Polymeric prodrugs of antibiotics with improved efficiency

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Macromolecular prodrugs of the antibiotic norfloxacin were prepared by coupling the drug via a peptide spacer onto a mannosylated dextran. The tetrapeptide gly-phe-gly-gly-gly-OMe was selected as substrate for lysosomal enzymes. The drug was coupled on the α-C of the terminal glycine. *In vitro* degradation studies demonstrated the release of the parent drug in the presence of cathepsin B. *In vivo* experiments on mice showed a promising therapeutic effect.

1. Introduction

In order to be effective, antibiotics must reach adequate concentrations at the appropriate location. To destroy intracellular organisms, the drug must be able to enter not only the cells, but also the subcompartment where the micro-organisms reside [1]. One way to solve the bioavailability problem is to use a drug delivery system capable of specifically targeting the drugs into the macrophages [2]. Norfloxacin covalently linked onto a polymeric carrier is one approach to improve its pharmacological properties. Prodrug uptake by cells is then restricted to the mechanism of endocytosis, thus allowing for cell-specific targeting of the drug and intracellular controlled release at the target site. In previous work [3] macromolecular prodrugs dextran-gly-phe-ala-leu-norfloxacin and dextran-gly-phe-leu-gly-norfloxacin were prepared. However, degradation studies revealed the release of not free norfloxacin but leu-norfloxacin and gly-norlfoxacin.

In this paper, the preparation of a tetrapeptide derivative, namely gly-phe-gly-gly in which norfloxacin is the α-substituent of the C-terminal glycine residue and its subsequent coupling onto mannosylated dextran are described. Mannosylated dextran is known to be recognized by macrophages [4]. The *in vitro* and *in vivo* properties of the prodrug were investigated.

2. Materials and methods

2.1. Materials

Norfloxacin was obtained from Sigma. Peptides were purchased from Bachem Chem. Co. Dextran was obtained from Pharmacia. Paranitrophenylchloroformate was obtained from Merck. Bovine cathepsin B was obtained from Fluka. All other chemicals were purchased from Across.

2.2. Synthesis of Z-gly-gly(α-norfloxacin)-OMe

Z-gly-ser-OMe: Z-gly-paranitrophenylester (1 g, 3 mmol), ser-OMe (0.46 g, 3 mmol) and methylmorpholine (3 ml) were dissolved in *N,N*-dimethylformamide (DMF) (15 ml). The solvent was evaporated after 48 h and the residue was purified by chromathography (dichloromethane/isopropanol: 98/2 → 88/12).

Z-gly-gly(α-OAc)-OMe: Z-gly-ser-OMe (200 mg, 0.65 mmol), $Pb(OAc)_4$ (340 mg, 0.97 mmol) and molecular sieves (0.4 nm, 550 mg) were refluxed in dry ethyl acetate (20 ml) for 3 h. After cooling, the mixture was filtered on Celite and the solvent was evaporated.

Z-gly-gly(α-norfloxacin)-OMe: Z-gly-gly(OAc)-OMe (200 mg, 0.59 mmol) was dissolved in DMF (10 ml). Triethylamine (82 μl, 0.59 mmol) and norfloxacin (189 mg, 0.59 mmol), dissolved in DMF, were added and stirred for 36 h. The solvent was evaporated and

extracted with dichloromethane, HCl-solution (3 × 0.05 M) and water (3 ×).

2.3. Coupling of the dipeptides

After removal of the Z-protecting group Boc-gly-phe-pentafluorophenylester (137 mg, 0.28 mmol) and gly-gly(α-norfloxacin)-OMe (130 mg, 0.28 mmol) were dissolved in DMF (10 ml) and some drops of *N*-methylmorpholine were added. DMF was evaporated after 48 h. Extraction with dichlormethane and evaporation was followed by chromatography using dichloromethane/methanol (9/1) → dichloromethane/methanol/acetic acid (9/1/0.1) as eluents.

2.4. Coupling of gly-phe-gly-gly(α-norfloxacin)-OMe with activated dextran

After removing the Boc-group, the product was coupled onto chloroformate-activated dextran prepared as described before [5]. The degree of linear carbonates was measured by u.v. Chloroformate-activated dextran was dissolved in dimethylsulfoxide (DMSO)/pyridine (1/1) the mannose derivative and gly-phe-gly-gly(α-norfloxacin)-OMe were added. After 48 h the conjugate was precipitated in ethanol/ether (1/1) dissolved in NaOH-solution (0.1 M, 8 ml) and purified with preparative gel permeation chromatography (GPC) (G15). The degree of substitution was 7.7 mol% of norfloxacin and 3.2 mol% of mannose.

2.5. Degradation of the polymeric norfloxacin conjugate

To study the hydrolytic stability, about 1 mg/l ml of the polymeric norfloxacin conjugate was dissolved in phosphate buffers of pH 7.4 and 5.5, at 37 °C. At regular time intervals samples were taken and analyzed by high-pressure liquid chomatography (HPLC) (PLRP-S column, 10 nm, 5 μm), eluent: a mixture of acetonitrile/methanol/tetrahydrofuran/acetic acid/water (228/39/16/138/1582 ml), flow: 0.75 ml min^{-1}, u.v. detected at 278 nm. To study the enzymatic stability, the conjugate (about 1 mg) was dissolved in a mixture of buffer pH = 5.5 (1500 μl), reduced glutathion in buffer (200 μl, 50 mM), ethylenediaminetetra-acetic acid (EDTA) in buffer (200 μl, 10 mM) and cathepsin B (100 μl, 2.5 mg ml^{-1} bovine cathepsin B). Samples were regularly taken out of the incubation mixture and analyzed by HPLC.

2.6. *In vivo* study of the polymeric norfloxacin conjugate

The following protocol was used: day 1 : 25 mice (C57 BI/6, female) were injected with 10^6 bacteria that cause tuberculosis. Days 6-9: each group of five animals was injected with: DEX_{64}-$mannose_2$ (0.5 mg ml^{-1}); phosphate-buffered saline (PBS) (0.5 ml): blanco; isoniazid (0.5 mg ml^{-1}); DEX_{64}-$mannose_2$-$norfloxacin_7$ (0.5 mg ml^{-1}); norfloxacin 0.5 mg ml^{-1}). After 13 days the animals were sacrificed and the bacteria were counted in liver, spleen and lungs.

3. Results and discussion

In a previous publication [3], we reported about the synthesis and *in vitro* evaluation of dextran-gly-phe-leu-gly-norfloxacin and dextran-gly-phe-ala-leu-norfloxacin

For these prodrugs the linkage between norfloxacin and the peptide spacer was an amide bond. Consequently, the prodrug was completely stable under hydrolytic conditions. However, in the presence of cathepsin B, a lysosomal protease gly-norfloxacin and respectively leu-norfloxacin were released. The study of the antibacterial activity gly-norfloxacin and leu-norfloxacin reveals that the MIC and MBC values of the amino acid conjugates are significantly higher that those of the drug itself. Therefore, an alternative prodrug was designed. Norfloxacin is linked onto gly-phe-gly-gly-oMe as α-substituent of the terminal glycine residue. This approach was first reported by Kingsbury and co-workers [6, 7]. The tetrapeptide was finally coupled with mannosylated dextran.

3.1. Synthesis of gly-phe-gly-gly(α-norfloxacin)-OMe

The general *reaction scheme* for the synthesis of the polymeric norfloxacin conjugate is shown in Fig. 1. In a first step, the seryl peptide Z-gly-ser-OMe (**3**) was obtained by the reaction of the paranitrophenylester of Z-protected glycine (**1**) and serine methylester (**2**). In the second step, the serine residue was converted into an α-acetoxyglycyl residue (**4**). The α-substituted dipeptide was synthesized following the strategy of Apitz and co-workers [8, 9], who reported the conversion of different types of seryl peptides into the corresponding α-acetoxyglycine derivatives by treatment with lead tetraacetate in the presence of molecular sieves. The products obtained were 1 : 1 mixtures of diastereoisomers. The α-acetoxyglycine derivative is suitable for reaction with a nucleophile. In the presence of triethylamine the acetate group was displaced by the piperazine aminogroup of norfloxacin [10–12]. A racemic mixture of Z-gly-gly(α-norfloxacin)-OMe (**5**) was obtained in a good yield (89%). The following step in the synthesis of the norfloxacin conjugate was the lengthening of the dipeptide to a tetrapeptide.

3.2. Stability of the polymeric norfloxacin conjugate

In order to investigate the hydrolytic stability of the polymeric prodrug, the conjugate was incubated in buffers, physiological pH and lysosomal pH (Fig. 2). Dextran-gly-phe-gly-gly(α-norfloxacin)-OMe was not completely stable under hydrolytical conditions: norfloxacin was gradually released. After 24 h at pH 7.4, 27% norfloxacin was released. At pH 5.5, 40% norfloxacin was released over the same time period. The enzymatic stability of the polymeric prodrug was studied by incubating the substrate in buffer pH 5.5 in the presence of the lysosomal enzyme cathepsin B (Fig. 2). It

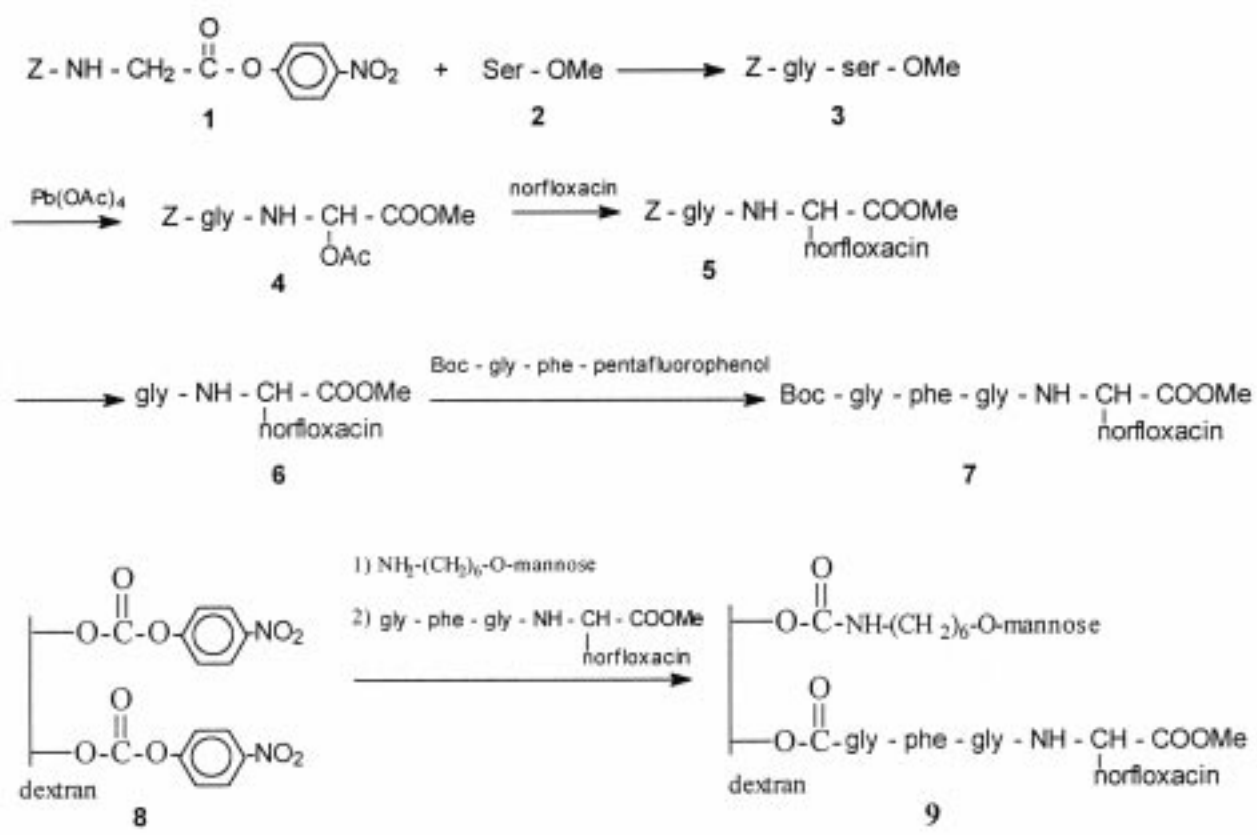

Figure 1 Reaction scheme for the preparation of mannosylated dextran-gly-phe-gly-gly(α-norfloxacin)-OMe.

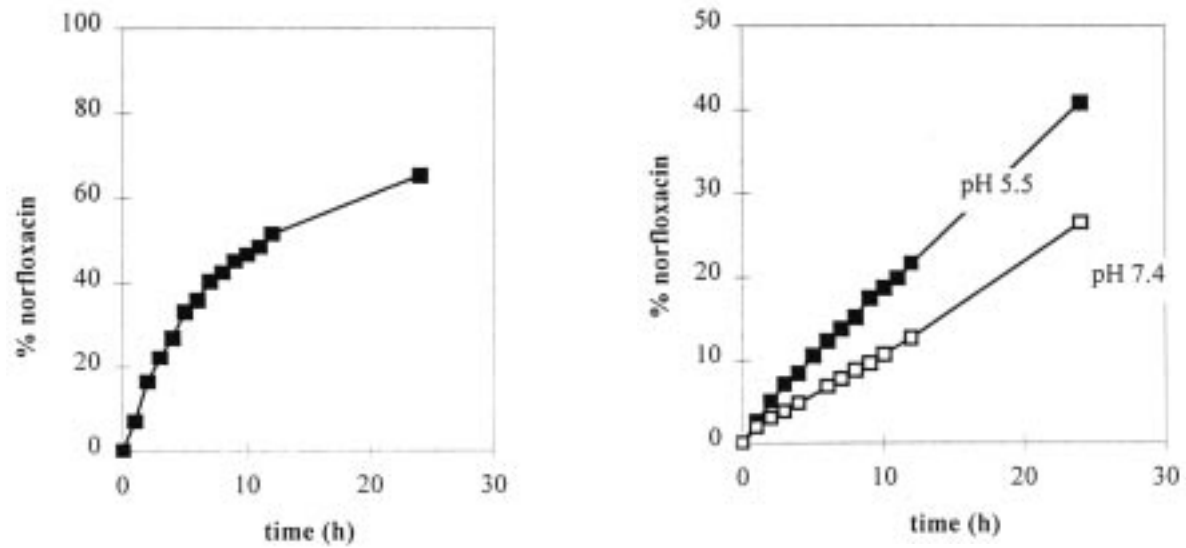

Figure 2 Hydrolytic and enzymatic stability of the polymeric norfloxacin conjugate and enzymatic stability of the polymeric norfloxacin conjugate.

is important to note that the enzymatic hydrolysis leads to the release of norfloxacin itself. The release starts off fast. After almost 50% norfloxacin is released, the release rate slows down. This can be explained by the fact that the polymeric prodrug of norfloxacin is a mixture of two stereoisomers, one of them probably inert to cathepsin B. Half of the norfloxacin conjugate is a substrate for enzymatic hydrolysis, the other half is only sensitive to hydrolytic hydrolysis. The curve obtained is the result of the combination of both phenomena.

3.3. *In vivo* evaluation on mice infected by *mycobacterium tuberculosis*

In vivo tests against *Mycobacterium tuberculosis* demonstrated a much better efficacy of the conjugate as compared to unmodified norfloxacin (Fig. 3). Only a small difference is observed between the amount of bacteria counted in the mice treated with norfloxacin and those treated with a buffer solution. Norfloxacin is not a particularly good candidate for the treatment of tuberculosis. On the other hand the mannosylated

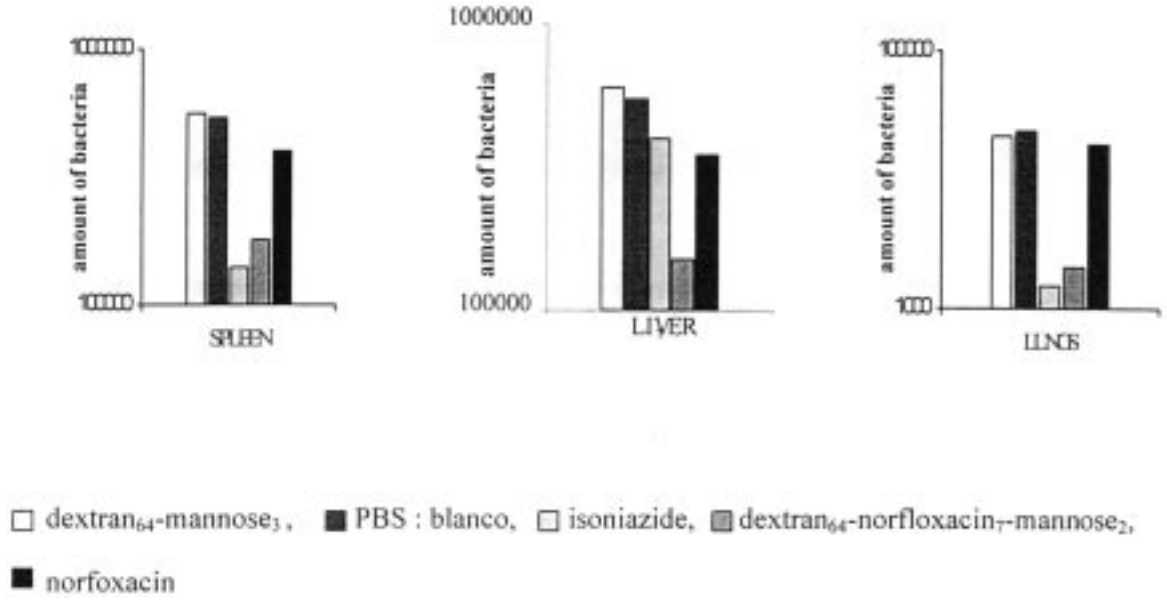

Figure 3 In vivo evaluation on mice infected by *Mycobacterium tuberculosis*.

dextran–drug conjugate shows a much higher therapeutic effect. In the liver, the conjugate has an even higher efficacy then the reference drug isoniazid. It is believed that the pronounced liver uptake is due to the glycosyl side groups along the polymeric carrier promoting macrophage uptake by receptor mediated endocytosis.

4. Conclusion

Dextran-gly-phe-ala-leu-norfloxacin and dextran-gly-phe-leu-gly-norfloxacin are suitable substrates for lysosomal enzyme cathepsin B, releasing leu-norfloxacin and gly-norfloxacin. On the other hand a polymeric prodrug having the drug incorporated via a tetrapeptide spacer as the α-substituent of the terminal glycine residue released the parent drug in medium containing cathepsin B.

In vivo tests against *Mycobacterium tuberculosis* demonstrated a much better efficacy of the conjugate as compared to the unmodified norfloxacin. The conjugate proves to be very promising in treating microorganisms residing in the liver.

Acknowledgments

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