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Mode of Action (MOA) for Liver Tumors Induced by Oral Exposure to 1,4-Dioxane

1,4-dioxane Consortium

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Update: Mode of Action (MOA) for Liver Tumors Induced by Oral Exposure to 1,4-Dioxane

Abstract

A reanalysis of data from two older mouse cancer bioassays on 1,4-dioxane, one 13-week mouse study, seven rat cancer bioassays, coupled with other data such as 1,4-dioxane's negative mutagenicity, its lack of DNA repair, and the appearance of liver tumors with a high background incidence, leads to the conclusion that these rodent tumors are evoked by a regenerative hyperplasia mode of action (MOA) that stimulates existing background mutations. Regenerative hyperplasia in this context is due to an overwhelming toxicity in the rodent liver as evidenced by an increase in blood levels of enzymes indicative of liver cell damage and associated histopathology due to 1,4-dioxane exposure that occurs in a dose and time related manner throughout the lifespan. Findings also include similarities in noncancer liver toxicity between shorter term/high dose and longer term/lower dose studies, which is recognized as typical for other chemicals. Importantly, the observed liver toxicity is related to the metabolic saturation of 1,4-dioxane, which is expected to have a threshold in the dose scale. It follows that threshold approaches to the assessment of this chemical's toxicity, and specifically for its liver tumor development, can be made in a confident manner.

Introduction

The proper way to assess the risk for 1,4-dioxane exposures has befuddled the risk assessment community. As a result, there have been contrasting toxicological assessments from different regulatory authorities [Health Canada, 2005; NICNAS, 1998; ATSDR, 2012; U.S. Environmental Protection Agency (EPA), 2013]. In part because of this lack of clarity, EPA (2013) defaulted to a linear approach for the development of the oral cancer slope factor, despite the fact that EPA did not consider this chemical to be mutagenic nor to cause DNA repair, two hallmarks which routinely lead to application of a linear approach. Moreover, EPA (2013) described information on alternative modes of action (MOA) that appeared credible.

EPA's dependence on a default linear approach, rather than a science-based alternative, was due in large part to the general lack of noncancer histopathology in the livers of male and female mice after lifetime exposures in two separate studies (NCI, 1978; JBRC, 1990a [published as Kano et al., 2009]). However, noncancer toxicity in the liver was shown to occur in subchronic studies for mice and rats (JBRC, 1990b [published as Kano et al., 2008]). In addition, noncancer toxicity was observed in lifetime studies in rats. These latter studies support a regenerative hyperplasia MOA for the development of liver tumors, which led to the conclusion that a threshold approach was appropriate for 1,4-dioxane by both Health Canada and NICNAS. The use of such an approach would yield a "safe" dose in the assessment of 1,4-dioxane, or at least to its most sensitive endpoint, liver tumors.

During the external peer review of EPA's IRIS file (2011-2012), EPA's review panel suggested that a re-read of the National Cancer Institute (NCI, 1978) chronic mouse liver slides might be helpful in resolving this apparent lack of noncancer toxicity data. Specifically:

> "The EPA should explore the possibility that slides from the NCI studies on 1,4-dioxane are available and in adequate condition to evaluate possible linkages between toxic effects and tumor outcome in the drinking water carcinogenicity studies in rats and mice."[1]

This suggestion was based on the fact that NCI pathologists in 1978 were more interested in finding tumors, and when tumors were found were not as concerned about recording available noncancer toxicity (McConnell, 2011). Some evidence that this might be occurring is found in the NCI (1978) report, where female mice are shown to have liver hyperplasia in the low dose group, but do not have this effect at the high dose where most animals have had liver tumors.

Based on this recommendation from EPA's review panel, scientists with Toxicology Excellence for Risk Assessment (TERA) and PPG Industries worked with Dr. Gene McConnell and scientists from the National Toxicology Program to re-read the 1978 NCI slides. The EPA National Center for Environmental Assessment and Integrated Risk Information System (IRIS) peer review panel for 1,4-dioxane were kept informed of the progress of this work. The older mouse liver slides were re-stained and then blindly re-read. The liver noncancer toxicology findings from the re-read were in stark contrast to the minimal noncancer findings reported in the original NCI report. Dr. McConnell reported noncancer toxicity that was evident at all doses and in a manner (i.e., hypertrophy, necrosis, inflammation, foci, adenoma, carcinoma) that was consistent with regenerative hyperplasia evoking the liver tumors. The reanalysis of the NCI (1978) mouse slides was published as Dourson et al. (2014), and the underlying pathology report of McConnell (2013), completed with the support of NCI staff, was made available to EPA and its peer review panel. The journal article is open access and the pathology report is available upon request.

A second long-term oral mouse bioassay conducted by the Japan Bioassay Research Center (JBRC, 1990a). A summary of this report and shorter term studies were subsequently published in English as Kano et al. (2008, 2009). Like the NCI (1978) bioassay, the Japanese work reported little noncancer toxicity in the mouse liver after long-term exposure, yet noncancer liver toxicity was reported in its 13-week studies (JBRC, 1990b).

Because the JBRC reports (1990a,b) were not available in English, TERA worked with a consortium of government scientists (TERA, 2014) to request full access to the lab reports and to have them translated.[2] Taken together, these translated reports include observations of additional noncancer effects in the liver of rats and mice. These data

[1] PEER REVIEWER COMMENTS. External Peer Review on the *Toxicological Review of 1,4-Dioxane* (CASRN No. 123-91-1). Versar, Inc. Contract No. EP-C-07-025 Task Order 118 (May 2012)

[2] The full translations of these Japanese findings can be obtained by any investigator (TERA, 2015).

allow for a more comprehensive MOA analysis as shown in this text. The use of data, rather than defaults is recommended by EPA (2005) cancer risk assessment guidelines.

Methods

The U.S. EPA (2005) guidelines for cancer risk assessment state that the MOA should be evaluated in determining the approach for dose response assessment from positive human or experimental animal tumor data. This evaluation is accomplished by first proposing a MOA, including identification of key events as shown in Figure 1, which is adapted from EPA (2013). Data on these key events, including available in vivo, in vitro, and mechanistic studies are then evaluated relative to the modified Hill criteria. When sufficient data are available, a biologically based dose-response (BBDR) model is the preferred method for low dose extrapolation. Absent such data, low dose extrapolation usually proceeds via a linear model (if the chemical acts via a direct DNA-reactive MOA or the MOA is not known) or a non-linear model (for a non-DNA-reactive MOA) based on one or more combinations of relevant tumors. Afterwards, determination of the human equivalent dose from the experimental animal dose is accomplished by a comparison of human and experimental animal kinetics or a default procedure.

These guidelines were followed by Dourson et al. (2014) where they analyzed two potential MOAs for liver tumor development from exposure to 1,4-dioxane: a heritable mutation to liver and/or nasal cell DNA, or liver cytotoxicity followed by regenerative cell proliferation and stimulation of endogenously mutated DNA. In this publication, analyses were performed on the basis of pooled results[3] from both males and females. This was appropriate since these two sexes did not get the same mg/kg-day doses when 1,4-dioxane was administered, and the overall results of the various cancer and noncancer effects were roughly similar. In this paper, we again followed a pooled approach in data for both sexes, and we specifically enhanced the investigation of the MOA on regenerative cell proliferation and stimulation of endogenously mutated DNA through the use of the translated Japanese study reports of the JBRC (1990a,b).

Results

The translated study reports of JBRC (1990a,b) confirm information found in the publications Kano et al. (2008 and 2009) and add some new information not found in the published articles. From these Japanese studies, the NCI (1978) bioassay, and the re-read of the mouse liver slides from the NCI (1978) study by McConnell (2013), we have further developed the hypothesized regenerative hyperplasia MOA, to the point where it is convincing in rats, and compelling in mice.

[3] Data are considered "pooled" when individual group level information is maintained in any analysis, such as the development of a dose response curve. In contrast, data are considered combined, when individual group level information is combined at the same or similar dose for subsequent analysis.

Review of the Japanese Translations and Integration with Other Findings: Rats

Figure 2a shows hyperplasia preceding the development of liver foci in rats when measured as a pooled percent of control. These same data are shown to precede the development of liver adenomas and carcinomas in Figure 2b when measured as pooled incidence. Figure 3 shows the pooled incidence of two additional effects in rats, that of centrilobular swelling and single cell liver necrosis from the 13 week studies. Here, the doses from the 13 week studies have been reduced by a 3-fold factor to address the well known differences in effect level among durations (Dourson and Stara, 1983).[4]

The overall result of this data overlay is to show that liver cell swelling and necrosis precede hyperplasia, which precedes the development of foci, which precedes the development of adenomas, which precedes the development of carcinomas. Liver enzyme changes in rats shown in Figure 4 pattern the histology shown in the first 3 figures.[5] Not to be forgotten, Figure 5 shows the histopathology results from the NCI (1978) study in rats (corresponding liver enzyme changes were not monitored). Although the overall incidences of the various effects are lower in the NCI (1978) rat bioassay, the form of these results match the findings in rats from the JBRC (1990a).

All of these findings in rats show the expected changes due to a regenerative cell proliferation and stimulation of endogenously mutated DNA, and the observed effects occur in the expected dose sequence. This sequence also matches the finding from the laboratory study report of Kociba et al. (1971), which was subsequently published by Kociba et al. (1974) (see Appendix 1 figures).

Review of the Japanese Translations and Integration with Other Findings: Mice

Figure 6 shows the results of a similar sequence of effects in mice found in the McConnell (2013) reread of the NCI (1978). Here, hypertrophy and necrosis precede the development of foci, which precedes the development of tumors, similar to what is found in the rat data. The information from the Japanese translated study reports and publication on mice are found in Figure 7a, with information from the 13-week studies also plotted. Centrilobular liver cell swelling appears to precede necrosis, and both of these effects appear to precede others, but hyperplasia and foci are nearly absent and adenomas and carcinomas appear early in the dose sequence. When the data for mice from both bioassays are conflated as in Figure 7b, the results are mixed.

Centrilobular liver cell swelling, hypertrophy and necrosis more clearly lead to tumor development in mice from the NCI (1978) study as re-read by McConnell (2013). And the McConnell (2013) results are consistent with the well-established sequence observed in all rat studies. Unfortunately, the Japanese histopathology findings in mice

[4] Some might argue that a 10-fold uncertainty factor would be more appropriate here. If so, the use of this factor would shift the data points for centrilobular swelling and single cell liver necrosis to the left, making the pattern of noncancer effect proceeding the development of tumors even more apparent.

[5] Here, the doses from the 13 week studies have been divided by a 10-fold uncertainty factor; caveats as in footnote 3 still apply.

(JBRC 1990a,b) are not consistent with either McConnell (2013) or any of the rat studies.

However, Figure 8 shows the corresponding changes in mouse liver enzymes from the Japanese work, where the 13-week doses are adjusted by 10-fold uncertainty factor (the NCI studies did not monitor these enzymes). These mouse enzyme results in the Japanese work are actually consistent with the sequential progression of noncancer effects from McConnell (2013) and all of the rat studies. This internal inconsistency in the Japanese mouse study, that is, negative noncancer liver histopathology but positive liver enzyme changes, adds to the conflicting findings in the mouse data from the JBRC study (1990a,b). Specifically, the lack of noncancer histopathology in the mouse study does not match the rest of the changes in liver enzymes. An additional inconsistency is that the tumor response in the low dose female mice of JBRC (1990a) does not match the tumors findings in the McConnell (2013) re-read, nor the original tumor findings of NCI (1978).

The results from the individual mouse studies of McConnell (2013) and (JBRC 1990a,b) are sufficiently different so as to question one data set or the other. Towards this point, Kano et al. (2009, page 2777) states that they changed their histopathology findings:

> The hepatic hyperplasia of rats and mice diagnosed in the previous report (Yamazaki et al., 1994) [which was a presentation of the JBRC, 1990a] was re-examined histopathologically and changed to hepatocellular adenomas and altered hepatocellular foci including acidophilic, basophilic and clear cell foci in the present studies, according to the current diagnostic criteria of liver lesions in rats and mice (Mohr, 1997; Deschl et al., 2001).

This statement suggests that results from the JBRC (1990a) study report were subsequently changed for the publication. However, the translations of the Japanese study reports do not show an increase in hyperplasia in mice [incidence out of 50 males of 5, 7, 5, 6 and out of 50 females of 2, 2, 1, 1, for control, low, medium and high doses, respectively]; foci are likewise nearly absent. This suggests that the JBRC (1990a) study report has also been modified as stated above, and then this modified report was subsequently published as Kano et al. (2009). This imparts uncertainty in the reliability of the findings from the JRBC studies.

Review of the Hypothesized Regenerative Hyperplasia MOA

A reanalysis of rodent data on 1,4-dioxane, shown in Figures 2 through 8 can be used to construct key event tables as suggested by (IPCS and EPA MOA references here). Other information can be added to these tables, as applicable. These tables are arranged in dose, time, and severity of effect, following the hypothesized regenerative hyperplasia MOA shown in Figure 1.

Table 1 shows the key event sequence for the available rat data. Key event 1, non-linear metabolism, followed by liver weight increase and/or hypertrophy (cellular swelling), is shown to occur at administered 13-week doses as low as 126 mg/kg-day (chronic dose equivalent of 42 mg/kg-day), or 2-year doses as low as 55 mg/kg-day. Key

event 2, necrosis and/or inflammation, is shown to occur at administered 13-week doses as low as 657 mg/kg-day (chronic dose equivalent of 219 mg/kg-day), or 2-year doses as low as 94 mg/kg-day. Key event 3, DNA synthesis, is shown to occur at administered 11-week doses as low as 1000 mg/kg-day (chronic dose equivalent of 330 mg/kg-day). Key event 4a, hyperplasia, is also shown to occur at administered 11-week doses as low as 1000 mg/kg-day (chronic dose equivalent of 330 mg/kg-day, and is seen at administered 2-year doses as low as 55 mg/kg-day. Key event 4b, pre-neoplastic foci, is seen at administered 13-week doses as low as 1168 mg/kg-day (chronic dose equivalent of 389 mg/kg-day), or 2-year doses as low as 55 mg/kg-day. Finally, the apical effect, adenomas and/or carcinomas is not seen at 13 weeks, but does occur after two years at doses as low as 274 mg/kg-day.

Thus, the dose sequence of these key events is:

- Key event 1, non-linear metabolism, liver weight increase, and/or hypertrophy (cellular swelling) at 42-55 mg/kg-day
- Key event 2, necrosis and/or inflammation at 94-219 mg/kg-day
- Key event 3, DNA synthesis at 330 mg/kg-day (DNA synthesis was only evaluated at 3.3 mg/kg/day [negative] and at 330 mg/kg/day [positive])
- Key event 4a and 4b, hyperplasia and foci development at 55-389 mg/kg-day
- Apical effect, adenomas and carcinomas at 274-1015 mg/kg-day

This sequence of key events from seven rat bioassays, when coupled with 1,4-dioxane's negative mutagenicity, its lack of DNA repair (indicating no DNA damage), and the appearance of only naturally occurring liver tumors (EPA, 2013), leads to the inescapable conclusion that rat liver tumors are evoked by a regenerative hyperplasia that stimulates existing mutations. This hyperplasia is due to an overwhelming toxicity in the rat liver due to 1,4-dioxane exposure that occurs in a dose and time related manner throughout the lifespan as shown in Table 1. Findings include similarities in toxicity between shorter term/high dose and longer term/lower dose, which is recognized as typical for other chemicals. Thus, the expectation is that the shorter-term higher dose liver noncancer toxicity shown in Kano et al. (2008) would be expected to occur at lower doses and longer exposures shown in Kano et al. (2009). This is evident in Figure 2b for rats, where the adjustment of the shorter-term exposures by a 3-fold uncertainty factor matches the chronic study findings.

Table 2 shows the key event sequence for the available mouse data. Key event 1, non-linear metabolism, followed by liver weight increase and/or hypertrophy (cellular swelling), is shown to occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day) or 2-year doses as low as 191 mg/kg-day. Key event 2, necrosis and/or inflammation, is also shown to occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day), or 2-year doses as low as 191 mg/kg-day. Information on Key event 3, DNA synthesis, is not available in mice. Key event 4a, hyperplasia, is not shown to occur in the sole 13 week study, but is seen at administered 2-year doses as low as 380 mg/kg-day; interestingly this effect is not recorded for the high dose of the NCI bioassay (see previous discussion). Key event 4b, pre-neoplastic foci, is also not seen at administered 13-week doses, but is found at administered 2-year doses as low as 380 mg/kg-day in the McConnell re-read of

the NCI (1978) bioassay, but generally not found in (JBRC, 1990a). Finally, the apical effect, adenomas and/or carcinomas is not seen at 13-weeks, as expected, but does occur after two years at doses between 66-964 mg/kg-day.

Thus, the dose sequence of these key events is:

- Key event 1, non-linear metabolism, liver weight increase, and/or hypertrophy (cellular swelling) in the range of 190-200 mg/kg-day
- Key event 2, necrosis and/or inflammation in the same range of 190-200 mg/kg-day
- Key event 3, DNA synthesis has not been evaluated
- Key event 4a and 4b, hyperplasia and foci development at doses as low as 380 mg/kg-day in one study but not the other
- Apical effect, adenomas and carcinomas at doses of 66-1015 mg/kg-day

This sequence of key events from two chronic mouse studies and one subchronic mouse study generally support the hypothesized regenerative hyperplasia MOA. These collective results are not any stronger than this, however, because tumors in female mice from the JBRC (1990) report are found at the lowest dose of 66 mg/kg-day, which is lower than doses from suggested key events. It might be appropriate to adjust 13-week study doses by a 10-fold factor to estimate the chronic dose equivalent (rather than a 3-fold factor), which would allow a sequence in doses of the key events in mice to be more similar to that found in the rat studies. Alternatively, it might be noted that the results of the two chronic mouse bioassays are simply contrasting. This difference may be due in part to the change in the recording of the liver lesions reported by Kano et al. (2009).

Discussion

As discussed more extensively by Dourson et al. (2014), in making decisions about the potential MOA for 1,4-dioxane, the animal tumor findings often give important clues. Some of the factors EPA (2005) recommends in a review of such findings include tumor types, number of studies, and of tumor sites, similarity of metabolic activation and detoxification, influence of route of exposure on the spectrum of tumors, effect of high dose exposures on the target organ or systemic toxicity that may not reflect typical physiological conditions, presence of proliferative lesions, effect of dose and time on the progression of lesions, ratio of malignant to benign tumors as a function of dose and time, time of appearance of tumors, development of tumors, tumors at organ sites with high or low background historical incidence, biomarkers in tumor cells, and shape of the dose-response curve in the range of tumor observation.

In considering these criteria, 1,4-dioxane oral exposure appears to be a mutagenic carcinogen in some respects. It evokes multisite and multispecies tumors that are not restricted to one sex suggesting an influence that is not restricted to gender, strain, or species. In addition, tumors evoked by 1,4-dioxane are both benign and malignant. However, all but one of the tumor types (i.e., nasal tumors) are at sites with a high historical background incidence, and findings in mutagenicity bioassays, initiation bioassays, and DNA repair bioassays are predominantly negative as described by EPA (2013). Furthermore, EPA concludes that: “The results from in vitro and in vivo assays

do not provide overwhelming support for the hypothesis of a genotoxic MOA for 1,4-dioxane carcinogenicity."

In contrast, extensive toxicity is seen in the primary tumors sites (liver and nose) suggesting a growth-promoting, and specifically, a regenerative cell proliferation, mode of action. This MOA is also supported by positive findings in promotion bioassays and DNA replication bioassays suggesting growth stimulation. The modified Hill criteria of EPA (2005) for the proposed regenerative cell proliferation MOA hypothesis for liver tumors by Dourson et al. (2014) was further evaluated in light of the translations of JBRC (1990a,b) and was determined again to be met for strength, consistency, biological plausibility, and coherence. Moreover, dose response and temporal concordance for noncancer precursors to tumors were clearly evident for rats (Table 1), and generally supportive for mice (Table 2).

The reason that the findings in mice are not more supportive of the regenerative hyperplasia MOA is because the histopathological characterizations of McConnell (2013) and of JBRC (1990a) in mice do not agree. McConnell (2013) found extensive liver noncancer toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990a). In contrast, JBRC (1990a) reported more tumors and nearly an absence of liver noncancer histopathology in the chronic study. The lack of liver noncancer histopathology in JBRC (1990a) is unexpected, especially since the JBRC (1990b) 13-week study showed extensive liver noncancer histopathology at higher doses, and comparable, but adjusted, chronic doses would also be expected to show this effect. Moreover, JBRC (1990a) does indicate liver noncancer toxicity given the observed increase in liver enzymes associated with cell damage. This internal inconsistency has not yet been resolved.

The fact that Kano et al. (2009) states that their histopathology lesions of JBRC (1990a) were changed for their publication, coupled with our observation that neither hyperplasia nor foci are evident in JBRC (1990a), suggest to us that not only has the Kano et al. (2009) report been changed as stated above, but that the JBRC (1990a) report may have also been changed. The higher reported incidences of liver adenomas and carcinomas reported in JBRC (1990a), compared to both NCI (1978) and McConnell (2013), may be due in part to this change. Furthermore, the degree of contrast among the subchronic and chronic findings of JBRC (1990a,b), and the larger than expected mortality in female mice in the chronic study, suggest caution in the use of this information for reliable estimation of health risks.[6] In contrast, the use of either the McConnell (2013) mouse findings, or the findings from any of the several rat bioassays could be done with much greater confidence.

Opinions of two pathologists lend support to the use of the McConnell (2013) mouse findings, or the findings from any of the several rat bioassays for risk assessment. The first opinion is found on the International Toxicity Estimates for Risk (*ITER*) file for 1,4-dioxane, and specifically:

[6] We are seeking additional pictures of mouse liver slides from JBRC (1990a) in order to resolve this apparent difference.

> "One of the panelists stated that, in general, Japanese pathologists tend to diagnosis disease which most US pathologists would consider as background. This might explain why the Japanese mouse studies tend to show more toxicity [tumors] in the liver than the NCI (1978) bioassays."

Another pathologist stated that it would be unlikely for the MOA to differ among rodent species to a chemical that caused liver tumors. The implication is that if the MOA is well established in rats, which it appears to be for 1,4-dioxane, then it is likely to be the same MOA in mice. [Note, Michael Dourson will be asking this pathologist for a written statement to this effect].

Additional text to be developed in conjunction with other partners.

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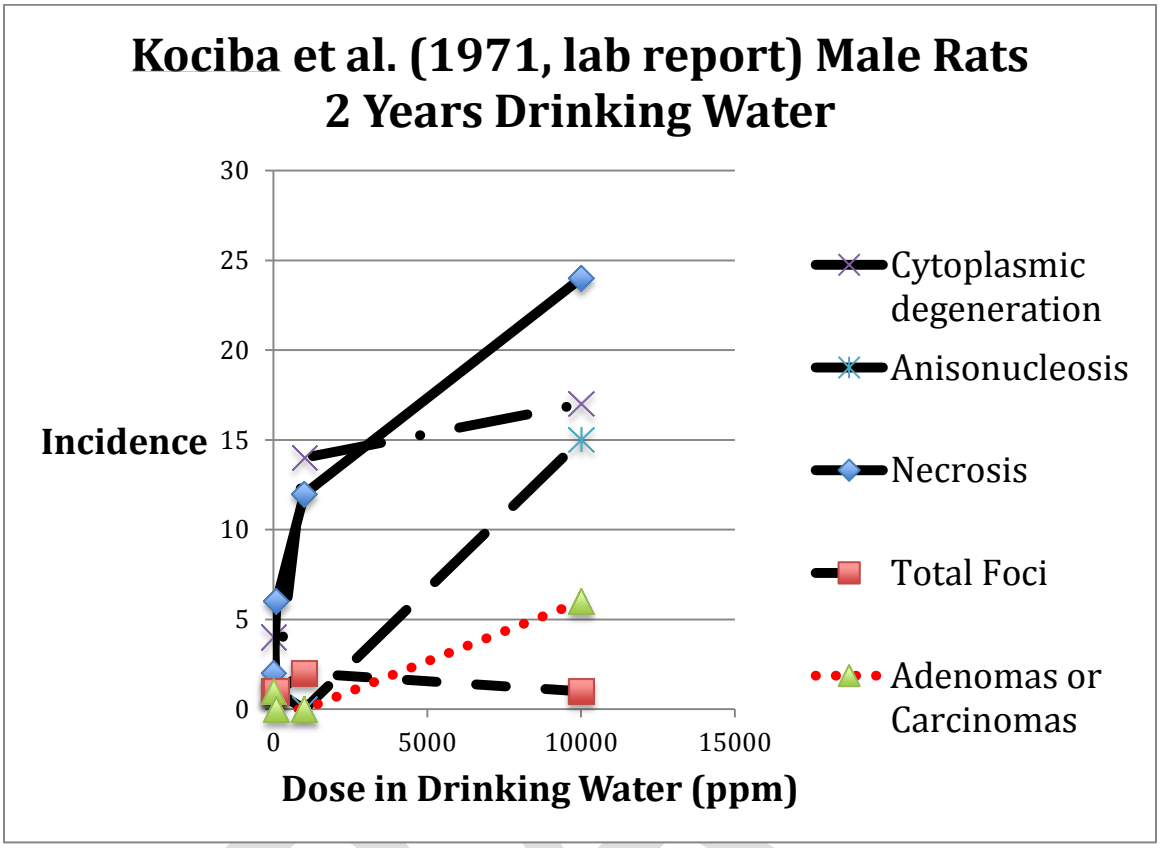
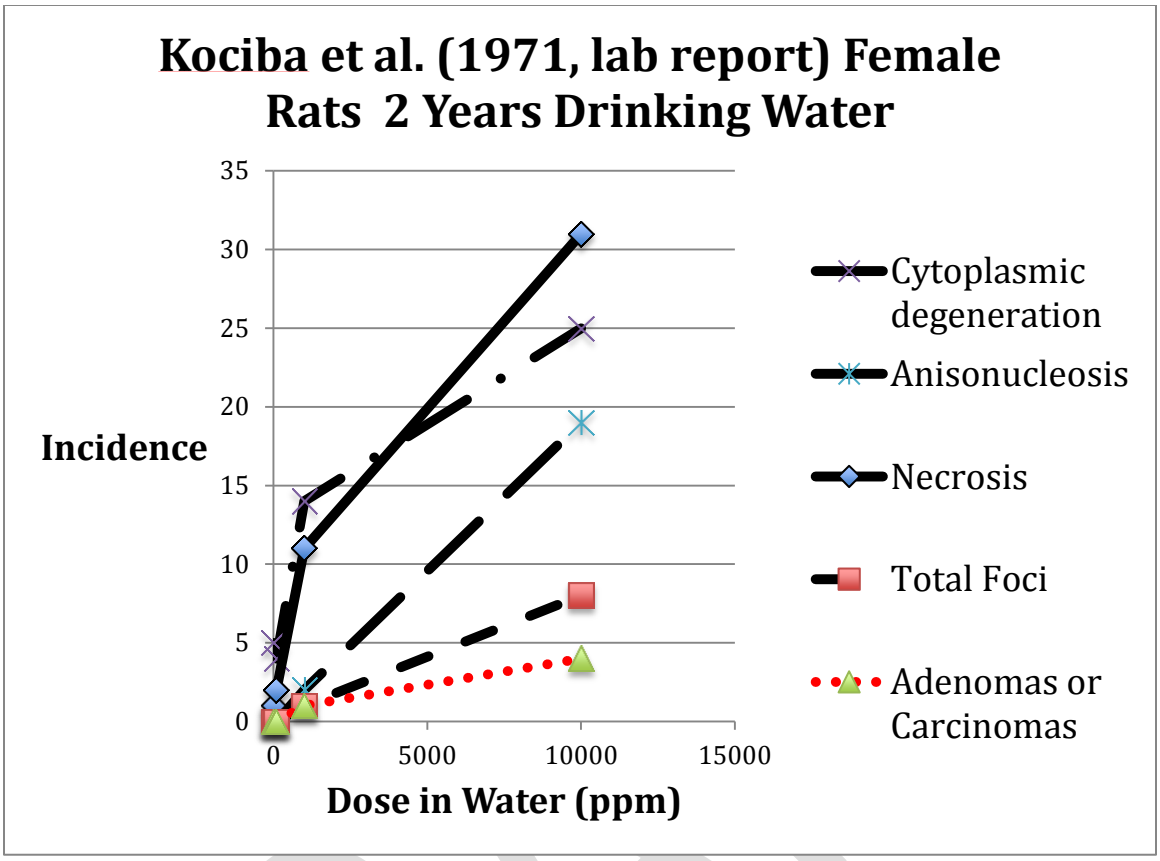
Appendix




Kociba et al. (1971, lab report) Female Rats 2 Years Drinking Water
Incidence
35
30
25
20
15
10
5
0
0
5000
10000
15000
Dose in Water (ppm)
Cytoplasmic degeneration
Anisonucleosis
Necrosis
Total Foci
Adenomas or Carcinomas