EXPERT OPINION

GI-4000 in *KRAS* mutant cancers

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Introduction: Cancer develops mainly as a result of accumulating mutations in genes controlling cell growth regulation. *RAS* is one of the most commonly mutated genes in cancer. While agents targeting the signaling aspects of *RAS* have met with some success, resistance to therapy remains a major issue. Another focus of drug development has been to harness the immune system to target cells harboring mutated proteins, which can appear 'foreign' to the immune system. It has been observed that cancer is able to avoid regular immune surveillance through local and systemic mechanisms leading to immune tolerance. One potential way of breaking immune tolerance is through vaccine therapy.

Areas covered: The authors review the current but limited available literature on KRAS vaccine therapy. The research reviewed was identified from PubMed and presentations from national oncology meetings related to KRAS vaccines in general and GI-4000 series specifically.

Expert opinion: While targeting KRAS has proven difficulties, developing novel vaccine approaches such as 'tarmogens' appear to be safe with early efficacy in subset of patients with *KRAS* mutations. However, further research is crucial to identify this group of patients and develop biomarkers.

Keywords: cancer, immunotherapy, *KRAS* mutation, vaccine

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1. Introduction

Activating mutation in the *KRAS* oncogene is a common event in cancer. The incidence of *KRAS* mutation is > 20% across all tumor types [1] and > 90% in pancreatic cancer. Mutation of *KRAS* results in several malignant phenotypes, including deregulated tumor cellular growth, resistance to programmed cell death and invasion. *KRAS* mutation is an early event in the pathogenesis of pancreatic cancer [2]. In lung and colon cancers, it confers resistance to EGFR-targeted therapy [3]. Several attempts to target *KRAS* have not been fruitful [4]. For the purpose of this paper, we will review GI-4000 (**Box 1**) series of vaccines in *KRAS* mutant malignancies and the clinical studies conducted to date.

2. KRAS signaling

RAS signaling has been reviewed extensively [5]. Hereby, we briefly highlight the function and signaling of RAS protein family. The RAS proteins are members of a large family of low molecular weight GTP-binding proteins, which include KRAS, HRAS and NRAS. KRAS is essential for normal development of a mouse but not HRAS or NRAS based on the knockout model [6]. Several factors are important for RAS signaling. RAS requires post-translational modification, which localizes the RAS protein to its effective location in the cell. The ratio of GTP:GDP plays an important role in the RAS activation. EGFR and other receptor tyrosine kinases that are part of an upstream complex that activates RAS also play an important role in aberrant RAS signaling in many cancers. RAS also activates a variety of downstream pathways that are themselves oncogenic and also potential targets for

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Box 1. Drug summary.

Drug name	GI-4000
Phase	II
Indication	*KRAS* mutant cancers
Pharmacology description	Anticancer, vaccine
Route of administration	Injectable, subcutaneous
Pivotal trial(s)	[20,21]

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therapy including the RAF/MAPK pathway and the phosphatidylinositol 3-kinase pathway.

Different *KRAS* mutations have been reported to vary prognostically. Data from the CRYSTAL and OPUS studies (that evaluated patients with colon cancer who received chemotherapy with or without the EGFR-targeted agent cetuximab), were analyzed with regard to response rate, progression-free survival and overall survival (OS) based on presence of a *KRAS* p.G13D mutation versus other *KRAS* mutations. Patients with *KRAS* p.G13D mutation appeared to experience a benefit from cetuximab but not the group with other KRAS mutations [7]. This is consistent with findings from another study, which demonstrated that *KRAS* codon 12 mutations (in particular, c.35G>T), but not codon 13 mutations, are associated with inferior survival in *BRAF* wild-type colorectal cancer [8]. These studies highlight our relatively incomplete understanding of the structure–function relationships of mutated *KRAS*.

3. Targeting KRAS

Targeting KRAS has been explored at several levels and continues to be a major focus of drug development. Drugs have been developed to target upstream signaling, downstream signaling and KRAS itself. Perhaps, the first 'KRAS inhibitors' were the farnesyltransferase inhibitors (FTIs). A common mechanism of action for the FTIs is targeting the carboxy-terminal CAAX motif of RAS resulting in competition for binding to farnesyltransferase. Preclinical studies demonstrated very promising results with little toxicity [9]. Unfortunately, these findings did not translate into clinical benefit in human trials, and the actual mechanism of action of studied FTIs was not clear [10]. The lack of toxicity was attributed to the incomplete inhibition of relevant RAS proteins.

Targeting pathways upstream of RAS has primarily involved targeting EGFR and other ERBB family members, which are closely linked. The activation of the EGFR pathway through excess ligand, receptor overexpression or an activating mutation results in a large variety of potentially oncogenic intracellular activities leading to proliferation of cancer cells, induction of angiogenesis and metastasis [11]. Targeting EGFR has been successful in colon and lung cancers; however *KRAS* mutation is a biomarker predictive of lack of benefit to EGFR-targeted therapy in colon cancer [3].

Bypassing the *KRAS* mutation to target the downstream pathways (i.e., BRAF, MAPK kinase [MEK] and extracellular signal-regulated kinases [ERK]) has shown promising results in early phase clinical trials in some tumor types but not in colorectal cancer or pancreatic cancer to date [12,13]. Disappointing results in these tumor types mean the search for novel means of targeting oncogenic *RAS* mutations must continue.

4. Experience with KRAS vaccines

Given that mutated proteins in general can be interpreted by the immune system as 'foreign', a potentially novel way of attacking *RAS* mutated cancers is to design immunotherapy strategies that exploit the creation of potentially immunogenic peptides when *RAS* is mutated. One of the earlier such strategies was utilization of synthetic mutant *KRAS* peptides. A small study evaluated the immunogenicity and the safety of synthetic mutant KRAS peptide in five patients with pancreatic cancer [14]. Peripheral blood mononuclear cells were collected by leukapheresis and isolated by density centrifugation. The cells were loaded with a synthetic RAS peptide encompassing residues 5 – 21 of p21 RAS. After overnight incubation, the peptide-loaded cells were washed to remove the unbound peptide, diluted in normal saline and supplemented with albumin and heparin prior to infusion through a peripheral vein. All patients were vaccinated on day 0 and then on days 14 and 35 with a boost vaccine every 4 – 6 weeks. This intervention induced a specific T-cell reaction in two of the five participants. There was no therapeutic response noted (which could have been related to large volume disease), but importantly, no safety concerns were raised in this small study.

Another study evaluating a mutant KRAS-specific peptide vaccine [15] that was designed using the same methodology of the previously mentioned study [14] enrolled 48 patients with pancreatic cancer; 10 patients with resected disease and 38 patients with advanced stage. This study evaluated a different delivery model and schedule. Eligible patients received four vaccinations at weekly intervals and a booster at weeks 6 and 10. Weekly intradermal injection of a single mutant *RAS* peptide corresponding to the *KRAS* mutation identified in resectable patients or a mixture of four mutant *RAS* peptides corresponding to the most frequent *KRAS* mutation found in pancreatic cancer was delivered in 0.1 ml of saline. GM-CSF was administered as an adjuvant therapy with the vaccine. Objectives of this Phase I/II study were to determine the safety and toxicity of the KRAS vaccine in combination with GM-CSF in addition to response rate and outcome. Twenty-five of 43 (58%) evaluable patients demonstrated peptide-specific immunity. Patients with advanced disease who demonstrated an immune response experienced a longer

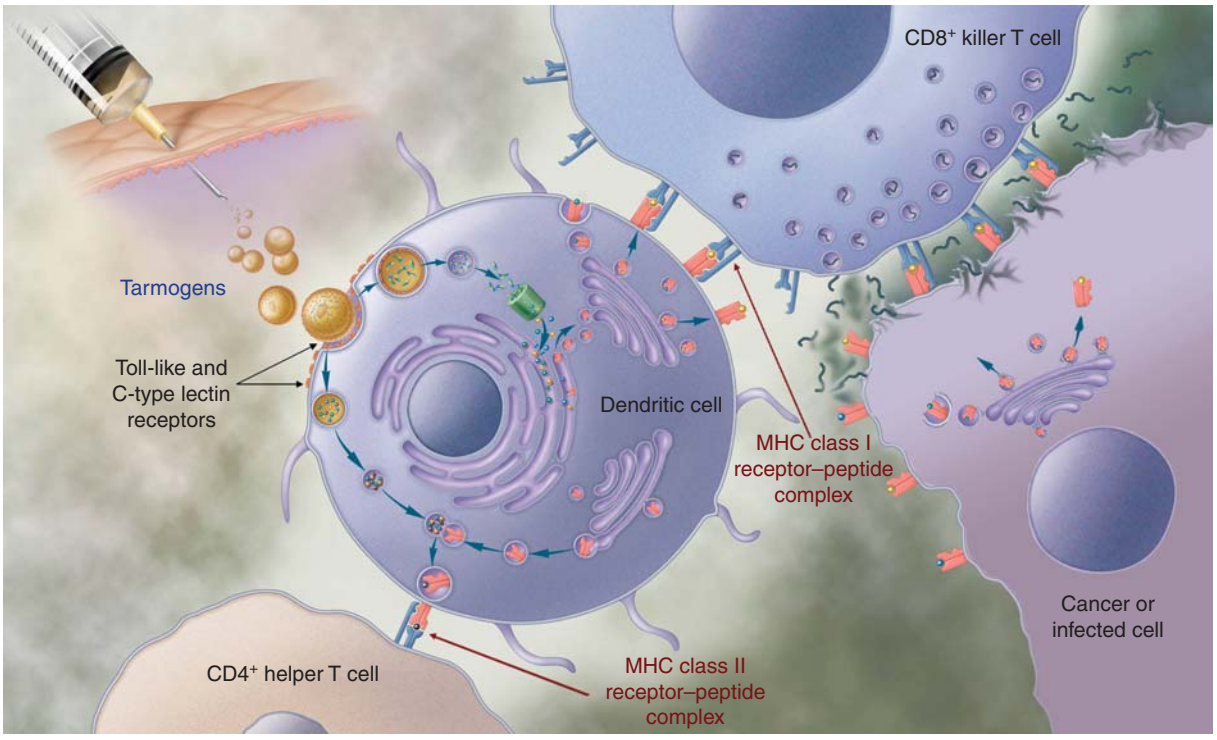

Figure 1. Tarmogens mechanism of action is shown. Yeasts are combined to a truncated RAS protein and avidly taken up by dendritic cells and macrophages. Dendritic cells mature and become activated after receptor-mediated yeast phagocytosis. The heterologous tumor antigens expressed in yeast are digested into peptides for presentation via class II and class I MHC receptors that trigger the activation of antigen-specific cytotoxic T lymphocytes and helper T cells.
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survival compared to non-responders (141 vs 61 days, p = 0.0002). In a follow up on 23 patients who were vaccinated in two Phase I/II studies against KRAS, a 10-year survival and immune response were evaluated [16]. After 10 years of follow up, a 9-year immune memory response and a 20% survival at 10 years were reported – very encouraging results for an early phase clinical trial.

Using a similar approach, 39 patients with various solid tumors harboring *KRAS* or *TP53* mutations were enrolled in a clinical trial [17]. Mononuclear cells were isolated from the peripheral blood, irradiated and loaded with a synthetic KRAS protein vaccine. The cells were subsequently infused intravenously into the patients, with repeat vaccination after 21 days and then every 2 months for four doses or more. Evidence of T-cell activation against *TP53* or *KRAS* was noted in 26% of patients. Median OS (mOS) of 393 versus 98 days for a positive versus negative T-cell activation (p = 0.04), respectively, and of 470 versus 88 days for a positive versus negative IFN response (p = 0.02), respectively, were detected.

Last, 24 patients with pancreatic cancer received an autologous KRAS vaccine. This vaccine was prepared on testing for codon 12 *KRAS* mutation in patients' tumors. Each patient was given a vaccine directed at the specific *KRAS* mutation present in their pancreatic cancer. GM-CSF was administered as an adjuvant immunotherapy [18]. Safety of this approach and its efficacy were the primary objectives of this study. Patients were treated with vaccine monthly up to 3 months and were permitted to receive neoadjuvant or adjuvant therapy for their disease. Only 25% of patients were evaluable for immunologic response, and one patient had an evidence of immunity. Median disease-free survival was 8.6 months (2.96 – 19.2) and mOS was 20.3 months (11.6 – 45.3). There was no evidence of meaningful immunogenicity in this study and no clear signal of efficacy.

5. GI-4000 and cancer

GI-4000 series tarmogens (**Figure 1**) are recombinant yeasts, *Saccharomyces cerevisiae*, each of which expresses a truncated and modified human RAS protein (representing different known mutant forms of RAS) that is recognized by dendritic cells and elicits cell-mediated immune response *in vivo* [19]. GI-4001 yeasts are engineered to express *KRAS* mutant epitopes. For example, a construct with the Q61R epitope was created by cloning the mutant gene from E9 mouse lung adenocarcinoma cell line. Total RNA was extracted from the E9 mouse lung adenocarcinoma cell line and was used for cDNA synthesis with a KRAS-specific reverse primer. An amplified DNA fragment encoding the KRAS Q61R protein was ligated to the episomal vector, pYEX-BX, and transfected

into W303α yeast. Other forms of this vaccine were developed to express different common *KRAS* mutations. GI-4014, GI-4015 and GI-4016 each express a truncated and modified human RAS protein containing one of the three most common mutations at codon 12 (p.G12V, p.G12C or p.G12D, respectively) and the two most common mutations at codon 61 (p.Q61R and p.Q61L).

GI-4000 was studied in a Phase I clinical trial [20] in patients with advanced colorectal, pancreatic and non-small-cell lung cancer. Patients received five subcutaneous weekly doses of the product corresponding to the patient's individual mutation. Patients were followed for safety, immunogenicity and tumor response. Nine had *RAS* mutations in their tumors, seven of which were contained in one of the three products. Six patients were treated. Of the three low-dose patients in whom cellular assay data are available, two have shown mutation-specific T-cell responses by proliferation and cytokine secretion assays. No treatment-related serious adverse events were noted and treatment-related adverse events were limited to mild fever and malaise. No radiographic responses were noted in the study but were not necessarily expected in a heavily pretreated Phase I population. Final reporting of this study is still awaited.

A bias has long existed that vaccine approaches should work best in 'low residual disease' situations. In keeping with this philosophy, GI-4000 was evaluated in a Phase II clinical trial as an adjuvant therapy for patients with resected pancreatic cancer. Patients were stratified based on their resection status (R0 vs R1) [21]. Patients received either GI-4000 in three weekly injections of 40 YU (Yeast Unit) in addition to standard dose gemcitabine of 1000 mg/m^2 i.v. on days 1, 8 and 15 of 28 days cycle or placebo in addition to the same dose and schedule of gemcitabine for 6 months. GI-4000 was continued on monthly basis for up to 5 years or disease recurrence, intolerance or death. A total of 176 patients were enrolled with a recurrence-free survival being the primary end point. The data from 39 patients with R1 resection was unblinded and reviewed (results in the entire population have not been published or reported to date). Patients who received GI-4000 in addition to gemcitabine demonstrated improvement in all parameters. The GI-4000 group had an 11.4 week advantage in mOS (524 vs 444 days), 16% advantage in 1-year survival (72 vs 56%) and a 4.6 week advantage in median relapse-free survival (287 vs 255 days). The GI-4000 group demonstrated a significantly higher rate of mutation-specific T-cell response to RAS with a more pronounced survival benefit in GI-4000 treated immune responders – 21.7 week advantage in median survival (596 vs 444 days) compared to placebo. No new or unexpected toxicities were observed. It is important to note that these findings were observed in an unplanned subset analysis. Although this is an interesting observation in patients with positive margins, it must also be considered hypothesis-generating.

Similarly, in another trial performed at a single institution, patients with p.G12C, p.G12D or p.G12V *KRAS* mutant stages I – III lung adenocarcinoma received GI-4000 as further adjuvant therapy after completing a standard curative-intent treatment [22]. GI-4000 was given for 3 weekly doses, 6 monthly doses and then every 3 months for up to 3 years. A total of 24 subjects were enrolled. Of the 17 patients, 8 (47%) patients developed an immune response to mutant RAS; 5 of the 9 (55%) patients developed a treatment emergent response and 3 of the 8 (37%) patients developed an improvement in a preexisting baseline response based on prespecified immunologic criteria. The investigators concluded that GI-4000 is immunogenic in targeting mutated *KRAS* as an adjuvant 'consolidation' therapy in patients with stages I – III lung adenocarcinomas harboring *KRAS* mutations. Data on relapse-free and OS are still pending from this study.

Finally, looking at a somewhat different strategy, GI-4000 is undergoing evaluation in patients with *KRAS*-mutant metastatic colon cancer. In this trial, patients with treatment-naïve metastatic colorectal cancer receive either FOLFOX (5-fluorouracil [5-FU], leucovorin and oxaliplatin) or FOLFIRI (5-FU, leucovorin and irinotecan) plus bevacizumab and GI-4000 [23]. GI-4000 is administered as three weekly injections and followed by eight cycles of bevacizumab + FOLFOX (or FOLFIRI) – on days 1 and 2 every 14 days. Subsequent doses of GI-4000 are administered on day 8 of each cycle. On completion of chemotherapy, GI-4000 is continued along with bevacizumab maintenance every 2 weeks for up to 5 years or until subjects experience intolerance, disease recurrence or death. This study will address in preliminary fashion the potential role of GI-4000 in combination with standard of care for the treatment of colon cancer. This study is still ongoing and these results are eagerly awaited.

6. Conclusion

GI-4000 series tarmogens represent a novel strategy for the delivery of immunogenic antigens to elicit an immune response. To date there is modest evidence of clinical activity, with several key trials ongoing. At present, however, limited information about its true efficacy exists. Importantly, there have not been any concerning safety findings in early phase clinical trials. Longer follow up on the adjuvant study of patients with pancreatic cancer is of particular interest, as is further study of potential predictive markers.

7. Expert opinion

Targeting KRAS via immunotherapy is certainly an intriguing idea, but to date, the number of clinical studies evaluating the technology remains small, and a wide range of outcomes and responses have been reported. This is likely related to the variability in methodology and settings these studies were conducted in. The advantage of peptide vaccines is the ease of manufacturing and administration; however, they are less immunogenic and might lead to immune tolerance as opposed to immunity [24], with complete failure of inducing

$CD8^+$ T cells memory [25]. Observations have been made that vaccines are more effective in the setting of minimal residual disease, explaining the focus of many trials on high-risk but clinically disease-free clinical situations. Additionally, vaccines require approximately 3 months to develop immune responses, so in theory combining vaccine therapy with cytotoxic chemotherapy might lead to a decrease in the degree of immune response. On the other hand, it has been observed experimentally that the timing and dosage of cytotoxic therapy may enhance the degree of immune response by sheering the physical burden of tumor cells, breaking down local and systemic immune tolerance [26]. KRAS seems to consistently elicit an immune reaction in a subset of patients, and therefore, identifying this group or developing a more immunogenic method of delivery might help induce a more robust immune response. It is important to evaluate if the immune response translates into a clinical benefit, and certainly, will be interesting to follow up on the results of GI-4000 studies' long-term outcome. The ideal vaccine will target several antigens that are crucial for the tumor progression. Adding checkpoint blockade inhibitors to the vaccine therapy might lead to activating cytotoxic T cells and augmenting further antitumor effect. Of course, diverse molecular factors influence clinical outcomes. In the case of RAS vaccines, diversity of mutations could well influence the degree of immune reactivity [27] and therefore the outcome. Last, a number of host and tumor factors can contribute to the mount of immune response in an individual patient for a specific tumor [28].

While targeting KRAS has proven difficult in the past two decades, it is still an appealing target. In fact the National Cancer Institute is developing a 'RAS project' to target RAS proteins due to its prevalence and overwhelming impact on several cancers, including one of the deadliest cancers, pancreatic cancer. Several attempts to bypass the KRAS oncogene by targeting downstream proteins such as MEK and ERK had shown some efficacy in early phase clinical trials in several malignancies, but not in some of the most common cancer types. GI-4000 represents a novel approach to induce an immune response against *KRAS* mutation using recombinant yeasts. Early phase clinical trials using GI-4000 series demonstrated acceptable toxicity profile and promising results that warrants further evaluation. With the positive results of the Phase II trial in patients with R1 resection, and awaiting further results from the entire population in this study, it will be crucial to evaluate this hypothesis in a larger randomized Phase II trial to see whether these results are reproducible and beneficial over standard of care. It is essential to continue the search for a biomarker to identify the group of patients who are likely to respond. Whereas 20% of patients with pancreatic cancer present with resectable disease at the time of diagnosis, up to 80% of them experience recurrence within the first 2 years of surgery. There will be a need for a better selection for surgical resection, and certainly, a more effective adjuvant therapy. Last, newer therapies aimed at inhibiting suppressor T cells have had very interesting outcomes in several diseases, with hints of response even in gastrointestinal cancers and lung cancers. Combinations of such 'non-specific' immunotherapy strategies with more specific strategies, such as RAS-targeted tarmogens, might be very interesting to study in the near future.

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Declaration of interest

The authors declare that they have no conflict of interest and have received no payment in the preparation of their manuscript.

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