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Original Article

Biodistribution, pharmacokinetics and toxicity of a *Vasconcellea cundinamarcensis* proteinase fraction with pharmacological activity

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ABSTRACT

Prior studies demonstrate that a proteinase fraction from *Vasconcellea cundinamarcensis* V.M. Badillo, Caricaceae, exhibits wound healing activity in gastric and cutaneous models and antitumoral/antimetastatic effects. Here, we present the toxicity, pharmacokinetics and biodistribution data for this proteinase fraction following a single dose into Swiss mice by *i.v.*, *s.c.* or *p.o.* routes. The *i.v.* and *s.c.* toxicity assays demonstrate that proteinase fraction at ≤20 mg/kg is non-lethal after single injection, while parental administration (*p.o.*) of ≤300 mg/kg does not cause death. Based on *p.o.* acute toxicity dose using Organisation for Economic Cooperation and Development protocols, proteinase fraction ranks as Class IV "harmful" substance. Proteinase fraction shows high uptake determined as Kp (distribution tissue/blood) in organs linked to metabolism and excretion. Also, high bioavailability (≈100%) was observed by *s.c.* administration. The blood contents following *i.v.* dose fits into a pharmacokinetic bi-compartmental model, consisting of high removal constants – k_{el} 0.22 h^{-1} and k_d 2.32 h^{-1} and a half-life – $t_{½}$ = 3.13 h. The Ames test of proteinase fraction (0.01–1%) demonstrates absence of mutagenic activity. Likewise, genotoxic evaluation of proteinase fraction (5 or 10 mg/kg, *i.p.*) shows no influence in micronuclei frequency. In conclusion, the acute doses for proteinase fraction lack mutagenic and genotoxic activity, clearing the way for clinical assays.

Introduction

The study of plant cysteine proteinases attracts interest because of their multiple alleged activities. The pharmacological action described for these proteins include anthelmintic action against nematode infection (Gomes et al., 2011), anti-inflammatory activity (Gaspani et al., 2002; Brien et al., 2004) wound debridant (Ford et al., 2002; Ayello and Cuddigan, 2004; Melano et al., 2004) and to antagonize animal morbidity due to septic shock (Lima-Filho et al., 2010). The antitumoral/antimetastatic activity is perhaps one of the most attractive properties of plant cysteine proteinases, as this effect was demonstrated in enzymes from different sources (Beuth, 2008; Wald, 2008).

Cysteine proteinases from *Vasconcellea cundinamarcensis* V.M. Badillo latex have been characterized biochemically (Teixeira et al., 2008) and pharmacologically by our research group. The species is member of the Caricaceae family common to many areas in South America. Until now, studies using P1G10, a proteinase fraction obtained by gel filtration on Sephadex G-10, shows interesting pharmacological activities, such as; wound healing of dermabrasions (Lemos et al., 2011), burns (Gomes et al., 2010), gastric ulcer protective and healing effects by *p.o.* route (Mello et al., 2006, 2008; Araujo e Silva et al., 2015). Besides, mitogenic, angiogenic and anti-inflammatory effects are evident along with the healing effect. Subcutaneous administration of P1G10 exhibits antitumor/antimetastatic activity. Apoptosis, inhibition of angiogenesis and a loss of cell adhesion to extracellular components are associated to the antitumoral effect (Dittz et al., 2010, 2015). In addition, *s.c.* proteolitically active P1G10 acts as antithrombotic (Bilheiro et al., 2013) and this effect is accompanied by a decline in platelet activation, increase in prothrombin (PT), thrombin (TT) and activated partial thromboplastin times (APTT), along with fibrinogenolytic and fibrinolytic action.

Previous *in vivo* studies (Lemos et al., 2011) demonstrate that P1G10 at concentrations 10-fold higher than the effective dose (0.1%, w/w in Polawax®) does not induce apparent changes on intact skin. Also, continuous topical application of P1G10 0.1%

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during 6-month did not induce skin damage as demonstrated by microscopic examination. Also, chronically (6-month) treated specimens showed no changes in weight or histology of organs. The absence of systemic effect was anticipated considering the small amount of active principle in Polawax® that permeates intact or scarified skin (<0.3% and 26% relative to the total amount applied, respectively) (Lemos et al., 2011).

In this study, we synthesize ^{99m}Tc-P1G10 to evaluate the pharmacokinetics parameters following a single injection, and study the toxicological and mutagenic effects aiming studies at prospective therapeutic use of the formulated product.

Material and methods

Materials

Unripe fruits of *Vasconcellea cundinamarcensis* V.M. Badillo, Caricaceae, 2–4 year-old, were the source of latex used in this study. A voucher specimen of the plant was deposited at the herbarium of the Universidad de La Serena, Chile, with #15063. Benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) was from Sigma Co. Sephadex G-10 and G-15 were from GE Healthcare, ^{99m}Tc was produced in a ^{99}Mo/^{99m}Tc generator and provided by IPEN (São Paulo, Brazil) and each reagent used was analytical grade.

Animals

Swiss mice (male and female), approximately 8-weeks old, were obtained from CEBIO-ICB, UFMG animal facility and housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) with a 12-h light-dark cycle (light from 7 a.m. to 7 p.m.). Food and water were available ad libitum. The project was approved by the Ethics Committee on Animal Experimentation (CETEA) from UFMG (Process nº. 236/2008 and 91/2009).

Methods

Isolation and characterization of P1G10 fraction

Latex was collected by making three longitudinal incisions onto the surface of unripe fruits with the aid of a sharp steel blade. Following collection into a plastic dark container, it was stored at −20 °C until lyophilized. Dried latex was dissolved and incubated at room temperature in buffer containing 25 mM L-cysteine, 5 mM DTT, 10 mM EDTA pH 5.0 in 1 M sodium acetate solution. The suspension was centrifuged at 4000 × *g* 10 min at room temperature and the filtered supernatant (Whatman #1) applied onto a Sephadex G-10 (25 mm × 400 mm) previously equilibrated with 1 M sodium acetate pH 5.0. The first A_{280} nm protein absorbing fraction containing the bulk proteolytic activity, designated as P1G10 was concentrated by ultrafiltration (10,000 Da pore size) and washed three times with an equivalent volume of distilled water, lyophilized and stored at −20 °C until used. The recovered yellowish powder was analyzed for its proteolytic and amidase activities, electrophoretic mobility by SDS-PAGE and the HPLC retention time, as described by Mello et al. (2008).

Protein radiolabeling

P1G10 (1 mg) was labeled with 37 MBq $Na^{99m}TcO_4$, 3.7 mM $SnCl_2$ and 2.6 mM $NaBH_4$ in a total volume of 200 μl and incubated for 20 min at room temperature as described elsewhere by Nunan et al. (2002). The labeling efficiency was assessed by the method adapted from the United States Pharmacopeia (USP 24, 2000). An aliquot of the reaction mix (2 μl) was initially cromatographed on ascending Silica gel 60 chromatography (TLC Merck) followed by Whatman #1 descending paper chromatography to monitor labeling efficacy. During the first TLC step 5 μl of the reaction mix were spotted onto a silica plate and after sample drying the chromatogram was developed with acetone. The strip was scrapped off and 1 cm^2 fractions placed in vials and counted in an Automatic Scintilator (ANSR-Abbot, USA). Under this condition, the reduced technetium plus the radiolabeled protein remain at the application site while oxidized Tc ($^{99m}TcO_4^-$) migrated with the front of the solvent allowing assessment of the percentage of $^{99m}TcO_4$. For descending chromatography 5 μl of reaction mix were applied onto a Whatman paper strip (15 cm × 36 cm) previously saturated with 1% BSA solution. Saline solution (0.9%) was used to develop the chromatogram. Again, the strip of paper was cut into fractions and each fraction placed into vials and the radioactivity measured in an Automatic Scintilator, as before. Under these conditions $^{99m}TcO_4^-$ and ^{99m}Tc-P1G10 migrate with the solvent front, while $^{99m}TcO_2$ remain at the origin. The percent $^{99m}TcO_2$ is determined by the distribution of radioactivity on the paper strip. The labeling yield was calculated as follows:

$$\text{Labeling yield (\%)} = \frac{\text{cpm}^{99m}\text{Tc-P1G10}}{\text{Total cpm}(^{99m}\text{Tc} - \text{P1G10} + ^{99m}\text{TcO}_4 + ^{99m}\text{TcO}_2)}$$

The proportion of labeled protein relative to free $^{99m}TcO_2$ was established after running parallel Sephadex G-15 (4.0 cm × 1.5 cm) chromatographies of the labeled protein (P1G10) and a control containing only the tracer equilibrated with 0.9% (w/v) NaCl (Nunan et al., 2002). Radioactive fractions of P1G10 were subsequently pooled and used in experiments within the next 24 h following protein labeling.

Pharmacokinetics and tissue distribution of ^{99m}Tc-P1G10

Swiss male mice (25–30 g, 8–10 weeks, $n = 48$) received 1 mg/kg ^{99m}Tc-P1G10 (0.1 ml/30 g body weight) intravenously (*i.v.*), subcutaneously (*s.c.*) or orally (*p.o.*). The animals were sacrificed by exsanguination at different intervals (0.25, 0.5, 1, 2, 4, 8, 12 and 24 h), under anesthesia with a xilazine (9 mg/kg) ketamine (60 mg/kg) mixture. Blood and urine were collected and spleen, bladder, brain, heart, stomach, liver, small- and large intestine, skin, lung, kidney, adipose tissue and thyroid were removed and weighed. The radioactivity in organs and blood was measured with an automatic scintillation counter (ANSR-Abbot, USA). The results were expressed as percentage of the injected dose per g of tissue or ml of blood. The correction for the radioisotope decay was done by simultaneous counting at a specific interval of the residual tracer equivalent to the initially injected dose. The resulting values were plotted using *Graph Pad Prism 5* software for determination of radioactivity versus time and their respective areas under the curve (AUC) and the rate constants k_{el} and k_d by a nonlinear regression model. Using the elimination constant – k_{el} (slow rate slope), we calculated the half-life time ($t_{½}$) = 0.693/k_{el}. The values for bioavailability (F) during *p.o.* and *s.c.* treatments were calculated by the equation:

$$F = \frac{[\text{AUC}\ \ (s.c.\ \ \text{or}\ \ p.o.) \times \text{Dose}\ \ (i.v.) \times 100]}{[\text{AUC}\ \ (i.v.) \times \text{Dose}\ \ (s.c.\ \ \text{or}\ \ p.o.)]}$$

The partition coefficient of tissue versus blood (Kp) was determined by the ratio; AUC_{organ}/AUC_{blood} as before by Gibaldi and Perrier (1982).

Acute oral toxicity test

Swiss female mice were allowed to adapt for 7 days before treatment. After this period, P1G10 (5, 10, 20 or 300 mg/kg) or the vehicle (0.9% NaCl–saline) were administrated intravenously (*i.v.*), subcutaneously (*s.c.*) or by gavage (*p.o.*) at a single dose. Mice survival and clinical signs were scored (abdominal contraction, tremors,