Pathway of Oral Absorption of Heparin with Sodium *N*-[8-(2-Hydroxybenzoyl)Amino]Caprylate

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Purpose. The oral bioavailability of heparin is negligible. Recent studies, however, have shown that sodium *N*-[8-(2-hydroxybenzoyl) amino]caprylate (SNAC) and other N-acylated amino acids enable oral heparin absorption. To investigate the mechanism by which heparin crosses the intestinal epithelium in the presence of SNAC, we have used fluorescence microscopy to follow the transport of heparin across Caco-2 cell monolayers.

Methods. The experiments were carried out on Caco-2 monolayers and Caco-2 cells grown to confluence on culture dishes, using different concentrations of SNAC. The localization of fluorescently labeled heparin was determined using epi-fluorescence and confocal microscopy. DNA dyes were used to determine the effect of SNAC on the plasma membrane integrity. F-actin was labeled with fluorescent phalloidin to investigate the stability of perijunctional actin rings in the presence of SNAC.

Results. Heparin was detected in the cytoplasm only after incubation of the cells with heparin and SNAC. No DNA staining was observed in cells incubated with a DNA dye in the presence of SNAC concentrations at which heparin transport occurred. In addition, no signs of actin redistribution or perijunctional ring disbandment were observed during the transport of heparin.

Conclusions. The results indicate that SNAC enables heparin transport across Caco-2 monolayers via the transcellular pathway. Heparin transport in the presence of SNAC is selective and does not involve permeabilization of the plasma membrane or tight junction disruption.

KEY WORDS: heparin; oral drug delivery; caco-2 cells, transcellular absorption.

INTRODUCTION

Heparin, a polydispersed glycosaminoglycan, is a powerful anticoagulant indicated for the prevention of deep venous thrombosis and pulmonary embolism in high-risk patients. Currently, heparin is administered parenterally because its oral bioavailability is negligible. The development of oral formulations is highly desirable, not only to improve patient convenience and compliance, but also to allow for the use of heparin for other clinical indications (1,2).

The low oral bioavailability of heparin is attributed mainly to its molecular size ($MW_{avg.}$ = 15 kDa) and high negative charge. The development of oral forms of macromolecules with these characteristics is certainly challenging (3). Passive diffusion across the intestinal epithelium is restricted to relatively small molecules (typically MW <500 Da). Hydrophilic molecules are absorbed paracellularly and hydrophobic molecules transcellularly. Some hydrophilic molecules of various sizes are also absorbed transcellularly, but only through specific active or facilitated mechanisms (4,5).Despite the difficulties, the demand for oral forms of macromolecules is increasing due to the growing number of macromolecule drugs in the market and in clinical development. In response to this demand, a variety of different approaches to enable oral macromolecule absorption are being developed and significant advances have been achieved in this field (6–8).

Over the past few years, Emisphere Technologies has developed a series of novel delivery agents that enable oral heparin absorption (9). Results from preclinical studies led to the selection of one of these delivery agents, sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC), for the development of an oral heparin formulation, which is currently in Phase III clinical trials (10). Previous non-clinical studies showed that SNAC causes no intestinal tissue damage and suggested that heparin is transported transcellularly (11). To further investigate the mechanism of heparin transport with SNAC, we used fluorescent microscopy to follow the transport of heparin across Caco-2 cell monolayers.

MATERIAL AND METHODS

Materials

Heparin Sodium USP was purchased from Scientific Protein Laboratories (Waunakee, WI, USA). YOYO®-1 iodide, ALEXA FLUOR® 488 hydrazide, and ALEXA FLUOR 488 labeled phalloidin were obtained from Molecular Probes, Inc. (Eugene, OR, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was obtained from Pierce (Rockford, IL, USA). Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum, non-essential amino acids, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were from Sigma.

Fluorescently Labeled Heparin

Heparin (30 mg/ml) was incubated, overnight, at pH 5 and room temperature, with ALEXA FLUOR 488 hydrazide (10 mg/ml) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (5 mg/ml). The mixture was then dialyzed twice overnight against 4 liters of 10 mM sodium phosphate buffer at pH 7.4, using 3500 Da MWCO membranes. The degree of labeling was 0.4 mol of dye/mol of heparin (estimated according to manufacturer's instructions and using 15 kDa for the molecular weight of heparin). Labeled samples were analyzed by gel filtration using NAP ™ 10 columns (Amersham Pharmacia Biotech AB, Sweden) to verify that all the free dye was removed during dialysis.

Cells

Caco-2 cells originating from a human colorectal carcinoma were obtained from American Type Culture Collection (Manassas, VA, USA). Cells, passage number 20 to 40, were cultured on collagen coated 35 mm cell culture dishes (Nunclon, Denmark) for 2 to 3 days or on 24 mm collagen coated

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polycarbonate filters, TRANSWELL® cell culture inserts, 0.45 μm pore diameter (Costar, Cambridge, MA, USA), for 3 to 4 weeks after seeding. Cells were cultured at 37°C and 5% CO_2 atmosphere in DMEM growth medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, penicillin (100 Unit/ml), and streptomycin (100 μg/ml). The integrity of the Caco-2 monolayers was determined by measuring the transepithelial electrical resistance (TEER) with an Epithelial Voltohmmeter EVOM-G (World Precision Instruments, Sarasota, FL, USA) and a pair of silver/silver-chloride pellet electrodes. The TEER was typically 300 to 400 Ω cm^2.

For transport measurements, six well plates containing the monolayers on TRANSWELL filters were mounted on an orbital shaker at 30 to 40 rpm and 37°C in an atmosphere of 5% CO_2. The growth medium was removed and the cells were incubated for 20 min in HBSS buffer, pH 7.4. Afterwards, the apical solution was replaced with HBSS containing heparin and SNAC. The basolateral solution was sampled every 10 min and the aliquots were analyzed using a FLUOstar fluorescence microplate reader (BMG LabTechnologies, Inc., Germany). The concentration of heparin in the basolateral solutions was determined by using a standard curve ($r = 0.9997$) obtained from fluorescence measurements of known heparin quantities. The basolateral solutions were also analyzed by gel filtration to confirm that the fluorescent material detected in these solutions was labeled heparin and not free dye. No detectable amount of free dye was found in these solutions.

Microscopy

Conventional brightfield and epi-fluorescence microscopy was done with a Nikon LABOPHOT®-2 upright microscope using 10/0.35 and 40/0.55 dipping lenses (Nikon, Inc. Garden City, NY, USA). The images were captured with CCD 72 camera (DAGE-MTI, Inc., Michigan City, IN, USA) and digitized by DT3155 frame grabber (Data Translation, Inc., Marlboro, MA, USA). Confocal laser scanning microscopy was carried on AXIOVERT®100M inverted microscope with LSM 5 PASCAL™ scanning module and 40/1.3 oil immersion objective (Carl Zeiss Inc., Germany). The cells were preincubated with HBSS buffer (pH 7.4) at 37°C and 30 to 40 rpm shaking for approximately 20 min to remove residual growth medium. Afterwards, they were incubated with HBSS containing SNAC and heparin (5 mg/ml). The cells were then washed twice and observed with the microscope. For membrane integrity assays, 1 μM of YOYO-1 iodide was added to the SNAC solution. All the experiments were repeated at least twice.

F-actin was stained as follows. After incubation for 30 min with heparin and SNAC, the cells were fixed for 10 min in 4% paraformaldehyde in HBSS, treated for 5 min with 2% Triton X-100, and stained with ALEXA FLUOR 488 phalloidin (~0.5 μM) for 20 min in the dark. Each step was separated by double rinsing with HBSS.

RESULTS

Heparin Uptake by Caco-2 Cells Grown on Culture Dishes

Intracellular uptake of heparin by Caco-2 cells grown to confluence on culture dishes was observed after incubation of the cells with heparin in the presence of SNAC concentrations ≥33 mM. No intracellular fluorescence was observed when the cells were incubated only with heparin or with SNAC concentrations below 33 mM. Interestingly, no intracellular fluorescence was observed when the cells were incubated with SNAC and the fluorescence probe, ALEXA FLUOR 488 hydrazide, instead of heparin. The uptake of heparin was observed as early as 5 min after incubation of the cells with heparin and SNAC. Heparin was localized both in the cytosol and nucleus and no endocytic vesicles were detected in the cytoplasm (Fig. 1).

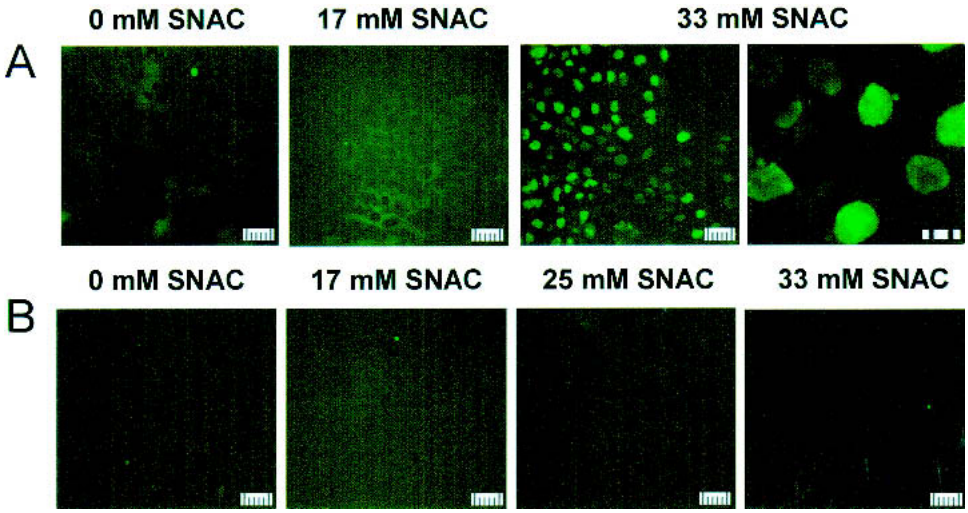

Fig. 1. (A) Uptake of fluorescent heparin by Caco-2 cells incubated on culture dishes with heparin (5 mg/ml) and different SNAC concentrations for 30 min. Heparin uptake was observed only in the presence of ≥33 mM SNAC. Heparin was detected in the cytoplasm and endocytic vesicles were not observed. (B) Cells incubated with SNAC and the fluorophore, ALEXA FLUOR 488 hydrazide (0.25 mM), instead of heparin. No intracellular fluorescence was observed after 30 min incubation. The scaling bar is in 10 μm increments.

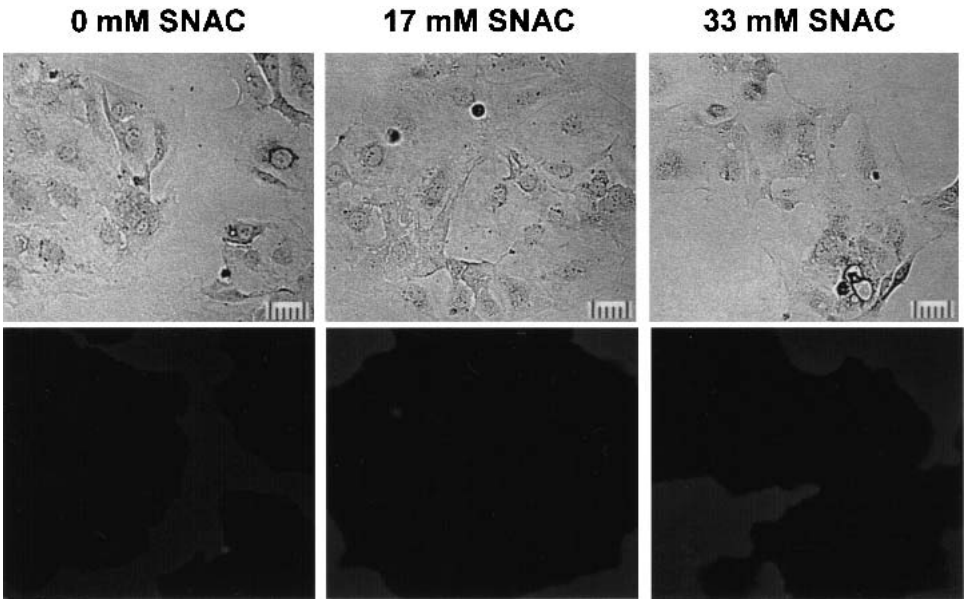

Fig. 2. Brightfield and fluorescence micrographs of Caco-2 cells grown on culture dishes after 30 minutes incubation with YOYO-1 iodide and different concentrations of SNAC. The scaling bar is in 10 μm increments.

YOYO-1 iodide, a membrane impermeant DNA dye, was used to investigate the effect of SNAC on the integrity of the plasma membrane. This DNA dye is used as an indicator of membrane permeabilization because it cannot enter the cells and stain DNA unless the plasma membrane has been permeabilized or damaged. Fig. 2 shows the micrographs of cells incubated with YOYO-1 iodide and SNAC under the same conditions of the experiments carried out with heparin. No signs of DNA staining can be observed, an indication that YOYO-1 iodide was unable to enter the cells and that the plasma membrane was not permeabilized.

Heparin Transport across Caco-2 Monolayers

Heparin was detected in the cytoplasm of the Caco-2 cells after heparin and SNAC were applied to the apical side of the monolayers. As in the case of the cells grown on culture dishes, no heparin uptake was observed in the absence of SNAC or at SNAC concentrations below 33 mM. The intra-

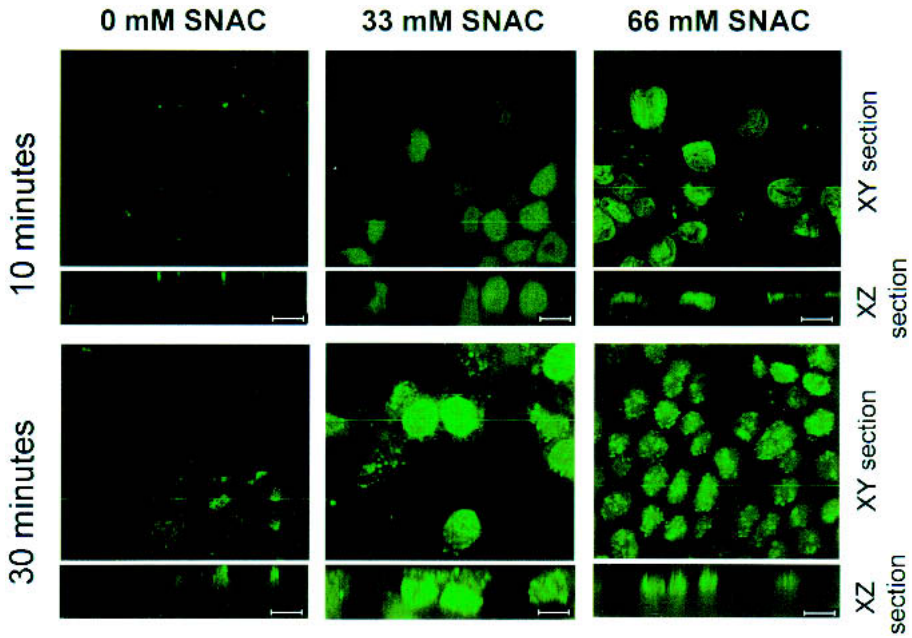

Fig. 3. Heparin uptake by Caco-2 monolayers at different time points after incubation with heparin and SNAC. The micrographs were obtained by Z-stack scanning of Caco-2 monolayers after 10 and 30 min incubation with SNAC and heparin. The XZ sections correspond to the cut along the indicated lines through 14 μm thick stack. The scaling bar is 10 μm.

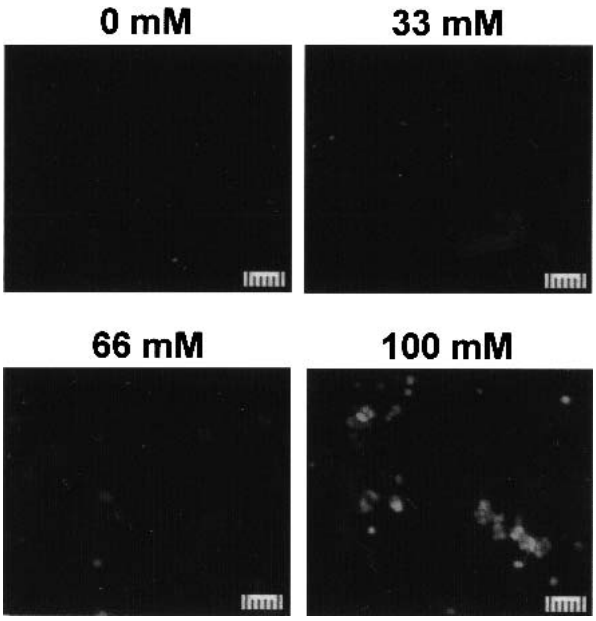

Fig. 4. Caco-2 monolayers after 30 minutes incubation with YOYO-1 iodide and different SNAC concentrations. DNA staining was observed in some cells only after 30 min incubation with 100 mM. The scaling bar is in 10 μm increments.

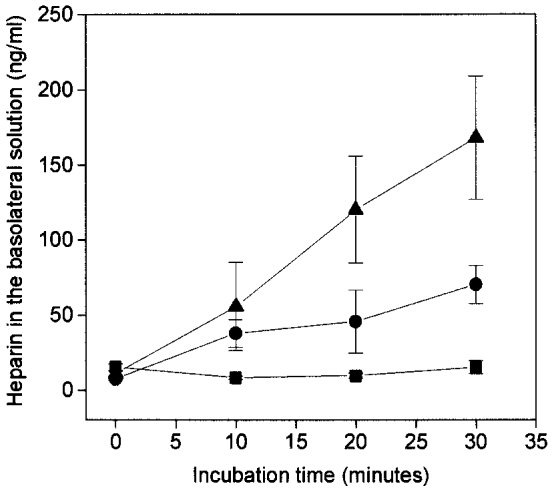

Fig. 5. Heparin concentration in the basolateral solutions of Caco-2 monolayers incubated with heparin in the absence (■) and in the presence of 33 (●) and 66 mM (▲) SNAC (n = 5, mean ± SD).

cellular fluorescence increased as the incubation time and SNAC concentration increased. No fluorescence was detected in the intercellular spaces (Fig. 3).

No DNA staining was observed when the monolayers were incubated with YOYO-1 iodide and SNAC under the same conditions of the experiments carried out with heparin. DNA staining was observed in some cells only after incubation of the monolayers with ≥100 mM SNAC for 30 min (Fig. 4).

Heparin was also detected in the basolateral solutions of monolayers that were incubated with heparin and 33 or 66 mM SNAC on the apical side. Fig. 5 shows that the amount of heparin in the basolateral solutions increased with the incubation time and the SNAC concentration. The TEER remained within 95% to 110% of the initial value at the end of the experiments. Additional experiments showed that the TEER remained within this range even after 2 h incubation with heparin and 33 or 66 mM of SNAC.

Effect of SNAC on the Perijunctional Rings

Actin filaments are closely associated with the proteins responsible for maintaining the tight junctions, thus disbandment of perijunctional rings and redistribution of actin are readily observed when tight junctions are disrupted and intercellular spaces dilated (12–14). To investigate the effect of SNAC on the stability of the perijunctional rings, F-actin was stained with ALEXA FLUOR 488 phalloidin after incubating the monolayers for 30 min with different SNAC concentrations on the apical side. Fig. 6 shows that the appearance and fluorescence intensity of the perijunctional rings of monolayers incubated with 33 and 66 mM SNAC were indistinguishable from those of the control monolayer, which was incubated without SNAC. The rings, however, were partially dis-

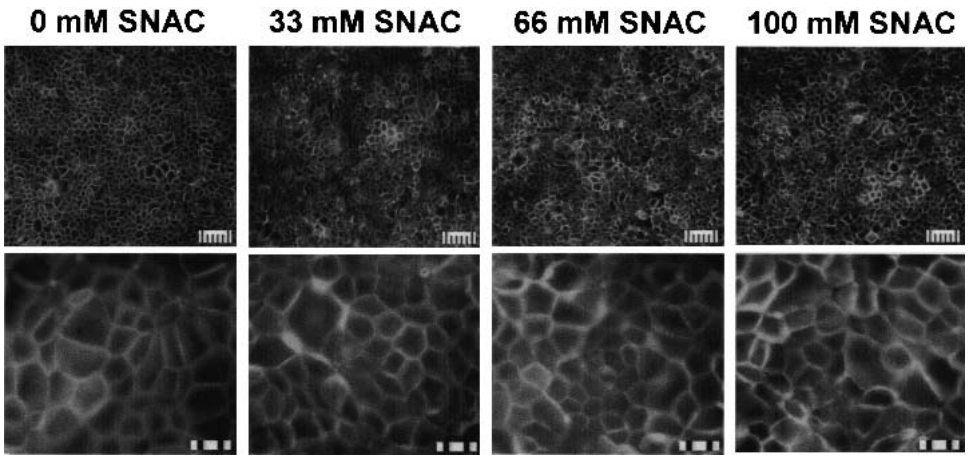

Fig. 6. Apical perijunctional rings of Caco-2 monolayers stained with ALEXA FLUOR 488 phalloidin after incubation for 30 min with different SNAC concentrations. Control monolayers and monolayers incubated with 33 and 66 mM SNAC were undistinguishable. Partial actin disbandment was observed in some of the cells of the monolayers incubated with 100 mM SNAC. The scaling bar is in 10 μm increments.

banded in some of the cells of the monolayers that were incubated for 30 min with ≥100 mM SNAC.

DISCUSSION

The results of this study indicate that SNAC enables the transcellular absorption of heparin. Furthermore, the fact that neither ALEXA FLUOR 488 hydrazide nor YOYO-1 iodide were able to penetrate the cells in the presence of SNAC concentrations at which heparin transport was observed, clearly indicates that the absorption of heparin occurs without damage or permeabilization of the plasma membrane. The possibility of paracellular transport under the conditions of our experiments is unlikely in view of the stability of the perijunctional rings and the TER during the transport of heparin. In addition, the Z-stack scans obtained by confocal microscopy clearly show that heparin was inside the cells and not in the intercellular spaces.

Slight signs of membrane permeabilization and tight junction disruption were observed after prolonged exposure to high concentrations of SNAC. However, the uptake and transcellular transport of heparin was observed already at lower concentrations and shorter incubation times, which did not affect the integrity of the plasma membrane or tight junctions. Because no evidence of necrosis or tissue damage was observed in previous *in vivo* and *in situ* experiments using higher SNAC concentrations and longer incubation times than those investigated here (9,11), it is likely that the slight cytotoxic effects observed in this study after prolonged exposure to high SNAC concentrations are due to the limitations of the Caco-2 model. Caco-2 monolayers are more sensitive and fragile than whole tissue models, which are provided with the mucus layer and in which exposure to the drug is limited by the transit times (15).

Heparin uptake and transport in the presence of 33 and 66 mM SNAC occurred without plasma membrane permeabilization or opening of the paracellular pathway. Under these conditions, the absorption of heparin was relatively fast. Heparin was detected inside the cells and in the basolateral solutions when first sampled, 10 min after SNAC and heparin were added to the apical surface of the monolayers. This rapid absorption agrees with results from *in vivo* experiments, in which heparin was detected in the bloodstream 15 to 20 min after oral dosing, with peak concentrations at 40 to 60 min (9,16).

The specific mechanism by which heparin is able to cross the plasma membrane without compromising its integrity remains to be elucidated. The possibility of endocytosis is unlikely because heparin was localized in the cytosol and no endocytic vesicles were observed inside the cells. This conclusion is further supported by the fact that heparin was detected also in the nucleus. It is well known that molecules smaller than 25 to 30 kDa or of less than 9 nm in diameter diffuse freely and rapidly across nuclear pores and are often observed in the nucleus (17–19). However, only molecules that are free in the cytosol and not entrapped inside vesicles are able to cross the nuclear pores.

Results from previous studies suggested that SNAC interacts with heparin forming non-covalent complexes, which are more lipophilic than heparin alone (9,11). These complexes could potentially diffuse passively across cell membranes and cross the intestinal epithelium via the transcellular pathway, dissociating later upon dilution in the bloodstream. Additional studies to confirm this hypothesis are currently in progress. The possibility of facilitated transport is also under investigation.

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REFERENCES

1. W. Klein, A. Buchwald, S. E. Hillis, S. Monrad, G. Sanz, A. Graham, G. Turpie, J. van der Meer, E. Olaisson, S. Undeland. and K. Ludwing for the FRIC Investigators. Comparison of low-weight heparin with unfractionated heparin acutely and with placebo for 6 weeks in the management of unstable coronary artery disease: Fragmin in Unstable Coronary Artery Disease Study (FRIC). *Circulation* **96**:61–68 (1997).
2. R. Kay, K. S. Wong, Y. L. Yu, W. Y. Chan, T. H. Tsoi, A. T. Ahuja, F. L. Chan, K. Y. Fong, C. B. Law, A. Wong, and J. Woo. Low-molecular-weight heparin for the treatment of acute ischemic stroke. *N. Engl. J. Med.* **333**:1588–1593 (1995).
3. V. H. L. Lee and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Del. Rev.* **4**:171–207 (1990).
4. A. L. Daugherty and R. J. Mrsny. Transcellular uptake mechanisms of the intestinal epithelial barrier. Part One. *Pharm. Sci. Technol. Today* **2**:144–151 (1999).
5. A. L. Daugherty and R. J. Mrsny. Regulation of the intestinal epithelial paracellular barrier. *Pharm. Sci. Technol. Today* **2**:281–287 (1999).
6. B. J. Aungst. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *J. Pharm. Sci.* **83**:979–987 (1993).
7. I. Gomez-Orellana and D. R. Paton. Advances in the oral delivery of proteins. *Exp. Opin. Ther. Patents* **8**:223–234 (1998).
8. I. Gomez-Orellana and D. R. Paton. Advances in the oral delivery of proteins. Update. *Exp. Opin. Ther. Patents* **9**:247–253 (1999).
9. A. Leone-Bay, D. R. Paton, B. Variano, H. Leipold, T. Rivera, J. Miura-Fraboni, R. Baughman, and N. Santiago. Acylated non-alpha-amino acids as novel agents for the oral delivery of heparin sodium USP. *J. Controlled Release* **50**:41–49 (1998).
10. G. F. Pineo, R. D. Hull, and V. J. Marder. Orally active heparin and low molecular weight heparin. *Curr. Opin. Pulm. Med.* **7**: 344–348 (2001).
11. D. Brayden, E. Creed, A. O'Connell, H. Leipold, R. Agarwal, and A. Leone-Bay. Heparin absorption across the intestine: effects of sodium N-[8-(2-hydroxybenzoyl)amino]caprylate in rat *in situ* intestinal instillations and in Caco-2 monolayers. *Pharm. Res.* **14**:1772–1779 (1997).
12. J. L. Madara, D. Barenberg, and S. Carlson. Effects of cytochalasin D on occluding tight junctions of intestinal absorptive cells: Further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. *J. Cell Biol.* **102**:2125–2136 (1986).
13. J. L. Madara, J. Stafford, D. Barenberg, and S. Carlson. Functional coupling of tight junctions and microfilaments in T84 monolayers. *Am. J. Physiol.* **254**:G416–G423 (1988).
14. J. L. Madara. Intestinal absorptive cell tight junctions are linked to cytoskeleton. *Am. J. Physiol.* **253**:C171–C175 (1987).
15. P. Artursson, K. Palm, and K. Luthman. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Del. Rev.* **46**:27–43 (2001).
16. R. A. Baughman, S. C. Kapoor, R. A. Agarwal, J. Kisicki, F. Castella-Lawson, and G. A. FitzGerald. Oral delivery of anticoagulant doses of heparin. A randomized, double blind, controlled study in humans. *Circulation* **98**:1610–1615 (1998).
17. P. L. Paine, L. C. Moore, and S. B. Horowitz. Nuclear envelope permeability. *Nature* **254**:109–114 (1975).
18. W. L. Jiang and M. Schindler. Chemical factors that influence nucleocytoplasmic transport: a fluorescence photobleaching study. *J. Cell Biol.* **102**:853–858 (1986).
19. D. W. Stacey and V. G. Allfrey. Microinjection studies of protein transit across the nuclear envelope of human cells. *Exp. Cell. Res.* **154**:283–292 (1984).