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5-Fluorouracil acetic acid/β-cyclodextrin conjugates: Drug release behavior in enzymatic and rat cecal media

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ABSTRACT

5-Fluorouracil-1-acetic acid (5-FUA) was prepared and covalently conjugated to β-cyclodextrin (β-CyD) through ester or amide linkage, and the drug release behavior of the conjugates in enzymatic solutions and rat cecal contents were investigated. The 5-FUA/β-CyD ester conjugate was slowly hydrolyzed to 5-FUA in aqueous solutions (half lives ($t_{1/2}$) = 38 and 17 h at pH 6.8 and 7.4, respectively, at 37 °C), whereas the amide conjugate was hardly hydrolyzed at these physiological conditions, but hydrolyzed only in strong alkaline solutions (>0.1 M NaOH) at 60 °C. Both ester and amide conjugates were degraded in solutions of a sugar-degrading enzyme, α-amylase, to 5-FUA/maltose and triose conjugates, but the release of 5-FUA was only slight in α-amylase solutions. In solutions of an ester-hydrolyzing enzyme, carboxylic esterase, the ester conjugate was hydrolyzed to 5-FUA at the same rate as that in the absence of the enzyme, whereas the amide conjugate was not hydrolyzed by the enzyme. On the other hand, 5-FUA was rapidly released when the ester conjugate was firstly hydrolyzed by α-amylase, followed secondly by carboxylic esterase. The results indicated that the ester conjugate was hydrolyzed to 5-FUA in a consecutive manner, i.e. it was firstly hydrolyzed to the small saccharide conjugates, such as the maltose conjugate, by α-amylase, and the resulting small saccharide conjugates having less steric hindrance was susceptible to the action of carboxylic esterase, giving 5-FUA. The *in vitro* release behavior of the ester conjugate was clearly reflected in the hydrolysis in rat cecal contents and in the *in vivo* release after oral administration to rats.

1. Introduction

Several colon-specific delivery methods have been developed: coating with biodegradable polymers (He et al., 2008; Saffran et al., 1986; Tozaki et al., 1999), coating with pH-sensitive polymers (Ibekwe et al., 2006; Klein et al., 2005), gastrointestinal pressure-controlled release (Jeong et al., 2001), and enzyme-based systems (Friend and Chang, 1984, 1985; MacLoad et al., 1993; Tozer et al., 1991; Xi et al., 2005). In the enzyme-based prodrug approach, Friend and Chang demonstrated the colon-targeting of steroid using glycoside prodrugs, where the drugs are liberated from their 21-β-glucosides after cleavage by bacterial glycosidase in rat colonic microflora (Friend, 2005; Friend and Chang, 1984, 1985).

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6–8 glucose units through α-1, 4-glycosidic bonds and have been utilized for improvement of certain properties of drugs such as solubility, stability and bioavailability, etc., through the formation of inclusion complexes (Stella and Rajewski, 1997; Uekama et al., 1998). CyDs are known to be barely hydrolyzed and only slightly absorbed in passage through the stomach and small intestine. However, they are fermented into small saccharides by colonic microflora (Antenucci and Palmer, 1984; Flourie et al., 1993; Gerloczy et al., 1985). Further, CyDs are rapidly excreted in an intact form into the urine after intravenous administration: more than 95% of α- and β-CyDs and 70% of γ-CyD were recovered within 6 h in the rat urine (Creminon et al., 1999; Frijlink et al., 1990; Yamamoto et al., 1991). These biodegradable properties of CyDs are useful as a colon-targeting carrier, and thus CyD prodrugs may serve as a source of site-specific delivery of drugs to the colon. In our previous studies, we prepared the colon-specific delivery prodrugs that anti-inflammatory drugs, biphenylyl acetic acid, prednisolone and ketoprofen, were introduced at one of the primary or secondary hydroxyl groups of CyDs (Hirayama et al., 1996; Kamada et al., 2002; Minami et al., 1998; Uekama et al., 1997; Yano et al., 2001a,b, 2002). These drug-CyD conjugates were negligibly absorbed in passage through the stomach and small intestine and reached the

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cecum and colon in an intact form, and released drugs after enzymatic actions of microflora existing in these lower intestinal tracts. In the present study, to expand the application of the prodrug of drug-CyD conjugates to anti-cancer drug, we newly synthesized 5-FU-appended β-CyD conjugates through ester or amide bond, and investigated the drug release behavior of the conjugates to evaluate as colon-specific delivery prodrugs for anti-cancer drug.

2. Materials and methods

2.1. Materials

β-CyD was donated from Nihon Shokuhin Kako (Tokyo, Japan). 5-Fluorouracil (5-FU) was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-Hydroxysuccinimide (HONSu), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and carbonyldiimidazole (CDI) were obtained from Nacalai Tesque (Kyoto, Japan). α-Amylase from *Aspergillus oryzae* (EC 3.2.1.1) and carboxylic esterase from *Porcine liver* (EC 3.1.1.1) were purchased from Sigma (St. Louis, MO). Mono-6-deoxy-6-amino-β-CyD (amino-β-CyD) was prepared according to the method reported (Bellanger and Perly, 1992). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

2.2. Preparation of 5-fluorouracil-1-acetic acid (5-FUA)

5-FUA was prepared according to the method reported previously (Tada, 1975). Briefly, 5-FU (1.3 g) was dissolved in water (11.5 mL) containing potassium hydroxide (KOH, 1.31 g), α-chloroacetic acid (950 mg) was added to the solution, and the mixture was stirred at 100 °C for 2 h, keeping pH of the solution 10 by KOH solution. After the reaction solution was cooled to room temperature (r.t.), hydrogen chloride (HCl) was added to adjust pH to 2, and the resulting precipitate was filtrated. Obtained powder was re-dissolved in a saturated potassium hydrogen carbonate solution, the solution was acidified to pH 2 by hydrogen chloride, and the resulting precipitate was filtrated. R_f = 0.54 (TLC: silica gel $60F_{254}$ (Merck, Darmstadt, Germany); ethyl acetate–water (100:2, v/v)); fast-atom bombardment (FAB) mass (*m*/*z*): $[M-H]^-$ 188; ^1H NMR (500 MHz, D_2O): δ (ppm) 4.63–4.87 (methylene), 7.81–7.84 (6-H).

2.3. Preparation of 5-FUA/β-CyD amide conjugate

5-FUA/β-CyD amide conjugate was prepared according to the method reported previously (Ouchi et al., 1991). Briefly, 5-FUA (0.189 g) and HONSu (0.115 g) were dissolved in DMSO, DCC (0.206 g) was added to the solution on ice, and the mixture was stirred at r.t. for 24 h. After filtration of the reaction solution, amino-β-CyD was added to the filtrate and the mixture was stirred at 50 °C for 5 days. Acetone (about 300 mL) was added to the reaction solution, and the resulting precipitate was filtrated. 5-FUA/β-CyD amide conjugate was purified by column chromatography (DIAION® HP-20), eluting with methanol-water solution with increasing methanol contents. The elutes were monitored by TLC, and the conjugate appeared in the elutes of 30–50% methanol. After methanol was removed under reduced pressure, the solution was lyophilized to obtain 5-FUA/β-CyD amide conjugate: R_f = 0.36 (TLC: silica gel $60F_{254}$ (Merck, Darmstadt, Germany); ethyl acetate-2-propanol-ammonium hydroxide-water (7:7:5:4, v/v); indicator *p*-anisaldehyde); FAB Mass (*m*/*z*): $[M-H]^-$ 1302; ^1H NMR (500 MHz, D_2O): δ (ppm) 7.57–7.59 (5-FUA, 6-H), 5.11 (CyD, 1′-H), 5.09–5.10 (CyD, 1-H), 4.40–4.51 (5-FUA, methylene), 3.60–4.01 (CyD, 2–6-H), 3.12-3.38 (CyD, 6′-H); ^{13}C NMR (126 MHz, D_2O): δ (ppm) 171.0 (5-FUA, 1-C), 130.4–151.2 (5-FUA, 3–6-C), 102.7–102.9 (CyD, 1-C), 82.2–84.0 (CyD, 4-C), 61.3–82.1 (CyD, 2–5-C), 61.2 (CyD, 6-C), 52.6 (CyD, 6′-C), 41.2 (5-FUA, 2-C).

2.4. Preparation of 5-FUA/β-CyD ester conjugate

5-FUA/β-CyD ester conjugate was prepared according to the method reported previously (Yano et al., 2001b). Briefly, 5-FUA (0.189 g) was dissolved in DMSO, CDI (0.486 g) was added to the solution, and then the mixture was stirred at r.t. for 3 h. After the addition of β-CyD (1.135 g) in DMSO (10 mL)/triethylamine (TEA, 12 mL), the mixture was stirred at room temperature for 48 h. Acetone (about 300 mL) was added, and the resulting precipitate was filtrated. 5-FUA/β-CyD ester conjugate was purified by column chromatography (DIAION® HP-20), under the same conditions described above. R_f = 0.39 (TLC: silica gel $60F_{254}$ (Merck, Darmstadt, Germany); ethyl acetate–2-propanol–ammonium hydroxide–water (7:7:5:4, v/v); indicator *p*-anisaldehyde); FAB mass (*m*/*z*): $[M-H]^-$ 1303; ^1H NMR (500 MHz, D_2O): δ (ppm) 7.89–7.98 (5-FUA, 6-H), 5.10 (CyD, 1-H), 5.04–5.05 (CyD, 1′-H), 4.42–4.45 (5-FUA, methylene), 4.11–4.32 (CyD, 5-H), 3.99–4.00 (CyD, 3-H), 3.66–3.98 (CyD, 2∼6-H), 3.60–3.63 (CyD, 4-H); ^{13}C NMR (126 MHz, D_2O): δ (ppm) 169.8 (5-FUA, 1-C), 131.9–151.6 (5-FUA, 3–6-C), 102.6–102.8 (CyD, 1-C), 82.2–82.4 (CyD, 4-C), 82.1 (CyD, 4′-C), 72.3–74.1 (CyD, 2, 3, 5-C), 61.0–61.2 (CyD, 6-C), 50.7 (CyD, 6′-C), 41.3 (5-FUA, 2-C).

2.5. Hydrolysis of 5-FUA/β-CyD conjugates

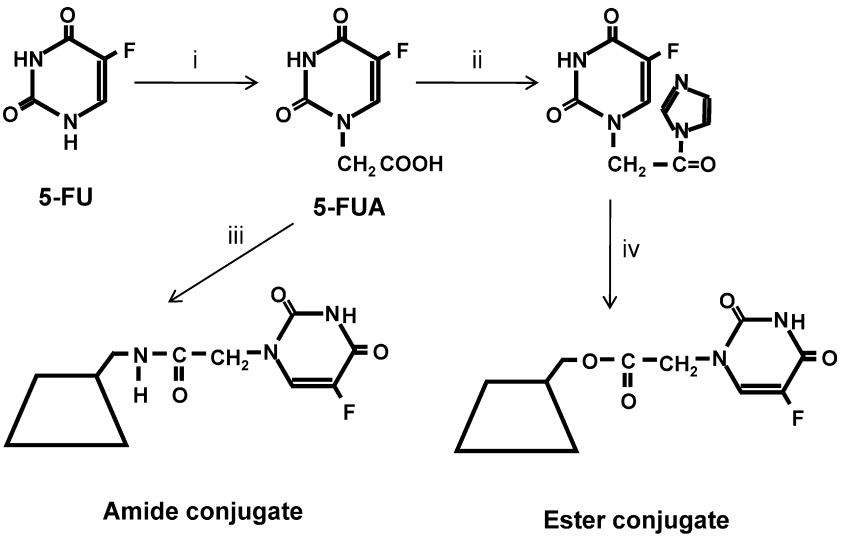
5-FUA/β-CyD amide conjugate solution in DMF was added to sodium hydroxide (NaOH) solutions of various concentrations at 60 °C. The final concentrations of the conjugate and DMF were 4.0×10^{-5} M and 1.0%, respectively. At appropriate intervals, 200 μL of the reaction solutions were collected and neutralized by HCl (100 μL), 60 μL of which was subjected to HPLC analysis of the amide conjugate and 5-FUA under the following conditions: YMC A303 ODS column (5 μm, 4.6 × 250 mm, Kyoto, Japan), a flow rate of 0.5 mL/min, a mobile phase of water/methanol (4/1, v/v), a detection at 267 nm. In the case of the ester conjugate, the hydrolysis was conducted in sodium phosphate buffer (I = 0.2) at 37 °C, and at appropriate intervals, 200 μL of the reaction solutions were collected, 60 μL of the aliquot was subjected to HPLC analysis of the ester conjugate and 5-FUA under the same HPLC conditions.

2.6. Hydrolyses of 5-FUA/β-CyD conjugates in enzymatic solution and rat colonic contents

Hydrolysis in α-amylase solutions: the conjugates in DMF were added to 0.01 M $CaCl_2$/0.2 M acetate buffer (pH 5.5) containing α-amylase (*A. oryzae*, 40 units/mL) at 37 °C. The final concentrations of the conjugates and DMF were 4.0×10^{-5} M and 1.0%, respectively. At appropriate intervals, 200 μL of the reaction solutions were collected, 60 μL of the aliquot was subjected to HPLC analysis of the conjugates and 5-FUA under the HPLC conditions described above.

Hydrolysis in carboxylic esterase solutions: the conjugates in DMF were added to *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES)–NaOH buffer (pH 7.4) containing carboxylic esterase (*Porcine liver*, 39 units/mL) at 37 °C. The final concentrations of the conjugates and DMF were 4.0×10^{-5} M and 1.0%, respectively. The conjugates and 5-FUA were analyzed under the same HPLC conditions.

Hydrolysis in rat cecal contents: the hydrolysis in cecal fluids of male Donryu rats (120 g, Japan SLC, Shizuoka, Japan) was carried out at 37 °C according to the method reported previously (Hirayama et al., 2000; Uekama et al., 1997), i.e. concentrations of the conjugates (4.0×10^{-5} M), DMF (1.0%) and cecum contents (10%, w/v, pH 6.8). At appropriate intervals, 5-FUA was extracted from the



i. α-chloroacetic acid in aq. KOH at 100°C, 2 h;
ii. CDI in DMSO at r.t., 3 h;
iii. Amino-β-CyD, DCC and HONSu in DMSO at 50°C, 5 days;
iv. β-CyD in DMSO, TEA, at r.t., 48 h.

Fig. 1. Preparation of 5-FUA/β-CyD ester and amide conjugates. (i) α-Chloroacetic acid in aq. KOH at 100 °C, 2 h. (ii) CDI in DMSO at r.t., 3 h. (iii) Amino-β-CyD, DCC and HONSu in DMSO at 50 °C, 5 days. (iv) β-CyD in DMSO, TEA, at r.t., 48 h.

reaction solution (100 μL) by ethyl acetate (5 mL) containing an internal standard (5-bromouracil, 10 μg/mL), after addition of 0.1N phosphoric acid (100 μL). The organic phase (4 mL) was evaporated under reduced pressure and the residue was dissolved in 100 μL of mobile phase (water/methanol = 4/1, v/v), 60 μL of the aliquot was subjected to HPLC analysis of 5-FUA under the same conditions described above.

2.7. In vivo release study of 5-FUA/β-CyD ester conjugate after oral administration to rats

Male Donryu rats (120 g) were fasted for 16 h prior to drug administration. 5-FUA/β-CyD ester conjugate (equivalent to 25 mg/kg 5-FUA) was dissolved in saline, and orally administered to rats. The concentrations of 5-FUA in the small and large intestines were also determined by HPLC within 24 h after oral administration of the ester conjugate, according to the method reported previously (Minami et al., 1998).

3. Results and discussion

3.1. Preparations of 5-FUA/β-CyD amide and ester conjugates

5-FUA/β-CyD amide and ester conjugates (Fig. 1) were prepared according to the methods reported previously (Ouchi et al., 1991; Tada, 1975; Yano et al., 2001b). 5-FU was carboxymethylated using α-chloroacetic acid. The amide conjugate was synthesized using OHNSu and DCC, while the ester conjugate was prepared by the direct coupling of the carboxylic acid of 5-FUA with a hydroxyl group of β-CyD using CDI. It is confirmed from ^{13}C NMR and mass spectroscopic data that one 5-FUA molecule was introduced into one of the primary hydroxyl groups of β-CyD through amide or ester bond, because the mass spectra gave the parent ion peaks corresponding to molecular weights of the conjugates and the ^{13}C NMR signal of the primary hydroxyl groups of β-CyD was split into two signals by the conjugation (Yano et al., 2001b).

3.2. Release behavior of 5-FUA/β-CyD conjugates in aqueous solutions

The colon-targeting prodrugs have to survive passage through stomach and small intestine, to reach colon as an intact form, and to be degraded by enzyme of colonic microflora. The physiological factors which affect on drug release from prodrugs are pH, secretion of esterase and bile acids, intestinal contents and microflora. Therefore, we investigated the effects of pH, enzyme and cecal contents on drug release behavior from the conjugates. Fig. 2 shows hydrolysis rate–pH profiles of the amide and ester conjugates in aqueous solutions. Both conjugates degraded according to the first order kinetics, giving quantitatively 5-FUA, under the conditions of Fig. 2. 5-FUA/β-CyD amide conjugate was markedly stable at physiological conditions such as pH 6–7 and 37 °C, but hydrolyzed in strong alkaline solutions at higher temperatures. The hydrolysis rate showed a first-order dependence on hydroxide concentration (Fig. 2A). On the other hand, the ester conjugate was hydrolyzed to 5-FUA at moderate rates at 37 °C. As shown in Fig. 2B, the ester conjugate was hydrolyzed at a constant rate at pH 1–5, whereas the rate increased with pH of the solutions above pH 6, the slope of the pH profile being 0.8. These results indicate that a water-catalysis and a specific base-catalysis are involved in the hydrolysis of the ester conjugate in acidic and alkaline solutions, respectively. According to the rate-pH profiles (Fig. 2B), the half-lives of the ester conjugate at pH 1.2, 6.8 and 7.4 were estimated to be about 500, 38 and 17 h, respectively. These results suggest that the ester conjugate is not significantly subject to the chemical degradation until it reaches to the colon, because it takes 1–5, 4–5 and 7–15 h for drug to pass stomach, small intestine and large intestine, respectively, after oral administration in human.

3.3. Release behavior of 5-FUA/β-CyD conjugates in enzyme and rat cecal media

The glycosidic bonds of CyDs are known to be subject to the hydrolysis by some sugar-degrading enzymes such as Taka-

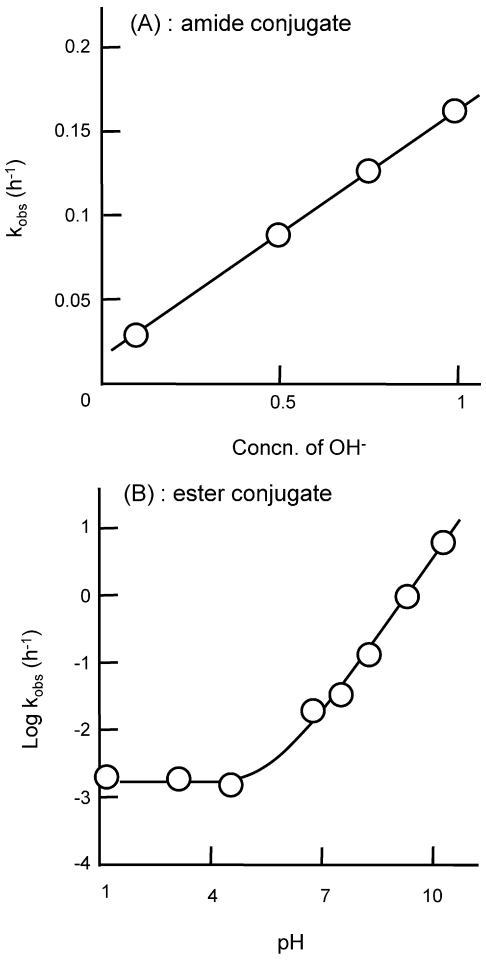


Fig. 2. Hydrolyses of 5-FUA/β-CyD amide (A) and ester (B) conjugates. (A) First-order dependence of hydrolysis rate constants of 5-FUA/β-CyD amide conjugate (4.0×10^{-5} M) on hydroxide ion concentrations at 60 °C. (B) pH-profile for hydrolysis rate constants of 5-FUA/β-CyD ester conjugate (4.0×10^{-5} M) at 37 °C. Each point represents the mean ± S.E. of 3 experiments.

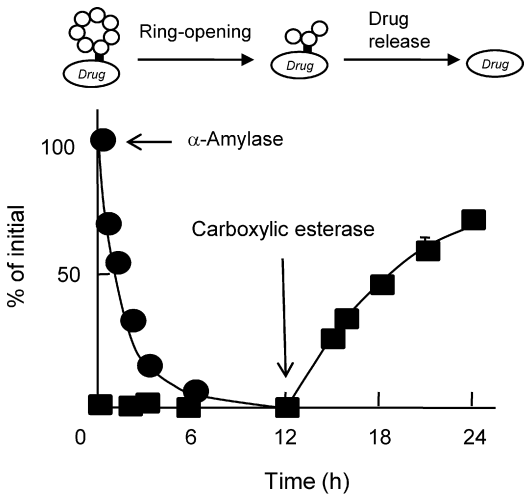


Fig. 4. Time courses of disappearance (●) of 5-FUA/β-CyD ester conjugate and appearance (■) of 5-FUA in α-amylase (pH 5.5)/carboxylic esterase (pH 7.4) solutions at 37 °C. The esterase (39 units/mL) was added 12 h after the α-amylase (40 units/mL) hydrolysis.

amylase and α-amylase (Fujita et al., 1987). α-Amylase (from *A. oryzae*) was used as a model enzyme for sugar-degrading enzymes in this study. In the presence of α-amylase, the amounts of 5-FUA/β-CyD amide and ester conjugates decreased to about 30% and 20% (Fig. 3A and B), respectively, 4 h after the reaction. However, no production of 5-FUA was observed under the α-amylase-catalyzed hydrolysis conditions. The α-amylase-catalyzed hydrolysis rate was similar between the amide and ester conjugates, suggesting that both conjugates are hydrolyzed in a same way, i.e. the ring-opening reaction of glycosidic bonds of the β-CyD giving 5-FUA/linear saccharide conjugates. In fact, several new peaks corresponding to the small saccharide conjugates were observed in HPLC chromatograms of the α-amlyase-treated solutions. Fig. 3C shows the time courses of disappearance of the ester conjugate in the absence and presence of carboxylic esterase. The ester conjugate was quantitatively hydrolyzed to 5-FUA according to a first order kinetics, whereas the amide conjugate was not hydrolyzed under the experimental conditions. The hydrolysis rate of the ester conjugate in the presence of carboxylic esterase was the same as that in the absence of the enzyme, indicating that the ester linkage of the β-CyD conjugate is less susceptible to the esterase hydrolysis than the linear saccharide conjugates, probably due to a steric hindrance of the large β-CyD ring.

Fig. 4 shows the hydrolysis behavior of the ester conjugate when the carboxylic esterase was added 12 h after the α-amylase-catalyzed hydrolysis. The α-amylase hydrolyzed the ester conjugate to the small saccharide conjugates, during which no release of 5-FUA was observed. However, 5-FUA was rapidly released when the carboxylic esterase was added after the α-amylase hydrolysis. These results supported the conclusion described above, i.e. the ester linkage of the β-CyD conjugate is less susceptible to esterase hydrolysis, but that of the small saccharide conjugates, which were produced after α-amylase hydrolysis,

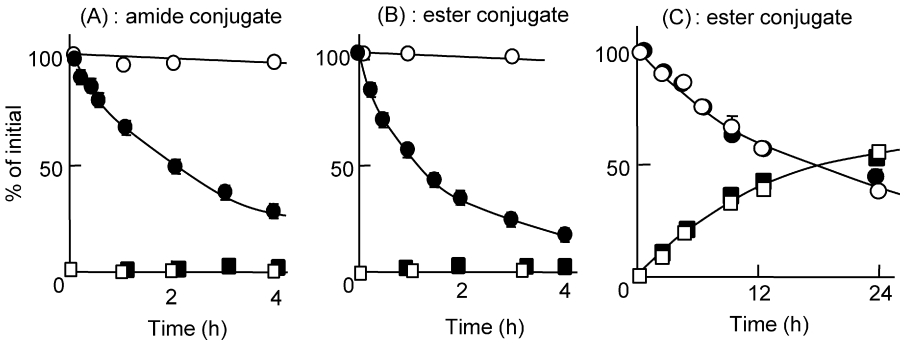


Fig. 3. Time courses of disappearances (○, ●) of 5-FUA/β-CyD amide (A) and ester (B) conjugates (4.0×10^{-5} M) and appearances (□, ■) of 5-FUA in the absence (○, □) and the presence (●, ■) of *Aspergillus oryzae* α-amylase (40 units/mL) in acetate buffer (pH 5.5) at 37 °C. (C) Time courses of disappearances (○, ●) of 5-FUA/β-CyD ester conjugate (4.0×10^{-5} M) and appearances (□, ■) of 5-FUA in the absence (○, □) and the presence (●, ■) of carboxylic esterase (39 units/mL, pH 7.4) at 37 °C. Each point represents the mean ± S.E. of 3–6 experiments.

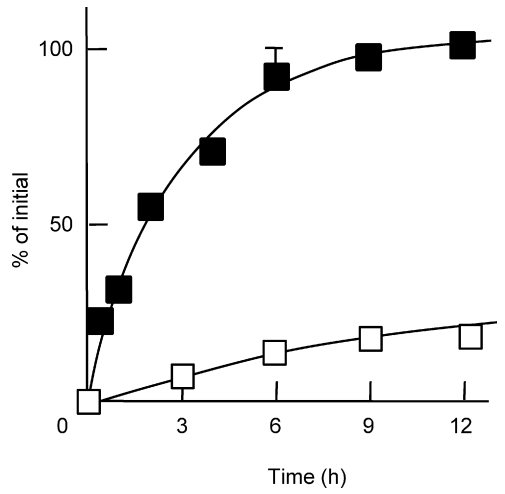


Fig. 5. Release profiles of 5-FUA from 5-FUA/β-CyD ester conjugate in the absence (□) and presence (■) of rat cecal contents (10%, w/v) in phosphate buffer (pH 6.8) at 37 °C.

is easily cleaved by the esterase, probably because of a relief of steric hindrance of the large β-CyD. The quantitative analysis of the carboxylic esterase-catalyzed hydrolysis of the small saccharide conjugates was not carried out because these conjugates were a mixture of maltose and triose conjugates. These kinetic studies suggest that two enzymes, sugar-degrading and ester-hydrolyzing, together with the spontaneous hydrolysis, are required for the release of 5-FUA from its β-CyD ester conjugate.

Fig. 5 shows the release behavior of 5-FUA from the ester conjugate in suspensions of rat cecal contents, in comparison with that in the absence of the contents. The release rate of 5-FUA in the contents was faster than that without the contents, and 5-FUA was almost completely released from the ester conjugate after 9 h in the medium of rat cecal contents, probably due to the enzymatic degradation by sugar-degrading and ester-hydrolyzing enzymes. These results suggest that 5-FUA/β-CyD ester conjugate releases 5-FUA in the large intestine after oral administration.

3.4. Release of 5-FUA/β-CyD ester conjugate after oral administration to rats

To confirm the *in vitro* release behavior of the conjugate qualitatively by *in-vivo* releases, we determined 5-FUA levels in small and large intestines, after oral administrations of the ester conjugate to rats. Fig. 6 shows the amounts of 5-FUA in small and large intestines 0.5, 6 and 24 h after the administration of the ester conjugate (equivalent to 25 mg/kg 5-FUA) to rats. The 5-FUA concentrations in the small and large intestines were low at 0.5 and 6 h after the administration. In contrast, the 5-FUA concentration was significantly increased in the large intestine at 24 h after the administration. The small amounts of 5-FUA in the small intestine at 6 and 24 h may be produced from the hydrolysis by intestinal microflora, because bacteria are present in relatively large numbers in the stomach and small intestine of rats, compared with those of humans and guinea pigs (Friend, 1992). Further, the concentration of the drug in the small intestine may be slightly overestimated, because it contains the amounts in both of the contents and membranes (Minami et al., 1998). These results suggested that the conjugate released 5-FUA predominantly in the large intestine, although the release rate was slow. The *in vitro* drug release behavior of 5-FUA/β-CyD ester conjugate was reflected in the *in vivo* release after oral administration to rats.

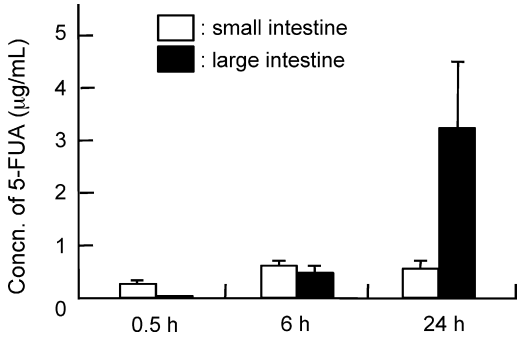


Fig. 6. Drug release behavior of 5-FUA/β-CyD ester conjugate after oral administration to rats. Concentrations of 5-FUA in contents of small and large intestines 0.5, 6 and 24 h after oral administration of 5-FUA/β-CyD ester conjugate (equivalent to 25 mg/kg 5-FUA) to rats. Each value represents the mean ± S.E. of 3 experiments.

In conclusion, 5-FUA/β-CyD ester conjugate may survive passing through stomach and small intestine and release 5-FUA preferentially in cecal and large intestinal tracts of rats after the fermentation of β-CyD to small oligosaccharides. The results indicate that CyDs can work as a pro-moiety for colon-specific targeting prodrugs.

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