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Screening and Testing Methodology

International Initiatives to Develop a Global Strategy on Endocrine Disruptors Testing and Assessment

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At the request of Member countries and the international industry OECD initiated in 1997 the Special Activity on Endocrine Disrupters Testing and Assessment with the objectives to provide a set of internationally recognised and harmonised testing guidelines and testing and assessment strategies for regulatory use that would avoid duplication of testing and thus save resources, including animals.

Managed by the Endocrine Disrupters Testing and Assessment Task Force (EDTA) and its two Validation Management Groups on mammalian tests (VMG-mammalian) and ecotoxicity tests (VMG-eco) respectively, several comprehensive test validation projects have been initiated and some of these are currently well underway or close to completion. In addition. Test Guidelines 414 and 416 have been updated and were subsequently adopted by Council in January 2001. The validation project of the Uterotrophic Assay for oestrogenic effect assessment is nearing its completion as the 3rd Meeting of VMG-mammalian in March 2001 agreed that no more experimental work is needed on this assay. The 2nd phase of the validation of the additional parameters added to enhance Test Guideline 407 is expected to be finalised at the end of 2001 and the results of the first phase of the validation of the Hershberger Assay for androgenic effect assessment are showing promising results. Another human health Test Guideline relevant for hazard identification of endocrine disrupters is also close to completion (Test Guideline 426 on Developmental Neurotoxicity).

In the area of ecotoxicity testing, two Expert Consultation Meetings have been held to discuss and rank currently available reproductive toxicity tests in fish and to recommend a screening test approach that would be ready for formal validation. In March 2001 the 1st Meeting of VMG-eco discussed technical issues and agreed on a work plan for this validation. In addition, the VMG-eco also discussed the outcome of an Expert Consultation Meeting on bird reproduction toxicity testing and recommended next steps towards the development of a bird test, relevant for the hazard identification of endocrine disrupters.

As this ongoing co-operative work is starting to bear fruit and the tools for testing and assessment of possible endocrine disrupters are taking shape, the next step in the process is to start using these tools. In June 2001 OECD Member countries considered it appropriate to find ways structured ways for sharing (at least some of) the testing and assessment work internationally for the following reasons:

- A vast amount of chemicals currently in use need to be considered,
- There is considerable time pressure to identify and assess endocrine disrupting chemicals as expeditiously as possible,
- The number of studies necessary for screening and, as appropriate, full hazard assessment is considerable, and
- There is an obvious lack of resources needed for this work,

It appeared that one area where international co-operation will be very useful is in grouping chemicals that, for one reason or another, have triggered a regulatory interest in a critical assessment of their endocrine disrupting potential. Each OECD country and/or region that has identified group(s) of chemicals of interest will now share these series of chemicals with other countries. In order for such information exchange to be useful, it is essential that the criteria on the basis of which particular chemicals have been grouped be provided. In addition, information on planned actions, including details of scheduled screening/testing is also considered very useful. A co-ordinated database of all chemical groupings, together with criteria and updates of scheduled work is being established to avoid duplication of testing and assessment.

Co-operation in the OECD work on test method development and validation is a major priority. In addition to the current activities mentioned above, Member countries agreed that current work on QSARS and the development of High Through Put Screens (HTPS) in Japan and the USA have provided pre-screening tools that could be very useful for other countries as well. In addition, the development of screening tests covering mechanisms other than receptor-mediated effects and the work on more definitive testing methods such as the mammalian two-generation reproduction test and reproduction tests in birds and fish are often of interest to other countries than those currently investing in these activities. Plans are underway to share all this information among all OECD Member countries.

The more definitive tests, in particular multi-generation reproductive toxicity tests in mammals, birds and even fish are so expensive that conducting these tests should all be internationally co-ordinated. This co-ordination, as agreed, will include: i) an early notice (so other countries considering the test would not start as well), ii) use of an internationally agreed protocol, and iii) being prepared to share the results. OECD is currently establishing a way that data could be easily shared among member countries.

There is reluctance among Member countries in going the route of international assessments. Experience with other international assessment programmes has learned that this may be a long and slow process. Member countries felt more comfortable with an approach based on sharing assessments rather than reaching agreement on them. In order to facilitate a common understanding of assessments, the first aim should be to reach agreement on a series of elements to be included in assessment reports. The development of guidance document(s) focussing on the interpretation of particular findings (e.g., fish histopathology, effects on hormone levels) could be a helpful tool. However, the sharing of all endocrine disrupter's assessment reports can only be achieved by a structured international co-operation. Therefore, providing such a structure is currently a high priority of the OECD Special Activity on Endocrine Disrupters.

International Harmonization of a Screening and Testing Framework for Estrogenic Chemicals

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International workshops have recommended that a tiered screening and testing framework be designed and validated to identify and then characterize the hazards of estrogenic chemicals. The framework objective is to first systematically reduce the large number of chemicals in commerce to a subset that require screening. From this subset, screening methods identify those chemicals with clear estrogenic characteristics and lead to a determination of testing needs for adverse reproductive and development effects. The hazard characterization from test results and an exposure assessment allow for an assessment of risks and, as necessary, the management of those risks.

These workshops proposed a screening and testing framework to achieve this objective where a series of methods are arranged in consecutive tiers. The conceptual basis for this framework is that an estrogen mode of action exists that is initiated by the binding of a ligand to the estrogen receptor, which regulates the transcription of estrogen target genes, and eventually leads to estrogen-mediated adverse effects.

The first tier reviews the available toxicological data and employs basic structure activity relationships to assess the possible binding affinity of a compound to the estrogen receptor. The second tier employs one of several candidate *in vitro* screens, which may based on the ability of a chemical to either bind the estrogen receptor or to initiate estrogen mediated gene transcription. The third tier employs an *in vivo* screen, the uterotrophic assay, to add the biological relevance of the intact animal such as pharmacokinetics that may enhance or modify the ability of a compound to induce the growth of a sensitive estrogen target organ, the uterus. The final tier is composed of traditional subchronic tests and the definitive multiple-generation reproductive and developmental bioassay, enhancing these tests as needed with estrogen sensitive endpoints. The methodology for each tier must be standardized, validated, and international harmonized. Already nearing completion is the validation of the uterotrophic bioassay under the auspices of the OECD. Work on a variety of structure activity models and other methods are proceeding under the management of various national regulatory agencies.

Ultimately, these various pieces must be coordinated and fitted into a functional, not theoretical, internationally agreed framework. This international framework must not only be constructed, but also validated and, most importantly, harmonized. Otherwise, significant resources will be necessary to conform to methods that may be duplicative, in most cases, and may be in conflict, in some cases. Then, due to the scale of even a harmonized screening and testing workload, international consensus and agreement is needed to plan, distribute and to conduct this large regulatory effort and to share its information.

This presentation briefly reviews the proposed framework and the status of the development, standardization, and validation of the individual screens and tests. The presentation then identifies the need to validate and compare several methodologies on an international basis, particularly structure activity relationships and *in vitro* screens. Otherwise, different lists of chemicals and different priorities will emerge and, rather than cooperative, regulatory programs may come into competition and even conflict.

The presentation notes that each tier and method must have both a clearly defined role and regulatory purpose, where that purpose guides the development, standardization, and validation of the method. In addition,

the scientific basis and capability of the method must be consistent with that purpose. The ultimate advantage of the tiered approach is to allow simple methods to rapidly and systematically reduce the large universe of chemicals to a workable subset. Then, more complex, but more informative, methods can be economically employed on the reduced subset. Recognizing that risk is a combination of hazard and exposure, it is essential to balance data on potency with estimates of exposure. Thus, prioritization decisions at each tier must be designed to move forward in a realistic fashion those compounds presenting the highest potential risks. Again, an international system to share data and to achieve broad consensus will be necessary. In summary, we face not just the task of developing and validating methods together, but the task of employing the screening and testing methods in a harmonized manner that allows the information to be generated in the most efficient manner and to be used by all.

Testing Strategies for Endocrine Disruption in the Aquatic Environment

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Field observations of endocrine disruption (ED) in fish and other aquatic species have contributed to a global effort to establish test methods for detecting ED effects in wildlife species. For ecological risk assessment, validated tests are needed for Amphibia, fish and aquatic invertebrates, supported by a tiered approach incorporating mechanistic data and exposure characterisation. The potential for extrapolation of ED data from mammalian to aquatic species may be limited, however, due to significant physiological differences in function and regulation of hormone systems in (aquatic) lower vertebrates and invertebrates. Presently, the OECD is considering a tiered approach for ED risk assessment, incorporating a fish 14-day screening assay (Tier 1); fish development and reproduction tests (both Tier 2); and a fish full life-cycle test (Tier 3). For detection of (anti-) oestrogens, the yolk-precursor protein vitellogenin is an ideal biomarker of exposure and functionally equivalent biomarkers are being sought for (anti-) androgens, aromatase inhibitors etc. At the two higher tiers, impacts are assessed in terms of apical endpoints (eg development, breeding behaviour and fecundity) and also gonadal histopathology. With respect to amphibian testing, the need for tests sensitive to thyroid-active compounds has generated interest at national and international level in developing an amphibian metamorphosis test. Given the variety of mechanisms through which thyroid hormone homeostasis may be altered, such tests may require biochemical or molecular endpoints in addition to apical parameters (e.g. larval development), to provide mechanistic specificity. Higher tier partial or full life cycle tests may then be required to further characterise effects in ecologically relevant species. Validation of these higher tier tests should include comparison of sensitivity of biochemical and apical endpoints to optimise the value of biomarkers for predicting adverse health effects (eg impaired reproduction). The specificity of future OECD fish and amphibian test guidelines for endocrine disrupters needs further consideration through inclusion of mechanistic endpoints based on state-of-the-art molecular endocrinology. A mechanism-based approach to endocrine disrupter screening in wildlife species may enhance our ability to extrapolate from regulatory test species to wildlife.

Screening and Testing of Endocrine Disruptors in Avian Species

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In May, 1999, a meeting was held in Leipzig University by a call of OECD secretariat and experts from member countries were gathered to discuss on avian reproduction toxicity testing. The experts made a sincere discussion to revise the existing test guideline 406 into new "Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite". The issues of discussion were 1) selection of endpoints, 2) statistical analysis of data, 3) validation studies and sensitivity comparison studies, 4) test species and the existing 206, and 5) other issues for discussion. We spent most of time allotted for the meeting for these issues and at the end of the meeting, we discussed "screens and tests for assessing endocrine disruption in birds". After discussion, we decided to have another test guideline for endocrine disruption in avian species using Japanese quail and the design should be two-generation test.

The US EPA volunteered to draft a two-generation protocol for assessment of endocrine disrupters in birds taking into account the recommendations from the Leipzig meeting. The proposed protocol would be based on research and studies conducted by Mary Ann Ottinger (Maryland University, USA) and the Wildlife International Ltd, USA. A first draft outline of protocol for an "Avian Two-generation Toxicity Test in the Japanese Quail" was circulated to the Expert Group in December 1999. This draft proposal also identifies a number of issues to be resolved.

According to a recommendation of EDTA 4, the second meeting was held in Nashville, Tennessee, United States in 2000 to solve the issues. The objective of the 2nd OECD Expert Meeting on Endocrine Disrupters Testing in Birds is to progress on the development of a two-generation avian reproduction test for the detection of endocrine disrupting effects. For that purpose, the following was addressed and discussed; (1) review of the December 1999 draft proposal for a new guideline on "Avian Two-generation Toxicity Test in the Japanese Quail", (2) key issues related to a two-generation study, (3) validation of the one-generation (6-week) study and a link to the development of a two-generation study, (4) making proposals for further work on developing test protocol and conducting appropriate research/pre-validation studies. We discussed about purposes of the test for risk assessment, reproductive and developmental toxicity versus endocrine disrupting mediated effects, exposure scenarios, dosing, etc. etc. but could not reach clear conclusions. At the end, we decided that two subgroups were made to discuss and obtain a revised draft and to make a pre-validation proposal of an avian two-generation toxicity test with the Japanese quail.

The pre-validation proposal was circulated to the members and now being compiled the opinions for further revision. So, at the moment, the revision of the existing 206 test guideline is still on the process and the developing a new guideline for assessing endocrine disruptors over avian species is on the way.

In the meantime, we Japanese avian endocrinologists conduct basic research to contribute for selecting endpoints and applied research to establish methods for screening and testing with supports from the Ministry of Environment, Japan. A part of successful results was that we have established an ELISA assay system to estimate circulating vitellogenin in avian species and confirmed a HPLC method to measure circulating VLDL to estimate estrogenic effects in male birds. In the process of making test guideline for endocrine disruptors in Japanese quail, we have to appeal the usefulness of our screening methods and reflect them into the process as well as the results of our basic researches.

Recent Research of Endocrine Disrupters Testing of Amphibians in Japan

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It has been reported that populations of many amphibians have declined in different habitats and some species have disappeared from certain localities around the world. Although the evidence for reduced numbers of amphibians is accumulating, the reasons for the worldwide decrease in number are less clear and may be complicated. In the life of amphibians, eggs, embryos and larvae grow in aquatic environments and then move on to land after metamorphosis. Breeding seasons for amphibians are spring and summer in most cases. Due to the heavy application of pesticides on agricultural land during these seasons, the environmental changes and chemicals in the environment may be apt to influence amphibians in nature. In Japan there are many amphibian species, with much scientific research being done in relation to embryology, endocrinology and so on in amphibians. However, there is currently little evidence of pollution-induced endocrine dysfunction in amphibians.

OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force was established in 1998 and manages the OECD activities on endocrine disrupters in human and wildlife. Expert Consultation on Endocrine Disrupter Testing in Amphibians was held in April 2001 in Paris. In the meeting, the purpose of endocrine disrupter tests with amphibians was discussed and participants agreed as follows, (i) The first purpose of a screening assay with amphibians is to identify chemicals with the potential to interfere with the hypothalamus-pituitary-thyroid axis in vertebrates; (ii) the second purpose is to assess endpoints relevant to adverse effects in amphibians. According to the report of this meeting, the first meeting of Development and Assessment Committee of Endocrine Disrupter Screening and Testing Methods in Amphibians was held at the Ministry of Environment of Japan in September 2001. It was agreed in the meeting that the German protocol (XEMA) using Xenopus laevis would serve as a basis for developing a frog metamorphosis assay. The assay is now focusing on X. laevis and Japanese native species. In this presentation, progress in the experiments of XEMA and the reports in relation to the existence of deformed frogs and the hermaphroditism and abnormal gonads observed in wild amphibians will be introduced and discussed.