Phase I Evaluation of Succinylated Acinetobacter Glutaminase-Asparaginase in Adults

Raymond P. Warrell, Jr., Ting-Chao Chou, Clara Gordon, Charlotte Tan, Joseph Roberts, Stephen S. Sternberg, Frederick S. Philips, and Charles W. Young

Developmental Chemotherapy Service [R. P. W., C. W. Y.], the Laboratories of Experimental Enzyme Therapy [J. P.], Pharmacology [T-C. C., F. S. P.], and Clinical Pharmacology [C. G.], and the Departments of Pathology [S. S. S.] and Pediatrics [C. T.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Succinylated Acinetobacter glutaminase-asparaginase (SAGA) has broader antitumor activity than Escherichia coli L-asparaginase in experimental systems; moreover, drug resistance does not develop in tumor cell lines initially sensitive to this enzyme. We have investigated the pharmacology and toxicology of SAGA after both single-dose and serial daily dose injections in 20 adult patients.

Glutaminase activity in plasma after i.v. injection of single doses did not follow simple first-order kinetics (half-life during the initial 24 hr was 21 ± 9 hr). A linear relation was observed between increasing doses of SAGA and resultant levels of plasma enzyme activity and blood glutamate. Assay of whole blood which had been deproteinized immediately following phlebotomy showed that single doses of SAGA lowered glutamine only transiently to nondetectable levels; serial daily doses were required to achieve and maintain continuous glutamine depletion. Reversible depression of the central nervous system, ranging from encephalopathy to coma, occurred in a dose-related manner and was dose limiting. Other prominent reactions included respiratory alkalosis, hyperglycemia, nausea, and vomiting. Transient antitumor effects were noted in two patients with solid tumors and in two patients with leukemia.

SAGA causes considerable neurotoxicity in adults which requires close patient monitoring. Phase II studies in leukemic patients are in progress.

INTRODUCTION

Enzymes which deplete one or more nonessential amino acids selectively exploit an important biological difference between normal and neoplastic tissues; whereas the growth of certain tumor cells depends upon an exogenous supply of these amino acids, normal tissues retain the capacity for their intracellular synthesis. The clinical prototype for this class of enzymes, Escherichia coli L-asparaginase, has substantial clinical utility only in lymphoblastic leukemia (2, 13) although recently its use in combination with antimetabolites was reported (1). Adverse reactions to L-asparaginase were frequent and unpredictable (12); moreover, after initial exposure to the drug, leukemic cells rapidly became resistant to retreatment through the introduction of an asparagine synthetase (18).

An enzyme from an Acinetobacter soil organism was isolated by Roberts et al. (16) which possessed both glutaminase and asparaginase activities. In preclinical testing, this preparation showed a much broader spectrum of antitumor activity than L-asparaginase (9, 17, 19); furthermore, several tumor cell lines resistant to L-asparagine deprivation were killed by depletion of L-glutamine (15, 17). Also, contrary to the experience with L-asparaginase, Roberts et al. found that tumor cell lines initially sensitive to the enzyme did not develop resistance upon retreatment with glutaminase-asparaginase (17).

While the plasma half-life of the native enzyme was short (t1/2, 1 hr in mice), succinylation of its free amino groups markedly prolonged the t1/2 without altering its other biological activities (10). Preclinical toxicological studies showed that rodents were relatively insensitive to the effects of native Acinetobacter glutaminase-asparaginase after i.p. injection. Using the succinylated enzyme, we found that renal damage, principally tubular necrosis, was prominent in dogs but not in rhesus monkeys who received multiple toxic doses. Dogs receiving 10 IU/kg/day (approximately 200 IU/sq m/day) in 10 daily injections became toxic (manifesting weight loss, diarrhea, gait disturbance, and azotemia), whereas those given one-half that dose were unaffected. Single doses of 20 IU/kg were tolerated by dogs with minimal evident toxicity.

Several preliminary clinical studies, using both a similar enzyme (8) and other bacterial glutaminases (6, 21), have been reported; however, patients in these studies were almost uniformly ill with acute leukemia making analyses for dose-effect relationships and toxicity (especially hematological toxicity) somewhat difficult. We report herein the results of a broad Phase I investigation which defines the toxicity of this enzyme and suggests a dose for disease-oriented trials.

MATERIALS AND METHODS

Enzyme Preparation. The glutaminase-asparaginase enzyme, prepared from broths of Acinetobacter glutaminasificans (American Type Culture Collection 27197) using procedures developed by Roberts et al. (16), was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, Japan. The enzyme is a tetramer with a molecular weight of 138,000 g/mol; L-asparagine is hydrolyzed at 0.8 times the rate of L-glutamine. The Km values for L-glutamine and L-asparagine are 5.8 × 10⁻⁶ M and 4.8 × 10⁻⁶ M, respectively (16).

The enzyme was succinylated using the following modification of a procedure described previously (10). One hundred ml of a solution containing approximately 5 mg of enzyme protein per ml in 0.1 M 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid buffer (pH 8.9 ± 0.1) were prepared just before use, and, if necessary, the pH was adjusted to the final figure with solid sodium carbonate. A finely ground mixture of succinic
Phase I Trial of Glutaminase

anhydride:sodium carbonate (1:2 by weight), in an amount providing 2.5-fold mol ratio of anhydride:amino groups (0.54 mg/mg protein), was placed in a 150-ml beaker with a stirring bar [the mol ratio is based on 0.7 µmol ε-NH₂ lysine groups per mg protein (16)]. The beaker was centered on a magnetic stirrer so that the stirring bar, rotating at a slow speed, spread the solid around its periphery. A combination pH electrode was then positioned in the beaker and connected to a recorder. The enzyme solution at room temperature was added rapidly, and, simultaneously, the stirring speed was increased. A uniform wetting and dispersal of the solid occurred with rapid dissolution. Usually the pH became constant within 8 min. The modified enzyme was then dialyzed overnight at 5° in 0.01 M potassium phosphate buffer, pH 7.2, and lyophilized. This treatment resulted in 35 ± 5% succinylation of the enzyme’s free amino groups and a plasma t₁/₂ in mice of 8.5 to 11.0 hr. The isoelectric point of the succinylated enzyme ranged from 4.3 to 5.3. Two batches of enzyme were used in this study with specific l-glutaminase activities of approximately 200 IU/mg protein each.

Analyses. Blood samples were collected from patients by venipuncture and withdrawal of 10 ml of whole blood into heparinized plastic syringes. In certain patients, blood collected by syringe was divided into 5-ml aliquots at the bedside and transferred immediately into heparinized glass tubes and into plastic tubes containing cold PCA (final PCA concentration, 10%). Samples were placed on ice and then centrifuged at 4° in the laboratory to obtain plasma and whole-blood PCA filtrates. The plasma samples were used for analysis of glutamine, glutamate, glutaminase activity, and ammonia; whole-blood PCA filtrates were used for glutamine:glutamate determinations in selected patients after injections of SAGA. Cerebrospinal and pleural fluids were assayed without deproteination. PCA in the filtrates was removed by extraction with 0.5 M tri-n-octylamine (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) in Freon (1,1,2-trichloro-1,2,2-trifluoroethane, Fisher Scientific Co., Pittsburgh, Pa.) until neutral pH was reached.

Since we and others (14) have noted unsatisfactory results using automated amino acid analyzers for quantitation of glutamine and glutamate, the following methods were developed for quantitative analysis of these amino acids. Glutamine and glutamate concentrations were measured spectrophotometrically by the glutamate dehydrogenase reaction. The reaction mixture in the cuvet contained 0.1 ml of 30 mM NADP, 0.2 ml of sample, and 2.9 ml of potassium phosphate buffer (0.5 M, pH 7.5). After reading the initial absorbance at 340 nm against the sample blank, 20 μl of glutamate dehydrogenase (EC 1.4.1.3, 910 IU/ml, type II; Sigma Chemical Co., St. Louis, Mo.) were added and mixed. After standing at 23° for 20 min, a second A₃₄₀ was recorded. Then, 20 μl of SAGA (50 IU/ml in Tris-HCl buffer, 5 mM, pH 7.5, containing bovine serum albumin, 5 mg/ml) were added and mixed. After standing for another 20 min at 23°, a third A₃₄₀ was recorded. The increase of absorbance between the first and second readings corresponds to the amount of glutamate, and the increase between the second and third readings corresponds to the amount of glutamine. Authentic L-glutamine and L-glutamic acid were used to construct the standard curves. The linear range was up to 400 nmol/ml of glutamate or glutamine, and thus appropriate dilutions were made in some samples for accurate measurement. Since each mol of glutamate and glutamine produces 1 and 2 mol of ammonia, respectively, and since ammonia has inhibitory effects on the glutamate dehydrogenase reaction, glutamine and glutamate do not follow the same standard curves at concentrations greater than 400 nmol/ml.

This procedure was tested by measuring recovery of the amino acids after addition of known amounts to whole blood before PCA deproteination. When the glutamine concentration in whole blood was increased by either 50 or 500 nmol/ml, recovery averaged 71 ± 7.1%; recovery of similar concentrations of glutamate averaged 106 ± 8.8%. Recovery in plasma of added glutamine and glutamate averaged 118 ± 23% and 104 ± 3.1%, respectively.

For assay of glutaminase in plasma samples, the radioactive tracer method was used. The reaction mixture, with a final volume of 5.0 ml in Tris-HCl buffer (1 mM, pH 7.5), contained L⁴⁻¹⁴Cglutamine (0.2 µCi, 1 mM) and 0.1 ml of glutaminase sample; incubation was carried out at 37° for 15 min. The reaction was stopped by placing the cuvet into an iced-water bath and immediately quenching with 0.5 ml ice-cold glutamine solution, 100 mM. The solution was then applied to a Chromo-lex column containing 0.7 x 6 cm of AG 1-ι-X8 (anionic exchange resin in acetate form; Bio-Rad Laboratories, Richmond, Calif.). After the column was drained by gravity, an additional 6 ml of cold distilled water were applied to the column to wash out the unreacted [¹⁴C]glutamine. The product, [¹⁴C]glutamate, retained on the column was eluted with 6 ml 1 N HCl, and an aliquot of the eluate was counted for radioactivity. Enzyme blanks were included in all assays, and a standard curve was prepared with serial dilution of a SAGA preparation of known glutaminase activity. Plasma glutamine activity as low as 0.001 IU/ml can be measured with this assay.

Ammonia content in clinical samples was assayed with the reversed reaction of glutamate dehydrogenase. In the presence of α-ketoglutarate and ammonia, NADH was oxidized to NAD, and the decrease in absorbance at 340 nm was recorded (Sigma Ammonia Assay Kit 170-B).

Clinical Protocol. The drug was administered as follows: each patient was skin tested with 0.1 IU of enzyme per kg injected i.d. using an equal volume of 0.9% NaCl solution as a control. The injected areas were observed over 1 hr for the presence of erythema or urticaria; if neither appeared, the dose of SAGA was administered in a 50-ml volume of 0.9% NaCl solution over 15 to 20 min. All therapy was given on an inpatient basis.

Patients were eligible for this study after they had failed conventional chemotherapy or radiation therapy; they were required to have a life expectancy without treatment in excess of 4 weeks and to have adequate renal and hepatic function (defined as serum creatinine, <1.5%, and total bilirubin, <1.5%). While patients with hematological cancers were not completely excluded from entry, an effort was made to treat sufficient numbers of patients with normal blood counts (WBC count, 4,000/cu mm; platelet count, >150,000/cu mm) at each dose level to insure adequate assessment for hematological toxicity.

All patients had a complete history and physical examination and underwent additional procedures as needed to define the extent of disease. While all patients were evaluable for toxicity,
the absence of bidimensionally measurable disease parameters did not exclude entry into this study. Patients were monitored with the following laboratory tests: complete blood and platelet count daily; serum electrolytes, blood urea nitrogen, and glucose every 3 days; and screening biochemical profile (including uric acid, total protein, albumin, alkaline phosphatase, glutamyl oxaloacetotransaminase, lactate dehydrogenase, calcium, phosphorous, and creatinine) every 5 days. Arterial blood gases were checked if the serum bicarbonate level fell below 20 mEq/ml. Coagulation profiles [including prothrombin time, activated partial thromboplastin time, thrombin time, reptilase time, fibrinogen, fibrin degradation products, factor VIII (coagulant activity)] were obtained pretreatment and every 3 days. All patients in the single-dose study and at least one patient at each daily dose level underwent detailed pharmacological study with serial measurements of blood glutamate and glutamine content and plasma ammonia and glutaminase activity. In patients receiving daily doses of SAGA, these determinations were made on blood drawn 24 hr following the previous dose. These determinations were also made on urine, CSF, and pleural fluid from selected patients.

The protocol was designed so that most patients received a single dose of SAGA with pharmacological studies performed over the succeeding 7 to 10 days; after their plasma levels of glutamine, glutamate, and ammonia returned to base-line values (minimum interval, 25 days), these patients were then entered into a daily dose schedule which was continued without escalation for a planned 10-day course. The daily schedule was to be interrupted only if unmanageable toxicity developed. The starting single dose level was 640 IU/sq m; anticipating greater toxicity in the daily dose schedule, a lower starting dose in rodents. A modified Fibonacci sequence was used to determine dose escalation (3).

RESULTS

Between April and October 1979, 28 courses of SAGA therapy were administered to 20 individual adult patients. Their diagnoses and pretreatment characteristics are presented in Table 1.

Pharmacological Analysis. The decrease in plasma glutaminase activity after single-dose injections did not follow simple first-order kinetics. The half-life during the initial 24 hr was 21 ± 9 hr, and between Days 2 and 5, the half-life approximated 37 ± 5 hr; this nonlinearity may reflect some variability in the degree of succinylation of the enzyme. The volume of distribution, calculated from 3 patients who underwent frequent plasma sampling during the initial 30 min after enzyme injection, was 43.0 ± 2.2 ml/kg; this value approximates the normal plasma volume in adults (4). A linear relation was noted between increasing doses of SAGA and resultant plasma levels of glutaminase activity 1 hr after injection (Chart 1). During the daily administration of a constant dose of SAGA, plasma levels of glutaminase activity typically increased to a plateau at Days 5 and 6, while the plasma glutamate level continued to rise throughout the full 10-day course; the plasma ammonia level peaked earlier (Days 1 and 2) and then leveled off for the remainder of treatment (Chart 2).

The assay for glutamine concentration in whole blood which had been immediately deproteinized following phlebotomy showed that a single dose of SAGA (640 IU/sq m) only transiently lowered glutamine to nondetectable levels (<5 μM) in one patient with rapid recovery thereafter; serial daily doses were required to achieve and maintain prolonged glutamine depletion in whole blood (Chart 3).

Specimens of cerebrospinal fluid were collected from 2 patients receiving 320 IU/sq m for 3 consecutive days; there was no detectable glutaminase activity in the CSF, whereas the glutamine and ammonia levels were elevated relative to reported normal values (23). An additional patient underwent a thoracentesis 4 days after receiving a single dose at 640 IU/sq m; evidence of enzyme activity 57% of the simultaneous plasma level was noted in the pleural fluid.

Toxicity. The dose escalations and major toxicities encountered on the single-dose and daily dose schedules are presented in Tables 3 and 4. There was no discernible hematolog-
Phase I Trial of Glutaminase

By far the most significant (and dose-limiting) toxicity was depression of the CNS; this reaction was seen in both the single-dose and daily dose regimens, and its severity was graded as follows: 1+, lethargy; 2+, disorientation; 3+, coma; and 4+, death from CNS depression (Tables 3 and 4). Except for patients at the lowest dose levels, most patients complained of fatigue and lack of energy 2 to 3 days into the daily dose trial; for the majority of individuals, this feeling persisted without change for the remaining 10-day course. In several patients, particularly those at the highest dose level, this syndrome progressed to a clinical picture indistinguishable from hepatic encephalopathy (i.e., disorientation with asterixis). The drug was generally stopped after the appearance of this syndrome. One patient who had already developed asterixis received an additional dose of SAGA; he rapidly became comatose and was responsive only to aversive stimuli for 48 hr, after which time he awoke and recovered completely. For the remainder of the daily dose study, all patients were observed for the appearance of asterixis, and therapy was stopped if it appeared.

Three patients developed encephalopathy after a single dose of SAGA (2 of 5 at 1280 IU/sq m and 1 of 2 at 2100 IU/sq m). This reaction appeared 24 hr after administration of the drug, at which time the patients developed confusion and asterixis. The severity of this reaction peaked at 36 to 48 hr and cleared by 72 hr in 2 of the 3 patients. The third patient was encephalopathic at 48 hr and expired 7 days after receiving a single dose of 2100 IU/sq m; while her death was unexpected, it was considered secondary to progressive lung cancer rather than a reaction to SAGA.

Other toxic reactions included transient prerenal azotemia observed in 2 patients (probably related to vomiting). One patient on the daily schedule (320 IU/sq m) developed asymptomatic hyperglycemia (serum glucose, 250 mg/100 ml); the drug was continued for 10 days without interruption, and the glucose returned to normal 48 hr after the last dose. Six patients on the daily schedule showed a gradual decline in serum bicarbonate (mean, 17 mEq/liter; range, 15 to 19); arterial blood gases in each case revealed a respiratory alkalosis which normalized 72 hr after discontinuance of the drug.

One patient developed fever and chills during his third course of SAGA therapy and demonstrated accelerated enzyme clearance from his plasma; conceivably, by this time, he had developed an antibody to the drug similar to those patients described by Holcenberg et al. (7). Several courses of therapy at least 25 days apart were administered to the same individuals which allowed an assessment for the development of hypersensitivity; except for the above-mentioned episode, there were no other clinical manifestations of allergy.

**Therapeutic Results.** There were no complete or partial

---

**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fluid analyzed</th>
<th>Glutamine (mM)</th>
<th>Glutamate (mM)</th>
<th>NH₃ (mM)</th>
<th>Glutaminase (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Plasma</td>
<td>389 ± 96⁵</td>
<td>67.8 ± 24.0</td>
<td>32 ± 12</td>
<td>0.0076 ± 0.0006</td>
</tr>
<tr>
<td>SAGA-treated</td>
<td>CSF</td>
<td>1000</td>
<td>30</td>
<td>109</td>
<td>0</td>
</tr>
<tr>
<td>2 CSF</td>
<td>490</td>
<td>36</td>
<td>86</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

⁵ CSF specimens collected after 3 consecutive daily doses of SAGA, 320 IU/sq m/day, i.v.
⁶ Pretreatment values, mean ± S.D. (n = 8).
⁷ Mean ± S.D. (n = 9) (23).

---

*R. P. Warrell, Jr., and T-C. Chou, unpublished data.*
responses as conventionally defined. Two patients with solid tumors (one each with adenocarcinoma of the colon and squamous carcinoma of the bladder) showed major symptomatic improvement and objective stabilization of disease for 4.5 months during this trial. Two patients with hematological cancers (acute lymphoblastic leukemia and chronic myelocytic leukemia in blast crisis) showed transient declines in circulating blast counts; the initial antileukemic effect was seen 48 hr after the first dose and persisted for 48 hr after the drug was stopped.

**DISCUSSION**

Early in the course of this trial, it appeared that the problem of CNS depression was unpredictable and potentially severe, thus possibly precluding further clinical investigations with this agent. Additional study, however, has shown it to be predictable, dose related, and reversible with careful patient monitoring.

Our results differ from studies published previously using a similar enzyme which reported severe CNS reactions generally in relation to leukemic meningitis or prior cranial radiation and intrathecal chemotherapy (7, 8). None of our patients who became encephalopathic had carcinomatous meningitis or brain metastasis, and none had received radiation to the CNS; likewise, all were of outpatient performance status and were not experiencing coincident sepsis, hemorrhage, or severe metabolic disorders. The single patient who had periodically received intrathecal methotrexate over a 2-year period developed no CNS toxicity whatsoever. One possible factor to account for the high frequency of CNS reactions in this study relative to previous reports based on pediatric populations may be that adults are generally more susceptible to these effects.

The similarity of this disorder to that of hepatic encephalopathy is striking; both states are characterized by elevated levels of ammonia in plasma and CSF and by increased CSF concentrations of glutamine (7, 23). Glutamine synthesis from glutamate and ammonia (via glutamine synthetase) presumably represents the major pathway for ammonia metabolism in brain (24), and thus the high CSF ammonia concentrations may account for the encephalopathy seen in both disorders.

Given the variety of toxic effects seen with *E. coli* L-asparaginase, we should point out several reactions prominently associated with that drug that were not seen in this study of SAGA. By virtue of its causing a marked decrease in protein synthesis, L-asparaginase has produced severe, occasionally fatal depression of circulating coagulation factors, particularly fibrinogen (5); none of the patients in this trial demonstrated levels of fibrinogen below normal control values at any point during their course. One patient was found to have a dysfibrinogenemia, presumably on an acquired basis (a phenomenon reported in association with the underlying disease, colonic...
trials suggest that a constant level of plasma glutaminase which could be attributed to SAGA therapy. Hypofibrinogenemia, episodes of pancreatitis, and random samples of serum amylopectinemia and hypocalcemia. There were no clinical carcinomas with hepatic metastases) (11). There was no substantial change in serum levels of total protein or albumin, and no patient developed hypocalcemia. There were no clinical episodes of pancreatitis, and random samples of serum amylase activity in 6 patients were all within the normal range. There were no biochemical abnormalities of liver function which could be attributed to SAGA therapy. Hypofibrinogenemia, hypoproteinemia, and hypocalcemia have been reported in a clinical study of a similar enzyme preparation (7), but the complicated nature of those patients’ illnesses (85% with acute leukemia) makes it uncertain that these effects resulted from drug toxicity.

During periods of enzyme therapy, it is important to perform amino acid analyses on blood in which the enzyme has been inactivated immediately after phlebotomy. Since SAGA appears confined to the intravascular space (excepting its partial entry into exudative effusions), blood glutamine content is determined by the level of plasma glutaminase activity and glutamine transit across a gradient from high extravascular to low intravascular concentrations. Even low levels of glutaminase activity in blood removed for analysis may cause glutamine depletion in the test tube and yield a lower determination relative to the actual in vivo blood glutamine concentration. Our data show that a single dose of SAGA causes only a transient fall in blood glutamine concentration to nondetectable levels with rapid recovery thereafter; serial daily doses are required to deplete glutamine content in deproteinized whole blood. These results conflict with a recent study which reported prolonged glutamine depletion following single injections of SAGA (7). This discrepancy cannot be attributed to our measurement of RBC glutamine concentrations in whole blood versus that of plasma alone since our data show that even whole-blood glutamine levels become nondetectable at least transiently. Furthermore, while the blood glutamine concentration is falling, simultaneous glutamate levels are several times the initial level of glutamine. Whether these favorable data can be translated into meaningful clinical responses in human cancer remains to be tested.

ACKNOWLEDGMENTS

The authors thank Alvin Donner for technical assistance, Orlando Hernandez for assistance with data collection, and Shelley Hantman for assistance with preparation of the manuscript.

REFERENCES

Phase I Evaluation of Succinylated Acinetobacter Glutaminase-Asparaginase in Adults

Raymond P. Warrell, Jr., Ting-Chao Chou, Clara Gordon, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/40/12/4546

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.