Published in final edited form as:

Surgery. 2010 August ; 148(2): 310–318. doi:10.1016/j.surg.2010.03.022.

BIOENGINEERED ARGINASE I INCREASES CASPASE-3 EXPRESSION OF HEPATOCELULAR AND PANCREATIC CARCINOMA CELLS DESPITE INDUCTION OF ARGININOSUCCINATE SYNTHETASE-1

E. S. Glazer, M.D.¹, W. D. Kaluarachchi, M.S.¹, K. L. Massey, B.S.¹, Cihui Zhu, B.S.¹, and S. A. Curley, M.D.^{1,2}

¹Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

²Department of Mechanical Engineering and Materials Science, Rice University, Houston, TX

Abstract

Background—Hepatocellular and pancreatic carcinomas are often auxotrophic for L-arginine, a semi-essential amino acid. The purpose of this study was to investigate cancer cell death using a significantly more active, cobalt-substituted bioengineered arginase.

Methods—Panc-1, a human pancreatic carcinoma cell line, and Hep 3B, a human hepatocellular carcinoma cell line, were exposed to L-arginase. Flow cytometry was used to measure expression of Ki-67, caspase-3, and argininosuccinate synthetase-1 (ASS-1) 4 days after treatment. An MTT assay measured proliferation. Student's t-test determined statistical significance.

Results—Viability decreased by 31% ± 2% for Panc-1 cells ($p < 0.0001$) and 34% ± 1% ($p < 0.0001$) for Hep 3B cells after treatment. Both cell lines demonstrated a 4-fold increase activated caspase-3 expression after high dose treatment ($p < 0.01$), and 5-fold increase in ASS-1 expression ($p < 0.002$). Ki-67 expression did not vary in Hep 3B cells but decreased for Panc-1 cells ($p < 0.015$). The 50% inhibitory concentration was 8-fold higher for Panc-1 cells than Hep 3B cells ($p < 0.03$).

Conclusion—Increased ASS-1 expression by these cells, in order to increase L-arginine concentration, is inadequate suggesting a mechanism by which arginine depletion can be used in multimodality therapy for arginine-dependent cancers.

Introduction

In adults, L-arginine is a nonessential amino acid produced from citrulline via the urea cycle enzymes argininosuccinate synthetase-1 (ASS-1) and argininosuccinate lyase (Fig 1).(1) Human hepatocellular carcinomas (HCC) and pancreatic carcinomas (PC) are both auxotrophic for arginine, and systemic deprivation of L-arginine may induce cell death in these malignancies.(1–5) Arginine, however, may promote other malignancies as a nutritional

© 2010 Mosby, Inc. All rights reserved.

Corresponding author, Steven A. Curley, M.D., scurley@mdanderson.org, Phone: 713-794-4957, Fax: 713-745-5235.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We have no conflicts of interest.

precursor and, more importantly, as a DNA synthesis promoter.(6) The exact mechanisms remain to be elucidated, but it appears that ASS-1 is not upregulated in susceptible cancers when arginine is deprived.(1- 7) A mycoplasmic enzyme, arginine deiminase (ADI), that converts arginine to citrulline has had mixed results in early Phase I and II trials for hepatocellular cancer, with most patients having stable disease.(8-10) The immunogenicity of ADI is the major limiting factor despite a nearly 20-fold increase in activity compared to arginase.(8- 9) Other examples of successful enzyme deprivation therapy include pegylated asparaginase that is approved for treatment of acute lymphoblastic leukemia.(11)

The other major enzyme typically utilized for arginine degradation is arginase, an enzyme with low activity in physiologic conditions.(12) There are 2 isoforms of arginase that convert arginine to ornithine. There is a metal cation, usually manganese, at the active enzyme site which adds stability.(12- 13) Interestingly, there are examples of increased endogenous arginase decreasing arginine levels clinically, such as after hepatic arterial embolization, with short-term, decreased tumor burdens.(14) Therefore, a bioengineered version of L-arginase I was utilized in these experiments.(15) It was engineered by substituting cobalt for manganese at the active site, and this resulted in an over 10-fold increase in activity at physiologic conditions relevant for clinical use.(15) Preliminary results demonstrated an approximately 15-fold decrease in the IC₅₀ when Hep 3B cells were treated with the bioengineered version compared to the native enzyme.(15) Based on these preliminary findings, it was felt that this enzyme warranted further study.

We hypothesize that HCC and PC cell lines treated with this bioengineered arginase will undergo apoptosis and decrease proliferation of susceptible cells in a dose-dependent manner. In addition, we hypothesized that ASS-1 upregulation plays an important role in the compensatory mechanism leading to resistance during arginine deprivation.

Materials & Methods

Cell cultures

A human PC cell line, Panc-1, and a human HCC cell line, Hep 3B, were acquired from the American Type Culture Collection (Manassas, VA) and kept in standard growth conditions (37°C, 5% CO₂). The cell line identities were confirmed by the Characterized Cell Line Core service (M.D. Anderson Cancer Center, Houston, TX, April 2009). During the experiment, all cells were maintained in Dulbecco's Modified Eagle's Medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The media is formulated with 84 mg/L L-arginine. Experiments were performed in standard 60 mm culture dishes or multiwall plates (Corning Inc., Corning, NY).

Proliferation assay

Cells were plated in 96 well flat bottom plates (800 cells/well) on day minus one in 200 µL complete DMEM. The next day, stock bioengineered arginase (100 µM) was added for final concentrations of 100 nM, 50 nM, 5 nM, 0.5 nM, 0.05 nM, and 0 nM (PBS controls) for each group (n = 4) and kept in standard condition until analysis. On days 1, 3, 5, and 7, 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated in standard conditions for 4 additional hours. Next, the solution was gently aspirated and 200 µL of DMSO was added to each well in addition to blanks (n = 4). Absorbance in each well was measured with automated plate reader at 570 nm corrected to the blanks (Synergy HT, Bio-Tek, Inc., Winooski, VT). Within each group, the mean and the standard error of the mean were calculated. These values were then normalized to the absorbance at 570 nm of the control cells by determining the ratio of the mean absorbance in each treatment group to the mean absorbance of the control group.

Flow cytometry

In quadruplicate, cells were plated at 5×10^5 cells/dish in 5 mL complete DMEM on day minus one. The next day, cells were treated with cobalt-substituted arginase at final concentrations of 100 nM, 1 nM, and 0 nM (phosphate buffered saline, PBS, only controls). Four days later, the media were collected with any floating cells while adherent cells were disassociated with trypsin-EDTA (Mediatech, Inc., Manassas, VA). The latter cells were collected after trypsin neutralization with media. Next, the cells were sequentially washed with PBS followed by Annexin Buffer 1 $\times$ (BD Biosciences, Franklin Lakes, NJ). The cells were suspended in 100 μ L of buffer, stained with 7-Aminoactinomycin D (7-AAD, BD Biosciences, Franklin Lakes, NJ) for necrosis/cell death, and stained with Annexin-V conjugated to Horizon-V450 (BD Biosciences, Franklin Lakes, NJ) to measure external phosphatidylserine expression. In order to remove excess unconjugated fluorophore prior to permeabilization, the cells were twice washed with buffer. The cells were then fixed and permeabilized with a commercially available, paraformaldehyde based kit (CytoPerm/CytoFix, BD Biosciences, Franklin Lakes, NJ).

After permeabilization, intracellular staining was performed with fluorophore labeled monoclonal antibodies according to the manufacturers' recommendations. A phycoerythrin-labeled antibody against the nuclear proliferation antigen Ki-67 (BD Biosciences, Franklin Lakes, NJ) and a fluorescein isothiocyanate labeled antibody against activated caspase-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each sample. At the same time, 5 μ L of a monoclonal antibody against ASS-1 conjugated to APC-H7 was added (provided as a kind gift from BD Biosciences). The cells were gently mixed and then kept in the dark for 15 minutes at 4°C.

After staining, the cells were suspended in ~200 μ L of permeabilization buffer and analyzed on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). At least 10,000 single cell events were recorded per sample, and often there were over 30,000 single cell events. Only single cell events were used for analysis. Compensation was performed according to the manufacture's recommendation. Data were analyzed and plotted with FlowJo 8.8.6 (TreeStar, Inc., Ashland, OR).

After indentifying the single cell population and excluding non-cellular debris based on the forward and side scatter plots, the single cells were gated into viable, apoptotic, dead, or necrotic cells based on a two-dimensional Annexin-V / 7-AAD plot. Neither the single cell gate of the forward/side scatter plots nor the two-dimensional gate of Annexin-V / 7-AAD plots were adjusted during analysis. After single cell events were classified according to viability status (viable, apoptotic, dead, or necrotic cells), a histogram of fluorescence intensity was created of all events in each sub-group for Ki-67, ASS-1, or activated caspase-3. Once again the same gating schema without change from the cell viability assessment was used for each fluorophore conjugate (Ki-67, ASS-1, or activated caspase-3) for all replicates. The percent of single cell events (gated) with high fluorescence intensity was found by applying the same gate to each subpopulation (Ki-67, ASS-1, or activated caspase-3 expression levels) across all sub-groups (viable, apoptotic, dead, or necrotic debris) for each treatment group (all samples). The mean as well as standard error of the mean was found for each measurement in each subgroup.

Cell cycle analysis

In a similar manner to above, a separate flow cytometry experiment was performed in order to analyze variations in the cell cycle after treatment. Four days after treatment with 100 nM of bioengineered arginase ($n = 3$), the cells were prepared as described. Controls were treated with equivolume PBS ($n = 3$). After permeabilization, the cells were stained with 250 μ L of

propidium iodide – RNAase buffer (BD Biosciences, Franklin Lakes, NJ) and incubated in the dark, at room temperature, for 15 minutes. Analysis proceeded after approximately 40,000 single cell events were collected for each sample. Again, only single cell events were used for analysis. Cell cycle analysis (Watson/Pragmatic fit model) was performed with Flow Jo 8.8.6 (Tree Star, Inc., Ashland, OR).

Western blotting

Western blot analysis was performed to measure intracellular protein expression after arginase treatment. After 4 days of treatment, cell lysates were obtained by exposing cells to cold radioimmunoprecipitation assay (RIPA) buffer and subsequently incubated on ice for 30 minutes, followed by centrifugation at 13,000 rpm for 30 minutes. The protein extracts were then electrophoresed on 6% Bis-Tris protein gel and transferred to a PVDF membrane. The membranes were incubated in 5% dry milk for 1 hour and then incubated with primary antibodies (BD Biosciences, Franklin Lakes, NJ) and β -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Next, the membranes were incubated with Peroxidase AffiniPure goat anti-rabbit IgG (H+L chains, Jackson Immuno Research Laboratories, West Grove, PA) and goat anti-mouse IgG (H+L chains, Jackson ImmunoResearch) secondary antibodies as appropriate. A peroxide solution (GenDEPOT, Inc., Barker, TX) detected the bands. Finally, the films were scanned using a high-resolution photo scanner (CanoScan 4400F, Canon, Inc., Lake Success, NY).

Statistical analysis

Data were analyzed and plotted with Microsoft Excel for Mac ver. 12 (Microsoft Corp., Redmond, WA) and SigmaPlot ver. 10 (Systat Software Inc., San Jose, CA). Means between groups were tested for statistical significance with a two-tailed, unpaired Student's t-test. Significance was set to $\alpha = 0.05$. Uncertainties are standard errors of the mean, unless otherwise stated. The 50% inhibitory concentration (IC_{50}) was determined by the Hill-Slope model. (16)

Results

Proliferation

After 1 day of treatment with bioengineered arginase, there was no significant decrease in proliferation by MTT assay in either cell line. This was true even for the highest concentration (100 nM). However, starting on the third day of treatment, there was a concentration dependent decrease in proliferation. This continued for days 5 and 7. After 3, 5, and 7 days duration, a single arginase treatment resulted in an IC_{50} of 0.36 ± 0.07 nM for Hep 3B cells and 1.22 ± 0.22 nM for Panc-1 cells ($p < 0.01$).

Viability

The viability of Panc-1 and Hep 3B cells decreased in a statistically significant manner as measured by flow cytometry 4 days after arginase treatment (Table 1). Apoptosis was the predominant mechanism of cell death (Table 1). For example, in Panc-1 cells, the apoptotic fraction increased from $3.2\% \pm 0.7\%$ to $25.3\% \pm 2.8\%$ ($p < 0.0002$, Fig 3). However, Hep 3B cells were much more sensitive as measured by the similar effects on viability at the low and high dose treatments (Table 1).

ASS-1 and Caspase-3 expression

ASS-1 expression was significantly increased in both cell lines after arginase treatment based on flow cytometric analysis. In addition, there was a dose response relationship (Table 2, Fig 4 and 5, $p < 0.01$). Of note, however, the distribution of ASS-1 expression varied by cell viability

(Fig 6). Activated caspase-3 expression increased 3 to 4 fold for Panc-1 cells ($p < 0.01$) and slightly more for Hep 3B cells ($p \sim 0.01$) after arginase treatment.

Ki-67 expression

A nuclear proliferation marker, Ki-67, was significantly decreased for 100 nM arginase treatment of Panc-1 cells ($p < 0.01$), but was not decreased at lower dosages of arginase treatment compared to control cells not treated with arginase (as measured with flow cytometry). Of note, Hep 3B cells that were apoptotic (Annexin V positive, PI negative) after arginase treatment had increased expression of Ki-67 in a concentration-dependent manner whereas Panc-1 cells had decreased Ki-67 expression (Fig 6). In addition, Panc-1 cells that remained viable after treatment decreased their relative proliferation whereas even viable Hep 3B cells increased their proliferation (Fig 2) as measured by Ki-67 expression.

Cell cycle analysis

Four days after high dose treatment, Panc-1 cells were halted at the G2/M phase of the cell cycle (Table 3, $p \sim 0.001$). Meanwhile, Hep 3B cells actually demonstrate a 3-fold decrease in the proportion of cells in the G2/M phase ($p \sim 0.0003$) with a significant increase in the sub-G0 phase (Table 3, Fig 7, $p \sim 0.0007$).

Discussion

The interesting findings of these experiments describe relationships between apoptosis and intracellular ASS-1 and Caspase-3 expression following arginase treatment. We demonstrate that arginine deprivation with a novel bioengineered human arginase disrupts the normal cellular behavior. Hep 3B cells, a very sensitive cell line, has global decreased cellular mass *in vitro* (as measured by MTT, Fig 2) because of cell death (as measured by FC, Fig 3), but in analyzing the single cell events on an event-by-event basis, Hep 3B apoptotic cells with inverted cell membranes (Annexin V surface positive) do not have decreased Ki-67 expression (Fig 6). The clinical importance of Ki-67 expression in hepatocellular carcinoma and pancreatic carcinoma is not fully understood. This discrepancy in protein expression compared to the Panc-1 cell line is likely related to the much higher arginase sensitivity of Hep 3B cells. Western blots do not allow this unique analysis as they are qualitative in nature. Furthermore, the crude nature of Western blot collection in conjunction with the lack of multiparameter analysis is a disadvantage of this technique. However, multiparameter flow cytometry permits analysis of a known population of cells without contamination from proteinaceous debris and in the context of cell viability. In this analysis, there are small discrepancies between the Western blot and flow cytometric data for ASS-1. Western blots, by their very nature, combine cells in aggregate-viable, dead, apoptotic, necrotic, and debris- whereas flow cytometry permits simultaneous investigation of multiple important characteristics. We suspect that the aggregated Western blot data is slightly different than the flow cytometry based on the relative different distributions of viability (and hence, different amounts of ASS-1) within each treatment group. Dead Panc-1 cells express minimal Ki-67, as one would expect- dead cells should not proliferate (Fig 6). However, Hep 3B “dead” cells continue to have expression of Ki-67 (Fig 6). This is an unexpected finding. We attribute the presence of Ki-67 to the Hep 3B cells not attempting to slow their proliferation prior to cell death. Whereas Panc-1 cells attempted to slow their cellular activity by decreased utilization of resources, Hep 3B cells do not. The MTT data suggests that the cells do die as measured by less mitochondrial mass (decreased absorbance) while the Ki-67 data provides some insight into how quickly the cells died and the viability data provides some insight into what mechanisms may have been involved. We hypothesize that arginine does not act as a promoter for DNA synthesis and proliferation on Hep 3B cells whereas it does on Panc-1 cells. Therefore, we theorize the lack of arginine signal decreases proliferation of Panc-1 cells in attempts at stress-induced survival whereas Hep 3B

cells continue to synthesize DNA in a nearly “normal” fashion, even though there is less than adequate substrate for ongoing protein synthesis. This is ultimately futile for both cell lines as there is inadequate arginine for normal protein synthesis for survival.

The discrepancy between MTT measurements of changes in proliferation and Ki-67 measurements has been described in other systems.(17–19) For example, Chen et al. described wide variations in Ki-67 immunostaining of individual cells within keratocyte spheroids despite an apparently uniform increase in proliferation based on MTT results.(17) Immunostaining or flow cytometry can assess differences in Ki-67 expression within subpopulations of the cells being assessed, whereas MTT assays are a much more gross measurement of the changes in proliferation in all cells in a given well of the culture plates. Smalley et al.(18) demonstrated large differences in inhibition of cellular proliferation (as measured by MTT absorbance) with specific small molecule inhibitors, while quantitative Ki-67 staining demonstrated specific groups of cancer cells adversely affected by the inhibitor molecules and allowed a more precise definition and identification of cells sensitive to these treatments when compared to the gross measurements of MTT absorbance. Finally, Al-Nasiry et al. demonstrated significant differences in MTT absorbance and Ki-67 staining results of choriocarcinoma cells after treatment with forskolin and TNF- α .(19) The relatively more crude measurement of MTT lacks the ability and accuracy to measure the molecular biologic complexities leading to changes in Ki-67 expression which can be demonstrated in subpopulations of cells in flow cytometric analysis.

Panc-1 cells increased ASS-1 expression by approximately 10-fold, while in Hep 3B cells it increased much less. In analyzing the distribution of ASS-1 by viability in flow cytometry in Hep 3B cells (Fig 5), it is clear that ASS-1 induction was inadequate to maintain viable cells. However, it is closer to adequate in Panc-1 cells. This suggests that ASS-1 induction can be an important component in subpopulations of cancer cells maintaining viability during arginine deprivation therapy, especially at later time points (i.e., after day 3). We believe that some Hep 3B cells (and to a lesser extent Panc-1 cells), can tolerate arginine deprivation via arginase treatment because it seems that arginine is not a major promoter for DNA synthesis in these cells. Removing arginine, therefore, does not decrease DNA synthesis, as one would expect (and survival would mandate). Arginine auxotrophic cancer cells may also be able to tolerate arginine deprivation by other, unknown mechanisms, however. This is in agreement with our findings and correlates the clinical situation encountered with massive endogenous arginase release. Therefore, Hep 3B cells continue to attempt normal proliferation (as measured by Ki-67) despite not having an adequate supply of arginine. Overall, cells die via apoptosis secondary to nutritional starvation without specific protective mechanisms one would expect. Panc-1 cells, at a low concentration of arginase, decrease proliferation in hopes of maintaining viability. However, eventually they cannot remain viable, and they undergo caspase-dependent apoptosis secondary to lack of arginine as a protein precursor (Tables 1 and 2).

Traditionally, the sub-G0 phase is considered the “apoptotic” cell fraction, but as refinements in the understanding of apoptosis continue, the sub-G0 phase does not yield the most information. Cell inversion, as measured by surface staining of Annexin-V, characterizes apoptosis in a very functional manner.(20) Likewise, DNA nicks are an early sign of pending apoptosis.(21) We chose to determine apoptosis with the Annexin-V staining, as this is a relatively simple and efficient method.

The slightly different effects of a single agent are interesting as well. We speculate that the two different cell lines utilize arginine in different ways. Cells, malignant or normal, from various organs have differences in susceptibility and tolerance of amino acid deprivation or generalized protein starvation. Panc-1 cells utilize arginine not only as a nutritional precursor, but arginine levels may modulate transcription and Panc-1 cells are relatively more resistant with slightly

longer survival times under arginine deprivation conditions compared to the Hep3B cells. Hep 3B cells do not utilize arginine in the same way and are more sensitive to specific reductions in intracellular levels of critical protein precursors, and as such, are much more susceptible to arginine deprivation therapy. Other groups have demonstrated variations in cellular response to arginine deprivation therapy *in vitro* and *in vivo* (5, 22), and we believe that variations between cancer types (and even within cancer types) results not only from a given cancer cell's ability to increase arginine concentration, but more importantly, any contribution that arginine has in proliferation (positive or negative) and cellular homeostasis in the untreated state.

Interestingly, Panc-1 cells increased the proportion of cells in the S phase ($p \sim 0.0001$) after arginase treatment while Hep 3B cells decreased that same proportion by over 40% ($p \sim 0.02$). Panc-1 cells are able to continue protein synthesis because they are able to increase ASS-1 expression, whereas Hep 3B cells do not compensate in a similar manner (Table 2). We suspect the variation in G2/M phase is a result of the same mechanisms.

Further studies will investigate the use of this novel bioengineered human arginase in animal models of cancer. Rationally selected DNA synthesis inhibitors and other treatments such as hyperthermic treatments may increase cancer cell death even further in concert with arginine deprivation. We suspect that the unusual pattern of some cancers to continue proliferation (albeit, in an inadequate manner) will allow for multimodality therapy by combining cell cycle phase-specific cytotoxic chemotherapeutic agents, small molecule inhibitors, hyperthermic treatment, and arginase to disrupt cancer cells from multiple signaling pathways. An added advantage of bioengineered human arginase compared to ADI which is currently in clinical trials will be a reduction or absence of immunogenicity.

Acknowledgments

The authors would like to thank Robert Balderas, Ph.D. and Li Liu, M.D., Ph.D., from BD Biosciences for advice regarding conjugation and kindly providing the ASS-1 conjugate. We would also like to acknowledge Kristine Ash and Yolanda Brittain for administrative support. The bioengineered arginase was kindly provided by Dr. George Georgiou and Dr. Everett M. Stone from The University of Texas-Austin.

Funding sources: This work was partially funded from the NIH (1 R01 CA139059-01). In addition, ESG is an NIH T32 research fellow (5 T32 CA09599).

References

1. Dillon BJ, et al. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers - A method for identifying cancers sensitive to arginine deprivation. *Cancer* 2004;100(4):826–833. [PubMed: 14770441]
2. Cheng PNM, et al. Pegylated recombinant human arginase (rhArg-peg(5,000mw)) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Research* 2007;67(1):309–317. [PubMed: 17210712]
3. Curley SA, Bomalaski JS, Ensor CM, Holtsberg FW, Clark MA. Regression of hepatocellular cancer in a patient treated with arginine deiminase. *Hepato-Gastroenterology* 2003;50(53):1214–1216. [PubMed: 14571701]
4. Feun L, et al. Arginine deprivation as a targeted therapy for cancer. *Current Pharmaceutical Design* 2008;14(11):1049–1057. [PubMed: 18473854]
5. Bowles TL, et al. Pancreatic cancer cell lines deficient in argininosuccinate synthetase are sensitive to arginine deprivation by arginine deiminase. *International Journal of Cancer* 2008;123(8):1950–1955.
6. Barbul A. Arginine and immune function. (Translated from eng). *Nutrition* 1990;6(1):53–58. discussion 59–62 (in eng). [PubMed: 2135757]
7. Yoon CY, et al. Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. (Translated from eng). *Int J Cancer* 2007;120(4):897–905. (in eng). [PubMed: 17096330]

8. Miyazaki K, et al. Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a Mycoplasma-infected cell line. *Cancer Research* 1990;50(15): 4522–4527. [PubMed: 2164440]
9. Izzo F, et al. Pegylated arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: Results from phase I/II studies. *Journal of Clinical Oncology* 2004;22(10):1815–1822. [PubMed: 15143074]
10. Glazer ES, et al. Phase II Study of Pegylated Arginine Deiminase for Non-resectable and Metastatic Hepatocellular Carcinoma. *JCO*. 2010 accepted.
11. 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). (Translated from eng). *Oncologist* 2007;12(8):991–998. (in eng). [PubMed: 17766659]
12. Junowicz-Kocholaty R, Kocholaty W. Arginase. (Translated from Eng). *Science* 1941;94(2432):144. (in Eng). [PubMed: 17744598]
13. Shi O, Kepka-Lenhart D, Morris SM Jr, O'Brien WE. Structure of the murine arginase II gene. (Translated from eng). *Mamm Genome* 1998;9(10):822–824. (in eng). [PubMed: 9745037]
14. 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. (Translated from eng). *Cancer Lett* 2005;224(1):67–80. (in eng). [PubMed: 15911102]
15. Stone E, et al. Replacing Mn²⁺ with Co²⁺ in Human Arginase I Enhances Cytotoxicity Towards L-Arginine Auxotrophic Cancer Cell Lines. *ACS Chemical Biology*. 2010 accepted.
16. Anonymous. Assay Guidance Manual Version 5.0. Eli Lilly and Company and NIH Chemical Genomics Center). 2008
17. Chen YH, Wang JJ, Young TH. Formation of keratocyte spheroids on chitosan-coated surface can maintain keratocyte phenotypes. (Translated from eng). *Tissue Eng Part A* 2009;15(8):2001–2013. (in eng). [PubMed: 19292684]
18. Smalley KS, et al. Ki67 expression levels are a better marker of reduced melanoma growth following MEK inhibitor treatment than phospho-ERK levels. (Translated from eng). *Br J Cancer* 2007;96(3): 445–449. (in eng). [PubMed: 17245336]
19. Al-Nasiry S, Spitz B, Hanssens M, Luyten C, Pijnenborg R. Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. (Translated from eng). *Hum Reprod* 2006;21(1):193–201. (in eng). [PubMed: 16210392]
20. Koopman G, et al. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. (Translated from eng). *Blood* 1994;84(5):1415–1420. (in eng). [PubMed: 8068938]
21. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. (Translated from eng). *Cancer Res* 1993;53(8):1945–1951. (in eng). [PubMed: 8467513]
22. Kim RH, Bold RJ, Kung HJ. ADI, autophagy and apoptosis Metabolic stress as a therapeutic option for prostate cancer. *Autophagy* 2009;5(4):567–568. [PubMed: 19276647]

Figure 1.

L-arginase is an integral protein in the urea cycle. Importantly, the bioengineered version is over 10-fold more active at physiologic pH. ASS-1 = arginosuccinate synthetase 1, OTC = ornithine carbonylase, ASL = argininosuccinate lyase.

Figure 2.

Proliferation of both Hep 3B cells (A) and Panc-1 cells (B) are decreased in a concentration-dependent and a time-dependent fashion as measured in this MTT assay. After 24 hours (blue), there is no decrease in proliferation. However, proliferation is decreased thereafter 3, 5, and 7 days after arginase treatment. MTT = Methylthiazolyldiphenyl-tetrazolium bromide. The normalized values are ratios of the average absorbance in each treatment group compared to the average absorbance of the control group. The units are arbitrary absorbance units.

Figure 3.

Treatment of Panc-1 cells with PBS (A) and 100 nM arginase (B) demonstrates a shift from viable (lower left quadrant) to apoptotic (lower right quadrant) or dead (upper right quadrant) fraction with arginase treatment. There was minimal variation in the necrotic fraction (upper left quadrant) after treatment. This variation was concentration dependent (C). Replicates of $n = 4$. Error bars are standard error of the mean.

Figure 4.

Four days after arginase treatment, both cell lines demonstrate a slight increase in argininosuccinate synthetase (ASS, *left*) as measured by flow cytometry. However, activated caspase-3 expression increased only slightly in Hep 3B cells, while it is similar for Panc-1 cells after treatment (*right*). Note that ASS is a larger (higher molecular weight) protein than β -actin while activated caspase-3 is smaller.

Figure 5.

Arginase treatment induced a significant increase in quantitative measurements of both activated caspase-3 and ASS-1 expression in Hep 3B cells based on flow cytometric analysis of single cell events ($p < 0.01$, Table 2). These quantitative results are more precise than the qualitative description gleaned from Western Blotting (Figure 4).

Figure 6.

The nuclear proliferation marker, Ki-67, decreased in viable Panc-1 cells after high dose arginase treatment, but it increased in viable Hep 3B cells. It increased more so in apoptotic cells. This data suggests that viable Panc-1 cells decreased proliferation in order to maintain viability whereas Hep 3B do not. Hep 3B cells are not able to maintain viability over long time courses (i.e., greater than 3 days) as demonstrated in the MTT assay (Figure 2).

Figure 7.

Control Hep 3B cells maintain a typical cell cycle pattern as seen in this PI flow cytometry cell cycle analysis. However, when treated with arginase, the portion of sub-G0 phase is equivalent to the G1 phase of the cell cycle. This suggests that the lack of arginine induces overwhelming apoptosis with corresponding loss of DNA.

The viability of human Panc-1 and Hep 3B cells is significantly decreased ($p \leq 0.0001$ compared to controls) four days after treatment with cobalt-substituted arginase as determined by flow cytometric analysis. There is negligible increase in the fraction of necrotic cells (7-AAD positive only) but a statistically significant increase in the apoptotic cell proportion (Annexin-V positive only). Viable cells are negative for both Annexin-V and 7-AAD while dead cells are positive for both. Figure 3 contains a demonstrative viability dot plot. SEM = standard error of the mean.

Table 1

	<i>Treatment</i>	<i>Viable</i>	<i>SEM</i>	<i>Apoptotic</i>	<i>SEM</i>	<i>Necrotic</i>	<i>SEM</i>	<i>Dead</i>	<i>SEM</i>
Panc-1	Control	91.7%	0.4%	3.2%	0.7%	1.0%	0.3%	4.1%	0.4%
	1 nM	73.4% *	2.1%	21.2% *	1.9%	0.4%	0.1%	5.0%	0.3%
	100 nM	60.7% *	0.8%	25.3% *	2.8%	0.6%	0.2%	13.4% **	1.9%
Hep 3B	Control	89.4%	1.2%	9.9%	1.1%	0.3%	0.2%	0.4%	0.2%
	1 nM	50.5% *	2.1%	34.8% **	2.2%	1.9%	0.4%	12.9% *	1.4%
	100 nM	49.0% *	1.3%	41.1% *	0.1%	0.9%	0.2%	9.0% **	1.1%

* $p \leq 0.0001$ compared to control
** $p \leq 0.005$ compared to control

Arginyl succinate synthetase-1 (ASS-1) expression increased in both cell lines after high dose treatment, but significantly more in the Panc-1 cell line compared to Hep 3B (p = 0.017). Caspase-3 (activated) increased in a similar manner for both cell lines. Finally, Ki-67 expression significantly decreased in Panc-1 cells after treatment, but did not significantly change in Hep 3B cells (p > 0.1). Data are based on flow cytometric analysis. SEM = standard error of the mean. Intracellular staining was performed after viability staining and permeabilization.

Table 2

	<i>Treatment</i>	<i>ASS-1</i>	<i>SEM</i>	<i>Caspase-3</i>	<i>SEM</i>	<i>Ki-67</i>	<i>SEM</i>
Panc-1	Control	4.1%	0.7%	10.5%	1.0%	23.5%	1.7%
	1 nM	39.3% ±	5.6%	32.5% ±	2.6%	27.9%	4.2%
	100 nM	43.5% ±	5.3%	45.4% ±	1.6%	10.1% ±	2.8%
Hep 3B	Control	3.5%	1.4%	12.9%	1.4%	4.4%	1.8%
	1 nM	14.0% ±	2.1%	33.5% ±	5.7%	9.3%	3.8%
	100 nM	20.6% ±	2.1%	57.4% ±	11.2%	10.7%	6.8%

± p ≤ 0.01 compared to control

The cell cycle distribution is significantly different after treatment with 100 nM arginase. However, the response is different for each cell line. While Panc-1 cells are halted at the G2/M phase after treatment, Hep 3B increased the Sub-G0 portion of the cell cycle. Despite the lack of Sub-G0 phase shift for Panc-1 cells, a significant portion of that population binds Annexin-V on the outer membrane surface, suggesting early apoptosis without major DNA destruction. Totals do not exactly equal 100% because (1) small variations in the modeling routine are used for cell cycle analysis, (2) rounding when aggregating data, and (3) a small percentage of cells found beyond the G2/M phase on the DNA histogram (Figure 7). SEM = standard error of the mean. Cell cycle analysis (Watson/Pragmatic fit model) was performed with Flow Jo 8.8.6. Statistical significance was calculated by unpaired, two tailed Student's t-test for differences between control and treatment groups for each cell cycle phase.

Table 3

	<i>Treatment</i>	<i>Sub-G0</i>	<i>SEM</i>	<i>p value</i>	<i>G0/G1</i>	<i>SEM</i>	<i>p value</i>	<i>S</i>	<i>SEM</i>	<i>p value</i>	<i>G2/M</i>
Panc-1	Control	1.56%	0.64%		68.50%	1.45%		20.50%	1.41%		13.80%
	100 nM	1.70%	0.24%	0.84	42.60%	0.47%	0.0001	29.60%	0.40%	0.003	32.50%
Hep 3B	Control	3.49%	0.79%		43.30%	1.71%		29.30%	0.15%		20%
	100 nM	52.30%	5.03%	0.0007	20.80%	2.71%	0.002	17%	1.66%	0.002	6.05%