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# *N*-benzyloxycarbonyl-L-proline: An in vitro and in vivo inhibitor of prolidase

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**Abstract**

Prolidase deficiency (PD) is a recessive disorder of the connective tissue caused by mutations in the prolidase, a specific peptidase, cleaving the dipeptides with a C-terminal prolyl and hydroxyprolyl residue. PD is a complex syndrome characterized mainly by intractable skin lesions, recurrent respiratory infections and mental retardation. The relation between prolidase biological functions and the disease is still largely unknown. We studied the effect of a prolidase inhibitor, *N*-benzyloxycarbonyl-L-proline (Cbz-Pro), in vitro on prolidase from human fibroblasts and in vivo on murine erythrocytes prolidase. A 90% inhibition was detected incubating cellular extracts at 1:1 ratio of Gly-Pro substrate: Cbz-Pro inhibitor. Pulse experiments performed incubating human fibroblasts with 6 mM Cbz-Pro revealed that the inhibitor uptake was completed in about 1 min. The Cbz-Pro uptake was saturable and pH dependent. Long-term incubation of fibroblasts with Cbz-Pro caused mitochondria depolarization and increased cellular death as reported for long-term culture of fibroblasts from PD patients. An inhibitory effect of Cbz-Pro has also been shown in vivo. Our results demonstrated that Cbz-Pro is a potent inhibitor of prolidase in cultured fibroblasts and it can be used in vivo to better characterize the prolidase enzyme and further investigate PD physiopathology.


*Keywords:* *N*-benzyloxycarbonyl-L-proline; Prolidase; Prolidase deficiency; Prolidase inhibition; Capillary electrophoresis

## 1. Introduction

Prolidase (EC 3.4.13.9) is an extremely specific cytosolic metallo-peptidase, cleaving Xaa-Pro, Xaa-Hyp dipeptides. It is involved in the final stage of endogenous and dietary proteins catabolism and its primary physiological role is though to be the recycling of proline from collagen. Prolidase has a wide tissue distribution, having been found in such diverse locations as intestinal mucosa, kidney, liver, brain, heart, thymus, uterus and prostate, as well as in erythrocytes, leukocytes, dermal fibroblasts and plasma [1–4].

In humans, a deficiency of the enzyme, caused by mutations in the prolidase gene, leads to prolidase deficiency (PD), a rare autosomal recessive connective tissue disorder characterized by complex clinical symptoms, which include chronic ulcerative dermatitis, mental retardation, recurrent infections, splenomegaly and dysmorphic facies. The age of onset of the disease varies from birth to 22 years of age, whereas some cases are asymptomatic and a late case has also been reported [5].

The inability to correlate many of the clinical outcome with the putative biological roles of the enzyme, combined with the existence of asymptomatic cases of PD [6], indicates that the complete physiological function of the enzyme is still not fully understood. In absence of animal models of PD, either naturally occurring or experimentally generated, a useful tool to study the prolidase biological roles will be the use of a potent in vivo inhibitor.

In the past, in absence of crystallographic evidence, inhibition studies of the prolidase had been performed to

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better understand the catalytic specificity, active-site characteristics and mechanism of hydrolysis. Using the evidence of phenylglyoxal and carbodiimide inactivation, with protection conferred by proline derivatives, Mock and Zhuang deduced the presence of arginine and carboxylate (aspartic/glutamic acid) residues at the active site of porcine kidney prolidase [7]. Mock and Liu on the basis of previous studies of prolidase inhibition proposed a catalytic cleavage mechanism for porcine kidney prolidase involving chelative activation of the substrate by one of the pair of active-site metal ions, with nucleophilic addition to the scissile carbonyl group by the second metal hydroxide [8,9].

*N*-benzyloxycarbonyl-L-proline (Cbz-Pro) has been reported in literature to be a good prolidase inhibitor of purified porcine kidney prolidase and sheep and human prolidase isolated from erythrocytes. It is effective at low concentrations and able to permeate cell membranes [10,11].

Because fibroblasts are the main cell type present in the skin, that is the most affected tissue in PD patients, in our study, we investigated the inhibition properties of Cbz-Pro on human fibroblasts prolidase. A deeper analysis of the properties of prolidase in this cell type will contribute to further understand PD physiopathology. We also demonstrated for the first time the efficiency of Cbz-Pro as prolidase inhibitor in vivo and as useful tool to generate in future an animal model for the disease.

## 2. Materials and methods

### 2.1. Cell culture

Control human fibroblasts were purchased from American Type Culture Collection (ATCC, Bethesda, MD, USA) and were used between the 7th and 14th passages. Cells were grown at 37°C in the presence of 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% (v/v) Fetal Calf Serum (FCS, Euroclone) or 1% (v/v) ITS+3 serum substitute (Sigma).

For in vitro inhibition experiments, cells were grown in DMEM supplemented with 10% (v/v) FCS.

For uptake experiments up to 1 h, 2 days or 10 days, cells were grown, respectively in buffer: (a) 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 16 mM HEPES; (b) DMEM, 16 mM HEPES; (c) DMEM, 1% (v/v) ITS+3, 16 mM HEPES with or without 6 mM Cbz-Pro (ICN).

For uptake experiments at different pH, fibroblasts were grown for 0, 1, 5, 10 min in buffer (a) at pH 6.0 or 7.5, respectively, in presence of 6 mM Cbz-Pro.

For uptake experiments at different inhibitor concentration, fibroblasts were grown in buffer (a) supplemented with Cbz-Pro at 5, 6, 8, 10 mM.

### 2.2. Enzyme assay

Prolidase activity was determined according to the procedure of Myara et al. [12]. Briefly, cell layers were scraped in 50 mM Tris–HCl pH 7.8 and lysed by freeze–thawing. Cell extracts were clarified by centrifugation. To estimate $K_m$ and $K_i$ supernatant aliquots were incubated with different concentrations of substrate Glycil Proline (2.5, 5, 10 mM Gly-Pro, ICN) and inhibitor (0, 1, 3, 6 mM Cbz-Pro). To evaluate prolidase activity, following Cbz-Pro inhibition, lysates of fibroblasts, grown for 0, 1, 5, 10, 15, 20, 30, 60 min in buffer (a) supplemented with 6 mM Cbz-Pro, were incubated with 45 mM Gly-Pro and the released proline was measured by Chinard's reagent [13].

### 2.3. Intracellular inhibitor quantitation

Cbz-Pro quantitation was performed by capillary electrophoresis, a Beckman (Palo Alto, CA, USA) P/ACE 2100 instrument equipped with a UV detector and an external nitrogen pressure was used. An untrated fused-silica capillary of 50 μm i.d. and 50 cm effective length was adopted. The samples were analyzed using 50 mM sodium tetraborate buffer pH 9.3 (BDH Chemicals) supplemented with 30 mM α-ciclodextrin. An injection time of 1 s was used, 25 kV current was applied and the absorbance was measured at 214 nm.

To ensure run-to-run reproducibility of separations, the capillary column was purged before each injection with 0.1 M sodium hydroxide and fresh buffer for 5 min.

The Beckman P/ACE Station Software was used for data analysis. Five experiments were performed in triplicate and expressed as nmol of Cbz-Pro/mg proteins. The protein determination was performed using the Bio-Rad Protein Assay using the bovine serum albumin as standard.

Peak identification was performed by co-injection of commercial Cbz-Pro.

### 2.4. Trypan blue exclusion test

Cells grown for 1 h, 2 days and 10 days as described above were trypsinized with 0.05% (v/v) trypsin, 0.02% (w/v) EDTA. Aliquots of cells were mixed with Trypan Blue (0.4% Solution, Sigma) at the ratio 1:1 (v/v), incubated at room temperature for 5 min and loaded onto a hemocytometer. A triplicate of each sample was performed. Values were calculated as percentage of non-viable cells on total cells. Significance was determined by *t*-test. $P<0.05$ was considered statistically significant.

### 2.5. Confocal microscopy analysis

Fibroblasts plated at density of $3\times10^4$ on 30 mm diameter Petri dishes were grown for 7 or 10 days in DMEM supplemented with 1% (v/v) ITS+3 with or without 6 mM Cbz-Pro. Mitochondria membrane potential was

measured by lipophilic cationic probe JC-1 (5,5′,6,6′-tetrachloro 1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Molecular Probes) as previously described [14]. Samples were examined with Nikon (Melville, N.Y., USA) PCM 2000 Confocal Microscope System and images were processed by Image Analysis System Zeiss (Jena, Germany) KS 400.

Results are reported as mean ± SD of $n = 90$ integrations for each sample collected in three different experiments. Data analysis was performed by $t$-test. $P < 0.05$ was considered statistically significant.

Fibroblasts cultured in the same conditions were also labeled for 3 min at room temperature in presence of Fluorescein Diacetate (FDA) and Propidium Iodide (PI) (0.2 μg/mL and 0.06 μg/mL, respectively), analyzed by confocal microscopy and counted. Values were expressed as a percentage of nonviable cells on total cells for samples collected in three different experiments.

### 2.6. Effect of Cbz-Pro in vivo

Four weeks old C57Bl/6J mice (Charles River, Italy) were injected daily with 60 mg/kg Cbz-Pro in PBS or with placebo (PBS) for 3 weeks.

Institutional and national guidelines for the care and use of laboratory animals were followed.

Blood (about 0.1 mL) was collected from the tail vein by withdrawing into tubes containing citrate solution as anticoagulant. Following repeated washing with PBS and low speed centrifugation (2000 rpm, 5 min at room temperature) 0.2 mL of 50 mM Tris–HCl pH 7.8, 10 mM $MnCl_2$, 0.75 mM reduced glutathione were added to the samples. To ensure complete lysis, the cell suspension was froze–thawed 3 times and clarified by centrifugation (14,000 rpm, 15 min, 4°C).

Hemoglobin concentration was determined by spectrophotometric measurement [15]. Prolidase was activated by incubating the lysate at 4°C for 24 h. To determine prolidase activity an aliquot of the mixture containing the enzyme (50 μL) was then incubated with 20 mM Gly-Pro in 50 mM Tris–HCl pH 7.8 for 5 min at 37°C, the reaction was stopped with TCA. Proline release was evaluated spectrophotometrically at 515 nm upon incubation with Chinard's reagent [13].

Results were reported as μmol of proline released per h per g of hemoglobin.

### 2.7. Cbz-Pro detection in urine samples

Urine samples collected from Cbz-Pro treated and untreated mice were collected by covering the bottom of the cage with absorbent filter paper for 24 h [16]. The paper was allowed to dry on a clean, flat, non-absorbent surface in room air and sent to the laboratory. Squares of 2.5 $cm^2$ (corresponding to yellow spot) were cut, fun folded and placed in a 1.5-mL tube. The soluble components were eluted with 1 mL of pure water by mechanical shaking for 5 min. Then the tube was left for 15 min at room temperature and finally centrifuged for 5 min at 3000 rpm. A second extraction for each sample was obtained from a new paper square using as extraction solvent the previously extracted solution. The samples obtained were stored at − 20°C. The sample concentration was normalized to the creatinine amount, measured spectrophotometrically [17]. A capillary electrophoresis (CE) method was optimized in order to detect Cbz-Pro inhibitor. The urine samples were diluted to a creatinine final concentration of 50 mg/mL and centrifuged prior to the run. The samples were analyzed by Capillary Zone Electrophoresis (CZE) using an untreated fused-silica capillary of 50 μm i.d. and 50 cm effective length and 50 mM sodium tetraborate buffer pH 9.3. Samples were injected hydrodynamically for 10 s, 25 kV direct current was applied and the absorbance measured at wavelength of 254 nm. To ensure run-to-run reproducibility of separations, the capillary column before each injection was purged with 0.1 M sodium hydroxide and fresh buffer for 5 min. Qualitative analysis was performed in triplicate. Peak identification was performed by co-injection of commercial Cbz-Pro.

## 3. Results

### 3.1. Cbz-Pro inhibition of human fibroblast prolidase

Inhibition properties of Cbz-Pro of human fibroblast prolidase were verified using Gly-Pro as substrate and fibroblast cell lysates as prolidase source.

Cbz-Pro at $[S_0] = [I] = 20$ mM inhibited 90% of human fibroblasts prolidase activity, but already at concentration $[S_0]/[I]$ of 20:1 the inhibition was over 50%. Experiments performed in absence of Gly-Pro showed that no Cbz-Pro degradation with proline release was detectable in our experimental conditions.

A detailed kinetic analysis of Cbz-Pro effect was undertaken. First, the kinetic parameters for the fibroblasts prolidase (FBP) hydrolysis of Gly-Pro were verified. The $K_m$ was 4.1 mM that is similar to the value of 4.7 mM [18] and 6.2 mM [10] reported for porcine kidney prolidase. The Dixon plot, obtained using Gly-Pro at three different concentrations (2.5, 5, 10 mM) and Cbz-Pro at 0, 1, 3, 6 mM showed that Cbz-Pro is a linear competitive inhibitor of FBP (Fig. 1). The inhibition constant ($K_i$) calculated from Lineweaver–Burk plot was 310 μM.

### 3.2. Cellular permeability of Cbz-Pro

The Cbz-Pro uptake in cultured fibroblasts at different times, measured by capillary electrophoresis, was fast and completed in about 1 min. In fact, after 1 min, the intracellular content of Cbz-Pro (282.5 ± 7.96 nmol/mg) was similar to the amount measured after 1 h and 2 days

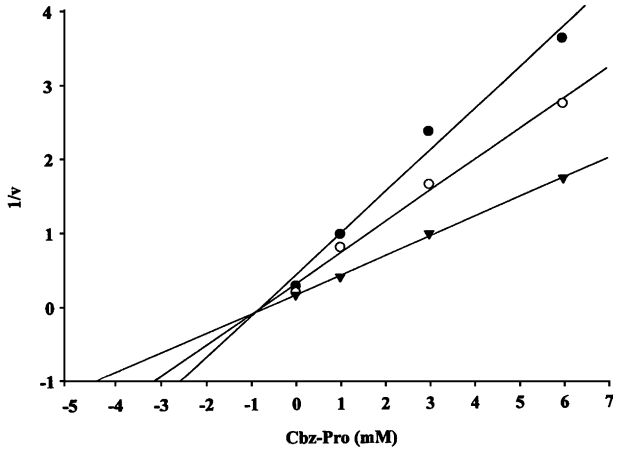


Fig. 1. A Dixon plot of reciprocal rates of proline formation (1/v μmol Pro/h/mg) as function of inhibitor concentration (mM). Each line represents linear regression analysis of reciprocal of average rates of proline formation for different Gly-Pro substrate concentration (2.5 mM (●), 5 mM (>) and 10 mM (▼)) versus different Cbz-Pro inhibitor concentrations (0, 1, 3, 6 mM) ($n = 3$). The pattern shown is representative of 5 independent experiments performed on triplicate.

(267 ± 41.22 nmol/mg; 299 ± 15 nmol/mg, respectively) (Fig. 2A). For uptake experiments up to 1 h, the maximum value measured was after 5 min (351 ± 101.04 nmol/mg).

The intracellular prolidase activity was already reduced of 80% after only 1 min of incubation (data not shown).

Cbz-Pro uptake was influenced by the pH of the uptake medium, the optimal pH was 6.0 (Fig. 2B). This result implies that protons may be involved as the driving force for the metabolic transport of Cbz-Pro in human fibroblast cells.

To examine the kinetic of Cbz-Pro uptake by human fibroblast cells, initial rate of Cbz-Pro uptake was evaluated at pH 6.0 over the concentration range of 5 to 10 mM in the medium at 37°C for 15 min. The uptake of Cbz-Pro tended to saturate as its concentration increased over 8 mM (Fig. 3).

### 3.3. Cellular viability of cultured human fibroblasts in presence or absence of Cbz-Pro

Cellular viability of cultured human fibroblasts in presence or absence of Cbz-Pro, evaluated by Trypan Blue assay, showed no difference after 1 h (3%) or 2 days (7%) of culture. After 10 days, the percentage of dead cells was 7% in absence of Cbz-Pro and 33% in presence of 6 mM inhibitor (Fig. 4).

### 3.4. Mitochondrial membrane potential

Mitochondrial membrane potential in long-term cultured human fibroblasts was measured by labelling the cells with the cationic dye JC-1 after 7 or 10 days of culture in presence or absence of 6 mM Cbz-Pro. The statistical analysis of the data showed a significant increase of green percentage area (JC-1 monomers), indicating low potential and depolarized mitochondria, for cells cultured in presence

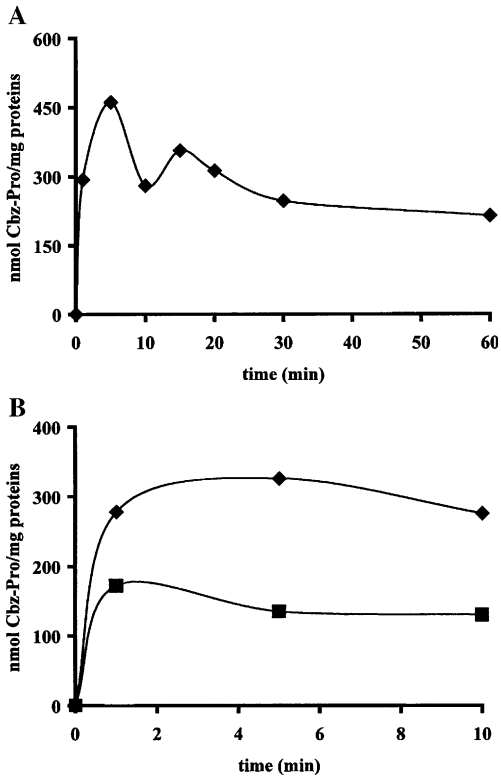


Fig. 2. Panel A: Cbz-Pro (nmol/mg) uptake in cultured fibroblasts as function of time (min). Cbz-Pro content was determined by capillary electrophoresis (as described in Materials and methods) on cells incubated with 6 mM Cbz-Pro buffer a) pH 6.0 for 0, 1, 5, 10, 15, 20, 30, 60 min. Panel B: Cbz-Pro (nmol/mg) uptake in cultured fibroblasts as function of pH. Cbz-Pro content was determined by capillary electrophoresis on cells incubated with 6 mM Cbz-Pro buffer (a) at pH 6.0 (♦) or pH 7.5 (■) for 0, 1, 5, 10 min. Patterns shown are representative of a triplicate of independent experiments.

of Cbz-Pro with respect to untreated cells ($P < 0.05$). Cell viability was also evaluated by FDA/PI double labelling. After 7 days, the percentage of dead cells in untreated and treated cells was, respectively, 9.5% and 27%; it increased after 10 days to 29.1% and 61%, respectively (Fig. 5).

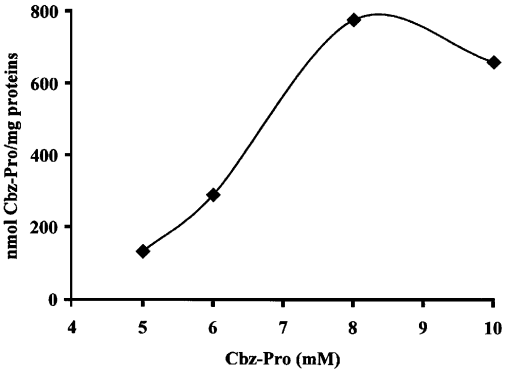


Fig. 3. Cbz-Pro (nmol/mg) uptake in cultured fibroblasts as function of inhibitor concentration (mM). Cbz-Pro content was determined by capillary electrophoresis on cells incubated with 5, 6, 8, 10 mM Cbz-Pro buffer (a) at pH 6.0 for 15 min. Pattern shown is representative of a triplicate of independent experiments.

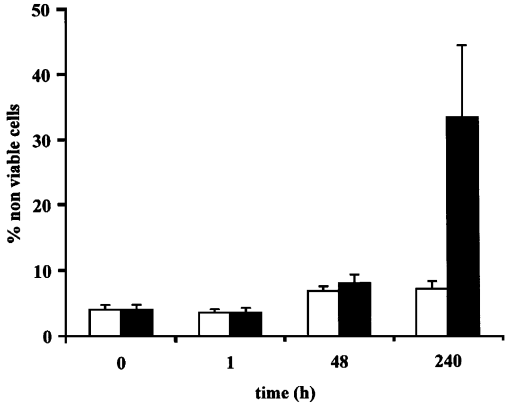


Fig. 4. Cellular viability of cultured fibroblasts in presence or absence of inhibitor measured at different times (h) is expressed as % of non-viable cells on total counted cells. Cellular viability was determined by Trypan Blue assay on cells incubated with buffer (a) at pH 6.0 in absence (white) or presence (black) of 6 mM Cbz-Pro for 0, 1, 48, 240 h. Results are the mean ± S.E.M. of three different experiments.

### 3.5. Cbz-Pro effect in vivo

A total of 19 mice 4 weeks old were injected daily, respectively, with the inhibitor Cbz-Pro ($n$ = 10) or with sterile PBS ($n$ = 9) for 3 weeks as described in Materials and methods. A statistically significant reduction of erythrocytes prolidase activity was detected starting from the 3rd week of treatment in Cbz-Pro treated mice ($P < 0.05$) (Fig. 6). Furthermore, the inhibitor was detected by capillary electrophoresis in the urine of the treated animal revealing that Cbz-Pro is not enzymatically degraded in vivo (data not shown). No alteration of viability or toxic effects had been reported in treated mice with respect the control group.

## 4. Discussion

This study was undertaken to identify and characterize an in vitro and in vivo inhibitor of the prolidase, a cytosolic enzyme involved in the final stage of protein catabolism. The major goal was to provide a unique and valuable system to better understand the biological functions of this enzyme and to further investigate the molecular bases of the Prolidase Deficiency, a rare recessive disorder caused by mutations in the prolidase gene.

Hui and Lajtha first demonstrated that Cbz-Pro inhibited calf brain prolidases (79% inhibition when $[S_0] = [I] = 1$ mM) [11]. Later, King et al. showed that Cbz-Pro inhibited the human erythrocytes enzyme (48% inhibition when $[S_0] = 10$ mM, $[I] = 2.5$ mM) [10].

King also studied some Cbz-Pro kinetic parameters for the porcine kidney prolidase. First, he evaluated the $K_m$ value (6.2 mM) and demonstrated that Cbz-Pro is a linear

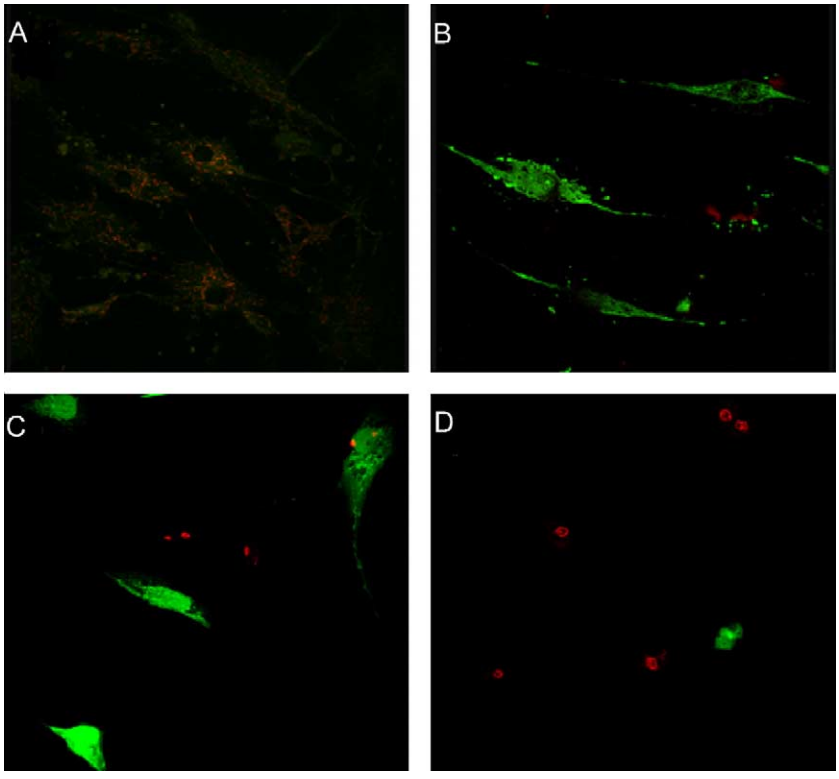


Fig. 5. Confocal microscopy analysis of 10 days cultured fibroblasts. Panels A and B: JC-1 labeling of untreated (A) and 6 mM Cbz-Pro treated (B) fibroblasts. The red color indicates a well-polarized mitochondrial membrane, the green color corresponds to less polarized mitochondria membrane. Panels C and D: FDA/PI labeling of untreated (C) and 6 mM Cbz-Pro treated (D) fibroblasts. The red color, corresponding to nuclei, indicates cellular death after membrane damaging, the green color indicates living cell with a normal intracellular pH. Images shown are representative of a triplicate of independent experiments.

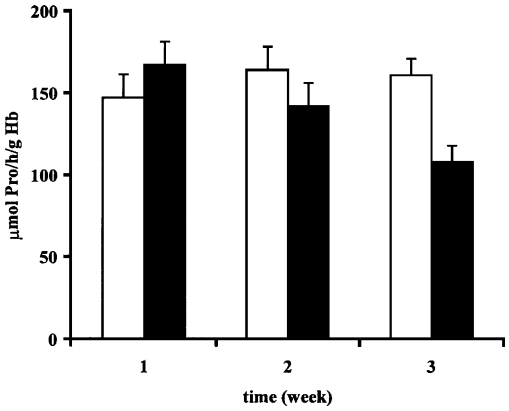


Fig. 6. Prolidase activity (µmol Pro/h/g Hb) in Cbz-Pro treated and untreated mouse erythrocytes as a function of injection time (week). Activity was determined by Chinard assay on erythrocytes from untreated (white) or treated (black) mice. Results are expressed as mean ± S.E.M. of $n = 9$ untreated and $n = 10$ treated mice.

competitive inhibitor of porcine kidney prolidase with $K_i$ = 90 µM. Transport studies performed on sheep and human erythrocytes showed a Cbz-Pro rapid protein-mediated permeation suggesting that it might be effective in vivo [10].

Considering the data available in literature, we tested the Cbz-Pro inhibitory effect on human fibroblast prolidase, a source directly related to the major outcome of PD patients that is chronic intractable lesions of skin, particularly located on the lower limbs.

The results reported in this study demonstrated for the first time that Cbz-Pro is a potent linear competitive inhibitor of human fibroblasts prolidase and resistant to degradation by other cellular enzyme.

We showed that Cbz-Pro permeates quickly the cell membrane of human fibroblasts and its uptake into the cells resulted saturable and influenced by extracellular pH, faster at pH = 6.0 than pH = 7.5. Cbz-Pro has a single ionizable group, the carboxylate on the pyrrolidine ring, which is hydroxylated at low pH, so under our experimental conditions, the inhibitor is an univalent anion. The fast entry of the molecule into human fibroblasts excluded a passive diffusion, but suggested an active or facilitated transport as major uptake pathway.

So we hypothesize that the presence of a $H^+$ gradient across the plasma membrane stimulates a possible transporter. Nakanishi et al. [19] excluded the existence of a dipeptide pH-dependent transporter in normal fibroblasts, however, King et al. [10] suggested the existence of a facilitate diffusion via a transport protein as a major cause of Cbz-Pro entry into erythrocytes [20]. Facilitate diffusion via a transport protein appears also responsible of Cbz-Pro uptake into human fibroblasts.

We have previously demonstrated that long-term cultured fibroblasts from PD patients showed the activation of necrosis-like cellular death [14].

Fibroblasts treated with 6 mM Cbz-Pro after 7 or 10 days of incubation showed some characteristics of fibroblasts obtained from PD patients as: mitochondria depolarization, increased cellular death and a round and branched shape (data not shown).

In cultured dermal fibroblasts incubated with the inhibitor, we found a strong reduction in the catalytic activity of the prolidase enzyme. A reduction in the prolidase activity is a common feature in PD patients [14,21].

We also demonstrated the efficiency of Cbz-Pro as in vivo inhibitor of erythrocytes prolidase by injecting it into control mice. Prolidase activity was reduced after few weeks of Cbz-Pro treatment and the inhibitor was not degraded but recovered in the urine samples of the treated mice.

On the basis of these evidences, we conclude that Cbz-Pro is an efficient human fibroblasts prolidase inhibitor in vitro and in vivo effective at low concentration, resistant to degradation by other enzyme and capable of permeating cell membranes. It might be useful in developing an experimental animal model of Prolidase Deficiency.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2005.03.008.

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