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# Cytotoxicological Effect of Tebufenozide, an Insect Growth Regulator (IGR): Stimulation of Filamentous Actin Reorganization and Enhancement of Hsp27 Expression in Drosophila Kc Cells

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**The cytotoxicological responses to insect growth regulator (IGR), using tebufenozide as ecdysteroid mimic, were investigated in Drosophila Kc cells. Treatment of Kc cells with tebufenozide showed significant growth inhibition and striking morphological changes including aggregation and elongation of the cells. In order to understand the cellular mechanism underlying the response of Drosophila cells to tebufenozide, immunofluorescence microscopy was performed. We found that treatment of Kc cells with tebufenozide enhanced the reorganization of f-actin and stimulated the expression of hsp27. These data suggest a possible association of filamentous actin (f-actin) and hsp27 in the cytotoxicological mechanisms of growth regulators in Drosophila cells.**

**Key Words: IGR, Tebufenozide, Drosophila, f-actin, hsp27**

## INTRODUCTION

As a class of powerful third-generation insecticides, insect growth regulator (IGR), specifically induces premature and lethal molt in many invertebrates. Tebufenozide, a class of IGR, was developed as a potent new ecdysone agonist. However, only a few reports have demonstrated the mechanisms of IGR specifically at the molecular and cellular levels to understand the cytotoxic effect from the points of cell biology and biochemistry.

Stress responses of cells universally conserved the combination of gene expression, protein synthesis, and reorganization of skeletal elements against environmental stress. Therefore, the exposure of cells and organisms to various environmental or pathophysiological stresses leads to the synthesis of a number of stress proteins including heat shock proteins (hsp) and filamentous actin (f-actin) (de Jong et al, 1993; Geneviève et al, 2000).

Heat shock proteins usually control cell death or protect damaged cells by minimization of protein aggregation for ensuring proper folding and transport. The belief that hsps protect the damage caused by stresses is supported by the fact that hsp expression parallels exactly the development and decay of thermotolerance (Li et al, 1991). The stress response can also be induced by adverse environmental situations, including exposure to heavy metals, oxidants, amino acid analogue, tissue trauma and microbial infections (Ritossa, 1962; Ropp et al, 1983). Drosophila hsp27 of hsp groups also reportedly plays a protective role in response to various stresses including free radicals (Mehlen et al, 1996; Wang et al, 2004). Moreover, Drosophila hsp27 has been known as an important modulator in metamorphosis induced by ecdysteroid hormone (Chen et al, 2002). Furthermore, f-actin reorganization is also one of the widely conserved cellular responses in eukaryotes against environmental stresses. In addition to an element of stress responses, f-actin formation has been shown to have a link to metamorphosis induced by the ecdysteroid hormone (Chen et al, 2004).

However, no report so far has been published on the roles of hsp27 and f-actin in the cytotoxicological effect of tebufenozide as an ecdysone agonist in the invertebrate system. Thus, it might be important to investigate the roles of f-actin and hsp27 in Drosophila Kc cells, in order to understand the cellular responses in terms of IGR as an insecticide.

## METHODS

### *Cell culture and treatment*

Drosophila Kc cells were cultured at 25°C in tissue culture flasks with D-22 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml penicillin and streptomycin (Sigma, USA). Tebufenozide (kindly provided by Dr. Yong-Man Yu, Kyungju Research Institute, Kyungnong Inc., Korea) dissolved in dimethyl sulfoxide (DMSO) was added directly to the culture flasks 3 days after subculture.

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**ABBREVIATIONS:** IGR, insect growth regulator; Hsp, heat shock protein; F-actin, filamentous actin; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide.

### *Cell growth inhibition assay*

The 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed for cell growth inhibition assay. Briefly, cells were seeded in 96-well tissue culture plates (Nunc Inc., USA), and were either untreated, or tebufenozide was added to the medium at the concentrations indicated. Viable cells remaining after the incubation time indicated were stained with yellow tetrazolium MTT. MTT reagent, 100 $\mu$l of 2 mg/ml solution in PBS, was added to the medium, and the plates were returned to the incubator for four hours. Visible blue precipitate correlated with cell density as viewed by a light microscope. Medium was centrifuged for 5 minutes at 500 g, aspirated, and the precipitate was then dissolved in 50 $\mu$l of DMSO per well. Absorbance of individual wells was determined at 490 nm by a microplate reader (Molecular Device Inc., USA). Absorbance of wells that received the treatments was expressed relative to that in untreated wells in the same experiment, and percent survival was obtained.

### *Measurement of apoptosis induction*

Morphological features of apoptotic phase were assessed with acridine orange/ethidium bromide staining. Condensed chromatin or fragment (dots) nucleus shows clearly in green as an indicator in apoptotic cells (Anne et al, 1995). According to these criteria, the evaluation of apoptotic phase was performed with a fluorescence microscope (Leica, Switzerland). After washing once with PBS, the treated cells were stained with 4 $\mu$g/ml acridine orange and 4 $\mu$g/ml ethidium bromide dissolved in PBS. Subsequently, apoptotic bodies were visually scored, and the fraction was determined as the percentage of cells.

### *Fluorescence microscopy*

Kc cells were allowed to remain on a cover glass in medium for 2 hours for better adherence of samples. Subsequently, they were rinsed gently in PBS, fixed with 3.7% formaldehyde in PBS for 30 minutes, and lysed with 1% Triton X-100 in PBS for 20 minutes. As blocking step, they were submerged in PBS containing 1% BSA (bovine serum albumin). In case of f-actin staining, they were incubated with 0.167 mM TRITC-phalloidin (Sigma) in PBS for 2 hours at room temperature. In case of detecting expression of hsp27, they were incubated with monoclonal anti-hsp27 (mouse anti-Drosophila hsp, diluted 1 : 100, kindly provided by Dr. Robert Tanguay at Laval Universite, Cananda) containing 0.1% BSA in PBS for 1 hour at room temperature, and then incubated with FITC-goat anti-mouse IgG (Fab specific, diluted 1 : 64, Sigma, USA) containing 0.1% BSA in PBS for 30 minutes. The samples were rinsed three times with PBS before examination. A Leica phase contrast microscope with an epifluorescence illuminator was used throughout the experiment. For statistical analysis, Sigma Plot software (SPSS Inc., USA) was employed.

## RESULTS AND DISCUSSION

The cytotoxicity of tebufenozide, a ecdysteroid mimic, was evaluated in Drosophila cell lines with MTT assay. As seen in Fig. 1A, the significant sensitivity to tebufenozide was observed, indicating the inhibitory effect of tebufenozide on proliferation of Drosophila cells. The $IC_{50}$ of tebufenozide on Kc cells was determined to be $1.866 \times 10^{-7}$ M, using Softmax Software (Molecular Device Inc, USA), from dose response curve. Indeed, tebufenozide has been reported to be more potent IGR in the field than other natural products. Interestingly, apoptosis was not observed in tebufenozide-treated cells (Fig. 1B), implying that cytotoxic mechanism might be through apoptosis-independent pathways, even though further investigations are in need.

It is clear that tebufenozide acts as an ecdysone agonist in Drosophila Kc cells. Therefore, the assay on the insecticide in vitro might be extended to other invertebrate cell culture systems, using ecdysone as IGR. Indeed, tissue culture techniques have been gaining the widespread use

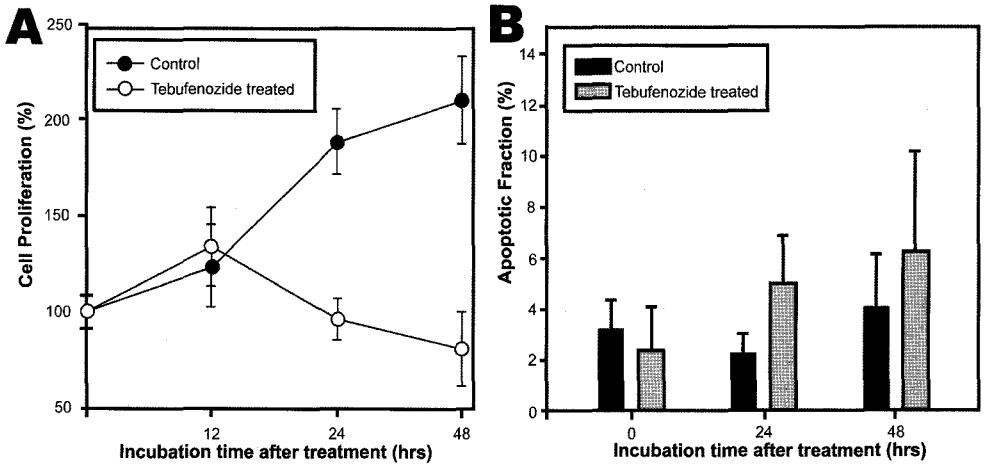


**Fig. 1.** Cytotoxic effect of tebufenozide on proliferation of Kc cells. (A) MTT assay showed significant inhibitory effect on cell growth ($p < 0.01$). (B) no significant effect on apoptosis induction has been observed in tebufenozide-treated cells ($p > 0.05$). Data points reflect mean of three independent experiments; bars, SD. Statistical analyses (t-test) were conducted with SigmaPlot Software (SPSS Inc., USA).

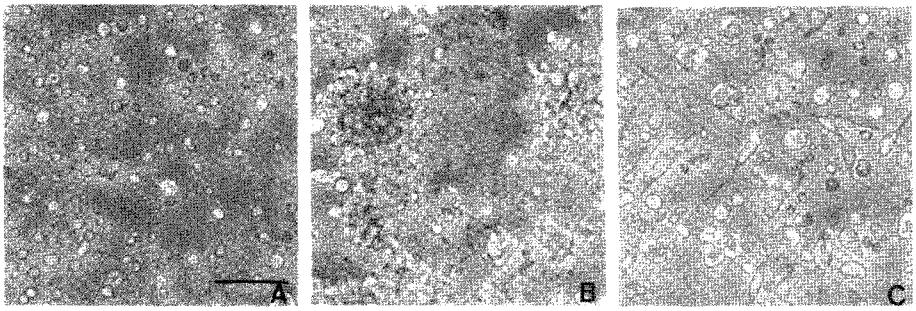


**Fig. 2.** Morphological alterations after treatment with tebufenozide. (A) Inverted micrographs of Drosophila Kc cells, incubated for 48 hours after treatment with DMSO-treated control, showing the roughly spherical shape as normal. (B) Incubated for 48 hours with $1\times10^{-6}$ M tebufenozide, showing the increase of clumping of the cells. (C) Incubated for 48 hours with $1\times10^{-6}$ M tebufenozide, disaggregated with gentle pipetting, showing the spindle shape as morphological change. Bar=50 $\mu$m.

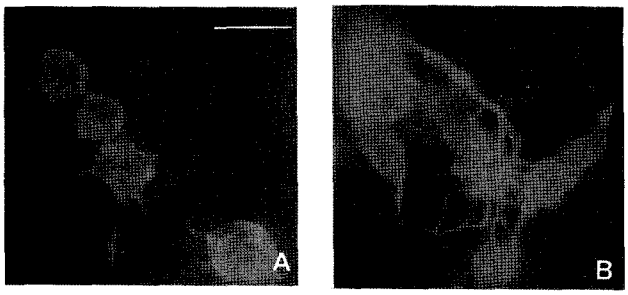


**Fig. 3.** Effect of tebufenozide on cytoskeleton of Drosophila Kc cells, showing significant alteration of f-actin pattern after the treatment with tebufenozide. (A) Fluorescence staining of f-actin in Kc cells, incubated with DMSO-treated control. (B) Incubated for 48 hours with $1\times10^{-6}$ M tebufenozide, showing significant increase of intensive f-actin labeling in cells. Bar=10 $\mu$m.

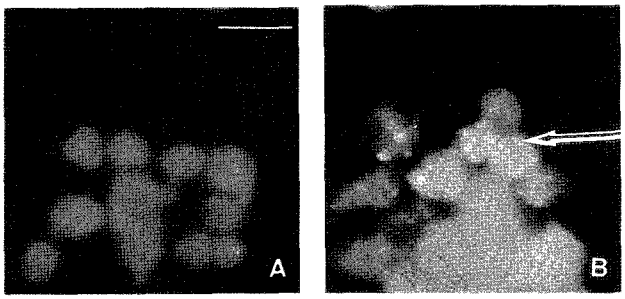


**Fig. 4.** Effect of tebufenozide on the expression of hsp27 in Kc cells, showing increase of hsp27 after treatment with tebufenozide (arrows indicate). (A) Immunofluorescence staining of hsp27 in Kc cells, incubated with DMSO-treated control for 4 hours as control. (B) Incubated for 4 hours with $1\times10^{-6}$ M tebufenozide, showing significant increase of intensive hsp27 labeling in cells. Bar=10 $\mu$m.

in the field of invertebrate endocrinology. Many of these investigations have utilized organs or tissues in short term cultures (Marks, 1980), and the majority of studies on insect hormones at the cellular level have been performed on cell lines derived from Diptera, particularly Drosophila. In these cells, hormone treatment induces various biochemical and cellular events, including elongation of cells, increase of acetylcholinesterase and galactosidase activity, cell clumping, and cessation of cell multiplication (Courgeon, 1972; Cherbas et al, 1977; Best-Belpomme et al, 1978). Particularly, Kc cell line, originally derived from Drosophila embryos (Echalier & Ohanessian, 1969), has been proved to be an excellent model system for the study of ecdysone on differentiation and morphogenesis (Cherbas et al, 1984) as well as for the investigation of early events in genomic regulation and protein synthesis (Cherbas et al, 1986; Riddiford, 1993; Hock et al, 2000). Therefore, Drosophila Kc cell lines might be one of the most useful tools not only for screening insecticides, but also for biochemical and physiological study of IGR (Nakagawa et al, 2002).

In the present study, dramatic morphological changes were observed by a Leica inverted microscope 48 hours after the treatment with $1\times10^{-6}$ M tebufenozide: The roughly spherical shape of Kc cells was changed into the spindle shape after the treatment (Fig. 2). As one of steroid molting hormones, tebufenozide is the mimic of 20-hydroxyecdysone, which is the physiological inducer of molting and metamorphosis in insects. In Drosophila Kc cells which are known as one of ecdysone-responsive systems, the insecticide tebufenozide was shown to mimic the action of 20-hydroxyecdysone by inducing morphological change and inhibiting cell proliferation.

In order to understand the mechanism of morphological alteration in response to tebufenozide, the effect on distribution of filamentous actin (f-actin) was investigated, using TRITC-phalloidine. As seen in Fig. 3, the increment of f-actin distribution was shown in tebufenozide-treated Kc cells. The result suggests that the major factor of morphological change induced by the treatment of tebufenozide might be stimulation of f-actin reorganization in Drosophila Kc cells. In order to investigate the effect of tebufenozide on hsp27 of Drosophila Kc cells, hsp27 was detected with immunofluorescence microscopy. As seen in Fig. 4, the induction of hsp27 was detected 4 hours after the treatment of $1\times10^{-6}$ M tebufenozide. These data suggest that the morphological change in Kc cells, induced by ecdysteroid treatment, has significant relevance with actin filament and hsp. These results are consistent with other reports on mammalian cells treated with steroid hormones, suggesting that the cellular pathways in response to steroid hormones are conserved in eukaryotes.

In conclusion, as one of the cytotoxiclogical mechanisms

of tebufenozide as a potent IGRs, we showed significant growth inhibition accompanied with dramatic morphological alteration in Drosophila Kc cells. Futhermore, a possible role of f-actin and hsp27 in response to the treatment of tebufenozide was firstly suggested. The present observation is expected to give an insight into understanding the cytotoxicological effects of insecticide as growth hormone regulator (IGR), although further studies are needed.

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## REFERENCES

Anne JM, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR. Cell Death. In: Schwartz L, Osborne B ed, *Methods in Cell Biology*. Academic Press, San Diego, CA, p 153−185, 1995

Best-Belpomme M, Courgeon AM, Rambach A. Galactosidase is induced by hormone in Drosophila melanogaster cell cultures. *Proc Natl Acad Sci USA* 75: 6102−6106, 1978

Chen L, Reece C, O'Keefe SL, Hawryluk GW, Engstrom MM, Hodgetts RB. Induction of the early-late Ddc gene during Drosophila metamorphosis by the ecdysone receptor. *Mech Dev* 114: 95−107, 2002

Chen GC, Gajowniczek P, Settleman J. Rho-LIM kinase signaling regulates ecdysone-induced gene expression and morphogenesis during Drosophila metamorphosis. *Curr Biol* 14: 309−313, 2004

Cherbas L, Bene H, Bourouis M, Burtis K, Chao A, Cherbas P, Crosby M, Garfinkel M, Guild G, Hogness D, Jami J, Jones CW, Koehler M, Lepesant JA, Martin C, Maschat F, Mathers P, Meyerowitz E, Moss R, Pictet R, Rebers J, Richards G, Roux J, Schulz R, Segraves W, Thummel C, Vijayraghavan K. Structural and functional analysis of some moulting hormone-responsive genes from *Drosophila*. *Insect Biochem* 16: 241−248, 1986

Cherbas L, Fristrom JW and O'connor JD. Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. In: Hoffmann J, Porchet M ed, *Proceedings in Life Sciences*. Springer-Verlag, Berlin, p 305−322, 1984

Cherbas P, Cherbas L, Williams CM. Induction of acetylcholinesterase activity by -ecdysone in a Drosophila cell line. *Science* 197: 275−277, 1977

Courgeon AM. Action of insect hormones at the cellular level: Morphological changes of a diploid cell line of Drosophila melanogaster treated with ecdysone and several analogs in vitro. *Exp Cell Res* 74: 327−336, 1972

de Jong WW, Leunissen JA, Voorter CE. Evolution of the alpha-crystallin/small heat-shock protein family. *Mol Biol Evol* 10: 103−126, 1993

Echalier G, Ohanessian A. Isolation in tissue culture, of Drosophila melangaster cell lines. *C R Acad Sci Hebd Seances Acad Sci D.* 268: 1771−1773, 1969

Geneviĕve M, Yutaka I, Kanefusa K, Robert MT. The small heat shock protein Hsp22 of Drosophila melanogaster is a mitochondrial protein displaying oligomeric organization. *J Biol Chem* 275: 31204−31210, 2000

Hock T, Cottrill T, Keegan J, Garza D. The E23 early gene of Drosophila encodes an ecdysone-inducible APT-binding cassette transporter capable of repressing ecdysone-mediated gene activation. *PNAS* 97: 9519−9524, 2000

Li GC, Li L, Liu YK, Mak JY, Lee C. Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding genes. *Proc Natl Acad Sci USA* 88: 1681−1685, 1991

Marks EP. Insect tissue culture: An overview. *Annu Rev Entomol* 73: 1971−1978, 1980

Mehlen P, Kretz-Remy C, Preville X, Arrigo AP. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNF alpha-induced cell death. *EMBO J* 15: 2695−2706, 1996

Nakagawa Y, Minakuchi C, Takahashi K, Ueno T. Inhibition of [$^3$H]ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem and Mol Biol* 32: 175−180, 2002

Riddiford LM. Atlas of Drospheila Development: Drosophila Third Instar Eye Disk Chart. In: Bate M, Arias AM ed, *The development of Drosophila melanogaster*. Cold Spring Horbor Lab Press, Plainview, NY, p 899−939, 1993

Ritossa F. A New puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia* 18: 571−573, 1962

Ropp M, Courgeon AM, Calvayrac R, Best-Belpomme M. The possible role of the superoxide ion in the induction of heat-shock and specific proteins in aerobic Drosophila cells during return to normoxia after a period of anaerobiosis. *Can J Biochem Cell Biol* 61: 456−461, 1983

Wang HD, Kazemi-Esfarjani P, Benzer S. Multiple-stress analysis for isolation of Drosophila longevity genes. *PNAS* 101: 12610−12615, 2004