The Mouse rasH2/BHT Model as an *in vivo* Rapid Assay for Lung Carcinogens

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We have demonstrated the utility of a 9-week *in vivo* two-stage assay for lung cancer initiating agents, using transgenic mice carrying the human prototype c-Ha-*ras* gene (rasH2 mice) and butyl-hydroxytoluene (BHT) as a potent lung promoter (rasH2/BHT model). In the present study, to ascertain appropriate conditions for BHT administration in this model, the effects of exposure on proliferation of alveolar type II cells in male rasH2 mice were examined. Additionally, use of BHT was validated for promotion of urethane (UR) carcinogenesis in male and female rasH2 mice. In a time-course study of a single intragastric administration of BHT at a dose of 400 mg/kg, increased bromodeoxyuridine-labeling index (LI) reached a maximum 3 days after treatment and was still observed after 7 days. In a dose-response study, effects were dose-dependent, the dose of 400 mg/kg causing eight-fold elevation as compared to the control. With repeated administration, whereas the LI was increased dramatically at first, effects gradually diminished with further exposure, and finally six BHT treatments failed to induce cell proliferation. In a two-stage model using UR as the initiator, although up to five consecutive doses of BHT were able to exert continued enhancing effects in terms of adenoma yield, no increment was evident with further treatments. The data overall indicate that a rasH2/BHT model with five weekly administrations of BHT at a dose of 400 mg/kg is most efficacious.

Key words: rasH2 mice — Butylhydroxytoluene — *in vivo* assay — Lung

For the purpose of detecting environmental carcinogens, 2-year long-term *in vivo* carcinogenicity tests using rodent species have generally been conducted. However, these tests require large numbers of animals, their long-term maintenance and large-scale facilities, leading to inefficiency in the search for potential carcinogens in the environment. While *in vitro* short-term screening assays such as the Ames test and chromosomal aberration test are powerful tools to detect mutagens and consequently are helpful to predict the carcinogenicity of compounds,[1, 2] interest has also been concentrated on transgenic mouse models, introduced in the expectation of detecting *in vivo* gene mutations.[3–5] Together with the lack of information on target sites with *in vitro* assays, the accumulation of data suggesting a lack of congruity between mutagenicity and carcinogenicity[6] indicates the importance of developing rapid *in vivo* assay systems with tumors themselves as endpoints. The Ministry of Health, Labour and Welfare, Japan, concerned with this issue, has set up a research project entitled "Studies on establishment of new methods for evaluation of carcinogenicity studies using animals and its implications."

In our previous papers, we have already demonstrated the utility of rapid *in vivo* assays for genotoxic carcinogens, developed using the rasH2 mouse, a transgenic strain

that expresses the human prototype c-Ha-*ras* gene.[7] In particular, we have paid attention to the extremely high susceptibility of rasH2 mice to lung carcinogens and the potential application of a potent lung promoter, butylhydroxytoluene (BHT) in the rasH2 mouse (rasH2/BHT model). In fact, carcinogenicity of genotoxic carcinogens targeting the murine lung, such as urethane (UR), diethylnitrosamine (DEN) and 4-nitroquinoline 1-oxide (4NQO), can be detected within a 9-week experimental period in our rasH2/BHT model.[8] To detect environmental chemicals acting as initiating agents in the murine lung is important for evaluation of possible harm to human lung, and thus the rasH2/BHT model is a promising tool for risk assessment.

It is considered that the tumor-promoting action of BHT depends on metabolite-induced cytotoxicity to alveolar type I cells and the consequent increase of cell proliferation by their alveolar type II counterparts.[9] Cell kinetics of alveolar cells in several strains of mouse following BHT exposure have been reported,[10, 11] and strain differences exist regarding susceptibility to BHT-induced effects.[12–14] Interestingly, repeated administration of BHT diminishes its potential to induce cell proliferation, though this may also depend on the mouse strain.[15]

The aim of the present study was to establish a standard protocol for the rasH2/BHT model, especially with regard to appropriate BHT administration. We therefore investi-

gated time- and dose-dependence, and repeated-dose effects of BHT on cell proliferation in alveolar type II cells of male rasH2 mice, with reference to bromodeoxyuridine (BrdU) incorporation. In addition, effective repeated BHT exposure to male and female rasH2 mice was explored in terms of modifying effects on UR carcinogenesis.

MATERIALS AND METHODS

Chemicals BrdU, UR and corn oil were obtained from Sigma Chemical (St. Louis, MO) and BHT from Wako Pure Chemical Co., Ltd. (Osaka). The former two were dissolved in saline and the last in corn oil, all at a concentration of 10% (w/v).

Animals Mice of the rasH2 strain, originally developed by Saito *et al.*[16] by insertion of the human prototype c-Ha-*ras* gene into the DNA of wild CB6F1 mice, were bred at the Central Institute for Experimental Animals (Kanagawa). In the present study, 7-week-old rasH2 mice were housed for 1 week under controlled conditions in polycarbonate cages (4–5 mice per cage) in a conventional animal facility, and given free access to CRF1 basal diet (Charles River Japan, Kanagawa) and tap water. The Animal Care and Utilization Committee for the National Institute of Health Sciences, Japan, approved the protocols for these studies.

Experimental design

Experiment 1: We examined the time-course of change in BrdU-labeling index (LI) of alveolar type II cells following a single exposure of BHT to male rasH2 mice. Groups of 5 mice were given a single intragastric administration at a dose of 400 mg/kg, this being selected based on preliminary studies as the highest with which no fatalities occurred up to 7 days after compound administration, in spite of the obvious decrease in body weight (data not shown). Animals were killed by exsanguination under ether anesthesia at 1, 3, 5 and 7 days after the treatment. Control animals were similarly administered corn oil at the same dose and killed after 7 days in the same manner. All animals were intraperitoneally injected with BrdU (100 mg/kg) once on the day of termination, 2 h before killing.

Experiment 2: We examined the dose-response with regard to the LI of alveolar type II cells following a single exposure of BHT at doses of 0, 100, 200 and 400 mg/kg to groups of 5 male rasH2 mice. They were killed 3 days after the treatment and then the same procedures were performed as for the time-course study.

Experiment 3: We examined the effects of repeated doses of BHT on the LI of alveolar type II cells in male rasH2 mice. Groups of 4 mice received 400 mg/kg of BHT by gavage, with one to six consecutive administrations. The interval between each administration was set to be 1 week. Controls were treated with corn oil alone and were

included for each treatment group. They were killed 3 days after each final treatment with BHT and the same procedures were performed as in the above two studies.

Experiment 4: We examined the modifying effects of repeated doses of BHT on UR lung carcinogenesis in rasH2 mice. A total of forty male and female rasH2 mice were intraperitoneally injected with UR at a dose of 250 mg/kg and then were randomly divided into 4 groups (groups 2–5), as shown in Fig. 1. Animals in groups 3–5 were given weekly administration of BHT intragastrically at a dose of 400 mg/kg from 1 week after UR treatment for 3, 5 and 8 weeks, respectively. Animals in group 2 were given corn oil instead of BHT with the same procedure as in group 5. Additionally, ten male and female rasH2 mice untreated with UR were given BHT with the same procedure as in group 5 (group 1). All animals were killed at 9 weeks after UR treatment, as in the previous studies. At necropsy in all cases, lungs were fixed by intratracheal instillation of 10% neutral buffered formalin, and routinely processed for embedding in paraffin. All lung lobes were horizontally sectioned at 4–5 μm thickness to ensure that maximum areas of each could be examined and five sections of different lung lobes were stained with BrdU in the first three studies and with hematoxylin and eosin (H&E) in the last.

Immunohistochemical procedures For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson, Mountain View, CA) (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4 N HCl for 30 min at room temperature. The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.
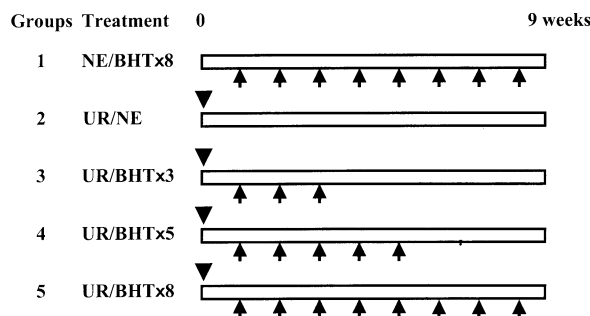

Fig. 1. Experimental design to detect modifying effects of weekly repeated injections of BHT on UR-induced pulmonary carcinogenesis in male and female rasH2 mice. NE, no exposure; ▼, UR 250 mg/kg, i.p.; ▲, BHT 400 mg/kg, i.g.

Quantification of cell proliferation in alveolar type II cells Type II cells were microscopically identified on the basis of their location, mostly in the corners of alveoli, their cuboidal shape, protrusion into alveoli, and their uniformly vacuolated cytoplasm.[17] Numbers of labeled type II cells in thirty randomly-selected square views (surrounded by a 25 mm²-micrometer attached to the eyepiece) of the alveolar region of each animal were counted.

Pathological assessment Pulmonary neoplastic lesions distinguishable from hyperplastic alveolar lesions maintaining the normal wall structure despite their epithelial cells being enlarged were classified into alveolar/bronchiolar adenomas as previously described.[18, 19] The areas of the examined sections were measured by means of an image analyzer with a microscope (IPAP, Sumika Technos, Osaka) in order to allow average numbers of neoplasias/cm² to be calculated from the data.

Statistical analysis The significance of differences in the results for LI and tumor multiplicity were evaluated with ANOVA, followed by Student's or Welch's *t* tests (experiments 1 and 3) or Dunnett's multiple comparison or Dunnett's type mean rank tests (experiments 2 and 4). Fischer's exact probability test was used to determine the significance of differences in tumor incidence (experiment 4).

RESULTS

Time-course of change in cell kinetics following a single BHT administration Data for LIs of alveolar type II cells of male rasH2 mice after a single intragastric treatment with BHT at a dose of 400 mg/kg are detailed in Fig. 2. A gradual rise occurred, and reached a peak 3 days after treatment. The LI after 7 days was still significantly higher than in the controls (38.2 ± 17.2 vs. 15.2 ± 5.0, $P < 0.05$).

Dose-response with regard to cell kinetics following a single BHT administration Changes in LIs of alveolar type II cells of male rasH2 mice exposed to BHT by single intragastric administration at various doses are shown in Fig. 3. The LIs were elevated in a dose-dependent manner, with significant increases at 400 (90.4 ± 20.6, $P < 0.01$) and 200 (72.6 ± 8.3, $P < 0.01$) mg/kg, as compared with the control value (11.0 ± 1.8).

Cell kinetics following repeated administrations of BHT As shown in Fig. 4, the first exposure of male rasH2 mice to BHT (400 mg/kg) resulted in dramatic increase of the LI in alveolar type II cells (76.6 ± 6.6 vs. 12.0 ± 1.7 (control), $P < 0.01$). The second and third exposures were also able to induce remarkable elevation of the LI, while with the fourth and fifth exposures only slight increments were evident, although they were still statistically significant (20.0 ± 5.1 vs. 10.0 ± 2.5, $P < 0.05$, 18.4 ± 3.8 vs. 10.2 ± 1.6, $P < 0.01$, respectively). The sixth
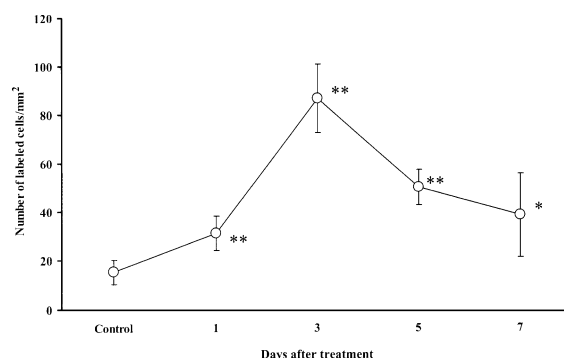

Fig. 2. Time-dependent changes of LI in alveolar type II cells of male rasH2 mice given BHT at a dose of 400 mg/kg by single i.g. administration. Values represent the mean±SD of data for five mice. Control data were obtained from animals killed 7 days after the administration of corn oil alone. ∗, ∗∗ $P < 0.05$, 0.01 vs. control.
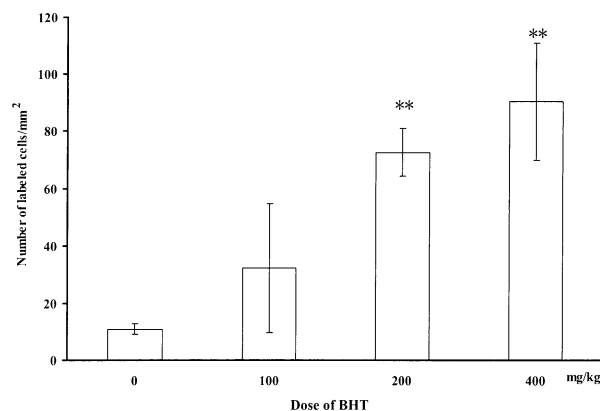

Fig. 3. Dose-dependent changes of LI in alveolar type II cells of male rasH2 mice 3 days after single i.g. administration of BHT at doses of 0, 100, 200 and 400 mg/kg. Values represent the mean±SD of data for five mice. ∗∗ $P < 0.01$ vs. 0 mg/kg.

exposure no longer augmented the numbers of S-phase alveolar type II cells.

Effects of repeated BHT injections on UR-initiated lung tumorigenesis Table I summarizes the incidences and multiplicities of neoplastic lesions in the lungs of male and female rasH2 mice at 9 weeks after single intraperitoneal injection of UR at a dose of 250 mg/kg followed by three, five or eight administrations of BHT at a dose of 400 mg/kg at 1-week intervals. No statistically significant increment in incidences or multiplicities of lung tumors was evident in male and female rasH2 mice given BHT three times after UR initiation, except for the incidence in males. In contrast, a statistically significant increase was
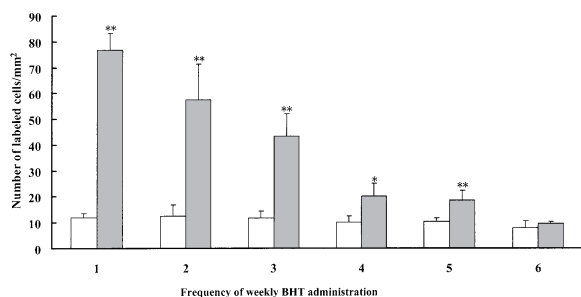
Fig. 4. The effects of weekly repeated injections of BHT at a dose of 400 mg/kg by gavage on LI in alveolar type II cells of male rasH2 mice. Animals were killed 3 days after each final treatment with BHT. Values represent the mean±SD of data for four mice. □, vehicle control; ▣, BHT treatment. *, ** $P<0.05$, 0.01 vs. the relevant control.

noted for both parameters in rasH2 mice of both genders given BHT five times. However, the incidences and multiplicities in male and female rasH2 mice given BHT eight times were comparable to those in the mice given five doses.

DISCUSSION

The results of the present study confirmed the utility of BHT application for promoting lesion development in the rasH2 mouse lung model for risk assessment of environmental compounds. BHT induces compensatory proliferation and differentiation of alveolar type II cells following destruction of alveolar type I cells.[9] Recent reports demonstrated inhibition of gap junctional intercellular communication[20] and apoptosis[21] in lung epithelial cells. It seems likely that metabolites of BHT generated by hydroxylation of the *tert*-butyl group by P450 isozymes are necessary to trigger the chain of events leading to enhance-

ment of lung tumor development.[22] Therefore, the appearance of biological effects may be dependent on the ability to metabolize BHT, which implies the existence of strain differences in susceptibility. In fact, it has been reported that there is major variation in the profiles of pulmonary DNA synthesis after BHT exposure among C57BL, BALB/c, ICR and SSIn mice.[10] The present results of time-course and dose-response studies suggest that weekly intragastric treatment with BHT at a dose of 400 mg/kg is sufficient to maintain increased proliferation of alveolar type II cells of rasH2 mice for at least 5 weeks. The previous reports demonstrated that three weekly administrations of BHT were unable to induce cell proliferation in alveolar type II cells of A/J mice, though single and double treatments were efficacious.[15] Clearly the proliferative effects due to repeated BHT exposure are more prolonged in rasH2 mice than in A/J mice. Although the reason for the strain difference is uncertain, destruction of Clara cells, in which BHT is mainly metabolized to the active form is presumably of importance.[23]

Regarding strain differences in sensitivity to BHT and the diminution of the response with repeated BHT exposures, it has been reported that six weekly administrations
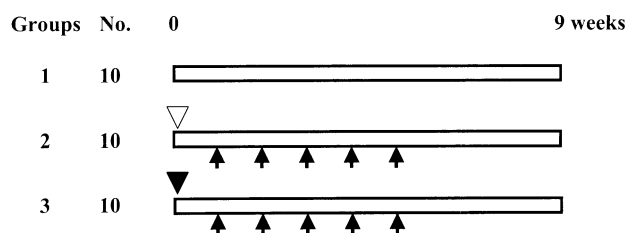

Fig. 5. Nine-week *in vivo* assay for lung carcinogens: rasH2/BHT model. ▽, vehicle; ▼, test compound; ▲, BHT 400 mg/kg, i.g. Animals: male and female rasH2 mice, 8 weeks old.

Table I. The Modifying Effects of Repeated Doses of BHT on UR-induced Pulmonary Carcinogenesis in Male and Female rasH2 Mice

Groups	Treatments	Male			Female		
		No. of adenomas	Incidence	Average number (no./cm²)	No. of adenomas	Incidence	Average number (no./cm²)
1	NE/BHT×8	0	0/10	0	0	0/10	0
2	UR/NE	1	1/10	0.05±0.16	2	2/10	0.12±0.26
3	UR/BHT×3	8	6/10[a]	0.47±0.67	6	6/10	0.22±0.29
4	UR/BHT×5	11	6/10[a]	0.65±0.67[a]	19	8/10[b]	1.06±1.03[b, c]
5	UR/BHT×8	10	6/10[a]	0.57±0.61[a]	17	9/10[b]	0.97±0.57[b]

a) $P<0.05$ vs. control (UR/NE).
b) $P<0.01$ vs. control (UR/NE).
c) $P<0.05$ vs. UR/BHT×3.
UR, urethane; NE, no exposure.

of BHT with BALB/c mice following UR elevated the multiplicity of lung tumors, whereas the number of tumors in CB6F1 mice (BALB/c×C57BL) F1 hybrids, the background strain of rasH2 mice, was unaffected.[24] In the present study with rasH2 mice both five and eight administrations of BHT exerted enhancing effects on UR carcinogenesis, but there were no differences in incidences and multiplicities between the two cases, suggesting that more than five exposures does not bring further benefit. The results for cell kinetics also provide support for the conclusion.

The data overall allow us to propose a standard protocol for the rasH2/BHT model, i.e., rasH2 mice of both genders are treated with test chemical and, starting 1 week later, are given BHT weekly five times at a dose of 400 mg/kg by gavage, and then killed at week 9 (Fig. 5). Further studies using various murine lung carcinogens appear warranted to confirm the validity of the rasH2/BHT model.

REFERENCES

1) Ames, B. N., McCann, J. and Yamasaki, E. Methods for detecting carcinogens and mutagens with *Salmonella*/mammalian microsome mutagenicity test. *Mutat. Res.*, **32**, 347–364 (1975).

2) Ishidate, M., Sofuni, T. and Yoshikawa, K. Chromosomal aberration test *in vitro* as a primary screening tool for environmental mutagens and/or carcinogens. *Gann Monogr. Cancer Res.*, **27**, 95–108 (1981).

3) Gossen, J. A., de Leeuw, W. J., Tan, C. H., Zwarthoff, E. C., Berends, F., Lohman, P. H., Knook, D. L. and Vijg, J. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. *Proc. Natl. Acad. Sci. USA*, **86**, 7971–7975 (1989).

4) Kohler, S. W., Provost, G. S., Fieck, A., Kretz, P. L., Bullock, W. O., Sorge, J. A., Putman, D. L. and Short, J. M. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. *Proc. Natl. Acad. Sci. USA*, **88**, 7958–7962 (1991).

5) Nohmi, T., Katoh, M., Suzuki, H., Matsui, M., Yamada, M., Watanabe, M., Suzuki, M., Horiya, N., Ueda, O., Shibuya, T., Ikeda, H. and Sofuni, T. A new transgenic mouse mutagenesis test system using Spi⁻ and 6-thioguanine selections. *Environ. Mol. Mutagen.*, **28**, 465–470 (1996).

6) Zeiger, E. Mutagens that are not carcinogens: faulty theory or faulty tests? *Mutat. Res.*, **492**, 29–38 (2001).

7) Umemura, T., Kodama, Y., Hioki, K., Inoue, T., Nomura, T. and Kurokawa, Y. Susceptibility to urethane carcinogenesis of transgenic mice carrying a human prototype c-Ha-ras gene (rasH2 mice) and its modification by butylhydroxytoluene. *Cancer Lett.*, **145**, 101–106 (1999).

8) Umemura, T., Kodama, Y., Hioki, K., Inoue, T., Nomura, T. and Kurokawa, Y. Butylhydroxytoluene (BHT) increases susceptibility of transgenic rasH2 mice to lung carcinogenesis. *J. Cancer Res. Clin. Oncol.*, **127**, 583–590 (2001).

9) Witschi, H. P. Separation of early diffuse alveolar cell proliferation from enhanced tumor development in mouse lung. *Cancer Res.*, **46**, 2675–2679 (1986).

10) Kehrer, J. P. and DiGiovanni, J. Comparison of lung injury induced in 4 strains of mice by butylated hydroxytoluene. *Toxicol. Lett.*, **52**, 55–61 (1990).

11) Witschi, H. P. and Lock, S. Toxicity of butylated hydroxytoluene in mouse following oral administration. *Toxicology*, **9**, 137–146 (1978).

12) Gressani, K. M., Leone-Kabler, S., O'Sullivan, M. G., Case, L. D., Malkinson, A. M. and Miller, M. S. Strain-dependent lung tumor formation in mice transplacentally exposed to 3-methylcholanthrene and post-natally exposed to butylated hydroxytoluene. *Carcinogenesis*, **20**, 2159–2165 (1999).

13) Malkinson, A. M. and Thaete, L. G. Effects of strain and age on prophylaxis and co-carcinogenesis of urethane-induced mouse lung adenomas by butylated hydroxytoluene. *Cancer Res.*, **46**, 1694–1697 (1986).

14) Malkinson, A. M., Koski, K. M., Evans, W. A. and Festing, M. F. W. Butylated hydroxytoluene exposure is necessary to induce lung tumors in BALB mice treated with 3-methylcholanthrene. *Cancer Res.*, **57**, 2832–2834 (1997).

15) Witschi, H. P. and Morse, C. C. Cell kinetics in mouse lung following administration of carcinogens and butylated hydroxytoluene. *Toxicol. Appl. Pharmacol.*, **78**, 464–472 (1985).

16) Saito, A., Kimura, M., Takahashi, R., Yokoyama, M., Nomura, T., Izawa, M., Sekiya, T., Nishimiura, S. and Katsuki, M. Most tumors in transgenic mice with human c-Ha-ras gene contain somatically activated transgenes. *Oncogene*, **5**, 1195–1200 (1990).

17) Witschi, H. P. and Morse, C. C. Enhancement of lung tumor formation in mice by dietary butylated hydroxytoluene: dose-time relationship and cell kinetics. *J. Natl. Cancer Inst.*, **71**, 859–866 (1983).

18) Mitsumori, K., Koizumi, H., Nomura, T. and Yamamoto, S. Pathological features of spontaneous and induced tumors in transgenic mice carrying a human prototype c-Ha-ras gene used for six-month carcinogenicity studies. *Toxicol.*

Pathol., **26**, 520–531 (1998).

19) Gunning, W. T., Castonguay, A., Goldblatt, P. J. and Stoner, G. D. Strain A/J mouse lung adenoma growth patterns vary when induced by different carcinogens. *Toxicol. Pathol.*, **19**, 168–174 (1991).

20) Dwyer-Nield, L. D., Thompson, J. A., Peljak, G., Squire, M. K. T., Barker, T. D., Parkinson, A., Cohen, J. J., Dinsdale, D. and Malkinson, A. M. Selective induction of apoptosis in mouse and human lung epithelial cell lines by *tert*-butyl hydroxylated metabolite of butylated hydroxytoluene: a proposed role in tumor promotion. *Toxicology*, **130**, 115–127 (1998).

21) Guan, X., Hardenbrook, J., Fernstrom, M. J., Chaudhuri, R., Malkinson, A. M. and Ruch, R. J. Down-regulation by butylated hydroxytoluene of the number and function of gap junctions in epithelial cell lines derived from mouse lung and rat liver. *Carcinogenesis*, **16**, 2575–2585 (1995).

22) Thompson, J. A., Schullek, K. M., Fernandez, C. A. and Malkinson, A. M. A metabolite of butylated hydroxytoluene with potent tumor-promoting activity in mouse lung. *Carcinogenesis*, **10**, 773–775 (1989).

23) Bolton, J. L., Thompson, J. A., Allentoff, A. J., Miley, F. B. and Malkinson, A. M. Metabolic activation of butylated hydroxytoluene by mouse bronchiolar Clara cells. *Toxicol. Appl. Pharmacol.*, **123**, 43–49 (1993).

24) Miller, A. C. K., Dwyer, L. D., Auerbach, C. E., Miley, F. B., Dinsdale, D. and Malkinson, A. M. Strain-related differences in the pneumotoxic effects of chronically administered butylated hydroxytoluene on protein kinase C and calpain. *Toxicology*, **90**, 141–159 (1994).