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Alkaloids from the Poisonous Plant *Ipomoea carnea*: Effects on Intracellular Lysosomal Glycosidase Activities in Human Lymphoblast Cultures

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There is natural intoxication of livestock by the ingestion of *Ipomoea carnea* (Convolvulaceae) in Brazil and other parts of the world. The alkaloidal glycosidase inhibitors swainsonine, 2-*epi*-lentiginosine, and calystegines B_1, B_2, B_3, and C_1 have been identified as constituents of this plant. Swainsonine is a potent inhibitor of rat lysosomal α-mannosidase, with an IC_{50} value of 0.02 μM, whereas calystegines B_1, B_2, and C_1 are potent inhibitors of rat lysosomal β-glucosidase, with IC_{50} values of 2.1, 0.75, and 0.84 μM, respectively. The action of swainsonine results in a lysosomal storage disorder that closely mimics α-mannosidosis in humans. To determine whether the toxicity of *I. carnea* to livestock is due to purely swainsonine or due to a combination of effects by swainsonine and calystegines, intracellular lysosomal glycosidase activities in normal human lymphoblasts grown with inhibitors in the medium were examined. Incubation of lymphoblasts with 0.1 μM swainsonine for 3 days resulted in ∼60% reduction of α-mannosidase activity. On the other hand, calystegines B_2 and C_1 showed no inhibition of β-glucosidase up to 1 mM; instead inclusion of calystegines B_2 and C_1 at 100 μM in the culture medium increased its activity by 1.5- and 1.6-fold, respectively. Calystegines B_2 and C_1 seem to act as chemical chaperones, enhancing correct folding of the enzyme and enabling smooth trafficking to the lysosome. The lysosomal β-glucosidase inhibitory calystegines seem to have little risk of inducing intoxication of livestock.

KEYWORDS: ***Ipomoea carnea*; toxic principle; swainsonine; calystegine; human lymphoblast**

INTRODUCTION

There are outbreaks of natural poisoning in livestock that chronically ingest *Ipomoea carnea* (Convolvulaceae), which is a plant of tropical American origin but is now widely distributed in the tropical regions of the world. Early poisoning reports are from Sudan (*1*) and India (*2*), and a recent one is from Mozambique (*3*). The toxicity has been confirmed in feeding experiments with goats and sheep (*3*, *4*). In the leaves collected in Mozambique where goats were intoxicated, the indolizidine alkaloid swainsonine and the nortropane alkaloids calystegines B_2 and C_1 were detected by gas chromatography−mass spectrometry (GC-MS) (*3*). In Brazil, natural poisoning of livestock by this plant is especially common in drought periods because it is one of the few plants that then stay green. Very recently, we have reported the isolation of swainsonine (**1**), 2-*epi*-lentiginosine (**2**), calystegines B_1 (**3**), B_2 (**4**), B_3 (**5**), and C_1 (**6**), and *N*-methyl-*trans*-4-hydroxy-L-proline (**7**) (**Figure 1**) from the leaves of *I. carnea* in Brazil (*5*). Swainsonine is a potent inhibitor of class II α-mannosidases and has been identified as the toxic principle in "pea struck" in sheep eating *Swainsona* species in Australia (*6*, *7*) and "locoism" in the western United States caused by *Astragalus* and *Oxytropis* species (*8*). Prolonged ingestion of swainsonine by animals leads to a phenocopy of the genetic mannosidosis of humans (*6*). Decreased α-mannosidase activity results in lysosomal accumulation of undegraded oligosaccharides, loss of cellular function, and ultimately cell death (*9*, *10*). The nortropane alkaloid calystegines are common in the Solanaceae and Convolvulaceae (*11*−*14*). There is a possibility that calystegines can poison livestock and induce lysosomal storage disorders because some calystegines are potent inhibitors of glycosidases (*15*−*17*). There are livestock poisonings, which symptomatically resemble "locoism", by *Solanum* species. For example, *S. dimidiatum* causes "crazy cow syndrome" in Texas (*18*) and *S. kwebense* causes "maldronksiekte" in South Africa (*19*). Although both plants contain high concentrations of a range of calystegines (*11*),

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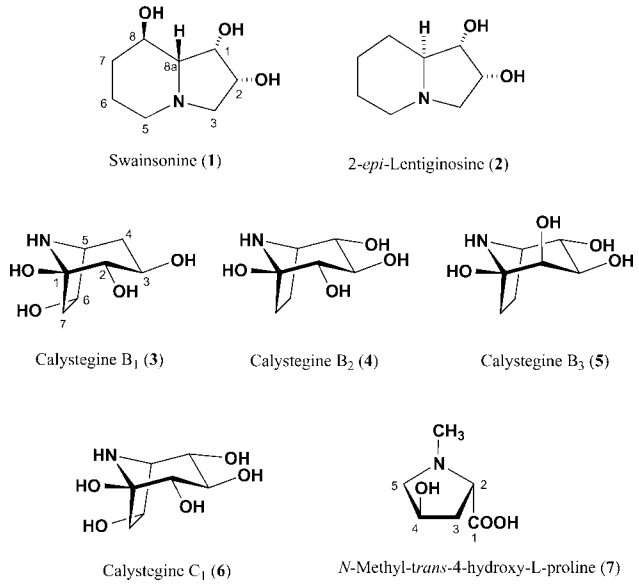

Figure 1. Structures of alkaloids isolated from *I. carnea*.

calystegines have not yet been proved to be toxic principles of the neurological disorders caused by both plants. Thus, it is very important to elucidate whether glycosidase-inhibiting calystegines induce lysosomal storage disorders and whether the toxicity of *I. carnea* to livestock is due to purely swainsonine or due to a combination of effects by swainsonine and calystegines.

In the present study we show that swainsonine inhibits lysosomal α-mannosidase in human lymphoblasts in culture at only 0.1 μM, whereas calystegines do not inhibit any lysosomal glycosidases even at high concentrations (100 μM), but rather enhance intracellular glycosidase activities.

MATERIALS AND METHODS

Alkaloids. Alkaloids were prepared from the leaves of *I. carnea* collected in May 2001 from the culture in the Research Center for Veterinary Toxicology (CEPTOX), School of Veterinary Medicine, University of São Paulo, Brazil, according to the method of our previous paper (*5*).

Culture of Human Lymphoblasts. Normal human lymphoblasts were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics at 37 °C under 5% CO_2.

Enzyme and in Vitro Enzyme Assay. Human lysosomal α-galactosidase A was prepared as described previously (*20*). β-Glucocerebrosidase (Ceredase) was purchased from Genzyme (Boston, MA). The cell lysate of normal human lymphoblasts was used as the source of lysosomal α-glucosidase, β-galactosidase, α-mannosidase, β-mannosidase, and α-fucosidase. The reaction mixture consisted of 50 μL of 0.15 M sodium phosphate−citrate buffer (pH 4.5), 50 μL of 2% Triton X-100 (Sigma Chemical Co.), 30 μL of the enzyme solution, and 20 μL of an inhibitor solution or H_2O. The reaction mixture was preincubated at 0 °C for 10 min and started by the addition of 50 μL of 6 mM 4-methylumbelliferyl glycoside (Sigma Chemical Co.) (1 mM in the case of β-galactoside), followed by incubation at 37 °C. The reaction was stopped by the addition of 2 mL of 0.1 M glycine buffer (pH 10.6). Liberated 4-methylumbelliferone was measured (excitation, 362 nm; emission, 450 nm) with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Intracellular Lysosomal Glycosidase Assay. The cells (1×10^6 cells/mL) were incubated in culture medium in the absence or presence of an inhibitor for 3 days. For the assay of lysosomal glycosidases other than β-glucocerebrosidase, the cells were washed twice with phosphate-buffered saline (PBS) and homogenized in 300 μL of 0.15 M sodium phosphate−citrate buffer (pH 4.5). The supernatant obtained after centrifugation at 10000*g* for 30 min was used for the determination of lysosomal glycosidases. For the β-glucocerebrosidase assay, the cells were homogenized in 300 μL of 0.04 M sodium phosphate−citrate buffer (pH 5.5) containing 0.1% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 4 mM β-mercaptoethanol. After centrifugation at 10000*g* for 30 min, the supernatant was used for the determination of β-glucocerebrosidase. The activity of α-galactosidase A was determined with 50 μL of the substrate solution (6 mM 4-methylumbelliferyl α-galactoside and 90 mM acetylgalactosamine in 0.15 M sodium phosphate−citrate buffer, pH 4.5), and the β-glucocerebrosidase activity was determined with 50 μL of the substrate solution (6 mM 4-methylumbelliferyl β-glucoside in the presence of 1% sodium taurocholate and 1% Triton X-100 in 0.15 M sodium phosphate−citrate buffer, pH 5.5). The other enzyme activities were determined with 50 μL of 6 mM 4-methylumbelliferyl glycoside (1 mM in the case of β-galactoside) in 0.15 M sodium phosphate−citrate buffer (pH 4.5).

Table 1. Effects of Alkaloids Isolated from *I. carnea* on Human Lysosomal Glycosidases

	IC_{50}[a] and K_i (μM)					
enzyme[a]	**1**	**2**	**3**	**4**	**5**	**6**
α-glucosidase	–[b]	–	–	–	–	–
β-glucosidase	–	–	2.5 (1.5)[c]	0.99 (0.49)	76	2.5 (0.9)
α-galactosidase	–	–	–	50 (30)	–	–
β-galactosidase	–	–	–	–	–	–
α-mannosidase	0.04	5.0	–	–	620	–
β-mannosidase	–	–	–	–	–	–
α-fucosidase	–	–	–	–	–	–

[a] Concentration giving 50% inhibition. [b] Less than 50% inhibition at 1000 μM. [c] In the measurement of the kinetic parameter for lysosomal β-glucosidase, Ceredase was used as the enzyme source.

RESULTS AND DISCUSSION

Inhibition of Human Lysosomal Glycosidases by Alkaloids in Vitro. We previously reported inhibitory activities of alkaloids isolated from *I. carnea* toward rat lysosomal glycosidases (*5*). The indolizidine alkaloids swainsonine (**1**) and 2-*epi*-lentiginosine (**2**) were potent inhibitors of rat lysosomal α-mannosidase, with IC_{50} values of 0.02 and 4.6 μM, respectively, whereas calystegines B_1 (**3**), B_2 (**4**), and C_1 (**6**) were potent inhibitors of rat lysosomal β-glucosidase, with IC_{50} values of 2.1, 0.75, and 0.84 μM, respectively. As shown in **Table 1**, the susceptibility of human lysosomal glycosidases toward the alkaloids is little different from that of the rat enzymes.

Swainsonine is known to be a powerful competitive inhibitor of human lysosomal α-mannosidase with a K_i value of 80 nM but not an inhibitor of lysosomal β-mannosidase (*20*). As seen in **Figure 2**, calystegines B_1, B_2, and C_1 inhibited human β-glucocerebrosidase in a competitive manner with K_i values of 1.5, 0.49, and 0.90 μM, respectively. Calystegine B_2 was also a competitive inhibitor of α-galactosidase A with a K_i value of 30 μM.

Effect of Alkaloids on Lysosomal Glycosidases in Culture of Human Lymphoblasts. When the leaf extract of *I. carnea* was incubated with normal human lymphoblasts for 3 days, intracellular glycosidase activities were determined with 4-methylumbelliferyl glycosides as substrate at pH 4.5. As shown in **Figure 3**, inclusion of the extract at 10 μg/mL in culture medium decreased intracellular α-mannosidase activity by >50%, and its inclusion at 1000 μg/mL completely nullified the enzyme activity. Interestingly, at concentrations below 100 μg/mL, other glycosidase activities were significantly elevated when compared with untreated cells. Exposure at 10 μg/mL enhanced enzyme activities 1.3-fold for α-galactosidase, 1.7-

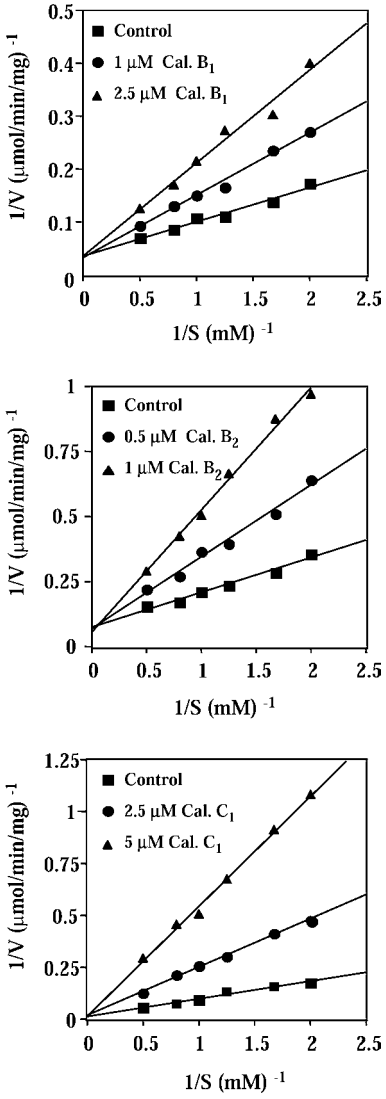

Figure 2. Lineweaver–Burk plots of calystegines B_1, B_2, and C_1 of human β-glucocerebrosidase. Increasing concentrations of 4-MU-β-D-glucoside were used to determine the K_i values.

fold for β-galactosidase, 1.2-fold for β-mannosidase, and 1.4-fold for β-glucosidase The β-galactosidase and β-glucosidase activities even at 1000 μg/mL were higher than those in untreated cells.

As shown in **Figure 4**, swainsonine and calystegines B_2 and C_1 that showed potent inhibitory activity toward lysosomal α-mannosidase and β-glucosidase, respectively, were tested for the effects on intracellular glycosidase activities in normal human lymphoblasts. When the cells were placed in contact with medium containing only 0.1 μM swainsonine, ~60% of intracellular α-mannosidase activity was inhibited. However, inclusion of swainsonine at 10 μg/mL in the culture medium enhanced intracellular β-mannosidase activity 1.3-fold. Calystegines B_2 and C_1 showed no inhibition toward α- and β-galactosidases, even at concentrations as high as 1000 μM and, to the contrary, slightly elevated intracellular β-galactosidase activity at the same concentrations. Surprisingly, β-glucosidase activity was significantly elevated by adding the potent β-glucosidase inhibitors calystegines B_2 and C_1, up to 1.5- and 1.6-fold at 100 μM, respectively. Furthermore, intracellular β-glucosidase activity was higher than that in untreated cells even at concentrations as high as 1000 μM of both compounds.

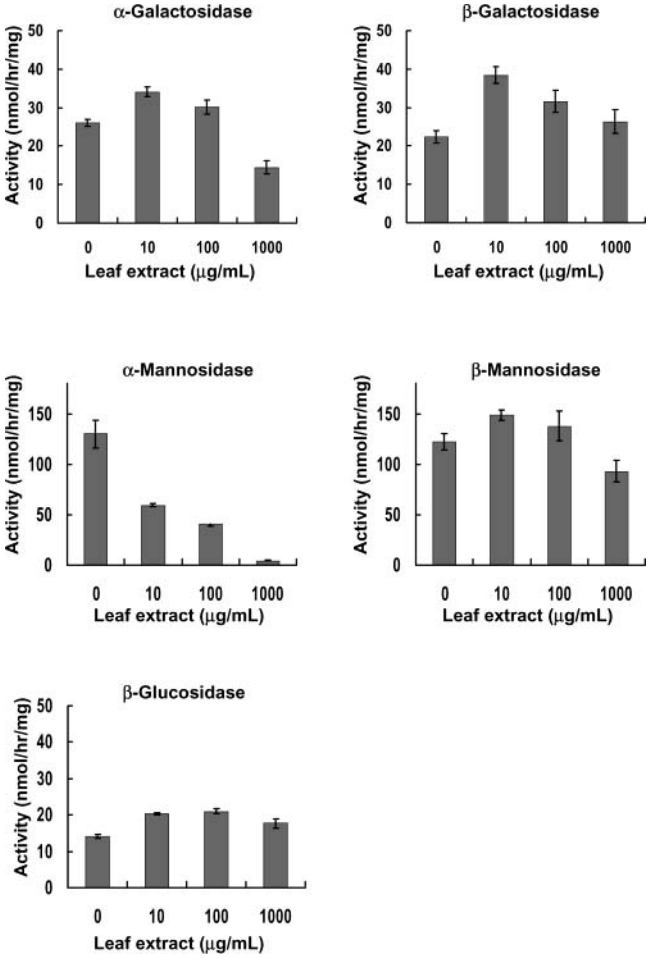

Figure 3. Effects of the leaf extract of *I. carnea* on lysosomal glycosidase activities in normal human lymphoblasts.

It has been shown that swainsonine is a lysosomotropic compound and accumulates rapidly in lysosomes of normal human fibroblasts in culture to produce inhibition of intracellular lysosomal α-mannosidase, resulting in the induction of a phenocopy of the genetic mannosidosis of humans (*20*). The low concentration of swainsonine ingested is made effective by its ability to permeate the plasma membranes freely, but once inside lysosomes it is protonated due to the low pH and becomes concentrated there (*20*). Our present result is consistent with this demonstration because treatment with 0.1 μM swainsonine for 3 days inhibited ~60% of intracellular α-mannosidase activity. On the other hand, calystegines B_2 and C_1, in vitro potent inhibitors of lysosomal β-glucosidase, seem to present little risk of inducing intoxication of livestock because they act as enhancers rather than inhibitors of lysosomal β-glucosidase in the cell culture system.

The endoplasmic reticulum (ER) possesses efficient quality control mechanisms to facilitate the selective elimination of improperly folded proteins and to ensure that only proteins folded correctly into their functional conformation are transported to the Golgi apparatus for further maturation (*21*). Misfolded and genetic mutant proteins are normally retained in the ER and eventually degraded by ER-associated degradation (ERAD) (*22*, *23*). Recent experimental data show that some human genetic diseases are due to mutations in proteins that influence their folding and lead to retaining of mutant proteins in the ER and successive degradation (*24*, *25*). Genetic defects in lysosomal enzymes lead to accumulation of their substrates, resulting in lysosomal storage diseases. Fabry's disease is caused

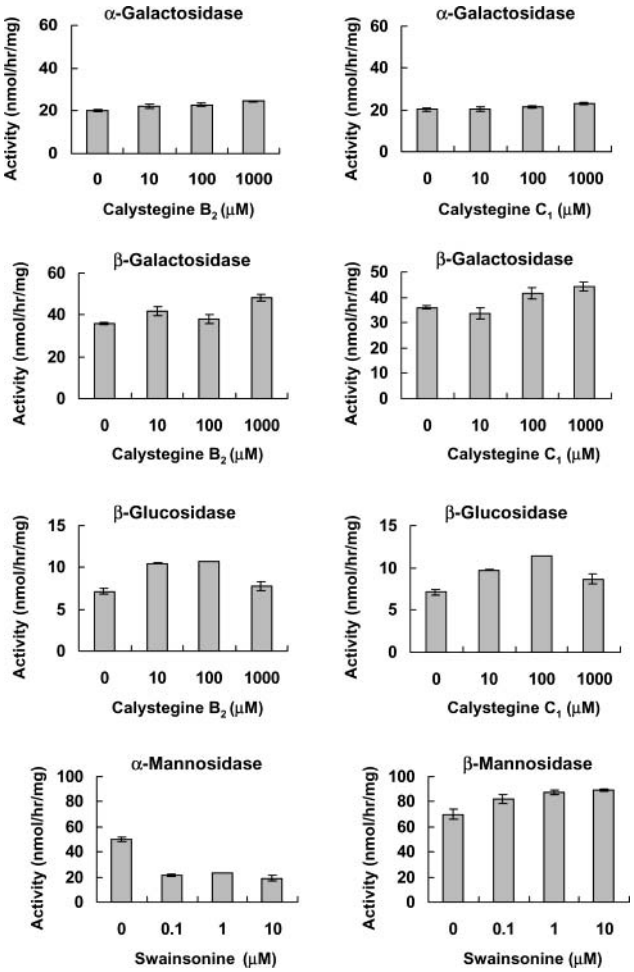

Figure 4. Effects of calystegines B_2 and C_1 and swainsonine on lysosomal glycosidase activities in normal human lymphoblasts.

by deficient activity of lysosomal α-galactosidase A (*26*). Recently, 1-deoxygalactonojirimycin, a potent competitive inhibitor of α-galactosidase A, effectively enhanced the mutant enzyme activity in lymphoblasts established from Fabry patients (*27*). 1-Deoxygalactonojirimycin binds to the catalytic domain of a mutant enzyme and induces the proper conformation as a specific "chemical chaperone" and prevents the retention of the mutant protein by the quality control mechanism in the ER and the successive degradation in ERAD. Competitive enzyme inhibitors are expected to be effective active-site-specific chaperones because of their high affinity for the catalytic domain.

In the present work, calystegines B_2 and C_1, potent competitive inhibitors of lysosomal β-glucosidase, were found to be enhancers rather than inhibitors of intracellular β-glucosidase in the cell culture system. This implies that they served as active-site-specific chaperones to assist the correct folding of the unfolded or misfolded normal enzyme and its successful transport from the ER to the Golgi apparatus, resulting in correct targeting to the lysosome. Furthermore, this indicates that calystegines do not appear to be lysosomotropic. If calystegines are rapidly taken up into the lysosomes in the cell culture system and concentrated after being protonated due to the low pH, they should inhibit intracellular β-glucosidase as effectively as swainsonine inhibits lysosomal α-mannosidase. It is now known that not only misfolded proteins but also normal proteins can be degraded in ERAD. It has been recently reported that one-third of newly synthesized proteins are rapidly degraded by ERAD, even using cells under the most physiological conditions (*28*). It can therefore be presumed that calystegines acted as active-site-specific chaperones in the cell culture system and rescued unfolded or misfolded enzyme proteins that are destined for intracellular degradation in ERAD. A chemically induced lysosomal storage disease seems to be caused only by inhibitors with potency of inhibition of lysosomal glycosidase and lysosomotropic behavior such as swainsonine. Calystegines, which are in vitro potent inhibitors of lysosomal β-glucosidase but nontoxic in the cell culture system, are expected to become candidates for the treatment of Gaucher's disease caused by deficient activity of lysosomal β-glucosidase, also known as β-glucocerebrosidase.

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