Pure Appl. Chem., Vol. 75, Nos. 11–12, pp. 2483–2489, 2003.

Workshop 4.2

Testing of endocrine active substances using an enhanced OECD test guideline 407: Experiences from studies on flutamide and ethinylestradiol*

Alexius Freyberger‡, Peter Andrews, Elke Hartmann, Rolf Eiben, Ingo Loof, Ulrich Schmidt, Michael Temerowski, Andree Folkerts, Michael Becka, Bernhard Stahl, and Martin Kayser

BAYER AG, PH PD P Toxicology and PH PD Biometry and Pharmacometry, D-42096 Wuppertal, Germany

Abstract: Groups of five male and five female Wistar rats were treated by gavage with 0, 1, 10, and 100 mg/kg body weight (b.w.) flutamide (FLU) or 0.01, 0.05, and 0.2 mg/kg b.w. of ethinylestradiol (EE2) for at least 28 days according to an enhanced Organization for Economic Cooperation and Development (OECD) test guideline (TG) 407 to investigate which of the current and/or additional parameters would detect effects on the endocrine system and to provide data on intralaboratory variability. Two identical studies were performed in parallel on each compound. Common enhancements were determination of thyroid hormones (T3, T4) and thyroid stimulating hormone (TSH), of the stage of the estric cycle to ensure necropsy of females in diestrus, of the number and morphology of epididymal sperm, and of additional organ weights (e.g., male accessory sex organs, MASO) and histopathology of additional organs (e.g., pituitary, vagina). Endocrine-mediated findings consistently observed in these studies were decreased relative weights of MASO at 100 mg/kg FLU and at 0.2 mg/kg EE2, histological changes in pituitary and testes at ≥10 mg/kg and in MASO, epididymis and adrenals at 100 mg/kg in FLU-treated males, histological changes in the mammary gland at ≥0.05 mg/kg and in testes, MASO and adrenals at 0.2 mg/kg in EE2-treated males, estrogenization of uterus and vagina (despite necropsy in diestrus) at ≥0.01 mg/kg EE2, and changes in the ovary at 0.2 mg/kg EE2. Spermatology was insensitive (EE2) or revealed changes only at the maximum tolerated dose (MTD). Determination of T3, T4, and TSH did not contribute to the detection of the endocrine effects (FLU) or provided equivocal results. Doubling the group size to 10 animals by combining the studies run in parallel did not increase the sensitivity of detection of endocrine-mediated effects above the level obtained by histopathological examination of groups of five animals. Only some of the proposed enhancements evaluated were helpful in detecting the endocrine-mediated effects of FLU and EE2. Evaluation of studies according to an enhanced TG 407 on 10 compounds with different endocrine activities will identify the most appropriate enhancements.

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* **75**, 1617–2615 (2003).

‡Corresponding author

INTRODUCTION

In response to concerns that current toxicological test guidelines (TGs) may not address the detection of interactions of chemicals with the endocrine system sufficiently, EMSG had proposed enhancements to the current subacute rodent Organization for Economic Cooperation and Development (OECD) TG 407 by parameters thought to be effective in detecting endocrine modulation. The enhancements had been discussed at the international level under the umbrella of OECD, and the resulting final version of the enhanced protocol [1] was the basis for a feasibility study on FLU [2]. Experiences from this and other studies resulted in a reduction of the enhancements. This streamlined protocol [3] was then used for the testing of a broad range of endocrine active substances. For each compound, two studies (designated A and B) were run in parallel with the conventional animal number of five rats per dose and sex in order to assess intralaboratory variability. Moreover, individual (n = 5) and combined (n = 10, designated as Combined) evaluation of the results was performed in order to investigate a potential increase in sensitivity when doubling animal numbers. Our laboratory was entrusted with the testing of the flutamide (FLU, feasibility study) and ethinylestradiol (EE2). In the following, we discuss the efficiency of current and additional parameters of the enhanced TG 407 protocol for the detection of endocrine-mediated changes induced by FLU and EE2. Only enhancements common to both studies will be considered, namely the additional determination of (a) thyroid-related hormones; (b) estrus cyclicity from vaginal smears starting in exposure week 4 to ensure necropsy of all females in the diestrus stage of the estrus cycle in week 5; (c) the number and morphology of cauda epididymal spermatozoa; (d) organ weights of ovaries, uterus, thyroid, male accessory reproductive organs and pituitary; and (e) histopathological investigation of pituitary, mammary gland, epididymides, pancreas, and vagina.

MATERIAL AND METHODS

SPF-bred Wistar rats (Hsd Cpb:WU) obtained from Harlan–Winkelmann (Borchen, Germany) were 7 weeks old at initiation of treatment. The study design followed the enhanced protocol [1,3]. The high dose was chosen to represent a mean therapeutic dose (MTD), the low dose was chosen to result in a no observed adverse effects level (NOAEL). Groups of 5 male and 5 female rats were orally gavaged with 0, 1, 10, or 100 mg/kg body weight (b.w.) FLU per day or with 0, 0.01, 0.05, or 0.2 mg/kg b.w. EE2 per day for at least 28 days. Two identical studies were run concurrently for each compound. Females were sacrificed beginning at day 28, provided they were in the diestrus stage. Animals which did not come into diestrus were sacrificed on day 32. In the FLU study, one male rat per group and study was necropsied per day from day 28 to 32, whereas in the EE2 study males were necropsied on days 28 and 29. Appropriate statistical tests were performed. Results of manipulative behavioral tests and incidences of abnormal spermatozoa and of histopathological findings were not subjected to statistical analysis [for details see refs. 2,4].

RESULTS

General findings

All animals survived to necropsy. Body weight gains were biologically significantly and consistently reduced by more than 30 % in high-dose males treated with FLU or EE2, whereas females were less affected. Treatment-related clinical signs (FLU only) and alterations in hematological and clinical chemical parameters were moderate and mainly restricted to the high dose [for details see refs. 2,4].

Effects on estrus cyclicity

All FLU-treated females were sacrificed in the diestrus stage of the female cycle according to vaginal smear cytology and subsequent histopathology of the vaginal epithelium. Two females (0.05 mg/kg,

study B) of the investigation on EE2 did not enter into diestrus during the observation period and were sacrificed in estrus, whereas all other females were classified as diestric by vaginal smear cytology when necropsied.

Effects on organ weights

FLU strongly increased liver weight in both sexes at the high dose. Other changes in organ weights were restriced to male rats (Table 1). Effects of EE2 treatment on organ weights in male animals are shown in Table 2. Remarkable changes in organ weights in EE2-treated female rats were a dose-dependent, strong increase of liver weights and an increased uterine weight observed only at the high dose and following combined analysis of studies A and B.

Table 1 Effects of FLU treatment on terminal body weights and relative organ weights in male rats.

		Control	1 mg/kg	10 mg/kg	100 mg/kg
Terminal body wt (g)					
	Study A	352 ± 15	318 ± 32	338 ± 38	308 ± 17
	Study B	327 ± 19	311 ± 36	313 ± 32	288 ± 20
	Combined studies	339 ± 21	315 ± 32	326 ± 36	298 ± 20**
Organ wt (mg/100 g)					
Pituitary	Study A	3 ± 0.2	3 ± 0.1	3 ± 0.3*	4 ± 0.3**
	Study B	3 ± 0.4	3 ± 0.5	3 ± 0.4	3 ± 0.8
	Combined studies	3 ± 0.3	3 ± 0.4	3 ± 0.3	3 ± 0.7
Adrenals	Study A	20 ± 3	22 ± 1	21 ± 2	24 ± 3
	Study B	19 ± 2	21 ± 3	24 ± 5	24 ± 3*
	Combined studies	19 ± 2	21 ± 2	22 ± 4*	24 ± 3**
Testis, left	Study A	469 ± 12	494 ± 41	462 ± 39	490 ± 5
	Study B	480 ± 24	421 ± 96	499 ± 42	496 ± 39
	Combined studies	475 ± 19	457 ± 80	480 ± 43	493 ± 26
Epididymis, left	Study A	185 ± 20	186 ± 18	152 ± 20	133 ± 35**
	Study B	198 ± 12	179 ± 29	166 ± 19	106 ± 18**
	Combined studies	191 ± 17	183 ± 23	159 ± 20*	120 ± 30**
Ventral prostate	Study A	124 ± 16	92 ± 14	92 ± 21	32 ± 14**
	Study B	121 ± 26	90 ± 11	93 ± 10	30 ± 21**
	Combined studies	123 ± 20	91 ± 12	93 ± 15	31 ± 15**
Seminal vesicles	Study A	473 ± 104	433 ± 86	333 ± 38*	103 ± 14**
plus dorso-lateral	Study B	467 ± 37	462 ± 102	391 ± 108	101 ± 21**
prostate	Combined studies	470 ± 73	448 ± 90	362 ± 82**	102 ± 17**

Tissues were weighed fresh or after fixation (pituitary). Enhancements are underlined. Asterisks indicate a significant difference from the corresponding controls at the $p \leq 0.05$ (*) and $p \leq 0.001$ (**) level.

Table 2 Effects of EE2 treatment on terminal body weights and relative organ weights in male rats.

		Control	0.01 mg/kg	0.05 mg/kg	0.2 mg/kg
Terminal body wt (g)					
	Study A	321 ± 25	311 ± 21	293 ± 11	277 ± 22**
	Study B	304 ± 25	336 ± 34	301 ± 42	269 ± 28
	Combined studies	312 ± 25	323 ± 30	297 ± 29	273 ± 24**
Organ wt (mg/100g)					
Pituitary	Study A	3 ± 0.4	3 ± 0.7	3 ± 0.6	4 ± 0.4
	Study B	3 ± 0.8	3 ± 0.7	4 ± 0.7	5 ± 1.2
	Combined studies	3 ± 0.7	3 ± 0.7	3 ± 0.7	4 ± 1.0*
Thyroid	Study A	5 ± 0.9	5 ± 0.8	6 ± 0.4	6 ± 0.4*
	Study B	5 ± 1.2	5 ± 1.0	5 ± 0.7	6 ± 0.9
	Combined studies	5 ± 1.0	5 ± 0.8	5 ± 0.7	6 ± 0.7*
Adrenals	Study A	15 ± 3	16 ± 2	19 ± 3*	24 ± 2*
	Study B	16 ± 2§	17 ± 1§	20 ± 4	25 ± 3
	Combined studies	16 ± 2	17 ± 2	20 ± 3**	25 ± 3**
Testes	Study A	1051 ± 123	1091 ± 196	1101 ± 57	1037 ± 155
	Study B	955 ± 90	959 ± 137	917 ± 299	1110 ± 50
	Combined studies	1003 ± 113	1025 ± 174	1009 ± 225	1073 ± 115
Epididymides	Study A	346 ± 16	336 ± 43	341 ± 22	310 ± 101
	Study B	324 ± 31	344 ± 45	283 ± 96	335 ± 32
	Combined studies	335 ± 26	340 ± 42	312 ± 72	323 ± 72
Ventral prostate	Study A	126 ± 12	144 ± 27	113 ± 26	67 ± 31*
	Study B	141 ± 29	143 ± 16	113 ± 24§	68 ± 14**
	Combined studies	133 ± 22	144 ± 21	113 ± 23	68 ± 23**
Dorso-lat. prostate	Study A	151 ± 17	159 ± 27	136 ± 42	99 ± 41
	Study B	163 ± 26	143 ± 24	144 ± 53§	85 ± 17**
	Combined studies	157 ± 22	151 ± 25	140 ± 44	92 ± 30**
Seminal vesicles	Study A	230 ± 39	234 ± 42	204 ± 26	102 ± 38**
	Study B	273 ± 58	253 ± 15	225 ± 60§	122 ± 33**
	Combined studies	252 ± 51	244 ± 31	213 ± 42	112 ± 35**
Coagulating glands	Study A	46 ± 9	40 ± 8	37 ± 5	17 ± 11*
	Study B	49 ± 13	50 ± 10	40 ± 16§	19 ± 9**
	Combined studies	48 ± 11	45 ± 10	39 ± 10	18 ± 9**

§, $n = 4$. No multiple comparisons were calculated. Tissues were weighed fresh or after fixation (pituitary, thyroid, male accessory sex organs). Enhancements are underlined. Asterisks indicate a significant difference from the corresponding controls at the $p \leq 0.05$ (*) and $p \leq 0.001$ (**) level.

Histological findings

In the liver, centrilobular hepatocellular hypertrophy and cytoplasmic change was induced by FLU in males and females at the high dose. Effects of FLU on endocrine organs and hormone sensitive tissues in males are shown in Table 3. In females, only a slightly increased incidence of increased interstitial glands in the ovaries was observed at the high dose. Effects of EE2 are shown in Table 4. Most striking was a discrepancy between the diagnosis of the stage of the female cycle by vaginal smear cytology (diestrus) and vaginal and uterine morphology (estrogenized tissue).

Table 3 Important histological findings in endocrine tissues of FLU-treated male rats.

Dose (mg/kg body wt)		1			10			100		
Study		A	B	A & B	A	B	A & B	A	B	A & B
Pituitary	Increased number of PAS-positive cells				⊕	⊕	⊕	⊕	⊕	⊕
	Intracytoplasmic inclusions and hypertrophic basophilic cells							⊕	⊕	⊕
Testis	Leydig cell hypertrophy				⊕	⊕	⊕	⊕	⊕	⊕
Epididymis	Decreased tubular size and increased interstitial tissue							⊕	⊕	⊕
Accessory sex organs	Atrophy							⊕	⊕	⊕
Adrenals	Microvesicular cytoplasmic vacuoles in zona fasciculata							⊕	⊕	⊕

A & B, combined evaluation of studies A and B; ⊕, changes detected. Enhancements are underlined. The asterisk indicates a significant difference from the corresponding controls at the $p \leq 0.05$ (*) level.

Table 4 Important histological findings in endocrine tissues of EE2-treated rats.

Dose (mg/kg body wt)		0.01			0.05			0.2		
Study		A	B	A & B	A	B	A & B	A	B	A & B
Changes in male rats										
Testes	Degeneration of germinal epithelium, Leydig cell atrophy							⊕	⊕	⊕
Prostate	Atrophy							⊕	⊕	⊕
Seminal vesicles	Atrophy					⊕	⊕	⊕	⊕	⊕
Coagulating gland	Atrophy				⊕			⊕	⊕	⊕
Mammary gland	Feminization	⊕			⊕	⊕	⊕	⊕	⊕	⊕
Adrenals	Reduced vacuolation in zona fasciculata							⊕	⊕	⊕
Changes in female rats										
Ovaries	Increase of early stage follicles							⊕	⊕	⊕
Uterus	Increased epithelial height and other correlates of estrogenic action	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Vagina	Reduced number of diestric animals	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	Keratinization and other correlates of estrogenic action	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Adrenals	Cytoplasmic eosinophilia								⊕	⊕

A & B, combined evaluation of studies A and B; ⊕, changes detected. Enhancements are underlined.

Hormone determinations

Determination of thyroid related hormones did not contribute to the detection of the endocrine activity of FLU [2]. In EE2-treated rats mainly combined analysis suggested increased thyroid stimulating hormone levels in both sexes and increased thyroxine levels in females. However, these findings were not clearly dose-related, and hormone levels of the individual studies greatly differed from one another [4].

Spermatological findings

Effects of FLU and EE2 treatment are given in Table 5.

Table 5 Effects of FLU and EE2 on epididymidal sperm count and morphology.

		Control	Low dose	Mid dose	High dose
Right epididymidal spermatozoa counts (10^3 spermatozoa/mg)					
Flutamide	Study A	602 ± 170	705 ± 286	780 ± 157	498 ± 221
	Study B	687 ± 210	701 ± 318	901 ± 269	270 ± 109
	Combined studies	645 ± 186	703 ± 286	840 ± 217	384 ± 203*
Ethinylestradiol	Study A	715 ± 160	664 ± 88	618 ± 210	743 ± 239[§]
	Study B	553 ± 153	496 ± 189	733 ± 214[§]	799 ± 249
	Combined studies	634 ± 170	580 ± 165	669 ± 207	774 ± 231
Abnorm spermatozoa (%)					
Flutamide	Study A	1.3	1.0	1.6	3.1
	Study B	1.3	1.3	1.4	7.9
	Combined studies	1.3	1.2	1.5	5.4
Ethinylestradiol	Study A	0.6	1.6	0.1	1.3[§]
	Study B	0.2	1.4	0.8[§]	0.4
	Combined studies	0.4	1.5	0.4	0.8

[§]One male with aspermia not taken into account. Enhancements are underlined.

CONCLUSIONS

It was feasible to include all enhancements into the testing routine. The endocrine activity of FLU and ethinylestradiol (EE2) was readily demonstrated using the enhanced OECD TG 407 protocol. Both already existing and newly added parameters contributed to the detection of endocrine-mediated changes and individual studies A and B corresponded well to one another. Additional organ weights (mainly male accessory sex organs) and additional histological investigation of pituitary, epididymis, male mammary gland, and vagina were helpful in detecting endocrine mediated changes. Thyroid related hormones showed high variability and did not contribute to the detection of the endocrine activity of FLU. Changes of these hormones in EE2-treated animals could not be clearly related to estrogenic activity and thus did not contribute to sensitivity. Spermatology was insensitive at all or revealed changes only at the MTD. The use of vaginal smear cytology as a measure to determine the female cycle appears to be problematic in estrogen treated animals. Increasing the animal number from five to ten animals per sex and per dose level did not increase the sensitivity of detection of flutamide or EE2 induced endocrine-mediated effects above the level already obtained by histological examination of groups of five animals.

Care must be taken to include in the future version of the OECD TG 407 only those additional endpoints that have been proven to contribute to a reliable and sensitive detection of endocrine-mediated effects. Thorough evaluation of the results of 20 studies on 10 different compounds known or suspected to interact with the endocrine system through different mechanisms that have been tested in the validation phase 2 will provide a sound basis for the identification of the most appropriate enhancements.

ACKNOWLEDGMENT

The authors wish to thank EMSG for substantial financial support of this investigation.

REFERENCES

1. OECD. *OECD Protocol for Investigating the Efficacy of the Enhanced TG 407 Test Guideline, Rationale for the Investigation and Description of the Protocol*, Paris (1999).

2. P. Andrews, A. Freyberger, E. Hartmann, R. Eiben, I. Loof, U. Schmidt, M. Temerowski, M. Becka. *Arch. Toxicol.* **75**, 65–73 (2001).
3. OECD. *OECD Protocol for Investigating the Efficacy of the Enhanced TG407 Test Guideline (phase 2), Rationale for the Investigation and Description of the Protocol*, Paris (2000).
4. P. Andrews, A. Freyberger, E. Hartmann, R. Eiben, I. Loof, U. Schmidt, M. Temerowski, A. Folkerts, B. Stahl, M. Kayser. *Arch. Toxicol.* **76**, 194–202 (2002).