HHS Public Access

Author manuscript

Cancer Res. Author manuscript; available in PMC 2019 March 15.

Published in final edited form as:

Cancer Res. 2018 March 15; 78(6): 1549–1560. doi:10.1158/0008-5472.CAN-17-2106.

A novel L-asparaginase with low L-glutaminase coactivity is highly efficacious against both T and B cell acute lymphoblastic leukemias *in vivo*

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Abstract

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Disclosure of Potential Conflicts of Interest

H.A. Nguyen, A.M. Schalk, Y. Su. and A. Lavie declare competing financial interest by being founders with equity stake in Enzyme by Design, Inc. a startup developing new L-asparaginases. Y. Saunthararajah is on the Scientific Advisory Board of Enzyme by Design, Inc. All other authors declare no competing financial interests.

Acute lymphoblastic leukemia (ALL) is the most common type of pediatric cancer, although about 4 of every 10 cases occur in adults. The enzyme drug L-asparaginase serves as a cornerstone of ALL therapy and exploits the asparagine-dependency of ALL cells. In addition to hydrolyzing the amino acid L-asparagine, all FDA-approved L-asparaginases also have significant L-glutaminase coactivity. Since several reports suggest that L-glutamine depletion correlates with many of the side effects of these drugs, enzyme variants with reduced L-glutaminase coactivity might be clinically beneficial if their anti-leukemic activity would be preserved. Here we show that novel low L-glutaminase variants developed on the backbone of the FDA-approved Erwinia chrysanthemi L-asparaginase were highly efficacious against both T and B cell ALL, while displaying reduced acute toxicity features. These results support the development of a new generation of safer L-asparaginases without L-glutaminase activity for the treatment of human ALL.

INTRODUCTION

Bacterial L-asparaginases are enzymes with dual activities. The predominant one, the L-asparaginase activity that gives these enzymes their name, is the ability to hydrolyze the amino acid L-asparagine (Asn) into L-aspartic acid (Asp) and ammonia. The secondary activity present in L-asparaginases is an L-glutaminase activity, which drives hydrolysis of L-glutamine (Gln) to L-glutamic acid (Glu) and ammonia. For the FDA-approved L-asparaginases (*Escherichia coli* (*Ec*A) and *Erwinia chrysanthemi* (*Er*A), approved in 1978 and 2011, respectively), the L-glutaminase activity ranges from 2 to 10% of their primary L-asparaginase activity (1). The dual L-asparaginase and L-glutaminase property of *Ec*A and *Er*A is expected to manifest itself in the depletion of both Asn and Gln in the patient's blood, a notion that is supported by several studies (2–5).

The anticancer effect of L-asparaginase is believed to be predominantly due to the depletion of Asn from the blood. Indeed, leukemic blasts from ALL patients completely depend on scavenging Asn from the blood, as they lack or display very low levels of the asparagine synthetase (ASNS) enzyme (6–8). In contrast, the clinical importance of the L-glutaminase activity present in all FDA-approved versions of L-asparaginases is still under debate, with conflicting reports in the literature about its putative anti-leukemic effect (9,10). On one hand, pharmacodynamic analyses showed that deamination of Gln is critically required for optimal Asn deamination (2) and other more recent studies indicated contribution of L-glutaminase activity to the cytotoxicity of L-asparaginase on leukemic cells (10,11). In contrast, others have found that the L-glutaminase activity is not required for the drug's *in vitro* anticancer effect, as long as the ALL cells lack ASNS (9).

Common side effects in patients treated with L-asparaginases, in addition to an immune response against the bacterial enzymes, include hepatotoxicity, hyperglycemia, dyslipidemia, perturbations in blood coagulation factors, and pancreatitis (12–14). Several clinical studies have documented the Gln depletion resulting from the L-glutaminase coactivity of current L-asparaginase preparations (4,15,16), and suggested that the aforementioned side effects can, at least in part, be attributed to this property of the drugs. For example, a link between the L-glutaminase activity and the immunosuppressive effects of these drugs have been reported

(17,18), as well as its role in hepatotoxicity (19) which was proposed to be due to deleterious effects on Gln homeostasis (3). Likewise, Gln depletion could likely contribute significantly to the disrupted protein synthesis in the liver and spleen that is a cause of the coagulopathy aspects of drug toxicity (20). Moreover, hydrolysis of both Asn and Gln will produce ammonia as a byproduct of the reaction. However, given that Gln concentrations are much higher in the blood as compared to Asn, Gln hydrolysis will have a more profound effect on the eventual concentration of ammonia in the blood. Indeed, hyperammonemia has been observed in patients undergoing L-asparaginase treatment (16,21–25), which has been associated with neurotoxicity.

Additional information on the putative interplay between L-glutaminase activity and drug toxicity came from at least four clinical trials of L-asparaginases. First, in the early 1980s, a clinical trial, which examined an L-asparaginase from *Acinetobacter* with very high L-glutaminase activity, was forced to terminate early due to central nervous system toxicity (26). Second, between 2001–2008, the L-asparaginase from *Wolinella succinogenes,* which was initially thought to be a low L-glutaminase enzyme, was evaluated clinically through a US National Cancer Institute Rapid Access to Intervention Development (NCI RAID) grant. However, the enzyme produced via this program was found to be toxic in patients and we recently showed that it actually does contain significant L-glutaminase activity (27). Third, in 2008, a phase II clinical trial examining the FDA-approved *Ec*A in ovarian cancer patients had to be terminated early due to excessive toxicities (28). Interestingly, while weight loss was reported as one of the main drug-related toxicities in the phase II ovarian cancer study, it is also a significant L-glutaminase-related toxicity indicator in our actual pre-clinical study. Finally and very recently, a clinical trial of eryaspase (red blood cell encapsulated *Ec*A) showed that, for a yet unclear reason, the encapsulation process reduced the L-glutaminase activity (i.e. increased the selectivity for Asn hydrolysis over Gln hydrolysis), a factor pointed out as an explanation for the decrease in adverse events in the eryaspase clinical trial compared to naked *Ec*A (29). Hence, these trials support the notion that certain side effects observed in patients undergoing L-asparaginase treatment might be associated with the level of L-glutaminase activity. Therefore, reducing the L-glutaminase activity of available L-asparaginases may be advantageous to lessen toxic side effects, but for now it is unclear whether this would be detrimental for the anti-leukemic efficacy of these drugs.

Previously, we engineered variants of *Er*A with decreased L-glutaminase activity while maintaining near wild-type L-asparaginase activity (30). Here we evaluated these novel *Er*A variants *in vitro* and *in vivo* for their ability to kill ALL cells, and compared them to their wild-type counterpart. It is important to appreciate the experimental complexity when comparing different L-asparaginases for their efficacy and toxicity, since in addition to the kinetic properties of the enzyme drugs, pharmacokinetics and immunogenicity (when tested in patients) play a major role in determining the outcome. To simplify the interpretation of the results, here we present the comparison of L-asparaginases that have similar L-asparaginase activities and that only differ by 1–3 residues, suggesting very similar pharmacokinetic properties, but that have vastly different L-glutaminase activity. Together, our results suggest that high L-glutaminase activity, as present in current FDA drugs, is not essential for efficient *in vivo* elimination of L-asparaginase sensitive ALL cells. Additionally, reduced toxicity was observed in the low L-glutaminase variants compared to

the high L-glutaminase enzymes. This sets up the rationale for further evaluation of such low L-glutaminase variants, which are predicted to have fewer side effects, as alternatives to the current FDA-approved bacterial L-asparaginases for the treatment of ALL.

Materials and Methods

Expression and Purification of L-asparaginases

Enzymes used for kinetic, NMR, and cell culture studies were expressed and purified as previously reported in Nguyen et al. (30,31) for *Er*A-WT, *Er*A-E63Q, *Er*A-DM, and *Er*A-TM; and as in Schalk et al. (32) for *Ec*A-WT.

Kinetic Assays

L-asparaginase and L-glutaminase activities were determined using a continuous spectroscopic enzyme-coupled assay as previously described (32,33).

Cell culture

The LOUCY cell line was established from the peripheral blood of a T-cell ALL patient (34). The luciferase-positive LOUCY cell line was generated as described previously (35). The SUP-B15 cell line was established from cells harvested from the bone marrow of a Philadelphia chromosome positive B-cell ALL patient (36). The luciferase-expressing SUP-B15 cell line was a kind gift from Dr. Michael Jensen, University of Washington School of Medicine. All cell lines were analyzed by STR (Short Tandem Repeat) and confirmed to match 100% to corresponding STR profile data from the Global Bioresource Center ATCC. All cell lines were verified to be mycoplasma free. The Alamar Blue assay for cell viability is described in Supplementary Methods. IC_{50} values were determined by GraphPad Prism 6.0 using sigmoidal interpolation model with 95% confidence intervals.

In vivo treatment of cell line xenografts with L-asparaginases

Non-obese diabetic/severe combined immune-deficient γ (NSG) mice (The Jackson Laboratory) were i.v. injected at 6 weeks of age with 150 μL DPBS containing 5×10^6 luciferase-positive LOUCY or 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, the mice were randomly divided into different groups that were administered via i.p. injection at a dose of 50 IU/mouse daily for 14 days with either *Er*A-WT, *Er*A-E63Q, *Er*A-DM, *Er*A-TM or the same volume of DPBS. In another experiment, LOUCY-engrafted mice were treated with 25 IU/mouse at days 0, 2, 4, 7, 9, 11, 12, 13 and 14 via i.p. injection. The bioluminescent imaging (BLI) signal was measured every two to three days as indicated in Fig. 2; Fig. S1, S2 and S3. During the experiment, the mice were observed and weighed every day. The ethical committee on animal welfare at University of Illinois at Chicago approved this animal experiment.

Acute toxicity study

The experimental design of this study incorporated a blinded strategy where the toxicologist was provided with samples labeled as #1 and #2, without knowing the identity of the

enzymes (*Er*A-WT or *Er*A-TM). In this dose escalation study, 6 animals (3 males, 3 females) per dose group were administered the enzymes i.v. at a starting dose of 40 IU/g, increasing to 80 and finally 160 IU/g. Due to a shortage of the enzymes, *Er*A-TM group 6 was limited to 4 animals (3 females, 1 male), and a few animals did not receive the full intended dose (one animal of *Er*A-WT group 3 received 136 instead of the intended 160 IU/g, one animal of the *Er*A-TM group 5 received 60 instead of the intended 80 IU/g). The unexpected shortage of enzymes was due to higher than expected loss during filtration through a 0.22 μm filter and the adjustment needed for bigger body weight of the mice. After enzyme administration, the animals were monitored daily and clinical signs (hunched posture, decreased activity, sunken eyes, and rough coat) were noted if observed. None of the animals died during the 4-day observation period, and all the animals were euthanized at the end of day 4.

In vivo asparaginase activity determination

C57BL/6 mice of 7–10 weeks old were i.p. injected with two batches of 50 IU of *Er*A-WT or *Er*A-TM. 24 hours after the injection, peripheral blood was collected (5 animals per group) via cardiac puncture under anesthesia (5% isoflurane in oxygen). Shortly after collection, blood was centrifuged in heparin-coated tubes (2000 g, 10 min, 4°C) for plasma preparation. Plasma L-asparaginase activity was quantified by incubating the samples with an excess amount of L-aspartic acid β-hydroxamate (AHA) (Sigma-Aldrich A6508) at 37.0°C. L-asparaginase hydrolyses AHA to L-aspartic acid and hydroxylamine, which was detected at 690 nm with a SpectraMax M3 (Molecular Devices) spectrophotometer, after condensation with 8-hydroxyquinoline (Merck 8.20261) and oxidation to indooxine. A detailed procedure can be found in the Supplementary Data. The ethical committee on animal welfare at Ghent University Hospital approved the experiment.

In vivo pharmacodynamics of amino acid

C57BL/6 mice of 7–10 weeks old were i.p. injected with 50 IU of *Er*A-WT or *Er*A-TM. For the pharamacodynamic study, peripheral blood was collected at days 1, 3, 7, and 14 (5 animals per group) via cardiac puncture under anesthesia (5% isoflurane in oxygen). In addition, blood of seven untreated mice was collected to determine the baseline value (day 0). Shortly after collection, blood was centrifuged in heparin-coated tubes (2000 g, 10 min, 4°C) for plasma preparation. Plasma was diluted with equal volume of a 10% 5'-sulfosalicylic acid dihydrate solution in water and stored at −80°C for the determination of amino acid levels.

For the amino acid analysis, the plasma samples (50 μL) were deproteinized by adding 100 μL of a 10% sulfosalicylic acid solution containing 50 μM internal standards mix. After vortexing, 50 μL of UPLC-grade water was added. Centrifugation occurred for 10 minutes at 9960 g. Derivatization of 10 μL of supernatant was performed according to the manufacturer's instructions of the AccQ-Tag® kit of Waters. The 4 amino acids (asparagine, aspartic acid, glutamine and glutamic acid) were measured on an Acquity UPLC with QDA detector of Waters and quantified based on a 5-points calibration curve. The ethical committee on animal welfare at Ghent University Hospital approved these experiments.

Patient-derived xenograft experiment

A xenograft of a pediatric primary human T-ALL sample was established in NSG mice. Upon establishment of disease, human leukemic cells were isolated from the spleen via Ficoll-Paque (GE Healthcare) density gradient centrifugation. Next, these cells were injected in the tail vein of 15 female NSG mice at 7 weeks of age. Each mouse received 150 μl PBS containing 1.2×10^6 cells. Engraftment of the cells was followed by measuring the percentage of human CD45 positive (%huCD45$^+$) cells in the blood. Upon evidence of leukemic cell engraftment, mice were randomly divided into 3 groups (day 0), and treated daily via i.p. injection with 50 IU/mouse for 13 days of either *Er*A-WT, *Er*A-TM or the same volume of PBS. At day 0, 7 and 13, blood was collected via the tail vein. At day 13, all mice were sacrificed and the spleen and bone marrow were collected. The %huCD45$^+$ cells in the blood, bone marrow and spleen was analyzed by staining with a phycoerythrin-labeled antibody for human CD45 (130–098-141; Miltenyi Biotec, Bergisch Gladbach, Germany), performing red blood cell lysis and measuring the percentage on a LSRII flow cytometer using FACSDiva software (BD Bioscience). During the experiment, mice were observed and weighed every day. The experiment was approved by the ethical committee on animal welfare at Ghent University Hospital.

qPCR experiments

Total RNA was isolated using the miRNeasy mini kit (Qiagen) and the RNAse-Free DNAse set (Qiagen). cDNA was synthesized with the iScript Advanced cDNA synthesis kit (Bio-Rad). The SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used and the PCR reactions were run on the LightCycler 480 (Roche, model LC480). Every sample was analyzed in duplicate. qBasePLUS software (Biogazelle, Zwijnaarde, Belgium) was used for analysis. Gene expression was normalized against 3 reference genes (GAPDH, TBP, YWHAZ). ASNS primers: (F) 5’-CCCTGCACGCCCTCTATG-3’, (R) 5’-GGATCCTGAGGTTGTTCTTCACA-3’; GAPDH primers: (F) 5’-TGCACCACCAACTGCTTAGC-3’, (R) 5’- GGCATGGACTGTGGTCATGAG-3’; TBP primers: (F) 5’- CACGAACCACGGCACTGATT -3’, (R) 5’-TTTTCTTGCTGCCAGTCTGGAC -3’ and YWHAZ primers: (F) 5’-ACTTTTGGTACATTGTGGCTTCAA-3’, (R) 5’- CCGCCAGGACAAACCAGTAT-3’.

Statistical Methods

See Supplementary data file for details on Biostatistics.

RESULTS

Design and characterization of *Er*A variants with high L-asparaginase and low L-glutaminase activities

To determine whether L-asparaginase variants with low L-glutaminase activity may hold clinical potential, we investigated several L-glutaminase-deficient *Er*A variants (denoted as *Er*A-E63Q, *Er*A-DM (double mutant) and *Er*A-TM (triple mutant)) that retain most of their wild-type L-asparaginase activity (30). Comparisons of kinetic parameters between these *Er*A variants and the FDA-approved wild-type versions of *Er*A and *Ec*A (denoted as *Er*A-

WT and *Ec*A-WT) are summarized in Table 1. We selected *Er*A-WT over *Ec*A-WT as the backbone to develop low L-glutaminase variants because of its superior L-asparaginase activity (~2.5-fold higher rate than *Ec*A-WT in hydrolyzing Asn at the physiological concentration of 50 μM). However, *Er*A-WT also has >70-fold higher L-glutaminase activity compared to *Ec*A-WT at the physiological concentration of 500 μM Gln. Since both k_{cat} (i.e. the rate at saturating substrate concentration) and Km influence the turnover of enzyme catalyzed reactions, the k_{cat}/Km ratio is often taken as a measure of enzyme efficiency, and this ratio is shown in Table 1 for the L-asparaginase and L-glutaminase activities of the examined enzymes. However, to assess the specificities of the enzymes, we also calculated the ratio between the k_{cat}/Km of the L-asparaginase reaction to the k_{cat}/Km of the L-glutaminase reaction (the larger the number, the higher is the specificity for the L-asparaginase reaction). From this calculation, it is clear that *Er*A-TM and *Er*A-DM are much more L-asparaginase specific (ratio = 68,750 and 4,842, respectively) compared to the original *Er*A-WT enzyme (ratio = 58.6), but that only *Er*A-TM is significantly more specific than *Ec*A-WT (ratio = 4,625). However, this analysis based on the ratios of the L-asparaginase and L-glutaminase k_{cat}/Km values may not best reflect the physiological conditions. Therefore, we also calculated specificity ratios based on the observed rates (k_{obs}) at physiological substrate concentrations (50 μM for Asn, 500 μM for Gln). These calculations show that our engineered *Er*A variants have significantly superior Asn:Gln specificity as compared to *Er*A-WT (Table 1). Using this calculation, even *Er*A-DM is about 2-fold more L-asparaginase specific compared to *Ec*A-WT, with *Er*A-TM being 47-fold more specific. The reduced rate of L-glutaminase activity for each *Er*A mutant was also demonstrated through measuring changes to Gln concentrations over time by NMR spectroscopy (Fig. 1A & B). Notably, while *Ec*A-WT completely hydrolyzed Gln in ~45 minutes, solutions with *Er*A-DM and *Er*A-TM contained >80% of the starting Gln after 1 hour, demonstrating their exceptionally low L-glutaminase activities (Fig. 1B).

In vitro testing of *Er*A-WT and *Er*A mutants in ALL cell lines

The development of *Er*A mutants with comparable L-asparaginase but variable L-glutaminase activity (*Er*A-E63Q > *Er*A-DM > *Er*A-TM) allowed us to test whether the high intrinsic L-glutaminase activity of *Er*A-WT is truly required for its clinical efficacy. Notwithstanding the limitations of evaluating L-asparaginase in cell culture, we first validated the anti-proliferative effect of wild type and mutant L-asparaginases *in vitro* on the human leukemic cell lines, LOUCY (T-ALL) and SUP-B15 (B-ALL). Results indicated that both ALL cell lines were similarly sensitive to *Er*A-WT, *Ec*A-WT, and to each of the L-glutaminase-deficient *Er*A mutants (Table 2 and Fig. S4). Since most cell lines depend on high Gln levels in culture, the slightly lower IC_{50} values for *Er*A-WT and *Ec*A-WT compared to the L-glutaminase-deficient *Er*A mutants is not surprising.

The His-SUMO tag acts to stabilize the *Er*A variants *in vivo*

Given that *in vitro* studies cannot unambiguously clarify whether L-glutaminase activity is required for *in vivo* effectiveness of L-asparaginases, we subsequently used xenograft models of luciferase-positive LOUCY and SUP-B15 cells to perform *in vivo* drug treatment experiments. Engraftment of human leukemic cells in mice is often considered successful when the percentage of peripheral blood (PB) cells positive for the human CD45 antigen

(%huCD45$^+$) is ≥1–2% (37,38). Four weeks after NOD-*scid* IL2Rgamma^{null} mice (NSG) received cell line injections, bioluminescence imaging (BLI) flux signals corresponding to a PB %huCD45$^+$ greater than 8% confirmed successful engraftment and showcased the high level of disease burden in the examined animals (Fig. S1, S2 and S3; Fig. S5 reports the calibration between BLI flux and PB %huCD45$^+$). With this level of engraftment, daily drug treatment with *Er*A-WT (intraperitoneal injection (i.p.) of 50 IU/day for 14 days) was initiated. Surprisingly, this FDA-approved L-asparaginase failed to reduce tumor cell growth *in vivo*. Of note, *Er*A-WT has a half-life of only 0.65 days in humans, compared to 1.24 days for *Ec*A-WT (39). Furthermore, half-lives of these drugs are dramatically shortened in mice (40). Thus, we hypothesized that the short half-life of *Er*A-WT prevented therapeutic efficacy. To evaluate whether drug instability indeed hindered the anticancer effect, we retained the N-terminal SUMO tag, which was originally incorporated to increase stability and facilitate the heterologous expression of the enzymes in *E. coli*. The SUMO tag has only moderate impact on the enzymatic activity of the variants (see Table 1), and dosing of the drugs according to their activity (i.e. adjusted to deliver the same IU) largely accounts for the effect of the SUMO tag on activity. Notably, treatment of the LOUCY cells xenografted mice with this adapted SUMO-*Er*A-WT enzyme (50 IU/day i.p. for 12 days) resulted in a marked decrease in tumor burden (Fig. S1), suggesting that maintaining the SUMO tag increased the stability of the enzyme with no or minimal effects towards the therapeutic enzymatic properties of *Er*A-WT. Therefore, this stability tag was incorporated in the wild-type and mutant *Er*A enzymes used in subsequent *in vivo* drug treatment experiments.

In vivo testing of *Er*A-WT and *Er*A mutants in ALL cell line xenograft models

We next evaluated the *in vivo* efficacy of the low L-glutaminase variants listed in Table 1. *Er*A-E63Q and *Er*A-DM were as efficient as *Er*A-WT in reducing the BLI signal in mice engrafted with LOUCY (Fig. S1) or SUP-B15 cells (Fig. S2). For a more stringent evaluation of the requirement for the L-glutaminase coactivity for L-asparaginase efficacy against ALL *in vivo*we compared *Er*A-TM, the variant with the lowest L-glutaminase activity, with *Er*A-WT, which has inherently high L-glutaminase activity. Results indicated that both enzymes are indistinguishable in their ability to rapidly reduce leukemic burden (Fig. 2A; Fig. S3 and Fig. S6). After 14 days of consecutive treatment, both *Er*A-WT- and *Er*A-TM-treated mice displayed BLI signals diminished to background levels (Fig. 2B). In addition, analysis of huCD45$^+$ cells in PB and bone marrow (BM) of *Er*A-WT- and *Er*A-TM-treated mice showed undetectable levels of leukemic cells at day 14 of treatment (Fig. 2, C and D). Smaller spleen size and reduced lymphoblastic invasion of liver tissue further confirmed the comparable anti-leukemic efficacy of *Er*A-WT and *Er*A-TM (Fig. 2, E and F).

Since the dosing regimen used for the above studies (50 IU/mouse daily) induced an abrupt decrease in the BLI signal for both *Er*A-WT and *Er*A-TM treated groups (BLI signal below 7% at day 3 and less than 1% relative to treatment start already by day 7, Fig. 2A, Fig. S3 and Fig. S6), we asked whether a less aggressive dosing regimen would reveal differences in efficacy between the two enzyme variants. Therefore, we conducted an additional efficacy study where the animals were treated with 25 IU/mouse (half the dose of the previous experiments). Since after the initial 6 drug treatments, which were administered on a Mon-Wed-Fri schedule, the BLI was not below background level for both groups (1 – 5% relative

to day 0), we added three daily drug injections at the same dose (for a total of 9 drug administrations per group). The ALL burden, quantitated by the BLI signal, was monitored during the treatment period followed by an additional 15 days after the last treatment (Fig 2G). Even at this reduced dose, both enzymes rapidly decreased the BLI signal. Multiple t-tests were calculated for every single imaging data point revealing no statistically significant difference in BLI between the two treated groups (p-value >0.01). The BLI signal was below 0.5% at the last day of treatment and a week later (day 22), and remained below 1% even at day 29 (15 days post last treatment).

However, the residual BLI signal indicated that the cancer was not eradicated in both treated cohorts, suggesting that further drug dosing would be needed. Whereas the *Er*A-TM treated group could tolerate additional doses, this was not the case for the *Er*A-WT-treated group, a point clearly illustrated by the condition of the shredding toys (untouched by the *Er*A-WT-treated group, shredded by the *Er*A-TM-treated group), shown both at the last day of drug treatment and 10 days later (Fig. S7). Collectively, these results convincingly show that the ultra-low L-glutaminase *Er*A-TM enzyme maintains a very similar ability to combat ALL cells *in vivo* as compared to the high L-glutaminase *Er*A-WT enzyme but with an improved tolerability profile.

Indication for reduced toxicity of the low L-glutaminase *Er*A variants

In addition to noting the reduced activity of the high L-glutaminase *Er*A-WT-treated mice, but not of the low L-glutaminase *Er*A-TM-treated mice, we also compared the impact of the drugs on the animals' body weight. At the end of the experiment we observed 27% mean weight loss in *Er*A-WT-treated mice (Fig. 2H), which is consistent with results from previous studies (41,42). This effect was largely mitigated in the animals treated with *Er*A-TM, which only experienced 10% mean weight loss. Hence, by treatment day 14, the *Er*A-WT group lost on average an additional 4 grams of body weight (7 versus 3) compared to *Er*A-TM (p-value <0.0001). In addition, the correlation between percentage weight loss and level of enzyme L-glutaminase activity level was reproducible across several independent experiments (Fig. S8, A and B). Finally, we examined the acute toxicity of *Er*A-WT and *Er*A-TM in a blinded single dose-escalation study in CD-1 mice, using a concentration range of 40 to 160 IU/g. For comparison, the dose used to treat the ALL-bearing mice was ~2.5 IU/g. No fatalities occurred in either group, but clear physical and behavioral signs of toxicity were observed in the *Er*A-WT-treated group (Table 3). These signs were virtually absent in *Er*A-TM-challenged mice (Table 3). Together, these data show a clear correlation between reduced L-glutaminase activity and reduced drug toxicity. As previously noted, a common side effect of L-asparaginase treatment is hepatotoxicity, which presents histopathologically as macrovesicular hepatic steatosis (43), coupled with abnormally elevated serum levels of the liver enzymes alanine aminotransferase (ALT) (44) and aspartate aminotransferase (AST) (45). However, blood chemistry analysis did not display a clear difference in ALT or AST levels between *Er*A-WT- and *Er*A-TM-treated mice. The absence of a difference in serum liver enzyme levels in the studied animals may not be surprising since L-asparaginase induced hepatotoxicity in humans has been linked to fatty liver disease (46), elevated BMI (12,47), and increased age (48), whereas the mice studied did not present histologically with hepatic fat accumulation (Fig. 2F), were lean, and young.

The differences in toxicity between *Er*A-WT and *Er*A-TM is not due to a difference in *in vivo* stability but correlates with the enzyme's impact on the blood glutamine levels

The similar anti-ALL power of *Er*A-WT and *Er*A-TM but dissimilar toxicity profile could be due to their different L-glutaminase activity, but could also be due to different pharmacokinetic properties. To investigate this possibility, mice were injected i.p. with 50 IU of *Er*A-WT or *Er*A-TM from two different batches - we chose this drug dose to be consistent with the majority of our efficacy studies. The L-asparaginase activity in blood plasma samples was measured 24 h after the initial injection (for both batches) and in addition at days 3, 7 and 14 for the second batch. We measured comparable L-asparaginase activity for both enzymes at all time points, indicating a similar *in vivo* stability (Fig. 3A and B). We also determined the levels of the amino acids asparagine, aspartate, glutamine, and glutamate prior to the treatment and at days 1, 3, 7 and 14 post administration of *Er*A-WT or *Er*A-TM. Both enzymes depleted the blood asparagine, which was below detection at day 1 and 3 and then slowly recovered (Fig. 3C). Consistent with this, the levels of aspartate increased at day 1 and 3, and then decreased to the pre-treatment level by day 7 (Fig. 3D).

Notably, when we examined the effect of the enzymes on glutamine, we noted that *Er*A-WT reduced the glutamine level from ~600 μM pre-treatment to ~250 μM at day 1, which then promptly recovered by day 3. In contrast, *Er*A-TM did not reduce the glutamine levels (Fig. 3E). As expected from this, the glutamate levels increased for the *Er*A-WT-treated mice, but not for the *Er*A-TM-treated animals (Fig. 3F). These results are consistent with the predictions from the kinetic properties of these enzymes (Table 1) and the NMR experiments (Fig. 1), showing that the *in vitro* observed similarity in the L-asparaginase activity but differences in the L-glutaminase activity translates into the *in vivo* setting. Moreover, whereas the glutamine levels recovered by day 3, we would expect that the daily dosing schedule, as used in our efficacy studies, would continuously impact the glutamine levels.

In vivo evaluation of *Er*A-WT and *Er*A-TM using a patient derived T-ALL xenograft model

The previous experiments demonstrated the anti-ALL equivalence between *Er*A-WT and *Er*A-TM in both T- and B-ALL cell lines. To further probe the potential clinical relevance of *Er*A-TM, we compared these enzymes using a patient derived xenograft (PDX) model from a primary human T-ALL. After verifying leukemia engraftment (%huCD45+ range 1.8 – 7.5%), animals (n=5/group) were randomized into control, *Er*A-WT-, and *Er*A-TM- treated groups (50 IU/dose daily for 13 days). PB %huCD45+ increased dramatically in the control group (average 4% increasing to 78% at day 13; Fig. 4A). In contrast, both of the L-asparaginase treated groups displayed very low %huCD45+ in PB at day 13. These data were further recapitulated when examining %huCD45+ in BM after the animals were sacrificed at day 13 (Fig. 4B), and in spleens at that time point (Fig. 4C). Likewise, analysis of the spleen weights showed enlarged spleens for the controls but normal sized spleens for the treated animals (Fig. 4D). These data demonstrate that both *Er*A-WT and *Er*A-TM carry strong pre-clinical *in vivo* activity, while bigger cohorts might be essential to demonstrate whether or not differences in efficacy between the enzymes are present. The seemingly moderate increase in potency of *Er*A-WT comes with increased toxicity, as indicated by the amount of weight lost by the animals. Indeed, recapitulating the weight loss trend we observed before (Fig. 2H), mice treated with *Er*A-WT lost nearly twice as much weight

compared to *Er*A-TM (30% versus 15% of starting body weight, p-value <0.05). (Fig. 4E). As in the previous experiment with the LOUCY cell line, we could not detect differences in ALT or AST levels between *Er*A-WT- and *Er*A-TM-treated mice.

Whereas treatment of mice engrafted with the T-ALL cell line LOUCY with *Er*A-WT or *Er*A-TM resulted in complete loss of the BLI signal and in undetectable %huCD45+ in the PB and BM (Fig. 2, A–D), treatment of mice engrafted with primary T-ALL cells resulted in lowered but still detectable leukemia burden (Fig. 4A–D). One possible explanation for the difference in efficacy between the human cell lines and the PDX could be the ASNS expression level. To probe this point, we used quantitative PCR to measure the ASNS mRNA levels in the LOUCY cell line, in the patient-derived cells used in the PDX experiment, and in 4 additional T-ALL patient samples. Indeed, the ASNS mRNA in the LOUCY cells is 20 to 30-fold lower than that measured in the patients' sample (Fig. S9). However, we note that a growing body of evidence conclusively revealed no correlation between ASNS expression and sensitivity to L-asparaginase in patients (49–52). Hence, the genetic reason(s) that make ALL susceptible to L-asparaginase therapy is/are not fully understood. Based on the data presented here, it appears that patients with measurable ASNS levels will have similar responsiveness but better drug tolerance to the low L-glutaminase *Er*A-TM variant compared to the FDA-approved *Er*A-WT drug.

DISCUSSION

Identifying the L-asparaginase with the best clinical properties is a challenge. Scoring L-asparaginases based solely on cell culture data, where L-glutamine is indispensable, may be misleading, due to the significant L-glutaminase activity of these enzymes. Scoring L-asparaginases based on *in vivo* data is much more clinically relevant but also presents several challenges. The enzyme's kinetic properties (k_{cat} and Km values) for both Asn and Gln are important parameters to consider. Additionally, the drug's persistence in circulation (*i.e.*, half-life) will determine the duration of enzymatic Asn and Gln depletion, a parameter that can be fine-tuned by appropriate dose and frequency of drug administration. Ideally, one would like to combine a prolonged half-life as seen with the introduction of pegylated *Ec*A, high L-asparaginase activity, and only low or absence of L-glutaminase activity, the latter predicted to be in part responsible for acute toxicities of the drug. Currently, a pegylated version of *Er*A-WT is being evaluated (53). While such a version is predicted to solve the problem of short *in vivo* persistence of native *Er*A, we caution that with the concomitant longer time for Asn depletion, such an enzyme will also have a longer duration of Gln depletion. Considering the very high L-glutaminase activity of *Er*A-WT, this would predict increased side effects.

Here we present *Er*A variants with significantly lower L-glutaminase but comparable L-asparaginase activities relative to *Er*A-WT and extended circulation time achieved by maintaining the SUMO tag. We demonstrated that our engineered low L-glutaminase *Erwinia* L-asparaginase variants have preserved cell killing properties, similar to *Er*A-WT. Comparable pharmacokinetic properties of the SUMO-tagged *Er*A-WT vs *Er*A-TM enzymes accompanied by similar Asn but remarkably different Gln depletion profiles convincingly discount the possibility that the observed anti-leukemic effect in *Er*A-TM was due to

glutamine depletion. Moreover, the comparable serum persistence and anti-leukemic properties of the enzymes indicate that the significant difference in the toxicity profile is linked to the difference in impact on Gln levels, which suggests a superior tolerability for the low L-glutaminase variant *Er*A-TM.

Others have also recognized the potential advantages of L-asparaginases with low L-glutaminase activity. One in particular is the low L-glutaminase S121 variant of the *Wolinella succinogenes* L-asparaginase (*Wo*A-S121) – for additional details about this enzyme see Supplementary material. The ratio of the L-asparaginase to L-glutaminase rates (measured at the physiological substrate concentrations of 50 μM for Asn and 500 μM for Gln) best conveys the clinically-relevant substrate specificity of these drugs (a high ratio describes a more specific L-asparaginase with low L-glutaminase). We recently investigated the properties of *Wo*A-S121 and discovered that this ratio is 62 (27), which is actually inferior to that for *Ec*A-WT (ratio = 188), but superior to *Er*A-WT (ratio = 6.6) – Table 1. However, this ratio for *Er*A-TM is ~9,000, showcasing the extremely high L-asparaginase preference of this variant.

In conclusion, this study convincingly shows that high L-glutaminase activity, as present in current FDA-approved L-asparaginase drugs, is not essential for efficient *in vivo* elimination of L-asparaginase sensitive ALL cells. Furthermore, our results suggest a decline in *in vivo* toxicity when the drug's L-glutaminase activity is reduced. Since the debilitating side effects of current L-asparaginases often result in treatment cessation, an event associated with inferior event-free survival (48,54), L-asparaginases with diminished toxicity are highly pertinent to improving ALL treatment outcome. In line with this notion, a recent pilot study, (55) which evaluated an intensified L-asparaginase treatment in a population of high-risk pediatric ALL patients using pegylated *Ec*A (the current standard of care in the USA with >20-fold higher L-glutaminase activity compared to *Er*A-TM), had to be aborted due to an unacceptable frequency of adverse-effects. However, since the patients were receiving additional chemotherapeutic drugs, the causative agent behind the increased toxicity cannot be directly linked to the more frequent dosing of the L-asparaginase. Notably, the ultra-low L-glutaminase *Er*A-TM variant, as presented in this study, now provides an alternative L-asparaginase that can directly probe this point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Michael Jensen (University of Washington School of Medicine) for the generous gift of the SUP-B15-luciferase cell line and Béatrice Lintermans for excellent technical assistance. The histology work was carried out by the UIC Research Resources Center's Research Histology and Tissue Imaging Core.

Financial support

A. Lavie. Was supported in part by NIH grant RO1 EB013685 and by Merit Review Award I01BX001919 from the United States (U.S.) Department of Veterans Affairs Biomedical Laboratory Research and Development Service, and by the UIC Cancer Center. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

P. Van Vlierberghe was supported by the Fund for Scientific Research Flanders (FWO) grant 3G0C4713, by the Belgian Foundation Against Cancer grant 365W3415W and by Stand up to Cancer, the Flemish Cancer Society research grant 365Y9115W. S. Piers was support by doctoral and postdoc FWO grant FWO17/PDO/111.

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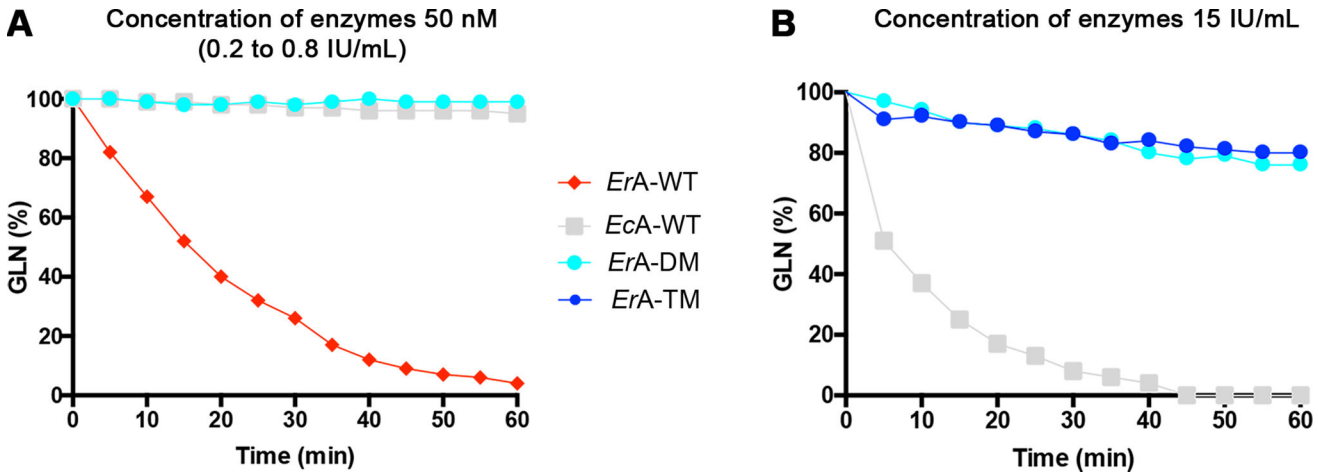

Fig. 1. Engineered *Er*A variants have reduced L-glutaminase activity

(**A**) NMR spectroscopy was used to monitor the ability of various L-asparaginases to hydrolyze Gln. The starting Gln concentration (600 μM; labeled as 100%) was chosen to reflect physiological Gln levels. All enzymes were added to the same final concentration of 50 nM, which depending on molecular weight and L-asparaginase rate translates to 0.2 – 0.8 IU/ml. Under these conditions, *Er*A-WT fully hydrolyses the Gln in an hour (red trace). In contrast, *Er*A-DM exhibits negligible Gln hydrolysis (green trace), reduced even compared to *Ec*A-WT (gray trace). (**B**) To further compare the L-glutaminase activity of the *Er*A variants relative to *Ec*A-WT, an experiment was conducted at a higher enzyme concentration relative to that shown in panel **A**. Enzyme amounts were based on matched L-asparaginase activity level; with all enzymes at 15 IU/ml, *Ec*A-WT completely depletes Gln within 45 minutes, whereas for both *Er*A-DM and *Er*A-TM, Gln levels are only reduced by 20% after 1 hour.

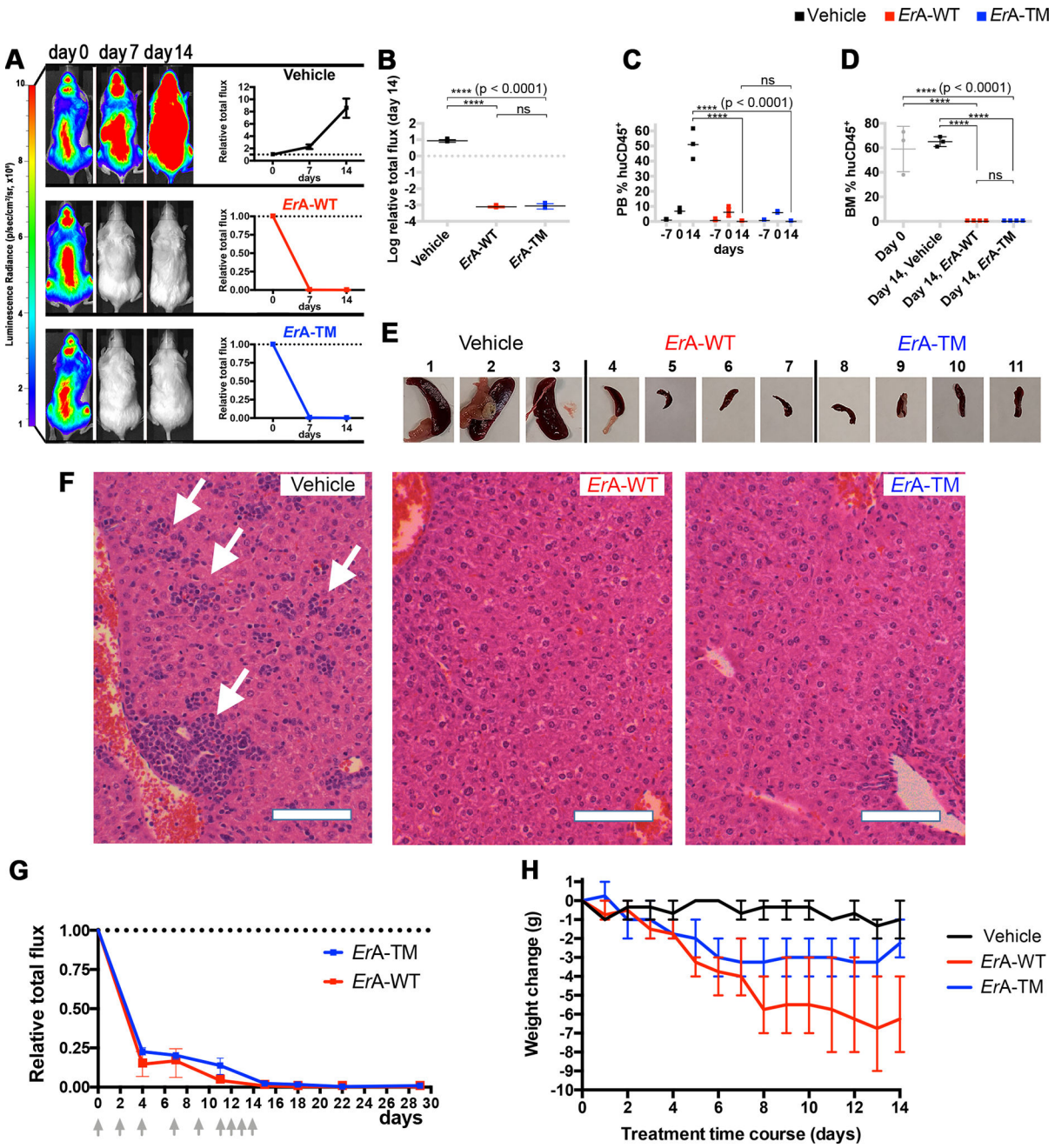

Fig. 2. The low L-glutaminase *Er*A-TM eliminates T-ALL LOUCY cells as effectively as the high L-glutaminase *Er*A-WT and with reduced toxicity

(**A**) Female mice tail vein-injected with luciferase-expressing LOUCY cells four weeks prior were treated daily with vehicle (n=3); *Er*A-WT (n=4); and *Er*A-TM (n=4) for 14 days (drug dose 50 IU/mouse/day; i.p.). For each group, the representative animal shown had the highest BLI signal at day 0 of treatment. BLIs from all animals are presented in Supplementary Fig. 4 and 6. The average BLI signal of each group at day 7 and 14 relative to the value at day 0 (day 0 = 1) was plotted with mean and standard deviation (SD). See Supplementary Biostatistics on imaging for detailed standard error analysis. (**B**) Relative BLI flux at day 14 between the vehicle, *Er*A-WT, and *Er*A-TM groups. The flux for the

vehicle mice increased 10-fold relative to day 0. For both treated groups, the flux decreased dramatically relative to vehicle control (p-value <0.0001), returning to background levels by day 14, with no significant (ns) difference between the treated groups. Mean with SD were plotted. See Supplementary Biostatistics on imaging for detailed standard error analysis. (**C**) PB %huDC45+ levels were determined one week prior to treatment initiation (day -7), at treatment start (day 0), and at end of treatment (day 14). At day 0, all animals were highly engrafted, as indicated by %huCD45+ >8%. By day 14, for the vehicle-treated mice, the %huDC45+ increased to 40–60%, whereas for both treatment groups, the %huCD45+ was undetectable (p-value <0.0001 between vehicle- and enzyme-treated groups; ns between the two enzyme-treated groups). Mean with SD were plotted. All tests were set at controlling for probability of Type I error of 0.05. See Supplementary Biostatistics for more details. (**D**) At day 0, assessment of BM %huDC45+ in 3 mice with similar BLI flux as the ones used for treatment revealed high engraftment (gray boxes). At day 14, BM %huDC45+ remained high in the vehicle-treated mice, but was undetectable in both enzyme-treated groups (p-value <0.0001 between vehicle- and enzyme-treated groups; ns between the two enzyme-treated groups). Mean with SD were plotted. All tests were set at controlling for probability of Type I error of 0.05. See Supplementary Biostatistics for more details. (**E**) Spleens from the vehicle-treated mice were highly enlarged, whereas spleens from the *Er*A-WT and *Er*A-TM groups resembled normal mouse spleens in size. (**F**) H&E-stained paraffin sections of livers from vehicle-, *Er*A-WT- and *Er*A-TM-treated mice. Vehicle-treated animals had livers filled with deposits of lymphoblastic leukemic cells (arrows). In contrast, livers of mice treated with *Er*A-WT or *Er*A-TM had no detectable leukemic cells present; bar = 10 μm. (**G**) Female mice tail vein-injected with luciferase-expressing LOUCY cells four weeks prior were treated i.p. with *Er*A-WT (n=3); and *Er*A-TM (n=3) for 14 days (a total of 9 drug doses of 25 IU/mouse on days indicated by gray arrows). The average BLI (+SD) signal of each group at day 0, 4, 7, 11, 15, 18, 22 and 29 relative to the value at day 0 (day 0 = 1) is plotted. (**H**) Correlation between L-glutaminase activity and toxicity of the *Er*A variants. Weight loss (in grams, relative to day 0), an indicator of toxicity, was monitored in mice treated with vehicle (black trace), *Er*A-WT (red trace, L-glutaminase@$Gln_{500\mu M}$=15.87 sec^{-1}) and *Er*A-TM (blue trace, $Gln_{500\mu M}$=0.01 sec^{-1}). The pronounced daily weight loss in the *Er*A-WT-treated group is ameliorated in the *Er*A-TM-treated group by 0.29 g/day, p-value <0.0001.

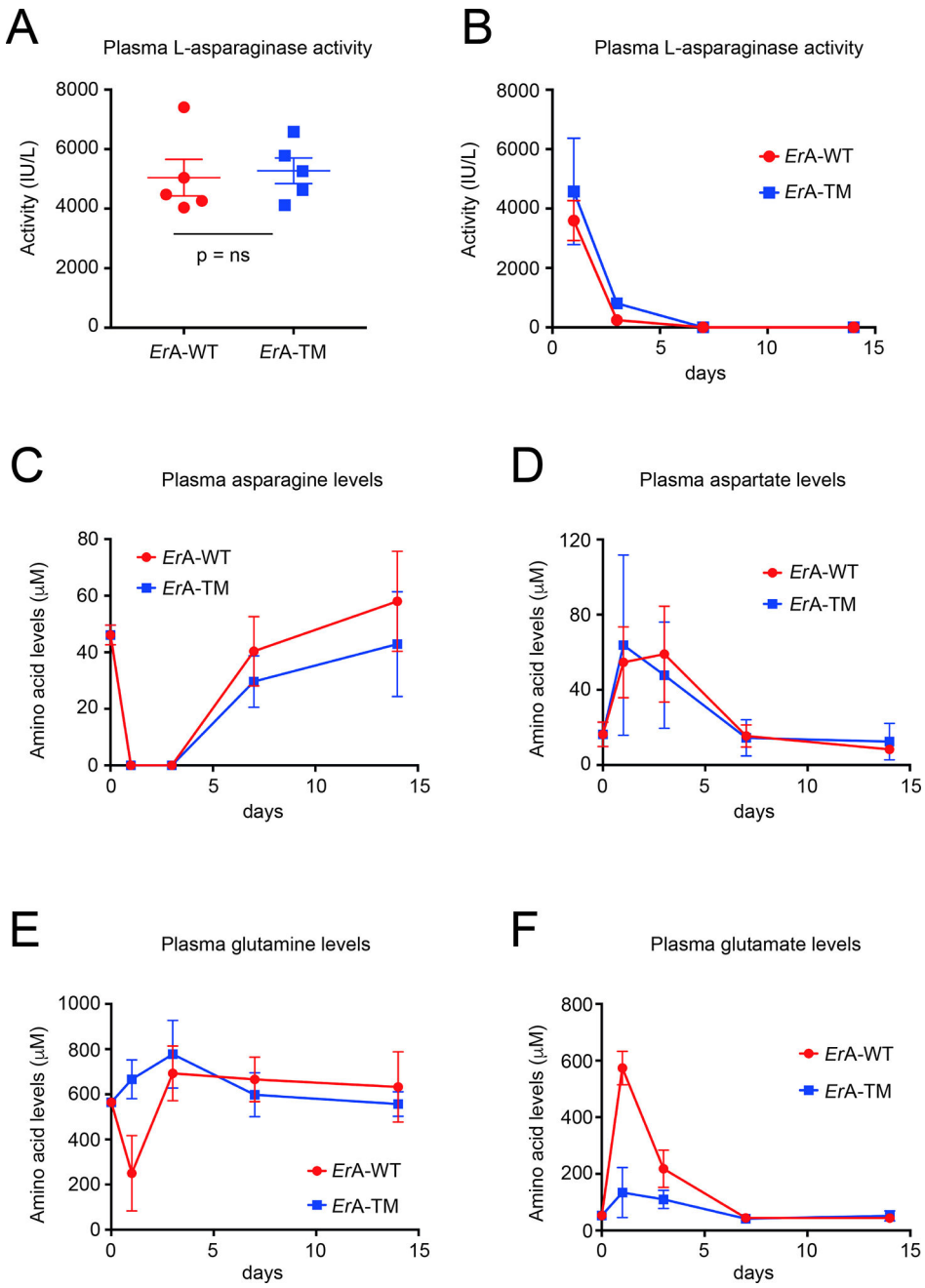

Fig. 3. Similar *in vivo* stability for *Er*A-WT and *Er*A-TM and ability to deplete blood asparagine but dissimilar impact on glutamine homeostasis

(**A**) L-asparaginase activity measurement in blood plasma samples of mice obtained 24 h after injection of 50 IU of *Er*A-WT or *Er*A-TM from batch #1. Mean with SEM (standard error of the mean) is shown for each group and non-parametric Mann-Whitney test was used for statistics. (**B**) L-asparaginase activity measurement in blood plasma samples of mice obtained 1, 3, 7 and 14 days after injection of 50 IU of *Er*A-WT or *Er*A-TM from batch #2. (**C**) Determination of asparagine levels in blood plasma samples of mice prior to the treatment and at days 1, 3, 7 and 14 post administration of *Er*A-WT or *Er*A-TM. For each

time point, the mean and standard deviation are shown. (**D**) Determination of aspartate levels in blood serum samples of mice prior to the treatment and at days 1, 3, 7 and 14 post administration of *Er*A-WT or *Er*A-TM. For each time point, the mean and standard deviation are shown. (**E**) Determination of glutamine levels in blood plasma samples of mice prior to the treatment and at days 1, 3, 7 and 14 post administration of *Er*A-WT or *Er*A-TM. For each time point, the mean and standard deviation are shown. (**F**) Determination of glutamate levels in blood plasma samples of mice prior to the treatment and at days 1, 3, 7 and 14 post administration of *Er*A-WT or *Er*A-TM. For each time point, the mean and standard deviation are shown.

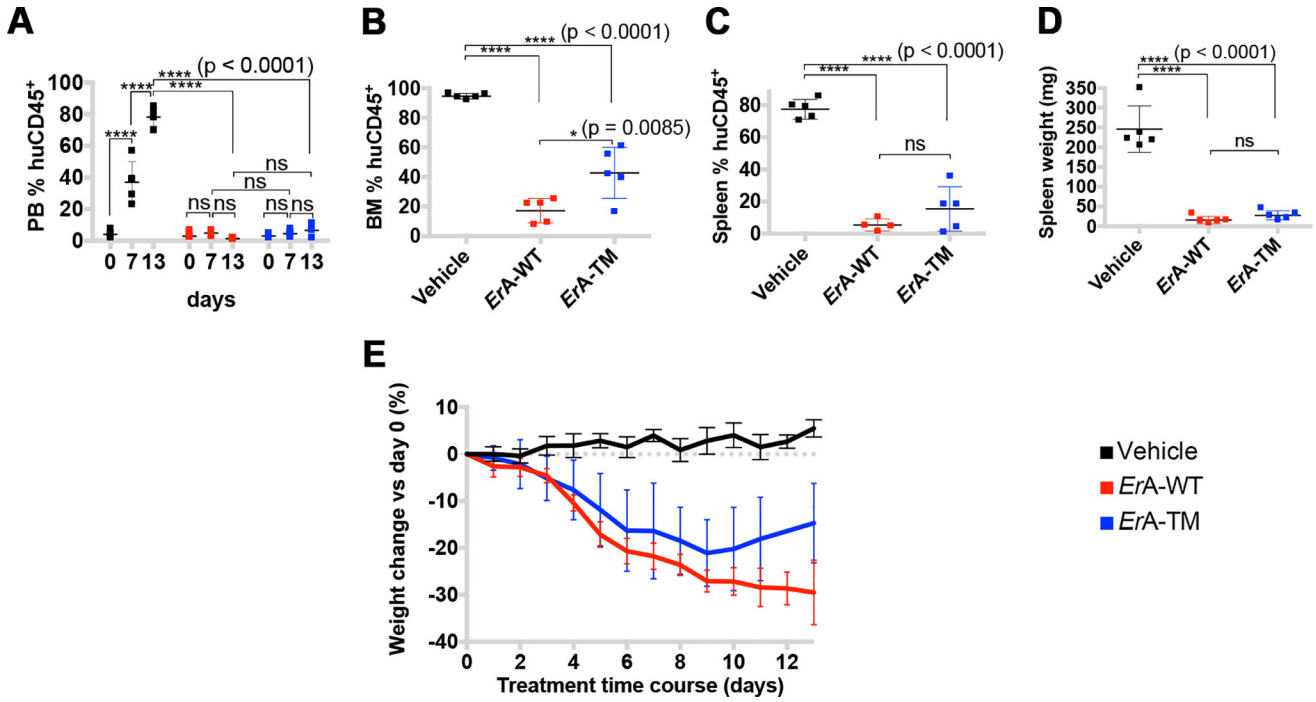

Fig. 4. In a T-ALL PDX model, *Er*A-TM displays similar cell killing combined with reduced toxicity compared to *Er*A-WT

Female mice injected with primary T-ALL cells five weeks prior were treated daily with vehicle; *Er*A-WT or *Er*A-TM (n=5 for each group) for 13 days (drug dose 50 IU/mouse/day; i.p.). Cell debris and duplicates were gated out during flow cytometry data analysis. (**A**) PB %huCD45+ levels were determined once every week and at day 0, all animals were engrafted, as indicated by %huCD45+ ~4%. For the vehicle-treated mice, the %huCD45+ increased to ~40% by day 7 and ~80% by day 13, whereas for both treatment groups, the %huCD45+ stayed statistically indifferent to day 0 (p-value >0.2). No difference was detected between the two enzyme-treated groups at day 7 and day 13 (p-value >0.6). The %huCD45$^+$ levels could not be analyzed for one mouse in the *Er*A-WT group and for one mouse treated with *Er*A-TM due to bad sample quality. (**B**) Similar to panel A, but for the BM at day 13. Whereas the BM of the vehicle group was full of cancer cells with %huCD45+ ~95%, the disease was largely controlled in the two enzyme-treated groups with %huCD45+ ~20 – 40%. (**C**) Similar to panel B, but for the spleen at day 13. The analysis of spleen sample of mouse 18 (*Er*A-WT-treated) was not included because of bad sample quality. Whereas the vehicle-treated group's spleens were ~80% invaded with cancer cells, less than 20% of %huCD45+ was detected in the enzyme-treated groups. No significant difference was detected between the *Er*A-WT- and *Er*A-TM-treated groups (p-value ~0.2). (**D**) On sacrifice, spleens were harvested and weighed. Shown are the spleen weights for the 3 groups. Consistent with the cancer cell invasion data in panel C, the weight of spleens from the vehicle-treated group are significantly higher than the enzyme-treated groups, but no significant difference was detected between the two enzyme-treated groups (p-value >0.8). (**E**) Mice weight change, shown as % change relative to day 0, over the course of the 13-day treatment period is shown. On average, at day 13, *Er*A-WT- treated mice lost ~30% of body weight, whereas *Er*A-TM- treated mice lost ~15% (p-value <0.05). n.s. = not

significant. In all panels, the color code depicting the vehicle-treated group, the *Er*A-WT-treated group and the *Er*A-TM- treated group is black, red and blue, respectively. Mean with SD were plotted.

Table 1

Enzyme kinetic parameters

	Enzyme name	**k_{cat} (sec^{-1})**	**Km (μM)**	**k_{cat}/Km ($sec^{-1}\mu M^{-1}$)**	**k_{obs} @50 μM[a] (sec^{-1})**	**k_{obs} @50 μM[b] (sec^{-1})**
L-asparaginase activity	*Er*A-WT	207.5 ± 3.6	47.5 ± 3.5	4.37	118.9	145.8
	*Er*A-TM	261.2 ± 2.8	95.0 ± 3.5	2.75	79.6	56.4
	*Er*A-DM	169.8 ± 1.5	185.3 ± 5.5	0.92	22.4	23.3
	*Er*A-E63Q	186.8 ± 1.7	50.7 ± 2.0	3.68	112.7	135.9
	*Ec*A-WT	44.4 ± 0.3	15.0 ± 0.5	2.96	41.3	
L-glutaminase activity	Enzyme name	k_{cat} (sec^{-1})	Km[c] (μM)	k_{cat}/Km[c] ($sec^{-1}\mu M^{-1}$)	k_{obs} @0.5 mM[a] (sec^{-1})	k_{obs} @0.5 mM[b] (sec^{-1})
	*Er*A-WT	26.84 ± 0.26	360 ± 20	74.56×10^{-3}	15.87	14.76
	*Er*A-TM	1.84 ± 0.11	47,460 ± 695	0.04×10^{-3}	0.01	0.04
	*Er*A-DM	2.93 ± 0.03	15,800 ± 300	0.19×10^{-3}	0.11	0.09
	*Er*A-E63Q	8.33 ± 0.16	3,860 ± 230	3.68×10^{-3}	0.74	1.05
	*Ec*A-WT	0.89 ± 0.01	1,380 ± 90	0.64×10^{-3}	0.22	
Specificity	Enzyme name	$k_{obs}[Asn_{phs}] / k_{obs}[Gln_{phs}]$[d]		k_{cat}/Km (Asn) / k_{cat}/Km (Gln)		
	*Er*A-WT	6.6		58.6		
	*Er*A-TM	8910		68,750		
	*Er*A-DM	330		4,842		
	*Er*A-E63Q	124.6		1,000		
	*Ec*A-WT	187.7		4,625		

[a] k_{obs} for enzymes without the SUMO tag

[b] k_{obs} for enzymes with the SUMO tag

[c] Concentrations are given in μM to facilitate comparison with the L-asparaginase data

[d] k_{obs} for Asn@50μM, k_{obs} for Gln@500μM

Table 2

Sensitivity of LOUCY and SUP-B15 cells to L-asparaginase

Enzyme name	LOUCY IC_{50} (mIU/mL)[a]	SUP-B15 IC_{50} (mIU/mL)
*Er*A-WT	0.33	0.16
*Er*A-E63Q	0.55	0.24
*Er*A-DM	0.65	0.31
*Er*A-TM	0.61	0.27
*Ec*A-WT	0.35	0.22

[a] mIU; milli International unit.

Table 3

Acute toxicity study showing correlation between L-glutaminase activity and toxicity of the *Er*A variants

	***Er*A-WT**			***Er*A-TM**		
Group	1	2	3	4	5	6
Dose (IU/g)	40	80	160	40	80	160
Hunched posture	6 (2-4)	6 (2-4)	6 (1-4)	0*	0*	4 (1)
Decreased activity	0	6 (3)	0	0	0*	0
Sunken eyes	1 (2-3)	6 (2-3)	6 (1-4)	0	0*	0*
Rough coat	6 (1-4)	6 (1-4)	6 (1-4)	4 (1-2)	0*	0*

Toxicity scale: None Mild Severe

CD-1 mice were subjected to an acute single dose-escalation toxicity study. Each group had 3 males and 3 females, except group 6 which had only 4 animals (3 females, 1 male) due to a shortage of ErA-TM. Animals administered ErA-WT presented significant physical and behavioral symptoms, whereas the ErA-TM administered animals were largely devoid of such symptoms. The number of animals (out of 6 total) presenting symptoms is indicated. In parenthesis, the day(s) on which the symptom was observed is noted. One male in group 3 was dosed with 136 IU/g, and one female in group 5 was dosed with 60 IU/g. *$p < 0.05$, analysis by Fisher's exact test: (Group 1 vs. Group 4, Group 2 vs. Group 5, Group 3 vs. Group 6). *$p < 0.05$, analysis by Mann-Whitney U Test: (Group 1 vs. Group 4, Group 2 vs. Group 5, Group 3 vs. Group 6). Mean with SD were plotted. See Supplementary Biostatistics on imaging for detailed standard error analysis.