Approach to Endocrine Disruptor Screening Program of Environmental Protection Agency

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Kenta Minami
Keiko Ose
Takafumi Yamaguchi

The existence of endocrine disrupting chemicals (EDCs) was suggested in the late 1990s, and concern about their effects on humans and wild animals has gathered a lot of public attention. This resulted in a worldwide reinforcement of regulations for EDCs, and the U. S. Environmental Protection Agency published their Endocrine Disruptor Screening Program (EDSP) and started an evaluation of EDCs.

In this article, we introduce our approach to and achievements in the evaluation of EDCs. In addition, we provide an overview of EDSP and the result of an EDSP assessment of Sumitomo Chemical's compound, Pyriproxyfen.

This paper is translated from R&D Report, "SUMITOMO KAGAKU", vol. 2016.

Introduction

With increases in awareness of safety for chemical products, suitable regulation of endocrine disrupting chemicals (EDCs) has become an important issue worldwide. In 2002, the World Health Organization (WHO) stated that "EDCs are exogenous substances or their mixture that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" through the International Program on Chemical Safety (IPCS).[1] However, there is still no procedure for determining suitable evaluation, classification, management or regulation of EDCs.

As a starting point for attracting the world's attention to EDCs we can look to *Our Stolen Future*,[2] a book published by Dr. Theo Colborn et al. in 1996. In this book, the hypothesis that EDCs were a cause of worldwide declines in organisms in the environment was proposed. Concern that EDCs were affecting the fertility of organisms in the environment and reducing the number of sperm in humans was shown based on various survey data. All of the information showing these reductions in the capacity for reproduction were not proven to be related to EDCs, and the discussion is continuing at present, but the impact of the book was significant; it can be said to be the trigger to strengthen regulation of EDCs by the regulatory authorities in various countries including Japan, U. S. and Europe.

Under these circumstances, the U. S. Congress passed the Food Quality Protection Act in 1996 to manage and establish the regulation of EDCs. With the passage of the act, the U. S. Environmental Protection Agency (EPA) published an overview of the Endocrine Disruptor Screening Program (EDSP)[3] in 1998 and started investigating pesticides and chemical substances. In 2009, 11 test method guidelines came into effect, and at the same time, evaluations of pesticides and chemical substances by the EDSP began. In this article, we will introduce details about the EDSP initiative of the EPA and also introduce the results of evaluations of pyriproxyfen, which is an insect growth regulator, as an example of work by Sumitomo Chemical for the EDSP. In 2015, the EPA generally agreed with the conclusions of Sumitomo Chemical regarding the evaluation of pyriproxyfen and determined that additional evaluations were unnecessary.

Problems in Evaluating and Regulating Endocrine Disrupting Chemicals (EDCs)

As is found in the definition proposed by WHO, EDCs act on the endocrine system in organisms and have adverse effects on individuals and progeny. On the other hand, it is also known that substances present in nature, such as phytoestrogens (daidzein, genistein, etc.) have effects on the endocrine system. Therefore, we think that, in suitable management and regulation

of EDCs, it is important to clarify their effects and tolerant amounts of exposure by chemically appropriate means.

After an introduction to a typical endocrine system as relates to endocrine disrupting activity, we will discuss problems in evaluation of EDCs based on the regulations.

1. Typical endocrine system and hormones

The typical endocrine organs are the gonads or ovaries and testes, which produce and secrete sex hormones (estrogens in females and androgens in males) necessary for acquisition and maintenance of reproductive capacity, and the thyroid which produces and secretes thyroid hormones necessary for development and growth of the brain. In addition, the production and secretion of sex hormones and thyroid hormones is regulated by the hypothalamus and the pituitary gland, which are in the center of the brain.

Therefore, it is important to appropriately evaluate the effects on the hypothalamic–pituitary–gonadal axis (HPG axis) and the hypothalamic-pituitary-thyroid axis (HPT axis). Representative endocrine systems and their hormones are shown in **Fig. 1**.

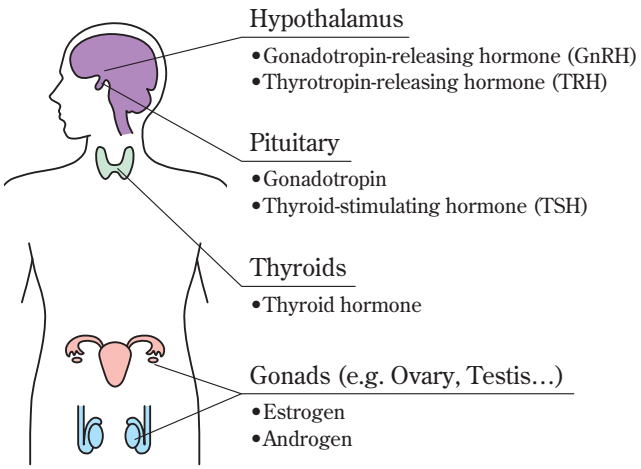


Fig. 1 Representative organs on endocrine system and typical hormones

In 1998, the Organization for Economic Cooperation and Development defined the activity to revise and supplement the guidelines as an important issue for screening potential EDCs. Effects on the HPG and HPT axes were regarded as important targets of EDCs. This can be understood by the fact that the development of test methods was started from the ones focusing on estrogenic and androgenic activities, effects on the thyroid, and inhibition of the production of steroid hormones.

2. Problems with the regulations

There are no established procedures for evaluating adverse effects of EDCs on the endocrine system. Worldwide discussions have continued for a long time, but there is no consensus of opinion even now, and currently, decisions on those procedures should be dependent on expert judgment. Therefore, there are examples where the regulatory authorities in various countries may conclude different evaluations for specific chemical substances.

As is discussed later, the U. S. EDSP is leading the world in working on systematically evaluating and regulating EDCs, and attention is being given to the progress by regulatory authorities and industries of many countries.

Approach of Sumitomo Chemical for EDC Evaluation

1. Participation in OECD test method guideline development

Sumitomo Chemical has worked on developing technology to evaluate EDCs since the 1990s, when there was a rapid increase in societal concern about EDCs. At the time, some media published inciting reports that EDCs are a threat to the survival of human beings and organisms in the environment, thus there were increasing needs to reassess the conventional safety evaluation methods. In particular, there was increasing concern focused on the effects on reproduction, thus establishment of technology to investigate the effects on reproductive organs (gonads, accessory sex organs, etc.) and sex hormones was being promoted.

In 1998, the OECD selected three guidelines, an uterotrophic assay (OECD TG 440), a Hershberger assay (OECD TG 441) and a 28-day repeated dose toxicity test (OECD TG 407), as primary guidelines to be validated. Following this, many test methods (*in vitro* tests such as an assay using cultured cells, tests using organisms in the environment, etc.) were validated. In this situation, Sumitomo Chemical participated in the development of the OECD guidelines, suggested opinions based on scientific evidence and also contributed to the establishment of test methods by providing data from international validation studies.[4)–6)] In addition, in the development of a transcription activation assay

(OECD TG 455) for estrogen receptor (ER), Sumitomo Chemical participated in a national project, and as a result of many repeated trials, recombinant cells produced by Sumitomo Chemical came to be used as standard cells (hER α-HeLa-9903) for those studies.

2. Development of Sumitomo Chemical internal evaluation system

We have exhaustively investigated the toxicological concerns in general toxicity tests, and supplemental evaluations were conducted for chemical substances for which endocrine disrupting effects could not be dispelled, with evaluation methods that incorporated toxicity mechanisms. For example, toxicity predictions based on structural similarities, various *in vitro* tests, and other studies using animals which can be evaluated in a short period (*in vivo*) have been conducted. In addition, where necessary, rat reproductive studies that evaluate adverse effects on the endocrine system over the long term are conducted for final confirmation. We have also established systems that can carry out a fish short-term reproduction assay and an amphibian metamorphosis assay for organisms in the environment. Regarding amphibians, the evaluation system was established based on our experiences of breeding, histopathological examinations, etc. This led Sumitomo to conduct exhaustive safety evaluation internally.[7] In addition, we have established a simple test system using Japanese quail embryos to evaluate the effects on androgenic activity in birds, for which there have been few reports.[8]

Endocrine Disruptor Screening Program (EDSP)

In 1998 the EPA published an overview of the EDSP, and began investigations into pesticides and chemical substances. Along with introducing the overview of the EDSP here, we will report our actions and the results of EPA evaluation for pyriproxyfen, which is an insect growth regulator developed by Sumitomo Chemical.

1. Overview of EDSP

In accordance with the requirements of the Food Quality Protection Act passed by the U. S. Congress in 1996, the EPA started to establish a screening program for evaluating the possibility of effect on estrogen systems and various endocrine systems in humans by certain chemicals using appropriate validated tests and chemical information. Then, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), was established. In the report[9] of the EDSTAC, a two-tiered approach was proposed; potential evaluation by screening tests (Tier 1) and assessments of adverse effects in comprehensive life cycle studies (Tier 2). In this system, Tier 2 might be required when a potential endocrine disrupting effect is observed in Tier 1 (**Fig. 2**). The OECD has already issued guidelines for many of these test methods and the EPA has issued them as EDSP test method guidelines. Generally speaking, EDSP can be said to be a screening program to detect EDCs through mechanisms of action such as production, dynamics, binding with receptors, and/or metabolism of hormones.

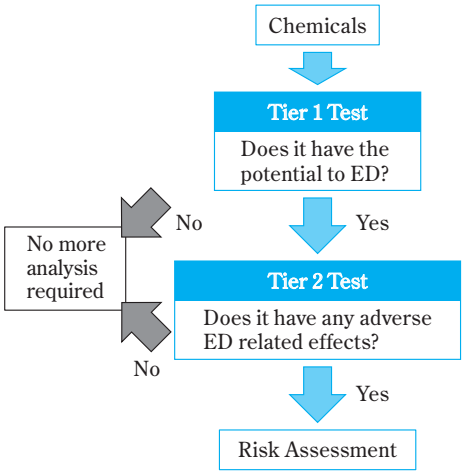
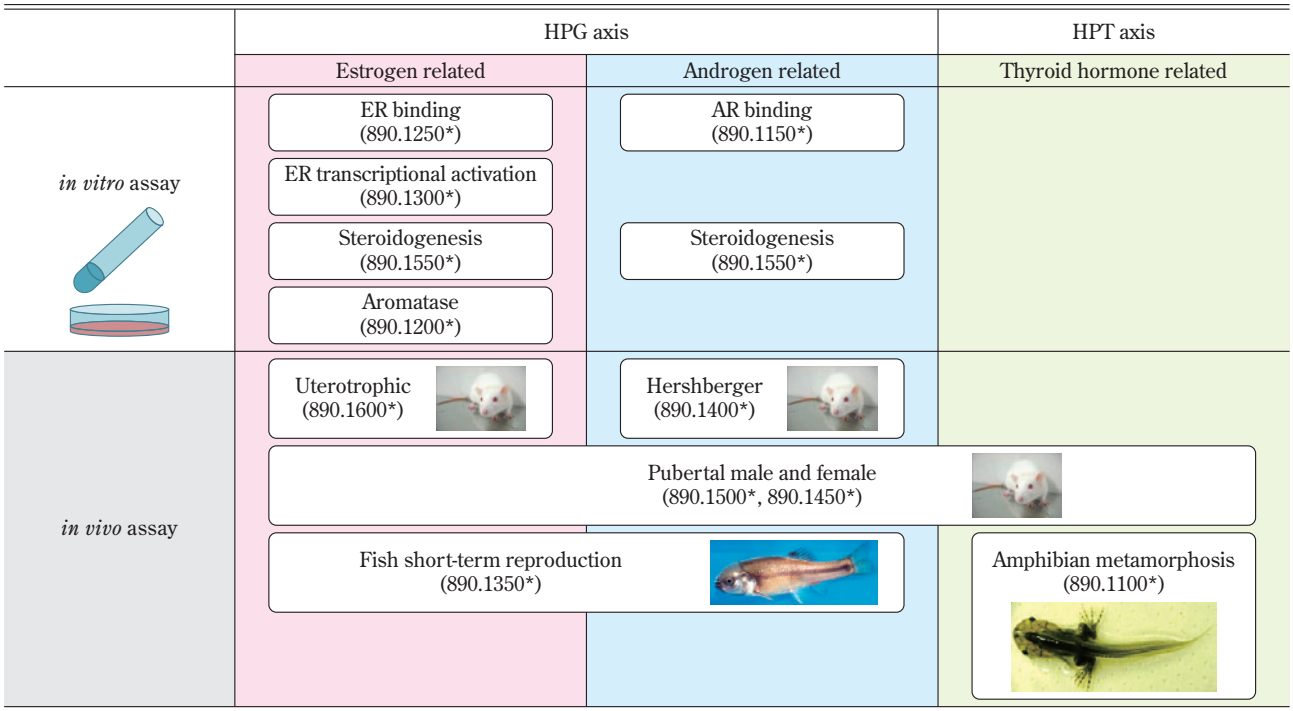


Fig. 2 Flow chart for EDSP evaluation (Tier 1 and Tier 2)

In 2009, 67 substances (List 1) were published as targets of initial evaluations and they were mainly pesticides. These were selected with a focus on chemicals with multiple exposure pathways for human (food, water, residential and occupational exposure) rather than the potential of being EDCs.[10] Tier 1 evaluations will be started by the test orders issued by the EPA to registrants for each substance. The registrants receiving the test orders will have choices to prepare for Tier 1; acquiring new data, using existing data, or participating in a consortium of multiple registrants. If submitting new data for Tier 1 evaluations, it has to be issued within two years from issuance of the test order. Analysis of the Tier 1 test results for the chemical substances in List 1 has already been completed by the EPA, and the evaluations were disclosed in June 2015.[11] Many other chemical substances are expected to become targets of the EDSP in the future.



HPG: Hypothalamus-Pituitary-Gonad
HPT: Hypothalamus-Pituitary-Thyroid
*: Guideline No.

Fig. 3 Overview of the EDSP Tier 1 Tests

2. EDSP Tier 1 tests

The purpose of the tests listed for Tier 1 is detecting substances that have effects on specific endocrine systems. The EPA is focusing on the HPG axis, HPT axis and steroid hormone production. In 2009, 11 test method guidelines (**Fig. 3**) were issued and came into effect for Tier 1 tests. Tier 1 tests consist of *in vitro* tests to detect the effects of chemical substances on cells and receptors and *in vivo* tests to detect the effects on the organisms. An overview of these tests is given as follows.

(1) *In vitro* tests

(i) ER binding assay (OPPTS TG 890.1250) and androgen receptor (AR) binding assay (OPPTS TG 890.1150)

Estrogens and androgens show hormone activity by binding with their respective receptors in an organism. These tests are for detecting the affinity of chemical substances and the receptors.

(ii) ER transcriptional activation assay (OPPTS TG 890.1300)

This test evaluates the transcriptional activation (estrogenic activity) based on the binding of a chemical substance with ERs. HeLa cells (hERα-HeLa-9903) which have a firefly reporter plasmid-bearing ER responsive element stably introduced by Sumitomo Chemical are used.

(iii) Steroidogenesis assay (OPPTS TG 890.1550)

In this test, human adrenal cortex-derived cells (H295R) are used, and effects of chemical substances on the ability to produce steroids are detected.

(iv) Aromatase assay (OPPTS TG 890.1200)

Aromatase (CYP19) contributes to estrogen production in steroidogenic systems. In this test, aromatase inhibitory activity of chemical substances is measured.

(2) *In vivo* tests

(i) Uterotrophic assay (OPPTS TG 890.1600)

The purpose of this test is to detect chemical substances having estrogenic activity. In this test, rats from which the ovaries have been removed or immature rats are used to eliminate the effects of estrogen present in the body. Rats from which the ovaries have been removed have no HPG axis function; therefore, there is a reduction in the estrogen level in the body, and the uterus has atrophied. Besides, the uterus of immature rats prior to the start of the estrous cycle is in a rudimentary state.

When animals in which the uterus has atrophied or

is rudimentary in this manner are repeatedly administered chemical substances having an estrogenic activity, the uterus becomes hypertrophic. In this test, the period for administration is three days, and changes in uterine weight are evaluated as an index.

(ii) Hershberger assay (OPPTS TG 890.1400)

The purpose of this test is to detect chemical substances having androgenic activity or anti-androgenic activity. In this test, rats in which the testes have been removed (castration) are used to eliminate the effects of androgens present in the body. Castrated rats have a reduction in the androgen concentration within the body because the HPG axis does not function. As a result, accessory sex organs such as the ventral prostate, seminal vesicles, coagulating glands, etc. atrophy dependent on the androgen concentration. When animals with atrophied accessory sex organs are repeatedly administered chemical substances having androgenic activity, the accessory sex organs become hypertrophic. In addition, when substances with anti-androgenic activity are repeatedly administered under conditions in which these animals are administered an exogenous androgen precursor (testosterone propionate) and the accessory sex organs are hypertrophic, the accessory sex organs atrophy. The administration period for this test is 10 days, and changes in accessory sex organ weight are evaluated as an index.

(iii) Male pubertal assay (OPPTS TG 890.1500)

This test repeatedly doses (31 days) immature male rats orally until the maturation period, and the effects on the endocrine system focused on the gonads and thyroid are comprehensively evaluated. Besides organ weight and histopathological examinations for the organs related to reproduction and the thyroid, items examined include measurements made for sex differentiation (preputial separation), and testosterone (typical androgen), thyroid hormone and thyroid stimulating hormone in the blood.

(iv) Female pubertal assay (OPPTS TG 890.1450)

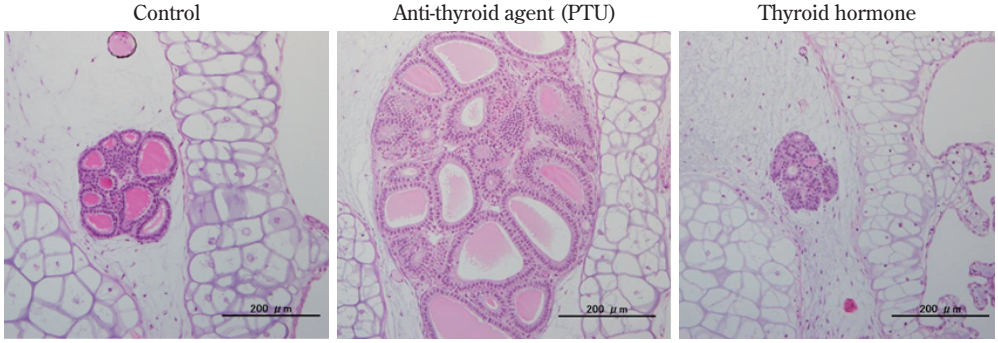
This test repeatedly doses (21 days) immature female rats orally until maturation, and the effects on the endocrine system focused on the gonads and thyroid are comprehensively evaluated. Besides organ weight and histopathological examinations for the organs related to reproduction and the thyroid, sex differentiation (vaginal opening), estrous cycle, and thyroid hormone and thyroid stimulating hormone in the blood are measured.

(v) Fish short-term reproduction assay (OPPTS TG 890.1350)

The purpose of this test is to detect chemical substances having estrogenic and androgenic activities as well as anti-estrogenic activity in fish. In this test, adult male and female fathead minnows are used to detect the effects on the number of eggs produced and fertility during the exposure period (21 days), and on gonad weights, blood vitellogenin level, secondary sexual characteristics and gonad histopathology at the end of exposure. Based on these results, the effects on sexual hormonal system are evaluated. Vitellogenin is a specific precursor protein of egg yolk in oviparous species and is used as an estrogenic activity biomarker.

(vi) Amphibian metamorphosis assay (OPPTS TG 890.1100)

This test evaluates effects of chemical substances on the HPT axis using amphibians. The progress of



Anti-thyroid agent induced thyroid grand hypertrophy, follicular cell hypertrophy and hyperplasia (middle), Thyroid hormone induced thyroid grand atrophy and decreased follicular lumen area (right).

Fig. 4 Thyroid histopathology of African clawed frogs (tadpoles)

amphibian metamorphosis is regulated by thyroid hormones, and the period of metamorphosis is hypersensitive to thyroid hormones. Tadpoles of African clawed frogs are exposed to chemical substances. In addition to measuring stage classification[12] based on morphological characteristics, measurements of hind limb length and snout-vent length, and thyroid histopathological examinations, as the most sensitive factor, are carried out. **Fig. 4** shows thyroid histopathology of African clawed frogs in prometamorphosis. Since the HPT axis is controlled similarly in mammals and amphibians, amphibian metamorphosis assay can evaluate the effects on the HPT axis for vertebrates including humans.

The *in vitro* tests among the Tier 1 tests as well as the *in vivo* tests using mammals and organisms in the environment have items for evaluation that are complementary to each other, and it is not adequate to evaluate the absence or presence of effects on specific endocrine systems using only results obtained from a single test. In particular, information on mode of action for specific endocrine systems can be obtained with the *in vitro* tests, but on the other hand, they do not reflect the effects due to metabolism, excretion of chemical substances in the whole body and other biological conditions. For the above reasons, all Tier 1 test data, including known toxicity information should be analyzed in detail in the Tier 1 evaluations, and based on the significance of the various tests, an overall evaluation is necessary.

The purpose of Tier 1 tests is evaluating potential for detecting substances that can affect endocrine systems. When, as a result of Tier 1 tests, it is determined that the extent of effects on endocrine systems must be further elucidated, Tier 2 tests must be carried out. Tier 2 tests are positioned as definitive tests, and multiple organism groups (mammals, birds, amphibians, fish and invertebrates) are used to evaluate the characteristics of endocrine related effects and their intensity. Existing two-generation reproductive toxicity studies (OECD TG 416) for rats or extended one-generation reproductive toxicity studies (OECD TG 443) for rats are used for the mammalian evaluations. In terms of organisms in the environment, guidelines have been produced for larval amphibian (African clawed frog) growth and development assay (OCSPP TG 890.2300), medaka extended one generation reproduction test (OCSPP TG 890.2200) and avian two-generation toxicity test in the Japanese quail (OCSPP TG890.2100), and establishment of guidelines for mysid shrimp two-generation tests is planned.

3. Work on Tier 1 evaluations of pyriproxyfen

An EDSP Tier 1 test order for pyriproxyfen was issued in 2009 as one of the chemical substances targeted in List 1. In response to this, Sumitomo Chemical submitted "Other Scientific Relevant Information (OSRI)" in which existing results of various toxicity tests and reference reports were analyzed and summarized in 2010 and, having sufficient information for Tier 1, had its application for waiver for the uterotrophic assay and ER transcriptional activation test accepted. In terms of new tests, the ER binding assay, AR binding assay, steroidogenesis assay, aromatase assay, Hershberger assay, male and female pubertal assays, fish short-term reproduction assay and amphibian metamorphosis assay were carried out. Furthermore, we submitted a comprehensive discussion emphasizing that pyriproxyfen was not an EDC based on existing tests, including additional tests conducted taking into consideration useful discussions of some test results and available data such as the medaka full life cycle test.

The EPA conducted Tier 1 evaluations of this information and Sumitomo Chemical's assertion was generally accepted. The following gives an overview of the various test results and shows the comprehensive discussion.

(1) *In vitro* test results

(i) ER binding assay and AR binding assay

In these test systems, no ER or AR binding was found.

(ii) ER transcriptional activation assay

Weak estrogen activity was found in existing reference reports.[13), 14)] However, it was an effect at concentrations much greater than the concentration in the blood in the *in vivo* tests.

(iii) Steroidogenesis assay

At concentrations where cytotoxicity for H295R cells was not found, no effect on testosterone production was found. On the other hand, since a slight enhancement in estradiol production was found, the results suggested estrogen production inducibility. However, in the *in vivo* tests that will be discussed later, no effects related to inducing estrogen production were found.

(iv) Aromatase assay

In this test system, no aromatase inhibitory activity was found.

(2) *In vivo* tests

(i) Uterotrophic assay

Even at the limit dose of 1000 mg/kg determined by the guidelines, no changes in uterine weight of immature rats were found, and no changes suggesting estrogenic activity were detected.

(ii) Hershberger assay

As a result of administration of the limit dose of 1000 mg/kg determined by the test method guidelines, no changes in the weight of any accessory sex organs were found. Likewise, no changes in the weight of accessory sex organs were found even in animals administered the testosterone precursor. Therefore, changes suggesting androgenic or anti-androgenic activity were not detected.

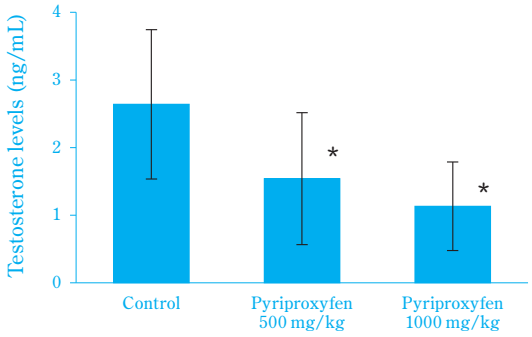
(iii) Male pubertal assay

Immature rats (aged 23 days at the start of administration) were orally administered 500 and 1,000 mg/kg by gavage for 31 days. As a result, reductions in testosterone concentration in the blood (**Fig. 5**) and reductions in the weight of accessory sex organs were observed. In the liver, changes in weight and histopathological changes (hepatocyte hypertrophy) were observed. It has been reported that pyriproxyfen induces drug metabolizing enzyme in the liver,[15] and in this test, the effects on the liver that were found can be thought as being caused by the same changes. The possibility that the reduction of testosterone concentration in the blood found in this test was a secondary change through induction of metabolism in the liver rather than a direct effect such as steroidogenesis inhibition can be considered. Additionally, in terms of effects on the kidneys, blood chemistry test values (urea nitrogen and creatinine), changes in weight and histopathological changes were observed.

In terms of the thyroid, a reduction of the colloid region and thickening of the follicular epithelial cells were found for histopathological changes. In rats, metabolism of thyroid hormone is enhanced by inducing drug metabolizing enzyme (UDP-glucuronyl transferase (UGT)) in the liver, and it is known that thyroid stimulating hormone (TSH) is secreted by the pituitary gland to maintain homeostasis.[16] As a result, the changes described above can be seen in histopathological examinations. In other words, the effect on the thyroid can be thought of as secondary changes through the induction of drug metabolizing enzyme in the liver.

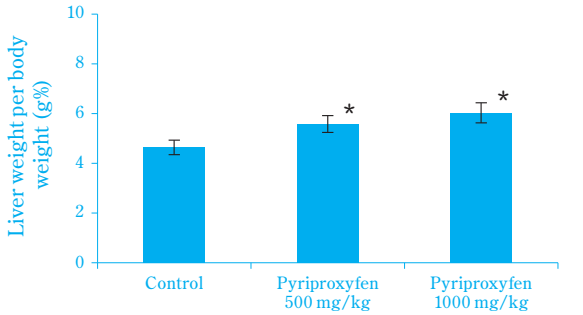
(iv) Male pubertal assay – additional study

In terms of the reduction in testosterone found in the male pubertal assay, an additional study was carried out to prove the hypothesis that they were secondary changes through effects on the liver. In the additional study, effects on drug metabolizing enzymes (CYP2B and CYP3A) in the liver and on testosterone production in the testes due to pyriproxyfen administration were examined. It has been reported that testosterone is metabolized by these drug metabolizing enzymes in the liver.[17)–19)] In addition to increase in organ weight of the liver (**Fig. 6**), induction of drug metabolizing enzymes (**Fig. 7**) was found, and there was no effect on the activity of enzyme (17β-HSD) related to testosterone production in the testes (**Fig. 8**). Additionally, since no effects on the androgen system were found in other Tier 1 tests, it was concluded that the reduction



*: p<0.01

Fig. 5 Serum testosterone levels of the pyriproxyfen administrated male rats in the pubertal assay



*: p<0.01

Fig. 6 Relative liver weight of the pyriproxyfen administrated male rats in the additional study

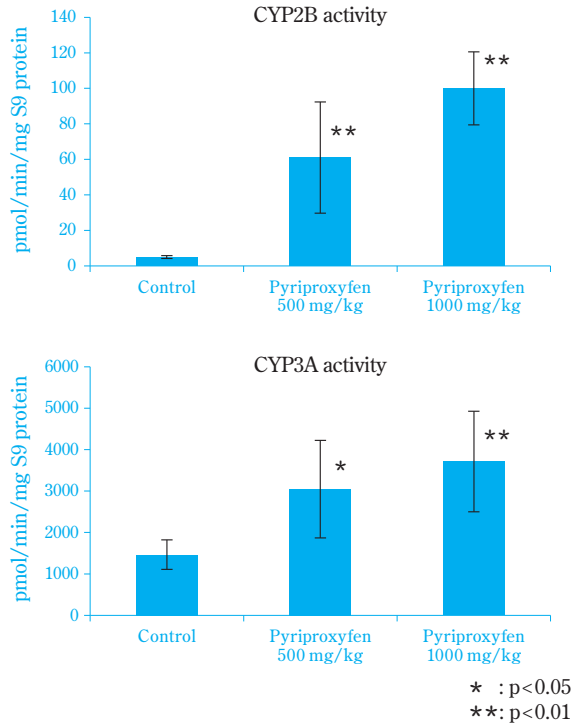


Fig. 7 Hepatic enzyme activities of the pyriproxyfen administrated male rats in the additional study

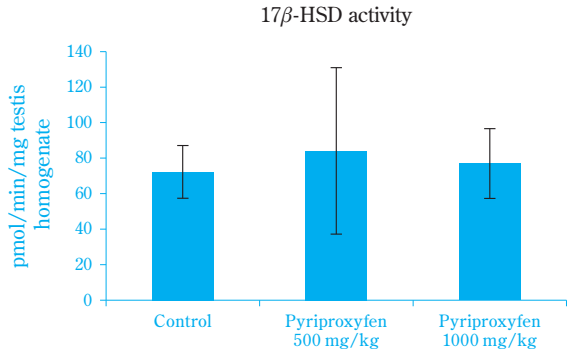


Fig. 8 Testicular enzyme activity of the pyriproxyfen administrated male rats in the additional study

in testosterone concentration due to pyriproxyfen administration was an indirect change because of effects on the liver rather than a direct effect on the endocrine system (**Fig. 9**).

(v) Female pubertal assay

Immature rats (aged 22 days at the start of administration) were orally administered 500 and 1000 mg/kg by gavage for 21 days. As with the male pubertal assay, changes that suggested effects on the liver and kidneys were found. In addition, decrease in the weights of the pituitary gland and ovaries was observed, but these were slight changes not accompanied by histopathological changes. In examinations of vaginal opening and estrous cycle, no abnormalities were found; therefore, it could be assumed that there were no effects on ovarian function. In addition, in various existing toxicity tests, no effects suggesting anti-estrogenic activity or effects on reproductive function in two-generation reproductive study using rats were found.

With the thyroid, a reduction of the colloid region and thickening of the follicular epithelial cells were found in histopathological examinations. As was also described in the items for the male pubertal assay, the effects on the thyroid can be thought of as secondary changes through the induction of drug metabolizing enzymes in the liver.

(vi) Fish short-term reproduction assay

The highest concentration was set at a nominal concentration of 300 µg/L, close to the water solubility limit, based on no lethal toxicity being observed in the short term at the upper limit of water solubility. Although increases in gonadosomatic index and

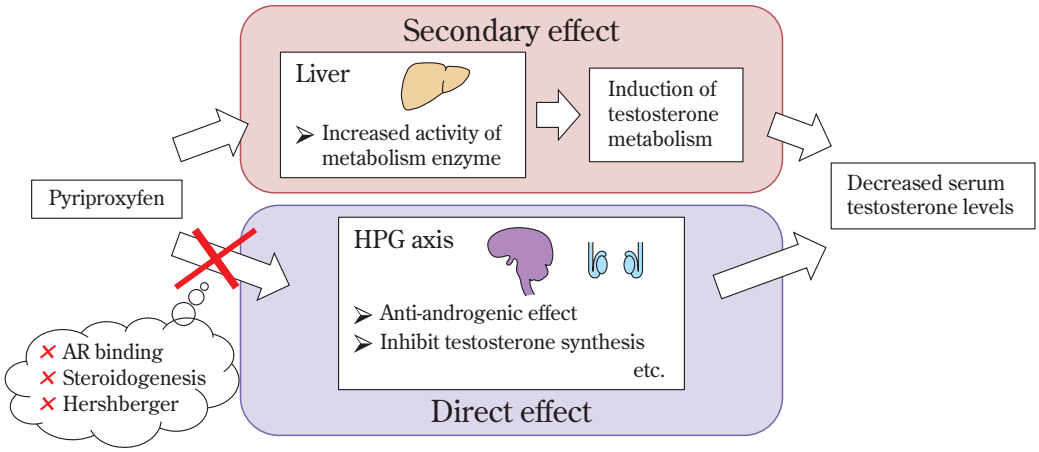


Fig. 9 Decreased serum testosterone levels caused *via* liver effect

decreases in secondary sexual characteristics were observed in males at the highest test concentration, there were no effects on gonadal histopathology, blood vitellogenin levels and fertility (egg laying rate and fertilization rate), all of which are important items for evaluation, as well as sex hormone levels.

(vii) Medaka full life cycle test – existing data

The purpose of this test is to examine the effects on reproduction in fish, which are exposed to the test substance in a period from fertilized eggs through the reproductive period of medaka (approximately 60 – 110 post-hatch days) up to the next generation fish (60 post-hatch days). No effects were observed in the main evaluation items (morphology including secondary sexual characteristics, reproduction and hatching, gonadal histopathology, and vitellogenin level) in both parent and offspring generations at the highest concentration (actual measured concentration of 8.6 µg/L).

(viii) Amphibian metamorphosis assay

The highest concentration was set at a nominal concentration of 300 µg/L, close to the water solubility limit, based on no lethal toxicity being observed in the short term at the upper limit of water solubility. Eeven though retardations of the development stage and growth in hind-limb length and body weights were observed at the highest test concentration, decreases in food consumption and no histopathological changes in the thyroid, which is the most sensitive indicator, were observed at that concentration. Therefore, it was concluded that there were no effects on the HPT axis due to the pyriproxyfen exposure.

In addition to the results of newly conducted EDSP Tier 1 tests, the additional study of the male pubertal assay, existing test results which were allowed a waiver, and reference information were also included for comprehensive consideration of endocrine disrupting activity by pyriproxyfen. Estrogenic activity was suggested in the *in vitro* tests and reference reports, effects on androgen systems were suggested in the male pubertal assay in mammals, and anti-androgenic activity was suggested in the fish short-term reproduction assay. Changes found in the *in vitro* tests were those found at extremely high concentrations that could not occur in the blood in the *in vivo* tests. From test results in additional examinations for mammals, they were thought to be indirect changes via drug metabolizing enzymes in the liver, and it was also assumed from the effects on the liver from low doses in various toxicity tests. In addition, since no exposure effects were found in the medaka full life cycle test whose test procedure was similar to that of the Tier 2 tests, it was thought that there was no endocrine disrupting activity in fish. Furthermore, based on existing test results and reference information, it was determined that pyriproxyfen was not an EDC, and the conclusion was that Tier 2 tests were unnecessary. In evaluation results published by the EPA in 2015, Sumitomo Chemical's opinion was generally accepted, and it was determined that Tier 2 tests were unnecessary in mammals and organisms in the environment.

4. Evaluation status of other List 1 substances

From the published evaluation results for the 52 substances undergoing Tier 1 evaluations of the 67 substances selected as List 1 substances in the EDSP, there were 14, 17 and 18 substances having the potential of effecting estrogens, androgens and thyroid hormone systems, respectively. In addition to the existing data for various toxicity tests most of the substances have undergone risk assessments, and additional testing was not required. On the other hand, additional tests and/or Tier 2 equivalent tests were recommended for one substance for which effects related to the androgen pathway in mammals were suggested, four substances for which effects related to the thyroid pathway in mammals were suggested, 13 substances for which effects related to the estrogen and androgen pathways in organisms in the environment were suggested and five substances for which effects related to the thyroid pathway were suggested. The results of the Tier 1 evaluations for these List 1 substances have been published on the EDSP website.[11)]

5. Future developments for EDSP

Chemical substances that are targets of EDSP evaluations can be thought of as substances managed and regulated by the Food, Drug and Cosmetic Act and the Safe Drinking Water Act in addition to the Food Quality Protection Act. Chemical substances for which there is concern of their being contained in drinking water are within the range of targets, and as of 2011 it has been postulated that approximately 10,000 substances may be targets of evaluations.[20)] For the 67 substances that were targets of evaluation in List 1, the EPA has spent approximately six years up to the disclosure (2015) of

the Tier 1 evaluation results since the issuing of test orders (2009). If evaluation progresses similarly in the future, it is expected to be virtually impossible to evaluate all of the chemical substances. Thus, the EPA has come up with the EDSP21[20] plan for effectively making progress with the EDSP. Within this plan, high throughput (HTP) models for the *in vitro* tests will be used in addition to toxicity predictions using various types of existing toxicity test data and computer models, and priorities of the chemical substances that are targets of evaluation or the replacing the HTP model for Tier 1 evaluations will be assigned (**Fig. 10**). A method known as integrated bioactivity exposure ranking (IBER) has been proposed as for setting priorities. With IBER, it is possible to select substances for which there is a high level of concern of their being EDCs based on bioactivity and prediction data for exposure. In addition, it has been reported that it is possible to make substitutions for multiple tests in EDSP Tier 1 by using the HTP model.[21] Furthermore, a complete substitution of these HTP models for EDSP Tier 1 tests has been put forth as a long-term goal, and if it is achieved, shortening of test periods and reductions in costs can probably be expected. However, the *in vitro* tests that constitute the HTP models do not reflect the effects of metabolism, excretion and other physiological conditions for the chemical substances in the body; therefore, more prudent investigations are necessary for complete substitution.

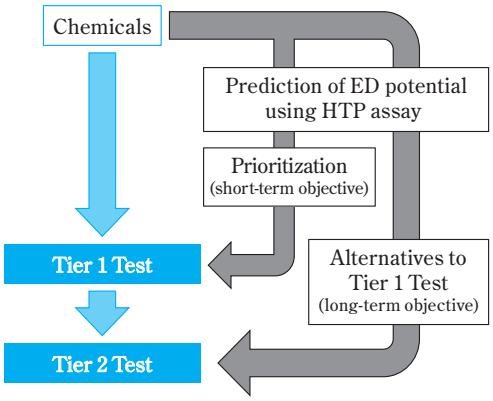


Fig. 10 Future scheme of the EDSP proposed in the EDSP21

Conclusion

The situation is one in which clear and uniform standards have yet to be established for the regulation of chemical substances for which there is concern about endocrine disrupting activity. On the other hand, the EPA has implemented the systematic program called EDSP and has started evaluating EDCs. Furthermore, progress on a new effort known as EDSP21 for achieving a structure for evaluating all chemical substances is as previously described. The EU is still making progress in the process of discussing the definitions and classifications of EDCs, and the future trend of regulation is in flux.[22] In Japan, the main relevant authority has become the Ministry of the Environment, and while investigational research and test method development are moving forward,[23] no specific regulations have been established. The regulatory authorities in various countries will be expected to work in coordination.

Most of the important issues for the adverse effects of EDCs are related to the HPG and HPT axes at this time from the historical background and the extent of the effects on health. On the other hand, endocrine systems are constructed of complex networks, and a large number of endocrine organs and hormones function within the body. Therefore, the range for evaluation of the adverse effect due to EDCs can be expected to diversify in the future and not stop at the HPG and HPT axes. Starting with the adrenal glands which secrete many types of hormones, we must bring the establishment of evaluation procedures for the adverse effects on various endocrine systems into our field of view. At Sumitomo Chemical, we are working on introducing new evaluation methods using OMICS analytical methods, etc. and continuing to improve the precision of evaluation techniques. We would like to continue making efforts toward developing test methods using the state-of-the-art scientific techniques and constructing an exhaustive safety evaluation structure.

References

1) WHO, “International Programme on Chemical Safety, Global Assessment of the State-of-the-Science of Endocrine Disruptors” (2002).
2) T. Colborn, D. Dumanoski and J. P. Myers, “Our Stolen Future”, Dutton/Penguin Books (1996).
3) U. S. Environmental Protection Agency, “Federal Register, Vol. 63, No. 248, Endocrine Disruptor Screening Program; Proposed Statement of Policy” (1998).
4) T. Yamada, O. Sunami, T. Kunimatsu, Y. Kamita, Y. Okuno, T. Seki, I. Nakatsuka and M. Matsuo, *Toxicology*, **162**, 103 (2001).

5) T. Yamada, T. Kunimatsu, K. Miyata, S. Yabushita, T. Sukata, S. Kawamura, T. Seki, Y. Okuno and N. Mikami, *Toxicol. Sci.*, **79**, 64 (2004).
6) T. Kunimatsu, T. Yamada, K. Miyata, S. Yabushita, T. Seki, Y. Okuno and M. Matsuo, *Toxicology*, **200**, 77 (2004).
7) K. Miyata and K. Ose, SUMITOMO KAGAKU, **2012**, 51 (2012).
8) T. Utsumi, M. Miyamoto and T. Katagi, SUMITOMO KAGAKU, **2011-I**, 49 (2011).
9) U. S. Environmental Protection Agency, "Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report" (1998).
10) U. S. Environmental Protection Agency, "Federal Register, Vol. 74, No. 71, Final List of Initial Pesticide Active Ingredients and Pesticide Inert Ingredients to be Screened Under the Federal Food, Drug, and Cosmetic Act" (2009).
11) U. S. Environmental Protection Agency, "Endocrine Disruptor Screening Program Tier 1 Screening Determinations and Associated Data Evaluation Records", Retrieved Jun. 14, 2016, from https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and
12) P. D. Niewloop and J. Faber, "Normal Table of *Xenopus Laevis* (Daudin)", Garland Publishing, Inc. New York & London (1994).
13) M. Kojima, K. Fukunaga, M. Sasaki, M. Nakamura, M. Tsuji and T. Nishiyama, *Int. J. Environ. Health Res.*, **15** (4), 271 (2005).
14) M. Manabe, S. Kanda, K. Fukunaga, A. Tsubura and T. Nishiyama, *Int. J. Hyg. Environ. Health*, **209**, 413 (2006).
15) H. Yoshino, H. Kaneko, I. Nakatsuka and H. Yamada, *J. Agric. Food Chem.*, **44** (6), 1578 (1996).
16) "Casarett and Doull's Toxicology-The Basic Science of Poisons (8th Edition)", C. D. Klaassen (Ed.), McGraw-Hill (2013).
17) D. B. Peakall, *Environ. Health Perspect.*, **13**, 117 (1976).
18) P. W. Harvey, K. C. Rush and A. Cockburn, "Endocrine and Hormonal Toxicology", John Wiley & Sons Ltd. (2009).
19) V. K. Turan, V. M. Mishin and P. E. Thomas, *Drug Metab. Dispos.*, **29** (6), 837 (2001).
20) U. S. Environmental Protection Agency, "Endocrine Disruptor Screening Program for the 21st Century: (EDSP21 Work Plan)" (2011).
21) P. Browne, R. S. Judson, W. M. Casey, N. C. Kleinstreuer and R. S. Thomas, *Environ. Sci. Technol.*, **49** (14), 8804 (2015).
22) European Commission, "Roadmap: Defining criteria for identifying Endocrine Disruptors in the context of the implementation of the Plant Protection Product Regulation and Biocidal Products Regulation" (2014).
23) Ministry of the Environment of Japan, "Further Actions to Endocrine Disrupting Effects of Chemical Substances: EXTEND 2010" (2010).

PROFILE

Kenta Minami
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Researcher

Takafumi Yamaguchi
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate

Keiko Ose
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate