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Review Article

The Mechanism of L-Canavanine Cytotoxicity: Arginyl tRNA Synthetase as a Novel Target for Anticancer Drug Discovery

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There is a clear need for agents with novel mechanisms of action to provide new therapeutic approaches for the treatment of pancreatic cancer. Owing to its structural similarity to L-arginine, L-canavanine, the δ-oxa-analog of L-arginine, is a substrate for arginyl tRNA synthetase and is incorporated into nascent proteins in place of L-arginine. Although L-arginine and L-canavanine are structurally similar, the oxyguanidino group of L-canavanine is significantly less basic than the guanidino group of L-arginine. Consequently, L-canavanyl proteins lack the capacity to form crucial ionic interactions, resulting in altered protein structure and function, which leads to cellular death. Since L-canavanine is selectively sequestered by the pancreas, it may be especially useful as an adjuvant therapy in the treatment of pancreatic cancer. This novel mechanism of cytotoxicity forms the basis for the anticancer activity of L-canavanine and thus, arginyl tRNA synthetase may represent a novel target for the development of such therapeutic agents.

Keywords: L-Canavanine; L-Arginine; Arginyl tRNA synthetase; Pancreatic cancer; Radiosensitization agent; Adjuvant therapy

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States and it is estimated that in the year 2003, 30,700 new cases will be diagnosed and 30,000 individuals will die of this disease.[1] Cancer of the exocrine pancreas is rarely curable with the highest cure rate occurring only if the tumor is localized to the pancreas. Even in this patient population, however, the five-year survival rate is only 17%.[1] Unfortunately, due to the non-specificity of the symptoms and the difficulty in early detection, less than 20% of patients have the disease confined to the pancreas at the time of diagnosis, rendering surgical and medical interventions ineffective. For patients with advanced disease, the five-year survival rate is 1% and most patients die within one year of initial assessment.[1] Conventional treatments of pancreatic cancer include surgery, radiation therapy, chemotherapy and palliation of secondary symptoms. Currently, the antimetabolites gemcitabine and 5-fluorouracil (5-FU) are the most effective single agents used in the treatment of pancreatic cancer,[2] but even with these agents the prognosis is poor. Consequently, there is a clear need for more effective agents with novel mechanisms of action to improve the therapeutic options for patients with pancreatic cancer.

Arginyl-tRNA Synthetase

Aminoacyl-tRNA synthetases are important enzymes in maintaining the fidelity of the protein synthesis process. In most organisms, there are 20 distinct aminoacyl-tRNA synthetases with each one being responsible for the aminoacylation of its cognate tRNA to a specific amino acid. Aminoacyl-tRNA synthetases recognize structural features of their cognate tRNA and catalyze the esterification of their complementary amino acid to the 2′- or 3′-ribose hydroxyl of tRNA (forming an aminoacyl-tRNA). The aminoacyl-tRNA not only activates the carboxylic acid of the amino acid to promote peptide bond formation, but also provides a mechanism for

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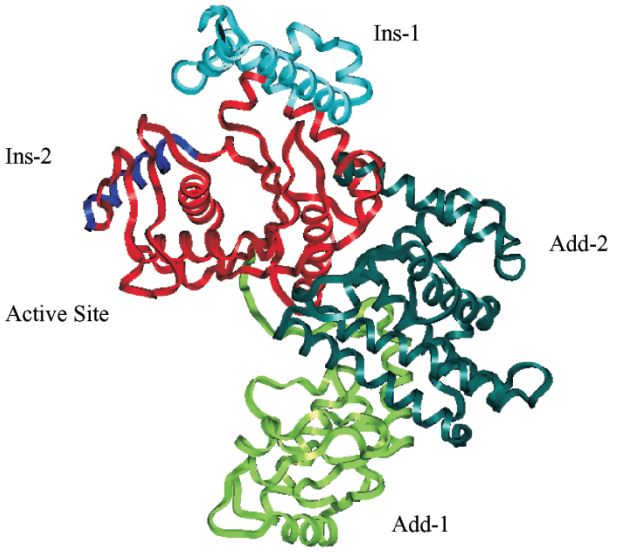


FIGURE 1 Structure of *S. cerevisiae* Arginyl-tRNA Synthetase. Add-1 and Add-2 are additional domains attached at the N-terminal and C-terminal sides of the active site, respectively. Ins-1 and Ins-2 are domains inserted into the catalytic core of the enzyme. The active site is depicted in red.

correct placement of the amino acid in a nascent peptide chain. The aminoacyl-tRNA proceeds to the ribosome where the codon of the mRNA recognizes the anticodon of the tRNA to accurately translate the genetic code. Thus, the specific amino acid attached to the tRNA is delivered to the protein synthesis machinery, and properly incorporated into a growing peptide chain.[3]

Arginyl-tRNA synthetase (Figure 1) is unique among tRNA synthetases for several reasons. The arginyl-tRNA synthetase mechanism (along with glutaminyl tRNA synthetase and glutamyl tRNA synthetase mechanisms) requires that the cognate tRNA be present for the first step of the aminoacylation reaction.[4–6] The binding of tRNA to arginyl tRNA synthetase induces a conformational change in the structure of the active site. These changes may be required for ATP to properly bind to the enzyme.[7]

Despite numerous studies by several researchers, the detailed mechanism of aminoacylation catalyzed by arginyl-tRNA synthetase is still not known. Arginyl-tRNA synthetase may load L-arginine to arginyl-tRNA either by a sequential reaction mechanism or in a concerted fashion. The sequential mechanism is a two-step process and is the aminoacylation mechanism that operates for most of the aminoacyl-tRNA synthetases. In the first step of this proposed mechanism, L-arginine is activated as an enzyme-bound aminoacyl-adenylate intermediate (Figure 2). In this reaction, the carboxyl group of the amino acid is bound in a mixed anhydride linkage with the 5′-phosphate of AMP, with displacement of pyrophosphate (Figure 3).

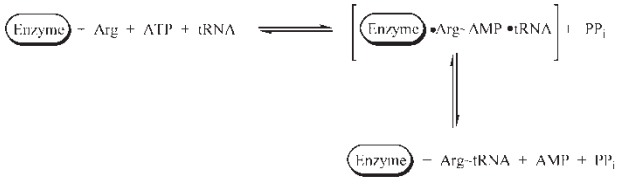


FIGURE 2 Sequential two-step aminoacylation reaction proceeding through an aminoacyl-adenylate intermediate.

The second step of the reaction involves the transfer of L-arginine to the 3′-hydroxyl of the cognate tRNA to afford arginyl-tRNA (Figure 4). Through nucleophilic attack at the carbonyl carbon of L-arginine, the 3′-ribosyl hydroxyl group of adenosine displaces AMP. Support for this mechanism comes from several kinetic studies showing that *Escherichia coli* arginyl-tRNA synthetase catalyzes the ATP-PP_i exchange in the presence of non-loaded arginyl-tRNA.[8,9] Furthermore, it was found that all three substrates bound to the enzyme before any product was released.[9] This indicates that the reaction does not proceed *via* a ping-pong mechanism, since in a ping-pong mechanism one or more of the products are released before all of the substrates are bound. In the case of arginyl-tRNA synthetase, all three substrates are bound to the enzyme *before* any product is released, indicating that the reaction proceeds either by a sequential mechanism or by a concerted mechanism.

Alternatively, as proposed in the second mechanism, ATP, L-arginine and tRNA may react in a concerted fashion leading to a one-step aminoacylation of tRNA,[10] as shown in Figure 5. Support for this mechanism comes from studies where the researchers have been unable to detect the arginyl–adenylate intermediate.[11] Despite numerous investigations by several groups, no definitive evidence favoring either one or the other of the two proposed mechanisms has emerged. It is hoped that recent studies elucidating the structure of the active site of arginyl-tRNA synthetase may afford information that will provide further insight into the mechanism of this enzyme.[7,12,13]

Due to its crucial role in protein synthesis, arginyl tRNA synthetase may be a novel target for the development of therapeutics. In particular, L-canavanine **(1)**, a non-protein amino acid analog of L-arginine **(2)** and substrate for arginyl tRNA synthetase, has shown promise as a possible novel therapy for pancreatic cancer (Figure 6).

NATURAL SOURCES AND FUNCTION OF L-CANAVANINE

L-Canavanine was discovered in 1929 when it was isolated from the seeds of the jack bean plant,

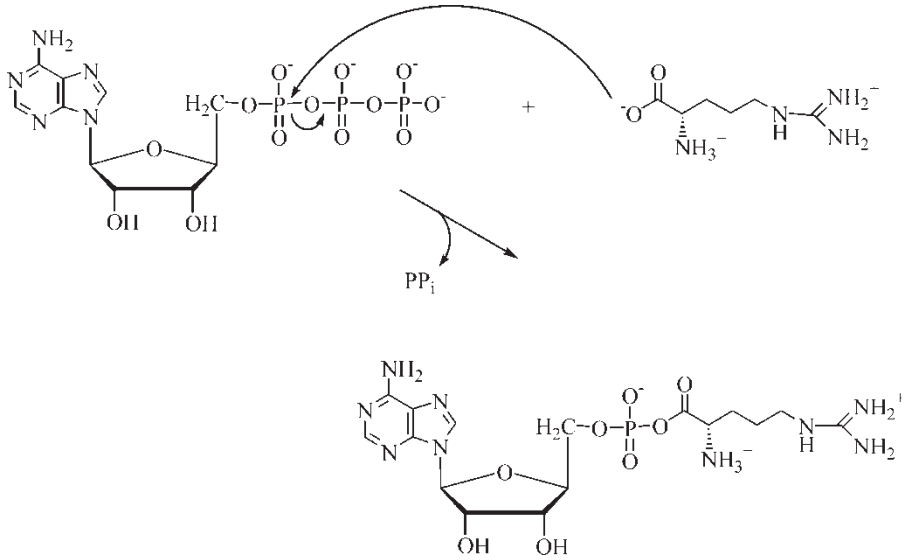


FIGURE 3 Formation of the aminoacyl-adenylate intermediate from L-arginine and ATP, with the concomitant displacement of pyrophosphate.

Canavalia ensiformis.[14] L-Canavanine is predominantly found in legumes, and particularly in members of the Lotaidea family, a major subdivision of Leguminosae. Many agronomically important crops, including alfalfa, clover, several *Lespedeza* species, and trefoils contain L-canavanine.[15] Alfalfa (*Medicago sativa* (Fabaceae)) is the most consumed L-canavanine-containing plant in North America[16] and is generally regarded as safe for human use by the FDA. In Asia, Africa, and areas in the tropics, *C. ensiformis* (L.) DC. (Fabaceae) and sword bean (*Canavalia gladiata* (Fabaceae)) are important dietary legumes that contain L-canavanine.[17]

L-Canavanine has two primary functions in the plant; it acts as a nitrogen storing metabolite and is an integral part of the plant's chemical defense system. As a result of its high nitrogen content (31.8%), L-canavanine is well suited to store and transport nitrogen in plant seeds. For example, L-canavanine accounts for up to 5% of the dry weight in the jack bean seed, 2.4% in the alfalfa plant, and 1.4–2.5% in *C. ensiformis* (L.) DC. (Fabaceae) and

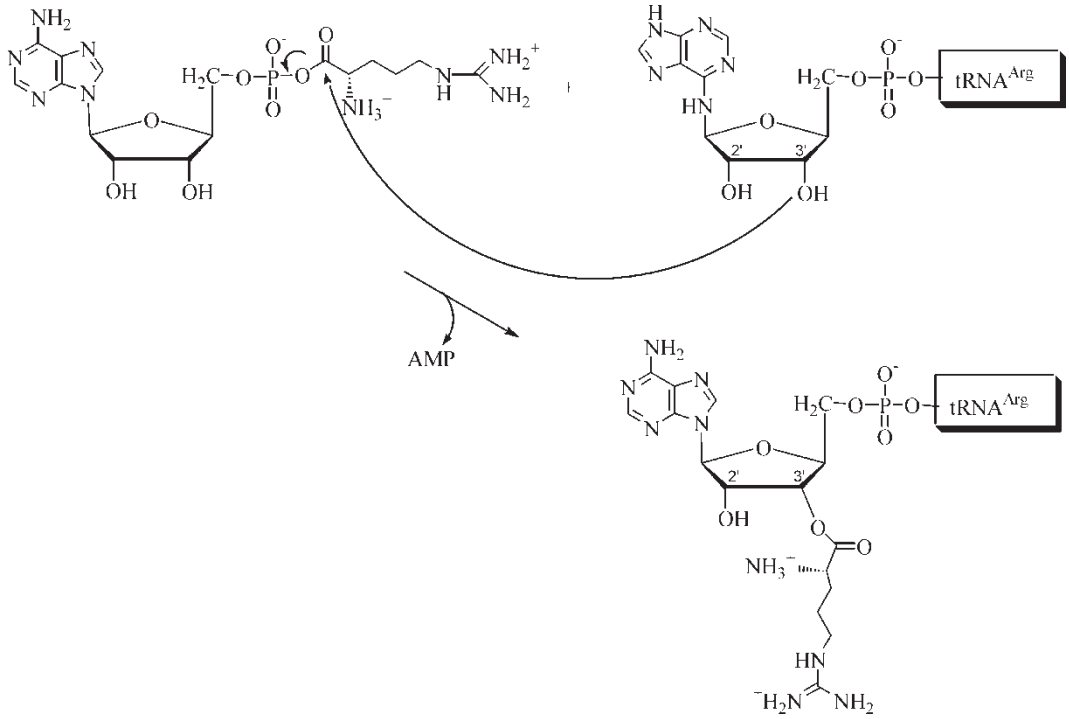


FIGURE 4 Formation of arginyl-tRNA from the aminoacyl-denylate intermediate with displacement of AMP.

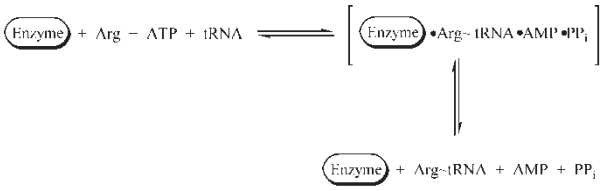


FIGURE 5 Concerted aminoacylation reaction of L-arginine to arginyl-tRNA.

sword bean (*Canavalia gladiata* (Fabaceae)).[16–18] The L-canavanine content of other leguminous seeds can be as high as 10%.[19] In the seed, L-canavanine is an important amino acid that supplies nitrogen for the needs of the developing plant and as the seed grows, the L-canavanine content declines sharply.

A second function of L-canavanine in plants is protecting the plant from insects and other herbivores.[20] L-Canavanine can deleteriously affect the growth and development of organisms that consume the L-canavanine-containing plant. For instance, when the tobacco hornworm, *Manduca sexta* ingests L-canavanine, the development of this plant-eating insect is stunted and growth attenuated in both pupae and adults.[20,21] Similarly, direct injection of L-canavanine into the hemolymph of *M. sexta* results in development aberrations. This property of L-canavanine provides a measure of defense for the plant against certain insects and herbivores that are sensitive to the detrimental effects of L-canavanine.

PHYSICOCHEMICAL PROPERTIES OF L-CANAVANINE

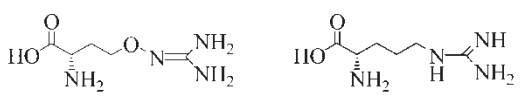
L-Canavanine is the δ-oxa-analog of L-arginine (Figure 6). Since the oxyguanidino group of L-canavanine is electronically and structurally different than the guanidino group of L-arginine, there are several distinct differences between L-canavanine and L-arginine. As seen in Table I, replacement of the δ-carbon of L-arginine with an electronegative oxygen atom dramatically alters the pK_a value of the oxyguanidino group compared to the guanidino group.[22] The destabilizing effect of the oxygen atom diminishes the electron density in the guanidino group. Consequently, the oxyguanidino group of canavanine has a pK_a value of 7.01 compared to 12.48 for the guanidino group of L-arginine.[22,23] As a result of the extreme basicity of the guanidino group in L-arginine, at physiological pH over 99% of L-arginine molecules are protonated. In contrast, only approximately 30% of L-canavanine molecules are protonated at physiological pH.

TABLE I The pK_a values for the ionizable moieties in L-arginine and L-canavanine

Functionality	pK_a in L-Canavanine	pK_a in L-Arginine
Carboxylic acid	2.35	2.18
α-Amino	9.22	9.04
Guanidino	7.01	12.48

In addition to decreasing the basicity of the guanidino moiety, the presence of the δ-oxygen in L-canavanine alters the tautomeric form of the guanidino group. As shown in Figure 7, the guanidino moiety can exist in both the amino **(3)** and imino **(4)** forms. A X-ray diffraction study on a single crystal of L-canavanine indicated that L-canavanine exists in the amino tautomeric form and is uncharged.[22] This same study of crystalline L-canavanine also showed that, like other amino acids, L-canavanine exists as a zwitterion with proton-transfer from the carboxylic acid moiety to the α-amino nitrogen. In contrast, X-ray diffraction studies of L-arginine dihydrate showed that, in the crystalline state, L-arginine exists predominantly in the imino form (2:1 imino:amino). The difference in tautomeric forms was clearly reflected in differences in the C—N bond distances of the guanidino group of L-arginine and L-canavanine. In L-arginine, all three C—N bonds in the guanidino moiety were approximately 1.34 Å. However, the bond distances in L-canavanine were not equal; the O—C=N double bond was 0.05 Å shorter than the terminal C—N bonds of the oxyguanidino moiety indicating that in crystalline L-canavanine, the double bond is in the amino tautomeric form.[22]

Despite the overall structural similarity of L-canavanine to L-arginine, the presence of the δ-oxygen in L-canavanine causes several significant differences between these analogous amino acids. When compared to L-arginine, the L-canavanine molecule is less basic, slightly longer and exists predominantly in the amino, rather than imino tautomeric form. Although these differences are



(1) **(2)**

FIGURE 6 Chemical structures of L-canavanine **(1)** and L-arginine **(2)**.

(3) **(4)**

FIGURE 7 Structures of the amino **(3)** and imino **(4)** forms of the guanidino moiety.

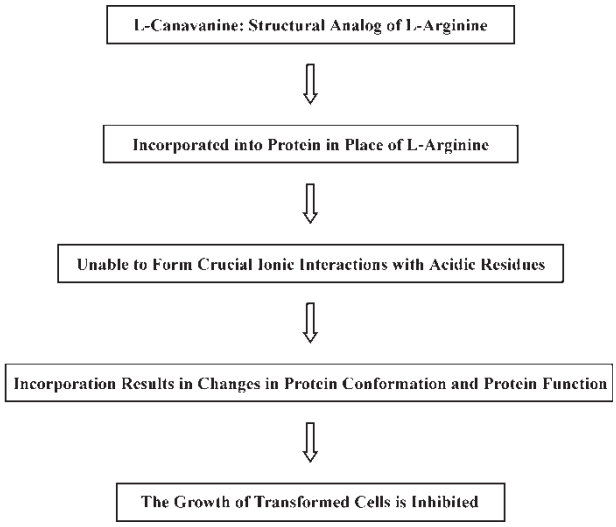


FIGURE 8 Proposed mechanism of action of L-canavanine.

subtle, they have a pronounced effect on the differing physiological actions of L-canavanine and L-arginine. L-Arginine is an important amino acid required for the normal growth and development of cells. In contrast, L-canavanine has been shown to be cytotoxic to transformed cells and holds promise as a novel anticancer agent.

MECHANISM OF ACTION OF L-CANAVANINE

It is hypothesized that the antimetabolite properties of L-canavanine are based on the inability of arginyl-tRNA synthetase to discriminate between L-canavanine and L-arginine.[24,25] In native proteins, positively charged L-arginine residues form ionic pairs with negatively charged amino acid residues such as aspartic acid or glutamic acid. These ionic interactions are often crucial in maintaining the structural integrity, and consequently the function of the protein. Replacement of an L-arginine residue in a protein with L-canavanine, which is less basic, slightly longer, and exists in the amino rather than imino form, can significantly affect key ionic interactions that determine the tertiary and quaternary structure of the protein. The erroneous incorporation of L-canavanine results in structural changes that alter protein function and form the basis of L-canavanine's anticancer activity.[24,26] (Figure 8)

L-Canavanine is Loaded to Arginyl-tRNA

Several *in vitro* experiments have provided preliminary and indirect evidence that L-canavanine could be loaded to arginyl-tRNA. In the presence of L-canavanine, there was a significant decrease (50%) in the amount of radiolabeled L-arginine loaded to *E. coli* arginyl-tRNA.[27] Similarly, concomitant exposure to L-canavanine and L-[^{14}C]-arginine resulted in a 28% reduction in the incorporation of labeled L-arginine into *E. coli* strain 961 tRNA.[28] In rat neurointermediate lobes, L-canavanine decreased the rate of L-[^{3}H]-arginine into proteins by 75%.[29] Although these experiments did not unequivocally prove that L-canavanine was incorporated into protein molecules, they provided the first evidence to suggest that L-canavanine may have the capacity to be loaded to arginyl-tRNA in place of L-arginine.

The first experiments to directly show that L-canavanine was loaded to arginyl-tRNA were performed in 1964 by Allende and Allende.[30] These researchers demonstrated that L-[^{14}C]-canavanine was loaded to yeast arginyl-tRNA, and they isolated the radioactive fraction containing the canavanyl-tRNA species. They determined that L-[^{14}C]-canavanine could be loaded to arginyl-tRNA, even in the presence of L-arginine.[30]

Recently, *in silico* modeling experiments were performed to determine the theoretical binding affinities of L-canavanine and L-arginine when docked into the active site of *Saccharomyces cerevisiae* arginyl tRNA synthetase.[31] These experiments provided additional evidence to demonstrate the capacity of L-canavanine to be loaded to arginyl tRNA. When the oxyguanidino group of L-canavanine was protonated, it had interaction energies with the active site residues of arginyl tRNA that approached those of the protonated form of L-arginine. The root mean square (RMS) of the structures also were analyzed to assess how conformationally similar the bound structures were to each other. The protonated forms of L-canavanine and L-arginine had a RMS of 0.164, suggesting that structurally, L-canavanine bound to the enzyme in a fashion that was almost analogous to that of L-arginine. Indeed, a visual inspection of the superimposition of the bound structures of L-arginine and the protonated form of L-canavanine revealed that both compounds bound to the active site in a virtually identical manner. In contrast, the interaction energy was over two-fold lower for the unprotonated form of L-canavanine. The RMS of the unprotonated form of L-canavanine and the protonated form of L-arginine was 1.107 indicating that L-canavanine (unprotonated form) bound to the enzyme in a quite different manner from that of the protonated form L-arginine. These data suggest that the enzyme recognizes only the protonated form of L-canavanine. It is important to note that at physiological pH less than half of the L-canavanine molecules will be protonated and able to successfully compete with L-arginine for access to the active site of arginyl-tRNA synthetase.[31]

L-Canavanine is Incorporated into Proteins in Place of L-Arginine

Several studies have provided both direct and indirect evidence that not only can L-canavanine be loaded to arginyl-tRNA, but also that it is subsequently incorporated into a nascent peptide chain. In Walker carcinosarcoma 256 cells treated with L-canavanine, the amount of L-arginine lost from the protein hydrolysate equaled the L-canavanine content found in the protein hydrolysate, suggesting L-canavanine may have specifically replaced L-arginine in the protein.[32] Schactele and Rogers used a similar experimental design to indicate that L-canavanine was incorporated into protein molecules of *E. coli*.[33] Although these studies were elegantly designed, they did not provide direct evidence that L-canavanine was incorporated into protein molecules in place of L-arginine. Later studies, utilizing amino acid analysis, amino acid sequencing techniques, and metabolic labeling with radioactive analogs have indeed confirmed that L-canavanine is incorporated into proteins in place of L-arginine. In Chinese hamster ovary cells, L-[^{14}C]-canavanine was taken up into the soluble pool and incorporated into macromolecules.[34] Similarly, L-[^{14}C]-canavanine was incorporated into protein molecules in HeLa S-3 cells.[35] Additional evidence that L-canavanine was incorporated into protein molecules was provided when Hep-G2 cells were incubated with L-[^{14}C]-canavanine and radioactive albumin was isolated and identified. The albumin produced from the L-canavanine-treated cells had lower electrophoretic mobility when analyzed by SDS-PAGE than the albumin from control cells. Moreover, the albumin from L-canavanine-treated cells was more acidic in nature than that from control cells, as evidenced by its markedly different chromatographic elution properties.[36] These results indicated that some of the basic L-arginine residues had been replaced by less basic L-canavanine residues. Similar results were seen in *E. coli* K12 cells exposed to L-[^{14}C]-canavanine. Using antibodies specific to alkaline phosphatase, [^{14}C]-labeled protein was isolated. This protein was partially purified and compared to the native protein, which contained 20–22 L-arginine residues. In the L-canavanyl protein, at least 13, and possibly as many as 18 or 19 L-arginine residues were found to have been replaced with L-canavanine.[37]

Indirect evidence for the incorporation of amino acid analogs into the amino acid sequence of protein molecules, as reflected in altered chromatographic and electrophoretic characteristics of the protein, has also been demonstrated. Isolated rat neurointermediate lobes were incubated in the presence of L-canavanine. Subsequently, the protein pro-opoimelanocortin (POMC), a common precursor to β-endotrophin and adrenocorticotropin, was resolved by two-dimensional gel electrophoresis. Since the oxyguanidino group of L-canavanine is much less basic than the guanidino group of L-arginine, replacement of L-arginine residues by L-canavanine should lower the isoelectric point of the POMC molecule and shift the position of the protein towards the acidic region of an isoelectric focusing gel without causing a change in the molecular weight of POMC. In the presence of L-canavanine, numerous alterations in the protein pattern were observed in the acidic region of the gel. The spots corresponding to various forms of POMC disappeared while new proteins migrating with approximately the same molecular weight were observed, indicating L-canavanine may have replaced some of the L-arginyl residues.[29] Additional studies, using two-dimensional electrophoresis, tryptic and chymotryptic peptide mapping, and analysis (on polyacrylamide gels in the presence of SDS) of the fragments resulting from partial digestion with chymotrypsin, confirmed that L-canavanine had replaced some of the L-arginyl residues in the POMC molecule.[29]

The capacity of L-canavanine to be incorporated into pancreatic proteins from anglerfish islets cells has been demonstrated. Native prohormones of glucagon, insulin, and somatostatin were easily hydrolyzed with trypsin. However, after the cells were exposed to L-canavanine, the prohormones resisted tryptic hydrolysis. Since trypsin cleaves the peptide chain at L-arginine residues, the authors suggested that the failure of the prohormones to be cleaved by trypsin indicated L-canavanine might have replaced L-arginine in these prohormones. Similarly, L-canavanine-containing prohormones failed to be cleaved in the islet secretory granule-converting assay that cleaves the prohormone at dibasic (two adjacent basic residues) sites in the chain.[38] The authors concluded that this result indicated that L-canavanine had replaced L-arginine at some of the crucial dibasic cleavage sites.

The most comprehensive studies investigating the capacity of L-canavanine to be incorporated into protein in place of L-arginine have been conducted in insects. As previously discussed, L-canavanine can adversely affect the growth and development of organisms that consume L-canavanine-containing plants. While the capacity of L-canavanine to be incorporated into protein in place of L-arginine has been demonstrated in a wide variety of insects, the extent of L-canavanine incorporation differs among insect species. The degree of substitution error frequency, in turn, often determines how toxic L-canavanine is to the insect. This observation reinforces the hypothesis that the detrimental effects of L-canavanine result from the capacity of

L-canavanine to be incorporated into protein in place of L-arginine.

The tobacco hornworm, *M. sexta*, is extremely sensitive to the antimetabolic effects of L-canavanine, and also has a high substitution error frequency. When newly emerged *M. sexta* larvae were administered L-*guanidinooxy*-^{14}C]-canavanine, the *de novo*-synthesized hemolymph proteins were labeled with significant amounts of carbon-14. In the hemolymph, L-canavanine replaced approximately one out of every three L-arginine residues. An even higher substitution error frequency was observed for *de novo* synthesized proteins in the fat body and body wall of the organism.[39]

In contrast, the tobacco budworm, *Heliothis virescen* (Noctuidae), is an L-canavanine-resistant organism. This destructive plant herbivore feeds on a variety of higher plants, but rarely feeds on L-canavanine containing plants. Despite this, *H. virescen* showed remarkable resistance to the insecticidal effects of L-canavanine. Larvae were reared on a L-canavanine-rich diet with only a minimal negative effect on larvae growth, and without any discernible development aberrations. The tobacco budworm had a substitution error frequency of only 1 in 65, meaning that less than 2% of L-arginine residues were replaced with L-canavanine. In addition, unlike the tobacco hornworm, no appreciable radiolabeled L-canavanine was incorporated into *de novo* synthesized hemolymph proteins. While L-canavanine still retained the ability to be incorporated into *H. virescen* proteins, the low frequency of this event presumably protected the insect from the detrimental effects of L-canavanine seen in other organisms.[40]

The bruchid beetle, *Caryedes brasiliensis* (Bruchidae), and the weevil, *Stemechus tuberculatus* (Curculionoidea), are examples of L-canavanine-utilizing organisms. Both of these organisms feed exclusively on legumes, which contain significant amounts L-canavanine. The L-canavanine in plants, however, does not produce toxicity in these organisms because they have adapted and can effectively discriminate between L-arginine and L-canavanine, thus avoiding substitution errors. *C. brasiliensis* and *S. tuberculatus* had a substitution error frequency of only one L-arginine in 365, and one L-arginine in 500, respectively. Indeed, *C. brasiliensis* actually utilizes L-canavanine as a nitrogen source for the biosynthesis of many essential amino acids. As a result of the low frequency of incorporation into protein, exposure to L-canavanine was not toxic to these insects.[41]

Incorporation of L-Canavanine Results in Altered Protein Conformation

Studies examining erroneous incorporation of L-canavanine into the large protein molecule, vitellogenin, clearly demonstrate the effect that erroneous L-canavanine incorporation into the amino acid sequence has on the conformation of a protein molecule. Vitellogenin is synthesized by the female locust, *Locusta migratoria migratoriodes* (Orthoptera), and is utilized in the synthesis of vitellin, an essential egg protein. When the locust was exposed to L-canavanine, L-canavanine was incorporated into vitellogenin in place of L-arginine. Although only 18 of the nearly 200-arginine residues (less than 10%) were replaced with L-canavanine, these substitutions caused significant changes in the conformation of vitellogenin.[26] These changes were manifested as significant differences in the protein fragmentation pattern with trypsin, as determined by electrophoretic analysis.

Physical and chemical studies further characterized the conformational changes induced by L-canavanyl vitellogenin. Since different protein conformations alter the specific amino acids exposed on the surface of the protein, an analysis of the surface amino acids can provide information regarding changes in protein conformation. Fluorescence studies revealed that twice as many tyrosine resides were surface-exposed in L-canavanyl vitellogenin than in native vitellogenin. Chemical studies also indicated a different amino acid composition on the surface of the L-canavanyl protein. When a protein is treated with cyanate, lysine residues exposed on the surface react with the cyanate to form homocitrulline. In L-canavanyl vitellogenin, 115 lysine residues were exposed on the surface compared to only 84 in native vitellogenin; this represents a 35% increase in lysine residues exposed to the surface in L-canavanyl vitellogenin when compared to the native protein. Similarly, the number of tyrosine residues on the surface of a protein can be determined by subjecting the protein to gentle acetylation to form O-acetyltyrosine. When using this method, there was a 13% increase in tyrosine residues on the surface of the protein in L-canavanyl vitellogenin when compared to native vitellogenin. These studies clearly indicate that L-canavanine incorporation into vitellogenin affords a three dimensional conformation of L-canavanyl-vitellogenin that is significantly different than that of native vitellogenin.[26] However, these results need to be interpreted with caution since the process and treatments required to measure the conformational changes have the potential themselves to induce conformational changes in vitellogenin.

There have been several other experiments, including the studies in Hep-G2 cells described above, where the electrophoretic mobility of proteins produced in the presence of L-canavanine has been altered. This effect has also been seen in proteins from IMR-90 fibroblasts,[42] *E. coli* K12,[37] murine leukemia virus,[43] and Moloney murine sarcoma virus 124.[43] It has been suggested that

the incorporation of L-canavanine into protein molecules causes conformational changes that persist, even in the presence of SDS. These conformational changes, in turn, may be responsible for the altered electrophoretic mobilities observed in protein molecules isolated from these cells.[36]

Incorporation of L-Canavanine Alters Protein Function

While the studies with vitellogenin were significant, in that they demonstrated that L-canavanine incorporation alters protein conformation, of greater importance is the effect that L-canavanine incorporation has on protein and enzyme function. The most comprehensive studies demonstrating the deleterious effects that L-canavanine incorporation has on protein and enzyme function have been conducted in an insect model. The larvae of the meat-eating fly, *Phormia terranovae* (Diptera), produces diptericin A, B, and C, a family of antibacterial proteins. When L-canavanine was incorporated into diptericins, the antibacterial activity of these proteins was abolished (diptericin B and C) or reduced (diptericin A).[44]

The function of the enzyme lysozyme was adversely affected by incorporation of L-canavanine into its structure in place of L-arginine. Lysozyme is induced in insects in response to a bacterial infection, and protects the organism by lysing the mucopolysaccharide structure of the bacteria's cell wall. When *M. sexta*was exposed to L-canavanine and stimulated to produce lysozyme, 21% of the L-arginine residues in the induced lysozyme molecule were replaced with L-canavanine. This residue substitution resulted in a 49.5% loss of the catalytic activity of lysozyme.[24]

Other studies have demonstrated that exposure of organisms to L-canavanine adversely affects enzymatic function, secretion, and post-translational modifications of peptides and proteins. For instance, the activity of the water-soluble β-galactosidase from *E. coli* was completely abrogated after treatment with L-canavanine.[45] Similarly, L-canavanine incorporation into proteins can disrupt post-translational modifications, especially when L-arginine is at a crucial cleavage or modification site. L-Arginine residues mark two of the processing sites for the conversion of preproalbumin to albumin. After Hep G2 cells were treated with 3 mM L-canavanine for four hours, albumin production was reduced by 67%. Additional studies indicated that Hep G2 cells exposed to L-canavanine retained the capacity to produce proalbumin, but only a minimal amount (21%) could be converted to the fully processed albumin.[36]

L-Arginine marks crucial cleavage sites in POMC. When L-canavanine was incorporated into neurointermediate lobe proteins isolated from rat, POMC molecules were processed into the end products at a much slower rate than the control precursor. After two hours, only 25% of the L-canavanyl POMC had been converted, while 83% of the native prohormone had been converted to the end products.[29]

The extensive body of work described above clearly demonstrates the capacity of L-canavanine to be loaded to arginyl-tRNA and to be a substrate for arginyl tRNA synthetase, resulting in its incorporation into protein in place of L-arginine. When L-canavanine replaces L-arginine in a nascent peptide chain, conformational and electronic changes are induced in the protein, which can lead to functional changes in the canavanine-containing protein. These effects constitute the biochemical basis for the anticancer activity of L-canavanine.

ANTICANCER PROPERTIES OF L-CANAVANINE

Both *in vitro* and *in vivo* studies have demonstrated the anticancer properties of L-canavanine. In 1959, it was found that L-canavanine significantly inhibited the growth of Walker carcinosarcoma 256 cells *in vitro*.[32] In subsequent studies, L-canavanine preferentially inhibited the *in vitro* growth of transformed canine kidney epithelial cells when compared to normal canine kidney epithelial cells. After five days of treatment, the non-transformed cell line showed little, if any, cell death. In contrast, after only four days of exposure, over 90% of the transformed kidney epithelial cells were killed.[46] It was demonstrated that the difference in cytotoxicity was not due to differences in growth rate, since both the normal and tumorigenic cell lines grew at identical rates. Interestingly, after treatment with L-canavanine, the transformed cell line lost its plating efficiency more rapidly when compared to the non-transformed cell line. This observation led the authors to postulate that the basis for the selectivity of L-canavanine may be L-canavanine incorporation into cell surface proteins. Since transformed cells have fewer attachment proteins on their cell membrane, the structural changes resulting from L-canavanine incorporation into cell surface proteins in the transformed cells may have had a greater effect on the capacity of the cells to adhere than in the non-transformed cells.[46] Although the mechanism of selectivity is still unclear, this study was pivotal since it indicated that L-canavanine exhibited selective cytotoxicity towards transformed cells, a highly desired property in anticancer agents.

After 24 hours of exposure to L-canavanine, the cell survival of the human colon cancer cell line, HT-29, decreased exponentially as a function of L-canavanine concentration.[47] L-Canavanine also inhibited the growth of Balb/c-3T3 cells; exposure to 0.1 mM L-canavanine for 48 hours caused

the [^{3}H]-thymidine labeling index to decrease to only 3.4%, indicating DNA synthesis had been severely inhibited. Moreover, L-canavanine completely abrogated the capacity of these cells to form colonies in culture.[48] Exposure to L-canavanine attenuated the growth of the human melanoma cell line M14 in culture. After 20 hours of exposure, the IC_{50} value for L-canavanine was between 1.2 and 0.6 mM.[49] L-Canavanine was cytotoxic to the human uterine sarcoma cell lines MES-SA (parental) and Dx-5 (MDR +), and to the human leukemia cells lines K562 (parental) and K562-R7 (MDR +). Notably, there was no difference in the cytotoxicity of L-canavanine in multidrug resistance (MDR) positive cells when compared to their respective parental cells. The data from these preliminary experiments are promising, since they indicate that L-canavanine may not be an MDR substrate and, unlike several other antineoplastic agents, may be effective against both non-drug resistant and MDR tumors.[50] More recently, L-canavanine was found to be cytotoxic in the human lung adenocarcinoma cell line A549, the human bladder cancer cell line HTB9, and a human cervical cancer cell line, HeLa. In each of these cells lines, exposure to 3 mM of L-canavanine for 48 hours killed greater than 75% of the tumor cells.[51]

L-Canavanine is not only cytotoxic when evaluated in *in vitro* systems, but also has the capacity to shrink tumor growth *in vivo*. Male mice (C57BL/6 × DBA/2 F_1) carrying L1210 leukemia cells received an initial intraperitoneal (i.p.) injection of 20 mg L-canavanine, followed by a continuous subcutaneous (s.c.) infusion at 20 mg/h for 24 hours, when the lifespan of the treated mice increased by 44% when compared to control animals.[52] Thomas and co-workers demonstrated the *in vivo* capacity of L-canavanine to not only increase lifespan, but also reduce solid tumor size.[53] In male Fisher rats carrying a xenograft rat colon carcinoma, tumor size was reduced by 22%, after five days of L-canavanine treatment (3 g/kg, s.c.), and by 60% after nine days of treatment. While the 60% reduction in tumor size after nine days of 3 g/kg L-canavanine treatment was impressive, this treatment regimen was deemed to be too severe, since two of the five animals died on the final day of the experiment. In all five animals treated, significant (31%) weight loss was observed.

A histological examination of tissues from rats receiving 2 or 3 g/kg of L-canavanine daily for five or nine days indicated that all tissues, including the pancreatic islet cells, appeared normal, with the notable exception that there were varying degrees of pancreatic acinar atrophy and fibrosis. When the red and white blood cell values of L-canavanine-treated rats were compared to control animals no differences in the number of red or white blood cells were observed.[53] This is highly significant, since it shows that L-canavanine may not have myelosuppressive effects. In contrast, myelosuppression is the primary toxicity of many antineoplastic agents, including gemcitabine and 5-FU.

In a subsequent series of experiments, L-canavanine (2 or 3 g/kg, s.c., daily) was administered to male Fisher rats with xenograft for five days followed by a six-day observation period. The results of this study indicated that both tumor regression and weight loss were reversible. Within three days after the termination of L-canavanine treatment, tumor growth returned to control values, and at the end of the six-day observation period, the weight loss had been reversed. Subsequent experiments determined that tumor reduction was not a direct consequence of the weight loss.[53]

L-Canavanine reduced both the volume and regrowth of transformed endothelial cells *in vivo*. The endothelioma line, H5V, was transplanted in syngenic female C57B1/6NCrlBR mice inducing the formation of subcutaneous tumors. The common course of development of these tumors is that after a period of growth, the host rejects the tumors. Following the rejection, 70–90% of the animals experience a second regrowth phase of the tumor. When mice carrying a H5V tumor were treated with L-canavanine (30 mg/kg, i.p.), a significant reduction in tumor volume in animals analyzed 8, 11, or 13 days after the tumor inoculation, was observed. Furthermore, only one of eight L-canavanine-treated mice showed a regrowth of the tumor, even up to 60 days after the initial tumor inoculation. In contrast, six of eight control animals exhibited tumor regrowth during the same period of time.[54] A recent report suggests that L-canavanine, as part of a natural product extract, may have a role in preventing spontaneous tumorigenesis in SHN mice. Additional work using the pure compound needs to be conducted to validate and characterize this potentially exciting property of L-canavanine.[55]

L-CANAVANINE IS ACTIVE AGAINST HUMAN PANCREATIC CELLS

The capacity of L-canavanine to inhibit the growth of human pancreatic cancer cells *in vitro* was first demonstrated in 1994 by Swaffar and coworkers.[56] L-Canavanine was cytotoxic in MIA PaCa-2 cells, and this cytotoxic effect was reversed with the addition of L-arginine. When MIA PaCa-2 cells grown in Dulbecco's modified Eagle Medium containing 0.4 mM L-arginine were exposed to L-canavanine, the IC_{50} of L-canavanine was approximately 2 mM. When the L-arginine content of the media was reduced to 0.4 μM, the IC_{50} value of L-canavanine dropped to 10 μM.[56] The capacity of L-canavanine to inhibit MIA PaCa-2 growth was confirmed by Na Phuket and collaborators.[57]

Recently, the effect of L-canavanine on pancreatic cancer cells grown in culture has been further characterized. L-Canavanine was cytotoxic not only in MIA PaCa-2 cells, but also in the human pancreatic cancer cell lines PANC-1, CFPAC-1, Capan-1 and BxPC-3. In PANC-1 and MIA PaCa-2 cells the overall growth inhibition induced by L-canavanine could not be attributed to apoptotic cell death.[31] Additionally, exposure to L-canavanine in these cells resulted in a significant accumulation of cells in the G_2/M phase of the cell cycle.[58] Cells in the G_2/M phase are known to be more sensitive to the effects of ionizing radiation,[59,60] and subsequent studies showed that when PANC-1 or MIA PaCa-2 cells were exposed to L-canavanine prior to being irradiated, L-canavanine was synergistic with the ionizing radiation.[58] The results of these studies may have important implications for the therapeutic potential of L-canavanine, since they suggest that L-canavanine may be an effective radiosensitization agent in the treatment of pancreatic cancer.

The capacity of L-canavanine to inhibit the growth of pancreatic tumors *in vivo* has been demonstrated. Seven athymic nude mice with xenograft human pancreatic carcinoma were treated daily with L-canavanine (4 g/kg, s.c.) for 28 days, while seven animals in a control group received 0.9% saline. Compared to control animals, the L-canavanine-treated animals showed a significant retardation in tumor growth. Two of the seven animals treated with L-canavanine showed a remarkable reduction in tumor size, while tumor growth in the other five animals was clearly inhibited. This experiment confirmed that L-canavanine could effectively inhibit the growth of pancreatic tumors *in vivo*.[61,62]

RATIONALE FOR THE USE OF L-CANAVANINE IN THE TREATMENT OF PANCREATIC CANCER

Numerous studies have indicated that L-canavanine is effective in inhibiting the growth of a variety of cancer types. L-Canavanine, however, may have particular utility in the treatment of pancreatic cancer. As described earlier, there are currently no effective agents for the treatment of pancreatic cancer, and both *in vitro* and *in vivo* studies have confirmed that L-canavanine can effectively inhibit the growth of pancreatic cancer cells and be incorporated into pancreatic proteins. Of greater importance, however, are the results of a crucial *in vivo* metabolism study that indicated that L-canavanine may be selectively taken up by the pancreas.[63]

L-Canavanine was administered to female neonatal and adult Sprague-Dawley rats. Adult rats were given L-[*guanidinooxy*-^{14}C]-canavanine (2 g/kg) orally, while the neonates received L-[*guanidinooxy*-^{14}C]-canavanine (2 g/kg) *via* subcutaneous injection. After 24 hours, the rats were sacrificed, and the amount of radiolabel in each of the major organs was quantified (Table II). In both adult and neonate rats the greatest amount of L-[*guanidinooxy*-^{14}C]-canavanine was found in the pancreas. The pancreas contained more than twice as many pCi/mg of protein than any other organ, indicating that L-canavanine appeared to be selectively sequestered by the pancreas.[63] Although the mechanism for the localization of L-canavanine in the pancreas is unknown, this important property of L-canavanine may have utility in clinical applications. Based on the selective localization of L-canavanine in pancreas, and the capacity of L-canavanine to preferentially inhibit tumor cell growth, L-canavanine may have potential as a treatment for pancreatic cancer.

TABLE II Incorporation of L-[*guanidinooxy*-^{14}C]-canavanine into adult and neonatal rat proteins. Adapted from Thomas *et al.*[63]

Organ	Adult (pCi/mg protein)	Neonate (pCi/mg protein)
Pancreas	49.90 ± 8.1	41.38 ± 2.4
Kidney	21.34 ± 1.1	18.39 ± 5.0
Thymus	15.44 ± 2.8	14.49 ± 0.8
Spleen	14.92 ± 1.0	19.23 ± 5.0
Salivary gland	14.30 ± 1.3	24.39 ± 0.4
Fat	12.32 ± 0.1	*
Heart	10.50 ± 0.8	16.79 ± 0.5
Lung	10.30 ± 0.4	19.46 ± 6.1
GI tract and contents	6.83 ± 0.5	8.39 ± 0.4
Brain	6.38 ± 0.5	14.98 ± 0.4
Muscle	4.50 ± 0.8	*
Liver	4.00 ± 0.1	4.89 ± 0.3

* Value not determined.

LIMITATIONS OF L-CANAVANINE AS AN ANTICANCER AGENT AND ITS POTENTIAL ROLE IN THERAPY

The capacity of L-canavanine to be incorporated into protein in place of L-arginine has been clearly demonstrated. It is important to note, however, that L-arginine is an important component in numerous cellular pathways that may regulate the growth and development of cells. These pathways include the urea cycle, the nitric oxide synthetase pathway, and the polyamine pathway. While L-canavanine has been shown to be a substrate for arginyl-tRNA synthetase and it is incorporated into protein; it may also be a substrate or inhibitor of other L-arginine utilizing enzymes and/or pathways.[64–68] Consequently, some of the cytotoxic effects of L-canavanine may result from the disruption of cellular processes that utilize L-arginine. While the incorporation of L-canavanine into proteins in place

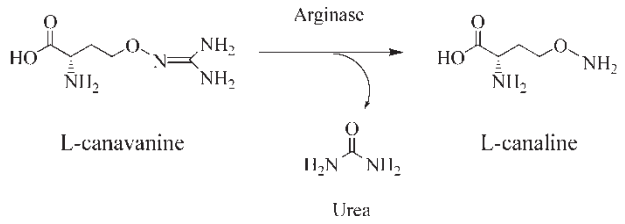


FIGURE 9 Conversion of L-canavanine to L-canaline by arginase.

of L-arginine may be the basis for the anticancer properties of L-canavanine, it is important to recognize that other L-arginine-utilizing pathways could also be involved.

Additionally, the L-canavanine metabolite, L-canaline (L-2-amino-[4-aminooxy]butanoic acid) may contribute to the cytotoxicity of L-canavanine. L-canaline, is formed when arginase hydrolyzes the oxyguanidino group of L-canavanine, generating the oxygen isostere of L-ornithine, as illustrated in Figure 9.[69] Although L-canaline is an inhibitor of pyridoxal phosphate (PLP)-dependent enzymes and has cytotoxic properties,[65,70,71] the *in vitro* growth inhibitory effects of L-canavanine in the pancreatic cell line MIA PaCa-2 do not appear to be attributable to the conversion of L-canavanine to L-canaline.[72] The contribution of L-canaline to the *in vivo* cytotoxicity of L-canavanine has not yet been determined.

The capacity of L-canavanine to act as a substrate for arginyl tRNA synthetase and be incorporated into proteins in place of L-arginine resulting in proteins with altered structure and function has clearly been demonstrated. However, it is still unclear whether L-canavanine incorporation disrupts the function of one particular protein crucial for cellular processes (e.g. cell surface adhesion proteins or arginine rich histones) or non-specifically competes with L-arginine and alters the function of multiple critical proteins and/or enzymes. Identifying the pathway that ultimately leads to cellular death is an important step in the development of L-canavanine into an effective therapeutic agent.

Despite the antitumor properties of L-canavanine and its selective localization in the pancreas, high doses of L-canavanine may be required for therapeutic effect since at low does L-canavanine may not efficiently compete with dietary L-arginine for incorporation into protein. In the *in vivo* studies describe above, the doses used in the evaluation of the antitumor properties of L-canavanine ranged from 30 mg/kg (for mice endothelial tumors),[54] to 4 g/kg for rat pancreatic tumors.[61] However, neither a dose escalation study with L-canavanine in animals, nor studies in humans, have been performed to identify the maximum tolerated dose or the maximum effective dose. Due to the high doses that may be required, the clinical potential of L-canavanine may be best realized in the adjuvant setting. The current mainstay of all cancer treatment, including pancreatic cancer, is to use combinations of multiple agents and modalities to maximize efficacy.[73] The dose-limiting toxicity of gemcitabine and 5-FU, currently the most effective drugs used to treat pancreatic cancer, is myelosuppression. In contrast, in the *in vivo* evaluations with L-canavanine the primary toxicities were alopecia and weight loss;[74,75] myelosuppression was not observed.[53] These preliminary observations indicate that L-canavanine might have non-overlapping toxicities with current therapies, making it an attractive adjuvant agent. Furthermore, L-canavanine has been shown to be synergistic with 5-FU in the human pancreatic cancer cell line MIA PaCa-2, significantly enhanced the activity of 5-FU in a male Fischer rat xenograft model, and is synergistic with ionizing radiation,[58,75] indicating it may enhance the efficacy of existing therapies.

L-canavanine may serve as a paradigm for the development of other anticancer agents that are substrates for aminoacyl-tRNA synthetases. For instance, the non-protein amino acid, azatyrosine, is a substrate for tyrosyl-tRNA synthetase and is incorporated into protein in place of tyrosine. Azatyrosine has the unique capability of converting cells with a transformed phenotype to a normal phenotype.[76–78] Thus, targeting aminoacyl-tRNA synthetases may prove to be a novel approach for the development of anticancer therapies. Indeed, our laboratory is exploring other L-arginine analogs to determine their capacity to act as substrates for arginyl tRNA synthetase and to evaluate their antineoplastic activities.[31,79]

Although additional studies are needed to further characterize the toxicity and efficacy profile of L-canavanine, this non-protein amino acid analog holds promise as a new agent with a novel mechanism of action, which hopefully may complement existing therapies in the treatment of pancreatic cancer.

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