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Structural effects of *N*-aromatic acyl-amino acid conjugates on their deconjugation in the cecal contents of rats: implication in design of a colon-specific prodrug with controlled conversion rate at the target site

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ABSTRACT: *N*-aromatic acyl-amino acid conjugates possess a colon-targeted property, implying that such conjugates are stable and are not absorbable until reaching the large intestine in which they are microbially converted (hydrolysed) to the parent drugs that are therapeutically active. To investigate the structural effect of *N*-aromatic acyl-amino acid conjugates on the large intestinal deconjugation, the hydrolysis of various *N*-aromatic acyl-amino acid conjugates was examined in the cecal contents. On incubation of conjugates with glycine, D or/and L forms of alanine or phenylalanine in the cecal contents, the conjugates with D amino acids were not hydrolysed. The other conjugates are susceptible to the hydrolysis, the rates of which decreased as the size of the substituent on the 2-position of the amino acids increased. The conjugates with alkyl analogs (2–4 carbons) of glycine and taurine were resistant to the hydrolysis, while taurine- and glycine-conjugates were hydrolysed effectively. The hydrolysis of *N*-aromatic acyl-glycine conjugates was enhanced by para-substitution of electron withdrawing groups on the aromatic acyl moiety and vice versa for electron-donating groups. While a methyl, methoxy or chloro group on the ortho-position retarded the hydrolysis, a hydroxyl group on the position accelerated it. Our data may provide useful information for the design of a colon-specific prodrug with controlled conversion rate in the large intestine. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: *N*-aromatic acyl-amino acid conjugates; colonic delivery; structural effect; colon specific prodrug; drug design

Introduction

Delivery of orally administered drugs specifically to the colon is highly desirable for several reasons. Firstly, for the efficient treatment of diseases developed locally at the colon such as ulcerative colitis, Crohn's disease, constipation or colorectal cancer. Being delivered specifically to the site of action with limited systemic absorption, only a small dose is needed, which subsequently results in reduced side effects. Secondly, the colon could be the preferential site of absorption for orally administered therapeutic peptides and proteins. Thirdly, delayed absorption by the colonic targeting may also be useful for the treatment of diseases susceptible to diurnal rhythm such as asthma, gastric ulcer or arthritis, which may have peak symptoms at bedtime, by adjusting the plasma concentration–time profile to the symptom [1–4].

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The development of a prodrug is a way to deliver drugs specifically to the colon. A colon-specific prodrug should be stable and non-absorbable in the upper intestine so that it could reach the colon in an intact form, and the linkage between drug and promoiety should dissociate to release the active drug in the colon for action [4]. In the prodrug approach, polymeric carriers have been used to prevent the absorption of prodrugs in the upper intestine [5–8]. There are cases in which water-soluble small molecules have been used as a colon-specific carrier [9–12]. After being delivered to the colon, the prodrug is presumed to be activated by the enzymes originating from the microbes which are especially abundant in that portion of the alimentary canal [13].

N-Aromatic acyl-amino acid conjugates are known to be stable in the upper intestine and to be dissociated by the microbial enzymes in the colon [14]. For this reason, amino acid can be used as a colon-specific promoiety for a drug with an aromatic carboxylic acid such as 5-aminosalicylic acid as reported by Jung *et al*. [15,16]. Lee *et al*. synthesized *N*-nicotinoyl-2-(5-fluorouracil-1-yl)-glycine (NFG) by substitution of 5-fluorouracil (with a nucleophile). The NFG was designed as a colon-specific prodrug of 5-fluorouracil because 2-(5-fluorouracil-1-yl)-glycine, which is formed after the cleavage of the amide bond in NFG in the cecal contents, decomposes spontaneously to release 5-fluorouracil [17]. Substitution of other drug with a nucleophile should produce a compound with a general structure of *N*-aromatic acyl-2-(drug)-glycine, which can be a colon-specific prodrug [18]. In this case, *N*-aromatic acyl-glycine acts as a colon-specific promoiety and bioactivation of the prodrug depends on the hydrolysis of the amide bond in the colon. The bioactivation processes and structures of the above prodrugs are shown in Figure 1A and B.

In the present study, the structural effects of the amino acids (or amino acid analogs) on the cecal deconjugation (amide hydrolysis) of *N*-benzoyl-amino acid conjugates were investigated. In addition, the effects of various substituents on the aromatic acyl moiety on the deconjugation

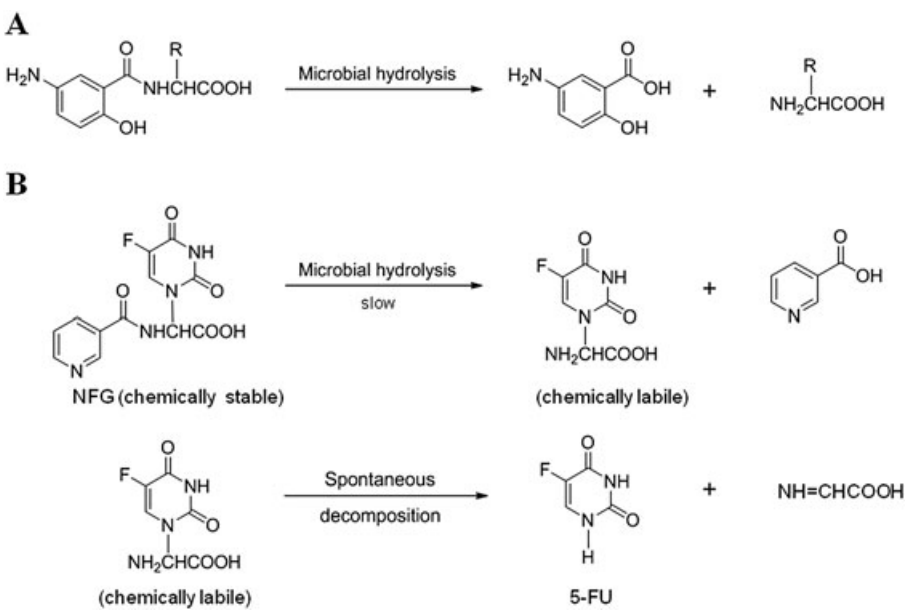

Figure 1. Bioactivation of *N*-aromatic acyl-amino acid conjugates in the large intestine. (A) Bioactivation of 5-aminosalicyloylamino acid conjugates in the large intestine. (B) Bioactivation of *N*-nicotinoyl-2-(5-fluorouracil-1-yl)-D,L-glycine (NFG) in the large intestine

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of *N*-aromatic acyl-glycine were scrutinized as well.

Materials and Methods

Materials and instruments

Benzoic acid, salicylic acid, benzoyl chloride, 4-methoxybenzoyl chloride, 2-methoxybenzoyl chloride, 2-chlorobenzoyl chloride, 4-chlorobenzoyl chloride, D-alanine, glycine, taurine (2-aminoethansulfonic acid), *N*-benzoyl-glycine, *N*-benzoyl-L-alanine, *N*-benzoyl-D,L-alanine, *N*-benzoyl-L-phenylalanine, *N*-benzoyl-D-phenylalanine, *N*-benzoyl-glycyl-D,L-phenylalanine, *N*-(4-nitrobenzoyl)-glycine, *N*-(2-hydroxybenzoyl)-glycine, aminomethansulfonic acid, 3-amino-1-propanesulfonic acid, *N*-(4-methylbenzoyl)-glycine, *N*-(2-methylbenzoyl)-glycine and *N*,*N*′-carbonyldiimidazole were purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA) and used as received. *N*-(4-Aminobenzoyl)-glycine, 4-amino-*n*-butyric acid, *N*-benzoyl-D,L-phenylalanine and *N*-benzoyl-β-alanine were purchased from Tokyo Kasei (Tokyo, Japan) and used as received. Solvents for NMR and HPLC were obtained from Merck Inc. (Darmstadt, Germany). All other chemicals were reagent grade, commercially available products. The IR spectra were recorded on a Bomem MB 100 FT-IR spectrophotometer (Bomem, Ontario, Canada). The ^{1}H-NMR spectra were taken on a Varian AS 500 spectrometer (Santa Clara, CA) and the chemical shifts are in ppm downfield from tetramethylsilane. Elemental analysis was carried out by an Elemental Analyzer System (Profile HV-3, Manchester, UK). The HPLC system consisted of Model 305, 306 pumps, a 117 variable UV detector, a Model 234 autoinjector, a Model 805 manometric module, and a Model 811C dynamic mixer from Gilson (Middleton, WI). Buffer solutions, hydrochloric acid buffer solution (pH 1.2), isotonic phosphate buffer (pH 6.8), 0.067M phosphate buffer (pH 4.5, pH 5.5) and 0.050M phosphate buffer (pH 6.0), were prepared as described in USP XXIII. The animal protocol used in this study has been reviewed and approved by the Pusan National University–Institutional Animal Care and Use Committee (PNU-IACUC) on their ethical procedures and scientific care. The cecal content of rats is used most frequently for *in vitro* experiments to examine activation of a colon specific prodrug because the animal has similar intestinal microflora composition to that of humans [19].

Preparation of N-aromatic acyl-amino acid conjugate

Compounds for structure activity relationship (SAR) study that are not commercially available were prepared in our laboratory.

1. Preparation of N-benzoyl-2-aminoethansulfonic acid. To the solution of 2-aminoethansulfonic acid (1.3g, 10.5mmol) in 10ml of 1 N-sodium hydroxide, benzoylchloride (1.4g, 10.0mmol) and 3 N-sodium hydroxide were added separately from a separatory funnel at such a rate that the solution was always slightly alkaline while stirring and cooling below 30°C. After the addition was completed, the mixture was stirred for 2h longer. It was then poured into 3ml of concentrated hydrochloric acid in a 100ml beaker. The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 251.3–253.8°C, IR (nujol)ν_{max}: 1193, 1051 (S=O, symmetric, asymmetric), 1640 (amide I: C=O), 1537 (amide II: N-H), 1307 (amide III: C-N), ^{1}H NMR (D_2O): δ 3.08 (t, 2H), 3.64 (t, 2H), 7.39 (t, 2H), 7.48 (t, 1H), 7.61 (d, 2H). Elemental analysis for $C_9H_{11}SO_4N$: Calculated: C; 47.12, H; 4.80, S; 13.99, N; 6.11. Found: C; 47.53, H; 4.62, S; 13.64, N; 5.81.

2. Preparation of N-benzoyl-aminomethanesulfonic acid. Aminomethanesulfonic acid (1.2g, 10.8mmol) was reacted with benzoyl chloride (1.4g, 10.0mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 260–305°C (dec.), IR (nujol)ν_{max}: 1187, 1047 (S=O, symmetric, asymmetric), 1649 (amide I: C=O), 1547 (amide II: N-H), 1297 (amide III: C-N), ^{1}H NMR (D_2O): δ 4.49 (s, 2H), 7.42 (t, 2H), 7.53 (t, 1H). 7.70 (d, 2H). Elemental analysis for $C_8H_9SO_4N$: Calculated: C; 44.60, H; 4.18, S; 14.90, N; 6.50. Found: C; 44.78, H; 4.53, S; 14.34, N; 6.87.

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3. Preparation of N-benzoyl-3-amino-1-propanesulfonic acid. 3-Amino-1-propanesulfonic acid (1.5 g, 10.7 mmol) was reacted with benzoyl chloride (1.4 g, 10.0 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 254.7–255.7°C, IR (nujol)ν_{max}: 1197, 1050 (S=O, symmetric, asymmetric), 1626 (amide I: C=O), 1543 (amide II: N-H), 1290 (amide III: C-N), ^{1}H NMR (D_2O): δ 1.95 (q, 2H), 2.88 (t, 2H), 3.37 (t, 2H), 7.39 (t, 2H), 67.48 (t, 1H), 7.60 (d, 2H). Elemental analysis for $C_{10}H_{13}SO_4N$: Calculated: C; 49.32, H; 5.34, S; 13.18, N; 5.75. Found: C; 49.57, H; 5.63, S; 13.64, N; 5.34.

4. Preparation of N-benzoyl-D-alanine. D-Alanine (0.95 g, 10.5 mmol) was reacted with benzoyl chloride (1.4 g, 10.0 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: >350°C (dec.); IR (nujol)ν_{max}: 1732 (carboxylic acid: C=O), 1604 (amide I: C=O), 1557 (amide II: N-H), 1288 (amide III: C-N), ^{1}H NMR (D_2O): δ 1.70 (d, 3H), 4.6 (m, 1H), 7.68 (t, 2H), 7.80 (t, 1H), 7.95 (d, 2H). Elemental analysis for $C_{10}H_{11}O_3N$: Calculated: C; 62.14, H; 5.70, N; 7.25. Found: C; 61.77, H; 5.98, N; 7.64.

5. Preparation of N-benzoyl-4-aminobutyric acid. 4-Aminobutyric acid (1.1 g, 10.9 mmol) was reacted with benzoyl chloride (1.4 g, 10.0 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 65.3–66.3°C, IR (nujol) ν_{max} 1691 (carboxylic acid: C=O), 1625 (amide I: C=O), 1529 (amide II: N-H), 1300 (amide III: C-N), ^{1}H NMR ($CDCl_3$): δ 1.92 (q, 2H, C-3), 2.43 (t, 2H, C-2), 3.47 (q, 2H, C-4), 7.36 (t/d, 2H), 7.45 (t/t, 2H), 7.76 (d/d, 2H). Elemental analysis for $C_{11}H_{13}O_3N$: Calculated: C; 63.69, H; 6.27, N; 6.76. Found: C; 63.57, H; 6.63, N; 6.34.

6. Preparation of N-(4-methoxybenzoyl)-glycine. Glycine (0.8 g, 10.5 mmol) was reacted with 4-methoxybenzoyl chloride (1.7 g, 10 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 166.4–170°C, IR (nujol)ν_{max} 1741 (carboxylic acid: C=O), 1609 (amide I: C=O), 1564 (amide II: N-H), 1263 (amide III: C-N), ^{1}H NMR (D_2O): δ 3.85 (s, 3H), 4.0 (d, 2H), 7.0 (d, 2H), 7.90 (d, 2H). Elemental analysis for $C_{10}H_{11}O_4N$: Calculated: C; 57.37, H; 5.26, N; 6.69. Found: C; 57.57, H; 5.23, N; 6.94.

7. Preparation of N-(2-methoxybenzoyl)-glycine. Glycine (0.8 g, 10.5 mmol) was reacted with 2-methoxybenzoyl chloride (1.7 g, 10 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 102.2–104.5°C, IR (nujol)ν_{max} 1693 (carboxylic acid: C=O), 1668 (amide I: C=O), 1599 (amide II: N-H), 1259 (amide III: C-N), ^{1}H NMR (DMSO-d6): δ 3.79 (5H), 4.1 (d, 2H), 6.98 (t, 1H), 7.10 (d, 1H), 7.49 (m, 1H), 7.61 (m, 1H). Elemental analysis for $C_{10}H_{11}O_4N$: Calculated: C; 57.41, H; 5.30, N; 6.70. Found: C; 56.98, H; 5.31, N; 6.88.

8. Preparation of N-(2-chlorobenzoyl)-glycine. Glycine (0.8 g, 10.5 mmol) was reacted with 2-chlorobenzoyl chloride (1.75 g, 10 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 186.9–188.2°C, IR (nujol)ν_{max} 1724 (carboxylic acid: C=O), 1626 (amide I: C=O), 1552 (amide II: N-H), 1228 (amide III: C-N), ^{1}H NMR (DMSO-d6): δ 3.88 (d, 2H), 7.38-7.49 (4H), 7.85 Elemental analysis for $C_9H_8O_3N$: Calculated: C; 50.60, H; 3.77, N; 6.56. Found: C; 51.20, H; 3.79, N; 6.61.

9. Preparation of N-(4-chlorobenzoyl)-glycine. Glycine (0.8 g, 10.5 mmol) was reacted with 4-chlorobenzoyl chloride (1.75 g, 10 mmol) according to the procedure described in '1'). The resulting precipitates were collected by suction filtration, washed with cold water, dried and recrystallized from ethanol–water. mp: 147.9–150.2°C, IR (nujol)ν_{max} 1745 (carboxylic acid: C=O), 1684 (amide I: C=O), 1560 (amide II: N-H), 1219 (amide III: C-N), ^{1}H NMR (DMSO-d6): δ 3.92 (d, 2H), 7.55 (m, 2H), 7.87 (m, 2H). Elemental

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analysis for $C_9H_8O_3N$: Calculated: C; 50.60, H; 3.77, N; 6.56. Found: C; 52.58, H; 3.57, N; 6.14.

TLC analysis

The TLCs were performed on Merck Kieselgel 60 F_{254} and spots were visualized by an UV monitor at 254 nm. The mobile phases consisted of chloroform/methanol in a ratio of either 70/30 or 50/50.

HPLC analysis

The concentration of *N*-aromatic acyl-amino acid conjugates, benzoic acid and salicylic acid was measured by a reverse-phase HPLC. Standard or blank solution (1 ml) was mixed on a vortex mixer for 2 min, centrifuged at 10000 rpm for 5 min and filtered through a membrane filter (0.45 μm). The filtrate (20 μl) was injected on a Lichrospher 100 RP-18 column (250 × 4.6 mm, 5 μm) from Merck and eluted with a mobile phase at a flow rate of 0.7 ml/min. The mobile phases consisted of various ratios of methanol/5 mM, pH 6.0 phosphate buffer solution, which were filtered through a 0.45 μm membrane filter before use. The eluate was monitored by measuring the absorption at 224 or 254 nm at a sensitivity of AUFS 0.01. The Gilson 712 software was employed for the data analysis. HPLC analytical data of the analytes are listed in Table 1.

Table 1. HPLC analytical data

No. of chemical (Retention time in min)	% Methanol in mobile phase (Methanol/5 mM, pH 6.0 PBS)
* **20** (22.2)	2.5
* **1** (14.6)	5
* **15** (12.3)[a]	10
* **17** (18.3)	12.5
* **14** (12.1), **22** (11.1), **3** (10.7)	15
* **2** (8.7), **4** (8.7), **5** (7.6), **11** (7.7) **16** (8.2), **21** (7.0), **19** (9.2)	20
* **10** (6.7)	30
* **8** (14.9), **9** (14.7)	gradient[b]
* **12** (5.76), **13** (12.05), **18** (11.09)	c
* **Salicylic acid** (12.5), **Benzoic acid** (15.1)	d

Flow rate: 0.7 ml/min, Column: Lichrospher 100 RP-18 column (250 × 4.6 mm, 5 μm).
[a]Mobile phase contains 5 mM tetrabutyl-ammonium chloride.
[b]0–4 min, 20%; 4–5 min, 20→50%; 5–12 min, 50%; 12–13 min, 50→20%; 13–25 min, 20%.
c, Acetonitrile/pH 4.5 PBS=15/85.
d, Acetic acid/methanol/pH 4.5 PBS=4/35/65.

Preparation of standard solutions in the rat cecal contents

A male Sprague-Dawley rat was anesthetized by diethyl ether and a midline incision was made. The cecal content obtained was suspended in the isotonic phosphate buffer (pH 6.8) solution to a concentration of 10 w/v% and filtered through medical gauze to remove the fibrous mass. To each 100 μl portion of the suspension, 20, 40, 60, 100 or 200 μl portion of each solution of either *N*-aromatic acyl-amino acid conjugates (100 μg/ml) or aromatic acids (benzoic acid and salicylic acid, 100 μg/ml) in isotonic phosphate buffer (pH 6.8) and an appropriate amount of methanol were added to make the final volume of 1 ml. The standard solutions in a concentration of 2.0, 4.0, 8.0, or 20.0 μg/ml were obtained by the above processes. A calibration curve was constructed from the concentrations of the standard solutions versus the peak areas on HPLC.

pH stability

The pH stability was determined by incubating test compounds (100 μg/ml) in either pH 1.2 hydrochloric acid buffer or 6.8 phosphate buffer for 24 h at 37°C. At a predetermined time interval, a 20 μl portion of the solution was removed to analyse the concentration of *N*-aromatic acyl-amino acid conjugates by HPLC as described previously.

Incubation of an N-aromatic acyl-amino acid conjugate with the rat cecal contents

A male Sprague-Dawley rat was anesthetized by diethyl ether and a midline incision was made, and the cecal content was collected in a glove box, which was previously displaced by nitrogen. To a 0.1 g portion of the cecal contents, each solution of 0.9 ml of *N*-aromatic acyl-amino acid conjugates in pH 6.8 isotonic phosphate buffer (100 μg /0.9 ml) was added and the mixture was incubated at 37°C. At appropriate time intervals, the samples were centrifuged at 5000 rpm for 3 min. To a 0.1 ml portion of the supernatants, 0.9 ml of methanol was added, vortexed for 2 min and centrifuged for 5 min at 10000 × *g*. The concentration of

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N-aromatic acyl-amino acid conjugates in a 20μl portion of the supernatants was determined by HPLC as described previously. In some experiments, benzoic acid and salicylic acid as the cecal metabolites of *N*-aromatic acyl-glycine conjugates were analysed by HPLC.

Results

Preparation of N-aromatic acyl-amino acid conjugates

Preparation of *N*-aromatic acyl-amino acid conjugates and *N*-aromatic acyl-aminoalkylsulfonic acid conjugates were prepared by the addition of acyl chloride to the solution of aqueous amino acid or aminoalkylsulfonic acid keeping the reaction mixture slightly alkaline throughout the reaction period. After acidification and collection of the resulting precipitates, the product was obtained in good yield. The structure of the product was confirmed from the data of IR and ^{1}H-NMR spectra and the results from the elemental analysis. Compounds used for the present study include *N*-benzoyl-glycine (**1**), *N*-benzoyl-L-alanine (**2**), *N*-benzoyl-D-alanine (**3**), *N*-benzoyl-D,L-alanine (**4**), *N*-benzoyl-β-alanine (**5**), *N*-benzoyl-L-phenylalanine (**6**), *N*-benzoyl-D-phenylalanine (**7**) *N*-benzoyl-D,L-phenylalanine (**8**), *N*-benzoyl-glycyl-D,L-phenylalanine (**9**), *N*-(4-methylbenzoyl)-glycine (**10**), *N*-(2-methylbenzoyl)-glycine (**11**), *N*-(2-chlorobenzoyl)-glycine (**12**), *N*-(4-chlorobenzoyl)-glycine (**13**), *N*-(2-hydroxybenzoyl) -glycine (**14**), *N*-(4-aminobenzoyl)-glycine (**15**), *N*-(4-nitrobenzoyl)-glycine (**16**), *N*-(4-methoxybenzoyl)-glycine (**17**), *N*-(2-methoxybenzoyl)-glycine (**18**), *N*-benzoyl-4-aminobutyric acid (**19**), *N*-benzoyl-aminomethanesulfonic acid (**20**), *N*-benzoyl-2-aminoethanesulfonic acid (**21**) and *N*-benzoyl-3 -aminopropanesulfonic acid (**22**). The structures of the above compounds are shown in Figure 2.

Chemical stability of N-aromatic acyl-amino acid conjugate

To investigate whether chemical hydrolysis of the *N*-aromatic acyl-amino acid conjugate takes place at the pHs of the various sections of the gastrointestinal tract, *N*-benzoyl-glycine, *N*-benzoyl-taurine, *N*-benzoyl-D,L-alanine, *N*-benzoyl-D,L-phenylalanine, *N*-(2-hydroxybenzoyl)-glycine, *N*-(4-nitrobenzoyl)-glycine or *N*-(4-aminobenzoyl)-glycine was incubated for 24h at 37°C in pH 1.2 hydrochloric acid buffer and pH 6.8 phosphate buffer solution. None of them was hydrolysed at these conditions, implying that the amount of hydrolysis product produced during the incubation with the rat cecal contents represents solely that of microbial origin, not from the chemical hydrolysis.

Incubation of N-aromatic acyl-amino acid conjugates with the rat cecal contents

N-Aromatic acyl-amino acid conjugates are known to be stable in the upper intestine and to be dissociated by the microbial enzymes in the colon [14]. Bioactivation (deconjugation) of colon-specific prodrugs that have the general structure occurs by the enzymatic hydrolysis of the amide bond in the large intestine [4].

To ensure whether the amide bonds in an *N*-benzoyl-amino acid conjugate and an *N*-aromatic acyl-glycine with a substituent on the aromatic ring could be hydrolysed in the cecal contents, either *N*-benzoylglycine or *N*-(2-hydroxybenzoyl)-glycine was incubated in the cecal contents and the hydrolysis of the conjugates and production of the aromatic acids were monitored. As shown in Figure 3A and B, the aromatic acids were produced as much as the conjugates disappeared. This result indicates that the amide bonds in *N*-aromatic acyl-amino acid conjugates should be hydrolysed in the cecal contents.

Effect of the amino acid moiety on the cecal hydrolysis of N-benzoyl-amino acid conjugates

Results from the incubation of *N*-benzoyl-amino acid conjugates with the cecal contents of rats are shown in Figure 4. It was observed that hydrolysis took place mostly with the L-amino acid conjugate. Hydrolysis hardly occurred when *N*-benzoyl-D-alanine or *N*-benzoyl-D-phenylalanine was incubated with the cecal contents of rats. It is seen from Figure 3 that the degree of hydrolysis reached approximately 90% for glycine, 52% for L-alanine, 37% for L-phenylalanine, 30% for D,L-alanine and 19% for D,L-phenylalanine at 8h after incubation. The degree of hydrolysis reached 94%, 88%, 85%, 45% and 47% for glycine, L-alanine,

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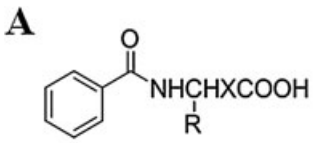
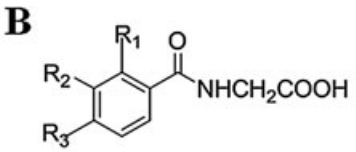
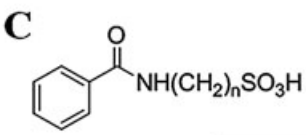


R	X	Compound No
H	H	(1)
CH_3	H	L (2), D (3), D,L (4)
CH_2Ph	H	L (6), D (7), D,L (8)
H	CH_2	(5)
H	$(CH_2)_2$	(19)
H	CONHCH(Ph)	(9)



R_1	R_2	R_3	Compound No
H	H	CH_3	(10)
CH_3	H	H	(11)
Cl	H	H	(12)
H	H	Cl	(13)
OH	H	H	(14)
H	H	NH_2	(15)
H	H	NO_2	(16)
H	H	CH_3O	(17)
CH_3O	H	H	(18)



n	Compound No
1	(20)
2	(21)
3	(22)

Figure 2. Structures of *N*-aromatic acyl-amino acid conjugates. (A) Structures of *N*-benzoyl-amino acid conjugates. (B) Structures of *N*-aromatic acyl glycine conjugates. (C) Structures of *N*-benzoyl-aminoalkylsulfonic acids

L-phenylalanine, D,L-alanine and D,L-phenylalanine, respectively at 24h. As the size of the substituent on the 2-position of amino acid increased from H to CH_3, $CH_2C_6H_5$, the rate of hydrolysis decreased, which appeared most prominently at 8h. To confirm this, the degree of hydrolysis of *N*-benzoyl-L-phenylalanine was compared with that of *N*-benzoyl-glycyl-D,L-phenylalanine with an insertion of a glycyl moiety between the benzoyl group and glycine as a spacer group. The degree of hydrolysis increased from 37% to 49% at 8h. Glycine and its isotere, taurine, are known to be good colon specific carriers of 5-aminosalicylic acid [16,20]. To examine an effect of alkyl group length of the amino acids on the hydrolysis, a series of alkyl homologs of glycine was incubated with the cecal contents of rats and the effect of chain length on hydrolysis was observed. The hydrolysis profiles are shown in Figure 5. Hydrolysis did not take place with *N*-benzoyl-β-alanine and *N*-benzoyl-4-aminobutyric acid. To compare the structural effect of the substitution of amino acid with aminoalkylsulfonic acid, a homologous series of *N*-benzoyl-aminoalkylsulfonic acids were prepared and incubated with the cecal contents of rats. The hydrolysis profiles of the conjugates are shown in Figure 6. While *N*-benzoyl-2-aminoethanesulfonic acid (*N*-benzoyl-taurine) was hydrolysed to the extent of 86% in 24h, the others were completely resistant to deconjugation in the cecal contents. These results suggest that the hydrolysis in the cecal contents of rats is largely confined to the amide bond originating from the natural amino acid or natural metabolite such as taurine.

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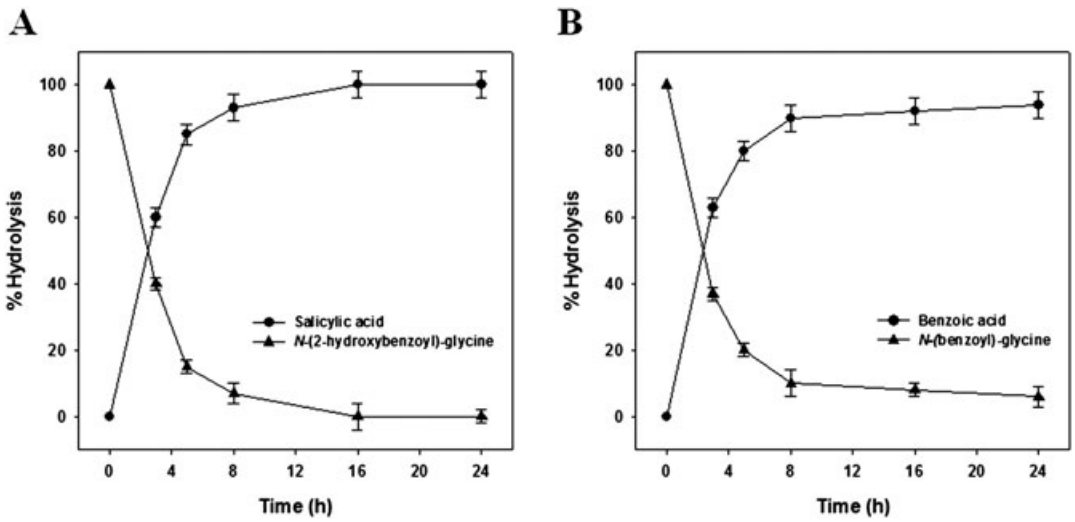

Figure 3. Hydrolysis of *N*-benzoyl glycine and *N*-(2-hydroxybenzoyl) glycine and production of the aromatic acids during incubation with the cecal contents of rats. Either *N*-benzoyl glycine or N-(2-hydroxybenzoyl) glycine was incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100μg of conjugates/0.1g cecal contents). The change in concentration of the conjugates and their metabolites was measured. Data are mean±SE (n=3–5)

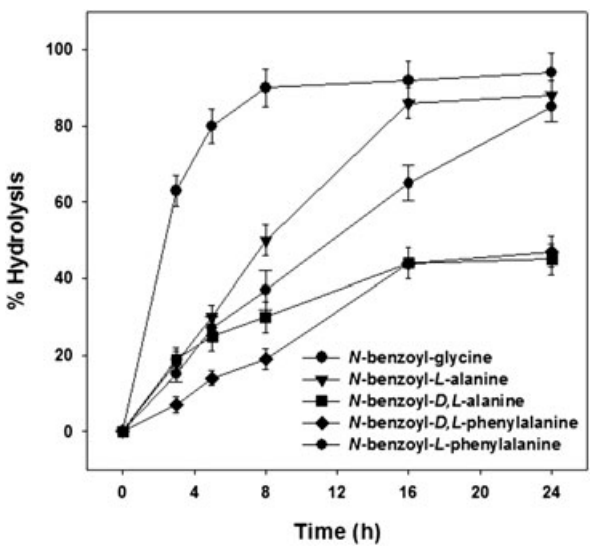

Figure 4. Hydrolysis (%) profiles of *N*-benzoyl-amino acid conjugates during incubation with the cecal contents of rats. *N*-benzoyl-amino acid conjugates were incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100μg of conjugates/0.1g cecal contents). The change in concentration of the conjugates was measured. Data are mean±SE (n=3–5)

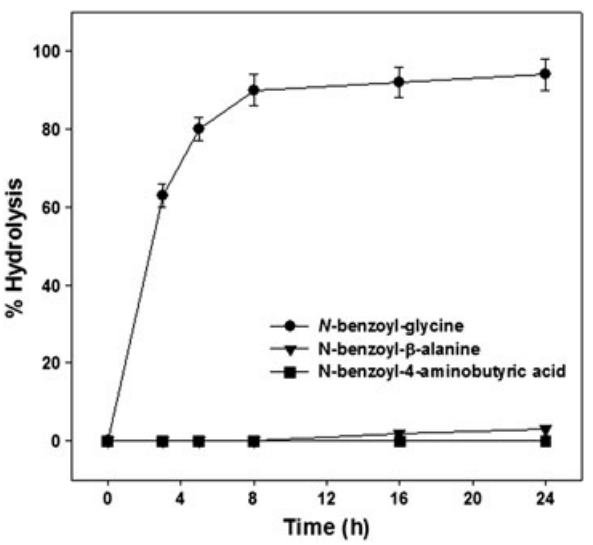

Figure 5. Hydrolysis (%) profiles of alkyl homologs of *N*-benzoyl-glycine conjugates during incubation with the cecal contents of rats. Alkyl homologs of *N*-benzoyl-glycine conjugates were incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100μg of conjugates/0.1g cecal contents). The change in concentration of the conjugates was measured. Data are mean±SE (n=3–5)

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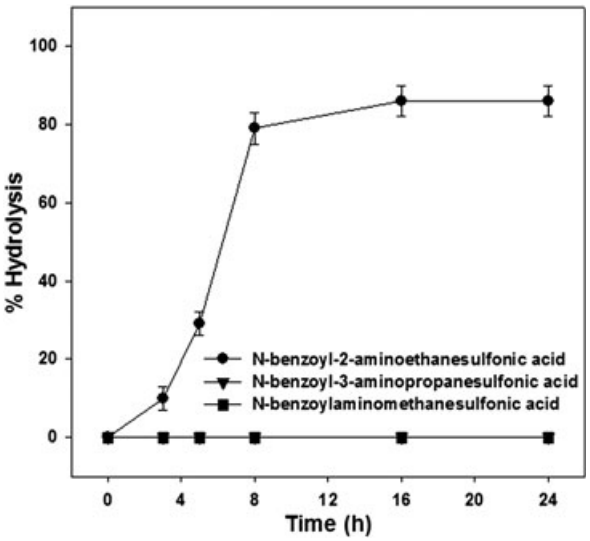

Figure 6. Hydrolysis (%) profiles of *N*-benzoyl-aminoalkylsulfonic acid conjugates during incubation with the cecal contents of rats. *N*-benzoyl-aminoalkylsulfonic acid conjugates were incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100 μg of conjugates/0.1g cecal contents). The change in concentration of the conjugates was measured. Data are mean±SE (n=3–5)

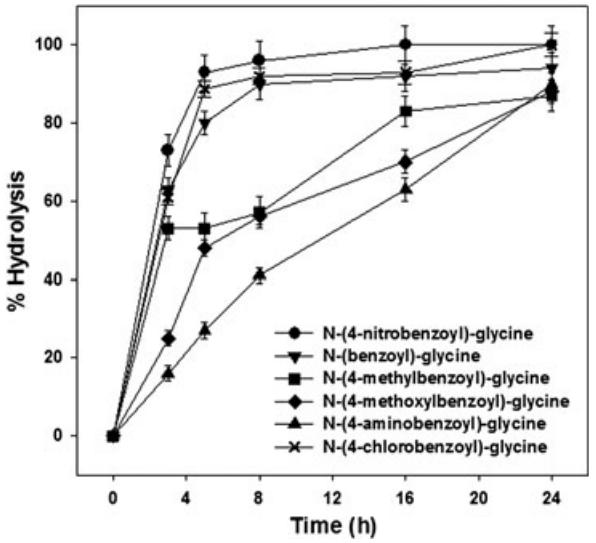

Figure 7. Hydrolysis (%) profiles of *N*-benzoyl-glycine conjugates with substituent on 4-position during incubation with the cecal contents of rats. *N*-aromatic acyl-glycine conjugates were incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100μg of conjugates/0.1g cecal contents). The change in concentration of the conjugates was measured. Data are mean±SE (n=3–5)

Effect of substituents on the aromatic acyl moiety on the cecal hydrolysis of N-aromatic acyl-glycine conjugates

N-(Aromatic acyl)-glycine conjugates with a substituent on the para position of the aromatic ring were incubated with the cecal contents of rats. Data from hydrolysis profiles are shown in Figure 7. It is seen from Figure 7 that the degree of hydrolysis reached 90% for *N*-(benzoyl)-glycine, 95% for *N*-(4-chlorobenzoyl)-glycine, 96% for *N*-(4-nitrobenzoyl)-glycine, 57% for *N*-(4-methylbenzoyl)-glycine, 56% for *N*-(4-methoxybenzoyl)-glycine and 41% for *N*-(4-aminobenzoyl)-glycine at 8h after incubation. The degree of hydrolysis reached 94% for *N*-(benzoyl)-glycine, 87% for *N*-(4-methylbenzoyl)-glycine, 88% for *N*-(4-methoxybenzoyl)-glycine and 90% for *N*-(4-aminobenzoyl)-glycine at 24h. *N*-(4-Nitrobenzoyl)-glycine and *N*-(4-chlorobenzoyl)-glycine were completely hydrolysed at 10h after incubation. Compared with the hydrolysis rate of *N*-(benzoyl)-glycine, the hydrolysis was accelerated when electron-withdrawing groups were substituted on the para-position and *vice versa* for electron-donating groups especially at an early stage of incubation. The strong electron-donating amino group elicited a greater retarding effect on the hydrolysis than the weak electron-donating groups methyl and methoxy group. However, the degree of hydrolysis became about the same level after 24h. On the other hand, when a methyl, chloro or methoxy group was introduced on the ortho-position, the hydrolysis was severely impaired. Ortho-substitution with a hydroxyl group on the ortho-position, namely *N*-(2-hydroxybenzoyl)-glycine, accelerated the hydrolysis. The results are shown in Figure 8.

Discussion

In this study, it was demonstrated that *N*-aromatic acyl-amino acid conjugates that possess various amino acids and substituents on the aromatic ring exhibit differences in the rates of hydrolysis in the cecal contents. The stereochemistry, size of a substituent at the 2 position and the alkyl chain length of amino acids affect the cecal conversion rates of the conjugates. Moreover, the rates are

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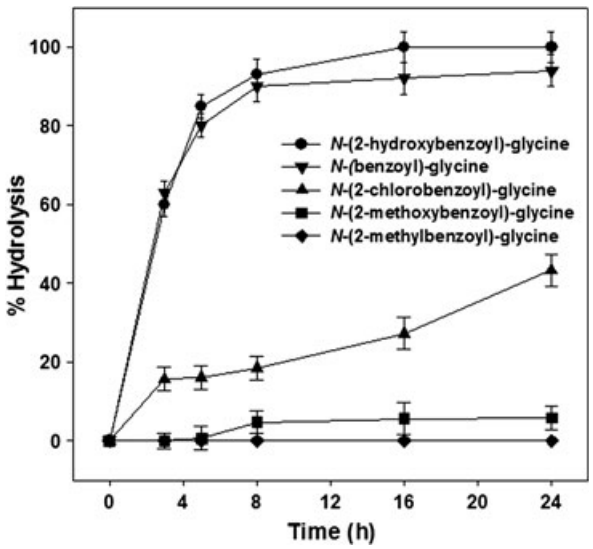

Figure 8. Hydrolysis (%) profiles of *N*-benzoyl-glycine with substituent on 2-position during incubation with the cecal contents of rats. *N*-aromatic acyl-glycine conjugates were incubated with 10% cecal contents in isotonic phosphate buffer (pH 6.8) at 37°C (100 μg of conjugates/0.1 g cecal contents). The change in concentration of the conjugates was measured. Data are mean±SE (n=3–5)

also modulated by the electronic and steric properties of a substituent on the aromatic ring.

Our data showing stereo-specificity of the hydrolysis for conjugates with L-amino acids are in line with a previous observation that the deconjugation of aromatic acid-amino acid conjugates occurs by microbial enzymes in the cecal contents [4]. As the degree of hydrolysis of the racemic conjugates was approximately half of the corresponding L-conjugates, the D-conjugates do not seem to affect the deconjugation of the respective L-conjugates. Considering that an enzymatic reaction is generally sensitive to steric hindrance, this microbial enzyme-dependent hydrolysis may rationalize our observation that as the size of substituents at the 2-position of amino acids increased, the rates of hydrolysis decreased. The steric effect at the 2-position of amino acids was confirmed by comparing the hydrolysis rate of *N*-benzoyl-L-phenylalanine with that of *N*-benzoyl-glycyl-D,L-phenylalanine with reduced steric hindrance at the 2-position. The hydrolysis rate of *N*-benzoyl-glycyl-D,L-phenylalanine was greater. It seems that the enzymes that are responsible for the hydrolysis of the conjugates recognize natural amino acids in the conjugates. This speculation is based on the findings that glycine and taurine, but not alkyl homologs of them, are susceptible to the hydrolysis.

In addition to amino acids in the conjugates, a substituent on the aromatic ring influenced the hydrolysis rate. Our results suggest that a substituent on the para-position of the aromatic ring elicited an electronic effect on the hydrolysis of the conjugates. The hydrolysis occurred faster when an electron-withdrawing group was substituted on the para-position and *vice versa* for electron-donating groups, especially at an early stage of incubation. The observation that substitutions on the ortho-position severely impaired the hydrolysis implies that substituents at the positions imposed steric hindrance. On the other hand, although the steric effect of the ortho substitution seemed to be predominant, an electronic effect still confers an influence on the hydrolysis rate. This is based on the observation that substitution with a chloro group, which is similar in size to a methyl group, exhibited faster hydrolysis than that with a methyl group. However, substitution on the ortho position does not always impose such a steric barrier as the conjugate with a hydroxyl group on the ortho position is susceptible to the hydrolysis, which occurred a little faster than the unsubstituted conjugate. This observation is in parallel with a previous finding that 5-aminosalicylglycine with an ortho-hydroxyl group is deconjugated effectively in the cecal contents [14]. The ortho-hydroxyl group effect might be caused by some special kind of interaction, such as intramolecular hydrogen bonding.

As mentioned in the introduction, colon-specific drug delivery technology is directed mainly for the efficient treatment of colonic diseases such as colorectal cancer, constipation, irritable and inflammatory bowel disease and intestinal infection, chronotherapeutic treatment of asthma, hypertension, arthritis and gastric ulcer and orally active peptide therapy. Therefore, all therapeutic agents available for the diseases are candidate drugs for modification to colon specific prodrugs [4]. A group of drugs with aromatic carboxylic acid or sulfonamide could be designed as a colon specific prodrug using various amino acids as a colon

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specific carrier (please see Figure 1A). On the other hand, *N*-aromatic acyl-2-(acetoxy)-glycine could be used as a platform for the design of a colon specific prodrug (*N*-aromatic acyl-2-(drug)-glycine) of drugs with a nucleophile (please see Figure 1B).

In a previous review [4], the rate of conversion of a colon specific prodrug to its parent drug is important in that it could affect the regional availability of the prodrug in the large intestine. The regional availability should influence the therapeutic effect of prodrugs for the treatment of colonic diseases whose lesion is located in different regions in the large intestine. If the conversion rate is rapid enough for a prodrug to liberate most of the parent drug in the proximal part of the large intestine, such a prodrug may not provide a sufficient amount of the drug for the distal part of the large intestine. This limitation in distribution of the drug would be aggravated if the drug is hydrophobic and/or is susceptible to colonic metabolism. While a hydrophobic drug would have trouble with diffusion in the contents in the distal part of the large intestine where the water content is very low, a drug susceptible to colonic metabolism may be metabolically inactivated before reaching the distal part. On the contrary, if the conversion rate is too slow, a sufficient amount of a drug would not be supplied at any target site in the large intestine. Thus, our data demonstrating structural effects of the conjugates on the rate of the cecal conversion might provide valuable information for the design of a colon specific prodrug with a controlled-release property at the target site. However, for the development of a clinically useful colon specific prodrug, other factors, species and inter-individual difference of microflora and the possibility of premature activation in small intestine, that influence the therapeutic activity of a colon specific prodrug should be considered as well. A review paper deals with these issues extensively [4].

Conclusion

The hydrolysis rates of *N*-aromatic acyl-amino acid conjugates in the cecal contents depend on the structure of the amino acid and a substituent in the aromatic ring. By introduction of an appropriate amino acid and/or a substituent in the aromatic ring, it is likely to design a colon specific prodrug with controlled conversion rates in the large intestine, which influences the regional therapeutic availability of the colon specific prodrug in the intestinal tract.

Acknowledgements

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