Progress in Implementing the Endocrine Disrupter Screening Program

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Thank you very much for that kind introduction, and I appreciate being asked to be here by the conference organizers.

I understand that we are running a little behind time, so I will try to skip over some of the introductory information fairly quickly, but I did want to give some brief background information on the Endocrine Disrupter Screening Program (EDSP). You can certainly find more information on our website, and Dr. Kobayashi has translated the EDSTAC report into Japanese for those of you who would prefer to see some information in Japanese.

Mainly today, I would like to talk about our work in validating the assays for the EDSP. I will give you a status report and then focus on the pubertal male and female screening assays, the fish screening assay, and the avian dosing study that we are about to start.

The U.S. EDSP is the only screening program that I know of for endocrine disrupters that is required by law. The Food Quality Protection Act of 1996 required that EPA screen pesticides for estrogenic effects that may affect human health, and it required us to use appropriate validated test systems or other scientifically relevant information. We have broadened the very narrowly focused charge in the law to include contaminants in drinking water, other endocrine effects, and other chemicals. Based upon the current state-of-the-science, the program really encompasses not only estrogen, but also androgen and thyroid hormone systems. The scope will be broadened from human health to also cover ecological effects.

The framework is fairly straightforward: We will take a large a number of chemicals and using existing information, we will sort them into different groups based upon the availability of information, and then set priorities using such things QSAR and HTPS in addition to existing data. We will then put those chemicals that have limited data in the Tier 1 Screen, whose purpose is to identify substances for further testing. Those chemicals that are positive will go on to Tier 2, which is to identify the adverse effects and establish dose-response relationship that can be used in hazard assessment.

You heard about the validation process from previous speakers, so I will not go through this. Basically the purpose of validation is to take experimental procedures and turn them into regulatory guidelines. It is a process to establish the reliability and relevance of those assays to be used in a regulatory context.

There are different ways that you can actually apply the validation criteria in a practical sense. Looking at the resources that EPA has to apply, we could not do as the OECD did and get 20 laboratories to do the studies. We believe that three or four laboratories will provide an adequate basis for determining interlaboratory variability.

We will select chemicals specific to each assay rather than, just say, taking a block of chemicals and running every assay through that block. We will validate individual assays rather than validating the battery. We will use more chemicals in the pre-validation step than in the validation step, and that is really for two reasons. First of all, since the pre-validation step is done in fewer laboratories, it is cheaper to test those chemicals there. Secondly, if an assay looks like it is not going to work, you would like to eliminate it fairly early in the validation program rather than spend a lot of money testing it in multiple laboratories only to find out that it is really not relevant or reliable.

The battery validation itself will be a paper exercise that will look at all of the data across all the assays and decide which of those worked best in concert with each other to give an efficient battery for screening.

The colors of the dots on this slide show the status of the assays in the validation process (detailed review paper, pre-validation, validation). The ER/AR binding assay is shown here as being in all three phases at once. The detailed review paper really focuses on what is known in the literature, and that is the basis for deciding what are the appropriate things to do in the pre-validation studies stage.

During pre-validation we develop the optimized transferable protocol and it goes to multiple laboratories in the validation stage as was discussed yesterday. The EPA and the ACC (the American Chemistry Council) are developing some data, which will go into the validation of these assays.

But the validation here is really being carried out by ICCVAM (the U.S. Interagency Coordinating Committee for the Validation of Alternative Methods). They are producing four different documents: one for ER binding, one for AR binding, one for ER reporter gene assays, and another for AR reporter gene assays. The uterotrophic and the Hershberger assays, as you heard, are both in the OECD program, so I will not spend any more time on those.

The cornerstone assays as we conceive it in our Tier 1 screen will be either the male pubertal assay or the female pubertal assay, and I will spend a bit more time later in the talk about where they are.

The frog metamorphosis assay: We have had one demonstration of the frog metamorphosis protocol, as was noted yesterday. We had some difficulties because we chose too late a time for the administration of the challenge dose, and so we were really getting in to the natural thyroid surge. The metamorphosis assay is meant to be an assay to detect anti-thyroid agents, so we will need to repeat it using an earlier stage of development. We are also considering the use of limb bud formation as an alternative to the tail resorption.

The fish reproductive screen: I will talk about this later in my talk.

The steroidogenesis and aromatase assays: we are writing detailed review papers now. They will go before our new advisory committee in March, and we hope to be into the laboratory and complete the pre-validation phase of those sometime in the fall and then move into validation probably early next year.

Mammalian 2-generation: we have demonstrated the additional thyroid endpoints on the mammalian 2-generation using PTU. We have also started to carry out some work on extending the observation period on the 2-generation, just using a 1-generation study to determine the adequacy of the current mammalian protocol.

We want to see whether in fact that needs to be modified to carry more pups forward, so that we can look at some of the effects on the male tract, which are not observable in small numbers of animals done early in their life cycle. It is much easier to look at those tissues in animals that have gone through puberty.

The avian 2-generation: we have started a detailed review paper and we have done some pre-validation work comparing the bob white quail and the Japanese quail.

Amphibian chronic: no work has been done yet.

The Invertebrate Chronic: There has been a demonstration of a two-generation protocol in mysids. We are stating on the detailed review paper.

Fish Chronic: We are preparing a detailed review paper that will be ready next summer.

The *in utero* lactation assay is an interesting assay that probably is too complicated for a Tier 1 screen, but will probably make a very excellent assay to be used in place of the 2-generation in circumstances where you do not need all of the other information that normally goes with a 2-generation study.

The female pubertal assay is designed to detect estrogen and thyroid active substances and also alterations in LH, FSH, GnRH, and the hypothalamic function. The protocol uses 15 immature rats per dose level, and when run as a screen, we would anticipate using two dose levels.

Here is a schematic which shows that the dosing would start at day 22: one starts checking for vaginal opening, and then conduct a daily lavage after vaginal opening — that is one of the hallmarks of puberty. In the end, we will be sacrificing the animals at day 43 and looking at T4, TSH, and the weights of the various organs: the ovaries, uterus, liver, kidneys, pituitary, adrenals, and also some histology on the uterus and the ovaries.

The male assay is also designed to look at estrogenic/androgenic in this case antiandrogenic and thyroid effects, as well as LH, FSH, GnRH and hypothalamic function. That again would use 15 rats per dose level, and two dose levels.

The schematic dosing is shown here: on day 22/day 23, we will be looking for preputial separation at about day 37 as the hallmark of male puberty, and then again in necropsy looking at T4, TSH, and weight of the testes, epididymides, ventral prostate, seminal vesicles, coagulating gland, the levator ani and bulbo cavernosus muscle and histology on the testes, epididymides and also on the thyroid. If the frog metamorphosis assay is not in the battery, the pubertal assays would be the only other place we would pick up thyroid.

The purpose of the initial pubertal demonstration which has been completed was to: assess the utility of the protocol and the endpoints for the detection of substances that interfere with estrogen, androgen, and thyroid; to assess the robustness of these protocols with regard to intra-laboratory variability; to assess the inter-strain sources of variation (we actually use two different strains); and to give us some indication of the level of documentation of the protocols needed for successful completion of the assay.

As I mentioned, we used two different strains of rats: the Sprague Dawley and the Long Evans. In this case, only a single high dose was used, and a much smaller number was used, only six animals per group. We used a two block design so we could measure the intra-laboratory variability.

These are the results of this study. I am sorry, I neglected to put in the dose levels, but they are really run at or near the MTD. We used ethinyl estradiol as the ER agonist, and here you can see that it accelerated vaginal opening and accelerated the first estrus. There were also signs in histopathology. Tamoxifen, which is an ER antagonist/partial agonist also accelerated vaginal opening, but, in fact, it delayed age of estrus. There were also some effects in histology.

These are rather strange: we are not sure why we are seeing some things in TSH and T4. There may be somebody in the audience who could comment on those things.

PTU is designed to inhibit T4 synthesis, but it also had some effects in the Sprague Dawley strain on vaginal opening and age of estrus. Histopathology behaved as one would expect. TSH increased and T4 was depressed.

Ketoconazole is an inhibitor of steroid synthesis. It caused a delay in vaginal opening only in the Sprague Dawley, and also led to a delay of age of estrus. We also picked things up in histopathology. It depressed T4 levels.

Pimozide, a dopamine antagonist, delayed vaginal opening. We saw effects in histopathology. There was a lowering of TSH in the Long Evans. It also decreased T4 in both strains.

Methoxychlor is an ER agonist. It accelerated both age of vaginal opening and estrus. There is an effect noted in histopathology, and a lowering of TSH in the Long Evans rat.

In the male, we used flutamide as the androgen receptor antagonist. It delayed preputial separation and we saw effects in histology. Methyl testosterone was the AR agonist chosen. It accelerated the time to preputial separation as one would predict, and we saw effects in histology. PTU is an inhibitor for T4

synthesis delayed preputial separation, as I think is expected, and of course, had the expected effects here on TSH/T4.

Ketoconazole, an inhibitor of steroid synthesis also delayed age of preputial separation. Pimozide, a dopamine antagonist, delayed preputial separation, and we saw some effects in histopathology in the Long Evans only. Dibutyl phthalate is an anti-androgen that does not act through the receptor; again, it delayed preputial separation in the Long Evans. It is interesting to note that the effects here are seen only in the one strain, not in the Sprague Dawley.

The conclusions of the pubertal study were that the protocols were successful and that they identified the expected endocrine-mediated effects in both the male and the female that the data did raise some important issues that must be addressed in the pre-validation studies as pre-validation continues. One issue was strain differences: should the protocol specify a strain? Our advisory committee, which met just last week, indicated that going forward with the Sprague Dawley is acceptable, that it is normal to expect strain differences, and there probably is no one optimal choice of strain. You can find advantages in some, but there will be disadvantages as well.

We found that in practice that there was a large variation associated with the means of the fluid filled and small tissue weights, so that is a clear indication that we need to tighten up the protocol in those areas and that we need to establish some performance criteria. We knew what we were administering, these are well-studied compounds, but when it comes to unknowns we clearly need to give some guidance on how to establish dose levels for this particular set of screens.

We will be moving forward with the body weight study because it is known that variations in body weight can affect the pubertal markers and we want to look at this so that we can eliminate the false positives. We will also carry on a dose response study using three dose levels with vinclozolin and flutamide in males, and methoxychlor and ethinyl estradiol in females. We will carry out a chemical array study using two dose levels. Eight chemicals are being used in the female and nine chemicals are being used in the male, and those were just chosen this last week.

Moving on to fish. The OECD work group discussed fish screening studies and there were several candidates that were discussed: a reproductive effects screen that was something that we had worked on in our Duluth laboratory and it was at that time a 42-day study. We plan on repeating that and chopping the time for acclimatization down from 21 to 14 days, but continuing with the protocol as it was written with a 21-day exposure.

One of the complaints we heard was that the screen was too long, so we are going to also try to reduce the acclimatization time down to 7 days and the exposure time down to 14 days, and see if we still have a valid screen. We will also compare that with the assay in juvenile fish, which was discussed yesterday, and vitellogenin is one of the key markers in that one. We will do this on only one species at this time; we use the fathead minnow. These are the chemicals that will be used: methoxychlor, fadrazole, flutamide, and methyltestosterone; we would hope that we could complete it by early this summer.

The other environmental test that we are working on — in fact this is a Tier 2 test — is the avian dosing study. When the OECD bird work group met, there was some disagreement as to how one should proceed with the dosing regimen for a 2-generation study, so the purpose of this really is to look at that issue. We will be using Japanese quail. We will be using one or two chemicals that will demonstrate both the effect on maturation of parents and transgenerational effects. We plan on using the protocol, or a version of the protocol, devised by Mary Ann Ottinger, and would hope to conclude at the end of this summer.

I can wrap up here very quickly. These are the four different dosing scenarios that were laid out. The approach here will be to take one block and expose animals at sexual maturation and egg laying, then split that block, and continue exposure to the F1 generation. For the second block there will not be

exposure at sexual maturation, but only at egg laying; so they can be the internal controls, essentially, at this stage, and then again split with exposure to the F1s and one can compare all four of those then that way.

I will finish up here with just a reminder of the timeline for implementation. What I have been talking about here today is validation. We would hope to be able to complete that at the end of 2003, and actually start screening. In the U.S. most of that screening will be done by the regulated industry; Tier 2 validation will take longer, until 2005. The priority setting system is being worked on in parallel, and you heard Weida Tong talk about the QSAR that will be used there. Thank you very much.

Q&A

Takei: Thank you. We have time for one question. Are there any questions? Yes, please.

Pickford: Don Pickford, AstraZeneca. In relation to the thyroid endpoints in the pubertal assay and the frog metamorphosis assay which you are revalidating, will you be using the same battery, the same panel of chemicals you have used in the pubertal assay for your validation of the frog metamorphosis assay, and what will be your strategy if you find no false-negatives or false-positives in either battery?

Will you still continue with the frog metamorphosis assay or just fall back to using the rat assay?

Timm: I think that we will have to come up with several different chemicals to test the system in thyroid in addition to PTU, which is, of course, a very strong inhibitor. Perchlorate has come up as something and we will probably use that fairly soon. I think that, in fact, is in this next group of chemicals to be tested in the pubertal assay.

The frog metamorphosis assay, right now we do not have any more work scheduled on it. I think there is work being done in Germany. We probably will want to look at what they are doing and then make a decision on whether to go forward with the frog metamorphosis or not.

Takei: Thank you very much.

Timm: Thank you.