Intramuscular Electroporation of an Optimized Rheoswitch®-Regulated Interferonα Plasmid Transgene Shows Long Term Persistence *In Vivo*: Implications for Therapy of Cancer

P Agarwal[1], T Chan[1], J Roeth[1], C Reed[1], B Merenick[1], JA Barrett[2], F Khazi[1], H Youssoufian[2], R Herberman[1], and WE Fogler[1]

[1]Intrexon Corporation, Germantown, MD; [2]ZIOPHARM Oncology Inc., Boston, MA

Intrexon Corporation
20358 Seneca Meadows Parkway, Germantown, MD 20876
Phone 301-556-9900 Fax 301-556-9901 www.dna.com

ZIOPHARM Oncology, Inc.
1 First Avenue, Parris Building #34, Navy Yard Plaza, Boston, MA 02129
Main 617-259-1970 Fax 617-241-2855 www.ziopharm.com

Abstract

Background: Pegylated IFNα2b was approved in early 2011 for malignant melanoma despite significant side effects in clinical trials. Alternate approaches to widen the therapeutic window of IFNα may afford a more pronounced effect as a cancer therapeutic. This study optimized an IFNα plasmid DNA for regulated secretion from skeletal muscle, using Intrexon's novel RheoSwitch® Therapeutic System® (RTS®) inducible expression platform activated by a small molecule activator ligand (AL). The anti-tumor efficacy of an optimized plasmid was assessed in a B16F0 melanoma model *in vivo* following a single intramuscular (IM) administration via electroporation.

Materials and Methods: Optimization of RTS controllable DNA vectors for murine or human IFNα was accomplished by evaluating interchangeable modular noncoding regulatory domains and secretion signal peptides, enabled by Intrexon's UltraVector® platform. To determine pharmacodynamics, plasmid DNA was (a) transfected into human HT1080 fibrosarcoma cells or C2C12 myoblasts and (b) plasmids that induced high levels of secreted protein in the presence of AL were electroporated into the skeletal muscle of normal or B16F0 tumor-bearing mice.

Results: Vector optimization increased the *in vitro* secretion of IFNα in response to AL by up to 23-fold, relative to an unoptimized RTS-IFNα plasmid. The single administration of an optimized RTS-mIFNα plasmid by IM electroporation and daily oral dosing with AL, led to persistent plasma expression of IFNα for approximately 4 months (study termination) with no detectable induction of an adverse immune response. This systemic expression was tightly regulated and removal of AL led to a return in expression to basal levels. The regulated expression and therapeutic benefit of this optimized plasmid following IM electroporation was examined in a syngeneic B16F0 melanoma model. IFNα gene therapy in the absence of daily oral AL did not result in inhibition of tumor growth compared to control, untreated tumors. In contrast, the single IM administration of RTS-IFNα and daily oral AL led to significant tumor growth inhibition that was comparable to the therapeutic benefits of DTIC (60 mg/Kg, ip, qdx5) or bolus recombinant mIFNα protein (50,000 IU, sc qod), without overt toxicity as assessed by body weight change. Plasma levels of IFNα obtained with the optimized plasmid were approximately 5000 pg/mL and increased the expression of the anti-angiogenic downstream effector molecule, IP-10.

Conclusions: The UltraVector and RTS technologies have been used to create and optimize pDNA for the regulated secretion of IFNα from skeletal muscle. This innovative approach offers the potential advantage of a single administration of optimized RTS-IFNα transgene, in combination with oral AL, for sustained therapeutic anti-tumor efficacy and increased safety by avoidance of fluctuating levels of circulating IFNα.

Introduction

- Recombinant pegylated interferon alfa 2b (Sylatron™) is approved for the adjuvant treatment of melanoma with microscopic or gross nodal involvement within 84 days of definitive surgical resection, including complete lymphadenectomy
- Alternate administration approaches to widen the therapeutic window of interferonα (IFNα) may afford a more pronounced effect as a cancer therapeutic
- This study optimized an IFNα plasmid DNA to allow for the regulated secretion of effective anti-tumor doses from skeletal muscle following administration
- The study utilized Intrexon's novel RheoSwitch Therapeutic System (RTS) inducible expression platform, activated by an orally available small molecule activator ligand (AL)

RheoSwitch Proteins and Activator Ligand Controls Timing and Level of Target Gene Expression

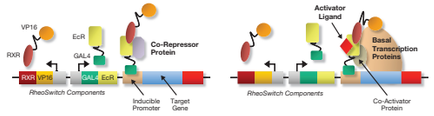

Figure 1a. The RheoSwitch Therapeutic System (RTS) inducible expression platform contains three basic components: (1) an inducible promoter; (2) constitutive transcription factor and a co-activation partner; and (3) a RheoSwitch activator ligand (AL). In the absence of AL, the switch proteins complex to provide an "off" signal. In contrast, in the presence of AL, the complex changes conformation and provides a dose-dependent "on" signal for target gene expression.

Vector Optimization Enabled by the Ultravector Platform Increases Regulated Mouse IFNα Expression *In Vitro*

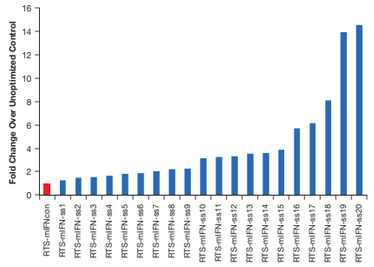

Figure 1b. A matrix of RTS platform-regulated mouse IFNα expression vectors, with modifications in the non-coding regulatory elements (Inducible Promoter, 5'REG, 3'REG) was generated to optimize mouse IFNα expression. The RTS-mIFNα plasmids were screened by transient transfection in C2C12 myoblast cells, and IFNα expression was induced by treatment with 100 nM of activator ligand (INXN-1001, AL). Supernatants were harvested 48 hours after transfection, and screened for mouse IFNα expression by ELISA. Expression levels are depicted as fold change over unoptimized pRTS-mouse IFNα (red bar). n = 3, mean ± s.e.m.

Summary: Intrexon's UltraVector and RTS platforms can facilitate vector optimization to achieve high levels of INXN-1001-regulated IFNα expression *in vitro*.

In Vitro Regulated Expression of pRTS-mIFNα in C2C12 Mouse Myoblast Cells

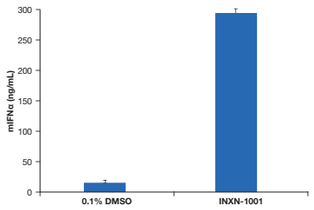

Figure 1c: pRTS-mIFNα-ss17 was selected from the matrix described above (Figure 1b) because it showed high INXN-1001-induced expression *in vitro*, and the lowest expression relative to the other high-expressing plasmids in the absence of ligand (data not shown). Here, C2C12 cells were transiently transfected with pRTS-mIFNα-ss17. Activator ligand (INXN-1001, AL) was added to the culture medium at a concentration of 100 nM, or cells were treated with 0.1% DMSO as a negative control. Supernatants were collected at 48h and assayed for mouse IFNα expression. n = 3, mean ± s.d.

Summary: pRTS-mIFNα-ss17, shows robust INXN-1001-regulated expression *in vitro*, and was selected for subsequent *in vivo* studies.

Single Intramuscular Administration of pRTS-mIFNα Leads to Sustained Regulated Plasma Expression *In Vivo*

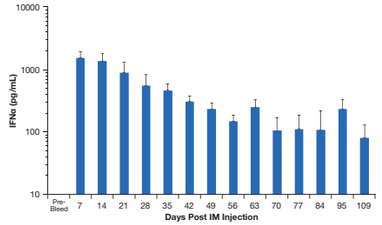

Figure 2: pRTS-mIFNα (250 μg) was administered to Balb/C female mice by intramuscular (IM) electroporation. AL (INXN-1001) was provided in the feed *ad libitum* (oral equivalent dose of approximately 50 mg/kg/day) starting on the day of plasmid injection. IFNα levels in the mouse plasma were assessed by ELISA (PBL Interferon Source). A control group injected with pRTS-mIFNα, received normal feed without AL (excipient). IFNα plasma levels in this control group were below the level of detection of the assay (LOD: 12.5 pg/mL) [Data not shown]. This study was terminated 110 days post IM electroporation. In a repeat of this study, removal of AL on Day 95 led to a return in serum expression to basal levels, and re-induction with AL on Day 109 led to a detectable increase in IFNα plasma levels (data not shown). n = 5 mice/group, mean ± s.e.m.

Summary: The RTS platform allows for the in vivo regulation of gene expression. No systemic expression is detected in pRTS-mIFNα injected animals in the absence of INXN-1001. In contrast, intramuscular delivery of pRTS-mIFNα, and induction with INXN-1001 results in robust and sustained plasma expression of IFNα.

Antitumor Activity of pRTS-mIFNα in the B16F0 Murine Melanoma

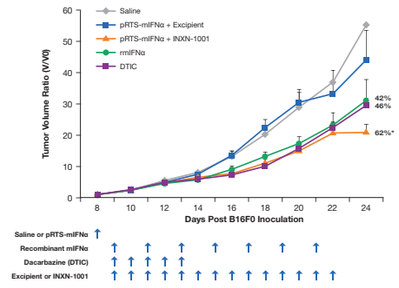

Figure 3a: C57/BL6 mice were injected subcutaneous with 1x10[5] B16F0 cells. On Day 8 post B16F0 inoculation, when tumors reached ~80 - 100 mm[3], all cohorts received either pRTS-mIFNα (250 μg in 40 uL) or saline (40 uL) by intramuscular (IM) electroporation. Activator ligand (AL) or excipient was administered daily by oral gavage to all groups, starting on Day 9 post cell inoculation. The mIFNα + INXN-1001 (orange line), saline (blue line), rmIFNα (grey line), and DTIC (purple line) cohorts received AL at a dose of 50 mg/Kg/day. The mIFNα – INXN-1001 control group (black line) received excipient only. Recombinant mIFNα (50,000 iu sc qod) and DTIC (60 mg/Kg ip qx5) treatments were initiated on Day 9. The data is plotted as a ratio of tumor volume on the indicated day (V) relative to tumor volume on Day 8. Percent tumor growth inhibition on Day 24 for rmIFNα, DTIC, and pRTS-mIFNα + INXN-1001 cohorts, relative to saline control, are indicated. (*p = 0.05, mIFNα + INXN-1001 vs. saline).

Generation of Systemic and Intratumoral mIFNα Following IM Administration with pRTS-mIFNα

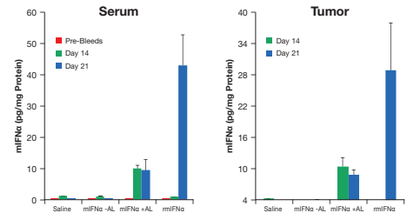

Figure 3b: Serum and tumor samples were collected 14 (D14) and 21 (D21) days post B16F0 inoculation. Since rmIFNα protein was administered every other day, tissue and serum collection on D21 but not D14 coincided with the day of rmIFNα treatment. Mouse IFNα levels were assessed using a MSD assay (Meso Scale Discovery). n = 7, mean ± s.e.m.

Summary: Transcription activation following the single IM administration of pRTS-mIFNα shows significant antitumor efficacy which is comparable to repeated bolus rmIFNα protein injection or treatment with the alkylating chemotherapeutic agent, decarbazine. In the absence of the transcription activator, INXN-1001, no antitumor activity is observed. Transcription activation of pRTS-mIFNα results in sustained, continuous levels of IFNα, in contrast to the excursion levels of mIFNα observed with bolus recombinant mIFNα treatment.

Production of Systemic and Intratumoral IP-10 Following IM Administration with pRTS-mIFNα

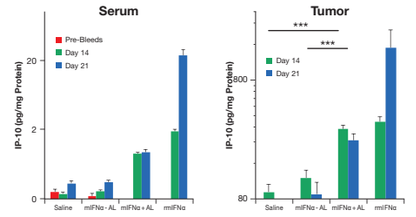

Figure 4: Serum and tumor samples were collected 14 and 21 days post B16F0 inoculation, corresponding to 5 and 12 days post treatment initiation. Tissue and serum collection on D21 but not D14 coincided with the day of rmIFNα treatment. Mouse IP-10 levels were assessed by ELISA. ** p < 0.05, *** p < 0.001, n = 7, mean ± s.e.m.

Summary: Treatment with pRTS-mIFNα and daily INXN-1001 results in sustained high levels of IP-10 in the serum and tumor, unlike the intermittent levels seen with every other day recombinant mIFNα treatment. In the absence of INXN-1001, IP-10 levels are unchanged relative to untreated controls.

pRTS-IFNα + INXN-1001 Treatment Increases CD8+ Effector Cell Population

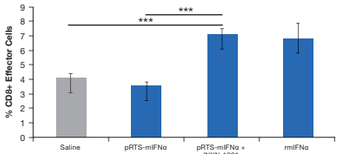

Figure 5: Single cell suspensions from mouse spleens, collected 14 days after B16F0 inoculation, were analyzed by flow cytometry using markers for CD4, CD8, NK, macrophages, and dendritic cells. pRTS-mIFNα treatment, in the presence of INXN-1001, resulted in a significant increase in CD8+ effector cells (CD44 high, CD62L low). As expected based on previous published reports, pRTS-mIFNα treatment also enhanced activation of CD4 T cells, CD8 T cells, and NK cells, and increased maturation of macrophages and dendritic cells, but only in the presence of INXN-1001 (not shown). *** p < 0.001. n = 7, mean ± s.e.m.

Summary: pRTS -IFNα systemic expression, when induced with INXN-1001, results in robust immune cell activation, comparable to repeated bolus recombinant IFNα treatment.

Vector Optimization Increases *In Vitro* Expression Of Human IFNα

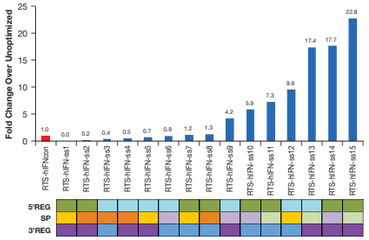

Figure 6: A matrix of RTS platform-regulated human IFNα expression vectors, with modifications in the non-coding regulatory elements (5'REG, 3'REG) and secretion signal peptides (SP), was generated to optimize human IFNα expression. The RTS-hIFNα plasmids were screened by transient transfection in HT1080 cells, and IFNα expression was induced by treatment with 100 nM of activator ligand. Supernatants were harvested 48 hours after transfection, and screened for human IFNα expression by ELISA. Expression levels are depicted as fold change over unoptimized pRTS-human IFNα (red bar). n = 3, mean ± s.e.m.

Summary : Vector optimization, enabled by Intrexon's UltraVector platform, can increase human IFNα expression by up to 23 fold over wild-type vector, affording a significant benefit for translation research.

Conclusions

- Using the Rheoswitch Therapeutic System inducible expression platform and the Ultravector platform a DNA vector encoding a regulated, secretory optimized, IFNα can be created
- The single IM electroporation of pRTS-IFNα combined with daily oral activator ligand treatment led to significant tumor growth inhibition, comparable to chemotherapy or repeated bolus injection with recombinant mIFNα protein
- No toxicity, as assessed by survival and changes in body weight, was observed in pRTS-IFNα treated groups
- Treatment with pRTS-IFNα resulted in sustained serum and tumor expression of mouse IFNα, the angiogenic biomarker IP-10, and activation of T cells (CD4 and CD8), NK cells, and dendritic cells
- Overall, our approach offers the potential advantage of a single administration of any optimized RTS-inducible transgene, in combination with oral AL, for sustained therapeutic anti-tumor efficacy and increased therapeutic window