

Absorption and Metabolism of *cis-9,trans-11-CLA* and of Its Oxidation Product 9,11-Furan Fatty Acid by Caco-2 Cells

Thorsten Buhrke · Roswitha Merkel ·
Imme Lengler · Alfonso Lampen

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Abstract Furan fatty acids (furan-FA) can be formed by auto-oxidation of conjugated linoleic acids (CLA) and may therefore be ingested when CLA-containing foodstuff is consumed. Due to the presence of a furan ring structure, furan-FA may have toxic properties, however, these substances are toxicologically not well characterized so far. Here we show that 9,11-furan-FA, the oxidation product of the major CLA isomer *cis-9,trans-11-CLA* (c9,t11-CLA), is not toxic to human intestinal Caco-2 cells up to a level of 100 μ M. Oil-Red-O staining indicated that 9,11-furan-FA as well as c9,t11-CLA and linoleic acid are taken up by the cells and stored in the form of triglycerides in lipid droplets. Chemical analysis of total cellular lipids revealed that 9,11-furan-FA is partially elongated probably by the enzymatic activity of cellular fatty acid elongases whereas c9,t11-CLA is partially converted to other isomers such as c9,c11-CLA or t9,t11-CLA. In the case of 9,11-furan-FA, there is no indication for any modification or activation of the furan ring system. From these results, we conclude that 9,11-furan-FA has no properties of toxicological relevance at least for Caco-2 cells which serve as a model for enterocytes of the human small intestine.

Keywords Furan fatty acid · Conjugated linoleic acid · CLA · Caco-2

Abbreviations

BSA Bovine serum albumin
CLA Conjugated linoleic acid
DMEM Dulbecco's modified Eagle's medium

DMSO Dimethylsulfoxide
FCS Fetal calf serum
Furan-FA Furan fatty acid
GC/MS Gas chromatography/mass spectrometry
LNA Linoleic acid

Introduction

Conjugated linoleic acids (CLA) are polyunsaturated fatty acids that are present in many natural food sources. The term CLA describes a group of positional and geometric isomers of linoleic acid (LNA) derivatives having conjugated double bonds. The conversion from LNA to CLA occurs in the rumen of ruminants by bacterial metabolic activity [1]. Therefore, the main source for CLA taken up by humans is foodstuff derived from ruminants such as milk or meat.

Over the last decade the interest in CLA has arisen because various publications suggested that CLA may have many different positive physiological effects. In vitro, CLA inhibited the growth of various human cancer cells such as colon [2], mammary [3] and prostate [4] cancer cell lines. This anticarcinogenic effect was also determined in vivo where supplementation with CLA resulted in a decreased size of chemically or genetically induced tumors in experimental animals (reviewed in [5]). In addition, CLA seem to prevent atherosclerosis [6, 7, 8] and may improve the immune system [9, 10]. On the other hand, some publications describe negative effects of CLA supplementation, i.e. the induction of non-alcoholic hepatic steatosis in mice [11], the decrease of insulin sensitivity in human subgroups [12], or the increase in proinflammatory cytokines in human adipocytes in vitro [13]. In the past decade,

T. Buhrke (✉) · R. Merkel · I. Lengler · A. Lampen
Department of Food Safety, Federal Institute for Risk
Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany
e-mail: thorsten.buhrke@bfr.bund.de

attention has predominantly been drawn on a potential anti-adipogenic role of CLA as these substances are proposed to have a positive effect on body composition by decreasing body fat mass and increasing lean muscle mass (reviewed in [14]). In the case of body composition, the CLA isomer *trans*-10, *cis*-12-CLA (t10,c12-CLA) seems to be responsible for reduction of body fat [15]. On the other hand, it was proposed that the isomer *cis*-9,*trans*-11-CLA (c9,t11-CLA) was more effective concerning the inhibition of tumor development [16]. Milk and meat of ruminant animals contain 28 different CLA isomers, and the most abundant isomer in food is the c9,t11-CLA isomer, which represents up to 80% of total CLA.

Due to the reported positive effects of CLA on body mass composition and despite controversial data and some observed negative effects of CLA, these compounds are already available in numerous formulas of food supplements and are currently being discussed for their use as food additives. CLA are classified as “novel foods” in Europe and a premarketing risk assessment of CLA is a mandatory part of the novel food approval. For a reliable risk assessment of CLA it is necessary to focus additionally on the oxidation products of CLA, the furan fatty acids (furan-FA).

Furan-FA are comprised of a furan ring flanked by one alkyl and one carboxyl side chain in the α -positions of the furan ring. It has been shown that in the presence of air CLA can react with dioxygen to form furan-FA [17] (Fig. 1). Therefore, if CLA-containing food supplements are consumed, significant amounts of furan-FA may be taken up by the consumer in parallel. Up to now, hardly anything is known about the toxicological potential of furan-FA. The presence of the aromatic furan ring system in furan-FA gives rise to the assumption that these compounds may have hazard potential since furan itself and numerous furan derivatives have shown themselves to have properties of toxicological relevance (reviewed in [18]). The aim of our study was to investigate the absorption and the metabolism of furan-FA by human intestinal cells. The well-established Caco-2 system was employed for this

study as it is a widely used in vitro model for the intestinal barrier. Differentiated Caco-2 cells are known to form a tight cellular monolayer with morphological and biochemical properties very similar to those of enterocytes of the small intestine [19, 20]. In addition, the Caco-2 system is a well-established model for intestinal lipid metabolism [21].

Experimental Materials and Methods

Chemicals and Fatty Acids

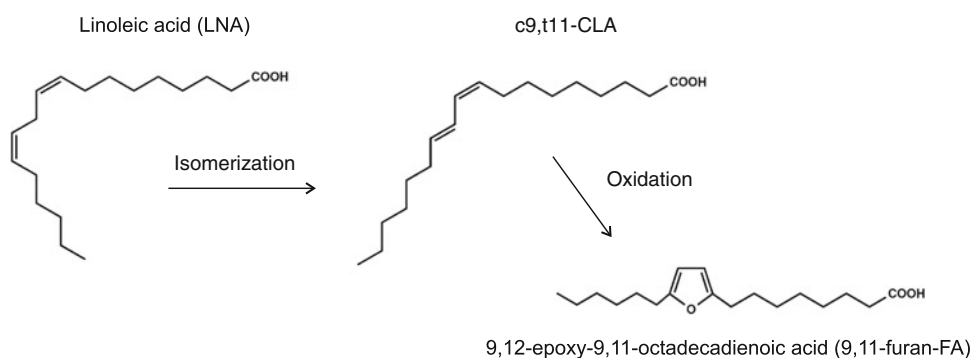
All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany) in the highest available purity. The CLA isomers *cis*-9,*trans*-11-octadecadienoic acid (c9,t11-CLA) and the furan fatty acid 9,12-epoxy-9,11-octadecadienoic acid (9,11-furan-FA) were obtained from Biotrend (Köln, Germany). LNA was purchased from Sigma-Aldrich (Taufkirchen, Germany). All fatty acids were dissolved in DMSO to get 0.1 M stock solutions.

Cell Culture and Treatment with Fatty Acids

Cells were obtained from the European Collection of Cell Culture (ECACC). Culture media and supplements were obtained from PAA Laboratories GmbH (Pasching, Austria). The human adenocarcinoma cell line Caco-2 (ECACC No. 860 10 202) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were passaged every 3–4 days by treatment with 0.1% trypsin and 0.04% EDTA and then plated at a density of $1.3\text{--}2 \times 10^4$ cells/cm².

For experimentally treatment with fatty acids, cells were allowed to attach by cultivating overnight in medium containing 10% FCS and were then incubated in serum-free medium supplemented with 1% insulin–transferrin–selenium

Fig. 1 Structures of LNA, c9,t11-CLA and 9,11-furan-FA



(ITS, Invitrogen, Karlsruhe, Germany) and 0.1 mg/ml fatty acid-free BSA (Sigma-Aldrich, Taufkirchen, Germany) with various concentrations of the respective fatty acid with a molar ratio of fatty acid to BSA of 4:1.

Oil-Red-O Staining

Caco-2 cells were cultivated on cover slips in 24-well plates with 40,000 cells/well. After 48 h of treatment with fatty acids staining with Oil-Red-O (Sigma-Aldrich, Taufkirchen, Germany) was performed. Cells were washed with Phosphate Buffered Saline (PBS) and fixed with 3.7% formaldehyde for 1 h. 0.3% Oil-Red-O in isopropanol was then added directly to the fixed cells and incubated for 1 h. The microscopy studies were performed with a Axio Observer microscope equipped with a digital camera (Zeiss, Jena, Germany).

Cell Viability Assay

Cells were plated in 96-well plates with 7,500 cells/well for Caco-2 cells in 100 μ l medium. After 2 days of treatment with fatty acids cell viability was estimated by the Cell-Titer-Blue[®] Cell Viability Assay (CTB Assay) (Promega, Madison, WI). Assays were performed by adding 20 μ l of a 1:4 dilution of the CTB reagent in phosphate-buffered saline (PBS) directly to the cells cultured in 100 μ l medium, incubating for 1 h at 37 °C and then measuring fluorescence at 540/590 nm with a Mithras Multimode Reader LB 940 (Berthold Technologies, Vienna, Austria). Values are expressed as percentages of the negative control which was exposed to medium containing 0.1% DMSO and which was taken as 100%. As a positive control for cytotoxicity: cells were exposed to medium containing 0.05% Triton X-100.

Extraction of Total Cellular Lipids and Fatty Acid Derivatization

Caco-2 cells were incubated with 100 μ M of LNA, c9,t11-CLA or 9,11-furan-FA for 48 h. Cells were washed twice with PBS, and after treatment with trypsin/EDTA cells were transferred to reaction tubes. 20 μ l of a 10 mM solution of butylated hydroxytoluene (BHT) in acetonitrile was applied to the cell suspensions to protect the lipids from oxidative damage. Subsequently, cells were lyophilized to completely remove the water and total lipids were extracted twice from the dried cell pellet with 0.7 ml of a 2:1 (v/v) chloroform:methanol mixture. 150 μ l of a 20% solution of BF₃ in methanol was applied to the lipid extracts, and transmethylation was conducted for 10 min at 70 °C. Methylated fatty acids were extracted with 1 ml of hexane, dried over anhydrous sodium sulfate, concentrated in vacuo and finally dissolved in 200 μ l hexane.

GC/MS Analysis

GC/MS analysis was conducted with an Agilent 7890A gas chromatograph coupled to an Agilent MSD 5975C mass spectrometer. Methylated fatty acids were separated with an Rt-2560 column (100 m \times 0.25 mm \times 0.2 μ m; Restek, Bad Homburg, Germany) by using helium as the carrier gas at a flow rate of 1 ml/min. 2 μ l of each samples was applied to the cold injection system (splitless mode, 15 °C) by taking advantage of an MP5 autosampler (Gerstel, Mülheim, Germany). The oven program was as follows: 6 min at 35 °C, ramp to 165 °C at 65 °C/min, 2 min at 165 °C, 1 min at 180 °C, 0.5 min at 190 °C, 1 min at 210 °C. Methylated fatty acids were identified by comparison of retention times with authentic standards and by the fragmentation pattern in the mass spectra. The artificial methylated fatty acid C19:0 was used as the internal standard. Mass spectra were recorded both in the SCAN modus and in the SIM modus to increase sensitivity. The quantitation limit of the method was 0.25 ng/ μ l for each individual fatty acid.

Results

Viability of Caco-2 Cells

In this study we focused on c9,t11-CLA, the major CLA isomer in foodstuff, and on its oxidation product 9,11-furan-FA. Moreover, the metabolic precursor of c9,t11-CLA, LNA, was included in the study.

To analyse potential cytotoxic effects of LNA, c9,t11-CLA and 9,11-furan-FA, Caco-2 cells were incubated with various concentrations of these substances. Subsequently, cellular viability was tested by using the CTB assay. As shown in Fig. 2, incubation of the cells with up to 100 μ M of the respective substance did not affect cellular viability whereas cellular viability was decreased to a level of about 3% upon incubation of the cells with medium containing 0.05% Triton X-100 which was used as positive control. Thus, none of the substances tested in this study displayed cytotoxic potential to Caco-2 cells up to a level of 100 μ M.

Fatty Acid Absorption by Caco-2 Cells

Caco-2 cells have the capacity to absorb free fatty acids and to incorporate them into triglycerides for storage in lipid droplets [22]. To analyze whether the enzymatic machinery of Caco-2 accepts furan-FA as a fatty acid-type substrate at all, lipid droplet staining was conducted with LNA-, CLA- and furan-FA-treated cells in order to visualize cellular absorption of the respective fatty acid. Caco-2 cells were incubated either with LNA, c9,t11-CLA, or

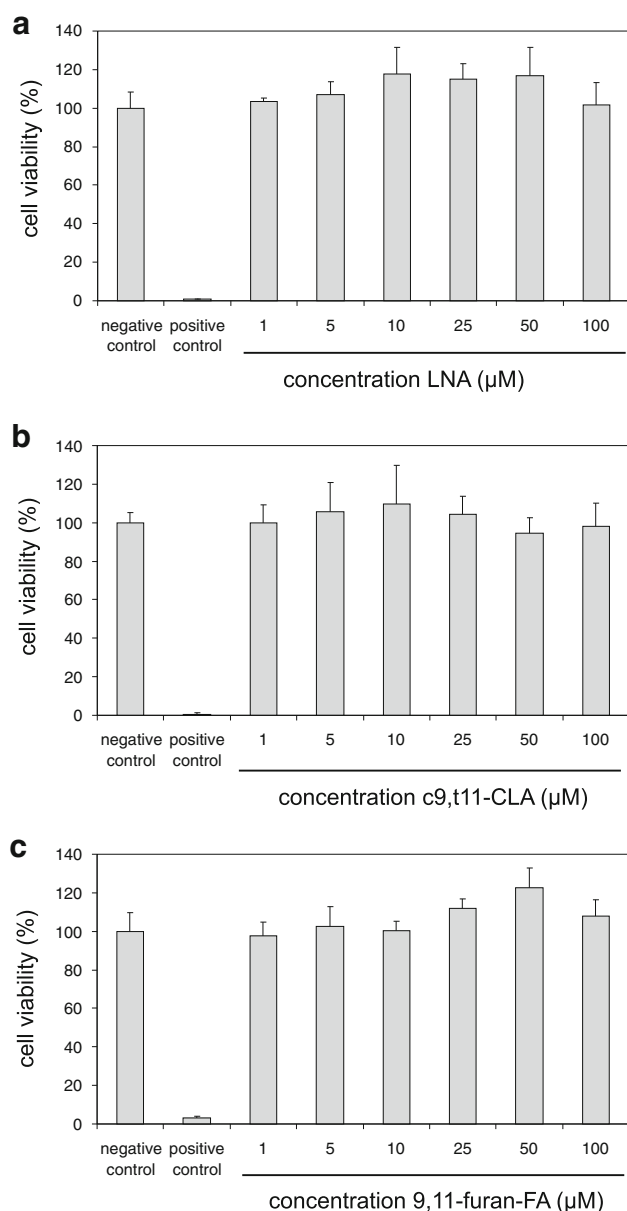


Fig. 2 Viability of Caco-2 cells. Cells were treated with various concentrations of (a) LNA, (b) c9,t11-CLA, and (c) 9,11-furan-FA for 48 h. Cellular viability was determined using the CTB assay and expressed as percentages of negative control which was exposed to medium containing 0.1% DMSO (set as 100%). Data are expressed as the means \pm SD; $n = 5$

9,11-furan-FA, and Oil-Red-O-staining of the cells was conducted after 48 h of incubation in order to visualize the formation of intracellular lipid droplets. In control cells, intracellular red stain was rarely visible, whereas numerous small lipid droplets were stained in cells that had been incubated with 10 μ M LNA, c9,t11-CLA or 9,11-furan-FA (Fig. 3). Incubation of the cells with 100 μ M of the respective free fatty acid yielded cells full of large lipid droplets (Fig. 3). Therefore, Fig. 3 clearly illustrates that

lipid droplet formation in Caco-2 cells increased with increasing concentrations of free fatty acid offered to the cells, independent of the type of fatty acid. Moreover, microscopic inspection of numerous cells indicated that Caco-2 cells treated with 100 μ M of the respective fatty acid tend to be enlarged in size due to lipid droplet formation. In these experiments there was no observation of specific alterations in cell morphology such as shrinking of the cells that might point to toxic effects induced by fatty acid treatment. Thus, in sum, Caco-2 cells seem to absorb 9,11-furan-FA and to store it as triglycerides in lipid droplets as they do with LNA and c9,t11-CLA.

Analysis of Fatty Acid Composition

To follow the fate of the fatty acids taken up by the cells, a GC/MS method was employed to analyze cellular fatty acid composition in more detail. For this purpose, total cellular lipids were extracted from Caco-2 cells that had been incubated with 100 μ M of LNA, c9,t11-CLA, or 9,11-furan-FA, respectively. After lipid hydrolysis and fatty acid derivatization, the resulting mixture of fatty acid methyl esters was separated by gas chromatography. Fatty acid identification was achieved by subsequent MS analysis of individual peaks. The relative amount of the respective fatty acid was calculated from the peak area in relation to the peak area of the internal standard. The results of this analysis are summarized in Table 1.

Incubation of the cells with LNA resulted in an approximately four-fold increase in the relative intracellular content of LNA and in a concomitant relative decrease of the amount of the other fatty acids with two exceptions. Compared to the control cells, the relative amount of the well-known LNA-metabolite 20:4n-6 increased upon incubation of the cells with LNA. In addition, a small amount of t9,t11-CLA was detectable in the samples obtained from LNA-incubated cells.

In the case of c9,t11-CLA, an approx. 20-fold increase in the relative intracellular amount of this substance was observed. In addition, the relative amount of the CLA isomers c9,c11-CLA and t9,t11-CLA increased whereas the relative content of the remaining fatty acids decreased.

Incubation of the cells with 9,11-furan-FA yielded an approx. 20-fold increase in the relative intracellular amount of this compound and a simultaneous decrease of the relative content of all other fatty acids. There was, however, an additional peak in the gas chromatogram that was neither present in the chromatogram of the control cells nor in the chromatograms of the cells incubated with LNA or c9,t11-CLA. The peak area of the additional peak was at a level of about 10% compared to the area of the 9,11-furan-FA peak (data not shown). Comparison of the mass spectra of 9,11-furan-FA and of the unknown substance from the

Fig. 3 Oil-Red-O staining. Caco-2 cells were incubated with LNA, c9,t11-CLA, or 9,11-furan-FA for 48 h. Oil-Red-O staining was conducted as described in the methods section. Red staining is indicative of lipid droplets (color figure online)

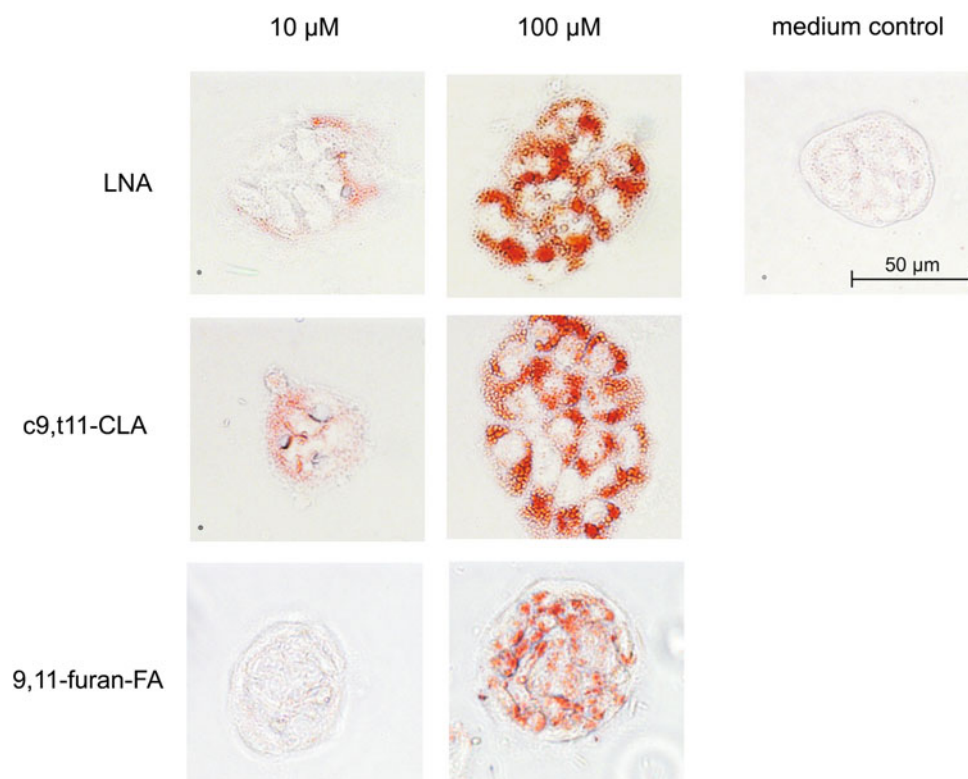


Table 1 Incorporation of LNA, c9,t11-CLA, and 9,11-furan-FA, respectively, into the total cellular lipids of Caco-2 cells after 48 h of incubation

Fatty acid (wt. %)	Control	LNA	c9,t11-CLA	9,11-furan-FA
16:0	6.10 ± 2.72	2.56 ± 1.22	0.92 ± 0.30	0.22 ± 0.06
18:0	50.87 ± 6.41	41.68 ± 4.83	30.21 ± 2.98	13.18 ± 1.61
18:1n-7	5.06 ± 0.57	4.53 ± 0.92	2.31 ± 0.81	2.61 ± 0.44
18:1n-9	5.07 ± 1.54	2.47 ± 0.93	1.98 ± 1.06	1.21 ± 0.33
20:0	4.27 ± 0.55	1.99 ± 0.75	1.31 ± 0.38	0.43 ± 0.14
20:1n-9	3.67 ± 0.46	1.18 ± 0.27	0.78 ± 0.21	0.72 ± 0.22
20:4n-6	12.10 ± 1.37	17.22 ± 3.88	4.00 ± 0.88	3.59 ± 0.52
c9,c11-CLA	nd	nd	2.55 ± 0.88	nd
t9,t11-CLA	nd	0.58 ± 0.26	6.57 ± 1.00	0.07 ± 0.04
LNA	6.44 ± 1.13	23.32 ± 4.29	2.80 ± 0.52	1.03 ± 0.26
c9,t11-CLA	2.19 ± 0.53	1.54 ± 0.49	44.50 ± 4.27	0.20 ± 0.09
9,11-furan-FA	4.23 ± 0.93	2.93 ± 0.79	1.92 ± 0.79	76.71 ± 2.58

nd not detected

additional peak revealed that this substance must be a metabolite of 9,11-furan-FA (Fig. 4). Two major peaks with m/z 165 and m/z 179 in the mass spectrum of 9,11-furan-FA (Fig. 4a) can be explained by the cleavage of the compound between carbon atoms C7 and C8, and between C6 and C7, respectively. Additional β -cleavage of these products between C13 and C14 results in masses of m/z 95 and m/z 107, respectively, additional major peaks in spectrum of Fig. 4a. Since these four masses are also present in the mass spectrum of the unknown metabolite (Fig. 4b), it can be concluded that this metabolite is a derivative of 9,11-furan-FA. Moreover, preservation of

these four masses indicates that the furan ring is still intact in the metabolite and that it was not altered upon cellular metabolic activity.

Since the molecule peak of 9,11-furan-FA with m/z 308 (Fig. 4a) was shifted to a mass of m/z 336 (Fig. 4b), the mass of the metabolite has increased by 28 atomic units (a.u.). In addition, the series of m/z 237, m/z 251, m/z 265 and m/z 277 of Fig. 4a has shifted by 28 a.u. to m/z 265, m/z 279, m/z 293 and m/z 305, respectively, in Fig. 4b. The increase of 28 a.u. can be explained by the addition of two methylene groups to 9,11-furan-FA, resulting in the C20-derivative as depicted in the insert of Fig. 4b.

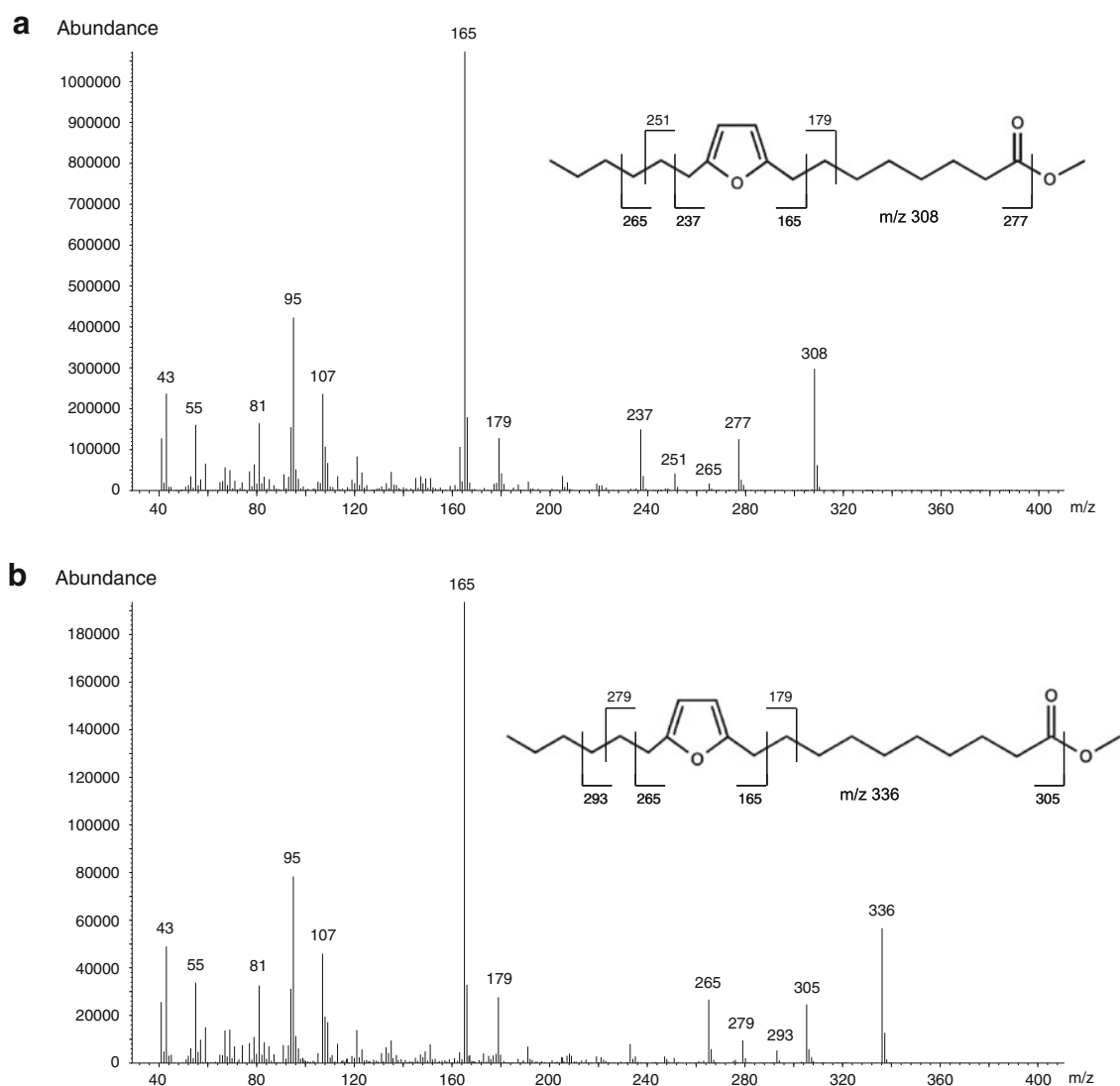


Fig. 4 Mass spectra of (a) 9,11-furan-FA and of (b) the metabolite that was formed upon incubation of Caco-2 cells with 9,11-furan-FA. The masses of the major cleavage products are assigned to the structures of the respective molecule

Discussion

In the past decades, CLA have been intensively investigated in numerous *in vivo* and *in vitro* studies. These substances are supposed to have various positive effects on human health. Therefore, CLA are already available as food supplements, and the risks and benefits of supplementation of foodstuff with CLA are currently under discussion. Surprisingly, the putative presence of furan-FA in CLA preparations plays no role in these discussions so far, although it was shown that furan-FA might arise from auto-oxidation of CLA in the presence of molecular oxygen [17]. From a toxicological point of view, furan-FA are of particular interest because furan-FA harbor a furan ring system, and furan and furan derivatives have been shown to

be toxic compounds (reviewed in [18, 23, 24]). In the liver, furan and furan derivatives are activated by CYP2E1 to form epoxides, and subsequently these epoxides are further converted to yield aldehydes in some cases [25–27]. Epoxides as well as aldehydes can directly react with lipids, proteins or DNA and are therefore toxic to cellular systems. Due to their reactivity to DNA, they are potent mutagens. Therefore, the toxicological characterization of furan-FA has to be conducted in parallel to the current risk and benefit assessment of CLA supplementation.

It is well-known that plants produce furan-FA as secondary metabolites. These furan-FA of plant origin carry methyl groups at one or both β -positions of the furan ring. If these compounds are ingested with food, the methyl groups are converted by liver metabolic activity to carboxylic acid

groups and the resulting urofuran acids are then excreted via the kidney (reviewed in [28]). In contrast to the plant furan-FA, the furan-FA analyzed in this study is an oxidation product of c9,t11-CLA and does not carry substituents at the β -positions of the furan ring. There are only few examples for non-substituted furan-FA in nature. A few non-substituted furan-FA have been isolated only from marine sponges so far, and preliminary in vitro studies revealed considerable cytotoxic potential of these isolates [29, 30]. The furan-FA analyzed in this study, however, showed no cytotoxic effect up to a concentration of 100 μ M.

The Caco-2 cell line used in this study serves as a model for human intestinal enterocytes. Caco-2 cells have been shown to be capable of lipid metabolism including triglyceride synthesis and lipid droplet formation. Moreover, these cells express numerous P450 enzymes and have the capacity to detoxify various compounds. Among others, the CYP2E1 enzyme has shown itself to be expressed by Caco-2 cells [31]. Therefore, the Caco-2 system was the ideal model to study uptake and metabolism of furan-FA by human intestinal cells, because triglyceride formation as well as β -oxidation and CYP-mediated activation of the furan ring could be examined by using this cell line. Our results indicate that furan-FA are taken up by the cells, esterified to triglycerides and stored in lipid droplets. At first glance, there seems to be no difference in the uptake and conversion of LNA, CLA and the corresponding furan-FA by Caco-2 cells. Hydrolysis of total cellular lipids and subsequent fatty acid quantification revealed that LNA and CLA were partly converted to related fatty acids by the cells (Table 1).

In the case of LNA, a relative increase in arachidonic acid (20:4n-6) was observed. It is well-known that mammalian cells have the capacity to use LNA as a precursor for the formation of 20:4n-6. Fatty acid elongation is facilitated by the activity of ELOVL5, a member of the ELOVL (elongation of very long chain fatty acid) protein family [32, 33] followed by the subsequent insertion of two additional double bonds. Thus, based on our lipid analysis, there is some indication that Caco-2 cells do not only take up fatty acid and store them as triglycerides in lipid droplets, but the cells also seem to elongate fatty acids prior to triglyceride formation.

Incubation of the cells with c9,t11-CLA resulted in a relative increase in the CLA isomers c9,c11-CLA and t9,t11-CLA (Table 1). It has recently been shown that the human colon cell line HT-29 can convert t11,t13-CLA into its isomer c9,t11-CLA probably by an enzymatic two-step saturation/desaturation reaction [34]. This might explain the appearance of the c9,c11-CLA isomer in our experiment but can not explain the presence of the t9,t11-CLA isomer as mammalian cells do not introduce *trans* double bonds into fatty acids. We can not exclude that *cis-trans* isomerization

might have occurred chemically during sample preparation or derivatization. By using a solution of c9,t11-CLA in control experiments, however, there was no indication of *cis-trans* isomerization under the given experimental conditions (data not shown). Finally, the unexpectedly high amounts of the different CLA isomers and of 9,11-furan-FA already in the control cells might have been due to the fact that the cells had been incubated in a medium containing 10% fetal calf serum (FCS) that may contain significant amounts of bovine-specific fatty acids.

Lipid extracts from cells incubated with 9,11-furan-FA yielded an additional peak in the gas chromatogram that was not detected with extracts from cells that had been treated with LNA or c9,t11-CLA. Based on the corresponding mass spectrum, we propose a structure for the furan-FA-derived metabolite as presented in Fig. 4b. From a biological point of view, the presence of this compound can simply be explained by the elongation of 9,11-furan-FA probably again by the action of a member of the ELOVL protein family and the subsequent reduction of the condensation product as described for LNA above.

Finally, GC/MS analysis of fatty acid extracts derived from furan-FA-treated Caco-2 cells gave no indication for any modification at the furan ring system. There were no metabolites detected that might have been formed upon epoxide formation or any other modification of the furan ring. Therefore, furan-FA might be either not accepted as a substrate for the cellular CYP machinery, or esterification of furan-FA to form triglycerides and subsequent storage in lipid droplets might be favoured in comparison to the postulated oxidative conversion of the furan ring. In conclusion, furan-FA taken up by human intestinal cells seem to end up preferentially in lipid metabolic pathways such as fatty acid elongation and triglyceride formation, whereas the furan ring present in furan-FA does not seem to be attacked by detoxifying enzymes. In vivo, intestinal cells pack triglycerides into chylomicrons which are VLDL-like particles and secrete them into the blood stream. These chylomicrons are then taken up by liver cells where they are further metabolized. Since our results indicate that the furan ring system of furan-FA was not affected by the metabolic activity of intestinal cells, we will address the question in future studies as to whether the furan ring might be attacked by liver metabolic activity.

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