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Response to Letter to the Editor by Cohen et al. (2014):

Re: Waalkes et al.: Lung tumors in mice induced by “whole-life” inorganic arsenic exposure at human-relevant doses, *Arch Toxicol*, 2014

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We appreciate the interest of Cohen et al. (2014) in our recent research on inorganic arsenic carcinogenesis in mice after whole-life exposure (Waalkes et al., 2014). However, we strongly disagree with their conclusion that this study does not show an arsenic-related lung tumor response because of comparison to historical controls in CD1 mice (Cohen et al., 2014). Cohen et al. (2014) assert this lack of an association despite the facts that our study includes fully satisfactory concurrent male and female control groups that show lung tumor rates equivalent to historical controls and that specific treatment groups show robust differences in lung tumor incidences when compared in a statistically appropriate fashion to these concurrent controls.

Cohen et al. (2014) employ two tactics when attempting to dismiss our observed arsenic treatment-related increase in lung tumors (Waalkes et al., 2014) in two males groups (500 and 50 parts per billion [ppb]) and one female group (50 ppb) of CD1 mice that they base on comparisons to historical control data. First, they compare the current work (Waalkes et al., 2014) to broad and highly variable historical control ranges of lung tumors in control groups of CD1 mice (Cohen et al., 2014) from various laboratories and studies as reported by Charles River Laboratories (Giknis and Clifford 2005), an organization that does not actually conduct tumor end-point studies. The International Agency of Research on Cancer (IARC) is a world-leading agency in assessment of carcinogenic potential of chemicals and has undeniable expertise in evaluation of adequacy of rodent tumor end-point studies for inclusion in such evaluations (IARC 2004, 2012). The recently updated IARC preamble states: “It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical controls show high between-study variability and are, thus, of little relevance to the current experiment” (IARC 2012). In the male CD1 mouse control groups from various studies, Giknis and Clifford (2005) report a range of 2% to 42% incidence of bronchiolo-alveolar adenoma and a range of 1.4% to 26% bronchiolo-alveolar carcinoma incidence, data which certainly indicate high between-study variability for which the application of range as an adequacy metric has little relevance to our current experiment. In addition, IARC also recommends that, if used as an adjunct to concurrent controls, historical rodent controls should be recent data and should resemble the concurrent controls as much as possible with regard to general laboratory environment, etc. (IARC 2004, 2012). The historical data Cohen et al. (2014) cite were collected from studies initiated from 1987 to 2000, which are hardly recent, and were conducted in a number of

different facilities such that variations of environmental conditions are inevitable (Giknis and Clifford 2005). For instance, beyond having little information on adequacy of these studies, specifically in this instance we know nothing about inorganic arsenic levels in the drinking water, since the mice were said to simply have “free access to water” (Giknis and Clifford 2005). In addition, the use of historical tumor range as the metric to determine study adequacy is unjustified (IARC 2012), in part because it over-emphasizes outlier studies, whereas the overall rates of lung tumors at least show a central tendency. In fact, Giknis and Clifford (2005) clearly report the overall rate of control male CD1 lung tumors in this historical CD1 mouse database as 14.3% for bronchiolo-alveolar adenoma (421 lesions/2945 lungs), and 7.4% for bronchiolo-alveolar carcinoma (421 lesions/2945 lungs), rates which are nearly identical to the concurrent control rate in males for our present study of 14% bronchiolo-alveolar adenoma and 8% bronchiolo-alveolar carcinoma (Waalkes et al., 2014). A similar close comparison of lung tumor rates occurs with the female CD1 mice (Giknis and Clifford, 2005; Waalkes et al., 2014). This shows how misleading the use of historical control ranges can be when they come from various laboratory environments. It is of interest that the work of Giknis and Clifford (2005) is cited for lung tumor ranges yet ignored for lung tumor rates although both metrics are juxtaposed in the same table.

A second tactic Cohen et al. (2014) use to dismiss the observed arsenic treatment-related increase in lung tumors in our current study (Waalkes et al., 2014) is to select only one of several of our prior tumor end-point studies concerning inorganic arsenic (Tokar et al., 2011) and to selectively compare the controls from this single prior study to the arsenic-treated mice in the current study (Waalkes et al., 2014). This comparison is neither justifiable nor valid. It is not justified because we have a fully satisfactory concurrent control which Cohen et al. (2014) choose to ignore. Furthermore, the validity of transplanting a control group from a completely separate prior study and using it for statistical analyses as a valid control in place of a robust concurrent control is highly dubious. Clearly the precise conditions could potentially be different at different times. Furthermore, with multiple prior study controls available selection bias could occur by subjectively choosing a control group from a study that performed as desired in statistical analyses over control groups from other available studies that did not. In this regard, we have conducted multiple studies concerning inorganic arsenic with CD1 mice from the same source (Charles River), conducted at the same facility (Frederick, Maryland; Tokar et al., 2012, 2011; Waalkes 2006a,b) including the one under discussion (Waalkes et al., 2014). The control animals in all these studies consumed control water (<0.5 ppb arsenic) during their entire time on test including as post-weaning adults, and via the mother during lactation and pregnancy, while the mothers also receive control water during breeding (Tokar et al., 2012, 2011; Waalkes 2006a,b) in a fashion identical to the present study (Waalkes et al., 2014). The accumulated control data from these studies show that, while there is variability in control background incidences, the concurrent control group from the present study has typical incidences for lung tumors. For instance, the accumulated control data are for male CD1 mice from our studies to date are 20.8% bronchiolo-alveolar adenoma, 7.4% carcinoma, and 28% adenoma or carcinoma (Tokar et al., 2012, 2011; Waalkes 2006a; Waalkes et al., 2014) which is similar to the rates in the present study (14% bronchiolo-alveolar adenoma, 8% carcinoma, and 22% adenoma or carcinoma; Waalkes et al., 2014). In essence, there is nothing highly unusual about our

concurrent control mice that would justify their replacement with selected controls from a single prior study that might introduce ex post facto selection bias.

The p-value opinion put forth by Haseman et al. (1986) was in the context of mitigating Type I errors when reporting tumor incidences in a large number of tissues. Our paper focuses only on lung tumors. Haseman et al. (1986) also repeatedly cautioned against over-generalizing this “rule”, as it is a statistical approximation to the more complex decision-making process employed by the National Toxicology Program.

Cohen et al. (2014) mention the lack of tumors induced by arsenic at sites other than the lung as noteworthy when compared to our prior work using doses in the parts per million (ppm) range (Tokar et al., 2011). We noted in the current study that only lung tumors were induced by arsenic exposure (Waalkes et al., 2014). It is possible that the lung is the most sensitive site for arsenic induced tumors in this model. There are non-neoplastic lesions in other tissues which we chose not to report because of the potential impact of the lung tumor data, as we clearly state in the paper (Waalkes et al., 2014). It is also possible that additional studies with other mouse strains, etc., will reveal additional tumors sites in this ‘whole life’ model. As we clearly state in the paper we are hopeful that our work will stimulate others to investigate the carcinogenic potential of additional human-relevant inorganic arsenic doses in mice.

Cohen et al. (2014) say that survival in the low dose groups was “considerably less than the other groups”. In point of fact, survival was not suppressed in multiple treatment groups as implied by Cohen et al. (2014). In only one group was survival to study termination lower than control, specifically the low dose females (50 ppb). Cohen et al. (2014) state “the survival differences might have impacted differences in tumor incidences”. Generally one is concerned that fewer tumors will occur in a shorter life-span. However, although few females in this low dose group (50 ppb) survived to termination, inspection of the survival curve (Figure 2; Waalkes et al., 2014) shows a majority of the female mice in this group survived to approximately 80 weeks, long enough to develop lung tumors and long after the first appearance of a lung tumor in this study (46 weeks; Waalkes et al., 2014). Indeed, a significant increase in lung bronchiolo-alveolar adenomas was seen in this group of females despite the issue with survival (Waalkes et al., 2014). If anything, diminished survival would be expected to diminish tumor incidence if the tumors were unrelated to arsenic treatment. Furthermore, as described in the statistical methods section, our analysis of tumor data with mixed effects logistic regression modeling took length of survival into account, so that any effects of differential survival were minimized. Cohen et al. (2014) then state that all groups in this study showed short survival. These survival rates are similar to our prior work and we feel would hardly keep these data from regulatory use.

Cohen et al. (2014) take for granted that our tumor data should fit a monotonic dose-response relationship, citing studies that used much higher arsenic concentrations, where we believe our data may be showing that something is different at low arsenic concentrations and that the dose-response relationship may not necessarily be monotonic (Waalkes et al., 2014). The fact is that we see a similar response pattern in both sexes with lower arsenic exposure levels producing more lung tumors while higher doses do not produce lung tumors.

This similar gender pattern helps support this occurrence of tumors as being related to arsenic exposure. In addition, arsenic shows antitumor effects in various systems, as we pointed out (Waalkes et al. 2014), and because of this effect it seems reasonable to suggest a potential to impact the shape of the dose-response curve for cancer development in a complex fashion.

In summary, the most appropriate and rigorous comparison of tumor incidence data from treated animals is a robust concurrent control. Such a comparison was used to show that arsenic exposure was associated with increased lung tumors in certain groups in our current study (Waalkes et al., 2014). In essence, Cohen et al. (2014) are judging the quality of this study (Waalkes et al., 2014) based on historical control data that they carefully select to conclude a lack of a response while ignoring the most important control data, that being the robust concurrent control.

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