Deprivation of arginine and lysine interferes with growth of patient-derived tumor xenografts

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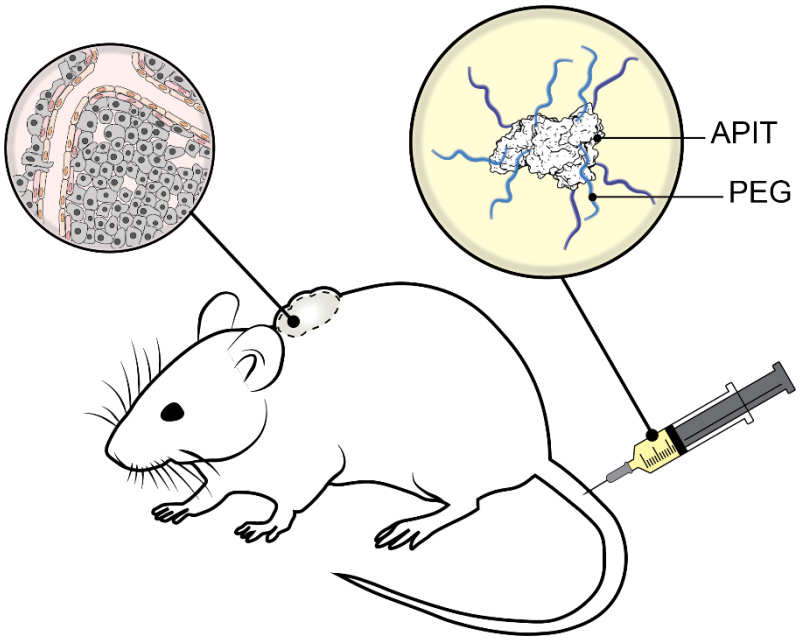
Abstract

In recent years, a novel treatment method for cancer 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 acids oxidase was described to oxidate the essential amino acid L-lysine and L-arginine to their corresponding deaminated alpha-keto acids. Here, we present the recombinant production and functionalization of 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 lead to deprivation of arginine was well tolerated.

Significance: Inhibiting the growth of human head and neck cancer in a mouse model by the depletion of amino acids L-lysine and L-arginine provides proof of principle for tumor therapy based on APIT-PEG.



Introduction

Increasing resistance of tumors towards chemotherapies can be observed with standard of care DNA-damaging agents, antimetabolites, mitotic inhibitors, nucleotide analogous or inhibitors of topoisomerase.(1) The cytotoxic effect of the drugs is primarily by 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 of activation of the apoptotic pathway.(2) This constitutes the need for alternative anti-tumor therapy approaches to tackle these limitations of current anti-tumor treatments.

An increasingly recognized strategy is the enzymatic depletion of amino acids in serum to target metabolic 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 semi-essential amino acid arginine occurs through epigenetic silencing of ***a**rgino**s**uccinate **s**ynthetase 1* (ASS1) or ***a**rginine **l**yase (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 **a**rginine **dei**minases (ADI), which deaminate arginine to citrulline and thereby deplete arginine. In combination with arginine auxotrophy, cells are then starved from 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 for this treatment method is ADI-PEG20. First clinical trials with a mycoplasma-derived PEGylated ADI demonstrated the sensitivity of tumors towards 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 cytostatically chemotherapy (cisplatin, pemetrexed-cisplatin, Nab-Paclitaxel with Gemcitabine, FOXLFOX6).(22-25) In the studies patients showed an increase of immunogenic reaction against ADI-PEG20 after few weeks, which decreased the effectiveness of the combination therapy.

To overcome these limitations, the necessity of alternative products for ADI-PEG20 emerges including an arginine and lysine specific **L-a**mino **a**cid **o**xidases (LAAO) in the defense ink of *Aplysia punctata* (***A**plysia **p**unctata* **i**nk **t**oxin = APIT). APIT was first isolated from crude ink, which deaminates L-lysine and L-arginine to their corresponding α-keto acids.(26) APITs tumor specific cytotoxicity was demonstrated on 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 **p**oly**e**thylene **g**lycol (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 exhibited 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 the 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.

Material and Methods

Recombinant expression of APIT

Competent *E. coli* HMS174 were transformed with the pET26b-based plasmid with inserted APIT encoding gene between *Nde*I and *Eco*RI restriction sites. Protein sequence of APIT is supplied in the SI. Expression was controlled by T7 promoter and induced by 1 mM 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, 4°C. Expression resulted in inclusion bodies. Pelleted cells were resuspended in 50 mM Tris 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 were solubilized in 100 mL 6 M Urea, 50 mM Tris, 200 mM NaCl, 100 mM DTT at pH 8.0 overnight at 4°C. Solubilized inclusion bodies were centrifuged at 5000 g, 4°C for 30 min and filtrated using a 0.2 µm 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.

80 **Purification of APIT**

81 The subsequent purification of refolded APIT was performed in two steps using an anion
82 exchange chromatography (HiTrap™ Q FF) and a size exclusion chromatography (HiLoad™
83 16/600 Superdex™ 200 pg) from Cytiva (Uppsala, Sweden).

84 Elution was performed with buffer A (50 mM Tris, pH 8.0) and buffer B (50 mM Tris, 1 M NaCl,
85 pH 8.0), starting with a washing step of 25% B in 10 CV, followed by an elution gradient of
86 25-60 in 10 CV. APIT was then loaded onto a HiLoad™ 16/600 Superdex 200 pg. Column was
87 then run using PBS pH 7.4 at flowrate of 1 mL/min.

88 **PEGylation and purification of APIT-PEG5kDa**

89 APIT was dialyzed in PBS pH 7.4 overnight at 4°C and concentration was adjusted to 1.1
90 mg/mL. 20 eq of PEG-NHS 5kDa (Celares GmbH, Berlin, Germany) was dissolved at a
91 concentration of 408 mg/mL in PBS pH 7.4 and 1:10 (v:v) added to APIT. The reaction was
92 performed overnight at 4°C continuously shaking. Reaction was purified using an anion
93 exchange chromatography (HiTrap™ Q HP, Cytiva, Uppsala, Sweden) with buffer A (50 mM
94 Tris, pH 8.0) and buffer B (50 mM Tris, 1 M NaCl, pH 8.0). The reaction was diluted 1:3 in
95 buffer A, a maximum of 10 mg of protein was loaded onto 1 mL of resin at a flowrate of 0.5
96 mL/min. Afterwards a washing step at 0% B for at least 10 CV and elution gradient from 0-
97 100% B was performed. Elution peak was pooled and dialyzed against PBS pH 7.4,
98 concentration was adjusted to 2 mg/mL PEGylated APIT was filtered using a 0.2 µm PS filter
99 and stored light protected.

100 **Enzyme activity**

101 The enzyme activity in the presence of the substate L-lysine was determined by measuring the
102 production of hydrogen peroxide. H_2O_2 is reduced stoichiometrically by the chromogenic
103 ABTS in a coupled HRP-catalyzed reaction as previously described by Butzke et al. (27). The
104 enzyme activity is expressed in units [U], 1 U is equivalent to the production of 1 µmol H_2O_2
105 per minute.

106 **HPLC Analysis**

107 Purity of produced monomeric APIT and PEGylated APIT was assessed by HPLC analysis
108 using an Agilent Technologies 1100 series system (Waldbronn, Germany). A ZORBAX

109 300SB-CN, 300 A, 5 µm, 4.6 x 250 nm was used. For elution was performed with Water in
110 HPLC grade + 0.1% TFA and increasing concentrations of Acetonitrile HPLC grade + 0.1 TFA.

111 **Degree of PEGylation**

112 The degree of PEGylation was assessed by determination of the mass increase measured by
113 MALDI mass spectra with an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen,
114 Germany) equipped with a 355 nm smartbeam-II™ laser. 20-50 µg of protein were desalted
115 using Sep-Pak Vac C18 cartridges. The recommended manufacturers instruction was followed.
116 Bound protein was eluted using 80% acetonitrile in ddH_2O+ 0.1% TFA and subsequently
117 lyophilized at -100°C, 0.001 mbar. For external calibration Bruker Protein II standard (Bruker
118 Daltonics #8207234) was mixed with sinapinic acid solution (saturated in a 1:2 mixture (v/v)
119 of acetonitrile and 0.1% TFA). Drops were measured on a stainless-steel target (MTP 384 target
120 plate ground steel, Bruker Daltonics #8280784). The proteins were analyzed after double layer
121 preparation. First a thin matrix layer of SA solution (saturated in ethanol) was applied onto the
122 stainless-steel target, second the sample solution (4 mg/mL in a 1:1 mixture of acetonitrile and
123 ddH2O) was mixed with sinapinic acid solution (saturated in a 1:2 mixture (v/v) of acetonitrile
124 and 0.1% TFA) and dropped onto the first layer. Several thousand shots were accumulated for
125 the spectrum.

126 **Pharmacodynamics of APIT-PEG on plasma L-arginine and L-lysine levels**

127 Depletion of arginine and lysine in blood serum was assessed after a single intravenous
128 administration of 250 U/kg of APIT, 1000 U/kg, 250 U/kg APIT-PEG or 250 mL/kg PBS as
129 control vehicle into 5 tumor-free mice each group. Blood was collected 1 h prior to injection
130 and 0.25 h, 1 h, 3 h, 6 h, 24 h and 48 h after injection from each mouse. Samples of each
131 timepoint were pooled.

132 **Dose-finding experiments and efficacy experiments of APIT-PEG in HNXF 536 PDX mice**
133 **model**

134 The maximum tolerated dose (MTD) of APIT-PEG was evaluated at three dose levels of 15
135 U/kg, 30 U/kg and 50 U/kg in 4 tumor-free mice per group. APIT-PEG was intravenously
136 injected every other day for one week and the tolerability was assessed by observing clinical
137 symptoms and body weight development.
138 Tumor fragments of HNXF 536 of a serial passage in nude mice were placed in PBS containing
139 10% penicillin/streptomycin and cut into fragments of 3-4 mm diameter. Prior to tumor

140 implantation, NMRI nu/nu (Crl:NMRI-*Foxn1*^{nu}) mice were anesthetized by inhalation of
141 isoflurane. Animals were monitored until the tumor implants reached an appropriate size of 50-
142 250 mm^3.
143 Mice bearing the HNXF 536 were randomly divided into control and treatment groups. The day
144 of randomization was designated as Day 0 of the experiment. Mice were treated with 1) control
145 vehicle 5 ml/kg/day every other day 2) APIT-PEG 15 U/kg/day every other day 3) APIT-PEG
146 40 U/kg/day every other day 4) doxorubicin-HCl 6 mg/kg every 7 days (See Tab. SI 1).
147 Injection was performed via the lateral tail vein. Intraperitoneal injections were carried out if
148 an intravenous injection was not possible due to an inflammation at the injection site. To control
149 arginine and lysine depletion during the therapy, blood was collected 6 h, 24 h and 48 h after
150 first treatment.

151 **Analysis of amino acids in serum**

152 For analysis of arginine and lysine levels in serum, blood was collected for by retrobulbar sinus
153 puncture under isoflurane anesthesia. Plasma was stored in lithium heparin vials on ice. Vials
154 were centrifuged at 2,000 x g for 5 min at 4°C. Separated plasma was transferred to a cooled
155 tube on ice. 60 µL sample was diluted with 15 µL precipitation buffer and stored at 5°C for 60
156 min to precipitate proteins present. After centrifugation at 2 000 x g for 5 min at 4°C, the
157 supernatants were diluted 1:1 with sample dilution buffer containing 200 nmol/ml Norleucin,
158 which was used as internal standard. For quantitative and qualitative analysis of the amino acids
159 serin, alanine, lysine and arginine at 570 nm the ARACUS Amino Acid Analyzer was used
160 (MembraPure, Hennigsdorf, Germany).

161 ***H&E staining of dissected tumors***

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

169 **Statistics**

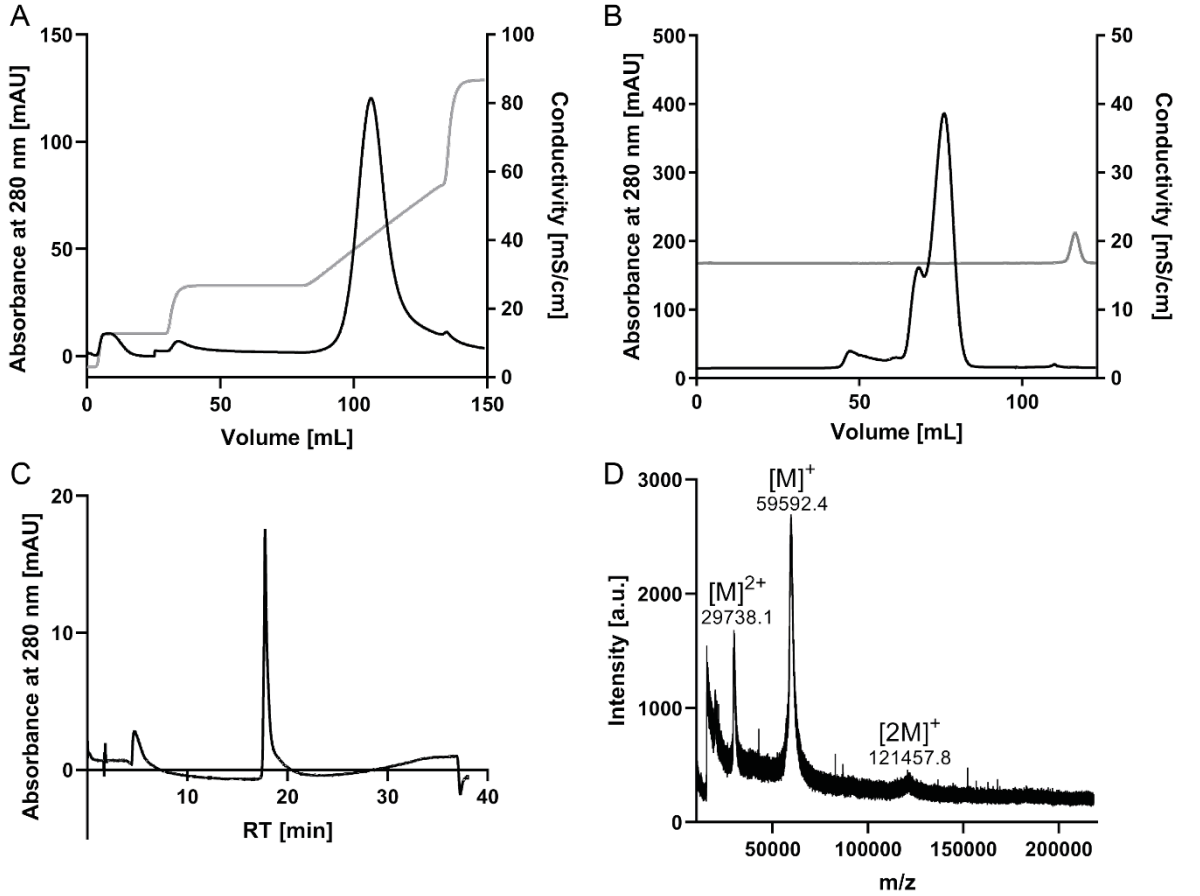
170 Relative tumor growth over time was compared to Day 0. The relative body weight changes
171 and relative tumor growth are depicted as the group median and 5-95% confidence interval.
172 Statistical analysis was performed on the relative tumor growth comparing the treatments
173 separately to the control using the unpaired, nonparametric Mann-Whitney-U test. A p value
174 less than 0.05 was considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

175 **Results**

176 **Expression and purification of monomeric APIT**

177 We have previously demonstrated that the arginine and lysine deaminase APIT, an enzyme
178 found in the *Aplysia punctata* ink toxin, induces cell death in cancer cells, but not in normal
179 cells. To develop APIT for potential future pharmaceutical application, the serum half-life of
180 APIT had to be increased through PEGylation. We therefore developed the recombinant
181 expression and subsequent purification of APIT, which resulted in approximately 8 mg/L
182 monomeric APIT after the last purification step via size-exclusion chromatography. The most
183 crucial part of the purification was the purity of the inclusion bodies prior to refolding, which
184 was optimized by a serial of sonification steps. Refolded APIT was purified via anion exchange
185 chromatography, which still contained high molecular APIT (Fig. 1A). To obtain pure
186 monomeric APIT, a reductive step using tris(2-carboxyethyl) phosphine (TCEP) was
187 introduced prior to size exclusion chromatography (SEC) (Fig. 1B). Over time, monomeric
188 APIT tended to form dimers, which did not interfere with the enzymatic activity. To avoid
189 dimerization, monomeric APIT was stored at -80°C with the addition of small amounts of
190 TCEP. Monomeric APIT had a mass of approximately 59592.4 g/mol and a purity of ≥98%
191 measured by HPLC (Fig. 1 C), with a small amount of dimeric APIT with a mass of

192 121457.8 g/mol (Fig. 1D) and a batch dependent enzyme activity of 80-100 U/mg



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

198 **Production of APIT-PEG for *in vivo* application**

199 To enhance the serum half-life, we PEGylated purified APIT at lysine residues and purified the
200 conjugates via anion-exchange chromatography (Fig. 2A). After conjugation, a protein
201 recovery of 60% of pure APIT-PEG was achieved. Compared to APIT the enzyme activity of
202 APIT-PEG was reduced by 20%. Purity of APIT-PEG was ≥95% measured by HPLC and SDS-
203 PAGE (Fig. 2B-C). APIT-PEG showed an average increase of mass by 55 507 g/mol indicating
204 a degree of PEGylation of approximately 8-10 mol PEG/ mol measured by MALDI-MS (Fig.
205 2D). APIT-PEG was formulated in PBS pH 7.2 and stored at 4°C. Prior to release for animal
206 experiments, the endotoxin level was determined and shown to have a concentration of 9
207 EU/mg, 0.14 EU/U. Masking of endotoxin by formulated APIT-PEG was controlled by the
208 spike-test. Spike recovery was 103-110%, concluding formulated APIT-PEG is not interacting
209 with endotoxins and therefore hiding endotoxins.

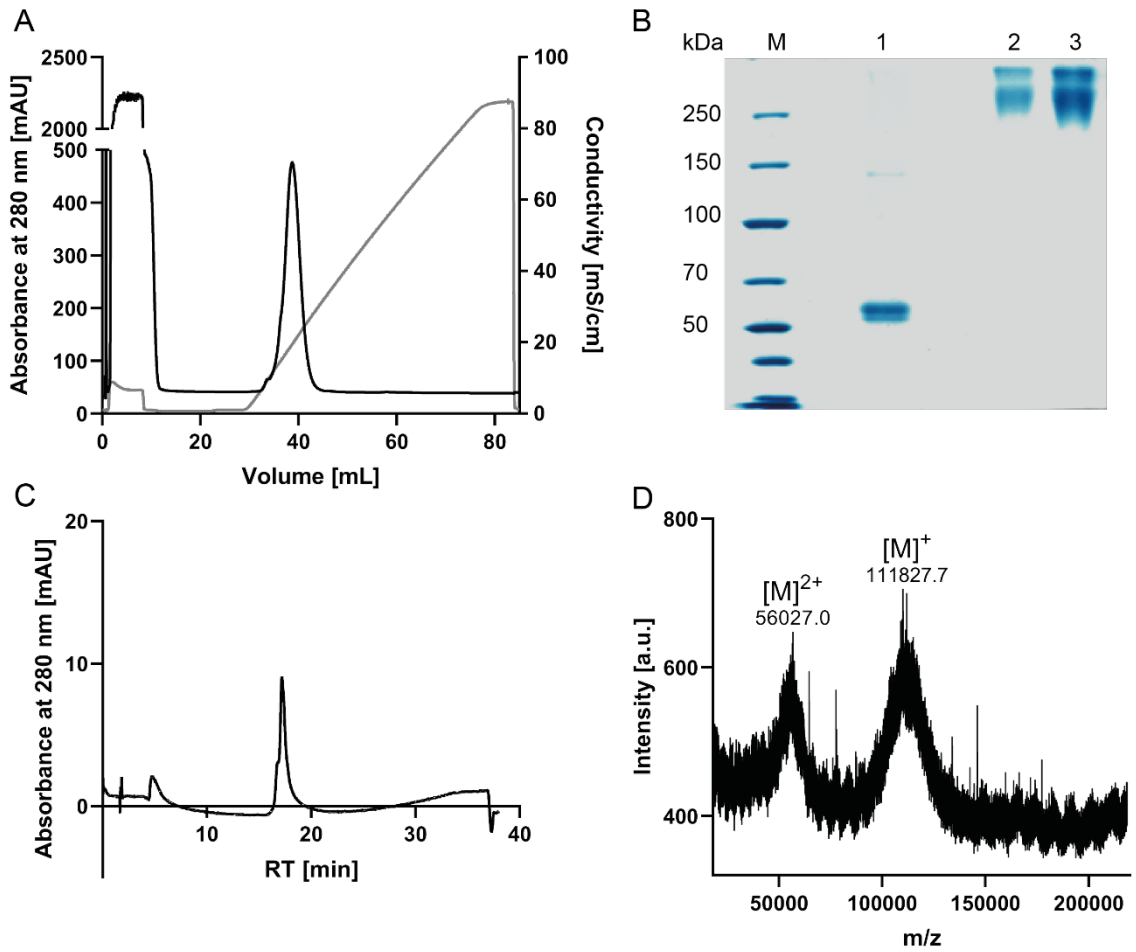
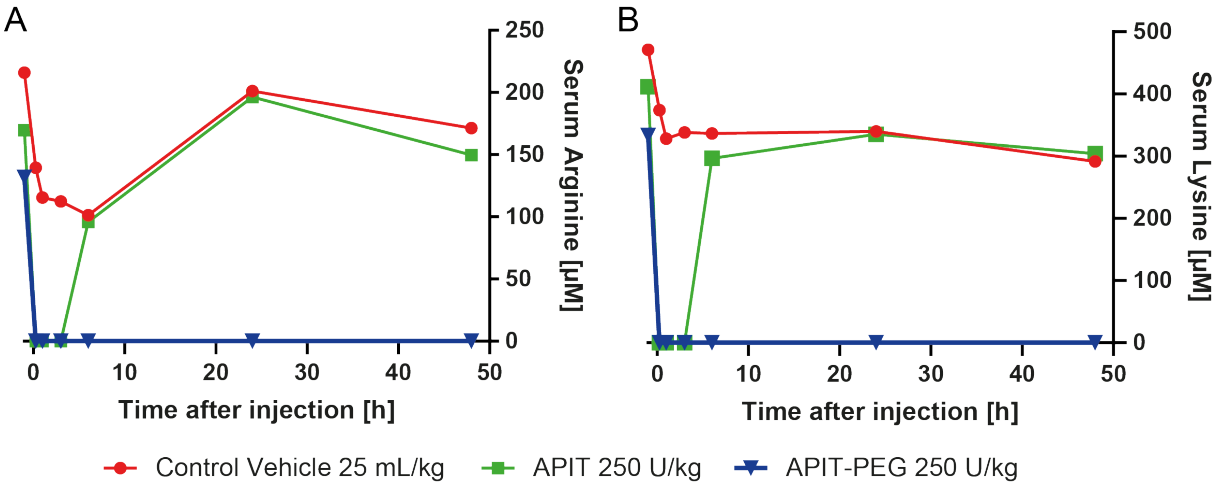


Figure 2: Purification of PEGylated APIT A) Chromatogram of Sepharose Q HP column B) 5-15% Tris-SDS-PAGE of non-reducing APIT (1) and APIT-PEG 2 µg (2) 4 µg (3) stained with Coomassie blue G250 C) CN-HPLC profile of PEGylated APIT after purification D) MALDI-mass spectrum of PEGylated APIT shows an increase of mass by 55507 g/mol, indicating a degree of PEGylation of 8-10 PEG per APIT.

216 **PEGylated APIT depletes serum arginine and lysine for at least 48 h**

217 Depletion of arginine and lysine in mice blood was tested after a single injection of A) 250
218 U/kg APIT B) 250 U/kg APIT-PEG or C) 25 mL/kg PBS as control vehicle for dilution effects.
219 Serum of mice treated with APIT showed a beginning recovery of arginine (Fig. 3 A) and lysine
220 levels after 6 h (Fig. 3 B). A single injection of 1000 U/kg APIT showed the depletion of
221 arginine and lysin for up to 6 h. Nevertheless, after 24 h a complete recovery of amino acids in
222 serum were observable (Fig. SI 1). Only the injection of APIT-PEG showed a complete
223 depletion of serum arginine and lysine during the 48 h period (Fig. 3).



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225 Figure 3: Pharmacodynamics of a single administration of 250 U/kg APIT or 250 U/kg APIT-
226 PEG on A) arginine concentration in Serum and B) lysine concentration in serum showing
227 beginning recovery of arginine and lysine concentration after 6 h, whereas APIT-PEG showed
228 a complete depletion of serum arginine and lysine at least 48 h.

229

230 **Tolerability experiments in tumor-free nu/nu mice**

231 Dose finding tolerability tests were performed to define the suitable dose that does not affect
232 the animal's health during the continuous treatment with APIT-PEG. Therefore, the relative
233 change in body weight was taken as an indicator of tolerability and health since weight loss or
234 gain should not exceed 10% in seven days during chemotherapy. Mice were treated with 15
235 U/kg, 30 U/kg or 50 U/kg of APIT-PEG every other day. The median relative body weight
236 change of mice administered with 50 U/kg APIT-PEG showed a body weight loss of 7.3% after
237 one week, which was declared as the upper limit (Fig. 4). The results of these experiments
238 demonstrated that doses up to 50 U/kg were well tolerated.

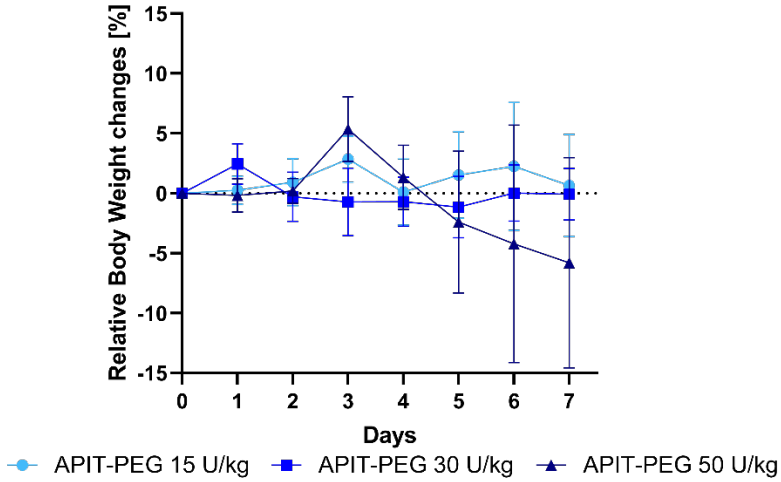
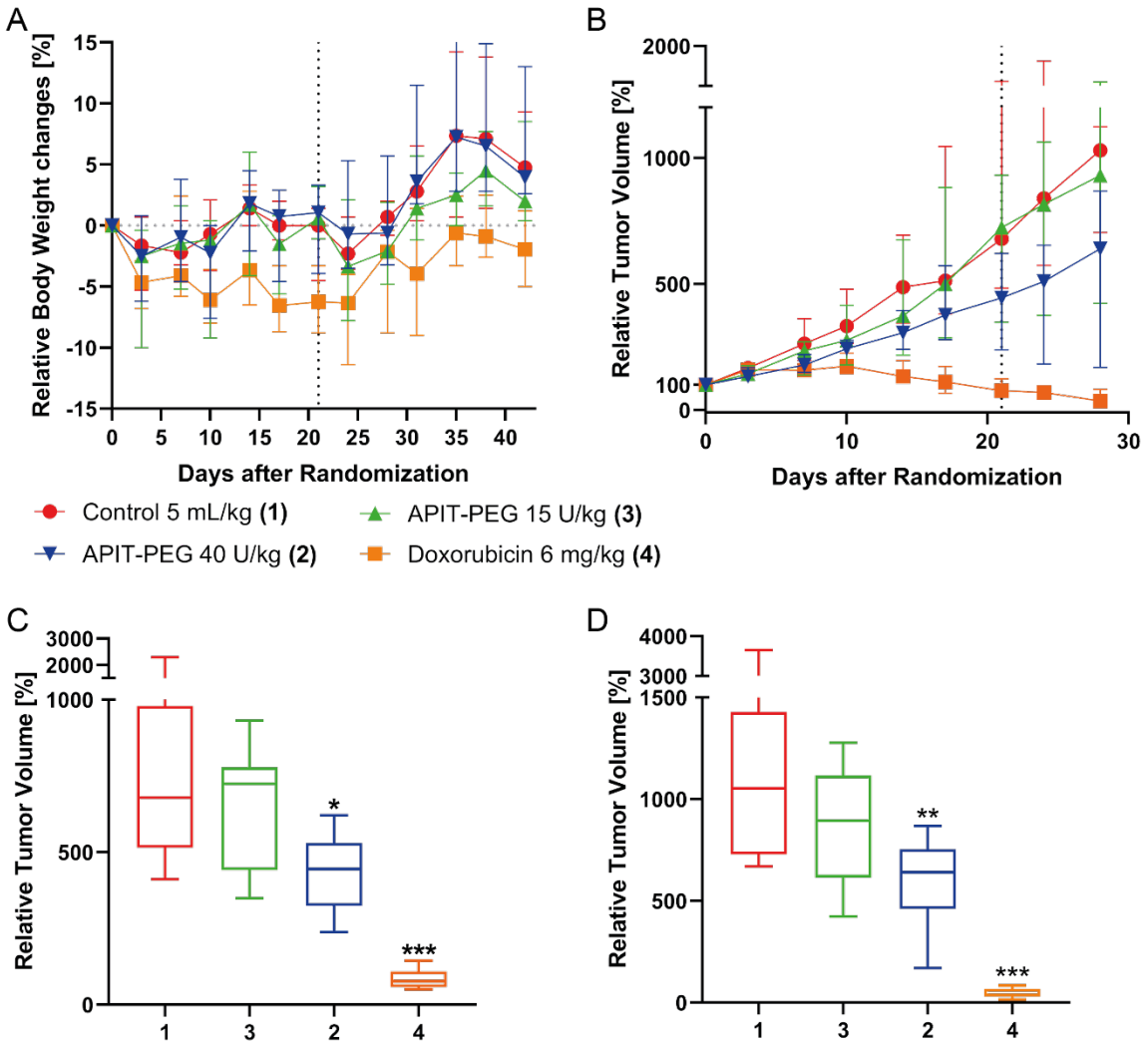


Figure 4: The median and 5-95% confidence interval (CI) of relative body weight change of mice treated with APIT-PEG every other day for one week shows a stronger impact of higher doses of APIT-PEG on the well-being of mice.

Efficacy experiment of APIT-PEG in the HNXF 536 PDX mouse model

To evaluate the anti-tumor effect of arginine and lysine deprivation, we tested APIT-PEG in mice xenografted with HNXF 536, a patient-derived head and neck cancer (SI Fig. 2). Based on the tolerability experiment APIT-PEG was applied intravenously at 15 U/kg and 40 U/kg every other day in this efficacy experiment in mice. As standard-of-care drug doxorubicin was administered once a week at a concentration of 6 mg/kg and the control group received PBS every other day (for details see Tab. SI 1). At day 21 the treatment was terminated due to the high tumor load in the control group and lower dose group of 15 U/kg APIT-PEG. Tumor growth was monitored until day 28 and last observations were carried forward. Mice were weighed until day 42, relative body weight of doxorubicin showed a loss of weight during the treatment, the well-known impact of standard chemotherapeutic treatment approaches on health (Fig. 5A). Body weight of mice was slowly regained after the end of the treatment. APIT-PEG treatment 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 from all mice as a measure for tumor growth (Fig. 5B). Treatment with doxorubicin resulted in significant loss of tumor volume starting at day 10. Treatment with 15 U/kg APIT-PEG did not show significant difference in relative tumor growth compared to the control group (Fig. 5B-D). Serum analysis

262 of amino acids showed an increase of serum arginine after 48 h, probably leading to the lag of
263 effectiveness due to incomplete arginine and lysine depletion (SI Fig. 3, 4). In contrast,
264 treatment with 40 U/kg APIT-PEG successfully depleted arginine and lysine in blood serum
265 during the treatment and showed a significant reduced tumor growth on the last day of injection
266 (p= 0.0160) and one week after the end of treatment (p= 0.0075) compared to the non-treated
267 control group (Fig. 5C-D).



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269 Figure 5: Efficacy experiment of treatment of patient derived HNXF 536 xenografted mice with
270 APIT-PEG or Doxorubicin the end of treatments is indicated with dashes lines A) Median and
271 5-95% CI of relative body weight changes for monitoring the mice's health B) Median and 5-
272 95% CI of relative tumor growth during and one week after the treatment C) Boxplot
273 representation of median and 5-95% confidence interval of relative tumor growth on day 21
274 (last day of treatment) D) and one week after the end of treatment on day 28. (Whiskers indicate
275 5-95% percentile values, the line in the middle of the box represents the median; Significance
276 was tested with the Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$).

277 **Discussion and Outlook**

278 Targeting amino acid metabolism is an attractive anticancer strategy since it may provide
279 alternatives for treatment of therapy resistant tumors (for review see (19)). Killing of therapy
280 resistant tumor cell lines has also been demonstrated for APIT, which kills cancer cells
281 independent of the induction of apoptosis (27), the mechanism induced by many of the
282 genotoxic agents used for cancer therapy.(1) Resistances of tumors to these canonical therapies
283 occur frequently by mutations in the general apoptosis signaling pathways.(2) This explains
284 why anti-tumor therapies provoke the emergence of broad resistance to drugs with the same
285 mode of action, the induction of apoptotic cell death. In addition, investigations in amino acid
286 depletion cancer therapies suggest that side effects like those elicited by current treatments with
287 genotoxic agents are less severe or even not observed, since amino acid auxotrophies are often
288 highly tumor specific.(3) A prime example of amino acid depletion cancer therapy is the
289 treatment of leukemias asparaginase curing more than 90% of pediatric acute lymphoblastic
290 leukemia patients.(4)
291
292 Since APIT depletes the essential amino acid lysine and the semi-essential amino acid arginine
293 (26), both activities interfere with tumor cell amino acid metabolism. Besides increased need
294 of proliferating cells for lysine, the enzymes involved in *de novo* synthesis of arginine,
295 argininosuccinate synthetase (ASS1) and argininosuccinate lyase (ASL) are frequently
296 deregulated in cancer cells (5, 6), leading to arginine auxotrophies in many different tumor cells
297 (for review see (9)) This metabolic Achilles heel of tumor cells can be used for therapeutic
298 approaches based on the activities of APIT. However, the blood circulation time of active
299 purified APIT is insufficient to achieve the long-lasting depletion of L-lysine and L-arginine
300 that is required to starve tumor cells.
301
302 Here we developed the PEGylation of purified APIT and thereby achieved a dramatic increase
303 in the blood circulation time of the enzyme. A single dose of PEG-APIT depleted L-lysine and
304 L-arginine for more than 50 hours whereas the amino acid oxidase activity of non-PEGylated
305 APIT was cleared in less than 10 hours. For the current experiments, the purification yield was
306 8 mg monomeric APIT per liter culture with a batch-dependent enzyme activity between 80-
307 100 U/mg. Further optimization of expression and large-scale production using fermenters will
308 enable the production of PEG-APIT at a scale required for clinical testing. So far, size-exclusion
309 chromatography step is necessary to yield pure monomeric APIT. Further optimization to
310 exclude higher molecular APIT might be achieved by selection of suitable hydrophobic

311 interaction chromatography media to improve the purification procedure also for large scale
312 approaches. Longer incubation times of APIT without reducing agents showed the formation
313 of dimers, without changes in enzyme activity. Monomeric APIT can be stored using reducing
314 agents like TCEP. Nevertheless, several other L-amino acids oxidases are reported to be
315 dimeric, the dimerization of APIT over time should further be evaluated.
316 APIT was PEGylated on lysine residues using monofunctionalized NHS-activated polyethylene
317 glycol. The high PEG concentration during the PEGylation resulted in minor precipitation of
318 protein, explaining the protein recovery of 60% APIT-PEG. PEGylated APIT was successfully
319 isolated by anion-exchange chromatography. The degree of PEGylation was further determined
320 by MALDI-mass spectrometry and was between 8-10 mol PEG per mol APIT. The enzyme
321 activity of APIT-PEG was about 20% reduced compared to APIT, indicating that PEGylation
322 causes a minor loss of enzyme activity. Furthermore, a critical step for the application in
323 animals is the endotoxin amount. Reduction of endotoxin might be achieved by endotoxin
324 reduced workflow via anion exchange chromatography and could be integrated in the existing
325 purification steps in larger scale productions.

326 For intravenous injection into mice a formulation of APIT-PEG in endotoxin-free PBS was
327 used. In this formulation no endotoxin masking was observed. Mice were administered different
328 concentration of APIT-PEG according to the previously tested tolerated doses. APIT-PEG
329 treatment had no negative effect on the well-being of mice, whereas doxorubicin treatment
330 resulted in weight loss. APIT-PEG showed dose-dependent, significant decrease of the head
331 and neck cancer HNXF 536 in patient derived xenografted mice compared to the control group.
332 Interestingly, treatment with the lower dose of 15 U/kg APIT-PEG caused a non-significant
333 reduction of the tumor volume and this correlated with a low but measurable level of L-arginine
334 and L-lysine in the plasma of these mice. Due to the very good tolerability of the treatment, the
335 dose and thus the efficacy could be optimized in further experiments. Due to its broader action
336 by depleting of two amino acids instead of only one, APIT-PEG could be an interesting
337 alternative for ADI-PEG20, which was granted the orphan drug status and is currently used for
338 the treatment of malignant pleural mesothelioma.

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