ELSEVIER

International Journal of Pharmaceutics 117 (1995) 139–146

international journal of pharmaceutics

Nasal absorption in the rat: IV. Membrane activity of absorption enhancers.

S.G. Chandler [a], N.W. Thomas [b], L. Illum [a,c,*]

[a] *Department of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, UK*
[b] *Department of Human Morphology, Queens Medical Centre, University of Nottingham, Nottingham NG7 2RD, UK*
[c] *DanBioSyst UK Ltd, Albert Einstein Centre, Highfields Science Park, Nottingham NG7 2TN, UK*

Received 12 May 1994; revised 13 July 1994; accepted 19 August 1994

Abstract

An erythrocyte model has been used to investigate the membrane activity of various agents which have been employed as enhancers of intranasal drug absorption. Enhancers were ranked according to haemolytic activity as follows: LPC-stearoyl > laureth-9 > STDHF > LPC-decanoyl > LPC-caproyl = DEAE-dextran = PBS (pH 7.0). The concentrations known to significantly enhance nasal absorption were generally, though not exclusively, in excess of that causing haemolysis, suggesting that membrane activity is involved in their mechanism of action, but that other mechanisms may also be important. In some cases, the effects on the erythrocyte membrane contrasted with those observed on the nasal epithelium in vivo, indicating the influence of the local environment of the nasal cavity on the activity of enhancing agents and the importance of histological studies in vivo.

Keywords: Absorption enhancer; Erythrocyte lysis; Membrane activity

1. Introduction

The nasal route of administration is one of several transmucosal routes under investigation for the systemic delivery of drugs with low oral bioavailabilities. In common with routes such as buccal, ocular, rectal and vaginal, nasal drug delivery often requires the co-administration of absorption enhancers to achieve therapeutic blood levels, particularly for large, hydrophilic drugs such as peptides and proteins (Daugherty et al., 1988; Hermens et al., 1990; Irie et al., 1992; Park et al., 1992). However, many of the agents used to increase transmucosal penetration have been associated with histological changes at the site of application which may limit their use.

Tissue analysis using light microscopy enables the identification of gross changes in epithelium integrity following exposure to absorption enhancers (Van Hoogdalem et al., 1990; Chandler et al., 1991b, 1994; Richardson et al., 1992). More subtle interactions which do not result in dramatic cell loss, obvious nuclear mutation or extensive mucus discharge are less easily distinguished. The higher level of magnification achieved using electron microscopy enables spe-

* Corresponding author.

SSDI 0378-5173(94)00308-4

cific cellular structures to be observed for toxic effects (Lee et al., 1988; Ennis et al., 1990).

In general, these techniques involve tissue exposure in vivo and rely on the accurate sampling and skillful preparation of sections for comparison with separate control tissue. Restriction of the dose volume and careful administration has enabled the two sides of the rat nasal cavity to be used as treated and control tissue for direct comparison in the same section (Chandler et al., 1991a). This avoids sampling errors and eases the identification of changes to the nasal epithelium, but the method, while an improvement, remains time consuming, labour intensive and costly in animals and materials.

Erythrocytes are commonly used as a model system for investigations into membrane interactions (Weltzien, 1979; Gill et al., 1994). They are readily available in large amounts and their lysis is easily measured by colourimetric determination of haemoglobin release. The relative membrane activity of different enhancing agents identified in this way provides further information about their biological effects and possible mechanisms of action, as well as giving an early indication of potentially unacceptable membrane toxicity.

Laureth-9 has been shown to be the most potent lytic agent in a range of non-ionic surfactants of similar structure (Hirai et al., 1981). This was parallelled by its ability to release protein from the nasal mucosa and its promoting effect on intranasal insulin absorption. This combination of effects suggested that the increase in membrane permeability was due to disruption of the structural integrity of the nasal epithelial barrier.

The effect of lysophospholipid structure on haemolytic activity has also been investigated (Cho and Proulx, 1971; Weltzien et al., 1977, 1979; Matsuzaki et al., 1988). Increased hydrocarbon chain length increased lytic activity to an optimal chain length of 16–18 carbon atoms; further increases resulted in a reduction of haemolysis (Weltzien et al., 1977). The introduction of a double bond in the side chain reduced activity relative to the compound of equivalent chain length (Matsuzaki et al., 1988).

This trend of reduced haemolytic activity with decreasing side chain length is similar to that found for intranasal absorption enhancing activity (Chandler et al., 1994); LPC-caproyl, with the shortest fatty acid chain of those investigated (C6), was inactive as an enhancer of intranasal insulin absorption and resulted in few signs of epithelium interaction. In contrast, the LPC preparation with a mixture of long chain fatty acids (stearoyl and palmitoyl) was an effective enhancer, but resulted in disruption of the nasal membrane. This suggests a relationship between haemolytic activity and interaction with the nasal membrane in vivo and also that membrane disruption is the predominant mechanism of activity for lysophospholipid enhancers.

However, LPC-decanoyl (of intermediate fatty acid chain length, C10) was shown to enhance insulin absorption without causing severe membrane disruption as assessed by light microscopy. Membrane interactions may have occurred as a mechanism of absorption enhancement, but at a level less than that resulting in gross epithelial damage. These more subtle effects are less easy to identify using the light microscope.

The present study, therefore, aimed to gain a more precise measure of the membrane activity of these lysophospholipids and other absorption enhancers over the range of concentrations used in vivo. The results could then be compared with the enhancing activity and epithelial disruption previously observed (Chandler et al., 1991b, 1994) to provide further information relating to the mechanism of action involved. Comparison of the results with the histological observations in the nasal cavity would also provide further evidence of the value of this in vitro technique in predicting nasal membrane activity and potential toxic effects in vivo.

2. Materials and methods

2.1. Materials

DEAE-dextran, L-α-lysophosphatidylcholines (caproyl (LPC_c) C6; decanoyl (LPC_d) C10; stearoyl (LPC_s) C18) and polyoxyethylene 9-lauryl ether (laureth-9) were purchased from Sigma

Chemical Co. Ltd, Dorset, UK. Sodium taurodihydrofusidate was obtained from California Biotechnology, CA, USA. Citrate phosphate dextrose adenine (CDP-A) buffer was used as supplied in a blood donor pack (Tuta Laboratories Pty Ltd, NSW., Australia). 10 mM phosphate-buffered saline (PBS, pH 7.0) was prepared using materials of reagent grade.

2.2. Preparation of erythrocyte stock suspension

The method used for the preparation of the erythrocyte suspension and incubation with test agents was that developed by Gill et al. (1994).

A stock suspension of erythrocytes was prepared on the day of each experiment. 4.5 ml of blood was collected from the carotid artery of a male Wistar rat (Medical School, University of Nottingham, UK) in a 15 ml plastic test tube containing 0.63 ml of CDP-A buffer as anticoagulant. The tube was carefully inverted a few times to ensure mixing.

As soon as possible after collection the blood was centrifuged at 1500 rpm for 5 min at room temperature in a Mistral 2000 centrifuge (MSE, Loughborough, UK) and the supernatant removed. The erythrocytes were washed by the addition of an equal volume of PBS, centrifuging at 1500 rpm for a further 5 min and removal of the supernatant. This washing procedure was repeated three more times.

A 2.0 ml sample of the washed cells was then added to 15.0 ml of PBS to produce the erythrocyte stock suspension which was used in experiments within 3 h of preparation.

2.3. Erythrocyte incubation procedure

A 0.125 ml aliquot of the stock erythrocyte suspension was added to 1.75 ml of the test media in a test tube. The tube was carefully inverted three times to ensure complete mixing and incubated in a water bath at 37°C for 10 min.

The incubation mixture was then centrifuged at 1800 rpm. for 2 min and the absorbance of the supernatant measured at 543 nm against a PBS blank (A_{543}). Each incubation was carried out in duplicate.

Deionised water and PBS were used as control media and were also incubated with erythrocytes in duplicate with each batch of test mixtures.

2.4. Test incubation media

Enhancer solutions were prepared in PBS by dilution of a stock solution. They were investigated over a range of concentrations which generally embraced the typical levels used in nasal absorption experiments (Chandler et al., 1991b):

(a) Laureth-9 (Mol. Wt 582): 0.010–5 mM (5.8×10^{-4}–0.3% w/v)
(b) LPC-stearoyl (LPC_s) (Mol. Wt 523.7): 0.001–20 mM (5.2×10^{-5}–1.1% w/v)
(c) LPC-decanoyl (LPC_d) (Mol. Wt 411.5): 0.001–20 mM (4.1×10^{-5}–0.8% w/v)
(d) LPC-caproyl (LPC_c) (Mol. Wt 355.4): 0.005–20 mM (1.8×10^{-4}–0.7% w/v)
(e) STDHF (Mol. Wt 647): 0.010–20 mM (6.5×10^{-4}–1.3% w/v)
(f) DEAE-dextran (mean Mol. Wt 500,000): 0.001–10% w/v

2.5. Treatment of results

The absorbance measurements from the PBS control incubations (A_{PBS}), reflected minimum haemoglobin release under the experimental conditions. Values from test incubations (A_{test}) were corrected for this background 'haemolysis' using the mean A_{PBS} from the corresponding PBS controls, i.e., those prepared in the same batch of incubations. The corrected values were then compared with the mean value from the deionised water control incubations (A_{dw}) in which total haemolysis was assumed to occur, for a measurement of the relative % haemolysis:

$$\% \text{ haemolysis} = \frac{A_{test} - A_{PBS}}{A_{dw}} \times 100$$

Haemolytic activity may be described by the molar amount required for 50% haemolysis (L_{50}) (Weltzien et al., 1977). This was determined from plots of % haemolysis against lysate concentration.

3. Results

Overall, the control incubations in deionised water resulted in a mean A_{543} value of 0.808 ± 0.047, indicating the reproducibility of the response on different days and between different batches of erythrocytes. Similarly, the mean A_{543} for the incubations in PBS was 0.040 ± 0.010. Comparison with the corresponding value in deionised water indicates the relative stability of the red blood cells in the buffer. The effect of the different enhancer systems on the stability of erythrocytes in PBS compared to deionised water was therefore considered to reflect their membrane activity.

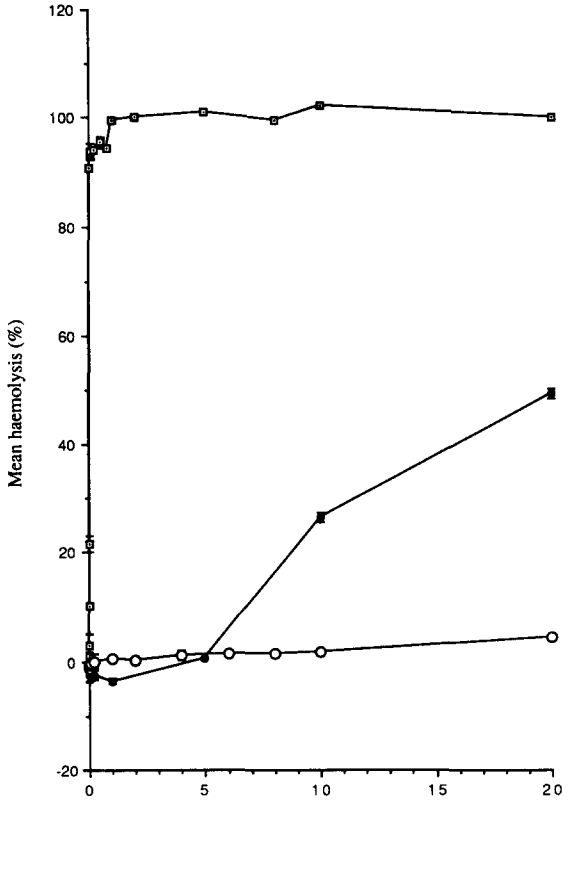

Fig. 1. The effect of LPC homologue concentration on erythrocyte lysis (±SD). (□) LPC-stearoyl; (●) LPC-decanoyl; (○) LPC-caproyl.

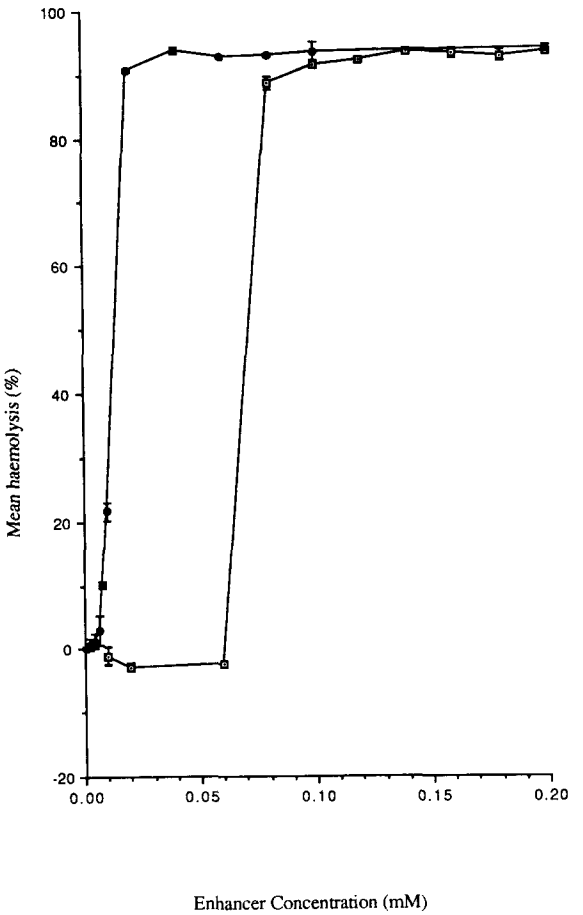

Fig. 2. Comparison of the effects of laureth-9 (□) and LPC-stearyl (●) on erythrocyte lysis at low concentrations (±SD).

The effects of the different LPC homologues investigated for lytic activity can be seen in Fig. 1. The haemolysis due to LPC-caproyl (LPC_c) was comparable to the control PBS incubations at all concentrations. Up to 5 mM (0.21% w/v), LPC-decanoyl (LPD_d) also resulted in no increase in haemolytic activity compared to the control. At higher concentrations however, haemolysis increased, although it did not reach maximum levels over the concentration range investigated.

In contrast, LPC-stearoyl (LPC_s) resulted in almost maximum haemolysis ($95.6 \pm 3.5\%$) at concentrations greater than 0.02 mM (1.05×10^{-3}% w/v). Again there was a sharp fall in erythrocyte lysis at lower concentrations of the

enhancer. This is seen more clearly in Fig. 2 in which the effects of LPC_s and laureth-9 are compared over the lower concentration range only. Laureth-9 resulted in the lysis of red blood cells (91.4 ± 2.1% haemolysis) at concentrations greater than 0.08 mM (4.7×10^{-3}% w/v). At lower concentrations the % haemolysis was comparable to background levels. This shows that LPC_s is a more potent lytic agent than laureth-9 as erythrocyte disruption occurred at lower molar concentrations of the lysophospholipid compared to the non-ionic surfactant.

Fig. 3 shows the haemolytic effects of STDHF. STDHF was a a potent lytic agent at concentrations of 0.8 mM (0.052% w/v) and above (88.4 ± 4.2%). Again the % haemolysis dropped sharply over a very narrow concentration range, although this change occurred at 10 times the enhancer concentration compared to laureth-9.

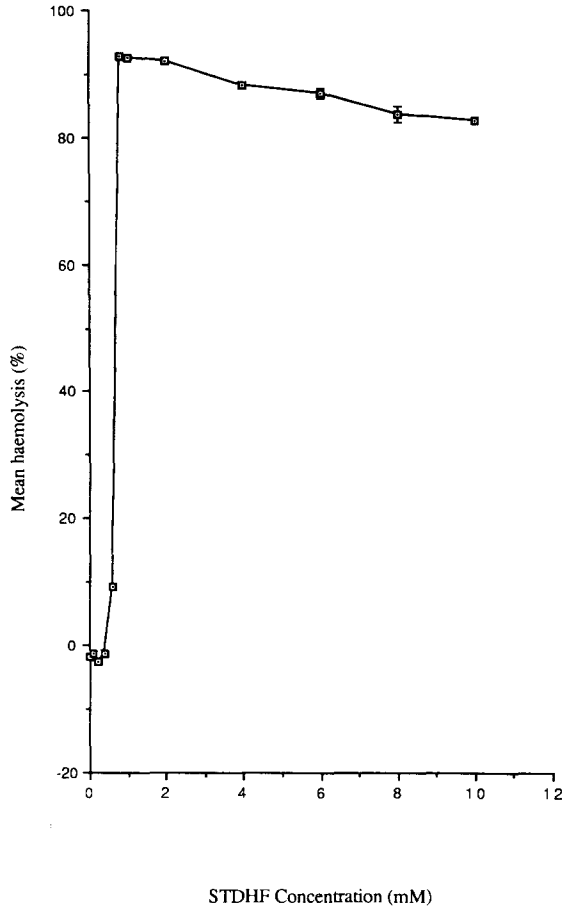

Fig. 3. The effect of STDHF concentration on erythrocyte lysis (± SD).

Table 1
Enhancer concentrations required for 50% erythrocyte lysis

Enhancer	L_{50}	
	mM	% w/v
Laureth-9	0.07	4.1×10^{-3}
LPC-stearoyl	0.01	5.2×10^{-4}
LPC-decanoyl	20	0.8
LPC-caproyl	–	–
STDHF	0.7	4.5×10^{-2}
DEAE-dextran	–	–

In contrast, DEAE-dextran resulted in haemolysis comparable to the PBS control incubations at all concentrations investigated (data not shown).

The L_{50} values determined from the % haemolysis vs enhancer concentration plots are given in Table 1.

These results enabled the enhancers to be ranked in terms of their haemolytic activity as follows: LPC-stearoyl > laureth-9 > STDHF > LPC-decanoyl > LPC-caproyl = DEAE-dextran = PBS (pH 7.0).

4. Discussion

Cell lysis may occur by the removal of membrane lipids into mixed micelles with the lysate molecule (Helenius and Simons, 1975; Matsuzaki et al., 1988) or by local high densities of the lysate incorporated into the membrane which leads to disruption of the normal molecular organisation and possibly the formation of hydrophilic channels (Weltzien, 1979; Israelachvilli et al., 1980); ion permeability changes and ultimately osmotic lysis and leakage of haemoglobin then follow.

For lysophospholipids, L_{50} values are thought to reflect equilibrium erythrocyte binding affinities (binding constants) (Weltzien et al., 1977) and are thus dependent on the cell density in the incubation mixture (Matsuzaki et al., 1988). In the present study the control incubations of erythrocytes in deionised water resulted in a reason-

ably low variation of A_{543} values (0.808 ± 0.047; CV = 5.8%). Assuming maximum haemoglobin was released from all cells, these values then reflected the total number of cells in the stock erythrocyte suspensions. Thus, the cell density was assumed to be the same in each incubation. The L_{50} values therefore reflected the number of lysate molecules bound to each cell for haemolysis to occur hence the relative membrane disrupting activity of the different absorption enhancers.

In this study laureth-9 was found to be a potent lytic agent. Expressing activity as the reciprocal of the minimum concentration (in mg/ml) to cause maximum haemolysis, the activity of laureth-9 was 21.5 ml/mg which is comparable to that of 25.0 ml/mg determined by Hirai et al. (1981). The difference might reflect the use of rat rather than rabbit erythrocytes in the present study, or variation in haematocrit as discussed above.

Previously reported absorption experiments in vivo (Chandler et al., 1991b) used a laureth-9 concentration of 1% w/v (approx. 17.2 mM) which is well above the haemolytic concentrations determined in this study. These results therefore support the theory that membrane disruption is the main mechanism of action for this absorption enhancing agent, as suggested by the extensive epithelium damage previously observed (Hirai et al., 1981; Daugherty et al., 1988; Chandler et al., 1991b;).

However, based on L_{50} values (Table 1), laureth-9 was found to be a slightly less potent haemolytic agent than LPC-stearoyl, whereas the disruption of the nasal epithelium by laureth-9 in vivo was considered to be more severe than that caused by a 0.625% w/v (approx. 11.9 mM) LPC (stearoyl/palmitoyl) enhanced insulin formulation (Chandler et al., 1991b). This may reflect the higher concentration of the non-ionic surfactant used in vivo, although both enhancers were used at concentrations in vast excess of the L_{50} values. Alternatively, the combination of homologues present in the natural LPC preparation might have resulted in a different level of activity in vivo or the results may reflect differences in the erythrocyte membrane and the nasal epithelium and their responses to the two enhancers.

Decreasing the LPC fatty-acid side-chain length resulted in reduced erythrocyte lysis which was in agreement with published reports (Weltzien, 1979; Matsuzaki et al., 1988). In particular, it was found that LPC_d had only moderate haemolysing activity at relatively high enhancer concentrations, while LPC_c with the shortest hydrocarbon side chain resulted in no erythrocyte lysis over the range of concentrations investigated.

The absorption-enhancing effects of the three LPC homologues were previously compared in vivo at a concentration of 11.9 mM (Chandler et al., 1994). LPC_s was an effective enhancer of insulin absorption at this concentration, which is well above the minimum concentration required for haemolysis and again suggests that membrane disruption was the major mechanism of enhancing activity. This theory is further supported by the complete lack of haemolytic activity determined with LPC_c across the entire concentration range tested, few signs of interaction with the nasal membrane in vivo and no insulin absorption enhancement at 11.9 or 17.6 mM (0.625% w/v) (Chandler et al., 1994).

However, at all of the LPC_d concentrations used in vivo (5.0, 11.9 and 15.2 mM), less than 50% erythrocyte lysis occurred, while insulin absorption was significantly increased from control levels in each case. Indeed, at a concentration of 5.0 mM, the effect on red blood cells was comparable to that of PBS alone. This may suggest that membrane disruption is not essential for the enhancement of insulin absorption and that a different mechanism of action is involved.

The degree of absorption enhancement tended to increase with the concentration of LPC_d, as did the extent of erythrocyte membrane interaction. Blood glucose measurements indicated that the enhancing effect 11.9 mM LPC_d was equivalent to that produced by the same concentration of LPC_s, although the % haemolysis was only about 1/3 of that of the homologue with the longer fatty acid chain. These results therefore imply that the extent of absorption enhancement may be increased by some membrane interaction, but that a more moderate degree of disruption may be equally effective, possibly in combination

with some other mechanism of enhancer action. However, extrapolating results from this model membrane system to the effect on the nasal epithelium and the use of glucose levels as an indirect measure of insulin absorption enhancement must be approached with caution.

STDHF resulted in a relatively high degree of membrane activity, although the concentration required to cause 50% cell lysis was 10 times higher than for laureth-9. Using the method of Hirai et al. (1981), Longenecker et al. (1987) found a 100-fold difference in haemolysing activity between STDHF and laureth-9. Expressing L_{50} as the reciprocal of the concentration (in mg/ml) required for 50% haemolysis, the result for STDHF was 0.25 ml/mg compared with 2.2 ml/mg in this study. This difference is difficult to explain, particularly as the L_{50} value determined by Longenecker and co-workers for laureth-9 (> 20 ml/mg) is assumed to be comparable to that of 24.4 ml/mg determined in the present study, and suggested similar erythrocyte sensitivity. STDHF may be more affected by differences in the experimental conditions than laureth-9, such as the use of 0.15 m NaCl (Longenecker et al., 1987) rather than PBS as the control incubation media and diluent. However, the relative haemolytic activities of different enhancers determined under the same experimental conditions as in this study may still be usefully compared.

Previously, the disruption of the nasal epithelium resulting from exposure to 1% w/v STDHF in vivo was found to be considerably less than that due to 1% w/v laureth-9 (Chandler et al., 1991b). Again this indicates a similarity in the membrane activity effects determined in vitro and in vivo. Good absorption enhancement has been found with 1% w/v STDHF (Lee et al., 1988; Chandler et al., 1991b), but this is in the range of maximum haemolysis which suggests that membrane activity was also involved in the mechanism of action of this bile salt derivative.

DEAE-dextran, on the other hand, resulted in negligible effects on red blood cells or on nasal epithelium in vitro, indicating that the absorption enhancement observed at 5% w/v was not the result of extensive membrane disruption.

The erythrocyte model is a useful tool for the assessment of membrane activity, but the contrasting effects observed in vivo and in vitro emphasize that the conditions and subsequent activity of lysate molecules in vivo may be quite different. The experiments demonstrated that agents which enhance intranasal insulin absorption do not necessarily exhibit a high degree of membrane perturbing activity and that those which result in disruption of a model membrane in vitro do not always have an equivalent effect in the nasal cavity. Thus, the investigation of formulations in vivo is vital.

The low level of membrane activity exhibited by some agents indicates the involvement of alternative or additional mechanisms of intranasal absorption enhancement which warrant further investigations. Moreover, the identification of enhancers with reduced membrane perturbing potential is promising from a toxicological standpoint and should encourage the development of enhanced transmucosal formulations.

References

Chandler, S.G., Illum, L. and Thomas, N.W., (1991a) Nasal absorption in the rat: I. A method to demonstrate the histological effects of nasal formulations. *Int. J. Pharm.*, 70 (1991a) 19–27.

Chandler, S.G., Illum, L. and Thomas, N.W., (1991b) Nasal absorption in rats: II Effect of enhancers on insulin absorption and nasal histology. *Int. J. Pharm.*, 76 (1991b) 61–70

Chandler, S.G., Illum, L. and Thomas, N.W., Nasal absorption in rats: III. Effect of lysophospholipids on insulin absorption and nasal histology. *Pharm. Res.*, 11 (1994) 1623–1630.

Cho, K.S. and Proulx P., Studies on the mechanism of haemolysis by acylcarnitines, lysolecithins and acylcholines. *Biochim. Biophys. Acta*, 225 (1971) 214–223

Daugherty, A.L., Liggitt, H.D., McCabe, J.G., Moore J.A. and Patton, J.S., Absorption of recombinant methionyl-human growth hormone (Met-hGH) from rat nasal mucosa. *Int. J. Pharm.*, 45 (1988) 197–206

Ennis, R.D., Borden, L. and Lee, W.A., The effects of permeation enhancers on the surface morphology of the rat nasal mucosa: a scanning electron microscopy study. *Pharm. Res.*, 7 (1990) 468–475

Gill, I.J., Illum, L., Farraj, N.F. and De Ponti, R., Cyclodextrins as protection agents against enhancer damage in nasal delivery systems: I. Assessment of effect by measure-

ment of erythrocyte haemolysis. *Eur. J. Pharm. Sci.*, 1 (1994) 229–236.

Helenius, A. and Simons, K., Solubilization of membranes by detergents. *Biochim. Biophys. Acta*, 415 (1975) 29–79

Hermens, W.A.J.J., Deurloo, M.J.M., Romeyn, S.G., Verhoef, J.C. and Merkus F.W.H.M., Nasal absorption enhancement of 17β-estradiol by dimethyl-β-cyclodextrin in rabbits and rats. *Pharm. Res.*, 7 (1990) 500–503

Hirai, S., Yashiki, T. and Mima, H., Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. *Int. J. Pharm.*, 9 (1981) 173–184

Irie, T., Wakamatsu, K. Arima, H., Artiomi, H. and Vekama, K., Enhancing effects of cyclodextrins on nasal absorption of insulin in rats. *Int. J. Pharm.*, 84 (1992) 129–139

Israelachvilli, J.N., Marcelja, S. and Horn, R.G., Physical principles of membrane organisation. *Q. Rev. Biophys.*, 13 (1980) 121–200

Lee, W.A., Ennis, R.E. and Foster, L.C., Histological studies of insulin absorption across the nasal mucosa in the presence of sodium taurodihydrofusidate (STDHF). *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 15 (1988) 70–71

Longenecker, J.P. Moses, A.C., Flier, J.S. Silver, R.D., Carey M.C. and Dubovi, E.J., Effects of sodium taurodihydrofusidate on nasal absorption of insulin in sheep. *J. Pharm. Sci.*, 76 (1987) 351–355

Matsuzaki, K. Handa, T., Miyajima, K., Mikura, Y., Shimizu, H. and Toguchi H., Quantitative analysis of haemolytic action of lysophosphatidylcholines in vitro: effect of acyl chain structure. *Chem. Pharm. Bull.*, 36 (1988) 4253–4260

Park, G.-B. Shao, Z. and Mitra, A.K., Acyclovir permeation enhancement across intestinal and nasal mucosa by bile salt-acylcarnitine mixed micelles. *Pharm. Res.*, 9 (1992) 1262–1267

Richardson, J.L., Illum, L. and Thomas, N.W., Vaginal absorption of insulin in the rat. *Pharm. Res.* 9 (1992) 878–883

Van Hoogdalem, E.J., Vermeij-Kerrs, C., De Boer, A.G. and Breimer, D.D., Topical effects of absorption enhancing agents on the rectal mucosa of rats in vivo. *J. Pharm. Sci.*, 79 (1990) 866–870.

Weltzien, H.U., Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. *Biochim. Biophys. Acta*, 559 (1979) 259–287

Weltzien, H.U., Arnold, B. and Reuther, R., Quantitative studies on lysolecithin-mediated haemolysis. Use of etherdeoxy lysolecithin analogs with varying aliphatic chain lengths. *Biochim. Biophys. Acta*, 466 (1977) 411–421