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Vanillic Acid as a Novel Specific Inhibitor of Snake Venom 5′-Nucleotidase: a Pharmacological Tool in Evaluating the Role of the Enzyme in Snake Envenomation

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Abstract—Vanillic acid has been investigated for its inhibitory effect on *Naja naja*, *Daboia russellii*, and *Trimeresurus malabaricus* venom 5′-nucleotidase activity. *Trimeresurus malabaricus* venom 5′-nucleotidase activity was 1.3- and 8.0-fold higher than that of *N. naja* and *D. russellii* venoms, respectively. Substrate specificity studies showed that for all the venoms tested, 5′-AMP was the preferred substrate for 5′-nucleotidase. This indicates the central role of adenosine in snake envenomation. Vanillic acid selectively and specifically inhibited 5′-nucleotidase activity among several enzymes present in the three venoms tested. The inhibitor was competitive, as the inhibition was relieved by increased substrate concentration. It appears that the COOH group in vanillic acid is the determining factor for inhibition as vanillin, a structurally similar compound with respect to vanillic acid, had no inhibitory activity. This study for the first time exemplifies vanillic acid as a pharmacological tool in evaluating the role of 5′-nucleotidase in snake envenomation.

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Key words: 5′-nucleotidase, *Naja naja*, *Daboia russellii*, *Trimeresurus malabaricus*, vanillic acid, purines

Snake venom is a complex mixture of biologically active components that has a diverse array of action on both prey and human victims. Snake envenomation is known to employ three well-integrated strategies: prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion [1]. Purines constitute the central element in these strategies exerting synchronous effect upon all cell types and ultimately bringing about death, thus acting as a multitoxin [1, 2]. Injection of exogenous (venom) nucleosides and releasing endogenous (prey tissue) nucleosides with venom enzymes are known to be complementary envenomation strategies [2].

5′-Nucleotidase is ubiquitously present in venoms [1, 2], suggesting its central importance in snake envenomation strategies. It is known to act through endogenous liberation of purines and also as a cofactor of hemorrhagic toxins, thus affecting the haemostatic system [1, 2]. It is reported to be one of the components of lethal fractions of snake species like *Trimeresurus purpuromaculatus* [3], *Trimeresurus sumatranus* [4], *Naja naja sputatrix* [5], and *Ophiophagus hannah* [6]. 5′-Nucleotidase is known to act synergistically with other toxins of Russell's viper venom and contribute to their toxicity [7]. Boffa and Boffa [8] showed 5′-nucleotidase as the most potent inhibitor of platelet aggregation from *Vipera aspis*. 5′-Nucleotidase purified from *Trimeresurus gramineus* is known to strongly inhibit platelet aggregation by removal of ADP and subsequent accumulation of adenosine through its enzymatic action [9]. We recently showed that 5′-nucleotidase is involved in inducing anticoagulant effect of *N. naja* venom by the use of a specific inhibitor [10].

Numerous studies have used specific inhibitors of enzymes as biochemical or pharmacological tools to characterize and establish the mechanism of enzyme action [11-13]. Furthermore, these inhibitors can be used as molecular tools for the development of new therapeutic agents in the treatment of ophidian accidents.

This study reports for the first time the effect of a novel non-nucleoside specific inhibitor: vanillic acid was checked on the activity of 5′-nucleotidase belonging to different families in order to evaluate the role of this enzyme in snake envenomation.

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MATERIALS AND METHODS

Materials. Cobra (*N. naja*) venom was purchased from Haffkins Institute, Bombay, India. Russell's viper (*Daboia russellii*) venom was purchased from Irula Snake catchers, Chennii, India. *Trimeresurus malabaricus* venom was from a snake of Bisllee Forest, India. *Escherichia coli* (lyophilized cells of strain W (ATCC 9637) and ^{14}C-labeled oleic acid were from Perkin Elmer Life Sciences Inc. (USA). Fat-free bovine serum albumin (BSA) fraction V was from PAA Laboratories GmbH (Austria). ConA, casein, and human fibrinogen were from Sigma Chemical Company (USA). Adenosine 5′-monophosphate (5′-AMP), α-methyl-D-glucoside, α-methyl-D-mannoside, and vanillic acid were from Sisco Research Laboratories (India). All other chemicals and reagents were of analytical grade. All the assays were done using double distilled water. For all the studies, venom and inhibitors were dissolved in saline.

5′-Nucleotidase assay. Venom 5′-nucleotidase activity was assayed by the procedure of Avruch and Wallach [14] with slight modifications. In brief, the reaction was carried out in a final volume of 1 ml containing 10 mM $MgCl_2$, 50 mM NaCl, 10 mM KCl, 50 mM Tris-HCl buffer, pH 7.4, 10 mM 5′-AMP with an appropriate amount of venom and incubated for 30 min at 37°C. The inorganic phosphate released was quantified by a colorimetric method using ascorbic molybdate reagent prepared by mixing equal volumes of 0.42% ammonium molybdate solution in 1 N sulfuric acid, 10% ascorbic acid solution, and water. This was left at room temperature for 30 min, and then absorbance at 660 nm was determined. This was calibrated by comparison with the reference curve established with KH_2PO_4. Results are expressed in nanomoles of inorganic phosphate liberated during 1 min per 1 mg protein.

For inhibition studies, venom samples were preincubated without or with different concentrations of vanillic acid (0-475 μM) or vanillin (0-600 μM) at 37°C for 15 min prior to determining enzyme activities. Appropriate controls were used, and the inhibition is expressed as percent activity taking activity of venom alone as 100%.

Phospholipase A_2 assay. Phospholipase A_2 activity was assayed according to the method of Vishwanath et al. [15]. Venom was added in a final volume of 320 μl containing 5 mM $CaCl_2$ and 100 mM Tris-HCl buffer, pH 7.5, and incubated for 1 h in the presence of 30 μl of [^{14}C]oleate-labeled autoclaved *E. coli* membrane (equivalent to 64 nmol of fatty acid). The reaction was terminated by adding 100 μl of 2 N HCl followed by 100 μl of 10% fatty acid-free BSA. The samples were then thoroughly vortexed and centrifuged at 10,000*g* for 10 min at room temperature. An aliquot (140 μl) of the supernatant (containing released ^{14}C-labeled oleic acid) was mixed with scintillation cocktail and counted in a Hewlett-Packard TRI CARB 2100 TR liquid scintillation analyzer. Activity is expressed as nanomoles of fatty acid released during 1 min per 1 mg protein.

Protease assay. Protease activity was determined using casein as substrate according to the method of Murata et al. [16]. Solution (0.4 ml) of casein (2% in 0.2 M Tris-HCl buffer, pH 8.5) was incubated with venom sample at 37°C for 2 h. The reaction was stopped by adding 1.5 ml of 0.44 M trichloroacetic acid and allowed to stand for 30 min. The tubes containing reaction mixtures were then centrifuged at 1500*g* for 15 min. An aliquot (1 ml) of the supernatant was mixed with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of Folin reagent (1 : 2 v/v). The color developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to give an absorbance of 0.01 at 660 nm per 1 h at 37°C.

Fibrinogenolytic assay. Fibrinogenolytic activity was measured according to the method of Ouyang and Teng [17]. The reaction mixture (40 μl) containing 50 μg of human fibrinogen in 10 mM Tris-HCl buffer (pH 7.6) was incubated with venom samples for 1 h at 37°C. The reaction was terminated by adding 20 μl of denaturing buffer containing 1 M urea, 4% SDS, and 4% β-mercaptoethanol. The hydrolyzed products were analyzed by 10% SDS-PAGE, and the protein pattern was visualized by staining with Coomassie Brilliant Blue R-250.

Hyaluronidase assay. Hyaluronidase activity was assayed by estimating the amount of N-acetylglucosamine released from hyaluronic acid, according to the method of Ressign et al. [18]. Venom samples were incubated with 50 μg of hyaluronan in 0.2 M sodium acetate buffer, pH 6.0, and 0.15 M NaCl in a reaction volume of 1 ml for 2.5 h at 37°C. The reaction was terminated by adding 50 μl of potassium tetraborate buffer, pH 9.1, followed by 1.5 ml of 1% *p*-dimethylaminobenzaldehyde. The stoppered tubes were boiled for 3 min in a water bath, and the color developed was monitored at 585 nm. Activity was expressed as millimoles of N-acetylglucosamine released during 2.5 h at 37°C.

L-Amino acid oxidase assay. L-Amino acid oxidase activity was determined as described in the Worthington Enzyme Manual [19] with some modifications. Peroxidase (250 NIH units/mg, 0.05 ml of 0.007% solution) was added to 1 ml of 0.2 M triethanolamine buffer, pH 7.6, containing L-leucine (0.1%) and *o*-dianisidine (0.006%), and then the reaction mixture was incubated for 30 min at room temperature. Venom samples were added, and the increase in absorbance at 420 nm was measured continuously. One unit of enzyme activity is defined as the amount of enzyme that causes an increase of 0.001 absorbance unit during 1 min per 1 mg protein.

Alkaline phosphomonoesterase assay. Alkaline phosphomonoesterase activity was assayed by a modification of the method described by Lo et al. [20]. Venom was added to the reaction mixture containing 0.4 ml of 0.01 M

p-nitrophenyl phosphate in 0.5 M Tris-HCl buffer, pH 8.5, 0.2 ml of 0.1 M $MgSO_4$ and made up to 1 ml with 0.5 M Tris-HCl buffer, pH 8.5, and then the mixture was incubated at 37°C for 30 min. At the end of the incubation period, 2 ml 0.2 N NaOH was added, and the sample was allowed to stand for 20 min. The absorbance at 440 nm was then measured. One unit of alkaline phosphomonoesterase activity is defined as amount of enzyme that causes an increase of 0.001 absorbance unit during 1 min per 1 mg protein.

Statistics. The results are expressed as mean ± SD of at least four independent experiments. Regression analysis was used to calculate the IC_{50} defined as the dosage of inhibitor necessary to produce a 50% inhibition of activities.

RESULTS AND DISCUSSION

Though 5′-nucleotidase is a ubiquitous enzyme, its involvement in snake envenomation strategies is unknown. The 5′-nucleotidase activity of *N. naja* (Elapidae family), *D. russelli* (Viperidae family), and *T. malabaricus* (Crotalidae family) was 653, 900, and 7200 μmol/min per mg. *Trimeresurus malabaricus* 5′-nucleotidase activity was thus 11- and 8-fold higher than in *N. naja* and *D. russelli* venoms, respectively. It has been observed that, on the average, viperine and crotaline venoms have 3.0- and 3.7-fold more 5′-nucleotidase activity, respectively, than elapid venoms [2]. From the results of this study and earlier studies [2], it appears that the 5′-nucleotidase activity in venoms varies with the family to which it belongs.

5′-Nucleotidase is known to hydrolyze various nucleoside 5′-monophosphates, releasing endogenous purines which are known to be hypotensive agents [1]. Substrate specificity studies show that for all the three venoms tested, 5′-AMP is the most preferred substrate followed by 5′-GMP and 5′-IMP. However, *N. naja* and *D. russelli* venoms showed varied preference regarding pyrimidine nucleosides. *Naja naja* venom 5′-nucleotidase preferred 5′-UMP over 5′-CMP and *vice versa* in the case of *D. russelli* venom (Fig. 1). It was observed that neither 3′-AMP and β-glycerophosphate nor *p*-nitrophenyl phosphate were acted upon in our assay system, suggesting that there is no involvement of nonspecific phosphatase activity under our assay condition. Nonspecific phosphatases are known to interfere in 5′-nucleotidase assay [21]. 3′-AMP hydrolyzing enzymes are reported to be present in mature lentil seeds and in promastigotes and amastigotes of *Leishmania mexicana* [22, 23]. However, the inability of snake venoms to hydrolyze 3′-AMP in our study and earlier studies [24] suggests that these enzymes are absent in venoms.

The substrate specificity studies show that venom 5′-nucleotidase in general hydrolyzes various purines and pyrimidine nucleoside 5′-monophosphates with different specificities. The preference of venom 5′-nucleotidase for 5′-AMP suggests central importance of adenosine in envenomation. Adenosine is known to be an active component of spider, ant, and snake venoms [2, 25-27]. In addition, it was observed that adenosine concentration was sufficiently high in *Bitis arietans*, *Bitis gabonica*, and *Bitis nasicornis* to bring about hypotensive effect observed in the prey envenomed by these snakes [28]. Purinergic activity has been reported for elapid, viperid, and colubrid venoms [1, 29]. Indeed, both hypotensive and purinergic activities have been suggested to play a role in envenomation strategies, such as prey immobilization [1]. Thus, it seems that 5′-nucleotidase primary function in venoms is to endogenously release adenosine and other purines (purinergic substances), causing hypotension in prey. As these are endogenous regulatory compounds in all vertebrates, it is virtually impossible for any prey organism to develop resistance. In this perspective, further study concentrated on finding a specific inhibitor of venom 5′-nucleotidase that would help in evaluation of its role in snake envenomation strategies and neutralize its pharmacological effects.

Various inhibitors reported so far seem to depend on the protein nature and lack specificity towards venom 5′-nucleotidase. Concanavalin A was shown to inhibit *Crotalus atrox* and *N. naja* venom 5′-nucleotidase activity by interacting with the glycol part of the protein [10, 30]. Citrate and EDTA inhibit venom 5′-nucleotidase activity by metal-ion chelating effect [31]. Nucleocidine, melanocidine A, and melanocidine B are polysaccharides in nature where steric effects are known to play a major role in inhibition [32]. Polyphenolic inhibitors isolated from wine grape Koshu and *Areca catechu* are known to possess tannic activity, i.e. ability to precipitate

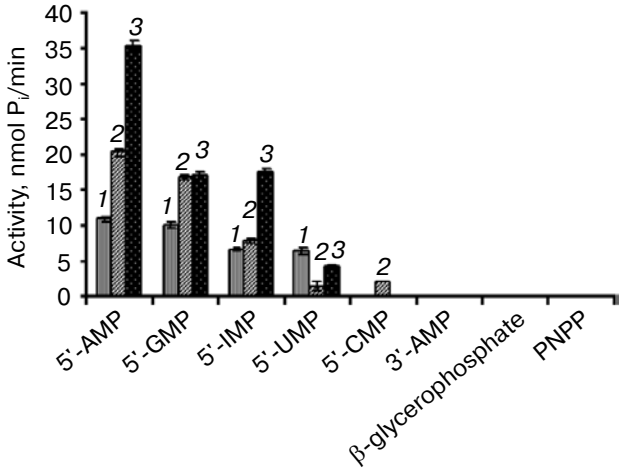

Fig. 1. Substrate specificity of venom 5′-nucleotidases: *N. naja* (*1*), *D. russelli* (*2*), and *T. malabaricus* (*3*). Reaction mixture containing 10 mM substrate was incubated with 20 μg of *N. naja*, *D. russelli*, or *T. malabaricus* venoms for 30 min at 37°C. PNPP, *p*-nitrophenyl phosphate.

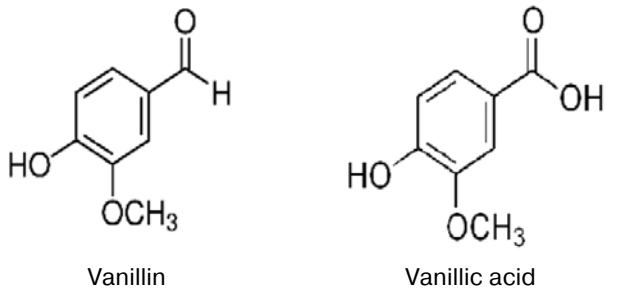

Fig. 2. Chemical structures of vanillin and vanillic acid.

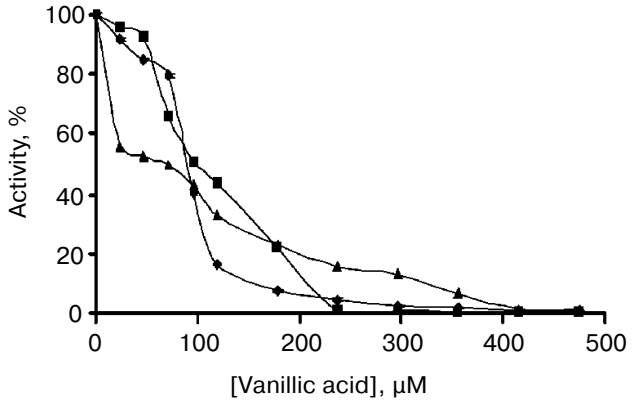

Fig. 3. Dose-dependent inhibition of venom 5′-nucleotidase by vanillic acid; *N. naja* (rhombi), *D. russelli* (squares), and *T. malabaricus* (triangles). *Naja naja* (30 μg), *D. russelli* (15 μg), and *T. malabaricus* (3 μg) venom samples were preincubated with vanillic acid in 0.9 ml assay buffer for 15 min at 37°C. Then the reaction was started by addition of 0.1 ml of 10 mM 5′-AMP and incubated for 30 min.

proteins [33]. Tannins are also known to be general inhibitors of snake venom toxins [34]. The reported substrate analog inhibitors [35], even though specific for 5′-nucleotidase, are known to act as ligands for receptors, thus restricting their use for *in vivo* studies. Further, as these studies were carried out in different contexts, we are not aware of anyone reporting a specific inhibitor of 5′-nucleotidase in relation to other enzymes present in venom.

In search of a specific inhibitor of venom 5′-nucleotidase, compounds such as vanillin (3-methoxy-4-hydroxybenzaldehyde) and vanillic acid (3-methoxy-4-hydroxybenzoic acid) (Fig. 2) where tested. It was observed that vanillic acid exhibited inhibitory effect on all venom 5′-nucleotidase activities tested in a dose-dependent manner. In contrast, vanillin did not exhibit any inhibitory activity even at the highest concentration tested. Vanillic acid inhibited *N. naja* and *D. russelli* venom 5′-nucleotidase activity up to 99% and *T. malabaricus* venoms up to 98% (Fig. 3). The IC_{50} values were 84, 98, and 65 μM for *N. naja*, *D. russelli*, and *T. malabaricus* venoms, respectively.

To check the possible inhibitory effect with reference to other enzymes present in venoms, the effect of vanillic acid on phospholipase A_2, protease, hyaluronidase, fibrinogenase, L-amino oxidase, and phosphomonoesterase activities of all the three venoms were tested. It was interesting to observe that it did not exhibit any inhibitory effect on any of these enzymes even at the highest concentration tested (data not shown). Phospholipase A_2, protease, fibrinogenase, L-amino acid oxidase, and hyaluronidase are known to be main enzymes involved in lethal and toxic effects of venoms. According to our study, it appears that vanillic acid selectively and specifically inhibits venom 5′-nucleotidase among the enzymes present in venoms. However, its action on other proteins cannot be completely ruled out.

To examine the mechanism of action or nature of the inhibition of vanillic acid on venom 5′-nucleotidase, inhibition was measured as a function of substrate concentration. The enzymes were always preincubated with the inhibitor and the assay was performed under steady-state conditions, except for L-amino acid oxidase, which was measured under presteady-state condition. The competitive nature of inhibition was evident as there was a relieving effect of inhibition upon increasing substrate concentration (Fig. 4). These data suggest that vanillic acid possibly competes with substrates for the active site of the enzyme to bring about inhibition. It appears that the COOH group in vanillic acid is the determining factor for the inhibitory activity on venom 5′-nucleotidase as vanillin, a structurally similar compound, differed in having CHO group had no inhibitory activity.

5′-Nucleotidase inhibiting platelet aggregation is known to consequently suppress blood coagulation without platelet lysis [9]. Recently we showed the role of *N.*

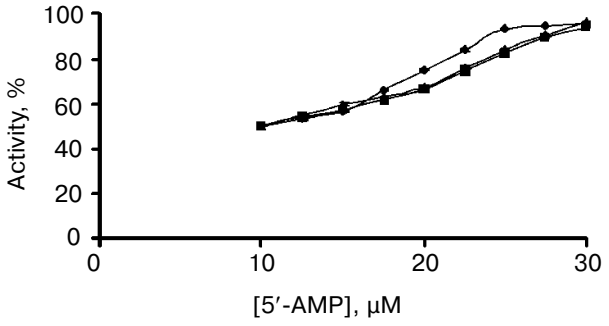

Fig. 4. Effect of substrate concentration on inhibition of venom 5′-nucleotidase activity by vanillic acid: *N. naja* (rhombi), *D. russelli* (squares), and *T. malabaricus* (triangles). *Naja naja* (30 μg), *D. russelli* (15 μg), and *T. malabaricus* (3 μg) venom samples were preincubated with respective IC_{50} concentrations of vanillic acid in 0.9 ml assay buffer for 15 min at 37°C and different concentrations of 5′-AMP were added and incubated for 30 min.

naja venom 5′-nucleotidase in the anticoagulant effect of the whole venom by the use of vanillic acid inhibition [10]. It is inferred that 5′-nucleotidase probably acts synergistically with other toxins, such as ADPase, phospholipases, and disintegrins to exert more pronounced anticoagulant activity [36]. However, its direct involvement in inducing hypotensive effects due to the endogenous liberation of purines in prey organisms is not clearly understood. As our study shows that irrespective of 5′-nucleotidase activity belonging to different families, vanillic acid specifically inhibits 5′-nucleotidase activity among all other enzymes present in venoms. Therefore, vanillic acid would be helpful in understanding the possible pharmacological/therapeutic role of venom 5′-nucleotidase in snake venoms. In addition, as vanillic acid is an intermediate metabolite product of vanillin that is excreted in urine [37], it would be an ideal biochemical or pharmacological tool to decipher the role of 5′-nucleotidase in snake envenomation strategies in *in vivo* studies. Also, since it is a normal metabolite, it could also prove to be a nontoxic anti-snake venom component for use in humans.

In conclusion, our study for the first time demonstrates that the novel non-nucleoside specific inhibitor vanillic acid can inhibit specifically venom 5′-nucleotidase activity among other enzymes present in venoms irrespective of family to which it belongs. We propose that further studies using this inhibitor as a biochemical or pharmacological tool would be helpful in evaluating the role of 5′-nucleotidase in venoms and in particular its involvement in endogenous liberation of purines.

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