Recombinant human arginase inhibits the in vitro and in vivo proliferation of human melanoma by inducing cell cycle arrest and apoptosis

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Summary

Melanoma has been shown to require arginine for growth, thus providing a potential Achilles' heel for therapeutic exploitation. Our investigations show that arginine depletion, using a *r*ecombinant form of *h*uman *arg*inase I (rhArg), efficiently inhibits the growth of mammalian melanoma cell lines in vitro. These cell lines are consistently deficient in ornithine transcarbamylase (OTC) expression, correlating with their sensitivity to rhArg. Cell cycle distribution of A375 human melanoma cells treated with rhArg showed a remarkable dual-phase cell cycle arrest in S and G_2/M phases, in contrast to the G_2/M single-phase arrest observed with arginine deiminase (ADI), another arginine-degrading enzyme. rhArg and ADI both induced substantial apoptosis in A375 cells, accompanied by global modulation of cell cycle- and apoptosis-related transcription. Moreover, PEGylated rhArg dramatically inhibited the growth of A375 and B16 melanoma xenografts in vivo. Our results establish for the first time that (PEGylated) rhArg is a promising candidate for effective melanoma treatment, with fewer safety issues than ADI. Insight into the mechanism behind the antiproliferative activity of rhArg could inform us in designing combination therapies for future clinical trials.

Introduction

Amino acid deprivation therapy has emerged as an effective means of treatment for some cancers. The most successful example to date of enzymatic amino acid depletion in cancer treatment has been the use of asparaginase to treat acute lymphoblastic leukemia (ALL). By lowering the circulating levels of asparagine, a non-essential amino acid, the enzyme efficiently attenuates the growth of ALL tumor cells, which have no endogenous means of synthesizing it from its precursor. Most normal human cells, however, do not require asparagine and are thus unaffected by the treatment (Verma et al., 2007). Hence, there is precedence for the use of amino acid-degrading enzymes to treat specific forms of cancer that are auxotrophic for non-essential amino acids. Arginine is a semi-essential amino acid; its synthesis from citrulline can be catalyzed in two steps

Significance

We propose recombinant human arginase (rhArg) as a novel, effective, and safe treatment for melanoma, based on its remarkable efficacy both in vitro and in vivo, as well as its human origin and relatively harmless enzyme reaction products. The rhArg enzyme induces a dual-phase cell cycle arrest and concurrent apoptosis in melanoma cells, providing a mechanistic explanation for its antimelanoma property.

by the urea cycle enzymes, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), mainly in the most proximal part of the kidney tubules (Levillain et al., 2000), but this conversion becomes limiting during development and when rapid growth occurs in adult body tissues, including tumors. ASS catalyzes the conversion of citrulline and aspartic acid to argininosuccinate, which is then converted to arginine and fumaric acid by ASL (Morris, 2004, 2007) (Fig. 1). Several murine and human melanomas as well as hepatocellular carcinomas (HCCs), however, lack ASS expression (Cheng et al., 2007; Dillon et al., 2004; Ensor et al., 2002). Therefore, these tumor cells are incapable of synthesizing arginine, rendering them potential targets of arginine depletion therapy (Wheatley, 2004). Ensor et al., (2002) have demonstrated that deprivation of arginine with the enzyme arginine deiminase (ADI) was highly effective in inhibiting the growth of melanoma and HCC in vitro and in vivo. Recently, we reported that another arginine-degrading enzyme, recombinant human arginase I (rhArg), also has significant antitumor activity against HCC (Cheng et al., 2005, 2007; Lam et al., 2009). Moreover, covalent attachment of polyethylene glycol (PEG) of molecular weight 5000 via a succinimidyl propionate (SPA) linker improved the stability and circulating half-life of rhArg without compromising its activity (Cheng et al., 2007; Tsui et al., 2009). Use of PEGylation to improve the pharmacokinetic or pharmacodynamic properties of proteins for clinical applications is common and has been generally accepted, thus strengthening the case for PEGylated rhArg as a systemic therapy for HCC (Pasut et al., 2008). Importantly, PEGylated rhArg has already progressed to clinical trial studies on liver cancer patients (http://cme.cancer.gov/drugdictionary/?CdrID=657226).

Research into novel treatments of malignant melanoma is especially urgent, given the low response rates to existing modalities (Crosby et al., 2000). Owing to their auxotrophy for arginine, melanomas should also be susceptible to rhArg treatment. Indeed, we have demonstrated that some melanoma cell lines might be sensitive to rhArg in vitro (Cheng et al., 2007; Wheatley and Campbell, 2003). In this study, we have explored in detail the potential of rhArg as a treatment for melanomas by testing its activity on cell lines as well as two mouse xenograft models. Furthermore, having previously proposed that the lack of expression of ornithine transcarbamylase (OTC) was linked to sensitivity of HCC cells to rhArg treatment (Cheng et al., 2007), we now address the question of whether (i) the correlation extends to melanoma cell lines and (ii) OTC expression is a reliable marker for pretreatment screening.

Beyond demonstrating the efficacy of rhArg against melanomas, we studied its mechanism of action, exploring whether rhArg inhibits melanoma growth by arrest of the cell cycle. Cell cycle arrest has been documented in tumor cells grown in arginine-free medium (Scott et al., 2000), and we have observed cycle arrest in rhArg-treated HCC cells (Lam et al., 2009), making it quite possible that a similar effect would be found with melanomas. Another common effect among antitumor drugs is the induction of apoptosis (Hickman, 1992): annexin V labeling was thus used to estimate any effect on apoptosis caused by rhArg. In addition, biologic manifestations at the cellular level are probably orchestrated by alteration in gene expression; for instance, a G_1 arrest induced by arginine deprivation in normal human fibroblasts is probably mediated by transcriptional repression of *cdk4* (Lamb and Wheatley, 2000). We therefore investigated the effects of rhArg treatment on the expression of a range of cell cycle- and apoptosis-related genes, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Better understanding of the mechanism could help future development of therapeutic regimes, especially in deciding potential combination therapies. Although both rhArg and ADI deplete arginine, their reaction products are distinctly different, the former producing ornithine and urea, whereas the latter produces citrulline and ammonia (Morris, 2004) (Fig. 1). Thus, for the first time, we compared the two

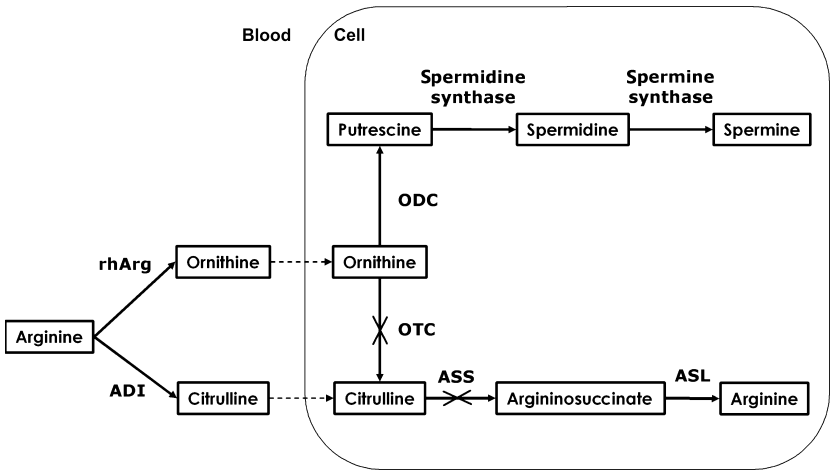

Figure 1. Schematic diagram showing the relationship between arginine depletion therapy and amino acid metabolism in a hypothetical melanoma cell. Absence of OTC and ASS renders the cell incapable of synthesizing arginine from ornithine, resulting in sensitivity to rhArg and ADI treatment, and possible accumulation of ornithine and/or polyamines as a result of rhArg-mediated arginine depletion. ARG, arginase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ODC, ornithine decarboxylase.

arginine-depleting enzymes to determine whether there were any mechanistic differences in their antiproliferative actions.

Evidence will be presented here that rhArg is an effective inhibitor of melanoma growth both in vitro and in vivo, possibly by eliciting a modulation of gene expression, followed by a dual-phase cell cycle arrest as well as apoptotic cell death.

Results

Recombinant human arginase inhibits proliferation of melanoma cell lines

rhArg was tested on five melanoma cell lines (four human and one murine), all of which showed marked attenuation of growth as a result. IC_{50} values for rhArg ranged from 0.058 to 0.616 U/ml (0.46–4.85 μg/ml), depending on cell type, demonstrating an efficient inhibition of melanoma cell growth by the enzyme (Table 1). RT-PCR results showed a lack of *OTC* expression in all five cell lines, consistent with the hypothesis that *OTC* deficiency confers sensitivity to rhArg (Fig. 2). On the other hand, positive results were obtained with controls including a human liver cDNA library as well as mouse liver tissue (Fig. 2), showing that the negative results in the melanomas were because of deficient expression. When SK-MEL-28 cells were transfected with a human *OTC* expression construct, *OTC*-encoding transcripts were also detected by RT-PCR (Fig. 2).

Because we have previously observed that overexpression of *OTC* in an OTC-negative HCC cell line could confer increased resistance toward rhArg, we proceeded to extend our investigation into a melanoma cell line. To that end, A375 cells were transfected with a construct encoding either a green fluorescent protein (GFP)–human OTC fusion protein or GFP alone. The transfections showed similar efficiencies judging by the abundant presence of green fluorescent cells in the cultures (Fig. S1A,B). We demonstrated that overexpression of GFP–OTC led to a significant reduction in the efficacy of rhArg in the inhibition of A375 cell proliferation, compared to transfection with GFP alone or mock transfection without plasmid (Fig. S1C). These results support our hypothesis that OTC expression is intimately linked with tumor resistance toward rhArg treatment. On the other hand, deficiency of ASS in A375 cells, as reported previously, might explain why rhArg still had a considerable inhibitory effect on the growth of GFP–OTC-transfected cultures; while OTC can convert ornithine to citrulline, ASS is required to convert citrulline back to arginine, in order for the tumor cells to overcome the arginine deprivation mediated by rhArg (Fig. 1). Nevertheless, the in vitro efficacy of rhArg against all the melanoma cell types tested indicated early promise of the enzyme as a potential treatment, thus motivating us to further investigate its mechanism of action and its antitumor potency in vivo.

Table 1. In vitro melanoma growth inhibition after rhArg treatment for 3 days (with 95% confidence intervals shown in parentheses)

	IC_{50} of rhArg	
Melanoma cell	U/ml	μg/ml
SK-MEL-2	0.166 (0.099–0.278)	1.30 (0.78–2.19)
SK- MEL-24	0.616 (0.388–0.979)	4.85 (3.05–7.71)
SK- MEL-28	0.157 (0.103–0.239)	1.23 (0.81–1.88)
A375	0.086 (0.063–0.117)	0.68 (0.50–0.92)
B16-F0	0.058 (0.019–0.175)	0.46 (0.15–1.38)

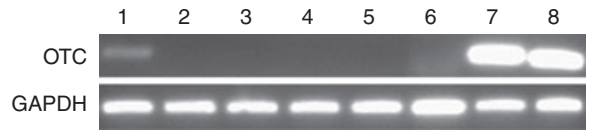

Figure 2. *OTC* expression was absent in all five melanoma cell lines. Positive results were obtained with the controls, human liver cDNA library, mouse liver tissue as well as SK-MEL-28 cells transfected with a human OTC expression construct. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression was used as an internal control. Lane 1, human liver cDNA library; lane 2, SK-MEL-2; lane 3, SK-MEL-24; lane 4, SK-MEL-28; lane 5, A375; lane 6, B16-F0; lane 7, liver tissue from ICR mouse; lane 8, *OTC*–transfected SK-MEL-28.

Recombinant human arginase induces a dual-phase cell cycle arrest in A375 cells

To elucidate the mechanism of the antiproliferative action of rhArg on melanoma cells, the cell cycle distribution of A375 cells was explored after treatment with rhArg, using propidium iodide (PI) staining and flow cytometry. We observed a significant rise in both the S and G_2/M phase subpopulations with increasing concentration of rhArg, suggesting that the enzyme inhibits A375 cell proliferation by effecting a dual-phase cell cycle arrest (Table 2). The S phase subpopulation sharply increased in response to the lowest dose of rhArg (0.188 U/ml), an effect that showed a plateau at higher doses (Table 2). In contrast, the G_2/M phase subpopulation steadily rose with increasing rhArg concentration (Table 2).

ADI leads to a G_0/G_1 arrest in neuroblastoma, lymphatic leukemia, and stomach adenocarcinoma cell lines, as well as in human umbilical vein endothelial cells (HUVEC) (Gong et al., 1999, 2000; Kim et al., 2009a; Noh et al., 2004). While it has been found effective against melanomas, its effect on their cell cycle remains unknown (Ensor et al., 2002). To compare whether the two arginine-depleting enzymes exert the same effect on the cell cycle level, we assayed the cell cycle profiles of ADI-treated A375 cells. Interestingly, ADI treatment resulted in a sharp increase in the proportion of G_2/M phase cells, but no significant effect on S phase (Table 3). Thus, even though rhArg and ADI both

Table 2. Cell cycle distribution of A375 after a 3-day treatment with rhArg, as measured with propidium iodide staining and flow cytometry

rhArg (U/ml)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	76.3 ± 3.75	20.0 ± 3.95	3.77 ± 2.12
0.1875	48.2 ± 2.57	44.0 ± 4.99**	7.87 ± 3.43
0.375	59.3 ± 5.54	35.2 ± 7.13**	5.52 ± 4.21
0.75	54.2 ± 6.70	34.3 ± 3.09**	11.3 ± 6.26
1.5	49.7 ± 8.12	33.6 ± 5.99**	16.7 ± 7.92**
3	44.8 ± 8.12	36.5 ± 4.43**	18.7 ± 5.97**

Data shown as means ± SD. One-way ANOVA revealed significant effects on S and G_2/M phase subpopulations ($P < 0.001$ in both cases; $n = 5–6$). *Post hoc* Dunnett's test of treatment versus control: **$P < 0.01$.

Table 3. Cell cycle distribution of A375 after a 3-day treatment with ADI, as measured with propidium iodide staining and flow cytometry

ADI (U/ml)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	73.1 ± 5.42	21.4 ± 3.42	5.55 ± 2.20
0.00625	63.9 ± 10.2	23.3 ± 11.1	12.8 ± 1.65**
0.0125	63.3 ± 11.1	20.2 ± 14.2	16.4 ± 3.15**
0.025	62.6 ± 12.1	21.4 ± 15.6	15.9 ± 4.21**
0.05	60.9 ± 9.51	24.7 ± 11.3	14.4 ± 2.97**
0.1	60.3 ± 10.4	23.9 ± 13.9	15.7 ± 4.19**

Data shown as means ± SD. One-way ANOVA revealed significant effects on G_2/M phase subpopulation ($P < 0.001$; $n = 4–5$). *Post hoc* Dunnett's test of treatment versus control: **$P < 0.01$.

deplete arginine and inhibit melanoma cell growth, their mechanism of action must differ in some respects.

Recombinant human arginase alters the expression profile of cell cycle genes of A375 cells

Because arginine depletion with rhArg and ADI led to divergent cell cycle arrests in A375 cells, their effects on expression of cell cycle genes might also be expected to differ. Therefore, qRT-PCR was used to examine the expression of several cell cycle- and apoptosis-related genes in A375 cultures treated with the two enzymes. Remarkably, rhArg and ADI elicited mostly similar responses in the genes examined, namely transcriptional upregulation of *cyclin D1*, *cyclin E1*, *cyclin A1*, *CDK6*, *CDK2*, and *caspase-3*, as well as a > 90% downregulation of *cyclin B1* mRNA expression (Figs 3 and 4). The latter is consistent with the G_2/M phase arrest observed with both enzyme treatments, because cyclin B forms a complex with the kinase cdc2, which mediates G_2 to M phase transition in the cell cycle (Arellano and Moreno, 1997). A few genes exhibited slightly different responses to the two drugs: for example, *cdc2* transcription was markedly reduced in ADI-treated A375 cells, but not in rhArg-treated cultures (Figs 3 and 4). This difference might explain why rhArg treatment apparently arrested the cells at G_2/M as well as S phases, whereas ADI treatment was only associated with G_2/M arrest (Tables 2 and 3). ADI also induced *CDK4* and *caspase 8* transcription, effects not observed with rhArg (Figs 3 and 4). Nonetheless, both enzymes effected a global modulation of cell cycle factor expression, which might in part account for their impact on the A375 cell cycle.

Recombinant human arginase induces apoptosis in A375 cells

Upregulation of caspase expression caused by rhArg and ADI treatment in A375 cells suggests that the enzymes might also induce apoptosis, in addition to eliciting cell cycle arrests. Further estimation of rhArg- and ADI-induced apoptosis, using annexin V staining, demonstrated that the enzymes both had strong pro-apoptotic effects, resulting in up to 33% apoptosis (annexin V-positive cells) when applied at the highest dose (3 and 0.1 U/ml, respectively) (Fig. 5A,B). Hence, rhArg and ADI might inhibit melanoma growth by inducing apoptosis as well as arresting the cell cycle.

PEGylated recombinant human arginase attenuates growth of melanoma xenografts in mice

Having established the efficacy of rhArg against melanoma cells in vitro, we examined its antitumor activity in vivo. rhArg was modified by covalent attachment of PEG of MW 5000 prior to injection into mice, because pegylation has been shown to improve the circulatory half-life of the enzyme while preserving its activity (Cheng et al., 2007; Tsui et al., 2009). Human melanoma A375 or mouse melanoma B16 solid tumors were implanted into BALB/c nude mice to generate xenografts. These tumor-bearing mice were treated with regular, twice weekly low (200 U) or high (400 U) doses of PEGylated rhArg (rhArg-PEG) per mouse, and the size of their tumors monitored over time. We observed significant and consistent tumor growth retardation in both A375 and B16 tumor-carrying mice when they were treated with either of these dosages of rhArg-PEG (Fig. 6). By the end of both experiments, the rhArg-PEG-mediated tumor inhibition, relative to the saline-treated control group, was >50% at both dosages (Fig. 6). Its remarkable efficacy in vitro and in vivo thus suggests great clinical potential for rhArg-PEG as an antitumor agent.

Discussion

Cutaneous melanoma is the most aggressive type of skin cancer, which commonly metastasizes to give a poor prognosis, with a median survival of 6 months (Crosby et al., 2000). Currently available systemic therapies produce little benefit: dacarbazine, regarded as the single chemotherapeutic agent of choice for metastatic melanoma, has partial response rates (>50% reduction

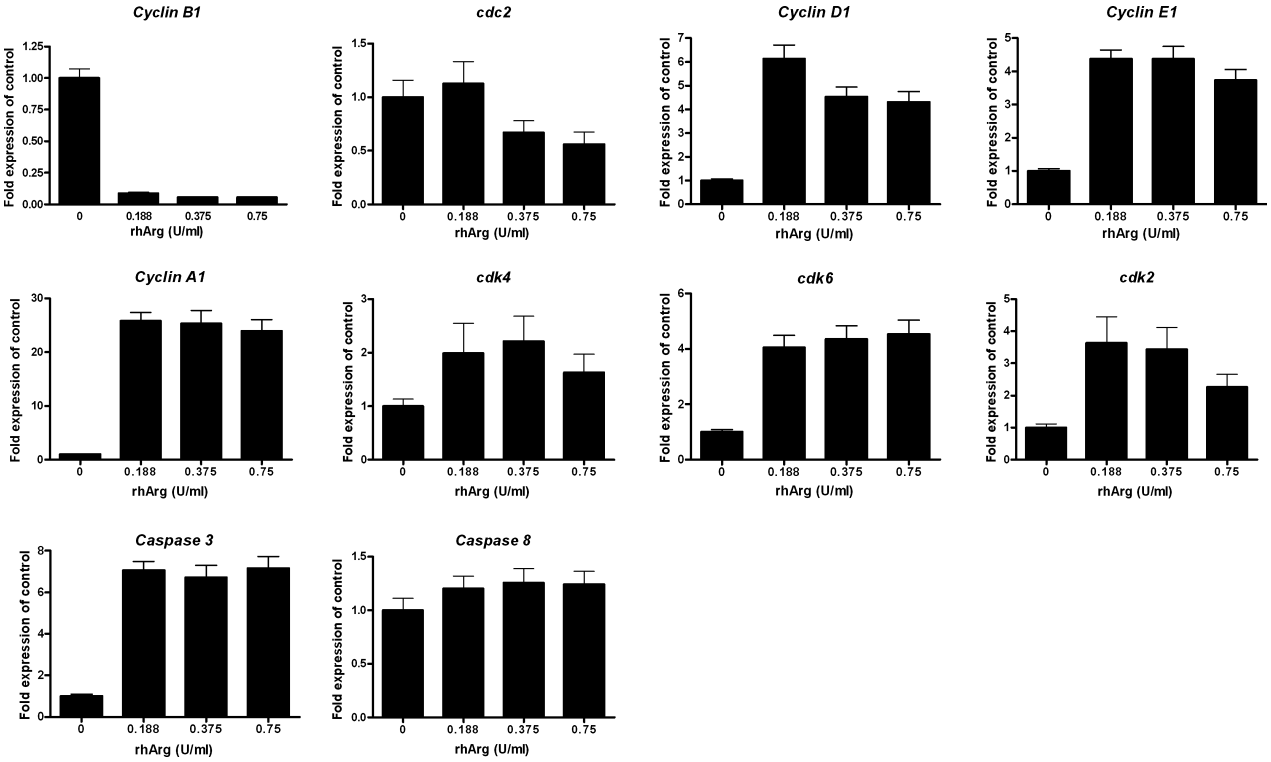

Figure 3. Impact of rhArg on cell cycle- and apoptosis-related gene expression in A375 cells, measured with quantitative RT-PCR (n = 9). Results are shown as means ± SEM.

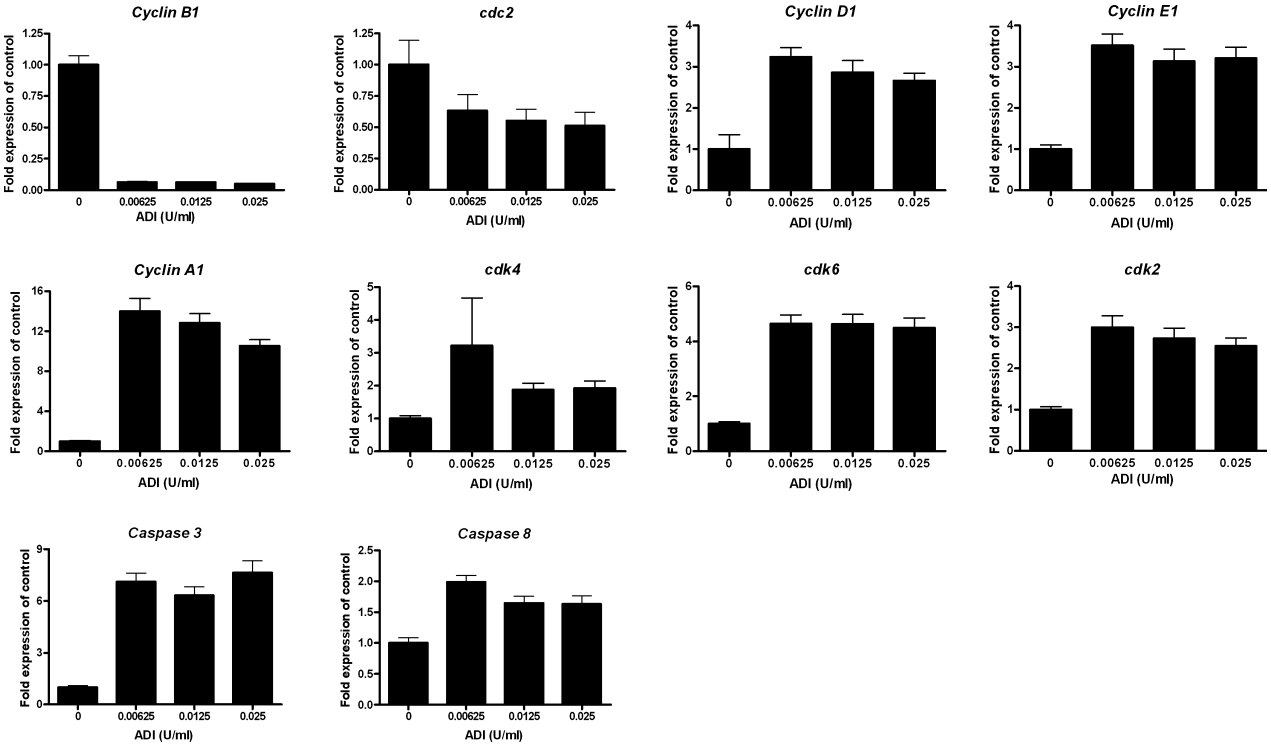

Figure 4. Impact of ADI on cell cycle- and apoptosis-related gene expression in A375 cells, measured with quantitative RT-PCR (n = 9–12). Results are shown as means ± SEM.

in tumor size over >4 weeks) of only 15–28%. Complete response was observed in only 3–5%, and long-term remission in <2%. Numerous other chemotherapeutic regimes, as well as immunotherapy with interferon α or interleukin-2, have also been tested singly or in combination, but showed no clear advantage and occasionally were accompanied by severe toxicity. Moreover, no effective adjuvant therapy has been identified for patients who have undergone surgical resection of their melanomas (Crosby et al., 2000; Jandus et al., 2009). A novel modality that would effectively inhibit the growth of melanomas with few deleterious side effects is therefore in great demand.

The discovery of melanoma auxotrophy for arginine opens up a new target for therapy. Systemic depletion of arginine using an arginine-degrading enzyme has been proposed as a possible means of inhibiting melanoma growth for more than 15 yrs. Arginine deiminase (ADI), an enzyme of *Mycoplasma* origin, was shown to have remarkable antitumor activity toward human melanomas, HCCs, and other cancers (Bowles et al., 2008, Ensor et al., 2002; Kim et al., 2007, 2009b; Miyazaki et al., 1990; Sugimura et al., 1992; Takaku et al., 1992, 1993, 1995; Yoon et al., 2007). However, its bacterial origin will inevitably induce immune reactions in patients, and the ammonia produced by its catalysis is toxic, especially when the nascent form is generated *in situ*. Resistance presents another problem. Expression of ASS, which renders tumors resistant to ADI, has been noted in some cancers (Cheng et al., 2007; Dillon et al., 2004; Ensor et al., 2002; Shen et al., 2003), and arginine depletion also induces ASS expression in cells (Feun et al., 2008; Jackson et al., 1988; Tsai et al., 2009). Arginase, on the other hand, presents an alternative that circumvents these problems (Bach and Swaine, 1965; Cheng et al., 2005). Previously, we demonstrated the efficacy of rhArg, in both its native and PEGylated forms, toward several HCC cell lines, as well as tumor xenografts in nude mice (Cheng et al., 2007; Lam et al., 2009). Here, we have shown that the antiproliferative activity of rhArg extends to melanomas. All five cell lines tested displayed significant growth inhibition when treated with the enzyme for 3 days, with IC_{50} values between 0.058 and 0.616 U/ml (Table 1). This confirms that arginine depletion is an effective way of reducing melanoma growth, regardless of the enzyme used. Moreover, rhArg, being a human enzyme, may offer a safer alternative than ADI, thus warranting further testing.

We previously reported that the expression of *OTC* was intimately linked with resistance toward rhArg (Cheng et al., 2007). The HCC cell lines we tested, all of which were rhArg-sensitive, lacked detectable *OTC* expression, whereas *ASS* expression was detected in all of them, thus rendering them resistant to ADI. This has led to a very important concept: cancer cells can be ADI-resistant but arginase-sensitive. Moreover, we have shown here (Fig. S1) as well as in previous reports that cells expressing endogenous or transfected *OTC* were consistently less sensitive to rhArg treatment (Cheng et al., 2007). We have again found no detectable *OTC* expression in the five melanoma cell lines tested (Fig. 2). These findings are once again consistent with our model which predicts that deficiency of any of the urea cycle enzymes ASS, ASL, or OTC is sufficient to render cancer cells sensitive to rhArg treatment (Fig. 1). Incidentally, deficiency of ASS has also been reported in a variety of melanomas (Dillon et al., 2004; Ensor et al., 2002). There has been evidence that promoter CpG methylation might be responsible for the repression of *ASS* in malignant pleura mesothelioma (Szlosarek et al., 2006), but similar epigenetic regulation of *ASS* expression was not observed in melanomas (Feun et al., 2008). It thus remains to be seen how *ASS* and *OTC* expression is suppressed in these tumors. Nevertheless, our results reported here and before suggest that the urea cycle enzymes (*OTC*, *ASS*, and *ASL*) could serve as a useful predictive marker for responsiveness to rhArg therapy, which therefore allows pretreatment screening. Such screening, using samples from tumor biopsies, could be valuable in identifying those cancer-bearing patients that would likely respond best to rhArg treatment.

How does arginine depletion lead to growth attenuation in tumors? There are two possible routes: by disrupting the cell division cycle and/or inducing cell death. We explored both of these possibilities in rhArg- and ADI-induced growth inhibition. We have demonstrated a divergence in the responses toward the two enzymes: rhArg induced a dual-phase cell cycle arrest

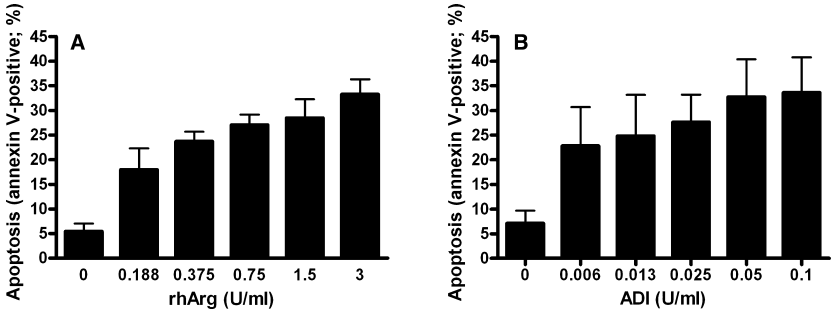

Figure 5. (A) rhArg and (B) ADI treatments induced substantial apoptosis in A375 cells, as detected by annexin V-FITC labeling and flow cytometry. One-way ANOVA, $P < 0.001$; $n \geq 3$ in both cases.

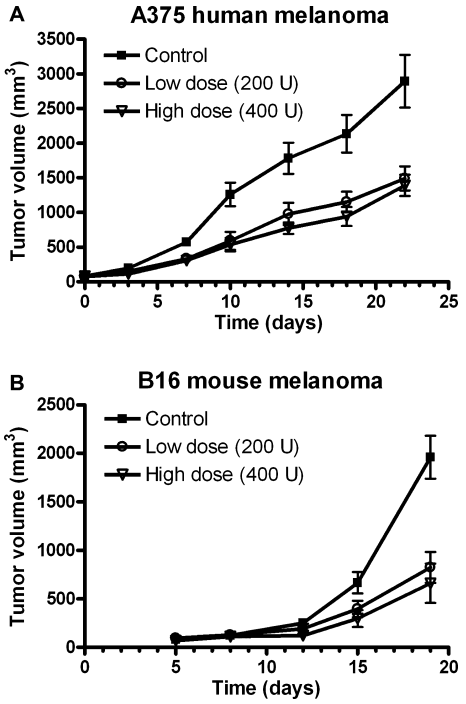

Figure 6. In vivo efficacy of rhArg-PEG against (A) A375 human melanoma and (B) B16 mouse melanoma xenografts in BALB/c nude mice. Data shown as mean values ± SEM. (n = 7–20 per group). Both low (200 U/mouse) and high (400 U/mouse) dosage levels of rhArg-PEG, administered twice weekly, efficiently inhibited tumor growth, relative to the saline-treated controls.

in A375 cells, namely a marked S phase arrest at all doses, together with a G_2/M arrest that became more apparent at higher doses (Table 1), whereas ADI elicited a significant G_2/M arrest with no accumulation of S phase cells (Table 2). The differences in their cell cycle effects could be attributed to the dissimilar products of the respective enzymatic reactions, rhArg converting arginine to ornithine and urea, whereas ADI produces citrulline and ammonia (Morris, 2004, 2007) (Fig. 1). Ornithine, for example, can be converted to polyamines (putrescine, spermidine, and spermine) by enzymes including ornithine decarboxylase (ODC) (Wallace et al., 2003) (Fig. 1). Polyamines are known to stimulate cell proliferation (Wallace et al., 2003), which would explain the drastic reduction (>25%) in G_0/G_1 phase cells induced by the lowest dose of rhArg (Table 2). ADI, in contrast, elicited only an approximately 13% decrease in G_0/G_1 cells even at the highest dose (Table 3). The proliferative drive caused by the polyamines could also account for the shift of the arrest to S phase. By pushing cells otherwise arrested at G_2/M phases through the cell cycle, the cells might eventually accumulate in a different phase where factors are limiting. This S phase arrest could compete with a G_2/M arrest caused by arginine depletion, with the former having a greater effect at the lower concentrations of rhArg, and the latter prevailing at higher doses (Tables 2 and 3).

Dual-phase arrests have been noted in several cell types treated with growth inhibitory factors, with concomitant alteration in gene expression being observed in all cases (Aranha et al., 2000; Kanazawa et al., 2003; Lamb and Wheatley, 1996; Tabruyn et al., 2005). Because the cell cycle is regulated by the cyclin/cyclin-dependent kinase (CDK) complexes, the expression of these proteins could link treatment with cell cycle arrest. Based on our qRT-PCR studies, rhArg- and ADI-treated A375 cells have very similar cell cycle-related gene expression profiles, in contrast to their different impacts on cell cycle distributions (Figs 3 and 4). Both treatments led to a dramatic downregulation of *cyclin B1* transcription (Figs 3 and 4). Because the cyclin B/cdc2 complex is a key mediator of the G_2 to M phase transition, the G_2/M arrests in both cases could at least partly be explained by the repression of *cyclin B1*. Other cyclins and cyclin-dependent kinases (CDKs) also showed altered expression when treated by the enzymes: cyclins D1, E1, and A1 were all upregulated as were *CDK6* and *CDK2* (Figs 3 and 4). Only a few differences were observed between the two treatments; whereas ADI led to a repression of *cdc2* and an induction of *CDK4* expression, rhArg had no significant effect on the expression of either gene (Figs 3 and 4). Their different effects on *cdc2* expression are consistent with the cell cycle profiles. Downregulation by ADI led to a G_2/M arrest, whereas the doses of rhArg which did not affect *cdc2* expression produced only slight G_2/M arrest (Figs 3 and 4, and Tables 2 and 3). Whether polyamines were responsible for these differences remains to be seen, and the complexity of the mammalian cell cycle forbids us from cost-effective analysis of all genes involved. Nonetheless, we conclude that arginine depletion with either rhArg or ADI elicits a global modulation of gene expression, which is probably responsible for the potent antiproliferative activity shown by the two enzymes. The molecular pathway leading from rhArg treatment to transcriptional modulation also remains to be elucidated. One possible candidate is the Mammalian Target of Rapamycin (mTOR) pathway, which is activated by amino acid starvation, notably of leucine and arginine (Bjornsti and Houghton, 2004). Indeed, ADI treatment activates this pathway in melanomas (Feun et al., 2008). Arginine starvation also signals via the GCN2 kinase pathway, resulting in modulation at transcriptional and post-transcriptional levels followed by G_0/G_1 phase arrest in T lymphocytes (Rodriguez et al., 2007). It would be of interest to study whether rhArg elicits the observed transcriptional responses via one or more of these pathways in melanomas.

Activation of the apoptotic pathways is another common consequence of antitumor treatments (Hickman, 1992). Indeed, both rhArg and ADI induced substantial apoptosis in A375 cells, with the highest doses tested

(3 and 0.1 U/ml, respectively) leading to 33% of the cell population being designated for apoptotic cell death, as shown by annexin V labeling (Fig. 5). Annexin V binds phosphatidylserine, a phospholipid whose translocation from the inner to outer leaflet of the plasma membrane is commonly regarded as an early sign of apoptosis (Van Engeland et al., 1998). Thus, annexin V selectively stains cells destined for, or are in the process of, apoptosis. Gene expression studies revealed a concomitant transcriptional upregulation of *caspase 3* in A375 after treatment with either enzyme, whereas *caspase 8* was upregulated by ADI but not rhArg (Figs 3 and 4). There are two apoptotic pathways – the extrinsic, death receptor pathway that involves caspase 8, and the intrinsic, mitochondrial pathway that is caspase 8 independent. Both pathways converge on the cleavage of downstream caspases (caspase-3, -2, and -7) (Li and Yuan, 2008; Okada and Mak, 2004; Riedl and Shi, 2004). Upregulation of either caspase-8 or -3 has been associated with the induction of apoptosis (Droin et al., 1998; Micheau et al., 1999). Moreover, upregulation of both caspases by ADI suggests that it induces apoptosis by the death receptor pathway, whereas induction of caspase-3 seems only to involve the mitochondrial pathway in the case of rhArg treatment. Whether an increase in intracellular polyamine concentration could explain the pathway taken by rhArg-treated cells remains to be tested. The mTOR pathway could also link arginine depletion with apoptosis. Amino acid starvation could induce apoptosis via the mTOR-ASK1-JNK pathway, although its role in rhArg- and ADI-induced apoptosis remains to be established (Bjornsti and Houghton, 2004).

Our findings imply that although both rhArg and ADI apparently inhibit melanoma growth by depleting arginine, there are significant differences between their mechanisms and effects. It is therefore important to investigate rhArg as a novel antitumor agent with distinct characteristics from ADI. Its efficacy in vitro is matched by impressive tumor growth attenuation in A375 and B16 tumor-bearing nude mice (Fig. 6). Its human origin minimizes immunogenicity, and no undesirable side effect has been noted in mouse models thus far (Tsui et al., 2009). Cell cycle arrest induced by rhArg could suggest potential synergy with cell cycle phase-specific chemotherapeutics: by lengthening the time a cell spends in a certain phase, it might render the cell vulnerable to drugs that function only in that particular phase (Noh et al., 2004; Wheatley, 2004). In theory, if the dual-phase arrest observed in A375 cells also applies to other melanomas, it would represent greater possibilities for synergistic combination therapy. S phase-specific modalities such as 5-fluorouracil (Cheng et al., 2007; Lam et al., 2009) and doxorubicin, as well as mitosis-targeting drugs such as pacliataxel and vinca alkaloids, could be tested in combination with (PEGylated) rhArg to achieve an optimal ratio with maximal efficacy and minimal toxicity. Our results, therefore, suggest that rhArg-PEG could be a promising candidate in the continuing quest for an effective treatment for metastatic melanoma.

Methods

Materials

Materials not specified here were obtained from Sigma Chemical Company (St. Louis, MO, USA). Cell proliferation kit with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, as well as all cell culture media and sera, were purchased from Invitrogen Life Technologies (San Diego, CA, USA). Methoxypolyethylene glycol succinimidyl propionate (mPEG-SPA), MW 5000 was purchased from Nektar Therapeutics (Huntsville, AL, USA).

Human malignant melanomas A375, SK-MEL-2, SK-MEL-24, and SK-MEL-28 (ATCC numbers CRL-1619, HTB-68, HTB-71 and HTB-72), as well as mouse melanoma B16-F0 (ATCC number CRL-6322), were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Mouse melanoma B16 cells used for in vivo study were obtained from the Cell Bank of Chinese Academy of Sciences. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin (Gibco, UK).

Protein expression and purification

Recombinant human arginase was expressed, purified, and PEGylated as described previously (Cheng et al., 2007). The arginine deiminase (ADI) gene was cloned from *Mycoplasma arginini* genomic DNA (ATCC accession number 23838D) using the primers S: 5′-ATGTCTGTATTTGACAGTAAATTTAAAGGA-3′ AS: 5′-CTACCACTTAACATCTTTACGTGATAAAGG-3′. For expression in *E. coli*, the gene was further amplified with the primers S: 5′-CATGCCATGGCTGTATTTGACAGTAAATT-3′ and AS: 5′-CGGGATCCCTACCACTTAACATCTTT-3′, digested with *Ncol*/*Bam*HI, and inserted into a pET-3d vector (Stratagene, CA, USA). 5 TGA codons (encoding Trp in *M. arginini*, stop in *E. coli*) within the insert were converted to TGG (Trp in *E. coli*), and second codon (GCT) converted back to TCT, by site-directed mutagenesis, as detailed previously (Noh et al., 2002). ADI protein purification was performed as described previously (Noh et al., 2002): the protein was expressed in the cytosol of *E. coli* BL21 DE3 as inclusion bodies, which were solubilized in 6 M guanidine hydrochloride for 1 h at 37°C and renatured by rapid 10-fold dilution in 10 mM potassium phosphate, pH 7.4. Refolded ADI was purified using DEAE- and phenyl-sepharose columns, followed by arginine-affinity column chromatography. rhArg and ADI activities were determined using the Diacetyl Monoxime (DAMO) method, a spectrophotometric assay that can be used to measure urea or citrulline concentrations (Boyde and Rahmatullah, 1980; Wybenga et al., 1971). The specific activities were 127 and 27 U/mg, respectively, where one unit of rhArg or ADI is defined as the amount that produces 1 μmol urea or citrulline, respectively, per min at 37°C, pH 7.4. rhArg-PEG activity was measured using a coupled spectrophotometric assay described by Ikemoto et al. (Ikemoto et al., 1989)

Cell proliferation assay

Cells (2.5×10^3) in 100 μl culture medium were seeded into each well of a 96-well plate and incubated for 24 h. The culture medium was replaced with medium containing different concentrations of rhArg. The plates were incubated for an additional 3 days at 37°C in an atmosphere of 95% air/5% CO_2. MTT assay was performed to estimate the number of viable cells in the culture. Non-linear

regression with Prism 4.0 (Graphpad Software) was used to fit a sigmoidal dose response curve, and the amount of rhArg needed to achieve 50% inhibition of cell growth was defined as IC_{50}.

OTC expression constructs

Preparation of the human OTC expression construct has been detailed before (Cheng et al., 2007). The GFP–OTC construct was produced by PCR amplification of OTC cDNA (primers: FOR 5′-GGCCCAAGCTTATGCTGTTTAATCTG-3′; REV 5′-ACGCGTCGACTC-AAAATTTAGGCTT-3′) from a human OTC cDNA clone (Origene, cat. no. SC119821), followed by ligation into the vector pcDNA3.1/NT-GFP (Invitrogen). Transfection of OTC construct into SK-MEL-28 was performed using Transfectin (Bio-Rad, CA, USA), and GFP–OTC was transfected into A375 cells using Lipofectamine LTX (Invitrogen), according to manufacturers' instructions. Expression of OTC gene in transfected cells was verified using RT-PCR analysis. Transfected cells were also analyzed for sensitivity to rhArg treatment for 3 days using the MTT assay.

Reverse transcription-polymerase chain reaction (RT-PCR) studies

Total RNA was extracted from cultured cancer cell lines using the Qiagen RNeasy kit, according to manufacturer's instructions. For RT-PCR, the RNA was first reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Briefly, 5 μg of total RNA was subjected to reverse transcription at 42°C for 30 min. A 2 μl portion of cDNA was then amplified using 50 μl of reaction mixture containing 0.5 units of iTaq DNA polymerase (Bio-Rad). PCR was performed in a DNA thermal MyCycler (Bio-Rad). Positive controls include a human liver 5′-stretch plus cDNA library (Clontech, CA, USA) and liver tissue from an ICR mouse.

The following flanking primers were used:
Human OTC (221-bp product):
S: 5′-GATTTGGACACCCTGGCTAA-3′;
AS: 5′- GGAGTAGCTGCCTGAAGGTG-3′
Human GAPDH (306-bp product):
S: 5′- TGAACGGGAAGCTCACTGG -3′;
AS: 5′- TCCACCACCCTGTTGCTGTA -3′
Mouse OTC (234-bp product):
S: 5′-CGTCTTCAAGCTTTCCAAGG-3′;
AS: 5′-AAACTTTGGCTTCTGGAGCA-3′
Mouse GAPDH (450-bp product):
S: 5′-ACCACAGTCCATGCCATCAC-3′;
AS: 5′-TCCACCACCCTGTTGCTGTA-3′

The reaction products were electrophoresed on 1% agarose gels. Band intensities were analyzed by Lumi-Imager (Boehringer Mannheim, Germany), and the relative mRNA expression levels were estimated by normalization with *GAPDH* expression.

Cell cycle analysis

To determine cell cycle distribution, 3×10^5 cells/well were plated in 6-well plates, grown overnight, and treated with various concentrations of rhArg or ADI for 72 h. After treatment, the cells were trypsinized, fixed in 70% ethanol, washed in PBS, resuspended in PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at 37°C, and analyzed by flow cytometry (FACSAria, BD Biosciences, San Jose, CA, USA). Data from 10 000 cells were collected for each data file. Analysis was performed using ModFit LT 3.1 (Verity Software House).

Real-time quantitative RT-PCR

Cells were seeded, grown overnight, and treated with different concentrations of rhArg or ADI for 3 days. Total cellular RNA was extracted as described earlier. Approximately 1 μg of total RNA was subjected to reverse transcription with iScript cDNA Synthesis kit (Bio-Rad) at 42°C for 30 min. The resulting cDNA was subjected to quantitative real-time PCR (Bio-Rad; IQ5 real-time PCR) using the QuantiTect SYBR Green PCR kit (Qiagen, Germany). The experimental and GAPDH reactions were carried out in separate wells in triplicates, and the average threshold cycle (C_T) for the triplicate was used in subsequent calculations. The mRNA level of each sample for each gene was normalized to that of the *GAPDH* mRNA. Gene expression was calculated by $\Delta\Delta C_T$ analysis, and one-way ANOVA was used to determine statistical significance. Primers are listed below.

Caspase 3: 5′-CTGGTTTTCGGTGGGTGT-3′;
5′-CAGTGTTCTCCATGGATACCTTTATT-3′
Caspase 8: 5′-ACTATGAAGAATTCAGCAAAGAGAGA-3′;
5′-GTATCCCCGAGGTTTGCTTT-3′
Cdc2: 5′-TGGATCTGAAGAAATACTTGGATTCTA-3′;
5′-CAATCCCCTGTAGGATTTGG-3′
CDK2: 5′-CCTCCTGGGCTGCAAATA-3′
5′-CAGAATCTCCAGGGAATAGGG-3′
CDK4: 5′-GTGCAGTCGGTGGTACCTG-3′
5′-TGTGTGGGTTAAAAGTCAGCA-3′
CDK6: 5′-TGATCAACTAGGAAAAATCTTGGAC-3′
5′-GGCAACATCTCTAGGCCAGT-3′
Cyclin A1: 5′-AATGGGCAGTACAGGAGGAC-3′
5′-CCACAGTCAGGGAGTGCTTT-3′
Cyclin B1: 5′-CATGGTGCACTTTCCTCCTT-3′
5′-AGGTAATGTTGTAGAGTTGGTGTCC-3′
Cyclin D1: 5′-GAAGATCGTCGCCACCTG-3′
5′-GACCTCCTCCTCGCACTTCT-3′
Cyclin E1: 5′-GGCCAAAATCGACAGGAC-3′
5′-GGGTCTGCACAGACTGCAT-3′
GAPDH: 5′-AGCCACATCGCTCAGACA-3′
5′-GCCCAATACGACCAAATCC-3′

Flow cytometry analysis for Annexin V binding assay

Evaluation of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the supplier's instructions. Briefly, cells grown in 6-well plates were treated with rhArg or ADI for 3 days, then harvested with trypsin and washed in PBS. Cells were resuspended in binding buffer (10 mmol/l HEPES/NaOH (pH 7.4), 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$), and stained with Annexin V-FITC at room temperature for >15 min in the dark. Cells were analyzed in a FACSAria flow cytometer (BD Biosciences) within 1 h after staining.

In Vivo efficacy of rhArg-PEG on nude mice bearing tumor xenografts

BALB/c nude mice (mean body weight approximately 20 g) were implanted with 3-mm^3 A375 human melanoma or B16 mouse melanoma solid tumors. When the tumor reached an average diameter of 5 mm, the mice were randomly divided into different groups. Treatment with rhArg-PEG (200 or 400 U/mouse), or vehicle (0.9% saline), was given intravenously (A375) or intraperitoneally (B16) into mice twice weekly during the course of the experiment. The solid tumor in each animal was measured *in situ* once every 3–4 days by digital caliper measurements to determine tumor volume, estimated using the formula $0.5 \times \text{length} \times (\text{width})^2$.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A375 cells were transfected with expression constructs encoding either GFP alone or a GFP–OTC fusion protein (A and B, respectively). Transfection efficiencies were similar judging by the number of green fluorescent cells observed (right panel). (C) MTT assay showed that GFP–OTC-transfected A375 cells were significantly less sensitive to rhArg-mediated growth inhibition after a 3-day treatment, when compared against overexpression of GFP alone or mock transfection without plasmid (n = 3, data plotted as means ± SEM).

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