Effects of the chitin synthetase inhibitor plumbagin and its 2-demethyl derivative juglone on insect ecdysone 20-monooxygenase activity

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Summary. The chitin synthetase inhibitor plumbagin and its 2-demethyl derivative juglone were found to inhibit in a dose-response fashion the cytochrome P-450 dependent ecdysone 20-monooxygenase activity associated with adult female *Aedes aegypti*, wandering stage larvae of *Drosophila melanogaster*, and fat body and midgut from last instar larvae of *Manduca sexta*. The concentration of these naphthoquinones required to elicit a 50% inhibition of the steroid hydroxylase activity in all the insects was approximately 1×10^{-4} M.
Key words. Cytochrome P-450; ecdysone 20-monooxygenase; juglone; plumbagin; steroid hydroxylase.

Insect steroid hydroxylases catalyze several reactions in the biosynthesis of ecdysteroids from cholesterol[1-3]. Although most of these insect steroid hydroxylases remain to be studied[3-5], several laboratories have extensively investigated ecdysone 20-monooxygenase (EC 1.14.99.22), the steroid hydroxylase responsible for the conversion of the insect molting hormone ecdysone to its more active metabolite 20-hydroxyecdysone[6-12]. These studies have revealed that isozymic variants of this enzyme may exist but that all of these C-20 hydroxylase systems essentially are NADPH requiring cytochrome P-450 dependent monooxygenases.

Given the importance of ecdysteroids in insect postembryonic development and reproduction, the cytochrome P-450 dependent steroid hydroxylases pose potential sites of vulnerability for possible exploitation by plants in the natural selection for their development of resistance to insects. Previous studies in our laboratory have demonstrated that numerous plant allelochemicals including flavonoids and terpenoids can affect insect steroid hydroxylase activity[13-15]. The most effective allelochemicals were found to be those previously reported to exhibit insect phagorepellent properties or to act as inhibitors of insect ecdysis[16]. Accordingly, in the present study we have investigated the effects of the insect ecdysis and chitin synthetase inhibitor plumbagin[17,18] and its phagorepellent 2-demethyl derivative juglone[19-22] on insect ecdysone 20-monooxygenase activity.

Materials and methods. Animals. The animals used in these investigations were: day-3–5 sucrose-fed posteclosion adult females of the Rock strain of *Aedes aegypti*; wandering stage third instar larvae of the Canton S strain of *Drosophila melanogaster*; and day-4 and day-5 non-diapausing gate II fifth instar larvae of the tobacco hornworm, *Manduca sexta*. Animals were reared and staged as previously described[11,23,24].

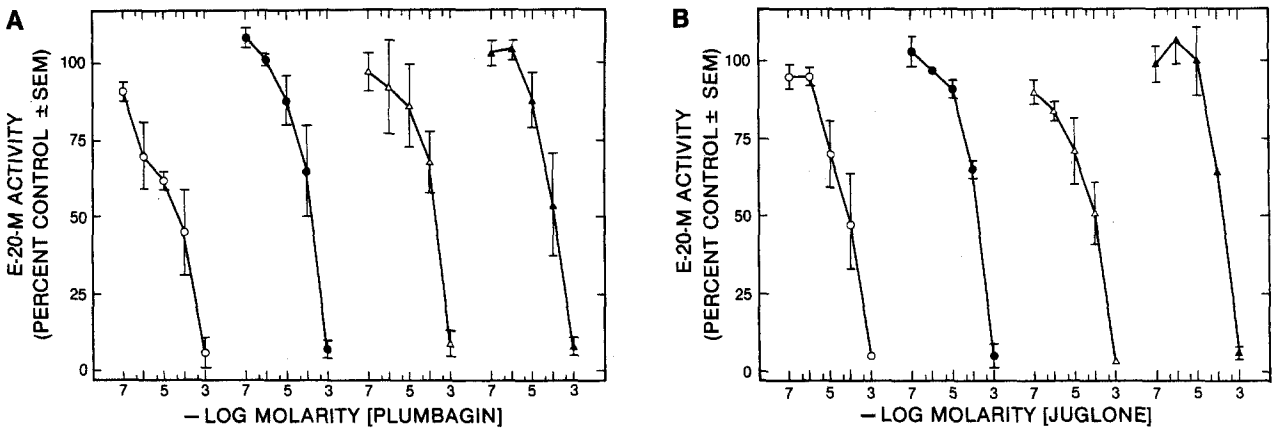
Ecdysteroids and chemicals. The radiolabeled ecdysteroid substrate for the monooxygenase assay was [23, 24-^{3}H]-ecdysone (stocks of 45, 70, and 80 Ci/mmol, radiopurity >99%) purchased from New England Nuclear, Boston, MA. The ecdysteroid standards, ecdysone and 20-hydroxyecdysone, were purchased from Fluka Chemical Corp., Ronkonkoma, NY; salts, organic solvents, and scintillation fluid (Scinti Verse E) were purchased from Fisher Scientific Co., Cleveland, OH; NADPH and the naphthoquinones juglone and plumbagin were purchased from Sigma Chemical Co., St. Louis, MO.

Tissue preparation and homogenization. Fat body from day-4 and midgut from day-5 gate II fifth instar larvae of *Manduca sexta* were dissected in a lepidopteran Ringer's at 4 °C[25]. Homogenates of the above tissues were made at 20 mg/ml for midgut and 100 mg/ml for fat body in sodium phosphate buffer (50 mM, pH 7.5, containing 250 mM sucrose) using a Potter-Elvehjem tissue grinder with a motor-driven Teflon pestle (275 rpm, 20 strokes, 0–4 °C). Abdomen homogenates of day-3–5 posteclosion adult female *Aedes aegypti* were made using the same buffer at 48 abdomen equiv/ml buffer. Homogenates of wandering stage third instar larvae of *Drosophila melanogaster* were made at a concentration of 33 mg/ml in 100 mM sodium phosphate buffer (pH 7.5, 250 mM sucrose).

Ecdysone 20-monooxygenase assay. Ecdysone 20-monooxygenase activity was detected and quantified using a radioassay. For the assay, 0.05-ml aliquots of insect homogenate (containing 2.4 mg abdomen equiv. of *Aedes*, 1.65 mg tissue equiv. of *Drosophila*, 5 mg tissue equiv. of *Manduca* fat body, or 1 mg tissue equiv. of *Manduca* midgut) were added to 0.05-ml aliquots of respective homogenization buffer (minus sucrose) containing [23, 24-^{3}H]-ecdysone (from 1.1×10^{-8} to 3.3×10^{-7} M assay concentration; 1.4–40 Ci/mmol), NADPH (1.6×10^{-3} M assay concentration) and varying concentrations of juglone or plumbagin (from 1×10^{-7} to 1×10^{-3} M assay concentration). Incubation was for 30 (*Drosophila, Manduca*) or 60 min (*Aedes*) at 35 °C with constant agitation. All assays were run in duplicate with zero time controls and were terminated by the addition of 2.0 ml ethanol. Following termination, the assay mixtures were centrifuged at $10{,}000 \times g$ for 10 min and 0.2-ml aliquots of the assay supernatant (containing from 5 to 10×10^3 dpm) plus 2 µg each of ecdysone and 20-hydroxyecdysone standards were evaporated to dryness. The residues were redissolved in methanol and streaked on analytical thin layer chromatography plates (0.25 mm silica gel 60, F-254; E. Merck, Darmstadt, Germany). The plates were developed in a solvent system of chloroform-95% ethanol (4:1, v/v) and the ecdysone and 20-hydroxyecdysone bands were visualized under short wavelength UV light. The ecdysteropid bands were scraped into scintillation vials, resuspended in scintillation fluid and counted using a Beckman Model 7000 scintillation counter (^{3}H counting efficiency, 60%). Control ecdysone 20-monooxygenase activity was expressed as pg 20-hydroxyecdysone formed/min/mg tissue equiv. or per abdomen equiv.; plumbagin and juglone effects on ecdysone 20-monooxygenase activity were expressed as percent of control (±SEM).

Results and discussion. The insect and chitin synthetase inhibitor plumbagin[17,18], was found to inhibit in a dose response fashion the ecdysone 20-monooxygenase activity associated with the abdomens of adult female *Aedes aegypti*, wandering stage larvae of *Drosophila melanogaster*, and the fat body and midgut from last instar larvae of *Manduca sexta* (fig. A). A similar dose-response profile of enzyme inhibition was obtained in all the insects with juglone, a 2-demethyl derivative of plumbagin (fig. B). To the best of our knowledge, this is the first demonstration that plumbagin and juglone, or naphthoquinones in general, can directly affect ecdysteroid biosynthesis or cytochrome P-450 dependent steroid hydroxylase activity.

At lower concentrations, 10^{-7} or 10^{-6} M, neither plumbagin nor juglone elicited significant levels of inhibition of ecdysone 20-monooxygenase activity in any of the insects (fig.). As the concentration of both naphthoquinones was raised to



Effects of increasing concentrations of plumbagin (A) and juglone (B) on the ecdysone 20-monooxygenase (E-20-M) activity in homogenates of abdomens from day-3–5 posteclosion adult female *Aedes aegypti* (open circles); wandering stage third instar larvae of *Drosophila melanogaster* (closed circles); fat body (open triangles) and midgut (closed triangles) from gate II fifth instar larvae of *Manduca sexta*. E-20-M activities are expressed as percent of control and each value is the mean (±SEM) of 3–6 determinations in duplicate. Control E-20-M activities were: *Aedes*, 2.3 pg 20-hydroxyecdysone (20-HE) formed/min/abdomen; *Drosophila*, 13.2 pg 20-HE formed/min/mg tissue; *Manduca* fat body, 39.5 pg 20-HE formed/min/mg tissue; and *Manduca* midgut, 263.5 pg 20-HE formed/min/mg tissue.

10^{-5} M, plumbagin effectively lowered the steroid hydroxylase activity in *Aedes* abdomens to 62% of the control value but was relatively ineffective with respect to the other insects (fig. A). Similarly, juglone at this concentration lowered the monooxygenase activity in *Aedes* abdomens and *Manduca* fat body to 70 and 71%, respectively, of their control values (fig. B) but was ineffective in *Drosophila* larvae and *Manduca* midgut.

Increasing the plumbagin and juglone concentration to 10^{-4} M resulted in significant levels of inhibition of enzyme activity in all the insects. This concentration of both plumbagin and juglone approximated the amount required to elicit 50% (I_{50}) inhibition of ecdysone 20-monooxygenase activity in the insect preparations. Raising the plumbagin and juglone assay concentration to 10^{-3} M resulted in almost complete inhibition of the steroid hydroxylase activity in all of the insects.

Based on the dose-response profiles of inhibition, it is clear that both plumbagin and juglone are effective inhibitors of insect ecdysone 20-monooxygenase activity. Previous studies have revealed that plumbagin is an inhibitor of larval ecdysis in such lepidopterans as *Pectinophora gossypiella, Trichoplusia ni,* and *Heliothis virescens* and *H. zea*[17,18]. It has furthermore been proposed that such ecdysis inhibition may be due to an inhibitory effect of plumbagin on chitin synthetase activity. However, in these earlier studies, plumbagin at a 3×10^{-4} M assay concentration was found to elicit an approximate 30% inhibition of chitin synthetase activity in vitro[17,18], whereas in the present study such an assay concentration of plumbagin would be graphically estimated to inhibit ecdysone 20-monooxygenase activity by approximately twice as much, i.e. by 65–75% (fig. A). Based on these data, it would appear that cytochrome P-450 dependent steroid hydroxylase activity may be more sensitive to plumbagin than is chitin synthetase activity. Accordingly, this suggests that the disruption of monooxygenase activity more so than chitin synthetase activity may contribute to the inhibition of insect ecdysis by plumbagin. However, in the present study juglone was as effective as plumbagin in inhibiting ecdysone 20-monooxygenase activity. Several earlier studies have demonstrated that juglone is an effective insect phagorepellent[19–22], but it has not previously been shown to inhibit insect ecdysis even at concentrations up to 2500 ppm[17,18].

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1 Smith, S. L., Bollenbacher, W. E., and Gilbert, L. I., in: Progress in Ecdysone Research, p. 139. Ed. J. A. Hoffmann. Elsevier/North Holland Biomedical Press, Amsterdam 1980.
2 Rees, H. H., in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7, p. 249. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
3 Smith, S. L., in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7, p. 295. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
4 Kappler, C., Kabbouh, M., Durst, F., and Hoffmann, J. A., Insect Biochem. *16* (1986) 25.
5 Kabbouh, M., Kappler, C., Hetru, C., and Durst, F., Insect Biochem. *17* (1987) 1155.
6 Johnson, P., and Rees, H. H., Biochem. J. *168* (1977) 513.
7 Feyereisen, R., and Durst, F., Eur. J. Biochem. *88* (1978) 37.
8 Smith, S. L., Bollenbacher, W. E., Cooper, D. Y., Schleyer, H., Wielgus, J. J., and Gilbert, L. I., Molec. cell. Endocr. *15* (1979) 111.
9 Weirich, G. F., Svoboda, J. A., and Thompson, M. J., Archs Insect Biochem. Physiol. *2* (1985) 385.
10 Smith, S. L., and Mitchell, M. J., Insect Biochem. *16* (1986) 49.
11 Mitchell, M. J., and Smith, S. L., Insect Biochem. *16* (1986) 525.
12 Srivatsan, J., Kuwahara, T., and Agosin, M., Biochem. biophys. Res. Commun. *148* (1987) 1075.
13 Smith, S. L., Ohio J. Sci. *83* (1983) 69.
14 Mitchell, M. J., and Smith, S. L., Am. Zool. *26* (1986) 56A.
15 Smith, S. L., and Mitchell, M. J., Biochem. biophys. Res. Commun., in press.
16 Reese, J. C., and Holyoke, C. W., in: CRC Handbook of Natural Pesticides, vol. III B, p. 21. Eds E. D. Morgan and N. B. Mandava. CRC Press, Boca Raton 1987.
17 Kubo, I., Uchida, M., and Klocke, J. A., Agric. biol. Chem. 47 (1983) 911.
18 Kubo, I., and Klocke, J. A., in: Natural Resistance of Plants to Pests-Roles of Allelochemicals, p. 206. Eds M. B. Green and P. A. Hedin. American Chemical Society, Washington, D.C. 1986.
19 Gilbert, B. C., Baker, J. E., and Norris, D. M., J. Insect Physiol. *13* (1967) 453.
20 Baker, J. E., and Norris, D. M., Experientia *28* (1972) 31.
21 Norris, D. M., and Chu, H. M., J. Insect Physiol. *20* (1974) 1687.
22 Rodriguez, E., and Levin, D. A., in: Biochemical Interaction Between Plants and Insects, p. 214. Eds J. W. Wallace and R. L. Mansell. Plenum Press, New York 1976.
23 Schlaeger, D. A., Fuchs, M. S., and Kang, S.-H., J. Cell Biol. *61* (1974) 454.
24 Vince, R. K., and Gilbert, L. I., Insect Biochem. *7* (1977) 115.
25 Smith, S. L., Bollenbacher, W. E., and Gilbert, L. I., Molec. cell. Endocr. *31* (1983) 227.

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