

A common pumiliotoxin from poison frogs exhibits enantioselective toxicity against mosquitoes

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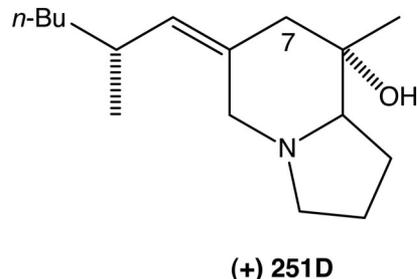
Neotropical poison frogs (Dendrobatidae) contain a variety of lipophilic alkaloids in their diffusely distributed cutaneous glands, including a major class of compounds known as pumiliotoxins. Pumiliotoxins are highly toxic and are believed to protect frogs against predators. Their potential activity against ectoparasites, however, has not been investigated. We tested female yellow fever mosquitoes (*Aedes aegypti*) for responses to 8-hydroxy-8-methyl-6-(2'-methylhexylidene)-1-azabicyclo[4.3.0]nonane, designated pumiliotoxin 251D [PTX (+)-251D], a skin alkaloid present in all genera of dendrobatids and in other anurans, and to its unnatural enantiomer, PTX (–)-251D. Both enantiomers of PTX 251D presented on silicone feeding membranes reduced landing and feeding by *A. aegypti*, but PTX (+)-251D did so at lower concentrations. PTX (+)-251D also induced toxicosis, shown when mosquitoes failed to fly off membranes. Similarly, mosquitoes confined with copper wires coated with PTX (+)-251D exhibited greater latencies to fly off the substrate and a higher incidence of leg autotomy than did those confined with the (–)-enantiomer. Our results on the contact toxicities of PTX 251D enantiomers parallel those reported for mice injected with them. The presentation of serial dilutions of PTX (+)-251D to *A. aegypti* revealed a minimum toxic concentration of 0.1 $\mu\text{g}/\text{cm}^2$. This value is substantially lower than that estimated for the cutaneous abundance of this compound in some frogs, an observation consistent the function of PTX 251D in anuran chemical defense against ectoparasitic arthropods.

alkaloids | chemical defense | dendrobatid poison frogs | insecticidal activity | skin secretions

Analyses of skin extracts of neotropical poison frogs (Dendrobatidae) reveal nearly 500 lipophilic alkaloids representing over 20 structural classes (1). Cutaneous alkaloids are believed to protect dendrobatids, as reflected by the many potential predators that avoid or reject them presumably in response to noxious skin secretions (2–7). Field experiments in Costa Rica, for example, reveal that ants (5) and spiders (6) typically release *Dendrobates pumilio* after biting it, suggesting that this frog's skin contains contact deterrents.

The responses to dendrobatid skin chemicals by ectoparasitic arthropods, such as mosquitoes and other biting flies, however, has not been investigated. Biting flies attack anurans (8–11), but apparently none has been reported to feed on dendrobatids. Nor have we (J.W.D., R. Saporito, and M. Donnelly, unpublished observations) observed flies attacking dendrobatids during field studies on these frogs, spanning >40 years, throughout Central and South America.

Pumiliotoxins/allopumiliotoxins (7-hydroxypumiliotoxins), represented by ≈ 80 compounds, comprise a major class of alkaloids found in most poison frogs (1, 12). We tested mosquitoes for responses to 8-hydroxy-8-methyl-6-(2'-methylhexylidene)-1-azabicyclo[4.3.0]nonane, designated pumiliotoxin 251D (PTX 251D) (Scheme 1). PTX 251D often is present in major or minor amounts in frogs of the genera *Dendrobates*, *Epipedobates*,



Scheme 1. PTX (+)-251D.

and *Minyobates*, and in trace amounts in *Phylllobates aurotaenia* (12, 13) (Fig. 1). This compound also occurs in the skins of Madagascar frogs (*Mantella* spp.) (14) and South American toads (*Melanophryniscus* spp.) (15).

PTX 251D and synthetic analogs of it were shown to be insecticidal in a study of the tobacco budworm (*Heliothis virescens*) (16). When injected into budworm larvae, PTX 251D was among the most toxic of the compounds tested, causing convulsions with only a dose of 10 ng per larva and exhibiting an LD_{50} of 150 ng per larva. These compounds also were toxic to larvae when applied topically. Interestingly, pumiliotoxin B [now designated PTX (+)-323A], which is highly toxic to mice (17), was weakly toxic to budworms (16).

Our subject was the yellow fever mosquito (*Aedes aegypti*), a circumtropical vector of viral, bacterial, and filarial diseases (11). *A. aegypti* feeds primarily on mammals, but it accepts anurans (8, 11). Thus, this species is appropriate to test with frog-derived compounds.

We first examined mosquitoes' responses to the naturally occurring skin alkaloid enantiomer, PTX (+)-251D, and the unnatural enantiomer, PTX (–)-251D, by using a module and membrane system designed to evaluate landing and feeding deterrents (18). We then confined mosquitoes with PTX 251D-coated wires to examine their responses to contact with this compound. Our results demonstrate that PTX 251D deters feeding *A. aegypti* and induces contact toxicosis, as evidenced by impaired flight and leg autotomy. We also show that the naturally occurring enantiomer, PTX (+)-251D, is more toxic to mosquitoes than is PTX (–)-251D, and that PTX (+)-251D is effective at levels observed naturally in dendrobatid frogs.

Author contributions: P.J.W. designed research; P.J.W. performed research; S.G. provided mosquitoes and facilities; T.F.S. and J.W.D. provided chemical solutions; M.K. analyzed data; and P.J.W., M.K., S.G., T.F.S., and J.W.D. wrote the paper.

The authors declare no conflict of interest.

Abbreviation: PTX 251D, pumiliotoxin 251D.

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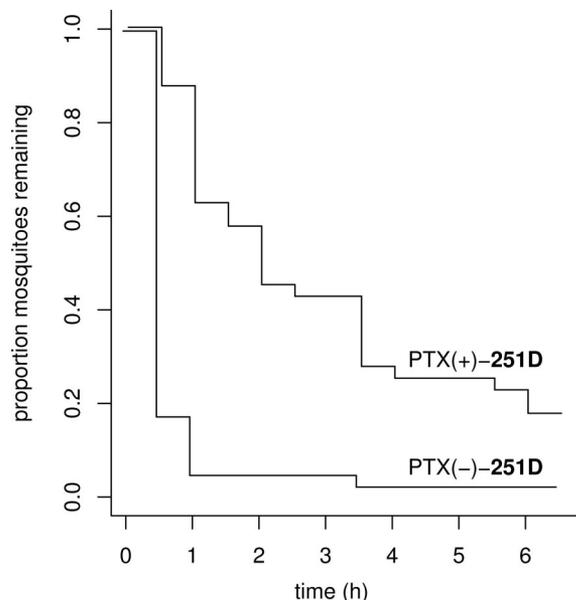


Fig. 3. Effect of PTX 251D on *A. aegypti* in wire-contact tests. Shown are proportions of mosquitoes ($n = 80$) remaining on the floor of a plastic container during 6 h after a 3-min exposure to wire treated with 1.2 mM methanolic solutions of PTX (+)-251D or PTX (-)-251D.

(+)-251D-coated wires exhibited greater latencies to fly and a higher incidence of leg autotomy than did those confined with the (-)-enantiomer. Mosquitoes are reported to autotomize their legs in response to other noxious compounds, including volatile repellents (22).

The minimum concentration of PTX (+)-251D that induced toxicosis in *A. aegypti* in our experiments was $0.1 \mu\text{g}/\text{cm}^2$. Precisely how this value relates to the amounts of PTX (+)-251D encountered on the skin surface of frogs is unclear because this and other skin alkaloids are concentrated in granular glands

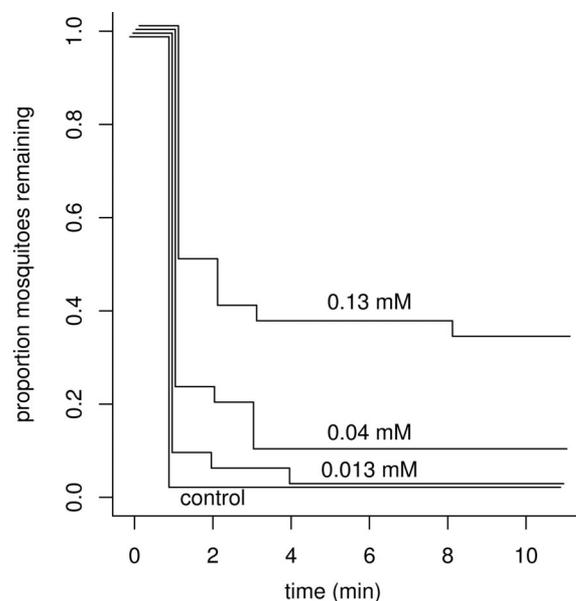


Fig. 4. Effect of PTX 251D on *A. aegypti* in wire-contact tests. Shown are proportions of mosquitoes ($n = 120$) remaining on the floor of a plastic container during 10 min after a 3-min exposure to a wire treated with methanol (control) or with 0.013, 0.04, and 0.13 mM methanolic solutions of PTX (+)-251D.

embedded in the epidermis. The cutaneous concentrations of PTX (+)-251D also vary greatly among dendrobatid populations and species (13).

With these caveats in mind, we consider the plausibility of PTX (+)-251D as a defensive agent for dendrobatids by referring to the abundance of this compound reported in *Epipedobates tricolor* from Ecuador. The average skin concentration of PTX (+)-251D from one population of this frog was $37 \mu\text{g}$ per individual (23). Assuming the total skin area of an adult *E. tricolor*, which attains a snout-vent length of ≈ 2 cm, to be $4\text{--}6 \text{ cm}^2$, we estimate cutaneous concentrations of PTX (+)-251D ranging from 6 to $9 \mu\text{g}/\text{cm}^2$. This range of values greatly exceeds the minimum toxic concentration of PTX (+)-251D that we observed for *A. aegypti*. Thus, we surmise that the amounts of this compound in the skin of *E. tricolor* are sufficient to deter biting mosquitoes. Further studies assessing the defensive value of cutaneous alkaloids should consider the combined and possibly synergistic effects of the diverse compounds present on various anuran species. In addition, deterrence of mosquitoes, such as *Uranotaenia* spp. (10), known routinely to feed on anurans could be tested with selected dendrobatid skin alkaloids.

Our results on the contact toxicity of PTX (+)-251D enantiomers with mosquitoes parallel those reported for mice injected with them. A 10 mg/kg dose of PTX (+)-251D administered s.c. to mice elicited convulsions, hyperalgesia, and death, whereas PTX (-)-251D at the same dose had no detectable effect (13). Pumiliotoxins are hypothesized to cause cardiac stimulation and, at high doses, convulsions, by prolonging the opening of voltage-sensitive sodium channels (13, 24). Other sites of action or selectivity at different sodium channel subtypes may underlie the diverse pharmacological activities of PTX (+)-251D. Whatever the mode of action, PTX (+)-251D appears to exhibit broad spectrum toxicity and, against arthropods, at least, contact toxicity consistent with a function in anuran chemical defense.

Materials and Methods

Mosquitoes. Laboratory colonies of *A. aegypti* were reared at 28°C and 80% relative humidity and kept on a 12:12 (light/dark) photoperiod. Adult female mosquitoes, 5–16 days old, were maintained on a 10% sucrose solution presented on cotton pads. For the membrane-feeding experiments, the sucrose-soaked pads were replaced with water-moistened pads 48 h before mosquitoes were tested.

Chemicals. PTXs (+)-251D and (-)-251D were synthesized as described in ref. 13.

Membrane-Feeding Tests. All experiments were conducted at 0900–2100 hours in a walk-in incubator (26°C , 63–80% relative humidity) illuminated by fluorescent lights. In membrane-feeding experiments, mosquitoes were tested in a two-piece Plexiglas module (18). The top piece of the module consisted of six $4.5 \times 4.0 \times 5.0$ -cm chambers, the floors of which were fitted with a sliding door positioned over a circular opening (diameter, 3.5 cm). For each test, five female mosquitoes were aspirated into each chamber through an aperture, which then was plugged with corks wrapped in plastic film.

The bottom piece of the module was a $40 \times 7 \times 4$ -cm hollow platform supporting six circular wells (diameter, 3.8 cm; depth, 6 mm). Water (40°C) flowed through the central cavity of the platform at a rate of 215 ml/min. The wells were filled with 7 ml of 10% sucrose solution containing ATP (2.9 mg/ml). Fifty microliters of green dye (McCormick Food Color, Hunt Valley, MD) was added to each well so that the mosquitoes imbibing the sugar solution through the membranes could be identified when crushed at the end of a test.

Twenty-five microliters of methanol or PTX (+)-251D or PTX (-)-251D in 0.4, 1.2, 3.7, 11.0, and 33.0 mM methanolic solutions

