Biodegradable Microspheres VI: Lysosomal Release of Covalently Bound Antiparasitic Drugs from Starch Microparticles

TIMO LAAKSO[‡], PETER STJÄRNKVIST[‡], AND INGVAR SJÖHOLM[*x]

Received October 2, 1986, from the [*]*Division of Pharmacy, Department of Drugs, National Board of Health and Welfare, S-751 25 Uppsala, Sweden,* and the [‡]*Department of Pharmaceutical Biochemistry, Biomedical Centre, University of Uppsala, S-751 23 Uppsala, Sweden.* Accepted for publication January 2, 1987.

Abstract □ The possibilities of using polyacryl starch microparticles as a carrier for low molecular weight drugs have been investigated. Two drugs containing primary amino groups, primaquine and trimethoprim, have been covalently coupled to the starch microparticles via tri-, tetra-, and pentapeptide spacer arms. The drug–particle complexes were prepared by coupling different drug–peptide derivatives to the particles after activation of the starch with carbonyldiimidazole. The activation process with subsequent removal of activated groups did not change the degradability of the starch microparticles. The resulting drug–carrier complexes were stable in serum, while free drugs were released in a lysosome fraction. Thus, the microparticle–peptide–drug conjugates fulfill the basic requirements for a drug carrier used to target drugs to the lysosomes (e.g., for the treatment of lysosomal parasitic diseases).

During the last ten years the development of drug carriers has received great interest (e.g., for the treatment of lysosomal diseases). This is especially true in the case of diseases caused by intracellular parasites, which often are difficult to treat with conventional drug formulations. So far, most of the work has been concerned with liposomes.[1] But lately, other systems have been presented, including small particles of polyisobutyl cyanoacrylate[2] or polyacryl starch,[3] and soluble carriers such as poly(*N*-[2-hydroxypropyl]methacrylamide)[4] or proteins.[5] The great potential in this field is exemplified by the treatment of experimental leishmaniasis with antimonial drugs in liposomes, where a dose reduction of ~700 times has been achieved.[1,6] Our research focuses on the evaluation of polyacryl starch microparticles as drug carriers. These microparticles are prepared from acryloylated starch and are readily dissolved in the lysosomal milieu.[3] Macromolecules (e.g., enzymes) can be entrapped in the microparticles and targeted to the lysosomes of the reticuloendothelial system.[7] However, low molecular weight drugs cannot be entrapped in the microparticles, but must be covalently attached to the microparticle matrix. This work was undertaken in order to develop methods to couple drugs containing amino groups (primaquine and trimethoprim) to starch microparticles. The drugs must remain bound to the carrier during the transport in the blood, but have to be released in free form at the target (i.e., the lysosomes of the reticuloendothelial system).

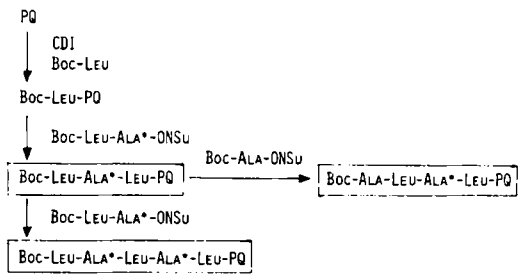
Experimental Section

Materials—*N-tert*-Butyloxycarbonyl-L-leucine (*N-tert*-Boc-L-leucine, Lot 14F 0270), *N-tert*-butyloxycarbonyl-L-alanine (*N-tert*-Boc-L-alanine, Lot 83F 0211), and 1,1′-carbonyldiimidazole, Lot 94F 3427, were obtained from Sigma Chemical Company (St. Louis, MO, USA). L-Alanine, Lot 115443110, was purchased from Fluka AG (Buchs, Switzerland). *N*-Hydroxysuccinimide, Lot 5213984, and dicyclohexylcarbodiimide were from E. Merck (Darmstadt, Germany). Primaquine (8-[(4-amino-1-methylbutyl)amino]-6-methoxyquinoline; PQ) was obtained from Janssen Chimica (Beerse, Belgium). Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine; TMP, Lot 20013] was a gift from Dr. Bo Ekman, Ferrosan (Malmö, Sweden). [^{14}C]Alanine, Lot 48, was from The Radiochemical Centre (Amersham, England). [^{14}C]Starch, Lot 1252132, prepared from *Nicotiana tabacum*, was obtained from New England Nuclear (Dreieich, W. Germany). The starch used in the microparticle preparation, MD6, Lot 58583, was a gift from Dr. Lars Svensson, Stadex AB (Malmö, Sweden).

Synthetic Section—The peptide–drug derivatives were synthesized as shown in the overall Scheme I. The methods used for the synthesis, described below, and the yield in each separate step are listed in Tables I and II. The purity and identity of the products were confirmed by TLC and NMR. Compounds which were subsequently coupled to microparticles (the tri-, tetra-, and pentapeptide derivatives) were also analyzed by HPLC and subjected to amino-acid analysis.

Condensation with Carbonyldiimidazole—*N-tert*-Boc-L-leucine (*N*-Boc-Leu) was activated with an equimolar amount of carbonyldiimidazole (CDI) for 30 min at room temperature in dry dimethylformamide (DMF).[8] An equimolar quantity of the appropriate drug (PQ or TMP) was added as the free base and the mixture was stirred overnight at either room temperature (Boc-Leu–PQ) or 100 °C (Boc-Leu–TMP). The reaction mixture was diluted with an equal volume of 0.2 M sodium carbonate, pH 11, and extracted several times with ether. The combined organic extracts were washed with 0.2 M citric acid, pH 2.0, dried over sodium sulphate, and evaporated to dryness under reduced pressure.

Condensation with Carbodiimide—Equimolar amounts of *N*-Boc-protected amino acid or peptide, *N*-hydroxysuccinimide (HOSu), and dicyclohexylcarbodiimide (DCC) were mixed in dioxane and stirred overnight at room temperature.[9] The dicyclohexylurea formed was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The resulting active ester in dioxane:water (1:1) was then treated with the appropriate amino acid or peptide derivative containing a free amino group (1.1 equivalent). Potassium bicarbonate (2 equivalents) was then added and the mixture was stirred overnight. The mixture was diluted with an equal volume of 0.2 M citric acid, pH 2.0, and extracted several times with ethyl acetate. The combined organic phases were washed with 0.2 M



Scheme I *—Synthetic pathways for peptide–primaquine (PQ) derivatives. The Leu-Ala-Leu–TMP conjugate was synthesized in principally the same way. The star (*) indicates the ^{14}C-label. The compounds in frames were subsequently deblocked and coupled to microparticles.*

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sodium carbonate (not done in the Boc-Leu-Ala synthesis), dried over sodium carbonate, and evaporated to dryness under reduced pressure. The Boc-Leu-Ala-Leu–TMP conjugate was further purified by flash chromatography on a silica gel column with chloroform containing 7% methanol and 1% formic acid.

Incorporation of [^{14}C]Alanine—When Boc-Leu-Ala was synthesized, trace amounts of [^{14}C]alanine were added, giving the product a specific radioactivity of 0.9 μCi/mmol.

Deblocking Procedure—The amino-protecting group *tert*-butyloxycarbonyl (Boc) was removed by treatment with concentrated trifluoroacetic acid (TFA) for 30 min at room temperature. The TFA was removed under reduced pressure, the resulting trifluoroacetate was dissolved in 0.2 M sodium bicarbonate, pH 11.0, and the deblocked compound was extracted with ethyl acetate. The combined organic phases were dried over sodium sulphate and evaporated.

Microparticles—*Preparation of Polyacryl Starch Microparticles*—Microparticles were prepared by polymerizing purified acryloylated starch[10] in an emulsion, as described previously.[9] Briefly, a solution of acryloylated starch was homogenized together with 300 mL of toluene:chloroform (4:1). Ammoniumperoxidisulphate (0.08 M) and tetramethylethylenediamine (TEMED) were used to initiate the polymerization. The microparticle composition is characterized by the D-T-C nomenclature,[11,12] where D represents acryloylated starch (g/100 mL), T represents the total concentration of acrylic groups (g/100 mL), and C represents the relative amount of crosslinking agent (% w/w). Generally, the D-T-C was 10-0.5-0 and 0.5 mL of TEMED was added, except for the microparticles used in the experiment described in Figure 1 where the D-T-C was 20-1-0 and 2 mL of TEMED was added.

^{14}C-Labeled starch microparticles were obtained by adding acryloylated [^{14}C]starch from *Nicotiana tabacum* to the monomer solution.

Activation of Polyacryl Starch Microparticles with Carbonyldiimidazole—Prior to the coupling with the peptide–drug complexes, the microparticles were activated with CDI.[13] Before activation, the microparticles were washed by repeated centrifugations in DMF that had been dried over molecular sieves. The CDI dissolved in dried DMF was added and the mixture was agitated end-over-end at room temperature for at least 30 min. After the reaction, the microparticles were washed 6 times with DMF.

Analysis of the Imidazole Content of Activated Microparticles—To measure the degree of activation, the imidazole content of the microparticles was determined. After the activation, the microparticles were transferred into physiological saline and hydrolyzed in 2 M NaOH overnight at 37 °C in a water bath with reciprocal shaking. The resulting clear solution was neutralized and the liberated imidazole was analyzed by HPLC, as described in the *Analytical Section*. The activation degree is expressed as the molar ratio between the imidazole and glucose units, as estimated from the dry weight of the microparticles. No compensation was made for the dry weight of the cross-linking hydrocarbon chains in the microparticles.

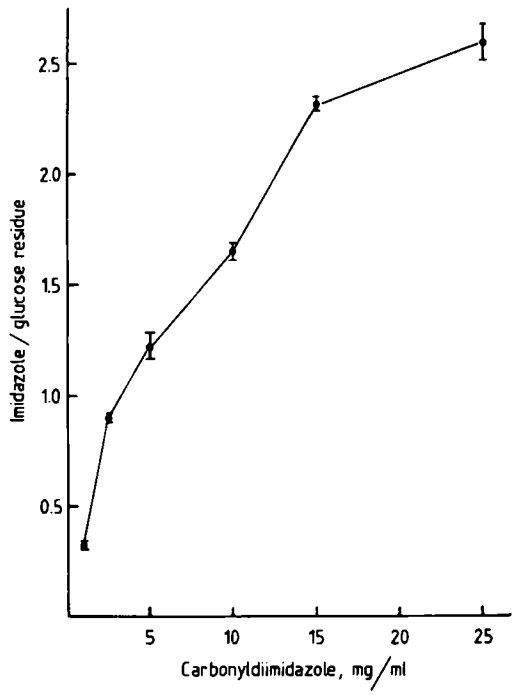


Figure 1—*Carbonyldiimidazole (CDI) activation of microparticles. Microparticles (5 mg) were reacted overnight with different concentrations of CDI in 2 mL of dimethylformamide (DMF). The activation level was determined by HPLC analysis of hydrolyzed particles (mean ± SD, n = 3).*

Coupling of Peptide–Drug Derivatives to Carbonyldiimidazole (CDI)-Activated Polyacryl Starch Microparticles—The method used is a modification of the coupling procedure described by Bethell et al.[13] The peptide–drug derivatives (0.28–2.22 × 10^{-4}mol) were added to 10 mg of activated microparticles in 5 mL of DMF containing 1 M TEMED. The reaction was carried out at 60 °C overnight in a water bath with reciprocal shaking.

The microparticles were washed 6 times with DMF after the coupling reaction and the excess of imidazole groups was hydrolyzed overnight in 0.2 M sodium carbonate buffer, pH 10, containing 0.5% (v/v) Tween 20. The released imidazole was removed by 4 washings in physiological saline containing 0.5% Tween 20. The microparticle–drug conjugates were stored at 4–6 °C until use.

Enzymatic Incubations—*Incubation of Peptide–Drug Derivatives in a Lysosomal Fraction*—The peptide–drug derivatives dissolved in 0.2 M sodium phosphate buffer (pH 5.5) containing 25% (v/v) methanol, 1% (v/v) DMF, and 5 mM reduced glutathion, were mixed with an equal volume of a lysosome-enriched fraction, prepared from rat liver as described earlier.[7] The final concentration of the derivatives was 200 μg/mL. The incubations were stopped by adding either acetonitrile (for analysis of PQ) or 10% trichloroacetic acid (TCA; for analysis of TMP).

Incubation of Microparticle–Drug Conjugates in Serum—Pooled human serum (200 μL) was mixed with the microparticle–drug conjugates (1 mg of microparticles in 200 μL of physiological saline). After 1 h at 37 °C with reciprocal shaking, either acetonitrile (PQ analysis) or 10% TCA (TMP analysis) was added to stop the incubation.

Incubation of Microparticle–Drug Conjugates in a Lysosome-Enriched Fraction—The microparticle–drug conjugates (1 mg in 200 μL of 0.2 M phosphate buffer, pH 5.5) were mixed with 200 μL of a lysosome-enriched fraction and incubated at 37 °C. The incubations were stopped by adding either acetonitrile (PQ analysis) or 10% TCA (TMP analysis).

Analytical Section—*Thin-Layer Chromatography*—The TLC was carried out on silica gel plates (Silica gel 60 F-254, layer thickness 0.25 mm, E. Merck, Darmstadt) using the solvent system chloroform:methanol:acetic acid (45:5:2). The spots were detected by UV absorption or by spraying with 0.3% ninhydrin in 1-butyl alcohol containing 3% acetic acid.

High-Performance Liquid Chromatography (HPLC) Equipment—The HPLC analyses were carried out with a system consisting of a Waters model 6000 A pump and a Shimadzu SPD-2 A spectrophotometric detector with variable wavelength. The samples (20 μL) were introduced through a Waters model U6K injector. The column used was a stainless steel tube, 150 mm × 4.6 mm I.D., packed in our laboratory with LiChrosorb RP8 with a medium particle size of 5 μm. When peptide–PQ derivatives were to be analyzed, LiChrosorb RP 18 with a medium particle size of 5 μm was used. The recorder was a Hitachi model 056. In each case, the flow rate of the mobile phase was 1 mL/min.

Imidazole Analysis—The particle hydrolysate was neutralized with 2 M HCl plus 0.2 M Na_2HPO_4, and injected onto the HPLC system. The mobile phase consisted of 0.100 M NaH_2PO_4, 0.050 M H_3PO_4, 0.005 M *n*-octyl sodium sulphate, and 20% acetonitrile (pH 2.5). The detector wavelength was 207 nm. The imidazole concentration was derived from a linear standard curve.

Primaquine Analysis—The samples containing serum or lysosomal fraction were treated with an equal volume of acetonitrile, left for 1 h in the dark, and centrifuged.[14] The clear supernatant was injected onto the HPLC system.

The recovery of PQ was 111% from serum incubations (n = 8, SD = 5.7), and 82% from lysosomal incubations (n = 8, SD = 8.1). The PQ concentration of the samples was derived from a linear standard curve. The lowest detectable concentration was 0.8 μg/mL. The mobile phase used was modified from Clark et al.[15] and consisted of

4.0 mM KH_2PO_4, 4.7 mM K_2HPO_4, and 70% (v/v) acetonitrile (pH 6.9). The detector wavelength was 254 nm. When samples containing peptide–PQ derivatives in addition to free PQ were analyzed, the concentration of acetonitrile in the mobile phase was decreased to 50% (v/v).

Trimethoprim Analysis—The samples containing either serum or lysosomal fractions were treated with an equal volume of 10% TCA in water and centrifuged. The clear supernatant was injected onto the HPLC system. The recovery of TMP was 72% from serum incubations (n = 9, SD = 4.4), and 104% from lysosomal incubations (n = 6, SD = 6.3). The TMP concentration was derived from a linear standard curve. The lowest detectable concentration was 0.4 μg/mL. The mobile phase consisted of 0.10 M NaH_2PO_4, 0.065 M H_3PO_4, and 20% acetonitrile (pH 2.4). The detector wavelength was 230 nm. When tripeptide–TMP in addition to free TMP were analyzed, the concentration of acetonitrile in the mobile phase was raised to 30% (v/v).

Results

Synthesis of Peptide–Drug Derivatives—The characteristics of the compounds synthesized according to the Scheme are summarized in Tables I and II. The derivatives used for coupling to microparticles were also submitted to amino acid analysis and the results are given in Table II. When chromatographed on an HPLC system, the tri-, tetra-, and pentapeptide–PQ derivatives used for the preparation of particle conjugates gave single peaks, separated from the other drug derivatives and from the peak produced by the corresponding free drug.

The synthesis and purification of the TMP–peptides were more complicated, probably owing to the presence of two amino groups in TMP. The Boc-Leu–TMP product showed two minor spots on the TLC, and the Leu-Ala-Leu–TMP product contained ~20% free TMP (as measured by peak area) after flash chromatography. However, the purity was sufficient for the release studies.

Lysosomal Incubation of Peptide–Drug Derivatives—To study the rate and pattern of degradation of the synthesized peptide–drug derivatives by lysosomal enzymes, the tetra- and pentapeptide–PQ derivatives and the tripeptide–TMP derivative were incubated in a lysosome-enriched fraction containing reduced glutathion. The incubations were stopped after different times and the supernatants were analyzed on a chromatographic system, where the different drug-containing degradation products, as well as the free drugs, could be separated and identified. When the degradation of Leu-Ala-Leu–TMP was studied, only the tripeptide derivative and free TMP were monitored.

Both the tetra- and pentapeptide–PQ derivatives, as well as the tripeptide–TMP derivative, released >90% of the drug content in free form after 3 h in a lysosome fraction (Figure 2). In addition, the tetrapeptide showed a faster degradation rate than the pentapeptide. When the pentapeptide–PQ derivative was incubated in a lysosome fraction without the addition of reduced glutathion, a slower degradation rate was seen; only 60% of the drug was released after 3 h.

Activation of Microparticles with Carbonyldiimidazole (CDI)—The hydroxyl groups on the starch microparticles can be activated with CDI in dry DMF overnight according to Scheme II. Figure I shows the resulting activation level when different concentrations of CDI were used. The degree of activation, given as the molar ratio between imidazole and glucose residues, varied between 0.3 and 2.6. The data indicate that a plateau value is approached above 20–25 mg CDI per mL. In addition, a kinetic study of the activation process showed that the reaction was essentially complete after 30 min. Therefore, in subsequent experiments, the microparticles (~5 mg/mL) were generally activated for 30 min using a CDI concentration of 25 mg/mL.

Dissolution of Activated Microparticles in Mouse Serum—The activation of starch microparticles with CDI can reduce the degradability of the microparticles (e.g., by cross-

Table I—*N-tert*-Butyloxycarbonyl (*N*-Boc)-Protected Compounds Synthesized

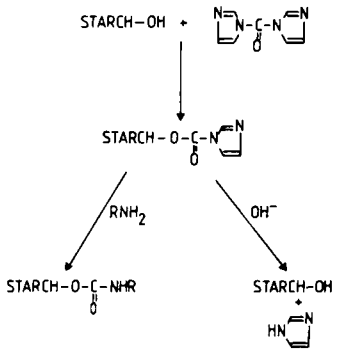
Compound	Starting Material and Reagents	Yield, %	Thin-Layer Chromatography, R_f
Boc-Leu-ONSu	Boc-Leu + DCC + HOSu	93	0.65
Boc-Ala-ONSu	Boc-Ala + DCC + HOSu	95	0.61
Boc-Leu–PQ	Boc-Leu + CDI + PQ	81	0.76
Boc-Leu–TMP	Boc-Leu + CDI + TMP	48[a]	0.62[b]
Boc-Leu-Ala	Boc-Leu-ONSu + Ala	98	0.54
Boc-Leu-Ala-ONSu	Boc-Leu-Ala + DCC + HOSu	84	0.66
Boc-Leu-Ala-Leu–PQ	Boc-Leu-Ala-ONSu + Leu–PQ	86	0.81
Boc-Leu-Ala-Leu–TMP	Boc-Leu-Ala-ONSu + Leu–TMP	45[a]	0.81[b]
Boc-Ala-Leu-Ala-Leu–PQ	Boc-Ala-ONSu + Leu-Ala-Leu–PQ	95	0.56
Boc-Leu-Ala-Leu-Ala-Leu–PQ	Boc-Leu-Ala-ONSu + Leu-Ala-Leu–PQ	89	0.65

[a] See comments on the purity in the text. [b] Main spot.

Table II—Deprotected Compounds Synthesized[a]

Compound	Yield, %	Thin-Layer Chromatography, R_f	Peptide Chain Composition, %	
			Found	Theoretical
Leu–PQ	94	0.18		
Leu–TMP	68[b]	0.07[c]		
Leu-Ala-Leu–PQ	60	0.09	Leu 65.8	66.7
			Ala 34.2	33.7
Ala-Leu-Ala-Leu–PQ	97	0.05	Leu 49.9	50.0
			Ala 50.1	50.0
Leu-Ala-Leu-Ala-Leu–PQ	31	0.06	Leu 58.6	60.0
			Ala 41.4	40.0
Leu-Ala-Leu–TMP	50[b]	0.03[c]	Leu 32.4	33.3
			Ala 67.6	66.7

[a] The compounds were obtained from the respective Boc-precursors by treatment with trifluoroacetic acid. [b] See comments on the purity in the text. [c] Main spot.



Scheme II—*Activation of the hydroxyl groups on starch with carbonyldiimidazole (CDI) and subsequent coupling of the amino group-containing ligands, or hydrolysis of the active group.*

linking the starch). Furthermore, in our experiments, very high activation levels are used and the excess of imidazole groups must be removed after coupling of the drugs. The following experiment was designed to study the degradability of activated microparticles before and after removal of the imidazole groups.

Activated microparticles were incubated at pH 7 or 10 in buffer solutions overnight. The bond between imidazole and starch was not affected at pH 7, but was quantitatively hydrolyzed at pH 10, as determined by HPLC analysis. After this step, the microparticles were washed with saline to remove the buffer substances and subsequently incubated for 15 min in mouse serum. The results are shown in Figure 3. The particles incubated at pH 7 already showed a reduction in the degradability at the lowest activation level. On the other hand, after removal of the imidazole groups by hydrolysis at pH 10, the microparticles were completely dissolved in mouse serum. Thus, CDI activation did not irreversibly reduce the degradability of the microparticles. The microparticles were consequently subjected to pH-10 treatment after activation and coupling in all the subsequent experiments.

Coupling of Primaquine (PQ)– and Trimethoprim (TMP)–Peptide Derivatives to Microparticles—The CDI-activated starch microparticles and the ^{14}C-labeled drug derivatives were allowed to react overnight at 60 °C. After washings in DMF and physiological saline, the drug content of the microparticles was determined from the incorporated radioactivity and the specific radioactivity of the different drug derivatives.

The amount of PQ–base in the microparticles, using different amounts of the tri-, tetra-, and pentapeptide derivatives of PQ in the coupling reaction, is shown in Figure 4. When 0.5 and 1 mol of drug derivatives were added per mol of glucose, the resulting drug content for all the peptide derivatives was 0.5–1.0% (w/w) PQ–base. However, when higher amounts of derivatives were added, the differences in microparticle drug content became more pronounced. The highest drug content, 4.6% (w/w) of PQ–base, was obtained with the pentapeptide derivative.

In a similar experiment, the tripeptide derivative of TMP consistently gave higher drug contents in the microparticles than the PQ–peptides (Figure 4). When 4 mol of the drug derivative was added per mol of glucose, 5.0% of the microparticle dry weight consisted of TMP–base.

Thus, by varying the amount of drug derivatives added to the reaction mixture, microparticles with different drug contents could be obtained. The amount of PQ– or TMP–base varied from 0.1 to 5.0% (w/w), as estimated from the microparticle dry weight.

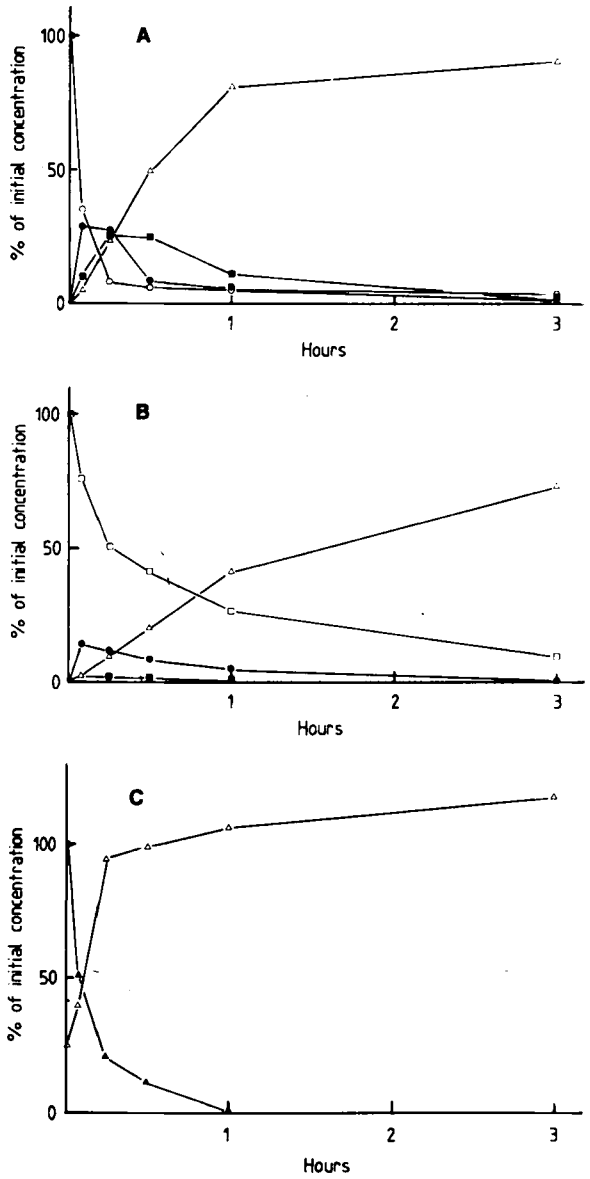


Figure 2—*Degradation kinetics of peptide derivatives of primaquine (PQ) and trimethoprim (TMP) in a lysosome-enriched fraction. The results are expressed as percent of the initial concentration (HPLC peak area) of the respective derivatives: (2a) tetrapeptide–PQ; (2b) pentapeptide–PQ; (2c) tripeptide–TMP. Key: (□) Leu-Ala-Leu-Ala-Leu–PQ; (○) Ala-Leu-Ala-Leu–PQ; (●) Ala-Leu–PQ; (■) Leu–PQ; (△) PQ; (▲) Leu-Ala-Leu–TMP; (△) TMP.*

Incubation of Microparticles Containing Peptide–Drug Conjugates in Human Serum—The stability in vitro of the linkage between the drugs and the microparticles was studied by incubation in human serum for 1 h. After the incubation, the microparticles were spun down and the content of free drugs or peptide–drug derivatives were analyzed by HPLC. No free drug or peptide drug derivatives could be detected in the supernatants, which shows that the different drug–microparticle conjugates are stable for at least 1 h in human serum.

Incubation of Microparticles Containing Peptide–Drug Conjugates in a Lysosome-Enriched Fraction—The release of free drug from the different microparticle–drug conjugates was studied by incubation in a lysosome–enriched fraction overnight (20 h). At the end of the incubation period, the samples were analyzed by HPLC to determine free and peptide-bound PQ or TMP.

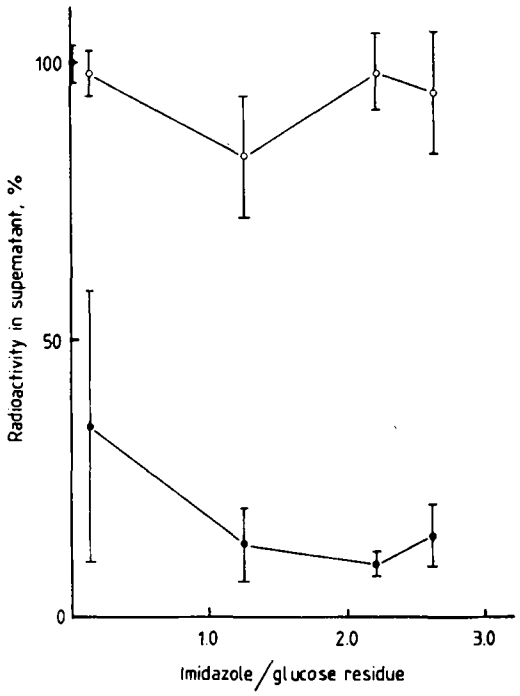


Figure 3—*Degradation of activated microparticles in serum after pH-7 (control) or pH-10 treatment. ^{14}C-Labeled microparticles (5 mg) were activated with different concentrations of carbonyldiimidazole (CDI) and the activation degree (imidazole/glucose residue) was determined. The particles were then subjected to pH-7 (●) or pH-10 (○) treatment overnight. The active groups were completely hydrolyzed at pH 10, but were not affected at pH 7. After washing, the microparticles were incubated in mouse serum for 15 min, and the radioactivity in the supernatant was determined after centrifugation (mean ± SD, n = 3).*

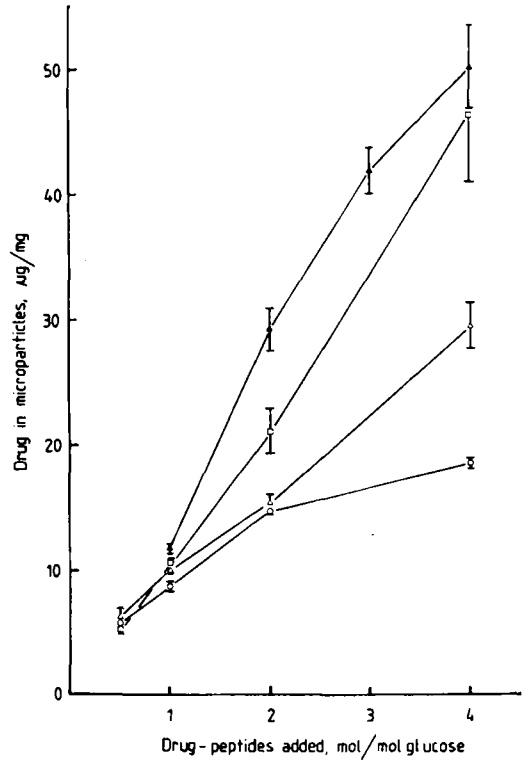


Figure 4—*Coupling of primaquine (PQ)- and trimethoprim (TMP)-peptide derivatives to activated starch microparticles. Different amounts of the ^{14}C-labeled drug–peptide derivatives were added to carbonyldiimidazole (CDI)-activated microparticles. After reaction overnight at 60 °C and repeated washings, the resulting microparticle drug content was determined by scintillation counting. Each value is the mean ± SD, n = 3. Key: (□) Leu-Ala-Leu-Ala-Leu–PQ; (○) Ala-Leu-Ala-Leu–PQ; (△) Leu-Ala-Leu–PQ; (▲) Leu-Ala-Leu–TMP.*

Figure 5 shows the release of free drug from the tri-, tetra-, and pentapeptide–drug derivatives versus the amount of drug bound to the microparticles. Particles with tri- and pentapeptide derivatives of PQ and the tripeptide derivative of TMP gave similar results, a 6–16% release within a 9-fold variation of the microparticle drug content. However, particles with the tetrapeptide–PQ derivative displayed a different picture. First, only a 3-fold variation in the microparticle drug content was reached, as described earlier. Second, the release of free drug was higher, 19–47%, than for the other peptide–drug derivatives. Third, the release increased with higher amounts of PQ–tetrapeptide bound to the microparticles.

The kinetics of drug release from microparticles is shown in Figure 6. The amount of free drug is expressed in percent units relative to the amount of drug initially bound to the microparticles. The tetrapeptide–PQ conjugate gave the most rapid release of free drug.

The influence of the drug content on the release kinetics was studied by increasing the microparticle content of tri- and pentapeptide–PQ derivatives 2-fold in a separate experiment. However, no differences were found. Furthermore, no change in the rate of drug release was seen when reduced glutathion was included in the incubation media.

Discussion

Polyacryl starch microparticles and other particulate drug carriers are essentially taken up by the macrophages of the reticuloendothelial system in the liver and, to a lesser extent, in the spleen after iv injection.[16] Their efficiency as carriers for macromolecules has been demonstrated by the treatment of an artificial lysosomal storage disease.[7] Thus, polyacryl starch microparticles are to be considered as an alternative to other lysosomotropic carrier systems (e.g., liposomes and soluble polymers) in the treatment of lysosomal parasitic diseases, such as leishmaniasis. However, low molecular weight drugs cannot be entrapped within the starch microspheres in the same way as macromolecules since they would leak out through the pores in the polymer matrix.[3] Therefore, they have to be covalently bound to the microparticle matrix. The same strategy also has to be applied to soluble drug carriers, such as proteins and polymers.[4,5] The linkage between the drug and the carrier has to be stable during the transport in the blood, but must be cleaved in the lysosomes to liberate free drug.

The present work shows that low molecular weight drugs can conveniently be covalently bound to the starch particles after activation of the hydroxyl groups. Carbonyldiimidazole (CDI) was chosen as the activating agent for several reasons. The imidazolylcarbamate formed is very reactive towards primary amino groups and the activation level is easily controlled and can be determined by imidazole analysis. Furthermore, after coupling of the ligand, it is possible to selectively remove unreacted imidazole groups. Blocking reactions, which often have to be employed using other activation methods, can thus be avoided. Using large amounts of CDI it was possible to obtain high activation levels, up to 2.6 imidazole groups per glucose residue.

The peptide derivatives of PQ and TMP were coupled to the microparticles after CDI activation of the starch. By using different concentration of the peptide–drug derivatives, it was possible to control the drug content in the microparticles.

Maximally, ~5.0% of the microparticle dry weight consisted of the base form of the drug. This corresponds to ~3 drug molecules per 100 glucose residues.

One potential drawback to the use of CDI is the possibility of crosslinking, as observed with soluble[17] and particulate[18] polysaccharides. This would decrease the degradability of the microparticles, and thus make them unsuitable as drug carriers. However, we found that activation with a large excess of CDI overnight, followed by removal of the imidazole groups by alkaline treatment, did not reduce the degradability of the microparticles. Later, kinetic experiments revealed that the CDI activation was essentially complete after 30 min, and activation overnight would have revealed any tendency to crosslink the microparticles. Thus, CDI seems to be a useful reagent for the activation of starch microparticles if unreacted imidazole is removed.

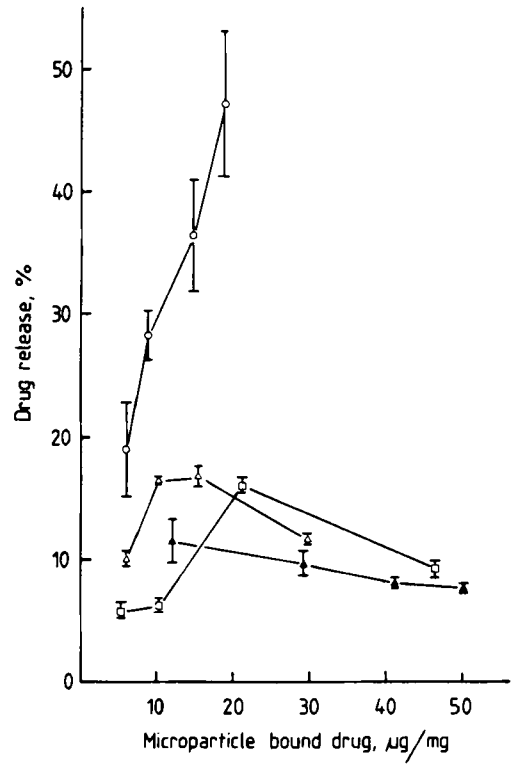


Figure 5—*Incubation of different microparticle-bound primaquine (PQ)– and trimethoprim (TMP)–peptides in a lysosome-enriched fraction overnight. The release of free drug was determined by HPLC analysis. Each value represents the mean ± SD, n = 3. Key: (□) Leu-Ala-Leu-Ala-Leu–PQ; (○) Ala-Leu-Ala-Leu–PQ; (△) Leu-Ala-Leu–PQ; (▲) Leu-Ala-Leu–TMP.*

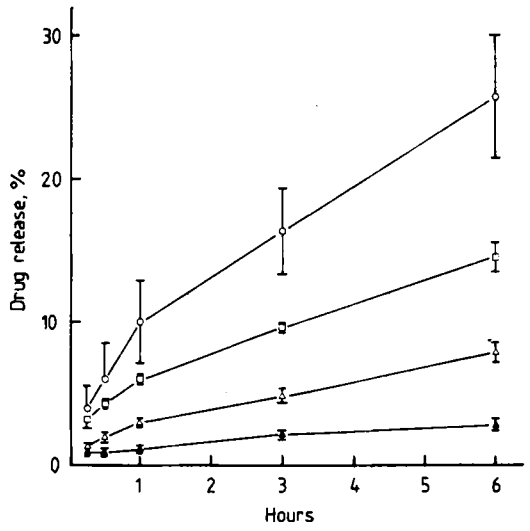


Figure 6—*Kinetics of drug release from microparticle-bound peptide–drug derivatives in a lysosome-enriched fraction. Microparticles containing peptide-bound drugs [primaquine (PQ), 15–20 μg/mg; trimethoprim (TMP) 50 μg/mg] were incubated for different times in a lysosomal fraction. The free drug released is expressed as the percent of the amount initially bound to the microparticles. Each value represents the mean ± SD, n = 3. Key: (□) Leu-Ala-Leu-Ala-Leu–PQ; (○) Ala-Leu-Ala-Leu–PQ; (△) Leu-Ala-Leu–PQ; (▲) Leu-Ala-Leu–TMP.*

When TMP was coupled directly to starch microparticles at 60 °C, the resulting drug-containing microparticles were dissolved by a lysosome-enriched fraction (data not shown). However, no release of free drug could be detected. This is in accordance with observations from experiments with protein–drug conjugates prepared by direct coupling of drugs to the protein; no free drug was enzymatically hydrolyzed from such preparations.[5] To circumvent this problem, a peptide spacer might be inserted between the drug and the carrier.[19]

The factors that determine the release of drugs bound via different peptide spacers are largely unknown, although it is recognized that the length and composition of the spacer is important.[5,20] It is not probable that unspecific hydrolases of the lysosome will cleave the bond between the C-terminal amino acid of the peptide and the drug to any significant degree. This implies that endopeptidases will be the enzymes which primarily hydrolyze the spacer arms. Possibly, in our case, after an initial dissolution, an aminoacyl– or peptidyl–drug derivative is obtained and aminopeptidases will subsequently release the free drugs. Several reports have already shown that such enzymes are effective in the lysosomes.[21] It is consequently important to select a composition (and length) of the spacer arms that can easily be attacked by endopeptidases and aminopeptidases. The Ala and Leu peptides are easily degraded by different amino peptidases.[22] The specificity of the endoproteases is incompletely known, but the lysosomal elastase shows high activity against synthetic substrates containing alanine.[21] Consequently, Ala-Leu peptides were tested as spacer arms in the present work. This strategy has been used by Trouet et al.,[5,23] who coupled primaquine to succinylated albumin using Ala-Leu spacers and demonstrated that the drug was efficiently released in the lysosomal milieu.

In our experiments, PQ and TMP were rapidly released in high yields from the soluble peptide conjugates in vitro in a lysosome-enriched fraction. The results show that free drug will be present as soon as the peptide arm in the particle conjugates is cleaved. The kinetic studies showed, moreover, that PQ is hydrolyzed particularly rapidly from the tetrapeptide derivative and no tetrapeptide–PQ was found in the digest of the pentapeptide conjugate. These findings are in accordance with the known presence of aminopeptidase, dipeptidylpeptidase, and endoprotease activities in the lysosomes.[21]

Primaquine and TMP were released from the microparticle–peptide–drug conjugates during incubation in a lysosome-enriched fraction. Primaquine was efficiently released from particles with a tetrapeptide arm and ~45% of the free drug was found in the lysosomal digest after overnight incubation. The relative extent of the release was related to the drug content of the microparticles and generally it increased with the drug content up to a level of ~20 μg per mg of particles. However, no upper limit was found for the tetrapeptide–particle complex of PQ. Thus, the tetrapeptide spacer arm seems to be better than the pentapeptide arm for PQ, at least as far as the rate of release in the lysosomal milieu in vitro is concerned. The reasons for this are not

understood at the moment. The stability in serum of the different drug–particle conjugates was good (no free drug or drug–peptides were detected after incubation for 1 h).

In several papers, Duncan et al. have demonstrated the importance of reduced glutathione for the release of drug analogues attached to the soluble polymer poly[*N*-(2-hydroxypropyl)methacrylamide] via different peptide spacers.[24,25] In our experiments, the presence of reduced glutathion in the lysosome-enriched fraction increased the release of free drug from the soluble peptide–drug derivatives, but not from the microparticle–drug conjugates. This indicates that the rate-limiting step in the hydrolysis of free drug from polyacryl starch microparticles is not catalyzed by a thiol-dependent enzyme. Possibly, this rate-limiting step is the dissolution of the microparticles. This could explain the different effects of thiols on the release of free drugs from polymer-bound drug peptides, compared with microparticle-bound drug peptides.

In this paper we demonstrate that low molecular weight antiparasitic drugs can be attached to polyacryl starch microparticles via peptide spacers and that the drugs remain stably bound in serum, but are released in the lysosomal milieu. Thus, the basic requirements for a useful lysosomotropic drug carrier are fulfilled. Further studies have to be performed to show the efficacy of this carrier system in the treatment of lysosomal parasitic diseases.

References and Notes

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