A Bioactively Modified Fatty Acid Improves Survival and Impairs Metastasis in Preclinical Models of Acute Leukemia

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Abstract

Purpose: Polyunsaturated fatty acids (PUFA) and the sulfur-substituted fatty acid tetradecylthioacetic acid (TTA) inhibit proliferation and induce apoptosis in lymphoma and leukemic cell lines, but it is unknown if they can modify leukemogenesis in the intact organism.

Experimental Design: We now examined the effects of PUFA and TTA in rats transplanted with either acute promyelocytic leukemia or acute T-cell leukemia. The rats were randomized to isenergetic diets containing either lard (control), ω3 (n-3) PUFA, or TTA.

Results: Whereas TTA prolonged survival (P < 0.05) in both types of rat leukemia, n-3 PUFA had no significant effect compared with controls. Only TTA inhibited (P < 0.05) leukemic infiltration in the bone marrow and spleen, probably due to apoptosis of the leukemic cells. Plasma metalloproteinase activity, a marker of metastatic activity, was significantly reduced in TTA-fed rats only.

Conclusions: Dietary intake of TTA, but not of n-3 PUFA, in rats with acute leukemia, prolonged their survival. TTA intake was also associated with reduced leukemic cell burden as well as diminished extramacular dissemination. TTA represents a modified fatty acid that exerts unique effects on malignant hematopoietic cells, and the present study indicates that TTA may have a therapeutic potential in patients with acute leukemias.

The prognosis of acute myeloid leukemia (AML) and acute lymphoid leukemia in the adult remains dismal. Most studies report a median 5-year survival of ~50% for adults (<60 years) that were offered optimal treatment (1, 2). The limited success of current therapeutic approaches for the acute leukemias might at least partly be explained by their lack of specificity; hence, there is a need for a more mechanism-based treatment.

Recent epidemiologic and clinical data suggest that obesity might be an important risk factor for the development of hematologic malignancies, including leukemia (3, 4). We have shown that inhibition of leptin, a key adipose tissue–derived cytokine involved in the regulation of energy expenditure and food intake, suppressed leukemic progression in rats with AML, suggesting that modification of a dysregulated energy metabolism might influence leukemogenesis (5).

A growing body of evidence indicate that polyunsaturated fatty acids (PUFA) might impair proliferation and exert proapoptotic and necrotic effects in leukemic cell lines (6, 7). To enhance the efficiency of fatty acids in controlling malignant growth and dissemination, various attempts have been made to modify their molecular structure. One such fatty acid analogue, tetradecylthioacetic acid (TTA), reportedly reduced the proliferation of AML blasts and other malignant cells (8, 9). The antiproliferative effects of TTA can, at least in part, be explained by induction of apoptosis, possibly mediated by mitochondrial alterations (10).

The use of PUFA and TTA in treating malignancy has mostly been restricted to malignant cell lines, including those of the lymphohematopoietic system, whereas studies in mammals are sparse. However, studies in the intact organism are mandatory to further establish the potential usefulness of fatty acid derivatives as antileukemic drugs. We have therefore examined the effects of dietary supplements of ω3 (n-3) PUFA and TTA to rats with either AML or acute lymphoid leukemia. Specifically, we wanted to determine the effect on survival, growth, and dissemination of the leukemic cells.

Materials and Methods

Rats and the transplantable acute leukemias. Animal care was in accordance with national legislation and institutional guidelines. We studied two rat models of human acute leukemia. The Brown Norway (BN) leukemic (BNML) rat is a well-defined model of human acute promyelocytic leukemia and share important characteristics regarding development and progression of the leukemic disease with human AML (11). Originally, this leukemic model was established by injecting a carcinogenic compound into BN rats (12). During progression to overt leukemia, the spleen became heavily infiltrated with malignant leukocytes, so that postmortem these spleen-derived cells could be isolated and frozen to be used for induction of leukemia into other BN.
rats. Similarly, splenic cells from a PVG rat originally given a β-emitting radionuclide have been used to induce and acute T-lymphocytic Roser leukemia (PVG-RL, ref. 13). This model bears close resemblance to human acute lymphoid leukemia (14). Male BN rats (range 260-280 g) and PVG rats (range 215-240 g) were obtained from Harlan (Allerod, Denmark). These animals tested negative for microbial agents according to the Federation of European Laboratory Animal Science Association recommendations (15). About 20 million splenic-derived leukemic cells were injected i.p. (16, 17). The leukemic rats were put to death if they developed paralysis or showed obvious signs of distress. The study was terminated at day 35 as recommended by our ethical committee.

Preparation of experimental diets. Each animal group was offered one of three semisynthetic diets: lard (19.5% lard), n-3 PUFA (9% lard and 10.5% Trimonial, EAPX5500; Pronova Biocare, Oslo, Norway), or TTA (19.1% lard + TTA 0.38%). In addition, 1.5% of soybean oil was provided to all dietary groups to avoid essential fatty acid deficiency. The percentage dietary composition by weight of total diet was as follows: sucrose, 20%; cornstarch, 31.5%; casein, 20%; cellulose, 1%; vitamin mixture, 1.5%; salt mixture, 5%, and fat, 21.5%. The diets (Table 1) provided ~40% of the energy from fat and they were kept at −20°C and given to the rats in portions of 17 g sufficient for 1-day supply.

Experimental protocol. The BNML and the PVG-RL rats were randomly assigned to one of the three experimental diets (n = 10 rats per diet). All 60 rats had free access to water and they were gradually adapted to their allocated diet, starting 1 week before injection of the leukemic cells. Rats were housed single and unrestrained in Macrolon adapted to their allocated diet, starting 1 week before injection of the leukemic cells. Rats were housed single and unrestrained in Macrolon II cages at constant temperature (21 ± 1°C) and relative humidity (55 ± 10%) on a 12-hour light-dark cycle with an air exchange rate of 20 changes/h.

On day 0, the leukemic cells were injected i.p. and the rats were observed closely until they were put to death (i.p. pentobarbitone, 100 mg/kg).

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Immediately before the rats were sacrificed, a blood sample was collected via a cardiac puncture during deep pentobarbitone anesthesia. Postmortem, we removed the liver, spleen, and femoral bones before the femoral bone marrow was isolated.

Determination of TTA and fatty acid composition. Lipids were extracted from plasma and diets using a mixture of chloroform and methanol (18). The extracts were added heneicosanoic acid as internal standard, and were transesterified with BF3-methanol. To remove neutral sterols and nonsaponifiable material, the extracts were heated in 0.5 mol/L KOH in ethanol-water solution. Recovered fatty acids were reesterified using BF3-methanol. The methyl esters were quantified as described (19).

Quantification of leukemic cell content. To quantify bone marrow infiltration of leukemic cells, we used a validated approach based on flow cytometric analysis after labeling with specific surface markers. Irrelevant antibodies were used as negative controls. The retrieved femoral bone marrow cells from the BNML rats were labeled with the monoclonal antibody (mAb) RM124 that specifically recognizes BNML cells even at very low frequencies and with very low binding to normal myeloid cells (20). This mAb was then linked to a secondary fluorescent antibody before the samples were sorted with a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). Similarly, leukemic cells from the PVG-RL rats were identified after labeling the bone marrow cell suspension with the mAb OX7 before flow cytometric analysis (17).

Measurement of apoptosis. Sorted leukemic cells were cultured in either RPMI 1640 (BNML cells) or Iscove’s medium (PVG-RL cells), and added with 300 μmol/L of either the n-3 PUFA eicosapentaenoic acid (EPA, C20:5n-3) or TTA for 24 hours, as separate experiments suggested this to be the optimal dose and incubation time before the cells were evaluated for apoptosis using staining of propidium iodide and subsequent flow cytometry. Before addition of fatty acids, the purity of the leukemic cell suspension exceeded 98% and the viability was always >96% when assayed with the trypan blue exclusion test.

Zymography. To measure matrix metalloproteinase (MMP) activity, we used a zymographic assay (21). Briefly, plasma samples were mixed with sample buffer before they were loaded onto an SDS-PAGE gel—containing gelatin. After electrophoresis, the gels were incubated in Triton X-100 to remove SDS before they were incubated in assay buffer overnight at 37°C to allow possible enzymes in the samples to degrade the gelatin matrix. The gel was then stained with Coomassie brilliant blue and the gelatinase activity was determined as unstained regions using ImageQuant 5.2 software (Amersham Biosciences, Oslo, Norway).

Protein levels and mRNA expressions of MMP-2 and MMP-9. MMP-2 and MMP-9 protein levels were measured with Western blotting (21). Samples were analyzed on 10% SDS-PAGE acrylamide gels and thereafter blotted onto a membrane before incubation in 5% nonfat dry milk in TBS and washed before incubation with primary mouse anti-rat mAb raised against either MMP-2 or MMP-9 (Oncogene, WVR, Oslo, Norway, and Ermotech, Ltd., Bergen, Norway). The membrane was washed and finally incubated with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences). Immunoreactivity
was detected by the enhanced chemiluminescence method (Amersham Biosciences).

We used Northern blotting to examine the mRNA expressions of MMP-2 and MMP-9 (21). In short, after extraction of RNA from sorted leukemic cells and transfer to a nylon filter membrane, it was UV cross-linked before hybridization with the cDNA probes. Following hybridization, blots were washed and radioactivity on the membrane was recorded. Densitometric analysis was carried out with the ImageQuant 5.2 software. The obtained mRNA signal was normalized to the signal obtained by hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe.

Statistics. Some rats died before we could perform adequate sampling of specimen; hence, for some variables, \( n < 10 \) per group. The various assays were done in triplicates. Values are presented as means and SE, and differences were evaluated with the Kruskal-Wallis test; when appropriate, a Bonferroni correction was made. Survival was examined with the Kaplan-Meier method. For correlation analyses, we applied Pearson’s correlation coefficient \((r)\). Significance was assumed for \( P < 0.05 \).

Results

Dietary intake of fatty acids. The three diets containing either lard, n-3 PUFA, or TTA were well tolerated and had no apparent adverse effects on the rats. Interestingly, none of the diets had any significant effect on the body weights in either the BNML or the PVG-RL rats throughout the observation period (Table 2). The leukemic rats consumed ~60% of the food offered (i.e., ~10 to 11 g daily). Compliance with the diets was evaluated by measuring the plasma concentrations of fatty acids. As expected, TTA was clearly present in the plasma of the TTA-fed rats, whereas no TTA could be detected in lard-fed or n-3 PUFA–fed animals (Table 2). We used EPA as a marker of compliance with the diet containing n-3 PUFA. Table 2 clearly shows that rats fed n-3 PUFA had a marked elevation of EPA in contrast with lard- or TTA-fed rats.

Dietary intake of TTA prolongs the survival of leukemic rats. It is evident from Fig. 1 that the dietary intake of TTA had a pronounced effect on the survival of both the BNML and the PVG-RL rats. Whereas median survival of PVG-RL rats was 16 days in the lard and n-3 PUFA groups, it increased \((P < 0.05)\) to 20 days among the TTA-fed rats (Fig. 1A). In comparison, the median survival in the BNML group was 30 and 31 days in the lard-fed and n-3 PUFA–fed groups, respectively, whereas median survival was not reached \((P < 0.05)\) in the TTA group when the study was terminated at day 35 according to protocol (Fig. 1B).

Table 2. Body, spleen, and liver weights, and plasma concentration of fatty acids

<table>
<thead>
<tr>
<th>Rat diet</th>
<th>Body weight (% change)</th>
<th>Spleen (g)</th>
<th>Liver (g)</th>
<th>EPA ((\mu)mol/L)</th>
<th>TTA ((\mu)mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNML</td>
<td></td>
<td></td>
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<tr>
<td>Lard</td>
<td>−0.9 ± 6.5</td>
<td>4.6 ± 0.6</td>
<td>28.9 ± 1.8</td>
<td>191 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>−7.1 ± 2.3</td>
<td>4.4 ± 0.7</td>
<td>25.4 ± 1.4</td>
<td>851.9 ± 132.7*</td>
<td>ND</td>
</tr>
<tr>
<td>TTA</td>
<td>6.0 ± 3.1</td>
<td>2.4 ± 0.5*</td>
<td>23.6 ± 1.8</td>
<td>14.2 ± 1.7</td>
<td>173.2 ± 15.2*</td>
</tr>
<tr>
<td>PVG-RL</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lard</td>
<td>1.7 ± 0.2</td>
<td>3.5 ± 0.4</td>
<td>14.8 ± 1.0</td>
<td>47.2 ± 9.1</td>
<td>ND</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>−2.3 ± 0.5</td>
<td>3.7 ± 0.6</td>
<td>15.7 ± 0.7</td>
<td>909.8 ± 330.9*</td>
<td>ND</td>
</tr>
<tr>
<td>TTA</td>
<td>7.9 ± 0.7</td>
<td>21 ± 0.5*</td>
<td>12.6 ± 0.5</td>
<td>10.3 ± 1.0</td>
<td>158.9 ± 23.9*</td>
</tr>
</tbody>
</table>

NOTE: Body weight difference was recorded between the day of leukemic cell transplantation and the day the rat was sacrificed. Values represent the means ± SE (\(n = 10\)). On the average, a normal liver weighs 14 and 10 g in the BN and the PVG rats, respectively.

Abbreviation: ND, not detected.

\(^*P < 0.05\) for all comparisons between the three experimental groups of either the BNML or the PVG-RL rats.
Diet-induced changes in plasma fatty acid profiles. Whereas the ratio of n-3 PUFA to n-6 PUFA was 1.4 in n-3 PUFA-fed BNML and PVR-RL rats, the corresponding ratio was 0.2 in BNML and PVG-RL rats fed either lard or TTA. In contrast, no apparent differences were noted for the total amount of saturated fatty acids among the three experimental groups (data not shown). This indicated that increased plasma concentrations of n-6, but not of n-3 PUFA, were associated with prolonged survival, as shown specifically for the C18:3n-6 versus the C18:3n-3 PUFAs depicted in Fig. 2A and B. Whereas the plasma concentration of the C16:1n-9 fatty acid significantly increased in the TTA-fed rats (Fig. 2C), the corresponding concentrations of the C14:0 fatty acid decreased ($P < 0.05$; Fig. 2D), both compatible with elevated mitochondrial fatty acid oxidation.

Decreased leukemogenesis in the bone marrow of TTA-treated rats. We quantified the content of intramedullar leukemic cells by using mAb labeling of specific surface antigens and flow cytometry. Figure 3 shows that the bone marrows of BNML and the PVG-RL rats fed either lard or n-3 PUFA were heavily infiltrated with leukemic cells at the end of the experiment. In contrast, the leukemic cell mass was markedly reduced in TTA-treated rats to $\sim 50\%$ in the BNML rats. An even more pronounced reduction was observed in the TTA-treated PVG-RL rats where the fraction of leukemic cells constituted $\sim 40\%$ of the intramedullar cell content compared with the lard or n-3 PUFA-fed rats at the end of the experiment.

In search for the possible mechanism of this profound antileukemic effect upon addition of TTA, we directly measured apoptosis of leukemic cells cultured in vitro and exposed to EPA or TTA. Interestingly, we found that whereas EPA exerted some proapoptotic effect on BNML cells as well as on PVG-RL cells, TTA clearly promoted apoptosis in both leukemic cell populations (Fig. 4). In contrast, no apparent effect on the viability of normal bone marrow cells cultured with EPA or TTA was found (data not shown).

Impaired extramedullar metastasis of leukemia in TTA-treated rats. We next examined if addition of the experimental diets would interfere with dissemination of the leukemic cells. We used splenic mass as one measure of extramedullar leukemic deposits because this variable seems to correlate well with disease severity. Moreover, in the later stages of the leukemia, the infiltrating leukemic cells distort the normal splenic architecture (22). Whereas the splenic weight was apparently unchanged in leukemic rats fed n-3 PUFA compared with lard-feeding, the addition of TTA in the diet approximately halved the splenic weights in the BNML rats as well as in the PVG-RL rats (Table 2). In contrast, none of the dietary regimens had any apparent effect on the liver weights.

Activation of MMP is a required initial step for malignant cells to traverse extracellular barriers within the bone marrow to enter the circulation before subsequently invading other...
tissues. MMP-2 and MMP-9 seem as likely candidates in this metastatic process (23). We used a gelatinase assay to measure plasma MMP activity. At the end of the experiment, the gelatinase activity among TTA-fed BNML rats was reduced to ~60% of the activity measured in lard-fed rats (Fig. 5A and B). The corresponding reduction among the TTA-fed PVG-RL rats was ~30%. Addition of n-3 PUFA had no apparent effect.

We then used Western blotting to evaluate if the protein levels of either MMP-2 or MMP-9 were altered. Figure 6A shows that TTA promoted a reduction of MMP-2 in the BNML rats. A similar reduction was also found in the PVG-RL group, but the protein level of MMP-9 was not altered in any feeding group (data not shown). The decrease in MMP-2 protein among the TTA-fed leukemic rats was accompanied by a similar reduction in the mRNA expression of MMP-2 (Fig. 6B).

**Discussion**

In the present study, we show a markedly reduced leukemogenesis in rats with acute leukemias by feeding a sulfur-modified fatty acid. Specifically, we also show that dietary intake of TTA, but not of n-3 PUFA, was associated with an increase in survival, in particular among the BNML rats. This may be due to an enhanced apoptotic rate among the population of leukemic cells residing within the bone marrow. Moreover, the extramedullar dissemination of the leukemic cells was clearly reduced in TTA-fed leukemic rats when measured as a reduction in the splenic mass and an inhibition of MMP activity, the latter being mostly due to a reduction in the mRNA expression and protein synthesis of MMP-2.

The possible clinical relevance of the present data is strengthened by the use of two different models of human acute leukemia. The BNML is a well-established model of human acute promyelocytic leukemia with respect to leukemic cell proliferation kinetics, dissemination pattern, and response to cytotoxic drugs. It has also served as a useful tool for studies on conditioning regimens before transplantation as well as for the development of methods to detect minimal residual disease (11, 12, 24). Although apparently not studied in similar detail, the PVG-RL model exhibits several important features in common with adult human acute lymphocytic leukemia of T-cell origin (14). Importantly, bone marrow blood flow is not.
responses to TTA feeding seem to vary from those of normal survival of rats with glioma xenografts (8, 25, 26). The cellular leukemia, and that it reduces tumor growth and prolongs malignant cell lines, e.g., from breast cancer, glioma, and survival rates.

duration probably only explain a small part of the differences different diets died on the same day. Hence, a variable diet diets for at least 3 weeks. Furthermore, some rats fed at least another 2 weeks; hence, they were all given their comparison of possible explanatory mechanisms somewhat difficult. However, all rats were given their respective diet 1 week before injection of the leukemic cells, and all rats lived at least another 2 weeks; hence, they were all given their diets for at least 3 weeks. Furthermore, some rats fed different diets died on the same day. Hence, a variable diet duration probably only explain a small part of the differences in survival rates.

There is evidence that TTA inhibits proliferation of various malignant cell lines, e.g., from breast cancer, glioma, and leukemia, and that it reduces tumor growth and prolongs survival of rats with glioma xenografts (8, 25, 26). The cellular responses to TTA feeding seem to vary from those of normal dietary fatty acids, like the PUFA's, indicating that TTA triggers different mechanisms. TTA cannot be β-oxidized; it is a ligand for peroxisome proliferator–activated receptors α, δ, and γ; and it causes changes in energy metabolism and mitochondrial functions, leading to increased fatty acid oxidation and altered lipid composition (27–29). Although the exact mechanism of the antimalignant activity of TTA remains to be more precisely defined, data support that the mitochondria may play a central role. In line with this, TTA activated the apoptotic program in IPC-81 leukemia cells via depolarization of the mitochondrial membrane potential, release of cytochrome c, and a depletion of mitochondrial glutathione, all compatible with a disruption of the normal regulation of the mitochondrial redox equilibriuim (10). Antiproliferative effects were also seen in primary human AML blasts (8). Whatever the exact mechanism is, the antileukemic properties of TTA is most likely caused by direct effects on the transplanted leukemic cells, and not an overall response of the host, as previously observed in L1210 leukemic mice fed a mixture of different PUFA's (30).

Various lines of recent published data collectively suggest a link between the development and progression of leukemic disease and a dysregulated energy metabolism, the latter being characterized primarily by overconsumption of energy combined with reduction in physical activity, thereby leading to obesity. For example, in their large case-control study, Pan et al. (4) found an increased risk of several hematologic malignancies, including leukemia, among obese women and men. Likewise, Estey et al. (3) noted a strong association between obesity and the incidence of acute promyelocytic leukemia, but curiously they found no association with obesity to other AML types. On a cellular level, we have previously found enhanced oxygen consumption and fatty acid metabolism in cultured BNML cells (31). Moreover, a neutralizing antileptin receptor mAb led to a substantial suppression of leukemic expansion and dissemination in the BNML model (5).

Data from in vitro and animal studies have suggested that increasing the ratio of n-3 to n-6 PUFA's might prevent carcinogenesis (32). However, epidemiologic studies have failed to consistently verify this assumption (32). Although not specifically designed to address this issue, the present data argue against this because an increase in the ratio of n-6 to n-3 PUFA's was associated with prolonged survival. Interestingly, prolonged survival correlated with increased plasma concentrations of γ-linolenic acid (18:3n-6; Fig. 2A), a biologically active fatty acid with antitumor properties in preclinical and clinical studies (9).

Previous animal data and the present study indicate that TTA may have a therapeutic potential in cancer, in particular because no apparent toxicity was recorded when TTA was given to healthy humans (8). TTA represents a modified fatty acid that exerts unique effects on malignant hematopoietic cells, and further investigation is needed to explore its therapeutic potential in leukemic patients.

Fig. 6. Reduced protein level and mRNA expression of MMP-2 in TTA-fed leukemic rats. Plasma samples from the leukemic rats (A) were subjected to Western blotting. Appropriate loading was checked with addition of an antialbumin antibody. Data are from three BNML rats fed one of the three experimental diets each. The mRNA expression of MMP-2 (B) from sorted BNML and PVG-RL cells in vitro were analyzed with Northern blotting and the obtained values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Columns, mean (n = 10 rats in all three dietary groups); bars, SE. *P < 0.05 compared with rats fed lard or n-3 PUFA.

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References


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