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Workshop 4.1

Simple, rapid assays for conventional definite testing of endocrine disruptor hazard: Summary and recommendations*

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Abstract: Study protocols for the characterization of endocrine active compounds presented in Workshop 4 included the enhanced Organization for Economic Cooperation and Development (OECD) test guideline (TG) 407, the medium-term rat liver and rat multi-organ carcinogenicity assays, and an enhanced one-generation reproduction study.

The outcome of rat studies on flutamide and ethinylestradiol indicated that these strongly active compounds can readily be detected even with a low animal number using the enhanced OECD TG 407. Both newly added (such as male accessory sex organ weights, histology of pituitary, vagina and male mammary gland) and already included parameters contributed to the detection of endocrine effects. Thorough evaluation of the results of 20 studies conducted with 10 compounds thought to interfere with the endocrine system by different mechanisms will identify the most appropriate enhancements to the current OECD TG 407.

Medium-term rat liver and rat multi-organ carcinogenicity assays are well recognized in the International Conferences on Harmonization for Pharmaceutical Chemicals. They have been successfully used to detect carcinogenic and modifying potentials of new chemicals within a relatively short time and can be applied to endocrine active compounds. Dose–response studies on nonylphenol, bisphenol A, and styrene using the rat liver carcinogenicity assay did not reveal effects of any of these compounds on the development of preneoplastic lesions in rat liver.

The enhanced one-generation reproduction study protocol included treatment of pregnant female rats from gestation day 0 through to lactation day 21, and examination of all offspring. Half of the animals were necropsied at weaning, the remaining animals were examined for vaginal opening, preputial separation, estrous cyclicity, and sperm characteristics and were necropsied at adulthood. In a pilot study ethinylestradiol inhibited maternal fertility at dose levels similar to those effective in the uterotrophic assay.

It is recommended to rapidly evaluate the conducted enhanced OECD TG 407 studies and to enhance the current OECD TG 407 appropriately. Further compounds with different mechanisms of action should be studied in the one-generation reproduction study to further investigate the usefulness of this protocol. The established medium-term carcinogenicity assays can be used to study carcinogenic potential rapidly. Use of female animals and inclusion of carcinogens targeting at breast and uterus should be considered in order to explore further the predictibility of this model.

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In the introduction to the workshop, Shoji Fukushima pointed to a number of areas that require particular emphasis when considering rapid assays for endocrine active substances (EASs) and the effects of these compounds on disease processes like reproductive lesions or neoplasia. First of all, and fundamental to the understanding of the mechanism of action of EASs, is the question of how an agent interacts with nuclear receptors or hormone synthesis, and which are the downstream events that then ensue. Related to this are metabolism of both EAS and endogenous hormones and the fate and potential activity of corresponding EAS metabolites and their environmental impact. In addition to studies on rodents, providing comprehensive information on a great diversity of endpoints, for human risk assessment due attention must be paid to any epidemiological evidence that may be available. It may also be helpful to take into account the effects of EASs in wildlife mammalian species should such information be available.

Of particular importance are the dose response and the appropriate definition of threshold doses. The possibility of homeostasis playing a role also deserves special consideration. Clearly, appropriate choice of representative test compounds and screening is essential, and given the likelihood of species specificity mammals must be the animals of choice for testing. Furthermore, the complex influence of hormones and EASs, acting simultaneously on many organs and tissues, means that in vivo approaches must be given high priority if a comprehensive understanding is to be obtained. Attention must also be concentrated on the stage in life when sensitivity would be expected to be highest on the basis of mechanistic and developmental considerations. This underlies the selection of models for presentation in our workshop.

In Workshop 4.1, by Freyberger et al., the enhanced OECD test guideline (TG) 407, was introduced. This subacute protocol for detecting endocrine modulation was discussed at the international level under the umbrella of the OECD, and the initial version was the basis for the reported feasibility study on flutamide (FLU). Furthermore, data on ethinyl estradiol (EE) using a modified protocol with fewer enhancements that is presently under investigation were presented for comparison. The enhancements considered were 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, male and female mammary gland, epididymes, pancreas. and vagina. For the study, groups of 5 male and 5 female rats were orally gavaged with FLU at 0, 1, 10, or 100 mg/kg body weight (b.w.)/day or with 0, 0.01, 0.05, or 0.2 mg/kg EE/day for at least 28 days. Two studies (A and B) were run in parallel to assess intra-laboratory variation and the potential increase in sensitivity with doubling of the animal numbers.

Endocrine activity of both FLU and EE was readily demonstrated with the enhanced OECD TG 407 protocol. Relative weights of the male accessory sex organs (MASO) were consistently decreased by the highest doses of FLU and EE. Treatment-related increase of relative uterine weights in EE-treated animals was only observed with the combined data at the high dose, indicating that uterine weight in young adult animals is not a sensitive measure of estrogenicity.

Consistent histopathological changes in studies A and B were atrophy of MASO and decreased tubular size of the epidiymus at 100 mg/kg FLU, along with increased numbers of PAS-positive cells from 10 mg/kg, hypertrophic basophilic cells at 100 mg/kg, and Leydig cell hypertrophy from 10 mg/kg, the latter indicating activation of the hypothalamic-pituitary-gonadal axis. Furthermore, microvesicular cytoplasmic vacuoles were observed in the zona fasciculata of the adrenals at the highest dose. With EE, similar atrophy of the MASO was recognized at the high dose, degeneration of the germinal epithelium and Leydig cell atrophy was apparent at 0.2 mg/kg, and feminization of the mammary gland was observed from 0.05 mg/kg. In contrast to the FLU study, reduced vacuolation in the zona fasciculata of the male adrenal at the high dose was seen with EE. In FLU-treated females, no endocrine-mediated histological changes were observed, while EE caused a striking discrepancy between the diagnosis of the stage of the female cycle by vaginal smear cytology (diestrus) and vaginal and uter-

ine morphology (estrogenized tissue) that was consistently observed in all treatment groups. Furthermore, an increase of early stage follicles was detected at the high dose. This findings underline the important role of histology in the detection of EASs.

Asssessment of thyroid-related hormones did not contribute to the detection of the endocrine activity of FLU, but the data from the combined analysis revealed an increase in thyroid stimulating hormone (TSH) levels in both sexes treated with EE and increased thyroxine levels in females. However, there was a lack of any clear dose relationship, and the data from the paired studies differed and changes could not be linked to estrogenic activity and thus did not contribute to sensitivity.

Treatment with EE did not affect sperm, whereas FLU at the highest dose increased the frequency of abnormal spermatozoa, especially in study B. A decreased epididymal sperm count was also noted on combined evaluation of studies A and B.

In general, the doubling of group size with analysis of combined data from the paired studies elevated the sensitivity although the additional significant alterations revealed had been already tentatively identified using the conventional group of five animals. This, however, was not the case with histopathology. Furthermore, doubling the animal number did not increase the sensitivity of detection of endocrine-mediated effects above the level already obtained by histopathological examination of groups of five animals.

From the present results for the strongly active compounds FLU and EE, inclusion of spermatology and determination of thyroid hormones and TSH in the final guideline cannot be recommended. Furthermore, use of vaginal smear cytology as a measure to determine the female cycle appears to be problematic in estrogen-treated animals. We must now await the evaluation of a phase 2 investigation that was performed in 13 laboratories in different countries using 10 compounds known or suspected to interact with the endocrine system through different mechanisms to finally decide which parameters should be included in the guideline and whether weakly active compounds can also sensitively be detected.

In Workshop 4.2, given by Imaida, medium-term in vivo tests for carcinogenicity or modifying potential of exogenous in rats were described, along with the body of results so far obtained. In the medium-term liver bioassay, animals are initiated with a single dose of diethylnitrosamine (DEN) then administered test compound starting two weeks thereafter with performance of partial hepatectomy in week 3. The effects of six weeks exposure to a compound on development of glutathione-*S*-transferase-positive putative preneoplastic foci are then assessed at the end of week 8. Distinction can be made between initating and promoting effects by inclusion of a group without DEN initiation. A total of 313 chemicals have already been analyzed, and the efficacy of the system for detection of hepatocarcinogens has thereby been well established. Comparison of results with this test and the two-year long-term rat assay confirmed similar dose-dependence and validated use of GST-P-positive foci as surrogate endpoint lesions. Dose–response studies of nonylphenol (doses of 25, 250, and 2000 ppm in the diet), bisphenol (40 and 160 mg/kg b.w., ig, six times per week) and styrene (250 and 1000 mg/kg b.w., ig, six times per week) did not reveal effects of any of these EASs on development of preneoplastic lesions, in terms of either number or area.

In the rat medium-term multiorgan bioassay described, male F344 rats are treated sequentially with five carcinogens, DEN, *N*-methyl-*N*-nitrosourea, dihydroxy-di-*n*-propylnitrosamine, *N*-butyl-*N*-(4-hydroxy-butyl)nitrosamine and dimethylhydrazine, primarily targeting the liver, lung, urinary bladder, and colon, respectively, then exposed to test compound for 24 weeks. A total of 63 chemicals have so far been tested in this model, all of 17 hepatocarcinogens and 19/22 carcinogens targeting other organs were positive. It is strongly recommended that representative EASs be now examined using this approach. Use of female animals and inclusion of carcinogens targeting the breast and uterus would be particularly beneficial in order to further explore the predictability of this model.

With both of these medium-term tests, particular advantages are the relatively small number of animals necessary, the short period of exposure, and the possibility of using surrogate markers for detecting carcinogenicity or modifying potential of EASs at low doses or in combination.

In Workshop 4.3, Aoyama described an enhanced one-generation reproductive study in rats for detecting endocrine-disrupting effects of chemicals, along with data obtained from pilot studies using EE. The presently used two-generation study protocol requires a relatively long period and only a small number of offspring is followed to adulthood. For the proposed new assay, pregnant females are treated with the test substance from gestation day 0 through to lactation day 21, the F1 offspring from half of the litters in each dose group then being killed for necropsy at weaning. The remaining half are examined for sexual maturation, estrous cyclicity, and/or sperm production. The parameters include fertility and gestation indices of dams, numbers of implants and pups delivered, sex ration and AGD of pups, the viability of pups during the lactation period, gross pathology of dams, and observations of F1 offspring such as organ weights at weaning and after maturation, sexual maturation (vaginal opening/preputial separation), estrous cyclicity, sperm characteristics, and gross and histopathological findings in all animals. Other additional endpoints that can be flexibly added include mRNA expression of appropriate genes. Following subcutaneous injection of EE effects on maternal fertility (inhibition of pregnancy and reduction of litter size) could be detected at dose levels corresponding to those effective in the uterotrophic assay. The same treatment of dams limited to the organogenetic or perinatal period did not disclose this effect. Thus, the days 0–5 of gestation are of prime importance. Findings observed in F1 offspring in the pilot study were slightly increased anogenital distance in female newborns and down- or upregulation of mRNAs of certain genes. Genes for androgen receptor in the prostate and IGF-1 in the uterus may be particularly sensitive markers for monitoring potential estrogenic effects.

Clearly, the different properties of the proposed single and two-generational approaches will require further comparative studies using the same representative test compounds at different doses, with appropriate consideration of data from other models.

Overall, the workshop pointed to potential variabilities of detecting EASs, especially for weakly active agents. It is recommended that several different approaches are evaluated in parallel to provide the most comprehensive profile possible before any conclusions are drawn.