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OFFICE OF
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SUBJECT: Review and Evaluation of Ciba-Geigy's Atrazine/Hormone Studies (MRID 427439-02 and -03), along with their Overview 429425-00 Document

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General Comments:

I have read the above three documents and will respond to your questions in your request dated December 8, 1993. In general, this reviewer has grave reservations concerning the development of an hypothesis based upon data that are presented. The hypothesis presented proposes that Atrazine treatment promotes the precocious appearance of mammary tumors in Sprague-Dawley via hormonal mechanisms, but not in the Fischer 344 rat. There are considerable discrepancies in the data provided (both within and between documents) and I have serious concerns that the results presented may be based upon flawed experimental design, suboptimal execution, and errors in these studies. Specific examples of discrepancies are addressed below.

General Issues:

1. Hypothesis that Atrazine promotes precocious appearance of mammary tumors.

Based upon the data presented in the overview document (429425-00), one interpretation of the data would be as the authors suggest. If the data are valid and the authors are correct in their interpretation, the hypothesis may be substantiated or negated by conducting several additional experiments. One experiment is to conduct similar investigations in a strain of rat that exhibits cycle cessation intermediate between Sprague-Dawley and the Fischer 344 rat (ex., Long-Evans rats that have an average cycle cessation of 10-15 months). If the authors hypothesis is correct, that Atrazine treatment promotes the precocious appearance of mammary tumors in Sprague-Dawley via hormonal mechanisms, then one would expect the appearance of mammary tumors in Long-Evans rats somewhat later than Sprague-Dawley. Of course, such new data in a different strain would not prove their hypothesis, but could support/or negate it. A more critical experiment that would provide a greater weight of support for their hypothesis would be to promote a hormonal imbalance, increasing the time of estrogen exposure and initiating premature cycle cessation in the Fischer 344 rat. In other words, to increase the days in estrus in the Fischer rat and thus mimic the Sprague-Dawley condition. This might be done by giving exogenous estradiol or by treating the Fischer rat with supra-ovulating concentrations of gonadotropins that would in turn elevate estrogens and deplete oocytes at times to mimic the Sprague-Dawley rat. Such a study in the Fischer rat would clearly strengthen the evidence in support of the authors working hypothesis.

2. Mechanism of action for Atrazine is through a threshold, Again, based upon the data presented, it is not clear that a threshold is operating. While admittedly there were no apparent increases in palpable mammary tumors over controls at the lowest dose (Table 8) in the Thakur study (1992), the 70 ppm dose clearly increased serum estradiol significantly at the 3 month sacrifice period over controls (see Table 6 of the overview draft) and increased significantly the % of days in estrus at the 1, 9, and 18th month sacrifice (times that may have been insufficient to promote mammary tumors). This study only presented data for two doses. This is indeed unfortunate, since earlier studies using the Sprague-Dawley rat provide conflicting data. The Rudzki, et al, 1991, showed no significant increases in mammary gland tumors at 500 ppm (a higher dose than the current study, which demonstrated a significant number of tumors), while Mayhew, 1986, showed a significant increase in malignant mammary tumors at both the 70 and 500 ppm. Therefore, the designation of a mechanism of action for Atrazine as "the mechanism" for relating increased estrogen changes with precocious appearance of mammary tumors in the Sprague-Dawley rat is premature and has yet to be determined with assurance.

3. Differences in mammary tumor response to Atrazine by Sprague-Dawley and Fisher 344 rats is attributed to differences in reproductive endocrinology between strains.

One interpretation of the data presented in the overview document is that Atrazine accelerates the appearance of mammary tumors in the Sprague-Dawley rat and not the Fischer 344 rat by promoting early cessation of estrous cycling, estrogen exposure and days in persistent estrus in the former rather than the latter. Clearly strain differences exist within a species, just as some individuals within a species are more susceptible to some toxicants than others. Whether differences in mammary tumor responses to Atrazine can be attributed specifically to reproductive hormonal differences between strains cannot be determined until more experiments as outlined above (General Issue #1) and below can be undertaken.

Specific Questions:

1. Were hormone assays conducted properly?

It is not possible to answer this question until more information is provided by the laboratory undertaking the assays. The fact that the serum samples were stored frozen at -80° C until assay and that peptide (prolactin) was assayed prior to steroids suggests adequate laboratory practice. No information was provided as to whether sera were extracted with appropriate organic solvents prior to radioimmunoassay for estradiol and progesterone (diethyl ether for estradiol, petroleum ether for progesterone). Was this done? If not, competing steroids in the radioimmunoassay could give erroneous measurements for the sex steroids. If not, did the investigators validate the RIA by looking to see whether different concentrations of Atrazine in rat sera interfere with the kit assays? Furthermore, if organic solvent extraction prior to RIA was not done, it is not likely that the investigators added preimmune sera containing appropriate concentrations of Atrazine to the standard curve tubes since they were using kits (see below). All in all, whether appropriate validation of the RIAs were actually done in the laboratories requires clarification.

Were the assay kits appropriate?

Radioimmunoassay kits are appropriate for estimating serum concentrations of peptide and steroid hormones. Of course each assay must be validated in each laboratory with known hormone concentrations added to "stripped" animal sera to be confident that the assay will detect what it is supposed to detect. No information was presented as to quality control. What was the intra and interassay % error for each of the hormones assayed? Because these investigators have published in refereed journals, it is assumed that the assays have been

validated in their laboratories. At the same time, the large standard deviations of the means between specimens of the same group is cause for some concern for the reliability of the hormone data.

2. From Table 6 of Summary Document:

- a. Were the blood samples chosen at the appropriate times?**

The authors report that an attempt was made to kill rats on the day of proestrus (defined as "the first appearance of well-defined cornified cells with possible nucleated cells present"). First, this definition is misleading, if not erroneous. Proestrus is defined by the presence of numerous small nucleated epithelial cells. It is this cell that is the dominant cell type. Once a few cornified cells appear within the field, late proestrus is the appropriate designation. Vaginal estrus is defined by the appearance of solely large cornified cells. Second, no mention of how many rats were actually killed on proestrus and what was the percentage of animals killed at other times. The high standard deviations of means for hormone concentrations could reflect that animals were killed at various stages of the cycle and consequently present considerable variability in steroid and prolactin concentrations.

It is not clear why proestrus was chosen as the day of interim sacrifice. No mention as to the rationale for selection of this stage was given in the methods section of the documents. While estradiol peaks around 9:00 am on proestrus, reaching a concentration of approximately 40-50 pg/ml, and would provide a hormone marker for confirmation of early proestrus, serum levels fall during late proestrus, reaching a nadir of 5-10 pg/ml during estrus. Consequently, it is important to know precisely when during the day vaginal smears were taken and when animals were killed and trunk blood collected for measurement of serum hormones. Were vaginal smears and blood collection procedures routinely undertaken at the same time of day for all sacrifices? If so, when? If not, then considerable variability in serum estradiol levels would contribute to the high standard deviations seen in the raw data, making statistical analyzes difficult.

Are the statistical data acceptable?

It would appear that the descriptive statistics and analysis of variance of the data are appropriate. I am unfamiliar with the Terpstra-Jonckheere Trend Test and do not know why this was selected over other trend tests.

What is the confidence that the statistically-significant increase in serum estradiol (E2) at the 400 ppm dose relative to the control at the 3 month period is real?

I have calculated the means and standard deviations for serum

estradiol concentrations of all three Atrazine dose groups and at 1, 3, 9, 12, 15, 18 and 24 months of the Sprague-Dawley study from the data provided in Appendix 3 of the 427430-02 document and confirm that they are essentially as listed in Table 11 of the -02 document. The high standard deviations of the means at the 3 month collection period for the control, 70 and 400 ppm groups are cause for concern with respect to confidence in the data. For the ten animals in the 400 ppm group, the range was from 1.5 to 47.1 pg/ml. These two latter values were obtained, incidentally, from rats in proestrus, where estradiol values should both have been elevated. Why was one so low? Furthermore, as mentioned below, there is a discrepancy between the means provided in the overview document from that given in the support document. These discrepancies are cause for concern in the validity of the hormone data provided.

c. Was the estrous cycling staging appropriately conducted?

It is the thinking of this reviewer that considerable discrepancies can be found in the raw data presented with respect to estrous cycle staging. The "reading" of vaginal smear exfoliate cytology should be done by experts. The examination of air dried specimens, as mentioned on page 13 in the 427439-02 and page 12 of the 427439-03 documents, is difficult to read by experts and contraindicated because of superimposition of cell types and considerable shrinkage, thus masking the true stage. Examination of vaginal smears should be done on freshly-prepared, wet specimens and observed quickly before drying at the periphery of the blister. There is considerable confusion as to the determination of estrous cycling stages reported in appendices 1 sections for both Sprague-Dawley and Fischer 344 rats based upon the cell identification data presented in Appendices 2 sections. I have made an extensive evaluation of estrous cycling stages based upon the raw data provided with respect to cell types present in the field and the designation of a specific stage (i.e., proestrus, estrus or diestrus) in both the Sprague-Dawley and Fischer rat studies. Using only the occurrence of either nucleated epithelial cell, cornified cell or leukocyte at high density (2), which would afford the least ambiguity in "reading the smear" and would define clearly a proestrus, estrus or diestrus stage, respectively, I have checked the stage designation reported against the dominant cell type in the vaginal smear field. For the Sprague-Dawley rat study, looking at the 0, 70 and 400 ppm groups at the 1, 3, 9, 12, 15, 18, and 24th month of sacrifice, I have found a 39, 22, 27; 30, 12, 36; 31, 13, 8; 17, 12, 6; 13, 18, 9.5; 14, 8, 7; and 0, 0, 6 % error in designation, respectively. For the Fischer rat study, looking at only the 0, 70 and 400 ppm groups at the 1, 3, 9, 12, 15, 18, and 24th month of sacrifice, I have found a 25, 9, 20; 39, 27, 26; 93, 79, 100;

92, 100, 67; 14, 100, 100; 0, 0, 0; and 0, 0, 0 & error in designation, respectively. These discrepancies are cause for serious concern, since this data are crucial to support the hypothesis that precocious hormonal imbalance as verified by vaginal smears in the Sprague-Dawley rat, but not in the Fischer 344 rat, following Atrazine treatment is related to development of mammary tumors. It is not clear to this reviewer how the discrepancies could occur. It may be due to inexperience in the execution by the technician, since it is apparent that at least 3 pseudopregnancies occurred in animal number 779, 848 and 863 in the Sprague-Dawley study. In at least two of the three animals, these pseudopregnancies were induced undoubtedly by the vaginal smear technician, since the animals were still cycling as evidenced by the fact that classical proestrous and estrous smears were recorded following the pseudopregnancy. It is not clear as to whether this was the case with animal number 863, since it was sacrificed during the pseudopregnancy (?) period at the end of 24 months. Because this strain does not normally exhibit repetitive pseudopregnancy during reproductive senescence, it is unlikely that this was a normal process of aging. Taken together, this reviewer has reservations concerning the vaginal smear cytology data.

Are the group means listed in Table 6 reliable?

The group means listed in Table 6 (page 22 of the overview document) are not reliable. For example, the group means of serum estradiol reported for the 3 month sacrifice period in the Table 6, for the Sprague-Dawley rat are listed as 2.1, 6.3 and 17.6 of the 0, 70 and 400 ppm groups, respectively. The group means of serum estradiol reported for the Fischer 344 rat at the same time period are listed as 9.6, 11.0 and 8.3 for the 0, 70 and 400 ppm groups, respectively. These numbers above are different from those reported in the supporting documents (see Table 11, pg 61 of 427439-02 and Table 12, pg 59 of 427439-03). In an effort to check this discrepancy, I have determined the group means from the raw data provided in the supporting documents. According to my calculations, the group means of serum estradiol reported for the 3 month sacrifice period in Table 6, for the Sprague-Dawley rat should be 3.5 +/- 5.9, 11.2 +/- 11.9 and 18.2 +/- 13.0 for the 0, 70 and 400 ppm groups, respectively. My calculations for the group means of serum estradiol for the Fischer 344 rat at the same time period are 10.1 +/- 5.3, 11.9 +/- 5.6 and 13.1 +/- 15.4 for the 0, 70 and 400 ppm groups, respectively. My calculations support the numbers presented in the two supporting documents and are in disagreement with what is reported in the overview document.

d. Was the premature senescence in the Sprague-Dawley adequately demonstrated?

It is well-established in the published literature that the

Sprague-Dawley female rat undergoes estrous cycling cessation on the average at 8-12 months of age. Like many aging rodents, the female exhibits persistent vaginal epithelial cornification associated with elevated serum estradiol and low levels of progesterone. The control groups of Sprague-Dawley rats in this study appear to conform to historical controls for this strain. While the mean values for days in estrus in the Atrazine-treated rats were higher than controls, they were statistically-higher only at the 9 and 18 month periods and not at the 12 and 15th interim months, according to the data provided. If there were indeed a persistent premature senescence brought about by Atrazine exposure, why wasn't it statistically significant at these two interim periods? No explanation was given for this. Furthermore, why was the mean $\frac{1}{2}$ days in estrus for the 70 ppm group at 24 months below the control mean? This reviewer thinks that the high standard deviations between group means is the explanation for these questions.

- e. What is the nature of the contradiction between the Ciba-Geigy study data and the data presented in the Loeb and Quimby reference provided?

There are a number of errors in the support document with respect to concentration units and levels for hormones. For example, pg 25, section 3.3.2.2, (-02), the concentration of progesterone in sera should be ng/ml not mg/ml. On page 23, line 1 of the summary document, on page 27, section 4.1, middle of page of the (-02) document, and again on page 25, section 4.1, line 13 of the (-03) document, the authors refer to the rising titer of serum estradiol with cycle stages. They say that estrogens are high at estrus, which in fact they are low (usually around 5-10 pg/ml). Therefore, the Loeb and Quimby reference is correct. The original reference is Smith, MS, Freeman, ME and Neill, JD. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96: 219-226, 1975. In the Smith, et al, study, 200-250g, Sprague-Dawley rats from Madison, Wisc. were used and estradiol (along with progesterone, FSH, LH and prolactin) were measured in blood collected within 10 sec of being removed from the cage. In the Smith, et al, report, estradiol was low (<10 pg/ml) during the early morning hours of estrus and high (40-50 pg/ml) during the day of proestrus.

If discrepancy exists and the Loeb and Quimby data are correct, what is the impact of the error on the integrity of the Ciba-Geigy study?

I think that the above discrepancies are errors that were not caught by the authors in the writing of the documents and probably have little impact on the substance or their interpretation of the data in these studies. Except to

indicate that more care should have been exercised in the reporting of the information, this reviewer would give the authors the benefit of the doubt.

f. Are the effects of treatment over time supportable? If the data are valid and the authors are correct in their interpretation, then this triazine herbicide may indeed promote the precocious appearance of mammary tumors in the Sprague-Dawley rat via a hormonal estrogen imbalance. In addition to the comments made above and below, this reviewer would like to know whether Atrazine has any intrinsic estrogenic activity. Have bioassay dose response curves in ovariectomized rats or mice been generated? If so, what were the results of this in vivo test? Next, have the investigators attempted to run competitive displacement estrogen radioreceptor assays, using ³H-estradiol and varying concentrations of Atrazine? If so, what were the results of this in vitro assay? Information from such experiments would provide greater evidence for elucidating possible hormonal mechanisms (i.e., increased estrogen exposure) of mammary tumor promotion.

3. Do the hormonal data support the authors conclusions (presented in Tables 7-11) of the summary document? For the same reasons as listed above, the hormonal data may or may not support the authors conclusions.

4. Do the data provided support the differences in the endocrine/estrous cycle effects between the two rat strains?

Within the context of the previous discussion, the data provided are suggestive that differences in cessation of estrous cycling, along with differences in hormone concentrations occur between strains.

5. What is the overall quality of the hormone studies with respect to:

a. Experimental design? Insufficient information to make a judgement.

b. Implementation? Questionable

c. Working hypotheses? Reasonable, but others might be formulated.

6. Do the findings in the two rat studies have applicability to the human female?

Although we know some of the risk factors in the etiology of human breast cancer, our understanding of cause and mechanism for initiation, promotion and progression (metastasis) for this disease in women is unknown or incomplete at best. At

present, it is the opinion of this reviewer that current testing paradigms in rodents has little scientific basis for applicability to human metastatic breast cancer. The justification for this, is that metastasis of mammary neoplasia is rare in rodents, but is the normal progression in women. At the same time, however, the initiation and development of mammary tumors in rodents and the human female may have some similarities with respect to cause. Temporal exposure to unopposed estrogens or the "Estrogen Window" hypothesis may explain many of the elements in the etiology of mammary cancer in both rodents and humans. The hypothesis by the authors (that Atrazine promotes in a susceptible strain of rat the precocious persistent vaginal cornification with elevated estrogen) as a suggested mechanism for the early development of mammary tumors in the Sprague-Dawley rat as indicated in the current documents is a reasonable possibility for explaining the data. Since humans have a functional corpus luteum of the menstrual cycle, where luteal progesterone may "oppose" follicular estrogen, and rodents lack a full functioning luteal stage of the estrous cycle, differences in hormone exposure and regulation of mammary tissue between species occur. Whether this difference or others may account for differences in the etiology of mammary neoplasia between rodents and the human female await further research.

Again in summary, this reviewer has considerable reservations for the development of an hypothesis based upon the data presented. Furthermore, because there are considerable discrepancies in the data provided (both within and between documents) and unanswered questions, I have serious concerns that the results presented may be based upon a number of errors in these studies. May I recommend a laboratory audit as a possible mechanism for resolving the discrepancies and answering some of the questions.