Information on 4-Methylimidazole Submitted to the
National Toxicology Program (NTP)
Office of the Report on Carcinogens (ORoC)

The American Beverage Association (ABA) appreciates the opportunity to submit information on 4-methylimidazole (4-MeI, CAS No. 822-36-6) to the Office of the Report on Carcinogens ("ORoC") in response to its September 20, 2013, Federal Register Notice (78 *Fed. Reg*. 57868). The ABA is the trade association that represents the non-alcoholic beverage industry. For the reasons stated below, the scientific data do not support a conclusion that 4-MeI is "reasonably anticipated to be a human carcinogen."

1. Data on Current Production, Use Patterns, and Human Exposure

4-MeI forms in small amounts through the Maillard Reaction process associated with heat and browning, and therefore is a byproduct found in many foods and beverages. Class III and IV caramel colorings contain 4-MeI which is subject to Food Chemical Codex (FCC) limitations. Contrary to information contained in the NTP Toxicity Report Series Number 67, no reliable data demonstrate that 2-methylimidazole (2-MeI) can be found in caramel colorings. 2-MeI is often reported in the literature associated with the analysis of caramel colorings, but it is used as a quality assurance marker in the analytical methodology used to measure the presence of 4-MeI.

The United States Food and Drug Administration (FDA) presented a poster at the 244th American Chemical Society National Meeting (Doell et al., August 21, 2012) entitled "Exposure Assessment of 4-Methylimidazole (4-MEI) from Caramel Coloring for the U.S. Population". FDA used what it described as a "worst case scenario" for this assessment, using the upper limit of 4-MeI content included in the FCC specifications for caramel colorings rather than data on the actual 4-MeI levels in food. This "worst case" estimate hypothesized overall mean exposure to 4-MeI resulting from the use of caramel colorings was 0.5 – 0.9 mg/person/day. FDA stated that it planned to refine this assessment with data from actual 4-MeI levels in food. If one were to estimate 4-MeI intake from caramel coloring Class III and IV by utilizing industry representative values, the FDA's estimated exposure to 4-MeI would be anticipated to be appreciably lower.

2. NTP Criteria for Listing

The NTP has identified the criteria it uses to determine whether a chemical should be listed as "Reasonably Anticipated To Be a Human Carcinogen":

There is limited evidence of carcinogenicity from studies in humans[1] , which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias, or confounding factors, could not adequately be excluded,

or

there is sufficient evidence of carcinogenicity from studies in experimental animals, which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset,

or

there is less than sufficient evidence of carcinogenicity in humans or laboratory animals; however, the agent, substance, or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

As discussed below, none of these criteria is met in the case of 4-MeI.

3. Carcinogenicity Information

a. NTP Criterion 1

There are no human studies concerning the carcinogenicity of 4-MeI. So the first NTP criterion for listing is not met.

b. NTP Criterion 2

In animals receiving 4-MeI, the only evidence of carcinogenicity was in one strain ($B6C3F_1$) of species (mice) at one tissue site (lung) by one route of exposure (ingestion). The only tumor type affected is the second most common spontaneous tumor in mice (Dixon et al. 2008). No evidence of an unusual degree of incidence, site, type of tumor, or age at onset was found. The standard bioassay for carcinogenicity in rodents was performed by NTP (2007) in $B6C3F_1$ mice and F344/N rats. In mice, 4-MeI was fed in the diet at 0, 312, 625 or 1250 ppm – equivalent to 0, 40, 80 and 170 mg/kg/day for 106 weeks. 4-MeI increased the incidence of alveolar/bronchiolar adenomas in all the treated groups of female mice, but not the incidence of alveolar/bronchiolar carcinomas. 4-MeI increased the incidence of alveolar/bronchiolar carcinomas in the 1250 ppm treated group of males and of alveolar/bronchiolar adenomas or carcinomas combined in the 1250 ppm treated males and the 625 and 1250 ppm treated female

[1] This evidence can include traditional cancer epidemiology studies, data from clinical studies, and/or data derived from the study of tissues or cells from humans exposed to the substance in question that can be useful for evaluating whether a relevant cancer mechanism is operating in people.

mice. Under the conditions of the study, NTP reported “clear evidence” of carcinogenic activity in male and female mice.

Haseman (2013), however, in a review of the NTP database for assigning a category of evidence of classification, stated that the “clear evidence” call made for lung tumors in the 4-MeI study was not consistent with the calls made by the NTP for lung tumor effects in mice in other similar NTP studies. He concluded that the proper “category of evidence” call should have been “some evidence” (Haseman 2013).

For example, Haseman found that the mouse lung tumor results in the 4-MeI bioassay were comparable to those observed in the NTP bioassays of ethylbenzene (TR-466), isobutyl nitrite (TR-488), molybdenum trioxide (TR-462), naphthalene (TR-410), and ozone (TR-440). Unlike for 4-MeI, the results from those studies were described by NTP as providing just “some evidence of carcinogenic activity.” In contrast, all substances described by NTP as showing “clear evidence of carcinogenic activity” based on mouse lung tumors alone showed stronger evidence of carcinogenicity than 4-MeI (Haseman 2013).

The European Food Safety Authority (EFSA) has also evaluated 4-MeI carcinogenicity in its re-evaluation of caramel colors (EFSA Journal 2011, 9(3):2004). EFSA stated that the type of tumor observed in the NTP bioassay could occur spontaneously at high incidence in these mice and that 4-MeI was not genotoxic and would show a threshold for carcinogenicity. EFSA assessed the risk to humans of 4-MeI in Europe from the use of caramel colors and concluded that human exposure was well below the threshold level reflected in the NTP study. EFSA stated that it had "no concern" about the carcinogenicity of 4-MeI for humans consuming foods with caramel color. In 2012, EFSA re-evaluated the consumer exposure to 4-MeI from caramel colors and reaffirmed its 2011 conclusion (EFSA Journal 2012, 10(12):3030).

In NTP's bioassay, the F344/N rats were reported to have shown “equivocal evidence of carcinogenic activity” in females based on increased incidence of mononuclear cell leukemia at the highest dose level and “no evidence of carcinogenic activity” in males. Mononuclear cell leukemia is common only in the F344 strain of rats in which it occurs spontaneously at high and variable incidence, and the spontaneous incidence has increased over time (Thomas et al. 2007). In contrast, the closest human equivalent is a rare form of large granular lymphocytic leukemia, of which a total of only 400 cases have been reported in the scientific and medical literature (Thomas et al. 2007). Interpretation of the implications of a slight increase in incidence of mononuclear cell leukemia in F344 rats is controversial (Caldwell 1999; Thomas et al. 2007).

The NTP report also mentioned the decreased incidence of certain tumors in both male and female rats. These decreases were further evaluated and elaborated on in a published paper by Murray (2011). Even after adjusting for the expected impact of reduced body weight in the 4-MeI-dosed animals, the decreased incidence of mammary, uterine, and clitoral gland tumors in female rats remains significant. These decreases also were not due to a difference in survival time between control and treated rats. The data indicate that 4-MeI *per se* may possess an ability to prevent tumor formation.

Thus, for the reasons presented above, 4-MeI does not meet the second criterion for listing as reasonably anticipated to be a carcinogen since it does not induce "tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset."

c. NTP Criterion 3

The third criterion NTP uses for identifying chemicals as reasonably anticipated to be carcinogens applies to substances for which there is less than sufficient evidence of carcinogenicity in humans or laboratory animals (as is the case for 4-MeI), but which "belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans."

4-MeI does not belong to "a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens." Although there are several substances listed in the RoC that contain an imidazole group, these do not form a well-defined, structurally related class with 4-MeI. For example, several heterocyclic amines are listed (NTP 2011), but these are all genotoxic amino-imidazoazaarenes, not simple imidazoles, like 4-MeI. Similarly, dacarbazine is a triazene prodrug with alkylating (methylating) properties, and metronidazole is a mutagenic nitro-imidazole (Speck et al. 1976), both quite unlike 4-MeI in chemical and biological properties (NTP 2011).

The structurally related, but non-genotoxic parent compound, imidazole (CAS No. 288-32-4), has not been tested for carcinogenicity potential (OECD 2003). Another imidazole that is structurally-related to 4-MeI is 2-MeI. Rats and mice exposed to 2-MeI in the diet exhibited clearly different and unrelated effects compared to those observed following 4-MeI exposure in the studies conducted by NTP (2004). In mice, 2-MeI did not produce the effects in the lungs that were observed with 4-MeI. In rats and mice, 2-MeI, unlike 4-MeI, was reported to produce effects in the thyroid. Additionally, the reductions in the numerous tumors observed in rats following exposure to 4-MeI were not observed with 2-MeI. These substantial differences in the responses of rats and mice to exposure to 2-MeI and 4-MeI preclude the extrapolation of 2-MeI toxicity data to 4-MeI. Therefore, 2-MeI and 4-MeI cannot be considered to be members of "a well-defined, structurally related class of substances" for the purposes of evaluating carcinogenicity.

Furthermore, there is no evidence that 4-MeI acts by "mechanisms indicating it would likely cause cancer in humans." In the bioassay report, NTP (2007) speculated on a mechanism whereby 4-MeI might be metabolized by Clara cells in the lung to reactive intermediates that might be responsible for the development of tumors. But NTP, itself, noted that this was unlikely because there is no evidence that 4-MeI is genotoxic. Such a mechanism would involve a genotoxic intermediate. NTP evaluated 4-MeI for genotoxicity in the *S. typhimurium* mutation

assay using 4 tester strains with and without hamster or rat liver metabolic activation enzymes. 4-MeI was not mutagenic in this assay. Also, no consistent or significant increases in micronucleated erythrocytes were seen in the bone marrow of male mice or rats treated with 4-MeI by i.p. injection or in peripheral blood samples for male and female mice administered the compound in dosed feed for 14 weeks (NTP 2007). Furthermore, 4-MeI did not induce mutations in five standard Ames strains of *S. typhimurium* using induced rat (F344/N) and mouse ($B6C3F_1$) liver and lung S9 as a source of exogenous metabolism, using both plate-incorporation and preincubation methodologies, together with 10% S-9 metabolic activation (Beevers and Adamson 2013).

Finally, the relevance of mouse lung tumors to human health risk is questionable. For example, the tuberculosis drug, isoniazid causes lung tumors in mice but human studies have shown no link to tumors in humans (Howe et al. 1979; Zheng et al. 1987).

4. Conclusion

4-MeI does not qualify and should not be further considered for listing as reasonably anticipated to be a human carcinogen or as a known human carcinogen. There are no studies in humans that address its carcinogenicity. 4-MeI induced tumors of the lung in one species (mice) and carcinomas in one sex (males), in one experiment, by one route of exposure. 4-MeI does not induce tumors in rats; rather, at several sites it results in reduced tumor incidence as compared to controls. 4-MeI does not induce chromosomal aberrations or mutations in experimental systems, and is not a member of a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens. It does not, therefore, meet any of the criteria for listing as reasonably anticipated to be a human carcinogen or as a known human carcinogen.

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5. Scientists with Expertise in 4-MeI

James Swenberg, DVM
Professor of Environmental Sciences and Engineering
University of North Carolina

David Longfellow, Ph.D., President
The Toxicology Forum
Washington, DC

James Felton, Ph.D,
Livermore Laboratory
Biology and Biotechnology Research Division
Livermore, CA

Eugene McConnell, DVM, President
ToxPath, Inc.
Raleigh, NC

Jay Murray, Ph.D.
Murray and Associates
San Jose, CA

Richard H. Adamson, Ph.D., President
TPN Associates, LLC
Germantown, MD and Walpole, MA

Joseph V. Rodricks, PhD, DABT, Principal
ENVIRON International Corporation
Arlington, VA

Submitted by: Richard H. Adamson, Ph.D.,
TPN Associates, LLC
13625 Esworthy Road
Germantown, MD 20874
and
7 Haynes Street
Walpole, MA 02081
301-221-0166
radamson.tpn@gmail.com

and

Joseph V. Rodricks, PhD, DABT
ENVIRON International Corporation
4350 North Fairfax Drive
Arlington, VA 22203
703-516-2316
jrodricks@environcorp.com

on behalf of the sponsoring organization:

American Beverage Association
1101 16th Street NW
Washington, DC 20036-4803