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Unsatisfactory quality of *E. coli* asparaginase biogenerics in India: Implications for clinical outcomes in acute lymphoblastic leukaemia

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Abstract

Background—The biotherapeutic asparaginase is a cornerstone of therapy in acute lymphoblastic leukaemia (ALL). With limited access to the original native *Escherichia coli*-derived asparaginase (EcASNase), a variety of EcASNase biogenerics are used in low-middle-income countries (LMICs). The variable quality of these biogenerics potentially influences clinical outcomes.

Procedure—Seven biogeneric EcASNases (P1–P7) marketed widely in India were evaluated, with P2 as an exemplar for in vivo monitoring. Therapeutic activity of P2 (10,000 IU/m²/dose, intramuscular, every 72 hours) was monitored during induction therapy, and drug-related toxicities recorded. Molecular identity, purity and in vitro drug activity of seven biogenerics were characterised using multimodal analyses, and findings compared with reference EcASNase (R).

Results—In patients ($N = 62$) receiving P2, subtherapeutic asparaginase activity (<100 U/L) was observed in 66% (46/70) of trough timepoints (72 hours postdose) during induction. Twelve patients (19%), 11 with high-risk ALL, developed hypersensitivity. Isoforms of EcASNase were identified in all seven biogenerics. All generic products contained impurities with batch-to-batch

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Shekhar Krishnan, Vaskar Saha and Jasmeet Sidhu designed the study. Praveen Agarwal, Tathagata Mukherjee, Debparna Saha, Priyanka Bose, Jasmeet Sidhu, Yogesh Phadke, Bhatu Sonawane and Pritha Paul performed experiments. Shekhar Krishnan, Vaskar Saha, Prakriti Roy, Manash Pratim Gogoi and Jasmeet Sidhu collected and analysed the data. Jasmeet Sidhu, Vaskar Saha and Shekhar Krishnan wrote the paper. All authors have reviewed and approved the final version of the manuscript submitted for publication.

variability. These included high levels of protein aggregates and host cell protein contamination. In vitro assays of EcASNase activity and leukaemia cell line cytotoxicity were not discriminatory.

Conclusions—Our findings confirm widespread concerns over the unsatisfactory quality and therapeutic activity of native EcASNase biogenerics marketed in LMICs. Appropriate use of these products requires monitored studies to identify clinical suitability and determine appropriate dosing and schedule. For large parts of the world, assured access to high-quality asparaginases remains an unmet therapeutic need.

Keywords

asparaginase; biogeneric; outcomes; quality

1 Introduction

Acute lymphoblastic leukaemia (ALL) is the commonest cancer of childhood. In high-income countries, contemporary treatment protocols achieve cure rates approaching 90%.^{1–3} Remarkably, this progress has been largely achieved using cytotoxic drug combinations discovered nearly half a century ago. The drug asparaginase (ASNase) is a critical component of multidrug treatment in ALL. Unique among cytotoxic drugs, ASNase is a therapeutic enzyme. The drug catalyses the breakdown of asparagine and to a lesser extent, glutamine—the resulting systemic depletion of these amino acids is relatively toxic to ALL lymphoblasts.^{4,5} The pharmacodynamic activity of ASNase thus depends on achieving sustained systemic depletion of asparagine. Current therapeutic formulations of ASNase are derived from bacterial strains of *Escherichia coli* and *Erwinia* species. ASNase extracted from *E. coli* (EcASNase) is the formulation used most widely in clinical practice. The reference EcASNase formulation (Kyowa Hakko, Japan) is derived from a modified high asparaginase-producing strain of *E. coli*. EcASNase has a short half-life (22–38 hours) and requires frequent dosing to maintain sustained asparagine depletion.⁶ As EcASNase is a non-human protein, repeated dosing of the drug can elicit a hypersensitivity response. The hypersensitivity may be subclinical or clinically overt, and is associated with ASNase-reactive antibodies that antagonise drug activity.⁷

Polyethylene glycol (PEG) conjugation of EcASNase substantially addresses the therapeutic limitations of the native molecule. The PEG-conjugated formulation (PEG-EcASNase) has a longer half-life (85–190 hours), requiring less frequent administration (typically every 14 days).⁸ The longer half-life of PEG-EcASNase ensures sustained amino acid depletion for extended durations, necessary for the drug's therapeutic activity.⁶ PEG-EcASNase is also associated with lower rates of hypersensitivity.^{9,10} The superior pharmacological attributes of PEG-EcASNase permits intensification of ASNase therapy, making it the formulation of choice in affluent economies. The switch to PEG-EcASNase has resulted in a decline in the supply of the standard formulation of native EcASNase worldwide.

PEG-EcASNase is expensive. *Erwinia*-derived ASNase is even more expensive, has a shorter half-life than EcASNase (13–22 hours), and its use is restricted to patients with hypersensitivity to EcASNase.⁷ Additionally, there is a global shortage of *Erwinia* ASNase. Thus in low- and middle-income countries (LMICs), native EcASNase is the only

affordable ASNase formulation. In these countries, limited availability of the reference native EcASNase product has led to the manufacture and supply of a number of EcASNase biogenerics. These biogenerics are not necessarily therapeutically equivalent to the reference product.^{6,11–13} The *E. coli* source of ASNase in these products are different, and the resulting EcASNase have amino acid sequence variations leading to variations in their substrate affinity, enzyme activity, stability and antigenicity. Differences in manufacturing processes also influence the quality of EcASNase biogenerics and their therapeutic activity.

Expanding on these observations, this paper reports the findings of a comprehensive examination of the physical, chemical, and in vitro biological characteristics of seven biogeneric EcASNases marketed widely in India. We then focus on one of these formulations, investigating asparaginase activity serially in a cohort of patients during the induction treatment phase and examining rates of drug-associated toxicity.

2 Methods

2.1 Patients and asparaginase treatment

Between 1 February 2017 and 31 March 2018, consecutive patients aged 1–18 years with newly diagnosed ALL were enrolled in the study. Patients were stratified into risk groups (T-cell ALL; standard-, intermediate- and high-risk B-cell precursor ALL) based on patient characteristics, disease features and treatment response. Treatment was based on the Indian Collaborative Childhood Leukaemia Group Study ALL 2014 (ICiCLE-ALL-14) treatment protocol (Clinical Trials Registry-India CTRI/2015/12/006434). In all risk groups, EcASNase was administered intramuscularly every 72 hours at 10,000 IU/m²/dose. All patients received EcASNase during induction (four doses in standard-risk ALL, eight doses in others) and delayed intensification treatment phases (four doses in all risk groups). Patients with high-risk B-cell precursor ALL and T-ALL received additional EcASNase during the consolidation phase (eight doses). Through the study period, one brand (P2) of native EcASNase was administered uniformly according to the protocol. Plasma asparaginase activity was evaluated in peripheral blood samples collected 48 and 72 hours after EcASNase administration during the induction treatment phase. The study was approved by the institutional review board and patients/families gave written informed consent for participation.

2.2 Analysis of asparaginase activity in vivo

Peripheral blood samples collected for measuring asparaginase activity were maintained at room temperature and processed within 24 hours.

The resulting plasma aliquots were stored immediately at –80°C and tested in batches. Asparaginase activity in banked plasma samples was analysed using a modified indoxine assay.¹⁴ Replicates of plasma aliquots were incubated with excess L-aspartic acid β -hydroxamate (AHA; Sigma) at 37°C in 96-well microtitre plates. Hydroxylamine generated from ASNase hydrolysis of AHA was condensed with 8-hydroxyquinoline (Sigma) and the resulting indoxine quantified spectrophotometrically at 710 nm (Spectramax, Molecular Devices). A trough asparaginase activity 100 IU/L was considered adequate.⁷ The dynamic

linear range of the assay was 100–1000 IU/L. For ASNase activity levels below the dynamic range, plasma samples were retested using a modification of the assay with a dynamic range of 5–100 IU/L. The assay standard curve was developed using serial dilutions of the reference asparaginase in pooled human plasma. Assay performance was monitored by including plasma samples with known asparaginase activity (low, mid and high activity) as calibrators.

2.3 In vitro ASNase cytotoxicity assays

The in vitro cytotoxic activity of six EcASNase products (PI–P6) was evaluated against that of the reference formulation (R, Medac GmbH). Two ALL cell lines, REH and SUP-B15, were used for cytotoxicity studies. Cell lines were authenticated, maintained in antibiotic-free growth medium (RPMI-1640) supplemented with 10% foetal bovine serum (FBS, Gibco) and tested routinely for mycoplasma contamination. Assessment of cytotoxic activity was performed in 100 μ l reaction volumes by adding serial 10-fold dilutions of ASNase products (20 μ l, 0.001–1000 IU/ml) to 80 μ l of cell line suspensions (REH, 50,000 cells/well; SUP-B15, 35,000 cells/well) in triplicates, followed by incubation for 72 hours (37°C, 5% CO₂). Proportion of live cells was determined by measuring the metabolic activity of intact cells using a microtitre plate-based WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) cell proliferation assay (Sigma). In the assay, mitochondrial dehydrogenases in intact cells reduce the water-soluble tetrazolium reagent WST-1 to formazan, which when measured at 440 nm provides an estimate of the proportion of live cells. Proportion of live cells in each well was estimated after incubation with WST-1 for 4 hours. Background absorbance (cell-free growth medium with WST-1) and untreated wells (with phosphate-buffered saline as vehicle control) were used as controls. Half-maximal inhibitory concentrations (IC₅₀) from three separate experiments were derived from a four-parameter nonlinear regression model using the GraphPad Prism software (v8.2.0, www.graphpad.com). Additional details are provided in the accompanying Supporting Information.

2.4 Physicochemical characterisation of commercial native *E.coli* ASNase products

Seven EcASNase products marketed commercially in India (brands P1– P7) were compared with the reference therapeutic EcASNase product (R, Medac GmbH). Where feasible, at least two different manufacturing batches were analysed for each brand (total 12 samples). Quality of the marketed brands was determined by investigating a range of physicochemical attributes. Drug quantity and activity were estimated, respectively, by measuring protein content (bicinchoninic protein assay, Thermo Scientific) and indoxine generation (described previously). Variations in protein characteristics of the test brands were examined using four orthogonal techniques—capillary isoelectric focussing, size exclusion chromatography (SEC) with multiangle light scattering (SEC-MALS), reversed phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Amino acid sequence of EcASNase biogenerics and the reference products were determined by peptide mass fingerprinting and compared with the theoretical EcASNase sequence. Host cell protein (HCP) content and bacterial endotoxin levels were analysed as measures of product contamination. Finally, suitability for administration was assessed both by evaluating ease of drug reconstitution

(appearance of the lyophilised drug cake) and testing product osmolality for parenteral use. Details of analytical techniques are presented in the accompanying Supporting Information.

2.5 Statistics

Descriptive statistics were used to report all observations, including the median and interquartile range (IQR) for continuous data. Groups were compared using the Mann–Whitney *U* test (two groups) and oneway analysis of variance (ANOVA; more than two groups). Two-sided *p*-values less than .05 were deemed significant.

3 Results

3.1 Subtherapeutic asparaginase activity of an EcASNase biogeneric

Over the 12-month study period, 65 children with newly-diagnosed ALL received the EcASNase biogeneric P2. Three died during the induction phase, and 62 patients were included in the final analysis. The clinical characteristics, treatment response and EcASNase-associated toxicities are shown in Table 1. A total 117 post-dose plasma samples were obtained from the 62 patients during the induction treatment phase. Forty-seven (40%) samples were collected at the 48-hour post-dose timepoint (post-dose 4 in SR patients; post-dose 7 in IR/HR patients) and the rest (70 samples) 72 hours after EcASNase administration (trough level; post-dose 2 in SR patients; post-dose 2 & postdose 4 in IR/HR patients).. At 48 hours postdose, the median ASNase activity was 149 IU/L (IQR, 93–217 IU/L), declining to 58 IU/L (IQR, 29– 139 IU/L) at 72 hours post-dose (Figure 1). Inadequate activity levels (<100 IU/L) were observed in 14 of 47 (30%) 48 hours post-dose and in 46 of 70 (66%) at the trough time of 72 hours post-dose.

Twelve patients (19%), 11 with high-risk disease, developed clinical hypersensitivity (Common Terminology Criteria for Adverse. Events version 5 (CTCAE) grade 3), requiring discontinuation of further asparaginase treatment. Clinical hypersensitivity in all high-risk patients were associated with the additional EcASNase doses during the consolidation treatment phase (EcASNase doses 9-13).

3.2 Shortcomings in critical quality attributes of EcASNase biogenics marketed in India

Seven EcASNase biogenics marketed widely in India (brands P1–P7) were examined for drug identity, specific activity, purity and quality.

Table 2 (and Table S1) summarises the findings of these physicochemical studies. No one brand met the minimum expected requirements for all characteristics. Batch-to-batch variability, examined in four brands, additionally demonstrated inconsistency in quality attributes between manufacturing batches. In five of seven brands, drug strength, evaluated by measuring specific activity per milligram of vial protein content, was lower than expected (<220 IU of ASNase activity/milligram of protein).

Identity of the therapeutic product was determined using a combination of ASNase activity assay, SDS-PAGE analysis, SEC, isoelectric point (pI) determination and peptide mass fingerprinting. In all brands, the ~36 kilodalton (kDa) band of the EcASNase monomer was detected on SDS-PAGE. SEC-MALS confirmed presence of the active enzyme tetramer

(~136 kDa) (Figure 2A, B). The pI of the EcASNase biogenics (5.6–5.86) differed from that of the reference standard (5.37 ± 0.30), suggesting amino acid sequence variations in the biogenics. RP-HPLC corroborated this observation, with all brands demonstrating retention time peaks different from that of the reference molecule (Figure 2C). Peptide mapping, covering 98.2% of the reference ASNase amino acid sequence, indicated sequence variations for EcASNase biogenics, suggested both by the reduced sequence coverage and distinct missing amino acid sequences (Table 3).¹⁵ An 11-amino acid sequence (residues 252–262) was not detected in two biogenic products, while a 22-amino acid sequence (residues 51–72) was not detected in one, suggesting differences in amino acid sequences in these segments. Both sequence segments represent potential EcASNase antigenic sites. Detailed sequencing of the P2 EcASNase used in clinical practice demonstrated amino acid substitutions at position 64 (D64N) and 252 (T252S), the latter within a potential antigenic region. Detailed analysis of amino acid sequence changes in the other EcASNase products was not carried out.

Purity of EcASNase biogenics was analysed using a combination of SDS-PAGE, SEC-MALS and RP-HPLC along with assays for HCP content and bacterial endotoxin contamination. SDS-PAGE indicated presence of additional higher and lower molecular weight bands in all EcASNase biogenics that were not observed in the reference EcASNase product (Figure 2A). While SEC showed a single peak in the reference sample, additional peaks (with shorter retention times, at 8.7 and 11.5 minutes) were observed in all EcASNase biogenics, indicating presence of higher molecular weight aggregates (5–18%) (Figure 2B). Multiangle light scattering confirmed that these aggregates were multimers of the EcASNase tetramer (molecular mass >280 kDa). Analysis by RP-HPLC demonstrated product impurities in all EcASNase biogenics (Figure 2C). HCP content was high in six of seven biogenics, nearing upper tolerable limits in two and exceeding recommended thresholds in four brands (Table 2).

In addition to the above, inconsistencies were observed in the appearance of the lyophilised cake in the biogenic products, with potential deleterious consequences for drug reconstitution, recovery and stability. A homogenous lyophilised cake was observed in only two products, with the rest as either free-flowing or crystalline powder. Product reconstitution time ranged from 30 to 100 seconds, resulting eventually in a clear colourless solution, although one brand was observed to have particle threads after reconstitution. Although the osmolality of all EcASNase formulations was below 600 mOsm/kg, osmolality values were inconsistent. Most formulations were hypotonic (exceedingly low in two, <100 mOsm/kg), while one product had an osmolality close to the upper tolerable limit.

3.3 Analysis of in vitro cytotoxicity of EcASNsases is not a discriminatory determinant of product quality and therapeutic activity

Malignant lymphoblast cell lines with relatively high (SUP-B15) and low (REH) sensitivity to EcASNase¹⁶ were tested in the WST-1 assay to examine the differential cytotoxic activities of biogenic and reference EcASNase products (Figure 3). In both cell lines, no differential sensitivities were observed to the EcASNase products, suggesting that

conventional in vitro assays of cytotoxicity do not serve as surrogate determinants of therapeutic suitability of these compounds.

4 Discussion

Our findings reiterate concerns over the quality of biogeneric EcASNases marketed in LMICs.^{12,13} None of the seven biogeneric formulations of native EcASNase marketed widely in India met the minimum manufacturing quality standards required for these products. These shortcomings in manufacturing quality conceivably impair the therapeutic activity of these biogeneric EcASNases as demonstrated by P2. Importantly, neither in vitro asparaginase activity nor cytotoxic activity of these products was a suitable surrogate determinant of poor therapeutic activity.

Observations with the P2 biogeneric serve as exemplar. Excess impurities were detected in vials of the P2 biogeneric (average purity, 89%), associated with high levels of protein aggregates (10%) and HCP contamination (~250 parts per million). Estimated activity of the product was ~25% lower than that specified in the product label. The unusually low osmolality of the batches tested renders the P2 product unsuitable for parenteral use. Unsatisfactory therapeutic activity was observed when the P2 biogeneric was administered to patients. Generic EcASNase dosed at 2500–5000 IU/m² every 72-hours has previously been reported to produce trough levels of >100 IU/L in 95% of patients.¹⁷ In contrast, nearly two thirds of patients administered intramuscular P2 EcASNase at standard doses (10,000 IU/m²/dose, every 72 hours) demonstrated inadequate drug activity at the 72-hour trough timepoint. Nearly one in five patients administered the P2 biogeneric developed clinical hypersensitivity, almost all of whom were treated on high-risk schedules that require intensified asparaginase therapy. Variations in amino acid sequence contribute to but do not probably account fully for the observed differences in therapeutic activity and antigenicity of the P2 biogeneric.

These observations have several implications. Asparaginase is primarily used early in the therapy of ALL. Inadequate asparaginase activity is likely to compromise the early response to therapy, a key prognostic determinant of survival in ALL.^{18–21} This is especially so in patients administered anthracycline-free schedules of asparaginase, vincristine and corticosteroid during the induction treatment phase. The variable quality of generic agents, in particular biologicals such as asparaginase, marketed in LMICs²² is potentially a significant determinant of the observed differences in outcomes between these countries and more affluent nations.^{23,24}

Nearly one in five patients administered P2 EcASNase, the majority with high-risk disease, had to discontinue asparaginase treatment due to clinical hypersensitivity. Erwinase is unaffordable in our context. The North American Children's Oncology Group reported higher risk of treatment failure in patients who required premature discontinuation of ASNase therapy, particularly in patients with high-risk disease who require intensive ASNase therapy.²⁵ ASNase discontinuation in these patients was predominantly due to clinical hypersensitivity. The Nordic Society of Paediatric Haematology and Oncology (NOPHO) group similarly reported increased risk of treatment failure in patients

with shortened ASNase schedules, including in patients with silent ASNase inactivation identified through therapeutic drug monitoring.²⁶

In our cohort, change to alternate-day dosing of P2 EcASNase (48-hour dose intervals) to obtain trough therapeutic levels would still be associated with inadequate drug activity in a third of patients. The additional drug doses with the alternate-day schedule are also likely to increase the risk of drug hypersensitivity and cost of treatment. Similar subtherapeutic activity has been observed with other biogeneric EcASNases in LMICs. Investigators from India have reported unsatisfactory drug activity in 75% of postdose plasma samples obtained from patients administered nine different biogeneric EcASNase formulations, with the proportion of subtherapeutic activity varying widely among the generic agents (from ~25% to ~100%).¹² Investigators in Brazil observed unsatisfactory asparaginase activity in vivo in a high proportion of patients administered a biogeneric EcASNase (97%) compared to the reference product (19%) with resultant inferior outcomes.²⁷ High level of HCP contamination (estimated at 19–37% of product content) was observed in biogeneric formulations, potentially accounting for the decreased drug bioavailability observed in patients and heightening the risk of immune reactions to EcASNase.¹³ Recent availability of PEG-conjugated EcASNase biogenics provides a potential therapeutic alternative. At least six biogeneric PEG-EcASNase brands are marketed in India currently. Switching to PEG-EcASNase biogenics is not a ready remedy. Manufacture of these compounds is a challenge, requiring stable uniform PEG-conjugation of EcASNase, minimising polydispersible complexes and diol impurities.²⁸ Inadequacies in PEG-conjugation will compound existing shortcomings in the manufacture of native EcASNase biogenics. The relatively high cost of PEG products and the limited stability of these liquid formulations are additional drawbacks. Added to this are the intrinsic variabilities associated with altered amino acid sequences of the EcASNase isoforms in these products. Clinical use of PEG-EcASNase biogenics will therefore require stringent tests of product quality and monitored studies to determine suitability for therapeutic use and the appropriate dose and schedule.

In summary, our findings echo deep concerns that across the globe for many patients with ALL, assured access to high-quality affordable asparaginases continues to remain an unmet therapeutic need.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AHA	L-aspartic acid β -hydroxamate
ALL	acute lymphoblastic leukaemia
ASNase	asparaginase
EcASNase	<i>E. coli</i> -derived asparaginase
HCP	host cell proteins
IQR	interquartile range
LMICs	low-middle-income countries
PEG-EcASNase	polyethylene glycol conjugated <i>E. coli</i> -derived asparaginase
pI	isoelectric point
RP-HPLC	reversed phase high-performance liquid chromatography
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC-MALS	size exclusion chromatography coupled with multiangle light scattering
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. Vora A, Goulden N, Mitchell C, et al. Augmented post-remission therapy for a minimal residual disease-defined high-risk subgroup of children and young people with clinical standard-risk and intermediate-risk acute lymphoblastic leukaemia (UKALL 2003): a randomised controlled trial. *Lancet Oncol.* 2014; 15 (8) 809–818. [PubMed: 24924991]
2. Toft N, Birgens H, Abrahamsson J, et al. Results of NOPHO ALL2008 treatment for patients aged 1–45 years with acute lymphoblastic leukemia. *Leukemia.* 2018; 32 (3) 606–615. [PubMed: 28819280]

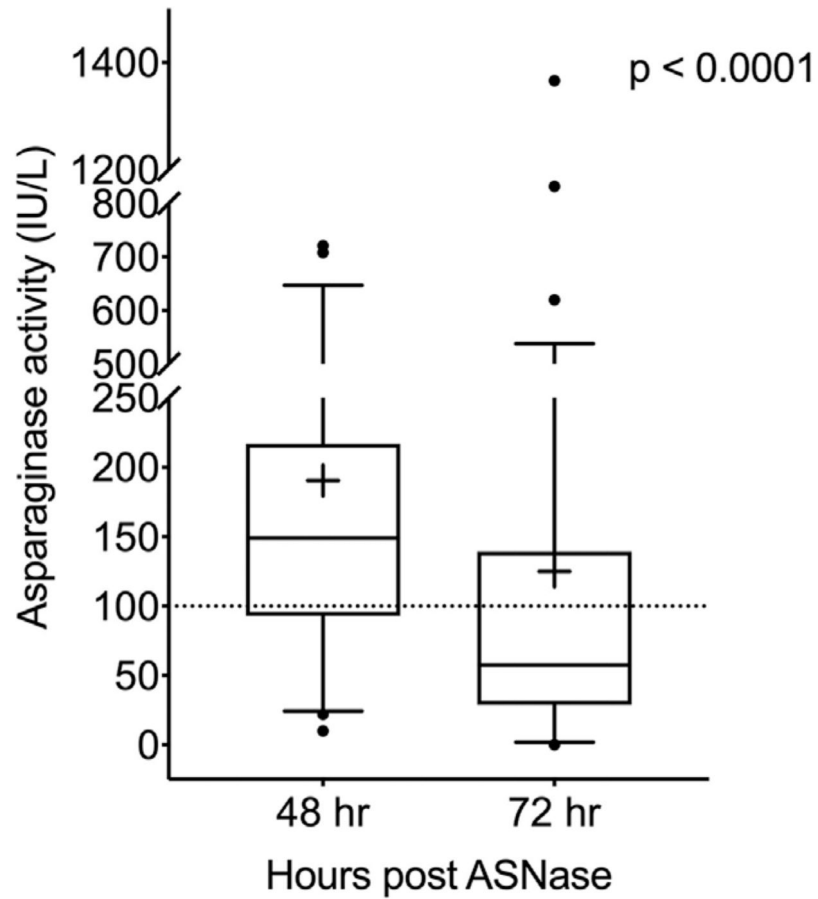
3. Jeha S, Pei D, Choi J, et al. Improved CNS control of childhood acute lymphoblastic leukemia without cranial irradiation: St Jude Total Therapy Study 16. *J Clin Oncol*. 2019; 37 (35) 3377–3391. [PubMed: 31657981]
4. Broome JD. Studies on the mechanism of tumor inhibition by L-asparaginase. Effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. *J Exp Med*. 1968; 127 (6) 1055–1072. [PubMed: 4871211]
5. Miller HK, Balis ME. Glutaminase activity of L-asparagine amidohydrolase. *Biochem Pharmacol*. 1969; 18 (9) 2225–2232. [PubMed: 4899451]
6. Boos J, Werber G, Ahlke E, et al. Monitoring of asparaginase activity and asparagine levels in children on different asparaginase preparations. *Eur J Cancer*. 1996; 32A (9) 1544–1550. [PubMed: 8911116]
7. van der Sluis IM, Vrooman LM, Pieters R, et al. Consensus expert recommendations for identification and management of asparaginase hypersensitivity and silent inactivation. *Haematologica*. 2016; 101 (3) 279–285. [PubMed: 26928249]
8. Hempel G, Muller HJ, Lanvers-Kaminsky C, Wurthwein G, Hoppe A, Boos J. A population pharmacokinetic model for pegylated-asparaginase in children. *Br J Haematol*. 2010; 148 (1) 119–125. [PubMed: 19821822]
9. Rizzari C, Citterio M, Zucchetti M, et al. A pharmacological study on pegylated asparaginase used in front-line treatment of children with acute lymphoblastic leukemia. *Haematologica*. 2006; 91 (1) 24–31. [PubMed: 16434367]
10. Liu WJ, Wang H, Wang WD, et al. Use of PEG-asparaginase in newly diagnosed adults with standard-risk acute lymphoblastic leukemia compared with E. coli-asparaginase: a retrospective single-center study. *Sci Rep*. 2016; 6 39463 [PubMed: 28000713]
11. Patel N, Krishnan S, Offman MN, et al. A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J Clin Invest*. 2009; 119 (7) 1964–1973. [PubMed: 19509471]
12. Sankaran H, Sengupta S, Purohit V, et al. A comparison of asparaginase activity in generic formulations of E.coli derived L-asparaginase: in-vitro study and retrospective analysis of asparaginase monitoring in pediatric patients with leukemia. *Br J Clin Pharmacol*. 2020; 86 (6) 1081–1088. [PubMed: 31925802]
13. Zenatti PP, Migita NA, Cury NM, et al. Low bioavailability and high immunogenicity of a new brand of E. coli L-asparaginase with active host contaminating proteins. *EBioMedicine*. 2018; 30: 158–166. [PubMed: 29550241]
14. Lanvers C, Vieira Pinheiro JP, Hempel G, Wuerthwein G, Boos J. Analytical validation of a microplate reader-based method for the therapeutic drug monitoring of L-asparaginase in human serum. *Anal Biochem*. 2002; 309 (1) 117–126. [PubMed: 12381370]
15. Swain AL, Jaskolski M, Housset D, Rao JK, Wlodawer A. Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. *Proc Natl Acad Sci U S A*. 1993; 90 (4) 1474–1478. [PubMed: 8434007]
16. Parmentier JH, Maggi M, Tarasco E, Scotti C, Avramis VI, Mittelman SD. Glutaminase activity determines cytotoxicity of L-asparaginases on most leukemia cell lines. *Leuk Res*. 2015; 39 (7) 757–762. [PubMed: 25941002]
17. Ahlke E, Nowak-Gottl U, Schulze-Westhoff P, et al. Dose reduction of asparaginase under pharmacokinetic and pharmacodynamic control during induction therapy in children with acute lymphoblastic leukaemia. *Br J Haematol*. 1997; 96 (4) 675–681. [PubMed: 9074406]
18. Campana D, Pui CH. Minimal residual disease-guided therapy in childhood acute lymphoblastic leukemia. *Blood*. 2017; 129 (14) 1913–1918. [PubMed: 28167658]
19. Pui CH, Pei D, Raimondi SC, et al. Clinical impact of minimal residual disease in children with different subtypes of acute lymphoblastic leukemia treated with response-adapted therapy. *Leukemia*. 2017; 31 (2) 333–339. [PubMed: 27560110]
20. Schrauder A, Stanulla M, Flohr T, et al. Prospective evaluation of MRD-kinetics in 274 children with high-risk ALL treated in trial ALL-BFM 2000: insights into development of resistance and impact on further refinement of treatment stratification strategies. *Blood*. 2007; 110 (11) 585.

21. Cazzaniga G, De Lorenzo P, Alten J, et al. Predictive value of minimal residual disease in Philadelphia-chromosome-positive acute lymphoblastic leukemia treated with imatinib in the European intergroup study of post-induction treatment of Philadelphia-chromosome-positive acute lymphoblastic leukemia, based on immunoglobulin/T-cell receptor and BCR/ABL1 methodologies. *Haematologica*. 2018; 103 (1) 107–115. [PubMed: 29079599]
22. Renner L, Nkansah FA, Doodoo AN. The role of generic medicines and biosimilars in oncology in low-income countries. *Ann Oncol*. 2013; 24 (Suppl 5) v29–v32. [PubMed: 23975702]
23. Arnold M, Rutherford MJ, Bardot A, et al. Progress in cancer survival, mortality, and incidence in seven high-income countries 1995–2014 (ICBP SURVMARK-2): a population-based study. *Lancet Oncol*. 2019; 20 (11) 1493–1505. [PubMed: 31521509]
24. India State-Level Disease Burden Initiative Cancer Collaborators. The burden of cancers and their variations across the states of India: the Global Burden of Disease Study 1990–2016. *Lancet Oncol*. 2018; 19 (10) 1289–1306. [PubMed: 30219626]
25. Gupta S, Wang C, Raetz EA, et al. Impact of asparaginase discontinuation on outcome in childhood acute lymphoblastic leukemia: a report from the Children’s Oncology Group. *J Clin Oncol*. 2020; 38 (17) 1897–1905. [PubMed: 32275469]
26. Gottschalk Hojfeldt S, Grell K, Abrahamsson J, et al. Relapse risk following truncation of PEG-asparaginase in childhood acute lymphoblastic leukemia. *Blood*. 2020; doi: 10.1182/blood.2020006583
27. Ceconello DK, Werlang ICR, Alegretti AP, et al. Monitoring asparaginase activity in middle-income countries. *Lancet Oncol*. 2018; 19 (9) 1149–1150. [PubMed: 30078698]
28. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. *J Pharm Sci*. 2016; 105 (2) 460–475. [PubMed: 26869412]

Meeting abstract

Part of the paper (in vivo activity of generic asparaginase) featured as an oral presentation at the National Paediatric Haematology Oncology Conference, 2017 at Kolkata.

Abstract published in *Pediatr Hematol Oncol J.* 2017;2(2):S2-S3 (<https://doi.org/10.1016/j.phoj.2017.11.132>).



	48 hr	72 hr
Number of values	47	70
Median (IU/L)	149	58
Inter-quartile range (IU/L)	93- 217	29 - 139
Mean (IU/L)	191	125
Std. Deviation	159	209

Figure 1. Subtherapeutic asparaginase activity in patients administered the P2 EcASNase biogeneric.

Box-plot representation of plasma asparaginase activity at trough (72-hour post-dose, $n = 70$ samples) and 48-hour post-dose timepoints ($n = 47$ samples) during the ALL induction treatment phase in patients ($N = 62$) administered P2 EcASNase intramuscularly at 10,000 IU/m²/dose every 72 hours. Adequate asparaginase activity (> 100 IU/L, horizontal dotted line) was observed in 34% (24/70) of trough timepoint samples and 70% (33/47) of 48-hour postdose samples (Mann–Whitney U test of significance, $p = .00002$). The interquartile ranges are indicated by boxes, whiskers indicate values 1.5 times the upper and lower

quartiles, filled circles indicate outliers, median values are indicated by horizontal bold lines within boxes and '+' within the boxes indicate mean values

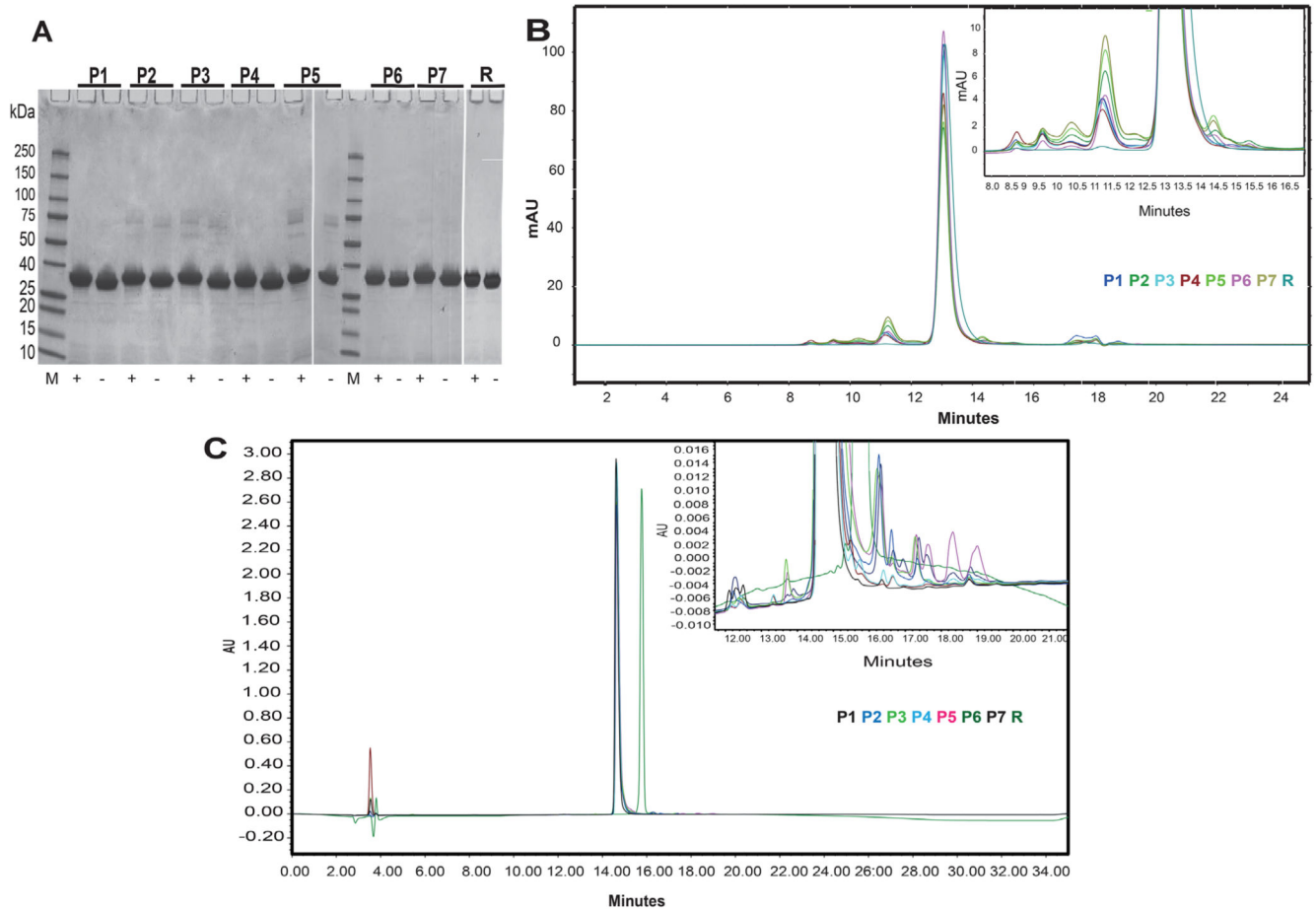


Figure 2. Multimodal analyses indicate impurities and amino acid sequence variations in EcASNase biogenics.

Examination of EcASNase biogenics P1–P7 and the reference EcASNase (R) using multimodal analytical techniques, including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, A), size exclusion chromatography (SEC, B) and reversed phase high-performance liquid chromatography (RP-HPLC, C). (A) Image of Coomassie-stained SDS-PAGE analysis of EcASNase products examined in reducing (+) and non-reducing (-) conditions confirms presence of the ~36 kDa EcASNase subunit in all products, and shows additional higher and lower molecular weight bands in the biogenics, suggesting presence of protein impurities (M, molecular weight marker). (B) Overlay of chromatograms from SEC analyses of EcASNase products indicates that in addition to the primary peak of EcASNase, biogenic products are characterised by additional smaller peaks with shorter retention times (inset, magnified view), suggesting the presence of higher molecular weight impurities, likely multimer aggregates of EcASNase (mAU, milli-absorbance unit). (C) Overlay of chromatograms from RP-HPLC analyses of EcASNase products indicate difference in retention times (~1 minute) between EcASNase biogenics and the reference product, likely related to differences in hydrophobicity owing to amino acid sequence variations in the biogenic EcASNases. Additional smaller peaks with varying

retention times are also observed in the biogeneric products, suggesting impurities (inset, magnified view). (AU, absorbance unit)

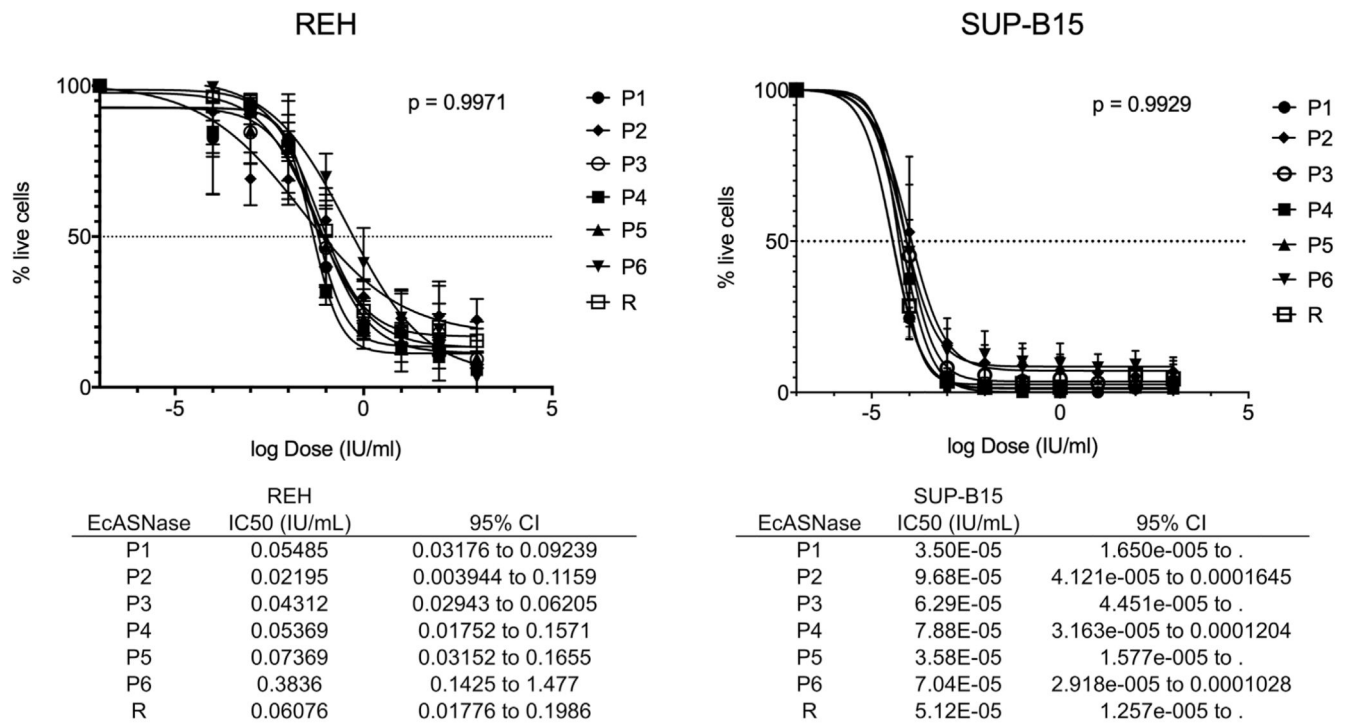


Figure 3. Unsatisfactory quality of EcASNase biogenics is not identified by standard in vitro cytotoxicity studies.

Drug response profile of malignant lymphoblast cell lines with relatively high (SUP-B15) and low (REH) sensitivity in vitro to EcASNase, treated for 72 hours with EcASNase products (biogenics P1–P6; reference product R) at serial 10-fold dilutions (0.001–1000 IU/ml) and assayed for viability using the WST-1 assay. Graph depicts pooled results from three separate experiments. Error bars represent standard errors of mean. ANOVA was used as the test for significance. Four-parameter logistic regression was used to obtain the dose response curve with the x -axis indicating log EcASNase concentration ($\log(\text{IU/ml})$) and the y -axis indicating residual live cells as a percentage of untreated controls. 95% CI, 95% confidence interval; ANOVA, one-way analysis of variance; IC50, half maximal inhibitory concentration (IU/ml)

Table 1
Clinical characteristics of the P2 EcASNase patient cohort (N = 62)

	N	%
Age (years)		
Median (IQR)	2.8 (3.9–6.1)	
Range	1.4–18.2	
Sex		
Male	41	66
Female	21	34
Immunophenotype		
Precursor B	62	100
WBC ($\times 10^9/L$)		
<50	45	73
50	17	27
Median (IQR)	22.3(7.1–51.5)	
Complete remission at end of induction		
Yes	58	94
No	4	6
Minimal residual disease at end of induction		
Low (<0.01%)	44	71
High (0.01%)	18	29
Risk groups^a		
Standard risk	16	26
Intermediate risk	10	16
High risk	36	58
Asparaginase-associated toxicities		
Hypersensitivity	12	19
Pancreatitis	2	3
Thrombosis	0	0

Note: All hypersensitivity reactions were CTCAE grade 3. For pancreatitis, one patient each for CTCAE grade 3 and CTCAE grade 4.

Abbreviations: CTCAE, Common Terminology Criteria for Adverse Events; IQR, interquartile range.

^aRisk groups: Standard risk (SR) = age 1 to <10 years at diagnosis, presenting WBC $<50 \times 10^9/L$, prednisolone good response, no high-risk cytogenetics, MRD $<10^{-4}$ end of induction and no CNS disease. Intermediate risk (IR) = age 10 years at diagnosis or presenting WBC $50 \times 10^9/L$ or bulky/testicular disease, prednisolone good response, no high-risk cytogenetics and no CNS disease. High risk (HR) = with high-risk cytogenetics or CNS disease or prednisolone poor response or MRD 10^{-4} end of induction.

Table 2
Analysis of critical quality attributes of native EcASNase biogenerics marketed in India

	Expected	Native <i>EcASNase</i> biogenerics marketed in India							Reference
		P1	P2	P3	P4	P5	P6	P7	EcASNase
Manufacturing batches tested		2	2	1	2	2	1	1	1
Drug strength									
Specific activity (IU/mg)	>220	220.2	243.2	157.8	211.2	189.8	154.0	162.0	256.3
Activity per 10,000 IU vial (IU)	10,000 ± 1500	9381.1	7446.3	5295.5	11,696.7	9215.5	5666.5	7080.5	9556.4
Drug identity									
Molecular weight, SEC (kDa)	134-136	135.8	136.2	135.9	134.6	135.6	136.6	135.5	134.6
Isoelectric point (pI)	5.37 ± 0.30	5.78	5.75	5.79	5.84	5.81	5.60	5.86	5.46
Drug purity									
Aggregates by SEC (%)	<5%	6.0	10.1	17.0	6.6	12.9	8.0	5.8	0.8
Purity by RP-HPLC (%)	>90%	93.8	88.5	79.4	92.0	85.4	91.8	92.6	99.2
Host cell protein (ppm)	<100	9.0	338.5	613.0	85.4	232.5	123.9	320.6	2.6
Bacterial endotoxin (EU/ml)	<50	2.0	1.3	5.1	1.2	0.4	11.0	41.0	0.2
Suitability for parenteral use									
Osmolality (mOsm/kg)	250–600	178.5	11	129	106.5	104.5	557	68	279

Note: Values outside acceptable limits are in bold font.

Abbreviations: EcASNase, *Escherichia coli* L-asparaginase; EU/ml, endotoxin Units/ml; kDa, kilodalton; ppm, parts per million; RP-HPLC, reversed phase high-performance liquid chromatography; SEC, size exclusion chromatography.

Table 3
Protein identification by peptide mass fingerprinting

EcASNase	Sequence coverage (%) ^a	Missing sequence regions
P1	91.1	51-72 ; 92-95; 302-303
P2	97.9	92-95;302-303
P3	97.9	92-95; 302-303
P4	94.5	92-95; 252-262 ; 302-303
P5	97.9	92-95; 302-303
P6	94.5	92-95; 252-262 ; 302-303
P7	97.9	92-95; 302-303
R	98.2	92-95;302-303

Note: Protein identification by peptide mass fingerprinting indicates presence of isoforms of EcASNase in biogeneric products, suggested both by the reduced sequence coverage and distinct missing amino acid sequences (highlighted in bold, P1, P4, P6) in these products. Amino acid sequence regions 92-95 and 302-303 were not detected in all samples, indicating technical limitations in the analysis

Abbreviations: EcASNase, *E. coli* L-asparaginase; P1-P7, biogeneric native EcASNases; R, reference EcASNase.

^aWith respect to the theoretical reference amino acid sequence of *E. coli* L-asparaginase.