Cancer Chemother Pharmacol (1993) 32: 310–314

Cancer Chemotherapy and Pharmacology

Pharmacokinetics of PEG-L-asparaginase and plasma and cerebrospinal fluid L-asparagine concentrations in the rhesus monkey

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Received 23 October 1992/Accepted 17 February 1993

Abstract. The pharmacokinetics of the polyethylene glycol-conjugated form of the enzyme L-asparaginase and the depletion of L-asparagine from the plasma and cerebrospinal fluid (CSF) following an i.m. dose of 2500 IU/m^2 PEG-L-asparaginase was studied in rhesus monkeys. PEG-L-asparaginase activity in plasma was detectable by 1 h after injection and maintained a plateau of approximately 4 IU/ml for more than 5 days. Subsequent elimination from plasma was monoexponential with a half-life of 6 ± 1 days. Plasma L-asparagine concentrations fell from pretreatment levels of 14–47 μM to <2 μM by 24 h after injection in all animals and remained undetectable for the duration of the 25-day observation period in four of six animals. In two animals, plasma L-asparagine became detectable when the PEG-L-asparaginase plasma concentration dropped below 0.1 IU/ml. Pretreatment CSF L-asparagine levels ranged from 4.7 to 13.6 μM and fell to <0.25 μM by 48 h in five of six animals. CSF L-asparagine concentrations remained below 0.25 μM for 10–14 days in four animals. One animal had detectable CSF L-asparagine concentrations within 24 h and another had detectable concentrations within 1 week of drug administration despite a plasma PEG-L-asparaginase activity profile that did not differ from that of the other animals. These observations may be useful in the design of clinical trials with PEG-L-asparaginase in which correlations among PEG-L-asparaginase pharmacokinetics, depletion of L-asparagine, and clinical outcome should be sought.

Introduction

L-Asparaginase is a bacterially derived enzyme that provides specific therapy for lymphoid malignancies such as acute lymphoblastic leukemia (ALL) by catalyzing the hydrolysis of L-asparagine to L-aspartic acid and depleting the circulating pools of this amino acid. Although the capacity to synthesize L-asparagine is constitutive in most normal tissues, malignancies of lymphoid origin lack the synthetic enzyme that catalyzes the transformation of L-aspartic acid to L-asparagine and therefore depend on exogenous sources of L-asparagine.

Despite this apparent specificity for lymphoblasts, however, therapy with L-asparaginase is often limited by significant toxicities such as pancreatitis, coagulation abnormalities, and hypersensitivity reactions. The latter range in severity from local reactions at the injection site to systemic allergic reactions including anaphylaxis. Hypersensitivity reactions lead to interruption or discontinuation of L-asparaginase therapy in up to 50% of patients with ALL [3]. In addition, antibody to L-asparaginase in allergic patients may lead to rapid elimination of the enzyme, which in turn may compromise the extent of L-asparagine depletion [3].

Modification of L-asparaginase by conjugation with polyethylene gylcol (PEG) appears to reduce the immunogenicity of this foreign protein and also conveys more favorable pharmacologic properties [1, 9, 18]. PEG-L-asparaginase (PEG-asp) has a considerably longer half-life and produces more prolonged depletion of plasma L-asparagine than does the native form of the drug. As a result, the dosing interval for PEG-asp is much longer than that for native L-asparaginase (every 2 weeks vs every 2–3 days) [9].

In the present study, plasma and cerebrospinal fluid (CSF) L-asparagine concentrations were measured after a single i.m. dose of PEG-asp had been given to nonhuman primates. The pharmacokinetic behavior of PEG-asp was also determined, and the duration of L-asparagine depletion from plasma and CSF was correlated with the plasma PEG-asp concentration. Our findings may have important implications for the rational design of future regimens containing PEG-asp.

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Materials and methods

Animals. Six adult male rhesus monkeys weighing 5.6–10.3 kg were used for this study. The animals were fed NIH Open Formula Extruded Nonhuman Primate Diet twice daily and were housed in accordance with the Guide for the Care and Use of Laboratory Animals [6]. Blood samples were drawn through a catheter placed in either the femoral or the saphenous vein. CSF samples were drawn through an indwelling fourth ventricular Pudenz catheter attached to a subcutaneous Ommaya reservoir [14].

Drug and inhibitor. PEG-asp was kindly provided by Enzon, Inc. (S. Plainfield, N. J.). The native *Escherichia coli* L-asparaginase used to prepare the PEG-asp was obtained by Enzon from Merck, Sharpe, and Dohme or from Kyowa Hakko of Japan and was conjugated to PEG by Enzon as previously described [1]. 5-Diazo-4-oxo-L-norvaline (DONV) was obtained by Enzon from Dr. R. Blakely, St. Jude Children's Research Hospital.

Experiments. Each of the six animals received a single i.m. injection of 2500 IU/m^2 PEG-asp on day 0 of the study. For confirmation of the bioequivalence of the two enzyme preparations, three animals received each product. Blood samples were obtained prior to drug administration, 1 h after drug administration, and on days 1–4, 7–11, 14, 16, 18, 21, 23, and 25. CSF samples were obtained prior to drug administration, 1 h after drug administration, and on days 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, and 25.

For L-asparagine determination, blood samples were drawn into sodium heparin-containing tubes to which the L-asparaginase inhibitor DONV had been added at a final concentration in whole blood of 46 m*M* to inhibit ex vivo L-asparagine hydrolysis. The plasma was immediately separated by centrifugation at 1500 rpm for 5 min. The plasma was then immediately loaded onto Centrifree Micropartition System devices (Amicon Division, W. R. Grace & Co., Danvers, Mass.) and centrifuged at 2000 *g* for 30 min to remove L-asparaginase. The plasma ultrafiltrates were then frozen at –20° C until analysis. CSF samples for L-asparagine determination were frozen immediately without the addition of DONV or ultrafiltration. Blood samples for PEG-asp determination were immediately separated by centrifugation at 1500 rpm and the plasma was frozen at –20° C until analysis.

L-Asparagine. L-Asparagine levels were measured with a high-pressure liquid chromatography (HPLC) technique following precolumn derivatization with *o*-phthaldialdehyde [12]. In all, 75 µl of the derivatizing atent, *o*-phthaldialdehyde (Sigma, St. Louis, Mo.) was mixed with 75 µl of the plasma ultrafiltrate or CSF for 30 s prior to the injection of 50 µl of this mixture onto a Dynamax Microsorb Type O 4.6 × 100-mm column with a guard module (Rainin Instrument Co., Woburn, Mass.). Fluorescence was monitored with a Spectroflow 980 fluorescence detector (ABI Analytical, Ramsey, N.J.) using an excitation wavelength of 340 nm and a cutoff of 440 nm. The mobile phase consisted of 0.025 *M* sodium acetate (pH 7.2)/methanol/tetrahydrofuran (95 : 4.5 : 0.5, by vol.). Elution was accomplished with a three-step gradient (initial conditions, 10% methanol and 90% mobile phase) involving a linear increase to 30% methanol over 12 min, followed by a linear increase to 80% methanol over 4 min, followed by a return to the initial conditions over 5 min. The system was allowed to reequilibrate for 20 min between injections. The flow rate was 1 ml/min throughout. The retention time for L-asparagine was 11 min. The limit of detection of L-asparagine was 2 µ*M* in plasma. In CSF, the assay was linear to 0.6 µ*M*; the limit of detection was 0.25 µ*M*. The coefficient of variation was 14%.

Standard curves were prepared by incubating normal human plasma or CSF with 1 IU L-asparaginase/ml (Sigma) at 37° C for 1 h to deplete endogenous L-asparagine. The plasma or CSF was then centrifuged through Centrifee micropartition filters (Amicon) to remove the enzyme. For plasma standards, 100 µl 0.5 *M* DONV was added to each milliliter of L-asparagine-depleted plasma prior to centrifugation. Appropriate amounts of L-asparagine were then added to the plasma or CSF ultrafiltrate to make standards. A fresh set of standards was prepared with each set of samples.

DONV protection. The efficacy of DONV inhibition of L-asparagine hydrolysis by L-asparaginase was assessed in vitro. DONV was added to normal plasma to yield a final concentration of 46 m*M*. *E. coli* L-asparaginase (Sigma) was then added at a final concentration of 0.25, 0.5, or 1.0 IU/ml, and the mixture was incubated at 37° C for 45 min. The L-asparagine remaining in the sample was then measured by HPLC after ultrafiltration and derivitization as described above.

L-Asparaginase. L-Asparaginase was measured by a modification of a previously described method [5]. A reagent mixture containing L-asparagine (1.5 mg/ml), glutamic oxaloacetate transaminase (2 U/ml), malic dehydrogenase (0.2 U/ml), and alpha-ketoglutarate (0.1 mg/ml) in TRIS-HCl (pH 8.35) was provided by Enzon, Inc. The working solution consisted of 10 ml reagent mixture and 1 mg β-reduced nicotinamide adenine dinucleotide (β-NADH, Sigma) prepared immediately prior to use. Then, 10 µl of a plasma or CSF sample was added to 1 ml working solution and incubated at 37° C for 3 min. The decrease in absorbance at 340 nm was measured over 3 min on a Gilford Response spectrophotometer (Ciba Corning Diagnostics Corp., Oberlin, Ohio). A standard curve was prepared with each set of samples by diluting PEG-asp (Enzon, Inc.) in phosphate-buffered saline solution. The standard curve was linear from 5 to 0.1 IU/ml. The coefficient of variation was <5%.

Pharmacokinetics. The postplateau (i.e., postaborption) plasma PEG-asp concentration-time data were fitted to the equation for a monoexponential curve with MLAB [13] using the formula

$$C(t) = C_0\, e^{-kel\, t},$$

where *C* is the drug concentration at time *t* and *kel* is the elimination rate constant. The half-life was calculated from 0.693/kel. The area under the concentration-time curve (AUC) was calculated using the trapezoidal method, and clearance was calculated from the relationship

$$\text{Clearance} = \text{Dose/AUC}.$$

Results

PEG-asp activity was detectable in the plasma of all six animals by 1 h after the i.m. injection, and maximal concentrations were reached by approximately 24 h after injection (Fig. 1). Mean PEG-asp activity in plasma then plateaued at approximately 4 IU/ml for more than 5 days. Subsequent elimination was monoexponential with a half-life of 6 ± 1 days. Clearance was 49 ml m^{-2} day^{-1}. The pharmacokinetic parameters for PEG-asp are summarized in Table 1. PEG-asp was not detectable in the CSF of any animal. There was no difference in pharmacokinetic parameters between the two enzyme preparations.

Pretreatment plasma L-asparagine concentrations ranged from 14 to 47 µ*M* (median, 33 µ*M*), and pretreatment CSF L-asparagine concentrations ranged from 4.7 to 13.6 µ*M* (median, 7.7 µ*M*). There was no correlation between the pretreatment plasma and CSF concentrations. Plasma L-asparagine was undetectable (<2 µ*M*) by 24 h after PEG-asp injection in all animals and remained undetectable for the duration of the 25-day observation period in four of six animals (Fig. 2). In two animals, plasma L-asparagine became detectable when the PEG-asp concentration dropped below 0.1 IU/ml.

CSF L-asparagine was undetectable (<0.25 µ*M*) by 48 h after PEG-asp administration in all but one animal and remained undetectable for 10–14 days in four animals (Fig. 3). In these four animals, CSF L-asparagine became

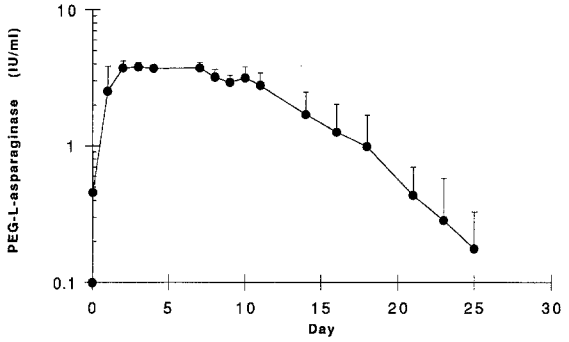

Fig. 1. Plasma PEG-asp activity (mean values ± SD for 6 animals) following an i. m. dose of 2500 IU/m²

Table 1. Pharmacokinetic parameters of PEG-asp following an intramuscular injection of 2500 IU/m²

Animal	AUC (IU ml⁻¹ day⁻¹)	$t_{1/2}$ (day)	Clearance (ml m⁻² day⁻¹)
1[a]	61.4	6.8	41
2[a]	51.9	7.2	48
3[a]	40.2	6.0	62
4[b]	50.0	5.6	50
5[b]	64.0	4.9	39
6[b]	45.1	6.0	55
Mean	52.1	6.1	49.2
SD	9.2	0.8	8.6

[a] Kyowa Hakko enzyme
[b] Merck, Sharpe, and Dohme enzyme

detectable when plasma PEG-asp activity dropped below 0.8 IU/ml. L-Asparagine became detectable in the CSF of a fifth animal within 1 week of drug administration even though plasma PEG-asp concentrations were >2 IU/ml. A sixth animal had detectable, albeit nonquantifiable, CSF L-asparagine levels on day 2 after PEG-asp injection, and concentrations became quantifiable (>0.6 μ*M*) within the 1st week despite plasma PEG-asp activity that did not differ from that of the other animals.

DONV provided partial protection against ex vivo hydrolysis of L-asparagine at all L-asparaginase concentrations studied (Fig. 4). After incubation with 0.25 IU L-asparaginase/ml, 75% of the pretreatment concentration of L-asparagine was detectable in the sample. In the absence of DONV, no L-asparagine was detectable after incubation with as little as 0.05 IU L-asparaginase/ml.

Discussion

Unlike most anticancer agents, which exert their cytotoxic activity by direct interaction with malignant cells, L-asparaginase works indirectly by depleting the environment of a nutrient essential to lymphoblasts. As a result, there is no clear dose-response relationship for L-asparaginase [2]. Instead, it appears likely that a minimum concentration of enzyme is required to deplete L-asparagine from the circulation. The concentration of L-asparaginase required for in vitro L-asparagine depletion, however, is unlikely to correlate directly with the concentration required in vivo, where ongoing L-asparagine synthesis and the presence of interstitial or intracellular L-asparagine makes complete depletion more difficult [2, 4]. Consequently, in pharmacologic studies of L-asparaginase, it is important not only to characterize the pharmacokinetic behavior of the enzyme but also to attempt to quantitate its effect on the circulating L-asparagine pool.

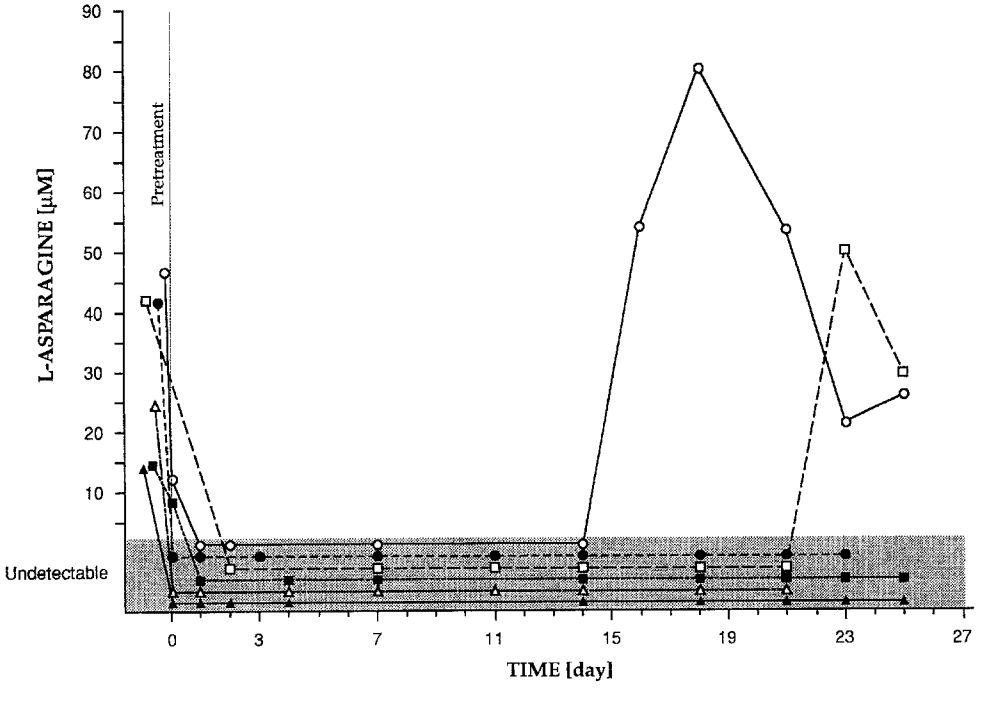

Fig. 2. Individual plasma L-asparagine concentration-time profiles in 6 animals following an i.m. dose of 2500 IU/m² PEG-asp

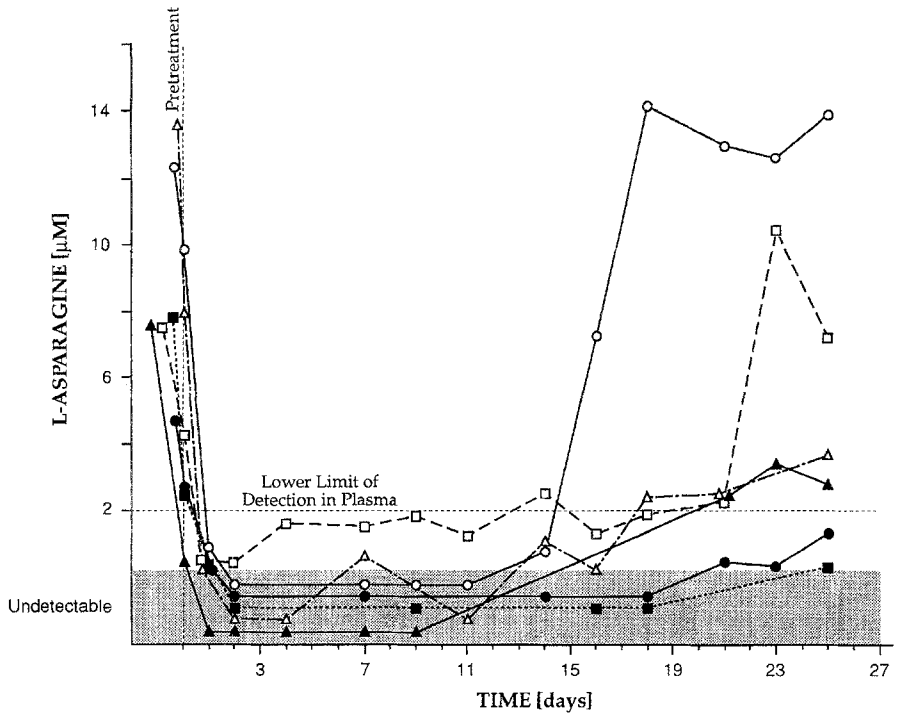

Fig. 3. Individual CSF L-asparagine concentration-time profiles in 6 animals following an i.m. dose of 2500 IU/m^2 PEG-asp

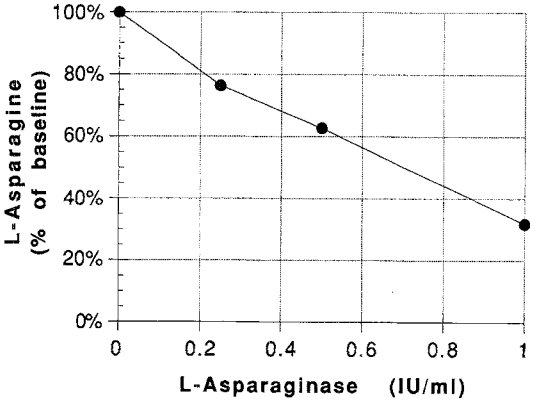

Fig. 4. Percentage of pretreatment L-asparagine remaining in normal plasma after incubation with 46 m*M* DONV and 0.25, 0.5, or 1.0 IU L-asparaginase/ml

The goal of therapy with L-asparaginase is to deplete L-asparagine for the entire treatment period, usually 2–4 weeks in patients with acute lymphoblastic leukemia. Native L-asparaginase, which is usually given three times weekly, has a half-life in nonhypersensitive patients of about 24 h [3, 17] and appears to reduce plasma L-asparagine concentrations effectively for a prolonged period on a thrice-weekly dosing schedule [3, 15, 17]. PEG-asp, with its longer half-life [1, 9, 18], appears to deplete L-asparagine pools as effectively on a less frequent dosing schedule.

The postplateau half-life of PEG-asp in the monkey was 6 days, and the clearance was 49 ml m^{-2} day^{-1}. Plasma L-asparagine was undetectable for at least 14 days and remained so as long as the PEG-asp activity in plasma was >0.1 IU/ml. These results are in good agreement with previously published data on PEG-asp pharmacokinetics in humans and animals [1, 8–11] and indicate prolonged depletion of plasma L-asparagine following a single dose of PEG-asp. Depletion of L-asparagine from CSF, however, was more variable and appeared to correlate best with a plasma PEG-asp concentration exceeding 1 IU/ml.

There are several possible explanations for the differences observed between plasma and CSF L-asparagine depletion. First, due to the nonspecific quenching of fluorescence by DONV, the present assay system is more sensitive for measuring L-asparagine in CSF, to which the addition of DONV is unnecessary, than in plasma (limit of detection, 0.25 μ*M* in CSF vs 2.0 μ*M* in plasma). If the limit of detection for L-asparagine in CSF were 2 μ*M*, for example, the persistence of low concentrations of L-asparagine in two animals would have been missed (Fig. 3), and a CSF L-asparagine depletion profile virtually identical to that of plasma would have been proposed. Second, since L-asparaginase does not appear to cross into the CSF following systemic administration, ex vivo hydrolysis of L-asparagine, a phenomenon that may result in an underestimation of the plasma L-asparagine concentration, is unlikely to be a factor in CSF. Finally, although CSF L-asparagine is believed to be primarily derived from the systemic amino acid pool [19], L-asparagine synthetase activity has been documented in the brain of several species, including the monkey [16]. Thus, it is possible that the central nervous system is capable of synthesizing L-asparagine locally despite depletion of the systemic pool.

The use of the plasma L-asparagine concentration as a pharmacodynamic correlate of L-asparaginase therapy has several potential drawbacks. In the presence of large amounts of L-asparaginase, DONV inhibition of ex vivo hydrolysis may be only partial, leading to an underestimation of the true plasma L-asparagine concentration. In addi-

tion, some malignant cell lines can grow in vitro in media containing L-asparagine concentrations of approximately 10 μM or even less [4, 7], such that even a 5- to 10-fold reduction in plasma L-asparagine concentration from the normal level of 40–50 μM may nonetheless be sufficient for tumor growth [4]. Finally, depletion of plasma L-asparagine by L-asparaginase therapy may not correlate with intracellular L-asparagine concentrations in the lymphoblast [4, 5]. Therefore, plasma L-asparagine depletion may be only an indirect marker for the effect of L-asparaginase therapy on the target cell, and it remains unclear as to whether an "undetectable" plasma L-asparagine concentration might be associated with a therapeutic effect.

In the present study, the pharmacokinetic behavior of PEG-asp following an i.m. dose of 2500 IU/m^2 was studied and the depletion of L-asparagine from the plasma and CSF was characterized. Our results suggest that caution should be used in the extrapolation of data on plasma L-asparagine depletion to conclusions about L-asparagine depletion in extramedullary sites. More detailed information correlating the pharmacokinetics of L-asparaginase, depletion of L-asparagine, and clinical outcome should be sought in future clinical trials of L-asparaginase in humans.

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