

Food-Borne Radiolytic Compounds (2-Alkylcyclobutanones) May Promote Experimental Colon Carcinogenesis

Francis Raul, Francine Gossé, Henry Delincée, Andrea Hartwig, Eric Marchioni,
Michel Miesch, Dalal Werner, and Dominique Burnouf

Abstract: Food irradiation is acknowledged as a safe process to improve food quality by reducing microbial contamination. Information on the toxicological potential of 2-alkylcyclobutanones (2-ACBs), radiolytic derivatives of triglycerides found exclusively in irradiated food, is scarce. Wistar rats received daily a solution of highly pure 2-tetradecylcyclobutanone (2-tDCB) or 2-(tetradec-5'-enyl)-cyclobutanone (2-tDeCB) at a concentration of 0.005% in 1% ethanol as drinking fluid, while control animals received 1% ethanol. All animals received a single intraperitoneal injection of the chemical carcinogen azoxymethane (AOM) at Weeks 3 and 4. At 3 mo after AOM injection, no significant changes were observed in the total number of preneoplastic lesions in the colon of AOM controls and 2-ACB-treated animals. After 6 mo, the total number of tumors in the colon was threefold higher in the 2-ACB-treated animals than in the AOM controls. The colon of four of six AOM control rats exhibited only one small tumor (~6 mm³). Multiple tumors were observed in four and three of six animals treated with 2-tDCB or 2-tDeCB, respectively. Medium (6 < S < 25 mm³) and larger (>25 mm³) tumors were detected only in 2-ACB-treated animals. This is the first demonstration that a compound found exclusively in irradiated dietary fats may promote colon carcinogenesis in animals treated with a chemical carcinogen.

Introduction

Food irradiation is considered a highly effective processing technology to improve and maintain food safety. Application of this process to food products effectively reduces the number of microbial pathogens, which are annually responsible for millions of food-borne illnesses worldwide (1). Furthermore, nutritional, genetic, and toxicological studies of shelf-stable chicken sterilized by ionizing radiation have failed to reveal any evidence of genotoxic effects in mice, rats, and rabbits (2).

Thirty years ago, LeTellier and Nawar (3) reported that a family of compounds, namely, the 2-alkylcyclobutanones (2-ACBs), is produced by high-dose irradiation of synthetic triglycerides. More recently, Stevenson et al. (4) detected these compounds in irradiated fat-containing food, such as minced chicken meat. Further work confirmed the presence of 2-ACBs in other irradiated meats such as pork, lamb, beef, and mechanically recovered meat, as well as in irradiated liquid whole egg (5). Recently, the 2-ACBs were identified in irradiated cheese, chicken, beef, fish (sardines and trout), mango, and rice (6), in mango, papaya, Camembert, and salmon (7), and in rice irradiated at a very low dose (0.1 kGy) and in pre-cooked meals containing small amounts of irradiated ingredients (8). The 2-ACBs are formed during the irradiation of fat-containing foods as a result of the radiation-induced cleavage of triglycerides. The 2-ACBs have the same number of carbons (n) as their fatty acid precursors, with an alkyl chain of (n-4) carbons in ring position 2 (4). They have been found exclusively in irradiated fat-containing food and have not been detected in nonirradiated foods treated by other food processes such as freezing, heating, microwave heating, ultraviolet irradiation, high-pressure processing, or simple preservation treatments (5,6,9,10). These compounds are thus considered unique markers for food irradiation. However, it would no doubt be more cautious to state that the amounts of 2-ACBs possibly present in the nonirradiated foodstuffs are always below the limit of detection of the analytical method used. Concerns about the safety of this group of radiolytic compounds have been raised with regard to public health risk, despite the fact that only very small amounts are present in the human diet. For example, the amount of 2-ACBs ingested by humans consuming 200 g of irradiated (at 3 kGy) chicken meat can be estimated to be ~80 µg. Preliminary results have indicated slight genotoxic effects of 2-dodecylcyclobutanone in vitro (11) and in vivo (12) studies. However, longer in vivo studies of the genotoxic potentials of 2-ACBs in animals have

F. Raul and F. Gossé are affiliated with the Laboratoire d'Oncologie Nutritionnelle, and D. Burnouf with the Groupe d'Epidémiologie Moléculaire du Cancer, F-67000 Strasbourg, France. H. Delincée is affiliated with the Bundesforschungsanstalt für Ernährung, D-76131 Karlsruhe, Germany. A. Hartwig is affiliated with the Institut für Lebensmittelchemie und Toxikologie, Universität Karlsruhe, D-76128 Karlsruhe, Germany. E. Marchioni is affiliated with the Laboratoire de Chimie Analytique et Sciences de l'Aliment (UMR 7512), Faculté de Pharmacie, Université Louis Pasteur, F-67400 Illkirch, France. M. Miesch is affiliated with the Laboratoire de Chimie Organique Synthétique (UMR 7123), Faculté de Chimie, Université Louis Pasteur, F-67000 Strasbourg, France. D. Werner is affiliated with Aériat, F-67300 Schiltigheim, France.

not been performed. Therefore, the aim of the present study was to determine whether the 2-ACBs, which can be considered markers for food irradiation, modulate carcinogenesis in an experimental animal model of colon carcinogenesis.

Materials and Methods

Chemicals

The 2-ACBs used for the experimental study were 2-tetradecylcyclobutanone (2-tDCB, derived from stearic acid) and 2-(tetradec-5'-enyl)-cyclobutanone (2-tDeCB, derived from oleic acid). These compounds were obtained as follows: the 2,2-dimethylhydrazone derived from cyclobutanone was treated with a base, and, after addition of the required primary alkyl halide (1-bromotetradecane for 2-tDCB and 1-bromotetradec-5-ene for 2-tDeCB), an acidic hydrolysis delivered the desired 2-ACB in a highly pure state (13,14).

Animals and Housing

The experiments were conducted according to National Research Council guidelines for the use and care of laboratory animals (authorization no. 67-49, French Ministry of Agriculture and Fishery).

Male Wistar rats ($n = 36$) weighing 260–270 g were housed under standardized conditions (22°C, 60% relative humidity, 12:12-h light-dark cycle, 20 air changes/h). The rats were randomly divided into three groups (12 rats/group), which received ad libitum the same isoenergetic diet (UAR A04, Villemoisson/Orge, France). This nonirradiated diet contained 16% protein as casein and fish protein, 60% carbohydrate as wheat starch, 3% lipid as soy and fish oil, 6% salt mixture, and 1% vitamin mixture. The fatty acid composition of the diet was palmitic acid at 2.6 mg/g, stearic acid at 0.5 mg/g, oleic acid at 8 mg/g, and linoleic acid at 14.5 mg/g.

Experimental Design

The two experimental groups received a freshly prepared aqueous solution of highly pure 2-tDCB or 2-tDeCB at a concentration of 0.005% (wt/vol) in 1% ethanol as drinking fluid daily at 5 PM throughout the experimental procedure; control animals received 1% ethanol. Fluid consumption was recorded daily, and the average consumption of 2-tDCB or 2-tDeCB was ~1.6 mg per rat per day. The 2-ACB molecules did not undergo any molecular transformation in 1% ethanol as observed with a ^1H nuclear magnetic resonance spectrum (200 MHz, CDCl_3) as well with an infrared spectrum (CCl_4).

The body weight was measured once a week throughout the experimental period. After an initial period of 2 wk, all rats were injected with azoxymethane (AOM, 15 mg/kg body wt ip) once a week for 2 wk (at Weeks 3 and 4). AOM is a chemical carcinogen that sequentially induces colon preneoplastic lesions [aberrant crypt foci (ACF)], adenomas, and adenocarcinomas in rats (15).

Assessment of Aberrant Crypts and Tumors in the Colon

At 3 and 6 mo after the last AOM treatment, six animals of each group were sacrificed, and the total number of aberrant crypts and ACF in the distal colon was recorded. The number and size of tumors throughout the colonic mucosa were measured under a dissecting microscope or a microscope with a low-power objective.

Determination of aberrant crypts was performed on a 5-cm-long segment corresponding to the distal half of the colon. These preneoplastic lesions, when present, are detectable only in the distal flat colonic mucosa. The segment was washed with physiological saline, cut open, pinned out flat, and fixed in 10% buffered formalin. The colon samples were stained with 0.2% methylene blue for 5 min, rinsed in Krebs-Ringer buffer, placed on a glass slide, and examined microscopically using a low-power objective ($\times 5$) for assessment of the number of aberrant crypts following a procedure described previously (16). All measurements were made by a blinded observer. The criteria for the identification of aberrant crypts were 1) increased size, 2) thicker epithelial cell lining, and 3) increased pericryptal zone relative to normal crypts.

Statistics

Values are means \pm SE. Statistical differences between groups were evaluated by one-way analysis of variance, and specific differences were identified using the Student's *t*-test.

Results

The two compounds (2-tDCB or 2-tDeCB) given at a concentration of 0.005% in the drinking fluid showed no toxic or adverse effects on rat growth and behavior throughout the experimental period. The body weight gain was similar for the 2-tDCB, 2-tDeCB, and AOM control groups [412 ± 30 , 437 ± 18 , and 416 ± 35 (SE) g, respectively] throughout the experimental period. These observations are in accordance with previous studies (2) showing that no acute toxicity was observed in animals fed a diet containing high-dose-irradiated chicken meat (i.e., 2-ACBs).

Assessment of the number of ACF is an early indicator of tumorigenic processes. At 3 mo after AOM injection, the surface density, expressed as the number of ACF per centimeter of colon, and the total number of aberrant crypts were similar in the AOM control, 2-tDCB, and 2-tDeCB groups (Table 1). These data indicate that, within 3 mo after injection of the carcinogen, neither 2-tDCB nor 2-tDeCB enhanced the number of preneoplastic lesions induced by AOM injection at this stage.

However, at 6 mo after AOM treatment, a significant increase (+80%) in the total number of aberrant crypts was observed in animals treated with 2-tDeCB (394 ± 37 vs. 218 ± 39 in AOM controls). In the group treated with 2-tDCB, the total number of aberrant crypts (261 ± 42) was not signifi-

Table 1. Preneoplastic Lesions (ACF and Total Number of ACs per Colon) in the Mucosa of the Distal Half (5 cm) of the Colon^{a,b}

Groups	No. of ACF/cm		Total No. of ACs	
	3 mo	6 mo	3 mo	6 mo
Controls	18 ± 2	14 ± 2	253 ± 24	218 ± 39*
+2-tDCB	17 ± 3	17 ± 2	221 ± 58	261 ± 42*
+2-tDeCB	20 ± 3	19 ± 2	260 ± 32	394 ± 37

a: Values are means ± SE of 6 rats/group at each time period (3 and 6 mo). ACF, aberrant crypt; ACF, AC foci; 2-tDCB, 2-tetradecylcyclobutanone; 2-tDeCB, 2-(tetradec-5'-enyl)-cyclobutanone.

b: Statistical significance is as follows: *, $P < 0.05$ vs. 2-tDeCB.

cantly enhanced compared with the control group (Table 1). Similarly, a higher number of ACF containing more than four aberrant crypts was recorded in the colon of 2-tDeCB-treated rats, whereas this effect was less marked in animals receiving 2-tDCB (Table 2). In contrast, the number of ACF was not increased significantly in the colon of rats receiving 2-tDCB or 2-tDeCB compared with the AOM control group (Table 1). These observations indicate that the size of the ACF was increased in the colon of rats receiving 2-ACBs because of an increased number of aberrant crypts per foci.

Tumors were only detected 6 mo after AOM treatment. Although no significant differences were observed in tumor incidence between the groups, individual differences in the total number and size of tumors were observed between AOM controls and 2-ACB-treated rats. The total number of tumors was 4 in the AOM control group, 14 in the 2-tDCB group, and 13 in the 2-tDeCB group. Only one small tumor was detected in four of six AOM control rats as a result of carcinogenesis initiation by the chemical carcinogen (Fig. 1). Their size never exceeded 6 mm³, and never was there more than one tumor per rat. In contrast, in the colon of rats additionally receiving 2-ACBs, the number of tumors increased dramatically compared with AOM controls. In the group receiving 2-tDCB, five animals exhibited tumors, and, among them, four rats presented multiple tumor formations. In the 2-tDeCB group, the colon of four animals exhibited tumors, with three rats showing multiple tumor formations. Medium (6 < S < 25 mm³) and large (>25 mm³) tumors were detected only in 2-ACB-treated animals.

Table 2. Distribution of ACF Containing 1, 2, 3, 4, or More Individual ACs in the Distal Half of the Colon^{a,b}

Groups	No. of AC per ACF				
	1	2	3	4	>4
Controls	24.5 ± 4	29.5 ± 3	23.6 ± 2	11.4 ± 2	11 ± 2
+2-tDCB	22 ± 2	32 ± 3	20 ± 2	13.5 ± 1	12.5 ± 2
+2-tDeCB	13 ± 2	30.3 ± 3	20.7 ± 2	14 ± 2	22 ± 3*

a: Values are means ± SE in percent of 6 rats/group 6 mo after azoxymethane (AOM) injection.

b: Statistical significance is as follows: *, significantly different from controls ($P < 0.05$).

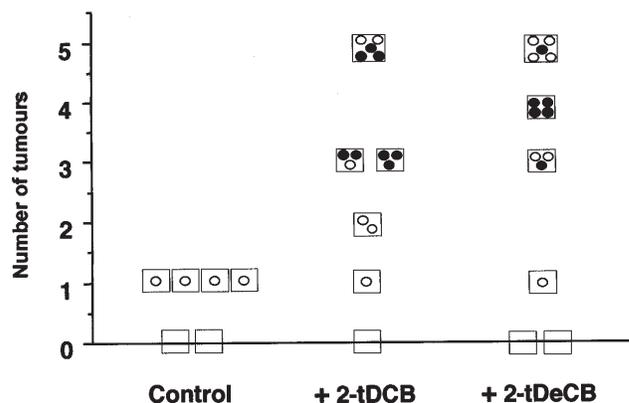


Figure 1. Number and size of colonic tumors observed in 2-alkylcyclobutanone (2-ACB)-treated and control rats 6 mo after azoxymethane treatment. 2-tDCB, 2-tetradecylcyclobutanone; 2-tDeCB, 2-(tetradec-5'-enyl)-cyclobutanone. Squares represent individual animals; open, grey, and black circles represent small (<6 mm³), medium (6 < S < 25 mm³), and large (>25 mm³) colonic tumors, respectively. Multiple medium and large tumors were observed only in 2-ACB-treated animals.

Discussion

Administration of AOM to rodents causes numerous morphological changes, ranging from normal intestinal epithelium to carcinoma, that are biologically and histologically quite similar to those seen in humans (17). Because of the potential progression of early changes to malignancy, the study of preneoplastic lesions (aberrant crypts) and tumor formation is crucial for the understanding of the pathogenesis of colon cancer. In this regard, identification of dietary constituents that are able to enhance or suppress the premalignant processes is an important issue in cancer prevention. The data reported in this study suggest that 2-ACBs may not initiate colon carcinogenesis per se, because the number of preneoplastic lesions 3 mo after AOM treatment was not significantly different between animals treated solely with AOM and those also receiving 2-ACBs. However, in the long term (>6 mo), the 2-ACBs potentiated the effects of the chemical carcinogen, as revealed by the development of preneoplastic ACF of larger size. These larger ACF, resulting from a higher number of aberrant crypts per foci, exhibit an increase in their growth autonomy and represent a more advanced stage of preneoplastic development (18). One may assume that 2-ACBs favor progression of the preneoplastic process by stimulating growth of preexisting ACF. This seems to be corroborated by a threefold increase in the number of tumors in the colon of 2-ACB-treated animals compared with AOM controls. Therefore, the main conclusion of this study is that 2-ACBs, which are unique radiolytic products formed specifically in irradiated foods, may be promoters of intestinal tumor formation. In a previous study carried out in the laboratory, Horvatovich et al. (19) specifically detected the 2-ACBs (2-tDCB or 2-tDeCB) fed to treated animals in body fat, and they also showed that ~1% of the ingested daily amount of 2-ACBs was not absorbed by the

digestive tract and remained in the feces, in direct contact with the colonic mucosa. This continuous exposure of the epithelial cells to the chemical could also account for the induction of colon tumors in the exposed animals.

Several fatty acids have been implicated in the modulation of tumorigenesis. In the present study, the fatty acid precursors stearic acid for 2-tDCB and oleic acid for 2-tDeCB do not seem to be involved in the procarcinogenic effects of the 2-ACBs. Indeed, it was reported that stearic and oleic acids inhibited tumor cell growth in vitro (20,21). Both fatty acids also exhibited antitumoral effects in vivo, either in a murine model (22) or in AOM-induced colon carcinogenesis in rats (23), a model similar that used in the present study. Therefore, the effects we observed on colon carcinogenesis appear to be directly related to the 2-ACB molecules.

The present report is the first demonstration that pure compounds, known to be exclusively produced on irradiation in dietary fats, may promote colon carcinogenesis in animals. The relevancy of these results for the risk assessment of human consumption of irradiated food remains to be elucidated. It must be emphasized that the daily amount of pure 2-ACB administered to rats corresponds to a pharmacological dose (3.2 mg/kg body wt), which is not comparable to the amount ingested by humans eating irradiated food products, which can be estimated to be $\leq 5\text{--}10\ \mu\text{g}/\text{kg}$ body wt. In addition, these food products may also contain several components that may reduce the bioavailability of 2-ACBs. The benefits of food irradiation to protect public health against food-borne pathogenic bacteria are becoming increasingly recognized (24). In light of the expected extended application of food irradiation, however, it seems necessary to further clarify the potential toxicity of 2-ACBs and their contribution to a possible risk associated with human consumption of irradiated fat-containing food.

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This work was supported in part by INTERREG II, Upper Rhine Centers Southern Programme Project 3.171. Address correspondence to F. Raul, 1, place de l'Hôpital, 67091 Strasbourg-Cedex, France. Phone: (33) 3 88 11 90 23. FAX: (33) 3 88 11 90 97. E-mail: francis.raul@ircad.u-strasbg.fr.

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