IMMUNE RESPONSES TO MUTATED RAS - DEVELOPMENT OF A YEAST BASED IMMUNOTHERAPEUTIC

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Introduction

GI-4000 is a product series designed to elicit an immune response against cells with activating *ras* mutations using heat-killed *Saccharomyces cerevisiae* yeast (named Tarmogens: Targeted Molecular Immunogens) genetically engineered to express Ras G12 and Q61 mutations.

Tarmogens activate antigen-specific T cell-mediated immune responses that have been shown to kill target cells expressing a number of cancer antigens including mutated Ras. Activating mutations in *ras* occur in > 90% of pancreas cancer cases and ~25% cases of NSCLC.

GI-4000 has been evaluated in phase 2 trials in both these indications and has previously demonstrated:

i) Protection in a murine model of lung cancer[1].

ii) In the phase 2 trial in pancreas cancer, GI-4000-02, improvements in median recurrence free survival and overall survival (OS) vs. placebo in subjects with a favorable proteomic signature[2] (see Figure).

iii) Also in the pancreas trial, 3 month improvement in median OS (p=NS) in R1 subjects, with 5 month improvement for immune responders (see Figure).

iv) Generation of interferon-γ (IFNγ) T cell responses in R1 subjects receiving GI-4000: 7/15 (46.7%) GI-4000 subjects vs. 1/12 (8.3%) placebo subjects (p=0.043) had an IFNγ response to their G12 mutation.

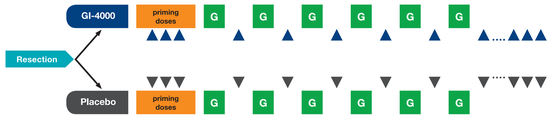
v) An indication of improved OS in subjects with NSCLC treated with GI-4000 compared to case-matched controls: HR=0.567, p=0.240.

In addition, Tarmogens specific for other oncologic targets have been shown to decrease the number and function of human regulatory T cells (Tregs) *in vitro* with a reciprocal increase in effector T cells[3]. The ability of Tarmogens to suppress Treg cells could be an important attribute for an immunotherapeutic in the treatment of cancer.

Here we present data showing that Tregs are reduced by GI-4000 administration in R0 subjects enrolled in the GI-4000-02 pancreas cancer trial and we also present data indicating that IFNγ T cell responses and OS are strikingly influenced by G12 mutation.

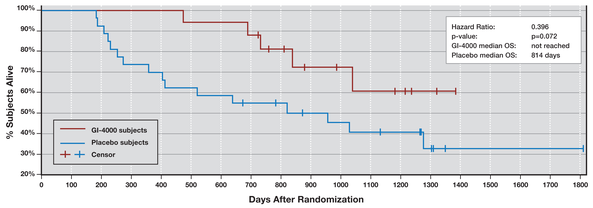
GI-4000 consists of four different heat-inactivated *S. cerevisiae* yeast GI-4014, GI-4015, GI-4016 and GI-4020 expressing seven common *ras* mutations in human cancers. Each of the four yeast expresses a fusion protein of three different *ras* mutations. Each protein product expressed in the yeast contains two mutations at codon 61 (glutamine to arginine [Q61R] or glutamine to histidine [Q61H], and glutamine to leucine [Q61L], plus one of four different mutations at codon 12 (either glycine to valine [G12V], glycine to cysteine [G12C], glycine to aspartate [G12D], or glycine to arginine [G12R]). Patient tumors are sequenced to identify the specific *ras* mutation contained in their tumor, and only the specific yeast with the matching mutation is administered to the patient.

GI-4000-02: Phase 2 study design

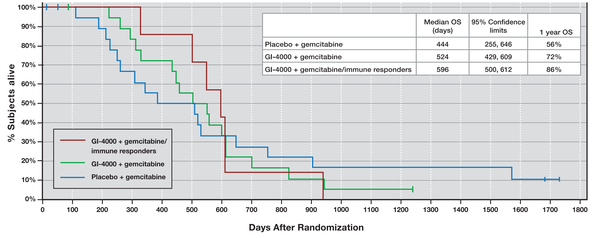


GI-4000-02 is a randomized, double-blind trial evaluating GI-4000 vs. placebo in combination with 6 cycles of adjuvant gemcitabine in subjects with resected pancreas cancer (R0 or R1). This study enrolled 176 subjects at 31 US centers and 8 international centers. Subjects received 3 priming doses of study drug or placebo prior to initiation of adjuvant gemcitabine therapy, followed by monthly doses of study drug or placebo in the 2 week holiday between cycles of gemcitabine. Study therapy is administered until disease recurrence or withdrawal, with recurrence-free survival (primary), overall survival and immune response as endpoints for the trial.

OS by treatment group for subjects with the late-recurrence proteomic signature



Overall Survival (R1 only; ITT)



	Median OS (days)	95% Confidence limits	1 year OS
Placebo + gemcitabine	444	255, 646	56%
GI-4000 + gemcitabine	524	429, 609	72%
GI-4000 + gemcitabine/immune responders	596	500, 612	86%

METHODS

IFNγ ELISpot assay: The production of IFNγ by T cells in response to mutated Ras peptides is a hallmark of antigen-specific $CD4^+$ and $CD8^+$ T cell activation to Ras mutations and was measured by ELISpot assay. Peripheral blood mononuclear cells (PBMCs) were collected pre-treatment and at various timepoints during treatment and were cryopreserved to enable longitudinal analysis. Thawed PBMCs were incubated with four pools of Ras peptides 10 and 15 residues in length that expressed the matched Ras mutation and with a control set of mismatched peptide pools, identical to the matched set except for the residue at Ras position 12. Single 9 mer peptides (Ras 6-14) were also used as stimulants[4].

Categorical ELISpot response criteria: at least one peptide pool or single peptide with an increase from baseline of >= 25 $IFNγ^+$ cells/10^6 PBMCs after subtraction of the control (mismatched peptide) response. Additionally the raw score for the specific peptides before subtraction of mismatch control must increase on treatment (increase of >= 25 $IFNγ^+$ cells/10^6 PBMCs).

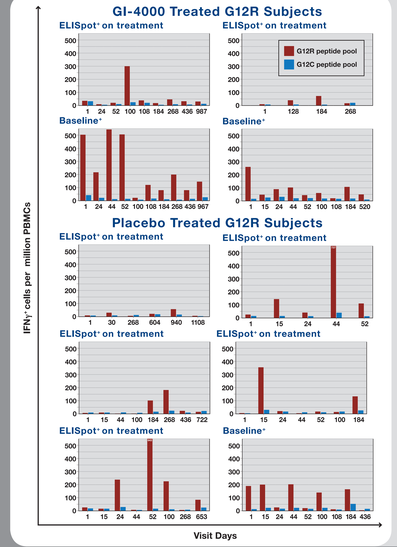
Baseline positive subjects: any G12 peptide pool or single peptide with a baseline response of >= 25 $IFNγ^+$ cells/10^6 PBMCs after subtraction of mismatched peptide response and a two-fold or greater increase in the response to that specific pool/peptide on treatment after subtraction of control, mismatched peptide response, PLUS a second product related peptide response on treatment of >=25 $IFNγ^+$ cells/10^6 PBMCs.

Regulatory T cell phenotyping: PBMCs from baseline and Visit Day 15 or 24 (pre-gemcitabine and one week following the second or third weekly immunization) were evaluated for frequencies of $CD4^+$, $CD14^-$, $CD45RA^+$, $Foxp3^+$ Tregs by flow cytometry using four color staining. Differential staining for these markers has previously been shown to divide Treg cells into three $CD4^+Foxp3^+$ fractions with distinct functionalities[5,6]:

Fraction I: $CD45RA^+$ $Foxp3^{low}$ = naïve Tregs
Fraction II: $CD45RA^-$ $Foxp3^{high}$ = activated Tregs
Fraction III: $CD45RA^-$ $Foxp3^{low}$ = non-Tregs, differentiation pathway skews to Th17 T cell phenotype

Response criteria: at least a two-fold change from baseline of the frequency of any Treg fraction. An increase or decrease in all $CD4^+/CD14^-$ T cells was defined as at least a 10% change from baseline.

GI-4000 Treated G12R Subjects



IFNγ ELISpot Response

1) For R1 subjects, GI-4000 treatment showed a greater rate of IFNγ responses to mutated Ras compared to placebo (see Introduction).

2) In contrast, in R0 subjects, GI-4000 treatment showed no increase in Ras mutation-specific IFNγ responses compared to placebo: 16/52 (30.8%) vs. 22/50 (44.0%) respectively.

3) R0 subjects had a two-fold higher frequency of pre-existing Ras mutation-specific IFNγ responses compared to R1 subjects: 22/102 (21.6%) R0 vs. 3/27 (11.1%) R1.

4) IFNγ ELISpot responses of R0 subjects were strikingly influenced by G12R mutation:

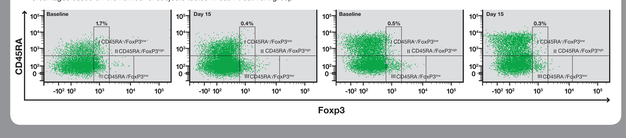
- Of 11 G12R subjects tested, irrespective of treatment arm, 7 (63.6%) had treatment-emergent G12R-specific responses and 3 (27.3%) had baseline responses that did not increase further on treatment and were thus deemed non-responders (Figures show responses of individual subjects).
- G12R-directed responses were highly focused on a single peptide pool, containing five peptides of 10 residues in length, suggesting a $CD8^+$-dominated T cell response (Figures show responses to this pool vs. G12C control pool).
- Subjects with G12V, G12C and G12D mutations had no focusing of the response to a single peptide pool and the frequency of immune responders was lower (33% across both treatment arms).

Regulatory T Cell Phenotyping

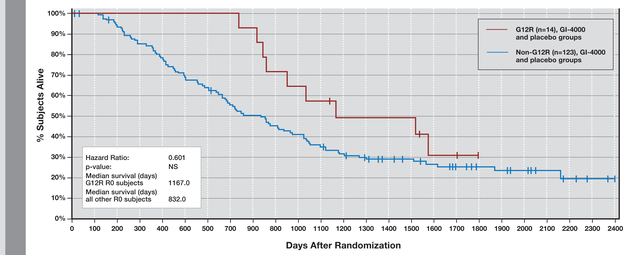
There was a three-fold greater frequency for a decrease in naïve Tregs (Fraction I) in the GI-4000 R0 treated group with 11/42 subjects (26.2%) showing a two-fold or greater decrease compared to 3/34 (8.8%) subjects in the placebo group (p=0.0476 Fisher's exact test) (see Figures below for examples). In contrast, Fraction II (data not shown) and Fraction III showed no difference between the two treatment arms. Changes in total $CD4^+$ T cells on treatment were similar between the arms.

	GI-4000 + gemcitabine	Placebo + gemcitabine	Total
Treg Fraction I, Categorical Change on Treatment			
Two-fold decrease	11 (26.2%*)	3 (8.8%)	14 (18.4%)
No change	23 (54.8%)	23 (67.6%)	46 (60.5%)
Two-fold increase	8 (19.0%)	8 (23.5%)	16 (21.1%)
Treg Fraction III Categorical Change on Treatment			
Two-fold decrease	8 (19.0%)	5 (14.7%)	13 (17.1%)
No change	30 (71.4%)	25 (73.5%)	55 (72.4%)
Two-fold increase	4 (9.5%)	4 (11.8%)	8 (10.5%)
$CD4+/CD14-$ T cells Categorical Change on Treatment			
10% or more decrease	12 (28.6%)	11 (32.4%)	23 (30.3%)
No change	19 (45.2%)	17 (50.0%)	36 (47.4%)
10% or more increase	11 (26.2%)	6 (17.6%)	17 (22.4%)

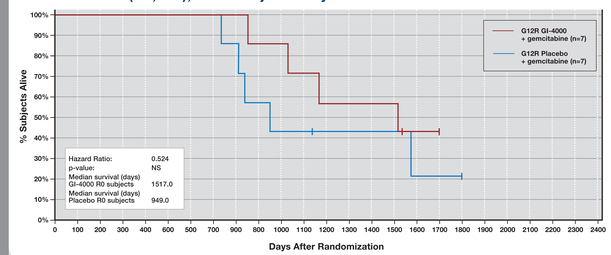
*Percentages based on the number of subjects tested in each treatment group



Overall Survival (R0; ITT), All Subjects



Overall Survival (R0; ITT), G12R Subjects Only



- For all G12R R0 subjects regardless of treatment arm, there was a pronounced improvement in overall median survival compared to all other mutations (335 days).
- Overall median survival was also greatly improved in GI-4000-treated G12R R0 subjects compared to placebo-treated G12R R0 subjects (568 days).
- Overall median survival was greatly increased for G12R vs. all other subjects for those subjects receiving GI-4000 (773 days, data not shown). For placebo subjects, there was only a 94 day improvement in overall median survival for G12R vs. non-G12R subjects (not shown).

These observations suggest that the presence of the Ras G12R mutation affords a survival advantage in pancreas cancer patients and that treatment with the GI-4000 can further improve survival for this subset of patients.

Conclusions

In the phase 2 trial of GI-4000 in R0 subjects with resected pancreas cancer:

- GI-4000 decreased frequencies of Tregs, which could be an important attribute for an immunotherapeutic in the treatment of cancer.
- Subjects with Ras G12R mutations had improved survival compared to all other mutations.
- Treatment of G12R subjects with GI-4000 improved survival compared to G12R subjects receiving placebo.
- The Ras G12R mutation was shown to be highly immunogenic compared to other G12 mutations (G12V, G12C and G12D).

References

1. Lu Y., et al. Cancer Research 64: 5084-5088, 2004.
2. Richards D.A., et al. ESMO 14th Congress GI Cancer, Barcelona, Spain, June 2012.
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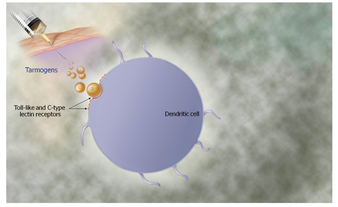
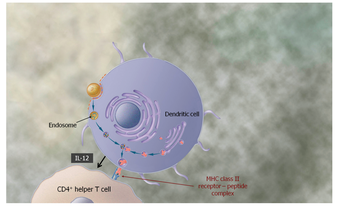
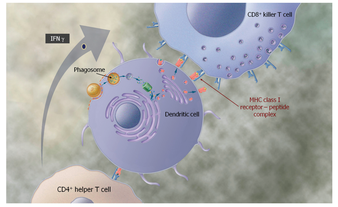
Active immunotherapy with yeast-based Tarmogens



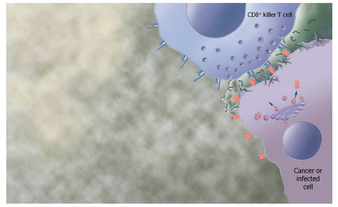
Administration of Tarmogens initially results in binding of the yeast to antigen-presenting cells, the most important of which are dendritic cells, near the injection site. The dendritic cells are activated as a result of the Tarmogens binding to Toll-like receptors and other receptor molecules on the surface of the dendritic cell, resulting in the activation of cytokine immune signaling molecules. The dendritic cell also engulfs the Tarmogen. Multiple Tarmogens may be taken up by the same dendritic cell.



The Tarmogen is processed by the dendritic cell in two ways. First, the Tarmogen is engulfed by endosomes and the protein inside the endosome is cut into shorter peptides fragments. These peptides are presented by Class II MHC molecules on the surface of the dendritic cell. In combination with IL-12, a cytokine that is produced by the dendritic cell, these MHC-peptide complexes on the surface of the dendritic cell are recognized by and activate cells involved in viral immunity called $CD4^+$ helper T cells.



Dendritic cells also process Tarmogens by engulfing them with phagosomes. This results in presentation of peptides, including the antigen from inside the Tarmogen, to $CD8^+$ killer T cells, via Class I MHC molecules on the surface of the dendritic cell, resulting in proliferation of identical antigen specific $CD8^+$ T cells. $CD4^+$ helper T cells are so named because one of their roles is to "help" activate killer T cells by expressing interferon gamma (IFNγ).



The newly activated $CD8^+$ killer T cells move throughout the body and identify any other cell that expresses the same disease protein as the one recognized by the $CD8^+$ killer T cells. Once the $CD8^+$ killer T cell finds another cell in the body containing the target protein, it can kill the cell using multiple mechanisms.

Immune Responses to Mutated Ras-Development of a Yeast Based Immunotherapeutic

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Background

While mutations in the *ras* gene and corresponding product are known to be etiologic in a number of human cancers, efforts to block the mutated protein have been largely unsuccessful leading to its characterization as "undruggable." We have taken an alternative approach involving generation of T cell responses to the mutated protein. GI-4000 is a series of proprietary immunotherapeutics designed to target cells with activating ras mutations using heat-killed *Saccharomyces cerevisiae* yeast (named Tarmogens: Targeted Molecular Immunogens) genetically engineered to express Ras G12 and Q61 mutations. Tarmogens activate antigen-specific T cell-mediated immune responses that kill target cells expressing a number of cancer antigens including mutated Ras. Activating mutations in ras occur in > 90% of pancreas cancer cases and >20% cases of NSCLC. GI-4000 has been evaluated in phase 2 trials in both these indications. GI-4000 has demonstrated: i) protection in a murine model of lung cancer (1), ii) in the pancreas trial, improvements in median RFS and OS vs placebo in subjects with a favorable proteomic signature (2), iii) also in the pancreas trial, 3 month improvement in median OS (p=NS) in R1 subjects, with 5 month improvement for immune responders (2), iv) an indication of improved OS in subjects with NSCLC treated with GI-4000 compared to case matched controls: HR=0.567, p=0.240. In addition, Tarmogens specific for other oncologic targets decreased human regulatory T cells (Tregs) *in vitro* with a reciprocal increase in effector T cells (3). Here we discuss immunologic outcomes in R0 subjects enrolled in the pancreas cancer trial.

Methods

In the pancreas cancer study 176 subjects with Ras mutant+ adenocarcinoma of the pancreas post resection were randomized 1:1 to GI-4000/gemcitabine or placebo/gemcitabine (stratified by resection status: R0/R1). Three weekly injections of GI-4000 or placebo were followed by 6 cycles of gemcitabine 1000 mg/m^2 iv (day 1, 8, 15, then every 28 days). Monthly GI-4000 or placebo were administered on gemcitabine off-weeks and continued monthly until disease recurrence, intolerable toxicity or death. R0 subjects (n=102) with adequate blood samples available from timepoints throughout the study were assayed for immune response by interferon-γ (IFNγ) ELISpot assay using Ras peptide pools containing the G12 mutation present in the subject's tumor and a mismatched peptide set identical to the mutation-specific set except at G12. Frequencies of Tregs at baseline and pre-gemcitabine were measured by flow cytometry (n=76).

Results

In contrast to the R1 group, there was no increase in Ras mutation-specific IFNγ responses in the R0 GI-4000 group compared to placebo: 16/52 (30.8%) vs 22/50 (44.0%) based on pre-specified criteria. However, naïve Tregs ($CD4^+/CD45RA^+/Foxp3^{low}$) in the GI-4000 treated group were significantly decreased compared to placebo: 11/42 (26.2%) vs 3/34 (8.8%) subjects had a >2-fold decrease in this fraction (p=0.048 Fisher's exact test). IFNγ responses and OS were strikingly influenced by G12 mutation and associations of specific Ras mutations with outcome will be discussed.

Conclusions

GI-4000 decreases frequencies of Tregs, which could be an important attribute for an immunotherapeutic in the treatment of cancer. GI-4000 also generates mutation-specific immune responses and appears to have clinical activity in pancreas cancer and NSCLC. Immune targeting of the activating mutation may be a promising approach to Ras mutated cancers.

References

1. Lu Y., et al. Cancer Research 64: 5084-5088, 2004.

2. Richards D.A., et al. ESMO 14th Congress GI Cancer, Barcelona, Spain, June 2012.

3. Cereda V., et al. Vaccine 29: 4992-4999, 2011.