Controlling cancer by restricting arginine availability—arginine-catabolizing enzymes as anticancer agents

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Interest has recently been revived in enzymes that degrade essential amino acids. Arginine-catabolizing enzymes now predominate and are discussed in this review. Apart from reducing tumor load through cell death occurring as a result of deprivation alone, these catabolic enzymes conveniently leave the remaining malignant cells vulnerable to other therapeutic modalities through combinatorial treatments with cycle-dependent drugs, the timing of additional treatment after deprivation being crucial. *Anti-Cancer Drugs* 15:825–833 © 2004 Lippincott Williams & Wilkins.

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Introduction: control through manipulation of the availability of essential nutrients in growth and the cell cycle

The body requires many nutrients; a fully mature individual turns over some 400 g of nitrogen per day in the process of protein breakdown and replenishment [1]. For cells to grow, whether malignant or not, nutrients have to be in constant supply and those of particular importance are the 20 or so amino acids making up proteins. Between 70 and 80% of body dry mass is protein, and therefore there is a high and continuing requirement for amino acids, of which we need about 11 amino acids in our diet.

Our own studies were preceded by work indicating a high requirement for arginine by tumor cells back in the last century [2–4] noting that rat tumors responded quickly and impressively within 4 days to deprivation of arginine. Since those observations, very little *concerted* work on arginine manipulation has been done until the last 5 years, probably because the early work was eclipsed by the advent of powerful anticancer drugs in the 1960s, the mustards, the nucleotide analogs and other 'bunkerbusters' of the modern therapeutic arsenal. Also, the rather isolated paper of Storr and Burton [5] had already sown seeds of doubt that amino acid manipulation held any promise.

Of the 20 or so amino acids that could sensibly be manipulated, arginine was the first to be seriously explored, but methionine, tryptophan, phenylalanine and other amino acids have had their champions (see [6]). One reason is that arginine is a nutrient that features in a plethora of metabolic pathways (Fig. 1). Without going into detail, extensive work on most amino acids led us eventually to choose arginine, probably because of its key position in so many metabolic pathways. It turned out to be the most difficult one on this account, but has since proved to be the best choice. In their recent study, Kondoh *et al.* [7] emphasize this point because they show that cells stressed by nutrient deprivation regulate arginine transport through ATR6 downregulation of the y+ carrier of arginine in the cell membrane. However, and paradoxically, G_0 arrest is dependent on having a tiny amount of arginine available because complete deprivation would not permit cells to reach this point of arrest. What Scott *et al.* [8] found was that arginine deprivation of tumor cells did not generally arrest most malignant cell types in G_1 cells, which inevitably led to imbalanced growth in a continued abortive cycle attempt, causing cells to die for as yet unexplained reasons *without any further intervention* (although some workers automatically, but probably wrongly, assume that it is by apoptosis, e.g. [9]). This was an unexpected bonus, but does not occur in all cell types. However, it was primarily these ideas that were in my thoughts when providing my title; if we could but control arginine availability, we might just be able to control malignant growth.

An approach based on cycle dynamic differences in normal and tumor cells

In essence, a cancer cell does not obey stop signals in its proliferative cycle, whereas the normal cell does. This is particularly noticeable in the response of the two phenotypes to amino acid deprivation. For normal cells, there are multiple checkpoints in the cell cycle, acting as guardians of cell survival and proliferative capacity by preventing the cells from progressing through further cycles when preparations for the next round have been

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Fig. 1

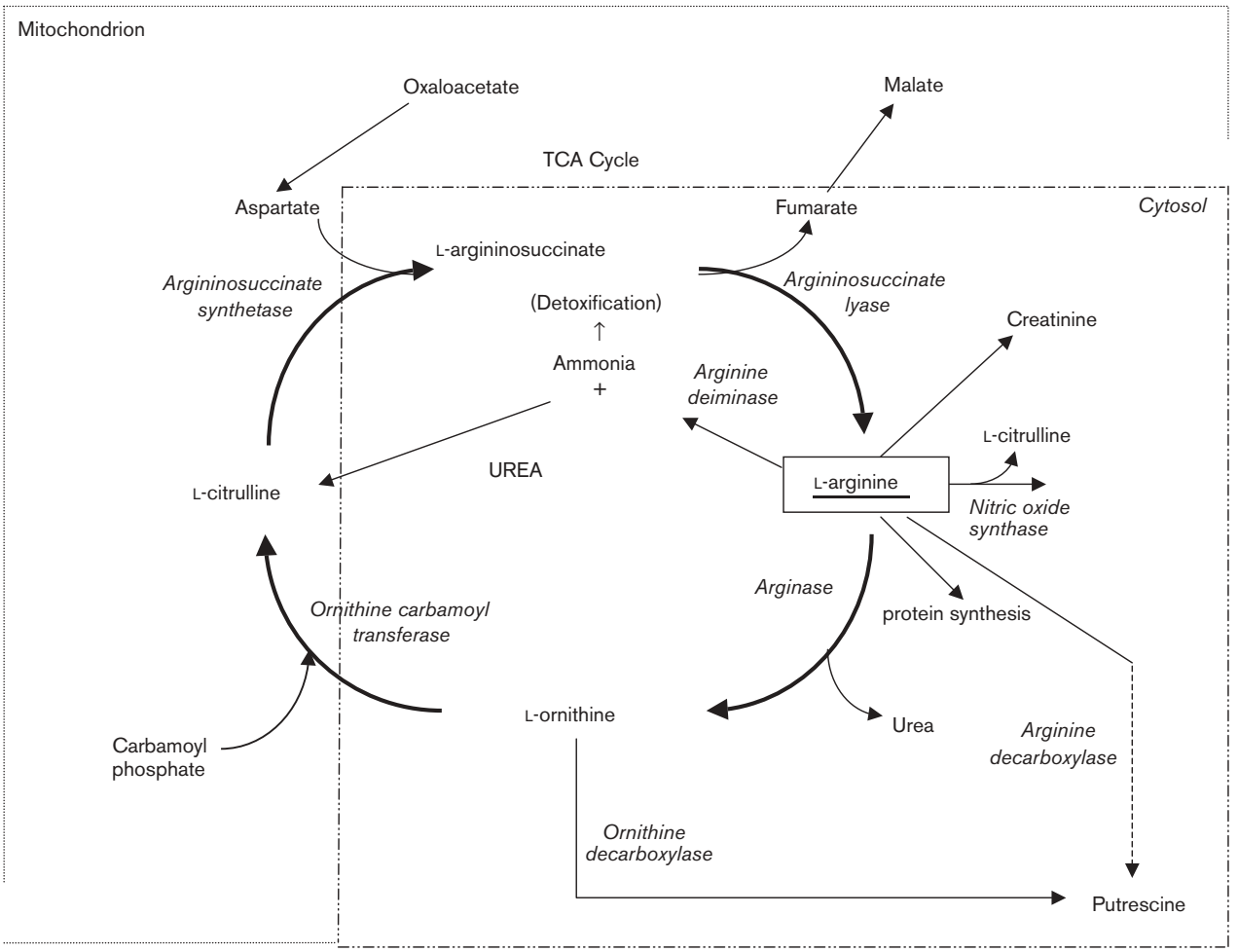


Diagram showing the complexity of arginine metabolism. There is a pleitropic response to arginine deprivation, with the associated stress in mammalian cells beginning to be severe after 48 h.

compromised or adverse conditions arise [10]. This difference has to be exploited, especially as mutations in genes in control of restriction points are among those that feature most prominently in malignant cells (e.g. p53). The ones to which we have already referred are those that can act at restriction points, such as G_1 [11], which have been fully identified in our previous publications [8,12,13]. It has long been known that cells will not advance when an (essential) amino acid is missing [13] and nutrient restriction will hold most organisms in check, until the missing element is replenished, as in the yeast GCN4 system [14]. The system that operates in mammalian cells has not been as well worked out as in yeast, but new data, such as that of Kondoh *et al.* [7], should make things clearer in the near future. What is even less understood from our own work and that of others is how cells that *cannot* enter a resting phase (a prolonged G_1 in the shorter term or G_0 in the longer term) die within a relative short period (several days), a process that may not be entirely confined to malignant cells. Taking it to be the latter case, then the only question that remains crucial is whether some particular types of normal cells might also suffer during deprivation stress from which they will not recover. So far the evidence suggests only that a small increase in normal cell drop-out occurs on top of that under non-deprivation conditions. The vast majority, unlike malignant cells (especially of the more anaplastic tumor types), survive and quickly recover. Thus, a window of opportunity presents itself in which the tumor cells will not move out of cycle and die, while normal cells remain quiescent and can be recovered by returning the missing nutrient. In addition, because malignant cell remain mostly in cycle, they are vulnerable to 'cycle-dependent' drugs, small doses of which will administer the *coup de gras* in simple combined therapeutic regimes. The use of a follow-up treatment with hydroxyurea is shown in Figs 2 and 3 (Wheatley and Philips, unpublished). In brief, having

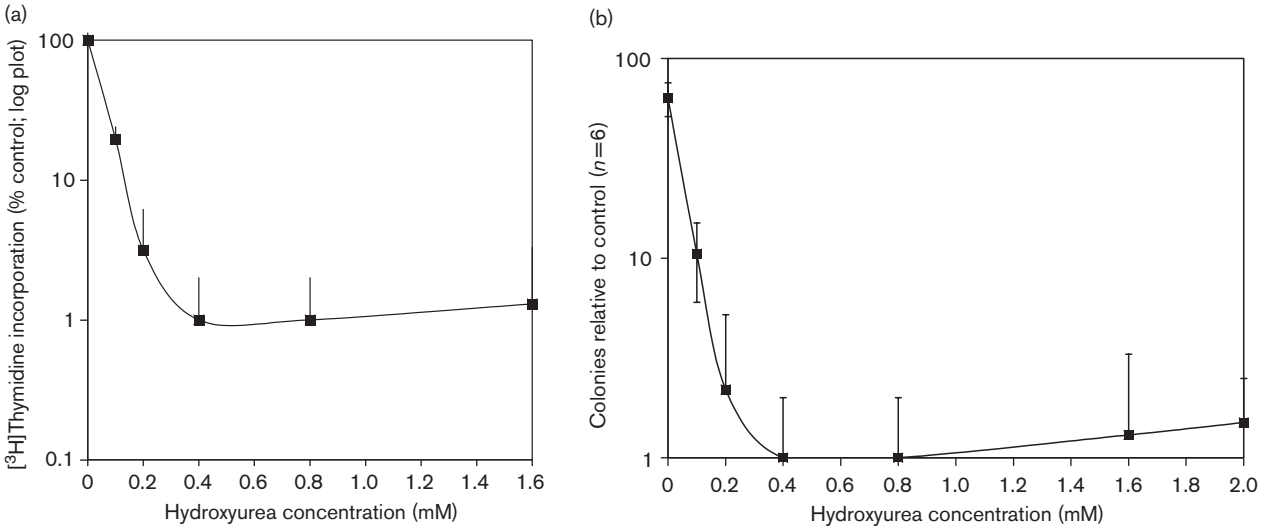
'staged' tumor cells by nutrient deprivation, we now show that those surviving the initial deprivation remain highly vulnerable (in a further *selective* manner) to cycle-dependent drugs.

Strategies other than the use of drugs for depriving cells of amino acids

It is possible to deplete cells or an organism of an essential amino acid without resorting to administering 'drugs' (i.e. enzymes). However, the protocols are complicated, but they have been employed in the treatment of animals and, in a few cases, terminal patients. One procedure is to dialyse the medium or blood so that the appropriate amino acid is depleted. This can be done either by degrading the chosen amino acid within the extracorporeal (extra medium) compartment using immobilized arginine-catabolizing enzymes before returning the medium/blood to the culture/patient, as described by Shettigar [15]. But the problem has largely been with making this system work, not just for hours, but for days. After surmounting many associated problems associated with platelet depletion, altered vascular tone due to arginine depletion, and hypovolemia amongst others, we devised a method whereby all low-molecular-weight materials were removed from the blood by extracorporeal dialysis, and everything was returned at physiological levels except arginine, citrulline and ornithine. This method proved satisfactory as some of the other associated problems were resolved by prostaglandin supplementation, control of vascular tone by balancing dilators and constrictors, and by 'clamping' the blood sugar level by controlled infusion of insulin and glucose. Dialysis could continue for days, but arginine levels seldom reached negligible levels, usually staying between 1 and 5 μM [16,17]. These are rather cumbersome procedures, which can nevertheless be applied where simpler treatment through the straightforward administration of drugs (i.e. enzyme) fails.

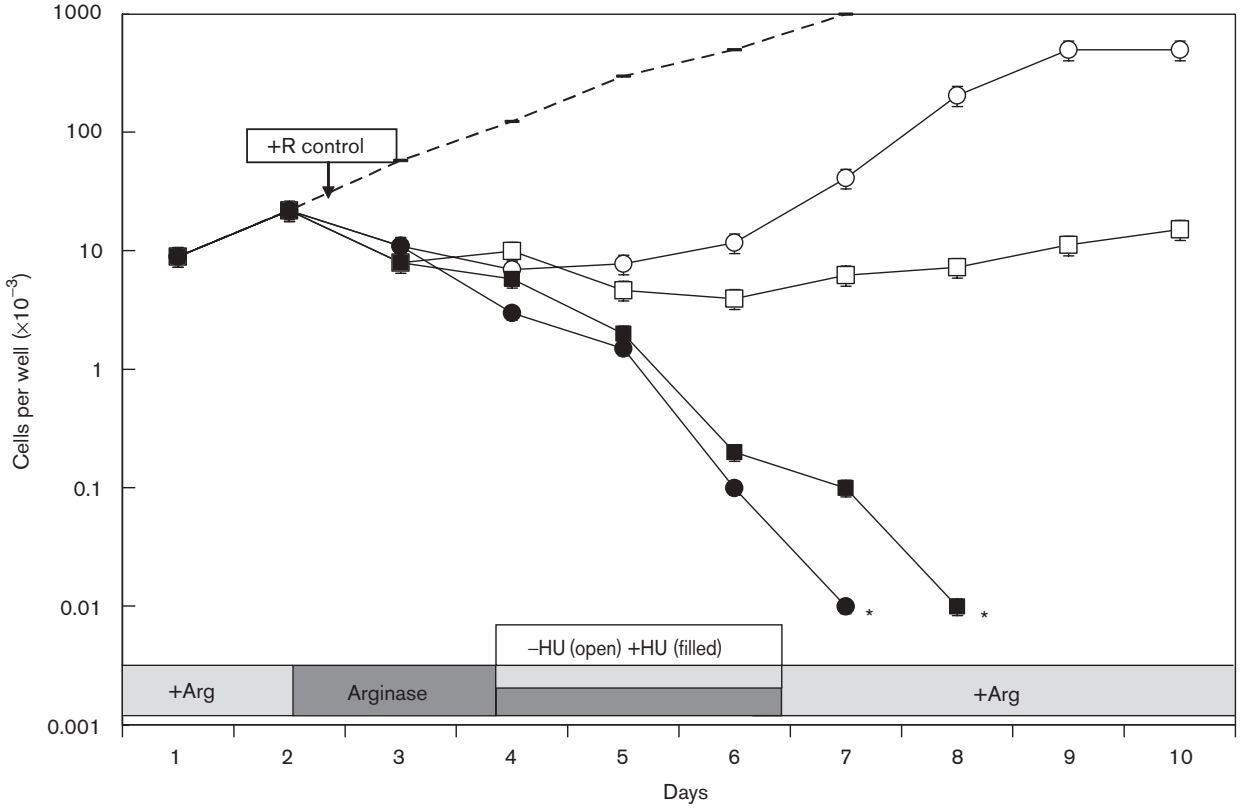
A couple of surgical interventions have been used to induce an arginine-deprivation state, but not as it happens for other amino acids. Both require damage of the liver, the first being by cryoablation, a procedure involving freezing of small spheres of liver tissue and then allowing them to 'recover' [18]. The other is to induce transient hepatic emboli [19]. Both procedures result in focal damage to hepatocytes rich in arginase that release significant quantities of arginase into the blood stream, resulting in transient, but profound, arginine deprivation in the plasma. Clearly these are some of the drastic measures we spoke of earlier, and they do not always work with the efficacy demanded and cannot be repeated more than a couple of times. The work does, however, vindicate all the studies since the 1960s that showed that arginase

Fig. 2



(a) [^{3}H]Thymidine incorporation into macromolecules of L1210 cells (300 000/well, six wells per time point) exposed for 1 h to 37 kBq/ml of the radioactive precursor with carrier thymidine present at 1×10^{-5} M. Hydroxyurea (HU) was added at zero time over the concentration range from 1×10^{-4} to 1.6×10^{-3} M. The curve shows around 70% inhibition within 1 h at 2×10^{-4} M. This was the dose chosen as lowest dose that exceeded the ID_{50} value for critical treatment of cells in combination with an arginine-deprived state (Fig. 4). Mean ± SD. (b) Cells used as in (a), but here they had been left for 2 days in HU over the same concentration range before washing the drug away and plating out 600 cells into soft agar (0.3 over 0.5%) suffused with RPMI 1640 medium containing 10% fetal calf serum in six-well plates. The numbers of foci after 7 days incubation were counted and scored relative to the control taken as unity (plating efficiency of 20–30% under these conditions and time). Again the level of 2×10^{-4} M proved to be close to the minimally effective dose. Large cells tolerate HU for several days and some clones will always form even after exposure to the highest doses levels (1–2% relative to the control). Mean ± SD.

Fig. 3



L1210 cells in culture were grown under arginine deprivation with 1 U/ml bovine arginase (Sigma, Poole, UK) for up to 5 days in fresh arginine-containing medium, except for one set of cultures which received no enzyme as the positive control (- - -). The remaining four parallel sets of cultures (three at each time-point) received arginase for 2 days (days 2–4) or 4 days (2–6 days, see bars). They were rescued in arginine-containing medium without arginase at day 4 in 2 cases (●, ○) and given either 2×10^{-4} M HU (●) or no HU from day 4–6 (○). In the last two sets of cultures, HU was added to one of them with continued arginase treatment (■), while the partner set received no HU (□). These cultures were rescued with fresh arginine-containing medium on day 6. Growth of cells in the cultures after recovery is shown. In the case of the control (- - -), growth was exponential up to almost 10^6 cells/well. None was seen for at least 2 days after arginase treatment. In cells exposed to the enzyme for either 2 or 4 days, no growth was seen in the following days and the cultures proved to have nothing more than dead husks of cells (asterisks), some of which were still being picked up as particles by the (Coulter) electronic counter. Cells not receiving HU showed good recovery of growth after a 2-day exposure (○) and slower recovery after 4 days (□), the latter soon thereafter recovering close to exponential growth (not shown in this experiment) after around day 10. Thus, combined treatment of arginase followed by a critically low level of HU was sufficient to prevent recovery of cells from the arginine deprivation state alone.

from liver extract has strong antiproliferative and anticancer action (e.g. [20,21]; see [7,13] for fuller accounts).

Enzymes: purified liver arginase

Bach and Maws [22] treated Jensen sarcoma in rats because they were utilizing large amounts of arginine for their growth, and thus they thought that arginase might compromise growth. Bach and Simon-Reuss [23] had earlier demonstrated that arginase slowed the growth of these tumor cells in culture. This group went diligently into the business of purifying bovine arginase (EC 3.5.3.1) for the experimental treatment of tumors, knowing two things—that although bovine liver is rich in this enzyme, it is an enzyme coming from a non-rat source and could set off an immune response. They purified a 138-kDa enzyme, and Bach and Swaine [4] subsequently treated CB rats with Walker 256 carcinoma i.p. daily or every other day for 4 days. Within those 4 days, a remarkable 31–71% reduction in tumor growth was recorded, but paradoxically high dose levels of enzyme were less effective. More surprising is that these findings were not followed up with more prolonged treatments at optimized dose levels. The work did not progress further, seemingly because attention was being diverted at that time to new classes of antinucleotide drugs with promise in cancer therapy.

Renewed interest in arginase as a cancer treatment

For the small handful of researchers that did continue to explore arginine deprivation, Osunkoya *et al.* [24] later reported that Burkitt's lymphoma responded to arginase. Storr and Burton [5] followed this by clearly demonstrating that mouse lymphoma cells newly taken into culture

in Fisher's medium (containing a low level of 80 μM arginine) died within 6–4 h of arginase treatment, a surprisingly short interval. However, they were unsuccessful in treating mice with L1210 and L5178Y ascitic tumors, but noted that arginase must have been actively working because of the generation of high levels of ornithine in the ascitic fluid. Since ornithine can be reconverted to arginine *in situ*, the effect not unexpectedly on tumor cells was poor and quite minimal, especially in mice, but the work was never followed up.

Arginase attack on L5178Y cells was greatly extended after 'pegylating' the enzyme [25]. But a real difference occurred between the *in vitro* and *in vivo* action of arginase in these two situations, which lies in the product of arginase activity and its fate. In culture, ornithine cannot be recycled to form arginine since the enzymes to do this are lacking, whereas in the body, ornithine can be converted to citrulline, and this regenerates arginine through argininosuccinate synthetase (AS) and argininosuccinate lyase (ASL), with the incorporation of aspartate. An intact urea cycle should effectively subvert efforts to kill tumor cells, so why quite large suppressions in tumor growth have been reported in some of the abovementioned experiments remains a mystery. However, there were other arginine catabolizing drugs on the scene, and we should now look at them and their relative merits.

We have used very high activity arginase purified by Ikemoto [26], which retains its high activity after pegylation, and therefore can be effective for periods of 5–7 days without the need to refresh the dose. Most of our data involves arginine deprivation of cells in culture, which shows a large number of cells lines (around 80%) of malignant phenotype do poorly under these conditions, whereas normal cells become quiescent and later recover when arginine is restored [27]. However, a strict comparison with other enzyme clearly shows that it makes little difference whether arginine deiminase or arginine decarboxylase (see following sections) is used, the object purely being to deplete the culture medium of arginine. All these enzymes at 0.5–1.0 U/ml destroy arginine in most culture media quickly and effectively. The *choice* of enzyme therefore depends on other factors, such as:

- Availability of a plentiful source
- Ease of isolation and purification
- Specific activity of the purified enzyme
- Loss of activity in subsequent storage and transportation
- Availability and stability of co-factors
- Stability of the administered enzyme (after optimal pegylation)
- Immunogenicity (with and without pegylation)
- Toxicity of by-products

Our work has shown arginase to be the enzyme of choice, at least as far as cell culture work is concerned, and *in vivo* studies are now accumulating that indicate it is also preferable to others amino acid-catabolizing enzymes [26, *cf.* 28]. But the most recent findings are as encouraging because a whole range of cancers appear to have inherent problems in recycling citrulline to arginine. Figures 1 and 4 show this pathway, particularly the enzymes involved in converting arginine from citrulline (ASS and ASL) that are tightly coupled. Many melanoma and hepatocellular carcinoma cells have been reported as deficient in this recycling process, and therefore are 'sitting targets' for destruction with the arginine deprivation protocols, as originally shown by Sugimura *et al.* [27]. It would be rash at this stage to go into further details here (and below, in the section on arginine deiminase), because the field is moving fast and therefore a review would be premature in this rapidly unfolding scenario. Also the fewer the generalization made on a small number of apparently promising trials, the less disappointed we will be should future trials be much less convincing in the percentage of these tumors categorized as deficient.

Arginine decarboxylase

Arginine decarboxylase (EC 4. 1.1.19) is found in bacteria and many plants, and finally in man, where some brain cells require the product, agmatine [29]. The bacterial

Fig. 4

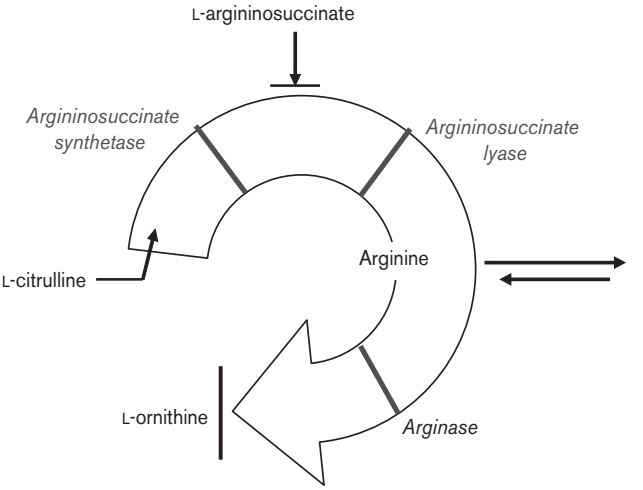
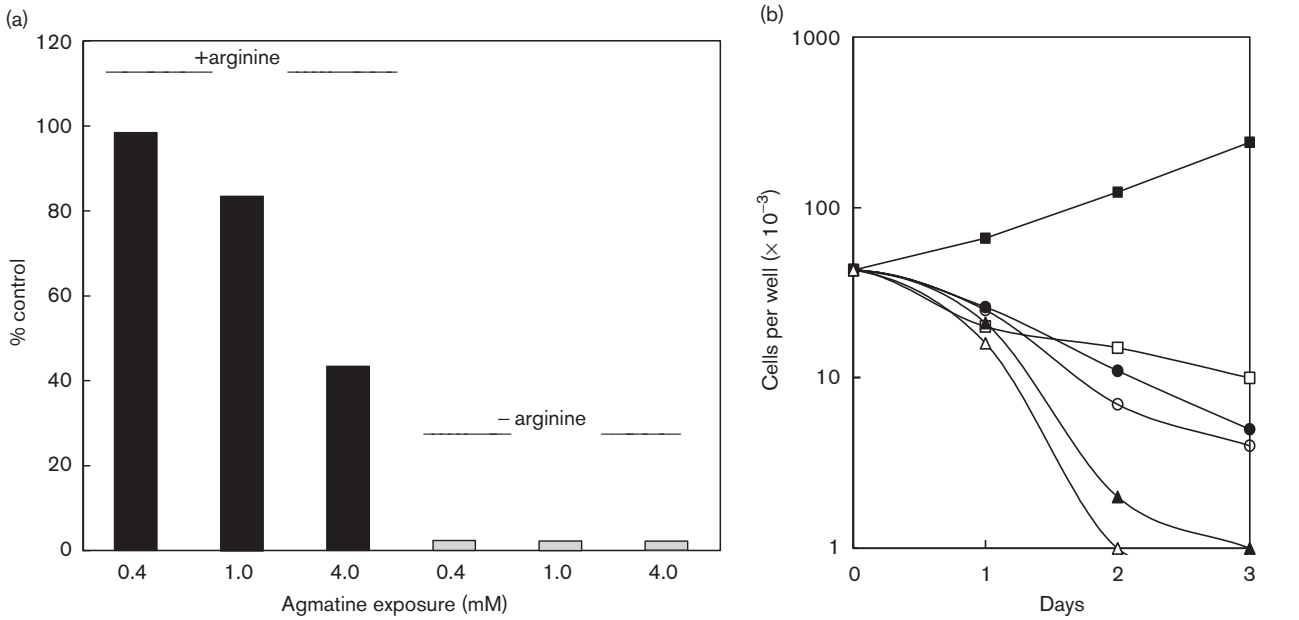


Illustration showing the tight coupling in the ASS/ASL part of the urea cycle (data from several sources [43,45,48]). Loss of this coupling is an exploitable defect in many cancer cells, i.e. of their inability to metabolize citrulline to arginine. Provision of the intermediate argininosuccinate exogenously can to a very limited extent sometimes enter the pathway, but in the normal cycle it is firmly excluded, confirming that the substrate of ASL has to be made *in situ* by ASS. Arginine made in the pathway exchanges with the rest of the cytoplasm and can later be acted upon by arginase to give ornithine. This is not a coupled reaction in this pathway. 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.

Fig. 5



Toxicity of exogenously added agmatine at up to 4 mM in the absence of arginine in A431 cells. When arginine at millimolar concentration is converted to a stoichiometrically equivalent concentration of agamatine by arginine decarboxylase, arginine will indeed be at low concentration and agmatine is relatively toxic, although much less so when arginine is present. A similar result was found with HeLa cells in arginine-deficient medium; after 3 days of exposure to 0.4 mM agmatine in arginine-free medium, cells were irrecoverable. Symbols: (■) arginine controls; (□) arginine-free; (●) 0.25 mM agmatine in arginine-free medium; (○) 0.5 mM agmatine in arginine-free medium; (▲) 1.0 mM agmatine in arginine-free medium; (△) 4.0 mM agmatine in arginine-free medium.

form has been synthesized by transfection [30]. Agmatine in the presence of arginine is not particularly toxic, but when its concentration reaches millimolar levels, especially in falling concentrations of free arginine, cell growth is markedly inhibited and cell death is prevalent (Fig. 5). This enzyme has advantages in that it (1) has a low K_m, (2) rapidly degrades arginine both *in vivo* and *in vitro*, and (3) produces one product that cannot be reconverted to arginine (agmatine) and another (CO_2) which is quickly removed. Recombinant arginine decarboxylase produced by *Escherichia coli* is considerably more active than Sigma enzymes prepared from natural (bovine articular cartilage) sources. One particular downside, however, is that pegylated arginine decarboxylase loses all its activity, probably because it has few lysine residues. The potential of arginine decarboxylase might otherwise be as good as other arginine-catabolizing enzymes and cloning the human gene may be the next step in the production of an immunogenetically acceptable product for treatment of human patients. Whether it is worth doing this seems dubious if it cannot be stabilized by pegylation.

Arginine deiminase—action on murine tumors

This is an enzyme secreted by several species of mycoplasma, notably *Mycoplasma arginini*. Barile and Levinthan [31] noted that *M. arginini* released an enzyme inhibiting lymphocyte proliferation, and Kraemer [32] made a similar observation on mycoplasma medium that inhibited the growth of lymphomas. Gill and Pan [33] identified the enzyme responsible as arginine deiminase (EC 3.5.3.6 or arginine dihydrolase). One advantage of arginine deiminase over arginase is its much lower K_m, Miyazaki *et al.* [34] estimating it around 1000 times more effective than the latter. It inhibited SV-40-transformed cell lines much more severely than untransformed control cells, and was also said to be inhibitory to 10 other tumor cell lines. However, their data do not in general support the latter contention, except perhaps in the case of one hepatoma cell line.

Arginine deiminase isolation, purification and degradative ability on arginine were reported by Sugimura *et al.* [27] and Takaku *et al.* [35], antiproliferative activity resulting from the conversion of arginine to citrulline and ammonia. However, citrulline is converted back to arginine by many mammalian cells in culture, and therefore it follows that a truly arginine-deficient state would be difficult to achieve. Nevertheless, inhibition of the growth of a number of murine tumors was noted [34–38] and this suggests that reconversion is a slow process in culture models where this data was obtained.

Takaku *et al.* [35] used the enzyme *in vitro* and found it actively suppressed growth in all six murine lines they used, with low IC_{50} values as low as around 10 ng/ml. They also showed that as little as 0.2 mg/mouse administered i.p prolonged the survival well beyond the untreated tumor-bearing controls. A bolus of 5 U i.v. catabolized free arginine to negligible level within 5 min, where it remained for 8 days. No untoward effects were reported of this remarkable suppression of free arginine. These are quite remarkable findings that should provide further incentive for exploring the antitumor activity of arginine deiminase.

Deiminase as an antileukemic agent

Recent attention has been on the antileukemic action of the deiminase. Gong *et al.* [36] suggested that arginine deiminase is more effective against mouse leukemia than L-asparaginase, which has had a place in human antileukemic protocols for 20 years. However, L-asparaginase has some undesirable side-effects, such as anaphylaxis and coagulopathy, which clinicians would rather avoid. Komada *et al.* [37] showed that T cells of the lymphoblastoid series were inhibited by their deiminase enzyme, while B cells and the myeloid line remained unaffected. Subsequently Gong *et al.* [38] found that four malignant (leukemic) cells lines were inhibited and underwent apoptosis as a result of deiminase administration. Their data were unclear as to whether inhibition of growth related specifically to arginine depletion rather than some 'more direct' (unspecified) action they suspected was involved. Indeed, this might explain why a generally acting enzyme that depletes arginine availability might affect T lymphoblast cells, but not touch the B series. Furthermore, they reported cycle arrest that in some cases seemed to be in G_1 and in others S phase (or an as yet unrecognized S_0 state). This leaves the possibility that cell cycle control has been compromised by arginine deficiency (or some 'direct action' of arginine deiminase) open to debate, with the possible consequences that cells might either recover (from arrested G_1) or progress into apoptotic cell death (if prolonged S phase occurred).

Although the authors claimed that the earlier papers from the Japanese groups 'confirmed their findings', their evidence provides only weak confirmation of these *preceding* results from the Japanese groups. Analysis of the claim of Gong *et al.* [38] that arginine deiminase is superior to asparaginase in the treatment of leukemia only reveals that they forgot to take into account the widely different specific activities of the two enzymes. Indeed, the experimental data they provide needs to be substantially more convincing and a rational explanation given for the supposed efficacy of the deiminase, because they also demonstrated that arginine deiminase was more toxic to leukemic cells *in the presence of* arginine than in its relative absence, which clearly indicates that another factor is at work, almost certainly the evolution of free ammonia, known to be deadly to cultured cells (see below). The importance of this cannot be ignored, and careful comparative work must be carried out before a new enzyme can be considered a potential replacement for the well-tried and tested L-asparaginase used in the therapy of human leukemia. Furthermore, for reasons already mentioned above, we believe that arginase remains a better choice of enzyme than arginine deiminase because its product (ornithine) cannot be reconverted to the substrate by most cultured cells (other than by a few minimal-deviation hepatoma cell lines) and ammonia is not evolved (see below). In the body the conversion of ornithine to arginine is much slower relative to citrulline.

The problem of free ammonia production

The issue of free ammonia generation is one that arose in the work reported by van Rijn *et al.* [39,40]. Although arginine deiminase still seems to show some promise, its main product in addition to citrulline is an equimolar amount of free ammonia. Since citrulline can be efficiently reconverted to arginine by most normal cells, it is still rather surprising that these reports and some subsequent ones (see below) were able to show not only rapid inhibition of tumor growth *in vivo* and *in vitro*, but extensive cell death—a problem remains to be resolved. A slow regeneration of arginine via citrulline should at least sustain a slow growth rate. The van Rijn data does in fact provide evidence that when a subclinical infection becomes manifest after irradiation, *M. arginini* proliferation leads to extensive cell death due to the presence of arginine deiminase [39,40]. The infecting organism releases a great deal of the same type of deiminase enzyme, which is always devastating to cultures when produced *in situ* or exogenously added [12]. Thus, the 'paradoxical' result of Gong *et al.* [36,38] that arginine deiminase works better on leukemic cells given arginine than those without arginine is simply explained and unfortunately these results [40] temporarily undermine the obvious promise of this enzyme.

Much is at stake here because arginine deiminase is now being used in clinical trials. It has been shown by Clark's group [41,42] to be tolerated well with little toxicity even when injected into guinea pigs, but only in a suitably pegylated form, the 20-kDa PEG conjugate being better than 5-kDa PEG. While data on toxicity, organ weights and other parameters were given, there was no straight comparison in this study of the activities of the 5- and 20-kDa pegylated enzymes relative to the native enzyme.

Value of impaired citrulline metabolism in enzyme therapy—exposing an Achilles' heel in many types of tumor cells

The way in which arginine deiminase can give the best results, therefore, is where tumor cells are deficient in the enzymes needed to reconvert citrulline to arginine, i.e. ASS and ASL. Schimke [43] examined this pathway in detail in tumor cells and noted its suppression at high arginine levels, but a 2- to 15-fold elevation soon after arginine fell back to limiting (low) concentrations. In contrast, Snodgrass and Lin [44] found no such elevation of biosynthetic enzyme of the urea cycle in hepatoma $7800C_1$ cells when made arginine deficient. Unless cells are truly defective in this pathway, citrulline production as a result of enzymatic catabolism of arginine (by deiminase or any other enzyme using it as a substrate) will always try to subvert attempts to achieve an adequately deficient arginine state. However, Sugimara *et al.* [27] claimed that human melanoma (and possibly some hepatoma) cell lines lack more generally the first of the converting enzymes, AS, whereas most other cell types have an intact pathway from citrulline to arginine. This purported feature of melanoma cells is not shared by murine melanomas, since arginine-deprived B16F10 cells, for example, can readily utilize citrulline and have a high ASS level [45]. Nevertheless, the ASS deficiency of some human melanomas and hepatomas could be the key to the specific application of arginine deprivation [46], especially as such tumors are currently on the rise and are highly malignant, killing patients within a short time-span. The therapeutic use of arginine deiminase deserves to be followed up, but the scope of target tumors has to be more comprehensively researched to see if ASS deficiency is more prevalent in other cancers than hitherto suspected. Our most recent data [45] and an extension of it covering in much greater detail tumors of all kinds (Wheatley and Campbell, submitted) totally vindicates this argument, and calls for the simple testing of tumors (biopsies of primary tumors) to assess whether they are deficient in ASS or have it in plentiful supply. This quickly monitors which are going to be most vulnerable to arginine deprivation. However, a worrying observation is that some normal cells of the body (e.g. kidney proximal tubule epithelial cells) are also unable to metabolize citrulline and the impact on their survival will have to be checked more assiduously.

Conclusions

In vitro data have almost got to the point where we know what is going on in arginine deprivation induced by enzymes as anticancer drugs. *In vivo* experiments should show, in due course, the extent to which arginine deprivation, especially through the use of pegylated enzyme, can provide courses of treatment that will inhibit and cause regression of not only such vicious malignancies such as melanoma and hepatocellular carcinoma, but perhaps many others. As already argued, all tumors are unique and therefore there seems good reason to test all tumors with regard to the ASS/ASL activity and coupling (Fig. 4) to see whether arginine deprivation therapy can suitably be applied, either as a single treatment or in combination with subtle doses of chemotherapy, as in Figs 2 and 3. Equally, a high level of ASS might indicate that the cells will be relative resistant to arginine deiminase, but not necessarily arginase treatment, because citrulline re-utilization could be sufficient to sustain an adequate to good growth rate, as shown by Shen *et al.* [46]. So a choice of enzyme remains open in such circumstances. We need to know much more about these problems, but the use of arginine-catabolizing enzymes may be of considerable value in the future across a broader spectrum of tumors than hitherto expected, and could provide relief irrespective of whether they are early or late, localized or disseminated, by bringing them systemically under control. In a recent article, Kitano [47] draws attention to the need for cancer therapy to include more analysis of systems-level strategies based on feedback control data on tumor cells that can show us in future how they can evade treatment, thereby helping us to understand how resistance develops. Throughout his article, he repeatedly uses the word 'control' and not 'cure' as the focus in cancer therapy. This is important to appreciate because cancer is so evasive and we need strategies to which they do not (or cannot) develop resistance. Arginine deprivation may allow us now to approach this level of control, to keep a tumor mass below a critical level, prevent its cells developing resistance, and leave them vulnerable to other more conventional chemotherapeutic modalities. In this way tumors may be suppressed to below a critical cell number that could lead to prolonged remission. There is a long road ahead, but one that seems well worth taking.

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