RE-EVALUATION OF DISCORDANT RESULTS IN RELATED OECD TG471 TESTER STRAINS

L.F. Stankowski Jr., B. Lallier, C. Tydrick, R. Baba, S. Tincher, J. Callupe and V.Y. Kwok
Charles River Laboratories – Skokie, 8025 Lamon Avenue, Skokie, IL 60077

charles river

1 ABSTRACT

The International Workshop on Genetic Toxicology (IWGT) recently met with one working group assessing the sensitivity/selectivity of tester strains used for the Bacterial Reverse Mutation (Ames) Test (OECD TG471). In part, results from large (>10,000-compound) databases were analyzed to assess relative responses between related tester strains: TA100/TA1535; TA97/TA1537; and TA102/WP2 *uvrA*/WP2 *uvrA* (pKM101). TA100/TA1535 comparisons were relatively straightforward, since both are required by OECD TG471 and the comparison data typically were generated concurrently in the same laboratory. In contrast, only one strain from other pairs/triplets are required, so comparisons typically compared results from different laboratories. Discordant results were often observed, especially when using the "2-fold" (or 3-fold) rule. Ten chemicals producing discordant results in related strains were re-evaluated concurrently: altertoxin I, chlorambucil, CI basic red 29, retrorsine and 2-methylpropanenitrile in TA97/TA1537; and N,N-diethylnitrosamine, folpet, acrylonitrile, $NiCl_2$, and p-toluenesulfonyl hydrazide in TA102/WP2 *uvrA*/WP2 *uvrA* (pKM101). Our results do not confirm previously reported differences. For example, altertoxin I was reported to be TA1537-positive and TA97-negative, but concurrent testing gave a higher response (revertants per µg/plate) in TA97. Similarly, folpet gave conflicting results in TA102/WP2 *uvrA* with and/or without S9, but was uniformly positive here in TA102/WP2 *uvrA*/WP2 *uvrA* (pKM101) ±S9. Chlorambucil, previously reported to be TA1537-positive and TA97-negative (+S9 only), was negative here in both. Results of the database analyses, and these, suggest: TA1535 adds little value to a set containing TA100; TA97 detects more unique mutagens than TA1537; and WP2 *uvrA* (pKM101) is more sensitive than TA102 or WP2 *uvrA*.

2 INTRODUCTION

During it's review on the equivalence of (and the need to keep all of) the current tester strains in the Bacterial Reverse Mutation (Ames) Test (OECD TG471[1]) the results from large (>10,000-compound) databases were analyzed to assess relative responses in related tester strains: TA100/TA1535; TA97/TA1537; and TA102/WP2 *uvrA*/WP2 *uvrA* (pKM101). TA100 and TA1535 are both are required by OECD TG471, and those comparisons were straightforward since data typically were generated concurrently in the same laboratory. In contrast, one can choose any one tester strain within the other pair/triplet. Many of those comparisons were of results from different laboratories, performed at different times and on different batches of a test article. Discordant results were often observed, especially when using the "2-fold" (or 3-fold) rule. Ten test articles previously reported to produce discordant results were re-tested here (five in each tester strain pair/triplet) and the results are reported below.

3 MATERIALS AND METHODS

Test Articles
The test articles were obtained and prepared as indicated (Table 1).

Treatments
The Ames tests were performed in tester strains TA97/TA1537, or TA102/WP2 *uvrA*/WP2 *uvrA* (pKM101), with and/or without S9 (Aroclor 1254-induced rat liver) as indicated (Table 1). Dose levels and treatment method(s) for the initial assays were based upon the previously discordance results (Table 2). Including subsequent re-tests, all test articles were evaluated up to 5000 µg/plate or solubility or toxicity limits. Plate incorporation and/or liquid preincubation (LPI; 20-minutes with shaking) treatments were performed according to standard methods[1,2]. Cultures were incubated for two days and scored using an automated colony counter or by eye.

Data Analysis
The responses between tester strains were compared using the historical "2-fold" (or 3-fold) rule, as well as by linear regression over the initial portion of the dose-response curves using Excel (Microsoft; Seattle, WA). An iterative process was used for the latter analysis, including only those data points producing the highest r^2 value (and the resulting slope is reported).

Guideline/ Regulatory Compliance
All assays were performed in general accord with OECD TG 471[1] (except that fewer than five tester strains were evaluated), and in compliance with the Good Laboratory Practices regulations of the US EPA[3] (except that the test articles were characterized by the manufacturer, and the stability, homogeneity and concentration of the dose formulations was not confirmed).

Table 1. Test Articles and Assay Conditions

Test Article	CAS No.	Vehicle	Source	Correction Factor	Tester Strains	S9	Method	Dose Range Evaluated (µg/plate)	
								Initial Assay	Retest (method)*
altertoxin I (ALT I)	56258-32-3	DMSO	LKT Labs	1.01	TA97, TA1537	±	Plate	3.13 - 100	n/a
chlorambucil (CBC)	305-03-3	DMSO	LKT Labs	-		+	LPI	7.81 - 1000	3.91 - 500
CI Basic Red 29 (CI BR 29)	42373-04-6	DMSO	Sigma	5.26		±	Plate	156 - 5000	9.38 - 300
2-methylpropanenitrile (2-MPN)	78-82-0	DMSO	Sigma	-		±	Plate	156 - 5000	156 - 5000 (LPI)
retrorsine (RET)	480-54-6	DMSO	Sigma	1.02		+	Plate	62.5 - 2000	156 - 5000
							LPI	3.91 - 500	
acrylonitrile (ACN)	107-13-1	DMSO	Sigma	-	TA102, WP2 uvrA, WP2 uvrA pMK101	±	Plate	39.1 - 5000	39.1 - 5000
folpet (FOL)	133-07-3	DMSO	Sigma	-		±	Plate	7.81 - 1000	7.81- 1000
nickel dichloride ($NiCl_2$)	7718-54-9	DMSO	Sigma	1.02		±	Plate	0.781 - 100	39.1 - 5000
N,N-diethylnitrosamine (DEN)	55-18-5	di-H_2O	Sigma	-		+	Plate	2.34 - 300	39.1 - 5000 (Plate/LPI)
p-toluenesulfonyl hydrazide (pTSH)	1576-35-8	DMSO	Acros	-		±	Plate	39.1 - 5000	39.1 - 5000

*if different from previous trial

4 RESULTS AND DISCUSSION

ALT I, CBC, CI BR 29, 2MPN and RET were previously reported to produce different qualitative results in tester strains TA97 and TA1537, while the same was true for DEN, FOL, ACN, $NiCl_2$, and pTSH in TA102, WP2 *uvrA and* WP2 *uvrA* (pKM101). Many of these previous characterizations were based upon the 2- or 3-fold "rules." For example, ALT I previously produced clear dose-dependent increases in revertant frequencies in TA97, even if they did not exactly reach the 2-fold threshold[5]. In this study, they clearly reached 2-fold in TA97, and the slopes (revertants/µg·plate^{-1}) were significantly greater in TA97 than TA1537 (Figure 1A). However, some of the other differences are inexplicable. CBC was previously reported to be positive in TA1537 +S9 and negative in TA97[6], but was uniformly negative here (two independent trials; Figure 1B). Conversely, CI BR 29 was previously reported to be negative in TA97[8], but reproducibly induced large increases here (two independent trials; Figure 1C and 1D). DEN also produced negative or inconsistent results in TA102[15] and WP2 uvrA[16], but produced bona fide positive responses here in all three related strains (two independent trials; Figure 2A). The same was true for folpet[17] (two independent trials; Figure 2B). All of the previous and current results are summarized below (Table 2).

Figure 1. Representative results (TA1537 +S9 ●, –S9 ○; TA97 +S9 ●, –S9 ○)

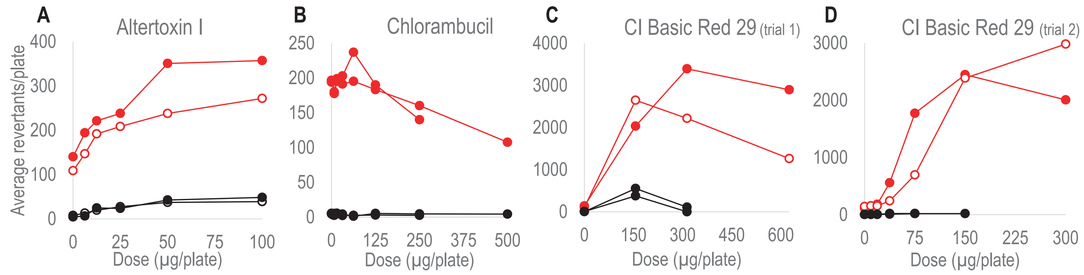
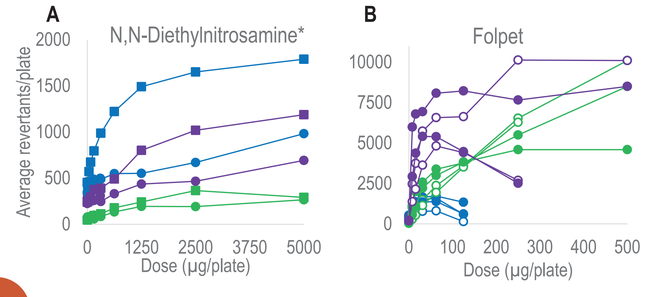


Table 2. Summary of Results

Test Article	Previous Reported Results		Current Results		
	TA1537	TA97	TA1537	TA97	
altertoxin I (ALT I)	8.7 - 10X↑-/+S9[4]	1.5 - 2.0X↑ -/+S9[5]	4.9 - 11X↑-/+S9	2.5 - 2.5X↑-/+S9	
chlorambucil (CBC)	4.5X↑ +S9[6]	negative +S9[6]	negative +S9	negative +S9	
CI Basic Red 29 (CI BR 29)	19 - 100X↑-/+S9[7]	negative -/+S9[8]	5.0 - 5.9X↑-/+S9	22 - 21X↑-/+S9	
2-methylpropanenitrile (2MPN)	2.1 - 3.1X↑-/+S9[9]	negative -/+S9[10]	negative -/+S9	negative -/+S9	
retrorsine (RET)	15X↑ +S9[11]	negative -/+S9[12]	negative -/+S9	negative -/+S9	
	TA102	WP2 uvrA	TA102	WP2 uvrA	WP2 uvrA pMK101
acrylonitrile (CAN)	negative -/+S9[13]	3.0 - 3.0X↑ -/+S9[14]	1.7 - 1.8X↑-/+S9	8.4 - 5.7X↑-/+S9	negative -/+S9
N,N-diethylnitrosamine (DEN)	negative -/+S9[15]	inconsistent -/+S9[16]	3.9X↑+S9	8.1X↑+S9	5.0X↑+S9
folpet (FOL)	mixed -/+S9[17]	mixed -/+S9[17]	3.2 - 4.7X↑-/+S9	170 - 110X↑-/+S9	24 - 31X↑-/+S9
nickel dichloride ($NiCl_2$)	negative -S9[18]	positive -S9[19]	negative -/+S9	negative -/+S9	negative -/+S9
p-toluenesulfonyl hydrazide (pTSH)	negative -/+S9[20]	2.5 - 5.2X↑ -/+S9[21]	negative -/+S9	1.9 - 2.2X↑-/+S9	negative - 2.1X↑-/+S9

Figure 2. Representative results (TA102 +S9, ●;–S9 ○, WP2 uvrA +S9 ●, –S9 ○; WP2 uvrA (pKM101) +S9 ●, –S9 ○



*circular symbols = plate incorporation
square symbols = liquid preincubation

5 REFERENCES

[1]OECD (1997) Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
[2] Maron, D.M and Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test, Mutation Research, 113-173-215.
[3]United States Environmental Protection Agency (1989) Good Laboratory Practice for Nonclinical Laboratory Studies, 40 CFR Part 792.
[4]Stack, M.E. and Prival, M.J. (1986) Mutagenicity of the Alternaria metabolites altertoxins I, II, and III, App. Environ. Microbiol., 52:718-722.
[5]Schrader, T.J et al (2006) https://doi.org/10.1016/j.mrgentox.2006.02.008
[6]Athanasiou, K. and Arzimanoglou, I. (1986) https://doi.org/10.1016/0165-7992(86)90117-X
[7]Cameron, T.P. et al (1985) https://doi.org/10.1016/0165-1218(85)90020-5
[8]NTP https://tools.niehs.nih.gov/cebs3/ntpViews/index.cfm?activeTab=detail&studyNumber=002-01770-0001-0000-8
[9]Japanese NITE http://anzeninfo.mhlw.go.jp/user/anzen/kag/pdf/B/B78-82-0.pdf
[10]NTP https://tools.niehs.nih.gov/cebs3/ntpViews/index.cfm?activeTab=detail&studyNumber=002-01362-0001-0000-5
[11]Wehner, F.C. et al (1979) https://doi.org/10.1016/0165-1218(79)90065-X
[12]Rubiolo, P. et al (1992) https://doi.org/10.1016/0165-7992(92)90050-R
[13]Muller, W. et al (1993) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1521147/pdf/envhper00380-0040.pdf
[14]Venitt, S. et al (1977) https://doi.org/10.1016/0027-5107(77)90028-8
[15]Aiub, C.A.F. et al (2003) https://doi.org/10.1016/S0378-4274(03)00263-7
[16]Dunkel, V.C. et al (1984) https://doi.org/10.1002/em.2860060702
[17]Arce, G.T. et al (2010) https://doi.org/10.3109/10408444.2010.481663
[18]Biggart N.W. and Costa, M. (1986) https://doi.org/10.1016/0165-7992(86)90056-4
[19]Seo, K.S. and Lee, C.S. (1993) Mutagenicity of metal compounds using *Escherichia coli* WP2uvrA, Misaengmul Hakhoechi, 31:527-531.
[20]Blakey, D.H. et al (1992) Mutagenic activity of p-toluenesulfonhydrazide, Toxicol. Ind. Health, 8:369-76.
[21]JETOC (1996) Japan Chemical Industry Ecology-Toxicology & Information Center.

6 CONCLUSIONS

- Concurrent testing did not confirm previously reported differences in response between the related tester strains
- Our inability to confirm previous responses is concerning; possible explanations include technical error, poor test article characterization, impurities, differences in exposure methods, etc
- Additional statistical analyses are warranted and ongoing