hydroxylase Activity of CYP3A in Human Liver Microsomes.** We examined whether gefitinib stimulated the metabolism of other CYP3A substrates. Midazolam is a representative substrate of human CYP3A. It is efficiently metabolized to 1'-hydroxymidazolam by human CYP3A. The effects of gefitinib on midazolam 1'-hydroxylase activity were examined with the use of human liver microsomes. The results are shown in Table 4. The addition of 10 \( \mu M \) gefitinib to the reaction mixture increased midazolam 1'-hydroxylase activity to 304% as compared with control. The substrate \( \alpha \)-naphthoflavone also increased midazolam 1'-hydroxylation catalyzed by CYP3A, consistent with the results of Maenpaa et al. (1998).

**Discussion**

This study was designed to test the hypothesis that gefitinib, a substrate of CYP3A, increases the bioavailability of irinotecan by inhibiting drug metabolism, as proposed by Stewart et al. (2004). We had initially anticipated that gefitinib would inhibit the CYP3A4-mediated formation of both NPC and APC. As shown in Figs. 2 and 3, NPC formation was inhibited by gefitinib as expected. However, APC formation was stimulated by the addition of gefitinib. In the liver microsomes, \( \text{CL}_{\text{int}} \) values for NPC and APC formation were 2.9 and 0.68 \( \mu M \) pmol/min/mg protein, respectively (Table 2), indicating that NPC formation was the major pathway for irinotecan metabolism by CYP3A4. The effects of gefitinib on \( \text{CL}_{\text{int}} \) for NPC formation were higher than those on \( \text{CL}_{\text{int}} \) for APC. Therefore, total metabolic clearance decreased according to the increase of gefitinib concentration. The NPC formation was also the major pathway for irinotecan metabolism by CYP3A4 in the intestinal microsomes (Table 3). However, total metabolic clearance was not necessarily decreased by the addition of gefitinib, since the effects of gefitinib on \( \text{CL}_{\text{int}} \) values for NPC and APC formation depended on the gefitinib concentrations. The effects of gefitinib on the bioavailability of orally administered irinotecan thus cannot be simply explained by our findings. Our results suggest that gefitinib-induced inhibition of mouse CYP3A enzymes present in the liver and the small intestine might not be responsible for the increase in the bioavailability of orally adminis-

**TABLE 2**

<table>
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<th>Gefitinib (( \mu M ))</th>
<th>NPC Formation</th>
<th>APC Formation</th>
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<tr>
<td></td>
<td>( K_m ) (( \mu M ))</td>
<td>( V_{\text{max}} ) (pmol/min/mg protein)</td>
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<tr>
<td>0</td>
<td>44</td>
<td>128</td>
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<td>5</td>
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<td>58</td>
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<td>40</td>
<td>93</td>
<td>69</td>
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</table>

Fig. 2. Velocity versus substrate concentration plots for irinotecan metabolism by CYP3A4 in human liver microsomes. A, NPC formation; B, APC formation. Each data point represents the mean of triplicate determinations and is shown with standard deviation bars. □, 0 \( \mu M \) gefitinib; ■, 5 \( \mu M \) gefitinib; ▲, 10 \( \mu M \) gefitinib; ○, 20 \( \mu M \) gefitinib; □, 40 \( \mu M \) gefitinib.
Effects of gefitinib on irinotecan metabolism by CYP3A4 in human small intestinal microsomes.

A. NPC formation; B. APC formation. Each data point represents the mean of triplicate determinations and is shown with standard deviation bars. ○, 0 μM gefitinib; ■, 5 μM gefitinib; △, 10 μM gefitinib; □, 20 μM gefitinib; O, 40 μM gefitinib.

The effects of gefitinib on the apparent kinetic parameters for NPC and APC formation by CYP3A4 in human small intestinal microsomes.

<table>
<thead>
<tr>
<th>Gefitinib (μM)</th>
<th>NPC Formation</th>
<th>APC Formation</th>
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<tr>
<td></td>
<td>$K_m$</td>
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<tr>
<td>0</td>
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<td>5</td>
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<td>20</td>
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<td>40</td>
<td>106</td>
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The effects of drug interactions further increasing the risk of drug interactions. The SN-38 concentration is affected by the metabolism of irinotecan by carboxylesterases to form SN-38 as well as by the metabolism of irinotecan by CYP3A4 to form NPC and APC. The SN-38 concentration is also influenced by glucuronidation catalyzed by UDP-glucuronosyltransferase 1A1 (detoxification) and by bile excretion by ABC2 transporter (Mathijssen et al., 2003). Any of these processes may be affected, leading to drug interactions. Our study focused on the effects of gefitinib on the CYP3A4-mediated metabolism of irinotecan. Our results showed that gefitinib had different effects on the formation of NPC and that of APC from irinotecan, making it difficult to predict changes in the plasma SN-38 concentration caused by gefitinib-induced alteration of CYP3A4 catalyzed metabolism. An understanding of the full picture of drug interactions between irinotecan and gefitinib thus requires consideration of the effects of gefitinib on other pharmacokinetic processes of irinotecan.

Gefitinib-induced inhibition of NPC formation and activation of APC formation from irinotecan occurred at concentrations of 5 μM or higher. The daily oral administration of gefitinib at recommended doses results in mean steady-state plasma concentrations ranging from 0.4 to 1.4 μM (Albanell et al., 2002). However, the local concentration of gefitinib in enterocytes immediately after oral administration is probably much higher than the plasma concentration. Therefore, if irinotecan and gefitinib are simultaneously administered, gefitinib present in enterocytes may affect the CYP3A4-mediated metabolism of irinotecan in the small intestine.

A progressive decrease in $V_{max}$ for NPC formation was induced by the addition of gefitinib in human liver microsomes (Table 2), whereas no effects were seen when human small intestinal microsomes were used (Table 3). These results suggest that differences in tissue from which CYP3A4 expresses may affect the enzymatic properties of CYP3A4. In the case of APC formation, progressive increases in $V_{max}$ values were observed by the addition of gefitinib in both microsomes, indicating the activation of the metabolism. However, $K_m$ values were somewhat random. At present, the reason is unclear.

CYP3A4 is a complex heme-containing enzyme that exhibits homogeneous and heterotropic cooperativity toward several substrates (Hutzler and Tracy, 2002). For example, α-naphthoflavone shows activation kinetics toward CYP3A4-mediated metabolism depending on the heterotropic positive cooperativity to substrates such as aflatoxin B, progesterone, and phenanthrene (Schwab et al., 1988; Shou et al., 1994; Ueng et al., 1997). Previous studies have suggested that CYP3A4 has a nancalytic effector site within the active-site cavity, capable of modulating its function (Shou et al., 1994). Similar to the results seen in the present study, pathway differential effects on CYP3A4-mediated metabolism of midazolam and triazolam by testosterone have been reported (Schrag and Wienkers, 2001; Galetin et al., 2003). Galetin et al. (2002) have also shown the similar effects of quinidine on the metabolism of midazolam by CYP3A4. Taking these results into account, the pathway differential effects on CYP3A4-mediated metabolism of irinotecan thus requires consideration of the effects of gefitinib on other pharmacokinetic processes of irinotecan.
simultaneously metabolized by CYP3A4 to form multiple metabolites, might be general mechanisms of CYP3A4 enzyme. The crystal structure of CYP3A4, directly supporting the presence of multiple substrate-binding active sites on CYP3A4 enzyme (Williams et al., 2004), may provide a clue to clarify the mechanisms.

We examined the effects of α-naphthoflavone in combination with gefitinib on the CYP3A4-mediated NPC formation. A 5 μM concentration of α-naphthoflavone and 40 μM gefitinib were simultaneously added to a reaction mixture containing 20 μM irinotecan and liver microsomes. These concentrations used were the same as those shown in Table 1. The addition of α-naphthoflavone did not alter the inhibitory effects of gefitinib on NPC formation (data not shown).

Gefitinib stimulated not only APC formation from irinotecan, but also midazolam 1'-hydroxylation catalyzed by CYP3A4 (Table 4). Gefitinib might also stimulate the metabolism of other CYP3A substrates in the liver and small intestine, thereby reducing drug bioavailability. Steroids such as dexamethasone (Gentile et al., 1996) and antiemetic agents such as ondansetron (Villikka et al., 1999), which inhibit the ability. Steroids such as dexamethasone (Gentile et al., 1996) and antiemetic agents such as ondansetron (Villikka et al., 1999), which inhibit CYP3A subfamily enzymes, might be general mechanisms of CYP3A4 enzyme. The crystal structure of CYP3A4, directly supporting the presence of multiple substrate-binding active sites on CYP3A4 enzyme (Williams et al., 2004), may provide a clue to clarify the mechanisms.

In conclusion, our in vitro studies demonstrated that gefitinib had opposing effects on the CYP3A4-catalyzed formation of NPC and that of APC from irinotecan, i.e., gefitinib inhibited the formation of NPC but stimulated the formation of APC.

### References


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