Alterations of mitochondrial electron transport chain and oxidative stress induced by alkaloid-like α-aminonitriles on *Aedes aegypti* larvae

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ABSTRACT

Aedes aegypti mosquitoes are responsible for dengue, chikungunya, and Zika virus transmission in tropical and subtropical areas around the world. Due to the absence of vaccines or antiviral drugs for human treatment, the majority of control strategies are targeted at *Ae. aegypti* elimination. Our research on mosquito control insecticidal agents has previously shown that the alkaloid girgensohnine and its analogues (α-aminonitriles) present *in vitro* acetylcholinesterase inhibition and *in vivo* insecticidal activity against *Ae. aegypti.* However, acetylcholinesterase inhibition may not be the only mechanism of action behind these effects. On this basis, the principal aim of this study was to elucidate the possible action mode of four α-aminonitriles on *Ae. aegypti* by studying other important enzymatic targets, such as mitochondrial electron transport chain complexes, catalase, and superoxide dismutase, key oxidative stress enzymes. Mitochondria were isolated from *Ae. aegypti* larvae by differential centrifugation, stored at $-70\,°C$, and fragmented using ultrasound for 10 min. The effects of α-aminonitriles (**1** to **4**) over enzymatic activities were evaluated using concentrations of 8 nM, 2 μM, 8 μM, and 40 μM. Results indicated that α-aminonitriles caused significant NADH dehydrogenase and succinate oxidase inhibition ($\sim 44\%$ at the highest concentration tested). Succinate dehydrogenase and cytochrome *c* oxidase activities were found to increase (162% and 106% at 40 μM, respectively). It was also observed that these compounds produced catalase inhibition and thus prevented H_2O_2 reduction, which induced the formation of reactive oxygen species (ROS). Moreover, NBT assay showed that compounds **3** and **4** (with 2-(pyrrolidin-1-yl) acetonitrile as substituent) increased by approximately 50% the $O_2^{•-}$ concentration in the mitochondrial respiratory chain. It was concluded that the tested compounds act as complex I inhibitors by blocking electron transport and causing electron leak, possibly between complex I and III. Furthermore, α-aminonitriles inhibited catalase activity; compounds **1** and **2** (with piperidine fragment) inhibited glutathione reductase activity and further promoted the accumulation of ROS, which probably induced oxidative stress.

1. Introduction

Insect-borne diseases are a leading cause of morbidity and mortality worldwide [1]. *Aedes aegypti* mosquitoes transmit numerous human and animal diseases, such as dengue, chikungunya and Zika [2]. Given that effective vaccines or drug treatments are yet to be developed, disease control is limited to mosquito population management, which currently focuses on eliminating the vector *Ae. aegypti* by using synthetic insecticides (organophosphates, carbamates, or pyrethroids). However,

the continuous and inadequate use of these pesticides has resulted in widespread insecticide resistance and environmental pollution [3–5].

Organophosphates and carbamates are the most commonly used insecticides against *Ae. aegypti* [6]; their insecticidal activity is associated with their ability to inhibit enzyme AChE, which results in overstimulation of cholinergic nerve transmission, paralysis, and insect death [7]. This inactivation of enzymes would increase its efficiency as a control method by using a different molecule design. In fact, the design of this insecticide is based on natural molecules in which alkaloid

metabolites play a key role [8].

We have recently reported that the girgensohnine alkaloid, a minor metabolite of the *Girgensohnia oppositiflora* (Amaranthaceae) [9], possesses weak AChE inhibition properties (IC$_{50}$ = 93 μM) *in vitro* [10]. We also found that their closest analogues, α-aminonitriles, displayed higher AChE inhibition at IC$_{50}$ values between 42 and 51 μM, and insecticidal activity *in vivo* in third and fourth instar of *Ae. aegypti* larvae at concentrations below 140 mg/L [11]. On this basis, it was suggested that the insecticidal activity of these compounds could be related to their ability to inhibit AChE. However, despite their AChE inhibition properties and acceptable *in vivo* insecticidal activity, these α-aminonitriles could cause insect mortality *via* another mechanism. Thus, in order to clarify the action mode of α-aminonitriles, the present study focused on mitochondria, an important biological target. For example, the mitochondrial electron transport complexes have been intensively studied as biochemical target sites for insecticides and acaricides [12].

Considering the role of mitochondria in the production of reactive oxygen species, regulation of calcium levels, ATP synthesis, signaling processes, and cell death [13–15], we inferred that mitochondrial tests could be useful in understanding the insecticidal effect of these molecules if the cellular respiration were to be affected [16]; these tests were based on the operating model of oxidative phosphorylation used by different enzyme complexes that make up the respiratory chain: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) [17].

The present study sought to understand the effects of **1**: 2-(3,4-dimethoxyphenyl)-2-(piperidin-1-yl)acetonitrile; **2**: 2-(3,4-dioxymethylenphenyl)-2-(piperidin-1-yl)acetonitrile; **3**: 2-(3,4-dimethoxyphenyl)-2-(pyrrolidin-1-yl)acetonitrile; and **4**: 2-(3,4-Dioxymethylenphenyl)-2-(pyrrolidin-1-yl)acetonitrile (Fig. 1) on each enzyme in the respiratory chain and to determine the activity of antioxidant enzymes as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase over the oxidative stress produced by these compounds on *Ae. aegypti* larvae.

2. Material and methods

The methodology developed in this work is summarized in the flow diagram of the experimental design (Fig. 1) and described in more detail below.

2.1. Reagents

Succinic acid, NADH, EGTA, EDTA, DCPIP, PMS, NBT, potassium ferricyanide (III), rotenone, sucrose, HEPES, BSA, and cytochrome *c*, were purchased from Sigma-Aldrich (St. Louis, USA); potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium heptamolybdate, and ferrous sulfate were purchased from Merck & Co. All reagents used were analytical grade and solutions were prepared in Milli Q water.

2.2. Biological material

Ae. aegypti mosquitoes of Rockefeller strain were reared within insectary in cages (40 × 40 × 40 cm) at 25 ± 5 °C and 70 ± 5% relative humidity with a photoperiod of 12 h. *Ae. aegypti* females were continually offered a 10% honey sugar solution 10%; when was necessary to obtain larvae for experiments, the insects were fed with blood from Wistar rats bred at the Universidad Industrial Santander. Once mosquito females were fed, a container coated on the inside with filter paper substrate was placed inside the mosquito cage for oviposition. The obtained eggs were placed in chlorine-free water containers where larvae subsequently hatched. Larvae were fed on a daily basis with TetraMin™ (tropical fish food flakes) for about one week until they reached the fourth instar.

2.3. α-aminonitriles with insecticidal action

The tested compounds **1–4** (Fig. 2) were synthesized by modified Strecker reaction using an equimolar mixture of the corresponding aldehyde (1 mmol) and cyclic amine (1.1 mmol), the source of cyanide (1.5 mmol) and the catalyst SSA (1:1 by weight) in acetonitrile (15–20 mL) at room temperature (16–24 h). The synthesized compounds were structurally characterized by instrumental techniques IR, GC–MS, ^{1}H, and ^{13}C NMR, and their physical constants were determined according to their aggregation state. All compounds were obtained as white crystals with high melting points. Different concentrations were used in order to evaluate the action of these molecules on the respiratory chain (8 nM, 2 μM, 8 μM, and 40 μM) of each compound [11].

2.4. Mitochondria isolation of Ae. aegypti larvae

Approximately, 10 g of larvae (N = 600–800) between third and fourth larval stages were used from breeding trays and filtered using surgical gauze and an isolation medium composed of 250 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM EGTA, and 0.1% BSA. All larvae were processed in a Van Potter homogenizer in order to rupture their membranes and obtain a homogeneous suspension for subsequent centrifugation. The obtained suspension was filtered using glass fiber, and the resulting solution was subjected to four centrifugations, the first at 300 × *g* at 4 °C for 5 min, and then the pellet was discarded and the supernatant was subjected to the second centrifugation at 10000 × *g* for 10 min at 4 °C. For the third centrifugation, the precipitate was suspended in extraction media and centrifuged at 300 × *g* for 5 min at 4 °C. Finally, the supernatant was centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting precipitate consisted of (partially fragmented) isolated mitochondria, which were maintained in extraction medium without BSA at − 70 °C until use.

2.5. Mitochondrial fragmentation

Isolated mitochondria and stored in 2 mL Eppendorf tubes at − 70 °C, were fragmented using ultrasound for 10 min at 4 °C; the obtained suspension was used as an enzyme source and kept at 4 °C.

2.6. Enzymatic activity of mitochondrial respiratory chain complex

NADH oxidase and succinate oxidase were polarographically evaluated using a Hansatech oxygraph (Hansatech Instruments, Norfolk, England) with a Clark-type electrode. NADH dehydrogenase (NADH: ubiquinone oxidoreductase) and succinate dehydrogenase were determined by spectrophotometry as described by Singer [18]. Cytochrome *c* reductase succinate (succinate: cytochrome *c* oxidoreductase) activity was measured by cytochrome *c* reduction at 550 nm, as described by Somlo [19]. Cytochrome *c* oxidase activity was determined at 550 nm, as recommended by Mason [20]. NADH-cytochrome *c* reductase enzyme activity was not determined, since our experiments revealed that the compounds inhibited complex I, so evaluating the passage from complex I to complex III was unnecessary.

2.7. Activity of antioxidant enzymes

The suspension of fragmented mitochondria was used as the source of enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase). The results are expressed as percentage of specific activity. SOD was also evaluated in total proteins, obtained in the supernatant of the first centrifugation, during the isolation of mitochondria.

Generation of superoxide radical and SOD activity were evaluated by the method described by Nishimiki [21]. A phenazine methosulphate PMS–NADH mixture at pH 8.0 generates superoxide radical