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ORIGINAL ARTICLE

**Charles W. Taylor · Robert T. Dorr · Paul Fanta
Evan M. Hersh · Sydney E. Salmon**

A phase I and pharmacodynamic evaluation of polyethylene glycol-conjugated L-asparaginase in patients with advanced solid tumors

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Abstract *Purpose*: To evaluate the in vitro activity of polyethylene glycol-conjugated L-asparaginase (PEG-L-asparaginase) against fresh human tumor specimens, using the human tumor clonogenic assay (HTCA), and to perform a phase I dose-escalation clinical trial of PEG-L-asparaginase. The goal of the clinical study was to determine the toxicity and optimum biologic dose of PEG-L-asparaginase based on depletion of serum L-asparagine in patients with advanced solid tumors. *Methods*: A modified method for determination of serum L-asparagine is described. PEG-L-asparaginase was administered by intramuscular injection every 2 weeks to 28 patients with various types of advanced solid tumor malignancies. At least 3 patients were evaluated at each dose level: 250 IU/m^2, 500 IU/m^2, 1,000 IU/m^2, 1,500 IU/m^2, 2,000 IU/m^2. *Results*: The in vitro HTCA studies suggested good antitumor activity against malignant melanoma and multiple myeloma. Serum L-asparagine was most consistently and profoundly depleted (up to 4 weeks) in patients treated with 2,000 IU/m^2. Patients receiving this dose level also showed more frequent grade 1, grade 2, and occasional grade 3 toxicities of fatigue/weakness, nausea/vomiting, and anorexia/weight loss. Three patients developed hypersensitivity reactions, but these were not dose related. Two patients developed deep vein thromboses. We saw no episodes of clinical pancreatitis, but there were minor fluctuations of serum amylase and lipase. We saw no partial or complete responses in patients treated in this study, including 11 patients with malignant melanoma. *Conclusions*: We conclude that PEG-L-asparaginase is generally well tolerated in patients with advanced solid tumors, and a dosage of 2,000 IU/m^2 by intramuscular injection every 2 weeks results in significant depletion of serum L-asparagine.

Key words Phase I · Clinical trial · Asparaginase · Asparagine · Melanoma

Sydney E. Salmon: deceased.

C. W. Taylor (✉) · R. T. Dorr · P. Fanta · E. M. Hersh
S. E. Salmon
Room 3945, Arizona Cancer Center,
The University of Arizona, 1515 N. Campbell Avenue,
Tucson, AZ 85724, USA
e-mail: ctaylor@azcc.arizona.edu
Tel.: +1-520-6262266; Fax: +1-520-6265462

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Introduction

The enzyme L-asparaginase has been used since the late 1960s as treatment for patients with lymphoid malignancies (acute lymphoblastic leukemia, non-Hodgkin's lymphoma) [4, 16]. Treatment with L-asparaginase results in depletion of the "nonessential" amino acid L-asparagine, which causes some degree of selective toxicity to many malignant cells because they lack the ability to synthesize L-asparagine de novo [11, 13, 15]. Previous reports suggest potential antitumor activity of L-asparaginase against solid tumors (e.g., gastrointestinal and breast cancer) [12, 19]. However, the utility of L-asparaginase is limited due to its toxicity profile, which includes hepatic toxicity, pancreatitis, central nervous system (CNS) toxicity, and decreased synthesis of blood clotting factors potentially leading to hemorrhage or thrombosis. In addition, the drug is both antigenic and immunogenic, and life-threatening hypersensitivity reactions can occur in 20–35% of patients [18]. The plasma half-life of L-asparaginase is relatively short (~20 h) and daily dosing is usually required [7].

The native L-asparaginase enzyme obtained from *E. coli* has been modified by covalent conjugation to polyethylene glycol (PEG-L-asparaginase) in an attempt to decrease the immune response and delay plasma clearance. The conjugate was active in preclinical tumor models and displayed a prolonged plasma half-life relative to the native enzyme [1]. The majority of previous clinical trials of PEG-L-asparaginase included heavily pretreated patients with lymphoid malignancies and to

date a phase I evaluation of the conjugate in patients with solid tumors has not been reported. The conjugate is less immunogenic than native L-asparaginase and some patients with a history of prior hypersensitivity reaction to native L-asparaginase tolerate PEG-L-asparaginase [10]. The plasma half-life for PEG-L-asparaginase is in the range of 2 weeks in adult patients (3–4 days in children), whereas the half-life for L-asparaginase in adults is approximately 20 h [7]. The doses of PEG-L-asparaginase administered in previous clinical trials (500–8,000 IU/m^2) were substantially lower than commonly used doses for L-asparaginase (10,000–25,000 IU/m^2). However, the minimum dose of PEG-L-asparaginase needed to achieve consistent depletion of plasma asparagine has not been adequately defined.

We here report a phase I dose-escalation trial of PEG-L-asparaginase in patients with advanced solid tumors, with special emphasis on patients with malignant melanoma. In vitro data suggest that malignant melanoma cells are sensitive to asparagine depletion [2] and in the early phase I studies of L-asparaginase, clinical antitumor activity was seen in patients with malignant melanoma [5]. In addition, we pursued an "in vitro phase II" evaluation of PEG-L-asparaginase against a spectrum of fresh human tumors in the human tumor clonogenic assay (HTCA), to potentially identify other sensitive tumor types for future phase II clinical testing of PEG-L-asparaginase. We attempted to identify not only the maximum tolerated dose (MTD) of PEG-L-asparaginase but also the optimum biologic dose (OBD), defined as the minimum dose needed to achieve prolonged (2 weeks or longer) depletion of serum asparagine.

Materials and methods

Human tumor clonogenic assay

The HTCA studies evaluated the in vitro antitumor activity of PEG-L-asparaginase against a variety of fresh human tumor specimens. The endpoint of the assay used tritiated thymidine incorporation into acid-precipitable DNA as previously described [17]. Each sample was tested in triplicate and the results reported as a percentage of control. PEG alone was not toxic to the tumor cells. Tumor samples for which the mean tritiated thymidine incorporation was less than 30% of control were considered sensitive to PEG-L-asparaginase. The concentrations of PEG-L-asparaginase tested included 0.075 IU/ml and 0.75 IU/ml.

Patient selection

To be eligible for this study, patients were required to have refractory solid tumor malignancies or those tumors for which no effective standard therapy existed. Additional eligibility requirements included: age 18 years or older at the time of study entry; score of 2 or less on the Zubrod scale; no chemotherapy or radiotherapy for at least 3 weeks prior to study entry; recovery from toxic effects of prior therapies; no more than two prior cytotoxic regimens and/or no more than two prior biologic regimens; life expectancy of at least 12 weeks; and nonchildbearing potential or using appropriate contraception. Exclusion criteria included: prior treatment with any form of L-asparaginase; clinical pancreatitis; history of pancreatitis; amylase or lipase elevation at least 1.5 times the upper limit of normal, uncontrolled hyperuricemia; history of coagulopathy; or history of intracranial hemorrhage. Laboratory value requirements included: white blood cell (WBC) count ≥2,000/mm^3; platelets ≥50,000/mm^3; hemoglobin ≥8 g%; bilirubin ≤ 2 mg/dl; SGOT and γ-glutamyltranspeptidase (GGT) ≤ 2 times the upper limit of normal; serum creatinine < 2.0 mg/dl; glucose ≤ 200 mg/dl. All patients provided written informed consent. This study was fully reviewed and approved by the University of Arizona Human Subject's Committee.

Study design and drug administration

This was an open-label study, with at least three patients enrolled into each of five different PEG-L-asparaginase dose levels: 250 IU/m^2, 500 IU/m^2, 1,000 IU/m^2, 1,500 IU/m^2, and 2,000 IU/m^2. Within each dose level cohort, patients received the same dose of PEG-L-asparaginase administered intramuscularly (IM) every 2 weeks for a total of four injections. Progression to the next dose level cohort was allowed if none of the patients at the previous dose level developed dose-limiting toxicity (NCI common toxicity criteria grade 3 or grade 4). If dose-limiting toxicity occurred, three additional patients were entered at that dose level. The toxicities of native L-asparaginase are not known to be dose dependent, and native L-asparaginase is not known to be myelosuppressive. Therefore, dose adjustments of PEG-L-asparaginase were not permitted. Patients were allowed to continue to receive PEG-L-asparaginase bimonthly after the fourth injection if there was no evidence of disease progression.

PEG-L-asparaginase was supplied in single-use vials containing 5 ml of an injectable solution with a potency of 750 IU/ml. The vials were stored under refrigeration at +2 °C to +8 °C. The volume of a single intramuscular injection was not allowed to exceed 3.0 ml. If multiple injections were required, each injection was given in a different limb.

Patient evaluations

All patients underwent the following evaluations at baseline: complete medical history and physical examination; complete blood cell count (CBC), with WBC differential and platelet count; prothrombin time (PT); partial thromboplastin time (PTT); and blood levels of fibrinogen, fibrin split products, glucose, blood urea nitrogen (BUN), amylase, lipase, creatinine, uric acid, cholesterol, total bilirubin, alkaline phosphatase, inorganic phosphorous, GGT, SGOT, lactic dehydrogenase (LDH), calcium, total protein, albumin, sodium, potassium, chloride, and carbon dioxide. Evaluations of PT, PTT, fibrinogen, fibrin split products, amylase, lipase, and GGT were repeated weekly. CBC and biochemical investigations were repeated at weeks 2, 4, 6, and 8. Tumor measurements by physical examination and/or imaging study (X-ray, CT, MRI) were recorded pretreatment and repeated at week 8. Blood samples for determination of L-asparagine were collected prestudy, twice weekly for 2 weeks after the first dose of PEG-L-asparaginase, and at weeks 4, 6, and 8. On dosing days, samples were collected immediately prior to administration of PEG-L-asparaginase. A volume of 10.0 ml of whole blood was drawn directly into a standard adult red-top tube, allowed to clot on ice, and rapidly processed as described below.

Response criteria

Tumor measurements included the longest diameter and its perpendicular applied at the widest portion of the tumor. Any response to therapy was required to last at least 4 weeks. Complete remission (CR) was defined as the complete disappearance of all clinical evidence of active tumor and the patient free of all symptoms. Partial remission (PR) required at least a 50% decrease in the sum of the diameters of the measured lesions, with no increase in size of any of the lesions and no new lesions. Stable disease indicated a steady state response less than PR or disease progression

that was less than "increasing disease." Increasing disease indicated unequivocal increase of at least 50% in the size of any lesion or the appearance of new lesions.

Serum L-asparagine measurement

The L-asparagine concentrations in patient serum were analyzed using the high-performance liquid chromatography method modified slightly from that of Jones and Gilligan [8]. This method utilizes precolumn derivation of L-asparagine to a fluorescent *thio*-substituted isoindole by reaction of the primary amino group of L-asparagine to *o*-pthaldialdehyde (OPA). This method has been successfully utilized to differentiate each amino acid in human serum [8] and has been applied to the analysis of serum L-asparagine levels following treatment with L-asparaginase [3].

An internal standard, aminoadipic acid (AAA) was used to control for extraction efficiency, and quantitation was performed by comparing the ratio of areas for the L-asparagine peak and the AAA peak. Patient's blood samples were allowed to clot on ice and a 450-µl aliquot was deproteinized with 500 µl of 4% sulfosalicyclic acid, then spun at 12,000*g* for 10 min in a microcentrifuge. A 0.5-ml aliquot was removed and neutralized with an equal volume of 0.1 M NaOH, followed by vortex mixing and removal of 150-µl aliquots for storage at −80 °C prior to derivation and analysis by HPLC. Derivation was performed on batches of samples 1–2 weeks later using a freshly prepared OPA commercial solution (*o*-Pthaldialdehyde Reagent solution, Sigma Chemical), which contains 50 mg OPA in 1.25 ml methanol, 50 µl of 2-mercaptoethanol, and 11.2 ml of 0.4 M sodium borate (pH 9.5) [9]. All reagents were from Sigma Chemical, St Louis, Mo., except for external standards of L-asparagine, which were obtained from Eastman Kodak Fine Chemicals, Rochester, N.Y. A set of highly purified L-amino acid standards were obtained from Sigma Chemical for authentication of each individual amino acid peak present in patient samples.

Chromatographic conditions utilized a gradient elution of 0.05 M sodium acetate buffer (pH 5.7) to acetonitrile (83%:17%) at time zero, with a continuous gradient to 30% to 70% buffer to acetonitrile at 6 min, followed by a reverse continuous gradient to the original 83% to 17% buffer to acetonitrile mixture at 8 min, which was then maintained isocratically to the end of the 25-min run time. The flow rate was 0.5 ml/min. The HPLC column was a C-18 reverse-phase column (150 mm × 4.6 mm), with 5 µm particles (Alltech HS C-18; Deerfield, Ill.). Equipment included a Varian Model 5020 chromatograph (Varian Associates, Walnut Creek, Calif.), with a Hitachi AS 2,000 autosampler, a Hewlett Packard Integrator, model 3394A (Hewlett Packard, Palo Alto, Calif.), and a Schoeffel Fluoresecnce Detector, model FS970. The detector was set for excitation and emission at 360 nm and 470 nm, respectively.

Results

In vitro tumor sensitivity to PEG-L-asparaginase

Fresh human tumors were evaluated in vitro for sensitivity to PEG-L-asparaginase using the HTCA. Tumor growth at two PEG-L-asparaginase concentrations (0.075 IU/ml and 0.75 IU/ml) was quantitated and recorded as a percentage of control growth. Tumor specimens were considered sensitive if exposure to PEG-L-asparaginase reduced tumor growth to less than 30% of control. The percentage of tumors meeting this criteria is shown in Table 1. Ovarian cancer and malignant melanoma were the most common tumor types tested (40 and 55 specimens, respectively). For each tumor type tested, greater sensitivity was seen at the higher concentration of PEG-L-asparaginase (0.75 IU/ml). For example, only 8% of lung cancer specimens were sensitive to 0.075 IU/ml, while 50% were sensitive to 0.75 IU/ml. Multiple myeloma and malignant melanoma were the most sensitive tumor types evaluated, with 74% and 85% sensitivity, respectively, to 0.75 IU/ml PEG-L-asparaginase. Colon cancer was the least sensitive, with only 38% of specimens sensitive to 0.75 IU/ml.

Patient characteristics and tumor response

A total of 28 patients were treated on this study (Table 2). Malignant melanoma (11 patients) and non-small cell lung cancer (6 patients) were the most common tumor types. The study included 2 or fewer patients with other disease types (Table 2). The majority of patients had received prior systemic therapy with a median of two prior regimens. The most common disease sites included lung (15 patients) and liver (5 patients). A total of 95 doses of PEG-L-asparaginase were administered in this study and the number of doses per patient varied from one to eight: 4 patients received one dose only; 7 patients, two doses; 3 patients, three doses; 10 patients, four doses; 2 patients, six doses; and 2 patients, eight doses. Of the 14 patients who received less than the planned four doses, 12 were removed from the study early owing to progression of disease. One patient treated with 1,000 IU/m^2 developed neurotoxicity after two doses and one patient treated with 1,500 IU/m^2 developed anaphylactic shock with ventricular tachycardia after the second dose. In the 14 patients who received four or more doses of PEG-L-asparaginase, we observed no partial or complete tumor responses. Four patients stabilized after four doses and went on to receive further PEG-L-asparaginase. Of the four patients in which the disease had stabilized, two with malignant

Table 1 Percentage of tumors demonstrating in vitro sensitivity (less than 30% survival compared with control) to polyethylene glycol-conjugated L-asparaginase (*PEG-L-asparaginase*)

Tumor type	PEG-L-asparaginase concentration (IU/ml)		Number of samples
	0.75	0.075	
Breast	50%	18%	18
Colon	38%	20%	13
Lung	50%	8%	14
Malignant melanoma	85%	53%	55
Multiple myeloma	74%	63%	19
Non-hodgkin's lymphoma	40%	30%	10
Ovary	60%	30%	40

melanoma received six and eight total doses, respectively, and then developed progressive disease; one with non-small cell lung cancer and one with colon cancer received eight doses each before the disease progressed.

Toxicities

The type, grade, and percentage of toxicities per dose level are shown in Table 3. There were no treatment-related patient deaths and only one grade 4 toxicity (allergic reaction) was observed. A total of three patients (11%) developed allergic reactions. A patient with malignant melanoma and lung metastases developed a macular/papular rash, eye edema, and mild shortness of breath (grade 1) after the fourth dose of PEG-L-asparaginase. Another patient with malignant melanoma with soft tissue disease developed facial flushing and severe rigors (grade 3), requiring meperidine and diphenhydramine after the sixth dose of PEG-L-asparaginase. The most severe allergic reaction was seen in a patient with bladder cancer with bone and lung metastases. She developed a total body rash, hypotension, bronchospasm, and ventricular tachycardia (grade 4) and required hospital admission following the second dose of PEG-L-asparaginase. The allergic side effects subsequently resolved in all of these patients and none were retreated with PEG-L-asparaginase.

Grade 2 and grade 3 elevations of amylase and lipase occurred, but were not dose related or associated with symptoms, and no patient developed clinical pancreatitis. Similarly, grade 1 and grade 2 elevations of PT and/or PTT levels and decreases in fibrinogen were observed, but these were not associated with clinical signs or symptoms. Two patients with advanced malignant melanoma developed lower-extremity deep vein thromboses, but had no abnormalities in PT, PTT, or fibrinogen levels. A patient treated with 1,000 IU/m^2 developed confusion and delirium (grade 3 neurotoxicity) after two doses, and another patient treated with 1,500 IU/m^2 developed confusion and somnolence (grade 2 neurotoxicity) after four doses of PEG-L-asparaginase. These neurotoxicities resolved after PEG-L-asparaginase was discontinued.

The side effects most consistently associated with increasing PEG-L-asparaginase dose included: nausea/vomiting, fatigue/weakness, and anorexia/weight loss. All patients treated at dose level 4 (2,000 IU/m^2) developed some degree of fatigue or weakness, 60% developed some degree of nausea/vomiting, and 20% developed grade 3 anorexia or weight loss. Considering these dose-related toxicities and the consistent depletion of serum L-asparagine at this dose level (see below and Fig. 1), further dose escalation was not pursued.

Table 2 Patient characteristics

	Number of patients (n = 28)[a]
Diagnosis	
Malignant melanoma	11
Non-small cell lung cancer	6
Sarcoma	2
Colon cancer	2
Bladder cancer	1
Cholangiocarcinoma	1
Multiple myeloma	1
Renal cell carcinoma	1
Salivary gland tumor	1
Small cell lung cancer	1
Unknown primary	1
Prior radiotherapy	10
Disease sites	
Lung	15
Liver	5
Brain	4

[a] Mean age, 55 years (26–83 years); 16 men, 12 women. Median prior systemic regimens, 2 (0–4)

Table 3 Percentage of patients with toxicities by dose level and grade

Dose level	250 IU/m^2				500 IU/m^2				1,000 IU/m^2				1,500 IU/m^2				2,000 IU/m^2			
Patients (*n*)	3				3				9				8				5			
PEG L-asparagine doses (*n*)	10				13				27				25				14			
Toxicity grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Allergic (%)									11		11					13				
Amylase elevation (%)		33																		
Anorexia, weight loss (%)									11				13						20	
Deep vein thrombosis (%)															25					
Diarrhea (%)									11				25	13			20			
Fatigue, weakness (%)									33		22		38	38			20	60	20	
Fibrinogen decreased (%)									22											
Lipase elevation (%)			33				33													
Nausea, vomiting (%)	33				33				33	33	11		50	13			40	20		
Neurologic (%)											11			13			20			
PT elevated (%)										11										
PTT elevated (%)									11	11							20			

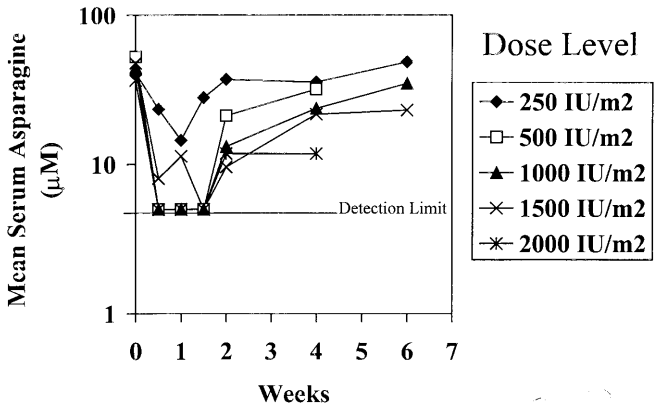

Fig. 1 Effect of PEG-L-asparaginase on asparagine levels. Data points represent mean asparagine levels at each time point and dose level: 250 IU/m^2, 3 patients studied; 500 IU/m^2, 3 patients; 1,000 IU/m^2, 9 patients; 1,500 IU/m^2, 8 patients; 2,000 IU/m^2, 4 patients. Patients received PEG-L-asparaginase at 0 weeks, 2 weeks, 4 weeks, and 6 weeks

Serum L-asparagine determinations

Mean retention times for the derived L-asparagine and the AAA internal standard were 5.26 min and 6.63 min, respectively. These were well separated from other endogenous amino acids, which were individually identified using the set of authentic L-amino acid standards. The standard curve for external standards of freshly prepared L-asparagine was linear over the range 1.07–17.09 μg/ml (7–114 μM), with an R^2 value of 0.9998, y-intercept −0.007, and slope 0.0366. All L-asparagine peaks were considered quantifiable at a signal-to-noise (S/N) ratio of at least 2:1, yielding a quantitation limit of 7.8 μM in th patients' blood. Recovery of L-asparagine from the blood samples averaged 95.8% (SD 8.7%).

Figure 1 shows the mean serum L-asparagine levels versus time, sorted by dose level. L-Asparagine levels became more suppressed with increasing dose levels of PEG-L-asparaginase. The most profound suppression of L-asparagine occurred at the highest dose level studied (2,000 IU/m^2). Despite continued dosing, L-asparagine levels began to increase after week 2 (i.e., after the second dose of PEG-L-asparaginase) in all dose levels except 2,000 IU/m^2. At 2,000 IU/m^2, L-asparagine levels remain suppressed at week 4. Data points beyond week 4 could not be obtained at this dose level, because the patients developed progressive disease and were removed from the study.

Discussion

PEG-L-asparaginase administered by intramuscular injection every 2 weeks in this group of patients with advanced cancer resulted in dose-related depletion of serum L-asparagine. L-Asparagine was most consistently and profoundly depleted at a dose of 2,000 IU/m^2. Consistent toxicities of fatigue/weakness, nausea/vomiting, and anorexia/weight loss also occurred at this dose level. While these toxicities were not severe (mostly grade 1 or 2), 2,000 IU/m^2 was felt to be the OBD of PEG-L-asparaginase.

Our experience in this study and other literature reports [10] suggests that hypersensitivity reactions associated with PEG-L-asparaginase are less frequent than with native L-asparaginase. However, three (11%) of our patients developed significant allergic symptoms, including: rash, facial flushing, and edema and, in one patient, hypotension, bronchospasm and ventricular tachycardia. Patients treated with PEG-L-asparaginase should still be observed closely for possible hypersensitivity reactions. Treatment with PEG-L-asparaginase resulted in minor, asymptomatic alterations of PT, PTT, fibrinogen, lipase, and amylase levels. While we saw no clinical pancreatitis, two of our patients developed lower-extremity deep vein thromboses. This is not, however, an unexpected complication in a group of patients with advanced cancer.

Normal serum asparagine concentrations range from approximately 30–60 μmol/l in untreated human cancer patients [3, 6, 14]. Prior studies of L-asparagine depletion with non-PEGylated L-asparaginase (Elspar; Merck Sharpe and Dohme, West Point, Pa.) have reported rapid lowering of L-asparagine to undetectable levels within minutes of drug injection. These levels remain undetectable for 7–10 days after such therapy [6], but this may be an artifact of continued L-asparagine depletion by L-asparaginase, which can remain active in whole blood samples stored on ice. Although an inhibitor of this residual enzymatic activity, 5-diazo-4-oxo-L-norvaline (DON), has been described [3], it is no longer available. Therefore, we chose to rapidly separate the plasma fraction and deproteinize it into a serum specimen in order to allow freezing at −80 °C prior to analysis. The two steps, deproteinization and freezing at −80 °C, should be sufficient to block the slow depletion of serum L-asparagine by residual L-asparaginase seen when samples are stored on ice as whole blood specimens [3]. This HPLC method also has much greater specificity than older spectrophotometric methods of analyzing serum amino acid levels following L-asparaginase treatment [6, 14].

Our in vitro studies using the HTCA in fresh human tumor specimens suggested potential for antitumor activity of PEG-L-asparaginase, particularly in patients with malignant melanoma and multiple myeloma. In addition, the previous preclinical and clinical experience with native L-asparaginase has suggested possible antitumor activity in patients with malignant melanoma 2, 5]. We treated 11 patients with malignant melanoma and 1 patient with multiple myeloma; we observed no partial or compete responses in any patient treated on the study. Four patients, including two with malignant melanoma, experienced disease stabilization after initial treatment with PEG-L-asparaginase and went on to

receive further dosing before eventually developing disease progression. The primary goal of this phase I trial was to assess the toxicity and determine the OBD of PEG-L-asparaginase. Only five patients were treated at the highest dose level (2,000 IU/m^2), which resulted in consistent depletion of L-asparagine. It is possible that the activity of PEG-L-asparaginase in our in vitro studies was partially artifactual, because there was a more efficient depletion of L-asparagine in tumor cell suspensions in vitro compared with in intact tumor tissue in vivo.

We conclude that PEG-L-asparaginase at a dosage of 2,000 IU/m^2 every 2 weeks results in consistent depletion of serum L-asparagine. Treatment with PEG-L-asparaginase was generally well tolerated, with grade 1 and grade 2 fatigue/weakness, nausea/vomiting, and anorexia/weight loss occurring more consistently at 2,000 IU/m^2. Further studies are needed to determine the antitumor activity of PEG-L-asparaginase in patients with solid tumors, especially those with malignant melanoma or multiple myeloma, since these tumor types demonstrated in vitro sensitivity.

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