Pegylated Recombinant Human Arginase (rhArg-peg5,000mw) Inhibits the In vitro and In vivo Proliferation of Human Hepatocellular Carcinoma through Arginine Depletion

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Abstract

Hepatocellular carcinoma (HCC) is believed to be auxotrophic for arginine through the lack of expression of argininosuccinate synthetase (ASS). The successful use of the arginine-depleting enzyme arginine deiminase (ADI) to treat ASS-deficient tumors has opened up new possibilities for effective cancer therapy. Nevertheless, many ASS-positive HCC cell lines are found to be resistant to ADI treatment, although most require arginine for proliferation. Thus far, an arginine-depleting enzyme for killing ASS-positive tumors has not been reported. Here, we provide direct evidence that recombinant human arginase (rhArg) inhibits ASS-positive HCCs. All the five human HCC cell lines we used were sensitive to rhArg but ADI had virtually no effect on these cells. They all expressed ASS, but not ornithine transcarbamylase (OTC), the enzyme that converts ornithine, the product of degradation of arginine with rhArg, to citrulline, which is converted back to arginine via ASS. Transfection of HCC cells with OTC resulted in resistance to rhArg. Thus, OTC expression alone may be sufficient to induce rhArg resistance in ASS-positive HCC cells. This surprising correlation between the lack of OTC expression and sensitivity of ASS-positive HCC cells shows that OTC-deficient HCCs are sensitive to rhArg-mediated arginine depletion. Therefore, pretreatment tumor gene expression profiling of ASS and OTC could aid in predicting tumor response to arginine depletion with arginine-depleting enzymes. We have also shown that the rhArg native enzyme and the pegylated rhArg (rhArg-peg5,000mw) gave similar anticancer efficacy in vitro. Furthermore, the growth of the OTC-deficient Hep3B tumor cells (ASS-positive and ADI-resistant) in mice was inhibited by treatment with rhArg-peg5,000mw, which is active alone and is synergistic in combination with 5-fluorouracil. Thus, our data suggest that rhArg-peg5,000mw is a novel agent for effective cancer therapy.

Introduction

Arginine has been known to influence the growth of transplantable mice tumor since 1930 (1). Diet supplemented with arginine enhances tumor growth in mice. Conversely, dietary restriction of arginine inhibits growth of metastatic tumor (2). Arginine is an indispensable amino acid to children but a semi-essential amino acid in adult humans, involved in the synthesis of a wide range of peptides and proteins, production of creatine and nitric oxide, and a myriad of metabolic pathways and cellular events (3). It is also a precursor of proline, polyamines, glutamine, glutamate, and other neurotransmitters, such as γ-aminobutyric acid. It also serves as a substrate for two important enzymes, arginase and nitric oxide synthetase.

The body obtains arginine from various sources, including dietary intake, muscle degradation with the proteosomal/ubiquitin pathway, and the "intestinal-renal axis." In the small bowel, ornithine transcarbamylase (OTC) catalyzes the synthesis of citrulline from ornithine and carbamoyl phosphate. In turn, this citrulline is converted to arginine in the proximal renal tubules by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), the so called "intestinal-renal axis," which accounts for up to 60% of all arginine endogenously generated in humans. OTC is expressed largely in the small bowel and liver. Despite its high degree of versatility within the cell, arginine is still regarded as a semi-essential amino acid in adult humans because somatic cells can synthesize it from citrulline via ASS and ASL, both of which are ubiquitous in somatic cells (1). This is because demand outstrips supply at certain times during growth and development; equally, the growth of a tumor can become a stress, which is relieved by supplementation of arginine (4).

The arginine-degrading enzyme in the urea cycle is arginase, the in vitro anticancer property of which has been well documented since the 1960s (5). Arginase enzymatically converts arginine to ornithine and urea. Currie et al. (6) showed that arginase released from lipopolyssacharide and zymosan-stimulated macrophages can be responsible for the death of V79 Chinese hamster lung cells, L5178Y lymphoma cells, and HSN hooded rat sarcoma in culture. Using murine and bovine liver arginases, Storr and Burton (7) showed the total destruction of lymphosarcoma cells when arginine was reduced to <8 μmol/L over 24 h. In vitro arginine depletion, either through arginase or preparation of arginine-free medium, leads to rapid tumor death in a wide range of tumor cell lines (7).

Another potent arginine-depleting enzyme is arginine deiminase (ADI), which is a microbial enzyme from Mycoplasma spp. The enzyme has both anti-hepatocellular carcinoma (HCC; ref. 8) and anti-malignant melanoma (4) properties. The pegylated form of ADI is now in phase II clinical trial stage, showing its strong anticancer activities against HCC and malignant melanoma (9, 10). These tumors are often dependent on exogenous arginine for...
growth because the cells generally cannot internally synthesize arginine (i.e., they are, or become, auxotrophic for arginine). The mechanism of arginine auxotrophy in these tumors is likely to be complex but primarily seems to be related to the down-regulation of the ASS gene at the transcriptional level by inhibition of its promoter sequence through methylation (11). This selective differential requirement in arginine between most somatic cells and tumor cells, which are auxotrophic for arginine, provides a rational basis that can be thoroughly exploited to develop new treatment methods not just for HCC and malignant melanoma but also for many types of malignancies (9–11).

ASS is a key enzyme in the synthesis of arginine from citrulline. Tumors (mainly malignant melanoma and HCC) that are sensitive to arginine depletion by ADI do not express ASS (4, 12, 13). Shen et al. (14) reported that many cell lines are resistant to ADI treatment, although most require arginine for proliferation. Their results indicate that resistance to ADI treatment may correlate with cellular ASS activity, either constitutive or inducible, allowing cell survival by conversion of the product of the ADI reaction (i.e., citrulline to arginine). Wheatley (15) suggested that the enzymes involved in converting arginine from citrulline (ASS and ASL) are tightly coupled, and in cultured cells, ornithine cannot be sent on round the urea cycle and, hence, is an end product except in some freshly isolated liver cells or some minimum deviation hepatomas.

Arginase converts arginine to ornithine and urea. Despite its strong in vitro antitumor properties, arginase was never seriously considered as a potential drug candidate for the treatment of human cancers for several reasons, including its much lower affinity for arginine ($K_m$, 6 mmol/L for the native enzyme; $K_m$, 12 mmol/L for the pegylated enzyme) at physiologic pH (16), a pH optimum of 9.6, and short circulatory half-life (a few minutes). These have been considered serious shortcomings since the 1980s, with the report from Savoca et al. (17) who observed no antitumor activity of bovine liver arginase in mice with Taper liver cancer. Other investigators also gave very negative reports on arginase (18). It is important to recognize, however, that these in vitro data were generated with bovine and murine arginases, which have different biochemical properties compared with the human liver arginase. Nowadays, one can use recombinant human arginase, which was not available for study until the advent of recombinant DNA technology (19).

We have previously shown (20) that arginine depletion with endogenous human hepatic arginase released from transhepatic arterial embolization could induce a systemic anti-HCC response. This convinced us that recombinant human arginase (rhArg) given exogenously, after suitable modification to lengthen its half-life such as pegylation, could induce a similar antitumor response. In the present study, the use of rhArg produced in a Bacillus subtilis expression system (21) is described. The isolated and purified enzyme is covalently attached via a succinimide propionic acid (SPA) linker to polyethylene glycol (PEG) of molecular weight 5,000. Unlike many other pegylated enzymes, this pegylated rhArg (rhArg-PEG_{5000mw}) was fully active. The $K_m$ value for arginine as the substrate of the pegylated rhArg was also much smaller ($K_m$, 2.9 mmol/L) than that of the pegylated bovine liver arginase ($K_m$, 12 mmol/L). Subsequently, the pegylated rhArg was found to have an in vivo half-life of ~3 days while maintaining sufficient enzyme catalytic activity at physiologic pH. The biochemical characteristics as well as the production methods have already been reported (21).

Using our rhArg and pegylated rhArg, we have done experiments to (a) compare the in vitro antitumor activities between rhArg and pegylated rhArg, (b) compare the in vitro antitumor activity of the rhArg native enzyme with that of the ADI native enzyme; (c) work out why some cancer cell lines are rhArg sensitive but ADI resistant in vitro; and (d) test the in vivo antitumor activity of rhArg-PEG_{5000mw} alone and in combination with 5-fluorouracil (5-FU), in nude mice bearing an ADI-resistant HCC xenograft. The questions “Is rhArg able to inhibit ASS-positive HCCs that cannot be treated by ADI?” and “Is rhArg-PEG_{5000mw} an effective anticancer agent?” have been investigated in this report.

Materials and Methods

Materials. Materials not specified here were obtained from Sigma Chemical Company (St. Louis, MO). Cell proliferation kit with 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was obtained from Promega (Madison, WI). All cell culture media and sera were purchased from Invitrogen Life Technologies, Inc. (San Diego, CA). Expression plasmid pcDNA3 was from Invitrogen (San Diego, CA). The Mycoplasma arginini ADI native enzyme was generously provided by Professor B.H. Min (Department of Pharmacology and BK21 Program for Medical Sciences, College of Medicine, Korea University, Seoul, South Korea). Methoxypolyethylene glycol succinimidyl propionate (mPEG-SPA; MW 5,000) was purchased from Nektar Therapeutics (Huntsville, AL).

The cell line HuH7 was provided by Professor R. Poon (Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong, China). All other cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). The details of the cell lines are as follows: HepG2 (HBV-negative hepatoblastoma, HB-8065); Hep3B (HBsAg-positive HCC, HB-8064); PLC/PRF/5 (primary hepatoma cells, contain hepatitis B, express hepatitis B virus surface antigen, CRL-8024); SK-HEP-1 (liver adenoscarcina, HTB-52); HuH7 (well-differentiated HBsAg-negative HCC); A549 (lung carcinoma, CCL-185); WiDr (colorectal adenocarcinoma, CCL-218); SK-MEL-28 (malignant melanoma, HB-72); and HeLa (cervix adenocarcinoma, CCL-2).

Preparation of rhArg and rhArg-PEG_{5000mw}. Recombinant human arginase (rhArg) was obtained by producing the His-tagged human arginase I (arginase) enzyme in B. subtilis (21). A coupled spectrophotometric assay was used to determine arginase activity as described by Ikemoto et al. (22). The specific activity of the purified enzyme was ~400 IU/mg protein. One international unit of arginase is defined as the amount of enzyme that can produce 1 mmol urea/min at 30°C, pH 8.5.

The purified enzyme (native rhArg) was used directly for conjugation with PEG as follows: mPEG-SPA of MW 5,000 was covalently attached to rhArg with the same methods that were used for formulating ADI with PEG (12). The resulting PEG-formulated (pegylated) arginase was termed rhArg-PEG_{5000mw} (Fig. 1). Only the purified M. arginini ADI (23) native enzyme (without pegylation) was used in our studies. The specific activity of the purified ADI was ~46 units/mg protein. One unit of the ADI native enzyme is the amount of enzyme activity that converts 1 mmol of arginine to 1 mmol of citrulline per minute at 37°C under the assay conditions (23).

To determine the number of PEG per arginase molecule, a PEG standard curve was constructed by the colorimetric assay as described by Nag et al. (24). Briefly, free PEG was detached from rhArg-PEG_{5000mw} by incubating with protease. The amount of free PEG in the resulting mixture was determined by comparing to the PEG standard curve. The number of PEG molecules attached to the primary amines of arginase was calculated by the molar ratio of free PEG and rhArg-PEG_{5000mw}. The number of PEG per rhArg was estimated to be 10 to 12.

Cell culture and cell proliferation assay. The cells were maintained either in DMEM or RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 100 units/mL penicillin/streptomycin. Cells (2.5 × 10³) in a volume of 100 μL of culture medium were seeded to each well of a 96-well plate and incubated for 24 h. The culture medium was replaced by medium
with varying concentrations of either rhArg (native or pegylated) or the ADI
native enzyme. The plates were incubated for an additional 3 or 7 days at
37°C in an incubator containing an atmosphere of 95% air and 5% CO₂.
Then, 0.02 mL of MTS reagent was added to each well and the plates were
incubated for another 4 h. The absorbance of the wells at 490 nm was then
determined. The amount of rhArg, native or pegylated, needed to kill 50% of
the cells in a culture was defined as IC₅₀. We used the CellTiter 96
AQueous Non-Radioactive Cell Proliferation Assay from Promega, which is a colori-
metric method for determining the number of viable cells in proliferation
assays. MTS is chemically reduced by cells to formazan, which is soluble
in tissue culture medium. The measurement of the absorbance of the
formazan can be carried out at 490 nm. The assay measures dehydrogenase
enzyme activity found in metabolically active cells.

Amino acid analysis. Cell culture medium after treatment was collected
and mixed with 50% trichloroacetic acid for precipitation of protein by
incubating on ice for 30 min. The samples were centrifuged at 13,000 × g
for 15 min, and arginine and ornithine levels were analyzed by high-speed
amino acid analyzer (model L-8800, Hitachi). The plasma arginine levels at the indicated time
points were determined using the amino acid analyzer as described by
Marshall and Cohen (25). Briefly, the standard assay mixture (3 mL)
contained 8 mmol/L Tris-HCl buffer (pH 8.5), 5 mmol/L ornithine, 5 mmol/L
arginine, and cell extract. The ornithine and carbamyl
carboxylic acid for precipitation of protein by incubating on ice for 30 min. The samples were centrifuged at 13,000 × g
for 15 min, and arginine and ornithine levels were analyzed by high-speed
amino acid analyzer (model L-8800, Hitachi). The plasma arginine levels at the indicated time
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Marshall and Cohen (25). Briefly, the standard assay mixture (3 mL)
contained 8 mmol/L Tris-HCl buffer (pH 8.5), 5 mmol/L ornithine, 5 mmol/L
carbamyl phosphate, and cell extract. The ornithine and carbamyl phosphatases were prepared just before use. The reaction was
started by adding the carbamyl phosphate solution and was allowed to
continue for 15 min at 37°C. Subsequently, the reaction was stopped by
the addition of 3 mL of 6.25% (v/v) trichloroacetic acid solution. The
centration of carbamyl phosphatase was measured by adding 1 mL
of the test solution into 2,3-butanedione monoxime/acid solution (1:3, v/v).
They were mixed with stirring and the tubes were transferred to boiling water
bath and incubated for 20 min. Distilled water (8 mL) was added and
absorbance was measured at 490 nm. Standard curves were constructed by
appropriately diluting a stock solution of carbamyl. One unit of OTC would
form 1 μmol of carbamyl from ornithine and carbamyl phosphate
per minute at pH 8.5 and 37°C.

Pharmacodynamics of rhArg-peg5,000mw. Sprague-Dawley rats were
obtained from the Chinese University of Hong Kong (Shatin, Hong Kong) for
use in the pharmacodynamic studies. Normal Sprague-Dawley rats (four females and four males), ~3 months old (average body weight, ~ 250 g),
were recruited and were randomly assigned into groups. Different dosages of
rhArg-peg5,000mw (500, 1,000, 1,500, and 3,000 IU per rat) were given i.p. on
day 0. Blood samples were drawn from their tail veins on day 0 before the
i.p. injection of rhArg-peg5,000mw as baseline, on days 1 to 6, and then every
2 days. Blood was collected in EDTA and mixed with 50% trichloroacetic acid
solution for protein determination by centrifugation on ice for 30 min. The samples were centrifuged at 13,000 × g
for 15 min, and arginine level in the
clar supernatant was analyzed by high-speed amino acid analyzer (model L-8800, Hitachi). The plasma arginine levels at the indicated time
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Implantation of tumor cells in nude mice. The BALB/c nude mice
used in this study were obtained from the Chinese University of Hong Kong
and were all males, 3 weeks old (average body weight, ~ 20 g), at the
beginning of the study. They were used in the in vivo drug efficacy tests of
rhArg-peg5,000mw. They were provided with standard mouse chow
and water ad libitum. Hep3B cells were removed from the tissue culture
flasks with trypsin in Dulbecco’s PBS (Invitrogen Life Technologies) and
resuspended in growth medium. Approximately 1 million cells were injected
eritum into 20 µL of Matrigel/water mixture s.c. near the axilla. When

Figure 1. SDS-PAGE analysis of purified rhArg and rhArg-peg5,000mw. Arginase
was purified with mPEG-SPA in a molar ratio of 1:50 for 2.5 h. Lane 1, low-range protein marker; lane 2, native rhArg, MW ~35 kDa; lane 3, purified
rhArg-peg5,000mw.

OTC expression construct. The coding region of the OTC gene was
amplified from the Marathon’s liver cDNA library (Clontech) at an annealing
temperature of 60°C with oligonucleotides 1 and 2, which contain the
cognition sites for restriction enzymes HindIII and EcoRI, respectively (underlined): oligo 1, 5’-CCCAAGCTTATGAGGGCATGAATGCT-3’, oligo 2, 5’-CCGATATCCTAGCTAGACTATCACGT-3’.
The PCR product was digested with HindIII and EcoRI and ligated into
HindIII/EcoRI-digested pcDNA3 vector (Invitrogen) to yield the OTC
expression plasmid pcDNA3-OTC. The plasmid was then introduced into
HCC cells by lipofection as follows: cells were plated 24 h before
transfection to reach 60% to 80% confluence in 12-well plates. Plasmid
DNA (1 μg), prepared using an endotoxin-free plasmid purification kit
(Promega), was mixed with 4 μL of TransFectin Reagent (Bio-Rad) and
incubated for 20 min at room temperature. The DNA/TransFectin
complexes were directly added to cells in serum-containing medium.
Following 6 h of incubation, the transfection medium was removed and
the complete medium was added. The expression of the OTC gene in
transfected HCC cells was verified by RT-PCR analysis using the primers
described above. These transfected cells were then analyzed again for
sensitivity to rhArg-peg5,000mw treatment for 3 days with MTS reagent.

OTC enzyme activity assay. Cells were harvested, washed, and
suspended in 0.1 mol/L Tris-HCl (pH 7.5). Extraction of cell proteins was
accomplished by incubating the cells with Cellytic MT reagent (Sigma) for
15 min on a shaker. Cell lysate was centrifuged and the supernatant was
transferred to clean tubes on ice. OTC activity was measured as the rate of
citrulline formation from ornithine and carbamyl phosphate as described by
Kong and were all males, 3 weeks old (average body weight,
500, 1,000, 1,500, and 3,000 IU per rat) were given i.p. on
day 0. Blood samples were drawn from their tail veins on day 0 before the
i.p. injection of rhArg-peg5,000mw as baseline, on days 1 to 6, and then every
2 days. Blood was collected in EDTA and mixed with 50% trichloroacetic acid
solution for protein determination by centrifugation on ice for 30 min. The samples were centrifuged at 13,000 × g
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the volume-doubling time was stabilized, the xenografts were then used for drug evaluation. In vivo efficacy of rhArg-peg5,000mw on nude mice bearing HCC xenografts and its synergy with 5-FU. Forty male nude mice (average body weight, ~20 g) were implanted with 3-mm solid tumors, which were allowed to grow until they reached an average diameter of 5 mm. The mice were then randomly divided into four groups (10 mice per group) and were treated i.p. once a week as follows: group 1, administered with 0.2 mL of 0.9% normal saline (control) per mouse; group 2, administered with 250 IU rhArg-peg5,000mw per mouse; group 3, administered with 250 IU rhArg-peg5,000mw per mouse and 10 mg/kg 5-FU; and group 4, administered with 10 mg/kg 5-FU.

The solid tumors in each animal were observed in situ once every 2 days by digital caliper measurements to determine the tumor size and weight. The size of tumor was calculated by the average of two perpendicular diameters and one diagonal diameter.

Statistical analysis. Statistical analysis was done with SPSS 11.0 software (SPSS, Chicago, IL). The differences in tumor sizes were determined by two-tailed Student's t test.

Results

In vitro arginine-degrading activity of rhArg-peg5,000mw. Figure 1 clearly shows that the rhArg native enzyme was very efficiently pegylated to form rhArg-peg5,000mw by the method we used. As can be seen from the SDS-PAGE data, the molecular weight of the pegylated rhArg-peg5,000mw was much higher than...
that of the rhArg native enzyme. Amino acid analysis of the culture medium contents showed that arginine was decreased, corresponding to an increase in ornithine level, indicating efficient enzymatic conversion of arginine to ornithine by rhArg-peg<sub>5,000mw</sub> in the culture medium (Fig. 2A and B). Asparaginase, which is currently used in the treatment of acute lymphoblastic leukemia, degrades both asparagines and glutamine (26). Its deleterious side effects were due to its degradation of glutamine (27). In contrast, the action of rhArg-peg<sub>5,000mw</sub> was specific in that we found no evidence of degradation of other amino acids (data not shown).

Ensor et al. (12) had reported that native ADI and pegylated ADI enzyme and the rhArg-peg<sub>5,000mw</sub> pegylated enzyme. After additional 3 d of incubation, viability of the cells was determined using MTS reagent, and the IC<sub>50</sub> values were determined. Several other human tumor cell lines were tested by the same method, including a human colorectal (WiDr) and a human lung cancer (A549), all of which were able to grow in the presence of 100 IU/mL, the highest concentration tested.

### Table 1. Arginase inhibition of HCC cell lines in vitro

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>Native rhArg IC&lt;sub&gt;50&lt;/sub&gt; (IU/mL)</th>
<th>Pegylated rhArg-peg&lt;sub&gt;5,000mw&lt;/sub&gt; IC&lt;sub&gt;50&lt;/sub&gt; (IU/mL)</th>
<th>OTC activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>0.20</td>
<td>0.24</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.10</td>
<td>0.10</td>
<td>Undetectable</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>0.21</td>
<td>0.21</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Huh7</td>
<td>0.23</td>
<td>0.21</td>
<td>Undetectable</td>
</tr>
<tr>
<td>SK-HEP-1</td>
<td>0.25</td>
<td>0.22</td>
<td>Undetectable</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.35</td>
<td>0.28</td>
<td>Undetectable</td>
</tr>
<tr>
<td>WiDr</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.120</td>
</tr>
<tr>
<td>A549</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.115</td>
</tr>
</tbody>
</table>

NOTE: The cells listed were grown in 96-well plates for 24 h and then challenged with 0 to 100 IU/mL of either the rhArg native enzyme or the rhArg-peg<sub>5,000mw</sub> pegylated enzyme. After additional 3 d of incubation, viability of the cells was determined using MTS reagent, and the IC<sub>50</sub> values were determined. Several other human tumor cell lines were tested by the same method, including a human colorectal (WiDr) and a human lung cancer (A549), all of which were able to grow in the presence of 100 IU/mL, the highest concentration tested.

**Inhibition of human HCC cell lines with the rhArg native enzyme and the rhArg-peg<sub>5,000mw</sub> pegylated enzyme.** Five HCC cell lines (HepG2, Hep3B, PLC/PRF/5, Huh7, and SK-HEP-1) were each incubated for 3 days with either native or pegylated rhArg to test their *in vitro* sensitivities to the enzyme (Table 1). Strikingly, all the HCC cell lines tested were sensitive to the rhArg native enzyme *in vitro* with IC<sub>50</sub> values between 0.10 and 0.25 IU/mL. Hep3B was the most sensitive tumor with an IC<sub>50</sub> of 0.10 IU/mL. Similar results were seen when rhArg-peg<sub>5,000mw</sub> was used, indicating that both the native rhArg and the pegylated rhArg (rhArg-peg<sub>5,000mw</sub>) have similar antitumor efficacy *in vitro*. On the other hand, HeLa cells (cervix adenocarcinoma) were also found to be sensitive to both rhArg and rhArg-peg<sub>5,000mw</sub> with IC<sub>50</sub> values between 0.28 and 0.35 IU/mL. However, a human colorectal adenocarcinoma cell line (WiDr) and a human lung cancer cell line (A549) were resistant to rhArg and rhArg-peg<sub>5,000mw</sub> treatment with IC<sub>50</sub> values >100 IU/mL, the highest concentration tested.

Next, we compared the *in vitro* efficacy of an established arginine-depleting enzyme, the ADI native enzyme, with the rhArg native enzyme on two HCC cell lines (HepG2 and Hep3B) and a melanoma cell line (SK-MEL-28; Table 2). Again, the rhArg native enzyme was highly effective against these cell lines, with IC<sub>50</sub> values between 0.10 and 0.20 IU/mL (or 0.35 and 0.70 μg/mL). Unexpectedly, HepG2 and Hep3B were highly resistant to the ADI native enzyme with IC<sub>50</sub> values well over 10 IU/mL (or 217 μg/mL). On the other hand, the SK-MEL-28 melanoma cell line was sensitive to ADI as well as to rhArg.

**Expression of ASS, ASL, and OTC mRNA in tested cell lines.** The mRNA products of the five HCC cell lines (HepG2, Hep3B, PLC/PRF/5, Huh7, and SK-HEP-1), as well as of four other cell lines (HeLa, WiDr, A549, and SK-MEL-28), were measured by RT-PCR. We were surprised that they all showed detectable and variable amounts of ASS and ASL mRNA (Fig. 3A). However, the melanoma cell lines (SK-MEL-28) did not produce detectable amount of ASS mRNA, consistent with previously published data (12). Intriguingly, OTC mRNA was not detected in most cell lines (Fig. 3A), except the colorectal (WiDr) and lung cancer (A549) cell lines, which were resistant to rhArg-peg<sub>5,000mw</sub>, even in the presence of high concentrations (100 IU/mL) of rhArg-peg<sub>5,000mw</sub> (Table 1). In addition, OTC enzyme activity was undetectable in the cells that were sensitive to rhArg-peg<sub>5,000mw</sub>. More importantly, OTC activity was detected in WiDr and A549, consistent with the RT-PCR results (Table 1). Thus, all OTC-negative cancer cell lines were much more sensitive to both rhArg and rhArg-peg<sub>5,000mw</sub> than OTC-positive cell lines and these cells exhibited a range of sensitivity to both rhArg and rhArg-peg<sub>5,000mw</sub> with IC<sub>50</sub> ranging from 0.10 to 0.35 IU/mL (or 0.35–1.23 μg/mL).

**OTC transfection of the HCC cell lines HepG2, PLC/PRF/5, and Huh7 and reversal of sensitivity to rhArg-peg<sub>5,000mw</sub>.** To confirm that it was the deficiency of OTC that caused sensitivity to rhArg or rhArg-peg<sub>5,000mw</sub> treatment, OTC mRNA-deficient cells were transfected with an expression plasmid carrying the OTC cDNA. Figure 3B clearly shows that OTC-transfected HepG2, PLC/PRF/5, and Huh7 cell lines expressed the OTC gene. These cell lines were grown in 96-well plates and incubated with rhArg-peg<sub>5,000mw</sub> of various concentrations. After 3 days, the growth of the cells was

### Table 2. A comparison between the IC<sub>50</sub> values of the rhArg native enzyme and the ADI native enzyme on HepG2, Hep3B, and the human melanoma cell line SK-MEL-28

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>Native ADI IC&lt;sub&gt;50&lt;/sub&gt; (IU/mL)</th>
<th>Native rhArg IC&lt;sub&gt;50&lt;/sub&gt; (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>&gt;10 (217 μg/mL)</td>
<td>0.20 (0.70 μg/mL)</td>
</tr>
<tr>
<td>Hep3B</td>
<td>&gt;10 (217 μg/mL)</td>
<td>0.10 (0.35 μg/mL)</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>0.0067 (0.13 μg/mL)</td>
<td>0.12 (0.42 μg/mL)</td>
</tr>
</tbody>
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measured with the MTS reagent. The cells transfected with the OTC gene were much more resistant to rhArg-peg5,000mw than the untransfected parental cells in vitro (Table 3). This suggested that OTC deficiency increased the sensitivity of these HCC cell lines to rhArg-peg5,000mw treatment. The results indicate that OTC expression alone may be sufficient to induce rhArg-peg5,000mw resistance in ASS-positive HCC cells.

Pharmacodynamic study of rhArg-peg5,000mw in rats. The effects of injection of rhArg-peg5,000mw on the plasma levels of arginine (the pharmacodynamics) were determined by administering rhArg-peg5,000mw to rats. As shown in Fig. 4, rhArg-peg5,000mw depleted the plasma arginine in rats in a dose-dependent manner. Administration of rhArg-peg5,000mw resulted in an immediate lowering of plasma arginine to zero on day 1 and, thereafter, arginine levels returned to normal level in the next 10+ days. A dose of 1,500 IU per rat (or 6 IU/g of rat) seemed to lower arginine levels to about zero for 4 to 5 days. This was deemed as the weekly optimal biological depletion dose for rats. As mice have a faster metabolic rate than rats, we estimated the weekly optimal biological depletion dose for mice to be approximately twice that for rats [i.e., ~ 250 IU per mouse (or 12 IU/g of mouse)].

Ensor et al. (12) reported that the circulation half-life of ADI (a few hours) was prolonged by pegylation. The pegylated ADI had a circulation half-life of a few days. Although the native ADI enzyme and the pegylated ADI were similar in their ability to inhibit the growth of melanoma and HCCs in vitro, only the pegylated ADI was found to be effective in inhibiting the growth of these tumor cells in vivo. It would seem that to achieve efficacy in vivo, serum arginine levels must be maintained at low levels for some time.

In vivo efficacy of rhArg-peg5,000mw on human HCC implanted in mice. The in vivo efficacy of rhArg-peg5,000mw on nude mice bearing human HCC xenograft was tested. The OTC-deficient Hep3B cell line (ASS-positive and ADI-resistant) was chosen for this study. In the control group, saline was used and progressive tumor growth was observed. In the treatment groups, rhArg-peg5,000mw was found to have significant tumor-retardation activity in Hep3B (Fig. 5), with early divergence of the tumor size curves, which seemed to parallel each other after the second or third week (P = 0.048). Intriguingly, the combination of 5-FU with rhArg-peg5,000mw seemed to augment the tumor regression rate on this HCC tested.

Discussion

Of all the amino acids, essential and nonessential, depletion of arginine causes the greatest havoc to the cells, in particular tumor cells. Because of their intact “R” checkpoint in the G1 phase of the cell cycle, normal cells generally enter into quiescence (G0) when depleted of arginine, awaiting the return of favorable conditions before resumption of normal cell division, a condition that many cells can tolerate for weeks. Tumor cells, on the other hand, with their defective “R” checkpoint or other cycle aberrations, continue to cycle despite the absence of arginine, which leads to gross imbalance and cell death (28). This is not unexpected because arginine occupies an extremely important position in a myriad of metabolic and enzymatic pathways (1).

For tumor cells in culture, total depletion of arginine is not survivable. Arginine is also unique in that it can be resynthesized by many somatic cells from citrulline. This is the built-in alternative arginine source of the body to ensure its survival in case the amino acid is in short supply or deficient (1). In vitro arginine depletion, whether through an arginine-degrading enzyme, such as arginase or ADI, or by using arginine-free medium, is survivable. Arginine is also unique in that it can be resynthesized by many somatic cells from citrulline. This is the built-in alternative arginine source of the body to ensure its survival in case the amino acid is in short supply or deficient (1). In vitro arginine depletion, whether through an arginine-degrading enzyme, such as arginase or ADI, or by using arginine-free medium, is particularly tumoricidal in those tumors that are highly dependent on exogenous arginine for growth (19). In vivo, enzymatic depletion of arginine offers the most logical and relatively simple approach to

<table>
<thead>
<tr>
<th>OTC-transfected HCCs</th>
<th>rhArg-peg5,000mw IC50 (IU/mL)</th>
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</thead>
<tbody>
<tr>
<td>OTC-HepG2</td>
<td>&gt;20</td>
</tr>
<tr>
<td>OTC-PLC/PRF/5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>OTC-Huh7</td>
<td>&gt;10</td>
</tr>
</tbody>
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NOTE: Human HCCs HepG2, PLC/PRF5, and Huh7 were transfected with an expression plasmid carrying the human cDNA encoding OTC under the constitutive expression of the cytomegalovirus promoter. The transfected cells were incubated with rhArg-peg5,000mw for 3 d and the viability of cells was measured by MTS assay.
arginine depletion. HCC and malignant melanoma are often auxotrophic for arginine (19). The mechanism of this arginine auxotrophy has been amply described in a number of reports (3, 15, 19, 29–31). In essence, these tumors reportedly lack ASS with which to regenerate arginine, which is indispensable for growth. This absolute dependence on exogenous arginine in these tumors makes them particularly vulnerable to arginine depletion (19, 29, 31).

ADI in its pegylated form, ADI-SS PEG20,000 mw (12, 32), has now been shown to have in vitro and in vivo activities in HCC and malignant melanoma as reported in the recent phase II studies (9, 10), although it does have a number of shortcomings. First, it is a bacterial enzyme and antigenicity may still be a problem despite pegylation. In phase II studies that have been reported, autoantibodies were detected as early as the 5th week and continued to increase with treatment (9, 10). This may potentially render the drug ineffective on prolonged treatment. Second, ADI converts arginine to citrulline and free ammonia, which could pose problems in patients with cirrhosis liver and hepatic decompensation with further elevation of ammonia levels (33), leading to prehepatic encephalopathy in man (9, 10, 19). Third, ADI product citrulline is readily recyclable and rescues cells not only from...
arginine-free medium but also from arginase-induced deficiency (34). This has led to the major limitation of ADI: it only kills cancer cells that are ASS deficient (9–12). Many cell lines are ASS positive and they are resistant to ADI treatment, although most require arginine for proliferation (14, 35).

Arginase is a good enzyme to use to degrade arginine in culture because its product is ornithine, which cannot be recycled to arginine because the urea cycle is incomplete in most cultured cells (34), but the mechanism for this phenomenon has been an unresolved area. In our present studies, both native and pegylated rhArg (Fig. 1) were highly active in vitro against all HCC cell lines, with IC_{50} values <0.3 IU/mL (Table 1). At a concentration between 0.3 and 0.4 IU/mL, rhArg-peg$_{5000}$ usually achieved maximal cell kill within ~72 h. However, it was apparent that not all the HCC cell lines exhibited the same degree of sensitivity to rhArg, with rhArg-peg$_{5000}$ to try and establish the most effective treatment different treatment chemotherapy combinations together with ongoing nude mice preclinical studies in our laboratory to test novel agent for effective cancer therapy.

Hep3B being most sensitive (IC$_{50}$, 0.10 IU/mL) and HepG2 least cell lines exhibited the same degree of sensitivity to rhArg, with IC$_{50}$ values <0.3 IU/mL (Table 1). At a concentration between 0.3 and 0.4 IU/mL, rhArg-peg$_{5000}$ usually achieved maximal cell kill within ~72 h. However, it was apparent that not all the HCC cell lines exhibited the same degree of sensitivity to rhArg, with Hep3B being most sensitive (IC$_{50}$, 0.10 IU/mL) and HepG2 least sensitive (IC$_{50}$, 0.24 IU/mL). Although the majority of the HCC cells died within 72 h, it was also apparent that some residual cells were still viable after this period of depletion. It is possible that once a critical level of arginine depletion is achieved, all the sensitive clones would have been totally annihilated, leaving behind residual cells that are relatively resistant to arginine depletion. Even when higher concentrations of arginase were added to complete the arginine depletion, no further cell kill was observed. This is consistent with our in vivo data (Fig. 5). When nude mice bearing HCC xenograft were treated with rhArg-peg$_{5000}$, there was a clear and rapid separation of the tumor size curves in the first 2 to 3 weeks, suggesting rapid killing of the sensitive clones. Thereafter, the curves seemed to diverge more slowly, suggesting continuous growth of a resistant clone of tumor cells. The mechanism of this relative resistance to arginine depletion awaits further elucidation.

Conceptually, the addition of cycle-dependent drugs such as 5-FU along with rhArg-peg$_{5000}$ might augment the antitumor effect of rhArg-peg$_{5000}$ by eradicates some of the resistant clones from the outset, in much the same way as acute lymphoblastic leukemia is treated with l-asparaginase and other cytotoxic agents (36). 5-FU also interferes with RNA and DNA synthesis (37). We used a very low concentration of 5-FU (10 mg/kg) such that this drug alone could not inhibit tumor growth. We were surprised that growth inhibition of HCC tumor cells was enhanced by the combined treatment of pegylated rhArg and 5-FU (Fig. 5). Arginase, in its pegylated form to enhance its arginine depletion activity, could be used as a means to destabilize cancer cells (in our case, HCC) so as to cause a perturbation in their cell cycling. This could account for the enhanced activity of 5-FU in vivo. The possible mechanisms of synergy will be investigated in future studies. Thus, our data suggest that rhArg-peg$_{5000}$ is a novel agent for effective cancer therapy.

The efficacy of combination treatment has already been shown with hydroxyurea given along with arginase (15). There are now ongoing nude mice preclinical studies in our laboratory to test different treatment chemotherapy combinations together with rhArg-peg$_{5000}$, to try and establish the most effective treatment combination. These data could serve as the scientific basis for the design of future phase III clinical studies.

When comparing the in vitro cytotoxic activities of the ADI native enzyme with the rhArg native enzyme, it was surprising to find that ADI was totally ineffective in all our HCC cell lines whereas rhArg remained highly effective (Table 2). The ADI native enzyme was effective on the SK-MEL-28 melanoma cell line, indicating that the ADI we used was functionally active. Ensor et al. (12) reported that there was a relatively wide range of sensitivity to ADI exhibited by ASS-negative cells, with IC$_{50}$s ranging from <0.01 to 0.3 mg/mL. Furthermore, our results showed that the rhArg IC$_{50}$ were 0.42 µg/mL for melanoma (SK-MEL-28) and 0.35 to 0.70 µg/mL for HCCs (HepG2 and Hep3B). Therefore, rhArg inhibited both melanoma and HCCs efficiently and it killed HCCs that could not be killed by ADI.

The reason for the inability of ADI to kill the HCC cells was immediately apparent from the gene profiles of these cell lines with regard to their ASS and ASL gene expression. Contrary to expectation, ASS levels were found to be uniformly present in all the cell lines tested (Fig. 3A), except SK-MEL-28. This would therefore explain the ineffectiveness of ADI on the HCC cell lines that we selected for study. This positive expression of ASS also indicated that the antitumor mechanism of rhArg and rhArg-peg$_{5000}$ was independent of ASS expression. This turned our attention to OTC, which catalyzes the conversion of ornithine to citrulline. Again, we unexpectedly found that OTC mRNA was absent in all the HCC cell lines (Fig. 3A) and OTC enzyme activity was undetectable (Table 1). Because OTC is responsible for the conversion of ornithine to citrulline, which is then converted to arginine via ASS and ASL, one must conclude that these HCC cell lines are auxothropic for arginine because of their inherent lack of OTC expression. Confirmation came by transfection with the OTC gene into the HCC cell lines HepG2, PLC/PRF/5, and HuH7, with the cell lines becoming resistant to arginine depletion with rhArg-peg$_{5000}$ (Fig. 3B; Table 3). As a positive control, we also did arginine depletion with rhArg-peg$_{5000}$ on known OTC-positive cell lines, WiDr (colorectal cancer) and AS49 (lung cancer), both of which are resistant to rhArg-peg$_{5000}$ (Table 1). This opens up an entirely new avenue for exploration with the burgeoning interest being shown in arginine deprivation as a means of bringing cancer cells under control, but it also calls for much more work on a wide spectrum of cell lines, normal and transformed, as well as on tumor biopsy samples, because information of the expression of this OTC enzyme will give valuable information about which tumors will be sensitive, as opposed to resistant, to a therapeutic procedure involving arginine depletion.

In the case of ADI-sensitive tumors, Sugimura et al. (4) had suggested that melanomas may be sensitive to ADI as a result of their inability to express ASS. Furthermore, Ensor et al. (12) reported that only the melanoma and HCC cell lines that lack ASS mRNA were sensitive to killing by ADI. They also found that cell lines that expressed ASS were resistant to ADI and were able to grow even in the presence of large amounts (100 µg/mL) of ADI. Because citrulline is converted to arginine by the sequential actions of ASS and ASL, an inability to express either enzyme would be expected to result in arginine auxotrophy and sensitivity to ADI. All the melanoma and HCC cell lines studied by Ensor et al. (12) did not produce detectable amounts of ASS mRNA but produced ASL mRNA. On the other hand, ornithine is converted to arginine by the sequential actions of OTC, ASS, and ASL, and all the cell lines tested thus far are ASS positive (Fig. 3A). Therefore, an inability to express either OTC or ASS would be expected to result in arginine auxotrophy and sensitivity to rhArg. Thus far, it is not known why human HCCs are unable to express OTC. It will be interesting to study the OTC gene structure and the promoter region to understand the molecular details. The use of pegylated rhArg to kill OTC-deficient, ASS-positive tumors has opened up many anticancer drug research and development possibilities.
In conclusion, we believe that the previous dogma that arginase is a poor drug candidate has now been disproven by our in vitro data as well as our in vivo HCC xenograft–bearing nude mice treated with rhArg-peg5,000mw. RhArg-peg5,000mw is potentially a better drug candidate than the pegylated ADI (ADI-SS PEG20,000mw) because of its predicted efficacy in both ASS-negative and OTC-negative tumors whereas ASS-negative only works in ASS-negative tumors. RhArg-peg5,000mw may be a safer drug candidate than ADI-SS PEG20,000mw with no free ammonia as a side product. Being a pegylated human hepatic enzyme, rhArg-peg5,000mw should be less immunogenic when compared with ADI-SS PEG20,000mw. We hope our studies will initiate a revival of interests in arginase research, in particular in the quest for a better arginine-depleting agent for the treatment of human malignancies.

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References


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Pegylated Recombinant Human Arginase (rhArg-peg 5,000mw) Inhibits the \textit{In vitro} and \textit{In vivo} Proliferation of Human Hepatocellular Carcinoma through Arginine Depletion

Paul Ning-Man Cheng, Tin-Lun Lam, Wai-Man Lam, et al.


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