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Biological effects of muristerone A and turkesterone on the epithelial cell line from *Chironomus tentans* (Diptera: Chironomidae) and correlation with binding affinity to the ecdysteroid receptor

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Muristerone A, turkesterone, ponasterone A, 20-OH-ecdysone, ecdysteroid receptor, insect cell line, acetylcholinesterase, Chironomidae, *Chironomus tentans*

Abstract. Ecdysteroids, which possess an α-hydroxyl group at C_{11}, bind with about the same affinity to the ecdysteroid receptor as compared to the corresponding compounds lacking the hydroxyl group. This is shown for turkesterone/20-hydroxyecdysone and muristerone A using receptor preparations from the epithelial cell line from *Chironomus tentans*. For all compounds tested, the potency to elicit a hormonal response corresponds with the affinity to the ecdysteroid receptor as determined by induction of acetylcholinesterase, a marker enzyme for cell differentiation.

INTRODUCTION

The phytoecdysteroid turkesterone, which has an additional α-hydroxylic group at C_{11} compared to 20-hydroxyecdysone (20E) exhibits moulting hormone activity in various test systems to quite different extents. Whereas in the lepidopteran *Galleria mellonella* nearly no effect was observed, turkesterone is equally active compared to 20E in the coleopteran *Dermestes vulpinus* and roughly tenfold more active in the dipteran *Sarcophaga bullata* (Sláma et al., 1993). In contrast, Clément et al. (1993) using the B2 cell line from *Drosophila melanogaster* found an approximately 200-fold lower activity of turkesterone than 20E. It is difficult to interpret these data, since the actual hormone concentration at the target site is unknown due to permeability problems, hormone metabolism and excretion. An even more active ecdysteroid with an additional α-hydroxylic group at C_{11} is muristerone A, which otherwise is identical to ponasterone A. Both ecdysteroids are highly active in a bioassay using *Drosophila* Kc-H cells (Cherbas et al., 1980).

Chironomus cells do not synthesize endogeneous moulting hormones but metabolize added ecdysteroids only very slowly, therefore this cell line is especially suited to determine the biological activity of moulting hormones (Spindler & Spindler-Barth, 1991). Among the numerous morphological and physiological responses related to moulting or tissue differentation elicited by ecdysteroids in this cell line (Spindler-Barth et al., 1988, 1989, 1992, 1995; Spindler-Barth, 1993; Fretz et al., 1993; Lammerding-Köppel et al., 1994; Quack et al., 1995), we choose induction of acetylcholinesterase as quantitative measure for moulting hormone activity, since the enhancement of enzyme activity is rather high – up to 30-fold stimulation – and allows, therefore, the detection of even small

differences in moulting hormone activity. In addition, a comparison of binding data and induction of acetylcholinesterase activity in this cell line using ecdysteroids and non-steroidal moulting hormone agonists revealed a positive correlation of hormone – receptor interaction and the extent of this biological response (Spindler-Barth, 1991; Spindler-Barth et al., 1991; Spindler et al., 1992, 1993).

Hormone binding properties of this receptor in *Chironomus* cells are well characterized (Turberg et al., 1988; Turberg & Spindler, 1992). Cloning of the gene revealed that the hormone binding domain is rather similar to the corresponding *Drosophila* receptor (Imhof et al., 1993), which may allow generalization of the observed effects to a certain extent. Independent of the species tested, the relative affinities to their cognate receptor are the same for all receptor preparations from invertebrates tested so far (Spindler-Barth & Spindler, 1987; Bidmon & Sliter, 1990).

Since expression of transfected genes in vertebrate cells under the control of ecdysteroids, ecdysteroid receptors and ecdysteroid responsive genes seems to be an interesting tool (Seagraves, 1994), we examined receptor affinity and biological activity of suited ecdysteroids.

MATERIAL AND METHODS

Cell line

The epithelial cell line, established by Wyss (1982) was kindly provided by M. Lezzi (ETH Zürich) and has been kept in our laboratory since 1986. Cells were grown at 25°C in medium according to Wyss (1982) and propagated by dissociation of the multicellular vesicles by pipetting and dilution with fresh medium 1 : 10 every 10 to 16 days.

Determination of acetylcholinesterase activity

Acetylcholinesterase was determined by a microfluorimetric assay essentially as described (Spindler-Barth et al., 1988).

Ecdysteroid binding assays

Cells were harvested by centrifuge (13,000 g, 4°C, 20 s), washed with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM K_2HPO_4, 1.5 mM KH_2PO_4, pH 6.7) and resuspended in HEPES-buffer (20 mM, 400 mM NaCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM 2-mercapto-ethanol, pH 7.9, freshly supplemented with the following protease inhibitors: 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin (final concentrations). All operations were carried out on ice except where indicated. Cells were homogenized by an all-glass homogenizer with ten strokes. NaCl was included in the homogenization buffer to extract ecdysteroid receptors from nuclei, since a considerable portion of the receptor is localized in the nuclei. The homogenate was centrifuged at 2°C at 100,000 g for 65 min and lipids carefully removed. The supernatant was desalted immediately with PD-10 columns, (Pharmacia, Freiburg, FRG) since ecdysteroid binding capacity of the receptor decreases after prolonged incubation under high salt conditions (Turberg et al., 1988).

Ecdysteroid binding was measured by a filter assay using nitrocellulose membranes (Schleicher and Schuell, BA 85) as described earlier (Turberg & Spindler, 1992). Receptor preparations were incubated for 3 hrs at 4°C with 2 nM [^{3}H]-ponasterone A (spec. act. 170 Ci/mmol) and 20-OH-ecdysone, turkesterone or muristerone as competitor. Tests were performed three times.

RESULTS AND DISCUSSION

The efficiency to compete with ^{3}H-ponasterone A binding is in the same range for ponasterone A (Turberg et al., 1988) and muristerone A, whereas 20-OH-ecdysone and turkesterone (Fig. 1) are clearly less potent competitors (Fig. 2). The corresponding compound without an α-OH-group at C_{11} is in both cases slightly less active compared to the

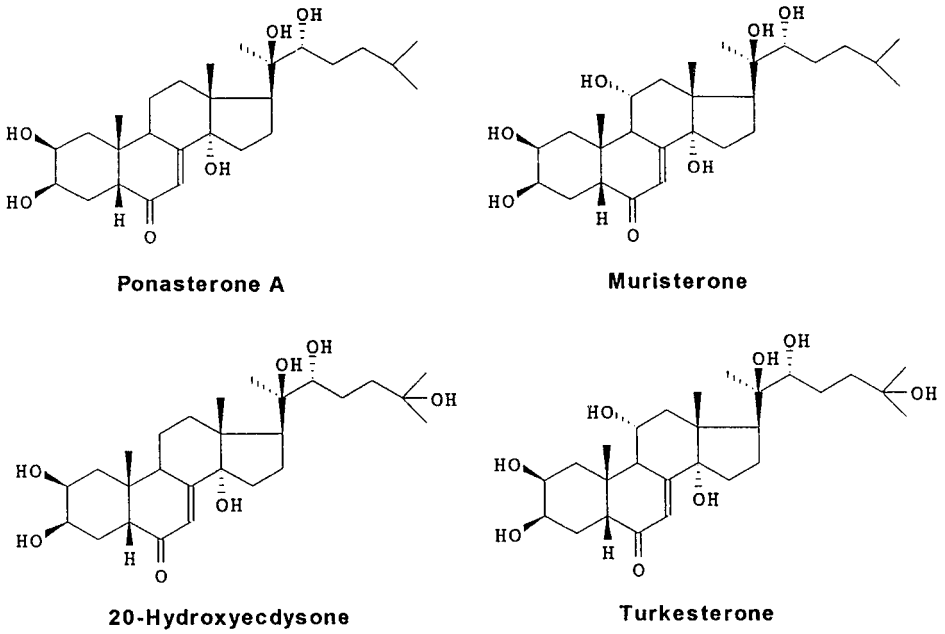

Fig. 1. Structures of ecdysteroids.

non-derivatized compound (Fig. 2), which is also in accordance with data from the ecdysteroid receptor of the tick *Amblyomma hebraeum* (Mao et al., 1995). These data demonstrate that the additional α-hydroxy group at C_{11} is unimportant for interaction of ecdysteroids with the receptor. This is further corroborated by studies on ajugasterone C, a phytoecdysteroid, which also has an α-hydroxy group at C_{11} and exhibits about the same biological activity as compounds lacking this additional hydroxyl-group (Imai et al., 1969).

Induction of acetylcholinesterase, a marker for tissue differentiation (Drews, 1975), is a rather late reponse to moulting hormones (Best-Belpomme & Courgeon, 1977; Cherbas et al., 1977; Spindler-Barth, 1991; Spindler-Barth, et al., 1988; Lammerding-Köppel et al., 1994). Nevertheless, a positive correlation between binding data and increase in enzyme activity is obtained as shown in Fig. 3. This is also reported for the benzoylhydrazine RH 5849, a nonsteroidal moulting hormone agonist (Spindler-Barth et al., 1991). Turkesterone induces AchE in a dose-dependent way with half-maximal induction at 0.8 μM. The potential of turkesterone to induce AchE is not distinguishable from that of 20E, whereas muristerone is more than an order of magnitude more effective (Fig. 3; Table 1). The induction of acetylcholinesterase by all ecdysteroids tested corresponds to their affinity to the ecdysteroid receptor (Fig. 3; Table 1). Half-maximal induction of enzyme activity is roughly in the same range as the K_D-value, calculated from competition data according to Cheng & Prusoff (1973).

Half-maximal induction of the biological reponse by turkesterone in the μmolar range is also reported by Clément et al. (1993) with an IC_{50}-value of 1.3 μmol using *Drosophila* B2

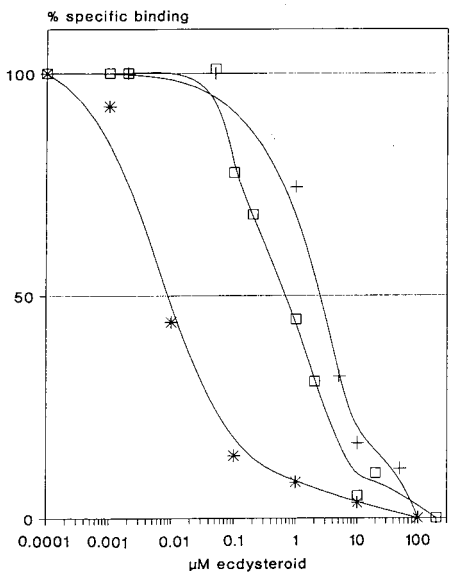

Fig. 2. Competition of ^{3}H-ponasterone A (2 nM) binding by muristerone A (✻), turkesterone (+) and 20-OH-ecdysone (□). n = 3, S.D. < 10 %.

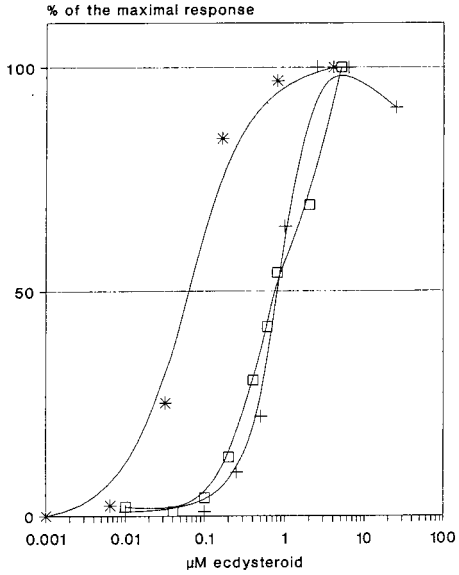

Fig. 3. Induction of acetylcholinesterase by muristerone A (✻), turkesterone (+) and 20-OH-ecdysone (□). n = 4, S.D. < 7 %.

cells. In this case hormone induced cell differentation was measured. From experiments obtained with a whole animal assay using *Sarcophaga* larvae (Sláma et al., 1993) we calculated an IC_{50}-value of 0.6 µmol. The ninefold lower biological activity of 20E in *Sarcophaga bullata* may be due to higher metabolic rates as compared to turkesterone. It is difficult, however, to understand why 20E is much more efficient in the *Drosophila* B2 bioassay (IC_{50}-value: 7.6 nmol), although the affinity to the ecdysteroid receptor seems to be considerably lower (half-maximum competition = 0.2 µmol) (Dinan, 1985) which corresponds to data reported in the literature (Bidmon & Sliter, 1990).

TABLE 1. Influence of turkesterone, muristerone and 20-OH-ecdysone on acetylcholinesterase activity and binding to the intracellular ecdysteroid receptor from *Chironomus tentans* cells. K_D-values were calculated according to Cheng & Prusoff (1973).

Compound	Ecdysteroid receptor binding		Acetylcholinesterase activity (half-maximal response) (µM)
	half-maximal competition* (µM)	K_D-values (µM)	
20-OH-ecdysone	0.700	0.172	0.75
Turkesterone	2.000	0.491	0.80
Muristerone A	0.010	0.003	0.06

* Binding of [^{3}H]-ponasterone A to the ecdysteroid receptor was competed with nonradioactive turkesterone, muristerone A or 20-OH-ecdysone.

Reporter genes being under the control of ecdysteroid responsive elements can be induced by ecdysteroids in vertebrate cells, which were transfected with EcR and USP expressing plasmids and the corresponding reporter gene construct (Seagraves, 1994). However, in contrast to invertebrate cells, 20-hydroxyecdysone or polypodine B do not induce the expression of the reporter gene and ponasterone A is only a rather weak inducer (Christopherson et al., 1992), in contrast to muristerone A. This may be explained due to the following reasons: (1) Ecdysteroids with an 11α-hydroxy group might be more stable in vertebrates. Ecdysteroids without this functional group are rapidly metabolized in mammals (Lafont et al., 1986, 1988). Since steroid hormones in vertebrates have only β-hydroxyl groups at C_{11} it is expected that steroids with an α-hydroxyl group at this position are metabolized to a considerably lower rate. (2) Ecdysteroid specificity of EcR might be changed after dimerization with RXR, present in mammalian cells used for transfection, instead of USP (Thomas et al., 1993). (3) In addition, other factors, necessary for full recovery of hormone binding and expression, may be present in *Drosophila* or yeast but absent from mammalian cells.

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