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Mechanism-Based Inactivation of Human Dihydropyrimidine Dehydrogenase by (*E*)-5-(2-Bromovinyl)uracil in the Presence of NADPH

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ABSTRACT

Purified recombinant human dihydropyrimidine dehydrogenase (hDPD) was incubated with ^{14}C-labeled (*E*)-5-(2-bromovinyl)-uracil ([^{14}C]BVU) in the presence of NADPH to investigate a possible mechanism in the 18 patient deaths caused by interactions of 5-fluorouracil prodrugs with the new oral antiviral drug, sorivudine. BVU is formed from sorivudine by gut flora and absorbed through intestinal membrane. hDPD, a rate-limiting enzyme for the catabolism of 5-fluorouracil and endogenous pyrimidines in the human, was NADPH dependently radiolabeled and inactivated by [^{14}C]BVU. Two radioactive tryptic fragments, I and II, isolated from radiolabeled hDPD were found by complete amino acid sequencing to originate from a common regional amino acid sequence located at positions 656 (Lys) to 678 (Arg) for I and positions 657 (Ser) to 678 (Arg) for II. However, only Cys^{671}, which should be present in the peptides, was not identified by amino acid sequencing. Mass spectrometric analysis of the tryptic fragments indicated that the sulfhydryl group of Cys^{671} in the hDPD was modified with 5,6-dihydro-5-(2-bromoethylydenyl)uracil (BEDU), a putative allyl bromide type of reactive molecule, to form a sulfide bond with loss of hydrogen bromide. The Cys^{671} sulfide bearing the debrominated BEDU had a 5,6-dihydrouracil ring highly strained by the exocyclic double bond at the 5-position, so that it underwent facile hydrolytic ring fission with alkali and heated acid treatments. A new proposal is also made for the amino acid sequence of the pyrimidine-binding domain, including Cys^{671}, of DPD in the human and other species.

^{14}C-Labeled (*E*)-5-(2-bromovinyl)uracil ([^{14}C]BVU) was demonstrated by us to rapidly bind covalently to purified rat (Okuda et al., 1997, 1998) and human (Ogura et al., 1998) dihydropyrimidine dehydrogenases (DPDs) in the presence of NADPH with concomitant rapid inactivation of their enzyme activity. The NADPH-dependent irreversible inhibition of DPD was also demonstrated by Desgranges et al. (1986) and Porter et al. (1992) with unlabeled BVU using the partially purified rat and purified bovine enzymes, respectively.

DPD is a homodimeric cytosolic protein with a molecular mass of 210 kDa having multiple Fe/S clusters and multiple FAD and FMN as an electron transfer system. In the presence of NADPH, DPD dihydrogenates the endogenous pyrimidines, uracil and thymine, and various 5-substituted exogenous pyrimidines, including the anticancer drug 5-fluorouracil (5-FU), for their further catabolism to β-alanine and α-substituted β-alanines (Shiotani and Weber, 1981; Diasio and Harris, 1989; Lu et al., 1993). In the human (Lu et al., 1992, 1993), rat (Shiotani and Weber, 1981; Lu et al., 1993), and mouse (Desgranges et al., 1986), hepatic DPD has been demonstrated to be a rate-limiting enzyme for regulating the tissue levels of 5-FU and the endogenous pyrimidines.

We have been trying to determine a possible mechanism for the acute deaths of 18 Japanese patients in 1993 that were caused by interactions between oral 5-FU prodrugs and sorivudine [1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil; SRV], the new oral antiviral drug used in the treatment of herpes zoster. These deaths occurred within 40 days after SRV was approved by the Japanese government for clinical use (Pharmaceutical Affairs Bureau, 1994). The patients who died from the drug-drug interaction had received SRV for the viral disease for only a few days while also receiving one of the 5-FU prodrugs every day for postsurgical anticancer chemotherapy. SRV caused no appreciable toxic symptom in patients who were receiving anticancer drugs other than 5-FU or its prodrugs.

SRV orally administered to rats (Nishimoto et al., 1990) and humans (Ogiwara et al., 1990) is decomposed in part by

ABBREVIATIONS: [^{14}C]BVU, ^{14}C-labeled (*E*)-5-(2-bromovinyl)uracil; BEDU, 5,6-dihydro-5-(2-bromoethylydenyl)uracil; BEU, 5-(2-bromoethyl)uracil; DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil; H_2-BVU, dihydro-BVU; hDPD, human DPD; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS, mass spectrometry (or mass spectrometric); SRV, sorivudine [1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil]; TFA, trifluoroacetic acid; ACTH, adrenocorticotropin; TPCK, tosylphenylalanyl chloromethyl ketone.

gut flora to generate BVU, which has no antiviral activity and appears in the plasma via the liver after being absorbed through intestinal membrane. Our toxicokinetic study with rats indicated that hepatic DPD activity was markedly decreased by the oral administration of SRV or BVU and that 5-FU concentrations in the plasma, bone marrow, and intestines were increased to a lethal level when the 5-FU prodrug tegafur [5-fluoro-1-(tetrahydro-2-furyl)-uracil], which was administered to most of the patients, was orally coadministered (Okuda et al., 1997, 1998). Therefore, the repeated coadministration of both drugs led all the rats to death within 10 days, after severe toxic symptoms such as diarrhea with bloody flux and marked decrease in white blood cell and platelet counts, as had been reported for the 18 patients who died.

Similar evidence was provided by Desgranges et al. (1986) for the marked increase in the plasma 5-FU level in the rat and mouse successively administered i.p. single doses of BVU and 5-FU. Potent inactivation of human DPD (hDPD) by SRV was also demonstrated by Yan et al. (1997) in the mononuclear cells of peripheral blood from patients who had herpes zoster and were repeatedly administered a clinical dose of SRV for 10 days. Our in vitro study indicated SRV to have no inhibitory effect on rat (Okuda et al., 1997, 1998) and human (Ogura et al., 1998) DPDs in the presence of NADPH under the same conditions used for the rapid and complete inactivation of these enzymes by BVU.

However, nothing is known of the molecular mechanism for the mechanism-based inactivation of hDPD by BVU in the presence of NADPH. In this study, we provide evidence for the molecular mechanism of hDPD inactivation, in which a cysteinyl residue located at position 671 in the pyrimidine-binding domain of the enzyme and 5,6-dihydro-5-(2-bromoethylydenyl)uracil (BEDU), an allyl bromide type of reactive molecule formed from BVU with NADPH in the domain, are involved.

Experimental Procedures

Materials. Adrenocorticotropin (ACTH) fragment 18–39, α-cyano-4-hydroxycinnamic acid, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). A PD-10 column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and immobilized TPCK-treated trypsin (200 U/mg) from Pierce Chemical Co. (Rockford, IL). [6-^{14}C]5-FU (2.1 MBq/μmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). [^{14}C]BVU was prepared as reported previously (Okuda et al., 1997). 5-(2-Bromoethyl)uracil (BEU) was prepared from 5-(2-hydroxyethyl)uracil by a previously described method (Griengl et al., 1985). Other reagents used were of analytical grade.

Enzyme Assay. Recombinant hDPD was purified from *Escherichia coli* cytosol as reported previously by Ogura et al. (1998). Enzyme activity of the purified hDPD was assayed using [6-^{14}C]5-FU as a substrate by a previously reported method (Okuda et al., 1997). The purified hDPD had a specific activity of 645 nmol/mg of protein/min. Inactivation of hDPD by [^{14}C]BVU and the incorporation of radioactivity into the enzyme were determined under previously reported conditions (Ogura et al., 1998). The inhibition constant (K_i) for BEU was determined using a Dixon plot obtained in the zero-order kinetics region of the enzyme reaction. Data were expressed as means of at least three experiments.

Inactivation of hDPD by [^{14}C]BVU and Tryptic Digestion. Purified hDPD (4 mg) was incubated with [^{14}C]BVU (50 μM; specific radioactivity, 2.0 MBq/μmol) in the presence of NADPH (200 μM) in a final volume of 1 ml of 5 mM potassium phosphate buffer (pH 7.4) containing 2.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1% (w/v) Triton X-100 at 37°C for 1 h. Unreacted [^{14}C]BVU was removed from the radiolabeled hDPD by gel filtration chromatography on a PD-10 column (16- × 50-mm) previously equilibrated in 0.2 M NH_4HCO_3, and the column effluent containing the radiolabeled hDPD (7.8 kBq/mg of protein) was lyophilized. The lyophilizate was dissolved in 2 ml of 0.5 M Tris-HCl buffer (pH 8.0) containing 2.7 mM EDTA, 6 M guanidine hydrochloride, and 45 mM dithiothreitol. The radiolabeled hDPD was alkylated with 0.5 ml of 0.5 M iodoacetic acid in 0.5 M Tris-HCl buffer (pH 8.0) at room temperature in the dark for 30 min. The alkylated radioactive hDPD was separated by gel filtration on a PD-10 column previously equilibrated in 0.2 M NH_4HCO_3 and digested with immobilized TPCK-treated trypsin (20 U) in 0.1 ml of 0.2 M NH_4HCO_3 at 37°C for 12 h. Thereafter, the same number of units of trypsin was added to the mixture three times at intervals of 12 h. After the reaction, the trypsin-coated beads were removed from the mixture by centrifugation at 5000*g* for 5 min.

Reverse-Phase HPLC of Tryptic Digest. The tryptic digest of the alkylated radioactive hDPD was separated by reverse-phase HPLC on an Inertsil ODS-2 column (4.6- × 250-mm, 5 μm; GL Science, Tokyo, Japan). The digest was loaded onto the column and eluted at a flow rate of 1 ml/min with a 0 to 30% (v/v) linear gradient of acetonitrile (0.33%/min) in water containing 0.1% (v/v) trifluoroacetic acid (TFA) in the eluant. Radioactive peptides I and II were eluted at retention times of 60 and 62 min, respectively, from the column used for the first step of HPLC. For additional purification, the peptides were separately subjected to the second step of HPLC on a Hi-Pore RP-304 C_4 column (4.6- × 250-mm, 5 μm; Bio-Rad Laboratories, Richmond, CA) eluted at a flow rate of 1 ml/min with a 10 to 20% (v/v) linear gradient of acetonitrile (0.17%/min) in water containing 0.1% (v/v) TFA in the eluant. Radioactive peptides I and II were eluted from the Hi-Pore RP column at retention times of 29 and 30 min, respectively. Peptide I was obtained in homogeneous form by the second step of HPLC. Peptide II was subjected to the third step of HPLC for purification on the Inertsil ODS-2 column and eluted under the same conditions used for the first step of HPLC. The elution of the peptides was monitored by absorbance at 214 nm. Fractions were collected every minute, and radioactivity was determined by radioluminography with a BAS 2000 bioimaging analyzer (Fuji Photo Film, Tokyo, Japan) as reported previously (Baba et al., 1994).

Amino Acid Sequencing. The purified radiolabeled peptides were sequenced by automated Edman degradation with an Applied Biosystems (Foster City, CA) 477A protein sequencer combined with an Applied Biosystems 120A analyzer. Radioactivity of the column effluent from the analyzer at each cycle was measured by liquid scintillation counting with an Aloka LSC-100 liquid scintillation counter (Tokyo, Japan).

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Molecular weights of purified radiolabeled peptides I and II were determined by MALDI-TOF MS with a Voyager Elite TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) operated in the reflectron mode. The samples were crystallized with α-cyano-4-hydroxycinnamic acid [10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) TFA, water]. All spectra were externally calibrated with the $[M+H]^+$ ions using ACTH fragment 18–39 (2465.69) as a standard peptide.

Results

Radiolabeling and Inactivation of hDPD by [^{14}C]BVU in the Presence of NADPH. Purified recombinant hDPD was rapidly radiolabeled and completely inactivated by preincubation with 5 μM [^{14}C]BVU in the presence of NADPH in a reciprocal manner (Fig. 1). The radioactivity incorporated into the enzyme protein was not removed by

washing the enzyme protein from the reaction mixture on a ProBond nickel-resin column (Invitrogen Co., Carlsbad, CA) as reported previously (Ogura et al., 1998). No radiolabeling or inactivation of hDPD occurred in the absence of NADPH, indicating that a reactive dihydro-derivative, H_2-BVU, formed from BVU with NADPH bound covalently to the enzyme. BEU, an alkyl bromide type of dihydro-derivative, had no irreversibly inhibitory effect on hDPD at concentrations up to 50 μM when preincubated in the presence and absence of NADPH. However, BEU was a potent competitive hDPD inhibitor with a K_i value of 2.2 μM in the reduction of 5-FU (Fig. 2).

The NADPH-dependent radiolabeling and inactivation of hDPD by [^{14}C]BVU were not retarded to any appreciable extent in the presence of the 10 mM thiols, dithiothreitol, 2-mercaptoethanol, cysteine, and glutathione (Fig. 1), indicating that the H_2-BVU formed as a reactive metabolite in the hDPD molecule may instantly react with the enzyme without being influenced by a high concentration of thiols.

Cys^{671} Unidentified in Radioactive Tryptic Fragments from hDPD Inactivated by [^{14}C]BVU. Radioactive peptides I and II were isolated and purified from a tryptic digest of radiolabeled and inactivated hDPD (4 mg, 7.8 kBq/mg of protein) by the second and third steps of HPLC, respectively (Fig. 3). Aqueous acetonitrile containing 0.1% (v/v) TFA was used for the chromatographic purification of the radioactive peptides. Complete amino acid sequencing of the purified tryptic fragments indicated that peptides I and II had N termini of Lys^{656} and Ser^{657}, respectively, and a common C terminus of Arg^{678} (Table 1). However, in their amino acid sequences, only Cys^{671}, which would be expected to be present in the molecularly cloned amino acid sequence of hDPD (Yokota et al., 1994), was unidentified. At cycles 16 and 15 for peptides I and II, respectively, in the automated amino acid sequencing (Table 1), ~90% of radioactivity of the radiolabeled peptide (equivalent to 70 and 30 pmol of [^{14}C]BVU for peptides I and II, respectively) was eluted from the chromatographic column used for identification of the amino acids.

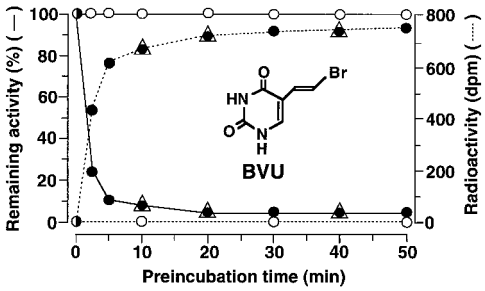

Fig. 1. Effect of thiols on time courses of enzyme activity of hDPD preincubated with BVU and of radioactivity incorporation into the enzyme from [^{14}C]BVU in the presence and absence of NADPH. Purified recombinant hDPD (0.5 μg/ml) was preincubated with 5 μM BVU or [^{14}C]BVU in the presence (●) and absence (○) of NADPH at 37°C. After preincubation, the remaining enzyme activity (solid lines) and radioactivity incorporated into the enzyme (broken lines) were determined by a previously reported method (Ogura et al., 1998). [6-^{14}C]5-FU was used as a substrate for measurement of enzyme activity. The thiol (10 mM, △), dithiothreitol, 2-mercaptoethanol, cysteine, or glutathione was added to the preincubation mixture containing NADPH. Only the effects of dithiothreitol on enzyme activity and radioactivity incorporation are shown because no appreciable difference was observed between dithiothreitol and the other thiols. Data are arithmetic mean values of at least three experiments.

MALDI-TOF MS Evidence for Covalent Binding of H_2-BVU to hDPD. MALDI-TOF MS analysis indicated that radioactive peptide I from [^{14}C]BVU-inactivated hDPD showed the major protonated parent ion signal $[M+H]^+$ at *m/z* 2556.40 (signal 1), which corresponded to the calculated molecular mass of a sulfide formed from H_2-BVU and the peptic moiety of Lys^{656} to Arg^{678}, including the Cys^{671} unidentified by amino acid sequencing (Fig. 4). The calculated value of $[M+H]^+$ for the unmodified peptide I with the amino acid sequence of Lys^{656} to Arg^{678} derived from hDPD was 2416.49, and the difference between the modified and unmodified peptides was 139.91, which corresponded to the molecular mass of debrominated H_2-BVU minus one proton from the sulfhydryl group of Cys^{671} for the sulfide formation.

The mass spectrum also indicated that the purified peptide I contained a peptide molecule showing a minor $[M+H]^+$ signal at *m/z* 2574.42 (signal 2), which corresponded to a hydrated molecule (+18) of peptide I (Fig. 4A). When the acidic HPLC column eluate [0.1% (v/v) TFA in aqueous acetonitrile] containing peptide I was heated at 100°C for 10 min before recording the mass spectrum, $[M+H]^+$ signal 1 markedly decreased with a concomitant marked increase in $[M+H]^+$ signal 2, indicating that the hydration of peptide I was accelerated by heating under an acidic condition of the column eluate (Fig. 4B). When the acidic HPLC column eluate was heated for 10 min, a new minor $[M+H]^+$ signal appeared at *m/z* 2592.28 (signal 3) in the mass spectrum, which corresponded to peptide I hydrated with 2 mol of water (+36). The $[M+H]^+$ signal 3 in the mass spectrum increased by heating the HPLC column eluate at 100°C for 30 min with a concomitant decrease in $[M+H]^+$ signal 2 (Fig. 4C).

Treatment of the HPLC column eluate containing peptide I with a final concentration of 0.4% (w/v) NaOH at room temperature for 5 min resulted in the facile formation of its monohydrated molecule appearing as $[M+H]^+$ signal 2 in the mass spectrum (Fig. 4D). No change was observed in the spectral signal pattern when the alkali treatment was continued for 60 min. The $[M+H]^+$ signal 3 observed when peptide I was treated for a prolonged period under acidic conditions did not appear in the spectrum by alkali treatment of the HPLC column eluate. After prolonged alkali treatment, $[M+H]^+$ signal 1 still remained as a minor signal

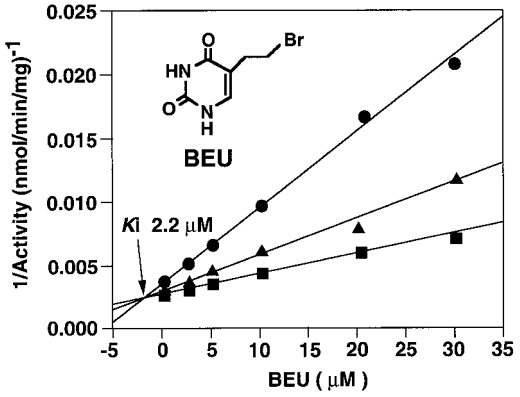

Fig. 2. Dixon plot of inhibition of hDPD by BEU. Purified hDPD (0.5 μg/ml) was incubated with 10 (●), 15 (▲), or 20 (■) μM [6-^{14}C]5-FU as a substrate at 37°C for 10 min in the presence of NADPH and various concentrations of BEU (0–30 μM). The enzyme activity toward 5-[^{14}C]FU was determined as reported previously by Ogura et al. (1998). Data are arithmetic mean values of at least three experiments.

in the spectrum. MS analysis of the HPLC column eluate containing the purified peptide II also showed very similar spectral profiles before and after acid or alkali treatment (data not shown).

Discussion

The mechanism-based inactivation of hDPD by BVU in the presence of NADPH was proved to be caused by covalent binding of the reactive metabolite H_2-BVU to the Cys^{671} residue located in the pyrimidine-binding domain of hDPD. BVU acts as a suicide inhibitor of hDPD.

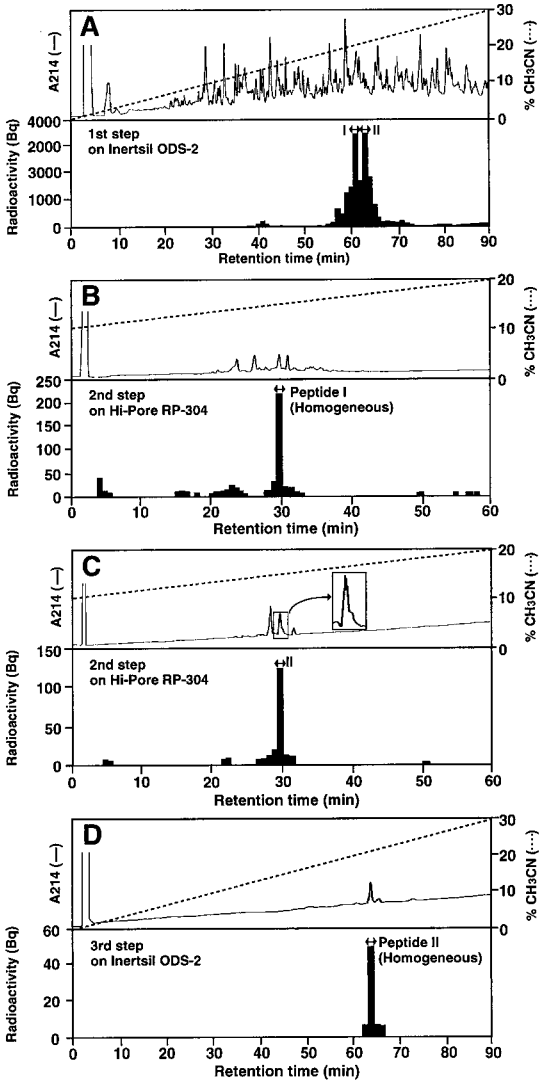

Fig. 3. Separation by reverse-phase HPLC of tryptic peptides I and II from hDPD inactivated by [^{14}C]BVU in the presence of NADPH. Chromatograms were monitored by absorbance at 214 nm (solid lines) and by radioactivity measurement of an aliquot of the eluate collected every minute (histograms). HPLC columns were eluted with 0.1% (v/v) TFA in water and an increasing amount of acetonitrile in a linear gradient manner (broken lines) as described under *Experimental Procedures*. Horizontal arrows above the radioactivity peaks (peptides) I and II represent the fractions pooled for additional purification or as homogeneous samples. Homogeneity of the separated peptides I and II was confirmed by their N-terminal amino acid sequencing as described under *Experimental Procedures*. Panel A represents separation on an Inertsil ODS-2 column of radioactive peptides I and II from a tryptic digest of hDPD (4 mg) inactivated by [^{14}C]BVU in the presence of NADPH. Panels B and C represent purification and partial purification of peptides I and II, respectively, on a Hi-Pore RP-304 column. Panel D represents rechromatography for purification of partially purified peptide II on the Inertsil ODS-2 column under the same conditions used in panel A.

Location of the pyrimidine-binding domain in the amino acid sequence of human and porcine DPDs was proposed by Yokota et al. (1994) through their molecular cloning studies of the enzymes based on evidence provided by Porter et al. (1991, 1992) (Fig. 5). Porter et al. indicated that bovine DPD was inactivated and radiolabeled by the reactive dihydrogenated metabolites formed from [6-^{3}H]5-iodouracil (1991) and [2-^{14}C]5-ethynyluracil (1992) in the presence of NADPH and also that only the Cys residue located in radioactive fragments from a common amino acid sequence in the radiolabeled DPD was modified. Later, a molecular cloning study (Albin et al., 1996) showed that bovine DPD was 92% identical with hDPD in total amino acid sequence and completely identical with hDPD in the sequence of the pyrimidine-binding domain proposed by Yokota et al. (1994), which contained the only Cys residue as indicated by Porter et al. (1991, 1992). Except for the N-terminal amino acid sequence including two different amino acid residues, radioactive peptides I and II isolated from the tryptic digest of hDPD inactivated by [^{14}C]BVU in the presence of NADPH had the same sequence, Ser^{660}-Arg^{678}, as the peptide from bovine DPD inactivated by [2-^{14}C]5-ethynyluracil in the presence of NADPH (Porter et al., 1992). Yokota et al. (1994) used this same sequence for proposing the pyrimidine-binding domain of hDPD excluding its N terminus Ser^{660}.

DPDs in four mammalian species, including the rat (Kimura et al., 1998), all consist of 1025 amino acid residues counting from the leading amino acid, Met, and have a completely identical sequence at positions Ser^{660} to Gln^{700}, including Cys^{671} (Fig. 5). This regional sequence is more than twice as long toward the C terminus as the pyrimidine-binding domain proposed by Yokota et al. (1994) who had only limited data available at that time (Fig. 5). Based on the currently available data obtained from the *Caenorhabditis elegans* gene (Wilson et al., 1994) and *Drosophila melanogaster* mRNA (Van Gelder et al., 1995), the long amino acid sequence of the pyrimidine-binding domain may be shortened to Leu^{665}-Gln^{686}, which contains one homologous amino acid substitution, Lys, in the worm instead of Arg at position 678 in mammals.

The local sequence Leu^{665}-Gln^{686}, proposed by us as a new pyrimidine-binding domain, contains another Cys residue at position 684. The Cys^{684} residue is most unlikely to react with the dihydrogenated reactive metabolite, H_2-BVU, formed from BVU in the hDPD molecule. No detectable amount of radioactive fragment containing the Cys^{684} was found in the tryptic digest of hDPD inactivated by [^{14}C]BVU after extensive survey of the digest by HPLC, followed by amino acid sequence analysis and MALDI-TOF MS. Therefore, the 5-substituted uracil, BVU, is assumed to tightly interact through its cyclic N^1-vinyl-N^3-acylureido moiety with a limited number of amino acid residues very near the Cys^{671} residue in the three-dimensional structure of the pyrimidine-binding domain of hDPD and to be reduced with NADPH to the reactive metabolite, H_2-BVU. The Cys^{684} in the pyrimidine-binding domain of hDPD may be distant from the immovable H_2-BVU formed in the enzyme molecule, sterically hindered whether located near H_2-BVU or masked as a disulfide by one of the other 35 Cys residues of hDPD, so that the sulfhydryl group of Cys^{684} cannot be modified with H_2-BVU. N^1- or N^3-methyl or alkyl-substituted uracils have

been demonstrated to interact with mouse hepatic cytosolic DPD to a much lesser extent than uracil and 5-substituted uracils (Naguib et al., 1989), strongly suggesting that DPD recognizes uracils with an unsubstituted acylureido moiety as the best substrates.

Two possible structures are considered with respect to the reactive metabolite H_2-BVU, formed from BVU with NADPH in hDPD. One is BEU, an alkyl bromide formed by reduction of the side chain vinyl group of BVU. However, BEU cannot be a candidate, because it had no irreversibly inactivating effect on hDPD, even when preincubated at a high molar concentration (50 μM) with the enzyme. BEU interacted with hDPD and inhibited the enzyme reversibly in a competitive manner at very low concentrations (Fig. 2). The K_i value for BEU was very low, 2.2 μM, in the reduction of 5-FU by hDPD. The other H_2-BVU is most likely to be BEDU, an allyl bromide type of reactive molecule formed by the attack of H^- from NADPH to the 6-uracil carbon with a shift of the 5,6-double bond to the exocyclic position of the side chain, followed by protonation to the 2′-carbon of the side chain (Fig. 6). BEDU may instantly form a sulfide bond by reacting as an alkylating agent with the sulfhydryl group of Cys^{671} with concomitant loss of hydrogen bromide. The sulfide bond formation between BEDU and the sulfhydryl group of Cys^{671} in hDPD was so facile that the inactivation and radiolabeling of hDPD by [^{14}C]BVU could not be retarded by a high concentration of various thiols, including dithiothreitol (Fig. 1). Similar evidence for the insufficient effect of dithiothreitol

TABLE 1
Amino acid sequence analysis of tryptic peptides I and II derived from hDPD inactivated with [^{14}C]BVU in the presence of NADPH

Peptide I			Peptide II			hDPD[a]
Cycle	Amino acid		Cycle	Amino acid		
		pmol			*pmol*	
1	Lys	65.9				Lys^{656}
2	Ser	29.6	1	Ser	25.2	Ser
3	Glu	27.5	2	Glu	8.8	Glu
4	Asp	19.9	3	Asp	10.3	Asp
5	Ser	14.7	4	Ser	6.0	Ser
6	Gly	24.4	5	Gly	10.6	Gly
7	Ala	21.5	6	Ala	5.9	Ala
8	Asp	11.3	7	Asp	5.8	Asp
9	Ala	24.6	8	Ala	6.4	Ala
10	Leu	14.2	9	Leu	5.3	Leu
11	Glu	9.9	10	Glu	2.6	Glu
12	Leu	18.6	11	Leu	7.1	Leu
13	Asn	14.5	12	Asn	5.3	Asn
14	Leu	18.7	13	Leu	0.4	Leu
15	Ser	6.9	14	Ser	2.2	Ser
16	X[b]		15	X[b]		Cys^{671}
17	Pro	7.5	16	Pro	2.2	Pro
18	His	0.6	17	His	0.2	His
19	Gly	8.9	18	Gly	5.6	Gly
20	Met	3.6	19	Met	1.3	Met
21	Gly	12.4	20	Gly	6.1	Gly
22	Glu	2.1	21	Glu	1.0	Glu
23	Arg	0.6	22	Arg	0.9	Arg^{678}

[a] Amino acid sequence of hDPD was taken from Yokota et al. (1994).
[b] This residue was not identified. About 90% of radioactivity of radiolabeled peptides I and II was eluted at cycles 16 and 15, respectively, and most of the remainder at next two cycles.

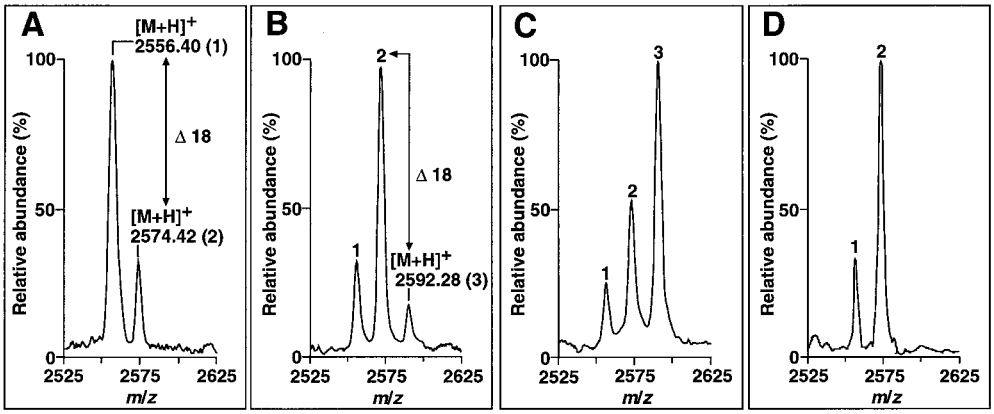

Fig. 4. MALDI-TOF mass spectra of radiolabeled peptide I. Mass spectra were recorded with a MALDI-TOF mass spectrometer operated in the reflectron mode. The mass assignment of the parent ions $[M+H]^+$ was performed using a one-point calibration with ACTH fragment 18–39 (2465.69) as an external standard. Panel A represents the mass spectrum of peptide I after separation by two-step HPLC eluted with 0.1% (v/v) TFA in aqueous acetonitrile as the eluant. Panels B and C represent the mass spectra of purified peptide I heated in the acidic HPLC column eluate [0.1% (v/v) TFA in aqueous acetonitrile] at 100°C for 10 and 30 min, respectively. Panel D represents the mass spectrum of purified peptide I after treatment with NaOH [0.4% (w/v)] at room temperature for 5 min. Before recording the spectrum, the alkali-treated sample was applied to a ZipTipC_{18} tip (Millipore Co., Bedford, MA) to remove the sodium ion according to the manufacturer's instruction.

has been obtained by Porter et al. (1992) in the mechanism-based inactivation of bovine DPD by 5-ethynyluracil in the presence of NADPH.

In addition to amino acid sequencing, MALDI-TOF MS analysis of radiolabeled tryptic fragments from hDPD inactivated by [^{14}C]BVU provided evidence for the modification of Cys^{671} by the reactive metabolite BEDU (Fig. 4). The major parent mass ion signal 1 of peptide I corresponded to the calculated molec-

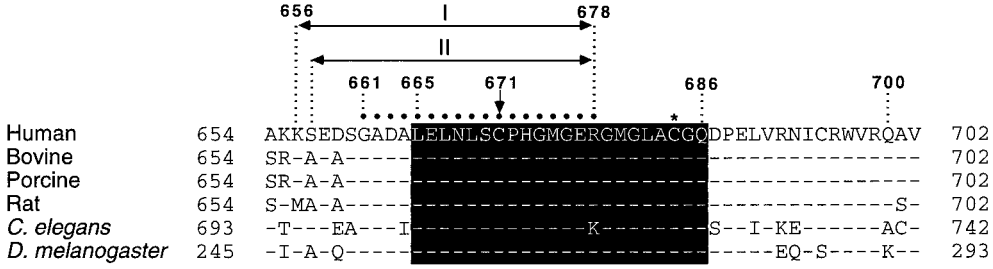

Fig. 5. Sequence alignment of DPDs in the region of the proposed pyrimidine-binding domain. The deduced amino acid sequence of hDPD (Yokota et al., 1994) is aligned with amino acid sequences of DPDs cloned from the bovine (Albin et al., 1996), porcine (Yokota et al., 1994), and rat (Kimura et al., 1998) DPDs, and of putative DPD encoded in the *C. elegans* gene (Wilson et al., 1994) and a DPD-like peptide in the *D. melanogaster* mRNA (Van Gelder et al., 1995). Amino acid residues identical with those in the sequence of hDPD are represented by dashes. Arabic numerals represent amino acid positions counted from the leading amino acid, Met. The pyrimidine-binding domain proposed by Yokota et al. (1994) is shown by dots above the sequence (Gly^{661}-Arg^{678}). Our new proposal for the pyrimidine-binding domain (Leu^{665}-Gln^{686}) is shown in white on black. Horizontal arrows I and II above the sequences represent tryptic peptides I (Lys^{656}-Arg^{678}) and II (Ser^{657}-Arg^{678}) from hDPD inactivated by [^{14}C]BVU in the presence of NADPH. The downward arrow represents the unidentified Cys^{671} residue modified by the reactive [^{14}C]BVU metabolite, H_2-BVU. The asterisk represents the Cys^{684} residue.

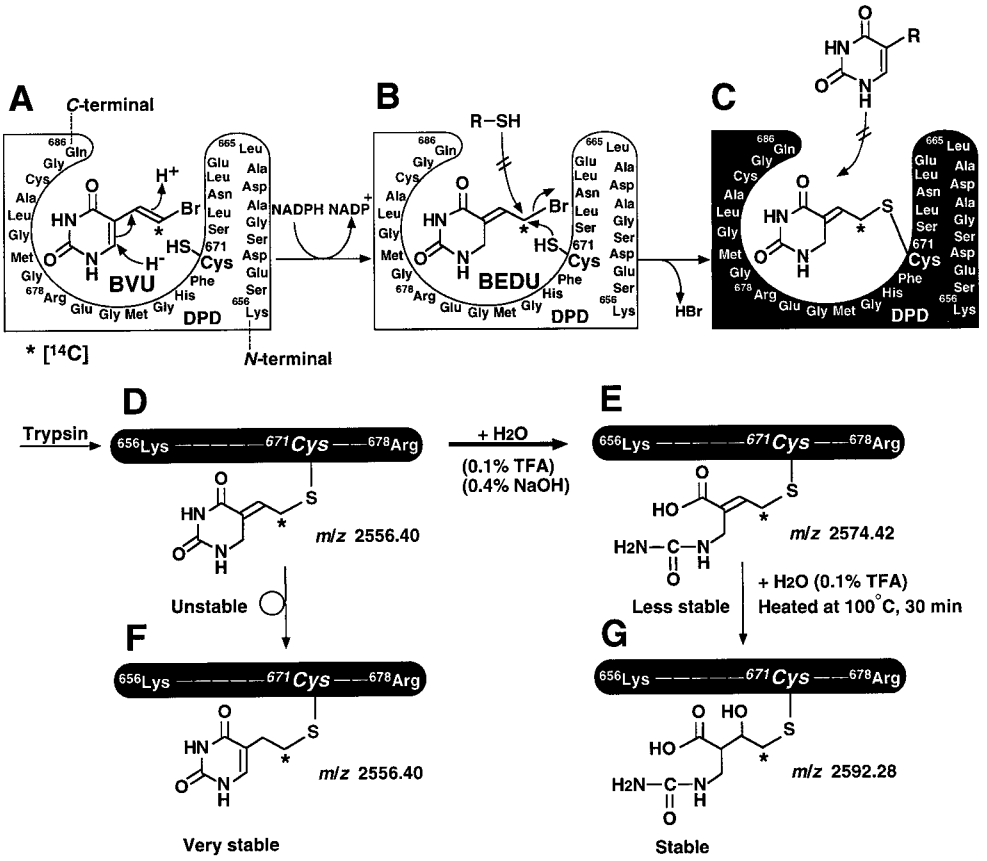

Fig. 6. A proposed mechanism for the mechanism-based inactivation of hDPD by [^{14}C]BVU as a suicide inhibitor in the presence of NADPH and characterization of radioactive tryptic peptide I from radiolabeled hDPD by MALDI-TOF MS analysis. A, [^{14}C]BVU interacts with the pyrimidine-binding domain of hDPD and is reduced with NADPH. B, by enzymatic reduction, [^{14}C]BVU is transformed to the allyl bromide type of reactive metabolite, BEDU, which instantly reacts with the sulfhydryl group of the Cys^{671} residue located in the pyrimidine-binding domain to form a sulfide with loss of HBr. Various thiol compounds (R-SH) added to the incubation mixture do not retard the inactivation and radiolabeling of hDPD by [^{14}C]BVU. C, the sulfide formed blocks the interaction of pyrimidine substrates, including 5-FU, with hDPD through its pyrimidine-binding domain. D, radioactive peptide I isolated by HPLC from a tryptic digest of radiolabeled hDPD bears an unstable debrominated BEDU moiety as a major component. E, the debrominated BEDU moiety of peptide I is slowly hydrolyzed during isolation by HPLC in 0.1% (v/v) TFA/aqueous acetonitrile. Hydrolysis of the dihydropyrimidine ring proceeds rapidly by heating the acidic HPLC eluate containing peptide I and instantly by the treatment of peptide I with NaOH at room temperature. F, peptide I isolated by HPLC also contained a very stable adduct as a minor component that is resistant to acid and alkali treatments. G, by prolonging the heating of peptide I in the acidic HPLC eluate, the ring-hydrolyzed adduct with an α,β-unsaturated moiety is hydrated with 1 mol of water.

ular mass of the sulfide formed by BEDU and the peptide Lys^{656}-Arg^{678}. The minor $[M+H]^+$ signal 2 in the spectrum of peptide I should be assigned as a hydrated molecule of peptide I bearing the debrominated BEDU whose 5,6-dihydrouracil ring is highly strained by the adjacent two *sp*2 carbons, a carbonyl group and an exocyclic double bond at the 4- and 5-positions, respectively (Fig. 6). Hydrated peptide I was likely to be formed at a slow rate during its separation by HPLC, requiring a long time, at least 30 h under acidic conditions, with 0.1% (v/v) TFA in aqueous acetonitrile used as an eluant. Radioactivity measurement of all the chromatographic fractions took 14 h for each step of HPLC. Intensity of $[M+H]^+$ signal 2 in the mass spectrum was increased slowly when the HPLC eluate containing peptide I was left to stand at room temperature (data not shown). The increase in intensity of signal 2 was accelerated by briefly (10 min) heating the HPLC eluate containing peptide I (Fig. 4B).

We expected that the hydrolytic ring opening of the debrominated BEDU in peptide I could be rapidly and completely accomplished by treatment of peptide I with NaOH rather than by acidic treatment. As expected, minor signal 2 rapidly became the major signal with a concomitant change of major signal 1 to the minor signal in the mass spectrum recorded after a brief (5 min) alkali treatment of peptide I at room temperature (Fig. 4D). However, signal 1 still appeared as an unchanged minor signal in the spectrum even after the alkali treatment was prolonged up to 60 min. Therefore, the remaining minor signal 1 was attributed to an alkali-resistant peptide, probably bearing a uracil ring (debrominated BEU) formed at a minor ratio as a thermodynamically stable isomer by the rearrangement of the exocyclic double bond into the dihydropyrimidine ring of the debrominated BEDU residue (Fig. 6).

By prolonging the heating of the acidic eluate containing peptide I, evidence was obtained that the ring-opening product had an α,β-unsaturated carboxylic acid moiety. Heat treatment gave a new mass spectral signal 3 corresponding in mass number to signal 2 plus 1 mol of water (Fig. 4C). The addition of a water molecule to the less stable ring-opening product is likely to be characteristic of the α,β-unsaturated carboxylic acid moiety of the sulfide, as has been reported for crotonic acid whose double bond has been demonstrated to be readily hydrated on heating in diluted mineral acids (Pressman and Lucas, 1939) and probably in aqueous TFA with strong acidity comparable to hydrochloric acid.

Thus, this study provides molecular evidence for the mechanism-based inactivation of hDPD by BVU derived from SRV, which caused many acute deaths in patients who were receiving 5-FU prodrugs. The patients were most likely to become extremely poor 5-FU metabolizers by the inactivation of hepatic DPD with BVU. Based on the occurrence of the patient deaths, this study teaches us that administration of 5-FU or its prodrugs must be avoided in patients whose DPD activity is genetically very low or deficient, as noted by Tuchman et al. (1985) and Diasio et al. (1988); otherwise patients will suffer from severe toxic symptoms or die due to extremely high tissue levels of unmetabolized 5-FU.

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