Azadirachtin Inhibits Proliferation of Sf 9 Cells in Monolayer Culture

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Azadirachtin A in ppm quantity inhibits proliferation and monolayer formation of *Spodoptera frugiperda* (Sf 9) insect cells in monolayer culture. The incubated cells demonstrate reduced rates of protein synthesis which finally leads to cell death. This growth inhibiting activity is compared with other botanicals such as ohchinin, salannin and volkensin. The high and specific activity of azadirachtin A on insect cells is discussed in comparison with its effect on a mammalian cell line, based on 2 D PAGE analysis of the total protein contents.

Azadirachtin A (**I,** Fig. 1) is the most prominent isomer of a series of limonoids (tetranortriterpenoids) which are present in the seed kernels of the tropical neem tree, *Azadirachta indica* A. Juss. The azadirachtins affect growth, reproduction and metamorphosis in diverse insect taxa [1–3]. However, whereas the *corpus cardiacum* has been identified as a target for azadirachtin [4], its mode of action on a molecular level and, consequently, its molecular target is still unknown. The standard bioassay for evaluating the growth-disrupting activity of azadirachtins follows the effects on metamorphosis of the Mexican bean beetle, *Epilachna varivestis* Muls. [5]. This highly reliable test, however, requires up to three weeks each, due to the retardation of larval and pupal development after azadirachtin treatment. There is a chance for overcoming this disadvantage, for routine screening primarily, by immunological or cell culture techniques.

No instance was found in literature of evaluating the biological activity of azadirachtin in an insect cell culture system, which would be the basis for a fast and reliable assay when compared with the existing bioassays. Cell lines have primarily been derived from a wide variety of lepidopteran species. Use of such cell cultures would also pave the way for answering several questions about the *in vivo* molecular targets of azadirachtin. Lepidopteran cell lines derived from *Manduca sexta* have, *e.g.*, been used to address questions about chitin synthesis and diflubenzuron activity [6].

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In our continuing efforts to demonstrate the molecular target of azadirachtin, we now provide evidence of the activity of azadirachtin A and several related compounds on the *Spodoptera frugiperda* ovarian cell line (Sf 9), which is currently in wide use as an *in vitro* eukaryotic protein expression system.

Materials and Methods

Insect cell culture

Sf 9 cells used in this study were derived from exponentially growing monolayer cultures (ATCA Cat. No. CRL 1711). About $2-2.5 \times 10^6$ cells were seeded into sterile 25 cm^3 cell culture flasks containing 4 ml of fresh TC 100 insect culture medium (Biochrom, Germany) and 10% fetal calf serum. The cells were allowed to attach to the bottom of the flask for 1 h.

Incubation with botanicals

Azadirachtin A (**I**), ohchinin (**II**), salannin (**III**), and volkensin (**IV**), with their structures presented in Fig. 1, were purified using standard protocols [7]. The compounds were dissolved in 30% ethanol/water and added to the culture flasks at a concentration of 1 µg/ml of the medium. A control flask was maintained containing only the solvents. The flasks were kept at 27 ± 1 °C for altogether 48 h and the cells monitored at every 12 h interval under an inverted microscope. Photomicrographs of the treated and control cells were made after 48 h.

Processing of the cells

The cells were harvested, washed in phosphate buffered saline and used for 2 D protein analysis or

stored for later use. The total protein content of the cells under various treatments was estimated using the Biorad DC protein assay kit. The 2D peptide maps of the total proteins were performed based on methods described in the literature [8]. The 2D gels developed after silver staining [9] were compared with respect to the total number of polypeptides.

Results

From the many limonoids which are present in meliaceae and which have structural similarities with azadirachtin A (**I**), three (**II–IV**) were selected for this study. They all share a similar structure of the two six-membered rings A and B, with two hydroxyl groups which are esterified with tiglic and acetic acid or, in case of ohchinin (**II**), with cinnamic acid and a free 3-OH group. **I** and **IV** have a free 7-OH group in the B-ring which by cyclization forms a tetrahydrofuran ring in **II** and **III**. From all the four limonoids, only azadirachtin A is active in the *Epilachna* bioassay.

The changes observed in the monolayer of the Sf 9 cells after treatment with a 1 ppm concentration of azadirachtin A are provided in Fig. 2. The Sf 9 cells have a normal population doubling time of 18–24 h and have to be split 2–3 times a week [10]. The characteristic changes observed in the azadirachtin treated flasks include the increase in the number of floating cells ("floaters") or loosely attached cells in the initial 24 h of treatment, followed by complete cell death (Fig. 2B). Already 24 h after treatment nearly 50% of the total cell population was noticed to aggregate and float. After 48 h the architecture of the monolayer was completely lost and only few cells were seen floating as compared to the control (Fig. 2A). In the case of cells treated with salannin (**III**), no characteristic change in the monolayer was discernible while a slighter change was seen in ohchinin (**II**) and volkensin (**IV**) treated flasks.

Estimation of the total protein contents in treated and normal cells showed considerable differences indicating the suppression of protein synthesis and, respectively, turnover in treated groups. In the case of normal cells the protein content was 249.0 µg/million cells, while it was only 138.0 µg in azadirachtin A treated cells. Salannin treated cells showed no significant reduction in their protein quantity (240.5 µg), while those after ohchinin and volkensin treatment showed 202.5 and 210.3 µg, respectively (Table I).

A critical examination of the 2D PAGE gels of the normal Sf 9 cells and after the various treatments also showed quantifiable differences in the

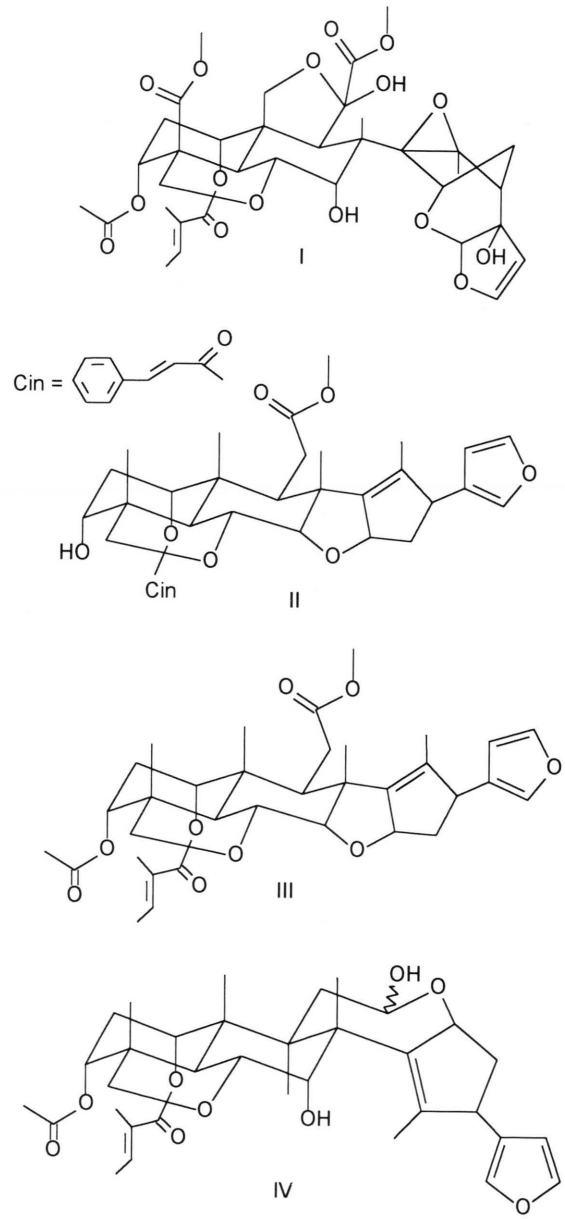

Fig. 1. Structures of the four limonoids, azadirachtin A (**I**), ohchinin (**II**), salannin (**III**), and volkensin (**IV**), as applied in this study.

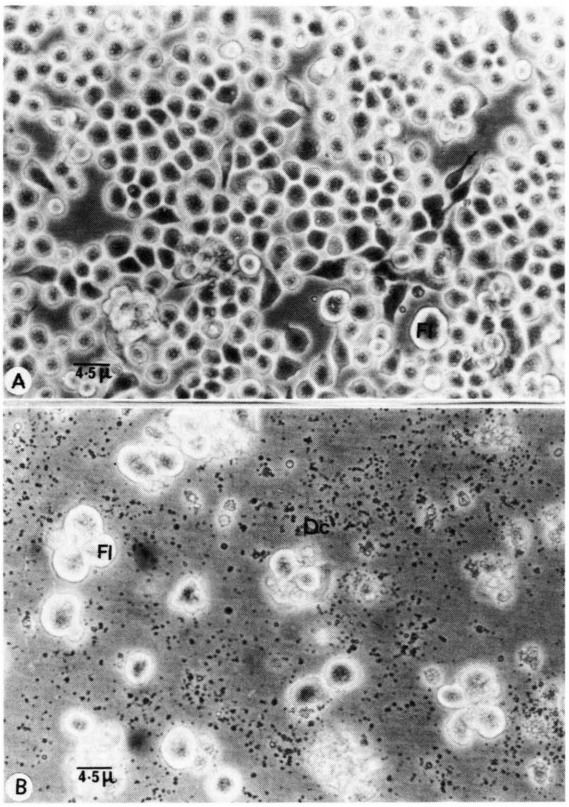

Fig. 2. Photomicrographs of monolayer cultures of (A) control and (B) azadirachtin A treated Sf 9 cells 48 h after treatment. Magnification 32 ×. Fl: floaters, Dc: dead cells.

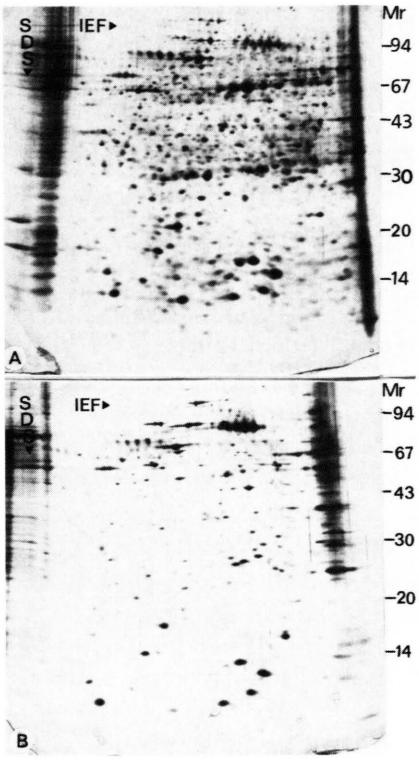

Fig. 3. 2D PAGE maps from whole cell lysates of (A) normal and (B) azadirachtin treated Sf 9 cells.

total number of polypeptides (Table I). The disappearance of several polypeptides was uniform to all the four treatments. However, azadirachtin treated cells show a reduction of 77.9% polypeptide spots in comparison with the control (Fig. 3). There are also polypeptides which resist as well azadirachtin as other treatments. The changes observed in the cases of ohchinin, salannin and volkensin treatments are provided in Fig. 4, which indicates a reduction in 35.0, 29.2 and 31.9% of the spots, respectively (Table I). A comparison made with these compounds using a mammalian cell line (Chinese Hamster Ovarian cells, ATCA Cat. No. 1001) did not bring out any change in the monolayer as well as in the 2D polypeptide pattern (data not shown).

Table I. Effect of azadirachtin A (aza A), ohchinin, salannin and volkensin on the total protein content and 2D polypeptide spots of Sf 9 cells.

Treatment	Total protein/ 1 million cells [µg]	% Loss	Number of 2D polypeptides	% Reduction
Control	249.0	–	489	–
Aza A (**I**)	138.0	44.6	108	77.9
Ohchinin (**II**)	202.5	18.7	318	35.0
Salannin (**III**)	240.5	3.4	346	29.2
Volkensin (**IV**)	210.3	15.5	333	31.9

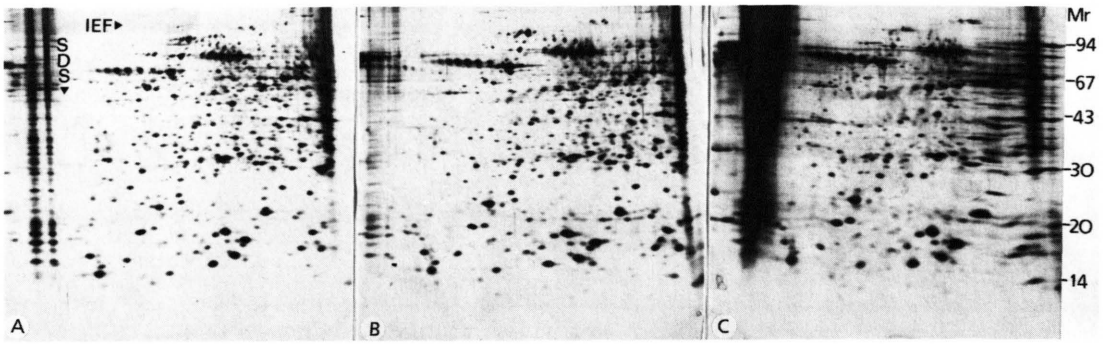

Fig. 4. 2D PAGE maps from whole cell lysates of (A) ohchinin, (B) volkensin and (C) salannin treated Sf 9 cells. Mr: Relative molecular mass × 10^3. Anode of the IEF separation is in all gels on the left.

Discussion

Many of the currently available bioassays for insect growth inhibitors provide the gross physiological effect of a particular compound on a species, but fail to indicate the actual mode of action. Davidson [11] indicates that cultured insect cells have proved very valuable in studies on the mode of action of bacterial toxins affecting insects, affording an opportunity to study the initial interaction of the toxins with their hosts in ways that would be impossible or difficult in the living hosts. Many aspects of the modes of action found in cultured cells appear to apply to the pathology in the host as well. However, caution must be exerted in extrapolating results from cultured cells to the living insect.

For azadirachtin A and its host of isomers the only standard bioassay demonstrated so far is the *Epilachna* assay. A diverse spectrum of insect species of agricultural and medical importance are reported to be sensitive to azadirachtin [12, 13]. They extremely differ in the response dose and in the type of response exhibited. A unique and reliable test protocol using an *in vitro* model will be of great value to extrapolate the results for laboratory and field situations. The Sf 9 cells offer such an opportunity due to the easy availability, established media and culture protocols. Current experiments with Sf 9 cells indicate the activity of azadirachtin on these ovarian cells. The moderate to no activity of other compounds such as ohchinin, salannin and volkensin reflect the level of activity *in vivo* in the *Epilachna* assay. The drastically reduced level of protein after azadirachtin A treatment and the suppressing effect on the number of polypeptides are reflected by a similar effect on the 2D polypeptide pattern in the desert locust, *Schistocerca gregaria,* brain and hemolymph [14]. But in no case such polypeptide spots were found in the insect cell culture which after azadirachtin treatment had been induced in these locusts. The arrest in growth and subsequent proliferation of azadirachtin treated Sf 9 cells indicate the efficiency of this substance to directly encounter the cell membrane without the aid of any carrier molecule as may be the case *in vivo*. Sterilization of the ovaries in locusts and other insect species due to azadirachtin treatment may also be due to a direct growth disrupting effect on the ovarian cells rather than indicating a hormonal suppression, or by both. The remarkably specific and high effect of azadirachtin on insect cells is also reflected through its inactivity against mammalian cells. Studies on the nature of suppressed (cytosolic/membrane) proteins, cell cycle specific interference and evaluation of other cell lines derived from diverse insect tissues can narrow down our current understanding towards the molecular target of azadirachtin. Further studies are in progress at our laboratory in this direction.

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