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

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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 (CL\text{int}) 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. CL\text{int} 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. CL\text{int} 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 anilinoquinazoline 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; McLellon 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 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)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-hydroxycamptothecin), 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-hydroxycamptothecin; NPC, 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin; APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; CL\text{int}, metabolic intrinsic clearance.

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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 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'-Hydroxymidazolam, 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 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 the 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 computerized 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. The intra- and interassay coefficient variations at 4.5 nM for NPC and 3.8 nM for APC were under 12% and 7.9%, 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

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**Fig. 1.** Major metabolic pathways for irinotecan.
Effects of Gefitinib on Irinotecan Metabolism by Human CYP3A4. The effects of gefitinib on the metabolism of irinotecan by CYP3A4 were evaluated. Gefitinib had opposing effects on the formation of NPC and APC from irinotecan. The reaction 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 \( \mu \)M 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 \( \mu \)M 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 \( \mu \)M. In contrast, an increment in the \( K_m \) value lowered the \( CL_{int} \) at a gefitinib concentration of 40 \( \mu \)M.

The \( K_m \) and \( V_{max} \) for the formation of APC were 68 \( \mu \)M 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 \( \mu \)M was caused by a decrease in the \( K_m \) value, whereas the increase at gefitinib concentrations ranging from 10 to 20 \( \mu \)M was by an increase of \( V_{max} \). The maximum \( CL_{int} \) was observed in the presence of 20 \( \mu \)M 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 \( \mu \)M, since the \( K_m \) value simultaneously increased with the \( V_{max} \) value. The inhibition of NPC formation by gefitinib was calculated to be 16 \( \mu \)M.

The effects of gefitinib on kinetic parameters for the formation of NPC and APC from irinotecan 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, CLint for NPC formation decreased as the gefitinib concentration increased (Table 3). The CLint in the presence of 40 μM gefitinib was about 45% of that in the absence of gefitinib. An increment in the CLint for NPC formation was the major pathway for irinotecan metabolism by CYP3A4. Gefitinib inhibited the formation of NPC but stimulated the formation of APC. Effects of Gefitinib on Midazolam 1'-Hydroxylase Activity of CYP3A4 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 μM gefitinib to the reaction mixture increased midazolam 1'-hydroxylation activity to 304% as compared with control. The substrate α-naphthoflavone also increased midazolam 1'-hydroxylation catalyzed by CYP3A4, 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, CLint values for NPC and APC formation were 2.9 and 0.68 μl/min/mg protein, respectively (Table 2), indicating that NPC formation was the major pathway for irinotecan metabolism by CYP3A4. The effects of gefitinib on CLint for NPC formation were higher than those on CLint 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 CLint 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

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<th>Gefitinib (μM)</th>
<th>NPC Formation</th>
<th>APC Formation</th>
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<tr>
<td></td>
<td>(K_m) μM</td>
<td>(V_{max}) pmol/min/mg protein</td>
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<tr>
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The apparent \(K_m\) and \(V_{max}\) values for the formation of NPC were 49 μM and 15 pmol/min/mg protein, respectively. Similar to the results with human liver microsomes, CLint for NPC formation decreased as the gefitinib concentration increased (Table 3). The \(CL_{int}\) in the presence of 40 μM 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 \(CL_{int}\). The \(K_m\) value of the inhibition of NPC formation by gefitinib was 48 μM.

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

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 μM gefitinib; ■, 5 μM gefitinib; ▲, 10 μM gefitinib; ●, 20 μM gefitinib; ○, 40 μM gefitinib.

The apparent \(K_m\) and \(V_{max}\) values for the formation of NPC were 49 μM and 15 pmol/min/mg protein, respectively. Similar to the results with human liver microsomes, CLint for NPC formation decreased as the gefitinib concentration increased (Table 3). The \(CL_{int}\) in the presence of 40 μM 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 \(CL_{int}\). The \(K_m\) value of the inhibition of NPC formation by gefitinib was 48 μM.

The \(K_m\) and \(V_{max}\) values for the formation of APC were 59 μM and 6.7 pmol/min/mg protein, respectively. There was no increase in CLint when 5 or 10 μM gefitinib was added to the reaction mixture, since the \(K_m\) value increased despite a rise in the \(V_{max}\) value. The CLint value for APC formation was increased by the addition of gefitinib at concentrations of 20 μM or higher. As the gefitinib concentration increased, the \(K_m\) value decreased and the \(V_{max}\) increased, thereby elevating the \(CL_{int}\). The CLint in the presence of 40 μM gefitinib was 2.8 times higher than that in the absence of the gefitinib.
Effects of Gefitinib on Irinotecan Metabolism by CYP3A4

In humans, the concentration of SN-38 in plasma is associated not only with the response to irinotecan, but also with the dose-limiting toxicity of irinotecan, such as leukopenia, diarrhea, or both (Negoro et al., 1991; Akabayashi, 1997). Severe, occasionally fatal toxicity occurs sporadically, even in low-risk patients participating in well-controlled clinical trials (Negoro et al., 1991; Kudoh et al., 1998; Rougier et al., 1998). Thus, the risk of drug interactions further increasing the treatment of irinotecan and the subsequent rise in the plasma concentration of SN-38 was reported by Stewart et al. (2004).

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Table 3

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<th>Gefitinib (μM)</th>
<th>NPC Formation</th>
<th>APC Formation</th>
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FIG. 3. Velocity versus substrate concentration plots for 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. a, 0 μM gefitinib; b, 5 μM gefitinib; c, 10 μM gefitinib; d, 20 μM gefitinib; e, 40 μM gefitinib.
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-bind- ing 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 N-oxidation. 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 I. The addition of α-naphthoflavone did not alter the inhibitory effects of gefitinib on N-oxidation (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 are also metabolized by CYP3A4, are usually coadministered with gefitinib in patients who require chemotherapy. Whether gefitinib alters the pharmacokinetics (metabolic clearance) and the pharmacodynamic effects of these drugs should be clarified in vivo.

In the present study, the 4'-hydroxymidazolam formation was detectable with the HPLC analysis used. However, unfortunately, the 4'-hydroxymidazolam was not quantified in the presence of gefitinib, since a peak derived from gefitinib or its metabolite disturbed the detection of the 4'-hydroxymidazolam. In conclusion, our in vitro studies demonstrated that gefitinib had opposing effects on the CYP3A4-catalyzed formation of APC and that of APC from irinotecan, i.e., gefitinib inhibited the formation of APC but stimulated the formation of APC.

**References**


