ELSEVIER

International Journal of Pharmaceutics 126 (1995) 139–145

international journal of pharmaceutics

In vitro study for the assessment of poly(L-aspartic acid) as a drug carrier for colon-specific drug delivery

Claudia S. Leopold*, David R. Friend

Controlled Release and Biomedical Polymers Department, SRI International, Menlo Park, CA, USA

Received 13 January 1995; accepted 18 April 1995

Abstract

Selective delivery of drugs to the colon for the treatment of large bowel disorders can be achieved using prodrugs which are cleaved by enzymes produced by microorganisms residing in the large intestine. Since it is known that enzymes of gut microflora are able to cleave certain peptide and ester bonds, the ability of poly(L-aspartic acid) (weight-average MW: 30000) to act as a drug carrier for the model drug dexamethasone (DX) was investigated. The ester prodrug (10% w/w drug loading) was synthesized in high yield using dicyclohexylcarbodiimide as dehydrating agent in dimethylformamide. Prodrug incubations were carried out in the homogenized luminal contents of various GI-tract segments of male Sprague–Dawley rats at 37°C and pH 6.5 (stomach at pH 4.5) and prodrug concentrations which provide enyzme saturated conditions. DX concentrations were measured by HPLC. In addition, incubations were done with the homogenized mucosa and muscle tissues at pH 4.5 (pH of the lysosomal compartment) and at pH 6.5. The half-life $t_{1/2}$ of chemical hydrolysis at these pH-values (37°C) was 114 days and 2.8 days, respectively. Enzymatic activity of the mucosa and the tissue samples was low ($<$ 10 μg DX/g mucosa or tissue per h); in contrast, markedly higher activities were found in the luminal contents. The cecum and colon contents showed significantly ($P < 0.01$) higher enzymatic activities (60 and 80 μg DX/g content per h, respectively) than the contents of the stomach or the small intestine (5–20 μg DX/g content per h). With the intestinal contents of colitic rats, lower enzymatic activities were found than with the contents of the conventional rats. Negligible enzymatic activities in the cecum and colon contents of germ-free animals indicate that bacterial enzymes are responsible for the cleavage of the prodrug. Incubations with human feces samples from colitic patients and non-colitic patients result in prodrug hydrolysis of 26–40 μg DX/g feces per h with no significant difference between the two groups. According to the present results, poly(L-aspartic acid) seems to be a suitable drug carrier for colon-specific drug delivery.

Keywords: Colon-specific drug delivery; Poly(L-aspartic acid); Dexamethasone; Ester hydrolysis; Germ-free rat; Colitic rat; Human feces

* Corresponding author at present address: Department of Pharmaceutical Technology, Heinrich-Heine-University, Universitätsstr.1, 40225 Düsseldorf, Germany. Tel/Fax: +49 211 3114225; E-mail: leopold@uni-duesseldorf.de

SSDI 0378-5173(95)04107-L

1. Introduction

During peroral treatment of large bowel disorders the selective delivery of drugs to the large intestine is desirable in order to lower the drug dose and to reduce side effects. Besides the administration of formulations with time-controlled and pH-controlled drug release, respectively, selective delivery to the colon may be achieved using prodrugs, which are cleaved by enzymes produced by microorganisms residing in the large intestine. These prodrugs include glycosides (Friend and Chang, 1984, 1985; Friend et al., 1991; Friend, 1992; Friend and Tozer, 1992), glucuronides (Haeberlin et al., 1993a,b,c), dextran esters (Harboe et al., 1989; Larsen et al., 1989, 1991; McLeod, 1992; McLeod and Tozer, 1992), amides (Nakamura et al., 1992a,b,c,d,e) and azo compounds (Chan et al., 1983; Fleig et al., 1988; Rijk et al., 1992). It is known that enzymes of gut microflora are able to cleave certain amide and ester bonds (Scheline, 1968, 1973; Williams, 1972; Boxenbaum et al., 1974; MacFarlane and Allison, 1986; Shamat, 1993). Therefore, the ability of poly(L-aspartic acid) (weight-average MW: 30000) to act as a drug carrier for steroids with a hydroxy group at C_{21} was investigated. Dexamethasone (DX) was used as model drug, since corticosteroids are widely used in the treatment of inflammatory bowel disease and it reportedly shows linear pharmacokinetics in man (Rohdewald et al., 1987). Because of the high molecular weight and the anionic structure of the prodrug created by the remaining free carboxy groups, early absorption in the small intestine is unlikely to occur.

2. Materials and methods

2.1. Materials

Dexamethasone (U.S.P., micronized) was purchased from Upjohn Co. (Kalamazoo, MI). Triamcinolone acetonide and poly(L-aspartic acid) (sodium salt, weight-average MW: 30000) were obtained from Sigma Chemical Co. (St. Louis, MO). Spectra/Por® CE molecularporous membrane (MWCO 1000) was purchased from Spectrum Medical Industries, Inc. (Houston, TX). All the other chemicals and solvents were of high purity or analytical grade and were used as received.

2.2. Preparation of dexamethasone-poly(L-aspartate) (DXPA)

The ester prodrug was synthesized in high yield using dicyclohexylcarbodiimide as dehydrating agent in dimethylformamide (Neises and Steglich, 1978). The sodium salt of poly(L-aspartic acid) was converted into the free acid form by adjusting the aqueous salt solution to a pH of 1.5 with hydrochloric acid. This solution was freeze-dried and the solid residue was dissolved in dimethylformamide. The insoluble sodium chloride was removed by centrifugation of the mixture and decanting the supernatant. A volume of that supernatant (about 4 ml), containing 130 mg of poly(L-aspartic acid), was transferred to a test tube and 0.3 mmol dexamethasone (DX) and catalytic amounts of 4-dimethylaminopyridine were added to the stirred solution under argon atmosphere. After cooling the mixture to 0°C, the reaction was started by adding 1.3 mmol of dicyclohexylcarbodiimide. The reaction mixture was then stirred for 5 min at 0°C and 3 h at 20°C. After pouring the mixture into 10 ml of water and extracting three times with 25 ml of diethyl ether, it was dialyzed for 15 h (Spectra/Por® CE molecularporous membrane, MWCO 1000) and then freeze-dried. The resulting white polymer powder was again washed twice with diethyl ether and dried. Under these conditions the drug loading was less than 20% (w/w), determined by prodrug hydrolysis under alkaline conditions. A higher drug loading leads to limited solubility at pH values below 7. The structure of the prodrug was confirmed by ^{1}H-NMR.

The hydrolysis experiments were performed with a batch of DXPA, containing 10 mg of dexamethasone per 100 mg prodrug.

2.3. Animals

The gastrointestinal (GI) tracts of three different groups of non-fasted male Sprague–Dawley rats (250–300 g, 7–8 weeks old) were used for the in vitro hydrolysis study. Conventional rats were purchased from Simonsen Labs (Gilroy, CA) and colitic rats were obtained from the Division of Gastroenterology of the University of Alberta (Edmonton, Canada). Pan-colitis was induced by flushing 4% acetic acid through the cecal-ascending junction and out the rectum (Fedorak et al., 1990). The rats were killed 48 h after induction of the colitis. Germ-free rats were obtained from Gnotobiote Laboratory of the University of Wisconsin (Madison, WI). After killing the animals by intraperitoneal injection of pentobarbital the GI-tracts were isolated. The latter were segmented into stomach, small intestine, cecum, and colon. The small intestine was further divided into proximal and distal segments of equal length. All GI-tract segments were stored at −30°C until required. For the experiments under anaerobic conditions, the GI-tract was segmented immediately before incubation under nitrogen atmosphere in order to prevent exposure to oxygen. The openings at the stomach and the colon were knotted with suture during the storage period.

2.4. Hydrolysis study

2.4.1. Chemical prodrug hydrolysis

For determination of the half-life $t_{1/2}$ of chemical hydrolysis, 10 μl of a DXPA stock solution (2 mg DXPA/1 ml water) were added to each of 24 test tubes, containing 965 μl of 0.1 M acetate buffer pH 4.5 and 0.1 M phosphate buffer pH 6.5, respectively. The test tubes were incubated at 37°C in a shaking water bath. Samples were withdrawn and analyzed with high performance liquid chromatography (see below) at 20 different time points over 4 days. The hydrolysis rate constants were calculated from the slope of the straight line, which resulted from a semi-logarithmic plot of the amount of released DX from the prodrug versus time.

2.4.2. Preparation of GI-tract homogenates

The amounts of DX released from DXPA were measured in the homogenized contents and tissues of rat stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon. Preparation of homogenates of the luminal contents was done following a procedure similar to that described by Haeberlin et al., 1993c. Briefly, thawed GI tract segments were squeezed out and the weight of the contents was determined. Contents of the rat small intestines and the human feces samples were diluted with chilled 0.9% NaCl to 20% slurries, and cecum and colon contents to 5–10% slurries, respectively. The diluted contents were homogenized under ice-cooling with an Ultra-Turrax homogenizer (type TP18/10SI, 20000 rev./min, Janke and Kunkel, IKA Werk, Staufen, Germany). These homogenates were stored at −30°C until required for incubation. For the experiments under anaerobic conditions, these steps were carried out in a plastic chamber with nitrogen flowing through. The mucosa samples were obtained by placing the rinsed intestines which had been cut open longitudinally on a cold surface and scraping off the mucosa with the edge of a microscopic slide. The mucosal scrapings were weighed and diluted to 8–10 ml with 0.9% NaCl before homogenization. After determining the weight of the remaining muscle layers they were cut in small pieces, then diluted with 20 ml of 0.9% NaCl and homogenized by a Kinematica Polytron (type PCU 1; Brinkman Instruments, Westbury, NJ).

2.4.3. Prodrug hydrolysis in GI-tract homogenates

Incubations were performed at pH 6.5, the average pH of the cecum and colon lumen, with all homogenates, except for the stomach contents which were incubated at pH 4.5. Homogenates of the GI mucosa and tissues were also incubated at pH 4.5 which is close to the pH optimum of many enzymes of the lysosomal compartment. The pH of the incubation mixtures was adjusted with 0.1 M sodium phosphate buffer pH 6.5, and 0.1 M sodium acetate buffer pH 4.5, respectively. All incubations were run at 37°C in a shaking water bath. For the incubations of the prodrug the homogenates (0.25 ml of stomach and large intes-

tinal contents, 0.75 ml of small intestinal contents and 1.0 ml of mucosa or tissue homogenate, respectively) were diluted with buffer to a volume of 1.80 ml as described by Haeberlin et al., 1993c. After preincubation at 37°C a 5 mM solution of DXPA was added (0.20 ml), resulting in a substrate concentration of 0.5 mM. Preliminary experiments with various substrate concentrations had been performed in order to confirm that the actual substrate concentration provides zero order hydrolysis kinetics. Saturated conditions are required in order to compare hydrolysis rates in the different homogenates investigated. Aliquots of the incubation mixture were withdrawn at zero time point and after 4 h and immediately mixed with chilled saturated aqueous sodium chloride and the internal standard. The extent of prodrug hydrolysis was determined by high performance liquid chromatography (HPLC) as described below. Blanks for all incubations were obtained by mixing the corresponding amounts of homogenate with the respective buffer. In order to ensure that chemical hydrolysis of the prodrug does not significantly contribute to the overall enzymatic activity the respective amount of prodrug was also incubated in buffer without adding homogenate. The hydrolytic activity present in the various homogenates was expressed as enzymatic activity, i.e. μg dexamethasone released per g homogenate and hour ($\mu g \times g^{-1} \times h^{-1}$), and was calculated by dividing the amount of released dexamethasone by the amount of GI tract (contents, mucosal scrapings, and muscle tissue, respectively) in the incubation mixture and the time period of incubation.

For statistical evaluation of the enzymatic activities ANOVA was used after logarithmic transformation of the data followed by Duncan test.

2.5. Analytical methods

After incubation all samples were extracted twice with 3 ml of a mixture of methyl-*t*-butyl ether/pentane (6:4, v/v) and the organic extracts were evaporated to dryness under argon atmosphere. The residue was reconstituted in acetonitrile/water (1:1, v/v) and after centrifugation, the supernatants (80 μl) were injected into the HPLC (Waters Associates, Inc., Milford, MA). DX concentrations in the samples were measured by reversed phase HPLC (UV-absorption at 246 nm) with triamcinolone acetonide as internal standard (Haeberlin et al., 1993c). A mixture of 0.02 M sodium acetate buffer (pH 4.8) and acetonitrile (68:32, v/v) was used as mobile phase. The separation was performed on a CRI C-102-R column (10 μm, 3.9 × 300 mm^2).

Since commercially available esterases were not able to hydrolyze DXPA, the remaining amount of unhydrolyzed prodrug in the samples was not determined.

3. Results and discussion

The presented in vitro study was done to evaluate the suitability of poly(L-aspartic acid) as polymeric drug carrier for colon-specific drug delivery. In the past, dextranes have been widely investigated for this purpose (Harboe et al., 1989; Larsen et al., 1989, 1991; McLeod, 1992; McLeod and Tozer, 1992). A high molecular weight of the prodrug leads to very limited absorption in the small intestine. In addition, the second carboxy group of the amino acid L-aspartic acid provides an anionic structure of the polymer molecule at higher pH values due to the remaining free carboxy groups since in the prodrug only few of them are linked to a drug molecule.

The half-life $t_{1/2}$ of chemical prodrug hydrolysis at pH 4.5 and 6.5 (37°C) was 114 days and 2.8 days, respectively. This finding indicates that chemical hydrolysis plays an insignificant role in the overall hydrolytic activity.

The results of the in vitro incubation experiments are shown in Figs. 1–5. High enzymatic activities were found in the luminal contents of the large intestine (Fig. 1). The cecum and colon contents showed significantly higher ($P < 0.01$) enzymatic activities than the contents of the stomach or the small intestine. This indicates a rather low contribution of host enzymes to prodrug hydrolysis. The prodrug ester bonds seem to be protected against hydrolysis by digestive enzymes due to the structure of the carrier poly(L-aspartic acid). It has been found with dextran-dexam-

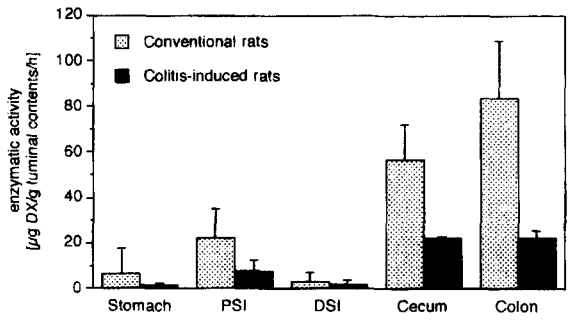

Fig. 1. Enzymatic activity (μg DX/g per h) of prodrug hydrolysis in homogenates of luminal contents from various GI tract segments of conventional and colitis-induced rats at pH 6.5 (stomach: pH 4.5) under enzyme saturated conditions. Data are means ± S.D. ($n = 3$).

ethasone prodrugs that the polymer backbone is first cleaved enzymatically into smaller fragments to allow esterases access to the polymer-drug ester bonds (McLeod, 1992). A statistically significant difference between the enzymatic activity of the proximal and the distal part of the small intestine could not be found.

The influence of atmospheric oxygen on the enzymatic activity of the large intestinal contents of conventional rats was investigated in preliminary experiments by measuring prodrug hydrolysis under anaerobic conditions (nitrogen atmosphere). No significant influence of oxygen on the extent of prodrug hydrolysis could be found (data not shown).

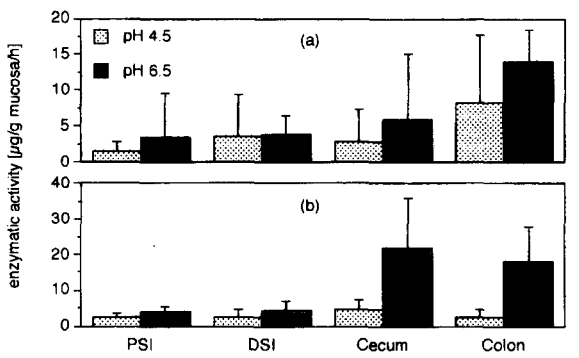

Fig. 2. Enzymatic activity (μg DX/g per h) of prodrug hydrolysis in homogenates of mucosal scrapings from various GI-tract segments of conventional (a) and colitis-induced (b) rats at pH 4.5 and 6.5 under enzyme saturated conditions. Data are means ± S.D. ($n = 3$).

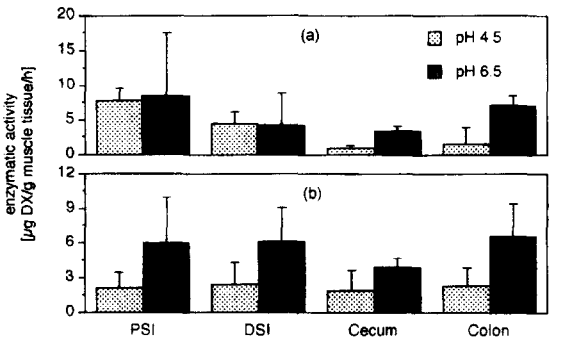

Fig. 3. Enzymatic activity (μg DX/g per h) of prodrug hydrolysis in homogenates of intestinal tissues (muscle layer) from various GI-tract segments of conventional (a) and colitis-induced (b) rats at pH 4.5 and 6.5 under enzyme saturated conditions. Data are means ± S.D. ($n = 3$).

The lower enzymatic activities in the intestinal contents of colitis-induced rats (Fig. 1) may be explained by dilution because of the increased secretory activity of the inflamed intestinal mucosa. A compromised hydrolytic activity of the bacterial enzymes, i.e. due to a change in the composition of the microflora, may contribute to the reduced hydrolytic activity. A low hydrolytic activity resulting in prolonged release from its carrier may be advantageous if the distal part of the colon has to be treated.

In contrast to the luminal contents the enzymatic activity of the mucosa and the muscle tissue samples was low (Figs. 2 and 3). However, the mucosal hydrolytic activity of the large intestine in colitic rats seems to be more pronounced than

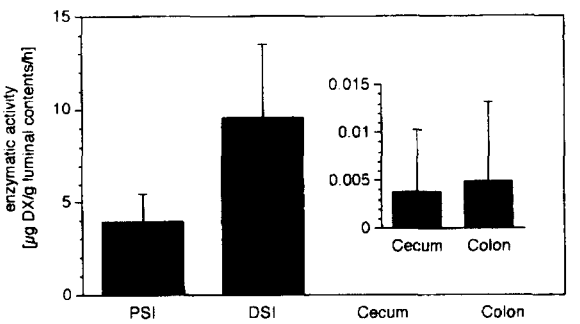

Fig. 4. Enzymatic activity (μg DX/g per h) of prodrug hydrolysis in homogenates of luminal contents from various GI-tract segments of germ-free rats at pH 6.5 under enzyme saturated conditions. Data are means ± S.D. ($n = 3$).

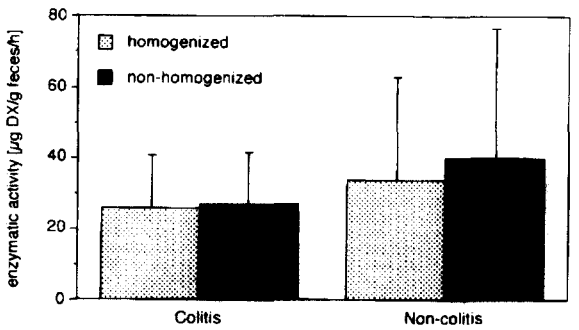

Fig. 5. Enzymatic activity (μg DX/g per h) of prodrug hydrolysis in homogenates of human feces from healthy volunteers and colitis patients at pH 6.5 under enzyme saturated conditions. Data are means $\pm$ S.D. ($n = 4$).

in conventional rats. This is advantageous in view of the fact that in ulcerative colitis the actual inflammatory lesions are located in the mucosa. Hydrolysis rates at pH 4.5 were slightly lower than at pH 6.5, probably due to the limited prodrug solubility at that lower pH. The higher enzymatic activity in the mucosal scrapings of the colon (conventional rat) may partly result from adsorption of colon contents to the mucosa. Negligible enzymatic activities in the large intestinal contents of germ-free animals (Fig. 4) confirm that gut microflora are responsible for prodrug cleavage. Activities in the contents of the proximal and distal small intestine are in the same order of magnitude as in the small intestinal contents of the conventional rats (Figs. 1 and 4). This finding means that host enzymes are mainly responsible for the observed prodrug hydrolysis in germ-free rats. Coprophagy does not seem to contribute significantly to the overall small intestinal enzymatic activity.

As expected, human feces samples show lower enzymatic activities than rat colon contents (Fig. 5). There is no significant difference between healthy volunteers and colitis patients in terms of fecal prodrug hydrolysis rate perhaps due to the low number of observations. Homogenization of the samples does not lead to a significant increase of the enzymatic activity which means that the contribution of intracellular bacterial enzymes to the total enzymatic activity is negligible (Fig. 5).

The extent of prodrug hydrolysis in the large intestinal contents of conventional rats indicate that the microorganisms of the large intestine are able to hydrolyze the prodrug. Low enzymatic activities in the cecum and colon contents of germ-free rats support the hypothesis that the bacterial microflora are responsible for the cleavage of the prodrug in the large intestine. The poly(L-aspartic acid) backbone apparently protects the prodrug ester bonds against hydrolysis by host enzymes as it is known to do for dextran prodrugs (McLeod, 1992). Prodrug cleavage in human feces is not as pronounced as in rat colon contents, but the inflamed colon does not seem to cause a significant decrease of the prodrug hydrolysis rate.

Acknowledgements

This work was supported by a fellowship from the German National Science Foundation and in part by NIH Grant 35147.

References

Boxenbaum, H.G., Jodhka, G.S., Ferguson, A.C., Riegelman, S. and MacGregor, T.R., The influence of bacterial gut hydrolysis on the fate of orally administered isonicotinuric acid in man. *J. Pharmacokin. Biopharm.*, 2 (1974) 211–237.

Chan, R.P., Pope, D.J., Gilbert, A.P., Sacra, P.J., Baron, J.H. and Lennard-Jones, J.E., Studies of two novel sulfasalazine analogs, ipsalazide and balsalazide. *Dig. Dis. Sci.*, 28 (1983) 609–615.

Fedorak, R.N., Empey, L.R., MacArthur, C. and Jewell, L.D., Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats. *Gastroenterology*, 98 (1990) 615–625.

Fleig, W.E., Laudage, G., Sommer, H., Wellmann, W., Stange, E.F. and Riemann, J., Prospective, randomized, double-blind comparison of benzalazine and sulfasalazine in the treatment of active ulcerative colitis. *Digestion*, 40 (1988) 173–180.

Friend, D.R., Glycosides in colonic drug delivery. In D.R. Friend (Ed), *Oral Colon-Specific Drug Delivery*, CRC Press, Boca Raton, FL, 1992, pp. 153–187.

Friend, D.R. and Chang, G.W., A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. *J. Med. Chem.*, 27 (1984) 261–266.

Friend, D.R. and Chang, G.W., Drug glycosides: potential prodrugs for colon-specific drug delivery. *J. Med. Chem.*, 28 (1985) 51–57.

Friend, D.R. and Tozer, T.N., Drug glycosides in oral colon-specific drug delivery. *J. Controlled Release*, 19 (1992) 109–120.

Friend, D.R., Phillips, S. and Tozer, T.T., Colon-specific drug delivery from a glucoside prodrug in the guinea-pig. Efficacy study. *J. Controlled Release*, 15 (1991) 47–54.

Haeberlin, B., Empey, L., Fedorak, R., Nolen III, H. and Friend, D., In vivo studies in the evaluation of glucuronide prodrugs for novel therapy of ulcerative colitis. *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, 20 (1993a) 174–175.

Haeberlin, B., Nolen III, H., Rubas, W. and Friend, D., In vitro studies in the evaluation of glucuronide prodrugs for novel therapy of ulcerative colitis. *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, 20 (1993b) 172–173.

Haeberlin, B., Rubas, W., Nolen III, H.W. and Friend, D.R., In vitro evaluation of dexamethasone-β-D-glucuronide for colon-specific drug delivery. *Pharm. Res.*, 10 (1993c) 1553–1562.

Harboe, E., Larsen, C., Johansen, M. and Olesen, H.P., Macromolecular prodrugs. XV. Colon-targeted delivery — bioavailability of naproxen from orally administered dextran-naproxen ester prodrugs varying in molecular size in the pig. *Pharm. Res.*, 6 (1989) 919–923.

Larsen, C., Harboe, E., Johansen, M. and Olesen, H.P., Macromolecular prodrugs. XVI. Colon-targeted delivery — comparison of the rate of release of naproxen from dextran ester prodrugs in homogenates of various segments of the pig gastrointestinal (GI) tract. *Pharm. Res.*, 6 (1989) 995–999.

Larsen, C., Jensen, B.H. and Olesen, H.P., Stability of ketoprofen-dextran ester prodrugs in homogenates of various segments of the pig GI tract. *Acta Pharm. Nord.*, 3 (1991) 41–44.

MacFarlane, G.T. and Allison, C., Utilisation of protein by human gut bacteria. *Fed. Eur. Microbiol. Soc. Microbiol. Ecol.*, 38 (1986) 19–24.

McLeod, A.D., Dextran prodrugs for colon-specific drug delivery. In D.R. Friend (Ed), *Oral Colon-Specific Drug Delivery*, CRC Press, Boca Raton, FL, 1992, pp. 213–231.

McLeod, A.D. and Tozer, T.N., Colon-specific delivery of steroids by dextran prodrugs: hydrolysis in rat gastrointestinal tract contents. *Pharm. Res.*, 9 (1992) S-242.

Nakamura, J., Asai, K., Nishida, K. and Sasaki, H., A novel prodrug of salicylic acid, salicylic acid-glutamic acid conjugate utilizing the hydrolysis in rabbit intestinal microorganisms. *Chem. Pharm. Bull.*, 40 (1992a) 2164–2168.

Nakamura, J., Asai, K., Nishida, K. and Sasaki, H., A novel prodrug of salicylic acid, salicylic acid-glycylglycine conjugate utilizing the hydrolysis in rabbit intestinal microorganisms. *J. Pharm. Pharmacol.*, 44 (1992b) 713–716.

Nakamura, J., Kido, M., Nishida, K. and Sasaki, H., Hydrolysis of salicylic acid-tyrosine and salicylic acid-methionine prodrugs in the rabbit. *Int. J. Pharm.*, 87 (1992c) 59–66.

Nakamura, J., Tagami, C., Nishida, K. and Sasaki, H., Development of a prodrug of salicylic acid, salicylic acid-L-alanine conjugate, utilizing hydrolysis in rabbit intestinal microorganisms. *J. Pharm. Pharmacol.*, 44 (1992d) 295–299.

Nakamura, J., Tagami, C., Nishida, K. and Sasaki, H., Unequal hydrolysis of salicylic acid-D-alanine and salicylic acid-L-alanine conjugate in rabbit intestinal microorganisms. *Chem. Pharm. Bull.*, 40 (1992e) 547–549.

Neises, B. and Steglich, W., Simple method for the esterification of carboxylic acids. *Angew. Chem. Int. Ed. Engl.*, 17 (1978) 522–524.

Rijk, M.C.M., Lier, H.J.J.v. and Tongeren, J.H.M.v., Relapse-preventing effect and safety of sulfasalazine and olsalazine in patients with ulcerative colitis in remission: a prospective, double-blind, randomized multicenter study. *Am. J. Gastroenterol.*, 87 (1992) 438–442.

Rohdewald, P., Möllmann, H., Barth, J., Rehder, J. and Derendorf, H., Pharmacokinetics of dexamethasone and its phosphate ester. *Biopharm. Drug Disp.*, 8 (1987) 205–212.

Scheline, R.R., Drug metabolism by intestinal microorganisms. *J. Pharm. Sci.*, 57 (1968) 2021–2037.

Scheline, R.R., Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol. Rev.*, 25 (1973) 451–523.

Shamat, M.A., The role of the gastrointestinal microflora in the metabolism of drugs. *Int. J. Pharm.*, 97 (1993) 1–13.

Williams, R.T., Toxicologic implications of biotransformation by intestinal microflora. *Toxicol. Appl. Pharmacol.*, 23 (1972) 769–781.