Toxicon 93 (2015) 68–78

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Inhibitory potential of three zinc chelating agents against the proteolytic, hemorrhagic, and myotoxic activities of *Echis carinatus* venom

Ankanahalli N Nanjaraj Urs [a], Manjunath Yariswamy [a], Chandrasekaran Ramakrishnan [c], Vikram Joshi [a], Kanve Nagaraj Suvilesh [a], Mysore Natarajan Savitha [a], Devadasan Velmurugan [b, c], Bannikuppe Sannanayak Vishwanath [a, *]

[a] Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore, Karnataka, India
[b] Bioinformatics Infrastructure Facility, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India
[c] Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form
25 October 2014
Accepted 4 November 2014
Available online 8 November 2014

Keywords:
Complementary therapy
Hemorrhage
Echis carinatus venom metalloproteases
Local tissue damage
Myotoxicity
Zinc chelating agents

ABSTRACT

Viperbites undeniably cause local manifestations such as hemorrhage and myotoxicity involving substantial degradation of extracellular matrix (ECM) at the site of envenomation and lead to progressive tissue damage and necrosis. The principle toxin responsible is attributed to snake venom metalloproteases (SVMPs). Treatment of such progressive tissue damage induced by SVMPs has become a challenging task for researchers and medical practitioners who are in quest of SVMPs inhibitors. In this study, we have evaluated the inhibitory potential of three specific zinc (Zn^{2+}) chelating agents; N,N,N′,N′-tetrakis (2-pyridylmethyl) ethane-1,2-diamine (TPEN), diethylene triamine pentaacetic acid (DTPA), tetraethyl thiuram disulfide (TTD) on *Echis carinatus* venom (ECV) induced hemorrhage and myotoxicity. Amongst them, TPEN has high affinity for Zn^{2+} and revealed potent inhibition of ECV metalloproteases (ECVMPs) *in vitro* (IC_{50}: 6.7 μM) compared to DTPA and TTD. The specificity of TPEN towards Zn^{2+} was confirmed by spectral and docking studies. Further, TPEN, DTPA, and TTD completely blocked the hemorrhagic and myotoxic activities of ECV in a dose dependent manner upon co-injection; whereas, only TPEN successfully neutralized hemorrhage and myotoxicity following independent injection. Histological examinations revealed that TPEN effectively prevents degradation of dermis and basement membrane surrounding the blood vessels in mouse skin sections. TPEN also prevents muscle necrosis and accumulation of inflammatory cells at the site of ECV injections. In conclusion, a high degree of structural and functional homology between mammalian MMPs and SVMPs suggests that specific Zn^{2+} chelators currently in clinical practice could be potent first aid therapeutic agents in snakebite management, particularly for local tissue damage.

1. Introduction

Chelation therapy is an important strategy employed to eliminate toxic heavy metals from the body. In addition, chelating agents are vital in restoring the physiological levels of metalloenzymes, particularly, the matrix metalloproteases (MMPs), as their dysregulated activity reflects in debilitating conditions such as cancer and arthritis (Gong et al., 2014; Shian et al., 2003). Similarly, upon envenomation, entry of snake venom metalloproteases (SVMPs) into victims causes dire consequences of local tissue damage such as hemorrhage, myonecrosis and in severe cases gangrene. Although anti-snake venoms are effective in neutralizing the snake venom induced systemic toxicity, they fail to neutralize local tissue damages often leading to permanent debilitated condition (Escalante et al., 2011; Markland and Swenson, 2013). In view of overcoming these persistent debilitating conditions, pharmacologically approved chelating agents such as ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA) and 1,

Abbreviations: DTPA, diethylene triamine pentaacetic acid; ECVMPs, *Echis carinatus* venom metalloproteases; MMPs, matrix metalloproteases; PPP, platelet poor plasma; TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethane-1,2-diamine; TTD, tetraethyl thiuram disulfide.

* Corresponding author. Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore, Karnataka 570 006, India.
E-mail address: vishmy@yahoo.co.uk (B.S. Vishwanath).

http://dx.doi.org/10.1016/j.toxicon.2014.11.224
0041-0101/

10-phenanthroline have been extensively studied for inhibition of SVMPs in addition to their role in regulating endogenous MMPs (Gowda et al., 2011; Howes et al., 2007; Kumar et al., 2010; Thompson et al., 2012). EDTA, EGTA and 1, 10 phenanthroline are non-specific divalent metal ion chelators and are very effective in neutralizing the activity of SVMPs *in vitro*. However, their non-specific binding with physiologically vital divalent metal ions, particularly calcium, poses an obstacle for their pharmacological use (Pereanez et al., 2013).

SVMPs being metzincin family proteases, possess a catalytically functional zinc (Zn^{2+}) at the active site bound to conserved Zn^{2+}-binding motif (Takeda et al., 2012). Chelating this Zn^{2+} metal ion by specific Zn^{2+} chelators rather than non-specific divalent metal ion chelators is more effective in the management of local toxicity as they can serve as adjunctive therapeutic molecules to aid antivenom therapy by limiting local tissue damage. In these lines, a high affinity membrane permeable Zn^{2+} chelator – TPEN (N,N,N′,N′-tetrakis (2-pyridylmethyl) ethane-1,2-diamine); an extracellular Zn^{2+} chelating agent – DTPA (Diethylene triamine pentaacetic acid) and an intracellular Zn^{2+} chelating agent – TTD (Tetraethyl thiuram disulfide) were selected for the study. Present study aims at evaluating the protective effects of afore said Zn^{2+} chelating agents against *Echis carinatus* venom (ECV) induced local tissue damage. Further, the specificity offered by these agents is an added advantage and will be of potential therapeutic value in management of viper snakebites.

2. Materials and methods

2.1. Venom

Lyophilized powder of *E. carinatus* venom (ECV) was purchased from Irula Snake-Catchers Co-operative Society Ltd., (Chennai, India). Required amount of venom was re-dissolved in saline and centrifuged at 6000 *g* for 10 min to remove debris. Protein content of crude venom was determined according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Aliquots were kept at −4 °C until further use.

2.2. Chemicals

N,N,N′,N′-tetrakis (2-pyridylmethyl) ethane-1,2-diamine (TPEN), diethylene triamine pentaacetic acid (DTPA), tetraethyl thiuram disulfide (TTD), gelatin, bovine fibrinogen, papain and bromelain were purchased from Sigma Aldrich (Saint Louis, USA). Fibri-prest® (thrombin reagent) was purchased from Diagnostica Stago Inc. (Paris, France). Casein, BSA, ethanol (HPLC grade), water (HPLC grade), dimethyl sulfoxide (DMSO), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), phenyl methyl sulphonyl fluoride (PMSF), and iodo acetic acid (IAA) were purchased from Sisco Research Laboratories (Mumbai, India). Polyvalent equine anti-snake venom serum (ASV) specific towards venom of *Naja naja, Bungarus caeruleus, Daoia russelii* and *E. carinatus* snakes of India (batch number: A5310035; expiry date: 09/14) was kind gift from Bharat Serums and Vaccines Ltd., (Mumbai, India). All the other chemicals and reagents used in this study were of analytical grade.

2.3. Animals

Swiss Albino mice (either gender; 25–30 g) were obtained from Central Animal House Facility, University of Mysore (UOM), Mysore, India. Animal care and handling were in compliance with National Regulations for Animal Research and the experiments were performed according to the protocols reviewed by the Institutional Animal Ethical Committee, UOM, Mysore (No: UOM/IAEC/25/2011).

2.4. Human plasma

Blood was drawn from healthy human volunteers (20–30 years; with consent), mixed with 3.2% trisodium citrate (9:1 v/v) and centrifuged at 250 *g* for 15 min. The plasma obtained was pooled and used as platelet poor plasma (PPP) for coagulation and fibrinolytic assays. The experiments conducted were in compliance with the protocols approved by the Institutional Human Ethical Committee, UOM, Mysore (IHEC-UOM No.62/Ph.D/2011-12).

2.5. Proteolytic activity

Proteolytic activities of ECV, trypsin, and bromelain were assayed according to the method of Murata et al. (1963) using casein as a substrate. For inhibition studies, similar reactions were performed after pre-incubating 25 μg of venom with various concentrations of Zn^{2+} chelating agents (TPEN, DTPA and TTD) for 10 min at 37 °C. Similarly, inhibition studies of trypsin and bromelain were performed after pre-incubating 500 μg of respective proteins with 1 mM chelating agents for 10 min at 37 °C. One unit of enzyme activity was defined as the amount of enzyme required to increase an absorbance of 0.01 at 660 nm/h at 37 °C. The proteolytic activity of ECV in the absence of inhibitors was considered as 100%.

2.6. UV-VIS spectral study

Specificity of Zn^{2+} chelating nature of inhibitors was evaluated by determining the changes in their absorbance spectra with $ZnCl_2$ and $CaCl_2$. The complex formation between the chelating agents and metal ions was analyzed by UV spectral scanning ($\lambda = 190–300$ nm) using BioMate™ 3S UV-VIS Spectrophotometer (Waltham, USA). TPEN, DTPA and TTD (0.1 mM) were incubated with various concentrations of divalent metal ions (0.1 mM–10 mM) in 1 ml of HPLC grade water. The absorption spectra of the chelating agents, divalent metal ions, chelating agent and metal ion complexes were compared to determine their specificity.

2.7. Gelatinolytic activity

Gelatinolytic activity of ECV was determined by substrate gel assay according to the method of Heussen and Dowdle (1980) with slight modifications. For inhibition studies, similar experiments were carried out with 10 μg ECV pre-incubated with various concentrations of TPEN, DTPA and TTD for 10 min prior to electrophoresis. Appropriate inhibitor and solvent controls were also processed in similar manner.

2.8. In vivo inhibition studies

Inhibition studies were carried out by 'co-injection method' where ECV and various concentrations of Zn^{2+} chelators were pre-