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Note

Validity Analysis of a Receptor Binding Assay for Ecdysone Agonists Using Cultured Intact Insect Cells

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INTRODUCTION

N-tert-Butyl-*N,N'*-dibenzoylhydrazine and its derivatives are known to be non-steroidal ecdysone agonists that possess insecticidal activity. They compete with insect molting hormone (20-hydroxyecdysone) against its receptor protein and cause disruption of the normal hormonal action.[1] In order to evaluate the activity of ecdysone agonists quantitatively, we recently developed a method in which the activity is expressed as inhibition of uptake of [^{3}H]ponasterone A ([^{3}H]PoA) into intact cultured cells, *Spodoptera frugiperda* Sf-9 and *Drosophila melanogaster* Kc.[2,3] However, whether this activity is equivalent to the ligand-receptor binding activity or not is unclear, since other factors such as permeability through the plasma membrane or exclusion from the cell can affect the uptake of a chemical. In this regard, it has been reported that two lines of dipteran cultured cells are capable of excluding tebufenozide, one of the dibenzoylhydrazine compounds.[4]

In this study, we measured the binding of a series of ecdysone agonists to the cellular homogenates of Sf-9 and Kc cells. Their binding potency was compared with the inhibitory activity against [^{3}H]PoA uptake into intact cells, in order to elucidate physicochemical characters that affect permeability through the plasma membrane.

MATERIALS AND METHODS

Sf-9 and Kc cell lines were cultured according to the reported method.[2,3] Confluent cells were scraped and centrifuged (200 g, 5 min). Cell pellets were washed three times with PBS(-) buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4), and once with EcR40 buffer (40 mM KCl, 25 mM HEPES, pH 7.0, 10% glycerol, 1 mM EDTA, 1 mM DTT and 10 mM $Na_2S_2O_5$).[5] Cell pellets were resuspended in EcR40 buffer containing 500 μM PMSF, 1 μM leupeptin and 1 μM pepstatin to make a cell concentration of 5×10^7 cells/ml. Cells were homogenized with a sonicator (ULTRA SONIC DISRUPTOR UR-200P, TOMY SEIKO, Tokyo, Japan) at an output of 4, with 8 cycles of 5-sec pulses on ice. It was microscopically confirmed that no intact cell remained in the cellular homogenates. Protein concentrations of the cellular homogenates were determined by the Bradford method,[6] and final concentrations were fixed at 9 mg/ml (Sf-9) and 3 mg/ml (Kc) respectively.

The cellular homogenate (100 μl) was placed in a disposable glass tube (12 × 75 mm) containing 1 μl of a dimethyl sulfoxide (DMSO) solution of each compound and 46 μl of EcR40 buffer. After 5 to 10 min on ice, 3 μl (ca. 90,000 dpm, 1.8 nM) of an ethanol solution of [^{3}H]PoA (120–200 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO) was added, and the mixture was incubated for 30 min at 25°C. The reaction mixture was filtrated rapidly using a Whatman GF/F glass-fiber filter pre-soaked with a 0.1% polyethylenimine solution, and washed three times with water. The radioactivity collected on the filter was measured in Aquasol-2 with a liquid scintillation counter (Aloka LSC-1000). From the concentration-response curve for the [^{3}H]PoA binding, the 50% inhibition concentration, IC_{50}(M), was evaluated by probit transformation.[7,8] The reciprocal logarithm value of the IC_{50} (pIC_{50}) was used as an index of the binding activity.

RESULTS AND DISCUSSION

The mean pIC_{50} values with their standard deviation (SD) for replications for test compounds are listed in Table 1 along with their log P values. The relationship between the binding activity against the cell-free preparation and the inhibitory potency against the uptake of [^{3}H]PoA into intact cells,[2,3] is shown in Fig. 1. The binding activity is well correlated to the activity to inhibit uptake of [^{3}H]PoA,[2,3] in both Sf-9 and Kc cells. In spite of the wide range of hydrophobicity values for the test compounds used in this study, no significant contribution of hydrophobicity to the correlation was observed. This result indicates that the uptake of a compound into cells is not affected by its transmembrane permeability, but depends solely on its binding activity for the cellular components in this system.

To date it has been shown that the structure-activity relationships (SARs) of ecdysone agonists in terms of *in vivo* activity, such as larvicidal activity, are very different among insect orders.[9,10] An analysis of SARs at the molecular level is required for a discussion of this issue. This study has shown that the assay system using intact cells is acceptable as a very simple, reproducible and labor-saving system for evaluating the binding activity of ecdysone agonists, and useful for clarifying the chemical basis of order-selectivity.

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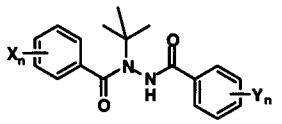
Table 1. Inhibition of ecdysone agonists for the [^{3}H]ponasterone A ([^{3}H]PoA) binding



Compounds			pIC_{50} (M)[a]				
			Sf-9		Kc		
No.	X_n	Y_n	Homogenates	Intact cells	Homogenates	Intact cells	log P
1	H	H	6.43±0.42 (4)	6.44±0.07 (3)[b]	5.51±0.23 (2)	5.24±0.03 (3)[c]	2.45[d]
2[e]	3,5-$(CH_3)_2$	4-C_2H_5	8.71±0.14 (4)	8.81±0.07 (4)[b]	6.27±0.30 (3)	6.39±0.17 (3)[c]	4.39[f]
3[g]	3,5-$(CH_3)_2$	2-CH_3, 3-OCH_3	8.24±0.16 (3)	8.46±0.06 (2)[b]	6.53±0.05 (2)	6.55±0.10 (2)[c]	3.93[f]
4	H	4-C_2H_5	7.33±0.27 (2)	7.64±0.11 (2)	5.75±0.05 (2)	5.69±0.09 (3)[c]	3.45[f]
5	2-Cl	H	6.70±0.06 (2)	7.08±0.14 (2)	N.D.	N.D.	2.59[d]
6	2-Cl	2-NO_2	5.93±0.08 (2)	6.04±0.21 (2)	N.D.	N.D.	1.99[f]
7	2-Cl	4-*t*-C_4H_9	7.46±0.05 (2)	7.66±0.13 (2)	N.D.	N.D.	4.48[f]
8	2-Cl	2,6-F_2	7.47±0.15 (3)	7.52±0.27 (2)	N.D.	N.D.	2.35[f]
9	3,5-$(CH_3)_2$	4-*n*-C_4H_9	6.88±0.03 (3)	7.08±0.04 (2)	N.D.	N.D.	5.39[f]
10	Ponasterone A		7.58±0.18 (3)	8.05±0.03 (3)[b]	8.54±0.20 (3)	8.89±0.07 (2)[c]	1.00[h]
11	20-Hydroxyecdysone		6.34±0.20 (3)	6.78±0.07 (3)[b]	6.98±0.30 (2)	7.34±0.02 (2)[c]	-1.21[h]
12	Inokosterone		5.86±0.06 (2)	6.25±0.15 (3)[b]	6.79±0.00 (2)	7.04±0.08 (2)[c]	-0.99[h]
13	Ecdysone		5.08±0.03 (2)	5.63±0.13 (2)[b]	5.34±0.04 (2)	5.59±0.09 (3)[c]	0.15[h]

[a] Mean±SD. Values in parentheses indicate the number of replications. N.D. : not determined. [b] See Ref. 2. [c] See Ref. 3. [d] Experimentally measured (See Ref. 11 and 12). [e] Tebufenozide. [f] Estimated empirically (See Ref. 11 and 12). [g] Methoxyfenozide. [h] Calculated from the CLOGP method.[13]

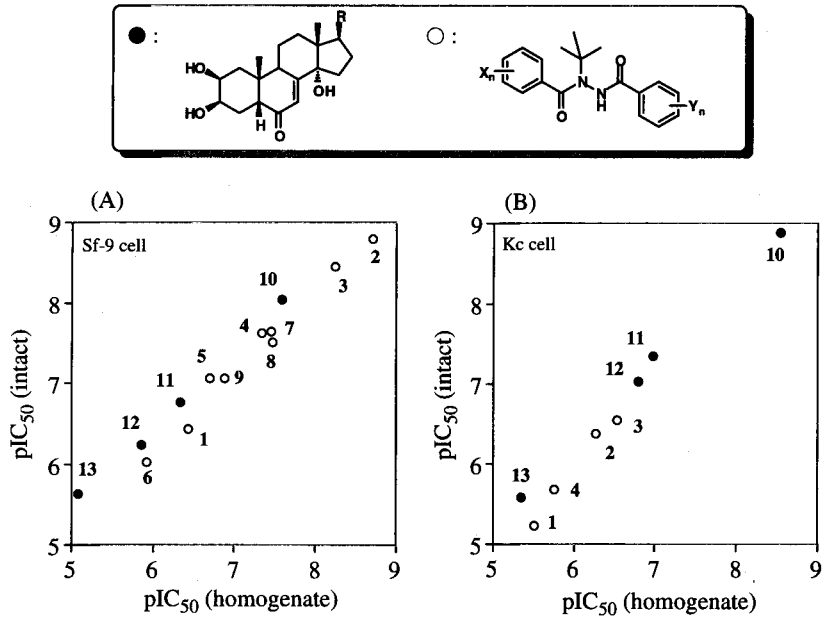


Fig. 1. Relationship between activities measured in cellular homogenates [pIC_{50}(homogenate)] and intact cells [pIC_{50}(intact)][2,3] of steroidal ecdysteroids (●) and dibenzoylhydrazines (○) against Sf-9 (A) and Kc (B) cell lines, respectively.
Numbers in figures (A) and (B) correspond to the compound numbers in Table 1.

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