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MINI-REVIEW

Arginine deiminase: recent advances in discovery, crystal structure, and protein engineering for improved properties as an anti-tumor drug

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Abstract Arginine deiminase (ADI) is an important arginine-degrading enzyme with wide applications, in particular as an anti-cancer agent for the therapy of arginine-auxotrophic tumors. In recent years, novel ADIs with excellent properties have been identified from various organisms, and crystal structures of ADI were investigated. To satisfy the requirements of potential therapeutic applications, protein engineering has been performed to improve the activity and properties of ADIs. In this mini-review, we systematically summarized the latest progress on identification and crystal structure of ADIs, and protein engineering strategies for improved enzymatic properties, such as pH optimum, K_m and k_{cat} values, and thermostability. We also outlined the PEGylation of ADI for improved circulating half-life and immunogenicity, as well as their performance in clinical trials. Finally, perspectives on extracellular secretion and property improvement of ADI were discussed.

Keywords Arginine deiminase (ADI) · Protein engineering · Directed evolution · PEGylation · Anti-tumor

Introduction

Arginine deiminase (ADI; EC 3.5.3.6), an arginine-degrading enzyme, catalyzes the hydrolyzation of arginine to citrulline and ammonium by deamination of guanidino group (Shirai et al. 2001). Generally, the arginine hydrolysis by ADI is considered to be the first step of the ADI system, which comprises two additional reactions: converting citrulline into ornithine and carbamoyl phosphate catalyzed by ornithine transcarbamylase (OTC) and degrading carbamoyl phosphate to ammonia and CO_2 by carbamate kinase (CK) (Zúñiga et al. 2002) (see in Fig. 1). The ADI system provides a major energy source for many microorganisms since it generates ATP by consuming equimolar arginine (Zúñiga et al. 2002).

Arginine, one of nonessential amino acid in humans, is synthesized from citrulline by the catalysis of argininosuccinate synthetase 1 (ASS1) and argininosuccinate lyase (ASL) (Caldara et al. 2008). ASS1 is usually regarded as a house-keeping gene in normal cells and a rate-limiting enzyme for the biosynthesis of arginine in hepatocytes and endothelial cells. However, many tumors, such as hepatocellular carcinomas (HCCs) and melanomas, lack ASS1 expression and thereby are auxotrophic for arginine (Dillon et al. 2004; Szlosarek et al. 2007). Therefore, the ASS1-deficient tumors strictly depend on exogenous arginine, and ASS1 deficiency is considered to be both a prognostic biomarker and predictor of sensitivity to arginine deprivation therapy (Delage et al. 2010). ASL, immediately downstream of ASS1, catalyzes the conversion of argininosuccinate into arginine and fumarate. The methylation of ASL often leads to the arginine auxotrophy in glioblastoma multiforme (Syed et al. 2013). Numerous studies have confirmed that arginine depletion by ADI is effective in many ASS1-deficient tumors (Hernandez et al. 2010; Kelly et al. 2012). Therefore, ADI is generally regarded as one potential cancer therapy agent for the treatment of arginine-auxotrophic tumors, and has attracted increasing interests in the past few decades.

In our previous review (Ni et al. 2008), we have focused on the anti-tumor activity, cloning and expression, structure

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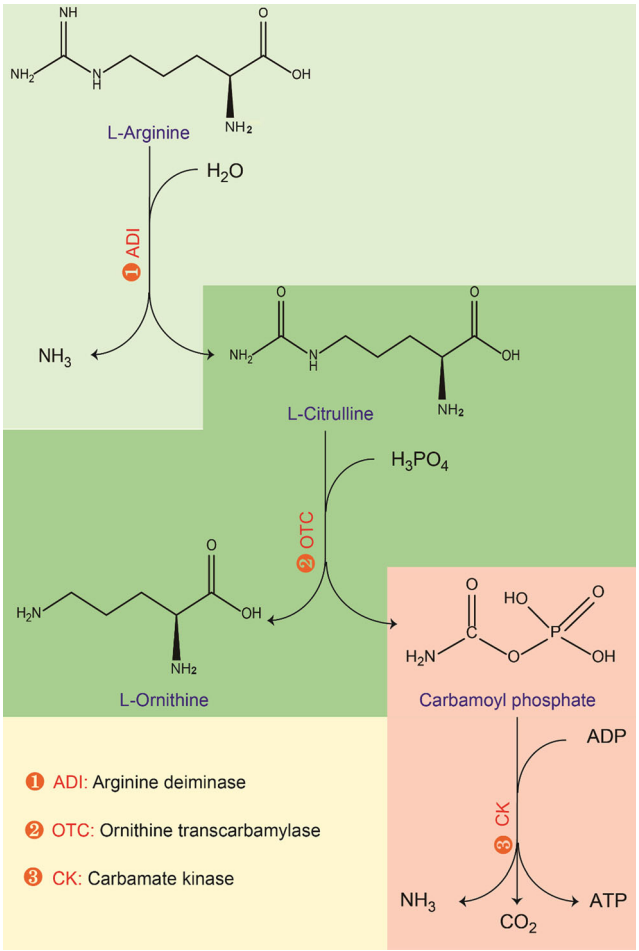

Fig. 1 Scheme of ADI pathway

analysis and clinical applications of ADI, as well as the importance of heterogeneous expression and protein engineering on ADI to overcome insufficient properties. In this mini-review, we summarized the latest progress on ADIs from novel sources, their crystal structures, protein engineering approaches, and PEGylated modifications for improved properties in pharmaceutical applications. The prospects for ADI-related investigations were also discussed.

Recent discoveries of ADI

Since ADI was first discovered from *Bacillus pyocyaneus* by Horn (1933), more and more ADI source organisms have been reported, such as *Pseudomonas putida*, *Halobacterium salinarium*, *Mycoplasma arginini*, *Mycoplasma hominis*, *Pseudomonas aeruginosa*, *Lactococcus lactis* ssp. *Lactis*, and *Pseudomonas plecoglossicida*. The characterizations of these ADIs have also been discussed (Ni et al. 2008). In recent years, various organisms capable of producing ADIs have been isolated, which possess excellent properties such as high inhibiton rate, good pH tolerance, and thermostability for clinic therapy application. For instance, an ADI producing strain, *P. plecoglossicida* CGMCC2039, has been isolated from Wuxi canal. ADI from *P. plecoglossicida* (PpADI) showed excellent inhibitory effect to human HCC cell line HEPG2, and the inhibiton rate was as high as 93.4 % with 0.5 U/ml of ADI (Liu et al. 2008). Amer and the coworkers isolated three novel *lactobacilli probiotic* strains from human infant feces, which were identified as *Lactobacillus gasseri* NM112 harboring the *ansA* gene (encoding L-asparagiase), *Lactobacillus fermentum* NM112, and *Lactobacillus casei* NM312 harboring the *acr*A gene (encoding ADI), respectively. These stains were sugested to be probiotics with potential therapeutic effect against cancer due to their good tolerance to low pH (pH 1.5), 0.3 % bile salts, and moderate tolerance to pancreatic enzymes as well as antagonistic action (Amer et al. 2013). ADI from group A *Streptococcus* (GAS, *Streptococcus pyogenes*) was regarded as a GAS vaccine candidate due to its location on the cell surface and production of opsonic antibodies capable of protecting mice against lethal challenges from GAS strains (Henningham et al. 2012). A novel thermostable ADI with relatively lower antigenicity has been purified from thermophilic *Aspergillus fumigatus* KJ434941, exhibiting great potential in clinical trials (El-Sayed et al. 2015).

Furthermore, some pathogenic organisms harboring ADI system have been discovered recently to elucidate infection pathogenesis. For instance, Ryan and coworkers revealed the pathogenesis of *Listeria monocytogenes* infection in the murine model, in which ADI system plays an important role in acid tolerance and conduce to the survival and growth of *L. monocytogenes* under acidic conditions (Ryan et al. 2009). Hitzmann and coworkers identified a surface-exposed localization of the ADI system enzymes from *Streptococcus canis* by genetic composition and in silico analysis, and elucidated the contribution of ADI system to the development of *S. canis* infection pathogenesis (Hitzmann et al. 2013). Similarly, Caparon's group recently demonstrated that the ADI pathway in *S. pyogenes* contributes to pathogenesis in murine models by modulation of innate immunity through depletion of arginine (Cusumano et al. 2014). Furthermore, they confirmed that arginine and citrulline catabolism have distinct contributions to virulence during an infection. Especially, a concerted action between citrulline catabolism and F_1F_O-ATPase could help to protect bacteria against the low-pH environment, which contributes to the pathogenesis of bacterial infection (Cusumano and Caparon 2015).

Crystal structure of ADI

To reveal the catalytic mechanism of ADI and provide molecular basis for protein engineering, crystal structures

of ADI from different sources are investigated and analyzed. The first crystallization of ADI was performed using multiple-step chromatography method (Kakimoto et al. 1971), and the crystallization procedure was then optimized (Shibatani et al. 1975). So far, nine crystal structures of ADI have been reported. As shown in Table 1, five crystal structures (tetrameric subunits) are from *P. aeruginosa*, two (homodimeric subunits) are from *M. arginini*, one (homodimeric subunits) is from *Mycoplasma penetrans*, and one (homodimeric subunits) is from GAS. Galkin et al. (2004) determined the crystal structure of ADI of *P. aeruginosa* with a 2.45-Å resolution (PDB ID: 1RXX) by multi-wavelength anomalous diffraction. The crystal structure reveals the main domain models of ADI and its probable catalytic mechanism. Subsequently, the catalytic mechanism was further confirmed by the crystal structure of four mutant ADIs C406A, H278A, D280A, and D166A (Galkin et al. 2005). Das and coworkers also determined the crystal structure of ADI from *M. arginini* in two different forms (1.6 and 2.0 Å, PDB ID: 1S9R and 1LXY) by multiple isomorphous replacement methods (Das et al. 2004). Commonly, ADI is formed by two or four subunits, which constitute a homodimeric or tetrameric structure (Ni et al. 2008). The single subunit is highly similar among ADIs from different microorganisms. As shown in Fig. 2, using ADI from *P. aeruginosa* (PDB: 2A9G) as an example, one core domain comprises five ββαβ modules with an additional α-helical inserted between the first and second ββαβ modules. A typical catalytic triad (Cys-His-Glu/Asp), around the substrate (such as L-arginine), is conserved in all ADIs as well as other arginine-degrading enzymes, such as N^{ω}, N^{ω}-dimethylarginine hydrolase (DDAH) (Murray-Rust et al. 2001)and human peptidylarginine deiminase (PAD4) (Arita et al. 2004).

ADI pathway has been proved to contribute to pathogenesis of *S. pyogenes* due to its protection against acidic stress during infection (Cusumano and Caparon 2015). Therefore, understanding the metabolic mechanism of ADI system is extremely important for preventing bacterial infection. Furthermore, ADI system in *M. penetrans* is considered to be a major energy source in anaerobic conditions. Recently, the crystal structure of three ADI system enzymes from *M. penetrans*, ADI, OTC, and CK, were presented to explain the metabolic mechanism of the ADI pathway (Gallego et al. 2012). The crystal structure (2.3 Å) of ADI from *M. arginini* was resolved in its apo-form, which showed an "open" conformation of the active site in comparison with previous "closed" conformation in a covalent complex with two L-arginine substrate intermediates (Das et al. 2004; PDB: 1LXY and 1S9R). The occurrence of "open" and "closed" conformations allows the entrance of substrate and release of product, which accords with the induced-fit mechanism. Additionally, similar substrate induced-fit mechanism was also recognized in *P. aeruginosa* ADI (Galkin et al. 2004; Galkin et al. 2005). Understanding ADIs' crystal structures and their catalytic mechanism will be useful for protein engineering of ADI (such as catalytic kinetics, activity and stability under physiological conditions) for further clinical applications. Administration of a GAS vaccine preparation involves the wild-type ADI from GAS. However, its inherent enzymatic activity may present a safety risk. To improve the vaccine safety, the crystal structure of GAS ADI (2.48 Å) and the structure immunogenic epitope mapping was used in vaccine design. Based on its crystal structure, several amino acid residues (such as D166, E220, H277, and C401) were selected for alanine scanning mutagenesis. These mutants abolished ADI activity while retained their three-dimensional structure for recognition by antisera and immunogenic epitopes. Two inactivated D166A and

Table 1 Crystal structures of ADIs from different sources

Organism source	PDB ID	Subunits	Resolution (Å)	Properties	Citation
P. aeruginosa	1RXX	Tetrameric	2.45	ADI	Galkin et al. 2004
	2A9G	Tetrameric	2.30	ADI mutant C406A in complex with L-arginine	Galkin et al. 2005
	2AAF	Tetrameric	2.30	ADI mutant H278A in complex with L-arginine	Galkin et al. 2005
	2ABR	Tetrameric	2.90	ADI mutant D280A in complex with L-arginine	Galkin et al. 2005
	2ACI	Tetrameric	2.50	ADI mutant D166A	Galkin et al. 2005
M. arginini	1LXY	Homodimeric	2.00	ADI in complex with L-citrulline	Das et al. 2004
	1S9R	Homodimeric	1.60	ADI in complex with a reaction intermediate	Das et al. 2004
M. penetrans	4E4J	Homodimeric	2.30	ADI	Gallego et al. 2012
GAS	4BOF	Homodimeric	2.48	ADI	Henningham et al. 2013

Fig. 2 The crystal structure of ADI from *P. aeruginosa* (PDB: 2A9G). (Cys406 is displayed in the crystal structure of ADI (PDB: 1RXX), with 2A9G superposition)

D277A ADI mutants have been finally selected in a GAS vaccine preparation (Henningham et al. 2013).

Protein engineering of ADI

Although various organisms could produce ADI, the enzyme yield and characterization are usually unsatisfactory. Since the first complete nucleotide sequence of ADI gene was cloned from *M. arginini* (Kondo et al. 1990), most studies have been focused on the heterologous expression and protein engineering of ADIs (especially for the PpADI) for improved properties (Ni et al. 2009; Zhu et al. 2010a, b; Ni et al. 2011; Zhu et al. 2014; Cheng et al. 2015a; Jamil et al. 2015). The detailed engineering methods and results are summarized in Table 2.

Intensive studies have been carried out on the protein engineering of *P. plecoglossicida* ADI (PpADI) by Schwaneberg's group and our laboratory (Fig. 3) (Ni et al. 2009; Ni et al. 2011; Zhu et al. 2010a, b; Zhu et al. 2014; Cheng et al. 2015a; Jamil et al. 2015). ADI encoding gene (*arcA*) from *P. plecoglossicida* CGMCC2039 was cloned and expressed in *Escherichia coli* (Liu et al. 2008; Ni et al. 2009). The recombinant PpADI showed effective inhibition on H22 tumor growth in mice. However, the recombinant PpADI had a specific activity of 4.76 U/mg at optimum pH 6.0 and lost approximately 90 % activity when pH shift to 7.4, which limits its application as an antitumor drug (the physiological pH of human plasma is 7.35 to 7.45). Additionally, its K_m value was 2.88 mM, which was more than 20 times of the serum arginine level (100–120 μM). Therefore, improving the properties of ADI is urgently necessary.

High-throughput screening approaches

Directed evolution is a common and versatile method applied in protein engineering, and appropriate High-throughput screening (HTS) methods are often considered to be one crucial factor. To obtain ADI mutants with excellent properties, a number of HTS methods have been investigated and established. For instance, based on the carbamido-diacetyl reaction method (Archibald 1944), Zhu et al. (2010a) developed and validated an adapted citrulline-screening protocol in microtiter-plate format for ADI directed evolution. This microtiter-plate-screening procedure was improved by simplifying the handling process, such as lowering the color-development temperature, increasing the diacetylooxime concentration and avoiding the use of the sensitizer thiosemicarbazide. To isolate ADI variants with high activity at low arginine concentrations, the HTS method was further modified by lowing the arginine concentrations (from 100 to 1 mM) and adjusting ADI reaction time (from 15 to 10 min) (Zhu et al. 2010b). Subsequently, to isolate ADI mutants with higher activity and lower K_m values under physiological pH, a rapid and precise two-step plate to plate screening procedure was developed, in which colonies on IPTG-agar plate instead of tedious liquid cultivation was directly used for activity assay (Ni et al. 2011). Recently, a more sensitive screening system based on ammonia detection was established in a 96-well microtiter plate to reliably detect ≥0.005 mM ammonia (Cheng et al. 2015a). Cheng and coworkers also developed a novel flow cytometry screening approach based on a competitive conversion/binding of arginine between ADI and arginine repressor at low substrate concentrations under physiological conditions (Cheng et al. 2015b).

Improving pH optimum

Low pH optimum of ADI is one major limitation factor for its clinical application, many efforts have been performed to improve the pH optimum. For example, one mutant M2 (K5T/D44E/H404R) with an optimum pH 7.0 was obtained by directed evolution, which was enhanced by 1.0 pH units compared with wild-type PpADI. Additionally, at the physiological pH 7.4, the mutant M2 retained about 50 % of its maximal activity (at pH 7.0), compared with about 10 % in the case of the WT PpADI (Zhu et al. 2010a). It has been reported that the mutagenesis at position 405 played an important role in modulating the pH optimum of ADI from *P. aeruginosa*. One mutant H405R exhibited enhanced pH optimum from 5.5 to 6.5. However, these mutants exhibited increased K_m values compared with the WT (Ding et al. 2012).

Table 2 Protein engineering of PpADI for improved enzymatic properties

Improvement	Method	Mutants	Properties	Citation
Activity K_m and k_{cat}	Heterologous expression	WT PpADI	Low specific activity (4.76 U/mg at pH 6.0), low optimum pH (6.0), 90 % activity loss at pH 7.4, and high K_m value (2.88 mM)	Ni et al. 2009
	Directed evolution with an adapted citrulline-screening protocol in microtiter plate	M1 (H404R)	1.8-fold of WT PpADI activity at pH 7.4; 4.7-fold of WT PpADI activity ratio (pH 7.4/pH 6.4)	Zhu et al. 2010a
		M2(K5T/D44E/H404R)	4-fold of WT PpADI activity at pH 7.4; 12.4-fold of WT PpADI activity ratio (pH 7.4/pH 6.4); 4-fold higher K_{cat} than that of PpADI	
	Directed evolution under physiological conditions and low arginine concentration	M3 (K5T/D38H/D44E/A128T)	K_m = 2.01 mM, k_{cat} = 10.13 s^{-1}, (pH 7.4);	Zhu et al. 2010b
		M4 (K5T/D38H/D44E/E296K/A128T)	K_m = 0.81 mM, k_{cat} = 5.88 s^{-1}, (pH 7.4);	
		M5 (K5T/D38H/D44E/A128T/H404R)	K_m = 1.48 mM, k_{cat} = 17.56 s^{-1}, (pH 7.4);	
		M6 (K5/D38H/D44E/A128T/E296K/H404R)	K_m = 0.81 mM, k_{cat} = 11.64 s^{-1}, (pH 7.4)	
	Directed evolution using a rapid and precise plate to plate procedure based on citrulline measurement	M314 (A128T/H404R/I410L)	Specific activity 9.02 U/mg at pH 7.4, K_m = 0.65 mM (pH 7.4)	Ni et al. 2011
	Semi-rational engineering	M13 (A128T/H404R/I410L/D38H/E296K)	Specific activity 15.23 U/mg, K_m = 0.52 mM, k_{cat} = 21.27 s^{-1} (pH 7.4), T_{50} = 52 °C, $t_{1/2}$ [50 °C] = 6.1 h, $t_{1/2}$ [60 °C] = 4 min	Jamil et al. 2015
		M13–2 (A128T/H404R/I410L/D38H/E296K/S45D)	Specific activity 21.19 U/mg, K_m = 0.59 mM, k_{cat} = 22.40 s^{-1} (pH 7.4);	
		M13–5 (A128T/H404R/I410L/D38H/E296K/R243L)	Specific activity 31.20 U/mg, K_m = 0.16 mM, k_{cat} = 30.25 s^{-1} (pH 7.4)	
	Sequence saturation mutagenesis	M19 (K30R/C37R/L148P/V291L)	K_m = 0.35 mM, k_{cat} = 21.10 s^{-1} (pH 7.4);	Cheng et al. 2015a
		M21 (K30R/C37R/L148P/V291L/G129S)	K_m = 0.33 mM, k_{cat} = 18.27 s^{-1} (pH 7.4)	
Thermostability and half-life	Directed evolution based on M6	M9 (K5T/D38H/D44E/A128T/V140L/E296K/F325L/H404R)	T_m value increased from 47 °C (M6) to 54 °C (M9), half-life increased from 2 days (M6) to 3.5 days (M9)	Zhu et al. 2014
	Semi-rational engineering	M13–9 (A128T/H404R/I410L/D38H/E296K/A276W)	Specific activity 18.68 U/mg, K_m = 0.62 mM, k_{cat} = 20.94 s^{-1} (pH 7.4), T_{50} = 78 °C, $t_{1/2}$ [50 °C] = 6.6 h, $t_{1/2}$ [60 °C] = 17.5 min.	Jamil et al. 2015

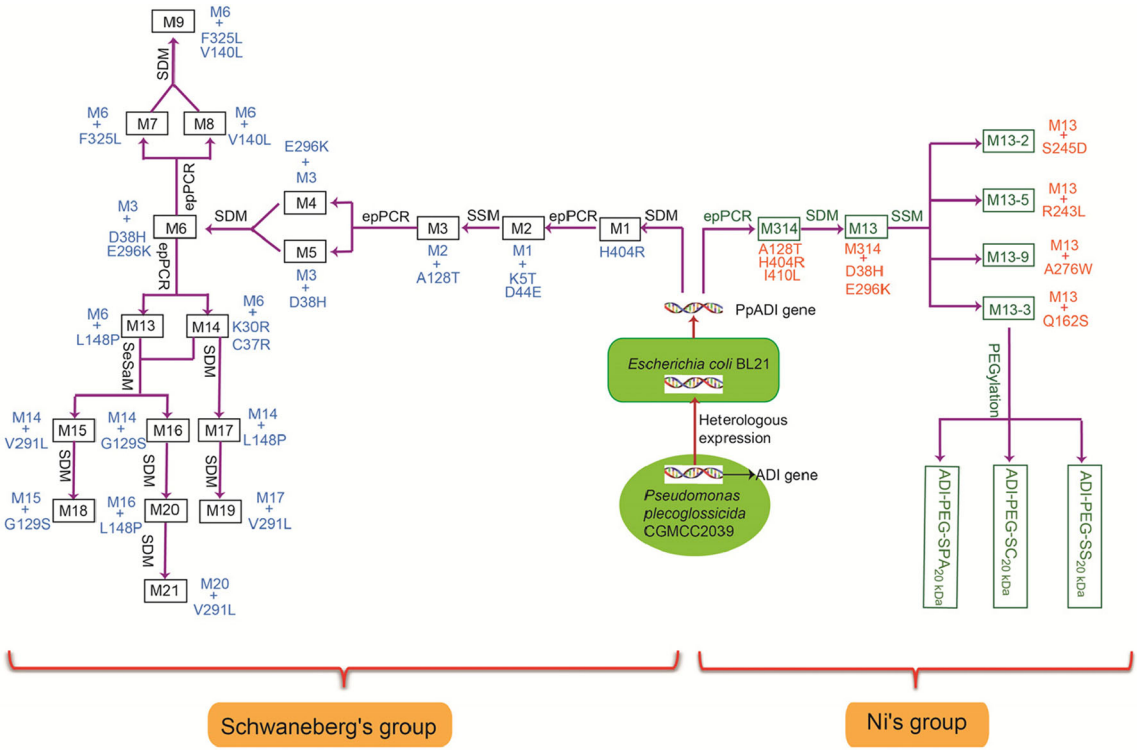

Fig. 3 Schematic presentation of heterologous expression and protein engineering of ADI from *P. plecoglossicida* CGMCC2039. (*epPCR* error prone PCR; *SDM* site-directed mutagenesis; *SSM* site-saturation mutagenesis; *SeSaM* sequence saturation mutagenesis)

Improving K_m and k_{cat} values

To isolate PpADI variants with low K_m value and high k_{cat} value, M6 (K5T/D38H/D44E/A128T/E296K/H404R) was obtained using improved HTS protocol, and its K_m value was decreased by 47.6 % while k_{cat} value was increased for 63.7-fold compared with the WT PpADI (Zhu et al. 2010b). In our previous study, one excellent mutant M314 (A128T/H404R/I410L) exhibited 20-fold increased specific activity and the decreased K_m value of 0.65 mM (at pH 7.4) (Ni et al. 2011). To further reduce the K_m value and improve the activity of PpADI, the mutant M19 (K30R/C37R/L148P/V291L) with higher k_{cat} value (21.1 s^{-1}; 106.5-fold of WT PpADI) and lower K_m value (0.35 mM) at physiological pH was obtained (Cheng et al. 2015a). Lately, one mutant M13-5 (D38H/A128T/E296K/H404R/I410L/R243L) with the lowest K_m value (0.16 mM) was identified by semi-rational engineering in our laboratory, and its specific activity was 31.2 U/mg at physiological conditions (Jamil et al. 2015).

Improving the thermostability

In addition to high activity and low K_m under physiological conditions, thermostability is an important prerequisite. ADI with high thermostability at 37 °C is favorable in medical application and capable of reducing dosage in each treatment. Using mutant PpADI M6 (K5T/D38H/D44E/A128T/E296K/H404R) as a template, one directed evolution based on citrulline detection system was employed to enhance its thermostability. One mutant M9 (K5T/D38H/D44E/A128T/V140L/E296K/F325L/H404R) was obtained, and its T_m value was increased from 47 (M6) to 54 °C (M9), corresponding to an prolonged half-life from about 2 days (M6) to 3.5 days (M9). The possible explanation is that two substitutions V140L and F325L are favorable for the formation of tetrameric PpADI with greater thermal resistance than dimeric one (Zhu et al. 2014). In addition, semi-rational engineering was performed on mutant PpADI M13 (D38H/A128T/E296K/H404R/I410L) to yield a thermostable mutant M13-9 (D38H/A128T/E296K/H404R/I410L/A276W), whose half-life was enhanced from 4 min (M13) to 17.5 min (M13-9) at 60 °C. Furthermore, M13-9 also displayed a remarkable enhancement on pH stability, which retained over 90 % activity over pH range from 4.5 to 8.5.

PEGylation of ADI for improved circulating half-life, immunogenicity, and thermostability

Generally, native ADIs exhibit short plasma half-life in vivo due to proteolytic degradation, fast kidney clearance, and intense host immunoresponse. To overcome this problem, modification of ADI with polyethylene glycols (PEGs) has been developed and confirmed to increase circulating half-life and decrease immunogenicity (Pasut and Veronese 2009). ADI

PEGylation is a technology that ADI is conjugated with PEGs of appropriate molecular weight, shape, and linkers for protein attachment. Based on comparison of PEGs with various molecular weights, the formulation of ADI with PEG of 20, 000 mw (ADI-$PEG_{20\ kDa}$) showed a best pharmacokinetic (pK) and pharmacodynamic (pD) properties. Furthermore, the structure (linear or branched chain) and linker chemistries of PEG did not significantly affect the specific activity of the ADI-PEG as long as one mole of ADI is conjugated with 8–12 mol of PEG (Holtsberg et al. 2002).

In our previous study, the PEGylation and pharmacological properties of an engineered PpADI M13-3 (D38H/A128T/E296K/H404R/I410L/Q162S) have been investigated. The enzyme was modified by bioconjugating different polyethylene glycol (PEG) reagents with succinimidyl ester groups varied in size and linkers, and yielded products ADI-PEG-$SS_{20\ KDa}$, ADI-PEG-$SC_{20\ KDa}$, and ADI-PEG-$SPA_{20\ KDa}$. The pharmacodynamic/pharmacokinetic (PD/PK) analysis showed that ADI-PEG-$SPA_{20\ KDa}$ exhibited the most significant improvement in circulating half-life (from 4.7 to 53.2 h) and serum arginine depletion compared with free ADI. Furthermore, in mice implanted with H22 tumors, ADI-PEG-$SPA_{20\ KDa}$ showed a greater inhibition (95.02 % of inhibition rate) on tumor growth than the free ADI (98.34 % of inhibition rate) with the same dosage (15 U) (Zhang et al. 2015). Additionally, PEGylation of ADI has been also proved with enhanced thermal stability recently. A novel thermostable, ADI from thermophilic *A. fumigatus* KJ434941 was purified and PEGylated. Comparison of the free ADI and PEG-ADI, PEG-ADI showed higher thermal stability although its specific activity was slightly reduced (El-Sayed et al. 2015).

Application of ADI in clinical trials

ADI treatment for ASS1-deficient cancer

ADI has been intensively investigated as a potential antitumor drug. A number of clinical investigations confirmed that arginine depletion by ADI or PEGylated ADI (from *Mycoplasma* species) played an important role in the treatment of various ASS1-deficient xenograft tumor models (as seen in Table 3). For example, PEGylation of *Mycoplasma* ADI was found to be a safe, well tolerated drug for the treatment of unresectable HCC (Glazer et al. 2010, Yang et al. 2010). It has been demonstrated that ADI-PEG 20 could consistently deplete arginine and effectively cure advanced melanoma (Ott et al. 2013). ADI-$PEG_{20\ kDa}$ could also selectively eliminate arginine from the circulation and against small-cell lung cancer (Kelly et al. 2012, Walts et al. 2015). ADI-$PEG_{20\ kDa}$ treatment in ASS1-deficient breast cancer could induce cell autophagy-dependent death by generating mitochondrial damage, mTOR and 3KP13K pathways inhibition, or nucleotide and protein synthesis impairment (Phillips et al. 2013; Qiu et al. 2014). In addition, ADI has also been proved to have a good efficacy on mesothelioma (Szlosarek et al. 2013), sarcomas (Van Tine et al. 2013), lymphomas (Delage et al. 2012), and glioblastoma (Fiedler et al. 2015).

However, in some clinic therapy, insensitivity to ADI treatment was found to associate with the induced expression of ASS, which may contribute to failure of ADI treatment (Feun and Savaraj 2006). Subsequently, ASS expression was found to be transcriptionally induced by ADI in melanoma cell lines A2058 and SK-MEL-2 and correlated with resistance to ADI treatment. The transcription mechanism may be related to the region near AS promoter that contains an E-box recognized by c-Myc and HIF-1α and a GC-box by Sp4. The E-box is bound by HIF-1α under noninduced conditions while HIF-1α is replaced by c-Myc under arginine depletion conditions. Thus, overexpression of c-Myc by transfection could upregulate ASS expression, whereas co-transfection with HIF-1α could suppress c-Myc-induced ASS expression. It suggests that regulation of ASS expression relates to interplay among positive transcriptional regulators c-Myc and Sp4, and negative regulator HIF-1α which confers resistance to ADI treatment (Tsai et al. 2009).

ADI treatment for other diseases

Besides multiple ASS1-deficient tumors, ADI is also a potential therapeutic treatment for other diseases (as seen in Table 3). For instance, as a potential anti-angiogenic agent, the recombinant ADI exhibited an inhibitory effect on the growth of human umbilical vein endothelial cells, which could lead to solid tumors (Beloussow et al. 2002). Recombinant ADI was also well applied for neurodegenerative diseases therapy by protecting cells from inducible nitric oxide synthase-mediated toxicity, although ADI has deleterious effects on nNOS-activated neurons (Lin et al. 2014). Furthermore, PEGylated ADI has been confirmed to have a therapeutic efficacy in the treatment of relapsed/refractory and/or elder acute myeloid leukemia (Ariza-McNaughton et al. 2013; Tsai et al. 2014). It has been indicated that PEGylated ADI was able to significantly decrease disease index, serum amyloid A level, and inflammatory cytokines in colonic explants to provide protection against colitis (Oz et al. 2012). Henningham and coworkers indicated that ADI from GAS could be used as a GAS vaccine candidate to protect mice against GAS infection (Henningham et al. 2012; Henningham et al. 2013). In addition, Marini and Didelija (2015) confirmed that ADI-PEG 20 therapy would not affect whole protein metabolism or muscle fractional protein synthesis rate during ADI treatment.

Table 3 Application of ADI and PEGylated ADI in clinical trials

Application	ADI type	Source	Treatment schedule	Disease	Trial model	Outcomes	references
ASS1-deficient cancer treatment	ADI	*Mycoplasma*	Intramuscular injection; dose: 80 IU/m^2 or 160 IU/m^2 weekly; duration: 6 months	Hepatocellular carcinoma (HCC)	69 humans patients	① 4 patients were excluded due to exclusion criteria after randomization ② Mean survival for all subjects was 15.8 months from time of diagnosis ③ Arginine levels remained below baseline for 50 days while antibodies against ADI reached a plateau at approximately the same time ④ No deaths ⑤ 2 patients were withdrawn for immunogenic-related adverse events	Glazer et al. 2010
	ADI-PEG 20	*Mycoplasma*	Intramuscular injection; dose: 160 IU/m^2 or 320 IU/m^2 weekly; duration: 8 or more weeks	Hepatocellular carcinoma (HCC)	71 Asian humans patients	① There were no objective responders ② Disease-control rate (DCR) was 31 % ③ Median overall survival (OS) was 7.3 months ④ Median OS of patients with undetectable circulating arginine for more than or equal to and <4 weeks was 10.0 and 5.8 months respectively	Yang et al. 2010
	ADI-PEG 20	*Mycoplasma*	Abdominal area subcutaneous tissue injection; dose: 1, 2 and 5 IU per animal once every 5 days; duration: 20 days	Small cell lung cancers (SCLC)	Mice	① 45 % of SCLC tumors and 50 % of cell lines assessed were negative for ASS1 ②ADI-PEG 20 treatment of ASS1-negative SCLC xenografts caused significant, dose-dependent inhibition of tumor growth of both small and established tumors	Kelly et al. 2012
	ADI-PEG 20	*Mycoplasma*	Intramuscular injection; dose: 160 IU/m^2/week; duration: 4 weeks	Lymphoid malignancies	One patient with refractory lymphoma	① After one dose, the patient's pruritus and skin oedema resolved that correlated with a minor reduction in the total white cell count (35–31 × 10^9/l; normal range 4–11 × 10^9/l) ② The plasma arginine concentration decreased with a corresponding increase in plasma citrulline	Delage et al. 2012
	ADI-PEG 20	*Mycoplasma*	Intramuscular injection; dose: 320 IU/m^2/week; duration: 6 weeks	Mesothelioma	One man with an epithelioid malignant mesothelioma	① A partial metabolic response with a 40 % reduction in the maximum standardized uptake value (5.5 decreasing to 3.5) after 3 doses ADI-PEG 20 treatment ② The patient experienced stable symptoms for an additional 3 weeks before developing increasing breathlessness and chest-wall pain with marked subdiaphragmatic extension of disease by CT	Szlosarek et al. 2013
	ADI-PEG 20	*Mycoplasma*	Intramuscular injection; dose: 40, 80 or 160 IU/m^2 weekly;	Melanoma	31 humans patients	① No objective responses were seen ② 9 patients achieved stable disease (SD), with 2 of them durable for >6 months, 4 of them with SD had uveal melanoma	Ott et al. 2013

Table 3 (continued)

Application	ADI type	Source	Treatment schedule	Disease	Trial model	Outcomes	references
						③ PD analysis showed complete plasma arginine were depleted in 30/31 patients by day 8 ④ Immunohistochemical ASS1 expression analysis was negative in 24 patients, 5 patients had <5 % cells positive	
	ADI-PEG 20	*Mycoplasma*	*In vivo* experiments: tail vein injection; dose: 4 IU of ADI-PEG 20 (0.2 ml) weekly; duration: 3 weeks	Breast cancer	*In vitro* experiments: breast cancer cells from patients; *In vivo* experiments: mice.	① The tumors from ADI-PEG 20 treated mice were smaller than control mice ② ASS1 was either low in abundance or absent in more than 60 % of 149 random breast cancer bio-samples	Qiu et al. 2014
	ADI-PEG 20	*Mycoplasma*	Unknown	Pulmonary neuroendocrine carcinomas (PNEC)	69 PNEC patients (49 SCLC and 20 large cell neuroendocrine carcinoma (LCNEC)) were retrospectively studied.	① 58 % of PNEC including 61.2 % of SCLC and 50 % of LCNEC were ASS1 negative ② The ASS1 negative tumors include 63 % of the primary and 40 % of the metastatic lesions tested	Walts et al. 2015
Other diseases treatment	ADI	*Mycoplasma*	*In vitro* experiment: 0.3×10^{-3}, 1×10^{-3} and 3×10^{-3} U ADI, incubated with human umbilical vein endothelial cells at 37 °C, 95 % air/ 5 % CO_2. ADI was removed after 4 days, and replaced with fresh medium and cultured for an additional 3 days	Angiogenesis	Human umbilical vein endothelial (HUVE) cells	ADI-treated HUVE cells were unable to complete the microvascular structure formation over the 24 time period.	Beloussow et al. 2002
	ADI	GAS	Unknown	GAS infection vaccine	Mice	ADI was identified to confer protection against lethal GAS challenge in a variety of mouse models	Henningham et al. 2012
	ADI	*M. arginini*	In vitro experiment: the cells were incubated with 1 mM NMDA, 1 mU/mL ADI for 24 h.	NO-related neurodegenera-tive disorders	SH-SY5Y neuroblastoma cells	ADI not only reduced NO production but also caused cellular toxicity in nNOS-activated SH-SY5Y cells, suggesting a dual role for ADI in NOS-mediated neurotoxicity	Lin et al. 2014
	ADI-PEG 20	*Mycoplasma*	Intramuscular injection; dose: 320 IU/m^2/week; duration: 4 weeks as one cycle, 2~6 cycles	Acute myeloid leukemia	9 human patients	① 2 patients achieved CR after 3 and 1 cycles of ADI-PEG 20 ② 6 patients had disease progression after an average of 1 cycles of treatment ③ 1 patient was not evaluable for response due to withdrawal of consent after the first 2 doses of treatment ④ 2 patients died within 2 weeks after the first doses of treatment, and were considered to have progressive disease	Tsai et al. 2014

Table 3 (continued)

Application	ADI type	Source	Treatment schedule	Disease	Trial model	Outcomes	references
	ADI-PEG	*Mycoplasma*	Dose: 5 IU/m^2/week and 10 IU/m^2/week; duration: 5 days	Colitis	Mice	① ADI-PEG treatment significantly protected DSS-exposed mice from weight loss and rectal bleeding ② ADI-PEG treatment significantly protected DSS-exposed mice against diarrhea, occult blood and prolapse ③ADI-PEG treatment animals exhibited significant reduction at the levels of SAA biomarker ④ADI-PEG treatment decreased distal and proximal colonic histological damage scores	Oz et al. 2014
Synergistic therapy with ADI and other agents	Combination of ADI-PEG with MEK inhibitor or cisplatin	*Mycoplasma*	ADI-PEG 20: 0.05 μg/ml; MEK inhibitor: 5 μM	Melanoma	*In vitro* experiment	The combination of ADI-PEG with other agents which can drive the cells towards apoptosis or inhibit the autophagic process could increase the therapeutic efficacy of melanoma	Savaraj et al. 2010
	Combination of ADI-PEG and gemcitabine	*Mycoplasma*	ADI 5 IU/week; gemcitabine 125 mg/kg, 2 days per week; combination of ADI and gemcitabine	Pancreatic cancer	Mice	The combination with gemcitabine in vivo yielded significant anti-tumor effects, with the benefit potentially involving the ribonucleotide reductase subunit M2 (RRM2) induced gemcitabine resistance in pancreatic ductal adenocarcinoma	Daylami et al. 2014
	Combination of ADI-PEG with cisplatin	*Mycoplasma*	Unknown	HCC	Human HCC cell lines (*In vitro* experiment)	The combination of ADI-PEG 20 and cisplatin restored ASS1 protein levels in most of the cell lines, and the combination therapeutic strategy is good for the management of HCC	McAlpine et al. 2014
	Combination of ADI-PEG 20 and cytarabine chemotherapy	*Mycoplasma*	ADI-PEG 20: peritoneal injection, 5 IU /week for 4 weeks; cytarabine: subcutaneous injection, 0.2 mg/day, for 10 days.	Acute myeloid leukemia (AML)	Mice	The combination of ADI-PEG 20 with cytarabine chemotherapy is more effective than either treatment alone resulting in responses in 6 of 6 AMLs tested in vivo	Miraki-Moud et al. 2015

ADI synergistic therapy with other agents

PEGylated ADI treatment combining with other drugs together often leads to better efficacy (Table 3). For instance, cytarabine chemotherapy with an anthracycline antibiotic is the backbone of acute myeloid leukemia (AML) induction treatment, which has remained unchanged during the past 50 years. ADI-PEG 20 may confer caspase activation, leading to apoptosis in sensitive AML. The combination of ADI-$PEG_{20\ kDa}$ treatment and cytarabine chemotherapy was more effective than either treatment alone in the therapy of AML in vivo (Miraki-Moud et al. 2015). Synergistical combination of ADI-PEG and gemcitabine could cause growth arrest, leading to increased tumor response in vivo. Therefore, PEGylated ADI could inhibit the gemcitabine-induced expression of ribonucleotide reductase subunit M2 (RRM2) levels, which confers gemcitabine resistance (Daylami et al. 2014). Savaraj and coworkers found that arginine deprivation could inhibit mTOR signaling while activate MEK and ERK with no changes in BRAF, resulting in cell survival by recycling intracellular arginine. Combination of ADI-PEG 20 and cisplatin or MEK inhibitor was proved to increase apoptosis or inhibit the autophagic process in melanoma cell lines, which could increase the therapeutic efficacy of melanoma treatment (Savaraj et al. 2010). Furthermore, McAlpine and coworkers indicated that ASS1 silencing in HCC cell lines is associated with simultaneous sensitivity to ADI-PEG 20 and resistance to cisplatin, and the combination strategy of ADI-PEG 20 and cisplatin is effective in HCC treatment (McAlpine et al. 2014).

Conclusion and perspectives

ADI is commonly considered to be a potential agent for various arginine-auxotrophic tumors therapy. Although many studies on ADI have been reported in the past few decades, work still needs to be performed for the more effective application of ADI. Here, simple perspectives on ADI are also provided (Fig. 4).

Improving extracellular secretion of ADI

In previous reports, most recombinant ADIs were heterologously expressed in *E. coli* hosts as intracellular enzyme, which increases difficulties for the further massive production and purification. Therefore, improving the extracellular secretion of ADI is critical for its efficient production and application. Several possible approaches may be used for improving the extracellular expression of ADI. Non-*E. coli* hosts, such as *Bacillus subtilis*, could be selected as ADI expression hosts. Compared with the Gram-negative bacterium, *B. subtilis* has the naturally highly secretory capacity for extracellular expression, and avoids codon preference and inclusion body problems. Besides, signal peptide could mediate the extracellular expression of proteins (Mergulhao et al. 2005). Choosing the suitable signal peptide for different proteins is important for the extracellular

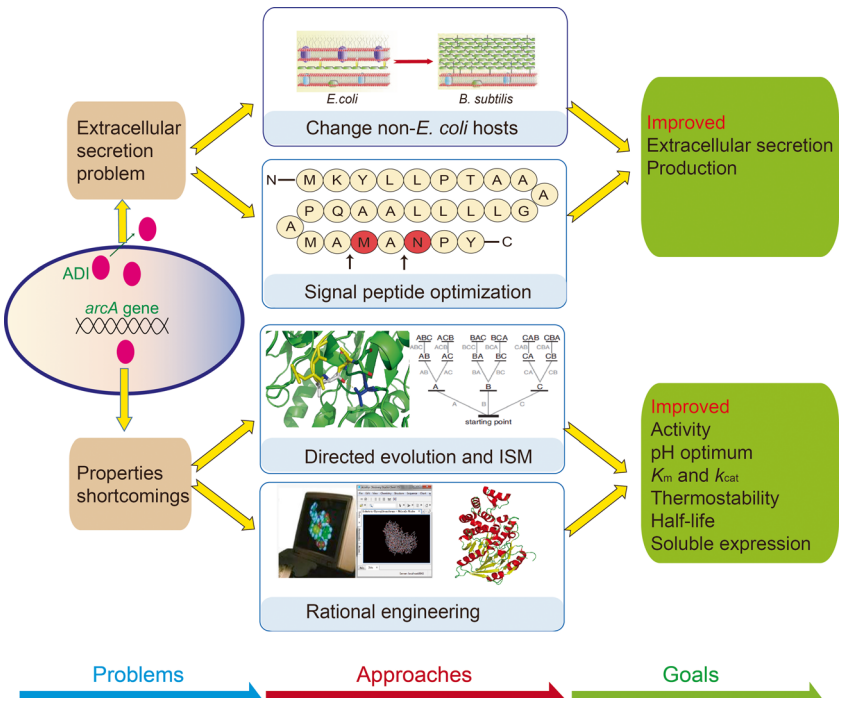

Fig. 4 Our perspectives on ADI study

secretion system, which has been previously summarized (Low et al. 2013). Modification of signal peptides is another important approach. Wu's group demonstrated that increasing the signal peptide cleavage sites (Chen et al. 2011) and translational initial region (TIR) degeneracy mutagenesis of pelB signal peptide (Liu et al. 2011) could improve the extracellular production of recombinant enzyme in *E. coli*.

Improving enzymatic properties of ADI

As a potential candidate for the therapy of arginine-auxotrophic tumors, ADI's properties under physiological conditions directly affect its clinical applications. Although many marvelous successes in ADI engineering for properties improvement have been reported during the past few decades, more work still needs to be done to improve its defective properties. In previous reports, great improvements on ADI's properties have been achieved by directed evolution and site-directed mutagenesis approaches. For example, in PpADI, positions 44 and 404 are confirmed to be important for pH optimum and k_{cat} value improvements (Zhu et al. 2010a); positions 30, 37, 148, and 291 contribute to K_m value improvement (Cheng et al. 2015a); positions 140 and 325 are key sites for improving the thermostability of ADI (Zhu et al. 2014); and position 162 possibly affects the soluble expression of ADI (Jamil et al., 2015). However, one mutant with multiple property improvements, including physiological pH optimum, high activity and k_{cat} value, high thermostability, half-life and soluble expression, and low K_m value, has not been attained. Therefore, iterative saturation mutagenesis could be employed on the beneficial sites to evolve mutants with multiple improved properties. Furthermore, rational and semi-rational engineering based on bioinformatics and structure analysis are necessary to identify more possible key site residues and regions for improved properties. Taken together, the achievements on ADI obtained so far are still limited, and considerable efforts on ADI properties improvement are necessary in future.

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