

**Story of the 15-Day Intact Adult Male Rat Assay
in EPA's Endocrine Disruptor Screening Program (as of 7-21-05)
By John O'Connor**

Background

In their 1998 report, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended to EPA a Tier I screening battery that comprised 8 assays, but EDSTAC also proposed two alternative batteries that were deemed worthy of further evaluation. All three EDSTAC batteries incorporate an array of 6-8 *in vitro*, *in vivo* mammalian, and environmental assays for examining effects of endocrine active compounds (EACs). In alternate Battery 1, the Hershberger, female pubertal, and *in vitro* steroidogenesis assays are replaced by the 15-day intact adult male rat assay. It is expected that the adult male assay will be run in parallel with the uterotrophic assay and the *in vitro* receptor binding and/or transcriptional activation assays; both are assays which are also in the EDSTAC-recommended Tier I screening battery. The primary purpose of the uterotrophic assay is identification of estrogenic/antiestrogenic compounds. The adult male battery is designed to complement the female battery by identifying several endocrine activities that alter the hypothalamic-pituitary-gonadal axis, primarily through a comprehensive hormonal assessment. The *in vitro* receptor/transactivation assays will aid in the identification of substances that directly interact with the steroid hormone receptors.

The 15-day intact adult male rat assay is designed as a mode-of-action screening assay. The mode-of-action screening approach advances scientific understanding over assays based on apical tests, as these essentially are reproductive effects screens that are not necessarily specific for endocrine activity. By identifying the potential mode of action, critical endpoints can be included in Tier II studies that will be used to define dose-response curves and no observed adverse effect levels (NOAELs)/no observed effect levels (NOELs) for the compound. Another advantage of the adult male assay is that it can easily be adapted for the detection of other endocrine activities by the inclusion of other hormone measurements, organ weights, and/or histopathology evaluations.

Protocol Development and Rationale

The approach of using a comprehensive hormonal assessment is based on the observation that different classes of EACs produce distinct hormonal profiles. Industry has used this approach to identify modes of action for different compounds using components of the male *in vivo* battery. For example, ammonium perfluorooctanoate (C8), a peroxisome proliferator, was shown to induce aromatase and increase serum estradiol levels in two-week studies similar in design to the current Tier I battery. Using a similar design to the Tier I male battery, linuron (a herbicide) was identified as being a weak androgen receptor antagonist. In a mechanistic study, 1-methyl-3-propylimidazole-2-thione (PTI) was shown to alter thyroid function by directly inhibiting thyroid hormone synthesis and by enhancing thyroid hormone excretion via UDP-

glucuronyltransferase induction. In addition, the modes of action for several proprietary compounds were identified using this two-week study design, all of which produced endocrine tumors in two-year rat bioassays but did not produce adverse responses in developmental or multigeneration reproduction studies.

In the assay, adult male rats (15/group) are dosed daily by oral gavage for 15 days with the test substance and euthanized on the morning of test day 15, approximately 2 hours after the last administered dose. At the terminal necropsy, the liver, thyroid gland, and reproductive organs [testes, epididymides, prostate, seminal vesicles with fluid, ASG (prostate and seminal vesicles)] are weighed, and the testes, epididymides, and thyroid gland are saved for histopathological evaluation. Blood is collected and serum is prepared for hormonal evaluation (testosterone, estradiol, DHT, LH, FSH, prolactin, T₃, T₄, TSH). Since the in-life portion of the adult male assay is performed similarly to routine toxicology studies, there are no serious logistical issues with performing the assay. The one potentially challenging aspect of the adult male assay is inclusion of serum hormone analyses, as these may not be routinely performed in many toxicology testing laboratories.

By comparing the “fingerprint” of the organ weight and hormonal endpoints for an unknown compound to a series of positive controls, the 15-day intact adult male rat battery not only identifies potential EACs, but aids in the characterization of their mode-of-action. The “fingerprints” that were obtained for flutamide and ketoconazole provide a useful example. Flutamide, an AR antagonist, competes with testosterone and DHT for binding to the AR. Essentially, flutamide blocks the recognition of testosterone and DHT. ASG weights, which are androgen-dependent, are decreased by flutamide treatment. The inability of the hypothalamus and pituitary to recognize androgens stimulates the secretion of GnRH and LH by these two organs, which in turn, stimulates testosterone production by the Leydig cells. Therefore, AR antagonists such as flutamide decrease ASG weights and increase serum testosterone and LH concentrations. Ketoconazole, a steroid biosynthesis inhibitor, inhibits testosterone production by binding to the heme iron of the three cytochrome P₄₅₀ isozymes of the testosterone biosynthetic pathway. Similar to flutamide, ketoconazole decreases ASG weights. However, since ketoconazole acts directly at the testis to inhibit testosterone production, serum testosterone concentrations are decreased, and secondary to the decreased serum testosterone concentrations, serum concentrations of LH are increased. Therefore, steroid biosynthesis inhibitors such as ketoconazole decrease ASG weights and serum testosterone concentrations, and increase serum LH concentrations. Hence, while organ weight measurements alone cannot distinguish between these two modes of action, the two different modes of action are easily distinguishable when the changes in organ weights are coupled with the serum hormone data.

Protocol Standardization and Prevalidation

O'Connor and co-workers completed a pre-validation exercise for an integrated Tier I screening battery which included the 15-day intact adult male rat assay. The two primary goals of the pre-validation exercise were to test the hypothesis that distinct “fingerprints” could be identified for each type of endocrine activity, and to determine which of the endpoints evaluated in the pre-validation exercise should be included in a final screen. To accomplish these goals, 15

positive controls with known endocrine activities were examined. Each endpoint was evaluated for variability, stability over time, predictability, and dose-dependency for each of the positive endocrine controls. The assay was effective at detecting a wide range of endocrine activities. In studies using the adult male battery, EACs that were identified include ER agonists and antagonists, AR agonists and antagonists, PR agonists and antagonists, thyroid modulators, steroid biosynthesis inhibitors (aromatase, 5 α -reductase, and testosterone biosynthesis), and prolactin modulators. Since the initial pre-validation exercise, additional EACs have been examined in the adult male assay using the standardized protocol, bringing the total number of compounds examined to over 25. Laboratories performing the 15-day intact adult male assay include both industrial laboratories (BASF, Syngenta, Dow) and contract laboratories (RTI and WIL).