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Synthesis and characterisation of D-amino acid-based oligopeptides for use as probes of the influence of molecular structure on the paracellular route of gastrointestinal drug uptake

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Summary. A series of peptides based on D-amino-acids, and with an N-terminal D-phenylalanine residue, was synthesised by solution methods using t-butoxycarbonyl amino protection. These peptides were designed to resist metabolism in the gastrointestinal tract and to serve as probes of the effects of molecular shape and charge on the paracellular route of drug uptake in the gut. The peptides were characterised by NMR spectroscopy, FAB mass spectrometry, optical rotation and purified by HPLC.

Keywords: D-amino acids – Paracellular – Drug delivery – Oral absorption

Abbreviations: HBTU, *o*-Benzotriazolyl-tetramethyluronium hexafluorophosphate; OSu, N-hydroxysuccinimide ester.

Introduction

Oral administration of drugs is restricted by the molecular limitations on the permeability of the intestinal epithelium which provides a barrier to their access to the systemic circulation. Drugs and other molecules present in the lumen of the gut can reach the blood by active or facilitated transport, passive transcellular transport or passive paracellular transport. Entry by the paracellular route is limited by the ease of passage between the cells lining the gut, the so-called tight junctions (Hochman and Artursson, 1994). The influence of chemical structure on paracellular intestinal permeability of drugs and related molecules is poorly understood. Permeability decreases with increasing size (Krugliak et al., 1989; Ma et al., 1990; Soria and Zimmermann, 1994), but the role of charge and shape is less well established. There is a reported charge specificity (Meadows and Dressman, 1990; Reeves et al., 1978) with a view of the paracellular route as negatively charged (Adson et al., 1994). To probe the structural requirements for paracellular absorption, we designed a series of structurally related polar oligopeptides, based on D-amino acids,

which were expected to be metabolically stable and absorbed via the paracellular route. Oligopeptides were chosen to permit systematic changes in shape, charge, size and other molecular properties.

Materials and methods

Materials

For coupling reactions, anhydrous reagents, solvents and conditions were used. D-Ala, D-Asp, D-Val, DCC and HOBt were purchased from Aldrich. D-AlaOMe.HCl, D-Glu(OMe)$_2$.HCl, D-GlyOBu^t and D-Val(OMe).HCl were purchased from Bachem (Switzerland). The remaining materials were purchased from Nova Biochem (UK). All other chemicals were of reagent grade. It was possible to synthesise radiolabelled [^{3}H] compounds (not reported here but custom-synthesised by Zeneca Specialities (Gadbrook Park, Northwich, Cheshire, UK)), utilising the same synthetic route as for the unlabelled material, from the appropriate D-phenylalanine derivative tritiated at the 4-position of the phenyl ring.

Synthesis of D-oligopeptides

Peptides were synthesized from the required D-amino acids or derivatives by standard solution techniques (Bodanszky and Bodanszky, 1994), summarised in Scheme 1. The compounds were characterised by ^{1}H NMR spectroscopy, FAB-MS, optical rotation and HPLC. FAB-MS were recorded on a Kratos Concept instrument using Xe beam bombardment in a *m*-nitrobenzyl alcohol matrix. ^{1}H NMR spectra were recorded on a Jeol JNM EX 270 spectrometer operating at 270 MHz using d^6-dimethylsulfoxide as solvent and tetramethylsilane as internal standard. Splitting patterns are abbreviated as: s, singlet; d, doublet; t, triplet; m, unresolved multiplet and br, broad. Optical rotations were measured in aqueous solution with an AA-100 Polarimeter (Optical Activity Ltd, UK).

HPLC analysis for peptide purification and characterisation was performed on a system consisting of a Kontron 32X pump, Kontron 332 detector usually operating at 210 nm linked to a Philips PM 8251 chart recorder. Preparative HPLC was effected using a Whatman Magnum 20 column (22 mm × 50 cm) with ODS3 Partisil 10 mm packing at a flow rate of 10 ml/min using a gradient elution system consisting of 0.1% trifluoracetic acid (TFA) in water (solvent A) and 60% acetonitrile and 0.1% TFA in water (solvent B). The elution was increased from 0–30% solvent B in 150 min. Analytical HPLC used a Hichrom Kromasil 100-5C18 column (4.6 mm × 25 cm) with 5 mm ODS3 packing. The flow rate was 1 ml/min and the gradient elution system used was 0–80% solvent B in 32 min.

$$\text{BocPheOH} + \text{H-XOCH}_3 \xrightarrow[\text{CH}_3\text{CN}]{\text{HBTU/}} \text{BocPhe-X-OMe} \xrightarrow[\text{ii) TFA/CH}_2\text{Cl}_2]{\text{i) 1N NaOH/CH}_3\text{OH}} \text{Phe-X} \quad (1)$$

$$\text{BocPheOSu} + \text{H-X} \xrightarrow[\text{DMF}]{\text{NaHCO}_3\text{/}} \text{BocPheX} \xrightarrow[\text{CH}_2\text{Cl}_2]{\text{TFA/}} \text{Phe-X} \quad (2)$$

$$\text{BocPhe} + \text{H-XOBu}^t \xrightarrow[\text{CH}_2\text{Cl}_2]{\text{DCC/HOBt}} \text{BocPheXOBu}^t \xrightarrow[\text{CH}_2\text{Cl}_2]{\text{TFA/}} \text{Phe-X} \quad (3)$$

Scheme 1. Synthetic route to peptides. H-X is the appropriate amino acid

Results

The synthetic and analytical details of the products are summarised in Table 1.

NMR spectral assignments are:

H-D-PheGly-OH: 8.20–8.30 (m, 1H, NH-Gly), 8.20 (br s, 2H, NH_2-Phe), 7.20–7.40 (m, 5H, Ph), 3.70 (d, J = 5Hz, CH_2-α-Gly), 3.60 (m, 1H, CH-α-Phe), 4.05 (br s, 1H, CH-α-Ala), 2.60–3.10 (m, 2H, CH_2-β-Phe).

H-D-PheSar-OH: 7.24–7.07 (m, 5H, Ph), 4.59 (t, J = 7Hz, 1H, CH-α-Phe), 3.93 (m, 2H, CH_2-α-Sar), 3.01 (m, 2H, CH_2-β-Phe), 2.74 (s, 3H, N-CH_3-Sar).

H-D-Phe-D-Ala-OH: 8.85 (d, J = 7Hz, 1H, NH-Ala), 8.20 (br s, 2H, NH_2-Phe), 7.25–7.35 (m, 5H, Ph), 4.30 (t, J = 9Hz, 1H, CH-α-Phe), 4.05 (br s, 1H, CH-α-Ala), 2.90–3.20 (m, 2H, CH_2-β-Phe), 1.35 (d, J = 9Hz, 3H, CH_3-β-Ala).

H-D-Phe-D-Val-OH: 8.60 (d, J = 6Hz, 1H, NH-Val), 8.10 (br s, 2H, NH_2-Phe), 7.25–7.35 (m, 5H, Ph), 4.05–4.25 (m, 2H, CH-α-Phe and CH-α-Val), 2.85–3.20 (m, 2H, CH_2-β-Phe), 2.10 (m, 1H, CH-β-Val), 0.93 (d, J = 6Hz, 6H, $(CH_3)_2$-γ-Val).

H-D-Phe-D-Ser-OH: 8.90 (d, J = 6Hz, 1H, NH-Ser), 7.25–7.35 (m, 5H, Ph), 4.35 (m, 1H, CH-α-Phe), 4.15 (m, 1H, CH-α-Ser), 2.85–3.85 (m, 4H, CH_2-β-Phe and CH_2-β-Ser).

H-D-Phe-D-Asp-OH: 8.95 (d, J = 6Hz, 1H, NH-Asp), 8.20 (br s, 2H, NH_2-Phe), 7.30–7.40 (m, 5H, Ph), 4.55–4.70 (m, 1H, CH-α-Asp), 4.0–4.20 (m, 1H, CH-α-Phe), 2.80–3.25 (m, 2H, CH_2-β-Phe), 2.67–2.80 (m, 2H, CH_2-β-Asp).

Table 1. Synthesis and characterisation of D-oligopeptides

D-peptide	Synthetic route Scheme 1	HPLC Retention time, min	FAB-MS Major molecular ions
PheGly	1	14	223($M+H^+$)
PheAla	2	11.5	237($M+H^+$)
PheVal	2	11	265($M+H^+$)
PheSer	2	10	253($M+H^+$)
PheAsp	2	15	281($M+H^+$)
PheGlu	3	10.5	295($M+H^+$)
PheLys	2	10	294($M+H^+$)
PheAlaVal	3	22	336($M+H^+$), 292($M-CO_2$), 218(M-Val), 188(AlaVal)
PheAlaValAla	3	18	407($M+H^+$), 318(M^+-Ala), 260(M^+-Phe), 219(M^+-ValAla), 189(M^+-PheAla)

H-D-Phe-D-Glu-OH: 12.60 (br s, 1H, CO_2 H-Glu), 8.80 (d, 1H, J = 5Hz, NH-Glu), 8.20 (br s, 2H, NH_2-Phe), 7.25–7.35 (m, 5H, Ph), 4.30 (m, 1H, CH-α-Phe), (m, 1H, CH-α-Glu), 2.85–3.20 (m, 2H, CH_2-β-Phe), 2.30–2.40 (m, 2H, CH_2-γ-Glu), 1.70–2.15 (m, 2H, CH_2-β-Glu).

H-D-Phe-D-Lys-OH: 8.85 (d, J = 6Hz, 1H, NH-Lys), 8.20 (br s, 2H, NH_2-Phe), 7.80 (br s, 2H, NH_2-Lys), 7.20–7.40 (m, 5H, Ph), 4.25 (m, 1H, CH-α-Phe), 4.10 (m, 1H, CH-α-Lys), 2.85–3.20 (m, 2H, CH_2-β-Phe), 2.70–2.85 (m, 2H, CH_2-ε-Lys), 1.30–1.80 (m, 6H, CH_2-β-Lys, CH_2-γ-Lys and CH_2-δ-Lys).

H-D-Phe-D-Ala-D-Val-OH: 8.70 (d, J = 8Hz, 1H, NH), 8.15 (m, 4H, NH_2-Phe and NH), 7.20 (br s, 5H, Ph), 4.50 (t, J = 7Hz, 1H, CH-α-Ala), 4.20 (m, 1H, CH-α-Phe), 4.10 (m, 1H, CH-α-Val), 2.85–3.20 (m, 2H, CH_2-β-Phe), 2.10 (m, 1H, CH-β-Val), 1.30 (d, J = 7Hz, 3H, CH_3-β-Ala), 0.90 (d, J = 6Hz, 6H, $(CH_3)_2$-γ-Val).

H-D-Phe-D-Ala-D-Val-D-Ala-OH: 8.70 (d, J = 8Hz, 1H, NH), 8.30 (d, J = 7Hz, 1H, NH), 8.10 (br s, 2H, NH_2-Phe), 8.0 (d, J = 10Hz, 1H, NH), 7.25 (m, 5H, Ph), 4.0–4.55 (m, 4H, CH-α), 2.85–3.20 (m, 2H, CH_2-β-Phe), 2.0 (m, 1H, CH-β-Val), 1.20–1.30 (m, 6H, CH_3-β-Ala), 0.85 (m, 6H, $(CH_3)_2$-γ-Val).

Discussion

The ten oligopeptides of varying molecular weight and molecular properties synthesised (Table 1) all had D-Phenylalanine as the N-terminal amino acid. The attempted synthesis of non-radiolabelled PheGlu proved problematic, producing material which showed signs of racemisation (by NMR and HPLC). In this case the radiolabelled compound was prepared by a solid-phase technique. We have described elsewhere the spontaneous cyclisation conditions for PheSar (Speers et al., 1996).

The choice of peptides was guided by several principles. The series was based on the unnatural D-series of amino acids to minimise metabolism from intestinal peptidases (Krokan et al., 1985). For larger peptides (PheAlaVal and PheAlaValAla) two contiguous D-Ala residues were avoided to minimise potential interaction with D,D-aminopeptidases of gut flora. D-Phenylalanine was chosen as a common residue at the N-terminus to facilitate radiolabelling the tracer compounds. The remaining peptides were chosen to provide a wide range of charge, polarity and size. The peptides were also believed likely to be transported predominantly by the paracellular route, due to their hydrophilic, zwitterionic nature, despite the presence of relatively hydrophobic residues in some cases. The vast majority of each peptide was excreted within the first 6-hr urine collection after iv administration to rats, which implies that these compounds are rapidly eliminated from the body (He et al., 1996). Oral bioavailability decreased with increasing molecular weight, falling from 30% for PheAla to only 1% for PheAlaValAla (He et al., 1996).

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References

Adson A, Raub TJ, Burton PS, Barsuhn CL, Hilgers AR, Audus KL, Ho NFH (1994) Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers. J Pharm Sci 83: 1529–1536

Bodanszky M, Bodanszky A (1994) The practice of peptide synthesis. Springer, Berlin Heidelberg New York Tokyo

He Y, Murby S, Gifford L, Wilcock R, Speers P, Collett A, Warhurst G, Douglas KT, Rowland M, Ayrton J (1996) Oral absorption of D-oligopeptides by the paracellular route. Pharm Res (in press)

Hochman J, Artursson P (1994) Mechanisms of absorption enhancement and tight junction regulation. J Controlled Rel 29: 253–267

Krokan H, Grafstrom RC, Sundqvist K, Esterbauer H, Harris CC (1985) Cytotoxicity, thiol depletion and inhibition of O^6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. Carcinogenesis 6: 1755–1759

Krugliak P, Katz K, Hollander D, Ma TY (1989) Mechanisms of polyethylene glycol 400 permeability in perfused rat intestine. Gastroenterology 97: 1164–1170

Ma TY, Hollander D, Krugliak P, Katz K (1990) PEG400, a hydrophilic probe for measuring intestinal permeability. Gastroenterology 98: 39–46

Meadows KC, Dressman JB (1990) Mechanism of acyclovir uptake in rat jejunum. Pharm Res 7: 299–303

Reeves PR, Barnfield DJ, Longshaw S, McIntosh DAD, Winrow MJ (1978) Disposition and metabolism of atenolol in animals. Xenobiotica 8: 305–311

Soria I, Zimmermann CL (1994) Intestinal absorption of (−)-carbovir in the rat. Pharm Res 11: 267–271

Speers P, Chan C, Wilcock R, Douglas KT (1996) Synthesis and comparative conformational energetics of D-phenylalanylsarcosine and its cyclic dehydration product, (R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione. Amino Acids (in press)

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