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Research Article

Low Molecular Weight Proteins as Carriers for Renal Drug Targeting: Naproxen–Lysozyme

Eric J. F. Franssen,[1,3] Ronald G. M. van Amsterdam,[1] Jan Visser,[1] Frits Moolenaar,[1] Dick de Zeeuw,[2] and Dirk K. F. Meijer[1]

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Low molecular weight proteins (LMWPs), such as lysozyme, may be suitable carriers to target drugs to the kidney. In this study the antiinflammatory drug naproxen was covalently bound to lysozyme (1:1). Pharmacokinetics of the conjugate, naproxen–lysozyme (nap-LYSO), were compared to that of an equimolar mixture of uncoupled naproxen with lysozyme in freely moving rats. Similar plasma kinetics and organ distribution for native lysozyme and the drug conjugate were observed (Cl_p = 1.2 and 1.1 ml/min; $t_{1/2,\beta}$ = 85 and 75 min, respectively). In case of the uncoupled naproxen–lysozyme mixture, a monoexponential plasma disappearance of naproxen with a $t_{1/2}$ of 2.8 hr was observed, coinciding with urinary excretion of naproxen metabolites (mainly 6-desmethylnaproxen sulfate; 6-DMN-S) between 2 and 8 hr after injection. Urinary recovery of total metabolites was 59% of the naproxen dose. In contrast, after injection of covalently bound naproxen, plasma levels of the parent drug were below the detection level, whereas naproxen was recovered as 6-DMN-S in urine over a period from 4 to 30 hr. However, only 8% of the administered dose was recovered as 6-DMN-S in urine, whereas 50% of the dose was recovered as naproxen metabolites in feces. Incubation experiments using purified renal tubular lysosomal lysates revealed that naproxen–lysozyme degradation ultimately results in a stable naproxen amino acid catabolite, naproxen–lysine (nap-lys). Hepatic uptake and biliary excretion of this catabolyte were demonstrated in isolated perfused rat livers. Further, an equipotent pharmacological activity relative to parent naproxen was observed. We conclude that LMWPs such as lysozyme are indeed suitable carriers for site-specific delivery of drugs to the kidney. Although naproxen covalently bound to lysozyme did not release the parent drug, it did result in renal release of a stable and active catabolite, naproxen–lysine.

KEY WORDS: drug targeting; low molecular weight proteins (LMWPs); carrier; naproxen; renal; lysosomes.

INTRODUCTION

Effects of drugs can be limited to target cells by linking them to specific carrier molecules. Recent development of carriers as drug delivery systems (1,2) has focused largely on those suitable for relatively toxic agents such as antineoplastic, antiprotozoal, and antiviral drugs (3,4).

Studies on renal drug delivery are scarce, although the kidney is a target organ for vasodilators, immunosuppressives, diuretics, antibiotics, and antiinflammatory drugs. Various prodrug approaches have been described on the basis of kidney-selective enzymes, e.g., γ-glutamyl transferase and phosphatase. These enzymes were supposed to mediate drug regeneration in the kidney by cleavage of the prodrug moiety from the drug. However, kidney accumulation may be low because of plasma protein binding or limited transport to the kidney (5). Thus, there is a demand for a general carrier for renal specific drug delivery.

Low molecular weight proteins (LMWPs) may provide attractive carrier properties. After intravenous injection, LMWPs rapidly and extensively accumulate in the proximal tubular cells of the kidney (6). Following glomerular filtration, these endogeneous proteins are endocytosed into these cells and hydrolyzed into amino acids in the lysosomal compartment. By covalently attaching drugs to these LMWPs, one may obtain kidney-specific drug delivery. The proximal tubular cells may function as a compartment for drug regeneration, and the released drug can act locally or be transferred into the tubular lumen of the kidney.

In the present study lysozyme was chosen as the LMWP for the following reasons:

(1) The renal disposition of lysozyme has been extensively studied (7–9).

(2) Lysozyme contains six lysine residues. The terminal

[1] Department of Pharmacology and Therapeutics, State University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands.

[2] Department of Nephrology, University Hospital of Groningen, Oostersingel 59, 9712 EZ Groningen, The Netherlands.

[3] To whom correspondence should be addressed at University Centre for Pharmacy, Department of Pharmacology and Therapeutics, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands.

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α-NH_2 and ϵ-NH_2 groups are potential sites for covalent linkage of drugs.

We chose the nonsteroidal antiinflammatory drugs (NSAID) as test drugs. Beneficial effects in patients with renal disease often coincide with both central and gastrointestinal side effects (10). Among these agents, naproxen was chosen for the following reasons:

(1) Naproxen (Fig. 1) is a relatively simple molecule; the carboxylic group is the only reactive functional group and can be used for an amide linkage with the amino groups of LMWPs (11).
(2) Naproxen and its metabolites can be analyzed with a high sensitivity and specificity.
(3) The pharmacokinetics of naproxen have been extensively studied in animals and in man (12,13).

Naproxen was covalently coupled to lysozyme, and the pharmacokinetics of the conjugate (nap-LYSO) were compared to those of parent drug coadministered with native lysozyme.

MATERIALS AND METHODS

Reagents and Chemicals

Naproxen, lysozyme, ketoprofen, β-glucuronidase, arylsulfatase, and *micrococcus*-cell substrate were purchased from Sigma; 6-desmethylnaproxen (6-DMN) and ϵ-naproxen–lysine were synthesized in our own laboratory [see Franssen *et al.* (11)]. Acetonitrile and other reagents were of analytical or reagent grade. Water for HPLC analysis was of Millipore quality.

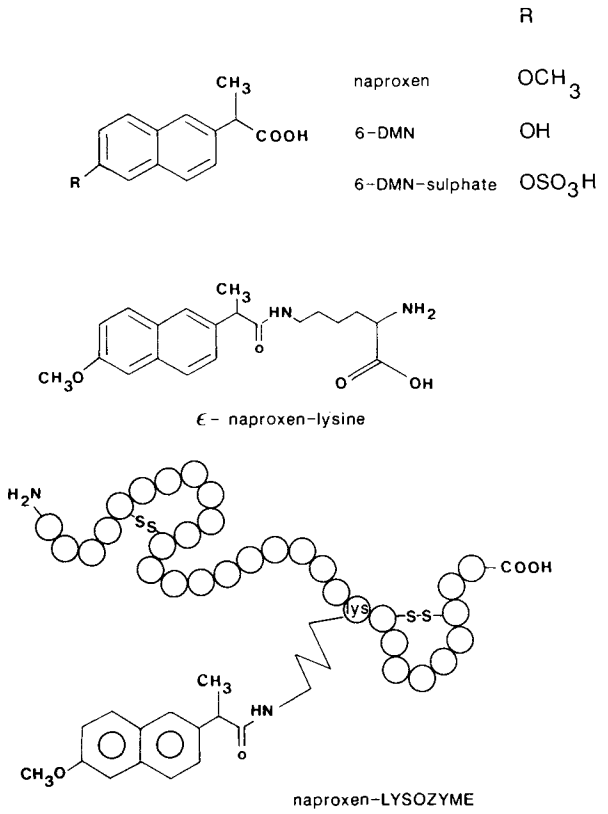

Fig. 1. Structural formulas.

Synthesis and Purification of the Conjugates

Naproxen was coupled to lysozyme by a *N*-hydroxysuccinimide method (11). Pure soluble naproxen–lysozyme (1:1) was obtained by fast protein liquid chromatography (Pharmacia) using cationic ion-exchange separation, using MONO-S (Pharmacia). After each preparative run 1.2 mg of pure soluble conjugate was obtained. For radioactive studies 100 μg of this product was labeled with either iodine-123 or iodine-125 by the chloramine-T method (14).

Characterization of the Drug Protein Conjugates

The amount of covalently bound naproxen to lysozyme was assayed after alkaline hydrolysis of the conjugate. Briefly, 10 mg of the conjugate with 6 *N* NaOH was incubated at 80°C for at least 72 hr. After acidification with 6 *N* HCl the hydrolysate was extracted with dichloromethane, and after evaporation of the organic solvent under a nitrogen atmosphere, the residue was dissolved in HPLC eluant and analyzed by HPLC. The molar coupling ratio was calculated to be approximately 1; i.e., 10 mg of the conjugate contains 160 μg of covalently bound naproxen. Noncovalently bound naproxen was less than 0.01%. The synthesis, purification, and characterization of naproxen–lysozyme conjugates have recently been described in more detail (11).

Analysis of Lysozyme and Its Drug Conjugates

Lysozyme and its conjugates were assayed by an enzymatic method described by Gorin *et al.* (15). The detection limit of lytic activity was 2.5 μg/ml, whereas data were corrected for individual endogeneous lysozyme activity. Proteins were also analyzed by counting radioactivity after labeling with iodine-123 (organ distribution) and iodine-125.

Analysis of Naproxen, 6-DMN-(S), and Naproxen–Lysine

We developed a rapid and sensitive method in order to analyze naproxen and its metabolites. Naproxen, 6-DMN-S, and lysozyme–lysine (nap-lys) were determined by HPLC with fluorescence detection. The mobile phase consisted of water–acetonitrile–HAc (60:40:1 or 65:35:1); the flow rate was 1.5 ml/min. The column was a μBondapack C-18 (Waters/Millipore Corp., Milford, MA). The detector was a Waters 470 dual monochromator, operating at excitation wavelength 330 nm and emission wavelength 360 nm. Ketoprofen was used as an internal standard, added prior to extraction and monitored simultaneously by UV detection (Waters 440; 254 nm). Naproxen was determined after an extraction procedure. Briefly plasma (100 μl) or urine (1 ml) were mixed with 1 *M* phosphate buffer, pH 4.6 (freshly prepared containing 20 μg/ml ketoprofen), until a final volume of 2.0 ml; 6.0 ml of dichloromethane was added. The mixture was vortexed for 1 min and centrifuged at 3000*g* for 10 min. The organic phase was evaporated under a nitrogen atmosphere. The residue was dissolved in 300 μl eluant and 100 μl was injected into the HPLC. Naproxen in the form of covalently bound conjugates was analyzed after alkaline hydrolysis of samples of plasma, urine, tissue homogenates, or lyophilized feces as described under *Characterization of the Drug–Protein Conjugates*.

Characterization of the Drug–Protein Conjugate

6-Desmethylnaproxen (6-DMN) was assayed as its parent form or its conjugated form 6-desmethylnaproxen sulfate. In short, urine samples were treated with arylsulfatase at pH 5.0 and 37°C for 18 hr. During this incubation period 6-DMN-S was quantitatively converted to 6-DMN. An aliquot (100 μl) was injected into the HPLC. Nap-lys was assayed by mixing plasma with 2 vol of acetonitrile, centrifuged as described above, and injected into the HPLC. Retention times were 3.3 min (nap-lys), 5.0 min (6-DMN), and 12.0 min (naproxen) (mobile phase, water–acetonitrile–acetic acid, 60:40:1; flow, 1.5 ml/min). Detection limits of naproxen, 6-DMN, and 6-DMN-S were 10 ng/ml (plasma) and 1 ng/ml (urine). R^2 of calibration curves was >0.999.

Qualitative Analysis of 6-DMN (as Sulfate) in Urine of the Rat and of ε-Naproxen–Lysine in Lysosomal Preparations

After derivatization the presence of the main urinary metabolite 6-DMN-S was checked using GC–MS–MS (gas chromatography combined with mass spectrometry, double serially equipped). Briefly, urine samples were hydrolyzed with sulfatase in acetate buffer, pH 5.2. 6-DMN was extracted by solid-phase extraction on a C-18 column at pH 5. 6-DMN was eluted with methanol. Derivatization to render an ethyl derivative was done with ethyliodide in acetone and potassium carbonate. Electron bombardment-ionization and chemical-ionization spectra were run on a Finnigan MAT TSQ-45 (Finnigan MAT Ion Trap Detector 700 and Carlo Erba 5160 GC, Multiplier 1700 V; interface and open-split temperature, 250°C; CI reaction gas, methane (0.45 Torr); collision gas, argon (1.8 mTorr); emission current, 0.20 mA). The presence of ε-naproxen–lysine was shown by the use of liquid chromatography (LC)–MS with an ion-spray device by the method of Bruins *et al.* (16).

Experiments in Vivo

Kinetics in Freely Moving Rats. Male Wistar rats (280–310 g), which had free access to food and water, were placed in metabolic cages during the experiments. The experimental setting allowed simultaneous blood and urine sampling. Ten milligrams (n = 6) and 1 mg (n = 2) of naproxen–lysozyme conjugates (containing 160 μg, respectively, 16.0 μg coupled naproxen) or a mixture (n = 6) of uncoupled naproxen (160 μg) and lysozyme (10 mg) was freshly dissolved in freshly prepared blood plasma and injected in freely moving, heparinized (500 IU), heart-cannulated rats (17). Plasma samples (0.5 ml) were taken at indicated times; urine samples were collected in 2- and 24-hr fractions. During the 2-hr urine fractionation experiments the rats were infused with a sterile 5% glucose solution (3 ml/hr) in order to obtain an adequate diuresis. All samples were immediately analyzed or stored at −20°C.

Organ Distribution of Naproxen-^{123}I-Lysozyme in Anesthetized, Immobilized Rats by Gamma-Camera Imaging. Gamma-camera imaging after injection of naproxen-^{123}I-lysozyme (1 mg; n = 2) and native ^{123}I-lysozyme (1 mg; n = 2) in heart-cannulated anesthetized rats was performed by the method of Haas and de Zeeuw (18). In short, native lysozyme and naproxen–lysozyme were labeled with iodine-123. Male Wistar rats (280–310 g) were anesthetized with sodium pentobarbital (60 mg/kg; intraperitoneally). Thereafter the rats were immobilized on a temperature-controlled heat pad. The iodinated proteins were injected (dorsal penile vein, intravenously) and monitored by extrarenal counting with a gamma-camera. The total body, kidneys, liver, and bladder were measured for 8 hr. Organ distribution is expressed as percentage of administered dose. The renal activity–time profiles of the iodine-123 reflect the combination of uptake and degradation of the proteins.

Experiments in Vitro

Stability of the Drug–Protein Conjugate in Plasma and Urine. Conjugates were incubated in rat plasma and urine at 37°C up to 2 days and analyzed by HPLC. In order to detect conjugated metabolites of naproxen, samples were also incubated at 37°C with β-glucuronidase at pH 6.0 and arylsulfatase at pH 4.6.

Degradation of Naproxen–Lysozyme by Lysosomal Lysates of the Kidney Cortex. Protein degradation was determined by incubation of the radiolabeled iodine-125 protein (concentration, 48.3 μg/ml, 1.5 μCi) in 0.1 *M* acetate buffer (pH 5.0) in the presence of lysosomal lysates of the kidney cortex [see Franssen *et al.* (11)]. At variable intervals, samples were drawn and the incubation stopped by the addition of trichloroacetic acid (TCA). Degradation of lysozyme and its drug conjugates was measured as the percentage of TCA-soluble radioactivity. Pellets and supernatants were processed in a gamma-counter (LKB, Bromma, Sweden) at a counting efficiency of 80%.

Drug Release of Naproxen–Lysozyme and ε-Naproxen–Lysine After Incubation with Lysosomal Lysates of the Kidney Cortex. Naproxen–lysozyme and ε-naproxen–lysine were incubated with lysosomal lysates of the kidney cortex [see Franssen *et al.* (11)]. Samples (100 μl) were drawn at various time intervals. The incubation was stopped by the addition of 200 μl acetonitrile to the incubation medium. After centrifugation (3000*g*, 2 min), 100 μl of the supernatant was injected into the HPLC system as described above.

Experiments in the Isolated Perfused Rat Liver. Native lysozyme (10 mg), naproxen–lysozyme (10 mg), naproxen (160 μg), and ε-naproxen–lysine (249 μg) were studied in the isolated perfused rat liver (n = 3) according to the procedure of Meijer *et al.* (19) using 100 ml of Krebs–bicarbonate solution with 1% bovine serum albumin (BSA). Perfusate and bile samples were drawn at indicated times. Perfusate profiles and biliary excretion were determined by analysis of the parent compounds and metabolites.

Cyclooxygenase Inhibiting Potency of ε-Naproxen–Lysine. The release upon antigen challenge of immunoreactive prostaglandin E_2 (i-PgE_2) from actively sensitized guinea pig trachea was used as a parameter of cyclooxygenase activity. Quantitation of cyclooxygenase activity was done by the method of van Amsterdam *et al.* (20). Naproxen (100 μ*M*) and ε-naproxen–lysine (100 μ*M*) were added to the buffer running through separate superfusion chambers, followed after 30 min by antigen, 10 μg ovalbumin/ml. Fractions of 3 min preceding and from 0 to 2, 2 to 5, and 5 to 8 min after antigen challenge were collected in separate polypropylene tubes. Before and after the superfusion, samples were collected for HPLC analysis for naproxen and naproxen–lysine.

Pharmacokinetic Analysis

Kinetic studies were performed using the multifit modified CFT3 program (21), which was implemented on the Olivetti PC.

Statistical Analysis

Statistical comparisons were made with Student's t test after checking equality of variances with an F test; $P < 0.05$ was selected as the minimal level of statistical significance.

RESULTS

Synthesis and Characterization of the Drug–Protein Conjugates

Coupling of naproxen to lysozyme (3:1) resulted in a conjugate of approximately 1.2 mol of naproxen to 1.0 mol lysozyme. The solubility of this conjugate at physiological pH was clearly decreased as compared to native lysozyme. Therefore we decided to purify and upscale a soluble naproxen–lysozyme conjugate (1:1). This product was obtained after reacting equimolar amounts of naproxen-*N*-hydroxysuccinimide ester and lysozyme, followed by the removal of unreacted native lysozyme using ion-exchange FPLC chromatography. These conjugates were freshly prepared and filtered through a 0.2-μm filter prior to infusion into the animals. For further details see Franssen *et al.* (11).

Experiments in Vivo

Experiments in Freely Moving Rats. Plasma clearance of the naproxen–lysozyme conjugate closely resembled that of lysozyme itself (Cl_p = 1.2 and 1.1 ml/min; $t_{1/2,\beta}$ = 85 and 75 min; n = 6) (Fig. 2).

In the case of the uncoupled naproxen–lysozyme mixture a plasma disappearance of naproxen with a $t_{1/2}$ of 2.8 hr was observed (Fig. 2), coinciding with a urinary excretion of naproxen metabolites (mainly 6-desmethylnaproxen sulfate) between 2 and 8 hr after injection (Fig. 3). The urinary recovery of this metabolite was 59% of the naproxen dose (Table I).

In contrast, after injection of covalently bound naproxen, plasma levels of the parent drug (also monitored as its glucuronated or sulfated metabolites) were below the detection limit. Yet 6-DMN-S could be detected in the urine of these rats. The presence of 6-DMN-S was verified by mass spectrometry; a characteristic mass-to-charge pattern was observed (m/z 273 with fragment 199) as analyzed by GC–MS–MS. 6-DMN-S was excreted into urine mainly over a period from 4 up to 30 hr, i.e., exhibiting a sustained-release profile as compared with the injection of unbound naproxen. Total urinary recovery, however, was only 12 and 6% of the administered dose, calculated as parent naproxen, after administration of 10 and 1 mg of protein conjugates, respectively (Table I). No parent naproxen was detected in the urine, with 8 and 4% of the dose, respectively, recovered as 6-DMN-S. Further, 4 and 2%, respectively, were recovered as covalently bound naproxen, representing unchanged excreted conjugate, excreted naproxen-containing catabolites, or other naproxen metabolites. HPLC analysis of collected feces revealed the presence of naproxen and 6-DMN metabolites, corresponding to 50 ± 20% (mean ± SD; n = 3) of the administered dose. These data indicate an extrarenal

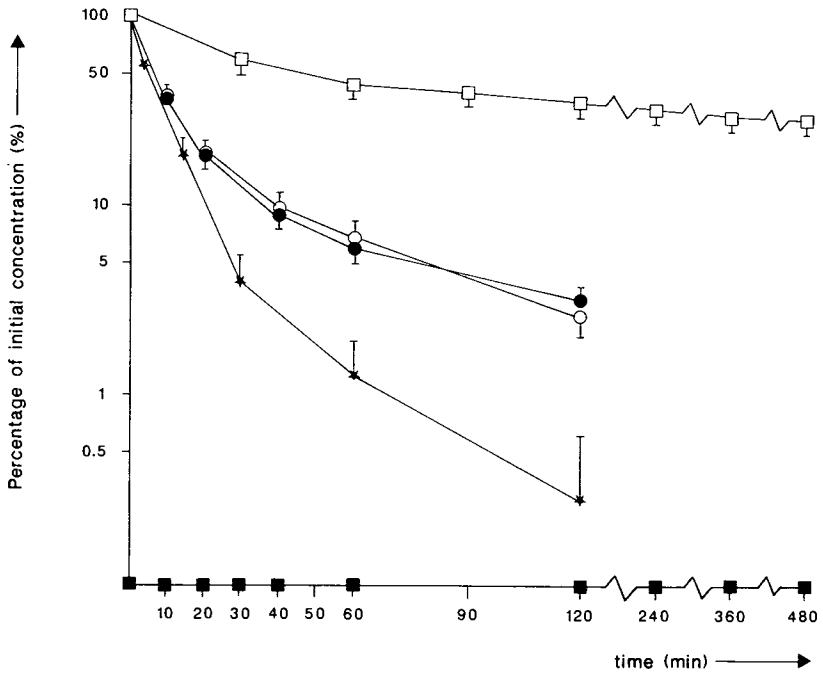

Fig. 2. Plasma concentration versus time profiles in rats of the following: native lysozyme, 10 mg, n = 6, ○——○; nap-LYSO, 10 mg, n = 6, ●——●; naproxen, 160 μg, as a mixture with native lysozyme (10 mg), n = 6, □——□; naproxen, 160 μg, as nap-LYSO (10 mg), n = 6, ■——■; and nap-lys, 249 μg, n = 3, ●——●. Initial concentration (1 min after injection) was set at 100%. Values represent mean ± SD.

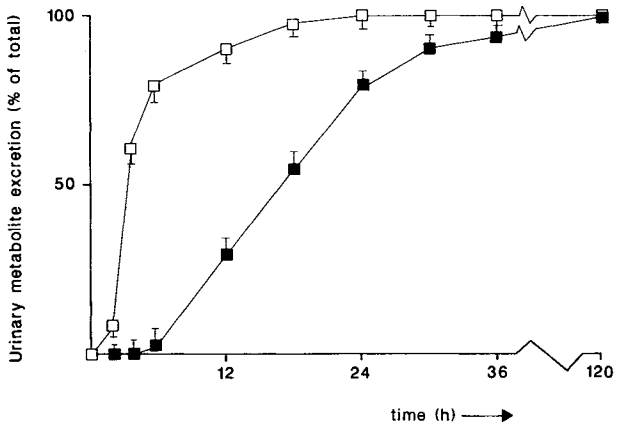

Fig. 3. Urinary excretion profile of naproxen, measured as its main metabolite 6-DMN-S, after administration of naproxen, 160 μg, as a mixture with native lysozyme (10 mg; □) or naproxen, 160 μg, covalently coupled to lysozyme (10 mg; ■). Values represent the fraction of the cumulative amount excreted (mean ± SD).

excretion route of naproxen, in spite of the primary renal distribution.

Injection of naproxen–lysine resulted in a very rapid plasma elimination as compared to both naproxen and naproxen–lysozyme. No unchanged naproxen was detectable in the urine of these rats, while 7% of the dose was excreted as 6-DMN-S in the urine.

Organ Distribution Studies of Soluble Naproxen–^{123}I-Lysozyme in Anesthetized and Immobilized Rats by Gamma-Camera Imaging. To extend the obtained pharmacokinetic observations of naproxen–lysozyme, the drug protein conjugate was also labeled with iodine-123 to get an impression of organ distribution *in vivo*. Kinetics of naproxen–^{123}I-lysozyme were comparable to those of native ^{123}I-lysozyme. Analysis of kidney accumulation revealed a t_{max} of 20 min (native lysozyme, 20 min), corresponding to 68 and 74% of the injected dose, respectively (mean of two separate experiments). Furthermore, the conjugate did not accumulate in the liver (less than 1%), whereas only 2% was excreted as unchanged protein (i.e., not absorbed in the kidney).

Table I. Urinary Recovery, Expressed as Percentage of Administered Dose (Mean ± SD) of Naproxen, Covalently Bound Naproxen,[a] and the Main Metabolite 6-DMN-S After Administration of a Mixture of 10 mg Lysozyme and 160 μg Naproxen (Uncoupled, 10; n = 6), 10 mg Naproxen–Lysozyme (Corresponding to 160 μg Covalently Bound Naproxen; Coupled, 10; n = 6), and 1 mg Naproxen–Lysozyme (Corresponding to 16.0 μg; Coupled, 1; n = 2).

Compound	Uncoupled, 10	Coupled, 10	Coupled, 1
Naproxen	0.02 ± 0.004	<0.01	<0.01
Bound naproxen		4 ± 0.3	2
6-DMN-S	59 ± 3.4	8 ± 1.3	4

[a] Bound naproxen is calculated by subtracting the free naproxen from the total amount of naproxen. The total amount of naproxen is determined after alkaline hydrolysis of individual urine samples.

Experiments in Vitro

Stability of the Conjugate in Plasma and Urine. Incubation of the conjugate with plasma and urine for 2 days did not result in the liberation of any detectable naproxen or naproxen metabolites.

Degradation of the Conjugate in Lysosomal Preparations of Renal Cortex Homogenates. The lysosomal preparations showed a 4.5 to 6 times enrichment of lysosomal enzymes as determined by the lysosomal enzyme marker acid phosphatase. Further, the renal lysosomal lysates showed considerable aminopeptidase and carboxypeptidase activity (11). To verify biodegradability of the carrier, both native ^{125}I-lysozyme and naproxen–^{125}I-lysozyme were incubated with these lysosomal preparations. Table II shows that both naproxen–lysozyme and native lysozyme are degraded into smaller peptide fragments as indicated by the increase in TCA-soluble radioactivity. Similar results were obtained by determining the enzymatic activity (toward lysis of the *Micrococcus* cell membrane): complete loss of activity was observed after 50 min of incubation. Therefore these results indicate potential biodegradability into amino acids by lysosomal enzymes.

Characterization of Catabolites After Incubation of the Conjugate with Lysosomal Preparations. Prolonged incubation (up to 120 hr) of nap-LYSO with lysosomal lysates did not reveal any detectable naproxen per se or its main metabolite 6-DMN-S. However, further analysis of the chromatograms of the obtained supernatants showed an increasing fluorescent peak eluting at t = 3.3 min (Table III), representing a low molecular weight degradation product of the conjugate that contains naproxen. Analysis of combined preparative fractions by mass spectrometry revealed a characteristic mass pattern, which corresponded to the naproxen–amino acid catabolite naproxen–lysine (MW = 358) and which was obviously resistant to further lysosomal enzymatic hydrolysis. To test this hypothesis we synthesized ε-naproxen–lysine (i.e., the amino acid derivative of naproxen by which it is attached to the protein) (see structure, Fig. 1). This compound also showed a characteristic peak eluting at 3.3 min. Further, incubation of this compound with the lysosomal lysates did not reveal any detectable naproxen or 6-desmethyl metabolites. The hypothesis of a locally generated naproxen–lysine fragment, resistant to enzymatic degradation, was further tested by injection of the conjugate into rats, followed by extirpation of the kidneys after several time

Table II. Protein Degradation of ^{125}I-Lysozyme and Naproxen–^{125}I-Lysozyme After Incubation with Lysosomal Lysates of the Kidney Cortex[a]

Time (hr)	^{125}I-LYSO (%)	Nap–^{125}I-LYSO (%)
0	0	0
1	23 ± 2.3	20 ± 2.2
2	32 ± 3.1	24 ± 2.3
5	39 ± 2.8	28 ± 2.3
21	40 ± 2.3	30 ± 2.2
25	39 ± 3.0	30 ± 2.2

[a] Values are expressed as percentage TCA-soluble radioactivity (mean ± SD; n = 8).

Table III. Release of Naproxen, 6-DMN, and Naproxen–Lysine from Naproxen–Lysozyme, Expressed as Percentage of Total Bound Naproxen, After Incubation of Naproxen–Lysozyme with Lysosomal Lysates of the Kidney Cortex (A; $n = 4$) and a Similar Incubation After Intraveneously Injecting 10 mg of Naproxen–Lysozyme in Rats, 30 min Prior to Removal of the Kidneys (B; $n = 4$)[a]

Time (hr)	A (%)			B (%)		
	Nap	6-DMN	Nap–lys	Nap	6-DMN	Nap–lys
0	0	0	0	0	0	4 ± 1.1
2	0	0	3 ± 1.3	0	0	16 ± 1.4
5	0	0	18 ± 1.8	0	0	18 ± 1.3
20	0	0	35 ± 1.4	0	0	26 ± 1.2
42	0	0	39 ± 1.9	0	0	30 ± 1.3
50	0	0	40 ± 1.5	0	0	30 ± 1.4

[a] Nap and 6-DMN were also measured as possible glucuronides or sulfates.

intervals. After incubating the lysosomal fraction of the kidney cortex we could not detect any naproxen or 6-DMN-(S), however, an increasing amount of naproxen–lysine was observed (Table III).

Stability of the Conjugate After Perfusion in the Isolated Perfused Liver. To study a possible role of the liver in the clearance of the conjugate, both native lysozyme and naproxen–lysozyme were added to an isolated rat liver perfusion (Fig. 4). The experiments in the isolated perfused liver showed no disappearance of either naproxen–lysozyme or native lysozyme from the perfusate during a 2-hr perfusion. Further, we could not detect any naproxen or 6-DMN-(S) in perfusate and bile.

On the other hand, the catabolite ϵ-naproxen–lysine was rapidly taken up from the perfusate by the liver as compared to naproxen, while the catabolite together with abundant (nonidentified) naproxen metabolites was found in the bile. No naproxen itself or 6-DMN-(S) was detectable in the bile. In contrast, after 2 hr of perfusion with naproxen itself, only small amounts of unchanged drug and 6-DMN-S were excreted into the bile.

Effect of Naproxen–Lysine on Antigen-Induced PgE_2 Release in the Guinea Pig Trachea. To test for possible vasoactive effects of the locally generated catabolite naproxen–lysine, the inhibition of antigen (i.e., ovalbumin)-induced PgE_2 release was determined in guinea pig trachea; a 91 and 87% inhibition of the i-PgE_2 was observed for naproxen–lysine and naproxen, respectively (mean of two separate experiments), suggesting an equipotent effect of the amino acid derivative and the parent drug. During the superfusion no free naproxen was detectable (<0.05 μ*M*). Therefore the observed effect is associated with the presence of naproxen–lysine itself.

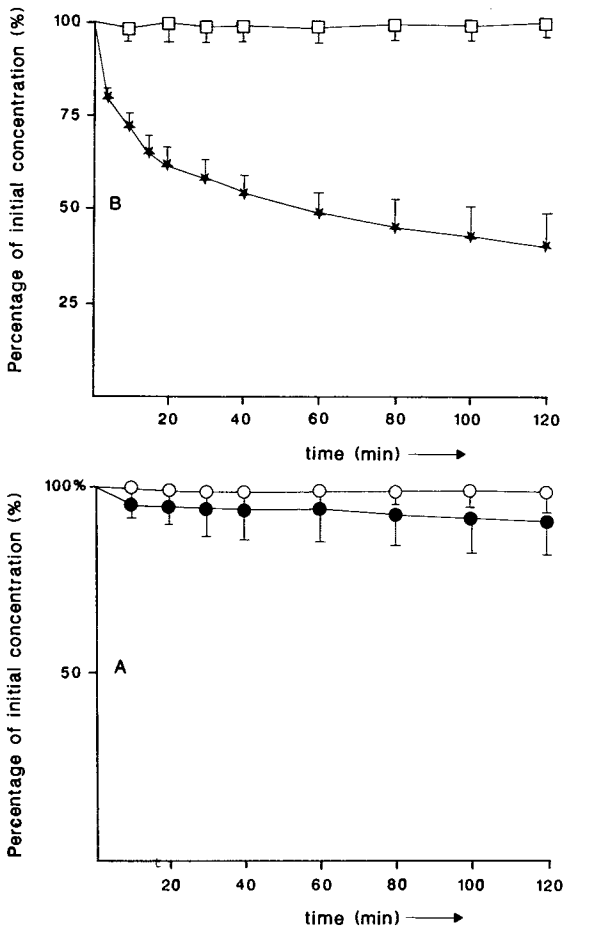

Fig. 4. Perfusate concentration versus time profiles in the isolated perfused liver. (A) Native lysozyme, 10 mg, $n = 3$, ○——○; naproxen–lysozyme, 10 mg, $n = 3$, ●——●. (B) Naproxen, 160 μg, $n = 3$, □——□; Naproxen–lysine, 249 μg, $n = 3$, ●——●.

DISCUSSION

Synthesis and subsequent purification of the drug–protein conjugate resulted in a soluble product with a molar coupling ratio of approximately 1. The removal of noncovalently coupled naproxen is important, since it might obscure the kinetic profile of the covalently bound drug or its main metabolite 6-DMN after metabolism of the conjugate in the kidney. The conjugate was found to be stable in plasma and urine for up to 2 days *in vitro*. These observations suggest that naproxen is not released from the carrier (by, e.g., plasma enzymes) before arrival in the proximal tubular cells.

The present pharmacokinetic studies show that naproxen bound to lysozyme is actually delivered into the kidney. Our data show identical pharmacokinetic patterns of the conjugate compared to native lysozyme, with respect to both rapid clearance from the plasma compartment and accumulation in the kidney. In similar experiments it is calculated that 80% of a tracer amount of lysozyme is internalized within 30 min (26). These observations are in agreement with other studies of lysozyme and other LMWPs by Maack *et al.* (6) and Hansen *et al.* (22), indicating extensive renal clearance and metabolism of lysozyme and complete absence of clearance by the liver. However, attaching moieties may change specific characteristics of the native protein, e.g. its molecular size, charge, and hydrophobicity. In general, by changing these properties, one may also alter the pharmacokinetic profile (23,24). Since we coupled only one drug mol-

ecule to one protein molecule, the pharmacokinetics in freely moving rats (Fig. 2), the lack of liver uptake in the isolated perfused liver (Fig. 4), and the *in vivo* distribution of the conjugate, measured by gamma-camera imaging, were all unchanged.

The pharmacokinetics of naproxen after administration as a mixture with lysozyme are comparable to single doses of naproxen and are in agreement with earlier reports of naproxen kinetics in animals (12,13). The administration of the naproxen–lysozyme conjugate did not result in any detectable plasma levels of naproxen. Further, the parent drug was not detectable in urine, whereas small amounts of drug and metabolite were recovered in urine over a period of 4 to 30 hr. The sustained-release profile (Fig. 3) suggests that lysosomal digestion is rate limiting in naproxen excretion. Approximately 50% of the naproxen dose was recovered in the feces, indicating an extrarenal route of elimination. For additional data with respect to organ distribution and elimination of naproxen, experiments with radiolabeled naproxen are required, especially since metabolites may be formed that were not detectable by the HPLC analysis.

The incubation of the conjugate in the lysosomal preparations demonstrated its potential biodegradability into amino acids. Biodegradability is essential with respect to toxicity and may also be required for drug liberation. Degradation, measured as release of TCA-soluble radioactivity, was incomplete, possibly as a result of precipitation of oligopeptide fragments in the procedure, depletion of essential cofactors, or heterogeneity in lysozyme itself for lysosomal digestion (9,25). Differences between the native protein and the drug–protein conjugate seem small and may also be related to precipitation artefacts. On the other hand, the extent and rate of lysosomal degradation of proteins may be influenced by their binding to low molecular weight substances (26).

In vitro incubations of the conjugate did not result in release of parent naproxen or 6-DMN-(S), but in naproxen–lysine, which appeared to be resistant to further lysosomal hydrolysis. The same was observed *in vivo* by removal of the kidneys after injection of drug–protein conjugate in rats. Therefore, these *in vivo* studies confirm our *in vitro* experiments (11). The kidneys apparently are not able to cleave the amide bond between naproxen and lysine. Lysozyme has six ε-amino-groups and only one α-amino group. Although the structure of the fragment was not further subjected to analysis, it is reasonable to assume that the prepared conjugate contains an amide bond between the carboxylic function of naproxen and the ε-amino groups of the lysines of the protein. This unphysiological ε-amide bond may not be recognized by the various carboxypeptidases in the lysosomes. Generation of the drug in the parent form often requires a suitable cleavable spacer between drug and protein. Various kind of spacers have been described for different drugs (3,27). In this respect, intercalating an oligopeptide spacer between naproxen and lysozyme might result in more physiological α-amide bonds. However, we have shown that for naproxen, and other structurally related drugs, α-amide bonds between drug and various natural amino acids are not rapidly cleaved by renal lysosomal enzymes. The ester bond, using L-lactic acid, was shown to be promising *in vitro* (11).

Since our *in vitro* incubations have shown that the amide bond between naproxen and lysine will not be cleaved in the kidney, we hypothesized that the low recovery of the naproxen metabolite in the urine and the presence of naproxen metabolites in the feces are the result of extrarenal clearance (probably by the liver) of naproxen–lysine after its reentry into blood from the kidney.

To test this hypothesis, the metabolite naproxen–lysine was also studied in freely moving rats and in isolated perfused livers. Injection of naproxen–lysine into rats showed a rapid clearance from the blood (Fig. 2) and an equally small amount of the dose was recovered as 6-DMN-S in urine, as occurred with injection of the conjugate. The naproxen–lysine fragment was rapidly taken up by the isolated perfused liver. Uptake was followed by metabolism and excretion of various naproxen metabolites into bile. These findings indicate a major role of the liver in the elimination and metabolism of naproxen–lysine, ultimately resulting in excretion of 6-DMN-S into urine and excretion of other products into feces. Therefore, also the observed 6-DMN-S in urine after administration of the conjugate may, in principle, be the result of liver metabolism of the renally generated naproxen–lysine fragment. After perfusion of naproxen–lysine in the isolated perfused liver, no free naproxen or 6-DMN-S was observed in the perfusate and bile. Therefore, we found no evidence for the cleavage of naproxen from lysine in the liver. Other extrarenal sites, such as the intestine, may be involved in partial conversion of naproxen into naproxen or 6-DMN (Fig. 5).

Since naproxen–lysine is a dipeptide-like degradation product and dipeptides are capable of passing the lysosomal membrane into the cytoplasm by either passive diffusion or

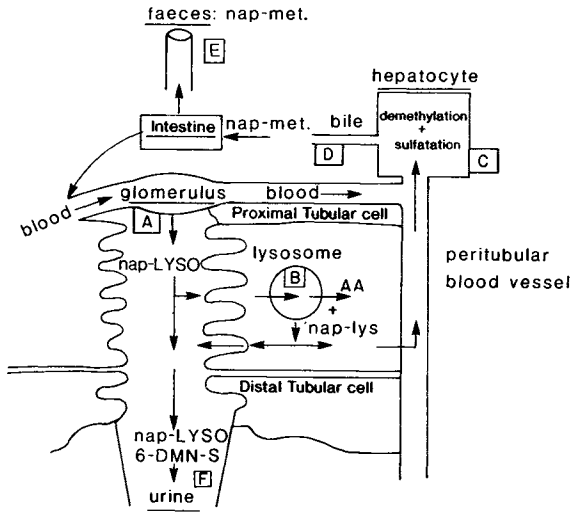

Fig. 5. Schematic presentation of the kinetics of nap-LYSO and nap-lys in the rat. Nap-LYSO is filtered and reabsorbed in the kidneys (A). Nap-LYSO is degraded into amino acids and nap-lys in the lysosomes of the proximal tubular cells (B). The generated nap-lys may locally act in the proximal or distal parts of the kidney; it may also be further metabolized and reenter the bloodstream. Nap-lys is rapidly cleared by the liver, resulting in the presence of this catabolite and other naproxen metabolites (nap-met.) in bile (D) and, ultimately, in the presence of naproxen metabolites in the feces (E) and 6-DMN-S in the urine (F).

active transport (28), this metabolite may exert a local effect in the kidney, i.e., inhibit cyclooxygenase synthesis. The activity of naproxen–lysine in guinea pig trachea was similar to that of the parent drug. Consequently regeneration of naproxen itself is not obligatory for a local drug action. Drug–peptide fragments may still retain the activity of the parent drug as reported for methotrexate and daunorubicin fragments (29,30).

In conclusion, the results indicate a selective delivery of naproxen to the kidney. After endocytosis the drug–protein conjugate is metabolized into amino acids in the lysosomes. At this particular site, naproxen is not regenerated as the parent drug but as a naproxen amino acid fragment, naproxen–lysine. Since this fragment has a pronounced pharmacological activity, regeneration of the parent drug is not required for successful targeting in this particular case. This lysosomal product may reenter the bloodstream and is rapidly cleared by the liver, resulting in low plasma concentrations. In order to regenerate drugs in their parent form, appropriate spacers between the drug and the protein should be used.

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