Low Molecular Weight Proteins as Carriers for Renal Drug Targeting: Naproxen Coupled to Lysozyme via the Spacer L-Lactic Acid

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Low molecular weight proteins (LMWPs) are potential carriers for targeting drugs to the kidney. To test whether ester bonds are suitable for the reversible drug conjugation, the antiinflammatory drug naproxen (Nap) was conjugated to the LMWP lysozyme (LYSO) via an ester bond using an L-lactic acid spacer (Nap-lact-LYSO, 1:1:1). The distribution and degradation of the conjugate in rats were compared to those of an equimolar mixture of free drug and LMWP and of a directly coupled conjugate without spacer (Nap-LYSO). The plasma clearance of Nap-lact-LYSO closely resembled that of Nap-LYSO and LYSO itself. Its major accumulation site appeared to be the kidney as demonstrated by extracorporal γ-camera counting of the LMWP. Renally released naproxen was excreted in the urine as 6-desmethyl-naproxen-sulfate (6-DMN-S). Apparently the kidneys represent the main sites of demethylation and sulfation after administration of the LMWP-coupled drug. In addition, the renal excretion of naproxen (including its metabolites) was significantly delayed and sustained as compared to that after injection of uncoupled naproxen. Using the L-lactic acid spacer LMWP conjugation, the renal selectivity of Nap was increased 5.6 ± 0.41-fold. Additional *in vitro* studies with Nap-lact-LYSO revealed that renal generation of the parent drug coincided with formation of low molecular weight catabolites, mainly as naproxen-L-lactic acid-lysine (Nap-lact-Lys). This indicated that *in vitro* the rate of cleavage of the ester bond is significantly slower than digestion of the carrier backbone itself. It is concluded that for drugs with free carboxyl groups the coupling to LMWPs via α-hydroxy acids can result in renal-specific delivery and endorenal drug release.

KEY WORDS: drug targeting; low molecular weight proteins; carrier; naproxen; spacer engineering; renal; lysosomes; L-lactic acid.

INTRODUCTION

Site-specific drug delivery to target cells can be achieved by covalent coupling of the drug to macromolecular carriers (1–3). The kidney is the target organ for many drugs, including vasodilators, diuretics, antibiotics, and antiinflammatory drugs (NSAIDs). Therapy with these agents in patients with renal disease is often accompanied by unwanted side effects. For instance, treatment with NSAIDs may relieve renal protein excretion via specific hemodynamic effects, whereas serious central and gastrointestinal side effects may occur (4). Recently, we have demonstrated that low molecular weight proteins (LMWPs) can serve as carriers for targeting antiinflammatory drugs and antibiotics (5–7). After intravenous injection, LMWPs rapidly accumulate in the proximal tubular cells of the kidney. Following glomerular filtration, these endogenous proteins are endocytosed from the primary urine into proximal tubular cells and hydrolyzed into amino acids in the lysosomes. By covalently linking drugs to the LMWPs, kidney-specific drug delivery may be achieved. Consequently, the proximal tubular cells function as compartments for drug regeneration, and the released drug can act locally or be transferred into the tubular lumen of the kidney.

Although the LMWPs are biodegradable, spacers between the drug and the carrier are required for the release of the coupled drugs in their parent forms (5,6). For drugs with carboxyl groups, such as NSAIDs, neither ε-amide bonds (introduced by direct coupling) nor α-amide bonds (by indirect coupling via oligopeptide spacers) are lysosomally cleaved. In contrast, ester bonds can be enzymatically cleaved, by esterases present in lysosomes and/or the cytoplasm of proximal tubular cells. The insertion of α-hydroxy acids, like L-lactic acid, between the drug (e.g., naproxen) and the protein (e.g., lysozyme) represents an attractive coupling technique. The aim of the present study was to evaluate this spacer concept *in vivo*. Naproxen was covalently coupled to lysozyme via L-lactic acid (Fig. 1) and its pharmacokinetics were compared to those of an equimolar mixture of the individual components.

MATERIALS AND METHODS

Animals

Male Wistar rats (250–300 g) were fed a standard lab chow (Hope Farms N.V., Woerden, The Netherlands) and had free access to water.

Reagents and Chemicals

Naproxen, L-lactic acid, lysozyme, flurbiprofen, and arylsulfatase were purchased from Sigma Chemical Co. (St. Louis, MO); Z-Asp-(OtBu)-Lys(BOC)-Arg(OH) (Org 2208) was donated by Dr. P. Boon (Organon, Oss, The Netherlands); 6-desmethyl-naproxen (6-DMN) was synthesized in our own laboratory (6). Acetonitrile and other reagents were of analytical or reagent grade. Water for HPLC analysis was of Millipore quality.

Synthesis and Characterization of Naproxen-L-Lactic Acid-Lysozyme (Nap-Lact-LYSO), Naproxen-L-Lactic Acid-Lysine (Nap-Lact-Lys), and Naproxen-L-Lactic Acid-Tripeptide [Z-Asp-ε-(Nap-Lact)-Lys-Arg(OH)]

Naproxen-L-lactic acid-lysozyme (Nap-lact-LYSO) (1:1:1) was synthesized as described previously (6); 1.0 mg of the conjugate contained 12.0 μg naproxen. The percentage of noncovalently bound naproxen or naproxen-L-lactic acid

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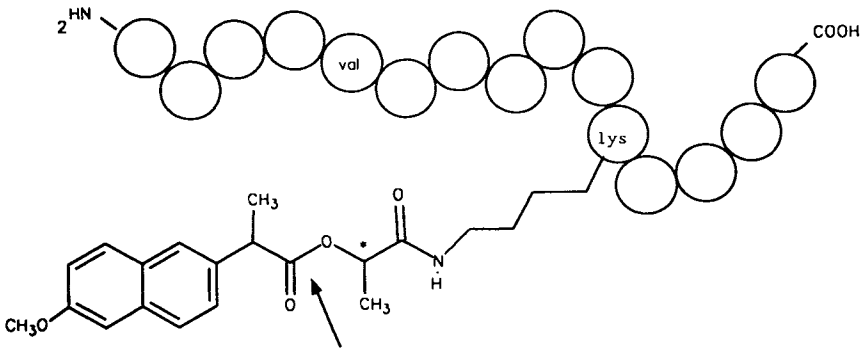

Fig. 1. Schematic representation of naproxen covalently coupled to lysozyme via L-lactic acid. The arrow denotes the ester bond.

[(Nap-Lact(OH)] was less than 4%, as established by HPLC. Naproxen-L-lactic acid-lysine (Nap-lact-Lys) and Z-Asp-ε-(Nap-lact)-Lys-Arg(OH) were synthesized following a similar procedure (6). Briefly, the *N*-hydroxysuccinimide ester of Nap-lact(OH) (Nap-lact-NHS) was dissolved in 10 mL dimethylformamide (DMF) and allowed to react with either lysine or the tripeptide in a DMF/borate (0.025 *M*, pH 8.5) (20/80) mixture for 24 hr. Z-Asp-(OtBu)-Lys(BOC)-Arg(OH) was previously treated with trifluoroacetic acid (30 min) for deprotection. The reaction mixture was partly evaporated *in vacuo* (20°C) and Z-Asp-Lys-Arg(OH) was isolated by HPLC and identified by mass spectral analysis (MW 654). The naproxen products were isolated by preparative HPLC and identified by mass spectral analysis as described previously [Nap-lact-Lys, MW 448; Z-Asp-ε-(Nap-lact)-Lys-Arg(OH), MW 835 (6)]. The HPLC conditions were similar as those described below for the analysis of naproxen and naproxen catabolites. The retention times were 4.6 min (Nap-lact-Lys), 7.1 (naproxen), 10.7 [Nap-lact(OH)], and 14.5 [Z-Asp-ε-(Nap-lact)-Lys-Arg(OH)]. The R_f values as established by TLC (Silicagel 60F254; ethylacetate/hexane/acetic acid, 33/66/1) were 0.39 [Z-Asp-ε-(Nap-lact)-Lys-Arg(OH)], 0.20 (naproxen), and 0.65 [Nap-lact(OH)].

Radioiodination

Nap-lact-LYSO was labeled with ^{123}I to a specific activity of 0.1 μCi/μg by using a mild chloramine-T method (8). Free ^{123}I was removed by gel filtration on a Sephadex G25 column. Immediately prior to the experiments any free ^{123}I was removed on a PD-10 column and radioactivity recovered in the void volume was >98% precipitable with TCA (added to a final concentration of 10%). The labeled protein was further purified from unreacted native lysozyme by chromatography on the FPLC (Pharmacia), using Mono-S 5/5 (Pharmacia) as cation exchanger as described previously for naproxen-lysozyme (6).

Analysis of Naproxen, 6-DMN-(S), Nap-Lact(OH), Nap-Lact-Lys, and Z-Asp-ε-(Nap-Lact)-Lys-Arg(OH)

Naproxen, 6-DMN in its free or sulfated form [6-DMN-(S)], Nap-lact(OH), Nap-lact-Lys, and Z-Asp-ε-(Nap-lact)-Lys-Arg(OH) were determined by RP-HPLC with fluorescence detection as described previously with only minor adaptations (5). The flow rate was 2 mL · min^{-1}. Flurbiprofen (2 μg/mL) was used as internal standard. Naproxen in the form of total naproxen was analyzed after alkaline hydrolysis of samples of plasma and urine as described previously (5). Bound naproxen was calculated by subtracting the amount of free naproxen, as established by HPLC without the prior hydrolysis of the samples.

Experiments *in Vivo*

Experiments with Freely Moving Rats

Surgery. Male Wistar rats (260–280 g) were prepared with a heart cannula using the method of Steffens (9). A 1-week recovery period was allowed before the experiments. During surgery halothane/N_2O/O_2 (5%) was administered for general anesthesia.

Experimental Conditions. The rats were placed in metabolic cages (Techniplast, Italy). The experimental setting allowed simultaneous blood and urine sampling without touching the animals. For regular urine sampling, the animals were infused with 5% glucose (1.2 mL · hr^{-1}). The metabolic cages were equipped with fraction collectors and recorders (Kipp) for automatic and timed urine sampling. Nap-lact-LYSO (10 mg; $n = 4$) was freshly dissolved in 2 mL saline and slowly injected (1 min). The urine was collected in cooled (4°C), preweighed tubes. The urine samples were weighed (accuracy of 10 mg), centrifuged (3000 rpm, 10 min), and stored at −20°C until analysis. Blood samples were drawn at indicated times and collected in heparinized polyethylene tubes. The blood samples were immediately centrifuged (3000 rpm, 3 min) and plasma samples were kept at −20°C until analysis. Consecutive studies in the same animals were carried out, with 1 week recovery in between.

Organ Distribution of Nap-Lact-123-I-LYSO and Urinary 6-DMN-S Excretion in Anesthetized and Immobilized Rats by Gamma-Camera Imaging

Gamma-camera imaging of Nap-lact-^{123}I-LYSO in heart-cannulated anesthetized (halothane/O_2/N_2O) rats was performed by the method of Haas and de Zeeuw (10). In addition, both ureters of each animal were cannulated. For a regular urine production, the rats were infused with 5% glucose (1.2 mL · hr^{-1}). In the specific experiments addressing carrier administration to a single kidney, the arteria renalis was temporarily (15 min) ligated immediately prior to injec-

tion of the conjugate. Nap-lact-^{123}I-LYSO (5 mg, 30 μCi; n = 2) was injected and monitored by external counting with a gamma camera. Three hours after the first injection, the experiment was repeated in the reversed setting: the animals were reinjected with an equal dose of the conjugate to check the viability of the previously ligated kidney. The total body, kidneys, liver, and bladder were monitored for γ-radiation for 6 hr. Organ distribution was expressed as percentage of the total administered dose. The renal activity–time profiles of the iodine-123 reflect the combination of uptake and degradation of the proteins. Urine collections were done every 30 min after injection of the conjugate. Radioactivity measurements in these samples were done with a gamma-counter (LKB, Bromma, Sweden) at a counting efficiency of 80%. Urinary 6-DMN-S excretion was established by HPLC as described above.

Experiments *in Vitro*

Renal Degradation of Nap-Lact-LYSO in Renal Lysosomal Lysates and Cortex Homogenates

Male rat lysosomal lysates and renal cortex homogenates were prepared as described previously (6). In the catabolism studies, the lysosomal lysates (100 μL) or cortex homogenates (250 μL) were diluted with 500 μL buffer (containing either 0.5 *M* HAc/NaAc, 4 m*M* DTT, 10 μg/mL flurbiprofen, pH 5.0, or 0.5 *M* phosphate, 4 m*M* DTT, 10 μg/mL flurbiprofen, pH 7.4). Naproxen, Nap-lact(OH), Z-Asp-ε-(Nap-lact)-Lys-Arg(OH), and Nap-lact-LYSO were dissolved in the appropriate buffer and added until a final volume of 1.0 mL and a final concentration of 10 μ*M* (as calculated for naproxen). This solution was incubated at 37°C. Samples (100 μL) were taken at indicated times and diluted with 3 vol of methanol. The mixtures were vortexed and centrifuged (2 min, 3000 *g*) and stored at 4°C until HPLC analysis. The supernatants (50 μL) were injected into the HPLC and further analyzed for naproxen and its metabolites as described above.

Pharmacokinetic Analysis

Kinetic studies were performed using the multifit modified CFT3 program (11).

Statistical Analysis

Statistical comparisons were made with the Wilcoxon paired rank sum test; $P < 0.05$ was selected as the minimal level of statistical significance.

RESULTS

Experiments *in Vivo*

Experiments in Freely Moving Animals

Figure 2 shows the plasma profiles of naproxen after administration of the conjugate (Nap-lact-LYSO) (left) and the uncoupled mixture of naproxen and LMWP as the control (right). The plotted values represent naproxen either in its covalently bound form or in its free form. The plasma levels and time profile are very different from those after

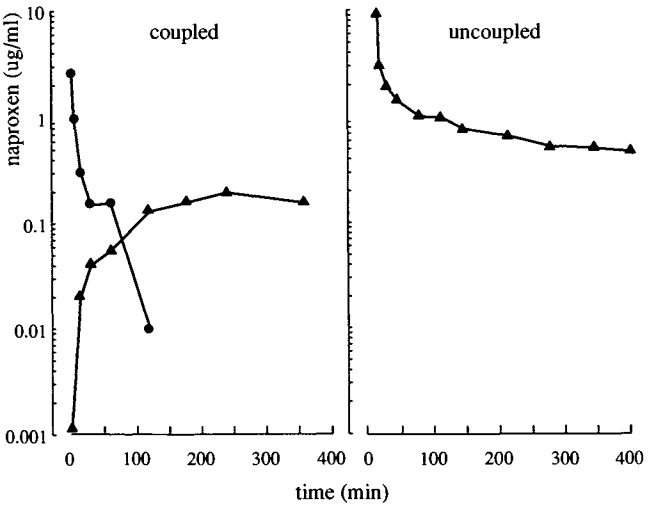

Fig. 2. Plasma curves of naproxen measured in its bound form (●——●) and its free form (▲——▲). Left: Curves obtained after injection of naproxen in its lysozyme-conjugated form coupled via the L-lactic acid spacer. Right: Curves obtained after injection of a mixture of naproxen and lysozyme.

injection of the uncoupled mixture. After administration of naproxen coupled to lysozyme, the conjugate is rapidly cleared from the plasma (Cl_p = 1.2 ± 0.38 mL · min^{-1}; n = 4). Plasma levels of the free naproxen were observed starting from 15 min after injection (t_{max} = 4 ± 0.2 hr; C_{max} = 7.6 ± 0.12% of the initial total naproxen concentration; n = 4). In contrast, after administration of naproxen in its uncoupled form, a biexponential disappearance curve of free naproxen was observed ($t_{1/2,\beta}$ = 2.8 ± 0.14 hr). In addition, in the case of administration in its coupled form, the area under the curve of free naproxen appeared to be reduced approximately eightfold compared to administration as a mixture [0.85 ± 0.32% (n = 4) versus 6.68 ± 0.32% (n = 8) dose · hr · mL^{-1}, respectively].

Administration of Nap-lact-LYSO resulted in a urinary excretion of naproxen in the form of its main metabolite 6-DMN-S, corresponding to 46% of the administered dose, mainly excreted during 2 until 21 hr after injection (t_{max} = 4 ± 0.3 hr; n = 4) (Fig. 3). Only 2.5 ± 0.41% of the dose was excreted in the urine in the form of parent naproxen (Table I). The renal excretion of 6-DMN-S after injection of the Nap-lact-LYSO conjugate was significantly delayed and showed a more sustained profile compared to that of the equimolar mixture ($t_{50\%}$ = 410 ± 11 versus 210 ± 8 min, respectively). The urinary excretion rate profile of 6-DMN-S showed a distinct similarity with the concentration–time profile of the free naproxen in plasma. It was inferred that the rate-limiting cleavage of the drug-L-lactic acid ester bond in the kidney is followed by both luminal and basolateral excretion of the drug from the tubular cells.

From the combined plasma clearance data of the free drug and the urinary excretion data of its main metabolite, an apparent renal clearance for naproxen was calculated: $Cl_{app,nap}$ = 37.42 ± 0.32 mL · hr^{-1} (in the case of the coupled form; n = 4) versus 6.71 ± 0.12 mL · hr^{-1} (in the case of the uncoupled form; n = 8). These apparent clearances were calculated as the quotients of the amount renally ex-

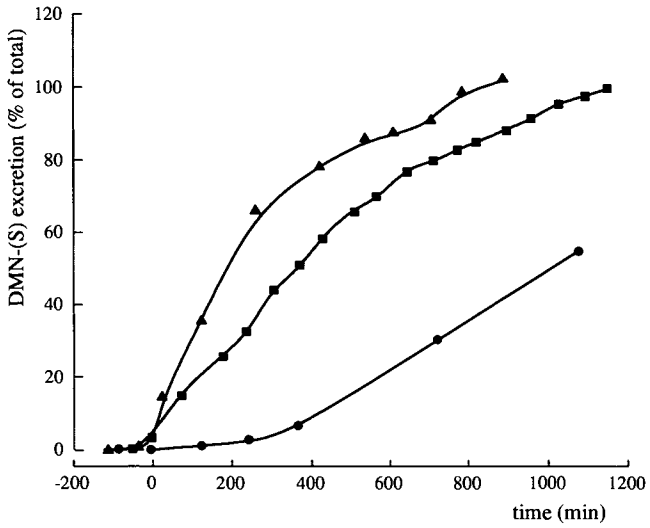

Fig. 3. Cumulative urinary excretion profile of naproxen in the form of its main metabolite 6-DMN-S, after administration of naproxen, 120 μg, as a mixture with native lysozyme (10 mg; ▲——▲), as a directly coupled conjugate with lysozyme (10 mg; ●——●), and conjugated to lysozyme via the L-lactic acid spacer (■——■).

creted naproxen [in the form of DMN-(S)] and the area under the curve (AUC) of the corresponding levels of free naproxen in the plasma (Fig. 2). The renal selectivity index was defined as the ratio of these clearances. Thus, by coupling to lysozyme the renal selectivity of naproxen was increased 5.6 ± 0.41 (n = 4)-fold (Table II). Additionally, these data indicate renal metabolism of the regenerated naproxen into 6-DMN-S.

Experiments in Anesthetized Animals

The predominant renal clearance of the Nap-lact-LYSO conjugate was further investigated by radioiodination of the protein of the conjugate. The iodine-123-labeled conjugate (100 μg; n = 2) was followed noninvasively in anesthetized and immobilized rats by gamma-camera imaging. The kinetics of naproxen-L-lactic acid-123I-LYSO were comparable to those of the native ^{123}I-LYSO. Analysis of the kidney time activity curve revealed a t_{max} of 20 min (native LYSO, 20 min), corresponding to 66 and 74% of the injected dose, respectively. Furthermore, the conjugate did not accumulate in the liver (less than 1%).

To confirm that 6-DMN-S formation of the released naproxen indeed occurred in the kidney, the following experiment was performed: the ureters of the animals (n = 2) were cannulated, and prior to intravenous injection of the iodine labeled conjugate (5 mg), one of the kidneys was temporarily ligated (15 min), allowing conjugate distribution only to one of the kidneys. After releasing the ligation, urinary 6-DMN-S excretion was monitored from both kidneys separately. The results are presented in Fig. 4. 6-DMN-S was excreted mainly by the kidney containing the conjugate, indicating a major 6-DMN-S formation by this particular kidney. Note that in the case of a major redistribution of naproxen metabolites to the bloodstream, 6-DMN-S excretion would be anticipated to occur at a similar rate in the contralateral kidney that had not "received" the conjugate. To validate that the kidney that had been ligated was in fact still viable and would have been able to generate 6-DMN-S, the experiment was repeated after 3 hr, now ligating the other kidney: this resulted in a similar protein accumulation and 6-DMN-S excretion in the previously ligated kidney. This indicates that a kidney ligated for 15 min is not irreversibly damaged and is able to produce and excrete 6-DMN-S metabolites.

Table I. Recovery of Naproxen, Covalently Bound Naproxen, and 6-DMN-(S) After Administration of Uncoupled Naproxen (n = 8), Directly Coupled Naproxen (n = 6), and Indirectly Coupled Naproxen via L-Lactic Acid (n = 4) to Freely Moving Rats[a]

Compound	Uncoupled	Coupled	
		Directly (ε-amide-bond)	Indirectly (ester bond)
Urinary excretion			
Naproxen	0.02 ± 0.004	<0.01	2.5 ± 0.41
Bound naproxen		4 ± 0.3	19 ± 1.0
6-DMN-(S)	59 ± 3.4	8 ± 1.3	46 ± 1.6
Fecal excretion			
Naproxen metabolites	nm	50 ± 20	nm
Total recovered drug	59 ± 3.5	62 ± 21	67.5 ± 1.6

[a] Values are expressed as the percentage of the administered dose (mean ± SD). nm, not measured.

Table II. Renal Apparent Naproxen Clearances[a] ($Cl_{R,app,nap}$) of Coupled (via L-Lactic Acid) Versus Uncoupled Naproxen (Equimolar Mixture) and the Renal Selectivity Index (RSI)[b]

Pharmacokinetic parameter	Uncoupled (n = 8)	Coupled (n = 4)
Amount renally excreted as DMN-(S) (% dose)	44.8 ± 0.62	31.8 ± 0.58
AUC (% dose · hr · mL^{-1})	6.68 ± 0.32	0.85 ± 0.09
$Cl_{R,app,nap}$ (mL · hr^{-1})	6.71 ± 0.12	37.42 ± 0.32
RSI		5.6 ± 0.41

[a] The apparent clearances were calculated as the quotient of the amount renally excreted of naproxen in the form of DMN-(S) and the area under the curve (AUC) of the corresponding levels of free naproxen in the plasma.

[b] RSI is the ratio of $Cl_{R,app,nap}$-coupled and $Cl_{R,app,nap}$-uncoupled.

Experiments *in Vitro*

Degradation of the Conjugate in Lysosomal Lysates and Cortex Homogenates

The cleavage of the ester bond was further examined *in vitro*. Figure 5 shows the HPLC patterns obtained after incubation of the conjugates with renal lysosomal lysates at pH 5.0. Besides formation of parent naproxen, other (intermediate) metabolites were also observed. ε-Naproxen-L-lactic acid-lysine was isolated as a rather stable intermediate metabolite. Obviously, under these conditions, the rate of cleavage of the ester bond was lower than that of the carrier

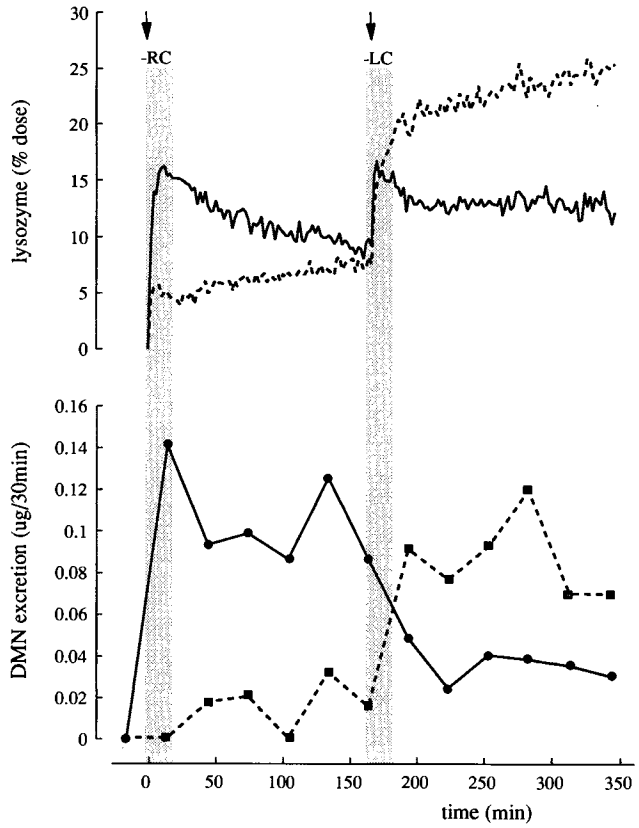

Fig. 4. Top: Time–activity curve of the left (solid line) and right (dotted line) kidney after injection (arrows) of Nap-L-lact-^{123}I-LYSO. Bottom: Urinary 6-DMN-(S) excretion curve collected from the left (●——●) and right kidney (■---■) after injection (arrows) of Nap-L-lact-^{123}I-LYSO. The arrow on the left denotes injection (5 mg) immediately after temporarily ligation (15 min) of the right kidney (−RC). The arrow on the right denotes reinjection (5 mg) after a similar ligation of the left kidney (−LC).

backbone itself. As controls the naproxen tripeptide Z-Asp-ε-nap-L-lactic acid-Lys-Arg(OH) and also ε-naproxen-L-lactic acid-lysine were incubated. In the former case also ε-nap-lact-lys was observed, which was itself relatively slowly converted to parent naproxen. Naproxen regeneration was found to be significantly enhanced by incubation in whole cortex homogenates at pH 7.4. This may indicate an involvement of other cytoplasmic esterases in the conversion of conjugates or derivatives of Nap-L-lact (OH) to parent naproxen.

DISCUSSION

The results presented in this study show the renal distribution, release, and predominantly luminal excretion of naproxen, when the drug is coupled to lysozyme via an ester bond, using a L-lactic acid spacer. The renal selectivity index of the drug was enhanced approximately sixfold, compared to equimolar uncoupled mixtures of the constituents.

LMWPs carrying drug molecules are, like native LMWPs, rapidly cleared from the plasma and rapidly accumulated in the kidney. Only 20% of the dose was excreted in the urine in the form of covalently bound naproxen, representing either nonreabsorbed conjugate or renally generated naproxen containing peptide catabolites.

A disadvantage of using ester bonds for drug targeting preparations might be their relative instability in plasma during transfer to the target site because of the presence of plasma esterases. The present study shows that, at least for the LMWP carrier used, this is not a major problem. After intravenous injection, LMWPs are rapidly cleared from the bloodstream by glomerular filtration and almost completely reabsorbed and catabolized by the renal lysosomes without reentering the bloodstream (12). The observation that ester bonds of NSAIDs are rather stable in plasma is in agreement with that of Bundgaard *et al.*, who demonstrated that ester derivatives of benzoic acid are stable in plasma (13,14). Therefore we conclude that, at least in the case of LMWP conjugation of naproxen via a L-lactic acid spacer, ester bonds are indeed sufficiently stable in the bloodstream during transport of the LMWP-conjugated drug to the kidneys.

Release of the parent drug naproxen successfully occurs from the conjugate within the kidney when using the L-lactic acid spacer. We previously reported on the kinetics of the directly coupled naproxen-LYSO (5), in which naproxen was attached via an ε-amide bond to the lysine residues of the protein. This drug-LMWP conjugate appeared to be renal selective. However, only 8% of the administered dose was recovered in the urine as naproxen metabolites, whereas 50% of the dose was recovered as naproxen metabolites in the feces (Table I). Lysosomal digestion resulted in the stable catabolite naproxen-lysine. By choosing a different coupling method (the L-lactic acid spacer), resistance to renal tubular lysosomal hydrolysis can be partly overcome, as demonstrated by the enhanced urinary recovery of released naproxen in the form of 6-DMN-S (49% of the dose instead of 8% after injection of the directly coupled product). These *in vivo* results agree with our earlier *in vitro* observations on conjugates of carboxyl group-containing drugs: ester bonds turn out to be cleavable, whereas amide bonds are resistant to hydrolysis (6). However, at least half of the conjugated naproxen was not recovered as 6-DMN-S in the urine. At least 20% of the dose was recovered in the urine still bound to the protein or catabolites. The remainder may be eliminated as undetected naproxen metabolites.

Reentry of the parent naproxen from the kidney into the blood is suggested by the observed appearance of free naproxen levels in the plasma. The plasma levels of the free drug appeared to be delayed and sustained (starting from 15 min until at least 24 hr after injection, with a t_{max} of 4 hr). This finding contrasted with the coinciding rapid plasma clearance of the drug in its bound form (within 2 hr after injection) and its corresponding rapid uptake in the kidney (t_{max} = 20 min). Therefore, the observed plasma levels of free drug may reflect transport of the released naproxen from kidney to blood. This reentry of the parent drug could result from transport of the renally liberated drug in tubular cells or from its tubular reabsorption. Passive or active reabsorption of NSAIDs (15) might modify the favorable tubular distribution of the drug. Since local retention of the drug is important for the ultimate success of drug targeting (16), drug reabsorption may be detrimental for renal targeting. On the other hand, these processes may also present the released drug to more distal parts of the renal tubuli and

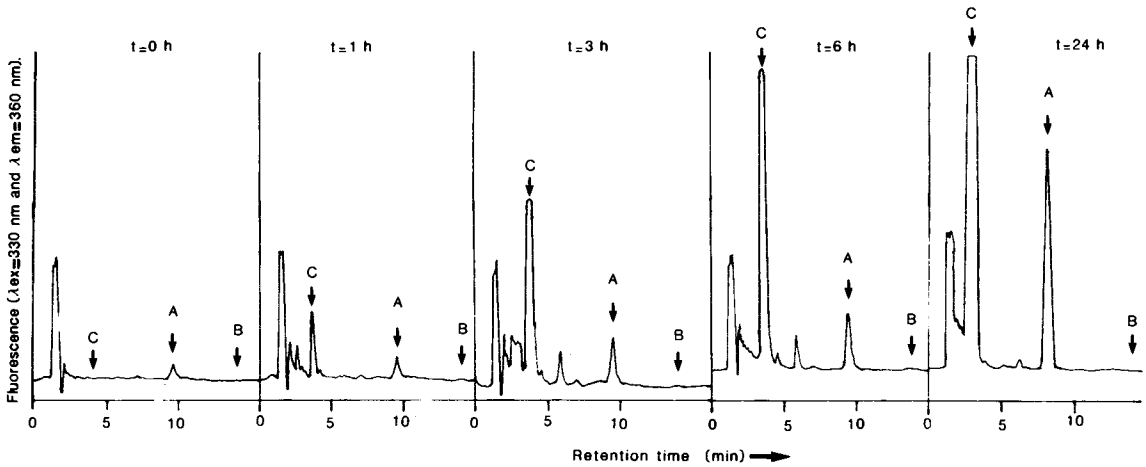

Fig. 5. HPLC chromatograms obtained after incubating Nap-L-lact-LYSO (10 μ*M*) with renal lysosomal lysates at pH 5.0. Arrow A denotes naproxen; arrow B denotes naproxen-L-lactic acid, and arrow C denotes Nap-L-lact-Lys.

could provide suitable drug levels in the interstitium. Since naproxen is also rapidly metabolized before it (re)enters the urine, renal retention is maintained.

The urinary recovery of naproxen (metabolites) might be enhanced further by using different spacers, since the renal cleavage of the ester bond might be suboptimal within the tubular cells. A suboptimal release of the drug can be inferred by the observation of intermediate lysosomal naproxen catabolites, such as Nap-lact-Lys, at least *in vitro*. For a more rapid drug release, two approaches can be considered. The first is chain lengthening by using a tetra-L-lactic spacer. This approach may make the bond more accessible to esterases as was demonstrated *in vitro* (6). The second option is the use of other α-hydroxyacids, such as citramalic acid, tartaric acid, and citric acid. These α-hydroxy acids have extra carboxyl groups available and this may enhance the affinity of the spacer for (carboxy)esterases. If a more gradual drug release is required, glycolic acid may be of use, since this spacer was slowly removed by tubular esterases *in vitro* (6).

The present study indicates that the kidney is involved in the metabolism of NSAIDs. 6-DMN-S has been identified as the main metabolite of naproxen in rats (5,17). The altered kinetics of naproxen by coupling it to lysozyme suggest a renal site of demethylation and subsequent sulfation of the drug. This finding is supported by the data obtained in the experiment with the single ligated kidney, also suggesting direct luminal transport and urinary excretion of naproxen in the form of 6-DMN-S. Renal demethylation of naproxen has also been observed *in vitro* in the isolated perfused rat kidney by Cox *et al.* (15). Sulfation can, in principle, also occur in the kidney (18–20). Apart from sulfation, abundant renal "phase two" metabolism of NSAIDs (glucuronidation) has recently been demonstrated in humans by Moolenaar *et al.* (21).

In conclusion, naproxen covalently coupled to lysozyme via a lactic acid spacer yields a specific renal delivery and local release by cleavage of the drug–lactic acid ester bond. Parent drug regeneration in the kidneys is followed by local demethylation and sulfation, followed by a predominant luminal transport and urinary excretion in the form of 6-DMN-S. The possibility of coupling drugs with carboxyl groups via ester bonds using α-hydroxy acids may therefore render the LMWP targeting concept applicable for a broad variety of therapeutic agents.

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