
Active-transport peptides induce GIT transport of nanoparticles containing leuprolide and insulin in an *in vivo* rat model

Executive Summary

Cytogen Corporation and Elan Pharmaceuticals collaborated on a joint research program using Cytogen's proprietary complex random-peptide phage library to identify peptide sequences that enhance transport across the gastro-intestinal tract (GIT) and to utilize these peptides on the surface of insulin- and leuprolide-loaded nanoparticles to demonstrate *in vivo* drug delivery. The screening step utilized a novel dual approach, with initial *in vitro* screening designed to pre-select peptides that bound GIT receptors followed by functional *in vivo* selection in a rat closed-loop intraduodenal model. More than 25 peptides were identified, and selected peptides were formulated with nanoparticles containing leuprolide in one experiment and insulin in another. Again using the *in vivo* rat closed-loop intraduodenal model, these particles were shown to enable *in vivo* delivery of insulin and leuprolide.

Background

Macromolecules are not amenable to oral delivery. Oral delivery of macromolecules, including proteins and peptides, continues to be a desirable but difficult goal (Ref. 1-3). The oral route of administration is preferable to intravenous or subcutaneous injection because of ease of administration and patient compliance, but this route is very difficult because macromolecules can be destroyed by the harsh intestinal environment and are often not absorbed through the gastro-intestinal tract. It is possible to prepare coated nano- or micro-particles which can protect sensitive drugs through the stomach, but efficient transport across the intestinal membranes into the blood supply has remained a difficult problem.

Complex random peptide libraries may contain active transport molecules.

We postulated that complex random peptide libraries may hold the key to identifying peptide sequences which can be actively transported across these membranes, and we had available to us a complex library with long, 38- and 43-amino acid, random inserts (see references 4 and 5). Such libraries were expected to be valuable because they may have conformation motifs that could interact with receptors that actively transport molecules across the gastro-intestinal tract (GIT).

Novel dual-screening method: in vitro pre-screening followed by in vivo functional selection. We used a dual-screening method to obtain active-transport peptides.

- In the first step, *in vitro* screening against cloned GIT receptors was used to obtain pre-selected libraries. The GIT receptors were chosen based on their expression in the GIT, expression along the length of the small intestine, high concentration of expression, the presence of an extracellular domain facing into the lumen of the GIT, and extracellular domains that would permit easy access and bio-adhesion by targeting particles.
- In the second step, *in vivo* functional screening further selected these phage by injection into a rat closed-loop duodenal model, which actively selected for phage which could cross the GIT. After injection into the closed loop, samples were taken from both the portal and systemic circulation at various time points.

Peptide-coated nanoparticles and animal models for drug delivery. Elan brought extensive experience in nano-particle technology and animal models of oral drug delivery, providing for an excellent collaboration. If peptide sequences could be identified from the dual screening method, they would be candidates for “active transport peptides,” and could be coupled to the surface of bio-degradable micro- and nano-particles. The goal was to load particles with peptide or protein drugs, coat their surface with the targeting peptides, and then demonstrate in an animal model that the peptide or protein drugs were delivered into the blood stream of the animals.

Materials, Methods, and Results.

Complex Peptide Libraries. Two long, complex random peptide phage-display libraries were used in this study (see references 4 and 5 for a detailed description), one with a random 38-amino acid insert and the second with a 43-amino acid insert, as shown in **Figure 1**. These libraries were designed to express complex binding patterns, including disulfide bonds, and thus should be advantageous over smaller random-peptide libraries.

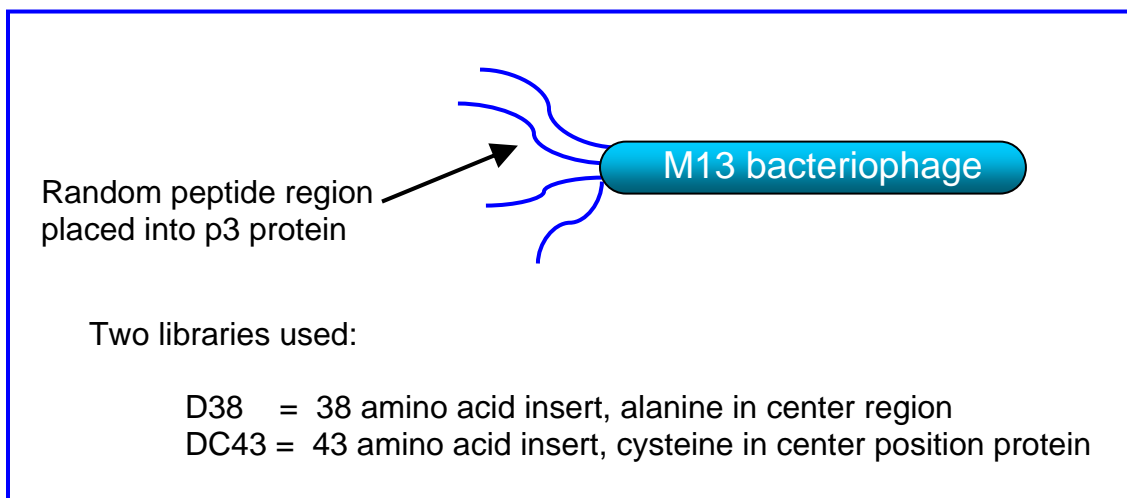


Figure 1. Peptide libraries used in Cytogen-Elan Studies

In vitro screening. The extracellular portions of 4 receptors (references 6-9) found in the GIT were cloned and expressed (see Table 1), and then used as the first step of panning against the peptide library. This yielded a “sub-library” of phage with specific binding to each receptor. These sub-libraries were then amplified and used in the *in vivo* rat model.

In vivo screening. The sub-libraries were injected into a closed-loop intraduodenal portion of the rat intestine, and blood samples taken at various time points. As shown in Table 1, there was selective transport of the phage across the GIT. These phage were sequenced and further evaluated, and candidate peptides finally were synthesized using solid-phase peptide synthesis.

Receptor	Type of receptor	Number of phage recovered from <i>in vitro</i> screen	Number of phage transferred into blood from <i>in vivo</i> screen
hSI	Sucrase-isomaltase transporter	10	3
D2H	Transporter of neutral and basic amino acids	16	7
HPT1	Peptide transporter	6	4
hPEPT1	di-/tri-peptide transporter	18	11

Table 1. Number of phage bound to each receptor and number transferred across the GIT into the blood.

Peptide-coated, drug-loaded nanoparticles. Elan Corporation has extensive experience in the manufacture of small particles commonly used for drug delivery. The ones used in these experiments were made up of a polymer called “poly-lactic-glycolic acid (PLGA).” These particles were loaded with either insulin or leuprolide following standard protocols, and then coated with one of the selected optimal peptides discovered in the screen described above.

Example 1: Oral Delivery of Leuprolide in Rat Model.

Leuprolide, also referred to as LHRH, is a decapeptide that is currently prescribed in injectable form for the treatment of prostate cancer and endometriosis. LHRH agonists are not active orally but can be administered as nasal sprays, subcutaneous injections daily, or deep intramuscular depot injections monthly (Ref. 10)

For the leuprolide study, it was decided to use two peptides from the hPEPT1 screen, PAX2 and P31. PLGA nanoparticles were first synthesized with leuprolide and the particles were either not coated (control) or coated with one of the peptides.

The ability of these particles to deliver leuprolide into the blood was studied in a rat “closed-loop” intraduodenal model. In this model, a section of the duodenum is tied off, and the particles injected into this “closed loop.” “Free” leuprolide, not encapsulated in the particles, was also used as a control. Samples of blood are then taken at various times and tested for the presence of leuprolide, which would indicate successful delivery of this peptide by the oral route. Ten (10) rats were used for each coating. The figure below indicates the results of this study

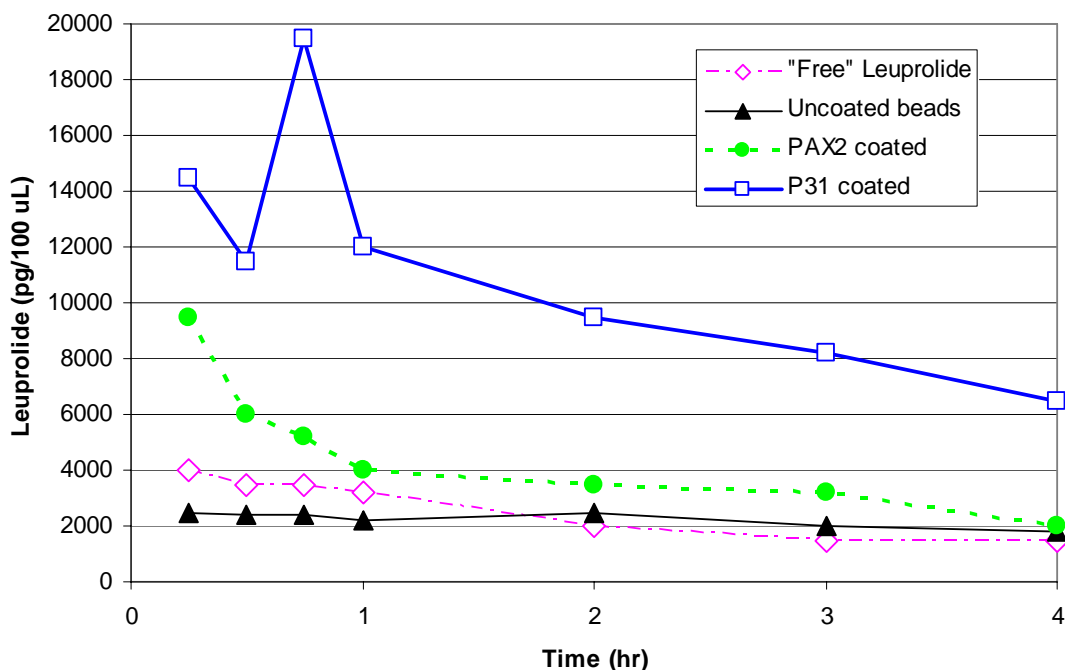


Figure 1. Leuprolide transport into blood from closed intra-duodenal loop

Both peptides enhance oral delivery of leuprolide. Figure 1 clearly shows that particles coated with either peptide P31 or PAX2 were able to “deliver” leuprolide significantly more effectively than either uncoated beads or injection of “free” leuprolide (not encapsulated into beads). Particles coated with peptide P31 were the most efficient in this study, with more than 4-fold delivery of leuprolide at the peak and a much higher over-all delivery than all other groups. This result is notable, because the method was not optimized. For instance, it is possible to stabilize the peptides on the surface of the particles and achieve even greater transport across the GIT (data not shown here).

Example 2: Oral Delivery of Insulin in Rat Model.

A second example of a macromolecule that cannot be taken orally is insulin, which must be injected daily in diabetic patients. An oral version would be welcomed by the many

patients who inject themselves daily, but again the peptide nature of insulin makes it impossible to deliver orally. We prepared control and peptide-spiked PLGA particles with encapsulated insulin, and then compared them in a rat model of diabetes in which anesthesia induces an insulin-sensitive hyper-glycemic state. Ten (10) animals were used for each sample, which were injected into a closed intra-duodenal loop. Samples were taken from the blood at various time points, and both the insulin levels and the blood glucose levels were measured at various time points. The glucose was measured to insure that insulin was delivered in a biologically active state. The specific groups were as follows

ID	Peptide coating	Insulin amount
Control (PBS)	None	None
Insulin solution (insulin injected as a solution)	None	300 IU
Particles (un-coated)	None	300 IU
hPEPT1	hPEPT1	300 IU
HPT1	HPT1	300 IU

The results for insulin are shown in Figure 2, and the glucose levels in Figure 3. Significantly, the insulin levels from the rats injected with both of the targeting peptides showed enhanced “delivery” of insulin compared to either insulin injected as a solution or to the un-coated insulin particles. The HPT1-coated particles were especially effective at delivering insulin to the blood.

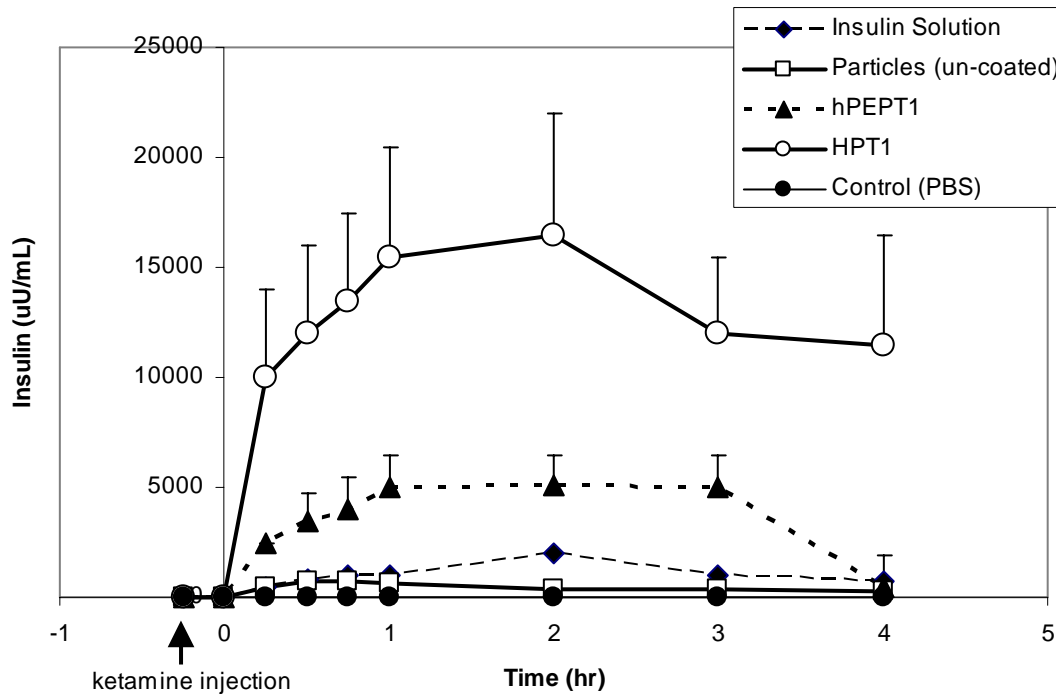


Figure 2. Insulin levels in rats after intra-duodenal injection of insulin-loaded particles and controls.

The levels of blood glucose give an indication of the biological activity and efficacy of the delivered insulin, and these values are shown in Figure 3. This figure shows that the injection of ketamine induced a hypoglycemic state in which the glucose level rose. Insulin injected as a solution decreased this amount slightly, as did the un-coated particles coated with insulin. Both targeting peptides decreased the insulin levels substantially, and in this assay were almost equally effective.

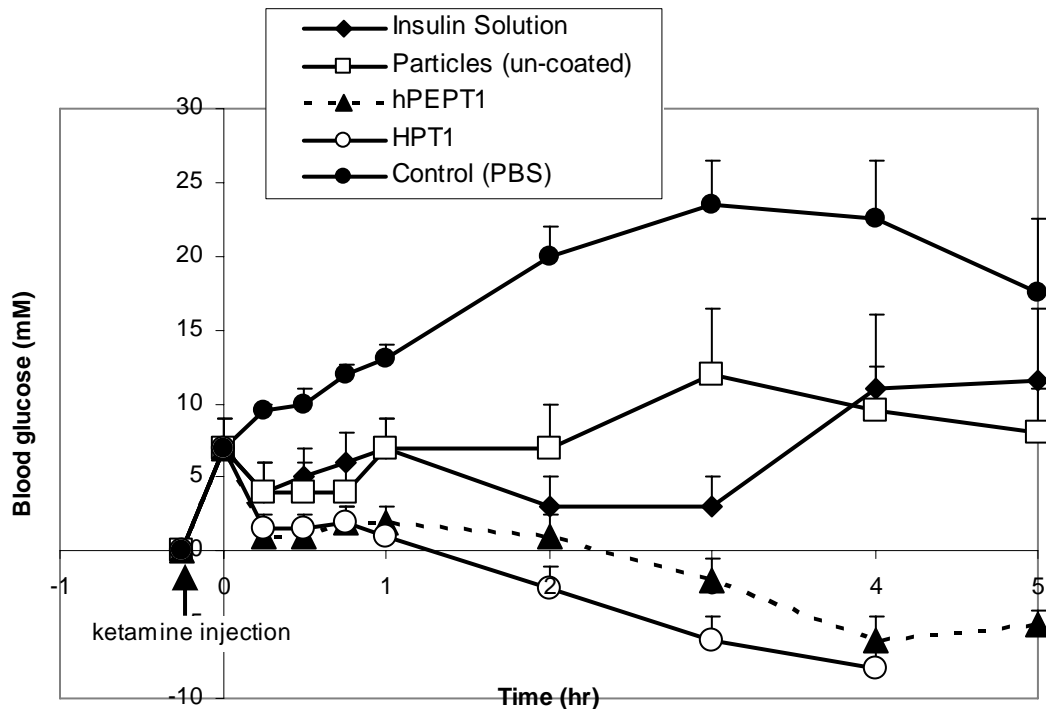


Figure 3. Blood glucose levels in rats after intra-duodenal injection of insulin-loaded particles and controls.

Summary: Successful development of Active Transport Peptides

A dual-screening approach was used to screen a random peptide library to search for peptides which would actively transport particles across the GIT. After an initial screening against cloned receptors found in the gut, the random-peptide library with more than 10^8 members was reduced to 50 specific binding peptides. The second selection, involving an *in vivo* rat model, demonstrated that 25 of these would cross the GIT into the bloodstream. Several of these were incorporated into drug-loaded PLGA nanoparticles and tested in an *in vivo* rat model. Successful delivery of both leuprolide and insulin were demonstrated.

Additional Feature: Brain Targeting Phage

In addition to evaluating the phage in the blood, several other organs, including the brain, were taken from the experimental animals, and evaluated for the presence of phage. In the case of the brain, seven (7) specific sequences were recovered from the brain. These phage therefore had dual functionality, the first being transport across the gastro-intestinal track wall into the systemic blood, and the second being transport across the blood-brain barrier. Such phage may be extremely important for delivery of chemotherapeutics into the brain, which is often a privileged site and thus difficult to treat with standard therapies.

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Relevant Issued US Patents:

US 6,361,938

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