**Engineered prolonged-acting prodrugs via albumin-binding probes**

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| **Project Number:** | #1555 |
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| **Patent Status:** | Pending |

**Overview and unmet need**

Therapeutic protein and peptide based drugs are an important class of medicines serving patients most in need of novel therapies. Recently approved recombinant protein therapeutics have been developed to treat a wide variety of clinical indications, including cancers, autoimmunity/inflammation, exposure to infectious agents, and genetic disorders. Currently, the market for peptide and protein drugs is estimated to be greater than US$40 billion/year, or 10% of the pharmaceutical market. However, most polypeptide drugs, in particular non glycosylated proteins of molecular mass less than 50 kDa, are short-lived species in vivo having circulatory half-lives of 5–20 min. One of the approaches applied for extending the actions of short-lived peptide/protein drugs is to endow them with affinity to serum albumin.

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.

One approach used to bind drugs to albumin is by covalently linking them to ligands that bind albumin, such as long-chain fatty acids (LCFAs). This approach was successfully used for designing an insulin derivative in which an LCFA-like probe is integrated into the insulin molecule, increasing its residence time.

Though successfully used in the past, a major drawback of linking ligands to target molecules is that conjugating a probe to a peptide or a protein could result in an inactive product. To overcome this last drawback, Prof. Shechter and prof. Fridkin designed a set of probes capable of binding albumin with high affinity. A first set of probes are designed to hydrolyse under physiological conditions, thus reactivating the prodrug. Conversely, another set of designed probes are non-hydrolysable but have more binding options and thus can be attached to the target drug in a manner that minimizes interference with its pharmacological activity.

**The Technology**

The team at Prof. shechter’s and Prof. Fridkin’s labs have development of a set of novel probes capable of binding human serum albumin (HSA) with high affinity (Ka = 105), sufficient to turn short-lived molecules into long-lived species in vivo. The first set of probes, LCFA-like sulfonated derivatives, are capable of selectively reacting with any drug containing an amino/mercapto group (e.g. protein or peptides containing a lysine or unbonded cysteine residues). Although peptide and protein drugs can undergo a considerable degree of lysine modification before being substantially inactivated, in case more than one amino group is present in the drug, the amino group to be linked to the albumin-binding probe could be selected as to minimally interfere with the pharmacological activity of the drug. A second set of probes are similar to the first albeit an addition of a hydrolysable heterobifunctional spacer which goes through spontaneous hydrolysis under physiological conditions. The latter set of probes can be conjugated to any amino group containing drugs. These probes undergo controlled reactivation in case the albumin-associated new product loses its biological/pharmacological potency upon conjugation. This conjugate reactivation only takes place in body fluids at a slow rate, with a desirable pharmacokinetic pattern.

Testing done at Prof. shechter’s lab demonstrated that all conjugates prepared using either set of probes exhibited considerably extended in vivo residence time compared to non-conjugated drugs and were pharmacologically active.

***Advantages***

* Up to a 6-fold increase in peptide/protein drug residence time in the blood.
* Significantly prolongs the life time of the drug without substantially interfering with its pharmacological activity
* Any short-lived amino-containing molecule including: proteins, peptides, amino acids and derivatives, catecholamines, aminoglycosides, nucleotides and derivatives is expected to be exploitable by this technology.

***Applications***

* Significantly prolonging the life time of short-lived amino/mercapto group containing drugs (e.g. proteins, peptides, amino acids and derivatives, catecholamines, aminoglycosides, nucleotides and derivatives) without substantially interfering with their pharmacological activity.

**Development Status**

A number of protein and peptides drug conjugates have been prepared and tested in vivo in mice and rat models. Fig. 1 compares the glucose-lowering pattern obtained, in mice, after a single subcutaneous administration of insulin conjugate containing the hydrolysable heterobifunctional spacer (insulin–FMS–MAL–S–(CH2)15–COOH) compared to that of Zn+2-free insulin at similar doses (0.17 nmol/mouse). As shown, the insulin-derivative had a flat glucose-lowering pattern, about twofold more prolonged than that of insulin. In terms of area under the curve, insulin–FMS–MAL–S–(CH2)15–COOH resembled the native hormone. Similarly, Fig. 2 shows a comparison between the glucose-lowering pattern obtained after a single administration of a non-hydrolyzable insulin conjugate (insulin-CO-(CH2)15-SO3H to that of Zn2+-free insulin, both administered subcutaneously at a low dose to CD1-mice (0.69 nmole/ mouse). As shown in Fig. 2, insulin-CO-(CH2)15-SO3H had a flat glucose-lowering pattern (t1/2 6.4±0.3 hrs), which was about 3-fold more prolonged than that of insulin (t1/2 2±0.2 hrs). In terms of area under the curve, insulin-CO-(CH2)15-SO3H resembled that of the native hormone.

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| **Fig. 1.** Comparison of circulating glucose levels in mice following a single subcutaneous administration of insulin–FMS–MAL–S–(CH2)15–COOH or Zn2+-free insulin | **Fig. 2.**Comparison of circulating glucose levels in mice following a single subcutaneous administration of insulin-CO-(CH2)15-SO3H or Zn2+-free insulin |

To further demonstrate that insulin–FMS–MAL–S–(CH2)15–COOH has a prolonged residence time compared to Zn2+-free insulin, radioactively labeled native insulin and insulin–FMS–MAL–S–(CH2)15–COOH were administered intravenously to rats. Fig. 3 shows that while Zn2+-free insulin has a blood t½ value of 3.3±0.4 h, insulin–FMS–MAL–S–(CH2)15–COOH has a t½ value of 17±1 h, and was still present in the blood upto 30 hours post injection.

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|  | **Fig. 3.** Comparison of residence time of 125I-Insulin–FMS–MAL–S–(CH2)15–COOH and Zn2+-free insulin following intravenous administration into rats |

Another important peptide used to treat type 2 diabetes mellitus is exendin-4. Exendin-4 is a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion. Fig. 4 shows the glucose lowering effect of native-exendin-4 compared to that of exendin-4–FMS–MAL–S– (CH2)15–COOH, both administered at a dose of 0.24 nmol/CD1 mouse. Following subcutaneous administration of Exendin-4–FMS–MAL–S–(CH2)15–COOH, circulating glucose reached its lowest concentration 3h after administration and this level was maintained over a period of 20h. 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. 5 shows a comparison done using exendin-4-CO-(CH2)15-SO3H, a non-hydrolysable probe. Using this probe demonstrates an even higher a t½ value of 32+2 hr.

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| **Fig. 4.** Comparison of the Glucose lowering pattern of Exendin-4–FMS–MAL–S–(CH2)15–COOH and native peptide following a single subcutaneous administration to CD1-mice. | **Fig. 5.** Comparison of the Glucose lowering pattern of Exendin-4-CO-(CH2)15-SO3H and native peptide following a single subcutaneous administration to CD1-mice. |

Lastly, the team were able to demonstrate the efficacy of this technology on low-molecular weight targets. Low molecular-weight amino containing compounds tend to suffer a massive loss of pharmacological potency upon conjugation. Gentamicin is commonly used antibiotic with a molecular weight of only 0.4 kDa. Fig. 6. shows the reactivation over time of Gentamicin–FMS–MAL–S–(CH2)15–COOH. at t=0 the compound has ~3±0.7% the antibacterial potency of native gentamicin. Upon incubation in PBS buffer (pH 7.4) containing 20 mg/ml human serum albumin, the antibacterial potency was generated with a t½ value of 7.1±0.2 h. Gentamicin–FMS–MAL–S–(CH2)15–COOH regained its full (100%) antibacterial potency following 30 h of incubation.

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|  | **Fig. 6**. Time course of in vitro reactivation of Gentamicin–FMS–MAL–S–(CH2)15–COOH incubated in PBS. |

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