In vivo stabilization of a less toxic asparaginase variant leads to a durable antitumor response in acute leukemia

Maaike Van Trimpont,[1,2,3] Amanda M. Schalk,[4] Yanti De Visser,[1,2,5] Hien Anh Nguyen,[4] Lindy Reunes,[1,2] Katrien Vandemeulebroecke,[1,6,7] Evelien Peeters,[1,3] Ying Su,[4] Hyun Lee,[8,9] Philip L. Lorenzi,[10] Wai-Kin Chan,[10] Veerle Mondelaers,[1,6] Barbara De Moerloose,[1,6,7] Tim Lammens,[1,6,7] Steven Goossens,[1,3] Pieter Van Vlierberghe[1,2#] and Arnon Lavie[4,11#]

[1]Cancer Research Institute Ghent (CRIG), Ghent, Belgium; [2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium; [3]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium; [4]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL, USA; [5]Department of Imaging and Pathology, KU Leuven, Leuven, Belgium; [6]Department of Pediatric Hemato-Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium; [7]Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium; [8]Department of Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago (UIC), Chicago, IL, USA; [9]Biophysics Core at Research Resources Center, University of Illinois at Chicago (UIC), Chicago, IL, USA; [10]Metabolomics Core Facility, Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA and [11]The Jesse Brown VA Medical Center, Chicago, IL, USA.

#PVV and AL contributed equally as co-senior authors

Correspondence: A. Lavie
Lavie@uic.edu

Received: May 11, 2022.
Accepted: August 4, 2022.
Prepublished: August 18, 2022.

https://doi.org/10.3324/haematol.2022.281390

Abstract

Asparagine is a non-essential amino acid since it can either be taken up via the diet or synthesized by asparagine synthetase. Acute lymphoblastic leukemia (ALL) cells do not express asparagine synthetase or express it only minimally, which makes them completely dependent on extracellular asparagine for their growth and survival. This dependency makes ALL cells vulnerable to treatment with L-asparaginase, an enzyme that hydrolyzes asparagine. To date, all clinically approved L-asparaginases have significant L-glutaminase co-activity, associated with non-immune related toxic side effects observed during therapy. Therefore, reduction of L-glutaminase co-activity with concomitant maintenance of its anticancer L-asparaginase effect may effectively improve the tolerability of this unique drug. Previously, we designed a new alternative variant of *Erwinia chrysanthemi* (*ErA*; Erwinaze) with decreased L-glutaminase co-activity, while maintaining its L-asparaginase activity, by the introduction of three key mutations around the active site (*ErA*-TM). However, Erwinaze and our *ErA*-TM variant have very short half-lives *in vivo.* Here, we show that the fusion of *ErA*-TM with an albumin binding domain (ABD)-tag significantly increases its *in vivo* persistence. In addition, we evaluated the *in vivo* therapeutic efficacy of ABD-*ErA*-TM in a B-ALL xenograft model of SUP-B15. Our results show a comparable long-lasting durable antileukemic effect between the standard-of-care pegylated-asparaginase and ABD-*ErA*-TM L-asparaginase, but with fewer co-glutaminase-related acute side effects. Since the toxic side effects of current L-asparaginases often result in treatment discontinuation in ALL patients, this novel *ErA*-TM variant with ultra-low L-glutaminase co-activity and long *in vivo* persistence may have great clinical potential.

Introduction

L-asparaginases (L-ASNases) are cornerstone drugs in the treatment of acute lymphoblastic leukemia (ALL) and have greatly improved patients' outcomes over the last 40 years.[1,2] Since the introduction of L-ASNases, mortality rates in pediatric ALL patients have drastically decreased, currently reaching overall 5-year survival rates of almost 90%.[3] While the depletion of blood asparagine (Asn) represents a critical component of the multiple-drug ALL treatment regimen,[3-5] the use of L-ASNase is also associated with a variety of toxic side effects.[6-9] A large part of these unwanted effects can be attributed to the secondary L-glutaminase co-activity displayed by all clinically approved bacterial L-ASNases.[10] These L-ASNases not only convert Asn into L-aspartate (Asp) and ammonia, but also hydrolyse glutamine (Gln) into L-glutamate (Glu) and ammonia.[11-13] For all clinically approved L-ASNases, this L-glutaminase co-activity ranges from 2-10% of their primary L-ASNase activity depending on their microbiological origin.[10,14,15] Importantly, Gln is much more abundant than Asn in the blood, with levels that range between 500-800 μM compared to ~50 μM for Asn.[16,17] Because Gln levels are much higher, extensive amounts of ammonia are produced which

might contribute to L-ASNase-induced neurotoxicity.[18-20] The L-glutaminase co-activity also has an impact on protein synthesis in the liver and pancreas resulting in hepatotoxicity, pancreatitis, hyperglycemia, leukopenia and thrombosis.[6,14,21]

Over the past years, several attempts have been made to develop less toxic, low-glutaminase variants from various biological sources with better enzymatic parameters.[10,21,22] Important criteria to keep in mind while designing alternative L-ASNases are efficacy, toxicity, kinetic properties, pharmacokinetics (i.e. *in vivo* half-life) and immunogenicity.[22-24] The active degradation of native L-ASNase enzymes and their short half-life represents one of the biggest problems observed in the L-ASNase research field.[25] Currently, the first-line L-ASNase used in the clinic is polyethylene glycol (PEG)-asparaginase, a L-ASNase derived from *Escherichia coli* (*EcA*) which is conjugated to a PEG-tag that not only improves the half-life of the enzyme, but also the solubility and masks the bacterial enzyme from the immune system.[25-31] However, studies have shown that PEG itself can be immunogenic and that this anti-PEG immune response facilitates clearance *in vivo*.[10,32,33] Considering the bacterial origin of clinically used L-ASNases, immune responses against the enzyme itself are also commonly observed during therapy, including bronchospasms, anaphylactic shock, and hypersensitivity reactions.[21,34,35]

Currently, debate is ongoing whether the L-glutaminase co-activity of clinically approved L-ASNases is essential, contributory or fully dispensable for their anticancer effect based on contradictory results.[10,36-39] Several studies have shown that the cancer cell killing potential of L-glutaminase co-activity has been linked to asparagine synthetase (ASNS) expression levels.[10] Chan *et al.* found that L-glutaminase co-activity is not required for the cell killing potential of L-ASNase against ASNS-negative ALL cancer cells *in vitro* using a newly designed L-glutaminase-deficient *EcA* Q59L mutant (EcA^{Q59L}),[40] while other groups reported that Gln depletion contributed to the overall anti-leukemic effect.[38,40,41] In addition, Panosyan *et al.* showed that deamination of Gln may enhance the anti-leukemic effect of L-ASNase.[42] Recently, Nguyen *et al.* engineered a variant of *Erwinia chrysanthemi* (*ErA*) with three mutations, *ErA*-TM, that acquired ultra-low L-glutaminase activity while maintaining its L-ASNase activity.[15] *ErA* has a very short *in vivo* half-life, but the authors reported that the addition of a His-SUMO tag N-terminally to *ErA*-TM enhanced the drug's *in vivo* stability.[43] Using short-term His-SUMO-*ErA*-TM treatment, *in vivo* antileukemic efficacy was successfully demonstrated in a B-ALL xenograft model of SUP-B15 cells and a patient-derived T-ALL xenograft model,[43] confirming the previous *in vitro* results from Chan *et al.*[40] However, it was recently shown that the EcA^{Q59L} of Chan *et al.* did not induce a durable *in vivo* anticancer effect against xenografts of the ASNS-negative SUP-B15 leukemia cell line in NOD/SCID gamma mice (NSG) compared to the L-ASNase wild-type ($L\text{-}ASNase^{WT}$),[44] therefore again contradicting previous reports.[40]

To clarify whether L-glutaminase co-activity is required for a durable long-term anti-leukemic response, we took advantage of the superior tolerability of our low-glutaminase *ErA*-TM variant. To further improve the *in vivo* persistence (even longer than that afforded by the His-SUMO tag[43]), we introduced an albumin-binding domain (ABD) N-terminal to *ErA*-TM, which we refer to as ABD-*ErA*-TM. We show that the introduction of an ABD-tag stabilizes *ErA*-TM and dramatically enhances its half-life. In addition, we evaluated the *in vivo* efficacy of this low-glutaminase variant in a SUP-B15 ALL xenograft model. Our results show a comparable long-lasting durable antileukemic effect between the mice treated with the standard-of-care PEG-asparaginase and ABD-*ErA*-TM, but with fewer co-glutaminase related non-immunological toxicities. In the future, the implementation of this new L-ASNase variant in the clinic could improve ALL patients' outcomes and quality of life.

Methods

Expression and purification of ABD-*ErA*-TM and ABD-*ErA*-WT, crystal structure of ABD-*ErA*-TM, surface plasmon resonance and isothermal titration calorimetry

For detailed methodology, see the *Online Supplementary Appendix*.

Enzyme activity assay

The catalytic activities of the L-ASNase variants were determined using the aspartic acid β-hydroxamate (AHA) method as previously described.[45-47] A detailed protocol can be found in the *Online Supplementary Materials and Methods*.

In vivo ABD-*ErA*-TM and PEG-asparaginase pharmacokinetics and pharmacodynamics study

The pilot pharmacodynamic study was performed at The University of Texas MD Anderson Cancer Center under protocol ACUF #00001658-RN01 following published methods.[44,48] For the other pharmacodynamic and pharmacokinetic study, healthy wild-type C57BL/6J were randomly divided into vehicle phosphate-buffered saline (PBS) (n=5), PEG-asparaginase (n=15) and ABD-*ErA*-TM (n=15) treatment groups. One single administration/dose of 2,500 IU/kg PEG-asparaginase or ABD-*ErA*-TM was administered via intraperitoneal injection. For the activity determinations, 50 μL of peripheral blood was collected and samples were processed according to the protocol described by Nguyen *et al.*[43] For the amino acid level determinations, 120 μL of peripheral blood was collected and amino acids were measured as previously described.[43] For a more detailed

description of the methods used, see the *Online Supplementary Appendix*.

Single-dose acute toxicity study

Healthy wild-type C57BL/6J (Charles River) mice (9 weeks old; n=18) received one single administration/dose (2,500 IU/kg) of PEG-asparaginase or ABD-*ErA*-TM via intraperitoneal injection. After 96 hours, blood was collected and hematologic (Vetscan® HM5 Hematology Analyzer; Abaxis) and biochemical analyses (VetScan® VS2 Chemistry Analyzer; Abaxis) were performed. For detailed methodology, see the *Online Supplementary Appendix*.

In vivo treatment of a SUP-B15 cell line xenograft model with L-ASNases

NSG (#005557; The Jackson Laboratory) female mice (n=15) were intravenously injected at 6 weeks of age with 150 μL PBS containing 5 × 10^6 luciferase-positive SUP-B15 cells. At regular time points, the bioluminescence was measured using the IVIS Lumina II imaging system (PerkinElmer). After evidence of leukemic cell engraftment in the spleen and/or bone marrow, the mice were randomly divided into vehicle PBS (n=5), PEG-asparaginase (n=5) and ABD-*ErA*-TM (n=5) treatment groups. PEG-asparaginase was administered every 10 days via intraperitoneal injection at a dose of 2,500 IU/kg (total of 5 administration/doses), while ABD-*ErA*-TM was given at the same dose every 7 days (total of 7 administration/doses). During the experiment, leukemic burden was evaluated via bioluminescent imaging at regular time points.

The ethical committee on animal welfare at Ghent University Hospital approved all the *in vivo* mice experiments.

Statistical analysis

Statistical power was calculated before every experiment (80% statistical power, α=0.05). The Shapiro-Wilk test was used to check for normality of the data. The amino acid level data were not normally distributed and therefore non-parametric Mann-Whitney U tests were performed. A two-sided *P* value <0.05 was considered statistically significant. For the toxicity data, Mann-Whitney U tests, followed by Bonferroni correction for multiple comparisons, were performed. Results are expressed as mean ± standard deviation where appropriate. GraphPad Prism 9 (GraphPad Software Inc., La Jolly, CA, USA) was used to analyze the data.

Results

Introduction of an ABD-tag extends the *in vivo* persistence of *ErA*-TM L-ASNase variants

Dennis *et al.*[49] used phage display to select peptides that bind to serum albumins. The 18-residue peptide SA21 with the highest binding affinity to the human serum albumin (467 nM) contains two cysteines speculated to form an intramolecular disulfide bond that would stabilize the binding to albumin. We fused this SA21 sequence, from now on referred to as ABD, to our low-glutaminase *ErA*-TM variant (Figure 1A). The challenge in the purification was the undesired buildup of inter-ABD disulfide bonds instead of the desired intra-ABD disulfide bonds. The lack of cysteine residues in *ErA*/*ErA*-TM suggested that any undesired intermolecular disulfide bridges are between the cysteine of an ABD in a protomer and a cysteine of an ABD in another protomer. In fact, using gel filtration we could see that ~25% of the protein eluted as an octamer instead of the physiological tetramer, presumably due to this incorrect disulfide bond formation between ABD and cysteine residues located in different tetramers (*Online Supplementary Figure S1*). For the experiments described, only the physiological tetrameric fraction was used.

We first verified if the k_{cat} and K_m values of ABD-*ErA*-TM were comparable to the ones from untagged *ErA*-TM and our previously reported His-SUMO-*ErA*-TM variant[43] (Table 1). The K_m property is key for obtaining complete Asn depletion *in vivo*. The introduction of the triple mutations to *ErA* increased the K_m from ~50 μM to ~100 μM, and the fusion of *ErA*-TM to ABD further increased the K_m to ~150 μM.

To verify that ABD-*ErA*-TM can efficiently deplete Asn levels *in vivo*, we conducted a pilot pharmacodynamic properties study that showed a significant decrease of Asn levels upon ABD-*ErA*-TM administration (single administration/dose 2,500 IU/kg) (*Online Supplementary Figure S2*). At the same time, we were encouraged to see a significant increase in half-life compared to that of the His-SUMO-*ErA*-TM protein[43] (38.3 *vs*. 15.7 hours) (Table 2). Presumably, this increase in half-life is due to the interaction between the ABD moiety and the mouse serum albumin, and that this interaction slows down the degradation of the enzyme. To verify that our molecule binds serum albumins, we used surface plasmon resonance and isothermal titration calorimetry to determine the *in vitro* interaction with mouse and human serum albumins. Whereas *ErA*-TM shows no binding of either mouse or human serum albumin, ABD-*ErA*-TM binds with a low micromolar affinity to both (Table 3; *Online Supplementary Figures S3* and *S4*). To test the prediction that a disulfide bridge between the two ABD cysteines is required for the interaction with albumins, we generated a variant in which these cysteines were mutated to serines (ABD(C→S)-*ErA*-TM). As expected, this variant exhibited no affinity to either mouse or human serum albumin. Indeed, the *in vivo* half-life of the ABD(C→S)-*ErA*-TM variant was essentially identical to that of *ErA*-TM (2.5 *vs*. 2.3 hours) (Table 2).

Finally, to obtain a molecular understanding of the ABD, we solved the crystal structure of ABD-*ErA*-TM at 1.75 Å

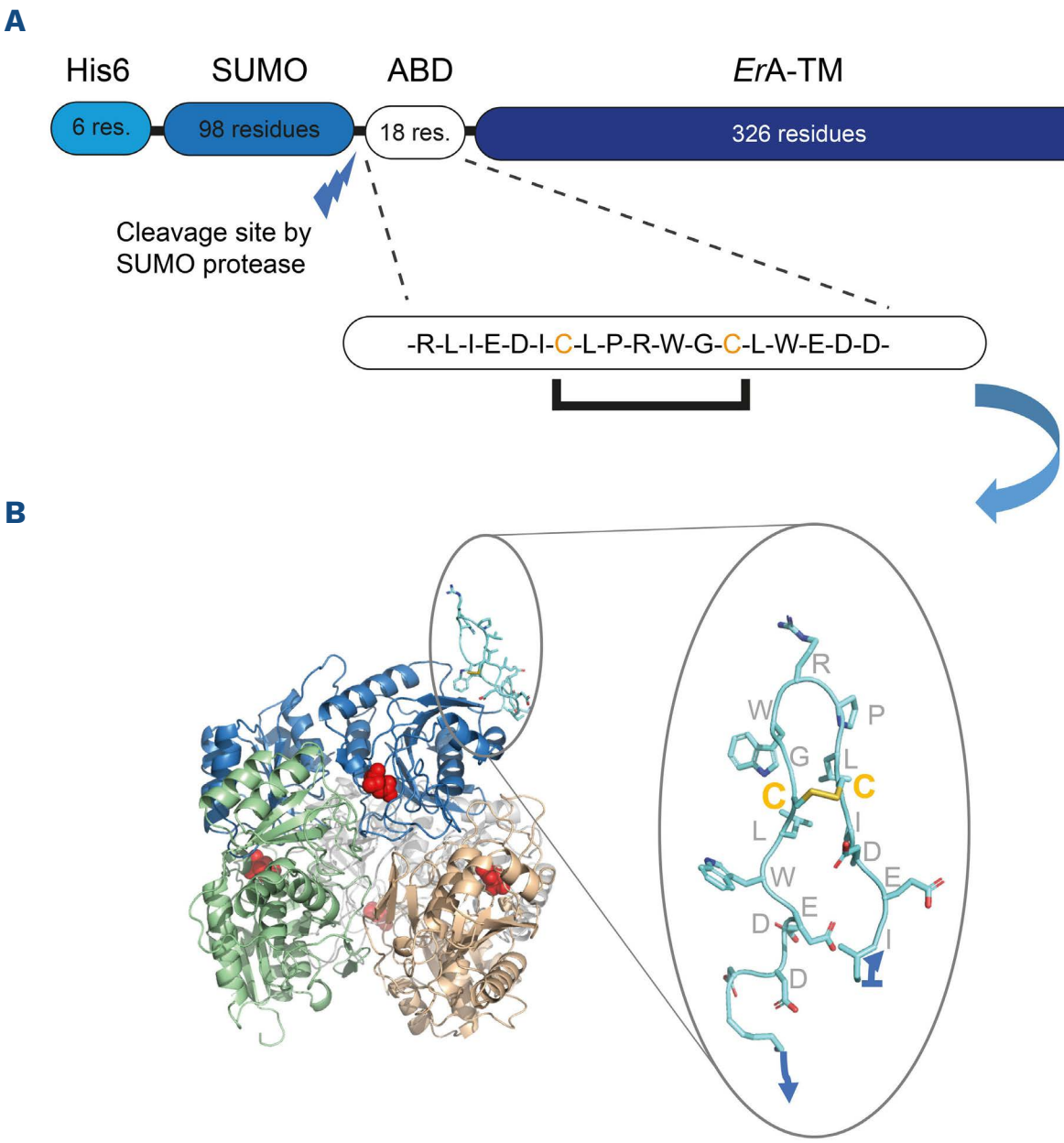


Figure 1. Visualization of ABD-*ErA*-TM. (A) ABD-*ErA*-TM is expressed as a His-SUMO fusion protein. Cleavage with SUMO protease releases the molecule used in these studies. Two cysteine residues in the ABD region build the disulfide bond required for albumin binding. (B) The tetrameric ABD-*ErA*-TM as observed in the crystal structure solved at 1.75 Å resolution. A disulfide bridge is formed between two cysteines of ABD that acts to rigidify the loop structure. Thus, for the first time, we have a view of the ABD-conformation that is required for the interaction with serum albumins.

Table 1. L-ASNase kinetic parameters for ABD-ErA wild type and select mutants. This table shows the K_m and k_{cat} values of our low-glutaminase alternative ABD-ErA-TM variant compared to the ones from untagged ErA-TM and our previously reported His-SUMO-ErA-TM variant.[43]

Enzyme	k_{cat} s^{-1}±SD	K_m µM±SD	k_{cat}/K_m s^{-1} $µM^{-1}$	Specific activity[a] IU/mg±SD
ABD-*ErA*-WT	311.3±5.5	39.3±3.0	7.92	504.9±30.1
ABD-*ErA*-TM	289.2±2.6	151.8±4.8	1.91	453.1±6.2
ABD-*ErA*-TM C7S/C13S	272.8±3.9	138.9±6.2	1.96	417.7±13.6
ErA-WT[b]	207.5±3.6	47.5±3.5	4.37	353.2±6.1
His-SUMO-*ErA*-TM[b]	261.2±2.8	95.0±3.5	2.75	443.5± 4.8

[a]1 IU of enzyme is defined as the amount of enzyme used to produce 1 µmol of aspartate in 1 minute at 37°C. [b]Kinetic parameters reported by Nguyen *et al.*[15] The introduction of an albumin binding domain (ABD) increases the K_m value, a parameter for asparagine specificity, from ~40 µM (ABD-*ErA*-WT) to ~150 µM. The specific activity (IU/mg of protein) is a commonly used parameter to express specific L-ASNase activity. SD: standard deviation.

resolution. Data collection and refinement statistics are shown in *Online Supplementary Table S1*. In the tetrameric molecule, one protomer had clear electron density for the ABD. Presumably, the ABD of the other three protomers were not seen due to being highly flexible. As shown in Figure 1B, the two cysteines of the ABD form a disulfide bridge that acts to rigidify the loop structure. Thus, for the first time, we have a view of the ABD-conformation that is required for the interaction with serum albumins.

In vivo pharmacokinetic and pharmacodynamic profiling of ABD-*ErA*-TM in comparison to PEG-asparaginase

To further document the pharmacokinetic profile and pharmacodynamic properties of our ABD-stabilized L-ASNase variant in comparison to those of the standard-

of-care, healthy C57BL/6J mice were injected intraperitoneally with 2,500 IU/kg of PEG-asparaginase or ABD-*ErA*-TM. The L-ASNase activity was measured at days 1, 3, 5, 7, 10 and 14. Although a similar pharmacokinetic profile was seen with the PEG-asparaginase, the activity of ABD-*ErA*-TM decreased more rapidly compared to that of the PEG-asparaginase (Figure 2A). In addition, we determined the Asn, Asp, Gln, and Glu levels in blood plasma prior to the treatment and at days 1, 3, 5, 7, 10 and 14 after L-ASNase administration. Both enzymes efficiently depleted the blood Asn in a comparable manner (P=0.0079; Mann-Whitney U test), with levels slowly recovering after day 5 for ABD-*ErA*-TM and after day 7 for PEG-asparaginase (Figure 2B). In line with these results, plasma Asp levels increased significantly at days 1 and 3 (P=0.0079; Mann-Whitney U test) compared to baseline levels and then decreased to the pretreatment level by day 14 for both treatment groups (*Online Supplementary Figure S5A*). For Gln, only at 24 hours after administration was a minimal but significant reduction in Gln plasma levels seen for PEG-asparaginase, while no decrease of Gln levels was observed in ABD-*ErA*-TM mice (*Online Supplementary Figure S5B*). On the other hand, a consistent differential effect was demonstrated on the plasma Glu levels, with a significant increase in the PEG-asparaginase mice up to day 7 after administration, followed by a decrease towards baseline levels again. These increased Glu levels upon PEG-asparaginase treatment point out that long-term L-glutaminase co-activity is maintained. Notably, a minimal increase was observed in the ABD-*ErA*-TM group but not to the extent as in the PEG-asparaginase mice, as to be expected from this low-glutaminase variant (*Online Supplementary Figure S5C*).

Toxicity profiling of ABD-*ErA*-TM

Next, we evaluated the acute toxicity of ABD-*ErA*-TM *versus* PEG-asparaginase in healthy wild-type C57BL/6J female mice at a dose of 2,500 IU/kg. We compared the impact of both enzymes on the animals' body weight, one of the main parameters to evaluate L-ASNase toxicity. At the end of the experiment, PEG-asparaginase mice lost a greater percentage of their starting body weight (P=0.0022; Mann-Whitney U test), while the ABD-*ErA*-TM mice showed no significant decrease in body weight (Figure 2C). In addition, we also analyzed hematologic parameters, including white blood cell count, red blood cell count and blood platelet levels. While no effect was observed on red blood cell counts, significant differences were seen in white blood cell and blood platelet levels (*Online Supplementary Figure S6A-C*). As previously mentioned, side effects of L-ASNase treatment, in particular liver and pancreas toxicity, are often observed. Blood chemistry analysis showed a significant decrease in globulin and albumin levels, both markers of hepatic dysfunction, in the PEG-asparaginase group compared to the vehicle group (P=0.0366 and P=0.0084, respectively) (*Online Supplementary Figure S6D-E*). Other blood parameters, including alanine aminotransferase (*Online Supplementary Figure S6F*), total bilirubin (*Online Supplementary Figure S6G*), blood urea nitrogen (*Online Supplementary Figure S6H)* and amylase (*Online Supplementary Figure S6I*) did not show any significant changes between groups.

In vivo therapeutic evaluation of ABD-*ErA*-TM in a B-cell acute lymphoblastic leukemia cell line xenograft model

We evaluated the *in vivo* efficacy of long-term Asn deprivation using ABD-*ErA*-TM in a luciferase positive SUP-B15 xenograft mouse model. To examine the need for co-glu-

Table 2. Half-life of *ErA* variants.

Protein	Half-life, (hours)
ErA-TM	2.3
His-SUMO-*ErA*-TM	15.7
ABD-*ErA*-TM	38.3
ABD(C→S)-*ErA*-TM	2.5

ABD-*ErA*-TM shows a significant increase in half-life compared to the His-SUMO-ErA-TM protein[43] (38.3 *vs.* 15.7 hours). The *in vivo* half-life of the ABD(C→S)-*ErA*-TM variant was essentially identical to that of *ErA*-TM (2.5 *vs.* 2.3 hours), demonstrating the need for a disulfide bridge between the two ABD cysteines.

Table 3. Binding affinities determined by surface plasmon resonance and isothermal titration calorimetry.

	Human serum albumin				Mouse serum albumin			
	SPR			ITC	SPR			ITC
	k_a ($\times 10^3$ $M^{-1}s^{-1}$)	k_d ($\times 10^{-2}$ s^{-1})	K_D (μM)	K_d (μM)	k_a ($\times 10^3$ $M^{-1}s^{-1}$)	k_d ($\times 10^{-2}$ s^{-1})	K_D (μM)	K_d (μM)
ErA-TM	NB	NB	NB	NT	NB	NB	NB	NT
ABD-*ErA*-TM	1.78±0.55	1.41±0.30	8.11±0.80	4.37	1.71±0.64	0.88±0.30	5.20±0.41	4.95±0.49
ABD(C→S)-*ErA*-TM	NB	NB	NB	NT	NB	NB	NB	NT

Surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) were used to determine the *in vitro* interaction with mouse serum albumin (MSA) and human serum albumin (HAS). While *ErA*-TM shows no binding of either MSA or HSA, ABD-*ErA*-TM binds with a low micromolar affinity to both MSA and HSA. Values are means ± standard deviations from four measurements each for SPR, two measurements for ITC with MSA, and a single measurement for ITC with HSA. NB: no binding; NT: not tested.

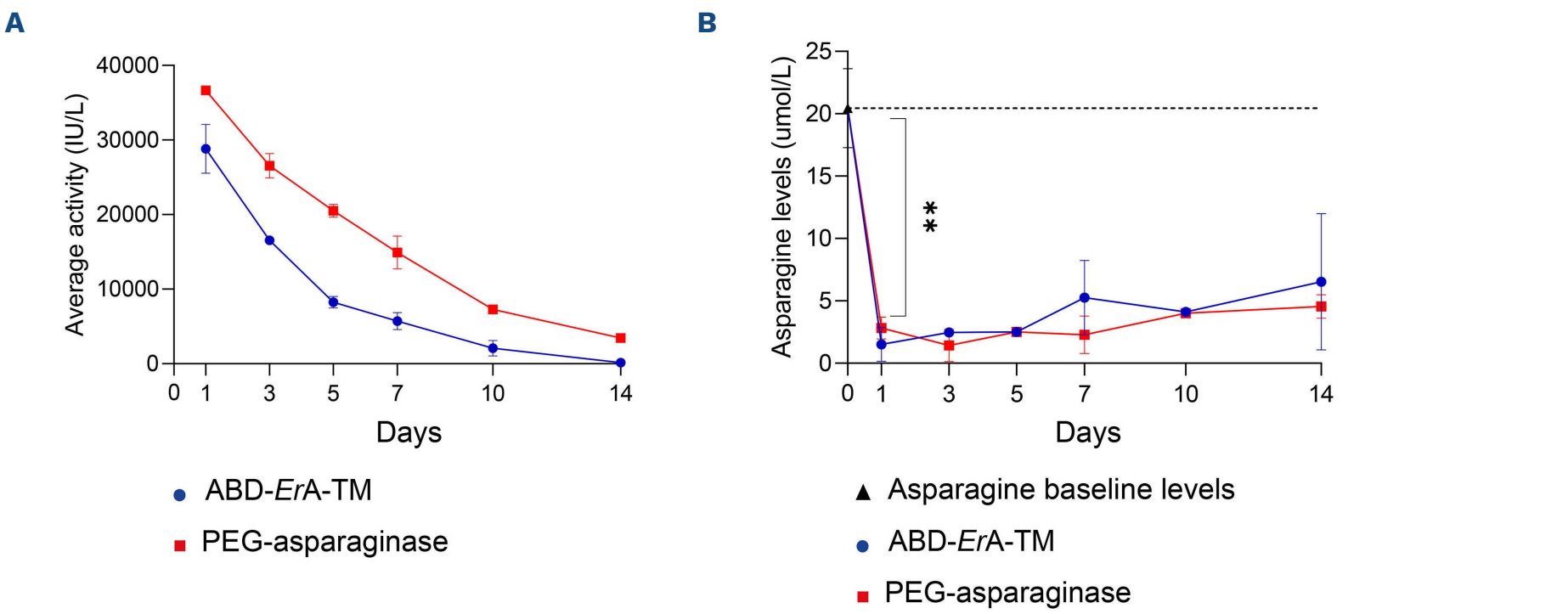


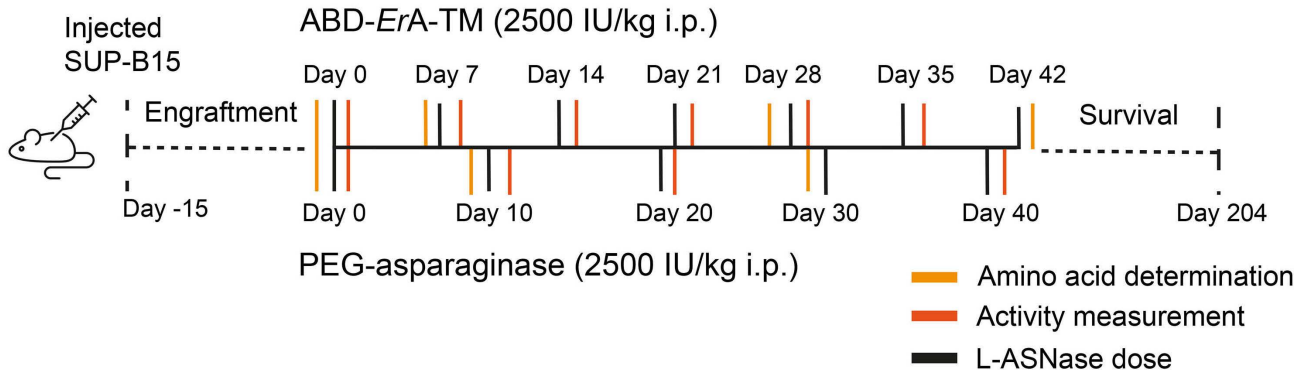
Figure 2. ABD-*ErA*-TM shows reduced toxicity in mice compared to PEG-asparaginase but displays a similar pharmacokinetic profile. (A) L-ASNase activity measurements in blood plasma samples of mice obtained at days 1, 3, 5, 7, 10 and 14 after 2,500 IU/kg of L-ASNase show a comparable pharmacokinetic profile for both enzymes, although the activity of ABD-*ErA*-TM decreased more rapidly compared to that of the PEG-asparaginase. (B) Measurement of asparagine levels in blood plasma samples of mice obtained at days 1, 3, 5, 7, 10 and 14 after 2,500 IU/kg of PEG-asparaginase or ABD-ErA-TM. A significant depletion of asparagine is observed for both enzymes (P=0.0079; Mann-Whitney U-test, with levels slowly recovering after day 5 for ABD-*ErA*-TM and day 7 for PEG-asparaginase. (C) After 96 hours, PEG-asparaginase mice lost a greater percentage of their starting body weight (P=0.0022; Mann-Whitney U test), while the ABD-*ErA*-TM mice showed no significant decrease in body weight after L-ASNase administration. For each figure, the mean and standard deviation are shown.

taminase activity to kill ALL cells, we compared our stabilized low-glutaminase variant ABD-*ErA*-TM with PEG-asparaginase. Based on the results of our pharmacokinetic and pharmacodynamic studies, a dose of ABD-*ErA*-TM (2,500 IU/kg) was administered every 7 days, while PEG-asparaginase (2,500 IU/kg) was given every 10 days guaranteeing continuous Asn deprivation in the blood with minimal residual L-ASNase activity building up before each new dose. In total, mice received either five doses of PEG-asparaginase or seven doses of ABD-*ErA*-TM (Figure 3A). Results not only show that both ABD-*ErA*-TM and PEG-asparaginase were equally capable of rapidly lowering the leukemic burden, but also that a comparable long-term survival could be accomplished (Figure 3B). During the treatment time course, both PEG-asparaginase and ABD-*ErA*-TM treated mice displayed lower bioluminescent signals, below the background detection levels at the end of the treatment, while PBS control mice clearly showed an increase in leukemic burden (Figure 3C). At day 55, control mice had to be euthanized due to ethical humane endpoints, while both PEG-asparaginase and ABD-*ErA*-TM mice showed no sign of ALL burden up to day 204, with the exception of one PEG-asparaginase treated mouse that relapsed at day 180 and clearly showed clinical signs of tumor formation.

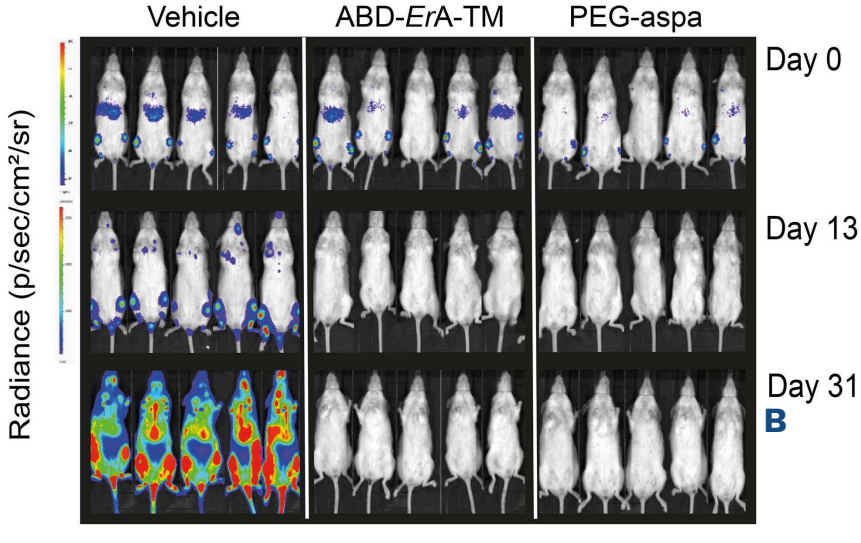
The L-ASNase activity in blood plasma samples was measured 24 hours after administration of ABD-*ErA*-TM (at days 1, 8, 15, 22, 36 and 43) and PEG-asparaginase (at days 1, 11, 21 and 41). Results indicate that acceptable stable enzyme activity levels were maintained during the whole experiment (threshold for adequate activity >100 IU/L) without any excessive build-up of L-ASNase activity (Figure 3D). In addition, we also determined the amino acid levels in treated mice to confirm efficacy of Asn depletion during L-ASNase treatment. Both enzymes depleted the blood Asn, which was below detection at every time point (Figure 3E).

Besides the therapeutic effect, the impact and tolerability of both drugs was evaluated on the body weight of the animals. At the end of treatment, we observed a mean weight loss of 8.8% in the ABD-*ErA*-TM treatment group, while the PEG-asparaginase mice had a mean weight loss

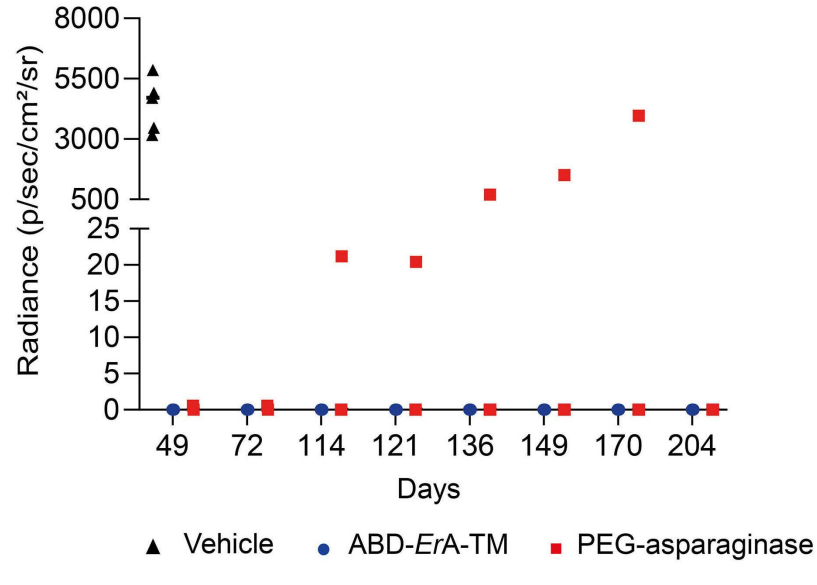
A



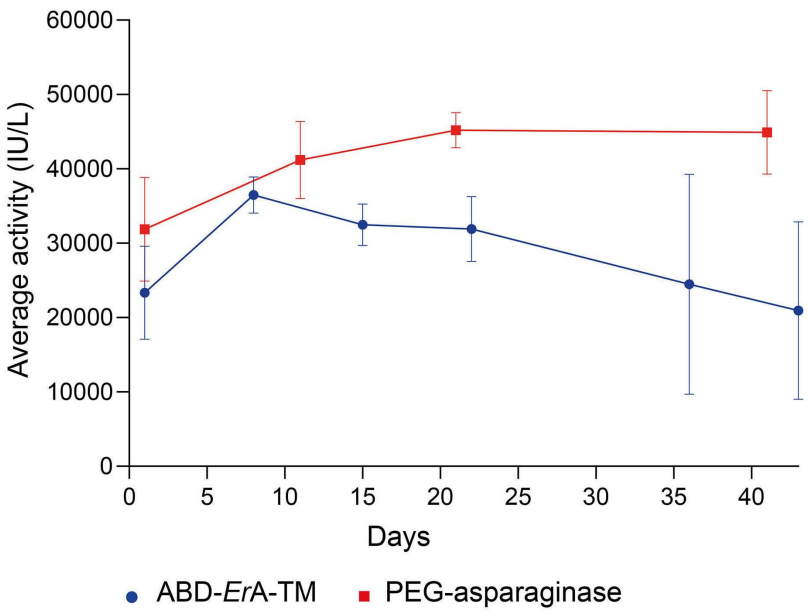
B



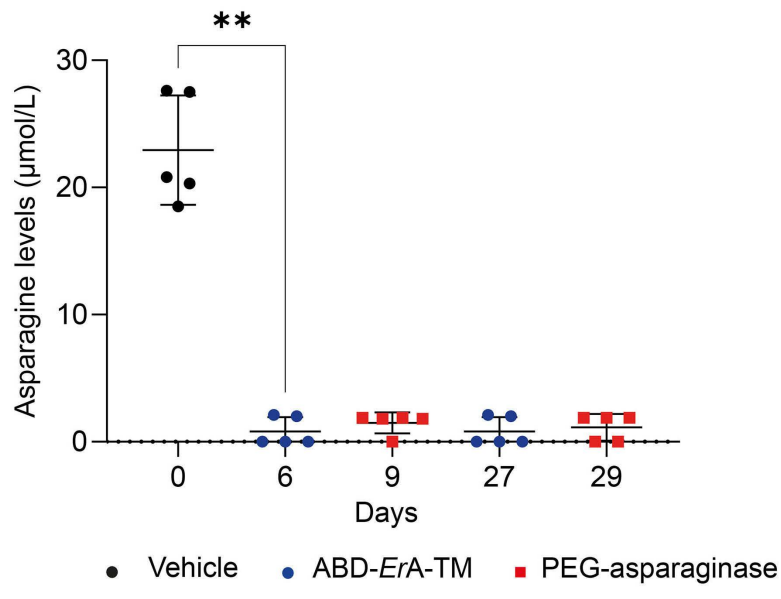
C



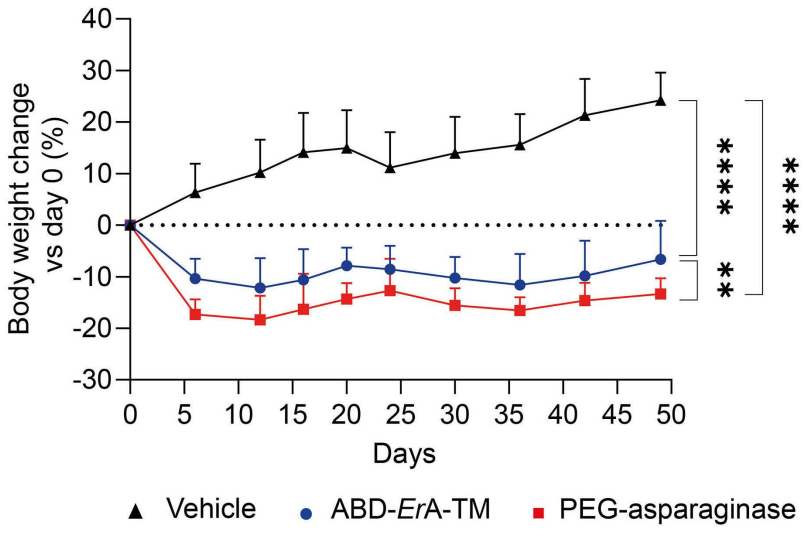
D



E



F



Continued on following page.

Figure 3. The low L-glutaminase ABD-*ErA*-TM eliminates SUP-B15 cells as effectively as the high L-glutaminase PEG-asparaginase, but with reduced toxicity. (A) Mice were injected with luciferase-expressing SUP-B15 cells and monitored for engraftment via bioluminescent imaging (BLI). Once engraftment was observed, mice were treated intraperitoneally (i.p.) every 10 days with PEG-asparaginase (n=5), every 7 days with ABD-*ErA*-TM (n=5) (drug dose for both enzymes 2,500 IU/kg) or vehicle (phosphate-buffered saline, PBS) (n=5). At regular time points during treatment, blood plasma samples were collected for amino acid determinations or activity measurements. (B) For each group, the BLI at regular time points is shown. Before the start of treatment, mice clearly show engraftment of SUP-B15 cells in the bone marrow and spleen area. At days 13 and 31, vehicle PBS-treated mice show a very strong BLI signal consistent with a high disease burden, while ABD-*ErA*-TM and PEG-asparaginase mice show no sign of leukemia. After treatment termination, mice were followed up for survival up to day 204 after initiation of treatment. Only one PEG-asparaginase-treated mouse relapsed and had to be euthanized due to ethical reasons. (C) For both L-ASNase treatment groups, the radiance dramatically decreased returning to background levels by day 31, with the exception of one PEG-asparaginase-treated mouse that relapsed during the survival monitoring. (D) The L-ASNase activity in blood plasma samples was measured 24 hours after each L-ASNase administration. Results clearly show that L-ASNase activity was maintained during the treatment time course. For each time point, the mean and SD are shown. (E) Asparagine levels were measured prior to L-ASNase administration at days 6 and 27 for ABD-*ErA*-TM and at days 9 and 29 for PEG-asparaginase. Our data show that asparagine levels significantly decreased during L-ASNase therapy in the same manner for both treatment groups compared to day 0 (P=0.0079; Mann-Whitney U test). For each time point, the mean and SD are shown. (F) At the end of treatment, a mean weight loss of 8.8% in ABD-*ErA*-TM-treated mice was observed, while the PEG-asparaginase-treated mice had a mean weight loss of 13.9% (ABD-*ErA*-TM *vs*. PEG-asparaginase P=0.0011; Mann-Whitney U test), demonstrating a better tolerability of ABD-*ErA*-TM in these mice. For each time point, the mean and SD are shown.

of 13.9% (Figure 3F). At the end of this survival experiment (day 204), mice were sacrificed and analyzed for signs of leukemia development. No significant difference between spleen/body weight in both treated groups was observed (*Online Supplementary Figure S7A*). In addition, analysis of human CD45$^+$ cells in peripheral blood, spleen and bone marrow showed undetectable levels of leukemic cells in both PEG-asparaginase- and ABD-*ErA*-TM-treated mice, with exception of the PEG-asparaginase mouse that relapsed during survival monitoring (*Online Supplementary Figure S7B*).

Together, these results convincingly show that the ABD-tag stabilizes this *ErA*-TM variant *in vivo* and ABD-*ErA*-TM can lower ALL leukemic disease burden in a manner comparable to that of the high-glutaminase standard-of-care PEG-asparaginase, but with a better tolerability.

Discussion

Although L-ASNase is a key chemotherapeutic agent in childhood ALL, particularly during the induction phase, its administration is associated with multiple and severe toxicities. Unfortunately, these side effects prevent many patients from receiving their prescribed course of treatment, resulting in a detrimental impact on their outcome.[50,51] Several studies showed that the L-glutaminase co-activity of clinically used L-ASNases contributes partially to these unwanted side effects.[21] Therefore, lowering L-glutaminase co-activity while maintaining its anticancer effect might provide the required safety profile that reduces treatment interruptions.

Immune reactions during L-ASNase therapy occur frequently together with the development of anti-L-ASNase antibodies.[52] Patients suffering from hypersensitivity reactions or silent inactivation to *EcA* are switched to native *Er*A.[50,52-54] Although the latter is a useful second-line therapy in ALL, some disadvantages are associated with this drug, including a much shorter half-life compared to that of PEG-asparaginase.[6,55] As patients can also develop antibodies against the PEG-tag itself, thereby limiting tolerance and efficacy,[33,56] additional research on more immunotolerant tags such as XTEN[57] and PASylation[58,59] is currently ongoing. In our previous study,[43] we presented an *ErA*-TM variant with significantly lower L-glutaminase but comparable L-ASNase activity relative to *ErA*-WT. We also achieved an extended circulation time by the fusion of a SUMO tag to these molecules. With the goal of further increasing blood circulation time, we analyzed several other tags, and discovered that the fusion of an ABD to *ErA* (WT or TM variant) resulted in respective molecules with much longer *in vivo* half-lives. In this study, we present ABD-*ErA*-TM, a novel *ErA* derived variant that is superior to our previous ones[43] by combining both low L-glutaminase co-activity and an exceptionally extended *in vivo* persistence.

Acute toxicological evaluation of ABD-*ErA*-TM revealed reduced non-immunological toxicity compared to the current first-in-line EcA. Our toxicity data show a clear correlation between reduced L-glutaminase co-activity and reduced drug toxicity, especially on the average percentage of body weight loss. We note that other biochemical parameters of liver and pancreas toxicity including alanine aminotransferase, total bilirubin, blood urea nitrogen and amylase levels did not show any changes. This may not be surprising since L-ASNase toxicity in patients is often only observed after multiple L-ASNase administration/doses.

The clinical relevance of L-glutaminase co-activity, present in all clinically Food and Drug Administration (FDA)-approved bacterial L-ASNases, in the context of ALL remains largely unclear. First, it was shown that this L-

glutaminase co-activity was not required to induce an *in vivo* L-ASNase effect in the leukemic SUP-B15 cell line.[43] However, Chan *et al.*[44] showed that a durable and long-lasting antileukemic effect in the same SUP-B15 xenograft could only be obtained using a L-ASNase variant that retained its L-glutaminase co-activity.[44] Our results clearly contradict these findings and demonstrate that SUP-B15 leukemic cells can be durably killed with a L-ASNase variant with ultra-low glutaminase co-activity. We speculate that the lack of durable response noted by Chan *et al.*[44] was due to insufficient long-term Asn depletion that resulted from using a non-stabilized L-ASNase variant, which means that it needs to be injected more frequently to observe a therapeutic effect. Our results clearly show a similar long-lasting durable anti-leukemic effect between PEG-asparaginase and ABD-*ErA*-TM.

Industrial production problems recently resulted in a shortage of *ErA* and reduced treatment options for patients who developed hypersensitivity or silent inactivation against PEG-asparaginase. To overcome these manufacturing issues, a recombinant version of *ErA*, JZP-458, was recently developed. While this JZP-458 has been approved by the FDA as part of a treatment regimen for children and adults with ALL or lymphoblastic lymphoma,[60] our ABD-*ErA*-TM has multiple benefits over the JZP-458 preparation. One is avoiding the toxicities that arise due to the glutaminase co-activity. Secondly, the *in vivo* persistence of JZP-458 is short whereas the albumin binding, provided by the ABD domain, increases the half-life >10-fold. This will provide a dramatic clinical advantage, allowing patients to be dosed once a week instead of three times a week as currently required for Erwinaze or JZP-458. In addition, our new Erwinia L-ASNase preparation requires no additional post-translational modifications resulting in high protein yields. As our ABD-*ErA*-TM would only need to be administered once a week, smaller amounts will be necessary to treat the same number of patients compared to current standard-of-care L-ASNases.

Until now, all immune-related side-effects were ignored in our experimental setup, as the *in vivo* efficacy study was done with human ALL cells xenotransplanted in immunodeficient NSG mice. Unfortunately, good preclinical models to evaluate the immunogenicity of L-ASNases are not available and this is one of the biggest challenges for future L-ASNase research.

In conclusion, this study convincingly shows that high L-glutaminase co-activity present in standard-of-care PEG-asparaginase is not essential for durable anti-leukemic properties *in vivo*, therefore contradicting previous reports. Furthermore, our findings also suggest a decline in *in vivo* toxicity when L-glutaminase co-activity is reduced. In addition, the fusion of an ABD-tag to our alternative *ErA*-TM variant increases its half-life and *in vivo* stability. Identifying a L-ASNase variant with fewer side effects will not only improve the quality of life of ALL patients directly, but will also allow treatment protocols to be maintained or prolonged, which will translate into improved outcomes, especially for adult ALL patients. In addition, a few aggressive solid cancer subtypes with poor prognosis and a clinical unmet need have been identified as sensitive to Asn deprivation, including ovarian and metastatic breast cancer. However, because of the adverse side effects in adults, clinical trials have been discontinued so far. By identifying a L-ASNase variant with fewer unwanted toxicities, we hope to revive the clinical trials and provide extra therapeutic opportunities for these patients.

Disclosures

AMS, AL and HAN declare competing financial interests by being founders with equity stake in Enzyme by Design Inc.: a start-up developing new L-asparaginases. AL and HAN are inventors of the Int. Patent. Appl. PCT/US2017/020090 (WO2017151707A1) that describes the ABD-ErA-TM variant. None of the other authors declare any competing financial interests.

Contributions

MVT, AMS, HAN, TL, SG, PVV and AL designed the study; MVT, AMS, HAN, HL and WC performed experiments; LR and KV provided excellent technical assistance; TL, SG, PVV and AL supervised this study; MVT and AL drafted the manuscript; MVT, AL, HUN and YDV edited the figures; MVT, AMS, YDV, HAN, LR, KV, EP, YS, HL, PLL, WC, VM, BDM, TL, SG, PVV and AL proofread the original draft and made valuable adjustments; AMS, PLL, TL, SG, PVV, and AL obtained funding for this study. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to thank Sri Ramya Donepudi for assistance with assessment of pharmacodynamics. We also thank the Biostatistics Unit of the Faculty of Medicine and Health Sciences of Ghent University for assistance with the statistics.

Funding

This work was supported by The Belgian Foundation Against Cancer (project grants to PVV and SG), Belgian Stand Up to Cancer Foundation (project grants to PVV and SG) and vzw Kinderkankerfonds (grant to TL); and in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development Biomedical Laboratory Research and Development MERIT Award I01BX001919 and Chicago Biomedical Consortium Accelerator Award (AL), and NIH SBIR grant R43-CA232798 (AMS). WKC and PLL are supported in part by NIH grants

S10OD012304-01 and P30CA016672. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817). The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs or the United States government.

Data-sharing statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. More detailed data are available from the co-senior authors PVV and AL upon reasonable request.

References

1. Radadiya A, Zhu W, Coricello A, Alcaro S, Richards NGJ. Improving the treatment of acute lymphoblastic leukemia. Biochemistry. 2020;59(35):3193-3200.
2. Malard F, Mohty M. Acute lymphoblastic leukaemia. Lancet. 2020;395(10230):1146-1162.
3. Kumar K, Kaur J, Walia S, Pathak T, Aggarwal D. L-asparaginase: an effective agent in the treatment of acute lymphoblastic leukemia. Leuk Lymphoma. 2014;55(2):256-262.
4. Micallef B, Nisticò R, Sarac SB, et al. The changing landscape of treatment options in childhood acute lymphoblastic leukaemia. Drug Discov Today. 2022;27(5):1483-1494.
5. Endicott M, Jones M, Hull J. Amino acid metabolism as a therapeutic target in cancer: a review. Amino Acids. 2021;53(8):1169-1179.
6. Cecconello DK, Magalhães MR, Werlang ICR, Lee MLM, Michalowski MB, Daudt LE. Asparaginase: an old drug with new questions. Hematol Transfus Cell Ther. 2020;42(3):275-282.
7. Hijiya N, van der Sluis IM. Asparaginase-associated toxicity in children with acute lymphoblastic leukemia. Leuk Lymphoma. 2016;57(4):748-757.
8. Thu Huynh V, Bergeron S. Asparaginase toxicities: identification and management in patients with acute lymphoblastic leukemia. Clin J Oncol Nurs. 2017;21(5):E248-E259.
9. Schmidt MP, Ivanov AV, Coriu D, Miron IC. L-asparaginase toxicity in the treatment of children and adolescents with acute lymphoblastic leukemia. J Clin Med. 2021;10(19):4419.
10. Fonseca MHG, Fiúza TDS, Morais SB, Souza T, Trevizani R. Circumventing the side effects of L-asparaginase. Biomed Pharmacother. 2021;139:111616.
11. Grigoryan RS, Panosyan EH, Seibel NL, Gaynon PS, Avramis IA, Avramis VI. Changes of amino acid serum levels in pediatric patients with higher-risk acute lymphoblastic leukemia (CCG-1961). In Vivo. 2004;18(2):107-112.
12. Ollenschläger G, Roth E, Linkesch W, Jansen S, Simmel A, Mödder B. Asparaginase-induced derangements of glutamine metabolism: the pathogenetic basis for some drug-related side-effects. Eur J Clin Invest. 1988;18(5):512-516.
13. Emadi A, Law JY, Strovel ET, et al. Asparaginase Erwinia chrysanthemi effectively depletes plasma glutamine in adult patients with relapsed/refractory acute myeloid leukemia. Cancer Chemother Pharmacol. 2018;81(1):217-222.
14. Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Crit Rev Oncol Hematol. 2007;61(3):208-221.
15. Nguyen HA, Su Y, Lavie A. Design and characterization of Erwinia chrysanthemi l-asparaginase variants with diminished l-glutaminase activity. J Biol Chem. 2016;291(34):17664-17676.
16. Cruzat V, Macedo Rogero M, Noel Keane K, Curi R, Newsholme P. Glutamine: metabolism and immune function, supplementation and clinical translation. Nutrients. 2018;10(11):1564.
17. Watford M. Glutamine and glutamate: nonessential or essential amino acids? Anim Nutr. 2015;1(3):119-122.
18. Tong WH, Pieters R, de Groot-Kruseman HA, et al. The toxicity of very prolonged courses of PEGasparaginase or Erwinia asparaginase in relation to asparaginase activity, with a special focus on dyslipidemia. Haematologica. 2014;99(11):1716-21.
19. Jaing TH, Lin JL, Lin YP, Yang SH, Lin JJ, Hsia SH. Hyperammonemic encephalopathy after induction chemotherapy for acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 2009;31(12):955-956.
20. Frantzeskaki F, Rizos M, Papathanassiou M, et al. L-asparaginase fatal toxic encephalopathy during consolidation treatment in an adult with acute lymphoblastic leukemia. Am J Case Rep. 2013;14:311-314.
21. Van Trimpont M, Peeters E, De Visser Y, et al. Novel insights on the use of L-asparaginase as an efficient and safe anti-cancer therapy. Cancers (Basel). 2022;14(4):902.
22. Beckett A, Gervais D. What makes a good new therapeutic l-asparaginase? World J Microbiol Biotechnol. 2019;35(10):152.
23. Avramis VI, Panosyan EH. Pharmacokinetic/pharmacodynamic relationships of asparaginase formulations: the past, the present and recommendations for the future. Clin Pharmacokinet. 2005;44(4):367-393.
24. Asselin B, Rizzari C. Asparaginase pharmacokinetics and implications of therapeutic drug monitoring. Leuk Lymphoma. 2015;56(8):2273-2280.
25. Brumano LP, da Silva FVS, Costa-Silva TA, et al. Development of L-asparaginase biobetters: current research status and review of the desirable quality profiles. Front Bioeng Biotechnol. 2019;6:212-212.
26. Müller HJ, Boos J. Use of l-asparaginase in childhood ALL. Crit Rev Oncol Hematol. 1998;28(2):97-113.
27. Kawedia JD, Rytting ME. Asparaginase in acute lymphoblastic leukemia. Clin Lymphoma Myeloma Leuk. 2014;14 Suppl:S14-17.
28. Soares AL, Guimarães GM, Polakiewicz B, de Moraes Pitombo RN, Abrahão-Neto J. Effects of polyethylene glycol attachment on physicochemical and biological stability of E. coli L-asparaginase. Int J Pharm. 2002;237(1-2):163-170.
29. Meneguetti GP, Santos J, Obreque KMT, et al. Novel site-specific PEGylated L-asparaginase. PLoS One. 2019;14(2):e0211951.
30. Verhoef JJF, Anchordoquy TJ. Questioning the use of PEGylation for drug delivery. Drug Deliv Transl Res. 2013;3(6):499-503.
31. Heo YA, Syed YY, Keam SJ. Pegaspargase: a review in acute lymphoblastic leukaemia. Drugs. 2019;79(7):767-777.
32. Zhang F, Liu M-R, Wan H-T. Discussion about several potential drawbacks of PEGylated therapeutic proteins. Biol Pharm Bull.

2014;37(3):335-339.
33. Kloos R, van der Sluis IM, Mastrobattista E, Hennink W, Pieters R, Verhoef JJ. Acute lymphoblastic leukaemia patients treated with PEGasparaginase develop antibodies to PEG and the succinate linker. Br J Haematol. 2020;189(3):442-451.
34. Krishna M, Nadler SG. Immunogenicity to biotherapeutics - the role of anti-drug immune complexes. Front Immunol. 2016;7:21.
35. Mondelaers V, Ferster A, Uyttebroeck A, et al. Prospective, real-time monitoring of pegylated Escherichia coli and Erwinia asparaginase therapy in childhood acute lymphoblastic leukaemia and non-Hodgkin lymphoma in Belgium. Br J Haematol. 2020;190(1):105-114.
36. Chiu M, Tardito S, Pillozzi S, et al. Glutamine depletion by crisantaspase hinders the growth of human hepatocellular carcinoma xenografts. Br J Cancer. 2014;111(6):1159-1167.
37. Long S, Zhang X, Rao Z, et al. Amino acid residues adjacent to the catalytic cavity of tetramer l-asparaginase II contribute significantly to its catalytic efficiency and thermostability. Enzyme and Microbial Technology. 2016;82:15-22.
38. Offman MN, Krol M, Patel N, et al. Rational engineering of L-asparaginase reveals importance of dual activity for cancer cell toxicity. Blood. 2011;117(5):1614-1621.
39. 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.
40. Chan WK, Lorenzi PL, Anishkin A, et al. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. Blood. 2014;123(23):3596-3606.
41. Maggi M, Chiarelli LR, Valentini G, Scotti C. Engineering of Helicobacter pylori L-asparaginase: characterization of two functionally distinct groups of mutants. PLoS One. 2015;10(2):e0117025.
42. Panosyan EH, Grigoryan RS, Avramis IA, et al. Deamination of glutamine is a prerequisite for optimal asparagine deamination by asparaginases in vivo (CCG-1961). Anticancer Res. 2004;24(2c):1121-1125.
43. Nguyen HA, Su Y, Zhang JY, et al. A novel l-asparaginase with low l-glutaminase coactivity Is highly efficacious against both T- and B-cell acute lymphoblastic leukemias in vivo. Cancer Res. 2018;78(6):1549-1560.
44. Chan WK, Horvath TD, Tan L, et al. Glutaminase activity of L-asparaginase contributes to durable preclinical activity against acute lymphoblastic leukemia. Mol Cancer Ther. 2019;18(9):1587-1592.
45. Fernandez CA, Cai X, Elozory A, et al. High-throughput asparaginase activity assay in serum of children with leukemia. Int J Clin Exp Med. 2013;6(7):478-487.
46. Hejazi M, Piotukh K, Mattow J, et al. Isoaspartyl dipeptidase activity of plant-type asparaginases. Biochem J. 2002;364(Pt 1):129-136.
47. Lanvers-Kaminsky C, Rüffer A, Würthwein G, et al. Therapeutic drug monitoring of asparaginase activity-method comparison of MAAT and AHA test used in the international AIEOP-BFM ALL 2009 trial. Ther Drug Monit. 2018;40(1):93-102.
48. Horvath TD, Chan WK, Pontikos MA, et al. Assessment of l-asparaginase pharmacodynamics in mouse models of cancer. Metabolites. 2019;9(1):10.
49. Dennis MS, Zhang M, Meng YG, et al. Albumin binding as a general strategy for improving the pharmacokinetics of proteins. J Biol Chem. 2002;277(38):35035-35043.
50. Pieters R, Hunger SP, Boos J, et al. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. Cancer. 2011;117(2):238-249.
51. 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.
52. Burke MJ. How to manage asparaginase hypersensitivity in acute lymphoblastic leukemia. Future Oncol. 2014;10(16):2615-2627.
53. Salzer WL, Asselin BL, Plourde PV, Corn T, Hunger SP. Development of asparaginase Erwinia chrysanthemi for the treatment of acute lymphoblastic leukemia. Ann N Y Acad Sci. 2014;1329:81-92.
54. Tong WH, Pieters R, Kaspers GJ,et al. A prospective study on drug monitoring of PEGasparaginase and Erwinia asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia. Blood. 2014;123(13):2026-2033.
55. Salzer WL, Asselin B, Supko JG, et al. Erwinia asparaginase achieves therapeutic activity after pegaspargase allergy: a report from the Children's Oncology Group. Blood. 2013;122(4):507-514.
56. Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty G. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer. 2007;110(1):103-111.
57. Ding S, Song M, Sim BC, et al. Multivalent antiviral XTEN-peptide conjugates with long in vivo half-life and enhanced solubility. Bioconjug Chem. 2014;25(7):1351-1359.
58. Schlapschy M, Binder U, Börger C, et al. PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. Protein Eng Des Sel. 2013;26(8):489-501.
59. Gebauer M, Skerra A. Prospects of PASylation® for the design of protein and peptide therapeutics with extended half-life and enhanced action. Bioorg Med Chem. 2018;26(10):2882-2887.
60. Maese L, Rizzari C, Coleman R, Power A, van der Sluis I, Rau RE. Can recombinant technology address asparaginase Erwinia chrysanthemi shortages? Pediatr Blood Cancer. 2021;68(10):e29169.