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Intratumoral-Regulated Expression of IL-12 as a Potential Immunotherapeutic Strategy for the Treatment of Breast Cancer

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Abstract

A major obstacle for the development of immunotherapeutics is the ability of tumors to escape the immune system coupled with toxicity associated with systemic administration of cytokines. To overcome these challenges we have developed an adenoviral vector with regulated expression of IL-12, Ad-RTS-mIL-12 (Ad), administered intratumorally under the control of the RheoSwitch Therapeutic System® (RTS®) expression platform. Previously we have shown that a single intratumoral injection of Ad combined with oral administration of the activator ligand, INXN-1001 (AL), led to significant inhibition of tumor growth and increased survival. Herein, we show that localized delivery of IL-12 is well tolerated and its mechanism of action is through an increase in tumor infiltrating leukocytes concomitant with a reduction in tumor growth. *In vitro* studies using HT1080 human fibrosarcoma cells transduced with Ad and incubated with AL have shown a concentration-related increase in IL-12 mRNA concomitant with an increase in expression of IL-12 protein. Removal of AL from the media resulted in return of IL-12 expression to baseline within 48 hours. In the 4T1 BALB/c mouse model, oral administration of AL elicited a dose- related increase in plasma AL levels which correlated with increasing tumor levels of AL. The increase in tumoral AL in turn activated the RheoSwitch® portion of the adenoviral vector leading to a dose-related increase in tumor IL-12 production with virtually no increase in systemic IL-12. The maximal tumoral IL-12 protein level of ~280 pg/mg protein was achieved on Day 4 of AL dosing with a concomitant serum IL-12 level of 0.5 pg/mg protein following a single intratumoral injection of Ad-RTS-mIL-12 ($1x10^{10}$ vp) on Day 1 combined with once daily oral administration of AL at 150 mg/m^2 on Days 1-7. This increase in tumoral IL-12 levels correlated with a dose-related increase in tumor-infiltrating CD4$^+$ and CD8$^+$ lymphocytes in and adjacent to the tumor, coupled with an increase in apoptotic marker caspase 3 in the tumor when compared with vehicle. No increase in IL-12 protein expression in mice treated with Ad or AL alone was observed. Moreover, this therapeutic strategy appears to be well-tolerated as no change in clinical signs or body weight was observed in the treated animals when compared with vehicle alone.

These results support our hypothesis that localized delivery of IL-12 is well tolerated and results in an increase in tumor infiltrating leukocytes concomitant with a reduction in tumor growth. These findings suggest the applicability of our immunotherapeutic strategy for the treatment of metastatic breast cancer.

Hypothesis

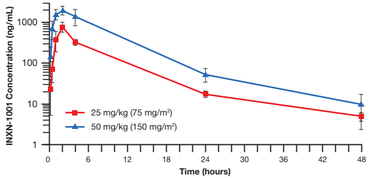
The combination of intratumoral (IT) delivery of Ad-RTS-mIL-12 + oral INXN-1001 generates a localized immune response which requires the careful coordination of a number of signals. An intricate system of checks and balances has evolved to ensure the appropriate response to the localized cytokine stimuli. The activation of naïve T cells in response to a specific antigen or cytokine requires multiple signals including tumor cells' expression of tumor associated antigens, the localized production of IL-12 which results in systemic stimulation of the host immune system, resulting in the influx of cytotoxic CD8$^+$ T cells coupled with a reduction in CD4$^+$ regulatory T cells. The end result is targeted tumor cytotoxicity as well as the induction of T cell memory resulting in systemic immune modulation.

Inducible Gene Regulation—RheoSwitch Therapeutic System

RheoSwitch Therapeutic System (RTS) is a Three-Component Transcriptional Regulator

1. **The Switch Components:** The RTS gene program includes two receptor protein fusions: VP16-RXR and Gal4-EcR. They form unstable and unproductive heterodimers in the absence of any ligand.
2. **The Inducible Promoter:** A customizable promoter to which basal transcription proteins are recruited and the target gene is transcribed.
3. **The Activator Ligand:** An ecdysone analog, diacylhydrazine-based small molecule functions as an activator. In the presence of the ligand, the protein heterodimer changes to a stable conformation and binds to the inducible promoter.

Dose-Dependent Increase in Plasma Exposure of the Oral Activator Ligand INXN-1001 in Mice



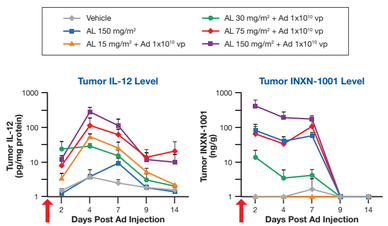
Plasma concentration vs. time profiles of INXN-1001 in BALB/c mice after a single dose administration of INXN-1001 via oral gavage. Depicted are the mean (± SD) concentration vs. time profiles for two dose groups of 3 animals/sex/time point/group at dose levels of 25 and 50 mg/kg (75 and 150 mg/m^2).

PK Parameters of INXN-1001 In BALB/C Mice After a Single Dose Administration of INXN-1001 via Oral Gavage

Dose (mg/m^2)	75	150
T_{max} (h)	2	2
C_{max} (ng/mL)	751	1898
AUC_{0-48h} (ng-h/mL)	5449	20656
$AUC_{0-\infty}$ (ng-h/mL)	5501	20745
Terminal $t_{1/2}$ (h)	7.4	6.1

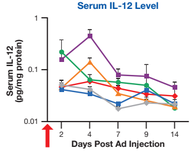
Note: PK parameters were determined based on mean concentration-time profile for each dose group (n=3/sex/time point/group)

Dose-Dependent Increase in Expression of Tumor IL-12 in Response to Activator Ligand (AL) INXN-1001



- Tumor IL-12 (p70) level with AL alone (150 mg/m^2) is baseline IL-12 level since no vector was administered and tumor was not manipulated
- The difference in tumor AL levels at 150 mg/m^2 alone vs. AL + vector is likely related to localized immune response associated with vector administration

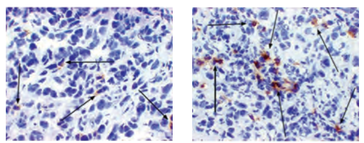
Low Levels of IL-12 in the Serum



- Minimal serum IL-12 (p70) level at all doses
- No detectable serum AL level at INXN-1001 doses <75 mg/m^2 (~24 hours post prior dose)
- Note Y-axis changed for serum IL-12 levels

120 BALB/c mice were subcutaneously inoculated with $1x10^6$ 4T1 tumor cells. When tumors reached ~100-200 mm^3, animals were randomly assigned to one of the treatment groups (N=20 each): vehicle control, INXN-1001 (AL) alone 150 mg/m^2, and Ad-RTS-mIL-12 ($1x10^{10}$ vp) in combination with INXN-1001 (15, 30, 75 or 150 mg/m^2). INXN-1001 dosing began on Day 1 and continued through Day 7. At ~2 hr after INXN-1001 dosing on Day 1, a single dose of Ad-RTS-mIL-12 $1x10^{10}$ vector particles was administered into the tumor in a constant volume of 100 µL. On Days 2, 4, 7, 9 and 14, four animals from each group were weighed, euthanized, tumor size recorded, tumor excised and assessed for INXN-1001, tumor immunohistochemistry and tumor and serum IL-12 via ELISA.

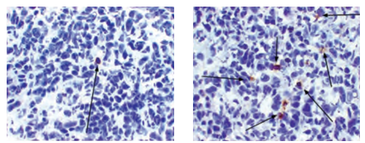
Ad-RTS-mIL-12 + INXN-1001 Increases CD8$^+$ TILs in the 4T1 Syngeneic Mouse Model



Group 1 Animal 1 stained with rat anti-mouse CD8 at 5 ug/mL. Note plasma membrane staining of very rare mononuclear cells (arrow) within the tumor. 40x

Group 6 Animal 11 stained with rat anti-mouse CD8 at 5 ug/mL. Note plasma membrane staining of very rare mononuclear cells (arrow) within the tumor. 40x

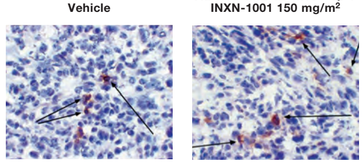
Ad-RTS-mIL-12 + INXN-1001 Increases CD4$^+$ TILs in the 4T1 Syngeneic Mouse Model



Group1 Animal 1 stained with rat anti-mouse CD4 at 5 ug/mL. Note plasma membrane staining of very rare mononuclear cells (arrow) within the tumor. 40x

Group 6 Animal 11 stained with rat anti-mouse CD4 at 5 ug/mL. Note plasma membrane staining of very rare mononuclear cells (arrow) within the tumor. 40x

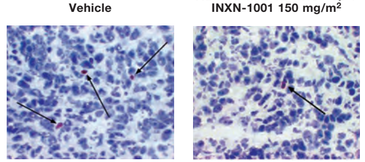
Ad-RTS-mIL-12 + INXN-1001 Does Not Effect CD25$^+$ TILs in the 4T1 Syngeneic Mouse Model



Group 1 Animal 1 stained with Bio-RtaMsCD25 at 1 µg/mL. Note plasma membrane staining of rare mononuclear leucocytes (arrows) within the tumor. 40X

Group 6 Animal 11 stained with Bio-RtaMsCD25 at 1 µg/mL. Note plasma membrane staining of rare to occasional mononuclear leucocytes (arrows) within the tumor. 40X

Ad-RTS-mIL-12 + INXN-1001 Decreases CD4$^+$ FoxP3$^+$ TILs in the 4T1 Syngeneic Mouse Model

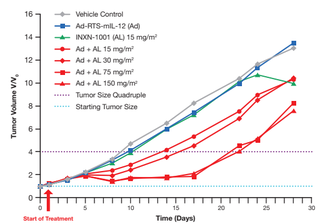


Group 1 Animal 1 stained with Bio-RtaMsFoxP3 at 20 µg/mL. Note nuclear staining of rare mononuclear leucocytes (arrows) within the tumor. 40X

Group 6 Animal 11 stained with Bio-RtaMsFoxP3 at 20 µg/mL. Note nuclear staining of very rare mononuclear leucocytes (arrow) within the tumor. 40X

120 BALB/c mice were subcutaneously inoculated with $1x10^6$ 4T1 tumor cells. When tumors reached ~100-200 mm^3, animals were randomly assigned to one of the treatment groups (N=20 each): vehicle control, INXN-1001 alone 150 mg/m^2, and Ad-RTS-mIL-12 ($1x10^{10}$ vp) in combination with INXN-1001 (15, 30, 75 or 150 mg/m^2). INXN-1001 dosing at the assigned dose began on Day 1 and continued through Day 7. ~2 hr after INXN-1001 dosing on Day 1, a single dose of $1x10^{10}$ vector particles Ad-RTS-mIL-12 was administered into the tumor in a constant volume of 100 µl. Illustrated are representative slides of selected tumor infiltrating T cells on Day 7. Depicted on the left are the effects of vehicle and on the right for mice treated with Ad-RTS-mIL-12 $1x10^{10}$ vp + INXN-1001 150 mg/m^2. Note increase in CD8$^+$ and CD4$^+$ TILs coupled with a decrease in CD4$^+$ FoxP3$^+$ TILs in the treated group.

Dose-dependent Anti-Tumor Activity of Ad-RTS-mIL-12 + INXN-1001 in Murine 4T1 Model



The effect of Ad-RTS-mIL-12 ($1x10^{10}$ vp) + INXN-1001 on tumor growth rate in the murine 4T1 syngeneic breast carcinoma model. When tumor volumes reached 100-200 mm^3, mice were randomized into 7 treatment groups (14 animals/group): vehicle control, Ad-RTS-mIL-12 alone ($1x10^{10}$ vp), INXN-1001 alone (15 mg/m^2), and Ad-RTS-mIL-12 ($1x10^{10}$ vp) in combination with INXN-1001 (15, 30, 75 or 150 mg/m^2). Ad-RTS-mIL-12 was administered as a single intratumoral injection on Day 1 while INXN-1001 was administered via oral gavage once daily on Days 1-5. Depicted are mean change in tumor volume relative to the initial tumor volume V_0 on Day 0 for each of the treatment groups.

Summary

- Orally administered INXN-1001 resulted in a dose-related increase in tumor INXN-1001 levels
- The increase in tumor INXN-1001 levels in combination with Ad-RTS-mIL-12 resulted in a dose-related increase in expression of IL-12p70 in the tumor with minimal increase in serum IL-12
- The increase in mIL-12 in the tumor resulted in an increase in tumor CD8$^+$ cytotoxic T cells concomitant with a decrease in tumor T_{regs}
- Ad-RTS-mIL-12 + INXN-1001 elicited dose-related decrease in tumor growth rate, with no significant change in body weight
- These findings suggest the applicability of our immunotherapeutic strategy for the treatment of breast cancer