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Rapid Communication

Renal specific delivery of sulfamethoxazole in the rat by coupling to the low molecular weight protein lysozyme via an acid-sensitive linker

Eric J.F. Franssen [1], Frits Moolenaar [1], Dick de Zeeuw [2] and Dirk K.F. Meijer [1]

[1] *University Center for Pharmacy, Department of Pharmacology and Pharmacotherapy, Ant. Deusinglaan 2, 9713 AW Groningen (The Netherlands) and* [2] *Department of Nephrology, Academic Hospital, Oostersingel 59, Groningen (The Netherlands)*

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Summary

Sulfamethoxazole (SM) was converted to a renal specific drug targeting preparation by coupling the drug to egg-white lysozyme via an acid-sensitive *cis*-aconityl linker (1 : 1). Due to this chemical manipulation SM was rapidly distributed to the kidney. Both in vitro and in vivo data indicate that SM was uncoupled from the carrier by chemical hydrolysis in the lysosomes of proximal tubular cells, resulting in parent active drug at the target site. This concept is applicable to other drug-polypeptide conjugates which rapidly distribute to the kidney and might enable selective manipulation of renal (patho)physiology.

Drugs can be made renal specific by linking them to so-called low molecular weight proteins (LMWP). These proteins (with molecular weights lower than 20 000) are freely filtered through the renal glomerular basement membrane and subsequently reabsorbed and hydrolysed by the proximal tubular cells (Maack et al., 1979). In principle, drugs covalently attached to these proteins can be selectively delivered to the tubular cells. Upon arrival in the lysosomes of the tubular cells, in principle drug-protein conjugates may be catabolized by proteolytic enzymes into their single amino acid constituents and active drug. This new concept for site-specific drug delivery to the kidney has recently been tested by coupling the model drug naproxen to the LMWP lysozyme (Mol. Wt 14 400; pI = 11) (Franssen et al., 1991). The pharmacokinetic profile of this drug-protein conjugate after intravenous administration in the rat resulted in the renal specific distribution and catabolism of the conjugate. However, renal delivery and degradation of the conjugate did not lead to the parent drug naproxen, but gave rise to the catabolite naproxen-lysine, both in vitro and

Correspondence: E.J.F. Franssen, University Center for Pharmacy, Department of Pharmacology and Pharmacotherapy, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands.

Abbreviations: LMWP, low molecular weight protein; LYSO, native lysozyme; SM, sulfamethoxazole; aco-SM, *N-cis*-aconityl sulfamethoxazole; SM-aco-LYSO, *N-cis*-aconityl sulfamethoxazole linked to egg-white lysozyme (1 : 1); TCA, trichloroacetic acid.

in vivo. Interestingly, this liberated catabolite exhibited full pharmacological activity as compared to the parent drug. Yet, parent drug release may be essential for other drugs for pharmacological activity at the target site. Thus, in exploiting this concept for a broader variety of drugs, we have focused on the design of drug-protein conjugates that potentially can produce the parent active drug. In principle, release of parent active drug may occur by either enzymatic or chemical hydrolysis in vivo. Enzymatic hydrolysis of the chemical bond between the drug and the protein is often limited by the narrow specificity of the renal lysosomal enzymes as we have shown for different model drugs and model bonds in renal lysosomal preparations (Franssen et al., 1992). Therefore, chemical hydrolysis seems to be an attractive alternative approach for achieving rapid and more complete release of parent drug. In this concept, effective chemical hydrolysis may be achieved by the use of a pH-sensitive linkage, which is stable in the bloodstream and which hydrolyses spontaneously in the pH range prevalent in lysosomes (pH 4 – 5). In this respect a pH-sensitive *cis*-aconityl linkage has been described for coupling daunorubicin to poly(D-lysine) by Shen and Ryser (1981). We tested this modality of pH-dependent release by linking the model drug sulfamethoxazole (SM) to the LMWP lysozyme via a *cis*-aconityl bond. The fate of this drug-protein conjugate (SM-aco-LYSO) was studied both in vitro and in vivo. In the present paper, we present data about the pharmacokinetics of this drug-protein conjugate as compared to equimolar amounts of uncoupled drug and native protein. Our results validate the LMWP pH-sensitive spacer concept: i.e. rapid and predominant distribution of the drug-protein conjugate to the kidney, pH-dependent release of the parent drug in the lysosomal compartments and luminal transport of parent drug.

Synthesis and characterization of the drug-protein conjugate: Coupling of sulfamethoxazole to *cis*-aconityl anhydride was achieved using a procedure similar to that described for the coupling of daunorubicin to poly(D-lysine) (Shen and Ryser, 1981). The SM-aco-LYSO conjugate was synthesized via a two-step reaction procedure. Initially, an acid-sensitive amide linkage was formed between amine functional groups of SM and carboxylic functional groups of *cis*-aconityl acid. Thereafter, the τ-carboxylic group of *cis*-aconityl sulfamethoxazole (aco-SM) was further reacted with the ϵ-amine groups of lysozyme. Unreacted drug or drug spacer was removed by gel-permeation chromatography on a Bio-gel P-2 column and washing by Amicon® ultrafiltration. Finally, the drug-protein conjugate was lyophilized and stored at −20°C. The drug load of the conjugate was determined by HPLC analysis of the drug as described below and protein content was assayed according to the method of Bradford (1976). Total bound drug was determined after acid hydrolysis (30 min) by the addition of 1 vol. of 6 N HCl. Non-covalently bound drug was assayed after the addition of 2 voles of acetonitrile. The mixtures were centrifuged and the supernatants were analyzed by HPLC. The degree of drug substitution was 1 : 1 on a molar basis. The amount of non-covalently bound drug was less than 1% of the total amount of drug present. The preparation and characterization of drug-LMWP conjugates have been described recently (Franssen et al., 1992).

Experiments in vitro: The drug-protein conjugates were studied with respect to release of parent drug in different (biological) media. Briefly, 1.0 mg of the protein (corresponding with 18.0 μg of sulfamethoxazole) was incubated with plasma, urine and renal lysosomal lysates, followed by dilution with appropriate buffers to give a final volume of 1.0 ml. The results are presented in Table 1. 84% of the parent drug was released in the renal lysosomal preparation (pH 5) after 24 h, whereas less than 10% was regenerated during a similar incubation with plasma and urine. These results indicate selective drug release under the acid conditions of the renal lysosomes and pronounced stability in plasma and urine. The data are in accordance with those reported concerning the stability of the corresponding daunomycin-poly(D-lysine) conjugates (Shen and Ryser, 1981).

Experiments in vivo: To test this targeting concept in vivo, 10 mg of the SM-aco-LYSO drug-protein conjugate was injected into four freely moving, heart-cannulated rats. Its pharmacoki-

TABLE 1

In vitro parent drug regeneration for the drug-LMWP conjugate SM-aco-LYSO

Time (h)	Release of sulfamethoxazole (%)							
	B5	H5	L5	B7	H7	L7	P	U
0	2	3	0	0	0	0	0	0
1	41	49	48	2	1	2	1	2
4	56	58	57	3	2	3	2	3
24	80	84	84	7	8	8	7	10

Drug release is expressed as the percentage of total drug present after incubation at 37°C. Total drug present was determined after acid hydrolysis (6 N HCl; 24 h) of the individual incubation vials; values represent the means of at least three separate incubations. Incubation media were: B5 and B7: pH of buffer 5.0 and 7.4, respectively; H5 and H7: renal cortex homogenate at pH 5.0 and 7.4, respectively; L5 and L7: lysosomal lysates buffered at pH 5 and 7.4, respectively; P, rat plasma buffered at pH 7.4; U, rat urine buffered at pH 6.8.

netics were compared to those of an equimolar mixture of uncoupled SM (180 μg) and native lysozyme (10 mg) in these same animals (1 week after the first experiment). The experimental setting allowed sampling of blood and urine over a period up to 5 days (Franssen et al., 1991). For regular urine production and sampling, rats were infused with 5% glucose (1.2 ml h^{-1}). The pH of the freshly collected urine samples was always greater than 6.8. The urine fractions were buffered (pH 7.4) and immediately frozen until analysis. Plasma and urine samples (100 μl) were analyzed for free sulfamethoxazole (SM), its main (hepatic) metabolite N^4-acetyl sulfamethoxazole (N4-Ac-SM) and SM-aco-LYSO. Plasma and urine levels of parent SM and N4-Ac-SM were determined by HPLC. The column was a μBondapack C-18 (Waters). The eluent used was water/acetonitrile/acetic acid (80:20:1) at a flow rate of 2 ml min^{-1}. Naproxen (1 μg/ml) was used as internal standard. The retention times of SM and N4-Ac-SM were 6 and 8.4 min, respectively. Briefly, plasma and urine samples (100 μl) were mixed with 2 vols of acetonitrile. The mixture was centrifuged and 100 μl of the supernatant was injected into the HPLC column. Calibration curves were constructed by adding known amounts of SM and N4-Ac-SM to plasma and urine. The values of R^2 for these curves were > 0.999. The amount of SM-aco-LYSO was calculated from data representing acid-labile bound SM. Acid-labile bound SM was assessed via HPLC analysis of SM by subtracting the total amount of SM (obtained after acidic hydrolysis of the samples) from the amount of free SM. Briefly, plasma and urine samples were mixed with 1 vol. of 6 N HCl. The mixture was left to stand at room temperature for 2 h. Thereafter, the mixture was centrifuged and analyzed by HPLC, exactly as described above. The results are listed in Table 2. In the case of an uncoupled SM:lysozyme mixture, the disappearance of a plasma with a $t_{\frac{1}{2},\beta}$ of 4.8 h was observed, coinciding with urinary excretion of parent SM (15 ± 4%) and N4-Ac-SM (43 ± 7%) between 0 and 24 h after injection. These data are in accordance with earlier reports of the kinetics of single doses of SM (Vree et al., 1978). In contrast, after injection of covalently bound SM, plasma levels were below the detection limit (50 ng/ml), whereas the parent drug was present in the urine from 1 to 10 h after injection (6.3 ± 2.5%). Furthermore, its N^4-acetyl metabolite was absent in the urine (< 1%). 57 ± 19% of the drug dose was already excreted in the urine in the form of unchanged (i.e. unabsorbed) drug-protein conjugate within 2 h after injection of the conjugate. The observed differences in pharmacokinetics between the conjugate and the mixture in a representative rat are visualized in Fig 1. The absence of the hepatic N^4-acetyl metabolite in the urine after injection of the SM-aco-LYSO conjugate indicates that the neither the conjugate

TABLE 2

Urinary covery, expressed as percentage of administered dose after 24 h of free SM, N4-Ac-SM and SM-aco-LYSO after injection of 180 μg of coupled SM and 180 μg of uncoupled SM

Compound	SM-aco-LYSO (10 mg)	SM + LYSO (180 μg/10 mg)
SM	6.3 ± 2.5% (4)	15 ± 4% (4)
N4-Ac-SM	< 1% (4)	43 ± 7% (4)
SM-aco-LYSO	57 ± 19% (4)	–

Values represent means ± S.D.; values between parentheses denote the number of experiments.

nor its attached drug is hepatically cleared from the bloodstream. Since free SM was not detectable in plasma, the presence of parent SM in the urine can only be the result of pH-dependent release in the lysosomes of proximal tubular cells. Apparently, very little of the renally released parent drug entered the general circulation. SM liberated in the kidneys may also be excreted in the form of other (new) metabolites which were not measured with the present HPLC method. Experiments with radiolabelled SM were programmed to investigate this point. In the protocol used in the present study, a bolus dose of 10 mg protein was administered to obtain a maximal amount of SM in the kidneys. At this dose the transport maximum for protein reabsorption was exceeded and therefore roughly 50% of the coupled SM was excreted in the bound form in the urine. This portion of the dose is not reabsorbed by the tubular cells and consequently will not be available for parent drug regeneration.

In addition, the kinetics and organ distribution of SM-aco-LYSO were compared to those of native lysozyme by labelling the proteins with I123. Conjugate and native lysozyme (100 μg) were labelled with I123 using the chloramine-T method (Greenwood et al., 1963). After injection of the conjugate or the uncoupled mixture (10 mg

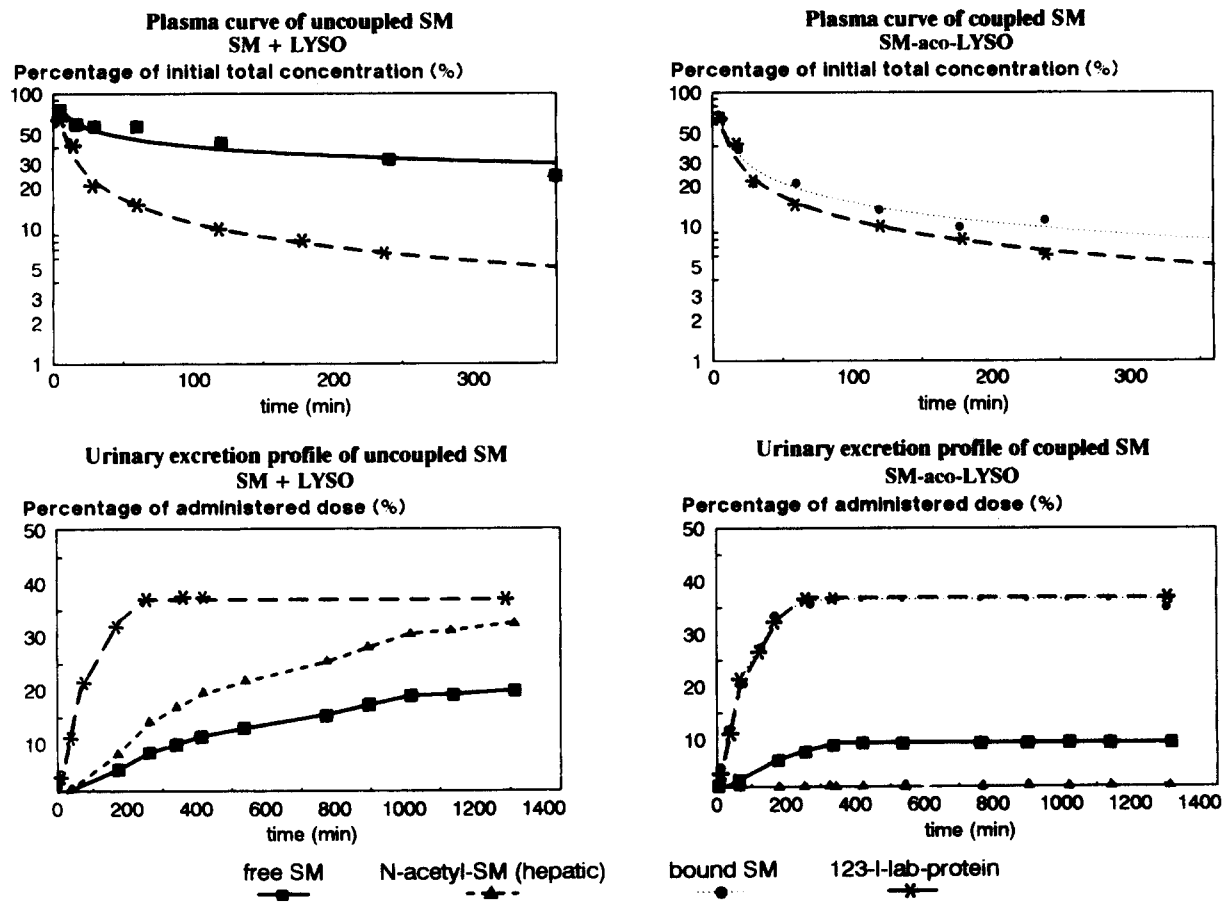

Fig. 1. Plasma curves and urinary excretion profiles of coupled SM (curves on the right) vs uncoupled SM (curves on the left) in a representative rat. The values are plotted for: free SM (■); its hepatic metabolite N^4-acetyl-SM (▲); bound SM (●); and trichloroacetic acid-precipitable counts (*).

protein, 3 μCi) into freely moving rats, plasma and urine samples were collected at the indicated time points (Fig. 1). After treating these samples with 3 vols of 20% trichloroacetic acid and centrifugation ($3000 \times g$, 2 min), the pellets and supernatants were processed in a gamma-counter (LKB, Bromma, Sweden). Their organ disposition was further studied by gamma camera imaging of the labelled conjugate or native lysozyme (100 μg protein, 30 μCi) after intravenous injection into three anesthetized rats. The kinetics (Fig. 1) and organ distribution of the conjugate were similar to those of native lysozyme. Gamma camera imaging revealed a predominant distribution to the kidney. A rapid t_{max} of 20 min in the renal tissue content profile was observed after injection of both labelled SM-aco-LYSO and native lysozyme. We conclude from these data that not only specific distribution to the kidneys was achieved but also that efficient renal degradation of the conjugated protein occurred. These results confirm the rapid and predominant distribution of bound SM to the kidneys and also the rapid and roughly 50% urinary excretion of unchanged bound SM (Table 2 and Fig. 1). Further, they indicate that the coupling of 1 mol of SM to 1 mol of LYSO does not basically change the overall disposition of the LMWP lysozyme.

Therefore, we conclude that the SM kinetics are altered by covalent coupling of the drug to lysozyme by an acid-sensitive linker such that:
(a) SM, in the form of SM-aco-LYSO, is rapidly and predominantly distributed to the kidney and during this transport free SM does not enter the plasma compartment.
(b) Renal uptake of SM, in the form of SM-aco-LYSO, occurs and lysosomal catabolism of the LMWP carrier coincides with the pH-sensitive release of parent active SM.
(c) Renally liberated parent SM is predominantly transported to the tubular lumen and finally excreted in the urine.

General conclusion: The combined data of the present study indicate that drugs covalently coupled to a LMWP using an acid-sensitive linker can be rapidly and predominantly filtered by the kidney, reabsorbed and chemically hydrolysed in their parent active form, due to the favourable pH circumstances (pH 4 – 5) in the lysosomes.

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