

# Induction of androgenic ablation and inhibition of prostate hormone-sensitive tumors by immunization with the GnRHm1-TT synthetic peptide adjuvated in VSSP

✉ Jesús A Junco<sup>1</sup>, Roberto Basulto<sup>1</sup>, Franklin Fuentes<sup>1</sup>, Eddy Bover<sup>1</sup>, María D Castro<sup>1</sup>, Osvaldo Reyes<sup>2</sup>, Eulogio Pimentel<sup>1</sup>, Lesvia Calzada<sup>1</sup>, Yovisleidis López<sup>1</sup>, Ricardo Bringas<sup>2</sup>, Niurka Arteaga<sup>1</sup>, Héctor Hernández<sup>1</sup>, Hilda Garay<sup>2</sup>, Luis Agüero<sup>1</sup>, Gerardo Guillén<sup>2</sup>

<sup>1</sup>Centro de Ingeniería Genética y Biotecnología de Camagüey, Ave. Finlay y Circunvalación Norte, CP 70100, Camagüey, Cuba

<sup>2</sup>Centro de Ingeniería Genética y Biotecnología, CIGB, Ave. 31 e/ 190 y 158, AP 6162, CP 10 600, Playa, Ciudad de La Habana, Cuba  
E-mail: jesus.junco@cigb.edu.cu

## Introduction

Prostate cancer is the second cause of cancer-related mortality among the male population in the western hemisphere [1]. In Cuba, it represents a significant health problem that causes over 2 000 deaths per year. The androgenic suppression of prostate cancer by treatment with gonadotropin releasing hormone analogs (GnRH) constitutes one of the most efficient therapeutic interventions currently available, and the use of therapeutic vaccines based on the combination of this hormone with potent adjuvants for the stimulation of the immune system is being hailed as one of the most promising, cost-effective alternatives under development [2]. However, so far the evaluation of these vaccines in clinical trials has yielded modest results, with low immunogenicity, epitope suppression processes triggered by carrier molecules and a limited therapeutic effect [3].

In this work we describe the design and synthesis of a new molecule derived from GnRH and its use as the main active ingredient of an immunogen aimed at the ablation of testosterone, and therefore at the inhibition of hormone-sensitive prostate tumors. It consists of a new, more immunogenic GnRH mimetic peptide named GnRHm1-TT, where glycine at position 6 is substituted by proline in order to increase the rigidity of the molecule, which is covalently linked during synthesis to the 830-844 T helper cell epitope of tetanus toxoid (TT) for increased immunogenicity. Once completely synthesized, GnRHm1-TT [4] is formulated in an oil emulsion that also contains VSSP (very small size proteoliposomes) [5]. VSSP were included to guarantee a long-lasting and potent immune response against GnRH and led to the demonstration, for the first time, of the usefulness of this immunomodulator for the generation of an immune response against GnRH-derived autologous peptides. The immunization with adjuvated GnRHm1-TT generated significant levels of GnRH-neutralizing antibodies, which correlated with a significant decrease in testosterone levels equivalent to castration and also reduced the size of testicles and prostate in rats, pigs, dogs and monkeys ( $p < 0.01$ ). In the rat model, the immunization was able to inhibit the growth of implanted hormone-sensitive Dunning R3327-H prostate tumors, also increasing two-fold the survival rates of the immunized animals.

Additionally, this work demonstrates, using an *in vitro* inositol phosphate inhibition assay, that the biological activity of the GnRHm1-TT peptide in immunized animals is mediated mainly by the generation of neutralizing antibodies against endogenous GnRH.

The preclinical studies described above in healthy or tumor-implanted animals, together with the identification of the mechanism of action of GnRHm1-TT, have been the basis for the authorization, by the regulatory entities, of the use of this vaccine preparation in a Phase I Clinical Trial in prostate cancer patients.

## Results

### Design and synthesis of GnRHm1-TT

GnRHm1-TT is a mimetic variant of mammalian GnRH-I modified by substituting the glycine in position 6 by proline, covalently linked during chemical synthesis to a T helper epitope from TT [4]. The sequence of the peptide is shown in Figure 1.

### First results in prepuberal pigs

The potential of peptide GnRHm1-TT as an immunogen for the induction of testosterone ablation and immunocastration was first evaluated in male prepuberal Landrace pigs. The animals were immunized twice bimonthly with a 1 mg dose of GnRHm1-TT formulated in complete Freund's adjuvant (CFA). A significant decrease was observed in the size of testicles ( $p < 0.01$ ), prostate ( $p < 0.01$ ) and seminal vesicles ( $p < 0.01$ ), leading to testosterone levels equivalent to castration (0.05 nmol/mL;  $p < 0.01$ ). Additionally, the erectile capacity of the pigs as well as their mating reflexes and capacity were significantly reduced. This first proof of concept study demonstrated the efficacy of the designed peptide in an animal model [6]. Table 1 summarizes some of the obtained results.

GnRH natural - pEHWSYGLRPG

GnRHm1-TT - pEHWSY**PLRPGG**GGQYIKANSKFIGITEL

Figure 1. Primary structure of native LHRH/GnRH and the GnRHm1-TT peptide. The substitution of proline per glycine at position 6 (bold) and peptide subunits (boxes) corresponding to GnRH and T helper cell epitope from the tetanus toxoid are shown.

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**Results in adult Beagle dogs**

The positive results obtained by immunizing prepuberal pigs were successfully reproduced in a model of healthy adult Beagle dogs. The immunization schedule was similar to that used in pigs and the formulation included the same adjuvant, achieving significant levels of antibodies against the natural GnRH molecule. In the case of male dogs, these antibody levels decreased the number of spermatozoids and led to the increased appearance of spermatozoids bearing deformities. Additionally, the epididymal lumen decreased and was invaded by interstitial tissue, leading to sterility and severe alterations in prostate architecture for the immunized animals [6].

**Results in healthy adult rats**

The appearance of signs of toxicity due to the use of CFA, the modest effect found in the intended target organs and the limitations of dogs as an animal model for prostate cancer led to the selection of Copenhagen rats for the establishment of a new preclinical model. This model would be used for enhancing the immunogenicity of the peptide through the manipulation of the number of immunizations and the vaccination schedule, as well as for the selection of a different adjuvant that could be used in humans with results equivalent to those obtained with incomplete Freund's adjuvant or CFA.

The first assays in healthy adult rats used three different doses (100, 300 and 750 mg) of GnRHm1-TT adjuvated in Montanide ISA 51, administered with three different immunization schedules (every week, two or four weeks). These assays established that the use of the 750 mg dose with a monthly schedule induced the highest anti-GnRH antibody titers and the steepest decrease in testosterone levels, resulting in the complete atrophy of prostate, testicles and accessory glands. This effect was not observed at doses below 750 mg, which were poorly immunogenic in this study (manuscript in preparation).

**Immunoenhancing effect of VSSP during the administration of GnRHm1-TT to healthy adult rats**

Once the optimal schedule and dosage for the immunization with GnRHm1-TT adjuvated in Montanide ISA 51 were established, further work was aimed at the introduction of VSSP into the formulation as a component which might provide an additional increase in the immune response against the hormone and, therefore, the efficacy of the vaccine. This decision was based in previous reports from other authors [7, 8] and our experiences while trying

to obtain a highly homogenous and effective immune response against an inherently poor immunogen such as GnRH, of small size and high sequence conservation in mammals.

The results from these assays are shown in figure 2. As shown in the picture, the inclusion of VSSP as an additive to the GnRHm1-TT peptide significantly increased the antibody titers against the native hormone and, particularly, the seroconversion kinetics and its effects on target organs (prostate and testicles;  $p < 0.01$ ).

**Inhibition of tumor growth and increased survival rate in rats implanted with a hormone-sensitive prostate tumor and therapeutically immunized with GnRHm1-TT**

After demonstrating that the immunization of healthy animals with GnRHm1-TT together with VSSP and Montanide ISA 51 induced immunocastration and atrophy of prostate and testicles, it was necessary to corroborate these results in an animal prostate cancer model. For this purpose, Copenhagen rats previously implanted with murine Dunning's R3327-H prostate tumor cells were immunized with GnRHm1-TT. In spite of the presence of the tumor, 90% of the animals developed antibody titers against GnRH that decreased testosterone values to castration levels. A direct correlation was observed between the level of castration and tumor growth inhibition, with statistically significant differences when compared to the placebo group. Additionally, the survival rate in the immunized and castrated groups was 2-fold higher than in the placebo animals.

Despite the sustained castration generated in the immunized or castrated animals, there was tumor growth in a small number of animals from both groups even in the absence of testosterone. This suggests that the behavior of the chosen tumoral model closely mimics that of prostate cancer in human patients.

**Inhibition of inositol phosphate production by endogenous GnRH-neutralizing antibodies generated by immunization with the GnRHm1-TT peptide**

The design of the GnRHm1-TT peptide and its administration schedule were aimed at exploiting its immunological properties as a vaccine. Although the antibodies induced by the vaccine preparation were previously shown to bind the natural GnRH peptide absorbed to a polystyrene plate, and a correlation was demonstrated between this binding and the levels of castration, these findings do not constitute conclusive proof about the capacity of these antibodies for neutralizing native GnRH.

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**Table 1 . Comparison of testosterone levels and weight of prostate, testicles, bulbourethral glands and other accessory glands of the male reproductive system in pigs immunized with the synthetic peptide GnRHm1-TT emulsified in complete Freund's adjuvant**

Experimental Group	No. of pigg	Prostate	Seminal vesicles	Bulbourethral glands	Epididymides	Testides	Testosterone (nmd/L)
GnRHm1-TT	5	0.12 ± 0.08**	1.32 ± 2.06*	2.79 ± 3.78*	3.88 ± 2.75*	7.43 ± 7.65**	0.05 ± 0.03**
Control	5	0.56 ± 0.30	4.22 ± 2.72	6.54 ± 2.96	7.36 ± 0.92	21.1 ± 4.54	17.63 ± 2.71

\*Statistically significant difference at 95% confidence interval.

\*\* Statistically significant difference at 99% confidence interval.

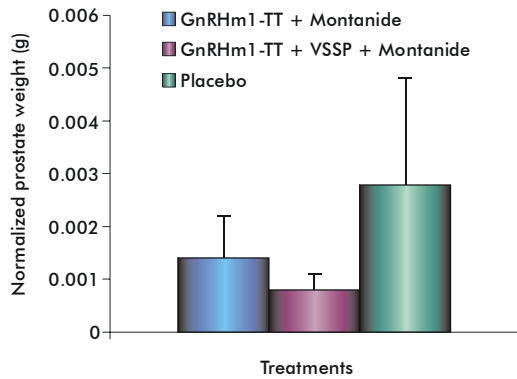


Figure 2. Administration of 4 immunizations to adult healthy rats with the synthetic peptide GnRHm1-TT formulated with VSSP and adjuvated with Montanide ISA 51.

For this reason, an inhibition experiment was carried out in COS-7 cells transformed with the gene for the GnRH receptor, using GnRH at a concentration of  $10^{-9}$  M. In this assay, 12.5 mg of anti-GnRH antibodies completely inhibited the production of inositol phosphate, with the subsequent and almost complete sequestration of GnRH from the cellular media (manuscript in preparation). Nonetheless, this peptide was able to bind to promiscuous receptors present in Dunning's R3327-G prostate tumor cells and other

tissues when administered in the absence of VSSP and Montanide ISA 51 adjuvants.

#### Toxicological assays on GnRHm1-TT. Authorization for a Phase I clinical trial in patients with locally advanced prostate cancer

The use of the GnRHm1-TT peptide adjuvated in VSSP and Montanide ISA 51 was shown to be innocuous in acute, local tolerance and repeated dose toxicity assays. These results, together with the evidences of an effective biological action in the previously mentioned preclinical models, allowed us to present the dossier for a Phase I clinical trial in patients with locally advanced prostate cancer, which was finally approved by the regulatory authorities.

#### Conclusions

Significant antibody titers against endogenous GnRH can be obtained by immunization with a designed GnRHm1-TT peptide variant, emulsified with VSSP in an oil-based adjuvant. These titers correlate with testosterone ablation, decreased size of the target organs (testicles and prostate) and growth inhibition of hormone-sensitive prostate tumors in murine models. Based on the preclinical *in vitro* and *in vivo* results obtained with the emulsified GnRHm1-TT peptide, authorization was granted to advance into Phase I clinical trials in patients with advanced prostate cancer.