APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 5, 409–415 (1991)

Evaluation of the genotoxic potential of six triorganotin compounds by the mouse micronucleus and spermhead abnormality tests

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Six triorganotin compounds—Ph_3SnOH, $BuPh_2SnOH$, $(p\text{-}ClC_6H_4)Ph_2SnOH$, $(cyclo\text{-}C_5H_9)Ph_2SnOH$, $Ph_3SnO_2CCH_2CS_2NMe_2$ and Bu_3SnOSO_2Et—were tested for their mutagenic potential in somatic and germinal cells in ICR mice by using the micronucleus and spermhead abnormality assays, respectively. In somatic cells, the compounds significantly induced chromosomal disorders at half their respective estimated LD_{50} (i.p.) values (5.00–6.25 mg kg^{-1} body wt), and one compound, Ph_3SnOH, even at 1/20 of its estimated LD_{50} value. In germinal cells, five compounds significantly induced chromosomal disorders at 1/64 of their respective estimated LD_{50} (i.p.) values, whereas one, $(p\text{-}ClC_6H_4)Ph_2SnOH$, caused such disorders only at 1/8 of its estimated LD_{50} value.

Keywords: Triorganotin, mutagenicity, micronucleus, spermhead

INTRODUCTION

Organotin compounds have achieved commercialization in a wide variety of applications,[1] and as a result of their increased use in the last two decades[2] they have become the subject of a growing number of studies aimed at determining their potential negative effects on environmental[2–4] and biological[5–8] systems.

Among the organotin compounds, the biological activity is maximal with the trisubstituted organotins such as triphenyl- and tricyclohexyl-tin species, formulated as agricultural chemicals, and tributyltin compounds, incorporated in wood preservatives and in antifouling coatings for ships and marine installations.[1] The butyl- and phenyltins are especially important since they combine high biological activity with low mammalian toxicity.[1,9] Representative members of this class of compounds have shown no evidence for induced cytogenetic effects especially when tested by the *in vitro* salmonella-microsome assay.[10,11] An *in vivo* micronucleus test on mice has also confirmed the absence of any mutagenic effect for bis(tributyltin) oxide (TBTO),[11] a well-established industrial biocide and wood protectant. The micronucleus test is based on the observation that mitotic cells having chromatid breaks or chromatid exchanges suffer from disturbances in the anaphase distribution of their chromatin and that, after telophase, a sizeable proportion of such chromatin is not included in the nuclei of the daughter cells but instead forms single or multiple micronuclei in the cytoplasm of the cells.[12,13]

In the present investigation, six triorganotin compounds, including triphenyltin hydroxide, an FAO/WHO-approved fungicide that has been used as a crop protectant for over 30 years, were selected for the evaluation of their mutagenic potential by using the micronucleus and spermhead abnormality tests on ICR mice. The mouse spermhead abnormality assay is aimed at determining the ability of a compound to disrupt the normal morphology of the mouse spermhead, and has been successfully applied in the detection of several organic carcinogens.[14,15]

EXPERIMENTAL

Materials and methods

Analytically pure samples of the following six triorganotin compounds were prepared for the *in vivo* mutagenic screening tests: triphenyltin hydroxide,[16] **D1** $[(C_6H_5)_3SnOH]$; butyldiphenyltin hydroxide,[17] **D2** $[BuPh_2SnOH]$; (*p*-

0268-2605/91/050409-07$05.00

Received 14 January 1991
Revised 14 June 1991

chlorophenyl)diphenyltin hydroxide,[18] **D3** [(*p*-Cl_6H_4)Ph_2SnOH]; cyclopentyldiphenyltin hydroxide,[18] **D4** [(c-C_5H_9)Ph_2SnOH]; triphenyltin *N*,*N*-dimethyldithiocarbamylacetate,[19] **D5** [$Ph_3SnO_2CCH_2CS_2NMe_2$] and tributyltin ethanesulphonate,[20] **D6** [Bu_3SnOSO_2Et]. The compounds were dissolved in dimethylsulphoxide (DMSO), and were made into 1 % stock solutions.

Outbred ICR mice, 9–10 weeks old and weighing 25–35 g each, were kindly supplied by the Animal Facilities Centre, Faculty of Medicine, University of Malaya.

Experimental design

Acute toxicity determination

The estimated LD_{50} of each of the six triorganotin test compounds was determined by injecting the mice intraperitoneally (i.p.) with 5 cm^3 of various concentrations of each compound, beginning with 1 % stock solutions. The concentrations were halved in a stepwise manner until the level was reached where half of the treated mice died within the period between day 3 and day 7 after the injections; this concentration was taken as the estimated LD_{50} of the compound under test.

Micronucleus assay

Three dose levels (estimated LD_{50}, HD and LD) were used in this assay. Dosage selection was made as follows: half the estimated LD_{50} value was assigned as the high dose (HD) and 1/20 of the estimated LD_{50} as the low dose (LD).[21] Each compound was injected as 5 cm^3 suspensions intraperitoneally into a group comprising four male and four female mice. For the controls, 5 cm^3 of DMSO was injected. The mice were sacrificed 24 h after the injections, and slides of their bone marrow preparations[22] were stained in May–Gruenwald and giemsa, air-dried and mounted with DPX before the micronuclei were scored under a light microscope.

Spermhead abnormality assay

Dose selection was as follows: 1/8 of the estimated LD_{50} was assigned as the high dose and 1/64 of the estimated LD_{50} as the low dose. Three dose levels (estimated LD_{50}, HD and LD) were used in this assay. The design of the assay was similar to that of the micronucleus assay, except that two or three male mice were used per dose, and the mice were sacrificed 56 days after the injections. Sperm preparations were made as previously described[23] and the slides were stained in aceto-orcein.

Mode of scoring and statistical evaluation

Only the young polychromatic erythrocytes (PCEs) were scored for the presence of micronuclei in the micronucleus test.[24–27] One thousand PCEs were examined per slide and, for each mouse, at least two slides were analysed. The results were expressed as the average number of micronucleated PCE per 100 PCE (%). The significance of the difference between the treated and the control was calculated by using a corrected chi-square test.[28] Doses that induced a statistically significant increase in the number of micronucleated PCE over those of the controls at the $P<0.01$ level were considered to cause marrow toxicity.[25, 26, 29]

For the spermhead abnormality assay, at least two slides were analysed per mouse studied. For each slide, 1000 sperms were examined for the occurrence of spermhead abnormalities. The abnormalities were categorised as described previously;[23] the folded-head type was excluded from the counts.

All the slides were coded prior to scoring, and scored blind. The results of both assays were analysed statistically.

RESULTS

The estimated LD_{50} values for the six triorganotin compounds tested intraperitoneally for their acute toxicity in ICR mice are given in Table 1, along with two other dosages (which are fractions of the estimated LD_{50} values) used for the micronucleus and spermhead abnormality assays. A comparison of the estimated LD_{50} values shows that the more toxic compounds were **D1**, **D4** and **D6** (estimated $LD_{50}=5.00\ mg\ kg^{-1}$), followed by **D2** and **D3** (estimated $LD_{50}=5.50\ mg\ kg^{-1}$) and **D5** (estimated $LD_{50}=6.25\ mg\ kg^{-1}$). The results of the micronucleus assay are summarized in Table 2. The micronuclei were identified as dark-blue bodies against the light-blue cytoplasm of PCE; the normal erythrocytes, by way of contrast, were red (Fig. 1). The frequencies of the micronucleated PCE in the background and control (DMSO) ICR mice were, respectively, 0.66 and 0.89 %, the difference between the

Table 1 Estimated LD_{50} (i.p.) and two other selected dosages of six organotin test compounds assayed for mutagenicity

Compound	Estimated LD_{50} (mg kg^{-1})	Dosage selection (mg kg^{-1} body wt)			
		Micronucleus assay		Spermhead abnormality assay	
		HD[a]	LD[a]	HD	LD
D1	5.00	2.50	0.25	0.62	0.08
D2	5.50	2.75	0.28	0.69	0.09
D3	5.50	2.75	0.28	0.69	0.09
D4	5.00	2.50	0.25	0.62	0.08
D5	6.25	3.10	0.31	0.78	0.10
D6	5.00	2.50	0.25	0.62	0.08

[a]Key: HD, high dose; LD, low dose.

background and the control being statistically insignificant at the $P<0.01$ level. The estimated LD_{50} and HD of all six compounds induced a statistically significant increase in the frequency of micronucleated PCE over that of the control at the level of $P<0.01$. However, at the LD levels, the compounds did not cause any significant increase in micronucleated PCE, except for Ph_3SnOH (**D1**). There was no noticeable difference in the results obtained between male and female mice.

The *in vivo* induction of spermhead abnormalities in the organotin-injected ICR mice is summarized in Table 3. The morphology of the normal spermhead, and some of the more frequently encountered types of abnormal spermheads are shown in Fig. 2. The chi-square test indicated that all three dose levels of **D2**, **D5** and **D6**, and the estimated LD_{50} and HD of **D1**, **D3** and **D4**, induced significant increases in the frequencies of abnormal spermheads over those of the DMSO controls at the $P<0.01$ level. The increase in the frequency of abnormal spermheads induced by **D4** was significant only at the $P<0.05$ level, but that induced by **D3** at the LD level was clearly not significant.

The frequencies of abnormal spermheads in the background and the control mice were 0.25 % and 1.08 %, respectively. The difference between the background and the control is statistically significant at the $P<0.01$ level. Therefore, DMSO itself is also capable of inducing a significant amount of spermhead abnormalities.

Table 2 Micronucleus assay in ICR mice 24 h after intraperitoneal injections

Compound	Dose (mg kg^{-1})	No. of mice[a]	No. of PCE	No. of m/n PCE[b]	Percentage of m/n PCE[c]	$\chi_c^{2\,d}$
Background	—	8	16000	106	0.66	—
DMSO	—	8	16000	143	0.89	—
D1	0.25	8	16000	215	1.34**	14.24
	2.50	8	16000	286	1.79**	47.64
	5.00	8	16000	258	1.61**	32.82
D2	0.28	8	16000	174	1.09ns	2.88
	2.75	8	16000	246	1.54**	27.07
	5.50	8	16000	341	2.13**	81.42
D3	0.28	8	17000	178	1.05ns	1.86
	2.75	8	16000	250	1.56**	28.95
	5.50	8	16000	274	1.71**	41.06
D4	0.25	8	16000	127	0.79ns	0.84
	2.50	8	16000	202	1.26**	9.86
	5.00	8	16000	225	1.41**	18.04
D5	0.31	8	17000	189	1.11ns	3.72
	3.10	8	17000	223	1.31**	12.75
	6.25	8	17000	528	3.11**	201.37
D6	0.25	8	16000	138	0.86ns	0.06
	2.50	8	16000	231	1.44**	20.48
	5.00	8	16000	266	1.66**	36.86

[a]Comprising four males and four females. [b]Mononucleates PCE (see text). [c]Key: ns, non-significant; **, $P<0.01$. [d]χ_c^2 denotes Yate's correction.[28]

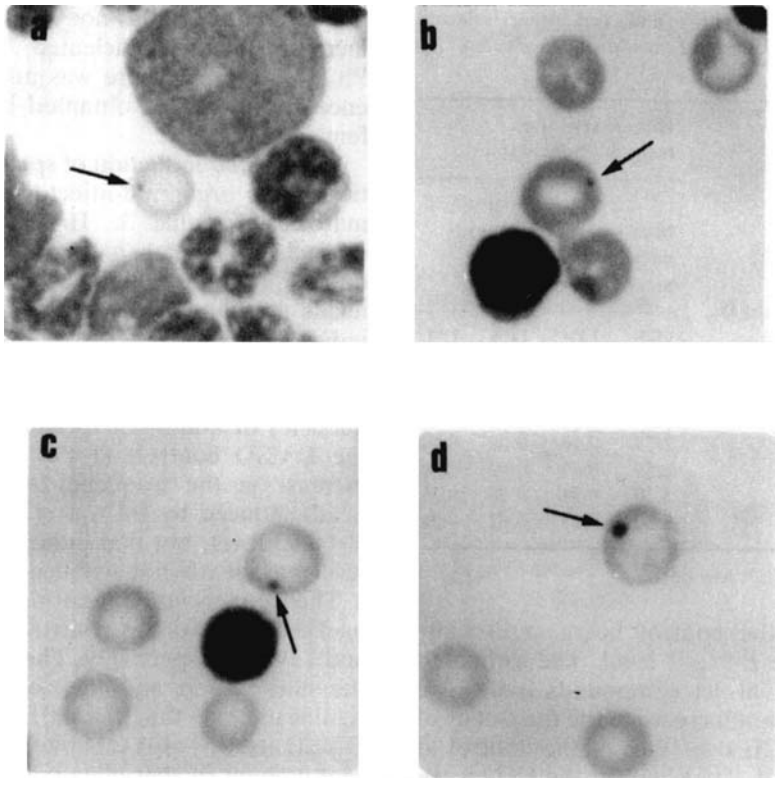

Figure 1 Mouse micronucleus assay: a, micronucleated normal erythrocyte; b, c and d, micronucleated PCE (magnification: ×1000).

DISCUSSION

In vivo effects in somatic cells of mice

The micronucleus test is a genotoxicity assay aimed at evaluating the possible deleterious effects posed by environmental chemical contaminants.[30,31] This assay is comparable in sensitivity with the conventional but more tedious chromosomal analysis.[32]

All six organotin compounds tested in this study were found to be capable of producing micronuclei at certain dose levels. At the respective LD levels, only **D1** induced a significant increase in the production of micronuclei, the rank of decreasing potency being **D1 > D5 > D2 > D3 > D6 > D4.** At the respective HD levels, all six compounds induced significantly elevated amounts of micronuclei relative to the control.

The production of micronucleated PCE indicates the occurrence of chromosomal aberrations induced during preceding mitotic divisions of erythroblasts. However, it is to be cautioned here that negative results in the micronucleus test may not prove an absence of mutagenicity: the compounds could be acting as specific-point mutagens or they apear inactive merely because of their inability to reach target cells used in the test system.

Our *in vivo* findings in mice can only be cautiously extrapolated to humans exposed to these compounds. Studies on the effects arising from exposure to, for example, ionizing radiation[33,34] have demonstrated corresponding findings in mice and man, although differences in metabolism hinder any direct extrapolation to humans from animal studies.[35] Furthermore, the actual dose levels in the environment are often much lower than those used under laboratory conditions. Triorganotins in biocidal formulations are used at relatively low concentrations compared with many organic-based biocides and, to date, have revealed no major risks to humans. Our results on Ph_3SnOH (**D1**) and the five other developmental organotin compounds, while suggesting their capability for causing chromosomal aberrations in intraperitoneally treated mice, perhaps represent an extreme situation, since

Table 3 Spermhead abnormality assay in ICR mice 56 days after intraperitoneal injections

Compound	Dose (mg kg^{-1})	No. of mice	No. of sperms	No. of abnormal sperms	Percentage of abnormal sperms	$\chi_c^{2\,b}$
Background	—	2	4000	10	0.25	—
Control	—	2	4000	43	1.08	—
D1	0.08	3	6000	94	1.57*	3.94
	0.62	3	6000	145	2.42**	22.70
	5.00	3	6000	242	4.03**	74.80
D2	0.09	3	6000	107	1.78**	7.78
	0.69	3	6000	180	3.00**	39.91
	5.50	3	6000	282	4.70**	99.15
D3	0.09	3	6000	93	1.55ns	3.69
	0.69	3	6000	204	3.60**	52.89
	5.50	3	6000	243	4.05**	76.32
D4	0.08	3	6000	97	1.62*	4.72
	0.62	3	6000	144	2.60**	22.25
	5.00	3	6000	171	2.85**	35.26
D5	0.10	3	6000	121	2.02**	12.62
	0.78	3	6000	228	3.80**	66.56
	6.25	3	6000	317	5.28**	121.16
D6	0.08	3	6000	95	1.58**	4.19
	0.62	3	6000	146	2.43**	23.15
	5.00	3	6000	245	4.08**	76.58

[a]Key: ns, not significant; *, $P<0.05$; **, $P<0.01$. [b]χ_c^2 denotes Yate's correction.[28]

organotin compounds probably enter the body of a mammal orally only through accidental ingestion. In a report on the mutagenic effects of TBTO orally administered at the 125 mg kg^{-1} concentration level, no mutagenic effects were observed over a three-day period.[11] The mutagenic effects on somatic cells are of concern for the present generation, but the mutagenic effects on the germ cells are of even greater concern, since germ-cell mutations are only manifested in future generations.

In vivo effects in germinal cells of mice

The spermhead abnormality test that evaluates chemical effects on exposed mice is based on the scoring of abnormal head forms in smears of sperms. The faulty differentiation of spermatozoa that leads to the formation of abnormal spermhead morphology reflects genetic damage in the male germ cell.[36] Virtually all murine germ-cell mutagens tested induced spermhead abnormalities in mice[14,29,37,38] and some carcinogens have been identified by using this method.[14,15,39] Compounds yielding positive results in the mouse spermhead morphology test should be regarded as suspect germ-cell mutagens in mammals. It was shown that this assay has moderate sensitivity (40–55 %) and high specificity (100 %) with respect to carcinogenic potential.[36]

The aprotic solvent DMSO used in the present study was found to be capable of inducing a significant amount of spermhead abnormalities although no significant increase in micronuclei was found. DMSO is thus not an ideal solvent for such mutagenicity studies, but water and solvents such as ethanol and acetone were incapable of dissolving the organotin compounds to the concentrations required for the tests. Where use of DMSO appears necessary, it is especially important therefore that as small a quantity as possible be used, and that DMSO controls be also included in the tests.

The present study showed that, at the respective low dose levels, all the compounds except **D3** induced significant increases in the number of abnormally formed spermheads against the DMSO control. The results indicated five of the compounds (but not **D3**) are capable of causing genotoxic damage to the germinal cells. Using the frequencies of abnormal spermheads induced at the low dose levels as reference points, the following rank of decreasing potency was deduced:

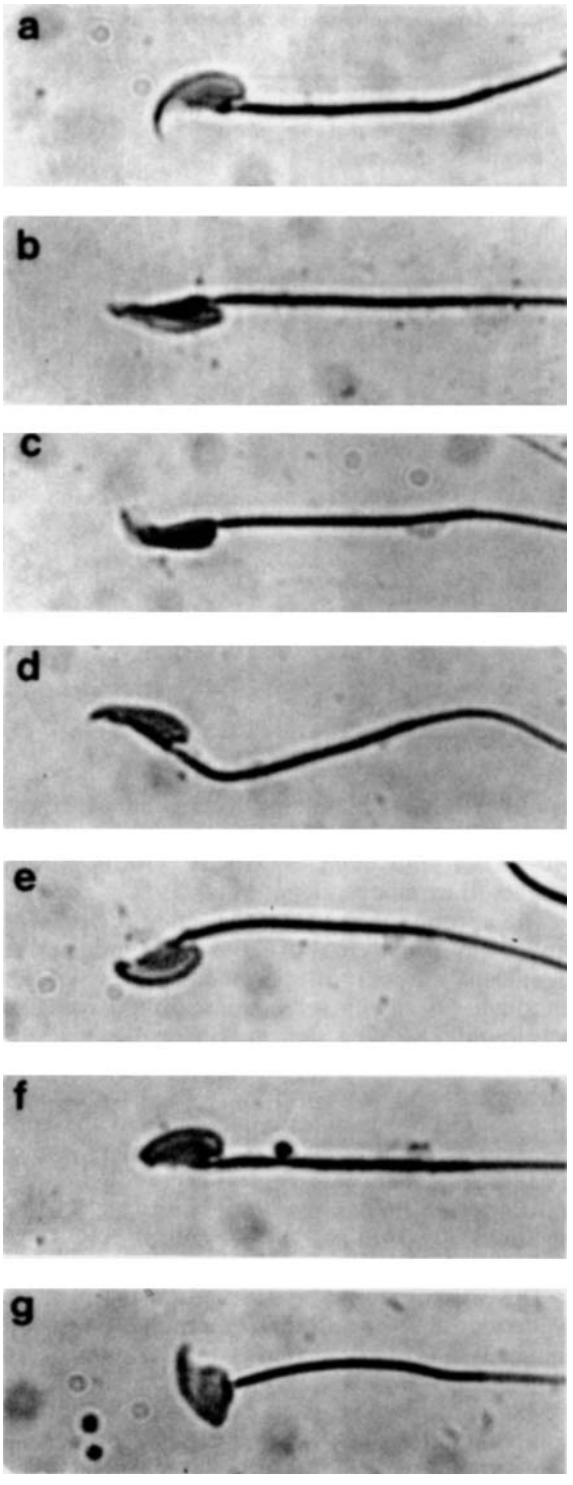

Figure 2 Mouse spermhead morphology: a, normal spermhead; b, c, d, e, f and g, types of abnormal spermhead (magnification: ×1000).

D5 > D2 > D4 > D6 > D1 > D3. The ranking, however, does not correlate with that obtained from the micronucleus assay, indicating that the mode of action of the six triorganotin compounds on the germinal cells differs from that on the somatic cells.

CONCLUSION

The present study demonstrates that when intraperitoneally administered, all the six triorganotin compounds are capable of inducing chromosomal disorders in somatic cells of ICR mice at doses corresponding to half their respective estimated LD_{50} values. One of the compounds, Ph_3SnOH (**D1**), a well-established crop protectant, is capable of inducing such disorders at 1/20 of its estimated LD_{50} value. Five compounds (**D1**, **D2**, **D4**, **D5**, and **D6**) were capable of inducing chromosomal disorders in germinal cells of ICR mice at doses that were as low as 1/64 of the respective estimated LD_{50}, again when administered intraperitoneally. The compound (p-ClC_6H_4)Ph_2SnOH (**D3**) was found to be the least potent, causing germ-cell disorders at only 1/8 of its estimated LD_{50} value. This compound has recently been claimed to be less phytotoxic than Ph_3SnOH (**D1**), besides being also superior in its fungitoxic action.[40] Since, in general, all the compounds tested intraperitoneally were found to be capable of causing *in vivo* genotoxic damage in both somatic and germinal cells of the ICR mice, due consideration must be given to their potential genetic hazard when contemplating applications that are based on their useful biocidal effects.

Acknowledgements The authors thank Dr G Baskaran of the Animal Facilities Centre, University of Malaya, for supplying the ICR mice, Prof Yong Hoi Sen for helpful discussions, and Mr Teh Kok Leng for technical assistance. Financial support from the National Science Council for R&D (Grant No 2-07-04-06) is gratefully acknowledged.

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