Canavanine augments proapoptotic effects of arginine deprivation in cultured human cancer cells

Bozhena O. Vynnytska, Oksana M. Mayevska, Yuliya V. Kurlishchuk, Yaroslav P. Bobak and Oleh V. Stasyk

Arginine deprivation achieved by means of recombinant arginine-degrading enzymes is currently being developed as a novel anticancer enzymotherapy. In this study, we showed that arginine deprivation *in vitro* profoundly and selectively sensitized human cancer cells of different organ origin to low doses of canavanine, an arginine analogue of plant origin. In sensitive cancer cells arginine starvation led to the activation of caspase-9, caspase-3 and caspase-7, cleavage of reparation enzyme, polyADP ribosyl polymerase, and DNA fragmentation, which are the typical hallmarks of intrinsic apoptosis realized by the mitochondrial pathway. Co-administration of canavanine significantly accelerated and enhanced apoptotic manifestations induced by arginine deprivation. The augmentation of canavanine toxicity for cancer cells was observed when either a formulated arginine-free medium or complete medium supplemented with bovine arginase preparation was used. Cycloheximide efficiently rescued malignant cells from canavanine-induced cytotoxicity under arginine deprivation, suggesting that it results mainly from canavanine incorporation into newly synthesized proteins. Cancer cells sensitive or resistant to arginine deprivation alone were not capable of restoring their proliferation after 24 h of combined treatment, whereas pseudonormal cells retained such ability. Our data suggest that the incorporation of canavanine into anticancer treatment schemes based on artificially created arginine starvation could be a novel strategy in tumor enzymochemotherapy. *Anti-Cancer Drugs* 22:148–157 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:148–157

Keywords: apoptosis, arginase, arginine deprivation, canavanine, combined treatment

Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine

Correspondence to Oleh V. Stasyk, Department of Cell Signaling, Institute of Cell Biology; Drahomanov str., 14/16, 79005, Lviv, Ukraine
Tel: +38032 2612146; fax: +38032 2612108;
e-mail: stasyk@cellbiol.lviv.ua

Received 10 March 2010 **Revised form accepted** 8 July 2010

Introduction

Amino acid deprivation is an efficient way to control malignant cell proliferation *in vitro* and *in vivo*. A number of recombinant amino acid-degrading enzymes can be used to substantially lower blood concentration of certain amino acids, for which particular types of tumors are auxotrophic. A well-known example from clinical practice is intravenous administration of L-asparaginase for the treatment of acute leukemias [1–4]. Another example, although not that successful, is the use of bacterial methioninase to deprive cancer cells of methionine, leading to so-called methionine deprivation stress [5,6]. During the last decade, arginine deprivation with recombinant bacterial arginine deiminase or human arginase I has also been receiving renewed scientific attention [7,8]. The common drawback of all potential enzymotherapies based on amino acid deprivation is a relatively narrow spectrum of sensitive tumors and frequent reappearance of therapy-resistant tumor clones [9]. Nevertheless, it was shown in a number of reports that metabolic stress triggered through arginine deprivation could be an efficient and relatively nontoxic way to selectively inhibit proliferation of malignant cells, induce apoptosis in certain sensitive tumors and potentiate the anticancer effect of a number of chemotherapeutic agents (e.g. 5-fluorouracil) [9–11].

Arginine is classified as a conditionally essential amino acid in humans. In addition to being required for protein biosynthesis and urea production, arginine serves as a key precursor for such important metabolites as nitric oxide, agmatine, creatine and polyamines [12,13]. It has been observed that many tumor cells have a higher nutritional demand for exogenous arginine in comparison with normal cells. Thus, arginine may be a growth-limiting factor for such malignant cells [14,15]. Because of the complexity of arginine-dependent metabolic networks, the general molecular mechanism that triggers cell death in sensitive tumors on arginine deprivation is still not well understood. It is assumed that deviant regulation of cell cycle on amino acid restriction may be the main trigger [10,14].

It was shown in animal models and clinical trials that arginine deprivation *in vivo* is well tolerated by the organism, but appears to be very detrimental for some tumors (e.g. hepatocellular carcinomas [16] and melanomas) [17]. Recent in-vitro studies on mesotheliomas [18], renal cell carcinomas [19], retinoblastomas [20],

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833e0334

pancreatic carcinomas [21] and prostate carcinomas [11] suggest that they may also be potentially sensitive targets of arginine deprivation-based anticancer therapy. There are, however, several unsolved problems that hamper the introduction of arginine deprivation-based therapy into wide clinical practice. For example, many cancer cells are able to convert citrulline (which is readily synthesized in the human intestine) back to arginine and remain viable on the administration of arginine-degrading enzymes *in vitro* and *in vivo* [22,23]. Nevertheless, it may be expected that the combination of arginine deprivation with rationally selected chemotherapeutic agents could enhance the anticancer effect of this therapy.

Canavanine is a natural toxic arginine analogue of leguminous plant origin that can be incorporated into newly synthesized proteins in the place of arginine [24]. The cytotoxic properties of canavanine have been shown on cells from different organisms and of different tissue origin. It has been also established that canavanine possesses profound anticancer activity both *in vitro* and *in vivo* [25–29]. The antineoplastic properties of canavanine were observed when it was used as a single agent [25,27] or in combination with chemotherapeutic drugs or radiotherapy [30–33]. Nevertheless, interest in the therapeutic use of canavanine eventually declined because of its general toxicity observed during in-vivo experiments and high effective doses that may be required for therapeutic applications [24,34].

In this study we aimed to analyze whether arginine deprivation may potentiate the antiproliferative effect of canavanine *in vitro*. We hypothesized that on arginine deprivation the cytotoxic concentration of canavanine should be profoundly lower because of the absence of substrate competition with arginine for transport, metabolic reactions and protein synthesis. This suggestion is based on data by Swaffar and Ang [35], who reported that excess arginine profoundly reduced the antiproliferative potential of canavanine. Although canavanine exhibits selectivity towards malignant cells *in vitro* [36], it was unclear whether this compound would be significantly more toxic for cancer, relative to normal cells in the absence of arginine. We addressed this question in a systematic study with a number of human epithelial cancer cell lines of different organ origin, both sensitive and resistant to arginine deprivation, as well as human pseudonormal cells. Our data suggest that a combination of arginine deprivation with canavanine treatment has the potential for further development as a new therapeutic approach.

Materials and methods

Cell lines and culture conditions

Human cell lines were obtained from different sources – keratinocytic carcinoma A431, lung adenocarcinoma A549, cervical carcinoma HeLa, hepatocellular carcinoma HepG2, pancreatic carcinomas MIA PaCa-2 and PANC1, breast adenocarcinoma MCF7 and embryonic kidney cells HEK293 from R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine); normal lung fibroblasts WI-38 from Center of Oncology, Maria Sklodowska Curie Memorial Cancer Center and Institute of Oncology (Gliwice, Poland); normal skin fibroblasts N1 were kindly provided by Professor L.A. Kunz-Schughart [37].

All cells were cultured as monolayers at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, Utah, USA), supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 2 mmol/l glutamine and 50 µg/ml gentamycin (Sigma-Aldrich, Steinheim, Germany). The cells were subcultivated every 2–3 days by trypsinization and split in a 1:3 ratio.

In all experiments the DMEM medium was formulated such that it could either contain 0.4 mmol/l arginine (complete arginine-rich medium) or be arginine-free. For the experiments dialyzed fetal bovine serum (HyClone Laboratories) was used, and its level was reduced to 5%.

Trypan blue exclusion test

The cells were seeded in 96-well tissue culture plates at a density of 2×10^4 cells per well in a complete DMEM medium. The medium was discarded 12 h after plating; the cells were washed with phosphate-buffered saline (PBS) and a fresh control or testing medium was added to each well. The cells were trypsinized after the indicated periods of time. Aliquots of cells were mixed with the trypan blue dye solution (Sigma-Aldrich) to its final concentration of 0.05%. Viable (unstained) and nonviable (blue-stained) cells were counted on a hemocytometer by light microscopy.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell seeding and medium change were performed as described earlier (see *Trypan blue exclusion test*). Five hours before the end of the experiments 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml; Sigma-Aldrich) was added to each well and the cells were further incubated at 37°C. Then the growth medium was removed and dimethylsulfoxide was added to each well to dissolve the purple crystals of formazan. Formazan concentration was determined by measuring the absorbance at 490 nm on a plate reader (BioTek, Winooski, Vermont, USA). The percentage of viable cells was calculated as a ratio of optical density determined for the treated cells to that for untreated controls, multiplied by 100. The cell viability data obtained from this assay are not influenced by changes in cell growth rate because of arginine starvation or cycloheximide treatment, which abrogates cell proliferation.

DNA fragmentation analysis

After the indicated incubation time in given treatment conditions, the cells were harvested by trypsinization, pelleted by centrifugation at 1000 *g* for 5 min and then treated with lysis buffer (0.2% Triton X-100, 10 mmol/l Tris–HCl pH 8.0, 1 mmol/l EDTA) at 4°C for 10 min. After centrifugation at 12 000 *g* for 15 min, a DNA-containing supernatant was transferred to a new micro-centrifuge tube, treated with RNase A (Fermentas, Vilnius, Lithuania) at 37°C for 1 h and subsequently with Proteinase K (Fermentas) at 50°C for another 1 h. DNA was precipitated with an equal volume of ice-cold isopropanol in the presence of 0.5 mol/l NaCl solution. The DNA pellet was dissolved in TAE buffer and analyzed by electrophoresis in 2% agarose gel. The DNA fragments were visualized by ethidium bromide staining under UV illumination.

Western blot analysis

The treated cells and their corresponding controls were washed with ice-cold PBS and lysed in extraction buffer (10 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 1% NP-40, 5 mmol/l EDTA, 50 mmol/l NaF, 1 mmol/l Na_3VO_4, 5 mmol/l benzamidine, 1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin) at 4°C for 20 min. Cell extracts were obtained after centrifugation at 12 000 *g* at 4°C for 30 min. Protein content in the extracts was determined according to Peterson's method [38].

For western blot analysis proteins were separated on 10% SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Bedford, Massachusetts, USA). The membranes were blocked in 5% non-fat dried milk in PBS containing 0.05% Tween-20 and immunoblotted with primary antibodies to cleaved caspase-3, caspase-7, caspase-9, cleaved polyADP ribosyl polymerase (PARP) (Cell Signaling Technologies, Danvers, Massachusetts, USA) and β-actin (Sigma, St. Louis, Missouri, USA) as the loading control. Secondary goat horseradish peroxidase-conjugated anti-rabbit (Cell Signaling Technologies) or anti-mouse (Millipore Corporation) antibodies and an enhanced chemiluminescence system (Millipore Corp., Billerica, Massachusetts, USA) were used for the detection of immunoreactive proteins.

Arginase assay

Bovine liver arginase (Sigma-Aldrich) was dissolved in complete DMEM medium (0.4 mmol/l of arginine), with or without 0.1 mmol/l canavanine, to the final concentration of 2 U/ml and incubated at 37°C for 1, 2, 3, 6, 9 and 24 h. The amount of urea produced during the indicated time intervals was used as an index of arginase activity. The standard urea solution and DMEM medium were used as positive and negative controls, respectively. The urea that was formed was assessed spectrophotometrically at 490 nm after the addition of a color reagent (diacetylmonoxime) and heating at 100°C for 10 min, as described in reference [39].

Statistics

In one experiment triplicate wells were used for each treatment and control. All experiments were repeated at least three times. Statistical analyses were performed using Student's *t*-test. Results were expressed as means ± SD. Significance was established when the *P* value was less than 0.05.

Results

Arginine starvation increases canavanine cytotoxicity more profoundly for cancer relative to pseudonormal cells

We compared the cytotoxic effect of canavanine for seven human cancer (A431, A549, HeLa, HepG2, MCF7, MIA PaCa-2, PANC1) and three pseudonormal (HEK293, N1, WI-38) cell lines under arginine-sufficient and arginine-deficient culture conditions. For this purpose the cells were treated with canavanine at a broad range of concentrations (0, 0.001, 0.01, 0.1, 1 and 10 mmol/l) in both arginine-rich (0.4 mmol/l) and arginine-free medium. Cell viability was determined after 72 h of incubation by the trypan blue exclusion test. The amount of canavanine needed to kill 50% of the cells in a culture was defined as the inhibitory concentration (IC50). The established canavanine IC50 values for all tested cell lines are given in Table 1.

We observed that the canavanine IC50 in arginine-rich medium did not differ drastically between tested malignant and pseudonormal cell lines and ranged around 3–10 mmol/l. In contrast, the effect of canavanine in the arginine-free medium differed markedly between cancer and pseudonormal cells. The inhibitory concentration for canavanine in arginine-free medium for cancer cells was approximately two orders of magnitude lower relative to

Table 1 Inhibitory concentrations (IC50) of canavanine determined under arginine-rich and arginine-free culture conditions

		CAV IC50, mmol/l	
Cell line		Arg-rich	Arg-free
	Cancer		
A431	Keratinocytic carcinoma	4	0.03
A549	Lung adenocarcinoma	8	0.07
HeLa	Cervical carcinoma	10	0.05
HepG2	Hepatocellular carcinoma	5	0.05
MCF7	Breast adenocarcinoma	10	0.07
MIA PaCa-2	Pancreatic carcinoma	3	0.03
PANC1	Pancreatic carcinoma	10	0.05
	Pseudonormal		
HEK293	Embryonic kidney cells	10	0.7
N1	Normal skin fibroblasts	5	0.8
WI-38	Normal lung fibroblasts	5	0.2

The cells tested were grown in 96-well plates and challenged with 0, 0.001, 0.01, 0.1, 1 and 10 mmol/l of canavanine in either arginine-rich (Arg-rich) or arginine-free (Arg-free) culture medium. After 72 h of treatment cell viability was determined using the trypan blue exclusion test and the IC50 values were calculated.

the IC50 in an arginine-rich medium and was between 0.03 and 0.07 mmol/l. At the same time, for pseudonormal cells, N1 and WI-38, the canavanine IC50 in an arginine-free medium was 0.8 and 0.2 mmol/l, respectively, thus approximately one order of magnitude lower than that in an arginine-rich medium. For pseudonormal HEK293 cells it remained significantly higher (7 mmol/l) and similar to the canavanine IC50 in an arginine-rich medium. The data obtained suggest that during arginine deprivation most cancer cells become much more sensitive to canavanine treatment than do pseudonormal cells.

Human pseudonormal but not cancer cells are able to restore growth after a combination of arginine starvation and canavanine treatment

We further examined the growth potential of malignant and pseudonormal cells after arginine starvation alone or in combination with canavanine treatment. For this purpose the ability to restore cell growth after different periods of arginine starvation or arginine starvation combined with 0.1 mmol/l canavanine treatment was assayed by the trypan blue exclusion test. The 0.1 mmol/l concentration of canavanine was chosen because this concentration was cytotoxic for all the tested cancer cells on arginine-deprived conditions but not in an arginine-sufficient environment, as documented by the IC50 analysis (Table 1).

As expected, none of the cell lines incubated in an arginine-free medium for 24, 48 and 72 h exhibited significant growth (Fig. 1). Moving cells to a fresh medium supplemented with 0.4 mmol/l arginine and subsequent incubation for an additional 72 h restored growth of all tested cell lines to a different extent, except for HepG2 hepatocellular carcinoma cells (Fig. 1). Importantly, in the case of A431 and HeLa cells the ability to recover growth after arginine starvation decreased time-dependently, whereas for A549, MCF7, HEK293 and N1 cells this ability remained largely unaltered during the whole experiment (Fig. 1). These data allow us to suppose that cancer HepG2, A431 and HeLa cells are sensitive, whereas cancer A549, MCF7 and pseudonormal HEK293 and N1 cells are relatively resistant to arginine deprivation during the time periods indicated.

In contrast, the effect of a combination of arginine starvation with 0.1 mmol/l canavanine treatment on the ability of the cells to restore growth differed greatly between malignant and pseudonormal cells. Although none of the cancer cell lines that were tested was able to restore growth after combined treatment, canavanine removal, resupplementation with arginine and subsequent cultivation for extra 72 h rescued growth of pseudonormal N1 cells after 24 h and HEK293 cells after 48 h of such treatment (Fig. 1). These results suggest that a combination of arginine starvation and canavanine treatment is effective, even against those cancer cells that are not sensitive to arginine starvation alone (A549 and MCF7 cells).

Low-dose canavanine treatment augments mitochondrial apoptosis induced by arginine starvation

To examine whether arginine starvation alone triggers apoptic cell death, A431 keratinocytic carcinoma cells were incubated in an arginine-free medium for different periods of time (6, 12, 24, 48 and 72 h) and afterwards a DNA fragmentation assay as well as western blot analysis with antibodies against the apoptotic markers, activated effector caspase-3 and caspase-7 and cleaved PARP protein, were performed.

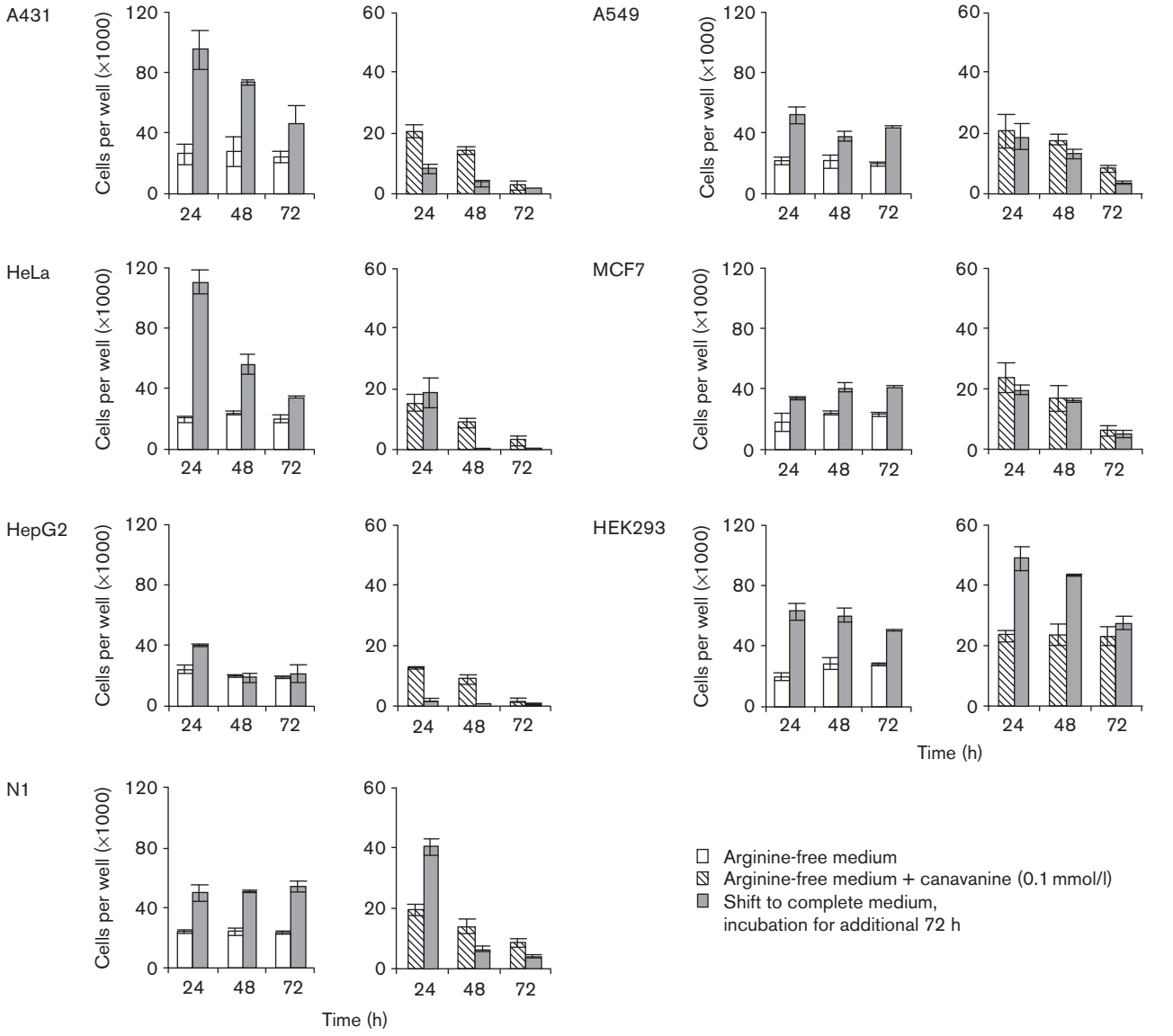
As can be seen in Fig. 2a, an apoptotic DNA ladder pattern was already clearly detectable in A431 cells after 24 h of arginine starvation. We also observed the appearance of large fragments of caspase-3 and caspase-7 cleaved proteins (19–20 and 17 kDa, respectively) after 12-h incubation of A431 cells in an arginine-free medium (Fig. 2b). The cleavage of PARP was detectable in accordance with the activation of caspase-3 and caspase-7 after 12 h of arginine starvation (Fig. 2b).

As arginine deprivation is a kind of nutrient stress, one can expect that it would induce apoptosis through an intrinsic pathway. To check this hypothesis, the cleavage pattern of the initiator caspase-9, the apical protease in the intrinsic apoptic pathway, was examined in A431 cells on arginine deprivation. Western blot analysis showed that in A431 cells caspase-9 was clearly activated after 12 h of cultivation in an arginine-free medium (Fig. 2b). These data suggest that the intrinsic mitochondrial apoptic pathway is activated under arginine starvation in A431 keratinocytic carcinoma cells.

To examine whether arginine starvation and canavanine treatment will exhibit a mutually enhancing effect with regard to the induction of apoptosis, their combined influence on DNA fragmentation, effector and initiator caspases activation, as well as PARP cleavage in A431 cells was studied. For this experiment the cells were incubated in an arginine-free medium supplemented with canavanine (0.1 mmol/l) for 6, 12, 24, 48 and 72 h. As in the case of arginine starvation alone, an apoptotic DNA fragmentation pattern was already observed after 24 h of treatment but was much more pronounced (Fig. 2a). Western blot analysis showed that, as with arginine starvation alone, a combination of arginine starvation and canavanine treatment induced initiator caspase-9, effector caspase-3 and caspase-7 activation and cleavage of PARP protein (Fig. 2b). However, when arginine starvation and canavanine treatment were applied in combination, caspase activation and PARP cleavage were already detectable after 6-h incubation, that is, earlier relative to arginine deprivation as the single treatment (Fig. 2b).

Essentially the same results on the pattern of initiator and effector caspases activation and protein PARP fragmentation were obtained for HepG2 hepatocellular

Fig. 1



Diagrams showing the degree of growth recovery of A431, A549, HeLa, MCF7, HepG2, HEK293 and N1 cells after exposure to arginine-free medium alone or in combination with 0.1 mmol/l canavanine for 24, 48 and 72 h. After the indicated periods of treatment, the medium was changed to complete arginine-rich and cells were allowed to grow for additional 72 h. Viable cell numbers were determined by the trypan blue exclusion test.

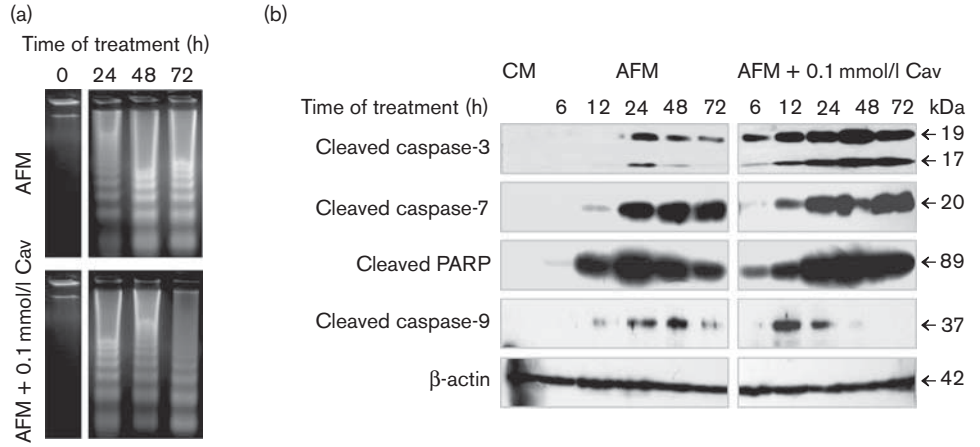
carcinoma cells cultivated in an arginine-free culture medium alone or supplemented with 0.1 mmol/l canavanine (data not shown).

Canavanine cytotoxicity results mainly from its incorporation into nascent proteins

To evaluate the contribution of canavanine incorporation in proteins to its cytotoxic activity, we determined cell viability upon combined treatment with canavanine and cycloheximide, a well-known inhibitor of protein biosynthesis.

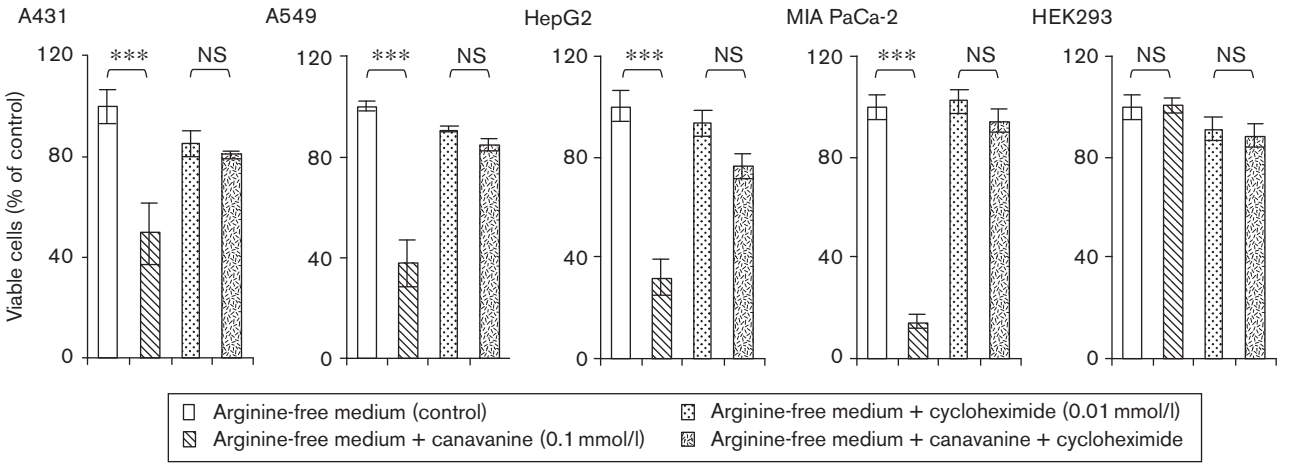
A431, A549, HepG2, MIA PaCa-2 and HEK293 cells were cultured in arginine-free medium supplemented with canavanine (0.1 mmol/l), cycloheximide (0.01 mmol/l) or a combination of both agents for 72 h. After the incubation period, an MTT assay was carried out to determine cell survival. As could be expected from our canavanine IC50 data (Table 1), under arginine starvation canavanine at a concentration of 0.1 mmol/l significantly decreased the viability of all tested cancer cells but had no significant effect on HEK293 cells (Fig. 3). At the same time, cycloheximide was not cytotoxic at the concentration of 0.01 mmol/l under arginine-free culture

Fig. 2



Canavanine accelerates and enhances arginine deprivation-induced apoptosis in arginine starvation-sensitive cell line. Effect of arginine starvation or combination of arginine starvation with 0.1 mmol/l canavanine treatment on DNA fragmentation (a) and activation of caspase-3, caspase-7 and caspase-9 and polyADP ribosyl polymerase (PARP) fragmentation (b) in A431 keratinocytic carcinoma cells. DNA fragmentation and the protein levels of activated caspases and cleaved PARP in the cell lysates were determined as described in 'Materials and methods' section. AFM, arginine-free medium; Cav, canavanine; CM, complete medium.

Fig. 3



Cycloheximide protects cells against canavanine-induced cytotoxicity upon arginine starvation. A431, A549, HepG2, MIA PaCa-2 and HEK293 cells were treated with canavanine (0.1 mmol/l), cycloheximide (0.01 mmol/l) or combination of both agents in arginine-free medium during 72 h. Viable cell numbers were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. ***$P < 0.001$, NS, non significant.

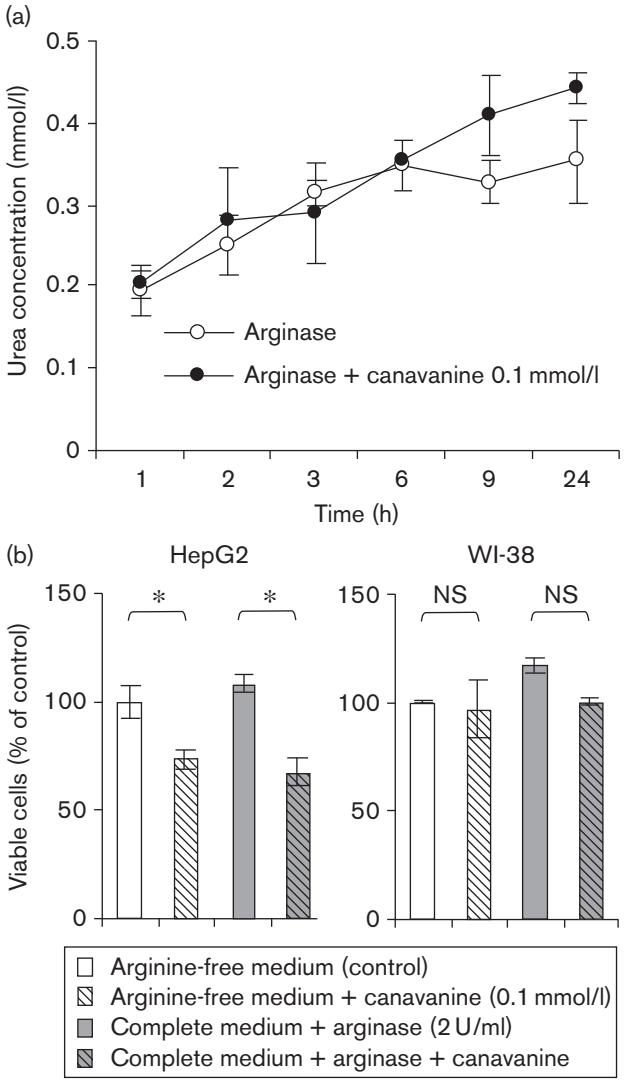
conditions. Co-incubation with both substances showed that cycloheximide completely protected cancer cells from canavanine cytotoxic activity under arginine deprivation (Fig. 3). Essentially the same results were obtained for HeLa, MCF7 and PANC1 cells upon cycloheximide administration (data not shown).

Arginase enhances canavanine cytotoxicity for cancer but not pseudonormal cells

In a human organism arginine deprivation could be artificially achieved by the administration of recombinant arginine-degrading enzymes (e.g. human arginase I, bacterial arginine deiminase) into the blood stream. As canavanine is a structural analogue of arginine, it can be expected that canavanine may inhibit arginase enzymatic activity, thus rendering a proposed combined approach of arginine starvation with canavanine treatment inefficient *in vivo*. To address this possibility, we measured in-vitro bovine liver arginase activity in the DMEM medium in the presence of canavanine. We revealed that incubation with canavanine at a 0.1 mmol/l concentration did not inhibit arginase enzymatic activity as determined by urea production during 24 h (Fig. 4a).

We also investigated whether an in-vitro combination of canavanine with bovine liver arginase treatment in a complete culture medium will yield the same effect on cell survival as the application of the formulated arginine-free medium supplemented with canavanine. In this experiment HepG2 hepatocellular carcinoma cells sensitive to arginine deprivation (Fig. 1) and WI-38 normal lung fibroblasts were used as models. The cells were challenged with bovine liver arginase (2 U/ml) and canavanine (0.1 mmol/l) in a complete arginine-rich medium for 72 h and afterwards cell viability was assessed by an MTT assay. It was shown that the application of canavanine with both an arginine-free medium or arginase in a complete medium significantly decreased the viability of malignant HepG2 cells, although the viability of normal WI-38 cells remained unaltered under the same conditions (Fig. 4b). The relatively higher number of viable cells for both cell lines in the samples in which arginase was added to a complete medium, which was observed in comparison with the samples in which an arginine-free medium was used, can be explained by the fact that a certain time is needed to enzymatically reduce the arginine level in a complete medium.

Fig. 4



(a) Canavanine at 0.1 mmol/l concentration does not inhibit arginase activity. Bovine liver arginase (2 U/ml) was incubated in Dulbecco's modified Eagle's medium medium with or without 0.1 mmol/l canavanine. Arginase activity was assessed by the level of urea production. (b) Combined treatment with bovine liver arginase (2 U/ml) and canavanine (0.1 mmol/l) yields the same effect on viability of HepG2 hepatocellular carcinoma cells and WI-38 normal lung fibroblasts as application of arginine-free medium supplemented with 0.1 mmol/l canavanine. Cells were incubated under defined treatment conditions for 72 h. Viable cell numbers were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *$P < 0.05$, NS, non significant.

These data suggest that, as in the case with the defined arginine-free medium, arginase significantly enhances canavanine toxicity for cancer cells, but does not exert such an effect for noncancer controls.

Discussion

In this study we examined *in vitro* the potency of the combination treatment of single amino acid arginine starvation with canavanine, a toxic arginine analogue of plant origin. Several human malignant cell lines of different organ origin, sensitive or resistant to arginine deprivation alone, as well as pseudonormal cells were used as experimental models.

We observed that, as expected, canavanine toxicity was strongly dependent on arginine availability in the culture medium. In arginine-deprived culture conditions the IC50 of canavanine decreased dramatically for all cells but was considerably lower for cancer cells than for pseudonormal cells (Table 1). Importantly, canavanine exerted its cytotoxic effect even on those cancer cells that were relatively resistant to arginine deprivation alone. For instance, although lung adenocarcinoma, A549, and breast adenocarcinoma, MCF7, cells restored growth after prolonged 72-h arginine deprivation, they were unable to recover after the 24-h combined treatment with canavanine (Fig. 1).

It was observed earlier in the rat model that on subcutaneous injection, canavanine was selectively taken up and accumulated in the pancreas [40]. Although the mechanism for the sequestration has not been deciphered, this phenomenon was proposed as a rationale for the use of canavanine in the treatment of pancreatic tumors [24]. To elucidate whether cancer cells of pancreatic origin will be more sensitive to canavanine relatively to other malignant cells, we included human MIA PaCa-2 and PANC1 cells in our analysis. We observed that they did not differ dramatically from other examined cancer cell lines with respect to canavanine IC50 upon incubation in a complete or arginine-free

medium (Table 1). This fact, however, does not preclude the possibility that pancreatic cancer cells will be more sensitive to this combined treatment *in vivo*.

In the models of human cancer cells sensitive to arginine deprivation, keratinocytic carcinoma A431 and hepatocellular carcinoma HepG2, we showed that arginine deficiency triggered apoptic cell death of mitochondrial type as judged by a DNA fragmentation assay and activation of the corresponding initiator and effector caspases. The levels of active caspase-9 (specific for intrinsic apoptosis), caspase-3 and caspase-7 increased time-dependently starting approximately after 12 h of arginine starvation, along with rapid accumulation of the cleaved PARP fragment (Fig. 2). We observed that upon arginine deprivation combined with 0.1 mmol/l canavanine treatment, caspase activation and PARP cleavage occurred significantly earlier, already after 6 h of treatment (Fig. 2). These results support the earlier findings of other investigators, showing that canavanine alone is able to induce caspase-3-mediated apoptosis in Jurkat T cells [27]. Therefore, in our tumor models, canavanine accelerated and augmented mitochondria-dependent apoptosis compared with that observed under arginine starvation as the single treatment.

In addition to protein biosynthesis, arginine is involved in numerous regulatory networks, which significantly affect cell growth and viability [12]. Thus, one can expect that canavanine cytotoxicity may arise not solely from its incorporation into nascent proteins, but also be because of its involvement in other arginine-dependent pathways [24]. We observed that the cytotoxic effect of canavanine under arginine starvation was completely abolished when cycloheximide, an inhibitor of protein synthesis, was co-administered (Fig. 3). This protective effect of cycloheximide confirmed the hypothesis suggested by Swaffar and Ang [35], that the main portion of the cytotoxic effect of canavanine against human pancreatic cancer cells was attributable to its incorporation into newly synthesized proteins, leading to the formation of canavanyl-bearing proteins with altered structural and impaired functional activities. As no correlation between canavanine cytotoxicity and intracellular arginase activity was observed in different cancer cell lines (our unpublished observation), we suppose that the conversion of canavanine to its toxic catabolite canaline [41,42] is not responsible for the observed phenomenon. It is likely, therefore, that under arginine starvation cancer cells exhibit much higher sensitivity toward canavanine relative to pseudonormal cells, mainly because of the deviant regulation of protein synthesis in response to amino acid withdrawal.

One feasible way to starve tumor cells for arginine *in vivo* is the application of recombinant arginine-degrading enzymes. Stabilized by pegylation bacterial arginine deiminase and human arginase have been successfully evaluated *in vitro* and *in vivo* [9–11,43] and are being further developed as anticancer agents [16,17]. However, in the case of a combination of arginine starvation with canavanine treatment, it should be taken into consideration that canavanine, as an arginine analogue, is also a substrate of these enzymes [44,45]. When a recombinant enzyme and the drug are administered simultaneously, this could lead to partial detoxification of canavanine, or, alternatively, canavanine, as the competitive arginine analogue, can inhibit activity of the enzymes that are mentioned [46,47]. However, we showed that at a 0.1 mmol/l concentration canavanine did not inhibit bovine liver arginase activity *in vitro* (Fig. 4a). The probable reason for this is the considerably higher arginase K_m for canavanine relative to arginine [45,48]. We also analyzed whether the administration of bovine liver arginase to an arginine-sufficient medium along with canavanine would lead to an increase in a canavanine-effective dose relative to that in the defined arginine-free medium. It was observed that for hepatocellular carcinoma HepG2 cells and lung fibroblast, WI-38 cells the viability upon combined treatment with arginase and canavanine was the same as when arginine starvation was achieved by means of an arginine-free medium (Fig. 4b). Essentially the same results were obtained with the preparation of recombinant human arginase I (to be reported elsewhere). In contrast, our preliminary data suggest that the effective canavanine dose increased considerably when the preparation of recombinant *Mycoplasma hominis* arginine deiminase was co-administered with canavanine, making this combination less feasible (data not shown). This may be explained by the fact that arginine deiminase can be efficiently inhibited by canavanine [46,49]. As the catalytic properties of pegylated recombinant human arginase do not differ significantly from those of the native human enzyme [9], our data suggest that canavanine is a suitable agent for combined treatment with arginase preparations and warrant further studies on its clinical potential. It remains to be validated in animal models whether canavanine cytotoxicity at its minimal effective doses in the absence of arginine will be well tolerated by the organism.

One of the currently unsolved problems in antitumor enzymotherapy with arginine-degrading enzymes is the appearance of therapy-resistant clones (because of the restored ability to convert exogenous citrulline to arginine). As we showed that canavanine augments and accelerates apoptosis in tumor cells during arginine deprivation, it can be expected that such treatment will eliminate tumor cells faster, thus leaving a shorter time window for the occurrence of new prosurvival genetic alterations. Another important feature of the proposed combination treatment shown in this study is its effectiveness against various cultured cancer cells that originate from different organs. It can be expected, therefore, that the application of the proposed combinational strategy *in vivo* may potentially broaden the spectrum of

tumors sensitive to arginine deprivation-based therapy and may also be compatible with other rationally selected chemotherapeutic agents specific for certain types of tumors.

Acknowledgement

This study was partly supported by the West Ukrainian BioMedical Research Center (WUBMRC) grant awarded to B.O. Vynnytska.

References

1 Broome JD. L-asparaginase: the evolution of a new tumor inhibitory agent. *Trans N Y Acad Sci* 1968; **30**:690–704.
2 Hill JM, Roberts J, Loeb E, Khan A, MacLellan A, Hill RW. L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. *JAMA* 1967; **202**:882–888.
3 Goodsell DS. The molecular perspective: L-asparaginase. *Stem Cells* 2005; **23**:710–711.
4 Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Crit Rev Oncol Hematol* 2007; **61**:208–221.
5 Kreis W. Tumor therapy by deprivation of L-methionine: rationale and results. *Cancer Treat Rep* 1979; **63**:1069–1072.
6 Miki K, Al-Refaie W, Xu M, Jiang P, Tan Y, Bouvet M, *et al.* Methioninase gene therapy of human cancer cells is synergistic with recombinant methioninase treatment. *Cancer Res* 2000; **60**:2696–2702.
7 Wheatley DN. Controlling cancer by restricting arginine availability:S arginine-catabolizing enzymes as anticancer agents. *Anticancer Drugs* 2004; **15**:825–833.
8 Cheng PN, Wheatley DN. Arginine deprivation addresses the Achilles heel of cancer: urea cycle deficiencies in melanoma, HCC and other cancers. *Oncology News* 2008; **2**:6–8.
9 Cheng PN, Lam TL, Lam WM, Tsui SM, Cheng AW, Lo WH, *et al.* Pegylated recombinant human arginase (rhArg-peg5000 mw) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res* 2007; **67**:309–317.
10 Lam TL, Wong GK, Chong HC, Cheng PN, Choi SC, Chow TL, *et al.* Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest. *Cancer Lett* 2009; **277**:91–100.
11 Kim RH, Coates JM, Bowles TL, McNerney GP, Sutcliffe J, Jung JU, *et al.* Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. *Cancer Res* 2009; **69**:700–708.
12 Morris SM. Arginine: beyond protein. *Am J Clin Nutr* 2006; **83** (**Suppl**):508S–512S.
13 Choi BS, Martinez-Falero IC, Corset C, Munder M, Modolell M, Muller I, *et al.* Differential impact of L-arginine deprivation on the activation and effector functions of T cells and macrophages. *J Leukoc Biol* 2009; **85**:268–277.
14 Scott L, Lamb J, Smith S, Wheatley DN. Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer* 2000; **83**:800–810.
15 Wheatley DN, Kilfeather R, Stitt A, Campbell E. Integrity and stability of the citrulline-arginine pathway in normal and tumour cell lines. *Cancer Lett* 2005; **227**:141–152.
16 Izzo F, Marra P, Beneduce G, Castello G, Vallone P, De Rosa V, *et al.* Pegylated arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: results from phase I/II studies. *J Clin Oncol* 2004; **22**:1815–1822.
17 Ascierto PA, Scala S, Castello G, Daponte A, Simeone E, Ottaiano A, *et al.* Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. *J Clin Oncol* 2005; **23**:7660–7668.
18 Szlosarek PW, Klabatsa A, Pallaska A, Sheaff M, Smith P, Crook T, *et al.* In vivo loss of expression of argininosuccinate synthetase in malignant pleural mesothelioma is a biomarker for susceptibility to arginine depletion. *Clin Cancer Res* 2006; **12**:7126–7131.
19 Yoon CY, Shim YJ, Kim EH, Lee JH, Won NH, Kim JH, *et al.* Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. *Int J Cancer* 2007; **120**:897–905.
20 Kim JH, Kim JH, Yu YS, Kim DH, Min BH, Kim KW. Anti-tumor activity of arginine deiminase via arginine deprivation in retinoblastoma. *Oncol Rep* 2007; **18**:1373–1377.
21 Bowles TL, Kim R, Galante J, Parsons CM, Virudachalam S, Kung HJ, *et al.* Pancreatic cancer cell lines deficient in argininosuccinate synthetase are sensitive to arginine deprivation by arginine deiminase. *Int J Cancer* 2008; **123**:1950–1955.
22 Shen LJ, Lin WC, Beloussow K, Shen WC. Resistance to the anti-proliferative activity of recombinant arginine deiminase in cell culture correlates with the endogenous enzyme, argininosuccinate synthetase. *Cancer Lett* 2003; **191**:165–170.
23 Dillon BJ, Prieto VG, Curley SA, Ensor CM, Holtsberg FW, Bomalaski JS, *et al.* Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation. *Cancer* 2004; **100**:826–833.
24 Bence AK, Crooks PA. The mechanism of L-canavanine cytotoxicity: arginyl tRNA synthetase as a novel target for anticancer drug discovery. *J Enzyme Inhib Med Chem* 2003; **18**:383–394.
25 Swaffar DS, Ang CY, Desai PB, Rosenthal GA. Inhibition of the growth of human pancreatic cancer cells by the arginine antimetabolite L-canavanine. *Cancer Res* 1994; **54**:6045–6048.
26 Ding Y, Matsukawa Y, Ohtani-Fujita N, Kato D, Dao S, Fujii T, *et al.* Growth inhibition of A549 human lung adenocarcinoma cells by L-canavanine is associated with p21/WAF1 induction. *Jpn J Cancer Res* 1999; **90**:69–74.
27 Jang MH, Jun DY, Rue SW, Han KH, Park W, Kim YH. Arginine antimetabolite L-canavanine induces apoptotic cell death in human Jurkat T cells via caspase-3 activation regulated by Bcl-2 or Bcl-xLq. *Biochem Biophys Res Commun* 2002; **295**:283–288.
28 Green MH, Brooks TL, Mendelsohn J, Howell SB. Antitumor activity of L-canavanine against L1210 murine leukemia. *Cancer Res* 1980; **40**:535–537.
29 Thomas DA, Rosenthal GA, Gold DV, Dickey K. Growth inhibition of a rat colon tumor by L-canavanine. *Cancer Res* 1986; **46**:2898–2903.
30 Swaffar DS, Ang CY, Desai PB, Rosenthal GA, Thomas DA, Crooks PA, *et al.* Combination therapy with 5-fluorouracil and L-canavanine: *in vitro* and *in vivo* studies. *Anticancer Drugs* 1995; **6**:586–593.
31 Worthen DR, Chien L, Tsuboi CP, Mu XY, Bartik MM, Crooks PA. L-canavanine modulates cellular growth, chemosensitivity and P-glycoprotein substrate accumulation in cultured human tumor cell lines. *Cancer Lett* 1998; **132**:229–239.
32 Green MH, Ward JF. Enhancement of human tumor cell killing by L-canavanine in combination with γ-radiation. *Cancer Res* 1983; **43**:4180–4182.
33 Bence AK, Adams VR, Crooks PA. L-canavanine as a radiosensitization agent for human pancreatic cancer cells. *Mol Cell Biochem* 2003; **244**:37–43.
34 Thomas DA, Rosenthal GA. Toxicity and pharmacokinetics of the nonprotein amino acid L-canavanine in the rat. *Toxicol Appl Pharmacol* 1987; **91**:395–405.
35 Swaffar DS, Ang CY. Growth inhibitory effect of L-canavanine against MIA PaCa-2 pancreatic cancer cells is not due to conversion to its toxic metabolite canaline. *Anticancer Drugs* 1999; **10**:113–118.
36 Berjis M, Green MH. Selective cytotoxicity of L-canavanine in tumorigenic Madin-Darby canine kidney T1 cells. *Chem Biol Interact* 1986; **60**:305–315.
37 Kunz-Schughart LA, Heyder P, Schroeder J, Knuechel R. A heterologous 3-D coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblasts differentiation. *Exp Cell Res* 2001; **266**:74–86.
38 Peterson GL. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* 1977; **83**:346–356.
39 Marsh WH, Fingerhut B, Miller H. Automated and manual direct methods for the determination of blood urea. *Clin Chem* 1965; **11**:624–627.
40 Thomas DA, Rosenthal GA. Metabolism of L-[guanidinooxy-14C]canavanine in the rat. *Toxicol Appl Pharmacol* 1987; **91**:406–414.
41 Rosenthal GA. L-canaline: a potent antimetabolite and anti-cancer agent from leguminous plants. *Life Sci* 1997; **60**:1635–1641.
42 Bence AK, Worthen DR, Adams VR, Crooks PA. The antiproliferative and immunotoxic effects of L-canavanine and L-canaline. *Anticancer Drugs* 2002; **13**:313–320.
43 Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. Pegylated arginine deiminase (ADI-SS PEG20 000 mw) inhibits human melanomas and hepatocellular carcinomas *in vitro* and *in vivo*. *Cancer Res* 2002; **62**:5443–5450.
44 Smith DW, Ganaway RL, Fahrney DE. Arginine deiminase from *Mycoplasma arthritidis*. Structure-activity relationships among substrates and competitive inhibitors. *J Biol Chem* 1978; **253**:6016–6020.

45 Downum KR, Rosenthal GA, Cohen WS. L-arginine and L-canavanine metabolism in Jack Bean, Canavalia ensiformis (L.) DC. and Soybean, Glycine max (L.) Merr. *Plant Physiol* 1983; **73**:965–968.
46 Li L, Li Z, Chen D, Lu X, Feng X, Wright EC, *et al.* Inactivation of microbial arginine deiminases by L-canavanine. *J Am Chem Soc* 2008; **130**:1918–1931.
47 Hrabak A, Bajor T, Temesi A. Comparison of substrate and inhibitor specificity of arginase and nitric oxide (NO) synthase for arginine analogues and related compounds in murine and rat macrophages. *Biochem Biophys Res Commun* 1994; **198**:206–212.
48 Colleluori DM, Ash DA. Classical and slow-binding inhibitors of human type II arginase. *Biochemistry* 2001; **40**:9356–9362.
49 Lu X, Li L, Feng X, Wu Y, Dunaway-Mariano D, Engen JR, *et al.* L-canavanine is a time-controlled mechanism-based inhibitor of *Pseudomonas aeruginosa* arginine deiminase. *J Am Chem Soc* 2005; **127**: 16412–16413.