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Report

The antiproliferative and immunotoxic effects of L-canavanine and L-canaline

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L-Canavanine and its arginase-catalyzed metabolite, L-canaline, are two novel anticancer agents in development. Since the immunotoxic evaluation of agents in development is a critical component of the drug development process, the antiproliferative effects of L-canavanine and L-canaline were evaluated *in vitro*. Both L-canavanine and L-canaline were cytotoxic to peripheral blood mononucleocytes (PBMCs) in culture. Additionally, the mononucleocytes were concurrently exposed to either L-canavanine or L-canaline and each one of a series of compounds that may act as metabolic inhibitors of the action of L-canavanine and L-canaline (L-arginine, L-ornithine, D-arginine, L-lysine, L-homoarginine, putrescine, L-ω-nitro arginine methyl ester and L-citrulline). The capacity of these compounds to overcome the cytotoxic effects of L-canavanine or L-canaline was assessed in order to provide insight into the biochemical mechanisms that may underlie the toxicity of these two novel anticancer agents. The results of these studies suggest that the mechanism of L-canavanine toxicity is mediated through L-arginine-utilizing mechanisms and that the L-canavanine metabolite, L-canaline, is toxic to human PBMCs by disrupting polyamine biosynthesis. The elucidation of the biochemical mechanisms associated with the effects of L-canavanine and L-canaline on lymphoproliferation may be useful for maximizing the therapeutic effectiveness and minimizing the toxicity of these novel anticancer agents. [© 2002 Lippincott Williams & Wilkins.]

Key words: Amino acid metabolism, L-canaline, L-canavanine, immunotoxicity.

Introduction

The comprehensive immunotoxicological evaluation of novel therapeutic agents is a critical component of the pharmaceutical development process.[1] Indeed, the immunotoxic effects of both novel agents and drugs long-standing in clinical use are often evaluated and re-evaluated, as dictated by new clinical indications and the needs of special patient populations.[2] In the case of many anticancer agents, immunosuppression and reduced cellular proliferation are commonly observed untoward effects. As a result, rigorous examination of the immunotoxic properties of novel anticancer agents is of particular interest as their therapeutic potential is explored.[2]

Two novel anticancer agents currently under development are L-canavanine, (L-2-amino-[4-guanidinooxy]butanoic acid), a naturally occurring L-arginine analog, and its arginase-catalyzed hydrolytic metabolite, L-canaline (L-2-amino-[4-aminooxy]-butanoic acid.[3,4] The enzymatic conversion of L-canavanine to L-canaline by arginase with the concomitant release of urea is illustrated in Figure 1.

L-Canavanine, an L-arginine analog, is produced by a variety of leguminous plants and is incorporated in place of L-arginine into newly synthesized proteins in a wide variety of organisms, resulting in the formation of non-functional proteins.[3–7] These non-functional proteins may be variously manifested as structural and functional defects, including morphological and developmental aberrations, altered protein conformation and structure, and impaired enzymatic activity, as well as decreased cellular tolerance to heat, radiation and other stressors.[6–9] L-Canavanine also adversely affects DNA replication, histone and heat shock protein function, RNA synthesis, and RNA translation into protein.[10–12] L-Canaline, the cytotoxic metabolite of L-canavanine, is an oxygen isostere of L-ornithine and is an inhibitor of pyridoxal phosphate (PLP)-dependent enzymes, including the enzyme that catalyzes the rate-limiting step in polyamine biosynthesis, ornithine decarboxylase (ODC).[13,14,20]

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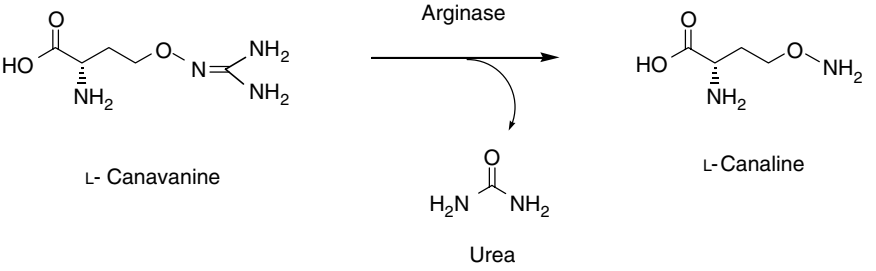

Figure 1. Conversion of L-canavanine to L-canaline by arginase with the concomitant release of urea.

The diverse biological activities of L-canavanine and L-canaline have led a number of investigators to evaluate the potential of these non-protein amino acids as anticancer agents. The antineoplastic activity of L-canavanine has been investigated in a number of studies, either as a single agent,[15–17] or in combination with either 5-fluorouracil[18] or radiation therapy.[19] In addition, the capacity of L-canavanine to sensitize both parental and multidrug-resistant human tumor cells to a variety of standard antineoplastic drugs has been demonstrated.[3] L-Canaline also shows promise as a novel anticancer agent. The antineoplastic activities of L-canaline and several congeners have been evaluated, and it has been suggested that the mechanisms underlying the observed cytotoxic activity of L-canaline may differ from those of the parent compound, L-canavanine.[20] The extensive body of work clearly demonstrates the potential of both L-canavanine and L-canaline as antineoplastic, radiosensitizing and chemosensitizing agents.

Previous research suggests that L-canavanine possesses immunomodulating activity. The capacity of L-canavanine to suppress nitric oxide-associated antigen-specific and antigen-non-specific T lymphocyte proliferation has been demonstrated.[21] L-Canavanine is incorporated into B cell surface proteins, causing alterations in the physico-chemical characteristics of these cells.[21,22] Exposure to L-canavanine may alter the isotype and quantity of antibodies produced by B cells, and appears to suppress T cell function by unknown mechanisms.[23–25] Notably, the ingestion of L-canavanine or L-canavanine-containing legumes may induce anemia and reversible, lupus-like autoimmune responses in primates.[26,27] These studies attribute complex immunological effects to L-canavanine, although the precise mechanisms underlying these effects remain unexplained. Further, no examination of the potential immunological effects of the L-canavanine metabolite, L-canaline, has been reported. Thus, an immunotoxicological evaluation of L-canavanine and its metabolite L-canaline is warranted if these compounds are to be developed as anticancer agents.

While it has been established that L-canavanine is incorporated into protein in place of L-arginine, resulting in aberrant and dysfunctional proteins, there are several other L-arginine-utilizing pathways that L-canavanine and L-canaline can disrupt that may also contribute to their observed cytotoxicity. These include L-arginine and/or L-ornithine antagonism, inhibition of amino acid transport,[28,29] nitric oxide synthase (NOS) inhibition,[30] and the inhibition of ODC and polyamine biosynthesis,[14] as well as the inhibition of other pyridoxal phosphate-dependent enzyme pathways.[13] Therefore, it is postulated that L-canavanine and L-canaline may disrupt one or more L-arginine-utilizing metabolic pathways, some of which are illustrated in Figure 2.

Due to the lack of information regarding the immunotoxicity of L-canaline and the need to fully characterize the immunotoxic effects of L-canavanine as part of the drug development process, the current studies were regarded as important in providing insights into the mechanisms of toxicity of L-canavanine and L-canaline.

Materials and methods

Materials

L-Canavanine was isolated from the seeds of the Jack bean, *Canavalia ensiformis*, by repetitive recrystallization.[31] L-Canaline was synthesized according to known procedures.[32] L-Arginine, D-arginine, L-homoarginine, L-lysine, L-ornithine, L-citrulline, L-ω-nitro arginine methyl ester (L-NAME), putrescine and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma (St Louis, MO). All media and cell culture reagents were obtained from Gibco/BRL (Gaithersburg, MD). The Ficoll-Hypaque used to separate lymphocytes was Ficoll/Lite LymphoH

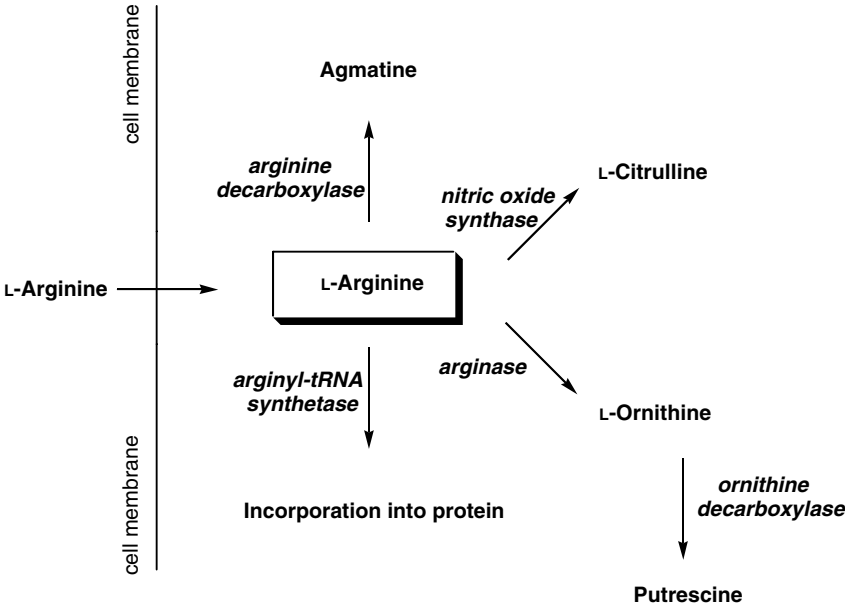

Figure 2. Potential L-arginine-utilizing pathways that may be disrupted by L-canavanine and contributing to its immunological effects.

from Atlantic Biologics (Norcross, GA). MTS ([4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) was obtained from Promega (Madison, WI).

Cell preparation and cell culture

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll/Lite LymphoH density gradient sedimentation of heparinized peripheral blood obtained from 12 healthy volunteers. Briefly, whole blood was diluted with Hank's Balanced Salt Solution (HBSS) and PBMCs were isolated with Ficoll/Lite LymphoH. The interfacial layer was carefully removed and resuspended in HBSS. The sample was centrifuged, washed twice, and taken up in media supplemented with 10% fetal bovine serum, glutamine and a penicillin/streptomycin solution (100 U/ml and 100 μg/ml, respectively).

In lymphocytes stimulated by PMA, 200 μg/ml was added the cell suspension for a final PMA concentration of 0.2 μg/ml. The cells were plated at a density of 1×10^5 cells/well in 96-well microtiter plates. For the mixed lymphocyte reactions, isolated PBMCs from two different subjects were plated (5×10^4 cells from each subject) in 96-well microtiter plates. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After plating, the cells were exposed to serial concentrations of L-canavanine (8 mM to 125 μM) or L-canaline (500 to 7.8 μM) in quadruplicate. In the biochemical inhibitor studies, 0.05, 0.1, 0.2 or 1 mM of the indicated inhibitor was added along with L-canavanine or L-canaline. After incubation for 72 h, proliferative capacity was assessed using the MTS cell proliferation assay.[33]

Statistical analysis

The data are presented as the mean $\pm$ SEM and a one-tailed Student's *t*-test was used to determine statistical significance in all of the cell culture experiments. $p < 0.05$ was considered significant.

Results

L-Canavanine or L-canaline and lymphocyte viability and proliferation

The effect of L-canavanine and L-canaline exposure on the proliferative capacity of human PBMCs was assessed. L-Canavanine inhibited the proliferation of PBMCs stimulated by PMA or by the mixed lymphocyte reaction. The greatest antiproliferative effect was observed when the cells were stimulated via a mixed lymphocyte reaction and subsequently exposed to

L-canavanine. The IC_{50} for cell proliferation under these conditions was 1.18 ± 0.07 mM. L-Canavanine also inhibited the proliferation of cells stimulated with PMA ($IC_{50}=1.64 \pm 0.06$ mM).

L-Canaline was significantly more immunotoxic than L-canavanine, inhibiting the proliferation of PBMCs after stimulation by PMA or via the mixed lymphocyte reaction. The greatest effect was seen with PMA-stimulated cells, where L-canaline had an IC_{50} of 0.26 ± 0.02 mM. L-Canaline was slightly less toxic to PBMCs stimulated via the mixed lymphocyte reaction ($IC_{50}=0.54 \pm 0.08$ mM).

The effect of biochemical modulators on the cytotoxicity of L-canavanine

In order to gain insight into the mechanism by which L-canavanine exerts its antiproliferative effects, human PBMCs were concurrently exposed to L-canavanine in the presence of L-arginine and several amino acid analogs of L-arginine. Alone, the analogs had no effect on the proliferative capacity of PBMCs. The capacity of the analogs to reverse the immunotoxic effects of L-canavanine was measured as an increase in the IC_{50} value observed after coadministration of L-canavanine with the indicated biochemical modulator, as compared to that observed with exposure with L-canavanine alone (Figure 3). When PBMCs were stimulated via the mixed lymphocyte reaction, the antiproliferative effects of L-canavanine were significantly reduced when L-arginine was coadministered. When PBMCs were supplemented with 1 mM L-arginine, the IC_{50} of L-canavanine rose from 1.22 ± 0.14 mM to greater than 8 mM. Similarly, when as little as 200 μM L-arginine was added to L-canavanine treated cells, the IC_{50} value increased significantly to 2.87 ± 0.38 mM.

Coadministration of L-ornithine (the structural analog of L-canaline) also attenuated the cytotoxic effect of L-canavanine in PBMCs. The addition of both 1 mM and 200 μM L-ornithine increased the IC_{50} of L-canavanine to approximately 150% of control values. Also, 1 mM agmatine, the decarboxylation product of L-arginine, attenuated the toxic effects of L-canavanine, significantly increasing the IC_{50} of L-canavanine from 1.22 ± 0.14 to 1.89 ± 0.14 mM. The inclusion of other cationic amino acids (i.e. D-arginine, L-homoarginine and L-lysine) in the culture had no statistically significant effect on the antiproliferative properties of L-canavanine in PBMCs stimulated via the mixed lymphocyte reaction. Similarly, putrescine, citrulline and L-NAME all had no significant effect on the cytotoxicity of L-canavanine.

In PMA-stimulated PBMCs, the antiproliferative effect of L-canavanine was abrogated when either 200 or 100 μM of L-arginine was added to the cell suspensions (L-canavanine $IC_{50} >$ 8 mM). Even in cells supplemented with only 50 μM of L-arginine, the effect was statistically significant, with the IC_{50} of L-canavanine increasing almost 3-fold, from 1.64 ± 0.06 to 4.43 ± 0.32 mM. Similarly, L-ornithine reduced the cytotoxicity of L-canavanine. The IC_{50} of L-canavanine in the presence of 200 μM L-ornithine rose from 1.64 ± 0.06 to 2.54 ± 0.88 mM. In contrast, concurrent exposure of L-canavanine with either L-NAME or putrescine did not affect the cytotoxic effects of L-canavanine (Table 1).

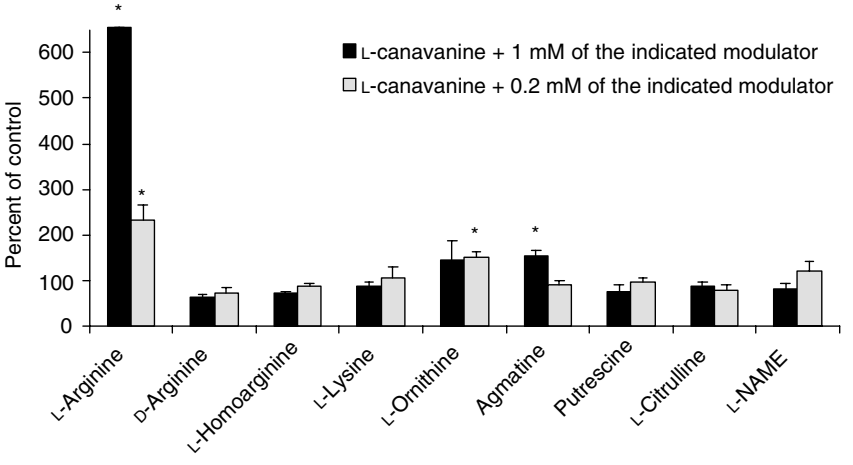

Figure 3. The antiproliferative effect of L-canavanine in combination with 1 or 0.2 mM of a series of potential biochemical modulators. The results are expressed as percent of control proliferation in the presence of L-canavanine without the addition of a biochemical modulator. *Statistically significant ($p < 0.05$) reduction over the control.

Table 1. The antiproliferative effect [$IC_{50} \pm$ SEM (mM)] of L-canavanine or L-canaline on PBMCs stimulated with PMA (L-canavanine was administered alone, or in combination with 0.2, 0.1 or 0.05 mM of a series of potential biochemical modulators)

	Modulator concentration			
	No modulator	0.2 mM	0.1 mM	0.05 mM
L-Canavanine +	1.64 ± 0.06			
L-arginine		>8[a]	>8[a]	4.31 ± 0.32[a]
L-ornithine		2.54 ± 0.88	1.23 ± 0.01	1.19 ± 0.03
putrescine		2.16 ± 0.33	1.42 ± 0.30	1.37 ± 0.28
L-NAME		2.21 ± 0.39	1.98 ± 0.22	1.82 ± 0.18
L-Canaline+	0.23 ± 0.02			
L-arginine		0.26 ± 0.03	0.24 ± 0.01	0.26 ± 0.01
L-ornithine		>0.5[a]	0.34 ± 0.04[a]	0.25 ± 0.01
putrescine		0.30 ± 0.07	0.27 ± 0.08	0.24 ± 0.02

[a]Statistically significant ($p < 0.05$) reduction over the control.

The effect of biochemical modulators on the cytotoxicity of L-canaline

In an effort to differentiate between the cytotoxic effects of L-canavanine and L-canaline, a series of L-arginine and L-ornithine analogs that were considered to be potential biochemical modulators of the cytotoxicity of L-canaline, were added together with L-canaline to the suspension of human PBMCs, in order to evaluate their effect, if any, on L-canaline toxicity.

In PBMCs stimulated via the mixed lymphocyte reaction (Figure 4), the cytotoxic effects of L-canaline could be reversed by the addition of either L-ornithine or putrescine. The IC_{50} value of L-canaline alone was 0.54 ± 0.08 mM; with the addition of L-ornithine, however, the IC_{50} rose significantly to 1.57 ± 0.22 mM (1 mM L-ornithine) and 1.06 ± 0.11 mM (200 μM L-ornithine). While the effect was not as pronounced as that observed with the addition of L-ornithine, the concomitant addition of putrescine and L-canaline to PBMCs also was statistically significant in attenuating the cytotoxic effect of L-canaline alone. The IC_{50} value of L-canaline in the presence of 1 mM putrescine was 1.38 ± 0.27 mM. L-Lysine, L-arginine and agmatine did not significantly affect the cytotoxicity of L-canaline in PBMCs stimulated by the mixed lymphocyte reaction.

Similarly, the toxicity of L-canaline to PBMCs stimulated by PMA was also modulated by the addition of certain potential inhibitors (Table 1). The inclusion of 200 μM of L-ornithine in PBMC cultures containing L-canaline more than doubled the effective IC_{50} of L-canaline from 0.23 ± 0.02 mM to greater than 0.50 mM. Similarly, the addition of 100 μM of L-ornithine increased the IC_{50} value of L-canaline to 0.34 ± 0.04 mM. The addition of L-arginine and putrescine did not significantly alter the IC_{50} value of L-canaline in PMA-stimulated lymphocytes.

Discussion

L-Canavanine is a promising anticancer agent currently in development. This study investigated the *in vitro* immunotoxicity of L-canavanine, and provides the first report of the effects of the L-canavanine metabolite, L-canaline, on lymphocyte proliferation. To better understand the lymphopenic potential of these novel antineoplastic agents and to elucidate the biochemical mechanisms that may contribute to the antiproliferative effects of these compounds, a number of *in vitro* immunotoxicological studies were performed. Isolated human PBMCs were stimulated to proliferate via two different mechanisms. The cells were exposed to serial concentrations of L-canavanine or L-canaline in the presence or absence of a series of potential biochemical antagonists of L-canavanine and L-canaline; these agents represent key intermediates in L-arginine-utilizing pathways. The agents included the natural isosteres of L-canavanine and L-canaline (L-arginine and L-ornithine, respectively), the enantiomer of L-arginine (D-arginine), the competitive y+ amino acid transporter substrates (L-lysine, and L-homoarginine),[34] the ODC product and key intermediate in the polyamine synthesis pathway (putrescine), a commercial NOS inhibitor (L-NAME), and the product of L-arginine oxidation by NOS, L-citrulline.

The above biochemical modulators were chosen in order to further elucidate the possible biochemical mechanisms of toxicity of L-canavanine and L-canaline in human lymphocytes. It was postulated that the addition of these biochemical modulators might cause a partial reversal of the observed immunotoxicity of

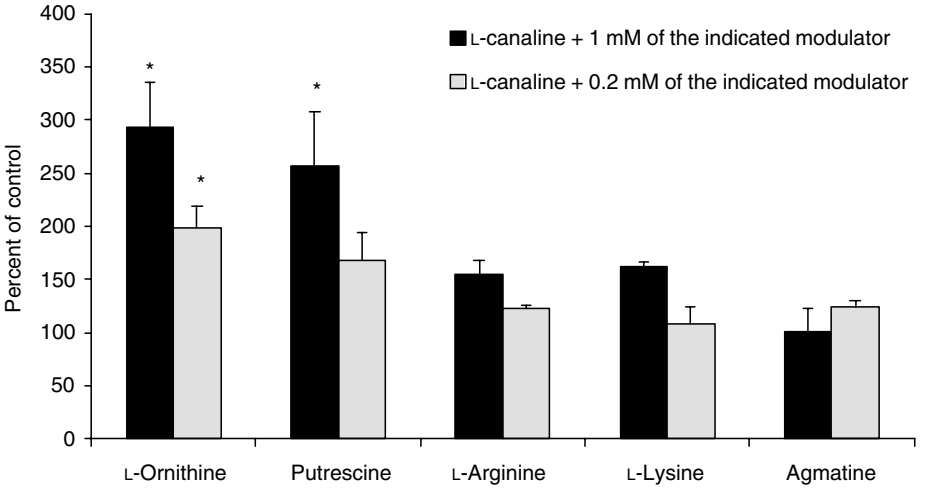

Figure 4. The antiproliferative effect of L-canaline in combination with 1 or 0.2 mM of a series of potential biochemical modulators. The results are expressed as percent of control proliferation in the presence of L-canaline without the addition of a biochemical modulator. *Statistically significant ($p < 0.05$) reduction over the control.

L-canavanine or L-canaline, providing evidence as to which L-arginine-utilizing pathways were adversely affected by exposure to L-canavanine or L-canaline.

Additionally, lymphocytes were stimulated by two different methods to evaluate whether the method of lymphocyte stimulation impacted the immunotoxic effects of L-canavanine and/or L-canaline. PMA stimulates lymphocytes through protein kinase C (PKC)-dependent mechanisms. In contrast, a mixed lymphocyte reaction induces a strong allogenic immune response. From the results, it is evident that L-canavanine and L-canaline inhibit the proliferation of stimulated PBMCs, irrespective of the manner in which the cells are stimulated. The method of lymphocyte stimulation (PMA or via the mixed lymphocyte reaction) did not have a significant impact on the immunotoxicity of either L-canavanine or L-canaline, suggesting that a common pathway or multiple pathways are affected by these two agents. Unpublished results from our laboratory have also demonstrated the capacity of L-canavanine to inhibit the *in vitro* proliferation of lymphocytes in stimulating assays utilizing phytohemagglutinin (PHA), Staph protein A, interleukin (IL)-2 or CD3A6. These data indicate that, like many other antineoplastic agents, L-canavanine and its metabolite, L-canaline, may have immunotoxic properties regardless of the method of stimulation.

These studies also provide a preliminary assessment of the mechanistic pathway(s) that may be involved in the immunotoxicity of both L-canavanine and L-canaline. In this evaluation, several components of biochemical pathways that may be adversely affected by L-canavanine or L-canaline were assessed. The addition of L-arginine completely reversed the immunotoxicity of L-canavanine. This result is not surprising, since L-canavanine has been shown to compete with L-arginine in many L-arginine-utilizing metabolic pathways.[4] L-Canavanine is a substrate for arginyl tRNA synthetase and is incorporated in place of L-arginine into newly synthesized proteins, resulting in the formation of non-functional proteins.[3–7] Although it is not possible from this data to differentiate between competition of L-canavanine with L-arginine for incorporation into protein and competition with L-arginine for other L-arginine-utilizing enzymatic pathways that may be involved in the immunotoxic mechanism for L-canavanine, the results of the current studies are consistent with the hypothesis that the cytotoxicity of L-canavanine is due primarily to its incorporation into protein in place of L-arginine.

Notably, the addition of L-ornithine to both L-canaline- and L-canavanine-treated cells partially attenuated the cytotoxic effects of both drugs. The effect of L-ornithine, however, was greatest in cells treated with L-canaline, suggesting that exposure to L-ornithine may preferentially reduce the cytotoxicity of L-canaline when compared to L-canavanine. The addition of putrescine to L-canaline-treated cells and agmatine to L-canavanine-treated cells also diminished the immunotoxicity of L-canaline and L-canavanine, respectively. L-Ornithine, putrescine and agmatine are all components of the polyamine biosynthesis pathway. It has previously been suggested that the cytotoxic effects of L-canaline

result from inhibition of ODC and the subsequent disruption of polyamine biosynthesis.[14,20] The results of the experiments described in this work are consistent with these findings and indicate that a mechanism of toxicity for L-canaline in lymphocytes may be the inhibition of polyamine biosynthesis. The capacity of L-ornithine and agmatine to attenuate the cytotoxicity of L-canavanine allows speculation that some of the immunotoxic effects of L-canavanine may be attributed to its capacity to affect polyamine biochemistry, either directly as a polyamine biosynthesis inhibitor or indirectly as a result of arginase-catalyzed conversion to L-canaline.

Since L-canavanine is a substrate for the y+ transport system that transports cationic amino acids into the cell,[29] it was postulated that L-canavanine may inhibit cationic amino acid transport and that this inhibition may contribute to the immunotoxic effects of L-canavanine. The data from the present study indicate that the capacity of L-canavanine to inhibit lymphocyte proliferation is not a consequence of inhibiting cationic amino acid transport; the addition of L-homoarginine and L-lysine, which are also substrates of system y+, did not reduce the cytotoxic effects of L-canavanine.

The addition of either L-NAME or citrulline to the PBMC medium did not alter the toxicity of either L-canavanine or L-canaline. While L-canavanine is known to be an inhibitor of NOS, the data from the present studies indicate that NOS inhibition, and consequently the NOS pathway, may not be an important factor regulating the immunotoxicity of L-canavanine or L-canaline in PBMCs.

While the experiments evaluating the potential biochemical inhibitors of L-canavanine and L-canaline toxicity provide insight into mechanisms of toxicity in human PBMCs, the results from these studies may also have potential clinical implications. L-Canavanine inhibits the proliferation of a variety of tumor cells, including pancreatic, lung, colon and leukemic cancers both *in vitro* and *in vivo*.[15–17] As a result of the lack of effective therapies for the treatment of pancreatic cancer and the capacity of L-canavanine to be selectively taken up by the pancreas,[35] particular efforts have been focused on the use of L-canavanine as a treatment for pancreatic cancer.[36]

The observation that the immunotoxic effect of L-canavanine in PBMCs can be attenuated by L-ornithine indicates that in PBMCs, some of the cytotoxicity of L-canavanine may be attributed to an interaction with the biochemical pathways involving L-ornithine. Recently, it has been shown that the cytotoxic effect of L-canavanine in the human pancreatic cancer cell line Mia PaCa-2 is independent of its conversion to L-canaline.[37] Indeed, recent unpublished results from our laboratory show that in two types of human pancreatic cancer cell lines (Panc-1 and Mia PaCa-2), the cytotoxicity of L-canavanine can be overcome by concurrent exposure to increasing concentrations of L-arginine, but not upon concurrent exposure to L-ornithine. These *in vitro* results suggest that the therapeutic coadministration of L-ornithine with L-canavanine may reduce the immunosuppressive and immunotoxic effects of L-canavanine, without affecting L-canavanine-mediated inhibition of pancreatic cancer cell proliferation.

In conclusion, this study, which is the first to investigate the immunotoxic effects of L-canaline, shows that the mechanism of toxicity of L-canaline may be related to the disruption of biochemical pathways involving L-ornithine and the polyamines. The results of these studies indicate that the immunotoxic effects of L-canavanine are mediated primarily through L-arginine-utilizing pathways and are independent of NOS pathways. In addition, the antiproliferative effects of L-canavanine may result from its conversion to the arginase-mediated metabolite, L-canaline. These important findings have potential value in furthering the development of L-canavanine and L-canaline for the treatment of neoplastic diseases, as well as affording insight into the differences in the way these two agents interact with transformed and non-transformed cells, and provide information of potential use in the development of drugs with large therapeutic indices. Ongoing studies are currently focused on improving our understanding of the antiproliferative and immunotoxic mechanisms of these novel antineoplastic agents in order to promote their development for the treatment of cancer.

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