

The ex vivo production of ammonia predicts L-asparaginase biological activity in children with acute lymphoblastic leukemia

Shizuka Watanabe · Kumiko Miyake · Chitose Ogawa · Haruna Matsumoto · Kenichi Yoshida · Shinsuke Hirabayashi · Daisuke Hasegawa · Tadao Inoue · Junko Kizu · Reiko Machida · Akira Ohara · Ryota Hosoya · Atsushi Manabe

Received: 18 June 2009 / Revised: 14 August 2009 / Accepted: 24 August 2009
© The Japanese Society of Hematology 2009

Abstract Patients with acute lymphoblastic leukemia (ALL), who develop antiasparaginase antibodies without clinical allergic reactions (“silent inactivation”) during L-asparaginase (L-Asp) treatment, have poor outcomes. Ammonia is produced by hydrolysis of asparagine by L-Asp. We postulated that plasma ammonia level might reflect the biological activity of L-Asp. Five children with ALL treated according to the Tokyo Children’s Cancer Study Group (TCCSG) protocol were enrolled. Plasma ammonia levels were analyzed immediately and 1 h after incubation at room temperature and “ex vivo ammonia production” was defined as increase in ammonia concentration. Ex vivo ammonia production well correlated with L-Asp activity ($r = 0.882$, $P < 0.01$, $n = 23$). It always exceeded 170 $\mu\text{g/dL}$ (170–345 $\mu\text{g/dL}$) in induction therapy. We found 3 patients whose ammonia production was

negligible during later phases of therapy. Antiasparaginase antibody was detected and L-Asp activity decreased in these patients. Ex vivo ammonia production is a surrogate marker of L-Asp biological activity.

Keywords Asparaginase · Ammonia · Acute lymphoblastic leukemia · Children · Silent inactivation

1 Introduction

L-Asparaginase (L-Asp) is a key drug in the treatment of childhood acute lymphoblastic leukemia (ALL). Leukemic blast cells are unable to synthesize sufficient amounts of asparagine, and the resulting asparaginase-induced deficiency impairs cellular function and eventually causes cellular death of ALL cells [1].

Because L-Asp is produced by bacteria, antiasparaginase antibodies are frequently induced, which not only causes hypersensitivity reactions (IgE class) but also leads to the inactivation of L-Asp (IgG class). The frequency of clinical hypersensitivity reactions to *Escherichia coli* (*E. coli*) L-Asp is above 50% [2, 3]. The antiasparaginase antibodies development varied from 30 to 70% of children after *E. coli* L-Asp administration [4, 5], and the appearance of antiasparaginase antibodies correlated with the occurrence of allergic reactions [2, 6]. However, inactivation of L-Asp by asparaginase-specific IgG antibody has not been well understood [7].

Recently, Panosyan et al. [8] showed that patients that produce neutralizing antiasparaginase antibodies but who do not suffer from clinical allergic reactions during native *E. coli* L-Asp treatment have a poorer outcome. This phenomenon is called “silent inactivation”. Although

S. Watanabe · K. Miyake · C. Ogawa · H. Matsumoto · K. Yoshida · S. Hirabayashi · D. Hasegawa · R. Hosoya · A. Manabe (✉)
Department of Pediatrics, St. Luke’s International Hospital,
9-1 Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan
e-mail: manabe-luke@umin.ac.jp

S. Watanabe
Department of Pharmacy, St. Luke’s International Hospital,
Tokyo, Japan

S. Watanabe · K. Miyake · H. Matsumoto · J. Kizu
Department of Practical Pharmacy,
Keio University, Tokyo, Japan

T. Inoue
Department of Pharmacy, International University
of Health and Welfare, Tokyo, Japan

R. Machida · A. Ohara
First Department of Pediatrics, Toho University, Tokyo, Japan

consecutive measurement of antiasparaginase antibody levels and L-Asp activity in real time is necessary to detect the occurrence of silent inactivation, it is not easy to perform in all patients.

Ammonia, which is produced by L-Asp biochemical reactions, can be easily measured in a routine laboratory setting. It is possible that the plasma level of ammonia is closely related to the biological activity of L-Asp. Since ammonia is immediately removed from the liver via its incorporation into the urea cycle, the elevation of ammonia in serum should reflect either the effects of L-Asp administration or hepatic dysfunction. Recently, there was a report that showed that the ammonia concentration increased when the serum of patients receiving L-Asp was incubated *ex vivo* [9]. In our preliminary experiment, we added L-Asp to the plasma samples of healthy adults and measured the ammonia concentration before and 1 h after incubation. The concentration of L-Asp used ranged from 0.01 to 5 U/mL. The ammonia concentration increased in a dose-dependent manner. In addition, Asn was completely depleted at all doses of L-Asp (Miyake et al., unpublished).

In this study, we investigated the ammonia increase in children with ALL receiving L-Asp and compared it with other parameters such as L-Asp activity and antiasparaginase antibody production. We prospectively assessed the clinical significance of ammonia increases in children with ALL.

2 Patients and methods

2.1 Patients

Five children who had been newly diagnosed with ALL in our institution between May and December of 2007 were enrolled in this study. The patients were treated according to protocol L04-16 of the Tokyo Children's Cancer Study Group (TCCSG). The median age at initial diagnosis was 4 years (range 2–7 years). The study was approved by the institutional review board, and written informed consent was obtained from the patients' guardians. Three patients were placed into the standard-risk group, and 2 patients were placed into the intermediate-risk group based on

their age, leukocyte counts at diagnosis, immunophenotype, prednisolone response, and cytogenetics (Table 1). Figure 1 shows an outline of the induction and reinduction therapies given to the two groups. In induction therapy, 6,000 U/m² native *E. coli* L-Asp was given as a 1-h intravenous infusion. In reinduction therapy, 10,000 U/m² L-Asp was given by intramuscular injection. The chemotherapy regimens were basically identical to those described in the TCCSG L99-15 [10]. The native *E. coli* L-Asp preparation used in this study was Leunase® (Kyowa Hakko Kogyo, Tokyo, Japan). It is known that the L-Asp activity is comparable when L-Asp is administered either by intravenous or by intramuscular [11]. Therefore, we analyzed the data obtained by both administration routes together.

2.2 Blood sampling points

First, we collected blood sample at various time points after the administration of L-Asp to elucidate the relation between L-Asp activity and ammonia production *in vitro*.

Next, we collected blood samples before and 24 h after L-Asp to demonstrate the fluctuation of L-Asp activity and ammonia production in each patient.

L-Asp activity was measured 24 h after L-Asp administration. The antiasparaginase antibody level was assessed before each phase of therapy.

2.3 Measurement of ammonia concentrations

Plasma ammonia concentrations were analyzed by a standard photometric assay, immediately after the blood collection and, an hour later, were incubated at room temperature (20°C). We defined “*ex vivo* ammonia production” as the following:

Ex vivo ammonia production = ammonia concentration at 1 h after incubation – ammonia concentration before incubation.

2.4 Measurement of asparagine

The plasma was immediately deproteinized by adding an equal volume of 10% sulfosalicylic acid (SSA) and was

Table 1 Patient characteristics

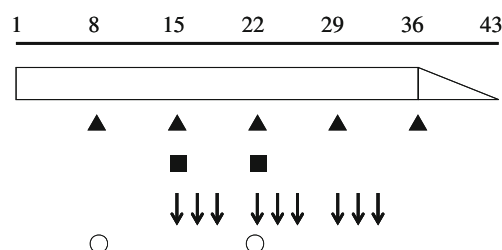
	No.	Sex	Age (years)	WBC/ μ L of diagnosis	Immunophenotype	Prednisolone response ^a	Risk group
WBC white blood cell count	1	F	4	6,100	B-precursor	Good	SR
	2	F	3	8,000	B-precursor	Good	SR
	3	M	6	5,900	B-precursor	Good	SR
	4	M	2	40,700	B-precursor	Good	IR
	5	F	7	33,500	B-precursor	Good	IR

^a Day 8 blast cells. The cut off is 1,000/ μ L

Fig. 1 Outline of induction therapy and reinduction therapy in SR and IR groups

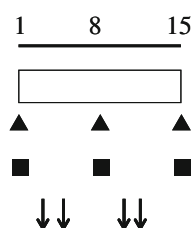
(A-1) Induction therapy in SR

Prednisolone (60 mg/m²/day max 80mg) PO
Vincristine (1.5 mg/m² max 2.0mg) IV
Pirarubicin (20 mg/m²) IV
L-asparaginase (6,000 U/m²) IV
Triple intrathecal therapy
1y 2y ≥3y
MTX 7.5mg 10mg 12.5mg
HDC 15mg 20mg 25mg
Ara-C 15mg 20mg 25mg



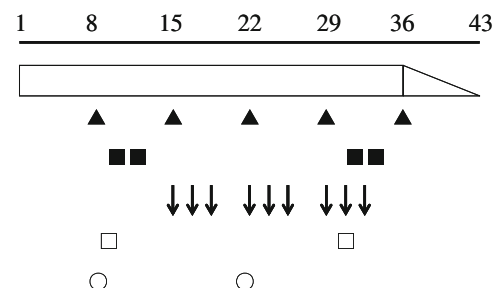
(A-2) Reinduction therapy in SR

Prednisolone (40 mg/m²/day max 60mg) PO
Vincristine (1.5 mg/m² max 2.0mg) IV
Pirarubicin (20 mg/m²) IV
L-asparaginase (10,000 U/m²) IM



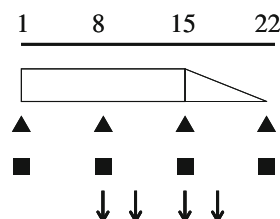
(B-1) Induction therapy in IR

Prednisolone (60 mg/m²/day max 80mg) PO
Vincristine (1.5 mg/m² max 2.0mg) IV
Pirarubicin (20 mg/m²) IV
L-asparaginase (6,000 U/m²) IV
Cyclophosphamide (1g/m²) IV
Triple intrathecal therapy
1y 2y ≥3y
MTX 7.5mg 10mg 12.5mg
HDC 15mg 20mg 25mg
Ara-C 15mg 20mg 25mg



(B-2) Reinduction therapy in IR

Dexamethasone (6 mg/m²/day max 8mg) PO
Vincristine (1.5 mg/m² max 2.0mg) IV
Adriamycin (25 mg/m²) IV
L-asparaginase (10,000 U/m²) IM



then stored at -80°C until the measurements were performed [12]. The asparagine levels were measured by high-performance liquid chromatography (HPLC) using pre-column derivatization with *o*-phthalaldehyde. The lower detection limit of this method was 0.2 $\mu\text{mol/L}$.

2.5 Measurement of L-Asp activity

L-Asp activity was measured by the enzyme coupling methods described by Tsurusawa et al. [13]. The detection limit of this method was 0.002 U/mL.

2.6 Measurement of anti-asparaginase IgG antibody

Anti-asparaginase IgG antibody was measured by an enzyme-linked immunosorbent assay (ELISA), according to the method described by Tsukimoto et al. [7]. The lower detection limit of this method was 4 U/mL.

2.7 Statistical analysis

The correlation between ex vivo ammonia production and L-Asp activity was measured by Spearman's rank

correlation test. SPSS statistical analysis software (SPSS 11.5 J) was used to do this.

3 Results

3.1 Ex vivo ammonia production correlates with L-Asp biological activity

We prospectively enrolled 5 children with ALL in our study and tested 23 samples at various time points after L-Asp administration. Ex vivo ammonia production correlated well with L-Asp activity according to Spearman's rank correlation test ($r = 0.882$, $P < 0.01$, $n = 23$) (Fig. 2).

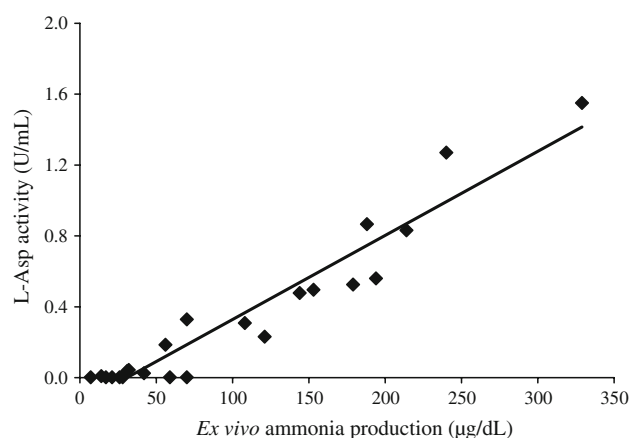


Fig. 2 The correlation between ex vivo ammonia production and L-Asp activity. Ex vivo ammonia production correlated with activity according to Spearman's rank correlation test ($r = 0.882$, $P < 0.01$, 23 samples)

3.2 Longitudinal changes of ex vivo ammonia production and L-Asp activity in individual patients

The ex vivo ammonia production in the 5 patients always exceeded 170 µg/dL (median 230 µg/dL, range 170–345 µg/dL) after 24 h of native *E. coli* L-Asp administration in the induction phase of treatment (Table 2). The ammonia production level of 170 µg/dL correlates well the L-Asp activity of 0.7 U/mL, which exceeds the reported effective dose of 0.4 U/mL (Fig. 2) [14].

Figure 3 shows representative changes in ex vivo ammonia production, L-Asp activity, and Asn levels during the reinduction phase of treatment (patient 1). The patient did not have measurable antibody levels before the reinduction phase of treatment, and no allergic reactions were observed. Ex vivo ammonia production correlated with L-Asp activity and was inversely related to Asn concentration after each administration of L-Asp. The parameter changes in patient 4 were similar to those in patient 1 (data not shown).

Figure 4 shows the changes in ex vivo ammonia production, L-Asp activity, Asn level, and antiasparaginase antibody level in patient 2. No ex vivo ammonia production was observed after the first and third doses of L-Asp. The antiasparaginase antibody levels increased during L-Asp treatment. There were no hypersensitivity reactions in this patient. Interestingly, ex vivo ammonia production was reinstated after the second and fourth doses of L-Asp. This suggests that the antiasparaginase antibodies had become saturated with L-Asp. The clinical course in patient 3 was similar to that in patient 2. He also produced antibodies but did not suffer from allergic reactions. Ex vivo ammonia production was also reduced in this patient. We hypothesized that “silent inactivation” had occurred in patients 2 and 3. In patient 5, ex vivo ammonia production was suboptimal after the third and fourth doses of L-Asp,

Table 2 The ex vivo ammonia production after 24 h of L-Asp treatment and antiasparaginase antibody during induction and reinduction therapy

No.	Induction therapy ^a			Reinduction therapy ^b				Antiasparaginase antibody ^c
	1	2	3	1	2	3	4	
Ex vivo ammonia production (µg/dL)								
1	252	216	170	194	329	188	240	<1
2	211	345	252	21	144	32	179	188
3	197	320	230	14	121	70	216	130
4	212	172	262	260	197	160	229	<1
5	225	239	261	165	204	35	85	27

^a The samples of ex vivo ammonia production in induction therapy were collected after every third dose of L-Asp

^b The samples of ex vivo ammonia production in reinduction therapy were collected after each administration of L-Asp

^c Antiasparaginase antibody was examined before the initiation of reinduction therapy

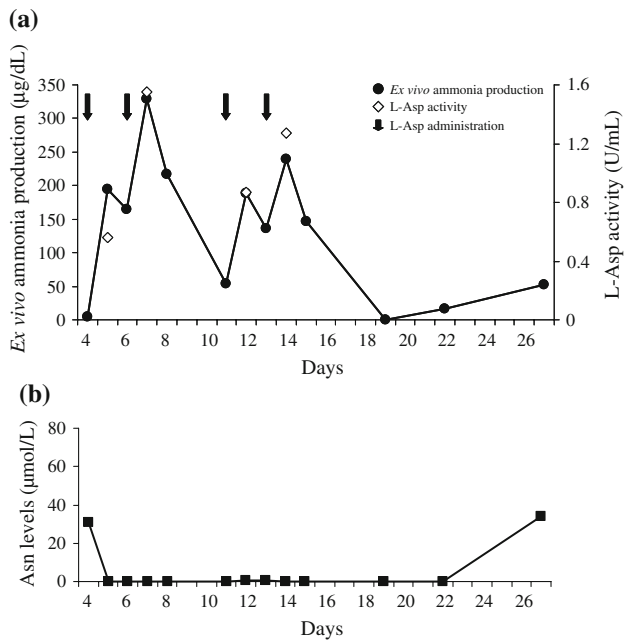


Fig. 3 Representative changes in ex vivo ammonia production, L-Asp activity, and Asn levels (patient 1). **a** Ex vivo ammonia production and L-Asp activity. **b** Asn levels

while L-Asp biological activity was undetectable after the third dose and was not available after the fourth dose. In fact, this patient had an intermediate level of antibody before the reinduction phase. The patient did not develop an allergic reaction (data not shown).

4 Discussion

L-Asp is an essential component of the standard treatment regimens for ALL. The unique mechanism of L-Asp is that the drug depletes the Asn in the serum. Its mechanism is different from that of any other cytotoxic drug. Unfortunately, one of the side effects of L-Asp treatment is the development of anti-asparaginase antibodies, which often means that therapy cannot be completed. It was reported that patients that produce anti-asparaginase antibodies have lower event-free survival (EFS) as well as overall survival (OS) in comparison with children that do not produce the antibodies [15]. However, patients that suffer from allergic reactions could still have a comparable prognosis, if another preparation of asparaginase was used instead [5].

Recent reports suggested that patients that produce anti-asparaginase antibodies but do not suffer clinical allergic reactions have poor outcomes [8]. Silent inactivation may be detected by serial monitoring of the biological activity of L-Asp; however, this method is cumbersome. During the induction phase of treatment, the ex vivo ammonia production of all 5 patients exceeded 100 µg/dL.

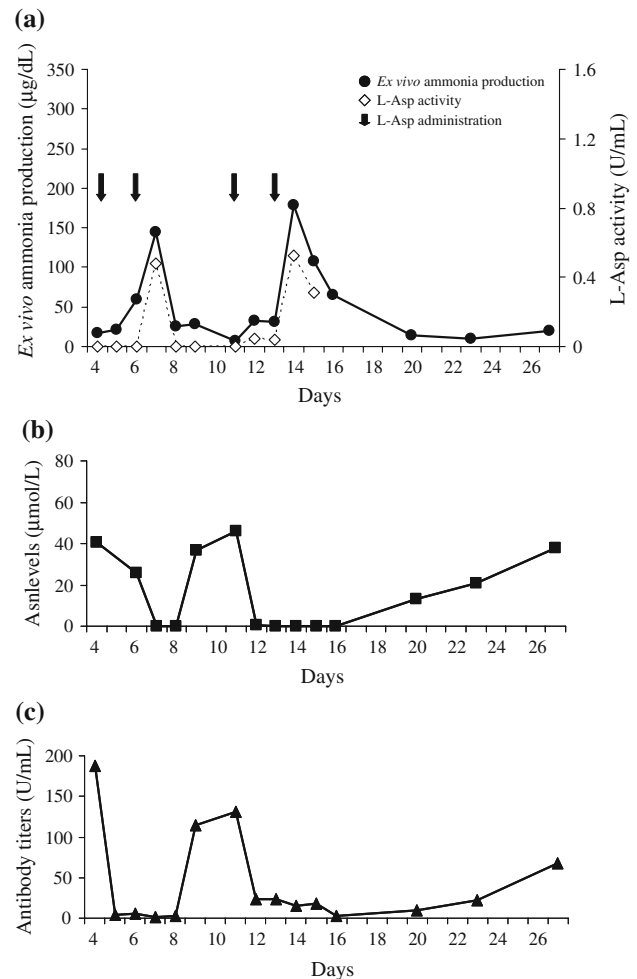


Fig. 4 Representative changes in ex vivo ammonia production (patient 2). **a** Ex vivo ammonia production and L-Asp activity. **b** Asn levels. **c** Antibody titers

However, in the reinduction phase of treatment, the ex vivo ammonia production of 3 patients was below 100 µg/dL, and anti-asparaginase antibodies were detected in these patients. Recently, Patel et al. reported that a proportion of patients with ALL possess blasts with a high asparaginyl endopeptidase (AEP) expression level. AEP is known to cleave *E. coli* L-Asp [16]. Therefore, it is necessary to prospectively monitor the efficacy of L-Asp treatment.

Steiner et al. [17] reported that ammonia levels were elevated 1 day after intravenous native *E. coli* L-Asp administration. Their observation was in our line but they did not study other important parameters such as the activity of the drug or antibody production. We systematically examined the ex vivo ammonia production, Asn level, L-Asp activity, and anti-asparaginase antibody level during treatment for ALL. We defined “ex vivo ammonia production” as the increase in the ammonia concentration 1 h after incubation. In this study, we could distinguish the

increase in ammonia by L-Asp administration from hepatic failure.

Ex vivo ammonia production was correlated with L-Asp activity ($r = 0.882$, $P < 0.01$; Fig. 2). On the other hand, the ammonia concentration immediately after blood sampling and L-Asp activity were more weakly correlated ($r = 0.627$, $P < 0.01$, data not shown). In fact, the longitudinal change of ex vivo ammonia production was correlated with L-Asp biological activity and was inversely correlated with Asn level in patients with ALL.

We decided that it would be interesting to observe ex vivo ammonia production after L-Asp administration using *Erwinia chrysanthemi* (*Erwinia*) instead of native *E. coli* L-Asp, and so we investigated it in two patients who were given 10,000 U/m² of *Erwinia* L-Asp. The result was that ex vivo ammonia production was observed in response to *Erwinia* L-Asp treatment always exceeded 361 µg/dL (median 395 µg/dL, range 361–480 µg/dL, 7 samples). Thus, ex vivo ammonia production can also be used to monitor the biological effects of *Erwinia* L-Asp.

In patients 2 and 3, additional administration of native *E. coli* L-Asp reinstated the increase in ammonia production, suggesting that saturation of the antiasparaginase antibodies with excessive L-Asp had occurred. Currently, utilizing another preparation of asparaginase, that is, *Erwinia* L-Asp, may be the only way to manage silent inactivation. One might introduce an additional dose of native *E. coli* L-Asp to overcome the neutralization of asparaginase by antibodies. Ex vivo ammonia production should be measured before considering a switch to *Erwinia* L-Asp.

In conclusion, measurement of ex vivo ammonia production is a simple method that predicts in vivo L-Asp activity. It can be used to easily identify patients that are suffering from silent inactivation of L-Asp and suggest a suitable treatment.

References

- Muller HJ, Boos J. Use of L-asparaginase in childhood ALL. *Crit Rev Oncol Hematol*. 1998;28:97–113.
- Woo MH, Hak LJ, Storm MC, Evans WE, Sandlund JT, Rivera GK, et al. Anti-asparaginase antibodies following *E. coli* asparaginase therapy in pediatric acute lymphoblastic leukemia. *Leukemia*. 1998;12:1527–33.
- Muller HJ, Beier R, Loning L, Bulteers-Sawatzki R, Dorff W, Maass E, et al. Pharmacokinetics of native *Escherichia coli* asparaginase (Asparaginase medac) and hypersensitivity reactions in ALL-BFM 95 reinduction treatment. *Br J Haematol*. 2001;114:794–9.
- Oettgen HF, Stephenson PA, Schwartz MK, et al. Toxicity of *E. coli* L-asparaginase in man. *Cancer*. 1970;25:253–78.
- Woo MH, Hak LJ, Storm MC, Sandlund JT, Ribeiro RC, Rivera GK, et al. Hypersensitivity or development of antibodies to asparaginase does not impact treatment outcome of childhood acute lymphoblastic leukemia. *J Clin Oncol*. 2000;18:1525–32.
- Cheung N-KV, Chau IY, Coccia PF. Antibody response to *Escherichia coli* L-asparaginase: prognostic significance and clinical utility of antibody measurement. *Am J Pediatr Hematol Oncol*. 1986;8:99–104.
- Tsukimoto I, Matsui J, Iwashita H, Shigeta K, Suzuki H, Hashimoto T. Improved measurements of anti-L-asparaginase IgG antibody and its clinical applications. *Jpn J Clin Hematol*. 1992;33:9–24. (in Japanese).
- Panosyan EH, et al. Asparaginase antibody and asparaginase activity in children with high-risk acute lymphoblastic leukemia children's cancer group study CCG-1961. *J Pediatr Hematol Oncol*. 2004;26:217–26.
- Laterza OF, Gerhardt G, Sokoll LJ. Measurement of plasma ammonia is affected in patients receiving asparaginase therapy. *Clin Chem*. 2003;49:1710–1.
- Manabe A, Ohara A, Hasegawa D, Koh K, Saito T, Kiyokawa N, et al. Significance of the complete clearance of peripheral blast after 7 days of prednisolone treatment in children with acute lymphoblastic leukemia: the Tokyo Children's Cancer Study Group Study L99-15. *Haematologica*. 2008;93:1155–60.
- Rizzari C, Zucchetti M, Conter V, Diomedea L, Bruno A, Gavazzi L, et al. L-Asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving i.m. or i.v. *Erwinia C. or E. coli* L-asparaginase as first exposure. *Ann Oncol*. 2000;11:189–93.
- Gentili D, Zucchetti M, Conter V, Masera G, D'Incalci M. Determination of L-asparagine in biological samples in the presence of L-asparaginase. *J Chromatogr B Biomed Appl*. 1994;657:47–52.
- Tsurusawa M, Chin M, Iwai A, Nomura K, Maeda H, Taga T, et al. L-Asparagine depletion levels and L-asparaginase activity in plasma of children with acute lymphoblastic leukemia under asparaginase treatment. *Cancer Chemother Pharmacol*. 2004;53:204–8.
- Avramis VI, Panosyan EH. Pharmacokinetic/pharmacodynamic relationships of asparaginase formulations. *Clin Pharmacokinet*. 2005;44:367–93.
- Zalewska-szewczyk B, Andrzejewski W, Mlynarski W, Jedrychowska-danska K, Witas H, Bodalski J. The anti-asparaginase antibodies correlate with L-asparaginase activity and may affect outcome of childhood acute lymphoblastic leukemia. *Leukemia Lymphoma*. 2007;48:931–6.
- Patel N, Krishnan S, Offman MN, Krol M, Moss CX, Leighton C, et al. A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J Clin Invest*. 2009;119:1964–73.
- Steiner M, Attarbaschi A, Kastner U, Dworzak M, Haas OA, Gadner H, et al. Distinct fluctuations of ammonia levels during asparaginase therapy for childhood acute leukemia. *Pediatr Blood Cancer*. 2007;15:640–2.