Published in final edited form as:

Insect Biochem Mol Biol. 2011 September ; 41(9): 645–652. doi:10.1016/j.ibmb.2011.04.006.

Antiviral, antiparasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response

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Abstract

Phenoloxidase (PO) and its activation system are implicated in several defense responses of insects. Upon wounding or infection, inactive prophenoloxidase (proPO) is converted to active PO through a cascade of serine proteases and their homologs. PO generates reactive compounds such as 5,6-dihydroxyindole (DHI), which have a broad-spectrum antibacterial and antifungal activity. Here we report that DHI and its spontaneous oxidation products are also active against viruses and parasitic wasps. Preincubation of a baculovirus stock with 1.25 mM DHI for 3 h near fully disabled recombinant protein production. The LC₅₀ for lambda bacteriophage and eggs of the wasp *Microplitis demolitor* were 5.6 ± 2.2 and 111.0 ± 1.6 μ M, respectively. The toxicity of DHI and related compounds also extended to cells derived from insects that serve as hosts for several of the aforementioned pathogens. Pretreatment of *Sf9* cells with 1.0 mM DHI for 4 h resulted in 97% mortality, and LC₅₀ values of 20.3 ± 1.2 μ M in buffer and 131.8 ± 1.1 μ M in a culture medium. Symptoms of DHI toxicity in *Sf9* cells included DNA polymerization, protein crosslinking, and lysis. Taken together, these data showed that proPO activation and DHI production is strongly toxic against various pathogens but can also damage host tissues and cells if not properly controlled.

Keywords

insect immunity; phenoloxidase; melanization; quinone; *Manduca sexta*

1. Introduction

Phenoloxidase (PO) and its activation system is a conserved component of the immune system of insects and other arthropods (Gillespie et al., 1997; Cerenius et al., 2008; Ragan et al., 2009). Active phenoloxidase (PO), which has a broad substrate specificity on monophenols and diphenols, catalyzes the formation of quinones which spontaneously cyclize and oxidize to yield other reactive intermediates (Sugumaran, 2002; Nappi and Christensen, 2005). Among them, semiquinones may produce free radicals while quinones polymerize to form eumelanin. Due to the high reactivity and assumed toxicity of quinolic compounds, protease-mediated proPO activation has always been considered as a mechanism for pathogen immobilization and killing.

Do proPO activation and PO-generated compounds play a role in insect immune defense? Several lines of evidence suggest yes. In response to bacteria, for example, our previous study shows that PO-catalyzed oxidation of dopamine generates reactive compounds that immobilize, aggregate, and kill *Escherichia coli* and *Bacillus subtilis* (Zhao et al., 2007). Against viruses, Trudeau et al. (2001) reported that *Helicoverpa zea*, a semipermissive host of *Autographa californica* multi-capsid nucleopolyherdovirus (AcMNPV), melanizes infection foci, whereas this response was not observed in the fully permissive host *Heliothis virescens* due to AcMNPV reducing proPO levels in the hemolymph (Li et al., 2008). Shelby and Popham (2006) elaborated on these results by showing that PO inhibitors abolish the virucidal activity of *H. virescens* plasma against a baculovirus, while Clarke and Clem (2002) report that hemocytes also affect spreading of AcMNPV in *Trichoplusia ni* and *Spodoptera frugiperda*. Melanization and other immune mechanisms also play essential roles in resistance against endoparasitoids (Beckage, 1998; Schmidt et al., 2001). Oxidation of eumelanin precursors (e.g. dopamine and DHI) affects parasite survival and growth in *Drosophila melanogaster* (Kohler et al., 2007), while several parasitoids suppress melanization and other host defense responses to prevent the immune system from killing offspring (Nappi et al., 2009). For example, a venom protein from the parasitic wasp *Cotesia rubecula* encodes a clip-domain serine protease homolog that blocks melanization of host hemolymph by likely interfering with proPO activation (Zhang et al., 2004). The bracovirus (MdBV) carried by the wasp *Microplitis demolitor* encodes a 26 kDa protein named Egf1.0 that suppresses melanization by inhibiting proPO-activating proteases (PAPs) (Beck and Strand, 2007; Lu et al., 2008). A related protein, encoded by MdBV Egf1.5, also suppresses melanization (Lu et al., 2010), while functional assays show that suppression of the host proPO activation system by Egf proteins greatly enhances the survival of both *M. demolitor* and MdBV (Beck and Strand, 2007).

These results collectively suggest a critical role for PO-mediated melanization in host defense, yet it remains unclear which PO-generated compounds are responsible for killing pathogens and parasites (Kanost and Gorman, 2008). Prior studies with vertebrates indicate that the melanin precursors 5,6-dihydroxyindole (DHI) and DHI carboxylic acid are cytotoxic to human cells (Pawelek and Lerner, 1978; Urabe et al., 1994), while our own data indicate that DHI kills selected bacteria and fungi (Zhao et al., 2007). In this study, we extend our survey of DHI antibiotic activity to two kinds of viruses, a parasitic wasp, and an insect cell line. Our results provide new insights on the effects of DHI and its oxidation products against different targets while also providing evidence that improper regulation of the PO cascade can cause serious damage to hosts.

2. Materials and methods

2.1. Effect of DHI on a baculovirus

A recombinant stock of *Autographa californica* nucleopolydrosis virus (AcNPV) (30 μ l, $1-2 \times 10^8$ pfu/ml) expressing *Schizaphis graminum* acetylcholinesterase-1 (AChE1) (Zhao et al., 2010) was treated with 50 μ l, 1.25 mM DHI in PB (50 mM sodium phosphate, pH 6.5) or PB alone at room temperature for 3 h. The treated and control samples (80 μ l) were separately added to *S. frugiperda* Sf9 cells (10^5 cells/ml, 2.0 ml) in fresh Sf900-III serum-free medium (Life Technologies, Inc) in a 6-well plate. After incubation at 27°C for 72 h, the AChE activity in the conditioned culture medium (20 μ l) was measured in triplicate using the modified Ellman method. Sf9 cells infected with DHI- or buffer-treated virus were examined by fluorescence microscopy using a monoclonal antibody against a hexahistidine tag at the carboxyl-terminus of *S. graminum* AChE1. The cells were collected, added to a microscope slide, fixed in cold methanol for 5 min, and permeabilized with cold acetone for 5 min. After washing with ethanol once and phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) three times, 1:1000 diluted anti-

(His)₅ antibody (Qiagen) was added to the treated cells and incubated at 37°C for 1 h. Following washing with PBS three times, 1:1000 fluorescein-labeled goat-anti-mouse secondary antibody (Sigma-Aldrich) was added to the slide. After incubation and washing, the cells were observed under a fluorescence microscope, Olympus BX-51.

2.2. Effect of DHI on bacteriophage lambda

Aliquots (10 µl) of 0, 0.2, 2, 20, 200, and 2000 µM DHI in sterile SM buffer (0.0001% gelatin, 0.1 M NaCl, 7 mM MgSO₄, 50 mM Tris-HCl, pH 7.5) were individually incubated with 10 µl, 1:100 diluted lambda bacteriophage stock (10⁹ pfu/ml) for 4 h at room temperature. After being diluted 1:10²-10⁶ in SM buffer, 10 µl of treated samples were separately incubated at 37°C for 15 min with 200 µl fresh *E. coli* X11-Blue cells suspended in 10 mM MgSO₄ (OD₆₀₀ = 1.0). Each reaction mixture was mixed with 4.0 ml, 55°C NZY broth containing 0.75% agarose, poured onto a pre-warmed NZY agar plate, and incubated at 37°C. Numbers of plaques formed on plates were counted to calculate viral titers: pfu/ml = plaque number × dilution factor × 100. An LC₅₀ was determined by plotting bacteriophage titer against final DHI concentration (0-1.0 mM) using Prism 3.0 (GraphPad Software).

2.3. Effect of DHI on the eggs of a parasitoid wasp

Newly laid (< 1 h old) eggs from the wasp *Microplitis demolitor* were collected from host larvae as described (Beck and Strand, 2007), and incubated with 0, 2, 20, 200 and 2000 µM DHI in TC-100 insect cell medium (Sigma-Aldrich) at room temperature for 1 h. After washing with the medium three times, the embryos were cultured in TC-100 supplemented with 30% heat-treated *Pseudoplusia includens* plasma. An egg was scored as alive if it hatched to form a first instar parasitoid after 30 h. An LC₅₀ value was determined by plotting viability of the wasp embryos against DHI concentration (0-2 mM) as described in Section 2.2.

2.4. Cytotoxicity of DHI to insect cells

Sf9 cells (1×10⁵ cells/ml, 200 µl) in a 96-well plate were incubated with 0, 1, 10, 20, 50, 100 µM DHI in PB at 27°C for 4 h. After the cells were washed with PB, 200 µl Sf900-III medium and 20 µl, 5 µg/µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well. Following incubation at 27°C for 4 h, the medium-dye mixture was removed and the cells in each well were washed with PB. Aliquots of DMSO (150 µl) were added to wells to dissolve reduced MTT taken up by live cells. A_{492nm} readings, which correlate with cell viability, were taken using a VersaMax microplate reader (Molecular Devices) and plotted against DHI concentration for determination of LC₅₀ values as described in Section 2.2.

Viability of DHI-treated *Sf9* cells was further examined by flow cytometry: 1×10⁵ *Sf9* cells were first treated with 0, 31, 63, 94, 125, 188, 250, 375 and 500 µM DHI in Sf900-III medium at 27°C for 5 h in a shaker-incubator (100 rpm). The cells were then harvested by centrifugation at 500×g for 5 min and resuspended in 50 µl propidium iodide (PI) and fluorescein-labeled Annexin V for 15 min in the staining buffer (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences). After addition of 400 µl buffer, cell suspensions were analyzed on a BD FACS Calibur flow cytometer. Data analysis and LC₅₀ calculation were performed as described in Section 2.2.

2.5. Morphological changes of DHI-treated *Sf9* cells

Sf9 cells (2×10⁴) in a 6-well plate were treated with 200 µl PB or 0.5 mM DHI in PB at 27°C for 6 h, stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 15 min, and observed by fluorescence microscopy. Additionally, *Sf9* cells (2×10⁴) were incubated with

Sf900-III SFM or 0.5 mM DHI in the medium at 27°C for 5 h, washed with 0.85% NaCl twice and staining buffer once, and stained with Evans Blue, a membrane-impermeable dye. The control and treated cells were observed under bright field to detect differences in staining patterns.

2.6. Staining of phosphatidylserine

Sf9 cells (2×10^4) were treated with Sf900-III or 0.5 mM DHI in the medium at 27°C for 6 h and washed with 0.85% NaCl and staining buffer. Fluorescein-labeled Annexin V was added to stain phosphatidylserine, a phospholipid exposed on the surface of cells undergoing early-stage apoptosis. PI was also included to stain dead cells for 15 min. Stained cells were washed with buffer and observed under the fluorescence microscope. The images were processed by adjusting brightness and contrast using Auto Levels in Photoshop 6.0 (Adobe Systems Inc).

2.7. Detection of fragmented genomic DNA

Sf9 cells (2×10^5) were treated with Sf900-III or 0.25 mM DHI in the medium at 27°C for 5 h, washed with PBS once, and resuspended in the same buffer. The control and treated cells were collected by centrifugation at $500 \times g$ and lysed in 0.7 ml buffer (1% *N*-lauroylsarcosine, 100 mM NaCl, 8.6 mM EDTA, 100 mM Tris-HCl, pH 8.5) by vortexing for 1 min. The suspensions were gently but thoroughly mixed with 0.7 ml phenol:chloroform:isoamylalcohol (25:24:1). After centrifugation at $15000 \times g$ for 5 min, the upper phase was collected and mixed with equal volume of cold chloroform and then spun at $15000 \times g$ for 2 min. The aqueous phase, with 1/10 volume of 3 M sodium acetate (pH 4.8) added, was mixed with equal volume of cold isopropanol. The DNA precipitate was collected using a pipette tip or, if too little, by centrifugation at $15000 \times g$ for 10 min. After washing with cold 75% ethanol, the DNA pellet was air-dried and then dissolved in 10 mM EDTA, 0.1 M Tris-HCl, pH 8.0. Following RNase treatment, the DNA samples were analyzed by 1% agarose gel electrophoresis.

2.8. Effect of DHI on DNA and protein aggregation

NcoI-HindIII digested H₆pQE60 DNA was separated by 1% agarose gel electrophoresis and recovered using QIAquick Gel Extraction Kit (Qiagen). Aliquots of the circular and linearized plasmid DNA samples (0.5 µg/µl, 10 µl) were added to an equal volume of DHI at 0, 0.02, 0.1, 0.2, 1.0, and 2.0 mM. After incubation at room temperature for 1.5 h or overnight, the reaction mixtures were separated by gel electrophoresis and stained with ethidium bromide.

The purified recombinant *S. graminum* AChE1 (0.038 µg/µl, 50 µl) was incubated with equal volume of PB or 2.0 mM DHI in PB overnight at room temperature. Residual enzymatic activities in the control and DHI-treated samples were determined. In addition, bovine serum albumin (BSA, 500 µg/µl, 50 µl) was mixed with equal volume of 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mM DHI in PB. After overnight incubation at 25°C, the samples were subjected to 10% native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE under non-reducing and reducing conditions. Sizes of the control and DHI-treated BSA were further analyzed on a Zetasizer (Malvern), which measures protein size by light scattering.

3. Results

3.1. Antiviral and antiparasitic activity of DHI

To quantitatively assess the virucidal activity of DHI against baculoviruses, we took advantage of an existing recombinant variant of AcNPV that secreted *S. graminum* AChE1 into culture medium (Zhao et al., 2010). After treating the viral stock with buffer or 1.25

mM DHI for 3 h, we incubated *Sf9* cells with these two mixtures for 72 h and determined AChE activity in the conditioned media. In comparison with the control, only 3% of the enzymatic activity was detected in the DHI-treated culture (Fig. 1), indicating a severe loss in viability and/or the ability of virions to infect *Sf9* cells. We did not observe any sign of viral infection in the *Sf9* cells. Neither was there fluorescence signal of *S. graminum* AChE1 in cells infected with DHI-treated viruses.

We further assessed the antiviral activity of DHI by conducting assays with lambda bacteriophage. As shown in Fig. 2, almost all the bacteriophages were inactivated by 1.0 mM DHI after 4 h incubation while reducing the DHI concentration increased phage viability. Based on the Sigmoidal dose-response curve, we estimated the LC_{50} of DHI against lambda bacteriophage to be $5.61 \pm 2.21 \mu\text{M}$.

To test whether DHI kills parasitoid wasps, we incubated eggs from *M. demolitor* in DHI at different concentrations for 1 h and then measured viability by determining the proportion of eggs that hatched into first instars. In the control, 77% of the eggs hatched into first instars within 30 h (Fig. 3). In contrast, egg hatching dose-dependently decreased with increasing concentration of DHI. The LC_{50} of DHI to *M. demolitor* embryos was $111.0 \pm 1.6 \mu\text{M}$ with no eggs surviving treatment with 2.0 mM DHI.

3.2. Cytotoxic effects of DHI

While PO-generated reactive chemicals kill pathogens, they also could cause damage host tissues and cells (Nappi and Christensen, 2005). We assessed the effects of DHI on host insect tissues by examining the cytotoxic activity of DHI against *Sf9* cells that derive from the moth *Spodoptera frugiperda*: a host susceptible to infection by AcNPV, *M. demolitor*, and several of the bacteria and fungi also killed by DHI (Zhao et al., 2007). Using a cell viability assay based on MTT reduction, we found that buffer-treated cells readily reduced MTT as measured by OD_{492} values while DHI dose-dependently decreased OD_{492} values (Fig. 4A). We estimated the LC_{50} of DHI toward *Sf9* cells in buffer as $20.3 \pm 1.2 \mu\text{M}$. To confirm these values, we also treated *Sf9* cells with different amounts of DHI in the Sf900 medium followed by vital dye staining and analysis by flow cytometry. Again, we detected a large reduction in cell survival with increasing concentration of DHI although LC_{50} values were higher ($131.8 \pm 1.1 \mu\text{M}$) than determined in buffer using the MTT assay (Fig. 4B).

DHI-treated *Sf9* cells also exhibited dramatic changes in their morphological features. Untreated cells were mostly round, stained by DAPI in the middle, and had a good distribution of cell sizes (Fig. 5, left panels). Only a small portion of them were dead and stained by Evens Blue, a membrane-impermeable dye living cells exclude. After DHI-treatment, nuclei of many DHI-treated cells were released into the medium and stained by DAPI, whereas the rest of these cells took the shape of a bubble attached to cellular contents (Fig. 5, right panels). Some of the membranous sacs remained intact and translucent, while others were filled with Evans blue. Most of the cells were dead and their cellular structures, colored brown due to DHI eumelanin, seemed to undergo polarization, membrane rupture, or partial-to-complete content release.

To test if apoptosis was involved in the DHI-mediated cell death and to further examine the membrane integrity of *Sf9* cells, we used Annexin V to specifically label phosphatidylserine, a component of inner layer of the plasma membrane in normal cells. At an early stage of apoptosis, plasma membrane flips phosphatidylserine to the outer layer without membrane destruction. By combining FITC-labeled Annexin V and PI (a membrane-impermeable DNA dye), we were able to distinguish early-stage apoptotic cells from late-stage apoptotic or dead cells (Fig. 6). The early-stage apoptotic cells were Annexin V positive and PI negative, whereas the latter two were Annexin V and PI double positive. In the control

group, most of the *Sf9* cells were colorless and translucent under bright field (Fig. 6A). Little FITC signal was detected under fluorescence, indicating most cells were healthy. Only a small fraction of them were dead and, thus, stained by PI (red). In the DHI-treated sample, a few cells (e.g. the ones surrounded by a *white* circle) were healthy and not stained by either dye (Fig. 6B). More cells were Annexin positive (*green*), polarized (as marked by *white* arrows), and intact (no red light emission). A majority of the cells were in late-stage apoptosis (marked by *red* arrows) or already ruptured as nuclei attached with some cellular components, which were stained by the two dyes at various levels. We also observed small, black bodies not labeled by either dye (*blue* arrows). They may represent released cellular contents heavily melanized by DHI.

To confirm apoptosis as a factor contributing to the cell rupture and death, we extracted DNA samples from buffer- and DHI-treated *Sf9* cells and separated them by gel electrophoresis. The control DNA did not run much into the gel while a portion of the other sample migrated as a series of bands at 180, 360, 540, 720 bp, and so on (Fig. 7) – the fragmentation of genomic DNA down to ~180 bp units is a characteristic feature of apoptosis.

3.3. Possible mechanisms for DHI's antibiotic and cytotoxic activities

To further explore the antibiotic activity and cytotoxicity of DHI, we treated circular and linearized plasmid DNA with DHI at various levels. Electrophoretic analysis showed that the amount of original-sized DNA decreased as DHI concentration increased (Fig. 8). There was a concurrent increase in DNA species at larger sizes, suggesting that DHI caused the DNA molecules to form high M_r aggregates. The longer the incubation time, the more aggregates formed. After overnight treatment, circular and linear DNA treated with 0.5 and 1.0 mM DHI completely disappeared at their original sizes.

We also examined the effect of DHI on two proteins, *S. graminum* AChE1 and BSA. After incubating the recombinant protein with DHI, we detected a loss of nearly 50% of its activity (Fig. 9), in comparison with the control. This suggests that access to the enzyme active site was affected by DHI treatment. In the other experiment, DHI-treated BSA samples showed a fainter band at the monomer position on 7.5% gel under native and denaturing conditions (Fig. 10, A and B). The higher the DHI concentration, the less abundant the monomer became. We also noted an increase in the deposition of dark substances near the bottom of sample wells. Thus, DHI caused BSA to form high M_r SDS-stable aggregates in a concentration-dependent manner. The covalent linkage in the aggregates was not reduced by DTT (Fig. 10C). Particle size measurement on a Zetasizer showed that BSA crosslinked by 2 mM DHI had an average molecular weight of about 10^8 kDa. After treatment using 0.5 mM DHI, two peaks were detected: one at $\sim 10^6$ kDa and the other at $\sim 10^7$ kDa. In the negative control, BSA had a molecular mass of ~ 50 kDa (Fig. 10D).

4. Discussion

4.1. DHI and melanin formation during defense responses

Melanin is a pigment produced by a wide variety of organisms via enzymatic conversion of mono- or diphenols (Nappi and Christensen, 2005). Melanin primarily exists in melanocytes, retinal cells, and macrophages of mammals for photoprotection (Riley, 2003), and it also protects cells against reactive oxygen species (ROS) in situations where a significant amount of ROS is present. As previously noted, both DHI and DHI carboxylic acid are cytotoxic (Pawelek and Lerner, 1978; Urabe et al., 1994), although at low concentrations DHI provides protective effects to retinal cells (Heiduschka et al., 2007).

DHI carboxylic acid also enhances production of nitric oxide generated by lipopolysaccharide-induced nitric oxide synthase (D'Acquisto et al., 1995).

While many aspects of melanin formation have been studied in arthropods (Kanost and Gorman, 2008; Cerenius et al., 2008), our understanding of the potential effector functions of DHI and other melanin precursors are more limited. Tyrosine hydroxylase, dopa decarboxylase, and dopachrome conversion enzyme are each induced upon immune challenge to catalyze the formation of dopa, dopamine and DHI. PO is a versatile enzyme that acts in multiple steps of eumelanogenesis to generate highly reactive quinones. Its precursor, proPO, is activated by a network of serine proteases and their homologs after pathogen-associated molecular patterns are recognized by receptor proteins in the plasma or on the surface of hemocytes (Jiang, 2008). Although it has long been assumed that the production of toxic quinone intermediates after proPO activation are involved in the killing of pathogens, only recently has any experimental evidence demonstrated that dopamine oxidation and DHI are antibacterial and antifungal (Zhao et al., 2007; Cerenius et al., 2010). Results of the current study provide further support for the role of DHI in immune defense by showing that it also exhibits antiviral and antiparasitic activities.

4.2. An extended survey for the antibiotic activity of DHI

Encapsulation, often accompanied by melanin deposition, is an effective defense mechanism against multicellular parasites or parasitoids (Strand, 2008). Parasitic wasps have reciprocally evolved strategies to evade or suppress host immune responses, which allow them to survive and develop in a hostile environment (Pennacchio and Strand, 2006; Nappi et al., 2009). For example, the wasp, *Leptopilina boulardi*, produces an immune suppressive factor to reduce the oxidation of dopamine and DHI in *D. melanogaster* (Kohler et al., 2007). A suite of proteins, encoded by wasp-associating polydnviruses, interfere with host immune responses such as proPO activation (Beck and Strand, 2007; Lu et al., 2008 and 2010), NF- κ B phosphorylation and translocation (Thoetkiattikul et al., 2005), phagocytosis (Beck and Strand, 2005), and apoptosis (Suderman et al., 2008). While these results underscore the importance of such anti-parasitoid responses, our data directly demonstrate the killing of wasp embryos by PO-generated reactive intermediates (Fig. 3). Moreover, since DHI-related compounds have to penetrate the egg chorion to kill the wasp embryo, we speculate these chemicals are likely also effective against other metazoan parasites like filarial worms and protozoans like *Plasmodium* (Collins et al., 1986; Lanz-Mendoza et al., 2002; Beerntsen et al., 2000).

In contrast to its anti-parasitic activity, the role of PO in insect antiviral responses has been less clear. On one hand, melanization of AcMNPV occurs in semipermissive but not fully permissive hosts (Trudeau et al., 2001). PO inhibitors also abolish the virucidal activity of *H. virescens* plasma against *H. zea* single-capsid NPV (Shelby and Popham, 2006). In *Lymantria dispar*, MNPV infection generates more PO activity and tissue melanization, which in turn reduces viral infection (McNeil et al., 2010). On the other hand, Saejeng et al. (2010) reports that plasma PO activity remains unchanged after infection of resistant *Plodia interpunctella* by a granulosis virus, yet significantly increases after infection of susceptible *P. interpunctella*. Here, we present *ex vivo* data showing that DHI and its oxidation products are virucidal (Figs. 1 and 2). This antiviral activity is also consistent with the polymerization of biological macromolecules such as DNA and proteins (Figs. 8-10). Protein crosslinking may decrease mobility of viruses or abolish interaction with receptors for entering host cells. DHI-related compounds may also modify viral DNA or RNA to interfere with genome replication and gene transcription.

4.3. Cytotoxic effects of DHI-related compounds

Since chemical modification of proteins and nucleic acids underlies, at least in part, the antibiotic activity of DHI against pathogens and parasites, it is difficult to imagine DHI does not also damage host cells. Indeed, we observed dramatic effects of DHI on *Sf9* cells (Figs. 4-7), which also underscores the importance of various regulatory mechanisms that restrict proPO activation to sites of infection. For example, various mutations in components that regulate the proPO activation system including necrotic, serpin-27A, and Persephone cause larval death in large measure because they result in global melanization of the insect which then likely dies from systemic damage to cells caused by DHI and other reactive intermediates (Ayres and Schneider, 2008).

DHI-induced death of *Sf9* cells shares certain features of apoptosis (*e.g.* phosphatidylserine flipping and DNA fragmentation). While a small population of cells went through membrane blebbing and apoptotic body formation, more cells formed of larger vesicles containing nuclei and cytoplasmic organelles to rupture from cells (Figs. 5 and 6). We also observe that DHI treatment causes filamentous actin to shift from an even distribution in cells to a condensed distribution that is associated with the formation of spikes that penetrate into the cytoplasmic membrane (data not shown). We thus suggest that DHI and its oxidization products enter cells, where they interact with actin, other cellular proteins, and nucleic acids in a manner that causes rapid death.

In summary, our studies demonstrate the broad-spectrum antiparasitic activity of DHI and other reactive intermediates against pathogens and parasites, which is in agreement with a defense role for the proPO activation system. However, we still need to interpret such *in vitro* data with caution. Dopamine, a key precursor of DHI, is a major catechol in plasma but its low concentration (0.2 mM) may limit its antimicrobial and/or antiparasitic activity (Hopkins et al., 1984). DHI is also detected in the hemolymph of *Drosophila* larvae parasitized by *L. bouhardi*, yet its concentration remains unknown (Nappi et al., 1992). Further studies are thus needed to determine the levels of DHI produced *in vivo* under different conditions and whether DHI production might be localized to sites of infection in order to minimize damage to host cells.

Acknowledgments

This work was supported by National Institutes of Health Grant GM58634 (to H.J.) and grants from the National Science Foundation (IOS 0749450) and United States Department of Agriculture (2007-04549) (to M.R.S.). This article was approved for publication by the Director of Oklahoma Agricultural Experimental Station and supported in part under project OKLO2450. We thank Drs. Michael Kanost, Jack Dillwith, and Stephen Marek for their helpful comments on the manuscript.

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Fig. 1. Effect of DHI on baculoviruses expressing *S. graminum* AChE1

A) Relative AChE activities in conditioned medium from infected *Sf9* cell cultures. The recombinant viral stock was treated with buffer (- DHI) or 1.25 mM DHI (+ DHI), and the mixtures were directly used to infect *Sf9* cells as described in *Section 2.1*. Enzymatic activities were measured and plotted as means $\pm$ SEM ($n = 3$) in the bar graph. **B)** Immunofluorescence detection of *S. graminum* AChE1 in *Sf9* cells infected with the control (*left*) and DHI-treated (*right*) viral stocks. The anti-(His)₅ monoclonal antibody and FITC-linked secondary antibody were used to label intracellular *S. graminum* AChE1 (*arrows*).

Fig. 2. Antiviral activity of DHI to lambda bacteriophages

A) Plaque formation on a lawn of *E. coli* XL1-blue cells infected with λ phage. The phage stock was treated with buffer alone (*top*) or 1.0 mM DHI in SM buffer (*bottom*). Plaque assays were carried out as described in *Section 2.2*. **B)** Concentration-dependent killing of λ phage. A sigmoidal dose-response curve was used to fit experimental data (mean $\pm$ SEM, $n = 4$) and calculate LC_{50} .

Fig. 3. Effect of DHI on viability of *M. demolitor* embryos

Newly laid wasp eggs were collected from hosts and treated with DHI. Their viability was then determined as described in *Section 2.3*. Data were fit to a sigmoidal dose-response curve as described in Fig. 2. Error bars equal SEM ($n = 3$).

Fig. 4. Cytotoxicity of DHI on *Sf9* cells

A) PB: *Sf9* cells were treated by 0, 1, 10, 20, 50, 100 μM DHI in 50 mM sodium phosphate (pH 6.5) and stained with MTT as described in *Section 2.4*. OD_{495} readings of DHI-treated cells were normalized against the control (100%, no DHI). **B)** Medium: *Sf9* cells were treated by 0, 31, 63, 94, 125, 188, 250, 375, 500 μM DHI in Sf900-III medium. Following PI and Annexin V staining, survivals of *Sf9* cells were quantified by flow cytometry, normalized against that of the control (100%, no DHI), and shown as mean $\pm$ SEM (**A**, $n = 6$; **B**, $n = 2$). A sigmoidal dose-response curve was used for data fitting and LC_{50} calculation.

Fig. 5. Morphological changes in *Sf9* cells after DHI treatment

In the upper panels, nuclei of control (- DHI) and treated (+ DHI) cells were stained with DAPI. The cells were observed under bright field on a fluorescence microscope. In the lower panels, cells (- or + DHI) were stained with the vital dye Evans Blue and observed under bright field as described in *Section 2.5*. Images were processed with Auto Levels in Photoshop. Cells stained blue have damaged membranes that allowed the dye to enter. Note that most of these cells also contain brown/black pigment indicative of DHI entry and melanin formation. *Arrowheads* point to membranous sacs (vesicles) that commonly formed after exposure of cells to DHI: the *red* ones for vesicles stained weakly by Evans Blue and the *black* ones for vesicles stained strongly.

Fig. 6. Early-stage apoptosis signal in DHI-treated *Sf9* cells

After buffer (A) or DHI (B) treatment, the cells were stained with FITC-Annexin V and PI (Section 2.6) and observed under bright (*left*) or dark (*right*) field with fluorescence. Images were processed with Auto Levels in Photoshop. *White circles* mark a group of healthy cells (Annexin V- and PI-negative). *White arrows* point to Annexin V-positive and PI-negative cells, whose contents were polarized and about to be released. *Red arrows* mark PI-positive cells that were releasing contents and, thus, PI-positive. *Blue arrows* indicate small, melanized bodies that are Annexin V- and PI-negative.

Fig. 7. Formation of DNA ladder in *Sf9* cells treated by DHI

Genomic DNA was isolated from control and DHI-treated cells as described in *Section 2.7* and separated by 1% agarose gel electrophoresis. *Left* lane, DNA of the control cells; *middle* lane, 1 kb DNA size markers (12.2, 1.6, 1, 0.85, 0.65, 0.5, 0.4, 0.3, 0.2, 0.1 kb); *right* lane: DNA from DHI-treated cells.

Fig. 8. Effect of DHI on mobility of circular and linear plasmid DNA after 1.5 h (A) and overnight (B) incubation

Left, circular DNA; right, linearized plasmid DNA. M, 1 kb DNA size markers. DHI concentrations are indicated on top of individual lanes.

Fig. 9. Enzyme activity of control and DHI-treated *S. graminum* AChE1

Fig. 10. Concentration-dependent reduction of monomeric BSA after DHI treatment
A) 7.5% native gel; **B)** 7.5% SDS-PAGE (nonreducing); **C)** 7.5% SDS-PAGE (reducing) with sizes of the M_r standards marked on the right; **D)** particle sizes of BSA after incubation with 0, 0.25, and 0.50 mM DHI. The DHI-treated BSA samples were mixed with DTT and SDS before size measurement on a Zetasizer. Peaks represent size distributions of protein particles (r , nm).