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Engineering prolonged-acting prodrugs employing an albumin-binding probe that undergoes slow hydrolysis at physiological conditions

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ABSTRACT

Here we describe the design and application of OSu–FMS–MAL–S–$(CH_2)_{15}$–COOH, an agent that associates with albumin while linked to a peptide or a protein with sufficient affinity (Ka = 2 to $2.6\times10^5 M^{-1}$) to protract the action of short- lived peptides and proteins in vivo. Under physiological conditions this probe undergoes spontaneous hydrolysis with the concomitant reactivation of inactive conjugates. Intravenously administered ^{125}I-labeled-Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH to rats shows half-life of 17 ± 2 h, exceeding 5.2 times that obtained with intravenously administered ^{125}I-labeled Insulin. In mice this derivative facilitates glucose-lowering effect over a period of 24 h, yielding AUC five times greater than that obtained by a similar dose of insulin-detemir. Similarly, subcutaneous administration of Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH into mice facilitated prolonged and stable reduction in glucose level, yielding a *t*½ value of 28 ± 2 h, exceeding the effect of exendin-4 4.7 folds. The inactive derivative gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH regained its full antibacterial potency upon incubation at physiological conditions yielding a t½ value of 7.1 ± 0.2 h.

In conclusion, the albumin-binding probe we introduced enables to prolong the action of any amino containing molecule in vivo, without the drawback of inactivation that often occurs upon such derivatization.

1. Introduction

Most polypeptide drugs, in particular nonglycosylated proteins of molecular mass less than 50 kDa, are short-lived species in vivo having circulatory half-lives of 5–20 min. The short life-time of such proteins in vivo is attributed to several mechanisms including glomerular filtration in the kidney and proteolysis at several levels [1].

Albumin is a long-lived protein in vivo. Likewise, drugs and endogenous substances, including proteins that associate tightly with albumin have lower clearance rates than the unbound equivalents and exhibit prolonged lifetime profiles in vivo [2]. The finding that long-chain free fatty acids (LCFA) bind tightly to albumin [3] provided the impetus for designing an insulin derivative in which an LCFA-like probe is integrated into the insulin molecule [4–6]. The optimal derivative thus obtained (insulin-detemir) exhibited protracted action in vivo, partly due to its associating affinity to endogenous albumin [4–6].

Here we used the principle of drug association with endogenous albumin for designing an approach suitable to protract the action in vivo of, potentially, any short-lived peptide/protein drug. In doing so two principal obstacles had to be overcome: (1) following its conjugation, the probe introduced into a peptide or a protein should have sufficient affinity to albumin to manifest prolonged action in vivo, and (2) in case such covalent introduction results in an inactive product, the latter should be capable to undergo slow reactivation at physiological conditions. Our plan was to overcome this last drawback by using a hydrolysable heterobifunctional agent previously developed in our laboratories [7–13].

In this study we have designed, prepared, and studied a unique albumin binding probe for its efficacy to turn short-lived peptide/protein drugs into inactive reactivable prodrugs having prolonged life-time profiles in vivo.

2. Experimental procedures

2.1. Materials

Human (Zn^{++}-free) insulin was donated by Novo Nordisk, Bagsvalrd, Denmark or by Biotechnology General (Rehovot, Israel).

Abbreviations: OSu–FMS–MAL, 2-sulfo-9-hydroxymethyl-7(amino-3-maleimido-propionate)-fluorene-N-hydroxysucinimide; HSA, human serum albumin; HPLC, high-performance liquid chromatography; PBS, phosphate buffer saline; TFA, trifluor-oacetic acid; PEG_5, polyethyleneglycol chain of a 5 kDa; BSA, bovine serum albumin; MAL, maleimide; ESMS, electrospray single quadrupole mass spectroscopy; t_R, retention time; PEG_5-MAL, PEG_5-maleimide; PEG_5-OSu, PEG_5-N-hydroxysuccinimide ester; AUC, area under the curve; BGL, blood glucose level; LCFA, long chain fatty acid.

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Insulin-detemir, a Novo Nordisk product, was extensively dialyzed against 0.01 M $NaHCO_3$ and stored at 7 °C until used. The concentration of this derivative was determined by its absorbance at 280 nm ($\varepsilon_{280} = 5900$) and/or by acid hydrolyzing an aliquot (in 6 M HCl for 22 h at 110 °C) followed by quantitative amino acid analysis. D-[U-^{14}C] glucose (4–7 mCi/mol) was obtained from Du Pont-NEN (Boston, Ma). Type I collagenase (134 U/mg) was purchased from Worthington (Freehold, NY). Gentamicin sulfate was from Sigma Chemical Co. (Ness-Ziona, Israel), and PEG_5-MAL was obtained from Shearwater Group Inc. (Ra'anana, Israel). Exendin-4 (HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2) was synthesized by the solid phase method using a multiple peptide synthesizer, AMS 422 (Abimed Analyser Technik, GmbH). All other materials used in this study were of analytical grade.

2.2. Chemical and biological procedures

OSu–FMS–MAL was synthesized as described in detail in ref. 7. The synthesis was initiated from 9-hydroxymethyl-2-aminofluorene and the final product was obtained in 65% yield following four steps of synthesis.

PEG_5-NH_2 was prepared by dissolving PEG_5-OSu (Shearwater product) at a concentration of 20 mg/ml in 0.1 M $NaHCO_3$, containing 1 M of 1.3-diaminopropane Dihydrochloride (Aldrich). The reaction was carried out for 1 h at 25 °C. The product was extensively dialyzed against H_2O, lyophilized and kept at 7 °C until used.

Isothermal Scanning Calorimetry measurements were performed with ITC_{200} microcalorimeter (Micro Cal LLC, Nothampton MA 01060 USA). Experimental details were carried out according to the ITC_{200} microcalorimeter user's manual.

2.3. Preparation of PEG_5-MAL–S–$(CH_2)_{10}$–COOH

To a stirred solution of PEG_5-MAL (50 mg in 2.0 ml H_2O) 2.3 mg of 11-sulfanylundecanoic acid was added (0.1 ml from a fresh solution of HS-$(CH_2)_{10}$-COOH in DMF 23 mg/ml). After 7 min, 10 mg solid $NaHCO_3$ was added. The reaction was carried out for 1 h, and after centrifugation the supernatant was dialyzed overnight against H_2O and lyophilized. The product thus obtained is DTNB-negative.

2.4. Preparation of monomodified FMS–MAL–S–$(CH_2)_{15}$–COOH-containing derivatives of insulin and Exendin-4

OSu–FMS–MAL 58.3 mg (100 μmol) and 16-sulfanylhexadecanoic acid [HS-$(CH_2)_{15}$-COOH, 38.5 mg,120 μmol] were dissolved in 1.0 ml dimethylformamide and pyridine, 20 μl (248 μmol) was added. The reaction mixture was stirred for 40 min at 25 °C, and product formation was monitored by the decrease in the maleimide moiety in aliquots withdrawn during synthesis. Following completion of the reaction (at 4 h) the derivative thus formed OSu–FMS–MAL–S–$(CH_2)_{15}$–COOH was added to an aqueous solution of insulin 6 mg/ml (1 μmol/ml) dissolved in 0.1 M $NaHCO_3$ (pH 8.5) at three molar excess over the protein (30 μl). The reaction was carried out for 2 h at 0 °C, and the mixture was than dialyzed against H_2O at 7 °C. Monomodifed derivative of insulin-linked to FMS–MAL–S–$(CH_2)_{15}$–COOH, was purified from unreacted insulin and from residual bismodified derivative, using semi-preparative HPLC (RP-4 column, Hesperia CA 20-100% solution B over 60 min with a flow rate of 10 ml/min). The fraction corresponding to monomodified Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH was collected, redialyzed against H_2O and lyophilized.

Monomodified derivative of exendin-4 [Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH], was prepared under identical conditions, by adding 3 fold molar excess of the reagent to an aqueous solution of exendin-4 (4.2 mg/ml in 0.1 M $NaHCO_3$, pH 8.5). The reaction was carried out for 3 h at 0 °C. The reaction mixture was than dialyzed overnight, purified by preparative HPLC, redialyzed and lyophilized.

2.5. Iodination of peptides/proteins

This was performed using [^{125}I] iodine following the chloramine-T method [14].

Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion [15].

Lipogenesis (during which [U-^{14}C] glucose was incorporated into lipids) was carried out by the procedure of Moody et.al. [16].

Antibacterial potency of gentamicin and derivatives was determined by inhibiting replication of *E. Coli*. Native gentamicin arrests replication of *E. Coli* half-maximally (IC_{50}) at a concentration of 2.1 ± 0.2 μM [17,18].

Blood glucose levels were determined at varying time points following administration of insulin, exendin-4, and their derivatives in blood aliquots taken from the tail vein. Glucose analyzer (Beckman Instruments Fullerton, CA) was used. Groups consisted of five or six mice each. Data are presented as means ± SE.

Radioactive content in rat blood following intravenous administration of ^{125}I-labeled peptides/proteins was monitored by withdrawing blood samples at varying time points from the tail vein. Blood aliquots (50–70 mg) were absorbed onto pre-weighed Whatman 3 MM filters that were re-weighed immediately after immersion in blood samples.

3. Results and discussion

3.1. Applying isothermal scanning calorimetry (ITC) for evaluating ligands' associating affinity to HSA

Several procedures have been used in the last three decades to evaluate associating affinities of varying ligands to albumins (reviewed

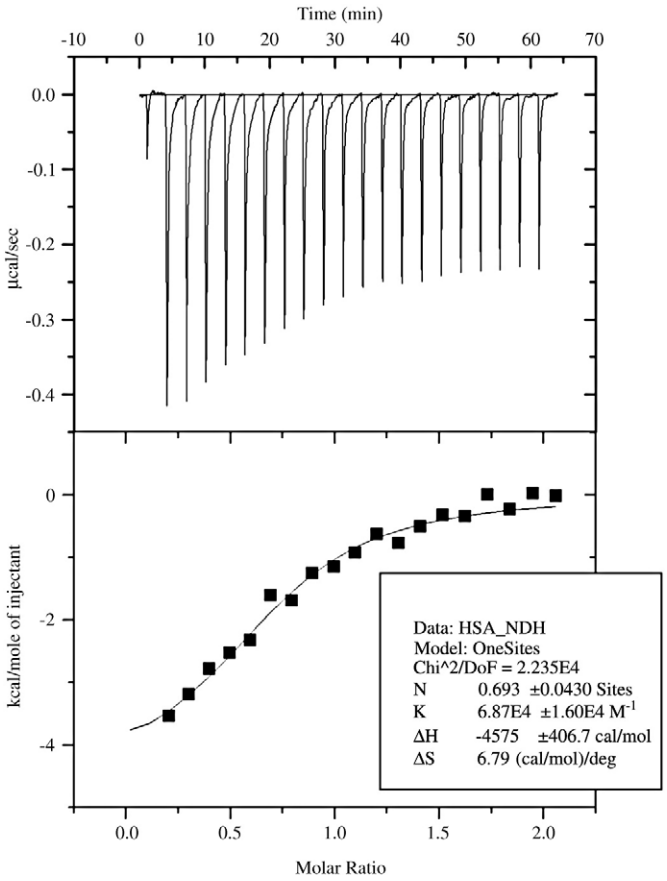
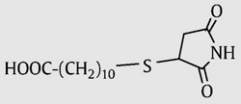
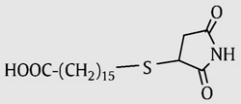
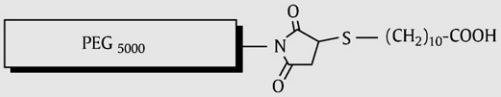
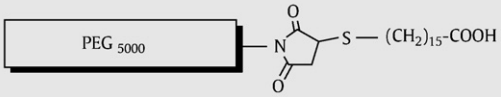
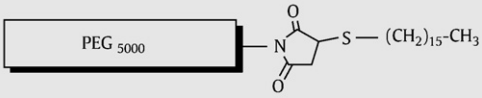
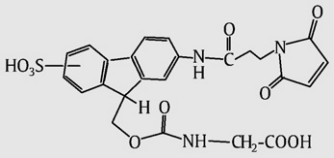
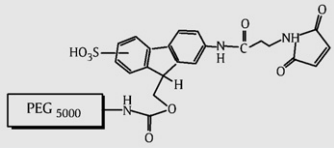
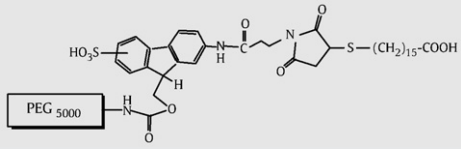
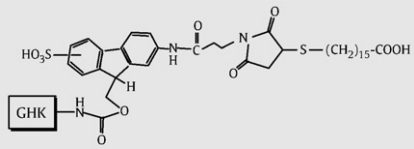
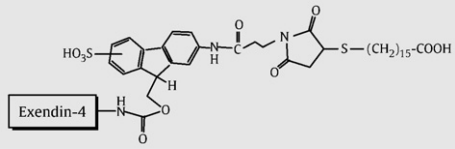
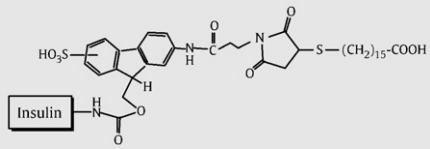


Fig. 1. Binding affinity of Insulin-detemir to human-serum albumin as determined by ITC-200. The processed data has been derived from 20 automatic injections (2.4 μl each, upper panel) of insulin-detemir (400 μM in PBS buffer, pH 7.4) into the sample cell containing HSA at a concentration of 10 μM in PBS buffer.

Table 1
Binding affinities of compounds and derivatives prepared in this study to HSA.

Compound	Structure	Association-constant to HSA $K\ M^{-1}$
MAL–S–$(CH_2)_{10}$–COOH		$1.3 \pm 0.2 \times 10^5$
MAL–S–$(CH_2)_{15}$–COOH		$1.55 \pm 0.15 \times 10^5$
PEG_5-MAL–S–$(CH_2)_{10}$–COOH		NONE
PEG_5–MAL–S–$(CH_2)_{15}$–COOH		$1.95 \pm 0.317 \times 10^5$
PEG_5–MAL–S–$(CH_2)_{15}$–CH_3		NONE
Glycine–FMS–MAL		$1.4 \pm 0.15 \times 10^5$
PEG_5–FMS–MAL		NONE
PEG_5–FMS–MAL–S–$(CH_2)_{15}$–COOH		$2.57 \pm 0.44 \times 10^5$
Gly-His-Lys–FMS–MAL–S–$(CH_2)_{15}$–COOH		$1.99 \pm 0.28 \times 10^5$
Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH		$2.2 \pm 0.2 \times 10^5$
Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH		$2.4 \pm 0.3 \times 10^5$

Simulated binding isotherms for the association of all compounds and derivatives prepared were carried out under the experimental conditions specified in the legend to Fig. 2. Each value is the arithmetic mean ± SEM of 4–6 determinations.

in [19]). Here we applied isothermal scanning calorimetry (ITC), a modern approach for determining binding enthalpies, stochiometries and constants for ligand-protein interactions (reviewed in [20]). Before applying this approach, we confirmed that the obtained Ka values for ligand-HSA associations are in close proximity to those obtained using other binding strategies. Fig. 1 shows the ITC data for the binding of

insulin-detemir to HSA. When the primary ITC data is translated to a binding isotherm it yields a binding constant of Ka = 0.687 ± 0.16 × 10^5 M^{-1} (Fig. 1). A significant higher value of Ka = 2.4 ± 0.7 × 10^5 M^{-1} was previously obtained for the binding of insulin-detemir to HSA using the immobilized HSA binding strategy [21]. The latter procedure assumes the same binding constants to either immobilized or free albumin [22]. It now appears that ITC yields more accurate binding constants than those obtained by partitioning procedures between macromolecules and free ligands [23]. With ITC measurements, ligands or macromolecules "signal" directly the accuracy of the binding process. Spectroscopic signaling can also directly reflect associating events depending, however, on the presence of chromophores or fluorophores that alter their properties in the binding state [23].

3.2. Towards developing an optimal reversible HSA binding probe

In the course of designing the optimal HSA binding probe, we have searched first for long-chain fatty acids (LCFA) — like molecules containing sulfhydryl moieties that ultimately can be linked to the MAL function of our hydrolysable heterobifunctional reagent, OSu–FMS–MAL. A required part of any such selected molecule is a terminal carboxylate (–CH_2–COOH), an essential entity of LCFA for their association with albumin [19]. Once such compound is found, a key question to be addressed is whether it preserves its HSA-associating affinity following its attachment to a macromolecule. As a model for a macromolecule, we have used PEG_5-maleimide, a 5 kDa polyethylenglycol chain that reacts with sulfhydryl containing molecules in a 1:1 stoichiometry. PEG_5-NH_2 has been used as well for linking the appropriate HSA binding probe through our hydrolysable heterobifunctional agent.

Both MAL–S–$(CH_2)_{10}$–COOH and MAL–S–$(CH_2)_{15}$–COOH associate with HSA yielding Ka values of 1.3 to 1.6 × 10^5 M^{-1} (Table 1). The former, however, has lost this capability when linked to PEG_5-MAL. In contrast, PEG_5-MAL-S-$(CH_2)_{15}$-COOH associates effectively with albumin yielding a Ka value of 1.95 ± 0.317 × 10^5 M^{-1} (Fig. 2, Table 1). PEG_5–MAL–S–$(CH_2)_{15}$–CH_3 does not bind to HSA (Table 1). Thus, the terminal carboxylate is undoubtedly essential for macromolecule-ligand association with this carrier protein. Therefore, HS–$(CH_2)_{15}$–COOH has been selected for further designing the optimal HSA-associating probe.

Glycine–FMS–MAL associated with HSA with a Ka value of 1.4 × 10^5 M^{-1} but lost its associating affinity toward this carrier protein when linked to a 5 kDa PEG-NH_2 chain (Table 1). This association capacity is regained upon linking HS–$(CH_2)_{15}$–COOH to the MAL-moiety of the spacer. Thus, PEG_5–FMS–MAL–S–$(CH_2)_{15}$–COOH associates with HSA with a Ka value of 2.57 × 10^5 M^{-1} (Table 1). Similar HSA-associating affinities were obtained when FMS–MAL–S–$(CH_2)_{15}$–COOH was linked to an amino side chain of either a small peptide (Gly–His–Lys), a larger polypeptide such as exendin-4 (4.2 kDa), or to insulin (5.8 kDa, Table 1). Thus, our binding studies revealed that the covalent introduction of this specific HSA-binding probe to peptide/protein of variable size conferred them with HSA associating affinity, all in the range of Ka = 2.0 to 2.6 × 10^5 M^{-1}. This value exceeds about 3.3 times the associating affinity of insulin-detemir to this carrier protein (Table 1, Fig. 1).

3.3. Chemical and biological features of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH

Table 2 summarizes the characteristic features of the HPLC-purified insulin-derivative relevant to this study. Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH is a monomodified derivative having molecular-weight of 6570 Da (calculated value is 6565.5 Da), as verified by mass spectroscopy. The derivative is soluble in PBS-buffer at a concentration of ~4 ± 1 mg/ml. It has high absorbance at 280 nm, ε_{280} = 23,300 ± 1000, amounting to 86% of the calculated value. Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH emerged as a symmetric peak on analytical HPLC-column with t_R = 9.052 min (Fig. 3). Native insulin elutes under the same experimental conditions with t_R = 7.28 min. Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH activates lipogenesis in rat adipocytes at about 10% the efficacy of insulin, yielding a half-maximal effect (ED_{50}) at a concentration of 1.03 ± 0.1 nM (summarized in Table 2). However, in

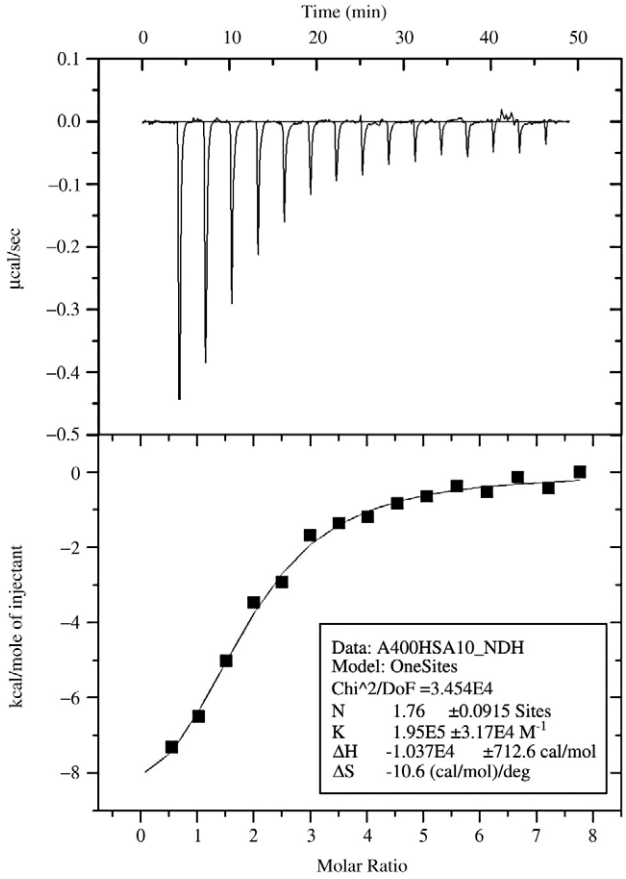


Fig. 2. Simulated binding isotherm for the association of PEG_5-MAL-S-$(CH_2)_{15}$-COOH with human serum albumin. The raw data was obtained for 15 automatic injections each of 2.7 µl. The total duration of the experiment was 45 min (upper panel). The concentration of PEG_5-MAL-S-$(CH_2)_{15}$-COOH in the injection syringe was 400 µM. Sample cell contained HSA at a concentration of 10 µM. Both components were dissolved in PBS-buffer, pH-7.4. The experiment was conducted at 23°C.

Table 2
Chemical and biological features of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH.

Characteristic	Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH
MS (*m/z*) calculated	6566 Da
MS (*m/z*) [a] found	6570 Da
Solubility in aqueous buffer pH 7.4	4 ± 1 mg/ml
molar extinction coefficient	
calculated [b]	ε_{280} = 26,800
found [c]	ε_{280} = 23,300 ± 1000
HPLC analysis retention time [d]	9.052 min
Lipogenic potency in rat adipocytes	ED_{50} = 1.03 ± 0.1 nM (~10%)
Lipogenic potency following incubation (pH 10.3, 4 h, 25 °C)	ED_{50} = 0.1 ± 0.02 nM(~100%)

[a] Mass spectra were determined using ESMS.
[b] Molar extinction coefficient for Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH was calculated by combining the ε_{280} values for Insulin (ε_{280} = 5800) and OSu–FMS–MAL (ε_{280} = 21,200).
[c] Derivative concentration was determined by hydrolyzing an aliquot in 6 M HCl (110 °C, 22 h) followed by quantitative amino-acid analysis.
[d] Analytical HPLC-analysis was carried out under the experimental conditions specified in the legend to Fig. 3. Under these conditions, insulin elutes with Rt = 7.28 min and has a surface area of 240,600 ± 8000 mav/µg of insulin.

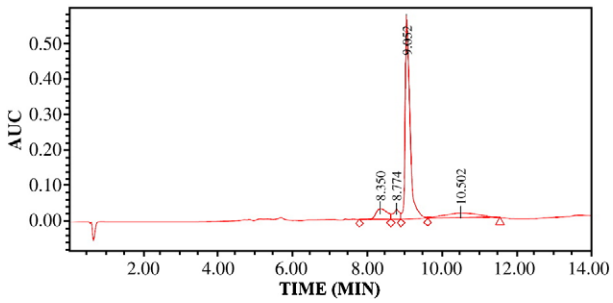


Fig. 3. HPLC-analysis of purified Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH. HPLC-purified Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH (50 μg) was loaded onto a Chromolith Rp-18e (100 mm × 4 mm) column and run with a linear gradient from 0 to 100% solution A (0.1% TFA) to solution B (acetonitrile-H_2O 75:25, in 0.1% TFA) over 10 min and then 4 min in solution B at a rate of 3 ml/min. The effluent was monitored at 220 nm. Under the same experimental conditions insulin elutes with Rt = 7.28 min.

this specific assay, the biological potency of such albumin- associated insulin derivative may be significantly reduced due to the presence of BSA (10 mg/ml) in the assay, as we previously noted with insulin-detemir (unpublished observations). Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH has regained its full lipogenic potency ($ED_{50} = 0.1 \pm 0.02$ nM) following 4 h of incubation at pH 10.3 at 25 °C, (Table 2), namely, under incubating conditions that release insulin fully from the conjugate [13].

3.4. Prolonged residence time of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH following intravenous administration into rats

In the experiments summarized in Fig. 4, either ^{125}I-Insulin or ^{125}I-Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH were administered intravenously to rats. At various time points, blood aliquots were withdrawn and quantitated for their radioactive content in cpm/ml blood. The circulating level of radioactively labeled insulin has been declined yielding a t½ value of 3.3 ± 0.4 h (Fig. 4). With intravenously administered Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH, radioactive content in blood has increased over a period of 2 h reaching a value of 31,000 ± 1000 cpm/ml blood. This high level has been stably maintained over a period of 6 h and then declined with a t½ value of 17 ± 1 h. A significant amount (~10,000 cpm/ml blood) was still evident 30 h after intravenous administration (Fig. 4).

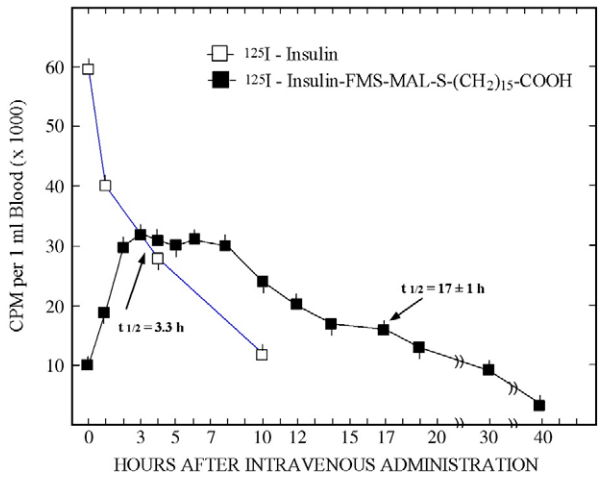


Fig. 4. Prolonged residence time of ^{125}I-Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH following intravenous administration into rats. Two groups of rats ($n = 3$ per group) received either ^{125}I-Insulin or HPLC-purified ^{125}I-Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH (8.4 ± 0.4 × 10^6 CPM per 220 ± 10 g rat). At the indicated time points blood aliquots (50-70 mg) were drawn and counted for their radioactive content (calculated in the Figure for 1 ml blood).

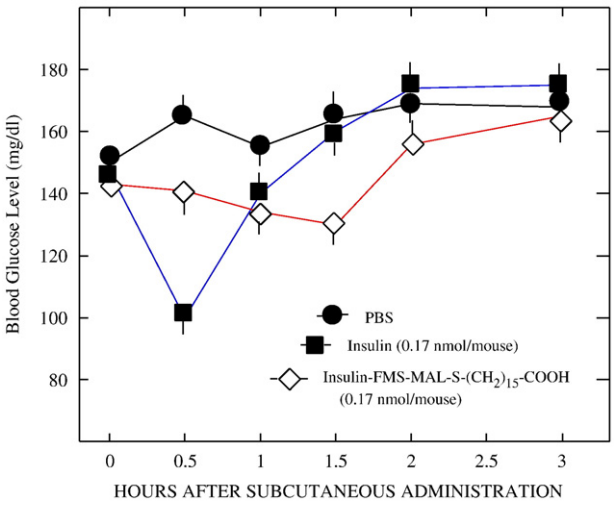


Fig. 5. Circulating glucose levels in mice following a single subcutaneous administration of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH. CD1-mice were injected subcutaneously with PBS-buffer (0.2 ml/mouse), Zn^{2+} free Insulin (0.17 nmol/mouse in 0.2 ml PBS buffer), or with Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH(0.17 nmol/mouse in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point is the arithmetic mean of $n = 5$ mice ± SE.

3.5. Glucose lowering pattern of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH following subcutaneous administration in mice

In Fig. 5 we compare the glucose-lowering pattern obtained after a single subcutaneous administration of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH to that of Zn^{+2}-free insulin, both administered at low and similar doses (0.17 nmol/mouse). As shown, the insulin-derivative had a flat glucose-lowering pattern, about two- fold more prolonged than that of insulin. In terms of area under the curve, Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH resembled the native hormone (integrated from Fig. 5), although in vitro this derivative has only 10% the biological potency of insulin (Table 2). Thus, Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH equals native insulin in terms of units/mg. Historically, the parameter of glucose lowering effect was determined for new insulin preparations in rabbits (reviewed in [27]). We, however, do not recommend doing so in this species with an insulin derivative that associates with albumin. Rabbit albumin exhibits extraordinarily high affinity to fatty-acid acylated insulins [5].

3.6. Glucose lowering pattern of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH in mice: comparison to insulin-detemir

Insulin-detemir is an insulin derivative possessing protracted action in vivo partly due to its associating affinity to serum albumin [6,21]. Our ITC measurements revealed that Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH has 3.5 times higher binding affinity to HSA than insulin-detemir (Fig. 1, Table 1) Fig. 6 shows the glucose lowering pattern of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH compared to that of Zn^{2+} free-insulin and insulin-detemir, when all three species were subcutaneously administered at the same dose (0.68 nmol/mouse). At this dose, Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH is highly potent in reducing BGL, maintaining it over a prolonged period with a t½ value of 6 ± 1 h. Low BGL was still evident 24 h following administration (Fig. 6). The AUC could not therefore be accurately integrated, however, it exceeded over five times values obtained by a similar dose of subcutaneously administered insulin-detemir. Subcutaneous administration of Zn^{+2}-free insulin to mice at this dose showed a *t½* value of about 1.8 h and quite hypoglycemic glucose-lowering pattern (Fig. 6).

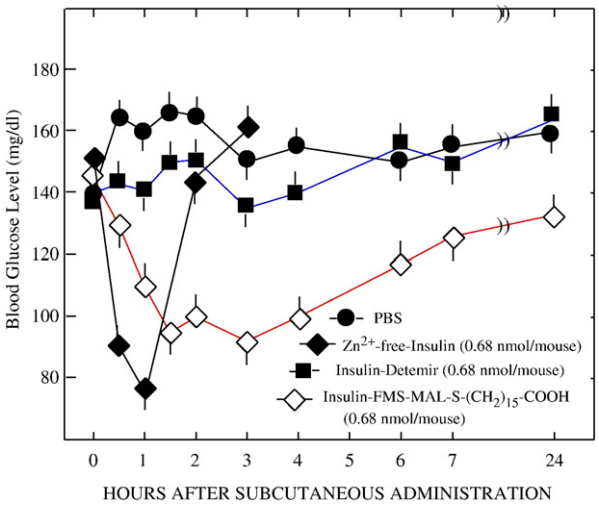


Fig. 6. Circulating glucose levels in mice following a single subcutaneous administration: Comparison of Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH to insulin-detemir. CD1-mice were injected subcutaneously with PBS-buffer, Insulin–FMS–MAL–S–$(CH_2)_{15}$–COOH or insulin-detemir (0.68 nmol/mouse in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point is the arithmetic mean of $n=5$ mice ± SE.

3.7. Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH facilitates prolonged glucose-lowering effect in CD1 mice

Fig. 7 shows the glucose-lowering profile of subcutaneously administered native-exendin-4 and of exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH, both administered at a dose of 0.24 nmol/CD1 mouse. Previously we reported that this strain of mice reflects well the action of this glucagon-like peptide-1 agonist in healthy and in Type II diabetic patients in the sense that at any dosage applied, circulating BGL never falls below a threshold level, which in CD1-mice amounts to a decrease of 27 ± 3% [7,25]. Following subcutaneous administration of Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH, circulating glucose reached its lowest concentration 3 h after administration and this level was maintained over a period of 20 h. Returning to initial glucose level took place with a t½ value of 28 ± 2 h, which is 4.7 times longer than that obtained by the same dose of the native hormone (Fig. 7).

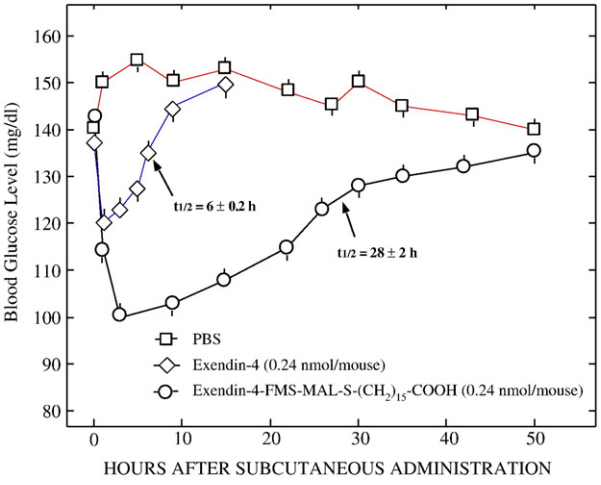


Fig. 7. Glucose lowering pattern of Exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH following a single subcutaneous administration to CD1-mice. Three group of CD1 mice ($n=6$ per group) underwent one subcutaneous administration of PBS buffer, pH 7.4, native exendin-4 (0.24 nmol/mouse) or exendin-4–FMS–MAL–S–$(CH_2)_{15}$–COOH (0.24 nmol/mouse). Circulating glucose levels were then monitored at the time points indicated in the Figure. Each point is the arithmetic mean of $n=6$ mice ± SE.

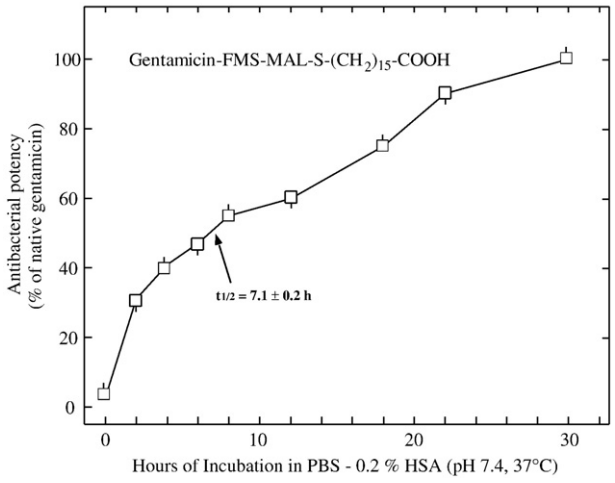


Fig. 8. Time course of in vitro reactivation of Gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH. Gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH (0.16 μmol/ml) was incubated in PBS, pH 7.4, containing 2% (w/v) HSA at 37 °C. Aliquots were withdrawn at the indicated time points and analyzed at several concentrations in the antibacterial assay. Gentamicin inhibits *E.Coli* replication with an $I.C_{50}=2.1\pm0.2$ μM. Aliquots with $I.C_{50}=21\pm2$ μM are considered having 10% of the native gentamicin antibacterial potency.

3.8. Gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH is an inactive, reactivable (prodrug) compound

Large protein drugs are expected to accommodate one or two such HSA-binding probe(s) of this size (760 Da) with the preservation of significant amount of their biological/pharmacological potencies [12,26]. This, however, does not seem to be the case with regard to low molecular-weight amino containing compounds. The latter category is likely to suffer a massive loss of pharmacological potency upon conjugation. Under these circumstances, this approach is impractical unless reactivation occurs.

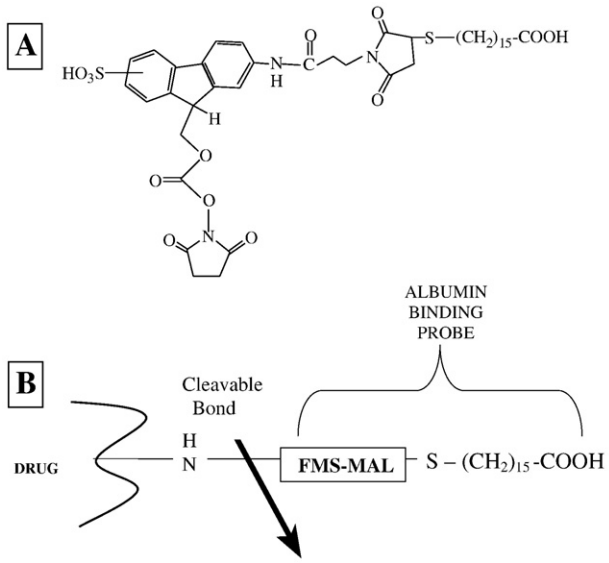


Fig. 9. (A) Structure of 16-(1-(3-(9-(((2,5-dioxypyrrolidin-1-yloxy) carbonyloxy)-methyl)-6-sulfo-9H-fluorene-2-yalamino)-oxopropyl)-2,5-dioxopyrrolidin-3-ylthio) hexadecanoic acid, an optimal albumin-binding probe, designed, synthesized and investigated in this study. (B) Schematic model describing the conversion of short-lived drugs to long-lived species in-vivo. Inactive albumin-associated conjugates bind to serum albumin and therefore exhibit prolonged residence time *in situ*. During this period the parent amino containing molecules are released from the inactive conjugates in their native-active form, an event that takes place at a slow rate, over many hours following administration.

Gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH has ~3 ± 0.7% the antibacterial potency of native gentamicin (time 0 in Fig. 8). Upon incubation in PBS buffer (pH 7.4) containing 20 mg/ml HSA, the antibacterial potency was generated with a t½ value of 7.1 ± 0.2 h. Gentamicin–FMS–MAL–S–$(CH_2)_{15}$–COOH regained its full (100%) antibacterial potency following 30 h of incubation (Fig. 8).

4. Concluding remarks

Here we introduced an albumin-binding compound of the structure given in Fig. 9. When this 872 Da molecule (760 Da following linkage due to elimination of the N-hydroxysuccinimide moiety) is introduced to, potentially, any amino-containing substance, it forms a conjugate having associating affinity to albumin in the range of $Ka = 2.0–2.6 \times 10^5 M^{-1}$ (Table 1). Such albumin-associating affinity is sufficient to turn short-lived molecules into long-lived species in vivo (Figs. 4–7). From pharmacological standpoint, any short-lived amino-containing molecule including in proteins, peptides, amino acids and derivatives, catecholamines, aminoglycosides, nucleotides and derivatives is expected to be exploitable by this development.

Why this strategy has not been materialized earlier? We assume that two main barriers may account for it: (i) The difficulty to obtain an albumin-binding probe having sufficient associating affinity to albumin, as most small molecules that bind tightly to this carrier protein lose this capability when linked to a macromolecule (i.e. Table 1 this study); and (ii) the lack of "reversibility principle" of the type developed in our laboratories in recent years. The latter is a prerequisite condition in case the albumin-associated new product loses its biological/pharmacological potency upon conjugation. This conjugate reactivation becomes relevant only if it takes place in body fluids at a slow rate, with a desirable pharmacokinetic pattern. This condition is fulfilled with all conjugates prepared by us that were linked through this specific hydrolysable heterobifunctional spacer (Fig. 8, references [7–13,18,24]).

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