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Novel bioadhesive drug delivery system protecting (poly)peptides from gastric enzymatic degradation

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Abstract

We have been developing a new generation of a drug delivery system based on the covalent attachment of a pepsin inhibitor (pepstatin A) to a bioadhesive carrier matrix. This approach involves covalent coupling of pepstatin to sodium carboxymethyl cellulose with the use of an appropriate spacer (1,8-diaminooctane). The protective effect of this novel matrix system was quantified by an enzyme assay, determining the degree of pepsinic degradation of inserted horseradish peroxidase. The result demonstrated a reduction of only 18.5 ± 4% (mean of three experiments; ± S.D.) of enzyme activity after 8 h of incubation with 0.05 N HCl containing 1.25 mg of pepsin per ml. Substitution of the conjugate with sodium carboxymethyl cellulose led to a 83 ± 15% (mean of three experiments; ± S.D.) loss of enzyme activity with the same approach. Inhibition of pepsinic degradation of inserted (poly)peptides could also be demonstrated by SDS-Page analysis. The pepstatin-matrix conjugate formed the basis for the development of a bioadhesive drug delivery system providing a controlled release of incorporated peptide drugs. Epidermal growth factor is a potent candidate for our system, which, when perorally administered, should maximize therapy in the treatment of gastric ulcers.

Keywords: Bioadhesive drug delivery system; Pepstatin; Epidermal growth factor; Sodium carboxymethyl cellulose; Pepsin; Gastric ulcers

1. Introduction

The majority of (poly)peptide drugs are most commonly administered by parenteral routes that are often complex, difficult, painful and occasionally dangerous (Meyer et al., 1990). The need for novel systems for an effective peroral delivery of these drugs is becoming increasingly recognised particularly as the number of potential candidates increases. Bioadhesive polymers have received considerable attention as platforms for controlled drug delivery because they can be localized in

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specified regions and have the ability to enhance the bioavailability of therapeutic agents by a prolonged residence time and an optimal contact with the absorbing surface. Moreover, a drug delivery system for the oral administration of therapeutic (poly)peptides should also exhibit a protective effect from luminal proteolytic inactivation. The development of such a bioadhesive drug delivery system protecting incorporated (poly)peptides from gastric enzymatic degradation was realized in this work to generate a system for the peroral administration of epidermal growth factor (EGF).

Because it is secreted from the submandibular glands into saliva, the significant role of luminal available EGF in the maintenance of the gastric mucosal defense system has already been discussed (Konturec et al., 1981). It is recognized as an important factor for acceleration of ulcer healing and has a peculiar biological property to repair tissue damage by an enhanced proliferation and differentiation of epithelial tissues (Hollenberg, 1979). The accelerated healing of gastroduodenal ulcers in animals after parenteral or oral application has already been shown in the past decade (Konturec et al., 1981; Skov et al., 1984; Poulsen et al., 1985).

Recently, Itoh and Matsuo (1994) demonstrated in a double-blind controlled clinical study the enhanced healing of rat gastric ulcers after oral administration of EGF. This effect could even be significantly increased by combining EGF with a hydroxypropyl cellulose matrix system as a vehicle for sustained-release. The result demonstrated the essential need for an appropriate galenic for the oral administration of EGF in the treatment of gastric ulcers.

Incubation of EGF with pepsin results in peptide degradation which is forced by increasing amounts of pepsin (Slomiany et al., 1990). This partial pepsinic proteolysis of perorally administrated EGF could play a significant role in the ability to prolong the luminal availability of intact EGF. To prevent this luminal degradation, the slow and tight-binding aspartyl protease inhibitor, pepstatin A (Cho et al., 1994), was covalently bound to the matrix system. On one hand this immobilization of pepstatin should keep the inhibitor concentrated on the matrix and on the other hand, it should make the inhibitor unabsorbable to avoid systemic side effects caused by the inhibition of physiological essential, but pepstatin A-sensitive enzymes (McCaffrey and Jamieson, 1993; Carmel, 1994; Plumpton et al., 1994).

The synthesis of such a bioadhesive drug delivery system protecting from pepsinic proteolysis should promise new aspects in the treatment of gastric ulcers with perorally administrated EGF.

2. Materials and methods

2.1. Synthesis of the pepstatin-spacer conjugate

The spacer 1,8-diaminooctane (Sigma, St. Louis, MO) was coupled to pepstatin (Calbiochem, San Diego, CA) by a condensation reaction with the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) catalysed by sulfo-*N*-hydroxysuccinimide (SNHS) following the procedure described by Staros et al. (1986). The carboxylic acid group of the inhibitor was covalently bound to the NH_2-group of the spacer by constituting an amide binding. The molar proportion of 1,8-diaminooctane to pepstatin was 12:1 to promote the binding of pepstatin to only one primary amino group of the spacer. The pH value of a solution containing 40 mg of EDAC, 2.5 mg of SNHS and 10 mg of 1,8-diaminooctane in 1.7 ml of demineralized water was adjusted to pH 6.5 with 2 N HCl. To this solution 4 mg of pepstatin in 0.3 ml of ethanol were added and the reaction mixture was stirred for 2 h at room temperature. After this, the conjugate was isolated by preparative TLC (layer: aluminium sheets silica gel 60 F_{254} (Merck, Darmstadt, Germany); layer thickness: 0.2 mm; mobil phase: n-butanol/acetic acid/H_2O (8 + 2 + 2); migration distance: 8 cm; detection: spraying with ninhydrin-reagent (0.3 g ninhydrin, 100 ml n-butanol, 3 ml acetic acid) or sodium nitroprusside-reagent (5 g of sodium nitroprusside, 10 ml of acetaldehyde and 90 ml of demineralized water added to 2 g of sodium carbonate in 100 ml of demineralized water before spraying) and heating to 100°C for 5 min; R_f of

the conjugate: 0.65–0.74). Between a R_f of 0.6 and 0.8, the silica gel was scraped off the aluminium sheets. The conjugate was eluted from the separated silica gel with methanol and brought to dryness (rotavapor: Heidolph WB 2001; 25°C).

2.2. Synthesis of the pepstatin-matrix conjugate

In the second step of the synthesis the isolated pepstatin-spacer conjugate was coupled to the matrix (sodium carboxymethyl cellulose, Na-CMC) by a condensation reaction as described above. The remaining NH_2-group of the spacer was covalently bound to the carboxyl group of the Na-CMC by constituting an amide binding (Fig. 1). To activate the carboxyl groups of the polymer, 4.0 mg of Na-CMC (Kwizda, Vienna, Austria), 3.0 mg of SNHS and 40 mg of EDAC were dissolved in 1.4 ml of demineralized water and stirred for 1 h at room temperature. To this reaction mixture the isolated pepstatin-spacer conjugate dissolved in 0.6 ml of ethanol was added and the reaction allowed to proceed for 2 h. After this, the reaction mixture was incubated with 1 mg of glycin (Merck, Darmstadt, Germany) for 0.5 h to saturate the remaining activated carboxyl groups of the polymer. The pepstatin-matrix conjugate was isolated by dialysis against demineralized water at 10°C for 16 h. The remaining unbound pepstatin was separated by adding methanol to the dialysate to a final concentration of 85% (v/v), incubating for 15 min and isolating the conjugate by centrifugation (Hermle Z 323 K, 13 000 rev./min, 20°C, 10 min). Washing the pepstatin-matrix conjugate with methanol was repeated four times to guarantee the separation of the whole unbound inhibitor. The purified coupling product was lyophilized and stored at 4°C.

2.3. Analysis of the conjugate

2.3.1. Enzyme assay

Pepsin inhibition studies of the conjugate were carried out with the following experiment: 12.5% of the isolated conjugate, 0.01 mg of pepsin (1:10 000; United States Biochemical, Cleveland, OH) and 0.05 mg of horseradish peroxidase (Sigma, St. Louis, MO) in 200 μl 0.05 N HCl were stirred for 1 h at 37°C; 100 μl of this mixture were transferred to the first well of a microtitration plate (96-well, not binding) and diluted in 1:2 steps with 0.05 N HCl in the following wells; 50 μl of the substrate medium (20 mg o-phenylendiamine dihydrochloride, 7.77 ml 0.1 M Na_2HPO_4, 2.33 ml H_2O and 20 μl 30% H_2O_2) were added and the enzymatic reaction was allowed to proceed at room temperature for 10 min. Optical densities were read at 492 nm with a microtitration plate reader (Anthos reader 2001). Reaction mixtures containing pure Na-CMC and different pepstatin concentrations instead of the inhibitor-matrix conjugate, were used as references. For a positive control pepsin was omitted during 1 h preincubation with 0.05 N HCl.

2.3.2. SDS-Page

With the same approach as described above, horseradish peroxidase was either substituted by 0.2 mg of bovine serum albumine (BSA) (Sigma, St. Louis, MO) or 0.2 mg of bovine insulin (Sigma, St. Louis, MO). After 1 h incubation at 37°C, 10-μl samples of the enzymatic reaction mixtures were analyzed by SDS-Page. On Coomassie stained gels, the degree of proteolysis was determined by comparing bands of these proteins with bands of gradually degraded BSA or bovine insulin resulting from different pepstatin concentrations during the enzymatic reaction.

2.4. Preparation and analysis of the drug delivery system

Ten milligrams of pepstatin-matrix conjugate, 50 mg of Na-CMC, 30 mg of mannit (Merck, Darmstadt, Germany), 9 mg of bovine insulin and 1 mg of horseradish peroxidase were homogenized and pressed (Hanseaten Type EI, Hamburg, Germany) to tablets (diameter: 5.0 mm; depth: ~4 mm). The pressing power was kept constant during the preparation of all tablets.

The protective effectiveness of this drug delivery system was determined by an enzyme assay as well as SDS-Page analysis. For this, tablets were incubated for 8 h at 37 ± 0.5°C in flow through

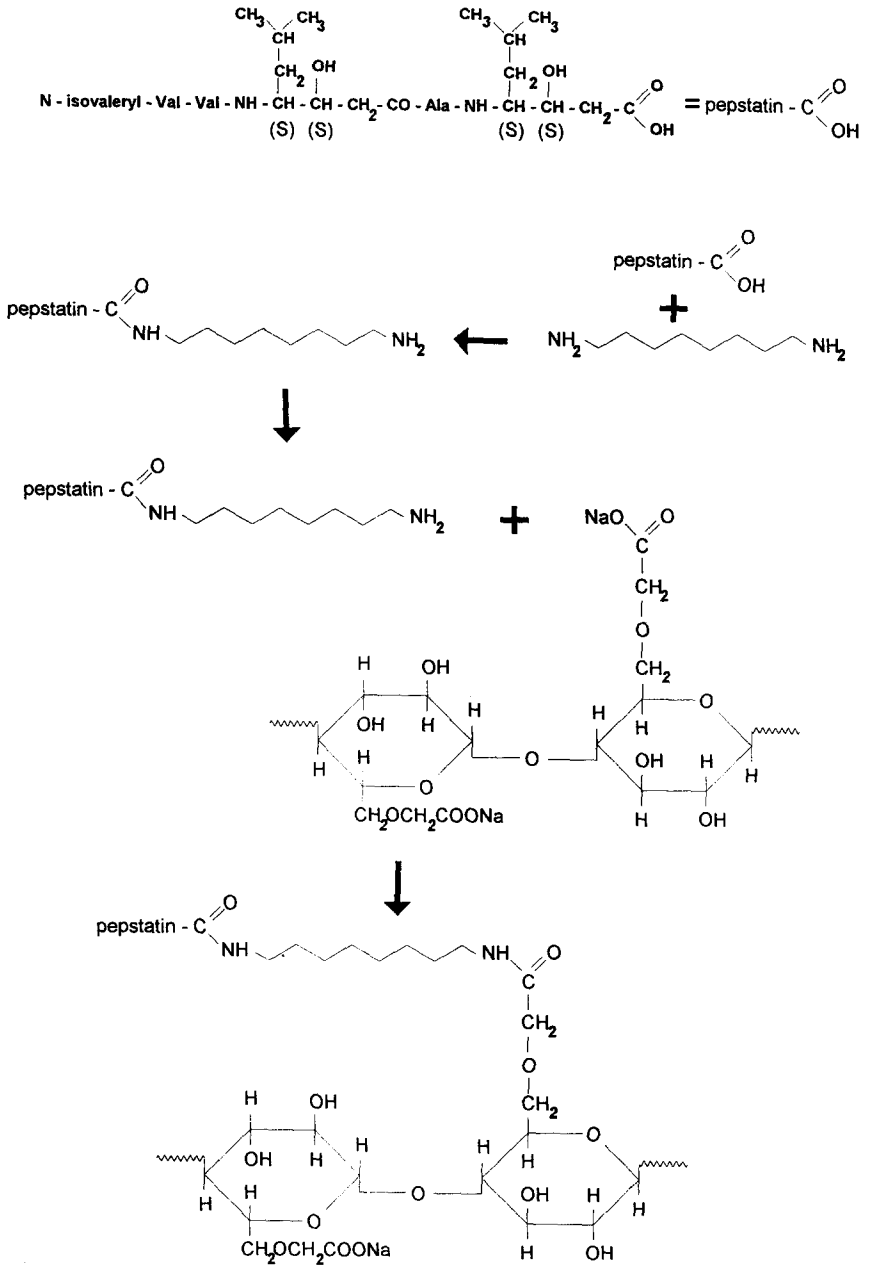

Fig. 1. Synthetic pathway of the covalent attachment of pepstatin A to the polymer matrix sodium carboxymethyl cellulose.

cells (Desaga, Heidelberg, Germany) with 100 ml of circulating 0.05 N HCl containing 1.25 mg of pepsin (1:10 000) per ml. The swollen matrices were frozen for 1 h at −20°C and 10% aliquots were withdrawn by dividing the matrices with a scalpel in well defined parts. These samples were diluted 1:17 with 0.05 N HCl and aliquots of 100 μl were transferred to the first well of the microtitration plate, followed by the procedure of the enzyme assay as described above. SDS-Page

analyses were done on 5-μl samples of the frozen matrices.

2.5. Release studies

The in vitro release rate of bovine insulin and pepstatin from the drug delivery system was analyzed by a method which does not conform to the United States Pharmacopeia (USP). Because of the small amount of protein or inhibitor in the delivery system, tablets were placed in a 25-ml beaker (Schott, Duran 25 ml, Germany) containing 10 ml of release medium (0.05 N HCl). The vessels were closed, placed on a waterbath-shaker (GFL 1092; 30 rev./min) and incubated at 37 $\pm$ 0.5°C; sink conditions were maintained throughout the study; 1.0 ml samples of released protein or enzyme inhibitor respectively were withdrawn at 1-h intervals and replaced with an equal volume of release medium preequilibrated to temperature. For release studies of bovine insulin the amount of horseradish peroxidase in the tablet was substituted by bovine insulin. The liberation behavior of unbound pepstatin from tablets containing 60 mg of Na-CMC, 39 mg of mannit and 1 mg of unbound pepstatin instead of the pepstatin-matrix conjugate was determined. The inhibitor and bovine insulin were assayed by measuring the absorbance spectrophotometrically (Perkin-Elmer) at 220 nm and 280 nm, respectively. Concentrations were calculated by interpolation from standard curves. Withdrawn samples of released pepstatin were previously brought to dryness and suspended in a mannit saturated ethanolic solution. After centrifugation the supernatant and a mannit saturated ethanolic solution — used as reference — were brought to dryness, dissolved in demineralized water and quantified.

3. Results

3.1. Preparation of the pepstatin-Na-CMC conjugate

The pepstatin-sodium carboxymethyl cellulose conjugate was prepared according to the synthetic scheme shown in Fig. 1. The isolated pepstatin-spacer conjugate had a R_f of 0.65–0.74, which is between the R_f of pepstatin (0.9) and 1,8-diaminooctane (0.2–0.3). Although pepstatin A was not detectable with ninhydrin spraying reagent, the band of the conjugate was ninhydrin positive. Because of the selectivity of this reagent for primary amines, the successful coupling of the spacer to the inhibitor as well as the free availability of the remaining NH_2-group after the coupling reaction could be verified. TLCs of reaction mixtures prepared at -20°C were used as negative control and did not exhibit the band of the conjugate. After isolation by preparative TLC the conjugate appeared as a single ninhydrin band as well as a sodium nitroprusside positive band on the gel. It could be dissolved in methanol, ethanol or DMSO and showed an inhibition of pepsinic degradation of peroxidase, bovine insulin and BSA.

The coupling of this conjugate to Na-CMC was largely influenced by the concentration of ethanol during the reaction. On one hand ethanol concentrations higher than 30% caused partial to complete insolubility of Na-CMC and led to lower or no inhibitory effect of the isolated pepstatin-matrix conjugate, respectively. On the other hand, ethanol concentrations lower than 30% caused insolubility of pepstatin which also decreased the inhibitory effects of the synthesis product. For the isolation of the conjugate it was necessary to purify it first of all from coupling reagents by dialysis against demineralized water and then from unbound pepstatin by washing it several times with ethanol. A synthesis product prepared and isolated in the same way but using pepstatin instead of the pepstatin-spacer conjugate during the coupling reaction showed no inhibitory effect in the enzyme assay, demonstrating the efficiency of this purification method. However, 1 mg of isolated pepstatin-matrix conjugate exhibited an inhibitory effect in the enzyme assay which was equivalent to 37 μg of pure pepstatin. This result could be confirmed by SDS-Page analysis of inserted proteins.

3.2. Analysis of the drug delivery system

To guarantee a sustained release of EGF over a period of at least 8 h, we investigated the release profile of our system with Na-CMC concentrations between 20% and 80%. Because of financial reasons, we used bovine insulin instead of human EGF as a model drug for release studies which has almost the same molecular mass (EGF: 6045 Da; bovine insulin: 5734 Da). The percentage of model drug released within the first 8 h correlates with the percentage of polymer in the tablet. A 60% portion of the polymer on the whole drug delivery system demonstrated a release of 22.3% of the model drug (bovine insulin) within the first hour and in the following 7 h a release rate of 7.7% per hour. The release of bovine insulin from the drug delivery system is illustrated in Fig. 2.

Release studies of unbound pepstatin demonstrated within the first 3 h a liberation of 98 ± 1.9% (mean of three experiments; ± S.D.) inhibitor per hour. The remaining pepstatin amount in the matrix system could be determined from the concentration at the plateau phase in the release profile (Fig. 3); 2 ± 1.9% of the inserted pepstatin remained after 8 h in the matrix system.

Tablets containing 10% conjugate were incubated for 8 h with a pepsin containing medium and showed a protective effect from enzymatic degradation of inserted horseradish peroxidase or bovine insulin. The enzymatic activity of horseradish peroxidase after incubation was reduced to 81.5 ± 4% (mean of three experiments; ± S.D.). Substituting the conjugate by Na-CMC in the same approach demonstrated a remaining activity of 17 ± 15% (means of three experiments; ± S.D.). Results could be confirmed by SDS-Page analysis of inserted bovine insulin and are illustrated in Fig. 4.

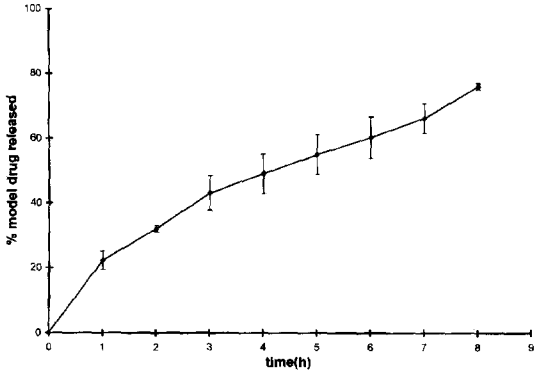

Fig. 2. Release profile of bovine insulin from the drug delivery system containing 10 mg of the pepstatin-matrix conjugate, 50 mg of Na-CMC, 30 mg of mannit and 10 mg of bovine insulin. The system was incubated in 10 ml release medium (0.05 N HCl) at 37 ± 0.5°C. Each point represents the mean ± S.D. of three experiments.

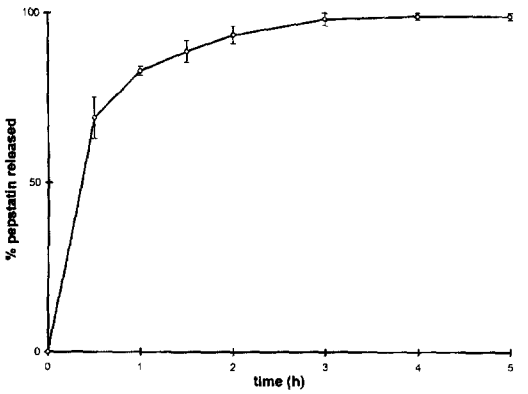

Fig. 3. Release profile of pepstatin A from the drug delivery system containing 60 mg of Na-CMC, 39 mg of mannit and 1 mg of unbound pepstatin. The system was incubated in 10 ml release medium (0.05 N HCl) at 37 ± 0.5°C. Each point represents the mean ± S.D. of three experiments.

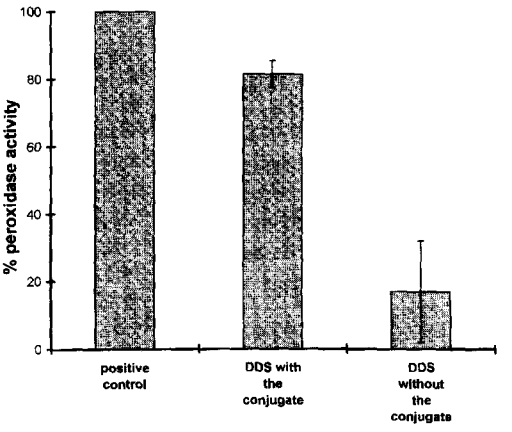

Fig. 4. Protective effect of the drug delivery system (DDS) with and without the pepstatin-polymer conjugate from enzymatic degradation of horseradish peroxidase incorporated into the matrix (means of three experiments; ± S.D.). DDSs (except the positive control) were incubated for 8 h in 0.05 N HCl containing 1.25 mg of pepsin (1:10 000) per ml.

4. Discussion

In the treatment of gastric ulcers with perorally administered EGF, a prolonged residence time of the delivery system in the stomach has an important influence on the bioavailability of the drug. Bioadhesive polymer systems seem to be able to guarantee a specific localisation of the drug delivery system on affected regions of the gastrointestinal tract. Ch'ng et al. (1985) demonstrated in this connection that about 50% of a bioadhesive polymer (poly(acrylic acid-divinylglycol)) remained in rat stomach 12 h after administration. Sodium carboxymethyl cellulose is also an anionic bioadhesive polymer with almost the same mucoadhesive force as poly(acrylic acid-divinylglycol) (Junginger and Lehr, 1990); it has no toxic effects and is commonly used as a vehicle for controlled release. For these reasons, it was selected for the synthesis of our drug delivery system. 1,8-Diaminooctane was used as a spacer to provide the free availability of matrix bound pepstatin. Structural analysis of pepstatin by testing the inhibitory activity of several synthetic analogues demonstrated the major structural function of the statine components (Fig. 1), which seem to be responsible for the inhibitory activity (McConnell et al., 1991). Hence, the coupling of the spacer to the COOH-group of pepstatin by forming an amide should not influence its inhibitory activity. The solubility of pepstatin during the coupling reaction was guaranteed by an ethanol concentration of 30%. Although alcohols can interfere with carbodiimide mediated coupling reactions of primary amines with carboxylic acids (Goodfriend et al., 1966), a successful covalent binding of pepstatin to the polymer in the presence of 30% ethanol was possible. The inhibition of pepsinic degradation of horseradish peroxidase, BSA and bovine insulin in the presence of the pepstatin-Na-CMC conjugate demonstrated on one hand the free availability of the immobilized inhibitor and on the other hand the preservation of pepstatin activity after having modified its structure by forming an amide binding to the spacer.

According to the method of the United States Pharmacopeia (USP), pepsin activity is determined by measuring the peptic hydrolysis of hemoglobin. But to investigate the protective effect of the conjugate from enzymatic inactivation of therapeutic (poly)peptides incorporated in the matrix system, we had to develop a new method. Measuring the reduction of peroxidase activity inside the delivery system caused by peptic degradation offers several advantages. It allows an exact determination of the pepsin inhibitory activity, samples withdrawn from different parts of the matrix system can be directly and also easily investigated and the reduction of peroxidase activity inside the delivery system is comparable with a possible activity loss of inserted therapeutic agents.

Because of earlier investigations about gastrointestinal transit times of bioadhesive polymers (Ch'ng et al., 1985), our drug delivery system was generated under the assumption of an at least 8-h residence time of the pepstatin-sodium carboxymethyl cellulose conjugate in the stomach. During this time, EGF should be on one hand protected from pepsinic degradation and on the other hand sustainedly released from the matrix system. The release of (poly)peptides from sodium carboxymethyl cellulose mainly depends on their molecular mass (Achleitner, 1995). Therefore we used a model drug of almost the same molecular mass as EGF for release studies. A controlled release of 7.7% model drug per hour should guarantee the availability of the drug during the adhesion in the stomach. Itoh and Matsuo (1994) combined EGF with only 2% of hydroxypropyl cellulose which caused a 30 times higher gastric drug level than without this adjuvant within the first 3 h after administration. Our release studies demonstrated that there are much higher cellulose derivative concentrations necessary to maintain a sustained release during the whole period of virtual gastric bioadhesion. With the novel drug delivery system described here, increased gastric EGF levels for a period of at least 8 h should become possible but have to be verified by subsequent in vivo studies.

The adherence ability of the drug delivery system could perhaps be improved by the covalent binding of bacterial adhesins to the polymer. Bacterial adhesins are able to bind specifically to GI epithelial receptors and should therefore ensure a

very intimate contact of the drug delivery system with the absorbing membrane. Proteolytic degradation of EGF on the way out of the drug delivery system to the gastric epithelium should become negligible. Bernkop-Schnürch et al. (1995) have already bound fimbriae to a drug delivery system and demonstrated the specific receptor-binding capacity of the coupled adhesin. With regard to this, it should be possible to generate similar drug delivery systems especially designed for a specific attachment to the gastric mucosa. The isolated cell wall lipopolysaccharide of *Helicobacter pylori*, binding specifically to the laminin receptor on the gastric epithelial surface (Valkonen et al., 1994), seems to be a suitable candidate. Direct adhesion of such delivery systems to the mucosa should be possible at breaches of the mucus barrier, which are often responsible for gastric ulcers (Copeman et al., 1994). An only local reduction of the remaining mucus could be provided through the addition of mucolytic substances i.e. *N*-acetylcysteine (Harms and Bertele-Harms, 1994; Sangaletti et al., 1994) to the drug delivery system. In this case, the pepstatin-matrix polymer should take over the protective effect of the lacking mucus barrier from peptic degradation.

In summary, the novel drug delivery system described here offers numerous potential advantages in the treatment of gastric ulcers. The covalent coupling of pepstatin to the matrix system should guarantee a protective effect of inserted EGF from peptic degradation. The immobilization keeps the inhibitor concentrated on the matrix system and prevents its absorption followed by possible toxic side effects. The bioadhesive polymer provides a prolonged residence time in the stomach and a sustained release of the therapeutic agent for at least 8 h.

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