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Strategies for Optimizing the Serum Persistence of Engineered Human Arginase I for Cancer Therapy

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Abstract

Systemic L-Arginine depletion following intravenous administration of L-Arginine hydrolyzing enzymes has been shown to selectively impact tumors displaying urea-cycle defects including a large fraction of hepatocellular carcinomas, metastatic melanomas and small cell lung carcinomas. However, the human arginases display poor serum stability ($t_{1/2}$ = 4.8 hrs) whereas a bacterial arginine deiminase evaluated in phase II clinical trials was reported to be immunogenic, eliciting strong neutralizing antibody responses. Recently, we showed that substitution of the Mn²⁺ metal center in human Arginase I with Co²⁺ (Co-hArgI) results in an enzyme that displays 10-fold higher catalytic efficiency for L-Arg hydrolysis, 12–15 fold reduction in the IC₅₀ towards a variety of malignant cell lines and, importantly a $t_{1/2}$ = 22 hrs in serum. To investigate the utility of Co-hArgI for L-Arg depletion therapy in cancer we systematically investigated three strategies for enhancing the persistence of the enzyme in circulation: (i) site specific conjugation of Co-hArgI engineered with an accessible N-terminal Cys residue to 20 KDa PEG-maleimide (Co-hArgI-C^{PEG-20K}); (ii) engineering of the homotrimeric Co-hArgI into a linked, monomeric 110 KDa polypeptide (Co-hArgI ×3) and (iii) lysyl conjugation of 5 KDa PEG-N-hydroxysuccinimide (NHS) ester (Co-hArgI-K^{PEG-5K}). Surprisingly, even though all three formulations resulted in proteins with a predicted hydrodynamic radius larger than the cut-off for renal filtration, only CohArgI amine conjugated to 5 KDa PEG remained in circulation for sufficiently long durations. Using CohArgI-K^{PEG-5K} labeled with an end-terminal fluorescein for easy detection, we demonstrated that following intraperitoneal administration at 6 mg/kg weight, a well tolerated dose, the circulation $t_{1/2}$ of the protein in Balb/c mice is 63 ± 10 hrs. Very low levels of serum L-Arg (<5 μM) could be sustained for over 75 hrs after injection, representing a 9-fold increase in pharmacodynamic efficacy relative to similarly prepared Mn²⁺-containing hArgI conjugated to 5 KDa PEG-NHS ester (Mn-hArgI-K^{PEG-5K}). The favorable pharmacokinetic and

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pharmacodynamic properties of Co-hArgI-K^{PEG-5K} reported here, coupled with its human origin which should reduce the likelihood of adverse immune responses, make it a promising candidate for cancer therapy.

Keywords

Serum persistence; L-Arg depletion; Systemic therapeutic; Antineoplastic

1. Introduction

Many tumors exhibit metabolic deficiencies in one or more amino acid biosynthesis or salvage pathways and as a result, are forced to rely upon uptake of these amino acids from the serum for growth. Systemic depletion of tumor-essential amino acid results in apoptosis of the malignant cells with minimal side effects to normal cells. Enzyme-mediated depletion of extracellular amino acids has the added benefit that it can target sensitive tumors even when those that are poorly vascularized or are sequestered in inaccessible locations such as CNS or testes. Thus unlike antibody drugs, the function of enzyme therapeutics is not dependent on tumor penetration.

A large number of hepatocellular carcinomas, melanomas, renal cell and prostate carcinomas [1–3] do not express the urea cycle enzyme, argininosuccinate synthase (ASS) and thus are sensitive to L-arginine (L-Arg) depletion. Recently, Cheng et al. [4] demonstrated that many HCC cells are deficient in ornithine transcarbamylase expression and thus also rely on external sources of L-Arg. Whereas non-malignant cells enter into quiescence (G₀) when depleted of L-Arg and remain viable for several weeks, hepatocellular carcinomas experience cell cycle defects that lead to the re-initiation of DNA synthesis even though protein synthesis is inhibited, in turn resulting in major imbalances and rapid cell death [5, 6]. The selective toxicity of L-Arg depletion for HCC, melanoma and other urea-cycle deficient cancer cells has been extensively demonstrated *in vitro*, in xenograft animal models and in clinical trials [1, 5, 7, 8].

Arginine deiminase (ADI) (EC 3.5.3.6) an enzyme found in prokaryotes and archaea but not in mammals, catalyzes the efficient hydrolysis of L-Arg to L-Citrulline (L-Cit) plus ammonia [9]. The bacterial *Mycoplasma arginini* enzyme, a homodimer of two 48 KDa polypeptide chains, has been selected for clinical evaluation due to its high catalytic rate under physiological conditions [5]. The anti-tumor activity of ADI is likely related to the induction of apoptosis under conditions of amino acid stress, although additional mechanisms such as localized ammonia toxicity and inhibition of neovascularization may play a role [10]. Since ADI is cleared rapidly from circulation, conjugation to 20 KDa MW polyethylene glycol (PEG) was employed to generate a molecule suitable for clinical applications. However, meta-analysis of phase II clinical data indicated that ADI is immunogenic, leading to the generation of neutralizing antibodies that coincided with an increase in serum L-Arg to pre-treatment levels [11, 12]. This is not surprising since adverse antibody responses to heterologous enzymes have been reported in numerous instances [13–15].

Since human proteins are much less likely to be immunogenic than their heterologous counterparts, arginine hydrolyzing enzymes of human origin have also been considered for L-Arg depletion therapy [16]. Humans produce two Mn²⁺-dependent L-Arginase isozymes (EC 3.5.3.1) that catalyze the hydrolysis of L-Arg to urea and L-ornithine (L-Orn). The Arginase I (hArgI) gene, located on chromosome 6 (6q.23), is highly expressed in the cytosol of hepatocytes and functions in nitrogen removal in the final step of the urea cycle. The Arginase II gene, located on chromosome 14 (14q.24.1), is localized to the

mitochondria in tissues such as kidney, brain, and skeletal muscle where it is thought to provide a supply of L-Orn for proline and polyamine biosynthesis [17]. In early clinical studies, administration of Mn²⁺-human arginase I (Mn-hArgI) by transhepatic arterial embolisation led to partial remission of HCC in several patients [18]. Unfortunately, under physiological conditions (pH 7.4 & serum L-Arg ~120 μM) Mn-hArgI has minimal activity due to a high pH optimum (~9.5), high K_M (2.3 mM) and low stability (t_{1/2} in serum=4.8 hrs) [19].

We recently reported that substitution of the Mn²⁺ cofactor with Co²⁺ in hArgI (Co-hArgI) markedly improves its pharmacological properties, namely: (i) lowers the pH optimum to 7.4; (ii) lowers the K_M from 2.3 mM to 120 μM without an appreciable effect on k_{cat} and (iii) increases the t_{1/2} in serum to ~22 hr. The enhanced pharmacological properties of Co-hArgI translate into a 12–15 fold improvement in the killing of melanomas and hepatocellular carcinomas *in vitro* [19].

hArgI is a homotrimer with a subunit MW of ~35 KDa. Even though the M.W. of the trimer is 105 KDa and therefore larger than the limit for glomerular filtration, in mice, the enzyme is cleared from circulation within 30 minutes [20]. This may be due to the filtration of monomeric hArgI in equilibrium with trimer. Although sedimentation experiments did not reveal the formation of appreciable amounts of monomer at equilibrium, the continuous removal of the monomeric form in the kidneys could result in the gradual loss of the protein [21]. Cheng and coworkers reported that conjugation of multiple lysyl residues in hArgI to PEG 5KDa, extended its circulation half-life (PD) in mice to 12 hrs [22]. More recently the same group reported that Mn-hArgI conjugated to PEG 5KDa maintained low concentration of L-Arg for approximately 3 days in a single patient [23, 24].

Here we evaluated three strategies for conferring a long circulation half-life to the serum stable CohArgI form of arginase: (i) Site specific conjugation of Co-hArgI engineered with an accessible N-terminal Cys residue to 20 KDa PEG-maleimide, a process that necessitated dissociation of the trimer into monomers after reaction with 20 KDa PEG-maleimide by low pH size exclusion chromatography followed by reassembly of the PEGylated trimer. (ii) Engineering of a linked, monomeric 110 KDa Co-hArgI polypeptide which could be expressed at preparative levels in bacteria following genetic optimization (Co-hArgI ×3). (iii) Amine conjugation with 5 or 10 KDa PEG-N-hydroxysuccinimide (NHS) ester (Co-hArgI-K^{PEG-5K} or Co-hArgI-K^{PEG-10K}). Even though the first two approaches resulted in near homogenous mono-PEGylated and homotrimeric Co-hArgI respectively, surprisingly, they failed to confer long circulation persistence in Balb/c mice. In contrast, Co-hArgI-K^{PEG-5K} resulted in a circulation t_{1/2} of 63 ± 10 hrs. In balb/c mice we determined that the optimal dose for sustained L-Arg depletion is 6–8 mg/kg. IP injection of Co-hArgI-K^{PEG-5K} at 6 mg/kg weight was shown to deplete serum L-Arg to below the detection limits for 78 ± 10 hrs. The improved pharmacodynamics of this molecule correspond to a 9 –fold longer serum persistence than an equivalent dose of the authentic, Mn²⁺-hArgI conjugated to 5K PEG in an identical fashion [19]. Thus, Co-hArgI-K^{PEG-5K} exhibits PK and PD characteristics suitable for clinical development.

2. Materials & Methods

2.1. Materials

DNA modifying enzymes and reagents were from NEB (Ipswich, MA), L-Arginine and various buffers and chemicals were obtained from Sigma (St.Louis, MO), various PEG materials were obtained from JenKem Technology USA (Allen, TX) and Nanoc Inc (New York, NY).

2.2 Molecular Biology Techniques

2.2a Construction of an hArgI-C gene for Site-Specific Cys-PEGylation—We constructed a gene (named *hArgI-C*) containing the previously described *E. coli* codon optimized gene of human Arginase I (hArgI) containing a codon for Cys on the third residue (N terminal sequence Met-Gly-Cys), followed by an N-terminal 6 × His tag by overlap extension PCR amplification using the oligonucleotide primer NTC-F `5-GATATACCATGGGTTGTTCTCACCATCATCAC-CACCACAGCTCTGGCG and additional primers as previously described [19]. The PCR product was cloned into the pET28a vector (Novagen).

2.2b Construction of hArgI E356Q—The E256Q mutation has been shown to disrupt trimerization resulting in the formation of monomeric Arginase I enzyme [25]. The E256Q gene containing an N-terminal cysteine residue (above) was constructed using two mutagenic primers: `5-ggttaacgtatcgcCAGggcctgtatcacgg, and `5-CCGTGATATACAGGCCCTGGCGATACGTTAAACC, and two specific end primers by overlap extension PCR, and the gene product was cloned into pET28a vector as above.

2.2c Construction of a Human Arginase linked trimer (hArgI × 3)—An hArgI × 3 expression vector was constructed in several stages, such that the coding sequence for each hArgI subunit (hArgI 1–3) was cloned separately into pET28a vector before consolidation as a three part gene fusion. (See supplemental figure S1 for a schematic representation) as follows: First, hArgI-1 was constructed by replacing the stop codon in the hArgI gene with a synthetic fragment containing a sequence encoding the 31 aa linker GAGTGSGTGSGAGSGTGAGSGTGSGAGSGTG). The resulting gene also contained a flanking 5' NcoI restriction site and a 3' EcoRI restriction site. hArgI-1 was then cloned into NcoI-EcoRI digested pET28a to produce pET28-hArgI-1. Second, we constructed an hArgI-2 gene containing the aforementioned 31 aa linker and flanked by 5' NcoI followed by an EcoRI restriction site and 3' in-frame BamHI and NotI restriction sites. The gene fragment was cloned between NcoI and NotI in pET28a to produce pET-hArgI-2. Finally, hArgI-3 contained a 5' BamHI restriction site and a sequence encoding a 6 × His tag in-frame to the C-terminus of hArgI followed by 3'NotI restriction site hArgI-3. hArgI-3 was cloned between the BamHI and NotI in pET28a. The hArgI × 3 gene assembly from the three monomer-encoding fragments described above was performed as follows: The hrgA I-2 was excised from pET-hArgI-2 using EcoRI and NotI and then ligated to pET-hArgI-1 plasmid that had been digested with EcoRI and NotI. Similarly, hArgI-3 gene digested with BamHI and NotI was ligated C-terminal to pET-hArgI-1-2 digested with BamHI and NotI to create the *hArgI × 3 gene*. The resulting ~3.5 kB insert was verified by DNA sequencing.

2.3 Protein Expression and Purification

hArgI or hArgI E356Q were expressed in *E.coli* BL21 (DE3) containing the appropriate expression vectors and grown at 37 °C in Terrific Broth (TB) media containing 50 µg/ml kanamycin in shake flasks at 250 rpm to an OD₆₀₀ of 0.5–0.6 at which point protein synthesis was induced by adding IPTG to a concentration of 0.5 mM. After an additional 12 hrs of growth, cells were harvested and proteins were purified by IMAC as described previously [19] except that following binding, the IMAC column was washed extensively (80–90 column volumes) with buffer containing 10 mM NaPO₄/10 mM imidazole/300 mM NaCl, pH 8 containing 0.1 % Triton 114 to remove endotoxin [26]. The purified protein was then PEGylated as discussed below.

hArgI × 3 was expressed in *E.coli* BL21 (DE3) transformed with pET-hArgI × 3. Cells were grown with shaking at 250 rpm in 2 L shake flasks containing 500 mL minimal media (Sigma, St. Louis MO) supplemented with 2% glucose, 2 mM MgSO₄, 100 µM CaCl₂, and

50 µg/ml kanamycin. The cells were grown to an OD₆₀₀ of ~ 1 at 37°C and protein expression was induced by adding 100 µM IPTG. 100 µM CoCl₂ was also added to the growth media to facilitate the replacement of the Mn²⁺ metal cofactor in hArg I with Co²⁺ and the cells were incubated overnight at 25°C. The hArgI ×3 enzyme was purified by IMAC as above, the purified protein was incubated with 0.5 mM CoCl₂ for 15 min at 50°C and then excess Co²⁺ ions were removed by multiple buffer exchange steps with PBS buffer containing 10% glycerol using a 10,000 MWCO centrifugal filter device (Amicon). The resulting protein is hitherto referred to as (Co-hArgI ×3). After sterile filtration through a 0.22 µm syringe filter, aliquots of Co-hArgI ×3 were flash-frozen in liquid nitrogen and stored at – 80°C.

2.4 Protein PEGylation

2.4a Site Specific PEGylation of hArgI-C—Purified hArgI-C was buffer exchanged into 100 mM NaPO₄ buffer, pH 8.3, using a 10,000 MWCO filtration device (Amicon) to a concentration of 2–4 mg/ml. Subsequently, 3 molar equivalents of tris-2-carboxyethylphosphine (TCEP) were added to the protein solution for 90 min at 25 °C to reduce the N-terminal cysteine. Then three molar equivalents of Methoxy PEG Maleimide 20,000 Da (Peg20K) (Jenkem Technologies, Location) were then added and allowed to react for an additional 90 min at 25°C under constant stirring. At the end of the reaction, any precipitates were removed by centrifugation, and the supernatant was buffer exchanged into 100 mM sodium acetate, pH 4.5 using a 10,000 MWCO filtration device as above. The protein was then loaded onto a Superdex 200 column (GE), pre-equilibrated with 100 mM sodium acetate, pH 4.5. Fractions having the appropriate MW were mixed with an equal volume of 200 mM HEPES, 1 mM CoCl₂, pH 8.5 and incubated at 50 °C for 15 minutes to ensure full incorporation of Co²⁺. The fractions containing PEGylated arginase as assessed by SDS-PAGE, were pooled and was buffer exchanged into sterile PBS, 10% glycerol, pH 7.4, sterilized by passing through a 0.2 micron syringe filter (VWR, Radnor PA) and aliquots were flash frozen in liquid nitrogen and stored at –80 °C. This protein is hitherto referred to as Co-hArgI-C^{PEG-20K}.

2.4b PEG amine conjugation—hArg I was mixed with methoxy PEG succinimidyl carboxymethyl ester of either 5,000 Da or 10,000 Da (JenKem Technology) in 100 mM NaPO₄ buffer, pH 8.3, at 40:1 molar ratio and allowed to react for 1 hr at 25°C under constant stirring. 10 mM of either CoCl₂ or MnCl₂ was then added and the solution was heated to 50°C for 10 minutes. After centrifugation to remove any precipitates, the enzyme conjugate was extensively buffer exchanged (in PBS with 10 % glycerol) using a 100,000 MWCO filtration device (Amicon), and sterilized with a 0.2 micron syringe filter (VWR, Radnor PA). Pegylated enzyme preparations were analyzed for lipopolysaccharide (LPS) and aliquots were flash frozen in liquid nitrogen and stored at – 80°C. The 5,000 Da and 10,000 Da PEGylated enzymes are hitherto referred to as Co-hArgI-K^{PEG-5K} or Co-hArgI-K^{PEG-10K} respectively

Fluorescently-labeled amine-PEGylated hArgI was prepared by reacting hArgI (~ 100 µM) with 2 molar equivalents of fluorescein isothiocyanate (FITC)-appended PEG succinimidyl ester MW 5000 Da (FITC-PEG-5K) (Nanoc Inc, New York NY) for 1 hr at 25°C under constant stirring. Then, an additional 40 molar equivalents of methoxy-PEG succinimidyl ester MW 5,000 Da or MW 10,000 Da was added at a 40:1 molar ratio and allowed to react for 1 hr at 25°C under constant stirring. The resulting solution was incubated with CoCl₂ and buffer exchanged as described above. The amount of FITC-PEG-5K incorporated into hArgI was quantified using a standard curve with known amounts of FITC-PEG-5K constructed by monitoring fluorescence in a microtiter plate reader with an excitation filter of 485 nm and an emission filter of 528 nm.

2.5 Protein Characterization

PEGylation was evaluated via Coomassie staining of SDS-PAGE gels followed by a Kurfurst stain [27] for PEG detection. Briefly, the Coomassie-stained gel was immersed in 0.1% perchloric acid for 15 minutes, followed by the addition of 5 ml BaCl₂ 5% w/v and 2 ml of 3-fold diluted in Lugol's solution (Fluka, Milwaukee WI) and subsequent destaining in deionized H₂O [27]. The apparent M.W.s of all the enzymes and enzyme conjugates injected into animals were determined by size exclusion chromatography using a Superdex 200 column (GE, Piscataway NJ) equilibrated in PBS. M.W. were calculated from a standard curve determined using proteins of known mass (Biorad, Hercules CA).

Steady state enzyme kinetics for Co-hArgI-K^{PEG-5K}, Co-hArgI-K^{PEG-10K}, Mn-hArgI-K^{PEG-5K}, Co-hArgI-C^{PEG-20K} and hArgI-3 $\times$ were determined at pH 7.4, 37°C as described previously [19]. The stability of the proteins in pooled human serum at 37°C was also evaluated as previously described [19]. For comparison with previously reported data [24] the stability of Mn-hArgI-K^{PEG-5K} was also determined by first buffer exchanging the protein into 125 mM boric acid buffer, pH 8.3 and then adding pooled human serum at a 1:3.3 v/v ratio. Activity was followed as a function of time at 37°C. The concentration of LPS in all the samples injected into animals was first determined using a Limulus Amebocyte Lysate (LAL) kit (Cape Cod Incorporated).

2.6 Animal studies

All procedures were performed according to IACUC and institutional guidelines. Six groups of female balb/c mice (5 mice/group) (Jackson laboratories) were given 32, 16, 8, 6, 4 mg/kg intraperitoneal (IP) injections of Co-hArgI-K^{PEG-5K} or saline as a control. Baseline blood samples were collected one week prior to drug injection and samples after treatment were collected at 6, 24, 48, 72, 100, and 168 hrs post treatment. A second dose of Co-hArgI-K^{PEG-5K} or saline was given at day 7 and mice were observed until day 14 at which point the mice were sacrificed for organ and tissue harvest. Blood samples (20 – 30 μ l) were collected by tail venipuncture and immediately mixed on ice with 75 μ l of a 200 mM sodium citrate buffer, pH 4. Mice were fed water and mouse chow *ad libitum* and monitored for weight changes and general appearance during the course of the experiment as assessed by body condition scores (BCS)[28].

Serum was separated from the blood samples immediately after collection by addition of 50 μ l dH₂O by application onto a 10K MWCO filtration device (Microcon) and centrifuged at 4°C for 10 min. The serum and whole blood fractions were stored at –80°C until further analysis.

In one study a group of 5 female balb/c mice was treated with a 16 mg/kg IP injection of Co-hArgI-K^{PEG-5K} that had previously been inactivated by extensive incubation with EDTA. Baseline blood samples were collected one week prior to drug injection and samples after treatment were collected at 6, 24, 48, 72, 100, and 168 hrs post-treatment and processed as above.

The pharmacokinetics and pharmacodynamics of Mn-hArgI-K^{PEG-5K}, Co-hArgI-K^{PEG-10K}, Co-hArgI-C^{PEG-20K}, and Co-hArgI $\times$ 3 were determined as follows: 5 groups of female balb/c mice (5 mice/group) were given a 6 mg/kg intraperitoneal (IP) injection of Mn-hArgI-K^{PEG-5K}, Co-hArgI-K^{PEG-10K}, Co-hArgI-C^{PEG-20K}, Co-hArgI $\times$ 3 or saline as a control. Baseline blood samples were collected one week prior to drug injection and after treatment samples were collected at 6, 24, 48, 72, 100, and 168 hrs post treatment. A second dose of drug or saline was given at day 7 and mice were observed an additional 7 days and were subsequently sacrificed for organ and tissue harvest.

2.7 Analytical Techniques

2.7a Pharmacodynamics—L-Arg in serum was derivatized with o-phthalaldehyde (OPA) and detected on a C18 reverse phase HPLC column (5 μ m, 4.6 $\times$ 150 mm), essentially as described by Agilent Technologies (Publication Number: 5980-3088) except that the flow rate was reduced by 50% to 1 ml/min and the acquisition time was doubled to 20 min to obtain better peak separation. Peak areas corresponding to the retention time of L-Arg were measured and serum concentrations determined using a standard curve. Where applicable, serum L-Arg concentrations as a function of time were fit to a modified Gompertz equation [29] to estimate total depletion time (lag time).

2.7b Pharmacokinetics—The half life of amine conjugated *PEG 5 K* or *PEG 10K hArgI* whole blood fractions collected from mice treated with Co-hArgI-*K^{fPEG-5K}* or Co-hArgI-*K^{fPEG-10K}* were analyzed by running samples on a SDS-PAGE gel along with purified *fPEG5K-Co-hArgI* standards. In gel fluorescence was measured by excitation at 488 nm on a Typhoon scanner (GE Healthcare). The resulting fluorescent bands from standards and samples were analyzed by standard curve densitometry using a quantification program (ImageQuant, Amersham Biosciences). Additionally, Co-hArgI-*K^{fPEG-10K}*, Co-hArgI-*C^{PEG-20K}*, and Co-hArgI $\times 3$ were detected by western blotting using either anti-FITC-hrp anti-penta-His Alexa Fluor 647 (Qiagen,) for Co-hArgI-*C^{PEG-20K}*, and Co-hArgI $\times 3$ respectively. Protein bands were detected and quantified using the SuperSignal Chemiluminescent Kit (Pierce, CA) or by excitation at 633 nm using a Typhoon scanner as appropriate. Where applicable serum hArgI concentrations were fit as a function of time to an extravascular model of administration (Equation 1) where C_p is the serum concentration of drug, K is the elimination rate, F is the fraction absorbed, $(X_a)_0$ is the administered dose, V is the volume of distribution, K_a is the absorption rate and t is time [30] using the software program, Kaleidagraph (Reading PA). An extravascular model was used to fit the data because we used an IP route of drug administration and could thus follow drug appearance and disappearance in the serum compartment. The clearance half-life was estimated directly from the fitted elimination rate K such that $t_{1/2} = \ln(0.5)/-K$.

$$C_p = \frac{K_a F (X_a)_0}{V (K_a - K)} (e^{-Kt} - e^{-K_a t}) \quad \text{Equation 1}$$

3. RESULTS

3.1 Construction of high M.W. Co-hArgI variants

Earlier studies have revealed that bovine arginase is cleared from circulation in mice with a $t_{1/2}$ of < 1 hr and that increasing its hydrodynamic radius via amine conjugation to PEG resulted in markedly increased persistence in circulation (12 hrs) [20]. However, in contrast to reports by Cheng and coworkers [24] we had reported earlier that Mn-hArgI is rapidly deactivated in serum with a $t_{1/2}$ of only 4.8 hours and that this phenomenon is due to the chelation of the Mn^{2+} metal cofactor of the enzyme by serum components. The reason for this discrepancy is discussed later. Because of the rapid deactivation of hArgI in serum, solely improving the pharmacokinetic behavior of Mn-hArgI conjugated to 5 KDa PEG would be highly unlikely to mediate a profile of L-Arg depletion in serum that is favorable for cancer therapy.

We recently reported that substitution of the Mn^{2+} cofactor of hArgI by Co^{2+} results in an enzyme that displays more than an order of magnitude better catalytic activity and 4-5 fold greater serum stability. The *in vitro* properties of Co-hArgI suggested that this enzyme might be much more suitable for L-Arg depletion therapy relative to the Mn-hArgI

employed in earlier studies. To use Co-hArgI *in vivo* we constructed, characterized biochemically and compared the pharmacokinetics and pharmacodynamics of three different types of enzyme formulations designed to increase the hydrodynamic radius and reduce renal filtration:

- (i) Construction of a covalently linked, hArgI $\times 3$ monomer. First, we deployed a protein engineering strategy to generate a single polypeptide that encodes three identical hArgI monomers joined together via flexible 31 aa linkers (GAGTGS GTGSGAGSGTGAGSGTGSGAGSGTG) (Figure 1 (A)). A 6 $\times$ His tag was appended onto the C-terminal, creating a single polypeptide with a calculated molecular mass of 110.3 KDa. Thus, Co-hArgI $\times 3$ has a MW that exceeds the cut-off for renal filtration which is estimated to be around 70 KDa [31]. The hArgI $\times 3$ gene was constructed in a four step process, as described in the Materials and Methods section. Under optimal expression conditions between 2 and 3 mg of purified Co-hArgI $\times 3$ per L culture were obtained in shake flasks. Co-hArgI $\times 3$ could be easily purified by IMAC to $\geq 95\%$ homogeneity as assessed by SDS-PAGE and staining with Coomassie Brilliant Blue. Co-hArgI $\times 3$ catalyzed L-Arg hydrolysis with a k_{cat}/K_M of $3,070 \pm 430 \text{ mM}^{-1} \text{ s}^{-1}$ compared to $1,270 \pm 330 \text{ mM}^{-1} \text{ s}^{-1}$ per monomer of Co-hArgI [19]. The higher k_{cat}/K_M is a direct result of the presence of three active-sites per polypeptide. Furthermore, the activity over time in pooled human serum was assessed and found to be essentially identical to our previously reported value for CohArgI indicating that the fusion of the monomers into the Co-hArgI $\times 3$ does not negatively affect protein stability [19]. Analytical size exclusion chromatography (SEC) indicated that apparent MW of Co-hArgI $\times 3$ is about 100 KDa (Figure 2 (A)) consistent with its calculated MW of 110.3 KDa.
- (ii) **Site-Specific PEGylated Co-hArgI-C^{PEG-20K}**. The PEGylation of biopharmaceutical products has become a useful means to prolong circulation half-life, improve solubility, and help protect from proteolysis and potential immune responses [32]. Structural analysis of hArgI shows that the enzyme contains three native Cys residues, none of which appears to be solvent exposed. Thus, we hypothesized that introduction of an additional Cys residue in front the N-terminal 6 $\times$ His tag would be a convenient site for specific conjugation to PEG. The resulting protein Co-hArgI-C could be produced at a high yield (approx. 200 mg/L shake flask) in *E.coli*. Following purification and reduction of the solvent-exposed Cys residue with TCEP, the protein was conjugated to PEG-20KDa-maleimide used at a 3 molar excess. Omitting TCEP from the reaction led to non-specific PEGylation demonstrating that after purification the N-terminal Cys was oxidized, presumably by forming a disulfide with L.M.W. thiols produced in *E.coli*. However, following reduction with TCEP, only about 50% of the Co-hArgI-C was found to be PEGylated as determined by SDS-PAGE. Conjugation reactions using a higher molar excess of PEG-20KDa-maleimide led to non-specific PEGylation as assessed by using hArgI lacking the N-terminal Cys residue as a control. The relatively low reaction yield with PEG-20KDa-maleimide suggested that a significant fraction of the N-terminal Cys residues in Co-hArgI-C had been oxidized to a non-reducible species such as sulfinic acids or sulfoxides. Attempts at separating PEGylated from unPEGylated material using size exclusion chromatography under conditions that did not affect the catalytic activity of the enzyme were unsuccessful. Further analysis indicated that the inability to separate PEGylated from un-PEGylated protein was due to the formation of mixed trimers in which only one or two of the Co-hArgI monomers had been conjugated to PEG-20KDamaleimide (Figure S2 A). We reasoned that this problem might be avoided by expressing a

monomeric form hArgI which could then be PEGylated to completion. The E256Q mutation had been shown to convert hArgI into a monomer which displayed near wild-type activity [25]. Co-hArgI-C E356Q was expressed and purified in *E.coli* and gel filtration chromatography confirmed that it is predominantly monomeric with a low amount of dimeric protein and no observable higher order oligomerization species. Co-hArgI-C E356Q was then conjugated to PEG-20KDa-maleimide as described above. Following gel filtration chromatography in PBS pH 7.4, we were able to prepare homogeneous PEGylated enzyme which was designated Co-hArgI-E256Q-C^{PEG-20K} (Figure S2 B). Unfortunately, the stability of Co-hArgI-E256Q-C^{PEG-20K} to deactivation in pooled human serum was greatly diminished, with a $t_{1/2}$ of only 20 ± 8 min.

Since E256 makes an ionic bond with R255 from an adjacent subunit we hypothesized that protonating E256 in WT hArgI could disrupt that bond and mimic the effect of E256Q in promoting the formation of monomeric enzyme. After evaluating SEC in a variety of low pH buffers (pH 3 – 5) we found that optimal preparation of monomeric and highly active enzyme could be obtained in sodium acetate buffer at pH 4.5. This strategy allowed good separation of Co-hArgI-C^{PEG-20K} from non-pegylated hArgI-C (Figure S2 C). Under these conditions we obtained a protein preparation comprised of ~90 % monomeric and ~10 % dimeric hArgI without irreversible loss of catalytic activity (Figure S3). Upon return to physiological pH Co-hArgI-C^{PEG-20K} resumes the trimeric form. Following incubation with CoCl₂ the measured specificity constant was virtually identical to previously measured values with $k_{cat}/K_M = 1,390 \pm 290$ s⁻¹mM⁻¹. The activity over time in pooled human serum was also assessed and found to be essentially identical to previously reported values [19].

- (iii) Multiply PEGylated hArgI-K^{PEG-5K} or Co-hArgI-K^{PEG-10K}. Purified Co-hArgI or Mn-hArgI was reacted for 1 hr with a 40-fold molar stoichiometric excess of PEG 5KDa succinimidyl ester at pH 8.3 yielding an apparently homogeneous product as assessed by SDS-PAGE and gel filtration (Figure S4). The respective proteins were designated Co-hArgI-K^{PEG-5K} and Mn-hArgI-K^{PEG-5K}. Analytical SEC of Co-hArgI-K^{PEG-5K} gave a calculated apparent MW of 750 KDa (Figure 2 (C)). We also partially incorporated FITC-labeled PEG 5K onto hArgI to facilitate quantification of the protein in serum samples. We obtained about 1 FITC-PEG 5K molecule per hArgI trimer as calculated from a standard curve of FITC-PEG-5K. Although all hArgI constructs described here have a 6 × His tag that can be detected by an anti-His antibody we found that FITC-PEG was a much more sensitive label for monitoring the concentration of enzyme in serum by immunochemical techniques. Additionally, Co-hArgI-K^{FITC-PEG-5K} could be detected directly by in gel fluorescence measurements.

The kinetics and serum stability of Co-hArgI-K^{PEG-5K} and Co-hArgI-K^{PEG-10K} was found to be essentially the same as previously reported values for non-PEGylated Co-hArgI [19]. However, Mn-hArgI-K^{PEG-5K} had a $k_{cat}/K_M = 65 \pm 9$ s⁻¹mM⁻¹, about 2-fold lower than non-PEGylated enzyme. On the other hand, Mn-hArgI-K^{PEG-5K} was found to be slightly more stable in serum, with an apparent $t_{1/2}$ of 9 hrs as opposed to a 4.8 hr for the unPEGylated enzyme [19]. In earlier studies PEGylated Mn-hArgI had been reported to have a half-life in serum of at least 3 days [24]. We observed that in those experiments enzyme stability had been determined using an enzyme preparation stored in 125 mM boric acid buffer that was diluted ~ 4 fold into plasma. Borate is a non-competitive inhibitor of arginase isozymes with a $K_I \sim 300$ μM and has been observed (crystallographically) to displace the nucleophilic water molecule and

to bridge the dinuclear metal center [33, 34]. Therefore, the presence of a large excess of borate might be able to complex the dinuclear metal center and aid in retention of the catalytic metal preventing deactivation by serum components. Consistent with this hypothesis when we incubated Mn-hArgI-K^{PEG-5K} in serum with borate buffer exactly as reported previously [24], 30 mM final concentration) it displayed less activity but greatly increased stability and was deactivated with a $t_{1/2}$ of more than 3 days.

Pharmacodynamics and Pharmacokinetics in Balb/c mice

Co-hArgI-K^{PEG-5K}, Mn-hArgI-K^{PEG-5K}, Co-hArgI-K^{PEG-10K}, Co-hArgI-C^{PEG-20K}, or Co-hArgI $\times 3$ were injected into mice ($n=5$ per group) by IP injection at a dose of 6 mg/kg weight. 20 microliter blood samples were collected by tail venipuncture at 6, 24, 48, 72, 100, and 168 hrs after injection and the concentration of L-Arg was determined by HPLC following derivatization with o-phthalaldehyde. We used an IP administration route because of its facile application and due to the fact that other enzyme therapeutics such as PEGylated and native Asparaginase have been demonstrated to partition to the serum compartment following IP injection [35]. Special caution was applied to ensure no L-Arg hydrolysis occurred after sample collection, either due to residual hArgI activity in the sample or due to the release of mouse arginase from erythrocyte lysis during cell separation. (We had observed that L-Arg from mouse blood collected in a neutral buffer was degraded over time which could be prevented by collection in a low pH buffer to inactivate endogenous or exogenous arginase activity [36]. Specifically, arginase activity was completely inhibited by immediately mixing blood samples with an excess of 400 mM citrate buffer at pH 4.0).

The time course of L-Arg concentration in blood as a function of time is shown in Figure 3. Surprisingly, we found that even though Co-hArgI $\times 3$ is stable in serum and has a higher expected M.W. than the renal filtration cutoff [31] IP administration of this enzyme preparation at 6 mg/kg weight had no effect on serum L-Arg levels. No enzyme could be detected by Western blot even within 6 hours after administration. These results suggested that Co-hArgI $\times 3$ was being rapidly cleared from circulation.

Administration of site-specifically PEGylated, Co-hArgI-C^{PEG-20K}, reduced the serum L-Arg levels to $\sim 12 \mu\text{M}$ at $t = 6$ hrs but returned to near normal by $t = 24$ hrs. Additionally and similar to Co-hArgI $\times 3$ we failed to detect Co-hArgI-C^{PEG-20K} by Western blotting even in the $t=6$ hr sample. These results indicate that the site-specifically PEGylated Co-hArgI is also cleared rapidly from circulation.

Co-hArgI-K^{PEG-5K} and Co-hArgI-K^{PEG-10K} were able to greatly reduce serum L-Arg to a level that remained below detection for 78 ± 10 hrs and 73 ± 8 hrs, respectively. In contrast, administration of the same dose of Mn-hArgI-K^{PEG-5K} reduced L-Arg to below detection limits for only 9 ± 3 hrs. (Figure 3 A–D)

To determine the pharmacokinetics of Co-hArgI-K^{PEG-5K} and Co-hArgI-K^{PEG-10K} we used protein labeled with FITC-appended PEG-5 KDa so that the protein could be easily detected by either in gel fluorescence assays or by western blot analysis using an anti-FITC antibody. Using an in-gel fluorescence assay of proteins from blood samples collected from mice treated with a 6 mg/kg dose of Co-hArgI-K^{PEG-5K}, we determined a clearance $t_{1/2}$ of 63 ± 10 hrs (Figure S5 shows a representative in-gel fluorescence assay). The pharmacokinetics of Co-hArgI-K^{PEG-10K} was nearly identical to Co-hArgI-K^{PEG-5K} (Figure S6) with Co-hArgI-K^{PEG-10K} yielding a similar elimination $t_{1/2}$ of 69 ± 20 hrs but with a slightly slower apparent absorption rate (K_a) (Figure 4).

These results demonstrate that amine conjugation of 5 KDa PEG offers a large pharmacokinetic advantage, greatly extending the circulation time of hArgI.

Dosage/Toxicity Study of Co-hArgI-K^{PEG-5K}—Consistent with recent reports, balb/c mice treated with high doses Co-hArgI-K^{PEG-5K} (> 8 mg/kg by IP injection) experience significant weight loss and signs of distress [37]. Mice treated with an inactivated 16 mg/kg dose showed no weight loss suggesting that the effects observed following administration of active Co-hArgI-K^{PEG-5K} were related to the enzyme function and the accompanying L-Arg depletion rather due to the presence of residual LPS and trace host cell proteins in the injected protein formulation (Figure S7 A). As reported previously, groups treated with 8 mg/kg or less displayed a moderate degree of weight loss around day four but did not show any other apparent signs of distress. The inactivated drug regained some activity *in vivo* as seen by a partial lowering of serum L-Arg levels (Figure S7 B). It appears, at least in balb/c mice, that toxicity is associated with long term L-Arg depletion (> 3 days) and suggests that the optimal biological dose is about 6–8 mg/kg.

4. DISCUSSION

Previously, non-PEGylated bovine arginase was reported to have a very short circulation half-life of < 1hr in the mouse [20]. Similarly, in a more recent report it was also found that even large doses of recombinant human hArgI could not fully deplete L-Arg in a rat model [38]. Thus, to capitalize on the high catalytic activity and serum stability of Co-hArgI it was necessary to formulate the enzyme in a manner that confers much longer circulation half-life and results in prolonged depletion of systemic L-Arg.

PEGylation represents a useful and widely applicable strategy to prolong circulation half-life, improve solubility, and provide protection from proteolysis and potential immune responses [32]. Of the therapeutic enzymes that are either FDA approved (adenosine deaminase, asparaginase) or in preclinical development (arginine deiminase, catalase, superoxide dismutase, uricase) multiple PEGylation of lysyl residues has been found to increase the circulatory half-lives by 10–24 fold in mice and in some cases to also reduce or delay antibody responses and proteolysis [39]. However, conjugation of PEG to exposed amines or carboxyls can in some cases lead to significant losses of activity and inevitably leads to a heterogeneous protein mixture that may be problematic from a regulatory standpoint. In the case of therapeutic antibody fragments (Fab's) or cytokines, where binding a target or receptor is important for biological activity, single site directed PEGylation is typically used for increasing circulatory persistence and can be more easily purified to homogeneity. Another clever strategy to increase circulatory longevity was recently developed through the use a 864 aa unstructured polypeptide, called XTEN, that is expressed as a fusion protein with a therapeutic protein or peptide and has been shown to increase plasma half-lives by one or two orders of magnitude [40].

We hypothesized that rapid clearance of hArgI was a consequence of the fact that the trimeric enzyme (105 KDa) exists in equilibrium with the monomer which is smaller than the exclusion limit for glomerular filtration. Hence, we sought to evaluate the increase in M.W. of Co-hArgI by joining the three polypeptide monomers that comprise the native trimeric enzyme via flexible peptide linkers. The resulting Co-hArgI ×3 enzyme could be readily expressed in *E.coli*, albeit at a lower yield relative to hArgI, was purified to near homogeneity, and was shown to display catalytic activity and serum stability nearly identical to the native enzyme. Unfortunately the pharmacokinetic properties of Co-hArgI ×3 in mice were found to be unsuitable for *in vivo* applications. We found that the protein is completely cleared within 6 hours after IP injection and results in essentially no reduction in the serum L-Arg concentration. Thus even though at >100 KDa Co-hArgI ×3 is larger than the size

limit for renal filtration it is nonetheless eliminated rapidly from circulation. Given that the protein is stable in serum the most likely explanation for its rapid clearance is that it is able to readily pass through the glomeruli. In an effort to prepare a pharmaceutical preparation of Co-ArgI that displays a high degree of homogeneity we explored the generation of site-specifically PEGylated enzyme. An accessible and readily reduced Cys residue was introduced to allow for maleimide specific conjugation of a PEG chain. However, despite extensive efforts to identify optimal reaction conditions, we obtained a mixture of mono, di and tri-PEGylated multimer in which one, two or all three of the hArgI monomers had been conjugated to PEG-20K maleimide. Efforts to separate the fully PEGylated trimeric enzyme from the mono- or di- PEGylated forms proved unsuccessful whereas prolonged reaction with PEG 20K-maleimide resulted in the conjugation of multiple PEG molecules per monomer (data not shown). A close examination of the mechanism for trimer formation suggested that incubation at low pH favors the dissociation of the enzyme trimer into monomers. Accordingly, we showed that at pH 4.5 the protein exists almost exclusively as a monomer and that the PEGylated monomer can be separated from un-PEGylated monomer with a very high efficiency. Returning the pH to normal then favors re-formation of the trimer. In this manner, we succeeded in preparing Co-hArgI-C^{PEG-20K} that was nearly 90% homogeneous. However, in mice at a dose of 6 mg/kg, Co-hArgI-C^{PEG-20K} resulted only in transient L-Arg depletion and the protein could not be detected in serum samples even at t=6 hrs following administration. Although few reports compare the pharmacokinetics of mono-PEGylated vs. multiply PEGylated proteins, one study compared human growth hormone (hGH) conjugated to either one PEG MW 20 KDa or to 4–5 PEG MW 5 KDa molecules. These two formulations were of nearly identical size by formula weight and SEC analysis but the multiply PEGylated hGH had at least a 4 fold increased serum retention suggesting that clearance is more than a function of size and may correlate with the number and/or sites of attachment [41].

In contrast to Co-hArgI $\times 3$ and Co-hArgI-C^{PEG-20K}, Co-hArgI-K^{PEG-5K} and Co-hArgI-K^{PEG-10K} formulations were able to deplete serum L-Arg to below detection levels for 78 ± 10 hrs and 73 ± 8 hrs, respectively. This also closely agreed with their pharmacokinetic profile with an elimination $t_{1/2}$ of 63 ± 10 hrs and $t_{1/2}$ of 69 ± 20 hrs, although it should be noted that Co-hArgI-K^{PEG-10K} had a slower apparent rate of absorption. One mechanism that may account for the much slower clearance of Co-hArgI-K^{PEG-5K} relative to the mono-PEGylated Co-hArgI-C^{PEG-20K} could be that more negatively charged proteins are retained in circulation serum longer than neutral or positively charged proteins due to the negative charge barrier in the glomeruli [42]. Co-hArgI has a net charge of $\sim +3$ at physiological pH, but PEGylating 8–10 lysyl residues confers a net charge of -5 to -7 . Thus, one possibility is that the much higher retention Co-hArgI-K^{PEG-5K} in circulation compared Co-hArgI-C^{PEG-20K} may be due to the much higher negative charge of the former resulting from multiple-lysyl PEGylation.

The PEGylation of arginase was first attempted over 30 years ago with bovine arginase and it was found to greatly increase the serum half-life, but did not greatly reduce the growth of a tapir liver tumor model in the mouse [20, 22]. This was undoubtedly due to the bovine enzyme's very high K_M for L-Arg (~ 12 mM), and the fact that it lost significant activity after PEGylation. Very recently a PEG-hArgI (PEGylated with O-[2-(N-succinimidyl)oxycarbonyl]-ethyl]-O'-methylpolyethylene-glycol (PEG) 5000 mw) was used in a lymphoblastic T cell leukemia (T-ALL) NOD-SCID mouse xenograft model where it appeared to be well tolerated and prolonged overall survival time when used in combination with the anti-metabolite, cytarabine [43]. This group's lowest reported dose (~ 5 mg/kg) shows a serum L-Arg clearance curve in good agreement with our dosing at 6 mg/kg with Mn-hArgI-K^{PEG-5K}. We found that a 6 mg/kg dose of Mn-hArgI-K^{PEG-5K} resulted in a serum L-Arg depletion time of 9 ± 3 hrs followed by a return to normal levels over the

next few days. In contrast a 6 mg/kg dose of Co-hArgI-K^{PEG-5K} gives a serum L-Arg depletion time of 78 ± 10 hrs (Figure 3A). This ~ 9 fold increase in dosage efficacy is well aligned with the greater serum stability, and the 10–15 fold increase in k_{cat}/K_M and *in vitro* cytotoxicity we reported previously with Co²⁺ substituted enzyme.

One area of confusion in the literature is the differential toxicities seen in rodent models with various L-Arg depleting regimens. Clark and coworkers reported that mice were very tolerant of L-Arg depletion even at 800 U/m² (~ 12 mg/kg) of PEG-ADI but that the enzyme is extremely toxic to rats at a 240 U/m² dose (~2 mg/kg) requiring euthanasia at 5 days [44]. In contrast to the PEG-ADI toxicity in rats, Cheng et al. reported that the use of ~30 mg/kg rhArgI-PEG5,000 mw in rats depletes L-Arg for over 5 days with no apparent toxicities [23]. In rats, L-Arg is considered essential during early growth but not for adults [45] thus some discrepancy in L-Arg depletion toxicity may be due to the age of the rats used in the studies above [23, 44]. Recently, Hernandez et al. dosed NOD-Scid mice with ~ 20 mg/kg (peg-Arg I) every 4 days $\times$ 3 cycles, depleting L-Arg for 12 days with almost no toxicity as assessed by body mass [43]. However in our study when balb/c mice were injected with $\geq$ 16 mg/kg Co-hArgI-K^{PEG-5K} we found significant weight loss after 4 days of L-Arg depletion, an effect not seen when Co-hArgI-K^{PEG-5K} was inactivated, indicating that L-Arg depletion causes weight loss. Another possibility is that the low K_M of ADI (~ 25 μ M) and Co-hArgI-K^{PEG-5K} (120 μ M) allows depletion of L-Arg to a lower absolute level than Mn²⁺ substituted hArgI which has a K_M of ~2.3 – 3 mM. A very low L-Arg level may also explain the differential toxicities reported for ADI and Mn-hArgI in rats and mice. Also of consideration are the reaction products of L-Arg hydrolysis, L-Cit and L-Orn from ADI and arginase respectively. Regeneration of L-Arg from L-Orn requires ornithine transcarbamylase for conversion to L-Cit, and is expressed mainly in the liver and intestine, whereas regeneration from L-Cit requires argininosuccinate synthase which is expressed in a wide variety of tissues [46]. Experimentally we have also found that care must be taken in the collection of blood samples; if adequate measures are not taken to quench arginase activity released from lysed red blood cells can lead to erroneously low L-Arg measurements.

We have found that failure to acid quench arginase activity from serum samples of untreated control mice can lead to samples devoid of L-Arg, a small but important detail.

As part of the present study we employed use of PEG-fluorescein labeled enzyme to enable the highly sensitive detection of PEGylated protein in serum and possibly in tissues, eliminating the need for specific antibodies to measure pharmacokinetics. Our hypothesis is that simple diffusion of L-Arg will allow it to be depleted from all extracellular spaces, but there is some debate that some tumors may exist in a protected environment. Visualizing where FITC Co-hArgI-K^{FITC-PEG-5K} physically partitions may shed some light on the issue as well as aid in determining physiological routes of elimination.

5. CONCLUSION

Arginase is a promising chemotherapeutic agent for the treatment of L-Arg auxotrophic tumors. In this work we compared three different approaches for improving the pharmacokinetic and pharmacodynamic behavior of Co²⁺ substituted hArgI. Modification with PEG-5K NHS esters was shown to increase the retention of the enzyme in circulation by about 2 orders of magnitude. Moreover, in the mouse model Co-hArgI-K^{PEG-5K} resulted in a 9-fold better pharmacodynamics, i.e. complete depletion of L-Arg in serum compared to identically prepared hArgI but containing the native metal cofactor of human arginase, Mn²⁺. Cobalt substitution gives hArgI virtually identical kinetics (k_{cat}/K_M) with the bacterial ADI in clinical trials. Excitingly, in recently published work, the use of Co-hArgI-K^{PEG-5K}

has translated into effective control of hepatocellular and pancreatic carcinomas in xenograft models[37]. Importantly, the fact that the former is a human protein for which immunological tolerance must pre-exist in patients should ameliorate the deleterious immune responses seen with ADI. Data from two recent clinical trials revealed that the use of PEG-ADI elicits strong neutralizing antibody responses and therefore L-Arg depletion is maintained for only a few days. Within 4–6 weeks the serum L-Arg concentration in patients was shown to return to near physiological level, a finding that may explain the relatively low percentage of objective responses reported in the clinical trials, [12, 47].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo. *Cancer Res.* 2002; 62(19):5443–5450. [PubMed: 12359751]
- [2]. Feun L, You M, Wu CJ, Kuo MT, Wangpaichitr M, Spector S, Savaraj N. Arginine deprivation as a targeted therapy for cancer. *Current pharmaceutical design.* 2008; 14(11):1049. [PubMed: 18473854]
- [3]. Yoon CY, Shim YJ, Kim EH, Lee JH, Won NH, Kim JH, Park IS, Yoon DK, Min BH. Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. *Int J Cancer.* 2007; 120(4):897–905. [PubMed: 17096330]
- [4]. Cheng N, Chytil A, Shyr Y, Joly A, Moses HL. Enhanced hepatocyte growth factor signaling by type II transforming growth factor-beta receptor knockout fibroblasts promotes mammary tumorigenesis. *Cancer Res.* 2007; 67(10):4869–4877. [PubMed: 17495323]
- [5]. Shen LJ, Beloussow K, Shen WC. Modulation of arginine metabolic pathways as the potential anti-tumor mechanism of recombinant arginine deiminase. *Cancer Lett.* 2006; 231(1):30–35. [PubMed: 16356828]
- [6]. Scott L, Lamb J, Smith S, Wheatley DN. Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer.* 2000; 83(6):800–810. [PubMed: 10952786]
- [7]. Izzo F, Marra P, Beneduce G, Castello G, Vallone P, De Rosa V, Cremona F, Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA, Ng C, Curley SA. Pegylated arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: results from phase I/II studies. *J Clin Oncol.* 2004; 22(10):1815–1822. [PubMed: 15143074]
- [8]. Feun L, Savaraj N. Pegylated arginine deiminase: a novel anticancer enzyme agent. *Expert Opin Investig Drugs.* 2006; 15(7):815–822.
- [9]. Ni Y, Schwaneberg U, Sun ZH. Arginine deiminase, a potential anti-tumor drug. *Cancer Lett.* 2008; 261(1):1–11. [PubMed: 18179862]
- [10]. Wheatley DN. Arginine deprivation and metabolomics: important aspects of intermediary metabolism in relation to the differential sensitivity of normal and tumour cells. *Semin Cancer Biol.* 2005; 15(4):247–253. [PubMed: 15886013]
- [11]. Ascierto PA, Scala S, Castello G, Daponte A, Simeone E, Ottaiano A, Beneduce G, De Rosa V, Izzo F, Melucci MT, Ensor CM, Prestayko AW, Holtsberg FW, Bomalaski JS, Clark MA, Savaraj N, Feun LG, Logan TF. Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. *J Clin Oncol.* 2005; 23(30):7660–7668. [PubMed: 16234528]

- [12]. Glazer ES, Piccirillo M, Albino V, Di Giacomo R, Palaia R, Mastro AA, Beneduce G, Castello G, De Rosa V, Petrillo A, Ascierto PA, Curley SA, Izzo F. Phase II Study of Pegylated Arginine Deiminase for Non-resectable and Metastatic Hepatocellular Carcinoma. *JCO*. 2010
- [13]. Sarkissian CN, Gamez A. Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria, where are we now? *Mol Genet Metab*. 2005; 86(Suppl 1):S22–26. [PubMed: 16165390]
- [14]. Yang Z, Wang J, Yoshioka T, Li B, Lu Q, Li S, Sun X, Tan Y, Yagi S, Frenkel EP. Pharmacokinetics, methionine depletion, and antigenicity of recombinant methioninase in primates. *Clinical Cancer Research*. 2004; 10(6):2131. [PubMed: 15041734]
- [15]. Dinndorf PA, Gootenberg J, Cohen MH, Keegan P, Pazdur R. FDA drug approval summary: pegaspargase (oncaspar) for the first-line treatment of children with acute lymphoblastic leukemia (ALL). *Oncologist*. 2007; 12(8):991–998. [PubMed: 17766659]
- [16]. Dillon BJ, Holtsberg FW, Ensor CM, Bomalaski JS, Clark MA. Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. *Med Sci Monit*. 2002; 8(7):BR248–253. [PubMed: 12118186]
- [17]. Lopez V, Alarcon R, Orellana MS, Enriquez P, Uribe E, Martinez J, Carvajal N. Insights into the interaction of human arginase II with substrate and manganese ions by site-directed mutagenesis and kinetic studies. Alteration of substrate specificity by replacement of Asn149 with Asp. *Febs J*. 2005; 272(17):4540–4548. [PubMed: 16128822]
- [18]. Cheng PN, Leung YC, Lo WH, Tsui SM, Lam KC. Remission of hepatocellular carcinoma with arginine depletion induced by systemic release of endogenous hepatic arginase due to transhepatic arterial embolisation, augmented by high-dose insulin: arginase as a potential drug candidate for hepatocellular carcinoma. *Cancer Lett*. 2005; 224(1):67–80. [PubMed: 15911102]
- [19]. Stone E, Glazer ES, Chantranupong L, Cherukuri P, Breece RM, Tierney DL, Curley SA, Iverson BL, Georgiou G. Replacing Mn 2+ with Co 2+ in Human Arginase I Enhances Cytotoxicity Towards L-Arginine Auxotrophic Cancer Cell Lines. *ACS Chemical Biology*. 2010
- [20]. Savoca KV, Abuchowski A, Van Es T, Davis FF, Palczuk NC. Preparation of a non-immunogenic arginase by the covalent attachment of polyethylene glycol. *Biochimica et Biophysica Acta (BBA)-Protein Structure*. 1979; 578(1):47–53.
- [21]. Lavulo LT, Sossong TM, Brigham-Burke MR, Doyle ML, Cox JD, Christianson DW, Ash DE. Subunit-Subunit Interactions in Trimeric Arginase. *Journal of Biological Chemistry*. 2001; 276(17):14242. [PubMed: 11278703]
- [22]. Savoca KV, Davis FF, Van Es T, McCoy JR, Palczuk NC. Cancer therapy with chemically modified enzymes. II. The therapeutic effectiveness of arginase, and arginase modified by the covalent attachment of polyethylene glycol, on the taper liver tumor and the L5178Y murine leukemia. *Cancer biochemistry biophysics*. 1984; 7(3):261. [PubMed: 6488153]
- [23]. Cheng PNM, Lam TL, Lam WM, Tsui SM, Cheng AWM, Lo WH, Leung YC. Pegylated recombinant human arginase (rhArg-peg5, 000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Research*. 2007; 67(1): 309. [PubMed: 17210712]
- [24]. Cheng NM, Leung YC, Lo WH. Pharmaceutical preparation and method of treatment of human malignancies with arginine deprivation. *Google Patents*. 2008
- [25]. Sabio G, Mora A, Rangel MA, Quesada A, Marcos CF, Alonso JC, Soler G, Centeno F. Glu-256 is a main structural determinant for oligomerisation of human arginase I. *FEBS Lett*. 2001; 501(2–3):161–165. [PubMed: 11470277]
- [26]. Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein expression and purification*. 2006; 46(2):483–488. [PubMed: 16290005]
- [27]. Kurfürst MM. Detection and molecular weight determination of polyethylene glycol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Analytical biochemistry*. 1992; 200(2):244–248. [PubMed: 1378701]
- [28]. Ullman-Culleré MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. *Laboratory animal science*. 1999; 49:319–323. [PubMed: 10403450]

- [29]. Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K. Modeling of the Bacterial Growth Curve. *Appl Environ Microbiol.* 1990; 56(6):1875–1881. [PubMed: 16348228]
- [30]. Williams, DA.; Foye, WO.; Lemke, TL. Foye's principles of medicinal chemistry. Lippincott Williams & Wilkins; 2002.
- [31]. Lote, CJ. Principles of renal physiology. Kluwer Academic Pub; 2000.
- [32]. Jevševar S. PEGylation of therapeutic proteins. *Biotechnology Journal.* 2010; 5(1):113–128. [PubMed: 20069580]
- [33]. Baggio R, Elbaum D, Kanyo ZF, Carroll PJ, Cavalli RC, Ash DE, Christianson DW. Inhibition of Mn²⁺ 2-arginase by borate leads to the design of a transition state analogue inhibitor, 2 (S)-amino-6-boronohexanoic acid. *J. Am. Chem. Soc.* 1997; 119(34):8107–8108.
- [34]. Colleluori DM, Morris SM Jr, Ash DE. Expression, purification, and characterization of human type II arginase. *Arch Biochem Biophys.* 2001; 389(1):135–143. [PubMed: 11370664]
- [35]. Inada Y, Matsushima A, Hiroto M, Nishimura H, Kodera Y. Chemical modification of proteins with polyethylene glycols. *Microbial and Enzymatic Bioproducts.* 1995:129–150.
- [36]. Hosoyama Y. The Reversible Inactivation of Rat Liver Arginase at Low pH. *European Journal of Biochemistry.* 1972; 27(1):48–52. [PubMed: 5049056]
- [37]. Glazer ES, Stone EM, Zhu C, Massey KL, Hamir AN, Curley SA. Bioengineered Human Arginase I with Enhanced Activity and Stability Controls Hepatocellular and Pancreatic Carcinoma Xenografts. *Translational Oncology.* 2011; 4(3):138. [PubMed: 21633669]
- [38]. Tsui SM, Lam WM, Lam TL, Chong HC, So PK, Kwok SY, Arnold S, Cheng PNM, Wheatley DN, Lo WH. Pegylated derivatives of recombinant human arginase(rhArg 1) for sustained in vivo activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics in vivo and in vitro and action upon hepatocellular carcinoma cell(HCC). *Cancer Cell International.* 2009; 9:9–9. [PubMed: 19374748]
- [39]. Fishburn CS. The pharmacology of PEGylation: Balancing PD with PK to generate novel therapeutics. *Journal of pharmaceutical sciences.* 2008; 97(10):4167–4183. [PubMed: 18200508]
- [40]. Schellenberger V, Wang C, Geething NC, Spink BJ, Campbell A, To W, Scholle MD, Yin Y, Yao Y, Bogin O. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. *Nature Biotechnology.* 2009
- [41]. Finn RF. PEGylation of human growth hormone: strategies and properties. *PEGylated Protein Drugs: Basic Science and Clinical Applications.* 2009:187–203.
- [42]. Sörensson J, Ohlson M, Lindström K, Haraldsson B. Glomerular charge selectivity for horseradish peroxidase and albumin at low and normal ionic strengths. *Acta Physiologica Scandinavica.* 1998; 163(1):83–91. [PubMed: 9648626]
- [43]. Hernandez CP, Morrow K, Lopez-Barcons LA, Zabaleta J, Sierra R, Velasco C, Cole J, Rodriguez PC. Pegylated arginase I: a potential therapeutic approach in T-ALL. *Blood.* 2010
- [44]. Bomalaski JS, Ivett JL, Vegarra M, Holtsberg FW, Ensor CM, Clark MA. Comparative toxicity of arginine deiminase formulated with poly(ethylene glycol) 5000 or 20,000 and the effects of arginine. *Preclinica.* 2003; 1:284–293.
- [45]. Ball RO, Urschel KL, Pencharz PB. Nutritional consequences of interspecies differences in arginine and lysine metabolism. *Journal of Nutrition.* 2007; 137(6):1626S. [PubMed: 17513439]
- [46]. Husson A, Brasse Lagnel C, Fairand A, Renouf S, Lavoinne A. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *European Journal of Biochemistry.* 2003; 270(9): 1887–1899. [PubMed: 12709047]
- [47]. Yang TS, Lu SN, Chao Y, Sheen IS, Lin CC, Wang TE, Chen SC, Wang JH, Liao LY, Thomson JA. A randomised phase II study of pegylated arginine deiminase (ADI-PEG 20) in Asian advanced hepatocellular carcinoma patients. *British Journal of Cancer.* 2010

Figure 1.

Schematic representation of the pharmacological formulations of hArgI: A) hArgI $\times 3$: three polypeptides of arginase linked N-terminal to C-terminal with a flexible amino acid linker. B) hArgI-C^{PEG-20K}: a Cys residue was engineered into the N-terminal for conjugation with PEG-20K maleimide. C) hArgI-K^{PEG-5K} or Co-hArgI-K^{PEG-10K}: the lysyl residues of hArgI were conjugated with PEG 5 or 10K succinimidyl-ester.

Figure 2. Analytical SEC of hArgI Formulations: (A) The Co-hArgI $\times 3$ fusion protein with an apparent MW of 100 KDa. (B) The singly PEGylated Co-hArgI-C^{PEG-20K} with an apparent mass of 520 KDa. (C) The multiply PEGylated Co-hArgI-K^{PEG-5K} with an apparent MW of 750 KDa.

Figure 3.

in vivo serum L-Arg levels in mice following IP injection at a 6 mg/kg dose each of: A) Co-hArgI-K^{PEG-5K} (●), B) Mn-hArgI-K^{PEG-5K} (○), C) Co-hArgI-C^{PEG-20K} (◆) and D) Co-hArgI x3 (▲). Co-hArgI-K^{PEG-5K} (●) yielded a calculated serum L-Arg depletion time of 78 ± 10 hrs. B) Mn-hArgI-K^{PEG-5K} (○) with a calculated serum L-Arg depletion time of 9 ± 3 hrs. C) Co-hArgI-C^{PEG-20K} with an estimated serum L-Arg depletion time of ~ 0.5 hrs and Co-hArgI x3 (▲) showing no apparent effect upon serum L-Arg levels. (Figures C & D: the fitting lines were extrapolated to 200 hrs to provide a sense of scale with Figures A & B)

Figure 4.

Pharmacokinetics in balb/c mice following IP administration at 6 mg/kg. Each sample contained a portion of protein PEGylated with FITC terminating polyethylene glycol and the concentration of protein was monitored by in-gel fluorescence: of (●) Co-hArgI-K^{PEG-5K} (n=5). The absorption rate (K_a) was too fast to estimate, but the elimination rate (K_{el}) was well fit by equation 1, yielding a serum $t_{1/2}$ life of 63 ± 10 hrs. (□) Co-hArgI-K^{PEG-10K}. Absorption rate $K_a = 0.13 \pm 0.05 \text{ hr}^{-1}$; $t_{1/2} = 69 \pm 20$ hrs.