Inhibition of the Growth of Human Pancreatic Cancer Cells by the Arginine Antimetabolite L-Canavanine

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Introduction

L-Canavanine (CAV), the L-2-amino-4-guanidinooxy structural analogue of L-arginine (ARG), is a potent ARG antagonist which occurs in the jack bean, Canavalia ensiformis. This ARG antimetabolite is active against L1210 murine leukemia and a solid colonic tumor in the rat. Our initial studies using a microtiter assay show that CAV exhibits a 50% inhibitory concentration of approximately 2 mM against the human pancreatic adenocarcinoma cell line, MIA PaCa-2, when these cells are grown in Dulbecco's modified Eagle's medium containing 0.4 mM ARG. When the ARG concentration is reduced to 0.4 μM, the 50% inhibitory concentration for CAV falls precipitously to 0.01 mM. The pronounced increase in the ability of CAV to inhibit MIA PaCa-2 cell growth at the lower ARG concentration may result from enhanced CAV competition with ARG for incorporation into newly synthesized cellular proteins. At 0.4 μM ARG, 30 mM CAV almost completely inhibits cell growth by 6 h. In contrast, with 0.4 mM ARG, complete inhibition does not occur until after 48 h. A dramatic reversal of growth inhibition caused by a very high concentration of CAV was observed when cells treated with CAV were replenished with a high concentration of ARG. Our results suggest that CAV has real potential as a lead compound for the development of analogues with enhanced activity against human pancreatic cancer.

Materials and Methods

Drugs and Chemical Reagents. CAV, isolated from seeds of the jack bean, Canavalia ensiformis (1), is a structural analogue of ARG originally isolated from the jack bean, Canavalia ensiformis (1). It differs structurally from ARG in that oxygen replaces the terminal methylene group of ARG.

CAV is synthesized by many leguminous plants and plays a seminal role in higher plant chemical defense against insects (2). CAV, a substrate for arginyl-tRNA synthetase (3), is incorporated readily into proteins in place of ARG (4). In insects, Rosenthal et al. (5–7) and coworkers have shown that this incorporation produces structurally aberrant "canavanyl" proteins which exhibit altered protein conformation (5) and impaired function (6, 7). This nonprotein amino acid is much less basic than ARG; the guanidinooxy group of CAV has a pKb of 7.05 (8), while that of ARG is 12.48 (9). Incorporation of CAV into newly synthesized proteins can decrease residue basicity and alter residue interactions, thereby disrupting tertiary and/or quaternary protein structure.

Numerous studies on the effects of CAV have been conducted in insects and prokaryotes (reviewed in Refs. 2 and 6) with fewer reports of effects on mammalian cells. Kruse and McCoy (10) demonstrated that CAV affects Walker carcinosarcoma 256 cells; it was shown later to have antitumor activity against murine L1210 leukemia (11). CAV was also found to sensitize human colon tumor cells to the lethal effects of γ-irradiation (12). These investigators reported that the lethal effects of CAV occur preferentially in rapidly dividing cells. In other studies with monkey kidney cells whose transformation was temperature dependent, CAV inhibited DNA synthesis to a greater extent in transformed versus normal (untransformed) cells (13, 14).

CAV3 [L-2-amino-4-(guanidinooxy)butyric acid] is a structural analogue of ARG originally isolated from the jack bean, Canavalia ensiformis (1). It differs structurally from ARG in that oxygen replaces the terminal methylene group of ARG.

L-canavanine
content were prepared. For the initial experiments, this ARM contained 10-fold (0.04 mM), 100-fold (0.004 mM), or 1000-fold less (0.4 μM) ARG than that contained in the commercial DMEM (0.4 mM). For later experiments, ARG concentrations that fell between the above concentrations were used in the ARMs. Cells were maintained routinely in commercial DMEM and then exposed to ARM as described during assays.

**MTT Assay for Cytotoxicity.** The protocol used for the MTT assay was similar to those reported previously by Swaffar et al. (20, 21) with several modifications. MIA PaCa-2 cells grown in DMEM were trypsinized with 0.05% (w/v) trypsin and 0.53 mM EDTA prior to centrifugation at 1000 rpm for 5 min. After cells were washed twice with ARM, the cell pellet was resuspended in fresh ARM. Cells were counted with a hemacytometer, and viability was determined by trypan blue exclusion (only cells having a viability >95% were used for assays). The cell suspension was adjusted to a concentration of 3 × 10^5 cells/ml by the addition of the appropriate amount of ARM. Using a multichannel pipettor, 100 μl of this cell suspension were plated into a 96-well microtiter plate. Wells containing only medium served as blanks. Microtiter plates were then incubated in a humidified 5% CO₂ atmosphere at 37°C for 4 h to allow the cells to adhere. Eight replicate samples, consisting of two sets of quadruplicate wells, were used for each CAV concentration (11 μl/well); control wells received PBS. Plates were incubated at 37°C in a humidified 5% CO₂ atmosphere. After 6 h, the contents of the wells were aspirated and replaced with fresh, unsupplemented McCoy’s medium (ARM and DMEM interfered with the MTT assay). A 5-mg/ml (11-μl) stock solution of MTT in PBS was added to all wells. Plates were incubated for 4 h, and 100 μl of 0.04 N HCl in isopropyl alcohol was added and thoroughly mixed to solubilize the formazan produced by viable cells. Well absorbance was measured at 540 nm with a Bio-Rad MP450 plate reader. The mean absorbance of quadruplicate drug-treated wells was compared to that of control wells and expressed as a percentage of control absorbance ± SEM. For each ARG concentration, at least three independent experiments were performed.

**Timed-Exposure Studies.** In order to determine the time course of CAV-mediated growth inhibition at varying ARG concentrations, MIA PaCa-2 cells were exposed to CAV in ARM or DMEM and were plated as above. After an incubation period of at least 4 h, the upper four rows of the plate received 11 μl of CAV solution, and quadruplicate wells on the lower four rows of the plate received 11 μl PBS. Plates were incubated for the specified period of time. At each time point, CAV- or PBS-containing medium was aspirated from the appropriate wells and replaced with fresh medium. Plates were then incubated for the remaining period of the assay, the medium was aspirated and replaced with unsupplemented McCoy’s medium, MTT added, and absorbances were measured as above. The data were processed as described above.

In order to determine if cell growth inhibition due to CAV was reversed by ARG, cells were exposed to CAV in a medium containing 0.4 μM ARG (1000-fold less ARG than that of DMEM). MIA PaCa-2 cells in ARM containing 0.4 μM ARG were plated as above, treated with either CAV or PBS as described above, and incubated for the indicated time periods. At each time point, CAV- or PBS-containing medium was aspirated from the appropriate wells and replaced with fresh DMEM or ARM with 0.4 μM ARG. Plates were incubated for the remaining period of the assay and processed as above.

**Results**

The sensitivity of MIA PaCa-2 cells to CAV after a 72-h exposure in DMEM is depicted in Fig. 1. In this medium, CAV had only moderate cytotoxicity against MIA PaCa-2 cells, as demonstrated by an IC₅₀ of around 2.5 mM. CAV at 0.1 mM did not inhibit the growth of the cells, but nearly 80% of the cells were killed by approximately 3 mM CAV; complete inhibition of cell growth was seen at 30 mM CAV.

Fig. 2 illustrates the results of varying the concentration of ARG on MIA PaCa-2 cell growth. As the ARG concentration of the medium decreased, CAV toxicity was amplified. CAV toxicity increased about 10-fold from a 10-fold reduction in ARG concentration (0.04 mM), but CAV toxicity did not increase exponentially when the ARG concentration was lowered. At 0.004 mM, CAV was nearly 200-fold less toxic when assays were performed at the lowest ARG concentration (0.4 μM) evaluated in our study. In this instance, the IC₅₀ level was approximately 15 μM. At an ARG concentration of 0.8 μM, 0.004 mM, or 0.008 mM, the CAV IC₅₀ was only slightly higher, but when the ARG concentration reached 0.04 mM, the CAV IC₅₀ rose to 0.35 mM. At 0.4 mM ARG, the CAV IC₅₀ reached about 2.5 mM.

MIA PaCa-2 cells were exposed to a concentration of CAV (30 mM) that was shown in earlier experiments to inhibit the growth of 100% of the cells after 72 h in DMEM. At each of the indicated time periods of Fig. 3, CAV-containing medium was removed and replaced with fresh medium; the cells were further incubated for a total exposure period of 72 h. When cells were exposed to 30 mM CAV (in DMEM), growth inhibition exceeded 50% after 18 h. In contrast, at 0.04 mM ARG, 50% of the cells were killed after only 1 h, and by 24 h, over 80% growth inhibition was seen. When the ARG concentration was lowered to 0.4 μM, cell growth inhibition attained 80% after 1 h. Nearly all cells were killed by 18 h when CAV exposure occurred with the lowest ARG concentration evaluated.

The dramatic reversal of CAV toxicity by ARG is depicted in Fig. 4. This figure depicts the results of a timed-exposure study conducted with cells exposed to 30 mM CAV in 0.4 μM ARG-containing medium. The CAV-containing medium was removed at the indicated times and replaced with either ARM (0.4 μM ARG) or DMEM. Cells were then incubated for a total exposure period of 72 h. These results clearly showed that, at earlier time points, CAV toxicity was reversed by ARG. Interestingly, over 80% of the cell growth was inhibited when cells were exposed to the low ARG media for 1 h and then replaced with this same media. However, when cells were exposed to the low ARG media for 1 h and then replaced with DMEM containing 0.4 mM ARG, no cell growth inhibition was seen. This dramatic reversal continued up to about 12 h, and by 18 h, there was still partial reversal of cell growth inhibition by ARG. After 24 h of exposure of cells to CAV, ARG could no longer reverse CAV-mediated cell growth inhibition. In contrast, nearly complete reversal of growth inhibition was still seen when cells were exposed to CAV in the presence of 0.4 mM ARG for 5 h and then replaced with DMEM.

**Discussion**

CAV inhibits the growth of MIA PaCa-2 cells in a dose-dependent manner, but the degree of growth inhibition is dependent on the ARG concentration of the cell growth medium. Concentrations of ARG from 0.04 mM to 0.4 mM assuage CAV toxicity. The high IC₅₀ of CAV
varying concentrations of ARG: V, 0.4 mM; L, 0.08 mM; A, 0.04 mM; , 0.03 mM; •, 0.02 mM; V, 0.008 mM; 0, 0.004 mM; •, 0.08 µM; and 0, 0.04 µM.

Fig. 2. Sensitivity of MIA PaCa-2 cells exposed to CAV for 72 h in ARM containing varying concentrations of ARG: V, 0.4 mM; , 0.08 mM; A, 0.04 mM; 0, 0.03 mM; •, 0.02 mM; V, 0.008 mM; 0, 0.004 mM; •, 0.08 µM; and 0, 0.04 µM. Points, the means of results from 3 to 10 independent experiments; bars, SE. This clearly showed that, at earlier time points, CAV toxicity was reversible by ARG. After 24 h, it became irreversible.

The question of why CAV-mediated inhibition of MIA PaCa-2 cells is reversible with ARG up to 12 h posttreatment but then becomes irreversible is intriguing and worthy of detailed investigation.

Fig. 3. The time course of growth inhibition of MIA PaCa-2 cells by CAV in ARM containing varying concentrations of ARG. CAV-containing medium was removed after the indicated times and replaced with fresh medium. Cells were further incubated for a total exposure period of 72 h, and cell survival was determined. •, DMEM; V, ARM (0.4 mM); 0, ARM (0.04 mM); 0, ARM (0.004 mM); and •, ARM (0.4 µM). Points, the means of results obtained from at least three independent experiments; bars, SE.

Fig. 4. Reversal of cell growth inhibition by ARG. MIA PaCa-2 cells were exposed to 30 mM CAV in ARM containing 0.4 µM ARG for the indicated time periods. CAV-containing medium was removed and replaced with either fresh ARM with 0.4 µM ARG or with fresh DMEM (0.4 mM ARG). Cells were incubated for a total exposure period of 72 h. •, replaced with DMEM; •, replaced with 0.4 µM ARG media. Points, the means of results obtained from at least three independent experiments; bars, SE. This clearly showed that, at earlier time points, CAV toxicity was reversible by ARG. After 24 h, it became irreversible.

seen in DMEM was undoubtedly due to direct competition with ARG in the cell growth medium. When ARG is present in an adequate concentration, it probably competes effectively with CAV for uptake into cellular proteins, and this attenuates the toxic effects of CAV. However, the issue of whether the rate of CAV incorporation into cellular protein has actually been altered by the ARG concentrations used awaits CAV uptake experiments. Our results suggest that more CAV was probably incorporated into protein when ARG concentrations were low. Under these conditions, it is likely that a greater extent of CAV incorporation would result in a greater degree of cell growth inhibition.

Timed-exposure studies were performed to determine when CAV begins to exhibit growth inhibitory effects, both with high and trace amounts of ARG. In the presence of 0.4 mM ARG, a concentration of CAV, known to inhibit the growth of 100% of the cells by 72 h, inhibits the growth of 50% of the cells around 18 h. Up to 12–18 h, CAV toxicity appears to be readily reversed by ARG. Even the effects of a highly toxic dose such as 30 mM CAV can be nearly completely reversed by 0.4 mM ARG. For example, when cells exposed to CAV for 6 h in the low ARG media were then washed free of CAV and replenished with a high amount of ARG (as in DMEM), the ARG apparently was able to almost completely reverse the cell growth inhibition (compared to that seen when cells were exposed to CAV and replenished with a lower amount of ARG).

We found that replenishment of MIA PaCa-2 cells with normal amounts of ARG (as in DMEM) can reverse completely cell growth inhibition at early time points and that this becomes irreversible after approximately 18 h. Finally, our study provides the first evidence of the activity of CAV in a human pancreatic carcinoma and of ARG reversibility of growth inhibition in a tumor cell line. The results of our study of CAV and MIA PaCa-2 cells suggest that CAV and ARG compete directly for activation and aminoacylation by arginyl-tRNA synthetase. We plan to evaluate this point by providing these cells with L-[guanidinoxy-14C]CAV and monitoring its uptake into newly synthesized proteins in the presence of varying amounts of ARG.

These results also pose the question of what influence ARG concentrations may have on the rate of turnover of proteins that have incorporated CAV. Several investigators have reported that some CAV-containing proteins degrade faster than normal proteins (22–26). Cells then may have the ability to recognize aberrant proteins and destroy them; however, our results suggest otherwise. Our hypothesis is that, if proteins that have incorporated CAV are rapidly degraded, at a continuing low ARG concentration, insufficient ARG is present to preferentially be taken up into protein, and this is a toxic insult to the cells. Pazlarova et al. (27) have recently reported that, in Saccharomyces cerevisiae, CAV-containing proteins were degraded more slowly when glucose was in the medium as compared to ethanol (as a carbon source) in the medium when ARG was added after a 2-h ARG-free incubation. This interesting observation warrants further investigation of protein degradation in the MIA PaCa-2 cells treated with and without CAV in the presence of varying ARG concentrations.
tion. These cells would be expected to preferentially degrade their CAV-containing proteins and thereby remove such structurally aberrant proteins. Perhaps, there is a threshold for effective error correction, and the production of excess aberrant protein counterbalances cellular ability to remove these defective macromolecules. Alternatively, CAV may disrupt nucleic acid metabolism, and a point may be reached where ARG-driven cellular recovery is no longer possible. We plan to examine radiolabeled thymidine and uridine uptake by CAV-treated and control cells and to determine the turnover and degradation of \[^{3}H\]ARG- and \[^{14}C\]CAV-containing proteins.

CAV has been reported to induce the synthesis of heat shock proteins (28, 29). It is not known what effect ARG concentrations in the medium have on levels of heat shock proteins, but based on these reports, it would be reasonable to expect that lower concentrations of ARG in the medium would increase the synthesis of heat shock proteins, i.e., at the lower ARG concentrations, more hsps would be produced, and this may enable cells to survive at higher temperatures. However, other investigators have reported that CAV renders melanoma cells more sensitive to heat (17). Also, Laszlo and Li (30) have recently reported that, in Chinese hamster fibroblasts, CAV induces synthesis of hsps, but these hsps are nonfunctional and sensitize the cells to heat. It is not known what effect heat has on our MIA PaCa-2 cells exposed to CAV, since all our studies were conducted at 37°C.

Overall, our preliminary report of the activity of CAV against a human pancreatic cancer cell line suggests that CAV may be incorporated in place of ARG in ARG-rich pancreatic proteins and contribute to the demise of these cells. We have also clearly demonstrated that CAV toxicity to MIA PaCa-2 cells is reversible by ARG but after 18 h becomes irreversible. Studies to address these interesting observations are in progress.

References
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