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Cytochrome P-450 Inducers and Inhibitors Interfering with Ecdysone 20-Monooxygenases and Their Activities during Postembryonic Development of *Neobellieria bullata* Parker

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Abstract: The cytochrome P-450-dependent microsomal and mitochondrial ecdysone 20-monooxygenase systems convert ecdysone into 20-hydroxyecdysone. The microsomal fraction of fat bodies of zero h wandering stage fleshfly larvae (*Neobellieria bullata*; Diptera: Sarcophagidae) has a high ecdysone 20-monooxygenase activity. The effects of cytochrome P-450 inhibitors were investigated *in vitro* on microsomal ecdysone 20-monooxygenase. Metyrapone, fenarimol and certain imidazole derivatives (KK-42, KK-110, KK-135 and PIM) are strong inhibitors. The IC_{50} value of KK-110, which is the strongest inhibitor, is 2×10^{-7} M. A triazolyl and two cyclopropylamine derivatives have low activity. The activities of different NADPH-cytochrome c (P-450) reductase inhibitors were also assessed; diquat dibromide is a moderate inhibitor of microsomal ecdysone 20-monooxygenase, while paraquat dichloride has no activity.

In-vivo experiments with cytochrome P-450 inducers and inhibitors gave the following results: (a) fenarimol, FI-121, precocene-2 caused 'permanent' first-instar larvae; (b) barbital, phenobarbital and their sodium salts caused significant delay in larval development; (c) PIM, PTM, metyrapone, KK-42, KK-135, J-2710, RH 5849 and colchicine caused moulting disturbances; (d) J-2710, PIM, PTM, KK-42, KK-135, RH 5849 and colchicine caused lethal spiracle and mandible malformation; (e) KK-110, fenarimol, barbital and phenobarbital caused precocious pupariation.

INTRODUCTION

It is widely believed that the appearance of the cytochrome P-450 system coincided with the appearance of life on Earth.[1] The food of insects usually contains appreciable cytochrome P-450 inducers and inhibitors[2] (as allelochemicals), which are detoxified by poly-substrate monooxygenase (PSMO; E.C.1.14.14.1) systems in a phase I reaction.[3]

Insects cannot synthesize sterols *de novo* and have a dietary sterol requirement which is met by phytosterols in phytophagous species, or by cholesterol in carnivorous insects. Phytosterols/cholesterol are converted into ecdysteroids,[4,5] mainly by different cytochrome P-450-dependent monooxygenases.[6] Phytosterols are converted into cholesterol via several steps, two of which are catalyzed by fucosterol-24(28)-epoxidase and by fucosterol epoxide-lyase, respectively.[7,8] Synthesis of the insect moulting hormone, ecdysone, from cholesterol involves introduction of a 6-oxo group, saturation of the

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Δ^5 bond, followed by a series of hydroxylations catalyzed by cytochrome P-450 isozymes: E-14-M, E-25-M, E-22-M, E-2-M.[9] 20-Hydroxyecdysone, an active metabolite of ecdysone, is formed in an ecdysone 20-monooxygenase-catalyzed reaction (E-20-M).[10] Degradative metabolism of ecdysone may occur in different ways, but an ecdysone 26-hydroxylase can be involved.[11] Cytochrome P-450-dependent monooxygenase systems play a crucial role in insect steroidogenesis. Mitochondrial E-2-M isozyme,[12,13] and microsomal (E-20-M_{ER}) and mitochondrial (E-20-M_{MT1}, E-20-M_{MT2}) ecdysone 20-monooxygenase systems[14] have been partially characterized.

A possible classification of agonists/antagonists/inducers of ecdysteroids is as follows:[15] (a) steronoids (steroid-like compounds): azadirachtins, alfalfa saponins; (b) anti-cholesterol agents: triparanol, 20,25-diazacholesterol; (c) ecdysteronoids (ecdysteroid-like compounds): ajugalactone, ajugasterone C, cyasterone, castasterone, 22*S*,23*S*-homocastasterone, 22*S*,23*S*-homobrassinolide; (d) ecdysteroid agonists: RH 5849; (e) inhibitors of nicotinamide-adenine dinucleotide phosphate (NADPH)-cytochrome c (P-450) reductase (and cytochrome b_5): diquat dibromide, 1-adamantylamine, plumbagin, juglone; (f) inhibitors of cytochrome P-450: KK-42, KK-110, KK-135, fenarimol, metyrapone, PIM, 20-hydroxy- and 20,25-dihydroxy-22-propargylcholesterol, xanthurenic acid; (g) inducers of metabolic enzymes (cytochrome P-450 systems, glutathione *S*-transferases, epoxy hydrolases): barbital, phenobarbital.

Our aim was the in-vivo and in-vitro examination of different chemicals which interfere in some way with the cytochrome P-450 isozymes, especially with the ecdysone 20-monooxygenase systems.

2 MATERIALS AND METHODS

2.1 Insect

Stock cultures of *Neobellieria bullata* Parker (Diptera: Sarcophagidae) were reared in a constant temperature room at 25(±0·5)°C and 50% R.H. with a daily photophase of 16 h. Groups of about 200 adults were fed on sucrose + milk powder (1 + 1 by weight). Larvae were reared under the same conditions on homogenized beef liver + 1% agar (1 + 1 by volume).

2.2 Cytochrome P-450 inhibitors and inducers

The inhibitors/inducers used were as follows: (a) *cytochrome P-450 inducers*: barbital, barbital-Na, phenobarbital, phenobarbital-Na, butobarbital, amobarbital, hexobarbital (all from Alkaloida, Hungary), α-pinene (Aldrich Chem., Germany), sinigrin, menthol, camphor (all from Sigma, USA), digitoxin, 3-methylcholantrene, mitotane (all from Aldrich Chem., Germany); (b) *NADPH-cytochrome c (P-450) reductase inhibitors*: paraquat dichloride, diquat dibromide (both ICI, UK), 1-adamantylamine (Aldrich Chem., Germany); (c) *cytochrome P-450 inhibitors*: proadifen HCl (SKF 525A; Eli Lilly, USA), piperonyl butoxide (Aldrich Chem., Germany), safrol (Merck, Germany), J-2710 (5-methoxy-6-[1-(4-methoxyphenyl)ethyl]-1,3-benzodioxole; gift of A. de Loof, synthesized by L. Jurd),[16] disulfiram (Chinoin, Hungary), metyrapone (Sigma, USA), bisacodyl (Chinoin, Hungary), fenarimol, nuarimol (both DowElanco, USA), phenyl-triazolyl-metyrapone (PTM; synthesized by I.B.),[17] acenocoumarole (Alkaloida, Hungary), 2-methyl-1-phenyl-2-(1,2,4-triazol-1-yl)-1-propanone (PIM; synthesized by I.B.),[17] KK-42 (1-benzyl-5-[*E*-2,6-dimethyl-1,6-heptadienyl]imidazole; synthesized by E.K.),[18] KK-110 (1-neopentyl-5-(2-ethoxyphenyl)imidazole; synthesized by E.K.),[19] KK-135 (1-neopentyl-5-(4-chlorophenyl)imidazole; synthesized by E.K.),[19] clotrimazole (Bayer-Egis, Hungary), cimetidine (Richter Gedeon Co., Hungary), NKI-42016 (*N*-cyclopropyl-4-*tert*-butylbenzylamine; synthesized by I.B.),[17] NKI-42109 (*N*-cyclopropyldodecanamide; synthesized by I.B.),[17] 1-aminobenzotriazole (Aldrich Chem., Germany), FI-121 (7-propargyloxy-3,4-dichloro-2,2-dimethylchromene; synthesized by T.T.);[20] (d) *ecdysteroid agonist*: RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine; gift of K. D. Wing);[21] (e) *juvenoid*: methoprene (Egis, Hungary); (f) *anti-juvenile hormone agent*: precocene-2 (also termed pro-allatocidin; synthesized by T.T.); (g) *anti-mitotic agent*: colchicine (Sigma, USA), vincamine (Carl Roth K. G., Germany). For details of chemical structures not given see the *Merck Index*[22] or *Pesticide Manual*.[23]

2.3 In-vivo experiments

Active ingredient was added to the diet, larvae being fed on the diet from hatching. On day 14 of adult life, the females were forced to lay eggs by slight lateral abdominal pressure. Twenty-five larvae (repeated twice) were used in each case. The mortality of the larvae and puparia was checked daily until adult emergence and the weight of 10 larvae/puparia per treatment was measured. The data were analyzed using a one way analysis of variance (ANOVA) and probit analysis. Cuticles and cephalopharyngeal sclerites of dead larvae, after treatment for three days in lactic acid at room temperature, were examined with a light microscope to determine effects of inhibitors on development.

2.4 In-vitro experiments

Five-day-old larvae were subjected to wet synchronization[24] for 48 h. Fat bodies were dissected at 4°C

TABLE 1

LC_{50} Values for Mortality Related to Adult Emergence of *Neobellieria bullata* Raised on Inhibitor-Treated Food during Larval Development[a, b]

Active ingredient[c]	*Slope* (±*S.E.*)	LC_{50} (*mg kg*$^{-1}$)	
		Mean	*95% C.L.*
Methoprene (juvenoid)	3·3 (±0·1)	1·4	1·3–1·5
Colchicine (anti-mitotic agent)	4·4 (±0·8)	3·1	3·0–3·2
RH 5849 (ecdysteroid agonist)	3·4 (±0·1)	10·2	7·0–10·8
J-2710	5·1 (±1·2)	61·7	59·6–63·8
Fenarimol	2·3 (±0·1)	109·7	99·6–120·7
PIM	6·1 (±2·0)	148·3	144·2–152·4
1-Adamantylamine	4·7 (±0·1)	153·5	148·0–159·1
Precocene-2 (anti-JH agent)	6·6 (±1·9)	198·2	192·9–203·6
KK-110	2·3 (±0·1)	218·3	198·0–240·6
Mitotane	5·2 (±1·2)	248·9	240·9–257·2
KK-42	2·5 (±0·1)	254·1	234·8–275·0
Piperonyl butoxide	4·5 (±1·4)	322·1	310·2–334·5
PTM	4·0 (±0·2)	469·9	452·0–488·5
KK-135	5·0 (±0·9)	495·5	478·9–512·6
Metyrapone	5·1 (±0·6)	561·1	542·2–580·6
FI-121	6·2 (±2·1)	604·0	587·7–620·7
Phenobarbital	15·9 (±0·8)	1086·5	1075·2–1097·9

[a] Values are estimates of probit regressions of corrected mortality percentage (Abbott's formula).

[b] A minimum of four concentrations of each compound with 25 insects per dose was replicated twice ($n = 200$).

[c] LC_{50} values of compounds tested but not mentioned here were higher than 1000 mg kg^{-1}.

under isosmotic Neobellieria-Hepes buffer, pH 7·2 (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Hepes) 20 mM; potassium fluoride, 20 mM; sucrose, 260 mM; 340 mOsm) and homogenized with a Potter-Elvehjem homogenizer in the same buffer (fat bodies + medium: 1 + 12 by volume). The homogenate was successively centrifuged at 600*g* for 5 min (3×), 15000*g* for 10 min and 200000*g* for 75 min to yield a 'microsomal' pellet, which was resuspended in hypotonic Neobellieria-Hepes buffer, pH 7·2 (Hepes, 20 mM; potassium fluoride, 20 mM; sucrose, 50 mM; 120 mOsm). The E-20-M_{ER} assay mixture (200 µl) consisted of microsomal fraction (25 µl; approx. 0·22 mg protein; equivalent to 2·5 larval fat bodies) preincubated with inhibitor (155 µl) for 10 min. NADPH-regenerating system (10 µl; NADPH $3{\cdot}4 \times 10^{-3}$ M; G6P $2{\cdot}6 \times 10^{-3}$ M final concentration, G6P-dehydrogenase 0·6 IU), and substrate (10 µl; 0·25 µCi, ^{3}[H]ecdysone 4·22 pmol) were then added and incubation was carried out at 30°C for 30 min. Each inhibitor was examined in duplicate at a minimum of four concentrations. Incubations were terminated by addition of ice-cool ethanol (1 ml), the mixtures kept at −20°C for 30 min before centrifugation at 8000*g* for 10 min. The ethanolic extracts were fractionated by TLC (0·25 mm, Kiesel GF-254) developing twice with chloroform + ethanol (4 + 1 by volume). Radioactivity in the bands of ecdysone and 20-hydroxyecdysone was measured directly by LSC.[25] The ecdysone 20-monooxygenase activity of the appropriate solvent/medium system without inhibitor was used as a specific untreated control (i.e. the 20-monooxygenase activity in the presence of inhibitor was expressed as a percentage of that in its absence in the same solvent).

The inhibitors used and the methods employed for their solution were as follows (the final concentrations were never more than 0·1 ml litre^{-1} acetone, 0·1 ml litre^{-1} dimethylsulfoxide (DMSO), 0·1 ml litre^{-1} methanol, 0·1 ml litre^{-1} PEG 6000, 0·01 ml litre^{-1} 'Tween 80' in the medium): (a) cytochrome P-450 inducers: phenobarbital (dissolved in acetone + methanol, 1 + 1 by volume, added to 'Tween 80'-containing medium and sonicated for 5 min); (b) NADPH-cytochrome c (P-450) reductase inhibitors: paraquat dichloride (in medium only), diquat dibromide (in medium only); (c) cytochrome P-450 inhibitors: J-2710 (dissolved in acetone), metyrapone (dissolved in methanol), fenarimol (dissolved in DMSO), PTM (dissolved in methanol, added to 'Tween 80'-containing medium and sonicated for 5 min), PIM (dissolved in methanol), KK-42 (dissolved in 'Tween 80'- and PEG 6000-containing medium and sonicated for 5 min), KK-110 (dissolved in methanol, added to 'Tween 80'-containing medium and sonicated for 5 min),

KK-135 (dissolved in methanol, added to 'Tween 80'-containing medium and sonicated for 5 min), NKI-42016 (dissolved in methanol), NKI-42109 (dissolved in 'Tween 80'- and PEG 6000-containing medium and sonicated for 5 min).

3 RESULTS AND DISCUSSION

3.1 In-vivo effects of different compounds which interfere with the cytochrome P-450 system

3.1.1 Toxicity

The LC_{50} values of the most active cytochrome P-450 inhibitors examined ranged from 100 to 300 mg kg^{-1} (ppm) (Table 1). The levels of activity of fenarimol, PIM and 1-adamantylamine were somewhat higher than that of the pro-allatocidin compound, precocene-2.

Feeding deterrent activity was not found in any of the treatments used. The crops and guts were always full with treated diet. Sarcophagid species fill their crops with diet during the first few movements.

TABLE 2
Extension of Larval Development Caused by Sub-lethal Concentrations of Different Active Ingredients Fed during Larval Development of *Neobellieria bullata*

Active ingredient[a]	*Conc.* (*mg* kg^{-1})	*Time from egg hatching to pupariation*[b] (*day*) (± *S.E.*)
Barbital	1250	10·8 (±0·9)g
Phenobarbital	1000	10·7 (±0·5)g
Barbital-Na	1250	10·1 (±0·5)f
3-Methylcholanthrene	1000	9·9 (±0·3)ef
Nuarimol	2000	9·5 (±0·5)e
Phenobarbital-Na	1250	9·4 (±0·5)de
Mitotane	250	9·1 (±0·3)de
Digitoxin	1000	8·9 (±0·3)d
J-2710	125	8·8 (±0·5)cd
PIM	250	8·8 (±0·5)cd
NKI-42016	2000	8·8 (±0·8)cd
Butobarbital	1250	8·3 (±0·3)c
Metyrapone	500	8·0 (±1·0)bc
Aminobenztriazole	2000	7·9 (±0·4)bc
Diquat dibromide	1000	7·8 (±0·6)bc
FI-121	500	7·7 (±0·5)b
PTM	1000	7·7 (±0·6)b
Control		6·7 (±0·5)a
S.D. 1%		0·51

[a] Compounds tested but not mentioned here did not increase significantly the duration of larval development.
[b] Values in a column followed by the same letters are not significantly different at < 1% level (one way ANOVA).

TABLE 3
Decrease in Puparium Weight Caused by Sub-lethal Concentrations of Different Active Ingredients Fed during Larval Development of *Neobellieria bullata*

Active ingredient[a]	*Conc.* (*mg* kg^{-1})	*Weight*[b] (*mg*) (± *S.E.*)
Safrol	1000	42·5 (±9·0)e
Barbital	1250	49·3 (±5·9)e
Metyrapone	500	50·0 (±9·2)de
J-2710	125	64·0 (±5·0)de
Nuarimol	2000	64·8 (±48·6)d
NKI-42016	2000	65·3 (±24·0)cd
Phenobarbital	1000	68·8 (±10·3)cd
Barbital-Na	1250	72·7 (±13·1)cd
Proadifen HCl	2000	77·5 (±11·6)cd
PTM	1000	79·0 (±41·3)cd
Digitoxin	1000	80·5 (±16·7)c
Camphor	2000	85·5 (±25·3)bc
Diquat dibromide	1000	87·4 (±8·9)bc
FI-121	500	100·0 (±9·2)b
Control		132·0 (±12·4)a
S.D. 1%		15·21

[a] Compounds tested but not mentioned here did not reduce the weight significantly.
[b] Values in a column followed by the same letters are not significantly different at < 1% level (one way ANOVA).

The activities of the compounds presumably relate to their effects on PSMO. None of the cytochrome P-450 inhibitors used reached the level of activity of certain neuro-active zoocides (dimethoate, LC_{50}: 0·1 mg kg^{-1}; carbaryl, LC_{50}: 30 mg kg^{-1}). It seems that the very efficient and adaptive properties of the insect PSMO system may detoxify different cytochrome P-450 inhibitors.

3.1.2 Delay in larval development

Fenarimol at 2000 mg kg^{-1} and FI-121 at 1000 mg kg^{-1} (as well as precocene-2) caused 'permanent' first-instar larvae for 4–6 days with lethal consequences (data not shown). The first-instar larvae only passed the stage of apolysis of anterior spiracles (which represents 16-h-old larvae), but no further development occurred. This phenomenon concerns the relatively specific inhibition of a cytochrome P-450 isozyme, which is essential for larval development.[26] The larvae could not pass the pre-ecdysial processes and moult. A similar effect observed with the pro-allatocidin, precocene-2 and the methyl-farnesoate epoxidase inhibitor, FI-121[20] suggests that a decreased level of juvenile hormone is involved in this effect.

Barbital and phenobarbital at sub-lethal concentrations caused significant delays (3 d) in larval development (Table 2), causing precocious pupariation. Most of the other compounds delayed the first moult in particular, without causing lethal consequences (Table 2).

TABLE 4
Special Lethal Syndromes Caused by Different Active Ingredients Fed during Larval Development of *Neobellieria bullata*

Active ingredient	*Effective concentration (mg kg* $^{-1}$*)*					
	Pre-ecdysial disturbance			*Moulting disturbance*[d]	*Precocious pupariation*[e]	*Others*
	AS[a]	*PS*[b]	*M*[c]			
Barbital	—	—	—	—	1250	
Phenobarbital	—	—	—	—	1250	
Piperonyl butoxide	—	—	—	—	—	MP[f] 500
Safrol	—	—	—	—	—	MP[f] 1000
J-2710	125	125	125	125	—	M[g]
Metyrapone	—	—	—	1000	—	RS[h] 2000
Fenarimol	—	—	—	1000	500	PL[i] 2000
PTM	2000	2000	2000	2000	—	
PIM	250	250	250	250	—	
KK-42	2000	2000	2000	2000	—	EW[j] 1000
KK-110	—		—	2000	1000	EW[j] 500
KK-135	2000	2000	2000	2000	—	EW[j] 500
FI-121	—	—	—	—	—	PL[i] 1000
RH 5849	25	—	25	25	—	PM[k] 250
Methoprene	—	—	—	—	—	MP[f] 4
Precocene-2	—	—	—	—	—	PL[i] 1000
Colchicine	—	—	16	60	—	O[l] 8

[a] Malformation of the anterior spiracles.
[b] Malformation of the posterior spiracles.
[c] Malformation of the mandibles.
[d] Moulting disturbances, dying within double cuticles.
[e] Precocious pupariation.
[f] Mortality before adults' emergence.
[g] Different malformations.
[h] Red spots on the body.
[i] Permanent first larval stage during 4–6 days.
[j] Extra weight L_3.
[k] Provoked moult.
[l] Mortality as old larvae.
Compounds tested but not mentioned here had no effects until adult emergence.

3.1.3 Decreased body weight
At sub-lethal concentrations, safrol, barbital (resulting in some precocious puparia), metyrapone and J-2710 all markedly decreased body weight (to about one-third; Table 3). This parameter was reflected in the viability of adults and their progeny. Triadimefon caused a similar effect on *Spodoptera littoralis* (Boisd.) larvae.[8]

3.1.4 Disturbances in pre-ecdysial processes and malformations
J-2710, PTM, PIM, KK-42,[27] KK-135, and RH 5849[28] caused lethal malformations of mandibles and the anterior and posterior spiracles (Table 4). The abnormally shaped doubled mandibles suggest that mandible formation may have started twice in the cases of PTM and PIM.[29] The anterior spiracles were abnormally shaped and contained many vacuoles. The stigmata of the posterior spiracles had an unusual structure.

J-2710 is known as an inhibitor of tubulin polymerization,[30] while RH 5849 is an ecdysteroid agonist.[21] One β-tubulin gene is induced by 20-hydroxyecdysone.[31] Mandible and spiracle formation may be dependent on 20-hydroxyecdysone.[32] The new structures arise by mitoses from mother stem cells. For these reasons pre-ecdysial processes may be very sensitive to anti-mitotic agents, and, indirectly via tubulin synthesis, to the anti-ecdysteroid agents and ecdysteroid agonists. Colchicine caused malformations of mandibles. In contrast, vincamine did not cause any malformations in pre-ecdysial processes, and J-2710 and PTM had a very slight effect on E-20-M_{ER}. J-2710,[16] KK-42,[18] and PIM[17] are also known anti-juvenile hormone agents.

3.1.5 Moulting disturbances
J-2710, PTM, PIM, metyrapone, KK-42, KK-110, KK-135, RH 5849, and colchicine caused dose-dependent

TABLE 5
IC_{50} Values of Inhibitors Related to Microsomal Ecdysone 20-Monooxygenase of Fat Bodies of *Neobellieria bullata* Wandering Stage Larvae[a,b]

Active ingredient	*Slope* (±*S.E.*)	IC_{50}[c] (M)	
		Mean	*95% C.L.*
KK-110	1·65 (±0·85)	$1·99 \times 10^{-7}$	$1·79–2·21 \times 10^{-7}$
Metyrapone	1·12 (±0·16)	$4·96 \times 10^{-7}$	$4·31–5·70 \times 10^{-7}$
Fenarimol	1·07 (±0·02)	$6·62 \times 10^{-7}$	$5·57–7·63 \times 10^{-7}$
PIM	0·95 (±0·13)	$7·89 \times 10^{-7}$	$6·75–9·21 \times 10^{-7}$
KK-135	1·09 (±0·13)	$1·15 \times 10^{-6}$	$1·06–1·32 \times 10^{-6}$
KK-42	1·33 (±0·24)	$1·88 \times 10^{-6}$	$1·67–2·12 \times 10^{-6}$
Diquat dibromide	1·69 (±0·07)	$3·79 \times 10^{-5}$	$3·40–4·23 \times 10^{-5}$
Phenobarbital		$> 10^{-4}$	
Paraquat dichloride		$> 10^{-4}$	
J-2710		$> 10^{-4}$	
PTM		$> 10^{-4}$	
NKI-42016		$> 10^{-4}$	
NKI-42109		$> 10^{-4}$	

[a] A minimum of four concentrations of each compound were incubated with microsomal fraction from 2·5 *N. bullata* 0 h wandering stage larvae fat bodies per dose, all incubations being replicated twice.
[b] Average activity of control: 14·33 pmol h^{-1} mg^{-1} protein.
[c] Values are estimated from probit regressions of corrected inhibitory percentage.

moulting disturbances (Table 4). At high concentrations, the larvae died within the unshed old cuticle, mainly at the first moult. At a lower concentration (63 mg kg^{-1}) the larvae died at the second moult, and at the lowest concentration (8 mg kg^{-1}) they died before pupariation. RH 5849 at a high concentration (250 mg kg^{-1}) caused a premature moult, which preceded the pre-ecdysial processes. The larvae started to moult before mandible and spiracle formation. It may be that the pre-ecdysial processes and moulting require different levels of 20-hydroxyecdysone, and, for this reason, a high level of RH 5849 can provoke the moult without pre-ecdysial processes, while a low level of RH 5849 causes pre-ecdysial malformations. The metyrapone caused rusty red spots on the cuticle, which were not observed in any other case.

The sensitivity of the first moult was always conspicuous in our experiments. Many of the inhibitors which were very effective during L_1 lost their activity (at the same concentration) when we started the treatment at L_3.[33] It is accepted that the cytochrome P-450 complement of the L_1 is poor,[34] and is completed via induction by different food components. This may possibly be true in the case of the PSMO system, which handles xenobiotics, but we found that at the time of birth, *N. bullata* has very high E-20-M_{ER} activity.[33] Thus, at birth, there is a weak PSMO system and an active, but not satisfactorily protected, E-20-M system in fleshfly larvae. For this reason L_1 may be the most sensitive period for inhibition of steroid hydroxylases.

3.1.6 Precocious pupariation

Barbital (1250 mg kg^{-1}), phenobarbital (1250 mg kg^{-1}), KK-110 (1000 mg kg^{-1}) and fenarimol (500 mg kg^{-1}) all caused precocious pupariation of 4–10% of treated *N. bullata* larvae. The treated larvae remained in the second instar, usually reached an approximately doubled weight (35–40 mg), showed wandering behaviour (purged their crops) in normal time (KK-110 and fenarimol) or 1–3 days later (barbital and phenobarbital), and pupariated directly. Precocious puparia had posterior spiracles with two stigmata and died before head evagination (Table 4).

In endopterygote insects (e.g. Lepidoptera), a few anti-juvenile hormone agents, such as EMD,[35] fluoromevalonate,[36] J-2710 and J-2922,[37] KK-42,[18] KK-110 and KK-135[19] induce a precocious pupation. Precocious pupariation of dipterous insects is a lethal mode of post-embryonic development.[38,39] Fenarimol and KK-110 may cause precocious pupariation via inhibition of methylfarnesoate epoxidase.[26,40,41] A different mode of action presumably brings about precocious pupariation in the cases of barbital and phenobarbital. Phenobarbital is a known inducer of PSMOs,[3] glutathione *S*-transferases,[12] and possibly epoxy hydrolases.[43] A cytochrome P-450 isozyme of wide specificity is phenobarbital-inducible in vertebrates[1] and a phenobarbital receptor may be a part of the 4-aminobutyric acid (GABA) system.[44] 4-Aminobutyric acid plays a role in the release of ecdysone from ring glands.[45] It is possible that the barbital/phenobarbital-induced PSMO, glutathion *S*-transferase and epoxy hydrolase systems (via extended

xenobiotic detoxification) simultaneously catabolize the juvenile hormone.

3.1.7 Mortality at the pupal stage

Piperonyl butoxide and safrol (as well as methoprene) caused mortality before adult emergence. Mortality before adult emergence seems to be a particular effect of an elevated JH titre.[35] These two methylenedioxyphenyl compounds possibly inhibit the metabolism of JH.[46] JH-diepoxides may play a JH function in fly species (Cyclorrapha sub-order).[47]

3.2 In-vitro effects of different substances on microsomal ecdysone 20-monooxygenase

KK-110, metyrapone, fenarimol, PIM, KK-135, KK-42, and diquat dibromide were the most potent inhibitors of E-20-M_{ER} (Table 5). PTM, NKI-42016, NKI-42109, paraquat dichloride, J-2710 and phenobarbital had no significant effect at 10^{-4} M.

KK-110 was found to be approximately 1000 times more active as an inhibitor of E-20-M than azadirachtin,[18] plumbagin or juglone.[49] E-20-M_{ER} is sensitive to imidazole, pyridine and pyrimidine compounds. E-20-M may be inhibited by diquat dibromide via NADPH-cytochrome c (P-450) reductase. Our results are the first direct evidence that the Kuwano compounds may exert their anti-hormonal action via cytochrome P-450 inhibition whereas the Jurd compounds possibly have a different mode of action.

3.3 Correlation between inhibition of microsomal ecdysone 20-monooxygenase and symptoms/syndromes *in vivo*

Unfortunately, no clear correlation was found between in-vitro and in-vivo results. No doubt, the relative selectivity of the inhibitors used, and multiply connected and highly organized developmental processes are the main reasons for the different phenotypic expression of E-20-M inhibitors (for example KK-42 and PIM cause malformations, but KK-110 and fenarimol cause precocious pupariation), or the same phenotypic expression of chemicals with different modes of action (for example fenarimol and KK-110 as cytochrome P-450 inhibitors, and barbital and phenobarbital as cytochrome P-450 inducers also cause precocious pupariation). In addition pre-ecdysial malformations are caused by tubulin biosynthesis inhibition and precocious pupariation may be caused by juvenile hormone deficiency syndrome which occurs frequently with E-20-M inhibition. In a sense, our results call attention to the fact that some widely believed specific effects may arise in rather unspecific ways, for example the inhibitory effect of a contact desiccant and non-selective herbicide, diquat dibromide acting via NADPH-cytochrome c (P-450) reductase inhibition on microsomal ecdysone 20-monooxygenase.[50]

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