[36] B. van Loon, E. Markkanen, U. Hubscher, Oxygen as a friend and enemy: how to combat the mutational potential of 8-oxo-guanine, DNA Repair (Amsterdam) 9 (2010) 604–616.
[37] X. Tan, A.P. Grollman, S. Shibutani, Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2′-deoxyadenosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine DNA lesions in mammalian cells, Carcinogenesis 20 (1999) 2287–2292.
[38] H. Kamiya, K. Miura, H. Ishikawa, H. Inoue, S. Nishimura, E. Ohtsuka, c-Ha-ras containing 8-hydroxyguanine at codon 12 induces point mutations at the modified and adjacent positions, Cancer Research 52 (1992) 3483–3485.
[39] T. Arai, V.P. Kelly, O. Minowa, T. Noda, S. Nishimura, High accumulation of oxidative DNA damage, 8-hydroxyguanine, in Mmh/Ogg1 deficient mice by chronic oxidative stress, Carcinogenesis 23 (2002) 2005–2010.
[40] J.P. McGoldrick, Y.C. Yeh, M. Solomon, J.M. Essigmann, A.L. Lu, Characterization of a mammalian homolog of the *Escherichia coli* MutY mismatch repair protein, Molecular and Cellular Biology 15 (1995) 989–996.
[41] T. Nguyen, D. Brunson, C.L. Crespi, B.W. Penman, J.S. Wishnok, S.R. Tannenbaum, DNA damage and mutation in human cells exposed to nitric oxide in vitro, Proceedings of the National Academy of Sciences of the United States of America 89 (1992) 3030–3034.
[42] J.P. Henderson, J. Byun, M.V. Williams, M.L. McCormick, W.C. Parks, L.A. Ridnour, J.W. Heinecke, Bromination of deoxycytidine by eosinophil peroxidase: a mechanism for mutagenesis by oxidative damage of nucleotide precursors, Proceedings of the National Academy of Sciences of the United States of America 98 (2001) 1631–1636.
[43] T. Asahi, H. Kondo, M. Masuda, H. Nishino, Y. Aratani, Y. Naito, T. Yoshikawa, S. Hisaka, Y. Kato, T. Osawa, Chemical and immunochemical detection of 8-halogenated deoxyguanosines at early stage inflammation, Journal of Biological Chemistry 285 (2010) 9282–9291.
[44] R. Eritja, D.M. Horowitz, P.A. Walker, J.P. Ziehler-Martin, M.S. Boosalis, M.F. Goodman, K. Itakura, B.E. Kaplan, Synthesis and properties of oligonucleotides containing 2′-deoxynebularine and 2′-deoxyxanthosine, Nucleic Acids Research 14 (1986) 8135–8153.
[45] H. Kamiya, T. Sakaguchi, N. Murata, M. Fujimuro, H. Miura, H. Ishikawa, M. Shimizu, H. Inoue, S. Nishimura, A. Matsukage, et al., In vitro replication study of modified bases in ras sequences, Chemical and Pharmaceutical Bulletin 40 (1992) 2792–2795.
[46] M. Yasui, N. Suzuki, H. Miller, T. Matsuda, S. Matsui, S. Shibutani, Translesion synthesis past 2′-deoxyxanthosine, a nitric oxide-derived DNA adduct, by mammalian DNA polymerases, Journal of Molecular Biology 344 (2004) 665–674.
[47] V.A. Bohr, C.A. Smith, D.S. Okumoto, P.C. Hanawalt, DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall, Cell 40 (1985) 359–369.
[48] B.A. Donahue, S. Yin, J.S. Taylor, D. Reines, P.C. Hanawalt, Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template, Proceedings of the National Academy of Sciences of the United States of America 91 (1994) 8502–8506.
[49] P.C. Hanawalt, G. Spivak, Transcription-coupled DNA repair: two decades of progress and surprises, Nature Reviews Molecular Cell Biology 9 (2008) 958–970.
[50] I. Kuraoka, M. Endou, Y. Yamaguchi, T. Wada, H. Handa, K. Tanaka, Effects of endogenous DNA base lesions on transcription elongation by mammalian RNA polymerase II. Implications for transcription-coupled DNA repair and transcriptional mutagenesis, Journal of Biological Chemistry 278 (2003) 7294–7299.
[51] N. Charlet-Berguerand, S. Feuerhahn, S.E. Kong, H. Ziserman, J.W. Conaway, R. Conaway, J.M. Egly, RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors, EMBO Journal 25 (2006) 5481–5491.
[52] S. Tornaletti, L.S. Maeda, R.D. Kolodner, P.C. Hanawalt, Effect of 8-oxoguanine on transcription elongation by T7 RNA polymerase and mammalian RNA polymerase II, DNA Repair (Amsterdam) 3 (2004) 483–494.
[53] E. Larsen, K. Kwon, F. Coin, J.M. Egly, A. Klungland, Transcription activities at 8-oxoG lesions in DNA, DNA Repair (Amsterdam) 3 (2004) 1457–1468.
[54] M. Pastoriza-Gallego, J. Armier, A. Sarasin, Transcription through 8-oxoguanine in DNA repair-proficient and Csb(−)/Ogg1(−) DNA repair-deficient mouse embryonic fibroblasts is dependent upon promoter strength and sequence context, Mutagenesis 22 (2007) 343–351.
[55] H. Kamiya, Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary, Nucleic Acids Research 31 (2003) 517–531.
[56] L.B. Bloom, X. Chen, D.K. Fygenson, J. Turner, M. O'Donnell, M.F. Goodman, Fidelity of *Escherichia coli* DNA polymerase III holoenzyme. The effects of beta, gamma complex processivity proteins and epsilon proofreading exonuclease on nucleotide misincorporation efficiencies, Journal of Biological Chemistry 272 (1997) 27919–27930.
[57] T.A. Kunkel, Frameshift mutagenesis by eucaryotic DNA polymerases in vitro, Journal of Biological Chemistry 261 (1986) 13581–13587.
[58] S. Shibutani, A.P. Grollman, On the mechanism of frameshift (deletion) mutagenesis in vitro, Journal of Biological Chemistry 268 (1993) 11703–11710.
[59] T. Yamaguchi, M. Wei, N. Hagihara, M. Omori, H. Wanibuchi, S. Fukushima, Lack of mutagenic and toxic effects of low dose potassium bromate on kidneys in the Big Blue rat, Mutation Research 652 (2008) 1–11.
[60] J.L. Parsons, G.L. Dianov, Co-ordination of base excision repair and genome stability, DNA Repair (Amsterdam) 12 (2013) 326–333.

Mutation Research 770 (2014) 95–104

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxic potential and *in vitro* tumour-promoting potential of 2-dodecylcyclobutanone and 2-tetradecylcyclobutanone, two radiolytic products of fatty acids

Kohji Yamakage[a,*], Hajime Sui[a], Ryo Ohta[a], Tomoyasu Toyoizumi[a], Kumiko Kawakami[a], Hirotaka Matsumoto[a], Toshitaka Takahashi[a], Kiyoshi Sasaki[a], Mayu Ikezumi[a], Saki Negishi[a], Keisuke Izumi[b], Setsuko Todoriki[c], Kondo Takashi[d], Masakazu Furuta[e]

[a] *Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan*
[b] *Department of Molecular and Environmental Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*
[c] *Food Safety Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*
[d] *Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan*
[e] *Laboratory of Quantum-Beam Chemistry and Biology, Radiation Research Center, Osaka Prefecture University, 1-2 Gakuen-cho, Sakai, Osaka 599-8570, Japan*

ARTICLE INFO

Article history:
Received 25 March 2013
Received in revised form 16 March 2014
Accepted 10 April 2014
Available online 8 June 2014

Keywords:
Radiolytic products
2-Dodecylcyclobutanone
2-Tetradecylcyclobutanone
Genotoxicity
Bhas 42 cell-transformation assay
Tumour-promoting effect

ABSTRACT

The DNA-damaging and tumour-promoting effects of two 2-alkylcyclobutanones (2-ACBs), which are found in irradiated fat-containing foods, were investigated by use of the comet assay and in an azoxymethane (AOM)-induced colon-carcinogenesis study in rats, respectively. We conducted genotoxicity tests of 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB) according to the test guidelines for chemicals or drugs. In addition, a cell-transformation assay with Bhas 42 cells was performed to investigate their promoting potential *in vitro*.

The *Salmonella typhimurium* mutagenicity assay (Ames test), conducted with five tester strains, revealed that neither 2-dDCB nor 2-tDCB possessed mutagenic activity. Moreover, both in the *in vitro* chromosomal aberration test on CHL/IU cells and the *in vivo* bone-marrow micronucleus test where mice were given 2-dDCB and 2-tDCB (orally, up to 2000 mg/kg bw/day), we did not detect any clastogenic effects. Furthermore, DNA strand-breaks were not detected in the *in vitro* comet assay with CHL/IU cells, and DNA adducts derived from 2-dDCB and 2-tDCB were not detected in the colon tissues of the mice used for the micronucleus tests, in rats from a repeated dose 90-day oral toxicity test (0.03% 2-tDCB in the diet), or in rats from the AOM-induced carcinogenesis study (0.025% 2-tDCB in the diet). An *in vitro* tumour-promotion assay with Bhas 42 cells revealed that the number of transformed foci increased significantly following treatment of cells in the stationary phase with 2-dDCB or 2-tDCB for 10 days.

Our results indicate that neither 2-dDCB nor 2-tDCB were genotoxic chemicals. However, they exhibited promoting activity, at least *in vitro*, when Bhas 42 cells were continuously exposed to these chemicals at toxic doses.

Abbreviations: 2-ACBs, 2-alkylcyclobutanones; 2-dDCB, 2-dodecylcyclobutanone; 2-tDCB, 2-tetradecylcyclobutanone; 2-tDeCB, 2-(tetradec-5′-enyl)-cyclobutanone; AOM, azoxymethane; SA, stearic acid; PA, palmitic acid; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; 2AA, 2-aminoanthracene; SoA, sodium azide; 4NQO, 4-nitroquinoline-1-oxide; 9AA, 9-aminoacridine; CP, cyclophosphamide monohydrate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MMC, mitomycin C; MMS, methyl methanesulfonate.

* Corresponding author at: Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 259-8523, Japan. Tel.: +81 463 82 4751; fax: +81 463 82 9627.
E-mail address: yamakage.k@fdsc.or.jp (K. Yamakage).

http://dx.doi.org/10.1016/j.mrgentox.2014.04.021
1383-5718/

1. Introduction

Irradiation of fat-containing food generates 2-alkylcyclobutanones (2-ACBs), which result from radiation-induced breakage of triglycerides [1]. These compounds contain the same number of carbons (n) as their fatty acid precursors, as well as an alkyl chain of carbons (n-4), which is branched at ring-position 2. These molecules have been found exclusively in irradiated fat-containing foods and are thus considered unique markers of food irradiation [2,3]. The yield of 2-ACBs is dependent on the original fatty acid profile of the irradiated food [4]. The concentrations of 2-ACBs in food are on the order of parts per million; for example, the amount of 2-dDCB, which is derived from palmitic acid (PA), has been estimated to be 12.9 μg per 100 g of chicken meat irradiated at 3 kGy [5].

The genotoxic potential of pure 2-dDCB has been previously tested in the comet assay [6]. The toxic potential of 2-ACBs has been previously investigated by a French–German research group in the EU interregional programme [7], in which the tumour-promoting potential of 2-tDCB and 2-(tetradec-5′-enyl)-cyclobutanone (2-tDeCB) was reported [8]. However, further studies are required to resolve remaining uncertainties regarding the toxicity/carcinogenicity of 2-ACBs, because very few studies have been conducted to investigate the genotoxicity of pure 2-ACBs according to specific test guidelines. In particular, apart from the Ames test [9–11], *in vivo* tests have not been conducted.

To elucidate the genotoxic potential of 2-ACBs, we performed standard genotoxicity tests (reverse-mutation assay, *in vitro* chromosomal-aberration assay, and *in vivo* micronucleus test). In addition, we conducted an *in vitro* comet assay and a DNA-adduct detection test in mouse colon tissue to investigate the reproducibility of DNA strand-break induction and tissue-specific responses. The tumour-promoting activities of 2-dDCB and 2-tDCB, which were not detected in the genotoxicity tests, were investigated in a cell-transformation assay in Bhas 42 cells, which has been previously validated by international validation studies [12–14].

2. Materials and methods

2.1. Chemicals

Pure 2-dDCB (purity, 99.2%) and 2-tDCB (purity, 99.1%) were synthesized by Hayashi Pure Chemical Ind. (Osaka, Japan). Stearic acid (SA, CAS no. 57-11-4) and PA (CAS no. 57-10-3), which are the precursors of 2-dDCB and 2-tDCB, were purchased from MP Biomedicals, LLC (OH, USA). For the positive control substances, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, CAS no. 3688-53-7), 2-aminoanthracene (2AA, CAS no. 613-13-8), sodium azide (SoA, CAS no. 26628-22-8), and 4-nitroquinoline-1-oxide (4NQO, CAS no. 56-57-5) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 9-aminoacridine (9AA, CAS no. 90-45-9) was purchased from MP Biomedicals, LLC (Solon, OH, USA). Cyclophosphamide monohydrate (CP, CAS no. 6055-19-2) and 12-O-tetradecanoylphorbol-13-acetate (TPA, CAS no. 16561-29-8) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Mitomycin C (MMC, CAS no. 50-07-7) and methyl methanesulfonate (MMS, CAS no. 66-27-3) were purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan) and Kanto Chemical Co. Inc. (Tokyo, Japan), respectively.

2.2. S9 mix

Rat liver S9 (Kikkoman Corporation, Chiba, Japan) was prepared from the liver homogenates of male SD rats treated with phenobarbital and 5,6-benzoflavone. For the Ames test, S9 was mixed with co-factors and then used as the S9 mix (final concentrations, 10% [v/v] of S9, 5 mM glucose-6-phosphate, 4 mM nicotinamide-adenine dinucleotide [reduced form, disodium salt], 4 mM nicotinamide-adenine dinucleotide phosphate [reduced form, tetrasodium salt], 33 mM KCl, 8 mM $MgCl_2$, and 100 mM sodium-phosphate buffer). For the chromosomal-aberration test, S9 was used at a final concentration of 5% (v/v) after mixing with co-factors [15,16].

2.3. Tester strains and culture conditions

Salmonella typhimurium strains TA100, TA1535, TA98, and TA1537 [17] and *Escherichia coli* WP2 *uvrA* [18] were used according to the "Guideline for Tests on Drugs (in Japanese)," which is based on the ICH Harmonized Tripartite Guideline [19]. These strains were inoculated into nutrient broth No. 2 (Oxoid Ltd., Hampshire, UK) and cultured at 37 °C for 10 h while shaking. The number of viable bacteria in the culture was maintained at a minimum of 1×10^9 cells/mL for all strains.

2.4. Cell lines and culture conditions

CHL/IU cells originating from the lung of a female Chinese hamster were used for the chromosome-aberration test and the comet assay. The CHL/IU cells were cultured in Eagle's minimum essential medium (MEM, Nissui Pharmaceuticals Co., Tokyo, Japan) supplemented with 10% (v/v) calf serum (CS, Biological Industries Israel Beit Haemek Ltd., Israel) in a humidified CO_2 incubator (5% CO_2, 37 °C). The cells had a modal chromosome number of 25 and were mycoplasma-free; the doubling-time was about 15 h.

Bhas 42 cells (v-*Ha-ras*-transfected Balb/c 3T3 cells) [20] were used for the cell-transformation assay. The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, GIBCO) supplemented with 5% (v/v) fetal bovine serum (FBS, GIBCO) in a humidified CO_2 incubator (5% CO_2, 37 °C).

2.5. Animals and housing conditions

Male 8-week-old CD1 (ICR) mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were observed daily for at least seven days (i.e., quarantine period). Nine-week-old healthy mice were then randomly assigned to either the control or the treatment groups (5 mice/group). The animals were individually housed in TPX cages (CLEA Japan, Inc., Tokyo, Japan) on paper-based bedding (Paperclean, Japan SLC, Inc.), where pellet chow and tap water were provided ad libitum in an air-conditioned room (12 h light/12 h dark cycle; 21–25 °C; 40–75% humidity). The animal experiments were conducted in accordance with the Japanese law for Animal welfare and the guidelines for animal experiments at our institute (Animal Experiment Approval nos. 1090259A and 1110298A).

2.6. Bacterial reverse-mutation test

2-tDCB and 2-dDCB were dissolved in ethanol. AF-2 (0.1 and 1 μg/mL), 9AA (800 μg/mL), and 2AA (10 and 100 μg/mL) were dissolved in dimethyl sulfoxide (DMSO), and SA (5 μg/mL) was dissolved in water for injection. In accordance with the "Guideline for Tests on Drugs" (in Japanese), the reverse-mutation test was performed by use of the pre-incubation method [21] (37 °C for 20 min) in the presence or absence of the S9 mix. Two plates were used for each group. The mean values for the two plates were calculated and determined as the number of revertants. The results were evaluated as positive when the number of revertants for each dose was increased 2-fold or more compared with the negative (ethanol) control. This evaluation method has been most widely used instead of a statistical analysis [22]. All solvents (ethanol, DMSO and water) used in this test are compatible with the test system, and historical control data of the solvents have been accumulated. Historical control data of positive control chemicals have also been collected. Therefore, the results of the positive control were compared with the results of the negative (ethanol) control, which were in the range of the historical control data.

2.7. In vitro chromosomal aberration test

2-tDCB and 2-dDCB were dissolved in acetone, and the test solution was applied at 1% (v/v). MMC and CP were dissolved in water for injection at concentrations of 20 μg/mL and 1 mg/mL, respectively. The chromosomal aberration test was performed according to the OECD guideline for the testing of chemicals, No. 473 [23] and a previous report [24]. The CHL/IU cells (2×10^4 cells) were plated on plastic dishes (diameter: 6 cm) and treated with test compounds for 6 h in the presence or absence of the S9 mix, washed in MEM, and then cultured for an additional 18 h in the MEM supplemented with 10% (v/v) CS. The CHL/IU cells were also continuously treated with the test compounds for 24 h. Two dishes were used for each group. After treatment with colcemid, which led to accumulation of metaphase cells, a portion of the detached cells was quantified with a Coulter Counter. The remaining cells were used to make chromosome specimens using the conventional air-dry method. Chromosomal analysis was performed on coded slides that were stained with Giemsa solution. Two hundred well-spread, but not dispersed, metaphase cells (centromere numbers: 23–27, 100 cells/dish) were observed for structural chromosomal aberrations in each group. Four hundred metaphase cells (200 cells/dish) were observed for polyploid cells (centromere numbers: 38 or more) in each group.

The number of cells with structural chromosomal aberrations (excluding gaps) and polyploid cells in the treatment groups were statistically compared with those in the negative (acetone) control groups by means of Fisher's exact probability test (one-sided, $p < 0.01$). Clastogenicity and induction of polyploidy by the test compounds were judged to be positive or negative on the basis of the results of the statistical analysis and the biological significance. Both solvents (acetone and water) used in this test are compatible with the test system, and historical control data of solvents have been accumulated. Historical control data of positive control chemicals have also been collected. Therefore, the results of the positive control were statistically compared with the results of the negative (acetone) control, which were in the range of the historical control data.

2.8. In vitro comet assay

The CHL/IU cells were exposed to the test compounds for the comet assay under the same testing conditions and at the same time as the chromosomal aberration test. After a 6-h treatment in the absence of the S9 mix, the cells were immediately harvested, and an alkaline comet assay was performed according to previous methods [25,26]. MMS was dissolved in DMSO at a concentration of 10 mg/mL. Two dishes were used for each group. The cells were embedded in 0.5% (w/v) low-melting agarose gel on slides, which were coated with 1.0% (w/v) normal-melting agarose gel. The slides were immersed in chilled lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris hydroxymethyl aminomethane acid, 1% [v/v] Triton-X100 and 10% [v/v] DMSO; pH 10) overnight at 4 °C. Next, the slides were left for 20 min in the electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13 or more) to unwind the DNA and were electrophoresed for 20 min at approximately 0.7 V/cm at a constant voltage and approximately 0.3 A. The temperature of the electrophoresis solution during the unwinding and electrophoresis was kept below 10 °C. After electrophoresis, the slides were immersed in cold neutralization buffer (400 mM Tris hydroxymethyl aminomethane acid, pH 7.5) for 20 min. All the coded slides were dehydrated by immersion in ethanol for 10 min and then air-dried. One hundred cells (50 cells/dish) were analysed, after staining with SYBR Gold solution, by means of an image-analyser system (Comet Assay IV version no. 4.11; Perceptive Instruments, Suffolk, UK). The percentage of DNA (%DNA) in the tail was used as an index of the degree of DNA damage. Heavily damaged cells, which are referred to as hedgehogs, were excluded from the image analysis; however, the number of hedgehogs was counted.

The %DNA in tail in the treatment groups was statistically compared with that in the negative control (acetone or DMSO) group by Dunnett's test (two-sided, $p < 0.05$). The frequencies of hedgehogs in the treatment groups were statistically compared with the negative control group by Fisher's exact probability test (one-sided, $p < 0.05$). Induction of DNA damage by the test compounds was judged to be positive or negative on the basis of the results of the statistical analysis and the biological significance.

2.9. Mouse bone-marrow micronucleus test

2-tDCB and 2-dDCB were dissolved in olive oil. CP was dissolved in physiological saline at a concentration of 5 mg/mL. The micronucleus assay was performed according to the OECD guideline for the testing of chemicals, No. 474 [27] and a previous method described by Hayashi et al. [28]. Briefly, olive oil (i.e., vehicle control), 2-dDCB, or 2-tDCB was orally administered once daily for 2 days at an interval of 24 h. CP was orally administered once at a dose of 50 mg/kg bw. The mice were sacrificed by cervical dislocation at 24 h after administration of the last dose, the bone-marrow cells were collected, and slide specimens for microscopic observation were prepared. Each coded specimen was stained with an acridine-orange solution immediately before observation. For each mouse, 2000 polychromatic erythrocytes (PCEs) were analysed for the presence of micronucleated PCEs (MNPCEs). The frequency of PCEs represented the index of bone-marrow toxicity.

The frequencies of MNPCEs in the treatment groups were statistically compared with that in the negative (olive oil) control by Fisher's exact probability test (one-sided, $p < 0.01$). As an index of suppression of bone-marrow cell proliferation, statistical analyses were carried out for the proportion of PCEs in the total erythrocytes. The difference between the control groups and the treatment groups, including the positive control group, was analysed by Student's *t*-test (two-sided, $p < 0.05$). Both solvents (olive oil and physiological saline) are compatible with test system, and historical control data of the solvents have been accumulated. Historical control data of the positive control chemical have been also collected. Therefore, the results of the positive control were statistically compared with the results of the negative (olive oil) control, which were in the range of the historical control data.

2.10. ^{32}P-postlabelling assay

DNA-adduct formation was examined by use of the ^{32}P-postlabelling TLC method, after treatment of the DNA with nuclease P1 [29].

Mouse colon-tissues were obtained from all the mice that received the vehicle or 2000 mg/kg bw/day of 2-dDCB or 2-tDCB in the micronucleus test. The colon tissues were immediately frozen in liquid nitrogen, and stored at −70 °C until use in the ^{32}P-postlabelling assay. The colon tissues (three samples from each group) of rats that were either fed 2-tDCB at 0.025% for 25 weeks for the 2-stage carcinogenicity test, or 2-tDCB at 0.03% for 90 days [30], were used for the ^{32}P-postlabelling assay. CHL/IU cells treated with DMSO (0.5% [v/v]) and 4NQO (4.8 μg/mL) for 1 h, respectively, were used as control samples.

Genomic DNA was extracted from frozen samples by use of the phenol/chloroform extraction method [31]. The genomic DNA (10 μg) was treated with RNase A (0.012 mg; Sigma–Aldrich Chemical Co.) and RNase T1 (0.24 U; Sigma–Aldrich Chemical Co.) at 37 °C for 1 h. DNA (10 μg) was hydrolysed to 2′-deoxyribonucleotide-3′-monophosphates at 37 °C for 3 h with micrococcal endonuclease (3.6 U; Sigma–Aldrich Chemical Co.) and spleen phosphodiesterase II (0.0375 U; Sigma–Aldrich Chemical Co.) in 0.1 M sodium succinate buffer (pH 6.0) containing 0.05 mM $CaCl_2$. The digested DNA sample (5 μL) was mixed with nuclease P1 (4 mg/mL; Wako Pure Chemical Industries) in 0.3 M sodium acetate (pH 5.3) containing 1 mM $ZnCl_2$. The reaction was terminated by adding 3 μL of 0.5 M Tris-base. After addition of 0.25 mCi of [γ-^{32}P]ATP (7000 Ci/mmol; MP Biomedicals Inc.) and T4 polynucleotide kinase (5 U; Wako Pure Chemical Industries), the mixture (15 μL) was incubated at 37 °C for 1 h for ^{32}P-postlabelling. Next, potato apyrase (0.05 U; Sigma–Aldrich Chemical Co.) was added and incubated at 37 °C for 30 min. The mixture was applied to a pre-washed PEI-TLC plate (10 × 10 cm; Macherey-Nagel, Düren, Germany), which was developed with 2.3 M sodium-phosphate buffer (pH 6.0) (first dimension, D1) for 14 h. A piece of the plate around the application point was cut off and attached to another PEI-TLC plate (10 × 10 cm), which was developed with 4.5 M lithium formate (pH 3.5) containing 7 M urea (D2). The plate was turned 90° (second dimension) and developed with 0.5 M Tris–HCl buffer (pH 8.0) containing 7 M urea and 1.1 M LiCl (D3). The plate was then developed in the same direction as D3 with 2.3 M sodium-phosphate buffer (pH 6.0) (D4). The PEI-TLC plate was washed with purified water after each development. The ^{32}P-labelled DNA adducts on the TLC plate were visualized by screen-enhanced autoradiography with X-ray film.

2.11. Bhas 42 cell-transformation (tumour-promotion) assay

2-tDCB and 2-dDCB were dissolved in acetone, and the test solution was applied at 0.5% (v/v). TPA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 μg/mL.

The tumour-promotion assay with Bhas 42 cells was performed as previously described [32]. Cells (1.4×10^4 cells) were plated in 6-well plates (2 mL/well, 3 wells/group for the cytotoxicity test; 6 wells/group for the transformation test). On days 4, 7, and 11 after plating, the medium in each well was replaced with medium containing the test compounds. On day 7 after plating, the Bhas 42 cells that had been treated for 3 days were fixed with formaldehyde and stained with crystal violet to measure cell density. On day 14 after plating (10 days after treatment), the medium in each well was replaced with fresh medium, and the cells were cultured for an additional 7 days. On day 21 after plating, the cells were fixed with methanol and stained with Giemsa solution. The transformed foci per well were counted.

The number of transformed foci in the treatment groups was compared with that in the negative control (acetone or DMSO), to verify the statistical significance of the difference, by means of Dunnett's test (two-sided, $p < 0.05$).

3. Results

3.1. In vitro genotoxicity tests

3.1.1. Bacterial reverse-mutation test

First, we performed a reverse-mutation test with five tester strains and one plate per dose to determine the appropriate dose, and obtained negative results in the presence or absence of the S9 mix for up to 5000 μg/plate of 2-dDCB and 2-tDCB (data not shown). In the second test, conducted on the basis of these results, induction of revertants after exposure to 2-tDCB or 2-tDCB was not observed in the presence or absence of the S9 mix (Tables 1 and 2). Growth inhibition was observed in the plates treated with 2-dDCB, but not with 2-tDCB. However, reproducible negative results were obtained in the bacterial reverse-mutation tests.

3.1.2. In vitro chromosomal aberration test

On the basis of the preliminary experiments, several concentrations were established at a common ratio of 1.5 or 2 in each treatment system (6-h treatment in the presence or absence of the S9 mix, and 24-h continuous treatment in the absence of the S9 mix). When CHL/IU cells were treated with 2-dDCB for 6 and 24 h without S9 mix, 55% and 48% cell growth at the maximum concentration of 0.1 mg/mL were observed, respectively (Table 3). Chromosomal analysis at the maximum concentration was possible for the 6-h treatment without S9 mix and impossible for the 24-h continuous treatment on the basis of the mitotic index results. We judged that a concentration of approximately 0.1 mg/mL is the maximum analysable concentration for a 6-h treatment without the S9 mix, because cell growth was <20% at 0.15 mg/mL, which was 1.5-fold the maximum concentration of 0.1 mg/mL (data not shown). Precipitates were observed in the 6-h treatment in the presence of the S9 mix for 2-dDCB and in all treatment systems for 2-tDCB. During these treatments, growth inhibition of 50% or more was not observed at the test concentrations, and a concentration-dependent inhibition of cell growth

Table 1
Results of bacterial reverse mutation test in five tester strains treated with 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB) without S9 mix.

Groups	Dose (μg/plate)	No. of revertants/plate (average[a])									
		TA100		TA1535		WP2 *uvrA*		TA98		TA1537	
Ethanol[b]	100 μL	87, 86	(87)	8, 8	(8)	24, 27	(26)	21, 15	(18)	6, 11	(9)
2-dDCB	19.5	74, 79	(77)	NT		NT		NT		5, 10	(8)
	39.1	80, 101	(91)	NT		NT		NT		7, 6	(7)
	78.1	104, 97	(101)	13, 7	(10)	NT		NT		5, 3	(4)
	156[c]	93, 61	(77)	6, 10	(8)	NT		NT		9, 11	(10)
	313[c]	79[d], 78[d]	(79)	7, 8	(8)	25, 27	(26)	21, 17	(19)	11[d], 6[d]	(9)
	625[c]	106[d], 91[d]	(99)	10, 12	(11)	18, 12	(15)	20, 27	(24)	6[d], 2[d]	(4)
	1250[c]	NT		10[d], 7[d]	(9)	25, 19	(22)	13, 16	(15)	NT	
	2500[c]	NT		NT		28, 28	(28)	23, 12	(18)	NT	
	5000[c]	67[d], 73[d]	(70)	8[d], 7[d]	(8)	17, 16	(17)	14, 15	(15)	4[d], 1[d]	(3)
2-tDCB	78.1	NT		11, 9	(10)	NT		NT		NT	
	156	NT		6, 14	(10)	NT		NT		NT	
	313	111, 107	(109)	13, 10	(12)	17, 32	(25)	23, 19	(21)	12, 9	(11)
	625[c]	80, 88	(84)	8, 10	(9)	29, 29	(29)	22, 24	(23)	11, 12	(12)
	1250[c]	89, 87	(88)	9, 11	(10)	26, 33	(30)	11, 30	(21)	13, 13	(13)
	2500[c]	83, 83	(83)	5, 4	(5)	30, 31	(31)	23, 24	(24)	12, 13	(13)
	5000[c]	65, 65	(65)	5, 10	(8)	29, 31	(30)	20, 19	(20)	11, 15	(13)
AF-2	0.01	298, 304	(301)[e]	NT		89, 93	(91)[e]	NT		NT	
	0.1	NT		NT		NT		392, 375	(384)[e]	NT	
SoA	0.5	NT		575, 543	(559)[e]	NT		NT		NT	
9AA	80	NT		NT		NT		NT		217, 265	(241)[e]

AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; SoA, sodium azide; 9AA, 9-aminoacridine; NT, not tested.
[a] Average revertants of two plates were shown in parentheses.
[b] Ethanol was used as the solvent control.
[c] Precipitation was observed.
[d] Growth inhibition was observed.
[e] Biologically different from the negative control (ethanol) because of more than 2-fold increases of the number of revertants.

was not observed because of the limited solubility of 2-dDCB and 2-tDCB in the medium. Chromosomal analysis was performed at 2–4 concentrations, which were selected on the basis of mitotic index and concurrent cell growth. Numbers of cells with structural aberrations, such as gaps, breaks, and exchanges and polyploid cells, were not statistically increased in any of the treatment groups (Table 3). On the other hand, the positive control chemicals (MMC and CP) clearly induced structural chromosomal aberrations. Thus, 2-dDCB and 2-tDCB did not induce chromosomal aberrations in CHL/IU cells.

3.1.3. *In vitro comet assay*

To compare the genetic damage induced by 2-dDCB or 2-tDCB at the DNA and chromosomal levels, the comet assay was performed at the same concentrations as those used in the chromosomal aberration test. The %DNA in tail in the CHL/IU cells

Table 2
Results of bacterial reverse mutation test in five tester strains treated with 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB) with S9 mix.

Groups	Dose (μg/plate)	No. of revertants/plate (average[a])									
		TA100		TA1535		WP2 *uvrA*		TA98		TA1537	
Ethanol[b]	100 μL	105, 87	(96)	13, 11	(12)	32, 30	(31)	32, 39	(36)	15, 16	(16)
2-dDCB	4.88	NT		NT		NT		NT		12, 17	(15)
	9.77	NT		NT		NT		NT		15, 16	(16)
	19.5	NT		NT		NT		NT		13,9	(11)
	39.1	NT		NT		NT		NT		13, 10	(12)
	78.1	NT		NT		NT		NT		13, 13	(13)
	156	NT		NT		NT		NT		11[d], 10[d]	(11)
	313	70, 69	(70)	5, 4	(5)	31, 25	(28)	34, 32	(33)	6[d], 3[d]	(5)
	625	68, 51	(60)	4, 11	(8)	30, 22	(26)	24, 30	(27)	NT	
	1250[c]	73, 62	(68)	8, 6	(7)	29, 31	(30)	36, 32	(34)	6[d], 4[d]	(5)
	2500[c]	71, 77	(74)	10, 10	(10)	30, 29	(30)	28, 29	(29)	NT	
	5000[c]	80, 66	(73)	8,7	(8)	20, 20	(20)	23, 23	(23)	4[d], 5[d]	(5)
2-tDCB	313	101, 97	(99)	16, 10	(13)	26, 23	(25)	31, 37	(34)	12, 13	(13)
	625	74, 103	(89)	12, 13	(13)	22, 24	(23)	32, 33	(33)	9, 8	(9)
	1250[c]	82, 81	(82)	8, 11	(10)	26, 17	(22)	43, 32	(38)	10, 13	(12)
	2500[c]	86, 105	(96)	11, 11	(11)	16, 18	(17)	36, 35	(36)	13, 14	(14)
	5000[c]	111, 83	(97)	4, 12	(8)	21, 24	(23)	36, 35	(36)	9, 10	(10)
2AA	0.5	NT		NT		NT		376, 361	(369)[e]	NT	
	1	618, 642	(630)[e]	NT		NT		NT		NT	
	2	NT		329, 349	(339)[e]	NT		NT		185, 192	(189)[e]
	10	NT		NT		501, 530	(516)[e]	NT		NT	

2AA, 2-aminoanthracene; NT, not tested.
[a] Average revertants of two plates were shown in parentheses.
[b] Ethanol was used as the solvent control.
[c] Precipitation was observed.
[d] Growth inhibition was observed.
[e] Biologically different from the negative control (ethanol) because of more than 2-fold increases of the number of revertants.

Table 3
Results of chromosomal aberration test in CHL/IU cells treated with 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB).

Groups	Concentration (mg/mL)	S9 mix	Time of exposure (h)	No. of dishes used	Concurrent[a] cell growth (%)	Mitotic[b] index (%)	No. of cells with aberrations[c]		No. of[d] polyploid cells (%)
							+gap (%)	−gap (%)	
Acetone[e]	1% (v/v)	−	6–(18)	2	100	NA	4 (2.0)	4 (2.0)	1 (0.3)
2-dDCB	0.044	−	6–(18)	2	98	NA	5 (2.5)	3 (1.5)	3 (0.8)
	0.067	−	6–(18)	2	90	NA	7 (3.5)	6 (3.0)	5 (1.3)
	0.10	−	6–(18)	2	55	3.4, 1.6	3 (1.5)	2 (1.0)	3 (0.8)
MMC	0.0001	−	6–(18)	2	91	NA	80 (40.0)	79 (39.5)*	1 (0.3)
Acetone[e]	1% (v/v)	+	6–(18)	2	100	NA	5 (2.5)	4 (2.0)	5 (1.3)
2-dDCB	0.44[pe]	+	6–(18)	2	59	NA	6 (3.0)	5 (2.5)	1 (0.3)
	0.67[pe]	+	6–(18)	2	70	NA	1 (0.5)	1 (0.5)	8 (2.0)
	1.0[pe]	+	6–(18)	2	65	7.0, 5.2	3 (1.5)	2 (1.0)	12 (3.0)
CP	0.01	+	6–(18)	2	89	NA	72 (36.0)	69 (34.5)*	0 (0.0)
Acetone[e]	1% (v/v)	−	24	2	100	NA	3 (1.5)	2 (1.0)	1 (0.3)
2-dDCB	0.030	−	24	2	104	NA	NA	NA	NA
	0.044	−	24	2	104	NA	7 (3.5)	6 (3.0)	2 (0.5)
	0.067	−	24	2	96	7.8, 3.4	9 (4.5)	6 (3.0)	2 (0.5)
	0.10	−	24	2	48	Tox, Tox			
MMC	0.00005	−	24	2	87	NA	82 (41.0)	78 (39.0)*	2 (0.5)
Acetone[e]	1% (v/v)	−	6–(18)	2	100	NA	6 (3.0)	5 (2.5)	1 (0.3)
2-tDCB	0.13[pbe]	−	6–(18)	2	77	NA	3 (1.5)	3(1.5)	3 (0.8)
	0.20[pbe]	−	6–(18)	2	84	NA	NA	NA	NA
	0.30[pbe]	−	6–(18)	2	80	NA	NA	NA	NA
	0.44[pbe]	−	6–(18)	2	96	NA	3 (1.5)	3 (1.5)	0 (0.0)
	0.67[pbe]	−	6–(18)	2	85	NA	3 (1.5)	3 (1.5)	2 (0.5)
	1.0[pbe]	−	6–(18)	2	69	7.2, 3.8	4 (2.0)	3 (1.5)	2 (0.5)
MMC	0.0001	−	6–(18)	2	69	NA	136 (68.0)	132 (66.0)*	3 (0.8)
Acetone[e]	1% (v/v)	+	6–(18)	2	100	NA	4 (2.0)	4 (2.0)	3 (0.8)
2-tDCB	0.50[pbe]	+	6–(18)	2	85	NA	NA	NA	NA
	1.0[pbe]	+	6–(18)	2	81	NA	2 (1.0)	1 (0.5)	3 (0.8)
	2.0[pbe]	+	6–(18)	2	84	NA	5 (2.5)	3 (1.5)	2 (0.5)
	4.0[pbe]	+	6–(18)	2	87	4.2, 3.4	7 (3.5)	6 (3.0)	4 (1.0)
CP	0.01	+	6–(18)	2	69	NA	90 (45.0)	87 (43.5)*	2 (0.5)
Acetone[e]	1% (v/v)	−	24	2	100	NA	7 (3.5)	6 (3.0)	1 (0.3)
2-tDCB	0.088	−	24	2	92	NA	NA	NA	NA
	0.13[pbe]	−	24	2	78	NA	10 (5.0)	7 (3.5)	1 (0.3)
	0.20[pbe]	−	24	2	84	NA	10 (5.0)	9 (4.5)	1 (0.3)
	0.30[pbe]	−	24	2	93	1.6, 1.6	9 (4.5)	8 (4.0)	4 (1.0)
	0.44[pbe]	−	24	2	86	1.2, 0.6	10 (7.3)[f]	10 (7.3)[f]	0 (0.0)[g]
	0.67[pbe]	−	24	2	82	0.0, 1.2	NA	NA	NA
	1.0[pbe]	−	24	2	78	1.0, 0.4	NA	NA	NA
MMC	0.00005	−	24	2	89	NA	104 (52.0)	104 (52.0)*	0 (0.0)

+gap, total number of cells with aberrations including gaps; −gap, total number of cells with aberrations excluding gaps; MMC, mitomycin C; CP, cyclophosphamide monohydrate; Tox, cytotoxic; NA, not analysed.

[a] Cell number, representing cytotoxicity, was measured with a Coulter Counter.

[b] Metaphase frequency was calculated by counting 500 cells in each dish.

[c] Two hundred cells for structural aberrations were analysed in each group.

[d] Four hundred cells for polyploidy were analysed in each group.

[e] Acetone was used as the solvent control.

[f] One hundred and thirty-seven cells were analysed.

[g] Three hundred and thirty-four cells were analysed.

* Significantly different from the negative control (acetone) at $p < 0.01$ (one-side) by Fisher's exact probability test.

[pe] Precipitation was observed at the end of the treatment in the medium by the naked eye.

[pbe] Precipitation was observed at the beginning and the end of the treatment in the medium by the naked eye.

Table 4
Results of comet assay in CHL/IU cells treated with 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcylobutanone (2-tDCB) for 6 h without S9 mix.

Groups	Concentration (mg/mL)	Dish no.	Hedgehog No. of cells analysed	No. of hedgehog	Comet No. of cells analysed	%DNA in tail[a]
Acetone[b]	1% (v/v)	1	50	3	50	5.3±6.2
		2	50	0	50	4.3±9.1
		Total	100	3	100	4.8
2-dDCB	0.044	1	50	0	50	4.5±4.5
		2	50	0	50	4.2±4.9
		Total	100	0	100	4.3
	0.066	1	50	0	50	4.6±5.8
		2	50	0	50	4.9±5.3
		Total	100	0	100	4.8
	0.1	1	50	5	50	4.1±4.9
		2	50	5	50	3.4±6.5
		Total	100	10	100	3.7
DMSO[c]	1% (v/v)	1	50	0	50	7.8±6.1
		2	50	0	50	7.6±6.6
		Total	100	0	100	7.7
MMS	0.05	1	50	23	50	66.5±10.3
		2	50	5	50	51.0±15.8
		Total	100	28[#]	100	58.7[*]
Acetone[b]	1% (v/v)	1	50	0	50	7.4±6.5
		2	50	0	50	9.0±8.2
		Total	100	0	100	8.2
2-tDCB	0.13	1	50	0	50	7.4±6.7
		2	50	0	50	5.2±9.2
		Total	100	0	100	6.3
	0.44	1	50	0	50	6.5±6.9
		2	50	0	50	5.3±6.0
		Total	100	0	100	5.9
	0.67	1	50	0	50	5.3±5.9
		2	50	1	50	9.5±10.4
		Total	100	1	100	7.4
	1	1	50	0	50	7.9±7.9
		2	50	0	50	6.9±8.3
		Total	100	0	100	7.4
DMSO[c]	1% (v/v)	1	50	0	50	8.2±8.2
		2	50	0	50	6.1±5.0
		Total	100	0	100	7.2
MMS	0.05	1	50	0	50	65.7±10.1
		2	50	0	50	60.7±10.4
		Total	100	0	100	63.2[*]

DMSO, dimethyl sulfoxide; MMS, methyl methanesulfonate.
[a] Mean ± SD.
[b] Acetone was used as the solvent control to test compounds.
[c] DMSO was used as the solvent control to the positive control chemical, MMS.
[*] Significantly different from the negative control (acetone or DMSO) at $p < 0.05$ by Dunnett's test.
[#] Significantly different from the negative control (acetone or DMSO) at $p < 0.05$ by Fisher's exact probability test.

treated with 2-dDCB or 2-tDCB was not statistically increased at any concentration, which was the same as in the chromosomal analysis (Table 4). On the other hand, the positive control chemical (MMS) clearly induced DNA damage. The results suggest that 2-dDCB and 2-tDCB did not induce genetic damage at the DNA level.

3.2. *In vivo genotoxicity tests*

3.2.1. *Mouse bone marrow micronucleus test*

We administered 2-dDCB or 2-tDCB to the mice twice orally, and no toxicological signs were observed in any of the treated mice (data not shown). Statistically significant increases were also not observed in the frequency of the MNPCEs after doses of up to 2000 mg/kg bw/day of 2-dDCB or 2-tDCB (Table 5). Moreover, the proportion of PCEs in the total erythrocytes showed no significant differences between the 2-dDCB or 2-tDCB dose-groups and the vehicle-control group. On the other hand, the positive control chemical (CP) clearly induced MNPCEs. Thus, 2-dDCB and 2-tDCB did not induce chromosomal aberrations *in vivo*.

3.2.2. *^{32}P-postlabelling assay*

No spots indicating DNA-adduct formation were found in any of the DNA samples that were obtained from the colon tissues of mice given 2-dDCB or 2-tDCB at 2000 mg/kg bw/day (Fig. 1a). In addition, DNA samples from the colon tissues of rats given a diet containing 2-tDCB at 0.025% for 25 weeks or a diet containing 2-tDCB at 0.03% for 90 days showed no DNA-adduct spots (Fig. 1b). In the positive control samples, DNA from CHL/IU cells treated with 4NQO showed two DNA-adduct spots (Fig. 1c).

3.2.3. *Bhas 42 cell-transformation (tumour-promotion) assay*

On the basis of the preliminary results of a test for cell growth in Bhas 42 cells treated for 3 days, the concentrations for the promotion assay were determined. Bhas 42 cells in the stationary phase were treated with the test compounds for 10 days. In cells treated with 2-dDCB, a significant increase in the number of transformed foci was observed at concentrations of 2-dDCB that were approximately the same (0.012 mg/mL and 0.014 mg/mL) as the cytotoxic concentration (0.015 mg/mL), which resulted in a range of responses, from formation of transformed foci to insufficient cell growth (Table 6). The tumour-promotion assay using 2-dDCB was

Table 5
Results of micronucleus test in male CD1(ICR) mice after oral administrations of 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB).

Groups	Dose (mg/kg/day)	No. of dosing	No. of animals	No. of polychromatic erythrocytes		% of MNPCEs[a] ± SD	% of PCEs in ERYs[b] ± SD
				Observed	Micronucleated		
Olive oil JP[c]	10 mL/kg/day	2	5	10,000	10	0.10 ± 0.10	55.8 ± 5.3
2-dDCB	500	2	5	10,000	18	0.18 ± 0.10	53.9 ± 6.4
	1000	2	5	10,000	12	0.12 ± 0.09	53.9 ± 4.9
	2000	2	5	10,000	17	0.17 ± 0.08	50.8 ± 5.5
CP	50	1	5	10,000	252*	2.52 ± 0.38	52.1 ± 10.6
Olive oil JP[c]	10 mL/kg/day	2	5	10,000	9	0.09 ± 0.05	58.3 ± 4.6
2-tDCB	500	2	5	10,000	12	0.12 ± 0.06	57.6 ± 9.5
	1000	2	5	10,000	7	0.07 ± 0.08	63.3 ± 3.1
	2000	2	5	10,000	6	0.06 ± 0.07	58.6 ± 7.2
CP	50	1	5	10,000	236*	2.36 ± 0.80	51.6 ± 11.6

CP, cyclophosphamide monohydrate (single oral administration).
[a] % of micronucleated polychromatic erythrocytes in polychromatic erythrocytes observed.
[b] % of polychromatic erythrocytes in erythrocytes (5000 cells) observed.
[c] Olive oil for JP was used as the vehicle control.
* Significantly higher than the negative control at $p < 0.01$ (Kastenbaum and Bowman's table).

repeated with the same concentrations, and consistent results were obtained (data not shown). Moreover, the number of transformed foci induced by treatment with 2-tDCB also increased at concentrations of 2-tDCB that were one fourth (0.005 mg/mL) and half (0.01 mg/mL) of the cytotoxic concentration (0.02 mg/mL), which resulted in a range of responses, from formation of transformed foci to severe cytotoxicity (Table 6). However, treatment with the fatty acids SA and PA, the precursors of 2-dDCB and 2-tDCB, respectively, did not increase the number of transformed foci (Table 6). The positive control chemical (TPA) clearly induced transformed foci.

4. Discussion

Carcinogenesis is a multi-step process, involving at least three steps: initiation, promotion, and progression [33]. Genetic changes are related to initiation, and non-genetic factors are related to promotion. Thus, we investigated the genotoxic and tumour-promoting effects of 2-dDCB and 2-tDCB.

To examine the genotoxic effects of 2-dDCB and 2-tDCB, we performed three assays according to a standard test battery.

First, the bacterial mutation assay (Ames test) showed negative results, which was consistent with the results of previous

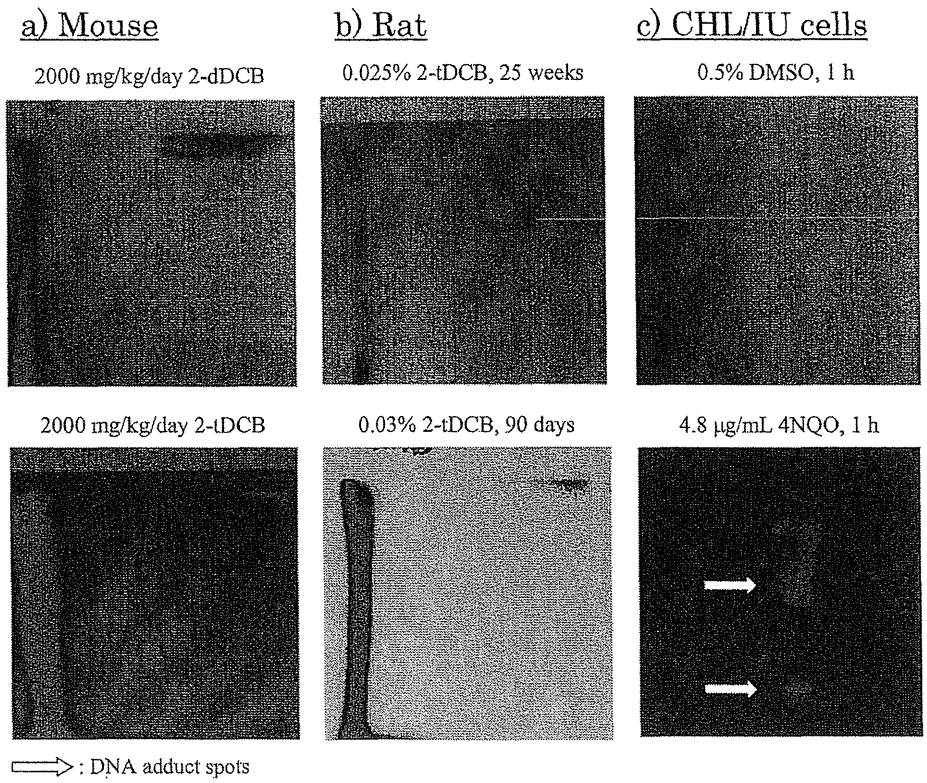


Fig. 1. Results of DNA-adduct analysis by the ^{32}P-postlabelling/TLC method in colon tissues of mice and rats, and in CHL/IU cells.

Table 6
Results of promotion assay in Bhas 42 cells treated with 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB) for 10 days.

Groups	Concentration (mg/mL)	Cell growth		Transformants[c]/well
		OD (%)[a]/wellAverage ± SD	% of control[b]	Average ± SD
Acetone[d]	0.5% (v/v)	96.0 ± 5.3	100.0	6.8 ± 2.4
2-dDCB	0.003	95.0 ± 2.6	99.0	7.3 ± 2.0
	0.006	98.7 ± 3.1	102.8	11.8 ± 2.9
	0.012	97.7 ± 4.0	101.7	24.7* ± 2.7
	0.013	100.7 ± 2.9	104.9	3.7 ± 3.8
	0.014	102.3 ± 1.5	106.6	25.2* ± 2.9
	0.015	104.3 ± 3.1	108.7	Insufficient growth[f]
	0.02	81.3 ± 11.7	84.7	Insufficient growth[f]
Palmitic acid	0.0025	85.3 ± 1.2	88.9	5.7 ± 3.1
	0.003	91.0 ± 1.7	94.8	7.8 ± 2.4
	0.005	88.3 ± 4.0	92.0	5.8 ± 2.9
	0.0075	85.0 ± 3.6	88.5	6.5 ± 1.8
	0.01	79.3 ± 4.2	82.6	3.7 ± 1.6
	0.02	49.7 ± 0.6	51.7	Insufficient growth[f]
DMSO[e]	0.5% (v/v)	89.3 ± 1.5	100.0	6.7 ± 2.8
TPA	50 ng/mL	116.7 ± 1.2	130.6	26.5* ± 3.6
Acetone[d]	0.5% (v/v)	98.3 ± 2.9	100.0	5.2 ± 1.6
2-tDCB	0.000625	102.7 ± 2.5	104.4	5 ± 2.4
	0.00125	95.7 ± 2.1	97.3	4.3 ± 1.0
	0.0025	103.3 ± 1.2	105.1	7.3 ± 1.2
	0.005	100.3 ± 1.5	102.0	13.3* ± 3.3
	0.01	112.0 ± 3.0	113.9	24.7* ± 2.4
	0.02	0.3 ± 0.6	0.3	Cytotoxic
Stearic acid	0.0016	97.7 ± 0.6	99.3	5.5 ± 2.2
	0.0031	101.7 ± 3.1	103.4	5.2 ± 2.5
	0.0063	97.0 ± 2.0	98.6	7.3 ± 1.2
	0.013	99.3 ± 2.1	101.0	5.8 ± 1.9
	0.025	77.0 ± 1.7	78.3	3.5 ± 1.4
	0.05	52.7 ± 2.1	53.6	1.7 ± 0.8
	0.1	38.0 ± 3.6	38.6	1.5 ± 1.0
DMSO[e]	0.5% (v/v)	93.3 ± 2.1	100.0	9.3 ± 2.0
TPA	50 ng/mL	109.7 ± 2.5	117.5	25.5* ± 3.3

DMSO, dimethyl sulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate.
[a] Cell density 3 days after stating treatment was measured with a Monocellater.
[b] Relative cell density to the solvent control, acetone or DMSO, was calculated.
[c] Bhas 42 cells were fixed and stained 21 days after starting treatment, then transformed foci were counted in each well.
[d] Acetone was used as the solvent control to test compounds.
[e] DMSO was used as the solvent control to the positive control chemical, TPA.
[f] Cell density was not enough to form transformants.
* Significantly different from the negative control (acetone or DMSO) at $p < 0.05$ by Dunnett's test.

studies [34–38]. Furthermore, 2-dDCB was reported not to induce gene mutations (*HPRT* gene) in human TK6 lymphoblasts [39].

Second, in the *in vitro* chromosomal aberration test, negative results were obtained for 2-dDCB or 2-tDCB treatment in CHL/IU cells. Regarding the clastogenic potential of 2-dDCB, Sommers [39] previously reported that micronuclei were induced in TK6 cells following treatment with this agent. This micronucleus induction was not 2-dDCB-specific because PA, a precursor of 2-dDCB, also induced micronuclei in TK6 cells [40]. There has been no previous report regarding clastogenicity of 2-tDCB. Thus, our result is the first, indicating a direct observation of the non-clastogenicity of 2-tDCB at the chromosomal level.

DNA damage may also induce gene mutations and/or structural chromosome aberrations. However, the DNA damage detected in test systems such as the comet assay and the UDS assay, may be partially repaired. Delincée and Pool-Zobel [6] and Knoll et al. [41] previously reported that tail intensity (DNA damage) in the *in vitro* comet assay increased in primary rat colon cells and human cells treated with 2-dDCB for 30 min. Thus, we performed the *in vitro* comet assay on CHL/IU cells under the same conditions as the chromosomal aberration test. However, we could not detect DNA damage in the comet assay, despite the treatment of the cells with 2-dDCB or 2-tDCB during 6 h. Moreover, there have been no positive results of 2-tDCB in the comet assay [38,42], and negative results for 2-dDCB in the comet assay have been reported in human colon-tumour cells [38,42,43]. The results of the comet assay with 2-dDCB might be associated with the cytotoxicity of 2-dDCB, because an increased tail intensity was observed in cells for which the viability had decreased after treatment for 60 or 90 min [41]. Previously we also demonstrated that 2-dDCB induced apoptosis [44]. Apoptotic or necrotic cells can result in comets with small or nonexistent heads and large diffuse tails (hedgehogs) [45]. We excluded hedgehog images from the comet analysis according to the international validation-study protocol; however, it is difficult to distinguish between quantifiable comet images and hedgehog images. Thus, these apoptotic effects may have contributed to the discrepancy of the results obtained in the comet assay.

Third, the *in vivo* micronucleus test was performed in mice after oral administration of 2-dDCB or 2-tDCB at 2000 mg/kg bw/day, and negative results for both chemicals were obtained in the bone-marrow cells. However, it is possible that the target organs of 2-dDCB and 2-tDCB are not bone-marrow cells: Raul et al. [8] previously reported that 2-tDCB and 2-tDeCB might promote colon carcinogenesis. We investigated *in vivo* DNA-adduct formation in the colon and did not detect adduct formation in DNA obtained from colon tissues of mice or rats that had been given 2-dDCB or 2-tDCB orally.

To verify the tumour-promoting activities of 2-dDCB and 2-tDCB *in vitro*, we performed a cell-transformation assay with Bhas

42 cells. This test system can detect both initiators and promoters [20,32,46,47], and international validation studies have been performed [12–14]. When Bhas 42 cells were treated with 2-dDCB or 2-tDCB for 10 days at near-toxic or growth-inhibiting concentrations, the number of transformed foci increased. The tumour-promotion process was reversible [48], and continuous stimulation by promoters used at doses close to the threshold for treatment may be necessary to promote transformation into tumour cells [46,49]. Our transformation assay suggested that continuous treatment at near-toxic concentrations may be essential for the promoting effects of 2-dDCB and 2-tDCB.

We could not detect any tumour-promoting activity of 2-tDCB in F344 rats, and toxicological signs were not observed in our *in vivo* tests [30], suggesting that a diet containing 0.025% 2-tDCB may result in a different exposure level from the *in vitro* test. The total amount of 2-dDCB and 2-tDCB recovered from the feces and adipose tissue of the animals was very low [50,51], and 2-dDCB was metabolized into 2-dodecylcyclobutanol [52]. Thus, 2-tDCB that is orally administrated *in vivo* may be metabolized and/or eliminated before reaching the exposure concentration or duration necessary to promote conversion of initiated cells into tumour cells.

Taken together, our *in vitro* and *in vivo* genotoxicity test results suggest that 2-dDCB and 2-tDCB are not genotoxic chemicals. However, these compounds showed tumour-promoting activity, at least *in vitro*. Since a tumour-promoting effect was not confirmed in the AOM-induced two-stage carcinogenesis study in F344 rats, the context where the cells are continuously exposed at toxic doses may not occur *in vivo*.

Conflict of interest statement

None declared.

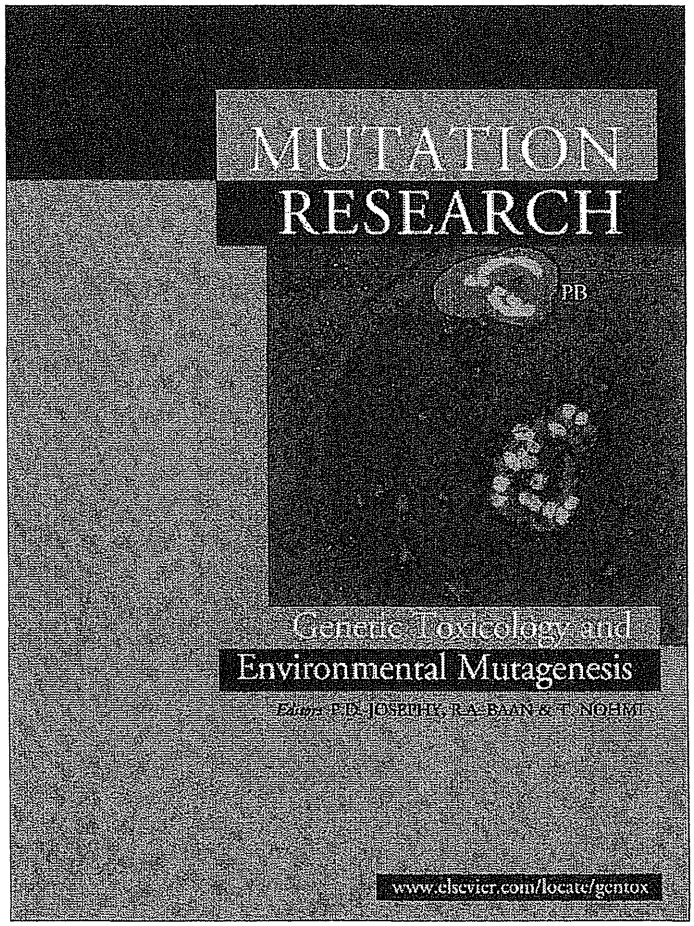
Acknowledgements

This work was supported by the Food Safety Commission, the Cabinet Office, Japan (#0906).

References

[1] P.R. Letellie, W.W. Nawar, 2-Alkylcyclobutanones from radiolysis of triglycerides, Lipids 7 (1972) 75–76.
[2] M.H. Stevenson, A.V.J. Crone, J.T.G. Hamilton, Irradiation detection, Nature 344 (1990) 202–203.
[3] B. Ndiaye, G. Jamet, M. Miesch, C. Hasselmann, E. Marchioni, 2-Alkylcyclobutanones as markers for irradiated foodstuffs II. The CEN (European Committee for Standardization) method: field of application and limit of utilization, Radiat. Phys. Chem. 55 (1999) 437–445.
[4] H. Obana, M. Furuta, Y. Tanaka, Analysis of 2-alkylcyclobutanones with accelerated solvent extraction to detect irradiated meat and fish, J. Agric. Food Chem. 53 (2005) 6603–6608.
[5] Health Canada, Evaluation of the Significance of 2-Dodecylcyclobutanone and Other Alkylcyclobutanones, 2002, http://www.hc-sc.gc.ca/food-aliment/fpi-ipa/e_cyclobutanone.html
[6] H. Delincée, B.-L. Pool-Zobel, Genotoxic properties of 2-dodecylcyclobutanone, a compound formed on irradiation of food containing fat, Radiat. Phys. Chem. 52 (1998) 39–42.
[7] D. Burnouf, et al., Etude toxicologique transfrontalière destinée à évaluer le risque encouru lors de la consommation d'aliments gras ionisés – Toxikologische Untersuchung zur Risikoberwertung beim Verzehr von bestrahlten fetthaltigen Lebensmitteln – Eine französisch-deutsche Studie im Grenzraum Oberrhein, Rapport final d'étude Interreg II, project No 3171, 2002.
[8] F. Raul, F. Gossé, H. Delincée, A. Hartwig, E. Marchioni, M. Miesch, D. Werner, D. Burnouf, Food-borne radiolytic compounds (2-alkylcyclobutanones) may promote experimental colon carcinogenesis, Nutr. Cancer 44 (2002) 88–191.
[9] European Commission, Statement of the Scientific Committee on Food on a Report on 2-Alkylcyclobutanone, SCF/CS/NF/IRR/26 ADD 3 Final (expressed on 3 July 2002).
[10] WHO Statement on 2-Dodecylcyclobutanone and Related Compounds, 2003.
[11] EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Scientific opinion on the chemical safety of food irradiation, EFSA J. 9 (4) (2011) 1930 [57 pp.]. http://www.efsa.europa.eu/efsajournal.htm
[12] K. Ohmori, M. Umeda, N. Tanaka, H. Takagi, I. Yoshimura, K. Sasaki, S. Asasda, A. Sakai, H. Araki, M. Asakura, H. Baba, Y. Fushiwaki, S. Hamada, N. Kitou, T. Nakamura, Y. Nakamura, H. Oishi, S. Sasaki, S. Shimada, T. Tsuchiya, Y. Uno, M. Washizuka, S. Yajima, Y. Yamamoto, E. Yamamura, T. Yatsushiro, An interlaboratory collaborative study by the non-genotoxic carcinogen study group in Japan, on a cell transformation assay for tumour promoters using Bhas 42 cells, ATLA 33 (2005) 619–639.
[13] N. Tanaka, K. Sasaki, K. Hayashi, A. Sakai, S. Asada, D. Muramatsu, S. Kuroda, F. Mizuhashi, M. Nagai, H. Suzuki, T. Imamura, M. Asakura, H. Satoh, A. Sakamoto, R. Nakao, H. Hirose, N. Ishii, M. Umeda, An interlaboratory collaborative study on a cell transformation assay using Bhas 42 cells, AATEX 14 (2009) 831–848.
[14] A. Sakai, K. Sasaki, K. Hayashi, D. Muramatsu, S. Arai, N. Endou, S. Kuroda, A. Poth, S. Bohnenberger, T. Kunkelmann, M. Asakura, H. Hirose, N. Ishii, F. Mizuhashi, S. Kasamoto, M. Nagai, K. Pant, S.W. Bruce, J.E. Sly, S. Yamazaki, M. Umeda, N. Tanaka, An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity, Mutat. Res. 725 (2011) 57–60.
[15] M. Ishidate Jr., Data Book of Chromosomal Aberration Tests In vitro, LIC/Elsevier, Tokyo/Amsterdam, New York, Oxford, 1988.
[16] K. Yamakage, H. Kusakabe, N. Tanaka, Comparative studies of MCL-5 cells and human lymphocytes for detecting indirect-acting clastogens, Mutat. Res. 412 (1998) 55–61.
[17] D.M. Maron, B.N. Ames, Revised methods for the Salmonella mutagenicity test, Mutat. Res. 113 (1983) 173–215.
[18] M.H.L. Green, Mutagen testing using Trp$^+$ reversion in *Escherichia coli*, in: B.J. Kilbey, M. Legator, W. Nichols, C. Ramel (Eds.), Handbook of Mutagenicity Test Procedures, Elsevier, Amsterdam, 1984, pp. 161–187.
[19] ICH Harmonised Tripartite Guideline, S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, 1995.
[20] K. Sasaki, H. Mizusawa, M. Ishidate, Isolation and characterization of ras-transfected BALB/3T3 clone showing morphological transformation by 12-*O*-tetradecanoyl-phorbol-13-acetate, Jpn. J. Cancer Res. 79 (1988) 921–930.
[21] T. Matsushima, T. Sugimura, M. Nagao, T. Yahagi, A. Shirai, M. Sawamura, Factors modulating mutagenicity in microbial tests, in: K.H. Norpoth, R.C. Garner (Eds.), Short-Term Test Systems for Detecting Carcinogens, Springer, Berlin, Heidelberg, New York, 1980, pp. 273–285.
[22] E. Zeiger, Bacterial mutation assays, in: A. Dhawan, M. Bajpayee (Eds.), Genotoxicity Assessment: Methods and Protocols, Methods in Molecular Biology, vol. 1044, Springer Science + Business Media, New York, 2013, pp. 3–26.
[23] OECD Guideline for the Testing of Chemicals, No. 473, In vitro Mammalian Chromosome Aberration Test, 1997, http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
[24] H. Kusakabe, K. Yamakage, S. Wakuri, K. Sasaki, Y. Nakagawa, M. Watanabe, M. Hayashi, T. Sofuni, H. Ono, N. Tanaka, Relevance of chemical structure and cytotoxicity to the induction of chromosome aberrations based on the testing results of 98 high production volume industrial chemicals, Mutat. Res. 517 (2002) 187–198.
[25] B. Burlinson, R.R. Tice, G. Speit, E. Agurell, S.Y. Brendler-Schwaab, A.R. Collins, P. Escobar, M. Honma, T.S. Kumaravel, M. Nakajima, Y.F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, Fourth International Workgroup on Genotoxicity testing: results of the *in vivo* comet assay workgroup, Mutat. Res. 627 (2007) 31–35.
[26] International Validation of the *In vivo* Rodent Alkaline Comet Assay for Detection of Genotoxic Carcinogens (Draft Version 14.2).
[27] OECD Guideline for the Testing of Chemicals, No. 474, Mammalian Erythrocyte Micronucleus Test, 1997, http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
[28] M. Hayashi, R.R. Tice, J.T. MacGregor, *In vivo* rodent erythrocyte micronucleus assay, Mutat. Res. 312 (1994) 293–304.
[29] M.V. Reddy, K. Randerath, Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts, Carcinogenesis 7 (1986) 1543–1550.
[30] M. Sato, S. Todoriki, T. Takahashi, E. Hafez, C. Takasu, Y. Fujii, H. Uehara, K. Yamakage, T. Kondo, K. Matsumoto, M. Furuta, K. Izumi, A 90-day oral toxicity test and investigation of the azoxymethane-induced carcinogenesis of 2-tetradecylcyclobutanone, a radiolytic product of stearic acid, in F344 rats, Food Chem. Toxicol. (2014) (in press).
[31] J. Sambrook, D.W. Russell, Molecular Cloning, A Laboratory Manual, 3rd ed., vol. 1, Cold Spring Harbor Laboratory Press, New York, 1989.
[32] A. Sakai, K. Sasaki, D. Muramatsu, S. Arai, N. Endou, S. Kuroda, K. Hayashi, Y.M. Lim, S. Yamazaki, M. Umeda, N. Tanaka, A Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity, Mutat. Res. 702 (2010) 100–122.
[33] T. Sugimura, Multistep carcinogenesis: a 1992 perspective, Science 259 (1992) 603–607.
[34] C.H. Sommers, 2-Dodecylcyclobutanone does not induce mutations in the *Escherichia coli* tryptophan reverse mutation assay, J. Agric. Food Chem. 51 (2003) 6367–6370.
[35] P. Gadgil, J.S. Smith, Mutagenicity and acute toxicity evaluation of 2-dodecylcyclobutanone, J. Food Sci. 69 (2004) 713–715.
[36] C.H. Sommers, R.H. Schiestl, 2-Dodecylcyclobutanone does not induce mutations in the Salmonella mutagenicity test or intrachromosomal recombination in *Saccharomyces cerevisiae*, J. Food Prot. 67 (2004) 1293–1300.
[37] C.H. Sommers, Recent advances in food irradiation: mutagenicity testing of 2-dodecylcyclobutanone, in: V.K. Juneja, J.P. Cherry, M.H. Tunick (Eds.), Advances

in Microbial Food Safety, ACS Symposium Series, vol. 931, American Chemical Society, 2006, pp. 109–120.
[38] A. Hartwig, A. Pelzer, D. Burnouf, H. Titéca, H. Delincée, K. Briviba, C. Soika, C. Hodapp, R. Raul, M. Miesch, D. Werner, P. Horvatovich, E. Marchioni, Toxicological potential of 2-alkylcyclobutanones – specific radiolytic products in irradiated fat-containing food – in bacteria and human cell lines, Food Chem. Toxicol. 45 (2007) 2581–2590.
[39] C.H. Sommers, Induction of micronuclei in human TK6 lymphoblasts by 2-dodecylcyclobutanone, a unique radiolytic product of palmitic acid, J. Food Sci. 70 (2006) 254–256.
[40] C.H. Sommers, Induction of micronuclei by palmitic acid and its unique radiolytic product 2-dodecylcyclobutanone, in: Proceedings of the United States–Japan (UJNR), 36th Annual Meeting, October 21–25, Tsukuba, Ibaraki, Japan, 2007, pp. 59–62, http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=216513
[41] N. Knoll, A. Weise, U. Claussen, W. Sendt, M. Marian, M. Glei, B.L. Pool-Zobel, 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary human colon cells and in cells from preneoplastic lesions, Mutat. Res. 594 (2006) 10–19.
[42] H. Delincée, C. Soika, P. Horvatovich, G. Rechkemmer, E. Marchioni, Genotoxicity of 2-alkylcyclobutanones, markers for an irradiation treatment in fat-containing food—Part I: cyto- and genotoxic potential of 2-tetradecylcyclobutanone, Radiat. Phys. Chem. 63 (2002) 431–435.
[43] E. Marchioni, F. Raul, D. Burnouf, M. Miesch, H. Delincee, A. Hartwig, D. Werner, Toxicological study on 2-alkylcyclobutanones—results of a collaborative study, Radiat. Phys. Chem. 71 (2004) 147–150.
[44] D.Y. Yu, Q.L. Zhao, M. Furuta, S. Todoriki, K. Izumi, K. Yamakage, K. Matsumoto, T. Nomura, T. Kondo, Molecular mechanisms of apoptosis induction by 2-dodecylcyclobutanone, a radiolytic product of palmitic acid, in human lymphoma U937 cells, Apoptosis 17 (2012) 636–645.
[45] P.L. Olive, J.P. Banath, Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent, Exp. Cell. Res. 221 (1995) 19–26.
[46] K. Ohmori, K. Sasaki, S. Asada, N. Tanaka, M. Umeda, An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells, Mutat. Res. 557 (2004) 191–202.
[47] S. Asada, K. Sasaki, N. Tanaka, K. Takeda, M. Hayashi, M. Umeda, Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-*ras*-transfected BALB/c 3T3 cells (Bhas 42 cells), Mutat. Res. 588 (2005) 7–21.
[48] H. Yamasaki, Reversible inhibition of cell differentiation by phorbol esters as a possible mechanism of the promotion step in chemical carcinogenesis, IARC Sci. Publ. 27 (1980) 91–111.
[49] T. Tsuchiya, M. Umeda, Relationship between exposure to TPA and appearance of transformed cells in MNNG-initiated transformation of BALB/c 3T3 cells, Int. J. Cancer 73 (1997) 271–276.
[50] P. Horvatovich, F. Raul, M. Miech, D. Burnouf, H. Delincee, A. Hartwig, D. Werner, E. Marchioni, Detection of 2-alkylcyclobutanes, markers for irradiated foods, in adipose tissues of animals fed with these substances, J. Food Prot. 65 (2002) 1610–1613.
[51] P. Gadgil, J.S. Smith, Metabolism by rats of 2-dodecylcyclobutanone, a radiolytic compound present in irradiated beef, J Agric. Food Chem. 54 (2006) 4896–4900.
[52] F. Hijaz, T.B. Shrestha, S.H. Bossman, F. Hussain, J.S. Smith, In vitro and in vivo metabolism of the radiolytic compound 2-dodecylcyclobutanone, J. Food Sci. 75 (2010) 72–80.



Mutation Research 749 (2012) 97–100

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres

Short communication

In vitro clastogenicity and phototoxicity of fullerene (C_{60}) nanomaterials in mammalian cells

Masamitsu Honma[a,*], Toshitaka Takahashi[b], Shin Asada[b], Yuzuki Nakagawa[b], Atsuko Ikeda[b], Kohji Yamakage[b]

[a] Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Food and Drug Safety Center, Hatano Research Institute, 729-5 Ochiai, Hadano-shi, Kanagawa 257-8523, Japan

ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 31 July 2012
Accepted 24 August 2012
Available online 31 August 2012

Keywords:
Fullerenes (C_{60})
Clastogenicity
Phototoxicity
Polyploidy

ABSTRACT

Carbon nanomaterials such as carbon nanotubes, graphene, and fullerenes (C_{60}) are widely used in industry. Because of human health concerns, their toxic potential has been examined in vivo and in vitro. Here we used mammalian cells to examine the in vitro clastogenicity as well as the phototoxicity of C_{60}. While C_{60} induced no structural chromosome aberrations in CHL/IU cells at up to 5 mg/ml (the maximum concentration tested), it significantly induced polyploidy at 2.5 and 5 mg/ml with and without metabolic activation. In BALB 3T3 cells, C_{60} showed no phototoxic potential but the anatase form of titanium oxide did. Since insoluble nanomaterials cause polyploidy by blocking cytokinesis rather than by damaging DNA, we concluded that the polyploidy induced by C_{60} in CHL/IU cells was probably due to non-DNA interacting mechanisms.

1. Introduction

A variety of nanomaterials have been recently developed. They are used in industry and in medicine (for drug delivery) because of their unique physical and chemical properties [1,2]. But nanoparticles in diesel exhaust and nano-sized asbestos particles have toxic effects [3–5], with the latter being particularly hazardous because they lead to mesothelioma and bronchogenic carcinoma with high frequency [6,7]. This raises concerns about the health effects of newly developed nanomaterials. Here we report on the in vitro clastogenicity and phototoxicity of fullerene (C_{60}).

2. Materials and methods

2.1. Test chemicals

We obtained fullerene (C_{60}, Nanom purple SUH, purity >99.9%) from Frontier Carbon Corporation, Tokyo, and titanium dioxide (TiO_2) in the anatase form (lot # p-25, average size 21 nm) from Nippon-Aerosol Corporation, Tokyo, and in the rutile form (lot# LU175, average size 20 nm) from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan. We purchased mitomycin C (MMC) from Kyowa-Kirin Co., Ltd., Tokyo, and cyclophosphamide monohydrate (CP) and chlorpromazine hydrochloride salt (CPZ) from Sigma–Aldrich Japan, Tokyo. For use, we prepared C_{60} sample by suspending them with 0.5% carboxymethylcellulose sodium solution (CMC-Na). TiO_2 samples were also prepared by suspending them with 0.5% CMC-Na. MMC, CP and CPZ were dissolved in distilled water. The state of C_{60} in the suspension was observed through a transmission electron microscope (TEM) (TEM-1010; JOEL Datum Ltd., Japan). The size distribution was measured by particle size distribution analyzer LA-920 (Horiba Ltd., Japan).

2.2. In vitro chromosome aberration test

We conducted the chromosomal aberration test according to OECD guideline TG 473 [9] and the procedure of Kusakabe and colleagues [10]. We grew CHL/IU cells obtained from the National Institute of Health Sciences, Tokyo, on culture medium which was Eagle's minimum essential medium (MEM) supplemented with 10% calf serum (Hyclone Inc., USA) and maintained them in culture plates at 37 °C in a humidified 5% CO_2 atmosphere. We treated the cells with the test chemicals for 6 h in culture medium with or without S9 mix and then cultured them for 18 h in fresh culture medium. We mixed S9 (Kikkoman Inc., Chiba, Japan) with co-factors immediately before use and added the mix to the cultures. We then treated the cells with 0.1 μg/ml colcemid for 2 h and prepared chromosome specimens by the air-dry method. We stained them with 3% Giemsa solution for 8 min and scored the number of cells with chromatid- and chromosome-type breaks and exchanges per 100 cells for each culture (200 cells at each concentration). We also scored polyploid cells per 800 cells at each concentration. We used the Fisher exact test to compare the frequency of aberrant cells in treated versus untreated cells. All tests were performed in duplicate.

2.3. In vitro phototoxicity test

We conducted the in vitro phototoxicity test according to OECD guideline TG 432 [11] and the procedure of the European Cosmetic Industry Association joint validation project [12]. We obtained BALB3T3 clone A31 cells from ATCC, grew them on Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated calf serum (Hyclone), and maintained them on culture plates at 37 °C in a humidified 5% CO_2 atmosphere. For the study, we seeded the cells into 96-well plates (10^4 cells/well), maintained them for 24 h, and then treated them with the test chemicals for 1 h in Earle's balanced salt solution (EBSS) at 37 °C in a

* Corresponding author. Tel.: +81 3 3700 1141x435; fax: +81 3 3700 2348.
E-mail address: honma@nihs.go.jp (M. Honma).

1383-5718/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mrgentox.2012.08.006

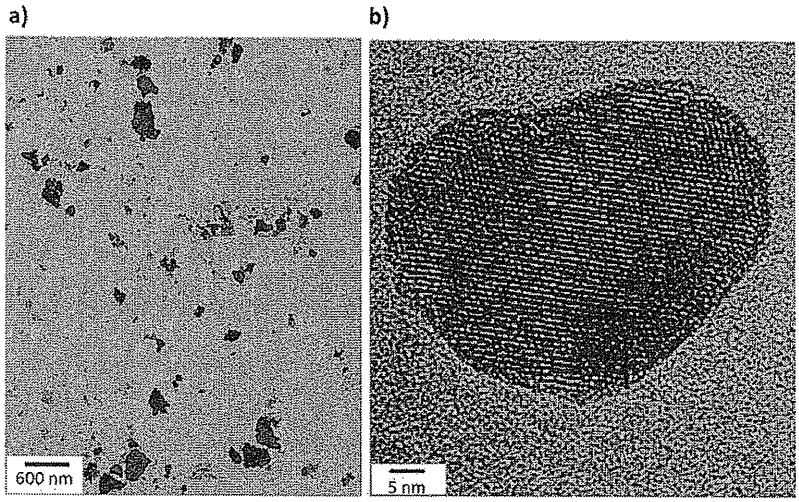


Fig. 1. TEM pictures of the C_{60} nanomaterial in the aqueous suspension used in this study. The left picture (a) shows the image of whole suspension (32,000×). The right picture (b) shows one of fine nanoparticle (210,000×).

humidified 5% CO_2 atmosphere. As they were being treated, we irradiated them with UVA (2.5 mW/cm^2) for 50 min using a sunlight simulator (SOL 500 Dr. Honle, Martinsried, Germany, or SLX 2500V, SERIC, Tokyo) (total, 7.5 J/cm^2) and measured the emitted energy with a UVA meter (UVR-3036/S, Topcon, Tokyo). After the treatment, we washed the cells, placed them in fresh medium, and cultured them for 17 h. We then placed them in fresh medium containing Neutral Red (NR) (final concentration, 50 μg/ml), cultured them for another 3 h, and fixed them with 1% $CaCl_2$/1% formaldehyde solution. We extracted NR with desorb solution (freshly prepared with 49 parts water, 50 parts ethanol, and 1 part acetic acid), measured its optical density at 540 nm with a spectrophotometer for the 96-well microplate, and determined the concentration responses obtained in the presence and absence of irradiation and the concentration required to reduce cell viability to 50% relative to the untreated controls (IC50).

3. Results

3.1. Characterization of the C_{60} nanomaterial suspension

The TEM pictures of C_{60} nanomaterial in the aqueous suspension are shown in Fig. 1. Most of C_{60} was agglomerated forming large particles (>100 nm in diameter) (Fig. 1a). Some fine particles (<50 nm in diameter) were also observed infrequently (Fig. 1b). The size of diameter was widely distributed ranging from 20 to 2000 nm (data not shown). The average diameter is approximately 300 nm.

3.2. In vitro cytotoxicity and chromosome aberration test

We conducted chromosomal aberration tests with CHL/IU cells to evaluate the clastogenicity of C_{60}, which was prepared by 0.5% CMC-Na. We treated the cells with C_{60} for 6 h in the presence and absence of rat S9-mix. Both with and without S9-mix, C_{60} exhibited moderate cytotoxicity in a concentration-dependent manner up to 5 mg/ml (Fig. 2), but it did not induce structural chromosome aberrations at any concentration although MMC (0.1 μg/ml) and CP (10 μg/ml), which are positive controls for the absence or presence of S9-mix, respectively, clearly produced structural chromosome aberrations (data not shown). On the other hand, it significantly increased the frequency of polyploid cells at 2.5 and 5 mg/ml treatments in both conditions.

3.3. In vitro phototoxicity test

We conducted in vitro phototoxicity tests with BALB3T3 cell for C_{60}, TiO_2 (rutile form), and TiO_2 (anatase form). We treated the cell with the chemicals prepared by 0.5% CMC-Na until 1 mg/ml. C_{60} and the rutile form of TiO_2 were not phototoxic at any concentration, but the anatase form of TiO_2 was phototoxic in a concentration-dependent manner starting at the lowest concentration (Fig. 3). The IC50 was calculated to be 0.09 mg/ml. CPZ, which is a positive control for phototoxicity tests, yielded clear phototoxicity with 0.46–0.67 μg/ml of IC50 (data not shown).

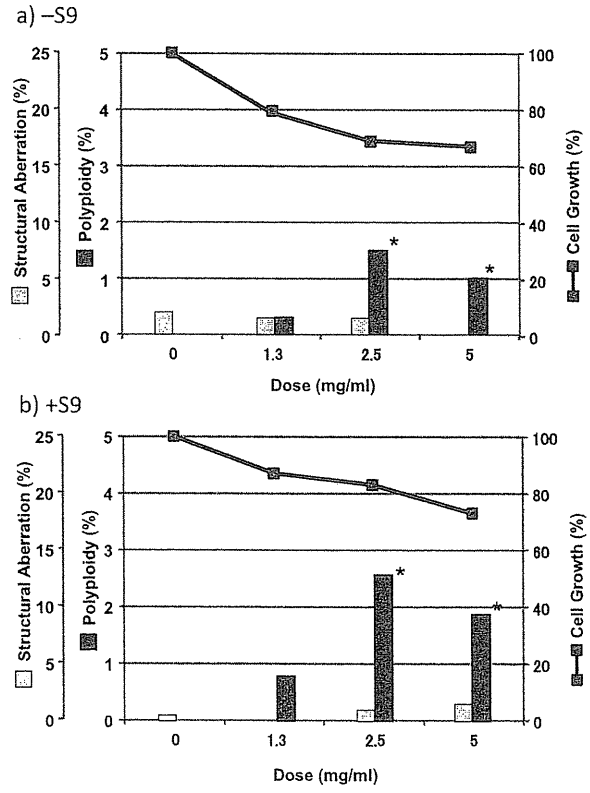


Fig. 2. Chromosome aberrations and cytotoxicity in CHL/IU cells exposed to C_{60} in (a) the absence of S-9 mix and (b) the presence of S-9 mix. Structural chromosome aberrations and polyploidy were examined independently. *$P<0.01$, Fisher exact test.

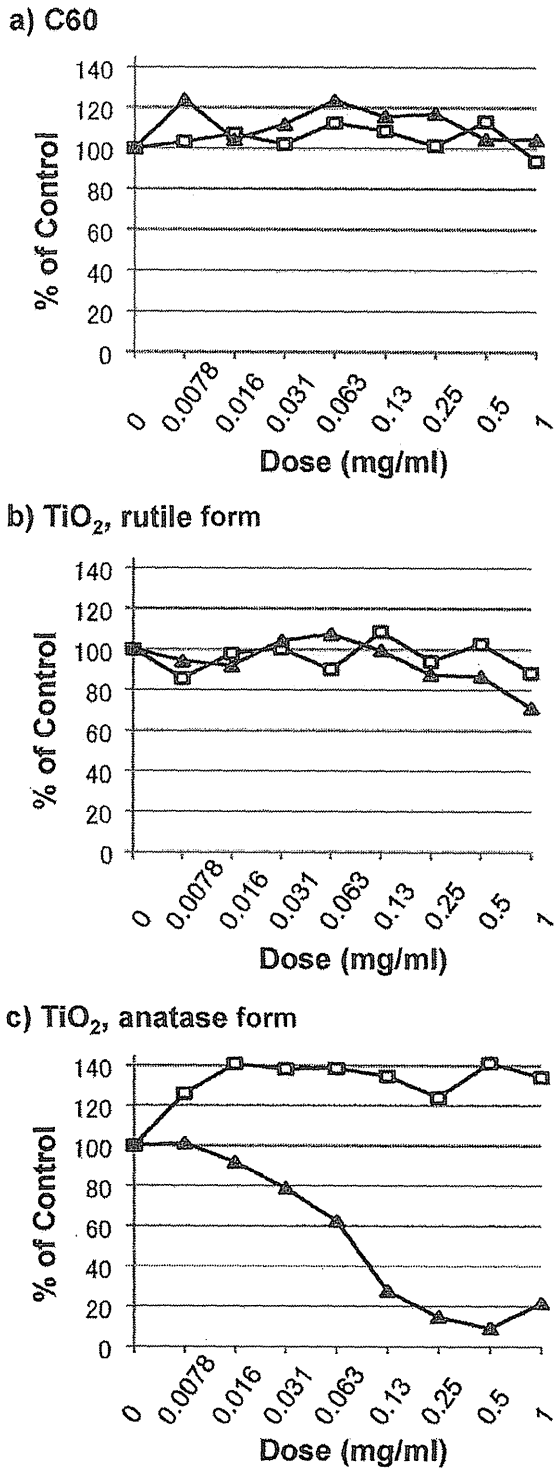


Fig. 3. Phototoxicity in BALB3T3 cells exposed to various concentrations of (a) C_{60}, (b) TiO_2 (rutile form), and (c) TiO_2 (anatase form). Open squares, without photoirradiation; closed triangles, with photoirradiation.

4. Discussion

After obtaining negative results in standard genotoxicity tests (Ames, chromosome aberration, and in vivo micronucleus tests) conducted according to OECD guidelines [9], Shinohara and colleagues concluded that C_{60} had no in vitro or in vivo genotoxic potential [8]. The Ames test, however, may not be appropriate for nanomaterials because bacterial cells lack the capacity for endocytosis and the nanomaterial may not be able to pass through the cell wall, resulting in misleading negative results [13]. Therefore, the in vitro genotoxicity of nanomaterials should be examined in mammalian cells.

Although C_{60} genotoxicity has been studied in mammalian cells in vitro, the results are conflicting. C_{60} significantly induced micronuclei in a concentration-dependent manner in human lung cancer cell line A549 [14] and weakly induced gene mutations in MEF cells isolated from gpt-delta mice [15], but it was negative in the chromosomal aberration test [8,16] and the comet assay [17].

In the present study, we conducted the chromosomal aberration test according to the OECD guideline [9], and demonstrated that the C_{60} clearly induced polyploidy. This result is contrast to that by Shinohara and colleagues [8]. We used the same lot of C_{60} and prepared the sample by the suspension with CMC-Na. The disparity is likely due to the size of the C_{60} in the treatment. Shinohara and colleagues suspended the C_{60} into 0.1% CMC-Na and extensively pounded in an agate mortar for 30 min, while we suspended the C_{60} into 0.5% CMC-Na in usual manners. As the results, the size of prepared C_{60} for the chromosomal aberration test was much different between them; 50 nm in Shinohara and colleagues vs. 300 nm in ours. Larger size of the C_{60} may specifically induce polyploidy. Totsuka et al. also demonstrated that C_{60} prepared by 0.05% Tween 80 induced micronuclei in human lung cancer cell line A549 [14]. The most abundant sizes were two peaks at 234 and 867 nm.

The induction of polyploidy may have been due to a physical interaction between C_{60} nanoparticles and the spindle apparatus during cytokinesis. Jensen and colleagues demonstrated that asbestos fiber induces polyploidy and aneuploidy by sterically blocking cytokinesis in monkey epithelial cells [18], and Asakura and colleagues recently reported that multiwall carbon nanotubes (MWCNTs) induce polyploidy but not structural chromosome aberrations in CHL cells [19]. Because asbestos and MWCNT did not induce micronuclei, but increase the number of bi-nucleated and multi-nucleated cells, the induction of polyploidy is considered to the result by the interference with components of the mitotic spindle during chromosome segregation or by blocking of cytokinesis. It is not clear whether C_{60} produces polyploidy by the similar mechanism. Because larger sizes of C_{60} only induced polyploidy, C_{60} heavily overlaying cell surface may physically block cell division. Anyway, these nanomaterials look not directly interact with DNA, and there may be a threshold for that activity [20]. C_{60} did not induce mesotheliomas in mice by intraperitoneal administration, while asbestos and MWCNT exposure cause mesotheliomas at high frequency [21,22].

Fullerene derivatives produce an excited triplet state by photoexcitation, yielding both singlet molecular oxygen and superoxide through energy and electron transfer to molecular oxygen [23]. They may thus cause oxidative DNA damage resulting in cytotoxicity and genotoxicity. In the present study in BALB3T3 cells, however, we did not observe any C_{60} phototoxicity, nor did Shinohara and colleagues find that UV-irradiation affected the in vitro cytotoxicity and genotoxicity of C_{60} [8]. On the other hand, we found that the anatase form (but not the rutile form) of TiO_2 was clearly phototoxic, which is in agreement with the finding of Nakagawa and colleagues who demonstrated in vitro photogenotoxicity of the anatase form of TiO_2 by the comet assay and the chromosomal aberration test [24]. The oxidative stress generated by the photocatalytic activity of anatase TiO_2 may contribute to cell killing and DNA damage [25].

In conclusion, C_{60} did not, by mutagenic or phototoxic activity, cause direct DNA damage in mammalian cells in vitro. It did,

however, induce polyploidy that was probably due to non-DNA interacting mechanisms.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by Health and Labor Sciences Research Grant, Japan, grant number H21-chemical-general-008, and Human Science Foundation, Japan, grant number KHB1006. We think Mr. Suemura in Frontier Carbon Corporation, Tokyo for providing of the C_{60} nanomaterial and its information. We thank Miriam Bloom (SciWrite Biomedical Writing & Editing Services) for professional editing.

References

[1] L. Mazzola, Commercializing nanotechnology, Nat. Biotechnol. 21 (2003) 1137–1143.
[2] R. Paull, J. Wolfe, P. Hebert, M. Sinkula, Investing in nanotechnology, Nat. Biotechnol. 21 (2003) 1144–1147.
[3] T.W. Hesterberg, C.M. Long, C.A. Lapin, A.K. Hamade, P.A. Valberg, Diesel exhaust particulate (DEP) and nanoparticle exposures: what do DEP human clinical studies tell us about potential human health hazards of nanoparticles, Inhal. Toxicol. 22 (2010) 679–694.
[4] H.J. Johnston, G.R. Hutchison, F.M. Christensen, S. Peters, S. Hankin, K. Aschberger, V. Stone, A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: the contribution of physico-chemical characteristics, Nanotoxicology 4 (2010) 207–246.
[5] G. Oberdorster, Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology, J. Intern. Med. 267 (2010) 89–105.
[6] G.W. Gibbs, G. Berry, Mesothelioma and asbestos, Regul. Toxicol. Pharmacol. 52 (2008) S223–S231.
[7] H. Nagai, S. Toyokuni, Biopersistent fiber-induced inflammation and carcinogenesis: lessons learned from asbestos toward safety of fibrous nanomaterials, Arch. Biochem. Biophys. 502 (2010) 1–7.
[8] N. Shinohara, K. Matsumoto, S. Endoh, J. Maru, J. Nakanishi, In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles, Toxicol. Lett. 191 (2009) 289–296.
[9] OECD Guideline for Testing of Chemicals No. 473, In Vitro Mammalian Chromosome Aberration Tests, Paris, 1997, Available at: http://www.oecd.org/env/testguidelines.
[10] H. Kusakabe, K. Yamakage, S. Wakuri, K. Sasaki, Y. Nakagawa, M. Watanabe, M. Hayashi, T. Sofuni, H. Ono, N. Tanaka, Relevance of chemical structure and cytotoxicity to the induction of chromosome aberrations based on the testing results of 98 high production volume industrial chemicals, Mutat. Res. 517 (2002) 187–198.
[11] OECD Guideline for Testing of Chemicals No. 432, In Vitro 3T3 NRU Phototoxicity Test, Paris, 2004, Available at: http://www.oecd.org/env/testguidelines.
[12] H. Spielmann, M. Balls, M. Brand, B. Doring, H.G. Holzhutter, S. Kalweit, G. Klecak, H.L. Eplattenier, M. Liebsch, W.W. Lovell, T. Maurer, F. Moldenhauer, L. Moore, W.J. Pape, U. Pfanenbecker, J. Potthast, O. De Silva, W. Steiling, A. Willshaw, EEC/COLIPA project on in vitro phototoxicity testing: first results obtained with a Balb/c 3T3 cell phototoxicity assay, Toxicol. In Vitro 8 (1994) 793–796.
[13] N. Singh, B. Manshian, G.J. Jenkins, S.M. Griffiths, P.M. Williams, T.G. Maffeis, C.J. Wright, S.H. Doak, NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials, Biomaterials 30 (2009) 3891–3914.
[14] Y. Totsuka, T. Higuchi, T. Imai, A. Nishikawa, T. Nohmi, T. Kato, S. Masuda, N. Kinae, K. Hiyoshi, S. Ogo, M. Kawanishi, T. Yagi, T. Ichinose, N. Fukumori, M. Watanabe, T. Sugimura, K. Wakabayashi, Genotoxicity of nano/microparticles in in vitro micronuclei, in vivo comet and mutation assay systems, Part. Fibre Toxicol. 6 (2009) 23.
[15] A. Xu, Y. Chai, T. Nohmi, T. Hei, Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells, Part. Fibre Toxicol. 6 (2009) 3.
[16] T. Mori, H. Takada, S. Ito, K. Matsubayashi, N. Miwa, T. Sawaguchi, Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis, Toxicology 225 (2006) 48–54.
[17] N.R. Jacobsen, G. Pojana, P. White, P. Moller, C.A. Cohn, K.S. Korsholm, U. Vogel, A. Marcomini, S. Loft, H. Wallin, Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade markMouse lung epithelial cells, Environ. Mol. Mutagen. 49 (2008) 476–487.
[18] C.G. Jensen, L.C. Jensen, C.L. Rieder, R.W. Cole, J.G. Ault, Long crocidolite asbestos fibers cause polyploidy by sterically blocking cytokinesis, Carcinogenesis 17 (1996) 2013–2021.
[19] M. Asakura, T. Sasaki, T. Sugiyama, M. Takaya, S. Koda, K. Nagano, H. Arito, S. Fukushima, Genotoxicity and cytotoxicity of multi-wall carbon nanotubes in cultured Chinese hamster lung cells in comparison with chrysotile A fibers, J. Occup. Health 52 (2010) 155–166.
[20] I.D. Mitchell, J.B. Carlton, M.Y. Chan, A.A. Robinson, J. Sunderland, Noscapine-induced polyploidy in vitro, Mutagenesis 6 (1991) 479–486.
[21] A. Takagi, A. Hirose, T. Nishimura, N. Fukumori, A. Ogata, N. Ohashi, S. Kitajima, J. Kanno, Induction of mesothelioma in p53+/– mouse by intraperitoneal application of multi-wall carbon nanotube, J. Toxicol. Sci. 33 (2008) 105–116.
[22] K. Donaldson, F.A. Murphy, R. Duffin, C.A. Poland, Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma, Part. Fibre Toxicol. 7 (2010) 5.
[23] B. Zhao, Y.Y. He, C.F. Chignell, J.J. Yin, U. Andley, J.E. Roberts, Difference in phototoxicity of cyclodextrin complexed fullerene [(gamma-CyD)2/C60] and its aggregated derivatives toward human lens epithelial cells, Chem. Res. Toxicol. 22 (2009) 660–667.
[24] Y. Nakagawa, S. Wakuri, K. Sakamoto, N. Tanaka, The photogenotoxicity of titanium dioxide particles, Mutat. Res. 394 (1997) 125–132.
[25] C.Y. Jin, B.S. Zhu, X.F. Wang, Q.H. Lu, Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells, Chem. Res. Toxicol. 21 (2008) 1871–1877.

Apoptosis
DOI 10.1007/s10495-012-0698-1

ORIGINAL PAPER

Molecular mechanisms of apoptosis induction by 2-dodecylcyclobutanone, a radiolytic product of palmitic acid, in human lymphoma U937 cells

Da-Yong Yu · Qing-Li Zhao · Masakazu Furuta · Setsuko Todoriki · Keisuke Izumi · Kohji Yamakage · Kozo Matsumoto · Takaharu Nomura · Takashi Kondo

Abstract The irradiation of fat-containing food forms 2-dodecylcyclobutanone (2-DCB) from palmitic acid (PA). In this study, we investigated whether 2-DCB and PA induce apoptosis in human lymphoma U937 cells. We found that cell viability decreased by 2-DCB and apoptosis was induced by 2-DCB and PA. 2-DCB and PA significantly enhanced the formation of intracellular reactive oxygen species (ROS). Apoptosis induced by 2-DCB and PA was strongly prevented by an antioxidant, *N*-acetyl-L-cysteine. The treatment with 2-DCB and PA resulted in the loss of mitochondrial membrane potential, and Fas, caspase-8 and caspase-3 activation. Pretreatment with a pan-caspase inhibitor (z-VAD) significantly inhibited apoptosis induced by 2-DCB and PA. Moreover, 2-DCB and PA also induced Bax up-regulation, the reduction in Bcl-2 expression level, Bid cleavage and the release of cytochrome *c* from the mitochondria to the cytosol. In addition, an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was observed after the treatment with 2-DCB and PA. Our results indicated that intracellular ROS generation, the modulation of the Fas-mitochondrion-caspase-dependent pathway and the increase in $[Ca^{2+}]_i$ involved in apoptosis are induced by 2-DCB and PA in U937 cells.

Keywords 2-Dodecylcyclobutanone · Palmitic acid · Apoptosis · Reactive oxygen species · Calcium

Abbreviations

2-DCB	2-Dodecylcyclobutanone
PA	Palmitic acid
ROS	Reactive oxygen species
NAC	*N*-Acetyl-L-cysteine
MMP	Mitochondrial membrane potential
z-VAD	Pan-caspase inhibitor

D.-Y. Yu · Q.-L. Zhao · T. Kondo (✉)
Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan
e-mail: kondot@med.u-toyama.ac.jp

M. Furuta
Laboratory of Quantum-Beam Chemistry and Biology, Radiation Research Center, Osaka Prefecture University, 1-2 Gakuen-cho, Sakai, Osaka 599-8570, Japan

S. Todoriki
Food Safety Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

K. Izumi
Department of Molecular and Environmental Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

K. Yamakage
Division of Alternative Toxicology Test, Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hatano, Kanagawa 257-8523, Japan

K. Matsumoto
Department of Animal Medical Sciences, Faculty of Life Sciences, Kyoto Sangyo University, Motoyama Kamigamo, Sakyo-ku, Kyoto 603-8555, Japan

T. Nomura
Radiation Safety Research Center, Central Research Institute of Electric Power Industry, Komae 201-8511, Japan

Published online: 04 February 2012

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
2-ACBs	2-Alkylcyclobutanones
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
PI	Propidium iodide
FITC	Fluorescein isothiocyanate
HE	Hydroethidine
H_2DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
TMRM	Tetramethylrhodamine methyl ester

Introduction

Food irradiation has been considered as a safe processing technology for improving food safety and reducing pathogen population. When food is treated with ionizing radiation, a group of 2-alkylcyclobutanones (2-ACBs) is formed [1]. These alkylcyclobutanones are unique radiolytic products from fatty acids [2], and contain the same number of carbon atoms as their precursor fatty acids, the alkyl group is located in ring position 2 [3] (Fig. 1). 2-Dodecylcyclobutanone (2-DCB) derived from PA is radiation-specific and has never been detected in any non irradiated or microbiologically spoiled food; therefore, there is increasing interest in 2-DCB in food toxicology.

Previous experiments using comet assay and measuring single-strand DNA breaks indicated a slight genotoxic potential of 2-DCB in both rat and human colon cells in an in vitro study [4], as well as in an in vivo experiment using rat colon cells [5]. In addition, DNA damage has been detected by comet assay and an increased frequency of translocations has been determined by 24-color fluorescence in situ hybridization predominantly in LT97 human colon adenoma cells as well as in primary human colon cells after treatment with 2-DCB [6]. Recently, the cytotoxic and genotoxic potentials of various highly pure synthetic 2-ACBs were studied in bacteria (*Salmonella* strains TA97, TA98, and TA100) and human colon cell lines.

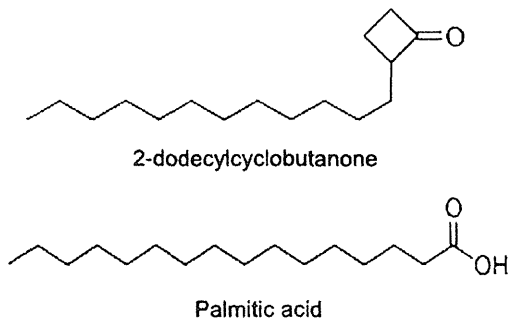


Fig. 1 Chemical structures of 2-DCB and PA

Although pronounced cytotoxicity is evident in bacteria, no mutagenic activity has been revealed by the Ames test in the *Salmonella* strains TA97, TA98, and TA100. In mammalian cells, genotoxicity has been demonstrated mainly by the induction of DNA base damage recognized by the formamidopyrimidine–DNA glycosylase (Fpg), as determined by the comet assay. The degrees of cytotoxicity and genotoxicity are dependent on fatty acid chain length and the degree of unsaturation of the fatty acid chain [7]. Recent studies showed that PA could induce apoptosis in human lung carcinoma cells, hepatocytes, renal cells, smooth muscle cells and endothelial progenitor cells [8–12]. The mechanisms of apoptosis induced by PA are involved in endoplasmic reticulum stress, mitochondrial dysfunction, PKC activation and increased oxidative stress [10, 13–16]. However, the effects of 2-DCB, a radiolytic product of PA, on apoptosis induction have never been shown.

In this study, we investigate the effects of 2-DCB and PA on apoptosis and the molecular mechanism of such apoptosis in human lymphoma U937 cells. To our knowledge, this is the first report on apoptosis induced by 2-DCB.

Materials and methods

Chemicals

Pure 2-DCB was synthesized by Hayashi Pure Chemical Inc. (Osaka, Japan). Palmitic acid (PA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-DCB and PA were dissolved in acetone to achieve the desired concentrations for experimental use. Maximum concentration of acetone is 0.5%. And we found that low concentration acetone (0–1.0%) have no toxicity in U937 cells (data not shown). We used untreated cells as control.

Cell line and culture

A human lymphoma cell line (U937) (obtained from Human Sciences Research Resource Bank, Tokyo) was maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, USA). Human colon carcinoma cells (HCT116) (purchased from DS Pharma Biomedical Co., Ltd., Osaka, Japan) were cultured in Macoy's 5A (Gibco, Grand Island, NY) with 10% FBS. The human cervical cancer cell line, HeLa cells (obtained from Human Sciences Research Resource Bank, Tokyo) were maintained in MEM with 10% FBS at 37°C in humidified air with 5% CO_2. The culture medium for all the cell lines except for U937 cells were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Measurement of cell viability

Cell viability induced by 2-DCB was determined by MTT assay (Sigma), as described previously [17].

Assessment of apoptosis

To detect early apoptosis and secondary necrosis, the Annexin V-FITC kit (Beckman Coulter, Inc. Miami, FL) was used according to the manufacturer's instructions. Briefly the cells were stained simultaneously with propidium iodide (PI) and fluorescein isothiocyanate (FITC) labeled Annexin V and assessed by flow cytometry (Beckman-Coulter EPICS XL^{TM}). The morphology of nuclei in cells treated with 2-DCB was examined by florescence microscopy (ELIPSE E600, Nikon, Tokyo, Japan) with Hoechst 33258 staining. The method was described in detail in a previous paper [18].

Measurement of intracellular reactive oxygen species (ROS)

Hydroethidine (HE, Molecular Probes, Eugene, OR) was used to determine superoxide generation using the method of Gorman et al. [19]. Briefly, the cells were incubated with 5 μM HE for 15 min at 37°C. Intracellular levels of superoxide anion radicals were assessed by flow cytometry with excitation and emission wavelengths of 488 and 600 nm, respectively.

The detection of intracellular peroxide (H_2O_2) was performed by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes). After treatment, H_2DCF-DA was added to the cells at a final concentration of 5 μM, and the cells were incubated at 37°C for 30 min. The cells were then harvested and the fraction of DCF-positive cells was determined by flow cytometry (excitation at 488 nm; emission at 525 nm) as the proportion of cells containing intracellular peroxide [20, 21].

Measurement of mitochondrial membrane potential (MMP)

To measure changes in MMP, U937 cells were stained with 10 nM tetramethylrhodamine methyl ester (TMRM) (Molecular Probes) for 15 min at 37°C in PBS containing 1% fetal bovine serum. The fluorescence of red TMRM was analyzed by flow cytometry (excitation at 488 nm; emission at 575 nm). The percentage of low-MMP cells was determined from the cell count falling into the 0.1–12 low window of the TMRM log scale [20].

Assessment of intracellular caspase-3 and caspase-8 activities

The CaspGlow^{TM} Fluorescein Active Caspase-3 Staining kit (MBL, Nagoya, Japan) was used to monitor the intracellular caspase-3 activity following the manufacturer's instructions. Briefly, the cells (1×10^6/ml) were subjected to the treatment. 300 μl of each of the samples and control cultures was placed into a microtube, and 1 μl of FITC-DEVD-FMK was added into each tube, followed by incubation for 0.5 h at 37°C in a 5% CO_2 incubator. Then the samples were analyzed by flow cytometry.

To measure caspase-8 activity, we used the FLICE/Caspase-8 Colorimetric Protease Assay kit (MBL, Nagoya, Japan). The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the labeled substrate IETD-pNA. pNA light emission can be quantified using a spectrophotometer at 400 nm (Beckman Instruments Inc., Fullerton, CA) [22].

Western blot analysis

The cells were collected, washed with cold PBS and lysed at a density of 10^6 cells/50 μl of lysis buffer. After a brief sonication, the lysates were centrifuged at 12,000×*g* 10 min at 4°C, and the protein content of the supernatant was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). The protein lysates were denatured at 96°C for 5 min after mixing with 5 μl of SDS-loading buffer, applied on an SDS polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) for electrophoresis, and transferred to nitrocellulose membranes (Amersham Biosciences, Buchinghamshire, UK). Western blot analysis was carried out to detect the expressions of Bcl-2, Bax, Bid and β-actin using specific anti-Bcl-2 monoclonal antibody mAb (Santa Cruz Biotech, Santa Cruz, CA), anti-Bax polyclonal antibody pAb (Santa Cruz), anti-Bid pAb (Cell Signaling Technology, Beverly, MA), and anti-β-actin mAb (Sigma), respectively. With secondary HRP (horseradish peroxidase)-conjugated anti-rabbit and anti-mouse IgGs, band signals were visualized on an X-ray film using chemiluminescence ECL detection reagents (Amersham Biosciences) [23].

To prepare the cytosolic extract, the cells were harvested and suspended in an ice-cold solution containing 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 250 mM sucrose. The cells were disrupted using a Dounce homogenizer. The samples were centrifuged at 1,500×*g* for 5 min at 4°C to remove nuclei and intact cells. The supernatant was centrifuged at 105,000×*g* for 30 min at 4°C. The resulting supernatant

was used as the soluble cytosolic fraction. The protein content of the cytosolic fraction was determined as described earlier. Following SDS-PAGE, Western blotting was performed to detect the cytochrome *c* released to the cytosol using anti-cytochrome *c* pAb (Santa Cruz Biotech) and anti-β-actin mAb (Sigma).

Flow cytometric detection of Fas on the cell surface

The cells were washed twice with PBS, suspended in 20 μl of washing buffer containing 2.5 μg/ml FITC-labeled anti-Fas monoclonal antibody (clone: UB3, MBL), incubated for 30 min at 37°C, and then analyzed by flow cytometry [24].

Determination of intracellular concentration of calcium ions in single cells

The method of determining $[Ca^{2+}]_i$ was described previously [24]. The cells were collected by centrifugation and washed with HEPES-buffered Ringer (HR) buffer. Then the cells were suspended in supplemented HR, and loaded with 5 μM Fura-2-AM (Dojindo Lab, Bethesda, MD) for 30 min at 25°C. After washing the cells, digital images of Fura-2 fluorescence were acquired and analyzed using a digital image processor (Argus 50/Ca, Hamamatsu Photonics, Hamamatsu, Japan) coupled to an inverted fluorescence microscope. The ratio of the 510 nm emission fluorescence at 340 nm excitation to that at 380 nm excitation, F (340/380), was used as an indicator of $[Ca^{2+}]_i$ in single cells. Fura-2 pseudocolor images of individual cells were also obtained.

Statistics

The results are expressed as means ± SD. Significance of differences between means was tested using Student's *t* test and assumed for $p < 0.05$. All the experiments were performed in triplicate.

Results

Effects of 2-DCB and PA cell viability and apoptosis

We measured cell viability cells induced by 2-DCB (0, 25, 50, 100, 200 and 400 μM) for 12 and 24 h in U937 cells. As illustrated in Fig. 2, 2-DCB (25–400 μM) significantly inhibited cell viability for 24 h in U937 cells ($p < 0.05$). And 2-DCB induced more cell viability decrease for 24 h compared to that for 12 h in the concentration range 25–100 μM. The IC50 values (amount of 2-DCB to inhibit cell viability by 50%) is 53.9 μM for 12 h and 29.5 μM for 24 h on U937 cells. 2-DCB induced a significant decrease in viability in a dose- and time-dependent manner in U937 cells.

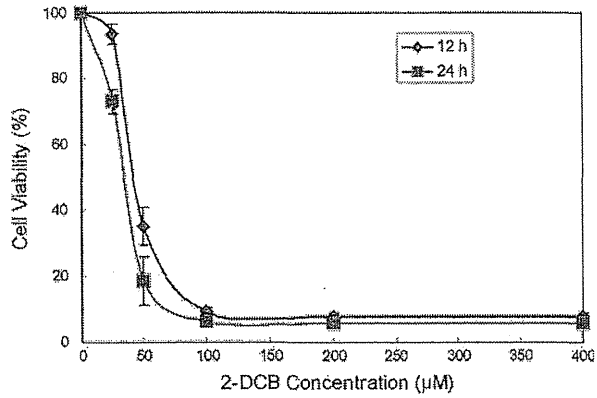


Fig. 2 2-DCB inhibited the cell viability of U937 cells. Cells were treated with 2-DCB at various concentrations for 12 and 24 h. Cell viability rate was estimated by MTT assay. The results are expressed as means ± SD ($n = 3$)

The combination of Annexin-FITC with PI has been used extensively to distinguish living cells in early and late apoptosis. In this study, we measured the early apoptosis (Annexin-V FITC positive and PI negative) and late apoptosis (Annexin-V FITC and PI double positive). Then we got the apoptosis (early apoptosis plus late apoptosis) induced by 2-DCB (50, 100 and 200 μM) and PA (500 μM) for 12 and 24 h in U937 cells. Flow cytometry using Annexin V-FITC and PI staining revealed that the percentages of apoptosis after the treatments with 2-DCB and PA were significantly higher than that of the control cells (Fig. 3a) ($p < 0.05$). As shown in Fig. 3b, early apoptosis (green fluorescence) and late apoptosis (green and red fluorescence) induced by 200 μM 2-DCB treatment for 24 h were observed under fluorescence microscopy. The changes in cell morphology after treatment with 200 μM 2-DCB were examined by fluorescence microscopy after staining with Hoechst 33258. Hoechst 33258 staining revealed that nuclei with chromatin condensation and apoptotic bodies were formed in cells that were treated with 2-DCB; however, these features were present in only a few control cells (Fig. 3c). In addition, the effects of 2-DCB on HCT116 and HeLa cells after 24 h were observed. 2-DCB decreased cell viability in a dose-dependent manner in HCT116 cells. We found that 200 μM 2-DCB led to an almost 60% cell killing in the HCT116 cells after 24 h. Also 200 μM 2-DCB induced a significant enhancement of apoptosis (Annexin V-FITC and PI double staining) in the HCT116 and HeLa cells ($p < 0.05$).

Generation of ROS by 2-DCB and PA in U937 cells

Our previous studies of the above chemical compounds showed that ROS play a key role in the apoptosis of U937 cells [20, 22–26]. Therefore, we determined the effects of 2-DCB and PA on ROS production by flow cytometry. Our results demonstrated that 2-DCB and PA significantly