Cytotoxicity of human recombinant arginase I (Co)-PEG5000 in the presence of supplemental L-citrulline is dependent on decreased argininosuccinate synthetase expression in human cells

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Human recombinant arginase I cobalt [HuArgI (Co)] coupled with polyethylene glycol 5000 [HuArgI (Co)-PEG5000] has shown potent in-vitro depletion of arginine from tissue culture medium. We now show that HuArgI (Co)-PEG5000 is toxic to almost all cancer cell lines and to some normal primary cells examined. In contrast, HuArgI (Co)-PEG5000 in combination with supplemental L-citrulline is selectively cytotoxic to a fraction of human cancer cell lines in tissue culture, including some melanomas, mesotheliomas, acute myeloid leukemias, hepatocellular carcinomas, pancreas adenocarcinomas, prostate adenocarcinomas, lung adenocarcinomas, osteosarcomas, and small cell lung carcinomas. Unfortunately, a subset of normal human tissues is also sensitive to HuArgI (Co)-PEG5000 with L-citrulline supplementation, including umbilical endothelial cells, bronchial epithelium, neurons, and renal epithelial cells. We further show that cell sensitivity is predicted by the level of cellular argininosuccinate synthetase protein expression measured by immunoblots. By comparing a 3-day and 7-day exposure to HuArgI (Co)-PEG5000 with supplemental L-citrulline, some tumor cells sensitive on short-term assay are resistant in the 7-day assay consistent with the induction of argininosuccinate synthetase expression. On the basis of these results, we hypothesize that HuArgI (Co)-PEG5000 in combination with L-citrulline supplementation may be an attractive therapeutic agent for some argininosuccinate synthetase-deficient tumors. These in-vitro findings stimulate further development of this molecule and may aid in the identification of tissue toxicities and better selection of patients who will potentially respond to this combination therapy. *Anti-Cancer Drugs* 23:51–64 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Chemotherapeutic regimens have limited activity in most patients with metastatic cancers. The poor clinical performance of traditional treatments has led to the development of agents that specifically target molecular pathways that are relatively unique to cancer cells. Nonessential amino acid auxotrophy has been observed for a number of different malignancies [1]. Depletion of such amino acids should inhibit auxotrophic tumor growth. One such nonessential amino acid with tumor cell dependency is L-arginine. The growth of rodent breast and colon carcinomas was observed to be associated with extracellular arginine levels [2,3]. Further, mycoplasma arginini infections of tumors induced cell cycle arrest and cell death because of arginine deiminase secreted by these organisms [4–6]. Subsequent studies have established a urea cycle defect in these arginine auxotrophic neoplasms, namely, deficiency of cytoplasmic argininosuccinate synthetase (ASS) [7–9]. Arginine auxotrophy has been observed in a fraction of hepatocellular carcinomas, malignant melanomas, mesotheliomas, pancreatic adenocarcinomas, and prostate adenocarcinomas [10–13]. This molecular defect provides an attractive target for biological therapy.

Three protein agents have been developed to target arginine auxotrophic tumors. Pegylated recombinant arginine deiminase (ADI-PEG20) degrades plasma arginine to citrulline and ammonia, but it is immunogenic [14–16]. One drawback of ADI-PEG20 is onset of hyperammonemia, which was reported in 5.9% of patients at a dose of 320 IUm^{-2} [16]. Elevated ammonia levels may be toxic *in vivo* [17]. Pegylated recombinant human arginase [ArgI (Mn)-PEG5000] metabolizes arginine to ornithine and urea and has shown antitumor activity *in vitro* and *in vivo* [15,18–21]. However, past studies have found that the HuArgI (Mn)-PEG5000 enzyme is unstable in serum and displays low catalytic activity at physiological pH [22]. Recently, pegylated recombinant human Arg I containing two Co^{2+} atoms instead of Mn^{2+} in the native human

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enzyme [HuArgI (Co)-PEG5000] was prepared [22]. The reaction chemistry catalyzed by HuArgI (Co)-PEG5000 is identical to that of the Mn^{2+} enzyme, but the kinetics of the arginine hydrolysis and the stability of the enzyme in serum are markedly improved by the Co^{2+} substitution [22,23]. Given the safety and efficacy of arginine deprivation previously reported in patients with melanomas and hepatocellular carcinomas [16,24,25]; here, we investigate in detail the anticancer efficacy of HuArgI (Co)-PEG5000 and identify a potential predictive biomarker for response. The molecularly targeted agent likely has advantages over current arginine depletion enzymes because of the anticipated reduced immunogenicity of the human enzyme and the greater enzyme activity and stability due to Co^{2+} substitution.

We analyzed both tumor and normal cell sensitivity to HuArgI (Co)-PEG5000 in 3-day and 7-day cytotoxicity assays and also cell levels of ASS and ornithine transcarbamylase (OTC) [26]. We tested cell sensitivity in the presence and absence of supplemental L-citrulline. Normal blood L-citrulline levels range from 12 to 61 μmol/l [27]. As we used 10% fetal bovine serum in our media, the baseline L-citrulline concentration in the media was 1.5–3.8 μmol/l. In some experiments, L-citrulline was added to a final concentration of 38 mmol/l. This level of L-citrulline was consistent for all cell lines tested. L-citrulline only requires the presence of ASS and argininosuccinate lyase to replenish cellular arginine stores [28]. Coadministration of HuArgI (Co)-PEG5000 with L-citrulline may improve the therapeutic index by protecting normal tissues unable to rescue arginine from ornithine. Furthermore, because amino acid depletion can induce cell cycle arrest, protein synthesis inhibition, and apoptosis, we measured the inhibition of both cellular DNA and protein synthesis, as well as overall growth inhibition measured by metabolic conversion of tetrazolium salt to a formazan [19,29].

Materials and methods

Reagents

HuArgI (Co)-PEG5000 and HuArgI (Mn)-PEG5000 were produced from purified HuArgI expressed in *Escherichia coli* [22]. In brief, the enzyme in 100 mmol/l of sodium phosphate, pH 8.3, was reacted with a 40-fold excess of methoxy PEG succinimidyl carboxymethyl ester 5000 MW (JemKem Technology, Allen, Texas, USA) for 1 h at 25°C. The mixture was then incubated with 10 mmol/l of cobalt chloride hexahydrate (MP Biomedicals, Solon, Ohio, USA) and heated to 50°C for 10 min. HuArgI (Co)-PEG5000 was then centrifuged, dialyzed into phosphate-buffered solution (PBS) with 10% glycerol, sterile filtered, aliquoted, and stored at −80°C until use. L-Citrulline was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). A monoclonal antibody to ASS was obtained from BD Biosciences (San Jose, California, USA); a polyclonal rabbit anti-OTC antibody was obtained from GeneTex (cat no GTX105140; Irvine, California, USA); a monoclonal antibody to β-actin (cat no 47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig antibody was from GeneTex; HRP-conjugated goat anti-mouse Ig light chain antibody was purchased from Jackson ImmunoResearch (West Grove, Pennsylvania, USA); HRP-conjugated goat anti-biotin antibody, Lumiglo reagents and peroxide, nonfat milk, biotinylated protein ladder, and prestained protein ladder were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA).

Cell lines and cell culture

Human cancer cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Normal human primary cells were purchased from ScienCell Research Laboratories (Carlsbad, California, USA). Media, sera, and trypsin were obtained from the same vendors as the cells. Cells were grown as recommended by the vendors. All cells were maintained in a 37°C/5% CO_2 environment, except cells requiring Leibovitz media, which were grown at 37°C/1% CO_2.

Cytotoxicity assays

Three-day cell proliferation and protein synthesis inhibition assays were performed. In brief, 10^6 cells were resuspended in 20 ml of complete growth medium at a density of 3×10^4 cells/ml. A volume of 100 μl was plated per well in two Costar 96-well flat-bottom plates. Cells were allowed to recover for 1–6 h. Serial three-fold dilutions of HuArgI (Co)-PEG5000 at final concentrations of 0–1 μmol/l in complete medium or complete medium and 114 mmol/l of L-citrulline was prepared, and 50-μl aliquots were added to wells containing cells. Cells were incubated for 54 h at 37°C with either 5 or 1% CO_2. ^{3}H-Thymidine (1 μCi; NEN DuPont) or ^{3}H-leucine (1 μCi; NEN DuPont) in 50 μl of complete medium or complete medium with 114 mmol/l of L-citrulline was added and incubated at 37°C/5% CO_2 or 1% CO_2 for an additional 18 h. Plates were frozen for 2 h at −80°C and then thawed for 2 h at 37°C. Cells were harvested onto glass fiber mats on a Molecular Devices Micro 96 Cell Harvester (Molecular Devices, Sunnyvale, California, USA). Mats were then wetted with Betaplate Scint liquid scintillation fluid and tritium counted on a Perkin Elmer MicroBetaLux counter (Waltham, Massachusetts, USA). All assays were performed in triplicate. Mean and standard errors of the mean were determined for each data point. Data were plotted as the percentage of incorporation inhibition relative to control wells versus arginase concentration. The concentration of arginase producing 50% inhibition of thymidine or leucine incorporation (IC_{50} values) was determined by interpolation from adjacent points on the curve. Maximal percentage inhibition was the value at 0.33 μmol/l of arginase. These parameters have been described previously [30].

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Three-day cell metabolic inhibition assays were performed. Aliquots of 2×10^4 cells/ml were plated in 96-well flat-bottom Costar plates with 0.33 μmol/l of HuArgI (Co)-PEG5000, 142 mmol/l of cyclohexamide, or media alone and incubated for 3 days at 37°C with 5% CO_2. Cells were then incubated with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS; Promega Fisher, Madison, Wisconsin, USA) for 1–4 h with absorbance readings at 490 nm using the Molecular Devices VersaMax microplate reader (Molecular Devices). Background absorbance readings were averaged and subtracted from measured values. The percentage of cell metabolic inhibition was determined by calculating percentage absorbance in treated wells relative to nontreated controls, followed by subtraction from 100%.

For 7-day metabolic inhibition assays, cells were suspended at a final density of 3×10^4 cells/ml. Aliquots of 100 μl were placed in Costar 96-well round-bottom or flat-bottom plates. Flat-bottom plates were used for adherent cells and round-bottom plates for nonadherent cells. A volume of 50 μl of media with 114 mmol/l of L-citrulline or media alone was added to all wells. Cells were then treated with either 0.33 μmol/l of HuArgI (Co)-PEG5000 or 142 mmol/l of cyclohexamide (Sigma-Aldrich) or left untreated. The plates were incubated for 3 days at 37°C with 5% CO_2 or 1% CO_2, as described above. After 3 days of incubation, 0.33 μmol/l of HuArgI (Co)-PEG5000 was reintroduced to the wells treated initially with 0.33 μmol/l of HuArgI (Co)-PEG5000. The plates were incubated for 3 more days. After 6 days, media were removed from all wells, followed by a wash with 200 μl of PBS. Media with or without citrulline were then readded. Nonadherent cells were centrifuged at $716 \times g$ for 5 min in a Beckman Coulter Allegra 25R centrifuge to pellet cells. Media were removed, and cells were resuspended with gentle pipetting in media with or without supplemental L-citrulline. A volume of 142 μmol/l of cyclohexamide was reintroduced into wells initially treated with cyclohexamide. Cells were incubated for an additional 24 h, followed by addition of media with or without L-citrulline to empty wells in the 96-well plates for background absorbance. A volume of 20 μl of MTS was added to all wells, including background wells, and incubated at 37°C with 5% or 1% CO_2 for 1–4 h. Readings were taken for 1–4 h at 490 nm using the Molecular Devices VersaMax microplate reader (Molecular Devices). Background absorbance readings were averaged and subtracted from measured values. The percentage of cell survival was determined by calculating percentage absorbance in treated wells relative to nontreated controls. Molar excess of cyclohexamide was used to serve as a positive control, and nontreated wells were used as the negative control. The percentage of metabolic inhibition was calculated by subtracting averaged background absorbance from both nontreated and cyclohexamide-treated group, followed by subtraction from 100%. All assays were performed in triplicate.

To more accurately estimate the concentration of L-citrulline required to prevent nonspecific toxicity, we repeated the HuArg (Co)-PEG5000-resistant SKBr3 cell sensitivity proliferation assay with seven different concentrations of L-citrulline ranging from 0.052 to 38 mmol/l. Both IC_{50} values and percentage of inhibition were determined.

Arginine concentrations of the different cell growth media were obtained from the manufacturers. Arginine levels were 0.10, 0.10, 0.10, 0.24, 0.30, 0.30, 0.33, 0.48, 0.50, 0.73, 1.20, 2.0, 2.4, or 2.90 mmol/l for endothelial cell media, renal epithelial cell media, hepatocyte media, modified McCoys 5a, neuron media, cardiomyocyte media, CMRL 1066, Iscove's, Dulbecco's modified Eagle's medium, Eagle's minimal essential medium, Roswell Park Memorial Institute medium 1640, bronchial epithelium media, F12K, and Leibovitz L-15, respectively.

Western blot

Cells were seeded in T-75 or T-150 flasks. At confluence, the cells were washed with PBS, trypsinized and collected in media, washed twice with PBS, and lysed with 1.5 × cell pellet volume of 1% Triton X-100, 50 mmol/l of NaCl, and 25 mmol/l of Tris, at pH 7.5. Additional lysates for OTC assay were prepared using the radioimmunoprecipitation assay buffer with 0.1% SDS and protease inhibitors (Sigma, St. Louis, Missouri, USA). Lysates were centrifuged at $15\,000 \times g$. The protein concentration in the supernatant after centrifugation was determined using a Pierce Protein BCA kit (Thermo Fisher Scientific Inc., Rockford, Illinois, USA). Lysates with 18–23 mg/ml of protein were mixed 1 : 1 with 2 × Laemmli sample buffer containing 5% β-mercaptoethanol and stored at −80°C until use. Normal tissue lysates were purchased from ScienCell Research Laboratories. A volume of 5 μl of tumor cell lysates or 25 μl of normal tissue lysates (approximately 50 μg of protein) was loaded on 10 or 12% SDS-polyacrylamide gel electrophoresis Tris–glycine gels, electrophoresed at 150 V for 80 min, equilibrated for 5 min in 48 mmol/l of Tris–HCl, pH 8.3; 39 mmol/l of glycine; 0.037% SDS; and 20% methanol, transferred to nitrocellulose with an Amersham Biosciences Multiphor II semi-dry electroblotter (GE, Pittsburgh, Pennsylvania, USA), and blocked for 30–45 min in 5% nonfat dry milk/Tris-buffered solution (TBS). For OTC immunoblots, blocking buffer consisted of 5% nonfat dry milk/0.1% Tween-20/TBS. Blots were washed three times with TBS and 0.05% Tween-20 (TBST), reacted overnight at 4°C with 1 : 1000 mouse monoclonal anti-ASS in TBST or 1 : 400 rabbit anti-OTC in blocking buffer, rewashed with TBST, reacted with HRP-conjugated goat anti-mouse Ig (1 : 2000) for 1 h at room temperature or with HRP-conjugated goat anti-rabbit Ig (1 : 5000) for 2 h at room

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temperature, rewashed with TBST, and developed with Lumiglo reagents following the recommendations of the manufacturer. Band intensity was determined by a Fluorchem SP densitometer (Cell Biosciences/Alpha Innotech, Santa Clara, California, USA). Blots were then washed, stripped with 40 ml of 63-mmol/l Tris, pH 6.8; 2% SDS; and 0.7% β-mercaptoethanol at 55°C for 30 min, rewashed, blocked for 60 min in blocking buffer with or without Tween-20, washed, reacted overnight at 4°C with mouse monoclonal anti-β-actin antibody (1 : 500 in TBST), rewashed, incubated with HRP-conjugated goat anti-mouse Ig, washed, and finally developed as above. ASS and OTC levels were standardized to lysate β-actin levels.

Arginine levels in media

SKBr3 cells were incubated at 20 000 cells/ml in McCoys5a+10% fetal bovine serum media with or without 0.33 µmol/l of HuArgI (Co)-PEG5000 or HuArgI(Mn)-PEG5000 for 3 days at 37°C/5% CO_2. Media were then harvested and centrifuged at 10 000 × *g* for 5 min at 21°C. Supernatants were assayed for arginine by C18 high-performance liquid chromatography as previously described [23].

Statistical analysis

Analyses were performed using the SPSS Statistical software (version 18.0; SPSS Inc., Chicago, Illinois, USA) and R 2.12.1 (2010, R Core Development Team). Spearman's correlation coefficients were assessed as measures of association between variables of interest. For scatter plots between percentage of inhibitions and ASS, we fit a linear regression line after taking natural log transformation of ASS. *P* values of less than 0.05 were considered statistically significant.

Results

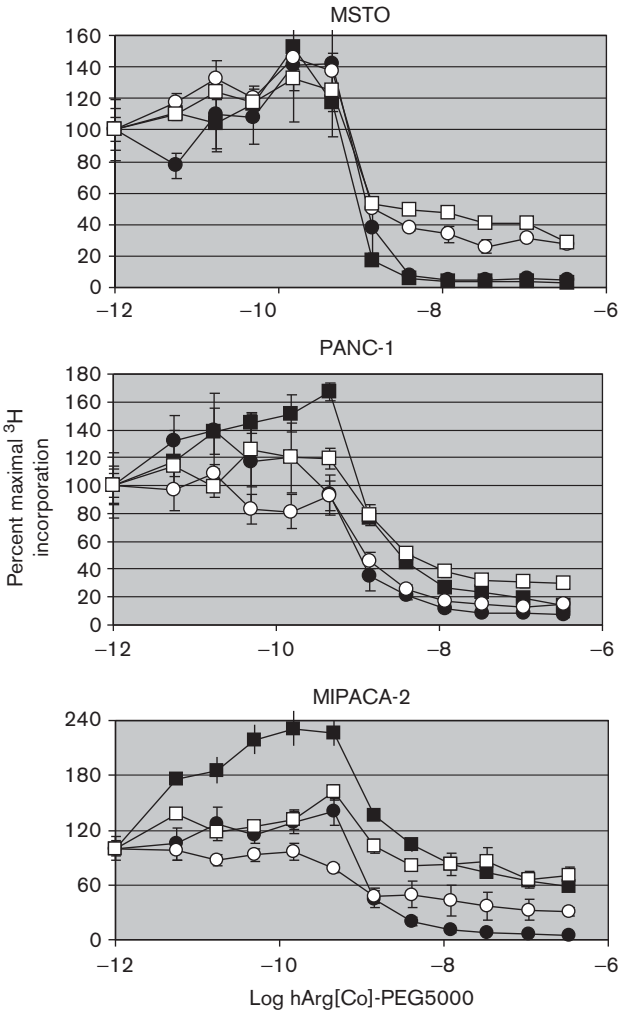
HuArgI (Co)-PEG5000 characterization

HuArgI (Co)-PEG5000 fast protein liquid chromatography size exclusion Sephedex 200 column chromatography revealed that the protein eluted as a single peak with an apparent molecular weight of 750 kDa and contained less than 1% aggregated material (unpublished data). The k_{cat}/K_m displayed by HuArgI (Co)-PEG5000 was determined to be 1270 ± 130 mmol/l^{-1} s^{-1} in serum compared with wild-type HuArgI (Mn)-PEG5000 prepared under identical conditions, which yielded a k_{cat}/K_m value of 65 ± 9 mmol/l^{-1}s^{-1} (unpublished data).

HuArgI (Co)-PEG5000 3-day human tumor cell proliferation and protein synthesis inhibition

We tested a panel of 22 human cancer cell lines for sensitivity to HuArgI (Co)-PEG5000 in the absence or presence of supplemental 38 mmol/l of L-citrulline (Fig. 1; Tables 1 and 2). Remarkably, arginine depletion for 3 days resulted in uniform inhibition of proliferation for all human cells examined, with maximal inhibition of 49–99% at 0.33 µmol/l of HuArgI (Co)-PEG5000 (Fig. 2; Table 1). Protein synthesis was inhibited to a lesser degree but nonetheless was observed for all cells examined, with the exception of one osteosarcoma cell line (SaOS2), with a range of 40–89% inhibition.

Fig. 1



Three-day HuArgI (Co)-polyethylene glycol (PEG) 5000 cell proliferation and protein synthesis inhibition assays. Cell lines shown are MSTO, PANC-1, and MIAPACA-2. Plots of percentage maximal incorporation versus HuArgI (Co)-PEG5000 concentration. ●, thymidine; ■, thymidine and L-citrulline; ○, leucine; □, leucine and L-citrulline.

To identify conditions under which HuArgI (Co)-PEG5000 is selectively toxic for tumor cells, we tested the agent in combination with an excess of L-citrulline. Under these conditions, as measured by both proliferation and protein synthesis inhibition, some cells were sensitive to arginine depletion, whereas some were not (Fig. 2; Table 1). Twelve human cancer cell lines showed

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Table 1 HuArgI (CO)-polyethylene glycol 5000 3-day proliferation inhibition

		Absence of L-citrulline		Addition of L-citrulline	
Tumor	Cell line	IC_{50} (nmol/l)	INH (%)	IC_{50} (nmol/l)	INH (%)
Melanoma	A375	1	98	1	99
Melanoma	SKMEL28	1	98	1	98
Mesothelioma	MSTO	1	96	1	97
Lung cancer	A549	1	99	3	95
AML	KASUMI-1	1	80	3	90
Osteosarcoma	SAOS2	5	91	5	89
SCLC	SW1271	8	84	20	88
Pancreas cancer	PANC-1	1	93	3	86
AML	KG-1	1	99	3	80
Liver cancer	HEPG2	2	99	3	76
Osteosarcoma	SW1353	100	79	100	72
Prostate cancer	PC3	1	99	3	70
Liver cancer	HEP3B	1	95	2	68
ALL	JURKAT	0.4	95	1	68
Pancreas cancer	MIAPACA-2	1	95	>1000	41
Colon cancer	WIDR	1	99	>1000	40
Thyroid cancer	BHT101	2	98	>1000	6
ALL	CEM	3	99	>1000	0
Cervical cancer	A431	2	64	>1000	0
Breast cancer	SKBR3	1	94	>1000	0
Cholangioca	MZCHA1	>1000	49	>1000	0
Osteosarcoma	U2OS	10	81	>1000	0

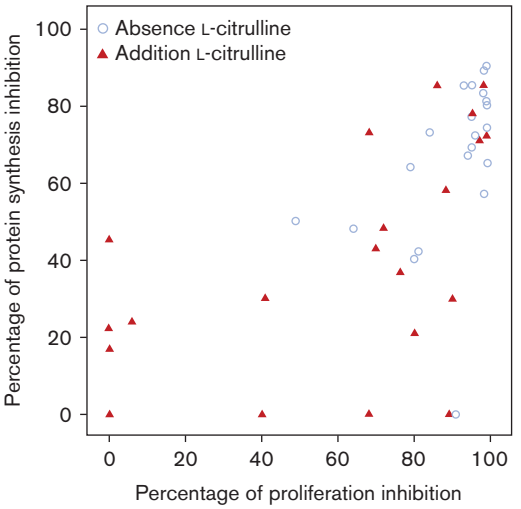
Incubation for 54 h and then addition of isotope for 18 h, followed by harvesting and counting.
AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; IC_{50}, concentration of arginase yielding 50% thymidine incorporation inhibition; INH, inhibition of thymidine proliferation incorporation relative to control; L-citrulline, 38 mmol/l of supplementation in media; SCLC, small cell lung cancer.

Table 2 HuArgI (CO)-polyethylene glycol 5000 3-day protein synthesis inhibition

		Absence of L-citrulline		Addition of L-citrulline	
Tumor	Cell line	IC_{50} (nmol/l)	INH (%)	IC_{50} (nmol/l)	INH (%)
Melanoma	SKMEL28	3	89	3	85
Pancreas cancer	PANC-1	2	85	2	85
Lung cancer	A549	4	81	6	78
ALL	JURKAT	1	85	3	73
Melanoma	A375	4	83	3	72
Mesothelioma	MSTO	1	72	3	71
SCLC	SW1271	8	73	12	58
Osteosarcoma	SW1353	100	64	>1000	48
ALL	CEM	1	90	>1000	45
Prostate cancer	PC3	5	80	>1000	43
AML	KASUMI-1	6	40	>1000	30
Liver cancer	HEPG2	1	74	>1000	37
Pancreas cancer	MIAPACA-2	1	69	>1000	30
Thyroid cancer	BHT101	800	57	>1000	24
Cholangiocarcinoma	MZCHA1	1000	50	>1000	22
AML	KG-1	15	65	>1000	21
Cervical cancer	A431	>1000	48	>1000	17
Osteosarcoma	SAOS2	>1000	0	>1000	0
Colon cancer	WIDR	4	81	>1000	0
Liver cancer	HEP3B	2	77	>1000	0
Breast cancer	SKBR3	20	67	>1000	0
Osteosarcoma	U2OS	>1000	42	>1000	0

Incubation for 54 h and then addition of isotope for 18 h, followed by harvesting and counting.
AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; IC_{50}, concentration of arginase yielding 50% leucine incorporation inhibition; INH, inhibition of protein synthesis leucine incorporation relative to control; L-citrulline, 38 mmol/l of supplementation in media; SCLC, small cell lung cancer.

Fig. 2



Scatterplot of percentage of proliferation inhibition versus percentage of protein synthesis inhibition only for tumor cell lines ○ in the absence of L-citrulline; ▲ in the presence of 38 mmol/l of L-citrulline. Spearman's correlation coefficients are 0.598 ($P=0.003$) and 0.611 ($P=0.002$) with and without L-citrulline, respectively.

70% or greater proliferation inhibition in the presence of L-citrulline, as opposed to modest inhibition in others. Of the 12 tumor cell types identified as sensitive by DNA synthesis inhibition, only five showed 70% or greater protein synthesis inhibition. Correlation between proliferation inhibition and protein synthesis inhibition was modest. Spearman's correlation coefficients were 0.598 ($P = 0.003$) and 0.611 ($P = 0.002$) with and without citrulline, respectively.

HuArgI (Co)-PEG5000 3-day normal tissue cell proliferation and protein synthesis inhibition

We tested seven different normal tissue primary cells for sensitivity to HuArgI (Co)-PEG5000 for 3 days in the absence or presence of 38 mmol/l of supplemental L-citrulline (Table 3, Fig. 3). Again, arginine depletion for 3 days resulted in uniform inhibition of proliferation for all normal human tissues examined, with maximal inhibition of 66–93% at 0.33 μmol/l of arginase. Protein synthesis was inhibited to a lesser degree, with maximal inhibition of 10–81%. In the presence of L-citrulline, umbilical endothelium, renal epithelium, bronchial epithelium, neurons, fibroblasts, hepatocytes, and cardiomyocytes displayed 88, 67, 61, 47, 26, 0, and 0% proliferation inhibition, respectively. These results indicate that L-citrulline protects hepatocytes and cardiomyocytes from the cytotoxic effect of HuArgI (Co)-PEG5000 treatment. Similarly, protein synthesis inhibition was more limited with supplemental L-citrulline. Whereas umbilical endothelium, renal epithelium, and bronchial epithelium showed 71, 70, and 60% protein synthesis inhibition, no

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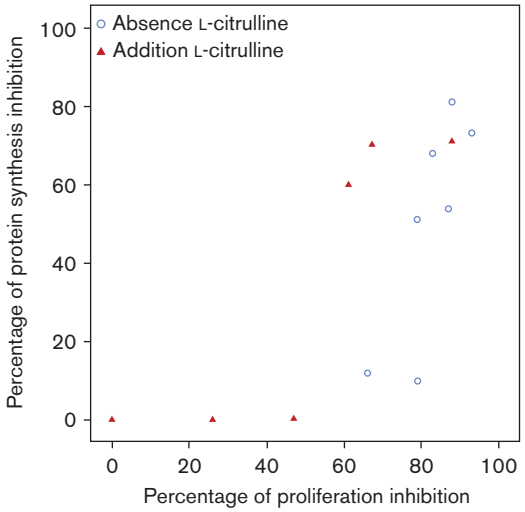
Table 3 HuArgI (CO)-polyethylene glycol 5000 3-day normal tissue proliferation and protein synthesis inhibition

	Percentage proliferation inhibition		Percentage protein synthesis inhibition (%)	
Tissue	Absence of L-citrulline	Addition of L-citrulline	Absence of L-citrulline	Addition of L-citrulline
Umbilical endothelium	83	88	68	71
Renal epithelium	87	67	54	70
Bronchial epithelium	88	61	81	60
Neurons	66	47	12	0
Fibroblasts	79	26	51	0
Hepatocytes	79	0	10	0
Cardiomyocytes	93	0	73	0

Percentage proliferation inhibition, percentage inhibition of thymidine incorporation relative to control; Percentage protein synthesis inhibition, percentage inhibition of leucine incorporation relative to control; L-citrulline, 38-μmol/l supplementation in media. Incubation for 54 h with 0.33 μmol/l of HuArgI (Co)-PEG5000 with or without citrulline, then addition of isotope for 18 h, followed by harvesting and counting.

Fig. 3



Scatterplot of percentage of proliferation inhibition versus percentage of protein synthesis inhibition for normal tissue cells ○ in the absence of L-citrulline; ▲ in the presence of 38 mmol/l of L-citrulline. Spearman's correlation coefficients are 0.915 (P=0.004) and 0.865 (P=0.012) with and without L-citrulline, respectively.

inhibition of protein synthesis was observed for neurons, fibroblasts, hepatocytes, and cardiomyocytes under these conditions.

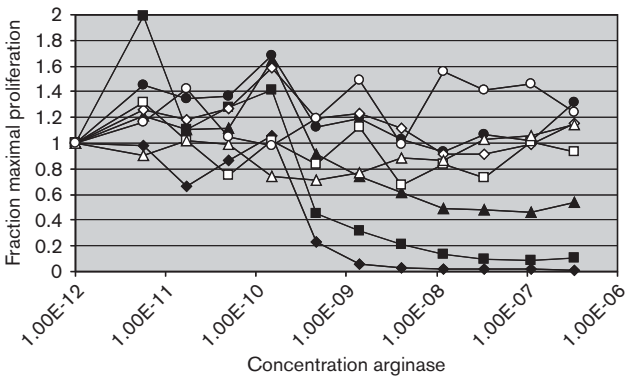
HuArgI (Co)-PEG5000 3-day tumor cell metabolic inhibition

Four of the six cell lines tested, namely, CEM, PC3, Kg-1, and PANC-1, had 59–97% 3-day metabolic inhibition as opposed to 62–97% 7-day metabolic inhibition, 65–90% 3-day protein synthesis inhibition, and 93–99% 3-day proliferation inhibition. The remaining two cell lines, namely, A549 and WiDr, had 0–1% metabolic inhibition at 3 days as opposed to 95–100% metabolic inhibition at 7 days, and 81% protein synthesis inhibition at 3 days, and 99% proliferation inhibition at 3 days in the absence of citrulline (Table 4).

Table 4 HuArgI (Co)-polyethylene glycol 5000 3-day human tumor cell line metabolic inhibition

Tumor cell line	Percentage of 3-day metabolic inhibition
CEM	97
Kg-1	92
PC3	84
PANC-1	59
A549	1
WIDR	0

Fig. 4



Three-day HuArgI (Co)-polyethylene glycol (PEG) 5000 SKBr3 cell proliferation-inhibition assay with different concentrations of L-citrulline: ◆, 0; ■, 0.052 mmol/l; ▲, 0.158 mmol/l; ●, 0.470 mmol/l; ◇, 1.4 mmol/l; □, 4 mmol/l; Δ, 13 mmol/l; and ○, 38 mmol/l.

L-Citrulline protection concentrations

As shown in Fig. 4, addition of L-citrulline at a concentration of 470 μmol/l or higher yielded full protection of SKBr3 cells from HuArgI (Co)-PEG5000 cytotoxicity. Percentage cell kill was 99, 90, 47, 0, 0, 7, 0, and 0 with 0, 52 mmol/l, 158 mmol/l, 470 mmol/l, 1.4 mmol/l, 4 mmol/l, 13 mmol/l, and 38 mmol/l of L-citrulline supplementation, respectively. On the basis of this and our earlier experiments, we estimate 400 μmol/l as the minimum protective supplemental L-citrulline concentration.

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Table 5 HuArgI (CO)-polyethylene glycol 5000 7-day recovery by MTS tumor cell assay[a]

		Absence of L-citrulline	Addition of L-citrulline
Tumor	Cell line	Percentage inhibition	Percentage inhibition
Melanoma	A375	97	100
Osteosarcoma	SAOS2	95	93
Mesothelioma	MSTO	100	89
Liver cancer	HEPG2	100	87
AML	KASUMI-1	85	84
Prostate cancer	PC-3	82	81
Liver cancer	HEP3B	96	79
Osteosarcoma	SW1353	96	79
Melanoma	SKMEL28	93	78
ALL	JURKAT	99	76
Pancreas cancer	PANC-1	97	65
Pancreas cancer	MIAPACA-2	95	55
AML	KG-1	62	49
Lung cancer	A549	95	43
Thyroid cancer	BHT101	87	38
ALL	CEM	100	23
Cervical cancer	A431	89	8
Colon cancer	WIDR	100	7
Breast cancer	SKBR3	98	0
Osteosarcoma	U2OS	100	0

Percentage inhibition, percentage inhibition of MTS incorporation relative to control wells.
AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
[a]Six-day incubation with 0.33 μmol/l of arginase ± 38 mmol/l of L-citrulline, then 1-day incubation with full media, followed by addition of MTS and reading. SW1271 and MZCHA1 failed to give measurable values with MTS assay.

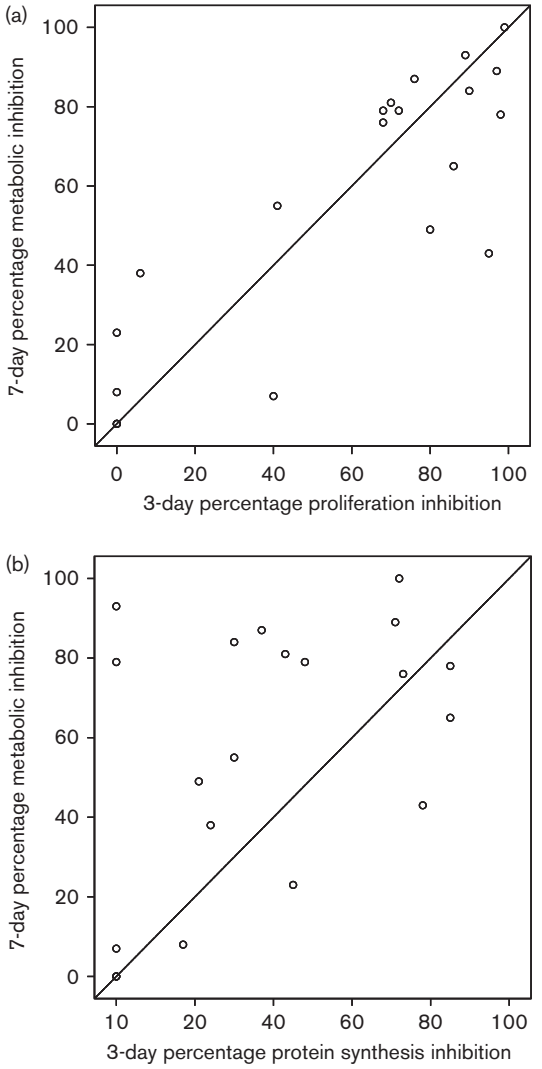
Effect of media arginine levels on HuArgI (Co)-PEG5000 3-day cell proliferation inhibition

A scatterplot of media arginine concentration versus percentage maximal proliferation inhibition for tumor and normal cells did not show any correlation (Fig. 9).

HuArgI (Co)-PEG5000 7-day human tumor cell metabolic inhibition

We tested the same panel of 22 human tumor cell lines for sensitivity to HuArgI (Co)-PEG5000 in the 7-day metabolic inhibition assay (Table 5). Two cell lines, SW1271 and MZCHA-1, could not be assayed by MTS. There was an excellent correspondence between 3-day cell proliferation inhibition sensitivity and 7-day cell metabolic sensitivity with addition of L-citrulline, except for three cell lines, namely, A549, KG-1, and PANC-1, which showed relative resistance to cell killing by HuArgI (Co)-PEG5000 in the 7-day metabolic-inhibition assay (Fig. 5 and Table 6). A weaker association of 3-day cell protein synthesis inhibition with 7-day metabolic inhibition may have been due to the synthesis of proteins or enzymes needed to cope with stress or amino acid imbalances. The 3-day and 7-day human tumor cell metabolic inhibition assays revealed strikingly different results for A549 and WiDr in the absence of citrulline. Both of these cell lines were resistant to metabolic inhibition in the 3-day assay but were sensitive in the 7-day assay. These same cell lines showed day 3 proliferation and protein synthesis inhibition but may have required additional days to show metabolic inhibition.

Fig. 5



Scatterplot of 7-day metabolic inhibition versus 3-day proliferation and protein synthesis-inhibition assays in the presence of 38 mmol/l of L-citrulline only for tumor cell lines with Y = X as a reference line. (a) Three-day proliferation inhibition. (b) Three-day protein synthesis inhibition. Spearman's correlation coefficients are 0.760 and 0.348, yielding *P* values of less than 0.001 and 0.133, respectively. Also found in Table 4.

HuArgI (Co)-PEG5000 7-day normal human cell metabolic inhibition

Five human primary cells could be cultured for the 7-day assay (Table 7). Without L-citrulline supplementation, the percentage cell kill for renal epithelium, umbilical endothelium, hepatocytes, fibroblasts, and cardiomyocytes was 81, 93, 41, 34, and 100, respectively. In contrast, with L-citrulline, the percentage cell kill for renal epithelium, umbilical endothelium, hepatocytes, fibroblasts, and cardiomyocytes was 86, 85, 21, 27, and 10, respectively. Thus, L-citrulline protected many of the normal tissues from

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Table 6 Spearman's correlation coefficients between association of 3-day proliferation and protein synthesis inhibition and 7-day metabolic-inhibition assay in the presence of L-citrulline

	Overall			Normal			Tumor		
	3-day PI vs. 3-day PSI	3-day PI vs. 7-day MTS	3-day PSI vs. 7-day MTS	3-day PI vs. 3-day PSI	3-day PI vs. 7-day MTS	3-day PSI vs. 7-day MTS	3-day PI vs. 3-day PSI	3-day PI vs. 7-day MTS	3-day PSI vs. 7-day MTS
Correlation	0.640	0.623	0.335	0.915	0.051	0.335	0.598	0.760	0.348
P value	<0.001	0.001	0.101	0.004	0.935	0.581	0.003	<0.001	0.133
N	29	25	25	7	5	5	22	20	20

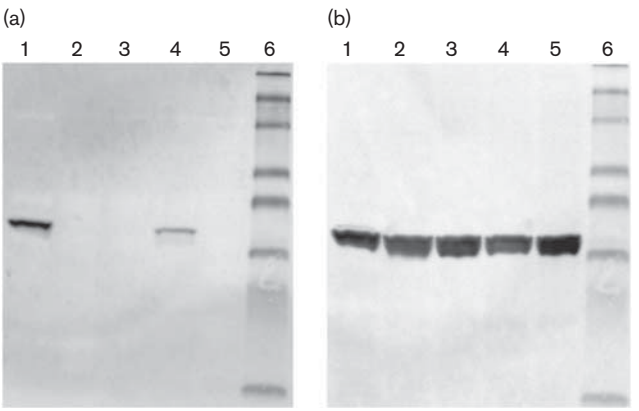
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PI, proliferation inhibition; PSI, protein synthesis inhibition.

Table 7 HuArgI (CO)-polyethylene glycol 5000 7-day recovery by MTS normal cell assay[a]

Tissue	Percentage inhibition in the absence of L-citrulline	Percentage inhibition in the presence of L-citrulline
Renal epithelium	81	86
Umbilical endothelium	93	85
Fibroblasts	34	27
Hepatocytes	41	21
Cardiomyocytes	100	10

Percentage inhibition, percentage inhibition by MTS incorporation relative to control wells.
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.
[a]Six-day incubation with 0.33 μmol/l of arginase ± 38 mmol/l of L-citrulline, then 1-day incubation with full media, followed by addition of MTS and reading. Neurons and bronchial epithelium failed to give measurable values with MTS assay.

Fig. 6



Human tumor cell line lysate immunoblots. (a) Anti-argininosuccinate synthetase (ASS) immunoblot. (b) Anti-β-actin immunoblot. Lane 1, Jurkat; lane 2, SKMEL28; lane 3, SW1353; lane 4, U2OS; lane 5, SAOS2; and lane 6, biotin.

arginase toxicity as was observed in the 3-day proliferation and protein synthesis inhibition assay.

Argininosuccinate synthetase protein expression of human cells

To test the expression of ASS in the cultured tumor cells, we performed immunoblots on cell lysates and verified protein loading with β-actin levels (Fig. 6). Relative to β-actin, the level of ASS among different cell lines varied by a 333-fold range (Table 8). Repeat measurements on different preparations of cells showed interassay and intra-assay variance of less than 30%. Normal tissue cells showed a 15-fold variation in ASS expression, with the highest being hepatocytes, fibroblasts, and cardiomyocytes and the lowest being umbilical endothelium and renal epithelium.

Ornithine transcarbamylase protein expression of human cells

OTC immunoblots of cell lysates showed a band corresponding to OTC only in human liver. Twenty-one human tumor cells and six other normal human tissues were OTC negative (Table 9).

Correlation of HuArgI (Co)-PEG5000 cytotoxicity with the argininosuccinate synthetase level

A strong correlation between tumor cell ASS levels and IC_{50} with HuArgI (Co)-PEG5000 in the presence of supplemental L-citrulline was observed, with Spearman coefficients and *P* values of −0.854 and < 0.001, −0.496 and 0.019, and −0.729 and < 0.001 for 3-day proliferation inhibition, 3-day protein synthesis inhibition, and 7-day metabolic inhibition, respectively (Table 6 and Fig. 7). A statistically significant correlation was observed for both tumor cells and normal tissues; however, for the latter, the statistical significance was lower because of the few specimens of normal tissue tested. Specifically, the Spearman coefficients and *P* values for normal cell ASS levels and HuArgI (Co)-PEG5000 cytotoxicity in the presence of supplemental L-citrulline were −0.685 and 0.09, −0.709 and 0.074, and −0.6 and 0.285 for 3-day proliferation and inhibition, 3-day protein synthesis inhibition, and 7-day metabolic inhibition, respectively (Table 10).

Media arginine levels

Both HuArgI (Co)-PEG5000 and HuArgI (Mn)-PEG5000 depleted media arginine from 121.7 ± 3.3 to 1.89 ± 0.1 and 2.22 ± 0.3 μmol/l, respectively.

Discussion

Cell cycle arrest after arginine depletion has been reported for a spectrum of cancer cell lines with ArgI

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Table 8 Argininosuccinate synthetase levels by lysate immunoblots

Category	Tissue	Cell line	Ass (STD by β-actin)
Tumor	Melanoma	A375	0.03
	SCLC	SW1271	0.05
	Osteosarcoma	SAOS2	0.06
	AML	KASUMI-1	0.07
	Melanoma	SKMEL28	0.08
	Mesothelioma	MSTO	0.09
	Osteosarcoma	SW1353	0.1
	Pancreas cancer	PANC-1	0.11
	AML	KG-1	0.18
	Lung cancer	A549	0.18
	Prostate cancer	PC3	0.22
	Thyroid cancer	BHT101	0.30
	Liver cancer	HEPG2	0.35
	Pancreas cancer	MIAPACA-2	0.36
	Osteosarcoma	U2OS	0.60
	ALL	CEM	1.7
	Cholangiocarcinoma	MZCHA1	1.8
	Liver cancer	HEP3B	2.0
	ALL	JURKAT	4.0
	Colon cancer	WIDR	4.7
	Breast cancer	SKBR3	7.0
	Cervical cancer	A431	10
Normal	Umbilical endothelium	Primary	0.10
	Renal epithelium	Primary	0.20
	Cardiomyocytes	Primary	0.50
	Neurons	Primary	0.54
	Bronchial epithelium	Primary	0.66
	Fibroblasts	Primary	1.0
	Hepatocytes	Primary	1.5

ASS, argininosuccinate synthetase; ALL, acute lymphoblastic leukemia; SCLC, small cell lung cancer; STD, standardized to same β-actin level.

Table 9 Ornithine transcarbamylase levels of human tumor cells and normal tissues

Tissue	OTC (STD by β-actin)
Normal hepatocytes	1.0
AML, Kasumi-1	<0.1
Cholangiocarcinoma, MZCHA1	<0.1
Thyroid cancer, BHT101	<0.1
Liver cancer, HEP3B	<0.1
Osteosarcoma, U20S	<0.1
Mesothelioma, MSTO	<0.1
ALL, Jurkat	<0.1
Breast cancer, SKBR3	<0.1
Lung carcinoma, A549	<0.1
Colon carcinoma, WIDR	<0.1
Pancreas carcinoma, MIPACA-2	<0.1
Pancreas carcinoma, PANC-1	<0.1
Liver carcinoma, HEPG2	<0.1
Osteosarcoma, SW1353	<0.1
AML, KG1	<0.1
ALL, CEM	<0.1
Melanoma, SKMEL28	<0.1
SCLC, SW1271	<0.1
Osteosarcoma, SAOS2	<0.1
Normal fibroblasts, HSP27	<0.1
Melanoma, A375	<0.1
Normal umbilical endothelium	<0.1
Cervical carcinoma, A431	<0.1
Normal renal epithelium	<0.1
Normal cardiomyocytes	<0.1
Normal neurons	<0.1
Normal bronchial epithelium	<0.1

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; OTC, ornithine transcarbamylase; STD, standardized to same β-actin level.

(Mn)-PEG5000 and arginine deiminase. Arrest occurred at either G_1/S or G_2/M and was shown to be associated with diminished cyclins including cyclin B1 and D3 [18,31–33]. Thus, our observed DNA synthesis inhibition associated with S phase arrest and protein synthesis inhibition associated with G_2 phase arrest are consistent with previous reports.

The mechanism by which amino acid starvation results in cell cycle inhibition has been reported to be multifactorial [34]. In our study, we observed a global reduction of translation in all cells that we examined. In eukaryotes from yeast to man, amino acid depletion results in derepression of GCN2, which recognizes uncharged tRNAs through binding to its regulatory histidyl tRNA synthase-like domain and phosphorylates eukaryotic initiation factor 2α (eIF2α) at Ser-51. Phosphorylated eIF2α then inhibits recycling of eIF2-GTP by binding to the guanine nucleotide exchange factor eIF2B, leading to a reduced global translation including translational repression of cyclin D. Consistent with the induction of this pathway, CEM cells exposed to Mn^{2+} arginase resulted in phosphorylation of eIF2α and reduction of cyclin D3 [30]. Another process that mediates cell cycle arrest after amino acid depletion is the inhibition of the preinitiation complex formation by sequestration of eIF4E by dephosphorylated 4E-BP1 [34]. For example, L-asparagine depletion has been shown to suppress eIF4E and cap-dependent mRNAs in transcripts that include those of cyclins [35].

The cytotoxic effects of HuArgI (Co)-PEG5000 on examined cells suggest failure of recovery from arginine depletion. As shown in Fig. 8, arginase yields ornithine and urea as products. To synthesize arginine from ornithine, cells must have enzymes OTC, argininosuccinate lyase, and ASS [36]. OTC deficiency is frequently observed in cultured cell lines. Six of eight, 12 of 13, 14 of 40, and one of one cell lines lacked OTC expression and/or showed sensitivity to arginase in previous reports [19,20,26,35]. We were able to observe OTC expression in normal human liver but not in 21 human tumor cell lysates or six additional normal human tissues. Our cytotoxicity results and the previous reports are consistent with frequent absence of OTC. Addition of L-citrulline to the media bypasses OTC and allows cells with argininosuccinate lyase and ASS to recover in the presence of arginine-depleted media.

In our studies, three of four assays did not find any cell lines displaying resistance to HuArgI (Co)-PEG5000. We tested some of the same cell lines reported to be OTC positive and resistant to Mn^{2+} arginase by others (e.g., cell lines WiDr and A549) and found that, although these cells lines displayed arginase toxicity in our 3-day proliferation and protein synthesis-inhibition assays and 7-day metabolic-inhibition assay, these two cell lines displayed only 0–1% metabolic inhibition in our 3-day

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metabolic inhibition assay similar to the previous report [19]. The previous report used 0.04–16 μmol/l of HuArgI (Mn)-PEG5000 with a 72-h incubation. In this study, we tested levels of arginine achieved after 72-h treatment for both HuArgI (Mn)-PEG5000 and HuArgI (Co)-PEG5000 at a maximal enzyme concentration of 0.33 μmol/l and observed comparable arginine depletion. We also examined different media arginine concentrations relative to cytotoxicity and did not observe any correlation (Fig. 9). An increased metabolic inhibition at day 7 and greater proliferation and protein synthesis inhibition at day 3 may be explained by the greater time needed for metabolic inhibition with arginine depletion in these two cell lines. They may have low levels of OTC that were not detected by our immunoblot assay but were measurable with the RT-PCR and enzyme activity methods previously reported [19]. The low-level OTC may protect the cell lines for several days; however, it fails after prolonged incubation with HuArgI (Co)-PEG5000 for 7 days.

Many different assays for amino acid deprivation-induced cell cytotoxicity have been used. Our use of proliferation and protein synthesis inhibition assays was based on their interassay and intra-assay reproducibility and the large differences observed between sensitive and resistant cells (10–1000-fold ratios). Nevertheless, these assays miss two critical phenomena. First, cell proliferation and protein synthesis may be inhibited without cell death. Second, after removal of the amino acid starvation stress, cells may have differential rates of recovery. Glazer *et al.* [35] compared HuArgI (Co)-PEG5000 cytotoxicity with Hep3B and PANC-1 cells by MTT and flow cytometry with annexin V, activated caspase 3, propidium iodide/RNase, and Ki-67. They found discrepancies between proliferation inhibition (Ki-67 staining, propidium iodide/RNase, and MTT) and apoptosis induction (annexin V and activated caspase 3 staining). Their data are supported by our studies, as we found discrepancies between the 3-day metabolic inhibition and 3-day proliferation inhibition results. Glazer *et al.* [35] suggested that different tumor cells may handle arginine deprivation differently. Interestingly, their study found that the Hep3B cell line, which had the least proliferation inhibition, also had maximal apoptosis. It is possible that phosphorylation of eIF2α and the resulting inhibition of protein translation may differentially affect proliferation regulators such as cyclins and apoptosis regulators such as IκB and nuclear factor-κB. In addition, inadequate

Fig. 7

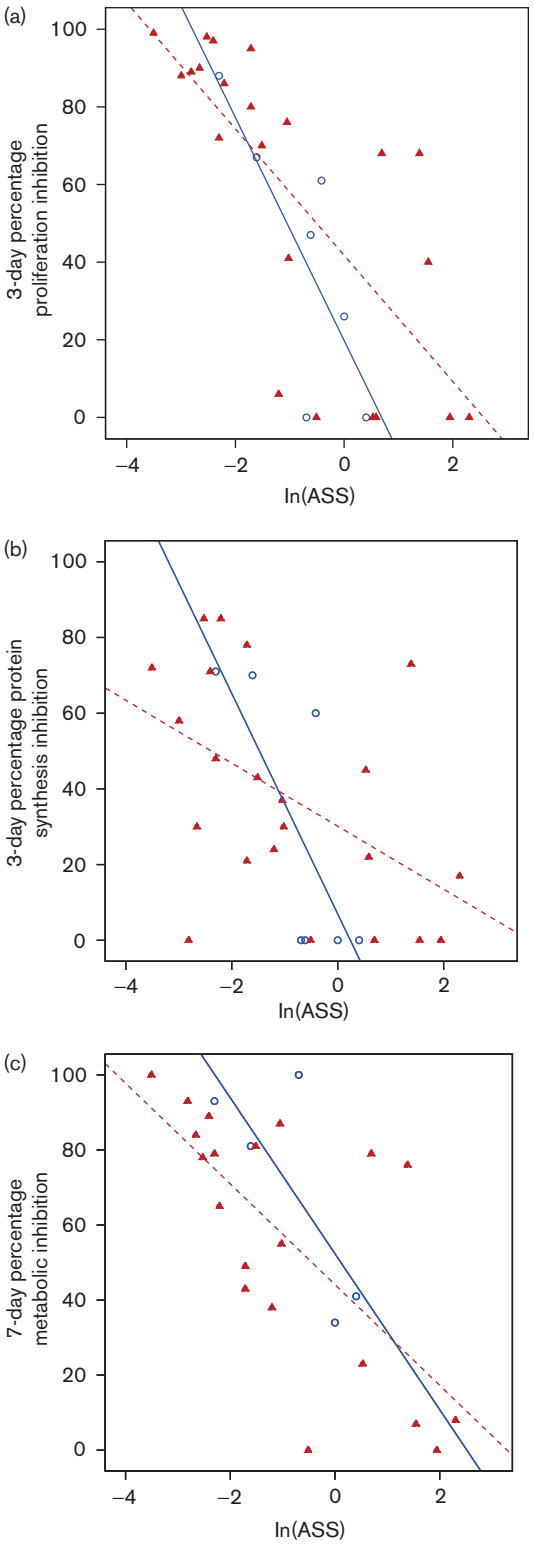

Fig. 7

Scatterplots of HuArgI (Co)-polyethylene glycol (PEG) 5000 growth inhibition in the presence of L-citrulline versus ASS concentration. (a) Three-day proliferation inhibition versus natural log ASS level for ▲ tumor cell and ○ normal cell. (b) Three-day protein synthesis inhibition versus natural log ASS level for ▲ tumor cell and ○ normal cell. (c) Seven-day metabolic inhibition versus natural log ASS level for ▲ tumor cell and ○ normal cell. A linear regression line is fitted with solid line for normal cells and with dotted line for tumors. Spearman's correlation coefficients are shown in Table 10.

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GCN2/P-eIF2α 'stress' response could overtax the cell and cause cell death. Mutations in this 'stress' pathway occur in the autosomal recessive disorder Wolcott–Rallison syndrome and are associated with epithelial and mesenchymal cell apoptosis [37]. Thus, differences between results with proliferation inhibition, protein synthesis inhibition, and metabolism inhibition are not unexpected. Overall, we were surprised by the overall good correlation observed between our assays.

Table 10 Spearman's correlation coefficients of association between proliferation and protein synthesis inhibition and argininosuccinate synthetase in the presence of L-citrulline

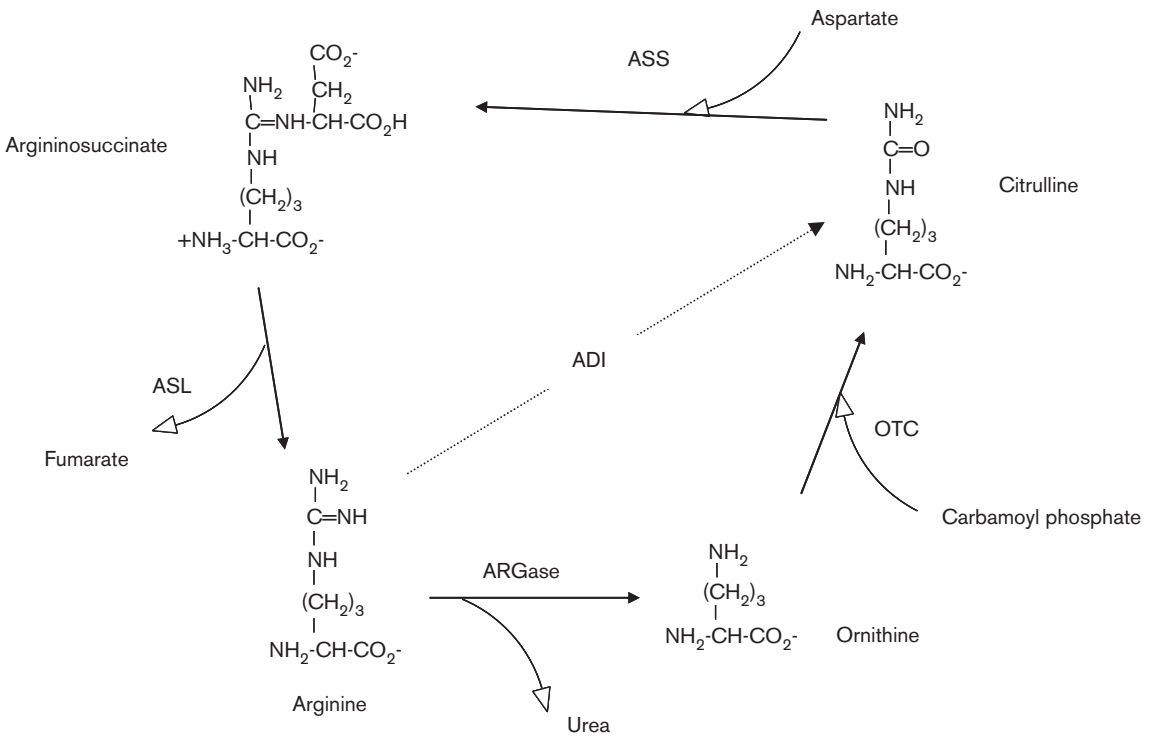
	Group		
ASS correlation	Overall	Normal	Tumor
3-day % proliferation inhibition			
Correlation	−0.827	−0.685	−0.854
P value	<0.001	0.090	<0.001
N	29	7	22
Percentage of protein synthesis inhibition			
Correlation	−0.509	−0.709	−0.496
P value	0.005	0.074	0.019
N	29	7	22
7-day percentage metabolic inhibition			
Correlation	−0.679	−0.600	−0.729
P value	<0.001	0.285	<0.001
N	25	5	20

ASS, argininosuccinate synthetase.

We found selective HuArgI (Co)-PEG5000 cytotoxicity with supplements of L-citrulline. Cytotoxicity mediated by HuArgI (Co)-PEG5000 under these conditions has similar effect to arginine deiminase and depends only on ASS and argininosuccinate lyase levels. We observed a similar pattern of tumor-type sensitivity with arginase and L-citrulline to arginine deiminase, with potent activity on some melanomas, hepatocellular carcinoma, pancreas cancer, mesothelioma, and prostate cancers and new findings of activity in lung carcinoma, osteosarcoma, and acute myeloid leukemia. Similar broad tumor inhibition by amino acid depletion has been observed with L-asparagine deprivation in acute myeloid leukemia, natural killer lymphoma, and acute lymphoblastic leukemia [38–40].

The potent cytotoxicity observed in tissue culture in this study has not been replicated to date in clinical trials. Only one report shows tumor regression in mice after arginine depletion. Ensor *et al.* [41] treated SK-Mel-28 and SK-Mel-2 melanomas grown subcutaneously in nude mice with weekly administration of ADI-PEG20 and observed tumor regressions for 2 months. Two additional studies were conducted of ADI-PEG20 administered to

Fig. 8



Schema of molecular mechanism of arginine auxotrophy. Urea cycle shown with intermediates. Deficiency of argininosuccinate synthetase (ASS) leads to reduced arginine levels. ADI, arginine deiminase; ARGase, arginase; ASL, argininosuccinate lyase; OTC, ornithine transcarbamylase. Adapted from Feun [42].

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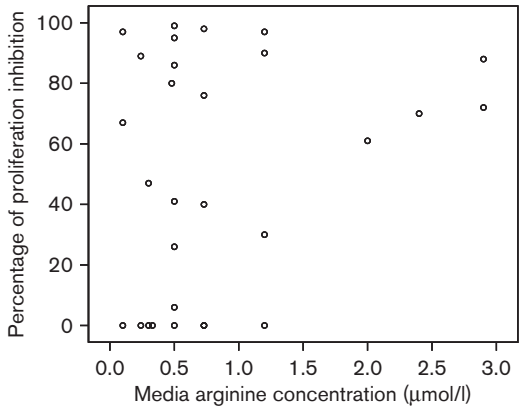
Fig. 9



Scatterplot of different media pretreatment arginine concentrations versus 3-day proliferation inhibition in the presence of 38 mmol/l of L-citrulline. Correlation coefficients and *P* values are shown.

nude mice bearing CWR22Rv1 prostate tumors or MIAPACA-2 pancreas tumors in which only 50–80% tumor growth inhibition was achieved [12,13]. Two studies of HuArgI (Mn)-PEG5000 given to nude mice bearing Hep3B or PLC/PRF/5 hepatocellular carcinoma tumors produced 50 and 90% tumor growth inhibition, respectively [18,19]. Three clinical studies of ADI-PEG20 produced no remissions in over 150 patients with hepatocellular carcinoma and transient or partial remissions in six of 24 melanoma patients [16,24,25]. The modest in-vivo antitumor efficacy observed in these studies is likely due to pharmacologic barriers and cell resistance mechanisms. The pharmacologic barriers include the short-enzyme half-life in vivo due to poor stability in serum for the Mn^{2+} arginase and the immunogenicity of the ADI, which results in neutralization or rapid clearance of the enzyme once a high antibody titer has been induced. Cell resistance may arise from the induction of ASS, stromal cell production of arginine, autophagy, or increased antiapoptotic proteins. The latter cell resistance mechanisms were addressed by comparing our 3-day and 7-day cytotoxicity results. Earlier studies have reported ASS induction after 4–6 days of arginine depletion [20,42–46]. The ASS gene expression may be regulated by several mechanisms involving binding of various transcription factors to the promoter. These may bind to the amino acid response element regions or other elements. c-Myc, HIF-1α, SP4, and ATF4 are candidate regulatory transcription factors in ASS gene expression [44,45,47]. Animal tumors and patient tumors after arginine depletion therapy have shown increased ASS levels [48]. We found three tumor cell lines that were sensitive in the 3-day assays but insensitive in the 7-day assays in the presence of citrulline, namely, PANC-1, KG1, and A549. The failure of these three cell lines to show toxicity in the 7-day assay may reflect ASS induction with prolonged treatment. Some of the other potential resistance mechanisms have been explored by others, including changes in apoptotic and antiapoptotic proteins (noxa, survivin, and death receptors), and bear an additional study [49].

Although 3-day proliferation and protein synthesis inhibition were observed for all normal tissues without citrulline supplementation, we still observed toxicity in the presence of L-citrulline to endothelium, kidney epithelium, and bronchial epithelium among the seven normal tissue types tested. A modest effect of HuArgI (Co)-PEG5000 was observed with neurons and fibroblasts, whereas no proliferation inhibition occurred with hepatocytes and cardiomyocytes. Interestingly, in the 7-day metabolic inhibition assay, only two of five tested normal tissues were sensitive to the combination of HuArgI (Co)-PEG5000 and L-citrulline, that is, umbilical endothelium and renal epithelium. These cumulative results point to blood vessels, kidney, and potentially lung as sites of normal tissue injury in arginine depletion therapy. As all normal tissues were not tested and in-vitro assays may poorly model in-vivo side effect profiles, animal toxicity studies are imperative. In preliminary work, administration of HuArgI (Co)-PEG5000 at 6 mg/kg resulted in only transient weight loss in Balb/c mice, whereas treatment with doses greater than 12 mg/kg was detrimental (unpublished work). L-citrulline supplementation has not been tested to date with this agent. These results contrast with the in-vivo safety of HuArgI (Mn)-PEG5000 in animals and patients [41,48,50]. This is in addition to the safety and efficacy of ADI-PEG20 in humans [24,25,48]. ADI-PEG20 yields citrulline and ammonia as products of arginine metabolism rather than ornithine and urea. Our tissue culture results suggest that citrulline addition may improve the therapeutic index of HuArgI (Co)-PEG5000 and, hopefully, reproduce the safety but not immunogenicity of ADI-PEG20 in animals and humans. In summary, these results combined with those of others suggest that the further development of HuArgI (Co)-PEG5000 is merited.

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Conflicts of interest

There are no conflicts of interest.

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