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Research paper

A novel synthetic quinolinone inhibitor presents proteolytic and hemorrhagic inhibitory activities against snake venom metalloproteases

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ABSTRACT

Metalloproteases play a fundamental role in snake venom envenomation inducing hemorrhagic, fibrige-n(ogen)olytic and myotoxic effects in their victims. Several snake venoms, such as those from the *Bothrops* genus, present important local effects which are not efficiently neutralized by conventional serum therapy. Consequently, these accidents may result in permanent sequelae and disability, creating economic and social problems, especially in developing countries, leading the attention of the World Health Organization that considered ophidic envenomations a neglected tropical disease. Aiming to produce an efficient inhibitor against bothropic venoms, we synthesized different molecules classified as quinolinones – a group of low-toxic chemical compounds widely used as antibacterial and antimycobacterial drugs – and tested their inhibitory properties against hemorrhage caused by bothropic venoms. The results from this initial screening indicated the molecule 2-hydroxymethyl-6-methoxy-1,4-dihydro-4-quinolinone (Q8) was the most effective antihemorrhagic compound among all of the assayed synthetic quinolinones. Other in vitro and *in vivo* experiments showed this novel compound was able to inhibit significantly the hemorrhagic and/or proteolytic activities of bothropic crude venoms and isolated snake venom metalloproteases (SVMPs) even at lower concentrations. Docking and molecular dynamic simulations were also performed to get insights into the structural basis of Q8 inhibitory mechanism against proteolytic and hemorrhagic SVMPs. These structural studies demonstrated that Q8 may form a stable complex with SVMPs, impairing the access of substrates to the active sites of these toxins. Therefore, both experimental and structural data indicate that Q8 compound is an interesting candidate for antiophidic therapy, particularly for the treatment of the hemorrhagic and necrotic effects induced by bothropic venoms.

1. Introduction

Snake venoms are complex mixtures of several proteins including phospholipases A_2, phospholipases A_2-like, metalloproteases, serineproteases, L-amino oxidases, disintegrins, C- type lectins and others. Their combination are able to unleash a complex series of events responsible for the pathophysiology of snake envenomation and the consequent emergence of deleterious

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pharmacological/biological effects like neurotoxicity, myotoxicity, edema-inducing activity, hemorrhage, coagulation and several other properties [1–5].

Recent proteomic analyses indicated that snake venom metalloproteases (SVMPs), which are composed by a group of zinc-dependent enzymes, are the most representative toxins present in viperid species [1–9] and also are found in Colubridae [10–12]; Elapidae [13–16] and Atractaspididae [17] families. According to the revised classification of SVMPs by Fox and Serrano [18,19], these molecules are distributed in three different classes, depending on their molecular weight and domain structure. i) small SVMPs (20–30 kDa) with only a single catalytic domain in the P-I class; ii) medium SVMPs (30–60 kDa) composed by catalytic and disintegrin-like domains in the P-II class; and iii) large SVMPs (60–100 kDa) with catalytic, disintegrin-like and cysteine-rich domains in the P-III class (the large SVMPs with an extra lectin-like domain are also included in the P-III class).

Initially, the main role attributed to SVMPs was related to the remarkable hemorrhagic activity presented by many of these molecules (particularly the large SVMPs). This biological property is clearly linked to their ability to interact with specific receptors on endothelial cells [20,21] and fibroblasts [22] and is also involved in the degradation of extracellular matrix components [23]. However, a wide spectrum of other SVMP functions have been described in the last years, comprising very distinct capabilities as fibrin(ogen) olytic activity, inactivation of blood serine proteinase inhibitors, prothrombin and blood coagulation factor X activation, platelet aggregation inhibition, apoptosis induction, and pro-inflammatory action [24]. All of these activities presented by SVMPs contribute to the systemic actions observed after envenomation, but the influence of these toxins on the local effects is especially critical, since they contribute strongly to severe tissue necrosis resulting from their hemorrhagic and proteolytic activities [25]. Hence, as serum therapy is not effective enough to avoid the occurrence of local effects caused by viperid venoms, it is quite interesting to search for new molecules which could be also used in anti-snake venom treatments.

In many countries, including Brazil, plant extracts are traditionally used for the treatment of snakebite envenomations, although there is scientific validation only in a few cases [26–30]. On the other hand, several isolated plant alkaloids have been described as in vitro and *in vivo* anti-snake venom inhibitors [30–35]. Thus, we synthesized different quinolinone molecules – low-toxic chemical compounds [36] which are already widely used as antibacterial and antimycobacterial drugs [37,38] – and tested their inhibitory properties against hemorrhage caused by *Bothrops jararacussu*, *Bothrops moojeni* and *Bothrops alternatus* venoms and the isolated P-III class SVMP BjussuMP-I from *B. jararacussu*. The results obtained from this initial screening pointed out the most effective antihemorrhagic molecule was the compound 2-hydroxymethyl-6-methoxy-1,4-dihydro-4-quinolinone (identified herein as Q8). Subsequently, other in vitro and *in vivo* experiments showed the novel Q8 compound was able to significantly inhibit, even at lower concentrations, the hemorrhagic and/or proteolytic activities of four bothropic crude venoms (*B. jararacussu*, *Bothrops neuwiedi*, *B. moojeni* and *B. alternatus*) and two isolated SVMPs (P-III class BjussuMP-I from *B. jararacussu* and P-I class neuwiedase from *B. neuwiedi*).

Additionally, docking and molecular dynamics simulations involving BjussuMP-II, a P-I SVMP from *B. jararacussu* venom [39] with a very well characterized proteolytic activity, and Q8 compound were performed to get some insights into the inhibitory activity of this novel 4-quinolinone molecule against the action of SVMPs. These theoretical studies are justified since proteolysis is a common feature of SVMPs related to local tissue damage and hemorrhage. Therefore, the structural basis related to the harmful activities of the SVMP catalytic domains is a important topic for the identification of potential compounds for snake envenomation treatment.

2. Materials and methods

2.1. Materials and chemicals

All commercially available reagents were purchased from Aldrich Chemical Co. Reagents® and solvents were purified when necessary according to the usual procedures described in the literature. *B. jararacussu*, *B. neuwiedi*, *B. moojeni* and *B. alternatus* venoms were purchased from Bioagents Serpentarium Ltda. (Batatais, São Paulo State, Brazil). The SVMPs (P-III class BjussuMP-I, P-I class BjussuMP-II and P-I class neuwiedase) were isolated and biochemically characterized as previously described [39–41]. The licenses for scientific purposes are from *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA* and *Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio*. Numbers: 11094-2, 11094-1, 10394-1 and 15484-1.

The infrared spectra were measured on a Bomem M102® spectrometer (4000 - 400 cm^{-1}). NMR data assignment was based on ^{1}H and ^{13}C and the spectra were recorded on a Bruker DRX-200® and ARX-400®. Mass spectral analyses were carried out with a Shimadzu GCMS-QP5000® spectrophotometer. Analytical thin-layer chromatography was performed on a 0.25 mm film of silica gel containing a fluorescent indicator UV_{254} supported on aluminum sheet (Sigma–Aldrich®). Flash column chromatography was executed using a silica gel (Kieselgel 60, 230–400 mesh, E. Merck®). Gas chromatography was performed in a Shimadzu GC-17A® chromatograph equipped with a DB-5 column, employing H_2 as a carrier. Elemental analyses and the melting points were performed, respectively, with Fisons EA1108 CHNS-O® analyzer and FISATOM 430® equipment. Melting points were determined using a Microquimica MQAPF-301® device.

2.2. Compounds and procedures employed for 2-hydroxymethyl-6-methoxy-1,4-dihydro-4-quinolinone (Q8) synthesis

2.2.1. General procedure for the synthesis of enamines

In a flask under N_2 at 55 °C containing aniline (5.0 g, 0.054 mol) or *p*-anisidine (5.0 g, 0.041 mol) in dry methanol (1 mL/mmol aniline), DMAD (1 eq.) was added and the mixture was stirred overnight. Then methanol was removed under reduced pressure, and CH_2Cl_2 (30 mL) was added followed by a saturated solution of NH_4Cl (3 × 5 mL). The organic layer was dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was purified on a silica gel column, using hexane:ethyl acetate 9.5:0.5 as eluent to afford desired enamine compounds 3 or 4 in 50% yield.

*2.2.1.1. 2-Phenylamino-2-enediocic acid dimethyl ester (compound **3**).* 6.3 g. IR (ν_{max}, film, cm^{-1}): 3457; 3380; 2953; 1739; 1668; 1282; 1031. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.67 (sl, 1H); 7.32–7.25 (m, 2H); 7.13–7.05 (m, 1H); 6.9 (m, 2H); 5.39 (s, 1H); 3.74 (s; 3H); 3.70 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 169.8; 164.7; 147.9; 140.2; 129.0; 124.1; 120.6; 116.6; 93.5; 52.6; 51.1. MS (% rel. intensity) *m/z*: 235.25 (6.9%); 144.15 (93.3%); 77.10 (100%).

*2.2.1.2. 2-(4-Methoxy-phenylamino)-2-enediocic acid dimethyl ester (compound **4**).* 5.5 g. IR (ν_{max}, film, cm^{-1}): 3284; 3210; 2952; 2836; 1742; 1637; 1033. ^{1}H NMR (200 MHz, $CDCl_3$): δ. 9.57 (sl, 1H); 6.91–679 (m, 4H); 5.30 (s, 1H); 3.78 (s, 3H); 3.73 (s, 3H); 3.67 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 182.4; 170.0; 164.8; 156.9; 149.0;