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PEGylation and pharmacological characterization of a potential anti-tumor drug, an engineered arginine deiminase originated from *Pseudomonas plecoglossicida*

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ABSTRACT

Arginine deiminase (ADI) has been studied as a potential anti-cancer agent for arginine-auxotrophic tumors. PEGylation is one of the best methods to formulate a bioconjugated protein with extended physical stability and reduced immunogenicity. Here, PEGylation and pharmacological properties of an engineered ADI originated from *Pseudomonas plecoglossicida* were studied. Among polyethylene glycol (PEG) reagents with succinimidyl ester groups varying in size and linkers, three PEGylated products with high yield and catalytic activity were further characterized, named ADI-$SS_{20\ kDa}$, ADI-$SC_{20\ kDa}$, and ADI-$SPA_{20\ kDa}$. In the pharmacodynamic/pharmacokinetic (PD/PK) studies with ADI-$SPA_{20\ kDa}$, a remarkable improvement in circulating half-life compared with native ADI was observed. ADI-$SPA_{20\ kDa}$ injections via intravenous, intramuscular and subcutaneous routes all exhibited superior efficacy than native ADI on depleting serum arginine. Additionally, our results demonstrated that single ADI-$SPA_{20\ kDa}$ administration of 5 U/mouse via intravenous injection could maintain serum arginine at an undetectable level for 5 days with a half-life of 53.2 h, representing 11-fold improvement in half-life than that of the native ADI. In a mice H_{22} hepatocarcinoma model, ADI-$SPA_{20\ kDa}$ dosage of 5 U per 5 days showed an inhibition rate of 95.02% on tumor growth during 15-day treatments.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers and is now the third leading cause of cancer-related deaths worldwide. Since HCC is often diagnosed at an advanced stage when chemotherapeutic treatment has low positive response rate, most patients merely have a life expectancy of less than one year. Consequently, effective treatments for these malignant diseases are required urgently [1–4]. Some enzymes have inherent abilities as pharmaceuticals, owing to their substrate specificity and high catalytic efficiency under physiological environments. Biological therapy with amino-acid degrading enzymes has been demonstrated as a promising anti-cancer treatment of amino acid auxotrophic cancers, such as asparaginase therapy for acute lymphoblastic leukemia [5–8]. HCC and melanoma cells do not express argininosuccinate synthetase (ASS), and have been confirmed to be arginine-auxotrophic cancers. Therefore, it has been suggested that an arginine-degrading enzyme may be effective in controlling these arginine-auxotrophic cancers [9–12].

Arginine deiminase (ADI, EC 3.5.3.6) is an arginine-degrading enzyme. It catalyzes the conversion of L-arginine into L-citrulline and ammonia [5,13,14]. ADI has been studied as a potential anti-cancer agent for inhibiting arginine-auxotrophic tumors, such as HCCs and melanomas. A single dose of ADI could extend the survival of tumor bearing animals, daily injections of ADI are however not a favorable solution due to its short serum half-life (approximately 4 h) [15]. In recent years, a number of PEG conjugated biomolecules have been reported to exhibit clinically useful properties superior to their unmodified parent molecules [16,17]. PEGylation has become one of the best drug delivery methods owing to its extended serum half-life and lower immunogenicity. At least 8 PEGylated proteins are on the market, including Adagen®, Oncaspar®, and Pegasys® [17–20] etc. Therefore, PEGylation has emerged as a dominant strategy for enabling or improving novel therapeutics [21].

Clark and co-workers reported the formulation of recombinant ADI (from *Mycoplasma arginini*) with mPEG-$SS_{20\ kDa}$ for extended serum half-life from 4 h to nearly 7 days, and a weekly dose of 5 IU could keep the serum arginine under detectable levels [4]. Later, a 20 kDa mPEG with succinimidyl succinate linker was selected among PEG reagents varying in size and linker [22,23]. ADI-PEG-20 developed by Phoenix Pharmacologics Inc. has been granted by the FDA and EMEA as orphan drug status for potential treatment of two lethal

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diseases, HCC and melanoma. Clinical trials for the treatment of HCC (Phase III) and melanoma (Phase II) using ADI-PEG-20 are ongoing in the United States and Italy [24]. In 2006, Polaris Group acquired the intellectual property of Phoenix Pharmacologics Inc., and now holds the worldwide rights to ADI-PEG-20.

In previous studies, a *Pseudomonas plecoglossicida* CGMCC2039 strain exhibiting high ADI activity was isolated from the local canal [25]. Our results showed that ADI from *P. plecoglossicida* (PpADI) was effective in inhibiting HCC cell line HepG2 *in vitro*, as well as mice implanted with H_{22} tumor [10,18]. In our further studies, directed evolution of PpADI was carried out to improve the enzymatic properties and activity under physiological pH. M314$^{A128T/H404R/I410L}$ was obtained after one round of ep-PCR, showing 20-folds increase in activity (9.02 U/mg at pH 7.4) and an enhanced pH optimum of 6.5 compared with WT-PpADI [3]. After another round of random mutagenesis in combination with semi-rational design, an excellent variant M13-3$^{D38H/A128T/E296K/H404R/I410L/Q162S}$ was selected, which exhibited a specific activity of 13.59 U/mg (at pH 7.4) and a pH optimum of 6.5 (unpublished data).

In this study, to increase the efficacy of ADI as a potential anti-cancer drug, ADI M13-3 was modified by PEGylation to improve its serum half-life, aiming at developing a novel anti-cancer agent of high efficiency and low cost. Seven different PEGylation reagents varying in linker chemistry, size, and structure were selected. These PEG polymers possess succinimidyl ester linkers which could react with the primary amines on the surface of ADI. Furthermore, the pharmacodynamics (PD) and pharmacokinetics (PK) of the ADI bioconjugates were also investigated in mice. Then, a mice model implanted with H_{22} hepatocarcinoma cells was used to evaluate the performance of PEGylated ADI *in vivo*.

Materials and methods

Materials

Activated PEG reagents were purchased from Laysan Bio, Inc. (AL, USA) and Kaizheng Biotech (Beijing, China). Protein markers (170 and 260 kDa) were obtained from Thermo Scientific (MA, USA). 2,4,6-Trinitrobenzene sulfonic acid (TNBS) was obtained from Sigma-Aldrich Co. LLC. (MO, USA). HiPrep 16/10 DEAE FF column and Superdex 200 gel filtration columns were supplied by GE Healthcare (NJ, USA). Other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Female Kunming (KM) mice (4 weeks old) were obtained from Slac Laboratory Animal (Shanghai, China). Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committees of Jiangnan University (JN NO 20130327-0702 [7]).

Recombinant expression and purification of ADI M13-3

Recombinant expression of ADI M13-3 in *E. coli* was performed as described for M314 [3]. Cells were collected and resuspended in 20 mM PBS buffer, pH 7.4, then disrupted with Constant Cell Disruption Systems (Constant Systems Limited, United Kingdom). Crude enzyme extracts were subjected to anion-exchange chromatography on a HiPrep 16/10 DEAE FF column. ADI was eluted by a NaCl gradient in PBS buffer (20 mM, pH 7.4). The obtained ADI fraction was then concentrated by a 10 kDa Amicon Ultra-50 (Millipore, MA, USA), and subjected to Superdex 200 gel filtration column chromatography. All the purification processes were carried out on an ÄKTA avant (GE Healthcare, NJ, USA).

Electrophoresis analysis

The expression and purification of ADI M13-3 were confirmed by SDS-PAGE on a 12% polyacrylamide separation gel. A native-PAGE was also carried out to determine the number of subunits. The pI value of ADI M13-3 protein was determined by IEF electrophoresis using PhastSystem (GE Healthcare, NJ, USA) on Invitrogen precast vertical pH 3–10 gels (CA, USA).

Activity assay

ADI activity was determined by measuring the formation of L-citrulline from L-arginine using a modified DAM-TSC method as described in our previous study [3]. The amount of protein was determined using Bradford reagent with bovine serum albumin as a standard. One unit (U) of ADI activity is defined as the amount of enzyme required for converting 1 μmol of L-arginine to 1 μmol of L-citrulline per minute under the assay conditions.

PEGylation of ADI

An ADI solution of 0.5 mg/mL was prepared in 20 mM PBS buffer, pH 8.0. PEG reagents were added into the ADI solution to a molar ratio of PEG to ADI of 60:1. The PEGylation reaction was carried out for 2 h at room temperature, with magnetic stirring. The reaction mixture was washed repeatedly with PBS (20 mM, pH 7.4) through a 100 kDa Amicon Ultra-50 (Millipore, MA, USA) to remove free PEGs. When free ADI was detected on SDS-PAGE, the concentrated PEG-ADI was further purified by gel filtration chromatography using a Superdex 200 column.

Characterization of ADI and ADI-PEG

The PEGylation reaction mixtures were analyzed by SDS-PAGE on an 8% polyacrylamide separation gel. TNBS reagent was used to determine the modification ratio. First, ADI and ADI-PEG solutions were prepared in 100 mM borate buffer (pH 8.5) with a protein concentration of 1 mg/mL. Then 0.1% TNBS solution was added and the reaction mixture was further incubated in 40 °C for 2 h. The absorbance at 420 nm was measured and the modification ratio of PEGylation was defined as follows:

$$\text{Modification ratio} = [(1 - \text{OD}_{420}\text{ of ADI} - \text{PEG})/\text{OD}_{420}\text{ of ADI}] \times 100\%$$

The effects of pH and temperature on the activity of ADI and ADI-PEG, as well as their plasma stability, were investigated. The pH optimum of ADI and ADI-PEGs was determined over pH 4.0 – 9.0 in 0.5 M different buffers (acetate buffer, pH 4.0–5.5; PBS buffer, pH 6.0–8.0; Tris–HCl buffer, pH 8.5–9.0). The pH stability of ADI and ADI-PEGs was measured after incubating with the above various pH buffers for 2 h. The optimal temperature was measured at various temperatures from 20 to 65 °C. For thermostability, ADI and ADI-PEGs were incubated at 20–65 °C for 2 h before the activity assay. Furthermore, the stability of ADI and ADI-PEGs in mice plasma (Ruite BIO-tec, Guangzhou, China) was also evaluated. The enzymatic activity assay was performed as described in the Activity Assay section.

Pharmacodynamics of ADI and ADI-PEG in mice

Fifty four female KM mice were assigned into 18 groups, with 3 mice in each group. Single intravenous (i.v.), intramuscular (i.m.), or subcutaneous (s.c.) injections of 0.2, 1, 5 U/mouse of ADI or ADI-SPA$_{20\ kDa}$ were administered on day 0. Blood was collected from the tail vein 1 h after the injection (ADI injection only), on each day for 1 week. Blood samples were incubated on ice for 30 min after clotting at room temperature. The samples were then centrifuged at 5000 rpm for 10 min and the supernatant fraction was collected as serum. Sulfosalicylic acid (10%) was added into the serum samples to precipitate protein on ice for 30 min. The samples were then centrifuged at 12,000 rpm for 10 min, and the supernatant fractions were analyzed by an amino acid analyzer (L-8900 Amino Acid Analyzer, HITACHI, Tokyo, Japan) to measure concentrations of arginine and citrulline.

Pharmacokinetic analysis of ADI-PEG in mice

As described in "Pharmacodynamics of ADI and ADI-PEG in mice", 54 female KM mice were assigned into 18 groups and single injections of ADI or ADI-SPA were administered. The residual concentration of ADI and ADI-SPA in the serum samples was determined by activity assay. Descriptive statistics (mean, SD) and compartmental analysis were performed to calculate the pharmacokinetic parameters using *PKSolver* V2.0 (30) (China Pharmaceutical University).

Evaluation of ADI and ADI-PEG in mice H_{22} tumor model

H_{22} mice hepatocarcinoma model was established in female KM mice. First, H_{22} tumor cells in cryopreservation were resuscitated at 37°C and were adjusted to a concentration of 1×10^7 cells/mL. Then, a 0.3 mL cell suspension was implanted into mice via intraperitoneal (i.p.) injection. After 7 days, ascites tumor cell suspension was obtained, and a 0.2 mL cell suspension of 1×10^7 cells/mL was implanted into 60 KM mice via underarm s.c. injection. Finally, 48 mice bearing H_{22} tumors were selected and divided into 6 groups to evaluate the effect of ADI and ADI-SPA treatments. Here, 5-Fluorouracil (5-FU) was used as a positive control.

$$\text{Inhibition rate} = [1 - \text{Average tumor weight of treatment group}/\text{Average tumor weight of control group}] \times 100\%$$

Table 1
Two-step purification of ADI M13-3.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery ratio (%)
Crude enzyme	17,383.46	3596.98	4.83	–	–
Hiprep DEAE FF	10,603.92	1203.62	8.81	1.82	61
Superdex 200	7952.83	585.2	13.59	2.81	45.75

Results

Purification and characterization of ADI M13-3

Using an improved ADI variant M314$^{A128T/H404R/I410L}$ as parent [3], a superior ADI mutant M13-3$^{D38H/A128T/E296K/H404R/I410L/Q162S}$ was obtained through another round of random mutagenesis and combination with semi-rational design. M13-3 exhibited a specific activity of 13.59 U/mg at pH 7.4 and a pH optimum of 6.5 (unpublished data), and was used in this study. In a 3-L bioreactor, the activity of ADI M13-3 reached the highest level of 17.38 U/mL broth after 14 h of fermentation. After two-step purification, 585.2 mg purified ADI with specific activity of 13.59 U/mg was obtained from 1 L fermentation broth. ADI protein was purified for 2.81-fold with a recovery ratio of 45.75% (Table 1).

As shown in Fig. 1, the molecular weight of purified M13-3 was determined by Superdex 200 gel filtration and SDS-PAGE. The results showed that the M13-3 tetramer has a subunit of approximately 46 kDa (Fig. 1B), and a molecular weight of around 188 kDa (Fig. 1A) which is 2-fold of its dimeric WT-PpADI (Fig. 1C) [4]. Previous studies indicate that both substitutions D38H and E296K affected the formation of PpADI dimer or tetramer, as well as its population distribution. It is speculated that the substitution E296K promotes the tetramer formation, probably by introducing an electrostatic stabilizing interaction with the residue Glu38 instead of His38 [26]. In this study, M13-3 consists predominantly of tetramers compared to WT-PpADI (Fig. 1C), demonstrating a more complicated effect of various substitutions including D38H and E296K, etc.

Purified ADI M13-3 from gel filtration chromatography has a purity of approximately 95% (by HPLC). The pI value of ADI M13-3 was determined to be 5.25 by IEF, close to its theoretical pI 5.51 (ExPASy Proteomics Server, ProtParam).

PEGylation of ADI M13-3

A series of PEG reagents with various succinimidyl ester groups were used in our study, including mPEG20-SS, mPEG40-SS, mPEG20-SC, mPEG20-SPA, mPEG20-SCM, mPEG20-NHS, and mPEG40-NHS. These PEGs could formulate amide bonds with lysine residues on protein surface. Studies have shown that PEGs of 20 kDa usually have a larger distribution volume with a low clearance compared with smaller PEGs such as 5 kDa. Holtsberg and co-workers reported that 20 kDa derivatives exhibited longer pharmacokinetics than those of 5 kDa ones [5,22]. Currently, the largest PEG size approved for PEG-conjugated drugs in human therapy (i.e., Pegasys®) is 40 kDa [17]. In this study, both 20 kDa PEGs and 40 kDa PEGs were tested.

Primary structure analysis and three-dimensional structure prediction by Pymol (DeLano Scientific LLC, CA, USA) indicate that there are 19 lysine residues on the surface of one ADI M13-3 subunit. Theoretically, each lysine residue could be covalently attached to a PEG molecule by amide bond formed under alkaline condition (pH > 8). Based on the number of lysine residues and pI value (5.25), the reaction condition was optimized for a better PEGylation yield. Results of different PEGs formulated ADI are shown in Table 2. Similar types

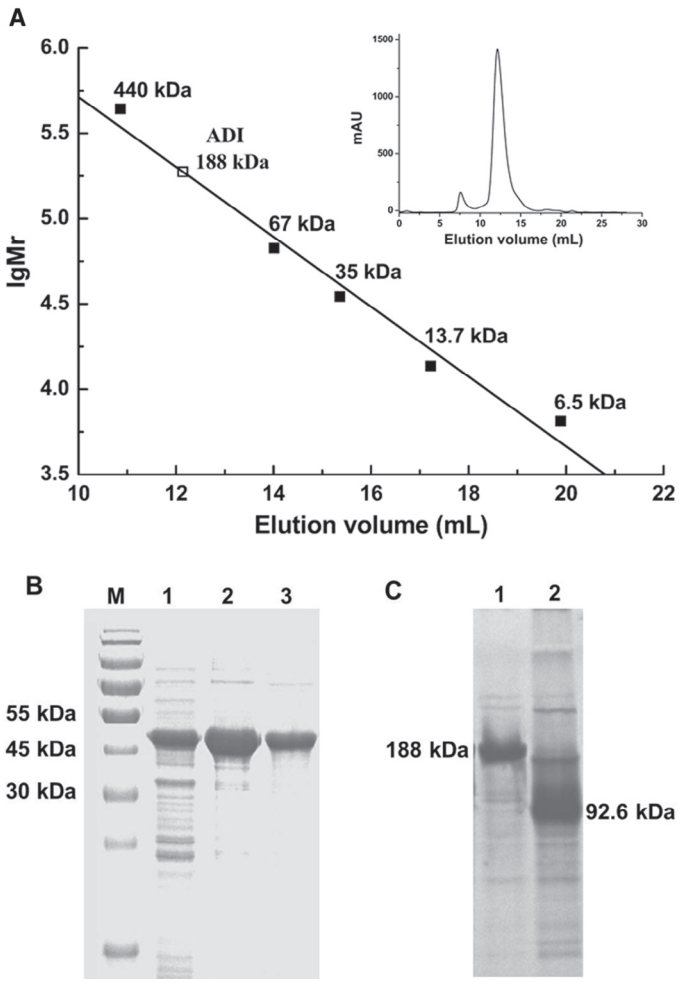

Fig. 1. Purification and molecular weight of ADI M13-3. (A) Native molecular weight of ADI on Superdex 200 column. Protein elution profile by OD_{280} is shown in the *inset*. Standard curve on Superdex 200 was measured using five standard proteins as general. (B) SDS-PAGE (12%) analysis of ADI M13-3 purification. *Lanes*: M, protein marker; lane 1, crude extract of M13-3; lane 2, eluted fraction from Hiprep DEAE FF; lane 3, purified enzyme from Superdex 200. (C) Native-PAGE analysis of ADI M13-3 and WT-PpADI. *Lanes*: lane 1, M13-3; lane 2, WT-PpADI (92.6 kDa).

of PEGs with different sizes, mPEG-SS and $mPEG_2$-NHS of 20 or 40 kDa, did not result in ADI-PEGs with distinct specific activities, but affected the yield significantly. Our results suggest that 20 kDa PEGs give a relatively higher yield compared with 40 kDa PEGs. Moreover, ADI conjugates prepared using linear or branched 20 kDa PEGs showed similar specific activities. It is presumed that the

Table 2
Comparison of ADI modification using various PEGs.

PEG linker	PEG mass (kDa)	Specific activity (U/mg)	Yield[a] (%)
Succinimidyl succinate (SS)	20	6.96 ± 0.34	100
	40	7.32 ± 0.49	45
Succinimidyl propionate (SPA)	20	7.53 ± 0.38	100
Succinimidyl carbonate (SC)	20	6.15 ± 0.35	100
Succinimidyl carboxymethylate (SCM)	20	6.38 ± 0.31	70
N-Hydroxysuccinimide[b] (NHS)	20	6.82 ± 0.36	40
	40	7.17 ± 0.62	10

ADI-PEGs were prepared with seven PEG reagents.
[a] Estimated by scanning densitometry of SDS-PAGE using Quantity One.
[b] $mPEG_2$-NHS is a branched PEG, differing from other linear PEGs used in this study.

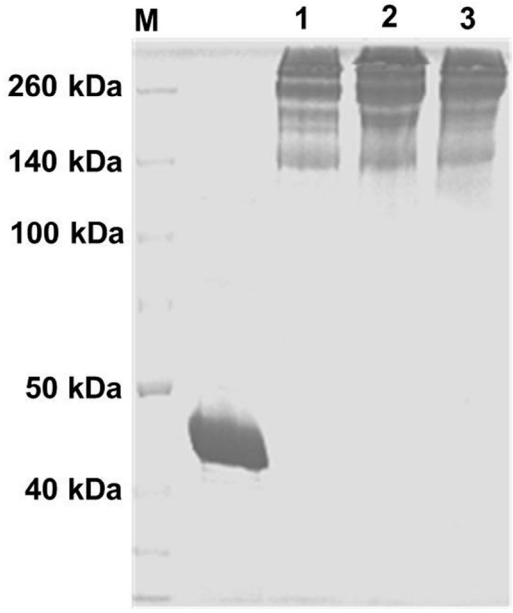

Fig. 2. SDS-PAGE (8%) analysis of PEGylation of ADI with various 20 kDa mPEG. *Lanes*: lane 1, purified ADI; lane 2, ADI-$SS_{20\ kDa}$; lane 3, ADI-$SC_{20\ kDa}$; lane 4, ADI-$SPA_{20\ kDa}$.

increased steric hindrance of larger or branched PEGs could prevent its subsequent covalent attachment to the lysine residues and result in lower yields.

Three ADI-PEG conjugates with 100% yield, ADI-$SS_{20\ kDa}$, ADI-$SC_{20\ kDa}$ and ADI-$SPA_{20\ kDa}$, were further characterized for their enzymatic properties. SDS-PAGE results show that all three PEGylated ADIs exhibited the most abundant band at 260 kDa and above (Fig. 2). It is conjectured that each ADI subunit is covalently attached with at least 10 PEG molecules. Although the PEGylation process is a random modification of lysine residues on the protein's surface, different batches of PEGylation reactions actually resulted in similar average modification ratios of around 50% and relative activity of 45 – 55% compared with free enzyme (13.59 U/mg) (Table 3). Similar results have been reported in other PEGylated proteins, for example PEG–Superoxide dismutase [27]. And different batches of PEGylation showed similar activities and modification ratios of ADI-PEGs.

The effect of pH on the enzymatic activity of PEGylated ADI M13-3 was studied (Fig. 3A). The highest activity was observed at pH 6.5 for both ADI-PEGs and free ADI, while about 70% of activities were retained at pH 7.0. Both free and PEG-conjugated ADI showed a good stability under pHs ranging from 5.5 to 8.5, while less than 10% activity was maintained at pH 5.0 and below (Fig. 3B). Maximum activity was observed at 35 °C (near physiological temperature) for both free ADI and its PEGylated form (Fig. 3C). For free ADI, 90% activity was retained after incubation at 40 °C for 2 h, whereas the activity dropped rapidly as temperature increased. However, PEGylated ADI preserved nearly full activity after a 2 h incubation at 40 °C (Fig. 3D), suggesting PEGylation could improve the thermostability of ADI. Moreover, stability studies in mice plasma also indicate that PEGylated ADI is more stable than free ADI. Merely 10% activity of ADI M13-3 was preserved in mouse plasma after 5 days, while ADI-PEGs showed over 30% residual activities on the 7th day (Fig. 3E).

Table 3
Activity and modification ratio of ADI bioconjugates.

Bioconjugates	Relative activity (%)[a]	Average modification ratio (%)[b]	Molecular weight of subunit (kDa)[c]
ADI-$SS_{20\ kDa}$	51.21 ± 2.50	52.23 ± 8.32	>260
ADI-$SC_{20\ kDa}$	45.25 ± 2.57	57.30 ± 9.05	>260
ADI-$SPA_{20\ kDa}$	55.41 ± 2.80	53.69 ± 7.64	>260

[a] Relative activity = (specific activity of ADI-PEG/specific activity of unmodified ADI) × 100%.
[b] Measured by TNBS method. Each ADI subunit has 19 lysine residues available for PEG conjugation. The modification ratio is average of random modification ratio.
[c] From the results of SDS-PAGE in Fig. 2.

Pharmacodynamics of ADI-$SPA_{20\ kDa}$

For further pharmacological evaluation of ADI-PEGs, ADI-$SPA_{20\ kDa}$ was selected, using free ADI M13-3 as control. First, the pharmacodynamics of ADI-$SPA_{20\ kDa}$ was investigated by measuring the arginine and citrulline levels at various time points following different injection routes and dosages. KM mice were grouped and injected with single i.v., i.m., or s.c. injections of 0.2, 1, 5 U/mouse of ADI-$SPA_{20\ kDa}$ or ADI. As shown in Fig. 4A, single i.v. administration of 1 and 5 U ADI per mouse could decrease the serum arginine to an undetectable level (0.2 μM) within 1 h, and it returned to a normal level after 24 h while 0.2 U/mouse of ADI injection gave an undesirable effect. In comparison, ADI-$SPA_{20\ kDa}$ injection showed more sustained effects on serum arginine depletion. As shown in Fig. 4B, i.v. administration of 1 and 5 U of ADI-$SPA_{20\ kDa}$ led to a decrease in serum arginine to undetectable levels for 4 days, and recovering to normal level after 7 days. Even the lowest dose of 0.2 U ADI-$SPA_{20\ kDa}$ could achieve a similar effect for 2 days, which is much more efficacious and lasting than a free ADI injection. ADI-$SPA_{20\ kDa}$ dose of 5 U via i.m. injection showed similar effects as the i.v. injection, depleting serum arginine to undetectable levels for 4 days (Fig. 4D). S.c. administration is not a quick-acting route, and usually causes a delay in drug action. Compared with those of i.v. and i.m., ADI administration via s.c. was a little less effective, depleting serum arginine to undetectable levels for 2 days at 5 U dosage (Fig. 4F).

Since ADI catalyzes the conversion of arginine into citrulline, serum citrulline level increases as arginine decreases. In our pharmacodynamics studies, serum citrulline from i.v. injection was also measured to evaluate the efficacy of ADI-$SPA_{20\ kDa}$. As shown in Fig. 5, serum citrulline levels increased along with the decrease of arginine (Fig. 4A and B) after a single i.v. injection of native ADI and ADI-$SPA_{20\ kDa}$, and then returned to its physiological level after 2–6 days.

Pharmacokinetics of ADI-$SPA_{20\ kDa}$

PK analysis plays a guiding role in drug design, drug evaluation and dose schedule optimization. Pharmacokinetic properties of ADI-$SPA_{20\ kDa}$ were investigated for further drug evaluation. Blood drug concentrations were measured by enzymatic activity assays of ADI and ADI-$SPA_{20\ kDa}$. As shown in Fig. 6, PEGylated ADI derivatives maintained detectable enzyme activities for a longer time as compared with native ADI in mice serum following single injections. The enzymatic activity of i.v. and i.m. injection decreased rapidly to nearly undetectable levels after 1 day (Fig. 6A, C). Whereas the ADI activity decreased much slower with s.c. injection (Fig. 6E), dropping to the bottom on day 2. As expected, ADI-$SPA_{20\ kDa}$ injection revealed a significantly prolonged time period of enzyme reaction. Apparently, i.v. injection manifested the fastest blood drug diminishing rate compared with other two routes. Regardless of different injection routes, ADI activities could be detected until day 6 following single doses of 5 U/mouse ADI-$SPA_{20\ kDa}$, which is much longer than that of native ADI. Similar to pharmacodynamic studies, there is also a dose-dependent manner in pharmacokinetic profiles.

The pharmacokinetic properties of ADI-$SPA_{20\ kDa}$ and native ADI at 5 U/mouse via 3 injection routes are summarized (Table 4). The half-life of ADI was significantly prolonged after PEG modification. In i.v. administration, ADI-$SPA_{20\ kDa}$ had a circulating half-life

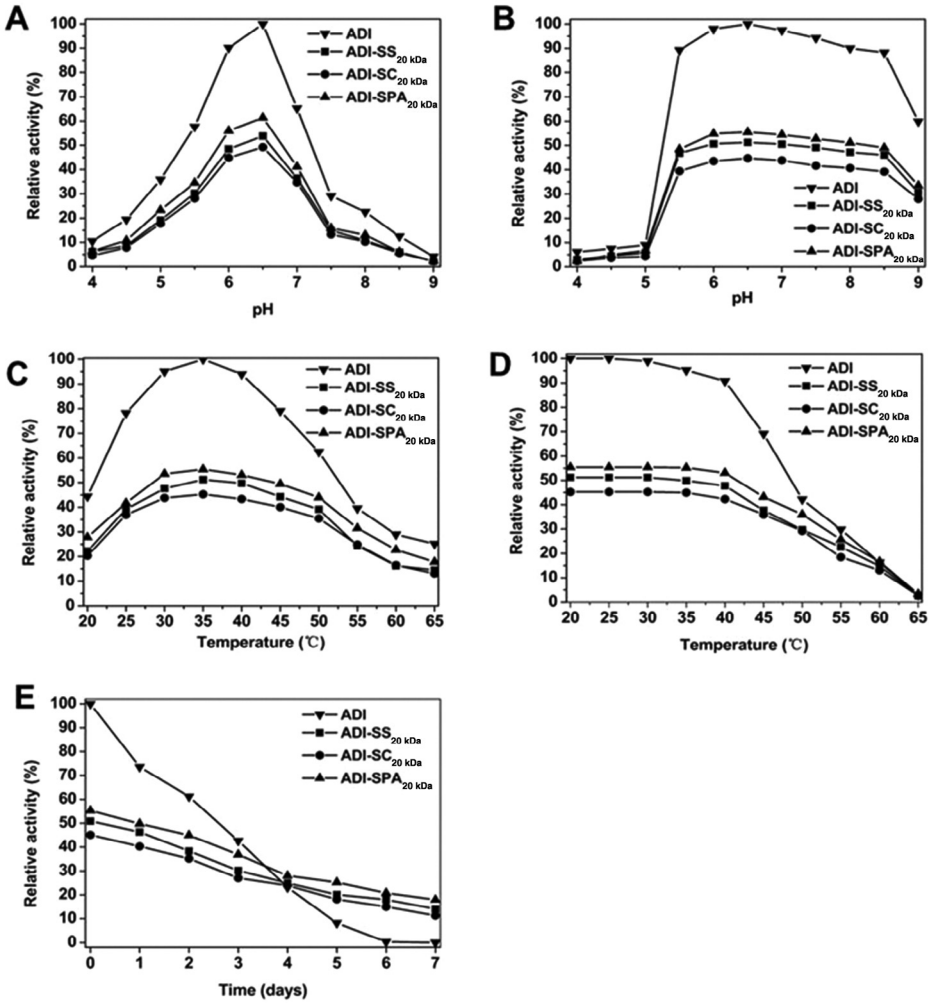

Fig. 3. The (A) pH optimum, (B) pH stability, (C) temperature optimum, (D) thermostability, and (E) plasma stability of ADI and various PEGylated ADIs. (▼) ADI; (■) ADI-$SS_{20\ kDa}$; (●) ADI-$SC_{20\ kDa}$; (▲) ADI-$SPA_{20\ kDa}$.

of 53.2 h, representing 11-fold that of unmodified ADI. And results of C_{max} and AUC demonstrate that ADI-$SPA_{20\ kDa}$ renders a much stronger response than unmodified ADI. In addition, mean retention time (MRT) and clearance rate (CL) also indicate that ADI-$SPA_{20\ kDa}$ had a longer acting time. Concerning different injection routes, i.v. administration presented better pharmacokinetic properties than i.m. and s.c. injections. In the above PD/PK studies, the results of PD/PK profiles with a single-does course can be a guide for further multiple-dose analysis.

Anti-tumor activity of ADI-$SPA_{20\ kDa}$ on mice H_{22} model

In our previous study, ADI originated from *P. plecoglossicida* has shown strong inhibitory effect on HCC tumor cells both *in vitro* and *in vivo* [18]. To further evaluate the efficacy of PEGylated ADI *in vivo*, mice H_{22} hepatocarcinoma models were established. Based on the results of PD/PK analysis, treatments on the H_{22} tumor model were divided into six groups (Table 5). During a 15-day course, ADI and 5-FU were injected daily while ADI-$SPA_{20\ kDa}$ of 3 different dosages (totally 0.6 U, 3 U and 15 U) was administered every 5 days.

Here, the average inhibition rate of ADI and ADI-$SPA_{20\ kDa}$ on H_{22} tumor was measured for drug evaluation. After 15-day treatment, H_{22} tumors were separated from mice and 6 tumors were selected in each group (except one death in control group) (Fig. 7). Based on average tumor weight, inhibition rates of ADI and ADI-$SPA_{20\ kDa}$ on H_{22} tumor were calculated (Fig. 8). Both ADI-$SPA_{20\ kDa}$ and free ADI showed evident inhibitory effect on H_{22} tumor growth. Different dosages of ADI-$SPA_{20\ kDa}$ showed a distinct effect on H_{22} tumor. High-dosage course of 5 U/5 days injected via i.m. exhibited better efficacy on tumor growth with an inhibition rate of 95.02%, which is similar to that of the 5–FU chemotherapy group (98.34%). In contrast, a daily ADI injection of 1 U (15 U) only exhibited 87.19% inhibition rate.

Discussion

PEG conjugation of protein prolongs the circulation time of protein drugs *in vivo* by protecting against enzymatic digestion, slowing down kidney clearance, and reducing the generation of neutralizing antibodies [6]. PEG conjugated drugs have been established as an effective strategy to overcome the application limitations of protein drugs. In this study, the PEGylation and pharmacological properties of a PpADI mutant M13-3 originated from *P. plecoglossicida* were investigated.

Three ADI-PEG bioconjugates, ADI-$SS_{20\ kDa}$, ADI-$SC_{20\ kDa}$ and ADI-$SPA_{20\ kDa}$, exhibited higher enzyme activity and yield. Stability analysis results confirm that the catalytic property of ADI was well preserved in its corresponding bioconjugates. This may be attributed to the shielding effect of PEGylation on ADI, which helps

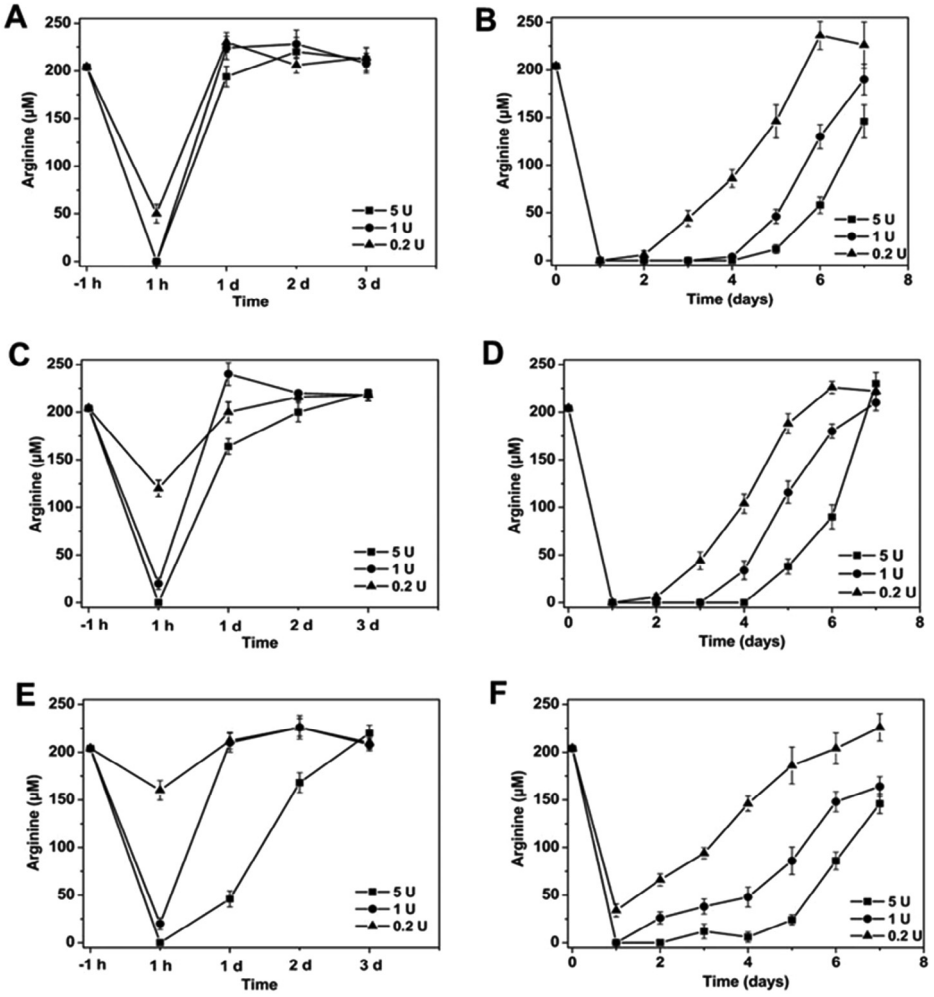

Fig. 4. Pharmacodynamics of ADI and ADI-$SPA_{20\ kDa}$ in KM mice following single i.v. (A, B), and i.m. (C, D), and s.c. (E, F) administration. Data on ADI and ADI-$SPA_{20\ kDa}$ injections are shown in A, C, E, and B, D, F, respectively. Serum arginine levels were measured following injection of 5 U (■), 1 U (●), 0.2 U (▲) per mouse. Data are presented as means ± SD.

to protect certain accessible residues from proteolysis. The increased stability of ADI-PEGs is of great importance for their therapeutic applications, such as drug storage, delivery and activity retention.

ADI-$SPA_{20\ kDa}$ was selected for pharmacodynamic/pharmacokinetic studies. Based on a pharmacodynamics study, a remarkable improvement in circulating half-life and efficacy compared with native ADI via all three routes was observed. Our results indicate that i.v. administration with a 5 U ADI-$SPA_{20\ kDa}$ provided the most lasting efficacy in arginine degrading among the 3 injection routes in mice, which is related to the biodistribution and metabolism of different injection routes. PK analysis demonstrated that single ADI-$SPA_{20\ kDa}$ administration of 5 U/mouse via i.v. injection could maintain serum arginine at an undetectable level for 5 days with

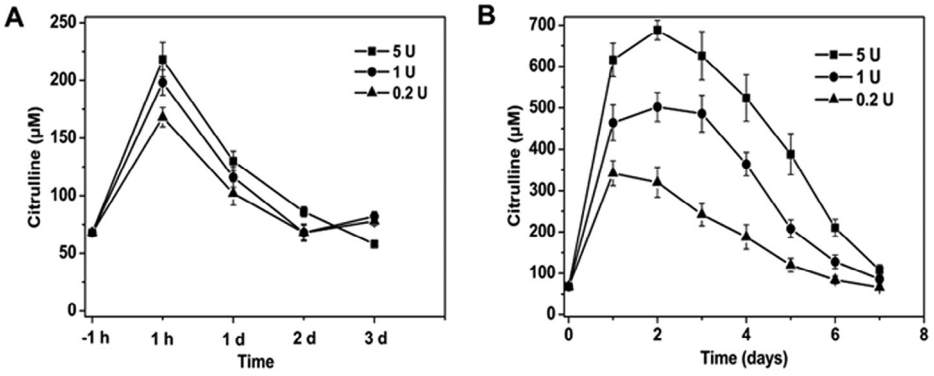

Fig. 5. Pharmacodynamics of ADI (A) and ADI-$SPA_{20\ kDa}$ (B) in KM mice following single i.v. administration. Serum citrulline levels were measured following injection of 5 U (■), 1 U (●), 0.2 U (▲) per mouse. Data are presented as means ± SD.

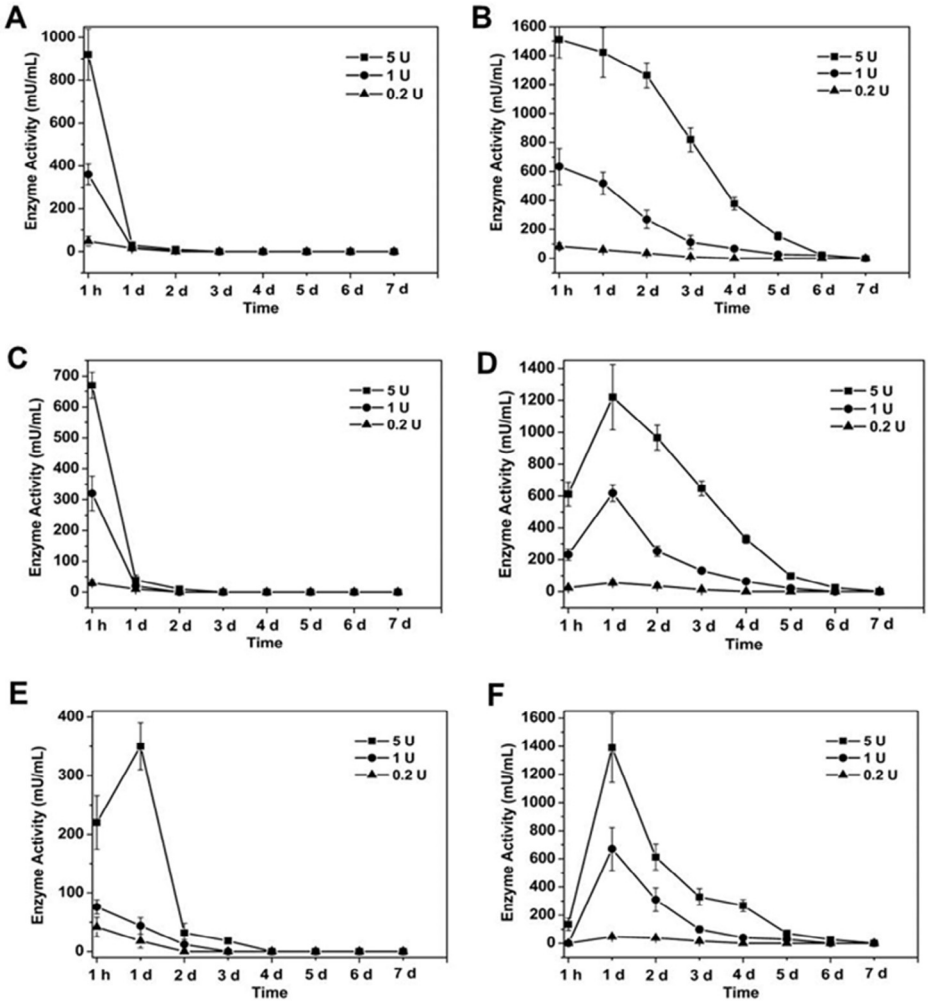

Fig. 6. Pharmacokinetics of ADI and ADI-SPA$_{20\ kDa}$ in KM mice following single i.v. (A, B), i.m. (C, D), and s.c. (E, F) administration. Data on ADI and ADI-SPA$_{20\ kDa}$ injections are shown in A, C, E, and B, D, F, respectively. Residual enzyme activities in serum were measured following injection of 5U (■), 1U (●), 0.2U (▲) per mouse. Data are presented as means ± SD.

a half-life of 53.2 h, representing 11-fold of that of native ADI. In clinical trials by Phoenix Pharmacologics, ADI-PEG-20 exhibited a half-life of 7 days compared with 5 h of native ADI [4]. Our results indicate that PEGylation is effective in improving the pharmacodynamic profiles of PpADI variant M13-3, and thus in increasing its therapeutic effects.

In *in vivo* evaluations in mice implanted with H22 tumors, ADI-SPA$_{20\ kDa}$ injection of 5 U dosage/5 days via i.m. showed an inhibition rate of 95.02% on tumor growth during 15-day treatments, which is similar to the chemotherapy group (98.34%). In contrast, ADI injection of 1 U dosage/1 day only showed 87.19% inhibition rate, indicating the same dosage of ADI-SPA$_{20\ kDa}$ (15 U) could inhibit the tumor growth with lasting effects. Due to the toxicity and strong side effects of chemotherapeutics, biological therapy using ADI bioconjugates could become an important strategy in future clinical trials.

Currently, we are carrying out toxicity and immunogenicity studies on ADI and ADI-SPA$_{20\ kDa}$, and some preclinical experiments in mice and rabbits. Additionally, the pharmacology profiles of ADI-PEGs will be further investigated under a multiple-dose course. As substitutes for ADI-PEG-20 in current clinical trials, the PEGylated PpADI in our study (ADI-SPA$_{20\ kDa}$, as well as ADI-SS$_{20\ kDa}$ and ADI-SC$_{20\ kDa}$) provides novel and promising therapeutic agents for arginine-auxotrophic cancers.

Table 4
Pharmacokinetic properties of ADI and ADI-SPA$_{20\ kDa}$ in mice at a dosage of 5 U/mouse.

	Route	HL (h)	C_{max} (U/mL)	T_{max} (h)	AUC $_{(0-t)}$ (h*U/mL)	MRT (h)	CL (mL/h)
ADI-SPA$_{20\ kDa}$	i.v.	53.2	1.724	–	113.545	76.8	0.037
ADI-SPA$_{20\ kDa}$	i.m.	34.5	1.220	24	85.555	49.9	0.057
ADI-SPA$_{20\ kDa}$	s.c.	26.7	1.311	24	68.543	38.6	0.072
ADI	i.v.	4.7	0.920	–	7.199	6.7	0.69
ADI	i.m.	5.7	0.712	0.4	6.136	8.3	0.81
ADI	s.c.	11.6	0.780	8	17.725	16.7	0.28

Table 5
Evaluation of ADI and ADI-SPA$_{20\ kDa}$ treatment on mice H22 model.

	Control	5-FU	ADI	ADI-SPA (0.6 U)	ADI-SPA (3 U)	ADI-SPA (15 U)
Drug	–	5-FU	ADI	ADI-SPA		
Route		i.p.	i.m.			
Dosage		0.05–0.1 mg	1 U	0.2 U	1 U	5 U
Course		Daily		Every 5 days		

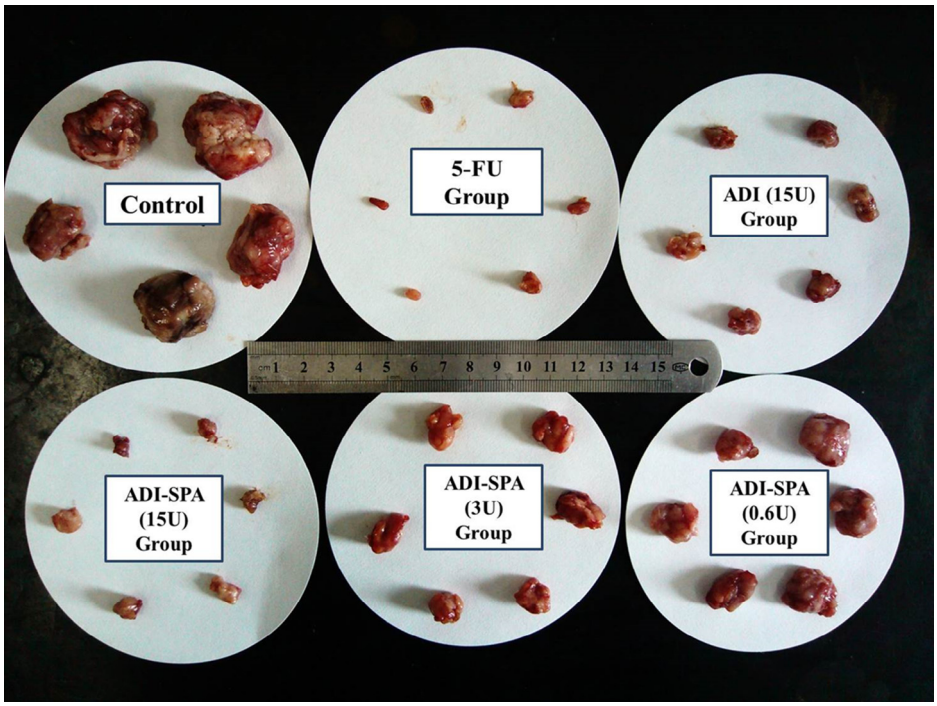

Fig. 7. Tumor sizes in H_{22} tumor-bearing mice treated with ADI and ADI-$SPA_{20\ kDa}$.

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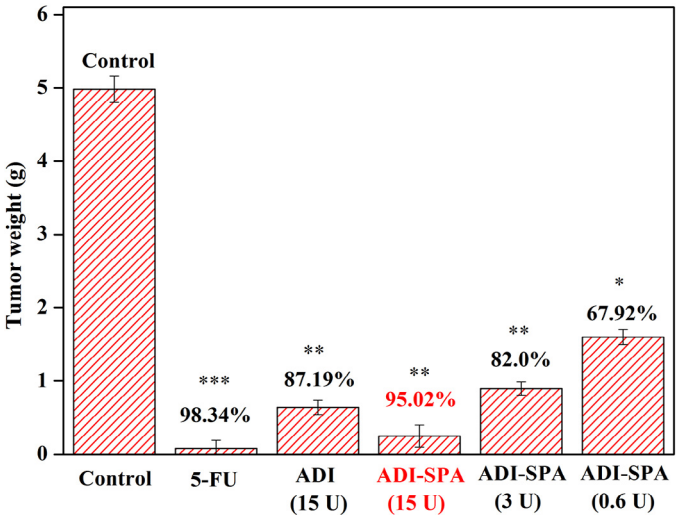

Fig. 8. Comparison of average tumor weight and inhibition rate in H_{22} tumor-bearing mice treated with ADI and ADI-$SPA_{20\ kDa}$. Inhibition rate (in percentage) and statistical significance *p*-value are shown above each column. The significant difference of tumor weight compared with that of control group was evaluated by independent t-test analysis. ***: $p < 0.001$; **: $p < 0.01$; *: $0.01 < p < 0.05$.

Conflict of interest

I hereby state that potential conflicts do not exist.

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