Inhibition of Neurotoxic Secretory Phospholipases A₂ Enzymatic, Edematogenic, and Myotoxic Activities by Harpalycin 2, an Isoflavone Isolated from Harpalyce brasiliana Benth


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Secretory phospholipases A₂ (sPLA₂) exert proinflammatory actions through lipid mediators. These enzymes have been found to be elevated in many inflammatory disorders such as rheumatoid arthritis, sepsis, and atherosclerosis. The aim of this study was to evaluate the effect of harpalycin 2 (Har2), an isoflavone isolated from Harpalyce brasiliana Benth., in the enzymatic, edematogenic, and myotoxic activities of sPLA₂ from Bothrops pirajai, Crotalus durissus terrificus, Apis mellifera, and Naja naja venoms. Har2 inhibits all sPLA₂ tested. PrTX-III (B. pirajai venom) was inhibited at about 58.7%, Cdt F15 (C. d. terrificus venom) at 78.8%, Apis (from bee venom) at 87.7%, and Naja (N. naja venom) at 88.1%. Edema induced by exogenous sPLA₂ administration performed in mice paws showed significant inhibition by Har2 at the initial step. In addition, Har2 also inhibited the myotoxic activity of these sPLA₂s. In order to understand how Har2 interacts with these enzymes, docking calculations were made, indicating that the residues His48 and Asp49 in the active site of these enzymes interacted powerfully with Har2 through hydrogen bonds. These data pointed to a possible anti-inflammatory activity of Har2 through sPLA₂ inhibition.

1. Introduction

The flavonoids are a group of plant secondary metabolites widely distributed in nature. They are divided in two main classes: the flavonoids and the isoflavonoids. Many pharmacological activities have been described for both classes such as anti-inflammatory and antioxidant, antiallergic, antiviral, and anticancer [1, 2]. Many works have shown the phospholipase A₂ (PLA₂) inhibitory effects of flavonoids such as rutin [3], morin [4], and quercetin [5], however, only few works have been shown the antiphospholipasic A₂ activity of isoflavonoids [6].

The PLA₂s are a class of enzymes (EC 3.1.1.4) that catalyzes the hydrolysis of the sn-2 ester bond of phospholipids...
to produce lysophospholipids and free fatty acids, which are substrates for the synthesis of proinflammatory eicosanoids and platelet aggregating factor (PAF) [7, 8], in addition to reactive oxygen species produced during the synthesis of eicosanoids (which play a role as a positive feedback of the enzymatic active of the PLA₂) [9]. They are divided in two major groups: cytosolic phospholipase A₂ (cPLA₂) and secretory phospholipase A₂ (sPLA₂). The last are divided in ten groups as discussed elsewhere [10]. In many inflammatory diseases, as in sepsis, atherosclerosis, and rheumatoid arthritis, group IIA sPLA₂ are found in the inflammatory areas and play a role not fully understood up to date [11, 12].

The sPLA₂ present in animal venoms share structural features with mammalian (including human) group IIA sPLA₂, mainly in the active site, being for that reason useful tools for the study of sPLA₂ inhibitors [7–12]. The exogenous administration of these enzymes to experimental animals provokes an inflammatory response similar to that observed with administration of endogenous sPLA₂, besides more specific responses as myonecrosis [5, 13].

Current anti-inflammatory therapies include nonsteroidal anti-inflammatory drugs that inhibit either LOX or COX-1/2 enzymes and have serious side effects such as gastrointestinal ulceration, bleeding, and cardiovascular complications. In addition to these problems, COX-1/2 and LOX inhibitors cannot regulate the production of the PAF, which continues causing inflammation [7, 8]. Effective inhibitors of sPLA₂ could be capable of depleting the downstream proinflammatory metabolites of arachidonic acid as well as PAF, without the adverse effects of the current corticosteroids therapy since these enzymes are secreted only in pathological conditions [7–10].

Harpalyce brasiliana Benth. (Fabaceae) is a Brazilian folk medicine, popularly known as “raiz-de-cobra” (Port. Lit. snakeroot). Its roots have been used in the Northeast of Brazil to treat snakebite [14], while its leaves are claimed to be anti-inflammatory (personal unpublished data). In this paper, the effect of harpalycin 2 in structure and enzymatic, edematogenic, and myotoxic activities of four sPLA₂ isolated from animal venoms was evaluated. In addition, the analysis of the interaction between harpalycin 2 and the active site of the tested sPLA₂ was performed by docking calculations.

2. Material and Methods

2.1. Material. Secretory PLA₂ from Bothrops pirajai (PrTx-III) and Crotalus durissus terrificus (Cdt F15) were purified as described by Toyama et al. [15, 16], respectively. sPLA₂ from Apis mellifera venom was purchased from BIOMOL International. Bovine pancreas and Naja naja venom sPLA₂ were purchased from Sigma-Aldrich. The COX-1, COX-2, LOX 15hrc, and LOX 15syP1 came from Cayman Chemical. Other salts, reagents, solvents were ultrapure grade, HPLC grade, or sequencing grade purchase from the BIORAD, Sigma-Aldrich (Supelco) and Pharmacia.

2.2. Plant Material. Leaves of Harpalocyce brasiliiana Benth. were collected at the Chapada do Araripe, Barbalha (Ceará, Brazil) by Prof. E. R. Silveira. Botanical authentication was made by Prof. E. P. Nunes of the Department of Biology, Federal University of Ceará. Voucher specimen (number: 32525) has been deposited at the Prisco Bezerra Herbarium (EAC), Department de Biology, Federal University of Ceará, Fortaleza (Ceará, Brazil).

2.3. General Procedures. The mass spectra were obtained on a Hewlett-Packard 5971 mass spectrometer by electron impact ionization (70 eV). 1H and 13C NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz for 1H and 125 MHz for 13C); chemical shifts were expressed in scale and were referenced to residual DMSO (2.5 and 39.5 ppm). Silica Gel 60 (Merck, 70–230 mesh) was used for analytical TLC. Column chromatographies were performed over silica gel (Merck, 60 F254 230–400 mesh).

2.4. Extraction and Isolation of Harpalycin 2. Leaves of Harpalocyce brasiliiana were pulverized and extracted with EtOH at room temperature. The solvent was removed under reduced pressure which produced a dark viscous extract (HBFE). Liquid-liquid partition of a water suspension of HBFE (110 g) using petrol ether, CHCl₃, EtOAc, and n-BuOH yielded five fractions after solvent evaporation: HBFE (24.5 g), HBFE (22.4 g), HBFE (6.8 g), HBFE (30.4 g), and HBFE (21.2 g).

Flash chromatography of HBFE (12.0 g) using n-hexane and EtOAc as binary mixtures of increasing polarity afforded 30 fractions, which were pooled in 9 fractions after thin layer chromatography (TLC) analysis. HBFE (10–12) presented a yellow precipitate, yielding 120.0 mg after recrystallization. NMR and Mass-spectrometric analysis showed the structure of the isoflavone harpalycin 2 (Har2). The fractions HBFE (8–9) and HBFE (13–17) were purified, using the same method, yielding more 200.0 mg of Har2.

2.5. Inhibition of sPLA₂ Activity. sPLA₂ activity was measured following the protocols described by Hernandez-Oliveira et al. [17] and modified by Toyama et al. [18] for 96-well plate. The standard assay mixture contained 200 mL of buffer (10 mM Tris-HCl, 10 mM CaCl₂, 100 mM, and NaCl, pH 7.8), 20 µL of substrate (4-nitro-3-octanoyloxy-benzoic acid (4NO3BA) 1 mg/mL, manufactured by BIOMOL, USA), 20 µL of water, and 20 µL of sPLA₂ solution (1 mg/mL). Enzymatic activity was calculated based on the increase in absorbance at 425 nm after 20 min, at 37°C, as a direct result of the cleavage of the synthetic substrate. All assays were done using n = 12 and absorbance was measured using a SpectraMax 340 multwell plate reader (Molecular Devices, Sunnyvale, CA). Evaluation of Har2 effect on sPLA₂ enzymatic activity was performed after incubation of Bothrops pirajai (PrTx-III), Crotalus durissus terrificus (Cdt F15), Apis mellifera (purified, without the mellitin component), and Naja naja sPLA₂ with Har2 at equal mass (1 : 1; w: w) for a period of 30 minutes. The final concentration of the inhibitor in the reaction mixture was the same of the substrate due to the kinetic behavior of the sPLA₂.
2.6. Animals. Male Swiss mice (20–25 g) obtained from the Animal Facilities of Federal University of Ceará were used in this study. The animals were maintained under standard conditions (22 ± 2°C; 12 h light/dark cycle) with food and water ad libitum. All experiments with animals were guided in accordance with Brazilian laws for Care and Use of Laboratory Animals and all the study protocols were approved by Committee of Ethics from Federal University of Ceará (Fortaleza, Brazil) protocol number 68/08.

2.7. Neutralization of the Edema Inducing Activity. Neutralization of sPLA2-induced paw edema by Har2 was performed according to Iglesias et al. [4], using male Swiss mice (20–25 g, n = 6). The edema was induced by a single subplantar injection of 25 µL of sPLA2 (25 µg/paw). Paw volume was measured immediately before the injection of the samples and at selected time intervals thereafter (30, 60, 120, 240, and 480 minutes) using a plethysmometer (Ugo Basile, Italy). All samples were dissolved in sterile PBS. Results were expressed as the increase in paw volume (µL) and calculated by subtracting the basal volume. Evaluation of Har2 effect on sPLA2 edema-inducing activity was carried out after incubation of Bothrops pirajai (PrTx-III), Crotalus durissis terrificus (Cdt F15), Apis mellifera (purified, without the mellitin component), and Naja naja sPLA2 with Har2 at equal mass (1:1; w:w) for 30 minutes at 37°C. The negative controls were performed by administration of Har2 (25 µg/paw). These values were subtracted from the volume of the paws treated with sPLA2 incubated with Har2 for clarity reasons.

2.8. Neutralization of Myotoxic Activity. Plasma creatine kinase (CK) activity was measured using a CK-UV kinetic kit (Sigma Chemical Co.). Native sPLA2 (1 mg/mL) or those previously incubated with Har2, as described above, were injected intramuscularly (25 µL) in the gastrocnemius of male Swiss mice (20–25 g, n = 6). The control group was injected with sterile PBS, and the negative control with Har2. After 3 hours, a blood sample was collected from the tails using heparinized capillary tubes and centrifuged for plasma separation. CK activity was determined in triplicate using 4 µL of plasma according to the manufacturer’s instructions, and its activity was expressed in U/L.

2.9. Characterization of IC50 of Har2 against Several sPLA2 and Inflammatory Enzymes. The inhibitory capability of Har2 against COX-1/2, LOX 15hrc, and 15sryP1, bovine pancreas, and human group V sPLA2 was investigated according to the manufacturer’s instructions (Cayman Chemical). All assays were carried out using n = 12 and the data measured using a SpectraMax 340 multwell plate reader (Molecular Devices, Sunnyvale, CA). Har2 was added in different concentrations and IC50 values were calculated using GraphPad Prism 5.0.

2.10. Circular Dichroism Spectroscopy. Native sPLA2, and Har2-treated sPLA2 were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and final protein concentrations were adjusted to 8.7 mM. After centrifugation at 4000 g for 5 min, samples of 20 µL were injected into molecular exclusion column TSK G4000SWXL (0.7 × 300 mm) coupled in the LC-2000Plus Series HPLC Systems (Jasco, USA), which have been previously equilibrated with same buffer used for the preparation of the samples sPLA2, Har2-treated sPLA2. In this case, the chromatographic run of each samples was simultaneously monitored using a CD-2095 Circular Dichroism HPLC detector (Jasco, USA), FP-2020 Fluorosence detector, and UV-2075 190 to 600 nm detector. Circular dichroism spectra were obtained by adjust the wavelength range 220–260 nm and 260–320 nm to measure the presence of random coil and tertiary protein folding, respectively. Data collection was performed with a bandwidth of 1 nm, response time of 1 s at room temperature with 100 nm/min scanning speed. The fluorescence detection was adjusted specifically for monitoring the fluorescence emission of tryptophan, which was measured between 300 and 450 nm after excitation at 280 nm.

2.11. Molecular Modeling (Docking). The structural optimization of the harpylactic 2 ligand was initially achieved using the AM1 method [19] implemented in the BioMedCache program (BioMedCache, 1989) with default values for the convergence criteria. Docking calculations were performed with the GOLD 4.0 program [20] in order to obtain the relative in silico affinities of the Har2 ligand with respect to the sPLA2 targets. The sPLA2 structures were taken from the RCSB Protein Data Bank (http://www.pdb.org/), under the PDB ID: 1GMZ, ZQOG, 1PSH, and 1POC, respectively, for PrTx-III, Cdt F15, Naja, and Apis.

The docking calculations were performed taking advantage of the flexibility of the Har2 ligand, by activating its rotational degrees of freedom. The active site was defined as all atoms within a radius of 10.0 Å from the residue 48 (His or Asp), which is an important residue according to the literature [21, 22].

2.12. Statistical Analysis. Results were expressed as the mean ± SEM of replicated experiments. The significance of differences between means was assessed by an analysis of variance, followed by a Dunnett’s test where several experimental groups were compared with the control group. The confidence limit for significance was set at P < 0.05.

3. Results and Discussion

Natural products from plants are of potential interest for the treatment of a number of inflammatory diseases. They serve as template molecules for the development of new drugs and prototypes [12]. Flavonoids are polyphenolic compounds widely distributed in plants. Due to their various effects on immune and inflammatory systems, these compounds are currently of great pharmacological interest [3]. There are several reports demonstrating that flavonoids are able to inhibit PLA2 activity, arachidonic acid release, and the formation of arachidonic acid metabolites [3, 4, 13]. In particular, isoflavones have also been reported to show...
anti-inflammatory activities, including inhibition of phospholipases A$_2$ and COX-1/2 [17, 18]. Harpalycin 2 (Har2) was isolated as a white amorphous solid with m.p. 232.6–234.4°C. Its molecular formula of C$_{21}$H$_{18}$O$_7$ was established by the molecular ion at m/z 382 Daltons in the MS spectrum. Structure elucidation was performed by spectroscopic means, including 1D and 2D NMR, and comparison with the data from literature [14]. The structure of Har2 is shown in Figure 1.

Group IIA secretory phospholipases A$_2$ may be catalytically active or inactive depending on the amino acid residue 49. In catalytically active isoforms, this residue is occupied by an aspartic acid [24]. Cotrim et al. [5] showed through docking calculations that the chemical treatment of crototoxin B (Cdt F15) with quercetin lead to an inhibition of enzymatic activity due to the fact that quercetin binds in the vicinity of His48 and Asp49 residues. Herein, Har2 inhibits all sPLA$_2$ tested when the treatment was made before the substrate addiction, with percentages of inhibition at about 58.7% for PrTX-III, at 78.8% for Cdt F15, at 87.7% for Apis, and at 88.1% for Naja secretory phospholipase A$_2$ (Figure 2). These percentages of inhibition were greater than p-bromophenacyl bromide (40%), a well-known sPLA$_2$ inhibitor [5].

Neutralization of edema induced by exogenous sPLA$_2$ administration performed in mice paws by harpalycin 2 showed significant inhibition of the edema initial step induced by PrTX-III, Cdt F15, Apis, and Naja (Figure 3). This first step is correlated with histamine/serotonin involvement [24]. The edema-inducing effect of sPLA$_2$s could be attributed to their ability to hydrolyse phospholipids; however Lys49 phospholipase A$_2$-homologues can also induce edema in the absence of PLA$_2$ activity, which implies a different mechanism of action for this pharmacological effect [24]. In this case, all sPLA$_2$ tested were catalytic active and the partial inhibition of edema formation may be correlated to the inhibition of phospholipids catalysis by the isoflavone.

Harpalycin 2 was able to inhibit the myotoxic activity of the venom secretory phospholipases A$_2$ tested in this study (Figure 4), which is a pharmacological activity shared by several types of snake venom sPLA$_2$ [25]. This model, when studied using Asp49 sPLA$_2$, is interesting for evaluation of the interfacial activation of the sPLA$_2$s, since myotoxicity is directed linked with enzymatic activity in the catalytic sPLA$_2$ isoforms [7]. Recently, involvement of potassium, ATP, calcium, and purinergic receptors in the myotoxic activity of snake venom sPLA$_2$ was described by Cintra-Francischinelli et al. [23]. The authors emphasized the role of the purinergic receptor P2X and its inhibitors in the extent of muscle tissue damage, giving an additional explanation to the finding that the antitrypanosomal drug suramin provides protection from the toxic effect of the Lys49 myotoxins of the Bothrops jararacussu and Bothrops asper venoms. Suramin also binds to P2X channels, and this property could, at least in part, account for its myotoxic inhibitory activity. Several flavonoids and isoflavones antagonize purinergic receptors [26] which could explain in part the inhibitory effect of these compounds in the myotoxic activity of snake venom sPLA$_2$.

In order to elucidate the anti-inflammatory activity of harpalycin 2, we further examined the ability of Har2 to inhibit the enzymatic activity of COX-1/2 and LOX 15hrc and 15syP1 as well as bovine pancreas PLA$_2$ and human group V PLA$_2$. Har2 showed lower IC$_{50}$ values for both bovine and human sPLA$_2$ than for COX-1/2 and LOX enzymes, as shown in Table 1. These results showed that harpalycin 2 has probably more affinity for sPLA$_2$ than for other proinflammatory enzymes.

Aiming to understand how harpalycin 2 interacts with these sPLA$_2$s, CD spectroscopy, fluorescence analysis, and docking calculations were performed. Fluorescence profile analysis of Har2, sPLA$_2$s, and Har2:sPLA$_2$s showed significant changes in the spectral fluorescence profile among Har2 and the sPLA$_2$ treated with Har2. CD analysis of native and Har2-treated sPLA$_2$ revealed that the treatment induced discrete unfolding of sPLA$_2$, which did not modify the tridimensional structure of the proteins (See supplementary material, available at doi: 10.1155/2012/987517).

The sPLA$_2$ structures were represented by the PDB IDs 1GMZ, 2QOG, 1PSH, and 1POC, respectively, for PrTX-III, Cdt F15, Naja, and Apis. The calculated docking score values
Figure 3: Effect of harpalycin 2 (Har2) on edema formation after a single subplantar injection of sPLA₂ (25 µg/paw) from (a) Bothrops pirajai (PrTX-III), (b) Crotalus durissus terrificus (Cdt F15), (c) Apis mellifera (Apis), and (d) Naja naja (Naja) venom expressed as the increase in paw volume (µL). Native sPLA₂ edematogenic activity is represented by circles whereas harpalycin 2 treated sPLA₂ edematogenic activity is showed as squares. Data expressed as mean ± S.E.M. and analyzed by ANOVA followed by Dunnett’s test, with *P ≤ 0.05.

Table 1: Inhibition of enzymatic activity of proinflammatory enzymes by harpalycin-2.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrTX-III</td>
<td>11.34</td>
</tr>
<tr>
<td>BPPLA₂</td>
<td>11.90</td>
</tr>
<tr>
<td>HGVPLA₂</td>
<td>27.42</td>
</tr>
<tr>
<td>COX-1</td>
<td>131.90</td>
</tr>
<tr>
<td>COX-2</td>
<td>32.73</td>
</tr>
<tr>
<td>LOX 15HRC</td>
<td>55.96</td>
</tr>
<tr>
<td>LOX 15SY</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Note: PrTX-III (piratxin-III); BPPLA₂ (bovine pancreas PLA₂); HGVPLA₂ (human group V PLA₂); COX-1 (cyclooxygenase-1); COX-2 (cyclooxygenase-2); LOX 15HRC (Lipoxygenase 15hrc); LOX 15SY (Lipoxygenase 15sy). N/D (Not determined).

(GOLD scores) for these targets were 33.93, 44.12, 55.26, and 51.23, respectively, for PrTX-III, Cdt F15, Naja, and Apis. One can see in Figure 5 that an interesting trend between the in silico (docking scores) and in vitro (percentages of inhibition) results was observed. This means that greater stability (the most positive docking score values) of the complex between the harpalycin 2 and the four sPLA₂ enzymes is related to greater inhibition percentage of the enzymatic activities. Thus, harpalycin 2 has inhibitory capacity against the enzymatic, edematogenic, and myotoxic activities from neurotoxic venom secretory phospholipases A₂, probably due to the interaction with residues 48 and 49 in the active site of these toxins. Comparing the amino acid sequences alignment performed by Clustal X [27] among the four sPLA₂, we found that the Apis has a more specific sequence (with an identity of 24% as compared with PrTX-III) while
Figure 4: Inhibition of myotoxic activity of sPLA₂ (25 µg/mice) from Bothrops pirajai (PrTX-III), Crotalus durissus terrificus (Cdt F15), Apis mellifera (Apis) and Naja naja (Naja) venom expressed as creatine kinase release on plasma. (a) Native sPLA₂ myotoxicity and (b) harpalycin 2 (Har2) treated sPLA₂ myotoxicity. Data expressed as mean ± S.E.M. and analyzed by ANOVA followed by Dunnett’s test, with *P set at 0.05.

Figure 5: Trend between docking scores performed by GOLD 4.0 (in silico) and percentages of inhibition of the enzymatic activity (in vitro). The values near the points represent the percentage of inhibition for each PLA₂, with the respective PDB ID below. The PDB ID: 1GMZ, 2QOG, 1PSH, and 1POC were used, respectively, for PrTX-III, Cdt F15, Naja, and Apis.

the other three sequences are similar to each other (identity values of 44% for Naja and 57% for Cdt F15, when compared to PrTX-III), Figure 6(a). Considering the secondary and tertiary structures, the difference between the Apis and the others is more relevant. Therefore, we performed a structural alignment only for PrTX-III, Cdt F15, and Naja, using PyMOL [28], as shown in Figure 6(b). The great similarity among these three PLA₂ is probably related to their evolutionary origin from snake venoms, where PrTX-III and Cdt F15 are classified as GIIA, while Naja is classified as GIA). Using the 1PSH (Naja) target as example, because it represents the best result, a detailed inspection for the molecular reasons of the good inhibition behavior of the harpalycin 2 in this target can be found in Figure 7. The most important residues in the active site, including HIS48 and ASP49, are labeled and are involved in polar interactions with harpalycin 2.

This data corroborates both ethnopharmacological uses of this plant by Brazilian northeast population: the treatment of snake bites, as sPLA₂ s are the main toxins in these venoms and, as anti-inflammatory, due to the main role of sPLA₂ in the inflammatory cascade of events. The data also pointed to a possible anti-inflammatory activity of this isoflavone mainly in disorders which involve sPLA₂, such as asthma and rheumatoid arthritis.

**Abbreviations**

Cdt F15: Crotoxin B from Crotalus durissus terrificus
COX-1/2: Cyclooxygenase-1/2
Figure 6: (a) Multiple sequence alignment of PrTX-III, Cdt F15, Naja and Apis, with the PDB ID: 1GMZ, 2QOG, 1PSH and 1POC, respectively. ClustalX was used with default setup. (b) Structure alignment of the PLA2 (cartoon model) with their respective docking solutions for harpalycin 2 (stick model): PrTX-III (green), Cdt F15 (red), and Naja (blue). Figure generated using PyMOL [28].

Figure 7: Panoramic (a) and detailed (b) view of the best docking solution obtained with the 1PSH (Naja) target. Important residues, directly involved in intermolecular interactions with the inhibitor (harpalycin 2), are labeled. The Ca$^{2+}$ atom of the catalytic center was also represented in the figure.

CK: Creatine kinase

gVPLA2: Group V phospholipase A2

Har2: Harpalycin 2

LOX: Lipoygenase

PAF: Platelet activation factor

PLA2: Phospholipase A2

PrTX-III: Piratoxin-III

sPLA2: Secretory phospholipase A2.
Authors’ Contribution

R. M. Ximenes and M. M. Rabello have contributed equally to this work.

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