A Phase I Study of the Natural Killer T-Cell Ligand α-Galactosylceramide (KRN7000) in Patients with Solid Tumors

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ABSTRACT

Purpose: α-galactosylceramide (KRN7000) is a glyco-sphingolipid that has been shown to inhibit tumor growth and to prolong survival in inoculated mice through activation of natural killer (NK) T cells. We performed a dose escalation study of KRN7000 in advanced cancer patients.

Experimental Design: Patients with solid tumors received i.v. KRN7000 ($50–4800$ μg/m^2) on days 1, 8, and 15 of a 4-weekly cycle. Patients were given 1 cycle and, in the absence of dose-limiting toxicity or progression, treatment was continued. Pharmacokinetics (PK) and immunomonitoring were performed in all patients.

Results: Twenty-four patients were entered into this study. No dose-limiting toxicity was observed over a wide range of doses ($50–4800$ μg/m^2). PK was linear in the dose range tested. Immunomonitoring demonstrated that NKT cells (CD3+Vα24+Vβ11+) typically disappeared from the blood within 24 h of KRN7000 injection. Additional biological effects included increased serum cytokine levels (tumor necrosis factor α and granulocyte macrophage colony-stimulating factor) in 5 of 24 patients and a transient decrease in peripheral blood NK cell numbers and cytotoxicity in 7 of 24 patients. Importantly, the observed biological effects depended on pretreatment NKT-cell numbers rather than on the dose of KRN7000. Pretreatment NKT-cell numbers were significantly lower in patients compared with healthy controls ($P = 0.0001$). No clinical responses were recorded and seven patients experienced stable disease for a median duration of 123 days.

Conclusion: i.v. KRN7000 is well tolerated in cancer patients over a wide range of doses. Biological effects were observed in several patients with relatively high pretreatment NKT-cell numbers. Other therapeutic strategies aiming at reconstitution of the deficient NKT-cell population in cancer patients may be warranted.

INTRODUCTION

α-GalCer[3] (KRN7000) is a synthetic glycolipid, originally extracted from the marine sponge *Agelas mauritianus*. KRN7000 consists of a galactose and a ceramide molecule linked in an α-configuration (Fig. 1; Ref. 1). On the basis of structure-activity relationship studies, KRN7000 was selected as the most potent agent of a series of compounds (2).

KRN7000, which is not directly cytotoxic for tumor cells, is presented by CD1d, a monomorphic, HLA class I-related, antigen-presenting molecule, to NKT cells (3, 4). NKT cells are evolutionary highly conserved lymphoid cells, characterized by the coexpression of NK receptors and an invariant T-cell receptor, and capable of rapidly producing large amounts of cytokines on triggering (5–8). KRN7000-activated NKT cells were reported to directly attack tumor cells (9), and to secrete large amounts of cytokines such as IFN-γ and IL-4, thereby activating other effector cells including NK cells, T cells, B cells, and macrophages (10–12). The administration of α-GalCer in animals also induced the production of other cytokines, such as IL-2, IL-3, IL-6, IL-12, GM-CSF, and stem cell factor, which may also reinforce the immune system (13).

In *in vivo* animal studies, KRN7000 showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma, and lymphoma) in hepatic and lung metastases models (14, 15). Repeated administration with intervals was superior to single injections. Interestingly, apart from microgranulomas in liver and spleen in the highest dose level, no side

[3] The abbreviations used are: α-GalCer, α-galactosylceramide; NK, natural killer; NKT, NK T (cell); TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; PK, pharmacokinetic(s); MTD, maximum tolerated dose; OBAD, optimal biologically active dose; ACTH, adrenocorticotropic hormone; PB, peripheral blood; PBMC, PB mononuclear cell; MLR, mixed lymphocyte reaction; HPS, human pooled serum; RPE, R-phyco erythrin.
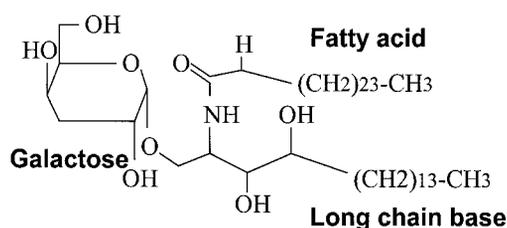
Fig. 1 Chemical formula of KRN7000, or (2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol. Molecular formula: $C_{50}H_{99}NO_9$.

Table 1 Dose escalation scheme and number of patients treated at each dose level

Dose level	Dose ($\mu g/m^2$)	No. of patients
1	50	6
2	150	3
3	300	3
4	600	3
5	1200	3
6	2400	3
7	4800	3

effects were reported in mice, rats, and monkeys with doses up to 2200 μg/kg, 440 μg/kg, and 660 μg/kg, respectively, given for 28 consecutive days.[4] The administration of α-GalCer (100 μg/kg) in mice resulted in a transient increase in alanine aminotransferase activity that was found to be mediated by activated NKT cells (16, 17). Antitumor activity has been observed in mouse models at doses ranging from 0.01 to 100 μg/kg (14, 15, 18).

We performed a Phase I study of KRN7000 given weekly i.v. for 3 consecutive weeks to patients with refractory solid tumors. The PK and biological effects of KRN7000 were monitored. The major end point of this study was to identify the MTD and the OBAD of KRN7000.

PATIENTS AND METHODS

Patients. Patients with a histological or cytological diagnosis of progressing solid tumor for which no standard therapy was available were eligible for this study. Patients had to be older than 17 years; have WHO performance status of 0, 1, or 2; life expectancy of >3 months; no chemotherapy or radiation therapy in the past 4 weeks; no prior immunotherapy in the past 3 months; normal bone marrow reserve; and normal organ functions. Patients were not to be pregnant, and patients with immunodeficiency or positive for HIV or HBsAg, or a history of autoimmune disease were excluded. Written informed consent was obtained from all of the patients.

On-Study Evaluations. History and physical examination were taken at study entry and before every cycle of treatment. Hematology, chemistries and urinalysis, electrocardiogram, and chest X-ray were also assessed before study entry. Proper diagnostic techniques were used to adequately assess the tumor extension. An ACTH test (synachten, 250 μg i.v.; blood sampling for serum cortisol were taken immediately before the injection and 30 min thereafter) was performed, and antithyroid antibodies were measured at study entry. Chemistries and full blood cell counts were repeated every week, and radiological assessment of tumor response was performed every two cycles.

Study Design. This was an open-label nonrandomized dose-escalation study. The aims of the study were to evaluate the safety profile of KRN7000, to determine the MTD; to study the biological and immunological changes induced by KRN7000, and to identify an OBAD. Furthermore, drug con-

centrations in blood and urine and possible antitumor activity were investigated over time. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria (CTC), and antitumor activity according to the WHO criteria. The MTD was defined as a dose associated with dose-limiting toxicity in at least two of six patients. Dose-limiting toxicity was defined as any adverse drug reaction (ADR) of ≥CTC grade 3, excluding nausea, vomiting, and grade 3 fever or any ADR that caused interruption of the weekly schedule administration. OBAD was to be defined on the basis of results obtained by immunological monitoring.

At least three patients were entered on each dose level, and increased to six if dose-limiting toxicities were encountered. Expansion of one or more cohorts was also planned in case of identification of biological activities.

Drug Formulation and Administration. The clinical dosage form of KRN7000 was supplied as vials containing lyophilized KRN7000 (0.2 mg), sucrose (56 mg), L-histidine (7.5 mg), and polysorbate 20 (5 mg) and was stored at 2°C to 8°C. Just before administration, KRN7000 was reconstituted with 1 ml of water. KRN7000 was administered by slow i.v. infusion on days 1, 8, and 15 of a 4-weekly cycle. Before drug administration, signs and symptoms of toxicity had to have disappeared. Doses administered and number of patients included at each dose level are summarized in Table 1.

PK. Serial serum sampling (2 ml PB collected in tubes containing clot activator) for PK of KRN7000 were performed before the first administration; then 15 and 30 min and 1, 2, 4, 6, 10, 24, 48, 96, and 168 h after the first administration; before the third administration; and 15 min and 1, 4, 10, 24, 96, and 168 h after the third administration.

Urines were collected in 24-h aliquots both before and after drug administration. All of the samples were stored at −20°C until analysis. KRN7000 concentrations in urine and serum were determined with ^{13}C-labeled KRN7000 as an internal standard using validated high-performance liquid chromatography tandem mass spectrometry. After thawing, urine, to which 10% Tween 20 was added, and serum samples were mixed with an internal standard in phosphate buffer with 1 M NaOH. Then KRN7000 and an internal standard in samples were extracted twice into ethylacetate. The organic phase was evaporated after phase separation. The residue was reconstituted with methanol, and the aliquot was injected into a liquid chromatography system with a tandem mass spectrometry, either a Quattro II (Micromass, Manchester, United Kingdom) or an API 365 (PE Sciex, Foster City, CA), with electrospray, by which product

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[4] G. Giaccone *et al.*, unpublished results.

ions, galactose of KRN7000 and ^{13}C-labeled KRN7000, from deprotonated ions were detected.

Immunomonitoring. To assess biological activities of KRN7000, extensive immunomonitoring was performed, including phenotyping of PBMCs, NK cell lytic activity, stimulation of the MLR, and serum cytokine levels. A total of 20 ml of heparinized PB were collected before the first administration (day 1), and on days 2, 3, 5, and 8, then before the third administration (day 15) and on days 16, 19, and 22, to perform cellular assays. Serum samples for cytokine evaluation were stored at -20° before, and 1, 2, 4, 6, 8, 10, and 24 h after, the first KRN7000 injection and before, and 2, 4, 10, and 24 h after, the third injection.

Preparation of PBMCs for Cryopreservation. PBMCs were isolated from heparinized diluted PB by Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. Cells were washed and resuspended at 4°C at 6×10^6 viable cells/ml in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 25% FCS and 12.5% DMSO. Cells were then cryopreserved and stored in liquid nitrogen until use, allowing for simultaneous testing of all of the PBMC samples from each individual patient.

PBMC Proliferation Assay. Thawed PBMCs were cultured in Iscove's modified Dulbecco's medium (BioWhittaker) containing 20% heat-inactivated HPS (CLB, Amsterdam, the Netherlands). Two hundred thousand PBMCs were cultured in the presence of 0, 1, 10, and 100 ng/ml KRN7000 (150 μl/well) for 7 days at 37°C with 5% CO_2 in humidified air. Then [3H]thymidine (0.4 μCi; Amersham Pharmacia Biotech, Roosendaal, the Netherlands) was added to each well, and cells were incubated for an additional 18 h. Cells were harvested (Skatron micro96 harvester; Skatron Instruments, Lier, Norway), and radioactivity was measured by a beta counter (Microbeta counter; Wallac Inc., Gaithersburg, MD).

Allogeneic MLR Assay. Thawed PBMCs of the patients were used as allogeneic stimulatory cells for healthy responder PBMCs in the MLR. For each patient, optimal responder PBMCs were first selected from a panel of three donors. Triplicate cultures were set up with 0.25×10^4, 1.0×10^4, and 4.0×10^4 irradiated (30 Gy) stimulator cells and 2×10^5 responder cells in Iscove's modified Dulbecco's medium containing 10% HPS in a 96-well round-bottomed plate (Corning Inc., Corning, NY). After 5 days of culture, lymphocyte proliferation was measured as [3H]thymidine uptake as described above.

Flowcytometric Cell Surface Analysis. Antibodies used in this study were: FITC-labeled anti-CD1c (Ancell, Bayport, MN); RPE-Cy5-labeled anti-CD3 (Dako, Carpinteria, CA); FITC-labeled anti-CD4 (Becton Dickinson & Co., Franklin Lakes, NJ); PE-labeled anti-CD8 (Becton Dickinson); RPE-Cy5-labeled anti-CD14 (Coulter, Fullerton, CA); PE-labeled anti-CD56 (Becton Dickinson); PE-labeled anti-CD86 (PharMingen); FITC-labeled anti-Vα24 (Immunotech, Marseille, France); PE-labeled anti-Vβ11 (Immunotech); and FITC-labeled anti-HLA-DR (Becton Dickinson). Isotype controls used: were FITC-labeled mIgG1 (Becton Dickinson); PE-labeled mIgG1 (Becton Dickinson); RPE-Cy5-labeled mIgG1 (DAKO); FITC-labeled mIgG2a (Becton Dickinson); and RPE-Cy5-labeled mIgG2a (Coulter). All of the samples from a pa-

tient were tested in the same run and one vial of a large batch of control cells was thawed and stained simultaneously in every experiment. Thawed cells were suspended in PBS and fixed with 1% paraformaldehyde. After washing, cells were resuspended in PBS containing, as blocking agent, 10% HPS, mIgG1 (1:10 dilution) and mIgG2a (1:10 dilution) for 30 min at room temperature. After washing, antibodies and isotype controls were added to the cell pellets. Cells were kept in darkness at 4°C until analysis. Analysis was done using a FACStar plus (Becton Dickinson).

NK Cell Cytotoxicity Assay. PBMCs were tested for NK cell cytotoxic activity in a ^{51}Cr release assay. After thawing, PBMCs (0.5×10^6 cells/ml in RPMI 1640 with 10% heat inactivated FCS were precultured overnight to recover. NK-sensitive target cells (K562) were labeled with 100 μCi $Na_2[^{51}Cr]O_4$ (Amersham Pharmacia Biotech) at 37°C for 1 h. ^{51}Cr-labeled K562 cells were washed three times and resuspended at 0.1×10^6 cells/ml in RPMI 1640 with 10% FCS. PBMCs and ^{51}Cr-labeled target cells (1×10^4 cells/well) were added to 96-wells round-bottomed plates at E:T ratios of 10:1, 20:1, and 40:1 (triplicate cultures). Additional wells were set up for the measurement of spontaneous and total [^{51}Cr] release by adding target cells to wells with 100 μl of medium or 1% Triton X-100, respectively. Plates were centrifuged ($150 \times g$) for 1 min and then incubated at 37°C with 5% CO_2 for 4 h. One hundred μl of supernatant were harvested from each well and transferred to a 96-well sample plate (Beta plate, Wallac Inc.), 100 μl of scintillation fluid were added, and plates were shaken for at least 15 min. Radioactivity was measured by a beta counter. The percentage of specific ^{51}Cr release was calculated as follows: [(test cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] $\times$ 100. A large batch of frozen healthy PBMCs were used in all of the experiments to evaluate interassay variation.

Serum Cytokine Measurement. All of the samples were kept at -80°C until testing. IL-1β, IL-2, IL-4, IL-10, IL-12, IFN-γ, GM-CSF, and TNF-α in serum were measured using ELISA kits according to the manufacturer's instructions. The IL-2 ELISA was purchased from Cell Sciences Inc. (Diaclone; Norwood, MA) and all of the other ELISAs (high sensitive kits except for IFN-γ) from R&D Systems Europe Ltd. (Abingdon, United Kingdom).

RESULTS

From October 1998 to February 2000 a total of 24 patients were recruited in the study from two institutions. Patient characteristics are reported in Table 2. On all dose levels, three patients were entered, with the exception of dose level 1, in which six patients were entered because distinct biological changes were seen in this first cohort of patients.

Toxicity. A total of 69 cycles were given (median, 2 per patient; range, 1–6). All of the side effects occurred after or during the first administration. One patient on dose level 1 developed grade 3 fever (temperature up to 40.1°C), associated with headache, vomiting, chills, and malaise, 2–3 h after KRN7000 administration, which abated after about 7 h with the use of paracetamol. Prophylactic paracetamol was used thereafter, and these side effects did not recur even when the patient

Table 2 Patient characteristics

Total number entered	24
Median age (range)	54 (31–69)
Gender (male/female)	14/10
Performance status (0/1/2)	5/16/3
Prior chemotherapy	22
Prior immunotherapy	6
Tumor types	
Melanoma, esophagus	3 each
Breast, thymus, head and neck, stomach, lung, kidney sarcoma	2 each
Bladder, pancreas, prostate, biliary tract	1 each

Fig. 2 NKT-cell numbers (absolute numbers) in PB of patients ($n = 21$) and healthy volunteers ($n = 37$; 17 males, 20 females; age range, 21–52 years). The number of NKT cells in cancer patients (median, 333 cells per ml of blood) was significantly lower than that of healthy volunteers (median, 1013 cells per ml of blood; $P = 0.0001$).

forgot to use paracetamol. One patient on dose level 6 experienced transient flush immediately after injection, which occurred after each administration. On dose level 7, one patient experienced sneezing during each drug administration. Importantly, although KRN7000 induces liver toxicity in mice (16, 17), no signs of KRN7000-induced liver toxicity were found in the current study.

In conclusion, no serious drug-related adverse event occurred during this study. The ACTH test was not negatively affected in the patients who were tested again after therapy discontinuation. Further dose escalation could not be performed using the current drug formulation; therefore, the MTD could not be identified.

Immunological Monitoring. Pretreatment, KRN7000-induced *in vitro* lymphocyte proliferation was observed in 4 of 24 patients (doubling of cpm counts induced by at least one dose of KRN7000): in 2 with high NKT cells at the start and 2 with low NKT cells (see NKT cells below). There were no consistent KRN7000-induced changes in the numbers of granulocytes, monocytes, B cells, and CD4+ or CD8+ T cells. Effects on the expression of HLA-DR, CD86, and CD1c on either CD14-positive or CD14-negative nonlymphocytic (based on light scatter) PBMCs were also inconsistent. Moreover, there were no KRN7000-related effects on the stimulatory capacity in MLR assays.

NKT Cells. NKT cells, expressing the invariant T-cell receptor consisting of a $V\alpha24$ chain paired with a $V\beta11$ chain (8), were measured in the PB of all of the patients except in two of the first- and one of the second-dose level. The number of circulating NKT cells in cancer patients was significantly lower than that of healthy volunteers (Fig. 2; $P = 0.0001$, Wilcoxon two-sample test). Using the median number of NKT cells in patients (333 cells/ml PB) as the cutoff value, we defined an NKT-high group and an NKT-low group (Fig. 2). The NKT cells in the PB in the NKT-high group decreased to undetectable levels within 24 h after the first administration of KRN7000 at all of the dose levels, and recovery to the preadministration levels was not observed within a week (Fig. 3).

Serum Cytokines. NK cells have been reported to play an essential role in the inhibition of tumor metastases in mice treated with KRN7000 (11, 14). Production of IFN-γ and IL-12, both of which can trigger NK cells, increased when KRN7000 was administered to mice (18). Here, only one patient (patient 3 on the first cohort), who had a relatively high pretreatment NKT cell count, showed a faint but detectable increase in serum IFN-γ at 6 h after the first KRN7000 administration (Fig. 4A),
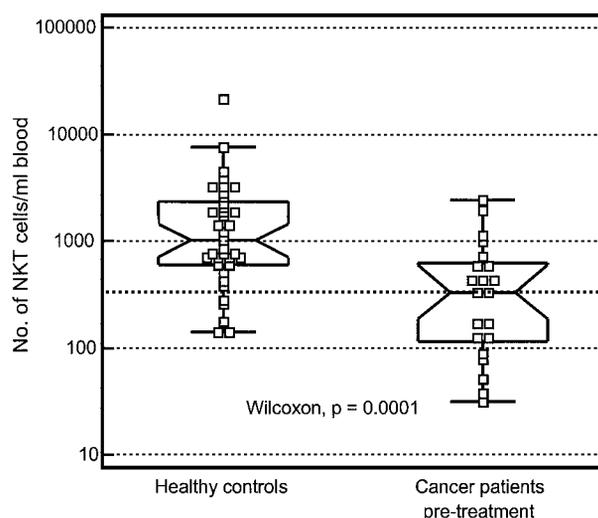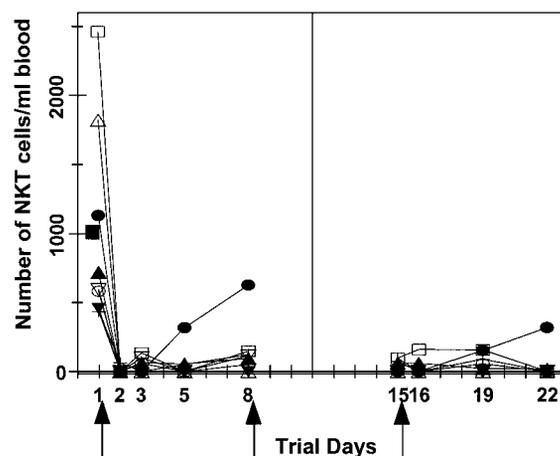

Fig. 3 Reduction of PB NKT-cell numbers (absolute numbers) on KRN7000 injection in patients with high (>333/ml) pretreatment NKT-cell numbers ($n = 10$). No follow-up data were available from Patient 3. PBMCs were collected on day 1 (before the first administration), on days 2, 3, 5, 8 (before the second administration), on days 15 (before the third administration), 16, 19, and 22. *Arrows*, the three first weekly injections. *Closed symbols*, the four patients with TNF-α and GM-CSF responses on KRN7000 (■, patient 3; ▲, patient 5; ●, patient 9; ▼, patient 17).

and an increase in serum IL-12 peaking 8 h after KRN7000 administration (Fig. 4B). Detectable levels of IFN-γ or IL-12 (>15 or 0.8 pg/ml, respectively) were never observed in the NKT-low group (data not shown).

Of the NKT-high group, 5 of 10 patients showed an increase in both GM-CSF and/or TNF-α levels, peaking 4–6 h after KRN7000 injection (Fig. 5, *A* and *C*). Of these, patient 3, who experienced severe fever after the first administration of KRN7000, besides showing both IL-12 and IFN-γ production,
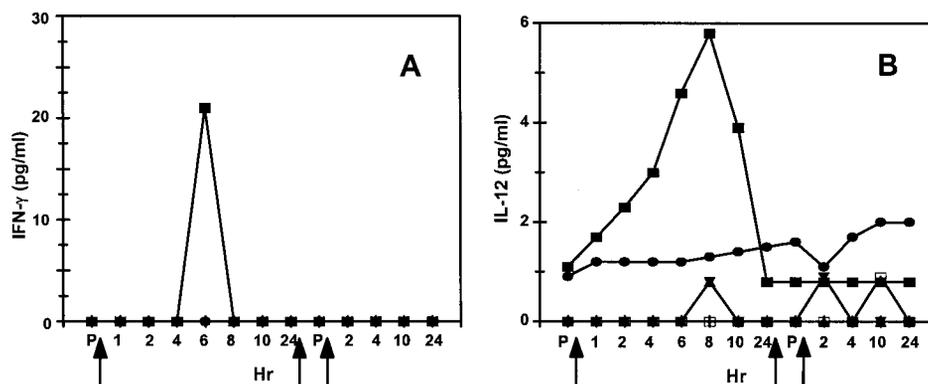
Fig. 4 Serum IFN-γ (*A*) and IL-12 (*B*) in patients with high pretreatment NKT-cell numbers (>333/ml; *n* = 10). Serum samples were collected before the first administration; at 1, 2, 4, 8, 10, and 24 h after the first administration; and on day 15 (before the third administration); and at 2, 4, 10, and 24 h after administration. Data represent the means of duplicate ELISA assays. Patient 3 showed an increase of both IFN-γ and IL-12 levels after the first administration. *Closed symbols*, the four patients with TNF-α and GM-CSF responses on KRN7000 (■, patient 3; ▲, patient 5; ●, patient 9; ▼, patient 17). *Arrows*, the three first weekly injections.
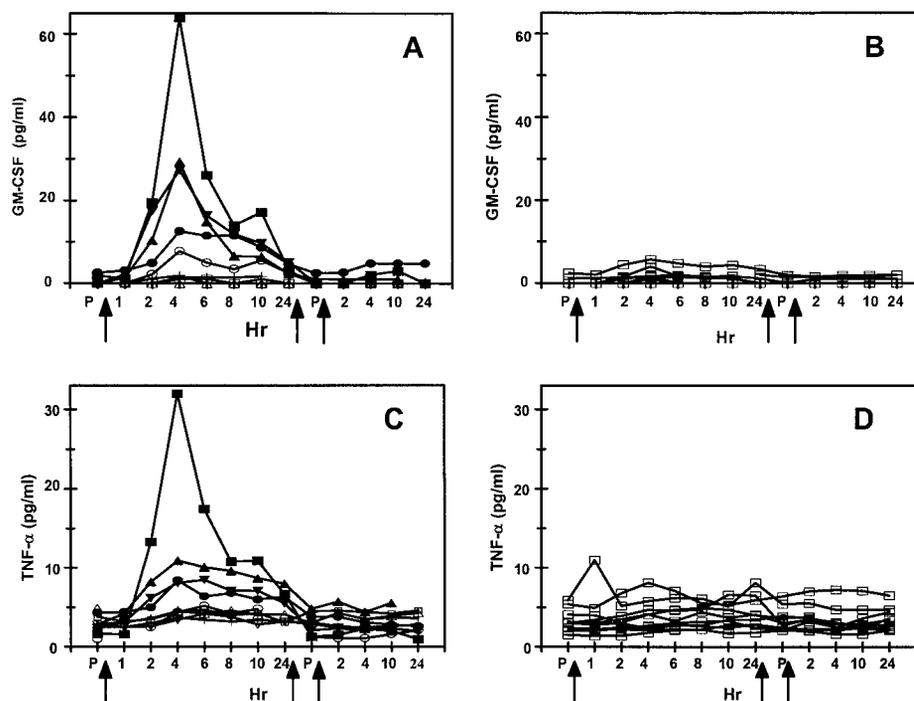

Fig. 5 Serum GM-CSF and TNF-α levels in patients with high (>333/ml, *n* = 10, *A* and *C*) and low (<333/ml, *n* = 11, *B* and *D*) pretreatment NKT-cell numbers. Serum samples were collected before the first administration and at 1, 2, 4, 8, 10, and 24 h after the first administration, and on day 15 (before the third administration), and at 2, 4, 10, and 24 h after administration. Data represent the means of duplicate ELISA assays. *Closed symbols*, the four patients with TNF-α and GM-CSF responses on KRN7000 (■, patient 3; ▲, patient 5; ●, patient 9; ▼, patient 17). *Arrows*, the three first weekly injections.

also developed the highest levels of GM-CSF and TNF-α. In contrast, in the NKT-low group, only slight fluctuations of GM-CSF and TNF-α serum levels were seen after KRN7000 administration (Fig. 5, *B* and *D*).

IL-1β, IL-6, and soluble IL-2R were detected in all of the patients tested but there were inconsistent responses to KRN7000 administration (data not shown). Only one patient produced IL-4 (detection limit, 0.25 pg/ml), but levels were not influenced by KRN7000 administration. No patient had detectable (>15 pg/ml) IL-2 levels.

NK Cells. The number of NK cells as well as the NK cytotoxicity of the NKT-high group are shown in Fig. 6. Seven of 10 patients with high pretreatment NKT cell numbers showed a transient decrease in NK cell numbers and NK cell cytolytic activity 24 h after KRN7000 treatment. No apparent response in NK cells was observed in patients in the NKT-low group (data not shown).

In conclusion, none of the immunological parameters tested showed clear KRN7000 dose-dependent effects; therefore, an OBAD could not be identified.
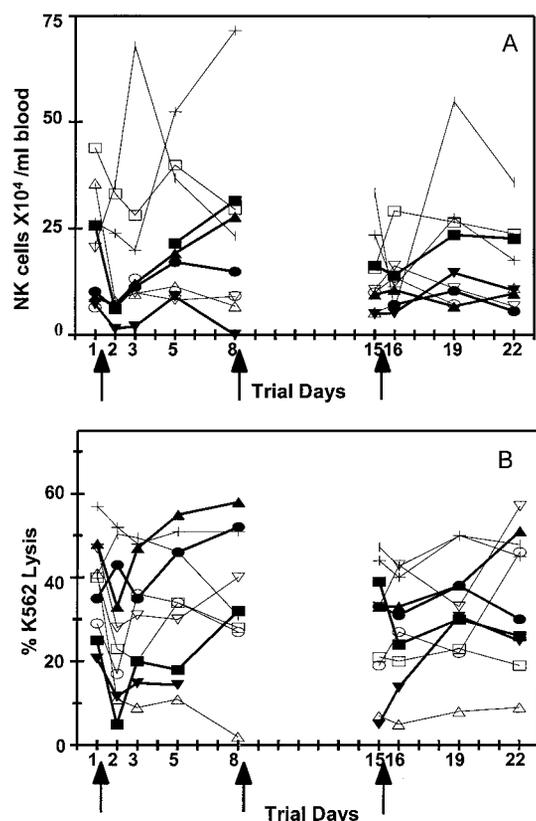
Fig. 6 NK-cell numbers (*A*) and NK lytic activities (*B*) in patients with high pretreatment NKT-cell numbers (>333/ml, *n* = 10). Analysis were performed using PBMCs collected before the first administration, and on days 2, 3, 5, 8 (before the second administration), 15 (before the third administration), 16, 19, and 22. *Closed symbols*, the four patients with TNF-α and GM-CSF responses on KRN7000 (■, patient 3; ▲, patient 5; ●, patient 9; ▼, patient 17). *Arrows*, the three first weekly injections.

PKs. Table 3 summarizes the major PK parameters. There was no indication of drug accumulation or saturation in the serum PK. Of note, plasma concentrations associated with activity in mice (18) were already achieved at dose level 3. There was a linear correlation between dose administered and area under the curve (AUC). Fig. 7 shows the serum PK at all of the dose levels. No apparent drug accumulation was observed after multiple dosing. KRN7000 was not detectable in urine at any dose level.

Antitumor Response. Two patients were not evaluable for response: one received only one cycle, and the general conditions deteriorated, preventing treatment continuation and tumor evaluation; in the other patient, the tumor could not be properly assessed. No partial or complete responses were observed; 7 stabilizations for a median of 123 days (range, 83–216 days) and 15 tumor progressions were recorded.

DISCUSSION

In this Phase I study, we attempted to define the MTD and OBAD of KRN7000, a synthetic α-GalCer, with potent tumor-inhibitory activity in metastatic tumor models in mice (1, 2, 14, 15, 18). In the dose range tested, spanning almost a 100-fold range in doses, we did not observe any dose-limiting toxicity. No accumu-

lation was observed with weekly administrations, and urine excretion of KRN7000 was essentially under the detection limit of our method. In only one patient, treated at the lowest-dose level of 50 μg/m^2, did we observe occurrence of high fever, with shivering, which was clearly drug related. In this patient, we also detected increased serum levels of IFN-γ, IL-12, GM-CSF, and TNF-α. Although cytokine production was infrequently observed in the study, this was limited to patients with relatively high circulating NKT-cell numbers before treatment.

NKT cells have been shown to play an important role in antitumor immune responses. A protective role of NKT cells in tumor-immunosurveillance was demonstrated (19), and NKT cells were reported to be essential for the antitumor effects of IL-12 (20). The antitumor effects of KRN7000 are crucially dependent on NKT cells, because no antitumor activity was observed in NKT cell-deficient mice (9). Although KRN7000-activated NKT cells are able to directly lyse a wide variety of tumor cell lines (9, 21, 22), they also enhance antitumor immune responses by rapidly releasing large amounts of cytokines that secondarily activate NK cells, T cells, B cells, dendritic cells, and monocytes (10–12, 14, 23). Clinical benefit for cancer patients was anticipated, because both human and mouse CD1d can present KRN7000 to NKT cells from either species, illustrating a strong conservation of recognition (5, 6).

An important finding of our study was the observation that the initial size of the circulating NKT-cell pool in cancer patients was significantly lower compared with that in healthy controls. This was previously observed in patients with melanoma and prostate cancer (21, 24), but our data suggest that the decrease is more widespread among cancer patients. Although the mechanism of this decrease has not been investigated, it could be caused by tumor-derived immunosuppressive factors or the intra- or peritumoral accumulation of NKT cells. Alternatively, the size of the NKT-cell population could have been reduced before tumor development, thereby acting as a risk factor. Another important finding was the observation that (residual) PB NKT cells rapidly disappeared from the circulation when KRN7000 was administered. Interestingly, a similar KRN7000-induced depletion of NKT cells from liver, spleen, and lymph nodes has been reported in mice and has been attributed to apoptosis after NKT-cell activation (and subsequent cytokine production; Refs. 11, 17, 25). The exact duration of the NKT-cell depletion after KRN7000 administration was not investigated in our study, but NKT-cell repopulation was not observed in the week after the injection of KRN7000. The 1-week intervals for this study were based on the best antitumor activity obtained by i.v. administration of KRN7000 on days 1, 5, and 9 in mice (15). However, weekly i.v. injections may well be too frequent because they may suppress NKT-cell repopulation. This is supported by our observation that individuals showing increased serum levels of GM-CSF and TNF-α in response to KRN7000 showed this response only after the first administration, when relatively high numbers of NKT cells were still present in the circulation. Interestingly, because human NKT cells were indeed shown to produce GM-CSF and TNF-α on activation *in vitro*, a role for KRN7000-activated NKT cells in the recruitment and maturation of dendritic cells has been inferred (26).

In mice, NKT cells were reported to rapidly activate NK cells on i.v. administration of KRN7000 (10). Other studies demonstrated that KRN7000 administration resulted in an increase in NK

Table 3 Serum pharmacokinetics

A three-compartment structural kinetic model with first-order elimination was used to model the serum-time profiles of KRN7000. Data represent mean ± SD. Intercompartmental clearance for the first (Q2) and second (Q3) peripheral compartment were 6.856 liters/h and 0.145 liter/h, respectively. Interpatient variabilities of Q2 and Q3 were not determined in this analysis.

Dose (µg/m²)	V1[a] (liter)	Cl (liter/h)	V2 (liter)	V3 (liter)	AUC (ng·h/ml)
50	1.88 ± 0.73	0.08 ± 0.03	1.47 ± 0.60	1.24 ± 0.48	1,517 ± 871
150	2.88 ± 1.08	0.14 ± 0.02	2.75 ± 0.38	2.24 ± 0.28	1,934 ± 163
300	1.96 ± 0.45	0.11 ± 0.01	2.02 ± 0.49	1.68 ± 0.38	5,144 ± 449
600	2.67 ± 0.76	0.23 ± 0.13	4.00 ± 2.13	3.13 ± 1.55	6,251 ± 3,940
1200	2.57 ± 0.70	0.14 ± 0.04	3.61 ± 0.67	2.87 ± 0.49	18,137 ± 6,704
2400	3.33 ± 0.91	0.20 ± 0.06	4.67 ± 2.37	3.61 ± 1.69	23,225 ± 6,700
4800	3.09 ± 1.77	0.18 ± 0.07	3.78 ± 2.35	2.96 ± 1.71	54,539 ± 22,020

[a] V1, volume of distribution of the central compartment; Cl, clearance from the central compartment; V2, volume of distribution of the first peripheral compartment; V3, volume of distribution of the second peripheral compartment; AUC, area under the curve per administration.
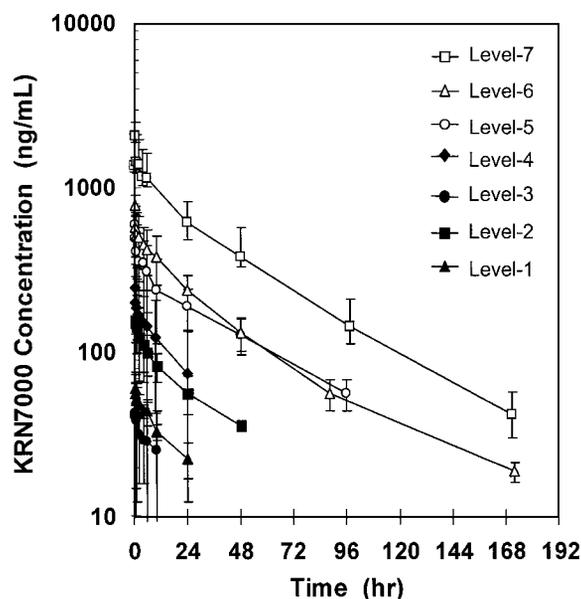

Fig. 7 Serum concentration profile of all of the dose levels, after the administration of one dose of KRN7000. Sampling times are reported in "Patients and Methods." PK parameters are summarized in Table 3.

cell numbers and cytotoxicity in the liver (11, 17). Here, we could not identify any sign of NK cell activation or an increase in NK cell lytic activity on KRN7000 administration; actually, in the majority of patients with high pretreatment NKT cell numbers, a decrease in NK cell cytotoxicity was observed 24 h after KRN7000 administration, whereas no apparent change was observed in patients with low NKT cell counts. Although this decrease in PB NK cell numbers and cytotoxicity, at first glance, contrasts with murine studies, it should be noted that a temporary loss of NK cells in the spleen, accompanied the KRN7000, induced an increase in liver NK cells in mice (17). Recently, KRN7000 was shown to enhance the *in vitro* tumor-killing activity of human hepatic CD56⁺ NK cells, underscoring the strong functional similarities in function between mouse and human NKT cells (27).

No clinical responses were observed in our study, although a number of patients had relatively long-lasting stable disease. The absence of clinical responses in combination with the low frequency of patients in which signs of immunological activation were

observed, may suggest that this schedule and/or route of administration should not be further investigated in a Phase II study. The low number of NKT cells in cancer patients most likely contributed to the lack of immunoreactivity after KRN7000 injections. This view is supported by the fact that immune activation was observed in only those patients who had relatively high pretreatment NKT-cell numbers. An intriguing observation was, however, that even at the lowest dose tested (50 µg/m²), cytokine production and NKT-cell depletion were observed. This may support the study of lower doses than those tested in our trial. However the fact that α-GalCer inhibits the development of autoimmune diabetes (28, 29) and multiple sclerosis (30–32) in mice, and that the elimination of CD1-dependent NKT cells has been shown to improve tumor rejection (33), adds further complexity, which suggests that additional preclinical studies are needed to allow optimal clinical development of this compound.

In conclusion, this Phase I study of KRN7000 did not reach the MTD and did not record substantial toxicities across a broad range of doses. Moreover, no OBAD was defined, despite a very intensive immunological monitoring in all of the patients included in the study. However, the strong, and evolutionarily highly conserved, similarities between mouse and human NKT cells warrant intensive research into alternative approaches and routes of administration (*e.g.,* s.c.), to try to promote KRN7000-induced antitumor effects in humans. Among these are adoptive transfer of NKT cells and the treatment of cancer patients with KRN7000-pulsed dendritic cells; both strategies have resulted in strong antitumor immune responses in mice (34, 35).

ACKNOWLEDGMENTS

REFERENCES

1. Natori, T., Morita, M., Akimoto, K., and Koezuka, Y. Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritianus.* Tetrahedron, *50:* 2771–2784, 1994.

2. Morita, M., Motoki, K., Akimoto, K., Natori, T., Sakai, T., Sawa, E., Yamaji, K., Koezuka, Y., Kobayashi, E., and Fukushima, H. Structure-activity relationship of α-galactosylceramides against B16-bearing mice. J. Med. Chem., *38:* 2176–2187, 1995.

3. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H., and Taniguchi,

M. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. Science (Wash. DC), *278:* 1626–1629, 1997.

4. Exley, M., Garcia, J., Balk, S. P., and Porcelli, S. Requirements for CD1d recognition by human invariant Vα24+ CD4-CD8- T cells. J. Exp. Med., *186:* 109–120, 1997.

5. Spada, F. M., Koezuka, Y., and Porcelli, S. A. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J. Exp. Med., *188:* 1529–1534, 1998.

6. Brossay, L., Chioda, M., Burdin, N., Koezuka, Y., Casorati, G., Dellabona, P., and Kronenberg, M. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J. Exp. Med., *188:* 1521–1528, 1998.

7. Chen, H., and Paul, W. E. Cultured NK1.1+ CD4+ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. J. Immunol., *159:* 2240–2249, 1997.

8. Godfrey, D. I., Hammond, K. J., Poulton, L. D., Smyth, M. J., and Baxter, A. G. NKT cells: facts, functions and fallacies. Immunol. Today, *21:* 573–583, 2000.

9. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Sato, H., Kondo, E., Harada, M., Koseki, H., Nakayama, T., Tanaka, Y., and Taniguchi, M. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells. Proc. Natl. Acad. Sci. USA, *95:* 5690–5693, 1998.

10. Carnaud, C., Lee, D., Donnars, O., Park, S. H., Beavis, A., Koezuka, Y., and Bendelac, A. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J. Immunol., *163:* 4647–4650, 1999.

11. Hayakawa, Y., Takeda, K., Yagita, H., Kakuta, S., Iwakura, Y., Van Kaer, L., Saiki, I., and Okumura, K. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. Eur. J. Immunol., *31:* 1720–1727, 2001.

12. Nishimura, T., Kitamura, H., Iwakabe, K., Yahata, T., Ohta, A., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M., Nakui, M., Sekimoto, M., and Koda, T. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int. Immunol., *12:* 987–994, 2000.

13. Inoue, H., Koezuka, Y., Nishi, N., Osawa, M., Motoki, K., Kobayashi, E., Kabaya, K., Obuchi, M., Fukushima, H., and Mori, K. J. α-galactosylceramide (AGL-517) treatment protects mice from lethal irradiation. Exp. Hematol., *25:* 935–944, 1997.

14. Nakagawa, R., Motoki, K., Ueno, H., Iijima, R., Nakamura, H., Kobayashi, E., Shimosaka, A., and Koezuka, Y. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an α-galactosylceramide. KRN7000. Cancer Res., *58:* 1202–1207, 1998.

15. Kobayashi, E., Motoki, K., Uchida, T., Fukushima, H., and Koezuka, Y. KRN7000, a novel immunomodulator, and its antitumor activities. Oncol. Res., *7:* 529–534, 1995.

16. Nakagawa, R., Nagafune, I., Tazunoki, Y., Ehara, H., Tomura, H., Iijima, R., Motoki, K., Kamishohara, M., and Seki, S. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice. J. Immunol., *166:* 6578–6584, 2001.

17. Osman, Y., Kawamura, T., Naito, T., Takeda, K., Van Kaer, L., Okumura, K., and Abo, T. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. Eur. J. Immunol., *30:* 1919–1928, 2000.

18. Nakagawa, R., Motoki, K., Nakamura, H., Ueno, H., Iijima, R., Yamauchi, A., Tsuyuki, S., Inamoto, T., and Koezuka, Y. Antitumor activity of α-galactosylceramide. KRN7000, in mice with EL-4 hepatic metastasis and its cytokine production. Oncol. Res., *10:* 561–568, 1998.

19. Smyth, M. J., Thia, K. Y., Street, S. E., Cretney, E., Trapani, J. A., Taniguchi, M., Kawano, T., Pelikan, S. B., Crowe, N. Y., and Godfrey, D. I. Differential tumor surveillance by NK and NKT cells. J. Exp. Med., *191:* 661–668, 2000.

20. Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y., Koseki, H., Kanno, M., and Taniguchi, M. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. Science (Wash. DC), *278:* 1623–1626, 1997.

21. Kawano, T., Nakayama, T., Kamada, N., Kaneko, Y., Harada, M., Ogura, N., Akutsu, Y., Motohashi, S., Iizasa, T., Endo, H., Fujisawa, T., Shinkai, H., and Taniguchi, M. Antitumor cytotoxicity mediated by ligand-activated human Vα24 NKT cells. Cancer Res, *59:* 5102–5105, 1999.

22. Metelitsa, L. S., Naidenko, O. V., Kant, A., Wu, H. W., Loza, M. J., Perussia, B., Kronenberg, M., and Seeger, R. C. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J. Immunol., *167:* 3114–3122, 2001.

23. Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S., Ohta, A., Ohmi, Y., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M., and Nishimura, T. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J. Exp. Med., *189:* 1121–1128, 1999.

24. Tahir, S. M., Cheng, O., Shaulov, A., Koezuka, Y., Bubley, G. J., Wilson, S. B., Balk, S. P., and Exley, M. A. Loss of IFN-γ production by invariant NK T cells in advanced cancer. J. Immunol., *167:* 4046–4050, 2001.

25. Matsuda, J. L., Naidenko, O. V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C. R., Koezuka, Y., and Kronenberg, M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J. Exp. Med., *192:* 741–754, 2000.

26. Yang, O. O., Racke, F. K., Nguyen, P. T., Gausling, R., Severino, M. E., Horton, H. F., Byrne, M. C., Strominger, J. L., and Wilson, S. B. CD1d on myeloid dendritic cells stimulates cytokine secretion and cytolytic activity of Vα24JαQ T-cells: a feedback mechanism for immune regulation. J. Immunol., *165:* 3759–3762, 2000.

27. Ishihara, S., Nieda, M., Kitayama, J., Osada, T., Yabe, T., Kikuchi, A., Koezuka, Y., Porcelli, S. A., Tadokoro, K., Nagawa, H., and Juji, T. α-glycosylceramides enhance the antitumor cytotoxicity of hepatic lymphocytes obtained from cancer patients by activating CD3⁻CD56⁺ NK cells *in vitro*. J. Immunol., *165:* 1659–1664, 2000.

28. Sharif, S., Arreaza, G. A., Zucker, P., Mi, Q. S., Sondhi, J., Naidenko, O. V., Kronenberg, M., Koezuka, Y., Delovitch, T. L., Gombert, J. M., Leite-De-Moraes. M., Gouarin, C., Zhu, R., Hameg, A., Nakayama, T., Taniguchi, M., Lepault, F., Lehuen, A., Bach, J. F., and Herbelin, A. Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset of autoimmune type I diabetes. Nat. Med., *7:* 1057–1062, 2001.

29. Hong, S., Wilson, M. T., Serizawa, I., Wu, L., Singh, N., Naidenko, O. B., Miura, T., Haba, T., Scherer, D. C., Wei, J., Kronenberg, M., Koezuka, Y., and Van Kaer, L. The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat. Med., *7:* 1052–1056, 2001.

30. Jahng, A. W., Maricic, I., Pedersen, B., Burdin, N., Naidenko, O., Kronenberg, M., Koezuka, Y., and Kumar, V. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. J. Exp. Med., *194:* 1789–1799, 2001.

31. Singh, A. K., Wilson, M. T., Hong, S., Olivares-Villagomez, D., Du, C., Stanic, A. K., Joyce, S., Sriram, S., Koezuka, Y., and Van Kaer, L. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. J. Exp. Med., *194:* 1801–1811, 2001.

32. Miyamoto, K., Miyake, S., and Yamamura, T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature (Lond.), *413:* 531–534, 2001.

33. Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D. D., Carbone, D. P., Paul, W. E., and Berzofsky, J. A. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat. Immunol., *1:* 515–520, 2000.

34. Toura, I., Kawano, T., Akutsu, Y., Nakayama, T., Ochiai, T., and Taniguchi, M. Cutting edge: Inhibition of experimental tumor metastasis by dendritic cells pulsed with α-galactosylceramide. J. Immunol., *163:* 2387–2391, 1999.

35. Shin, T., Nakayama, T., Akutsu, Y., Motohashi, S., Shibata, Y., Harada, M., Kamada, N., Shimizu, C., Shimizu, E., Saito, T., Ochiai, T., and Taniguchi, M. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Vα14 NKT cells. Int. J. Cancer, *91:* 523–528, 2001.

A Phase I Study of the Natural Killer T-Cell Ligand α -Galactosylceramide (KRN7000) in Patients with Solid Tumors

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