ELSEVIER

International Journal of Pharmaceutics 172 (1998) 97–101

international journal of pharmaceutics

The enzymatic degradation and transport of leucine–enkephalin and 4-imidazolidinone enkephalin prodrugs at the blood–brain barrier

L. Lund [a], A. Bak [b], G.J. Friis [b], L. Hovgaard [a], L.L. Christrup [a,*]

[a] *Department of Pharmaceutics, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark*
[b] *Department of Analytical and Pharmaceutical Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark*

Received 6 October 1997; received in revised form 7 May 1998; accepted 2 June 1998

Abstract

In this study, the stability in and transport across a cell culture model of the blood–brain barrier (BBB) is investigated for leucine–enkephalin (Leu–enkephalin) and four 4-imidazolidinone prodrugs of Leu–enkephalin. The results show that Leu–enkephalin is degraded in the cell culture model of the BBB both by cytosolic and plasma membrane bound enzymes. It is likely that aminopeptidase is the predominant enzyme responsible for the degradation of Leu–enkephalin. All four 4-imidazolidinone prodrugs of Leu–enkephalin examined showed an increased stability of Leu–enkephalin against degradation by both plasma membrane bound and cytosolic enzymes. Consequently, the transport properties of Leu–enkephalin is also improved by up to 60% by formation of 4-imidazolidinone prodrugs.

Keywords: Blood–brain barrier; 4-Imidazolidinone prodrugs; Leucine–enkephalin

Enkephalins are potential future analgesics as they possess analgesic activity (Chang et al., 1976); however, because of transport (Banks and Kastin, 1984, Banks et al., 1987) and stability (Dupont et al., 1977, Craves et al., 1978, Thompson and Audus, 1994) problems, the prodrug principle is used either to increase permeation across the blood–brain barrier (BBB) and/or to stabilise against degradation (Weber et al., 1993, Greene et al., 1996). It has earlier been shown that 4-imidazolidinone prodrugs of leucine–enkephalin (Leu–enkephalin) possess increased stability in human plasma, in the intestinal mucosae of rat and in pure enzymatic solutions (Rasmussen and Bundgaard, 1991, Bak et al.,

* Corresponding author. Tel.: +45 35 370850; fax: +45 35 371277; e-mail: LLC@mail.dfh.dk

0378-5173/98/$ - see front matter
PII S0378-5173(98)00192-6

1997). In this study, a cell culture model of the BBB is used to examine both the stability at and transport across the BBB of Leu–enkephalin and four different 4-imidazolidinone prodrugs. Cell culture models of the BBB have previously been shown to express the degrading enzymes that are found in vivo (Weber et al., 1993, Thompson and Audus, 1994).

Microvessel endothelial cells (BBEC) were isolated from bovine brain as previously described (Lund et al., 1998). The integrity of the BBEC monolayer was evaluated by the transport of [^{14}C]sucrose from the apical to the basolateral side. Lowry assay was used to determine the amount of protein in the cluster dishes (Lowry et al., 1951). The average value determined for a six-well cluster dish was 2.44 ± 0.23 mg protein ($n = 5$). The synthesis of the 4-imidazolidinone prodrugs (acetone = I, cyclopentanone = II, cyclohexanone = III, 4-methylcyclohexanone = IV) was performed as described by Bak et al. (1997). The degradation of Leu–enkephalin and prodrugs I, II, III and IV in BBEC homogenate was investigated at 37°C. Endothelial cells from six wells were used for each compound. Cells were washed twice with phosphate buffered saline (PBS), the cluster scraped off in PBS, centrifuged for 5 min at $5000 \times g$ at 4°C and resuspended in Hank's buffer containing 10 mM HEPES, pH 7.4 (Hank's–HEPES).

The extracellular stability of Leu–enkephalin and prodrugs I, II, III and IV was evaluated using BBEC grown in cluster dishes. For the transport studies, 10^{-4} M Leu–enkephalin, prodrugs I, II, III and IV or 0.1 μCi/ml [^{14}C]sucrose dissolved in Hank's–HEPES were added on either the apical side or the basolateral side of the filter, with Hank's–HEPES on the other side. Quantitation of the compounds was done by reversed phase high-performance liquid chromatography. The degradation in BBEC homogenate and the extracellular degradation for Leu–enkephalin and prodrugs I, II, III and IV were evaluated by the pseudo-first-order rate constant (k_{obs}) obtained for the degradation and the half-life ($t_{1/2}$). For the transport studies, the cumulated amount of substances permeated was plotted against time and the steady-state flux dQ/dt estimated as the slope of the curve. The apparent permeability coefficient (P_{app}) was calculated by dividing dQ/dt by the initial concentration and the diffusion area.

From Fig. 1A,B it is seen that the logarithmic degradation of Leu–enkephalin is initially linear, indicating first-order kinetics. The calculated k_{obs} values (Table 1) indicate that Leu–enkephalin is degraded more rapidly after incubation in the BBEC homogenate than following exposure to intact BBEC, suggesting the existence of both plasma membrane bound and cytosolic/organelle membrane bound enzymes. Thompson and Audus (1994) have examined the degradation of Leu–enkephalin in both a cytosolic fraction and a membrane fraction of cultured cerebral microves-

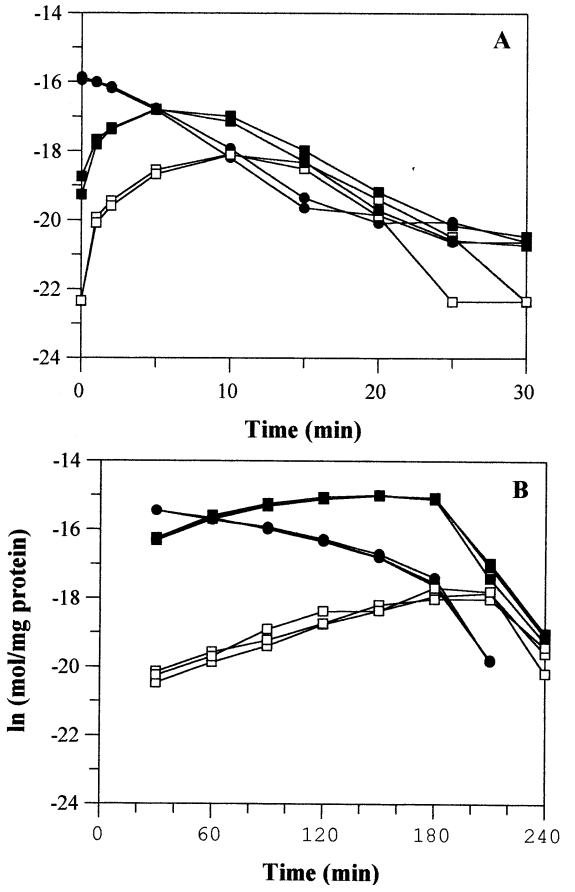

Fig. 1. Degradation of Leu–enkephalin (●), expressed as ln (mol/mg protein), in BBEC homogenate (A) and on intact BBEC (B). The amounts of GGPL (■) and PL (□) were also measured in both experiments ($n = 2$).

Table 1
Apparent k_{obs} and $t_{1/2}$ values of Leu–enkephalin, prodrugs I, II, III and IV degraded by BBEC homogenate ($n = 1$) and by intact BBEC grown in six-well clusters ($n = 3$)

	Homogenate		Intact BBEC	
	k_{obs} (1/h)	$t_{1/2}$ (h)	k_{obs} (1/h)	$t_{1/2}$ (h)
Leu–enkephalin	12 [a]	0.059	0.51 ± 0.03	1.4 ± 0.08
Prodrug I	0.076	9.2	0.028 ± 0.006	26 ± 6
Prodrug II	0.24	2.9	0.13 ± 0.06	5.3 ± 0.2
Prodrug III	0.11	6.1	0.046 ± 0.02	16 ± 5
Prodrug IV	0.11	6.6	0.024 ± 0.01	37 ± 23

[a] $n = 2$.

sel endothelial cells, and found the metabolism of Leu–enkephalin to be much higher in the cytosol than in the membrane fraction. Results from studies on the degradation of Leu–enkephalin and Met–enkephalin in different cerebral subcellular fractions from mouse and rat also showed maximal metabolic activity in the non-membrane fractions (Craves et al., 1978). It is likely, therefore, that Leu–enkephalin is mainly degraded by cytosolic enzymes in the BBEC homogenate used in the present study. During the initial degradation of Leu–enkephalin, Gly-Gly-Phe-Leu (GGPL) are formed to a larger extent than Phe-Leu (PL) (Fig. 1A,B), indicating that Leu–enkephalin is degraded primarily by aminopeptidase M and to a lesser extent by angiotensin converting enzyme (ACE) or enkephalinase. These results are consistent with the results obtained by Thompson and Audus (1994), who showed complete inhibition of Leu–enkephalin degradation by the ACE inhibitor ramipril. On the other hand, Weber et al. (1993) reported an effective inhibition of the degradation of Met–enkephalin by the enkephalinase inhibitors thiorphan and phosphoramidon in the same type of cell culture used by Thompson and Audus (1994). This discrepancy could arise from a difference in affinity between Met–enkephalin and Leu–enkephalin towards ACE and enkephalinase. During transport across BBEC (Fig. 2), Leu–enkephalin is degraded since GGPL and PL are measured in the receptor chamber. Probably the plasma membrane bound peptidases are responsible for this, as Leu–enkephalin is expected to permeate by paracellular diffusion. The transport of Met–enkephalin across cultured microvessel BBEC has previously been studied by Greene et al. (1996), who found that Met–enkephalin was not degraded during the transport experiment, while Weber et al. (1993) were only able to estimate the permeability coefficient if enkephalinase inhibitors were added. The reason for this discrepancy cannot readily be explained. Comparison of P_{app} in the two directions shows a P_{app} value 42% higher in the apical to basolateral direction, possibly due to the existence of an active transport mechanism in the basolateral to apical direction. The hypothesis has been confirmed by the in vivo experiments of Banks and Kastin, 1984 and Banks et al., 1987, whose results suggested the active transport of Leu–enkephalin out of the brain. In our study, only 30% of Leu–enkephalin was left on the apical side and 80% on the basolateral side (data not shown) after 4 h, indicating a higher level of peptidase activity on the apical side. This is supported by the results showing the formation of four times higher GGPL levels at the apical side than at the basolateral side. The logarithmic degradation of prodrugs I, II, III and IV was linear during the experiment, both in the BBEC

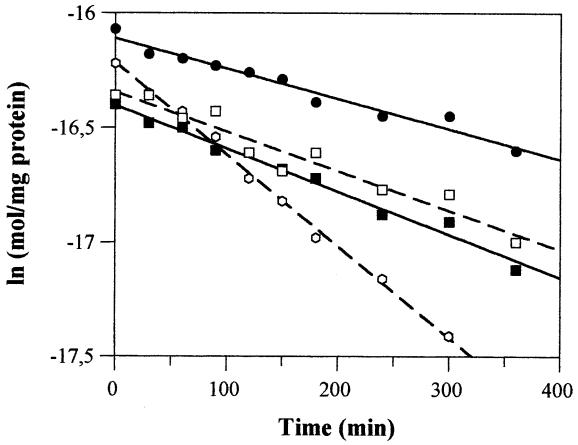

Fig. 2. Degradation of prodrugs I (●), II (○), III (■) and IV (□), expressed as ln (mol/mg protein), in BBEC homogenate ($n = 1$).

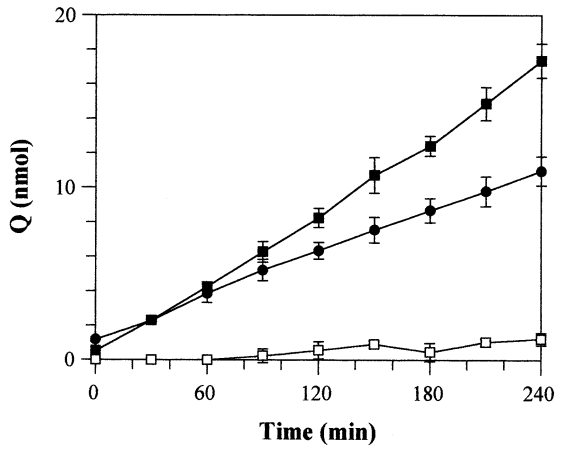

Fig. 3. Transport profile of Leu–enkephalin from the apical to the basolateral side. Q is the accumulated amount of Leu–enkephalin (●), GGPL (■) and PL (□) on the basolateral side ($n = 3$).

Table 2
Apparent permeability coefficients for Leu–enkephalin, prodrugs I, II, III and IV transported apical to basolateral and basolateral to apical across BBEC monolayers

	P_{app} (cm/min $\times 10^{-4}$) [a]	
	Apical to basolateral	Basolateral to apical
Leu–enkephalin	3.60 ± 0.27	5.11 ± 0.24
Prodrug I [b]	4.16 ± 0.15	7.23 ± 1.7
Prodrug II [b]	3.12 ± 0.043	7.92 ± 0.40
Prodrug III [b]	3.89 ± 0.29	5.14 ± 0.10
Prodrug IV [b]	5.80 ± 0.11	7.31 ± 0.96

[a] P_{app} values are given as mean $\pm$S.D.
[b] For prodrugs I, II, III and IV, the P_{app} is determined from the cumulated amount of substances detected on the receptor side, calculated as the sum of intact prodrug and prodrug degraded into Leu–enkephalin.

homogenate (Fig. 3) and on intact BBEC (data not shown). This indicates first-order kinetic from which k_{obs} and subsequently $t_{1/2}$ can be calculated (Table 1). All the prodrugs investigated were more stable than Leu–enkephalin. The half-lives in BBEC homogenate are comparable with the half-lives of hydrolysis in buffer and in pure samples of aminopeptidase N and ACE (Rasmussen and Bundgaard, 1991, Bak et al., 1997), indicating that prodrug formation stabilises the peptide against enzymatic degradation. The P_{app} values (Table 2) representing apical to basolateral and basolateral to apical transport express the sum of the prodrug and Leu–enkephalin transported, as Leu–enkephalin is the active drug substance which should be available for action in the brain under in vivo conditions. In all cases, the P_{app} values were higher for basolateral to apical transport than vice versa. For prodrugs II, III and IV, the increase in lipophilicity (Bak et al., 1997) is followed by an increase in transport. As the apparent permeability coefficient found in this study was determined from the amount detected on the receptor side, both transport and degradation are taking place, and no linear correlation between lipophilicity and P_{app} can be expected. Others have examined the permeability of enkephalin analogues (Weber et al., 1993) and enkephalin analogue prodrugs (Greene et al., 1996) of cyclic [D-Pen^5,D-Pen^5]enkephalin across a cerebral endothelial cell culture, and found increases in the permeability with increases in the lipophilicity.

Acknowledgements

This work was supported by the Pharmabiotec Research Centre. The authors would like to thank Bettina Dinitzen and Mads Bjerknæs Larsen for technical assistance.

References

Bak, A., Fich, M., Frokjaer, S., Larsen, B.D., Friis, G.J., 1997. 4-Imidazolidinone prodrugs of the N-terminal in Leu–enkephalin. Evaluation of the chemical and enzymatic stability as well as the lipophilicity with respect to delivery ascross the blood–brain barrier (BBB). Eur. J. Pharm. Sci. (accepted).

Banks, W.A., Kastin, A.J., 1984. A brain-to-blood carrier-mediated transport system for small *N*-tyrosinated peptides. Pharmacol. Biochem. Behav. 21, 943–946.

Banks, W.A., Kastin, A.J., Michals, E.A., 1987. Tyr-MIF-1 and Met–enkephalin share a saturable blood–brain barrier transport system. Peptides 8, 899–903.

Chang, J.K., Fong, B.T., Pert, A., Pert, C.B., 1976. Opiate receptor affinities and behavioral effects of enkephalin: structure–activity relationship of ten synthetic peptide analogues. Life Sci. 18, 1473–1481.

Craves, F.B., Law, P.Y., Hunt, C.A., Loh, H.H., 1978. The metabolic disposition of radiolabeled enkephalins in vitro and in situ. J. Pharmacol. Exp. Ther. 206, 492–506.

Dupont, A., Cusan, L., Garon, M., Alvarado Urbina, G., Labrie, F., 1977. Extremely rapid degradation of [^{3}H]methionine–enkephalin by various rat tissues in vivo and in vitro. Life Sci. 21, 907–914.

Greene, D.L., Hau, V.S., Abbruscato, T.J., Bartosz, H., Misicka, A., Lipkowski, A.W., Hom, S., Gillespie, T.J., Hruby, V.J., Davis, T.P., 1996. Enkephalin analog prodrugs: assessment of in vitro conversion, enzyme cleavage characterization and blood–brain barrier permeability. J. Pharmacol. Exp. Ther. 277, 1366–1375.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193, 265–275.

Lund, L., Krarup, L.H., Hovgaard, L., Christrup, L.L., 1998. Comparison of the BBEC to the Caco-2 as in vitro models for assessment of passive diffusion of drugs. S.T.P. Pharma Sciences (submitted).

Rasmussen, G.J., Bundgaard, H., 1991. Prodrugs of peptides. Part 15. 4-Imidazolidinone prodrug derivatives of enkephalins to prevent aminopeptidase-catalyzed metabolism in plasma and absorptive mucosa. Int. J. Pharm. 76, 113–122.

Thompson, S.E., Audus, K.L., 1994. Leucine–enkephalin metabolism in brain microvessel endothelial cells. Peptides 15, 109–116.

Weber, S.J., Abbruscato, T.J., Brownson, E.A., Lipkowski, A.W., Polt, R., Misicka, A., Haaseth, R.C., Bartosz, H., Hruby, V.J., Davis, T.P., 1993. Assessment of an in vitro blood–brain barrier model using several [Met^5]enkephalin opioid analogs. J. Pharmacol. Exp. Ther. 266, 1649–1655.