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PEGylated Recombinant *Aplysia punctata* Ink Toxin Depletes Arginine and Lysine and Inhibits the Growth of Tumor Xenografts

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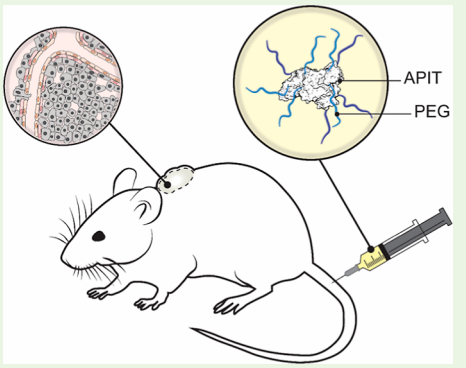
ABSTRACT: In recent years, a novel treatment method for cancer has emerged, which is based on the starvation of tumors of amino acids like arginine. The deprivation of arginine in serum is based on enzymatic degradation and can be realized by arginine deaminases like the L-amino acid oxidase found in the ink toxin of the sea hare *Aplysia punctata*. Previously isolated from the ink, the L-amino acid oxidase was described to oxidate the essential amino acids L-lysine and L-arginine to their corresponding deaminated alpha-keto acids. Here, we present the recombinant production and functionalization of the amino acid oxidase *Aplysia punctata* ink toxin (APIT). PEGylated APIT (APIT−PEG) increased the blood circulation time. APIT−PEG treatment of patient-derived xenografted mice shows a significant dose-dependent reduction of tumor growth over time mediated by amino acid starvation of the tumor. Treatment of mice with APIT−PEG, which led to deprivation of arginine, was well tolerated.



KEYWORDS: *L-amino acid oxidase, PEGylation, arginine-auxotrophy, arginine lysine starvation, tumor therapy, patient-derived tumor*

INTRODUCTION

Increasing resistance of tumors toward chemotherapies can be observed with standard of care DNA-damaging agents, antimetabolites, mitotic inhibitors, nucleotide analogues, or inhibitors of topoisomerase.[1] The cytotoxic effect of the drugs is primarily attributed to the induction of apoptosis. In treated tumor cells, mutations of the general apoptosis signaling pathways can be observed, which leads to broad resistance to drug treatment due to the lag in activation of the apoptotic pathway.[2] This constitutes the need for alternative antitumor therapy approaches to tackle these limitations of current antitumor treatments.

An increasingly recognized strategy is the enzymatic depletion of amino acids in serum to target metabolically deficient malignances. A prime example of amino acid depletion cancer therapy is the treatment of leukemias with asparaginase. Normal cells convert aspartate to asparagine; however, this pathway is insufficient in leukemic cells. Treatment with asparaginase cures more than 90% of pediatric acute lymphoblastic leukemia patients.[3,4]

Another highly discussed therapeutic approach is the depletion of arginine in arginine auxotroph tumors. Auxotrophy to the semiessential amino acid arginine occurs through epigenetic silencing of *arginosuccinate synthetase* 1 (ASS1) or *arginine lyase* (AL) genes, preventing the synthesis of arginine through citrulline in the urea cycle.[5−8] In many cancer types, arginine-auxotrophy reaches an average of 60−100%.[9] ASS1-deficient cells have a generally poor survival rate and an increased requirement for aspartate used for pyrimidine synthesis to support the proliferation of cells.[10−17] This property can be therapeutically exploited by treating these tumor cells with arginine deiminases (ADI), which deaminate arginine to citrulline and thereby deplete arginine. In combination with arginine auxotrophy, cells are then starved of arginine and undergo apoptotic cell death.[17,18] Therefore, a novel strategy for targeting auxotrophic cancers by starvation of the tumor of arginine emerged.[19]

A promising example of this treatment method is ADI-PEG20. The first clinical trials with a mycoplasma-derived PEGylated ADI demonstrated the sensitivity of tumors toward arginine deprivation.[20,21] Based on this finding, the FDA and the EMA granted orphan drug designation for ADI-PEG20 for the treatment of hepatocellular carcinoma and melanoma. Phase I clinical trials combined arginine deprivation with conventional cytostatic chemotherapy (cisplatin, pemetrexed-cisplatin, Nab-Paclitaxel with Gemcitabine, FOXLFOX6).[22−25]

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In the studies, patients showed an increase of immunogenic reaction against ADI-PEG20 after a few weeks, which decreased the effectiveness of the combination therapy.

To overcome these limitations, the necessity of alternative products for ADI-PEG20 emerges including arginine- and lysine-specific L-amino acid oxidases in the defense ink of *Aplysia punctata* (*A. punctata* ink toxin = APIT). APIT was first isolated from crude ink, which deaminates L-lysine and L-arginine to their corresponding α-keto acids.[26] APIT's tumor-specific cytotoxicity was demonstrated on an apoptosis-resistant tumor cell.[27] However, in preliminary in vivo studies, intravenous injection of APIT into mice had an unfavorable renal clearance shortly after application.[28] To increase serum half-life, polymer modifications such as the coupling of polyethylene glycol (PEG) to the protein are well-known strategies. Unspecific PEGylation of APIT, in which the lysine residues of the enzyme are targeted by *N*-hydroxysuccinimidyl chemistry, was performed, leading to increased serum half-life and exhibiting comparable anticancer activity as ADI-PEG20.[28]

In this study, the effect of arginine and lysine deprivation mediated by APIT−PEG treatment was tested in a patient-derived HNXF 536 head and neck xenograft model (PDX). The results show that APIT−PEG efficiently and continuously depleted L-arginine and L-lysine levels in treated animals and significantly inhibited tumor growth in this model.

EXPERIMENTAL SECTION

Materials and Methods. *Recombinant Expression of APIT.* Competent *E. coli* HMS174 were transformed with the pET26b-based plasmid with an inserted APIT encoding gene between *Nde*I and *Eco*RI restriction sites. The protein sequence of APIT is provided in Supporting Information. Expression was controlled by the T7 promoter and induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Steinheim, Germany) at an OD_{600} of 0.8−1.0. After 4 h, cells were harvested by centrifugation at 5000*g* at 4 °C. Expression resulted in inclusion bodies. Pelleted cells were resuspended in 50 mM tris (hydroxymethyl)aminomethane (Tris) at pH 8.0. Inclusion bodies were isolated in several sonification steps using a Bandelin Sonoplus ultrasonic device (Berlin, Germany).

Refolding of APIT. 0.5 g of purified inclusion bodies was solubilized in 100 mL of 6 M urea, 50 mM Tris, 200 mM NaCl, 100 mm dithiothreitol at pH 8.0 overnight at 4 °C. Solubilized inclusion bodies were centrifuged at 5000*g* at 4 °C for 30 min and filtrated using a 0.2 μm polyethersulfone (PS) filter. The protein was diluted in refolding buffer 1 m Tris, 1 m NaCl, 1 mm EDTA, 25.6 μm FAD, 20 μm cysteine, 0.5% PEG6000 pH 8.6 and stirred at 4 °C for 7 days.

Purification of APIT. The subsequent purification of refolded APIT was performed in two steps using anion exchange chromatography (HiTrap Q FF) and size exclusion chromatography (SEC) (HiLoad 16/600 Superdex 200 pg) from Cytiva (Uppsala, Sweden).

Elution was performed with buffer A (50 mM Tris, pH 8.0) and buffer B (50 mM Tris, 1 M NaCl, pH 8.0), starting with a washing step of 25% B in 10 CV, followed by an elution gradient of 25−60 in 10 CV. APIT was then loaded onto a HiLoad 16/600 Superdex 200 pg. The column was then run using phosphate-buffered saline (PBS) pH 7.4 at a flow rate of 1 mL/min.

PEGylation and Purification of APIT−PEG5 kDa. Since tris(2-carboxyethyl) phosphine (TCEP) added prior to the storage of APIT at −80 °C (see the Results section) interfered with the PEGylation reaction, APIT was dialyzed in PBS pH 7.4 overnight at 4 °C and the concentration was adjusted to 1.1 mg/mL. For the PEGylation of APIT, the 5 kDa linear form was selected in a preliminary experiment, in which the serum stability of native APIT modified with a variety of branched and different-sized PEG forms was tested (Figure S1). A 20 equiv amount of PEG-*N*-hydroxysuccinimide (NHS) 5 kDa (Celares GmbH, Berlin, Germany) was dissolved at a concentration of 408 mg/mL in PBS pH 7.4 and 1:10 (v/v) added to APIT. The reaction was performed overnight at 4 °C while continuously shaking. The reaction was purified using anion exchange chromatography (HiTrap Q HP, Cytiva, Uppsala, Sweden) with buffer A (50 mm Tris, pH 8.0) and buffer B (50 mm Tris, 1 m NaCl, pH 8.0). The reaction was diluted 1:3 in buffer A, and a maximum of 10 mg of protein was loaded onto 1 mL of resin at a flow rate of 0.5 mL/min. Afterward, a washing step at 0% B for at least 10 CV and an elution gradient from 0 to 100% B were performed. The elution peak was pooled and dialyzed against PBS pH 7.4, and the concentration was adjusted to 2 mg/mL. PEGylated APIT was filtered using a 0.2 μm PS filter and stored light-protected.

Enzyme Activity. The enzyme activity in the presence of substate L-lysine was determined by measuring the production of hydrogen peroxide. H_2O_2 is reduced stoichiometrically by the chromogenic 2,2′-azino-di(3-ethylbenzthiazolin-6-sulfonic acid in a coupled horseradish peroxidase-catalyzed reaction as previously described by Butzke et al.[27] The enzyme activity is expressed in units [U], and 1 U is equivalent to the production of 1 μmol H_2O_2 per minute.

HPLC Analysis. The purity of the produced monomeric APIT and PEGylated APIT was assessed by high-performance liquid chromatography (HPLC) analysis using an Agilent Technologies 1100 series system (Waldbronn, Germany). A ZORBAX 300SB-CN, 300 A, 5 μm, 4.6 nm × 250 nm was used. The elution was performed with water in HPLC grade +0.1% trifluoroacetic acid (TFA) and increasing concentrations of acetonitrile in HPLC grade +0.1 TFA.

Degree of PEGylation. The degree of PEGylation was assessed by the determination of the mass increase measured by matrix-assisted laser desorption ionization (MALDI) mass spectra with an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 355 nm SmartBeam-II laser. 20−50 μg of protein was desalted using Sep-Pak Vac C18 cartridges. The recommended manufacturer's instructions were followed. Bound protein was eluted using 80% acetonitrile in ddH_2O + 0.1% TFA and subsequently lyophilized at −100 °C, 0.001 mbar. For external calibration, Bruker Protein II standard (Bruker Daltonics #8207234) was mixed with sinapinic acid (SA) solution [saturated in a 1:2 mixture (v/v) of acetonitrile and 0.1% TFA]. Dried drops were measured on a stainless-steel target (MTP 384 target plate ground steel, Bruker Daltonics #8280784). The proteins were analyzed after double-layer preparation. First, a thin matrix layer of SA solution (saturated in ethanol) was applied onto the stainless-steel target, and second, the sample solution (4 mg/mL in a 1:1 mixture of acetonitrile and ddH2O) was mixed with SA solution [saturated in a 1:2 mixture (v/v) of acetonitrile and 0.1% TFA] and dropped onto the first layer. Several thousand shots were accumulated for the spectrum.

Pharmacodynamics of APIT−PEG on Plasma L-Arginine and L-Lysine Levels. Depletion of arginine and lysine in blood serum was assessed after a single intravenous administration of 250 U/kg of APIT, 1000 U/kg of APIT, 250 U/kg APIT−PEG, or 250 mL/kg PBS as a control vehicle into 5 tumor-free mice per group. Blood was collected 1 h prior to injection and 0.25 1, 3, 6, 24, and 48 h after injection from each mouse. Samples from each time point were pooled.

Ethics Statement. Mouse experiments were approved by the German Committee on the Ethics of Animal Experiments of the regional council (permit nos.: G-21/100 and G-20/163). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Society of Laboratory Animals (GV SOLAS) in an AAALAC-accredited animal facility.

Dose-Finding Experiments and Efficacy Experiments of APIT−PEG in HNXF 536 PDX Mice Model. The maximum tolerated dose of APIT−PEG was evaluated at three dose levels of 15, 30, and 50 U/kg in 4 tumor-free mice per group. APIT−PEG was intravenously injected every other day for 1 week, and the tolerability was assessed by daily monitoring the mice for symptoms and body weight development.

Tumor fragments of HNXF 536 from a serial passage in nude mice were placed in PBS containing 10% penicillin/streptomycin and cut into fragments of 3−4 mm in diameter. Prior to tumor implantation,

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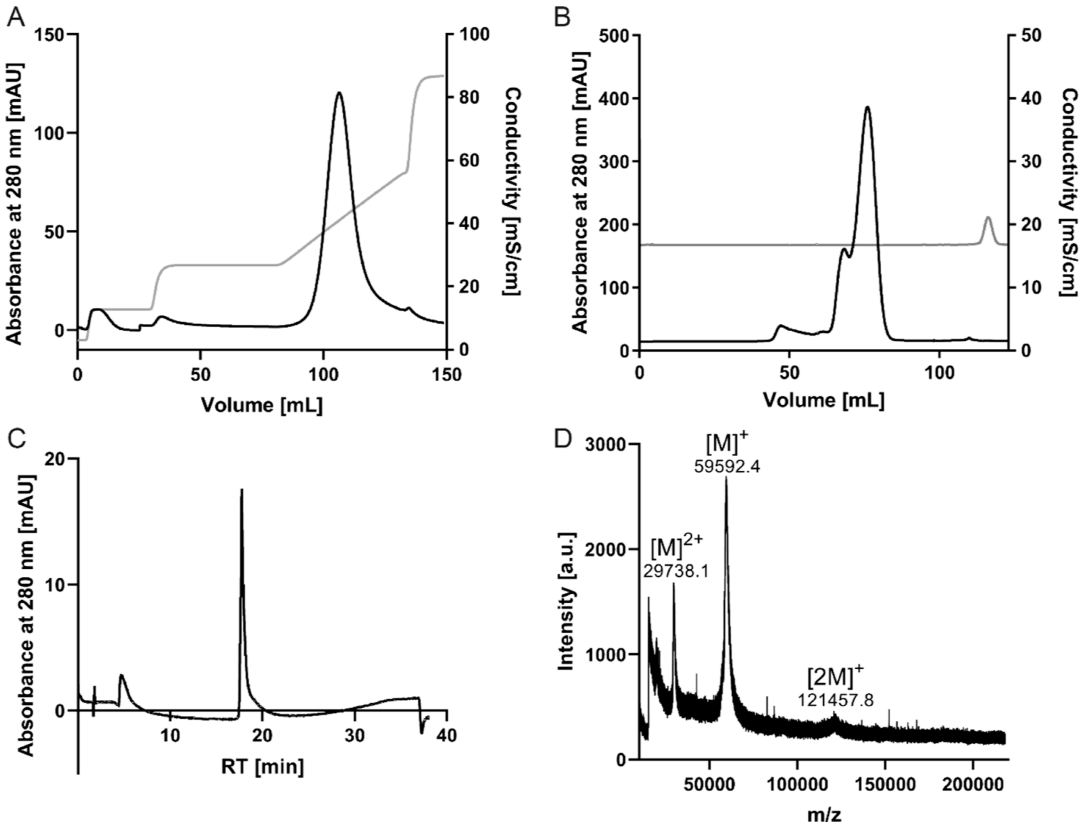

Figure 1. Purification and characterization of recombinant APIT. (A) Chromatogram of anion exchange after refolding of APIT. (B) Size-exclusion chromatogram of pooled and reduced APIT. (C) HPLC analysis of monomeric recombinant APIT after purification on a normal phase column. (D) MALDI mass spectrum of purified APIT showing small amounts of remaining dimeric APIT.

NMRI nu/nu (Crl:NMRI-*Foxn1*^{nu}) mice were anesthetized by the inhalation of isoflurane. Animals were monitored until the tumor implants reached an appropriate size of 50−250 mm^3.

Mice bearing HNXF 536 were randomly divided into control and treatment groups. The day of randomization was designated as day 0 of the experiment. Mice were treated with the (1) control vehicle (5 mL/kg/day) every other day; (2) APIT−PEG (15 U/kg/day) every other day; (3) APIT−PEG (40 U/kg/day) every other day, and (4) doxorubicin HCl (6 mg/kg) every 7 days (see Table S1 in Supporting Information). The injection was administered via the lateral tail vein. To control arginine and lysine depletion during the therapy, blood was collected 6, 24, and 48 h after the first treatment.

Analysis of Amino Acids in Serum. For analysis of arginine and lysine levels in serum, blood was collected by retrobulbar sinus puncture under isoflurane anesthesia. Plasma was stored in lithium heparin vials on ice. Vials were centrifuged at 2000*g* for 5 min at 4 °C. The separated plasma was transferred to a cooled tube on ice. 60 μL of sample was diluted with 15 μL of precipitation buffer and stored at 5 °C for 60 min to precipitate the proteins present. After centrifugation at 2000*g* for 5 min at 4 °C, the supernatants were diluted 1:1 with sample dilution buffer containing 200 nmol/mL norleucine, which was used as an internal standard. For quantitative and qualitative analysis of the amino acids serine, alanine, lysine, and arginine at 570 nm, the ARACUS amino acid analyzer was used (MembraPure, Hennigsdorf, Germany).

H&E Staining of Dissected Tumors. Tumors were dissected on day 42 after randomization. Tumor blocks were cut into 5 μm sections by using a Leica RM2135 microtome (Leica Biosystems, Wetzlar, Germany). Slides were dried overnight at 40 °C. Prior to staining, slides were heated at 60 °C for 30 min. Paraffin was removed by using xylene and rehydrated in ethanol solutions and washed with distilled water. Sections were then stained with hematoxylin for 8 min and washed with water for 15 min. Prior to eosin staining for 2 min, the slide was dipped in 96% ethanol. The slide was washed with water, dehydrated in 100% ethanol and xylene, and mounted using the Merck Neo-Mount solution.

Statistics. Relative tumor growth over time was compared to that on day 0. Statistical analysis of the curves was performed by fitting a linear regression and a comparison of the mean beta-coefficient by ordinary one-way ANOVA in multiple comparisons to the control group. Statistical analysis on specific days was performed on the relative tumor growth by comparing the treatments separately to the control using the unpaired, nonparametric Mann−Whitney *U* test. A *p*-value less than 0.05 was considered significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

RESULTS

Expression and Purification of Monomeric APIT. We have previously demonstrated that the arginine and lysine deaminase APIT, an enzyme found in the APIT, induces cell death in cancer cells but not in normal cells. To develop APIT for potential future pharmaceutical applications, the serum half-life of APIT had to be increased through PEGylation. We therefore developed the recombinant expression and subsequent purification of APIT, which resulted in approximately 8 mg/L monomeric APIT after the last purification step via SEC. The most crucial part of the purification was the purity of the inclusion bodies prior to refolding, which was optimized by a series of sonification steps. Refolded APIT was purified via anion exchange chromatography, which still contained high-molecular-weight APIT (Figure 1A). To obtain pure monomeric APIT, a reductive step using TCEP was introduced prior to SEC (Figure 1B). Over time, monomeric APIT tended to form dimers, which did not interfere with the enzymatic activity. To avoid dimerization, monomeric APIT

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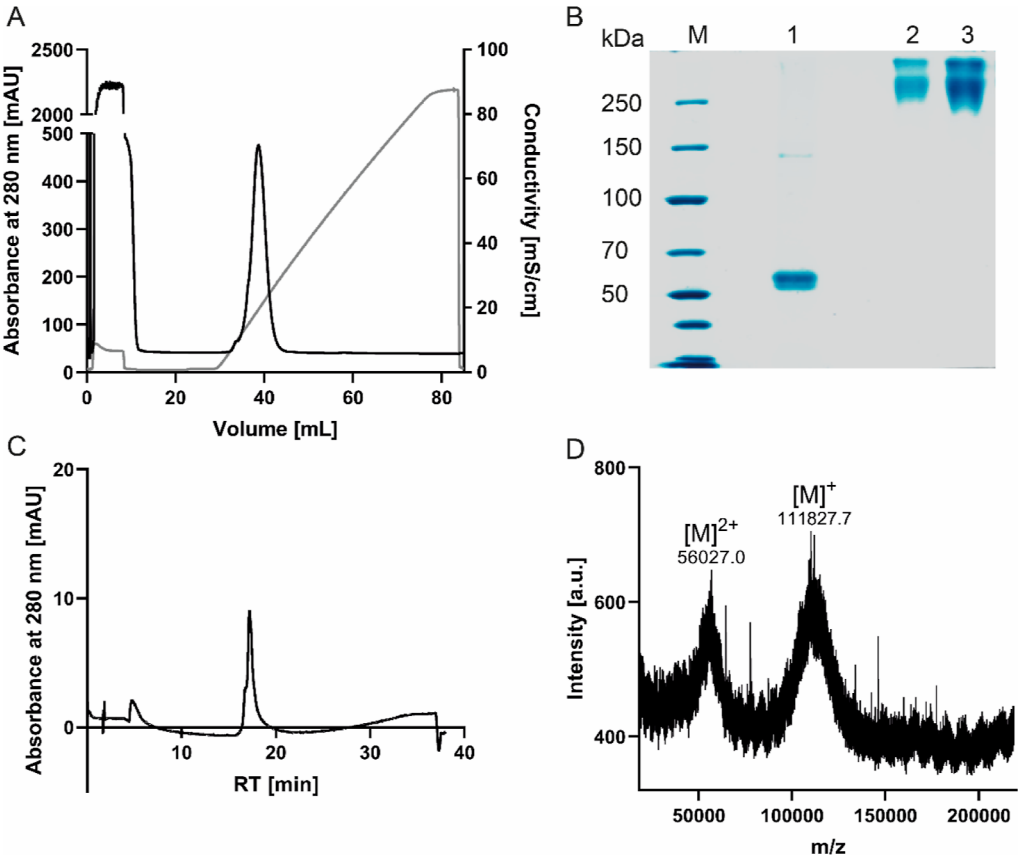

Figure 2. Purification of PEGylated APIT. (A) Chromatogram of the sepharose Q HP column. (B) 5−15% Tris-SDS−PAGE of nonreducing APIT (1) and APIT−PEG [2 μg (2) and 4 μg (3)] stained with Coomassie blue G250. (C) CN-HPLC profile of PEGylated APIT after purification. (D) MALDI mass spectrum of PEGylated APIT shows an increase of mass by 55,507 g/mol, indicating a degree of PEGylation of 8−10 PEG per APIT.

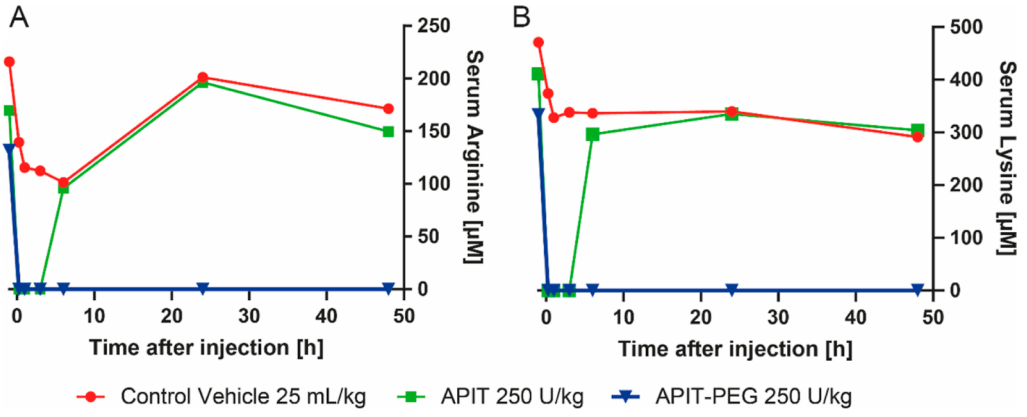

Figure 3. Pharmacodynamics of a single administration of 250 U/kg APIT, 250 U/kg APIT−PEG, or a control vehicle (n = 5) on the (A) arginine concentration in serum and (B) lysine concentration in serum showing a beginning recovery of arginine and lysine concentrations after 6 h, whereas APIT−PEG showed a complete depletion of serum arginine and lysine for at least 48 h. Blood sera were pooled prior to amino acid quantification due to low serum amounts drawn.

was stored at −80 °C with the addition of small amounts of TCEP. Monomeric APIT had a mass of approximately 59592.4 g/mol and a purity of ≥98% as measured by HPLC (Figure 1C), with a small amount of dimeric APIT with a mass of 121457.8 g/mol (Figure 1D) and a batch dependent enzyme activity of 80−100 U/mg.

Production of APIT−PEG for In Vivo Applications. To enhance the serum half-life, we PEGylated purified APIT at lysine residues and purified the conjugates via anion-exchange chromatography (Figure 2A). After conjugation, a protein recovery of 60% pure APIT−PEG was achieved. Compared to that of APIT, the enzyme activity of APIT−PEG was reduced by 20%. The purity of APIT−PEG was ≥95% as measured by HPLC and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Figure 2B−C). APIT−PEG showed an average increase of mass by 55,507 g/mol, indicating a degree of PEGylation of approximately 8−10 mol PEG/mol as measured by MALDI−MS (Figure 2D). APIT−PEG was formulated in PBS pH 7.2 and stored at 4 °C. Prior to release for animal experiments, the endotoxin level was

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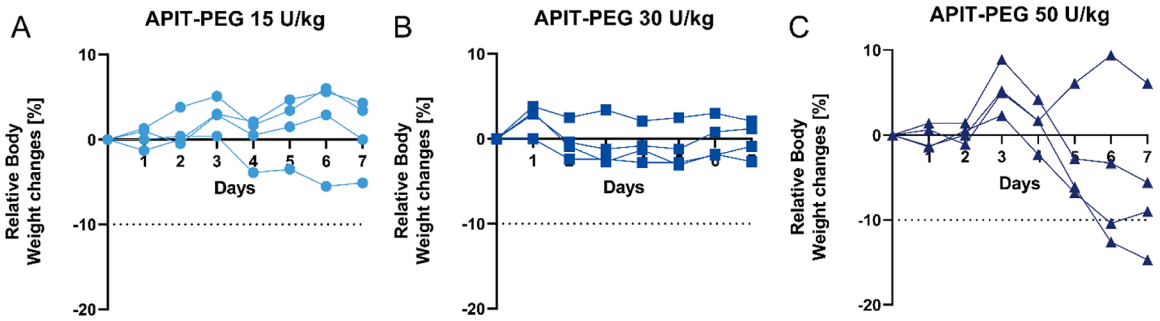

Figure 4. Median and 5−95% CI of relative body weight change of mice treated with APIT−PEG in doses of (A) 15, (B) 30, and (C) 50 U/kg (each $n = 4$) every other day for 1 week shows a stronger impact of higher doses of APIT−PEG on the well-being of mice.

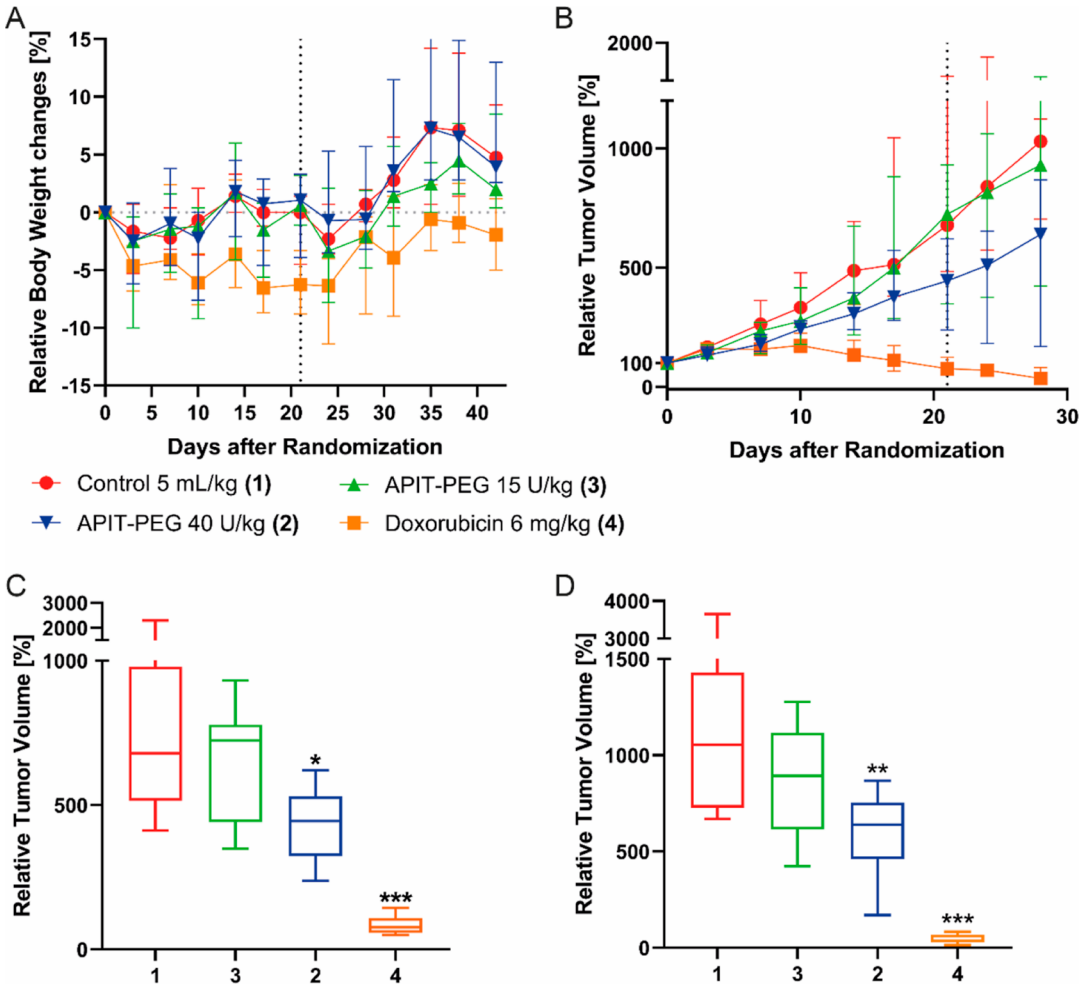

Figure 5. Efficacy experiment of treatment of patient-derived HNXF 536 xenografted mice with APIT−PEG 15 U/kg ($n = 8$), APIT−PEG 40 U/kg ($n = 6$), doxorubicin ($n = 10$), or the control vehicle ($n = 10$); the end of treatments is indicated with dashed lines. (A) Median and 5−95% CI of relative body weight changes for monitoring the mice's health; (B) median and 5−95% CI of relative tumor growth during and 1 week after the treatment; and (C) boxplot representation of the median and 5−95% CI of relative tumor growth on day 21 (the last day of treatment) and (D) 1 week after the end of treatment on day 28. (Whiskers indicate 5−95% percentile values and the line in the middle of the box represents the median; significance was tested with the Mann−Whitney U test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.)

determined and shown to have a concentration of 9 EU/mg, 0.14 EU/U. Masking of endotoxin by formulated APIT−PEG was controlled by the spike test. Spike recovery was 103−110%, concluding that the formulated APIT−PEG does not interact with endotoxins and therefore hides endotoxins.

PEGylated APIT Depletes Serum Arginine and Lysine for At Least 48 h. Depletion of arginine and lysine in mice blood was tested after a single injection of 250 U/kg APIT, 250 U/kg APIT−PEG, or 25 mL/kg PBS as a control vehicle for dilution effects. The serum of mice treated with APIT showed a beginning recovery of arginine (Figure 3A) and lysine levels after 6 h (Figure 3B). A single injection of 1000 U/kg of APIT showed the depletion of arginine and lysine for up to 6 h. Nevertheless, after 24 h, a complete recovery of amino acids in serum was observable (Figure S2). Only the

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injection of APIT−PEG showed a complete depletion of serum arginine and lysine during the 48 h period (Figure 3).

Tolerability Experiments in Tumor-Free nu/nu Mice. Dose-finding tolerability tests were performed to define the suitable dose that does not affect the animal's health during the continuous treatment with APIT−PEG. Therefore, the relative change in body weight was taken as an indicator of tolerability and health since weight loss or gain should not exceed 10% in 7 days during chemotherapy. Mice were treated with 15, 30, or 50 U/kg of APIT−PEG every other day. Due to a high variance of body weight changes of the mice, especially in the group with the highest dose, the median of relative body weight change with a 5 to 95% confidence interval (CI) was used for evaluation to accurately depict results. The mice administered with 50 U/kg APIT−PEG showed a median body weight loss of 7.3% after 1 week, with one mouse above the limit of 10% showing a loss of 14.3%. The dose of 50 U/kg APIT−PEG was therefore declared the upper limit (Figure 4). The results of these experiments demonstrated that doses of up to 50 U/kg were well tolerated.

Efficacy Experiment of APIT−PEG in the HNXF 536 PDX Mouse Model. To evaluate the antitumor effect of arginine and lysine deprivation, we tested APIT−PEG in mice xenografted with HNXF 536, a patient-derived head and neck cancer (Figure S3). Based on the tolerability experiment, APIT−PEG was applied intravenously at 15 and 40 U/kg instead of 50 U/kg since individual mice in the 50 U/kg group lost more weight than the limit of 10% (Figure 4). APIT−PEG was administered every other day in this efficacy experiment in mice and doxorubicin as standard-of-care drug once a week at a concentration of 6 mg/kg. The control group received PBS every other day (for details, see Table S1 in Supporting Information). At day 21, the treatment was terminated due to the high tumor load in the control group and the lower dose group of 15 U/kg APIT−PEG. Tumor growth was monitored until day 28, and the last observations were carried forward. Mice were weighed until day 42, and the relative body weight of doxorubicin showed a loss of weight during the treatment, indicating the well-known impact of standard chemotherapeutic treatment approaches on health (Figure 5A). Mice slowly regained body weight after the end of the treatment. APIT−PEG treatment, in contrast, did not result in severe changes of body weight and did not affect the mice's health when compared to the control group. After the end of the treatment, all mice gained weight comparable to the control group. The tumor volume was determined in all mice as a measure of tumor growth (Figure 5B). Treatment with doxorubicin resulted in a significant loss of tumor volume starting at day 10. Treatment with 15 U/kg APIT−PEG did not show a significant difference in relative tumor growth compared to the control group (Figure 5B−D). Serum analysis of amino acids showed an increase of serum arginine after 48 h, probably leading to the lag in effectiveness due to incomplete arginine and lysine depletion (Figures S4 and S5). In contrast, treatment with 40 U/kg of APIT−PEG successfully depleted arginine and lysine in blood serum during the treatment. Statistical analysis of the curves (Figure 5B) was performed by fitting a linear regression and a comparison of the mean beta-coefficient by ordinary one-way ANOVA in a multiple comparison to the control group (Figure S6). The curve of the relative tumor growth of 15 U/kg was not significant, whereas the 40 U/kg ($p = 0.0493$) and doxorubicin ($p < 0.0001$) treatment curves were significantly different from the control group. Evaluation of the tumor size on the last day of injection ($p = 0.0160$) and 1 week after the end of treatment ($p = 0.0075$) confirmed a significant reduction in the 40 U/kg APIT−PEG group compared to the nontreated control group (Figure 5C,D).

DISCUSSION

Targeting amino acid metabolism is an attractive anticancer strategy since it may provide alternatives for treatment of therapy-resistant tumors (for review, see ref 19). Killing of therapy-resistant tumor cell lines has also been demonstrated for APIT, which kills cancer cells independent of the induction of apoptosis,[27] the mechanism induced by many of the genotoxic agents used for cancer therapy.[1] Resistance of tumors to these canonical therapies occurs frequently due to mutations in the general apoptosis signaling pathways.[2] This explains why antitumor therapies provoke the emergence of broad resistance to drugs with the same mode of action, the induction of apoptotic cell death. In addition, investigations into amino acid depletion cancer therapies suggest that side effects like those elicited by current treatments with genotoxic agents are less severe or even not observed since amino acid auxotrophies are often highly tumor-specific.[3] A prime example of amino acid depletion cancer therapy is the treatment of leukemias with asparaginase, which cures more than 90% of pediatric acute lymphoblastic leukemia patients.[4]

Since APIT depletes the essential amino acid lysine and the semiessential amino acid arginine,[26] both activities interfere with tumor cell amino acid metabolism. Besides the increased need of proliferating cells for lysine, the enzymes involved in *de novo* synthesis of arginine—argininosuccinate synthetase (ASS1) and argininosuccinate lyase—are frequently deregulated in cancer cells,[5,6] leading to arginine auxotrophies in many different tumor cells (for review, see ref 9). This metabolic Achilles heel of tumor cells can be used for therapeutic approaches based on the activities of APIT. However, the blood circulation time of active purified APIT is insufficient to achieve the long-lasting depletion of L-lysine and L-arginine that is required to starve tumor cells.

Here, we developed the PEGylation of purified APIT and, thereby, achieved a dramatic increase in the blood circulation time of the enzyme. A single dose of PEG−APIT depleted L-lysine and L-arginine for more than 50 h, whereas the amino acid oxidase activity of non-PEGylated APIT was cleared in less than 10 h. Despite the long-lasting amino acid depletion, APIT−PEG treatment had no negative effect on the well-being of mice, whereas doxorubicin treatment resulted in weight loss. APIT−PEG induced a dose-dependent, significant decrease in the head and neck cancer HNXF 536 in patient-derived xenografted mice compared to the control group. Interestingly, treatment with the lower dose of 15 U/kg APIT−PEG caused a nonsignificant reduction of the tumor volume, and this correlated with a low but measurable level of L-arginine and L-lysine in the plasma of these mice. This is in line with the requirement of a long-lasting efficient depletion of L-lysine and L-arginine to achieve an antitumor effect. Due to the very good tolerability of the APIT−PEG treatment, the dose and thus the efficacy could be optimized in further experiments. Due to its broader action by depleting of two amino acids instead of only one, APIT−PEG could be an interesting alternative to ADI-PEG20, which was granted orphan drug status and is currently used for the treatment of malignant pleural mesothelioma.

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APIT was PEGylated on lysine residues using monofunctionalized NHS-activated polyethylene glycol. The high PEG concentration during the PEGylation resulted in minor protein precipitation, explaining the protein recovery of 60% APIT−PEG. PEGylated APIT was successfully isolated by anion-exchange chromatography and contained between 8 and 10 mol of PEG per mol of APIT. Furthermore, the enzyme activity of APIT−PEG was about 20% reduced compared to APIT, indicating that PEGylation causes only a minor loss of enzyme activity.

For the current experiments, the purification yield was 8 mg of monomeric APIT per liter of culture with batch-dependent enzyme activity between 80 and 100 U/mg. Further optimization of the expression and large-scale production using fermenters will enable the production of PEG−APIT at a scale required for clinical testing. So far, a SEC step is necessary to yield pure monomeric APIT. Further optimization to exclude higher molecular APIT might be achieved by selecting suitable hydrophobic interaction chromatography media to improve the purification procedure for large-scale approaches as well. Furthermore, a critical step for application in clinical testing is the endotoxin amount. Reduction of endotoxin might be achieved by endotoxin-reduced workflow via anion exchange chromatography and could be integrated into the existing purification steps in larger-scale productions. Our work therefore gives us hope that preclinical testing will soon be completed and manufacturing and clinical testing can begin.

CONCLUSIONS

This study presents the recombinant synthesis and purification of arginine- and lysine-deaminase APIT. Due to a low serum half-life, PEGylation of the lysine residues of APIT was performed. Increased serum half-life was proven in an in vivo study. Furthermore, APIT−PEG was tested in vivo in a dose-escalation study to determine the tolerated doses in healthy mice. Subsequently, the effect of arginine and lysine depletion in the serum of mice carrying patient-derived head and neck cancer resulted in a dose-dependent inhibition of tumor growth. In conclusion, this initial proof-of-principle study demonstrated the effect of APIT−PEG as a potential tumor therapeutic agent.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.4c00473.

> Screening of APIT−PEG conjugates on the pharmacodynamics; pharmacodynamics of administration of APIT and APIT−PEG on arginine and lysine concentrations in serum; representative histology of patient-derived head and neck cancer as a xenograft in mice; amino acid concentration in serum of treated xenografted mice; serine, alanine, lysine, and arginine concentrations in the serum of different treated groups of xenografted mice; and comparison of beta coefficients of the curves of relative tumor growth performed by a linear regression fit (PDF)

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A.M.W.: investigation and writing—original draft. B.B.: investigation. J.A.: validation and resources. M.E.: investigation. E.G.: funding acquisition. M.G.: validation and resources. L.H.: validation and resources. R.H.: conceptualization and methodology. R.K.: PEGylation and methodology. F.L.: PEGylation and methodology. T.L.: supervision. J.S.: investigation. L.S.: validation. M.T.: project administration. L.M.: supervision, funding acquisition, and writing—review & editing. T.R.: conceptualization, project administration, funding acquisition, and writing—review & editing.

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Notes

The authors declare no competing financial interest.

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