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FINAL REPORT
UNIDO Contract N° 91/243P
Project No:DP/HUN/86/006

Practical Development of Non-Toxic Anti-Insect Agents: Activities in other Areas based on Interference with Molting Hormonal Activity

Objectives:

To determine relative biological activities of synthetic non-steroidal mimics of natural molting hormones of insects, ecdysteroids.

Methods:

An insect epidermal cell line established from imaginal wing discs of the indian meal moth, *Plodia interpunctella* , was proved to be responsive to natural ecdysteroids. Active forms of the molting hormones induced cessation of growth of cell population, aggregation of the cells and stimulation of uptake of N-acetyl-D-glucosamine (GlcNAc), a precursor of chitin which the major component of insect exoskeletton. These properties of the cell line have been used for monitoring the relative activities of 9 different non-steroidal potent mimics of ecdysteroids synthesized by Dr. Màtolczy and for comparing them to the activies of true ecdysteroids and an active non -steroidal mimic.

Reagents:

Ecdysteroids used in the study were kindly provided by Dr. R. Lafont. Non-steroidal agonists were supplied by Dr. G. Matolczy.

Cell culture:

IAL-PID2 cells were maintained at 26°C in 25-cm2 tissue culture flasks with 4 ml of antibiotic-free Grace's medium with 10% heat-inactivated fetal bovine serum and otherwise modified according to Yunker et al..

Experiments were initiated by seeding flasks with $5x10^5$ cells in 4ml of medium. Hormones and potent agonists were added 3 days after subculture for a further 3 day period except in three flasks used as controls. Three replicates of each treatments were performed and three untreated flasks served as controls. At the end of this period, three different tests were performed on the cells

Effect on growth of cell population:

Cells were exposed to final concentrations of 10^{-7}M, 10^{-6}M or 10^{-5}M of test compounds.

Cell numbers were determined at the end of the treatment by hemocytometer counts on the 3 replicate cultures ran under identical conditions. Inhibitory effect of hormones and agonists on growth of cell population was calculated as follows:

$$\frac{\text{mean number of cells in treated flasks-mean number of cells in controls}}{\text{mean number of cells in controls}} \times 100$$

Effect on cell morphology:

Upon continuous exposure to increasing concentrations of 20-hydroxyecdysone for 3 days the isolated fibroblastic cells first lost their apical expansions and rounded, then formed aggregates and finally were embedded in a fibrillar material. Morphology was studied at the end of each experiment by phase-contrast microscopy on living cultures utilizing an inverted microscope (Nikon)reverse phase and the aspect of the culture thoroughly checked. Activity of the treatment for each concentration of hormone or agonist was scored as follows:

0: no change, mostly single fibroblastic cells.
1: loss of cellular expansions
2: formation of aggregates
3: embedding of aggregates in fibrillar material

Final activity of each compound was determined by adding scores obtained for the three concentrations used.

Effect on GlcNAc uptake:
Cells were first exposed to final concentration of 10-6M of each test compound for 3 days. At the end of the treatment, uptake was measured by adding 5 µCi of [^{3}H]-GlcNAc (Spec. Act.: 11.2 Ci/mmol, Amersham, Les Ulis, France) to the tissue culture flasks, and harvesting the cells after a 1h30 incubation. The cells were removed gently from the flasks by pipetting, and were centrifuged at 200g for 5 min. They were then washed in 2ml of plain Grace's medium, centrifuged,and resuspended in 1.5ml of the same.A 0.5 ml aliquot of the suspension was centrifuged at 200g for 5 min, the supernatant was discarded, and the pellet was resuspended in 150 ml of Soluene 350 (Packard, Downers Grove,IL, USA). The pellets were stored in the Soluene for 24h at 4°C. The samples were then transferred to scintillation vials that contained 3ml of scintillation cocktail; and their radioactivity was measured with a liquid scintillation counter (Beta IV, Kontron Instruments, St Quentin en Yvelines, counting efficiency: 63%). Another aliquot of the suspension was used to determine cell number by countig them in a Malassez hemocytometer on a Wild microscope. Radioactivity is expressed as cpm/cell. On this cpm/cell basis uptake is then expressed as percentage of control.

Results:

Inhibition of growth of cell population:

	CONCENTRATIONS TESTED		
	10^{-7}M	10^{-6}M	10^{-5}M
20-HYDROXYECDYSONE	-16.5%	-30%	-39.5%
ECDYSONE	0%	0%	-25%
MAKISTERONE A	0%	-31%	-56%
PONASTERONE A	-51%	-49%	-47%
RH 5849	-7%	-24%	-51%
NKI 43597	-4%	-15%	-36.5%
NKI 43598	-5%	-27%	-44%
NKI 43897	-2.5%	-35%	-52.5%
NKI 43898	0%	0%	-19%
NKI 43639	0%	-10.5%	-16.25%
NKI 43640	0%	0%	-10%
NKI 43418	-0.5%	-9%	-37.5%
NKI 43720	-1.5%	-9.5%	-37.5%
NKI 43770	0%	0%	0%

Morphological changes:

	CONCENTRATIONS TESTED		
	10^{-7}M	10^{-6}M	10^{-5}M
20-HYDROXYECDYSONE	0	1	2
ECDYSONE	0	0	1
MAKISTERONE A	0	1	2
PONASTERONE A	1	2	3
RH 5849	0	1	2
NKI 43597	0	0	1
NKI 43598	0	1	2
NKI 43897	0	1	2
NKI 43898	0	0	1
NKI 43639	0	0	1
NKI 43640	0	1	2
NKI 43418	0	1	2
NKI 43720	0	1	2
NKI 43770	0	0	0

Stimulation of GlcNAc uptake:

	CONCENTRATION TESTED 10^{-6}M
20-HYDROXYECDYSONE	**+284%**
ECDYSONE	**+105%**
MAKISTERONE A	**+128%**
PONASTERONE A	**+257%**
RH 5849	**+4.4%**
NKI 43597	**0%**
NKI 43598	**+1.7%**
NKI 43897	**+104%**
NKI 43898	**+14%**
NKI 43639	**+38%**
NKI 43640	**0%**
NKI 43418	**+29%**
NKI 43720	**+55%**
NKI 43770	**+42%**

Only one concentration for each compound was assayed in this experiment. This concentration was chosen since it gave the major diversity of effects on cell proliferation for the various compounds.

General conclusions:

The study undertaken in this sub-contract intended to compare, *in vitro*, biological effects of different natural ecdysteroids and non-steroidal potent agonists.
The results obtained so far clearly indicate that different compounds exhibit differential actions in the 3 tests used.
The whole set of results is to be sent to Prof. G. Matolcsy who will compare them to its own *in vivo* results by detailed computer assisted graphic method and conformation analysis. From this comparison, I hope he will be able to predict the best structure for the active site in each type of assay and which of this *in vitro* assays correlate best with *in vivo* activity of non-steroidal mimics of the molting hormone.