Updated evaluation (Oct. 2015) of the genetic toxicology of tert-butanol (TBA) and tert-Butyl acetate (TBAC) - James S. Felton, Ph.D.

The overall response to TBA and TBAC in genetic toxicology assays is very weak to negative. There are only three papers that are relevant to TBA with purported positive responses. These results are far out-numbered by negative results in the mouse lymphoma assay, Chinese hamster ovary assessment for SCEs, aberrations and micronuclei, and negative responses in Salmonella TA 98, 100, 104, 1535, and 1537. The overall data supports a negative overall assessment of genotoxicity for TBA and TBAC (see below and Tables 1 & 2).

Table I Summary of Salmonella Results for *TBA*

Test	Results	Ref
TA 100	-	Hüls AG (1979)
TA 100	-	EG&G Mason (1981)
TA 100	-	Zeiger et al. (1987)
TA 98	-	Hüls AG (1979)
TA 98	-	EG&G Mason (1981)
TA 98	-	Zeiger et al. (1987)
TA 1537	-	Hüls AG (1979)
TA 1537	-	EG&G Mason (1981)
TA 1537	-	Zeiger et al. (1987)
TA 1538	-	Hüls AG (1979)
TA 1538	-	EG&G Mason (1981)
TA 1538	-	Zeiger et al. (1987)
TA 1535	+	EG&G Mason (1981)
TA 1535	-	Zeiger et al. (1987)
TA 102	-	McGregor et al. (2005) a
TA 102	-	McGregor et al. (2005) b
TA 102	±	Williams-Hill et al. (1999)
TA 104*	-	Caprino et al. (1998)

*MTBE

Table II. Salmonella mutagenicity of TBAC (Tert-butyl acetate)

Test	Results	Ref
TA 100	-	McGregor et al. (2005)
TA 98	-	McGregor et al. (2005)
TA 1537	-	McGregor et al. (2005)
TA 1535	-	McGregor et al. (2005)
TA 102	-	McGregor et al. (2005)
WP2uvrA/pKM (E. coli)	-	McGregor et al. (2005)

One purported positive assay by Williams and Hill (Mut. Res., 1999) examines TBA response in Salmonella strain TA 102. TA 102 is UVRB positive which means it does not have a defect in excision repair of DNA. This strain, which has 30 copies of the hisG 428 gene on a plasmid, is typically positive for oxidative mutagens like x-rays, bleomycin, peroxide and quinones. If TBA causes oxidative damage to DNA, as suggested by Sgambato et al., then strains TA 102 and TA 104 should be very sensitive. However, this is not the case for TA 102, suggesting TBA causes cellular damage instead of mutations. The mutagenic response in two studies independent from Williams and Hill are clearly negative and did not confirm these reported positive results. A number of variations of the assay were tested with no positive outcomes.

In the Williams and Hill study there are some irregular results that should be considered. One, the background levels (420-460 rev) are much higher than either the Williams/Hill historical controls «400 rev) or the controls found in other studies using TA 102 (41-317 (mean = 194»(McGregor et al. 2005). Thus, the two-fold background requirement, which is barely met, is the result of a three-point linear response on the dose-response curve starting with a historically high background. Each point on this curve has overlapping error bars with the adjacent dose, which suggests a lack of repeatable colony counts for each plate at any given dose. This could be considered a very weak mutagenic response, but given the high background levels and overlapping error bars, the response is probably not strong enough to be considered positive.

All other Salmonella strains are negative with TBA. The TA 104 strain, which has the same basic DNA target as TA 102 and the repair mutation to make it more sensitive, was negative for MTBE (a similar compound that also metabolizes to TBA). If TBA caused oxidative DNA damage, one would expect this strain to be positive since the metabolite is the same, but it is not. Budroe et al. have made the argument that TBA, TBAC, and MTBE when dissolved in DMSO will have their free radical activity attenuated because of the radical scavenging properties of this solvent. If all the

bacterial genotoxicity was done with DMSO this argument might be justified. But McGregor et al. (2005) have reported that the Salmonella assay when done with water as solvent, as well as the DMSO, was also negative. This suggests that the solvent was not the determinant of the negative result. The mutagenic response to TBA using TA 102 in the two studies independent of Williams and Hill was negative, both in water and DMSO. Therefore, the Williams and Hill study has little credibility because of the caveats of high background, weak response (just two-fold background), and large error bars for each dose. In conclusion, TBA should not be considered a Salmonella mutagen based on the current published studies.

In addition to the Salmonella genotoxicity data, there is a report by Tang at al. that claims TBA is positive in an alkaline elution test. This test, which is commonly called the "comet assay", looks for DNA breaks in individual cells. Typically this set of experiments is done under very high stringency so that the tail of the comet can be measured using a number of different geometric parameters. In the case of the Tang paper this was not done in the standard way; only the appearance or lack of appearance of the tail was noted. The choice of
cells, tumor cells in this case, makes one wonders if normal cells (even in a transformed state) would behave in the same manner. Tumor cells typically divide at a faster rate, meaning cells are in S-phase, and thus, more sensitive to DNA damage. Chemo drugs clearly have more efficacy in fast dividing tumor cells. In conclusion, this assay is not only poorly done, but is done in tumor cells having characteristics not found in normal cells. This study does not add credible evidence for TBA inducing DNA damage.

Most recently (Aug 2015) TBAC was tested by Charles River, Edinburgh, UK, in the Mouse Lymphoma Mutation Assay (OECD 476). This test scores for forward mutations at the thymidine kinase locus. The results were clearly negative in two trials. TBAC was tested with and without Aroclor 1254 induced liver S9. Positive controls, historical controls, DMSO controls, dose response, toxicity testing, chemical dose analysis, and colony size examination all check out; indicating a well-designed study. Again, as reported above, the genotoxicity testing does not support any genotoxic mechanism involved in tumor initiation or promotion for these compounds, especially in mammalian tumor cells in culture.

Another study purported to offer evidence of DNA adduct formation following TBA exposure is the AMS study by Yuan et. al. The appearance of DNA adducts is an important step in determining whether a chemical causes DNA damage at an in vivo organ or site. Numerous methods are available to analyze this response and characterize the DNA adducts including: P-32 post labeling, mass spectrometry, immunoassay, and HPLC separation followed by scintillation counting. In the case of Yuan et aI., accelerator mass spectrometry
was used to detect DNA binding. This is a very sensitive method that has some of the same pitfalls as scintillation counting. One must characterize the product or chemical before analyzing the carbon-14 content. One advantage of accelerator mass spectrometry is that it is exquisitely sensitive.

In the case of Yuan et at. the characterization of the DNA adducts was primarily done by UV, recording a 260/280 ratio. Yuan et at stated they had a ratio of 180, which is acceptable. However, it is important to make sure that only DNA and its suspected adduct(s) are quantified. In these experiments there was no indication of a positive linear dose response for adducts. It is curious that most of the curves even in different tissues had the same shape, but were not linear as should be expected for loglog plots. In the same paper the MTBE adduct responses look linear, so why the deviation for TBA? This anomaly further supports the possibility that these AMS measurements are not quantifying TBA DNA adducts.

The metabolism of TBA in the intact mouse may also lead to metabolites that still have the incorporated C 14 label (based on the position of the labeled carbon). If the C14 is incorporated into acetone, for example, this metabolite of TBA could potentially be reincorporated into the DNA as a C 14 labeled base. This, of course, would add a large background to the DNA adduct counts or even suggest adducts that don't exist. This possibility makes it important to characterize the adducts chemically, to ensure that adducts are actually formed. Unfortunately, this was not done in the Yuan study so one cannot conclude with certainty from this study that DNA adducts were in fact formed.

Overall, based on the weight of the evidence, TBA cannot be considered a genotoxin. There are too many issues present for each one of the weakly positive assays to influence my overall conclusion - that TBA is not a genotoxin (this is also based on the recent mouse lymphoma negative mutation assay).

In my opinion, the negative response of TBA in bacterial mutagenicity tests could be further backed-up with TA 104 testing ± S9 and dissolved in both water and DMSO. At this point (10/15) we can also see the same negative gentox response in mammalian cells in culture, making the gentox effect of these compounds almost consistently negative.

The negative genotoxicity results for TBAC support the above conclusions, as similar metabolites are seen for both TBA and TBAC and are consistent with no mutation and DNA damage from either compound. TBAC was negative in all strains tested. Combining the genotoxicity results for TBA and TBAC, there is very strong evidence that this class of chemical compound is not genotoxic.

The lack of genotoxic results for TBA and TBAC has important implications for the interpretation of the rat kidney tumor data in the 1995 NTP chronic study with TBA. In the IARC criteria for assessment (Swenberg and Lehman-McKeeman, 1999) of male rat kidney tumors associated with alpha-2u globulin production there should be negative genotoxicity associated with the tumor inducing chemical (criteria #6). This is exactly what we see with TBA and TBAC. This is a very specific tumor type associated with male rats only and cannot be considered to translate directly to the human.