

**Story of the Steroidogenesis Assay**  
**in EPA's Endocrine Disruptor Screening Program**  
**by Gary Timm OSCP, OPPTS, EPA**

Background

Because it is not clear whether the *in vivo* assay that may be selected for the Tier 1 screening battery (e.g., female pubertal assay) will be adequately sensitive to detect effects on the steroid system, the *in vitro* steroidogenesis assay was devised to identify chemicals that have the ability to interfere with the synthesis of steroid hormones. In mammals, testosterone plays an essential role in sexual differentiation at a very specific time period during embryonic development. While testosterone also plays a role in females, a female *in vivo* assay may not be sufficiently sensitive to detect the effects of a steroid agonist or antagonist at the low levels that may affect sexual differentiation of the male.

Protocol Development

In developing an appropriate steroidogenesis screening assay, several different protocols were closely examined and discussed in a Detailed Review Paper (DRP). In compiling the DRP, entire protocols were dismissed for various reasons. EPA examined *ex vivo* methods (a combination of whole animal and organ methods), as well as tissue-based methods, including the testicular sliced/minced testes and minced ovary (i.e., granulosa) methods. *Ex vivo* methods were dismissed as not suitable for a screening program. It was determined that the minced ovary method was not ideal to detect chemicals that may disrupt the steroid system, as measuring steroid disruption in the ovary is complicated by the large natural variation in steroid hormones due to the estrous cycle. Historically, the sliced testes method has been most successful in detecting steroid effects, and it was also judged to be the protocol most likely to be validated in a relatively short period of time.

In addition to whole animal and tissue-based methods, the DRP also examined cell-line methods, which would eliminate the use of animals for this assay. Cell-based assays also offer the advantage over tissue-based methods of demonstrating enzyme induction effects in addition to competitive inhibition. While a myriad of methods were discussed and dismissed, three candidate cell-line protocols were seriously considered using the following cell lines: the mouse Leydig cell tumor line (MA-10), the rat Leydig cell tumor line (R2C), and a human adrenocortical carcinoma cell line. The mouse and rat Leydig tumor cell lines lack a critical enzyme, 17 ketosteroid reductase (17KSR), and as a consequence, they can only identify substances that interfere with the first half of the steroid synthesis pathway. Therefore, the most promising cell line was judged to be the human adrenocortical carcinoma cell line (H295R) because it possesses all of the enzymes in the steroid synthesis pathway and can, therefore, identify substances that modulate the entire steroidogenesis pathway from cholesterol recruitment by the STAR protein to conversion of androgens to estrogens .

**The Sliced Testes Assay**

## Optimization

Several parameters were examined for optimization of the sliced testes method. The lead laboratory assessed factors such as the size of the tissue slice, incubation temperature, vessel type and size, shaker speed, media volume, and the concentration of Human Chorionic Gonadotropin (HCG), which is used to stimulate hormone production in the tissue. Sampling time factors and composition of the gas in the incubation chamber were also optimized for the sliced testes protocol.

Besides the studies to optimize the parameters listed above, three other small special studies were conducted to further optimize the sliced testes protocol and to provide definitive answers to questions that arose from attempts to interpret data in the original optimization studies. First, there was a need to analyze the combination of media volume and vessel size (these had been examined as separate parameters in the first study). Second, collection and analysis of four separate samples proved to be time consuming and costly. Thus, a small experiment was carried out to determine if a single measurement could be taken at the end of the four-hour period. Because it was found that fresh media replacement was necessary, a compromise ultimately resulted: samples were collected hourly but pooled and analyzed at the end of the four-hour exposure period. Third, the question of the degree to which the testes fragments contribute to the variability of the assay surfaced as a key element in further reducing the variability of the assay. Therefore, an experiment was designed to examine the variability of testosterone production in fragments taken from different testes and different locations within a testis. The same experiment was used to optimize the dose level of the positive control, aminoglutethimide.

## Additional Prevalidation Studies

The Endocrine Disruptor Methods Validation Subcommittee (EDMVS), the predecessor of the EDMVAC, was concerned that cytotoxicity may confound the results of the assay. In response, EPA requested Battelle to investigate several cytotoxicants including 2,4-dinitrophenol, dipropylene glycol, sodium azide and ethane dimethane sulfonate for use as possible cytotoxicant positive controls. The cytotoxicity studies using an LDH detection system showed few of the expected signs of cytotoxicity within the four-hour exposure period. Therefore, special studies further examining cytotoxicity were undertaken at EPA's laboratory in Research Triangle Park.

Three laboratories participated in this study of the transferability of the protocol. They ran the basic assay in order to determine if they could 1) induce testosterone production with hCG to demonstrate that they could perform the basic techniques required in the assay; 2) run the assay with the positive control, aminoglutethimide; and 3) run several the cytotoxicity positive control assay to detect potential cytotoxic chemicals. The inter- and intra-laboratory special studies were carried out several times. The fairly high variability seen in some of these studies and the multichemical study is a concern and will be a topic of discussion at the April meeting.

A study was conducted in the lead laboratory to test the protocol with other test chemicals suggested in the DRP-- 2-4 dinitrophenol, flutamide, ketoconazole, vinclozolin, atrazine, dimethoate, finasteride, spironolactone, verapamil, and prochloraz-- in order to determine their effect on inhibiting the synthesis of testosterone in the sliced testes system. The results of this study showed high within-laboratory variability and raised concerns about the ability of the assay to correctly distinguish positive from negative compounds

#### April 2005 EDMVAC Meeting

At the April meeting, EPA asked the EDMVAC its recommendation regarding the next steps for the sliced testes assay. The Committee formally recommended 1) that the EPA discontinue efforts to further validate the sliced testes assay on the basis of their concern that EPA would never be able to develop a cytotoxicity assay that was specific only to Leydig cells and 2) that the Agency should continue its work to develop and validate the H295R cell based assay.

#### **H295R Cell-Based Assay**

This assay is being developed as an alternative to the sliced testes assay. It employs a subpopulation of the H295 human adrenocortical carcinoma cell line that forms a monolayer in culture which has been shown to express all of the key enzymes necessary for steroidogenesis. Preliminary data shows that the H295R assay detects both stimulators and inhibitors of the enzymes involved in the production and transformation of steroid hormones.

EPA initiated assay development in March 2004 under a contract with Entrix and Michigan State University. MSU evaluated several commercially available ELIZA kits for measuring progesterone, testosterone, 17 $\beta$ -estradiol, and estrone to determine which performed best in detecting the hormones in the cell culture medium. MSU also optimized the culture conditions, developed a protocol for the assay, and reproducibly measured baseline hormone production. In a second series of experiments, MSU evaluated the performance of the assay with four model chemicals which include both enzyme inducers and inhibitors: prochloraz, forskolin, aminoglutethimide, and ketoconazole.

At a meeting of the Organization of Economic Cooperation and Development (OECD) non-animal test method Validation Management Group (VMG-NA) in November 2004, EPA presented an overview as to why the Agency selected the H295R assay for steroidogenesis and of the progress made in developing and refining the assay. EPA invited Member Countries to join the US in further refining and validating this assay as a potential OECD test method. Danish and Japanese laboratories volunteered to join the U.S. in demonstrating the transferability of the method and optimization of the protocol, and work began in April 2005. The following laboratories are participating in these initial studies:

Lead Laboratory:

Dr. John P. Giesy, Principle Investigator

*Last updated: 4/1/05*

Dr. Markus Hecker, Study Coordinator  
Department of Zoology, National Food Safety and Toxicology Center  
Michigan State University  
East Lansing, MI  
U.S.A.

Participating Laboratories:

Dr. Yumi Akahori and Dr. Makoto Nakai  
Chemicals Assessment Center  
Chemicals Evaluation and Research Institute  
1-4-25 Kouraku  
Bunkyo-ku  
Tokyo 112-0004  
Japan

Dr. Ralph Cooper  
Dr. John Laskey  
Endocrinology Branch  
National Health and Environmental Effects Laboratory  
U.S. Environmental Protection Agency  
Research Triangle Park, NC 27711  
U.S.A.

Dr. Anne Marie Vinggard  
Department of Toxicology and Risk Assessment  
Mørkhøj Bygade 19  
DK-2860 Soborg  
Denmark

Inter-laboratory studies are being conducted in two phases.

Phase I

In Phase I each laboratory established the necessary procedures and test conditions for the test and measured basal hormone production using the measurement method of their choice. This phase did not use a prescribed protocol but was conducted on a performance basis. Thus each laboratory was free to use different culture conditions and measurement systems for the hormones. Work on Phase I began in April 2005 and was completed in October 2005.

Phase II

The protocols used in Phase I were used in testing the four reference chemicals: prochloraz, forskolin, aminoglutethimide, and ketoconazole. Phase II compares the results each laboratory obtained for progesterone, testosterone and 17 $\beta$ -estradiol.

The results of Phases I and II will be discussed at the November 2005, EDMVAC meeting.

### Phase III

Phase III will identify sources of interlaboratory variability in basal hormone production and test system response observed with model compounds in Phase II. These data will assist in choosing an optimum protocol which will be used in formal interlaboratory testing. Phase III will be planned at the December meeting of the VMG-NA and is expected to commence in early 2006.