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Preclinical paper

Growth inhibitory effect of L-canavanine against MIA PaCa-2 pancreatic cancer cells is not due to conversion to its toxic metabolite canaline

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L-Canavanine (CAV) is an arginine (ARG) analog isolated from the jack bean, *Canavalia ensiformis*. CAV becomes incorporated into cellular proteins of MIA PaCa-2 human pancreatic cancer cells and the aberrant, canavanyl proteins are not preferentially degraded. Hydrolytic cleavage of CAV to canaline (CAN) and urea is mediated by arginase. CAN is a potent metabolite that inactivates vitamin B_6-containing enzymes and may inhibit cell growth. To determine the presence of arginase and its specificity for ARG and CAV in MIA PaCa-2 cells, a radiometric assay, which quantifies the ^{14}C released from the hydrolytic cleavage of L-[*guanidino*-^{14}C]ARG or L-[*guanidinooxy*-^{14}C]CAV mediated by arginase, was employed. Insignificant amounts of $^{14}CO_2$ were released when cells were exposed to [^{14}C]CAV or to [^{14}C]ARG. Pancreatic cancer cells secrete a negligible amount of arginase. Cytotoxic effects of CAN and CAV were compared on cells exposed to varying concentrations of ARG. These studies provide evidence that CAV's cytotoxic effects on MIA PaCa-2 cells cannot be attributed to conversion to the active metabolite CAN. A slower and decreased hydrolysis of CAV by arginase allows CAV to persist and increases its chances of incorporating into proteins in these cells. Lack of considerable amounts of arginase in the pancreas makes CAV a worthy candidate for further studies in pancreatic cancer. [© 1999 Lippincott Williams & Wilkins.]

Key words: Arginase, canaline, canavanine, MIA PaCa-2, pancreatic cancer.

Introduction

L-Canavanine (CAV) is a natural analog of L-arginine (ARG) isolated from jackbean seeds of *Canavalia ensiformis*. Since CAV is considerably less basic than ARG, its erroneous incorporation into newly synthesized proteins can disrupt catalytic activity of some proteins (reviewed in Rosenthal[1]). CAV has chemotherapeutic potential against human pancreatic cancer with the MIA PaCa-2 cell line as a model, and it exhibits its cytotoxic effects in a CAV dose- and time-dependent manner; these were also dependent on the ARG concentration of the cell growth medium.[2] Synergistic effects were observed when these cells were exposed to CAV in combination with equal amounts of 5-fluorouracil (5-FU),[3] the most commonly used chemotherapeutic agent for pancreatic cancer today. CAV was also recently found to incorporate readily into the cellular proteins of these cells and the degradation of CAV-containing proteins was not significantly faster than the degradation of native ARG-containing proteins (data submitted elsewhere).

Studies of CAV metabolism in rats[4] revealed that L-canaline (CAN) [L-2-amino-4-(aminooxy) butyric acid] is the major metabolite. The formation of CAN from CAV is mediated by the sequential hydrolytic cleavages of arginase (EC 3.5.3.1) and urease (EC 3.5.1.5). Arginase, which is secreted primarily by hepatocytes, ordinarily hydrolyzes ARG to ornithine (ORN) and urea. CAN has been reported to react vigorously with the pyridoxal phosphate moiety of vitamin B_6-containing enzymes to form a covalently bound oxime that inactivates the enzyme irreversibly (reviewed in Rosenthal[5]). While ORN stimulates cell growth, CAN inhibits it. Some of the toxicity observed in the rat was assumed to be due to CAV's conversion to CAN, which subsequently can inhibit ORN-utilizing, pyridoxal phosphate-dependent enzymes.[4,6]

Cann *et al.*[7] have questioned the contention that CAV's incorporation into cellular proteins is the key factor in its growth inhibition of MIA PaCa-2 cells.

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Instead, they are of the opinion that arginase may play an important role in CAV's activity. To ascertain whether CAV's mode of action in MIA PaCa-2 cells is solely from the incorporation of CAV into cellular proteins, it becomes necessary to delineate whether CAV is metabolized by these cells, particularly when the metabolite CAN is a potentially potent inhibitor of vitamin B_6-containing enzymes. There have been no reports of arginase activity of pancreatic adenocarcinoma cells; however, it has been reported that arginase activity in the rat insulinoma cells, RINm5F, is significantly lower than that in normal rat islet cells.[8] To examine the arginase activity and its specificity for ARG and CAV in the MIA PaCa-2 cells, a radiometric assay that conveniently quantifies the $^{14}CO_2$ released from the cleavage of [^{14}C]ARG or [^{14}C]CAV by arginase,[9] was employed. We now report that CAV's growth inhibitory effects on MIA PaCa-2 pancreatic cancer cells are not due to its toxic metabolite, CAN.

Materials and methods

Drugs and chemical reagents

L-[*guanidinooxy*-^{14}C]CAV (specific activity 2.146 MBq/mol) was obtained from Dr Gerald A Rosenthal (University of Kentucky), synthesized and purified by the method of Ozinskas and Rosenthal.[10] Purity of [^{14}C]CAV was confirmed using the ion-exchange chromatography method described by Rosenthal and Thomas,[9] and was found to be greater than 96%. L-[*guanidino*-^{14}C]ARG (specific activity 1.905 MBq/mol) was obtained from DuPont NEN (Boston, MA) and the scintillation cocktail (Scintisafe 30%) was obtained from Fisher Scientific (Pittsburgh, PA). Bovine arginase, jack bean urease (Type IV) and benzethonium hydroxide (hyamine hydroxide) were purchased from Sigma (St Louis, MO). Stopper tops (also referred to as 'blood tube' septums) and center wells were purchased from Kontes Glass (Vineland, NJ). Media reagents were purchased from Gibco/BRL (Grand Island, NY).

Cell cultures

MIA PaCa-2 cells were purchased from ATCC (Rockville, MD). Monolayer cultures were maintained in DMEM as previously reported.[2] ARG-free medium (AFM) and ARG-reduced media (ARMs) of varying ARG concentrations were prepared as described previously.[2] Cells were confirmed to be free of mycoplasma contamination by the sensitive Gen-Probe kit employing the hybridization of a labeled cDNA probe for mycoplasma (Gen-Probe, San Diego, CA).

Arginase assay

Using a modification of the method of Rosenthal and Thomas,[9] MIA PaCa-2 cells were harvested by trypsinization, centrifuged and washed twice with AFM. A volume of 100 μl containing 1×10^5 viable cells in AFM was transferred into a 16×100 mm culture tube. For each of the arginase standards, equal volumes of bovine arginase solution and the corresponding urease solution to give three final concentrations of 0.01, 0.10 or 1.0 U each of arginase and urease were transferred into tubes (positive controls). A tube that contained no arginase and urease served as background counts (negative control). A final concentration of either 3.285 kBq/ml [^{14}C]ARG or 3.7 kBq/ml [^{14}C]CAV was then added to the tubes. These equimolar concentrations were previously determined to be optimal in incorporation and degradation studies (data not shown). The assay was carried out in triplicate tubes. Each tube was immediately sealed with a rubber 'blood tube' septum holding a center well suspended from the top. The center well contained a 7×1 cm 'accordioned' filter paper soaked in 200 μl of benzethonium hydroxide to trap any $^{14}CO_2$ released. After a 2 h incubation at 37°C, reactions were stopped by the addition of 6N HCl to the tubes. The center wells were snipped off and placed into 20 ml borosilicate vials containing 10 ml of scintillation fluid. The vials were vortexed thoroughly and left at room temperature for 4 days to reduce phosphorescence. Radioactivity was determined with a Beckman LS 3801 liquid scintillation counter. Results were expressed as pmol $^{14}CO_2$ released $\pm$ SEM.

MTT assay for *in vitro* cytotoxicity versus MIA PaCa-2 cells

The question about the possible potent cytotoxicity of CAN has led to the direct examination of CAN's effects on the MIA PaCa-2 cells. A comparative cytotoxicity study of the parent compound (CAV) and metabolite (CAN) was investigated with the MTT assay. The protocol used was essentially as described previously[2] with few modifications. Cells were incubated in DMEM on 96-well plates at 37°C in a humidified 5% CO_2 atmosphere for 24 h. Medium was removed and replaced with the appropriate ARM followed by the addition of either CAN or CAV. After 72 h drug exposure medium was removed, replaced with fresh

serum-free medium and MTT added to plates. Following 4 h incubation at 37°C well absorbances were measured with a BioRad MP450 plate reader. For each ARG concentration, three to four independent experiments consisting of eight replicate samples each were performed.

Results

Arginase assay

Any $^{14}CO_2$ released and trapped in the absence of exogenously introduced arginase and urease would be indicative of the presence of arginase activity in the cells. Tubes with varying amounts of exogenously introduced arginase and urease served as positive controls. To ensure that the $^{14}CO_2$ trapped did not come from the spontaneous breakdown of [^{14}C]CAV or [^{14}C]ARG, a set each of negative controls with no cells, and no arginase and urease was used; only [^{14}C]CAV or [^{14}C]ARG was added to the medium. These negative controls also served as background counts.

The amount of CAN produced was indirectly calculated from the $^{14}CO_2$ released and trapped using the stoichiometric equation of CAV metabolism of a 1:1:1 ratio of [^{14}C]CAV:CAN:$^{14}CO_2$. Hence, the amount of $^{14}CO_2$ trapped reflects the arginase activity of the cells and indicates CAN production by the cells.

$$[^{14}C]CAV \xrightarrow{\text{arginase}} CAN + [^{14}C]urea \xrightarrow{\text{urease}} NH_3 + {}^{14}CO_2$$

Similarly, the amount of ORN produced was calculated from the $^{14}CO_2$ trapped.

$$[^{14}C]ARG \xrightarrow{\text{arginase}} ORN + [^{14}C]urea \xrightarrow{\text{urease}} NH_3 + {}^{14}CO_2$$

A direct comparison of $^{14}CO_2$ trapped from the [^{14}C]ARG and the [^{14}C]CAV exposed groups ascertained whether ARG or CAV was preferentially metabolized by the arginase present in the MIA PaCa-2 cells.

The averaged results from three independent experiments involving trapping of $^{14}CO_2$ in the presence of [^{14}C]ARG or [^{14}C]CAV are illustrated in Figure 1. ARG was preferentially metabolized in the presence of arginase. Metabolism of CAV was marginal under arginase activity. For example, at 1.00 U of arginase, 69.78±2.01 pmol of ARG and 3.17±0.2 pmol of CAV were metabolized, which translated to a preference of approximately 22-fold higher for ARG than for CAV. This result corroborated with earlier findings that arginase preferentially metabolizes ARG as compared to CAV (reviewed in Rosenthal and Thomas[9]).

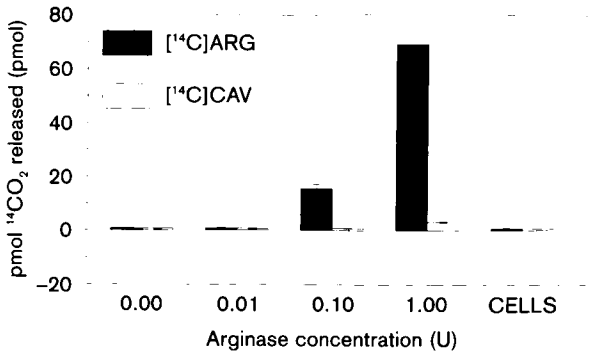

Figure 1. Arginase activity in the MIA PaCa-2 cells. A radiometric assay involving the trapping of $^{14}CO_2$ generated by arginase-mediated hydrolysis of [^{14}C]CAV or [^{14}C]ARG was employed as described in the text. The bars labeled 'CELLS' did not contain any exogenous arginase/urease. Note that the arginase activity in these cancer cells is negligible. There is preferential metabolism of ARG by arginase. Bars represent means and SEM for three independent experiments.

Since the MIA PaCa-2 cells exposed to either [^{14}C]ARG or [^{14}C]CAV did not liberate any notable amounts of $^{14}CO_2$, the arginase activity in these human pancreatic cancer cells was presumed to be insignificant. The average number of pmol of $^{14}CO_2$ released from the 'background control' (i.e. 0.00 U arginase) was 0.67±0.15 and that released from [^{14}C]CAV incubated with MIA PaCa-2 cells was 0.67±0.09. For ARG, there were 0.74±0.18 pmol of $^{14}CO_2$ released from the 'background control' and 0.72±0.16 released from cells incubated with [^{14}C]ARG. Apparently there was less than 0.01 U of arginase in the cells and this would not be sufficient to metabolize ARG. Hence, an insignificant amount of $^{14}CO_2$ released correlated to a negligible amount of CAN or ORN produced. This finding also indicates that none of the intracellular [^{14}C]ARG or [^{14}C]CAV was metabolized by arginase in these cells during our incorporation and degradation studies.

MTT cytotoxicity assay

The sensitivity of MIA PaCa-2 cells to CAV or CAN after a 72 h exposure in media of varying ARG concentrations is depicted in Figure 2. Figure 2(A) shows that CAV and CAN were about equally cytotoxic with an IC_{50} of about 1.5 mM when cells were exposed in DMEM (0.4 mM ARG), an ARG-rich environment. CAV began to exhibit growth inhibitory activity at lower

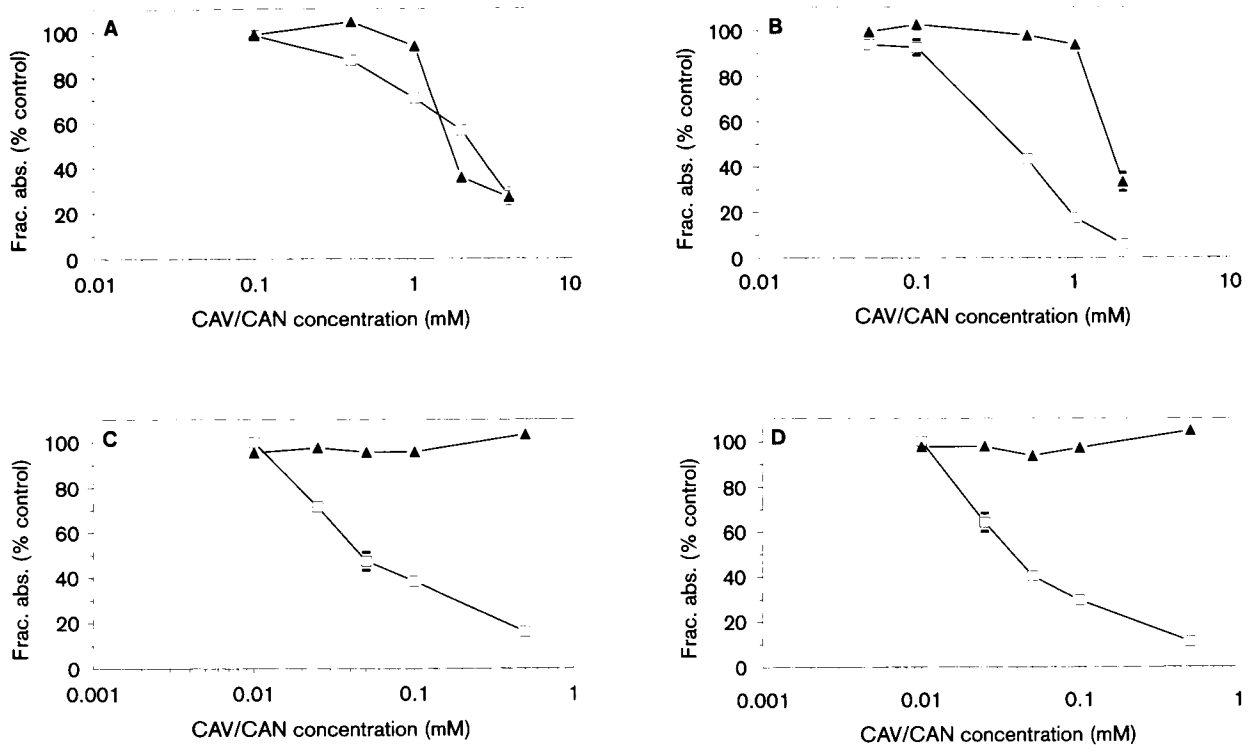

Figure 2. Sensitivity of MIA PaCa-2 cells to CAV and CAN in (A) DMEM (0.4 mM ARG), (B) 0.04 mM ARM, (C) 0.004 mM ARM and (D) 0.4 μM ARM. MIA PaCa-2 cells were exposed to CAV or CAN for 72 h, and cell survival was determined by the MTT cell cytotoxicity assay as described in the text. ▲, CAN; □, CAV. Points represent the means for three to four independent experiments of eight replicates each. SEM is shown when it exceeded the area occupied by the data point.

concentrations than CAN. The curve for CAN showed a much steeper slope with nearly a 70% difference being seen between 1 and 2 mM. Figure 2(B) shows that in 0.04 mM ARM, the IC_{50} of CAN remained about the same as that observed in D-MEM, i.e. 1.5 mM. However, CAV cytotoxicity increased; the IC_{50} was about 0.3 mM. As the ARG concentration of the medium was reduced to 0.004 mM (Figure 2C), the IC_{50} of CAV fell to about 0.04 mM. Over the same range of concentrations as CAV, CAN was not cytotoxic, even at 1 mM. Figure 2(D) shows that in 0.4 μM ARM, CAN was considerably less cytotoxic than CAV. The IC_{50} of CAV decreased to about 0.03 mM. It was apparent that CAN cytotoxicity, unlike that of CAV, was independent of the ARG concentration in the medium.

Discussion

Our data establish that CAV-mediated inhibition of the growth of MIA PaCa-2 cells is not due to the active metabolite, CAN. If it was due to the metabolite, then CAV would have to be converted to CAN by arginases in the cells. The current study was undertaken to determine the extent of CAN formation, with a consequent release of CO_2. Our finding of a lack of significant arginase activity in pancreatic cancer cells is not unexpected. Arginase is found mainly in the liver (98%), while lesser concentrations (1-2% of the concentration present in the liver) are found in the red blood cells, kidney, gastrointestinal tract, brain and lactating mammary glands.[11] There are two human arginase isozymes, AI and AII. The AI isozyme constitutes about 98% or more of the liver and red blood cell-arginase activity while AII constitutes the remaining arginase activity in the other organs.[11] Grody *et al.*[12] noted that human liver arginase activity was 100 times higher than that in the kidney, gastrointestinal tract or brain. In chickens it has been reported that kidney arginase activity is decreased by CAV.[13] It was thus expected that pancreatic adenocarcinoma cells would have very low arginase activity.

Human arginases are not readily available commercially. However, as reported by Rosenthal and Thomas,[9] bovine, porcine and rat liver arginases also exhibit high catalytic activity toward ARG and are suitable for this radiometric assay. The optimal pH of

arginase is relatively high at pH 9.0-9.5. In order to maintain cell viability, experimental conditions for the arginase assay with MIA PaCa-2 cells mimicked the physiological pH of 7.0-7.4. It was evident that with only 1.00 U of bovine arginase, about one-third of the [^{14}C]ARG was metabolized within 2 h, even under sub-optimal pH of arginase (1 U of arginase converts 1.0 μmol of ARG to ORN and urea per minute at pH 9.5 at 37°C).

The specificity of arginase is significantly higher for ARG (the normal substrate) than for CAV. Jack bean arginase has been shown to exhibit slightly more than 4-fold higher catalytic activity toward ARG than CAV.[9] The unpublished observation of Bleiler (as noted in Rosenthal and Thomas[9]) revealed a significantly higher catalytic activity of bovine, porcine and rat liver arginase than jack bean arginase. With our current results using this assay, bovine arginase was shown to have a 22-fold higher catalytic activity toward ARG than CAV.

Because of the negligible arginase activity in the MIA PaCa-2 cells, it is reasonable to assume that the metabolism of CAV in these human pancreatic cancer cells is not very likely. Hence, the cytotoxicity observed earlier with CAV in these cells[2] was not likely attributable to the metabolite (CAN) but rather was from the consequences of the incorporation of the parent compound (CAV) into cellular proteins. By examining the cytotoxicity of CAN along with CAV, it was observed that CAN was not cytotoxic at all CAV concentrations tested in the ARG-reduced media. It only exhibited a parallel IC_{50} with that of CAV in 0.4 mM ARG-containing medium (DMEM). Since CAN is not an antimetabolite of ARG, its cytotoxicity was independent of the ARG concentration in the cellular medium. With this important finding, it is apparent that the viability of these cells is unaffected by CAN concentrations up to about 1 mM. Furthermore, in the absence of any significant arginase activity in these pancreatic cancer cells, it is unlikely that CAV would be metabolized to CAN to achieve a cytotoxic millimolar (mM) concentration. CAV was significantly more cytotoxic in low ARG-containing media and all of its cytotoxicity must have derived from its increased incorporation into proteins of the cancer cells.

In summary, these results imply that human pancreatic adenocarcinoma cancer cells secrete a negligible amount of arginase. These data are also in agreement with previous reports that arginase has a higher affinity for ARG (the normal substrate) than for CAV. The dilemma of CAN cytotoxicity in these cells is resolved since CAN is only cytotoxic at a relatively high (mM) concentration. A slower and decreased cleavage of CAV by arginase would allow CAV to persist and would increase its chances of incorporating into the cellular proteins of these cells. Incorporation of a 'faulty' amino acid like CAV will ultimately form aberrant and non-functioning proteins.[14-16] The current results, along with our recent finding (submitted elsewhere) that CAV readily incorporates into cellular proteins and that the canavanyl proteins are not preferentially degraded, may aid in explaining the sensitivity of these human pancreatic cancer cells to CAV and in establishing the fate of CAV in the cells. The lack of considerable amounts of arginase in the pancreatic cancer cells makes CAV a worthy consideration for further studies in human pancreatic cancer. A potential study for examining CAV *in vivo* could entail site-specific targeted delivery of CAV to pancreatic tumors. It has recently been reported that some pancreatic cancer cell lines express high levels of an oligopeptide transporter (which accepts many different substrates) and this may be useful in targeting the delivery of antineoplastic agents to the pancreas.[17]

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