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Research Paper

Low Bioavailability and High Immunogenicity of a New Brand of *E. coli* L-Asparaginase with Active Host Contaminating Proteins

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ABSTRACT

The drug L-asparaginase is a cornerstone in the treatment of acute lymphoblastic leukemia (ALL). The native *E. coli* L-asparaginase used in Brazil until recently has been manufactured by Medac/Kyowa. Then a decision was taken by the Ministry of Health in 2017 to supply the National Health System with a cheaper alternative L-asparaginase manufactured by Beijing SL Pharmaceutical, called Leuginase®. As opposed to Medac, the asparaginase that has been in use in Brazil under the trade name of Aginasa®, it was not possible to find a single entry with the terms Leuginase in the Pubmed repository. The apparent lack of clinical studies and the scarcity of safety information provided to the hospitals by the drug distributor created a debate among Brazilian pediatric oncologists about issues of safety and efficacy that culminated eventually in a court decision to halt the distribution of the new drug all over the country. Boldrini Children's Center, a non-profit pediatric oncohematology hospital, has conducted its own evaluation of Leuginase®. Mass spectrometry analyses found at least 12 different contaminating host-cell proteins (HCP) in Leuginase®. The presence of two HCP (beta-lactamase and malate dehydrogenase) was confirmed by orthogonal methodologies. The relative number of HCP peptides ranged from 19 to 37% of the total peptides identified by mass spectrometry. *In vivo* studies in mice injected with Leuginase® revealed a 3 times lower plasma bioavailability and the development of higher antibody titres against L-asparaginase in comparison to Aginasa®-injected animals. The decision to buy a new drug based on its price alone is not safe. Developing countries are especially vulnerable to cheaper alternatives that lack solid quality assurance.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. According to the last Brazilian cancer incidence data, approximately 3000 children (up to 19 years of age) are diagnosed with ALL annually (Estimativa 2018, 2017). Brazilian children enrolled in prospective cooperative clinical trials achieve survival rates comparable to those reported by centres in Western Europe and North America (Ribeiro et al., 2007; Möricke et al., 2010; Silverman et al., 2010; Hunger et al., 2012; Brandalise et al., 2010, 2016). ALL treatment is based fundamentally on combination chemotherapy. One essential drug is L-asparaginase, an enzyme produced in *Escherichia coli* that catalyzes the hydrolysis of asparagine into ammonia and aspartic acid (Pieters et al., 2011). The clinical effectiveness of this drug is based on asparagine depletion and the selective vulnerability of lymphoblasts whose survival is dependent on extracellular sources of asparagine (Müller and Boos, 1998).

Public health services are provided freely to almost 75% of the Brazilian population by the Government (Montekio et al., 2011). In the beginning of 2017 the Brazilian Ministry of Health acquired a new L-asparaginase produced by Beijing SL Pharmaceutical (China) with the trade name of Leuginase®. Due to eminent risk of shortage, the Ministry of Health purchased this new drug based on price and under less stringent import conditions, *i.e.* solely on registration in the country of origin and Good Manufacturing Practices certificate, without comparability studies. The virtual absence of clinical studies with the drug prompted our institution to investigate the purity, bioavailability and immunogenicity of Leuginase®, provided to the hospitals directly by the Government, in comparison to Medac L-asparaginase – the drug in prior use in the Country under the trade name of Aginasa® (Medac/Kyowa).

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2. Methods

2.1. Reagents

Leuginase® (Beijing SL Pharmaceutical) vials used in this study were from batch number 2016100101, validation date 10/23/2018. Aginasa® (Medac/Kyowa) vials were from batch number G140371A, validation date 07/31/2017. Antibodies used in ELISA were: anti-L-asparaginase (Abcam, ab55824), HRP-conjugated goat anti-mouse IgG (KPL, 074-1806), and HRP-conjugated goat anti-rabbit IgG (KPL, 04-15-16).

2.2. Protein Quantification

Protein mass quantifications of the L-asparaginase preparations were done using a fluorescence assay (Qubit Protein Assay, ThermoFisher Scientific), according to the manufacturer recommendations.

2.3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Assay

The LC-MS/MS method adopted for the analysis conducted at LNBio (Campinas, Brazil) is described here. The corresponding method used at MS Bioworks (Ann Arbor, MI, USA) can be provided on request. L-asparaginase samples were reconstituted in saline (0.9% NaCl), 30 μg of protein were aliquoted and 10 μL Ureia 8 M and 0.4 μL 250 mM DTT were added. This mix was then incubated at 56 °C for 25 min, followed by addition of 0.57 μL 500 mM iodoacetamide and incubation for 30 min at room temperature in the dark. After alkylation, 0.4 μL 250 mM DTT was added again and incubated for 15 min. After these steps of reduction and alkylation, samples were digested by addition of 53.25 μL 50 mM ammonium bicarbonate, 0.74 μL 100 mM $CaCl_2$ and 1 μg of trypsin or chymotrypsin (Sequence Grade Modified, Sigma Aldrich) and incubated at 37 °C for 13 h. The reaction was stopped by addition of trifluoroacetic acid to a final concentration of 1%. Samples were then de-salted by the method of Stage Tips (Rappsilber et al., 2007). The samples were dried in a vacuum concentrator and reconstituted in 135 μL of 0.1% of formic acid. Two μL containing 0.44 μg of the resulting peptide mixture was analyzed on an ETD enabled LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with LC-MS/MS by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source.

Peptides were separated by a 2–30% acetonitrile gradient in 0.1% formic acid using a C18 PicoFrit Column (20 cm × ID75 μm, 5-μm particle size; New Objective) and an EASY-nLC at a flow rate of 300 nL/min over 30 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275 °C. The scan MS spectra (m/z 300–1600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1×10^6 (Brandalise et al., 2010). Resolution in the Orbitrap was set to $r = 60{,}000$ (m/z 400). Peptide ions were sequentially isolated to a target value of 80,000 and fragmented in the HCD (high collisional dissociation) energy (normalized collision energy of 40%). The signal threshold for triggering an MS/MS event was set to 7500 counts. An activation time of 0.1 ms was used.

The raw files were processed using Proteome Discoverer 1.4 (Thermo Scientific), and the MS/MS spectra were searched using the Sequest software against the Uniprot SwissProt *E. coli* database (Release: March 31th, 2017; 10,082 entries), with a tolerance of 10 ppm for precursor ions, 0.02 Da for fragment ions, and a maximum of 1 missed cleavage for protein identification. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was chosen as a variable modification. Both peptide and protein identifications were filtered at a maximum of 1% false discovery rate. Raw data for LNBio and MS Bioworks may be provided on request. Results from Butantan Institute can be found by entering the code 15705121420402650000000001247715 in the following link: http://pje1g.trf3.jus.br:80/pje/Processo/ConsultaDocumento/listView.seam

2.4. N-Terminus Region Analyses

The raw data from MS Bioworks were processed using Mascot Distiller 2.3 and the resulting MGF file was used in Mascot server 2.3 (Matrix Science Ltd) to search for non-specific cleavages using the same parameters described above. The scoring function model was not specific to any particular digestion enzyme, so that all peptides (including but not restricted to those generated by trypsin) were accessible.

2.5. L-Asparaginase Activity

L-Asparaginase (EC 3.5.1.1) activity in Leuginase®, Aginasa®, and murine plasma samples was measured as described previously (Lanvers et al., 2002). Leuginase® and Aginasa® were diluted in Tris buffer, pH 7.3 (0.015 M), supplemented with 0.015% (w/v) bovine serum albumin fraction V (BSA) at concentrations of 5 IU/L, 10 IU/L, 20 IU/L, and 30 IU/L. For the determination of L-asparaginase activity, 20 μL of each enzyme dilution was mixed with 180 μL of 2 mM L-aspartic beta-hydroxamate (AHA) solution dissolved in Tris/BSA buffer (64.5 mM Tris, pH 7.3, 0.15 mg/mL Bovine Serum Albumin fraction V). Assays were performed in triplicates, in 96-well plates. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 60 μL trichloroacetic acid (24.5%, w/v), and the samples were centrifuged for 5 min at 2500 rpm. Fifty microliters of the supernatant were transferred to a new well and 200 μL of freshly made Oxin reagent (1 vol. of 2% 8-hydroxyquinoline in ethanol and 3 vol. of 1 M sodium carbonate solution) was added. After heating at 95 °C for 1 min and cooling down the plate for exactly 10 min, absorbane was measured at 690 nm in a Synergy H1 Hybrid Reader (Biotek).

2.6. Beta-Lactamase Activity

Beta-lactamase (EC 3.5.2.6) activity was assessed indirectly by a modification of the antibiotic sensitivity assay. Briefly, antibiotic discs (10 μg to 30 μg) were impregnated with Aginasa® (50 to 100 IU) or Leuginase® (0.5 to 100 IU) or control and placed on agar plates inoculated with *Escherichia coli* (ATCC 25922). After 18 h incubation at 37 °C inhibition rings were compared.

2.7. Malate Dehydrogenase Activity

Malate dehydrogenase (EC 1.1.1.37) activity was assayed in the forward direction (NADH production) as previously described (Dasika et al., 2015). Mass inference was obtained by comparison to a standard curve made with recombinant *E. coli* malate dehydrogenase (Sigma-Aldrich, cat# SRP6105). Reactions were assembled in 96-well flat-bottom plates kept on ice. Sixteen microliters of different amounts of Leuginase®, Aginasa® and recombinant *E. coli* malate dehydrogenase (Sigma-Aldrich, cat# SRP6105) were added to 100 μL of a reaction mixture containing 1 mM NAD, 1 mM Malic acid, 90 mM KCl, 100 mM Tris, pH 8.9. NADH concentration was immediately measured by fluorescence at 470 nm with excitation at 340 nm, at 37 °C, using a Synergy H1 Hybrid Reader (Biotek). Data were measured over time. Data presented refer to 2:08 min, when a plateau was reached for the highest concentrations.

2.8. L-Asparaginase Bioavailability and Immunogenicity Assays in Mice

The study was registered and approved by CEUA/UNICAMP under #4556-1/2017. Animals were maintained with food and drink *ad libitum* in ventilated racks. Ten Balb/c female mice (6 to 8-week-old) were distributed randomly in two groups of five animals and received injections of 525 IU/kg of Leuginase® or Aginasa®. Blood was collected in EDTA

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