Pure Appl. Chem., Vol. 75, Nos. 11–12, pp. 2491–2495, 2003.

Workshop 4.3

Application of rat medium-term bioassays for detecting carcinogenic and modifying potentials of endocrine active substances*

Katsumi Imaida[1,‡], Seiko Tamano[2], Akihiro Hagiwara[2], Shoji Fukushima[3], Tomoyuki Shirai[4], and Nobuyuki Ito[5]

[1]Faculty of Medicine, Kagawa Medical University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan; [2]Daiyu-kai Institute of Medical Sciences, 64 Gura, Nishiazai, Azai-cho, Ichinomiya 491-0113, Japan; [3]Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan; [4]Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, 467-8601, Japan; [5]Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, 467-8601, Japan

Abstract: Two in vivo bioassay methods, a rat medium-term liver bioassay and a rat multi-organ bioassay, can be used for detecting carcinogenic or modifying potentials of endocrine active substances (EASs) on endocrine disruption (ED). The first bioassay, the rat medium-term liver bioassay, is fundamentally based on the two-step hypothesis of liver carcinogenesis; initiation with diethylnitrosamine (DEN, 200 mg/kg b.w., ip) is followed by test chemical administrations during the second stage, in combination with 2/3 partial hepatectomy. It requires only eight weeks for animal experimental treatment and a further few weeks for quantitative analysis of immunohistochemically demonstrated gluthathione-*S*-transeferase placental form positive hepatic foci. A total of 313 chemicals/substances have already been analyzed, and the efficacy of the system for hepatocarcinogenesis has thereby been well established. This bioassay also provides information concerning dose responses and inhibitory potentials of test chemicals. Several possible EASs, most of them categorized as pesticides, have already been examined in this bioassay, and dose–response studies of nonylphenol, bisphenol A, and styrene have also been tested. Another bioassay, a medium-term, multi-organ bioassay system, using five different chemical carcinogens—DEN, MNU, BBN, DMH, and DHPN—has also been established for rapid detection of not only hepatocarcinogens, but also other organ-targeted carcinogens. These medium-term bioassays are particularly useful and reliable methods for detecting carcinogenic or modifying potentials of low doses of test chemicals, such as EASs, and these methods can be used for the effects of chemical mixtures of EASs.

INTRODUCTION

The risk of carcinogenic or modifying potentials of endocrine active substances (EASs) on endocrine disruption (ED) to humans is of great concern. It is urgently necessary to establish practical and reli-

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* **75**, 1617–2615 (2003).

‡Corresponding author

able bioassay methods for detecting carcinogenic and modifying potentials of EASs, which are effective at very low doses. Two in vivo bioassay methods, a rat medium-term liver bioassay and a rat multi-organ bioassay, can be used for detecting those risks from EASs.

RAT MEDIUM-TERM LIVER BIOASSAY FOR CARCINOGENS

Male 6-week-old F344 rats were initially given a single ip injection of diethylnitrosamine (DEN, 200 mg/kg body weight, b.w.) dissolved in saline to initiate hepatocarcinogenesis (Fig. 1). Two weeks later, animals received test chemicals and were subjected to two-thirds partial hepatectomy (PH) at week 3. The animals were sacrificed for quantitative analysis of glutathione-*S*-transferase placental form (GST-P) positive liver foci at week 8. Carcinogenic or modifying potentials of test chemicals are scored by comparing the numbers and areas per cm^2 of induced GST-P-positive foci in the livers of test chemical-treated groups with those of corresponding control groups given DEN alone. A positive response is defined as a single increase in the quantitative values of GST-P-positive foci, a negative response is defined as no change or a decrease. The results obtained have been compared with reported *Salmonella*/microsome and long-term carcinogenicity test findings for the same compounds.

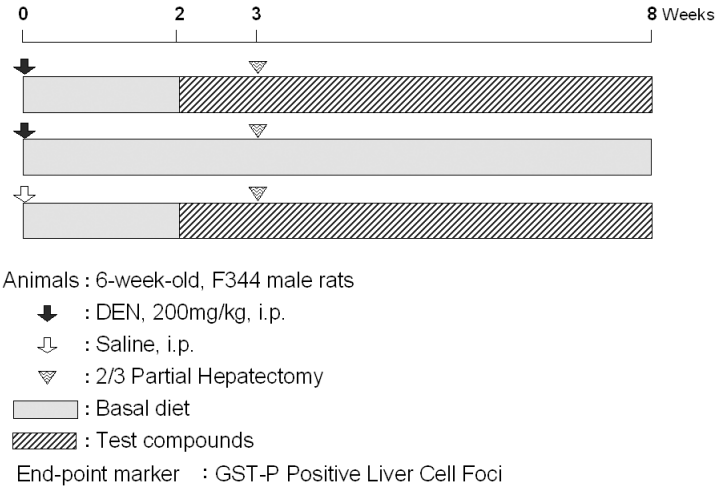

Fig. 1 Rat medium-term liver bioassay for carcinogens.

Results of 313 chemicals in the medium-term liver bioassay

Of a total of 313 chemicals examined, 60 out of 65 known hepatocarcinogens (92 %) gave positive results (Table 1). Five hepatocarcinogens that proved negative all belonged to the peroxizome proliferator group that depresses GST-P expression. Carcinogens targeting organs other than liver gave fewer positive results (10 out of 43, 23 %). One of the 48 chemicals reported as noncarcinogenic was found to be positive in this assay, but this might suggest that the chemical is a liver tumor promoter rather than the finding being a false-positive.

Table 1 Positive rates for 313 compounds of different categories (%).

Test compound	Ames test			Total
	+	–	Unknown	
Hepatocarcinogen	30/31 (97)	29/33 (88)	1/1 (1000)	60/65[a] (92)
Nonhepatocarcinogen	7/26 (27)	2/15 (13)	1/2 (50)	10/43 (23)
Noncarcinogen	0/6 (0)	1/40 (3)	0/2 (0)	1/48[b] (2)
Unknown	4/14 (29)	30/86 (36)	14/57 (24)	48/157 (31)
Total	41/77 (53)	62/174 (35)	16/62 (25)	119/313 (38)

[a]Negative; 5 peroxisomal proliferators, such as clofibrate, and DEHP, etc.
[b]Positive; malathione.

Results of possible EASs, which have been tested in the rat medium-term bioassay

Table 2 showed the results of possible EAS compounds, which have already been examined in the bioassay. Most of all chemicals are categorized as pesticides, but some other chemicals, such as hormone-related medicines, have also been examined. Eight chemicals have been showed positive, and two chemicals showed negative in this bioassay.

Table 2 Results of possible EASs analyzed in rat medium-term liver bioassay.

EAS	Dose (ppm)	Route	GST-P positive foci				Results
			Number	(cm^2)	Area (mm^2/cm^2)		
Alachlor	2000	D	10.49***	(6.98)	1.33**	(0.69)	↑
Aldrin	50	D	8.46***	(4.35)	0.83***	(0.33)	↑
Atrazine	500	D	7.72	(6.98)	0.79	(0.67)	–
Benomyl	5000	D	6.86	(6.46)	0.46	(0.44)	–
Chlordane	500	D	3.59	(3.25)	0.49**	(0.25)	↑
DDT	10	D	12.17***	(7.09)	0.88**	(0.52)	↑
Dieldrin	100	D	14.41***	(9.09)	1.41***	(0.77)	↑
Permethrin	4000	D	11.51**	(8.86)	1.03	(0.80)	↑
Trifluralin	5000	D	14.36***	(6.82)	1.26*	(0.52)	↑
Vinclozolin	2000	D	16.08***	(8.32)	1.48***	(0.72)	↑

*$P < 0.05$
**$P < 0.01$
*** $P < 0.001$
( ): Respective control values

Dose–response study of nonylphenol in the medium-term liver bioassay

Dose–response studies of nonylphenol have been completed in this medium-term liver bioassay. Male F344 rats were given a single ip injection of DEN at a dose of 200 mg/kg b.w., and starting two weeks later, received nonylphenol at doses of 2000, 250, and 25 ppm in the basal diet. Quantitative data (numbers and areas) for GST-P-positive liver foci in each treated group and the control group were 4.7, 4.2, 6.1, and 6.1/cm^2; and 0.31, 0.30, 0.41, and 0.44 mm^2/cm^2, respectively. These results indicate that nonylphenol did not show clear dose response on induction of GST-P-positive foci, preneoplastic liver lesions, at the doses examined.

Dose–response study of bisphenol A and styrene in the medium-term liver bioassay

Male F344 rats were given DEN, ip, and then animals received bisphenol A at doses of 40 and 160 mg/kg b.w., ig, or styrene at doses of 250 and 1000 mg/kg b.w., ig, six times per week, for six weeks. Quantitative values of GST-P-positive liver foci in bisphenol A created groups and in the control group were 4.9, 5.2, and 6.0/cm^2 and 0.39, 0.42, and 0.42 mm^2/cm^2, respectively. Those values are not statistically different between groups. The GST-P values in styrene-treated and the control groups were 3.3, 1.5, and 6.0/cm^2 and 0.37, 0.10, and 0.42 mm^2/cm^2, respectively, and these values are statistically different between groups. Therefore, the results indicate that styrene showed dose–response inhibitory effects on induction of GST-P-positive liver foci at doses examined.

Since styrene showed inhibitory effects in the previous study, further low-dose studies were conducted in the same bioassay. After injection of DEN, animals received styrene at doses of 0.0006, 0.006, and 0.6 mg/kg b.w., ig, six times per week, for six weeks. The lowest dose of styrene examined was the almost same level found in our environment. The GST-P values in styrene-treated and the control groups were 8.3, 8.7, 9.0, and 8.4/cm^2 and 0.80, 0.67, 0.84, and 0.74 mm^2/cm^2, respectively. These values were not statistically different, and indicate that at the levels found in our environment, styrene did not show any modifying effect on the induction of GST-P liver foci.

RAT MEDIUM-TERM MULTIORGAN BIOASSAY

Male 6-week-old F344 rats were treated sequentially with five carcinogens (DEN, 100 mg/kg b.w. in saline, ip, single dose at the commencement; *N*-methyl-*N*-nitrosourea, 20 mg/kg b.w. in citrated-buffered solution, ip, four doses on days 2, 5, 8, 11; dihydroxy-di-*N*-propylnitrosamine, 0.1 % in drinking water during weeks 3 and 4, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, 0.05 % in drinking water during weeks 1 and 2; 1,2-dimethylhydrazine, 40 mg/kg b.w. in saline, sc, four doses on days 14, 17, 20, 23 (DMBDD treatment) (Fig. 2). After those treatment, the animals were given test substances for 24 weeks from week 5. All animals were sacrificed and subjected to complete necropsy, and all organs/tissues were histopathologically and immunohistochemically examined.

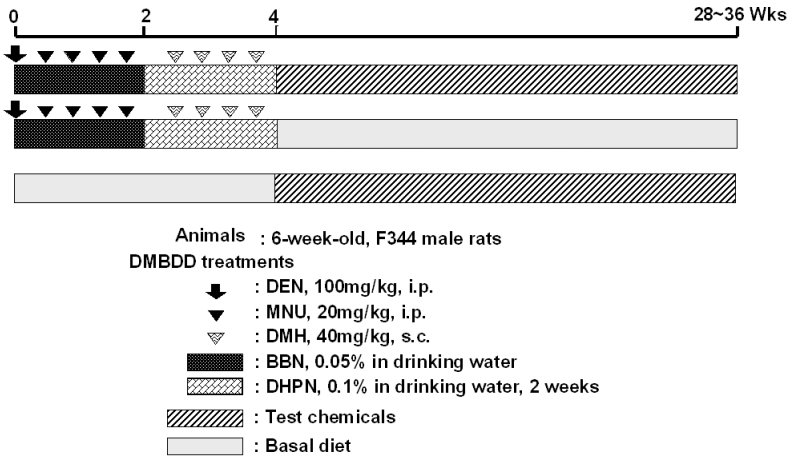

Fig. 2 Rat multi-organ bioassay (DMBDD methods).

Results of 63 chemicals in the medium-term multiorgan bioassay

Sixty-three chemicals have been tested in this bioassay (Table 3). All 17 hepatocarcinogens (100 %) and 19/22 (86 %) of the nonhepatocarcinogens were positive in the bioassay. Five noncarcinogens were neg-

ative. For chemicals with unknown carcinogenicity, the positive rate was 9/19 (47 %). This bioassay is useful for analysis of carcinogenic or modifying potential of test chemicals when their target organs are other than liver. This bioassay system can also be useful for dose–response studies, including at very low doses, and can be used for analyzing risk of carcinogenic potentials of EDCs at low doses.

Table 3 Results of 63 test compounds in a rat multiorgan bioassay for carcinogens.

Category of chemicals	Ames' test (%)			Total
	Positive	Negative	Unknown	
Hepatocarcinogen	12/12 (100)	5/5 (100)	0/0 (0)	17/17 (100)
Nonhepatocarcinogen	10/11 (91)	8/10 (80)	1/1 (100)	19/22 (86)
Noncarcinogen	0/1 (0)	0/4 (0)	0/0 (0)	0/5 (0)
Unknown	0/1 (0)	6/11 (55)	3/7 (43)	9/19 (47)
Total	22/25 (88)	19/30 (63)	4/8 (50)	45/63 (71)

CONCLUSIONS

These medium-term liver and multiorgan bioassay systems are very useful tools for detection of not only genotoxic but also nongenotoxic carcinogens. Positive results obtained in a relatively short period closely correlate with the long-term carcinogenicity test. The bioassays are particularly useful and reliable methods for detecting carcinogenic or modifying potentials of low doses of test chemicals, such as EASs, and these methods can also be used for the effects of chemical mixtures of EASs.

REFERENCES

1. N. Ito, K. Imaida, M. Asamoto, T. Shirai. *Mutat. Res.* **462**, 209–217 (2000).
2. M. A. Moore, H. Tsuda, S. Tamano, A. Hagiwara, K. Imaida, T. Shirai, N. Ito. *Toxicol. Pathol.* **27**, 237–242 (1999).
3. T. Shirai, M. Hirose, N. Ito. *IARC Sci. Publ.* **146**, 251–272 (1999).
4. N. Ito, R. Hasegawa, K. Imaida, M. Hirose, T. Shirai. *Exp. Toxicol. Pathol.* **48**, 113–119 1996 (1996).
5. N. Ito, R. Hasegawa, K. Imaida, M. Hirose, M. Asamoto, T. Shirai. *Crit. Rev. Oncol. Hematol.* **21**, 105–133 (1995).
6. N. Ito, R. Hasegawa, K. Imaida, S. Takahashi, T. Shirai. *Drug Metab. Rev.* **26**, 431–442 (1994).
7. R. Hasegawa and N. Ito. *Food Chem. Toxicol.* **30**, 979–992 (1992).
8. N. Ito, T. Shirai, R. Hasegawa. *IARC Sci. Publ.* **116**, 353–388 (1992).
9. N. Ito, K. Imaida, R. Hasegawa, H. Tsuda. *Crit. Rev. Toxicol.* 19, 385–415 (1989).
10. N. Ito, H. Tsuda, M. Tatematsu, T. Inoue, Y. Tagawa, T. Aoki, S. Uwagawa, M. Kagawa, T. Ogiso, T. Masui, et al. *Carcinogenesis* **9**, 387–394 (1988).